Genetic studies of *Phytophthora* on *Theobroma cacao* from East New Britain and Bougainville (Papua New Guinea)

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ABSTRACT

DNA was extracted from 14 *Phytophthora* isolates from *Theobroma cacao* plants (SG2 hybrids, hybrid derived clones and Trinitario varieties) collected from New Britain and Bougainville in Papua New Guinea (PNG). A fragment of the mitochondrial genome cytochrome b (cytb) region was amplified from these DNAs using the polymerase chain reaction (PCR) and compared to cytochrome b sequences from *Phytophthora palmivora* and other *Phytophthora* (two isolates previously isolated from cocoa lesions and two *P. palmivora* isolates obtained from culture collection-Australia). All isolates were identical in their cytb gene sequence and similar to *P. palmivora*. Additionally, we sequenced the mitochondrial genomes of four isolates from PNG. The syntenic arrangement of genes in one complete assembly was compared with other published mitochondrial genomes. The sequences of four mitochondrial genes (COII, nad2, rps10 and SecY) from the four PNG isolates were aligned with orthologues from accessions of *P. palmivora* and other *Phytophthora* species available in the NCBI Genbank reference database. A concatenated data matrix was produced with 2,295 homologous sequence positions. 34 accessions of *Phytophthora* (including 14 *P. palmivora*) were used to construct a maximum likelihood tree of phylogenetic relationships. This reconstruction recovered all 10 major clades of *Phytophthora* previously reported. In this phylogenetic reconstruction, the four PNG isolates were clearly identifiable as *P. palmivora* and these were closely related to the Clade 4 *Phytophthora* species *P. megakarya* and *P. quercetora*. Of the genes analysed, COII showed greatest variability, resolving *P. palmivora* into three sub groups. COII was sequenced in all *P. palmivora* isolates from PNG and used to reconstruct an ML tree. The phylogenetic analyses suggested a potential origin for the PNG strain of *P. palmivora* in Samoa. Syntenic comparisons of *P. palmivora* and other clade 4 species identified a potential target for developing a Loop Mediated Amplification (LAMP) assay for *P. palmivora* near the atpH gene region. DNA amplification primers were designed for this region using PrimerExplorer, V4, Eiken Chemical CO.Ltd) and validated against available DNAs for Clade 4 and other *Phytophthora* species.

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CHAPTER1: INTRODUCTION

1.1 Cocoa history and uses

To the ancient Aztecs and Mayas, Cocoa (*Theobroma cacao*, L.) was the '*Food of the Gods'*. It belongs to the Steculiaceae family of flowering plants and is known to have originated as a small understory tree in the Amazon basin from the southern and central region of the American continent (Motamayor *et al*. 2002*;* Coe *et al*. 2006*;* Motamayor *et al*. 2008*;* Argout *et al*. 2011). Historically, cacao trees were cultivated by the Mayans (now Guatemala) and Aztecs of Mexico, and the beans were used as currency as well as for the production of a spiced drink called "chocolatl" (Beckett, 1994*;* World Cocoa Foundation, 2016). This story of chocolate making dates back 1200 years to 1900 BC (Watson, 2013). The story is fascinating, and origins of chocolate are deeply rooted to Aztec and Mayan cultures and linked to betrothal and marriage ceremonies (World Cocoa Foundation 2016, ICCO 2011). Cocoa fascinated Christopher Columbus during his discovery voyage to the Mesoamerica and he brought back cocoa beans to Europe (Beckett, 1994). Cocoa beans later became commercially important as the basis for a popular drink in Spain and the commodity quickly spread to Central and Northern Europe (Beckett 1994). According to the World Cocoa Foundation (2016) and Hart (2010), by 1657 London's first chocolate house had opened and the café society advocated the drink as a cure (medicine) for treating Tuberculosis. This recorded history tells how cocoa beans (roasted and grinded) were mixed with sugar, cocoa butter and milk to make a solid chocolate bar, and by the 1700s, every country, from England to Austria, was producing confectionary from the fruit of the cocoa tree. During this period, the introduction of the steam engine mechanized cocoa bean grinding, reducing production costs and making chocolate affordable to all (World cocoa foundation, 2016; Beckett, 1994). Today chocolate production involves many companies from all continents of the world and involves people from Europe, Africa, Asia, North America, South America, Australia and Oceania/Pacific. People around the world enjoy chocolate in thousands of different forms, consuming more than three (3) million tons of cocoa annually (World Cocoa Foundation, 2016). Figure 1.1 illustrates a sample of products produced by different manufacturers.

Some research has suggested that the cocoa bean does have medicinal properties, most notably, cocoa is thought to provide significant benefits for the cardiovascular system,

helping to reduce the risk of heart attack and stroke, reduce the risk of high blood pressure, and even reduce the risk of cancer (Mathur *et al*. 2002*;* Kilham, 2012). It is widely known that cocoa is rich in anti-oxidants similar to wine and tea, and cocoa butter is used in the manufacture of cosmetics (Prance 2006, Williams *et al*. 2009).

There are various cultivated populations and varieties of *Theobroma cacao* that currently exist including Criollo, Forastero, Amelonado, Amazonian, and Trinitario (Wood and Lass, 1985). Although, this classification has been in existence for centuries, an alternative classification of cocoa types (races) was more recently proposed based on 106 microsatellite markers that identified ten (10) genetically distinct groups (Motamayor *et al*., 2008). Despite the new classification system, many farmers and researchers in PNG still use the old system because it is simple and practical to implement.

Figure 1.1 Chocolate and cosmetic products made from cocoa beans A) Chocolate bars are sold according to cocoa content (e.g. 70% cocoa, 40% cocoa etc). B) Physical appearance and packaging of chocolate products varies considerably. C) A chocolate bar handmade in Wellington from beans sourced in Bougainville. D) Cocoa butter is frequently used as a component of medicinals and cosmetics**.**

The Trinitario germplasm was the first cocoa planting material introduced into PNG. It was brought to PNG from Samoa by early settlers, missionaries and German colonists () in the early 1900s (Woods and Lass, 1985). Initial cocoa production in PNG was based only on the Trinitario germplasm (variety), which was cultivated on large estate plantations. Similar germplasm was introduced from Java in 1932 (Efron *et al*. 1996). The upper Amazonian germplasm was introduced in the 1960s. Evaluation of these introductions resulted in the selection of improved clones, including KEE 42, KEE 43 and KEE47, which were found to be tolerant to *Phytophthora* pod rot disease while KEE 5 and KEE5 were found to be susceptible (McGregor, 1981). Several of these clones have been used as parental stocks for a cocoa improvement breeding program in PNG. Several commercial poly-cross hybrids namely: Seed Garden One and Seed Garden Two (SG1 and SG2) have been developed and released in PNG (Efron *et al*. 1996). In addition, there are now two series of hybrid derived clonal varieties grown for the PNG cocoa industry.

1.1.1 Cocoa and PNG economy

Agriculture plays a major role in the development of many countries including PNG. Cocoa is one on the main agricultural commodities and agriculture contributes significantly to total value of exports. Figure 1.2 indicates this value at 17.4%, in comparison to the mineral (53.4%) and petroleum (15.9%) sectors (Data from http://atlas.media.mit.edu/en/ profile/country/png/). While overshadowed by energy and mining investments, PNG's agricultural sector is nevertheless seen as a key sector for attracting foreign currency and as a primary employer for many of its citizens (Oxford Business Group, 2015). Smallholder farmers began to cultivate cocoa as a cash crop in the 1950s, (Efron *et al*. 1996*;* Eskes *et al*. 2000*;* Efron *et al*. 2005). Currently, PNG produces about 39, to 40,000 000 metric tonnes of dry bean per year (ICC0, 2014) worth over K380 million (U$ 127 million). More than two thirds of the total annual cocoa is produced by small farm holders. At the current average world price of $U3200 per tonne, which is equivalent to approximately K9696 per tonne, cocoa farmers earn much needed cash for food, clothing, school fees and other goods and services that improve their standard of living (Allen *et al*. 2009). Figure 1.3 indicates the comparative earnings from different crops in PNG. Cocoa (10.9%) is the third (3rd) largest revenue earner in the agriculture sector after palm oil (41.9%) and coffee (13.2%).

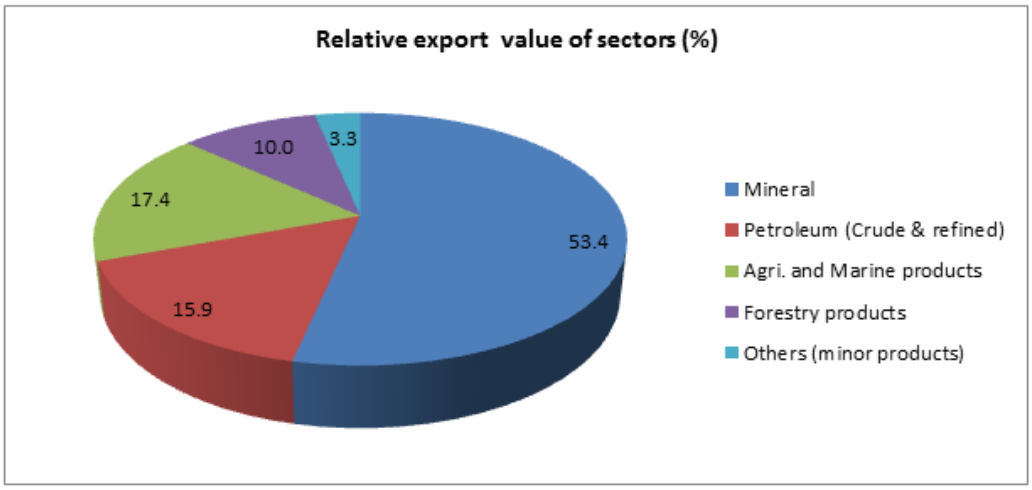

Figure 1.2 Proportion (%) of total merchandised export (K19484 million or NZ$ 9917 million) in 2013. **Source:** **http://atlas.media.mit.edu/en/profile/country/png/**.

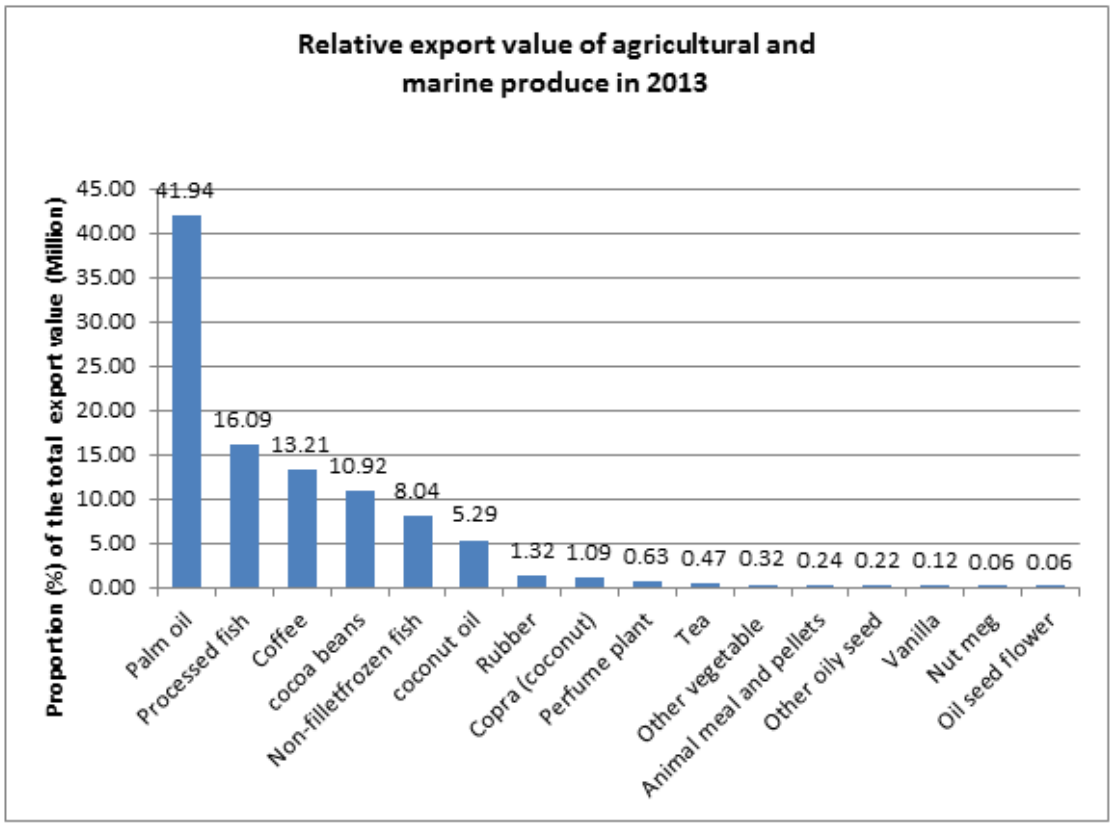

Figure 1.3 Relative export value of agricultural and marine produce in 2013

Source: http://atlas.media.mit.edu/en/profile/country/png/

1.1.2 Cocoa diseases pose a major constraint to increasing yield and income

Globally, the production of cocoa, is constrained by the prevalence of numerous destructive pests and diseases. Wherever cocoa has been introduced, it has encountered new pests and diseases (Buddengen, 1977). The most common diseases in cocoa growing regions include black pod disease (Bp) and *Phytophthora* pod rot (Ppr). Termed the 'plant destroyer' *Phytophthora* are a cosmopolitan genus of Oomycete obligate plant pathogens (Erwin & Ribiero, 1996).

This group of ubiquitous fungus-like pathogens infect many agricultural and horticultural plants as well as forest and ornamental plants (Brasier *et al*. 1979; Vanegtern *et al*. 2015*;* Jung *et al*. 2007).

Two major diseases thought to have co-evolved with cocoa at its center of origin (the Amazon basin) are witches broom (*Ernipellis pernisiosa* (stahel) Singer) and Monoliasis (*Moniliopthora roreri*). Following its translocation to new growing regions, Theobroma plants have also been challenged by pests and diseases recorded only from outside of the center of origin of cocoa. These include viruses (Cocoa swollen shoot virus), Myrids, Pantorythes weevils, Longicorn trunk borer, the Cocoa pod borer and *Phytophthora* (Keane *et al*.1991). The latter include *P. megakarya* in West Africa (Braiser *et al.* 1981), *P. capsici* Leon in Brazil and *P. palmivora* in the Pacific and Africa. Of most economic significance is the Vascular Streak Dieback (VSD) caused by *Oncobasidium theobromae* and *Phytophthora* pod rot (Ppr) caused by *P. palmivora* (Keane *et al.*1991; Efron *et al.* 1996).

In many South East Asia countries, *Phytophthora* results in crop losses of rubber, durian, coconut, pepper, citrus fruit, potato, and cocoa (Drenth and Guest, 2004; Figure 1.4). The estimated loss in monetary value due to the crop failure in various countries has been estimated at 2.4 billion USD (Drenth and Guest, 2004). In cocoa production, *Phytophthora* is responsible for *Phytophthora* pod rot (PPR) or black pod disease, stem canker, leaf and seedling blight, chupon wilt and flower cushion infections (Akrofi, *et al*. 2003*;* McMahon and Purwantara, 2004) as well as diseases of cocoa plant roots (Drenth and Sendall, 2001, Drenth and Guest, 2004). PPR causes 10–30% of the annual losses in yield of cocoa beans production globally, and sometimes this is as high as 40 % (Saul, 1993) in PNG (Guest 2006) particularly during wet and humid conditions (Drenth and Sendall, 2001*;* McMahon and Purwantara, 2004*;* Vanegtern *et al*. 2015). Due to their devastating effects worldwide, and more specifically in PNG, *P. palmivora* and other *Phytophthora* sp. have been constantly monitored for their spread and evolution for over a century ago, beginning in the 1800s.

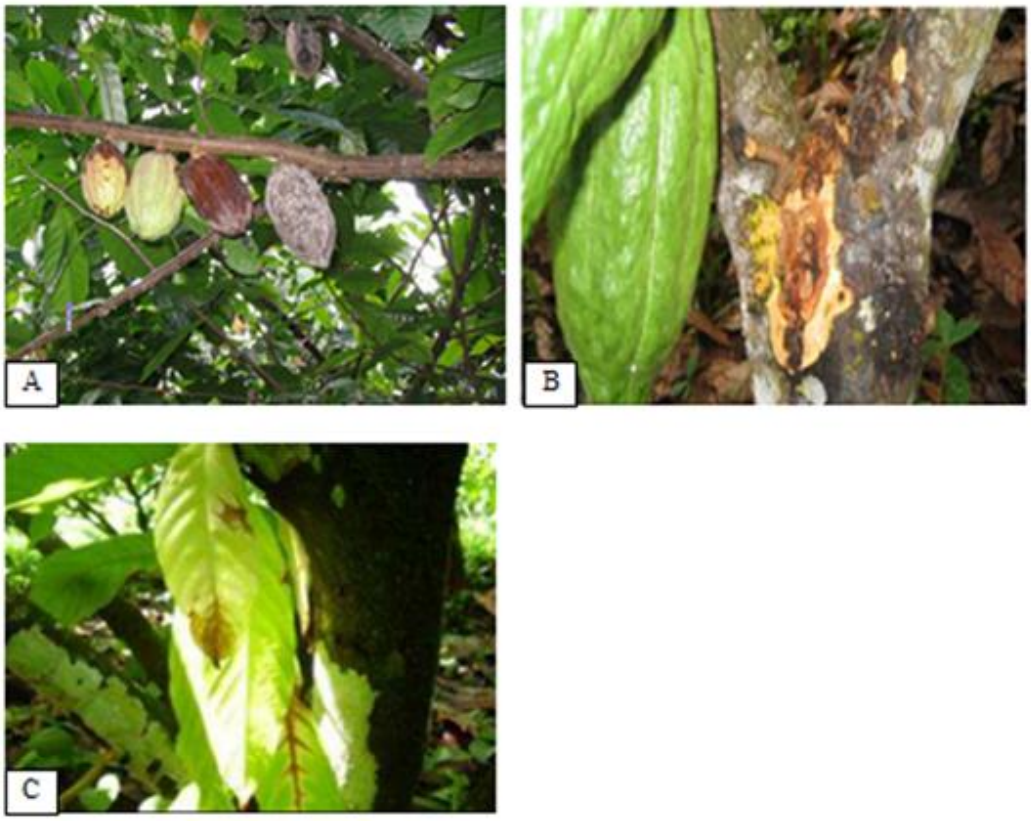

Figure 1.4 Types of cocoa diseases caused by *P.palmivora*

A) Black pod, B) Canker and C) seedling blight.

1.2 A history of research on *Phytophthora*

Phytophthora has a wide geographical distribution throughout the world (Martins *et al*. 2007). The genus is famously remembered as responsible for the late blight disease of potato that caused the Irish potato famine of 1845–1852 in Europe. The causative agent for this disease was *P. infestans*, and it was the first formally described species within the genus (Erwin and Ribiero, 1996*;* Drenth *et al*. 2006).

Since Anton de Bary first described *P. infestans*, 70 new species have been identified and formally described. All species of *Phytophthora* are plant pathogens and they can cause severe devastation to a large variety of ornamental trees and crop plant species. *P. palmivora* which causes wide spread pod rot (Bp) disease, exists in almost all cocoa producing countries around the world, with the exception of West African countries, where the close phylogenetic relative *P. magakarya* occurs.

