# Towards the diagnosis of two intracellular pathogens of grapevine in South Africa

By

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Submitted in fulfilment of the requirements of the degree

**MAGISTER SCIENTIAE**

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# DECLARATION

I declare that the dissertation/thesis, which I hereby submit for the degree M.Sc (Microbiology) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature: _____

Date: _____

***"What the hell wine is THAT?"***

The other night, while eating Asian
Take-away, I had occasion
To try a cheeky little red -
"Pinot noir" I think it said.
This of course then got me thinking
Just what was it that I was drinking?
"Pinot noir", whilst sounding haughty,
Sounds maybe just a little naughty -
In parts of Asia, be construed
As maybe something rather rude.
The names of types and kinds of wine
Then rattled round my tiny mind.
And actually, I'm rather keen
To find out what they really mean.
I did some drinking, called 'research' -
I nearly even went to church.
I looked up books - to no avail.
The CD's are another tale...
My senses steeled, my mind was set
I surfed and trawled the Internet.
The list is not exhaustive, true,
But what I've found I'll share with you.
It certainly, most have agreed,
Makes for an interesting read.
**Burgundy** is named in honour
Of the thirteenth century's famous scholar.
**Malbec**, it seems was wont to dance
With the son of the cousin of the King of France.
**Merlot** too, he loved to sing -
A special favourite of the King.
In the Louvre he had his quarters,
He bonked the Queen and all her daughters.

**Shiraz**, a middle-English word,
Its meaning now is seldom heard.
Amidst the laughter, hale and hearty,
Put very simply, means "Let's Party!"
The Duke of **Cabernet**, it's true,
Knew how to drink a thing or two.
His parties always had pizzazz -
Nicknamed the 'Cabernet Shiraz'.
It's said that **riesling** really means
What Germans do without their jeans.
Those suffering from **Durif** will know
The ins and outs of feeling low.
The wine was named, among the wattles
By one who suffered several bottles.
The Spanish writer, El **Tarrango**
Was fascinated by the Tango.
His fondness for the grape was famed,
So after him a wine was named.
**Chardonnay**, I've heard them say,
Will make it really big some day.
If only everybody knew
That 'Chardonnay' means 'wombat poo'.
**Moselle** was named, so I believe,
On a catwalk last year's New years Eve.
The Body moseyed down the plank,
The judges checked the wine they drank.
While all were charged with hormones fearsome,
They named it after Elle Macpherson.
The **Grenache** soldiers, it is said,
Really liked a drop of red.
So President, young Charles de Gaulle
Bought them wine - they had a ball!
A house of ill-repute in Spain,
Hatched a plan, though quite insane,

To knock their noisy patrons out
With several wines, while lights were out.
The wine they chose, it wasn't yellow, but
A vivid green they called **Verdelho**.
(I'd like to note I'm well-intentioned,
'Bordello' wasn't even mentioned.

...D'OH!)
Hubble, rubble, toil and trouble,
**Champagne** always likes to bubble.
Hangover cure is always tricky -
Most attempt the 'sham pain' sickie.
Well there they are. They may astound,
Though these I swear I really found. But
As Bill Gates said to the pleb
"Who believes the World Wide Web?"

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  ("Red red wine…")

# SUMMARY

## Towards the diagnosis of two intracellular pathogens of grapevine in South Africa

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A survey was conducted, from 2001 to 2004, of viruses spreading within certified grapevine material in South Africa. As far as possible, viruses were identified and detection methods established. However, unknown spherical virus-like particles were observed in samples that also contained Grapevine Leafroll Associated Virus-Type 3. The unknown spherical particles were thought to most likely be Grapevine Fleck Virus, which was previously found in South Africa.

A PCR method to be used locally for the routine detection of Grapevine Fleck Virus was established and first used to determine whether any of the greenhouse and field samples with the unknown spherical viruses were infected with Grapevine Fleck Virus.

During the 2001 to 2004 survey, plants with leafroll and reddening symptoms unlike classical grapevine leafroll disease were also observed. No grapevine

leafroll-associated viruses could be detected in these, but the symptoms observed resembled symptoms induced by phytoplasmas in Europe.

A PCR method for the routine universal detection of phytoplasmas was established and this method was used to determine if phytoplasmas were associated with the symptomatic plants found. Sequence information from PCR amplicons suggest the presence of *Candidatus* phytoplasma solani, found for the first time in South Africa. This important finding however requires conformation by a second laboratory.

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# ABBREVIATIONS

| | |
|---|---|
| ℃ | Degrees Celsius |
| bp | Basepairs |
| cDNA | Complementary DNA |
| CTAB | Cethyl-trimethyl-ammonium bromide |
| DEPC | Diethylpyrocarbonate |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxyribonucleotide triphosphate |
| ds | Double stranded |
| ELISA | Enzyme Linked Immunosorbent Assay |
| FD | Flavescence doree |
| g | Gram |
| GFkV | Grapevine Fleck Virus |
| GFLV | Grapevine Fanleaf Virus |
| GLRaV-3 | Grapevine Leafroll Associated Virus Type 3 |
| IPTG | Isopropyl β-D-thiogalactosidase |
| ISEM | Immunosorbent Electron Microscopy |
| kb | Kilobase pairs |
| kDa | KiloDalton |
| LB | Luria-Bertani |
| M | Molar |
| min | Minutes |
| mm | Millimetre |
| mM | Millimolar |
| M-MLV | Moloney-Murine Leukaemia Virus |
| MTR | Methyltransferase |
| µg | Microgram |
| µl | Microliter |
| µm | Micrometer |
| nm | Nanometer |
| nt | Nucleotides |
| ORF | Open Reading Frame |

| | |
|---|---|
| PCR | Polymerase Chain Reaction |
| pmol | Picomol |
| pNPP | para-Nitrophenylphospate |
| PPRI | Plant Protection Research Institute |
| PVP | Polyvinylpyrrolidone |
| RdRp | RNA dependent RNA polymerase |
| RNA | Ribonucleic acid |
| rpm | Revolutions per minute |
| sec | Seconds |
| Spp | Species |
| STOL | Stolbur |
| TNA | Total nucleic acid |
| U/µl | Units per microliter |
| UV | Ultraviolet |
| V | Volt |
| W/v | Weight per volume |
| xg | Gravitation force |
| X-gal | 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside |

# Chapter 1

# Review of Literature

# PART I: CERTIFICATION, DIAGNOSTICS AND VIRUS ELIMINATION OF GRAPEVINE

## 1.1 GENERAL INTRODUCTION

The art of wine making is one of the oldest industries known to civilization. Vineyards existed for the use of Egypt's rulers and nobles in the pre-dynastic and early dynastic periods (~3200 BC).

Reviewed in Kruger (2000) this industry was established in 1652 in South Africa, shortly after the arrival of the Dutch at the Cape of Storms. After 300 years of wine making, the average yield of South African wines in the 1960's was amongst the lowest in the world, and the low yield was accompanied with low quality wines. It is likely that virus and virus combinations were responsible for the low production and quality of wine in South Africa during the 1960's (Kriel, 2000).

According to The American Phytopathological Society (1998), most plant viral infections are latent but cause huge economic losses worldwide. Virus infections cause the plants to grow slower, influence the fruit production and in due course the production time of a crop. As viral infections do not always induce visible symptoms, this dramatic effect could only be noticed once an infected crop was compared with a healthy crop (Martelli and Walter, 1998). The "Koöperatieve Wijnbouwers Verening van Zuid-Afrika Bpkt. (KWV)" realized if they want to improve their wine – they first have to improve the condition of their vineyards (Kriel, 2000).

In 1986 KWV initiated the formation of the Vine Improvement Association (VIA) to improve the quality of South African vineyards, and it is still the official propagation organization in the Government certification scheme for wine grapes (Kriel, 2000).

According to the South African Wine Industry Statistics (SAWIS) nr.29 (2005), South Africa comprises of 124 749-hectare area under vines, has 4406 primary wine producers and 561 wine cellars. South Africa is also ranked 9th in the world regarding wine production and is responsible for 2.7% of the total world wine production. Figure 1.1 is a schematic representation of the South African wine regions.

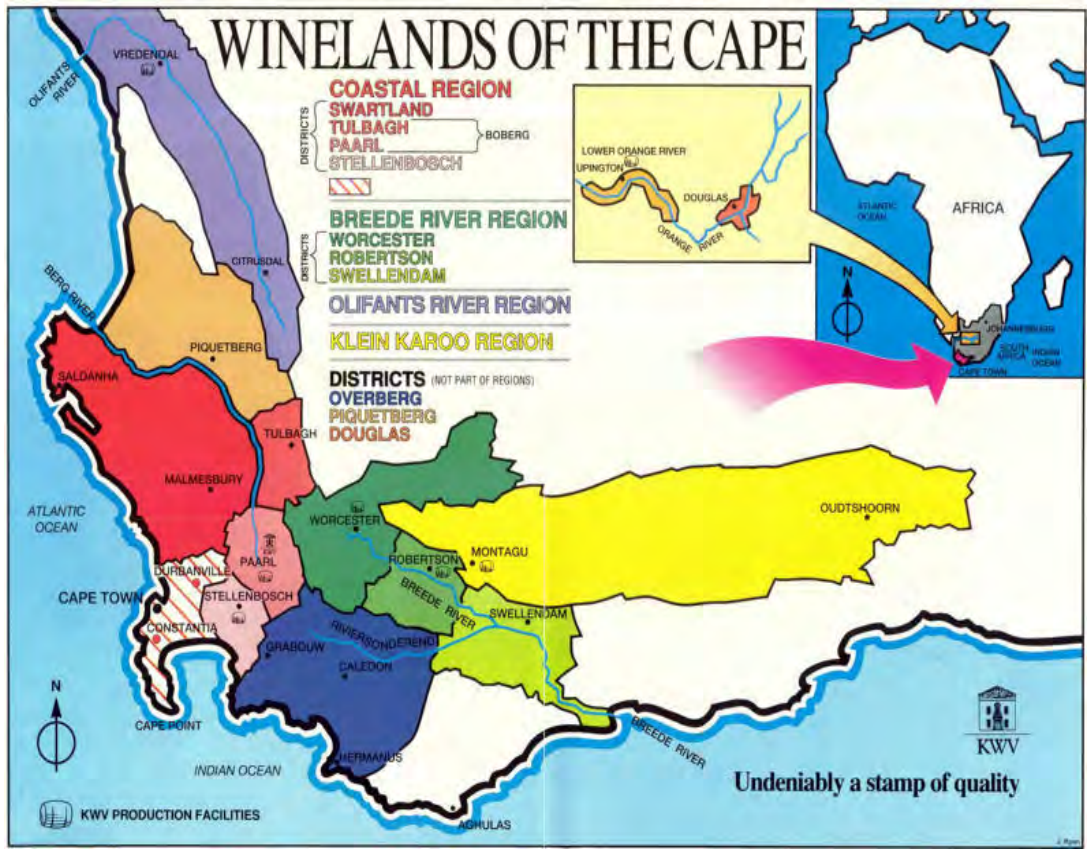


*Figure 1.1: Map of South African wine regions (Image: KWV)*

# 1.2 CERTIFICATION SCHEME OF GRAPEVINES WORLDWIDE

## 1.2.1 Introduction

Certification is the procedure where candidate mother plants, that are going to be used as a source of propagation material, are subjected to treatments to protect trueness-to-type and absence from any number of pathogens, as specified by certain regulations, which differs from country to country (Martelli and Walter, 1998). However, little has been done to promote internationally recognized certification schemes that would allow free trading of high-quality nursery materials among participating countries (Rowhani *et al*., 2005).

Within specific countries within the modern era there has been a substantial increase in demand for voluntary certification. For this reason, voluntary certification has become widespread. This kind of certification is closely controlled by regulations issued by a certifying authority and is not imposed forcefully (Martelli and Walter, 1998).

Compulsory certification is imposed whenever it becomes essential to prevent the distribution of destructive diseases. There are no restrictions to the kind and number of pathogens that may be considered for elimination in a certification program (Martelli and Walter, 1998).

Certification is a long-lasting effort to attain the desired health level of a crop and to maintain this level through time. Availability of technology for the reliable detection of diseases and their agents and the effective elimination of disease and pathogens are required to implement a certification program (Martelli and Walter, 1998).

Clonal selection is done in selected vineyards with desirable characteristics. Selection is done based on varietal conformity, vegetative vigor, bud fertility, quality and quantity of the field, timing and uniformity of ripening. Vines are

kept under observation for a few years and the best performing and least infected ones are chosen as candidate clones (Martelli and Walter, 1998).

Various virus elimination procedures are currently available for selected clones. Dormant canes are treated with hot water, 50°C, for 45 minutes to eliminate prokaryotes (Hot water treatment). Vegetating vines are grown at a constant temperature of 36-37°C for 100 days (Figure 1.2), where after excision and rooting of shoot tips take place to eliminate virus and virus-like diseases (Hot air treatment) (Martelli and Walter, 1998).

*Figure 1.2: Example of plants in the heat treatment chamber (Photo: L. Nel)*

*In vitro* culture is often used in conjunction with hot air treatment (Martelli and Walter, 1998). *In vitro* culture involves the excision of the upper most part of the meristem (0.24mm). The meristem is placed on sterile agar and left to develop a root system. The principle behind the combined methods is that virus replication is suppressed at 36-38°C while the plant grows optimally and theoretically the upper part of the meristem is virus-free. Micrografting of meristem tips are also done onto *in vitro*-grown seedlings (Martelli and Walter, 1998). Once the *in vitro* cultures are large enough, the plants are transferred to greenhouses (Figure 1.3). From there the plants are transferred to containers and placed in nuclear blocks (Figure 1.4).

*Figure 1.3: Greenhouse with small grapevine plants (Photo: L. Nel)*

From the nuclear block the grapevines are planted into small foundation blocks, which are strictly controlled open blocks. Because of mass-propagation, the grapevines are planted from the foundation blocks to bigger, less controlled mother blocks.

*Figure 1.4: Nuclear blocks at KWV, Paarl (Photo: L. Nel)*

## 1.2.2 South Africa

The South African Plant Certification scheme for wine grapes (Schedule 1, Article 10) demands specific requirements for the three different units of propagation (Nuclear, Foundation and Mother).

Nuclear units

Nuclear units must be covered in an insect proof greenhouse. The plants in nuclear units must be established in containers, where the soil is free from Grapevine Fanleaf Virus (GFLV) vectors. The floor surface of the greenhouse containing nuclear plants must be covered in such a way that the roots of the plants in the containers cannot penetrate the soil on which the greenhouse was build. The greenhouse must be isolated through means of an isolation area at least two meters wide of other vineyards and virgin soil. No plants, of any kind, may be planted in such an isolation area, unless the authority gives written consent. Irrigation water being used in nuclear units must be free of GFLV vectors and may not be contaminated with drainage- or runoff water from other vineyards.

Foundation units

Foundation units must be isolated through means of an isolation area. If the foundation unit is situated in a greenhouse, the isolation area must be at least one meter wide, otherwise if the foundation unit is situated in the field the isolation area must be twenty-five meters wide. No plants, of any kind, may be planted within five meters of the foundation unit, unless the authority gives written consent.

Mother units

Mother units must be isolated through means of an isolation area. If the mother unit is situated in a greenhouse, the isolation area must be at least one meter wide, otherwise if the mother unit is situated in the field the isolation area must be three meters wide. If the mother unit is not in a greenhouse, it must be at least three hectares in size, unless the authority stipulates otherwise.

Plant material from the nuclear blocks are tested on a yearly basis, with virus specific tests, for pathogens that might be present in the grapevines. Plant material from the foundation blocks are also tested on a regular basis for pathogens. Plant material from the bigger mother blocks are not tested with virus specific tests, but inspectors monitor the blocks for any symptoms of known pathogens.

In addition, certain phytosanitary requirements are also prescribed by the South African Plant Certification scheme for wine grapes (Schedule 2, Article 11(1) (b) (i), 3(a), 12(b)).

Establishment and certification requirements

Plant material that has been established in a unit must comply with certain requirements during the registration term and the presentation of the plants for certification. Plant material and plants of rootstock varieties must be free from Grapevine Fanleaf, Grapevine Fleck, Grapevine Leafroll, Grapevine Corky Bark, Grapevine Stem grooving and Shiraz-disease. The plant material and plants of rootstock varieties must also be visually free from *Agrobacterium radiobacter* pv *tumefaciens, Pythium spp, Phytophthora spp* and *Xylophilus ampelinus.* For the presentation of the plants for certification, the plant material and plants of rootstock varieties must also be visually free from *Margarodes spp, Meloidogyne spp, Pseudococcus spp* and *Viteus vitifoliae.* Plant material and plants of the scions must comply with the requirements as stipulated by the authorities, as far as the diseases and pathogens mentioned for the establishment and certification requirements for rootstocks are concerned.

### 1.2.3 Europe

In European Union (EU) countries with active viticulture, the incidence of virus and virus-like diseases is high and their spread has been rapid due to the uncontrolled distribution and use of infected cultivar and rootstocks, that took place in the post-war period (Martelli and Walter, 1998).

The alarming sanitary deterioration of grapevines encouraged the EU Council to issue directives for the improvement of the Union's grapevine industry in 1968 and 1971 (Martelli and Walter, 1998). The grapevine is the only woody crop that, since 1968, is the objective of a compulsory certification by the EU (Directive 68/93 EEC) (Rowhani *et al*., 2005). This regulation prescribes only the absence of harmful virus diseases, notably fanleaf and leafroll from nursery material (Martelli and Walter, 1998). Although some EU member states (France, Germany, Italy, Portugal and Spain) have implemented certification schemes with sanitary requirements that are more restrictive than those of the existing Directive, and use the same virus detection, national protocols are still far apart. It is unlikely that the production with such a low sanitary status would be acceptable to any viticultural country aware of and concerned with the serious virological problems associated with this crop. A recently issued Directive (2002/11 EU) is supposed to harmonize the system (Rowhani *et al*., 2005). The technical annex to the 2005 Directive complied that the lowest possible level of harmful organisms required the absence of Grapevine fanleaf virus, Arabis mosaic virus, Grapevine leafroll-associated virus 1, Grapevine leafroll-associated virus 3 and for rootstocks only Grapevine fleck virus (Martelli, 2006). The European certification schemes are required to operate under European and Mediterranean Plant Protection Organization (EPPO) guidelines. These include the Italian schemes at the University of Bari, International Council for the Study of Viruses and Virus Diseases of the Grapevine (ICVG), Mediterranean Agronomic Institute and the French certification scheme, Institut National de la Recherche Agronomique (INRA) (Constable and Drew, 2004).

### 1.2.4 America and Canada

The Canadian certification scheme is required to operate under the Canadian Food Inspection Agency (CFIA), while the American certification scheme (FSP) is supported by the US Department of Agriculture (USDA), the California Department of Food and Agriculture (CDFA) and the University of California, Davis. Canada and America comply with the North American Plant Protection Organization (NAPPO) guidelines for regional risk management

regarding entry, establishment and spread of regulated pathogens (Constable and Drew, 2004). In addition, NAPPO participates with other regional plant protection groups within the western hemisphere and globally to develop international standards (Rowhani *et al*., 2005). The document "Guidelines for the Importation of Grapevines into a NAPPO Member Country RSPM #15 Part 1: Viruses and Virus-like Pests, Viroids, Phytoplasmas and Bacteria" is the initial regional guideline for the development of harmonized North American Standards for grapevine nursery stock (Rowhani *et al*., 2005). Canada has a formal national certification program, which is voluntary. The United States operates under voluntary state certification, which combined with strict quarantine regulations have resulted in high-quality nursery stock with a minimum of regulatory infrastructure (Rowhani *et al*., 2005).

## 1.3 DIAGNOSTIC TESTS USED FOR PLANT PATHOGENS

A common objective shared by certification schemes worldwide is to identify healthy sources for propagation through the application of time-tested indexing procedures as well as more recently developed molecular assays (Rowhani, *et al.,* 2005). Detection methods in the laboratory are a valuable tool for investigating grapevine pathogens, since diagnosis of grapevine diseases in the field can be inaccurate. Symptoms displayed in the field are seldom exclusive to a particular disease and some infected vines may not show any symptoms at all. This can either be due to low concentration of the disease-causing agent or the infection may be in cultivars that are tolerant to the specific disease-causing agent (Weber *et al*., 2002).

In addition, some of the infected grapevines only display seasonal symptoms and it is therefore necessary to have detection methods to test vines even if they appear to be healthy. This is very important where material for

propagation is collected and prepared during the dormant season (Weber *et al.*, 2002).

Various detection methods are available, each with its own advantages and disadvantages.

### 1.3.1 Biological methods

Biological methods are time consuming and require large greenhouse and field space, but are very useful if the vines tested are valuable and a high level of confidence is needed in the diagnosis (Weber *et al.*, 2002).

Biological indexing

Two different groups of indicator plants are used during biological indexing.

The first group of plants are herbaceous, maintained in greenhouses and used to detect mechanically transmissible viruses. These tests can be completed in few weeks time. If the virus transmission is successful, the indicator plants may develop primary symptoms, including localized lesions, after a few days of incubation. Thereafter systemic symptoms appear, including vein clearing and leaf deformation (Rowhani *et al.*, 2005; Martelli, 1993).

The second group of plants are woody and requires a lengthier incubation period, up to three years. Inoculation is either done by cleft grafting, chip-bud grafting, bench grafting or green grafting (Martelli and Walter, 1998). These woody incubator plants usually belong to the same genera as the plant under evaluation and are selected based on the specific diagnostic symptoms it elicits. The indicator plants are inspected annually for two to three seasons for any visible symptoms (Rowhani *et al.*, 2005). This technique has been used for detection and identification of various plant pathogens (Rowhani *et al.*, 1997; Credi, 1997; Habili *et al.*, 1992).

### 1.3.2 Serological methods

Serological methods are rapid, inexpensive, very specific and a cost-effective way for detecting viruses in woody plants (Weber *et al*., 2002). These methods cannot however be applied to unknown particles and lacks sensitivity to detect viruses present in low concentrations. (Weber *et al*., 2002).

Enzyme Linked Immunosorbent Assay (ELISA)

ELISA can be used to simultaneously detect various pathogens on using a single plate with different antibodies coated to each well, in triplicate for reproducibility. The major limitation of ELISA is the necessity for polyclonal or monoclonal antibody sera specific for each pathogen (Webster *et al*., 2004). ELISA utilizes antibody reactions with disease agents, like viruses and bacteria (Weber *et al*., 2002). In the final step of the test an enzyme and substrate are used to produce a colour reaction, which indicates the presence or absence of the virus in the sample (Weber *et al*., 2002). A modification of ELISA named voltametric enzyme immunoassay, detects the change in electrical conductivity of the substrate, rather than a color change, when acted upon by an enzyme attached to a secondary antibody. This method is claimed to be an order of magnitude more sensitive than ELISA (Webster *et al*., 2004). This technique has been used for detection and identification of various plant pathogens (Moris and Bertwick, 1996; Ling *et al*., 2000; Forsline *et al*., 1996).

Immunosorbent electron microscopy (ISEM)

ISEM is the trapping of virus particles onto grids, which have been coated with specific virus antibodies, and their subsequent observation under the electron microscope. This technique is costly due to the need for an electron microscope, the labor-intensive sample preparation and the visual identification required. The electron microscope plays a critical role in virus diagnostics because of its sensitivity and its ability to detect viruses in plants, when no antisera or other detection systems are available (Van der Merwe, 2001).

Tissue blot immunoassay (TIBA)

Tissue blotting utilizes antibodies raised against pathogens. Sap from the plant tissue is expressed onto blotting paper, nitrocellulose or nylon membranes and the pathogen is detected by labeled probes (Webster *et al*., 2004). The procedure is less labor intensive than ELISA, rapid, sensitive, simple because no virus extraction is required, inexpensive, suitable for surveys of 1000-2000 samples per day and the samples can be taken in the field and processed some time later (D'Onghia *et al*., 2001).

Quartz crystal microbalance (QCM) immunosensors

During this technique a quartz crystal disk is coated with virus specific antibodies. Voltage is applied across the disk, making the disk warp slightly via a piezoelectric effect. Adsorption of virus particles to the crystal surface changes its resonance oscillation frequency in a concentration-dependent manner (Eun *et al*., 2002). This method is therefore qualitative and quantitative. It is apparently as sensitive but more rapid than ELISA and economical (Webster *et al*., 2004).

### 1.3.3 Nucleic acid analysis

Nucleic acid analysis has higher sensitivity and specificity than the other methods. It can be applied to non-immunogenic pathogens and a disease with unknown etiology can be investigated. It can detect multiple viruses and detect plants with mild or even no symptoms. These methods are however quite expensive and specialized facilities are necessary. One of the methods most commonly used to explore molecular genetic identification of pathogens, is the PCR and modifications thereof.

Polymerase Chain Reaction (PCR)

PCR involves the selective amplification of a small part of a pathogen's genome (Weber *et al*., 2002). Some disadvantages of this technique are the insufficient viral genome sequences available to design primers (although the situation is improving as more information is submitted to

public domains like Genbank), the fact that some plant extracts contain inhibiting substances to PCR and the need for a thermocycler, which can be expensive (Webster *et al*., 2004). This technique is extremely sensitive, fairly inexpensive and requires minimal skill to perform (Weber *et al*., 2002). Both RNA and DNA can be detected and identified using this technique. For RNA viruses, a cDNA strand complementary to the virus is made with reverse transcriptase. Oligonucleotide primers, flaking part of the genome of the virus, are extended by a thermostable DNA polymerase in a series of denaturing and extension steps that exponentially increase the target DNA. For DNA viruses, no reverse transcription step is required. There are a number of variations on the basic technique, designed to increase sensitivity, alter specificity or allow automation of detection (Webster *et al*., 2004). Some of these are listed below:

Multiplex PCR

Multiple strains can be detected in a single reaction by combining oligonucleotide primers specific for different pathogens. The design of a multiplex procedure requires that the same PCR conditions amplify two different sized DNA fragments with balanced efficiency and no cross-reactivity (Clair *et al*., 2003). This technique has been used for detection and identification of various plant pathogens (Grieco and Gallitelli, 1999; Clair *et al*., 2003)

Real-time PCR (Fluorescence PCR using Taqman™ technology)

Two primers flank the sequence of interest and a third fluorescently labeled primer anneals between them. As the flanking primers extend, the labeled primer is released and fluorescence occurs. The advantages of this method are that no post-reaction processing is required to detect the reaction product and that it is quantitative (Webster *et al*., 2004). The disadvantage of using real-time PCR include the inability to

monitor amplicon size without opening the system, the incompatibility of some platforms with some fluorogenic chemistries, and the relative restricted multiplex capabilities of current applications (Mackay *et al*., 2002). Also, unless large-scale testing is envisioned, the cost of a Taqman™ ABI Prism 7700 Sequence Detection System and the labeled primers may be too expensive (Webster *et al*., 2004). This technique has been used for detection and identification of various plant pathogens (Roberts *et al*., 2000; Eun *et al*., 2000).

Competitive fluorescence PCR

This is a variation on real-time PCR. Using this method, virus and multiple virus infections can be differentiated simultaneously. A number of primer sets are each labeled with a different fluorescent marker and added to the reaction mixture. Virus strains are differentiated with primers that differ only at the 3' end, complementary to a nucleotide position that is polymorphic between strains. Only where the 3' nucleotide is complementary, extension will take place. Only primers that generate amplicons fluoresce and the wavelength emitted identifies the primers that have been extended (Walsh *et al*., 2001).

Immunocapture PCR

This technique combines the capture of the pathogen particles by antibodies together with amplification by PCR. The virus is adsorbed by the antibody, which is bound to a surface, then removed by heating with a non-ionic surfactant. The nucleic acids of the pathogen are then amplified (Harper *et al*., 1999). This method is useful in concentrating virus particles from plant species where virus titer is low or where compounds that inhibit PCR are present (Webster *et al*., 2004). This technique has been used for detection and identification of various plant pathogens (Chevalier *et al*., 1995; Nolasco *et al*., 1993).

Nested PCR

Two consecutive PCRs are carried out with the first reaction increasing the amount of template for the second reaction. The method is particularly useful where the pathogen is present in very low titer or inhibitors of DNA polymerase are present in the plant extract. Low–specificity oligonucleotides, usually degenerate, are used in the first rounds of amplification. An aliquot of the first reaction is then placed in a fresh tube for a second PCR with specific primers that anneal within the first amplicon (Webster *et al*., 2004). This technique has been used for detection and identification of various plant pathogens (Dovas and Katis, 2003; Bertaccini *et al*., 1999).

