The molecular identification and characterisation of Eutypa dieback and a PCR-based assay for the detection of *Eutypa* and Botryosphaeriaceae species from grapevine in South Africa

By

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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Signature Date

This thesis is dedicated to the memory of my grandparents, and my family for their love and support.

SUMMARY

Grapevine trunk diseases are caused by invasive pathogens that are responsible for the slow decline of vines. In particular, Eutypa dieback of grapevine has had a devastating impact on vineyards worldwide, reducing growth and yield, eventually killing the grapevine. The causal organism of Eutypa dieback was first described as *Eutypa armeniacae* Hansf. & Carter, the pathogen that causes dieback of apricots, but since 1987 this species has been considered a synonym of *Eutypa lata* (Pers.:Fr.) Tul & C. Tul (anamorph *Libertella blepharis* A. L. Smith). Recently, it was proposed that at least two species that are capable of infecting grapevines are responsible for Eutypa dieback. Consequently, the molecular identification and characterisation of Eutypa dieback was used to delineate the species occurring on infected grapevines in South Africa. This involved the molecular analyses of three molecular markers, namely, the internal transcribed spacer (ITS) and large subunit (LSU) regions of the ribosomal DNA operon, and the β-tubulin gene. The results obtained revealed the presence of a second species, namely, *Eutypa leptoplaca* (Mont.) Rappaz, that occurred together with *E. lata* on infected grapevines.

Also co-habiting with these pathogens were related fungi form the Diatrypaceae family, *Cryptovalsa ampelina* (Nitschke) Fuckel and *Eutypella vitis* (Schwein.) Ellis & Everhart. Pathogenicity tests conducted on isolates representing *C. ampelina, E. lata*, *E. leptoplaca*, and *E. vitis* revealed that all were pathogenic to grapevine. Several species of Botryosphaeriaceae that commonly invade the woody tissue of grapevines are also pathogenic to grapevine. The symptoms in grapevine commonly associated with Botryosphaeriaceae are easily confused with the symptoms produced by Eutypa dieback which prompted the need for the development of a detection method that can correctly identify the presence of multiple pathogens.

A reverse dot blot hybridisation (RDBH) method was subsequently applied to provide a rapid, accurate and reliable means of detecting the *Eutypa* species involved in the *Eutypa* disease complex, as well as those species of Botryosphaeriaceae known to cause disease

in grapevines. The method involved the use of multiplex PCR to simultaneously amplify and label the regions of DNA that are used as pathogen specific probes. Consequently, membrane immobilised species-specific oligonucleotides synthesised from the ITS, β-tubulin and LSU molecular data were evaluated during the application of this diagnostic method to detect *Eutypa* species. It was found that the species-specific oligonucleotides, designed from ITS sequence data, could consistently detect *E. lata* and *E. leptoplaca*. The application of the RDBH method for the detection of these *Eutypa* species, based on β-tubulin and LSU sequence data, however, proved to be unsuccessful. Subsequently, a RDBH method, utilising species-specific oligonucleotides designed from elongation factor-1α sequence data, was successfully applied for the detection of *Botyrosphaeria dothidea* (Moug.:Fr.) Ces. & De Not., *Neofusicoccum luteum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips, *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers, A.J.L. Phillips and *Neofusicoccum ribis* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips. The method, however, was unsuccessful for the detection of *Diplodia seriata* De Not.

In addition to the above-mentioned shortcomings, the RDBH was not amenable to the detection of pathogens directly from field or environmental samples, but required preparation of DNA from pure cultures. The method, however, allows for the identification of multiple pathogens in a single assay. As DNA extraction methods are amended, improved and honed to obtain DNA from environmental samples, so would it increase the usefulness of RDBH.

OPSOMMING

Wingerd stamsiektes word veroorsaak deur patogene wat die vermoë het om wingerdplante te infekteer en dan stadige agteruitgang van dié wingerde te veroorsaak. Veral Eutypa terugsterwing het ‘n vernietigende effek op wingerde wêreldwyd deurdat dit groeikrag en oesmassa verlaag, maar ook omdat dit uiteindelik wingerdstokke kan dood. Die veroorsakende organisme is aanvanklik as *Eutypa armeniacae* Hansf. & Carter beskryf, die patogeen wat terugsterf by appelkose veroorsaak, maar sedert 1987 word hierdie spesies beskou as ‘n sinoniem van *Eutypa lata* (Pers.:Fr.) Tul & C. Tul (anamorph *Libertella blepharis* A. L. Smith). Dit is egter onlangs voorgestel dat ten minste twee spesies die vermoë het om wingerd te infekteer om Eutypa terugsterwing te veroorsaak. Gevolglik is molekulêre identifikasie- en karakteriseringstudies geloods om te bepaal watter spesies Eutypa terugsterwing in Suid-Afrikaanse wingerde veroorsaak. Dit het die molekulêre analise van drie molekulêre merkers behels, naamlik die interne getranskribeerde spasiëerderarea (“ITS”), die groot ribosomale subeenheid (“LSU rDNA”) en β-tubilien geen. Resultate van die filogenetiese analise dui daarop dat ’n tweede spesies, naamlik *Eutypa leptoplaca* (Mont.) Rappaz, saam met *E. lata* in geïnfekteerde plante voorkom.

Saam met bogenoemde twee spesies het daar ook verwante spesies van die Diatrypaceae familie voorgekom, naamlik *Cryptovalsa ampelina* (Nitschke) Fuckel en *Eutypella vitis* (Schwein.) Ellis & Everhart. Patogenisiteitstudies wat uitgevoer is met verteenwoordigende isolate van *C. ampelina, E. lata*, *E. leptoplaca*, en *E. vitis* dui daarop dat almal patogene van wingerd is. Verskeie Botryosphaeriaceae spesies wat gereeld in houtagtige wingerdweefsel aangetref word, is ook patogene van wingerd. Interne simptome wat algemeen met Botryosphaeriaceae infeksies geassosieer word, kan baie maklik met dié van Eutypa terugsterwing verwar word en dit het die nood laat ontstaan om ‘n opsporingsmetode te ontwikkel wat akkuraat genoeg is om tussen veelvoudige infeksies te onderskei.

'n Omgekeerde-stippelklad-hibridisasie (OSH) metode is gevolglik aangewend om *Eutypa* spesies betrokke in die Eutypa-siektekompleks op 'n vinnige, akkurate en betroubare manier op te spoor, sowel as die Botryosphaeriaceae species wat bekend is as patogene van wingerd. Die metode behels 'n saamgestelde PKR vir die vermeerdering en merk van DNS areas wat gebruik word as patogeen spesifieke peilers. Spesies-spesifieke oligonukleotiede ontwikkel vanaf die ITS, β-tubilien en LSU molekulêre data is op 'n membraan vasgeheg en gebruik om 'n diagnostiese toets te ontwikkel vir *Eutypa* species. Merkers ontwikkel vanaf die ITS kon *E. lata* and *E. leptoplaca* konsekwent opspoor. Die opspoor van *Eutypa* spesies met merkers vanaf die β-tubulien en LSU gene met OSH was onsuksesvol. Die OSH metode met merkers vanaf die verlengingsfaktor-1α kon susksesvol gebruik word om *Botyrosphaeria dothidea* (Moug.:Fr.) Ces. & De Not., *Neofusicoccum luteum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips, *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers, A.J.L. Phillips and *Neofusicoccum ribis* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips op te spoor. Dié metode kon egter nie *Diplodia seriata* De Not. opspoor nie.

Bykomend tot bogenoemde tekortkominge, kon die omgekeerde-stippelklad-hibridisasie metode ook nie aangepas word om patogene direk vanuit plantmateriaal op te spoor nie en word DNS afkomstig vanaf suiwer kulture benodig. Dié metode laat egter identifikasie van verskeie patogene in 'n enkele toets toe. Soos DNS ekstraksie metodes aangepas, verbeter en verfyn word om DNS vanuit plantmateriaal te verkry, sal die bruikbaarheid van die omgekeerde stippelklad hibridisasie metode ook verbeter.

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ABBREVIATIONS

AFLP	amplified fragment length polymorphism
ANOVA	analysis of variance
BLAST	basic local alignment search tool
bp	base pair
CBS	Centraal Bureau voor Schimmelcultures
cDNA	complementary DNA
CI	consistency index
CsCl	caesium chloride
CSPD	disodium 3-(4-methoxy-spiro{1,2-dioxetane-3,2'-(5'-chloro) tricyclo [3.3.1.1^{3,7}]decan}-4-yl)
CTAB	cetyltrimethylammonium bromide
dATP	deoxyadenine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dTTP	deoxythymidine triphosphate
dUTP	deoxyuridine triphosphate
EDTA	ethylene diamine tetra acetic acid
fig	figure
g	gram
h	hour
HI	homoplasy index
HPLC	high-performance liquid chromatography

ITS	internal transcribed spacer
kg/ha	kilogram per hectare
l	litre
LSU	large subunit
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
NaCl	sodium chloride
ng	nanogram
P	partition value
PAUP	phylogenetic analysis using parsimony
PCR	polymerase chain reaction
PDA	potato dextrose agar
RC	rescaled consistency index
RDBH	reverse dot blot hybridisation
rDNA	ribosomal DNA
RFLP	restriction fragment length polymorphism
RI	retention index
RNA	ribonucleic acid
s	second
SCARs	sequence characterised amplified regions
SDS	sodium dodecyl sulphate
SSC	sodium chloride and sodium citrate solution
STE-U	Stellenbosch University
TBR	tree-bisection-reconnection
Tris-HCl	Tris[hydroxymethyl]-aminmethane hydrochloric acid

µl	microlitre
µm	micromolar
µM	micromolar
v/v	volume per volume
w/v	weight per volume

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MOTIVATION

Grapevine trunk diseases involve a complex of pathogens that are responsible for the death and decline of grapevines throughout the grape growing regions of the world. Of these diseases, Eutypa dieback in particular has evoked a great deal of interest due to the severity and extensive damages and losses it has incurred. It is reported to have cost the US industry $260 million per annum (Siebert, 2001), while in South Africa losses of almost R1.7 million have been recorded for the 2000/2001 season (Van Niekerk *et al*., 2003). Eutypa dieback is thus recognised as one of the main limiting factors to the productivity and lifespan of a vineyard with the impact of this disease most significant on older, more established vines (Carter, 1988).

Eutypa dieback develops slowly on grapevines with symptoms apparent in one season then appearing absent in the next, or healthy parts of the vine will cover them up. Consequently, it will have appeared as though the vines have recovered; however, the symptoms may persist for several years until the infected portion of the vine dies. Normally, the infected portion is only on one cordon arm bearing stunted shoots with shortened internodes and small leaves (Munkvold, 2001). Bunches on the affected shoots also appear normal at the beginning of the season but tend to ripen late, producing a mixture of large and small berries (Creaser and Wicks, 1990). The shoot and foliar symptoms are characteristic of Eutypa dieback and can be traced back to a canker surrounding an old pruning wound (Trese *et al.,* 1980; Petzold *et al.,* 1981).

The causal organism of Eutypa dieback is *Eutypa lata* (Pers.:Fr.) Tul & C. Tul. It is an ascomycetous fungus with a wide host range occurring on 88 hosts in 28 plant families (Bolay and Carter, 1985; Carter, 1986). *Eutypa lata* has been reported to cause infection on agriculturally important crops like apricot (*Prunus armeniaca* L.), the host in which it was first described, and on almond (*Prunus dulcis* [Miller] D.A. Webb), cherry (*Prunus avium*), olive (*Olea europaea* L.), peach (*Prunus persica* L.), and walnuts (*Juglans regia*) (Carter *et al*., 1983; Munkvold and Marois, 1994). In South Africa, little was known about the incidence of the disease in our vineyards. It is, however, known that the fungus

is prevalent in high rainfall areas, with ascospores disseminated by rain, and responsible for invading pruning wounds (Carter, 1988). Such conditions occur typically in the vineyards of the Western Cape which is situated in a winter rainfall region. Consequently, it was decided to investigate occurrences of Eutypa dieback on grapevines in South Africa and while *E. lata* is largely responsible for the damages to grapevine, the importance of other fungi which contribute to the death and decline of grapevines could not be ignored. Species of Botryosphaeriaceae found on grapevines in South Africa (Crous *et al.,* 2000) are responsible for several diseases. The symptoms commonly associated with Botryosphaeriaceae species are the formation of cankers, dieback of shoots and branches, decline, brown streaking and the V-shaped lesion (Phillips, 1998 and 2000; Larignon *et al.*, 2001; Van Niekerk *et al.,* 2004). These symptoms are easily confused with the symptoms occurring in Eutypa dieback thus complicating disease identification and detection.

With the above as background, the aim of this study was thus twofold, i.e. (1) to correctly identify and characterise the pathogen(s) responsible for Eutypa dieback in South Africa and, (2) to develop a molecular detection method to screen infected grapevine material for the presence of *Eutypa* and Botryosphaeriaceae species.

It was decided to use molecular methods to identify and characterise the isolates obtained from diseased plants because identification methods solely based on cultural and morphological characteristics are insufficient for identifying the species responsible for Eutypa dieback (Glawe and Rogers, 1982; Glawe *et al*., 1982). Morphological characters may be lost or reduced in number when *E. lata* is cultured in the laboratory, but identification based on molecular characters uses stable DNA or protein sequence data to compare and evaluate the relationship among organisms. DNA-based molecular methods have been used extensively to facilitate the accurate identification of ascomycetous fungal species (Samuels and Seifert, 1995). Consequently, the sequence data from three molecular markers were analysed to identify and characterise Eutypa dieback as it occurs on grapevines in South Africa (Chapter 2). A reverse dot blot hybridisation method for disease detection was subsequently applied to screen infected grapevine material for

Eutypa and Botryosphaeriaceae species (Chapter 3), since this molecular technique was previously successfully used in the medical field to detect mutations related to human disorders (Saiki *et al.,* 1989), to assess bacteria from environmental samples (Voordouw *et al.,* 1993) and to identify *Phytophthora* species (Levesque *et al.,* 1998).

