Development and validation of a robust and rapid isothermal loop-mediated amplification (LAMP) assay for the detection of Black Sigatoka Disease *(Pseudocercospora fijiensis)* in bananas

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Luania Koi Bob

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Abstract

Black Sigatoka disease is a serious threat to banana and plantain production. However, the causal agent of the disease, *Pseudocercospora fijiensis,* is difficult to distinguish from related species associated with yellow Sigatoka disease (*P. musicola*) and eumusae leaf spot disease (*P. eumusae*) on the basis of symptomology or morphology. These similarities complicate pathogen identification. Molecular methods such as conventional PCR have been used in diagnosing this disease, but the time and cost of testing as well as the requirement for specialised equipment and infrastructure have limited its usefulness. New molecular approaches are reducing the barriers to molecular testing by allowing testing to be conducted in non-laboratory settings. Given the serious threat of black Sigatoka disease, a simple and rapid isothermal loop-mediated amplification (LAMP) assay has been developed to improve pathogen detection. A potential target region within the mitochondrial small ribosomal subunit (ssRNA) gene was identified and a set of four LAMP primers were developed. Initial trials established optimal reaction conditions for the *P. fijiensis* LAMP assay. These were a 1:8 primer ratio with an amplification temperature of 60˚C and an amplification time of 60 minutes. Laboratory validation of the assay suggests specific amplification in the presence of the target organism with no cross-reaction with several related species and other fungal pathogens of bananas and it detects anything above 9.1×10^6 copies of the target. The LAMP assay was used against foliar and mycelial samples from Papua New Guinea and foliar samples from Fiji. Testing of foliar samples identified *P. fijiensis* in >90 % of the samples. These results were confirmed using PCR and sequencing. Nuclear ribosomal ITS sequencing suggested that non-target pathogens dominated the mycelium samples. However, LAMP testing indicated *P. fijiensis* was present in 50% of these samples. These results were confirmed using a PCR marker specific to *P. fijiensis*. In combination it suggests *P. fijiensis* was present on culture plates but competing species grew more rapidly. The testing performed suggests the LAMP assay provides a specific test for *P. fijiensis* that is sensitive enough to identify this pathogen from symptomatic tissue. Further work is needed to develop protocols for field testing and to examine the potential for testing non-symptomatic material.

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Chapter 1
Introduction

1.1 Introduction

Bananas and plantains (Musaceae) were initially domesticated in South East Asia and New Guinea (Donohue & Denham, 2009; Perriera et al., 2009), but are now cultivated in more than 100 countries. Cultivated primarily for their fruits, these crops are rich sources of energy, minerals and vitamins as well as providing antioxidants, protein and dietary fibre (Brown et al., 2017). Ranked as the sixth most important staple crop, bananas and plantains play an important role in global food security with an annual production of 114 million tonnes (FAOSTAT, 2019). Large plantations of, primarily, Cavendish bananas in Ecuador and the Philippines supply much of the global market whereas production in many other developing countries provides food security and income for subsistence farmers.

Fungal diseases of bananas and plantains are a key cause of crop losses for both smallholders and commercial operations (Almeida, Rodrigues & Coelho, 2019; de Bellaire et al., 2010; James et al., 2011; Jeger et al., 1995; Nelson, Ploetz & Kepler, 2006; Ploetz, Kema & Ma, 2015). In particular, black Sigatoka disease seriously limits banana production in many countries. This disease, caused by *Pseudocercospora fijiensis,* damages the leaf surface lowering photosynthetic capacity and ultimately leading to reduced yield and marketable value (Thompson, 2011). Various methods including the application of fungicides have been used to control the disease (Churchill, 2011; Henderson et al., 2006; Marin et al., 2003; Romero & Sutton, 1997; Tinzaara et al., 2018). The high virulence of the pathogen has made eradication difficult (Churchill, 2011) and black Sigatoka disease is now recognised as a critical threat to the banana industry.

It is difficult to distinguish *P. fijiensis* from the related species *P. musicola* (yellow Sigatoka disease) and *P. eumusae* (eumusae leaf spot disease) based on visual disease symptoms and morphological characteristics in culture. Distinguishing between these diseases typically requires molecular diagnosis (Arzanlou et al., 2008; Vázquez-Euán et al., 2012; Zandjanakou-Tachin et al., 2009). Currently a PCR-based assay that requires specialist infrastructure and equipment as well as highly trained staff is used. However, new molecular technologies have emerged over the last 20 years that remove some of the barriers to wider uptake of molecular diagnostics. In particular, there is a growing interest in isothermal methods for DNA amplification that have the potential to reduce the need for specialist equipment and facilities. One such method is loop-mediated isothermal amplification (LAMP) (Notomi et al., 2000). This approach has rapidly increased in popularity due to the relative simplicity and speed of the reactions (Fu et al., 2010). Over the last decade LAMP diagnostics have been developed for a wide range of applications (e.g., infectious disease diagnosis, food safety, and plant disease diagnosis). That LAMP assays are simple, robust and portable means they can potentially be used on-site, substantially reducing the time needed for diagnosis and therefore to implement disease controls.

1.2 Research Statement

Traditional diagnosis of plant fungal diseases involves the visual recognition of symptoms on the host and morphological identification of the pathogen following *in vitro* isolation. Molecular approaches to disease diagnosis, in particular those involving PCR, have become increasingly important over the last 25 years. Although molecular tools are now widely used for the diagnosis of plant diseases, uptake of these tools has been slower in developing countries such as Papua New Guinea (PNG). Generally, factors such as the availability and cost of equipment, the lack of appropriate facilities, and the lack of trained staff to carry out testing are substantial barriers to the use of molecular diagnostics in developing countries.

The difficulty diagnosing *P. fijiensis* using non-molecular approaches and obstacles to accessing standard molecular diagnostics in PNG has motivated the development of a diagnostic LAMP assay for this pathogen. A test of this type would remove some of the obstacles to the use of molecular diagnostics in low infrastructure settings such as PNG.

1.3 Thesis structure

This thesis describes the development and laboratory validation of a LAMP assay for the detection of *P. fijiensis*, the causal agent of Black Sigatoka disease. The thesis consists of five chapters; these provide an overview of the literature, the approach taken and the findings of the research. Table 1.1 lists the chapters and provides a brief summary of each chapter.

Table 1.1 Chapter highlights in brief

Chapter	Description
Chapter 1: Introduction	Provides a general overview of the thesis as well as a research statement.
Chapter 2: Literature Review	Provides a description of the importance of bananas and plantains, a description of black Sigatoka disease and the causal agent, *P. fijiensis*, and, finally, a description of both traditional and molecular diagnostics.
Chapter 3: Materials and Methods	The approach used during the development of the LAMP assay is discussed. Includes descriptions of both field and lab work.
Chapter 4: Results	The findings of the study are reported.
Chapter 5: Discussion	Discusses the findings and places these in a broader context.

Chapter 2

Literature review

2.1 Bananas and plantains

2.1.1 Morphological description

The family Musaceae consists of three herbaceous angiosperm genera, distributed mostly in tropical and sub-tropical regions (Brown et al., 2017). Members of the genus *Musa* L. are large, tree-like plants 2-9 m tall (Karamura & Karamura, 1995; Nelson, Ploetz, & Kepler, 2006). Plants typically have a tall, tough pseudostem – consisting of the stem proper encased by sheathing petiole bases of the leaves – topped with large, broad leaves (Nayar, 2010) (Figure 2.1). Both the pseudostem and a fibrous root system develop from a corm that sits on the soil surface (Price, 1995; Thompson, 2011).

The emergence of a flower spike indicates plant maturity; usually 25-40 leaves emerge before a plant will flower (Nayar, 2010). Initially, the immature inflorescence develops within the pseudostem (Thompson, 2011). Clusters of male and female flowers form two rows on a hanging inflorescence that is enclosed by a large bract (Nayar, 2010). Up to 20 fruits, described as "leathery" berries due to their thick skin, ripen per inflorescence (Khanvilkar et al, 2016). Ripe fruits are either yellow, red, or green in colour, 6-35 cm long, and 2.5-5 cm thick (Nayar, 2010). The size and quantity of fruits depends on the species or variety and growing conditions.

2.1.2 Taxonomy

The name *Musa* is derived from the Arabic word, "mauz", for banana (Linnaeus, 1753). Based on culinary use Linnaeus (1753) distinguished dessert bananas and plantains as *Musa sapientum* L. and *Musa paradisiaca* L., respectively. The classification of edible bananas has however been controversial as morphology does not consistently differentiate them. Early studies suggested that both *M. sapientum* and *M. paradisiaca* were derived from the wild seed-producing *Musa* species*, M. acuminata* Colla and *M. balbisiana* Colla by interspecific hybridisation. Cheesman (1947) distinguished banana cultivars based on vegetative morphology identifying them as *M. acuminata, M. balbisiana* or a hybrid (*M. acuminata* × *M. balbisiana*).

Later, Simmonds and Shepherd (1955) constructed an informal classification of cultivars that categorises bananas based on characteristics of their karyotype. The species *M. acuminata* and *M. balbisiana* were recognised as having distinctive karyotypes, denoted A for *M. acuminata* and B for *M. balbisiana*. Simmonds and Shepherd (1955) then recognised

Figure 2.1 Labelled diagram of a typical fruiting banana plant. Source, Bakry et al. (2009).

diploids (e.g., Lady Finger bananas, AA) and triploids (e.g., Cavendish bananas, AAA). Subsequent classifications based on both morphological and genetic analysis have also identified tetraploids (e.g., Goldfinger bananas, AAAB) (Manthey & Jaitrong, 2017; Nsabimana & Staden, 2006).

2.1.3 History of banana cultivation

Bananas and plantains are currently cultivated throughout the tropics and subtropics. Currently, over 1000 cultivars of bananas and plantains are cultivated in over 120 countries (De Langhe et al., 2009; Tinzaara et al., 2018; FAO, 2019). However, the natural range of *M. acuminata* is primarily South East Asia (i.e., India and Myanmar to Indonesia), New Guinea and northern Queensland while *M. balbisiana* occurs naturally in the northern Philippines, Taiwan and China (De Langhe et al., 2009; Wong et al., 2001)(Figure 2.2). Archaeological, linguistic and genetic evidence suggests that the early domestication of bananas and plantains occurred in New Guinea and South East Asia (Donohue & Denham, 2009; Perriera et al., 2009). Specifically, Perriera et al. (2009) concluded that *M. acuminata* was first domesticated in the highlands of New Guinea whereas *M. balbisiana* was first domesticated in the Philippines. The tropical rainforest areas of Australia may also have provided an initial site of banana and plantain cultivation (De Langhe et al., 2009).

Most of the tropical and subtropical regions where bananas and plantains are now produced are considered secondary sites of cultivation. During the domestication and cultivation of bananas and plantains intra- and interspecific hybridisation within and between *M. acuminata* and *M. baibisiana* has led to novel cultivars (Perriera et al., 2009). Genetic evidence suggests that the movement of humans has influenced patterns of hybridisation. For example, although no wild species of banana or plantain occur in Africa or the Americas (Nsabimana & Staden, 2005; De Langhe et al., 2009) these areas have been an important source of novel hybrids and cultivars. Two hypotheses have been suggested for the introduction of bananas to Africa; one suggests arrival via India and the Middle East whereas the other a route via Madagascar (Neumann & Hildebrand, 2009). Archaeological evidence supports that latter (Mindzie et al., 2001) and morphology comparisons the former (Fuller &

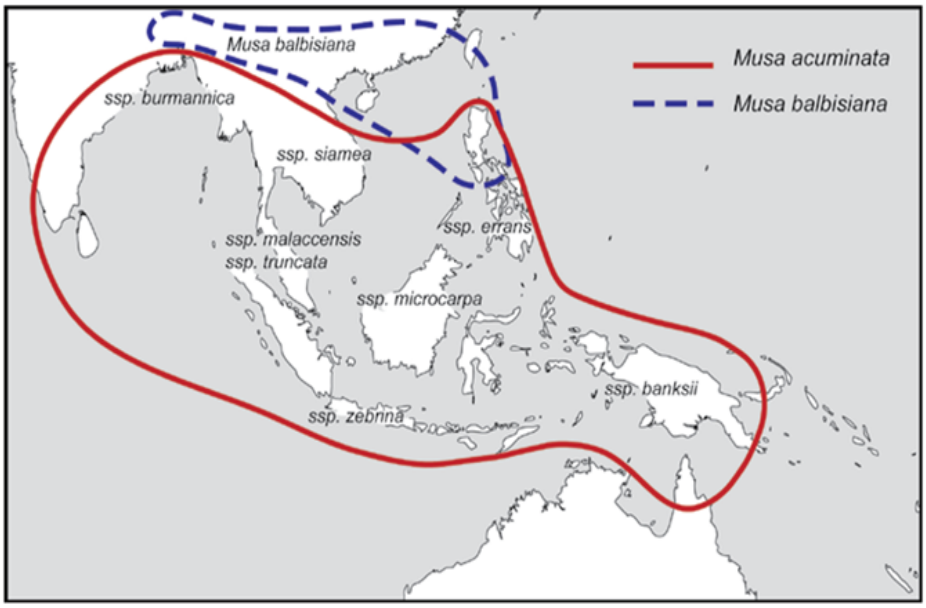

Figure 2.2 Map showing the distributions of the two wild, seed producing ancestors of cultivated bananas, *Musa acuminata* and *M. balbisiana*. Source, De Langhe et al. (2009).

Madella, 2009). Bananas and plantains were introduced to Central and South America during the 16th century by the Portuguese (Arvanitoyannis & Mavromatis, 2009).

The early spread of bananas involved parthenocarpic diploids. However, most of these varieties were later replaced by sterile triploids (Stover & Simmonds, 1987). Perrier et al. (2011) have suggested several stages in the domestication of bananas, including an initial transition phase from wild to edible diploids and the development of edible triploids. The wild species of both bananas and plantains produce seeds but seedless fruits have being selected for over time. In edible banana varieties seedlessness has arisen as the result of parthenocarpy (Simmonds, 1953), sterility (Simmonds, 1960), mutation and hybridisation (Heslop-Harrison, J. S., & Schwarzacher, 2007). In some cases seedless forms have arisen spontaneously in natural populations and have been taken into cultivation by farmers (Heslop-Harrison & Schwarzacher, 2007). These initial collections were cultivated and distributed through vegetative propagation, ultimately giving rise to different banana cultivars. Currently, commercial production of bananas and plantains is dominated by triploid varieties (Thompson, 2011).

2.1.4 Uses and economic importance

Ranked as the sixth most important staple crop (FAO, 2019), bananas and plantains play an important role in global food security (Tinzaara et al., 2018) and are economically important crops in many developing countries (Brown et al., 2017). Bananas and plantains grow well in the humid, lowland tropics (Anderson, 1998; Thompson, 2011) and are mainly produced in Asia, Africa and the Latin in America (FAO, 2019).

