Improvement of phytoplasma diagnostic techniques

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Abstract

Phytoplasmas are wall-less, non-culturable, phloem-limited bacterial pathogens that belong to the Mollicutes. They cause many diseases in lots of plant species (wild and cultivated) belonging to many different plant families, resulting in significant losses in important crops, and economically damaging epidemics worldwide. Phytoplasmas infect major cultivated crops such as many annual crops, fruit trees, grapevines and palms, which makes control of these diseases a priority, and the first important step for management is efficient and effective phytoplasma diagnosis. Detection of phytoplasmas is difficult because of their irregular distribution within the diseased plants and low concentration inside infected plants. In the last two decades most research toward the detection of phytoplasmas has used nucleic acid-based techniques such as PCR, which is used to amplify regions of phytoplasma genomes existing inside infected plants. However, most routine diagnostics has moved from general PCR to real-time PCR, due to the improved sensitivity and reduced risk of contamination due to the use of a closed system for product detection. Also the method can be developed into a semi-quantitative method.

In this project the aim has been to improve the specificity and reliability of phytoplasma diagnostic techniques by using primers that detect specific genes in phytoplasma genomes, and designing new universal primers for conventional and real-time PCR. This has involved developing new assays for 16Sr groups II, III, V, VI, XI, and XII, to facilitate analysis of changes in levels of different phytoplasmas in mixed infections. In addition, this work has involved the evaluation of LAMP (loop mediated isothermal amplification) diagnostic assays for different phytoplasma groups (I, II, III, V, VI, X, and XII) and also validating a rapid DNA extraction method and

whether this is effective for all plant species (i.e. Madagascan periwinkle versus Napier grass and other grasses). The second main objective was to investigate the rate of evolution of phytoplasma genomes. For this, infected plants (of phytoplasma groups 16SrI, II, III, VI, and X) were grafted onto fresh plants at 3-4 month intervals throughout the project. Once the phytoplasma had re-established, DNA was extracted and a range of genes including 16S rRNA, *secA*, *tuf*, and *rp* were amplified and sequenced. The aim was to determine whether there was any evidence of genome evolution over time. However, the results suggested that in these genes at least, the rate of change due to point mutations and/or insertion of potential mobile elements (PMUs) was slow, with no sequence changes being detected over the three years of study.

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Contents

List of Abbreviations

AP	Apple Proliferation Phytoplasma
AY-WB	Aster Yellows Witches' broom Phytoplasma
BLL	Brinjal Little Leaf Phytoplasma
Ca. P.	'*Candidatus* Phytoplasma'
CSP	Crotalaria Saltiana Phyllody Phytoplasma
CSPWD	Cape Saint Paul Wilt Disease
C_T	Cycle Threshold
CTAB	Cetyltrimethylammonium bromide
COX	Cytochrome Oxidase
ELISA	Enzyme-linked Immunosorbent Assay
EY	Elm Yellows Phytoplasma
ICSB	International Committee on Systematic Isothermal Bacteriology
IRPCM	International Research Project for Comparative Micro Mycoplasmology
LAMP	Loop-Mediated Isothermal Amplification
Leu	Leucyl tRNA synthetase gene
MEGA	Molecular Evolutionary Genetics Analysis
MLOs	Mycoplasma-like Organisms
NGS	Napier Grass Stunt Phytoplasma
NLS	Nuclear Localisation Signal
OY-M	Onion Yellows Phytoplasma Mild Strain
PAa	Australian Grapevine Yellows Phytoplasma
PCR	Polymerase Chain Reaction
PMU	Potential Mobile Unit
PWB	Potato Witches' Broom Phytoplasma
PWX	Peach Western X Phytoplasma
qPCR	Real-time Quantitative PCR, or Real-Time PCR
RG	Rehmannia Glutinosa Phytoplasma
RIV	Ribes in Vinca Phytoplasma
SGP	Strawberry Green Petal Phytoplasma
SLY	Strawberry Lethal Yellows Phytoplasma
SRP	Signal-Recognition Particle
SPLL	Sweet Potato Little Leaf Phytoplasma
T-RFLP	Terminal restriction fragment length polymorphism
VCP	Vinca Coconut Phyllody Phytoplasma

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Chapter 1: Introduction

1.1 Discovery of phytoplasmas

Phytoplasmas are wall-less, non-culturable bacteria and are a group of prokaryotic plant pathogens that exist in the phloem of plant hosts and the salivary glands of their insect vectors (Lee *et al.,* 1998; Christensen *et al.,* 2005). They were first identified when in 1967, Doi and co-workers discovered pleomorphic bodies in infected samples from mulberry trees and other plants which were infected by yellows diseases (Doi *et al.,* 1967). Previously, it had been believed that most yellowing diseases were caused by viruses because they are generally transmitted by insects, but many yellows-type diseases are now known to be caused by phytoplasmas (Bertaccini and Duduk, 2009).

Initially phytoplasmas were named MLOs (mycoplasma-like organisms) due to ultrastructural and morphological similarities to mycoplasmas and because the mycoplasmas that cause diseases in humans and animals have similar sensitivity to tetracyclines, and that name (MLOs) was used for phytoplasmas until 1994 (Doi *et al.,* 1967). In 1994, the International Committee on Systematic Bacteriology (ICSB) sub-committee on the taxonomy of *Mollicutes* agreed to rename them as 'Phytoplasmas' to identify them from other bacteria which belong to the class *Mollicutes*. Phytoplasmas were given this new name because of specific characteristics, such as their phytopathogenic properties, which do not exist in either mycoplasmas or other MLOs that inhabit phloem sieve tubes (ICSB, 1993; 1997).

1.2 Morphology and genomic characteristics

The morphology of phytoplasmas is different to other bacteria and they have a range of sizes from 200 to 800 nm and appear under an electron microscope as filamentous or pleiomorphic bodies (Lee *et al.,* 2000) (Fig. 1.1). It has been suggested that there are filamentous forms of phytoplasmas and a reason for the phytoplasma size differences is that these represent different developmental stages in their plant hosts. In addition their cells lack a rigid cell wall and have just a single unit cell membrane (Lee *et al.,* 2000). Phytoplasmas have amongst the smallest genomes in bacteria, between 530 kb and 1350 kb; the tomato strain of the stolbur phytoplasma and a Bermuda grass white leaf phytoplasma represent the largest and smallest genomes known (Firrao *et al.,* 2007).

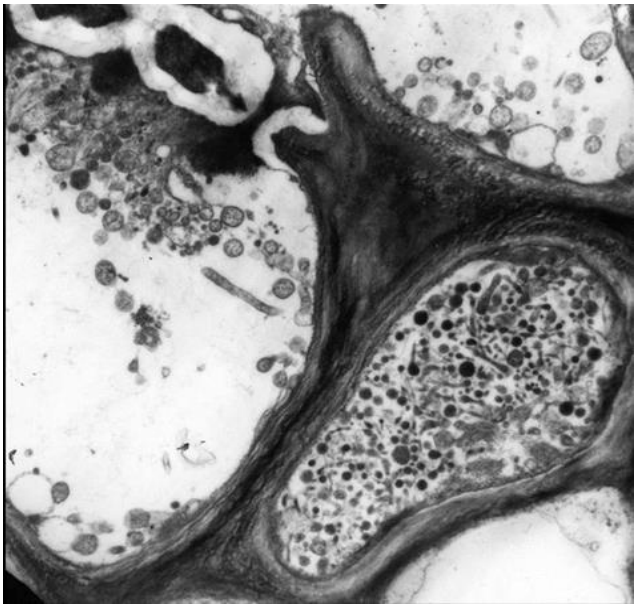


Figure 1.1 Electron microscopy picture (6000x) of a cross section of sieve tubes with phytoplasmas (Bertaccini *et al.,* 2014).

Generally, the main reason for the limited studies of phytoplasma diseases has been the lack of ability to culture them *in vitro,* which may be related to their lack of essential genes, such as genes for biosynthesis of nucleotides, fatty acids and amino acids (Oshima *et al.,* 2004; Christensen *et al.,* 2005). In addition, whilst most bacteria have a phosphotransferase system, which is important for phosphorylation and import of sugars (sucrose, fructose and glucose), phytoplasmas do not have this which makes them highly dependent on their plant hosts to get nutrients (Hogenhout *et al.,* 2008).

1.3 Pathogenicity of phytoplasmas

The study of phytoplasmas has been difficult because no reliable phytoplasma culturing has been accomplished in *in vitro* conditions such that understanding of phytoplasma pathogenicity is limited. The majority of results of gene expression and genome studies in *in vitro* conditions have been based on creating transgenic plants expressing phytoplasma genes in *Nicotiana benthamiana* or *Arabidopsis thaliana*. Evidence suggests two main factors affect phytoplasma pathogenicity, which are virulence factors and protein secretion.

1.3.1 Virulence factors

There are two main factors that appear to give the phytoplasmas their severity; the first factor is that several genes in phytoplasmas encode effector proteins that act directly on the host plant. The second is the side effects of phytoplasma infection.

For the first factor, a number of genes have been found in phytoplasmas that act as virulence factors and appear to be responsible for pathogenicity. For example, Bai *et al.* (2009) reported that the SAP11 protein, which is one of 56 secreted AY-WB phytoplasma proteins (SAPs), contains a nuclear localisation signal (NLS) and targets the nuclei of host cells, and also contains an N-terminal signal protein (SP). In addition, Sugio *et al.* (2011) reported that SAP11 expression increased in the AY-WB insect vector *Macrosteles quadrilineatus* offspring.

For the second factor, since phytoplasmas lack many important genes such as for metabolite synthesis and metabolic pathways, phytoplasmas must obtain many nutrients from their hosts and the depletion of nutrients from plants causes some of the symptoms of phytoplasma diseases (Oshima *et al.,* 2007). Furthermore, the OY-W strain of the onion yellows phytoplasma has been shown to possess two glycolytic gene clusters which is a higher copy number than in the OY-M strain. It has been suggested that the harsh symptoms caused by the OY-W strain compared with the mild strain is because of the higher utilization of the carbon source resulting from possessing this increased number of glycolytic genes (Oshima *et al.,* 2007).

1.3.2 Protein secretion

When phytoplasmas are transmitted to plants by insect vectors, they enter into the cytoplasm of phloem cells (Hogenhout *et al.,* 2008). After that, phytoplasmas start multiplying and secreting proteins inside the cytoplasm of phloem cells and some of these proteins are small enough to be able to move to other plant cells (Hogenhout and Loria, 2008). Phytoplasmas have two main systems to secrete proteins; the first system is called the Sec system for integrating and secreting proteins into cells of hosts, and the

second one is the YidC system for integrating membrane proteins (Oshima *et al.,* 2013).

For the Sec protein secretion system, this system in *Escherichia coli* involves three stages; the first stage is called 'targeting' in which a protein is guided by the SecB protein and the signal-recognition particle (SRP) to exit locations in the membrane. The second stage is called 'translocation' in which the guided protein uses the action of the SecA, SecE and SecG proteins and nonessential subunits (SecD and SecF) to cross the lipid bilayer. The third stage is called 'release' in which the translocated protein is released and allowed to be natively folded in the periplasm or to proceed to the outer membrane for integration. It has been shown from whole genome sequencing of phytoplasmas including Strawberry lethal yellows (SLY) (Anderson *et al.,* 2013), '*Candidatus* Phytoplasma australiense' strain PAa (Tran-Nguyen *et al.,* 2008), '*Candidatus* Phytoplasma mali' strain AT (Kube *et al.,* 2008), '*Candidatus* Phytoplasma asteris' strain witches' broom (AY-WB) (Bai *et al.,* 2006), '*Candidatus* Phytoplasma asteris' strain OY-M (Oshima *et al.,* 2004) that genes encoding SecA, SecE, and SecY are present in all five genomes.

The YidC protein secretion system is also believed to be a common system in phytoplasmas, because the gene encoding the YidC protein has been found in all sequenced phytoplasma genomes (Oshima *et al.,* 2013). In addition, Serek *et al.* (2004) reported that in *E. coli,* YidC facilitates newly synthesized membrane proteins to be accurately inserted into membranes and functions separately as a Sec-independent protein.

1.4 Phytoplasma classification

Because phytoplasmas cannot as yet be reliably grown in culture, they are classified in the '*Candidatus* (*Ca*.) Phytoplasma (P.)' taxon. In this system, if the similarity of the 16S rRNA gene is less than 97.5%, a new *'Ca.* P. species' can be designated and separated from previously classified '*Ca.* P. species'. Sometimes, if there is a sufficiently different host range and/or insect vectors, a novel '*Ca.* P. species' can also be created, even if the similarity of the 16S rRNA gene is more than 97.5% (IRPCM, 2004).

For additional information and to get a clearer and deeper classification for closely related phytoplasmas, DNA sequences of other genes have been used, such as the spacer between the 16S and 23S rRNA, the ribosomal protein gene (*rp*), *secA*, *secY* and *tuf* genes (Jarausch *et al.,* 2000; Lee *et al.,* 2004; Hodgetts *et al.,* 2008).

Using the '*Ca.* P. species' system, phytoplasmas have been classified into thirty-three 16S rRNA groups (Bertaccini and Duduk, 2009; Bertaccini *et al.,* 2014). Table 1.1 provides details of these 16Sr groups, including the name, geographic distribution, and strain/'*Ca.* P. species'.

Table 1.1 Classifications of phytoplasmas based on the 16S rRNA gene (modified from Bertaccini *et al.,* 2014).

16Sr group	Name	Geographic Distribution	Strain/*Ca.* P. species
I	Aster yellows	America, Europe, Asia, Africa	➢ Aster yellows witches' broom (AY-WB) '*Ca.* P. asteris' ➢ Onion yellows mild strain (OY-M) '*Ca.* P. asteris' ➢ Clover phyllody ➢ Rehmannia glutinosa (RG) ➢ Chrysanthemum yellows (ChY) ➢ Ribes in Vinca (RIV)
II	Peanut witches' broom	America, Africa , Europe, Asia, Australia	➢ Peanut witches' broom (PnWB) ➢ Sweet potato little leaf (SPLL) ➢ Vinca coconut phyllody (VCP) ➢ Crotalaria saltiana phyllody (witches' broom) (CSP)
III	X-disease	America, Europe, Asia,	➢ Peach X-disease '*Ca.* P. pruni' ➢ Poinsettia branching factor (PBF) ➢ Plum leptonecrosis (PL)
IV	Coconut lethal yellows	America, Africa	➢ Coconut lethal yellows
V	Elm yellows	America, Asia, Africa, Europe	➢ Elm yellows (EY) '*Ca.* P. ulmi' ➢ Flavescence dorée ➢ Rubus stunt '*Ca.* P. rubi'
VI	Clover proliferation	America, Asia, Europe	➢ Clover proliferation '*Ca.* P. trifolii' ➢ Potato witches' broom (PWB) ➢ Brinjal little leaf (BLL)

16Sr group	Name	Geographic Distribution	Strain/Ca. P. species
VII	Ash yellows	America, Europe	➢ Ash yellows '*Ca.* P. fraxini'
VIII	Loofah witches' broom	Asia	➢ Loofah witches' broom
IX	Pigeon pea witches' broom	America, Asia, Europe	➢ Pigeon pea witches' broom ➢ Almond witches' broom '*Ca.* P. phoenicium'
X	Apple proliferation	America, Europe	➢ Apple proliferation (AP) '*Ca.* P. mali' ➢ European stone fruit yellows '*Ca.* P. prunorum' ➢ Pear decline '*Ca.* P. pyri'
XI	Rice yellow dwarf	Africa , Asia, Europe	➢ Rice yellow dwarf '*Ca.* P. oryzae' ➢ Sugarcane white leaf ➢ Napier grass stunt (NGS)
XII	Stolbur	Africa , America, Asia, Australia, Europe	➢ Stolbur (Stobur 284/09) '*Ca.* P. solani' ➢ Australian grapevine yellows (PAa) ' *Ca.* P. australiense' ➢ Strawberry lethal yellows (SLY) '*Ca.* P. australiense'
XIII	Mexican periwinkle virescence	America	➢ Mexican periwinkle virescence ➢ Strawberry green petal (SGP)
XIV	Bermuda grass white leaf	Europe	➢ Bermuda grass white leaf '*Ca.* P. cynodontis'
XV	Hibiscus witches' broom	America	➢ Hibiscus witches' broom '*Ca.* P. brasiliense'

Continued,

16Sr group	Name	Geographic Distribution	Strain/*Ca.* P. species
XVI	Sugarcane yellow leaf syndrome	America	➢ Sugarcane yellow leaf syndrome '*Ca.* P. graminis'
XVII	Papaya bunchy top	America	➢ Papaya bunchy top '*Ca.* P. caricane'
XVIII	American potato purple top wilt	America	➢ American potato purple top wilt '*Ca.* P. americanum'
XIX	Chestnut witches' broom	Asia	➢ Chestnut witches' broom '*Ca.* P. castaneae'
XX	Rhamnus witches' broom	Europe	➢ Rhamnus witches' broom '*Ca.* P. rhamni'
XXI	Pinus phytoplasma	Europe	➢ Pinus phytoplasma '*Ca.* P. pini'
XXII	ND	ND	➢ Lethal yellow disease Mozambique '*Ca.* P. palmicola' ➢ Cape St. Paul wilt disease
XXIII	ND	ND	➢ Buckland valley grapevine yellows
XXIV	ND	ND	➢ Sorghum bunchy shoot

Continued,

16Sr group	Name	Geographic Distribution	Strain/*Ca.* P. species
XXV	ND	ND	➢ Weeping tea witches' broom
XXVI	ND	ND	➢ Sugarcane phytoplasma D3T1
XXVII	ND	ND	➢ Sugarcane phytoplasma D3T2
XXVIII	ND	ND	➢ Derbid phytoplasma
XXIX	Cassia witches' broom	Asia	➢ Cassia witches' broom '*Ca*. P. omanense'
XXX	Salt cedar witches' broom	Asia	➢ Salt cedar witches' broom '*Ca.* P. tamaricis'
XXXI	Soybean stunt	America	➢ Soybean stunt '*Ca.* P. costaricanum'
XXXII	Malaysian periwinkle virescence and phyllody	Asia	➢ Malaysian P. virescence '*Ca.* P. malaysianum' ➢ Malayan oil palm phytoplasma
XXXIII	Allocasuarina muelleriana phytoplasma	Australia	➢ Allocasuarina phytoplasma '*Ca.* P. allocasuarinae'

ND: Not Designated.

1.5 Symptoms of phytoplasma diseases

Symptoms in plants can depend on the season, pathogen species and the stage of disease development, and different organs will exhibit different symptoms; in addition, a few plants can harbour phytoplasmas without showing any symptoms.

Symptoms of diseased plants caused by phytoplasma infections include yellowish foliage (discolorations of leaves and shoots) (Fig. 1.2 and Fig. 1.3), shoot proliferation (witches' broom) (Fig. 1.4), stunting (small leaves and shortened internodes), proliferations (growth of shoots from floral organs) and phyllody (the development of leaf-like structures instead of flowers) (Fig. 1.5) (Hogenhout *et al.,* 2008).

As examples, aster yellows phytoplasma infected plants show witches' broom symptoms throughout the plant or on flower stalks (Fig. 1.4), and most of the time only the symptoms on flowers are visible (Grabwski, 2012). Elm yellows phytoplasma can cause several different symptoms such as witches' broom, epinasty, leaf yellowing and decline of Eurasian species (*Ulmus* spp.) (Griffiths *et al.,* 1999). Napier grass stunt shows clear symptoms of phytoplasma infection on Napier grass plants, such as proliferation of tillers and shortening of internodes, little leaves, leaf yellowing, stunt and eventual death (Fig. 1.2) (Jones *et al.,* 2004).

Sometimes the symptoms can be different or very hard to recognize because of complex interference between them and host physiology (Marzachi *et al.,* 2004).

Figure 1.2 Symptoms of Napier grass stunt phytoplasma on Napier grass plants showing leaf yellowing and stunting (International Society for Infectious Diseases, 2014).

Figure 1.3 Symptoms of Elm yellows phytoplasma on infected *Ulmus americana* showing yellowing leaves and epinasty compared with a healthy plant on the left side in the picture (Albanese *et al.,* 2012).

Figure 1.4 Phyllody symptoms on a goldenrod flower infected with aster yellows (Pracros *et al.,* 2006).

(a) (b)

Figure 1.5 Shortened internodes, witches'-brooms and little leaves (a) hypertrophied inflorescences (b) on olive trees affected by phytoplasmas (Albanese *et al.,* 2012).

1.6 Phytoplasma transmission and lifecycle

Transmission of phytoplasmas is generally from infected plants to healthy plants by insect vectors feeding on phloem tissue of diseased plants. Insect vectors can be hoppers (Auchenorrhyncha, Homoptera) or psyllids (Sternorrhyncha, Homoptera) and transmission occurs in a persistent manner. Relationships between phytoplasmas and their insect vectors are a key driver in the host range of phytoplasmas (Bertaccini and Duduk, 2009). Some insect vectors can transit more than one phytoplasma stain, and particular phytoplasmas can be transmitted by more than one vector. However, the mechanisms of insect vector-phytoplasma specificity or compatibility are not well understood (Weintraub and Beanland, 2006).

The lifecycle of a phytoplasma starts with acquisition feeding; the phytoplasma passes into the vector through the vector stylet during feeding. The phytoplasma then passes through the intestine and is absorbed into the haemolymph, before moving to the salivary gland, which takes a few weeks from the initial feeding. During these weeks phytoplasmas keep multiplying until their concentrations reach a high enough level to be transmitted when the insects start feeding on healthy plants (inoculation feeding) (Fig. 1.6) (Hodgetts *et al.,* 2008). Furthermore, in some combinations of insect vectors and phytoplasmas, it has been discovered that there is transovarial transmission, which means the phytoplasma can be transmitted from parent insect vectors to offspring. Examples of this are the combinations of the leafhopper *Matsumuratettix hiroglyphicus* (Matsumara) and sugarcane white leaf phytoplasma (Hanboonsong *et al.,* 2002), and the leafhopper *Hishimonoides sellatiformis* and mulberry dwarf phytoplasma (Kawakita *et al.,* 2000).

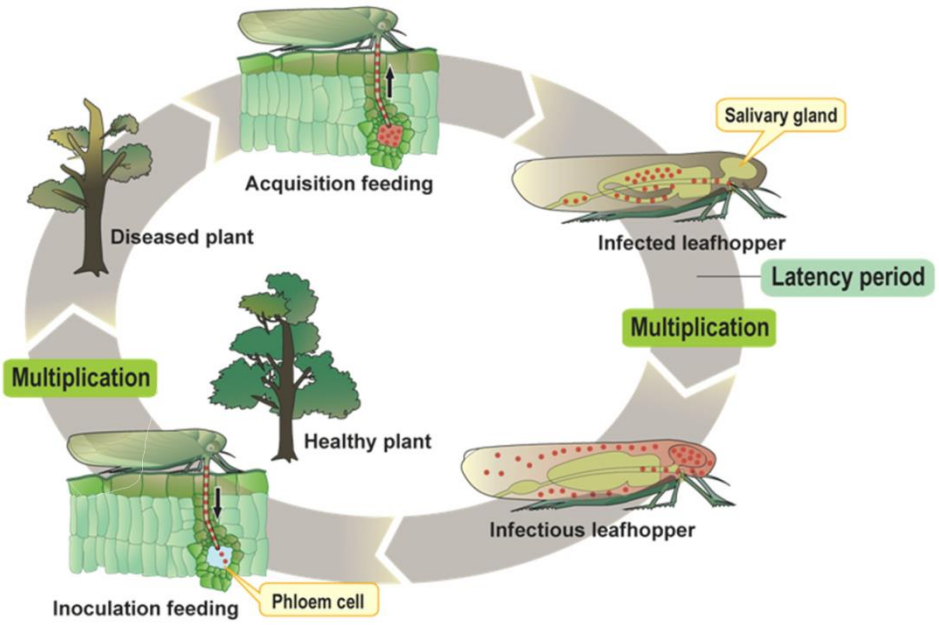


Figure 1.6 Life cycle of phytoplasmas. Phytoplasma is shown as a red dot. Acquisition feeding is when the insect (leafhopper) takes up phytoplasmas. There is then a latency period (which means the time from phytoplasma uptake to getting an infection titre for the phytoplasma transmission), and the last stage is infection feeding; phytoplasma transmitted from insects to healthy plants (Oshima *et al.,* 2011).

It is also possible for phytoplasmas to be transmitted by the parasitic plant, dodder (*Cuscuta* spp.) in experimental conditions. Once dodder plants start growing on infected plants, phytoplasmas enter inside the dodder plant through haustoria and proliferate within the dodder plant, and then move to healthy plants via haustoria of dodder (Pribylova and Spak, 2013). For example, European stone fruit yellows, pear decline, and other phytoplasmas have been experimentally transmitted from naturally infected plants to healthy periwinkle plants (*Catharanthus roseus*) using dodder plants (Marcone *et al.,* 1999). Pribylova *et al.* (2001) reported that dodder plants transmitted RG phytoplasma successfully from infected *Rehmannia glutinosa* plants to healthy periwinkle plants.

Experimentally, grafting can also be used for phytoplasma transmission and maintenance of infected plants by transmitting phytoplasmas from naturally infected plants to healthy plants (mostly periwinkle plants). For example, phytoplasmas have been transmitted from infected grapevine to healthy periwinkle plants by wedge grafting (Tanne and Orenstein, 1997), and chip and bark grafting has been used to transmit the apple proliferation (AP) phytoplasma from infected apple plants to healthy periwinkle plants (Aldaghi *et al.,* 2007). However, grafting has not been successful for all phytoplasmas, and other transmission methods sometimes need to be used.

In addition phytoplasmas can be transmitted by vegetative propagation such as storage tubers, cuttings, bulbs or rhizomes (Lee *et al.,* 2000).

1.7 Phytoplasma diagnostics

Detection of phytoplasmas is difficult because of their irregular distribution within the diseased plants and also, especially in woody hosts, because the concentration of phytoplasmas is often low. In the past, researchers tried to use techniques to detect phytoplasmas such as grafting onto indicator hosts, electron microscopy observations, and using DNA-specific dyes to stain the nucleic acid of the pathogen, but these approaches often gave unreliable results. In the last two decades most research toward the detection of phytoplasmas has used serology, or more often, nucleic acid-based techniques such as PCR, which is used to amplify regions of phytoplasma genomes existing inside infected plants. However, most routine diagnostics has moved from general PCR to real-time PCR, due to the improved sensitivity and reduced risk of contamination due to the use

of a closed system for product detection. Also the method can be developed into a semi-quantitative method (Hodgetts *et al.,* 2009).

DNA extraction is generally the first step in diagnosis of phytoplasmas, and many protocols are reported for DNA extraction from potentially infected plants and vectors. Each protocol is different from another and depends on sampling procedures and on the species of plant host / insect or condition of storage in which the collected samples were placed (Daire *et al.,* 1997; Green *et al.,* 1999; Bosco *et al.,* 2002; Boudon *et al.,* 2003).

1.7.1 Serological assays

Enzyme-linked immunosorbent assays (ELISA) are one serological method that has been used, but it is little used because of the limitations in specificity and sensitivity compared with nucleic acid-based assays. A commercial monoclonal anti IgG kit based DAS-ELISA has been developed, but it was unable to detect all '*Ca*. P. mali' (Canik and Ertuce, 2007).

1.7.2 Nucleic acid-based assays

Polymerase chain reaction:

In the last two decades most research toward the detection of phytoplasmas has used nucleic acid-based techniques such as PCR. Researchers have tried to obtain sequences of specific regions of phytoplasma DNA and design primers for these regions. Some of the design has involved specific primers for a particular group, but most are trying to design universal or semi universal primers to detect all groups of phytoplasma or at least most of these groups, of which there are at least thirty-three 16S rRNA groups (Bertaccini and Duduk, 2009; Bertaccini *et al.,* 2014). Ribosomal RNA primers are one of the sets of universal primers

that are generally used to detect phytoplasmas and amplify a specific fragment of phytoplasma DNA (16S rRNA gene). Because of the low concentration of phytoplasma DNA, most laboratories use universal primers in the first round of PCR reactions and then use another internal primer (second round) which is often more specific (nested PCR). There are also 'universal primers' for other genes such as the *rp* (ribosomal operon), *secA, tuf, secY, hlg, gyr* and nitroreductase genes (Chuang *et al.,* 2000; Hodgetts *et al.,* 2008).

Practically, a first round PCR (direct PCR) is performed with a pair of primers, then followed by a second pair of primers which targets primer sites on first round PCR products. Second round PCR assays are created which increase sensitivity and specificity of the diagnosis compared to first round PCR. Figure 1.7 shows an example of first round and nested PCR primers for the 16S rRNA gene or the spacer region between 16S rRNA and 23S rRNA (Smart *et al.,* 1996).

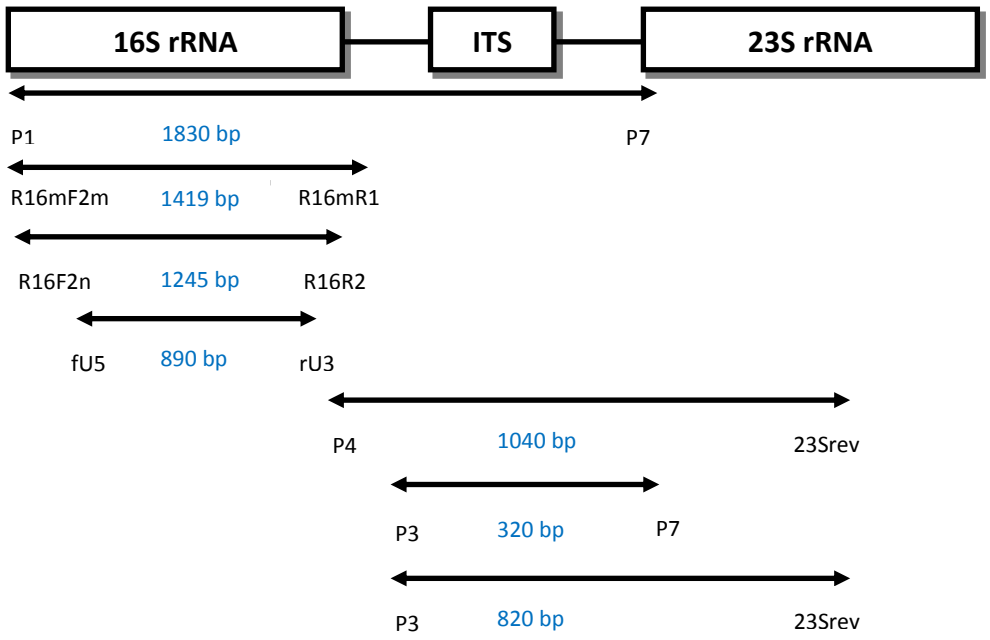


Figure 1.7. Diagrammatic representation of phytoplasma 16S-23S rRNA operon (ITS) primers (Hodgetts *et al.,* 2008).

From the spacer region (rRNA operon) it is also possible to design specific, semi universal and universal primers. In addition, other genes have been used to obtain high specificity of assays and distinguish between closely related phytoplasma strains, such as ribosomal operon (*rp*), *secA*, *secY*, *tuf* (Jarausch *et al.,* 2000; Lee *et al.,* 2004; Hodgetts *et al.,* 2008). In addition, PCR products can be sequenced and compared against reference phytoplasma sequences on the NCBI database.

Terminal restriction fragment length polymorphism (T-RFLP):

T-RFLP is a method that has also been developed for phytoplasma identification in their host (plants and vector insects) and for assigning them to phylogenetic groups. In this method, one of the PCR primers is labelled with a fluorescent tag such that following PCR and restriction enzyme digestion with an appropriate enzyme, it is the size of the terminal restriction fragment (TRF) containing the labelled primer that can be analysed on an automated DNA machine (Hodgetts *et al.,* 2007).

Real-time PCR:

Increasingly there are new methods to diagnose phytoplasmas, but improvements in diagnostics are needed to produce methods that are more economic, quicker and more robust (Christensen *et al.,* 2004). Sensitivity of nested PCR protocols is high, but the problem with nested PCR is obtaining high levels of sensitivity without the risk of false positive results due to contamination of samples during the various procedures and opening of tubes (Hodgetts *et al.,* 2009). In the last few years, real time PCR (qPCR) has been used as a tool for phytoplasma diagnosis, and this technique was introduced to replace standard PCR because of direct reading and high sensitivity of the result. A key characteristics of qPCR is

that gel electrophoresis is not needed, which reduces the risk of sample contamination (Christensen *et al.,* 2004; Jarausch *et al.,* 2004).

To use qPCR for phytoplasma diagnosis, there are two alternative systems, the first system using SYBR Green (Ponchel *et al.,* 2003), and the second uses TaqMan probes (Heid *et al.,* 1999).

The SYBR Green system requires a binding dye for double-standed DNA, and during qPCR cycles the dye can be detected when it binds double stand qPCR products. The way the SYBR GREEN assay works is as follows. While PCR is happening, PCR products are created because the DNA polymerase amplifies the target sequences. Once the dye is added, it immediately binds to any double-stranded DNA and fluoresces – therefore every new copy of DNA generated will bind, and as more amplification occurs the fluorescence intensity increases, and this can be used as a measure of the amount of product (Paudel *et al.,* 2011).

Advantages of the SYBR-GREEN assay are that it can be used to monitor the amplifications for any sequences of double-stranded DNA, and no probes are required, which reduces the costs of assay set-up. The disadvantage is that because the SYBR-GREEN dye can bind to any double-stranded DNA (target DNA and non-target DNA), it can also bind to nonspecific double-stranded DNA sequences, which may generate false positive signals. Thus, to avoid inaccurate results, it is very important to have well-designed primers that will improve specificity of SYBR-GREEN assays, and validations of qPCR reactions must be done though melt curve analysis (Galetto *et al.,* 2010; Paudel *et al.,* 2011).

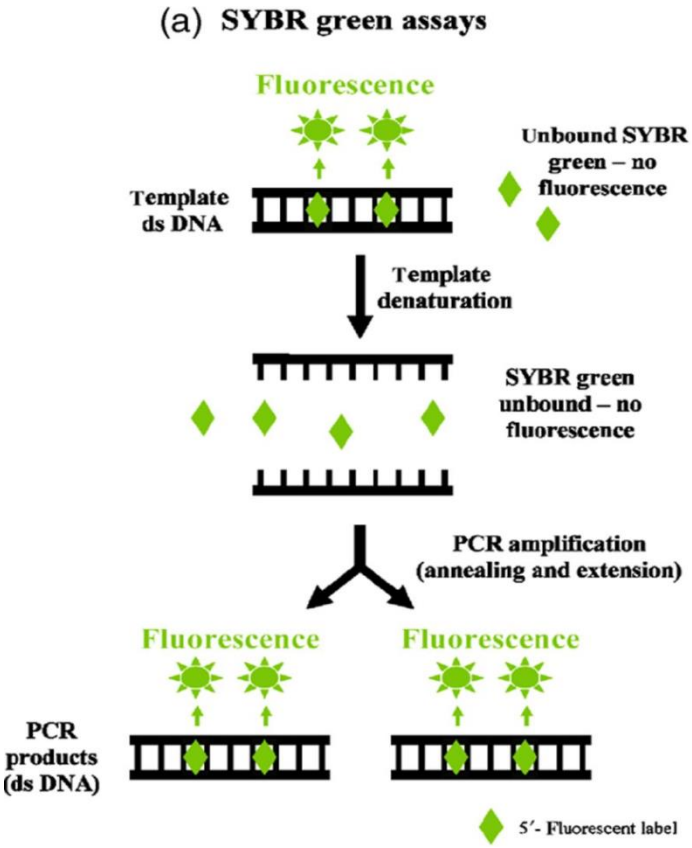


Figure 1.8 How the SYBR GREEN assay works step by step (Paudel *et al.,* 2011)

In the TaqMan system instead of using binding dyes, probes and reporter dyes are used. Probes encode from 5' to 3' to target a specific site between a forward qPCR primer site and a reverse qPCR primer site. During cycles of TaqMan reactions, primers start amplifying the DNA target, then probes bind the qPCR products, and the reporter dye is freed from the quencher and detected by the instrumentation of qPCR (Heid *et al.,* 1999; Ikten *et al.,* 2016).

For Taqman assays, the probe normally contains a fluorescent reporter dye on the 5' end and a quencher dye in the 3' end. When the target DNA is added, the probe will anneal downstream from one primer site and is

cleaved by the 5’ end of the *Taq* DNA polymerase as this primer is extended. In this step (cleavage of probe), the reporter dye separates from the quencher dye, increasing the signal of the reporter dye. Removing the probe from the target DNA will allow primers to continue extending till the end of the template strand. This prevents the probe from inhibiting the overall PCR process. In each cycle, the reporter dye molecules are cleaved from respective probes, and this leads to an increase in fluorescence intensity linked to the amount of amplicon produced (see Fig 1.9) (Pang *et al.,* 2014)

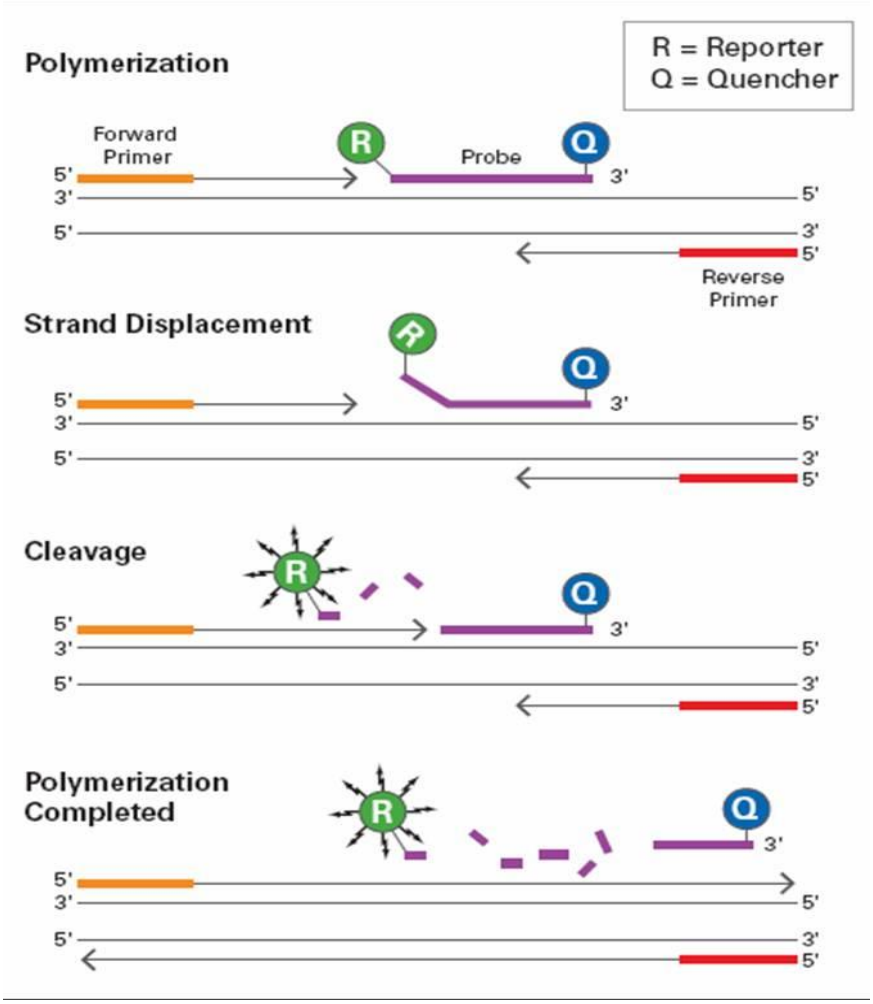


Figure 1.9 How Taqman assays work step by step (Pang *et al.,* 2014)

Advantages of Taqman assays are that the fluorescent signal is generated only when specific hybridization between probe and target DNA takes place. There is also the capacity to label probes with different and distinguishable reporter dyes, which potentially allows amplification and detection of two or more different sequences in one reaction tube. In addition, no further PCR processing is required, which reduces material costs and assay labour (Pang *et al.,* 2014). The main disadvantage of Taqman assays is the expense of the synthesis of the different probes required for different DNA sequences (Pang *et al.,* 2014).

TaqMan assays have been developed as universal assays and assays for a number of 16Sr groups (Fig. 1.9) (Hodgetts *et al.,* 2009). In addition, SYBR Green assays have also been developed especially for apple proliferation (AP), bois noir, and flavescence dorée phytoplasmas, and other universal assays to detect phytoplasmas in groups 16SrV, 16SrX, and 16SrXII. When comparing between SYBR Green systems and other techniques of phytoplasma detection such as the TaqMan system and conventional nested PCR, the SYBR Green system can give rapid and specific detection of the DNA target of phytoplasmas. For example, assays have been developed to detect quarantine phytoplasmas quantitatively, for example '*Ca.* P. pyri', '*Ca.* P. mali', and '*Ca.* P. prunorum' (members of 16SrX group) affecting fruit trees (Torres *et al.,* 2005).

Loop-mediated isothermal amplification:

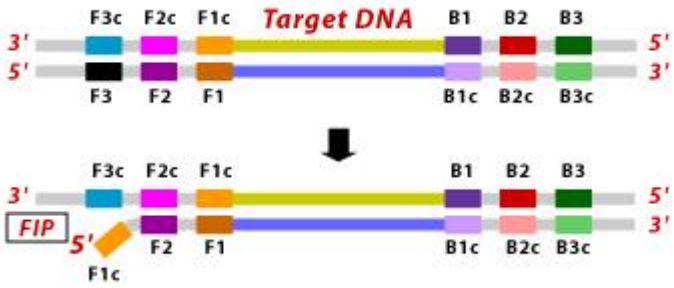
Recently, the LAMP technique (loop-mediated isothermal amplification) has been used a lot for phytoplasma detection because it is easy to use and takes a very short time to get the results. This technique amplifes DNA targets under isothermal conditions, and generally uses a set of six primers

for each assay (external primers, internal primers, loop primers) and a DNA polymerese with strand displacement activity (Fig. 1.10). Results of amplifications generally take less than an hour, and can be observed by agarose gel, hydroxyl napthol blue colorimetric assays, or intercalating fluorescent dyes performed in instruments for real-time PCR or LAMP (Tomlinson *et al.,* 2010; Bekele *et al*., 2011).

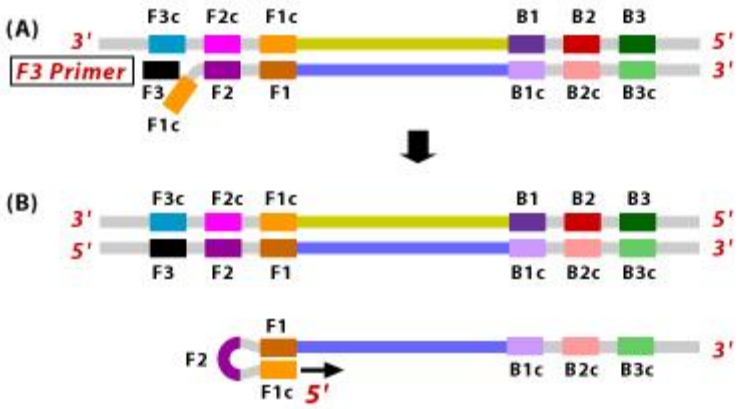
For example, LAMP assays for two different specific phytoplasma groups have been published; from 16SrI group (aster yellows phytoplasma) and from 16SrXXII group (Cape St Paul wilt). Sensitivity of these assays was compared to nested PCR, and assays were specific for target DNA phytoplasma (Tomlinson *et al.,* 2010). Other LAMP assays have subsequently been developed and published for 16SrII and 16SrXII phytoplasma groups in fruits and weeds of the Ethiopian Rift Valley (Bekele *et al.,* 2011).

How Loop-mediated Isothermal Amplification works:

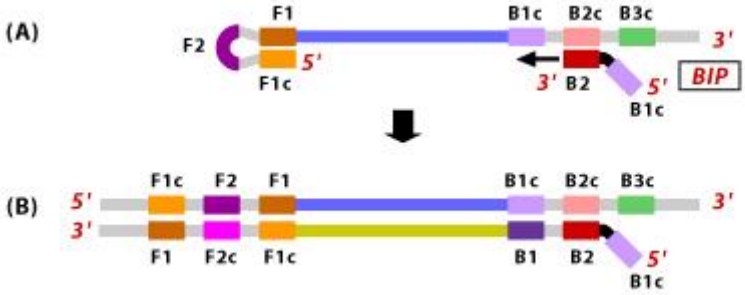
1. The F2 region (part of FIP primer) hybridizes to region of F2c (part of target DNA) and initiates complementary strand synthesis.



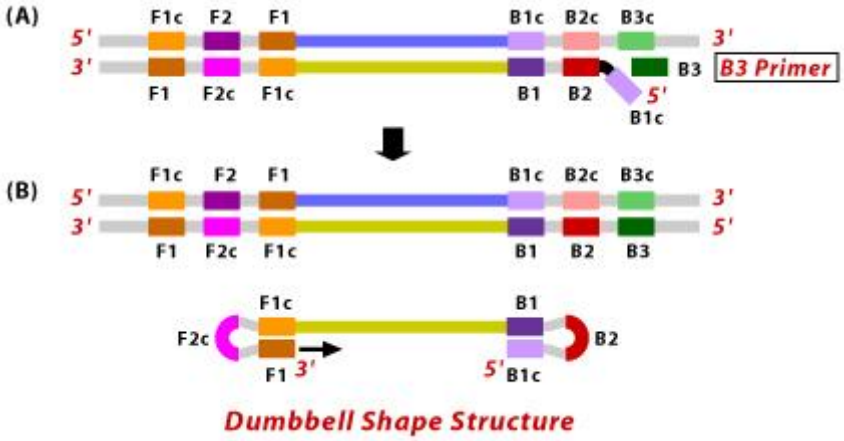
2. Similarly, with external primer F3 hybridizes to the F3c (part of target DNA) and extends; this leads to removal of the FIP linked complementary stand. Those removed stand forms will make a loop at the 5′ end.



3. This DNA with a loop at the 5’ end becomes a template for BIP, and the same process will happen with B2 and B2c, with hybridization between the B2 region and B2c region of the template DNA.

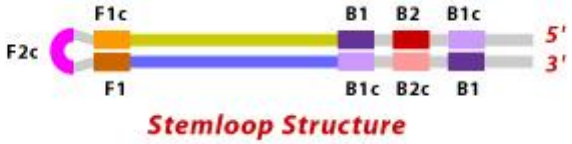


4. External primer B3 hybridizes to B3c (from target DNA) and then extends, leading to removal of the BIP linked complementary stand. This results in the formation of dumbbell sharped DNA.

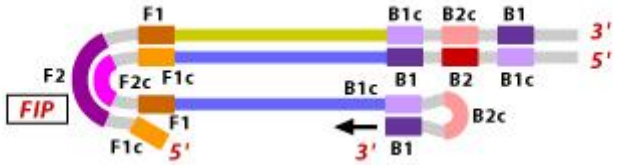


5. DNA polymerase adds nucleotides to the 3’ end of F1, and extends to open the loop at the 5’ end. The dumbbell shaped DNA now gets converted

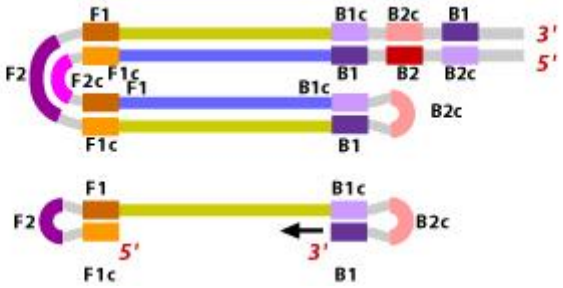
to a stem loop structure and this structure will be useful in the second stage of the LAMP reaction as an initiator for LAMP cycling.



6. In LAMP cycling, the FIP hybridises to the stem-loop DNA. In this step strand synthesis in initiated. Because the FIP hybridizes to the loop, the F1 strand is removed and forms to a new loop at the 3' end.



7. Again, DNA polymerase adds nucleotides to the 3' end of B1, and extends which removes the FIP strand. This new strand again forms a dumbbell shaped DNA. Subsequent self-primed stand displacement DNA synthesis yields one complementary structure of the original stem loop DNA and one gap repaired stem loop DNA.



8. After getting two products these start serving as template for a BIP primed strand displacement reaction in the subsequent cycles. Therefore, a LAMP target sequence is amplified 13 fold every half cycle.