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CHAPTER 1

INTRODUCTION

Grapevine trunk diseases are caused by invasive pathogens that are responsible for the slow decline of grapevines (Gubler *et al*., 2005; Halleen *et al*., 2005). The diseases associated with the decline are Petri disease, esca and Eutypa dieback. Several species of Botryosphaeriaceae that commonly invade the woody tissue of diseased grapevines are also responsible for diseases occurring on grapevines. Eutypa dieback, however, is one disease in particular that has evoked a great deal of interest due to the severity and extensive damages and losses it has incurred. Few of the grape growing areas worldwide (Fig. 1-1) have escaped invasion demonstrating the ubiquitous nature of Eutypa dieback. Incidences of the disease have been reported in grape producing countries in both hemispheres. From regions experiencing severe winters like central Europe and eastern United States, to temperate regions like California, southern Australia, southern France and the Western Cape of South Africa.

Eutypa dieback on grapevines (*Vitis vinifera* L.) was detected for the first time in Australia in 1973 (Carter and Price, 1973; Wicks, 1975). In France, Bolay identified the disease on grapevines in 1977 (Bolay and Moller, 1977), where it was commonly referred to as Eutypiose. The disease, however, had been described previously, where it had been implicated in "dieback" of apricots (*Prunus armeniacae* L.), also commonly referred to as "gummosis". In the United States, the first appearance of the disease was in 1974 in New York (Uyemoto, *et al*., 1976) and in California (Moller *et al.,* 1974), while in South Africa, where it is referred to as "tandpyn", it was assumed to be the cause of "dying arm" in vines (Matthee and Thomas, 1977). The disease had often also been referred to as "dead arm" because many of the symptoms described for dieback was attributed to the pathogen, *Phomopsis viticola* (Sacc.) Sacc. (Moller and Kasimatis, 1981). Since then it was proposed that the term Eutypa dieback be used to describe the disease in grapevines. In extensive experiments it was demonstrated that many symptoms ascribed to "dead arm" were actually characteristic of Eutypa dieback.

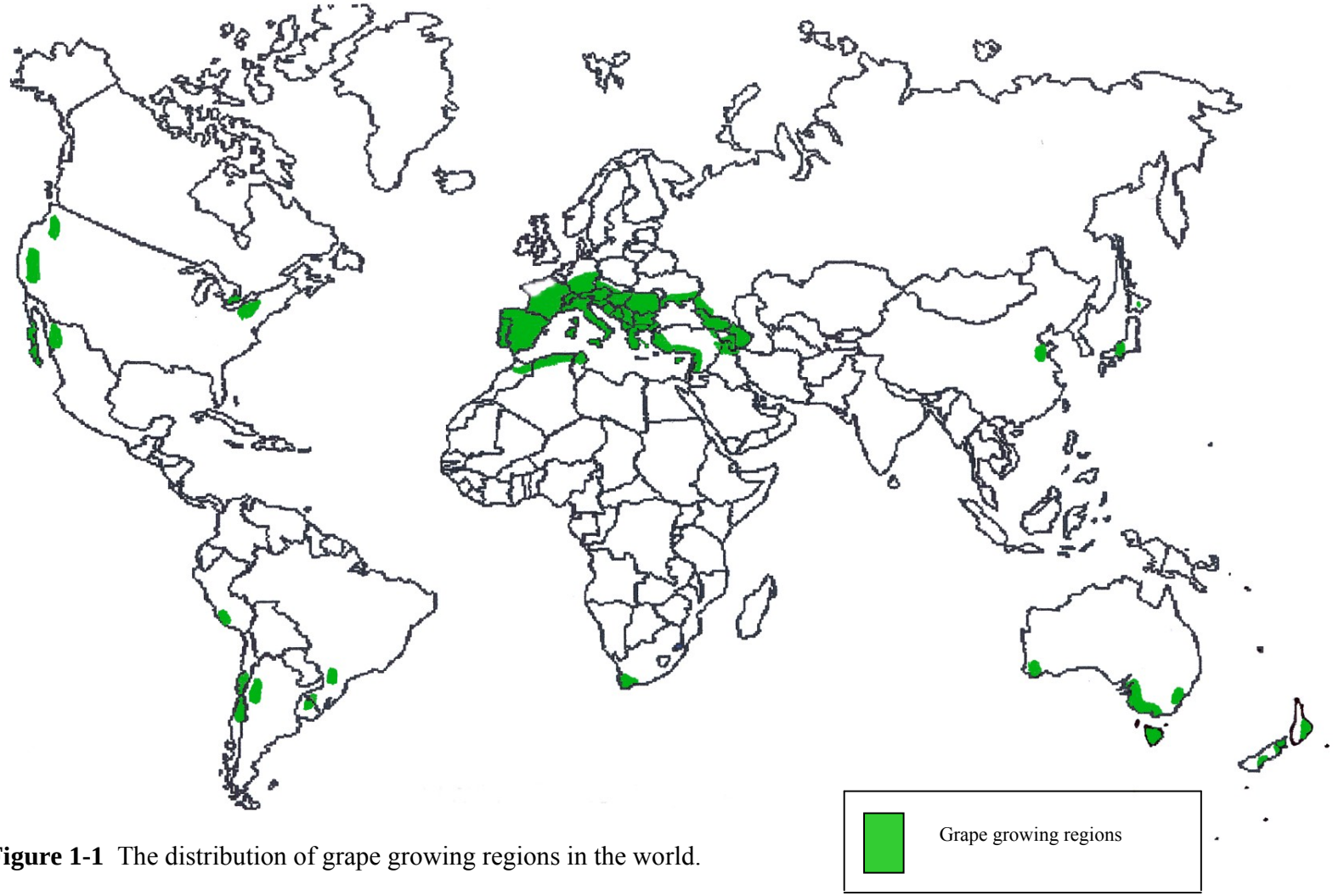

Figure 1-1 The distribution of grape growing regions in the world.

Thus, what do we know about the disease, Eutypa dieback? What are the symptoms, the disease cycle, how does the disease spread and what tools have been used to characterise and identify the causal organism?

1.1 Characterisation of Eutypa dieback

1.1.1 Symptoms

Eutypa dieback is chronic and slow to develop, with symptoms only appearing several years after infection (Munkvold *et al*., 1993). This could be six to eight years after infection (Chapuis *et al.*, 1998), but symptoms could become apparent as early as two to four years after infection (Creaser and Wicks, 2001). The earliest symptoms are the leaf and shoot symptoms (Fig. 1-2A) most apparent in spring, becoming more pronounced with each year (Carter, 1988). Even then symptoms may vary according to years, area and cultivars (Petzoldt *et al*., 1981; Péros *et al*., 1999, Creaser and Wicks, 2001). The symptoms can persist for several years until the infected portion of vine dies, resulting in "dead arm" (Fig. 1-2B).

The shoot symptoms are most apparent in spring when the shoots are 20 - 40 cm long (Munkvold, 2001). The shoots from infected wood are stunted with shortened internodes and small leaves (Fig. 1-2A). The leaves that become chlorotic (i.e. pale yellow or green) are cupped and tattered around the edges or margins (Carter, 1988; Kovacs, 2000; Munkvold, 2001). Some leaves are speckled with small brown lesions (Magarey and Carter, 1986) which, with time, develop a scorched appearance (Fig. 1-2C). These foliar symptoms often appear only on one cordon arm while the rest of the vine shoots appear unaffected. Often healthy shoots on adjacent cordons mask these symptoms. Towards the end of the season the leaf and shoot symptoms will all but disappear, with only the basal leaves of shoots affected. Consequently, it will have appeared as though the vines have recovered, but the infected trunk and the growth above it will wither and die.

Fig. 1-2. Symptoms of Eutypa dieback of grapevines.

A. Weak, stunted shoots with shortened internodes on a vine arm. **B.** An older vine severely affected by Eutypa dieback, resulting in "dead arm". **C.** Some leaf symptoms that can occur are leaves with tattered edges or margins, or leaves with speckled, brown lesions. With time the leaves develop a scorched appearance. **D.** Bunches on affected shoots producing mixture of large and small berries. These bunches shrivel and die on more severely affected shoots. (Photo: JHS Marais). **E.** A cankered area on wood surrounding an old pruning wound (Photo: JHS Marais). **F.** A cross-section of an *Eutypa lata* infected arm shows a brown wedged-shaped zone of dead wood (Photo: F. Halleen).

Similary, influorescences on the affected shoots appear normal but after flowering they often wither and die. Bunches on the affected shoots also appear normal at the beginning of the season but tend to ripen late, producing a mixture of large and small berries or bunches could shrivel and die (Fig. 1-2D) on the more severely affected shoots (Creaser and Wicks, 1990). Shoot and foliar symptoms are usually accompanied by canker formation (Fig. 1-2E).

A cross section of the trunk reveals a canker that appears as darkened or discoloured wood in a wedge shape (Fig. 1-2F), with a definite margin between live and dead wood. The cankered wood on the trunk has a distorted and flattened appearance and is normally covered by old dead bark. These cankers can develop up to three feet long downwards and can extend below the ground line on severely affected vines as determined in tests done on 14 year Shiraz vines in the spring of 1999 (Creaser and Wicks, 2001). Vascular streaking or discolouration from infected shoots can be traced back to a cankered area on the wood (Fig. 1-2E) surrounding an old pruning wound (Trese *et al*., 1980; Petzoldt *et al*., 1981). Surrounding the pruning wound is a dark stroma containing fungal fruiting bodies. From these fruiting bodies the causal organism of Eutypa dieback on grapevines can be identified.

1.1.2 Causal organism

The causal organism of Eutypa dieback on grapevines was first described as *Eutypa armeniacae* Hansf. & Carter (Carter, 1957), which causes dieback of apricots. In 1973, research by Carter and Price discovered grapevines (*Vitis vinifera* L.) as another economically important host of the pathogen. Since 1987, this species has been considered a synonym of *Eutypa lata* (Pers.: Fr.) Tul & C. Tul (anamorph *Libertella blepharis* A.L. Smith). In 1999, however, genetic analysis of *Eutypa* strains isolated from vineyards in California performed by Descenzo *et al*. (1999), presented the concept that the two species of *Eutypa* (*E. armeniacae* and *E. lata*) are not conspecific. In truth, prior to 1987, *E. armeniacae* and other taxa were not considered synonymous with *E. lata*. Interestingly, research conducted in California indicated that more than one species of *Eutypa*, and perhaps other genera in the same family, could also be pathogens of

grapevine capable of infecting pruning wounds (Smith, 2004). But, *E. lata* is the species that has been implicated most in Eutypa dieback and as a grapevine pruning wound invader.

1.1.3 Disease cycle

The initial or primary sites of infection are pruning wounds, where the fungus can survive in an infected trunk for a long period of time (Fig. 1-3). The pruning wounds are surrounded by a dark layer or stroma. The stromata are black, cracked and sometimes punctate (Munkvold, 2001). Embedded in the stromata are small black fungal fruiting bodies called perithecia. By scraping the surface of the stromata the perithecial cavities are revealed in which spores, called ascospores, reside in a gelatinous whitish mass (Teliz and Valle, 1979). The development of perithecia is favoured by an annual rainfall of at least 350 mm and is often only seen in areas with high rainfall. Infection is initiated when ascospores are deposited onto fresh pruning wounds. Rain or snowmelt is required for the release of the ascospores that become airborne and are deposited on the ends of exposed vessels. It has been suggested that viable ascospores can be aerially transported for 50 to 100 km (Carter, 1988). The ascospores travel through the xylem tissue to the cambium and phloem where they germinate in a matter of hours provided an optimal temperature of 20 to 25^{o}C is reached. Germination takes place 2 mm or more beneath the surface of the wound where the mycelia slowly multiply in the vessels and subsequently affecting those elements associated with the functioning of the wood. The disease develops slowly on grapevine and no symptoms will be apparent on the first or second season's growth. After an incubation period of about three years or more (Moller and Kasimatis, 1978) cankers form, which lead to the characteristic shoot and foliar symptoms of Eutypa dieback.

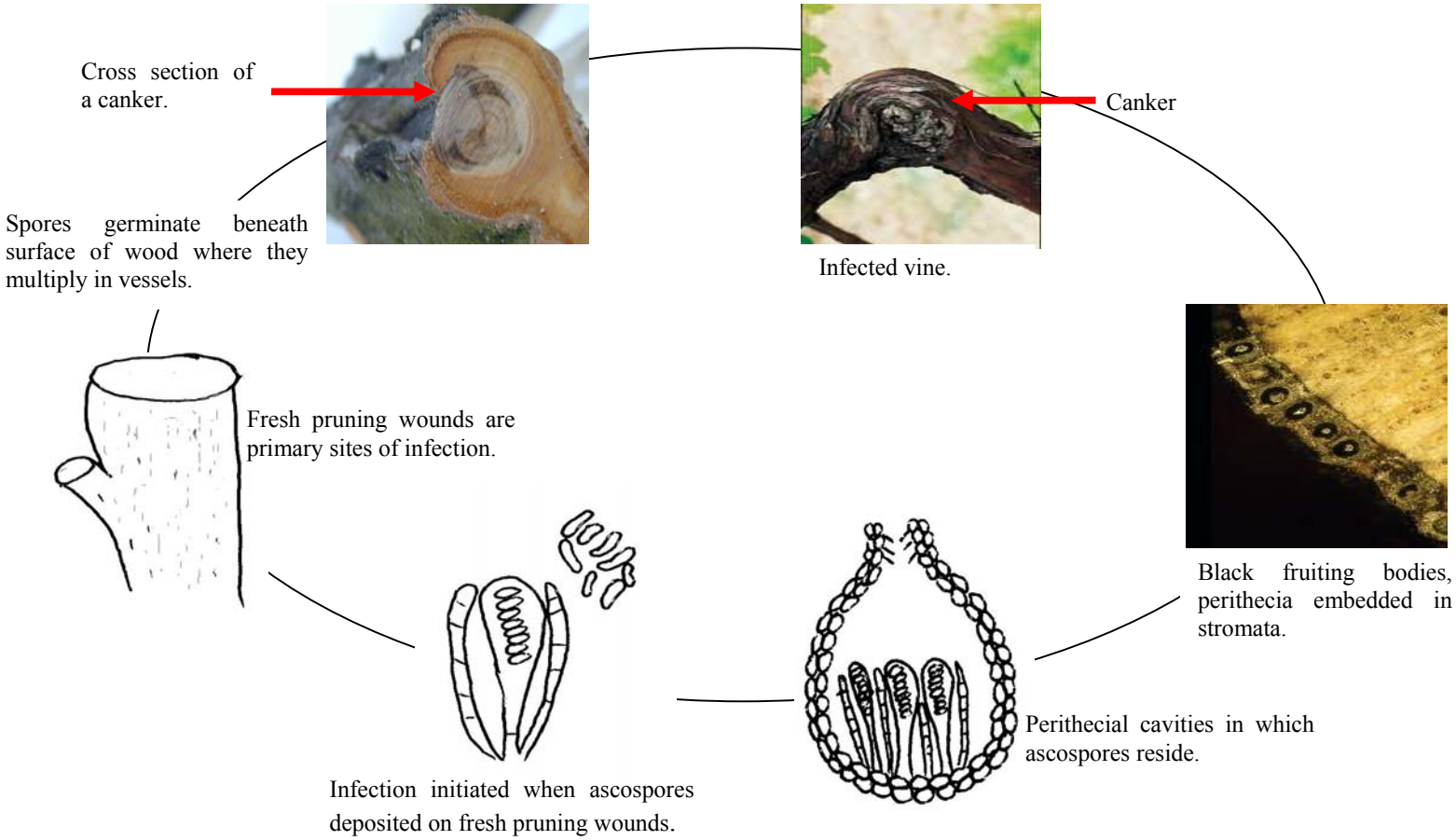

Fig. 1-3. Characteristic morphological stages in the disease cycle of Eutypa dieback.