Almost all the commercially available varieties of bananas and plantains are polyploid, developed via breeding programs and field selection (Thompson, 2011). For example, the most widely available variety, the Cavendish banana, is a triploid containing three copies of the A type genome (Bakry et al., 2009; Heslop-Harrison & Schwarzacher, 2007). In 2017 global production of bananas reached ~114 million tonnes with Asia accounting for half of the world's total production (FAOSTAT, 2019; Table 2.1). India produced 29 million tonnes followed by China with 11 million tonnes and the Philippines with 7.5 million tonnes. However, only about 15% of global banana production is traded on the international market and most of the bananas grown in Asia (Ploetz, Kema, & Ma, 2015), as well as Africa and Oceania are locally consumed (Table 2.2). Despite lower overall banana production in Central and South America these countries primarily contribute to the international market (Table 2.2).

Plantains are widely grown in the Pacific while both dessert bananas and plantains are common in the South East Asian countries and the Americans (Tinzaara et al., 2018). Although plantains account for a relatively small proportion of the international market, they

Figure 2.3 Some of the diversity of bananas and plantains available at a vegetable market in Port Moresby, Papua New Guinea. Photo source, L. Bob (2019).

are important for domestic markets (Ploetz, Kema, & Ma, 2015; Escobar-Tovar et al., 2015; Tinzaara et al., 2018).

In developing countries small scale farming of bananas and plantains provides an important food source for many people (Karamura et al., 1998; Nelson, Ploetz, & Kepler, 2006; Price, 1995; Tinzaara et al., 20018; Wambugu & Kiome, 2001). For example, over 60% of the bananas and plantains produced in Papua New Guinea (PNG) are consumed locally (Bourke & Vlassak, 2004) (Fig. 2.3). Small scale farming places greater emphasis on the cultivation of plantains as these can withstand climatic variations, have a range of uses and can be grown in backyards for local consumption. However, bananas and plantains contribute both to the economy and to food security in the countries that produce them (Thompson, 2011; Tinzaara et al., 2018).

Bananas and plantains both have high nutritional value. For example, commercially available bananas are a good source of vitamins (e.g., A, B, C) and minerals, also providing antioxidants, proteins and dietary fibre (Brown et al., 2017; Thompson, 2011). In developing countries this makes bananas and plantains an important part of the diet (Blades et al., 2003; Englberger et al., 2003; Kay, Grobin, & Track, 1981; Preethi & Balakrishna Murthy, 2013;

Table 2.1 Global production of bananas in 2017 by region. Source, FAO (2019).

Region	Percentage of production	Bananas produced (tonnes)
Asia	50.82	57,934,800
The Americas	32.97	37,585,800
Africa	14.09	16,062,600
Oceania	1.55	1,767,000
Europe	0.57	649,800
Total	100.00	114,000,000

Table 2.2 Global export of bananas in 2017 by region. Source, FAO (2019).

Region	Percentage of production	Bananas exported (tonnes)
The Americas	41.24	15,500,000
Asia	3.28	1,900,000
Africa	6.23	1,000,000
Oceania	0.00	0
Europe	0.00	0
Global	16.41	18,400, 000 tonnes

Wachirasiri, Julakarangka & Wanlapa, 2009).

Edible parts of the crop include the fruits and blossoms, also known as the "banana heart" (Florent, Loh & Thomas, 2015). Bananas are typically consumed raw when ripe (e.g., as a snack) whereas plantains are generally cooked (e.g., boiled, fried or baked) before consumption. The fruits of bananas and plantains may also be processed into products such as purees, powders, flour, chips, vinegar, jam, jelly and wine (Adams, 1980; Akubor et al., 2003; Guerrero, Alzamora & Gerschenson, 1994; Singh, Kaushik & Gosewade, 2018). The flowers are also eaten in South East Asia, Africa and the Pacific. The florets of the banana heart are plucked, cleaned and stir or deep fried (Nelson et al., 2006; Florent, Loh & Thomas, 2015).

Bananas and plantains have uses beyond being a food source. In several areas the leaves are used as wrappers to store food in or as plates to serve food on (e.g., Kora, 2019) (Fig 2.4). Other uses include as biomass for ethanol production, as cattle feed, as an antioxidant and as an insecticide (Mohapatra, Mishra & Sutar, 2010; Tinzaara et al., 2018). Bananas and plantains also have cultural significance. For example, in PNG bananas are an important part of traditional ceremonies especially in the lowlands where they are used as compensation, as bride prize in marriage ceremonies and as a peace offering. Additionally, bananas and plantains have been used in the treatment of human ailments for example on skin irritations, digestive disorders, to alleviate blood pressure and can be applied as an antibiotic agent. (Turner, 1997; Kumar et al., 2012; Almadhoun & Abu Naser, 2018).

2.2 Diseases of banana and plantains

Although banana and plantains are a staple crop in many developing countries, biotic and abiotic factors including pests and diseases, climatic events (e.g. cyclones and droughts) and post-harvest losses threaten production (e.g., Mugisha et al., 2008; Anap et al., 2014; Jones, 2007; Kumari, Singh & Atre, 2018; Nandwani, 2010). Production constraints imposed by disease control limit yield and, if unsuccessful, disease outbreaks can cause significant

Figure 2.4 Traditional use of banana leaves in the East New Britain Province, Papua New Guinea. Food may be wrapped in banana leaves and cooked (A), banana leaves may be used as a plate to serve food on (B) or as a wrapper to pack food in (C). Photo source, F. Vutia (2019).

crop losses (Tinzaara et al., 2018). Most banana cultivars are susceptible to fungal diseases; these are responsible for the majority of crop losses due to disease (Pagen & Garcia-Arenal, 2018). Key examples include Panama disease (or *Fusarium* wilt), banana bunchy top virus disease (BBTV) and black Sigatoka disease (or black leaf streak disease) (Table 2.3). These diseases affect the production of bananas and plantains across much of their cultivated range (Marin et al., 2003; Ploetz, 1994). For example, Panama disease, caused by the fungus *Fusarium oxysporum* Schlecht, has devastated production of the susceptible 'Gros Michel' cultivar in Honduras (Ploetz, 2000). Likewise, the black Sigatoka disease is considered a major threat to the banana industry globally (de Bellaire et al., 2010).

2.2.1 Black sigatoka disease

2.2.1.1 History of disease

Black Sigatoka disease was first reported from Viti Levu, Fiji in 1963 (Isaza et al., 2016; Stover, 1980). However, a survey between 1964 and 1967 suggests that black Sigatoka disease had been well established in the Pacific and South East Asia prior to its discovery in Fiji (Henderson et al., 2006). According to Henderson et al. (2006), the exact origins of the disease remains uncertain due to the emergence of another new disease and environmental and host factors during the survey period.

Stover (1978) initially suggested that the black Sigatoka disease had originated in PNG. This suggestion is consistent with PNG having high diversity of both host and pathogen (Carlier et al., 2000). However, based on Henderson et al., (2006) have since suggested a South East Asian origin based on the distribution of diversity across the pathogens entire range.

Table 2.3 Serious diseases of bananas and plantains

Disease common name	Causal agent
Panama disease (Fusarium wilt)	*Fusarium oxysporum* f. sp. *cubenseiiiii*
Black Sigatoka disease	*Pseudocercospora fijiensis*
Yellow Sigatoka disease	*Pseudocercospora musicola*
Eumusae leaf spot disease	*Pseudocercospora eumusae*
Moko disease (Bacterial wilt)	*Ralstonia solanacearum*
Banana anthracnose disease	*Gelosporium musae*
Head rot disease	*Erwinia carotovora*
Banana bunchy top disease	Banana bunchy top virus (BBTV)
Banana streak disease	Banana streak disease (BSV)

Black Sigatoka disease spread extensively during the 1970s and 1980s. some evidence suggests that the use of vegetative portions of the plant as packing material is a possible pathway for disease transmission over long distances (Henderson et al., 2006; Ploetz, 2000; Schlegel, 2009). The disease was reported from Central America in 1972. Initially arriving in Honduras, the disease had spread through Central America and into southern Mexico by 1980 (Stover, 1980). Despite spreading throughout Central America during the 1970s, the disease was not identified in the Caribbean until the early 1990s. Following an outbreak in Cuba during 1992 the disease spread to other islands in the region (Jones & Mourichon, 1993). Black Sigatoka disease also appears to have arrived in Africa during the 1970s, with evidence of outbreaks in Zambia during 1973 (Dabek & Waller, 1990) and Gabon in 1978 (Frossard, 1980). The disease was reported from Ghana and Nigeria in 1986 (Marin et al., 2003; Mourichon & Fullerton 1990; Wilson, 1987), Kenya and Madagascar in 1988 (Jones, 2003) and Malawi, Uganda and Rwanda during the late 1980s and 1990s (Tushemereirwe & Waller, 1993).

Black Sigatoka disease is now widely distributed in banana producing areas (Jacome 2003; Thompson, 2011). The disease has been reported from the Pacific Islands, Central and South America, the Caribbean, Asia and Africa. It was detected in Australia in 2001 but successfully eradicated (Henderson et al., 2006; Yonow et al., 2019). In the Pacific the disease has been reported from Papua New Guinea (Davis et al., 2000; Stover, 1976; Yonow et al., 2019), the Torres Strait Islands (Henderson et al., 2006), the Solomon Islands (Stover 1976; Yonow et al., 2019), New Caledonia (Mourichon & Fullerton, 1990; Yonow et al., 2019), Norfolk Island (Jones, 1990; Yonow et al., 2019), the Federated States of Micronesia (Jones & Mourichon, 1993), Niue, the Cook Islands, Vanuatu, Tahiti, Fiji, Tonga, Samoa, Hawaii and Wallis and Futuna (Yonow et al., 2019).

2.2.1.2 Pathogen associated with the disease

The causative agent of black Sigatoka disease is a filamentous, hemibiotrophic ascomycete (Chang et al., 2016; Churchill, 2011; Isaza et al, 2016). This fungus is currently referred to as *Pseudocercospora fijiensis* (Morelet) Deighton but was previously called *Mycosphaerella fijiensis* (Morelet). These names referred to the asexual (anamorph) and sexual (telomorph) forms, respectively (Churchill, 2011). Approximately 146 species of *Pseudocercospora* are currently recognised and these infect hosts belonging to 115 plant genera (Crous et al., 2013). *Pseudocercospora* infections are responsible for fruit and leaf spot diseases on commercially important crops, forest trees and ornamentals (Akinsanmi, Miles & Drenth, 2007; Crous et al., 2003, 2013; Darvas & Kotza, 1987; Hunter et al., 2006; Motohashi, Araki, & Nakashima, 2008; Yesuf, 2013; Yuan, 1996). About 20 species of *Pseudocercospora* including *P. fijiensis*, are known to infect bananas (Arzanlou et al., 2008).

The taxonomic placement of *P. fijiensis* is based on characteristic growth in culture as well as morphological and genetic analyses (Churchill, 2011). Two other members of *Pseudocercospora* are also associated with leaf spot disease in banana. Specifically, *P. musae* (Morelet), the causal agent of yellow Sigatoka disease, and *P. eumusae* (Morelet), the causal agent of eumusae leaf spot disease. These species are distinguished on the basis of asexual morphology (Crous & Mourichon, 2002), genome size and virulence on they manipulate the host immune system (Chang et al., 2016) difference. Of these three species *P. fijiensis* is the most virulent (Carlier et al., 2000; Churchill, 2011; Isaza et al., 2016).

2.2.1.3 Disease symptoms

The early signs of *P. fijiensis* infection are small narrow reddish-brown or yellowish-brown specks on the edges of older leaves, typically parallel to leaf veins. At this initial stage the leaf underside may show a more reddish-rust brown lesion (Churchill, 2011; Liberato et al., 2006; Marin et al., 2003). These streaks coalesce forming complex streaks that are dark brown to black in colour. Eventually, broad elliptical spots with “water-soaked” borders and surrounded by a yellow halo are formed (Liberato et al., 2006; Churchill, 2011). Finally, these areas become light greyish and necrotic.

Black Sigatoka disease symptoms have been reported on cultivated bananas and plantains as well as several wild *Musa* species (Churchill, 2011). Studies suggest that symptom expression differs between cultivars and these differences may be explained in terms of disease resistance (Carlier et al., 2000; Harelimana et al., 1997). Banana cultivars that are more vulnerable to the disease have more pronounced symptoms; for example, infection may lead to entire leaf death (Mourichon, Carlier & Fouré, 1997). Disease symptoms also been reported *Heliconia psittacorum*, which is considered an alternative host (Churchill, 2011; Gasparotto et al., 2005).

Although the symptoms of black Sigatoka and yellow Sigatoka allow these species to be differentiated, those of eumusae leaf spot disease overlap with both confounding identifications based on physical symptoms alone (Mourichon, Carlier, & Fouré, 1997). Laboratory testing is usually needed to confirm disease status (Crous et al., 2013).

2.2.1.4 Identification of P. fijiensis in culture

There are few morphological features that distinguish *Pseudocercospora* species (Crous et al., 2013). However, the lack of an obvious sporodochia (or hyphal mass) in *P. fijiensis* as well as characteristics of the conidia and conidiophores differentiate this species from *P. musicola* and *P.eumusae* (Churchill, 2011; Crous & Mourichon, 2002; Pérez-Vicente, 2012). The conidiophores of *P. fijiensis* are pale brown, 16-63 × 4-7 µm in size, straight to geniculate, and 0-5 septate. Conidiophores typically bear four conidia in *P. fijiensis* (Crous et al., 2013; Pérez-Vicente, 2012). These are obclavate to cylindro-obclavate in shape, hyaline to pale-green in colour, 1-10 septate and 30-132 × 3-5 µm in size. No single characteristic of the conidia and conidiophores distinguishes *P. fijiensis*; but in combination they do distinguish this species (Arzanlou et al., 2008; Churchill, 2011). For example, although the conidia of *P. fijiensis* and *P. musicola* overlap in size and number of septa (in *P. musicola* the conidia 0-8 septate and 10-109 × 2-6 µm in size) only *P. fijiensis* has a distinctive basal hilum.

Pseudocercospora fijiensis is slow growing on artificial media and takes about 8-14 days to sporulate (Leiva-Mora et al., 2008; Pérez-Vicente, 2012). Colony morphology differs depending on the growth medium (Stover, 1976). For example, on potato, dextrose agar colonies are compact with raised surfaces and range in colour from velvety pink to olive green and grey (Leiva-Mora et al., 2008; Mulder and Holliday, 1974). In contrast, on mycophyl agar colonies are greyish-brown to dark grey (Stover, 1980).

