University of Reading

Cacao swollen shoot virus in Nigeria

Analysis of a Pathogen and its Vectors

A Thesis submitted by

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For the degree of Doctor of Philosophy

School of Agriculture, Policy and Development

January 2016

DECLARATION

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

…… …… .....

Name Signature Date

DEDICATION

To my dear parents, Elder and Mrs Edet Ben Obok, who saw what education was; knew what higher education could be, but never had any personal experience.

To the memory of my teacher, friend and colleague, Late Dr Donald Ukeh.

ACKNOWLEDGEMENTS

I am grateful to the Commonwealth Scholarship Commission and the University of Reading for jointly sponsoring my PhD study. My appreciation goes to the Federal Scholarship Board, Abuja – Nigeria for nominating me for the Scholarship. The additional financial supports from MARS® Incorporated and the Cocoa Research UK are hereby duly acknowledged.

I especially thank my supervisor, Dr Andy Wetten, for his unique patience and stern direction throughout the course of this study. Like James Edward van der Plank (1908-1997) experienced as a student, I never sat in Virology, Pathology or an Entomology class, but the exceptional tutelage of Dr Andy Wetten granted me a grand experience during my doctoral studies. I also seize this opportunity to recognise the academic support that I received from Professor Paul Hadley during this period.

During the course of this journey, I have met and interacted with many people; some of whom I may not have fully remembered their names – laboratory technicians, fellow PhD students, friends and families, including Emeritus Professor Michael Aken'Ova, Emeritus Professor Helmut van Emden, Professor Godfrey Iwo, Professor Jim Dunwell, Professor Michael Shaw, Professor Gregory Walker, Prof Linda Walling, Professor Sylvia Umoetok, Prof Margaret Solomon, Dr Colin Campbell, Dr Freddy Tjallingii, Dr Peter Aikpokpodion, Dr Donatus Uwah, Dr Aniefiok Uko, Dr Christopher Idiong, Dr Paul Okon, Dr Joël Allainguillaume, Dr Andrew Daymond, Dr Peter Harris, Dr George Gibbings, Dr Suganya Shanmugavel, Dr Inibehe Ukpong, Dr Victor Okereke, Mr Lawrence Okojie, Mr Matthew Richardson, Val Jasper, Amanspreet Kaur, James Blackford, Liam Handley and Patrick Kalunda.

Thank you all for your earnest supports and concerns.

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ABSTRACT

Cacao swollen shoot virus (CSSV) causes the Cacao swollen shoot virus disease (CSSVD) and significantly reduces production in West African cacao. This study characterised the current status of the disease in the major cacao growing States in Nigeria and attempted a clarification on the manner of CSSV transmission. Two separate field surveys and sample collections were conducted in Nigeria in summer 2012 and spring 2013. PCR-based screening of cacao leaf samples and subsequent DNA sequencing showed that the disease continues to persist in Ondo and Oyo States and in new cacao sites in Abia, Akwa Ibom, Cross River and Edo States. Mealybug samples collected were identified using a robust approach involving environmental scanning electron microscopy, histology and DNA barcoding, which highlighted the importance of integrative taxonomy in the study. The results show that the genus *Planococcus* (*Planococcus citri* (Risso) and/or *Planococcus minor* (Maskell)) was the most abundant vector (73.5%) at the sites examined followed by *Formicococcus njalensis* (Laing) (19.0 %). In a laboratory study, the feeding behaviour of *Pl. citri*, *Pseudococcus longispinus* (Targioni-Tozzetti) and *Pseudococcus viburni* (Signoret) on cacao were investigated using electrical penetration graph (EPG) analysis. EPG waveforms reflecting intercellular stylet penetration (C), extracellular salivation (E1e), salivation in sieve elements (E1), phloem ingestion (E2), derailed stylet mechanics (F), xylem ingestion (G) and non-probing phase (Np) were analysed. Individual mealybugs exhibited marked variation within species and significantly differed ($p \leq .05$) between species for E1e and E1. PCR-based assessments of the retention time for CSSV in viruliferous *Pl. citri*, *Ps. longispinus* and *Ps. viburni* fed on a non-cacao diet showed that CSSV was still detectable after 144 hours. These unusually long durations for a pathogen currently classified as a semi-persistent virus have implications for the design of non-malvaceous barrier crops currently being considered for the protection of new cacao plantings.

Number of words: 300

Chapter 1 GENERAL INTRODUCTION

1.1 Origin of the cacao crop

The Amazon basin in South America and Mexico in Central America is described as cacao's (*Theobroma cacao* L.) centre of greatest genetic diversity (Bartley, 2005; Cheesman, 1944). The cacao crop thrives within 20°N and 20°S of the equator, an area known as the magic belt "20/20 Zone" (Figure 1.1). These areas have ample sunlight, rainfall, relative humidity and choice soil types which favour the cultivation of cacao (Wood and Lass, 1985). These factors explain why commercial cacao production has become concentrated in West Africa, South East Asia, Central and South America. *Theobroma* is a portmanteau of 'Theos' and 'Broma' in Greek that means 'the food of the gods'. *T. cacao* has been distinguished in several Mesoamerican languages with names including 'cacahuatl', 'kawkaw' and 'kagaw'. In West Africa, cacao is revered as the 'black gold' while the Europeans mostly know it as the 'chocolate tree'. Evidence of first patrons of cacao dates back to the Maya early in the century 400 BC (Rio Azul, Guatemala) and 600 BC (Colha, Belize) (Hurst *et al*., 2002). Here cacao beans were ground into fine powder, suspended in water and flavoured with spices and herbs as drinks. The Mayans, Toltec and Aztecs accorded the crop both sacred and monetary status at religious and ceremonial gatherings (Dillinger *et al*., 2000). A chronological report and the historical timeline of cacao are available at http://www.c-spot.com/atlas/historical-timeline/.

Today, the cacao bean is amongst the most sought after raw food materials. At least 62 countries in the world now produce cacao and the five major producers, ranked in terms of the volume of cacao beans produced, are Côte d'Ivoire, Indonesia, Ghana, Nigeria and Cameroon (FAOSTAT, 2015). These countries jointly account for over 75% of the world's cacao bean production output per annum (ICCO, May 2013). Though significant increases in cacao production is recorded in developing countries yearly (Aikpokpodion *et al*., 2009), huge importation of the beans (Figure 1.2) and apparent domestic cacao consumption (Figure 1.3) are greatest in the developed countries and this includes grindings, net imports of cocoa and

chocolate and chocolate products (cocoa processed into butter, paste, liquor, powder and cake). Net trade in chocolate and chocolate based products is converted to bean equivalents, based on general assumptions about the cocoa content of the chocolate products involved (ICCO, May 2013). An estimated 14 million people in the industrialized countries enjoy chocolate produced by 5.5 million smallholder cacao farmers in the developing countries who hardly ever get to experience the luxury of consuming chocolate (Olaiya *et al*., 2012). For these farmers, they have the raw cacao beans as their primary interest with an immediate marketable value.

Cacao beans are key to the production of chocolate, one of man's most sought after food sorts (Evans, 2007). Studies have demonstrated numerous health benefits associated with the consumption of chocolate which includes preventative and curative medicinal uses (Dillinger *et al*., 2000; Jalil and Ismail, 2008; Lippi, 2013). These benefits have been firmly linked to the crop's rich flavonoid profile (Badrie *et al*., 2015; Nabavi *et al*., 2015; Oracz *et al*., 2015).

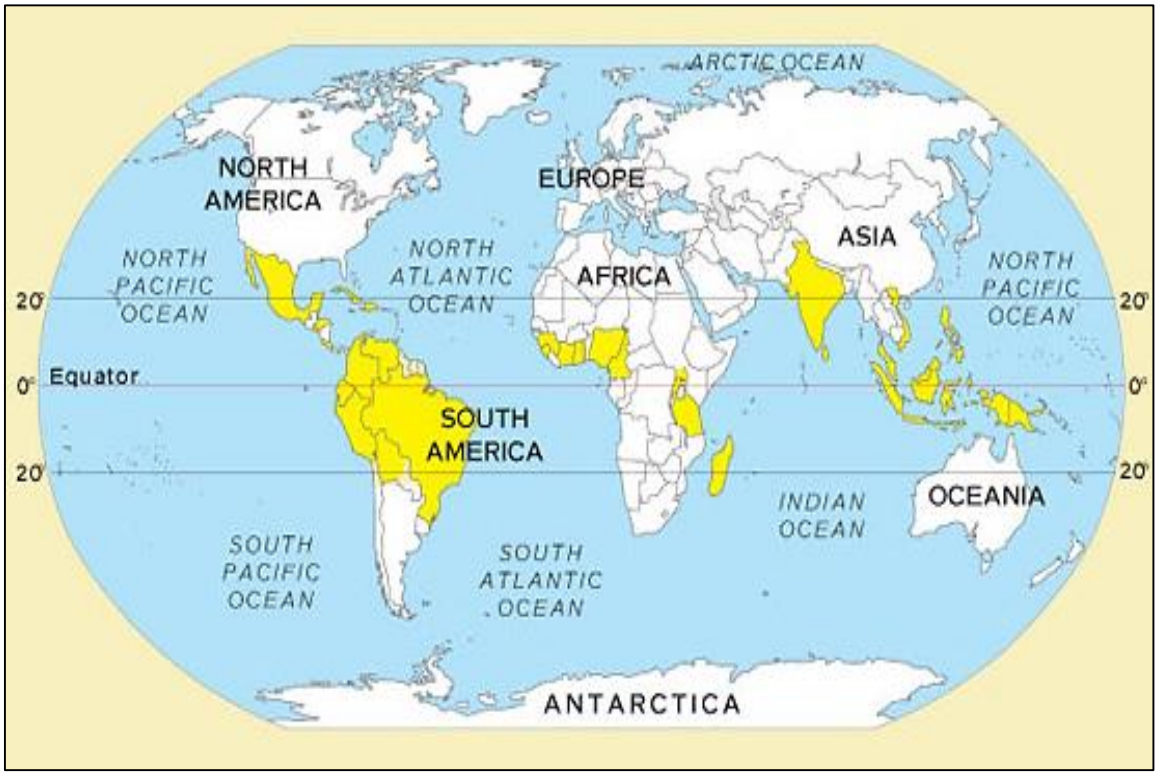

Figure 1.1: Map of the world showing the major cacao producing belt
Source: https://cacaonibs.wordpress.com/category/anique/ [Accessed on 3 August 2015].

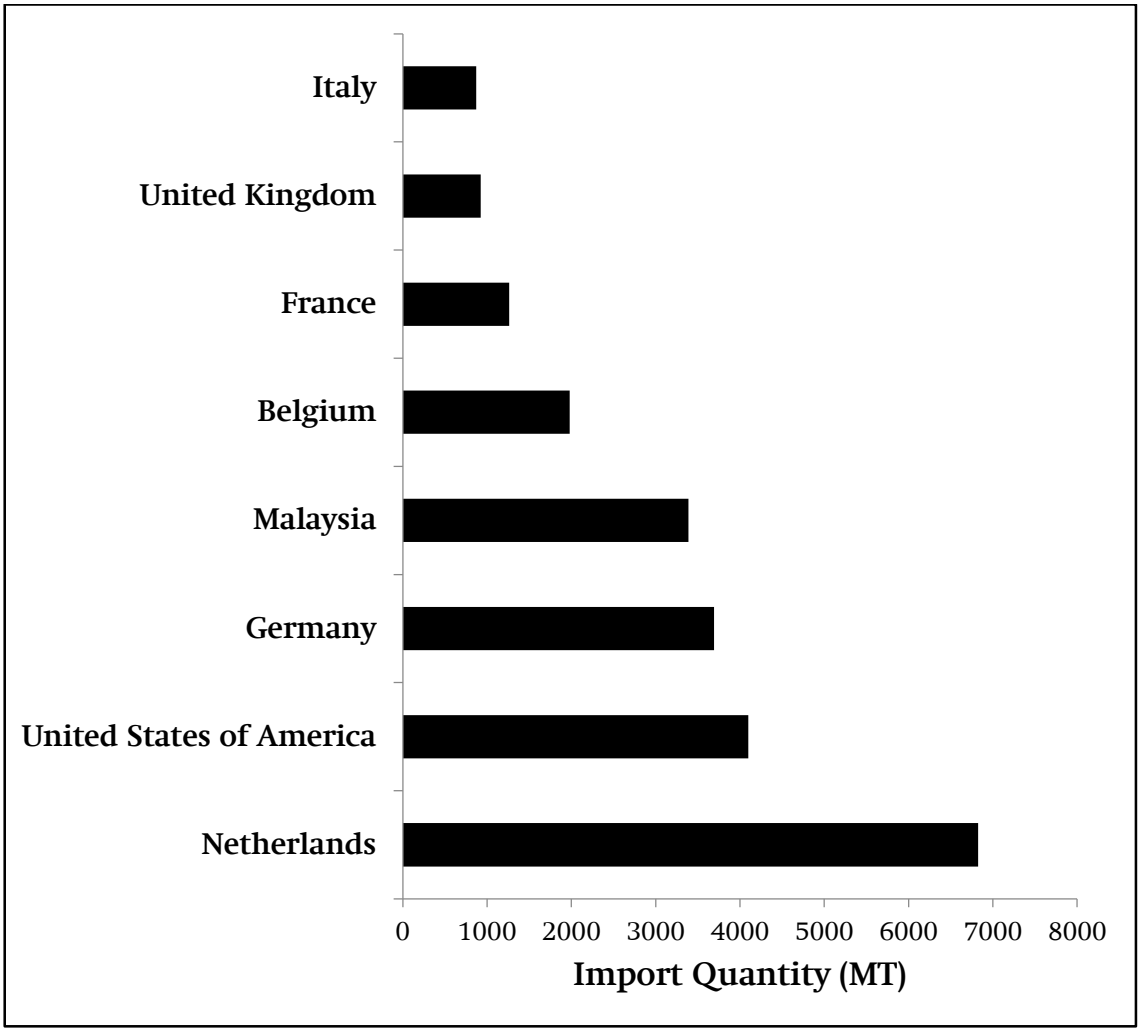

Figure 1.2: Major cacao bean importing countries (as at 2012) (FAOSTAT, 2015)
Source: http://faostat3.fao.org/download/T/TP/E [Accessed on 5 November 2015].

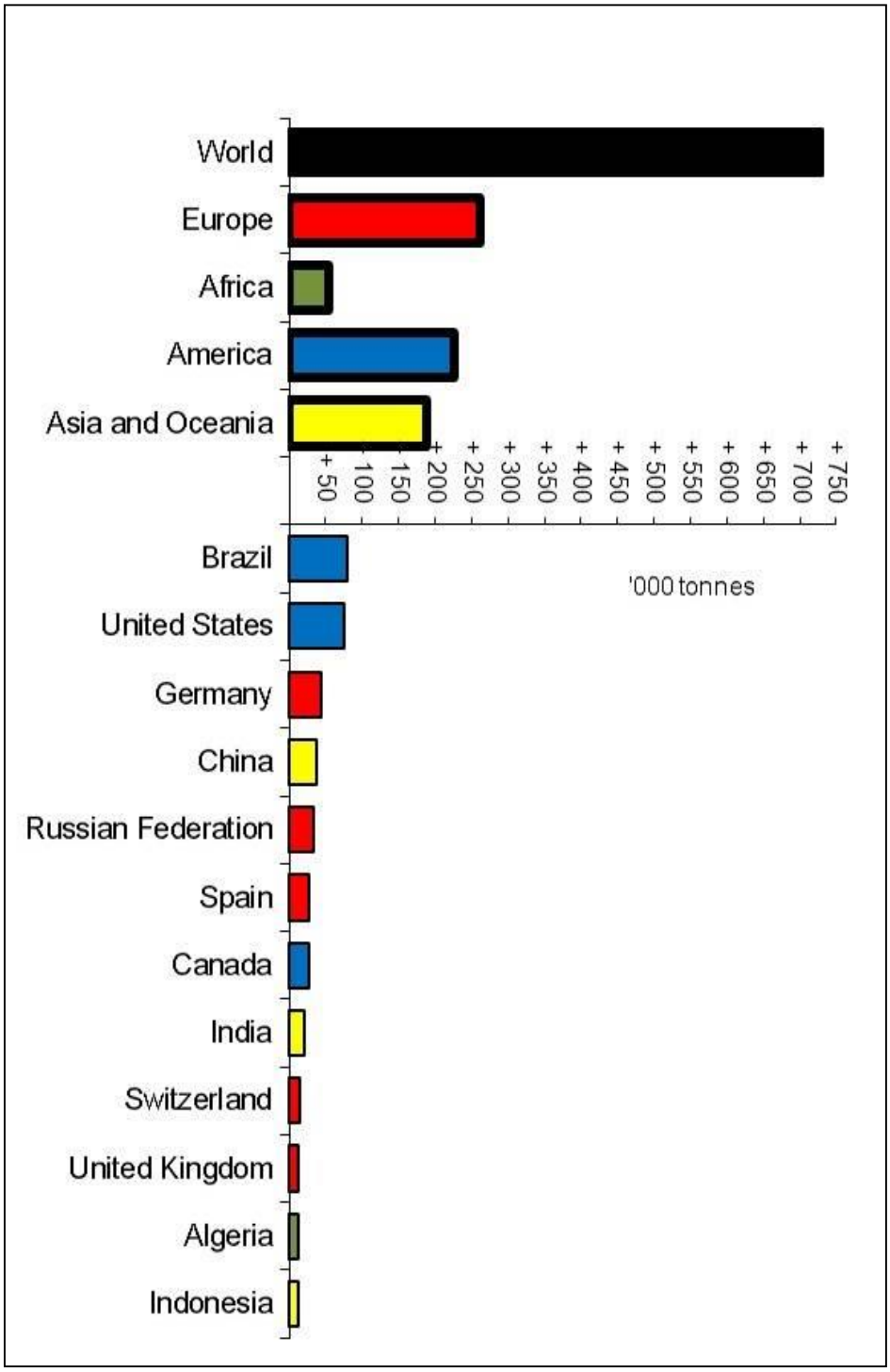

Figure 1.3: Global apparent domestic cacao consumption
Adapted from International Cocoa Organization (2012): World cacao consumption (bean equivalent) between 2002/2003 and 2010/2011.

1.2 Taxonomy

Theobroma cacao (L.) is a tropical perennial tree species. It belongs to the family *Malvaceae* (former *Sterculiaceae*) which, comprises 17 documented genera (Judd *et al*., 2002). The genus *Theobroma* is a close relative of the genus *Cola* (Cable and Cheek, 1998), which have other important tropical tree species like *Cola acuminata* (Schott & Endl.) and *C. nitida* (Schott & Endl.). There are 22 species of the genus *Theobroma* and *T. cacao* is the most widely cultivated of these (Wood and Lass, 1985). Other known species of the genus with some commercial value are *T. bicolor* (Humb. & Bonpl.), *T. speciosum* (Willd. ex Spreng.), *T. subincanum* (Mart.) and *T. grandiflorum* (Willd. ex Spreng.) K. Schum. (Hebbar *et al*., 2011; Zhang *et al*., 2011).

Sir Daniel Morris, a Jamaican, was able to class cacao into two major groups namely, Criollo and Forastero (Morris, 1882) which are still recognized to this day (Motamayor *et al*., 2008; Motamayor *et al*., 2003; Motamayor *et al*., 2002). The Forastero group has the largest number of cacao varieties including the Amelonado population which makes up 85% of the world's cacao germplasm (The Chocolate Society, 2010). Criollo is a relatively hardy cacao group characterised with a unique aroma and high-quality beans and accounts for about 3% of global cacao cultivation (Badrie *et al*., 2015; ICCO, 2012).

A third group, Trinitario, is a cross between Criollo and Forastero groups. This group contributes up to 12% of world's cacao cultivation (The Chocolate Society, 2010). Trinitario closely resembles the Forastero group (Toxopeus, 1985) and possesses a combination of high productivity, hardiness and an assortment of bean flavours inherited from both parents. Meanwhile, a recent study of the Trinitario using plastid genomics (chloroplast genomes) revealed that there could be a more complex hybrid origin of the Trinitario-type cacao conflicting with earlier evidence (Yang *et al*., 2013).

The robustness of cacao classification has, however, been challenged by the huge genetic diversity within *T. cacao*, suggesting the use of molecular marker techniques and fingerprinting for cacao classification (Dadzie *et al*., 2013; Guiltinan *et al*., 2008; Hebbar *et al*., 2011; Motamayor *et al*., 2008; Motamayor *et al*., 2002; Pugh *et al*., 2004; Sveinsson *et al*., 2013).

1.3 Genetics

Cacao is a diploid (2n = 2x = 20) species (Davie, 1935) with a genome size (~380 Mbp) (Figueira *et al*., 1992) almost thrice that of *Arabidopsis thaliana* (L.) (Argout *et al*., 2011; Lanaud *et al*., 1992). Based on their genetic origin a population of cacao could either be autogamous or allogamous.

1.4 Agronomy

Cacao grows as a relatively small, humid-tolerant and shade-loving understorey tree. When raised from seed, it grows into a mature evergreen tree in 2-3 years depending on the variety. The primary stem and the orthotropic shoots are arranged in a radial pattern, whereas the plagiotropic branches are monopodial and dorsiventral in orientation (Cuatrecasas, 1964). Cacao has a taproot system that could extend up to 2 m with a well-formed lateral root network. The cacao tree could attend a height of 6-12 m with 3-5 spreading branches (jorquette or fan) arising from the primary stem (Bartley, 2005). Erect suckers called the chupons sometimes arise from the main stem.

Mature cacao leaves are generally leathery, dark green and shiny in appearance and have an elliptical, obovate or an ovate shape with a total surface area of 140-280 cm^2. The tree produces cauliflorous small bright yellow or pale pink flowers throughout the year. The pollination of cacao flowers is naturally carried out by the *Forcipomyia* spp. midges (Glendinning, 1972; Young, 2007).

Up to 95% pollination failure occurs in cacao pollinated by midges (Lass, 1999); this has been identified to pose a significant problem with respect to pod bearing than general plant nutrition (Groeneveld *et al*., 2010). The use of hand-pollination markedly increases the success rates of pollination (Groeneveld *et al*., 2010; Valle *et al*., 1990) and subsequently, fertilization and mature fruits production in cacao.

Once the immature pods, cherelles, are formed on a cacao tree, a physiologically regulated mechanism in cacao naturally thins down the number of pod to nurture to maturity by wilting and mummifying some of the cherelles (McKelvie, 1956). Between 150 and 180 days after fertilization the remaining young pods mature into indehiscent drupes. The cherelles wilting process does not reduce yields in cacao (McKelvie, 1960). Ripe cacao pods contain an average of 30-40 cacao beans. The pods differ in shape (oblong, elliptical), colour (red, yellow, green, purple), and length (15-25 cm) depending on the variety.

1.5 Cacao production in West Africa: Nigeria

Early cacao production in Africa started in the islands of Sao Tome and Principe and Fernando Po (Bioko) in the North of Equatorial Guinea (Bartley, 2005). Cacao was introduced from Fernando Po into Nigeria in Bonny, Rivers State in 1874 by Chief Squiss Ibaningo (Are, 1969b; Opeke, 2005). Large scale cacao production started in the Western States of the country in 1887 and field trials were set up in Oyo with seedlings received from the old botanical garden at Lagos (Opeke, 1992).

Cacao production in Nigeria has had both economic and social impacts. Through the revenue accrued mainly from the exportation of cacao beans in the early 1960s, basic amenities and infrastructures (universities, hospitals, roads, and houses) were provided across the country. Cacao beans export was the primary source of income to the Nigerian economy before the discovery of crude oil in Nigeria in 1956.

Presently, cacao beans export is second to crude oil in its contribution to the country's export earnings and gross domestic product (GDP) (Aikpokpodion and Adeogun, 2011). In the ongoing Nigerian Agricultural Transformation Agenda which is directed at 'making agriculture the new oil', a renewed emphasis has been placed on agriculture as a business and specifically on cacao as a multi-billion dollar industry which could offer the country a sustainable alternative to oil in a diversified economy.

Nigeria makes a yearly contribution of 5-7% to the global total cacao production (ICCO, May 2013). As in other West African producing countries, Nigeria's cacao production is largely sustained by small-scale, fragmented (2-5 ha), family-owned cacao plantations. There are 14 out of the 36 States in Nigeria, where cacao is produced; the largest volume of cacao bean production coming from Ondo, Osun and Cross River States (Figure 1.4).

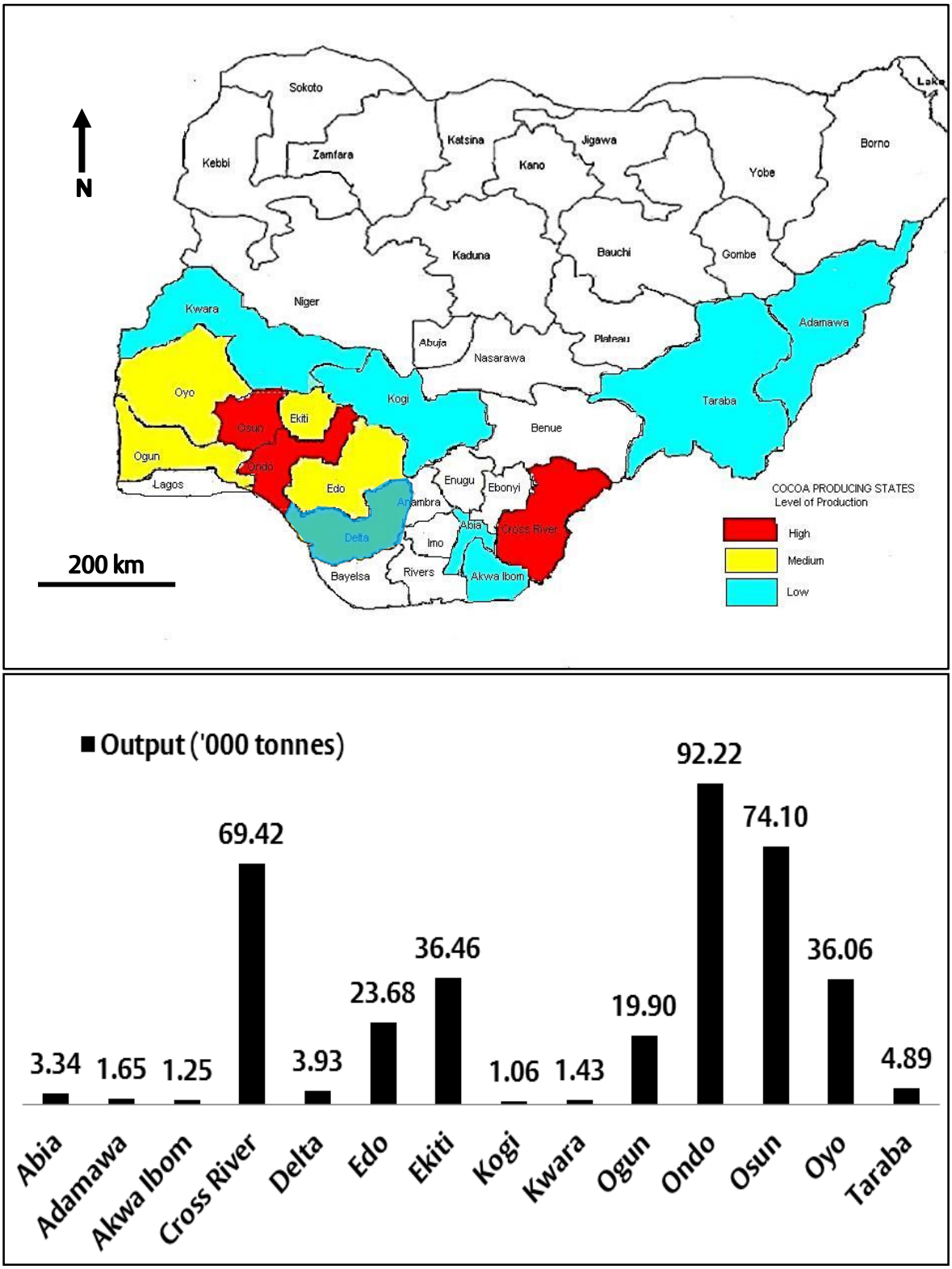

Figure 1.4: Nigeria's cacao bean (a) producing areas and (b) output by States (2011).

Source: National Bureau of Statistics, Nigeria in partnership with African Development Bank. (http://www.nigerianstat.gov.ng/) [5 November 2015].

Cacao beans extracted from harvested mature cacao pods are usually cured by fermentation. In Nigeria, cacao bean fermentation is mostly done in heaps and baskets lined and covered with leaves; sweat boxes are less frequently used. The fermentation activity could last for 5-8 days depending on the volume of beans and the required turning intervals. The fermented beans are sun-dried on raised flat surfaces, sometimes covered with mat materials. The cured beans are later sorted, bagged and ready to be marketed or sometimes further processed into grindings. Microflora presence, duration of fermentation and the method and temperature of the drying are key conditions needed to ensure an efficient fermentation and drying process (Lima *et al*., 2011). In addition to maintaining best farming practices, microbial fermentation is an important aspect in the processing of cacao beans (Saltini *et al*., 2013). It affects the final cacao beans quality (Lima *et al*., 2011; Schwan and Wheals, 2004), flavour and sensory characteristics in chocolate products (Afoakwa *et al*., 2008).

Cacao production is still a significant employer of labour for a huge number of rural farmers and enterprises in Nigeria and there have been increases in the production of cacao in Nigeria and rising annual revenue from cacao beans export (Figure 1.5).

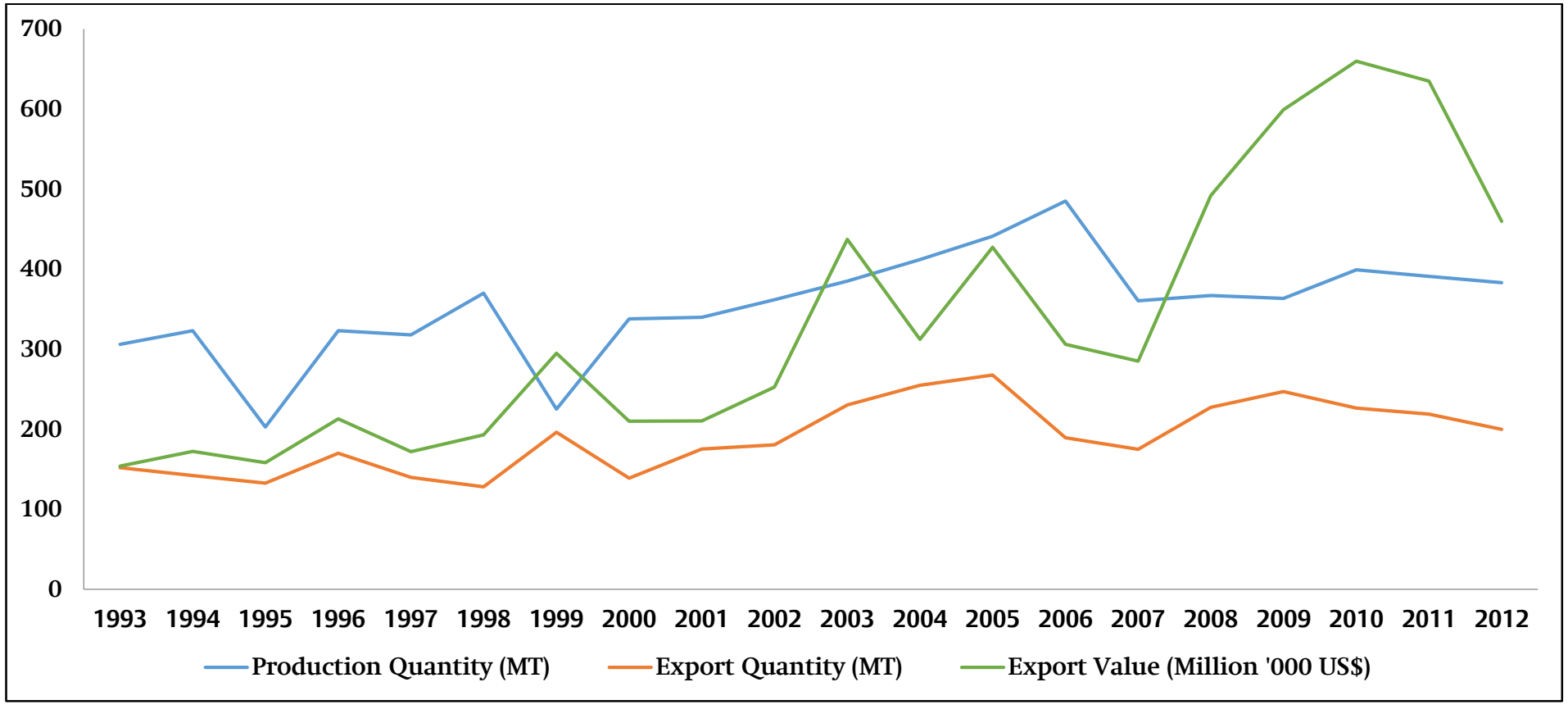

Figure 1.5: Nigerian cacao beans production, export and export value (from 1993 to 2012) (FAOSTAT, 2015).

1.6 Pests and diseases

The economic viability of cacao production faces great challenges from various pests and diseases (Hebbar, 2007; Ploetz, 2007). Global annual cacao yield reduction attributed to pests and diseases is 20-40% (Bowers *et al*., 2001; International Cocoa Organization, 2013; Ploetz, 2007). A common problem for cacao production in West Africa is the Cacao blackpod disease (CBD). CBD is caused by several fungi, especially *Phytophtora megakarya* (Brasier and M. J. Griffin), *Ph. palmivora* (Butler), *Ph. capsici* (Leonian), *Ph. citrophthora* (Smith and Smith) Leonian and *Ph. heveae* (Thompson) (Bowers *et al*., 2001; Kellam, 1986). There are other important field pests affecting cacao including capsids (*Miridae*) (*Distantiella theobroma* (Dist.) and *Sahlbergella singularis* (Hagl.), shoot-feeders (*Anomis leona* Shaus, *Earias biplaga* Wlk and *Sylepta retractalis* Hmps), shield bugs (*Bathycoelia thalassina* Herrich-Schaeffer), pod miners (*Mamara* species), root-feeding termites (*Macrotermes bellicosus* (Smeathman)), *Macrotermes natalensis* (Haviland), sap-sucking psyllids (*Mesohomotoma* (Tyora) *tessmani* Aulman), cacao thrips (*Selenothrips rubrocinctus* Giard) (DROPDATA, 2013), mistletoes (Olaiya *et al*., 2012), monkeys, rats, squirrels and birds. Post-harvest losses to cacao beans in storage are attributed to beetles and warehouse moths.

Cacao mottle leaf virus (CMLV), *Cacao necrosis virus* (CNV), *Cacao yellow mosaic virus* (CYMV) have less impact in terms of cacao yield losses in West Africa (Ollennu, 2001; Olunloyo, 2001; Thresh, 1960; Thresh and Tinsley, 1959) as opposed to the *Cacao swollen shoot virus* (CSSV). CSSV has been recognised as the cause of swollen shoot virus disease (CSSVD) in West Africa and for some time considered to be the most economically significant virus pathogen affecting cacao production in these areas (Dale, 1962). In CSSV infested cacao plantations, a 100% crop loss could be recorded in extreme cases of infection caused by a virulent strain of the virus and it is estimated that CSSV alone contributes over 25% loss to the annual global cacao production (DROPDATA, 2013).

The transmission and spread of CSSV in cacao are carried out by a number of mealybug species belonging to the scale insect group.

1.6.1 Mealybugs (Hemiptera: Coccoidea: Pseudococcidae)

Scale insects consist of over 8,000 species, 1,150 genera and 32 extant and 14 fossil families; they are ubiquitous, excluding the Arctic and Antarctic areas, could be polyphagous and/or cryptic (Kondo *et al*., 2008; Miller *et al*., 2014; Vea and Grimaldi, 2015). Many species are polyphagous and/or cryptic. On cacao, not less than 31 species of mealybugs have been reported to feed on the crop (Campbell, 1997). Diverse environmental conditions such as the presence of ants and shade have been found to affect mealybug population and abundance on cacao (Campbell, 1983, 1984, 1994). However, it is uncommon to find mealybugs on either ferns or mosses in spite of their widespread host range (Miller *et al*., 2014). Many species cause major problems in agricultural and ornamental ecosystems as there are commonly conveyed on plant materials. Their small size and pattern of feeding in hidden spaces eases their movement into new areas away from their native origins. If a mealybug species gets introduced and established in a new environment, it may eventually assume invasiveness, causing huge economic damage (Fand and Suroshe, 2015; Leimu *et al*., 2012; Miller *et al*., 2002).

1.6.1.1 General morphology

Mealybugs are typically small, flat and oval in shape (generally ≤5mm x ≤2mm) and morphologically characterisation is based on the adult female (Hardy, 2013; Kondo *et al*., 2008). They are characterized by the presence (or absence) of a single denticle on each leg, neotenic adult females, winged but non-feeding short-lived adult males (Thorold, 1975); with an unusual kind of lifecycle which typically involves a pre-pupa and pupa in the adult male. Usually, three to four instar stages occur in the paedomorphic females and up to five instars in the male.

At the adult stage the female mealybugs are morphologically distinct from males and could be mistaken for a different family (Kondo *et al*., 2008). Most scale insects (male and female) produce some kind of wax covering from specialized pores, which can vary from a mealy or powdery substance covering the body to elaborate waxy structures that are attached to the body or formed as domicile-like structures (Cox and Pearce, 1983). Female mealybugs have highly modified mouthparts called stylets which are absent in the alate males; these mean that the former may play a role as vectors of plant pathogens (Gray and Banerjee, 1999).

1.6.1.2 Taxonomy

Male and female mealybugs are genetically diploid (2n = 10), but the male is functionally haplodiploid and only transmits its maternal chromosome to the offspring after the elimination of the paternal heterochromatic chromosome (Brown and Nur, 1964; Nur, 1966, 1971). Mealybugs are grouped under the superfamily *Coccoidea* (Gullan and Cook, 2007; Kondo *et al*., 2008; Miller *et al*., 2014). The full generic taxonomic tree for mealybugs is given below.

Domain: Eukaryota
Kingdom: Metazoa
Phylum: Arthropoda
Subphylum: Uniramia
Class: Insecta
Order: Hemiptera
Suborder: Sternorrhyncha
Superfamily: Coccoidea
Family: Pseudococcidae

1.6.1.3 Biology: mealybug species under study

Three mealybug species (Hemiptera: Pseudococcidae), *Planococcus citri* (Risso), *Pseudococcus longispinus* (Targioni-Tozzetti) and *Ps. viburni* (Signoret), were initially selected for the present study. A brief overview of their biology and distribution (Figure 1.6) is given below.

1.6.1.3.1 *Planococcus citri* (Risso)

Pl. citri gained its common name, citrus mealybug, from its role as a significant pest of *Citrus* species. Though its actual origin remains speculative and debatable (Franco *et al*., 2004), *Pl. citri*

is a polyphagous species and now present in at least 183 countries worldwide (Figure 1.6); in temperate areas, it mostly occurs in glasshouses. Reproduction in hemipterans has been reported to occur both sexually by mating and asexually via facultative parthenogenesis (facultative haplodiploidy-thelytoky) (Normark, 2003), the latter having been reported to occur only in a single generation of unmated *Pl. citri* females (Padi (1995b). However, Borges da Silva *et al*. (2010) have proposed the existence of obligate amphimictic reproduction in *Pl. citri* while, Seabra *et al*. (2013) have reported the occurrence of polyandry in mealybugs using *Pl. citri* as a model species.

1.6.1.3.2 *Pseudococcus longispinus* (Targioni-Tozzetti)

Ps. longispinus is a cosmopolitan mealybug species with an Australian origin and is an important pest of apple and pear in New Zealand. It is polyphagous (Miller *et al*., 2002) with other hosts including citrus, taro, avocado, guava, eggplant and grapevine. It is widespread (Figure 1.6) but considered less of an important pest in some areas, for example the Mediterranean (Mendel *et al*., 1999). Adult females have on their posterior end waxy filamentous tails that can sometimes be twice as long as their vertical body length, thus the common name long-tailed mealybug. They reproduce sexually by mating (Waterworth *et al*., 2011b) and are reported to exhibit an absence of egg sacs; producing live crawlers (McKenzie, 1967).

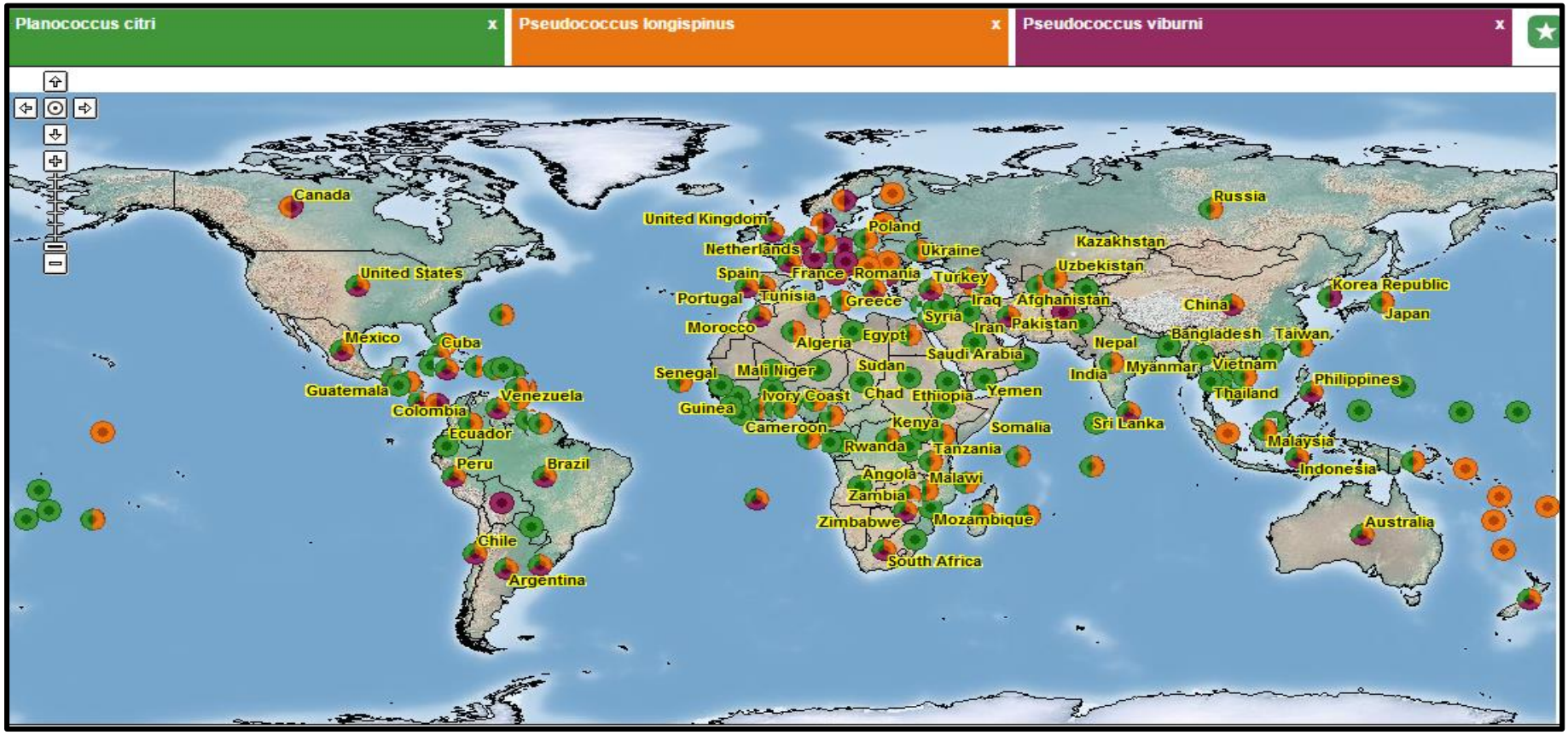

Figure 1.6: Global distribution of *Planococcus citri*, *Pseudococcus longispinus* and *Pseudococcus viburni*.
Compiled by the Plantwise knowledge bank based and reproduced by kind permission of CABI. ©CABI 2015.
http://www.plantwise.org/KnowledgeBank/Map/GLOBAL/Planococcus_citri/Pseudococcus_longispinus/Pseudococcus_viburni/

1.6.1.3.3 *Pseudococcus viburni* (Signoret)

Ps. viburni, commonly referred to as the obscure mealybug, is another polyphagous cosmopolitan mealybug species with a Nearctic (South American) origin (Ben-Dov and Hodgson, 1997; Miller *et al*., 2002; Williams and Granara de Willink, 1992). This is a non-afrotropical mealybug species (Figure 1.6) that has not been reported in cacao in the field in West Africa. They reproduce sexually and are shown to readily emerge from their cocoon to mate when exposed to pheromones as demonstrated by Waterworth *et al*. (2011b) in a comparison with *Pl. longispinus* and *Pl. ficus.*

1.6.2 *Cacao swollen shoot virus* (CSSV) and cacao swollen shoot virus disease

The *Cacao swollen shoot virus* (CSSV) is a bacilliform virus, 121 to 130 nm in length and up to 30 nm in diameter (Brunt *et al*., 1964) with a 7.2 - 7.5 kilo base pair (kbp) dsDNA genome (Lockhart *et al*., 1995; Lot *et al*., 1991; Uhde *et al*., 1993); with five putative functional open reading frames (ORFs) (Hagen *et al*., 1993). CSSV is a member of the *Caulimoviridae* virus family (Murphy *et al*., 1995) which comprises a total of eight genera: *Badnavirus*, *Caulimovirus*, *Cavemovirus*, *Orendovirus*, *Petuvirus*, *Solendovirus*, *Soymovirus* and *Tungrovirus* (King *et al*., 2012). A ninth genus, *Rosadnavirus*, was proposed following a phylogenetic analysis of the *Rose yellow vein virus* (RYVV) (Mollov *et al*., 2013). Viruses of the *Caulimoviridae* family are typical plant pararetroviruses in that they replicate their genomic DNA by an RNA intermediate transcription in the nucleus and a reverse transcription in the cytoplasm (Hohn and Rothnie, 2013; Medberry *et al*., 1990).

The natural host of *Badnaviruses* are mostly plants species of South Asian and Australasian origins (Borah *et al*., 2013; Lockhart and Olszewski, 1994). *Caulimoviridae* viruses of economic importance have been reported in a number of crops including *Banana streak virus* (Iskra-Caruana *et al*., 2014; James *et al*., 2011), *Pineapple bacilliform CO virus*-HI1 (Sether *et al*., 2012), *Sweet potato chlorotic stunt virus* (Cuellar *et al*., 2011) and *Dioscorea bacilliform virus* (Seal *et al*.,

2014). Endogenous pararetrovirus members of *Caulimoviridae* have also been reported in rice, tobacco and tomato (Geering *et al*., 2010), *Sugarcane bacilliform virus* (Karuppaiah *et al*., 2013; Lockhart, 1988), taro (Yang *et al*., 2003), citrus (Borah *et al*., 2013) and ornamentals (Eid and Pappu, 2013; van Regenmortel *et al*., 2000).

CSSV is one of the *Badnavirus* species that solely deleteriously affects cacao (King *et al*., 2012). The identification of CSSV as a serious impediment to cacao production occurred in the late 1930s in Ghana (Steven, 1936b). The Imperial Mycological Institute later adopted the name, 'swollen-shoot', after the description of the Cocoa swollen shoot virus disease (CSSVD) in cacao (Adegbola, 1971). CSSV is only naturally vectored by mealybugs of the family *Pseudococcidae* and is only endemic to West Africa (Posnette, 1941; Thresh, 1958; Thresh *et al*., 1988b). There have been no reports of this virus in the crop's centres of origin. CSSV currently causes an estimated annual loss of 50,000 tonnes of global cacao production (DROPDATA, 2013). Extreme cases of CSSV infection can result in drastic yield reductions (25-50%) and complete yield loss within 3-4 years (Crowdy and Posnette, 1947).

CSSVD presence was first reported in West Africa (Figure 1.7) in the Eastern region of Ghana in 1922 (Posnette and Strickland, 1948) and later described and distinguished from a similar disease, the dieback, by Steven (1936b, 1936a). Speculative reports on cacao virus (not confirmed to be CSSV) presence in non-West African countries have been made, for example in Sri Lanka (Peiris, 1953) and Indonesia (Turner, 1972). Though the primary host of CSSV remains the cacao tree, forest tree species of the family *Malvaceae* (previously separated as *Sterculiaceae*, *Malvaceae*, *Tiliaceae* and *Bombaceae*) have also been documented as alternative hosts for the virus (Posnette *et al*., 1950) and may well constitute the original reservoir of the virus prior to the introduction of cacao to West Africa (Posnette, 1981).