Restriction fragment length polymorphism (RFLP)

RFLP describes the patterns of different sizes of DNA that results from cutting the PCR amplicons with restriction enzymes. RFLP is used to identify differences between viruses based on the presence or absence of restriction enzyme-recognition sites. RFLP is a method for identifying pathogens after PCR. After amplification, the amplicon is digested with restriction enzymes and the fragment sizes analyzed by gel electrophoresis (Webster *et al*., 2004). RFLP has been used routinely for identification of phytoplasmas (Schneider *et al*., 1997; Gibb *et al*., 1995; Martini and Murari, 1999; Angelini *et al*., 2001).

Nucleic acid hybridization

The advantage of this method if that nucleic acid of the virus can be detected in both forms, single-stranded and double-stranded. cRNA probes are labeled with either isotopes or non-radioactive probes. cRNA probes are preferable to cDNA probes when used to detect RNA viruses, because RNA/RNA hybrids are more stable than DNA/RNA hybrids. A nucleic acid extraction from the infected tissue is blotted onto a membrane and the probe hybridized to it and detected (Webster *et al*., 2004). This technique has been used for detection and identification of carnation mottle virus (Sanchez-Navarro *et al*., 1996).

Microarrays and Macroarrays

Both these methods have been used for visualizing relative changes in global expression levels of mRNA, as well as single nucleotide polymorphism typing and host-pathogen interactions (Webster *et al*., 2004). This technique has the ability to simultaneously display the expression of thousands of genes at a time, making it a powerful tool for the simultaneous detection and identification of many plant pathogens (Hadidi *et al*., 2004). Single-stranded DNA probes are irreversibly fixed as an array of spots to a surface of glass, membrane or polymer (Webster *et al*., 2004). Base-paring of complementary sequences by hybridization if the underlying principle of arrays. Microarrays are high-density arrays, with a density of the order 1000-10000 spots per $cm^2$ or even higher. Macroarrays are generally membrane-based and low-density arrays, with a density of the order of 100 spots per $cm^2$ (Hadidi *et al*., 2004). Arrays printed with probes corresponding to a large number of pathogen can be utilized to simultaneously detect all those pathogens present within the tissue of an infected host. Viral/pathogen nucleic acids are extracted from the host and amplified, then labeled with a probe, either radioactive or fluorescently tagged nucleotides such as fluorescin, Cy3 or Cy5. The labeled target molecule is denatured and allowed to hybridize with the arrayed probes. Excess target is washed from the surface and spots where labeled target molecules have bound, become fluorescent under appropriate lighting conditions. The position of a visible spot corresponds to the presence of a particular pathogen in the plant sample (Webster *et al*., 2004).

The advantages of microarrays include the simultaneous detection and quantification of thousands of hybridization events and the great scope for high-throughput applications and the development of automated systems. The disadvantage is that this method is very expensive (Hadidi *et al*., 2004). This technique has been used for detection and identification of various plant pathogens (Boonham *et al*., 2003; Lee *et al*., 2003).

From the overview on the diagnostic methods, described above, one can understand that not all pathogens can be detected. Therefore certain pathogens can enter the certification scheme unnoticed and spread rapidly if this infected material is used as propagation material.

# PART II: SELECTED GRAFT-TRANSMISSIBLE INTRACELLULAR PATHOGENS INFECTING GRAPEVINE

## 1.4 GRAPEVINE FLECK VIRUS

Grapevine Fleck Virus (GFkV) is a member of the genus *Maculavirus*. *Maculavirus* (*Macula* – Latin for fleck) is one of three genera in the family *Tymoviridae*. This genus consists of a small group of phloem-limited isometric viruses that is not sap-transmissible and with no known vectors (Adams and Antoniw, 2005). GFkV has been found in Europe and the United States of America but with no evidence of spread (Brunt *et al*., 1996). It has been found with evidence of spread in South Africa in 1990 (Engelbrecht and Kasdorf, 1990).

No vector up to date has been associated with the transmission of GFkV (Sabanadzovic *et al*., 2000; El Beaino *et al*., 2001). GFkV is latent in *Vitis vinifera*, but in the indicator plant *Vitis rupestris* it induces specific foliar symptoms. The phloem cells of infected plant have highly characteristic cytopathic structures called vesiculated bodies, which are deranged mitochondria that have undergone peripheral vesiculation (Castellano and Martelli, 1984; Sabanadzovic *et al*., 2000; El Beaino *et al*., 2001).

The symptoms include localized clearing flecks in the veinlets of young leaves (Figure 1.5) and in older leaves the symptoms diffuse into a mosaic pattern

and the leaves wrinkle and curl upward. Grapevines may remain symptomless, but if symptoms do present, these may vary seasonally and could also disappear in time (Foundation Plant Services, 2002).

*Figure 1.5: GFkV symptoms on a leaf placed under a light source*

The morphology of this genus includes non-enveloped isometrical particles, ~30nm in diameter, with a rounded contour. The genome of GFkV is a monopartite, linear, single-stranded, positive sense RNA genome with a total size of 7564 nt excluding the poly-A tail. The genome has a high cytosine content (49.8%) and consists of four putative reading frames and untranslated regions of 291 nt and 35 nt at the 5' and 3' region, respectively (Sabanadzovic *et al*., 2000; El Beaino *et al*., 2001; Martelli *et al*., 2002) (Figure 1.6).

Open reading frame (ORF) 1 encodes a 215.4-kDa polypeptide, which plays a role during viral replication. ORF 1 has the conserved motifs of replication-associated proteins of positive stranded RNA viruses and a papain-like protease domain. ORF 1 of GFkV lacks a highly conserved 16 nt long subgenomic RNA promoter, known as the Tymobox, present in other members of the *Tymoviridae* family (Martelli *et al*., 2002).

ORF 2 encodes a 24.3-kDa polypeptide, which has been identified as the coat protein (Martelli *et al*., 2002).

ORF 3 and ORF 4 are located at the 3' end of the genome and encode proline-rich proteins of 31.4 kDa and 15.9 kDa with unknown functions (Martelli *et al*., 2002).

Replication is likely to occur in the cytoplasm, with association of vesicles in the mitochondria. This could be due to autoproteolytic cleavage of the 215-kDa polypeptide encoded by ORF 1 as well as the production of sub-genomic RNA (Martelli *et al*., 2002).


*Figure 1.6: Schematic representation of GFkV genome organization, showing the relative position of the ORF's and their products. MTR – Methyltransferase, PRO – Papain-like protease, HEL – Helicase, RdRp – Polymerase, CP – Coat protein, p31 and p16 – Proline rich proteins (Image: Martelli, 2002)*

## 1.5 PHYTOPLASMAS INFECTING GRAPEVINE

Phytoplasmas belonging to the genus *Candidatus* Phytoplasma, class Mollicutes, comprises of 26 species and 15 different phylogenetic groups (Firrao *et al*., 2004). It is believed that phytoplasmas have diverged from gram-positive eubacteria (Hogenhout, 2004). The reduction in genome size may have resulted from differential loss of genes during evolution. It appears that mollicutes may have lost genes encoding for the synthesis of macromolecule precursors such as cell-wall components, amino acids and long-chain fatty acids (Razin *et al*., 1998; Lee *et al*., 2000).

In 1967, Doi *et al*. discovered that particles in ultrathin sections of the phloem of cells affected by yellows diseases, resembled animal and human mycoplasmas. The agents associated with these plant yellows diseases were pleiomorphic in shape, with an average diameter of 200-800 µm. These agents also lacked rigid cell walls, were surrounded by a single unit membrane and were sensitive to tetracycline antibiotics (Doi *et al*., 1967; Lee *et al*., 2000).

From 1967 to 1994, the term mycoplasma-like organisms (MLO) were used to refer to the causal agents of many yellows diseases (Lee and Davis, 1992; McCoy *et al*, 1989). In 1994, the name phytoplasma was adopted by the Phytoplasma Working Team, at the 10$^{th}$ Congress of International Organization of Mycoplasmology (Lee *et al*., 2000).

Although phytoplasmas appeared as rounded pleiomorphic bodies during single cross sections, other studies revealed a filamentous morphology (Lee and Davis, 1992; Walters and Osborne, 1978; Haggins *et al*., 1978; Lee and Davis, 1983). Filamentous bodies were especially predominant in infected plant tissue during the early stages of infection (Lee *et al*., 2000).

Phytoplasmas infecting plants cause disturbances in the normal balance of plant hormones or growth regulators (Chang, 1998; Chang and Lee, 1995).
The symptoms include virescence (the development of green flowers and the loss of normal flower pigments), phyllody (the development of floral parts into leafy structures), sterility of flowers, proliferation of auxiliary shoots resulting in a witches'-broom appearance, abnormal elongations of the internodes resulting in slender shoots, generalized stunting (small flowers and leaves and shortened internodes), discoloration of leaves or shoots, leaf curling, bushy appearance of growth at the ends of the stems and generalized decline (stunting, dieback of twigs and unseasonal yellowing or reddening of the leaves). Internally, infections can cause extensive phloem necrosis and excess formation of phloem tissue, resulting in swollen veins (Lee *et al*., 2000).

Phytoplasmas are phloem-limited plant pathogens that are found primarily in the sieve elements of infected plants (Lee *et al*., 2000). Phytoplasmal diseases are spread primarily by sap-sucking insect vectors and including vertically between generation of insects (Hogenhout, 2004). These sap-sucking insects belong to the families *Cicadellidea* (Leafhoppers) and *Fulgoridea* (Planthoppers). Insects feed on phloem tissue, where the phytoplasma is acquired and transmitted from plant to plant (Lee *et al*., 2000). Phytoplasmas cannot be transmitted mechanically and are not seed transmissible, but are graft transmissible (Lee *et al*., 2000).

In the past few decades, detection and identification of phytoplasmas were never accurate, because of the inability to obtain pure cultures. In the 1980s, the development of molecular probes such as antibodies and cloned phytoplasma-specific DNA advanced the art of phytoplasma diagnostics. PCR-based assays developed in the late 1980s and early 1990s further advanced diagnostics for phytoplasmal diseases (Lee *et al*., 2000).

The rRNA genes in phytoplasmas are arranged in the same order as in other eubacteria: 5' 16S rRNA – spacer region – 23S rRNA 3'. Sequence analysis of the spacer region revealed that a single $tRNA^{ile}$ (isoleucine transfer RNA) is present in all phytoplasmas (Kirkpatrick *et al*., 1990; Kuske and Kirkpatrick, 1992; Lee *et al*., 2000). The 16S rRNA gene is best characterized, and PCR primers designed on the basis of these unique sequences have been used for specific detection of phytoplasmas in infected plant and insect vectors (Lee *et al*., 2000).

Geographically, phytoplasmas occur worldwide. The different groups of phytoplasma seem to be restricted to one continent or to a specific geographical region (Lee *et al*., 2000).

### 1.5.1 Grapevine Flavescence doree

Flavescence doree is associated with a phytoplasma in the genus *Candidatus* Phytoplasma vitis, in the phylogenetic Elm Yellows (16SrV)

group (Firrao *et al.*, 2004). The principle host of Flavescence doree is *Vitis vinifera* as well as *Vitis riparia* (Maixner and Pearson, 1992). During a study done in 1993 by Kuszala *et al.* on grapevines showing yellowing symptoms, from all parts of the world, this phytoplasma was only detected in material from southern France and northern Italy (Kuszala *et al.*, 1993; EPPO/CABI, 1997).

The Flavescence doree phytoplasma is located in the phloem tissue of the infected grapevine and can be obtained by its vector for transmission. No alternative host other than grapevine is known and it is therefore likely that the biological cycle is completed in grapevine and vector (EPPO/CABI, 1997).

The vector of Flavescence doree is a cicadellid, *Scaphoideus titanus*. In 1985, Caudwell and Dalmasso found that this vector was accidentally introduced into Europe from North America (Caudwell and Dalmasso, 1985). *S. titanus* has five larval instars and both the larval stages and adults are capable of acquiring the phytoplasma. The acquisition period is generally 7-8 days, followed by a long latent period, so that transmission takes 38-42 days in total (EPPO/CABI, 1997).

The symptoms of Flavescence doree (adapted from EPPO/CABI, 1997) can be divided into three groups:

Symptoms on the shoots

When early infection occurs, the shoots fail to lignify, are thin, rubbery and hang downward. The shoots later become brittle and there may be necrosis of the apical and lateral buds. During winter the non-lignified branches blacken and die. If the shoots become infected later in the season, the lignification is interrupted (Figure 1.7).

In more resistant cultivars, non-lignification is more pronounced and is limited to certain internodes. Numerous black pustules form along the diseased branches of susceptible cultivars.

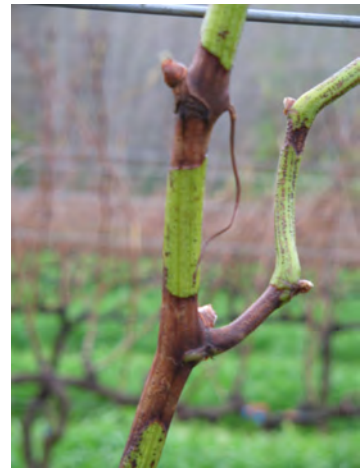

*Figure 1.7: Photo of limited non-lignification of internodes*

Symptoms on the leaves

In white-fruited cultivars there is a yellowing of the portion of the lamina exposed to the sun. Later in the season, distinct creamy-yellow spots (of a few mm in diameter) become visible along the main veins. These spots broaden and form continuous yellow bands along the veins.

In red-fruited cultivars similar patterns of colour change develop on the leaves, but the discoloration are reddish. The central portion of the discolored areas becomes necrotic and dries out. The discolored leaves stay on the grapevine longer than the healthy leaves (Figure 1.8).

*Figure 1.8: Photo of discolored leaves staying on the grapevine*

Symptoms on the fruit

If the grapevine is infected earlier in the season, the fruit setting is reduced and the inflorescence dry out and fall off. In later infection, bunches become brown and shriveled.

### 1.5.2 Grapevine bois noir

Bois noir (Stolbur) are associated with a phytoplasma in the genus *Candidatus* Phytoplasma solani, in the phylogenetic Stolbur (16SrXII) group (Firroa *et al.*, 2004).

It is thought that grapevine may not be the host of this pathogen and that this pathogen has accidentally been transmitted to grapevine from other hosts (EPPO/CABI, 1997). In 1994 Maixner *et al.* found the bois noir phytoplasma in the plant hopper *Hyalesthus obsoletus*, after years of speculation on whether a vector did exist. *H. obsoletus* feeds on various wild plants and weeds, but rarely on grapevine, and therefore vine-to-vine transmission rarely occurs (Maixner *et al.*, 1994; EPPO/CABI, 1997).

The symptoms of this phytoplasma are more or less the same as described above for Flavescence doree. Bois noir (black wood) refers

to the blackening of the non-lignified shoots in winter (EPPO/CABI, 1997).

### 1.5.3 Australian Grapevine Yellows

Australian Grapevine Yellows (AGY) phytoplasma is associated with a phytoplasma in the genus *Candidatus* Phytoplasma australiense, in the phylogenetic Stolbur (16SrXII) group (Firroa *et al*., 2004).

The symptoms include irregular veinal and interveinal yellowing and downward rolling of leaves that overlay one another in a shingled appearance. Shoots display abortion of the flowering bunches early in the season or shriveled berries later in the season. Affected shoots also often display tip death followed by dieback of the shoots, node by node. Leaf blades on affected shoots tend to fall early. The petioles remains attached to the shoots for longer than the leaf blades but would eventually abscise from the shoot. The stem of affected shoots often develops a blue, waxy appearance and remains rubbery later in the season (Constable *et al*., 2003).

Restricted growth disease (RG) can be expressed in grapevines with AGY. The aetiology of RG is unknown but phytoplasmas may be the possible cause (Constable *et al*., 2003). To determine the involvement of phytoplasmas, shoots from grapevines with RG have been tested by PCR but no association between phytoplasmas and RG was shown (Bonfiglioli *et al*., 1995; Padovan *et al*., 1995; Gibb *et al*., 1999). Symptoms of RG include retarded growth resulting in shortened shoots and smaller leaves. Affected grapevines have an overall appearance of stunting or lack of vigour throughout the season. Some grapevines with RG may have also display uneven or no bud development, resulting in canes and cordons that are bare in places or entirely bare with little or no bunch development (Constable *et al*., 2003).

Late season leaf curl disease (LSLC) can also affect grapevines with AGY (Constable *et al*., 2003). Shoots from grapevines with LSLC were

also tested to determine whether phytoplasmas might be involved (Bonfiglioli *et al*., 1995; Gibb *et al*., 1999). Some researchers found a high association with phytoplasmas and LSLC affected shoots (80%) (Bonfiglioli *et al*., 1995) but others found a low association (10%) (Gibb *et al*., 1999), thus the association between phytoplasmas and LSLC is still unclear (Constable *et al*., 2003). Symptoms include leaves on affected shoots, which are rolled tightly downward, remains green and overlays one another in a shingled appearance. The leaves are often tough, leathery and brittle (Constable *et al*., 2003).

## 1.6 OTHER DISEASES OF GRAPEVINE

### Shiraz decline

Shiraz Decline must not be confused with Shiraz disease. Up to now no correlation has been found between the disease and any fungus, bacteria, phytoplasma, soil type, climate, rootstock, clone or virus disease (Spreeth, 2005).

During a presentation at the meeting of the National Working Group at ENTAV in 2004, studies were related to the possible implication of a phytoplasma in this disease. The first analyses led to detection of phytoplasma belonging to groups 16SrI (Yellow Asters) and 16SrXII (Stolbur) in several samples with or without symptoms (Renault-Spilmont *et al*., 2005).

In France the symptoms have been observed for the past 10 years and Professor Denis Boubals, editor of the well-known French viticultural magazine "Le Progrès Agricole et Viticole" magazine, contends that all Shiraz vineyards older than 15 years display between 1 and 15% symptoms of the disease, depending on the location and the cultivation conditions of the vineyard (Spreeth, 2005).

The symptoms include thickened graft joints with cracks on the graft joint and red discoloration of the leaves from middle to late summer.

The symptoms are similar to those usually observed in girdled vines or shoots. The bark thickens above the graft joint and deep cracks can be seen on the stem and cordon arms. The vines weaken and usually die back between five to ten years (Spreeth, 2005).

**Shiraz disease**

Shiraz Disease apparently occurs only in South Africa and causes deterioration in the cultivars Shiraz, Merlot and Malbec (Carstens, 1999). The disease is graft-transmissible although natural transmission has previously been reported (Engelbrecht and Kasdorf, 1990). The disease may be latent in certain cultivars and symptoms will only occur when this latent infected material is grafted onto the above-mentioned indicator cultivars (Carstens, 1999).

Grapevine Virus A (GVA) infection of Shiraz and Merlot cultivars in South Africa has been shown to be associated with Shiraz disease (Goszczynski and Jooste, 2003). According to Habili and Randles (2004), Goszczynski and Jooste (2003) provided evidence that Shiraz disease was associated with GVA infection alone. However Koch's postulates has not yet been proven, and thus there is no evidence that this disease is associated with only one organism.

Symptoms of the disease include reddening of leaves and veins, curling of the leaves, poor lignification of canes, retention of leaves through the winter and restricted growth (Goszczynski and Jooste, 2003). These symptoms are essentially the same as described for Flavescence doree and Stolbur, although no black pustules form on the stem. The question remains if this disease is caused by a complex of viruses and phytoplasmas.

Shiraz Disease is graft transmissible, a typical characteristic of virus and phytoplasma diseases. In 1997 Carstens found with the help of a nucleic acid linked technique that grapevine leafroll associated virus-3 (GLRaV-3) often occurs in material showing symptoms of Shiraz

Disease (Carstens, 1999). In 1993, Burger and Spreeth found Grapevine leafroll associated virus-1 and 2, as well as grapevine virus A (GVA), in different combinations with GLRaV-3 in vines showing symptoms of Shiraz Disease (Carstens, 1999).

# PART III: AIMS OF THE STUDY

1. Establish a PCR method for the routine detection of Grapevine Fleck Virus in South Africa
2. Using this method to determine if the unknown spherical particles in greenhouse and field samples are Grapevine Fleck Virus
3. Establish a PCR method for the routine detection of phytoplasmas in South Africa
4. Using this method to determine if phytoplasmas are associated with the symptomatic plants found, which lack lignification

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# Chapter 2

# Optimization and implementation of two existing PCR detection systems for GFkV in South Africa

## 2.1 INTRODUCTION

The elimination of grapevine viruses in nuclear plant material for use in South Africa is effective and for a number of years no example of virus-like infections have occurred in these facilities (Oosthuizen, *per comm.*). However, because mass production is done in open fields, leafroll re-infection frequently occurs. As reviewed in Alkowni *et al.*, 2004, leafroll disease in grapevine alters fruit coloration and delays fruit maturation. Other symptoms include interveinal reddening in red grape varieties and yellowing in white grape varieties in late summer and fall leaves, as well as downward rolling of leaves and phloem disruption (Alkowni *et al.*, 2004). Winetech, in conjunction with the Agricultural Research Council – Plant Protection Research Institute (ARC-PPRI), drafted a project in 2001 to determine and identify leafroll-associated viruses spreading within certified grapevine material in South Africa and to develop/establish means to detect these viruses.

From 2001 to 2004, motherblocks were surveyed for leafroll symptoms associated with Grapevine Leafroll Associated Virus Type 3 (GLRaV-3), a very serious pathogen of grapevine in South Africa. Motherblocks are open blocks in which the grapevines are being mass propagated and are monitored annually for any symptoms of known pathogens. During Immunosorbent Electron Microscopy (ISEM) analysis of this material, it was found that 15 % of the GLRaV-3 infected plants also contained unknown spherical virus-like particles (Figure 2.1) of about 30nm in diameter (Pietersen, unpublished).

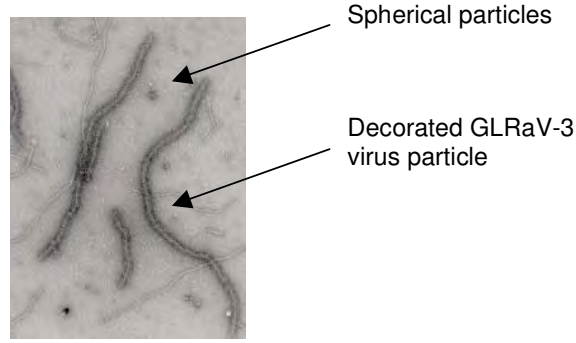


*Figure 2.1: Electron micrograph of a plant infected with GLRaV-3 and unidentified spherical particles following ISEM with Black Spanish antiserum. (Photo: K. Kasdorf)*

Grapevine Fleck Virus (GFkV) is a non-mechanical transmissible spherical virus of 30nm in diameter (Sabanadovic *et al*., 2000; El Beaino *et al*., 2001; Martelli *et al*., 2002) and has been found in South Africa, with evidence of spread (Engelbrecht and Kasdorf, 1990). Therefore, it was hypothesized that the unidentified spherical particles found were most likely GFkV.

Since no molecular detection method is available for GFkV in the current certification scheme for wine grapes in South Africa, GFkV could be present in certified material. Commercial ELISA kits are available for GFkV, but the high import cost makes routine use of these prohibitively expensive. An alternative was to establish a PCR method to detect this virus, which can be used in the South African certification scheme for wine grapes.

Over 40 spherical viruses can potentially infect grapevine. To narrow down the list of possible viruses, mechanical inoculations were done to determine whether these spherical viruses could be mechanically transmitted to various host range plants. ELISA was performed to determine the presence of GFkV, where after different RNA extraction methods were tested. In this study two PCR methods for the routine detection of GFkV within certified grapevine planting material as well as a field survey to determine the spread of GFkV in the field are presented.

# 2.2 MATERIALS AND METHODS

## 2.2.1 OPTIMIZATION OF RdRp GENE AND MTR GENE SPECIFIC PCR

### 2.2.1.1 Virus sources

The positive controls (BU4 and BU21) used during the optimization of the two PCRs, were lyophilized total nucleic acid (TNA) from GFkV-infected vines, obtained from Nina Abou Ghanem-Sabanadzovic, Istituto Agronomico Mediterraneo, Italy.

### 2.2.1.2 cDNA Synthesis

cDNA synthesis was done in a 20µl reaction following the USB First Strand cDNA Synthesis Protocol (USB Corporation, Ohio, USA) with slight modifications. 2-5µg of TNA were denatured together with 40pmoles of gene specific primer and DEPC-treated water at 65°C for 5 min and incubated on ice for 2min (i.e. for the RdRp system RD2 primer and for the MTR system MTR1 primer -also see Section 2.2.1.3)

To the denatured RNA mix the following were added as final concentrations in a 20µl reaction: 5x M-MLV Reaction Buffer (USB Corporation, Ohio, USA), 10mM dNTP mix, 25U/µl Ribonuclease Inhibitor and 200 units/µl M-MLV Reverse Transcriptase (USB Corporation, Ohio, USA). The mixture was incubated at 37°C for 30min where after the reaction was inactivated by 70°C for 10min.

### 2.2.1.3 PCR: RdRp and MTR genes

RdRp gene specific PCR (Sabanadzovic *et al.*, 2000)

In a 50µl reaction the following reagents were used as final concentrations: 10x Promega Reaction Buffer (Promega Corporation, Wisconsin, USA), 2mM $MgCl_2$, 2,5mM dNTP mix, 30pmol RD1 primer, 30pmol RD2 primer, 5U/µl Taq

DNA polymerase (Promega Corporation, Wisconsin, USA), 3µl cDNA from positive control and distilled water.

The PCR conditions were as follows:

94℃ for 120 sec;
94℃ for 30 sec;
52℃ for 30 sec; } x 35 cycles
72℃ for 60 sec;
72℃ for 300 sec.

10µl of the PCR products were electrophoresed in a 1% (w/v) agarose gel at 100V in 1 x SB buffer (0.004% NaOH, 0.0023% Boric Acid, pH8). The agarose gels were pre-stained with ethidium bromide (0.5µg/ml) and the DNA fragments visualized by UV fluorescence.

The RD primer set was modified, as non-specific bands were obtained after PCR. RD1 primer was extended by six nucleotides (RdRp1) and RD2 was extended by six nucleotides (RdRp2). Extension of the primers was done to increase the specificity of the primers during the annealing step. The modifications are shown in red in Table 2.1.

TABLE 2.1: **Modification of RdRp gene specific primer set**

| | | | |
|---|---|---|---|
| RD 1 | 5' CYC ARC AYA ARG TVA ACG A 3' | RdRp 1 | 5' CYC ARC AYA ARG TVA ACG ADV RCT C 3' |
| RD2 | 5' GCG CAT GCA BGT SAG RGG G 3' | RdRp 2 | 5' GCG CAT GCA BGT SAG RGG GCC RAA Y 3' |

The RdRp primer set was optimized using the exact same conditions as during the RD system.

MTR gene specific PCR (Sabanadzovic *et al.*, 2000)

Reactions were done as described above for RdRp using forward primer MTR1 and reverse primer MTR2, final concentration 3mM $MgCl_2$ and an annealing temperature of 58℃.

2.2.1.4 Purification of PCR products

The Promega Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, Wisconsin, USA) was used for purification of PCR products. The Wizard ® SV Gel and PCR Clean-Up System is designed to extract and purify DNA fragments of 100bp to 10kb from standard agarose gels. This system is based on the ability of DNA to bind to silica membranes in the presence of chaotropic salts. Purification was done according to manufacturers instructions (Promega Corporation, Wisconsin, USA, Technical Bulletin No.308).

## 2.2.2 PREPARATION OF RdRp GENE AND MTR GENE PCR POSITIVE CONTROLS

2.2.2.1 Preparation of competent cells

Competent cells were prepared using the method described by Hanahan *et al.* (1991) with slight modifications.