2.2.1.5 Life cycle of P. fijiensis

Pseudocercospora fijiensis is a hemibiotroph. That is, it initially parasitizes living tissue and later lives on the dead tissue. The disease cycle *of P. fijiensis* has four main stages (Churchill, 2011; Henderson et al., 2006; Pérez-Vicente, 2012) – spore germination, penetration of the host, symptom development and spore production.

Black Sigatoka disease has been reported on bananas and plantains growing under conditions ranging from arid to wet and tropical to cool temperate (Crous et al., 2013). Infection rates for the disease depend upon the prevailing weather conditions and susceptibility of cultivars (Carlier et al., 2000; Churchill, 2011; Henderson et al., 2006; Stover 1980). More susceptible cultivars exhibit disease symptoms more rapidly (Carlier et al., 2000; Churchill, 2011). Typically, symptoms appear 8-10 days following infection (Stover, 1980).

Spore gemination and onset of the disease cycle, is promoted by humid conditions (Henderson et al., 2006; Stover, 1980). The germinating hyphae penetrates the leaf through the stomatal opening and a hyphal mass develops within the air spaces between mesophyll cells (Churchill, 2011; Jones, 2000; Stover 1980). As the pathogen spreads through the leaf interior, disease symptoms become apparent on the leaf surface (Henderson et al., 2006; Leiva-Mora et al., 2008). The formation of necrotic lesions, a symptom of advanced infections, is associated with the production of asexual conidia and sexual ascopores.

Pseudocercospora fijiensis reproduces both asexually and sexually (Figure 2.4); both are important for disease initiation and dispersal (Marín et al. 2003). Asexual reproduction involves the production of conidia under favourable conditions. The conidia are released and are typically dispersed short distances in air currents or rain splashes (Churchill, 2011; Henderson et al., 2006). In contrast, sexual ascopores typically take four weeks to mature (Stover, 1980). Oval to globose spermagonia (23-55 µm in diameter) containing rod-shaped spermatia (2.0-5.0 × 1.5-3 µm in size) develop on the under surface of the leaf. Spermatia are released at maturity. However, it has not yet been shown how spermatia are involved in the production of sexual ascopores (Churchill, 2011). Spherical sexual fruiting bodies or perithecia (47-85 µm in diameter), are found on both the upper and lower leaf surfaces. These contains numerous fisstunicate, obclavate ascii that contain unequally septate ascopores (Pérez-Vicente, 2012). Ascospores are dispersed by air currents or rain splashes (Mulder & Holliday, 1974) and may not germinate immediately; they may remain dormant until weather conditions are favourable (e.g., temperature is 25-28 °C) (Churchill, 2011; Jacome & Schuh, 1992; Stover 1980).

2.2.1.6 Impact of black Sigatoka disease on bananas/plantains

A wide range of *Musa* sp. and cultivars are affected by the disease (Arzanlou, 2008). The widely grown cultivars of bananas and plantains differ in their susceptibility to black Sigatoka disease. For example, trials in Uganda and Tanzania suggest that the AAA and AAAB hybrids are more susceptible to the disease than other trialled groups (Kimunye et al., 2020). Other publications have suggested that the AAB group of plantains (Mobambo,1993) and Cavendish bananas (Fullerton & Casonato, 2019; Isaza et al., 2016; Yonow et al., 2019) are highly susceptibility.

Given the susceptibility of the Cavendish bananas it is not surprising that the disease has substantial impacts on commercial production of dessert bananas (Castelan et al., 2012; Ploetz, 2000). Although black Sigatoka disease primarily targets the leaves, fruit quality and shelf-life are both reduced (Stover & Simmonds, 1987; Carlier et al., 2000; Thompson,

Figure 2.5 The life cycle of *Pseudocercospora fijiensis*, the causal agent of black Sigatoka disease of banana. Source, Churchill (2011).

2011). These effects are a result of infected individuals having reduced photosynthetic capability. Specifically, the surface area available for photosynthesis can be substantially reduced due to the death of large sections of infected leaf (Churchill, 2011; Etebu & Young-Harry, 2011; Thompson, 2011). Reduced production of photosynthates limits overall yield and the development of those fruits that are produced.

The marketable value of commercial banana and plantain crops is adversely affected by any *P. fijiensis* infection (Carlier et al., 2000; Marin et al., 2003). Typically, black Sigatoka disease causes crop losses of 20-50% (Crous & Mourichon, 2002), although the Food and Agriculture Organisation (2013) reported that exports from Guyana were reduced by 100% and from Saint Vincent and the Grenadines by 90%. Black Sigatoka disease also impacts smallholder farmers who rely on bananas and plantains as a food source and a cash crop (Chang et al., 2016; Etebu & Young-Harry, 2011; Lemchi et al., 2005). In regions where bananas and plantains serve as a major food source for the local population this disease may compromise food security (Tinzaara et al., 2018; Mourichon & Fuller, 1990). Due to the high virulence of black Sigatoka disease – *P. fijiensis* can infect cultivars that are resistant to *P. eumusae* and *P. musicola* – it is recognised as a critical threat to the banana industry.

2.2.1.7 Control of black Sigatoka disease

A combination of systemic and protectant fungicides is used to control black Sigatoka disease in commercial plantations (Brahima et al., 2017; Marin et al., 2003). The main systemic fungicides include bencimidazols, morolines, strobirulines and triazoles (Peláez et al., 2006). Disease outbreaks in parts of Australia during the 1980s and 1990s were successfully controlled and these areas are now considered disease free (Henderson et al., 2006; Jones & Alcorn, 1982). Although used commercially chemical control agent are expensive and not generally accessible to smallholder farmers (Tinzaara et al., 2018).

In areas where black Sigatoka disease occurs chemical controls are applied weekly (Isaza et al., 2016). The need for such intensive use of chemical controls is a concern from both the environmental and human health perspective (Király, 1996; Nicolopoulou-Stamati et al., 2016; Wightwick et al., 2010). The repeated use of fungicides over large areas and with very short intervals between applications has the potential to cause environmental damage via impacts on microbiomes (Clark et al., 2019; Pérez-Vicente, 2012) and prolonged exposure to fungicides may also be harmful to farmer workers and the local population more generally (Churchill, 2011). Moreover, although alternating systemic and protectant fungicides has been used to delay the emergence of fungicide resistance (Churchill, 2011), fungicide resistant strains of *P. fijiensis* have begun to appear. Crop failures as a result of fungicide resistant strains of *P. fijiensis* have already been reported (Marin et al., 2003). At best fungicide resistant strains will require more expensive controls and at worst could severely limit production (Brahima et al., 2017; Ganry et al., 2012).

The challenge of controlling black Sigatoka disease has exacerbated secondary problems with the production of bananas and plantains (Thompson, 2011). In Australia, both cultural and chemical control measures were successfully implemented to control the disease (Henderson et al., 2006). Varieties of bananas and plantains tolerant of black Sigatoka disease are available and ongoing research is focused on enhancing tolerance (Curry, 2012).

2.3 Commonly used approaches for the diagnosis of fungal disease in plants

Methods for identifying plant pathogens range from visual inspection of plant material in the field for physical symptoms of disease to laboratory-based approaches for detecting the genetic signatures of individual pathogens (Balodi et al., 2017). Identifying *P. fijiensis* in the field is difficult because the symptoms of black Sigatoka disease are broadly similar to those of *P. eumusae* and *P. musicola*.

2.3.1 Culture-based methods

Culturing of fungal pathogens *in vitro* is a classical approach (Schmit & Lodge, 2005). The method involves isolating and then growing the fungus on an artificial medium; the fungus can then be identified morphologically (Choi, Hyde & Ho, 1999; Nevalainen, Kautto & Te'o, 2014). Usually culuring involves placing infected tissues onto the surface of an artificial growth medium in a petri dish (Choi, Hyde & Ho, 1999; Schmit & Lodge, 2005) but fruiting bodies collected from infected tissue or debris may be used (Carris, Little & Stiles, 2012; Schmit & Lodge, 2005). The petri dishes are then incubated. Often following initial incubation, the growing fungi will be sub-cultured to obtain pure cultures or a single spore (Choi, Hyde & Ho, 1999; Noman et al., 2018). Identification of the isolated fungi is based on visual morphology that is usually viewed with the aid of a microscope.

Despite our reliance on culturing, this approach has significant limitations. Perhaps most importantly not all fungi are amenable to *in vitro* culturing on artificial media and as a result not all plant pathogens can be identified using this approach (Crous, Hawksworth & Wingfield, 2015). Even for fungi that can be cultured the approach may be time consuming and expensive. Isolation and growth of the fungus to the stage at which diagnostic, often reproductive, characteristics appear may take weeks, require specialised equipment, and workers highly skilled in both culturing and identification (Ward et al., 2004). Moreover, even after isolating a fungus it may not be possible to distinguish amongst morphologically and physiologically similar pathogenic and non-pathogenic forms (Al-mohanna, 2016; Macher, 2001). These limitations have help drive a shift towards molecular approaches to fungal pathogen identification (Lievens & Thomma, 2005).

Currently, *Pseudocercospora fijiensis* is often isolated directly from infected leaf samples (Carlier et al., 2000; Peláez Montoya et al., 2006; Stover, 1978). Leaf tissue fragments with visual symptoms of black Sigatoka disease are first surface sterilised in a sodium hypochlorite or ammonia solution, then rinsed with water and plated on to solid media plates; once a hyphal mass or colony is observed the fungus can be sub-cultured in order to produce pure isolates (Cruz-Martín et al., 2011; Peláez Montoya et al., 2006). For identification of *P. fijiensis* initial isolation of infected tissue and sub-culturing can be performed typically on water agar, PDA, modified V8 juice, or mycophyl media (Cruz-Martín et al., 2011; Peláez Montoya et al., 2006; Stover, 1978). *Pseudocercospora fijiensis* cultures are typically incubated at 20-30°C and under these conditions the fungus will usually sporulate after 10-15 days. In culture *P. fijiensis* may produce predominantly conidia or ascospores, or some combination of both (Jacome & Schuh, 1992; Cruz-Martín et al., 2011). Although *P. fijiensis* can be identified morphologically based on conidia and ascospores, the distinguishing characteristics are subtle and in some cases *P. fijiensis* may be confused with *P. eumusae* or *P. musicola* (Crous & Mourichon, 2002).

2.3.2 Molecular methods

Using molecular diagnostics, the accuracy and speed of fungal pathogen identification has increased (Crous, Hawkworth & Wingfield, 2015). Methods including randomly amplified polymorphic DNA (RAPD), flow cytometry (FCM), restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphism (AFLP), serology, western blot assays, enzyme-linked immunosorbent assays (ELISA), DNA barcoding, fluorescent *in situ* hybridisation (FISH), DNA arrays and DNA amplification-based methods have all been used for fungal pathogen identification (e.g., Atkins & Clark, 2004; Aslam et al., 2017; Balodi et al., 2017; Bernreiter, 2017; Crous, Hawkworth & Wingfield, 2015; Fang & Ramasamy, 2015; Luna-Moreno et al., 2019; Sankaran et al., 2010). The following focuses on methods that have been used for diagnosis of *P. fijiensis*.

2.3.2.1 Enzyme-linked immunosorbent assay (ELISA)

The method is based on the reaction between antigen and antibody (Fang & Ramasamy, 2015; Sankaran et al., 2010). The target organism is typically identified on the basis of colorimetry, the colour change being due to the interaction between a liquid sample and immobilized enzymes (Fang & Ramasamy, 2015).

ELISA-based diagnostics are available for viruses, fungi and bacteria (Balodi et al 2017; Fang & Ramasamy, 2015; Hobbs et al., 1987). Although the ELISA method has potential, it typically lacks the specificity needed to differentiate plant fungal pathogens from closely related non-pathogenic forms. This method is now often used alongside methods such as PCR. Examples of diseases for which ELISA tests have been developed included *Botrytis cinera* in pear (Meyer, Spotts & Dewey, 2000), ascochyta blight of pea (Faris-Mokaiesh et al.,1995), smut of barley (Eibel, Wolf & Koch, 2005) and potato late blight (Hussain, Singh & Anwar, 2017). Various ELISA tests have been developed for the detection of *Pseudocercospora fijiensis* (Henderson et al., 2006; Luna-Moreno et al., 2019; Otero et al., 2007).

2.3.2.2 PCR-based diagnostics

DNA diagnostics based on the polymerase chain reaction (PCR) technology have been developed for the identification of bacterial, viral and fungal plant pathogens (Fang & Ramasamy, 2015). PCR-based tests are now a standard tool for the diagnosis of plant diseases (Hensen & French 1993; Mori et al., 2001), even allowing pathogens with similar physical symptoms to be distinguished (Arzanlou et al., 2007).

PCR is an enzymatic amplification technique that uses a pair of specific oligonucleotide primers to target regions of the DNA that are diagnostic for the organism of interest (Atkins & Clark, 2004). Briefly, the sample DNA is first denatured, the primers then anneal to the single stranded DNA at their target site, and the section of DNA between the two primers is copied using a DNA polymerase (Hensen & French, 1993) (Figure 2.5). Two forms of PCR are now widely used for the diagnosis of fungal plant diseases. Conventional PCR is typically used for qualitative diagnostics. That is, the presence or absence of the pathogen is indicated by the presence or absence of amplification products following thermocycling (Lorenz, 2012). Alternatively, real-time PCR can be used to provide both qualitative and quantitative assessments of infection. In this case the accumulation of amplification products is measured over the course of the reaction; by comparison to standard amplification curves the quantity of pathogen present in the sample can be estimated (Atkins & Clark, 2004). In some cases, PCR alone may be insufficient to confirm the identity of the pathogen, in such cases DNA sequencing of the resulting PCR products may be used to confirm the diagnosis (Gyllensten, 1989).

The nuclear ribosomal internal transcripted spacer (nrITS) region is now widely used for the identification of both pathogenic and non-pathogenic fungi (Martin, James & Lévesqu, 2000). In the case of *P. fijiensis*, Carlier et al. (2000) have shown that the nrITS locus can be used to differentiate *Pseudocercospora* species and Henderson et al. (2006) describe conventional and real-time PCR diagnostics targeting the nrITS locus of *P. fijiensis*.

2.3.2.3 Isothermal amplification diagnostics

Although PCR-based diagnostics offer high sensitivity and specificity their use is limited by the need for specialised equipment, infrastructure and staff (Ahmed et al., 2015). In particular, the infield application of PCR-based diagnostics is restricted by the need to cycle the reaction through three temperature steps for specific periods of time. Thermocycling the reaction is something that can typically only be achieved in a laboratory.