The number of *Phytopthora* identified continues to grow. Over a 100 species have been described so far. About a third of them are still not officially recognized as species (Kroon *et al*. 2012*;* Bilodeau *et al*. 2014) and it is estimated that 100-500 species remain to be described (Brasier, 2009). Brasier suggests that it is helpful to recognise two phases in the discovery and identification of *Phytopthora* species. These are identifications made pre-2000,that is, from 1870 to the 1990s,.

1.2.1 New species discovery prior to 2000

Figure 1.5 plots the number of described *Phytopthora* species over a 120 year time period. The data are from Drenth *et al*. (2006), Brasier, (2009), Vanegtern *et al*. (2015). It shows that there is an almost constant linear increase in identifications rising from one to 55 species by the end of the 1990s. Coinciding with the end of the pre-2000 phase of *Phytophthora* discovery was a book entitled '*Phytophthora Diseases worldwide*' (Erwin & Ribeiro, 1996)'. This treatment provided taxonomic descriptions, methods for isolations/detection, culture techniques, physiology/morphology of species, host species and geographic distribution of *Phytophthora*. It also included details of cultural practices, chemical applications and biological agents that can be used for control of *Phytophthora*.

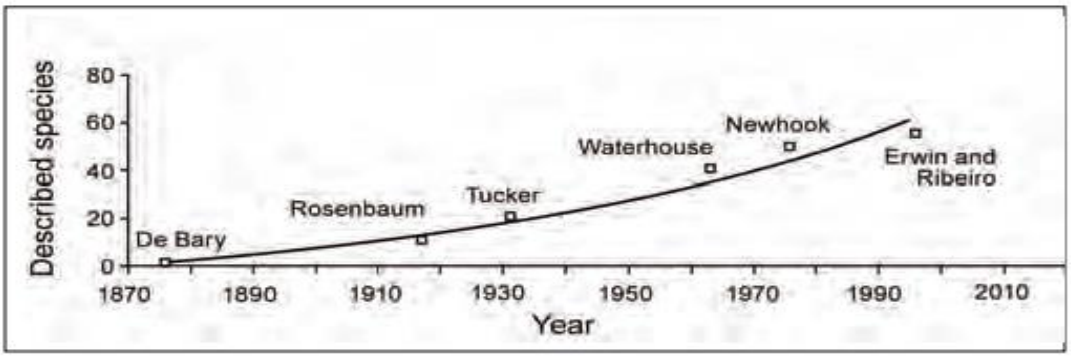

Figure 1.5 Number of described *Phytopthora* species over time, 1876-1995
Source: Braiser (2009)

1.2.2 New species discovery post 2000

Since 2000 there has been an exponential increase in the number of identifications and descriptions of *Phytophthora* species (Figure 1.6). This in part appears to reflect an increase in the number of field surveys and research activities of forestry and agricultural researchers as well as an increase in the number of (horticultural) plant species affected by the pathogen.

In addition, there has also been an emergence of more holistic, population based species concepts since the 1980s, as well as the rapid application of molecular tools (Braiser, 2009). In general, in recent years there has been a significant re-examination of species concepts in light of molecular data. The initiation of research projects and increase in the number of studies focused on *Phytophthora* species recognises the economic impact of these pathogens on crop plants and natural ecosystems (Miles *et al*. 2014).

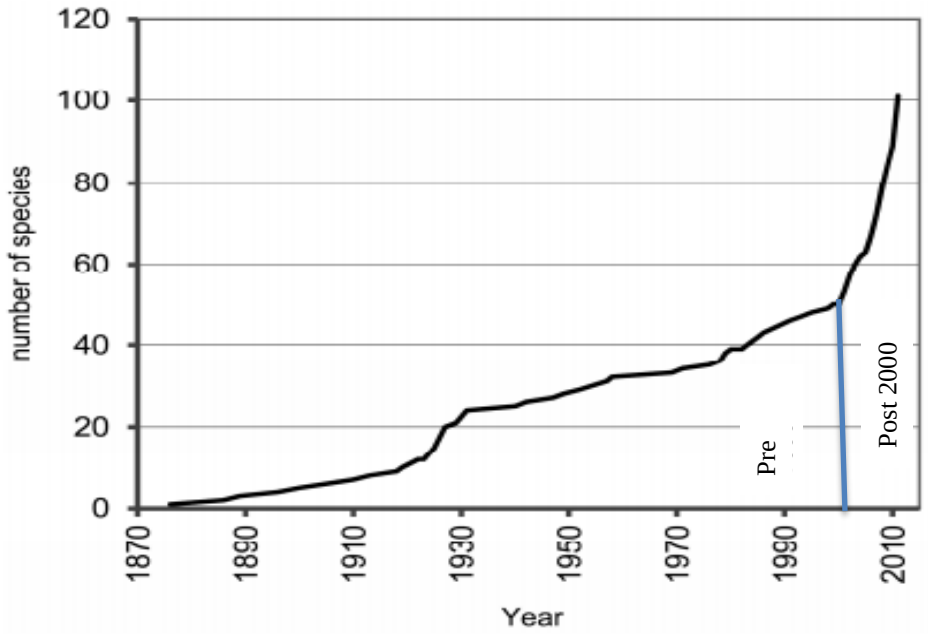

Figure 1. 6 Graph depicting number of *Phytophthora taxa* discovered pre and post 2000 Source: Braiser (2009)

There has being much debate on a taxonomic concept for *Phytopthora* species. As pointed out by Brasier *et al*. 1979, difficulties arise because of the lack of availability of type cultures and the absence of herbarium records for the collections first described by Butler in 1917. For example, several morphological groups within *P. palmivora* have been described by several authors (Drenth el al. 2001*;* Brasier *et al*. 1979). These distinct morphologies are now recognised as representing different species such the *P. megakarya* and *P. citophthora*. Morphological variation is also recognised within the species (Saul *et al*. 2009). For example, there is great variation in the sporangia shape, mycelium growth and branching on growth media within the *P. palmivora* population from PNG.

Originally classified in the kingdom 'Fungi', *Phytopthora* have been recently reclassified into the kingdom Stramenopila (Vanegtern *et al*. 2015). This has being done based on contemporary biochemical and molecular data which showed that Oomycetes have little affinity with the 'true' fungi such as *Ascomycetes* and *Basidiomycetes* (Baldauf *et al*. 2000). Thus while *Phytophthora* share some morphological similarities with Eumycotian fungi, these features are thought to have arisen independently. They differ from Eumycota fungi in features such as being diploid throughout their life cycle and forming motile, biflagellate spores called zoospores which are capable of swimming in water (Martin *et al*. 2007).

The most recent phylogenetic analyses with nuclear ribosomal DNA (rDNA) sequences and mitochondrial cytochrome oxidase II genes indicate a close affiliation of *Phytophthora* with downy mildews and white rusts (Albugo spp.) from the Peronosporales (a family of water moulds) (Beakes and Sekimoto 2009*;* Martins *et al*. 2012). Peronosporales are obligate biotrophic plant pathogens which parasitize their host plants as an intercellular mycelium using haustoria to penetrate their host cells.

1.3 *Phytophthora palmivora*

Eight (8) species of *Phytophthora* have been associated with cocoa diseases and isolated from diseased cocoa plant parts (pods, leaves and stems). Most production losses worldwide are caused by *P. palmivora*, and *P. megakarya* (Iwaro *et al*. 1998*;* Opoku *et al*. 2000). Additionally, *P. citrophthora* has also reportedly been commonly isolated from diseased *Theobroma* plants in Central and South America, and the West Indies (Brasier *et al*. 1979). All eight species have been found to be phylogenetically similar and are members of clade 4 (Widmer, 2014).

Saul (2008) studied the diversity of *Phytophthora* causing black pod disease in PNG recently based on random amplified microsatellite markers (RAMS). He confirmed that there is only one species (*P. palmivora*) causing black pod disease of cocoa in PNG. He also suggested that different sub–species exist in PNG which might reflect differences in geographic distribution, cross breeding of A1 type to A2 types or even an accelerated evolutionary process due to the climate change. Such hypotheses require further investigation. However, Saul's observations suggesting intra-specific diversity highlights the need for further investigation of *P. palmivora* in PNG.

1.3.1 Life cycle of P. palmivora.

P. palmivora is known to have four types of asexual spores that usually cause infection; sporangia, zoospores, oospores and chlamydospores (Widmer 2014*;* Vanegtern *et al*. 2015) (Figure 1.7). Diploid vegetative mycelium produces asexual sporangia, which may germinate directly, or differentiate to produce 8-32 flagellated, one celled and short lived zoospores which swim through thin films of water on leaf surfaces or water filled soil pores (Widmer, 2014), and this has been the main strategy of ensuring the cycle of dispersal and encystment before germination (Drenth el al. 2000*;* Widmer, 2014). The ssporangia can detach and be blown or splashed with water to nearby plants where they grow into hyphae or mycelium. Hyphae allow the pathogen to infect and grow within plant cells so that they can obtain food. Once *Phytophthora* infects the plant, it produces chlamydospores, oospores, and/or sporangia to complete its life cycle.

Oospores are produced through sexual reproduction in *P. palmivora* which require the presence of opposite mating types known as A1 and A2. These mating types can also produce zoospores capable of long term survival but zoospores do not generally play a significant role in the disease cycle because sexual reproduction requires the presence of opposite mating types and the chance of co-occurrence of these in nature is thought to be low (Braiser and Griffins,1979).

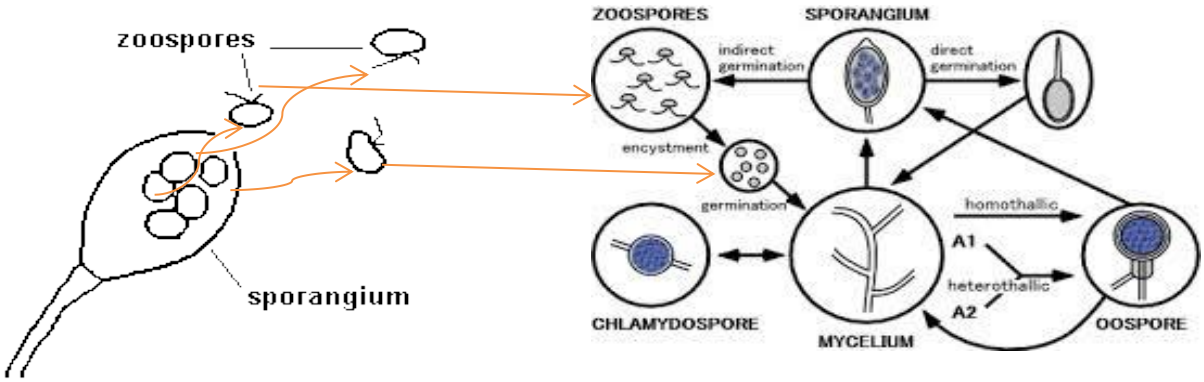

Figure 1.7 Different stages of the life cycle of the *P. palmivora*

1.3.2 Morphology of P. palmivora.

P. palmivora has its own distinct morphological features and reproductive cycle linked to various growth stages of its host plant species. Following infection of plant host tissues (leaves or stem) growth continues to a mature stage where fruiting bodies (sporangia)

containing zoospores are produced. Other spore types (chlamydospore and oospores) can also be produced depending on the growing conditions. Once developed, spores fall to the soil where they can remain viable for a long period of time, waiting for a conducive condition to germinate. This is often during the wet season when they then infect new plants.

A distinguishable feature of *P. palmivora* is the conspicuous papillate sporangia which are distinct from those of other *Phytophthora* species (Figure 1.8). In *P. palmivora* they are caduceus (easily detached and shed at an early stage) and have short pedicels. The sporangia form on sympodial sporangiophores (APPS, 2008).

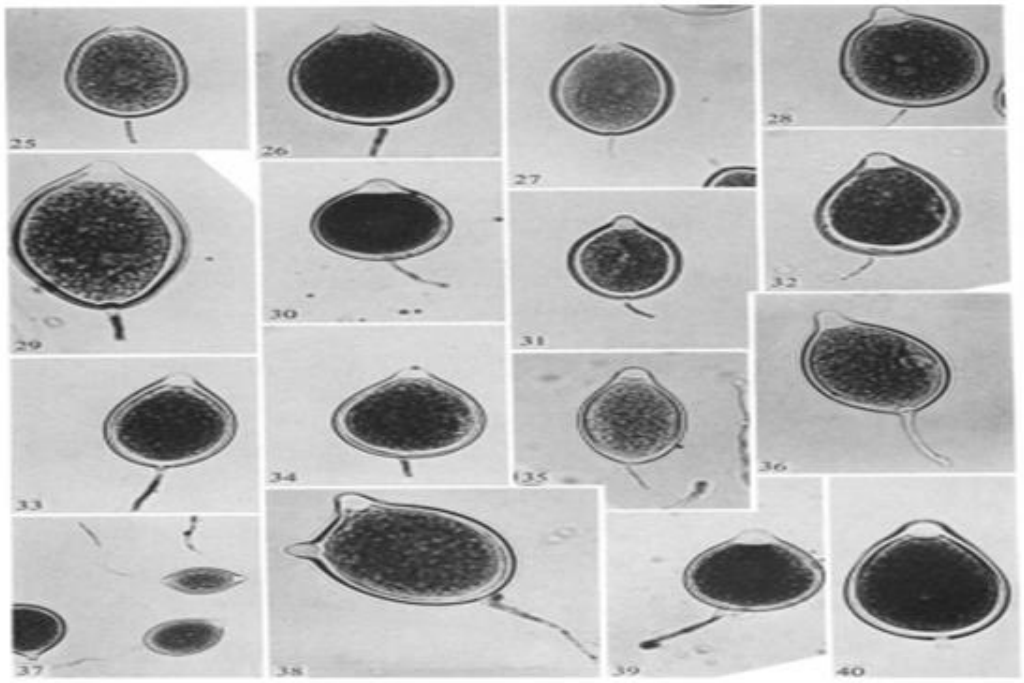

Figure 1.8 Sporangia types from Isolates collected in West Africa, Note typically thin non-occluded pedicels and variation in sporangial size, e.g 25-27 (Nigeria Isolate), 33-34 (Cameroon Isolate). Source: Braiser (1979).

1.3.3 Epidemiology of P. palmivora

The spread of black pod disease caused by *P. palmivora* involves a process that takes place between the hosts (cacao trees) and the environment (soil, climate, and insects) over a period of time. Infected pods, stems, infested flowers and contaminated soil provide a source of inoculum in the form of sporangia (zoospores) which arises from sporulation of growing mycelia on the plant tissues. Crawling or flying insects browsing on infected tissues, is thought to result in many sporangia or zoospores sticking to body parts and subsequent

transfer to healthy pods as the insects move between branches and trees. The inoculum is spread effectively during the night and day by flying beetles (Scolytids and Nitidulids) and during the day by tent-building ants. There have been many studies (Evan, 1973*;* Gregory and Maddison, 1981, Konan and Guest, 2004*;* Konam *et al*. 2008) that have indicated the role that these insects (invertebrates) play as vectors of black pod disease in cocoa. The building black ant (*Crematogaster striatula*) was found to be responsible for moving inoculum from the soil to the canopy. These ants also use old infected pods to construct tents around the pod peduncle, and this usually can lead to infection starting from the peduncle region. The soil is an important part of the disease cycle as it is the source of primary inoculum, the mycelium and chlamydospores that are spread by ants and termites to the canopy higher above, contributing to as high as 20% of primary infections as reported by Anon (1992). Sporangia are splashed from the surface of wet soil or piles of infected pod cases, picked up in turbulence and potentially spread as aerosol droplets that settle on developing pods or foliage (Konam and Guest 2004). As the new healthy pods become infected in higher branches, they provide an inoculum source (zoospores) which can be easily transferred to lower branches when it rains. Interventions, for example the removal of disease pods as a management practice, can break this cycle between cocoa trees, the environment and the pathogen.

1.4 Disease Management for *P. palmivora*

There are several means by which the disease is managed or controlled and this can involve cultural practices for growing plants and crop (tree) sanitation methods. The disease is also managed through disease management and breeding strategies that involve screening and the release of resistant varieties or cultivars (Efron *et al*. 1996*;* Saul *et al*. 2003). Integrated pest and disease management (IPDM) is employed. The aim of this being to prevent the build- up of the disease inoculum through the removal of infected pods, stem, branches and applying chemical treatments to cankers on tree trunks.

1.4.1 Improved tolerance to P. palmivora

One of the most effective and environmentally friendly control measures has been the introduction of resistant cocoa genotypes that can tolerate the infection and growth of *Phytophthora* on the cacao pods, leaves and stems (Zadoks 1996*;* Efron *et al*.1996). Resistant cultivars have been introduced in Cote d'Ivore (Kebe *et al*. 1996) and in the Cameroon

(Blaha,1974). Similar efforts have also been initiated in Nigeria as early as the 1950s (Toxopeus, 1996) and in Ghana also in the 1950s (Bell and Rogers 1956*;* Wharton, 1959). From these efforts good parental clonal materials were identified and tested in the production of improved hybrid varieties. Some have been used directly in commercial plantings as improved cultivars. Well-known clonal accessions include SCA6, SCA12, NA33 and NA34, P7 (Efron *et al*. 1996*;* Tahi *et al*. 2000*;* Efron *et al*. 2005). These have been distributed and shared worldwide by many national breeding programs. Breeding efforts have incorporated resistance genes into different genotypes which have other favourable traits such as high yield and large bean size.

In PNG several parental materials have been developed and used. Amongst them are K82 and KA2-106 which are used as female Trinitario parents with field resistance to black pod disease (*Phytophthora* pod rot) at rates of 8.8% and 8.1% respectively (Saul *et al*. 2003). In PNG, male parents used have been upper Amazonia clones KEE42, KEE43 and KEE47. These have respective pod rot infestation rates of 5.4, 1.8, and 4.5%. The hybrid progenies produced from these cultivar crosses have resulted in plants with a higher level of tolerance than the Amazonian clones and they are being produced and supplied to PNG farmers. According to Saul *et al*. (2003), the progenies derived from crosses with K82, a well-known resistant clone in PNG, have been superior to others. This finding is in agreement with earlier work reported by several researchers (McGregor 1981*;* Saul 1993). Despite this progress, there is still a need for good management and husbandry practices of the cacao trees in the field or plantation to increase yield through reducing disease pressure.

1.4.2 Management and sanitation of trees

Proper management of cacao trees is vital in reducing the *P. palmivora* diseases inoculum as well as the build-up of other pests in the field (farms) and to obtain better air circulation and light interception by the cacao trees. As the spread of *Phytophthora* pod rot (black pod disease) is associated with poor farm management practices, practicing good field sanitation such as removal of infected pod husks, proper pruning of the canopy, and judicious selection of shade species and associated crops can significantly reduce *Phytophthora* disease (Konam *et al*. 2008). According to Guest (2006), the discovery and recent development of antagonistic endophytes (a biological control agent) also offers promise in the future for protecting cocoa trees from black pod disease.