With the prevalence of cacao trees in many areas, these wild alternative host tree species are likely to have diminishing significance as inoculum reservoirs for the spread of the virus (Cooper and Jones, 2006). Most of these tree species are grown in close association with cacao trees (Todd, 1951) and in established plantations they may have previously served as shade trees to young cacao saplings.

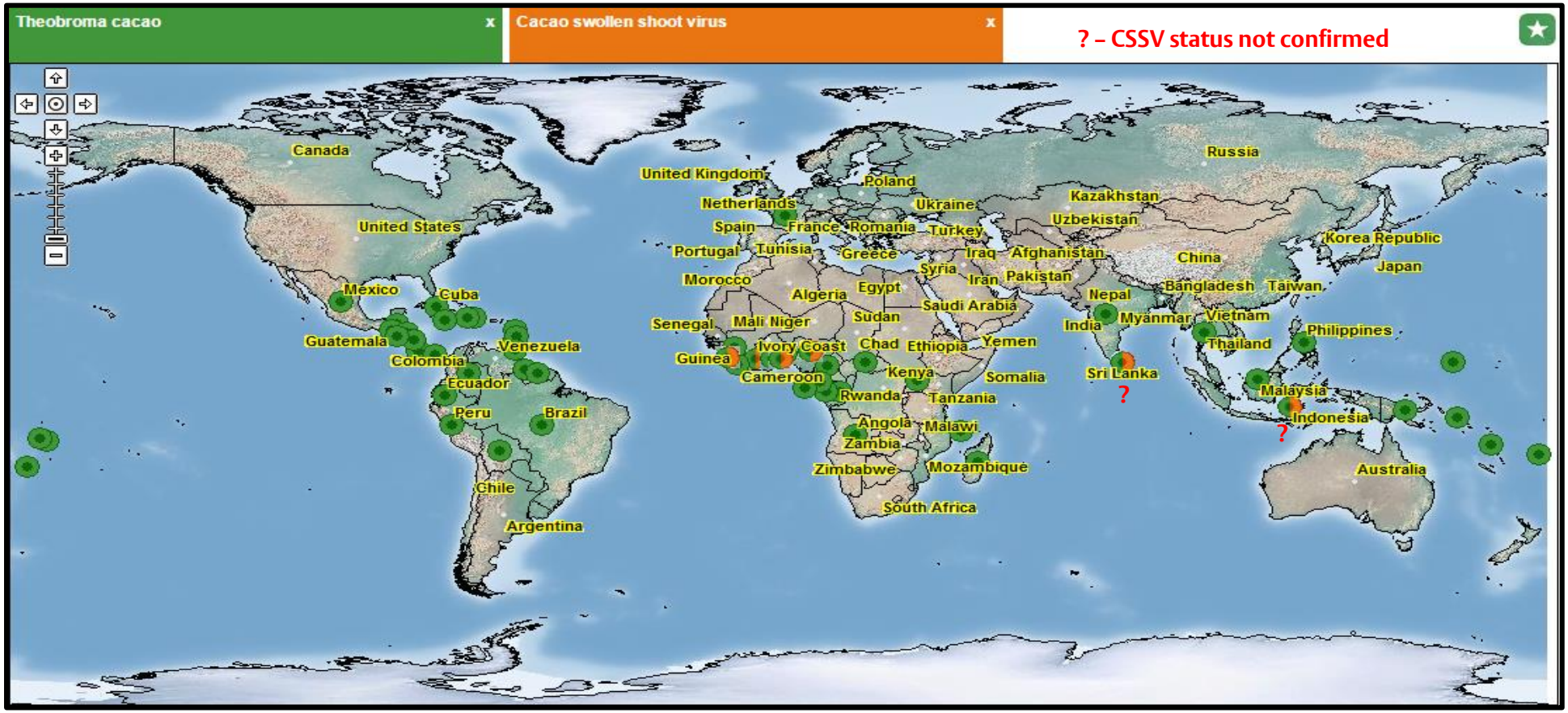

Figure 1.7: Reported CSSV presence in cacao growing areas of the world.
Compiled by the Plantwise knowledge bank and reproduced by kind permission of CABI. ©CABI 2015.
http://www.plantwise.org/KnowledgeBank/Map/GLOBAL/Theobroma_cacao/Cacao_swollen_shoot_virus/

1.6.2.1 Transmission of CSSV

In the field the transmission of CSSV between host plants is thought to be carried out by specific vector mealybug species (Box, 1945; Posnette, 1950a). CSSV could also be naturally transmitted from forest timber species like *Ceiba pentandra* (L.) Gaertn. , *Cola chlamydantha* K. Schum, *Co. gigantea* A. Chev., *Sterculia tragacantha* (Lindl.) and *Adansonia digitata* L. to cacao by the vector mealybugs species (Legg and Bonney, 1967; Posnette *et al*., 1950; Tinsley, 1971; Todd, 1951). Depending on the combined actions of wind and the mobility of vector mealybug species, CSSVD outbreaks could either have a jump-start or radial pattern of spread (Cornwell, 1960, 1958; Thresh, 1958; Thresh *et al*., 1988b). The viruliferous vector mealybug species could either be blown over long distances or crawl between contacting canopies of adjacent cacao trees. The slow spreading pattern of CSSVD makes it an exemplar 'crowd' disease (Thresh *et al*., 1988b; Vanderplank, 1949).

Under controlled conditions, mechanical transmission of the virus can be achieved through grafting and budding. The first mechanically transmitted CSSV inoculum (Brunt and Kenten, 1960) were sourced from the leaves of wild trees considered to be alternative host species for CSSV, *A. digitata* and *Bombax brevicuspe* Sprague. In a subsequent study, by using germinated and ungerminated Amelonado cacao beans as recipient tissues, Adomako and Owusu (1974) found that the concentration of CSSV inoculum and the total surface area of the infected tissues exposed to the inoculum could have an effect on the success of transmitting the virus mechanically. CSSV can also be transmitted via grafting and budding scion, bark 'patches' or buds from a previously infected seedlings to a healthy stock (Attafuah *et al*., 1963; Danquah, 2003; Posnette, 1940). The transmission of CSSV from an infected cacao tree to its seeds in the pod has raised concerns for international germplasm movements although no symptomatic expression of the pathogen has been observed via this route (Quainoo *et al*., 2008a).

Similarly, it was demonstrated that CSSV was not present in hybrid seeds obtained from crosses between virus-free (female parent) and CSSV-infected cacao trees (male parent) (Ameyaw *et al*., 2013).

1.6.2.2 Implication of mealybugs in CSSV transmission

CSSV transmission is naturally carried out by juvenile mealybugs and adult females. The female mealybugs are relatively sedentary and generally only move between neighbouring cacao trees with overlapping canopies. The nymphs are more readily carried by the wind over long distances (Cornwell, 1960). The acquisition and transmission of the virus by mealybugs occurs during their feeding activities. They feed at the phloem tissue level and their preference for vascular tissues is a characteristic shared with virus transmitting insects with piercing and sucking mouthparts (Stafford *et al*., 2012). Most of the CSSV vector mealybugs are reported to also transmit different isolates of the virus and could carry other viruses as well (Legg and Bonney, 1967; Posnette, 1950a).

Different species of vector mealybugs occur in an infested cacao tree (Bigger, 1981). *Formicococcus njalensis* Laing which has been reported to be the most common vector of CSSV in Ghana partly due to its high density of occurrence coupled with its low mobility rates in infested cacao trees while *Pl. citri* is the second most abundant vector mealybug species of CSSV (Bigger, 1981; Campbell, 1983). *Fo. njalensis* is regarded as a slow colonizing vector species of CSSV (Donald, 1955; Strickland, 1951). Bigger (1981) later opined that the total number of mealybug on infested cacao plots does not, however, give a true representation of its overall distribution within the trees. Estimates of mealybug species densities on cacao, therefore, should be interpreted with caution based on the sampling approach and technique used.

The presence of attendant ant species on cacao has been found to influence the population dynamics of vector mealybug species in concert with environmental factors such as shade and fertilizer application (Bigger, 1981, 1975; Campbell, 1983, 1984, 1990, 1994; Donald, 1955).

These attendant ants primarily feed on the honeydew secretions from the mealybugs while protecting the mealybug from other predatory species (Bigger, 1981; Campos and Camacho, 2014). However, they are not thought to be directly involved in the transmission of the viruses, although there are reports of their movement between host trees of potentially viruliferous mealybugs (Adenuga, 1975; Bigger, 1972).

1.6.2.3 Symptoms

Depending on the viral strain, a CSSV infected host plant can show banded chlorotic patterns on the veins and veinlets of young flushes, deformed cacao pods and swollen shoots and stems (chupons, fans or branches) (Steven, 1936b). At an advanced stage of the infection, an infected tree usually loses leaves extensively, typical of a dieback disease (Steven, 1936a). The expressed CSSVD symptoms can vary in severity and ease of detection within and between outbreaks in the field depending on the virulence of the virus strain, the genotype of the infected cacao and the duration of the latency period after infection (inoculation) (Dale, 1962). Often the observable leaf symptoms are either masked or mistaken for plant nutrient imbalances, for example Iron (Fe), Nitrogen (N) and Zinc (Zn) (Vlachopoulos *et al*., 2007) deficiencies. The visual identification of an asymptomatic cacao tree therefore, is a challenging task. This challenge has now being addressed with the use of high resolution molecular screening techniques.

1.6.2.4 CSSV detection

The most common approach for identifying CSSV presence in an infected host is through symptomatology (Naidu and Hughes, 2001). The virus is known to be serologically and genomically heterogeneous, producing varying degrees of symptoms on an infected cacao plant (Lockhart and Jones, 2000). However, the dependence on visual assessments to ascertain the presence of the virus is delimited where there are asymptomatic plants (Ollennu, 1989) and in plants showing nutrient deficiency symptoms.

Symptomatology requires great expertise, which is difficult to achieve and this tends to compromise the exclusive reliance on symptomatology as a primary approach for CSSV detection. Serological approaches for detection of CSSV presence in an infected cacao plant have been developed (Adomako, 1989; Adomako *et al*., 1983; Hoffmann *et al*., 1999; Hughes *et al*., 1995; Hughes and Ollennu, 1993).

An early attempt using an antisera raised against isolates of CSSV was performed by Kenten and Legg (1971). The use of enzyme-linked immunosorbent assay (ELISA) introduced by Clark and Adams (1977) later found great application in CSSV detection and diagnostic studies with higher sensitivity by addressing the limitations of low plant virus titre, incompatible particle morphology and the presence of inhibitors (inactivators). The combination of immunosorbent electron microscopy (ISEM) and ELISA further enabled the detection of additional isolates of CSSV (Adomako *et al*., 1983; Sagemann *et al*., 1983) and the results obtained from the ISEM and ELISA techniques showed a significant relationship (Sagemann *et al*., 1985).

Though ELISA was useful as a laboratory-based approach for the detection of CSSV, the subsequent use of virobacterial agglutination (VBA) test was aimed at detecting the presence of the virus in trees with latent infection in the field (Hughes and Ollennu, 1993). In practice, the sensitivity and reliability of these serological approaches were prone to failure with leaf tissues obtained from asymptomatic cacao trees. The use of enzyme-directed nucleic acid probes and a variety of the polymerase chain reaction (PCR) techniques (Saiki *et al*., 1988) have revolutionized molecular research and diagnostic studies with plant viruses (Dietzgen, 2002; Henson and French, 1993).

The PCR technique involves greatly enhanced sensitivity owing to its power to exponentially amplify small amounts of specific target DNA sequences *in vitro*, ranging from 100 to 10,000 times the power of ELISA (Dietzgen, 2002; Hoffmann *et al*., 1997).

Such diagnostic strength is useful for a high resolution detection, quantification and discrimination of closely related viral genomic DNA (Fox and Narra, 2006). PCR-based CSSV diagnosis began at the Cacao Research Institute of Ghana (CRIG), in the early 1990s (Sackey *et al*., 1995) following the first complete DNA sequence and organization of the CSSV genome (Hagen *et al*., 1993). PCR based screening is not however a panacea with drawbacks that can include both false positive and false negative results.

1.6.2.5 CSSV/CSSVD control

The removal of infected cacao trees and other putative hosts of the virus have been widely considered in the control of CSSV/CSSVD. A restriction in the movement of the vector mealybug species in addition to host-resistance breeding have also been explored as measures to control the spread of the disease. An eradication method was used for the control of CSSV spread (Lister and Thresh, 1957; Thresh, 1959) based on the original symptoms of the disease (Steven, 1936b). By surveys and inspections, eventually millions of CSSV infected cacao trees and the neighbouring and surrounding non-symptomatic cacao were removed in Ghana in a programme that began in 1940 (Posnette, 1943).

The CSSVD eradication campaign initiated in 1940 in Ghana eventually saw the removal of over 200 million trees resulting in huge economic burdens on the cacao farmers. Coppicing of contacts and complete removal of CSSV infected trees, was later introduced as a means of capturing new CSSVD outbreaks in cacao trees which were initially at their latent stages of infection while also encouraging the rehabilitation of already affected cacao farms (Are and Jacob, 1969; Legg *et al*., 1984; Thresh and Lister, 1960). Later other West African countries also adopted the 'cutting-out' strategy, for example in Nigeria in 1946 (Allnutt, 1953b; Johns and Gibberd, 1950; Thresh, 1960).

The combined use of pesticides to control the spread of the vector mealybug species in Ghana (Hanna *et al*., 1952; Hanna and Nicol, 1954) and its attendant ant species (Hanna *et al*., 1956) was expected to be a more effective approach (Hanna and Heatherington, 1957). Nonetheless, the ban of organophosphate pesticides use on cacao due to the associated health, food contamination and environmental concerns, not to mention the high cost associated with acquiring and applying pesticides, all contributed to the abandonment of the approach.

The planting of non-host trees of CSSV, for example citrus, coffee and oil palm, round cacao plantations could function as natural barriers against the movement and spread of viruliferous vector mealybug species (Ollennu *et al*., 1989). These tree species could create a *cordon sanitaire* expected to purge mealybug species carrying the virus as they settle to feed before moving onto CSSV-free hosts. This approach which has yet to be applied on a large scale in the field is based on the expectation that the loss of infectivity by the vector mealybug species would occur as they cross these boundaries.

Different strains of CSSV induce varying symptoms of CSSVD (Posnette, 1941, 1947b; Sagemann *et al*., 1985). The prevention of a susceptible host from becoming infected by a severe strain of a virus may occur if the host is already infected by a similar or closely related mild strain of the virus (Gal-On and Shiboleth, 2006; Zhou and Zhou, 2012). This concept has been exploited in CSSV control (Crowdy and Posnette, 1947; Hughes and Ollennu, 1994; Ollennu *et al*., 1996; Posnette and Todd, 1951). From the results, the success of using cross-protection greatly depends on the use of a mild strain showing a close serological relationship with the severe strain. Concerns associated with this approach include the possibility of infecting a non-target crop species and the later mutation of the mild virus strain into a more virulent strain.

The use of chemical and heat treatment therapies were attempted as crude measures to sanitise cacao planting materials considered being at risk of CSSV infection. For example, the immersion of infected cacao budwoods in hot water at relatively high temperatures (45-52 °C), raising of infected cacao seedlings in a heated growth chamber (38 °C) for a month (Lister and Thresh, 1956) and treating infected cacao seeds with organic chemicals before seeding (Holden, 1957). In recent developments, the application of somatic embryogenesis and cryotherapy techniques have been found to be effective in eliminating CSSV from secondary somatic embryos thereby potentially facilitating the exchange of disease-free clonal planting materials (Fang *et al*., 2004; Quainoo *et al*., 2008b).

The removal of CSSV infected cacao trees followed by the planting of healthy hybrid genotypes, selected either for their putative resistance or tolerance to CSSV infection, could be an effective means to control further spread of CSSVD (Adu-Ampomah *et al*., 1996). In order to facilitate the anticipated lasting solutions of breeding for resistance to CSSV in cacao, a comprehensive exploitation of the available cacao germplasm coupled with a detailed understanding of the biology of the vector mealybug species and concerted studies on the evolving molecular diversity of the virus across the West African cacao growing countries will be crucial.

1.7 CSSV/CSSVD in Nigeria

Klinkowski (1970) has described CSSVD as a catastrophic disease affecting cacao in West Africa. Following the introduction of cacao in West Africa, the vast majority of cacao plantations cultivated the Amelonado cacao types as their choice cultivar (Thresh, 2006). The Amelonado cultivar was highly susceptible to CSSV owing to the fact that at some point in Ghana and the rest of West Africa it was grown as the main cultivar; with its markedly uniform reproductive and vegetative characteristics, these subsequently paved the way for the wide spread presence of CSSVD in these areas (Thresh *et al*., 1988a).

The presence of CSSV in Nigerian cacao was reported in 1944 (Lister and Thresh, 1957). CSSV was found in the Western parts of the country in Egbeda, Abaku, Olanla, Offa-Igbo, Balogun, Ife, Ibadan, Ilaro, Araromi and Ikire (Lister, 1953; Lister and Thresh, 1957; Longworth and Thresh, 1963; Thresh, 1961). The infected cacao trees showed transient, mild and severe symptoms, which were attributed to the different CSSV isolates that were found in Nigeria (Thresh, 1961).

The 'cutting-out' policy introduced in Ghana in 1940 was subsequently implemented in Nigeria in February 1946. With this practice, the number of asymptomatic trees per symptomatic tree removed in Nigeria were more than was the case in Ghana (Lister and Thresh, 1957). However, this policy in Nigeria proved to be less effective partly because the CSSV strains in Nigeria were allegedly less virulent than those reported in Ghana and the missing-out of infected but asymptomatic cacao trees was supposedly widespread. The average ratio of symptomatic to asymptomatic trees destroyed in Nigeria was 1:10 (Thresh, 1959). Between 1946 and 1949, Nigerian cacao farmers expressed their outright rejection of the 'cutting-out' policy. Since the proposed complete removal of symptomatic CSSV infected trees was greeted with strong disapproval by Nigerian farmers, it was discontinued in June 1950 (Allnutt, 1953a).

As an alternative to the 'cutting-out' policy in areas with a small radius of infection, areas of mass infection in Nigeria were identified, demarcated and 'blocked-out' or 'sealed-off' with a clear-felled *cordon sanitaire* in an attempt to prevent the spread of the virus (Allnutt, 1953a; Lister, 1953). The 'sealing-off' policy was adopted in Nigeria in July 1950 to enable the control of CSSVD within an isolated area. The cacao emergency scheme was then established in 1950 which included a permanent cacao survey team that was directly saddled with the responsibility of controlling CSSVD by rehabilitating trees in affected areas (Johns and Gibberd, 1950). The problem then was the farmers' unwillingness to invite the team to inspect their farms.

The few farmers that responded did so only for the financial compensations and not for the intended objective of controlling the spread of the disease (Are, 1969b, 1969a; Are and Jacob, 1969). Perhaps as a consequence the development of new outbreaks and foci of infection from asymptomatic trees within the protected belt became an emerging issue. To reveal latent infection in asymptomatic cacao trees, coppicing was introduced in Nigeria as a CSSVD control strategy.

Depending on the size of the outbreak, symptomatic trees and neighbouring trees at a radius of 27m were coppiced (Thresh and Lister, 1960). The incidence of new disease outbreaks was assessed after a 12-month period following the regeneration of the coppiced trees. Still in the quest to tackle CSSVD in Nigeria, a CSSVD resistant breeding programme was attempted. Whereas general breeding activities on cacao in Nigeria started in 1912, breeding for CSSV resistance in the crop commenced in 1959 (Atanda, 1973; Badaru *et al*., 1996) albeit with no positive results to date. In all the attempts put together in the past to control the spread of the disease in Nigeria and West Africa as a whole, no lasting solution has emerged. This failure is inevitably at least in part due to the paucity of in-depth information on the behaviour of the virus and its vector mealybug species.

1.7.1 Challenges

Nigeria is dominantly an agrarian economy, with an average of 28.56% contribution to its yearly GDP coming from agriculture (1990 – 2013) (World Development Indicators, 2015); the agricultural sector remains the country's main employer of labour. Despite this, less than 2% of the total annual budget is typically allocated to the sector. For example, 1.7% (₦81.41billion) was allocated in 2013, an insignificant increase from the 1.66% (₦78.98billion) that was allocated in 2012 (National Association of Nigerian Traders, 2013). In addition, inconsistent administrative policies in Nigeria's research institutes are also a challenging issue.

Frequent industrial action and strikes at some of these institutes effectively truncates cacao research projects and programmes at institutes such as Cacao Research Institute of Nigeria (CRIN). On the other hand, the farmers have not been adequately enlightened by extension agents and encouraged to actively participate in on-going research conducted by CRIN thus justifying the farmers' laxity to cooperate in such studies. In making a specific case for CSSVD control in Nigeria, in the 1940s, it was not an easy task convincing Nigerian cacao farmers to destroy CSSV infected cacao trees as a means of eradicating the spread of disease. This problem still persists today. Farmers show reluctance in cooperating with researchers, partly because they do not perceive an immediate effect of the disease on their cacao crop, unlike other wide spread, high-profile diseases like blackpod.

Another restriction on effective CSSVD control in Nigeria is the limited number of recent national reports and publications on the disease which is a direct reflection of the extent of research studies carried out by relevant institutes such as CRIN and the International Institute of Tropical Agriculture (IITA) and agriculture-based universities. Also, while in Ghana where there is a dedicated Cacao Swollen Shoot Virus Disease Control Unit (CSSVDCU) of the Ghana Cacao Board solely involved in CSSV/CSSVD research alongside the Cacao Research Institute of Ghana (CRIG), there is no such equivalent designated establishment under CRIN. These factors indicate that as the impact of CSSV in the Nigerian cacao crop is set to expand, committed research and associated funding are needed. Recent surveys in combination with molecular-based evidence have shown that there is a widespread presence of CSSV in Ghana, Cote d'Ivoire and Togo despite several decades of control efforts in West Africa (Muller *et al*., 2014). There have, however, been no recent molecular-based studies or reports from Nigeria on the presence of the virus and its vector mealybug species. Therefore, a renewed effort in CSSV research in Nigeria, starting by surveying the health of the country's cacao, is of utmost importance.

1.7.2 Aims of the current study

The first part of this study (Chapter 1) aimed at presenting the current status of CSSV and mealybugs presence on cacao in selected major cacao-growing areas in Nigeria. The general materials and methods used in the study were reported in Chapter 2 and the molecular diversity study of CSSV and its vectors in the selected major cacao-growing areas in Nigeria is presented in Chapter 3. The subsequent part of the study (Chapters 4 and 5) aimed at understanding 'Cacao-CSSV-Mealybug' interactions. In Chapter 6, general discussions, conclusions and suggestions were made based on the results and findings in the present study.

The specific objectives of the current study were:

1. To survey and map the geographic distribution of the *Cacao swollen shoot virus* pathogen in the key cacao producing States in Nigeria.
2. To identify mealybug species occurring on Nigerian cacao.
3. To quantify the retention and transmission efficiencies of CSSV by putative CSSV vector mealybug species.
4. To characterise the feeding behaviour of mealybug putative CSSV vector species on cacao.

Chapter 2 General Materials and Methods

2.1 Introduction

The studies were carried out following standard operating conditions and procedures. Appropriate training on health and safety, quarantine facility use guidelines, alongside risk assessment evaluations was received. Modification and optimization of existing protocols and techniques were carried out where necessary. Suitable personal protective equipment (PPE) were worn in all working environments. All reagents were supplied by Sigma-Aldrich Co. LLC (Dorset, UK) unless otherwise specified. This section gives an account of all the generic steps during the laboratory and glasshouse experiments.

2.2 Plant materials

CSSV-infected cacao seedlings were used as the source of CSSV inoculum. The test plants were CSSV-free cacao seedlings (var. Amelonado) raised at the facility of the International Cocoa Quarantine Centre, Reading.

2.2.1 CSSV-infected cacao seedlings

Cacao seeds (var. Amelonado) already infected with the severe strain of CSSV, New Juaben, were obtained from Cacao Research Institute Ghana (GRIG), Tafo. Inoculation of seeds with CSSV was achieved by placing viruliferous mealybugs which had been previously fed on CSSV-infected plants onto the seeds after the testa had been removed. The cacao seeds were then sent to Reading following appropriate quarantine procedures. These were sown in compost (75%) - vermiculite (25%) mix in lightweight standard seed trays (34.4 cm x 21.4 cm x 5.2 cm). The growing medium was adequately wetted and maintained inside seedbed tunnels (25 ± 2°C, 60 - 65% RH) with automated watering and heating systems.

Two weeks after germination, successfully emerged seedlings were carefully removed into individual plastic plant pots (14 cm x 12 cm) (Figure 2.1). Water-based fertilizer supplement, Sangral 3:1:1 (William Sinclair Holdings Plc, Lincoln, UK) were supplied at intervals of four weeks.

2.2.2 CSSV-free cacao seedlings

Ripe cacao pods (var. Amelonado) were collected from the International Cacao Quarantine Centre (ICQC), Reading. The seeds were extracted and raised under the same conditions described in Section 2.2.1 in a separate section of the glasshouse.

Figure 2.1: CSSV inoculated cacao seeds at (a) 7 and (b) 30 days after sowing in individual plastic pots.

2.3 Screening of plant materials

To test for the presence of the viral DNA of CSSV in the cacao leaves, genomic DNA was first extracted from the leaves; the steps of which are described below.

2.3.1 Cacao DNA extractions and quantification

DNA extractions from cacao leaves were performed using the QIAGEN DNeasy Plant Maxi (24) (Cat. No.: 68163) and Mini (50) kits (Cat. No.:69104). Cacao leaf disc sections measuring 5 mm in diameter (80-100 mg in weight) were sampled and frozen (-20°C) in 1.5 ml Eppendorf tubes (MCT-150-C, Axygen, Inc., USA); each tube containing one 3 mm acid-washed tungsten carbide bead (QIAGEN, Cat. No.: 69997). The samples were loaded onto TissueLyser II adapters and flash frozen with liquid nitrogen (LN) for 20 - 30 s. The samples and beads inside the tube were placed inside adapters and secured in position onto the TissueLyser II (QIAGEN Cat. No.: 85300, Manchester, UK) for disruption. Disruption of the samples took 2-3 cycles, each cycle lasting for 30 s at 30Hz. The cycles were repeated accordingly and as quickly as possible until a finely-ground homogenous sample was obtained in each of the tubes.

The original protocols for DNA extractions were optimized for cacao leaf tissues lysis to improve DNA yields and concentrations as follows. Appropriate volumes of 100% ethanol were added to AW1 and AW2 buffer. 400 µl of pre-heated (65°C) buffer AP1 was added into each of the tubes with the disrupted sample. This was followed by the addition of 4 µl RNase and 4 µl Proteinase K (1 $mgml^{-1}$) (Sigma-Aldrich) The suspension was briefly vortexed on Ika® Vortex Genius 3 (Sigma-Aldrich) then loaded onto Eppendorf ThermoMixer®C (Eppendorf, Hamburg, Germany) fitted with a 1.5 µl SmartBlock™ for incubation at 60°C on 500 rpm for 10 min. A 130 µl aliquot of buffer P3 was added to the sample, mixed and incubated on ice for 6 min. The lysate was centrifuged on Eppendorf centrifuge 5424 with 24 × 1.5/2.0 ml aerosol-tight rotor (Eppendorf, Hamburg, Germany) at ≥ 14, 000 rpm for 5 min.

The sample and tube were carefully and immediately transferred into a microtube bench rack. The supernatant was pipetted into a QIAshredder spin column placed in a 2 ml collection tube (provided in the kit) and centrifuged for 2 min ≥ 14, 000 rpm. The flow-through was transferred into a new 2 ml tube without disturbing the pellet at the bottom of the tube, if formed. In each tube, the volume was noted and 1.5 volumes of pre-heated (65°C) buffer AW1 was added and mixed by pipetting for 5 – 20 s. The first 650 µl of the mixture was transferred into a DNeasy Mini Spin column placed in a 2 ml collection tube. This was centrifuged (≥14, 000 rpm) for 1 min.

The flow-through was discarded and the process was repeated with the remaining volume of the mixture. The flow-through from the second (final) centrifugation was also discarded with the collection tube. The spin column was placed in a new 2 ml collection tube; with the addition of 500 µl buffer AW2, this was centrifuged (≥14, 000 rpm) for 1 min. The flow-through was discarded and another 500 µl buffer AW2 was added to the spin column, centrifuged (≥14, 000 rpm) for 2 min. The spin column was carefully transferred, avoiding contact with the flow-through, into a new (autoclaved) 1.5 ml Eppendorf tube and centrifuged for 1 min to completely dry the column of the remaining solution before finally transferring the spin column into a new 1.5 ml Eppendorf tube. Where the previous step was not possible, the spin columns were opened and air dried at room temperature for 2 min. Buffer AE was used for the elution of DNA.

Where a final volume of 150 µl DNA was the target, 75 µl buffer AE was added, on two separate sessions, on to the centre of the spin column and incubated at room temperature for 10 min and 5 min on the first and second sessions, respectively. The column was centrifuged (≥14, 000 rpm) for 1 min for DNA collection in each session.

Concentrations of the extracted DNA samples were determined using a NanoDrop 2000 ver. 1.4.2 (Thermo Fisher Scientific, Wilmington, USA) spectrophotometer using 1.5 – 2 µl aliquots of the final DNA volume collected; 260 nm and 280 nm absorbance ratios were used as an assessment of the purity of DNA. The 260 nm and 230 nm absorbance ratios served as a secondary measure for DNA purity, giving an indication of the extent of contaminants present in the extracted DNA.

2.3.2 Cacao RNA extractions and quantification

The QIAGEN RNeasy extraction kit and protocol (Cat No.: 74104) was used for the extraction of RNA from CSSV infected/inoculated cacao leaves. Due to the need to modify the RNA extraction lysis buffer to suit RNA extraction from cacao leaves, the modified lysis buffer (50 ml volume) was prepared using 2% polyvinylpyrrolidone (PVP) (1.0g), 100mM Tris-HCl (pH 8.1, 5.0ml of 1M solution), 25mM ethylenedinitrilotetraacetic acid (EDTA) (2.5 ml of 0.5M solution), 2M NaCl (5.84 g), 0.05% Spermidine trihydrochloride ≥98% (TLC) (0.025 g), 2% Cetyltrimethylammonium bromide (CTAB) (1.0 g) and reverse osmosis (RO) H_2O. 20 ml RO H_2O was added into a 50 ml beaker followed by PVP. The solution was stirred for 5 – 10 min on a hotplate (set at 0°C) with a magnetic stirrer (Stuart, model UC152: Bibby Scientific Limited, Staffordshire, UK) at a moderate speed (set at 6). In a separate 25ml beaker, Tris-HCl and EDTA were added and stirred to mix while adding NaCl. Spermidine trihydrochloride ≥98% (TLC) and CTAB were also added in the process. The entire mixture was stirred to completely dissolve all the components added. In the end, the homogeneous solution in the 25 ml beaker was added to the previous solution in the 50 ml beaker, made up to 50 ml with H_2O and stirred to mix using the magnetic stirrer for 3 – 5 min. The final 50 ml RNA extraction lysis buffer was autoclaved at 121°C and later stored in room condition. RNA extractions from cacao leaves were done in two parts involving an overnight incubation lysis step and RNA purification step with modified QIAGEN protocol.

2.3.2.1 Cacao leaf sampling, preparation, lysis and RNA extraction

Cacao leaf samples for RNA extraction were sampled, placed either in 1.5 ml Eppendorf tube - for leaf disc or 15 ml centrifuge tube - for whole leaf, labelled and quickly frozen in liquid nitrogen (LN) inside a small laboratory Dewar. For an immediate RNA extraction, the leaf samples were processed without storage. Otherwise, the samples were stored in the freezer at – 80°C.

The lysis buffer was warmed up to 65°C in a water bath before use. 8M LiCl was placed on ice to cool. Each frozen whole cacao leaf was sampled, weighed (≤100mg) and ground in LN with a separate autoclaved pestle and mortar then emptied into a 1.5 ml Eppendorf tube. In the case of leaf disc initially sampled in 1.5 Eppendorf tubes, the samples were disrupted following the steps described in Section 2.3.1. In the fume hood, 600 µl of the lysis buffer and 12 µL of pre-heated 2% β-Mercapthoethanol were successively added to the ground sample and vortexed before incubation on a ThermoMixer®C at 65°C at 400 rpm for 20 min.

Still in the fume hood, 650 µl of chloroform: 3-methylbutanol (24:1) mixture was added to the sample, vigorously vortexed to form an emulsion and centrifuged for 10 min at 14,000 rpm. The supernatant was transferred into a new tube and the addition of 650 µl of chloroform: 3-Methylbutanol mixture was repeated in the fume hood. At this point, the supernatant was transferred into a new tube before adding 1/3 volume of the already cooled 8M LiCl. The mixture was gently inverted to mix and subsequently incubated on ice before leaving it overnight in the refrigerator at 4°C.

The sample was placed in a pre-cooled refrigerated centrifuge (Eppendorf Centrifuge 5810 R) at 4°C for 1 h at ≥10,000 rpm. The supernatant was removed, leaving behind a pellet. The subsequent steps were completed using the QIAGEN kit as described. 450 µl of RLT buffer was added, vortexed and transferred to a QIAshredder spin column in a 2 ml collection tube. The lysate in the column was centrifuged for 2 min at ≥14,000 rpm.

The supernatant of the flow-through in the collection tube was transferred to a new tube without disturbing the cell-debris pellet. 1/2 volume of 100% ethanol was added to the lysate and immediately mixed by pipetting for 5-10 times. The sample including any precipitate (≤650 µl) was transferred to an RNeasy mini spin column placed in a 2 ml collection tube. This was centrifuged for 15 s at ≥10,000 rpm and the flow-through discarded by pipetting.

For on-column DNase digestion using the RNase-free DNase set (Cat. No.: 79254), 350 µl of RW1 buffer was added to the spin column and inverted to let the buffer wash the lid of the column and roll the tube to wash the column thoroughly before centrifuging at ≥10,000 rpm for 15 s. The flow-through was discarded and the collection tube reused. 10 µl DNase I stock solution was added to 70 µl buffer RDD and mixed gently and briefly centrifuged. The DNase I incubation mix (80 µl) was added to the RNase spin column membrane and placed on a ThermoMixer®C at 37°C for 15 min. 350 µl buffer RWI was added to the RNeasy spin column and centrifuged at ≥10,000 rpm for 15 s; the flow-through was then discarded.

500 µl of RPE buffer was added to the spin column, inverted and the column rolled as previously done before centrifuging for 30 s at ≥10,000 rpm. The flow-through was discarded and the collection tube was reused. 500 µl of RPE buffer was added to the spin column, inverted, rolled and centrifuged for 2 min at ≥10,000 rpm. The spin column was placed in a new 2ml collection tube, discarding the old collection tube with the flow-through, centrifuged at ≥14,000 rpm for 1 min. The spin column was placed in a new 1.5 ml collection tube and 30-50 µl of RNase-free water was directly added to the centre of the spin column membrane and centrifuged for 1 min at ≥10,000 rpm to elute the RNA. To increase the RNA concentration, the eluate was pipetted out of the collection tube and added directly onto the same spin column membrane and centrifuged for 1 min at ≥10,000 rpm. The quality, concentration and integrity of the RNA were determined using the NanoDrop 2000 spectrometer and/or 0.8% Agarose (Sigma-Aldrich) gel electrophoresis as described in Section 2.3.1.

2.3.2.2 Complementary DNA synthesis and reverse transcription PCR

The RNA concentrations from Section 2.3.2.1 were normalised to 1 µg of total RNA per 10 µl reactions. Total RNA templates were converted with reverse transcriptase into its DNA complement strand (cDNA) following the High Capacity RNA-to-cDNA™ kit protocol (Applied Biosystems Cat. No.: 4387406) (Life Technologies Ltd, Paisley, UK). The yield of the cDNA from the reverse transcription of RNA was determined by RT-PCR. RT-PCRs were performed with the cDNA templates using SensiFAST™ SYBR® Hi-ROX kit protocol (Bioline Reagents Ltd, London, UK). A 5-point 1:10 dilution RT-PCR standard curve was prepared to measure the efficiency (≥ 90% ≤ 110%) and sensitivity of the PCR assay.

2.3.3 Primer design and selection

Primer design for the study was done with the assistance of Dr Joël Allainguillaume, University of the West of England, Bristol, UK. The cacao leaves were screened for CSSV using degenerate primers designed for four gene regions, the open reading frame 1 (ORF1), Reverse transcriptase (RTase), Ribonuclease (RNase) and movement protein (MP) (Table 2.1).

Table 2.1: CSSV primer pairs used for PCR

Name	Orientation	Sequence (5'-3')*
ORF1	Forward	AGTATCCARGARTGGTAYGA
	Reverse	TCATTGACCACCCAYTGRTC
RTase	Forward	GCTGTGTAYATHGAYGAYAT
	Reverse	CATCCTTCCATRCANCCRTC
RNase	Forward	GAAAGCGAYGGNTGYATGGA
	Reverse	TCCTTTCCRTTDATRTGYTC
MP	Forward	TTCGCACARATGGARATHGA
	Reverse	CAACCAGTNGCDATDATYTG

*Symbols for degenerate bases: R = A or G; Y = C or T; H = A or T or C; D = G or A or T; N = A or C or G or T. [A = adenosine, C = cytosine, G = guanine and T = thymine].

2.3.4 DNA polymerase chain reaction (PCR)

Unless otherwise specified PCR reactions were conducted as 25 µl volumes prepared with the extracted genomic DNA samples. A positive control sample was made with an already confirmed CSSV-positive DNA template derived from a symptomatic plant. The negative control samples included virus-free DNA extractions from quarantined Amelonado seedlings and DNA-free double distilled water (ddH_2O) blanks. Each PCR reaction mix consisted of 2 µl genomic DNA (average concentration range 5-50 ng μl^{-1}), 2.5 µl CSSV specific primer (2 µM), 8 µl ddH_2O and 12.5 µl BioMix (Taq polymerase and oligonucleotides) (Bioline). All PCRs were performed in a G-STORM GS2 thermal cycler (Gene Technologies Ltd, Essex, UK) under the following conditions: initial denaturation for Taq activation at 94°C for 4 min, then 40 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, elongation at 72°C for 2 min, with a final elongation at 72°C for 5 min and storage at 10°C for 30 s.

2.3.5 Gel electrophoresis

Gel electrophoresis procedure enables the separation of different size fragments of DNA (measured in base pair number) for visualisation and subsequent purification. The PCR products (amplified target DNA segments) were analysed by electrophoresis on 1% agarose gels prepared in Tris acetate ethylenediaminetetraacetic acid buffer (1X TAE) containing ethidium bromide (EtBr) (10 mg ml^{-1} stock solution) at a ratio of 2.5 µl 100 ml^{-1}. Before loading the PCR products into the well in the gel, 20% volumes of HyperLadder™ 100bp loading dye was added to each PCR product and mixed by pipetting (i.e. 2 µl loading dye + 10 µl PCR product). 2 µl of HyperLadderTM 100bp (Bioline) was also loaded into the first and last wells on each row for size referencing. Using a PowerPac™ Basic the electrophoretic gels were usually run at 80V (45 min) and 140V (60 min) depending on gel size. After binding the DNA to EtBr, the images were viewed over a UV light source in a closed chamber with GelDoc-It^{TS2} Imager run with UVP TS2 Software (Ultra-Violet Products Ltd., Cambridge, UK).

2.3.6 CSSV DNA sequencing and alignment

PCR products of the expected size were purified with the NucleoFast® DNA, RNA and protein purification kits (Macherey-Nagel GmbH & Co. KG, Düren, Germany) and sent to SourceBioScience (Oxford) for direct DNA sequencing. Using geneious software version 9.0 (Auckland, New Zealand), a phylogenetic tree of CSSV sequences using the Tamura-Nei model was generated for comparisons with *Citrus yellow mosaic virus* (CYMV) and *Cucumber mosaic virus* (CMV) badnaviruses outgroups using the neighbour-joining method.

2.4 Mealybug husbandry

The three mealybug species, *Pl. citri*, *Ps. longispinus* and *Ps. viburni* used in the study were collected and mass-reared to ensure a continuous supply of individual mealybug species throughout the study period.

2.4.1 Collection of mealybugs

Several egg sacs and adult females of *Pl. citri* and *Ps. longispinus* were sourced from the plant quarantine glasshouses in the Royal Botanic Gardens, London. The obscure mealybugs, *Ps. viburni* were collected from leaves of sweet potato, *Ipomoea batatas* (L.) Lam., and old cocoyam, *Colocasia esculenta* (L.) Schott grown in the tropical glasshouse, University of Reading, UK. Each group of mealybug specimens was collected off the leaves and stems with the aid of round-end paint brushes (sizes 2 and 4); one dedicated to each species. The leaves and stems carrying the mealybug were gently and repeatedly tapped for 1-2 minutes to disturb the feeding mealybugs and allow ample time for withdrawal of their stylets from the plant tissues. An additional 3-5 minutes were allowed before carefully picking and brushing off the female mealybugs and their egg sacs (where present) into labelled 1.5ml Eppendorf tubes. The collected specimens were preserved in re-sealable Ziploc® bags (S C Johnson, Wisconsin, USA) and kept at 4°C.

Table 2.2: General visual morphological characteristics of the three mealybug species used in the study

Mealybug Species	Field characters (observed with unaided eyes)
Pl. citri	Body oval; slightly rounded in lateral view; body yellow when newly moulted, pink or orange-brown when fully mature; legs brown-red; mealy wax covering body, not thick enough to hide body colour; with dorsomedial bare area on dorsum forming central longitudinal stripe; ovisac ventral only, may be two times longer than body when fully formed; with 18 pairs of lateral wax filaments, most relatively short, often slightly curved, posterior pair slightly longer, filaments anterior of posterior pair small, posterior pair about 1/8 length of body. Primarily occurring on foliage of host. Oviparous, eggs yellow. The surface of lateral filaments is rough.
Ps. longispinus	Body oval; slightly rounded in lateral view; body colour variable from light yellow to grey, pink, or light purple; mealy wax covering body, thin enough so that body colour shows through; with three longitudinal lines on dorsum, with single, broad dorsomedial line, with two, thin sub-marginal lines; ovisac absent, sometimes with a few waxy strands under body; with 17 lateral wax filaments, with exception of posterior two pairs all about same size, about 1/2 as wide as body, posterior pairs conspicuously longer than others, posterior pair as long as or longer than body, next pair about half as long as posterior pair. Primarily occurring on foliage of host. Usually ovoviviparous, rarely producing eggs which hatch soon after being laid, eggs yellow. The surface of lateral filaments is rough.

See the continuation below.

Continuation of Table 2.2: General visual morphological characteristics of the three mealybug species used in the study

Ps. viburni	Body oval; slightly rounded in lateral view; pink or light purple; mealy wax covering usually thin enough so that body colour shows through; without longitudinal line on dorsum; ovisac encloses all but head of female; with 17 lateral wax filaments, becoming progressively longer posteriorly, anterior pair about 1/8 width of body, straight, unusually thin, posterior pair longest, varying from 1/4 to 1/2 length of body. Occurring on most parts of host including main roots, fruit, leaves, stems, and under bark. Oviparous, eggs yellow. The surface of lateral filaments is smooth.

Adapted from Miller *et al*. (2014).

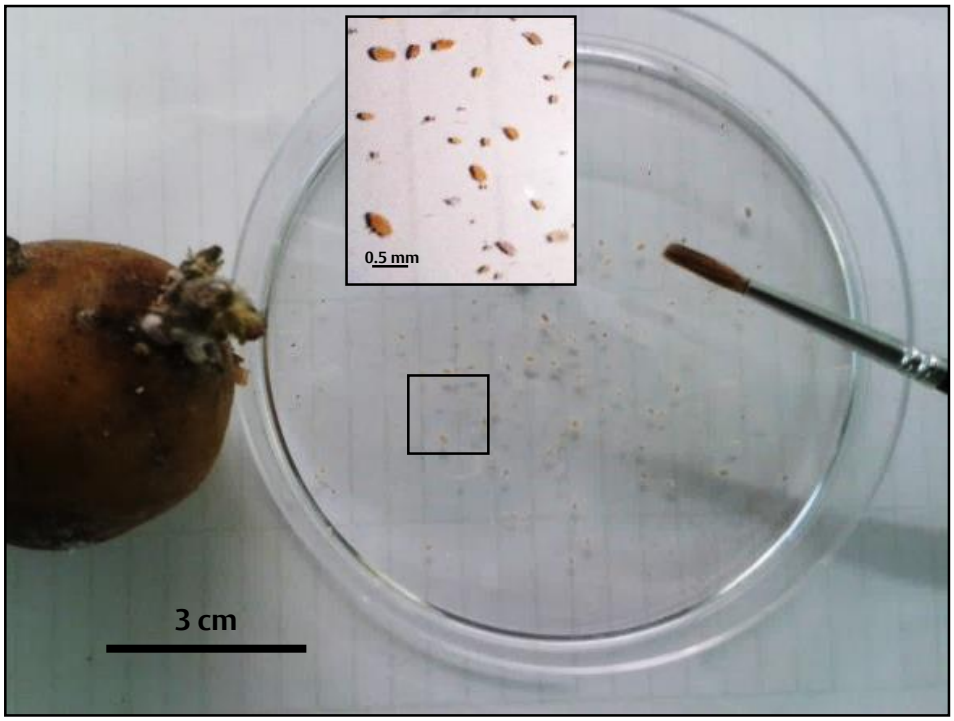

Figure 2.2: A collection of 1st and 2nd instars from potato sprouts inside a petri dish.

2.4.2 Mass rearing of mealybugs

Clean potato (*Solanum tuberosum* L.) tubers (average size, 6 cm x 4 cm) were purchased from the local grocery in Reading. Potato tubers with ≥3 nodes ('eyes') were selected and kept in dark conditions to induce sprouting (Figure 2.3a). After sprouting the potato tubers for 1-2 weeks, about 3-5 tubers were placed in a plastic lunch box (30 cm x 10 cm x 10 cm). The boxes were lined on the inside with paper towels. The lid was cut open (7 cm x 5 cm) in the middle and sealed with a breathable paper to ensure adequate ventilation (Figure 2.3b). Colonies of female mealybugs and groups of egg sacs previously collected were then placed directly on each of the sprouts for mass rearing (Figure 2.3a). The mealybug cultures in each box were retained in screen cages (40 cm x 40 cm x 70 cm) inside growth chambers (Sanyo 600G3/TTL, Sanyo Gallenkamp PLC, Loughborough, UK) (Figure 2.3b) at 25 ± 2°C, 50 - 55% RH, 14:10 - L:D.