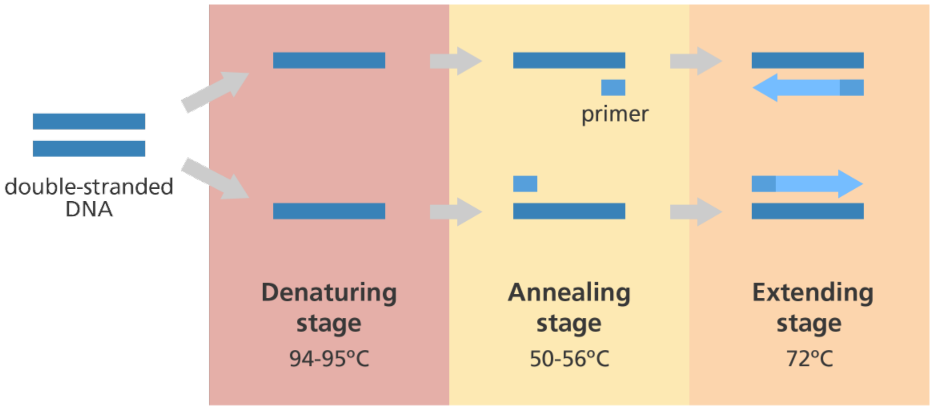

Figure 2.6 Schematic diagram of the polymerase chain reaction method showing the main steps. Source, Genome Research Limited (https://www.yourgenome.org/facts/what-is-pcr-polymerase-chain-reaction).

Alternative isothermal amplification technologies are now available. These approaches amplify DNA without the need for thermocycling, the entire reaction occurs at a constant temperature (Mori & Notomi, 2009). Examples include loop-mediated isothermal amplification (LAMP; Notomi et al., 2000), recombinase polymerase amplification (RPA; Boyle et al., 2013) and helicase-dependent amplification (HAD; Lau & Botella, 2017; Kong, Vincent & Xu, 2007). Isothermal methods are rapid, robust and have lower infrastructure requirements than PCR (Njiru, 2012). Diagnostics based on isothermal amplification are rapidly becoming important tools, especially for point of care applications. Various pathogens have been detected using these isothermal amplification methods. Examples of the isothermal amplification diagnostics performed include; tomato spotted wilt virus disease (Wu et al., 2016,), begomovirus diseases of beans and tomatoes (Londoño, Harmon & Polston, 2016), banana bunchy top virus disease (Kapoor et al., 2017), *Fusarium* head blight of small cereals (Niessen & Vogel, 2010), *Fusarium* wilt (Li et al., 2013), plum pox virus (Zhang et al., 2014) and root-infecting fungal pathogens of turfgrass (Karakkat et al., 2018).

4.0 Loop-mediated isothermal amplification (LAMP) detection methods

4.1 LAMP principle

Loop mediated isothermal amplification (LAMP) was developed two decades ago as a simple and rapid method for disease diagnosis (Fu et al., 2011; Notomi et al., 2000). Unlike traditional PCR-based amplification the LAMP reaction is catalysed by a polymerase with strand displacement capability; that is, a high temperature step is not required to denature the DNA strands before the enzyme can synthesise a new strand. As a result, the LAMP reaction does not need to be thermocycled and is instead performed at a constant temperature usually close to 65˚C, the optimal reaction temperature of the enzyme (Mori et al., 2001; Nagamine, Hase & Notomi, 2002: Njiru, 2012). Also, unlike PCR, the LAMP reactions require two or three primer pairs. These are commonly designed using a dedicated software tool, for example PrimerExplorer V5 (Torres et al., 2011).

A basic LAMP reaction requires four primers. An outer primer pair, commonly referred to as F3 and B3, and a pair of longer inner primers referred to as FIP and BIP. The three main stages of a LAMP reaction are shown in Figure 2.6. The non-cyclic reaction begins with strand annealing and displacement. One of the inner primers invades the target DNA and initiates amplification. Secondly, strand displacement occurs as the polymerase separates the target DNA while extending the inner primer. The initial product is then displaced through synthesis from an outer primer that has annealed upstream of the inner primers binding site. The annealing and displacement cycle are repeated (cyclic reaction) at the opposite end of the target sequence and a "dumbbell" shaped DNA structure is formed. This DNA dumbell forms the starting point for exponential amplification. Amplification is initiated at multiples site on this dumbbell resulting in the rapid accumulation of long, often branched DNA structures composed of many concatenated copies of the DNA target (Mori & Notomi, 2009; Mori et al., 2001; Notomi et al., 2000). The reaction speed can be further increased by the addition of one or more loop primers (Notomi et al., 2000). These latter primers play no role in reaction specificity but can result in increased sensitivity (Fu et al., 2011; Nagamine, Hase & Notomi, 2002).

The LAMP approach has several features that mean it can be carried out in a non-laboratory setting (Keikha, 2018). First, as a result of its strand displacement activity the DNA polymerase that catalyses the LAMP reaction does not require a thermocycler (Notomi et al., 2000). Second, the DNA polymerase used is less sensitive to inhibitors than the *Taq* polymerase used in PCR reactions (Schrader et al., 2012). Finally, LAMP products can be

Figure 2.7. An illustration of the main steps of the loop mediated isothermal amplification process. Source, Bruce et al. (2015).

visualised using colorimetry or turbidity; complex laboratory equipment for evaluating reaction results are not necessary (Njiru, 2012).

4.2 Examples of LAMP

The LAMP approach has rapidly become an important diagnostic tool due to the relative simplicity and speed of reactions (Fu et al., 2011). LAMP diagnostics have been developed for the diagnosis of infectious diseases (Fu et al., 2010; Lucci et al., 2010; Parida et al., 2008; Poon et al., 2005; Seki et al., 2018), for applications in food safety (Kokkinos et al., 2014; Niessen et al., 2013) and for the detection of phytopathogenic organisms including viruses (He et al., 2016; Fukuta et al., 2003; Lai et al., 2018; Li et al., 2010; Nie, 2005), viroids (Boubourakas, Fukuta & Kyriakopoulou, 2009; Park et al., 2013; Tsutsumi et al., 2010), bacteria (Bühlmann et al., 2013; Kubota et al., 2008; Ravindran et al., 2012; Rigano et al., 2010), phytoplasmas (Hodgetts et al., 2011; Tomlinson, Boonham & Dickinson, 2010; Sugawara et al., 2012) and fungi (Duan et al., 2014; Ghosh et al., 2015; Niessen, 2015; Niessen, & Vogel, 2010; Tomlinson, Dickinson & Boonham, 2010).

Chapter 3

Materials and Methods

3.1 Introduction

This chapter describes the various procedures carried out during this study. This includes work carried with the Papua New Guinea National Agriculture Quarantine and Inspection Authority (PNGNAQIA) and at Massey University, New Zealand.

Tissue samples were collected from banana plants displaying symptoms of Black Sigatoka disease at sites in the Central Province of PNG and from Fiji. For the PNG leaf samples fungal isolation and DNA extraction from leaf and mycelium samples was performed at the Kilakila Plant Heath Laboratory (Port Moresby, National Capital District). Leaf tissue samples from Fiji were DNA extracted at the University of the South Pacific.

Work at Massey University involved identification of potential genetic targets from publicly available sequence data, design and optimisation of LAMP primers, as well as LAMP testing and PCR validation of results for symptomatic leaf tissue samples.

3.2 LAMP assay establishment

3.2.1 Identification of potential genetic targets for a LAMP assay

Publicly available DNA sequences for *P. fijiensis* and related taxa were obtained from the NCBI nr database (https://www.ncbi.nlm.nih.gov). Database searches used the key words "*Pseudocercospora fijiensis*", "*Pseudocercospora*" and "Mycosphaerellaceae". Sequences were compared using Geneious V9.1.2 (Kearse et al., 2012) and multiple sequence alignments for potential targets carried out using MUSCLE (Edgar, 2004).

3.2.2 LAMP primer design

PrimerExplorer V5 (http://www.eiken.co.jp/en) was used to design LAMP primers. Initial searches used the *P. fijiensis* sequence and default parameters; conditions were relaxed in subsequent searches until at least one primer set was recovered.

Primer sets were then compared to available *P. fijiensis* sequences and queried against the NCBI nucleotide database (https://www.ncbi.nlm.nih.gov) using BLAST (Altschul et al., 1990) to investigate non-specific annealing.

3.3 LAMP assay optimisation

3.3.1 Template for LAMP assay optimisation

As a template for optimising the LAMP assay, a PCR fragment 1024 nucleotides in length was produced. Amplifications were performed in 20 µl reaction volumes containing 1× EmeraldAmp GT PCR Master Mix (Takara Bio Inc., Kusatsu City, Shiga Prefecture, Japan) and 0.5 µM of each primer (MFssRNAF 5'-GCAATTTGGGGGAATGGAGG-3' and MF ssRNAR 5'-ACAACCTTTTGCCCATATCATG-3'). Thermocycling was performed using a T1 Thermocycler (Biometra GmbH, Göttingen, Germany) and with standard cycling conditions including an initial 3 min denaturation at 94°C, then 35 cycles of denaturation at 94°C for 30 secs, annealing at 56°C for 30 secs and extension at 72°C for 30 secs, with a final 5 min extension at 72°C.

Following thermocycling a15µl aliquot of the PCR reaction as well as an aliquot of 1Kb Plus DNA Ladder (ThermoFisher Scientific) was electrophoresised on 1.5% agarose/TAE gels at 100 v for 45 mins. Following electrophoresis the amplification products were visualised using SYBR Safe DNA Gel Stain (ThermoFisher Scientific) and an Invitrogen SafeImager (ThermoFisher Scientific). Amplification products were using a sterile scapel blade and purified using a Zymoclean™ Gel DNA Recovery Kit (Zymo Rearch) following the manufacturers protocol.

3.3.2 Optimising the LAMP assay reaction conditions

Three reaction conditions were optimised; the ratio of external to internal primers, the amplification temperature, and the amplification time. Reactions were performed in 25µl volumes consisting of 15µl Optigene Isothermal Master Mix (ISO-DR001, http://www.optigene.co.uk), 2 ng of the PCR template and 5-9 µl of primer mix depending on the assay conditions being tested. Negative controls (i.e., no DNA) controls were included with each amplification set.

The ratio of external (i.e., F3/B3) to internal (i.e., FIP/BIP) primer pairs was optimised first. Specifically, ratios of the F3/B3 to FIP/BIP primer pair of 1:4, 1:6 and 1:8 were tested. For these tests, the final concentrations of primers F3 and B3 were always 0.2 µM, with those of the FIP and BIP primers ranging from 0.8 µM to 1.6 µM. The volume of milli-Q added to each tube was adjusted to maintain a 25µl reaction volume. Initial testing was performed at 65˚C for 30 mins followed by an enzyme denaturation step of 5 min at 80˚C. LAMP assays were carried out using a BioRanger LAMP device (Diagenetix, http://diagenetix.com).

Using the optimised primer ratio both amplification temperature and time were evaluated. To evaluate amplification temperature LAMP reactions were performed at 57˚C, 60˚C, 62˚C, and 65˚C. To evaluate amplification time LAMP reactions were performed for 30, 45, and 60 mins.

The LAMP reaction was monitored in real time with the outcome (i.e., amplification positive or negative) and the time to detection recorded for each sample. The outcome was also confirmed using gel electrophoresis. Typically, 3μl aliquots of each LAMP reaction as well as an aliquot of 1Kb Plus DNA Ladder were electrophoresised on 1.5% agarose/TAE gels at 100 v for 45 mins. Following electrophoresis results were visualised using SYBR Safe DNA Gel Stain and a UVI DOC HD6 gel documentation system (UVITEC Cambridge).

3.3.3 Sensitivity of the LAMP assay

The sensitivity of the LAMP assay was tested using a ten-fold dilution series from 1 ng/μl to 1 fg/μl of the PCR fragment. These tests were performed in typical 25μl reaction volumes using the optimised primer ratio, amplification time, and amplification temperature. Positive (i.e., 2 ng of gel cleaned PCR amplified target fragment) and negative controls (i.e., no DNA) controls were included with each amplification set.

The LAMP reaction was monitored in real time with the outcome (i.e., amplification positive or negative) and the time to detection recorded for each sample. The outcome was also confirmed by gel electrophoresis. Typically, 3μl aliquots of each LAMP reaction together as well as 1Kb Plus DNA Ladder were electrophoresised on 1.5% agarose/TAE gels at 100 v for 45 mins. Following electrophoresis results were visualised using SYBR Safe DNA Gel Stain and a UVI DOC HD6.

3.3.4 Specificity of the LAMP assay

LAMP assay specificity was assessed against total DNA extracts from samples obtained from culture collections (i.e., *Pseudocercopora* sp. [ICMP 22731], *Dothistroma pini* [NEB2], and *Strasseria geniculata* [NZFS 1023]) as well as those isolated as part of this thesis (i.e., *Nigrospora sphaerica* and *Fusarium* sp.). Testing was carried out using the optimal primer ratio, amplification temperature and reaction time; 1 μL aliquots of sample DNA (ng) were added to reaction mixes. Positive (i.e., 2 ng of gel cleaned PCR amplified target fragment) and negative controls (i.e., no DNA) controls were included with each amplification set.

The LAMP reaction was monitored in real time with the outcome (i.e., amplification positive or negative) and the time to detection recorded for each sample. The outcome was also confirmed by gel electrophoresis. Typically, 3μl aliquots of each LAMP reaction together as well as 1Kb Plus DNA Ladder were electrophoresised on 1.5% agarose/TAE gels at 100 v for 45 mins. Following electrophoresis results were visualised using SYBR Safe DNA Gel Stain and a UVI DOC HD6.

3.4 Testing infected leaf tissue samples

3.4.1 Sampling for P. fijiensis in PNG

Symptomatic banana plants were sampled from eight sites in two main districts in the Central Province, PNG; specifically, National Capital District (NCD) and Kairuku-Hiri (Table 3.1). Sites were selected on the basis of foliar symptoms consistent with black Sigatoka disease (Fig 3.1). Samples comprised an approximately 225 cm^2 section of leaf tissue displaying the yellow to brown streaks typical of black Sigatoka disease (Fig 3.1). Leaf material was collected from two diseased plants per site, with an additional four samples collected from asymptomatic plants; two each from NCD and Kairuku-Hiri districts.

Foliar samples were excised from leaves using sterilised scissors or blades. The tissue samples were stored in a herbarium press between labeled newspaper sheets for transport to the lab for further processing (Fig 3.1).

3.4.2 Culturing for P. fijiensis

3.4.2.1 Initial culturing of fungi from plant material

The leaf samples were first washed with distilled water and air dried at RT on laboratory benches. Leaf samples were then cut into 16 cm^2 fragments and surface sterilised in 0.5% sodium hypochlorite for 3-5 minutes. The leaf tissue fragments were then rinsed in distilled water for 3-5 minutes and air dried in a laminar flow cabinet. Once dry the leaf samples were cut into 1 cm^2 fragments.