There have been many reports that chemical control methods are being used. Chemicals, including application of metalaxyl (an acylalanine fungicide with systemic function; its chemical name is methyl N--N--DL-alaninate) and injection of phosphonates (organophosphorus compounds containing C−PO(OH)2 or C−PO(OR)2 groups (where R = alkyl, aryl); Figure 1.9), have proved successful in combating root rot in avocados and cocoa into (Drenth el al. 2001). Copper-based fungicides are the most commonly used chemicals for the control of black pod disease of cocoa caused by *Phytophthora* sp. (McGregor, 1984). The latest development and packaging of the 'Integrated Pest and Disease Management (IPDM)' strategy (Konam *et al*. 2008) describes low, medium, and high input options (involving pruning, pest control and use of chemical fungicides, shade tree management and use of resistant varieties) to reduce yield losses in cocoa.

Figure 1.9 A cocoa tree trunk affect by canker caused by *P. palmivora* and treatment with chemical fungicide (metalaxyl).

1.4.3 Screening for P. palmivora resistance

Genetic parameters of resistance have being studied, in order to more effectively guide the selection of resistance to black pod disease (Ppr). Some work has being done on resistance screening (e.g. Tan and Tan, 1990, Efron *et al*. 1996*;* Efron *et al*. 2002). Different ways of assessing resistance have been tested mainly by observing infection levels in the field and by carrying out artificial inoculation tests on attached or detached pods, natural infection in the field (Iwaro *et al*.1998). Assessing resistance at an early stage, by testing plant tissue other than pods (e.g. organs such as leaf) have been given a priority for most breeding programmes (Nyass´e *et al*. 1995). In PNG, Leaf discs and pods have been used for routine assessment. In this case, cultivars are screened with inoculations of zoospores extracted from *P. palmivora* isolates, usually collected from a specific site (e.g. such as from the CCI plantation field in the East New Britain province of PNG). Screening has been confirmed as a

promising technique in Cote 'D' Ivore (Tahi *et al*. 2000). There they have shown that field resistance of cacao genotypes to black pod can be predicted using early screening tests, and this can accelerate the selection process of resistant cultivars.

1.4.4 Pod Inoculation screening method

Immature unripe pods are collected as test materials in the field and brought to the laboratory for disinfection and sterilisation process. The pods are then put into trays sealed with plastic covering to maintain humidity or moisture and stored in a 24-25C^0 dark room overnight. The following day, previously cultured *P. palmivora* mycelia is extracted, washed and filtered to extract the zoospores and sprayed on the pods or the leaf discs that were

Figure 1.10 Screening resistance to black pod disease (*P.palmivora*), A) Detached pod inoculation test in the lab, B) Leaf discs inoculation test in the lab.

prepared the previous day. Following artificial inoculation, lesion formation and growth of mycelium on pods (or leaf discs) is assessed three, five and seven days after inoculation (Figure 1.10).

1.5 Diagnostics for *Phytophthora*

For timely decision making in managing fungal and fungal-like (e.g. Oomycete) pathogens and disease, rapid field deployable diagnostic tests are needed which can provide, accurate and reliable identification of *Phytophthora* species on plant tissues and in the soil. Often symptoms of different diseases look similar to each other. For example, the seedling

blight of small cocoa seedlings in the nursery may be mistaken for dieback symptoms of cocoa, and this means decisions concerning appropriate actions are more difficult to make.

Drenth *et al*. (2006) and Brasier *et al*. (1979) describe one approach for identification of *Phytophthora* pathogens that involves inducing the formation of spores from sporangia by placing small blocks of agar containing the *Phytophthora* into growth medium, incubating these and analysing the morphology of the spores through microscopic examination (Figure 1.11). Similarly, other characters such as the presence of chlamydospores, hyphal swellings and characters associated with the formation of oospores have also been used for identifying *Phytophthora* to species level (Braiser *et al*. 1979). However, such diagnostic investigations all require isolating the fungal pathogen from the diseased plant tissue using culture media containing a cocktail of antibiotics. This is a labour intensive process, which requires a considerable skill and time (Drenth *et al*. 2006). As an alternative for disease identification of *P. palmivora*, there have been reports of baiting (Fig.1.12) from the soil, immune-detection assays, and identification of species using polymerase chain reaction (PCR) and real- time PCR assays (Drenth *et al*. 2006*;* Jeffers and Martin 1986*;* Martin *et al*. 2012).

The enzyme – linked immuno-sorbent assay (ELISA) has also been used successfully to detect *Phytophthora* at the genus level from plant material, water and soil (Miles *et al*. 2014). While useful, species specific information has not been attainable using this approach.

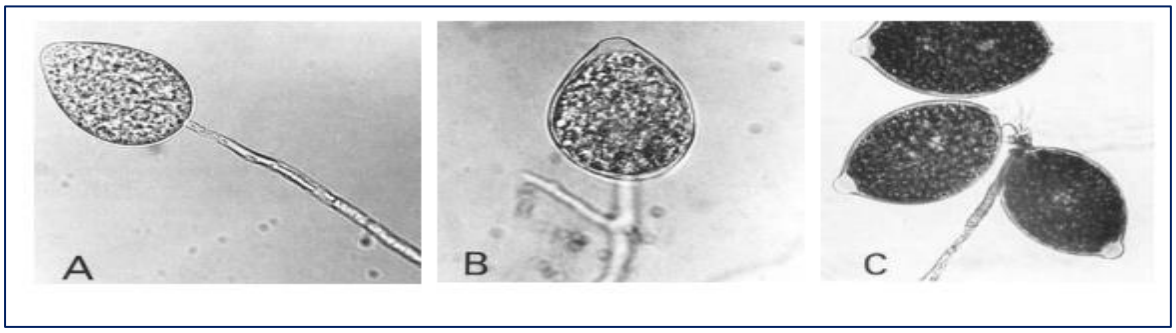

Figure 1.11 Morphological forms of sporagispores of *P. palmivora,* A) Non-papilate, B) semi-papilate and C) papillated sporangia. Source: Drenth (2001).

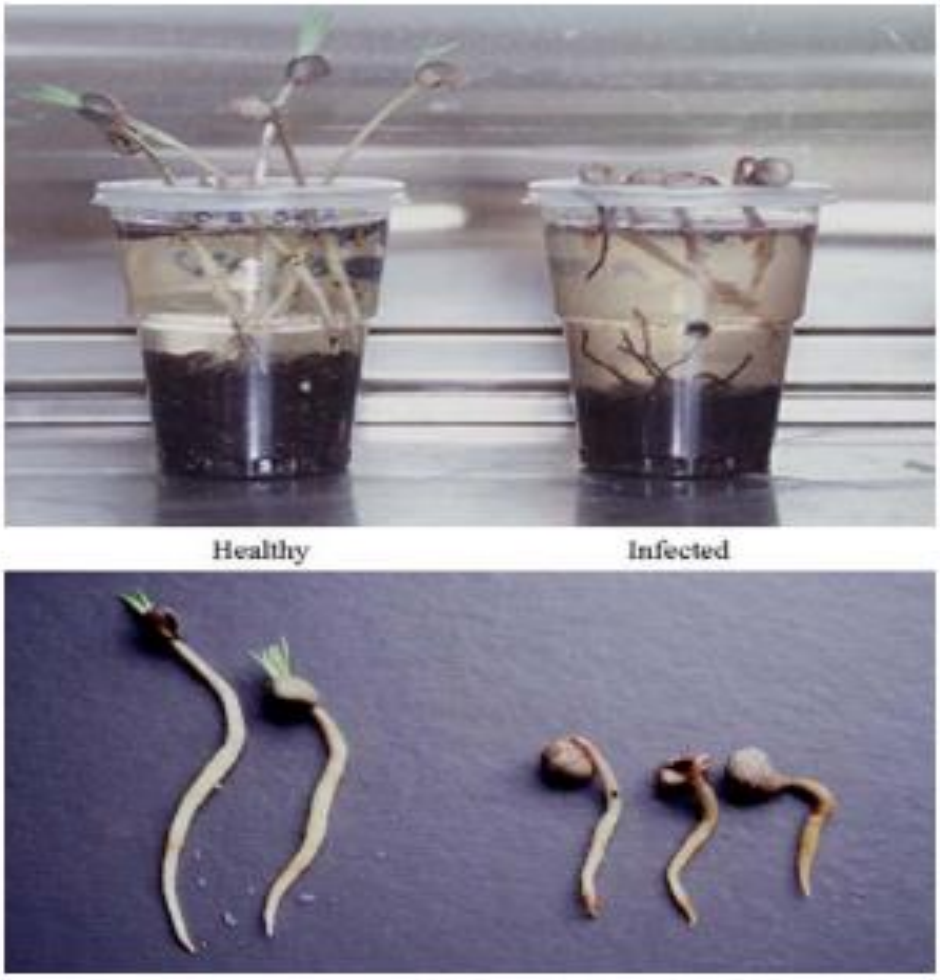

Figure 1.12 Lupin baiting of *Phytophthora* from soil samples. Source: Drenth (2001).

1.5.1 The Polymerase Chain Reaction

Molecular genetics has opened up new avenues for the detection and identification of fungal and fungal-like pathogens (Drenth, 2001). In particular, the development of the Polymerase Chain Reaction (PCR) (Mullis *et al*. 1986*;* Mullis *et al*. 1994*;* Saiki *et al*. 1985) has revolutionised diagnostics of these and other microorganisms. Furthermore, high levels of specificity can be achieved through the design of oligonucleotide amplification primers, and reaction conditions used in cyclic DNA amplification process.

PCR and it quantitative real-time PCR (qPCR) implementation have to-date provided a means for routine diagnosis of many infectious diseases, particularly bacterial diseases (Maurin, 2012). One reason for their popularity is that these methods are much faster that culture based methods of identification that rely on morphology and biochemical tests (Barken *et al*. 2007). The advantage is most significant when working with fastidious and

slow growing microorganisms. PCR based methods also offer more sensitivity and specificity over diagnostics based on traditional microbiological techniques (Mothershed and Whitney 2006). Using PCR, detection can be based on DNA amplification alone when species specific primers are used. Alternatively, detection can be based on restriction endonuclease digestion of the amplified DNA or sequencing of the amplified DNA (PCR product).

PCR technology has been made possible because of elucidation of the structure of DNA (Watson and Crick, 1953), by determining that DNA replicates by a semiconservative mode of replication (Kongberg, 1956; Meselson and Stahl, 1958), by identifying enzymes that catalyses the DNA replication process (e.g. Lehman *et al*. 1958) and understanding the role of oligonucleotide primers in initiating DNA synthesis (Okazaki *et al*. 1967). Together these ingredients formed the basis and the principles used in PCR technology (Figure 1.13; Mullis *et al*. 1986*;* Mullis *et al*. 1994) where a DNA is first denatured into its component two strands at 94C^{o} (high temperature), where oligonucleotide fragments (primers) are annealed of primers at ~50 C^{o} to initiate DNA synthesis, where DN synthesis (extension or elongation) on both strands occurs in the 5' to 3' direction at 72C^{o}. This process is cycled ~ 30x which is sufficient to produce millions of copies the targeted DNA region within a couple of hours. This process has enabled molecular biologists to manipulate and genotype small regions of the genome quickly and in many cases replace earlier DNA cloning methods which were being used to make copies of particular DNA regions. Genotyping of PCR products, which often involves subsequent DNA sequencing of the PCR product has proved useful in phylogenetic investigations and for identification purposes. To determine if PCR amplification has been successful, PCR products are often subjected to gel electrophoresis to visualise the DNA fragments as bands on a gel. These bands represent co-migrating DNA fragments of the same length. Gel electrophoresis is described in more detail below.

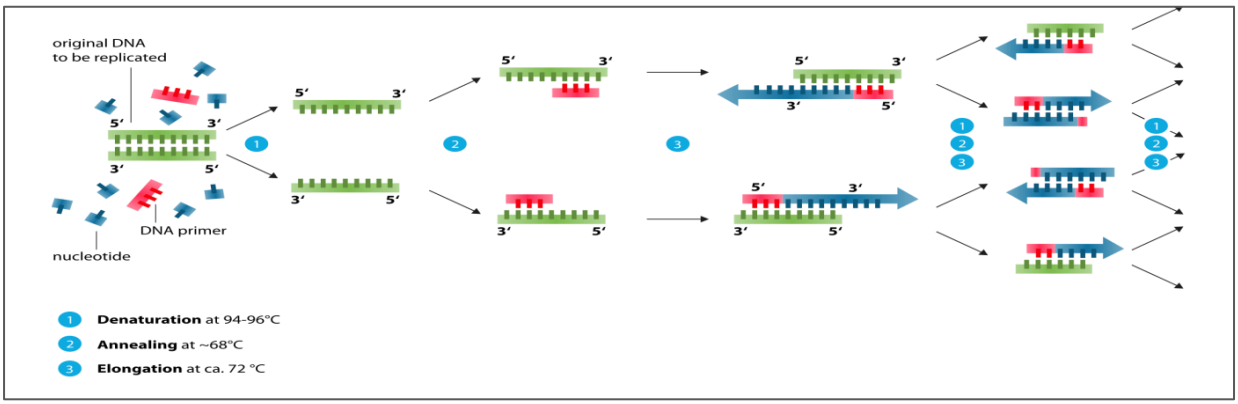

Figure 1.13 Principle of the Polymerase Chain Reaction

Where PCR primers are made highly specific for a particular pathogen genome, the resulting PCR products should be unique (this can be determined in a number of ways) and thus are useful for pathogen identification (Ersek *et al*. 1994). PCR tests are now widely used for diagnosing viral pathogens in mammals and plants.

In their research article, Drenth *et al*. (2006) emphasize that detection and identification of the causal agents of disease are an essential part of effective disease management. These researchers also developed and validated a PCR-based diagnostic assay that could detect and identify 27 different *Phytophthora* species. In their protocol, PCR amplicons were subjected to digestion by restriction enzymes to yield a specific restriction pattern unique to each species which could be visualized using gel electrophoresis. Cooke & Duncan (1997), Miles *et al*. (2014) and Martin *et al*. (2012), have also developed PCR based identification protocols targeting several nuclear genes in *Phytophthora* for species identification.

1.5.2 Di-deoxy DNA Sequencing

One of the most significant developments in molecular biology concerns the ease with which DNA sequence information can be obtained. One of the spin-offs of the human genome project has been the development of automated sequencers now widely available and used to rapidly and cheaply obtain DNA sequence data. Furthermore, such data has proved invaluable for the development of PCR based diagnostics. This is because it has enabled comparative analysis of DNA sequences from closely related pathogens to reveal their evolutionary relationships and provide a basis for the design of highly specific oligonucleotide primers.

DNA sequencing involves determining the order of DNA nucleotides of nitrogenous bases: (adenine (A), guanine (G), thymine (T) and cytosine (C) in stretches of an organism's genome. One of the most widely used methods for DNA sequencing has been the di-deoxy method chain termination method developed by Sanger *et al.* (1977). This approach enables the determination of DNA fragment sequences up to 1000 bases in length, and was the method used in the sequencing of the human genome.

Developed by Sanger *et al* (1977), the dideoxy chain-termination method of DNA sequencing makes use of 'dideoxynucleotides'(ddNTPs) which result in termination of DNA synthesis when they are incorporated into growing DNA fragment that is being synthesized. Figure 1.14 illustrates a common structure for one of these ddNTPs,.

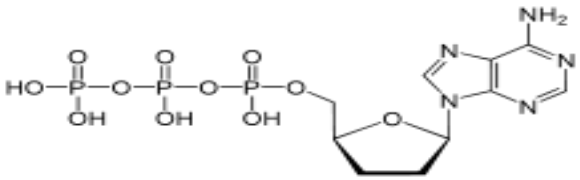

Figure 1.14 Molecular structure of 2',3-dideoxyadenosine triphosphate (ddATP)

The ddNTP–is a deoxynucleotide triphosphate with no OH group on carbon 3' of the sugar (http://biochem.co/2008/08/dna-sequencing-and-amplification/). As illustrated in Figure 1.15 the position or site on the molecule where a phosphate group would normally bind (to an OH group) as part of a DNA backbone is no longer available, and this property causes the replication to stops when the ddNTP is incorporated into the growing chain.

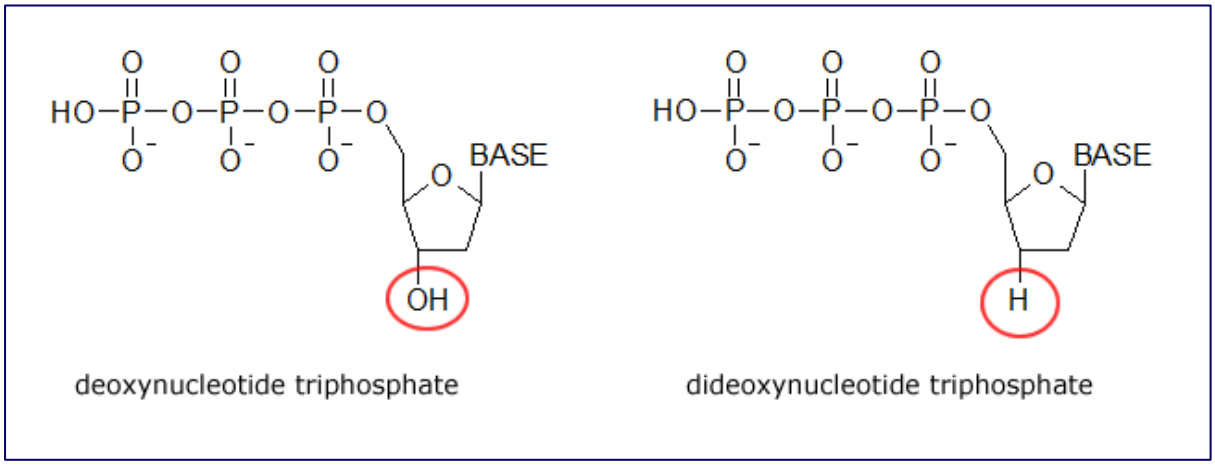

Figure 1.15 Form of dideoxynucleotide triphosphate and dideoxynucleotide triphosphate used to terminate sequencing reactions

In principle, four separate reactions are carried out simultaneously in which each reaction contains all the four dNTPs and a single ddNTP (ddATP in one case, ddCTP in another and so on), as well as a template DNA and a short DNA primer (about 20 nucleotides). A low ratio of ddNTP to dNTPs is maintained and DNA synthesis allowed to proceed. DNA synthesis produces fragments of different length, each terminating in a ddNTP. The fragments from the four reactions can be electrophoresed on a high resolution acrylamide gel containing urea, where a ladder like structure can be observed. In this visualization the migrating bands differ by length of 1 base. Reading the ladder from the fastest migrating band to the slowest gives the DNA sequence. DNA bands can be visualised using attached fluorophores or radioisotopes (Karger and Guttman, 2009).

In the example shown (Figure 1.16), incorporation of ddATP after synthesis of CGT would terminate the reaction.