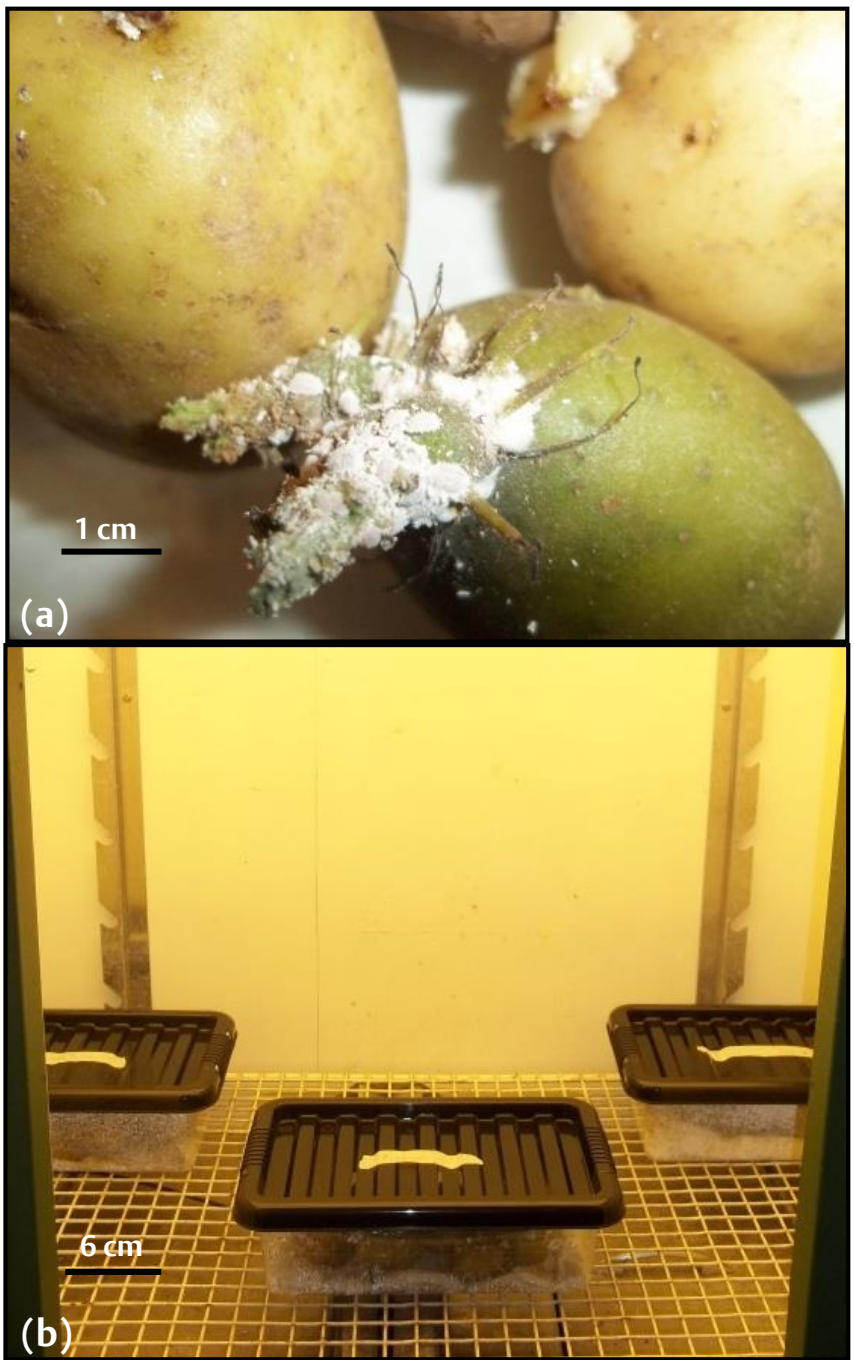

Figure 2.3: Colonies of mealybugs reared on (a) potato sprouts and (b) maintained under controlled conditions.

2.5 Morphological identification of mealybug species

2.5.1 Environmental scanning electron microscopy (ESEM)

Waxy coverings on the bodies of sampled mealybug species from were cleaned off prior to preparations for microscopy following the procedures described by Banks and Williams (1972) but with a few modifications. Two to three adult female mealybugs per species were each placed inside a 1.5 ml Eppendorf. 0.5 ml 2% DECON 90 surfactant (DECON Laboratories Ltd, East Essex, UK) was added to the specimen and incubated at room temperature. After a minimum of 24 h, *ca*. 0.4 ml was carefully pipetted off and 0.5 ml ddH_2O was added into the tube containing the mealybug sample. The sample was rinsed 2-3 times to completely remove as much as possible of the waxy meal covering the mealybug surfaces. After the last wash, each tube was carefully tipped over to dislodge the sample onto a Whatman blotting paper and left to dry for 15 – 20 s. A separate procedure was taken to ensure the preparation and preservation of very fine morphological features of the mealybug sample. This involved the use of the gold-coating procedure described by Sirisena *et al*. (2015). At the Environmental Microscopy Laboratory, University of Reading, using a Leica MZ6 Stereomicroscope (Leica Microsystems Ltd, Milton Keynes, UK) each sample was mounted laterally on a labelled SEM aluminium alloy pin stub (Ø12.7 mm x 8 mm pin height) with double sided adhesive tape. The pin stub with the sample was handled with a stainless steel pin stub mount gripper into the sample chamber of Field Emission Inc. (FEI) Quanta 600F (FEI UK Ltd, Cambridge, UK) machine. The ESEM images were captured at high resolutions at various magnifications under low vacuum, constant pressure (0.68 Torr) and high voltage (5.0 – 12.5 kV). Images of the gold-coated samples were captured at higher resolutions and magnifications under high vacuum at 20 kV.

Table 2.3: General microscopic morphological characteristics of the three mealybug species used in the study

Mealybug species	Validation characters (observed with scanning electron microscope)
Pl. citri	More than five ventral oral-collar tubular ducts between antennae; six or more ventral oral-collar tubular ducts lateral of middle coxae (count both sides); translucent pores on hind coxa and tibia; usually with 1 or more dorsolateral tubular ducts that often appear as oral rims; no auxiliary setae; anal bar present; 18 pairs of cerarii.
Ps. longispinus	More than a dorsal oral-rim tubular duct near most abdominal cerarii; ventral multilocular pores restricted to segments VIII and VII; penultimate and anal lobe cerarii with conspicuous basal sclerotisation; ventral oral-collar tubular ducts absent or uncommon in marginal areas of thorax and head; 17 pairs of cerarii; auxiliary setae present in cerarii; without discoidal pores near eye.
Ps. viburni	Ventral oral-collar tubular ducts in clusters between antennae and lateral of front coxae, absent or with fewer than three ducts lateral of middle coxae; with few dorsal oral-rim tubular ducts on abdomen (with an average of 13 and a range of 10 to 18); without oral-rim tubular duct on dorsum between cerarius 15 and 16; with dorsal oral-rim tubular duct near frontal cerarius; with an average of two and range of one to three discoidal pores associated with eye, not set in a sclerotized rim; translucent pores on hind femur and tibia.

Adapted from Miller *et al*. (2014).

2.5.2 Histology

Adapting with modifications, the protocols of European and Mediterranean Plant Protection Organization (2005), Williams and Granara de Willink (1992) and Ben-Dov and Hodgson (1997), individual slides of whole mealybug were prepared and stained with acid fuchsine-saturated Aman's lactophenol (phenol crystals – 5 g w/v, lactic acid – 5 g v/v, glycerol – 10 g v/v, water – 5 g v/v). The handling of mealybugs, slides and stains followed Watson and Chandler (2000). All phenol handling was done in the fume hood with PPE.

2.5.2.1 Mealybug preparation

Individual adult female mealybugs were obtained from the insectary and placed inside a U-base shaped 2ml Eppendorf tubes. 200 µl of 90% alcohol was added to the tube, and then heated for 20 min on a ThermoMixer®C. The mealybug was transferred to a new Eppendorf tube and 200 µl of 10% solution of KOH was added for incubation for 20 min at 40°C (on ThermoMixer®C) to 72 h (at room temperature) depending on the size and maturity of the specimen. After incubation, the specimen and the KOH were emptied into a square plain watchglass (41.2 mm).

With the help of a stereo microscope, a dorso-lateral incision was made with a fine tip stainless steel needle to aid in the maceration and expression of the internal tissues, gut and eggs (in the case of gravid females). The internal body contents were gently expelled by repeatedly pressing down the incised specimen with a 100 mm flat-end stainless steel Chattaway's microspatula. The expressed content was entirely pipetted off the watchglass into a new Eppendorf tube for subsequent DNA purification, PCR and CO1-based DNA barcoding.

The exoskeleton was twice cleaned in ddH_2O by retaining the sample in the solution for 20 min before pipetting out the water. 50 – 100 µl of the acid fuchsine-saturated Aman's lactophenol stain was added to the sample and allowed to stand in a 2 ml Eppendorf tube for 1 h. The stained sample was twice washed in glacial acetic acid to eliminate excess stain.

Each session involved adding 1-1.5 ml of glacial acetic acid to the sample in the staining solution and allowed to stand for 30 min before carefully pipetting out the washed out excess stain. Next, 100 – 150 µl lavender oil was added to the transparent exoskeleton in the Eppendorf tube for incubation, for a duration ranging from 1 to 72 h at room temperature. The oil was pipetted out and the Eppendorf tube with the exoskeleton was gently tipped on the side over a blotting paper to soak up the remaining excess oil. The exoskeleton was removed from the Eppendorf tube with a 10 µl pipette tip already dipped in the Canada balsam mountant. This was to enable an easy and quick recovery of the exoskeleton from the Eppendorf tube.

The recovered exoskeleton was carefully placed on a dry microscope slide and positioned under a microscope. An additional drop of Canada balsam (50 µl) was added over the sample on the slide before placing the cover slip. The slide was labelled on the side and held at room temperature for 1-4 week. The slide-mounted specimens were viewed and photographed under Leica Z6APO/DFC450 digital microscope camera with Leica LAS Live Image Builder (Leica Microsystems Ltd, Milton Keynes, UK) The features were examined and validated with the existing keys provided by McKenzie (1967) for species identification and subsequently compared with a voucher specimen.

2.6 Molecular characterisation of mealybug species

DNA purification was performed with both the KOH-incubated mealybug internal body content and whole mealybug body. These were subjected to PCR (Section 2.6.2) using CO1 and ITS2 specific primer pairs (Table 2.4).

2.6.1 Mealybugs DNA extraction (purification) and quantification

Individual mealybug species were placed inside Eppendorf tubes loaded with a single 3 mm tungsten carbide bead. This was sealed, labelled and loaded onto TissueLyser II adapters for disruption following the steps described in Section 2.3.1.

DNA extractions from the disrupted mealybugs were performed using QIAGEN DNeasy Blood and Tissue (50) kit with some modifications to the manufacturer's protocol that were found to improve the DNA yield and purity. The following modifications to the QIAGEN protocol were followed throughout the extraction and purification procedures of DNA from the mealybug samples. A single whole mealybug sample was added to 1.5 ml Eppendorf tube and disrupted as described in Section 2.3.1. 180 µl ATL buffer was added to the disrupted sample and followed with the addition of 20 µl Proteinase K. The sample was thoroughly vortexed for 2-3 min and incubated overnight (≥12 h) at 56°C on ThermoMixer®C at 300 rpm.

The sample was vortexed for 30 s before adding 200 µl, mixed and thoroughly vortexed. 200 µl 100% ethanol was added, mixed and thoroughly vortexed obtain a homogeneous solution (with precipitate). Where a larger number of samples were processed, AL and ethanol premix was made. The mixture was completely pipetted into a DNeasy Mini spin column placed in a 2 ml collection tube and centrifuged at ≥ 8,000 rpm for 1 min. The flow-through was discarded with the collection tube. The column was placed in a new 2 ml collection tube; 500 µl AW1 buffer was added and centrifuged at ≥ 8,000 rpm for 1 min. The flow-through and the collection tube were discarded while the column was placed in a new collection tube. 500 µl AW2 buffer was added and to the column and centrifuged at ≥ 14,000 rpm for 3 min. Only the flow-through was discarded after the previous step, the collection tube was reused to re-dry the column for 1 min at ≥ 14, 000 rpm. The column was then placed in a clean 1.5 ml Eppendorf tube (directly obtained from the Ziploc® carrier bag) for DNA elution from the membrane. A final total volume of 150 µl eluate was obtained in two steps. In each step, 75 µl AE buffer was directly added in the centre of the DNeasy membrane and incubated for 5 - 10 min at room temperature before the column was centrifuged at ≥ 8,000 for 1 min. The DNA concentration and purity was determined (Section 2.3.1) and the sample stored at – 80°C.

2.6.2 PCR for DNA barcoding

For each of the extracted genomic mealybug DNA samples a 25 µl PCR reaction was made up. The negative control sample had ddH_2O added in place of DNA template. Each PCR reaction mix consisted of 2 µl genomic DNA, 2.5 µl CO1 (or ITS2) primer (2 µM), 8 µl ddH_2O and 12.5 µl BioMix (Bioline). All PCRs were performed in a G-STORM GS2 thermal cycler (Gene Technologies Ltd, Essex, UK). The first step (5 cycles): initial DNA denaturation for Taq activation at 94°C for 1 min, annealing at 46°C for 1 min 30 s, elongation at 72°C for 1 min 30 s. The second step (35 cycles): denaturation at 94°C for 1 min, annealing at 50°C for 1 min 30 s and elongation at 72°C for 1 min with a final elongation at 72°C for 5 min and stored at 10°C for 30 s. PCR products were assessed by gel electrophoresis as described in 2.3.5. Phylogenetic tree of CO1 sequences was generated with Geneious (version 9); genetic distances were calculated using the Tamura-Nei model and neighbour-joining tree build.

Table 2.4: CO1 and ITS2 specific PCR primer pairs used for mealybug

Name	Orientation	Sequence (5'-3')
MFCO1	Forward	ATATCTCAAATTATAAATCAAGAA
MRCO1	Reverse	ATTACACCTATAGATAAAACATAATG
ITS2-M-FF	Forward	CTCGTGACCAAAGAGTCCTG
ITS2-M-RR	Reverse	TGCTTAAGTTCAGCGGGTAG

A = adenosine, C = cytosine, G = guanine and T = thymine.

Chapter 3 DIVERSITY STUDIES OF CSSV AND MEALYBUGS ON NIGERIAN CACAO

3.1 Introduction

Thrips, mites, mirids and mealybugs act as vectors for 2% of virus diseases affecting plants (Andret-Link and Fuchs, 2005). Mealybugs damage to a host includes and is not limited to the following activities, loss of aesthetic qualities, sap uptake, secretion of honeydew, development of sooty moulds, injection of toxins and the transmission of plant viruses (Franco *et al*., 2009). *Cacao swollen shoot virus* (CSSV) is thought to be transmitted by at least 16 mealybug species (Roivainen, 1980) and the implementation of appropriate control measures for CSSV requires updated information on the pathogen's presence in West African cacao-growing countries, especially in Nigeria, where such data have been at best limited. Thus, a study was first conducted in the key cacao-producing areas in Nigeria to identify and map CSSV presence with the primary aim of establishing, with molecular-based evidence, the geographic distribution of CSSV in these areas. The second part of the study involved the identification, characterisation and mapping of CSSV vector mealybug species occurring on cacao in the major cacao-producing areas in Nigeria.

3.1.1 CSSV survey in Nigeria

The first documented report of CSSV in Nigeria was in 1944 (Murray, 1945) and the first field survey for CSSV presence in Nigeria took place between 1944 and 1949. The aim was to identify and eradicate, by cutting-out, CSSV-infected cacao plants with visually observed symptoms of infection. Eradication of infected plants commenced in 1946 and by 1956 a total of 1, 514, 750 infected trees had been cut out (Lister and Thresh, 1957). An attempt to re-establish information on the presence of CSSV in Nigeria was made by Dongo and Orisajo (2007) using enzyme linked immunosorbent assay (ELISA)-based screening.

By sampling cacao leaves for CSSV detection from only a very limited cacao-growing area in Nigeria, coupled with the less than optimal sensitivity of the ELISA technique, these findings could not be considered to be representative of, at least, CSSV status in the major cacao growing areas in Nigeria.

3.1.2 Mealybug survey in Nigeria

In the beginning of June 1951, in continuation of the work done by the defunct West African Cacao Research Institute (WACRI), Nigerian cacao trees in the western part of the country were surveyed for mealybug presence alongside attendant ants (Sutherland, 1953). The objectives of these surveys were to establish the distribution of known and putative vectors of CSSV in the cacao-growing areas of then Oyo, Ondo and Benin provinces and also study the indigenous parasites that attack mealybugs in Abeokuta and Ijebu. The inability to complete a detailed survey of the Nigerian cacao in even the western part of the country made extending these surveys to other parts of the region, including the south-western zone problematic.

In a report by Strickland (1947) *Fo. njalensis* was found to be the most abundant mealybug species on cacao in Ghana. In Nigeria, *Fo. njalensis* was the second most important mealybug species in the Western part of the country after the dominant mealybug species, *Pl. citri* (Strickland, 1951). Other mealybug species recorded included *Pl. kenyae* (Le Pelley), *Pseudococcus* sp. nr. *celtis* (Strickland), *Ferrisia virgata* (Cockerell), *Ps. concavocerarri* (James), *Ps. bukobensis* = *Phenacoccus (Pseudococcus) hargreavesi* (Laing) and *Ps. longispinus*. Sutherland (1953) reported that there were cacao trees in areas without CSSV that had *Fo. njalensis*, especially in Ondo. However, there were no reports for CSSV or *Fo. njalensis* in the Benin Province. *Planococcus* spp and *F. virgata* were present in all the Provinces surveyed in Western Nigeria and accounted for 92% of the total number of colonies in these areas (Sutherland, 1953).

The contrasting species abundance results of these surveys raises the possibility of misidentification, in what is a notoriously challenging sub-order to characterise on the basis of morphology. Such misidentification would have inevitable implications for determining the dynamics of the pathogen-vector-host interactions (Posnette, 1950a; Posnette and Strickland, 1948).

3.1.3 Challenges of mealybug species identification: DNA barcoding

The suprageneric classification of hemipterans within Coccoidea is poor (Hardy *et al*., 2008), only applicable to adult female species, alongside the challenges of morphological identification and classification at the species level for the existing cryptic members of *Pseudococcidae*. This poses a problem to the well-trained eyes of entomologists and taxonomists. To date 25 different mealybug species have been reported on West African cacao, but so far CSSV has been shown to be transmitted by only 16 of these species (N'Guessan *et al*., 2014; Roivainen, 1980). Generally, the misidentification of mealybug species on cacao is not uncommon due to the variability and complexity of morphological features. The introduction of DNA barcoding in biological sciences revolutionised taxonomic classification of animals (Hebert *et al*., 2003a). This approach is based on the use of mitochondrial gene cytochrome *c* oxidase subunit I (COI) (Hebert *et al*., 2003b) that plays an essential respiratory role including cellular respiration and the production of adenosine triphosphate (ATP), rich in energy and fundamental for aerobic metabolic cell activities.

The importance of COI over morphology is based on the mutating rates of mtDNA in eukaryotes, which are comparatively fast compared to nuclear genes, serving two purposes in its use for molecular classification of cryptic mealybug species, thus bringing about wide and narrow genetic variation between and within species respectively (Kondo *et al*., 2008).

Barcoding comes as a fast, economic and an accurate approach in addressing problems associated with misidentification of related species (Hebert and Gregory, 2005; Hebert *et al*., 2003b) with unresolved morphological matches. For example, the identification of closely related mealybugs species, *Pl. ficus* and *Pl. citri*, by random amplification of polymorphic DNA (RAPD)(Demontis *et al*., 2007) and PCR-based restriction fragment length polymorphism (PCR-RFLP) (Cavalieri *et al*., 2008) using COI gene. Molecular characterisation of mealybugs species have also been carried out using the ribosomal internal transcribed spacer genes 1 (ITS1) and 2 (ITS2) (Correa *et al*., 2012; Mansour *et al*., 2012).

3.2 Objectives

The objectives of this study were: (1) to survey and map the presence of CSSV and putative vector mealybug species in Nigerian cacao using PCR-based molecular screening evidence and (2) to use a combination of environmental scanning electron microscopy (ESEM), histology and CO1-based DNA barcoding to improve on the characterisation of mealybugs species occurring on cacao in Nigeria.

3.3 Materials and methods

All cacao leaf and mealybug samples were collected from cacao farms in several locations across Nigeria. The location information of every sample was recorded using a Garmin eTrex Venture HC navigator (Garmin International Inc., Kansas, USA) in a format indicating the hemisphere, longitude and latitude. The maps were created with the use of Google Earth Software (Google Inc., USA). The samples were presented for inspection and phytosanitary clearance at the Quarantine Division of the Institute of Agricultural Research and Training, Moore Plantation, Ibadan, Oyo State for cacao leaves and mealybug samples collected from Oyo, Ondo and Edo States.

The Federal Ministry of Agriculture, Cross River State was responsible for the inspection and phytosanitary clearance of samples obtained from Abia, Akwa Ibom and Cross River States. These samples were sent to the University of Reading, received and stored at 4°C for screening and subsequent analysis.

3.3.1 Field surveys and collection of cacao leaf samples

An initial three-month survey of the cacao-producing sites in four states, Abia, Akwa Ibom, Oyo and Ondo, took place from 1 June to 31 August 2012. Individuals and groups of cacao farmers were consulted and informed about the project's aims and permission was sought to conduct the survey and this was provided in most cases (Appendix A). Prior to the commencement of the trip, two permanent field assistants were trained in the use of pictorial aid in the identification of symptomatic CSSV-infected cacao plants. At field sites, additional assistants, mostly individual cacao farm owners, were recruited. In the field, two leaves each of symptomatic (Figure 3.1a) cacao trees were randomly collected from opposing sides of the tree canopy. The collection of leaves from asymptomatic cacao leaves was important for possible latent infection detection. A second field survey was carried out on new sites in two additional states, Cross River and Edo, from 1 February to 30 April 2013. The sampled cacao leaves were freed of debris; laid flat and placed in pairs inside write-on grip seal Ziploc® polyethylene bags (Figure 3.1a). Each bag was labelled with the respective GPS coordinates of the sampled cacao tree. Leaf samples were also collected from putative alternative host trees.

3.3.2 Collection and morphological identification of mealybug samples

During summer 2012 and spring 2013 surveys in Nigeria, samples of adult female mealybugs were collected from the stems, leaves and pods of cacao trees (Figure 3.1d-g)and they were stored in 1.5 ml Eppendorf tubes. The samples were shipped to the University of Reading where they were stored at 4°C while awaiting processing.

Scanning electron microscopy (SEM) was used for the morphological assessment of the samples based on the keys developed to distinguish female (Figure 3.2) mealybug species (Cox, 1989; Cox and Freeston, 1985; Miller *et al*., 2014; Williams and Watson, 1988).

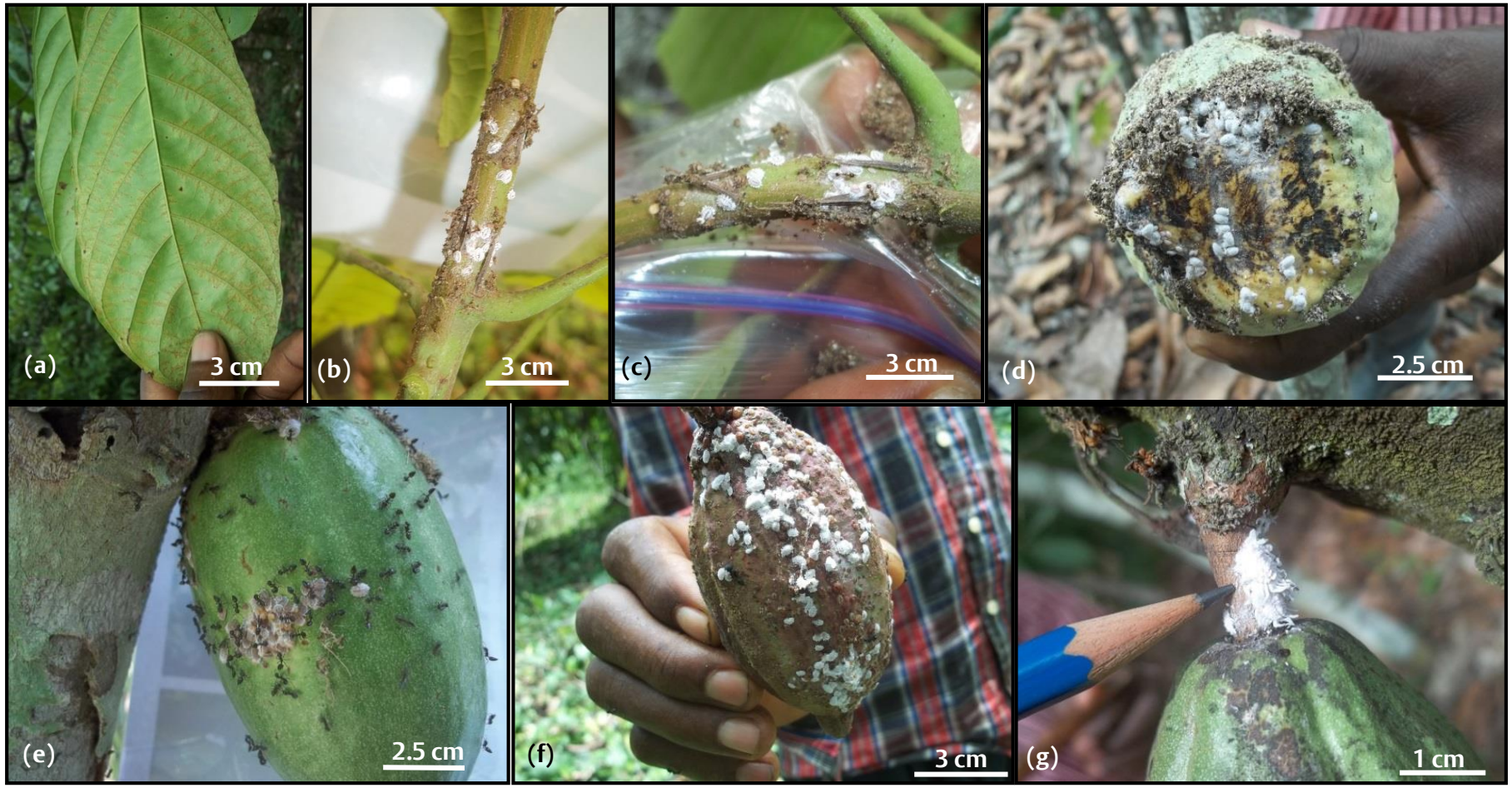

Figure 3.1: (a) Adaxial surface of symptomatic cacao leaf with CSSV infection; (b, c) mealybugs infestation on stems, (d – f) pods and (g) node/peduncle of cacao.

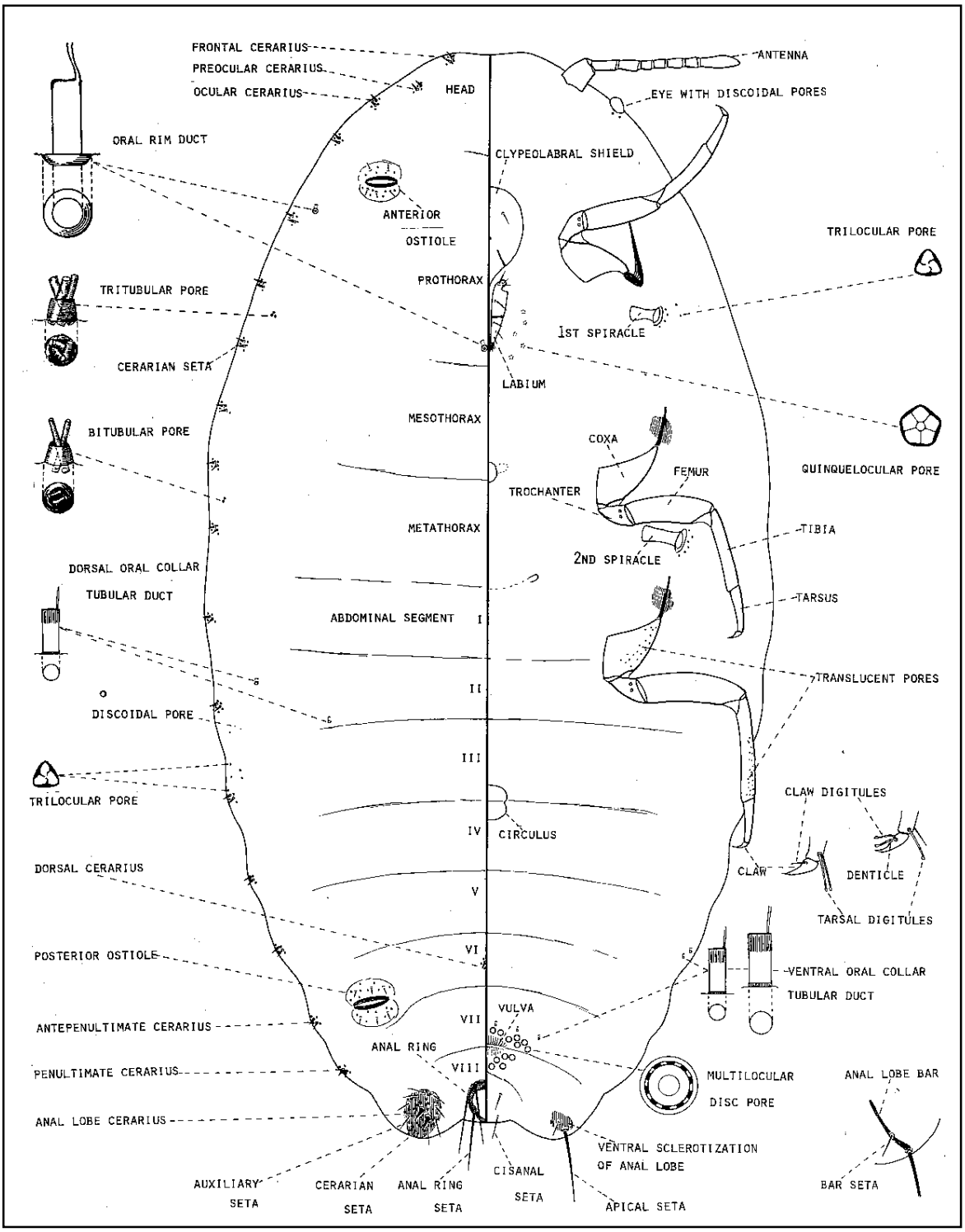

Figure 3.2: General morphology of an adult female mealybug (Williams and Watson, 1988).

3.3.3 Molecular characterisation of CSSV and mealybugs

DNA extractions for CSSV screening on sampled cacao leaves were performed with the 96-well plate QIAGEN DNeasy Plant Maxi kit; capable of handling a greater number (≥96) of cacao leaf samples at a time. DNA extractions and screenings for cacao and mealybug samples less than 50 in number, were performed with the QIAGEN DNeasy Plant Mini and QIAGEN DNeasy Blood and Tissue Mini kits as described under Sections 2.3 and 2.6, respectively. Dr Joël Allainguillaume of the University of the West of England in Bristol assisted with aspects of the molecular analysis including sequence alignments and phylogenetic analysis.

3.4 Results

The following results are based on CSSV-specific PCR-screening with the ORF1 primer and CO1-based DNA Barcoding of the sampled putatively CSSV-infected cacao and mealybug species occurring in Nigerian cacao, respectively. Though the cacao leaf samples were screened with the RTase, RNase and MP primers, but these were less reliable; RTase were prone to generating false positive PCR results. Only the results obtained for ORF1 primers have been presented for discussion owing to its high reliability and repeatability of results. From the results, comparisons were made with CSSV isolates (strains) and mealybug species obtained from other cacao producing countries. Average DNA concentrations ranged from 5 to 50 $ng\mu l^{-1}$ (cacao leaf, 80-100 mg) and 10 to 100 $ng\mu l^{-1}$ (whole mealybug).

3.4.1 Molecular characterisation of CSSV in Nigeria

The DNA sequences of PCR positive samples from CSSV screened cacao leaves obtained from cacao farms in Nigeria in the 2012 and 2013 field surveys were analysed for genetic similarity and relatedness. The phylogenetic tree of four CSSV DNA sequences from Nigeria, in comparison with three CSSV sequences from other two cacao-growing countries in West Africa and three *Badnavirus* outgroups, is presented below (Figure 3.3). Similar DNA sequences of CSSV from Nigeria were distinct from the sequences from Togo but closely related to sequences from Ghana (Figure 3.4).

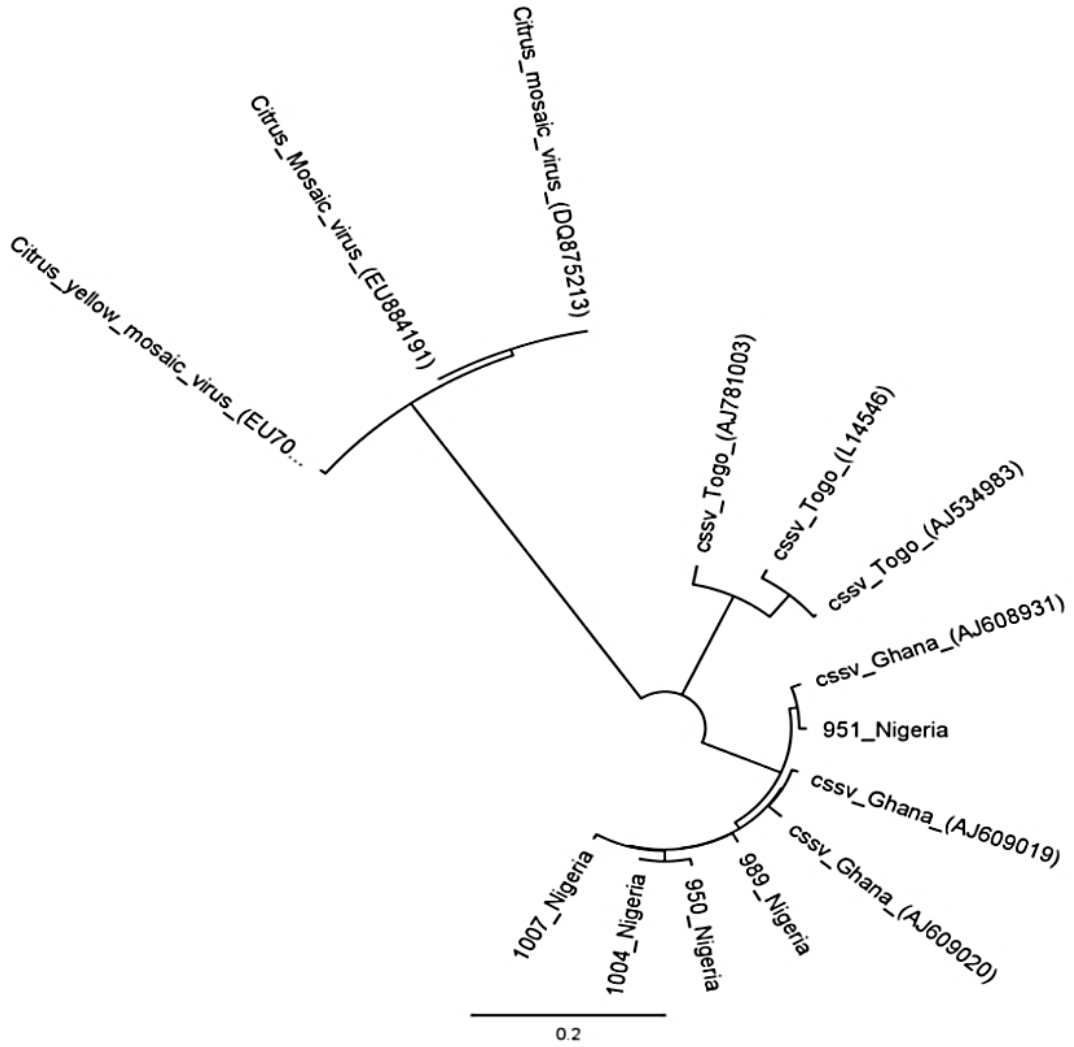

Figure 3.3: Phylogenetic relationships of CSSV ORF1 DNA sequences of isolates from Nigeria, Ghana and Togo.

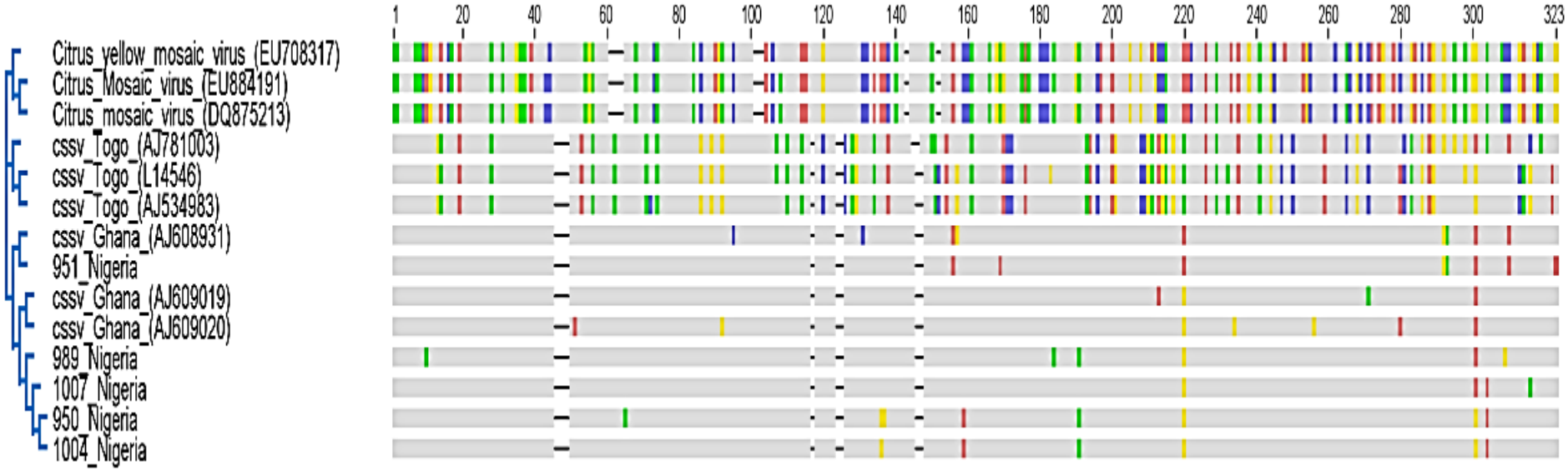

Figure 3.4: Alignment of CSSV ORF1 DNA sequences of isolates from Nigeria, Ghana and Togo.

3.4.2 Mapping of CSSV in major cacao-growing areas in Nigeria

PCR-based screening proved capable of CSSV detection in non-symptomatic trees and excluded apparently symptomatic false positives (the phenotype of which may have been due, for example, to nutrient deficiency). One alternative host tree, *Ceiba pentandra*, (out of the 13 sampled) and 42 (*ca.* 5.45%) out of 774 cacao trees (Appendix B) were CSSV positive based on the qualitative PCR screening. Screening revealed CSSV presence from 7°29'44"N on the north to 5°31'31"N on the south and from 5°57'48"E on the east to 4°01'39"E on the west (Figure 3.5). CSSV presence were detected in Ondo (Figure 3.6), Edo (Figure 3.7), Abia, Akwa Ibom and Cross River States (Figure 3.8).

Figure 3.5: Areas surveyed for CSSV presence using PCR based screening of leaf samples in Nigeria between June 2012 and April 2013.

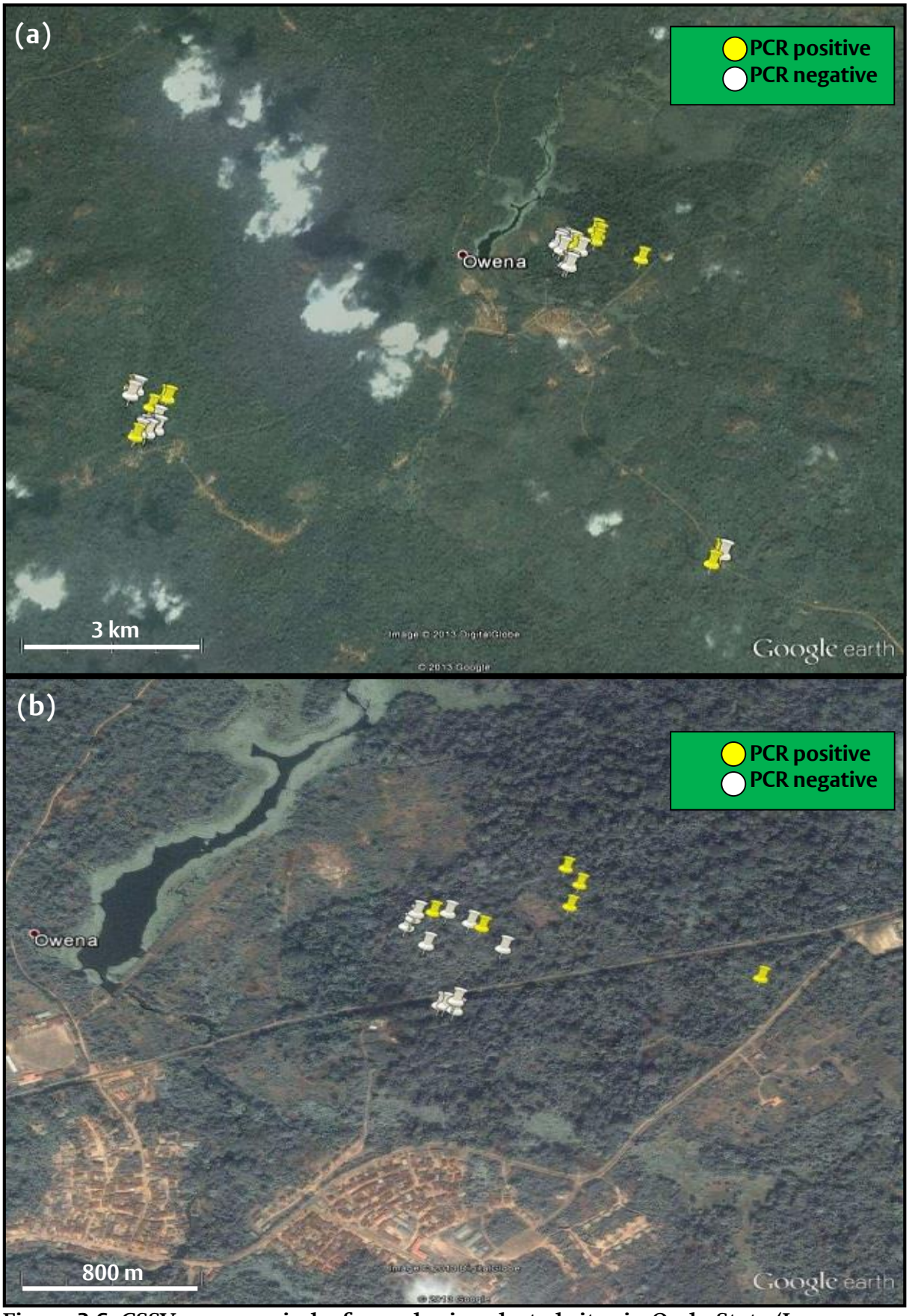

Figure 3.6: CSSV presence in leaf samples in selected sites in Ondo State (June 2012 - April 2013).

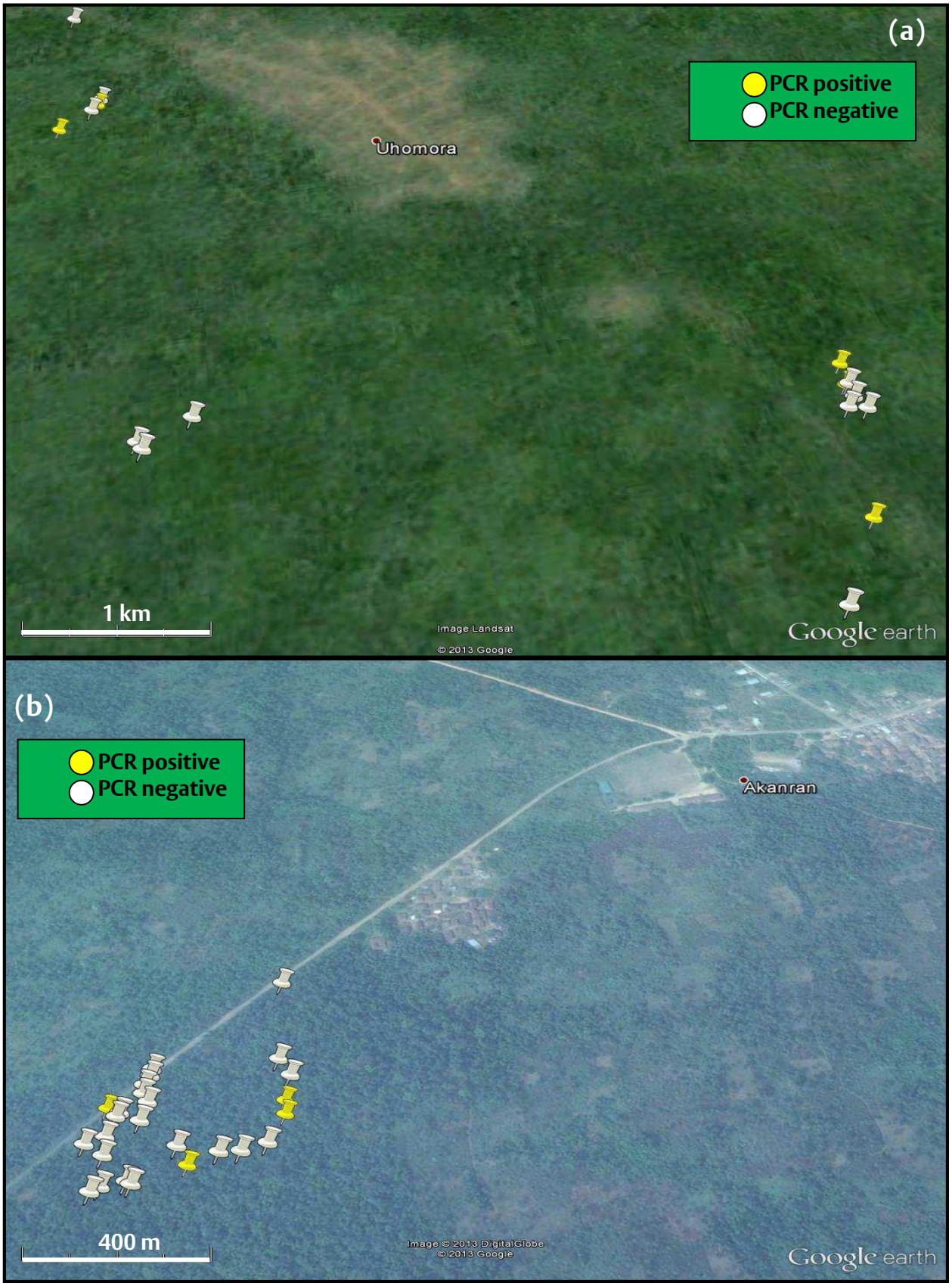

Figure 3.7: CSSV presence in leaf samples in selected sites in (a) Edo and (b) Oyo States (June 2012 - April 2013).

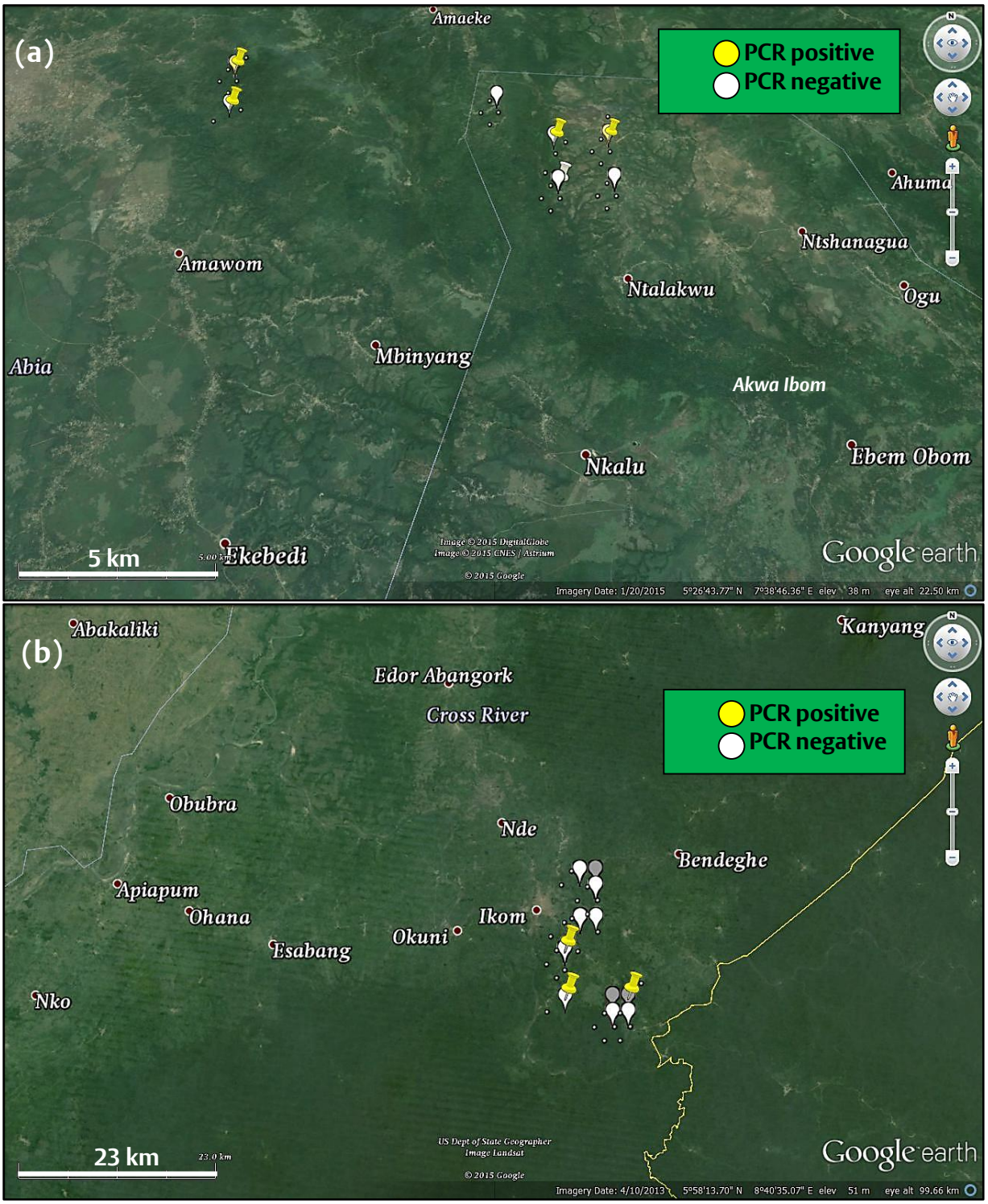

Figure 3.8: CSSV presence in leaf samples in selected sites in (a) Abia and Akwa Ibom (b) Cross River States (June 2012 - April 2013).