1.1.4 Epidemiology

The causal organism of Eutypa dieback exists in its perfect or teleomorphic (*E. lata* Pers.:Fr.) and in its imperfect or anamorphic (*Libertella blepharis* A.L. Smith) state. The anamorph, *L. blepharis*, produce filiform spores inside asexual fruiting bodies called pycnidia found on the inner bark or between the perithecia (Munkvold *et al*., 1993). The asexual spores or conidia have not been implicated in the infection process (Carter, 1957; Cortesi and Milgroom, 2001) as studies found that isolates sampled in a single vineyard was genetically different (Péros et al., 1997; Péros and Larignon, 1998) which is consistent with a sexual form of infection.

The teleomorph, *E. lata*, produces the perithecia in which ascospores reside and it is these spores that have been found to be the primary source of inoculum (Munkvold *et al*., 1993) particularly in areas with a mean annual rainfall higher than 330 mm and under optimal temperature conditions ranging from 20 to 25°C (Ramos *et al*., 1975). Perithecium formation is rare and the disease incidence lower in areas under sprinkler irrigation where the mean annual rainfall is lower than 279 mm (Ramos *et al*., 1975). In temperate regions these perithecia reach maturity in early spring (Carter, 1988) where a minimum rainfall of 2 mm is required to initiate the release of ascospores from dry stromata (Carter, 1957). By late autumn the contents of the perithecia will have been exhausted but enough ascospores will have been released to infect vines pruned in winter. In colder regions (below 0°C), dissemination of ascospores is greatest in late winter. Ascospores will, therefore, be in abundance during pruning time.

Most ascospores are released in winter (after rainfall or snowmelt) or early in spring while the numbers released in summer are less. The dissemination of the fungus coincides with the time when pruning is done. The chance of infection immediately after pruning is, therefore, higher in December than in January or February (winter and early spring in the northern hemisphere). This is similar to findings in the southern hemisphere where the chance of infection is higher in June than in July or August (winter and early spring in the south). Pruning wounds remain susceptible for up to two weeks (Magarey and Carter, 1986), after which susceptibility steadily declines (after three to four weeks,

Petzold *et al*., 1981). Perithecia can survive for long periods under favourable conditions and will continue to produce ascospores year after year.

1.1.5 Conditions favouring infection

The fungus has been known to develop on dead wood (Peros *et al.*, 1996) but in a study done by Cortesi and Milgroom (2001) on vineyards in Italy and Germany perithecia was found on living tissue as well. In winter rainfall regions with mild winter temperatures (e.g. Western Cape of South Africa) sporulation is encouraged and following a long, dry period the perithecia is "conditioned" for release following a long wet period (Ramos *et al*., 1975). Trese *et al.* (1980), stated after studying results from freezing and thawing tests, that ascospores can germinate in low temperatures and even at very low temperatures (such as -20°C). *Eutypa lata* favour and grow better in fast growing plant tissue than plants under stress conditions (Rumbos, 1987). The presence of alternative hosts would increase the chance of infection especially as viable ascospores can travel for up to 100 km on air currents.

1.1.6 Host range

Eutypa lata is an ascomycetous fungus with a wide host range, particularly on perennial tree species. Its host range includes 88 species distributed among 28 plant families of which most are tree species (Bolay and Carter, 1985; Carter, 1986). In all areas where *E. lata* has been isolated on alternative hosts it has always been associated with disease of grapevine in that area. This suggests that grapevine is the universally accepted host of *E. lata*, susceptible to a variety of its pathotypes, but with the fungus not necessarily pathogenic to nearby hosts (Carter *et al*., 1985). Pathogenicity studies (Carter *et al*., 1985; Munkvold and Marois, 1994) have supported that grapevines is the universal host.

Although *E. lata* is pathogenic to grapevines it does occur and severely affect some economically important crops like apricot (*Prunus armenicae* L.) and blackcurrant (*Ribus nigrum* L.) (Carter, 1988). Work by Magarey and Carter (1986) in Australia have shown

how *E. lata* can infect a variety of woody plants and has found alternative hosts in almond (*Prunus dulcis* [Miller] D.A. Webb), apple (*Malus domestica*), pear (*Pyrus communis* L.), tamarisk (*Tamarix parviflora*) and in at least 16 ornamentals which include *Ceanothus*, *Pittosporum* and the Guebler rose. In California, agriculturally important crops like almond (*Prunus dulcis* [Miller] D.A. Webb), sweetcherry (*Prunus avium* L.), olive (*Olea europaea* L.), peach (*Prunus persica* L.) and walnuts (*Juglans regia*) had been infected (Carter *et al*., 1983; Munkvold and Marois, 1994). The fungus has been known to cause rotting on olive and apple fruits (Rumbos, 1987) while in almond, where it was previously identified as a saprophyte, pathogenicity studies (Rumbos, 1985) indicated that it could cause infection. In the latter study it did not produce the characteristic shoot and foliar symptoms and dieback of arms had not been recorded. Munkvold (2001) has also stated that although *E. lata* occurs on approximately 88 species of woody dicots in 52 genera (including forest and ornamental species) not all isolates from these hosts need to be pathogenic. Pathogenicity has been ascertained for isolates originating from almond, apple, apricot, *Ceanothus* (as previously mentioned), chokecherry (*Prunus virginiana* L.), grapevine, olive, pear, sourcherry (*Prunus cerasus* L.), sweetcherry, walnut and possibly peach. Infection in peach has not been recorded but pathogenicity studies using *E. lata* isolates from apricot in the inoculation has shown some positive results. Other hosts not previously mentioned are lemon (*Citrus limon*) (Chapuis *et al.,* 1998) and pistachio (*Pistacia vera* L.) (Rumbos, 1986). *Eutypa lata* has, therefore, had quite an impact on many hosts other than grapevine but the symptoms of the disease in the latter are the most severe.

1.1.7 Impact of disease

Eutypa dieback of grapevines is a trunk disease that has a devastating impact on vineyards worldwide. The disease is slow to develop which makes it difficult to detect and the full implications are not felt until vineyards reach maturity (Carter, 1988). *Eutypa lata* infects propagating material, affects the growth of newly planted young vines and infection is especially threatening to established older vines. Once the disease has manifested in a vineyard, grapevines gradually decline and eventually die.

In Australia yield losses of 860 kg/ha (Shiraz) and 740 kg/ha (Cabernet Sauvignon) had been recorded (Wicks and Davies, 1999). In California losses were estimated at 30% to more than 60% for vineyards growing either Chenin Blanc or French Columbard while vineyards containing vines 20 years and older had recorded yield reductions of 83% (Munkvold *et al*., 1994). The cost to the Californian wine industry was estimated to be more than $260 million per annum (Siebert, 2001). The financial impact of the disease (Table 1-1) is the result of the cost of reworking, removing infected vines and, where necessary, the replanting of vineyards. Most threatening to vine productivity is susceptibility to pruning wounds made when mature vines are reworked to change the cultivar or to alter the growth pattern to a new training system.

In European countries Eutypa dieback is believed to be the chief limiting factor of the lifespan of vineyards. The reduction in yield is attributed to the decreased number of clusters per vine (Munkvold *et al.,* 1994) while reduced wine quality is due to uneven berry maturation (Wicks and Davies, 1999).

In the Western Cape of South Africa an average of 32% vineyards were found to show *Eutypa*-like symptoms (Halleen *et al.,* 2001a) with one 22 year old vineyard being the most severely affected (98%). Significant yield reductions are recorded annually even on vines showing minimal incidence of the disease. All *V. vinifera* cultivars are susceptible to *E. lata* and no remedial measures are available to effectively prevent the spread of the disease. Biocontrol agents investigated for the inhibition of *E. lata* (Ferreira *et al*. 1991; Schmidt *et al*., 2001a and b) showed some retardation of the fungus in laboratory experiments, but no field trials were conducted. Laboratory studies on the inhibitory effect of fungicides (Halleen *et al*., 2001b) proved benomyl to be the most effective. Benlate, Bavistin and acrylic paint, which proved to be successful on one-year old canes in the laboratory, are currently being tested in the field (Creaser and Wicks, 2002; Sosnowski *et al*., 2004). In California, field trials were conducted on pruning wounds using boron for the control of Eutypa dieback. The results indicated that boron could be used as a safe, economical and environmentally safe management strategy to control *E.*

Table 1-1. Impact of Eutypa dieback on vineyards worldwide.

WINE GROWING REGION	LEVEL OF INFECTION	LOSS RECORDED	PERIOD	REFERENCE
California, US	30 – 62%		1994	Munkvold *et al.*, 1994
		US$260 million	2001	Siebert, 2001
Southern Australia		A$20 million (Shiraz alone)	2000/2001	Sosnowski *et al.*, 2005
	24%	570kg or A$1150 per hectare	1999	Wicks and Davies, 1999
	47%	1500kg or A$3040 per hectare		Jalfon, 2005
South Africa	31 – 98% (highest level of infection recorded in 22 year old vineyard)		2000/2001	Halleen *et al.*, 2001a
		7.3% or 367 tons @ R4 610 per ton = R1.7 million R50 000 – R70 000 to replace vines	2003	Van Niekerk *et al.*, 2003

lata. However, formulations need to be optimised to increase the duration of control on the surface of the pruning wounds, while the effect of boron on bud failure of grapevine need to be confirmed (Rolshausen and Gubler, 2005).

1.1.8 Pruning wound susceptibility

The time of pruning influences the rate of contamination of pruning wounds. Moller and Kasimatis (1980) found that pruning wounds made in late winter are more susceptible than pruning wounds made in December in California while wounds made in March were less susceptible. Petzoldt *et al.* (1981) showed that pruning wounds made during late autumn were more susceptible than pruning wounds made in early spring with an intermediate period of susceptibility in winter. This coincides with increased spore dispersal during autumn and early spring with fewer spores in the air during winter (Trese *et al.,* 1980; Petzoldt *et al.*, 1981; Ramsdell, 1995). In South Africa, Ferreira (1999) attributed the increase in growth of the fungus during winter months to an increase in nutrients, thus pruning wounds made during this period could be more susceptible.

The age of the wound also plays a role in the rate of infection of pruning wounds. After the first pruning date pruning wounds are more susceptible to contamination than at the second pruning date. This could be because more sap is exuded when vines are pruned in the latter stages of the dormant seasons (Munkvold and Marois, 1995). Wound susceptibility decreased as the wound age increased (Gendloff *et al*., 1983). This could be attributed to the presence of other wound colonisers that could inhibit the growth of *E. lata* (Carter and Moller, 1970; Ferreira *et al.,* 1989). However, the decrease in wound susceptibility could be because of natural wound healing (Petzoldt *et al.*, 1981). These researchers also noted a 75 – 100% reduction in infection four weeks after pruning but Munkvold and Marois (1995) contend that the period of wound susceptibility could be longer. Ramsdell (1995) noted that pruning wounds in California were susceptible for up to a month while Trese *et al*. (1982) showed a reduced level of susceptibility over a 56 day period. Young plantings are more at risk to infection because pruning wounds go unprotected and the same holds true for older plantings because they require more severe pruning to rework the vine. Also, in older vines vigour will have declined.

Thus, from the above it is obvious that grapevines show a marked difference in susceptibility to infection and this could be because of the hosts' response, age, training system and the genotype of the vines. Cultural practices and climatic conditions could also be responsible for this variation in susceptibility. The tolerance of some cultivars to infection could be associated with differences in sensitivity to phytotoxic compounds.

1.1.9 Toxin production by *Eutypa lata*

The symptoms produced by *E. lata* would suggest that pathogenesis involves the production of a toxin (Tey-Rulh *et al*., 1991). Such a compound was isolated from diseased vines and identified as eutypine (Tey-Rulh *et al*., 1991). Eutypine [4-hydroxy-3-(3-methyl-3-butene-1-ynyl) benzaldehyde] (Fig. 1-4) was found in the sap, stem, leaves and influorescence of all grapevines infected with *E. lata*. It was stated that the presence of the toxin is largely responsible for the expression of symptoms in Eutypa dieback.