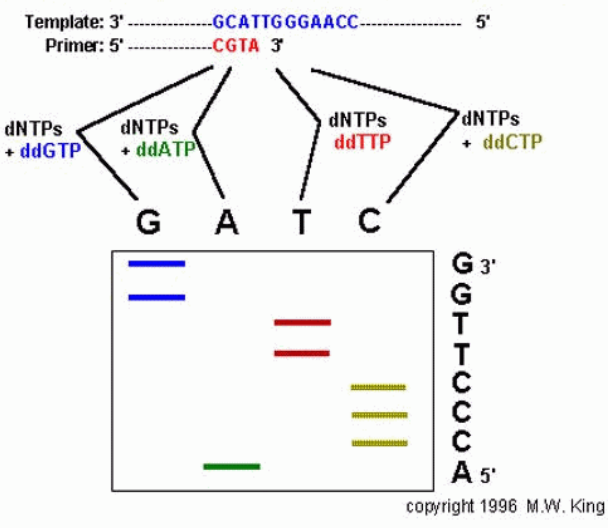

Figure 1.16 Illustration of the Chain Termination sequencing method

In some electrophoresis detection systems, such as with the ABI3700 and ABI3730 systems, an electric field is set across a porous gel tube within which the DNA moves towards the positive electrode. The gel acts like a sieve; where shorter DNA fragments move more quickly through the holes of the gel than do larger DNA fragments. As each DNA fragment reaches the end of the gel, a laser excites the attached fluorescent dye. The colour of emitted light is detected by a camera and the information is passed to a computer for image processing and base calling.

In the 1980s scientists began to discuss seriously how to sequence the entire human genome. It was argued that sequencing the complete genome would provide the most comprehensive collection of an individual's genetic variation (Ng and Kirkness, 2010) and this could be used to identify the genetic basis of traits including disease susceptibility. Because of its significance, with high levels of funding from the United State of America (about U$ three (3) billion) a consortium of international scientists (International Human Genome Sequencing Consortium) worked to complete a draft of the human genome which was eventually published in 2001 (International human genome sequencing consortium 2001). The speed at which the task was completed was greatly facilitated by the competition provided by a private company Celera Genomics which also raced to complete a draft

genome by 2001 (Venter *et al*. 2001*;* Primrose and Twyman, 2003). The public consortium used a strategy of subcloning from chromosomes, while Celera used a random shotgun cloning and sequencing approach. Random shotgun sequencing involves randomly shearing, sonicating or cleaving with restriction endonucleases genomic DNA into small pieces and then cloning the resulting DNA fragments into a self-replicating vector. If sheared or sonicated, adaptors can be added to the DNA fragments to improve their cloning efficiency.

The cloned fragments are then sequenced using the Sanger method and regions of overlap are matched to assemble larger regions of the genome.

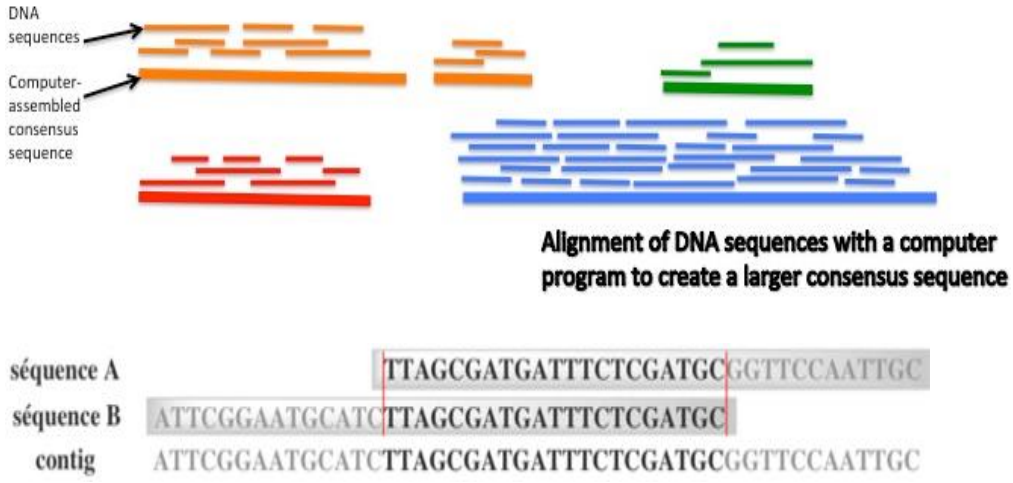

Figure 1.17 Sequencing, contig formation and alignment of contigs

Di-deoxy sequencing, accurate for lengths of DNA up to 1000 bases, can be used to determine the sequence of a DNA fragment one sequence at a time (up to 96 samples on an ABI3730 machine). This methodology is being quickly superseded with the development of high throughput (NGS) sequencing technologies.

1.5.3 High Throughput (NGS) DNA sequencing

Recent advances in technology have seen the development of so-called Next Generation Sequencing (NGS) methods such as represented by the Roche/454 FLX pyrosequencer, the Illumina/Solexa Genome Analyser, and the Applied Biosystems Solid ™ sequencer (Mardis 2008). Of these, the Ilumina sequencing protocols have emerged as the most cost efficient technology. In principle, Illumina protocols involve shearing or sonicating genomic DNA to into fragments of ~ 500 bases in length, ligating adaptors onto these sheared fragments, adding primer tails through PCR (although there is also a PCR free

protocol), attaching the fragments to the floor and walls of a flow cell, conducting bridge amplification to produce clusters of clone fragments and then sequencing either end of the fragments using DNA sequencing by synthesis protocol. Even the benchtop MiSEQ machine 25-35 million fragments (length up to 300 bp) can be sequenced in 26 hours. These data can be assembled into genomes and or the DNA fragments can be compared directly to sequences in public databases to identify the organisms represented by DNA fragments in the sample.

1.5.4 DNA sequence analysis

The database resources of the National Center for Biotechnology Information (NCBI) are commonly used as source for DNA reference sequences (Sayers *et al*. 2008) in genome analysis. NCBI maintains the GenBank® nucleic acid sequence database as well as providing bioinformatic analysis and retrieval tools for the data in GenBank and other biological data bases made available through the NCBI website. GenBank is a comprehensive database that contains publicly available nucleotide sequence for more than 300,000 organisms named at the genus level or lower, obtained primarily through submissions from researchers involved in large-scale sequencing projects (Benson *et al.* 2009). Some of the resources maintained in NCBI include Entrez, the Entrez Programming Utilities, MyNCBI, PubMed, PubMed Central, Entrez Gene, the NCBI Taxonomy Browser, BLAST, BLAST Link (BLink) and others. The BLAST program allows a researcher to compare his sequence data with available sequences. The output indicates how similar a query sequence is to those in the reference database. "E" values (expect values) indicate if the similarity, is sufficiently high that it cannot be explained by chance and must rather indicate homology (shared evolutionary ancestry). High identity matches can indicate the identity of the organism from which a query sequence has been obtained. BLAST was originally developed for comparing a single nucleotide or protein sequence to a database. In the case of environmental metagenomics studies where researchers use NGS sequencing to determine what organisms are present in an environmental sample, faster methods of database searching are needed, and this is an active area of research (Wang *et al*. 2012).

The advancement of NGS DNA sequencing technology has benefited and enabled *Phytophthora* research. In recent years the availability of DNA sequencing technology has led to the sequencing of nuclear and mitochondrial genomes for a number of *Phytophthora* species and well as the sequencing of expressed sequence tags (EST) (Judelson, 2007). There

have being major sequencing projects undertaken for *P.infestans, P.ramorum, P.sojaep, P.parasistica, P.nicotianae* and *P.capisici.*

1.5.5 Targeting the nuclear genome

Phytophthora species have a diploid genome with a variable chromosome number ranging from 8-20 (Braisser 1979). In most eukaryotic organisms including *Phytophthora*, the ribosomal RNA (rDNA) genes in the nuclear genome, comprise a multigene family in which the copies are arranged in tandem repeats. This has made them ideal targets as each repeat consists of a single transcription unit that includes the small subunit, the 5.8 S and the large subunit genes separated by two internal transcribed spacers "ITS1" and "ITS2". Because of their high copy number, the conservative nature of some rDNA regions to design primers, as well as the presence of slower (e.g. the LSU region) and faster evolving regions (nITS reion), these loci have been useful for reconstructing phylogenies and determining evolutionary relationships over different evolutionary time scales (Woese and Fox, 1977). Recently, mitochondrial DNA markers have also proved informative for species identification in *Phytophthora* (Martin and Tooley, 2003; Martin *et al.* 2007; Bilodeau *et al.* 2014; Martin *et al*. 2014).

1.5.6 Targeting the mitochondrial genome

The mitochondria are double membrane-bound organelles found in the cytoplasm of eukaryotic cells. They were first discovered and described about a century and half ago in 1857 by Albert von Kölliker as granules in muscle cells (Chavis and Arnold, 2009). Richard Altman identified the organelles using a dye technique and dubbed them ‘bioblasts’ He described the structures as basic units of cellular activity (http.www.brighthub.com/science/genetic /articles/26365.aspx). Although mitochondria in eukaryotes can be functionally diverse and ecologically specialised, their genomes are relatively conserved amongst close phylogenetic relatives. Mitochondria (and therefore their mt genomes) are also high in copy number in pathogens and so mt DNA also provides potential targets for evolutionary studies and in developing DNA diagnostics.

The detection of DNA in mitochondria was first reported by Nass and Nass, (1963) and after 30 years, mtDNA sequence was determined (approximately 17000 base pairs) in several species including human (Anderson *et al*. 1981). The mt DNA is localised in the

mitochondria matrix and seems to be associated with lipids and proteins (Bogenhagen and Clayton, 1974). In recent times the mt genome is being increasingly used in molecular systematic investigations of *Phytophthora* and mitochondrial genome analysis was conducted in the present study of *P. palmivora* from PNG.

Complete mitochondrial genome sequences were recently used to study the relationship of *P. ramorum* and *P. sojae.* Their mitochondrial genome sequences were determined during the course of complete nuclear genome sequencing (Tyler *et al*. 2006). Both mitochondrial genomes are circular with sizes of 39,314 bp for *P. ramorum* and 42,977 bp for *P. sojae.* A similar size is expected for species *P. palmivora* (i.e. genome size ranging from 39000bp - 42000bp) given their relatively close phylogenetic relatinship. In the analysis of *P. sojae* and *P. ramorum,* Tyler *et al*. (2006) reported 37 recognizable protein-encoding genes, 26 or 25 tRNAs *(P. ramorum* and *P. sojae*, respectively) specifying 19 amino acids, and six more open reading frames (ORFs) that are conserved, presumably due to functional constraint, across *Phytophthora* species (*P. sojae, P. ramorum,* and P. *infestan*s). Six ORFs were also found unique for *P. sojae* and one that was unique for *P. ramorum*. The non-coding regions comprised about 11.5 and 18.4% of the genomes of *P. ramorum* and *P. sojae*, respectively. Comparisons of these genomes with published sequences of the *P. infestans* mitochondrial genome revealed a number of similarities, but the gene order in *P. infestans* differed in two adjacent locations due to inversions and specific regions of the genomes exhibited greater divergence than others.

1.5.7 Isothermal DNA amplification tests

DNA sequencing and even the PCR require significant lab infrastructure, time and expense for genome characterisation (Miles *et al*. 2014). For countries that lack the necessary infrastructure, for example PNG, the time required to send samples to overseas laboratories and await results for molecular identification prohibits timely decision making. In PNG, the ability to identify a *Phytophthora* sp. using molecular techniques but with minimal laboratory equipment would be very helpful for management decisions. One recent advance that could help make this possible is the development of so called isothermal DNA amplification tests. These tests make use of genome science in developing primers, but once these primers have been developed rapid quantitative diagnostic tests deployed in the field that do not require a PCR machine. Isothermal DNA amplification tests have recently been developed to diagnose infectious diseases and these are now having uptake in many disciplines, including

agriculture. Of these methods, the Loop Mediated AMPlification (LAMP) method (Notomi *et al.* 2000) is currently emerging as the most popular isothermal DNA amplification method for pathogen detection.

Developed by scientist at Eiken Chemical Company of Japan (http//www.eiken.co.jp/en, Notomi *et al*. 2000), LAMP protocols are currently being used to develop real time diagnostics tool for early detection and diagnosis of pathogens that cause diseases such as Malaria (Cook *et al*. 2015*;* Morris *et al*. 2015*;* Bosward *et al*. 2016), fungal infestations (Su *et al*. 2016) bacterial and viral disease. More recently LAMP technology is also being used to detect genetic variation linked to cancer, to identify genetically modified food, identify drug resistance in bacteria and also for other non-conventional applications (Kundapur and Nema, 2014).

1.6 Aims and objectives of the present study

The present reports investigations on the mt genome of *Phytophthora* isolates (species) collected from Cocoa plantations in PNG, where this pathogen causes disease in plantation, contaminates soils and water. Of particular interest has been in characterising the genetic diversity of these isolates, better understanding the origin of the pathogen and the possibility of developing a rapid diagnostic test that could be deployed to detect and quantify inoculum levels of *Phytophthora* on plants, in soils and in water. Such a test would also have application in cultivar screening programmes. Towards achieving this goal, mt genome loci have been sequenced in collected *P. palmivora* isolates and these have been compared with DNA sequences from published sequence data from *P. palmivora* as well as other *Phytophthora* species. The complete mitochondrial genome was also assembled for *P. palmivora* (PNG) and compared with other species. These data have been used in phylogenetic analyses to identify the origin of *P. palmivora* in PNG and to develop DNA amplification primers that can be used in PCR and isothermal DNA amplification tests.

CHAPTER 2: MATERIALS AND METHODS

2.1 Introduction

This chapter provides details of the collections and procedures used in this study. Briefly diseased cocoa pods were collected in the major cocoa producing areas of PNG (Bougainville and East New Britain). Samples were taken to the PNG Cocoa Coconut Institute Limited (PNGCCIL) plant pathology laboratory where *P. palmivora* was isolated and cultured. Samples were sent to Massey University where DNA was extracted and target mitochondrial loci amplified using the polymerase chain reaction. Amplified fragments were sequenced at the Massey Genome Service with the resulting data used to confirm the identity of isolates and conduct phylogenetic analyses. For four representative PNG isolates high-throughput sequencing was used to produce complete mitochondrial genome sequences. These sequences were combined with data from sequencing of individual loci in order to identify suitable targets for the development of rapid LAMP-based diagnostics for *P. palmivora*.

Figure 2.1 Regional Context of current study in New Britain and Bougainville
The location of Samoa (Apia) has also been indicated.

2.2 *Phytophthora palmivora* collections

For this study *P. palmivora* was collected from four sites in islands of PNG. Specifically, samples were collected from Bougainville and East New Britain (Figure 2.2) because they are the major cocoa producing areas of PNG. A total of ten (10) collections were made on Bougainville. This included five samples from farms around Buin in southern Bougainville and five samples from the Buka area in northern Bougainville.

Samples consisted of whole diseased pods (Figure 2.3). For transport to the PNGCCIL laboratory pods were individually wrapped in newspaper and placed in a plastic bag to maintain humidity. Wrapped pods were then placed in a cooler to prevent overheating during transport.

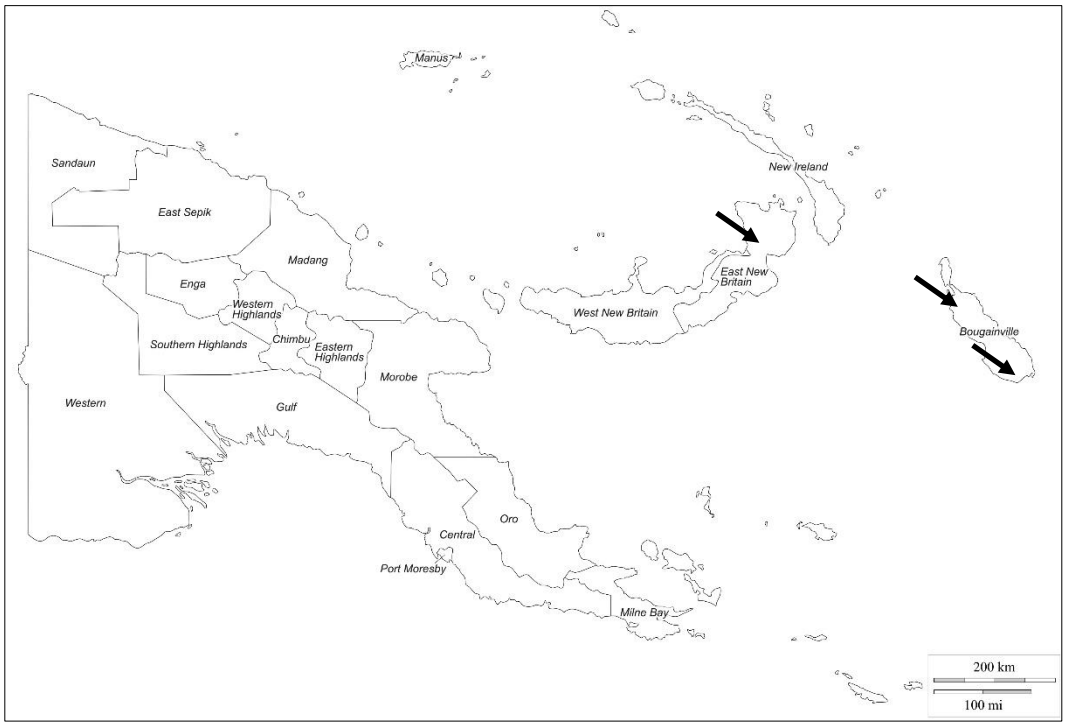

Figure 2.2 Map of PNG showing *P. palmivora* collection sites (base map from d-maps.com).

2.3 Culturing of *Phytophthora palmivora* isolates

2.3.1 Culturing of P. palmivora isolates

As an alternative to agar plating *P. palmivora* collected from sites on Bougainville and East New Britain were cultured using mature cocoa pods from clone KA2-101. Pods to be used for culturing (so-called 'media pods') were harvested and then prepared for inoculation. Media pods were first sprayed with insecticide (Mortein) and left to air dry on a bench top for 20

mins (Figure 2.4). They were then surface sterilised for 15 mins in a 10% hypochlorite (NaOCl) bath and finally rinsed twice in distilled water.

Prepared media pods were then inoculated with infected tissue from diseased pods. Diseased pods were washed with 75% ethanol before fragments of the advancing *P. palmivora* infection front were removed by scraping (Figure 2.4). In a laminar flow hood a small hole was drilled in the media pod and scrapings from a diseased pod packed into it. (Figure 2.4C). Inoculated pods were placed in a tray, covered with clear polythene plastic to maintain humidity and incubated in the dark at 24°C.

Figure 2.3 Photographs of *Theobroma cacao* pods from Bougainville (A-C) and East New Britain (d-F) with dark discolouration consistent with *P. palmivora* infection. *Phytophthora palmivora* isolated from these pods is among that analysed in the present study. Isolate codes (see Table 2.1) for pods are – A, Buin3; B, Buin4; C, Buka2; D, Kerv; E, Raul; F, Tav (office).

2.3.2 Microscopic examination of cultured P. palmivora

The presence of *P. palmivora* on media pods was evaluated using microscopic examination of spore morphology and concentration (Brasier *et al*. 1979; Drenth *et al*.

Figure 2.4 The process of isolating and culturing *P. palmivora* from diseased *Theobroma cacao* pods collected in the field. The media pods are surface sterilised by first spraying with an insecticide (A) and then washing in a NaOCl bath (B). Sterilised pods (C) then have a hole drilled into them; surface scrapings are taken from a diseased pod (D) and packed into this hole (E). The inoculated media pods are covered and incubated in the dark at 24°C to allow the *P. palmivora* infection to develop (F).