For each collection four tissue fragments were evenly distributed across the surface of a potato dextrose agar (PDA) plate with 100 mg/L streptomycin sulphate to limit bacterial growth. This process was repeated for a replicate set of malt extract agar (MEA) plates, again with 100 mg/L streptomycin. Lids were taped with parafilm and the plates incubated at room temperature (RT). Plates were checked daily for hyphal growth and sporulation.

3.4.2.2 Sub-culturing and isolation of pure cultures

Initial cultures were subcultured after visual observation of fungal sporulation but before bacterial contamination was noted; usually 5-7 days. Two subcultures were established from each initial culture using the hyphal tipping technique (Goh, 1999). For cultures with upright mycelial growth hyphal strands were tipped off from the hyphal mass using a sterilised wire loop and transferred to a nutrient agar (NA) plate with 100 mg/L streptomycin sulphate. A replicate subculture was established on a PDA with streptomycin plate. For cultures with flat mycelial growth, a sterile scalpel blade was used to cut a sliver of agar containing an area of high spore mass that was then transferred to a NA with streptomycin plate. Again, a replicate subculture was established on a PDA with streptomycin plate. Lids were taped with parafilm

Table 3.1. Details of sites where symptomatic foliar samples were collected in PNG

Location name	Districts	GPS Coordinates		Code
		Latitude, Longitude	Elevation	
Pacific Adventist University farm	Port Moresby North-East (NCD)	S09.40980˚, E147.27208˚	43m	PAU
14 mile rice field	Port Moresby North-East (NCD)	S09.39888˚, E147.26508˚	31m	RIC
Barakau	Hiri (Kairuku-Hiri)	S09.57245˚, E147.36768˚	10m	BAR
Gaire	Hiri (Kairuku-Hiri)	S09.66261˚, E147.41838˚	10m	GAI
Bomana Police College	Port Moresby North-East (NCD)	S09.38692˚, E147.24072˚	35m	BOM
Laloki Secondary School	Port Moresby North-East (NCD)	S9.39571˚, E147.30022˚	35m	LAL
Brown River	Hiri (Kairuku-Hiri)	S09.23.787˚, E147.13.775˚	53m	BRO
Sogeri Rouna 2	Koiari (Kairuku-Hiri)	S09.42707˚, E147.38992˚	477m	SOG

and the plates incubated at RT.

After 14 days a pair of pure cultures were estalished from each subculture using the same techniques as above with the exception that NA with streptomycin and water agar with streptomycin plates were used.

3.4.2.3 Characterization of the cultured fungi

After 3-5 days the characteristic morphological features of the fungal isolates were recorded from sub-culture and pure culture plates (e.g., colony colour and mycelium type). The cultures were also examined microscopically. For each culture a small portion of mycelium was scraped onto a microscope slide using a sterile inoculating needle, a drop of distilled water was added, and the sample covered with a cover slip. Prepared slides were examined for characteristic spore morphologies under 10× and 40× magnification.

Petri plates were then taped with parafilm and stored at 2˚C until DNA extraction.

3.5 Genetic testing for *P. fijiensis*

3.5.1 DNA extraction

3.5.1.1 DNA extraction from fungal cultures

Fungal culture plates were first grouped on the basis of morphological similarity and

Figure 3.1 Collection of symptomatic plant material in the field. (A) Inspection of symptoms in a typical banana garden in PNG. (B) Close up of symptomatic leaf. (C) Leaf sections were excised from banana leaves using sterilised scissors or kitchen knife. (D) Leaf midribs were removed and the leaf sections pressed between newspapers in a plant-press.

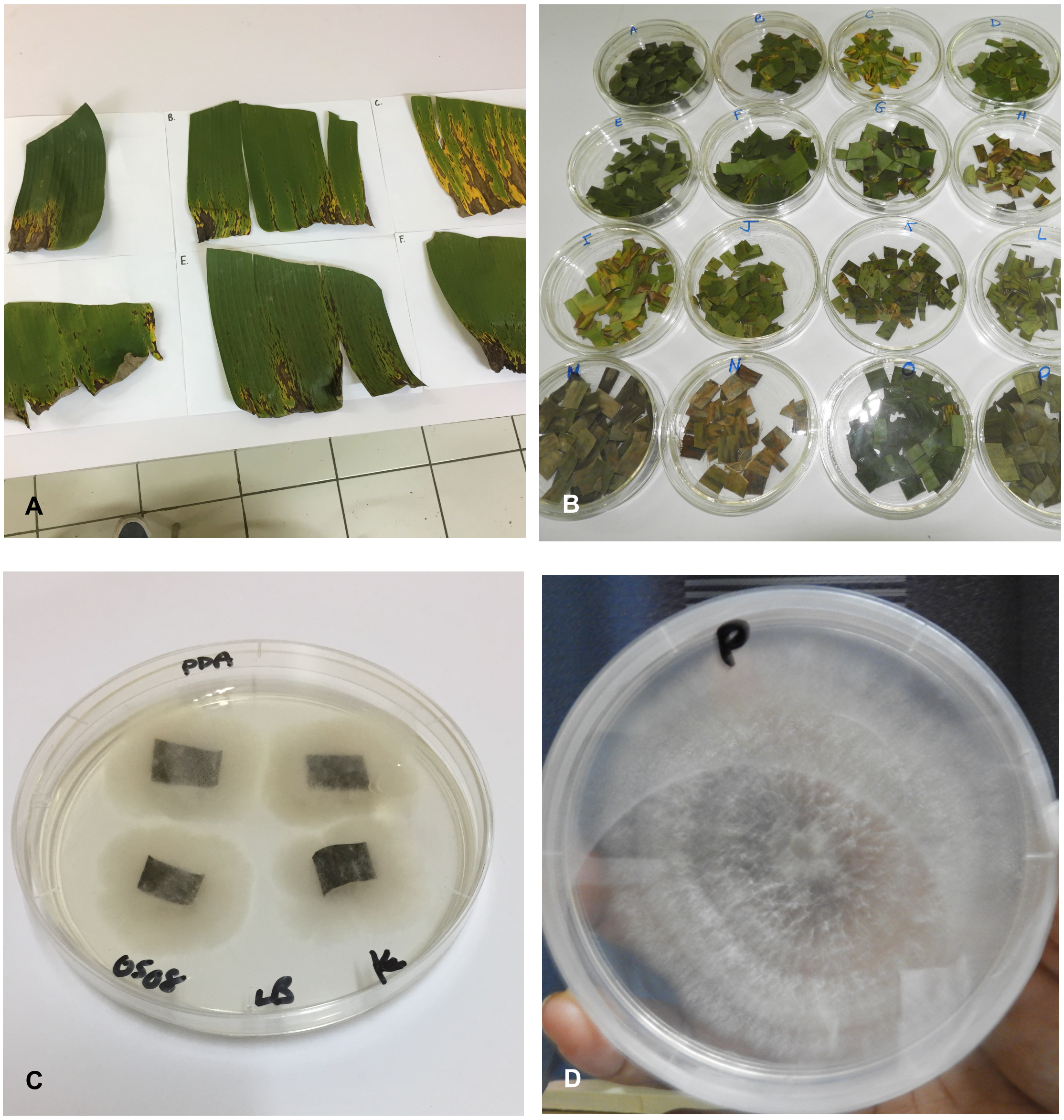

Figure 3.2 Lab processing of symptomatic leaf samples and isolation of fungal cultures. (A) Symptomatic leaf samples sorted on a clean bench top to air dry following washing. (B) Symptomatic leaf samples cut into pieces prior to DNA extraction. (C) Three to four day old after direct isolation of diseased leaf fragments on a PDA agar plate, fungal growth was observed. (D) Five days of mycelium growth on a sub-cultured PDA plate.

a subset of 32 cultures selected for DNA extraction. Approximately 100 mg of mycelium was scraped from the surface of each selected plates. These samples were first rinsed in 96% (v/v) ethanol and air dried on filter paper in a laminar flow cabinet. They were then combined with 300 µl of lysis buffer PA1 and ground using a pestle and mortar. Following the manufacturer's instructions, the BIOLINE Plant DNA Isolate II Kit was then used for the extraction of total genomic DNA.

Following manufacturer's instructions, the concentration of total genomic DNA in each extract was measured using a QuBit 2.0 Fluorometer (Invitrogen Life Technologies) and the Qubit dsDNA HS assay kit (Invitrogen Life Technologies).

3.5.1.2 DNA extraction from infected leaf samples

For leaf tissues, weighed leaf fragments were ground using either a bead mill or mortar and pestle. Following the manufacturer's instructions, the BIOLINE Plant DNA Isolate II Kit was then used for the extraction of total genomic DNA.

Following manufacturer's instructions, the concentration of total genomic DNA in each extract was measured using a QuBit 2.0 Fluorometer (Invitrogen Life Technologies) and the Qubit dsDNA HS assay kit (Invitrogen Life Technologies).

3.5.2 LAMP testing

Total genomic DNA samples from both the infected leaf samples and cultured isolates from PNG were tested for the presence of *P. fijiensis* using the LAMP assay. Additionally, 10 total genomic DNA samples from infected leaf samples collected in Suva, Fiji (GPS Coordinates, S18.23417 E9.63630) were also tested. Testing was carried out using the optimal primer ratio, amplification temperature and amplification time with 5 ng of sample DNA added to reaction mixes. Positive (i.e., 2 ng of gel cleaned PCR amplified target fragment) and negative controls (i.e., no DNA) controls were included with each amplification set.

LAMP reactions were monitored in real time with the outcome (i.e., amplification positive or negative) and the time to detection recorded for each sample. The outcome was also confirmed by gel electrophoresis. Typically, 3µl aliquots of each LAMP reaction together as well as 1Kb Plus DNA Ladder were electrophoresised on 1.5% agarose/TAE gels at 100 v for 45 mins. Following electrophoresis results were visualised using SYBR Safe DNA Gel Stain and a UVI DOC HD6.

3.5.3 PCR-based testing

To confirm the results of LAMP testing DNA samples from infected leaf samples (PNG and Fiji) and cultured isolates were also tested using PCR. For these tests both the nuclear ribosomal internal transcribed spacer (nrITS) locus and mitochondrial region targeted by the LAMP test were evaluated.

Amplifications were performed in 20 μl reaction volumes containing 1× EmeraldAmp GT PCR Master Mix and 0.5 μM of each amplification primer. The ITS4/ITS5 (White et al., 1990) and MF ssRNAF/MF ssRNAR primer pairs were used to amplify the nrITS and mitochondrial region, respectively. Thermocycling was performed using a T1 Thermocycler with standard cycling conditions including an initial 3 min denaturation at 94^{o}C, then 35 cycles of denaturation at 94^{o}C for 30 secs, annealing at 56^{o}C for 30 secs and extension at 72^{o}C for 30 secs, with a final 5 min extension at 72^{o}C.

The results of PCR amplifications were deterined using gel electrophoresis. Typically, 3μl aliquots of each LAMP reaction together as well as 1Kb Plus DNA Ladder were electrophoresised on 1.5% agarose/TAE gels at 100 v for 45 mins. Following electrophoresis results were visualised using SYBR Safe DNA Gel Stain and a UVI DOC HD6.

3.5.4 Sanger DNA sequencing of PCR amplification products

As a further check fragments amplified during PCR-based testing were subject to Sanger DNA sequencing. Prior to DNA sequencing the PCR fragments were purified enzymatically using a combination of shrimp alkaline phosphatase (ThermoFisher Scientific, Waltham, MA) and exonuclease 1 (ThermoFisher Scientific). Reactions were typically performed in a total of 20 μL consisting of 2 μL of the PCR reaction mix, 0.5 U of shrimp alkaline phosphatase, 0.25 U of exonuclease 1. The sample tubes were mixed and then incubated for 15 minutes at 37°C with a further 15 mins at 85°C to denature enzymes.

Purified PCR amplicons were used as template for sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit. Following manufacturer's instructions, the reaction was performed using 10pmol of primers and 5-39 ng/μl of purified PCR products in a 20 μl reaction volume. Reactions were thermocycled using a T1 Thermocycler (Biometra GmbH, Göttingen, Germany); conditions were an initial denaturation at 98°C for 2 mins, then 27 cycles of denaturation at 98°C for 10 secs, 54˚C for 10 secs (primer annealing) and 60°C for 4 mins (extension). Samples were then submitted to the Massey Genome Service for sequencing.

3.5.5 Analysis of sequenced DNA fragments

Sequence data were processed using Geneious V9.1.2 (Kearse et al., 2012). Forward and reverse sequences from each amplification product were aligned and consensus sequences generated for each. To confirm the identity of amplification fragments consensus sequences were queried against the NCBI nucleotide database (https://www.ncbi.nlm.nih.gov) using BLAST (Altschul et al., 1990).

Chapter 4

Results

4.1 Introduction

This chapter reports the development of a LAMP assay for the detection of *P. fijiensis* as well as its validation using field collected samples. A genetic target for the *P. fijiensis* LAMP test was identified from publicly available sequence data, a test was designed, the reaction conditions optimised, and the test validated, including an investigation of assay specificity and sensitivity.

The validated LAMP assay was then used to evaluate mycelial cultures and foliar samples with visual symptoms of disease from PNG as well as foliar samples with disease symptoms from Fiji. Results of the LAMP assay were confirmed using PCR amplification and DNA sequencing of the LAMP assay target and the nrITS region. The LAMP assay and DNA sequencing provided broadly consistent results, both confirming the presence of *P. fijiensis* on samples from PNG and Fiji.

4.2 LAMP assay establishment

Following initial keyword searches of the NCBI nr database a region of the mitochondrial small ribosomal subunit (ssRNA) gene was identified as a potential target for a LAMP assay. A multiple sequence alignment of publicly available DNA sequences for *P. fijiensis* and several related taxa suggested that at the target locus *P. fijiensis* was distinguished on the basis of both length and nucleotide substitutions (Figure 4.1).

Using PrimerExplorer V5 (http://www.eiken.co.jp/en) a single set of four basic LAMP primers was designed (i.e., F3/B3 and FIP/BIP) (Table 4.1; Figure 4.1). Despite relaxing several parameters no loop primers were generated in subsequent searches.