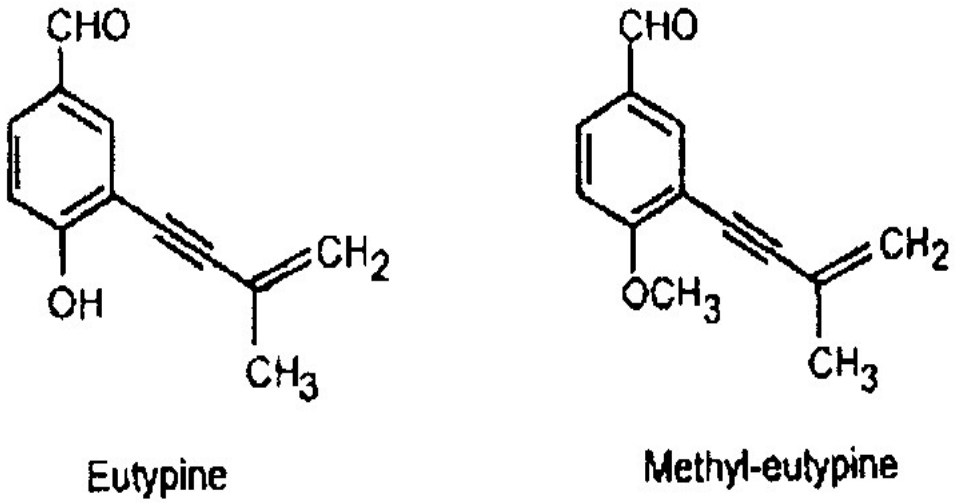

Fig. 1-4. Chemical structure of eutypine and methyl-eutypine (Deswarte *et al.*, 1996)

The toxin is known to accumulate in grapevine cells and result in the death of leaf protoplasts (Mauro *et al*., 1988). The toxin causes ultra-structural changes such as disruption of the cytoplasm, disorganisation of chloroplasts and breakage of the plasma membrane (Deswarte *et al*., 1994). Respiration and energy balances are also affected by the secretion and accumulation of the toxin (Deswarte *et al*., 1996a and b) and photosynthesis is inhibited (Amborabe *et al*., 2001). A protein encoding a eutypine reducing enzyme has been isolated and characterised (Roustan *et al.*, 2000) with the view to increase the tolerance of *V. vinifera* cells to *E. lata*. It has been used in transgenic grapevine research to impart increased resistance to grapevine plants to the toxin, eutypine (Legrand *et al*., 2003). It has also been suggested that the phytotoxicity of *E. lata* could be due to a group of structurally related compounds with varying degrees of activity (Molyneux *et al*., 2002; Smith *et al*., 2003) which could explain the variation in symptoms expressed in Eutypa dieback.

1.2 Identification of *Eutypa lata*

1.2.1 Identification using phenotypic characteristics

1.2.1.1 ***Morphological characteristics and cultural characteristics.*** The teleomorph *E. lata* of the family Diatrypaceae, class Pyrenomycetes of the Ascomycotina produces perithecia in a thin single layer, hidden in wood or bark (Rappaz, 1984). The bases of the perithecia are embedded at varying depths according to the plant host and age of the stroma (Rappaz, 1984). The stroma is black and continuous with irregular margins with slightly emergent necks or ostioles (Fig. 1-5, left). The asci (Fig. 1-5, right) are borne on pedicels of varying length (60–130 µm long) and measure 30-60 x 5–7.5 µm with an apical pore (Carter, 1988). An ascus contains eight ascospores that are subhyaline and allantoid measuring 6.5–11 x 1.8–2 µm (Rappaz, 1984; Carter, 1988). The teleomorph develops slowly and no perithecia are produced in culture. Under the latter conditions only the anamorph is produced.

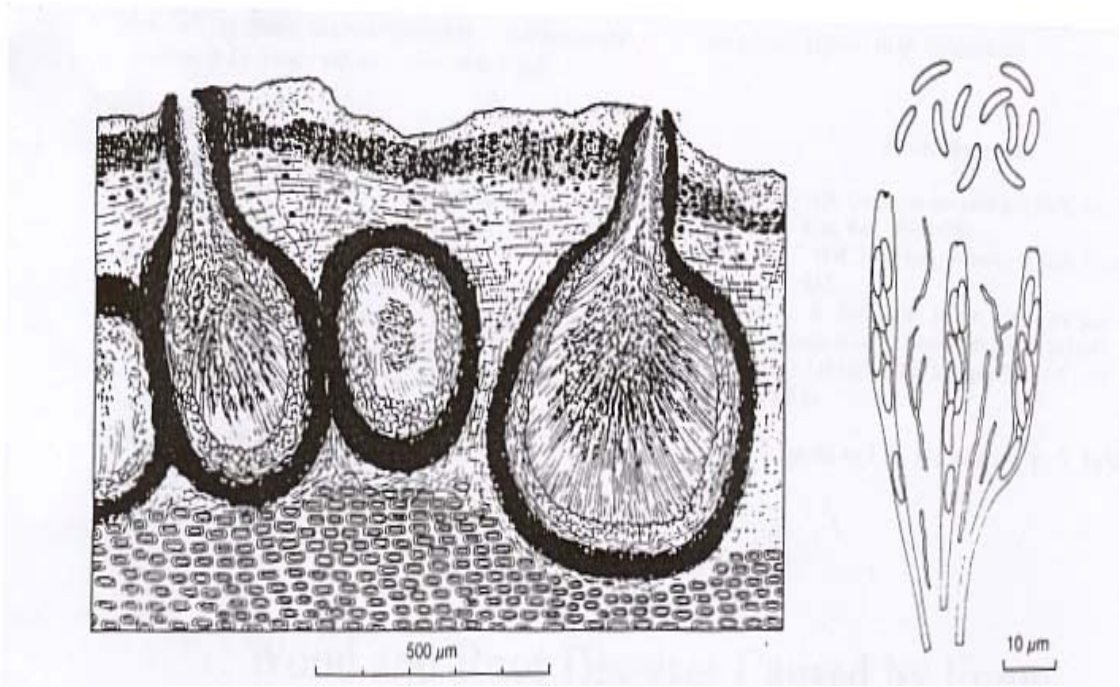

Fig. 1-5. Vertical section of perithecial stroma (left) and asci and ascospores (right) of *Eutypa lata.* (Adapted from Carter, 1988).

The anamorph, *Libertella blepharis* (= *Cytosporina* sp. Carter, 1957) form black pycnidia after four to six weeks (Glawe and Rogers, 1982) which exude a cream to orange coloured conidial mass. The conidia are filiform, straight or curved and numerous measuring 20-45 x 0.8–1.5µm (Munkvold, 2001) arising from septate hyphae that are branched, hyaline and smooth (Fig. 1-6).

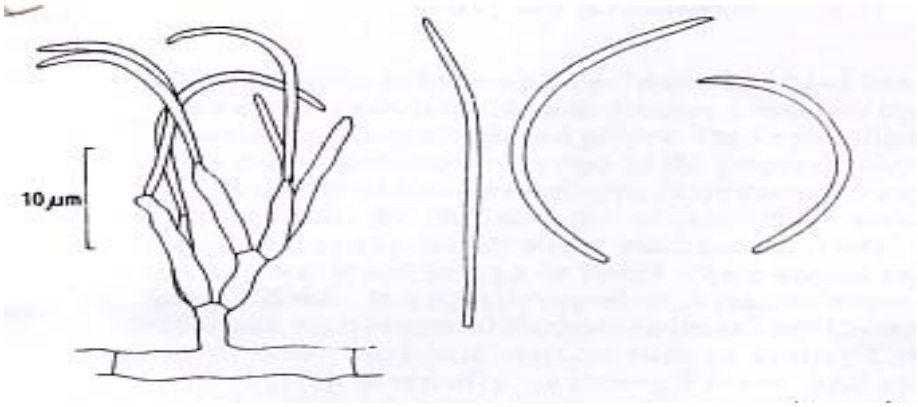

Fig. 1-6. Conidiogenous cells and conidiophores (left) and conidia (right) from a culture of *Libertella blepharis*. (Adapted from Carter, 1988)

Characteristically, under cultural conditions *E. lata* isolates produce mycelial colonies that are at first white and cottony, cream-coloured in reverse, then later (after approximately two weeks), cultures develop a grey pigment with the reverse side almost black (Munkvold, 2001).

Since it is known that taxonomic informative morphological characteristics may be lost or reduced in number when *E. lata* is cultured in the laboratory, identification procedures based on cultural and morphological characteristics alone are insufficient to correctly identify this fungal species. In contrast, identification based on molecular characters using DNA or protein sequence data is known to be a reliable manner to compare and evaluate the relationship among fungi. DNA-based molecular methods have been used extensively to differentiate genera, species, subspecies, races and strains (Glass and Donaldson, 1995).

1.2.2 Identification using molecular methods

The utility of molecular regions need to be taken into consideration when choosing a molecular marker in phylogenetic studies (Hillis and Dixon, 1991; Mitchell *et al.,* 1995). The regions should have sufficient levels of sequence conservation and variation. Regions that are too conserved have few nucleotide changes, therefore, limited resolving power. Similarly, regions that are too variable are inconsistent because of too many nucleotide changes. An ideal region should be large, abundant i.e. present in multiple copies yet evolve as a single copy (Guarro *et al*., 1999). The nuclear ribosomal RNA (rDNA) and protein coding genes like the β-tubulin gene are regions that fulfill these criteria (White *et al*., 1990; Guarro *et al*., 1999).

1.2.2.1 Nuclear ribosomal RNA

DNA sequence comparisons of the rDNA region have proved useful in determining relationships between fungal genera and species (Hillis and Dixon, 1991). Nuclear ribosomal DNA is comprised of three RNA genes: a small subunit (SSU), a large subunit (LSU) and the 5.8S subunit (Fig. 1-7). Interspersed between the rDNA regions which are

highly conserved are the internal transcribed spacer regions, ITS1 and ITS2, which are more variable and known to evolve at a faster rate than the three ribosomal gene sequences mentioned above. It was, however, found that the ITS regions are mostly highly conserved within a fungal species but are known to vary between species (White *et al*., 1990). Sequence analyses of the ITS regions have, therefore, been used in fungal taxonomy, including phylogenetic analyses (Mitchell *et al*., 1995)

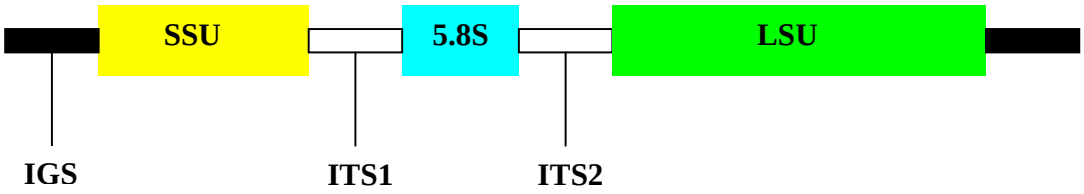

Figure 1-7. Gene arrangement within a eukaryotic rDNA unit. IGS = intergenic spacer; ITS = internal transcribed spacer; SSU = small subunit and LSU = large subunit

1.2.2.2 Phylogenies based on multiple genes

In the construction of phylogenetic trees a tree based on one set of sequence data (e.g. only from the ITS region) has limited resolving power (Mitchell *et al*., 1995). It is known that greater resolution would be achieved when trees are constructed from more than one set of sequence data. The development of methods using different molecules as phylogenetic markers was, therefore, used in comparing phylogenies generated by rDNA and other genes (Roger *et al*., 1999). Consequently, by combining the results from more than one set of sequence data it was possible to elucidate congruencies between data sets and eliminate any ambiguities (Roger *et al.,* 1999; Baldauf *et al*., 2000). Combinations of taxonomic informative gene sequences, such as the ribosomal gene cluster and the tubulin gene family have, therefore, been used with success in fungal taxonomy (Guarro *et al*., 1999; Roger *et al*., 1999; Baldauf *et al*., 2000).

1.2.2.3 Beta-tubulin genes

The tubulin gene family comprising of the alpha (α), beta (β) and gamma (γ) genes are widely distributed among the eukaryotes (Keeling and Doolittle, 1996). These genes code for components of microtubules which is a characteristic feature of eukaryotic cells.

The microtubules are major components of the cytoskeleton, the mitotic spindles and flagella. Of the tubulin gene family the sequence database of the β-tubulin is the largest. Beta-tubulin is a protein-coding gene with conserved exons and many introns (Fig. 1-8). It has sufficient length and level of sequence conservation to produce highly resolved trees. The β-tubulin gene was shown to have considerable sequence variation at the 5'-end (Dupont *et al*., 2000) and is useful as a phylogenetic marker because insertions and deletions which can lead to disparities in phylogenetic studies are rare (Edlind *et al*., 1996). Phylogenies based on α and β tubulin genes have taxonomic representatives from both basidiomycete and ascomycete fungi (Dupont *et al*., 2000; Keeling *et al*., 2000; Edgcomb *et al*., 2001; Dupont *et al*., 2002).

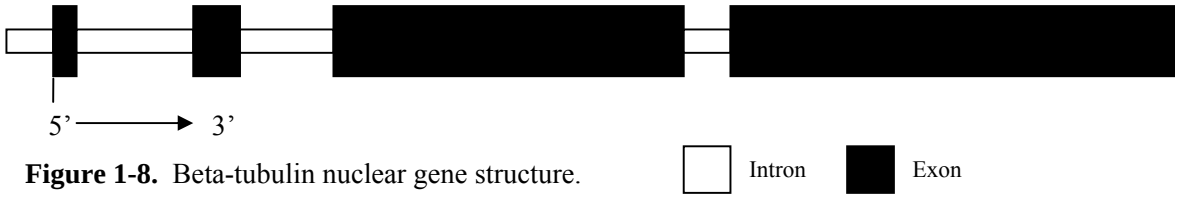

Figure 1-8. Beta-tubulin nuclear gene structure.

1.2.2.4 Large subunit of the rRNA genes

The divergent domains of the large subunit (LSU) regions of the rDNA operon (Fig. 1-9) have considerable sequence variation making this gene highly informative (Hillis and Dixon, 1991). The utility of the large subunit allows for comparison of organisms from a high taxonomic level down to species level. Comparison of the LSU sequence data can be used to infer phylogenetic relationships among closely related organisms. The use of sequence data has not only provided the means to analyse variation within fungal species to assess genetic diversity and phylogeny of species and genera but has had a far reaching impact on the detection and diagnosis of plant diseases (Henson and French, 1993).