3.4.3 Morphological identification of mealybugs occurring in Nigerian cacao

Some of the mealybug species were characterised using a combination of morphological keys for the identification of scale insects of the family *Pseudococcidae*. Basically, the identification was based on a combination of features, from each of the examined mealybug species, including and not limited to the number of setae, type and number of pores, absence of denticle on the claws, number of body segments (Figure 3.9) and number of segments on the antennae (Figure 3.10). Observed mealybug samples with similar morphological features which matched with the USDA keys for identical mealybug species were coded and labelled for subsequent CO1-based PCR screening followed by DNA sequencing and barcoding.

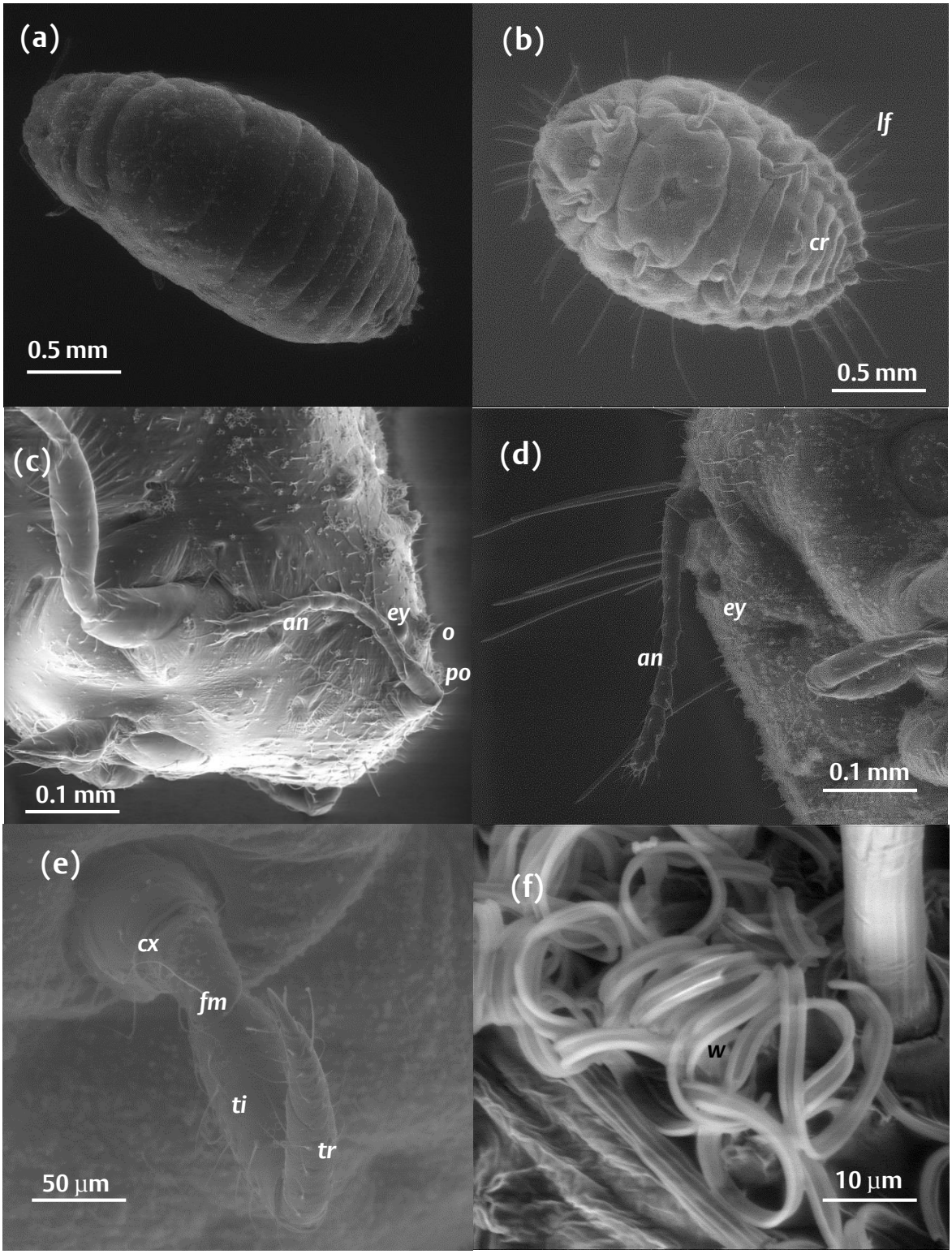

Figure 3.9: Low vacuum ESEM of whole adult female mealybug sample showing (a) body segments; (b) lateral filaments (*lf*), cerarii, 3-pairs of legs, circulus (*cr*) (3rd/4th abdominal segments); (c-d) head and eye (*ey*) with preocular (*po*) and ocular (*o*) cerarii and antenna (*an*); (e) coxa (*cx*), femur (*fm*), tibia (*ti*) and tarsus (*tr*); (f) wax from trilocular pores.

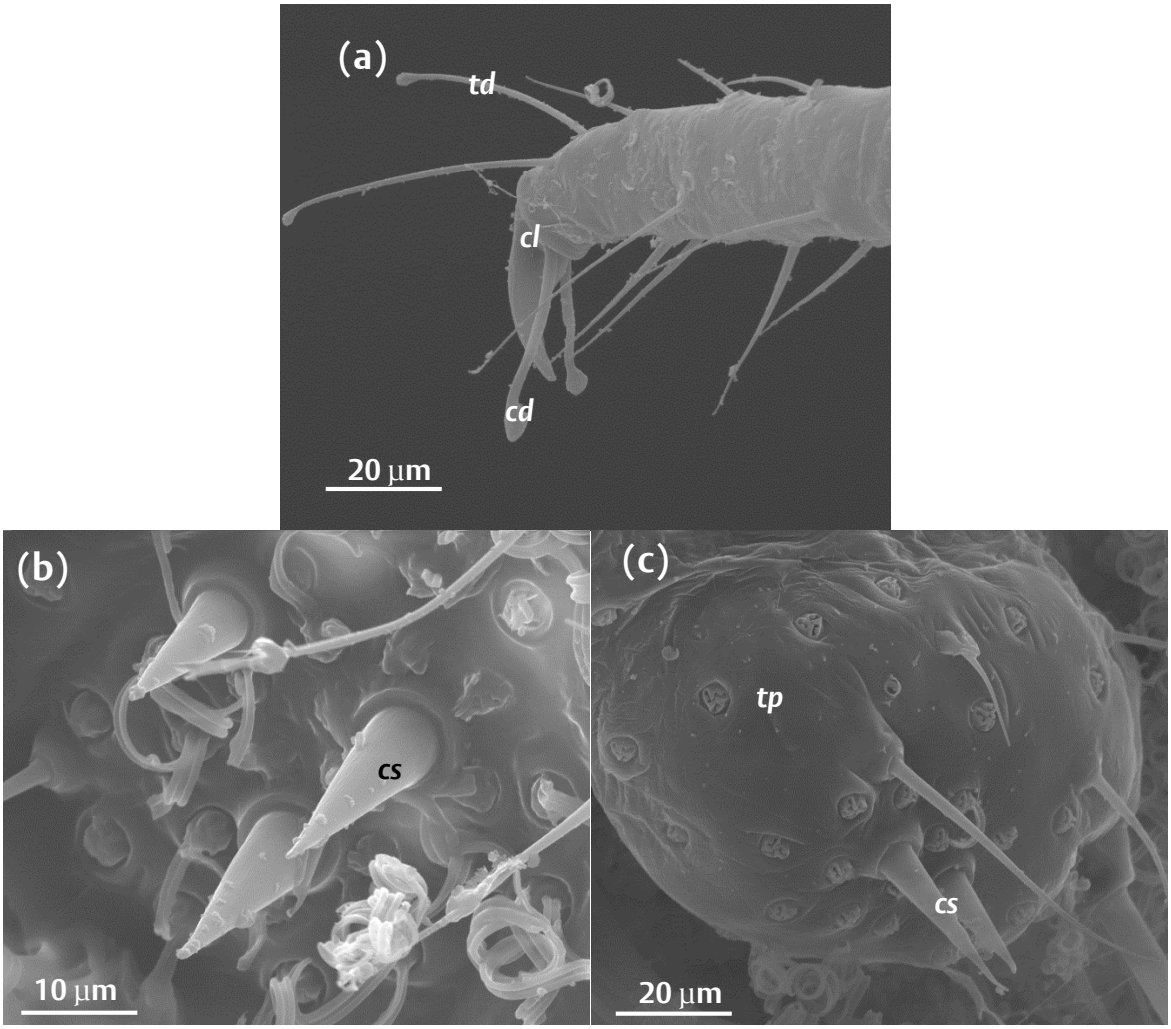

Figure 3.10: High vacuum gold-coated SEM images of adult female mealybug showing (a) claw (*cl*), claw digitules (*cd*) and tarsal digitules (*td*); (b) conical setae (*cs*); (c) trilocular pores (*tp*) and conical setae (*cs*) on anal lobe.

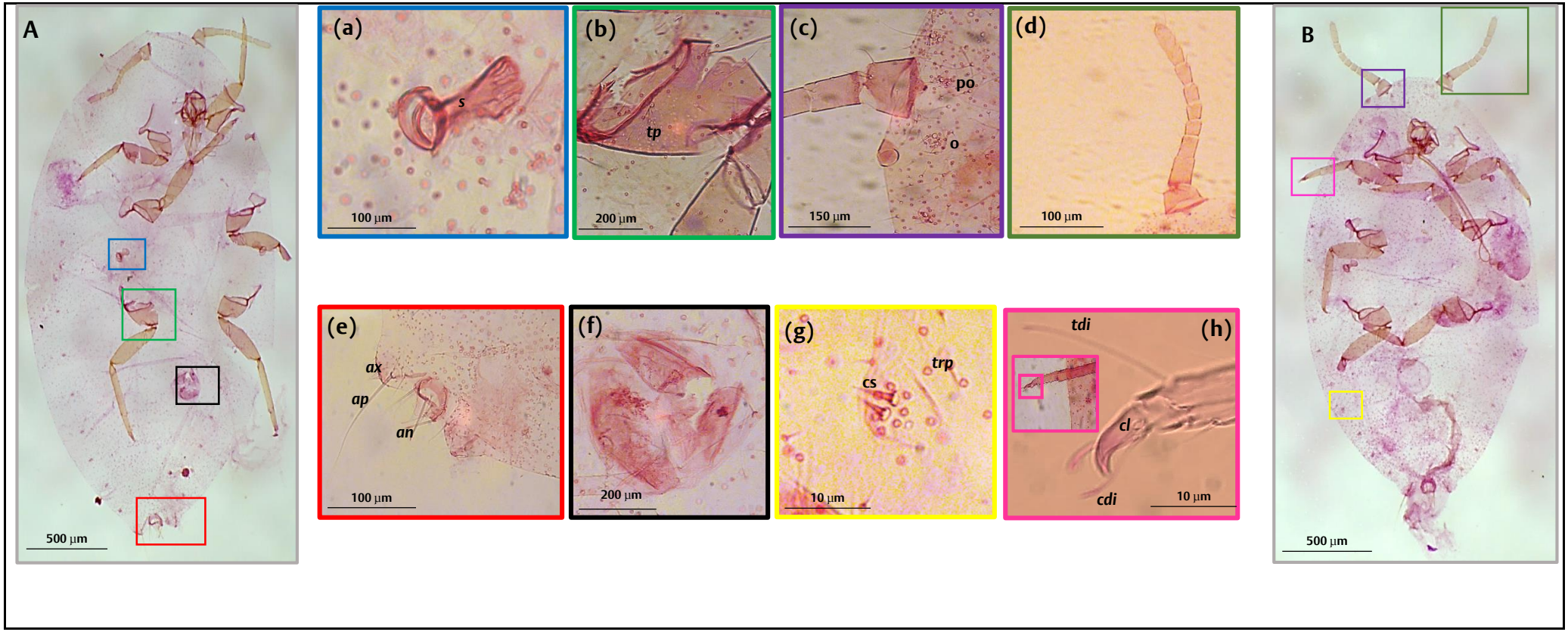

Figure 3.11: Light micrographs of adult female mealybugs showing, (a) spiracle (s); (b) coxa of the hind limb with translucent pores (*tp*); (c) base of the antenna, eye, frontal, pre- (*po*) and ocular (*o*) cerarii; (d) antenna with 8-segments; (e) sclerotised anal lobe with apical (ap), anal ring (*an*) and auxiliary (*ax*) setae; (f) circulus located on fourth/fifth body segment corresponding to the fifth cerarius from 'A' posterior; (g) conical setae (cs) and trilocular pores (*trp*); (h) tibia and tarsal of the fore limb (insert) showing the claw (*cl*), claw digitules (*cdi*) and tarsal digitules (*tdi*).

3.4.4 Molecular characterisation of mealybugs occurring in cacao in Nigeria

DNA barcodes of the morphologically characterised mealybug samples were generated following the procedures described in Chapter 2. Figure 3.12 shows the electrophoretic gel image for post-SEM mealybug samples run against CO1 and ITS2 primers. The use of single nucleotide polymorphism (SNPs) was also employed to discriminate the cryptic mealybug species (Figure 3.13). Figure 3.14 represents results from an on-going study characterising the mealybug fauna found on cacao in all major cocoa-producing areas throughout the tropics. The phylogenetic relationship between the mealybug species characterised in Nigeria and across other cacao growing countries is shown in Figure 3.15. As can be seen in Figure 3.16, while examples of *Pl. lilancinus* (Ckll.) have only been detected on cacao from the Far East (Indonesia and the Philippines), examples of *Fo. njalensis* have come exclusively from West African cacao (Côte d'Ivoire, Ghana and Nigeria in this study). Comparisons were made with a selection of NCBI reference samples for *Pl. citri* (e.g. accession: KM378731), *Pl. minor* (e.g. accession: EU250529), *Fo. njalensis* (e.g. accession: KM378733) and *Fe. virgata* (e.g. accession: GQ906765).

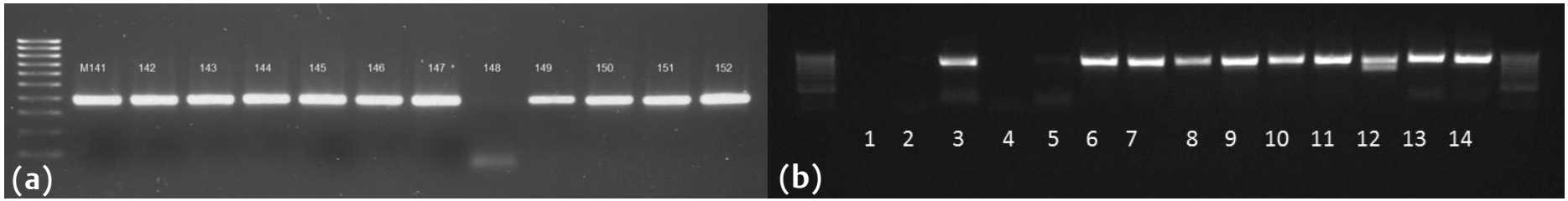

Figure 3.12: Nigerian mealybugs - Post-SEM (a) CO1 and (b) ITS2 PCRs showing the expected 400 bp and 850 bp fragments, respectively.

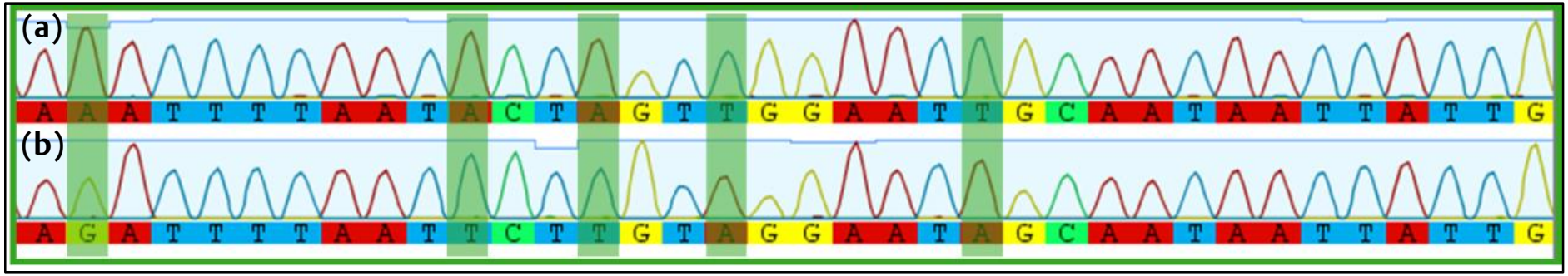

Figure 3.13: Alignment of CO1 sequences derived from SEM-analysed mealybugs highlighting SNPs (green vertical bars) that help to distinguish (a) *Planococcus citri* from (b) *Pseudococcus longispinus*.

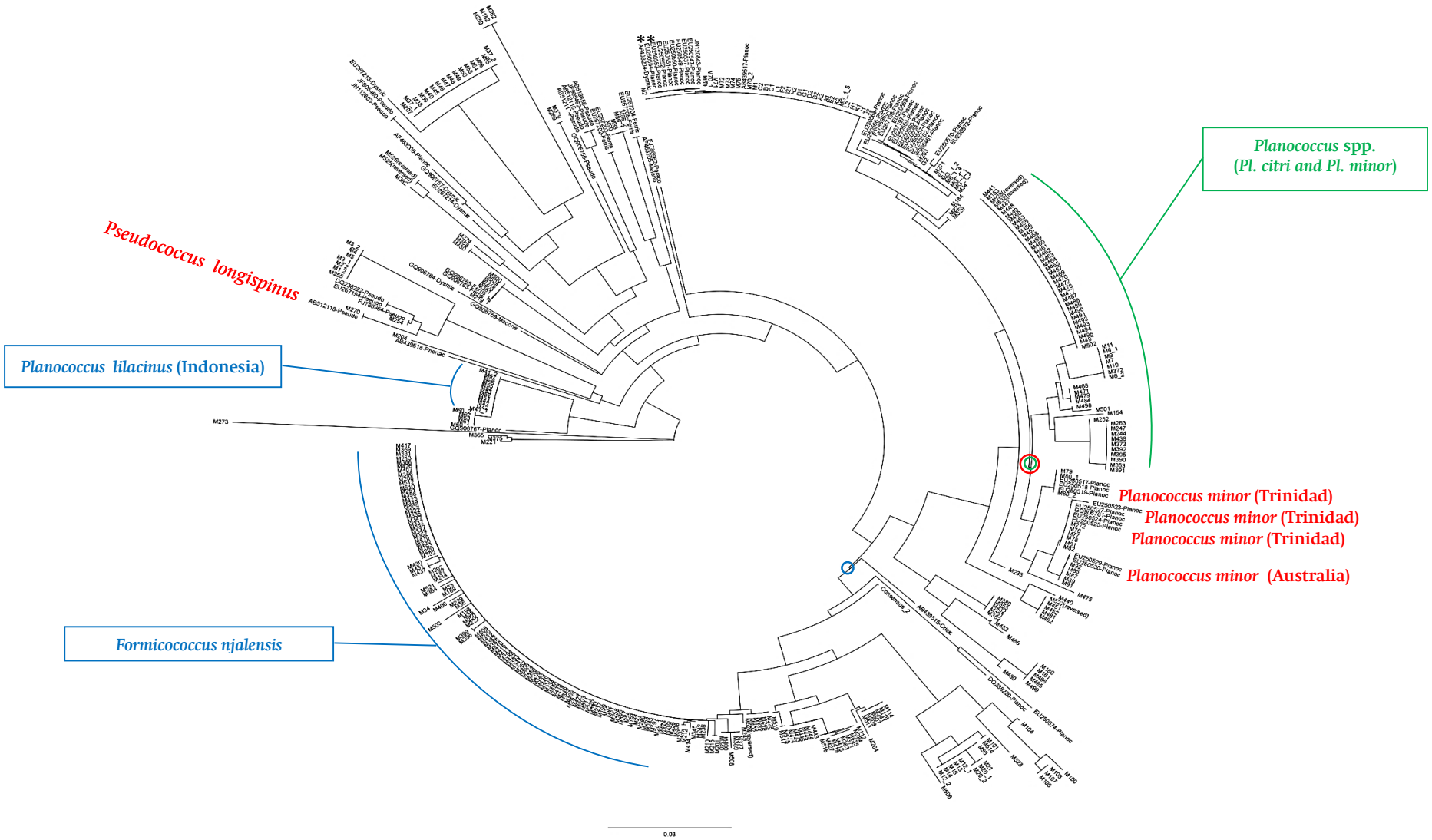

Figure 3.14: Phylogenetic tree of CO1 sequences of mealybugs (M codes) collected from cacao-growing areas in the world during the period 2010 to 2015. Additional published CO1 sequences from non-cacao mealybug samples (e.g. AF 483204) are included for reference.**

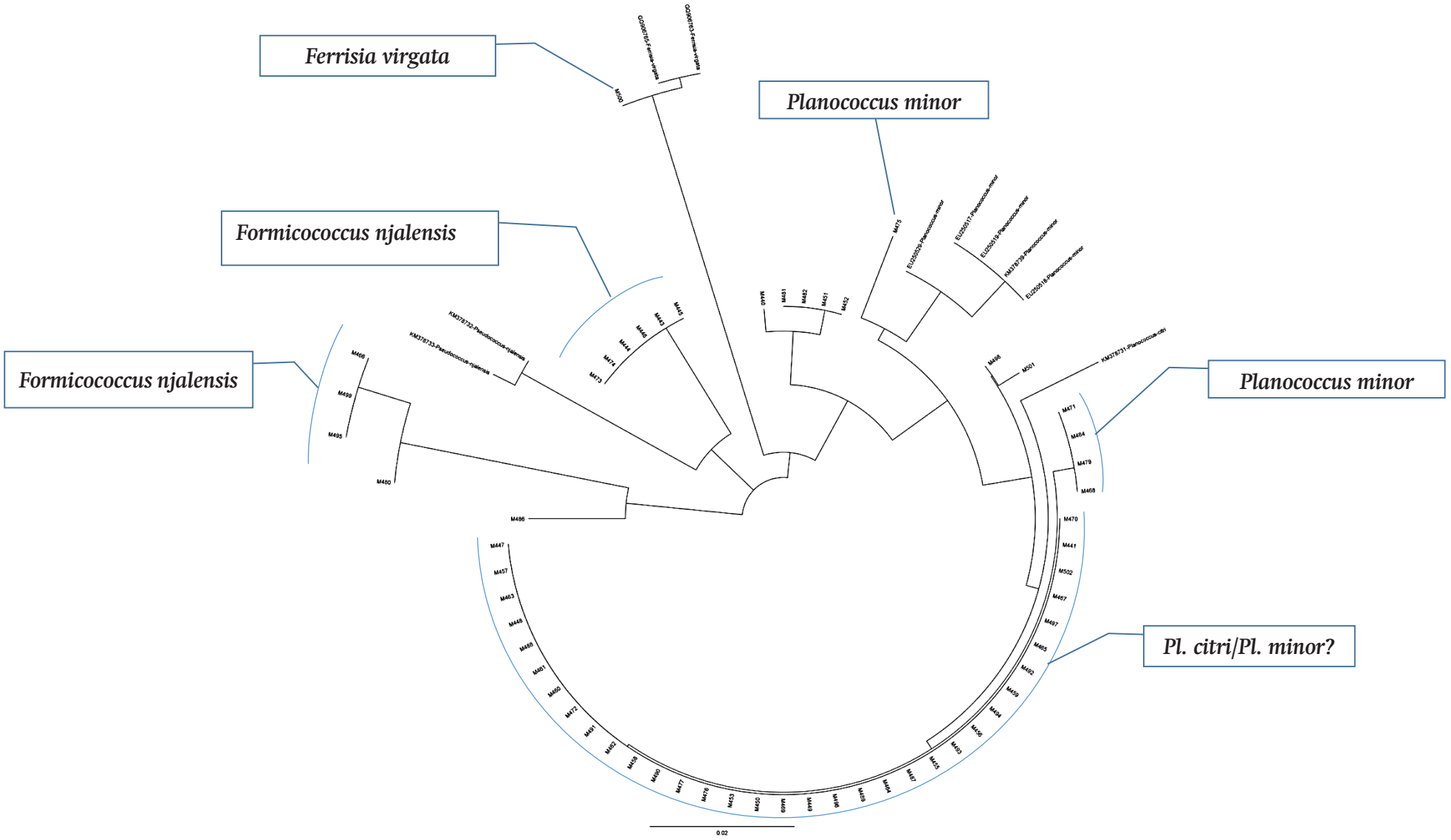

Figure 3.15: Phylogenetic tree of CO1 sequences (306 bp) from Nigerian mealybug samples (M-codes) taken from cacao.

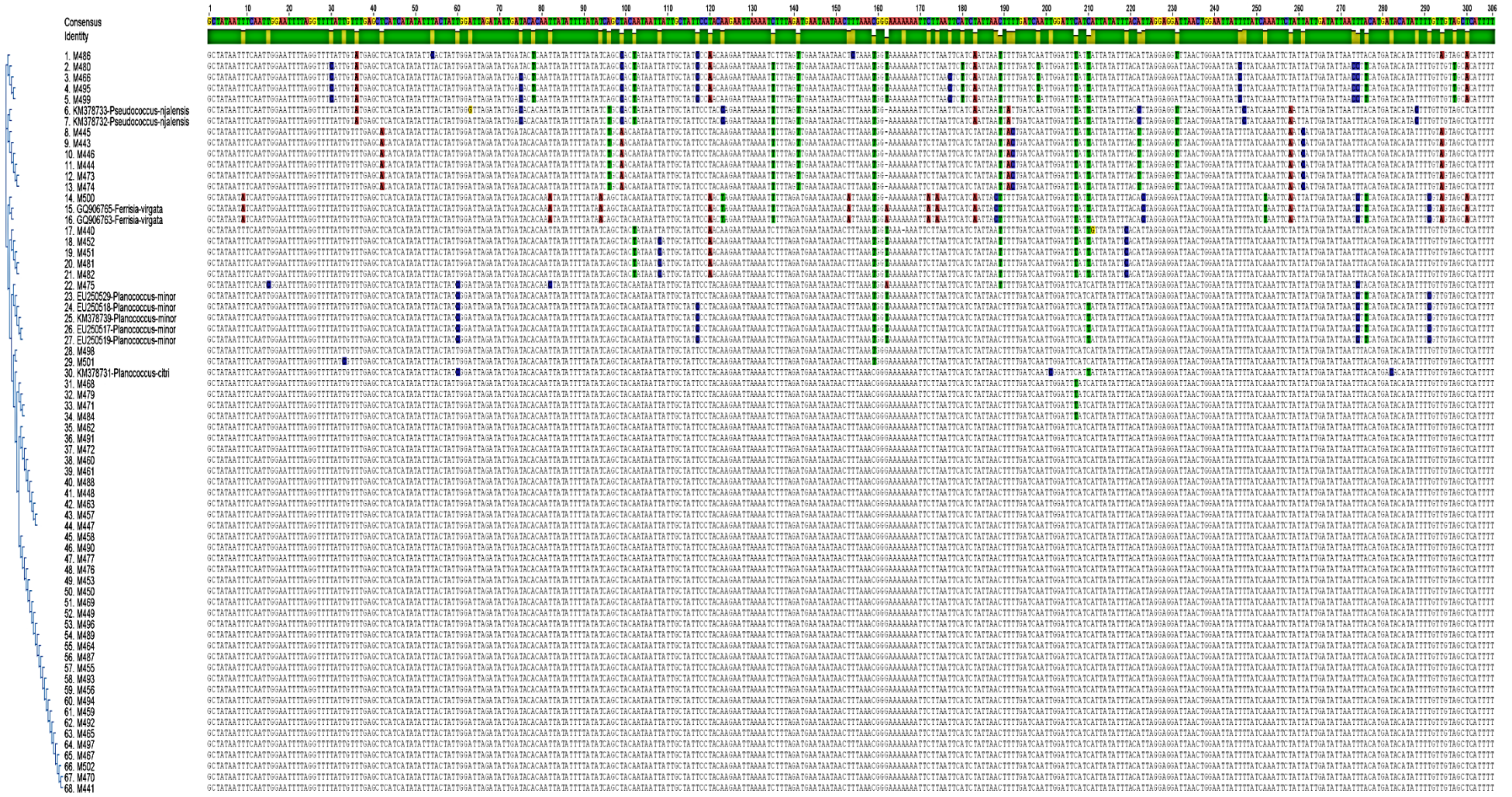

Figure 3.16: Alignment of CO1 sequences (306 bp) from Nigerian mealybug samples taken from cacao.

3.4.5 Mapping mealybugs occurring on cacao in selected sites in Nigeria

The overall geographic location of the identified mealybug species occurring on cacao at sampled sites in the major cacao-producing areas in Nigeria is represented in Figure 3.17**.** This shows the area of coverage during the survey of Nigerian cacao for mealybug collection, spanning from West to South of the country. Table 3.1 shows the abundance of mealybug species in the surveyed cacao-producing areas of Nigeria.

Table 3.1: Mealybug abundance on cacao at selected Nigerian sites based on CO1 PCR screening

***Mealybug species**	****Abundance (%)**
Planococcus citri/Planococcus minor	49.4
Planococcus citri	19.0
Planococcus minor	5.1
Formicococcus njalensis	13.9
Ferrisia virgata	1.3
Paraputo loranthi	1.3
†Unknown	10.1

*CO1 match based on nearest published CO1 sequence except †. **See Appendix C for individual mealybug samples.

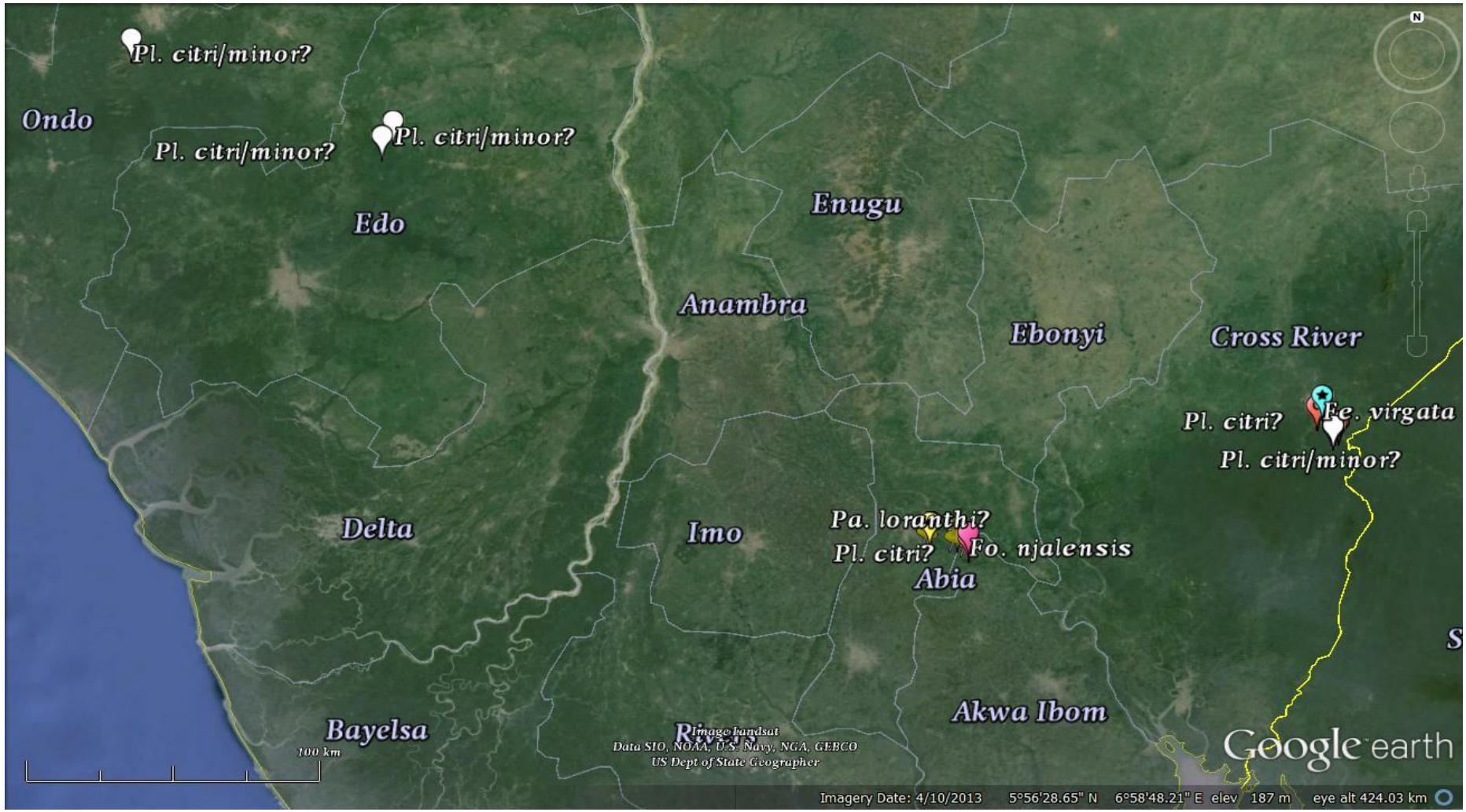

Figure 3.17: Locations of mealybugs collected on cacao in Nigeria in 2012 and 2013 field surveys: *Formicococcus njalensis* (pink), *Paraputo loranthi* (yellow), *Planococcus citri/minor* (white and red), *Ferrisia virgata* (blue).

3.5 Discussion

Information on the presence and geographical distribution of plant pathogens and its vectors are important for planning holistic pest management strategies. In cacao-producing countries around the world there have been regional reports on various pests and diseases of economic importance. The presence of the *Cacao swollen shoot virus* (CSSV) in West Africa was reported over nine decades ago and there have been research efforts directed at the establishment of its genetic details (Hagen *et al*., 1993; Lot *et al*., 1991), the need for early detection (Muller *et al*., 2001), its molecular variability (Muller and Sackey, 2005) with the review and update of information on CSSV presence and spread in the affected areas including Togo (Oro *et al*., 2012b), Côte d'Ivoire (Kouakou *et al*., 2012), Ghana (reviewed by Dzahini-Obiatey *et al*. (2010)) and Nigeria (Dongo and Orisajo, 2007). Interestingly, there has been no information on the molecular diversity of CSSV in Nigeria which is the world's fourth largest producer of cacao.

Observations of CSSV symptoms on an infected cacao plants can be misinterpreted in some cases. For example, the classical broad categorisation of CSSV symptoms into severe and mild infections according to the isolate (strain) of CSSV is barely sufficient in describing possible occurrence of complex viral infection. In addition, the visual identification of cacao plants with latent CSSV infection is also not feasible without the use of either a serological or molecular-based screening technique. As such, the importance of having information on the molecular diversity of CSSV have been highlighted to include the improvement of virus indexing diagnostics using PCR techniques, resistance breeding via screening of potentially virus-resistant cacao genotypes and applications in epidemiological studies, viral pathogen evolution and eradication control measures (Muller *et al*., 2014; Oro *et al*., 2012a).

In the present study, molecular-based screening results (qualitative PCR) have shown that CSSV was detectable in field-collected cacao leaf samples from all the six major cacao producing areas in Nigeria. The detection of CSSV in leaf samples obtained from previously unreported areas points at possible concerns on the current status of CSSV spread in Nigeria.

It was not uncommon to find Malvacean tree species in close association with cacao in farms in Nigeria and the PCR results showed that CSSV was also present in at least one *Ceiba pentandra* tree, one of the pathogen's alternative host species. The present reports from this study on CSSV outbreak in new areas is concerning given the importance of the cacao crop in these areas. The affected areas, Edo, Akwa Ibom and Abia States, have not previously been characterised as regions of CSSV presence and these results bring to attention the need to extensively study the progressive spread of CSSV within Nigerian cacao.

Future studies on the diversity of CSSV in Nigerian cacao should include quantitative PCR screening of already obtained cacao leaf samples from these areas; that could possibly enable the detection of low titre CSSV that may have remained undetected by qualitative PCR. These results could also be improved by putting in place a continuous field monitoring routine for CSSV outbreaks and identification of new and invasive mealybugs on cacao. Also, extending the survey areas to cover neighbouring cacao and non-cacao producing areas including the sampling of alternative hosts could help in exhaustively establishing the widespread presence of CSSV in Nigeria.

There is no published information available on the molecular diversity of CSSV vector mealybug species occurring on cacao in Nigeria and in West Africa as a whole, an area where CSSV is endemic. With the combination of morphological identification and DNA barcoding, the occurrence of the following mealybugs species, *Fo. njalensis*, *Fe. virgata, Paraputo loranthi* and *Planococcus* spp. (*Pl. citri* and/or *Pl. minor*) were confirmed in cacao in Nigeria; their identities and genetic relationships were closely matched with the nucleotides sequences of selected mealybug samples (with NCBI accession number) from America and South America. Though mealybug species were earlier reported to occur in cacao based on their morphological features, such reports may have been subjective due to the inherent limitations associated with the use of visual observations in the identification of similarly appearing mealybug species in a population.

In the past, where a large number of morphological characters were involved in the identification process for mealybugs, discriminant function analyses (DFA) and principal component analyses (PCA) were used and these analyses could barely be useful due to rounding up effects (Padi and Hollander, 1996). A typical example has been where *Pl. citri* species were possibly misidentified as variants, *Pl.* sp. nr. *minor* and *Pl.* sp. A, based on morphological discrimination using DFA and PCA (Padi, 1995a). This error was uncommon due to the lack of molecular tools to aid in the characterisation of these cryptic species with which coinciding morphological characters often exist.

The earliest molecular approach involving gel electrophoresis in mealybug taxonomy were used to determine enzyme inheritance in *Ps. obscurus* Essig. (Nur, 1977a) and to compare mealybug populations of *Ps. obscurus*, *Pseudococcus* spp. and *Pl. citri*, and variants of *Fe. virgata* and *Phenacoccus solani* (Ferris) (Nur, 1977b). The initial attempt to complement the use of conventional morphological features in the separation of diverse mealybug complexes in cacao was later performed using six enzyme groups, esterase (Est), acid phosphatase (Acph), alkaline phosphatase (Alph), fumerase (Fum), malic enzyme (Me) and malate dehydrogenase (Mdh), in a polyacrylamide gel isoenzyme electrophoresis system (Padi, 1994).

Six mealybug species, *Fo. njalensis*, *Pl. citri, Pl. kenyae*, *Ps. longispinus, Ph. madeirensis* and *Dysmicoccus brevipes* (Cockerell), were examined. With the expression of the genes coding the six enzymes which were displayed as electromorphs (bands representing alleles), the mealybug species were distinguished at intra- (nymphs and adults) and interspecific levels. But the challenges and limitations associated with the use of isoenzymes were its suitability for application with a wide range of mealybug species, problems of insufficient sensitivity, data interpretation for highly cryptic species and the absence of commercialised reagents and test kits.

Padi and Sackey (1999) later screened (using electrophoresis) adults and nymphs of *Fo. njalensis* and *Pl. citri* based on their nucleic acid molecular weights following phenol sodium dodecyl sulphate (SDS) extraction and DNase/RNase digest steps, but the expected bands (*ca.* 20 kbp) were indistinguishable for each of the species. They were only able to differentiate the species after using RNase treated DNA templates in PCR reactions involving 20 random oligonucleotide primers. It was then reported that adults and nymphs of *Pl. citri* and *Fo. njalensis* could be differentiated on the basis of the generated random amplified polymorphic DNA (RAPD) in terms of the number of products and molecular weight.

However, the use of RAPD in molecular taxonomy suffers a major setback of having single primers designed to target arbitrary nucleotide sequences; the expected results were prone to non-reproducibility of the assay in subsequent screening attempts. Both RAPD and isoenzyme techniques have been replaced with DNA barcoding which also utilizes PCR but with specific primers designed for the amplification of the mitochondrial cytochrome oxidase 1 (CO1) gene. Primarily, the use of CO1-based DNA barcode assay owes its superiority over isoenzyme to the rapid mutation rate of the CO1 gene coupled with its highly conserved genetic integrity which allows for the discrimination of closely-related species.

In the present study, the CO1 results shows that the mealybug species were distinctly identified showing exact phylogenetic relationship within and between species in Nigeria and mealybug species examined from other cacao-growing areas. The confirmation of the presence of non-indigenous mealybug species in new locations within the cacao growing areas in Nigeria is an indications of the potential spread of CSSV to new areas. Apparently, it is less likely that there are limitations to the extent of movements and hosts colonisation possible by these mealybug species, especially with the juveniles which have been implicated as the most efficient developmental stages in the transmission of CSSV between host plants.

The genus, *Planococcus*, was the most represented in the collection of mealybugs from Nigeria. Results relating to relative abundance of mealybug species within the contemporary Nigerian cacao crop contradicts the earlier of findings of Strickland (1951) which highlighted the dominance of *Pl. citri* in Nigeria. This also contrasts with findings of Bigger (1981) who examined mealybug diversity around Tafo in Ghana from 1971 to 1976 and found *Fo. njalensis,* occurring at the highest density on cacao trees, followed by *Pl. citri* which had a higher probability of occurrence (0.87) over *Fo. njalensis* (0.05). However, it could be argued that there were possibilities of inconclusive results from earlier studies on mealybug abundance which were conducted when conflicting reports on the morphological identity of mealybug species (Cox, 1989; McKenzie, 1967; Thorold, 1975) were commonplace in the literature.

Furthermore, there are practical challenges in accurately estimating mealybug species abundance within orchards given the distinct distribution of particular species and these include tree architecture, environment-related changes, labelling/numbering, costs, labour requirements and sampling methods (Campbell, 1983). One means of circumventing the problems of direct sampling is the use of female sex pheromone baited traps which have been employed to generate detailed information about geographical and temporal variation in the abundance of adult male mealybugs (Bahder *et al*., 2013; Millar *et al*., 2002; Walton *et al*., 2013).

The use of pheromone baited traps to detect, monitor and control mealybug population on hosts has been adopted for various species of mealybugs including *Pl. minor* (Roda *et al*., 2012), *Pl. citri, Ps. longispinus* and *Ps. viburni* (Waterworth *et al*., 2011a). These baits usually serve as lures with relatively long-lasting powerful activities. These lures have been found to be effective in the field for the control of male mealybugs over wide distances; they are cost effective as relatively small doses are required to achieve the same attractant effect as the large doses associated with coleopteran and lepidopteran pheromone lures (Evenden and Gries, 2010; Kawazu *et al*., 2004; Megido *et al*., 2013; Tewari *et al*., 2014; Waterworth *et al*., 2011a).

There is also that specificity in the composition and chemical structure of each pheromone which makes it possible for the attraction of a specific mealybug species to a dedicated lure. This attribute could serve as a veritable tool suitable for inclusion in integrative taxonomy of mealybugs. However, there are also generic pheromone baited lures adaptable for more than one mealybug species (Waterworth *et al*., 2011a).

While pheromone based trapping undoubtedly has an important role to play in terms of mealybug abundance monitoring, its value as a tool for the effective control of viral vectors is open to question. In crop-pest scenarios where the objective is to reduce spoilage, for example a mealybug-induced sooty mould on *Vitis vinifera* L. (Millar *et al*., 2002), any significant reduction in the pest population brought about by pheromone-based trapping is likely to have a beneficial effect. However, more needs to be known about the threshold mealybug vector population size required to achieve CSSV transmission before it would be possible to say whether the use pheromone-based mealybug control measure could fit into an integrated pest management strategy for cacao. This may be quite useful for host plants which often harbour more than one mealybug species, for example cacao. However, care is advised in terms of using custom generic lures as these could eventually attract non-target beneficial insect species that are of economic importance.

Chemical control through pesticide use is still seen as an effective way for mealybug control; especially where zero tolerance is expected in such places as in the quarantines facilities and nurseries. Though the use of foliar pesticides application may be effective in directly reducing mealybug population (abundance) especially where the waxy body coating (mealy) presents insignificant hindrance in the case of juveniles (crawlers), but prolonged usage could result in induced-resistance by the mealybug and detrimental effects on the economic yields of the host crop, for example tainting of cacao beans.

Soil-applied systemic pesticides seems to be favoured over foliar sprays as their effects in mealybugs control are indirect including acting as insect growth regulators, being environmentally-friendly and possibly having a reduced effect on non-target species. In considering mealybug management involving the use of a pesticide, its misuse could be of economic importance to mealybug species, non-target species and the cacao crop. The need to establish threshold levels application could be useful in the understanding the indirect relationships, effects and implications of mealybugs population control in cacao and CSSV transmission.

Further studies should consider exploring the electrical penetration graph (EPG) technology to examine the possible underlying effect of soil-imbibed systemic pesticide application (e.g. imidacloprid) on treated cacao acceptance (non-acceptance) by feeding mealybugs and subsequent transmission (non-transmission) of CSSV. The expected results should be discussed in the light of mealybug species abundance and diversity on cacao with emphasis on the spread of CSSV by vector mealybug species.

Advances have been made in the use of CO1-based DNA barcoding system for mealybug species differentiation. Recently, a rapid and inexpensive version of CO1 barcoding that uses the high-resolution melt analysis technique (Wetten *et al*., 2015), which eliminates the additional time required for DNA sequencing of PCR products, has been established. Wetten *et al*. (2015) have successfully employed the technique in the characterisation of mealybug species obtained from cacao. This approach is positioned as suitable in reducing the time required to characterise a huge number of cryptic mealybug species without losing precision and accuracy in species identification. For future studies where it might be expected that a larger number of mealybug species is characterised, the use of HRMA with CO1-based DNA barcoding could suffice as a cost-efficient and reliable option.

Dependable taxonomic information, once provided, could guide in the adoption of appropriate generic- and/or species-specific management strategies including pheromone-baited traps technology for mealybug species or groups of mealybug species on cacao, with the aim of controlling the spread of CSSV by vector mealybug species.

Chapter 4 MEALYBUG-CSSV-CACAO INTERACTIONS

4.1 Introduction

4.1.1 Highlights on mealybug-CSSV-cacao relationships

In cacao, mealybugs (Pseudococcidae: Hemiptera) have been identified as the only known natural vectors of the *Cacao swollen shoot virus* (CSSV) (Box, 1945; Cotterell, 1943; Posnette, 1941). Mechanical transmission by grafting and budding is also possible but with great difficulty (Adomako and Owusu, 1974; Brunt and Kenten, 1960, 1962; Glendinning *et al*., 1966; Posnette, 1940; Posnette and Strickland, 1948; Wessel-Riemens, 1965). The visual symptoms of CSSV infection on cacao have been the basis of assessing CSSV transmission by mealybugs (Box, 1945; Posnette and Strickland, 1948). Previous reports were based on placing groups of mealybugs to feed on CSSV-infected cacao for a specific duration before moving them onto a cacao plant free of CSSVD symptoms for a certain period of time (Dale, 1962; Posnette and Robertson, 1950; Roivainen, 1969, 1980).

The confirmation of transmission of CSSV by the 'viruliferous' mealybugs between the infected (source) and non-infected (test) cacao plants was based on observing the test plant for typical symptoms of CSSV infection. However, these observations (under field condition) were subjective and may have been prone to, for example, plant nutrient deficiencies being misconstrued as CSSV symptoms (Lockard and Asomaning, 1965). The relative efficiency of CSSV acquisition and its subsequent transmission by vector mealybugs in these studies were only determined based on the resulting symptomatic plants i.e. the infection rate (Posnette and Robertson, 1950); these trials may also have been underestimating transmission events due to their failure to detect all cases of latent CSSV infection in cacao and this might have had implications for CSSV resistance and tolerance studies.