2006). After eight days, mycelium on the exterior of each pod was harvested and placed on a filter paper. To separate the mycelium from sporangia and zoospores, the sample was then washed using 5ml distilled water and the flow-through collected in a conical flask. The flow-through was then checked under a light microscope for the presence and concentration of recognisably *P. palmivora* spores.

2.3.3 Harvesting and storage of P. palmivora mycelium for molecular analysis

The washed mycelium from each isolate was transferred to a 2 ml screw-top vials containing 70% ethanol. Additionally, scrapings of lesions from several diseased pods were preserved in the same manner. Samples were shipped to Massey University (New Zealand) at room temperature and then placed at -20°C until processed.

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2.4 DNA extraction

2.4.1 Sample homogenisation

One of two methods were used to homogenise mycelium samples prior to DNA extraction. For the first, 20-30mg of mycelium was placed in a pre-cooled mortar and covered with liquid nitrogen. Using a pestle the sample was then ground to a fine powder. While still frozen the powder was transferred to a clean 1.5ml eppendorf using a pre-cooled spatula. The second method used a similar amount (20-30mg) of tissue but in this case the sample was transferred to a 2ml screw-cap tube containing 6-10 siliconized glass beads (2mm diameter). The tubes were then processed for 90 secs at 5000 rpm in a MagNA Lyser instrument (Roche Life Science).

2.4.2 Genomic DNA extraction

Total genomic DNA was extracted from homogenised mycelium samples using the Macherey-Nagel NucleoSpin Plant II kit. The manufacturers recommended procedure for fungi was followed.

Table 2.1 Collection details for *P. palmivora* samples sent to Massey University

Isolate code	PNG Provinces	Tissue type	Host variety[1]	
Kerv	East New Britain	cultured mycelium	Trinitario type	6
Nari1	East New Britain	pod lesions	H/C37-13/1	1
Nari2	East New Britain	pod lesions	H/C25-6/3	2
Nari3	East New Britain	pod lesions	H/C63-7/3	–
Raul	East New Britain	cultured mycelium	H/C38-10/3	3
Tav (control)	East New Britain	cultured mycelium	H/C16-2/3	14
Tav (nursery)	East New Britain	cultured mycelium	Trinitario type	13
Tav (office)	East New Britain	cultured mycelium	Trinitario type	15
Buka1	northern Bougainville	cultured mycelium	H/C16-2/3	7
Buka2	northern Bougainville	cultured mycelium	H/C67-9/3	9
Buka3	northern Bougainville	cultured mycelium	H/C17-7/4	17
Buka4	northern Bougainville	cultured mycelium	H/C16-2/3	4
Buka5	northern Bougainville	cultured mycelium	H/C17-3/1	10
Buin3	southern Bougainville	cultured mycelium	SG2 hybrid	11
Buin4	southern Bougainville	cultured mycelium	SG2 hybrid	5
Buin5	southern Bougainville	cultured mycelium	Trinitario type	12
Buin6	southern Bougainville	cultured mycelium	H/C 34-13/2	16
Buin7	southern Bougainville	cultured mycelium	SG2 hybrid	8

[1] Trinitario, H/C and SG hybrid are cultivars of *Theobroma cacao* used by farmers in PNG and host of the *P.palmivora* isolates.

2.4.3 Evaluation of DNA quality and quantity

Extracted DNA was visualised by gel electrophoresis. Typically 5µl aliquots of each DNA sample was loaded onto a 1% agarose/TAE gel containing SYBR Safe (Invitrogen Life Technologies); an aliquot of 1Kb Plus DNA Ladder (Invitrogen Life Technologies) was loaded as a size standard. Gels were electrophoresed at 100 volts for approximately 40 mins after which PCR products were visualised on a Bio-Rad Gel Doc system. For each DNA sample the concentration of extracted DNA was evaluated using a QuBit 2.0 Fluorometer (Invitrogen Life Technologies).

2.5 PCR amplification of mitochondrial loci

Total genomic DNA was used as a template for the amplification of two mitochondrial loci. Specifically, a portion of the cytochrome b (*cytb*) gene as well as region containing portions of the cytochrome oxidase subunit 1 and 2 genes plus the intergenic spacer between them (*cox1-cox2*). These data were used to confirm the presence of *P. palmivora* in samples and evaluate the diversity of PNG samples, respectively.

Typically, PCR was performed in 20µl reaction volumes containing 10µl of Emerald Amp GT PCR master mix (TaKaRa), 10µM forward primer, 10µM reverse primer and 2-38ng template DNA. For cytb amplification primers were PTA-3F3 (5' accaccgttgttttcaactac 3') and PTA-3B3 (5' accaccgttgttttcaactac 3'); for cox2 primers FM 35 and Phy 10b of Martin *et al*. (2014) were used. Thermocycling was conducted on a BIOMETRA T1 Thermocycler. Conditions were an initial denaturation at 94°C for 3 mins, followed by 35 cycles of 94°C for 30 secs (denaturing), 50-55°C for 30 secs (primer annealing), and 72°C for 30 secs (extension) with a final 5 min extension at 72°C.

Following thermocycling PCR products were visualised by gel electrophoresis. Typically 4µl aliquots of each PCR mix was loaded onto a 1% agarose/TAE gel containing SYBR Safe. To allow the size of amplified fragments to be determined an aliquot of 1Kb Plus DNA Ladder was also loaded. Gels were electrophoresed at 100 volts for approximately 40 mins after which PCR products were visualised on a Bio-Rad Gel Doc system.

2.6 Enzymatic purification of PCR products

Prior to sequencing amplified fragments were purified enzymatically using shrimp alkaline phosphatase (SAP) and exonuclease III (EXO). These enzymes dephosphorylate dinucleotide triphosphates and digest the single stranded primers, respectively. Reactions were performed directly in PCR tubes. First, 0.5U SAP (Thermo Scientific) and 1.25U EXO (Thermo Scientific) were added. The tubes were then incubated at 37°C for 30 mins followed by 80°C for 15 mins to denature enzymes.

2.7 Sanger sequencing of PCR products

Cleaned PCR products were used as templates for Sanger sequencing reactions. Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) following manufacturer's instructions. Reactions were carried out in 20μl volumes and typically contained 25-45ng of purified PCR product plus 10pmol of the appropriate primer.

Thermocycling was conducted on a BIOMETRA T1 Thermocycler. Conditions were an initial denaturation at 98°C for 2 mins, followed by 26 cycles of 98°C for 10 secs (denaturing), 50°C for 10 secs (primer annealing), and 60°C for 4 mins (extension). After thermocycling samples were submitted to the Massey Genome Service for purification and electrophoresis on an ABI3730 Genetic Analyzer (Applied Biosystems).

2.8 Processing of Sanger sequence data

After sequencing electrophoretograms were inspected, edited and contigs assembled in Sequencher v4.9 (Gene Codes Corporation).

2.9 Next generation sequencing and assembly of whole mitochondrial genomes

To explore levels of genetic diversity among PNG isolates of *P. palmivora* and identify potential targets for molecular diagnostics whole mitochondrial genomes were generated for four representative isolates. Specifically, Buka1, Buin3, Raul and Kerv. These isolates were selected as they cover the geographical range of available sample and the extracted DNA met quality control criteria (e.g. quantity and purity) for high through-put sequencing.

2.9.1 High through-put sequencing of PNG P. palmivora isolates

The Illumina Nextera DNA library preparation method was used to prepare PNG *P. palmivora* libraries for sequencing on Illumina MiSeq machines. Briefly Nextera DNA library preparation involves enzymatically shearing the DNA into random fragments, to each end of which are added barcoded adapters during PCR enrichment (Figure 2.5). Library preparation and paired-end DNA sequencing was performed by New Zealand Genomics Ltd. (NZGL, http://www.nzgenomics.co.nz). Following sequencing the sequence reads were quality assessed by NZGL bioinformaticians using a standard workflow.

2.9.2 Mitochondrial genome assembly

Whole mitochondrial genome sequences for PNG isolate of *P. palmivora* were assembled from quality-assessed sequence reads using a bioinformatics pipeline developed by Lockhart and Winkworth. This pipeline utilises IDBA_ud (Peng *et al*. 2012), Geneious.

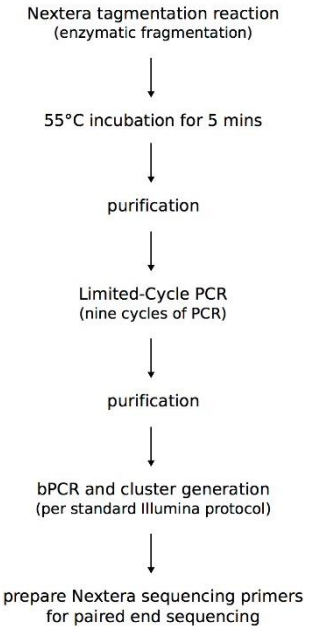


Figure 2.5 Flowchart for Nextera DNA library preparation.

(Biomatters) and BWA (http://bio-bwa.sourceforge.net/). Briefly, IDBA-ud is first used to assemble sequence reads into contigs. IDBA-ud is a de Bruijn graph assembler that is capable of assembling sequence reads when coverage of the genome by sequence reads is likely uneven. The resulting contigs were then filtered to remove those with low similarity to a reference collection of *Phytophthora* mitochondrial genome sequences. This was done using custom scripts, a local sequence database and BLAST+ 2.3. Filtered contigs were then assembled into whole mitochondrial genome sequences using the assembly tools implemented in Geneious. The draft genome was then evaluated using BWA (Burrows Wheeler Aligner), a fast gapped aligner. At this stage the quality-assessed sequence reads are mapped to the draft genome sequence. Gaps in read coverage were interpreted as assembly errors and the draft revised as appropriate.

2.9.3 Mitochondrial genome annotation

Phytophthora palmivora assemblies were annotated on the basis of similarity to published and unpublished *Phytophthora* mitochondrial genome sequences using a combination of Geneious (Biomatters) and DOGMA (Wyman *et al*. 2004; http://dogma.ccbb.utexas.edu/).

2.10 Relationships of PNG *P. palmivora* based on mitochondrial genes

Preliminary analyses of whole mitochondrial sequencing suggested very low levels of sequence diversity among *P. palmivora* isolates from PNG. To evaluate patterns of genetic diversity within a wider set of *P. palmivora* accessions independent and combined phylogenetic analyses of four mitochondrial loci were conducted. These loci were selected because in previous phylogenetic analyses of the genus they have been shown to resolve relationships within an between species (e.g. Martin *et al*. 2014). The loci used were *cox1-cox2*, a portion of gene encoding ribosomal protein S10 (*rps10*), a region containing portions of the ATP synthase F0 subunit 9 and NADH dehydrogenase subunit 9 genes plus the intergenic spacer between them (*atp9-nad9*) and a portion of the sec-independent transporter protein gene (*secY*). Sequences for these four regions were extracted from whole mitochondrial genome sequences for representative PNG *P. palmivora* isolates and were combined with sequence data from ten other *P. palmivora* isolates as well as sequences

representing other *Phytophthora* species. Details of the published sequences included in the present analyses are given in Table 2.2.

Table 2.2 Details of publically available DNA sequences used for phylogenetic analyses

Accession	Genbank accession numbers			
	atp9-nad9	*cox1-cox2*	*rps10*	*secY*
P. boehmeriae	JF771635	GU318298	JF770876	JF770500
P. brassicae	JF771639	JF771256	JF770881	JF770505
P. cactorum	JF771642	GU221951	JF770884	JF770507
P. cambivora	JF771650	JF771263	JF770888	JF770510
P. cinnamomi	JF771755	JF771337	JF770986	JF770609
P. colocasiae	JF771809	GU221985	JF771035	JF770660
P. erythroseptica	JF771832	JF771438	JF771050	JF770675
P. fallax	JF771837	GU222008	JF771054	JF770679
P. heveae	JF771855	GU222027	JF771073	JF770697
P. humicola	JF771858	GU222030	JF771075	JF770699
P. ilicis	JF771862	GU222033	JF771078	JF770703
P. infestans	JF771871	GU318302	JF771087	JF770712
P. irrigata	JQ439066	JQ439451	JQ439209	JQ439334
P. kernoviae	JF771913	GU222054	JF771127	JF770753
P. megakarya	JF771931	GU222067	JF771143	JF770768
P. multivesiculata	JF771949	JF771527	JF771154	JF770779
P. nemorosacearum	JF771957	GU222084	JF771158	JF770783
P. novaguineae	JF771973	GU222095	JF771168	JF770793
P. palmivora[1]				
American Samoa, *Theobroma*	JQ439122	JF771545	JF771176	JF770801
Costa Rica, *Theobroma*	JF771977	GU222099	JF771172	JF770797
Ghana, *Theobroma*	JF771979	JF771544	JF771174	JF770799

Guam, *Areca*[2]	JF771981	JF771546	JF771177	JF770802
Guam, *Areca*[3]	JF771982	JF771547	JF771178	JF770803
Guam, *Areca*[4]	JF771629	JF771245	JF770867	JF770493
Hawaii (USA), *Carica*	JF771976	GU222098	JF771171	JF770796
India, *Areca*	JF771978	JF771543	JF771173	JF770798
P. quercetorum	JF772008	GU222124	JF771201	JF770824
P. rosacearum	JQ439154	JQ439462	JQ439280	JQ439399

[1] Multiple isolates of *P. palmivora* were included in phylogenetic analyses. These were distinguished by geographic origin and host plant.

[2] *P. palmivora* isolate P10769 from *Areca catechu* (Guam).

[3] *P. palmivora* isolate P11010 from *Areca catechu* (Guam).

[4] *P. palmivora* isolate P11011 from *Areca catechu* (Guam).

Multiple sequence alignments were generated for each of the loci using ClustalO (Larkin *et al*. 2007) and edited in Mesquite version 3 (Maddison and Maddison, 2014) to remove portions where less than 50% of the accessions were represented or where the alignment was otherwise ambiguous. For each locus the best-fit substitution model was identified using the Bayesian Information Criterion (BIC; Schwarz, 1978) as implemented in JModelTest 2.2 (Guindon and Gascuel, 2003; Posada, 2008). Using PhyML (Guindon *et al*. 2010) and the best-fit model, maximum likelihood (ML) trees as well as ML bootstrap support (500 replicates) was estimated for each locus. A combined data set was compiled by concatenating the four individual matrices and analysed in the same way.

2.11 Designing primers for LAMP amplification of *P. palmivora* specific targets

Based on results from whole mitochondrial genome sequencing and phylogenetic analyses of mitochondrial loci two regions were selected as potential targets for a LAMP test. These were the *cox1-cox2* and *atp9-nad9* regions.

Based on the *P. palmivora* sequence PrimerExplorer V4 (Eiken Chemical) was used to generate external (F3/B3) and internal (FIP/BP) primer pairs for LAMP isothermal

amplification. To generate primers three parameters were modified from default settings. Specifically, the bounds on primer length, melting temperature and GC content were progressively lowered until primers were successfully generated. The LAMP primer sets were then visually compared to a multiple sequence alignment containing a range of *Phytophthora* species for the corresponding locus. Primer sets found to target regions of the sequence that distinguished *P. palmivora* from other *Phytophthora* species were further evaluated using PrimerExplorer V4 to identify loop primers.

2.12 Testing of the LAMP assay

2.12.1 Initial testing of LAMP primers using PCR

As a first developmental step the external and the internal primer pairs were trialled in standard PCR amplifications. Reactions were performed in 20µl volumes containing 10µl of Emerald Amp GT PCR master mix, 10µM forward primer, 10µM reverse primer and 2-38ng template DNA. Extracts from isolates Raul, Kerv and Buin5 were used for testing. Thermocycling was conducted on a BIOMETRA T1 Thermocycler. Conditions were an initial denaturation at 94°C for 3 mins, followed by 35 cycles of 94°C for 30 secs (denaturating), 50°C for 30 secs (primer annealing), and 72°C for 30 secs (extension) with a final 5 min extension at 72°C.

Following thermocycling PCR products were visualised by gel electrophoresis. Typically 4µl aliquots of each PCR mix was loaded onto a 1% agarose/TAE gel containing SYBR Safe. To allow the size of amplified fragments to be determined an aliquot of 1Kb Plus DNA Ladder was also loaded. Gels were electrophoresed at 100 volts for approximately 40 mins after which PCR products were visualised on a Bio-Rad Gel Doc system.

2.12.2 Optimisation of the LAMP assay

To optimise LAMP reactions the ratio of external (F3/B3) to internal (FIP/BIP) primer pairs as well as the effect of loop primer (LB) addition on amplification efficiency was evaluated. Specifically, three ratios of external to internal primer pairs (1:4, 1:6 and 1:8) were tested in the presence and absence of the loop primer.

For this experiment two sets of LAMP reactions were performed. Both used 25µl reaction volumes containing 15µl of LAMP mix (OptiGene), 5pM each of primers pp-F3 and

pp-B3, 20, 30 or 40μM each of primers pp-FIP and pp-BIP plus approximately 1ng template DNA. One reaction set also contained 10μM of primer pp-LB. For these tests DNA from Raul was used as a template. Reactions were incubated for 30 minutes at 60°C on a MJ Research PTC-150 Minicycler, the reactions were then heated to 80°C to deactivate the enzymes. The resulting LAMP products were visualised by gel electrophoresis. Typically 3μl aliquots of each LAMP mix was loaded onto a 1% agarose/TAE gel containing SYBR Safe. To allow the size of amplified fragments to be determined an aliquot of 1Kb Plus DNA Ladder was also loaded. Gels were electrophoresed at 100 volts for approximately 40 mins after which PCR products were visualised on a Bio-Rad Gel Doc system.

2.12.3 Specificity of the LAMP assay

The proposed *P. palmivora* LAMP assay was evaluated for specificity by testing it against DNA extracts from other *Phytophthora* species. LAMP reactions were performed in 25μl reaction volumes containing 15μl of LAMP mix 5pM each of primers pp-F3 and pp-B3, 30μM each of primers pp-FIP and pp-BIP, 10μM of primer pp-LB and approximately 1ng template DNA. Template were *P. palmivora* (Raul, ICMP 14577 and ICMP17706), *P. agathidicida*, *P. capitosa*, *P. cinnamomi* and *P. cryptogea*.

LAMP reactions were incubated and the reaction products visualised as described in section *2.15.2 Optimisation of the LAMP reaction.*

2.12.4 Sensitivity of the LAMP assay

The sentivitity of the LAMP assay was assessed in comparison to PCR amplifications using the pp-F3 and pp-B3 primer pair. Tests used DNA from the Raul isolate serially diluted in 10-fold steps with concentrations ranged from 100pg/μl to 0.1fg/μl.

PCR amplifications were performed in 20μl volumes containing 10μl of Emerald Amp GT PCR master mix, 10μM pp-F3 primer, 10μM pp-B3primer and 100pg-0.1fg template DNA. Reactions were thermocycled as described in section *2.15.1 Initial testing of LAMP primers using PCR*. LAMP reactions were performed in 25μl reaction volumes containing 15μl of LAMP mix 5pM each of primers pp-F3 and pp-B3, 30μM each of primers pp-FIP and pp-BIP, 10μM of primer pp-LB and 100pg-0.1fg template DNA. LAMP reactions were incubated as described in section *2.15.2 Optimisation of the LAMP reaction.*

PCR and LAMP products were visualised by gel electrophoresis. Aliquots of each reaction mix were loaded onto a 1% agarose/TAE gel containing SYBR Safe. To allow the size of amplified fragments to be determined an aliquot of 1Kb Plus DNA Ladder was also loaded. Gels were electrophoresed at 100 volts for approximately 40 mins after which products were visualised on a Bio-Rad Gel Doc system.