Figure 1-9. Gene arrangement within eukaryotic rDNA unit. LSU = large subunit.

1.3 Detection

Traditional diagnostic methods used in plant pathology are dependent on the observation of morphological and cultural characteristics of the organisms implicated in the disease (Samuels and Seifert, 1995). These methods are time consuming and laborious. Also, organisms like *E. lata* that lack comparable structural characters for its identification (Glawe and Rogers, 1984) and, thereby, its detection, is a complicating factor in the diagnosis of Eutypa dieback particularly as several species implicated in grapevine trunk diseases cause similar symptoms to those observed in Eutypa dieback.

1.3.1 Early methods for the detection of *Eutypa* species

One of the earliest methods for the detection of *Eutypa* species focused on the serological properties of the mycelium and ascospores of *Eutypa armeniacae* (Francki and Carter, 1970). It was, however, discovered that the ascospores of *Cryptovalsa ampelina* (Nitschke) Fuckel, a species that colonises infected grapevine tissue, are antigenically similar to that of *E. armeniacae*. The two species could thus not be distinguished. Later, an antiserum specific to *E. armeniacae* was described (Price, 1973). The antiserum, however, was not tested against other *Eutypa* species. The specificity of serological tests remains a questionable issue as the potential for false positive results is very real (Weber, 2002). Molecular methods involving polymerase chain reaction (PCR) have been developed to increase specificity and reliability.

1.3.2 Molecular methods of detection

The advances made by molecular methods in fungal systematics has resulted in the generation of large sequence databases from which information could be garnered to aid the development of detection methods. The fungal rDNA with its multiple units of variable and conserved regions has facilitated the design of universal primers (White *et al.*, 1990). Molecular markers like random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP) and restriction fragment length polymorphisms (RFLP) have been used extensively to study variation among fungal

genera and species (Chen *et al*., 1992; McDermott *et al.,* 1994; Sreenivasaprasad *et al*., 1996; Zhang *et al*., 1997; Lindqvist *et al*., 1998; Descenzo *et al*., 1999; Witthuhn *et al*., 1999; Dupont *et al*., 2002; Tan and Niessen, 2003; Martin and Tooley, 2004). These variations were further exploited in the subsequent design of species-specific primers (Brown *et al*., 1993, Lovic *et al*., 1995; Kageyama *et al*., 1997; Zhang *et al*., 1997; Lindqvist *et al*., 1998; Lecomte *et al*., 2000; Rolshausen *et al*., 2004). The development of sequence characterised amplified regions (SCARs) stemmed from this kind of research as well (Jiménez-Gasco and Jiménez-Diaz, 2003; Lardner *et al*., 2005).

Molecular-based detection methods have the advantage of being more sensitive, specific and reliable and with the added ability of processing large numbers of samples (Alvarez, 2004). However, detection methods that employ species-specific primers have limitations in that these PCR-based assays only detect one specific pathogen. If another pathogen is present it will not generate a positive PCR response. Furthermore, many of these primers can not be applied *in situ* to environmental samples.

Other molecular-based detection methods like nucleic acid hybridisation that have been in use for a long time now, have utilised the large sequence databases generated by fungal systematic studies to develop sequence-specific oligonucleotides as probes (Kawasaki *et al*., 1993; Levesque *et al*., 1998). Examples of these hybridisation methods, Southern blot and Northern blot, are widely used in molecular research. Previously, probes were obtained from cDNA and genomic clones labeled by nick translation (Kawasaki *et al*., 1993) but as DNA sequencing techniques have improved so have the ability to obtain probes that discriminate between closely related organisms. These methods, although a mainstay of molecular research, can be time-consuming and labour intensive. Consequently, the dot-blot method of hybridisaton was developed to simplify the analysis of samples.

In the dot-blot method of hybridisation, DNA (or RNA) obtained by PCR amplification is dotted onto membranes, fixed and then hybridised with specific oligonucleotides or probes (Kafatos *et al*., 1979). Dot-blots are widely applicable for the detection of genetic

mutations and polymorphisms (Bos *et al*., 1984; Dessauer *et al*., 1996) but as more different mutations and polymorphisms occur, the method becomes more cumbersome (Saiki *et al*., 1989). This is due mainly because more PCR products need to be fixed to a number of membranes to be hybridised to additional probes (Kawasaki *et al*., 1993). Dot-blots are applicable for the detection of only one particular pathogen, not the simultaneous detection of several pathogens occurring in a complex on the same infected plant material. These limitations were addressed with the development of the reverse dot blot hybridisation method.

1.3.2.1 ***Reverse dot blot hybridisation and multiple pathogen detection.*** Reverse dot blot hybridisation involves the use of multiplex PCR to simultaneously amplify and label the regions of DNA that are used to design specific oligonucleotides (Levesque *et al*., 1998). The labeled PCR products are used as probes for hybridisation with a membrane that contains an array of specific oligonucleotides. This is in "reverse" to the dot blot method of hybridisation where the PCR products are fixed to the membrane and the specific oligonucleotides are used as probes. A positive signal at a specific position on the membrane will indicate the presence of the particular pathogen against which the oligonucleotide was designed. In this way, because the species-specific or pathogen-specific oligonucleotides are fixed to one membrane, several pathogens can be detected.
Eutypa lata has been positively identified as the pathogen responsible for Eutypa dieback. However, *E. lata* is found on infected grapevine tissue in association with related fungi from the same family, the Diatrypaceae (Trouillas and Gubler, 2004). Also isolated from the V-shaped canker from infected grapevines are Botryosphaeriaceae species.

1.4 Botryosphaeriaceae species occurring on grapevines

In addition to the pathogen *E. lata* and related fungi, several species of Botryosphaeriaceae that commonly invade the woody tissue of diseased grapevines are responsible for diseases on grapevine. There are many species of *Botryosphaeria* Ces. & De Not. and as a genus it has been well-documented where it is found throughout the

world in temperate and tropical climates. *Botryosphaeria* spp. are ascomycetes with a wide host range, particularly on woody plant hosts (von Arx, 1987). Many species of *Botryosphaeria* are considered endophytic or saprophytic on many hosts including a number of species in the family Proteaceae and on *Fagus* spp. (Smith *et al*., 1996; Danti *et al*., 2002; Denman *et al*., 2003), though some species are pathogenic when plant hosts are growing under stress conditions (Brown and Britton, 1986). Some plant hosts to which *Botryosphaeria* spp. are pathogenic include *Arbutus menziesii* (Maloney *et al*., 2004), *Eucalyptus* spp. (Smith *et al*., 1994), *Pistacia vera* L. (Michailides, 1991; Ma *et al*., 2002; Ahimera *et al*., 2003), pome and stone fruit (Brown and Britton, 1986; Ogata *et al*., 2000; Slippers *et al*., 2007) and *Quercus* spp. (Shoemaker, 1964; Sanchez *et al*., 2003).

Symptoms commonly associated with the *Botryosphaeria* spp. are fruit and seed rots, leaf spots, stem and branch cankers, gummosis and dieback (Brown and Britton, 1986; Parker and Sutton, 1993; Pusey, 1993; Biggs and Miller, 2003). The *Botryosphaeria* spp. most associated with disease are *Botryosphaeria dothidea* (Moug.: Fr.) Ces. & De Not., *B. obtusa* (Schwein.) Shoemaker and *B. stevensii* Shoemaker, and to a lesser extent, *B. parva* (Pennycook and Samuels), *B. lutea* (A.J.L. Phillips) and *B. rhodina* (Berk. & M.A. Curtis) Arx.

Many *Botryosphaeria* spp. are commonly associated with diseases of grapevines. *Botryosphaeria stevensii* was associated with decline of mature grapevines in Canada (Shoemaker, 1964) and with black dead arm in Hungary in 1974 (Lehoczky, 1974). Later, researchers in Italy (Cristinzio, 1978; Rovesti and Montermini, 1987) ascribed black dead arm to *B. obtusa*, while Larignon and Dubos (2001) associated *B. obtusa* and *B. dothidea* with the disease when it was identified for the first time in 1999 in vineyards in France. Excoriose caused by *B. dothidea* is prevalent in grape-growing regions and results in severe damage and reductions in yield. Grapevine decline syndrome is caused by *B. parva*, but *B. obtusa*, *B. stevensii*, *B. lutea* and *B. rhodina* have also been associated with the syndrome (Phillips, 2002). Macrophoma rot has commonly been attributed to *B. dothidea*, but *B. ribis* Grossenb. & Duggar has also been isolated in association with this

disease (Mulholland, 1990). Diplodia cane dieback and bunch rot has been ascribed to *B. rhodina*. All of these diseases are responsible for severe losses and damages of grapevines but the *Botryosphaeria* species implicated as the causal organisms are considered as only weakly pathogenic (Phillips, 1998; Van Niekerk *et al*., 2004; Taylor *et al*., 2005) and as secondary invaders of damaged and stressed grapevines (Castillo-Pando *et al*., 2001).

It must be noted, however, that the taxonomy of species within the genus *Botryosphaeria* has recently undergone a re-evaluation with new lineages in the Botryosphaeriaceae now being recognised. This phylogenetic study was based on comparisons made using DNA sequence data from the large subunit of the rDNA operon and anamorph morphology (Crous *et al*., 2006). In accordance with the delineation obtained and with several new genera included in the Botryosphaeriaceae to represent these lineages, name changes were suggested for those fungi mentioned above. The genus *Botryosphaeria* was restricted to *B. dothidea* (Moug.: Fr.) Ces. & De Not. and *B. corticis* (Demaree & M.S. Wilcox) Arx & Müll. (Phillips *et al*., 2006) but is no longer valid for *B. obtusa*. Henceforth, *B. obtusa* will be referred to as *Diplodia seriata* De Not. As a new name for *B. rhodina* have not been proposed as yet, it will continue to be referred to by its traditional name. The genus *Neofusicoccum* is one of the lineages included in the Botryosphaeriaceae to accommodate fungi with *Fusicoccum*-like anamorphs. These include *B. lutea* which will henceforth be referred to by its anamorph *Neofusicoccum luteum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips comb. nov., *B. parva* will be referred to as *N. parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips comb. nov. and *B. ribis* will be referred to as *N. ribis* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips comb. nov. *Botryosphaeria stevensii* will in turn be referred to by its *Diplodia* anamorph, namely *Diplodia mutila* (Fries) Montagne.

In South Africa, several species of Botryosphaeriaceae have been identified where it is commonly associated with diseases on stone and pome fruit (Crous *et al*., 2000). Three *Botryosphaeria* species, namely; *B. obtusa*, *B. dothidea* and *N. ribis*, have been found on grapevines in South Africa (Crous *et al*., 2000) but during a recent study by van Niekerk

et al. (2004) on grapevines in South Africa only *B. obtusa* was consistently isolated. Two new *Fusicoccum* species were also isolated from grapevine during this study, namely *F. viticlavatum* (Niekerk and Crous) Crous, Slippers & A.J.L. Phillips comb. nov. and *F. vitifusiforme* (Niekerk and Crous) Crous, Slippers & A.J.L. Phillips comb. nov.

The symptoms commonly associated with the Botryosphaeriaceae species on infected grapevines are the formation of cankers, dieback of shoots and branches, decline, brown streaking and the V-shaped lesions (Phillips, 2000; Larignon *et al*., 2001; Van Niekerk *et al*., 2004). These symptoms are easily confused with the symptoms occurring in Eutypa dieback. The absence of morphological characters makes identification of the diseases attributed to Botryosphaeriaceae species difficult. The presence of Botryosphaeriaceae species in the V-shaped canker characteristic of Eutypa dieback makes disease identification and detection complicated. Here the reverse dot blot hybridisation method for disease detection may potentially be used to correctly identify the presence of multiple pathogens in a single assay.

In summary, it is clearly evident from the research done to date that *E. lata* is a major threat to vine productivity and longevity throughout the grape growing regions of the world. It has been said that Eutypa dieback or "tandpyn" of grapevines existed in South Africa since 1881 (Du Plessis, 1948). However, confirmation of the disease being similar to "dying arm" reported in Australia and the USA, was only obtained in 1976 (Matthee and Thomas, 1977). Then, *E. armeniacae* was accepted as the causal organism and identification was based largely on morphological characteristics. This method is generally insufficient to identify *E. lata*, especially in the presence of morphologically similar species, which is why molecular methods to identify and characterise Eutypa dieback in South Africa was important. How Eutypa dieback in South Africa compares with the disease elsewhere in the world is of great interest, particularly if more than one species of *Eutypa* is responsible for the disease. Hence, the use of molecular data and pathogenicity studies would aid in determining the presence of other organisms and their pathogenicity to grapevines, while shedding some light on the relationship between the organisms. This became the objective of the work done in Chapter 2.

In Chapter 3, the objective was to apply a reverse dot blot hybridisation method to detect *Eutypa* and Botryosphaeriaceae species. Symptoms in Eutypa dieback are slow to develop and vary from year to year and may go undetected. With more than one organism that can be isolated from the wedge-shaped canker characteristic of Eutypa dieback, it is important to have a diagnostic test that can accurately and correctly identify the presence of a particular pathogen.

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CHAPTER 2

MOLECULAR IDENTIFICATION AND CHARACTERISATION OF EUTYPA DIEBACK ON GRAPEVINES IN SOUTH AFRICA

2.1 INTRODUCTION

Eutypa dieback of grapevines (*Vitis vinifera* L.) is recognised as a serious disease of grapevines worldwide. The disease is responsible for a slow decline of vineyards, thereby reducing growth and yield, eventually killing the grapevines. Eutypa dieback is estimated to have cost the US wine industry alone approximately US$260 million in losses (Siebert, 2001), while in Australia it is estimated that it caused yield losses of approximately A$1000 – 2800 per hectare in Shiraz (Wicks and Davies, 1999). In South Africa, the losses are estimated to be in the region of R1.7 million for the Cabernet Sauvignon crop (2000/2001 season) and this value excludes the cost of replacing vines, manpower and the subsequent reestablishment period (van Niekerk *et al*., 2003).