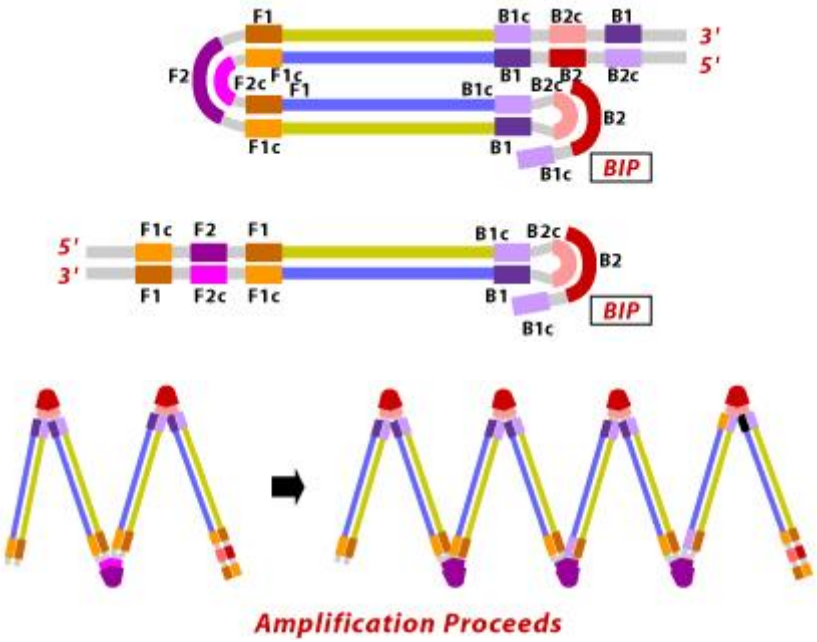


Figure 1.10 Schematic representation of the mechanism of LAMP assays; six primers include external primers (F3 and B3), internal primers (FIP and BIP), and loop primers (loopF and loopB (Mori *et al.,* 2012).

The advantages of isothermal amplifications are that they do not require any thermal cycling, making them easier to operate and the process needs less energy compared to PCR methods (no need for rapid heating and cooling steps), so small battery-operated portable machines can be developed. Additionally, as with real-time PCR, in real-time LAMP methods, the whole process is conducted in closed tubes, which reduces the risk of sample contamination (Macarthur *et al.,* 2009*).*

1.8 Phytoplasma genomics

In the last decade, four phytoplasma genomes have been sequenced completely; '*Ca*. P. asteris' strains OY-M (Oshima *et al.,* 2004), AY-WB (aster yellows witches'-broom) (Bai *et al.,* 2006), '*Ca*. P. mali' strain AT (Kube *et al.,* 2008) and '*Ca*. P. australiense' strain PAa (Tran-Nguyen *et al.,* 2008), and two others have good coverage that is publically available (strawberry lethal yellows SLY)(Anderson *et al.,* 2013), or have been sequenced at the University of Nottingham (Napier grass stunt NGS) (Kawicha and Dickinson, personal communication).

1.8.1 Genome features

According to these genome studies, phytoplasmas SLY, PAa, OY-M, and AY-WB have the larger genome sizes, which are in the order of 960, 880, 853, and 706 kbp. Phytoplasmas AT and NGS have smaller genome sizes of approximately 602 and 484 kbp. Generally, the G+C contents in the phytoplasmas are low; it is from 20.4% to 27.8%. From these six sequenced genomes the NGS has the lowest G+C content which is 20.4%, followed by the AT genome (21.3%), AY-WB genome (26.9%), SLY genome (27.2%), PAa genome (27.4%), and OY-M genome (27.8%). Interestingly, whilst most phytoplasma genomes appear to be circular, that of AT and possibly NGS are linear. The genomes have variable numbers of transfer RNA genes (from 31 to 35), but all of them have two ribosomal RNA operons. Furthermore, four plasmids are found in the genome of AY-WB, two plasmids are found in the genome of OY-M, and one plasmid is found in the genome of PAa and NGS (Oshima *et al.,* 2004; Bai *et al.,* 2006; Kube *et al.,* 2008; Tran-Nguyen *et al.,* 2008; Chung *et al.,* 2013; Praphat Kawicha PhD thesis, 2014).

1.8.2 Potential mobile units

Potential mobile units (PMUs) are repeated genes organized in units of approximately 20 kbp that occur in many genomes of phytoplasmas. These PMUs look like composite replicative transposons, and some are associated with putative 'virulence genes' and contain several genes for recombination (Dickinson, 2010). For example, aster yellows strain AY-WB has four different PMUs including PMU1, PMU2, PMU3, and PMU4. PMU1 is the largest unit at approximately 20 kbp, and the rest of the PMUs have similar gene structures to PMU1, but are smaller. Additionally, PMU1 in AY-WB strain is linear (Bai *et al.,* 2006; Toruno *et al.,* 2010).

Up to now, PMUs are regions that have been found and studied in phytoplasma genomes such as '*Ca.* P. asteris' strain OY-M, '*Ca.* P. australiense' strains PAa and SLY, and '*Ca.* P. mali' strain AT. But it is still unclear whether PMUs are present in all phytoplasma genomes (Jomantiene and Davis, 2006; Jomantiene *et al*., 2007).

1.9 Management and control of phytoplasmas

The main method for control or management of phytoplasmas is using insecticides to control insect vectors. Despite the widespread use of insecticides, phytoplasma diseases remain severe in many countries in the world (Firrao *et al.,* 2007). Furthermore, since the usage of chemical control is limited and being reduced worldwide (because of the other effects of chemical materials on humans, plants, animals, and the environment) other approaches are required. In addition, agricultural intensification and climate change are expected to raise insect vector populations, which may lead to more phytoplasma-related yield losses in

the future. From current research there is evidence that phytoplasma diseases are becoming widespread worldwide and spreading into new host plants (Walsh *et al.,* 2006).

Since antibiotics are too expensive and not a successful or practical strategy for long-time control, using them is not recommended for control of phytoplasmas. In an interesting study from 1985, Dickinson and Townsend, noticed from research on spiroplasmas (which are a related group of plant pathogenic *Mollicutes*) that remission of symptoms occurred during introduction of specific secondary isolates, with indications that this was because of the presence of lysogenic phages in certain isolates of spiroplasmas. Furthermore, there is evidence that when two different strains of phytoplasmas are mixed together to infect a healthy plant host, some strains can out-compete others. This evidence could be useful for developing the idea of using mild strains as a means of controlling some diseases (Schneider *et al.,* 2014). Recent experiments at the University of Nottingham have shown that inclusion of two different strains of phytoplasma into healthy periwinkle plants at the same time (RG+SPLL and RG+EY), can result in symptoms that belong to only one 16Sr group (RG) appearing on plants after 4-6 weeks. Following DNA extraction and testing of samples by real-time PCR and LAMP techniques, results confirmed that the RG phytoplasma was detected at a higher titre than others, which indicates that RG phytoplasma is able to outcompete the SPLL and EY phytoplasmas (Praphat Kawicha, PhD thesis, 2014).

Other methods that have been tried for control of phytoplasma diseases include roguing, which is the practice of removing diseased or infected plants as early as possible after symptoms become apparent (or as soon as phytoplasmas are detected using molecular detection tools) (Osler *et al.,*

2003). In addition, certification, which is a procedure where commercial production and nursery stocks undergo official regulation by competent government agencies for securing absence from phytoplasma diseases, can be used to prevent phytoplasma spread both within and between countries (Rowhani *et al.,* 2005).

1.10 Aims and objectives

The overall aim of this study was to improve and develop phytoplasma diagnostic techniques. Specific objectives were:

1. Evaluate different previously published primers for phytoplasma diagnostics: 16S rRNA, *secA, tuf* and *rp*. This was achieved by screening different isolates from the glasshouse and also DNA samples from the freezer of representative 16Sr groups (i.e. the ones for which fresh plant samples were unavailable). The aim of this part of the work was to determine which assays are most effective for different phylogenetic groups - i.e. which primers are universal; and also which ones are more sensitive versus which ones have to be used in nested PCR.

2. Study genomic evolution. At 3-4 month intervals throughout the project, grafts were taken from plants of 16Sr groups I, II, III, VI and X into fresh plants. Once the phytoplasmas had re-established, DNA samples were taken and the 16S rRNA, *secA*, *tuf*, and *rp* genes sequenced. The aim was to determine whether there is any evidence of genome evolution over time.

3. Identify primers for additional genes that could also be used for the genome evolution studies. These could then be used on the frozen samples of DNA from the grafting experiments to analyse variation in additional genes, again to look for evidence of genome evolution.

4. Develop use of TaqMan and SYBR Green real-time PCR assays and then develop new assays for groups II, III, IV, V, VI, X, XI, and XII. These could then be used for quantitation to be able to analyse changes in levels of different phytoplasmas in mixture infections.

5. Evaluate the LAMP diagnostic assays for different phytoplasma groups (I, II, III, V, VI, X, and XII), and in addition, check the rapid DNA extraction method and whether this is effective on all plant species (i.e. Madagascan periwinkle versus Napier grass and other grasses).

Chapter 2: Materials and Methods

2.1 Plant Materials

2.1.1 Healthy periwinkle plants:

Propagation of healthy Madagascar periwinkle plants (*Catharanthus roseus)* was done using the cutting method (Hodgetts *et al.,* 2013). Young shoots (6-8 cm in length and 2-3 mm in diameter) were cut by using a sterile scalpel blade and only the three top leaves were left with the rest of the leaves being cut away. A seedling tray was filled with seed compost and 25% perlite, and the bottom of the stem was dipped into hormone rooting powder (Doff Ltd, UK) and inserted into the seedling tray. Seedling trays were placed into seed trays and fitted with propagator lids. Watering was done 2-3 times a week. After six weeks, plants were transferred to individual pots, and after four months the plants were ready for graft transmission.

2.1.2 Infected plants

The School of Biosciences at the University of Nottingham has the largest collection of different phytoplasmas in the UK (thirteen different phytoplasmas which belong to different 16Sr groups). These are maintained as different clones in periwinkle plants (Table 2.1).

Table 2.1: List of phytoplasmas in the collection held in the School of Biosciences of the University of Nottingham (used in this study).

Phytoplasma name or host plant name	16Sr group	Source
Rehmannia glutinosa (RG)	I-B	Czech Republic
Strawberry Green Petal (SGP)	I-C	Czech Republic
Ribes in Vinca (RIV)	I-C	Czech Republic
Faba Bean Phyllody (FBP)	II-C	Fera, UK
Soybean Phyllody (SP)	II-C	Rothamsted, UK
Vinca Coconut Phyllody (VCP)	II	Rothamsted, UK
Plum Leptonecrosis (PL) - LNI	III	Fera, UK
Peach Western X (PWX)	III-A	Italy
Elm Yellows (EY)	V-A	Fera, UK
Brinjal Little Leaf (BLL)	VI-A	Rothamsted, UK
Potato Witches' Broom (PWB)	VI-A	Italy
Apple Proliferation (AP)	X-A	Ljubljana
Napier Grass Stunt (NGS)	XI	Kenya

In addition, there are many DNA samples that have been collected at the University of Nottingham from different regions of the world, and stored at -20°C (see appendix 3).

2.1.3 Plant growth conditions

All experimental plants including healthy and infected periwinkle plants were grown in a heated containment glasshouse (School of Biosciences,

Sutton Bonington Campus) with 16 hours day time at 28°C and 8 hours night time at 20°C.

2.2 DNA extraction

2.2.1 First method: QuickPick™ SML Plant DNA

To extract DNA, 2-3 small young leaves were taken as a sample from each plant in the glasshouse and put in a plastic bag for transport to the lab. In addition, samples were taken from healthy plants as a control. Total DNA was isolated from about 1.5 g leaf tissue. The QuickPick™ Plant DNA kit (Bio Nobile, Turku, Finland), which is based on the DNA-binding to magnetic particles was one method tested for total DNA extraction. Approximately 600 µl of lysis buffer and 25 µl of proteinase K were added to each leaf sample, and after homogenization the solution was put into a 1.5 ml tube, vortexed vigorously, incubated for 30 min at 65°C and centrifuged for 10 min at 5000 rpm in a bench top centrifuge. The supernatant was transferred to a second 1.5 ml tube, containing 5 µl magnetic particles and 125 µl binding buffer, and mixed gently then incubated at room temperature for 2-10 min. The magnetic particles were collected using the PickPen® 1-M and released into a third tube (the third, fourth and fifth tubes contained 750 µl wash buffer). The magnetic particles were washed by mixing the suspension gently for 10 – 20 seconds using the PickPen® tip. The washing steps were repeated in the fourth and fifth tubes, after which the magnetic particles were collected from the fifth tube with the PickPen® 1-M and released into tube 6 containing 50 µl elution buffer. The sixth tube was mixed continuously and incubated at room temperature for 2-10 min and the magnetic particles were then

collected from the sixth tube and discarded. The eluate in tube 6 contained the purified genomic DNA ready to be used or stored at -20°C until use.

2.2.2 Second method: CTAB buffer

This method was derived from Daire *et al.* (1997): 2-4 fresh young leaves of infected plants were taken and 800 µl of cetyltrimethyl ammonium bromide (CTAB) extraction buffer (20 mM EDTA pH 8.0, 100 mM Tris-HCl pH 8·0, 2% CTAB, 1.4 mM NaCl in sterile distilled water) was added. After homogenization at room temperature, 800 µl was transferred to a fresh tube and incubated in a waterbath at 65°C for 30 min, after which 500 µl of phenol was added to each tube, vortexed vigorously for 1 min and centrifuged 10 min at 5000 rpm in the bench top centrifuge. The supernatant (600 µl) was added to 600 µl of isopropanol, mixed and placed in the freezer for at least 2 hours. Following centrifugation for 15 min at 13000 rpm, the solution was discarded and the pellet washed with 70% ethanol and centrifuged 2-3 seconds. The ethanol wash was removed and the pellet dried for 30 min and dissolved in 50 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.6) and stored at -20°C until use.

2.2.3 Third Method: Alkaline Polyethylene Glycol-Based Method.

This method uses an alkaline PEG (polyethylene glycol) lysis reagent (Chomczynski and Rymaszewski, 2006). To prepare this buffer 60 g PEG 200 was combined with 0.93 ml 2M KOH and 39 ml water. The pH was adjusted to 13.3-13.5. Approximately 100 mg of plant material (leaves) was mixed with 1 ml PEG reagent, making sure that the amount of PEG reagent was at least 10 times that of the sample volume. Samples were incubated for 15 min at room temperature, vortexed and transferred to a 1.5-2 ml tube, after which the sample was ready to use for PCR or to store

in the freezer at -20°C (Chomczynski and Rymaszewski, 2006). This method was used successfully to extract DNA from infected samples that could be used directly in the LAMP technique to detect phytoplasmas without any further processing (Hodgetts *et al*., 2011).

2.2.4 Fourth Method: DNeasy Plant Mini Kit.

Amongst the advantages of using the DNeasy Plant Mini Kit (Qiagen Ltd, UK) to extract DNA are that it is simple, fast, does not contain a chloroform and phenol step (harmful chemicals) and has a minimum of handling steps. In this technique spin columns contain silica gel–based membrane are used which will bind the DNA. The membranes can be washed and cleaned from contaminants, after which DNA can be eluted from the membrane (column) by using water. The DNA obtained by this method will be clean and potentially more pure than DNA isolated by the other methods. However, the disadvantage of using this technique to extract DNA is the cost, approximately £3 per reaction. To use the method, sample material from infected plants (≤ 100 mg wet weight) was homogenised in a plastic bag by adding 400 µl of buffer AP1 and 4 µl of RNase A stock solution. The mixture was vortexed for 30 seconds and incubated for 10 min at 65°C. A volume of 130 µl of buffer AP2 was added to each tube, mixed gently, and incubated on ice for 5 min (this step precipitates proteins, polysaccharides, and detergent). Tubes were centrifuged for 5 min at 13000 rpm, the supernatant was transferred to the QIAshredder spin column set in a 2 ml collection tube then centrifuged for 2 min at 13000 rpm (the QIAshredder spin columns remove most cell debris and precipitates). The supernatant without pellet was transferred to a new 2 ml tube, 1.5 volumes of buffer P3 added in each tube and mixed. From this mixture, 650 µl of the mixture was put in the DNeasy mini spin

column in a 2 ml collection tube and centrifuged for 1 min at 8000 rpm. The supernatant was discarded and the last step repeated with the remaining sample and tubes centrifuged for 1 min at 8000 rpm. The DNeasy spin column was placed in a new 2 ml collection tube and 500 µl of buffer AW (wash buffer) added to the DNeasy spin column and centrifuged for 1 min at 8000 rpm. The supernatant was discarded and another 500 µl of AW added and centrifuged 2 min at 13000 rpm. The DNeasy spin column was placed in a new 2 ml collection tube, 30 µl of buffer AE added to each single DNeasy column tube which was then incubated for 5 min at room temperature, centrifuged for 1 min at 8000 rpm, and DNA samples were then ready to use or keep in the freezer at -20°C.

2.3 Using PCR to amplify DNA of phytoplasmas

Mastermixes for PCR reactions were prepared containing 12.5 µl mangomix (MangoTaq™ DNA polymerase, Bioline, USA), 10.5 µl sterile water, 1 µl forward primer (50 ng/µl) and 1 µl revise primer (50 ng/µl) per PCR reaction. Total DNA extracted by the CTAB or QiuckPick™ method was added (1 µl DNA sample to each reaction). Numerous different primer sets were used and amplification was normally carried out (unless otherwise stated in the results chapters) with an initial denaturing temperature of 94°C for 2 min, followed by 35 cycles of 94°C for 30 seconds, 53°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 10 min. For more sensitivity nested PCR was used for each primer with dilutions of the first round product 1:40 (1 µl of PCR product and 39 µl sterile water); all other PCR conditions were as before. PCR products were visualized on 1% agarose gels stained with ethidium bromide. Please see Appendix 1 for more details of all set pirmers that used in this thesis including *secA, tuf, 16S*, *rp,* and *Leu*.

2.4 Agarose gel electrophoresis

To prepare a 1 % (w/v) agarose gel, 1 g electrophoresis grade agarose (type III, Sigma) was weighed in an Erlenmeyer flask, and 100 ml of 1 x TBE buffer added (90 mM Tris-borate, 2 mM EDTA). The flask was plugged with cotton wool, and swirled to disperse the agarose then heated in a microwave oven until all the agarose has melted. The solution was cooled to 60°C, ethidium bromide added to a final concentration of 0.5 µg/ml, then poured into a casting tray containing a sample comb and allowed to solidify at room temperature. The comb was removed after the gel had solidified, and the gel was mounted horizontally into the gel electrophoresis tank, then covered with 1 x TBE buffer to about 1 mm depth. The samples for loading into the gel slots were prepared by mixing 6 µl from each sample with 2 µl of gel loading dye. Samples containing DNA mixed with loading dye were then pipetted into the sample wells. Electrophoresis was carried out for 90 V for 60 min, and the stained gel was visualised under ultra violet light examination and imaging was done on the UV (Ultraviolet radiation) transilluminator.

2.5 Grafting

Several grafting methods have been developed for phytoplasma transmission, which include top wedge, bark, whip and tongue, and chip-budding grafting. For example, the wedge method has been used to transmit phytoplasmas from infected grapevine to healthy periwinkle plants and is considerably easier to undertake than using dodder plants, and can be applied on a large scale (Tanne and Orestein, 1997).

Four-month-old healthy periwinkle plants were used for grafting by removing all stem leaves except the top 3-4 leaves with a sterile scalpel blade. Plant stems were vertically stabbed and a vertical wound opened approximately 4 cm in length. The wound was at least 5 cm above the compost.

The cut infected section from the plant shoot was inserted into the vertical wound of the healthy recipient plant and the whole wound area was wrapped by using Parafilm. Grafted plants were kept and observed in the same growing conditions as described above (see Fig 2.1).

Grafting was performed in the glasshouse with environment controls (day temperature 28°C, night temperature 22°C, and photoperiod 16 hours). Original diseased plants (0_grafting) were used as initial stocks, and healthy plants of a standard periwinkle genotype were then prepared by taking cuttings from a single mother plant. After two months the new healthy plants were ready for grafting. A small piece of diseased plant (3-5 mm thickness and 5 cm length) belonging to one of five different 16Sr groups (16SrI, 16SrII, 16SrIII, 16SrVI and 16SrX) (Table 2.2), was excised and inserted inside of the stem of a new healthy plant, and the wound was covered by wrapping with Parafilm. After 1-4 months symptoms could be observed and phytoplasmas detected by PCR methods (dependant on the season - summer or winter).

Figure 2.1: simple grafting procedures. A: four month old healthy periwinkle, B: phytoplasma grafting by inserting infected explant into the stem of healthy plant and C: covering the grafted wound by wrapping with Parafilm (Naofel Aljafer, this study).

Table 2.2: List of phytoplasma groups which have been used in grafting.

Phytoplasma name or host plant name	**16S rDNA group**	**Source**
Strawberry Green Petal (SGP)	I	Czech Republic
Faba Bean Phyllody (FBP)	II	Fera, UK
Plum Leptonecrosis (PL)- LNI	III	Fera, UK
Potato Witches' Broom (PWB)	VI	Italy

Apple Proliferation (AP)	X	Italy

2.6 Real-Time PCR (qPCR)

To validate the real-time PCR, serial dilutions of DNA samples were used. Two different methods were used, TaqMan real-time PCR and SYBR Green real-time PCR. Mastermixes were prepared by using TaqMan core reagents (Applied Biosystems) which consist of Buffer A (50 mM KCl, 10 mM Tris-HCl, pH 8.3, carboxy-X-rhodamine passive reference dye), 0.625 µl of AmpliTaq Gold, 5.5 mM $MgCl_2$, 1 µl of each primer, 1 µl of probe, and 9.75 µl of sterile water. From this, 24 µl of mastermix was placed in 96-well plates and 1 µl of DNA sample was added. Samples were run in the LightCycler Machine in the following protocol conditions; 2 min at 50°C and 10 min at 95°C, followed by 40 cycles consisting of 15 seconds at 95°C and 1 min at 60°C (Hodgetts *et al.,* 2009).

For SYBR green real-time PCR, in all reactions, 12.5 µl final volume amplifications were performed using a 1x concentration of the SYBR® Green JumpStart™ Taq ReadyMix™ (1.25 units *Taq* DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, 3.5 mM $MgCl_2$, 0.2 mM dNTP, stabilizers, JumpStart Taq antibody and SYBR Green I) (Sigma-Aldrich, Inc.), 0.5 µl DNA sample (approximately 200 ng) and the concentration of each primer varied between 50-300 nM. The thermal cycling parameters were: initial denaturation at 94°C for 2 min and 40 cycles of denaturation at 94°C for 15 seconds and annealing, extension and fluorescence reading at 65°C–69°C depending on Tm (melting temperature) of primer for 1 min.

Real-time PCR was performed with a LightCycler® 480 Instrument (Roche Diagnostic Ltd.) and the cycle threshold (C_T) value was identified using the second derivative maximum method with a LightCycler® 480 gene scanning software, version 1.5 (Roche Diagnostic Ltd.). This method identifies the C_T value of a sample as the point where the sample's fluorescence curve turns sharply upward. Melting curves of each reaction were examined in order to detect non-specific products.

Chapter 3: Evaluating different universal genes for phytoplasma diagnostics and studying genome evolution.

3.1 Introduction

One feature of phytoplasma genomes is that they often contain clusters of repeated gene sequences, named sequence-variable mosaics (Jomantiene *et al.,* 2007) or potential mobile units (PMUs) (Bai *et al.,* 2006). For example, the genome of '*Ca.* P. asteris' has PMUs that are approximately 20 kb in size, and consist of genes with similarity to *hflB, sigF, dnaB, dnaG, ssb, tmk, himA,* and the *IS3* family insertion sequence tra5 (Bai *et al.,* 2006). In addition, these genes have been found in multiple copies in other phytoplasma genomes in clusters or singly (Oshima *et al.,* 2013). One strategy that phytoplasmas are believed to use to adapt to different environments is the regulation of expression of PMU genes, and that expression of these genes is associated with a process that usually involves circularization and replicative transposition (Oshima *et al.,* 2013). Additionally, the expansions and deletions of PMUs might play a role in genome rearrangements, which increase the ability of phytoplasmas to adapt to diverse hosts and can be a major evolutionary factor allowing them to adapt to different insect vectors or to occupy broad plant host ranges (Oshima *et al.,* 2013). The ability of PMUs to move through phytoplasma genomes can affect genome sequences by reflecting on position of genes, size of genes, and sequences of genes.

In this chapter, primers for different phytoplasma genes, *16S* rRNA, *secA*, *tuf*, and *rp*, have been used to screen different isolates from glasshouse-grown plants and from DNA samples that have been obtained from a range of worldwide isolates over the years at the University of Nottingham and stored in the freezer. The aim of this part of the work was to determine which assays are most effective for different phylogenetic groups, which primers are universal, and also which ones are more sensitive versus which ones have to be used in nested PCR. The second aim of this chapter was to use these primers and those obtained in Chapter 4 for other genes to determine whether they can be used to examine genome evolution of phytoplasmas over time. The experiment was to transfer the phytoplasmas into fresh healthy periwinkle plants at 3-4 monthly intervals throughout the project, and then examine whether there were any changes in the genome sequences from one generation to the next. Grafts of plants from 16Sr groups I, II, III, VI and X were used, and the PCR approach on a range of genes was used as opposed to whole genome sequencing because of costs and because draft genome sequences are not available for most of these phylogenetic groups. Once the phytoplasmas had re-established, DNA was re-extracted and the 16S rRNA, *secA, tuf, rp* and leucyl tRNA synthetase genes were amplified and sequenced, to determine whether there was any evidence of genome evolution over time.

3.2 Materials and Methods

3.2.1 Grafting

Grafting was done in the glasshouse (the University of Nottingham) over two years (2012-2014). The environment in the glasshouse was controlled (day temperature 28°C, night temperature 22°C, and photoperiod 16 hours). The first generation was started as initial stocks from original

diseased plants (0_grafting), and fresh healthy plants were prepared by taking cuttings from a single healthy plant of a standard periwinkle genotype. The healthy plants were ready for grafting after 2-3 months. From plants of each of five different 16Sr groups (16SrI, 16SrII, 16SrIII, 16SrVI and 16SrX) (see Table 2.2) a small piece of diseased plant (3-5 mm thickness and 5 cm length) was cut and inserted inside of the stem of the new fresh healthy plant, after which the wound was covered by wrapping with Parafilm. Symptoms could be observed within 1-4 months and detection of phytoplasmas was done by PCR methods.

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3.2.2 DNA extraction

A range of methods, as outlined in chapter 2 were used to extract phytoplasma DNA from diseased and healthy plants, to determine which method was optimal for both PCR and LAMP, in terms of speed, reduced number of manual handling stages to reduce chances of contamination, and cost. The methods tested were the QuickPick™ SML Plant DNA kit, CTAB buffer, Alkaline Polyethylene Glycol-Based extraction and the DNeasy Plant Mini Kit.

3.2.3 Using PCR to amplify DNA of phytoplasmas

For PCR reactions, general mixes were prepared with 12.5 µl mangomix (MangoTaq™ DNA Polymerase), 10.5 µl sterile water, 1 µl forward primer and 1 µl reverse primer (concentration of each primer is 50 ng / µl) per PCR reaction. Total DNA was extracted from plants by different methods (see chapter 2) and was normally added as 1 µl DNA sample to each

reaction (total DNA concentrations were not generally determined since these give no indication of the concentration of phytoplasma DNA). Four different primer sets were used initially (16S rRNA, rp, tuf and secA), and amplification was carried out with an initial denaturing temperature of 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 53°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 10 min. For more sensitivity, nested PCR was used for each primer set with dilutions of the first round product 1:40 (1 µl of PCR product and 40 µl sterile water); all other PCR conditions were as before. PCR products were visualized on 1% agarose gels stained with ethidium bromide.

3.2.4 Molecular Evolutionary Genetics Analysis (MEGA)

Software: Molecular Evolutionary Genetics Analysis (MEGA) version 5 (http:// http://www.megasoftware.net/) was used for constructing multiple sequence alignments, distance matrix-based phylogenetic trees, maximum parsimony phylogenetic trees, maximum likelihood phylogenetic trees etc.

3.3 Results

3.3.1 Evaluating different genes 16S rRNA, secA, tuf, and rp.

Tests were performed using 16S rRNA, secA, tuf, and rp primer sets on many DNA samples that had been collected over the years at the University of Nottingham from different regions of the world, and are listed

in Table 3.2. Results of first round and nested PCR for representative samples are shown in Figures 3.1-3.4. Note that the negative controls used in these experiments were healthy Napier grass samples (first sample in table 3.2.

Table 3.1. List of DNA samples used for analysis

Extract No	Isolate	Group	DNA isolation date (if known)	Primer			
				16S	Tuf	SecA	Rp
1	Napier grass control plant (healthy)	control	Prior to 2006	–	–	–	–
2	Aster yellows dwarf	I	Prior to 2006	+	+	–	+
3	Hydrangea MLO	I	Prior to 2006	–	+	–	+
4	Aster yellows severe	I	Prior to 2006	–	+	–	–
5	Aster yellows gladiolus	I	Prior to 2006	+	+	+	+
6	Strawberry green petal	I	17/1/2007	+	+	+	+
7	Aster yellows	I-L	Unknown	+	+	+	+
8	Rehmannia glutinosa	I-B	25/10/2006	–	+	+	–
9	Rehmannia glutinosa2	I-B	30/10/2006	+	+	+	+
10	Faba bean phyllody	II	23/3/2006	+	+	+	+

11	Faba bean phyllody	II	31/10/2007	**+**	**+**	**+**	**+**
12	Lime witches' broom	II	3/7/2006	**–**	**+**	**–**	**–**
13	Poinsettia branching factor	III	16/1/2008	**–**	**+**	**+**	**+**
14	Peach X disease	III-A	28/2/2008	**–**	**+**	**+**	**+**
15	Elm witches broom (ULW)	V	28/2/2008	**–**	**+**	**+**	**+**
16	Flower stunting	XI	Unknown	**+**	**+**	**+**	**+**
17	Cordyline isolate from Cornwall, UK	XII	unknown	**+**	**+**	**+**	**–**
18	Candidatus phytoplasma fragariae, (Jersey)	XII	1/9/2006	**+**	**+**	**+**	**+**
19	Stolbur of potato (Romania)	XII	1/9/2006	**+**	**+**	**+**	**–**
20	Stolbur	XII	Unknown	**+**	**+**	**+**	**–**
21	Stolbur (Stol)	XII	28/2/2008	**+**	**+**	**+**	**+**
22	CSPWD (Ghana) isolate GH8	XXII	16/10/1992	**–**	**+**	**+**	**+**
23	CSPWD (Ghana) isolate 54	XXII	16/10/1992	**+**	**+**	**+**	**+**

24	CSPWD (Ghana) isolate Busua	XXII	27/2/1994	**+**	**+**	**+**	**+**
25	CSPWD (Ghana) isolate Ghana 6	XXII	4/12/1992	**+**	**+**	**+**	**+**
26	AWKA (Nigeria)	XXII	11/12/1992	**+**	**+**	**+**	**+**
27	AWKA (Nigeria)	XXII	17/8/1994	**+**	**–**	**+**	**+**
28	Tanzanian coconut lethal decline – Chambezi isolate	XXX	24/2/1993	**–**	**+**	**+**	**+**
29	Kenya coconut lethal disease	XXX	30/3/1993	**+**	**+**	**+**	**+**

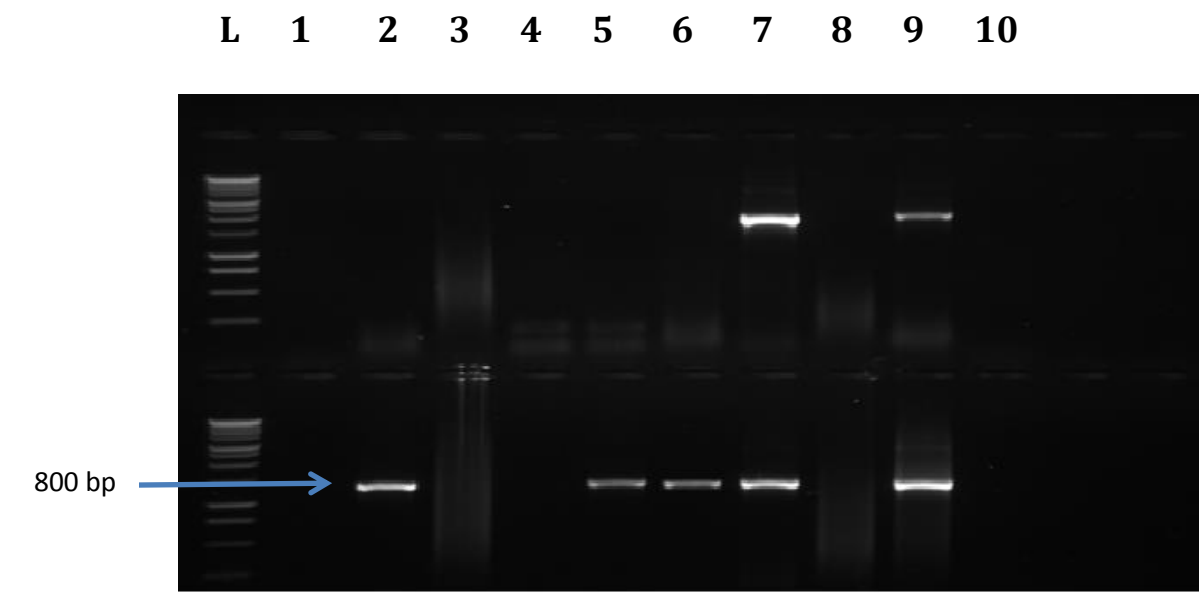


Figure 3.1 Polymerase chain reaction amplifications of representative DNA samples with 16Sr RNA primers. L: 1kb ladder; 1 - 9 (sample numbers 1 to 9 of table 3.1) amplification of phytoplasma (first round PCR) using P1/P7 primers; (second round PCR) using 16SF2/16SR2 Primers. Lane 10: Control samples (healthy periwinkle plant). Figure shows that sample numbers 2, 5, 6, 7, and 9 are positive (band sizes about 800 bp), and the remaining samples plus negative control show no bands.

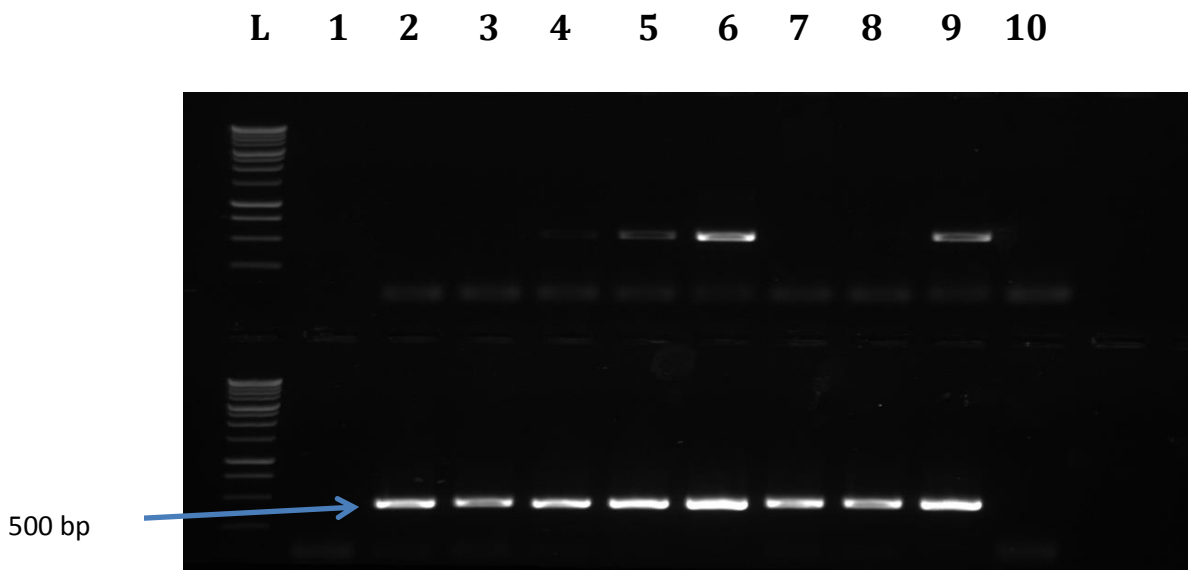


Figure 3.2 PCR amplifications of representative DNA samples with *tuf* primers. L: 1kb ladder; 1 - 9 phytoplasma samples (sample numbers from 1 to 9 in table 3.1) (first round PCR) using Tuf340/Tuf890 Primers; (second round PCR) using Tuf400/Tuf835 Primers. Lane 10: Control samples (healthy periwinkle plant). Figure shows that all samples apart from

sample number 1 and the healthy periwinkle plant are positive with a band size of about 500 bp.

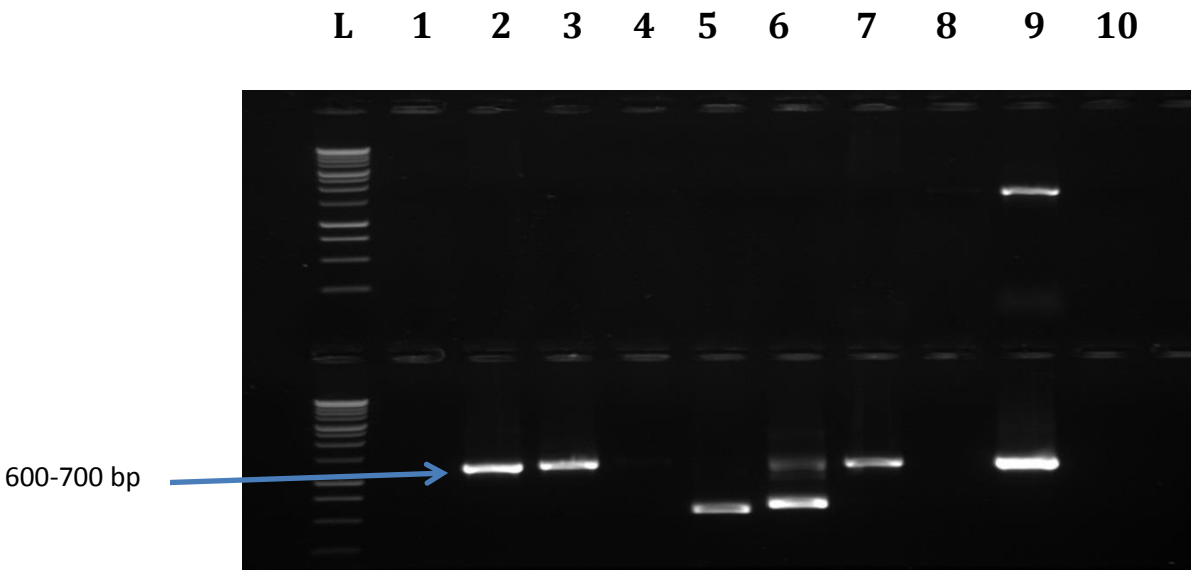


Figure 3.3: PCR amplifications of representative DNA samples with *rp* primers. L: 1kb ladder; 1 - 9 phytoplasma samples (sample numbers from 1 to 9 in table 3.1) (first round PCR) using $RPL_2F_3/Rp_{(1)}RIA$ Primers; (second round PCR) using RpFIC/ $Rp_{(1)}RIA$ Primers. Lane 10: control sample (healthy periwinkle plant). Figure shows that only sample numbers 1, 4, 8 and the control sample are negative (no bands) and the remaining samples are positive with a band size of about 600 – 700 bp. Sample numbers 5 and 6 show a different band size compared with other samples.

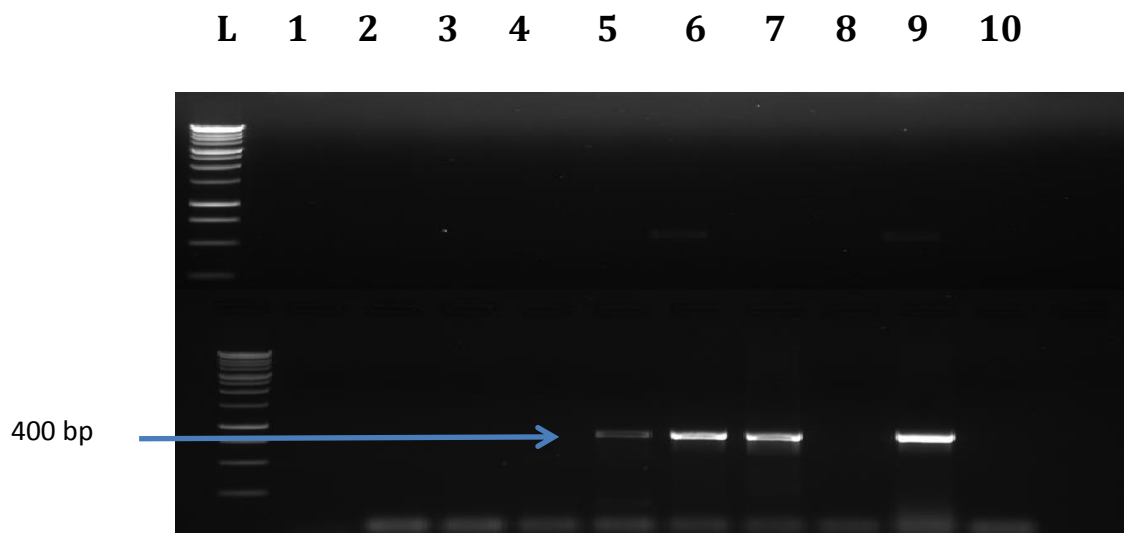


Figure 3.4: PCR amplifications of representative DNA samples with *secA* primers. L: 1kb ladder; 1 - 9 phytoplasma samples (sample numbers from 1 to 9 in table 3.1) (first round PCR) using SecAF1/SecAR3 Primers;

(second round PCR) using SecAF5/SecAR2 Primers. Lane 10: control sample (healthy periwinkle plant). Figure shows that only four samples (5, 6, 7, and 9) are positive (band size about 400 bp) and the other samples and the negative control show no band.

3.3.2 Phylogenetic analysis of isolates based on the different primer sets

Positive PCR samples with the different primer sets were purified and sequenced (all sequences for all samples that were obtained with the different universal primers are listed in appendix 5), and the sequences were then aligned into phylogenetic trees for each primer set using MEGA 5 software (Figs 3.5 - 3.8). This combined data can be used to evaluate which of the different primers can potentially be used as universal primers and which appear to be more group specific. For example, all the four different primers can detect and classify all samples from group 16SrI and XXII, but not all of them can detect and classify samples from group 16SrXXX (see Figs 3.5 – 3.8). For some samples it was difficult to obtain good quality sequences, for reasons that are unclear (although it is possible that some of the DNA samples had become contaminated with DNA from other isolates over the years in storage, which would have resulted in mixed PCR products, that would not have been resolved by direct sequencing), so not all positive samples for different set of primers in table 3.1 are in the phylogenetic trees.

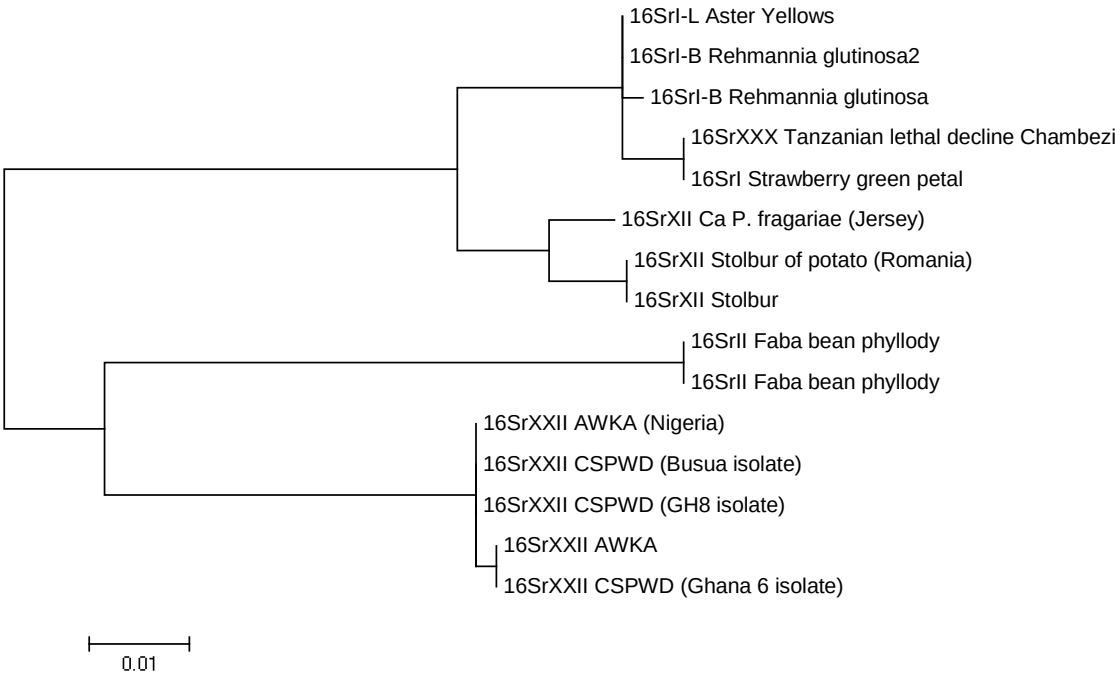


Figure 3.5: Phylogenetic distance tree of groups of phytoplasmas with the 16S rRNA primers. This gene shows a good alignment for samples that belong to specific groups, for example 16SrXII and 16SrXXII, but no reliable sequences were amplified from groups 16SrIII and 16Sr XI.

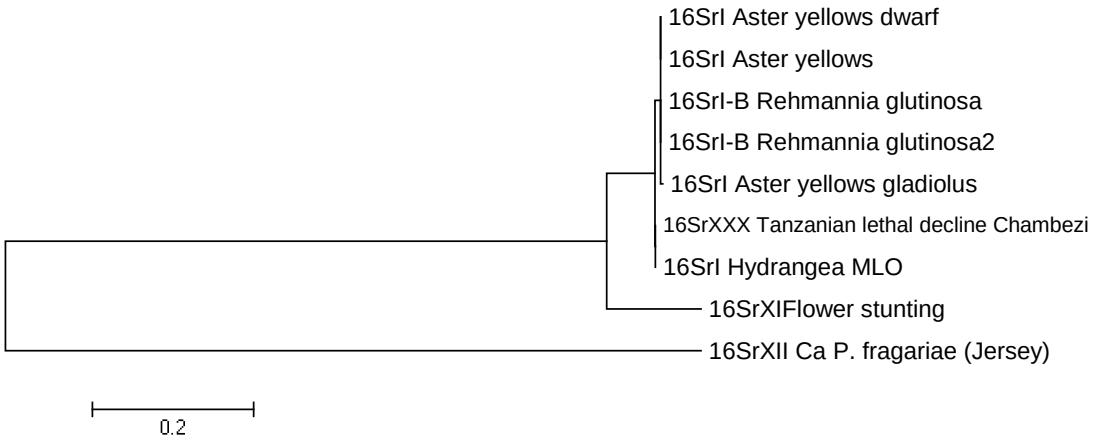


Figure 3.6: Phylogenetic distance tree of phytoplasma groups with *rp* primers. This set of primers was not able to amplify from a number of different groups, for example groups 16SrXXII and 16SrII.