*E. coli* (JM109) cells were streaked onto M9 minimal media agar plates (0.05M $Na_2HPO_4$-$2H_2O$, 0.02M $KH_2PO_4$, 8mM NaCl, 0.02M $NH_4Cl$, 2mM $MgSO_4$, 0.01M D-glucose, 0.1mM $CaCl_2$, 1mM thiamine hydrochloride, 1.5 % agar (w/v) pH 7.4) and grown overnight at 37°C. Single colonies were picked and streaked onto LB-agar plates (1% tryptone, 0.5% yeast extract, 1% NaCl pH7.5, 1.5% (w/v) agar) and grown overnight at 37°C. Several single colonies were picked and grown overnight in LB-broth (1% tryptone, 0.5% yeast extract, 1% NaCl pH7.5) with agitation at 37°C. 1:100 dilution of the overnight culture were inoculated in 100ml of pre-warmed LB-Broth and incubated with agitation at 37°C. The culture was grown until an optical density (OD) of 0.4-0.6 was reached at 550nm. The cells were transferred to a 50ml centrifuge tube and incubated on ice for 10min. The cells were collected by centrifugation at 6000xg for 10min (4°C). After the supernatant was aspirated, the cells were resuspended in 25ml of CCMB 80 medium (80mM $CaCl_2$-$2H_2O$, 20mM $MnCl_2$-$4H_2O$, 10mM $MgCl_2$-$6H_2O$, 10mM K-acetate, 10%

(v/v) redistilled glycerol, pH 6.4) and incubated on ice for 30min. The cells were collected by centrifugation at 6000xg for 10min (4℃), and the supernatant aspirated. The cells were resuspended in 1ml of CCMB 80 medium and incubated on ice for 30min. The cells were aliquoted and stored at –70℃.

2.2.2.2 Ligation reactions

The purified RNA dependent RNA polymerase gene, the amplified product by PCR with the use of the RdRp primer set, and the purified Methyltransferase gene, the amplified product by PCR with the use of the MTR primer set, were ligated and cloned as discussed below.

The vector used for ligation was the pGEM®-T Easy Vector System II (Promega Corporation, Wisconsin, USA). This vector has been prepared by cutting it with EcoRV and adding terminal thymidine residues to both ends. These single T overhangs improve the efficiency of ligation of a PCR product into a plasmid by preventing recircularization of the vector and providing a compatible overhang for PCR products, since Taq polymerase generates 3` adenine overhangs.

The ligation reactions were done according to manufacturers protocol (Promega Corporation, Wisconsin, USA, Technical Manual No.042). Briefly, in a 10µl reaction the following reagents were used: 2x Rapid ligation buffer, T4 DNA Ligase, 50ng of pGEM®-T Easy Vector, 30ng of MTR insert or 20ng of RdRp insert (to calculate the appropriate amount of insert to include in the ligation reaction, the following equation was used: [(ng of vector x kb size of insert)/(kb size of vector)] x insert: vector molar ratio (1:1) = ng of insert), 3 Weiss units/µl of T4 DNA Ligase and distilled water to final volume.
The reactions were mixed by pipetting and incubated at room temperature for one hour.

2.2.2.3 Transformation of competent cells

Before transformation of the ligation mixture, the competent cells were first test transformed with a plasmid of known concentration (pUC18, 10ng/µl) to calculate the transformation efficiency of the cells. The transformation was done using the heat shock method as described by Sambrook *et al.* (Sambrook *et al.,* 1989)

100 µl of the competent cells were placed in 3 pre-chilled Eppendorf tubes. To the first tube 10µl of the ligation reaction was added (RdRp and MTR, respectively), to the second tube a plasmid of known concentration was added (pUC18, 10ng/µl), to serve as a positive control, and to the third tube no DNA was added, to serve as a negative control. The tubes were incubated on ice for 30 min and then placed in a water bath at 42°C for 90 sec. After the heat shock, the tubes were chilled on ice for 2 min. After addition of 900 µl of pre-warmed LB-broth, the transformation mixture was incubated with agitation at 37°C for one hour to allow the cells to recuperate and express the ampicillin resistance gene. 100µl of the different mixtures were plated onto LB agar plates supplemented with 50µg/ml ampicillin. Recombinant transformants were selected by blue/white colour selection, based on the inactivation of the lac Z gene. To achieve this, 40µl of 2% X-gal solution and 10µl of 100mM IPTG solution were spread over the surface of the entire plate together with the cells. Recombinant plasmids with a $Gal^-$ phenotype were selected for further characterization and grown overnight at 37°C in LB-broth supplemented with ampicillin.

2.2.2.4 Plasmid DNA extractions

Recombinant plasmids were isolated using the alkaline lysis method as described by Sambrook *et al.* (1989) with slight modifications.

Recombinant transformants were streaked out onto LB agar supplemented with ampicillin and grown overnight at 37°C. Single colonies were picked and grown in LB-broth with ampicillin overnight with agitation at 37°C. 1.5ml of overnight culture was centrifuged for 1 min at 14000xg. The supernatant was

removed and the bacterial pellets were resuspended in 100µl of ice-cold Solution I (50mM glucose, 25mM Tris-HCl pH8, 10mM EDTA pH8), creating an environment in which the cells are prevented from plasmolysing. The resuspended cells were vortexed and incubated at room temperature for 5min followed by 1min incubation on ice. 200µl of Solution II (0.2M NaOH, 1% SDS) were added to the cells and mixed by inverting and incubated on ice for 5 min. 150µl of ice-cold 7.5M ammonium acetate was added to the cells and vortexed briefly. The cells were then incubated on ice for 15 min and centrifuged for 15 min. The supernatants were transferred to fresh tubes and 2 volumes of 95% ethanol were added. The solution was incubated at –20°C for 30min and centrifuged for 15 min at 14000xg. The DNA pellet was aspirated and washed with 70% ethanol. The pellet was dried and resuspended in 50µl of TE buffer (10mM Tris, 1mM EDTA, pH8). The plasmids were purified using the Promega Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, Wisconsin, USA).

2.2.2.5 PCR: Screening for recombinants

PCR was done on 4 possible MTR recombinants and 3 possible RdRp recombinants using the method described in Section 2.2.1.3.

2.2.2.6 DNA sequencing

The reaction was set up using the BigDye Terminator Version 3.1 (Greiner Labortchnik, Frikenhausen, Germany) according to manufacturers protocol.
In a 20µl reaction the following reagents were used: 2.5 x Ready Reaction Premix; 5 x BigDye Sequencing Buffer; 10 pmol primer (RdRp1/MTR1); 100 ng of plasmid DNA and distilled water to volume.

The cycling conditions were as follows:

96°C for 60 sec;
94°C for 10 sec;
50°C for 5 sec;
60°C for 240 sec;
} x 25 cycles

The sequencing reaction was added to a 1.5ml Eppendorf tube and 100µl of 60% ethanol was added. The tubes were briefly vortexed and incubated at room temperature for 15 min. The reaction was centrifuged for 20min at 14000xg and the supernatant carefully aspirated. The pellet was washed with 250µl of 70% ethanol and briefly vortexed. The tubes were centrifuged for 10 min at 14000xg in the same orientation. The supernatant was aspirated and the pellet was dried for 1 min at 90°C. Capillary electrophoresis was done (University of Pretoria, South Africa). The nucleotide sequences were analysed using the BLAST (Altschul *et al*., 1997) and DNAMAN version 4.13 (Lynnon Biosoft, Quebec, Canada) programs.

## 2.2.3 USE TWO PCRs TO TEST VARIOUS PLANT SAMPLES

### 2.2.3.1 Virus sources

The virus sources used in this investigation (Appendix 1) were cuttings rooted in sand and grown under greenhouse conditions. These 19 samples all contained unknown spherical particles observed during ISEM studies (Section 2.1). The positive controls used were the constructed recombinant MTR plasmids for the MTR region and for the RdRp region previous amplicons were used as positive controls.

### 2.2.3.2 Mechanical Inoculations

Mechanical inoculation were done with the 19 original samples to determine whether these particles were mechanically transmissible.

The 19 virus source samples were ground individually in 0.1M $PO_4$ buffer (0.1M $Na_2HPO_4$, 0.1M $NaH_2PO_4$, pH 7.2) containing celite and 2% nicotine. The extract was gently rubbed onto various indicator plants. The indicator plants used in this study included *Nicotiana tabacum* cv. Samsun, *Cucumis sativa* Ashley, *Nicotiana benthamiana*, *Phaseolus vulgaris* Bountiful, *Phaseolus vulgaris* Top Crop, *Lycopersicon esculentum* Red Khaki, *Chenopodium quinoa* and *Chenopodium amaranticolor*. After mechanical

inoculation, local and systemic symptoms were recorded weekly for one month.

2.2.3.3 ELISA

A DAS-ELISA was done on 14 of the 19 original samples along with the following samples 01/5008, 01/5009, 01/5011, 01/3380, 01/3381, 92/1028 and 98/0649 (See Appendix 1 for details).

The SEDIAG diagnosis kit for detection of GFkV (Sediag S.A.S, Dijon, France) was used following the manufacturers instructions with slight modifications during the conjugate step. During the conjugate step the antibodies (GFkV-IgE) was diluted 1/100 in the conjugate buffer (PVP (Mw 10 000 - 40 000), 0.2% BSA, 0.02% $NaN_3$) instead of 1/1000.

2.2.3.4 RNA extractions

Total RNA extractions were firstly done using the protocol by El Beaino *et al*. (2001) with slight modifications. 300mg of cortical scrapings were finely ground in liquid nitrogen. 3ml of STE buffer (0.1M NaCl, 0.05 M Tris, 0.001M EDTA, pH6.8) containing 1% SDS and 2% mercaptoethanol were added to the finely ground cortical scrapings. The extract was mixed with chloroform (1:1) and centrifuged for 5 min at 10 000xg. 30µl of silica powder and absolute ethanol (to reach a final concentration of 35% ethanol) was added to the supernatant. The mixture was incubated at room temperature with gentle shaking for 45 min. The mixture was washed two times with STE buffer containing 35% ethanol and centrifuged for 5 min at 10 000xg. The supernatant was aspirated and the pellet air-dried. The pellet was eluted in 100µl of nuclease free water.

Alternatively, RNA extractions were done using Promega Wizard® SV Total RNA System (Promega Corporation, Wisconsin, USA). Extractions were done according to manufactures protocol (Promega Corporation, Wisconsin, USA, Technical Manual N0.048). The extraction kit allowed a fast and simple technique for the preparation of purified and intact total RNA. cDNA synthesis

was done as described in Section 2.2.1.2 for the RdRp and MTR genes, respectively, followed by PCR as described in Section 2.2.1.3 for the RdRp and MTR genes, respectively.

2.2.3.5 Double stranded RNA extractions

The method of Valverde (1990) was used with slight modifications.
The sample used for ds-RNA extraction was 03/3381, a known GFkV-infected vine.

2 g of plant material was ground in 6ml 1 x STE Buffer (0.1M NaCl, 0.05M Tris, 0.001M EDTA, pH 6.8) and transferred to a 50ml centrifuge tube. 1ml of 10% SDS, 0.5ml of bentonite (25mg/ml) and 9ml of 1x STE-saturated phenol were added and the mixture shaken for 30min at 4°C. The mixture was then centrifuged at 8000xg for 15min at 4°C and 10ml of the upper aqueous phase was placed in a fresh 50ml centrifuge tube. 2.1ml of 95% ethanol was added to the tube and mixed well. Two columns were prepared. Using the barrel of a 20ml plastic syringe plugged with a disk of filter paper, a cellulose suspension consisting of 1g cellulose mixed with 25ml 1 x STE buffer containing 16% v/v ethanol was passed through. The STE buffer was allowed to drain thoroughly. The sample was added to one of the columns and eluted. The column was flushed with 40ml 1 x STE buffer containing 16% v/v ethanol and eluted. 2.5ml of 1 x STE buffer was added to the column and the eluant discarded. 10ml of 1 x STE buffer was added to the column and 10ml was collected in a fresh 50ml tube. 2.1ml of 95% ethanol was added to the 50ml tube and this mixture eluted. The eluant was discarded.

The elution step was repeated but only 6ml was collected in a fresh 50ml tube. 0.5ml of 3M sodium acetate, pH 5.5 as well as 20ml of 95% ethanol was added to the collected 6ml and stored overnight at –20°C. The samples were centrifuged at 8000xg for 25 min at 4°C, the supernatant poured off and the pellet air dried for 15 min. The pellet was resuspended in 40µl of TE buffer, labeled and stored at –20°C.

10µl of the double stranded RNA products were electrophoresed in a 0.8% (w/v) agarose gel at 60V in 1 x SB buffer (0.004% NaOH, 0.0023% Boric Acid, pH8). The agarose gels were pre-stained with ethidium bromide (0.5µg/ml) and the RNA fragments visualized by UV fluorescence. A ND-1000 spectrophotometer (Nanodrop Technologies, USA) was also used to determine the concentration of the ds-RNA. cDNA synthesis was done as described in Section 2.2.1.2 followed by PCR as described in Section 2.2.1.3 for RdRp.

## 2.2.4 USE OF ELISA TO TEST VARIOUS FIELD SAMPLES

### 2.2.4.1 Virus sources

The virus sources used in this investigation (Appendix 2 and Figure 2.2) were cane material and petioles collected from Block A and Block B using a judgment sampling method (White, 1998). In this sampling method, selections were made by human choice rather than at random. The advantage of this sampling method was a greater likelihood of getting infected sources, if present at low incidences.

### 2.2.4.2 ELISA

A DAS-ELISA was done on the 25 field samples.

The SEDIAG diagnosis kit for detection of GFkV (Sediag S.A.S, Dijon, France) was used following manufacturers instructions with slight modifications during the conjugate step.

During the conjugate step the antibodies (GFkV-IgE) were diluted 1/100 in the conjugate buffer (PVP (Mw 10 000 - 40 000), 0.2% BSA, 0.02% $NaN_3$) instead of 1/1000. During an optimization experiment, the highest absorbance was obtained using the 1/100 dilution.

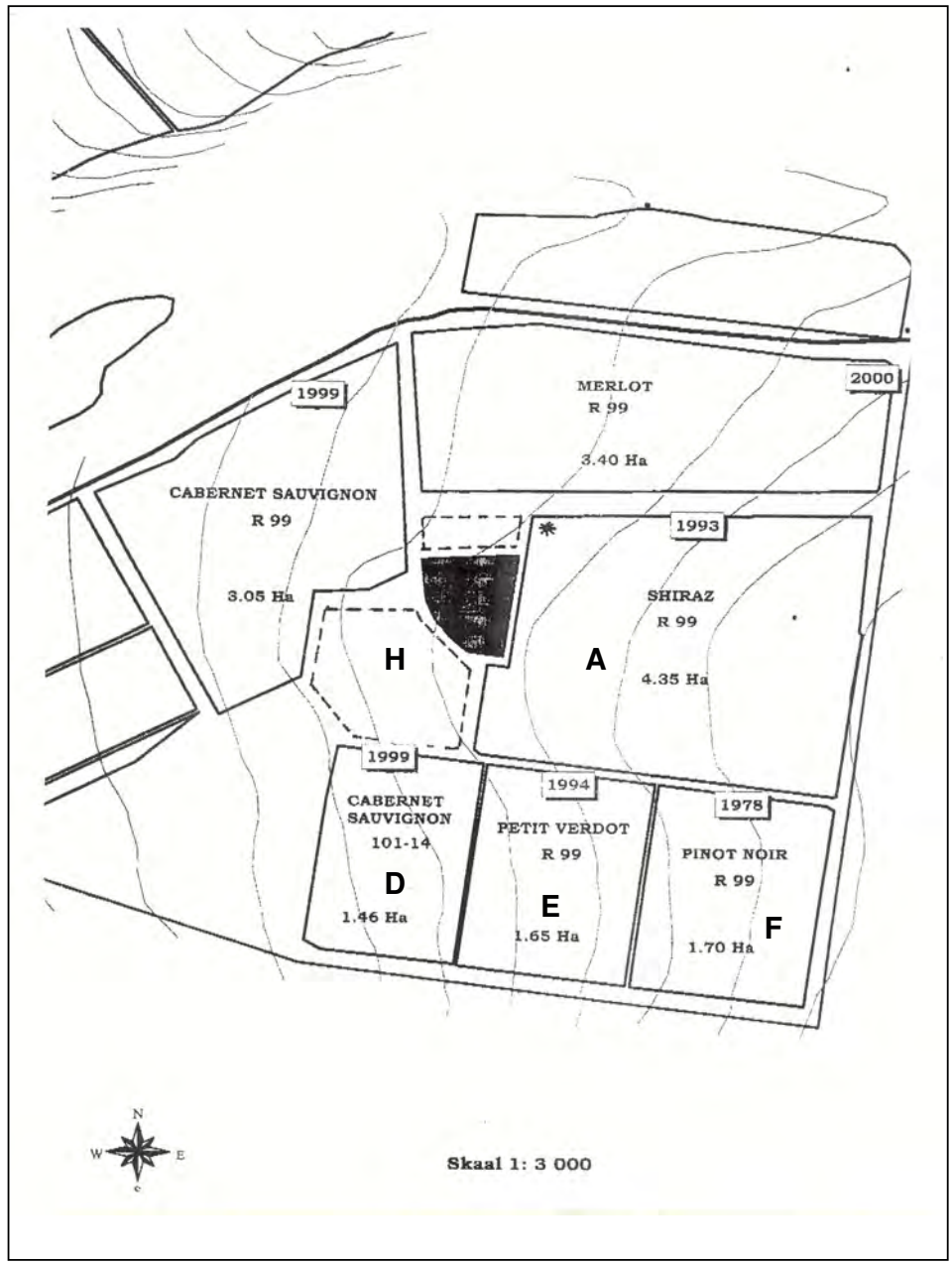


*Figure 2.2: Map of Groenhof farm, Stellenbosch (The red star indicates the position of the original GFkV-infected grapevine; subsequently removed)*

## 2.3 RESULTS

### 2.3.1 OPTIMIZATION OF RdRp GENE AND MTR GENE SPECIFIC PCR

RD system

After amplification with the RD primer set, amplicons of three sizes, ranging from ~200bp to ~400bp, were obtained. One of the bands was the expected size amplicon of 386 nt (Figure 2.3).

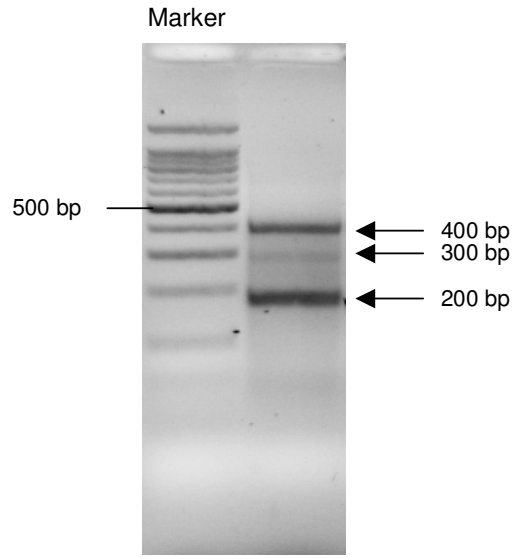


*Figure 2.3: Agarose gel electrophoresis of amplicons after PCR optimization with RD primer system*

The stringency of the reaction was increased by increasing the annealing temperature and lowering the $MgCl_2$ concentration, in an attempt to reduce the non-specific bands. The multiple bands were not reduced by increasing the stringency. Therefore, both the RD1 and RD2 primers lengths were increased by six nucleotides to increase the binding specificity of the primers (See Table 2.1 for modifications). Following the modification to primers RdRp1 and RdRp2, the expected size amplicon was obtained with no non-specific bands (Figure 2.4).

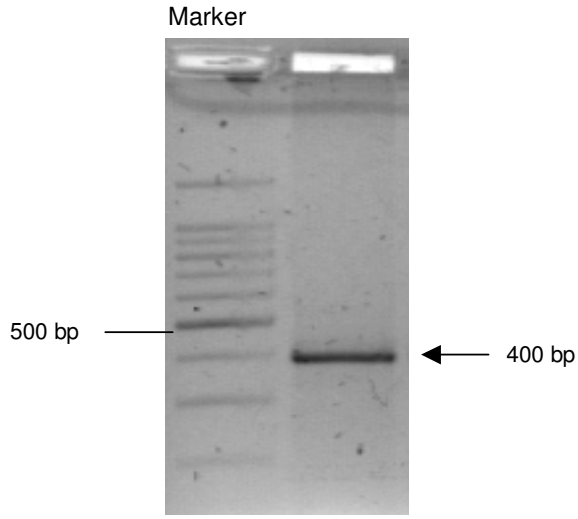


*Figure 2.4: Agarose gel electrophoresis of amplicons after PCR optimization with RdRp primer system*

MTR primers

After amplification with the MTR primer pair the expected size amplicon, ~600bp, was obtained (Figure 2.5). The MTR system was optimized using various annealing temperatures and $MgCl_2$ concentrations (Results not shown). The final optimal annealing temperature was 58°C and the $MgCl_2$ concentration 3mM.

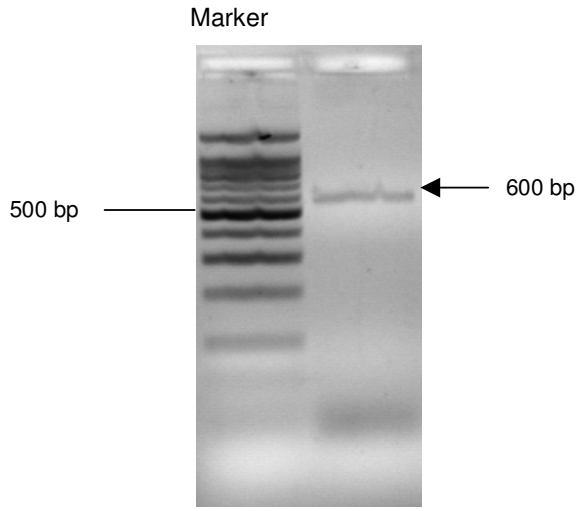


*Figure 2.5: Agarose gel electrophoresis of amplicons after PCR optimization with MTR primer system*

Both the amplified RdRp and MTR genes were cloned into pGEM®-T Easy Vector System II (Promega Corporation, Wisconsin, USA).

Plasmid extractions were done on recombinant colonies and PCR analysis followed. Two colonies were recombinants containing the MTR gene, while none were obtained for the RdRp gene. Sequencing was done on the positive amplicons obtained from the MTR PCR assay to confirm the presence of the GFkV MTR gene in the vector. These plasmids served as positive controls in subsequent studies.

## 2.3.2 USE OF TWO PCRs TO TEST VARIOUS PLANT SAMPLES

Mechanical inoculation

Mechanical inoculation was done on all samples to determine if some of the unknown spherical particles were mechanically transmissible. After the host plants were mechanically inoculated, symptoms were recorded for a period of one month. No local or systemic symptoms were observed during this period (Results not shown). Thus, it was concluded that these particles were non-mechanical transmissible viruses, incapable of infecting the selected host range plants.

ELISA

After testing the plants containing the unknown spherical particles with the GFkV-specific ELISA kit, none of the 19 samples was positive for GFkV (Figure 2.6). Four grapevine samples (01/3380, 01/3381, 92/1028 and 98/0649), maintained at the ARC-PPRI (Roodeplaat), tested positive for GFkV.

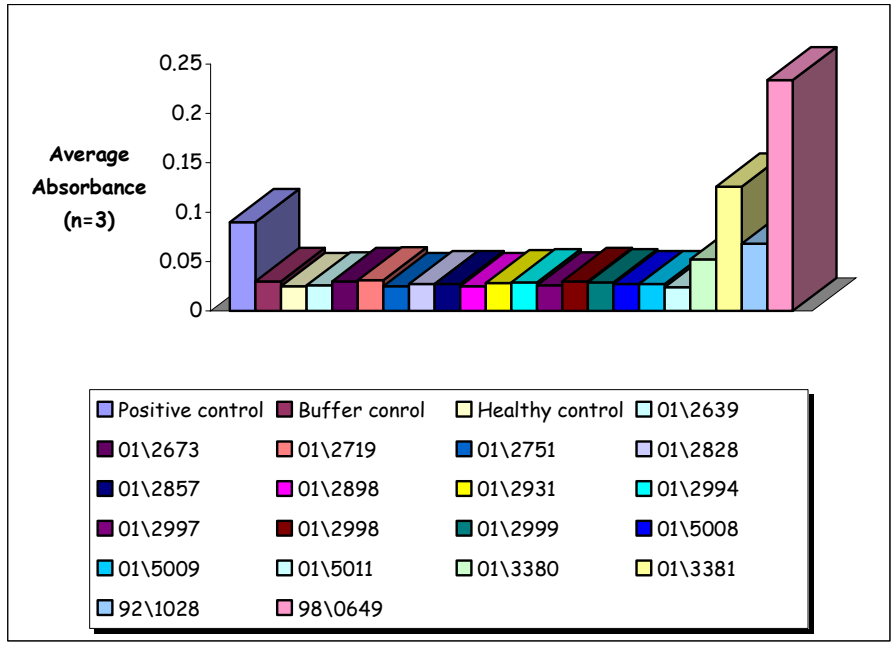


*Figure 2.6: Graph of the ELISA results done using a GFkV specific kit*

The low absorbance values could possibly be due to the fact that the ELISA was not sensitive enough, if the virus was present in low concentrations. Therefore the samples were tested using both the optimized RdRp and MTR PCR.

RdRp gene specific PCR

Negative results were obtained from the samples using both the extraction method described by Sabanadzovic *et al*. (2001) and the Promega Wizard ® SV Total RNA System. The TNA (BU4 and BU21) used as positive control did however amplify the expected amplicon using the RdRp primer set (Figure 2.7).

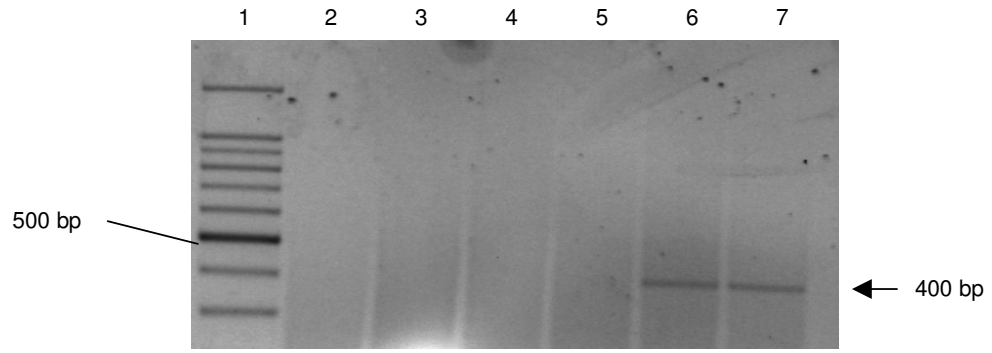


*Figure 2.7: Agarose gel electrophoresis of amplicons after PCR on greenhouse samples with RdRp primer system. Lane 1- Molecular marker, Lane 2- 01/2994, Lane 3- 01/2998, Lane 4- 01/2719, Lane 5- 01/5007, Lane 6- BU21, Lane 7- BU4*

MTR gene specific PCR

No amplicons could be obtained from the samples using the MTR primer pair, but the positive control did give the expected size band of ~600bp (Figure 2.8). Only one gel is presented.

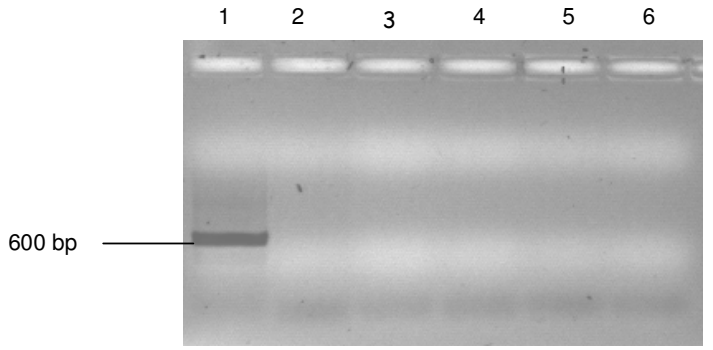


*Figure 2.8: Agarose gel electrophoresis of amplicons after PCR with MTR primer system. Lane 1- MTR recombinant plasmid, Lane 2- Buffer control, Lane 3-01/5008, Lane 4-01/5009, Lane 5-01/5011, Lane 6-01/2536*

Double stranded RNA extraction

Since the cDNA synthesis and both the PCRs were optimized, another RNA extraction method was tested. Double stranded RNA extraction was carried out on one sample known to be GFkV-infected, 03/3381, and a positive amplicon was obtained after cDNA synthesis and PCR with the MTR primer set (Figure 2.9). The yield of amplicon was not high but this system could be used.