The causal organism of Eutypa dieback on grapevines was first described as *Eutypa armeniacae* Hansf. & Carter (Carter, 1957), a fungus, that causes dieback of apricots (*Prunus armeniacae* L.). In 1973, Carter and Price discovered that grapevines represented another economically important host for the pathogen. Its host range presently includes 80 species distributed among 27 plant families of which most are tree species (Carter *et al.*, 1983; Bolay and Carter, 1985). Since 1987, *E. armeniacae* has been considered a synonym of *Eutypa lata* (Pers.: Fr.) Tul & C. Tul (anamorph *Libertella blepharis* A.L. Sm.). In 1999, however, genetic analysis of *Eutypa* strains isolated from vineyards in California (Descenzo *et al*., 1999) revealed that the two species of *Eutypa* (*E. armeniacae* and *E. lata*) were not conspecific. In truth, prior to 1987, *E. armeniacae* and other taxa were not considered synonymous with *E. lata* (Rappaz, 1987). Interestingly, research conducted in California indicated that more than one species of *Eutypa*, and perhaps other genera in the same family, could also be pathogens of grapevine, and were capable of infecting pruning wounds (Smith, 2004).

This research led to the recent discovery of *Eutypa leptoplaca* (Mont.) Rappaz, reported as a pathogen of grapevines, in California (Trouillas and Gubler, 2004). No other grape growing area worldwide where Eutypa dieback is a problem, have reported the same findings. In a South African context, it has thus become important to ascertain the pathogen or pathogens responsible for Eutypa dieback of grapevines. The correct identification and characterisation by use of molecular markers and pathogenicity testing would add greatly to the clarification of the causal organisms of Eutypa dieback in South Africa.

Molecular markers are commonly used to differentiate fungal taxa (Glass and Donaldson, 1995), and have proven particularly useful where cultural and morphological characteristics alone were insufficient to distinguish the causal organisms. The phylogenetic analysis embarked on in this study, with the aim of identifying and characterising the pathogen(s) responsible for Eutypa dieback in South Africa, used the internal transcribed spacer (ITS) and large subunit (LSU) regions of the ribosomal DNA (rDNA) operon, and the β-tubulin gene. Pathogenicity testing was also conducted on isolates to confirm Koch's postulates.

2.2 MATERIALS AND METHODS

2.2.1 Fungal isolates

Isolates were collected from grapevines and fruit trees with dieback symptoms, as well as from fruiting structures on infected wood (Table 2-1). *Eutypa lata* isolates were also obtained from Southern Australia and France, while reference cultures were obtained from the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands. Initial identification of *E. lata* isolates was based on comparison of the anamorph, *Libertella blepharis*, with published descriptions (Glawe and Rogers, 1982). The isolates were grown for 2 - 4 weeks on potato-dextrose agar (PDA; 39 g/l, Biolab, Merck) at 22^{o}C. All isolates were stored as colonised PDA plugs at 4^{o}C in sterile water and on agar slants. All isolates collected were deposited at the Stellenbosch University culture collection (STE-U), Stellenbosch, South Africa.

Table 2-1. Isolates collected and used in the phylogenetic study.

Isolate	Host	Cultivars	Origin
STE-U 5519	*Vitis vinifera*	Cabernet Sauvignon	South Africa
STE-U 5520	*V. vinifera*	Cabernet Sauvignon	South Africa
STE-U 5521	*V. vinifera*	Cabernet Sauvignon	South Africa
STE-U 5522	*V. vinifera*	Cabernet Sauvignon	South Africa
STE-U 5523	*V. vinifera*	Cabernet Sauvignon	South Africa
STE-U 5524	*V. vinifera*	Chenin Blanc	South Africa
STE-U 5525	*V. vinifera*	Chenin Blanc	South Africa
STE-U 5526	*V. vinifera*	Shiraz	South Africa
STE-U 5527	*V. vinifera*	Shiraz	South Africa
STE-U 5528	*V. vinifera*	Cabernet Sauvignon	South Africa
STE-U 5529	*V. vinifera*	Cabernet Sauvignon	South Africa
STE-U 5530	*V. vinifera*	Chenin Blanc	South Africa
STE-U 5531	*V. vinifera*	Chenin Blanc	South Africa
STE-U 5532	*V. vinifera*	Chenin Blanc	South Africa
STE-U 5534	*V. vinifera*	Cabernet Sauvignon	South Africa
STE-U 5535	*V. vinifera*	Cabernet Sauvignon	South Africa
STE-U 5536	*V. vinifera*	Cabernet Sauvignon	South Africa
STE-U 5537	*V. vinifera*	Cabernet Sauvignon	South Africa
STE-U 5538	*V. vinifera*	Chenin Blanc	South Africa
STE-U 5539	*V. vinifera*	Chenin Blanc	South Africa
STE-U 5540	*V. vinifera*	Chenin Blanc	South Africa
STE-U 5541	*V. vinifera*	Cinsaut	South Africa
STE-U 5542	*V. vinifera*	Cinsaut	South Africa
STE-U 5543	*V. vinifera*	Cabernet Sauvignon	France
STE-U 5544	*V. vinifera*	Unknown	France
STE-U 5545	*V. vinifera*	Unknown	France
STE-U 5546	*V. vinifera*	Unknown	France
STE-U 5547	*V. vinifera*	Unknown	France
STE-U 5548	*V. vinifera*	Unknown	France
STE-U 5549	*V. vinifera*	Unknown	France
STE-U 5550	*Prunus salicinia*	Angeleno	South Africa
STE-U 5551	*P. salicinia*	Angeleno	South Africa
STE-U 5552	*P. salicinia*	Angeleno	South Africa
STE-U 5553	*P. salicinia*	Angeleno	South Africa
STE-U 5554	*P. salicinia*	Angeleno	South Africa
STE-U 5555	*P. salicinia*	Larry Anne	South Africa
STE-U 5556	*P. salicinia*	Larry Anne	South Africa

STE-U 5557	*P. salicinia*	Angeleno	South Africa
STE-U 5558	*P. salicinia*	Angeleno	South Africa
STE-U 5559	*P. salicinia*	Angeleno	South Africa
STE-U 5560	*Prunus persica*	Armking	South Africa
STE-U 5561	*V. vinifera*	Merlot	South Africa
STE-U 5562	*V. vinifera*	Merlot	South Africa
STE-U 5315	*Prunus armeniaca*	Unknown	Southern Australia
STE-U 5316	*V. vinifera*	Merlot	Southern Australia
STE-U 5317	*V. vinifera*	Chardonnay	Southern Australia
STE-U 5318	*V. vinifera*	Shiraz	Southern Australia
STE-U 5319	*V. vinifera*	Cabernet Sauvignon	Southern Australia
STE-U 5580	*V. vinifera*	Cabernet Sauvignon	South Africa
STE-U 5581	*V. vinifera*	Chenin Blanc	South Africa
STE-U 5582	*V. vinifera*	Cabernet Sauvignon	South Africa
STE-U 5583	*V. vinifera*	Cabernet Sauvignon	South Africa
STE-U 5584	*V. vinifera*	Cabernet Sauvignon	South Africa
STE-U 5585	*V. vinifera*	Cabernet Sauvignon	South Africa
STE-U 5586	*V. vinifera*	Cabernet Sauvignon	South Africa
STE-U 5587	*V. vinifera*	Cabernet Sauvignon	South Africa
STE-U 5588	*V. vinifera*	Cabernet Sauvignon	South Africa
STE-U 5589	*V. vinifera*	Cabernet Sauvignon	South Africa
STE-U 5590	*V. vinifera*	Cabernet Sauvignon	South Africa
STE-U 5591	*V. vinifera*	Cabernet Sauvignon	South Africa
STE-U 5533	*V. vinifera*	Cabernet Sauvignon	South Africa
STE-U 5621	*V. vinifera*	Cabernet Sauvignon	South Africa
STE-U 5622	*V. vinifera*	Cabernet Sauvignon	South Africa
STE-U 5692	*V. vinifera*	Chenin Blanc	South Africa
STE-U 5693	*V. vinifera*	Chenin Blanc	South Africa
STE-U 5694	*V. vinifera*	Chenin Blanc	South Africa
STE-U 5695	*V. vinifera*	Chenin Blanc	South Africa
STE-U 5696	*V. vinifera*	Chenin Blanc	South Africa
STE-U 5697	*V. vinifera*	Chenin Blanc	South Africa
STE-U 5698	*V. vinifera*	Chenin Blanc	South Africa
STE-U 5699	*V. vinifera*	Sauvignon Blanc	South Africa
STE-U 5700	*V. vinifera*	Sauvignon Blanc	South Africa
STE-U 5701	*V. vinifera*	Sauvignon Blanc	South Africa
STE-U 5702	*V. vinifera*	Ruby Cabernet	South Africa
STE-U 5703	*V. vinifera*	Cabernet Franc	South Africa

CBS 622.84/STE-U 5627	*E. armeniacae*	*Vitis vinifera*	Italy
CBS 208.87/STE-U 5628	*E. lata*	*Tilia*	Switzerland
CBS 247.87/STE-U 5630	*E. lata*	*Lonicera xylosteum*	Switzerland
CBS 101932/STE-U 5632	*E. lata*	*Fraxinus excelsior*	Netherlands
CBS 286.87/STE-U 5633	*E. leptoplaca*	*Arundo donax*	France
CBS 288.87/STE-U 5634	*E. leptoplaca*	*Cissus hypoglauca*	South Australia

2.2.2 DNA isolation and PCR amplification

Fresh mycelium was harvested by scraping the surface of the agar with a scalpel and transferred to a microcentrifuge tube containing extraction buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 100 mM EDTA, pH 8.0, 2% w/v SDS). Total DNA was isolated according to the method of Lee and Taylor (1990). DNA was resuspended in sterile HPLC water (BDH, Merck) and examined on 0.8% agarose gels by electrophoresis. For PCR reactions the DNA samples were diluted 1:10 or 1:50 using sterile HPLC water.

All PCR amplifications were performed in 50 µl reactions on a MJ Research PTC 200 thermal cycler. Each DNA sample was amplified using universal ITS primers ITS4 and ITS5 (White *et al.,* 1990), β-tubulin primers Bt2b and T1 (Glass and Donaldson, 1995), and large subunit primers LROR and LR7 (Rehner and Samuels, 1994; Vilgalys and Hester, 1990). The cycling programs for the ITS PCR consisted of 35 cycles with a 45 s denaturation at 94°C, a 30 s annealing at 53°C, a 1 min extension at 72°C and a final extension period of 10 min at 72°C. The β-tubulin PCR program consisted of 36 cycles with a 30 s denaturation at 94°C, a 30 s annealing at 50°C, a 90 s extension at 72°C and a final extension period of 7 min at 72°C. The large subunit PCR program consisted of 35 cycles with an initial denaturation of 10 min at 95°C, followed by 30 s denaturation at 94°C, a 30 s annealing at 55°C, a 1 min extension at 72°C and a 10 min final extension period at 72°C. Amplified DNA fragments were visualized under UV light after electrophoresis at 70V on 1% agarose gels stained with ethidium bromide (10 mg/ml) and run in 1X Tris-borate-EDTA. PCR products were purified with PCR purification kits (GFX™ PCR DNA and Gel Band Purification Kit, Amersham Pharmacia Biotech Inc. N.J.) to remove any excess primers, nucleotides and polymerases. The concentration of the purified PCR products was determined by DNA fluorometer readings (DyNA Quant

200, Hoefer Pharmacia Biotech Inc. N.J.). All samples were sequenced in both directions directly from purified PCR products using the primers mentioned for amplification except for the large subunit that was sequenced using the primers LROR and LR5.

2.2.3 Phylogenetic analyses

Sequence data were edited and imported to Sequence Alignment Editor v.2.0a8 (Rambaut, 2002) which allows for manual alignment and manipulation of the forward and reverse sequences (see Appendix). Sequence homology was determined doing a BLAST search against GenBank sequence data. Phylogenetic analyses of all aligned sequence data were done using PAUP (Phylogenetic Analysis Using Parsimony) v.4.0b10 (Swofford, 2000). Gaps were treated as "fifth base" and all characters were unordered and of equal weight, while uninformative characters were excluded. A parsimony analysis was performed for all datasets using the heuristic search option with stepwise random addition and a tree-bisection-reconnection (TBR) as branch-swapping algorithm to find maximum-parsimony trees. Branches with a maximum branch length of zero were collapsed and all equally parsimonious trees were saved. Topological constraints were not enforced. Branch support was determined by 1000 bootstrap replicates. The consistency index (CI), retention index (RI) rescaled consistency index (RC) and homoplasy index (HI) were also calculated. Congruency between trees from different datasets was examined by partition homogeneity tests.

2.2.4 Pathogenicity tests

Fourteen isolates representing *E. lata* and other species collected from grapevines with dieback symptoms were selected for the pathogenicity tests (Table 2-2). The isolates were plated on PDA and incubated at 22^{o}C for 1 week. In the first experiment inoculations were made on one-year old rooted cuttings of Sauvignon Blanc planted in plastic bags [5l, 160 mm (length) × 120 mm (breadth) × 300 mm (height)] that were maintained in a glasshouse at +/- 26^{o}C and watered thrice a week. A V-shaped notch was cut through the cambium to a depth of approximately 1.5 mm above the first node. A 5

mm diam plug was cut from the margin of actively growing fungal isolates. The plug was placed mycelium-side down on the wound. A negative control of a non-colonised PDA plug was used to inoculate wounded cuttings serving as control treatments. The inoculation sites were covered with Parafilm (Pechiney Plastic Packaging, Menasha, WI) for 1 month after which it was removed. There were 10 replicates per treatment in a randomised design. The rooted cuttings were maintained in a greenhouse for 1 year. Results were collected by splitting the stems at the wound longitudinally and measuring the internal lesions.

In the second experiment green shoots were cut from one-year old Sauvignon Blanc and Red Globe vines 3 months after being potted in soil. The green shoots were cut between the internodes. Inoculations of the green shoots were made using the same fungal isolates selected for the first experiment. A V-shaped notch was cut through the cambium to a depth of approximately 1.5 mm above the internode. A 5 mm diam plug was cut from the margin of actively growing fungal isolates. The plug was placed mycelium-side down on the wound. A negative control of a non-colonised PDA plug was applied to the wounded shoots. The inoculation sites were covered with Parafilm for one week after which it was removed. There were 5 replicates per cultivar per treatment in a randomised design. Inoculated shoots were incubated under moist conditions in the laboratory for 3 weeks at 22^{o}C. Following the incubation period, the shoots were split longitudinally and the internal lesions measured. The data were statistically analysed using ANOVA and the mean lesion lengths are listed in Table 2-2.