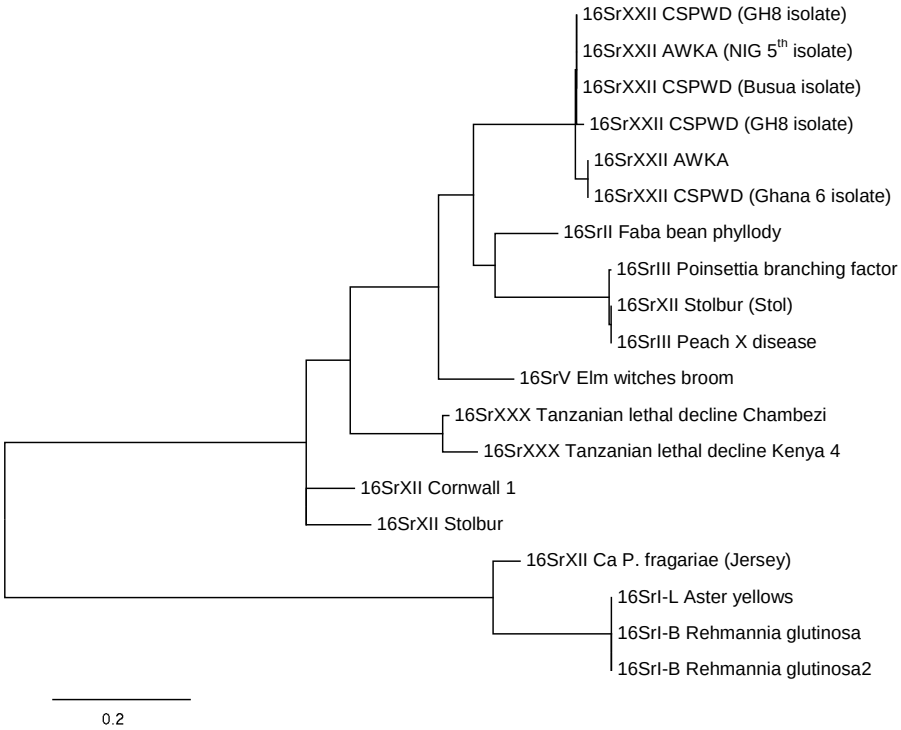


Figure 3.7: Phylogenetic distance tree of phytoplasma groups with *secA* primers. The *secA* primers amplified from most of the different groups and put them in very clear separate branches of the phylogenetic tree.

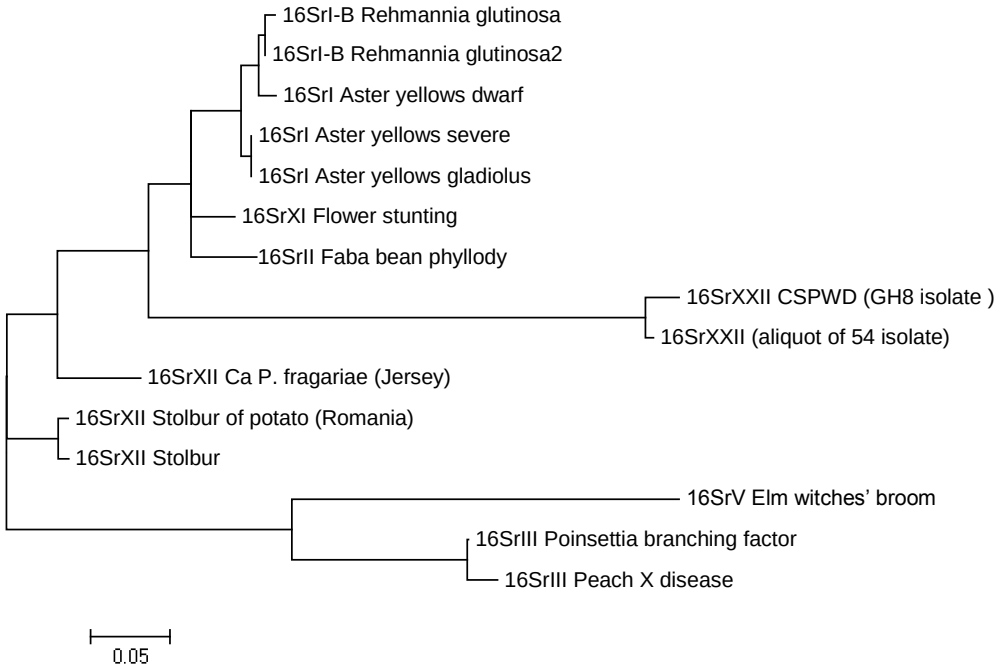


Figure 3.8: Phylogenetic distance tree of phytoplasma groups with *tuf* primers. This set of 13 mixed primers was able to amplify sequences for alignment from all 16Sr groups.

3.3.3 Studying genomic evolution

Infected plants from different phylogenetic groups (listed in Table 2.2) were established and DNA extracted. The first grafting into new healthy plants was performed on 01/02/2012 and once disease symptoms had established DNA was extracted from these new grafts. Second grafting was on 1/10/2012, third grafting was on 20/01/2013, fourth was in summer 2013 and the fifth one was done in autumn 2013. In each case DNA was extracted once symptoms had established and was stored prior to PCR amplification. The intention was that as new primers became available (eg the leucyl tRNA synthetase primer set developed in chapter 4) these could also be used on the DNA samples so that additional genes could be studied to attempt to find evidence of instability in the phytoplasma genomes, and evidence that they were evolving with time.

PCR reactions were done for 16S rRNA, *tuf, secA, rp,* and leucyl tRNA synthetase genes to amplify phytoplasma DNA. After sequences had been obtained for each gene and blasted on the NCBI website, it was discovered that the *rp* gene can amplify plant DNA as well as phytoplasma DNA for some samples, which caused problems in analyses, so it was decided to remove this gene (*rp*) from the analysis. Alignments of sequences from each generation for each gene set were examined using MEGA 5.2 software. It was found that for all the genes tested, the sequences were 100% identical after all generations (results not shown), and no evidence could be found of any genetic instability or random mutations occurring in any of the genes tested.

3.4 Discussion

In these studies, four different DNA extraction methods were tested and found to show no difference in their capacity to extract phytoplamsa DNA for use in PCR reactions. It was found that two methods (the QuickPick™ SML Plant DNA kit and the Alkaline Polyethylene Glycol-based method) took less than one hour to extract DNA, which potentially enabled a quick decision to be made on whether the phytoplasma had re-established inside grafted plants or not. However, these two methods did not give the best quality DNA, especially for samples with very low phytoplasma concentration. The DNeasy kit gave very good quality DNA that worked reliably in PCR, but it is expensive compared to the other methods. It is therefore probably the best method for obtaining high quality DNA for long-term storage and use in further experiments, but not when large numbers of samples need to be tested quickly. The fourth method using CTAB buffer is a cheaper method that is probably better to use when many samples need to be done at the same time, as it is more reliable than the first two method (QuickPick™ SML Plant DNA and Alkaline Polyethylene Glycol-based).

To evaluate different primer sets, the first gene used was 16S rRNA. As can be seen in Figure 3.1, these primers gave good amplifications for most groups, but gave negative results for some samples, eg some from groups 16SrII, 16SrXXII, and 16SrXXX. Generally, the phylogenetic analysis was consistent with expected positioning of the isolates into groups (Hodgetts *et al.,* 2008), except for isolate 16SrXXX Tanzanian lethal decline, which appears to have become contaminated with 16SrI aster yellows DNA (since it also appears in the 16SrI group in the rp tree). The reasons for this contamination could be down to human error since numerous people have had access to these samples over the years and this may have resulted in

contamination of this DNA sample with another DNA sample which belongs to 16SrI group.

The second gene tested was the ribosomal protein (*rp*), and fewer samples were positive with this primer set as shown in Table 3.2. For example samples Cordyline from Cornwall and Stolbur of potato (Romania) were negative. In addition, these primers gave two different band sizes (see Fig. 3.3 samples 5 & 6). Further analysis of some of these sequences showed that the rp primers amplified plant DNA (after blasting the sequences on the NCBI website, it was found those sequences belonged to the phenox genus) from some samples rather than phytoplasma DNA, suggesting that the primers lack the specificity required to be considered as universal primers. Based on the results shown in the phylogenetic tree (Fig. 3.6), it appears that these primers are effective on the 16SrI aster yellows group of phyoplasmas, but are of limited use on phytoplasmas from other phylogenetic groups.

The third gene tested was the *tuf* gene, and all phytoplasma samples from Table 3.1 samples from 2 to 29 were positive except one sample of Awka from Nigeria. This, combined with the phylogenetic tree analysis shown in Figure 3.8, suggests that the *tuf* gene primers amplify from a broad range of phytoplasma phylogenetic groups and could be considered as universal primers. However, it should be that whilst the 16S rRNA gene universal primers are a set of four primers (two for first round and two for the nested PCR), the *tuf* gene universal primers are a set of 13 primers. That is presumably why they can amplify from a high number of phytoplasma groups relative to other 'universal' primer sets.

The fourth gene tested was the *secA* gene. This also gave good results with phytoplasma samples from most phylogenetic groups, and resulted in good discrimination between different groups in the phylogenetic analysis,

consistent with the results of Hodgetts *et al.* (2008). In addition, the secA primer set is generally just a set of two primers for first round PCR and two primers for the nested PCR, and although the sequence amplified by these primers is much shorter than the sequence amplified by the 16S rRNA gene primers, because they come from a coding sequence, they give much better resolution between different phylogenetic groups.

To summarise the evaluation of different universal primers, it is proposed that whilst 16S rRNA gene primers have been the standard for discrimination between different groups and subgroups of phytoplasmas in the past, there are now additional sets of universal primers that can be used to provide better resolution, and form the basis of multi-locus sequence typing (MLST) of these organisms. The *tuf* gene primers, although a complex set of 13 primers, amplify from most groups, as do the *secA* gene primers. Furthermore, results from chapter also showed that the newly designed leucyl tRNA synthetase primers can also be added to this list of universal primers for MLST analysis. The results also showed that caution needs to be used with some sets of primers that have been suggested for use in the past. For example, the *rp* gene primers showed no amplification from many samples tested and in other cases showed poor specificity and amplified plant DNA.

Having identified universal primers, these were used to determine their value in studies of genome evolution. However, no evidence was found that these genes are showing rapid signs of change through successive generations within infected plants. Previous studies have suggested the presence of sequence-variable mosaics (Jomantiene *et al.,* 2007) or potential mobile units (PMUs) (Bai *et al.,* 2006) that can move and change their positions in phytoplasma genomes. These may be mechanisms that generate genome diversity in phytoplasmas, and the sequences of coding genes such as *secA* indicate a high level of diversity in sequence between

isolates from different phylogenetic groups and subgroups. However, much more sophisticated and better resourced studies, in which phytoplasmas are passaged through plants and insects over multiple generations, and whole genome sequences are analysed, will be required to further our understanding of phytoplasma genome evolution The systematic isolation and storage of DNA undertaken in this study starts to provide a resource that could be used in such future studies.

Chapter 4: Identifying primers for additional genes to use in genome evolution studies.

4.1 Introduction

In the last two decades most research toward the detection and classification of phytoplasmas has used nucleic acid-based techniques such as PCR, which are used to amplify regions of phytoplasma genomes existing inside infected plants. Researchers have tried to obtain sequences of specific regions of phytoplasma DNA and design primers for these regions. Some of the design has involved specific primers for a particular phylogenetic group, but many are trying to design universal or semi universal primers to detect all groups of phytoplasma or at least most of these groups, of which there are at least 33 currently recognised groups and 40 subgroups (Bertaccini *et al.,* 2014). Because of the low concentration of phytoplasma DNA in plants, most laboratories use universal primers in the first round of PCR reactions and then use another internal primer (second round) which may be universal or group-specific (nested PCR) to improve the sensitivity. Ribosomal RNA gene primers, which amplify specific fragments of phytoplasma DNA (the 16S rRNA gene), are the most commonly used sets of universal primers currently being used to detect and classify phytoplasmas. However, to increase the resolution of phylogenetic groupings, there are also universal or semi-universal primers for other genes such as the *rp* (ribosomal operon), *secA, tuf, secY, hlg, gyr* and the nitroreductase genes (Chuang *et al.,* 2000; Hodgetts *et al.,* 2008).

In this Chapter, the aim is to design universal primers for additional phytoplasma genes that could be used for development of diagnostics, phylogenetic analyses and also for looking at phytoplasma evolution.

4.2 Materials and Methods

4.2.1 Preparation of phytoplasma nucleic acids and PCR reactions.

DNA of phytoplasmas from diseased plants was extracted mainly by the CTAB method (see chapter 2). The leaves of infected plants were ground in 1.6 ml CTAB, and 800 µl of the plant extracts were incubated at 65°C for 30 min. Protein and cell debris were removed by phenol chloroform extraction. DNA samples were precipitated by isopropanol and centrifugation. DNA pellets were dissolved by 50 µl TE buffer and stored at -20°C.

PCR reactions were performed using 1x concentration of the MangoMix™ (Bioline Ltd, UK), 1 µl of DNA sample and 400 nM of each primer (first round:LeuF1 and LeuR1; second round:LeuF2 and LeuR2) (see appendix 1). The PCR conditions were initial denaturing temperature of 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 53°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 10 min. For more sensitivity, nested PCR was used for each primer set with dilutions of the first round product 1:40 (1 µl of PCR product and 39 µl sterile water); all other PCR conditions were as before. PCR products were imaged on 1% agarose gels stained with ethidium bromide.

4.2.2 Primer design from sequence alignments

Current work at the University of Nottingham is aimed at whole genome sequencing of the 16SrXI Napier Grass Stunt phytoplasma. This draft sequence was used for the design of new universal primers, and for selected genes, the sequences were aligned to the equivalent genes from the published 16SrI Onion yellows and Aster yellows witches' broom, 16SrXII 'Ca. Phytoplasma australiense', 16SrX Apple proliferation and *Acholeplasma laidlawii* by using MEGA5.2 software. Four different genes were selected for the initial analysis, leucyl tRNA synthetase, alanyl tRNA synthetase, RNA polymerase 1 and RNA polymerase 2.

To validate the new primer sequences, fourteen diverse phytoplasma samples were initially used, which belonged to seven different phylogenetic groups, along with a healthy periwinkle plant sample as control (Table 4.1).

Table 4.1. List of diseased plant samples tested by direct and nested PCR using the new primers (leucyl tRNA synthetase results shown).

Number	Samples of infected plants	16Sr group	Direct PCR	Nested PCR
1	Rehmannia glutinosa (RG)	I-B	+	+
2	Ribes in Vinca (RIV)	I-C	-	+
3	Strawberry Green Petal (SGP)	I-C	+	+

4	Crotalaria Saltiana Phyllody (witches broom) (CSP)	II-C	+	+
5	Faba Bean Phyllody (FBP)	II-C	+	+
6	Soybean Phyllody (SP)	II-C	-	+
7	Vinca Coconut Phyllody (VCP)	II	+	+
8	Peach Western X (PWX)	III-A	-	+
9	Plum Leptonecrosis (PL)- (LNI)	III-A	-	+
10	Potato Witches Broom (PWB)	VI-A	-	+
11	Brinjal Little Leaf (BLL)	VI-A	-	+
12	Apple proliferation (AP)	X-A	-	+
13	Elm Yellows (EY-C)	V-A	-	+
14	Napier Grass Stunt (NGS)	XI	-	+
15	Periwinkle Healthy plant		-	-

At the same time, reactions were performed on healthy control plants to evaluate whether the new sets of universal primers were able to amplify from different host plant DNAs or not. A list of the healthy plant samples tested (representative of the natural hosts for the key phytoplasmas used in this study) are listed in Table 4.2.

Table 4.2 list of healthy plant samples tested by COX primers and leucyl tRNA synthetase primers.

Serial number	Healthy plant sample	Soruce
1	Periwinkle (2011 sample from frozen DNA stock)	Nottingham, UK
2	Periwinkle fresh sample	Nottingham, UK
3	Coconut	Ghana
4	Oil palm	Malaysia
5	Wheat	Nottingham, UK
6	Barley	Nottingham, UK
7	Potato	Nottingham, UK
8	Tomato	Nottingham, UK
9	Apple	Nottingham, UK

4.2.3 Phylogenetic analysis

Phylogenetic trees were constructed by using the leucyl tRNA synthetase gene sequences. Alignments of sequences were performed using MEGA version 5.2 and also phylogenetic and molecular evolutionary analysis (Tamura *et al.,* 2011). Many samples were tested with the new gene primers which belonged to different phylogenetic groups from different countries. Phylogenetic trees developed from the leucyl tRNA synthetase were compared with trees based on the 16S rRNA and *secA* genes for the same or closely related phytoplasmas.

4.3 Results

4.3.1 Primer design for the new genes

To date, phylogenetic analyses of phytoplasmas have generally been based on the 16S rRNA gene, because universal primers that work on all phylogenetic groups are readily available for this gene. However, this gene only shows relatively limited genetic diversity between different 16Sr subgroups. For better resolution of phytoplasma subgroups, primers have been designed for a limited number of other genes but most of these do not appear to be universal. Therefore, the aim of this study was to attempt to develop an alternative set of universal primers that could be used for improved phylogenetic analyses. To achieve this, genes from the recently sequenced 16SrXI Napier grass stunt genome (Praphat Kawicha, University of Nottingham, personal communication) that were also present in the already sequenced 16SrI, 16SrX and 16SrXII genomes were selected, on the grounds that 16SrXI is probably about as different as it is possible to get from these groups. Therefore if we could find sequences to design primers that were common between these very diverse phylogenetic groups, it is possible that such primers would also work on all the other as yet unsequenced phylogenetic groups. Such potential primers were then analysed against the *Acholeplasma laidlawii* sequence to rule out any that might not be specific to just phytoplasmas. Sequences were then aligned using MEGA 5.2 software (http://www.megasoftware.net/).

Following use of the MEGA 5.2 software (see Figs 4.1 and 4.2), the following primers were designed for the leucyl tRNA synthetase and RNA polymerase I genes. No obvious regions were found in the alanyl tRNA synthetase or RNA polymerase II genes for primer design, since there was

too much divergence between sequences from the different phylogenetic groups, or the regions of commonality were too AT rich for effective primer design, so these two genes were not investigated any further.

Leu primer: For Direct PCR (PCR product size is 1460 bp)

- LeuF1 5' GAT ATG TTT CCT TAT CCT TC 3'
- LeuR1 5' TAC CAA GAR CTT CCW GC 3'

For Nested PCR:

- LeuF2 5' CAT CCT TTT GGT TGG GAT TC 3'
- LeuR2 5' CTS CCC AAT ATC TTT GRC G 3'

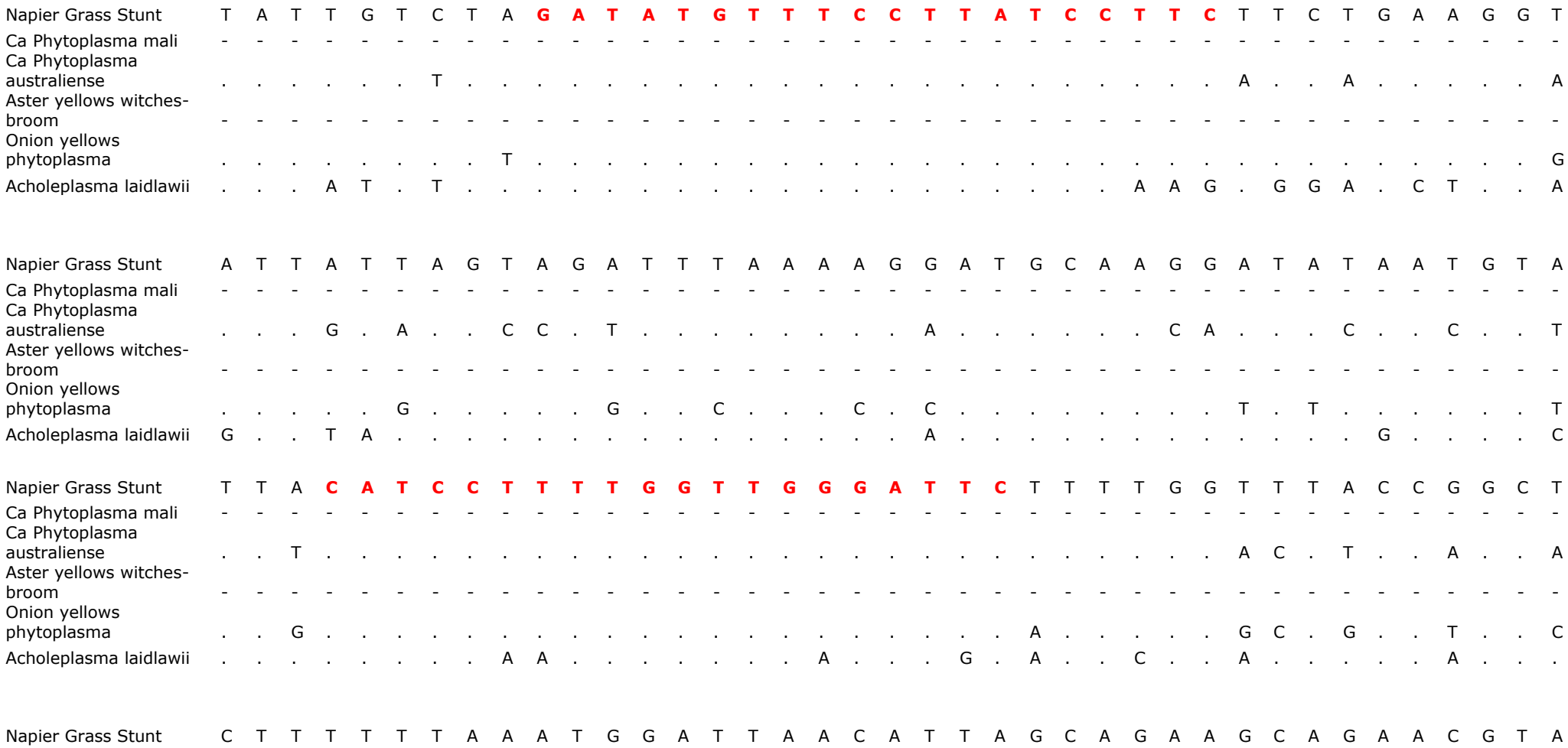
Pol primer: For Direct PCR (PCR product size is 1130 bp)

- PolF1 5' GAC ART GGT CTT ATG GTG 3'
- PolR1 5' CCR CCN GTA TGR AAA GTT CTC 3'

For Nested PCR:

- PolF2 5' GGA TGG TWA TGG ANG TTA TTC C 3'
- PolR2 5' GGT TCW CCR ATN GAT TGA GC 3'

Figure 4.1 The alignments for the leucyl tRNA synthetase gene and primers are marked by red colour.



Ca Phytoplasma mali	A	.	.	.	.	.	.	.	G	.	.	.	.	T	.	.	.	.	.	T	A	A	C	.	A	.	A	C	.	.	.	T	A	.	.	A	T	A	.
Ca Phytoplasma australiense	A	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	G	A	C	A	A	C	A	A	T	C	.	.	.	.	.	C	.	.	A	C	A	.
Aster yellows witches-broom	T	C	G	C	A	G	.	.	C	.	.	C	.	G	C	G	C	C	A	T	A	.	C	.	.	G	C	.	.	.	.	.	T	T	.	A	.	G	.
Onion yellows phytoplasma	A	.	.	.	.	.	.	.	.	.	.	.	.	T	C	.	T	.	A	T	A	A	C	.	A	.	C	.	.	.	.	T	A	.	.	A	C	.	.
Acholeplasma laidlawii	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Napier Grass Stunt	A	A	-	-	-	-	-	A	T	A	A	T	A	A	A	A	T	T	T	T	C	A	A	A	A	A	T	A	A	A	A	A	A	A	A	A	T	G	G
Ca Phytoplasma mali	.	.	-	-	-	-	-	.	.	T	.	.	T	G	.	.	.	.	.	.	T	.	G	.	.	.	A	.	.	.	T	.	.	.	T	T	A	.	.
Ca Phytoplasma australiense	.	G	-	-	-	-	-	.	.	G	.	.	G	C	.	.	.	.	.	.	T	.	.	.	.	G	A	.	.	.	.	.	.	.	T	T	A	.	C
Aster yellows witches-broom	.	G	C	A	C	T	T	.	C	T	C	C	G	T	T	G	C	C	.	.	T	G	G	.	T	T	.	G	G	.	C	.	T	T	T	T	A	.	-
Onion yellows phytoplasma	.	.	-	-	-	-	-	.	.	T	T	.	G	C	.	.	.	.	.	.	T	.	G	.	.	.	A	.	.	.	T	.	.	T	C	.	C	.	.
Acholeplasma laidlawii	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Napier Grass Stunt	T	T	A	T	A	T	T	T	A	T	T	C	T	A	C	T	T	A	T	C	A	A	A	T	G	C	A	T	G	A	T	T	G	G	G	T	T	T	T
Ca Phytoplasma mali	.	.	.	.	.	.	.	C	.	.	A	A	.	.	T	.	.	.	.	A	.	.	T	.	.	.	.	.	.	.	.	.	A	T	T	.	.	.	.
Ca Phytoplasma australiense	C	.	.	.	C	C	.	C	.	.	.	A	.	.	.	.	.	.	.	A	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Aster yellows witches-broom	-	.	G	.	G	A	.	C	.	.	G	T	C	C	.	A	A	.	A	T	.	.	T	A	C	.	T	.	.	.	.	.	A	A	C	.	A	A	.
Onion yellows phytoplasma	G	.	.	C	T	C	.	C	.	.	.	A	.	.	.	.	.	.	.	A	.	.	T	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.
Acholeplasma laidlawii	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Napier Grass Stunt	T	T	C	T	**C**	**G**	**T**	**C**	**A**	**A**	**A**	**G**	**A**	**T**	**A**	**T**	**T**	**G**	**G**	**G**	**G**	**A**	**G**	A	G	C	C	T	T	T	T	C	C	T	G	T	T	T	T
Ca Phytoplasma mali	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	A	.	.	A	.	.
Ca Phytoplasma australiense	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	A	.	.	A	T	.	.	A	.	.	.	.
Aster yellows witches-broom	.	.	T	-	T	.	A	A	.	.	G	.	T	.	C	.	.	T	.	.	T	.	.	.	A	A	.	C	A	A	A	.	.	C	A	G	A	.	C

Onion yellows phytoplasma	.	.	.	G	.	.	C	.	.	.	C	.	.	.	.	.	.	.	.	.	C	.	.	.	A	.	.	C	.	.	C	.	.	C	A	.	.	.	A
Acholeplasma laidlawii	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Napier Grass Stunt	A	A	A	A	G	T	A	T	A	A	A	A	G	A	G	A	-	T	T	C	A	A	A	T	A	C	T	A	T	G	C	C	T	C	A	A	G	T	A
Ca Phytoplasma mali	.	.	.	.	A	.	.	.	C	G	.	.	.	.	.	.	-	.	G	.	T	.	.	.	.	.	.	.	.	.	T	.	A	.	.	.	T	.	G
Ca Phytoplasma australiense	.	.	.	.	.	.	.	C	C	G	.	.	.	.	.	.	-	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	C	.	T
Aster yellows witches-broom	.	.	.	T	A	.	.	G	C	C	.	.	T	.	T	.	G	.	A	.	C	.	.	G	.	G	C	T	A	C	.	T	G	.	.	.	.	.	T
Onion yellows phytoplasma	.	.	.	.	A	.	.	.	C	G	C	.	.	.	.	.	-	.	.	.	T	.	.	C	.	.	.	.	.	.	.	.	A	.	.	.	C	.	T
Acholeplasma laidlawii	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Napier Grass Stunt	**G**	**C**	**T**	**G**	**G**	**A**	**A**	**G**	**T**	**T**	**C**	**T**	**T**	**G**	**G**	**T**	**A**	T	T	A	T	A	T	T	G	G	T	T	A	T	A	T	T	T	T	A	A	A	A
Ca Phytoplasma mali	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	A	.	C	.	.	.	.	.	.	.	.	.	G	.	.	.
Ca Phytoplasma australiense	.	.	A	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	G	.	.	.	.	.	.	.	.
Aster yellows witches-broom	.	T	-	.	.	C	.	-	-	.	G	G	.	.	T	.	.	C	A	.	.	C	.	C	T	.	C	G	.	.	.	.	.	.	.	T	T	G	C
Onion yellows phytoplasma	.	.	A	.	.	T	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	G	.	.	.
Acholeplasma laidlawii	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

The same analysis was done for the *pol* gene, and the alignment of the *pol* gene is shown in appendix 4.

4.3.2 Validation tests

The primers were tested using standard PCR conditions and a range of annealing temperatures to optimise the amplification. These tests indicated that an annealing temperature of 53°C was optimal for the leucyl tRNA synthetase gene primers (first round LeuF1/LeuR1, and second round LeuF2/LeuR2), whilst no temperature gave reliable amplification products for the RNA polymerase I gene. Having validated the Leu primers, they were then used to amplify products from the samples listed in Table 4.1. Representative results are shown in Figure 4.3.

When direct PCR primers were used for this gene (LeuF1/LeuR1) PCR products of around 900/1000 bp were sometimes amplified, but use of the LeuF2/LeuR2 in nested PCR was necessary for amplifications from all the phytoplasmas that were tested as shown in Table 4.3 and Figure 4.3. When these primers were initially used with samples from coconut, no products were amplified, so a new forward primer was designed for nested PCR that appears to cover all remaining phytoplasma groups (LeuF2LY: CATCCTTTTGGTTTTGATAG). This resulted in amplifications for samples LYAM, LYHV, and LYPR as shown in table 4.3.

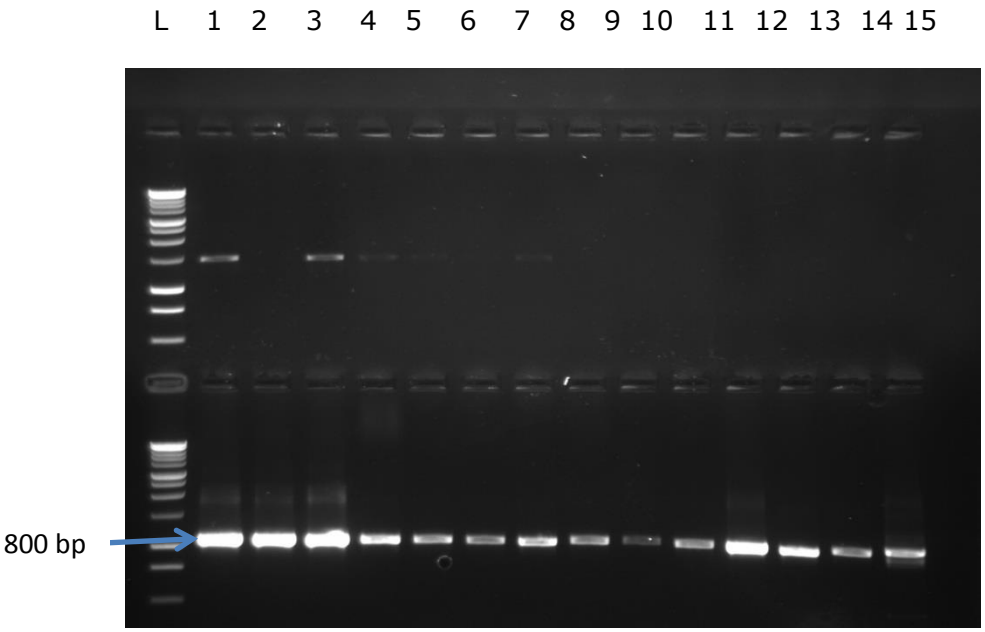


Figure 4.2 Polymerase chain reaction amplification of infected periwinkle samples with Leu primers. L: 1kb ladder; 1 - 14 amplification of phytoplasma samples listed in Table 4.1 (First round PCR - upper panel) using LeuF1/LeuR1 Primers; (Nested PCR – lower panel) using LeuF2/LeuR2 primers, and 15: control (healthy periwinkle plant).

In order to confirm that the primers only amplify phytoplasma DNA and do not have the potential to amplify from any of the natural host plant species of the phytoplasmas tested, DNA was extracted from a range of healthy plant species, and tested by nested PCR using the leucyl tRNA synthetase primers. No amplification products were detected (Fig. 4.3). To confirm that all the DNA extracts contained DNA that supported PCR and did not have PCR inhibitors, the same samples were tested with the cox primers that amplify a universal plant gene – cytochrome oxidase) (Hren *et al.,* 2007). All samples were positive with this primer set (Fig. 4.4).

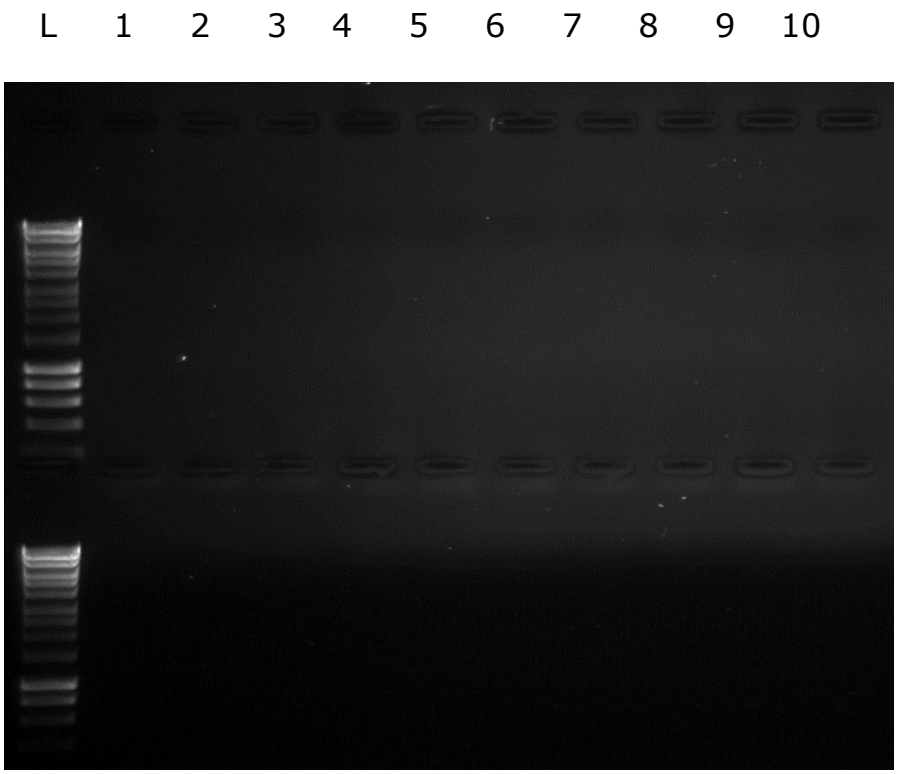


Figure 4.3 PCR amplifications from the healthy plant samples tested with the leucyl tRNA synthetase primers (direct PCR upper panel, and nested PCR low panel). Samples were as listed in table 4.2 plus negative control. All samples showed that no amplifications (no bands) occurred for any samples.

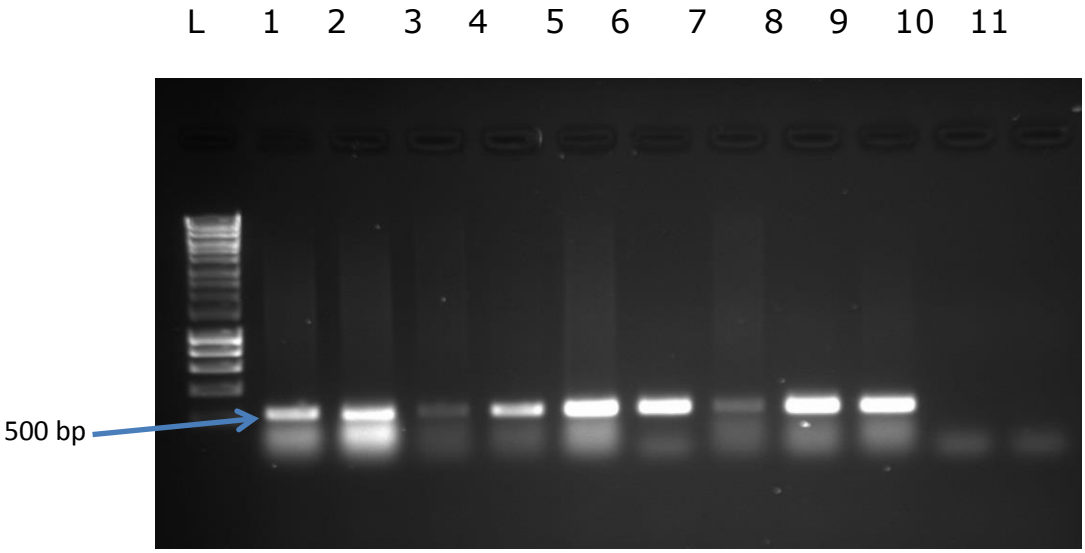


Figure 4.4 PCR amplifications with the cox primers of healthy plant samples as listed in table 4.2. All the samples had DNA of suitable quality for PCR amplification since bands of size 500 bp (samples from 1 to 9) were amplified with the COX primers from all plants and the water control (sample 10) showed no amplification.

4.3.3 Phylogenetic analysis

Having validated the leucyl tRNA synthetase primers on a range of phytoplasmas, they were subsequently tested on DNA from a more comprehensive set of isolates from a broad range of phylogenetic groups (Table 4.3). Following nested PCR, products were obtained from all samples, confirming that the primers were essentially universal, and the products were cleaned up using GenElute™ PCR Clean-Up Kit (Sigma-Aldrich). Purified PCR products were then sent to Eurofins (Germany) for sequencing.

Table 4.3 Strains of phytoplasma employed. Isolates of phytoplasmas from which DNA was used are listed according to which 16S rRNA gene group they belong to.

Extract No	Isolate	16Sr	Origin
1	[1]Onion yellows (Ac No NC005303)	IB	
2	[1]Aster yellows witches' broom (NC007716)	IA	
3	Rehmannia glutinosa	IB	Czech Republic
4	Ribes in Vinca	IC	Czech Republic
5	Strawberry green petal	IC	Czech Republic
6	Crotalaria saltiana phyllody	IIC	Rothamsted
7	Faba bean phyllody	IIC	Rothamsted
8	Soybean phyllody	IIC	Rothamsted
9	Vinca coconut phyllody	VI	Rothamsted
10	Peach western X	IIIA	Italy
11	Plum leptonecrosis	IIIB	Italy
12	[2]Coconut lethal yellows (from contig 103)	IV	
13	Elm yellows	VA	Fera
14	Potato witches' broom	VIA	Italy
15	Brinjal little leaf	VIA	Rothamsted
16	Apple proliferation	XA	Slovenia
17	[1]Candidatus *P. mali* (NC011047)	X	
18	Napier grass stunt 1	XI	Rothamsted
19	Napier grass stunt 2	XI	Rothamsted
20	[1]Ca. *P.australiense* (NC010544)	XII	
21	[1]*Acholeplasma laidlawii* (YP001620244)		
22	Cordyline phytoplasma	XII	Cornwall UK
23	Tanzanian coconut lethal decline	XXX	coconut, Tanzania

24	Pigeon pea witches' broom	IX	USA
25	Napier grass stunt 3	XI	Rothamsted
26	Napier grass stunt 4	XI	Rothamsted
27	Napier grass stunt 5	XI	Rothamsted
28	Flower stunting (BVK)	XI	Germany
29	Sugarcane whiteleaf	XI	Sri Lanka
30	Bermuda grass whiteleaf 34	XIV	Malaysia
31	Bermuda grass whiteleaf 1114	XIV	Malaysia
32	Bermuda grass whiteleaf 26	XIV	Sri Lanka
33	Bermuda grass whiteleaf 27	XIV	Sri Lanka
34	Bermuda grass whiteleaf 28	XIV	Sri Lanka
35	Bermuda grass whiteleaf 29	XIV	Sri Lanka
36	Bermuda grass whiteleaf 30	XIV	Sri Lanka
37	Sugarcane whiteleaf 31	XI	Sri Lanka
38	Sugarcane whiteleaf 32	XI	Sri Lanka
39	Sugarcane whiteleaf 34	XI	Sri Lanka
40	Sugarcane whiteleaf 35	XI	Sri Lanka
41	Bermuda grass whiteleaf DB1	XIV	Malaysia
42	Bermuda grass whiteleaf DB6	XIV	Malaysia
43	Sugarcane grassy shoot D6	XI	Sri Lanka
44	Sugarcane whiteleaf D3	XI	Sri Lanka
45	Sugarcane grassy shoot D4	XI	Sri Lanka
46	Sugarcane whiteleaf D2	XI	Sri Lanka
47	Cape St Paul Wilt Disease 1	XXII	Ghana
48	Cape St Paul Wilt Disease 2	XXII	Ghana
49	Awka5	XXII	Nigeria
50	Coconut lethal yellows	IV	Florida, USA
51	Cape St Paul Wilt Disease GH1	XXII	Ghana
52	Cape St Paul Wilt Disease GH6	XXII	Ghana
53	Cape St Paul Wilt Disease GH8	XXII	Ghana
54	Lethal yellows (*Adonidia merrillii*)	IV	USA
55	Lethal yellows (*Hyophorbe verschafetii*)	IV	USA
56	Lethal yellows (*Phoenix rubicola*)	IV	USA
57	Sugarcane grassy shoot Sri Lanka	XI	Sri Lanka
58	Sugarcane whiteleaf Sri Lanka	XI	Sri Lanka
59	Mozambique coconut lethal disease 2	XXII	Mozambique
60	Mozambique coconut lethal disease 9	XXII	Mozambique
61	Mozambique coconut lethal disease 23	XXII	Mozambique