CHAPTER 3: RESULTS

3.1 Summary

This chapter reports the results of several molecular analyses. First, genetic diversity within a short section of the cyt*b* gene was assessed for 14 *P. palmivora* isolates collected from Bougainville and East New Britain as well as two isolates obtained from the Landcare hosted culture collection ICMP. The cyt*b* gene data confirmed the identity of the PNG isolates as *P. palmivora* . High-throughput sequencing was then used to examine the complete mitochondrial genomes of four representative isolates. Further, sequence data from four gene loci (atp9-nad9, cox1-cox2, rps10 and secY) were extracted from the mitochondrial assemblies and combined with publically available sequences for the four gene loci to obtain a genus-wide sample of *Phytophthora*. These data comprising four combined genes were then used to investigate the evolutionary relationships of the isolates. Phylogenetic analyses suggested limited intraspecific variation within *P. palmivora*. They helped identify two loci, *cox1-cox2* and *atp9-nad9*, as potential targets for the development of rapid LAMP-based diagnostics for *P. palmivora*. Five primers that targeted a distinctive region of the *atp9-nad9* locus were evaluated for specificity and sensitivity under laboratory conditions.

3.2 Genetic diversity of *P. palmivora* isolates

DNA sequences were determined for a 183-nucleotide long region of the mitochondrial cyt*b* for all 14 isolates of *P. palmivora* from PNG. These were identical to each other, and also identical to sequences for the corresponding region from two *P. palmivora* isolates obtained from ICMP (Winkworth, unpubl.). Sources for these sequences were distinct in terms of host (i.e. *Carica* and *Pachira*).

A megablast search of the GENBANK nr database using the PNG *P. palmivora* cyt*b* sequence as the query term returned 43 hits with e-values of 1×10^{-50} or less with identities of 88-95%. All matches involved representatives of the Oomycetes and included the genera *Peronospora* (2 hits), *Phytophthora* (13 hits), *Plasmopara* (10 hits), *Pseudoperonospora* (1 hit) and *Pythium* (17 hits). Matches to *Phytophthora* included a single accession of *P. palmivora* (FJ810092)

3.3 Whole mitochondria genome sequencing and comparison

Levels of mitochondrial diversity among PNG isolates of *P. palmivora* were investigated using a high through-put sequencing approach. Specifically, four representative isolates – including two from Bougainville (Buka1, Buin3) and two from East New Britain (Raul, Kerv) – were subject to whole genome sequencing from which mitochondrial genome sequences were extracted.

3.3.1 Sequencing statistics

Sequence runs resulted in 0.67-1.95 million paired end reads for each of the *P. palmivora* samples. Individual reads were 32-251 nucleotides in length with average quality per read of 37-38.

3.3.2 Genome assemblies

Using the bioinformatics pipeline described in Chapter 2 (Section 2.9.2), a complete mitochondrial genome was assembled for one of the two *P. palmivora* isolates collected in East New Britain (Raul). This circular genome was 40,807 nucleotides in length (See Figure 3.1).

For the remaining three isolates the mitochondrial assemblies were incomplete. For the Buka1, Buin3 and Kerv 10, 14 and 9 contigs were recovered, respectively; these ranged in length from 164 to 25,148.

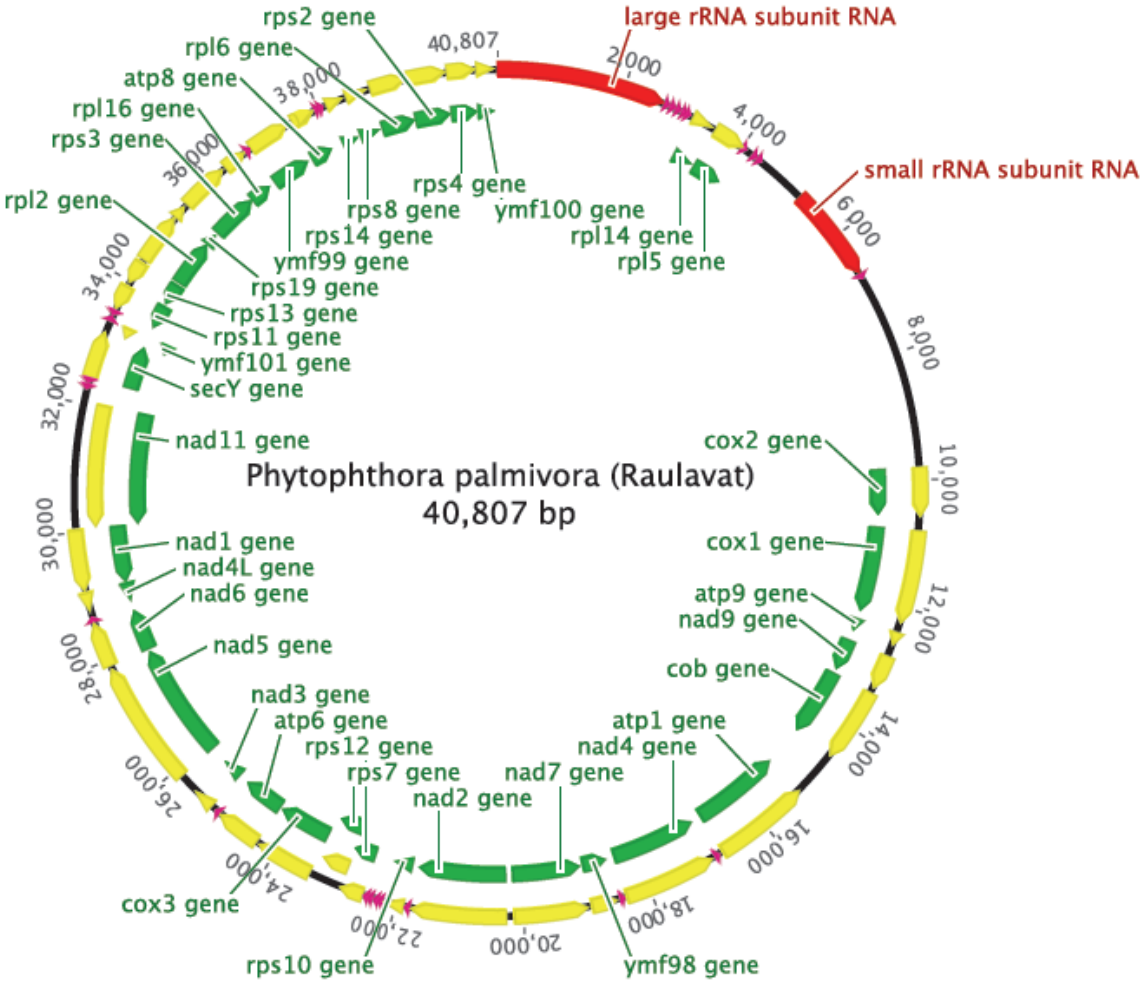


Figure 3.1 A visual presentation of the completed mitochondria gene map of *P. palmivora* isolate from Raulavat, East New Britain. The genes and rRNA, coding regions (green) area also shown. Arrows indicate transcriptional orientation.

3.3.3 Annotation of the P. palmivora genome

The annotation of the PNG *P. palmivora* complete genomes consisted of genes encoding 39 proteins, 25 transfer RNAs and two ribosomal RNAs. This set is conserved across *Phytophthora* based on a collection of more than 40 published and unpublished whole mitochondrial genome sequences. This set contains representatives of all ten major clades of *Phytophthora*. The protein coding gene set comprises 16 genes encoding ribosomal proteins (*rpl* and *rps* genes), 10 respiratory chain genes (*nad* genes), four ATP synthase genes (*atp* genes), three cytochrome oxidase genes (*cox* genes) and genes for the sec-independent transport protein (*secY* gene) and apocytochrome b (*cob* gene). Additionally, four genes of unknown function (*ymf* genes) were also included in this gene set. In total, protein coding genes, RNAs (rRNAs and tRNAs) and non-coding DNA account for 69%, 15% and 16% of the genome, respectively.

Arrangement of the genes within the genome is broadly consistent with that of other *Phytophthora*. Specifically, a number of gene blocks conserved across the set available whole mitochondrial genome sequences for *Phytophthora* were also identified for *P. palmivora*. However, the arrangement of these blocks is unique to *P. palmivora*.

3.4 Relationships of PNG *P. palmivora* based on mitochondrial genes

3.4.1 DNA sequences and data matrices

Sequences for the mitochondrial *atp9-nad9*, *cox1-cox2*, *rps10* and *secY* regions representing 21 *Phytophthora* species were obtained from Genbank. These were combined with homologous sequences extracted from whole mitochondrial genome sequencing of four *P. palmivora* accessions from PNG (Buka4, Buin3, Kerv, Raul) and the ICMP culture collection (Winkworth and Lockhart, unpublished data). In total aligned data matrices for each of the marker regions contained sequences from 14 *P. palmivora* isolates representing a wide geographical and host range. Summary statistics for the separate and combined data matrices has been provided in Table 3.1.

3.4.2 Nucleotide substitution models

Best-fit nucleotide substitution models were estimated for separate and combined data matrices using jModelTest 2.2. For the *atp9-nad9*, *cox1-cox2* and combined matrices the

best-fit model was the General Time Reversible with invariable sites and a gamma distribution of substitution rates (i.e., GTR+I+G). For *rps10* the Transitional model with invariable sites and a gamma distribution of rates was best-fit (TIM+I+G) whereas for *secY* the Transversional model (TVM) was preferred.

Table 3.1 Statistics for matrices and phylogenetic analyses

	atp9-nad9	*cox1-cox2*	*rps10*	*secY*	Combined
Matrices					
No. of taxa	34	34	34	34	34
Matrix length (nt)	558	684	327	726	2295
No. of varied sites	172	186	115	303	776
Maximum likelihood analysis					
Log likelihood	-2568.404	-3180.032	-1549.078	-4044.570	-11711.499

3.4.3 Relationships of PNG P. palmivora based on mitochondrial genes

Separate analyses of the four mitochondrial loci recovered generally similar patterns of relationship among *Phytopththora* species. In the majority of cases taxa recognised as belonging to the same clade (c.f. Blair *et al*. 2008) were sister in these analyses. There were exceptions. For example, in analyses of *cox1-cox2* and *secY* the representatives of clade 2, *P. colocasia and P. mutivesiculata,* did not form a clade. However, in both cases the reconstructed relationships received bootstrap support of less than 50%. In the remaining analyses these two taxa were resolved as sister with moderate to strong support.

Analysis of the combined four locus data consistently resolved the members of recognised clades as monophyletic (Figure 3.2). In each case, these relationships were strongly supported. Bootstrap support values for recognised clades was commonly 100% with clade 2 (*P. colocasia and P. mutivesiculata*), clade 6 (*P. brassicae* and *P. erythroseptica*) and clade 8 (*P. fallax* and *P. irrigata*) receiving more moderate support. Relationships among these groupings are more poorly resolved and supported.

In separate analyses, clades were consistently resolved as monophyletic although relationships among the *P. palmivora* isolates were generally poorly resolved. The exception was the *cox1-cox2* analysis which suggested that *P. palmivora* isolates form three sub-clades. This same set of relationships was also recovered in the combined four locus analysis. In this case the three clades were each well supported. Bootstrap support ranged from 80-98%. In these analyses the four included PNG isolates (Buka4, Buin3, Kerv, Raul) were placed together with isolates from American Samoa (also from *Theobroma*) and an isolate of unknown geographical origin but isolated from a *Pachira aquatica* (Guiana Chestnut). One of the remaining clades contained isolates exclusively from *Areca catechu* in Guam and the other contained isolates from a broad geographic and host range.

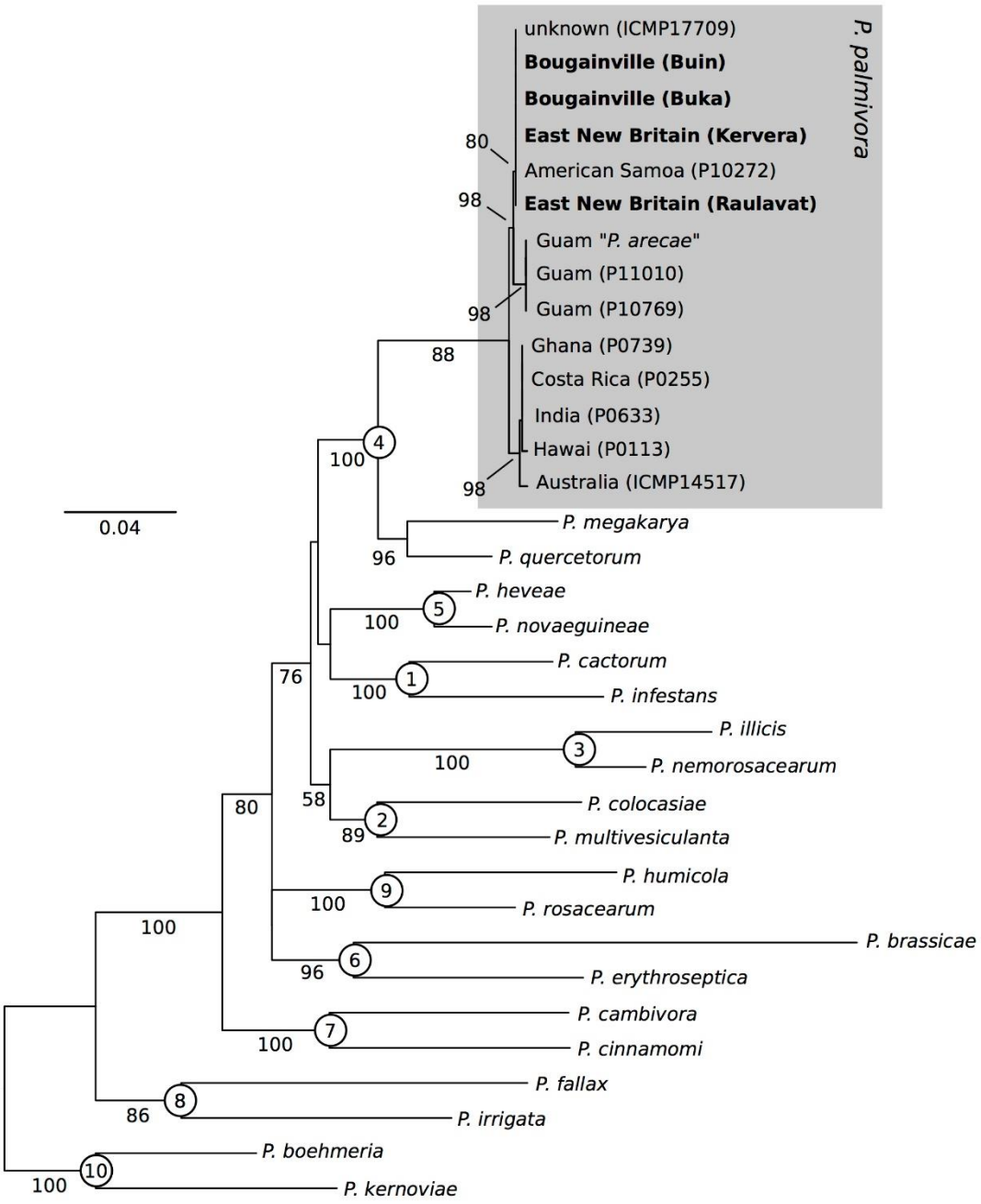


Figure 3.2 Maximum likelihood phylogeny from the combined analysis of *atp9-nad9*, *cox1-cox2*, *rps10* and *secY* regions. Numbered nodes indicate clades recognised by Blair *et al*. (2008), numbers associated with branches are bootstrap support values obtained for 500 replicates.

3.5 Characterising PNG *P. palmivora* isolates for *cox1-cox2* sequence variation

Given that phylogenetic analyses indicated the *cox1-cox2* region was variable across *P. palmivora* isolates, all 14 PNG *P. palmivora* isolates were characterised for this locus

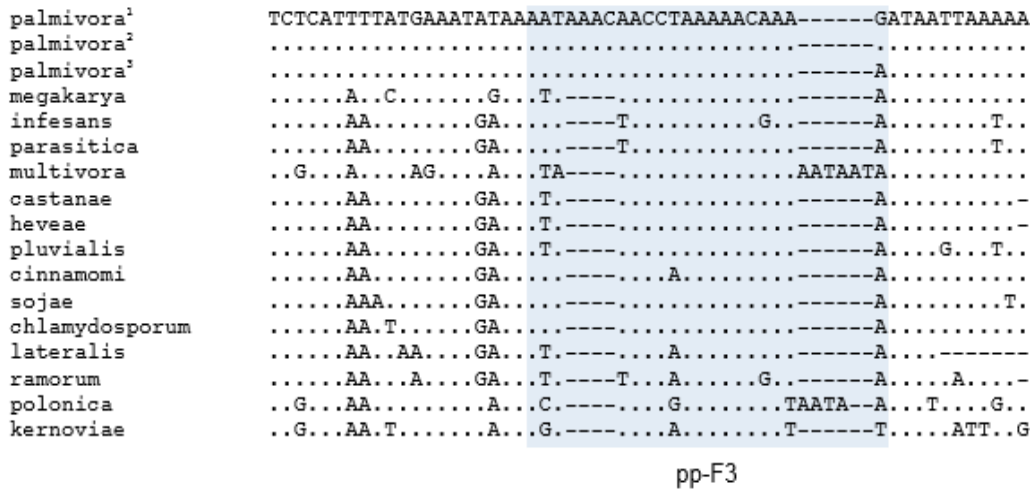
using Sanger sequencing. Sequences for all PNG isolates are identical over the length of the *cox1-cox2* locus.

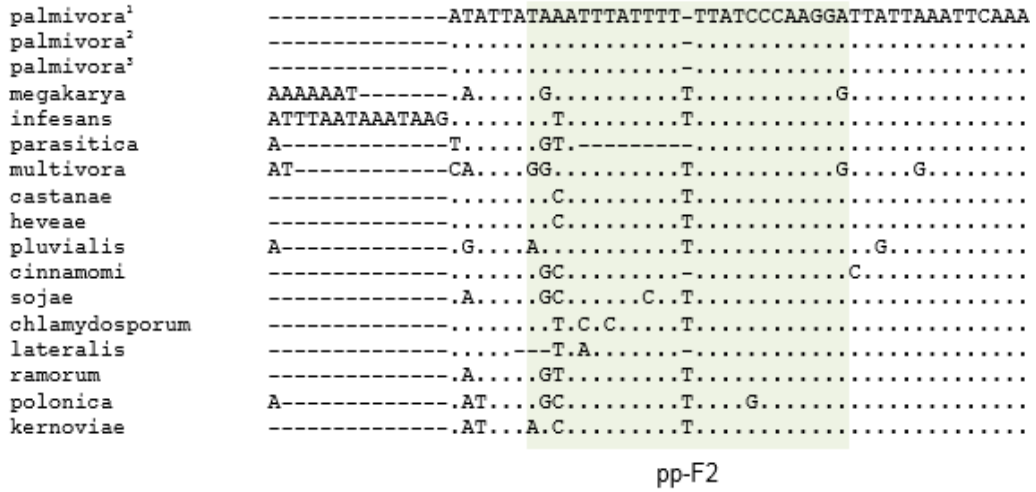
3.6 Designing primers for LAMP amplification of *P. palmivora* specific targets

Based on results from whole mitochondrial genome sequencing and phylogenetic analyses of mitochondrial loci the *cox1-cox2* and *atp9-nad9* regions were selected as potential targets for the development of *P. palmivora*-specific LAMP assays. PrimerExplorer v4 identified in excess of 1000 primer sets – each consisting of two pairs (F3/B3 and FIP/BIP) – for each of the selected loci. However, comparison to multiple sequence alignments for these two regions indicated that none of the LAMP primer sets identified for the *cox1-cox2* locus targeted portions of the sequence that distinguished *P. palmivora* from other *Phytophthora* species or differentiated between sub-clades of *P. palmivora*. In contrast for *atp9-nad9* various primer sets targeted a region containing both length and point mutations that differentiated *P. palmivora* and other *Phytophthora* species (Figure 3.3). Several of these were evaluated further using PrimerExplorer v4 and for one a single loop primer was identified. The primer set for experimental evaluation is described in Table 3.2.