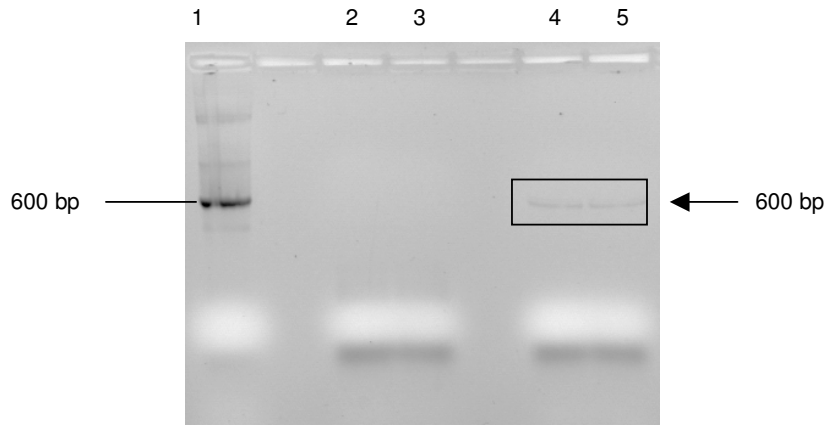


*Figure 2.9: Agarose gel electrophoresis of amplicons obtained from ds-RNA after PCR with MTR primer system. Lane 1- MTR recombinant plasmid, Lane 2+3- Buffer control, Lane 4+5- 03/3381*

## 2.3.3 USE OF AN ELISA TO TEST VARIOUS FIELD SAMPLES

A GFkV-specific ELISA kit was used to test Block A and Block B on Groenhof, Stellenbosch (Figure 2.2). No positive results were obtained from the field samples tested (Figure 2.10 and Figure 2.11).

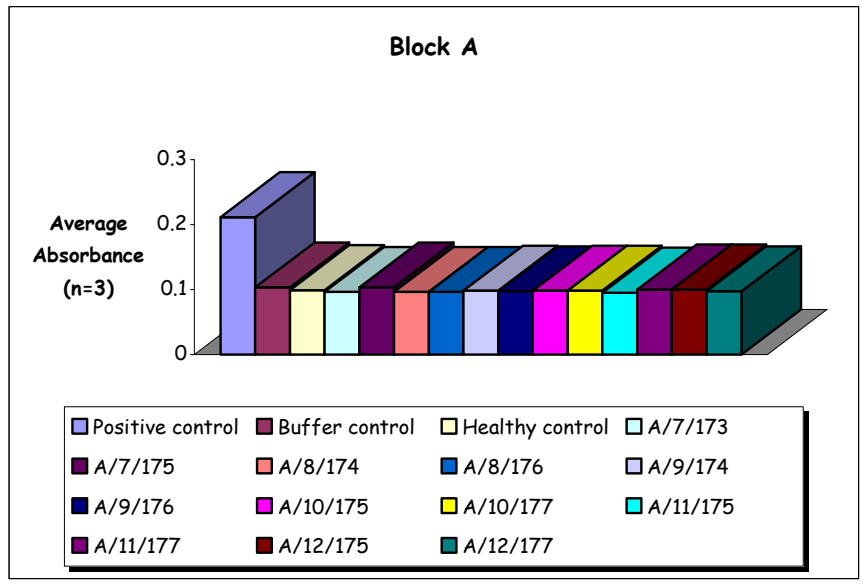


*Figure 2.10: Graph of the ELISA results done on block A using a GFkV specific kit*

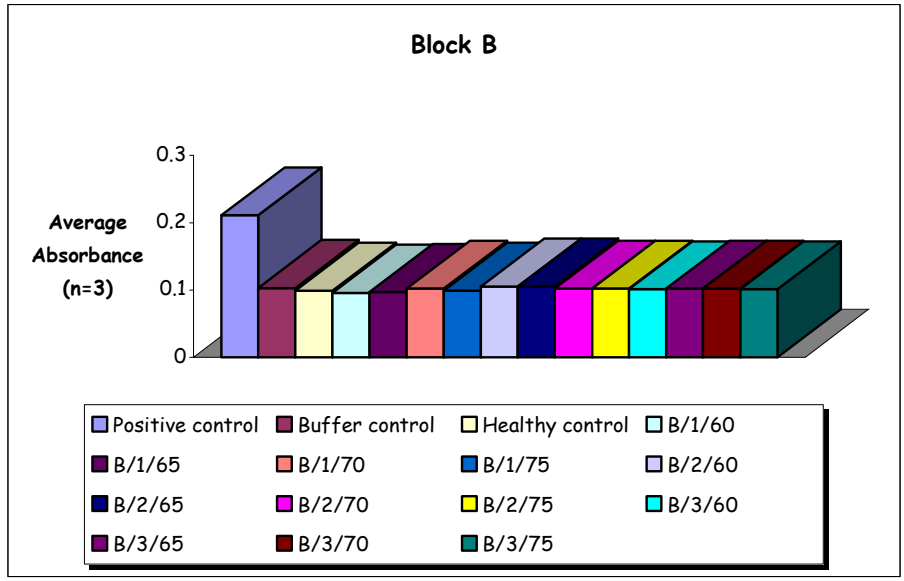


*Figure 2.11: Graph of the ELISA results done on Block B using a GFkV specific kit*

## 2.4 DISCUSSION

The re-infection of certified planting material with GLRaV-3 is a big concern for the wine industry in South Africa. During a project in 2001 to determine and identify leafroll-associated viruses spreading within certified grapevine material in South Africa and to develop/establish/implement means to detect these viruses, a number of unknown spherical virus-like particles were observed (Pietersen, unpublished). Since GFkV has been found in South Africa with evidence of spread (Engelbrecht and Kasdorf., 1990) and the morphology of GFkV correlated to that of the unknown spherical particles (Sabanadzovic *et al*., 2000), it was hypothesized that the unidentified spherical particles could be GFkV. Mechanical inoculation was done with the unidentified spherical particles to determine if they were mechanically transmissible. No local or systemic lesions could be noticed after a period of one month and it was concluded that the unidentified spherical particles were not mechanically transmissible. This finding supports the hypothesis that these agents may be GFkV, a virus well known to not be transmissible by mechanical inoculation (Sabanadzovic *et al*., 2000).

A commercial GFkV-specific ELISA kit was used to determine if these unknown spherical particles were GFkV. None of the 19 samples tested positive for GFkV, but this did not rule out the fact that GFkV could still be present in the plants. The low absorbance values obtained could be due to the lack of sensitivity to detect viruses present in low concentrations (Van der Merwe, 2001; Weber *et al*., 2002). Four plants situated at the ARC-PPRI did test positive for GFkV, and it was decided to include these plants as internal positive controls. Since no molecular detection method was available to test certified planting material for GFkV locally, two existing PCR detection methods were implemented in South Africa. The RdRp region of the GFkV genome has been used in previous phylogenetic studies (Sabanadzovic *et al*., 2000; El Beaino *et al*., 2001). In this study both the RdRp and the MTR region of the GFkV were included. The advantage of the MTR gene is that it is about

200bp longer than the RdRp gene, which makes it more suitable for phylogenetic studies.

The RdRp gene specific and MTR gene specific PCR methods, with higher sensitivity and specificity, were used to test these samples. No amplification could be obtained from the RdRp gene specific and MTR gene specific PCR, respectively. As samples 01/3380, 01/3381, 92/1028 and 98/0649 were known to be infected with GFkV, the RNA extraction method was suspected of being sub optimal. GFkV is generally present in low concentrations in the plant and the virus titres vary seasonally (Foundation Plant Services, 2002).

Since both the chloroform based RNA extraction method and the commercial RNA extraction kit delivered negative results, double stranded RNA extraction was done on sample 03/3381. Double stranded RNA is present in plants infected with RNA viruses (produced as an intermediate product) and is very resistant to enzymatic degradation (Valverde, 1990). The double stranded RNA template proved the most successful, as a positive amplicon of 600bp was obtained during the MTR gene specific PCR, but this method is labour intensive and too time consuming to use routinely. PCR inhibition could also be the reason for negative results during the previous two RNA extraction methods. The inhibition could be due to the presence of polyphenolic compounds present in grapevines (Koonjul *et al.,* 1998). These compounds are retained in plant vacuoles, but once the cells are broken open they are released and consequently co-purify with nucleic acids. It has been reported that these molecules inactivate certain enzymes, such as DNA polymerases (Koonjul *et al*., 1998)

During the field survey done on Groenhof farm to determine if GFkV spreads naturally through the field, a commercial GFkV-specific test was used. Even though none of the plants tested positive for GFkV, it cannot be concluded that GFkV is not present on Groenhof farm. Since the collection of the plants was done in November, early summer, the concentration of GFkV in the plants was low. Collection of plants must be done in early spring or late autumn (Weber *et al*., 2002).

Before the PCR method can be used as a routine diagnostic test, the RNA extraction method need to be optimized. Such optimization includes the dilution of possible inhibitors and using the appropriate parts of the plant, as the virus might be present in different concentrations throughout the plant. Future exploration of these PCR methods remain worthwhile since serological tests, like ELISA, cannot be applied to unknown particles and lacks the sensitivity to detect viruses when present in low concentrations (Weber *et al*., 2002; Van der Merwe, 2001). During this study two existing PCR methods were optimized and implemented for the detection of Grapevine Fleck Virus in South Africa, but the shortcomings in the preparation of RNA from sample plants have not allowed for a conclusion on the genetic similarities of the unidentified spherical particles with GFkV. Future work may include optimization of a rapid and reliable RNA extraction method. A field survey on Groenhof farm should be done in early spring and the optimized RNA extraction method together with the optimized MTR gene specific PCR system may be used to test the collected plants.

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# Chapter 3

# Implementation of an existing PCR detection system for phytoplasmas in South Africa

## 3.1 INTRODUCTION

Winetech, in conjunction the with Agricultural Research Council – Plant Protection Research Institute (ARC-PPRI), drafted a project to determine and identify leafroll-associated viruses spreading within certified grapevine material in South Africa and to develop/establish/implement means to detect these viruses (2001). From 2001 to 2004, motherblocks were surveyed for leafroll symptoms associated with Grapevine Leafroll Associated Virus Type 3 (GLRaV-3), a very serious pathogen of grapevine in South Africa. Motherblocks are open blocks in which the grapevines are being mass propagated and is monitored annually for any symptoms of known pathogens. Because mass production is done in open fields, leafroll re-infection frequently occurs. As reviewed in Alkowni *et al*., 2004, leafroll disease in grapevine alters fruit coloration and delays fruit maturation. Other symptoms include interveinal reddening in red grape varieties and yellowing in white grape varieties of late summer and fall leaves, downward rolling of leaves and phloem disruption (Alkowni *et al*., 2004).

A number of plants were observed with leafroll and reddening symptoms unlike classical grapevine leafroll disease. No grapevine Leafroll-associated viruses could be detected in these plants, and the common wisdom from the industry was that these symptoms were due to bark constrictions. In subsequent seasons, monitoring of the vineyards has shown that these plants slowly deteriorate and die. However, on a few occasions new symptomatic plants were found, sometimes in close proximity to the previous infected plants (Pietersen, unpublished), suggestive of an infectious agent. On recent, closer inspection of these symptoms, it appeared that in some instances the symptoms were similar to those induced by some phytoplasmas typically found in Europe (e.g. Flavescence doree and Bois noir) (Lee *et al*., 2000).

The symptoms included abnormal elongation of internodes, which resulted in slender shoots, generalized stunting and reddening of the leaves with downward curling. The diseases caused by both these phytoplasmas on

grapevines are destructive in Europe (EPPO/CABI, 1997) and the phytoplasmas are considered quarantine organisms as far as South Africa and some parts of Europe are concerned. It has long been feared that such an organism could enter South Africa. In order to prevent this, all imported material are subjected to heat treatment, however, no diagnostic tests were established to screen imported planting material for phytoplasmas.

In order to assess the possibility that these symptoms were due to phytoplasma infection, it was decided that a universal phytoplasma specific PCR needed to be established in South Africa. Because it is generally known that phytoplasma's occur in low titres in the plant (Schaff *et al*., 1992) and that polyphenolic compounds in the grapevine can act as inhibitors of the DNA polymerase (Koonjul *et al*., 1998), it was useful to establish a nested PCR, as this sequentially increases the target molecule and dilutes inhibitors (Webster *et al*., 2004). During this study, the P1/P7 as well as the 16R758f/m23Sr PCR were implemented in South Africa. Various samples with phytoplasma-like symptoms were collected and tested and it was found that 13 symptomatic plants yielded the expected amplicon during amplification and were sequenced. From these, nine were consistent with *Candidatus* Phytoplasma solani, two with common soil bacteria, one with Flavescence doree and one with Mollicutes from *Vitis vinifera*.

# 3.2 MATERIALS AND METHODS

## 3.2.1 Phytoplasma sources

The positive controls (ADN-J7 – Flavescence doree Isolate 92 DNA and ADN-I550 – STOL C DNA) used during the optimization of the PCRs, were total DNA from periwinkle infected with Flavescence doree (FD92) and Stolbur (STOLC), respectively. These samples were kindly supplied by Elizabeth Bourdon-Pardieu, Centre National De La Recherche Scientifique, France. Field samples were collected during winter 2005 (Appendix C) and are from various regions of the Western Cape, South Africa (Figure 3.1).

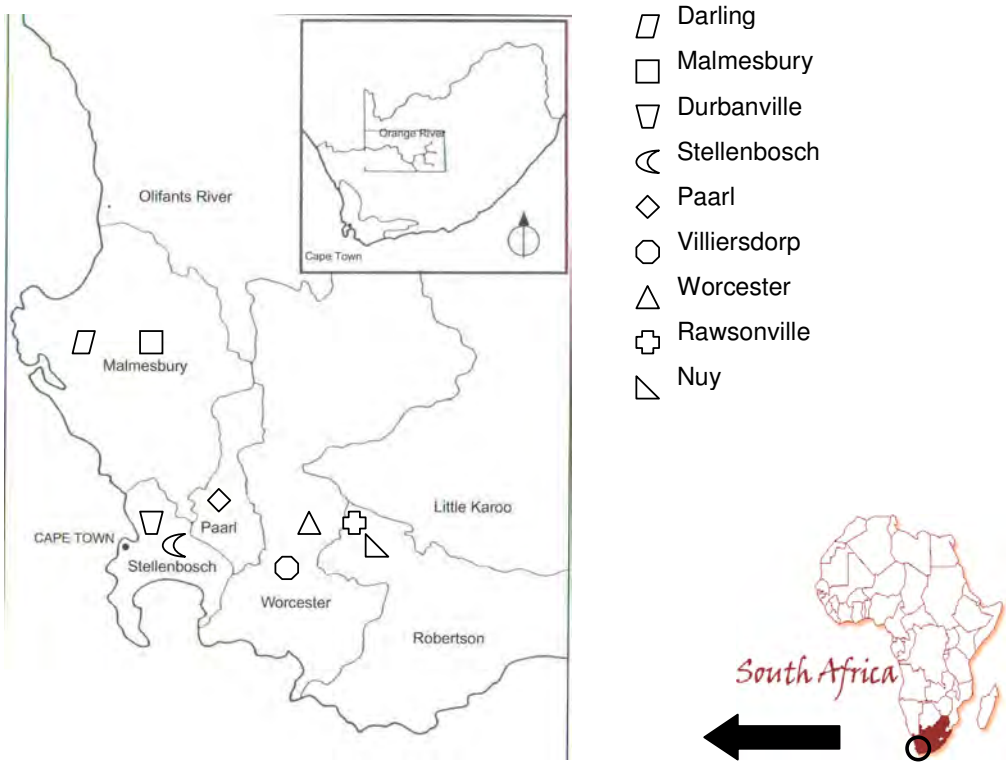


*Figure 3.1: Wine regions of South Africa, where field samples where collected (Image: SAWIS nr.29, 2005, with modifications).*

## 3.2.2 DNA extraction

DNA extractions were done based on the method described by Angelini *et al.* (2001). Briefly, 1g of petioles or cortical scrapings were placed into a maceration bag (Bioreba,) together with 5 ml of warm extraction buffer (2% CTAB, 10mM Tris-HCl pH8, 10mM EDTA, 1.4M NaCl, 0.2% 2-

mercaptoethanol). The contents of the bag were finely ground with a macerator (Homex 6, Bioreba) and the suspension (1ml) transferred to a 2ml Eppendorf tube. The suspension was incubated at 65°C for 20min, and thereafter allowed to cool for 3min. Chloroform was added to the tube at a 1:1 ratio and centrifuged at 14000xg for 10 min at room temperature. The chloroform extraction step was repeated. The upper aqueous phase was placed in a new 1ml Eppendorf tube. An equal volume of ice-cold iso-propanol was added and gently mixed. The solution was incubated overnight at room temperature and centrifuged at 14000xg for 10min. The supernatant was aspirated and 1ml 70% ethanol was added to the pellet. The pellet and ethanol were incubated for 20min at room temperature where after it was centrifuged at 14000xg for 5 min. After the supernatant was removed, the tubes were air dried at room temperature. The DNA pellet was resuspended in 150µl TE buffer.

### 3.2.3 PCR

The first primer pair used, P1 (Deng and Hiruki, 1991) and P7 (Smart *et al*., 1996), amplify 1800bp of the 16S rRNA gene extending through the 16S/23S spacer region and into the beginning of the 23S rRNA gene (Padovan *et al*., 1995). The second or nested primer pair used, 16R723f and m23Sr (Padovan *et al*., 1995), amplify 1076 bp, extending from the 16SR rRNA gene through the spacer region and into the start of the 23S rRNA gene (Padovan *et al*., 1995) (Figure 3.2).

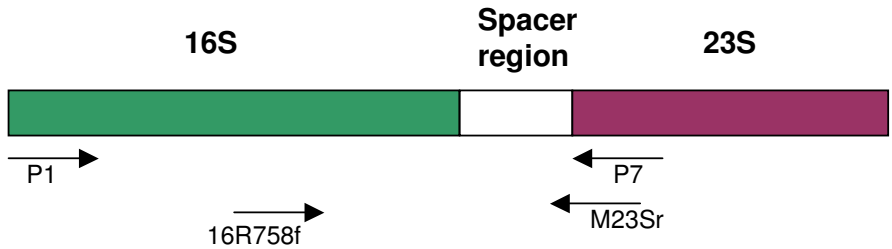


*Figure 3.2: Schematic representation of a phytoplasma rRNA operon, including the 16S and 23S rRNA genes and the intergenic spacer region. (Image: Smart et al., 1996, with slight modifications)*

The P1/P7 system (Smart *et al*., 1996) was done in a 25µl reaction using the following reagents as final concentrations: 10x Promega Reaction Buffer

(Promega Corporation, Wisconsin, USA), 3mM $MgCl_2$, 0.5mM dNTP mix, 0.5pmol P1 primer, 0.5pmol P7 primer, 1.5U/µl Taq DNA polymerase (Promega Corporation, Wisconsin, USA), 1µl DNA and distilled water.

The PCR conditions were as follows:

92℃ for 135 sec;
92℃ for 45 sec;
57℃ for 45 sec; } x 35 cycles
72℃ for 105 sec;

10µl of the PCR products were electrophoresed in a 1% (w/v) agarose gel at 100V in 1 x SB buffer (0.004% NaOH, 0.0023% Boric Acid, pH8). The agarose gels were pre-stained with ethidium bromide (0.5µg/ml) and the DNA fragments visualized by UV fluorescence.

The M23Sr/16R758f (nested) PCR was done using the conditions as described for the P1/P7 system. The universal P1/P7 primers were replaced with the forward primer, 16R758f, and reverse primer, M23Sr, and the template used in the nested PCR was 0.5µl PCR product from the first round.

### 3.2.4 Purification of PCR products

Purification was done as described in Section 2.2.1.4.

### 3.2.5 DNA sequencing

Sequencing was done as described in Section 2.2.1.10, using primers m23Sr and 16S758f. For each amplicon a forward and reverse sequencing reaction was done to confirm the results obtained.

## 3.3 RESULTS

PCR Optimization

Before testing could be done on field samples the PCR system had to be optimized, both for 1st and 2nd round of amplification. Different $MgCl_2$ concentrations were tested and amplicons of 1800bp and 1050bp were successfully amplified in the 1st and 2nd round, respectively. During optimization of the P1/P7 primer system (1st round), the best amplification was obtained using 3mM $MgCl_2$ in the PCR mixture. Optimal conditions for the m23Sr/16R758f primer system (nested) were the same PCR parameters and conditions as the P1/P7 system.

PCR on field samples

The field samples collected were tested in batches of 17 samples. None of the field samples yielded amplicons during the first round of amplification but the positive control gave the expected size amplicon of 1800bp. While this could mean that the samples did not contain phytoplasmas it could also be due to inhibition of the DNA polymerase, insufficient template DNA or low phytoplasma titers. During the 2nd (nested) round of amplification some of the field samples yielded amplicons of the expected size of 1050bp (Figure 3.3). A representative gel is shown in Figure 3.3.

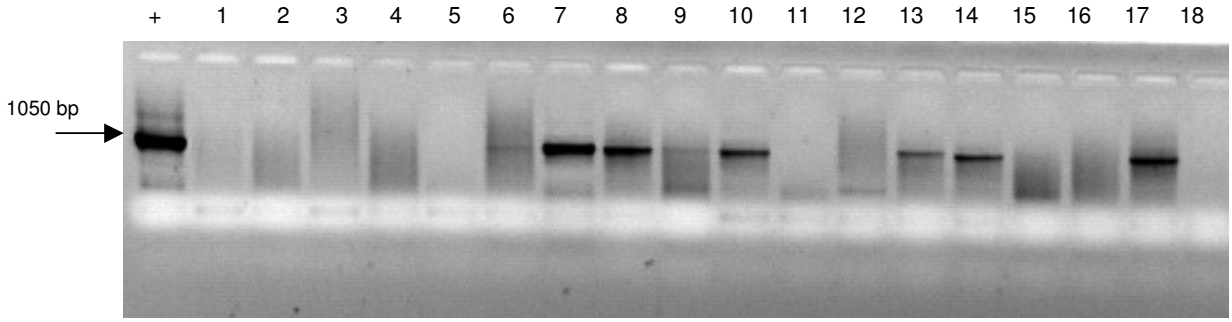


*Figure 3.3: Gel photo of 2nd round amplification using m23Sr/16R758f system. Lane +- Positive control (AND-J7), Lane 1- Sample 05/0019, Lane 2- Sample 05/0023, Lane 3- Sample 05/0036, Lane 4- Sample 05/0010, Lane 5- Sample 05/0035, Lane 6- Sample 05/0012, Lane 7- Sample 05/0033, Lane 8- Sample 05/0018, Lane 9- Sample 05/0024, Lane 10- Sample 05/0038, Lane 11- Sample 05/0020, Lane 12- Sample 05/0036, Lane 13- Sample 05/0022, Lane 14- Sample 05/0034, Lane 15- Sample 05/0021, Lane 16- Sample 05/0013, Lane 17- Sample 05/0028 and Lane 18- Buffer control*

Sequencing

Amplicons of the expected size from the nested PCR were purified using the Promega Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, Wisconsin, USA). The purified samples were sequenced using the m23Sr and 16R758 primer for each sample, to obtain a double set of sequence information for each sample. The forward and reverse sequences were trimmed and a consensus sequence was obtained. These consensus sequences were compared with known sequence information in the public domain (Genbank) in order to attempt gene-based identification of the sources of the amplicons.

Samples from 25 wine estates, representing 9 different geographical regions of the Western Cape, were tested. Of the 139 samples, 31 samples yielded amplicons of the expected size during the nested PCR. Thirteen of these samples were sequenced (Table A.3). The locations of the samples that yielded the expected size amplicon are shown in Figure 3.4.

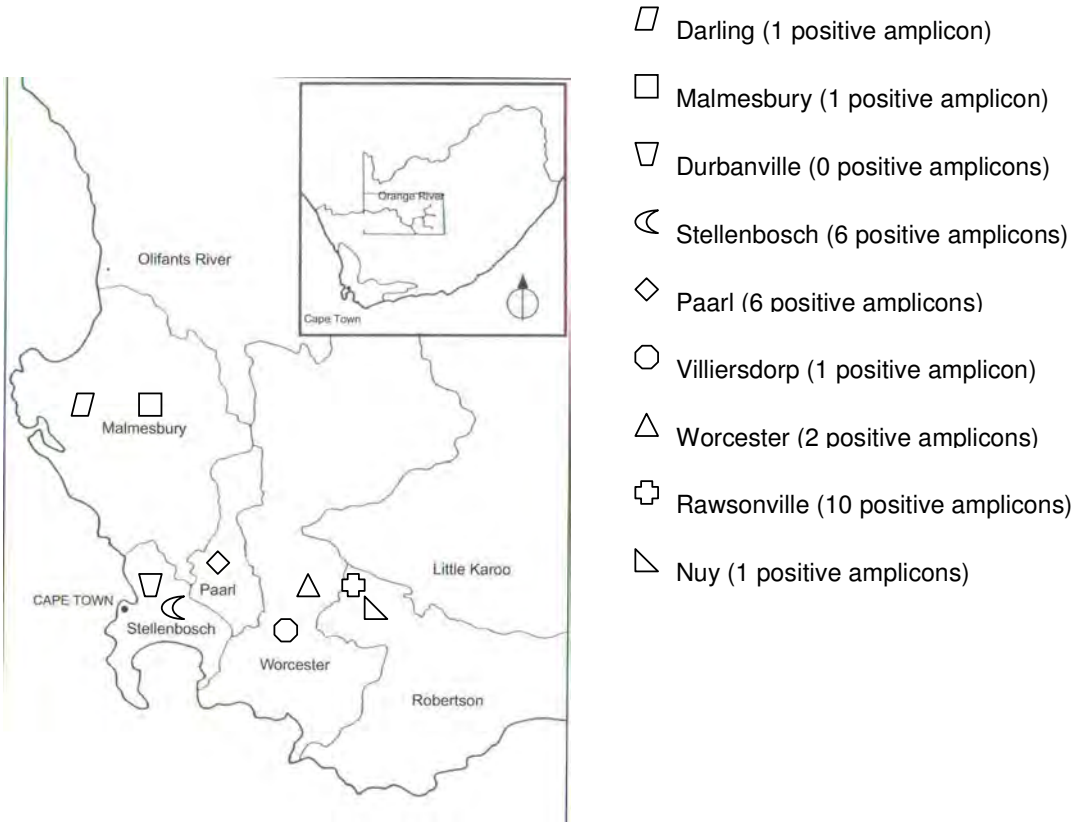


*Figure 3.4: Location of the positive amplicons obtained during nested PCR, shown on a map of South Africa (Image: SAWIS nr.29, 2005, with modifications).*

Pairwise comparisons were all done with a gap penalty of 20 and are shown in Appendix B. Eight samples yielded sequences similar to that of *Candidatus* Phytoplasma solani (Genbank accession no. AJ964960) (Torres *et al*., unpublished) (Table 3.1). *Candidatus* phytoplasma solani is the genus to which Stolbur is a member.

TABLE 3.1: **Similarity and position of samples to *Candidatus* phytoplasma solani**

| Sample | Similarity |
|---|---|
| 05-0014 | 99.23% |
| 05-0018 | 98.21% |
| 05-0038 | 99.00% |
| 05-0062 | 97.92% |
| 05-0102 | 98.93% |
| 05-0109 | 99.67% |
| 05-0111 | 98.82% |
| 05-0122 | 98.51% |
| 05-0127 | 99.22% |

Sample 05-0005 yielded a sequence identical (100%) to that of *Bacillus megaterium*, isolate AC46b1 (Genbank accession no. AJ717381) (Tiago *et al*., 2004). Sample 05-0077 yielded a sequence that was most similar (95.49%) to that of Mollicutes from *V. vinifera* (Genbank accession no. X76428) (Seemuller *et al*., 1994). Sample 05-0055 yielded an amplicon with a sequence most similar (97.59%) to that of uncultured bacterium clone 3 (Genbank accession no. DQ011250) (Wei *et al*., unpublished). However, one of the samples, 05-0033, yielded a sequence identical (100%) to that of Flavescence doree phytoplasma strain 1487 (Genbank accession no. AJ548787) (Torres *et al*., unpublished).