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#16srIB_Onion_yellows_NC005303        TCA CCA AAC CCG AA- --T ACC AAC AAG CAG TTA -AC AAT TAT TTG GAA TTA ACC AAA CAA AAA AAA
#16SrIA_AYWB_NC_007716                .A. ... ..A ... ..- --C ... ... ... ... ... -.. ... ... ... ... ... ..A ... ... ... ...
#16SrIB_Rehmannia_glutinosa           ... ... ... ... ..- --. ... ... ... ... ... -.. ... ... ... ... ... ... ... ... ... ...
#16SrIC_Ribes_in_Vinca                .A. ... ... ... ..- --. ... ... ... ... ... -.A ... ... ... ... ... ... ... ... ... ...
#16SrIC_Strawberry_green_petal        .A. ... ... ... ..- --. ... ... ... ... ... -.A ... ... ... ... ... ... ... ... ... ...
#16SrIIC_Crotalaria_saltiana_phyllody .A. .T. CTT .T. ..- --A .TA ... ..T T.. ..G -.T ... ... ..A C.. AA. ..T ... .GC ..G .GC
#16SrIIC_Faba_bean_phyllody           .A. .T. CTT .T. ..- --A .TA ... ..T T.. ..G -.T ... ... ..A C.. AA. ..T ... .GC ..G .GC
#16SrIIC_Soybean_phyllody             .A. .T. CTT .T. ..- --A .TA ... ..T T.. ..G -.T ... ... ..A C.. AA. ..T ... .GC ..G .GC
#16SrII_Vinca_coconut_phyllody        .A. .T. CTT .T. ..- --A .TA ... ..T T.. ..G -.T ... ... ..A C.. AA. ..T ... .GC ..G .GC
#16SrIIIA_Peach_wetern_X              .A. .T. CT. .A. .T- --C GTT GGA .T. A.A .AG -.T G.A ..C G.T A.. CGT ..T ... .GT ... .CT
#16SrIIIB_Plum_leptonecrosis          .A. ... CT. .A. .T- --C GTT GGA .T. A.. .AG -.T G.A ..C G.T ..C CGT ..T ... .GT ... .TG
#LY_from_contig_103                   .TG TT. ..A AGA ..- --. TTG CTG TTT ... .C. -.T G.A ... A.A ACT CA. .TT TC. ... ... .GC
#16SrVA_Elm_yellows                   .A. TTC ..A AA. ..- --. TTA TTG .TT .T. ... -.A .TA ... ..A ... GA. ..A ..G A.. ... .C.
#16SrVIA_Potato_witches_broom         .A. .T. ..A .A. .T- --. TTG TCG ... GT. .A. -.T TTA ... ..A ..C CA. .AT CG. ... ... .C.
#16SrVIA_Brinjal_little_leaf          .A. .T. ..A .A. .T- --. TTG TCG ... GC. .A. -.T TTA ... ..A ..C CA. ..T ... ... ... .C.
#16SrXA_Apple_proliferation           .A. .T. ..A .TT T.- --. TT. ..A ..T .CA ..T -TT G.. ... A.T ... CA. ..T ... ... ... C..
#P_mali_NC011047                      .A. .T. ..A .TT T.- --. TT. ..A ..T .CA ..T -TT G.. ... A.T ... CA. ..T ... ... ... C..
#16SrXI_Napier_grass_stunt            .A. .TT CT. AA. ..- --A .TA ..A TTC AG. .C. -.A ..C ... ..A A.G AA. ..A GTT AG. ... .GT
#ngs_from_sequence                    .A. .TT CT. AA. ..- --A .TA ..A TTC AG. .C. -.A ..C ... ..A A.G AA. ..A GTT AG. ... .GT
#16SrXII_P_aust_NC010544              .T. .T. CTT TAA ..- --C .G. ..G ... A.. ..T -TG G.. ... C.A ... .GC ..T ... ... ... ...
#Acholeplasma_laidlawii_YP_001620244  .TG TG. CT. AA. .T- --G .AG ..A ... AT. .AT -TA .GA ..C A.T ... CA. ..T ... ATG ..G TC.
#Group_XII                            .A. .T. CGA TAA .T- --C .T. ... ..A AT. ..T -CA TC. ... ..A ... AAT T.. ... ... ..G ...
#Group_XXX                            .A. .T. CT. .A. .T- --C GTT GGA .T. A.A .AG -.T G.A ..C G.T A.. CGT ..T ... .GT ... .CT
#Group_IX                             .TG TA- .CT .GA G.- --. .AT ..T G.A A.A .AC T.T GC. ... ..A A.. CA. ..T ... ... ... .CC
#NGS2                                 .A. .TT CT. AA. ..- --A .TA ..A TTC AG. .C. -.A ..C ... ..A A.G AA. ..A GTT AG. ... .GT
#BVK1                                 .A. .TT C.A AA. ..- --A .TG T.T CTC A.. .AC -.A G.A ... ..A A.T AA. ..T .TT AG. ... .CT
#SWLT                                 .G. .TT CT. AA. ..- --A .TA TTT T.A A.. ... -.T ... ... C.T ..T CA. .TA .G. AG. ... .GC
#BGWL34                               .AG TTT CTG AA. ..G AAA .TA TTT TTA A.. ... -.T TT. ... C.T ... CA. GTT CGT A.. ... .CT
#BGWL1114                             .AG TTT CTG AA. ..G AAA .TA TTT TTA A.. ... -.T TT. ... CAT ... CA. GTT CGT A.. ... .CT
#chamini_BGWL26                       .AG TTT CTG AA. ..- --A .TA TTT CTA A.. ... -.T GT. ... C.T ... CA. GTT CGT A.. ... .CT
#chamini_BGWL27                       .AG TTT CTG AA. ..- --A .TA TTT CTA A.. ... -.T GT. ... C.T ... CA. GTT CGT A.. ... .CT
#chamini_bgwl28                       .AG TTT CTG AA. ..- --A .TA TTT CTA A.. ... -.T GT. ... C.T ... CA. GTT CGT A.. ... .CT
#chamini_bgwl29                       .AG TTT CTG AA. ..- --A .TA TTT CTA A.. ... -.T GT. ... --- --- --- --- --- --- --- ---
#chamini_bgwl_30                      .AG TTT CTG AA. ..- --A .TA TTT CTA A.. ... -.T GT. ... C.T ... CA. GTT CGT A.. ... .CT
#chamini_sugar31                      .G. .TT CC. AA. ..- --A .TA TTT T.A A.. ... -.T ... ... C.T ..T CA. .TA .G. AG. ... .GC
#chamini_sugar_32                     .G. .TT CC. AA. ..- --A .TA TTT T.A A.. ... -.T ... ... C.T ..T CA. .TA .G. AG. ... .GC
#chamini_sugar_34                     .G. .TT CC. AA. ..- --A .TA TTT T.A A.. ... -.T ... ... C.T ..T CA. .TA .G. AG. ... .GC
#chamini_sugar35                      .G. .TT CC. AA. ..- --A .TA TTT T.A A.. ... -.T ... ... C.T ..T CA. .TA .G. AG. ... .GC
#BGWLDB1                              .AG TTT CTG AA. ..- --A .TA TTT CTA A.. ... -.T GT. ... C.T ... CA. GTT CGT A.. ... .CT
#SCGSD6                               .G. .TT CC. AA. ..- --A .TA TTT T.A A.. ... -.T ... ... ..T ..T CA. .TA .G. AG. ... .GC
#SCWLD3                               .G. .TT CC. AA. ..- --A .TA TTT T.A A.. ... -.T ... ... C.T ..T CA. .TA .G. AG. ... .GC
#SCGSD4                               .G. .TT CC. AA. ..- --A .TA TTT T.A A.. ... -.T ... ... C.T ..T CA. .TA .G. AG. ... .GC
#SCWLD2                               .G. .TT CC. AA. ..- --A .TA TTT T.A A.. ... -.T ... ... ..T ..T CA. .TA .G. AG. ... .GC
#CSPW1                                .TG TTC ....AA ..-  --. .TT CTT CTT T.. .G. -.T G.A ... ..A TTT AG. ..A ... ... ... .GT
#CSPW2                                .TG TTC ....AA ..-  --. .TT CTT CTT T.. .G. -.T G.A ... ..A TTT AG. ..A ... ... ... .GT
#EAT_4                                .T. ..A CTA AA. ..- --. .TT TTG ..C TT. ... -.A CTA ... A.T ... AA. ..T ... AC. ... .GT
#Florida_4                            .TG TT. ..AAGA .. - --. TTG CTG TTT ... .C. -.T G.A ... A.A ACT CA. .TT TC. ... ... .GC
#GH1                                  .TG TTC ....AA .. - --. .TT CTT CTT T.. .G. -.T G.A ... ..A TTT AG. ..A ... ... ... .GT
#GH6                                  .TG TTC ... AA .. - --. .TT CTT CTT T.. .G. -.T G.A ... ..A TTT AG. ..A ... ... ... .GT
#LYAM                                 .TG TT. ..A AGA ..- --. TTG CTG TTT ... .C. -.T G.A ... A.A ACT CA. .TT TC. ... ... .GC
#LYHV                                 .TG TT. ..A AGA ..- --. TTG CTG TTT ... .C. -.T G.A ... A.A ACT CA. .TT TC. ... ... .GC
#LYPR                                 .TG TT. ..A AGA ..- --. TTG CTG TTT ... .C. -.T G.A ... A.A ACT CA. .TT TC. ... ... .GC
#SCGSD_Sri_Lanka                      .G..TT  CC.  AA...- --A .TA TTT T.A A.. ... -.T ... ... C.T ..T CA. .TA .G. AG. ... .GC
#SCWL_Sri_Lanka                       .G..TT  CC.  AA...- --A .TA TTT T.A A.. ... -.T ... ... C.T ..T CA. .TA .G. AG. ... .GC
#Mozambique_2                         .TG TTC ... .AA ..- --. .TT CTT CTT T.. .A. -.T G.A ... ..A TTC CG. ..A ... ... ... .GT
#Mozambique_9                         .TG TTC ... .AA ..- --. .TT CTT CTT T.. .A. -.T G.A ... ..A TTC CG. ..A ... ... ... .GT
#Mozambique_23                        .TG TTC ... .AA ..- --. .TT CTT CTT T.. .A. -.T G.A ... ..A TTC CG. ..A ... ... ... .GT
```

Figure 4.5 Alignment of a part of the leucyl tRNA synthetase sequences between the LeuF2 / LeuR2 primers in phytoplasma genomes belonging to different groups.(.= the same nucleotide, - = missing)

.

Partial alignments for all the sequences are shown in Figure 4.5, and indicate the level of diversity that was found in the sequences between different isolates. Phylogenetic trees were constructed using MEGA 5.2 software, and show appropriate alignment of the samples into the 16Sr groups and subgroups (Fig. 4.6).

Comparison was further undertaken between this new leucyl tRNA synthetase gene phylogenetic tree and trees that had been constructed previously for rRNA and *secA* genes (Fig. 4.7) to confirm the similarities of groupings between isolates.

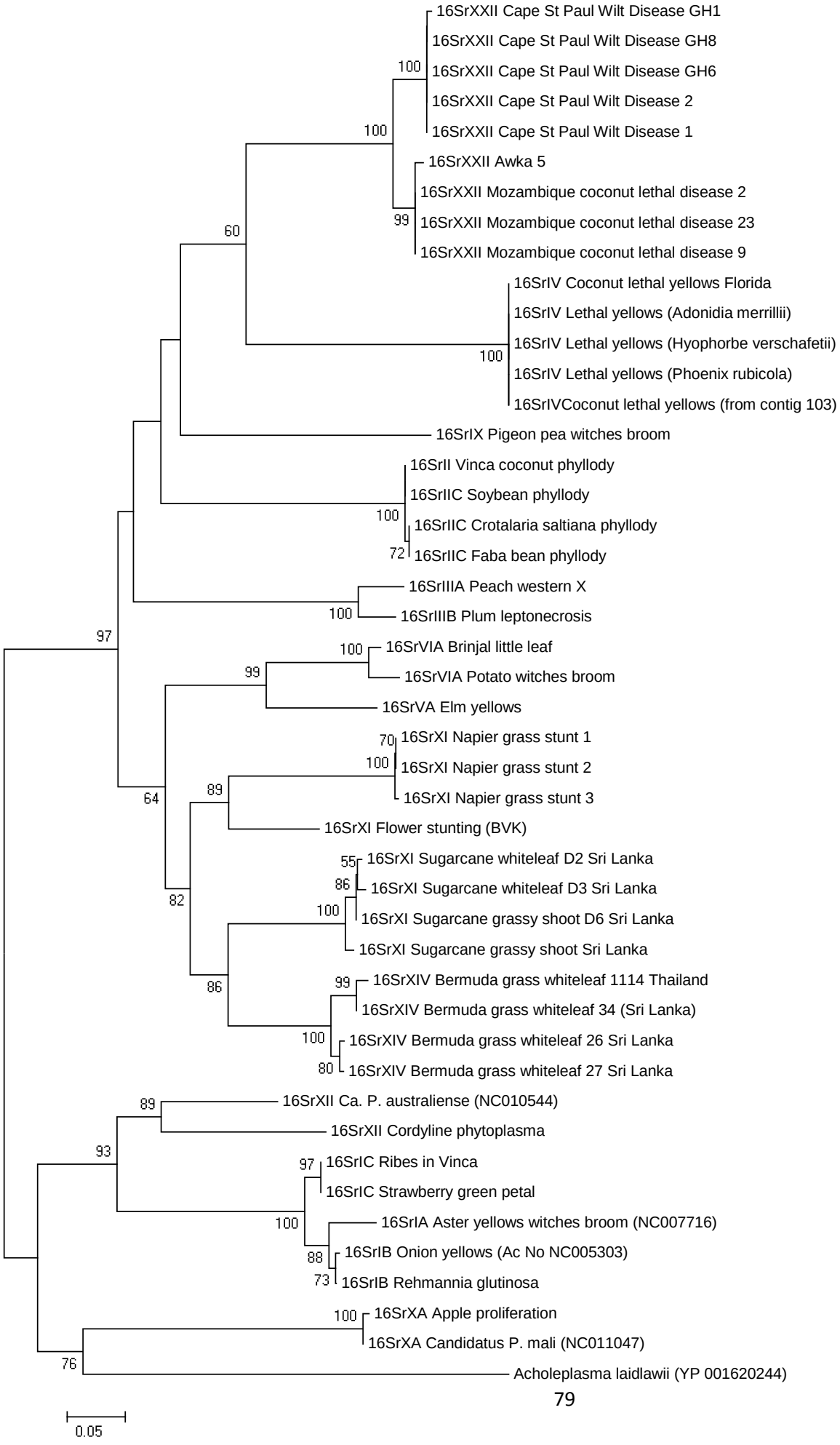

16SrXXII Cape St Paul Wilt Disease GH1
16SrXXII Cape St Paul Wilt Disease GH8
16SrXXII Cape St Paul Wilt Disease GH6
16SrXXII Cape St Paul Wilt Disease 2
16SrXXII Cape St Paul Wilt Disease 1
16SrXXII Awka 5
16SrXXII Mozambique coconut lethal disease 2
16SrXXII Mozambique coconut lethal disease 23
16SrXXII Mozambique coconut lethal disease 9
16SrIV Coconut lethal yellows Florida
16SrIV Lethal yellows (Adonidia merrillii)
16SrIV Lethal yellows (Hyophorbe verschafetii)
16SrIV Lethal yellows (Phoenix rubicola)
16SrIVCoconut lethal yellows (from contig 103)
16SrIX Pigeon pea witches broom
16SrII Vinca coconut phyllody
16SrIIC Soybean phyllody
16SrIIC Crotalaria saltiana phyllody
16SrIIC Faba bean phyllody
16SrIIIA Peach western X
16SrIIIB Plum leptonecrosis
16SrVIA Brinjal little leaf
16SrVIA Potato witches broom
16SrVA Elm yellows
16SrXI Napier grass stunt 1
16SrXI Napier grass stunt 2
16SrXI Napier grass stunt 3
16SrXI Flower stunting (BVK)
16SrXI Sugarcane whiteleaf D2 Sri Lanka
16SrXI Sugarcane whiteleaf D3 Sri Lanka
16SrXI Sugarcane grassy shoot D6 Sri Lanka
16SrXI Sugarcane grassy shoot Sri Lanka
16SrXIV Bermuda grass whiteleaf 1114 Thailand
16SrXIV Bermuda grass whiteleaf 34 (Sri Lanka)
16SrXIV Bermuda grass whiteleaf 26 Sri Lanka
16SrXIV Bermuda grass whiteleaf 27 Sri Lanka
16SrXII Ca. P. australiense (NC010544)
16SrXII Cordyline phytoplasma
16SrIC Ribes in Vinca
16SrIC Strawberry green petal
16SrIA Aster yellows witches broom (NC007716)
16SrIB Onion yellows (Ac No NC005303)
16SrIB Rehmannia glutinosa
16SrXA Apple proliferation
16SrXA Candidatus P. mali (NC011047)
Acholeplasma laidlawii (YP 001620244)
0.05

Figure 4.6 Dendrogram, constructed using the MEGA 5.2 software, for isolates listed in Table 4.3 using leucyl tRNA synthetase sequences attained in this project. Bar= 0.05 substitutions per nucleotide position in this phylogenetic tree.

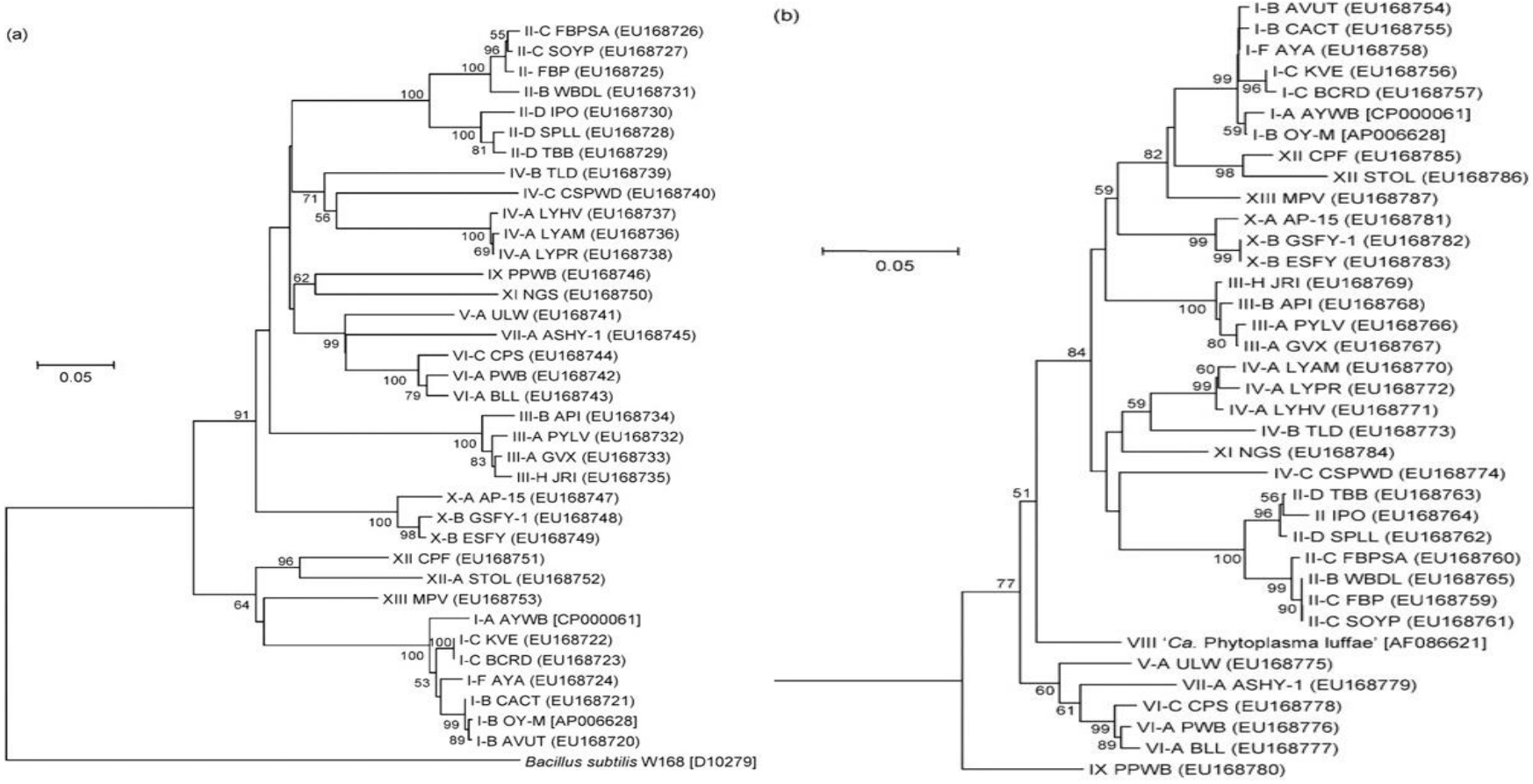

(a)
II-C FBPSA (EU168726)
II-C SOYP (EU168727)
II- FBP (EU168725)
II-B WBDL (EU168731)
II-D IPO (EU168730)
II-D SPLL (EU168728)
II-D TBB (EU168729)
IV-B TLD (EU168739)
IV-C CSPWD (EU168740)
IV-A LYHV (EU168737)
IV-A LYAM (EU168736)
IV-A LYPR (EU168738)
IX PPWB (EU168746)
XI NGS (EU168750)
V-A ULW (EU168741)
VII-A ASHY-1 (EU168745)
VI-C CPS (EU168744)
VI-A PWB (EU168742)
VI-A BLL (EU168743)
III-B API (EU168734)
III-A PYLV (EU168732)
III-A GVX (EU168733)
III-H JRI (EU168735)
X-A AP-15 (EU168747)
X-B GSFY-1 (EU168748)
X-B ESFY (EU168749)
XII CPF (EU168751)
XII-A STOL (EU168752)
XIII MPV (EU168753)
I-A AYWB [CP000061]
I-C KVE (EU168722)
I-C BCRD (EU168723)
I-F AYA (EU168724)
I-B CACT (EU168721)
I-B OY-M [AP006628]
I-B AVUT (EU168720)
Bacillus subtilis W168 [D10279]
0.05
(b)
I-B AVUT (EU168754)
I-B CACT (EU168755)
I-F AYA (EU168758)
I-C KVE (EU168756)
I-C BCRD (EU168757)
I-A AYWB [CP000061]
I-B OY-M [AP006628]
XII CPF (EU168785)
XII STOL (EU168786)
XIII MPV (EU168787)
X-A AP-15 (EU168781)
X-B GSFY-1 (EU168782)
X-B ESFY (EU168783)
III-H JRI (EU168769)
III-B API (EU168768)
III-A PYLV (EU168766)
III-A GVX (EU168767)
IV-A LYAM (EU168770)
IV-A LYPR (EU168772)
IV-A LYHV (EU168771)
IV-B TLD (EU168773)
XI NGS (EU168784)
IV-C CSPWD (EU168774)
II-D TBB (EU168763)
II IPO (EU168764)
II-D SPLL (EU168762)
II-C FBPSA (EU168760)
II-B WBDL (EU168765)
II-C FBP (EU168759)
II-C SOYP (EU168761)
VIII 'Ca. Phytoplasma luffae' [AF086621]
V-A ULW (EU168775)
VII-A ASHY-1 (EU168779)
VI-C CPS (EU168778)
VI-A PWB (EU168776)
VI-A BLL (EU168777)
IX PPWB (EU168780)
0.05

Figure 4.7 Dendrograms, constructed using the MEGA 5.2 software, showing the phylogenetic relationships amongst the phytoplasmas such as AYWB, OY-M and *Bacillus subtilis* strains based on DNA sequences of (a) the *secA* gene (between primers SecAfor2 and SecArev3), and (b) the 23S rRNA gene (between primers P7 and 23Ser) as published by Hodgetts *et al.* (2008).

4.4 Discussion

Phytoplasma classification by traditional taxonomy is currently not possible, because of the inability to culture them *in vitro*. This has led to the development of other systems based on gene sequences. Universal genes are targets for the molecular techniques both for detection of phytoplasmas and for phytoplasma phylogenetic analysis. For example, the first classification of phytoplasmas based on the 16S rRNA gene was by Lee *et al.* (1994) who divided phytoplasmas into 9 groups and 14 subgroups. Further analysis of the 16S-23S spacer region has improved group resolution (Wang *et al.,* 2003), and more recently several new 16Sr groups have been added to result in 33 current groups (Bertaccini *et al.,* 2014). Depending on different characteristics, for example plant host, vector specificity, and sometime on serological comparisons, some of the phytoplasma groups and subgroups have been named as '*Candidatus* Phytoplasma' (IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma Taxonomy Group, 2004; Firrao *et al*., 2005). Twenty three phytoplasma species have been renamed to '*Ca*. Phytoplasma status' and any new stain of phytoplasma which has less than 97.5% 16S rRNA gene sequence similarity to one of these species can potentially be assigned to a novel Candidatus species.

Additional genes for studying phytoplasmas, such as the *secA* gene (Hodgetts *et al.,* 2008), *secY* gene (Lee *et al.,* 2006), and *tuf* and *rp* genes (Marcone *et al.,* 2000; Botti & Bertaccini, 2003) have provided more details about primary groups and subgroups of phytoplasmas. For example, new universal primers based on the *secA* gene amplified a 482/488 bp region of phytoplasmas from all 13 groups on which they were

tested (Hodgetts *et al.,* 2008), and the first comprehensive analysis of phytoplasma between different groups was concluded by using *rp* genes (coding sequences) (Martini *et al.,* 2007).

In this study, attempts were made to design further sets of universal primers, and from the various genes analysed, one set of primers, those based on the leucyl tRNA synthetase gene, proved to be of value and amplify from all phytoplasmas tested. A second set of universal primers for the RNA polymerase (pol) gene was also designed, but these came out with negative amplifications for most samples that were used. The reason for this is unclear, but these primers were not pursued any further.

In addition to the leucyl tRNA synthetase primers working for all phylogenetic groups tested, the phylogenetic trees showed that most samples could be assigned to the expected positions in the trees and that groupings and subgroupings were similar when compared with 16S rRNA gene and *secA* gene phylogenetic trees. However, using the leucyl tRNA synthetase gene generally appears to give a better resolution between groups and subgroups of phytoplasmas, since the sequence being analysed is longer than the one that the previously designed *secA* gene primers amplify, and the sequence is for a coding region unlike the 16S rRNA gene, so the sequences between isolates are less well conserved.

Amongst the interesting features based on comparing the leucyl tRNA gene trees with other phylogenetic trees, it is noteworthy that all isolates gave appropriate results apart from one isolate, Tanzanian coconut lethal decline, which aligned with 16SrIII peach western X in the original tree, and was therefore removed from Figure 4.6, since this was presumed to be

due to the sample being old (it was originally collected in 1992) and having become contaminated. In Figure 4.5, this sample is labelled as IV-B TLD, and therefore is clearly not a 16SrIII phytoplasma. A further significant feature of the tree based on the leucyl tRNA synthetase gene is the positioning of the coconut lethal yellowing like diseases relative to each other. Based on the *secA* gene (Fig. 4.7a) and the 16S rRNA gene (Harrison *et al.,* 2014), the 16SrIV lethal yellows (now known as '*Ca.* Phytoplasma palmae'), clusters most closely with the Ghanaian / Nigerian / Mozambique coconut diseases (now known as '*Ca.* Phytoplasma palmicola') and the Tanzanian lethal decline phytoplasma ('*Ca.* Phytoplasma cocostanzaniae'). However, the 23S rRNA tree (Fig. 4.7b) indicates that this closeness might not be the case for all genes, since it places the Cape St Paul Wilt Disease ('*Ca.* Phytoplasma palmicola') closer to the 16SrII group than the other coconut phytoplasmas. The new tree based on the leucyl tRNA synthetase gene (Fig. 4.6) confirms this, and also places the 16SrIX pigeon pea witches' broom phytoplasma adjacent to '*Ca.* Phytoplasma palmicola', which is quite different from the position of 16SrIX in the 23S rRNA tree (Fig 4.7b). Interestingly, the leucyl tRNA synthetase tree also provides a clear distinction between the Ghanaian strains of the coconut disease and the Nigerian / Mozambique strain, which might suggest that the Mozambique strain, which was only detected for the first time in the early 2000's, may have originated from this older Nigerian type.

A further significant issue raised by the new leucyl tRNA synthetase tree is the positioning of the 16SrXI and 16SrXIV isolates. There are several diseases of sugarcane that are reported to be caused by phytoplasmas, including sugarcane white leaf (SCWL), sugarcane grassy shoot (SCGS), sugarcane yellow leaf (SCYL) and Ramu stunt disease (Marcone, 2002;

Blanche *et al.,* 2003; Arocha *et al.,* 2005). Taxonomically, the SCWL and SCGS phytoplasmas are closely related to phytoplasmas associated with rice yellow dwarf (RYD) disease, annual bluegrass white leaf (ABGWL), and Bermuda grass white leaf disease (BGWL) based on 16S rDNA phylogenetic analysis (Jung *et al*., 2003). Based on the 16S rRNA gene, SCWL and SCGS phytoplasmas have been classified under the 16SrXI-B subgroup of the rice yellow dwarf group (16SrXI; '*Ca.* Phytoplasma oryzae') whilst ABGWL and BGWL have been classified in the 16SrXIV ('*Ca.* Phytoplasma cynodontis') group (Lee *et al*., 1998; Harrison *et al.,* 2014). However, the leucyl tRNA synthetase tree (Fig. 4.6) indicates that whilst this classification may hold true based on the 16S rRNA gene, it is not the case for leucyl tRNA synthetase, where the sugarcane isolates are clearly more closely related to the 16SrXIV Bermuda grass white leaf phytoplasmas than to the other 16SrXI phytoplasmas such as flower stunting and Napier grass stunt. Taken together, this data continues to support the notion stated in Hodgetts *et al.* (2008) that analyses based on the rRNA gene alone should be treated with caution and that multi-locus sequence typing (MLST), and analyses based on combinations of coding and non-coding genes have higher resolution power in phytoplasma phylogenetics than studies based on single genes.

In summary, this chapter has shown the value of the new set of universal primers for the leucyl tRNA synthetase gene for phytoplasma classification and taxonomic restructuring. The sequences obtained (see table 4.3) show clearly and significant consistent variations in length for phytoplasmas from particular phylogenetic groups (see Fig 4.5 - 4.6), and even between phytoplasmas of the same group from different parts of the world. Such improved discrimination between samples will be invaluable in the future for monitoring the host range of particular phytoplasmas, the vector

relationships, and the spread of phytoplasmas through different parts of the world.

Chapter 5: Evaluating LAMP diagnostic assays and Real-time PCR assays for different phytoplasma groups

5.1 Introduction

Currently, most techniques for phytoplasma diagnostics use techniques such as PCR and primers to amplify phytoplasma DNA after extraction from diseased plants. In addition, there are alternative diagnostic methods that have been developed based on real time PCR (Hodgetts *et al.,* 2009) and microarrays (Nicolaisen & Bertaccini, 2007), as well as T-RFLP (terminal restriction fragment length polymorphism) (Hodgetts *et al.,* 2009), HMAS (Heteroduplex mobility assays) (Wang & Hiruki, 2005), and SSCP (Single-stand conformation polymorphisms) (Music *et al.,* 2008).

Real-time PCR or quantitative PCR is currently the optimal diagnostic assay, not only because it is a 'closed-tube' system, so has reduced risk of contamination of samples compared to conventional PCR, but also because it can be used for the quantification of phytoplasmas in diseased host plants. In addition, these assays can provide higher detection sensitivity compared to general detection assays, for example nested PCR.

The main idea of qPCR assays for detection and quantification includes amplification of the target pathogen and measurement of a fluorescent reporter molecule from the PCR product or amplicon as it accumulates. In qPCR the reporter employed can be SYBR Green (DNA-binding dyes) or TaqMan probes (fluorescently labelled sequence-probes). Comparing

between SYBR Green qPCR and TaqMan qPCR, the SYBR Green qPCR is less expensive and easier to design, and the difference in sensitivity, quantification and specificity is not significant as long as appropriate controls are undertaken (Andersen *et al.,* 2006). Both types of assays have been designed for phytoplasma diagnostics, including TaqMan assays that can be used to distinguish and quantify several target pathogens or genes in one reaction simultaneously, called multiplex qPCR (Hodgetts *et al.,* 2009). Basically, qPCR primers (universal and specific) for phytoplasma detection and quantification have been designed from the RNA operon and internal transcribed spacer region, because these regions are well conserved in each phytoplasma group (16Sr RNA groups) and consistently exist only as two copies in the genome, which is helpful for quantity calculations.

In general, the methods for PCR amplification of phytoplasma DNA and analysis of results require relatively expensive equipment, and involve use of buffers for DNA extraction such as cetyltrimethyl ammonium bromide buffer (CTAB) along with phenol/chloroform and isopropanol, such that they cannot be performed in the field. However, a new technique has been developed called loop-mediated isothermal amplification (LAMP) (Hodgetts *et al.,* 2009), for both generic and specific phytoplasma detection, which can potentially be used in the field, and which takes less than one hour to perform. Amongst the advantages of LAMP assays are that they are less prone to inhibition from compounds in DNA extracts, so relatively crude DNA preparations can potentially be used. In addition, real-time LAMP methods have been developed which have the same advantages as real-time PCR over conventional PCR-based diagnostic techniques in that they are closed tube systems, so once the mastermix and DNA samples are ready and the tubes closed, no further re-opening of tubes is required,

which avoids any chance to getting contamination of DNA samples with amplification product. A further advantage is that the equipment required is light weight and battery operated so is easy to use in fields and faster when compared to conventional or real-time PCR. The aims of this chapter were to design and validate some additional LAMP assays for different phytoplasma phylogenetic groups, and also to redesign real-time PCR assays for specific groups, in particular to develop SYBR-green assays.

5.2 Materials and Methods

5.2.1 Sources of phytoplasma and plant DNA samples.

Phytoplasma diseases belonging to various phylogenetic groups are held at the University of Nottingham, growing in Madagascar periwinkle (*Catharanthus roseus*) as the phytoplasma host plant. A collection is also held of DNA extracts that have been obtained from these plants along with from additional plant samples obtained from elsewhere, using the CTAB method as described in the materials and methods chapter 2. In addition DNA samples have been obtained from colleagues from the UK and overseas, including from Rothamsted and Fera (UK), Spain, Italy, Germany, the USA, Canada, Thailand, Nigeria, Ghana, Vietnam, Sri Lanka, Tanzania, and the Czech Republic. Examples of the DNA samples in the collections have been described previously (Hodgetts *et al.,* 2009). For this current study, initial confirmation of the presence of phytoplasma DNA in samples was done by using nested PCR using primers P1 & P7 followed by R16F2 & R16R2 as described in Smart *et al.* (1996). All DNA samples of phytoplasma were used in this chapter are listed in table 2.1 and appendix 3.

5.2.2 Design of primers for Loop-mediated isothermal amplification (LAMP)

LAMP primers were designed using LAMP Designer software from OptiGene http://www.optigene.co.uk/lamp-designer/. To get sequences to use in the LAMP designer software, nested PCR were done by using the new universal primers for phytoplasmas based on the leucyl tRNA synthetase gene (see chapter 4). The expectation was that use of this gene would provide more specific primers for the different phylogenetic groups than use of the 16S rRNA gene because of the greater sequence variation in this gene. Initial PCR used PCR primers LeuF1/LeuR1 in the first round followed by LeuF2/LeuR2 in the nested PCR to amplify phytoplasma DNA from specific isolates within 16Sr groups I, II, III, V, VI, IX, X, and XII. The PCR products were then sequenced (MWG, Germany) and the sequences were entered into the LAMP designer software. Six LAMP primers were designed for each group (loop primers loop-F and loop-B, external primers F3 and B3, and internal primers FIP and BIP).

For LAMP reactions all primer stocks were made up to 100 µM. A primer mastermix was then made by mixing 152 µl water with 4 µl F3, 4 µl B3 and 20 µl each of the other four primers (total volume 240 µl). Then for 8 reactions a reaction mastermix was made comprising 46 µl water, 23 µl primer mix and 115 µl enzyme Mastermix (Optigene, UK). Twenty µl of this mix was aliquoted into each of 8 tubes, and 1 µl DNA added, and the tubes were loaded into the Genie II machine for 30 min at 65°C, followed by an annealing temperature assay in which the samples were heated to 96°C and then allowed to cool, and the temperature at which the DNA reannealed (usually between 70°C - 90°C) could be detected through fluorescence detection. Note that this is different from the way in which

melt analyses are conducted in real-time PCR analysis, which is why the results from LAMP are presented as anneal temperatures rather than melt temperatures. Where necessary, results were confirmed by gel electrophoresis in 1% agarose gels.

5.2.3 Designing sets of primers and probes for real-time PCR assays.

Real-time PCR primers and probes were designed by Genscript Real-time PCR (TaqMan) Primer Designer software from Genscript (https://www.genscript.com/ssl-bin/app/primer). To get sequences to use in the real-time designer software, nested PCR were done by using different universal primers for phytoplasmas based on the leucyl tRNA synthetase gene, 16S rRNA gene, and *secA* gene. The same methods were used to obtain sequences from those genes as described in section 5.2.2. The sequences were entered into the Genscript designer software. Two real-time PCR primers and one probe were designed for each phylogenetic group (forward primer, reverse primer, and probe). However, in order to reduce the costs of assays, only the primers were ordered, so that these could then be validated in SYBR-Green assays alongside the previous primers and probes designed in the work of Hodgetts *et al.* (2009), which were used as both SYBR-Green assays and Taqman assays for comparative purposes.

For sensitivity assays, serial dilutions of DNA samples were made to see which DNA concentration was detectable. To perform the RT-PCR assays using the Taqman approach, mastermixes were prepared using TaqMan core reagents (Applied Biosystems) which consisted of Buffer A (50 mM

KCl, 10 mM Tris-HCl pH 8.3, carboxy-X-rhodamine passive reference dye), 0.625 µl of AmpliTaq Gold, 5.5 mM $MgCl_2$, 1 µl of each primer, 1 µl of probe, and 9.75 µl of sterile water. Twenty-four µl of mastermix was added into wells in 96-well plates and 1 µl of DNA sample was added. Samples were run in a LightCycler® 480 instrument (Roche Diagnostics Ltd) using the following protocol: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles each one consisting of 15 seconds at 95°C and 1 min at 60°C (Hodgetts *et al.,* 2009). This method identifies the C_T value of a sample as the point where the sample's fluorescence curve turns sharply upward. For the alternative SYBR-Green assays, 6.25 µl of 2X concentration of the SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma-Aldrich), 0.38 µl of each primer (primers concentration 10 µM), 5 µl nuclease-free water, 0.5 µl of DNA sample (DNA concentration approx 200 ng) was used in 12.5 µl final volume amplifications under the same amplification conditions.

5.3 Results

5.3.1 Design of LAMP primers based on leucyl tRNA synthetase gene sequences

Previous LAMP primers for phytoplasmas have generally been based on 16S-23S rRNA sequences; however, because of the relatively well-conserved nature of such sequences (especially the 16S and 23S RNA genes) it has often been difficult to find regions that are sufficiently unique for each of the different phytoplasma groups. In this work leucyl tRNA synthetase sequences were obtained by PCR and sequencing. Lengths of PCR products were generally ~ 840 bp, and the GC content was between 23–34 %. Sequences were analysed using the LAMP Designer software to come up with 6 primers for each different sequence. This was done for seven different phytoplasma groups (see Table 5.1).Because of the low GC

content in the leucyl tRNA sequences from 16SrXI phytoplasmas, the software was unable to come up with any primers for this group. Sequences of these primers are listed in Table 5.1.

Table 5.1 LAMP primers designed for 16SrI, II, III, V, VI, X, and XII-group phytoplasmas.

16Sr groups		Primers Sequences 5'-3'
Group 16SrI	F3	AAGAGACAATCATCCAGTTGTT
	B3	CTGTGCCATAATGAGGAAGAA
	FIP	AAAGAGTATTAGGGCGAGTGGTAATG CAAGTTAATTGGATTGGT
	BIP	CGCCTGAACACGAATTAGCCTTGTCT TTGTTGATGTCTCTTTC
	Loop_F	AACTAGGAAGGAGACGATTGC
	Loop_B	CCGAATACCAACAAGCAGTTAA
Group 16SrII	F3	GGAGTAGAGAATTAGCGACTTC
	B3	AGGATGTTCAGGAGCCAA
	FIP	CAGGATGACCACCACGTTCTGTTGT CCTCAATTAGGTACAGT
	BIP	TTGGCCAGAGTCTATTAAGGAAAG GCATAGTAGTGAAAACAGAC
	Loop_F	AGAAATAACCTCTTCATTCGCT
	Loop_B	AATGGATTGGCAAAACACCG
Group 16SrIII	F3	ATTAACTACTCCAGATCGTTGG
	B3	CGCTTCTTCATAATTAAGACCG
	FIP	CCCGTACCGTAATGAGGCAAAT GCTATTAACTTTGCTAACCAA
	BIP	CCATTATGGCCGTTCCTTATCAG AAATCTGGGTTTCTTCAGGTA
	Loop_F	AATCAGCGATCCAAATAGGGAT
	Loop_B	AGAGATTTTGAATTCGCTCGT
Group 16SrV	F3	TTTCCTTTCCTGTTTTATCAGA
	B3	TGACATCAAATGATACTTATCG
	FIP	AGGATGTTCTGGCGCTAAAACTAG AAGTTTTCACGACTAAACCA
	BIP	TGGTAGTTACGTTTTTCATCCT GAACCAACATAATAGCTCCTGT
	Loop_F	GCGCTTACTCCAAAAATAGTAC
	Loop_B	TTATGTCCTTCCTTCTTATGCT

Group 16SrVI	F3	TTCAAACAAATTGGATAGGCAA
	B3	GCATCATTAGTGGATTCGGA
	FIP	ACACCTTCGACAAAATCTGTTTTG GAAGTTTTTACGACAAAACC
	BIP	TGAACAAAGATCAAACAGGAGTC CGGTACCATAATAAGGCAAT
	Loop_F	AGGGTGTTCAGGAGATAAAACT
	Loop_B	TGGTAGTTATGCTATTCATCCT
Group 16SrX	F3	AGATAGAGTACAAATGGCAACG
	B3	ATCCATTGCGTCCAATGATAA
	FIP	CGCTGGCCGTATAACCTTCAATGT TTCCCTATCCCTCGG
	BIP	ACATCCTTTTGGTTGGGATTCTTTC TAGGATTGTTGCCAGTT
	Loop_F	TGACCTACATGTAAACCTTCGG
	Loop_B	TTACCAGCTGAACAATATGCTT
Group 16SrXII	F3	AGTTTTAGCTACCGAACATGAA
	B3	CGGAACGATTTCCTTAGTAAGA
	FIP	ATCGGCAACCCAAATAGGTATTGAAC AAAGATAAAAGTGGCGTT
	BIP	TGGTACAGGGATTTTGATGGGATG GTTGAATCACTTGAATCATT
	Loop_F	CCATTGCAAGGATTAATAGCGT
	Loop_B	GTTCCCTGCCACGATCAA

5.3.2 Validation of LAMP group-specific primers

Validation tests were undertaken using the LAMP primers for seven different groups of phytoplasma (I, II, III, V, VI, X, and XII) by using DNA samples from a broad range of different phylogenetic groups. The DNA samples were extracted using the DNeasy Plant Mini Kit (Qiagen Ltd) (see chapter 2), followed by preparing mastermixes then running phytoplasma DNA samples using the real-time LAMP machine (Genie II, Optigene Ltd, UK) and confirming that the primers only detected phytoplasmas in the specific groups for which they were designed. Results are presented in Table 5.2.

Table 5.2 Validation of primers for different phytoplasma groups. The first number is time (when amplifications started), and the number in brackets is the anneal temperature for each sample

Isolate	Group 16Sr	Name of phytoplasma group assay						
		I	**II**	**III**	**V**	**VI**	**X**	**XII**
RG	I	19:26 (84.74)	-	-	-	-	-	-
RIV	I	22:26 (84.35)	NT	NT	NT	NT	NT	NT
SGP	I	16:56 (84.27)	NT	NT	NT	NT	NT	NT
FBP	II	NT	16:38 (82.54)	NT	NT	NT	NT	NT
SP	II	-	16:53 (82.39)	-	-	-	-	NT
VCP	II	NT	15:53 (82.41)	NT	NT	NT	NT	NT
LNI	III	NT	NT	12:42 (84.67)	NT	NT	NT	NT
PWX	III	-	-	10:57 (85.47)	-	-	-	-
EY-C	V	-	-		18:32 (81.15)	NT	NT	NT
PWB	VI	-	NT	NT	NT	17:32 (82.24)	NT	NT
BLL	VI	NT	-	-	-	22:32 (82.34)	-	-
AP	X	-	-	-	-	-	9:23 (84.22)	-
NGS	XI	-	NT	-	-	NT	-	-
Cornwall	XII	NT	NT	NT	NT	NT	-	13:27 (84.47)