4.3 LAMP assay optimisation

Target copy number can vary between total DNA extractions from different

Table 4.1 Primer sequences for the *P. fijiensis* LAMP assay

Name	Sequence 5′-3′
PFF3	ATAGGATTAGATACCCTAGT
PFB3	GTCTAATGATTTCAGTTCCT
PFFIP	GCTGCGTTTCTAATATGATATTAATTGTCCAGGCAGAAAATTATGAATG
PFBIP	ATATGCTCTTGTTAATTAATGTATATGCCACATTACTCTTGAGG

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                                                 F3                    F2                                           F1
Septoria alnifolia           tggggagcgaataggattagataccctagtagtccaggcagaaaattatgaatgttatagac------------taataaaa--------------------------------------
Septoria alnii               tggggagcgaataggattagataccctagtagtccaggcagaaaattatgaatgttatagac------------taataaaa--------------------------------------
Septoria azaleae             tggggagcgaataggattagataccctagtagtccaggcagaaaattatgaatgttataggc------------taagaaat--------------------------------------
Septoria betulae             tggggagcgaataggattagataccctagtagtccaggcagaaaattatgaatgttatagac------------taataaaa--------------------------------------
Septoria canadensis          tggggagcgaataggattagataccctagtagtccaggcagaaaattatgaatgttatagac------------taagaaaa--------------------------------------
Septoria rubi                tggggagcgaataggattagataccctagtagtccaggcagaaaattatgaatgttatagac------------taagaaaa--------------------------------------
Cercospora sp. Q JZG-2013    tggggagcgaataggattagataccctagtagtccaggcagaaaattatgaatgttatagac------------caagaaaa--------------------------------------
Mycosphaerella sp. PB-2012b  tggggagcgaacaggatttgttaccctagtagtccaggcagaaaattatgaatgttatagac------------taagaaaa--------------------------------------
Mycosphaerella thailandica   tggggagcaaataggattagataccctagtagtccaggcagaaaattatgagtgttatagac------------taagtata--------------------------------------
Microcyclus ulei             tggggagcgaataggattagataccctagtagtccaggcagaaaattatgaatgttatagacta-----------aggagat--------------------------------------
Pseudocercospora assamensis  tggggagcgaataggattagataccctagtagtccaggcagaaaattatgaatgttatagacta-----------agtatat--------------------------------------
Pseudocercospora eumusae     tggggagcgaataggattagataccctagtagtccaggcagaaaattatgaatgttatagacta-----------actatat--------------------------------------
Pseudocercospora indonesiana tggggagcgaataggattagataccctagtagtccaggcagaaaattatgaatgttatagacta-----------agtatat--------------------------------------
Pseudocercospora longispora  tggggagcgaataggattagataccctagtagtccaggcagaaaattatgaatgttatagatta-----------agtatat--------------------------------------
Pseudocercospora mori        tggggagcgaataggattagataccctagtagtccaggcagaaaattatgaatgttatagacta-----------agtatat--------------------------------------
Pseudocercospora musae       tggggagcgaataggattagataccctagtagtccaggcagaaaattatgaatgttatagatta-----------agtatat--------------------------------------
Pseudocercospora fijiensis   tggggagcgaataggattagataccctagtagtccaggcagaaaattatgaatgttatatattaaatgcttttataataatatcatattagaaacgcagctattatttagtattgtagtt
Pseudocercospora fijiensis   tggggagcgaataggattagataccctagtagtccaggcagaaaattatgaatgttatatattaaatgcttttataataatatcatattagaaacgcagctattatttagtattgtagtt
Pseudocercospora fijiensis   tggggagcgaataggattagataccctagtagtccaggcagaaaattatgaatgttatatattaaatgcttttataataatatcatattagaaacgcagctattatttagtattgtagtt