A multiple alignment (Appendix C) was done in DNAMAN with the sequences of the eighteen samples. From the multiple alignment a phylogenetic tree was constructed in DNAMAN, using 1000 bootstrap replicates (Figure 3.5).

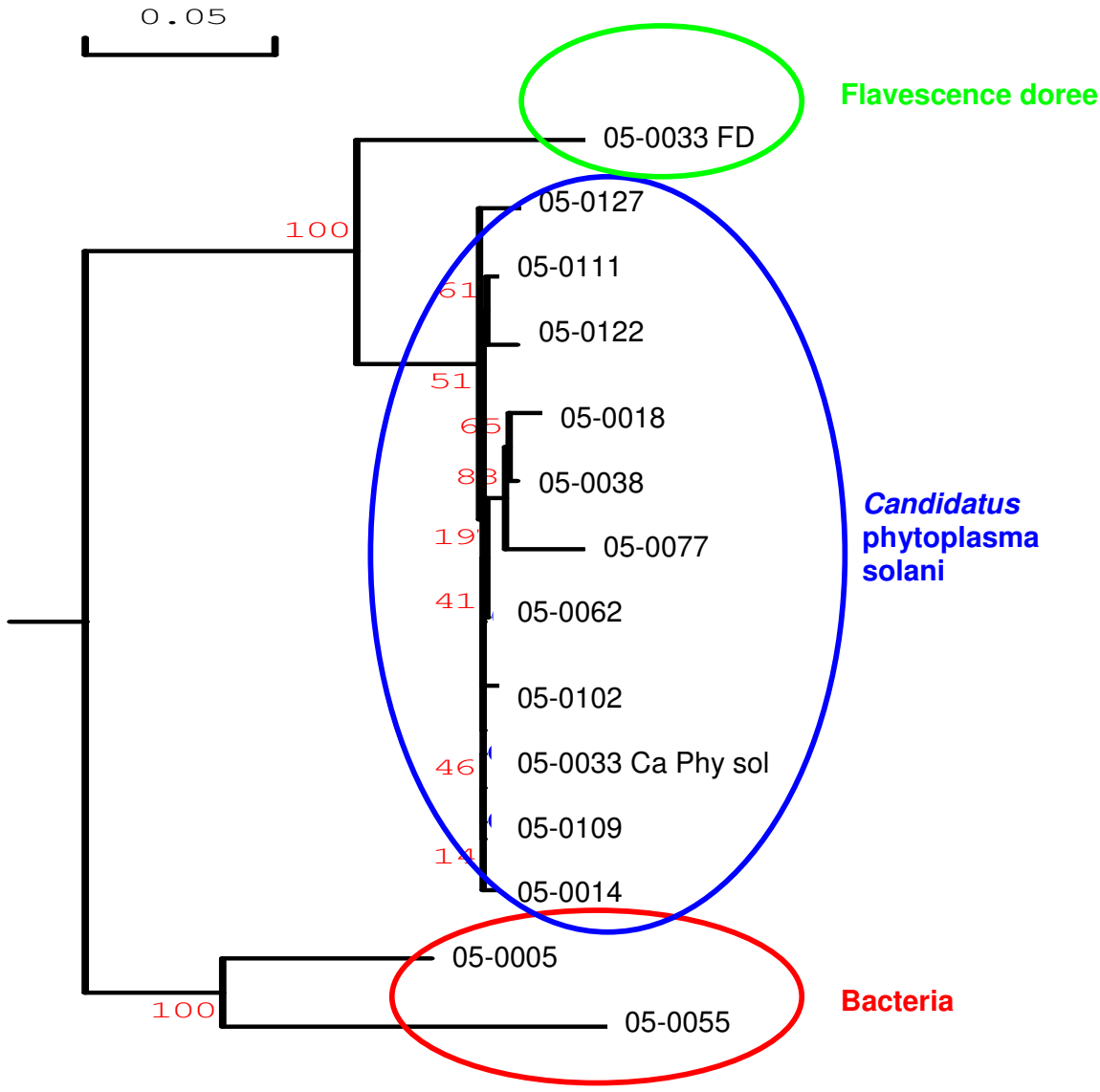


*Figure 3.5: Phylogenetic tree of thirteen samples sequenced. Bootstrap=1000.*

Sample 05-0033 was re-tested to confirm the results obtained due to the serious nature of this disease and phytosanitary implications of finding the etiological agent in South Africa. A positive amplicon of the expected size was obtained during the nested PCR, but on this occasion the amplicon had a

sequence most similar (99.67%) to that of *Candidatus* phytoplasma solani (Genbank accession no. AJ964960) (Torres *et al*., unpublished).
During the first DNA extraction procedure on sample 05-0033, that returned a sequence identical to Flavescence doree, petioles were used. During the second DNA extraction procedure however (*vide supra*), cortical scrapings were used. Both the petioles and cortical scrapings were collected during March 2005.

Some of the samples (05-0096, 05-0105, 05-0137, 05-0138 and 05-0139) presented smears after the nested amplification. Therefore, serial dilutions 1:10, 1:50 and 1:100 was done on the 1$^{st}$ round PCR products, serving as templates during the nested PCR, in order to reduce the concentration of the template. With the 1:100 dilution, amplicons of the expected size were obtained during the nested PCR, but the bands were too faint to purify and ultimately sequence.

## 3.4 DISCUSSION

From 2001 to 2004, a number of plants were observed with reddening symptoms, which appeared to be similar to symptoms induced by some phytoplasmas in Europe (Lee *et al*., 2000). These phytoplasmas are quarantine organisms in South Africa, but no detection method is available to screen imported planting material.

A universal nested PCR system (Deng and Hiruki, 1991; Smart *et al*., 1996; Padovan *et al*., 1995) was optimized and established in South Africa to detect phytoplasma, which can be used in the South African certification scheme for wine grapes. This optimized PCR was used in order to assess the possibility that the reddening symptoms observed were due to phytoplasma infection. During the first round of amplification, no amplification products could be observed when the plant material was used as template. This could be due to inhibitors present in the grapevine (Koonjul *et al*., 1998). However in spite of this, some of the plant samples gave the expected sized amplicon of 1050bp during the second round of amplification.

From 139 samples, amplicons of the expected size was obtained from 31 samples and 13 of these amplicons were sequenced. Nine of these samples yielded sequences that were most similar to that of *Candidatus* Phytoplasma solani. The phytoplasma belonging to this genus, Stolbur, is present all over the world and it is thought that grapevine may not be the original host of this pathogen and that this pathogen has accidentally been transmitted to grapevine from another host (EPPO/CABI, 1997).

Two samples were found to contain DNA most similar to that of common soil bacteria. Some saprophytic bacteria are present in soil, and the detection of these bacteria could be due to the universal nature of the primer pairs used. Different types of bacteria are present on the bark of the grapevine and since some of the samples did not show any lignification of the cane, the non-

lignified bark was used were normally the bark is removed and only the phloem is used during DNA extractions.

The finding that one of the samples contained DNA with a sequence identical to that of Flavescence doree phytoplasma was of particular concern. Flavescence doree only occurs in France, Italy and Spain (EPPO/CABI, 1997). Therefore, due to the phytosanitary implications of finding the organism in South Africa, the DNA extraction, nested PCR and sequencing was repeated. Surprisingly, the sequenced amplicon obtained from the repeated experiment was in this instance most consistent with *Candidatus* phytoplasma solani. However, different plant material components were used for the DNA extractions although the collection date was the same. Experimentally, a plant can be infected by more than one type of phytoplasma (Lee *et al*., 2000) and mixed infections in a single plant are evident in nature (Alma *et al*., 1996; Bianco *et al*., 1993; Lee *et al*., 2000). An alternative hypothesis however could be that a false positive was obtained from contaminating positive control. To investigate further, the Flavescence doree positive control for our experiments (ADN-J7) was sequenced and analyzed. It was found that the positive control corresponded with Flavescence doree strain 1487, rather than Flavescence doree isolate FD92 as previously thought, based on NCBI Genbank sequence archives. Therefore, the original amplicons and sequence obtained from the petiole sample 05-0033 may be due to laboratory contamination, since the sequence of the sample was identical to that of the Flavescence doree positive control used during the experiment (Appendix D). Therefore, the occurrence of Flavescence doree in sample 05-0033 is not likely due to a mixed infection, since the results could not be repeated and the sequence was identical to that of the positive control.

Sequence analysis of the amplicon obtained from sample 05-0077 demonstrated a high degree of similarity to that of Mollicutes from *V. vinifera* (Seemuller *et al*., 1994). This study suggests that the phytoplasma, *Candidatus* Phytoplasma solani, occur at many sites within the industry and may have been present in South Africa for a number years already. This is supported by the fact that sample 05-0127, from a Shiraz block established in

1977, was infected with *Candidatus* Phytoplasma solani. The disease does not seem to be spreading at most of the sites where the phytoplasmas were found, possibly due to the lack of an efficient vector. During the study, a PCR method was established to use for the routine detection of phytoplasmas in South Africa and phytoplasmas were associated with some of the symptomatic plants found. The *Candidatus* phytoplasma solani infected plants will be grafted onto periwinkle to maintain the sources and to prove Koch's postulates.

Even though Boudon-Padieu *et al*. (2003) highly recommends the current DNA extraction method, this method is too time-consuming for use during routine inspection and only a small portion of plant material can be tested. This poses a problem since phytoplasma infection may be limited to a certain part of the plant or double infection may occur and only the pre-dominant strain will be selected (Constable *et al*., 2003). The current PCR system is not optimal, since a nested PCR has to be performed to eliminate the inhibitory effect of the polyphenolic compounds (Webster *et al*., 2004). In addition, even when a positive amplicon is obtained, RFLP or nucleotide sequencing has to be done to confirm phytoplasmal infection, because of the universal nature of the primers. A multiplex nested PCR described by Clair *et al*. (2003) has been optimized and established in South Africa (Koch, unpublished) using specific primers for Flavescence doree and Stolbur, but the method is limited to these two organisms only and since the PCR has to be used in the certification scheme, the PCR has to detect all possible phytoplasmas.

A full-scale field survey is planned for all the grapevine production areas of South Africa, to determine the extent of this phytoplasma infection as well as spread, if any, of the phytoplasma. Should evidence of spread be observed, vector studies will be conducted. This survey might shed more light on the possibility that Shiraz disease might be caused by the phytoplasma detected in this study, as the two diseases elicit similar symptoms (Goszczynski and Jooste, 2003; EPPO/CABI, 1997). The possibly that the Stolbur phytoplasma can be responsible for Shiraz decline can also be explored since recent

studies has shown the presence of Stolbur phytoplasma in certain plants displaying Shiraz decline symptoms (Renault-Spilmont *et al.*, 2005). This is the first report of *Candidatus* Phytoplasma solani infecting grapevine in South Africa.

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## APPENDIX A.1

### TABLE A.1: **Field samples for testing in PCR**

| Accession number | Region | Estate | Cultivar clones And Rootstocks | Plant Year | Symptoms April/May 2002 |
|---|---|---|---|---|---|
| 01/2536 | Paarl | La Concorde | Merlot | 1992 | Leafroll |
| 01/2581 | Paarl | La Concorde | Merlot x Richter 101-14 | 1992 | Leafroll |
| 01/2639 | Paarl | La Concorde | Merlot x Richter 101-14 | 1992 | Healthy |
| 01/2673 | Stellenbosch | Rust + Vrede | Cabernet sauvignon x Richter 101-14 | 1997 | Leafroll |
| 01/2751 | Paarl | Klompzicht | Cabernet sauvignon x Richter 99 | 1997 | Leafroll |
| 01/2803 | Wellington | Soetendal | Pinotage x Richter 110 | 1995 | Leafroll |
| 01/2828 | Wellington | Soetendal | Pinotage x Richter 110 | 1995 | Leafroll |
| 01/2852 | Paarl | Picardi | Cabernet sauvignon x Richter 99 | 1998 | Leafroll |
| 01/2857 | Paarl | Picardi | Cabernet sauvignon x Richter 99 | 1998 | Leafroll |
| 01/2898 | Paarl | Plasir de Merle | Cabernet sauvignon | 1997 | Healthy |
| 01/2906 | Worcester | Memel | Shiraz x Richter 99 | 1993 | Leafroll |
| 01/2931 | Worcester | Meerlust | Pinotage x Richter 99 | 1997 | Leafroll |
| 01/2997 | Worcester | Merwida | Merlot x Richter 99 | 1994 | Healthy |
| 01/2999 | Worcester | Merwida | Merlot x Richter 99 | 1994 | Shiraz Disease |
| 01/5014 | Worcester | Merwida | Merlot x Richter 99 | 1994 | Leafroll |
| 01/2994 | Worcester | Merwida | Merlot x Richter 99 | 1994 | Leafroll |
| 01/2998 | Worcester | Merwida | Merlot x Richter 99 | 1994 | Leafroll |
| 01/2719 | Stellenbosch | Fransmanskraal | Cabernet sauvignon x Richter 110-14 | 1996 | Leafroll |
| 01/5007 | Worcester | Merwida | Merlot x Richter 99 | 1994 | Leafroll |
| 01/5008 | Worcester | Merwida | Merlot x Richter 99 | 1994 | Leafroll |
| 01/5009 | Worcester | Merwida | Merlot x Richter 99 | 1994 | Shiraz Disease |
| 01/5011 | Worcester | Merwida | Merlot x Richter 99 | 1994 | Leafroll |
| 92/1028 | Stellenbosch | Groenhof | Cabernet sauvignon | - | - |
| 98/0649 | Import number: I2979 | | Vitis rupestris St. George | - | - |

| Accession number | Region | Estate | Cultivar clones And Rootstocks | Plant Year | Symptoms April/May 2002 |
|---|---|---|---|---|---|
| 01/3380 | Import number: I2130 | | Michele Paliere | - | - |
| 01/3381 | Import number: I2133 | | Nedeltheff | - | - |

## APPENDIX A.2

### TABLE A.2: Field samples from Groenhof farm

| Block, Row, Plant position | Region | Estate | Cultivar | Plant Year |
|---|---|---|---|---|
| A/7/173 | Stellenbosch | Groenhof | Shiraz x Richter 99 | 1993 |
| A/7/175 | Stellenbosch | Groenhof | Shiraz x Richter 99 | 1993 |
| A/8/174 | Stellenbosch | Groenhof | Shiraz x Richter 99 | 1993 |
| A/8/176 | Stellenbosch | Groenhof | Shiraz x Richter 99 | 1993 |
| A/9/174 | Stellenbosch | Groenhof | Shiraz x Richter 99 | 1993 |
| A/9/176 | Stellenbosch | Groenhof | Shiraz x Richter 99 | 1993 |
| A/10/175 | Stellenbosch | Groenhof | Shiraz x Richter 99 | 1993 |
| A/10/177 | Stellenbosch | Groenhof | Shiraz x Richter 99 | 1993 |
| A/11/175 | Stellenbosch | Groenhof | Shiraz x Richter 99 | 1993 |
| A/11/177 | Stellenbosch | Groenhof | Shiraz x Richter 99 | 1993 |
| A/12/175 | Stellenbosch | Groenhof | Shiraz x Richter 99 | 1993 |
| A/12/177 | Stellenbosch | Groenhof | Shiraz x Richter 99 | 1993 |
| B/1/60 | Stellenbosch | Groenhof | Merlot x Richter 99 | 2000 |
| B/1/65 | Stellenbosch | Groenhof | Merlot x Richter 99 | 2000 |
| B/1/70 | Stellenbosch | Groenhof | Merlot x Richter 99 | 2000 |
| B/1/75 | Stellenbosch | Groenhof | Merlot x Richter 99 | 2000 |
| B/2/60 | Stellenbosch | Groenhof | Merlot x Richter 99 | 2000 |
| B/2/65 | Stellenbosch | Groenhof | Merlot x Richter 99 | 2000 |
| B/2/70 | Stellenbosch | Groenhof | Merlot x Richter 99 | 2000 |
| B/2/75 | Stellenbosch | Groenhof | Merlot x Richter 99 | 2000 |
| B/3/60 | Stellenbosch | Groenhof | Merlot x Richter 99 | 2000 |
| B/3/65 | Stellenbosch | Groenhof | Merlot x Richter 99 | 2000 |
| B/3/70 | Stellenbosch | Groenhof | Merlot x Richter 99 | 2000 |
| B/3/75 | Stellenbosch | Groenhof | Merlot x Richter 99 | 2000 |

## APPENDIX A.3

## TABLE a.3: Field samples collected during 2005

| Accession number | Date collected | Location | PCR results | Sequencing results | Direct pairwise comparison | BLAST Accession number |
|---|---|---|---|---|---|---|
| 05-0001 | May-05 | Merwida, Rawsonville | + | NS* | | |
| 05-0002 | May-05 | Merwida, Rawsonville | + | NS* | | |
| 05-0003 | May-05 | Merwida, Rawsonville | + | NS* | | |
| 05-0004 | May-05 | Merwida, Rawsonville | + | NS* | | |
| 05-0005 | May-05 | Merwida, Rawsonville | + | Bacillus megaterium 16S rRNA gene, isolate AC 46 b1 | 100% | AJ717381 |
| 05-0006 | May-05 | Merwida, Rawsonville | + | NS* | | |
| 05-0007 | May-05 | Merwida, Rawsonville | - | | | |
| 05-0008 | May-05 | Merwida, Rawsonville | + | NS* | | |
| 05-0009 | May-05 | Merwida, Rawsonville | + | NS* | | |
| 05-0010 | Mar-05 | Merwida, Rawsonville | - | | | |
| 05-0011 | May-05 | Merwida, Rawsonville | - | | | |
| 05-0012 | Mar-05 | Merwida, Rawsonville | - | | | |
| 05-0013 | Mar-05 | Rust en Vrede, Stellenbosch | - | | | |
| 05-0014 | May-05 | Rust en Vrede, Stellenbosch | + | *Candidatus* phytoplasma solani 16rRNA gene, partial 16S-23S IGS and $tRNA^{ile}$ gene | 99.23% | AJ964960 |
| 05-0015 | May-05 | Rust en Vrede, Stellenbosch | - | | | |
| 05-0016 | May-05 | Rust en Vrede, Stellenbosch | - | | | |
| 05-0017 | May-05 | Rust en Vrede, Stellenbosch | - | | | |
| 05-0018 | Mar-05 | Rust en Vrede, Stellenbosch | + | *Candidatus* phytoplasma solani 16rRNA gene, partial 16S-23S IGS and $tRNA^{ile}$ gene | 98.21% | AJ964960 |
| 05-0019 | Mar-05 | Rust en Vrede, Stellenbosch | - | | | |
| 05-0020 | Mar-05 | Rust en Vrede, Stellenbosch | - | | | |
| 05-0021 | Mar-05 | Rust en Vrede, Stellenbosch | - | | | |
| 05-0022 | Mar-05 | Rust en Vrede, Stellenbosch | + | NS* | | |
| 05-0023 | Mar-05 | Rust en Vrede, Stellenbosch | - | | | |
| 05-0024 | Mar-05 | Rust en Vrede, Stellenbosch | - | | | |
| 05-0025 | May-05 | Rust en Vrede, Stellenbosch | - | | | |
| 05-0026 | May-05 | Rust en Vrede, Stellenbosch | - | | | |
| 05-0027 | May-05 | Rust en Vrede, Stellenbosch | - | | | |
| 05-0028 | Mar-05 | La concordia, Paarl | + | NS* | | |
| 05-0029 | May-05 | Freedom Hill, Paarl | + | NS* | | |
| 05-0030 | May-05 | Freedom Hill, Paarl | - | | | |
| 05-0031 | May-05 | Freedom Hill, Paarl | - | | | |
| 05-0032 | May-05 | Freedom Hill, Paarl | - | | | |

| Accession number | Date collected | Location | PCR results | Sequencing results | Direct pairwise comparison | BLAST Accession number |
|---|---|---|---|---|---|---|
| 05-0033 | Mar-05 | Freedom Hill, Paarl | + | Flavescence doree phytoplasma isolate, 16S rRNA partial, 16S-23S IGS complete and 23 S ribosomal partial, strain 1487 | 100% | AF548787 |
| 05-0034 | Mar-05 | Freedom Hill, Paarl | + | NS* | | |
| 05-0035 | Mar-05 | Freedom Hill, Paarl | - | | | |
| 05-0036 | Mar-05 | Freedom Hill, Paarl | - | | | |
| 05-0037 | Mar-05 | Freedom Hill, Paarl | - | | | |
| 05-0038 | Mar-05 | Freedom Hill, Paarl | + | *Candidatus* phytoplasma solani 16rRNA gene, partial 16S-23S IGS and $tRNA^{ile}$ gene | 99% | AJ964960 |
| 05-0039 | May-05 | Nietvoorbij, Stellenbosch | - | | | |
| 05-0040 | May-05 | Nietvoorbij, Stellenbosch | - | | | |
| 05-0041 | May-05 | Du Toitskloof wine cellar, Rawsonville | - | | | |
| 05-0042 | May-05 | Kanonkop, Stellenbosch | - | | | |
| 05-0043 | May-05 | Location not known | - | | | |
| 05-0044 | May-05 | Location not known | - | | | |
| 05-0045 | May-05 | Location not known | - | | | |
| 05-0046 | May-05 | Location not known | - | | | |
| 05-0047 | May-05 | Location not known | - | | | |
| 05-0048 | Jun-05 | Overgaauw, Stellenbosch | - | | | |
| 05-0049 | Jun-05 | Overgaauw, Stellenbosch | - | | | |
| 05-0050 | Jun-05 | Overgaauw, Stellenbosch | - | | | |
| 05-0051 | Jun-05 | Overgaauw, Stellenbosch | - | | | |
| 05-0052 | Jun-05 | Overgaauw, Stellenbosch | - | | | |
| 05-0053 | Jun-05 | Overgaauw, Stellenbosch | - | | | |
| 05-0054 | Jun-05 | Cloof Wine Estate, Darling | - | | | |
| 05-0055 | Jun-05 | Cloof Wine Estate, Darling | + | Uncultured bacteria clone 3 16S rRNA gene and partial 16S-23S IGS | 97.59% | DQ011250 |
| 05-0056 | Jun-05 | Cloof Wine Estate, Darling | - | | | |
| 05-0057 | Jun-05 | Cloof Wine Estate, Darling | - | | | |
| 05-0058 | Jun-05 | Cloof Wine Estate, Darling | - | | | |
| 05-0059 | Jun-05 | Cloof Wine Estate, Darling | - | | | |
| 05-0060 | Jun-05 | Cloof Wine Estate, Darling | - | | | |
| 05-0061 | Jun-05 | Cloof Wine Estate, Darling | - | | | |
| 05-0062 | Jun-05 | La Rhine, Malmesbury | + | *Candidatus* phytoplasma solani 16rRNA gene, partial 16S-23S IGS and $tRNA^{ile}$ gene | 97.92% | AJ964960 |
| 05-0063 | Jun-05 | La Rhine, Malmesbury | - | | | |

| Accession number | Date collected | Location | PCR results | Sequencing results | Direct pairwise comparison | BLAST Accession number |
|---|---|---|---|---|---|---|
| 05-0064 | Jun-05 | La Rhine, Malmesbury | - | | | |
| 05-0065 | Jun-05 | La Rhine, Malmesbury | - | | | |
| 05-0066 | Jun-05 | La Rhine, Malmesbury | - | | | |
| 05-0067 | Jun-05 | La Rhine, Malmesbury | - | | | |
| 05-0068 | Jun-05 | Maastricht, Durbanville | - | | | |
| 05-0069 | Jun-05 | Maastricht, Durbanville | - | | | |
| 05-0070 | Jun-05 | Maastricht, Durbanville | - | | | |
| 05-0071 | Jun-05 | Maastricht, Durbanville | - | | | |
| 05-0072 | Jun-05 | Maastricht, Durbanville | - | | | |
| 05-0073 | Jun-05 | Maastricht, Durbanville | - | | | |
| 05-0074 | Jun-05 | Nitida, Durbanville | - | | | |
| 05-0075 | Jun-05 | Nitida, Durbanville | - | | | |
| 05-0076 | Jun-05 | Nitida, Durbanville | - | | | |
| 05-0077 | Jun-05 | Leipzig, Nuy | + | Mollicutes (from Vitis vinifera) 16S rRNA gene | 95.49% | X76428 |
| 05-0078 | Jun-05 | Leipzig, Nuy | - | | | |
| 05-0079 | Jun-05 | Leipzig, Nuy | - | | | |
| 05-0080 | Jun-05 | Leipzig, Nuy | - | | | |
| 05-0081 | Jun-05 | Leipzig, Nuy | - | | | |
| 05-0082 | Jun-05 | Leipzig, Nuy | - | | | |
| 05-0083 | Jun-05 | Leipzig, Nuy | - | | | |
| 05-0084 | Jun-05 | Leipzig, Nuy | - | | | |
| 05-0085 | Jun-05 | Leipzig, Nuy | - | | | |
| 05-0086 | Jun-05 | Du Vlei, Nuy | - | | | |
| 05-0087 | Jun-05 | Du Vlei, Nuy | - | | | |
| 05-0088 | Jun-05 | Du Vlei, Nuy | - | | | |
| 05-0089 | Jun-05 | Vrede, Worcester | - | | | |
| 05-0090 | Jun-05 | Vrede, Worcester | - | | | |
| 05-0091 | Jun-05 | Vrede, Worcester | - | | | |
| 05-0092 | Jun-05 | Vrede, Worcester | - | | | |
| 05-0093 | Jun-05 | Nooitgedacht, Worcester | NT* | | | |
| 05-0094 | Jun-05 | Nooitgedacht, Worcester | - | | | |
| 05-0095 | Jun-05 | Nooitgedacht, Worcester | - | | | |
| 05-0096 | Jun-05 | Nooitgedacht, Worcester | + | NS* | | |
| 05-0097 | Jun-05 | Statyn, Villiersdorp | NT* | | | |
| 05-0098 | Jun-05 | Statyn, Villiersdorp | - | | | |
| 05-0099 | Jun-05 | Statyn, Villiersdorp | - | | | |
| 05-0100 | Jun-05 | Statyn, Villiersdorp | - | | | |
| 05-0101 | Jun-05 | Statyn, Villiersdorp | - | | | |

| Accession number | Date collected | Location | PCR results | Sequencing results | Direct pairwise comparison | BLAST Accession number |
|---|---|---|---|---|---|---|
| 05-0102 | Jun-05 | Statyn, Villiersdorp | + | *Candidatus* phytoplasma solani 16rRNA gene, partial 16S-23S IGS and $tRNA^{ile}$ gene | 98.93% | AJ964960 |
| 05-0103 | Jun-05 | Statyn, Villiersdorp | NT* | | | |
| 05-0104 | Jun-05 | Tawny Acres, Worcester | - | | | |
| 05-0105 | Jun-05 | Tawny Acres, Worcester | + | NS* | | |
| 05-0106 | Jun-05 | Tawny Acres, Worcester | - | | | |
| 05-0107 | Jun-05 | Tawny Acres, Worcester | - | | | |
| 05-0108 | Jun-05 | Bothasguns, Rawsonville | - | | | |
| 05-0109 | Jun-05 | Bothasguns, Rawsonville | + | *Candidatus* phytoplasma solani 16rRNA gene, partial 16S-23S IGS and $tRNA^{ile}$ gene | 99.67% | AJ964960 |
| 05-0110 | Jun-05 | Bothasguns, Rawsonville | - | | | |
| 05-0111 | Jun-05 | Bothasguns, Rawsonville | + | *Candidatus* phytoplasma solani 16rRNA gene, partial 16S-23S IGS and $tRNA^{ile}$ gene | 98.82% | AJ964960 |
| 05-0112 | Jun-05 | Bothasguns, Rawsonville | - | | | |
| 05-0113 | Jun-05 | Hartenberg, Stellenbosch | - | | | |
| 05-0114 | Jun-05 | Hartenberg, Stellenbosch | - | | | |
| 05-0115 | Jun-05 | Hartenberg, Stellenbosch | - | | | |
| 05-0116 | Jun-05 | Hartenberg, Stellenbosch | - | | | |
| 05-0117 | Jun-05 | Hartenberg, Stellenbosch | NT* | | | |
| 05-0118 | Jun-05 | Hartenberg, Stellenbosch | - | | | |
| 05-0119 | Jun-05 | Hartenberg, Stellenbosch | - | | | |
| 05-0120 | Jun-05 | Hartenberg, Stellenbosch | - | | | |
| 05-0121 | Jun-05 | Elsenberg, Stellenbosch | - | | | |
| 05-0122 | Jun-05 | Elsenberg, Stellenbosch | + | *Candidatus* phytoplasma solani 16rRNA gene, partial 16S-23S IGS and $tRNA^{ile}$ gene | 98.51% | AJ964960 |
| 05-0123 | Jun-05 | Elsenberg, Stellenbosch | - | | | |
| 05-0124 | Jun-05 | Elsenberg, Stellenbosch | + | NS* | | |
| 05-0125 | Jun-05 | Elsenberg, Stellenbosch | - | | | |
| 05-0126 | Jun-05 | Elsenberg, Stellenbosch | - | | | |
| 05-0127 | Jun-05 | Lievland, Stellenbosch | + | *Candidatus* phytoplasma solani 16rRNA gene, partial 16S-23S IGS and $tRNA^{ile}$ gene | 99.22% | AJ964960 |
| 05-0128 | Jun-05 | Lievland, Stellenbosch | - | | | |
| 05-0129 | Jun-05 | Plaisir de merle, Paarl | + | NS* | | |
| 05-0130 | Jun-05 | Plaisir de merle, Paarl | - | | | |

| Accession number | Date collected | Location | PCR results | Sequencing results | Direct pairwise comparison | BLAST Accession number |
|---|---|---|---|---|---|---|
| 05-0131 | Jun-05 | Plaisir de merle, Paarl | - | | | |
| 05-0132 | Jun-05 | Plaisir de merle, Paarl | - | | | |
| 05-0133 | Jun-05 | Zorgvliet, Stellenbosch | - | | | |
| 05-0134 | Jun-05 | Blaauwklippen, Stellenbosch | - | | | |
| 05-0135 | Jun-05 | Cloof Wine Estate, Darling | - | | | |
| 05-0136 | May-05 | Vredendal | - | | | |
| 05-0137 | Aug-05 | Location not known | + | NS* | | |
| 05-0138 | Aug-05 | Location not known | + | NS* | | |
| 05-0139 | Aug-05 | Location not known | + | NS* | | |