C.p.fragariae.Jersey	XII	NT	NT	NT	NT	NT	-	11:42 (84.57)
Control		-	-	-	-	-	-	-

Data is presented as the time of amplification, with the anneal temperature given in parenthesis. NT = not tested,. **-** =no amplification.
A range of phytoplasma groups was tested with each primer set to confirm that each set can only amplify for the intended group.

Following LAMP reactions in the Genie II machine and real-time analysis, representative samples were also checked by gel electrophoresis (Fig. 5.1).

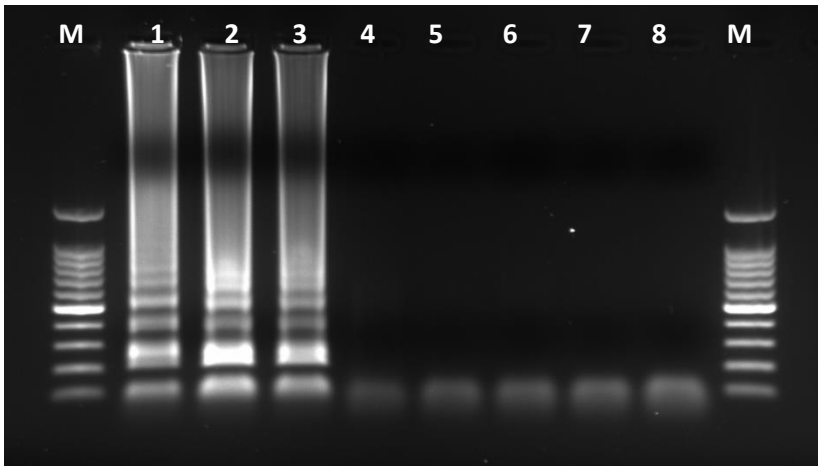


Figure 5.1: Picture of gel after running 16SrI LAMP products, M:100 bp DNA Ladder (Promega), 1: RG, 2: RIV, 3: SGP, 4: SP, 5, LNI, 6: AP, 7: NGS and 8: control (healthy periwinkle plant). The first three samples belong to 16SrI and gave positive results after running products on agarose gel and the remaining samples plus control gave negative results (no laddering), confirming the specificity of the 16SrI primers.

5.3.3 Sensitivity of LAMP assays

Evaluation of the sensitivity of the LAMP assays was performed by diluting DNA samples. The results below are for 16SrI Rehmannia glutinosa (RG) DNA. Total (plant and phytoplasma) DNA concentrations were determined by NanoDrop as 29.3 ng/µl, and serial dilutions were then made of 1/10, 1/100, 1/500, 1/1000, and 1/10000. The RG sample and dilutions were then tested by LAMP and compared with conventional PCR using 16S rRNA primers (P1 & P7) for first round and then diluting the first round products 1 in 40 of water and using 1 µl of this dilution for each sample for second-round PCR (see Fig. 5.2).

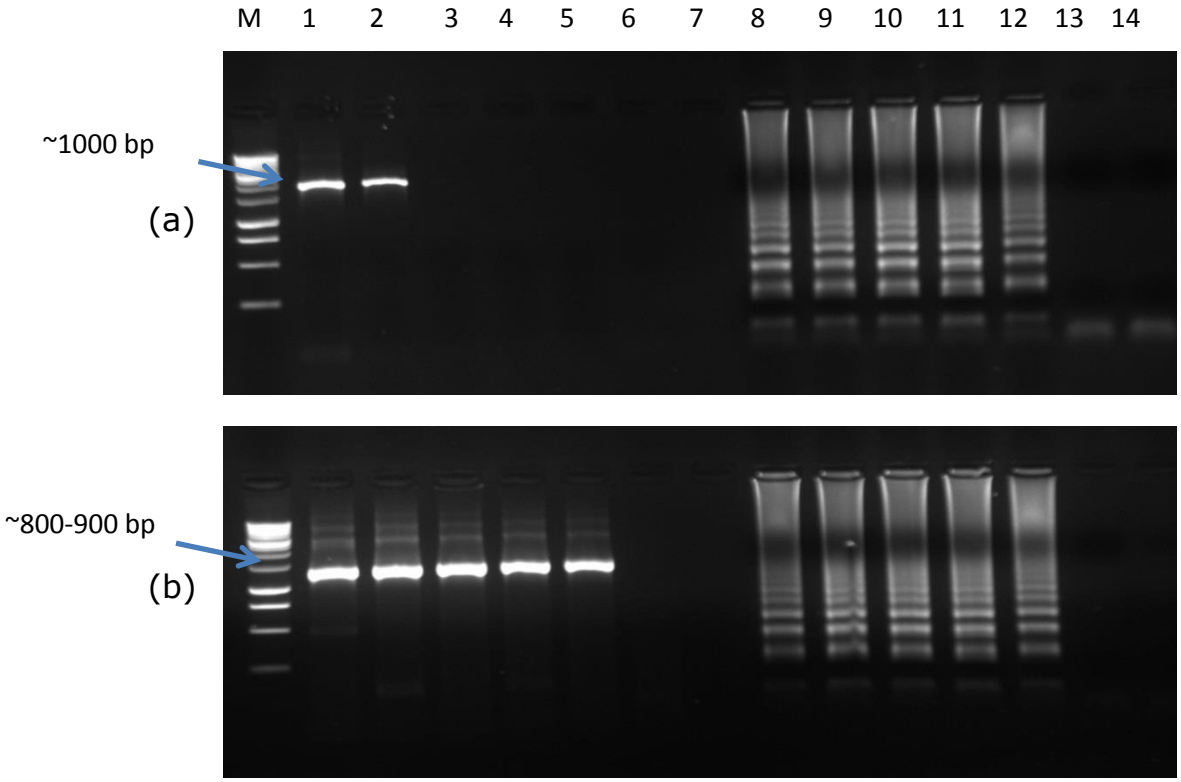
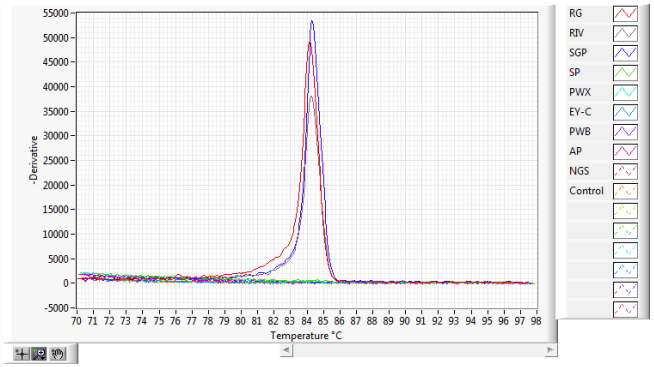


Figure 5.2: (a) Comparison of detection sensitivity of LAMP assays for group 16SrI compared with conventional PCR with 16S primers (P1 & P7). The samples were serially diluted from RG sample. Lane M: 1-kbp DNA ladder (Promega), Lanes 1 to 6 samples (1 and 8: original RG samples, 2 and 9: 1/10, 3 and 10: 1/100, 4 and 11: 1/500, 5 and 12: 1000, 6 and 13: 1/10000, 7 and 14: negative control (periwinkle healthy plant). 1 to 7 were tested by first-round PCR and 8 to 14 were tested by LAMP assays. (b) Comparison of detection sensitivity of LAMP assays of group I and nested PCR with 16S primers (16Sr F2/16Sr R2). Results show that LAMP assays can amplify DNA of phytoplasma better than first round PCR assays with dilutions from 1/10 to 1/1000 (first round can only amplify phytoplasma from original sample and first dilution sample 1/10). But second round PCR assays have similar sensitivity to the LAMP assays.

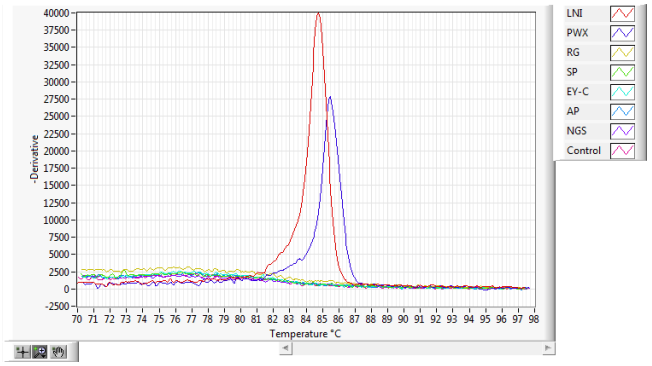
5.3.4 Anneal Analyses

The LAMP assays (Table 5.3) indicated that as well as positive samples showing amplification between 10 and 23 minutes, different 16Sr groups also had different annealing temperatures and shapes to the anneal temperature curves (Fig. 5.3 and Table 5.3). For a few groups such as group 16SrI, there was a clear single anneal temperatures for all isolates, whilst for others, e.g. 16SrIII, different isolates gave different anneals, and some groups gave double peaks (e.g. 16SrV and X).

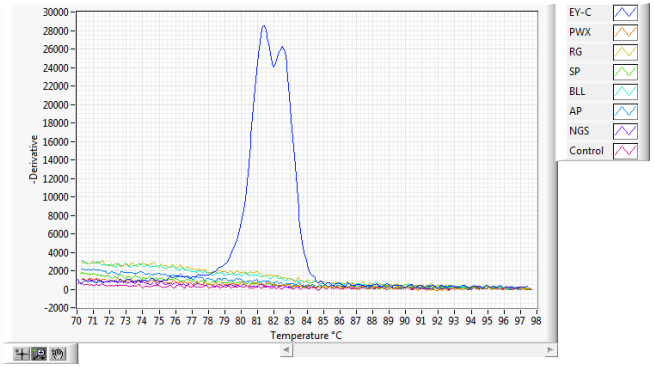
Based on this anneal analysis, an experiment was designed to determine whether anneal analysis could be combined with conventional PCR using universal primers, to discriminate between different phylogenetic groups. Because the 16S-23S ITS region shows the greatest difference in sequence between phylogenetic groups, this region was chosen for the analysis. PCR was performed using target DNA samples with SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma-Aldrich) instead of MangoMix ™ (Bioline Ltd), and 10 µM each of primers P4 (5'- GAAGTCTGCAACTCGACTTC-3') and P7 (5'-CGTCCTTCATCGGCTCTT-3') (Smart *et al.,* 1996). PCR thermal cycling parameters were: denaturation at 94°C for 30 sec, annealing at 53°C for 1 min and extension at 72°C for 1 min for 30 cycles. PCR products were then transferred to the Genie II machine for anneal analysis (Table 5.4 and Fig. 5.4). In general, there were a broad range of anneal temperatures, and different shapes to the anneal curves; with some good discrimination between different phylogenetic groups (e.g. 16SrI isolates had consistently higher annealing temperatures than 16SrII isolates).



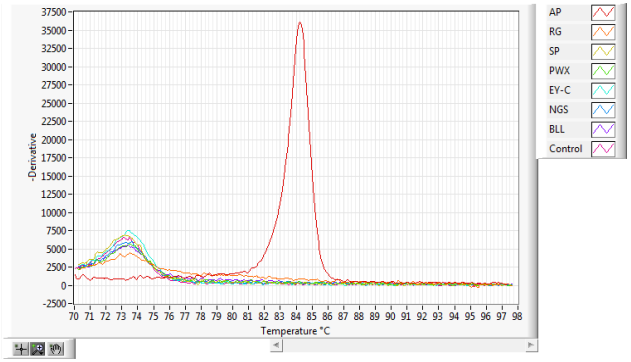
A: 16Sr I Group



B: 16Sr III Group



C: 16Sr V Group

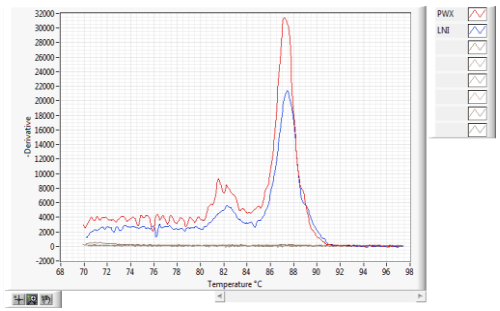


D: 16Sr X Group

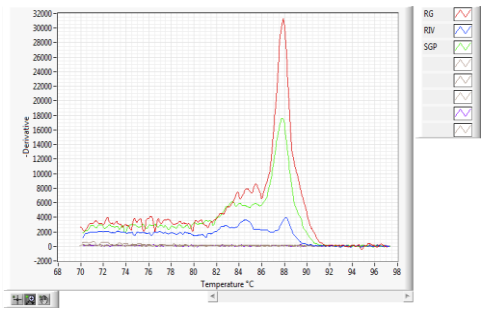
Figure 5.3 Annealing temperatures for different LAMP assays (A, B, C, and D) with different phytoplasma samples.

Table 5.3 Anneal readings of P4 & P7 PCR products for six different phytoplasma groups (different curves and different anneal value for each group).

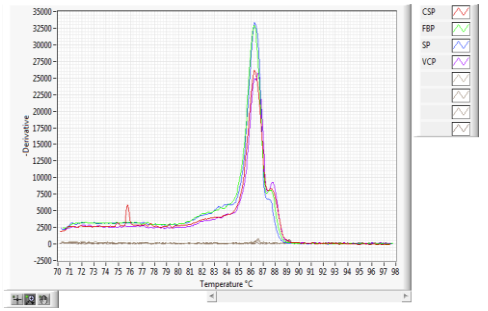
16Sr group	Well number	Sample name	Anneal value (℃)
Group I	A1	RG	87.88
	A3	SGP	87.80
Group II	A2	FBP	86.26
	A3	SP	86.30
	A4	VCP	86.60
Group III	A1	PWX	87.18
	A2	LNI	87.48
Group V	A1	EY-C	82.77 & 87.79
Group VI	A1	PWB	85.16
	A2	BLL	85.76
Group X	A1	AP	85.23



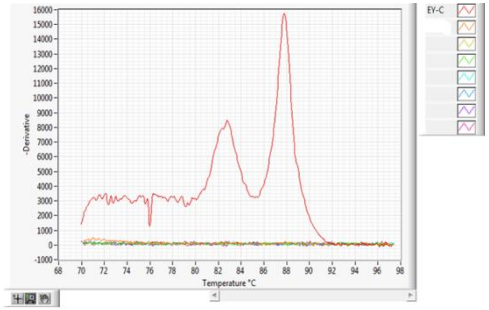
A: Group I



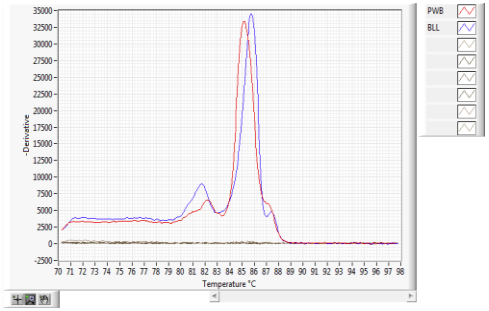
B: Group II



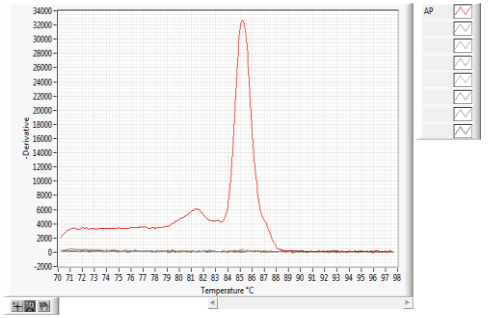
C: Group III



D: Group V



E: Group VI



F: Group X

Figure 5.4: Anneal curves for melting temperatures of P4/P7 PCR products for groups (A, B, C, D, E, and F).

5.3.5 Designing sets of primers and probes for real-time PCR

Most TaqMan real-time primers and probes have generally been based on 16S and 23S RNA genes. In this study, sequences obtained using the leucyl tRNA synthetase primers, *secA* gene sequences and 16S rRNA gene sequences were examined for suitability as primers using the Genscript Designer software to design two primers and one probe for each different sequence. Primers and probes were obtained for seven different phylogenetic groups, 16Sr III, IV, V, VI, X, XI and XII (see Table 5.4). The software could not provide specific primers and probes for group 16SrII, because there are four different strains and it was difficult to find specific primers that could amplify all strains of group II and at the same time not amplify from other groups. However, for these new assays, only the F3 and B3 primers were ordered since the aim was to validate new primers in the cheaper SYBR-Green assays, alongside testing the primers designed by Hodgetts *et al.* (2009) in SYBR-Green assays, even though these primers were originally designed for TaqMan assays.

Table 5.4. TaqMan primers and probes designed for 16SrIII, IV, V, VI, X, XI and XII group phytoplasmas.

16Sr group	Source/Strain	Target gene	Product length	Primers (5'-3')	Probe (5'-3')
Group III	Peach Western X-Disease	Leucyl gene	150 bp	**III-F** CGAAGAAGTTGTTCCTACTGAGGATG **III-R** TGAGGCGGCCATTCTAGTAAGTCT	**III-P** GACTACCGGATGAGACCCTCTTTCA
Group IV	Coconut lethal yellowing	16S gene	141 bp	**IV-F** ATCTCACGACACGAGCTGAC **IV-R** GCGGTGGATCATGTTGTTTA	**IV-P** CCATGCACCACCTGTATCCCTGA
Group V	Elm Yellows Phytoplasma diseases	secA gene	150 bp	**V-F** CACTCCGTTTAGCCCTTCTC **V-R** AAAGCCTTCTTGGCAAACAT	**V-P** CCCTATGTTGACTCATATCGCTTCGGA
Group VI	Potato Witches' Broom	16S gene	123 bp	**VI-F** GGGCTTGCGCCACATTAGTT **VI-R** GCCTCCCGTAGGAGTTTGGG	**VI-P** CAGTCCCAATGTGGCTGTTCAACC

Group X	Apple Proliferation	Leucyl gene	144 bp	**X-F** GCAACGTTATTGGCTTGAAA **X-R** ATATCGCTGGCCGTATAACC	**X-P** CCCTATCCCTCGGCCGAAGG
Group XI	Napier Grass Stunt	Leucyl gene	74 bp	**XI-F** CCAATTTGGATTTCTGATTATG **XI-R** ATATCACAAGAAGGCACGCA	**XI-P** ACTGCTCCTGTCCCGAAATGCA
Group XII	*Candidatus* Phytoplasma australiense	16S gene	117 bp	**XII-F** CATGCTGATTCGCGATTACT **XII-R** AAAGGGCAGCTAAAGCGTAA	**XII-P** CGAGTTGCAGACTTCAATCCGAACTG

5.3.6 Real-time PCR analysis

Previously, Hodgetts *et al.* (2009) have designed a universal assay for TaqMan real-time PCR to detect phytoplasmas along with a specific assay for group 16SrI. To avoid the high cost of ordering TaqMan probes, it was decided to test whether these same primers could also be used in SYBR-Green assays as an alternative to TaqMan. Therefore the 16SrI primers (JHF1 and JHR) with probe (JHP) were used in a Taqman assay and compared alongside use of JHF1 and JHR in a SYBR-Green assay, initially for the Strawberry green petal phytoplasma DNA. Results can be seen in Figures 5.5 and Fig 5.6).

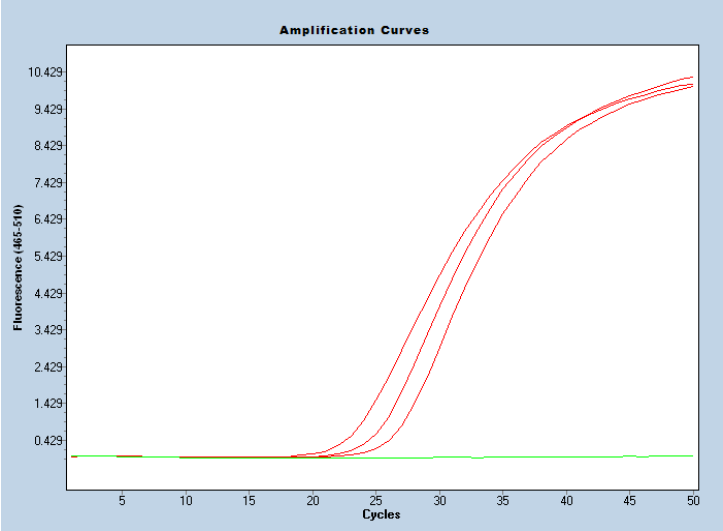


Figure 5.5: Amplification curves for Strawberry green petal DNA samples (16SrI) using the TaqMan real-time PCR assay (Naofel Aljafer, this study).

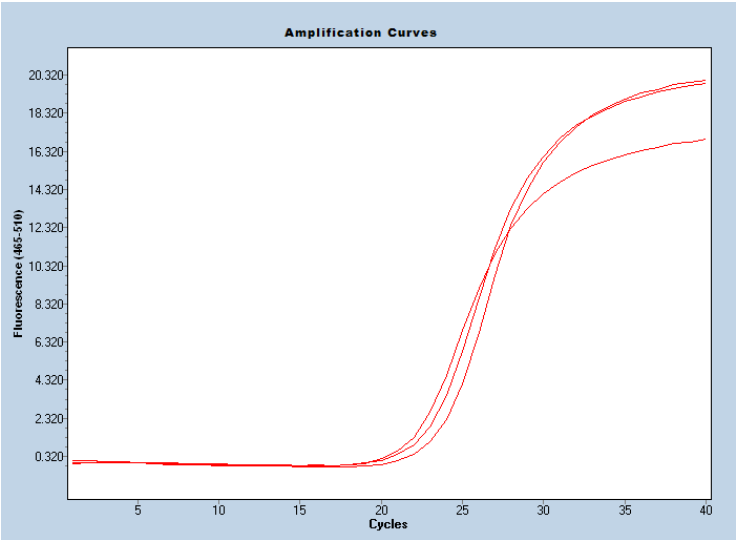
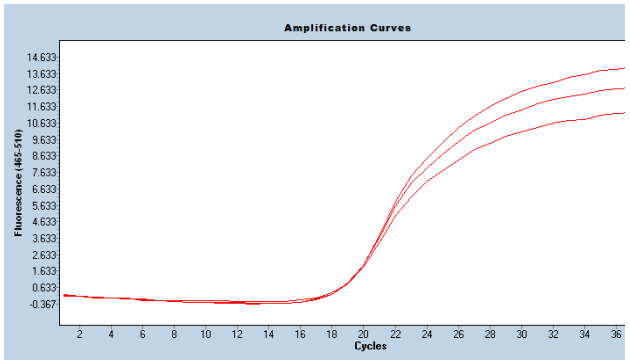


Figure 5.6: Amplification curves for Strawberry green petal DNA samples (16SrI) using the SYBR green real-time PCR assays (Naofel Aljafer, this study).

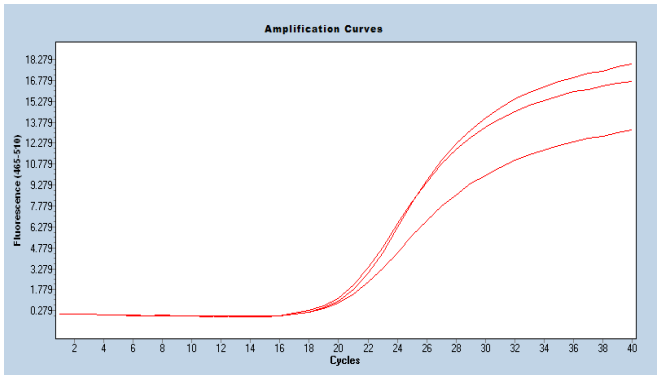
Since these initial results indicated that the primers worked effectively in SYBR-Green assays, it was decided to design primers for other phylogenetic groups using a range of different sequences, but using the same primer design software, simply omitting to order the probes. For each phylogenetic group for which we had isolates of phytoplasma maintained in the glasshouse, we designed and tested specific primers with SYBR-green, using three DNA replicates for each sample (Table 5.5). Amplification curves are shown in Figure 5.7.

Table 5.5 Results of testing phytoplasma DNA samples from different phylogenetic groups with the primers shown in Table 5.4 in SYBR-Green assays. The Ct values obtained for three replicates are shown in each case.

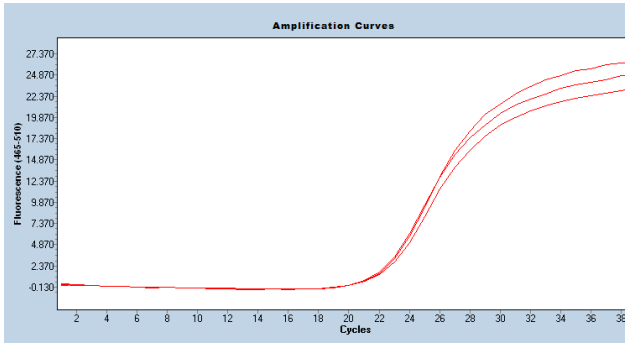
16Sr Group	Samples	C_T	Anneal value (℃)
Group III	LNI 1	18.13	79.90
	LNI 2	18.19	79.93
	LNI 3	18.31	79.94
Group IV	LYHV 1	20.28	82.56
	LYHV 2	20.05	82.54
	LYHV 3	20.56	82.69
Group V	EY-C 1	21.99	82.53
	EY-C 2	21.76	82.48
	EY-C 3	21.99	82.51
Group VI	PWB 1	18.66	83.70
	PWB 2	18.31	83.67
	PWB 3	18.06	83.70
Group X	AP 1	19.09	79.06
	AP 2	19.41	79.13
	AP 3	20.18	79.06
Group XI	NGS 1	19.12	80.75
	NGS 2	18.96	80.80
	NGS 3	19.34	80.83
Group XII	Cornwall, UK 1	19.84	85.72
	Cornwall, UK 2	19.93	85.08
	Cornwall, UK 3	23.44	85.29



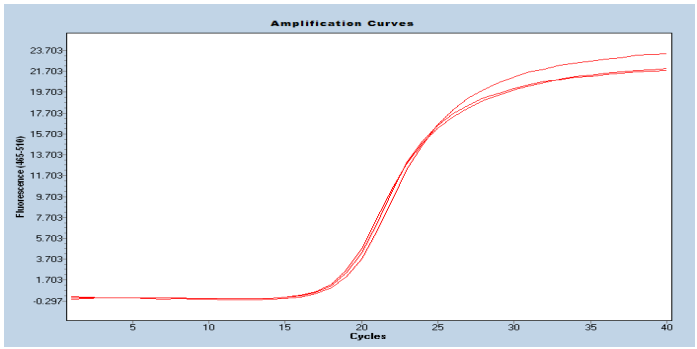
A: LNI samples (Group III)



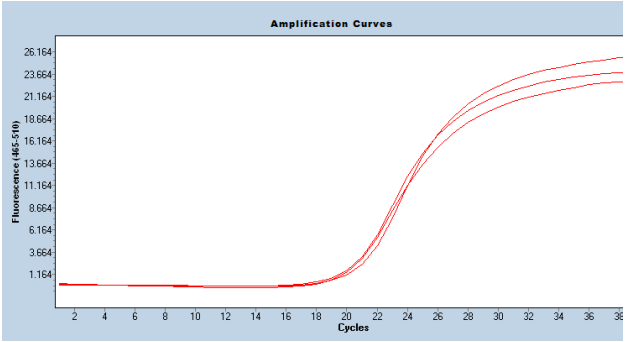
B: LYHV samples (Group IV)



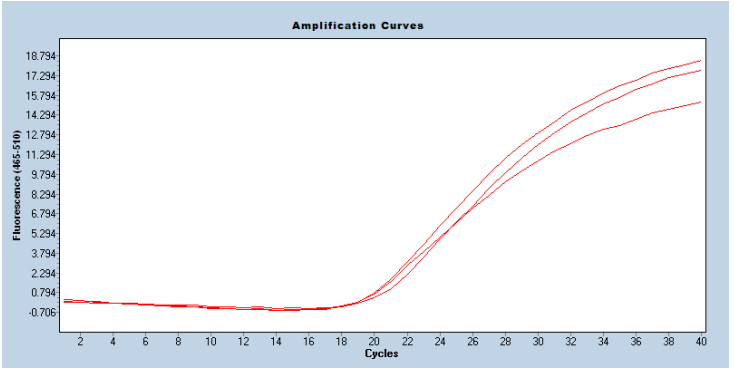
C: EY-C samples (Group V)



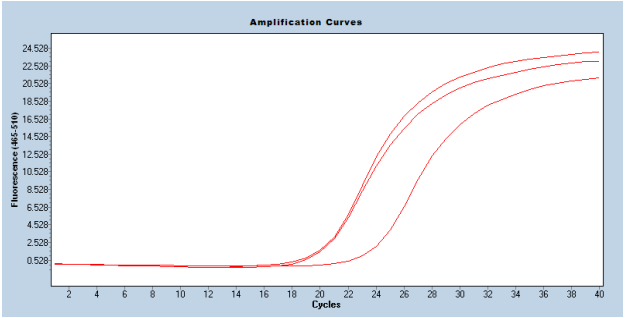
D: PWB samples (Group VI)



E: AP samples (Group X)

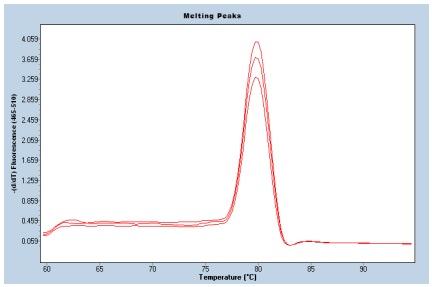


F: NGS samples (Group XI)

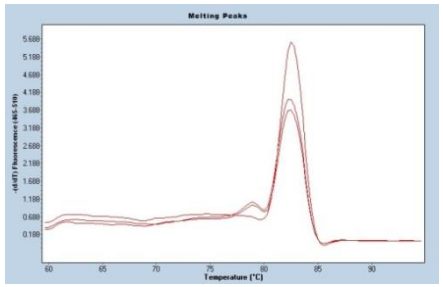


G: Cornwall, UK samples (Group XII)

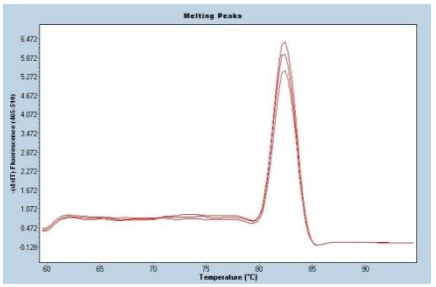
Figure 5.7 Amplifications curves obtained from running TaqMan primers in SYBR-green assays for phytoplasmas in groups 16Sr III, IV, V, VI, X, XI, and XII.



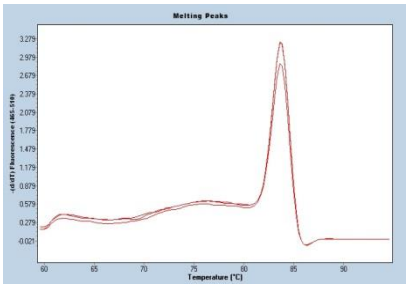
Group III



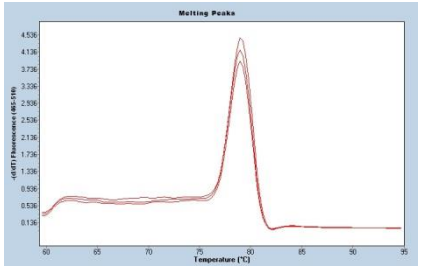
Group IV



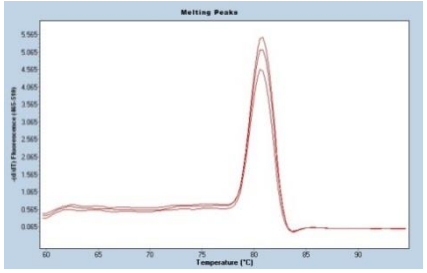
Group V



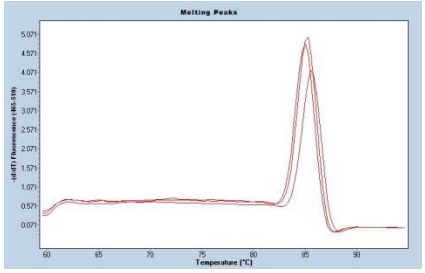
Group VI



Group X



Group XI



XII Group

Figure 5.8 Melt curves for melting temperatures of specific group assays of (Group III, V, IV, VI, X, XI, and XII) by running TaqMan primers with SYBR green set.

The results in table 5.5 and Figure 5.7 show that the values of C_T of different 16Sr groups III, IV. V, VI, V, X, XI and XII are from 18 to 23. Groups 16SrIII and VI have the lowest C_T values of about 18 and group 16SrXII has the highest C_T value of around 23. It was noted that there was a range of melt values. For example, groups 16SrIII and 16SrVI had the lowest melt temperature values of 79°C, and 16SrXII group has the highest melt temperature of 85°C.

In Fig 5.8, results of a melt curves for melting temperatures of specific groups confirm that all signals are only from every specific phytoplasma group and those signals come from those specific qPCR products and not from non-specific PCR amplifications.

5.4 Discussion

5.4.1 LAMP assay results

LAMP assays were developed for seven phytoplasma 16Sr groups based on the leucyl tRNA synthetase gene. Previously, several studies have been published on LAMP assays for phytoplasma detection, all of which had targeted the ribosomal RNA gene (Bekele *et al.,* 2011). However, it has often been difficult to find regions that are sufficiently unique for each of the different phytoplasma groups, because of the relatively well-conserved nature of such sequences (especially the 16S and 23S rRNA genes). The leucyl tRNA synthetase gene has a unique sequence for most groups of phytoplasma, and this has been reflected through the ability to design LAMP assays for phytoplasmas belonging to a range of different groups in this study. It should be noted that a study by Nakao *et al.* (2010) has suggested that the amplification efficiency of phytoplasma DNA in LAMP assays can vary between different targeted genes; however, in this study, the time to amplification for the different assays was usually within 20 minutes, with some assays, such as those for 16SrIII and 16SrX being around 10 minutes.

Of the assays designed, all appeared to give good specificity for the groups they were designed for. In previous studies, it was found that the rRNA assay for 16SrII is not able to detect all strains or subgroups of group II (Bekele *et al.,* 2011). However, based on the isolates tested, the leucyl tRNA synthetase based assay seemed to be able to pick all isolates in this group. Similarly, the 16SrIII assay was able to amplify several strains belonging to this group with different amplification times. In the studies of Tomlinson *et al.* (2010) it was found that the rRNA assay for the 16SrXXII

CSPWD, also amplified the closely related Mozambique lethal decline strain isolates, so was able to show good specificity and sensitivity. It is therefore clear from these various assays that it is not possible to predict which gene(s) will be best for assay design, and it is likely to be a question of trial and error, designing primer sets for a range of different genes and validating these against a broad range of isolates, that will determine which particular set of primers will be best for particular groups and subgroups. To date, good specific assays have now been designed in this study and others for a broad range of different phytoplasmas.

Results of detection sensitivities of LAMP assays show that such assays can amplify phytoplasma DNA with dilutions 1/10 to 1/1000 which is better than first round PCR assays, which can amplify only original samples and the first dilution. However, second-round (nested PCR) has similar sensitivity as the LAMP assays. Neither LAMP assays nor conventional PCR could amplify DNA from sample with 1/10000 dilution, which agrees with the results of Tomlinson *et al.* (2010).

5.4.2 Real-time assay results

This chapter also describes experiments aimed at designing specific primers and qPCR assays based on TaqMan design, but using SYBR green as a means of reducing the costs of the assays, with the aim of designing assays to detect particular phytoplasma phylogenetic groups. A number of specific primers have been designed previously based on sequences of the 16/23S rRNA genes; for example primers for the Apple proliferation (16SrX) group (Torres *et al.,* 2005), Flavescence dorèe, and Bois nois group (Angelini *et al.,* 2007), the 16SrVI group phytoplasmas (Crosslin *et al.,* 2006) and the 16SrI group (Hodgetts *et al.,* 2009).

In this study, the first test was to confirm that TaqMan designed primers could work in SYBR-green assays, and this was done by comparing the use of primers JHF1 and JHR designed by Hodgetts *et al.* (2009) for 16SrI with and without the TaqMan probe The results showed that the specificity and sensitivity is almost the same using either system, so the SYBR-Green system can potentially be used at much lower cost than the TaqMan system. Based on this, primers were designed and validated for a range of different 16Sr groups, III, IV. V, VI, V, X, XI and XII, and all results showed the values of C_T are between 18 and 23. These new primers will be useful to detect phytoplasmas especially in mixed infections and potentially also for quantification of phytoplasmas in plants.

Melting curves of qPCR amplicons is necessary after the PCR reactions when using the SYBR-Green assay, to confirm that the signal is from the specific product and not an artefact, and the analysis conducted in this study confirmed that the primers did have the required specificity and did not amplify non-target DNA.

One further set of experiments that was attempted was to determine whether LAMP-type anneal curve analyses could be combined with conventional PCR as a technique for being able to amplify phytoplasma DNA using universal primers, and then use the anneal analysis to assign the isolate to a particular phylogenetic group. The hypothesis was that if the sequences between the universal primers were sufficiently different between different isolates and groups, this would result in different anneal temperatures. Because the ITS region is more variable than either the 16S rRNA of the 23S rRNA, it was decided to use primer from either side of the ITS region to test this hypothesis. The results showed that there was

indeed variation in the anneal temperature for different isolates, and in some cases, these could be correlated with the 16Sr group. For example, the 16SrI isolates has anneal temperatures (87.88 and 87.80) higher than isolates in other phylogenetic groups, apart from the 16SrV isolate which had a very distinct double-peak. Both the 16SrIII isolates tested had similar, slightly lower anneal temperatures, and all three 16SrII isolates had anneals in the 86°C range (no other isolates had anneals in this range). There were also quite distinct differences in the shapes of anneal curves for different phylogenetic groups. Therefore, the method appears to have some potential for discrimination between isolates and groups, but more isolates and also different sets of universal primers probably need to be tested to identify a more robust discriminatory system that can combine conventional PCR with anneal analysis for 16Sr group discrimination.

Chapter 6: General Discussion

This study has led to the development of several diagnostic techniques (PCR, LAMP, and qPCR) for phytoplasmas which are easy to use, and offer improvements to established techniques. These sensitive and specific methodologies can be used for the detection of phytoplasmas within the host plant, and include both universal primers to improve group identification of phytoplasmas and specific primers / assays to detect specific 16Sr groups.

The essential element required to enable the development of new diagnostic techniques for any organism lies in the availability of good quality sequence data. For phytoplasmas from most 16Sr groups, such data is limited for genes other than the 16S rRNA and *secA* (Hodgetts *et al.,* 2008). This led to attempts to develop universal primers that could be used to amplify DNA from additional genes for a broad range of phytoplasmas, at the same time as using other published 'semi-universal' primers to see how successful these are at amplifying different 16Sr groups. The result was the development of a new universal set of primers for the leucyl tRNA synthetase gene, which in turn allowed for phylogenetic analysis to be performed (Chapter 3). Using first-round PCR primers (LeuF1/LeuR1) products were obtained of around 1460 bp, which when followed by nested PCR primers (LeuF2/LeuF2) gave PCR products of around 800 bp as shown in Table 3.2 and Figure 3.3. Although the primers worked on most isolates tested, there were initially no amplifications at the standard annealing temperature of 53°C when they were used to amplify phytoplasmas belonging to 16SrIV from coconut samples, so a new forward primer LeuF2LY was designed for nested PCR which, when mixed

with the original LeuF2 primer, resulted in nested PCR amplification at this standard temperature for these samples (see Table 3.3).

The phylogenetic tree based on these partial leucyl tRNA synthetase gene sequences is similar to the phylogenetic trees based on the 16S rRNA and *secA* genes, supporting the overall system used for phytoplasma classification (Bertaccini and Duduk, 2009; Bertaccini *et al.,* 2014). There was one anomaly in the initial phylogenetic tree, which was the location of the Tanzanian coconut lethal decline sample. In the phylogenetic trees based on 16S rRNA and *secA,* this isolate was classified as 16SrIV-B TLD, related but distinct from the coconut lethal decline phytoplasmas from Ghana, Nigeria and Mozambique (16SrIV-C) and lethal yellows phytoplasmas from the Caribbean (16SrIV-A). More recently, these coconut phytoplasmas have been reclassified (Harrison *et al.,* 2014) such that the 16SrIV group is now just the lethal yellows phytoplasmas (now known as *'Ca.* Phytoplasma palmae'), with the Ghanaian, Nigerian and Mozambique coconut diseases reclassified as 16SrXXII (now known as '*Ca.* Phytoplasma palmicola'), and the Tanzanian lethal decline (now known as '*Ca.* Phytoplasma cocostanzania') has no new 16Sr grouping in the revised classification system of Wei *et al.* (2007). Instead, in the initial leucyl tRNA synthetase tree, this TLD isolate aligned as being identical to peach western X (16SrIII group), so was removed from Figure 3.5. It is noteworthy that this is an old sample that has been stored since 1992, and we suspect that the DNA had become contaminated at some point with 16SrIII DNA, since it is highly unlikely that this phytoplasma would group in this position.

An interesting feature that does arise from the leucyl tRNA synthetase gene tree is that it provides a clear distinction between the Ghanaian

isolates of the coconut disease and the Nigerian / Mozambique isolates. The Ghanaian isolates GH1, GH6 and GH8 were originally collected in 1992 by Phil Jones (Rothamsted Research), whilst the Nigerian isolate (Awka5) was collected by Phil Jones in 1994. The other two Ghanaian isolates were collected by Matt Dickinson (University of Nottingham) in 2010, whilst the Mozambique isolates were collected by Simon Eden-Green (Natural Resources International) in 2007. This would suggest that the Mozambique disease (which was only detected for the first time in the early 2000's) probably originated from the Nigerian population of the disease rather than from the Ghanaian population.

The leucyl tRNA synthetase gene also provided some interesting information on the isolates classified as 16SrXI and 16SrXIV based on the 16S rRNA system. Based on previous 16S rDNA phylogenetic analysis (Jung *et al*., 2003), the sugarcane phytoplasmas sugarcane whiteleaf (SCWL) and sugarcane grassy shoot (SCGS) are closely related to phytoplasmas associated with rice yellow dwarf (RYD) in group 16SrXI, which is also where napier grass stunt (NGS) is positioned. Meanwhile the Bermuda grass whiteleaf group (BGWL) is classified as 16SrXIV (Lee *et al*., 1998; Wei *et al.,* 2007). The leucyl tRNA synthetase tree, however, indicates that the sugarcane isolates are more closely related to the 16SrXIV BGWL phytoplasmas based on this gene, rather than to other 16SrXI phytoplasmas such as napier grass stunt and flower stunting. These results support similar observations found for the *secA* gene for other isolates from the 16SrXI and 16SrXIV groups (Matt Dickinson and Saman Abeysinghe, unpublished observations), that some members of these groups appeared to be in one group based on 16S rRNA sequences but the alternative group based on *secA,* suggesting that some of these isolates may be recombinant phytoplasmas. Further work on more isolates

and using more genes would be required to confirm this hypothesis. However, designing universal primers for other genes is not an easy process as indicated in this study, where of the other three genes for which this was attempted, it was not possible to come up with regions to design primers for two of them, whilst for the third gene, RNA polymerase (pol), the primers failed to amplify from other phytoplasmas, presumably because of too much sequence diversity.

In the past, the 16S rRNA gene was the standard for discrimination between different groups and subgroups of phytoplasmas. However, in other bacteria multi-locus sequence typing (MLST) is now much more common for phylogenetic analyses (Larsen *et al.,* 2012). Other genes beyond the *secA* (Hodgetts *et al.,* 2008) and the leucyl tRNA synthetase gene (this study) have been attempted. Primers for the *tuf* gene (Makarova *et al.,* 2012) were shown in this study to be suitable for amplifying from a broad range of phytoplasmas, although these are a complex set of 13 primers (chapter 4). However, the rp primers (Martini *et al.,* 2007), which have also been proposed as a set of universal primers, were found in this study to amplify plant DNA as well as phytoplasmas when tested on other phylogenetic groups, so may not be appropriate as an easy-to-use set of universal primers.

Because of the previous work at Nottingham suggesting that some of the 16SrXI and 16SrXIV phytoplasmas might be recombinants, and the previous work of Jomantiene *et al.* (2007) who showed the presence of sequence-variable mosaics, and Bai *et al.* (2006) who found the presence of potential mobile units (PMUs) and evidence that these can move and change their positions in the phytoplasma genome suggesting there are mechanisms that generate diversity in phytoplasma genomes, experiments

were set up to investigate whether this is a rapid and easily-detectable process. Isolates from different phylogenetic groups were subcultured by grafting at intervals throughout the project and DNA extracted and tested using these sets of universal primers (16S rRNA gene, *secA* gene, *tuf* gene and leucyl tRNA synthetase gene) to look for any signs of diversity. However, no evidence was found for any rapid signs of changes during continual generations by using infected plants. What is required is more extensive studies in which different phytoplasmas are mixed in plants over multiple generations and also passaged through insects as well as between plants for many generations, along with whole genome sequence analysis once more draft sequences of phytoplasmas become available, to further our understanding of phytoplasma genome evolution. However, the systematic isolation and storage of DNA undertaken in this study starts to provide a resource that could be used in such future studies.

In chapter 5, improved diagnostic techniques for phytoplasmas were investigated. Assays based on the LAMP technique have been used to diagnose a number of plant pathogens (Tomlinson *et al.,* 2007; Tomlinson *et al.,* 2010). LAMP, because it is carried out at a single temperature, can be run on any suitable equipment that can maintain a constant temperature, such as in a water bath or on a simple heated block. LAMP amplicons can be detected by a range of methods including real-time methods (Bekele *et al.,* 2011). This study evaluated LAMP assays run on a real-time Genie II instrument for its effectiveness at detecting specific phylogenetic groups of phytoplasmas. The method involves the detection of amplicons as fluorescent signals. The specialised machine can also perform an anneal curve analysis which provides a means of validating the results. All infected samples positively diagnosed by nested PCR were also

detected by the LAMP assays (chapter 5). LAMP primers are easy to design by the LAMP designer software which can be purchased from OptiGene.

In previous studies, it was found that the rRNA-based LAMP assay for 16SrII group phytoplasmas was not able to detect isolates from all subgroups of 16SrII effectively (Bekele *et al.,* 2011). However, in chapter 5, four different strains which belong to different 16SrII subgroups were tested using primers designed based on the leucyl tRNA synthetase gene, and all these isolates were detected. In addition, the new 16SrX group assay based on the leucyl tRNA synthetase gene gave amplifications which started within 10 minutes which is very rapid for a phytoplasma LAMP assay, indicating that there is no apparent loss in sensitivity in using assays based on single copy genes. Based on the results to check sensitivity of phytoplasma detection using the new LAMP primers, the primers could amplify phytoplasma DNA at dilutions as low as 1/10 to 1/1000, which is better than the first round of conventional PCR, and similar to the detection level using nested PCR, which agrees with the results of Tomlinson *et al.* (2010). All group primers designed based on the leucyl tRNA synthetase gene gave very good specificity to detect their group, so these newly developed primers provide a valuable tool for in-field phytoplasma detection assays.

Also in chapter 5, primers were designed for new group-specific qPCR assays using SYBR-Green to avoid the high costs of ordering Taqman probes for each phytoplasma groups. Primers were designed and validated based on the leucyl tRNA synthetase gene and other universal genes (16S rRNA, and *secA* genes) for groups III, IV, V, VI, X, XI, and XII. Previously, several specific primers have been designed based on sequences of the 16-23S rRNA gene, such as primers for the 16SrI group (Hodgetts *et al.,*

2009), 16SrX group (Apple proliferation) (Torres *et al.,* 2005), Flavescence dorèe, and Bois nois groups (Angelini *et al.,* 2007), and the 16SrVI group phytoplasmas (Crosslin *et al.,* 2006).

Initial experiments involved redesigning the original Taqman assays of Hodgetts *et al.* (2009) to see whether the primers (JHF1 and JHR) for the 16SrI group assay could be used without the Taqman probe in a SYBR Green assay, and the results showed that the specificity and sensitivity was almost the same using both systems. Since the SYBR-Green system can potentially be used at a much lower cost than the TaqMan system, it was decided to use Taqman primer design software to design new primers for the other 16Sr groups, III, IV. V, VI, V, X, XI and XII. Validation of these primers in the SYBR-Green assays showed C_T values between 18 and 29 and the appropriate specificity, with melting curves of qPCR amplicons that confirmed the signals were from PCR products, and not artefacts. These new primers are now being used in the laboratory to detect and quantify phytoplasmas in mixed infection experiments to analyse competition between phytoplasmas in plants, and investigate the possibility of using phytoplasmas in cross-protection, based on previous experiments showing competition between phytoplasmas by other groups (Seemuller *et al.,* 2010; Schneider *et al.,* 2014).

The final set of experiments was to determine whether LAMP-type anneal curve analyses could be combined with conventional PCR as a technique amplifying phytoplasma DNA using universal primers, and then using the anneal analysis to allocate the isolate to a particular phylogenetic group. Conceptually, this is similar to HMAS (Heteroduplex mobility assays) (Wang & Hiruki, 2005), and SSCP (Single-stand conformation polymorphisms) (Music *et al.,* 2008) in that it is based on discriminating

between phytoplasmas through the sequences between universal primers being sufficiently different between different 16Sr groups for them to result in different anneal temperatures. Because the ITS region is more variable than either the 16S rRNA of the 23S rRNA, it was decided to use primers from either side of the ITS region to test this approach. The results showed that there is indeed sufficient variation in the anneal temperature between different isolates. For example, the 16SrI isolates had anneal temperatures (87.88°C and 87.80°C) higher than isolates in other phylogenetic groups, apart from the 16SrV isolate which gave a unique double-peak. Both the 16SrIII isolates tested had similar, slightly lower anneal temperatures, and also all three isolates of the 16SrII group had anneals in the 86°C range, whilst no other isolates had anneals in that range. Therefore, this method appears to be useful for discrimination between isolates and groups, and could be used in analysis of mixed infection by more than one phytoplasma group. Although it was not tested in this study, it is possible that other universal primers such as the leucyl tRNA synthetase primers, could provide still better resolution, particularly as these primers amplify a longer sequence than the ITS region.

Suggestions for future research