In both experiments re-isolations were made from the margins of the lesions by plating the infected material on PDA with incubation at 22^{o}C. The plant material in both experiments was subsequently destroyed by incineration or autoclaving twice for 20 min.

2.3 RESULTS

2.3.1 Phylogenetic analyses

Amplification of the ITS region of the isolates collected in South Africa yielded a ~600 bp PCR product for those from grapevines and a ~560 bp PCR product for those form fruit trees. Variations in size of the amplified PCR product were resolved through alignment. Of the 528 characters in the aligned sequence, 349 characters were constant, 125 characters were parsimony informative and 54 variable characters were parsimony uninformative. A heuristic maximum parsimony analysis resulted in 300 most parsimonious trees of 319 steps (CI = 0.721, RI = 0.983, RC = 0.676, HI = 0.279). The bootstrap consensus tree (Fig. 2-1) was rooted using *Diatrype flavovirens* (Pers) Fr., *Diatrypella pulvinata* Nitschke and *Cryptosphaeria eunomia* var. *fraxini* (Richon) Rappaz as outgroups.

Amplification of a partial sequence at the 5'-end of the β-tubulin gene region yielded a single PCR product of ~800 bp for all isolates collected in South Africa. Of the 736 characters in the aligned sequences, 159 characters were constant, 487 characters were parsimony informative and 90 variable characters were parsimony uninformative. A heuristic maximum parsimony analysis resulted in 144 most parsimonious trees of 1692 steps (CI = 0.702, RI = 0.932, RC = 0.654, HI = 0.298). The bootstrap consensus tree (Fig. 2-2) was rooted using *Diatrype* Fr. and *Diatrypella* (Ces. & De Not) De Not. as outgroups.

Amplification of a partial sequence of the large subunit region yielded a single PCR product of ~1500 bp of which ~900 bp was sequenced using primers LR0R and LR5. Of the 814 characters in the aligned sequences, 477 characters were constant, 304 were parsimony informative and 33 variable characters were parsimony uninformative. A heuristic maximum parsimony analysis resulted in 72 most parsimonious trees of 477 steps (CI = 0.795, RI = 0.827, RC = 0.657, HI = 0.205). The bootstrap consensus tree (Fig. 2-3) was rooted using *Xylaria hypoxylon* (L.) Grev. as an outgroup for the *Eutypa*

lata isolates and *Cryptosphaeria eunomia* as outgroup for the *C. ampelina* and *Eutypa* isolates.

A partition homogeneity test of the combined datasets of ITS and β-tubulin sequences contained 984 characters of which 514 characters were constant, 463 characters were parsimony informative and seven variable characters were parsimony uninformative. A heuristic maximum parsimony analysis resulted in a single most parsimonious tree (Fig. 2-4) of 792 steps (CI = 0.723, RI = 0.854, RC = 0.667, HI = 0.217, *P* = 0.059).

One hundred and eight isolates were included in the ITS tree (Fig. 2-1). The tree consisted of five groups with high bootstrap support. Group I included 59 isolates, of which 44 were isolates collected from grapevines in South Africa with dieback symptoms. The remaining 15 isolates comprised of the reference isolates (STE-U 5627/CBS 622.84, STE-U 5628/CBS 208.87 and STE-U 5630/CBS 247.87), the French isolates (STE-U 5543-5549) and the Australian isolates (STE-U 5315-5319). The 59 isolates grouped with *E. lata / E. armeniacae* in a well-supported clade with a bootstrap support value of 83%. In group II an isolate (STE-U 5581) identified as *E. leptoplaca* grouped together with the reference isolate, STE-U 5633/CBS 286.87 with high (94%) support in one clade while the other *E. leptoplaca* reference isolate, STE-U 5634 CBS 288.87, grouped with other *E. leptoplaca* isolates in a separate clade (bootstrap support value of 93%). Group III included *E. lejoplaca* (Fr.) Cooke, *E. maura* (Fr.) Sacc., *E. astroidea* (Fr.) Rappaz, *E. crustata* (Fr.) Sacc. and *E. consobrina* (Mont.) Rappaz. In group IV, isolates (STE-U 5621, STE-U 5622, STE-U 5701 and STE-U 5703) identified as *Cryptovalsa ampelina* (Nitschke) Fuckel grouped together with this species in a well supported clade with a bootstrap support value of 100%. In group V isolates STE-U 5561, STE-U 5562 and STE-U 5702, isolated from grapevines, grouped with the fruit tree isolates, STE-U 5550-5560. These isolates shared sequence similarity to *Eutypella vitis* (Schwein.) Ellis & Everh. with a bootstrap support value of 98%.

Eighty isolates were included in the β-tubulin tree (Fig. 2-2). The tree consisted of five groups with high bootstrap support. Group I included 30 isolates collected from

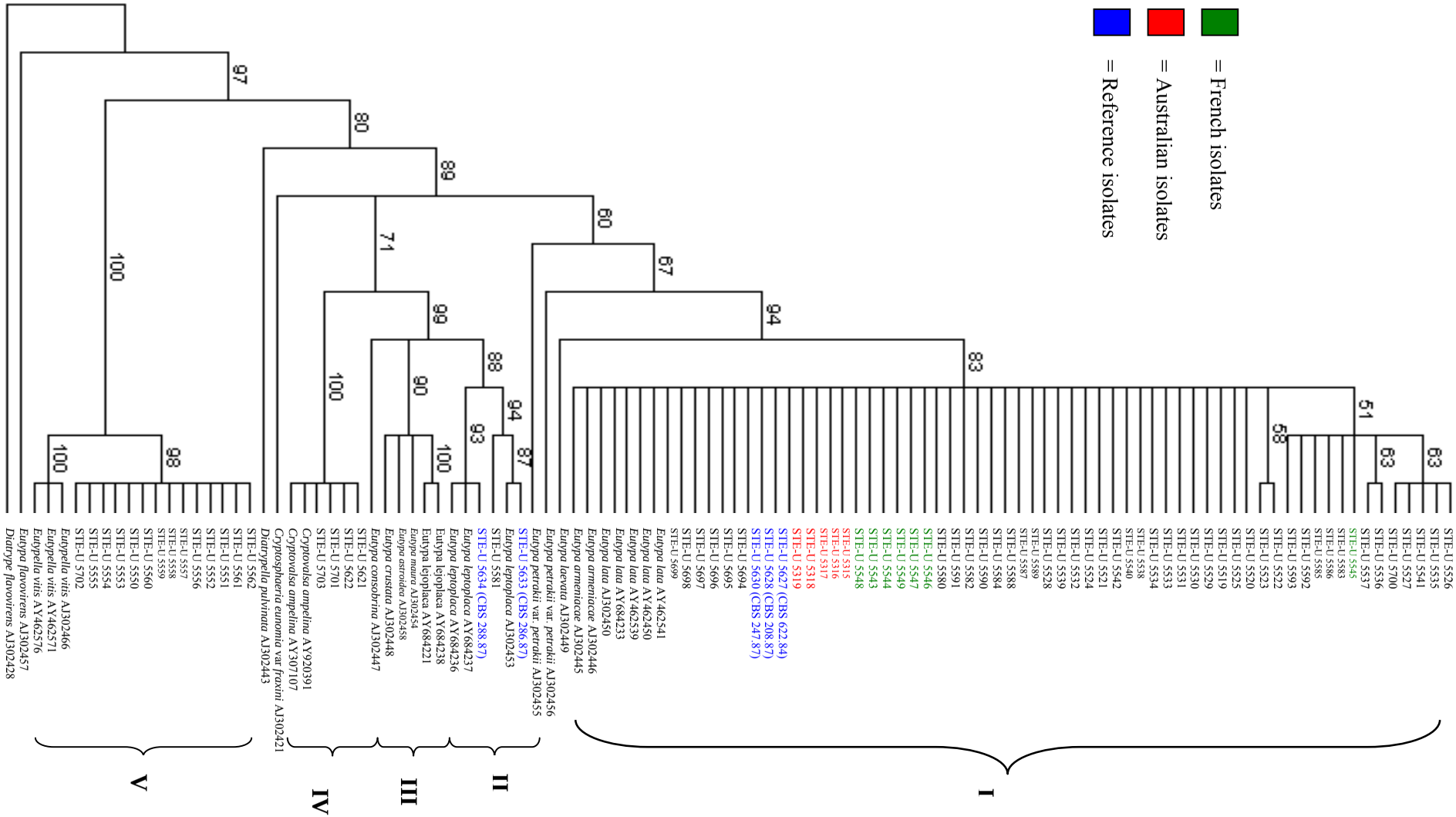
grapevines in South Africa which, grouping together with the French and Australian isolates, showed sequence homology with *E. lata*. The *E. lata* isolates STE-U 5546 and STE-U 5548, two of the seven French isolates used in this analysis, grouped together with STE-U 5590 in a cluster with low (62%) bootstrap support. The rest of the *E. lata* isolates grouped together in a separate cluster with 70% bootstrap support. STE-U 5581 and STE-U 5633/CBS 286.87 grouped with *E. leptoplaca* in group II with 100% bootstrap support. Group III included *E. lejoplaca, E. sparsa, E. maura* and *E. tetragona*. Group IV represented the *E. vitis* isolates, where STE-U 5552 (from plum) and STE-U 5561 (from grapevines) grouped apart with low (62%) bootstrap support, while the rest grouped together with 63% bootstrap support. In group V the *C. ampelina* isolates grouped together with 100% bootstrap support, except for STE-U 5701 and STE-U 5703 that grouped together with 51% bootstrap support.

Twenty-nine isolates were included in the LSU tree (Fig. 2-3), of which 26 isolates originated from South Africa. The sequences from these isolates proved to be identical and did not separate *E. lata* from *E. leptoplaca* or from *C. ampelina*. These isolates grouped together with low support.

The phylogenetic tree constructed of the combined ITS and β-tubulin datasets (Fig. 2-4) had a similar topology to the individual trees. The four groups of taxa comprised of *E. lata* (group I), *E. leptoplaca* (group II), *E. vitis* (group III) and *C. ampelina* (group IV) that yielded strong bootstrap support. The group III in the ITS phylogenetic tree which included *E. lejoplaca, E. maura, E. astroidea, E. crustata* and *E. consobrina*, was not included in the combined phylogenetic analysis, neither was group III of the β-tubulin phylogenetic tree. However, this did not significantly alter the overall topology of the tree.

Not included in the ITS phylogenetic tree was the reference isolate STE-U 5632/CBS 101932 identified by the CBS culture collection as *E. lata*. Sequence homology of this isolate was determined by doing a BLAST search against GenBank sequence data. STE-

Fig. 2-1. One of the most parsimonious trees with bootstrap support values using 1000 replicates generated in PAUP 4.0b10 from the 5.8S rDNA gene and flanking ITS1 and ITS2 regions (tree length = 319, CI = 0.721, RI = 0.983, RC = 0.676, HI = 0.279).



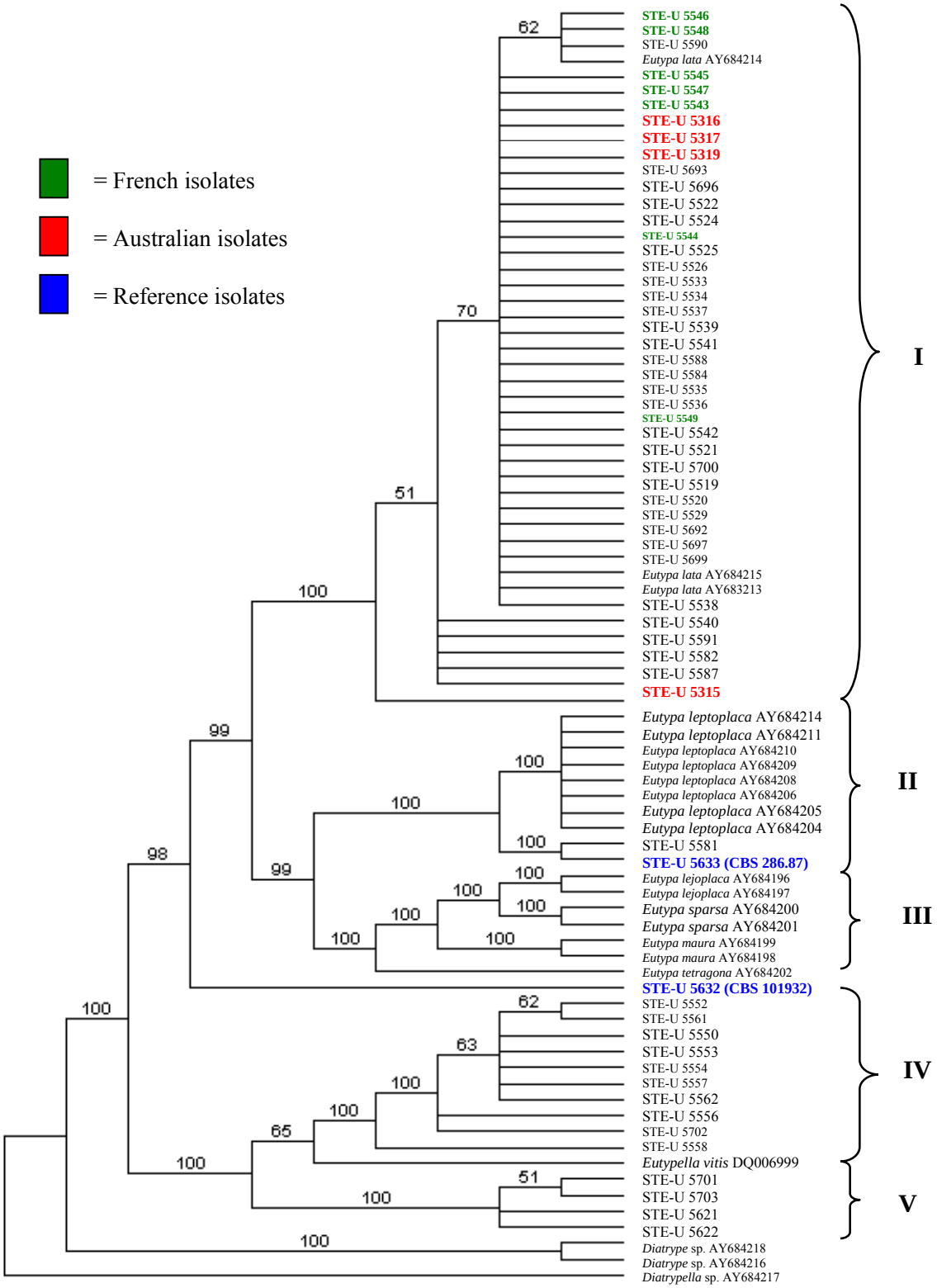


Fig. 2-2. One of the 72 most parsimonious trees with bootstrap support values using 1000 bootstrap replicates generated in PAUP 4.0b10 from the partial sequence of the β-tubulin gene region (tree length = 1692, CI = 0.702, RI = 0.932, RC = 0.654, HI = 0.298).