* NT: Not tested, NS: Not sequenced

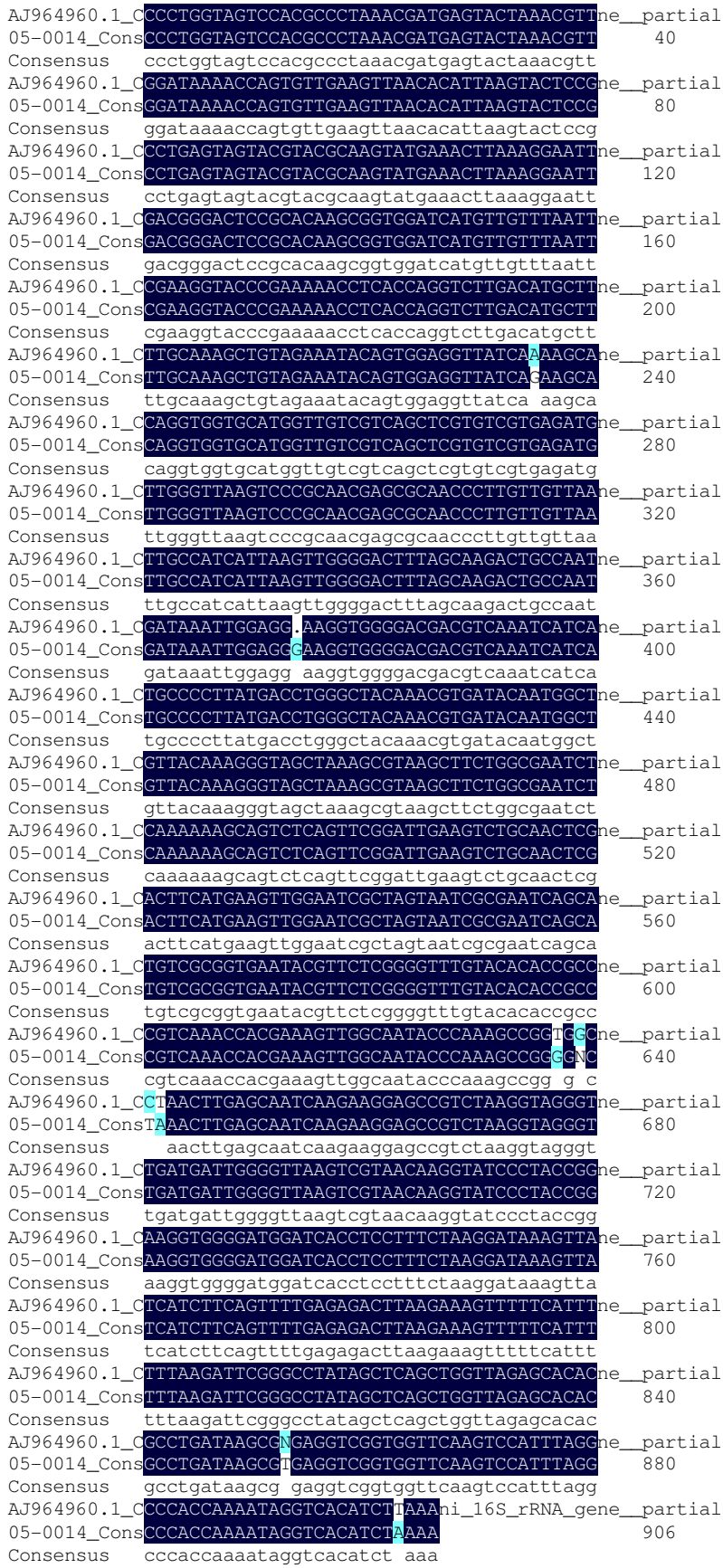
## APPENDIX B

## Pairwise comparisons of samples sequenced

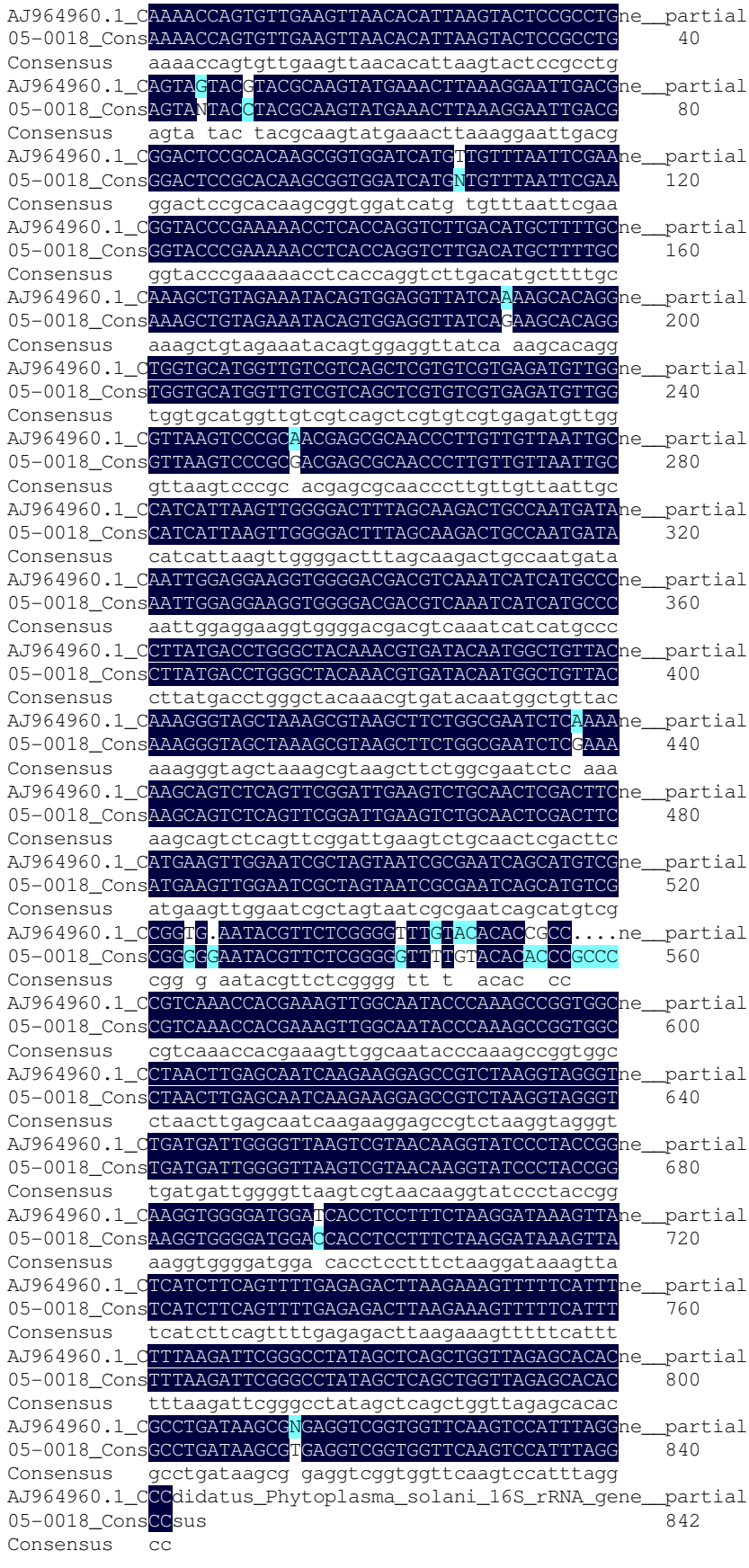
*Direct pairwise comparison of sample 05-0005 with Bacillus megaterium, isolate AC46b1 (Genbank accession no. AJ717381)*

```
AJ717381.1_BAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGe_AC46b1
05-0005_ConsAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAG     40
Consensus   aacgatgagtgctaagtgttagagggtttccgcccttag
AJ717381.1_BTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTAGe_AC46b1
05-0005_ConsTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTAC     80
Consensus   tgctgcagctaacgcattaagcactccgcctggggagtac
AJ717381.1_BGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGe_AC46b1
05-0005_ConsGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCG    120
Consensus   ggtcgcaagactgaaactcaaaggaattgacgggggcccg
AJ717381.1_BCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGe_AC46b1
05-0005_ConsCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCG    160
Consensus   cacaagcggtggagcatgtggtttaattcgaagcaacgcg
AJ717381.1_BAAGAACCTTACCAGGTCTTGACATCCTCTGACAACTCTAGe_AC46b1
05-0005_ConsAAGAACCTTACCAGGTCTTGACATCCTCTGACAACTCTAG    200
Consensus   aagaaccttaccaggtcttgacatcctctgacaactctag
AJ717381.1_BAGATAGAGCGTTCCCCTTCGGGGGACAGAGTGACAGGTGGe_AC46b1
05-0005_ConsAGATAGAGCGTTCCCCTTCGGGGGACAGAGTGACAGGTGG    240
Consensus   agatagagcgttcccccttcgggggacagagtgacaggtgg
AJ717381.1_BTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTe_AC46b1
05-0005_ConsTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTT    280
Consensus   tgcatggttgtcgtcagctcgtgtcgtgagatgttgggtt
AJ717381.1_BAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGe_AC46b1
05-0005_ConsAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAG    320
Consensus   aagtcccgcaacgagcgcaacccttgatcttagttgccag
AJ717381.1_BCATTTAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACe_AC46b1
05-0005_ConsCATTTAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAAC    360
Consensus   catttagttgggcactctaaggtgactgccggtgacaaac
AJ717381.1_BCGG.AGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTe_AC46b1
05-0005_ConsCGGCAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCT    400
Consensus   cgg aggaaggtggggatgacgtcaaatcatcatgcccct
AJ717381.1_BTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAAe_AC46b1
05-0005_ConsTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAA    440
Consensus   tatgacctgggctacacacgtgctacaatggatggtacaa
AJ717381.1_BAGGGCTGCAAGACCGCGAGGTCAAGCCAATCCCATAAAACe_AC46b1
05-0005_ConsAGGGCTGCAAGACCGCGAGGTCAAGCCAATCCCATAAAAC    480
Consensus   agggctgcaagaccgcgaggtcaagccaatcccataaaac
AJ717381.1_BCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGe_AC46b1
05-0005_ConsCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATG    520
Consensus   cattctcagttcggattgtaggctgcaactcgcctacatg
AJ717381.1_BAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGe_AC46b1
05-0005_ConsAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGG    560
Consensus   aagctggaatcgctagtaatcgcggatcagcatgccgcgg
AJ717381.1_BTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACe_AC46b1
05-0005_ConsTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACAC    600
Consensus   tgaatacgttcccgggccttgtacacaccgcccgtcacac
AJ717381.1_BCACGAGAGTTTGTAACACCCGAAGTCGGTGGAGTAACCGTe_AC46b1
05-0005_ConsCACGAGAGTTTGTAACACCCGAAGTCGGTGGAGTAACCGT    640
Consensus   cacgagagtttgtaacacccgaagtcggtggagtaaccgt
AJ717381.1_BAAGGAGCTAGCCGCCTAAGGTGGGACAGATGATTGGGGTGe_AC46b1
05-0005_ConsAAGGAGCTAGCCGCCTAAGGTGGGACAGATGATTGGGGTG    680
Consensus   aaggagctagccgcctaaggtgggacagatgattggggtg
AJ717381.1_BAAGTCGTAACAAGGTAGCCGTATCGGAAGGTGCGGCTGGAe_AC46b1
05-0005_ConsAAGTCGTAACAAGGTAGCCGTATCGGAAGGTGCGGCTGGA    720
Consensus   aagtcgtaacaaggtagccgtatcggaaggtgcggctgga
AJ717381.1_BTCACCTCCTegaterium_16S_rRNA_gene__isolate_AC46b1
05-0005_ConsTCACCTCCT                                    729
Consensus   tcacctcct
```

*Direct pairwise comparison of sample 05-0014 with Candidatus Phytoplasma solani (Genbank accession no. AJ964960)*



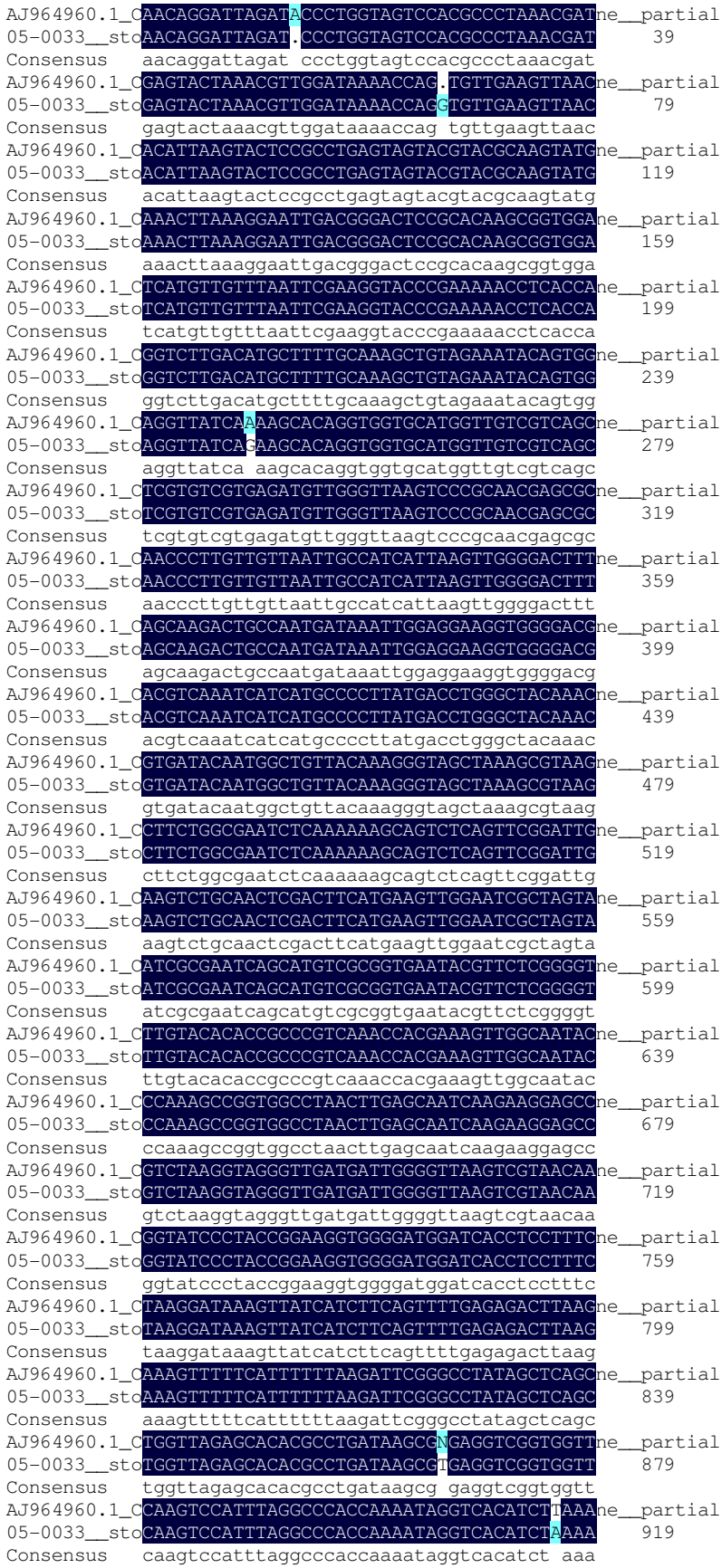
*Direct pairwise comparison of sample 05-0018 with Candidatus Phytoplasma solani (Genbank accession no. AJ964960)*



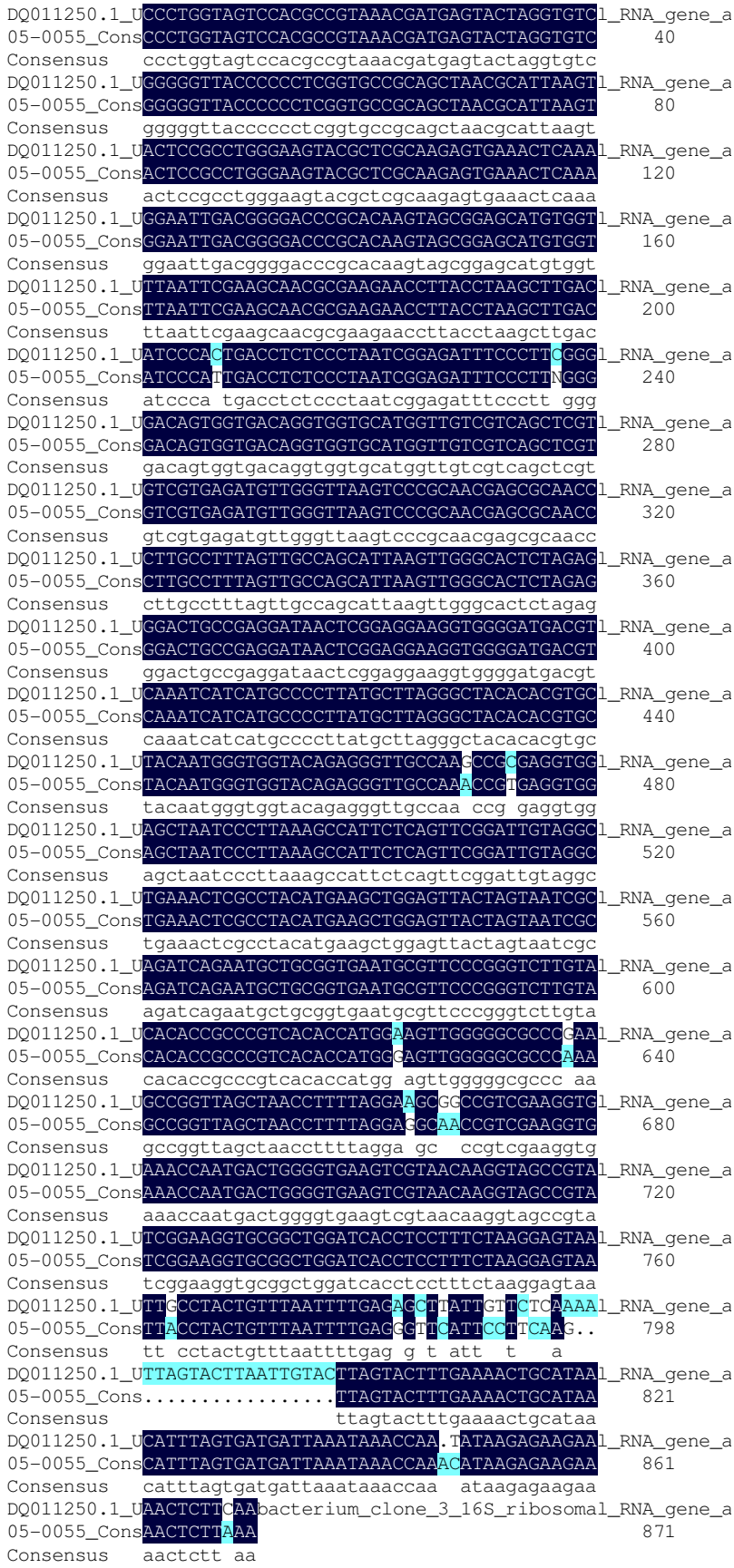
*Direct pairwise comparison of sample 05-0033 with Flavescence doree phytoplasma strain 1487 (Genbank accession no. AJ548787)*

```
AJ548787.2_FTTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAA_rRNA_gene_a
05-0033__FD_TTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAA     40
Consensus   ttaagtactccgcctgagtagtacgtacgcaagtatgaaa
AJ548787.2_FCTTAAAGGAATTGACGGGACTCCGCACAAGCGGTGGATCA_rRNA_gene_a
05-0033__FD_CTTAAAGGAATTGACGGGACTCCGCACAAGCGGTGGATCA     80
Consensus   cttaaaggaattgacgggactccgcacaagcggtggatca
AJ548787.2_FTGTTGTTTAATTCGAAGATACACGAAAAACCTTACCAGGT_rRNA_gene_a
05-0033__FD_TGTTGTTTAATTCGAAGATACACGAAAAACCTTACCAGGT    120
Consensus   tgttgtttaattcgaagatacacgaaaaaccttaccaggt
AJ548787.2_FCTTGACATACTCTGCAAAGCTATAGAAATATAGTGGAGGT_rRNA_gene_a
05-0033__FD_CTTGACATACTCTGCAAAGCTATAGAAATATAGTGGAGGT    160
Consensus   cttgacatactctgcaaagctatagaaatatagtggaggt
AJ548787.2_FTATCAGGGATACAGGTGGTGCATGGTTGTCGTCAGTTCGT_rRNA_gene_a
05-0033__FD_TATCAGGGATACAGGTGGTGCATGGTTGTCGTCAGTTCGT    200
Consensus   tatcagggatacaggtggtgcatggttgtcgtcagttcgt
AJ548787.2_FGTCGTGAGATGTTAGGTTAAGTCCTAAAACGAACGCAACC_rRNA_gene_a
05-0033__FD_GTCGTGAGATGTTAGGTTAAGTCCTAAAACGAACGCAACC    240
Consensus   gtcgtgagatgttaggttaagtcctaaaacgaacgcaacc
AJ548787.2_FCCTGTCGCTAGTTGCCAGCACGTAATGGTGGGGACTTTAG_rRNA_gene_a
05-0033__FD_CCTGTCGCTAGTTGCCAGCACGTAATGGTGGGGACTTTAG    280
Consensus   cctgtcgctagttgccagcacgtaatggtggggactttag
AJ548787.2_FCGAGACTGCCAATTAAACATTGGAGGAAGGTGGGGATAAC_rRNA_gene_a
05-0033__FD_CGAGACTGCCAATTAAACATTGGAGGAAGGTGGGGATAAC    320
Consensus   cgagactgccaattaaacattggaggaaggtggggataac
AJ548787.2_FGTCAAATCATCATGCCCCTTATGATCTGGGCTACAAACGT_rRNA_gene_a
05-0033__FD_GTCAAATCATCATGCCCCTTATGATCTGGGCTACAAACGT    360
Consensus   gtcaaatcatcatgccccttatgatctgggctacaaacgt
AJ548787.2_FGATACAATGGCTATTACAAAGAGTAGCTGAAACGCGAGTT_rRNA_gene_a
05-0033__FD_GATACAATGGCTATTACAAAGAGTAGCTGAAACGCGAGTT    400
Consensus   gatacaatggctattacaaagagtagctgaaacgcgagtt
AJ548787.2_FTTTAGCCAATCTCAAAAAGGTAGTCTCAGTACGGATTGAA_rRNA_gene_a
05-0033__FD_TTTAGCCAATCTCAAAAAGGTAGTCTCAGTACGGATTGAA    440
Consensus   tttagccaatctcaaaaaggtagtctcagtacggattgaa
AJ548787.2_FGTCTGCAACTCGACTTCATGAAGCTGG.AATCGCTAGTAA_rRNA_gene_a
05-0033__FD_GTCTGCAACTCGACTTCATGAAGCTGGGAATCGCTAGTAA    480
Consensus   gtctgcaactcgacttcatgaagctgg aatcgctagtaa
AJ548787.2_FTCGCGAATCAGCATGTCGCGGTGAATACGTTCTCGGGGTT_rRNA_gene_a
05-0033__FD_TCGCGAATCAGCATGTCGCGGTGAATACGTTCTCGGGGTT    520
Consensus   tcgcgaatcagcatgtcgcggtgaatacgttctcggggtt
AJ548787.2_FTGTACACACCGCCCGTCAAACCACGAAAGTTAGCAATACC_rRNA_gene_a
05-0033__FD_TGTACACACCGCCCGTCAAACCACGAAAGTTAGCAATACC    560
Consensus   tgtacacaccgcccgtcaaaccacgaaagttagcaatacc
AJ548787.2_FCGAAAGCAGTGGCTTAACTTCGAAAGAAGAGGGAGCTGTC_rRNA_gene_a
05-0033__FD_CGAAAGCAGTGGCTTAACTTCGAAAGAAGAGGGAGCTGTC    600
Consensus   cgaaagcagtggcttaacttcgaaagaagagggagctgtc
AJ548787.2_FTAAGGTAGGGTTGATGATTGGGGTTAAGTCGTAACAAGGT_rRNA_gene_a
05-0033__FD_TAAGGTAGGGTTGATGATTGGGGTTAAGTCGTAACAAGGT    640
Consensus   taaggtagggttgatgattggggttaagtcgtaacaaggt
AJ548787.2_FATCCTTACCGGAAGGTGAGGATGGATCACCTCCTTTCTAA_rRNA_gene_a
05-0033__FD_ATCCTTACCGGAAGGTGAGGATGGATCACCTCCTTTCTAA    680
Consensus   atccttaccggaaggtgaggatggatcacctcctttctaa
AJ548787.2_FGGACATACATATAAAAATCATCATCTTCAGTTTTGAAAGA_rRNA_gene_a
05-0033__FD_GGACATACATATAAAAATCATCATCTTCAGTTTTGAAAGA    720
Consensus   ggacatacatataaaaatcatcatcttcagttttgaaaga
AJ548787.2_FCTTAGGTTAAAATATAAGTTTTTCTTTTTATAAAAAAAGT_rRNA_gene_a
05-0033__FD_CTTAGGTTAAAATATAAGTTTTTCTTTTTATAAAAAAAGT    760
Consensus   cttaggttaaaatataagtttttctttttataaaaaaagt
AJ548787.2_FGTTTCTCTTATATAAAAGACCAAAGGGCCTATAGCTCAGT_rRNA_gene_a
05-0033__FD_GTTTCTCTTATATAAAAGACCAAAGGGCCTATAGCTCAGT    800
Consensus   gtttctcttatataaaagaccaaagggcctatagctcagt
AJ548787.2_FTGGTTAGAGCACACGCCTGATAAGCGTGAGGTCGGTGGTT_rRNA_gene_a
05-0033__FD_TGGTTAGAGCACACGCCTGATAAGCGTGAGGTCGGTGGTT    840
Consensus   tggttagagcacacgcctgataagcgtgaggtcggtggtt
AJ548787.2_FCAAGTCCACTTAGGCCCACCAATTTTATATCAGGAAAATA_rRNA_gene_a
05-0033__FD_CAAGTCCACTTAGGCCCACCAATTTTATATCAGGAAAATA    880
Consensus   caagtccacttaggcccaccaattttatatcaggaaaata
AJ548787.2_FTTvescence_doree_phytoplasma_partial_16S_rRNA_gene_a
05-0033__FD_TTonsensus                                   882
Consensus   tt
```