Both biological and genetic studies of phytoplasmas need to be continued to achieve effective phytoplasma disease control. This thesis provides useful techniques and information that can aid further research into phytoplasma diseases. Based on the results of this work, approaches have been tested and validated for designing new universal genes for use in MLST analysis of phytoplasmas, and the same approach of aligning genome sequences from diverse phylogenetic groups can now be used to identify potential primers for other genes. Whilst our results show that not all of these will necessarily work, it does indicate that some will, and these can provide better sets of primers than some of those currently available such as the rp primers that amplify plant DNA, and the tuf primers, that are a complex set of multiple primers. Further MLST analysis itself is also vital for being able to better discriminate between isolates of phytoplasmas, and potentially provide more detailed data on the epidemiology of phytoplasma diseases. For example, more detailed studies are required to identify the specific vectors and host ranges for particular subgroups, to identify the geographic distribution of isolates and how they spread, to identify where new incursions come from (for example the evidence in this thesis that suggests the Mozambique strain of coconut lethal disease originated from Nigeria), and to identify whether recombination between isolates is occurring (as suggested from the preliminary results on the sugarcane phytoplamas).

New unique sequences can also be used to design primers for different diagnostic techniques (conventional PCR, LAMP, and qPCR), and in turn these primers could be used for more detailed studies into the evolution of

new isolates of phytoplasmas, and also into the role of PMUs and recombination in this process. In addition, techniques that can better discriminate between different isolates could also be used to further investigations into control via cross protection, since the fate of isolates could be studied in mixed infection studies under controlled conditions. For example, because techniques are now available for detecting all the different groups and subgroups of phytoplasmas that are held in Nottingham in the Madagascan periwinkle, we are now able to introduce mixed infections and then study, using qPCR assys for the different 16Sr groups, how the different phytoplasmas compete with each other. This may lead to the identification of some isolates that produce mild symptoms but are particularly good at out-competing more severe strains.

Finally, the LAMP technique developed in this study, and in particular the real-time LAMP assays and use of a portable battery-operated detection system (the Genie II machine) offers the potential for LAMP assays to be used widely as a diagnostic technique for phytoplasmas in remote locations and in regions where limited sophisticated equipment is available. The provision of such a system, which avoids the need for samples to be sent to specialist labs for phytoplasma diagnostics, would facilitate much more rapid detection, which in turn could mean that control measures, such as roguing of infected trees to prevent spread to neighbouring trees, could be conducted in a more timely manner. Until new control techniques become available for phytoplasmas, such methods probably remain one of the most effective for limiting the spread of many phytoplasma diseases.

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Appendices

Appendix 1: List of primers that has been used in this thesis.

Primer name	Sequence 5'-3'	Reference
SecAF1	GARATGAAAACTGGRGAAGG	Hodgetts *et al.* (2008)
SecAR3	GTTTTRGCAGTTCCTGTCATNCC	Hodgetts *et al.* (2008)
SecAF5	ASTCGTGAAGCTGAAGG + AGCTAAAAGAGAATTTGAAGG	Bekele *et al.* (2011)
SecAR2	CCNTCRCTAAATTGNCGTCC	Bekele *et al.* (2011)
NGSsecfor1	TATACWACWAATAGTGAAATWGG	Abeysinghe *et al.* submitted
NGSsecrev1	GATAAGTAATAGTAGCAGCAATTTCAG	Abeysinghe *et al.* submitted
NGSsecfor2	CGATGAAGTWGATTCTGTC	Abeysinghe *et al.* submitted
NGSsecrev2	AGCTTCTAAAGCTTGATGTAATCC	Abeysinghe *et al.* submitted
Sugarsecfor2	GATTCTGTCTTAATAGACGAAGCTAG	Abeysinghe *et al.* submitted
Sugarsecrev2	GTAAATTGATCTATTATCAAAACATTATTT	Abeysinghe *et al.* submitted
Ricesecfor2	GATTCTGTCTTGATAGATGAAGCAAG	Abeysinghe *et al.* submitted
Rice secrev2	GTAAATTGGTCTATAATTAAAATCTGATTA	Abeysinghe *et al.* submitted

Ribosomal 16S rRNA gene set primers.

Primer name	Sequence 5'-3'	Reference
P1	AAGAGTTTGATCCTGGCTCAGGATT	(Deng and Hiruki, 1991)
P7	CGTCCTTCATCGGCTCTT	(Schneider *et al.*, 1995)
16s F2	GAAACGACTGCTAAGACTGG	(Gunderson and Lee, 1996)
16s R2	TGACGGGCGGTGTGTACAAACC	

Ribosomal protein gene set primers (Martini *et al.,* 2007).

Primer name	Sequence 5'-3'	Reference
rpL2F3	WCCTTGGGGYAAAAAAGCTC	(Martini *et al.,* 2007)
rp(I)R1A	ATGGTDGGDCAYAARTTAGG	(Martini *et al.,* 2007)
rpF1C	ATGGTDGGDCAYAARTTAGG	Lim & Sears (1992)

Leucyl tRNA synthetase gene set primers.

Primer name	Sequence 5'-3'	Reference
LeuF1	GAT ATG TTT CCT TAT CCT TC	Aljafer *et al.* (2016)
LeuR1	GTTTTRGCAGTTCCTGTC ATNCC	Aljafer *et al.* (2016)
LeuF2	CAT CCT TTT GGT TGG GAT TC	Aljafer *et al.* (2016)
LeuR2	CTS CCC AAT ATC TTT GRC G	Aljafer *et al.* (2016)
LeuF2LY	CATCCTTTTGGTTTTGAT AG	Aljafer *et al.* (2016)
PolF1	GAC ART GGT CTT ATG GTG	*This study*
PolR1	CCR CCN GTA TGR AAA GTT CTC	*This study*
PolF2	GGA TGG TWA TGG ANG TTA TTC C	*This study*
PolR2	GGT TCW CCR ATN GAT TGA GC	*This study*

Tuf gene set primers (Makarova *et al.,* 2012).

Barcode	Direct/nested PCR	Primer	Forward/reverse	Primer mix components	Sequence (5′–3′)
tuf	D	Tuf340	For	Tuf340a	GCTCCTGAAGAAARAGAACGTGG
				Tuf340b	ACTAAAGAAGAAAAAGAACGTGG
tuf	D	Tuf890	Rev	Tuf890a	ACTTGDCCTCTTTCKACTCTACCAGT
				Tuf890b	ATTTGTCCTCTTTCWACACGTCCTGT
				Tuf890c	ACCATTCCTCTTTCAACACGTCCAGT
tuf	N	Tuf400	For	Tuf400a	**GTAAAACGACGGCCAGT**GAAACAGAAAAACGTCAYTATGCTCA
				Tuf400b	**GTAAAACGACGGCCAGT**GAAACTTCTAAAAGACATTACGCTCA

				Tuf400c	**GTAAAACGACGGCCAGT**GAAACATCAAAAAGACAYTATGCTCA
				Tuf400d	**GTAAAACGACGGCCAGT**GAAACAGAAAAAAGACAYTATGCTCA
				Tuf400e	**GTAAAACGACGGCCAGT**CAAACAGCTAAAAGACATTATYCTCA
tuf	N	Tuf835	Rev	Tuf835a	*TAATACGACTCACTATAGGG*AACATCTTCWACHGGCATTAAGAAAGG
				Tuf835b	*TAATACGACTCACTATAGGG*AACACCTTCAATAGGCATTAAAAAWGG
				Tuf835c	*TAATACGACTCACTATAGGG*AACATCTTCTATAGGTAATAAAAAAGG

Appendix 2: Isolate collections of phytoplasma groups on periwinkles plants in glasshouses at University of Nottingham.

Isolate	Plant No	Group	Source	RESULT (Tm)	LAMP 10/2010	PCR: R16 10/2010	Lamp June/July 2011
Chrysanthemum Yellows	ChrymA	I-A			neg	pos	neg
Chrysanthemum Yellows	ChrymB	I-A	Jen / Fera		neg	neg	neg
Catharanthus virescence	CVT	I-B	Jen / Fera		neg	neg	neg
Rehmannia glutinosa	173	I-B	Czech Republic	pos. (85.0)	neg	pos	pos
Rehmannia glutinosa	35	I-B	Czech Republic	pos. (84.9)	pos	pos	pos
Rehmannia glutinosa	35 cut 1	I-B	Czech Republic				pos
Rehmannia glutinosa	35 cut 2	I-B	Czech Republic				pos
Clover phyllody	KVE-B	I-C	Jen / Fera		neg	neg	neg
Ribes in Vinca	159	I-C	Czech Republic	pos. (84.9)	pos	pos	pos
Ribes in Vinca	192	I-C	Czech Republic		neg	pos	pos
Strawberry Green Petal	160.2	I-C	Czech Republic				pos
Strawberry Green Petal	16.4.1	I-C	Czech Republic	neg. / neg.	neg	pos	pos
Strawberry Green Petal	16.4.2	I-C	Czech Republic		pos	pos	pos
Crotalaria Saltiana Phyllody (witches broom)	CSP1	II-C	Rothamsted	pos. (82.8)	pos	pos	pos
Crotalaria Saltiana Phyllody (witches broom)	CSP3	II-C	Rothamsted	pos. (82.7)	pos	pos	pos
Crotalaria Saltiana Phyllody (witches broom)	CSP4	II-C	Rothamsted	pos. (82.7)	neg	pos	pos
Faba Bean Phyllody	NC2/3	II-C	CSL	pos. (82.8)	pos	pos	pos
Faba Bean Phyllody	NC2	II-C	CSL	pos. (82.7)	pos	pos	pos
Faba Bean Phyllody	NC2/1	II-C	CSL	pos. (82.7)	pos	pos	pos
Soybean Phyllody	54	II-C	Rothamsted	pos. (82.7)	neg	pos	weak
Soybean Phyllody	68	II-C	Rothamsted	pos. (82.7)	pos	pos	pos

Soybean Phyllody	78	II-C	Rothamsted	pos. (82.7)	neg	pos	weak
Soybean Phyllody	p=1	II-C	Rothamsted	pos. (83.0)	pos	pos	pos
Sweet Potato Little Leaf	07/10 1	II-D	Rothamsted	pos. (83.3)	neg	pos	
Sweet Potato Little Leaf	16.6.1	II-D	Rothamsted		pos/neg	pos	
Sweet Potato Little Leaf	16.6.2	II-D	Rothamsted		neg	pos	
Sweet Potato Little Leaf	16.6.4	II-D	Rothamsted		neg	pos	
Sweet Potato Little Leaf	194	II-D	Rothamsted		ND	ND	
Australian Tomato Big Bud	165	II-D	CSL	neg.	neg	pos	
Vinca Coconut Phyllody	4	II	Rothamsted	pos. (82.9)	pos	pos	pos
Vinca Coconut Phyllody	15	II	Rothamsted	pos. (82.7)	ND	pos	pos
Vinca Coconut Phyllody	10 1	II	Rothamsted	pos. (82.8)	ND	pos	pos
Peach Western X	451	III-A	Italy		pos	pos	pos
	LNI	III-B	Jen / Fera		pos	pos	pos
Pointsettia Branching Factor	large	III			pos	pos	pos
	small	III			neg	pos	pos
	3	III			neg	pos	
Napier Grass Stunt	7	III	Rothamsted			pos	neg
Elm Yellows	EY-C	V-A	Jen / Fera			pos	pos
Potato Witches Broom	p=192	VI-A	Italy			pos	
Potato Witches Broom	cut 1	VI-A	Italy			pos	
Potato Witches Broom	cut 2	VI-A	Italy			pos	
Brinjal Little Leaf	2	VI-A	Rothamsted			pos	
Brinjal Little Leaf	73	VI-A	Rothamsted			pos	
Brinjal Little Leaf	59	VI-A	Rothamsted			neg	
Brinjal Little Leaf	41	VI-A	Rothamsted			pos	
Brinjal Little Leaf	8	VI-A	Rothamsted			pos	
Appled proliferation	AP	X-A	Ljubiana			pos	
German Stone fruit yellows	GSFY	X-B	Jen / Fera			pos	
Napier Grass Stunt	5	XI	Rothamsted			pos	pos
Napier Grass Stunt	S10	XI	Rothamsted			pos	pos
Napier Grass	183	XI	Rothamst	pos.		pos	pos

Stunt			ed	(84.4)			
Napier Grass Stunt	S10 /1	XI	Rothamsted	pos. (84.7)		pos	pos
Napier Grass Stunt	S10 /2	XI	Rothamsted	pos. (84.6)		pos	pos
Napier Grass Stunt	NGS 1	XI	Rothamsted	pos. (84.4)		pos	pos
on the floor							
sugar cane	007			neg.		neg	
Napier grass	A16840			neg.		pos	
Napier grassneg healthy							
Healthy C.roseus	H15						
	H2						
	SGP (P=166)						
	H23						
	H15/3						
	H172/L						
	H17/2A						
	H17/21/3						
oil palm							

Appendix 3: List of DNA samples that store at -20 ˚C at University of Nottingham.

Extract No	Isolate	Group	Plant ID/Source	H_2O / TE	Ext date	Ext method
	Box 1 DNA numbers 1-50					
1	Healthy Periwinkle		prior 2006	?	?	?
2	Burkholderia gladioli		?	?	?	?
4	Sweet Potato Little Leaf	II-D	prior 2006	?	?	?
5	Napier Grass Stunt	XI	prior 2006	?	?	?
6	Napier Grass Control		prior 2006	?	?	?
7	Stolbur	XII	prior 2006	?	?	?
8	Ramu Stunt	XI	prior 2006	?	?	?
9	Brinjal Little Leaf	VI	prior 2006	?	?	?
10	Elm Yellow	VI	prior 2006	?	?	?
11	Green Valley X	III	prior 2006	?	?	?
12	Peach Western X	III	prior 2006	?	?	?
13	Aster Yellows Cactus	I	prior 2006	?	?	?
14	Aster Yellows Dwarf	I	prior 2006	?	?	?
15	Hydrangea MLO	I	prior 2006	?	?	?
16	Crotalaria Saltiana Phyllody	II-C	prior 2006	?	?	?
17	Aster Yellows Severe	I	prior 2006	?	?	?
18	Aster Yellows Hydrangea	I	prior 2006	?	?	?
19	Stone Fruit Yellows	X	prior 2006	?	?	?
20	Aster Yellows Gladiolus	I	prior 2006	?	?	?
26	Healthy Coconut		Coconut trunk, Ghana	?	03-04 2005	?
27	Chambezi TLD (MYD1)	XXX	ex-Jones Rothamsted	?	24.02.1993	?
29	Clover Phyllody	I-C	UK	?	?	?
30	Sweet Potato Little Leaf (midribs)	II-D	G/H 003	H_2O	16.03.2006	CTAB
31	Sweet Potato Little Leaf (leaves)	II-D	G/H 003	H_2O	16.03.2006	CTAB
32	Healthy vinca seed extraction		G/H 032	TE		CTAB
34	Heakthy dodder seed extraction			TE		CTAB
35	Vinca Coconut Phyllody	II	G/H 016	H_2O	03.05.2006	CTAB
36	Healthy Vinca		G/H 035	H_2O	03.05.2006	CTAB
37	Tomato Big Bud (CSL)	II-D	CSL leaf material	?	23.03.2006	CTAB
38	Faba Bean Phyllody	II-C	CSL leaf material	?	23.03.2006	CTAB

39	Soybean Phyllody	II-C	G/H 001	H_2O	16.06.2006	CTAB
41	Healthy Vinca		G/H 032	H_2O	03.07.2006	CTAB
42	Faba Bean Phyllody	II-C	G/H 013	H_2O	03.07.2006	CTAB
43	Lime Witches Broom	II-B	CSL leaf material-86M	H_2O	03.07.2006	CTAB
44	Lime Witches Broom	II-B	CSL leaf material-86W	H_2O	03.07.2006	CTAB
45	Lime Witches Broom	II-B	CSL leaf material-88M	H_2O	03.07.2006	CTAB
46	Lime Witches Broom	II-B	CSL leaf material-88W	H_2O	03.07.2006	CTAB
47	Ribes in Vinca	I-C	G/H 140	TE	17.01.2007	CTAB
48	Rehmannia glutinose	I-B	G/H 141	TE	17.01.2007	CTAB
49	Strawberry Green Petal	I-C	G/H 142	TE	17.01.2007	CTAB
	Box 2 DNA numbers 51-100					
51	Cornwall 1	XII	Cordyline, Cornwall UK	?	?	?
53	EAT4	XXX	coconut, tanzania	?	02.11.1992	?
54	GH8	XXII	coconut, Ghana	?	16.10.1992	?
55	DIX4A	XXII	coconut, Ghana	?	01.02.1996	?
56	AGI	XXII	coconut, Ghana	?	27.02.1994	?
57	3441 WET	?	coconut, Vanuatu	boric acid?	?	?
58	11 (Rothamsted)	IV	coconut, Florida	?	03.04.2003	?
59	12 (Rothamsted)	IV	coconut, Florida	?	03.04.2003	?
60	14 (Rothamsted)	IV	coconut, Florida	?	03.04.2003	?
61	15 (Rothamsted)	IV	coconut, Florida	?	03.04.2003	?
62	17 (Rothamsted)	IV	coconut, Florida	?	03.04.2003	?
63	22 (Rothamsted)	IV	coconut, Florida	?	21.03.2003	?
64	25 (Rothamsted)	IV	coconut, Florida	?	21.03.2003	?
65	29 (Rothamsted)	IV	coconut, Florida	?	21.03.2003	?
66	2 (Rothamsted)	IV	coconut, Florida	?	01.04.2003	?
67	3 (Rothamsted)	IV	coconut, Florida	?	03.04.2003	?
68	Lethal yellows Phoenix rupicola	IV	Nigel, USA	TE	?	?
69	Lethal yellows Hyophorbe verscafelati	IV	Nigel, USA	TE	?	?
70	GH8 (aliquot of 54)	XXII	coconut, Ghana	?	16.10.1992	?
71	Hydrangea MLO	I	G/H 049	TE	23.08.2006	CTAB
72	Tomato Big Bud	II-D	G/H 039	TE	23.08.2006	CTAB
73	Tomato Big Bud	II-D	G/H 040	TE	23.08.2006	CTAB

74	Tomato Big Bud	II-D	G/H 023	TE	23.08.2006	CTAB
75	Tomato Big Bud	II-D	G/H 107	TE	23.08.2006	CTAB
76	Tomato Big Bud	II-D	G/H 080	TE	23.08.2006	CTAB
77	Tomato Big Bud	II-D	G/H 041	TE	23.08.2006	CTAB
78	European Stone Fruit Yellows (Kent, UK)	X	CSL ref; 8693. Apricot	?	Sep-06	?
79	Peanut Witches Broom	II-D	CSL ref; 4419	?	Sep-06	?
80	Peanut Witches Broom	II-D	CSL ref; 4420	?	Sep-06	?
81	Ipomea Phytoplasma unspecified	?	CSL ref;5395	?	Sep-06	?
82	European Stone Fruit Yellows (Kent, UK)	X-B	CSL ref; 8698. Apricot	?	Sep-06	?
83	European Stone Fruit Yellows (Kent, UK)	X-B	CSL ref; 8703. Apricot	?	Sep-06	?
84	European Stone Fruit Yellows (Kent, UK)	X-B	CSL ref; 8708. Apricot	?	Sep-06	?
85	Phytoplasma, unspecified	?	CSL ref; 8945	?	Sep-06	?
86	Western X Disease (UK)	III	CSL; 20411441. Delphinium	?	Sep-06	?
87	C. p. fragariae, (Jersey)	XII	CSL; 20611093 Cordyline	?	Sep-06	?
88	Stolbur of potato (Romania)	XII	CSL; 20612600 Potato	?	Sep-06	?
89	Stolbur of potato (Romania)	XII	CSL; 12600 A POT	?	Sep-06	?
90	Stolbur of potato (Romania)	XII	CSL; 12600 B POT	?	Sep-06	?
91	Bermuda grass (Mauritius)	XIV	CSL; 20611426	?	Sep-06	?
92	Phytoplasma unspecified (Cornwall UK)	XII ?	CSL; 20615399 Cordyline	?	Sep-06	?
93	Tabebuia Amostra 1	II	Barreto, Brazil	TE	Sep-06	CTAB
94	Tabebuia Amostra 2	II	Barreto, Brazil	TE	Sep-06	CTAB
95	Tabebuia Amostra 3	II	Barreto, Brazil	TE	Sep-06	CTAB
96	Tabebuia Amostra 4	II	Barreto, Brazil	TE	Sep-06	CTAB
97	Tabebuia Amostra 5	II	Barreto, Brazil	TE	Sep-06	CTAB
99	Aster yellows in tomato (T3L)	I	G/H T3	TE	25.5.2007	CTAB
100	Aster yellows in tomato (TS)	I	G/H T3	TE	25.5.2007	CTAB

Italian/Czech box DNA Nos 101-150

DIV	Diplotaxis virescence	I-B	Spain
AY21912	Aster Yellows	I-L	Germany
AVUT	Atypic Aster Yellows	I-M	Germany
AYA	Aprocit Chlorotic leafroll	I-F	Spain
CHRYM	Chrysanthemum yellows	I-A	Germany
KVE	Clover Phyllody	I-A	UK

CLP	Cleome Phyllody	II-A	Thailand			
FBP	Faba Bean Phyllody	II-C	Sudan			
TBBa	Tomato Big Bud	II-D	Australia			
PEP	Pichris echoides	II-E	Italy			
CoP	Cotton Phyllody	II-F	Burkina Faso			
CX	Peach X Disease	III-A	Canada			
API	Euscelidius variegatus	III-B	Italy			
JRI	Poinsettia Branching Factor	III-H	USA			
ULW	Elm Witches broom	V-A	France			
FD-C	Flavesence Dorree-C	V-C	France			
FD-D	Flavesence Dorree-D	V-C	France			
RuS	Rubus Stunt	V-E	Italy			
PWB	Potato Witches Broom	VI-A	USA			
CPS	Catharanthus Phyllody	VI-C	Sudan			
ASHY1	Ash Yellows	VII	USA			
AP15	Apple Proliferation	X-A	Italy			
GSFY1	German Stone Fruit Yellows	X-B	Germany			
PD	Pear Decline	X-C	Germany			
BVK	Flower Stunting	XI-C	Germany			
STOL	Stolbur	XII-A	Yugoslavia			
121	Lethal yellows Adonidia merrillii	IV	Nigel, USA	TE	?	?
122	Lethal yellows Hyophorbe verschafetii	IV	Nigel, USA	TE	?	?
123	Lethal yellows Phoenix rubicola	IV	Nigel, USA	TE	?	?
124	Lethal yellows Hyophorbe verschafetii	IV	Nigel, USA	TE	?	?
101	Ribes in Ribes	I	Jaroslava, Czech Repulbic	?	25.06.06	Phenol/chlo
102	Ribes in Vinca	I	Jaroslava, Czech Repulbic	?	25.06.06	Phenol/chlo
104	Strawberry Green Petal	I-C	Jaroslava, Czech Repulbic	?	21.10.06	Phenol/chlo
105	Rehmannia glutinosa	I-B	Jaroslava, Czech Repulbic	?	25.10.06	Phenol/chlo
106	Ribes in Vinca	I-C	Jaroslava, Czech Repulbic	TE	30.10.06	CTAB
107	Strawberry Green Petal	I-C	Jaroslava, Czech Repulbic	TE	30.10.06	CTAB
108	Rehmannia glutinosa	I-B	Jaroslava, Czech Repulbic	TE	30.10.06	CTAB
109	Napier Grass stunt	XI	G/H 008	TE	22.05.07	CTAB

110	Napier Grass stunt	XI	G/H 009	TE	22.05.07	CTAB
111	Primula blue	I	G/H 047	TE	22.05.07	CTAB
113	Sweet potato little leaf	II-D	G/H 158	TE	22.05.07	CTAB
114	EAT4 (aliquot of 53)	XXX	coconut, Tanzania	?	02.11.92	?
116	Rehmannia glutinosa	I-B	G/H 161	TE	22.05.07	CTAB
117	T3 tomato big bud in ailsa craig	II-D	G/H T3	TE	22.05.07	CTAB
118	Pigeon pea witches broom (2)	IX	Nigel, USA	TE	?	?
119	Mexican periwinkle virescence	XIII	Nigel, USA	TE	?	?
120	Pigeon pea witches broom (3) negative	IX	Nigel, USA	TE	?	?
	Box 3 DNA Nos 151-200					
151	Poinsettia branching (flower buds)	III	EP-O	TE	16.01.08	CTAB
152	Poinsettia branching (roots)	III	EP-1	TE	16.01.08	CTAB
153	Date palm phytoplasma	I	Khalid, Rothamsted	Water	?	CTAB
154	Stolbur (Ex-Jones)	XII	Rothamsted	TE	?	CTAB
155	Sweet potato little leaf	II-D	G/H 167	TE	7.07	CTAB
156	Australian tomato big bud	II-D	G/H 165	TE	7.07	CTAB
157	Pigeon pea witches broom (aliquot of 119)	IX	Nigel, USA	TE	?	?
159	Crotalaria saltiana phyllody	II-C	G/H 175	TE	06.11.07	CTAB
160	Crotalaria saltiana phyllody	II-C	G/H 176	TE	06.11.07	CTAB
162	Crotalaria saltiana phyllody	II-C	G/H 178	TE	06.11.07	CTAB
163	Ribes in vinca	I-C	G/H 174	TE	31.10.07	CTAB
165	Faba bean phyllody	II-C	G/H 184	TE	31.10.07	CTAB
167	Sweet potato little leaf	II-D	G/H 166	TE	06.02.08	CTAB
168	Faba bean phyllody	II-C	G/H 184	TE	06.02.08	CTAB
169	Soybean phyllody	II-C	G/H 1	TE	06.02.08	CTAB
170	Crotalaria saltiana phyllody	II-C	G/H 24	TE	06.02.08	CTAB
171	Napier grass stunt	XI	G/H 172	TE	06.02.08	CTAB
172	Rehmannia glutinosa	I-B	G/H 173	TE	06.02.08	CTAB
173	Poinsettia branching	III	EP-1	TE	09.01.08	CTAB
174	Poinsettia branching	III	EP-2	TE	09.01.08	CTAB
175	Poinsettia branching	III	EP-3	TE	09.01.08	CTAB
176	Healthy vinca		G/H	TE	16.01.08	CTAB
183	Stolbur (Stol)	XII	Bertaccini, Italy, plant	TE	28.02.08	CTAB

184	Elm witches broom (ULW)	V	Bertaccini, Italy, plant	TE	28.02.08	CTAB
185	Potato witches broom (PWB)	VI	Bertaccini, Italy, plant	TE	28.02.08	CTAB
186	Peach western X (CX)	III	Bertaccini, Italy, plant	TE	28.02.08	CTAB
187	Flower stunting (BVK)	XI	Bertaccini, Italy, plant	TE	28.02.08	CTAB
190	Lethal yellows Cocus nucifera (leaf)	IV	USA, Florida	Water	21.04.08	CTAB
191	Lethal yellows Cocus nucifera (leaf)	IV	USA, Florida	Water	21.04.08	CTAB
192	Cape St Paul wilt (leaf)	XXII	Takoradi, Ghana	Water	21.04.08	CTAB
193	Dixcove CSPWD (Ex Jones no 29)	XXII	Ghana	?	01.02.96	?
194	Peach X disease	III-A	G/H	TE	21.07.08	CTAB
195	Potato witches broom	VI-A	G/H 191	TE	21.07.08	CTAB
196	Potato witches broom	VI-A	G/H 192	TE	21.07.08	CTAB
197	Mexican periwinkle virescence	XIII	Nigel, USA	TE	?	?
198	Mungbean phyllody	II	Pakistan	TE	17.07.08	CTAB

Box Tanzania/India

10	Tanzanian Phyllody	XXX	Tanzania	0.3	15/09/1992
11	TAGNANAN TALL 2nd	XXX	Tanzania	1	15/09/1992
15	Sri lanka H1	?	Sri Lanka	1.7	08/01/1993
16	Sri lanka H2	?	Sri Lanka	1.3	08/01/1993
17	AY Sri Lanka S/N	I ?	Sri Lanka	0.8	08/01/1993
18	AY Sri Lanka	I ?	Sri Lanka	2	08/01/1993
19	Nig 4th	XXII	Nigeria	1.88	16/08/1994
20	Nigerian 2nd AWKA	XXII	Nigeria	0.6	11/12/1992
21	EAT 1st healthy		Tanzania	0.4	21/07/1992
22	EAT 2	XXX	Tanzania	1.4	11/12/1992
23	KY1 method 2	XXX	Tanzania	50ng	01/04/1993
24	KY2 method 2	XXX	Tanzania	50ng	01/04/1993
26	KY4 Kenya 4 method 1	XXX	Kenya	50ng	30/03/1993
27	SOTELE Diseased		Tanzania ?	?	?
33	MYDI-1 (3rd) CHAMBEZI (small-scale)	XXX	Tanzania	1.4	24/02/1993
34	MYD I (2nd) CHAMBEZI	XXX	Tanzania	1.6	15/09/1992
35	MYDI (3rd) CHAMBEZI (small-scale)	XXX	Tanzania	4	24/02/1993
36	MYD 3 1st CHAMBEZI	XXX	Tanzania	0.4	28/07/1992
37	MYD 5 KIFMANGAO (dna off)	XXX	Tanzania	3.5	28/09/1992
46	3rd TAG TALL	XXX	Tanzania	50ng	30/08/1993

47	Assuming original Ghana I	XXII	Ghana	50ng	30/03/1993	
54	NIG 4th 1 in 2 dilution	XXII	Nigeria			
55	NIGERIA 5th (mini prep)	XXII	Nigeria		17/08/1994	
56	NIG 5th 1 in 5 dilution	XXII	Nigeria			
58	AGONA I	XXII	Ghana			
59	AGONA 2A	XXII	Ghana			
60	AGONA 2B	XXII	Ghana	2.2		
61	BUSUA mini prep	XXII	Ghana ?		16/08/1994	
62	BUSUA 1 in 2 dilution	XXII	Ghana ?		27/02/1994	
63	BUS 2nd 1 in 2 dilution	XXII	Ghana ?		04/10/1994	
64	BUSUA I	XXII	Ghana ?			
65	ABAYEE H H5	XXII	Ghana ?			
66	AIYINASI H I	XXII	Ghana ?	2.4		
67	AGONA 2B 1 in 2 dilution	XXII	Ghana		16/08/1995	
68	DIX COVE IA	XXII	Ghana	0.23	28/10/1994	
69	KENYA I mini prep	XXX	Kenya		16/08/1994	
71	Florida palm #1	IV	USA			
72	Florida palm #4	IV	USA			
73	Ghana-8 (2nd) small-scale	XXII	Ghana		26/02/1993	
74	AGONA 2a 1 in 2 dilution	XXII	Ghana		27/02/1994	
75	AYENSUDU 2a 1 in 2 dilution	XXII	Ghana		27/02/1994	
76	AYENSUDU 2M 1 in 2 dilution	XXII	Ghana		27/02/1994	
79	Ghana I	XXII	Ghana	1.1	02/10/1992	
80	GHI-1 inflorescens	XXII	Ghana	0.7	02/10/1992	
85	Ghana 6	XXII	Ghana	0.4	04/12/1992	
86	Ghana 7	XXII	Ghana	0.6	04/12/1992	

Box 4 DNA Nos 201-250

201	Chickpea phyllody	II	Pakistan			CTAB
202	EAT3 Kifumangao	XXX	Tanzania		1992	CTAB
203	Poinsettia branching	III	G/H		2010	CTAB
204	Peach western X	III	G/H		2010	CTAB
205	Khalid date palm sample 1	I	Saudi Arabia		28.5.10	CTAB
206	Khalid date palm sample 2	I	Saudi Arabia		28.5.10	CTAB
207	Khalid date palm sample 3	I	Saudi Arabia		28.5.10	CTAB

208	Khalid date palm sample 4	I	Saudi Arabia	28.5.10	CTAB
209	Khalid date palm sample 5	I	Saudi Arabia	28.5.10	CTAB
210	Khalid date palm neg control		Saudi Arabia	28.5.10	CTAB
211	Khalid date palm sample 1	I	Saudi Arabia	28.5.10	Paper
212	Khalid date palm sample 2	I	Saudi Arabia	28.5.10	Paper
213	Khalid date palm sample 3	I	Saudi Arabia	28.5.10	Paper
214	Khalid date palm sample 4	I	Saudi Arabia	28.5.10	Paper
215	Khalid date palm sample 5	I	Saudi Arabia	28.5.10	Paper
216	Khalid date palm neg control		Saudi Arabia	28.5.10	Paper
217	Khalid alfalfa 7	II	Saudi Arabia	16.9.09	Paper
218	Khalid alfalfa 8	II	Saudi Arabia	16.9.09	Paper
219	Khalid alfalfa 9	II	Saudi Arabia	16.9.09	Paper
220	Khalid alfalfa 11	II	Saudi Arabia	16.9.09	Paper
221	Khalid alfalfa 12	II	Saudi Arabia	16.9.09	Paper
222	Napier grass stunt 9	III? Or XI	G/H		
223	Napier grass stunt 5	III? Or XI	G/H		
224	Napier grass stunt 6	III? Or XI	G/H		
225	Napier grass stunt bulk	XI	G/H		
226	Napier grass stunt bulk	XI	G/H		
227	Napier grass stunt bulk	XI	G/H		
228	Napier grass stunt bulk	XI	G/H		
229	Napier grass stunt 3	XI	G/H		
230	Vinca coconut phyllody 14	II	G/H		
231	Peach western X 190	III	G/H		
232	Faba bean phyllody	II-C	G/H		
233	Crotalaria saltiana phyllody 175	II-C	G/H		
234	Tomato big bud 37	II-D	G/H		
235	Sweet potato little leaf 166	II	G/H		
236	Bermuda grass whiteleaf	XIV	?		
237	Moz 150	XXII	Mozambique		
238	Kerala root wilt	XI	India		
239	Paenibacillus		Jen		
240	Lactobacillus		Jen		
241	Fructobacillus		Jen		
242	Soil DNA		Jen		

	Box Sri Lanka/Vietnam					
B36L	Sri lanka weligama wilt	XI	Sri Lanka		25.3.09	
B36I	Sri lanka weligama wilt	XI	Sri Lanka		25.3.09	
A5L	Sri lanka weligama wilt	XI	Sri Lanka		25.3.09	
R9L	Sri lanka weligama wilt	XI	Sri Lanka		25.3.09	
D	Sri lanka weligama insects	XI	Sri Lanka		26.8.09	
E	Sri lanka weligama insects	XI	Sri Lanka		26.8.09	
F	Sri lanka weligama insects	XI	Sri Lanka		26.8.09	
G	Sri lanka weligama insects	XI	Sri Lanka		26.8.09	
H	Sri lanka weligama insects	XI	Sri Lanka		26.8.09	
I	Sri lanka weligama insects	XI	Sri Lanka		26.8.09	
NA1	Vietnamese sugar cane	XI	Vietnam		2.11.09	
NA2	Vietnamese sugar cane	XI	Vietnam		2.11.09	
NA3	Vietnamese sugar cane	XI	Vietnam		2.11.09	
NA4	Vietnamese sugar cane	XI	Vietnam		2.11.09	
NA5	Vietnamese sugar cane	XI	Vietnam		2.11.09	
NA6	Vietnamese sugar cane	XI	Vietnam		2.11.09	
NA7	Vietnamese sugar cane	XI	Vietnam		2.11.09	
NA8	Vietnamese sugar cane	XI	Vietnam		2.11.09	
NA2/1	Vietnamese sugar cane	XI	Vietnam		2.11.09	
NA3/1	Vietnamese sugar cane	XI	Vietnam		2.11.09	
NA6/1	Vietnamese sugar cane	XI	Vietnam		2.11.09	
NA1P	Vietnamese sugar cane	XI	Vietnam		2.11.09	Paper
NA4P	Vietnamese sugar cane	XI	Vietnam		2.11.09	Paper
NA7P	Vietnamese sugar cane	XI	Vietnam		2.11.09	Paper
Per	Periwinkle virescence	New	Malaysia			
BGWL	Bermuda grass whiteleaf	XIV	Malaysia			
1337	Apple proliferation	X-A	Apple tree, Slovenia	Ct 24.4	28.7.10	
1338	Apple proliferation	X-A	Apple tree, Slovenia	Ct 25.3	28.7.10	
1340	Apple proliferation	X-A	Apple tree, Slovenia	Ct 26.8	28.7.10	
1339	Apple proliferation neg control		Apple tree, Slovenia		28.7.10	
1206	European stone fruit yellows	X-B	Apricot tree, Slovenia	Ct 26.6	28.7.10	
206	European stone fruit yellows neg control		Apricot tree, Slovenia		28.7.10	
1772	European stone fruit yellows	X-B	Plum tree, Slovenia	Ct 29.0	28.7.10	

1769	European stone fruit yellows neg control		Plum tree, Slovenia		28.7.10
834	European stone fruit yellows	X-B	Peach tree, Slovenia	Ct 23.6	28.7.10
1843	European stone fruit yellows neg control		Peach tree, Slovenia		28.7.10
1498	Pear decline	X-C	Pear tree, Slovenia	Ct 30.5	28.7.10
1580	Pear decline	X-C	Pear tree, Slovenia	Ct 35.8	28.7.10
1581	Pear decline	X-C	Pear tree, Slovenia	Ct 28.3	28.7.10

Applendix 4 the alignments for the *pol* gene with primer sites marked in red.

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#ngs          AAG AAA ATT TAG TAA AAA AAG ATT TTT TAA TAA AAA ATA AAA AAA ATA AAG AAT ATG GTA GTC GCG AAT ATG AAT ATT
#p_mali       TTC .C. ..C ..A ... TGT --- ... G.C ... .TC --- -.. ... ... G.. .-- --- -.T C.. A.A .AA .T. ..C ..C ...
#P_aust       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --. ... ..A ... .C. ... ..A .A. .T. ... .T. ...
#aywb          --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ... ... ... .C. ... ..A .A. .T. ... .T. ...
#oym           --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --. ... ... .C. ... ..A .A. .T. ... .T. ...
#achole_laid --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

#ngs          TTA AAA TTC GTT TAG CTT CTC CAC ATG AAA TAA GAC AAT GGT CTT ATG GTG AAA TAG TTA ATT ATG AAA CTA TTA ATT
#p_mali       ... ... ... ... ..T ... .AA GCG ... ... .T. ... ... ... ... ... ... ... .T. ... ... ... ... ... ... ...
#P_aust       .C. ... ... ... ... .A. ... .TG .A. ... .TC .T. ... ... ... ... .G. .GG .TA CA. G.. .C. ... .A. ... ...
#aywb          ... ... .C. ... ... ..G ... .TG .A. ... .C. ... .G. ... ... ... ... .GG .TA CC. GC. .C. ... .A. ... .C.
#oym           ... ... .C. ..C ... ..G ... .TG .A. ... .C. ... .G. ... ... ... ... .GG .TA C.. GC. .C. ... .A. ... .C.
#achole_laid --- --- --- --- --- --- -.. ..G ... ... .T. ..A .C. ... ... ... ... ..G .TA ... .CC ... ... ... ... .C.

#ngs          ATC GTA CTG CTA AAC CAG AAA TGG GAG GTC TTT TTT GTC CAA AAA TTT TTG GAC CTA TAA AAG ATT TAC AAT GTT TTT
#p_mali       ... ... ..T .A. ... .G. ... CT. .T. ... ... ... ... A.. ... ... ... ... .AG ... ... ... .T. ... ..A ...
#P_aust       ... ... ..T A.. ... .T. ..C CT. .C. .CT .A. ... ... AG. ... .C. ... .T. .AG ..G TT. ... ATA G.. ..G C..
#aywb          ... .G. .CT A.. ... ... .G. .AT C.. .AT .A. ... ... A.. .G. ... ... .C. .AG ..G TT. ... .C. ... ..G C..
#oym           ... .C. .CT A.. ... ... .G. .AT C.. .AT .A. ... ... A.. .G. ... ... .T. .AG ..G TT. ... .C. ... ..G C..
#achole_laid ..A .A. .AT .A. ... .T. ..C CA. .T. .A. .A. ..G A.G A.. G.. ... ... ... ... C.. G.. ... AC. ... ..G C..

#ngs          GTA CTA AAA AAG TAA CTT TGA GAA AAG GTC AAA TTT GTT CTA AAT GTG GAG TGG AAA TTA CAG AAC AAA AAG TTA GAA
#p_mali       ..T ... ... ..C A.. T.G .T. AC. ... .A. ... ... ... .A. GG. ..A AT. .A. ..T ... .C. ..T C.. ... ... ...
#P_aust       ..T .C. ... ..C A.. T.A GC. CC. ..A .C. ..T ... ..A A.. ... .CC AT. ... ..T ... .T. ..A CT. ... .C. ...
#aywb          ..T ... ... ..C A.G TCA GT. TC. ..A ... ..T ... ..A AC. ... ... ... .T. ..T ... .C. ..A CC. ... .GC .TC
#oym           ..T ... ... ..C A.G TCA GC. TC. ..A ... ..T ... ..A AC. ... ... ... .T. ..T ... .C. ..A CC. ... .GC .TC
#achole_laid ..G G.. .G. ..C A.. ..G .A. AC. ... .C. ... ... ..C .A. ... ... .TA .T. ... ... ... .GT CT. ... .AC .TC

#ngs          GAG AAA GAA TGG GGC ATA TCG AAT TAG CTG CTC CTA TAG TAC ATA CTT GGT ATT TGA ATA GTT CAC CTA GTA GAT TAG
#p_mali       ... ... ... ... CT. ... .T. .GC .C. .CA .A. ..G ... ... ... ... ... ... .T. ... AC. ... ... ..C .T. ..T
#P_aust       ... ... ... ... .A. .C. ... ... ..C AA. .G. .AG .T. ... ... ... ... ... .T. ..T C.. .T. ... ..C .T. .G.
#aywb          .T. ... ... ... ... .C. .T. ... ..C AAA ... .A. .C. .T. .C. ... ... .CC .T. ..T C.. .T. .C. .CC .T. ...
#oym           .T. ... ... ... .A. .C. ... ... ..C AAA .C. .C. .C. .C. .C. ... ... .CC .T. .CT C.. .T. .C. .CC .T. ...
#achole_laid .T. ..C .T. ... .A. ... .TA .C. ... AA. .A. ..G .T. .G. ... .A. ... .C. .A. .G. AC. .T. ..T CA. ... ...

#ngs          CTA TTT TAT TAG GTA TAA AAG CTA AAG AAT TAT CTG AAA TAG TTT ATT ATG CCT CCT ATA TTG TGT TTT ATC CAG G--
#p_mali       ... ... ... ... ... .T. ... .A. ... ..A .TG AA. ... ... ... ... ..A TG. .T. ... ... .AA ..G .A. .T. .--
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#P_aust       .CG ..A .T. ..A AC. .T. ... ... ... CT. ..G AA. ... ..A ... ... ..A TT. .T. ..G ... .AA ..G .C. .T. .--
#aywb         ... ..C .T. ..A A.. .C. ..A ... ..C ... ..G AA. ... ... ... ... ... TT. .T. .CG ... .AA ..G ... .T. .--
#oym          ... ..C .T. ..A AC. .C. ..A ... ..C ... ..G AA. ... ... ... ... ... TT. .T. .CG ... .AA ..G ... .T. .--
#achole_laid ... .CC ... ... .C. .T. ... ... ..C ... ..G AA. ..G .T. ... .CC ... .AA GT. ... ... ..A C.G .C. ... .TC

#ngs          -TA AAA CTA ATT TAG AAA AGA ATC AAA TAA TAA GCG AAA TTC AAT ATG GCC ACT ATT TAG AAC ATT ATG GT- --A ATA
#p_mali       -A. ..T ..T CA. ..A .T. .A. .A. ... .T. .TT CT. ... .GG ... ..A ATA .T. ... CT. ..T ... T.A ..- --. ...
#P_aust       -.. ... .AG ... .TA ..G .A. .AG ... .T. .TT C.A ... G.. ... ..A .T. .AA ... ... .TA T.. T.. .CC CT. ..G
#aywb         -A. ... ..G .A. .CA ..C CT. .AG ... .T. .T. CA. ... CC. ... .CA .TG .AG C.C .TT .TG .A. T.. .A- --. .C.
#oym          -A. ... .CG .A. .CA ..C CT. .AG ... .T. .T. CA. ... CC. ... .CA .TG .AG C.C .TT .TG .A. T.. .A- --. .CG
#achole_laid AA. .C. .AC CAC ... CT. .A. .A. ... .CT ..T C.. ..C AAG .C. ..T CTT TAC T.G .T. ..G .A. ... ..- --T CA.

#ngs          GTT TCA TTG CTT TAA GCG GAG CAG AAG CTA TTA AAA AAA TGT TAG AAG AAT TAA ATT TAA CTG AAA TAA TTC AAG AAT
#p_mali       ... .TG .A. ... .G. CT. ... ..A ... .A. ... ... C.. ... .GC ... CT. ..G ... ..G AAA ... AT. .AA ..A TT.
#P_aust       ... .TG ... ... ... CT. .T. .T. ... .A. ... G.. ..T .A. .G. ..A .CC .TG ... ..G AAA ... C.. ..A ..A TCC
#aywb         C.. .TG .A. ... ... CA. .T. .C. ... .GG ... ... ..T .AC ... ..A .TC .C. .C. ..G AAA ... CT. .CA ... TCC
#oym          C.. .TG .A. ... ... CA. .T. .C. ... .GG ... ... ..T .AC ... ..A .TC .C. ... ..G AAA ... CT. .CA ... TCC
#achole_laid .A. .T. CA. .GC .T. CT. .T. .T. ... .GG ... ... ..T .AC .TC ... .TC ..G ... ..G A.A ..G A.G ..A ..A .T.

#ngs          TAA GGT TTT TTT TAC CAA AAG TTT CTA AAC AAA AAA GAG ATA ATA TTA TTA AAA GAT TAG AGA TAA TTG AAT CTT TTA
#p_mali       ..C .TA AAA A.C .TA A.. T.. ..C ... ... CT. ... .GA .CG CC. ... .C. ... .G. ..A ... .TT ... ..G A.. ...
#P_aust       .T. .AA AAA AC. ..T ..G ... ... ... ... ... ..C ... G.G C.T .G. ... ... ... ... .A. .T. .G. ... ... .GT
#aywb         .TC .CA AA. C.. ..T .GG ..A A.. .C. ... ... ... ... .A. GC. .C. ... ... ..C ... .A. .C. .C. ... ... ..C
#oym          .TC .CA AA. C.. ..T .GG ..A A.. .C. ... ... ... ... .A. GC. ... ... ... ..C ... .A. .C. .C. ... ... ..C
#achole_laid ... .AA AAC G.C .TA A.. C.A G.. ... ... ... ..C .T. ..C G.. ... ... ..C .T. ... .TG .TG ... ..G .A. ...

#ngs          ATA ATT CAG ATA ATA AAC CTG AAT GGA TGG TTA TGG AGG TTA TTC CTG TAT TAC CAG CTG ATT TAA GGC CTA TAG TTC
#p_mali       ... .A. ... .A. ... ... .A. .G. ... ... ... ... .T. ... ... ... ..C .T. .TT G.. G.. ... .A. AA. .G. ...
#P_aust       ..C .A. .T. ... ... .T. ... .G. ... ... .A. ... .C. ... ... ... .T. .G. ..C .A. GA. ... .A. .C. .G. .C.
#aywb         ..C .A. ... .C. .C. ... ... ... ... ... .A. ... .T. ... ... ... ... .G. .TC .A. GA. ... .A. .G. .G. ...
#oym          ..C .A. ... .C. .C. ... ... ... ... ... .A. ... .T. ... ... ... ... .G. .TC .G. GA. ... .A. .G. .G. ...
#achole_laid .CC .A. ... .C. ... ... ... .G. ... ... ... ... .T. ... ... .A. .CA .T. ..C .A. ... ..C .T. .A. .G. ...

#ngs          CTT TGG ATG GTG GAC GTT TTG CTA CTA CTG ATA TTA ATG ATT TAT ACA GAA GAA TTT TAA ATC GTA ATA ATC GTT TGA
#p_mali       ... .A. ... ... .T. ... ... ... ... ... ..T CA. ... ... ... ... ... ... ... ... ... ... ... G.. ... .A.
#P_aust       ... .A. ... ... .T. ... .C. .C. ... ... .A. ... ... ... ... .TC .TC .T. ... ... ... .C. .C. ... ... .A.
#aywb         ... .A. ... ... .T. ... ... .C. ... ... .AG ... .C. ..C .T. ..C .CC .T. .CC .T. ... .C. .C. ... ... .A.
#oym          ... .A. ... ... .T. ... ... .C. ... ... .AG .C. .C. ..C .T. ..C .CC .C. .CC .T. ... .C. .C. ... ... .A.
#achole_laid .AC .A. ... ... .TA .A. ... ... .A. ... .CC ... .C. .C. ... .T. ... ... .C. ... .CA .A. .C. .C. ... .A.