The study of the mealybug-cacao-virus(es) relationship became the only well documented evidence of mealybug-virus-plant relationship at one period (Roivainen, 1980) and it was understood that there was no molecular-based evidence to substantiate the results from earlier studies (Owusu, 1971; Thresh *et al*., 1988b). For example, Roivainen (1969) reported that first instar nymphs of *Fo. njalensis* were still capable of transmitting CSSV, an assumed non-persistent stylet-borne plant virus, after ecdysis; the infection rate of which were not statistically different from unmoulted nymphs. These results from Roivainen's findings could have been validated with molecular tools by screening first for the availability of the virus in the source plants followed by the 'viruliferous' moulted vector mealybug alongside its exuviae and the unmoulted individuals to ascertain the integrity and persistence of the virus particles within the vectors.

In addition, the present day availability of fluorescence microscopy techniques would have also provided useful spatio-temporal information about the feeding efficiency, duration of infectivity, the location and presence of the viral particle within the stylets of the vector mealybug species in Roivainen's study (Roivainen, 1969). Since the use of these molecular screening approaches were not available in the early studies of mealybug-CSSV-cacao interactions, previously established information on the transmission and spread of CSSV infection between cacao plants by viruliferous mealybug species (Owusu, 1972; Roivainen, 1976; Thresh, 1958) could benefit from the application of a molecular-based re-validation approach without undermining the outcomes from the earlier studies.

The first step involved in a vector-virus transmission complex is the acquisition of virions from an infected source (Stafford *et al*., 2012), followed by a stable retention of the acquired versions at specific sites binding the virions to ligands which are then released from the retention sites upon salivation or regurgitation by the vector and delivered to the site of infection in viable cells of the host plant.

Virus transmission is an important step in the biological cycle of plant viruses because it ensures virus maintenance and survival (Andret-Link and Fuchs, 2005; Gutiérrez *et al*., 2013). Virus transmission involves vectors; this is different from the dissemination of plant viruses that is more often directed by human activities, vegetative propagation, global exchange of infected materials, cropping systems and introduction of novel crops either in existing or new agricultural areas.

In an attempt to understand the mealybug-CSSV-cacao interactions, the feeding activities of the vector mealybugs before CSSV acquisition (preliminary feeding or starvation), during (acquisition access period i.e. AAP) and after CSSV acquisition (inoculation access period i.e. IAP) (Roivainen, 1976) have significant roles to play in defining these interactions (Figure 4.1). Strickland (1947) presented the first detailed account of mealybug species occurring on cacao in West Africa. The initial search for the vectors of CSSV led to the identification of two mealybug species, *Fo. njalensis* Laing and *Fe. virgata* Ckll., an aphid (*Toxoptera coffeae* Nietner) and a psyllid (*Mesohomotoma tessmanii* Aulm) as vectors of CSSV 1A strain (Posnette and Strickland, 1948). In a subsequent confirmation study, *Pl. citri* was first pointed out by Box (1945) to transmit the CSSV 1C and 1D strains (Posnette, 1947b) of which Posnette (1947a) further confirmed the status of *Pl. citri* as a known vector of CSSV after the successful transmission of additional CSSV strains.

Inconsistent results (Box, 1945) showed that none of the non-mealybug vectors were capable of transmitting CSSV in subsequently repeated studies. *Pl. citri* is a highly polyphagous mealybug species; with conflicting reports on its origin as reviewed by Franco *et al*. (2004), it has a wider global distribution (CABI, 2015). Previous studies have shown that *Ps. longispinus*, native to Australia (Franco *et al*., 2004), was not among the dominant mealybug species found on cacao (Campbell, 1974, 1983; Cornwell, 1953; Donald, 1955; Strickland, 1947).

However, *Ps. longispinus* was enlisted as a vector mealybug species of CSSV following an extensive CSSV transmission study by Posnette (1950b) involving 17 groups ('strains') of *Theobroma* virus I (i.e. CSSV) from Ghana (8), Nigeria (7) and Cote d'Ivoire (2); this study used 10 species of mealybugs in addition to the already mentioned three species, *Fo. njalensis*, *Fe. virgata* and *Pl. citri*.

The third mealybug species used in the present study is *Ps. viburni*. Though it is not native to the afrotropical cacao-growing areas of West Africa (Miller *et al*., 2014), it is a polyphagous Nearctic (Franco *et al*., 2004; Miller *et al*., 2002) species which has been reported on grapevine in South Africa (Ben-Dov and Hodgson, 1997; Pieterse *et al*., 2010). *Ps. viburni* has not been found on cacao anywhere, neither has it been used in any demonstration study as a vector of CSSV.

4.2 Objectives

This chapter provides findings on molecular-based evidence relating to CSSV acquisition and retention within three mealybug species, two previously classified as 'CSSV-vectors' (*Pl. citri* and *Ps. longispinus*) and a 'non-CSSV vector' *Ps. viburni*, using PCR and qPCR analyses. The objectives of the study were to establish (1) the capacity (if any) for acquisition of CSSV by these species, fed on CSSV-infected cacao and (2) the retention time of CSSV in the viruliferous mealybug species fed on a non-malvaceous species (*S. tuberosum*).

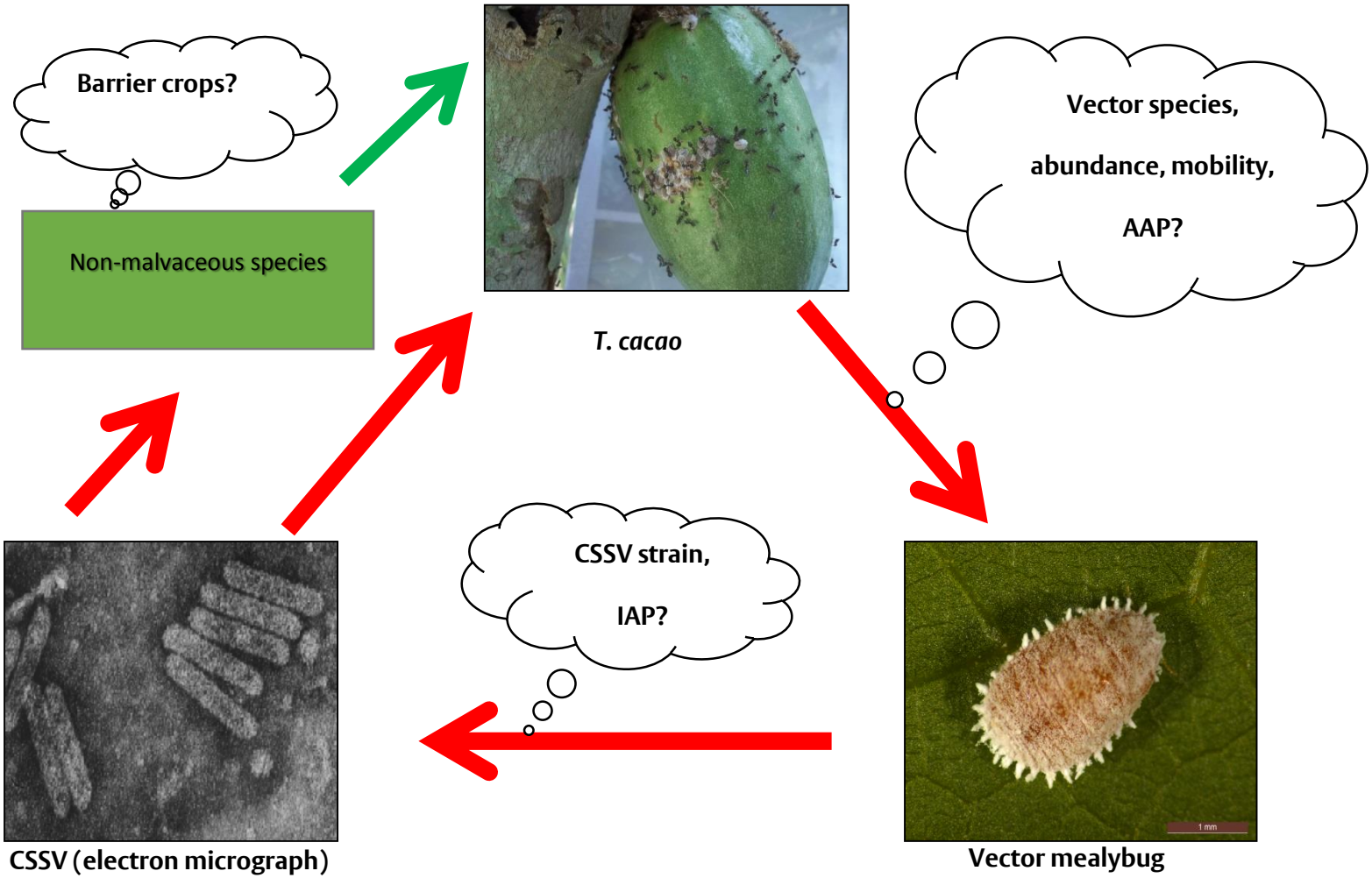

Figure 4.1: A hypothetical description of mealybug-CSSV-cacao interactions with the barrier crop interface. Red arrows show potential vector/CSSV movements and the green arrow indicates potential loss of mealybug infectivity/CSSV

4.3 Materials and methods

4.3.1 Mealybugs culture

Two mealybug species often found in West African cacao, *Pl. citri* and *Ps. longispinus*, were used for the study. The mealybug species, *Ps. viburni,* which has not been reported on cacao in the field, was included as a control for comparative purposes. Females of these three mealybug species were all obtained from cultures maintained in the mealybug husbandry described in Section 2.4.

4.3.2 CSSV-infected and CSSV-free cacao plants

Whole plants and/or leaves of young (3 – 4 months old) CSSV-infected and CSSV-free cacao seedlings (var. Amelonado) were used as source and test plants, respectively. These CSSV-infected plants were raised from CSSV-infected seeds obtained from the Cacao Research Institute, Ghana (CRIG) and the CSSV-free cacao seedlings were obtained from the International Cocoa Quarantine Centre, Reading (ICQC). These plants were maintained following the procedures described in Section 2.2.

4.3.3 CSSV acquisition by mealybugs species fed on CSSV-infected *Theobroma cacao*

Groups of newly moulted 2nd instar CSSV-free female mealybugs were first collected and starved for 24 h. These were loaded into a trap cage made up of an open-end transparent Perspex (acrylic) glass cylinder (h = 0.7 cm, ⌀ = 3 cm). One end of the cylinder was completely sealed off with a fine mesh (0.025 mm) which served as a platform for loading the mealybugs into the trap with 10 mealybugs in each trap. The open end of each mealybug-loaded trap was gently positioned placed over the abaxial side of a CSSV-infected cacao seedling, held in position with lightweight aluminium spring clips. The trap was tapped gently on the mesh fitted end, to dislodge the mealybugs onto the cacao leaf surface. The entire experimental setup, in four replications, was maintained in screen cages inside the growth chamber and the mealybugs allowed in this feeding position on CSSV-infected cacao seedlings for 72 h. These were set for the three species. The traps were then removed and entire individual mealybugs were DNA extracted and PCR-screened for CSSV following the steps described in Section 2.6.

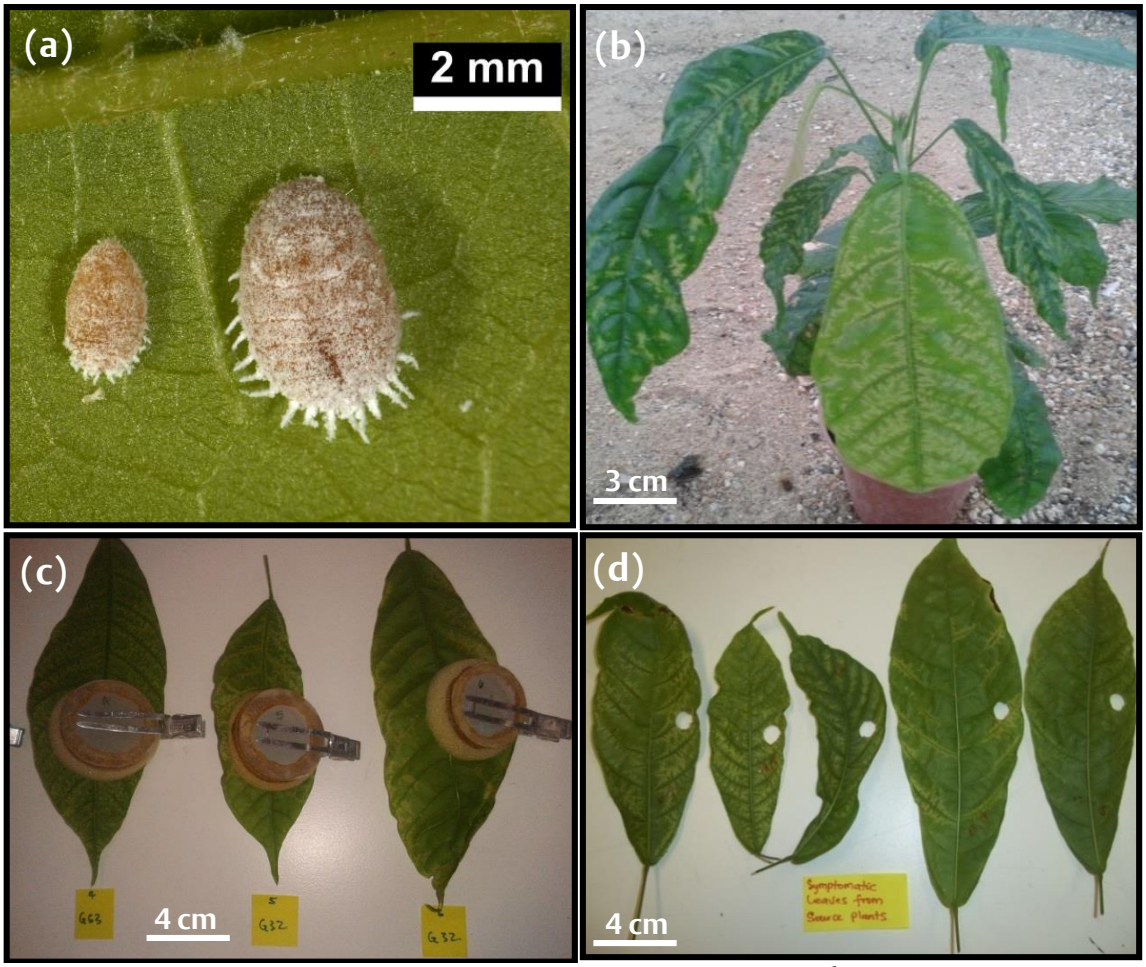

Figure 4.2: Mealybugs and CSSV acquisition feeding: (a) 2nd instar (left) and adult female (right) mealybug on cacao leaf (b) CSSV infected cacao seedling (c) trap-and-clip system (d) disc sampled symptomatic leaf.

4.3.4 CSSV retention by viruliferous mealybugs fed on CSSV-free *Solanum tuberosum*

Groups of newly moulted 2nd instar mealybugs were collected, starved for 24 h then given a 72 h virus Acquisition Access Period (AAP) on cacao seedlings (var. Amelonado) infected with the New Juaben strain of CSSV as described in Section 4.3.3. The 'viruliferous' mealybug individuals were then transferred to *S. tuberosum* sprouts (a non-CSSV host) (Figure 4.3). Thereafter, at daily intervals over a six day period, individuals were destructively sampled and PCR-screened for CSSV presence prior to qPCR analysis.

4.3.5 PCR and qPCR analyses

Mealybug DNA samples were first screened with conventional qualitative PCR using primers designed to produce a 500 bp fragment upon detection of a DNA sequence from Open Reading Frame 1 (ORF1) of CSSV isolate 'New Juaben' (Section 2.6). The qPCR analyses were performed using the following primer pair: forward (CSSVORF1Sf): 5'-ACC AAG CAG TTC CGA GAA AAC-3' and reverse (CSSVORF1Sr): 5'-GGC TCA TTG ACC ACC CAT TG-3'. These primers were designed within this sequence to yield a 130 bp fragment, a size optimised for real time PCR (qPCR) which was performed on the same mealybug DNA extractions using SensiMix™ SYBR® and Fluorescein kits (Bioline) on a QIAGEN Rotor-Gene 6000 thermocycler.

Each 25 µl qPCR reaction consisted of SensiMix™ (12.5 µl), ORF1 forward and reverse primers (2mM, 2.5 µl), ddH_2O (5 µl) and DNA (5 µl). Master mix solutions comprising SensiMix™, primers and water were prepared in excess for the 72 x 25µl reactions that were conducted for each run of the Rotor-Gene. Mealybug DNA samples were run in duplicate and each run also included a dilution series of the source plant (CSSV-infected) DNA diluted at 10^{-1}, 10^{-2} and 10^{-3} (each in duplicate).

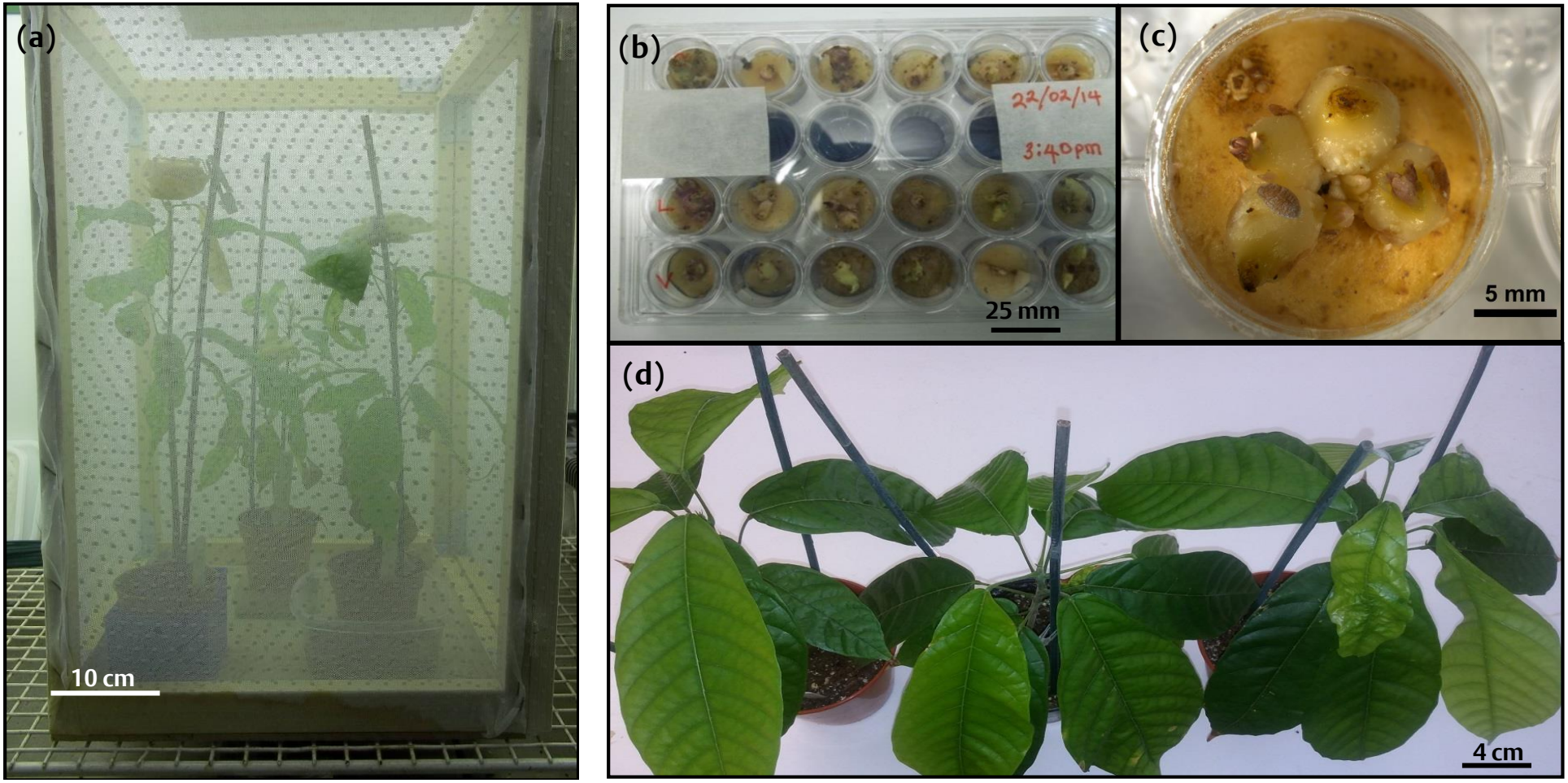

Figure 4.3: (a) Screen cage with *in situ* CSSV infected seedling carrying mealybugs in clip cages, (b and c) viruliferous mealybugs on cacao-free diet (*Solanum tuberosum* sprouts) and (d) CSSV-free cacao (var. Amelonado) seedlings (test plants).

4.4 Results

4.4.1 Characterisation of CSSV PCR and qPCR products

Primers for the qualitative PCR were designed in the conserved regions of the six published sequences of the CSSV genome (National Centre for Biotechnology Information (NCBI) accession number AJ608931) (Hagen *et al*., 1993; Muller and Sackey, 2005); with an expected product size of *ca.* 500 bp. Figure 4.4 shows the gel electrophoretic image of the expected product size for CSSV positive and negative control samples. A portion of the 180 bp fragment sequence used for the qPCR is shown in Figure 4.5.

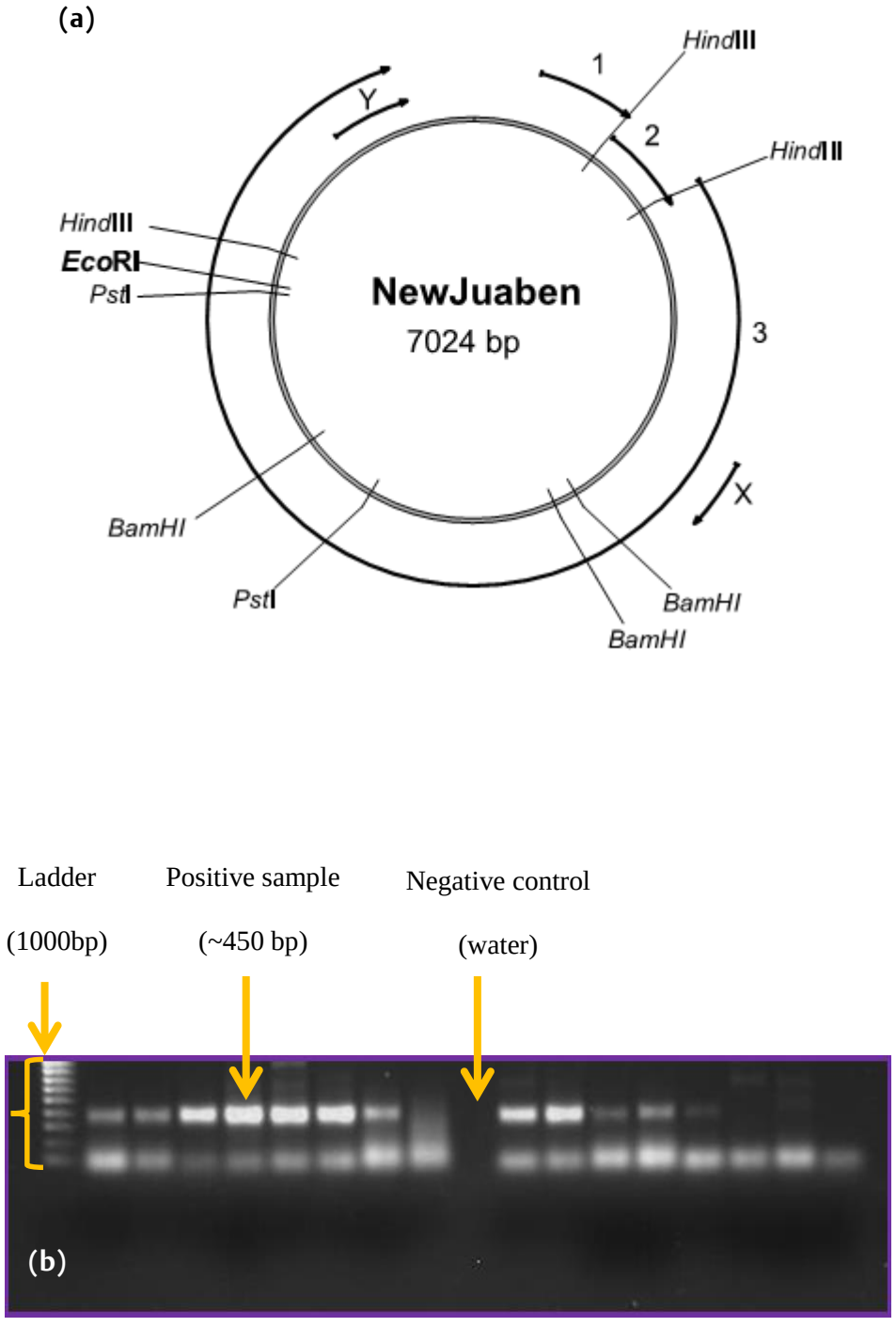

Figure 4.4: (a) The genome of the New Juaben isolate (Muller and Sackey, 2005) utilised in the present study, showing sites of the restriction endonucleases, *Bam*HI, *Eco*RI, *Hind*III, *Pst*I and the five open reading frames (ORFs) 1, 2, 3, X and Y. (b) An agarose electrophoretic gel image showing expected product size for OR1 primer against CSSV-positive samples and control (DNA template-free) sample.

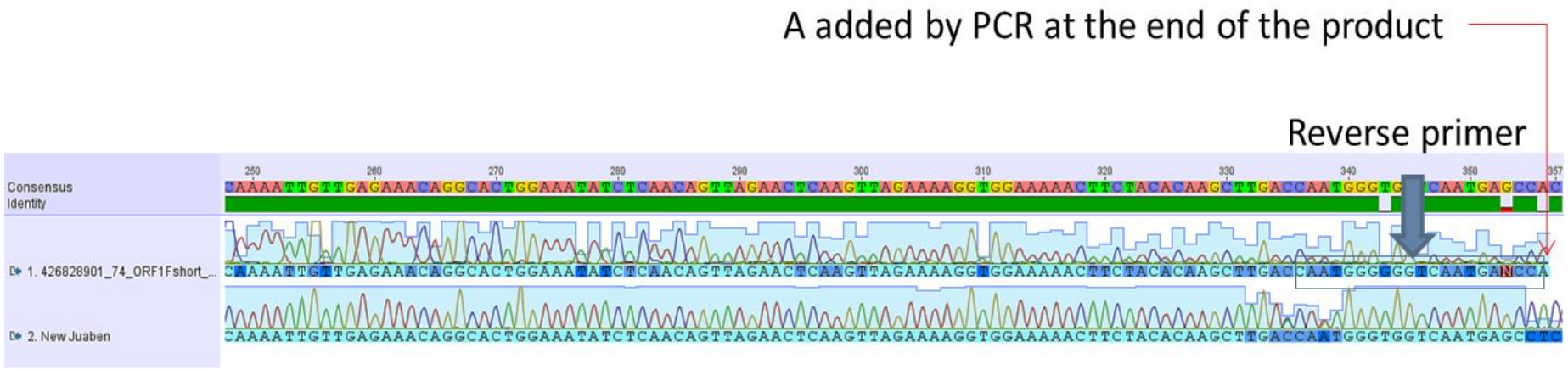

Figure 4.5: Portion of read from Sanger sequencing of 180 bp fragment produced with 'CSSVORF1Sf and CSSVORF1Sr primers with *Planococcus citri* sample 74 (144 h post-AAP on cacao-free diet) showing 100% homology with published sequence for the New Juaben strain of CSSV (NCBI accession number AJ608931).

4.4.2 Molecular evidence of CSSV acquisition and retention by mealybugs

It was demonstrated that *Ps. viburni* can acquire and also retain CSSV in a similar manner to *Pl. citri* and *Ps. longispinus* (Figure 4.6). The potential infectivity status of viruliferous mealybugs for all the three species was apparently not lost after feeding on the non-cacao diet as shown by the ORF1 PCR and qPCR screening results. Individuals of all three mealybug species showed evidence of CSSV retention after transfer to a potato tuber diet (in which the virus will not proliferate) (Figure 4.6). PCR products of the expected ca. 500 bp size (comparable with the positive controls from the CSSV+ donor cacao) generated from mealybug extractions, when purified and submitted for DNA sequencing (conducted by SourceBioscience, Oxford) were 100% matches with that of the CSSV isolate 'New Juaben'. Products of a non-target size (e.g. sample 6 in Figure 4.6 which can be seen to be below the 300 bp size maker) did not produce sequences that matched any badnavirus DNA sequences.

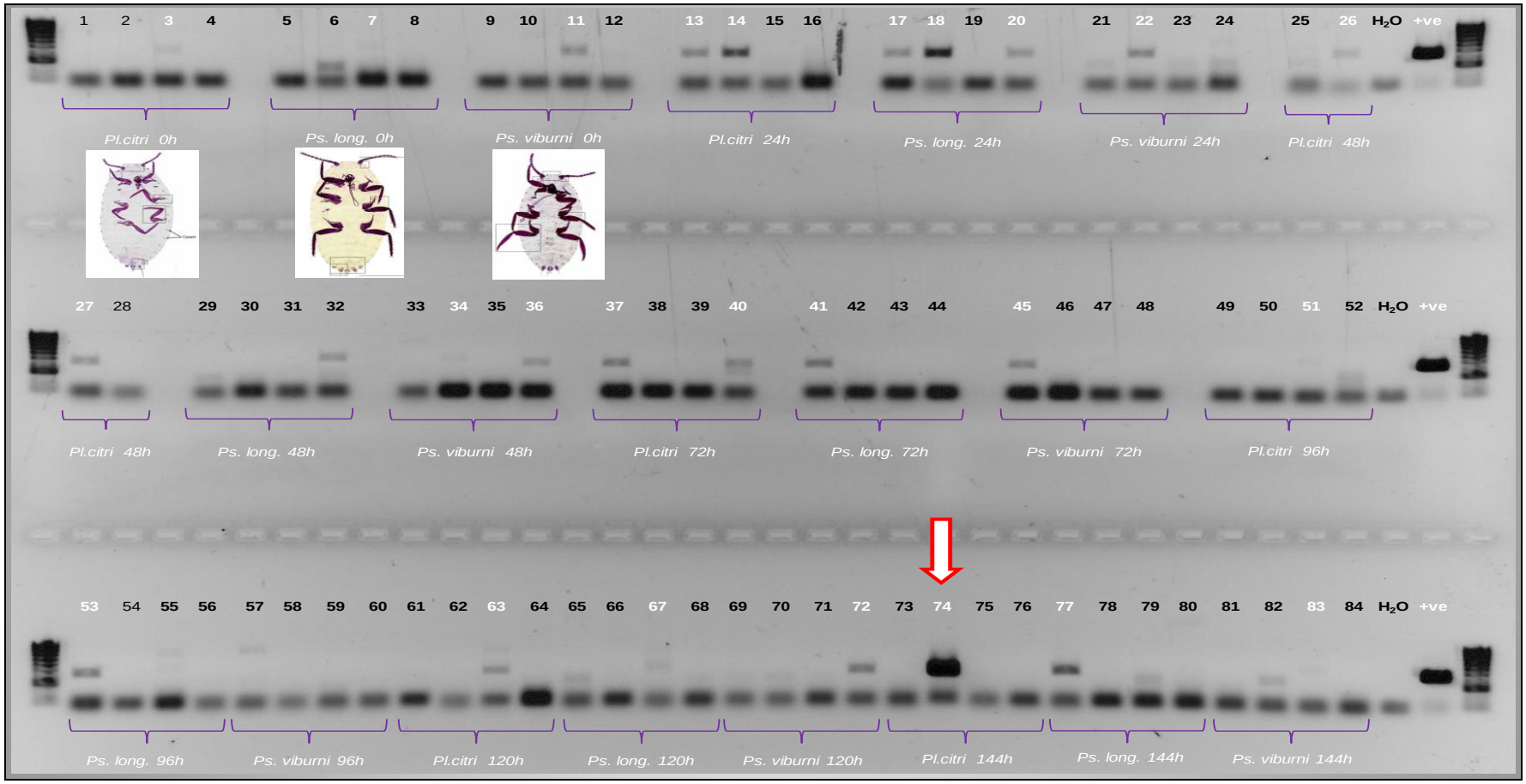

Figure 4.6: Retention of CSSV by 3 mealybug species fed on virus-infected cacao, then transferred to CSSV-free diet for 0 to 6 days as determined by qualitative PCR (white numbers = CSSV+ mealybugs). Arrow highlights particularly strong product from 144 h *Planococcus citri* individual (sample 74).

Real time PCR analysis of the same samples began with a dilution series of the source CSSV+ cacao leaf (Figure 4.7). The quantitative analysis worked as expected with duplicated samples amplifying at approximately the same position and the increasingly dilute CSSV samples (10^{-2} and then 10^{-3}) not amplifying until after further cycles of PCR amplification (Figure 4.7a). Products from mealybug samples were most frequently generated at levels that fell between those of the 10^{-2} and 10^{-3} dilutions of the source leaf DNA values (Figure 4.7b). The qPCR primers were used to generate products from these mealybug samples for sequencing using conventional PCR (because real time PCR products are not directly available for sequencing) and when the resultant sequences of the 130 bp products were assessed they were 100% matches with that of the CSSV isolate 'New Juaben'. Melt analysis of the real time PCR products was conducted and only those that showed a melt temperature that did not diverge significantly from that of the CSSV+ positive control (*ca.* 80°C) were recorded as positive.

There was consistency between the two methods of sample analysis with, for example, sample 74 (a *Pl. citri* individual fed on potato for 144 h) producing the particularly intense band on agarose following conventional PCR (Figure 4.6) and also generating the highest real time PCR signal of all the 84 mealybug samples analyzed (Figure 4.7c). However, the greater sensitivity of the real time PCR analysis meant that for each species time point the number of mealybug samples positive for the presence of CSSV DNA was equivalent to or greater than that shown by conventional PCR with all three species exhibiting viral retention six days after leaving their CSSV-infected food supply (Table 4.1).

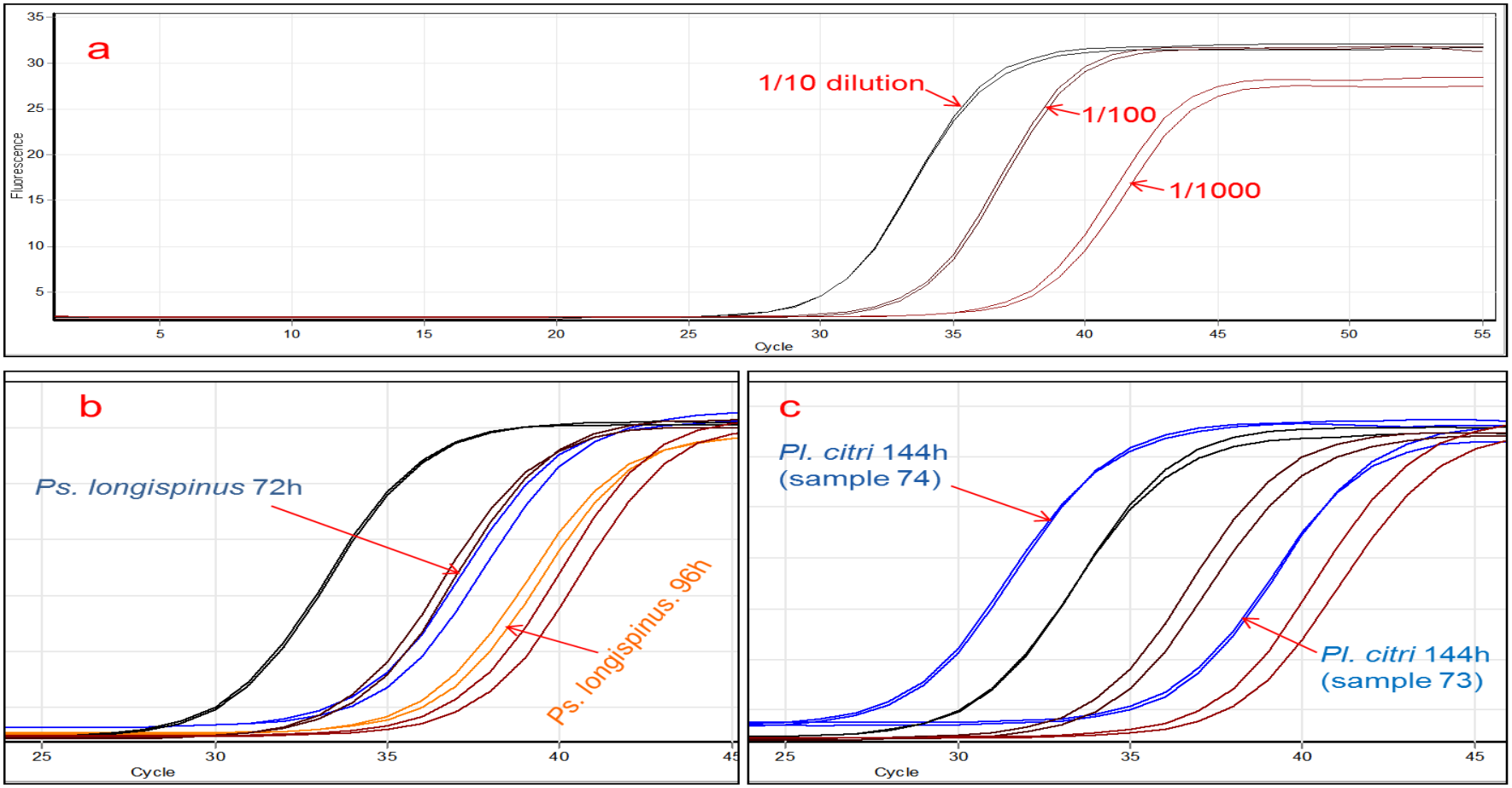

Figure 4.7: Retention of CSSV by mealybugs as determined by real time PCR. (a) Each run of up to 72 reactions included duplicated reference samples from the CSSV infected source leaf dilutions 10^{-1}, 10^{-2} and 10^{-3}. (b) CSSV levels in these 72 h and 96 h *Pseudococcus longispinus* samples were between those of the 10^{-2} and 10^{-3} leaf dilutions. (c) Sample 74 showed the highest CSSV concentration and samples such as 73 (apparently virus-free according to the qualitative PCR) showed detectable CSSV via real-time analysis.

Table 4.1: Retention of CSSV in three mealybug species fed on CSSV+ cacao for 72 h followed by transfer to a potato diet for 0 to 144 h, as detected by conventional and real-time PCR (data in parentheses). n=4

	Duration on potato diet after transfer from CSSV source plant (h)						
Mealybug species	**0**	**24**	**48**	**72**	**96**	**120**	**144**
Planococcus citri	1/4 (3/4)	2/4 (2/4)	2/4 (4/4)	2/4 (2/4)	1/4 (3/4)	1/4 (3/4)	1/4 (3/4)
Pseudococcus longispinus	1/4 (2/4)	3/4 (4/4)	-/4 (2/4)	1/4 (4/4)	1/4 (4/4)	1/4 (2/4)	-/4 (2/4)
Pseudococcus viburni	1/4 (3/4)	1/4 (3/4)	2/4 (3/4)	1/4 (3/4)	-/4 (3/4)	1/4 (1/4)	1/4 (1/4)

'-' = PCR negative

4.4.3 Screening of cDNA from CSSV infected cacao with CSSV-specific primers

Total genomic DNA and cDNA from leaf samples of CSSV infected seedlings we screened against the four CSSV specific primers (Table 2.1), ORF1, MP, RNase and RTase. Electromorphs of the PCR products are shown in Figure 4.8. The result demonstrated that the approach for detecting CSSV presence from the DNA extracted from an infected cacao leaf could work as well with cDNA synthesis from the RNA extracted from the same infected cacao leaf.

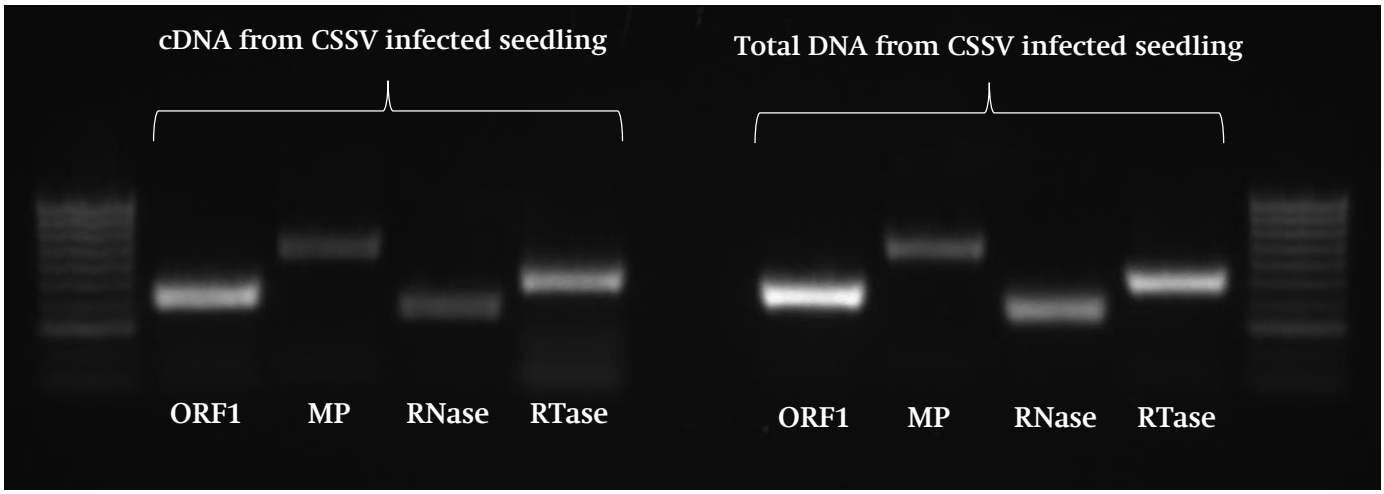

Figure 4.8: Comparison of CSSV-screened PCR products from total genomic DNA and cDNA of CSSV infected cacao seedling

4.5 Discussion

The effectiveness of mealybugs as virus vectors is said to be species dependent and varies according to their favoured feeding sites on the cacao plant and with respect to the age of the plant (Roivainen, 1980) and the determinants of the mode of virus transmission and infection by vectors are retention time, site of retention and internalization of viral particles (virions) by vectors (Andret-Link and Fuchs, 2005). In non-circulative stylet-borne viruses, the virions are dislodged during a sustained feeding duration and it is presumed that on subsequent ingestion, these virions are not reacquired by previously occupied positions on the stylets once they have been released (Gray and Banerjee, 1999). Differences in cacao infection rates, for example, have been observed between the mealybug vectors *Formicococcus njalensis* and *Ferrisia virgata* with distinct stylet dimensions and frequency of phloem penetration being proposed as the cause (Entwistle and Longworth, 1963).

In previous studies, *Fo. njalensis* and *Pl. citri* were considered to be the most important viral vectors on cacao as, at the time, they were generally the predominant mealybugs on the crop in the Afrotropical region (Bigger, 1981; Campbell, 1974, 1983; Strickland, 1947; Sutherland, 1953) respectively. Persistence of the virus within the vectors is reported to differ between the two species with *Fo. njalensis* showing a gradual decline in infectivity up to 18 h post acquisition feeding while *Pl. citri* transmission rates over a similar period were constant (Posnette and Robertson, 1950).

However, of these two species, while *Pl. citri* is usually present in lower numbers in West Africa (Strickland, 1951), its greater mobility and ability to infest new cacao trees make it potentially the more important virus vector. Reports have indicated that, in keeping with its classification as a semi-persistent virus, CSSV is retained by its mealybug vectors for only a matter of hours or days after feeding on an infected source plant ceases.

Roivainen (1969) (reviewed by Roivainen (1980)) recorded the longest period of CSSV retention to date when he reported that infectivity persisted in starved nymphs of *Fo. njalensis* until 72 h after virus acquisition. The present study demonstrated that all three mealybug species under test were capable of retaining viral DNA until at least 144 h after virus acquisition and that was in a scenario more analogous to a cacao plantation-border crop i.e. with mealybugs continuing to feed on a non-malvaceous crop. The current work also demonstrated that conventional qualitative PCR can be used to detect CSSV DNA in mealybugs that have fed on virus infected cacao. This approach also indicated that for two of the three test species when mealybugs were switched from CSSV+ cacao hosts to a potato tuber diet it was still possible to detect CSSV DNA in their bodies six days later.

When qPCR was applied to the same samples the superior sensitivity of that screening approach revealed that many of the apparently virus-free mealybugs still contained detectable levels of CSSV DNA. This suggests that the majority of viruliferous juvenile mealybugs retain at least some of their viral load until they next undergo ecdysis (skin shedding) when they lose the lining of the foregut where the virus is thought to be retained. This will be investigated in more detail with planned qPCR analysis of samples from a trial in which mealybugs were fed on a CSSV+ cacao source and then DNA extracted following ecdysis (the putative semi-persistent nature of the virus will be confirmed if CSSV is exclusively detected in the shed skins).

However, it has been established in the present study that *Ps. viburni* has the potential of acting as a vector of CSSV between cacao plants as shown by its ability to acquire and retain the virus. Interestingly it was reported that *Ps. viburni* and *Ps. longispinus* have a similar 16S rRNA sequence of the same primary endosymbiont, '*Candidatus* Tremblaya princeps' (Gatehouse *et al*., 2012), a β-proteobacterium present in *Pl. citri* (Lopez-Madrigal *et al*., 2011; Thao *et al*., 2002; von Dohlen *et al*., 2001).

These three species also act as vectors of *Grapevine leafroll-associated virus 3* (Gatehouse *et al*., 2012; Tsai *et al*., 2010) and this could partly explain why *Ps. viburni*, not native to the cacao producing areas of West Africa, is also able to acquire and retain CSSV. The information obtained in the present study on the acquisition and retention of CSSV by mealybugs previously implicated as vectors has now been validated with molecular evidence. This will be useful in planning future transmission studies targeted at other mealybug species with the aim of testing their 'potential' infectivity. Although *Ps. viburni* is not present in the main West African cacao growing countries, its presence in areas relatively close to the cacao growing belts, in Africa (Morocco, Saint Helena, South Africa and Zimbabwe), South America (Brazil, Costa Rica, Guatemala, Jamaica, Mexico, Panama, Peru, Venezuela) and Asia (Indonesia, Philippines, Sri Lanka) (EPPO, 2015) and its demonstrated ability to acquire and retain CSSV could jointly have implications for its emergence as an invasive pest on cacao of which its ecological adaptation could be driven by climate change towards the cacao-growing areas.

The influence of climate change on mealybug distribution has been reported on 'potential' invasive species including the pink hibiscus mealybug, *Maconellicoccus hirsutus* (Green) in Chile (Jara *et al*., 2012), the cassava mealybug, *Ph. manihoti* Matile-Ferrero in Asia (Parsa *et al*., 2012) and the cotton mealybug, *Ph. solenopsis* Tinsley (Fand *et al*., 2014) in India. Given that the capacity to control climate change effects on agroecology is limited, it may be appropriate to consider measures and management options for *Ps. viburni* invasion in the future, possibly including eradication, containment and integrated pest management strategies.

Together with the results from the present work that indicate long viral retention times for *Ps. viburni* and the two known vectors these findings suggest efforts to locally eliminate individual mealybug species (e.g. through the use of a sterile male release approach) may be undermined by the possible subsequent occupation of niches by sympatric mealybug species and/or immigration of species from further afield.

The use of predators and parasitoids in the control of mealybugs, for example *Pl. citri*, has met with minimal success partly due to the fact that its origin remains unknown (Franco *et al*., 2004; Franco *et al*., 2009) and there is the difficulty to acclimatize its natural enemies (predators and parasitoids) under different temperature requirements to match the mealybug population on susceptible host plant (Mendel *et al*., 1999). In addition to the expense associated with the rearing and release of mealybugs predators and parasitoids, it was also reported by Mendel *et al*. (1999) that the augmentation of the release of mealybug parasitoids could not bring about the expected result of reducing the population of *Pl. citri* on *Citrus* spp on the field.

With their hydrophobic wax coating (Cox and Pearce, 1983), defending attendant ant species (Hanna *et al*., 1956) and herbivory of a crop the fruit of which must be protected from chemical tainting, mealybug CSSV vectors present a challenging target for control by insecticide application. With the use of the most widely employed chemical control (Imidacloprid) becoming increasingly restricted, precise information about the effectiveness of new alternative pesticides becomes a priority. The application of the EPG monitoring technique will be extended to study insecticide impact on mealybug feeding. EPG monitoring of feeding hemipterans provides the most sensitive means of determining the effectiveness of candidate insecticides and can support the more targeted, environmentally sensitive, and economical use of such chemicals. By time course EPG analysis of mealybugs feeding on cacao treated with, for example, soil drench insecticide applications, threshold control concentrations may be accurately determined; this may reduce the occurrence of insecticide resistance which has been reported in mealybugs, for example *Pl. citri* (Mendel *et al*., 1999).