*Direct pairwise comparison of sample 05-0033 with Candidatus Phytoplasma solani (Genbank accession no. AJ964960)*



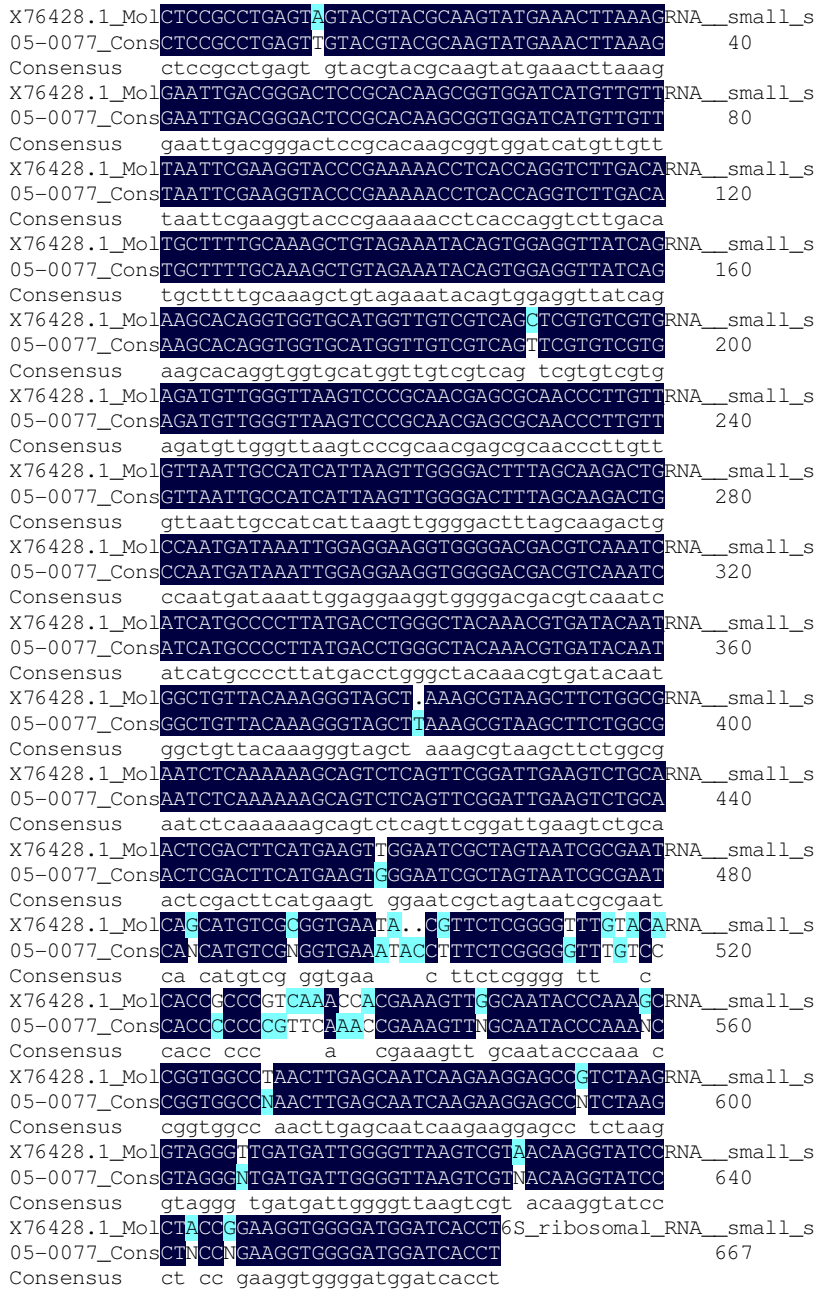
*Direct pairwise comparison of sample 05-0055 with uncultured bacterium clone 3 (Genbank accession no. DQ011250)*



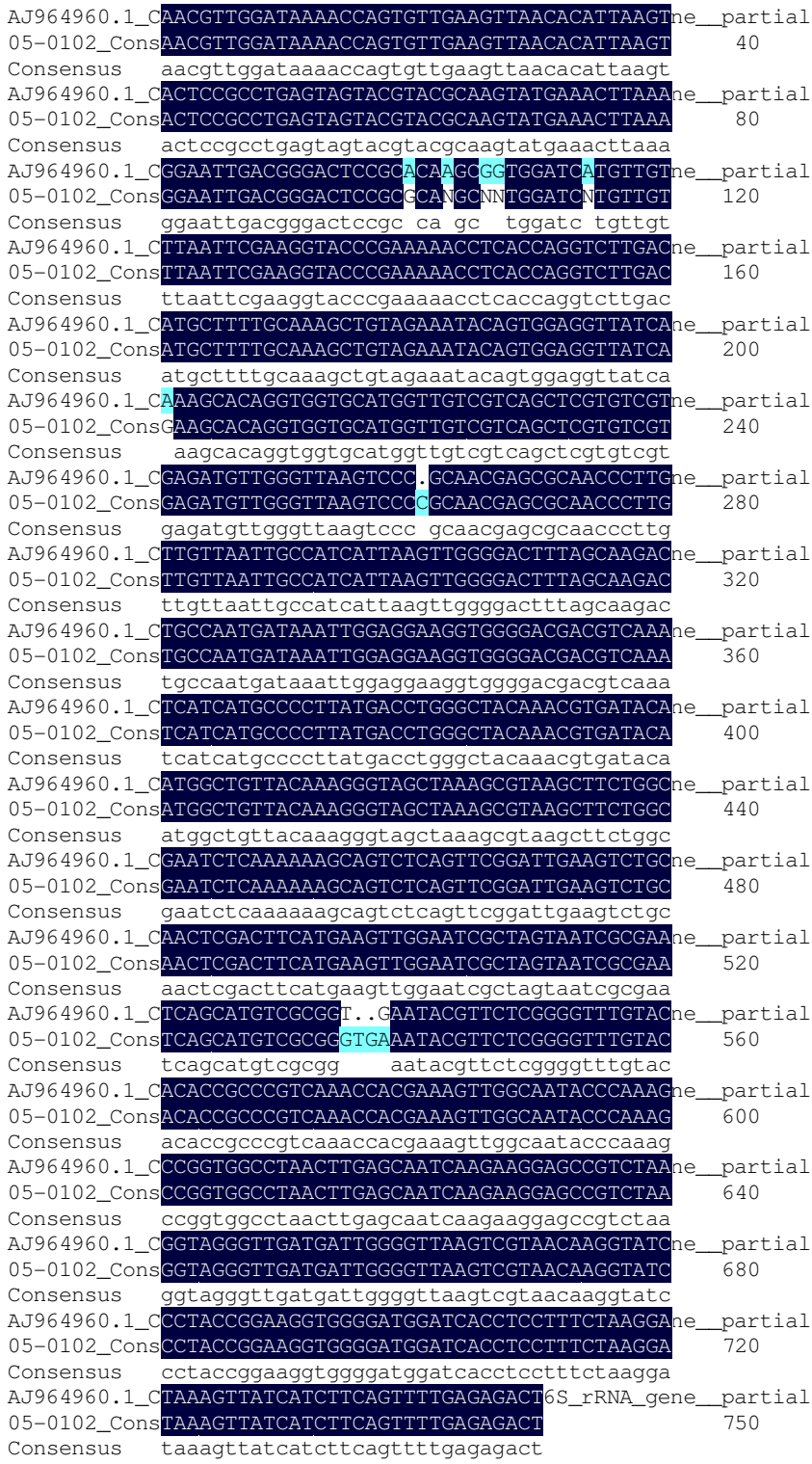
*Direct pairwise comparison of sample 05-0062 with Candidatus Phytoplasma solani (Genbank accession no. AJ964960)*

```
AJ964960.1_C ATTAGATACCCTGGTAGTCCACGCCCTAAACGATGAGTACne__partial
05-0062_Cons ATTAGAT.CCCTGGTAGTCCACGCCCTAAACGATGAGTAC      39
Consensus    attagat ccctggtagtccacgccctaaacgatgagtac
AJ964960.1_C TAAACGTTGGATAAAACCAGTGTTGAAGTTAACACATTAAne__partial
05-0062_Cons TAAACGTTGGATAAAACCAGTGTTGAAGTTAACACATTAA      79
Consensus    taaacgttggataaaaccagtgttgaagttaacacattaa
AJ964960.1_C GTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTAne__partial
05-0062_Cons GTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTA     119
Consensus    gtactccgcctgagtagtacgtacgcaagtatgaaactta
AJ964960.1_C AAGGAATTGACGGGACTCCGCACAAGCGGTGGATCATGTTne__partial
05-0062_Cons AAGGAATTGACGGGACTCCGCACAAGCGGTGGATCATGTT     159
Consensus    aaggaattgacgggactccgcacaagcggtggatcatgtt
AJ964960.1_C GTTTAATTCGAAGGTACCCGAAAAACCTCACCAGGTCTTGne__partial
05-0062_Cons GTTTAATTCGAAGGTACCCGAAAAACCTCACCAGGTCTTG     199
Consensus    gtttaattcgaaggtacccgaaaaacctcaccaggtcttg
AJ964960.1_C ACATGCTTTTGCAAAGCTGTAGAAATACAGTGGAGGTTATne__partial
05-0062_Cons ACATGCTTTTGCAAAGCTGTAGAAATACAGTGGAGGTTAT     239
Consensus    acatgcttttgcaaagctgtagaaatacagtggaggttat
AJ964960.1_C CAAAAGCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCne__partial
05-0062_Cons CAGAAGCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTC     279
Consensus    ca aagcacaggtggtgcatggttgtcgtcagctcgtgtc
AJ964960.1_C GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTne__partial
05-0062_Cons GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTT     319
Consensus    gtgagatgttgggttaagtcccgcaacgagcgcaaccctt
AJ964960.1_C GTTGTTAATTGCCATCATTAAGTTGGGGACTTTAGCAAGAne__partial
05-0062_Cons GTTGTTAATTGCCATCATTAAGTTGGGGACTTTAGCAAGA     359
Consensus    gttgttaattgccatcattaagttggggactttagcaaga
AJ964960.1_C CTGCCAATGATAAATTGGAGGAAGGTGGGGACGACGTCAAne__partial
05-0062_Cons CTGCCAATGATAAATTGGAGGAAGGTGGGGACGACGTCAA     399
Consensus    ctgccaatgataaattggaggaaggtggggacgacgtcaa
AJ964960.1_C ATCATCATGCCCCTTATGACCTGGGCTACAAACGTGATACne__partial
05-0062_Cons ATCATCATGCCCCTTATGACCTGGGCTACAAACGTGATAC     439
Consensus    atcatcatgcccccttatgacctgggctacaaacgtgatac
AJ964960.1_C AATGGCTGTTACAAAGGGTAGCTAAAGCGTAAGCTTCTGGne__partial
05-0062_Cons AATGGCTGTTACAAAGGGTAGCTAAAGCGTAAGCTTCTGG     479
Consensus    aatggctgttacaaagggtagctaaagcgtaagcttctgg
AJ964960.1_C CGAATCTCAAAAAAGCAGTCTCAGTTCGGATTGAAGTCTGne__partial
05-0062_Cons CGAATCTCAAAAAAGCAGTCTCAGTTCGGATTGAAGTCTG     519
Consensus    cgaatctcaaaaaagcagtctcagttcggattgaagtctg
AJ964960.1_C CAACTCGACTTCATGAAGTTGGAATCGCTAGTAATCGCGAne__partial
05-0062_Cons CAACTCGACTTCATGAAGTTGGAATCGCTAGTAATCGCGA     559
Consensus    caactcgacttcatgaagttggaatcgctagtaatcgcga
AJ964960.1_C ATCAGCATGTCGCGGTGAATACGTTCTCGGGGTTTGTACAne__partial
05-0062_Cons ATCAGCATGTCGCGGTGAANACGTTCTCGGGGNTTGTACA     599
Consensus    atcagcatgtcgcggtgaa acgttctcgggg ttgtaca
AJ964960.1_C CACCGCCCGTCAAACCACGAAAGTTCGCAATACCCAAAGCne__partial
05-0062_Cons CACCNCCCNTCAAACCACGAAAGTTNGCAATACCCAAANC     639
Consensus    cacc ccc tcaaaccacgaaagtt gcaatacccaaa c
AJ964960.1_C CGGTGGCCTAACTTGAGCAATCAAGAAGGAGCCGTCTAAGne__partial
05-0062_Cons CGGTGGCCNAACTTGAGCAATCAAGAAGGAGCCNTCTAAG     679
Consensus    cggtggcc aacttgagcaatcaagaaggagcc tctaag
AJ964960.1_C GTAGGGTTGATGATTGGGGTTAAGTCGTAACAAGGTATCCne__partial
05-0062_Cons GTAGGGNTGATGATTGGGGTTAAGTCGTNACAAGGTATCC     719
Consensus    gtaggg tgatgattggggttaagtcgt acaaggtatcc
AJ964960.1_C CTACCGGAAGGTGGGGATGGATCACCTCCTTTCTAAGGATne__partial
05-0062_Cons CTNCCNGAAGGTGGGGATGGATCACCTCCTTTCTAAGGAT     759
Consensus    ct cc gaaggtggggatggatcacctcctttctaaggat
AJ964960.1_C AAAGTTATCATCTTCAGTTTTGAGAGACTTAAGAAAGTTTne__partial
05-0062_Cons AAAGTTATCATCTTCAGTTTTGAGAGACTTAAGAAAGTTT     799
Consensus    aaagttatcatcttcagttttgagagacttaagaaagttt
AJ964960.1_C TTCATTTTTTAAGATTCGGGCCTATAGCTCAGCTGGTTAGne__partial
05-0062_Cons TTCATTTTTTAAGATTCGGGCCTATAGCTCAGCTGGTTAG     839
Consensus    ttcatttttttaagattcgggcctatagctcagctggttag
AJ964960.1_C AGCACACGCCTGATAAGCGNGAGGTCGGTGGTTCAAGTCCne__partial
05-0062_Cons AGCNCACNCCTGATAAGCGTGAGGTCGGTGGTTCAAGTCC     879
Consensus    agc cac cctgataagcg gaggtcggtggttcaagtcc
AJ964960.1_C ATTTAGGCCCACCAAAATAGGTCACATCTTAAArRNA_gene__partial
05-0062_Cons ATTTAGGCCCACCAAAATAGGTCACATCNTCTA           912
Consensus    atttaggcccaccaaaataggtcacatc t  a
```

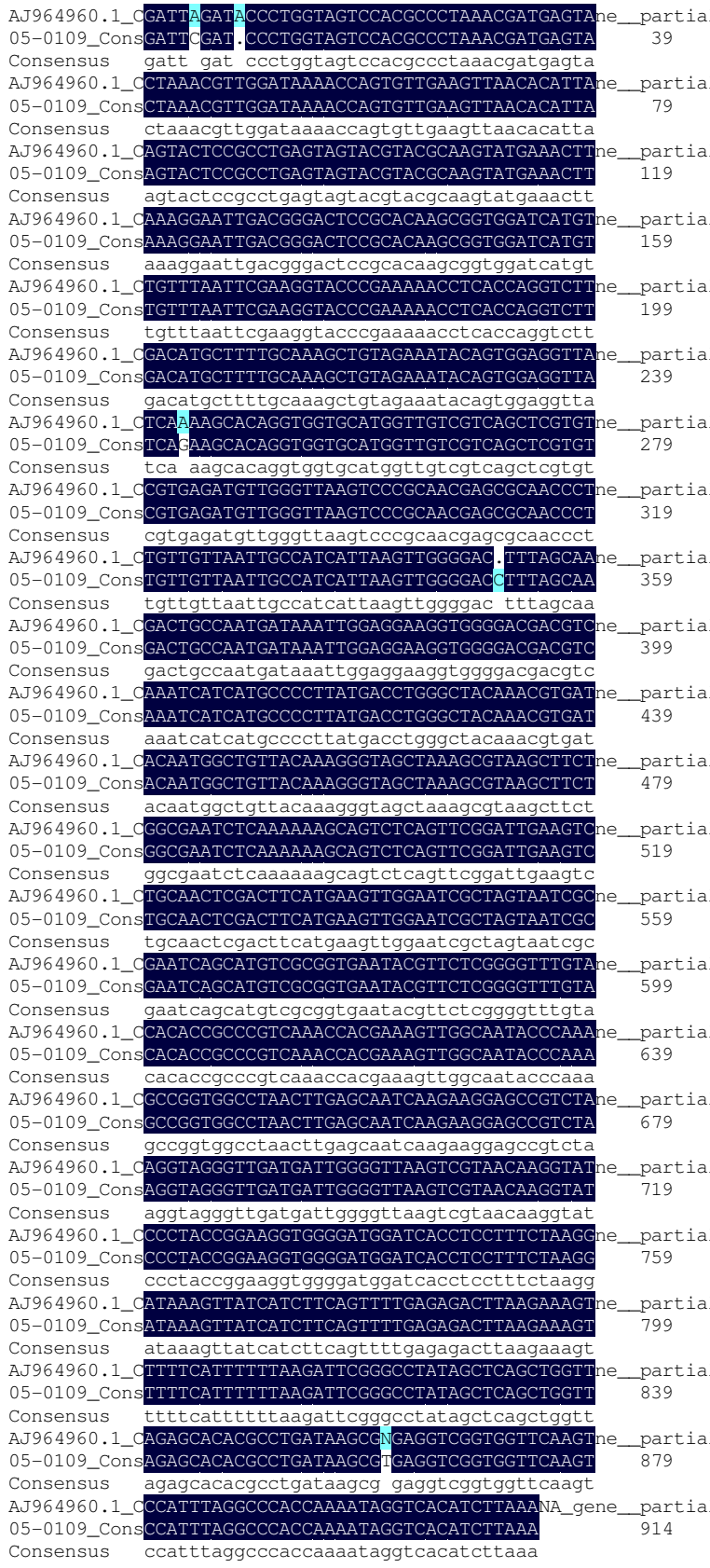
*Direct pairwise comparison of sample 05-0077 with Mollicutes from V. vinifera (Genbank accession no. X76428)*



*Direct pairwise comparison of sample 05-0102 with Candidatus Phytoplasma solani (Genbank accession no. AJ964960)*



*Direct pairwise comparison of sample 05-0109 with Candidatus Phytoplasma solani (Genbank accession no. AJ964960)*