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#ngs          AAA AAC AAA TTA AAC AAA AAG CTC CTA GAT TAA TTA TAA AAA ATG AAA AAA GAA TGT TAC AAG AAG CTG TTG ATG CTC
#p_mali       ... ... ..T .GC ... ... ... .A. ... AG. ... ... .T. ... ... ... ... ... ... ... ... .G. .A. .G. ... ..T
#P_aust       ... ... ... .GC TT. ... ... ... .A. .T. ... ... .CC ... ... ... ... ... ... ... .G. ... ... .A. ... ..T G.T
#aywb         ... ... ... .GC TC. ... ... ... ..C .T. ... .C. .T. ... .C. ... ... ... ..C ... ... ... .A. ... ... ..T
#oym          ... ... ... .GC TC. ... ... ... ..C .T. ... .C. .T. ... ... ... ... ... ..C ... ... ... .A. ... ... ..T
#achole_laid .G. ... ... AAG ... ... TG. .G. ..C .T. ... ... CT. .G. .C. ... .GC .T. ... ... ... ... .A. .A. ... .AT

#ngs          TAT TTG ACA ATG TTA AAA CCA ATA AAA AAA ATA GTA CTA ATA TAG AAA GAA ATA AAA ATT TAA AGT CTT TAT CTG AAA
#p_mali       .G. ... .T. .CA C.. ..C .T. ... ... ... ..T A.T ... G.. .TA .T. ... ... ... C.. ... .AC ... ... .A. ...
#P_aust       .G. ... .TG G.. A.. G.. A.G T.. .G. ... ..G T.. A.. ... ... ... A.. .CC GCC CGC .T. .A. ... ... .G. ...
#aywb         .G. ... .T. ..A CC. ... .T. GC. ... ... .CG T.. AC. ..G .C. ... A.. .CC GTC CAC .T. .A. ... ... .C. ...
#oym          ... ... .T. ... CC. ... .T. GC. ... ... .CG T.. AC. .CG .C. ... A.. ..C GCC CGC .T. .A. ... ... .C. ...
#achole_laid ... .C. .T. .C. CG. ..C G.G G.. .G. .GG --- --- ..G C.G .T. ..C .T. .CC GCC .C. ... .A. .A. ... ... .TT


#ngs          TGT TAA GAG GTA AAC AAG GTC GTT TTA GAC AGA ATC TTT TAG GAA AAA GAG TTG ATT ATT CTG CTC GTT CTG TAA TTA
#p_mali       CA. ... .G. ... ..A CT. ... ... ..C .T. .A. ... ... ... .C. .G. ... ... ... ... ... GA. ... .A. ... ...
#P_aust       ... .GC .T. .A. ... ... .A. .C. ..C .T. .A. ... ... ... ... ... .G. ... ... ... ... GC. ... .A. ... ...
#aywb         ..C ..C .T. .C. ... ... .G. ... .CC .T. .A. ... ... ... .C. ... ... ... ... ... .G. GA. .C. .C. ... ...
#oym          ..C ..C .C. ... ... ... .G. ... .CC .T. .A. ... ... ... .T. ... ... .G. ... ... .G. GA. .A. .C. ... ...
#achole_laid .AC ..C .T. ... ... ... ... ... .CC .T. .A. .CT .A. ... ... ..C .T. .G. .C. .C. ... GA. ... .A. .T. ...

#ngs          TAG TAG GAC CAG ATT TAA AAA TTC ATC AAT GTG GTA TAC CTA GAC AAA TGG CAT TAA TTT TAT TTA AAC CTT TTA TTT
#p_mali       ... .T. AT. .TT ... ... .G. .G. ... ... .C. ... .T. .A. ... ... ... .GG ... ... ... ... ... ... ..G ...
#P_aust       .T. ... .T. .T. .CC .T. ... .G. ... ... ... ... .T. ..C ..G ... ... ..A .T. .C. ... .C. ... ... ... ...
#aywb         .T. ... .T. .T. ... ..G ... .G. .C. ... .C. .CG .G. ..C .TG ... ... ..A .T. ... .G. .C. ... ... .C. ...
#oym          .T. ... .T. .T. ... ..G ... .G. .C. ... .C. ..G .G. ..C .TG ... ... ..A .T. ... .G. .C. ... ... .C. ...
#achole_laid .T. .T. .T. ... .C. ..G .G. .GT .C. ... ... ... .T. ..C .TG ... ... ..A .T. CA. ... ... ... .A. ... .C.

#ngs          TAA ATA AAT TAC AAG AAG TTA AGG GTA TCG ATA GAA AGA ATG CTA CTA TTT TAT ATG AAC AAA TGA ATG AAT ATG TTT
#p_mali       .G. GA. ... ... ... ..- --C .A. ... .T. .C. A.. CT. .CA T.G TCC A.G .T. ... .TA ... ..G ... ..C .A. .A.
#P_aust       .G. .A. ... .G. ... ..A C.. ... .A. .T. .C. A.. .A. ... .C. A.T C.A .T. ... ..A ... ... ... ..G .A. ...
#aywb         .G. .A. ... ... ... ..A CC. .A. ... ... ... A.. .A. .C. ... AC. CCA .T. ... ..A ... ... ... ..G .G. ...
#oym          .G. .A. ..C ... ... ..A CC. .A. .A. .T. .C. A.. .A. .C. .C. AC. CCA .T. ... ..A ... ... ... ..G .A. ...
#achole_laid ..C G.G ... ... ..C T.A CAC .T. ..G CA. .A. AG. .A. ... .A. ACG C.A A.. .C. ..A G.C GCG ... .CG .CA CA.

#ngs          TAA GTA TTT TAG AAG ATG TAG TTA AAG AAC ATC CTG TTT TAT TAA ATA GAG CTC CTA CAT TGC ATC GTC TAG GGA TAC
#p_mali       GG. AAG C.. ... ... .A. .G. .A. ... ... ... .A. .A. ... ... ... ... .G. .A. .T. ... ... ..T ... .A. ...
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#P_aust      GG. ACG ... ... ... .A. ... .C. ... ... ... ... ..C .TC .T. ..C .C. .C. ... .T. ... .C. ..T ... ... .T.
#aywb        GG. ACG CC. ... ... .A. ... .C. ... ... .C. .A. ..C .T. ... .CC .C. .C. .A. .GC .A. .C. ..T ... .A. .T.
#oym         GG. ACG CC. ... ... .A. ... .C. ... ... .C. .G. .CC .T. ... .CC .C. .C. .A. ..C .A. .C. ..T ... .A. .T.
#achole_laid GGC ..G CAC .T. ..A .A. .T. .AC GT. .G. .C. .A. .A. ..C ... .CC .T. .G. .A. .GC .A. ..A .A. .T. .T. .T.

#ngs         AAG CTT TTG AAC CAA AAT TAA TAG ATG GAA AAG CTA TTA GAT TAC ATC CTT TAG TCA CTC CAG CTT TTA ATG CTG ATT
#p_mali      ... ... ... ... ... ... ... ... .T. ... .A. ..C .T. ... ... ... ... .A. .G. ... ... ... ... ... ...
#P_aust      ... ... ... .T. .T. ... ... .C. .C. .C. ... ... ..C .T. .G. ... ... ... .A. .C. .T. .C. ... ... ... ...
#aywb        ... ... ... .T. .T. ... ... .T. .C. .C. ... .C. ..C .C. ... ... ... ... .T. .G. .T. .C. ... ... .C. ...
#oym         ... ... ... .T. .T. ... ... .T. .C. .T. ... .C. ..C .CC .C. .C. ... ... .T. .G. .T. .C. ... ... .C. ...
#achole_laid ... .A. .C. ..G TG. ... ... .T. ... .T. ... .G. ..C .TC ... .C. .AC .T. .T. .A. .T. .G. ... .C. .G. .C.

#ngs         TTG ATG GTG ATC AAA TGG CTG TTT ATT TAC CAT TAT CTA TTG AAT CAC AAG CTG AAG CAC GTT TAT TGA TGT TAG TTT
#p_mali      ... ... ... ... ... ... .A. .A. ... ... .TC .T. ..T .A. ..G ... ... .G. ... .T. ... ... .A. ... ..A .G.
#P_aust      .C. ... ... .C. ... ... .C. ... ..A ... .TC .T. ..T .G. ..G .T. ... .C. ... .T. ... .G. .A. ..C .T. C..
#aywb        .C. ... .C. .C. .G. ... .AA ... ..G .T. .TC .T. ..T .A. ..G .G. .G. ... ... ... .C. ... ... ..C .T. ...
#oym         ... ... .C. .C. .G. ... .AA ... ..G .C. .TC .T. ..T .A. ..G .C. ... .C. ... ... .C. ... ... ..C .T. ...
#achole_laid ... .C. .G. .C. ... ... .G. ..C .C. ... ..C ... .AC CA. ..G .T. ... .A. ... ..A .A. ... .A. ..C .T. CA.


#ngs         CTA ATA ATA TTT TAG ATC CTA AAA ATG GTA GTC CTG TTT TAT CTC CTT CAC AAG ATA TGG TTT TAG GGA ATT ATT ATT
#p_mali      ... ... ... ... ... ... ... ... .C. ... A.. ... ..G ..A ... .A. ... ... ... ... ... ... .T. ... ... ...
#P_aust      ... ... ... ... ... .C. ... ... ... ... ... .C. ..G .TA .C. ... .G. ... ... ... ... ... ... .A. .C. ... ...
#aywb        .G. .C. ... ... ... .C. ... ... ... ... A.. .A. .AG ..A ... ... ... ... ... ... ... ... ... .C. ...
#oym         .G. .C. .C. ... ... .C. ... ... ... ... A.. .A. .AG ..A .A. ... .C. ... ... ... ... ... ... .A. .C. .C. ...
#achole_laid ... .C. .C. .C. ..A .C. ... G.G ... ... AA. .A. ..G .TA .A. .A. .T. ... ... ... ... ... ... .T. .C. ... .CC

#ngs         TAA CTA TGG AAA AAA GTA AAT ATT TTA TAT CGC AAG ATT ATC AAA ATA TCA TCA ATT ATA AAA AGG CAG AAG AGT ATA
#p_mali      ... ... .T. ..G ... AA. ..A GCC G.. .TC TAG .G. G.G G.T TT. ... .TG AAC .AA .A. .T. .A. A.C .TC .A. ...
#P_aust      ... ... .T. ... CT. AA. ..G ..C ..T .CG .TA ... .CC TCA ..G T.T CTC AAG .AG ..G C.G CTA ATC .CC .AC .A.
#aywb        ... .G. .T. ..G ... AA. ..G ..C G.. CTA TAA .CA G.. ..G .TT C.G C.C AA. GAA C.G C.G --- A.C .CC .A. TC.
#oym         ... .C. .T. ..G ... AA. ..G ..C GC. CTA TAA .CA GC. ..G .TG C.G C.C AA. GAA C.G C.G --- A.C .CC .A. .C.
#achole_laid ... ... .T. ..G ..T C.. ..G ..C G.. AT. T-- --- -.G G.G .TG ..C .TG AA. GAA C.. .G. --- A.C .CC .AG .A.

#ngs         AAC ATA GAA ATG AAG GTA AAA TAT TCT GTG ATT TAA AAG AAG CTG AGT TAT CAT TTT ATA ATA AAG AAA TTG AAA TTC
#p_mali      ... ..C .T. .A. ... ... ..T .T. .TA A.A ... ..C ... ... ... .AA .TG .T. ..G .G. .A. ... ... ... CTT .A.
#P_aust      ... ..C .C. ... .G. .AG CTT .T. .TG C.. ..A .C. .C. ... TAA .AA .CG .T. A.C .A. GC. ... ... .A. G.C ...
#aywb        ... ..C .T. ... ... .A. CCT .T. .TG C.. ..A .C. .C. ... .CA .AA CTG .T. A.C .A. ... ... ... .CC .TC ...
#oym         ... ..C .C. ... ... .AG CTT .T. .TG C.. .CA .C. .C. ... .CA .AA CCG .T. A.C .A. ... ... ... .CC .CC ...
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#achole_laid .G. ..C .CC ... ... ... .GT .C. ... CAT C.A .TG .T. ... T.A ..A .TG .T. A.G .G. ... ... .T. ..T CTT .A.

#ngs         ATA CAA GAA TTT TAG TGA AAG CTT CTA ATA TTG ATT CTT GTT TTA CTG AAG AAC AAA AAG AAA AAT ATT TAG TTA CTA
#p_mali      ... ... ... .AG .GA .T. ..C .AA GCG G.. ..A CA. ..A ... ... .A. .T. ... ... ... ... ... ... ... ... ...
#P_aust      ... .GC .T. ... .TA .C. ..C .GC GAT CA. ..A .C. TA. C.. ... ... .G. ... ... ... ... ... ... ..A .G. ...
#aywb        ... ... ... ... .TA .C. ..C .AC AA. CA. ..A ... TA. CA. ... .C. ... ... ..C GTC ... ... ... ..A .G. ...
#oym         .C. ... ... ... .TA .C. ..C .AC AAG CA. ..A ... TA. CA. ... .C. ... ... ..C GTC ... ... ... ..A .G. ...
#achole_laid ... ... ... ..A .CA .T. ..C .AG AAT CAG ..A .AG ACA CA. ... .A. T.. ... ... .GA .C. TG. ... ... .G. .A.

#ngs         CAT TAG GCA AGT TGA TTT TTA ATG AAA TTT TAC CAT CTA ATT TTA ATT ATA TAC AAG AAC CAA CAA TAT ATA ATT TAG
#p_mali      .T. ... .T. .AA ... ... ... ..A .T. ... ... .T. .A. ... ..C C.. ... .TA .T. ... .T. .T. A.. ... ... ...
#P_aust      .T. ... .T. .A. .A. ... ... ..A .C. ... ... .TC ... ... ..C C.. .C. .TA .T. ... ... .TG A.A C.. ... ..T
#aywb        .T. ... .A. .A. .A. ... ... ... .C. ... .G. .TC .A. GC. .CC CC. .C. .TA .T. ... ... .CC AG. T.. ... .G.
#oym         .T. ... .A. .A. .A. ... ... ... .C. ... .G. .TC .A. G.. .CC CC. .C. .TA .C. ... ... .TC AG. T.. ... .G.
#achole_laid ... ... .T. .AA .C. .C. ... .C. ... .C. ... .TG AA. CA. .CC C.. ..G ..A .T. ... .T. ... .G. C.. .C. ..A

#ngs         AAA TAA AAA CTC CTG ATC TTT ATT TTG TAC CTA AAA ACG TAA ATC CAA AAG ATA TTT TAC CTA ATA TTA ATA TTA CAC
#p_mali      ... .TC ... ... ... ..T G.. ... ..A .TA A.. GGG G.A .TG ... ... ..A CAG ... ..A A.G ... ..C CA. ..C ..A
#P_aust      .CG .T. ... ... ... .CA GA. ... ..T ..A A.C C.G GA. A.. .C. .T. ..T CAT ... .GA AA. .AT .AC CCC .CC ..A
#aywb        .TG .T. ... .C. ... ..G C.. ... ..T ... .CC C.G GAA CC. .C. .C. ..C .AT .C. ..A AA. .AC ..C CC. CCC .CA
#oym         .TG .C. ... .C. ... ..G C.. ... ..T ..G ..C C.G GAA CC. .C. .C. ..C .AT .CC ..A AA. .AC ..C CC. CCC .TA
#achole_laid GTG A.. .G. .A. .A. .CA ... .C. ..A .TA AA. .GG GT. .G. ... ... .G. ..G CAC ..A AGC ... ..C C.. CAC .TG


#ngs         AAC CTT TTA AAA AGA AAT TTT TAT CTA TGA TTA TTG ATT ATG TTT TTA AAA AAA GTA ATA -TT ACA GAA ACT TCA AGA
#p_mali      GT. ... ... ... ... ... ... ... ... ..G .A. ..A ..- ..A ... ..T ... ..G A.G CC. A.. .TC ..G ... ..T .A.
#P_aust      ... ... ... .T. .A. .G. .C. ... .G. ... ... ... C.. G.- ... ..T ... C.. A.G .A. A.. ... ... ... ..G .A.
#aywb        ... ... .C. .C. .A. ... .CC ... ... ... ... ... CA. G.- ... ..C ... C.. A.G .A. A.. ... ... ... ..T .A.
#oym         ... ... ... .C. .A. ... .CC ... ... ... ... ... CA. G.- ... ..C ... C.. A.G .A. A.. ... ... ... ..C .A.
#achole_laid .G. .A. ... .G. ... G.. .C. ... ... ..G ... ... C.C .A- G.A ..T ... TT. T.C CAC A.. T.T ... ..A ... ...

#ngs         ATG TTA GAT AAT ATT AAA GAT TTA GGA TTT AAA TAT TCA ACT ATA GCA GGT ATT ACT ATT TCT CAT TCT GAT ATT AAT
#p_mali      ... ... ... G.. ... ... TC. ... ..T ... ... ... ..T ..A C.T ..G ... ... ..C ... ... TT. GAA ... ..C ...
#P_aust      ... ... ... ..A ... ... ... ... ..T ... ... ... ..T ... ..T ... ..A ... ... G.. ... TT. G.. ... ... ...
#aywb        ... C.. ... C.. ..C ... A.C ... ..T ..C ..G ... ..C ..C ..T ... ..A ... ..A G.. ..A TT. G.A ..C ..C ...
#oym         ... ... ... C.C ..C ... A.C ... ..T ..C ..G ... ..T ... ..C ... ..A ... ..A G.. ..A TT. G.A ..C ..C ...
#achole_laid ... ... ... ..A T.A ... ..C ... ... ... ... ... ... ..A G.T ..T ... ... ..A ... ... T.. G.A ..C ..C ..C

#ngs         GTT TAC TCA AAA AAA AAA GAA TTA CTA ACA GAA GTA GAA AAC AAA ATA TTA AAA ATA GAA AAA TGG TAT GAT CAT GGT
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#p_mali      A.. ..T ... ... ..G ... ... ... T.. CA. A.. AA. ... ..A ..T ..T A.T ... ..C ... G.. ... ... ..A G.C ...
#P_aust      A.. ..T ..T G.. ... G.C A.. ... T.. T.T A.. ..T ... ..A CGC .AT AAT C.. ..T ... ..T ... ... A.A ..A ..A
#aywb        AC. ... ..C ..T ... C.. ... ..G T.. CA. ... ... ... .C. CGC .AT A.T C.. ..T ..G .CT ... C.. A.A G.. ...
#oym         AC. ... ..C ..T ... C.. ... ..G T.. CA. ... ... ... GC. CGC .AT A.T C.. ..T ..G .CT ... ..C A.C G.. ...
#achole_laid ..A ... ..C ..G ... ... ... A.G G.T GA. .C. ACC ... G.A G.. ..T AAT G.. ..T ... G.. ... .TC ..A G.. ..A

#ngs         TTA TTA ACT ATT TCT GAA AAA AAG AAT TTA GTT ATT CAA GAA TGG AAA AAT GTT CGT GAT AAG ATT CAA GAA GGT TTA
#p_mali      C.T ... ... .G. .A. ... CGT ..A ... ... ... ... A.. ... ... ... G.A ... ..A ... C.. ... ... ... ..A ...
#P_aust      ..T ... ..A GA. G.A ... CGT CGC CG. ... ... ... A.C ... ... ... .CA A.C ..C ..C C.A ... ... ... ..A ...
#aywb        ..T ... ..A GA. G.C ... CGT CGT CG. ... ..A ... A.C ... ... ... .C. A.. ... ... G.A ... ... ... ... ..G
#oym         ..T ... ..A GA. G.C ... CGT CGT CG. ... ..A ... A.C ... ... ... ... A.. ... ... G.A ... ... ... ..C ..G
#achole_laid ... C.. ..A GA. ..A ... CGT CGT ..A C.T ... ... G.T A.. ... .C. ... ... AAA A.C G.A ... ... TCT ... A.T

#ngs         ATG AAA GAA TTT AAA TCT AAT AAT CAT TTG TTT ATG ATA AGT GAC AGT GGT GCT CGT GGT AAT GCT TCC AAT TTT TCT
#p_mali      ... ..T ..T ..A ... CAA G.. ... ... G.A ... ... ..G ... ..T ... ..A ..A ... ..A .G. AT. ..T ... ... A..
#P_aust      ... ... ... ..C CC. AA. G.C ..C ... A.T ... ... ..G ..C ..A ... ... ... ... ... .G. .T. ..T ..C ... A..
#aywb        ... ... ... ..C ... CAA G.C ..C ..C A.T ... ... ..G ..C ..A ... ..A ..G ... ... .G. .T. ..T ..C ... A..
#oym         ... ... ... ... C.. CAA G.C ..C ..C A.T ... ... ..G ..C ..A ... ..C ..A ... ... .G. .T. ..T ..C ... A..
#achole_laid ... ... ... ..C G.T AAA G.. ..C A.C A.C ... ... ..G ... ... TC. ... ..C ..C ... ..C .TA ..T ..C ... A.A

#ngs         CAA TTA TCT GGT ATG AGG GGT TTA ATG AAT AGT CCT AAA GGA GAA ATT TTA GAA ATT CCG GTA AAA AGT TCT TTT AAA
#p_mali      ... ... ATG ... ... ..A ... ... ... ..C .A. ... ... ..T ... ... A.T ... G.. ... ... ..G .C. ... ... .T.
#P_aust      ... ... G.A ..G ... ... ..A ..G ... ... .AC ... ... ..G ... ... A.T ... G.. ..C ..G ... GCC ... ... .G.
#aywb        ... ... G.A ..G ... ..A ..A ... ... ... .AC ... ... ..T ... ... A.T ... G.C ..T ..T ... GC. ... ..C CG.
#oym         ... ... G.A ..G ... ..A ..G ... ... ... .AC ... ... ..T ... ... A.T ... G.C ..T ..T ... GC. ... ..C CG.
#achole_laid ..G ... GTC ... ... ..A ..A C.T ... .G. .AC ... ... ..G ... .CG A.T ... G.. ..A ..T C.. TCA ..A ... .G.


#ngs         GAA GGT CTT ACT GTA TCT GAA TTT TTT ATT TCT ACA CAT GGA GCT AGA AAA GTT TCT ACC GAT ACG GCT TTA AAA ACA
#p_mali      ... ..A T.A ... ... ..A ... ... ... ... ... ..T ... ..G ... C.T ... .GC ..A ..T ... ..T ... ... ... ...
#P_aust      ... ... T.G .AA ..T ... ... ... ..C ... ... ..T ..C ... ..C C.C ... .G. ..A ... ..C ..T ... ... ... ..T
#aywb        ... ... ..G .AA ..T ... ..G ... ... ..C ... ..T ..C ..C ..C C.T ... .G. ..A ..T ... ..T ..C C.C ... ...
#oym         ... ... ..G .AA ..T ... ..G ... ..C ..C ... ..T ..C ... ..C C.T ... .G. ..A ..T ..C ..T ..C C.C ... ...
#achole_laid ... ... ..A ..C ... ... ... ..C ... ..C ..C ..C ..C ..T ..G ... ... .G. ... ... ... ..C ..G C.T ..G ..C

#ngs         GCA GAA TCA GGT TAT TTA ACA CGT AGA TTA GTA GAT GTT GCT CAA GAT AAT ATA ATT ATG AAA GAA GAT TGT GGT TCA
#p_mali      ..T ... ..T ..A ... ... ..T ... C.T ..G ... ... ... ..A ... ... .T. ..T ..A ..T ... ..T ... ... ..A AGT
#P_aust      ..T ... ... ... ... ... ... ... C.T ... ..T ... ... A.. ... ... GT. G.G G.. ..T ... ... ... ..C ..A AGT
#aywb        ..T ..G ... ... ... C.. ... ... C.T ... ..T ..C ..A A.. ... ... .T. G.C G.. ..T ... ... ..C ... AA. AGT
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#oym          ..C ..G ... ... ... C.. ..G ... C.T ... ..T ..C ..A A.. ... ... .T. G.T G.. ..C ... ... ..C ..C AA. AGT
#achole_laid ... ... ..T ... ..C ... ... ... C.T ... ..C ..C ..A T.A ... ..C GT. ..T ... G.T G.G ..T ... ... ... ...

#ngs          GAT AAA GGT ATT TTT ATA GAA CCT ATG AAC AGA GAT --- GAA AAA GAA ATT ATT TCA TTA TCT CAA CGT ATT TTT GGT
#p_mali       ..A TT. ... T.. AA. G.T ... G.. ..T ..A .AT ... --- .GT ... ..G ... ..A ..T ... CG. G.C ..C ..A ..A ...
#P_aust       ... C.T A.. T.. ... G.T ... G.. ... .TG .TC ..C --- .GC ... ... ... ... ..T ... AAA A.C ... ... ..G ...
#aywb         ..C CGC ..G T.. A.. G.G ... G.. ... .TG ..T ... --- .GC ... ... ... G.A ..T C.C AAA A.. ..C ... A.G ..A
#oym          ..C CGC ... T.. G.. G.. ... G.. ... .TT ..C ..C --- .GC ... ... ... G.. ... C.C AAA ... ..C ... A.G ..A
#achole_laid AGC C.T ... G.. .A. G.. ... G.A ..T ..A GAT ..G TCA .GT ... ... ..C G.. C.. ... .AC G.T A.A ... .A. ..C

#ngs          CGT TTT TCG GCA GAA AAT ATT ATT AAT TCT AAA AAT AAA GAA ATT ATT TTA AAC AAA AAT GAA CTA ATT ACT AAG GAA
#p_mali       ... ... .TA AGT A.. G.. ... T.A C.. C.. C.. .G. ... A.. ... ... ..T T.T .G. ... C.. T.. G.A .A. G.A ..T
#P_aust       ... .G. G.T AGT CGT G.. G.. T.G C.. C.. ... .CA GGT ..T G.. ... G.. TCT TCT ... ... T.. ... .AC G.. AC.
#aywb         ..C ... G.T AGT TGC G.C ... TG. C.C C.C ... .CC ..C ACC C.. ... G.. GCG CGT ... ... T.. ... .TC G.. TCT
#oym          ..C ... G.T AGT TGC G.C ... TG. C.. C.C ... .CC ..C .CC C.. ... A.. GCG CGC ... ... T.. ... ... G.. TCC
#achole_laid ..C ... G.T ... C.T G.C ... ..C .G. C.A ... .C. GGT ... G.A T.. G.G ..A CGT ... ... ..G ... ... G.A ...

#ngs          ATT ATT GAT AAA ATA GTT AAA TTA AAT ATT AAA AAA GTT AAA ATA AGA AAT ATT TTA AAT TGT AAT TGT GAA CAT GGT
#p_mali       ... TG. A.. ... ..T A.. G.T ... GG. ... GG. ... ..A G.. ..C ... .G. ... ... ... ... ... ..C CC. .G. ..A
#P_aust       ..C GCA C.A G.. ..C A.. G.T GC. ..A ... ... ... ... CCT ..C C.T .G. G.. ..G ... ... .G. ... ... T.C ...
#aywb         .AA GCA C.A G.. ..T A.. .CT GCC ..A ... ... ... ... CCT ..T ... .G. ... ... .C. ... ... ... ... T.. ...
#oym          .AA GCC C.A G.. ..T A.. .CT GCC ..A ... ... ... ... CCT ..T ... .GC ... ... .C. ... ... ... ... T.. ...
#achole_laid ..C GG. TTG GC. ..C ... ... .C. GG. ..G C.. ... ... G.. ..T ... .G. ... A.G .CA ... .CA .CA AGT ... ...

#ngs          ATT TGT TCT AAA TGT TAT GGT TTG AAT TTA GCT ACA AAT AAA ATT GTT GAA ATA GGA GAA GCT GTT GGT GTT ATA GCA
#p_mali       G.. ... AT. ... GA. ... ... A.A ... ... ..A ..T ... ... C.A ..A ... ... ..C ... ..A ..A ..A ... ..C ..T
#P_aust       ... ... G.. ... GA. ... ... ..T ... ... ..C ..T ... ... CCA ... ... G.C ..T ... ..C ... ..A ... ..T ..C
#aywb         ... ... G.C ... GAC ... ..C G.A ... C.. ... ..T ..C ... T.A ..A ... ..T ..C ... ... ... ... ... ..T ..T
#oym          ... ..C G.C ... GAC ..C ..C G.C ... C.. ... ..C ... ... T.A ..A ... ..T ..C ... ... ... ... ... ..T ..T
#achole_laid ... ... G.G ... GAC ..C ... A.T ..C ... ... ... ..C C.. T.. ... ... G.T ..T ... ... A.. ..G ... G.T ..T

#ngs          GCT CAA TCT ATT GGT GAA CCC GGC ACT CAA TTA ACT ATG AGA ACT TTT CAT ACT GGC GGT GTT TTA GCT GTT TCT GAT
#p_mali       ... ... ... ... ... ... ..A ..T ..A ... ... ... ... ... ... ... ... ..C ..T ... ... GCT T.A ... ... ...
#P_aust       ... ..G ..A ..C ..A ... ..A ..A ... ... ... ... ... ... ... ... ..C ... ..G ..T ... ... GCC T.A .CA .TA ..C
#aywb         ... ... ..C ..C ... ... ..A ..A ... ... ... ... ... ... ... ... ..C ... ... ..T ..A ... GCT T.. .CC ..A ...
#oym          ... ... ..C ... ..G ... ..A ..A ... ... ... ... ... ... ... ... ..C ... ... ..A ..A ... GCT T.. .CC ..A ...
#achole_laid ... ... ... ... ... ..G ..A ..T ..A ..G ... ..C ... C.T ..A ..C ... ..A ..T ..G ... GCT ... .G. G.C ...

#ngs          ATT ACT CAA GGA TTA CCA AGA ATT CAA GAA TTA TTT GAA GCT AGA AAA CCT AAA GGA AAA GCA ATA ATT AGT GAA TGT
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#p_mali      ... ... ... ..T ... ... ... ... ... ... ... ... ... .TA ... ... ... ... ..G ... ..T ..T ... ... ..G .TG
#P_aust      ... ... ... ... C.T ... ..G ... G.. ... ... ... ... ..A ... ..G ..C ... ... ... ..T T.. ... ... ... .T.
#aywb        ... ... ... ..T C.T ... ..G ..C G.. ... ... ... ... .TA C.C ... ... ... ... ... ..G C.. ... ... ... .TA
#oym         ... ... ... ..T C.T ... ..G ..C G.. ... ... ... ... .TA C.C ... ... ... ... ... ..G C.. ..C ..C ... .TA
#achole_laid ... ... ... ..T ... ... C.T ... ... ..G ... ... ... ..A C.T ..T ..A ... ..T ... ... .CG ... TCA ... GT.

#ngs         AGT GGT TTT GTT AAA GAT ATT AAA AAT ATT AAT TCT AAT TTT TAT GAA ATT AAA ATT ATT TCA GAT TCT GAT CAA A--
#p_mali      .AA ... AAA ... ... A.. ... G.. ..A ..A .GA A.. TTA AA. CC. ... ... GT. ... T.A ..T ... --- ... G.. G--
#P_aust      .AA ..A AAA ... ... A.A ..C ..C ..A ..C ... ..A CCA CA. CCA ... ... .TT ... .C. A.. AC. AAC ..A ACC GGA
#aywb        .AA ..A AAA A.C ... A.A ... G.T ..A ..C CG. ..C C.A AA. CCA ... ... GT. ... .CA GA. ..A AA. ..C .CT G--
#oym         .AA ..A AAA A.C ... A.A ... G.T ..A ..C CG. ..C C.A AA. CCA ... ... GT. ..C .CA GA. ..A AA. ..C .CT G--
#achole_laid GAA ... AAA ... ... ... ..C --- --- -.C .CG ..G TGG .GG .TC --. ... .GC ... .CA ATT AC. GA. ACC ... G--

#ngs         -AA ACA GAA TTT ATT TAT AAT GCT CTT TTT AAT TCT GAA ATA TTA GTA AGC AAA AAT ATG TAT ATT AAA GCG GGT CAA
#p_mali      -.. CA. ... A.A CG. ... .T. ..G GA. GC. ..C ... ... ..T ... ..T TCT ... GG. ... A.A ..A ... T.T ... ...
#P_aust      G.. .A. ... AA. C.. ... GT. TTA GA. CC. ... GT. ..C ..T ... ..G ... ... ... GAT G.. G.. T.T C.A ..A ...
#aywb        -.C ... ... CAC CG. ... .T. TTG GAA CC. ... GT. ..T ..T ... ... ... ... ..C .AC AT. G.. T.T C.T ... ...
#oym         -.C ... ..G CAC CG. ... .T. TTG GAA CC. ... GTC ..T ..T ... ... ... ... ..C .AC AG. G.. T.T C.C ... ...
#achole_laid -GC ..T ... .AC .AA ..C .CG CT. GA. CCA ..C AT. ... GCT ... ..T .AA ... GG. CAA G.. G.. GTT ..A ... ...
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Appendix 5

- Sequences Alignment of 16Sr tRNA gene of box 1 samples.

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LYAM 16S                          ----------------------------------------------------------CGCGTCAAGGGAGG-GAGC
LYPR 16S                          ----------------------------------------------------------------------------
LYHV 16S                          ----------------------------------------------------------------------------
CHAMBEZI 16S                      ----------------------------------------------------------------------------
GH1 16S                           ----------------------------------------------------------------------------
GH6 16S                           ----------------------------------------------------------------------------
GH8 16S                           ----------------------------------------------------------------------------
Cornwall 16S                      ----------------------------------------------------------CGCGTCAAGGGAGG-GAGC
GH8- 16S                          ----------------------------------------GAATACATTTAAGGAGGG--GGC
C. p. fragariae (Jersey) 16S      GTTCAAGACCTAGCATAGGTATGCTTAAGGGAGG-GAGC
RG 16S                            ---ACAGACCTAGCATAGGTATGCTTAAGGGAGAGAGC
SGP-16S                           ----------------------ATTC-TATGCTTAAGGGAG-GAGC

LYAM 16S                          AG-TCGAGTTGCAGACTTCAATCCGAACTGAGACTAT
LYPR 16S                          ----------------------------------------
LYHV 16S                          ..-.....................................
CHAMBEZI 16S                      ..-.....................................
GH1 16S                           ..-.....................................
GH6 16S                           ..-.....................................
GH8 16S                           ..A.....................................
Cornwall 16S                      GAC.ATGA.GTGTAG.CGGGC.GAG..GG.TGA..GGC
GH8- 16S                          GACGATGA.GTGTAG..GGG.C.GAG..GG.TGA..AGC
C. p. fragariae (Jersey) 16S      GAC.ATGA.GTGTAG.CGGGC.GAG..GG.TGA..GGC
RG 16S                            GAC.ATGA.GTGTAG.CGGGC.GAG..GG.TGA..GGC
SGP-16S                           GAC.ATGA.GTGTAG.CGGGC.GAG..GG.TGA..GGC

LYAM 16S                          TGTAATAGCCATTGTATCACGTTTGTAG---CCCAGA
LYPR 16S                          -----A..................------.......
LYHV 16S                          ..........................---.......
CHAMBEZI 16S                      ..........................---.......
GH1 16S                           ..........................---.......
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GH6 16S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	-	-	.	.	.	.	.	.
GH8 16S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	-	-	.	.	.	.	.	.
Cornwall 16S	A	.	C	.	G	.	.	.	G	G	.	A	.	T	.	T	C	G	G	.	A	A	.	G	.	A	G	.	A	A	A	.	T	.	T	.	.
GH8- 16S	A	.	C	.	G	.	.	A	G	G	.	A	.	T	.	T	C	G	G	.	A	A	.	G	.	A	G	.	A	A	A	.	T	.	T	.	.
C. p. fragariae (Jersey) 16S	A	.	C	.	G	.	.	.	G	G	.	A	.	T	.	T	C	G	G	.	A	A	.	G	.	A	G	.	A	A	A	.	T	.	T	.	.
RG 16S	A	.	C	.	G	.	.	.	G	G	.	A	.	T	.	T	C	G	G	.	A	A	.	G	.	A	G	.	A	A	A	.	T	.	T	.	.
SGP-16S	A	.	C	.	G	.	.	.	G	G	.	A	.	T	.	T	C	G	G	.	A	A	.	G	.	A	G	.	A	A	A	.	T	.	T	.	.

LYAM 16S	C	C	A	A	T	T	T	T	T	-	C	A	T	T	-	-	G	G	C	A	G	T	C	T	C	G	C	T	A	A	A	G	T	C	C	C	C
LYPR 16S	.	.	.	.	.	.	.	.	.	-	.	.	.	.	-	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
LYHV 16S	.	.	.	.	.	.	.	.	.	-	.	.	.	.	-	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CHAMBEZI 16S	.	.	.	.	.	.	.	.	.	-	.	.	.	.	-	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
GH1 16S	.	.	.	.	.	.	.	.	.	T	.	.	.	.	-	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
GH6 16S	.	.	.	.	.	.	.	.	.	T	.	.	.	.	-	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
GH8 16S	.	.	.	.	.	.	.	.	.	T	.	.	.	.	-	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cornwall 16S	A	A	.	G	.	.	C	.	.	T	T	.	.	.	A	G	.	.	A	.	.	A	A	.	A	A	A	.	G	.	T	.	G	A	A	A	A
GH8- 16S	A	A	.	G	.	.	C	.	.	T	T	.	.	C	A	A	.	.	A	.	.	A	A	A	A	.	.	A	.	G	T	.	G	.	G	A	A
C. p. fragariae (Jersey) 16S	A	A	.	G	.	.	C	.	.	T	T	.	.	.	A	G	.	.	A	.	.	A	A	.	A	A	A	.	G	.	T	.	G	A	A	A	A
RG 16S	A	A	.	G	.	.	C	.	.	T	T	.	.	.	A	G	.	.	A	.	.	A	A	.	A	A	A	.	G	.	T	.	G	A	A	A	A
SGP-16S	A	A	.	G	.	.	C	.	.	T	T	.	.	.	A	G	.	.	A	.	.	A	A	.	A	A	A	.	G	.	T	.	G	A	A	A	A

LYAM 16S	T	T	T	A	G	A	A	C	T	T	A	A	C	C	T	A	A	C	A	T	C	T	C	A	C	G	A	C	A	C	G	A	G	C	T	G	A
LYPR 16S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
LYHV 16S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CHAMBEZI 16S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
GH1 16S	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
GH6 16S	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
GH8 16S	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cornwall 16S	.	A	.	G	T	G	C	.	-	-	-	.	G	.	A	G	C	.	G	C	G	G	T	.	A	T	.	.	.	T	A	G	.	G	-	.	G
GH8- 16S	.	A	.	G	T	G	C	.	-	-	-	.	G	.	A	G	C	.	G	C	G	G	T	.	A	.	.	.	.	T	A	.	.	G	-	.	G
C. p. fragariae (Jersey) 16S	.	A	.	G	T	G	C	.	-	-	-	.	G	.	A	G	C	.	G	C	G	G	T	.	A	T	.	.	.	T	A	G	.	G	-	.	G
RG 16S	.	A	.	G	T	G	C	.	-	-	-	.	G	.	A	G	C	.	G	C	G	G	T	.	A	T	.	.	.	T	A	G	.	G	-	.	G
SGP-16S	.	A	.	G	T	G	C	.	-	-	-	.	G	.	A	G	C	.	G	C	G	G	T	.	A	T	.	.	.	T	A	G	.	G	-	.	G

LYAM 16S	T	A	T	T	T	C	T	A	T	A	G	C	T	T	T	G	C	A	G	A	G	T	A	-	T	G	T	C	A	A	G	A	C	C	T	G	G

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LYPR 16S                          .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  -  .  .  .  .  .  .  .  .  .  .  .  .  .
LYHV 16S                          .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  -  .  .  .  .  .  .  .  .  .  .  .  .  .
CHAMBEZI 16S                      .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  C  .  .  .  .  .  A  .  .  -  .  .  .  .  .  .  .  .  .  .  .  .  .
GH1 16S                           .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  C  .  .  .  .  G  A  .  .  A  .  .  .  .  .  .  .  .  .  .  .  .  .
GH6 16S                           .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  C  .  .  .  .  .  A  .  .  A  .  .  .  .  .  .  .  .  .  .  .  .  .
GH8 16S                           .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  C  .  .  .  .  .  A  .  .  A  .  .  .  .  .  .  .  .  .  .  .  .  .
Cornwall 16S                      C  G  G  .  .  A  A  .  .  .  A  G  .  .  .  A  T  G  .  T  C  .  .  A  G  T  G  .  .  .  T  G  .  T  .  A  A
GH8- 16S                          C  G  G  .  C  T  A  G  .  .  A  G  .  C  A  .  T  G  .  T  .  .  .  A  .  .  G  .  .  .  C  G  .  T  C  A  A
C. p. fragariae (Jersey) 16S      C  G  G  .  .  A  A  .  .  .  A  G  .  .  .  A  T  G  .  T  C  .  .  A  G  T  G  .  .  .  T  G  .  T  .  A  A
RG 16S                            C  G  G  .  .  A  A  .  .  .  A  G  .  .  .  A  T  G  .  T  C  .  .  A  G  T  G  .  .  .  T  G  .  T  C  A  A
SGP-16S                           C  G  G  .  .  A  A  .  .  .  A  G  .  .  .  A  T  G  .  T  C  .  .  A  G  T  G  .  .  .  T  G  .  T  .  A  A

LYAM 16S                          C  C  G  C  T  T  G  T  G  C  G  G  G  A  T  C  C  C  G  T  C  A  A  T  T  C  C  T  T  T  A  A  G  T  T  T  C
LYPR 16S                          .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .
LYHV 16S                          .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .
CHAMBEZI 16S                      .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .
GH1 16S                           .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .
GH6 16S                           .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .
GH8 16S                           .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .
Cornwall 16S                      G  A  .  G  C  -  A  A  .  T  .  .  A  .  .  T  .  .  A  .  G  T  G  .  A  G  T  G  G  .  .  .  A  A  .  G  .
GH8- 16S                          G  A  .  G  C  -  .  A  .  T  .  .  A  .  .  T  .  .  A  .  G  T  G  .  A  G  .  G  G  .  .  .  A  A  .  G  .
C. p. fragariae (Jersey) 16S      G  A  .  G  C  -  A  A  .  T  .  .  A  .  .  T  .  .  A  .  G  T  G  .  A  G  T  G  G  .  .  .  A  A  .  G  .
RG 16S                            G  A  .  G  C  -  A  A  .  T  .  .  A  .  .  T  .  .  A  .  G  T  G  .  A  G  T  G  G  .  .  .  A  A  .  G  .
SGP-16S                           G  A  .  G  C  -  A  A  .  T  .  .  A  .  .  T  .  .  A  .  G  T  G  .  A  G  T  G  G  .  .  .  A  A  .  G  .

LYAM 16S                          T  A  A  C  T  T  C  A  G  T  A  C  C  G  A  -  G  T  T  T  A  A  C  C  C  C  G  A  C  A  C  T  T  A  G  T  A
LYPR 16S                          .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  -  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .
LYHV 16S                          .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  -  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .
CHAMBEZI 16S                      .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  -  .  .  .  G  -  -  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .
GH1 16S                           .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  -  .  G  A  A  .  C  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .
GH6 16S                           .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  -  .  G  A  A  .  C  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .
GH8 16S                           .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  -  .  G  A  A  .  C  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .
Cornwall 16S                      .  G  G  G  .  C  T  T  -  .  .  .  T  .  .  C  .  C  .  G  .  G  G  .  A  .  .  .  A  .  -  -  -  -  .  C  G
GH8- 16S                          .  G  G  G  .  C  T  T  -  A  .  .  T  .  .  C  .  C  .  G  .  G  G  .  A  .  .  .  A  .  -  -  -  -  .  C  G
C. p. fragariae (Jersey) 16S      .  G  G  G  .  C  T  T  -  .  .  .  T  .  .  C  .  C  .  G  .  G  G  .  A  .  .  .  A  .  -  -  -  -  .  C  G
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RG 16S                          .  G  G  G  .  C  T  T  -  .  .  .  T  .  .  C  .  C  .  G  .  G  G  .  A  .  .  .  A  .  -  -  -  -  .  C  G
SGP-16S                         .  G  G  G  .  C  T  T  -  .  .  .  T  .  .  C  .  C  .  G  .  G  G  .  A  .  .  .  A  .  -  -  -  -  .  C  G

LYAM 16S                        T  T  T  G  C  T  C  C  C  C  A  C  G  C  T  T  T  -  C  G  T  G  C  A  T  C  A  G  C  G  T  C  A  G  T  A  A
LYPR 16S                        .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  -  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .
LYHV 16S                        .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  -  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .
CHAMBEZI 16S                    .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  -  .  .  .  .  .  .  C  .  .  .  .  .  .  .  .  .  .  .  .
GH1 16S                         .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  -  .  .  .  .  .  .  C  .  .  .  .  .  .  .  .  .  .  .  .
GH6 16S                         .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  -  .  .  .  .  .  .  C  .  .  .  .  .  .  .  .  .  .  .  .
GH8 16S                         .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  -  .  .  .  .  .  .  C  .  .  .  .  .  .  .  .  .  .  .  .
Cornwall 16S                    .  A  A  A  .  G  A  T  G  A  G  T  A  .  .  A  A  A  T  .  .  T  G  G  G  T  .  A  A  A  C  .  .  .  .  G  T