                                                               B1                                         B2                     B3
Septoria alnifolia           -----------------------------------aattggtgtataaatgaaagtgtaagcattccacctcaagagtaatgtggcaacacaggaactgaaatcat??????????????
Septoria alnii               -----------------------------------aattggtgtataaatgaaagtgtaagcattccacctcaagagtaatgtggcaacacaggaactgaaatcat??????????????
Septoria azaleae             -----------------------------------aattggtgtataaatgaaagtgtaagcattccacctcaagagtaatgtggcaacacaggaactgaaatcat??????????????
Septoria betulae             -----------------------------------aattggtgtataaatgaaagtgtaagcattccacctcaagagtaatgtggcaacacaggaactgaaatcat??????????????
Septoria canadensis          -----------------------------------aattggtgtataaatgaaagtgtaagcattccacctcaagagtaatgtggcaacacaggaactgaaatcat??????????????
Septoria rubi                -----------------------------------aattggtgtataaatgaaagtgtaagcattccacctcaagagtaatgtggcaacacaggaactgaaatcat??????????????
Cercospora sp. Q JZG-2013    -----------------------------------aattggtgtataaatgaaagtgtaagcattccacctcaagagtaatgtggcaacgcaggaactgaaatcattagaccgtttgtga
Mycosphaerella sp. PB-2012b  -----------------------------------aattggtgtataaatgaaagtgtaagcattccacctcaagagtaatgtggcaacgcaggaactgaaatcattagaccgtttgtga
Mycosphaerella thailandica   -----------------------------------aattggtgtataaatgaaagtgtaagcattccacctcaagagtaatgcggcaacgcaggaactgaaatcattagaccgtttgtga
Microcyclus ulei             -----------------------------------aattagtgtataaattaaagtgtaagcattccacctcaagagtaatgtggcaacgcaggaactgaaatcattagaccgtttgtga
Pseudocercospora assamensis  -----------------------------------aattagtgtataaatgaaagtgtaagcattccacctcaagagtaatgtggcaacgcaggaactgaaatcattagaccgtttgtga
Pseudocercospora eumusae     -----------------------------------aattagtgtataaatgaaagtgtaagcattccacctcaagagtaatgtggcaacgcaggaactgaaatcattagaccgtttgtga
Pseudocercospora indonesiana -----------------------------------aattagtgtataaatgaaagtgtaagcattccacctcaagagtaatgtggcaacgcaggaactgaaatcattagaccgtttgtga
Pseudocercospora longispora  -----------------------------------aattagtgtataaatgaaagtgtaagcattccacctcaagagtaatgtggcaacgcaggaactgaaatcattagaccgtttgtga
Pseudocercospora mori        -----------------------------------aattagtgtataaatgaaagtgtaagcattccacctcaagagtaatgtggcaacgcaggaactgaaatcattagaccgtttgtga
Pseudocercospora musae       -----------------------------------aattagtgtataaatgaaagtgtaagcattccacctcaagagtaatgtggcaacgcaggaactgaaatcattagaccgtttgtga
Pseudocercospora fijiensis   attaaaactatataataatataatatgctcttgttaattaatgtataaatgaaagtgtaagcattccacctcaagagtaatgtggcaacgcaggaactgaaatcattagaccgtttgtga
Pseudocercospora fijiensis   attaaaactatataataatataatatgctcttgttaattaatgtataaatgaaagtgtaagcattccacctcaagagtaatgtggcaacgcaggaactgaaatcattagaccgtttgtga
Pseudocercospora fijiensis   attaaaactatataataatataatatgctcttgttaattaatgtataaatgaaagtgtaagcattccacctcaagagtaatgtggcaacgcaggaactgaaatcattagaccgtttgtga
```

Figure 4.1 Multiple sequence alignment for representative *Pseudocercospora* and related genera for a section of the mitochondrial genome containing the *P. fijiensis* LAMP assay target. (Previous page). LAMP primer binding sites are indicated by grey outlines; F3 and B3 are binding sites for the external primer pair (i.e., PFF3 and PFB3) with F2/F1 and B2/B1 form the binding sites for the internal primers (i.e., PFFIP and PFBIP, respectively).

sources and this variation may confound optimisation of the LAMP assay. To eliminate the effect of such variation on assay optimisation, a 1024 nucleotide long PCR product was amplified and used as an amplification template. This approach allows target copy number to be standardised between tests. In preliminary tests it was found that using 2 ng of PCR products purified using the Zymoclean™ Gel DNA Recovery Kit protocol amplified more efficiently than the same quantity of products purified using the SAP/EXO enzymatic method. For all subsequent evaluations gel purified PCR products were used as a template.

Using 2ng of the gel cleaned PCR product as template detection times were similar for all primer ratios (i.e., 1:4, 1:6 and 1:8), amplification temperatures (57˚C, 60˚C, 62˚C, 65˚C) and amplification time (30-60 minutes). Optimised reaction conditions must strike a balance between speed, sensitivity and reliability. For the *P. fijiensis* LAMP assay the optimal conditions were a 1:8 primer ratio with an amplification temperature of 60˚C and an amplification time of 60 minutes. All subsequent testing was performed using these conditions.

4.4 LAMP specificity test

Using the optimised reaction conditions, specificity testing was conducted using a leaf sample assumed to be infected with *P. fijiensis*, gel cleaned PF1 PCR product, and DNA from another *Pseudocercospora* species and several relatives. Real time fluorescent detection indicated amplification only occurred for Fijian sample PF6 and the positive control; there was no amplification for the remaining samples (Figure 4.2). This result was confirmed by gel electrophoresis (Figure 4.3).

4.5 LAMP sensitivity test

Using the optimised reaction conditions, specificity testing was conducted using a ten-fold dilution series with template quantities ranging from 1 ng to 1pg of gel cleaned PF1 PCR product. For both the real time (Figure 4.4) and endpoint (Figure 4.5) detection of DNA amplification the minimum amount of DNA template detected was 1 pg.

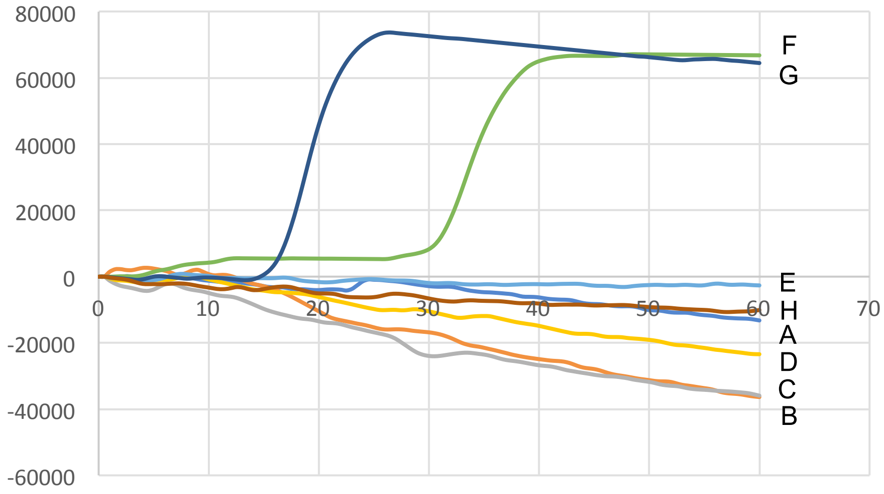

Figure 4.2 Real time visualisation of specificity testing for the *P. fijiensis* LAMP assay. A, 5 ng total DNA *Strasseria geniculata* (NZFS 1023); B, 5 ng total DNA *Dothistroma pini* (NEB2); C, 5 ng total DNA *Fusarium* sp.; D, 5 ng total DNA *Nigrospora* sp.; E, 5 ng total DNA *Pseudocercospora* sp. (ICMP 22731); F., 5 ng total DNA from symptomatic leaf tissue sample Fiji PF6; G, 2 ng gel purified PCR products from Fiji PF1; H, no DNA control.

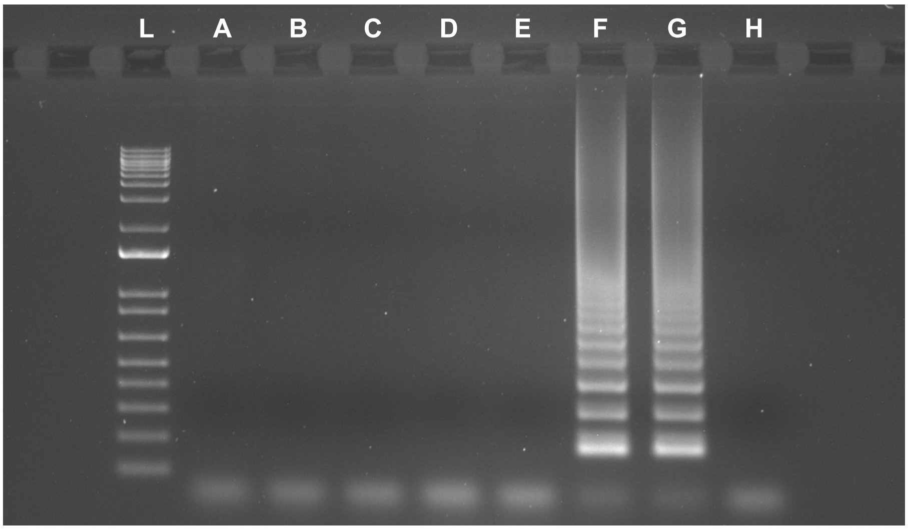

Figure 4.3 Endpoint visualisation of specificity testing for the *P. fijiensis* LAMP assay using SYBR Safe following electrophoresis on a 1% TAE agarose. L, 1 kb plus ladder; A, 5 ng total DNA *Strasseria geniculata* (NZFS 1023); B, 5 ng total DNA *Dothistroma pini* (NEB2); C, 5 ng total DNA *Fusarium* sp.; D, 5 ng total DNA *Nigrospora* sp.; E, 5 ng total DNA *Pseudocercospora* sp. (ICMP 22731); F, 5 ng total DNA from symptomatic leaf tissue sample Fiji PF6; G, 2 ng gel purified PCR products from PF1; H, no DNA control.

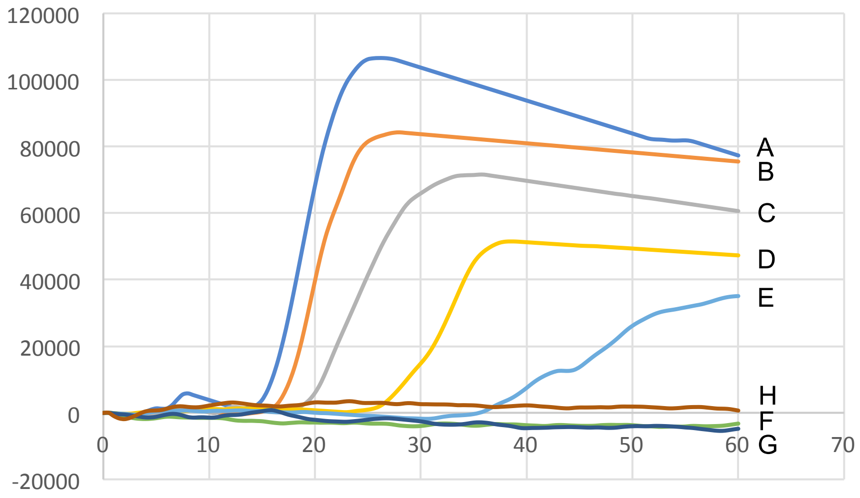

Figure 4.4 Real time visualisation of sensitivity testing for the *P. fijiensis* LAMP assay. L, 1 kb plus ladder; A, 2 ng gel purified PCR products from PF1; B, 1 ng gel purified PCR products from PF1; C, 100 pg gel purified PCR products from PF1; D, 10 pg gel purified PCR products from PF1; E, 1 pg gel purified PCR products from PF1; F, 100 fg gel purified PCR products from PF1; G, 10 fg gel purified PCR products from PF1; H, no DNA control.

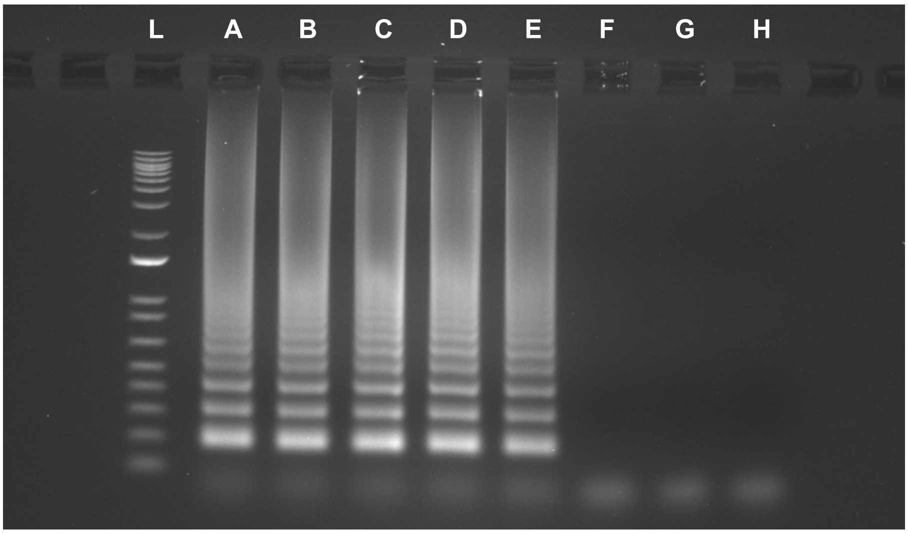

Figure 4.5 Endpoint visualisation of sensitivity testing for the *P. fijiensis* LAMP assay using SYBR Safe following electrophoresis on a 1% TAE agarose. L, 1 kb plus ladder; A, 2 ng gel purified PCR products from PF1; B, 1 ng gel purified PCR products from PF1; C, 100 pg gel purified PCR products from PF1; D, 10 pg gel purified PCR products from PF1; E, 1 pg gel purified PCR products from PF1; F, 100 fg gel purified PCR products from PF1; G, 10 fg gel purified PCR products from PF1; H no DNA control.

Table 4.2 Detection times from sensitivity analysis of the *P. fijiensis* LAMP assay

Quantity of target	Amplification detected	Detection time (mins)
2 ng (positive control)	Yes	19.0
1 ng	Yes	20.5
100 pg	Yes	25.0
10 pg	Yes	33.5
1 pg	Yes	50.0
100 fg	No	–
10 fg	No	–

The real time detection of DNA amplification suggested that detection times were longer for lower template concentrations (Table 4.2).

4.6 Sampling for *P. fijiensis* in PNG

4.6.1 Culturing for P. fijiensis

On initial culture plates of symptomatic leaf fragments fungal growth was observed after 3-5 days on both PDA and MEA plates. For subcultures on PDA and NA plates fungal growth was observed after 3-5 days. Based on the observation of sporulation, mycelial growth and development occurred faster on PDA than either MEA or NA. No fungal growth was observed on initial culture plates of asymptomatic leaf fragments.

Pure isolates from each of the eight PNG sampling locations were characterised as belonging to one of four morphological groups based on colony colour (e.g., white or pink) and growth characteristics (e.g., form of colony margin). These morphological groups are referred to as types I, II, II and IV. Colony types recovered at each of the PNG sampling locations are reported in Table 4.3.

4.6.2 DNA extractions

DNA was extracted from 20-100 mg of infected leaf or mycelium tissue. Extractions from cultured mycelium recovered 0.06-0.3 ng/µl total DNA whereas 0.08-0.8 ng/µl total DNA was recovered from infected leaf tissues.

4.6.3 Identification of PNG cultured isolates

The nrITS region was PCR amplified and DNA sequenced for each of the 14 available PNG mycelial samples. For samples SOG1 and SOG2 PCR fragments of two different sizes were recovered; sequencing of the products consistent in size with those from the remaining samples recovered usable sequence for only SOG2. Subsequent BLAST searches of the NCBI nucleotide database using the sequences recovered from 13 of the

Table 4.3 Morphological descriptions of fungal colonies isolated from PNG symptomatic leaf tissue samples

Sample codes	Colony description				Type
	Colour	Form	Elevation	Margin	
PAU1/PAU2	Whitish to light grey	Filamentous	Raised	Entire	Type I
RIC1/RIC2	Whitish to light grey	Filamentous	Raised	Entire	Type I
BAR1	White	Irregular	Flat	Entire	Type II
GAI1/GAR2	Pinkish-white	Filamentous	Raised	Entire	Type IV
BOM1/BOM2	Whitish to light grey	Filamentous	Raised	Entire	Type I
LAL2	Pinkish-white	Filamentous	Raised	Entire	Type IV
BRO1/BRO2	Whitish to light grey	Filamentous	Raised	Entire	Type I
SOG1/SOG2	White	Filamentous	Raised	Curled	Type III

cultured isolates identified hits with high coverage (e.g., 100 %) and identity scores (e.g., 99-100%) for each. None of the sequences from cultured isolates from PNG had high similarity to publicly available *P. fijiensis* nrITS sequences. Instead the sequences showed greater similarity to species of *Nigrospora* or *Fusarium* (Table 4.4).

4.7 Genetic testing for *P. fijiensis*

4.7.1 LAMP testing

Using the LAMP assay with optimised reaction conditions *P. fijiensis* was detected from symptomatic leaf tissue samples collected from PNG and Fiji as well as cultured fungal isolates from PNG. In all cases real time and endpoint visualisation of the LAMP assay provided consistent results (Figures 4.6, 4.7) and for symptomatic leaf tissue extracts detection times were consistently 30-55 minutes (Table 4.5). Although detected from both sample types detections were more common from the symptomatic leaf tissues – 14/16 (i.e., 87.5%) PNG and 9/10 (i.e., 90%) Fijian leaf samples (Table 4.6; Figure 4.6, 4.7) but only 7/14 (i.e., 50%) of the cultured fungal isolates (Table 4.7).

4.7.2 PCR testing

Results of confirmatory PCR testing of infected leaf tissue collected in both PNG and Fiji as well as cultured fungal isolates from PNG were broadly consistent with those of the *P. fijiensis* LAMP assay. Specifically, for symptomatic leaf tissue samples PCR amplification products were recovered for the same 14/16 PNG and 9/10 Fijian samples as were indicated to contain *P. fijiensis* by the LAMP assay (Table 4.6). In contrast, PCR amplification products were recovered for only 5/14 (i.e., 36%) of cultured fungal isolates (Table 4.7); this

Table 4.4 Results of BLAST queries against the NCBI nucleotide database using nrITS sequences recovered from PNG cultured isolates

Sample	Taxa matched	Results of comparison	Genbank accessions
PAU1	*Nigrospora sphaerica*	100% coverage, 100% identity	MK482388, MH793571, MH028054, MH645137, MH619724, HQ608063
PAU2	*N. sphaerica*	100, 100	MK482388, MH793571, MH028054, MH645137, MH619724, HQ608063
RIC1	*Fusarium equiseti, F. incarnatum, F. cf. incarnatum, F. sacchari*	100, 100	MH729012, MK990153, MK680159, MH879584, MH879250, MH857681, MH521295, LS479416, LS479415, MG655148, MH581383, MH578583, MH290470-MH290472, KR364598-KR364600, KP453980, KR047083, KM921664, KJ572780, EU595566, AY633745
RIC2	*N. sphaerica*	100, 100	MK482388, MH793571, MH028054, MH645137, MH619724, HQ608063
BAR1	*F. incarnatum, F. equiseti, F. oxysporum, F. sambucinam*	100, 100	MK336548, MN882828, MN559437, MN227262, MK990139, MH979697, MK733980, MK530522, MK163439, MH879583, MH866000, MH865892, MH854778, LS479410-LS479414, MK334366, MH581300, MH574897, MH567074, MF044047, KY508359, KY523100, KX588103, HQ332532
GAI1	*F. equiseti, F. incarnatum, F. cf. incarnatum, F. sacchari*	100, 100	MH729012, MK990153, MK680159, MH879584, MH879250, MH857681, MH521295, LS479416, LS479415, MG655148,

			MH581383, MH578583, MH290470-MH290472, KX845382, KY963137, KR364600, KR364599, KR364598, KP453980, KR047083, KM921664, KJ572780, EU595566, AY633745
GAI2	*F. equiseti,* *F. incarnatum,* *F. cf. incarnatum,* *F. sacchari*	100, 100	MH729012, MK990153, MK680159, MH879584, MH879250, MH857681, MH521295, LS479416, LS479415, MG655148, MH581383, MH578583, MH290470-MH290472, KX845382, KY963137, KR364598-KR364600, KP453980, KR047083, KM921664, KJ572780, EU595566, AY633745
BOM1	*F. incarnatum,* *F. equiseti,* *F. oxysporum,* *F. sambucinam*	100, 100	MK336548, MN882828, MN559437, MN227262, MK990139, MH979697, MK733980, MK530522, MK163439, MH879583, MH866000, MH865892, MH854778, LS479410-LS479414, MK334366, MH581300, MH574897, MH567074, MF044047, KY508359, KY523100, KX588103, HQ332532
BOM2	*N. sphaerica,* *N. osmanthi,* *N. oryzae*	100, 99.24	MK482388, MH793571, MH028054, MH645207, MH645137, MH619724, HQ608063, HQ608062
LAL2	*N. sphaerica*	100, 100	MK333938, MH854878, MG832537, MG832493, MG832486, MG832473,

			MG832454, MF434826, MF186859-MF186868, MF135664-MF135666, MG603658-MG603662, KX986004, KX985989, KX256179, KY883352, KX866880, KP731976, KT966515, HQ608030, FJ478134
BRO1	*F. equiseti, F. incarnatum, F. cf. incarnatum, F. sacchari*	100, 100	MH729012, MK990153, MK680159, MH879584, MH879250, MH857681, MH521295, LS479416, LS479415, MG655148, MH581383, MH578583, MH290470-MH290472, KX845382, KY963137, KR364598-KR364600, KP453980, KR047083, KM921664, KJ572780, EU595566, AY633745
BRO2	*N. sphaerica, N. osmanthi, N. oryzae*	100, 99.81	MK333946, MK333924, MH087476, MH087475, MK311280, MH619723, MH571758, MG832520, MG832511, MG832464, MG832461, MG832443, KM921666, MK131325
SOG2	*N. sphaerica*	100, 100	MK482388, MH793571, MH028054, MH645137, MH619724, HQ608063

pathogen had been detected using the LAMP assay for all five of these samples.

BLAST searches of the NCBI nucleotide database using the DNA sequence for these 23 PCR fragments identified each as sharing 100% identity with the corresponding region of the publicly available *P. fijiensis* mitochondrial genome (NC_044132). For the cultured fungal isolates PCR fragments were recovered for 5/14 samples. All five of these samples had also tested positive using the LAMP test and BLAST searches again indicated that the PCR

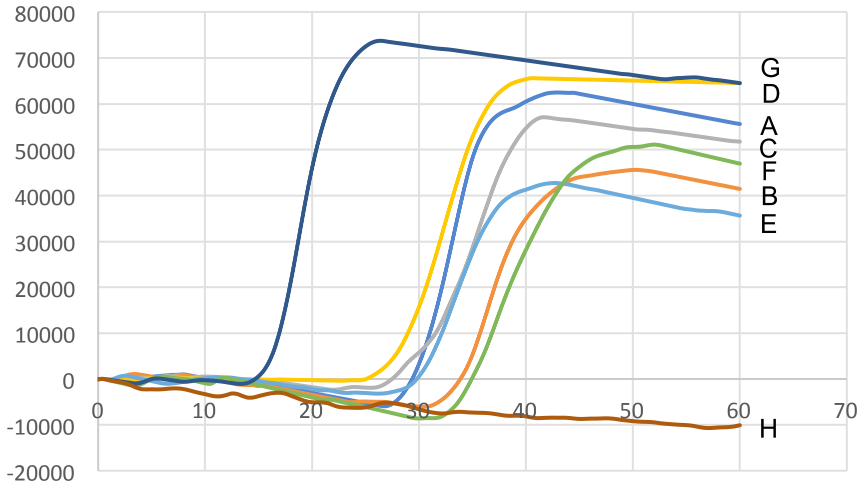

Figure 4.6 Real time visualisation of LAMP testing for *P. fijiensis*. L, 1 kb plus ladder; A, 5 ng total DNA from PAU1; B, 5 ng total DNA from RIC1; C, 5 ng total DNA from SOG2; D, 5 ng total DNA from PF5; E, 5 ng total DNA from PF6; F, 5 ng total DNA from PF9; G, 2 ng gel purified PCR products from PF1; H, no DNA control.

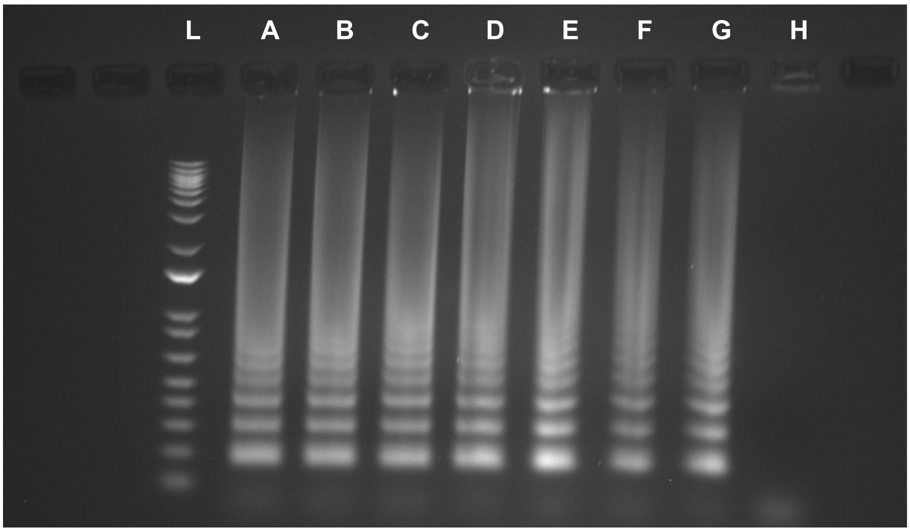