Further studies will be required to build knowledge of the vector and mechanism of CSSV infection. Since it has now been demonstrated in the present study that CSSV can be detected in both total genomic DNA and cDNA synthesised from the RNA extracted from CSSV infected cacao plant, it will then be suitable to use this approach straight away on testing cacao plants (CSSV-free) after CSSV inoculation by viruliferous mealybugs. Subsequently, this could be useful for CSSV screening purposes in breeding for resistance to CSSV (and its vectors) on different cacao genotypes.

Another suggestion will be the use of EPG to monitor and characterise mealybugs feeding (including analysis of the most widespread vector, *Fo. njalensis*) on different genotypes of cacao, and the time taken to achieve infection which is presented in the Chapter 5. In addition, the outcome of the combined EPG and systemic pesticide application study on cacao would inform parallel field experiments being set up in West African cacao-growing countries, for example in Côte d'Ivoire, an area that is presently responsible for the highest annual global cocoa bean production.

Chapter 5 Characterisation of Feeding Behaviour and Pattern of Mealybugs on Cacao Using Electrical Penetration Graph (EPG) Analysis

5.1 Introduction

The electrical penetration graph (EPG) technique provides real-time monitoring of feeding behaviour of insects with piercing/sucking mouthparts. The set-up basically consists of a gold wire-tethered insect, electrified substrate (the live plant, detached plant part or artificial diet chamber), an electrical circuit (DC or AC), and display monitor and waveform recorder. It allows for precise monitoring of stylet position and activity within the plant tissue. This chapter aimed to characterise the feeding behaviour and pattern of individual immature citrus mealybugs, *Planococcus citri* (Risso), long-tail mealybugs, *Pseudococcus longispinus* (Targioni-Tozzetti) and obscure mealybugs, *Ps. viburni* (Signoret) females on leaves of cacao plants using the DC-EPG technique.

5.1.1 History and development of EPG

Hemipteran feeding and probing behaviour was first monitored electrically in early 1960 following the development of electronic measuring/monitoring system (EMS) for aphid by D. L. McLean and M. G. Kinsey (McLean and Kinsey, 1964, 1965). This involved the introduction of a low voltage into a specific feeding substrate with the use of a 6V AC transformer alongside a potentiometer, attachment of insect (at the dorsum) to a thin wire onto a strip-chart recording device, and monitoring the patterns and voltage level fluctuation of the probing insect which was used in the description of the salivation and ingestion activities of the insect. Histological localisation of the stylets of the feeding (probing) insects was later used to bolster the evidence of the correlation between voltage fluctuations and a particular feeding activity within host plant tissues (McLean and Kinsey, 1967).

Though the initial apparatus for AC EMS was later modified (Backus and Bennett, 1992; Brown and Holbrook, 1976; Kimsey and McLean, 1987) and renamed electrical penetration graph (EPG) technique (Tjallingii, 1978), the operations of a typical AC EPG system are similarly governed in principle by the modulation (fluctuation) of the amplitude of a weak AC (low alternating voltage and high frequency) in a plant which synchronises with the changes in conductivity within the plant and insect in a complete circuit (McLean and Weigt, 1968). The processed voltage changes over time (i.e. signals) are then depicted as conductivity changes, devoid of the AC frequency, at the insect-plant interface.

The first DC powered system was originally designed to study the acquisition of a semi-persistently transmitted *Secoviridae* (Sadwavirus), *Strawberry mottle virus* (SMoV), by strawberry aphids, *Chaetosiphon* (*Pentatrichopus*) *fragaefolii* (Cockerell), feeding on *Fragaria vesca* L. (Schaefers, 1966). The system was further developed by adapting the amplifier's input specifications to aphids' stylet penetration (Tjallingii, 1978). The distinguishing features of the DC system included the exclusion of a transformer and potentiometer, avoidance of extensive grounding and the flexibility to construct a more portable device. The circuitries in the AC and DC systems are basically similar except for the replacement of DC's voltage source by an oscillator in the AC system (Tjallingii, 2000); there is an electromotive force (emf) component in addition to the resistance (R) component in the DC system. Backus and Bennett (2009) later designed a combined AC-DC EPG monitor system which could process signals from AC and/or DC voltage supplied to a substrate. Both AC and DC EPG systems enable the presentation of real-time information between biological phenomena and the fluctuations of electrical voltage (waveform) (Walker, 2000), especially for the feeding behaviours which characterises piercing and sucking insects such as aphids and mealybugs (Calatayud and Ru, 2006).

5.1.2 EPG analysis of mealybug feeding

In addition to the differences in stylet morphology and functioning, the nature of feeding in mealybugs is considered to be intermediate between aphids and whiteflies. For example, adult female mealybugs perform many intracellular cell punctures and transmit a greater number of semi-persistent viruses than whiteflies, but fewer than aphids (Andret-Link and Fuchs, 2005; Stafford *et al*., 2012). While the virus acquisition rate in whiteflies takes a longer duration than that of mealybugs, apparently, mealybugs have the lowest rate of virus inoculation (Cid and Fereres, 2010; Roivainen, 1980) in comparison to aphids and whiteflies. These characteristics may help explain why mealybugs are not reported as vectors of non-persistent, non-circulative plant viruses.

In understanding the virus transmission specificity in hemipterans with piercing and sucking mouthparts, there is an observational difficulty and molecular complexity in studying their feeding behaviour on various host plants. Though many of the initial applications of EPG were in aphid-plant-pathogen interaction studies, EPG recordings coupled with light microscopy for mealybugs was first reported by Calatayud *et al*. (1994) while monitoring cassava mealybugs (*Phenacoccus manihoti* Matile-Ferrero) on common and intermittent host plants.

It was also shown that the stylet penetration activity of *Ph. manihoti* is dominated by intercellular movement (Calatayud *et al*., 1994), in part facilitated by the degradation of the pectin layer bond between adjacent cells (middle lamellae) in leaf tissues by the secretion of the salivary enzyme, pectinesterase (Calatayud *et al*., 1996). Studies on characterising mealybug feeding behaviour (Huang *et al*., 2012), insect-host resistance and recognition have also been made possible with EPG in combination with live video recording (Renard *et al*., 1998).

It has been demonstrated using EPG and live recording of the stylet penetration activity performed by mealybugs that host selection is based on a series of chemically controlled sensory (Le Rü *et al*., 1995) and physical events including the vibration and tapping of their antennae tips and labium on the leaf surface (phylloplane) of the host plant (Renard *et al*., 1998).

Mealybug tending by ants in the field is a common mutualistic and symbiotic relationship between these species where the latter harvests the honeydew produced by the former while, in return, protection against predatory species is provided (Adenuga, 1975; Bigger, 1993; Way, 1963). The veracity of these relationships have been established through observed fluctuations in the population density of the ants as a result of either mealybug abundance or honeydew availability (production), while information on the actual feeding behaviour of the mealybugs either in the presence or absence of the ants were only passively obtained, both in field and laboratory conditions (Zhou *et al*., 2012a; Zhou *et al*., 2013; Zhou *et al*., 2012b).

However, the use of EPG made it possible to study the direct effects of ants' presence on the probing behaviour of mealybugs on a particular host plant (Wu *et al*., 2013). EPG-based analysis of mealybugs has also proved to be valuable in quantifying their host acceptance rate (Huang *et al*., 2014b), food resource preference under stressed conditions (Huang *et al*., 2014a), transmission mechanisms of plant viruses by mealybug vectors (Cid and Fereres, 2010; Sandanayaka *et al*., 2012) and its combination with molecular-based diagnostic tools has helped in studying the host's defence response systems (Zhang *et al*., 2015) which could be explored in resistance breeding studies. Overall, EPG studies on mealybugs are fewer than those with aphids which provide the basis for other hemipterans' EPG waveform comparisons.

5.2 Objectives

The objectives of studying the feeding behaviour of the three mealybug species, *Pl. citri*, *Ps. longispinus* and *Ps. viburni* on cacao were to (1) establish the basic EPG waveforms for mealybugs feeding on cacao (2) test the interspecific differences, based on EPG waveforms, between *Pl. citri*, *Ps. longispinus* and *Ps. viburni* feeding on cacao and (3) compare the EPG waveforms with those obtained from other hemipterans, especially *Pl. citri* and *Ps. longispinus* feeding on hosts other than cacao.

5.3 Materials and Methods

5.3.1 Mealybug preparation for EPG

Second instar female mealybug individuals were collected from the insectary and starved inside sealed sterile polystyrene petri dishes (90 mm x 15 mm), kept in the same conditions in the husbandry. After 24 h the mealybugs were immobilized by placing the petri dishes in the refrigerator at 4°C for 20 – 30 min. Under a stereomicroscope, a single individual was handled at a time for the cleaning and tethering procedures. An area on the dorsum of each of these was cleaned of excess wax covering to allow for an easy tethering of the gold wire using silver based glue (EPG Systems, Wageningen, Netherlands). The cleaning solution consisted of 2 µl Tween 20® (Polyoxyethylenesorbitan monolaurate) (Sigma-Aldrich, USA), 0.5 ml 99.9% ethanol and 1.5 ml ddH_2O. Young (4 - 6 weeks old) CSSV-free potted cacao seedlings (var. Amelonado) were set inside a Faraday cage for the EPG recordings following the procedures described in the EPG manufacturer's guidelines (EPG System Manual for Giga-4/8, March 2013 edition). The Faraday cage consisted of a cuboidal aluminium frame covered on all sides with a 1 mm mesh-size aluminium sheet. Its use was to shield the amplification of electromagnetic noise against the desired EPG waveform. The tethered individual mealybug was placed on the abaxial surface of a clamped, inverted fully-expanded single cacao leaf (positioned in the mid-stem) where it was capable of ambulation and settling to probe.

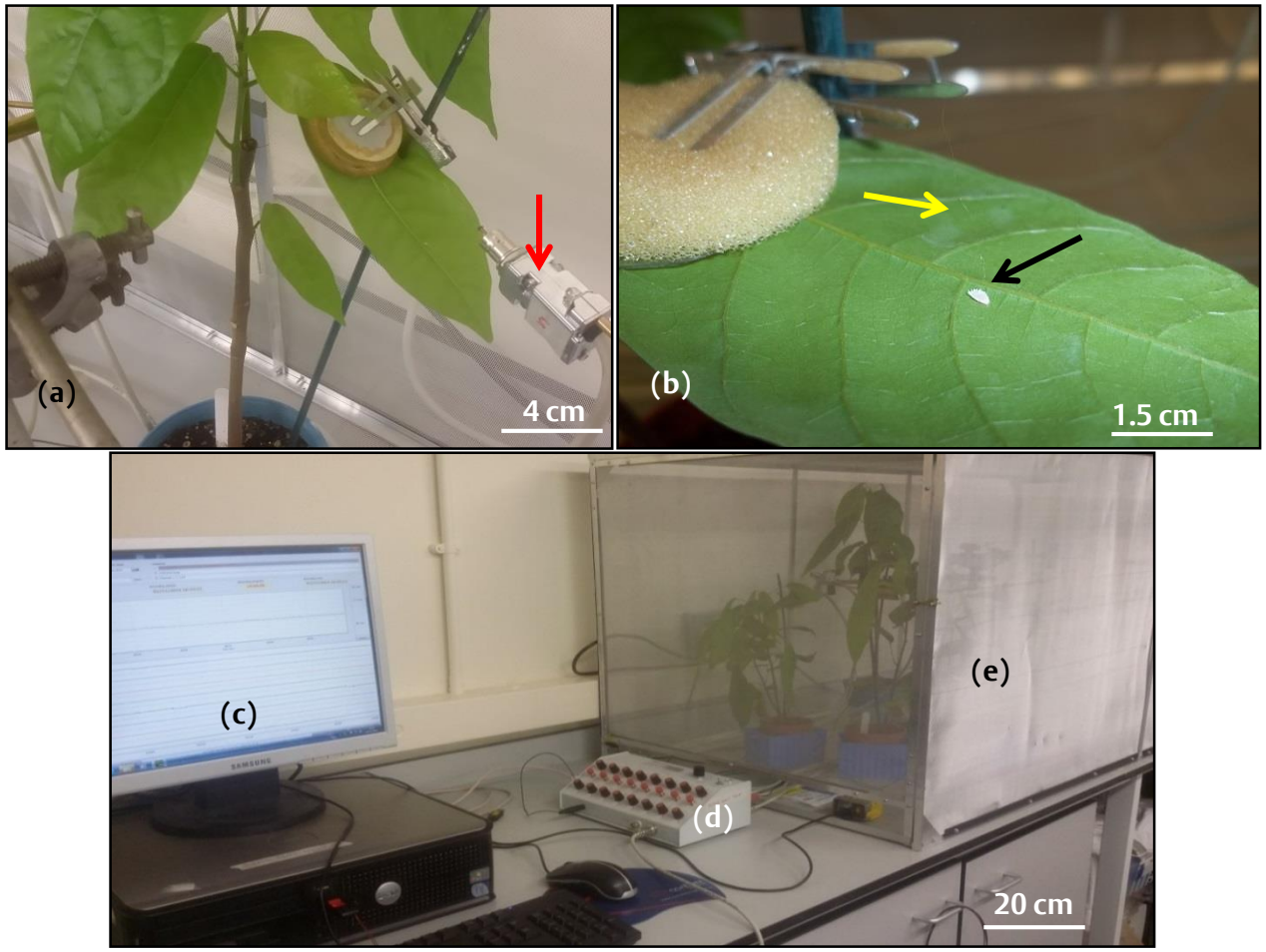

Figure 5.1 EPG Set-up for data acquisition showing (a) CSSV-free cacao plant with the EPG probe (red arrow), (b) gold-wire (yellow arrow) tethered mealybug (black arrow) on cacao leaf abaxial surface, (c) desktop personal computer and monitor (d) Giga-8 DC-EPG amplifier, (e) Faraday cage housing the feeding mealybugs during recordings.

5.3.2 EPG Data Acquisition

The EPG recording took 24 h for each of the 2nd stage instars on an 8-channel EPG apparatus, Giga-8 System (EPG Systems, Wageningen, Netherlands), suitable for eight simultaneous recordings. The entire EPG recording unit was set up as shown in Figure 5.1**.** Both sequential and non-sequential data types were acquired for each of the EPG variables during the study. The fluctuating voltages read as EPG signals (i.e. EPG waveforms) originated from the fluctuation of the electrical resistance (R) and 'generated' voltages, electromotive force (emf) at the plant-insect interface. On each of the eight channels, the R and emf signal components in the DC-EPG system were determined by adjusting and calibrating plant voltage supply (-5V to +5V) and gain (50x to 100x) after the commencement of recording. Tinytag Ultra 2 TGU-4017 (Gemini Data Loggers, UK), an indoor data logger with a built-in sensor, was used to record the temperature and relative humidity conditions during the EPG recordings. The EPG data acquisition was done with a built-in analogue-digital (AD) signal conversion device.

The AD device used Di710 (DATAQ Instruments, Inc., OH, USA) for data acquisition. STYLET+a software, version 01.24, was used for the EPG data analysis in the study (EPG Systems). The EPG signals, representing mealybug activities, were monitored in real-time and the data were concurrently held on the hard disk in the PC. At the end of each 24 h run, the data were automatically converted and saved in a designated file format as.aq8. The .aq8 file formats were later extracted into individual hourly EPG recordings for subsequent analysis.

5.3.3 Reference EPG waveforms for aphids and mealybugs

The present EPG waveform interpretations (similarities and deviations) and analysis were based on previously reported EPG waveform for aphid and mealybug species feeding on the host, non-host and artificial diets.

In this study, for a proper interpretation of the observed EPG waveforms for mealybug species feeding on cacao to be achieved, a waveform library of aphids (Figure 5.2) and mealybugs (Figure 5.3) specifically feeding on various species of the Malvacean family (none were available on cacao) were compiled from relevant literature as a key. However, these reference waveforms should not be regarded as being directly comparable for the interpretation of the observed EPG waveforms of mealybug feeding on cacao generated in the present study, but only to be used as a guide for waveforms annotation and subsequent analyses.

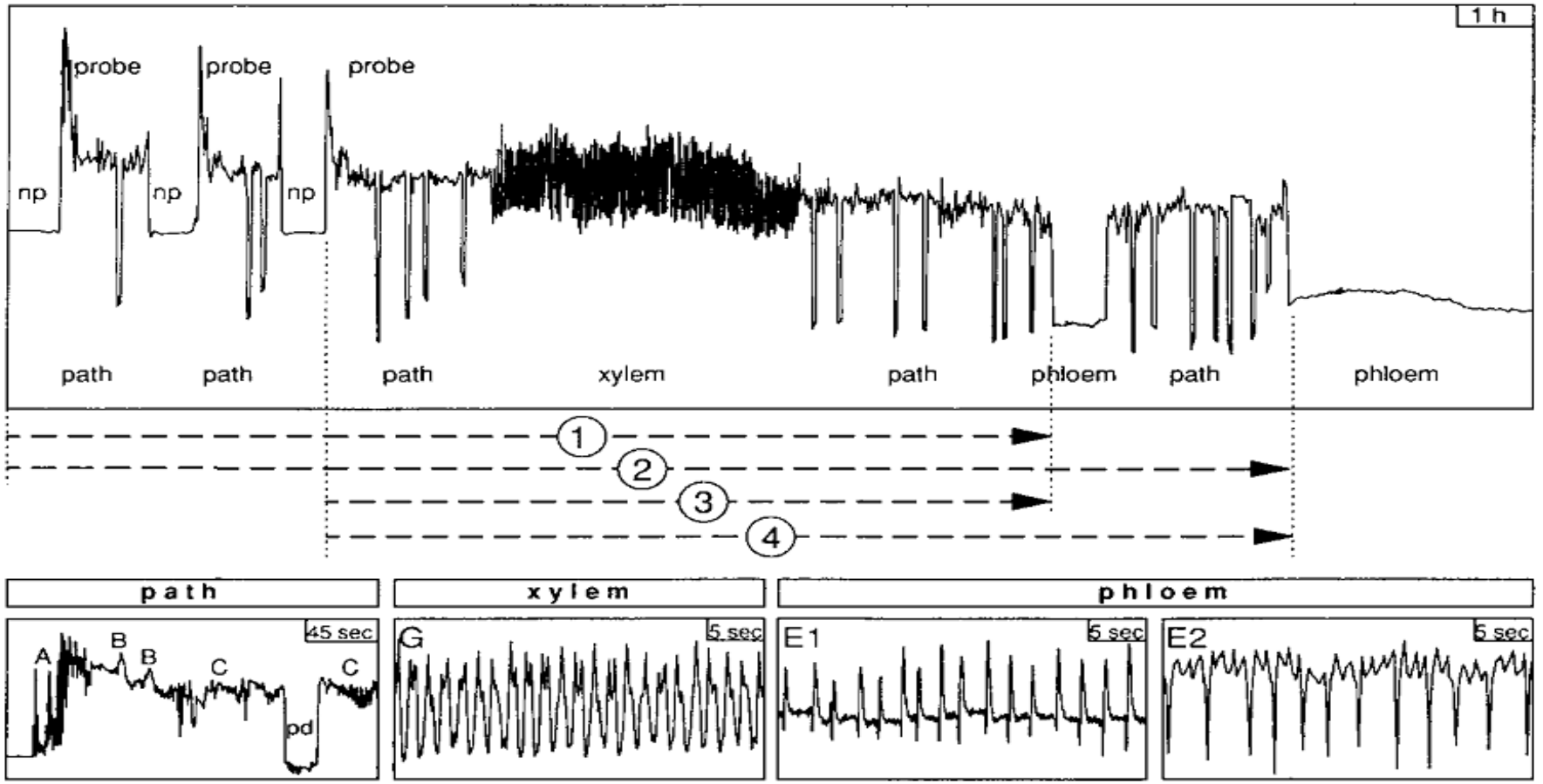

Figure 5.2: EPG waveforms Np, A, C, pd, G, E1 and E2 of aphid (*Aphis fabae*) on broad beans (*Vicia faba* L.) leaf (Tjallingii, 1994).

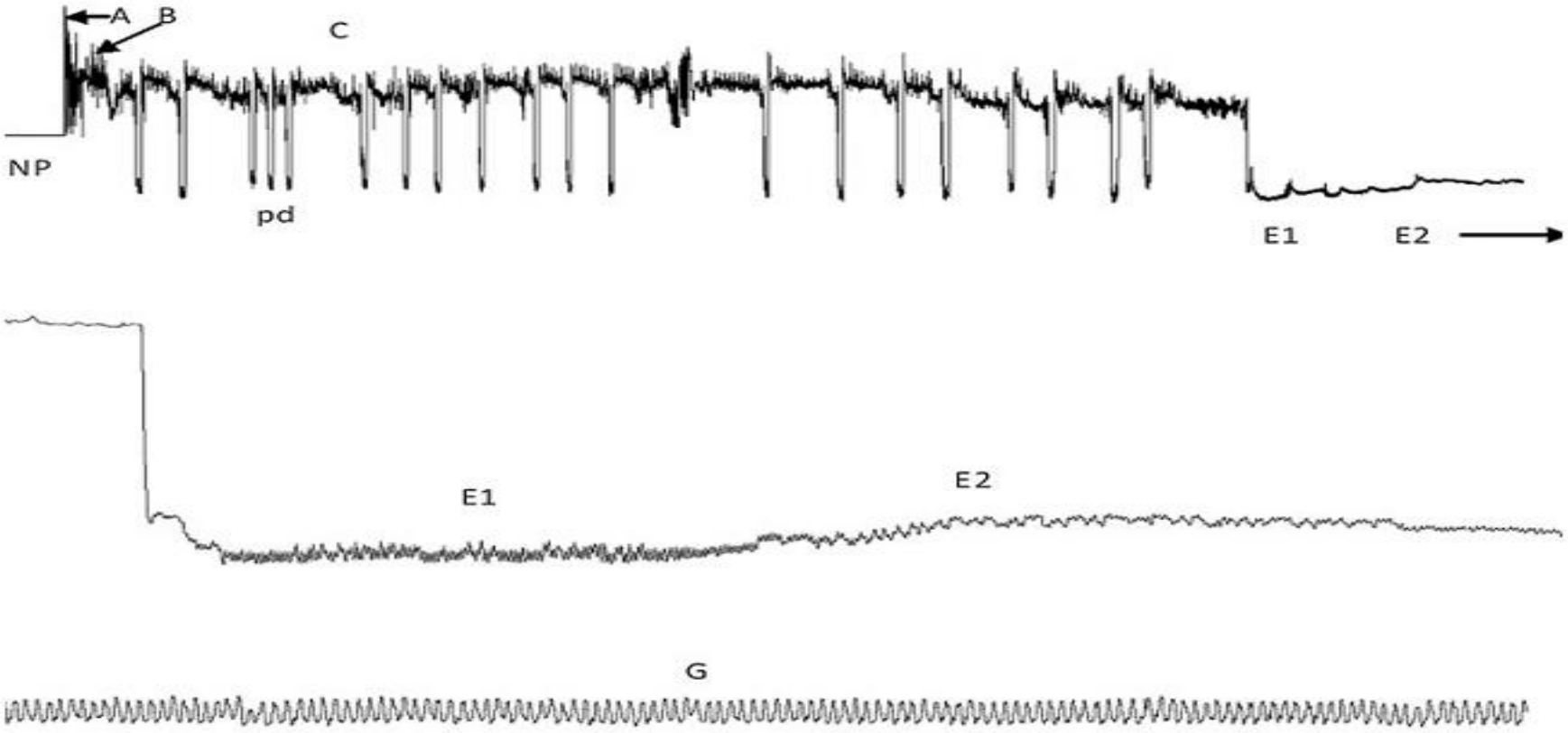

Figure 5.3: EPG waveforms Np, A, B, C, pd, E1, E2 and G of *Pseudococcus longispinus* on grapevine leaves (Sandanayaka *et al.*, 2012).

5.3.4 EPG Data Analysis

A separate EPG analysis module, the STYLET+d software ver. 01.24., was adapted to designate the EPG signals as waveform saved in .ana file format. Sequential and non-sequential parameters of the designated EPG waveform were processed by Microsoft Excel macros developed by the Julius Kuhn Institute (JKI, Quedlinburg, Germany) (Schliephake, 2014) and Consejo Superior de Investigaciones Científicas (CSIC, Madrid, Spain) (Sarria *et al*., 2009).

5.3.5 Statistical Analyses

All statistical analyses were performed with GenStat 16th edition version 16.1.0.10916 (VSN International Ltd, Hemel Hempstead, UK). The 'zero' value parameters represented a 'no-activity' case and counted towards the overall means of the observed waveform for each of the mealybug species. Shapiro-Wilk's test (applicable for sample size ≤ 50) (Shapiro and Wilk, 1965) was used to estimate data departure from normal distribution and Bartlett's test (Snedecor and Cochran, 1989) for homogeneity of variances. An appropriate data transformation was applied, where necessary. One-way analysis of variance (ANOVA) was then performed to determine significant differences between the observed means for each of the feeding activities carried out by the three mealybug species. A *post hoc* test on the significantly different means was performed using the Fisher's Least Significant Differences at ≤95% confidence level.

5.4 Results

The main variables of interest in these studies were C, E1e, E1, E2, F, G and Np (Table 5.1) and Table 5.2 presents the overall summary of the events of these variables for each of the mealybug species used in the EPG study on cacao.

Table 5.1: Description of basic EPG waveforms for mealybugs

Variable	Activity
A, B, C (combined as C)	Intercellular activities during penetration
E1e	Extracellular salivation
E1	Salivation in sieve element
E2	Phloem ingestion
F	Derailed stylet mechanics
G	Xylem ingestion
Np	Non-probing
pd	Intracellular stylet tip puncture

Adapted from Sarria *et al*. (2009).

Table 5.2: Summary of sequential and non-sequential EPG variables for *Pl. citri*, *Ps. longispinus* and *Ps. viburni* on CSSV-free cacao

Variable	Explanation	Unit	*Pl. citri*					*Ps. longispinus*					*Ps. viburni*				
			n	Mean	Min	Max	SE	n	Mean	Min	Max	SE	n	Mean	Min	Max	SE
s_C	sum of C	h	16	9.63	1.01	23.55	1.61	16	11.32	0.00	23.98	2.17	16	12.32	0.94	23.86	2.03
s_E12	sum of E12	h	5	0.55	0.00	6.80	0.42	1	0.39	0.00	6.22	0.39	1	0.68	0.00	10.94	0.68
s_E1e	sum of E1e	h	14	0.47	0.00	2.28	0.15	6	0.35	0.00	2.30	0.17	16	0.42	0.02	1.69	0.13
s_E2	sum of E2	h	5	0.49	0.00	6.73	0.42	1	0.39	0.00	6.22	0.39	1	0.67	0.00	10.74	0.67
s_F	sum of F	h	9	4.10	0.00	18.40	1.48	7	3.96	0.00	19.39	1.61	4	0.29	0.00	3.17	0.20
s_G	sum of G	h	14	8.17	0.00	22.88	1.94	11	5.53	0.00	24.00	2.29	15	8.92	0.00	23.02	2.15
s_Np	sum of non probing	h	16	1.02	0.02	4.41	0.28	16	2.42	0.00	21.01	1.30	16	1.35	0.00	12.47	0.76
t_1E	time to 1st E (always E1; from the 1st probe)	h	16	18.55	0.47	23.98	2.07	16	23.57	17.76	24.00	0.39	16	23.19	13.03	24.00	0.68
t_1E1_1E2	time from the 1st E1 to 1st E2	h	16	15.05	0.01	23.98	2.89	16	22.46	0.01	24.00	1.50	16	22.38	0.08	24.00	1.49
t_1E1_1sE2	time from the 1st E1 to 1st sustainable E2	h	16	19.86	0.48	23.98	2.08	16	23.57	17.77	24.00	0.39	16	23.20	13.11	24.00	0.67
t_1E12	time to 1st E12	h	16	18.75	0.47	23.98	2.10	16	23.57	17.76	24.00	0.39	16	23.19	13.03	24.00	0.68
t_1E2	time to 1st E2	h	16	18.80	0.48	23.98	2.09	16	23.57	17.77	24.00	0.39	16	23.20	13.11	24.00	0.67
t_1G	time to the first G (after first penetration)	h	16	5.28	0.16	23.98	1.94	16	10.01	0.00	24.00	2.70	16	4.01	0.06	23.98	1.84
t_1pd	time to 1st pd (from start of 1st probe)	h	16	0.21	0.01	0.89	0.06	13	0.33	0.00	2.58	0.16	16	0.70	0.00	7.40	0.46
t_1Pr	time to 1st probe (in recording; = d_1Np)	h	16	0.26	0.02	1.94	0.12	16	0.05	0.00	0.20	0.02	16	0.12	0.00	0.55	0.04
a_C	average C; with pd without E1e, F and G	min	16	40.57	7.12	113.35	7.10	16	232.70	0.04	1438.69	118.91	16	63.13	11.33	357.91	20.41
a_E1	average E1	min	6	3.28	0.00	31.63	2.01	1	0.03	0.00	0.41	0.03	1	0.11	0.00	1.72	0.11
a_E12	average E12	min	5	17.88	0.00	203.92	12.73	1	23.34	0.00	373.48	23.34	1	41.03	0.00	656.43	41.03
a_E1e	average E1e	min	14	2.14	0.00	19.23	1.15	6	1.53	0.00	11.87	0.76	16	2.35	0.52	8.14	0.57
a_E2	average E2	min	5	14.54	0.00	201.80	12.55	1	23.32	0.00	373.07	23.32	1	5.75	0.00	92.05	5.75
a_F	average F	min	9	97.68	0.00	326.13	30.69	7	130.88	0.00	799.91	59.62	4	15.14	0.00	190.10	11.84
a_G	average G	min	14	185.47	0.00	1061.76	75.33	11	251.63	0.00	1439.91	124.06	15	193.44	0.00	1371.44	86.69
a_Np	average non probing (period duration)	min	16	14.67	0.73	132.45	8.07	16	31.37	0.06	315.17	19.35	16	15.73	0.23	149.60	9.03
a_Pr	average probe	min	16	351.93	73.91	1434.49	90.02	16	622.69	59.77	1439.95	147.32	16	565.38	109.46	1439.78	117.02
d_1Pr	duration of 1st probe	min	16	7.08	0.06	23.91	2.11	16	11.23	0.11	24.00	2.60	16	9.11	0.13	24.00	2.42
s_E1	sum of E1 (sgE1 and E1)	min	6	4.83	0.00	31.63	2.41	1	0.03	0.00	0.41	0.03	1	0.75	0.00	12.06	0.75
s_pd	sum of pd	min	16	38.59	6.08	119.07	8.44	13	24.90	0.00	78.70	6.56	16	34.92	3.06	82.36	6.44
n_C	number of C periods	no.	16	18.81	1.00	56.00	3.38	16	11.63	1.00	44.00	3.40	16	17.13	1.00	44.00	3.05
n_E1	number of all E1 periods	no.	6	0.75	0.00	4.00	0.31	1	0.06	0.00	1.00	0.06	1	0.44	0.00	7.00	0.44
n_E12	number of E12 phloem periods i.e. with both, E1 and E2	no.	5	0.44	0.00	2.00	0.18	1	0.06	0.00	1.00	0.06	1	0.06	0.00	1.00	0.06
n_E1e	number of E1 extracellular (E1e) periods	no.	14	19.69	0.00	75.00	5.40	6	8.75	0.00	64.00	4.72	16	10.94	1.00	32.00	2.55
n_E2	number of E2 periods	no.	5	0.56	0.00	3.00	0.24	1	0.06	0.00	1.00	0.06	1	0.44	0.00	7.00	0.44
n_F	number of F	no.	9	1.50	0.00	8.00	0.54	7	0.88	0.00	4.00	0.33	4	0.56	0.00	5.00	0.33
n_G	number of G	no.	14	5.19	0.00	26.00	1.58	11	2.88	0.00	15.00	1.14	15	9.69	0.00	30.00	2.31
n_Np	number of non probing periods	no.	16	7.63	1.00	18.00	1.29	16	5.50	1.00	16.00	1.23	16	4.25	1.00	12.00	0.80
n_pd	number of pd	no.	16	85.00	14.00	275.00	19.13	13	49.75	0.00	165.00	13.51	16	83.19	8.00	208.00	15.81
n_Pr	number of probes	no.	16	7.63	1.00	18.00	1.29	16	5.31	1.00	16.00	1.24	16	4.13	1.00	12.00	0.77
n_Pr_1E	number of probes before the 1st E	no.	5	3.67	0.00	8.00	1.28	1	3.00	3.00	3.00	#DIV/0!	1	2.00	2.00	2.00	#DIV/0!
n_Pr_1pd	number of probes before 1st pd	no.	9	0.56	0.00	1.00	0.13	8	0.50	0.00	1.00	0.13	10	0.63	0.00	1.00	0.13
nPr_1G	number of probes before the first G	no.	14	1.81	0.00	7.00	0.42	11	1.81	0.00	13.00	0.79	15	1.13	0.00	3.00	0.15
d_1pd	duration of the first pd	s	16	33.11	11.02	180.43	10.05	12	21.84	0.00	117.55	7.31	15	19.90	0.00	46.83	3.43
d_2pd	duration of the second pd	s	16	24.55	3.90	38.99	2.38	13	21.69	0.00	44.42	3.80	14	20.39	0.00	54.02	4.00
d_pd5	mean duration of the first 5 pd	s	16	25.80	10.67	43.05	2.12	13	28.24	11.43	68.55	3.92	16	20.13	1.73	33.72	2.59

#DIV/0!: Max = Min; where n = 1

5.4.1 Feeding behaviour and pattern of *Planococcus citri* on cacao

The 24 h EPG recording showed that it took *Pl. citri* 15.54 ± 7.02 min to start probing once on a cacao leaf. The first cell puncture activity indicated by a potential drop, pd, followed after 12.6 ± 3.44 min. Xylem 'drinking' (G) and extracellular salivation (E1e) were each shown by 14 individuals. Time to first G was 5.28 ± 1.94 h and the total duration spent in G ranged from 0 to 22.88 h as exhibited by 14/16 individuals (Figure 5.5). 9/16 mealybugs expressed F and this lasted for 4.1 ± 1.48 h. The time spent in C was 9.63 ± 1.61 h. Only 6/16 individuals performed E1, ranging from 49.8 s to 31.63 min; of those six individuals five also exhibited E2 ranging in duration from 6.82 s to 6.73 h.

5.4.2 Feeding behaviour and pattern of *Pseudococcus longispinus* on cacao

Ps. longispinus spent an average of 2.72 ± 0.93 min on cacao leaves before making the first probe. The first potential drop was observed at 19.8 ± 9.6 min after the first probe. *Ps. longispinus* spent 11.32 ± 2.17 h in C and 3.96 ± 1.61 h in F and the time spent without probing was more than double for *Ps. longispinus* (2.42 ± 1.30 h) when compared with *Pl. citri* (1.02 ± 0.28 h). The duration to first G averaged at 10.01 ± 2.7 h and this was shown by 11 individuals. There were two instances out of the 16 trials when individuals completely remained in G phase throughout the entire feeding duration. Meanwhile, only a single individual performed E1 and subsequently E2 (Figure 5.5).

5.4.3 Feeding behaviour and pattern of *Pseudococcus viburni* on cacao

It took an average of 7.38 ± 2.43 min for individual *Ps. viburni* mealybugs to make the first probe on the cacao leaf surface. The first potential drop occurred after 41.82 ± 27.54 min. *Ps. viburni* spent, on average half (12.32 ± 2.03 h) of the total feeding duration in pathway phase. It took 4.01 ± 1.84 h to the first G waveform and this was exhibited by 15 of the 16 individuals; four of the individuals also showed the F pattern (Figure 5.5). A single individual showed E1 and this lasted for 12.06 h followed by E2 for 40.26 min.

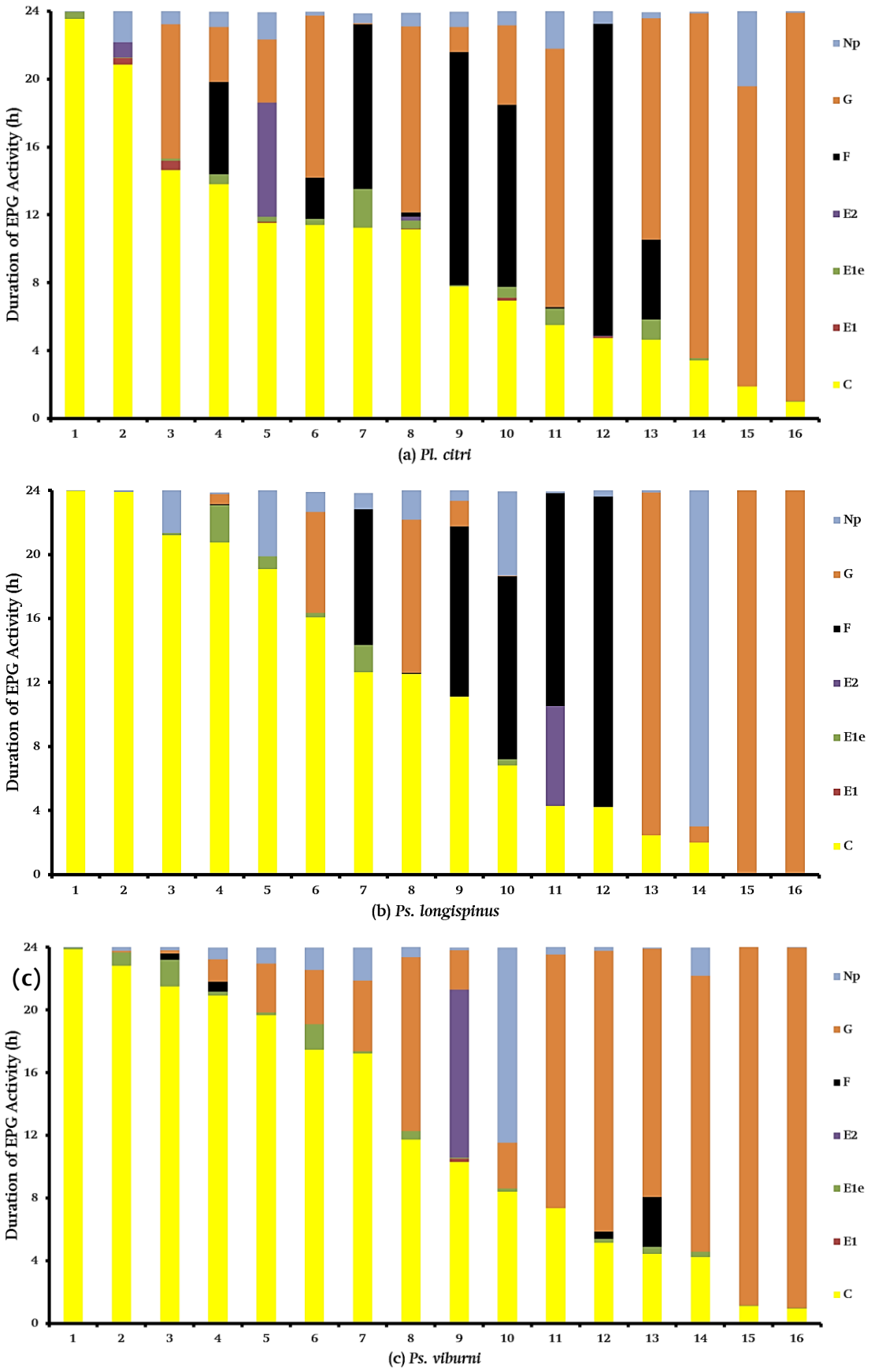

Figure 5.4: EPG activities of 16 individual mealybugs (a) *Planococcus citri*, (b) *Pseudococcus longispinus* and (c) *Pseudococcus viburni* feeding for 24 h on CSSV-free cacao leaves. C (pathway), E1e (extracellular salivation), E1 (intracellular salivation), E2 (phloem ingestion), F (derailed stylet mechanics), G (xylem drinking) and Np (non-probing).

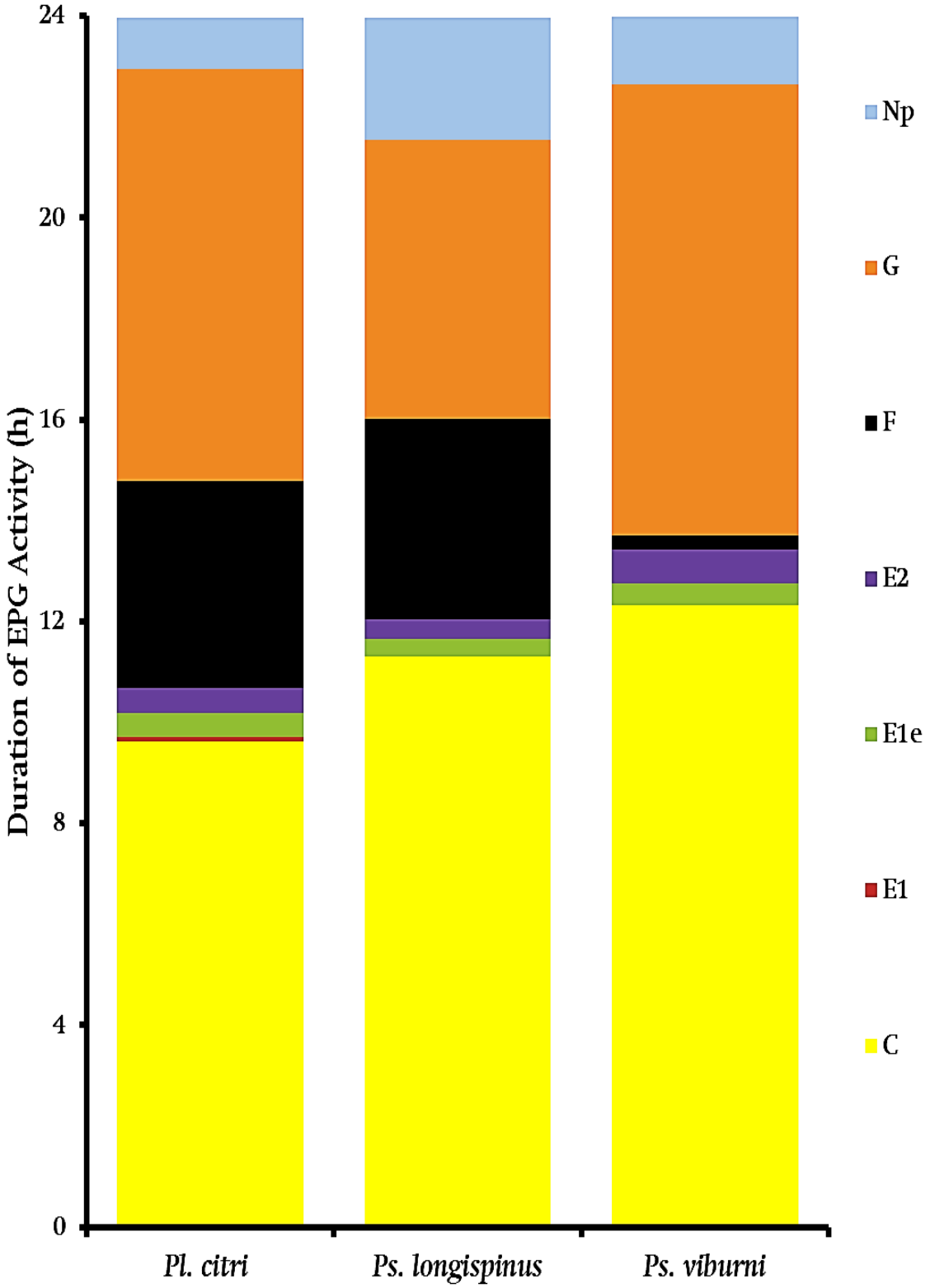

Figure 5.5: Mean EPG activities of 16 *Planococcus citri*, *Pseudococcus longispinus* and *Pseudococcus viburni* feeding for 24 h on CSSV-free cacao leaves. C (pathway), E1e (extracellular salivation), E1 (intracellular salivation), E2 (phloem ingestion), F (derailed stylet mechanics), G (xylem drinking) and Np (non-probing).

5.4.4 EPG waveform of *Planococcus citri*, *Pseudococcus longispinus* and *Pseudococcus viburni* on CSSV-free cacao

A minimum of 16 individual mealybug replicates were recorded over a 24-hour period for each of the EPG variables (feeding activity and pattern). Disrupted recordings due to eventual loss of mealybug-leaf contact were not included in the analysis. All results were presented in the format: mean ± standard error, except where stated differently; the total number of individuals performing the activities was also presented in parenthesis (Table 5.2). Based on the average proportion (in percentage) of individual activities performed by each group of the mealybug species, *Pl. citri* showed the order C>G>F>Np>E2=E1e>E1, *Ps. longispinus*: C>G>F>Np>E2>E1e>E1 and *Ps. viburni*: C>G>Np>E2>E1e>F>E1 (Figure 5.5). These observations were highly variable as indicated by the respective coefficients of variation.

5.4.4.1 Waveform C

Waveform A, B and C were the most performed extracellular EPG waveform for all the mealybug species after the initial non-probing phase following the mounting of the insects. These three waveforms showed repeated transition and similarity, and as such were bulked and labelled waveform C, representing the pathway phase. In the study, waveform C performed by *Pl. citri* and *Ps. longispinus* mealybug species on cacao leaves resembled those of *Pl. citri* (Figure 5.6) and *Ps. longispinus* (Figure 5.3) on grapevine leaves, in terms of the duration, irregularly high frequency and variable electrical origin (electromotive force (emf) and/or electrical resistance (R)). Based on these references, the C waveform was also determined and annotated for *Ps. viburni*.

5.4.4.2 Waveform F

Waveform F depicted the derailment (difficulty) experienced by mealybugs either to puncture or penetrate leaf tissues. In the study, waveforms F, with low amplitude and high frequency, were ascribed following Wu *et al*. (2013) (Figure 5.6).

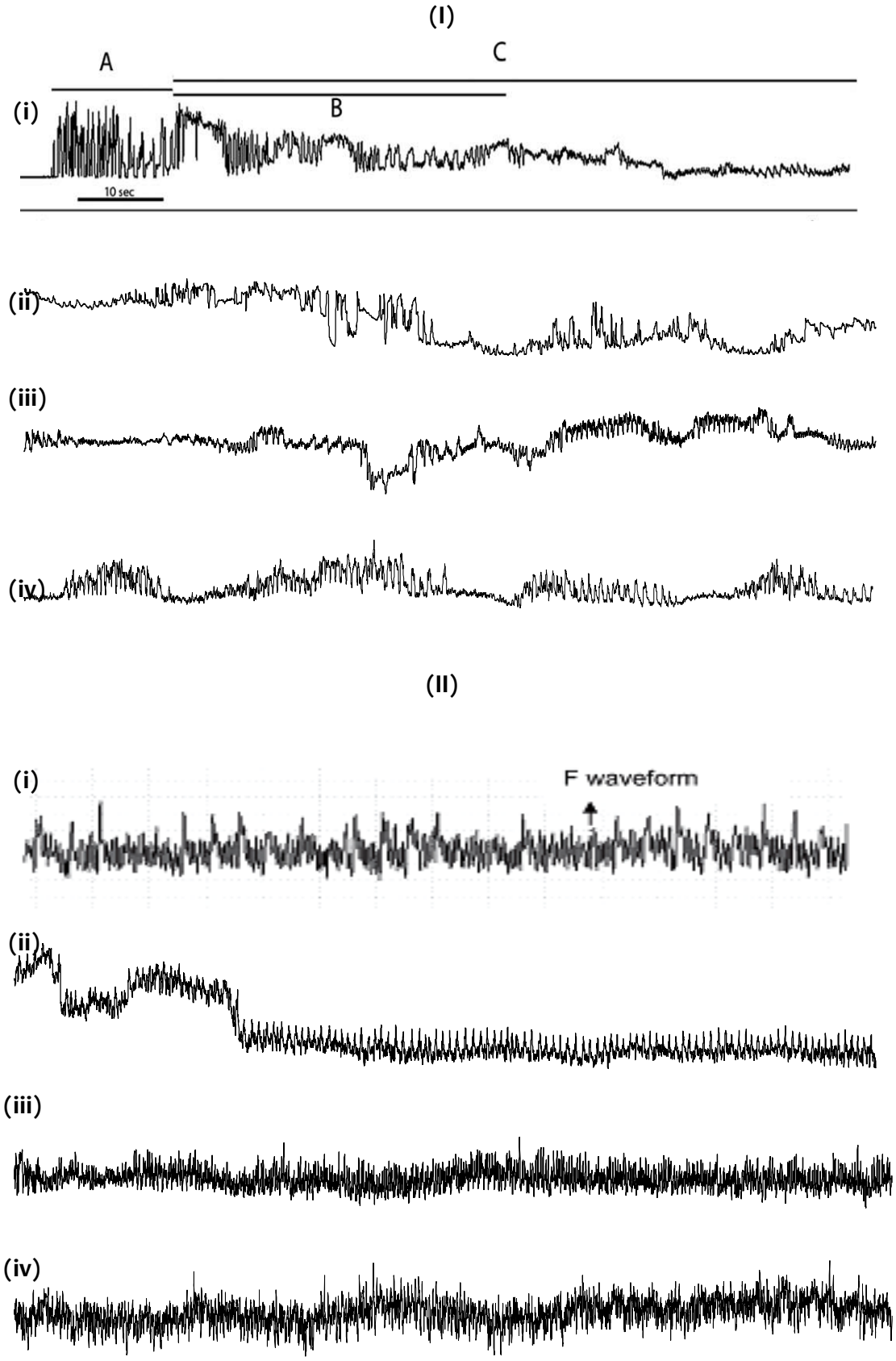

Figure 5.6: EPG waveform C (I): generated by (i) *Planococcus citri* on grapevine (Cid and Fereres, 2010), (ii) *Planococcus citri*, (iii) *Pseudococcus longispinus* and (iii) *Pseudococcus viburni* on *in situ* CSSV-free cacao leaves; waveform F (II): (i) *Phenacoccus solenopsis* Tinsley on *Hibiscus rosa-sinensis* (Wu *et al*., 2013), (ii) *Planococcus citri*, (iii) *Pseudococcus longispinus* and (iv) *Pseudococcus viburni* on *in situ* CSSV-free cacao

5.4.4.3 Waveform E1e

The extracellular E1e waveforms observed for *Pl. citri*, *Ps. longispinus* and *Ps. viburni* closely resembled waveform C and intercellular waveform E1/E2. E1e had relatively low amplitude and frequency and an emf electrical origin. The pattern of occurrence, the peaks and waves were similar for the three mealybug species, *Pl. citri*, *Ps. longispinus* and *Ps. viburni* on cacao in comparison to the E1e waveform described for *Ph. solenopsis* on leaves of the shoeblack host plant (Figure 5.7). The E1e waveform was generally preceded and also followed by potential drops while in some cases E1e was followed by a non-probing phase. This pattern was similar in all the three mealybug species and common to the reference waveform, *Ph. solenopsis*.