Table 3.1 Statistics for matrices and phylogenetic analyses

Name	Primer sequence	*Length*	*Tm*	*GC content*
pp-F3	AATAAACAACCTAAAAACAAAGA	24		21%
pp-B3	AATCATCCAGATTTACGAAG	21		33%
pp-FIP	AACTTAGCGCAACAATATCGAT-TAAATTTATTTTTTATCCCAAGGA	47		28%
pp-BIP	TTCTAAAAAACCACTTAAAGGAAAATC-AATTTTAACAGATTATGGTTTTGAAG	54		24%
pp-LB	TTTACGTAAAGGATGTC	18		33%


palmivora[1]
palmivora[2]
palmivora[3]
megakarya
infesans
parasitica
multivora
castanae
heveae
pluvialis
cinnamomi
sojae
chlamydosporum
lateralis
ramorum
polonica
kernoviae
pp-F3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palmivora[2]
palmivora[3]
megakarya
infesans
parasitica
multivora
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sojae
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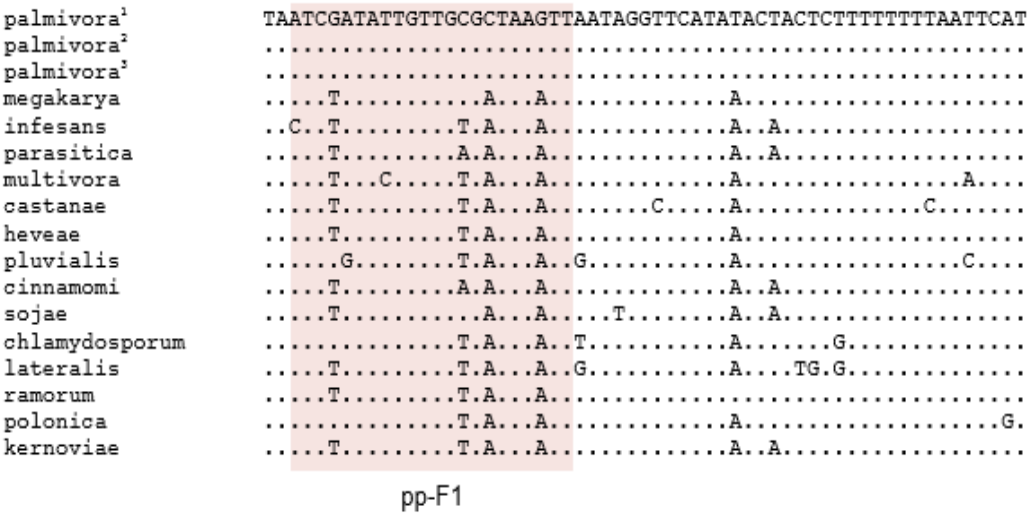
pp-F2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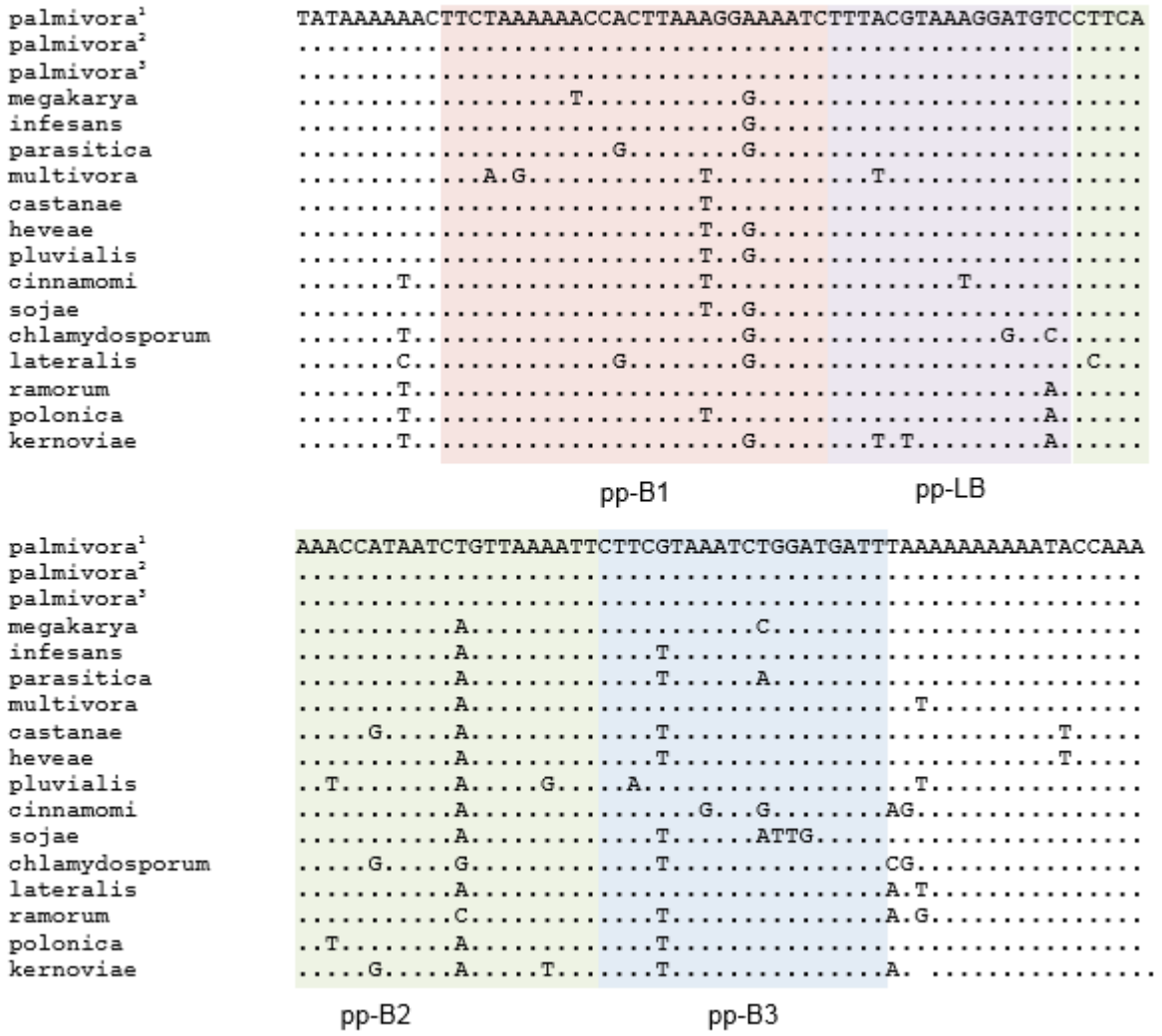

Figure 3.3 The regions coloured show where each primers sequence is located as identified by the primerexplorer software. Primer sequences are annotated with respect to the reference sequence (*P. palmivora*[1]). Dots indicate nucleotides identical to the reference genome.

1=*P. palmivora* isolate from PNG-(Raulavat)

2= *P. palmivora* from culture collection (ICMP14517), isolated from Papaya

3=*P .palmivora* (ICMP 17709) from culture collection, isolated from Guiana Chest nut

3.7 Testing of the LAMP assay

3.7.1 Initial testing of LAMP primers using PCR

Three *P. palmivora* isolates from PNG (Raul, Kerv and Buin5) were used for initial testing of the internal (pp-FIP/pp-BIP) and external (pp-F3/pp-B3) LAMP primer pairs using PCR. In both cases the LAMP primers gave positive amplifications for all three isolates . The negative controls (without DNA) were both clear (See Figure 3.4).

3.7.2 Optimisation of the LAMP assay

To optimise LAMP reactions the ratio of external (F3/B3) to internal (FIP/BIP) primer pairs as well as the effect of loop primer (LB) addition on amplification efficiency was evaluated. Amplification was visually detectable by agarose gel electrophoresis for each of the three primer ratios tested (Figure 3.6). However, amplification efficiency (as determined by intensity of fluorescence) appeared to was lower for the 1:4 ratio of external to internal primers than for those with higher concentrations of the internal primers (e.g. 1:6 and 1:8). Reactions conducted with and without the the loop primer were visually similar both in terms of the banding pattern and intensity of fluorescence (Figure 3.6). This suggests inclusion of the loop primer had little impact on DNA amplification efficiency.

3.7.3 Specificity of the LAMP assay

Specificity testing considered whether, under standardised conditions, the LAMP assay would consistently distinguish between *P. palmivora* and other *Phytophthora* species. These tests were conducted using a 1:6 ratio of external:internal primers, included the loop primer and involved a 30 min incubation at 60°C followed by 5 mins at 80°C. In multiple tests DNA from *P. palmivora* isolates consistently resulted in positive amplications whereas DNA from isolates representing other species were consistently negative (Figures 3.7-3.8).

3.7.4. Sensitivity of the LAMP assay

For comparison assay sensitivity was evaluated using both PCR and LAMP. These tests were conducted on DNA from the Raul isolate serially diluted in 10-fold steps with concentrations ranging from 100pg/µl to 0.1fg/µl. Using PCR with the pp-F3/pp-B3 primer pair the target region was amplified at DNA concentrations of between 100pg/µl and 10fg/µL

(Figure 3.9). The LAMP assay (with conditions as described in *3.7.3. Specificity of the LAMP assay*) was 10-fold more sensitive than PCR. Specifically, the target region was ampified at DNA concentrations of between 100pg/µl and 1fg/µL (Figure 3.10).

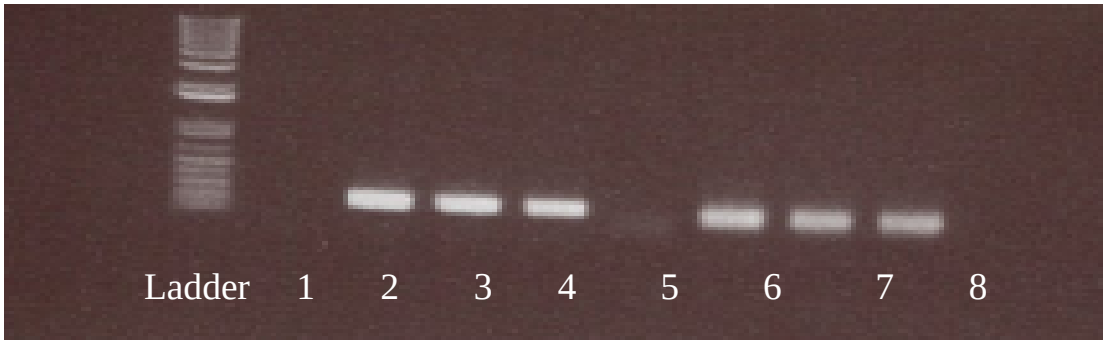

Figure 3.4 Electrophoresis of PCR products, Ladder, 1=negative control, 2=isolate 3, 4=isolate 6 and isolate 11 amplified by F3/B3 primers, 5=negative control, 6, 7, and 8 are same isolate but amplified with FIB/BIP primers.

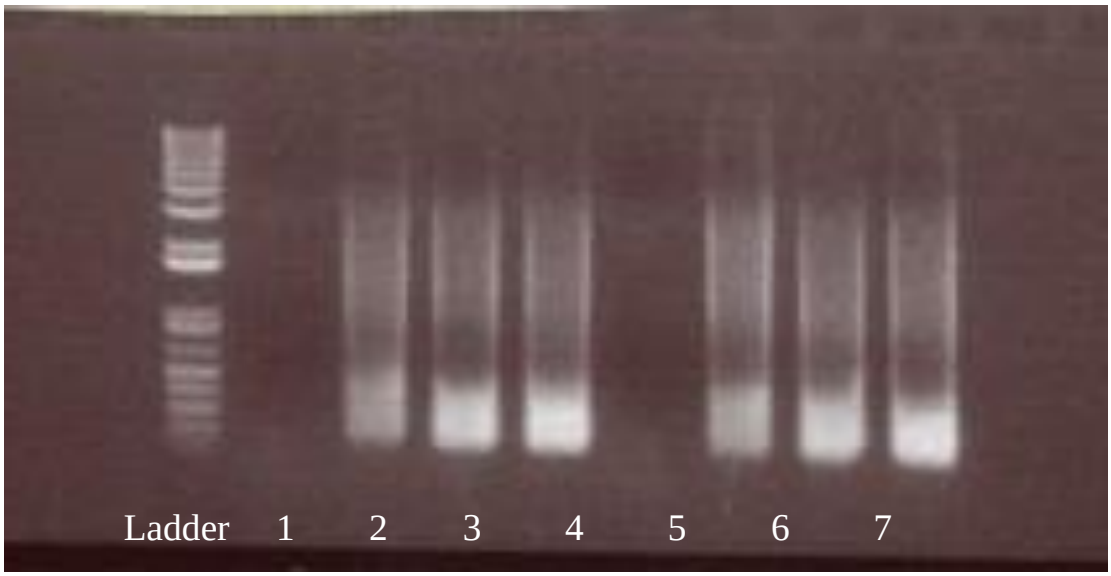

Figure 3.5 Electrophoresis of LAMP products. Ladder, 1=negative control, 2=ratio of 1: 4, 3=ratio of 1:6 and 4=ratio of 1:8 F3/B3 to FIB/BIP primer without Loop primer, 5=Negative control, 6= ratio of 1: 4, 7=ratio of 1:6, 8=ratio of 1:8 F3/B3 to FIB/BIP primer with loop primer, isolate but amplified with FIB/BIP primers. Only one isolate of *P. palmivora* was used.

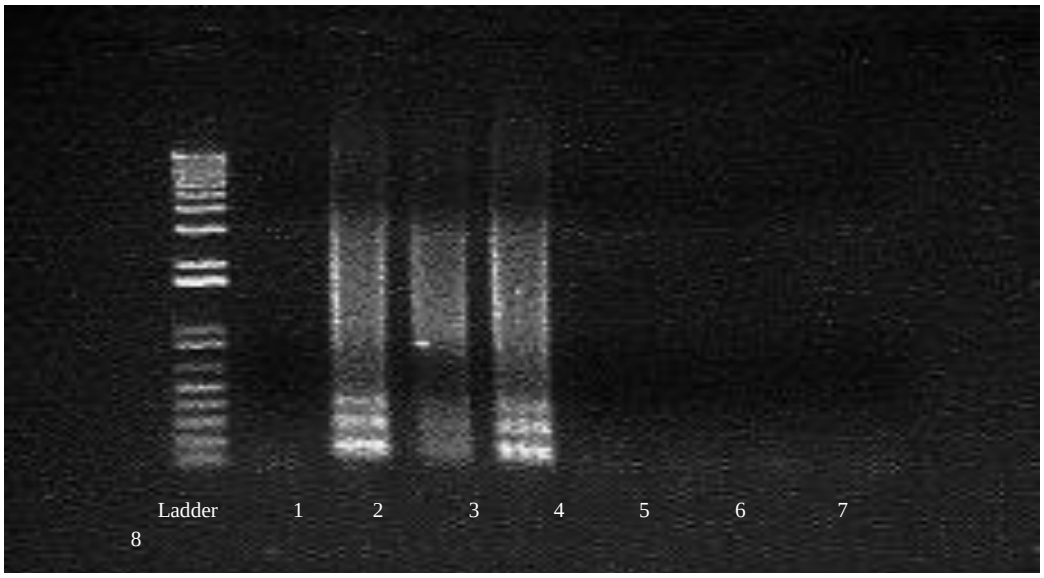

Figure 3.6 *Phytophthora* tested for DNA amplification using LAMP primers , Ladder, 1=negative control, 2= *P. palmivora* (PNG-Raul) , 3=*P. palmivora (*Chest nut-ICMP 1770), 4= *P. palmivora* (Papaya-ICMP14517) , 5=*P. capitosa*, clade 9, ICMP17567), 6=*P. agathicidae*, (NZFS3128, clade 5), 7=*P. cryptogea* NZFZ 4156-clade 8) and 8=*P. cinnamoni* (ICMP20276-clade 7)

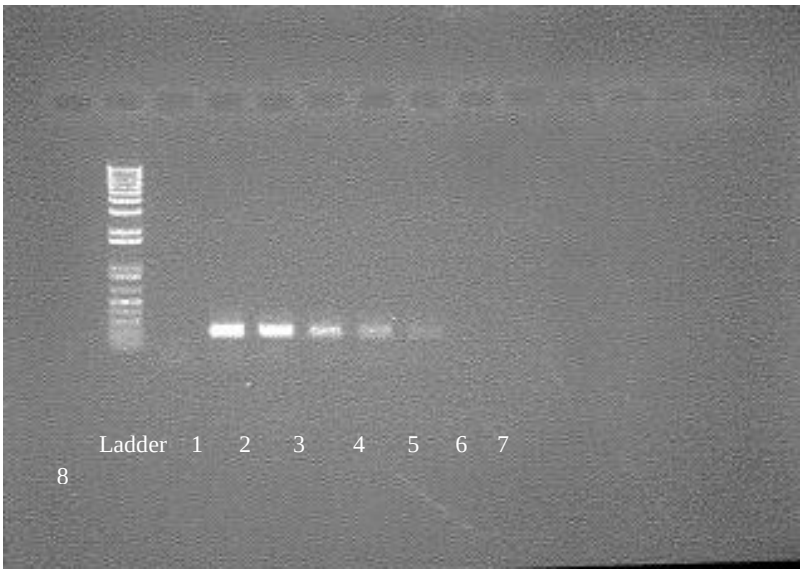

Figure 3.7 PCR amplification of the specified region of *P. palmivora*, DNA at different concentration (high –low), Ladder, 1 = negative control, 2 = 1ng/µL 3 =100pg/µL , 4 = 10pg/µL 5 = 1pg/µL, 6 = 100fg/µL 7 = 10fg/µL , 8 = 1fg/µL