GH8- 16S                        .  A  A  A  .  G  A  T  G  A  G  T  A  .  .  A  A  G  T  .  .  C  G  G  G  T  T  A  A  A  C  .  G  .  .  .  C
C. p. fragariae (Jersey) 16S    .  A  A  A  .  G  A  T  G  A  G  T  A  .  .  A  A  A  T  .  .  T  G  G  G  T  .  A  A  A  C  .  .  .  .  G  T
RG 16S                          .  A  A  A  .  G  A  T  G  A  G  T  A  .  .  A  A  A  .  .  .  T  G  G  G  T  .  A  A  A  C  .  .  .  .  G  T
SGP-16S                         .  A  A  A  .  G  A  T  G  A  G  T  A  .  .  A  A  A  T  .  .  T  G  G  G  T  .  A  A  A  C  .  .  .  .  G  T

LYAM 16S                        A  T  A  T  T  T  A  C  A  C  A  T  T  T  T  A  C  C  G  C  T  A  C  A  C  A  T  G  G  A  A  T  T  C  C  G  C
LYPR 16S                        .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .
LYHV 16S                        .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .
CHAMBEZI 16S                    .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .
GH1 16S                         .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .
GH6 16S                         .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .
GH8 16S                         .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .
Cornwall 16S                    G  C  .  A  G  .  .  T  G  A  .  A  C  .  .  .  A  A  .  G  A  .  T  T  G  .  C  .  .  G  .  C  .  .  .  .  .
GH8- 16S                        G  C  .  A  G  .  .  T  G  A  .  A  C  .  .  .  A  A  .  G  A  .  T  T  G  .  C  .  .  G  .  C  .  .  .  .  .
C. p. fragariae (Jersey) 16S    G  C  .  A  G  .  .  T  G  A  .  A  C  .  .  .  A  A  .  G  A  .  T  T  G  .  C  .  .  G  .  C  .  .  .  .  .
RG 16S                          G  C  .  A  G  .  .  T  G  A  .  A  C  .  .  .  A  A  .  G  A  .  T  T  G  .  C  .  .  G  .  C  .  .  .  .  .
SGP-16S                         G  C  .  A  G  .  .  T  G  A  .  A  C  .  .  .  A  A  .  G  A  .  T  T  G  .  C  .  .  G  .  C  .  .  .  .  .

LYAM 16S                        G  G  A  C  A  A  C  G  T  T  A  A  G  C  G  T  T  G  A  A  A  T  T  A  A  A  C  T  A  G  A  G  A  C  T  T  A
LYPR 16S                        .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .
LYHV 16S                        .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .
CHAMBEZI 16S                    .  .  .  .  .  G  .  .  .  .  .  .  .  .  .  C  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .
GH1 16S                         .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  T  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .
GH6 16S                         .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  T  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .
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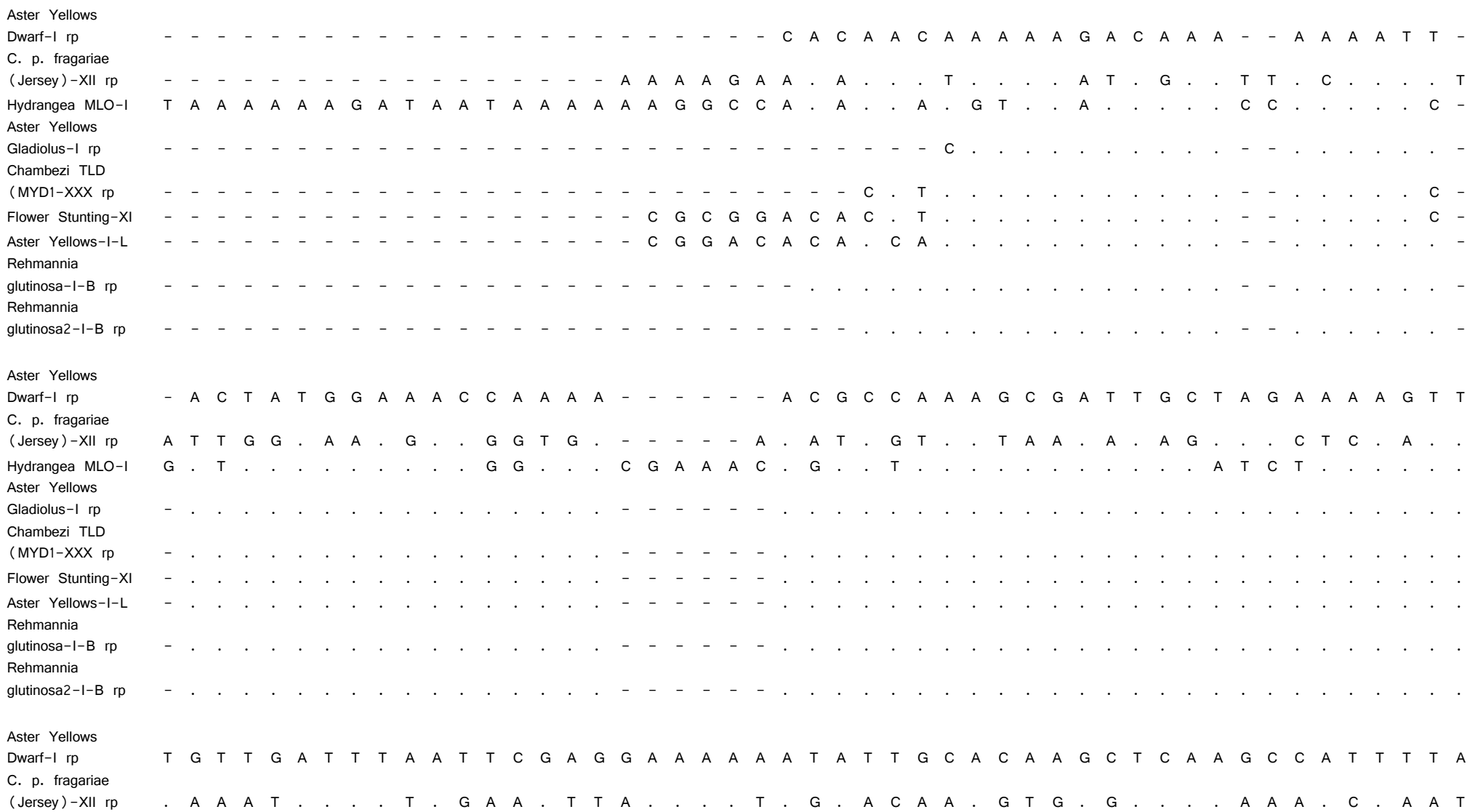
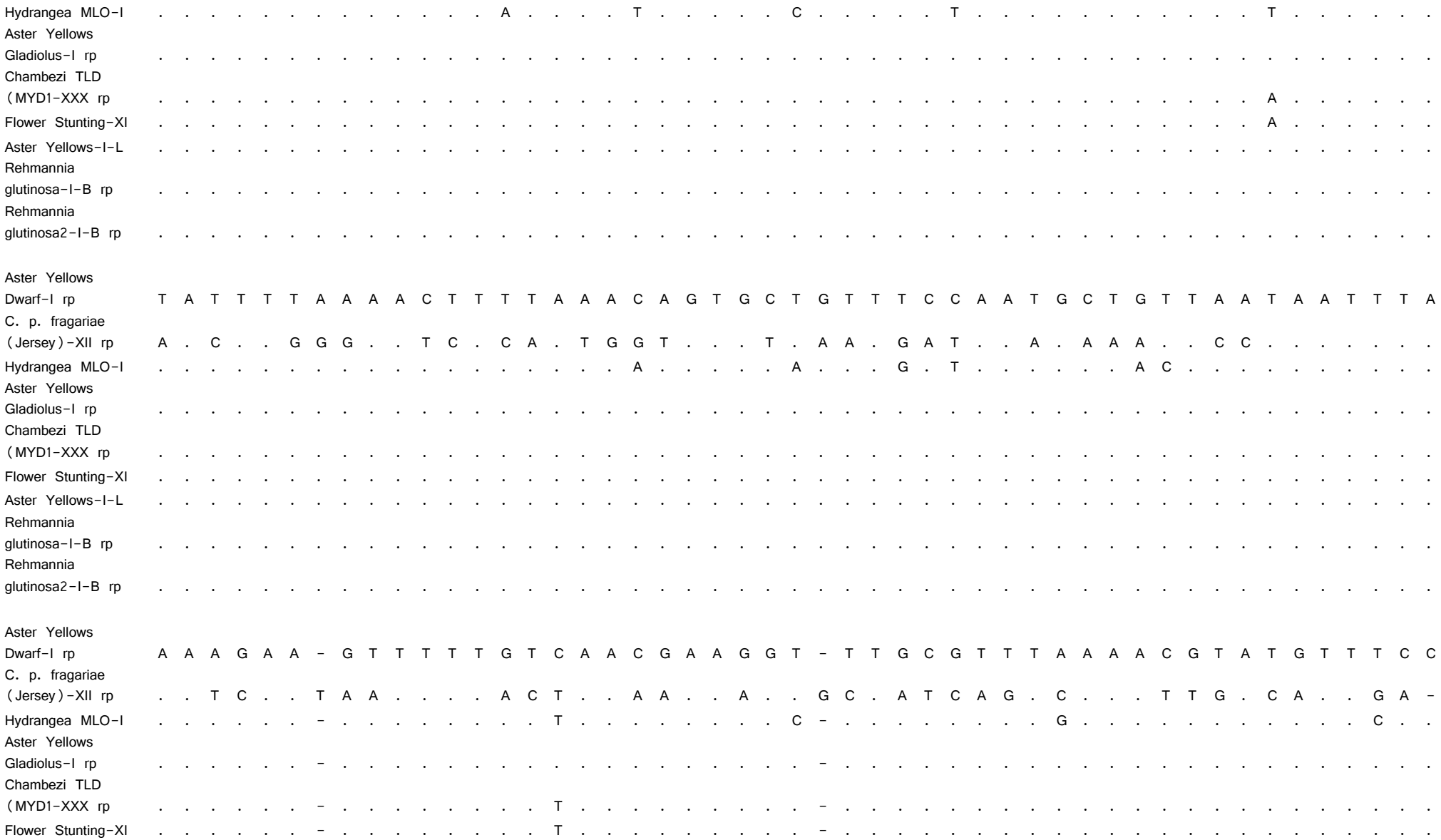
GH8 16S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cornwall 16S	A	A	.	.	C	T	.	A	C	C	.	G	.	T	C	.	.	.	.	C	.	.	G	C	T	T	.	.	G	C	.	A	.	G	C	.	G
GH8- 16S	A	A	.	.	C	T	T	A	C	C	.	G	.	T	C	.	.	.	.	C	.	.	G	T	T	T	T	.	G	C	G	A	.	A	C	G	G
C. p. fragariae (Jersey) 16S	A	A	.	.	C	T	.	A	C	C	.	G	.	T	C	.	.	.	.	C	.	.	G	C	T	T	.	.	G	C	.	A	.	G	C	.	G
RG 16S	A	A	.	.	C	T	.	A	C	C	.	G	.	T	C	.	.	.	.	C	.	.	G	C	T	T	.	.	G	C	.	A	.	G	C	.	G
SGP-16S	A	A	.	.	C	T	.	A	C	C	.	C	.	T	C	.	.	.	.	C	.	.	G	C	T	T	.	.	G	C	.	A	.	G	C	.	G

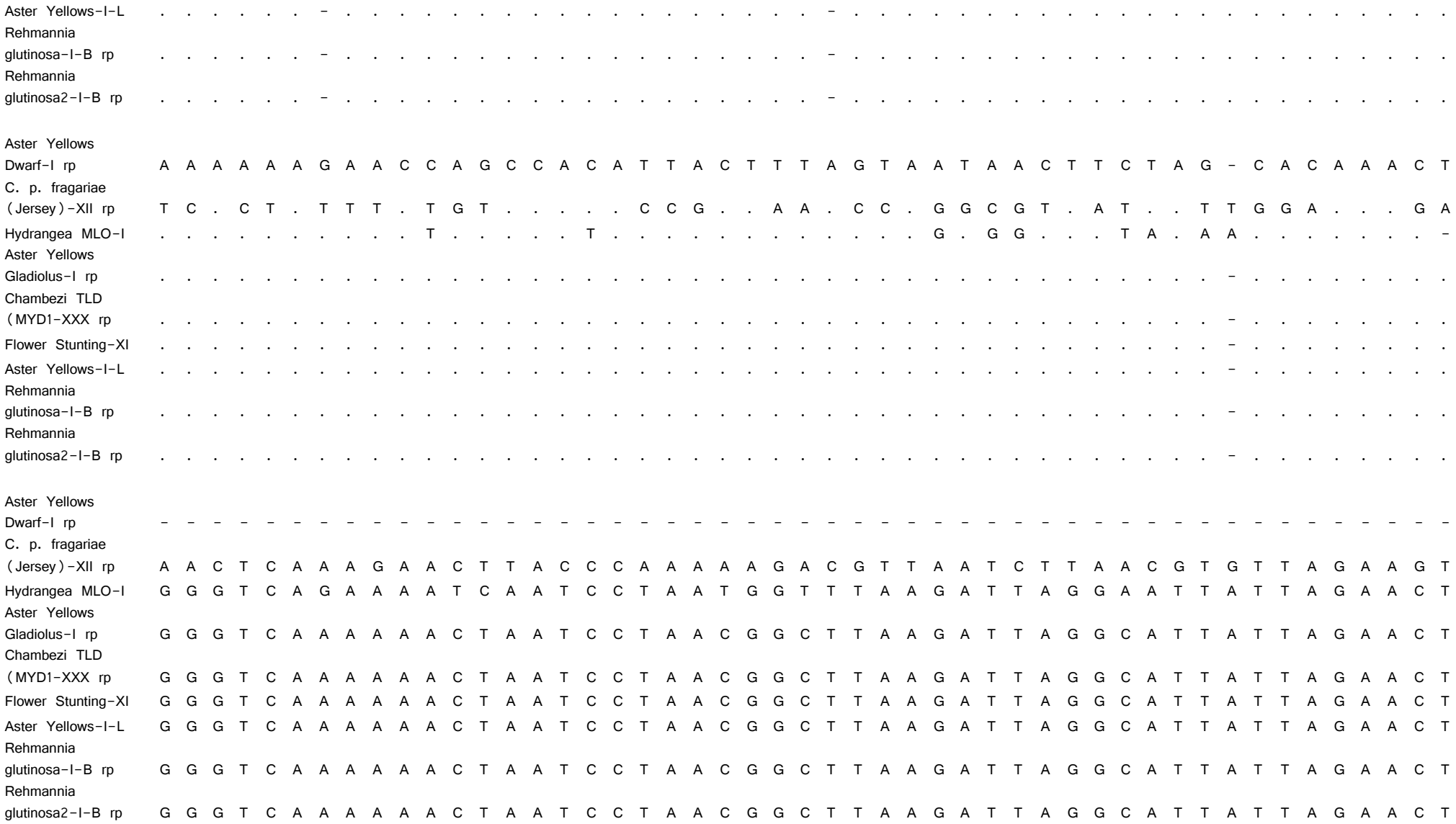
LYAM 16S	G	G	A	T	A	A	C	G	C	T	C	G	C	C	C	C	C	T	A	T	G	T	A	T	T	A	C	C	G	C	G	G	C	T	G	C	T
LYPR 16S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
LYHV 16S	.	.	.	.	.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CHAMBEZI 16S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GH1 16S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
GH6 16S	.	.	.	.	.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GH8 16S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cornwall 16S	.	.	T	.	-	G	T	C	G	.	.	A	G	.	T	.	G	.	G	.	C	G	T	G	A	G	A	T	.	T	T	.	G	G	T	T	A
GH8- 16S	.	.	T	.	T	G	T	C	G	.	.	A	G	.	T	.	G	.	G	.	C	G	T	G	A	G	A	T	.	T	T	A	G	G	T	T	A
C. p. fragariae (Jersey) 16S	.	.	T	.	-	G	T	C	G	.	.	A	G	.	T	.	G	.	G	.	C	G	T	G	A	G	A	T	.	T	T	.	G	G	T	T	A
RG 16S	.	.	T	.	-	G	T	C	G	.	.	A	G	.	T	.	G	.	G	.	C	G	T	G	A	G	A	T	.	T	T	.	G	G	T	T	A
SGP-16S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

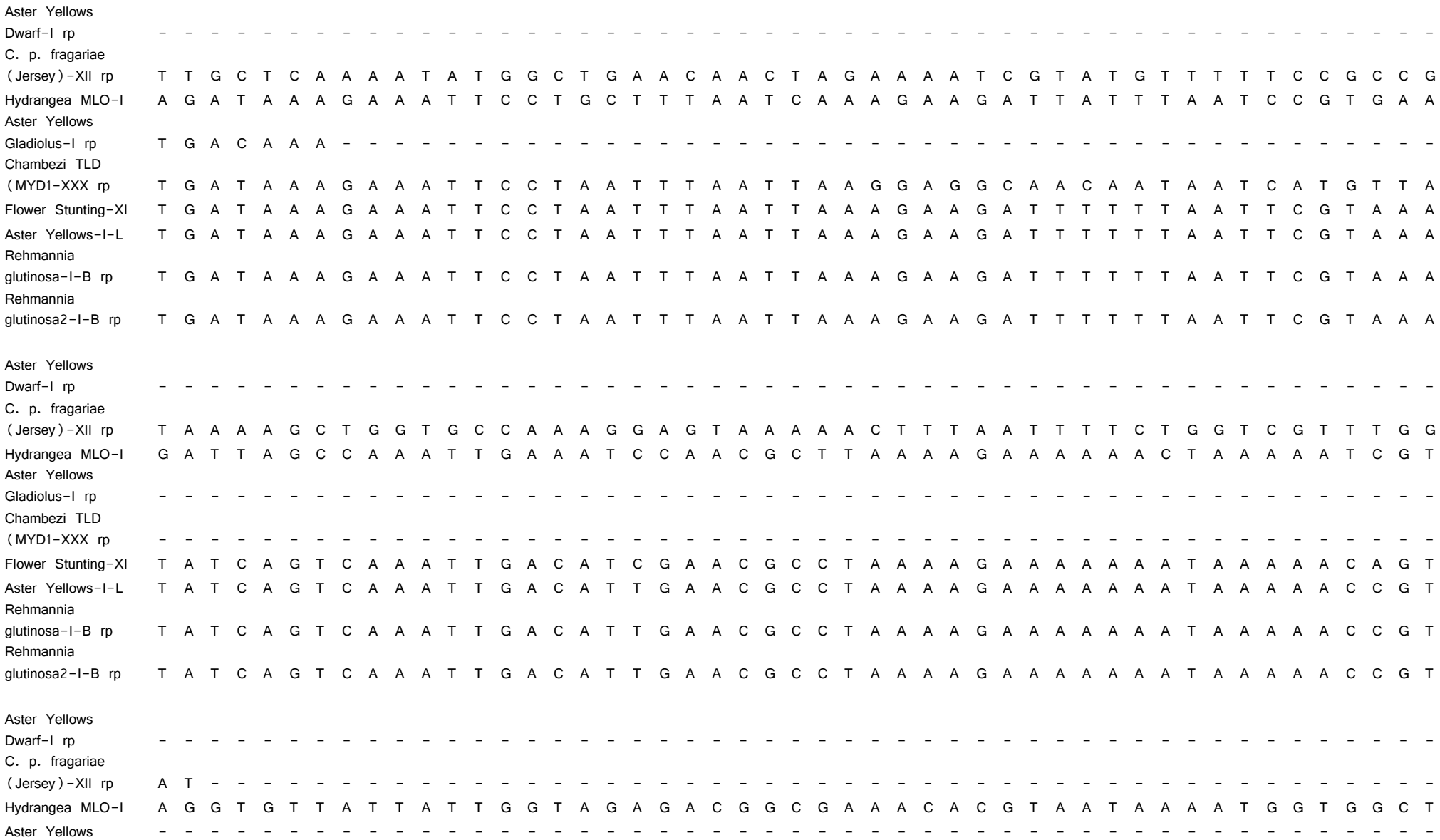
LYAM 16S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LYPR 16S	G	A	T	A	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LYHV 16S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CHAMBEZI 16S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GH1 16S	A	A	C	T	A	G	T	T	T	T	T	C	C	A	C	T	A	G	C	G	T	T	T	C	T	T	C	T	T	T	A	-	-
GH6 16S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GH8 16S	A	A	C	T	A	G	T	T	T	T	T	C	C	A	C	T	A	G	C	G	T	T	T	C	T	T	C	T	T	T	A	A	-
Cornwall 16S	C	G	T	A	A	T	G	G	T	G	G	G	G	A	C	T	T	T	A	G	C	A	A	G	A	C	T	G	C	C	A	G	T
GH8- 16S	C	G	T	T	A	T	G	G	T	G	G	G	G	A	C	T	T	T	A	A	C	G	A	G	A	C	T	-	-	-	-	-	-
C. p. fragariae (Jersey) 16S	C	G	T	A	A	T	G	G	T	G	G	G	G	A	C	T	T	T	A	G	C	A	-	G	A	C	T	G	C	-	-	-	-
RG 16S	C	G	T	A	A	T	G	G	T	G	G	G	G	A	C	T	T	T	A	G	C	A	A	G	A	C	T	G	C	-	-	-	-
SGP-16S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

- Sequences alignment of Rp gene that has been done on samples of box 1.




Hydrangea MLO-I
Aster Yellows Gladiolus-I rp
Chambezi TLD (MYD1-XXX rp
Flower Stunting-XI
Aster Yellows-I-L
Rehmannia glutinosa-I-B rp
Rehmannia glutinosa2-I-B rp
Aster Yellows Dwarf-I rp
C. p. fragariae (Jersey)-XII rp
Hydrangea MLO-I
Aster Yellows Gladiolus-I rp
Chambezi TLD (MYD1-XXX rp
Flower Stunting-XI
Aster Yellows-I-L
Rehmannia glutinosa-I-B rp
Rehmannia glutinosa2-I-B rp
Aster Yellows Dwarf-I rp
C. p. fragariae (Jersey)-XII rp
Hydrangea MLO-I
Aster Yellows Gladiolus-I rp
Chambezi TLD (MYD1-XXX rp
Flower Stunting-XI


Aster Yellows-I-L
Rehmannia glutinosa-I-B rp
Rehmannia glutinosa2-I-B rp
Aster Yellows Dwarf-I rp
C. p. fragariae (Jersey)-XII rp
Hydrangea MLO-I
Aster Yellows Gladiolus-I rp
Chambezi TLD (MYD1-XXX rp
Flower Stunting-XI
Aster Yellows-I-L
Rehmannia glutinosa-I-B rp
Rehmannia glutinosa2-I-B rp
Aster Yellows Dwarf-I rp
C. p. fragariae (Jersey)-XII rp
Hydrangea MLO-I
Aster Yellows Gladiolus-I rp
Chambezi TLD (MYD1-XXX rp
Flower Stunting-XI
Aster Yellows-I-L
Rehmannia glutinosa-I-B rp
Rehmannia glutinosa2-I-B rp


Aster Yellows Dwarf-I rp
C. p. fragariae (Jersey)-XII rp
Hydrangea MLO-I
Aster Yellows Gladiolus-I rp
Chambezi TLD (MYD1-XXX rp
Flower Stunting-XI
Aster Yellows-I-L
Rehmannia glutinosa-I-B rp
Rehmannia glutinosa2-I-B rp
Aster Yellows Dwarf-I rp
C. p. fragariae (Jersey)-XII rp
Hydrangea MLO-I
Aster Yellows Gladiolus-I rp
Chambezi TLD (MYD1-XXX rp
Flower Stunting-XI
Aster Yellows-I-L
Rehmannia glutinosa-I-B rp
Rehmannia glutinosa2-I-B rp
Aster Yellows Dwarf-I rp
C. p. fragariae (Jersey)-XII rp
Hydrangea MLO-I
Aster Yellows

Gladiolus-I rp	
Chambezi TLD (MYD1-XXX rp	- -
Flower Stunting-XI	A G G C G T T A T T A T T G G A A A A G A C G G C G A T A C A C G C A A C A A A T T A G T T G C C
Aster Yellows-I-L	A G G C G T T A T T A T T G G A A A A G A T G G C G A T A C A C G C A A C A A A T T A G T T G C C
Rehmannia glutinosa-I-B rp	A G G C G T T A T T A T T G G A A A A G A T G G C G A T A C A C G C A A C A A A T T A G T T G C C
Rehmannia glutinosa2-I-B rp	A G G C G T T A T T A T T G G A A A A G A T G G C G A T A C A C G C A A C A A A T T A G T T G C C

Aster Yellows Dwarf-I rp	- -
C. p. fragariae (Jersey)-XII rp	- -
Hydrangea MLO-I	T G A T T T A A A T A T T T T A G A A G T T A A A A A T T T T G A A A A A A T A G C T T T A T T A
Aster Yellows Gladiolus-I rp	- -
Chambezi TLD (MYD1-XXX rp	- -
Flower Stunting-XI	T A A T C T T A A C G T T T T A G A A G T T A A A A A C T C T G A T A A A A T C G C T T T A T T A
Aster Yellows-I-L	T A A T C T T A A C G T G T T A G A A G T T A A A A A C T C T G A T A A A A T C G C T T T A T T A
Rehmannia glutinosa-I-B rp	T A A T C T T A A C G T G T T A G A A G T T A A A A A C T C T G A T A A A A T C G C T T T A T T A
Rehmannia glutinosa2-I-B rp	T A A T C T T A A C G T G T T A G A A G T T A A A A A C T C T G A T A A A A T C G C T T T A T T A

Aster Yellows Dwarf-I rp	- -
C. p. fragariae (Jersey)-XII rp	- -
Hydrangea MLO-I	A A A T C G T A T G A A T T T C C G A C G T G T T C A A A A A A T G G C T A T T C A A A A G G C C
Aster Yellows Gladiolus-I rp	- -
Chambezi TLD (MYD1-XXX rp	- -
Flower Stunting-XI	A A A T C G T A T G T T T T T C C G C C G T G T T C A A A A A A T G G C A A T C C A A A A A G C C
Aster Yellows-I-L	A A A T C G T A T G T T T T T C C G C C G T G T T C A A A A A A T G G C A A T C C A A A A A G C C
Rehmannia	A A A T C G T A T G T T T T T C C G C C G T G T T C A A A A A A T G G C A A T C C A A A A A G C C

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glutinosa-I-B rp
Rehmannia
glutinosa2-I-B rp     A A A T C G T A T G T T T T T C C G C C G T G T T C A A A A A A T G G C A A T C C A A A A A G C C

Aster Yellows
Dwarf-I rp            - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
C. p. fragariae
(Jersey)-XII rp       - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Hydrangea MLO-I       C T T A G T C T C T G G A C G C T T A A A - T G G T G C T G A A A T A G C T C G T A G T G A A G G
Aster Yellows
Gladiolus-I rp        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Chambezi TLD
(MYD1-XXX rp          - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Flower Stunting-XI    T T T A A T T T C T G G T C G T T T G G G - T G G T G C T G A A A T A G C T C G A A G C G A A G G
Aster Yellows-I-L     T T T A A T T T C T G G T C G T T T G G G G T G G T G C T G A A A T A G C T C G T A G C G A A G A
Rehmannia
glutinosa-I-B rp      T T T A A T T T C T G G T C G T T T G G G T G G T G C T G A A T A G C T C G T A G C G A A G A C -
Rehmannia
glutinosa2-I-B rp     T T T A A T T T C T G G T C G T T T G G G - T G G T G C - - - - - - - - - - - - - - - - - - - - -
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- Sequences alignment of *secA* gene that has been done on samples of box 1.

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Chambezi TLD MYD1-XXX secA      - - - - - - - - T T G A T G G G A T G A C G G T A G G A C T T - A A T A C C A
Cornwall 1-XII secA             - - - - - - - - - - . T T A . . A C . T T . . . . T . . T . . . - . . . G T A .
GH8-XXII secA                   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
GH8 (aliquot of 54)-XXII SecA   - - - - - - - - - - - - - . A . T T . . T . A . . . . . T . . . - . . . G T T .
C. p. fragariae (Jersey)-XII SecA - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
tolbur (Ex-Jones)-XII secA      G G C T C G T T . . T T A . . T C . T T . . . . T . . . . . . - . . C . T T .
Poinsettia branching (flower buds)-III SecA - - - - - - - - - - - - T A . . C . . . . . T . . . . . T T . A - . . . . . T .
Elm witches broom (ULW)-V SecA  - - - - - - - - - - - - T A . A T T - - . . T . . . . . T T . A - . . . . . T T .
Stolbur (Stol)-XII SecA         - - - - - - - - - . . T T A . . C . . . . . T . . . . . T T . A - . . . . . T .
Peach X disease-III-A SecA      - - - - - - - - - - - - T T A . . C . . . . . T . . . . . T T . A - . - . . . T .
Aster Yellows-I-L SecA          - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Faba Bean Phyllody-II SecA      - - - - - - - - - - - - - T G . . T T . A . G T . . . . . T T . A - . . . C T T .
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Rehmannia glutinosa-I-B SecA                        ----------------------------------------------------------------------
Rehmannia glutinosa2-I-B SecA                       ----------------------------------------------------------------------
Nigerian 2nd AWKA-XXII secA                         ----------.....AT.A.....T.....T....GTT.
Tanzania box-KY4 Kenya 4 method 1-XXX               ----------------------------------------------------------------------
NIG 5th 1 in 5 dilution-XXII secA                   ----------.....T.A.....T.....-....GTT.
Tanzania box-BUS 2nd 1 in 2 dilution-XXII           ----------.....T.A.....T.....-....GTT.
Ghana 6-XXII SecA                                   ----------C.TT.AT.A.....T.....-....GTT.

Chambezi TLD MYD1-XXX secA                          GTGATATTCTCTACACTACTAACAGTGAATTAGGGTTTG
Cornwall 1-XII secA                                 .......T...T.........TTC.....C.T..T....
GH8-XXII secA                                       ----------------------------------------
GH8 (aliquot of 54)-XXII SecA                       C.....T..TT.A.....T.....G.....T....
C. p. fragariae (Jersey)-XII SecA                   ----------------------------------------
tolbur (Ex-Jones)-XII secA                          ....C..C..T..TG.....TTCC...C.T..A....
Poinsettia branching (flower buds)-III SecA         CC.....T..TT.....T.....T..C.
Elm witches broom (ULW)-V SecA                      .......T..TT....C.....C.....T..C.
Stolbur (Stol)-XII SecA                             CC.....T..TT.....T.....T..C.
Peach X disease-III-A SecA                          CC.....T..TT.....T.....T..C.
Aster Yellows-I-L SecA                              ----------------------------------------
Faba Bean Phyllody-II SecA                          .....T.A..TT.....T.....A.C..A....
Rehmannia glutinosa-I-B SecA                        ----------------------------------------
Rehmannia glutinosa2-I-B SecA                       ----------------------------------------
Nigerian 2nd AWKA-XXII secA                         CC.....T..TT.A.....T.....G.....T..C.
Tanzania box-KY4 Kenya 4 method 1-XXX               ----------------------------------------
NIG 5th 1 in 5 dilution-XXII secA                   C.....T..TT.A.....T.....G.....T....
Tanzania box-BUS 2nd 1 in 2 dilution-XXII           C.....T..TT.A.....T.....G.....T....
Ghana 6-XXII SecA                                   CC.....T..TT.A.....T.....G.....T..C.

Chambezi TLD MYD1-XXX secA                          TGGTAATGAAACGCCCTTACAGCTACGCTATTGTTGAATG
Cornwall 1-XII secA                                 .A..G.....T.....T.....T.....C....
GH8-XXII secA                                       ----------------------------------------
GH8 (aliquot of 54)-XXII SecA                       .AT.....AAAGAAA..T.AT..T.....T.A....
C. p. fragariae (Jersey)-XII SecA                   ----------ATAATT.AGACTTTG.TTT.GAA--CCC.CC
tolbur (Ex-Jones)-XII secA                          .A..G.....A.....T..T.....C...C....
Poinsettia branching (flower buds)-III SecA         .AA.G...C.T..TGA...T.A.....T.....A.....
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Elm witches broom (ULW)-V SecA                  .A.T.G.....T.A.AGAA..T.AT..........A.....
Stolbur (Stol)-XII SecA                         .A.A.G....C.T..TGA...T.A.......T.....A.....
Peach X disease-III-A SecA                      .A.A.G....C.T..TGA...T.A.......T.....A.....
Aster Yellows-I-L SecA                          ----------------------------------------
Faba Bean Phyllody-II SecA                      .T.T.......CT..TGAA..TG.T..T.TA....A.....
Rehmannia glutinosa-I-B SecA                    ----TCAT.TTA.AA...GGTC.AT.TTT.G.AA.CA.CT
Rehmannia glutinosa2-I-B SecA                   --------.TTA.AA...G-TC.AT.TTT.G.AA.CA.CT
Nigerian 2nd AWKA-XXII secA                     .AT.........AAAGAA..T.AT..T.........T.A.....
Tanzania box-KY4 Kenya 4 method 1-XXX           ----------------------------------------
NIG 5th 1 in 5 dilution-XXII secA               .AT.........AAAGAA..T.AT..T.........T.A.....
Tanzania box-BUS 2nd 1 in 2 dilution-XXII       .AT.........AAAGAA..T.AT..T.........T.A.....
Ghana 6-XXII SecA                               .AT.........AAAGAA..T.AT..T.........T.A.....

Chambezi TLD MYD1-XXX secA                      TAATTATTTCCCAAAGTGTCAAAGAAACTAAAAAATTTAT
Cornwall 1-XII secA                             .....C.....T.....C.A.G.....................
GH8-XXII secA                                   ..........AG.TGAAAAGCGTA...G..TT..A..T.
GH8 (aliquot of 54)-XXII SecA                   ..........AG.TGAAAAGCGTA...G..TT..A..T.
C. p. fragariae (Jersey)-XII SecA               A..GC.....T.TG.TGTGA.G..GC.GTGA.G..T.G..GG
tolbur (Ex-Jones)-XII secA                      .....C.....T.....C..A.....................
Poinsettia branching (flower buds)-III SecA     ..........GAGC.ACACTC..C..GG.......G..T.
Elm witches broom (ULW)-V SecA                  .........AGTCC..CG...C..GGA.TT..A..T.
Stolbur (Stol)-XII SecA                         ..........GAGC.ACACTC..C..GG.......G..T.
Peach X disease-III-A SecA                      ..........GAGC.ACACTC..C..GG.......G..T.
Aster Yellows-I-L SecA                          ----------------------------GAGGCGTG..C.
Faba Bean Phyllody-II SecA                      .....T.T.A.A.ACACC..CCTGG..TT..A..T.
Rehmannia glutinosa-I-B SecA                    AGGGCG...TTG.C.TG..GG.GC....GAGGCGTG..C.
Rehmannia glutinosa2-I-B SecA                   AGGGCG...TTG.C.TG..GG.GC....GAGGCGTG..C.
Nigerian 2nd AWKA-XXII secA                     ..........AG.TGAAAAGTGCA...A..TT..A..T.
Tanzania box-KY4 Kenya 4 method 1-XXX           ----------------------------------------
NIG 5th 1 in 5 dilution-XXII secA               ..........AG.TGAAAAGCGTA...G..TT..A..T.
Tanzania box-BUS 2nd 1 in 2 dilution-XXII       ..........AG.TGAAAAGCGTA...G..TT..A..T.
Ghana 6-XXII SecA                               ..........AG.TGAAAAGTGCA...A..TT..A..T.

Chambezi TLD MYD1-XXX secA                      CAGCCATTATCTTATTGAATTAGAAACTAAAACAATTGA
Cornwall 1-XII secA                             TCAA.....T.A.....G.........GC..............
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GH8-XXII secA A C A A . . . . . A . . . . . T G C . . . . T . A C . . T . T . . . . .
GH8 (aliquot of 54)-XXII SecA A C A A . . . . . A . . . . . T G C . . . . T . A C . . T . T . . . . .
C. p. fragariae (Jersey)-XII SecA T T A T T . A . G C . . . G . . C G G . . . A T T C A A T T G T T T T G C T T
tolbur (Ex-Jones)-XII secA . C A A . . . . . T . G G . . . . . G C . . . . . .
Poinsettia branching (flower buds)-III SecA A G A A . . . . . A . G . . C . . T . . . . . T . . . . . T . . C . .
Elm witches broom (ULW)-V SecA A . A . G G . . . . A . A . . . . . T . . . . . T . . . . . .
Stolbur (Stol)-XII SecA A G A A . . . . . A . G . . C . . T . . . . . T . . . . . T . . C . .
Peach X disease-III-A SecA A G A A . . . . . A . G . . C . . T . . . . . T . . . . . T . . C . .
Aster Yellows-I-L SecA T T . G T . A . T C . . . C . . C G G . A . . T T C A A T T G T T T T A G T T
Faba Bean Phyllody-II SecA T G A A . . . . . T . A . . C . . T . . . A . . T . A . . . . . T . . . . .
Rehmannia glutinosa-I-B SecA T T . G T . A . T C . . . C . . C G G . A . . T T C A A T T G T T T T A G T T
Rehmannia glutinosa2-I-B SecA T T . G T . A . T C . . . C . . C G G . A . . T T C A A T T G T T T T A G T T
Nigerian 2nd AWKA-XXII secA A C A A . . . . . A . . . . . T G C . . . . T . A C . . T . C . . . . .
Tanzania box-KY4 Kenya 4 method 1-XXX T . . A . . . . . T . . . . . T . . . . .
NIG 5th 1 in 5 dilution-XXII secA A C A A . . . . . A . . . . . T G C . . . . T . A C . . T . T . . . . .
Tanzania box-BUS 2nd 1 in 2 dilution-XXII A C A A . . . . . A . . . . . T G C . . . . T . A C . . T . T . . . . .
Ghana 6-XXII SecA A C A A . . . . . A . . . . . T G C . . . . T . A C . . T . C . . . . .

Chambezi TLD MYD1-XXX secA C C A A A T T G A T A A T T T A T A C A A C A T A G A A C A T G C C T C T T T
Cornwall 1-XII secA T . . . . . A C . A . . . . . G . T . . C C . . . . . T . T . . A C .
GH8-XXII secA T . . T . . G A . . . . . T . G . C C T C . . A . . T G T A A . . .
GH8 (aliquot of 54)-XXII SecA T . . T . . G A . . . . . T . G . C C T C . . A . . T G T A A . . .
C. p. fragariae (Jersey)-XII SecA G . . . . A C . . . T . G C . T C T T T G T . . . A . T T T . T A G . T . C .
tolbur (Ex-Jones)-XII secA T . . . . . A . . C . . . . . T . . T G . C . . G . . . . . A C .
Poinsettia branching (flower buds)-III SecA . . . . T . A A C . . . . . T . A T T C T . . C T . T . T A . .
Elm witches broom (ULW)-V SecA . . . . . A . A . . . . . T . G T G G C A . T A . . T A T . . . . .
Stolbur (Stol)-XII SecA . . . . T . A A C . . . . . T . A T T C T . . C T . T . T G . .
Peach X disease-III-A SecA . . . . T . A A C . . . . . T . A T T C T . . C T . T . T G . .
Aster Yellows-I-L SecA A . . . . A C . T . G T G C . T C T T T G T . . . A . T T C . T A G . T . C .
Faba Bean Phyllody-II SecA T A . . . . . A . . . . . T . G . . G T A . . A . . T . T . T . . .
Rehmannia glutinosa-I-B SecA A . . . . A C . T . G T G C . T C T T T G T . . . A . T T C . T A G . T . C .
Rehmannia glutinosa2-I-B SecA A . . . . A C . T . G T G C . T C T T T G T . . . A . T T C . T A G . T . C .
Nigerian 2nd AWKA-XXII secA T T . T . . G A . . . . . T . G . C C T C . . A . . T G T A A . . .
Tanzania box-KY4 Kenya 4 method 1-XXX . . . . . G . . A . . T . C . . . . . .
NIG 5th 1 in 5 dilution-XXII secA T . . T . . G A . . . . . T . G . C C T C . . A . . T G T A A . . .

Tanzania box-BUS 2nd 1 in 2 dilution-XXII	T . . T . . G A . . . . T . G . C C T C . . A . . T G T A A . . .
Ghana 6-XXII SecA	T T . T . . G A . . . . T . G . C C T C . . A . . T G T A A . . .
Chambezi TLD MYD1-XXX secA	G A T G C A C A A A G A C A A A G A T T A C T T A G T T G A T T A C A A A G A
Cornwall 1-XII secA	. . . . . . T . . . A . T . . . . T . . G . . G . . . C . A . . - - -
GH8-XXII secA	T . . . G C A C G T A . T . . . . T . . . . . A . . A A . A G . - - -
GH8 (aliquot of 54)-XXII SecA	T . . . G C A C G T A . T . . . . T . . . . . A . . A A . A G . - - -
C. p. fragariae (Jersey)-XII SecA	C . . C A . T T . . A . T . G . A -
tolbur (Ex-Jones)-XII secA	. . . . . . T . . . A . T . . . . . C . . T . . . . . A . . . A . T . . - - -
Poinsettia branching (flower buds)-III SecA	T G . . G . A . . . A . T . . . . T . . . . . G . . . A . A G G - - -
Elm witches broom (ULW)-V SecA	T . . . A . T . . . A . T . . . . T . . . . . A . . . A . T . . - - -
Stolbur (Stol)-XII SecA	T . . . G . A . . . A . T . . . . T . . . . . G . . . A . A G G - - -
Peach X disease-III-A SecA	T . . . G . A . . . A . T . . . . T . . . . . G . . . A . A G G - - -
Aster Yellows-I-L SecA	C . . C A . T T . . A . T . G . . T C . . . . . C A . C A . C A . T . G C A T
Faba Bean Phyllody-II SecA	T . . . G . A . . . . T . . . T . A C . A A . T G . - - -
Rehmannia glutinosa-I-B SecA	C . . C A . T T . . A . T . G . . T C . . . . . C A . C A . C A . T . G C A T
Rehmannia glutinosa2-I-B SecA	- -
Nigerian 2nd AWKA-XXII secA	T . . . G C A C G T A . T . . . . T . . . . . G . . A A C A G . - - -
Tanzania box-KY4 Kenya 4 method 1-XXX	. . . . C . . . .
NIG 5th 1 in 5 dilution-XXII secA	T . . . G C A C G T A . T . . . . T . . . . . A . . A A . A G . - - -
Tanzania box-BUS 2nd 1 in 2 dilution-XXII	T . . . G C A C G T A . T . . . . T . . . . . A . . A A . A G . - - -
Ghana 6-XXII SecA	T . . . G C A C G T A . T . . . . T . . . . . G . . A A C A G . - - -
Chambezi TLD MYD1-XXX secA	T T T G C C A G G A C G C C A T T T -
Cornwall 1-XII secA	. . . A A A G . . . . A G . T . . -
GH8-XXII secA	. . . A . A T . . . . . A T T A -
GH8 (aliquot of 54)-XXII SecA	. . . A . A T . . . . -
C. p. fragariae (Jersey)-XII SecA	- -
tolbur (Ex-Jones)-XII secA	. . . A A G . . . . . A . . T . -
Poinsettia branching (flower buds)-III SecA	. . . A A A T . . . . A . . -
Elm witches broom (ULW)-V SecA	. . . A . A . . . . A -
Stolbur (Stol)-XII SecA	. . . A A A T . . . . C . . -
Peach X disease-III-A SecA	. . . A A A T . . . . C . -
Aster Yellows-I-L SecA	. . . C . A T . T T G T . T C . . A A A T A A T C A C A C C C T - - - - - - -
Faba Bean Phyllody-II SecA	. . . . A T T . . . . A . . -

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Rehmannia glutinosa-I-B SecA                    . . . C . A T . T T G T . T C . . A A A T A A T C A A A C C C T A A T T C A C
Rehmannia glutinosa2-I-B SecA                   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Nigerian 2nd AWKA-XXII secA                     . . . A . A T . . . . A . . T - - - - - - - - - - - - - - - - - - - - - - -
Tanzania box-KY4 Kenya 4 method 1-XXX           - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
NIG 5th 1 in 5 dilution-XXII secA               . . . A . A T . . . . A . . T . - - - - - - - - - - - - - - - - - - - - - -
Tanzania box-BUS 2nd 1 in 2 dilution-XXII       . . . A . A T . . . . . . T T . - - - - - - - - - - - - - - - - - - - - - -
Ghana 6-XXII SecA                               . . . A . A T . . . . . . . . A . . - - - - - - - - - - - - - - - - - - - -

Chambezi TLD MYD1-XXX secA                      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Cornwall 1-XII secA                             - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
GH8-XXII secA                                   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
GH8 (aliquot of 54)-XXII SecA                   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
C. p. fragariae (Jersey)-XII SecA               - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
tolbur (Ex-Jones)-XII secA                      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Poinsettia branching (flower buds)-III SecA     - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Elm witches broom (ULW)-V SecA                  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Stolbur (Stol)-XII SecA                         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Peach X disease-III-A SecA                      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Aster Yellows-I-L SecA                          - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Faba Bean Phyllody-II SecA                      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Rehmannia glutinosa-I-B SecA                    G T T T T T G T G C G T G G T C T T T G T C T T T G G T A T T A A G T
Rehmannia glutinosa2-I-B SecA                   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Nigerian 2nd AWKA-XXII secA                     - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Tanzania box-KY4 Kenya 4 method 1-XXX           - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
NIG 5th 1 in 5 dilution-XXII secA               - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Tanzania box-BUS 2nd 1 in 2 dilution-XXII       - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Ghana 6-XXII SecA                               - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
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- Sequences alignment of TUF gene that was checked samples of box 1.

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Aster Yellows Dwarf-I tuf                       - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Aster Yellows Severe-I tuf                      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
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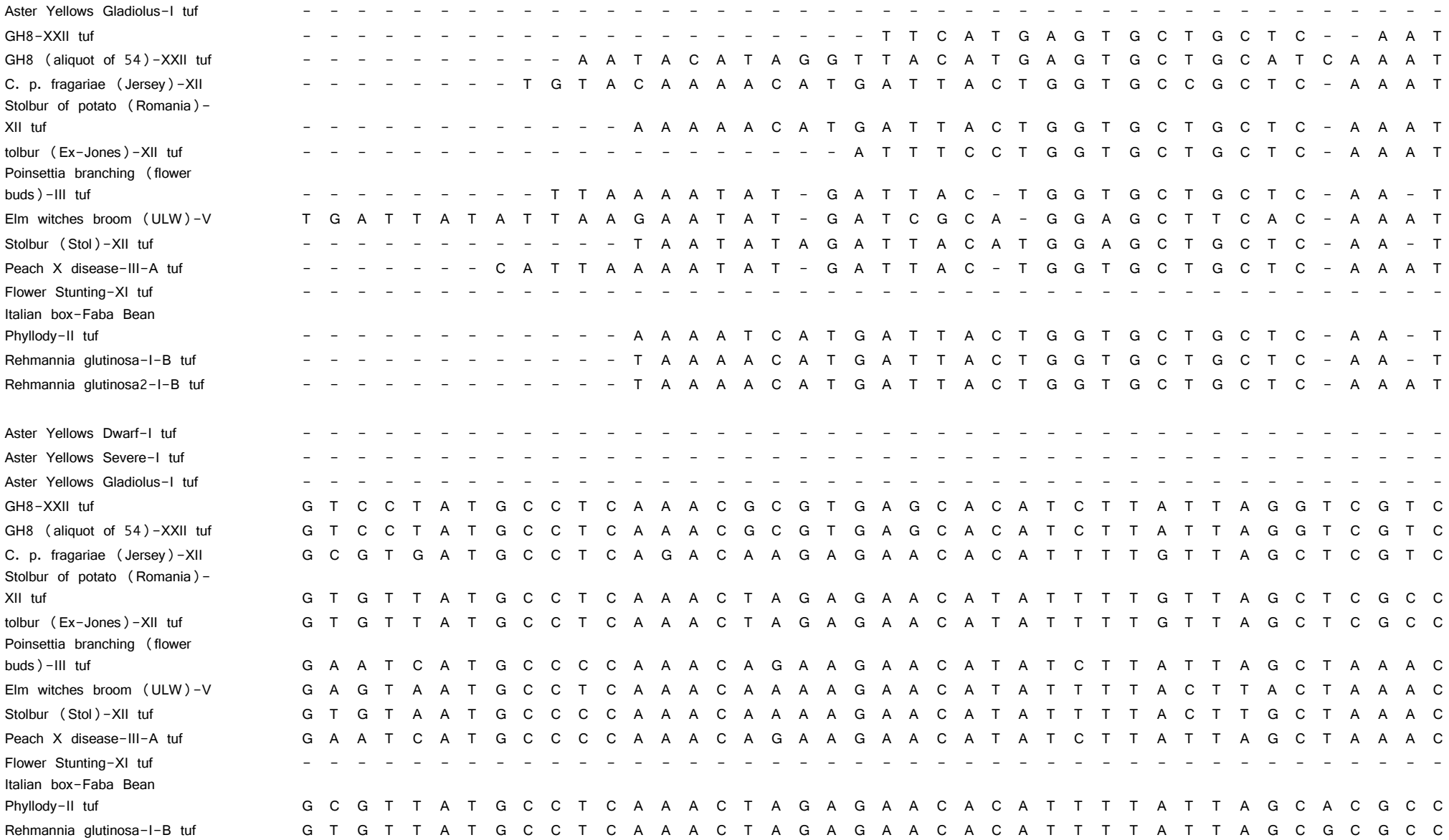
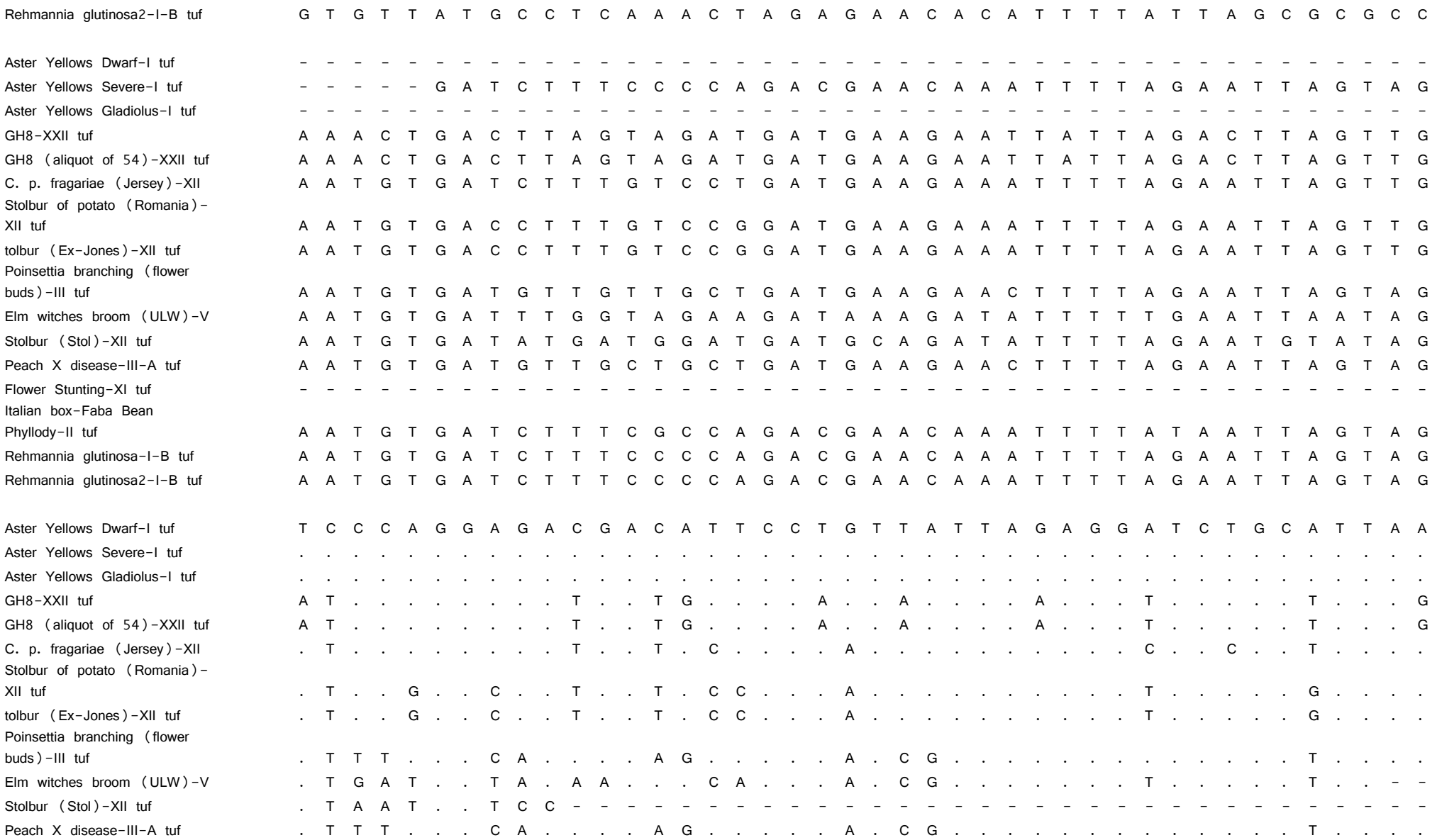

Aster Yellows Gladiolus-I tuf
GH8-XXII tuf
GH8 (aliquot of 54)-XXII tuf
C. p. fragariae (Jersey)-XII
Stolbur of potato (Romania)-XII tuf
tolbur (Ex-Jones)-XII tuf
Poinsettia branching (flower buds)-III tuf
Elm witches broom (ULW)-V
Stolbur (Stol)-XII tuf
Peach X disease-III-A tuf
Flower Stunting-XI tuf
Italian box-Faba Bean Phyllody-II tuf
Rehmannia glutinosa-I-B tuf
Rehmannia glutinosa2-I-B tuf
Aster Yellows Dwarf-I tuf
Aster Yellows Severe-I tuf
Aster Yellows Gladiolus-I tuf
GH8-XXII tuf
GH8 (aliquot of 54)-XXII tuf
C. p. fragariae (Jersey)-XII
Stolbur of potato (Romania)-XII tuf
tolbur (Ex-Jones)-XII tuf
Poinsettia branching (flower buds)-III tuf
Elm witches broom (ULW)-V
Stolbur (Stol)-XII tuf
Peach X disease-III-A tuf
Flower Stunting-XI tuf
Italian box-Faba Bean Phyllody-II tuf
Rehmannia glutinosa-I-B tuf


Rehmannia glutinosa2-I-B tuf G T G T T A T G C C T C A A A C T A G A G A A C A C A T T T T A T T A G C G C G C C
Aster Yellows Dwarf-I tuf -
Aster Yellows Severe-I tuf - - - - - G A T C T T T C C C C A G A C G A A C A A A T T T T A G A A T T A G T A G
Aster Yellows Gladiolus-I tuf -
GH8-XXII tuf A A A C T G A C T T A G T A G A T G A T G A A G A A T T A T T A G A C T T A G T T G
GH8 (aliquot of 54)-XXII tuf A A A C T G A C T T A G T A G A T G A T G A A G A A T T A T T A G A C T T A G T T G
C. p. fragariae (Jersey)-XII A A T G T G A T C T T T G T C C T G A T G A A G A A A T T T T A G A A T T A G T T G
Stolbur of potato (Romania)-XII tuf A A T G T G A C C T T T G T C C G G A T G A A G A A A T T T T A G A A T T A G T T G
tolbur (Ex-Jones)-XII tuf A A T G T G A C C T T T G T C C G G A T G A A G A A A T T T T A G A A T T A G T T G
Poinsettia branching (flower buds)-III tuf A A T G T G A T G T T G T T G C T G A T G A A G A A C T T T T A G A A T T A G T A G
Elm witches broom (ULW)-V A A T G T G A T T T G G T A G A A G A T A A A G A T A T T T T T G A A T T A A T A G
Stolbur (Stol)-XII tuf A A T G T G A T A T G A T G G A T G A T G C A G A T A T T T T A G A A T G T A T A G
Peach X disease-III-A tuf A A T G T G A T G T T G C T G C T G A T G A A G A A C T T T T A G A A T T A G T A G
Flower Stunting-XI tuf -
Italian box-Faba Bean Phyllody-II tuf A A T G T G A T C T T T C G C C A G A C G A A C A A A T T T T A T A A T T A G T A G
Rehmannia glutinosa-I-B tuf A A T G T G A T C T T T C C C C A G A C G A A C A A A T T T T A G A A T T A G T A G
Rehmannia glutinosa2-I-B tuf A A T G T G A T C T T T C C C C A G A C G A A C A A A T T T T A G A A T T A G T A G
Aster Yellows Dwarf-I tuf T C C C A G G A G A C G A C A T T C C T G T T A T T A G A G G A T C T G C A T T A A
Aster Yellows Severe-I tuf . . . .
Aster Yellows Gladiolus-I tuf . . . .
GH8-XXII tuf A T . . . . . T . . T G . . . . A . . A . . . . A . . . T . . . . . T . . . G
GH8 (aliquot of 54)-XXII tuf A T . . . . . T . . T G . . . . A . . A . . . . A . . . T . . . . . T . . . G
C. p. fragariae (Jersey)-XII . T . . . . . T . . T . C . . . . A . . . . . C . . C . . T . . . .
Stolbur of potato (Romania)-XII tuf . T . . G . . C . . T . . T . C C . . . A . . . . . T . . . . . G . . . .
tolbur (Ex-Jones)-XII tuf . T . . G . . C . . T . . T . C C . . . A . . . . . T . . . . . G . . . .
Poinsettia branching (flower buds)-III tuf . T T T . . . C A . . . . A G . . . . . A . C G . . . . . T . . . .
Elm witches broom (ULW)-V . T G A T . . T A . A A . . . C A . . . A . C G . . . . . T . . . . . T . . - -
Stolbur (Stol)-XII tuf . T A A T . . T C C -
Peach X disease-III-A tuf . T T T . . . C A . . . . A G . . . . . A . C G . . . . . T . . . .

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Flower Stunting-XI tuf                  . . . . . T . . A . . . . . .
Italian box-Faba Bean
Phyllody-II tuf                         . . . . . T . . T . . . . . .
Rehmannia glutinosa-I-B tuf             . . . . . .
Rehmannia glutinosa2-I-B tuf            . . . . . .

Aster Yellows Dwarf-I tuf               T A A A C G A A T T A A T C G A A A C T C T A G A C A C C T A C A T T G A A G A C C
Aster Yellows Severe-I tuf              . . . . . .
Aster Yellows Gladiolus-I tuf           . . . . . .
GH8-XXII tuf                            . T . T G . . . . . G . . T G . . G . T . . T . A . . . . . C C . A T . .
GH8 (aliquot of 54)-XXII tuf            . T . T G . . . . . G . . T G . . G . T . . T . A . . . . . C C . A T . .
C. p. fragariae (Jersey)-XII            . T . . T . . . . . T A . . . . T . . . . T T . T . . . . . C . . . . . T .
Stolbur of potato (Romania)-
XII tuf                                 . T . . . . . A . C G . . T . . . . T T . T . . . . . T .
tolbur (Ex-Jones)-XII tuf               . T . . . . . A . C G . . T . . . . T T . T . . . . . T .
Poinsettia branching (flower
buds)-III tuf                           . C C . A . . . . . C . T . . C T T A T . . . . . T . . . G . G . . . . . .
Elm witches broom (ULW)-V               . . . . A . . T . . . C . T . . T . . . T . . . . . . A . . T G . . . . . T .
Stolbur (Stol)-XII tuf                  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Peach X disease-III-A tuf               . C C . A . . . . . C . T . . C T T A T . . . . . T . . . G . G . . . . . .
Flower Stunting-XI tuf                  . . . . T . . . . . .
Italian box-Faba Bean
Phyllody-II tuf                         . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . .
Rehmannia glutinosa-I-B tuf             . . . . . .
Rehmannia glutinosa2-I-B tuf            . . . . . .

Aster Yellows Dwarf-I tuf               C A A A - -
Aster Yellows Severe-I tuf              T G . - - -
Aster Yellows Gladiolus-I tuf           T G . - - -
GH8-XXII tuf                            T G C . - -
GH8 (aliquot of 54)-XXII tuf            A G . . A -
C. p. fragariae (Jersey)-XII            T G . . - -
Stolbur of potato (Romania)-
XII tuf                                 A G . . - -
tolbur (Ex-Jones)-XII tuf               T G . - - -
Poinsettia branching (flower            T G . . - -
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buds)-III tuf

Elm witches broom (ULW)-V	T	G	G	.	-	-
Stolbur (Stol)-XII tuf	-	-	-	-	-	-
Peach X disease-III-A tuf	A	G	G	.	-	-
Flower Stunting-XI tuf	T	C	.	-	-	-
Italian box-Faba Bean						
Phyllody-II tuf	T	T	G	.	A	A
Rehmannia glutinosa-I-B tuf	A	.	G	.	-	-
Rehmannia glutinosa2-I-B tuf	A	.	.	.	-	-