5.4.4.4 Waveform G

Apparently the extracellular waveform G was an indication of how 'thirsty' the starved mealybug species were as they settled to commence feeding on the cacao leaves. The observed G waveforms for *Pl. citri*, *Ps. longispinus* and *Ps. viburni* had a similar electrical origin to waveform C (i.e. emf and/or R), especially waveform A but distinct with relatively high and uniform frequency. The peaks and waves closely matched with those of *Pl. citri* and *Ps. longispinus* on grapevines (figures in Cid and Fereres (2010) and Sandanayaka *et al*. (2012), repectively) and the reference, *Ph. solenopsis* on shoeblackplant (Wu *et al*., 2013) (Figure 5.7).

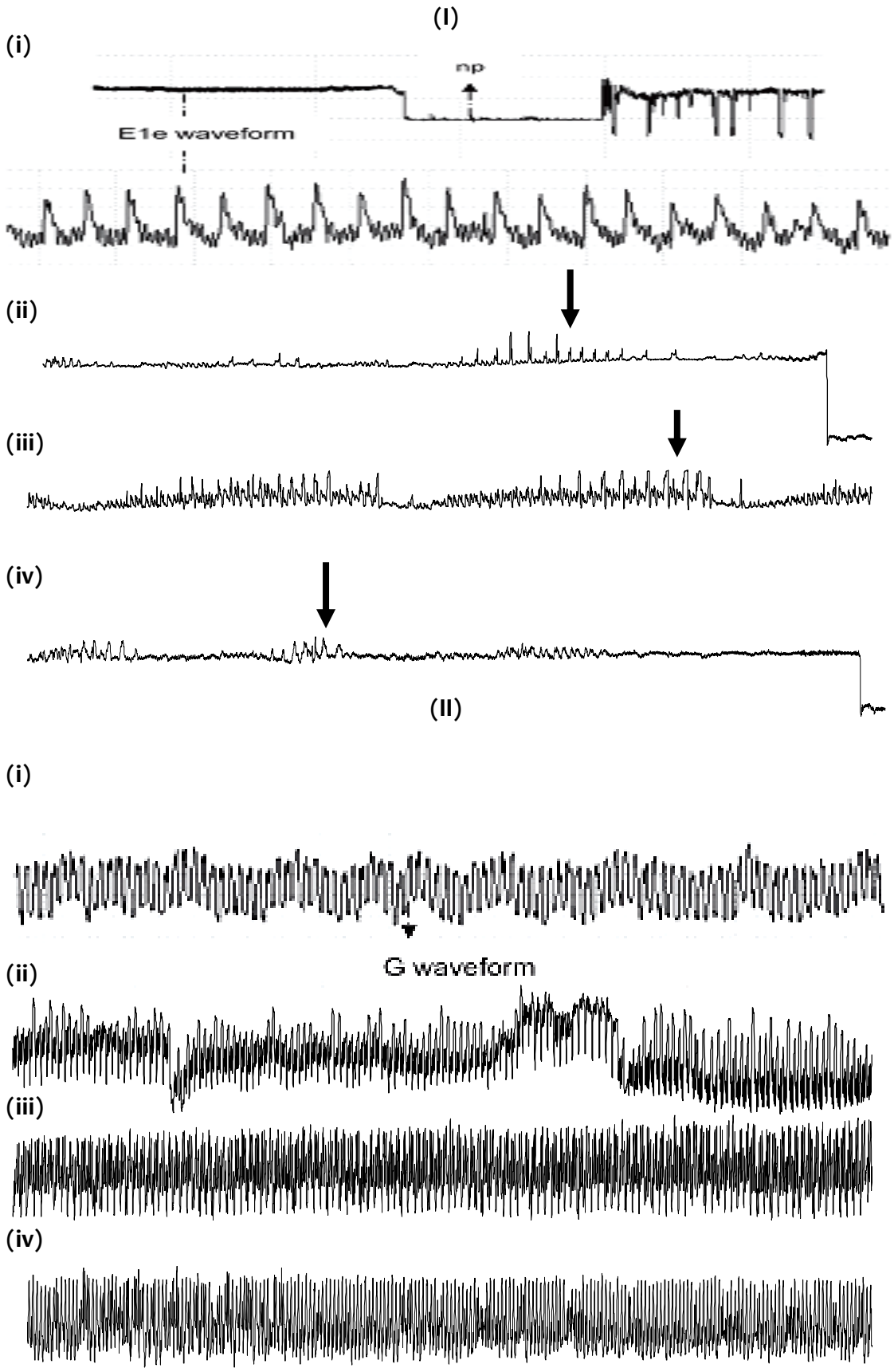

Figure 5.7: EPG waveform E1e (extracellular salivation) (I): generated by (i) *Phenacoccus solenopsis* on *Hibiscus rosa-sinensis* (Wu *et al*., 2013), (ii) *Planococcus citri*, (iii) *Pseudococcus longispinus* and (iv) *Pseudococcus viburni* on *in situ* CSSV-free cacao leaves; waveform G (II): (i) *Phenacoccus solenopsis* on *Hibiscus rosa-sinensis* (Wu *et al*., 2013), (ii) *Planococcus citri*, (iii) *Pseudococcus longispinus* and (iv) *Pseudococcus viburni* on *in situ* CSSV-free cacao leaves.

5.4.4.5 Waveforms E1 and E2

Sustained potential drop for *Pl. citri*, *Ps. longispinus* and *Ps. viburni* resulted in prolonged 'intercellular' EPG waveforms; E1 and E2 exhibited by insects and were synonymous with phloem access (sieve element salivation/ingestion) (Cid and Fereres, 2010). Generally, E1 and E2 waveforms occurred in succession and alternated. But at the point of transition from E1 to E2, the waveform patterns were not immediately distinguishable. In aphids, for example there are no known cues to the switching from E1 to E2 and vice versa at the phloem-feeding phase (Pettersson *et al*., 2007). While the E1 waveform showed variable and undetermined electrical origin, E2 originated from emf and R. These two waveforms are significant to phloem-limited virus upload and download by mealybugs. The regular frequency of the intracellular E1 was similar to the extracellular E1 (E1e) but they usually differed in duration; E2 has a longer duration of occurrence than E1 and also showed lower number of frequency peaks and waves. It was clear that *Pl. citri*, *Ps. longispinus* and *Ps. viburni* all showed these waveforms while feeding on cacao and that they were comparable with those generated by *Pl. citri* (Cid and Fereres, 2010) (Figure 5.8) and *Ps. longispinus* (Sandanayaka *et al*., 2012) on grapevine.

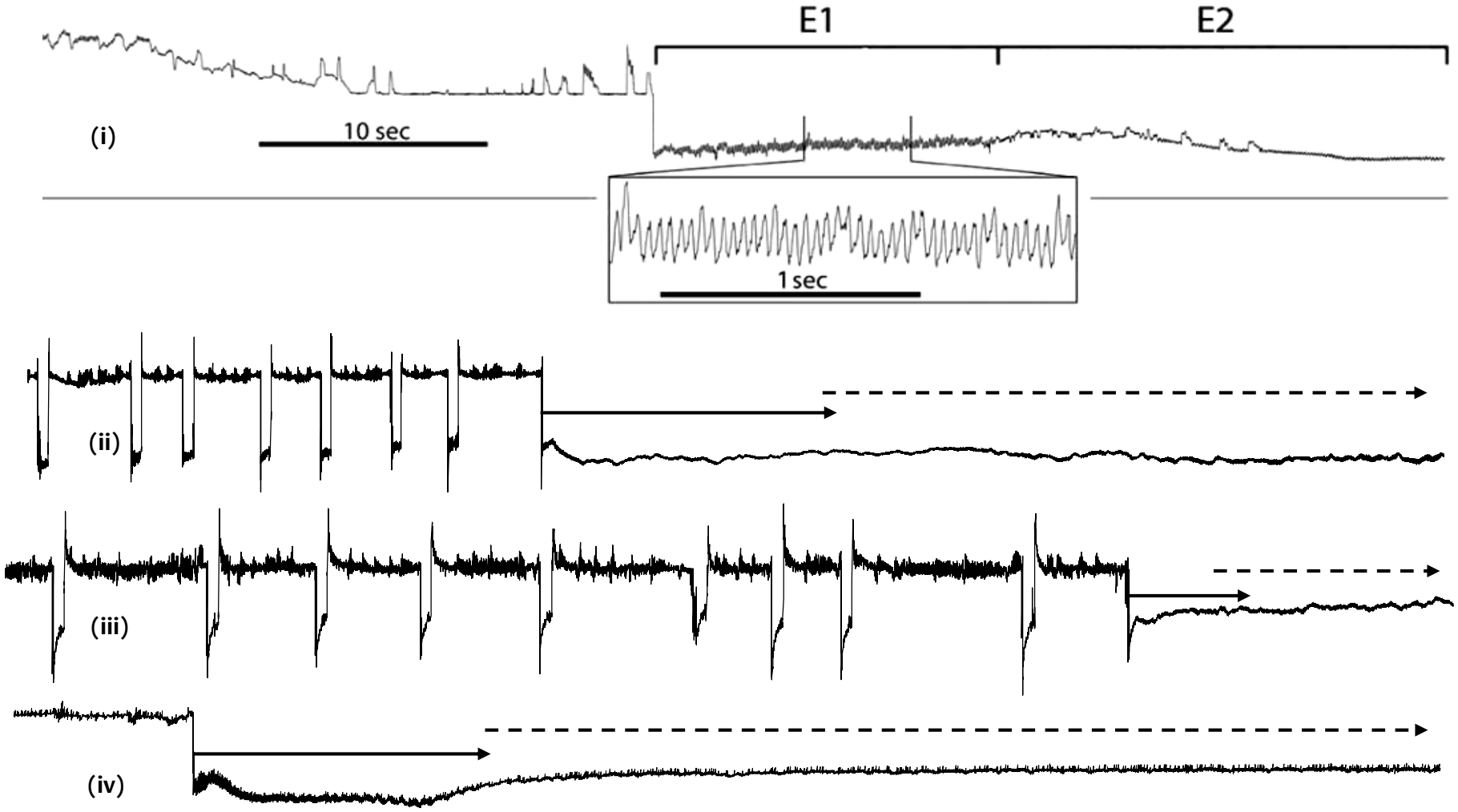

Figure 5.8: EPG waveform E1/E2 (i) generated by *Planococcus citri* feeding on grapevine leaves (Cid and Fereres, 2010), (ii) *Planococcus citri*, (iii) *Pseudococcus longispinus* and (iv) *Pseudococcus viburni* on *in situ* CSSV-free cacao leaves. [E1 = ——→; E2 = - - - - - →]

5.4.5 Analysis of EPG waveforms of *Planococcus citri*, *Pseudococcus longispinus* and *Pseudococcus viburni* on CSSV-free cacao

The pooled ANOVA of untransformed data for the seven waveforms (variables) observed for *Pl. citri*, *Ps. longispinus* and *Ps. viburni* on cacao showed no significant difference among the three mealybug species ($p \geq .052 \leq .924$) (See Appendix D). ANOVA for the natural log transformed data (variables) presented significant differences ($p \leq .05$) between the mealybugs for variables E1e and E1 (Table 5.3). *Pl. citri* significantly differed ($p \leq .05$) from *Ps. longispinus* but was similar to *Ps. viburni* for E1e. However, *Ps. longispinus* and *Ps. viburni* were not significantly different for E1.

5.4.6 Environmental conditions during EPG recording

The laboratory conditions during the EPG recordings were logged continuously. The following variables were recorded: mean, minimum and maximum daily ambient temperature, relative humidity and dew point. Their daily fluctuation throughout the study period is presented in Appendix F. During the 24-h EPG recording durations, the temperature ranged from 18.9°C to 24.6°C; relative humidity and dew point range were 72.6% RH to 82.7% RH and 14.3°C to 16.4°C, respectively.

Table 5.3: Mean duration of EPG waveforms observed for *Planococcus citri*, *Pseudococcus longispinus* and *Pseudococcus viburni* on cacao in 24 h feeding period (natural log transformed data)

Mealybug species	EPG waveform (log_e)						
	C	**E1e**	**E1**	**E2**	**F**	**G**	**Np**
Pl. citri	6.09	2.53	0.83	0.83	2.99	4.77	3.58
Ps. longispinus	5.65	1.35	0.02	0.37	2.27	2.98	3.30
Ps. viburni	6.26	2.61	0.16	0.40	0.97	5.23	3.08
p	.460	.050	.017	.682	.109	.051	.716
LSD ($p \leq .05$)	NS	1.124	0.586	NS	NS	NS	NS

NS = not significantly different at ≤ 95% confidence level.

5.5 Discussion

The daily temperature and relative humidity readings during the DC-EPG recording in laboratory conditions represented ranges that resemble conditions that support the survival of most Hemipterans (Pseudococcidae), including tropical mealybugs species in natural and glasshouse conditions (Goldasteh *et al*., 2009; Kumar *et al*., 2013; Prasad *et al*., 2012; Waterworth *et al*., 2011b). Although it is uncommon to find these parameters reported in detail in most previous electropenetrography studies (Garzo *et al*., 2015; Renard *et al*., 1998), the overall mean values observed in this study were similar to summary reports in studies involving the same or related mealybug species (Calatayud *et al*., 1994; Calatayud and Ru, 2006).

The bulk of this present study was devoted to establishing a large reference data set of characterised EPG recordings from which the feeding behaviour of mealybugs on virus-free cacao could be reliably described. As such, 16 x 24 h recordings were produced and analysed for *Pl. citri*. Under similar environmental and host plant conditions, further 16 x 24 h EPG recordings were made and analysed for each of the mealybug species *Ps. longispinus* and *Ps. viburni*.

By comparison with published EPG data for cassava mealybug, *Phenacoccus manihoti,* feeding on cassava (Calatayud *et al*., 1994) and grapevine (Cid and Fereres, 2010) with those of the *Pl. citri* data from the present cacao study similarities became apparent and it was possible to annotate the waveform recordings and distinguish six main patterns of stylet activity (plus 'non-probing' periods, Np). While the ranges of waveforms exhibited were highly consistent (at least one of the six types were present in each of the 48 24-hour recordings) a high degree of variability in their relative proportions was apparent between individuals.

In this study, it took an average of 15.6 ± 7.2 min for *Pl. citri* to start probing once at the phylloplane and the first pd was observed after 12.6 ± 3.6 min; the stylets are positioned in the epidermis and/or mesophyll tissues during the initial probing periods.

The first sustained pd (i.e. E1/E2) for *Pl. citri* in the study occurred 18.55 ± 2.07 h after the first probe, which was 12 h longer than the duration observed for *Pl. citri* on grapevine (6.39 ± 1.5 h, n = 14) (Cid and Fereres, 2010). Calatayud *et al*. (1994) reported a sustained pd range (n = 20) of 9.2 ± 3.1 to 10.8 ± 3.5 and 1.9 ± 0.4 to 7.9 ± 4.3 for *Ph. manihoti* on two occasional hosts (*Euphorbia pulcherrima* Willd. ex Klotzsch and *Talinum triangulare* (Jacq.) Willd.) and four cassava cultivars (including one hybrid), respectively.

However, the Cid and Fereres (2010) and Calatayud *et al*. (1994) EPG studies did not involve a 24 h starvation period for the mealybug species as was the case in the present study. Though there is no detailed EPG report on the effect of starvation on mealybug feeding on host plants, in the context of other hemipteran studies including those for aphid (Prado and Tjallingii, 1999) and psyllid (Bonani *et al*., 2010), the initial 24 h starvation period for *Pl. citri* in the present study could have contributed to an increase, not only in the time to first probe, but also in prolonging the time taken to achieve the first sustained pd (phloem-phase). Posnette and Robertson (1950), using *Fo. njalensis*, showed that there was a relationship between the morphology of mealybug stylets and the duration required for probing (feeding) in comparison with leaf-hoppers and aphids that happen to feed rabidly.

In starved hemipterans, an increase in the activity of waveform G is an indication of dehydration and stress during EPG monitoring (Pompon *et al*., 2010; Pompon *et al*., 2011; Ramírez and Niemeyer, 2000). Apparently, starved hemipterans would perform this activity in keeping with the need to maintain their internal homeostasis prior to feeding. The performance of the G waveform shows that the stylets have penetrated the parenchyma through to the xylem tissues, rich in water and soluble mineral nutrients (Lucas *et al*., 2013). The contribution of G to the 24 h feeding duration of *Pl. citri* on CSSV-free cacao ranged from 0 to 95.35%.

The positioning of the stylet, by feeding piercing-sucking hemipterans, within the sieve elements of host plant tissues is associated with E1 and E2 waveforms. Cid and Fereres (2010) reported a mean E1 duration of 45.7 ± 9.78 s for *Pl. citri* for 11 out of the 20 individuals fed on grapevine; nine of those resulted in E2, which lasted for over 4 h. But on CSSV-free cacao, 6/16 of the 24 h starved mealybugs were able to perform E1 for an average duration of 3.28 ± 2.01 min and five of those individuals proceeded to the sustained phloem ingestion phase (E2), ranging from 6.82 s to 6.73 h. This fell between the duration of E2 reported for *Pl. citri* on grapevine by Cid and Fereres (2010) and *Ph. manihoti* on cassava cultivars with a range of 7 ± 2.2 h to 17.5 ± 4.3 h (Calatayud *et al*., 1994).

Generally, *Ps. longispinus* (3 ± 1.2 min) and *Ps. viburni* (7.2 ± 2.4 min) individuals were comparably more active than *Pl. citri* (15 ± 7.2 min) in terms of the average time taken to make the first probe. But *Ps. longispinus* and *Ps. viburni* did achieve pd later than *Pl. citri*; after 19.8 ± 9.6 min and 42 ± 27.6 min, respectively. Sandanayaka *et al*. (2012) reported 80.7 ± 3.4% and 84.9 ± 4.8% of probing activity (24 h recording) for 24 (given 20 – 30 minutes starvation) first stage instar *Ps. longispinus* free of GLRaV-3 and with GLRaV-3, respectively. Ten of the 24 GLRaV-3-free *Ps. longispinus* individuals reached E1 and three proceeded to E2. Similarly, in the present study involving 16 24 h-starved *Ps. longispinus* individuals, the proportion of probing was 89.9 ± 2.42% with a single individual exhibiting E1 and E2.

Non-host acceptability and recognition behaviour could have been a factor that contributed to the prolonged delay by *Ps. viburni* in feeding on cacao, which is not one of its natural hosts. Renard *et al*. (1998) used a combination of EPG and a video camera recording to monitor how *Ph. manihoti* was able to distinguish three of its natural hosts from a non-host weed species while walking on the leaf surface.

The 'walking' activity of insects has been associated with the A, B, C waveform and at times Np. Though the average proportion of C ('pathway') in the EPG study of the three mealybugs species feeding on cacao ranged from 40.19 ± 6.72% (*Pl. citri*) to 51.35 ± 8.44% (*Ps. longispinus*), the highest C was shown by *Ps. viburni* with individual means ranging from 3.93% – 99.42%. Huang *et al*. (2014b) reported similar behavioural alternations when the cotton mealybug, *Phenacoccus solenopsis* (Tinsley) was transferred from cotton to tomato, the time to first probe increased 4 - 7 fold, there was more Np activity and it took 3 – 7 times longer to reach E2.

The changes in the feeding behaviour of a 'non-cacao' mealybug species, *Ps. viburni*, brought about by host plant shift was not uncommon and could as well be attributed to the dissimilarities in the morphology of the different phylloplanes, their internal tissue structure and cell arrangements. Being that the feeding behaviour of *Ps. viburni* on any of its hosts has never been monitored with EPG prior to this study, its EPG waveforms comparison were directly interpreted in the light of those of *Pl. citri* and *Ps. longispinus*.

All the three mealybug species demonstrated comparable waveform types which could be categorised into the aforementioned seven stylet activities. The dominant activity recorded for all species was C (Table 5.3) indicating the relatively protracted period spent by mealybugs establishing stylet contact with the vascular tissues of cacao leaves (compared with, for example the relatively brief pathway phase shown by rapidly feeding aphids) (Stafford *et al*., 2012; Tjallingii, 1995). For all three mealybug, species the next most sustained activity was G. The occurrence of waveform G is likely to be influenced by the water stressed nature of the individuals following the 24 h starvation period. Although this pre-treatment was necessary to establish a common baseline for all individuals at the beginning of the EPG recording, it is liable to be representative of the status of windblown/dislodged juveniles arriving on CSSV-free cacao plants in the field (i.e. the individuals of most importance in terms of long distance or jump virus spread) (Thresh, 1958; Thresh *et al*., 1988b).

The EPG data demonstrating cacao phloem access by *Ps. viburni* supports the earlier finding in this study of the species' capacity for CSSV transmission in spite of its apparent absence from cacao in the field. This species is however present on the African continent (Daane *et al*., 2012) and its range is expanding illustrating the potential for changes to the current CSSV vector fauna that could be investigated with the aid of EPG-based approaches.

Significant differences were apparent for the duration of the intra- and extracellular salivation events (E1 and E1e) for the three mealybug species. Given the intracellular salivation event is the stylet activity predominantly associated with virus transmission by hemipterans (Stafford *et al*., 2012) these results could aid in understanding the relative efficiency of CSSV transmission by its vector species.

Future work utilizing this EPG approach could address the potential for CSSV transmission during particular stylet activity phases. For example, EPG monitoring of viruliferous mealybugs feeding on previously virus-free plants and interrupting that feeding prior to phloem access could allow us to determine the susceptibility of plants to infection during the early probing events (when as the viral quantification studies have shown the vector's CSSV load is declining. Similarly, putative CSSV-resistant cacao genotypes could be assessed to determine if they possess characteristics that significantly alter the feeding behaviour of mealybug vectors; this approach has proved productive for the development of cucumber mosaic virus-resistant cucurbit varieties (Garzo *et al*., 2002) and the study of plant defence responses (Zhang *et al*., 2015).

The application of the EPG technique has gained importance in monitoring the highly modified mouthparts (stylets) of phloem-feeding hemipterans during the intracellular and intercellular navigations to locate feeding sites in sieve elements during pathogen acquisition and inoculation (Fereres and Collar, 2001; Garzo *et al*., 2015; Morris and Foster, 2008; Prado and Tjallingii, 1994; Sandanayaka *et al*., 2003; Tjallingii and Prado, 2001).

This has been made possible by the relationship that exists between stylet position and electrical charges across cell membranes (Walker, 2000) and the subsequent correlation with real-time feeding activities of piercing-sucking hemipterans. For phloem-limited plant pathogens, the efficiency of a potential vector to break the barriers of host plant and non-host plant resistance and act as vectors is associated with, among other factors, defence-signalling networks including the salicylic acid (SA), jasmonic acid (JA) and ethylene pathways (War *et al*., 2012). Gene expressions do occur in plants in response to both vector and pathogen presence (Zheng and Dicke, 2008) and these alterations, up and/or downregulated gene expressions, have shown a significant partitioning of plant resources for defence mechanism in response to vector and pathogen incidence (Thompson and Goggin, 2006; War *et al*., 2012). So, the combination of electrophysiology and the quantification of defence gene(s) responses by host plant, in the presence of a vector and its pathogen, could be studied simultaneously.

The EPG study by Serikawa *et al*. (2012) showed that the Asian citrus psyllid, *Diaphorina citri* (Kuwayama) performed fewer probes (100) on young and mature leaves of citrus seedling, sour orange (*Citrus aurantium* L.) pre-treated with imidacloprid via soil application (to whole plant) compared to untreated plants (360 probes) during EPG recording; the proportion of E2 events was 86% more for *D. citri* on imidacloprid-free *C. aurantium* as opposed to the imidacloprid-infected seedlings. These activities (E1 and E2) are linked to acquisition and transmission of '*Candidatus* Liberibacter asiaticus' (Las) by *D. citri*, though Serikawa *et al*. (2012) did not show molecular-based evidence for these findings. Recently, Garzo *et al*. (2015) have demonstrated that EPG technique, with molecular analysis and its validation, can be used to monitor the functional effects of systemic insecticides (sulfoxaflor LC_{50} and imidacloprid LC_{50}) on the feeding behaviour of two populations of *Myzus persicae* (Sulzer) on pepper leaves in addition to the detection of low to moderate level target-site resistance.

Kloth *et al*. (2015) have developed an automated video system that measures the feeding behaviour of aphid on leaf discs with the aim of assessing plant resistance levels. Further studies could benefit from the newly established EPG information obtained from this study of the feeding behaviour of mealybugs on cacao, integrated into the design of a testing system for systemic insecticide and defence response gene expression in cacao for resistance to CSSV and its vectors.

Chapter 6 General Discussion And Conclusions

This study highlighted the desirability of preliminary field and laboratory analysis of host-pest-pathogen interactions before the implementation of major changes to perennial, field crops intended to protect those crops. The following breakthroughs have been achieved during this study and are discussed in the light of the challenges that were faced during the study, the expected and observed results, practical application of the outcomes, suggestions and proposals for further studies.

6.1 Diversity studies of CSSV and mealybugs on Nigerian cacao: morphological and molecular characterisation of mealybugs

The first molecular evidence of CSSV presence in Nigeria, accompanied with geographic maps, have been presented in this study. The results of the study have also provided the first CO1-barcoding information on mealybug species obtained from Nigerian cacao. The discovery of CSSV and putative vector mealybug species in new cacao sites, previously not reported to harbour CSSV in Nigeria, has raised concerns about CSSV spread and its potential long-term implications on cacao production in these areas. Detailed environmental scanning electron microscopy combined with the updated USDA online histology interactive database of several mealybug species (Miller *et al*., 2014) was also used as an additional aid in the identification process. There is, however, conflicting morphological identification information and histological references available on that particular database for the identification the cacao mealybug, *Fo. njalensis*, a wide spread vector mealybug species of CSSV in the West African cacao. With the additional improvements made in this study to the histological methodology on mealybug slide preparation for Coccoidea: Pseudococcidae (Ben-Dov and Hodgson, 1997; Watson and Chandler, 2000; Williams and Granara de Willink, 1992), detailed morphological information for *Fo. njalensis* and other mealybug species can be established in conjunction with the generation of DNA barcodes from voucher specimens.

However, as Cox (1983) demonstrated individual species can vary in terms of morphology depending on diet and environment and so the use of DNA barcoding incorporated in to on-line keys such as the USDA database would be immensely beneficial. More exhaustive field work will be required to enhance the diversity data and the possibility of employing HRMA (Wetten *et al*., 2015) will hasten CO1-based PCR screening of mealybugs. Overall, this study has demonstrated the importance of the use of integrative taxonomy approach (Will *et al*., 2005) with the inclusion of more than one gene (Rubinoff *et al*., 2006) (e.g. CO1 and ITS) to improve the accuracy of species identification, especially the cryptic mealybug species. Integrative taxonomy is an approach which uses a combination of characters including DNA and many other types of data, to delimit, discover, and identify meaningful, natural species and taxa at all levels (Will *et al*., 2005).

There were a number of cases between *Pl. citri* and *Pl. minor* in this study where DNA-based authentication of these mealybug species was inconclusive. Though the possibility of misidentifying species from interbreeding populations was also not ruled out, this was not tested in this study primarily due to time constraints and costs. Interestingly, it has been reported that the use of synthetic sex pheromones could strengthen the identification of cryptic mealybug species (Ho *et al*., 2007) where, for example, conventional taxonomic keys have failed as in the case of *Pl. citri* and *Pl. minor* (Tu *et al*., 1988).

It is against this backdrop that it will be suggested that emphasis be made in future studies to improve on the use of integrative taxonomy including species-specific pheromone studies as an augmentation of existing identification keys (such as the frequently cited (Cox, 1989); Cox and Freeston (1985) and the USDA databases) rather than a replacement of the conventional morphological taxonomy with the DNA barcoding technology.

The involvement of dedicated research institutes, like the Cocoa Research Institute of Nigeria (CRIN), and agriculture-based Faculties in Universities involved in the routine monitoring of cacao growing areas for symptomatic CSSV-infected plants, organising farmers' awareness programmes on the identification and report of CSSV outbreaks and the training of more scientists in the area of molecular plant pathology and entomology will provide a far clearer picture of the status of the disease and its vectors.

6.2 Mealybug-CSSV-Cacao interactions

Molecular studies on the relationship and association between mealybugs, cacao and CSSV remains scant. This study presents the first molecular evidence of CSSV acquisition and retention by putative vector mealybug species after feeding on a CSSV-infected and then cacao-free diets. The capacity of all three vector mealybug species investigated in this study to retain CSSV for at least six days after removal from the virus host is unusually long for a pathogen characterised as semi-persistent. Thus, the observed retention times have implications for the breadth and composition of proposed barrier crops in order for them to be effective. Current work at Reading is focussing on the localisation of the virus within the vector to establish whether earlier assumptions of the non-circulative nature of the virus (Roivainen, 1976, 1969) are valid.

There is the need to further extend the molecular studies on CSSV acquisition and retention to cover the characterisation of the transmission efficiency of these mealybug species as CSSV vectors. Such information will help clarify and classify the mode of CSSV acquisition and transmission between hosts and the degree of variation, in terms of the transmission efficiency between its vectors. Studies on the implications for the control of mealybug populations in relation to CSSV infection using the new generation of molecular tools should be conducted as a priority.

6.3 Characterisation of feeding behaviour of mealybugs on cacao using electrical penetration graph (EPG) analysis

The first use of EPG to study mealybug feeding behaviour on cacao has been reported in this study. There are promising applications in the use of this technology in disease resistance breeding studies, especially defence gene expression in cacao genotypes and the possibility of using it as a rapid means of screening more cacao genotypes for CSSV resistance. There is the potential of applying EPG for studies of the efficacy of systemic pesticides in cacao.

EPG-derived information about the threshold quantities of pesticides required to control mealybugs would be valuable for a number of reasons. For example, imidacloprid (Elbert *et al*., 1991), which is the world's most widely used insecticide (Simon-Delso *et al*., 2015), is currently in use against mealybugs on cocoa in quarantine and nursery facilities, but the European Food Safety Authority (EFSA) restriction on the use of neonicotinoids due to possible effects on non-target pollinators like bees (EFSA 2013; Grimm *et al*., 2012; Walker, 2013) means that this may be curtailed.

In addition, neonicotinoids, which act as an inhibitors to the feeding activities of scale insects (Simon-Delso *et al*., 2015), have also been shown to have a lethal effect on dipteran insects (EFSA, 2012; Fryday *et al*., 2015) and may therefore have a detrimental effect on midges, *Forcipomyia* spp, the principal pollinators of cacao flowers. The nonselective organophosphate insecticide chlorpyrifos has been employed against mealybugs in Israel, but its frequent use led to resistance in *Pl. citri* (Mendel *et al*., 1999); in New Zealand *Ps. longispinus* also showed resistance to chlorpyrifos (Charles *et al*., 1993). The length of the useful life of neonicotinoid insecticides against mealybugs before the occurrence of resistance is debatable. It is likely the development of resistance to neonicotinoids will take longer to occur (over 15 years) (reviewed by Elbert *et al*. (2008)) with relatively reduced toxic effect on mealybug predators and parasitoids (Cloyd and Dickinson, 2006; Pisa *et al*., 2015) and the environment (Goulson

and Kleijn, 2013) in comparison with organophosphates and carbamates. It is expected that this would happened fairly rapidly with imidacloprid as anticipated in growth-regulating insecticides including buprofezin and pyriproxifen (Grafton-Cardwell *et al*., 2001).

Conversely, often accompanying novel pesticide use is secondary pest outbreaks and they cannot be ruled out in the case of the mealybug-cacao scenario due to the fact that almost every new insecticide introduction has an impact on other species useful either as natural enemies or pollinators. This highlights the potential role for monitoring, biological control and other environmentally friendly approaches that could contribute towards long-term management of mealybugs. Female sex pheromone traps have been shown to have several months of effective use, as attractants for the disruption of male mealybug populations. With a view to implementing such a management approach, it will be useful to have a female sex pheromone study in place to address the efficacy of these systems in West African cacao. This could provide useful detailed information about the seasonal activity of particular vector species so that any pesticide applications can be more targeted and thereby reduce the possibility of resistance build up.

It has been demonstrated that sustained feeding (salivation and ingestion) within the phloem tissues (indicated by EPG waveforms E1 and E2) of *S. tuberosum* by viruliferous psyllid, *Bactericera cockerelli* (Šulc), did not usually result in the transmission of the pathogen, '*Candidatus* Liberibacter solanacearum', which causes the zebra chip disease of potato (Mustafa *et al*., 2015). In aphids, the model EPG insects, it has also been demonstrated that the inoculation of turnip (*Brassica rapa* L. (var. Just-Right)) by the phloem-limited pathogen, *Cauliflower mosaic virus* (CaMV), by *Brevicoryne brassicae* (L.) and *Myzus persicae* (Sulzer) was possible after the initial intracellular stylet penetration of the epidermis and mesophyll (Moreno *et al*., 2005). CSSV is characterised as a phloem-limited mealybug-transmitted pathogen (Jacquot *et al*., 1999).

However, its mode of transmission by vector mealybugs still requires detailed studies to (1) test whether prolonged stylet penetration in vascular tissues (phloem phase) by viruliferous mealybugs on its host, cacao, usually results in CSSV transmission and (2) if shorter duration of tissue probes without access to the phloem by viruliferous mealybugs could cause the transmission of CSSV in cacao. In this way such studies could inform about the threshold size of viruliferous mealybug populations necessary for the effective transmission of CSSV and in turn elucidate the likely effectiveness of mealybug control regimes, be they pesticide, parasitoid or pheromone based, for the prevention of CSSV spread.

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APPENDICES

Appendix A: Survey of Nigerian cacao for CSSV screening and mealybug identification

(a) Preliminary interaction with individual cacao farmers* in Nigeria *via* an interpreter which also doubles as a field assistant (holding a pictogram of CSSV symptoms and description); (b) Sampled cacao tree growing in close proximity to *Ceiba pentandra*; (c) Hunting for cryptic mealybugs on cacao – perusing the underside of a cacao pod resting on one of the primary branches; (d) Taking a break with the field assistants* and a quintessential cacao farmer, an octogenarian* (second from the left) in Ondo State. **Consents were obtained to photograph individuals and include photos in any report/publication relating to the study.*

Appendix B: Log of Nigerian cacao leaf samples for CSSV screening at Reading

Reference Code	Field Code/Batch Date	Grid Reference
	24-8-12	
1027	Alt host	7°29'49.7"N 4°06'37.1"E
1028	Cocoa	7°21'49.7"N 4°02'31.9"E
1029	Cocoa	7°29'43.9"N 4°06'42.8"E
1030	Cocoa	7°29'40.9"N 4°06'53.7"E
1031	Cocoa	7°29'53.9"N 4°06'54.9"E
1032	Cocoa	7°29'42.6"N 4°06'45.8"E
1033	Cocoa	7°21'47.9"N 4°02'31.4"E
1034	Cocoa	7°29'41.4"N 4°06'54"E
1035	Cocoa	7°29'53.4"N 4°06'55.3"E
1036	Cocoa	7°29'46"N 4°06'57"E
1037	Cocoa	7°27'55.5"N 4°06'57.1"E
1038	Cocoa	7°29'54.2"N 4°06'55.4"E
1039	Cocoa	7°29'43.9"N 4°06'42.6"E
1040	Cocoa	7°29'43.8"N 4°06'43.4"E
1041	Cocoa	7°29'54.5"N 4°06'57.6"E
1042	Cocoa	7°29'50.5"N 4°06'57.5"E
1043	Cocoa	7°29'45.9"N 4°06'57.0"E
1044	Cocoa	7°29'51.1"N 4°07'00.9"E
1045	Cocoa	7°29'54.7"N 4°06'57.5"E
1046	Cocoa	7°12'56"N 4°01'40.3"E
1047	Cocoa	7°29'54.2"N 4°06'57.2"E
1048	Cocoa	7°29'43.8"N 4°06'43.6"E
1049	Cocoa	7°29'46.1"N 4°06'57.7"E
1050	Cocoa	7°29'44.0"N 4°06'58.3"E
1051	Cocoa	7°29'44.3"N 4°06'44.3"E
1052	Cocoa	7°29'42.5"N 4°06'53.5"E
1053	Cocoa	7°29'43.3"N 4°06'45.6"E
1054	Cocoa	7°29'54.4"N 4°06'57.0"E
1055	Cocoa	7°29'53.7"N 4°06'55.5"E
1056	Cocoa	7°29'51.9"N 4°06'56.4"E
1057	Cocoa	7°29'53.9"N 4°06'55.4"E
1058	Alt host? Cola (chlamydantha?)	7°21'50.2"N 4°02'31.0"E
1059	Alt host	7°21'46.9"N 4°02'27.9"E
1060	Cocoa	7°21'47.4"N 4°02'30.4"E
1061	Cocoa	7°12'56.8"N 4°01'43.0"E
1062	Cocoa	7°21'46.6"N 4°02'31.7"E
1063	Cocoa	7°29'40.5"N 4°06'53.7"E
1064	Cocoa	7°29'40.5"N 4°06'53.8"E
1065	Cocoa	7°29'50.9"N 4°06'57.3"E

Appendix B continued		
1066	Cocoa	7°29'41.8"N 4°06'45.6"E
1067	Cocoa	7°29'43.0"N 4°06'57.8"E
1068	Cocoa	7°29'44.4"N 4°06'45.3"E
1069	Cocoa	7°29'52.5"N 4°06'56.1"E
1070	Cocoa	7°13'03.3"N 4°01'42.5"E
1071	Cocoa	7°13'00.1"N 4°01'43.4"E
1072	Cocoa	7°21'47.0"N 4°02'30.8"E
1073	Cocoa	7°21'49.6"N 4°02'29.7"E
1074	Cocoa	7°12'58.2"N 4°01'40.4"E
1075	Cocoa	7°13'04.0"N 4°01'41.5"E
1076	Cocoa	7°13'00.8"N 4°01'43.8"E
1077	Cocoa	7°21'48.1"N 4°02'31.8"E
1078	Cocoa	7°21'49.1"N 4°02'30.0"E
1079	Cocoa	7°21'47.5"N 4°02'26.4"E
1080	Cocoa	7°12'58.9"N 4°01'42.3"E
1081	Cocoa	7°12'56.7"N 4°01'42.8"E
1082	Cocoa	7°13'04.0"N 4°01'40.5"E
1083	Cocoa	7°12'57.5"N 4°01'39.7"E
1084	Cocoa	7°12'58.8"N 4°01'39.7"E
1085	Cocoa	7°12'59.4"N 4°01'38.5"E
1086	Cocoa	7°13'03.0"N 4°01'43.1"E
1087	Cocoa	7°12'55.4"N 4°01'42.5"E
1088	Cocoa	7°21'49.7"N 4°02'32.1"E
1089	Cocoa	7°13'06.0"N 4°01'36.8"E
1090	Cocoa	7°21'46.9"N 4°02'31.8"E
1091	Cocoa	7°21'45.9"N 4°02'31.8"E
1092	Cocoa	7°21'50.1"N 4°02'30.6"E
1093	Cocoa	7°21'47.9"N 4°02'31.4"E
1094	Cocoa	7°12'56.8"N 4°01'40.4"E
1095	Cocoa	7°12'58.8"N 4°01'39.2"E
1096	Cocoa	7°21'49.0"N 4°02'30.1"E
1097	Cocoa	7°12'58.9"N 4°01'43.3"E
1098	Alt host? *Ceiba pentandra*	7°21'50.2"N 4°02'30.7"E
1099	Cocoa	7°21'49.2"N 4°02'33.0"E
1100	Cocoa	7°12'59.0"N 4°01'38.8"E
1101	Cocoa	7°13'01.8"N 4°01'44.0"E
1102	Cocoa	7°12'56.4"N 4°01'41.2"E
1103	Cocoa	7°12'57.7"N 4°01'39.6"E
1104	Cocoa	7°12'59.6"N 4°01'38.2"E
1105	Cocoa	7°12'55.8"N 4°01'42.5"E
1106	Cocoa	7°12'57.4"N 4°01'39.1"E
1107	Cocoa	5°31'31.1"N 7°39'17.9"E

Appendix B continued		
1108	Cocoa	5°32'20.5"N 7°34'54.2"E
1109	Cocoa	5°31'31.9"N 7°39'16.9"E
1110	Cocoa	5°32'22.7"N 7°34'50.6"E
1111	Cocoa	5°32'21.3"N 7°34'49.8"E
1112	Cocoa	5°32'25.9"N 7°34'51.7"E
1113	Cocoa	5°32'22.5"N 7°34'50.3"E
1114	Cocoa	5°32'20.4"N 7°34'55.7"E
1115	Cocoa	5°31'31.0"N 7°39'17.0"E
1116	Cocoa	5°31'32.0"N 7°39'16.7"E
1117	Cocoa	5°32'20.1"N 7°34'49.4"E
1118	Cocoa	5°32'20.9"N 7°34'56.0"E
1119	Cocoa	5°32'27.5"N 7°34'58.3"E
1120	Cocoa	5°32'21.8"N 7°34'50.4"E
1121	Cocoa	5°32'18.6"N 7°34'51.2"E
1122	Cocoa	5°32'28.3"N 7°34'55.5"E
1123	Cocoa	6°00'30.4"N 8°47'01.2"E
1124	Cocoa	6°00'16.8"N 8°47'21.0"E
1125	Cocoa	6°00'40.8"N 8°46'53.5"E
1126	Cocoa	5°59'51.0"N 8°47'40.6"E
1127	Cocoa	6°00'36.4"N 8°46'52.4"E
1128	Cocoa	6°00'37.3"N 8°46'51.0"E
1129	Cocoa	5°59'51.0"N 8°47'40.4"E
1130	Cocoa	6°00'31.0"N 8°46'59.7"E
1131	Cocoa	6°00'40.3"N 8°46'52.3"E
1132	Cocoa	6°00'29.3"N 8°47'04.3"E
1133	Cocoa	6°00'30.5"N 8°47'01.4"E
1134	Cocoa	6°00'37.9"N 8°46'50.2"E
1135	Cocoa	6°00'07.8"N 8°47'28.4"E
1136	Cocoa	6°00'16.5"N 8°47'21.8"E
1137	Cocoa	6°00'08.2"N 8°47'28.6"E
1138	Cocoa	6°00'33.7"N 8°46'52.7"E
1139	Cocoa	5°32'20.5"N 7°34'55.1"E
1140	Cocoa	6°00'25.2"N 8°47'08.5"E
1141	Cocoa	5°59'51.7"N 8°47'39.0"E
1142	Alt host?	6°00'17.0"N 8°47'21.1"E
1143	Cocoa	6°00'16.9"N 8°47'27.3"E
1144	Cocoa	6°00'08.4"N 8°47'28.7"E
1145	Cocoa	5°59'47.1"N 8°47'43.6"E
1146	Cocoa	6°00'36.1"N 8°46'52.3"E
1147	Cocoa	6°00'38.5"N 8°46'53.1"E
1148	Cocoa	5°59'52.7"N 8°47'38.0"E
1149	Cocoa	5°32'20.2"N 7°34'49.4"E

Appendix B continued		
1150	Cocoa	6°00'42.1"N 8°46'52.8"E
1151	Cocoa	6°00'29.4"N 8°47'04.0"E
1152	Cocoa	6°00'38.5"N 8°46'53.2"E
1153	Cocoa	6°00'30.3"N 8°47'01.4"E
1154	Cocoa	6°00'31.6"N 8°46'50.4"E
1155	Cocoa	6°00'16.5"N 8°47'22.0"E
1156	Cocoa	6°00'06.9"N 8°47'29.1"E
1157	Cocoa	6°00'38.6"N 8°46'53.2"E
1158	Cocoa	5°59'1.5"N 8°47'40.1"E
1159	Cocoa	6°00'38.5"N 8°46'49.3"E
1160	Cocoa	5°59'50.9"N 8°47'41.0"E
1161	Cocoa	6°00'16.9"N 8°47'21.1"E
1162	Cocoa	6°00'38.7"N 8°46'53.2"E
1163	Cocoa	6°00'30.4"N 8°47'01.7"E
1164	Cocoa	5°59'47.3"N 8°47'45.1"E
1165	Cocoa	6°00'08.0"N 8°47'27.0"E
1166	Cocoa	6°00'15.7"N 8°47'25.7"E
1167	Cocoa	6°00'36.0"N 8°46'52.1"E
1168	Cocoa	6°00'07.3"N 8°47'28.5"E
1169	Cocoa	6°00'16.3"N 8°47'22.8"E
1170	Cocoa	6°00'24.3"N 8°47'08.7"E
1171	Cocoa	6°00'38.4"N 8°46'49.9"E
1172	Cocoa	5°59'59.7"N 8°47'35.0"E
1173	Cocoa	5°59'47.0"N 8°47'44.4"E
1174	Cocoa	6°00'30.5"N 8°47'00.4"E
1175	Cocoa	6°00'37.6"N 8°46'50.8"E
1176	Cocoa	6°00'37.0"N 8°46'51.6"E
1177	Cocoa	5°29'07.7"N 7°41'00.6"E
1178	Cocoa	5°32'19.3"N 7°34'52.6"E
1179	Cocoa	5°32'19.8"N 7°34'53.2"E
1180	Cocoa	5°31'30.9"N 7°39'17.1"E
1181	Cocoa	5°29'59.8"N 7°40'59.5"E
1182	Cocoa	5°29'07.7"N 7°41'00.9"E
1183	Cocoa	5°29'59.3"N 7°41'00.0"E
1184	Cocoa	5°29'10.2"N 7°40'55.8"E
1185	Cocoa	5°29'58.5"N 7°40'58.9"E
1186	Cocoa	5°29'07.3"N 7°41'00.5"E
1187	Cocoa	5°29'10.7"N 7°40'56.2"E
1188	Cocoa	5°29'07.7"N 7°41'00.8"E
1189	Cocoa	5°29'59.1"N 7°41'00.2"E
1190	Cocoa	5°29'11.3"N 7°40'56.2"E
1191	Cocoa	5°29'59.9"N 7°41'01.3"E