Figure 4.7 Endpoint visualisation of LAMP testing for *P. fijiensis* using SYBR Safe following electrophoresis on a 1% TAE agarose. L, 1 kb plus ladder; A, 5 ng total DNA from PAU1; B, 5 ng total DNA from RIC1; C, 5 ng total DNA from SOG2; D, 5 ng total DNA from PF5; E, 5 ng total DNA from PF6; F, 5 ng total DNA from PF9; G, 2 ng gel purified PCR products from PF1; H, no DNA control.

Table 4.5 *Pseudocercospora fijiensis* LAMP assay detection times for DNA extracted from symptomatic leaf tissue

Sample	Amplification detected	Detection time (mins)
PNG	Yes	33
PAU1	Yes	33
RIC1	Yes	36.5
SOG1	Yes	33.5
Fiji		
PF5	Yes	37.5
PF6	Yes	36
PF9	Yes	33
Positive control	Yes	19.5
Negative control	No	–

Table 4.6 Comparison of results from LAMP- and PCR-based testing for *P. fijiensis* from infected leaf tissue

Sample	LAMP assay	PCR test	
	Amplification	Amplification	Sequence-based identification
PNG			
PAU1	Yes	Yes	*P. fijiensis*
PAU2	Yes	Yes	*P. fijiensis*
RIC1	Yes	Yes	*P. fijiensis*
RIC2	Yes	Yes	*P. fijiensis*
BAR1	Yes	Yes	*P. fijiensis*
BAR2	Yes	Yes	*P. fijiensis*
GAI1	No	No	–
GAI2	Yes	Yes	*P. fijiensis*
BOM1	Yes	Yes	*P. fijiensis*
BOM2	Yes	Yes	*P. fijiensis*
LAL1	Yes	Yes	*P. fijiensis*
LAL2	Yes	Yes	*P. fijiensis*
BRO1	No	No	–
BRO2	Yes	Yes	*P. fijiensis*
SOG1	Yes	Yes	*P. fijiensis*
SOG2	Yes	Yes	*P. fijiensis*
Fiji			
PF1	Yes	Yes	*P. fijiensis*
PF2	Yes	Yes	*P. fijiensis*
PF3	Yes	Yes	*P. fijiensis*

PF4	Yes	Yes	*P. fijiensis*
PF5	Yes	Yes	*P. fijiensis*
PF6	Yes	Yes	*P. fijiensis*
PF7	No	No	–
PF8	Yes	Yes	*P. fijiensis*
PF9	Yes	Yes	*P. fijiensis*
PF10	Yes	Yes	*P. fijiensis*

Table 4.7 Comparison of results from LAMP- and PCR-based testing for *P. fijiensis* from cultured fungal isolates

Sample	LAMP assay	PCR test	
	Amplification	Amplification	Sequence-based identification
PNG			
PAU1	Yes	Yes	*P. fijiensis*
PAU2	Yes	No	–
RIC1	No	No	–
RIC2	Yes	Yes	*P. fijiensis*
BAR1	No	No	–
GAI1	No	No	–
GAI2	Yes	No	–
BOM1	Yes	Yes	*P. fijiensis*
BOM2	No	No	–
LAL2	No	No	–
BRO1	No	No	–
BRO2	No	No	–
SOG1	Yes	Yes	*P. fijiensis*
SOG2	Yes	Yes	*P. fijiensis*

fragments shared identity with *P. fijiensis*. Samples B2 and H2 had tested positive using the LAMP assay but no PCR fragment was recovered (Table 4.7).

Chapter 5

Discussion

5.1 Introduction

This chapter discusses the development and implementation of a specific and sensitive loop mediated isothermal amplification test for *P. fijiensis*. Leaf tissue samples from PNG and Fiji with visual symptoms consistent with Black Sigatoka disease were tested using the assay developed. These tests indicated the presence of *P. fijiensis* in most of the field collected samples, with all positive results verified using a PCR test. In contrast the pathogen was identified with lower frequency from fungal isolates cultured from sections of the same PNG leaf tissue samples.

5.2 The LAMP assay

The *P. fijiensis* LAMP assay developed in this thesis targeted a region of the mitochondrial small ribosomal subunit (ssRNA) gene. Designed using the PrimerExplorer V5 the assay consisted of four primers – a forward outer primer (F3), backward outer primer (B3), forward inner primer (FIP) and backward inner primer (BIP) (Table 4.1). Using PrimerExplorer V5 loop primers could not be designed, likely because the distance between the F1/F2 and B1/B2 regions where loop primers would otherwise bind were short (i.e., 21 base pairs each) and GC content low (i.e., 21.4%) (Figure 4.1).

Optimisation of LAMP reaction conditions indicated that a 1:8 ratio of the outer to inner primer pairs with an amplification temperature of 60 ˚C was optimal for this assay. Based on sensitivity testing and preliminary results from direct testing of field collected tissue samples an amplification time of 60 minutes was consistently used. Using these conditions and a PCR amplification product as a template, the detection limit was 1 pg. Given Avogadro's number (i.e., 6.022 × 1023 molecules/mole), the predicted length of the amplification product (i.e., 1024 nucleotides), and average weight of a base pair (i.e., 650 Daltons) this limit corresponds to 9.1×10^6 copies of the target. For *Saccharomyces cerevisae* the number of mitochondrial genome copies per cell is 20-200 (Williamson, 2002). Although mitochondrial genome counts are likely to differ between taxa or life stages this count implies perhaps 45000 cells would be needed in order to detect *P. fijiensis*. Sensitivity differs between LAMP assays, however this count seems relatively high. In contrast, a recently reported LAMP assay for *Phytophthora agathidicida* detected as few as 116 copies of the assay target in 20-25 minutes (Winkworth et al., 2020). A key difference between this assay and the *P. fijiensis* LAMP assay presented here is the inclusion of loop primers. In

LAMP assays the loop primers do not contribute to reaction specificity, instead providing additional sites for primer binding and thereby increasing the efficiency of the LAMP reaction (Gadkar et al., 2018; Nagamine, Hase, Notomi, 2002; Rohatensky et al., 2018). The lack of loop primers in this case likely limits the sensitivity of the *P. fijiensis* LAMP assay.

Specificity testing used optimized reaction conditions as well as several other Ascomycotina (e.g., *Mycosphaerella pini* and *Pseudocercospora* sp.) and fungal pathogens of *Musa* species (e.g., *Nigrospora* sp. and *Fusarium* sp.). No amplification was observed using real-time or endpoint approaches for these non-target species. Clearly a larger set of taxa need to be examined in order to evaluate the potential for assay cross reactivity. That said, sequence comparisons using publicly available data suggest that primer binding sites for the LAMP assay are disrupted by length variations in even closely related *Pseudocercospora* species (e.g., *P. eumusae*, *P. musicola*). Specifically, the innermost portions of both the FIP and BIP primers have been partially or completely lost (i.e., sites F1 and B1 in Figure 4.1) along with the “stem” of the LAMP product. The loss of this portion of the sequence is likely to reflect differences in the conformation of the folded ssRNA molecule between *P. fijiensis* and closely related species. Given that size differences appear to differentiate the *P. fijiensis* target sequence from the corresponding region in even closely related taxa this increases the chances that the assay is specific to *P. fijiensis.*

5.3 Implementation of the LAMP test

Once the reaction conditions were optimised the *P. fijiensis* LAMP assay was used to evaluate samples collected from banana plantations in Fiji and PNG. Samples included DNA extracted from infected leaf tissue samples and DNA extracted from cultured fungal isolates.

Testing of infected leaf tissue samples using the *P. fijiensis* LAMP assay indicated the presence of *P. fijiensis* in all but two PNG samples (i.e., 87.5% positive) and all but one Fiji sample (i.e., 90% positive). In the case of the PNG leaf tissue samples, at least one sample from each site tested positive for *P. fijiensis*. A standard PCR test using primers that target the same region of the ssRNA as the LAMP assay was used to verify the results of the LAMP assay. Results of this test were consistent with those of the LAMP assay; *P. fijiensis* was again undetected from same two PNG and one Fiji sample. For each sample in which a PCR amplified fragment was recovered, BLAST searches of the NCBI nr database using the DNA sequence of the fragment recovered a *P. fijiensis* ssRNA sequence with 100% identity. These results strongly suggest that although the sensitivity of the *P. fijiensis* LAMP assay described here is lower than several other LAMP assays (e.g., Winkworth et al., 2020) the detection threshold is below that typically encountered when testing symptomatic plant material.

Traditional methods were used to isolate a pair of fungal cultures from each of the same symptomatic leaf tissue samples as used for direct testing. Standard PCR and sequencing of the nrITS region together with BLAST searches of the NCBI nr database were then used to identify each of these fungal isolates. Based on sequence similarity these isolates were identified as either *Fusarium* sp. or *Nigrospora* sp.; where BLAST searches identified both isolates from a single site they were often suggested to represent different genera despite growth characteristics suggesting no difference between them. For example, all the isolates from BOM, BRO and RIC were identified Type 1 (Table 4.3) although for each site one isolate was identified as *Fusarium* sp. and the other *Nigrospora* sp. based on sequence similarity. For seven isoaltes BLAST searches using the recovered nrITS sequence sequence matched representatives of *Nigrospora* sp. In five cases the sequence was uniquely matched to sequences from *Nigrospora sphaerica*, a pathogen that causes a red discolouration in the centre of the banana fruit; for the remaining two isolates this species was among the possible matches (Table 4.4). Six isolates were identified as *Fusarium* sp. For each of these isolates there were four possible matches and for at least two, *F. oxysporum*, the causal agent of Panama disease was among those species identified (Table 4.4).

Although this initial testing of the cultured isolates did not indicate that *P. fijiensis* had been recovered, subsequent testing of cultured isolates using the LAMP assay suggested the presence of this pathogen in 50% of the samples. At face value the results of nrITS sequencing and LAMP testing appear to be inconsistent. However, presence of the pathogen was also suggested by PCR amplification using *P. fijiensis*-specific primers; BLAST searches of the NCBI nr database using the DNA sequence of these fragments recovered a *P. fijiensis* ssRNA sequence with 100% identity.

A possible explanation for the apparent discrepancy between the results of nrITS sequencing and LAMP testing is that assay specificity is lower than suggested by initial testing. Results for the cultured isoalates are due to false positives. However, this seems unlikely as the LAMP assay was positive for isolates identified as both *Nigrospora* sp. and *Fusarium* sp. suggesting that amplification does not result from a sequence match charactistic of a particular group. Moreover, match scores between the LAMP target sequence in *P. fijiensis* and the corresponding region in *Nigrospora* sp. and *Fusarium* sp. are low. For example, comparisons to the complete mitochondrion genome of *Fusarium oxysporum* (NC_017930) matched the *P. fijiensis* target sequence in two sections one of 68 nucleotides with 97% identity and the other of 55 nucleotides with 80% identity; the second of these two segements was inverted in *F. oxysporum* relative to *P. fijiensis* suggesting that the primer interactions necessary for ampliciation would be disrupted.

An alternative and more likely explanation given the sequencing results is that the original isolates consisted of a mixture of fungal species. It is recognised that *P. fijiensis* grows more slowly on artificial media (Leiva-Mora et al., 2008; Mulder & Holliday, 1974; Pérez-Vicente, 2012) and it is therefore possible that this pathogen was present but not observed during the culturing process. As a result, DNA extracted from these isolates would have contributions from both the non-target species and *P. fijiensis*. Moreover, as *P. fijiensis* was not detected visually it is likely to have made up a relatively small proportion of the DNA extracted from these isolates. If so then it is perhaps not surprising that amplification and sequencing on the nrITS resulted in apparently unequivocal genus level identifications. The more common sequence type could have been preferentially amplified meaning that a single nrITS sequence type was recovered DNA sequencing. That said, it should be noted that several of the nrITS sequences were characterised by secondary peaks indicating that at least one other sequence type was present amongst the PCR products. Although it was not possible to determine the nucleotide sequence from these secondary peaks this observation is consistent with LAMP results suggesting the presence of *P. fijiensis* in these samples.

5.4 Implications of LAMP testing

Previously only Davis et al. (2000) appear to have recorded laboratory-based detections of *P. fijiensis* from PNG, these authors suggesting that the pathogen was already common. That said, prior to this study anecdotal evidence suggested that *P. fijiensis* was present at only one of the sites sampled in PNG. Consistent with Davis et al.'s (2000) suggestion that black Sigatoka disease was already common, symptoms consistent with this disease (Churchill, 2011) were noted at all the PNG samples sites. Although foliar symptoms were noted at all sites, the exact symptoms varied between them perhaps as a result of the differences in the age of the infection or pathogenicity of the infecting strain. That LAMP testing detected *P. fijiensis* in at least one sample from all PNG sampling sites certainly suggests that this pathogen is likely to be widely distributed across the two districts included in this study. Although more extensive sampling would be needed to fully characterise the distribution of *P. fijiensis* across PNG, given the results of Davis et al.'s (2000) and those presented here as well as the high dispersibility of this pathogen it seems likely that it will be widespread.

5.5 Prospects and limitations

The aim of the current study was to develop, and laboratory validate a simple, rapid LAMP assay for the detection of *P. fijiensis,* the causal agent of black Sigatoka disease of banana. A set of optimised conditions were established, and an evaluation of the assay suggests that it provides a specific diagnostic for the detection of the pathogen from field collected leaf samples and fungal cultures. The results of the current thesis are promising, however further work with field samples is now needed. In particular, selection of field samples for this study was based on the presence of visual symptoms. In the case of black Sigatoka disease sampling of diseased tissue is a reasonable initial goal as symptoms of this disease can be confounded with those of closely related pathogens. However, work is now needed to determine whether the pathogen can be detected prior to (e.g., of airborne spores) or early in the infection cycle. Being able to put in place interventions before symptoms begin to impact productivity may improve outcomes for growers.

Currently, molecular diagnostics for plant pathogens is not performed in PNG. Instead samples are regularly sent to laboratories in either Australia or New Zealand for testing. Such testing is difficult to carry out in developing countries like PNG because the requirements for specialised consumables, infrastructure and personnel are not easily met. LAMP assays provide an alternative; sensitive diagnostics that are cheap, simple and robust enough to be carried out in low infrastructure settings. Such assays, including that developed in the present thesis, have the potential to greatly improve the capacity and capability in countries like PNG. For example, staff at the Kilakila Plant Health Laboratory currently extract DNA from samples and then send these overseas for diagnostic testing with the results only received weeks or months later. Given the capacity and capability for DNA extraction it is not unreasonable to expect that these same staff members could be using LAMP assays to conduct testing for many plant pathogens at the Kilakila laboratory. Although the ultimate goal may be to bring diagnostic testing to field sites, in the case of PNG an important first step may be to make molecular diagnostic availabe in country. To do so would dramatically reduce the time needed to implement disease management protocols and potentially dramatic impacts on productivity and food security.

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