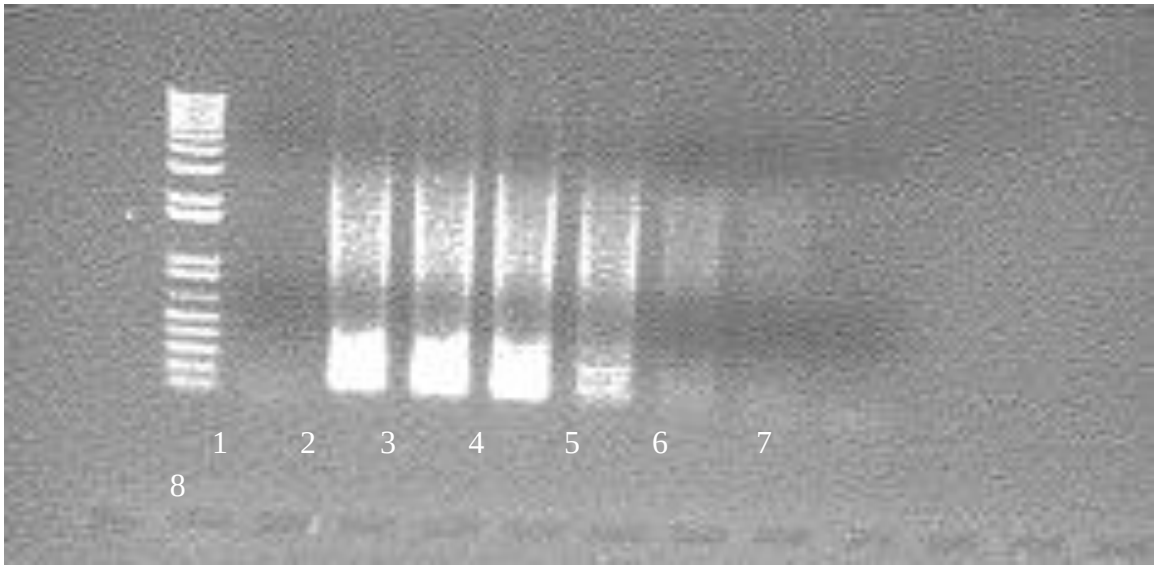

Figure 3.8 LAMP reaction of the specified region of *P. palmivora,* DNA at different concentration (high –low), Ladder, 1 = negative control, 2 = 1ng/µL 3 = 100pg/µL , 4 = 10pg/µL 5 = 1pg/µL, 6 =100fg/µL 7 = 10fg/µL , 8 = 1fg/µL

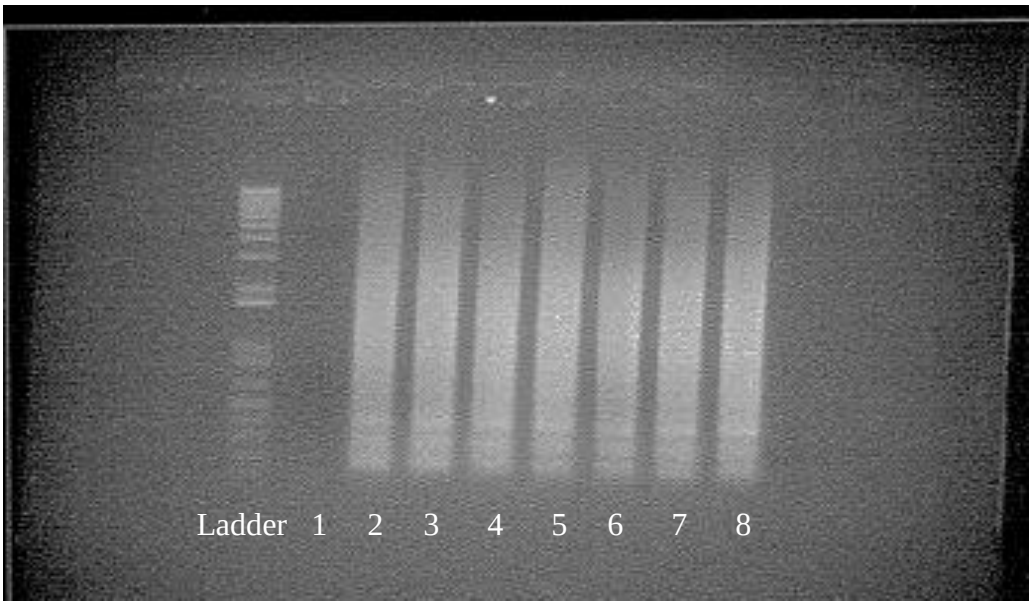

Figure 3.9 LAMP reaction detecting *P.palmivora* isolates (haplotypes) from DNA loaded in different Lanes, lane1=Negative control, lane 2 = Nari 1, lane 3 = Nari 2, lane 4 = Raulavat, lane 5 = (Buka 4), lane 6 = (Buka2), lane 7 = Buin3 and lane 8 = Tav.Office

CHAPTER 4: DISCUSSION

4.1 Introduction

This thesis reports an assessment of the genetic diversity of *P.palmivora* isolates collected in New Britain and Bougainville. Initial characterisation was based on the cytochrome *b* gene encoded in the mitochondrial (mt) genome. These analyses were extended to include phylogenetic analyses of additional mt genome loci. Analyses of these loci have shed light on a possible source of origin for *P. palmivora* in New Guinea. The complete mitochondrial genome was sequenced for one isolate and a comparison made of its gene organisation with that of other closely related *Phytophthora* species. This comparison facilitated the identification of genome regions unique to *P. palmivora* and the development of a loop mediated amplification test that has the potential to be deployed in the field as a management tool for *P. palmivora.*

4.2 Cytochrome *b* gene analyses of PNG isolates

Phytophthora species negatively impact agricultural production in Papua New Guinea, as they do elsewhere in the world. In particular, *P. palmivora* is of significant concern as it affects cacao bean production as well as other economically important crop plants. For many years it remained unclear as to how many species of *Phytophthora* were involved in causing BP disease of cocoa pods in PNG. However, recently, it was established that *P. palmivora* is the sole species causing disease in cocoa plantations in PNG (Saul *et al*. 2016). Furthermore, there has been reports of *P. palmivora* having being isolated from soil under non cultivated native forest in Ghana (Widmer, 2014) also support the idea that *P palmivora* may be a native causal agent of *Phytophthora* disease in palm tree and cacao trees in areas cacao is grown such as Ghana and PNG.

Work by Giresse et al. (2010) suggested that taxon specific mitochondrial cytochrome *b* (cyt*b*) region primers are suitable for phylogenetics and phylogeograhic studies of *Phytophthor*a. Thus initially, all isolates collected in PNG (East New Britain Province and Bougainville) for the present study were sequenced for a cyt*b* gene fragment to confirm their identity as *P. palmivora* and to investigate whether any genetic variation existed within the New Britain and Bougainville populations. BLAST pairwise sequence analysis results based on the ncbi available sequence records were consistent with identification of *P. palmivora*.

The identification was not conclusive as other species of *Phytophthora* produced similar high match scores for the short DNA fragment. Furthermore, no nucleotide sequence variation was detected for this marker among isolates. BLASTn searches against the public record identified several species including *P. palmivora* in the GenBank database with 95% identity to the query sequence. Comparison against a cyt*b* sequence from an isolate of *P. palmivora* sequenced from the New Zealand culture collection (coded ICMP14517) was more definitive. In a local BLAST comparison the identity was 100%.

The lack of variation in PNG isolates for the cyt*b* gene contrasts with the observations of Giresse *et al*. (2010), who reported a high proportion of polymorphic sites in the cyt*b* gene. However, this discrepancy is likely explained due to sequence length of the amplicon sequenced in the present study. Giresse *et al*. (2010) sequenced a 1420-1446bp gene fragment, whereas a short 183bp fragment was initially characterised in the current study.

4.3 The mitochondria genome of *P. palmivora*

The first mitochondrial genome from a *Phytophthora* to be sequenced was that for *P. infestans* (Avila-Adame *et al*. 2006). Since then, mitochondrial genomes have been sequenced as part of whole genome sequencing (WGS) projects for a number of *Phytophthora* species (Martin *et al*. 2014). These provided references for the present study. Using a WGS protocol four PNG isolates of *P. palmivora* were sequenced. The complete mitochondria genome was assembled for one of these and annotated using other *Phytophthora* reference genomes. Sequences from the other three *P. palmivora* isolates also sequenced were used to extract homologous gene sequences for phylogenetic analyses with other *Phytophthora* species representing different clades in the Phylogenetic tree (discussed next section).

The *P. palmivora* mitochondrial genome was found to be 40,807bp long, and is circular as with the mitochondrial genomes for other species of *Phytophthora*. It is similar in size to that of *P. romarum* (39,314bp) and *P. sojae* (42,977bp). Genome size differences among species of *Phytophthora* species are due to the addition or subtraction of specific genes such as open reading frames, such as for ymf 96. and differences in the length of non-coding intergenic regions (spacer regions) which are reported to comprise 30% of the mt genome of *Phytophthora* species (Martin *et al*. 2007). Another reason for variability in genome size among species of *Phytophthora* is reportedly due to the size of inverted repeated

(IR) and the presence of duplicated copies of some genes. IR length differences were first reported by Grayburn *et al*. (2004) in a study on a related Oomycete *S. fera.* This species has a genome size of 46,930bp and a 8618bp IR region. This latter region contributes most to the size difference with other Oomycete species. Its single copy sequences, comprising 38,312bp, are similar in size to that of *P. ramorum* and *P. infestans*. The genome of *P. palmivora* had no notable IR and the genome size was found to be within the normal size range of 38,000 to 42,000bp common for *Phytophthora* species.

Almost all protein coding gene loci with known functions identified in *P. ramorum* and *P. sojae* were also found in *P. palmivora* (Figure 3.2) with the exception of ymf96 and ymf16. Failure to identify these genes may reflect the higher level of sequence divergence (34-37%) reported for this locus (Martin *et al*. 2007). Of these coding genes, many have being selected and used in phylogenetic studies. Some of the most well-known genes utilised in this way have been nad9, Cox2, rps10, and SecY (Kroon *et al*. 2004, Martin & Tooley, 2003, Martin *et al*. 2014). Thus these genes were further characterised in the present study for all PNG isolates (discussed next section).

4.4 Phylogenetic relationship of *P. palmivora* based on four genes

One of the main goals of this study was to investigate the relationship of *P. palmivora* from cocoa plantations in PNG with *P. palmivora* from other plant species and locations. Four gene loci: nad9, Cox2, rps10, and SecY. were represented in the mt genome sequences of the four PNG isolates used for whole mt genome sequencing. These sequences were analysed individually and as part of a concatenated data matrix for taxa that included publically available homologous sequences from *P. palmivora* and other *Phytophthora* species.

The combined multi-gene phylogenetic analysis showed *P. palmivora* isolates from PNG clustered within clade 4 in agreement with previous studies (Martin & Tooley 2003, Kroon et al. 2004). All four genes (CoII, rps10, nad9 and secY) analysed individually recovered all 10 clades previously reported for *Phytophthora* species. However, only Cox2 was able to show distinct clusters within *P. palmivora.* Of particular note was the finding that the PNG isolates shared the same haplotype as an isolate of *P. palmivora* in GenBank (P10272) collected from *Theobroma cacao* in Western Samoa. This finding seemingly supports a well-known route in the historical movement and origin of PNG cocoa (*T. cacao*)

as reported by many authors (Efron *et al*. 1996*;* Efron *et al*. 2003*;* Wood & Lass, 1985). The PNG cocoa type known as the Trinitario was introduced from American or Western Samoa in the early 1900s and it may be that with this translocation there was also the simultaneous introduction *P. palmivora* into PNG. This idea was also supported by the results of the phylogenetic analysis that *P. palmivora*, (ICMP-17709, NZ culture collection) was recovered in the same group with PNG isolates. The isolate ICMP 17709 has been isolated from Guiana chest nut (*Pachira aquatic*) plant, native to South America and is from the same family as *T. cacao*. However, the above observations may contradict the suggestions by Widmer (2014) that the centre of origin of *P. palmivora* is in South East Asia where it was first discovered and described as affecting palms – hence its name *P. palmivora* (Butler, 1925).

Among the PNG isolates, there was only one haplotype identified in the concatenated gene analysis. This result seems fairly consistent with a recent study suggesting limited morphological, physiological and genetic diversity of *P. palmivora* on cocoa trees in PNG (e.g. Saul *et al*. 2008*;* Saul *et al*. 2016). Based on findings from microsatellite analyses, Saul *et al*. (2016) have suggested that *P. palmivora* in PNG belongs to one dominant clonal lineage, with restricted distributions for several other sub-populations.

4.5 Cox2 gene sequence variation

Specific primers (FM35 and Phy10) were designed to amplify a highly variable region of Cox 2 gene in seventeen (17) of the PNG isolates in order to assess diversity based on sequence variation. The sequenced PCR products (amplicons) of the isolates were aligned using ClustalX. Mesquite was then used to further manually edit and asses the single nucleotide polymorphism (SNPs), copy number variation and other variations in the amplicon sequence. The sequence analysis indicated no SNPs in the amplicon sequences for the Cox2 gene locus in the mitochondria across the *P. palmivora* isolates from PNG. The significance of this finding was that the region could be targeted for loop mediated amplification (LAMP), as identification of region conserved across all isolates would pave way for the development of *P. palmivora* specific primers. Ultimately, this region was not selected in the present study because of the availability of other genes regions sequenced from clade 4 species (Giresse *et al*. 2010, Martin *et al*. 2014). These comparative data were used to make the initial LAMP primers reported here.

4.6 The Design of LAMP primers

A loop mediated amplification (LAMP) test that is field deployable will have useful application in PNG as it could be used to inform about levels of inoculum on plants and in the soil. It could also be used to ensure germplasm and nursery stocks are free of *P. palmivora*. It would also have potential for disease identification from other plant hosts such as Durian, papaya, coconut, areca nut and many other horticulturally important plants in PNG.

The development of such a tool requires the designing species specific amplification primers (Notomi *et al*. 2000). In this study, from more than 3000 primers generated insilico, five primers were selected after considering base composition, GC content and melting temperature. These primers targeted the conserved region of loci atp9 (227 bp) to nad9 (557bp) and included part of the cob (cyt*b*) loci. The effectiveness of the primers was tested using *P. palmivora* and other *Phytophthora* DNA samples in the laboratory. They remain to be tested in the field condition.

The design of the primers followed those of Notomi *et al*. (2000) who first developed LAMP with two inner primers and one outer primer for both forward and backward targeting six distinct regions of the target gene. This design is similar to the approach taken here which produced two outer (F3 and B3), two forward inner primers (FIP), two backward primers (BIP) and a one loop primer (LP) for targeting the specified mt region between cox1/cox2 and cob (*cytb*) loci.

The efficiency and reliability of these LAMP primers for diagnosis of *P. palmivora* will still require further testing. Previous studies that have used four specific primers targeting a specific DNA (gene) for amplification have achieved good results (e.g. Su *et al*. 2016). In Su *et al*. 2016 different experiments (single factor experiment for LAMP, Orthogonal experiment of LAMP, and specificity and sensitivity tests) were conducted in order to evaluate the efficiency of their LAMP primers. The experimental evaluation used by these authors is rigorous and will be worth undertaking in the next stage of development for a *P. palmivora* LAMP kit.

4.7 Testing and implementation of LAMP in diagnostics

PCR and LAMP reactions were used to test the designed primers for the amplification of DNA from PNG isolates of *P. palmivora*. Of interest was the specificity and sensitivity of the primers. As mitochondrial genomes are highly variable among the Oomycetes including *Phytophthora* species it was anticipated the primers could be made highly specific (Giresse *et al*. 2010). The observation that mt genome gene regions are highly conserved among *P. palmivora* isolates was also encouraging that a test could be made specific for *P. palmivora.* Under laboratory conditions, the primers were found to be both specific and sensitive. This was shown by the results where among the different species of *Phytophthora* only the *P. palmivora* DNA was amplified.

It was also demonstrated in the study that the LAMP primers were able to detect the lowest DNA concentration of 1fg/µl of the *P.palmivora*. Even the DNA from cocoa pod lesion was successfully amplified thus showing high sensitivity as compared to PCR which would amplify from as low as 10fg/µl.

The promise of deploying the LAMP amplification technique as a field diagnostic for identification and quantification of *P. palmivora* is close to being realised now that specific primers have been developed and tested. However, further work is needed to develop real time monitoring of LAMP amplification. Here end point detection was used to visual positive results, and it is anticipated that real time monitoring will be both more quantitative and rapid. Fluorescence detection using affinity probes and 'SmartDart devices' coupled with phone or tablets offers this potential and is an exciting prospect for the future (Jenkins and Kuboto, 2015).

4.8 Concluding Statement

This study provides a further assessment of the level of genetic diversity of *P. palmivora* in East New Britain and Bougainville obtained by comparing mitochondrial genome sequence variation. No intraspecific variation was detected among *P. palmivora* isolates from different collection sites and different host cocoa varieties (races) and from two geographical locations or island provinces (Bougainville and East New Britain) of PNG. The PNG *P. palmivora* was found to be identical to the isolate (ICMP 17709) from the culture collection isolated from host Guina Chestnut (*Pachira aquatic*). Thus it can be concluded that *P. palmivora* is the sole species causing disease of cocoa in PNG.

The mt genome of the *P. palmivora* isolate (Raulavat) was successfully constructed as circular, and thus similar to other previously completed genomes for *P. sojae*, *P. ramorum* and *P. infestans* with a length (size) of 4080 7bp. Differences to these species has resulted from gene rearrangement (reverse and forward transcriptions) and absence of the ymf regions in *P. palmivora* genome.

Phylogenetic trees for different species of *Phytophthora* from all 10 clades including *P. palmivora* were reconstructed from four loci: coII, nad9, rps10, and secY. Three haplotype groups for *P. palmivora* were identified and the isolate from PNG and American Samoa *P. palmivora* (P10272) were in one group suggesting its introduction along with the host (cocoa seeds or plants) into PNG may be from American or Western Samoa.

This study has shown that it was possible to design LAMP primers from a mt genome region of *P. palmivora* flanked by the cox1/cox2 and cob as selected the Primerexplorer program. The final selected LAMP primer consists of forward (F3), backward (B3), two forward internal (FIP) and two backward (BIP) and one loop primers.

The designed primers were effective, specific and sensitive at isothermal temperature of $60C^0$ as shown by the result by effectively amplifying only *P. palmivora* DNA among other species of *Phytophthora*. It was shown that the primers were able to amplify extracted DNA from *P. palmivora* mycelia and pod lesions from a sample containing DNA concentration as low as 10fg/μl in a laboratory conditions using the LAMP. Further work is needed to before this LAMP diagnostic for *P. palmivora* can be used on plant and soil samples in field.

4.9 Future work

The following considerations are made for taking the present work forward:

- Once sensitivity and selectivity of the LAMP primers developed are evaluated further, attention needs to turn to developing the optimal assay conditions for field work. Both endpoint detection and real time detection methods are available (e.g. Parida *et al.* 2008; Kubota and Jenkins, 2015; Wang *et al.* 2016). Since real time detection may provide the best means of obtaining quantitative information on levels of infection investigating these methods may be preferable.
- Further research and development is also needed to investigate sampling protocols for LAMP assays from diseased tissues and different plant parts.

- The robustness of devices, the stability and availability of reagents under tropical conditions in PNG where they could be used by the cocoa and coconut institute ltd, the PNG Cocoa Board, the National Quarantine facilities and the training institutions such as Universities and Agricultural colleges is also an important consideration.

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