Appendix B continued		
1192	Cocoa	5°29'59.9"N 7°41'01.4"E
1193	Cocoa	5°29'59.3"N 7°41'00.0"E
1194	Cocoa	5°29'09.8"N 7°40'55.9"E
1195	Cocoa	5°29'58.5"N 7°40'59.0"E
1196	Cocoa	5°29'09.9"N 7°40'55.6"E
1197	Cocoa	5°29'09.6"N 7°40'55.3"E
1198	Cocoa	5°29'58.9"N 7°41'00.9"E
1199	Cocoa	5°29'10.3"N 7°40'55.9"E
1200	Cocoa	5°29'07.5"N 7°41'00.7"E
1201	Cocoa	5°32'20.7"N 7°34'54.0"E
1202	Cocoa	5°32'27.9"N 7°34'56.7"E
1203	Cocoa	5°31'32.1"N 7°39'18.2"E
1204	Cocoa	5°32'26.6"N 7°34'57.1"E
1205	Cocoa	5°32'26.7"N 7°34'57.0"E
1206	Cocoa	5°32'19.0"N 7°34'50.4"E
1207	Cocoa	5°31'31.6"N 7°39'17.7"E
	5-3-2013	
1491	cacao 1	7°12'01.9"N 5°01'52.3"E
1492	cacao - close to *Ceiba*	7°09'42.1"N 5°07'06.2"E
1493	cacao 8 - close to *Ceiba*	7°10'58.8"N 4°59'37.0"E
1494	cacao 4 - close to *Ceiba*	7°10'59.5"N 4°59'38.0"E
1495	cacao 3 - close to *Ceiba*	7°10'59.6"N 4°59'37.0"E
1496	*Ceiba*	7°09'42.1"N 5°07'06.1"E
1497	cacao - close to *Ceiba*	7°09'42.1"N 5°07'06.2"E
1498	cacao - close to *Ceiba*	7°09'41.8"N 5°07'03.1"E
1499	cacao - close to *Ceiba*	7°09'41.9"N 5°07'02.8"E
1500	*Ceiba*	7°09'41.8"N 5°07'02.9"E
1501	cacao 2	7°11'58.5"N 5°01'51.4"E
1502	cacao 3	7°11'58.5"N 5°01'51.4"E
1503	cacao 2	7°11'58.5"N 5°01'54.1"E
1504	cacao 2	7°11'58.2"N 5°01'53.5"E
1505	cacao 1	7°11'57.5"N 5°01'52.6"E
1506	cacao 2	7°10'21.0"N 5°02'17.3"E
1507	cacao 1	7°01'24.7"N 5°02'19.9"E
1508	cacao 6	7°09'37"N 5°07'09.4"E
1509	cacao 5	7°09'37.0"N 5°07'09.5"E
1510	cacao 4	7°09'36.9"N 5°07'09.6"E
1511	cacao 3	7°09'36.9"N 5°07'09.5"E
1512	cacao 2	7°09'37.1"N 5°07'09.7"E
1513	cacao 1	7°09'37.0"N 5°07'09.7"E
1514	cacao 6	7°10'41.8"N 5°08'56.5"E
1515	cacao	7°09'40.0"N 5°07'02.3"E

Appendix B continued		
1516	cacao	7°09'43.3"N 5°07'10.0"E
1517	cacao	7°09'34.8"N 5°07'06.6"E
1518	cacao	7°09'39.6"N 5°07'02.9"E
1519	cacao	7°09'43.8"N 5°03'33.8"E
1520	cacao	7°09'40.0"N 5°07'02.3"E
1521	cacao + mealybug	7°09'32.9"N 5°07'04.6"E
1522	cacao	7°09'41.4"N 5°07'05.0"E
1523	cacao	7°09'42.4"N 5°07'06.6"E
1524	cacao	7°10'03.1"N 4°57'35.6"E
1525	cacao	7°10'03.0"N 4°57'36.0"E
1526	cacao	7°09'39.5"N 5°07'02.8"E
1527	cacao	7°11'59.5"N 5°01'51.1"E
1528	cacao + mealybug (P2)	7°11'59.6"N 5°01'53.0"E
1529	cacao + mealybug (P3)	7°10'04.2"N 4°57'33.4"E
1530	cacao + mealybug (P1)	7°09'42.9"N 5°03'32.5"E
	7-3-2013	
1531	cacao 1 + mealybug (P5)	6°52'13.5"N 5°56'20.2"E
1532	cacao 2 + mealybug (P6)	6°50'41.1"N 5°57'36.1"E
1533	cacao + mealybug (P7)	6°52'41.2"N 5°52'18.8"E
1534	cacao 1 + mealybug (P8)	6°52'11.6"N 5°56'18.3"E
1535	cacao 1 + mealybug (P9)	6°51'13.3"N 5°56'25.6"E
1536	cacao + mealybug (P10) - close to *Ceiba*	6°52'51.2"N 5°51'55.8"E
1537	cacao + mealybug (P11)	6°49'46.6"N 5°54'08.4"E
1538	cacao 1 + mealybug (P12)	6°52'15.7"N 5°56'21.6"E
1539	cacao 1 + mealybug (P13)	6°49'44.4"N 5°54'04.0"E
1540	cacao + mealybug (P14)	6°50'46.4"N 5°57'43.1"E
1541	*Ceiba*	6°52'28.1"N 5°52'07.1"E
1542	*Ceiba*	6°51'09.5"N 5°57'45.3"E
1543	*Ceiba*	6°52'34.4"N 5°52'19.2"E
1544	cacao 2 + 2 sp? mealybug (P16)	6°51'02.4"N 5°57'43.9"E
1545	cacao 1 + mealybug (P17)	6°52'10.2"N 5°56'05.8"E
1546	cacao 7 + mealybug (P18)	6°52'40.2"N 5°52'20.2"E
1547	cacao + mealybug (P19)	6°50'11.4"N 5°54'30.0"E
1548	cacao 1 + mealybug (P20)	6°52'40.2"N 5°52'17.8"E
1549	cacao + mealybug (P21)	6°52'36.1"N 5°52'18.3"E
1550	cacao 1 + mealybug (P22) - new sp?	6°52'40.8"N 5°52'18.2"E
1551	cacao 1 + mealybug (P23)	6°52'41.2"N 5°52'19.0"E
1552	cacao 4	6°52'40.5"N 5°52'20.2"E
1553	cacao 4	6°49'44.4"N 5°54'04.0"E
1554	cacao 4	6°52'15.7"N 5°56'21.5"E
1555	cacao 4	6°49'44.8"N 5°54'04.8"E
1556	cacao 5	6°52'40.5"N 5°52'20.2"E

Appendix B continued		
1557	cacao 6	6°52'40.5"N 5°52'20.2"E
1558	cacao 3	6°52'41.2"N 5°52'19.0"E
1559	cacao 3	6°51'12.2"N 5°56'24.6"E
1560	cacao 3	6°52'40.2"N 5°52'17.8"E
1561	cacao 3	6°49'44.4"N 5°54'04.0"E
1562	cacao 3	6°52'40.8"N 5°52'18.2"E
1563	cacao 3	6°52'15.7"N 5°56'21.5"E
1564	cacao 3	6°52'40.5"N 5°52'20.2"E
1565	cacao 3	6°52'09.9"N 5°56'15.8"E
1566	cacao 3	6°49'44.8"N 5°54'04.8"E
1567	cacao 3	6°51'13.1"N 5°56'24.7"E
1568	cacao 3	6°49'43.2"N 5°54'08.7"E
1569	cacao 3	6°51'02.8"N 5°57'47.5"E
1570	cacao	6°50'45.2"N 5°57'42.8"E
1571	cacao	6°52'11.4"N 5°56'04.0"E
1572	*Ceiba*	6°52'19.5"N 5°56'23.9"E
1573	cacao	6°51'13.1"N 5°56'28.7"E
1574	cacao	6°52'14.1"N 5°56'20.5"E
1575	cacao	6°52'36.4"N 5°52'18.2"E
1576	cacao - dried out symptoms?	6°52'50.8"N 5°51'56.3"E
1577	cacao	6°50'42.6"N 5°57'38.2"E
1578	cacao 1	6°51'12.2"N 5°56'24.6"E
1579	cacao	6°52'36.3"N 5°52'17.9"E
1580	cacao	6°51'00.7"N 5°57'47.9"E
1581	cacao - under *Ceiba*	6°52'28.0"N 5°52'07.1"E
1582	cacao	6°52'10.1"N 5°56'15.6"E
1583	cacao	6°49'44.8"N 5°54'05.1"E
1584	cacao	6°50'10.0"N 5°54'29.0"E
1585	cacao	6°52'31.9"N 5°52'01.0"E
1586	cacao	6°50'42.5"N 5°57'38.9"E
1587	cacao	6°52'43.8"N 5°51'59.6"E
1588	cacao	6°51'13.5"N 5°56'28.4"E
1589	cacao - under *Ceiba* + photo	6°52'28.2"N 5°52'07.3"E
1590	cacao	6°52'36.2"N 5°52'18.3"E
1591	cacao 2	6°52'40.5"N 5°52'20.2"E
1592	cacao	6°50'10.4"N 5°54'28.4"E
1593	cacao	6°50'11.0"N 5°54'30.8"E
1594	cacao	6°51'13.5"N 5°56'28.6"E
1595	cacao	6°51'04.7"N 5°57'47.3"E
1596	cacao	6°52'40.5"N 5°52'17.8"E
1597	cacao	6°52'41.7"N 5°52'00.7"E
1598	cacao	6°50'37.5"N 5°57'35.8"E

Appendix B continued		
1599	cacao	6°50'43.2"N 5°57'39.5"E
1600	cacao	6°52'48.3"N 5°51'57.8"E
1601	cacao - close to *Ceiba*	6°52'45.7"N 5°51'58.4"E
1602	cacao	6°52'11.1"N 5°56'20.4"E
1603	cacao	6°51'02.3"N 5°57'43.8"E
1604	cacao	6°50'30.8"N 5°57'21.7"E
1605	*Ceiba*	6°52'28.2"N 5°52'06.8"E
1606	cacao	6°51'04.4"N 5°57'47.9"E
1607	cacao 1 + mealybug (P24)	6°52'12.3"N 5°56'20.7"E
1608	cacao	6°50'16.1"N 5°57'36.0"E
1609	cacao	6°51'07.9"N 5°57'47.8"E
1610	cacao	6°52'45.6"N 5°51'58.4"E
1611	cacao	6°50'10.0"N 5°54'28.8"E
1612	cacao	6°49'44.8"N 5°54'07.9"E
1613	cacao	6°50'46.4"N 5°57'43.1"E
1614	cacao	6°52'46.7"N 5°51'58.4"E
1615	cacao	6°51'02.2"N 5°57'44.0"E
1616	cacao	6°52'14.7"N 5°56'21.2"E
1617	cacao - close to *Ceiba*	6°51'18.2"N 5°56'32.1"E
1618	cacao	6°52'41.1"N 5°52'19.2"E
1619	cacao	6°52'42.2"N 5°52'00.7"E
1620	cacao	6°52'36.4"N 5°52'18.2"E
1621	cacao - under *Ceiba*	6°52'28.0"N 5°52'07.1"E
1622	cacao	6°52'51.4"N 5°51'55.8"E
1623	cacao	6°52'45.5"N 5°51'58.4"E
1624	cacao	6°51'15.9"N 5°56'31.6"E
1625	cacao	6°51'13.1"N 5°56'25.8"E
1626	cacao	6°49'42.2"N 5°54'05.4"E
1627	cacao	6°52'40.2"N 5°52'20.1"E
1628	cacao	6°52'49.7"N 5°51'55.4"E
1629	cacao 1	6°52'48.3"N 5°51'58.1"E
1630	cacao	6°51'13.6"N 5°56'27.4"E
1631	cacao	6°51'01.3"N 5°57'46.4"E
1632	cacao	6°50'42.5"N 5°57'39.0"E
1633	cacao + mealybug (P25)	6°51'00.8"N 5°57'47.4"E
1634	cacao	6°50'46.4"N 5°57'43.1"E
1635	cacao	6°51'02.1"N 5°57'43.9"E
1636	cacao - close to *Ceiba* (dry)	6°52'51.3"N 5°51'55.8"E
1637	cacao	6°49'42.4"N 5°54'04.7"E
1638	cacao	6°50'42.2"N 5°57'38.1"E
1639	cacao - under *Ceiba* + photo	6°52'51.3"N 5°51'55.8"E
1640	cacao	6°50'11.1"N 5°54'30.8"E

Appendix B continued		
1641	cacao	6°52'32.5"N 5°52'00.8"E
1642	cacao	6°51'11.3"N 5°56'25.2"E
1731	N193	5°30'00.0"N7°40'59.4"E
1732	194	5°31'30.9"N7°39'18.7"E
1733	195	5°31'31"N7°39'20.8"E
1734	196	5°32'04.5"N7°34'36.7"E
1735	197	5°31'30.2"N7°39'22.8"E
1736	198	5°30'41.2"N7°41'28.9"E
1737	199	5°32'27.0"N7°34'55.3"E
1738	200	5°32'26.0"N7°34'53.7"E
1739	201	5°29'59.5"N7°41'00.0"E
1740	202	5°32'27.6"N7°34'56.7"E
1741	203	5°32'26.3"N7°34'54.9"E
1742	204	5°30'32.8"N7°41'34.7"E
1743	205	5°30'30.9"N7°41'33.8"E
1744	206	5°30'27.9"N7°41'32.8"E
1745	207	5°29'58.8"N7°41'01.0"E
1746	208	5°29'59.7"N7°40'59.0"E
1747	209	5°30'32.6"N7°41'34.3"E
1748	210	5°32'23.9"N7°34'55.6"E
1749	211	5°29'59.5"N7°40'58.8"E
1750	212	5°30'25.4"N7°41'31.1"E
1751	213	5°32'26.3"N7°34'54.8"E
1752	214	5°30'33.7"N7°41'34.9"E
1753	215	5°32'26.9"N7°34'55.4"E
1754	216	5°32'25.7"N7°34'53.7"E
1755	217	5°30'33.6"N7°41'34.8"E
1756	218	5°30'23.4"N7°41'31.2"E
1757	219	5°32'26.3"N7°34'54.9"E
1758	220	5°29'58.9"N7°41'00.6"E
1759	221	5°32'26.9"N7°34'55.3"E
1760	222	5°29'59.6"N7°40'59.8"E
1761	223	5°30'42.2"N7°41'28.7"E
1762	224	5°30'41.3"N7°41'28.8"E
1763	225	5°30'00.0"N7°40'59.4"E
1764	226	5°30'27.3"N7°41'31.9"E
1765	227	5°32'24.2"N7°34'56.4"E
1766	228	5°30'29.4"N7°41'33.8"E
1767	229	5°29'58.3"N7°40'59.6"E
1768	230	5°32'05.8"N7°34'34.2"E
1769	231	5°32'02.1"N7°34'39.9"E
1770	N232	5°32'06.0"N7°34'33.5"E

Appendix B continued		
	4-6-13	
1963	N1	6°52'14.7"N 5°56'21.2"E
1964	N2	6°52'14.7"N 5°56'21.2"E
1965	N3	6°52'14.7"N 5°56'21.2"E
1966	N4	6°52'14.7"N 5°56'21.2"E
1967	N5	6°52'14.7"N 5°56'21.2"E
1968	N6	6°52'14.7"N 5°56'21.2"E
1969	N7	6°52'14.7"N 5°56'21.2"E
1970	N8	6°52'14.7"N 5°56'21.2"E
1971	N9	6°52'14.7"N 5°56'21.2"E
1972	N10	6°52'14.7"N 5°56'21.2"E
1973	N11	6°52'14.7"N 5°56'21.2"E
1974	N12	6°52'14.7"N 5°56'21.2"E
1975	N13	6°52'14.7"N 5°56'21.2"E
1976	N14	6°52'14.7"N 5°56'21.2"E
1977	N15	6°52'14.7"N 5°56'21.2"E
1978	N16	6°52'14.7"N 5°56'21.2"E
1979	N17	6°52'14.7"N 5°56'21.2"E
1980	N18	6°52'14.7"N 5°56'21.2"E
1981	N19	6°49'44.8"N 5°54'07.9"E
1982	N20	6°49'44.8"N 5°54'07.9"E
1983	N21	6°49'44.8"N 5°54'07.9"E
1984	N22	6°49'44.8"N 5°54'07.9"E
1985	N23	6°49'44.8"N 5°54'07.9"E
1986	N24	6°49'44.8"N 5°54'07.9"E
1987	N25	6°49'44.8"N 5°54'07.9"E
1988	N26	6°49'44.8"N 5°54'07.9"E
1989	N27	6°49'44.8"N 5°54'07.9"E
1990	N28	6°49'44.8"N 5°54'07.9"E
1991	N29	6°49'44.8"N 5°54'07.9"E
1992	N30	6°49'44.8"N 5°54'07.9"E
1993	N31	6°49'44.8"N 5°54'07.9"E
1994	N32	6°49'44.8"N 5°54'07.9"E
1995	N33	6°49'44.8"N 5°54'07.9"E
1996	N34	6°49'44.8"N 5°54'07.9"E
1997	N35	6°49'44.8"N 5°54'07.9"E
1998	N36	6°49'44.8"N 5°54'07.9"E
1999	N37	6°49'44.8"N 5°54'07.9"E
2000	N38	6°49'44.8"N 5°54'07.9"E
2001	N39	6°49'44.8"N 5°54'07.9"E
2002	N40	6°49'44.8"N 5°54'07.9"E
2003	N41	6°49'44.8"N 5°54'07.9"E

Appendix B continued		
2004	N42	6°49'44.8"N 5°54'07.9"E
2005	N43	6°51'07.9"N 5°57'47.8"E
2006	N44	6°51'07.9"N 5°57'47.8"E
2007	N45	6°51'07.9"N 5°57'47.8"E
2008	N46	6°51'07.9"N 5°57'47.8"E
2009	N47	6°51'07.9"N 5°57'47.8"E
2010	N48	6°51'07.9"N 5°57'47.8"E
2011	N49	6°51'07.9"N 5°57'47.8"E
2012	N50	6°51'07.9"N 5°57'47.8"E
2013	N51	6°51'07.9"N 5°57'47.8"E
2014	N52	6°51'07.9"N 5°57'47.8"E
2015	N53	6°51'07.9"N 5°57'47.8"E
2016	N54	6°51'07.9"N 5°57'47.8"E
2017	N55	6°51'07.9"N 5°57'47.8"E
2018	N56	6°51'07.9"N 5°57'47.8"E
2019	N57	6°51'07.9"N 5°57'47.8"E
2020	N58	6°51'07.9"N 5°57'47.8"E
2021	N59	6°51'07.9"N 5°57'47.8"E
2022	N60	6°51'07.9"N 5°57'47.8"E
2023	N61	6°51'07.9"N 5°57'47.8"E
2024	N62	6°51'07.9"N 5°57'47.8"E
2025	N63	6°51'07.9"N 5°57'47.8"E
2026	N64	6°51'07.9"N 5°57'47.8"E
2027	N65	6°51'07.9"N 5°57'47.8"E
2028	N66	6°51'07.9"N 5°57'47.8"E
2029	N67	6°51'07.9"N 5°57'47.8"E
2030	N68	7°10'59.5"N4°59'38.0"E
2031	N69	7°10'59.5"N4°59'38.0"E
2032	N70	7°10'59.5"N4°59'38.0"E
2033	N71	7°10'59.5"N4°59'38.0"E
2034	N72	7°10'59.5"N4°59'38.0"E
2035	N73	7°10'59.5"N4°59'38.0"E
2036	N74	7°10'59.5"N4°59'38.0"E
2037	N75	7°10'59.5"N4°59'38.0"E
2038	N76	7°10'59.5"N4°59'38.0"E
2039	N77	7°10'59.6"N4°59'37.0"E
2040	N78	7°10'59.6"N4°59'37.0"E
2041	N79	7°10'59.6"N4°59'37.0"E
2042	N80	7°10'59.6"N4°59'37.0"E
2043	N81	7°10'59.6"N4°59'37.0"E
2044	N82	7°10'59.6"N4°59'37.0"E
2045	N83	7°10'59.6"N4°59'37.0"E

Appendix B continued		
2046	N84	7°10'59.6"N4°59'37.0"E
2047	N85	7°10'59.6"N4°59'37.0"E
2048	N86	7°10'59.6"N4°59'37.0"E
2049	N87	7°10'59.6"N4°59'37.0"E
2050	N88	7°10'21"N5°02'17.3"E
2051	N89	7°10'21"N5°02'17.3"E
2052	N90	7°10'21"N5°02'17.3"E
2053	N91	7°10'21"N5°02'17.3"E
2054	N92	7°10'21"N5°02'17.3"E
2055	N93	7°10'21"N5°02'17.3"E
2056	N94	7°10'21"N5°02'17.3"E
2057	N95	7°10'21"N5°02'17.3"E
2058	N96	7°10'21"N5°02'17.3"E
2141	N233	5°32'05.7"N7°34'34.2"E
2142	234	5°32'05.7"N7°34'34.2"E
2143	235	5°32'02.9"N7°34'40.1"E
2144	236	5°31'30.2"N7°39'19.2"E
2145	237	5°32'02.2"N7°34'40.2"E
2146	238	5°32'01.9"N7°34'39.7"E
2147	239	5°32'00.5"N7°34'37.5"E
2148	240	5°32'01.7"N7°34'39.5"E
2149	241	5°31'31.3"N7°39'19.3"E
2150	242	5°32'04.5"N7°34'36.7"E
2151	243	5°32'05.4"N7°34'37.2"E
2152	244	5°32'02.9"N7°34'40.1"E
2153	245	5°32'00.1"N7°34'32.7"E
2154	246	5°31'29.5"N7°39'20.1"E
2155	247	5°32'04.5"N7°34'36.7"E
2156	248	5°32'06.1"N7°34'33.7"E
2157	249	5°31'30.2"N7°39'22.8"E
2158	250	5°32'01.0"N7°34'38.4"E
2159	251	5°32'05.9"N7°34'32.8"E
2160	252	5°31'29.5"N7°39'21.6"E
2161	253	5°32'04.2"N7°34'36.6"E
2162	254	5°32'04.5"N7°34'36.6"E
2163	255	5°32'01.9"N7°34'39.7"E
2164	256	6°50'46.0"N5°57'42.9"E
2165	257	6°50'46.3"N5°57'43.1"E
2166	258	6°50'42.3"N5°57'38.2"E
2167	259	6°50'36.9"N5°57'36.3"E
2168	260	6°50'15.4"N5°57'34.7"E
2169	261	6°52'45.2"N5°51'58.7"E

Appendix B continued		
2170	262	6°52'36.3"N5°51'59.9"E
2171	263	6°52'36.3"N5°52'17.7"E
2172	264	6°50'16.2"N5°57'35.9"E
2173	265	6°52'11.2"N5°56'16.4"E
2174	266	6°51'00.5"N5°57'47.4"E
2175	267	6°52'14.7"N5°56'21.2"E
2176	268	6°50'35.6"N5°57'32.6"E
2177	269	6°52'19.4"N5°56'23.9"E
2178	270	6°52'09.8"N5°56'15.3"E
2179	271	6°52'12.3"N5°56'20.9"E
2180	272	6°52'10.1"N5°56'05.8"E
2181	273	6°52'11.6"N5°56'18.3"E
2182	274	6°52'41.2"N5°52'19.0"E
2183	275	6°52'45.1"N5°51'58.7"E
2184	276	6°50'38.4"N5°57'35.3"E
2185	277	6°51'03.6"N5°57'48.2"E
2186	278	6°49'44.8"N5°54'08.0"E
2187	279	6°51'13.3"N5°56'25.6"E
2188	280	6°49'44.4"N5°54'04.0"E
2189	281	6°49'46.5"N5°54'08.5"E
2190	282	6°50'10.5"N5°54'28.6"E
2191	283	6°52'52.0"N5°51'57.8"E
2192	284	6°52'09.8"N5°56'15.8"E
2193	285	6°49'42.4"N5°54'04.7"E
2194	286	6°50'14.8"N5°57'36.0"E
2195	287	6°49'45.1"N5°54'08.1"E
2196	N288	6°51'13.2"N5°56'24.7"E
2399	N97	6°49'44.6"N5°54'08.0"E
2400	N98	6°49'44.8"N5°54'08.0"E
2401	N99	6°49'44.8"N5°54'08.0"E
2402	N100	6°49'44.6"N5°54'08.0"E
2403	N101	6°49'44.6"N5°54'08.0"E
2404	N102	6°49'44.8"N5°54'08.0"E
2405	N103	6°49'44.8"N5°54'08.0"E
2406	N104	6°49'44.8"N5°54'08.0"E
2407	N105	6°49'44.6"N5°54'08.0"E
2408	N106	6°49'44.6"N5°54'08.0"E
2409	N107	6°49'44.6"N5°54'08.0"E
2410	N108	6°49'44.8"N5°54'08.0"E
2411	N109	6°52'14.7"N5°56'21.3"E
2412	N110	6°52'14.4"N5°56'21.2"E
2413	N111	6°52'14.6"N5°56'21.3"E

Appendix B continued		
2414	N112	6°52'14.7"N5°56'21.3"E
2415	N113	6°52'14.7"N5°56'21.3"E
2416	N114	6°52'14.6"N5°56'21.3"E
2417	N115	6°52'14.6"N5°56'21.3"E
2418	N116	6°52'14.6"N5°56'21.3"E
2419	N117	6°52'14.6"N5°56'21.3"E
2420	N118	6°52'14.6"N5°56'21.2"E
2421	N119	6°52'14.6"N5°56'21.2"E
2422	N120	6°52'14.6"N5°56'21.2"E
2423	N121	6°52'14.6"N5°56'21.2"E
2424	N122	6°52'14.7"N5°56'21.3"E
2425	N123	6°52'14.7"N5°56'21.3"E
2426	N124	6°52'14.4"N5°56'21.2"E
2427	N125	6°52'14.7"N5°56'21.3"E
2428	N126	6°52'14.4"N5°56'21.3"E
2429	N127	6°52'14.4"N5°56'21.2"E
2430	N128	6°52'14.6"N5°56'21.3"E
2431	N129	6°52'14.4"N5°56'21.3"E
2432	N130	6°52'14.4"N5°56'21.2"E
2433	N131	6°51'08.1"N5°57'48.0"E
2434	N132	6°51'08.0"N5°57'47.9"E
2435	N133	6°51'08.0"N5°57'47.7"E
2436	N134	6°51'08.0"N5°57'47.7"E
2437	N135	6°51'08.2"N5°57'48.0"E
2438	N136	6°51'08.1"N5°57'48.0"E
2439	N137	6°51'08.0"N5°57'47.7"E
2440	N138	6°51'08.1"N5°57'48.0"E
2441	N139	6°51'08.3"N5°57'48.0"E
2442	N140	6°51'08.0"N5°57'48.0"E
2443	N141	6°51'08.0"N5°57'47.9"E
2444	N142	6°51'08.3"N5°57'48.0"E
2445	N143	6°51'08.3"N5°57'48.0"E
2446	N144	6°51'08.0"N5°57'47.6"E
2447	N145	6°51'08.2"N5°57'48.0"E
2448	N146	6°51'08.0"N5°57'47.6"E
2449	N147	7°10'20.8"N5°02'17.4"E
2450	N148	7°10'20.8"N5°02'17.4"E
2451	N149	7°10'20.8"N5°02'17.3"E
2452	N150	7°10'20.8"N5°02'17.3"E
2453	N151	7°10'21"N5°02'17.1"E
2454	N152	7°10'21"N5°02'17.1"E
2455	N153	7°10'51.5"N4°59'36.5"E

Appendix B continued		
2456	N154	7°10'51.5"N4°59'36.5"E
2457	N155	7°11'00.1"N4°59'37.9"E
2458	N156	7°11'00.2"N4°59'37.7"E
2459	N157	7°10'20.8"N5°02'17.2"E
2460	N158	7°10'20.8"N5°02'17.2"E
2461	N159	7°10'21.1"N5°02'17.2"E
2462	N160	7°10'21.0"N5°02'17.2"E
2463	N161	7°10'21.0"N5°02'17.2"E
2464	N162	7°10'21.0"N5°02'17.2"E
2465	N163	7°10'21.0"N5°02'17.2"E
2466	N164	7°10'21.0"N5°02'17.2"E
2467	N165	7°10'21.1"N5°02'17.2"E
2468	N166	7°10'21.1"N5°02'17.2"E
2469	N167	5°30'42.2"N7°41'28.7"E
2470	N168	5°30'41.2"N7°41'28.8"E
2471	N169	5°30'41.2"N7°41'28.8"E
2472	N170	5°30'40.6"N7°41'28.7"E
2473	N171	5°30'35.9"N7°41'34.2"E
2474	N172	5°30'36.7"N7°41'34.0"E
2475	N173	5°30'42.2"N7°41'29.1"E
2476	N174	5°30'41.2"N7°41'29.0"E
2477	N175	5°30'41.3"N7°41'28.9"E
2478	N176	5°30'39.8"N7°41'30.9"E
2479	N177	5°30'35.9"N7°41'34.3"E
2480	N178	5°30'40.6"N7°41'28.7"E
2481	N179	5°30'40.6"N7°41'28.7"E
2482	N180	5°30'38.8"N7°41'32.2"E
2483	N181	5°30'35.8"N7°41'34.2"E
2484	N182	5°31'59.3"N7°34'35.1"E
2485	N183	5°32'05.8"N7°34'34.2"E
2486	N184	5°31'59.6"N7°34'34.8"E
2487	N185	5°32'02.4"N7°34'39.9"E
2488	N186	5°31'30.9"N7°39'18.7"E
2489	N187	5°32'07.2"N7°34'44.4"E
2490	N188	5°32'01.7"N7°34'39.5"E
2491	N189	5°32'02.2"N7°34'40.2"E
2492	N190	5°32'04.5"N7°34'36.7"E
2493	N191	5°32'24.2"N7°34'56.4"E
2494	N192	5°29'59.9"N7°40'59.3"E
2495	N289	6°49'44.8"N5°54'04.8"E
2496	N290	6°51'12.2"N5°56'24.6"E
2497	N291	6°52'36.8"N5°51'59.8"E

Appendix B continued		
2498	N292	6°52'37.3"N5°51'59.5"E
2499	N293	6°52'48.3"N5°51'58.1"E
2500	N294	6°52'13.4"N5°56'20.2"E
2501	N295	6°52'36.3"N5°56'17.8"E
2502	N296	6°51'04.7"N5°57'47.6"E
2503	N297	6°52'15.7"N5°56'21.5"E
2504	N298	6°51'00.8"N5°57'48.4"E
2505	N299	6°51'14.6"N5°56'31.1"E
2506	N300	6°52'40.2"N5°52'17.8"E
2507	N301	6°51'01.4"N5°57'46.3"E
2508	N302	6°52'51.7"N5°51'59.8"E
2509	N303	6°50'36.6"N5°57'36.3"E
2510	N304	6°49'42.6"N5°54'07.3"E
2511	N305	6°51'11.1"N5°56'25.2"E
2512	N306	6°52'34.2"N5°52'00.3"E
2513	N307	6°52'35.1"N5°51'59.1"E
2514	N308	6°49'42.1"N5°54'05.1"E
2515	N309	6°52'51.3"N5°51'59.3"E
2516	N310	6°51'02.7"N5°57'47.5"E
2517	N311	6°49'42.0"N5°54'05.7"E
2518	N312	6°52'40.8"N5°52'18.2"E
2519	N313	6°52'45.6"N5°51'58.5"E
2520	N314	5°55'24.3"N8°45'37.7"E
2521	N315	5°57'38.0"N8°46'25.3"E
2522	N316	5°57'37.6"N8°46'24.1"E
2523	N317	5°52'38.7"N8°49'07.8"E
2524	N318	5°55'25.5"N8°45'37.6"E
2525	N319	5°57'34.0"N8°46'25.1"E
2526	N320	5°55'23.6"N8°45'37.6"E
2527	N321	5°55'25.1"N8°45'38.3"E
2528	N322	5°55'25.2"N8°45'38.6"E
2529	N323	5°52'01.0"N8°45'01.5"E
2530	N324	5°52'00.3"N8°45'01.6"E
2531	N325	5°51'44.0"N8°48'56.7"E
2532	N326	5°51'57.1"N8°49'02.0"E
2533	N327	5°57'41.3"N8°46'22.4"E
2534	N328	5°51'43.6"N8°49'00.1"E
2535	N329	5°55'23.6"N8°45'37.6"E
2536	N330	5°57'39.6"N8°46'19.4"E
2537	N331	5°52'38.7"N8°49'07.8"E
2538	N332	5°57'41.4"N8°46'22.4"E
2539	N333	5°55'25.6"N8°45'35.0"E

Appendix B continued		
2540	N334	5°57'38.9"N8°47'19.6"E
2541	N335	5°52'36.8"N8°49'06.6"E
2542	N336	5°55'24.4"N8°45'34.5"E
2543	N337	5°55'24.3"N8°45'34.2"E
2544	N338	5°55'25.5"N8°45'35.0"E
2545	N339	5°57'35.0"N8°46'24.4"E
2546	N340	5°55'24.6"N8°45'38.5"E
2547	N341	5°52'01.6"N8°48'57.7"E
2548	N342	5°52'40.9"N8°49'08.6"E
2549	N343	5°52'20.1"N8°48'56.9"E
2550	N344	5°57'40.4"N8°46'23.9"E
2551	N345	5°52'34.4"N8°49'01.7"E
2552	N346	5°51'42.7"N8°48'57.9"E
2553	N347	5°52'26.3"N8°48'57.8"E
2554	N348	5°52'17.8"N8°48'54.9"E
2555	N349	5°52'40.6"N8°49'08.3"E
2556	N350	5°51'44.3"N8°49'00.4"E
2557	N351	5°52'00.2"N8°49'01.5"E
2558	N352	5°51'43.5"N8°48'55.5"E
2559	N353	5°55'25.6"N8°45'36.3"E
2560	N354	5°51'44.3"N8°49'00.4"E
2561	N355	5°55'23.2"N8°45'37.2"E
2562	N356	5°52'34.3"N8°49'01.7"E
2563	N357	5°51'59.1"N8°49'00.6"E
2564	N358	5°55'24.3"N8°45'37.7"E
2565	N359	5°52'35.0"N8°49'05.4"E
2566	N360	5°51'43.4"N8°49'00.2"E
2567	N361	5°55'23.9"N8°45'35.4"E
2568	N362	5°57'40.4"N8°46'24.0"E
2569	N363	5°57'37.4"N8°46'24.0"E
2570	N364	5°55'24.4"N8°45'35.1"E
2571	N365	5°52'19.8"N8°48'58.0"E
2572	N366	5°52'36.8"N8°49'06.8"E
2573	N367	5°57'35.2"N8°46'25.3"E
2574	N368	5°57'37.4"N8°46'23.9"E
2575	N369	5°52'19.9"N8°48'58.0"E
2576	N370	5°55'23.4"N8°45'37.6"E
2577	N371	5°51'42.7"N8°48'58.0"E
2578	N372	5°52'00.8"N8°49'00.8"E
2579	N373	5°51'43.6"N8°48'55.9"E
2580	N374	5°51'43.6"N8°48'55.9"E
2581	N375	5°52'01.0"N8°49'01.5"E

Appendix B continued		
2582	N376	5°52'00.7"N8°49'00.6"E
2583	N377	5°57'34.0"N8°46'25.1"E
2584	N378	5°51'43.6"N8°48'55.9"E
2585	N379	6°51'01.4"N8°57'46.3"E
2586	N380	6°49'43.2"N5°54'08.7"E
2587	N381	6°52'37.3"N5°01'59.5"E
2588	N382	6°49'42.5"N5°54'04.8"E
2589	N383	6°52'52"N5°51'57.8"E
2590	N384	6°52'34.2"N5°52'00.3"E
NOTE	N385 - N412: Not Extracted!	
	N385	6°51'02.4"N5°57'43.9"E
	N386	6°52'45.1"N5°51'58.7"E
	N387	6°49'45.1"N5°54'08.1"E
	N388	6°51'11.2"N5°56'25.3"E
	N389	6°51'03.6"N5°57'48.1"E
	N390	6°52'36.3"N5°52'17.8"E
	N391	6°50'38.4"N5°57'35.3"E
	N392	6°52'36.8"N5°51'59.8"E
	N393	6°51'02.7"N5°57'47.5"E
	N394	6°49'42.0"N5°54'05.7"E
	N395	6°52'51.5"N5°51'59.4"E
	N396	6°49'42.6"N5°54'07.4"E
	N397	6°52'40.6"N5°52'20.2"E
	N398	6°49'44.7"N5°54'08.0"E
	N399	6°50'41.1"N5°57'36.2"E
	N400	6°52'51.8"N5°51'59.8"E
	N401	6°50'10.5"N5°54'28.6"E
	N402	6°49'43.2"N5°54'08.7"E
	N403	6°49'42.1"N5°54'05.1"E
	N404	6°51'14.6"N5°56'31.1"E
	N405	6°51'00.7"N5°57'48.5"E
	N406	6°52'35.1"N5°51'59.7"E
	N407	6°50'36.6"N5°57'36.3"E
	N408	6°51'13.2"N5°56'24.6"E
	N409	6°51'04.7"N5°57'47.6"E
	N410	6°49'44.8"N5°54'04.8"E
	N411	6°52'09.8"N5°56'15.8"E
	N412	6°50'14.8"N5°57'36.0"E

Appendix C: Identity and geographic location of mealybug species with matching CO1 sequences

Reference Code	Field Code	Grid Reference	CO1 match	Species match	Site
M153	P11d	5°30'33.6"N7°41'34.8"E	√	*Pl. minor*	Akwa Ibom
M157	P17a	5°32'04.5"N7°34'36.7"E	√	*Pl. minor*	Abia
M475	N12	5°32'25.7"N7°34'53.7"E	√	*Pl. minor*	Abia
M154	P11e	5°30'33.6"N7°41'34.8"E	√	*Pl. minor*	Akwa Ibom
M451	N16a	5°32'02.2"N7°34'40.2"E	√	*Pl. citri?*	Abia
M452	N16b	5°32'02.2"N7°34'40.2"E	√	*Pl. citri?*	Abia
M466	N29	5°55'23.4"N8°45'37.6"E	√	*Pl. citri?*	Cross River
M468	N2	5°32'04.5"N7°34'36.6"E	√	*Pl. citri?*	Abia
M471	N6	5°32'00.5"N7°34'37.5"E	√	*Pl. citri?*	Abia
M479	N14	5°32'02.4"N7°34'39.9"E	√	*Pl. citri?*	Abia
M480	N15	5°32'01.7"N7°34'39.5"E	√	*Pl. citri?*	Abia
M481	N16a	5°32'02.2"N7°34'40.2"E	√	*Pl. citri?*	Abia
M482	N16b	5°32'02.2"N7°34'40.2"E	√	*Pl. citri?*	Abia
M484	N17	5°32'04.5"N7°34'36.7"E	√	*Pl. citri?*	Abia
M486	N19	5°29'58.8"N7°41'01.0"E	√	*Pl. citri?*	Abia
M495	N23	5°52'01.0"N8°49'01.5"E	√	*Pl. citri?*	Cross River
M498	N28	5°55'24.4"N8°45'35.1"E	√	*Pl. citri?*	Cross River
M499	N29	5°55'23.4"N8°45'37.6"E	√	*Pl. citri?*	Cross River
M527	N16c (M483)	5°32'02.2"N7°34'40.2"E	√	*Pl. citri?*	Abia
M447	N13a	5°32'26.9"N7°34'55.4"E	√	*Pl. citri/minor?*	Abia
M448	N13b 1	5°32'26.9"N7°34'55.4"E	√	*Pl. citri/minor?*	Abia
M449	N13b 2	5°32'26.9"N7°34'55.4"E	√	*Pl. citri/minor?*	Abia
M450	N13b 3	5°32'26.9"N7°34'55.4"E	√	*Pl. citri/minor?*	Abia
M453	N20a 1	5°32'26.3"N7°34'54.8"E	√	*Pl. citri/minor?*	Abia
M455	N20b 1	5°32'26.3"N7°34'54.8"E	√	*Pl. citri/minor?*	Abia
M456	N20b 2	5°32'26.3"N7°34'54.8"E	√	*Pl. citri/minor?*	Abia
M457	N20b 3	5°32'26.3"N7°34'54.8"E	√	*Pl. citri/minor?*	Abia
M458	N20c 1	5°32'26.3"N7°34'54.8"E	√	*Pl. citri/minor?*	Abia
M459	N20c 2	5°32'26.3"N7°34'54.8"E	√	*Pl. citri/minor?*	Abia
M460	N20e 1	5°32'26.3"N7°34'54.8"E	√	*Pl. citri/minor?*	Abia
M461	N20e 2	5°32'26.3"N7°34'54.8"E	√	*Pl. citri/minor?*	Abia
M462	N21a 1	5°51'43.6"N8°48'55.9"E	√	*Pl. citri/minor?*	Cross River
M463	N21a 2	5°51'43.6"N8°48'55.9"E	√	*Pl. citri/minor?*	Cross River
M464	N24	5°51'43.6"N8°48'55.9"E	√	*Pl. citri/minor?*	Cross River
M465	N27	5°52'19.9"N8°48'58.0"E	√	*Pl. citri/minor?*	Cross River
M467	N32 1	5°52'36.8"N8°49'06.8"E	√	*Pl. citri/minor?*	Cross River
M469	N4	5°31'29.5"N7°39'21.6"E	√	*Pl. citri/minor?*	Akwa Ibom
M470	N5	5°32'05.9"N7°34'32.8"E	√	*Pl. citri/minor?*	Abia
M472	N7	5°32'26.9"N7°34'55.3"E	√	*Pl. citri/minor?*	Abia
M476	N13a	5°32'26.9"N7°34'55.4"E	√	*Pl. citri/minor?*	Abia

Appendix C continued					
M477	N13b	5°32'26.9"N7°34'55.4"E	√	*Pl. citri/minor?*	Abia
M487	N20a	5°32'26.3"N7°34'54.8"E	√	*Pl. citri/minor?*	Abia
M488	N20c	5°32'26.3"N7°34'54.8"E	√	*Pl. citri/minor?*	Abia
M489	N20d	5°32'26.3"N7°34'54.8"E	√	*Pl. citri/minor?*	Abia
M490	N20e	5°32'26.3"N7°34'54.8"E	√	*Pl. citri/minor?*	Abia
M491	(N20P x)	5°32'26.3"N7°34'54.8"E	√	*Pl. citri/minor?*	Abia
M492	N21a	5°51'43.6"N8°48'55.9"E	√	*Pl. citri/minor?*	Cross River
M493	N21b	5°51'43.6"N8°48'55.9"E	√	*Pl. citri/minor?*	Cross River
M494	N22	5°57'34.0"N8°46'25.1"E	√	*Pl. citri/minor?*	Cross River
M496	N24	5°51'43.6"N8°48'55.9"E	√	*Pl. citri/minor?*	Cross River
M497	N27	5°52'19.9"N8°48'58.0"E	√	*Pl. citri/minor?*	Cross River
M501	N31	5°57'35.2"N8°46'25.3"E	√	*Pl. citri/minor?*	Cross River
M502	N32	5°52'36.8"N8°49'06.8"E	√	*Pl. citri/minor?*	Cross River
-	-	7° 9'32.90"N 5° 7'4.60"E	√	*Pl. citri/minor?*	Ondo
-	-	6°49'46.6"N 5°54'8.4"E	√	*Pl. citri/minor?*	Edo
-	-	6°52'10.2"N 5°56'5.8"E	√	*Pl. citri/minor?*	Edo
M528	N20d	5°32'26.3"N7°34'54.8"E	√	*Pl. citri/minor?*	Abia
M532	N25	5°52'00.7"N8°49'00.6"E	√	*Pl. citri/minor?*	Cross River
M142	P2a2	5°32'01.9"N7°34'39.7"E	√	*Pa. loranthi?*	Abia
M141	P2a1	5°32'01.9"N7°34'39.7"E	√	*Fo. njalensis*	Abia
M144	P2a4	5°32'01.9"N7°34'39.7"E	√	*Fo. njalensis*	Abia
M145	P2a5	5°32'01.9"N7°34'39.7"E	√	*Fo. njalensis*	Abia
M146	P2a8	5°32'01.9"N7°34'39.7"E	√	*Fo. njalensis*	Abia
M159	P2ab	5°32'01.9"N7°34'39.7"E	√	*Fo. njalensis*	Abia
M443	N10a 1	5°30'23.4"N7°41'31.2"E	√	*Fo. njalensis*	Akwa Ibom
M444	N10a 2	5°30'23.4"N7°41'31.2"E	√	*Fo. njalensis*	Akwa Ibom
M445	N10b 1	5°30'23.4"N7°41'31.2"E	√	*Fo. njalensis*	Akwa Ibom
M446	N10b 2	5°30'23.4"N7°41'31.2"E	√	*Fo. njalensis*	Akwa Ibom
M473	N10a	5°30'23.4"N7°41'31.2"E	√	*Fo. njalensis*	Akwa Ibom
M474	N10b	5°30'23.4"N7°41'31.2"E	√	*Fo. njalensis*	Akwa Ibom
M500	(N30P x)	5°57'37.4"N8°46'23.9"E	√	*Fe. virgata*	Cross River
M143	P2a3	5°32'01.9"N7°34'39.7"E	√	+	Abia
M147	P4a	5°31'29.5"N7°39'21.6"E	√	+	Akwa Ibom
M149	P3b	-	√	+	-
M150	P11a dry	5°30'33.6"N7°41'34.8"E	√	+	Akwa Ibom
M151	P11b dry	5°30'33.6"N7°41'34.8"E	√	+	Akwa Ibom
M152	P11c	5°30'33.6"N7°41'34.8"E	√	+	Akwa Ibom
M155	P15	5°32'01.7"N7°34'39.5"E	√	+	Abia
M148	P3a	-	√	+	-

- unresolved (missing) information; + CO1 sequence matched but species unknown

Appendix D: Analysis of variance (untransformed data)

Variate: C

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Species	2	212199	106099	0.48	0.62
Residual	45	9870059	219335		
Total	47	10082258			
		s.e.	cv%		
	45	468.3	70.4		

Variate: E1

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Species	2	214.67	107.34	3.16	0.052
Residual	45	1527.78	33.95		
Total	47	1742.45			
		s.e.	cv%		
	45	5.827	311.5		

Variate: E1e

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Species	2	447	223	0.17	0.847
Residual	45	60189	1338		
Total	47	60636			
		s.e.	cv%		
	45	36.57	147.9		

Variate: E2

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Species	2	2362	1181	0.08	0.924
Residual	45	671814	14929		
Total	47	674175			
		s.e.	cv%		
	45	122.2	394.2		

Variate: F

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Species	2	537565	268783	2.91	0.065
Residual	45	4155515	92345		
Total	47	4693080			
		s.e.	cv%		
	45	303.9	181.9		

Appendix D (continued)

Variate: G

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Species	2	365691	182846	0.7	0.502
Residual	45	11755793	261240		
Total	47	12121484			
		s.e.	cv%		
	45	511.1	112.9		

Variate: Np

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Species	2	61319	30660	0.68	0.511
Residual	45	2026313	45029		
Total	47	2087632			
		s.e.	cv%		
	45	212.2	221.3		

Appendix E: Analysis of variance (log_e transformed data)

Variate: C

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Species	2	3.594	1.797	0.79	0.46
Residual	45	102.382	2.275		
Total	47	105.976			
		s.e.	cv%		
	45	1.508	25.2		

Variate: E1

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Species	2	6.0266	3.0133	4.45	0.017
Residual	45	30.4859	0.6775		
Total	47	36.5125			
		s.e.	cv%		
	45	0.823	243.2		

Variate: E1e

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Species	2	15.995	7.998	3.21	0.05
Residual	45	112.066	2.49		
Total	47	128.061			
		s.e.	cv%		
	45	1.578	72.9		

Variate: E2

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Species	2	2.062	1.031	0.39	0.682
Residual	45	120.294	2.673		
Total	47	122.356			
		s.e.	cv%		
	45	1.635	306.4		

Variate: F

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Species	2	33.649	16.824	2.33	0.109
Residual	45	324.639	7.214		
Total	47	358.288			
		s.e.	cv%		
	45	2.686	129.3		

Appendix E (continued)

Variate: G

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Species	2	45.454	22.727	3.18	0.051
Residual	45	321.816	7.151		
Total	47	367.27			
		s.e.	cv%		
	45	2.674	61.8		

Variate: Np

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Species	2	2.008	1.004	0.34	0.716
Residual	45	134.155	2.981		
Total	47	136.163			
		s.e.	cv%		
	45	1.727	52		

Appendix F: Daily indoor temperature (°C), relative humidity and dew point (°C) logs (October – December 2014) during EPG monitoring of *Planococcus citri*, *Pseudococcus longispinus* and *Pseudococcus viburni* feeding on cacao leaves.