*Direct pairwise comparison of sample 05-0111 with Candidatus Phytoplasma solani (Genbank accession no. AJ964960)*

```
AJ964960.1_CACGTTGGATAAAACCAGTGTTGAAGTTAACACATTAAGTAne__partial
05-0111_ConsACGTTGGATAAAACCAGTGTTGAAGTTAACACATTAAGTA     40
Consensus   acgttggataaaaccagtgttgaagttaacacattaagta
AJ964960.1_CCTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTAAAGne__partial
05-0111_ConsCTCCGCCTGAGTANTNCNTACGCAAGTNTGAAACTTAAAG     80
Consensus   ctccgcctgagta t c tacgcaagt tgaaacttaaag
AJ964960.1_CGAATTGACGGGACTCCGCACAAGCGGTGGATCATGTTGTTne__partial
05-0111_ConsGAATTGACGGGACTCCGCACAAGCGGTGGATCATGTTGTT    120
Consensus   gaattgacgggactccgcacaagcggtggatcatgttgtt
AJ964960.1_CTAATTCGAAGGTACCCGAAAAACCTCACCAGGTCTTGACAne__partial
05-0111_ConsTAATTCGAAGGTACCCGAAAAACCTCACCAGGTCTTGACA    160
Consensus   taattcgaaggtacccgaaaaacctcaccaggtcttgaca
AJ964960.1_CTGCTTTTGCAAAGCTGTAGAAATACAGTGGAGGTTATCAAne__partial
05-0111_ConsTGCTTTTGCAAAGCTGTAGAAATACAGTGGAGGTTATCAG    200
Consensus   tgcttttgcaaagctgtagaaatacagtggaggttatca
AJ964960.1_CAAGCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGne__partial
05-0111_ConsAAGCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTG    240
Consensus   aagcacaggtggtgcatggttgtcgtcagctcgtgtcgtg
AJ964960.1_CAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTne__partial
05-0111_ConsAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTT    280
Consensus   agatgttgggttaagtcccgcaacgagcgcaacccttgtt
AJ964960.1_CGTTAATTGCCATCATTAAGTTGGGGACTTTAGCAAGACTGne__partial
05-0111_ConsGTTAATTGCCATCATTAAGTTGGGGACTTTAGCAAGACTG    320
Consensus   gttaattgccatcattaagttggggactttagcaagactg
AJ964960.1_CCCAATGATAAATTGGAGGAAGGTGGGGACGACGTCAAATCne__partial
05-0111_ConsCCAATGATAAATTGGAGGAAGGTGGGGACGACGTCAAATC    360
Consensus   ccaatgataaattggaggaaggtggggacgacgtcaaatc
AJ964960.1_CATCATGCCCCTTATGACCTGGGCTACAAACGTGATACAATne__partial
05-0111_ConsATCATGCCCCTTATGACCTGGGCTACAAACGTGATACAAT    400
Consensus   atcatgcccctt atgacctgggctacaaacgtgatacaat
AJ964960.1_CGGCTGTTACAAAGGGTAGCTAAAGCGTAAGCTTCTGGCGAne__partial
05-0111_ConsGGCTGTTACAAAGGGTAGCTAAAGCGTAAGCTTCTGGCGA    440
Consensus   ggctgttacaaagggtagctaaagcgtaagcttctggcga
AJ964960.1_CATCTCAAAAAAGCAGTCTCAGTTCGGATT..GAAGTCTGCne__partial
05-0111_ConsATCTCAAAAAAGCAGTCTCAGTTCCGGATTGGAAGTCTGC    480
Consensus   atctcaaaaaagcagtctcagttc g  t  gaagtctgc
AJ964960.1_CAACTCGACTTCATGAAGTTGGAATCGCTAGTAATCGCGAAne__partial
05-0111_ConsAACTCGACTTCATGAAGTTGGAATCNCTAGTAATCGCGAA    520
Consensus   aactcgacttcatgaagttggaatc ctagtaatcgcgaa
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05-0111_ConsACCGCCCGTCAAACCACGAAAGTTGGCAATACCCAAAGCC    600
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AJ964960.1_CGGTGGCCTAACTTGAGCAATCAAGAAGGAGCCGTCTAAGGne__partial
05-0111_ConsGGTGGCCTAACTTGAGCAATCAAGAAGGAGCCGTCTAAGG    640
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AJ964960.1_CTAGGGTTGATGATTGGGGTTAAGTCGTAACAAGGTATCCCne__partial
05-0111_ConsTAGGGTTGATGATTGGGGTTAAGTCGTAACAAGGTATCCC    680
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AJ964960.1_CTCATTTTTTAAGATTCGGGCCTATAGCTCAGCTGGTTAGAne__partial
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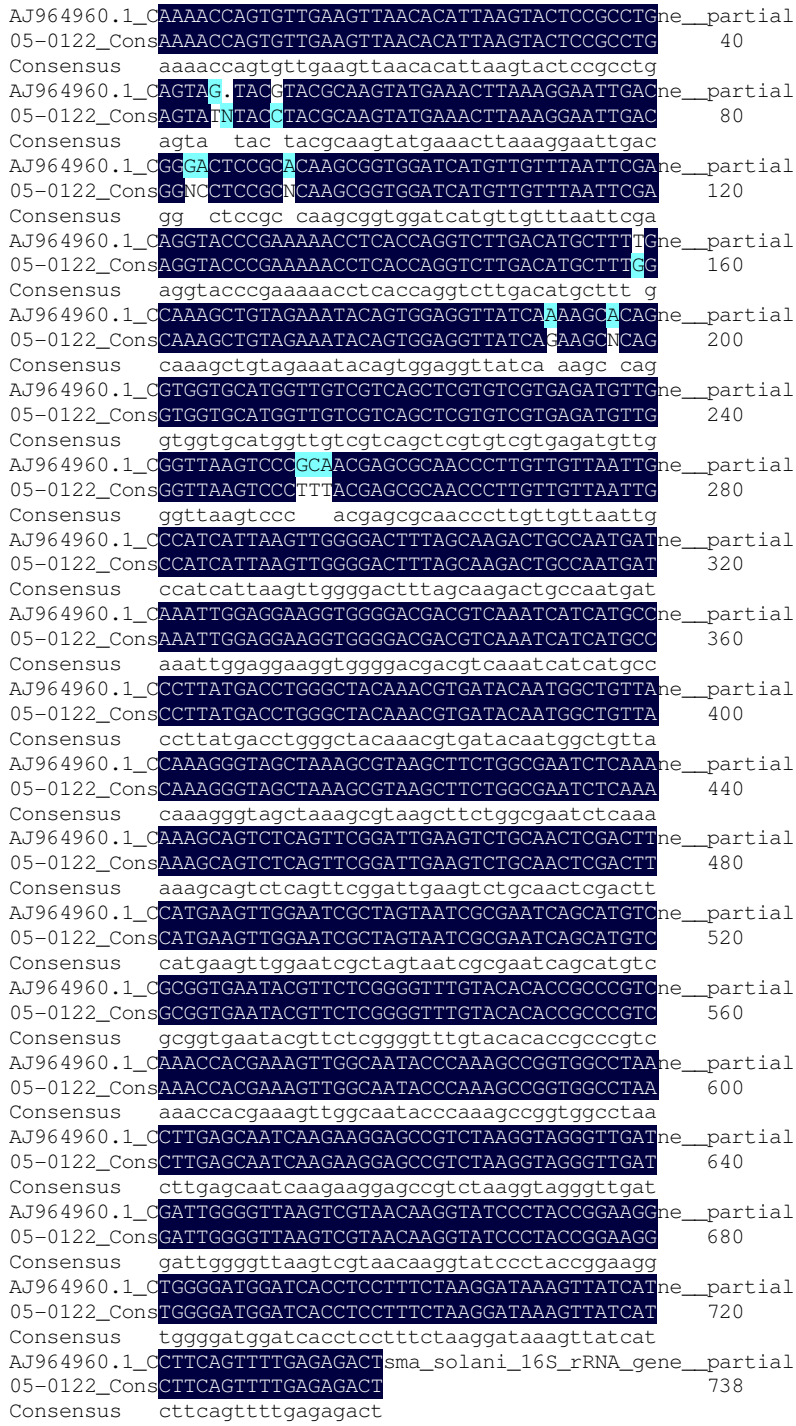
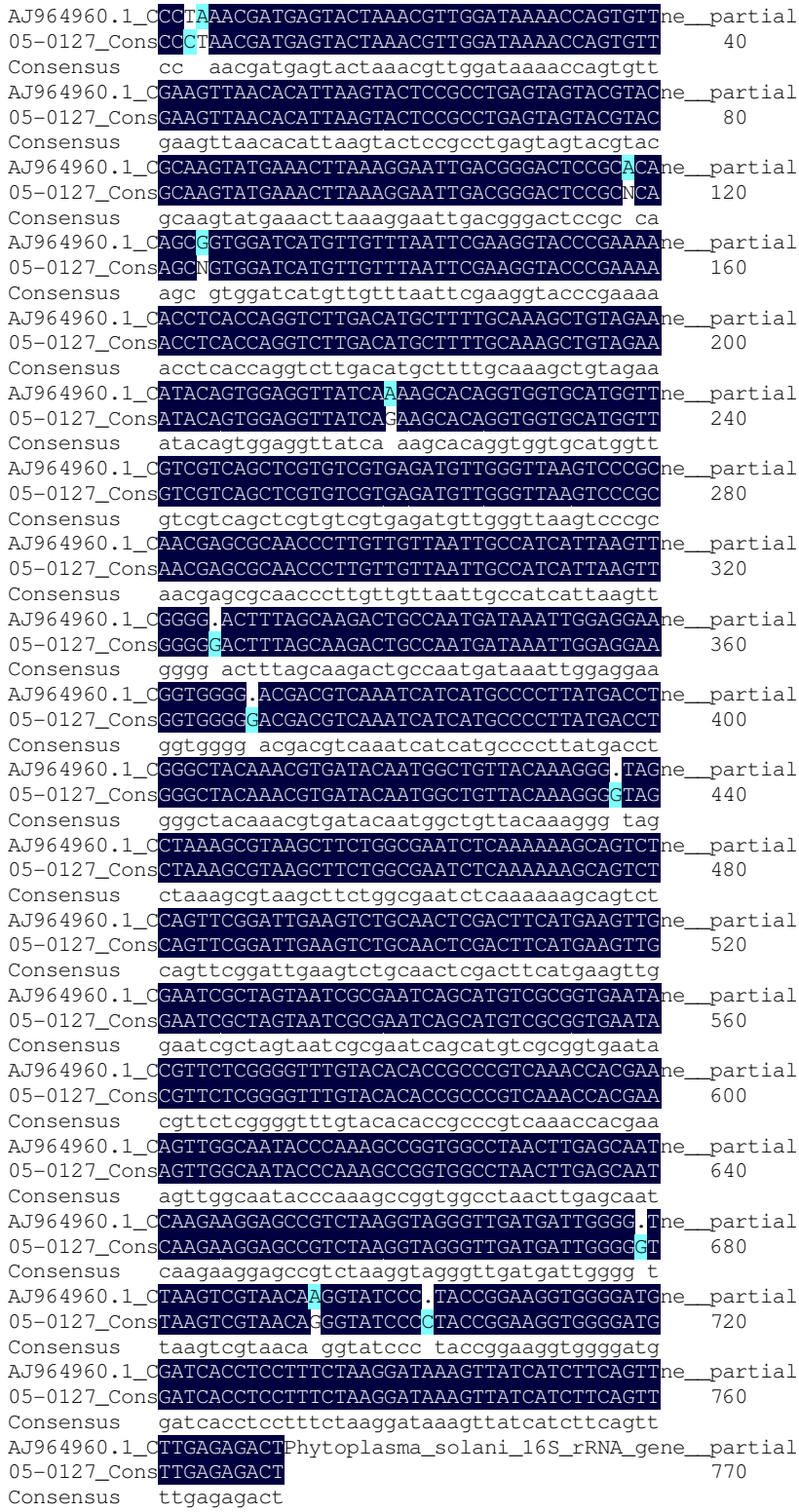
*Direct pairwise comparison of sample 05-0122 with Candidatus Phytoplasma solani (Genbank accession no. AJ964960)*

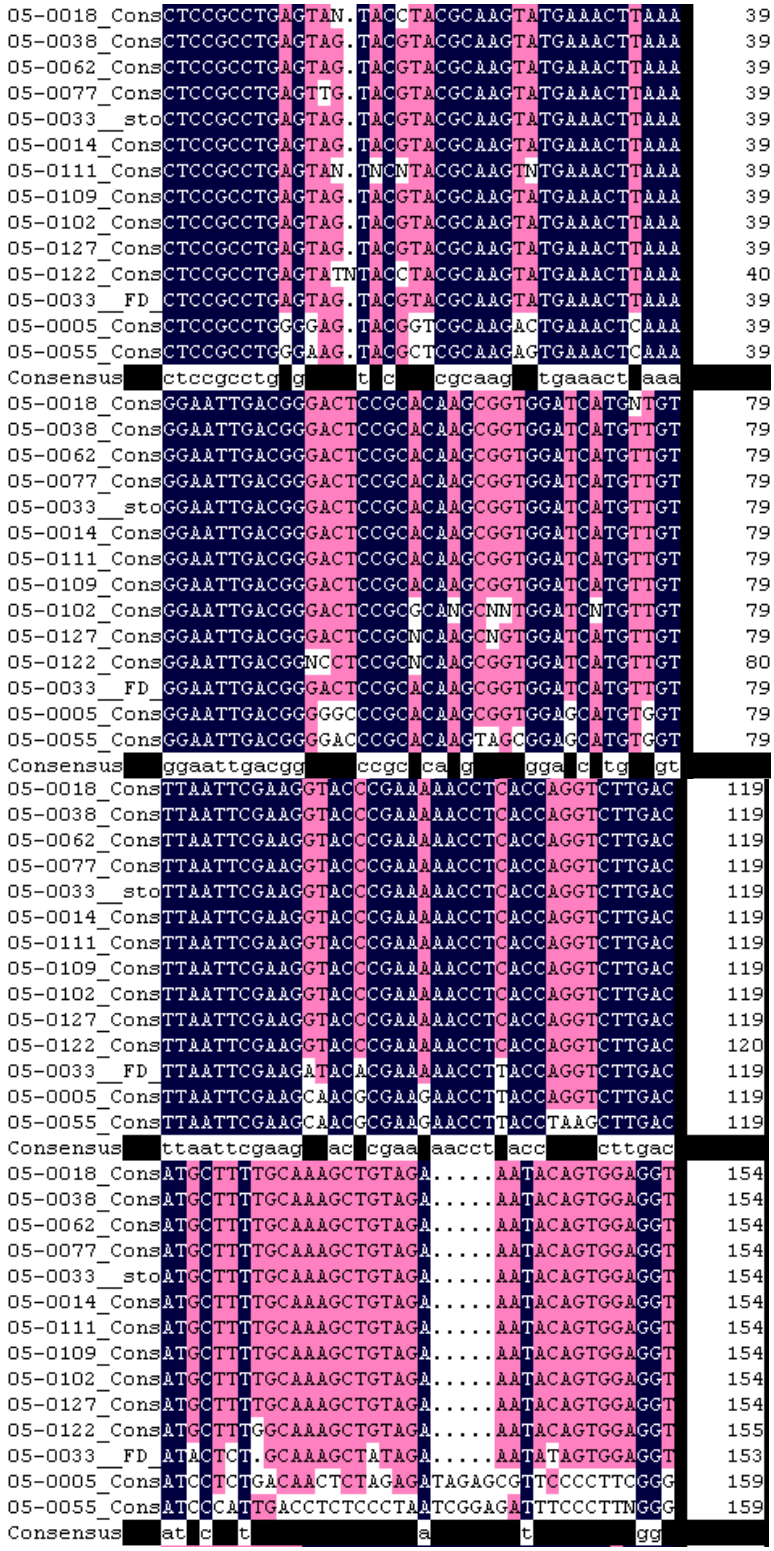


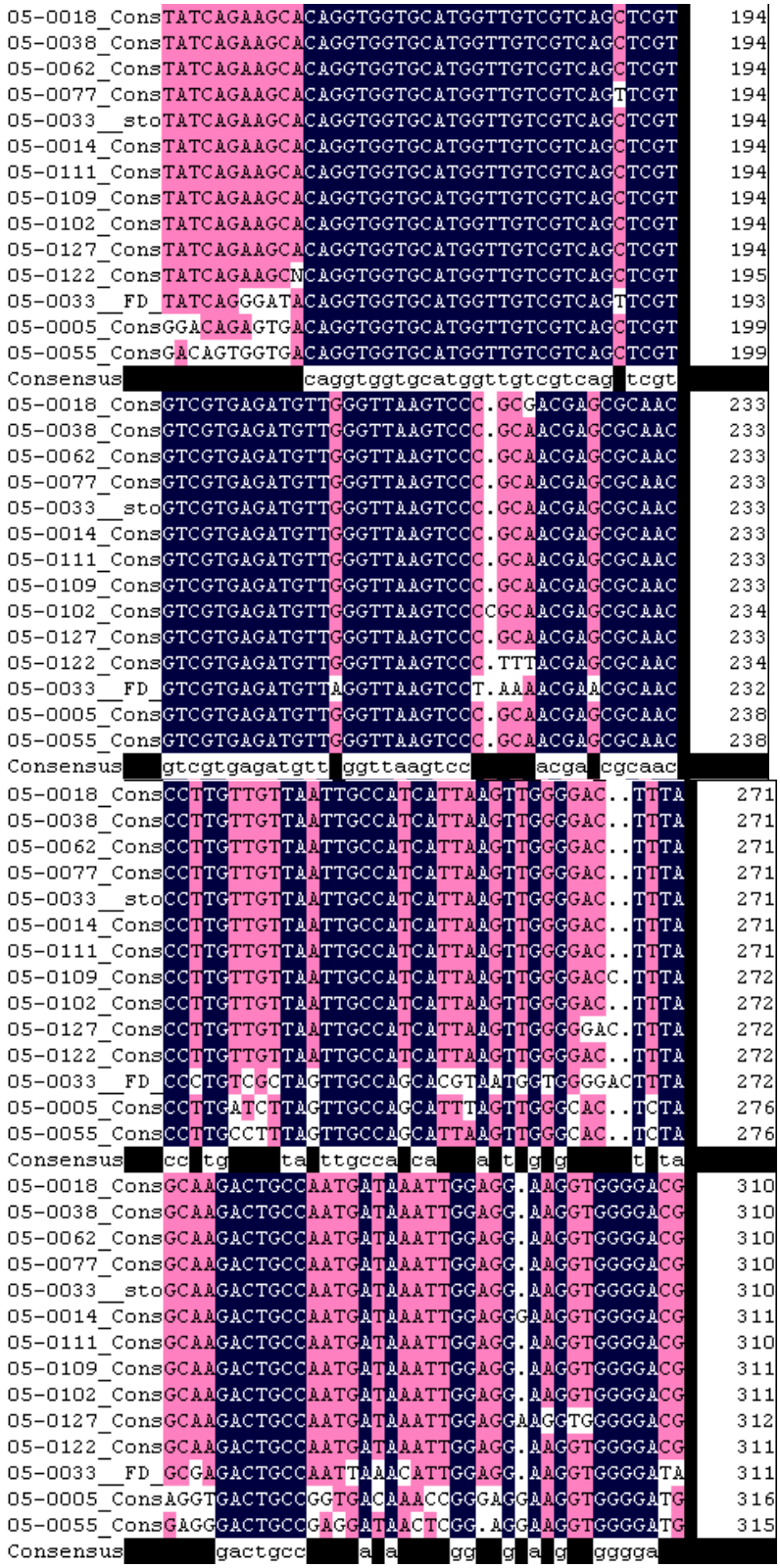
*Direct pairwise comparison of sample 05-0127 with Candidatus Phytoplasma solani (Genbank accession no. AJ964960)*



## APPENDIX C

### Multiple alignment of all the samples sequenced

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05-0077_Cons TATCAGAAGCACAGGTGGTGCATGGTTGTCGTCAGTTCGT 194
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05-0014_Cons TATCAGAAGCACAGGTGGTGCATGGTTGTCGTCAGCTCGT 194
05-0111_Cons TATCAGAAGCACAGGTGGTGCATGGTTGTCGTCAGCTCGT 194
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05-0127_Cons TATCAGAAGCACAGGTGGTGCATGGTTGTCGTCAGCTCGT 194
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05-0055_Cons GACAGTGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGT 199
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05-0038_Cons GTCGTGAGATGTTGGGTTAAGTCCC.GCAACGAGCGCAAC 233
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05-0077_Cons GTCGTGAGATGTTGGGTTAAGTCCC.GCAACGAGCGCAAC 233
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05-0102_Cons GTCGTGAGATGTTGGGTTAAGTCCCCGCAACGAGCGCAAC 234
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05-0005_Cons GTCGTGAGATGTTGGGTTAAGTCCC.GCAACGAGCGCAAC 238
05-0055_Cons GTCGTGAGATGTTGGGTTAAGTCCC.GCAACGAGCGCAAC 238
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05-0122_Cons CCTTGTTGTTAATTGCCATCATTAAGTTGGGGAC..TTTA 272
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05-0005_Cons CCTTGATCTTAGTTGCCAGCATTTAGTTGGGCAC..TCTA 276
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Consensus cc tg ta ttgcca ca a t g g t ta
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05-0055_Cons GAGGGACTGCCGAGGATAACTCGG.AGGAAGGTGGGGATG 315
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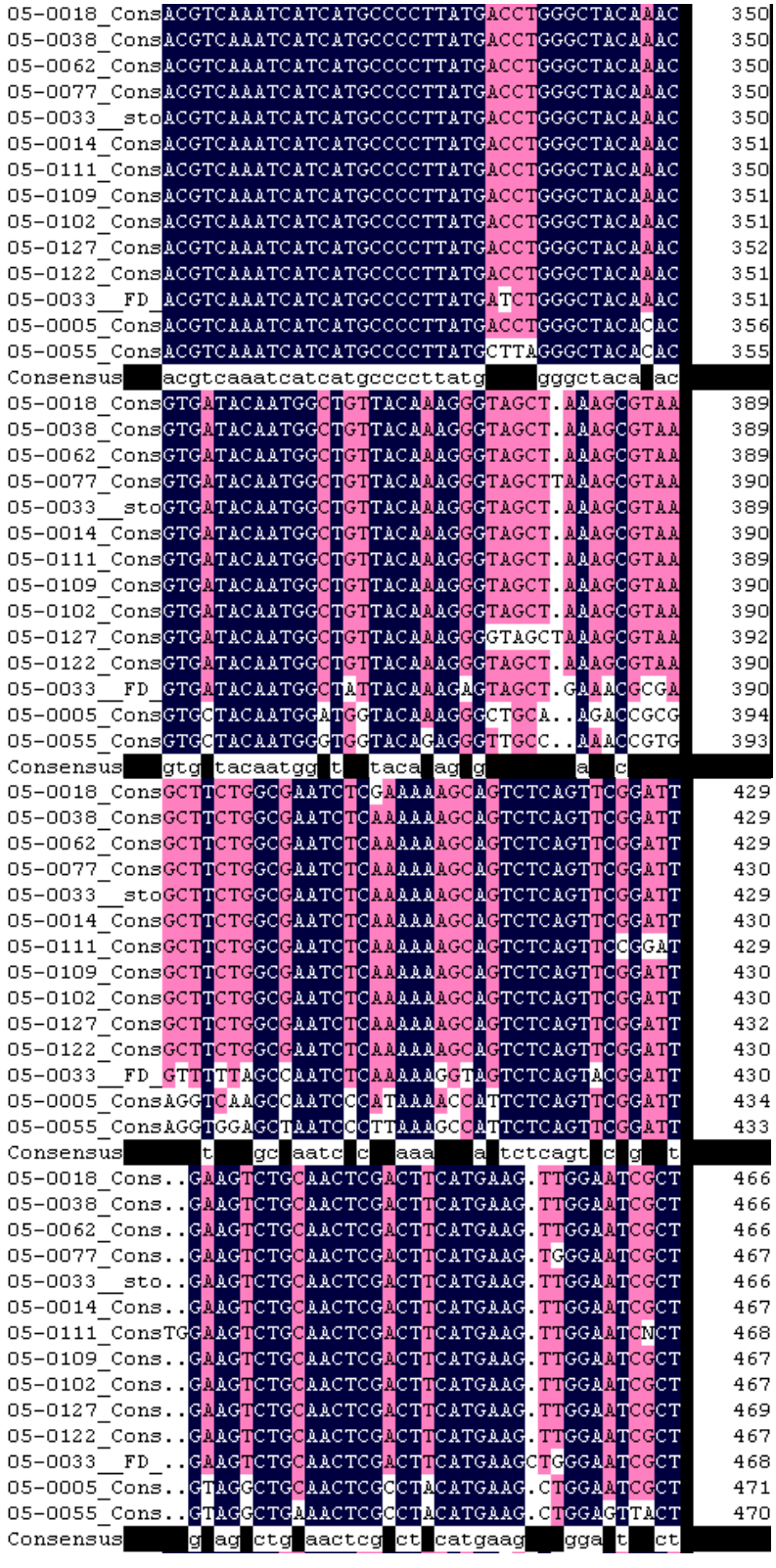

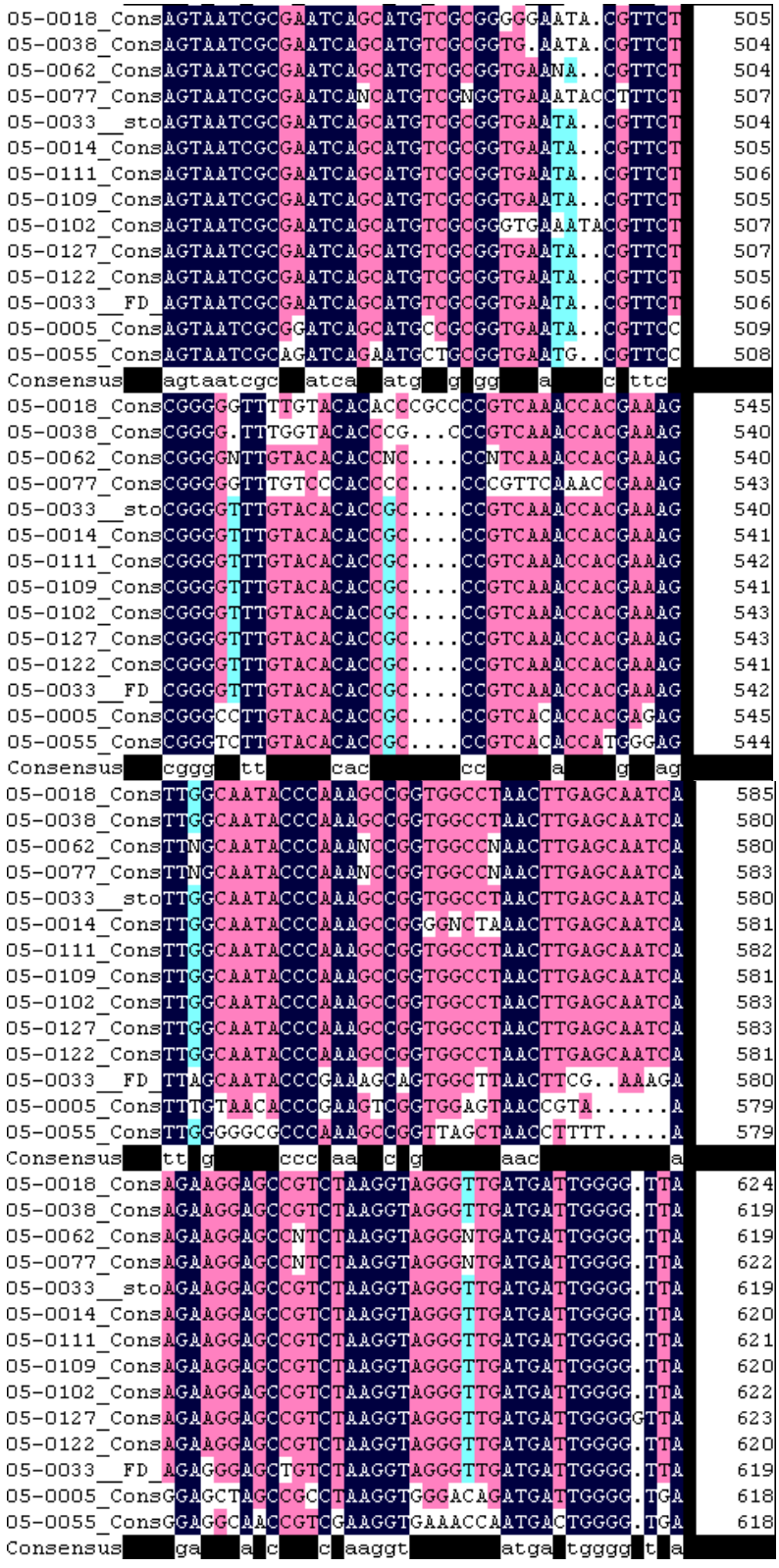

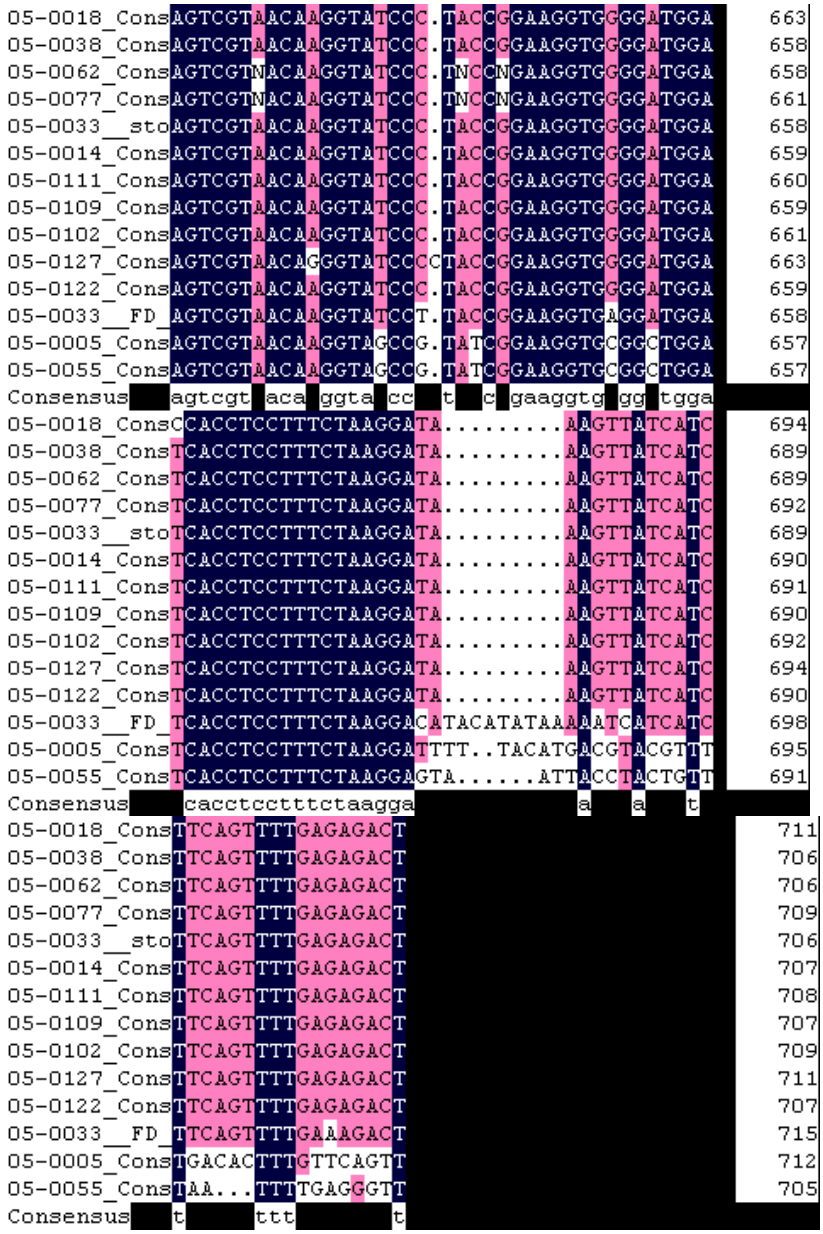
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05-0077_Cons AGTCGTNACAAGGTATCCC.TNCCNGAAGGTGGGGATGGA 661
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05-0109_Cons AGTCGTAACAAGGTATCCC.TACCGGAAGGTGGGGATGGA 659
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05-0122_Cons TTCAGTTTTGAGAGACT 707
05-0033__FD_ TTCAGTTTTGAAAGACT 715
05-0005_Cons TGACACTTTGTTCAGTT 712
05-0055_Cons TAA...TTTTGAGGGTT 705
Consensus t ttt t

## APPENDIX D

## Pairwise comparison of sample 05-0033 and Flavescence doree positive control

```
05-0033__FD_ TAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAAC  40
FDP_CS       TAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAAC  40
Consensus    taagtactccgcctgagtagtacgtacgcaagtatgaaac
05-0033__FD_ TTAAAGGAATTGACGGGACTCCGCACAAGCGGTGGATCAT  80
FDP_CS       TTAAAGGAATTGACGGGACTCCGCACAAGCGGTGGATCAT  80
Consensus    ttaaaggaattgacgggactccgcacaagcggtggatcat
05-0033__FD_ GTTGTTTAATTCGAAGATACACGAAAAACCTTACCAGGTC 120
FDP_CS       GTTGTTTAATTCGAAGATACACGAAAAACCTTACCAGGTC 120
Consensus    gttgtttaattcgaagatacacgaaaaaccttaccaggtc
05-0033__FD_ TTGACATACTCTGCAAAGCTATAGAAATATAGTGGAGGTT 160
FDP_CS       TTGACATACTCTGCAAAGCTATAGAAATATAGTGGAGGTT 160
Consensus    ttgacatactctgcaaagctatagaaatatagtggaggtt
05-0033__FD_ ATCAGGGATACAGGTGGTGCATGGTTGTCGTCAGTTCGTG 200
FDP_CS       ATCAGGGATACAGGTGGTGCATGGTTGTCGTCAGTTCGTG 200
Consensus    atcagggatacaggtggtgcatggttgtcgtcagttcgtg
05-0033__FD_ TCGTGAGATGTTAGGTTAAGTCCTAAAACGAACGCAACCC 240
FDP_CS       TCGTGAGATGTTAGGTTAAGTCCTAAAACGAACGCAACCC 240
Consensus    tcgtgagatgttaggttaagtcctaaaacgaacgcaaccc
05-0033__FD_ CTGTCGCTAGTTGCCAGCACGTAATGGTGGGGACTTTAGC 280
FDP_CS       CTGTCGCTAGTTGCCAGCACGTAATGGTGGGGACTTTAGC 280
Consensus    ctgtcgctagttgccagcacgtaatggtggggactttagc
05-0033__FD_ GAGACTGCCAATTAAACATTGGAGGAAGGTGGGGATAACG 320
FDP_CS       GAGACTGCCAATTAAACATTGGAGGAAGGTGGGGATAACG 320
Consensus    gagactgccaattaaacattggaggaaggtggggataacg
05-0033__FD_ TCAAATCATCATGCCCCTTATGATCTGGGCTACAAACGTG 360
FDP_CS       TCAAATCATCATGCCCCTTATGATCTGGGCTACAAACGTG 360
Consensus    tcaaatcatcatgcccccttatgatctgggctacaaacgtg
05-0033__FD_ ATACAATGGCTATTACAAAGAGTAGCTGAAACGCGAGTTT 400
FDP_CS       ATACAATGGCTATTACAAAGAGTAGCTGAAACGCGAGTTT 400
Consensus    atacaatggctattacaaagagtagctgaaacgcgagttt
05-0033__FD_ TTAGCCAATCTCAAAAAGGTAGTCTCAGTACGGATTGAAG 440
FDP_CS       TTAGCCAATCTCAAAAAGGTAGTCTCAGTACGGATTGAAG 440
Consensus    ttagccaatctcaaaaaggtagtctcagtacggattgaag
05-0033__FD_ TCTGCAACTCGACTTCATGAAGCTGCGAATCGCTAGTAAT 480
FDP_CS       TCTGCAACTCGACTTCATGAAGCTGG.AATCGCTAGTAAT 479
Consensus    tctgcaactcgacttcatgaagctgg aatcgctagtaat
05-0033__FD_ CGCGAATCAGCATGTCGCGGTGAATACGTTCTCGGGGTTT 520
FDP_CS       CGCGAATCAGCATGTCGCGGTGAATACGTTCTCGGGGTTT 519
Consensus    cgcgaatcagcatgtcgcggtgaatacgttctcggggttt
05-0033__FD_ GTACACACCGCCCGTCAAACCACGAAAGTTAGCAATACCC 560
FDP_CS       GTACACACCGCCCGTCAAACCACGAAAGTTAGCAATACCC 559
Consensus    gtacacaccgcccgtcaaaccacgaaagttagcaataccc
05-0033__FD_ GAAAGCAGTGGCTTAACTTCGAAAGAAGAGGGAGCTGTCT 600
FDP_CS       GAAAGCAGTGGCTTAACTTCGAAAGAAGAGGGAGCTGTCT 599
Consensus    gaaagcagtggcttaacttcgaaagaagagggagctgtct
05-0033__FD_ AAGGTAGGGTTGATGATTGGGGTTAAGTCGTAACAAGGTA 640
FDP_CS       AAGGTAGGGTTGATGATTGGGGTTAAGTCGTAACAAGGTA 639
Consensus    aaggtagggttgatgattggggttaagtcgtaacaaggta
05-0033__FD_ TCCTTACCGGAAGGTGAGGATGGATCACCTCCTTTCTAAG 680
FDP_CS       TCCTTACCGGAAGGTGAGGATGGATCACCTCCTTTCTAAG 679
Consensus    tccttaccggaaggtgaggatggatcacctcctttctaag
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Consensus    gacatacatataaaaatcatcatcttcagttttgaaagac
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05-0033__FD_ TConsensus                               881
FDP_CS       T                                        880
Consensus    t
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