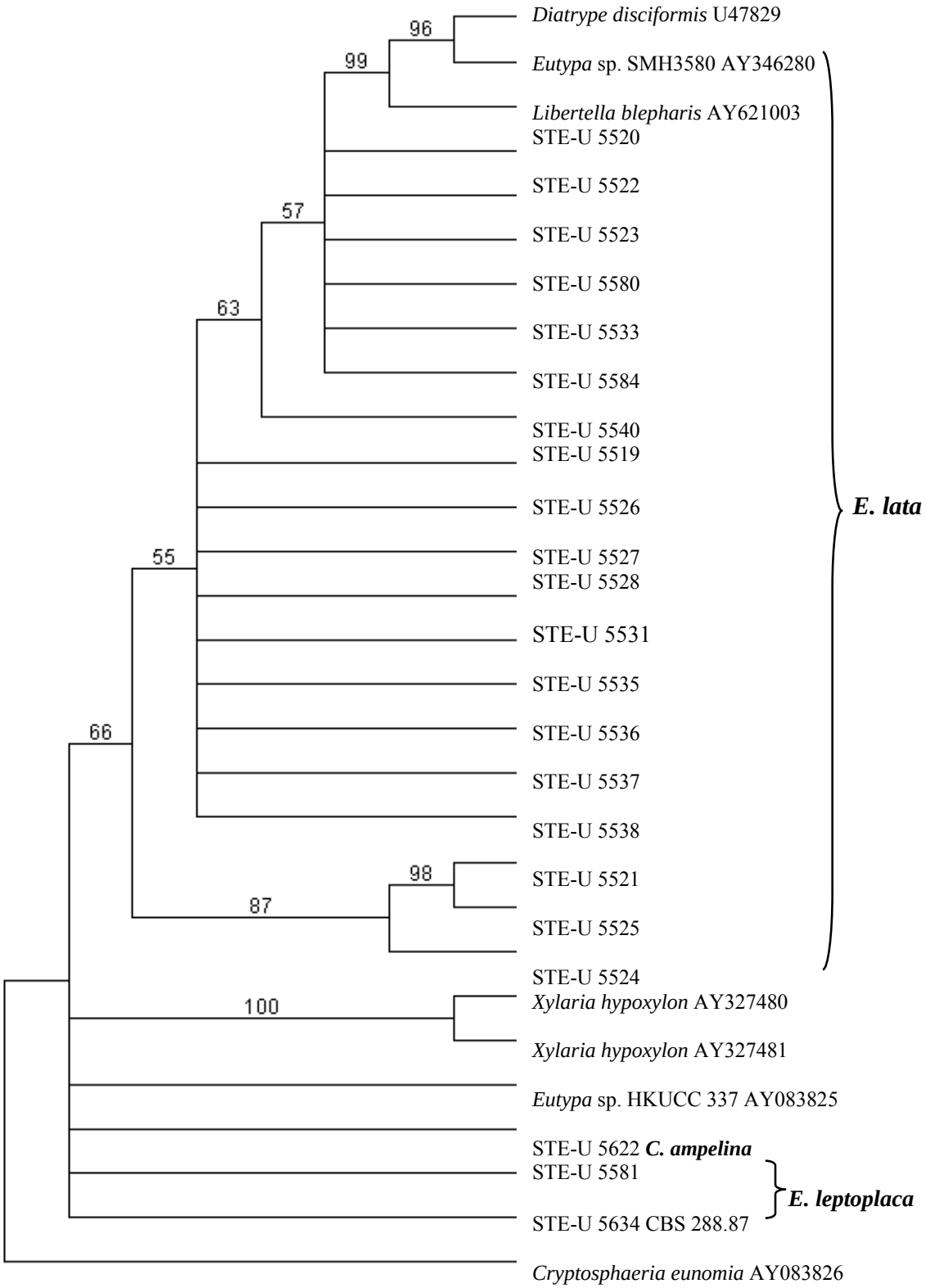


Fig. 2-3. One of the 72 most parsimonious trees with bootstrap support values using 1000 bootstrap replicates generated in PAUP 4.0b10 from the partial sequence of the large subunit region (tree length = 477, CI = 0.795, RI = 0.827, RC = 0.657, HI = 0.205).

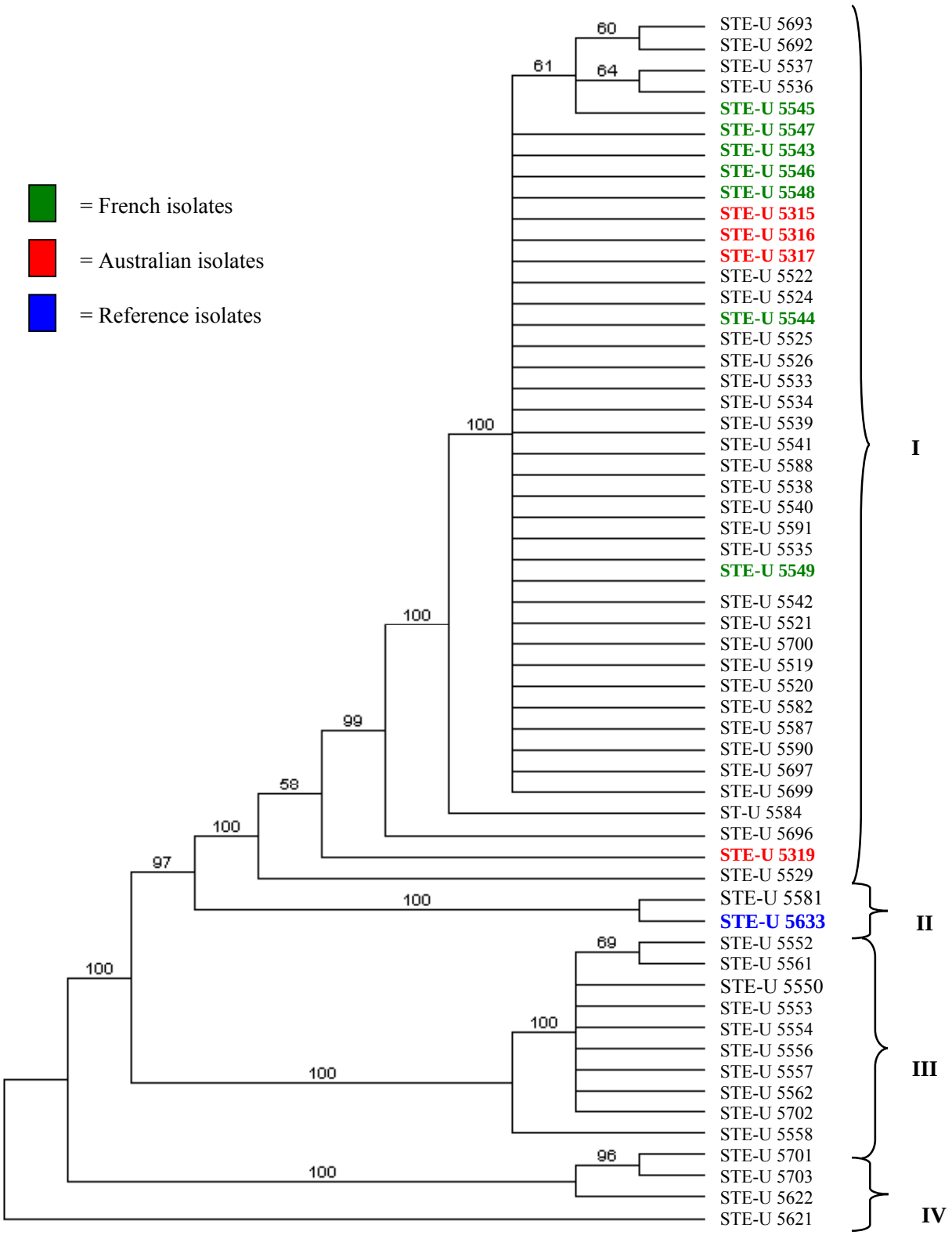


Fig. 2-4. One of the most parsimonious trees with bootstrap support values using 1000 replicates generated in PAUP 4.0b10 from the combined 5.8S rDNA gene and flanking ITS1 and ITS2 regions and β-tubulin gene (tree length = 792, CI = 0.723, RI = 0.854, RC = 0.667, HI = 0.217, P = 0.059).

Table 2-2. Mean lesion lengths in rooted cuttings and green shoots of grapevine cultivar "Sauvignon blanc", caused by inoculations with isolates of *Eutypa* and related species

Isolate	Mean lesion length (mm)*	
	Rooted cuttings	**Green shoots**
Eutypa lata STE-U 5519	24.2 a	14.2 b
E. lata STE-U 5520	24.5 a	13.9 b
E. lata STE-U 5521	24.8 a	13.6 b
E. lata STE-U 5522	23.5 ab	13.9 b
E. lata STE-U 5529	24.0 a	14.0 b
E. lata STE-U 5536	19.4 b	15.3 a
E. lata STE-U 5537	22.4 ab	15.3 a
E. lata STE-U 5540	25.3 a	13.6 b
E. lata STE-U 5585	25.8 a	13.9 b
E. leptoplaca STE-U 5581	9.75 d	6.88 d
Eutypella vitis STE-U 5551	17.7 b	12.8 bc
Cryptovalsa ampelina STE-U 5703	13.9 c	10.9 c
C. ampelina STE-U 5621	13.8 c	11.6 c
C. ampelina STE-U 5622	15.1 bc	11.7 c
Agar plug	3.74 e	3.6 e

* Values followed by the same letter are not significantly different from one other.

U 5632/CBS 101932 showed great sequence homology to *Eutypella caricae* (De Not.) Berl. (100%). However, when sequence homology was determined using the β-tubulin sequence data this isolate showed sequence homology to *Diatrypella* sp. and *Diatrype* sp. with a sequence identity of 88% and 89%, respectively, while the E values obtained would suggest that these matches are less significant (E values: 2e-173 and 4e-110, respectively). In the β-tubulin phylogenetic tree STE-U 5632/CBS 101932 was clearly distinct from *E. lata*, *E. leptoplaca* and the other species of *Eutypa* in group III. Additional reference isolates of *E. caricae* would be required to conclusively clarify the status STE-U 5632/CBS 101932.

2.3.2 Pathogenicity tests

The mean lesion lengths obtained for both experiments, one conducted with rooted cuttings and the other with green shoots, are listed in Table 2-2. In the first experiment *E. lata* caused longer lesions than *E. leptoplaca* on the rooted cuttings. The lesion lengths obtained for *C. ampelina* and *E. vitis* were shorter than most of the *E. lata* isolates tested, but longer than *E. leptoplaca*. In the second experiment, *E. lata* again caused longer lesions than *E. leptoplaca* on the green unrooted shoots. The lesion lengths obtained for *E. vitis* and *C. ampelina* were similar, and again shorter than most of the *E. lata* isolates tested, but longer than *E. leptoplaca*. Other than the stem lesions, no foliar symptoms were observed. Re-isolation of the isolates from lesions was successful. Isolations made further away from the inoculation site were not as successful due to the slow progression of the disease.

2.4 DISCUSSION

The molecular characterisation and identification of Eutypa dieback revealed that *E. lata* was present in all vineyards from which isolates were collected. This suggests that the disease is well established in South African grapevines, particularly on the cv. “Cabernet Sauvignon”. A study based on the visual identification of *Eutypa*-like symptoms from

vines in South Africa (Halleen *et al*., 2001) showed that the average level of infection was 31.7% with the highest level of infection (98%) being observed in a 22-year-old vineyard. This finding has serious economic implications in the long term for South African vineyards, as the disease is especially severe in older vineyards.

The molecular data gathered also showed that the isolates of *E. lata* were divergent from other species of *Eutypa*, but appeared synonymous with *E. armeniacae*. Alignment of the sequences of *E. lata* and *E. armeniacae* showed a 99% sequence homology between the two species. DeScenzo *et al*. (1999) used amplified fragment length polymorphisms (AFLP) and ITS sequence data in their genetic analysis of *Eutypa* strains. The isolates used in the genetic analysis were strains of *E. lata* from 10 host species which included grape (*V. vinifera* L.), apricot (*P. armeniaca* L.), oak (*Quercus lobata*) and madrone (*Arbutus menziesii*). Their analysis suggested that while *E. armeniacae* and *E. lata* are pathogenic on their hosts, the species found on grapevines and cultivated hosts is *E. armeniacae*, which is distinct from *E. lata*. However, the analysis did not include additional isolates representative of the Diatrypaceae and could thus not be compared. Carmarán *et al*. (2006) used ascal morphology as opposed to stromatal morphology to infer phylogenetic relationships among species and genera in the Diatrypaceae. The results obtained showed that *E. lata* is distinct from other species of *Eutypa* and from other genera like *Cryptosphaeria* Ces. & De Not., *Diatrype* (Ces. & De Not.) De Not. and *Eutypella* (Nitschke) Sacc. The molecular data in this present study and a similar study by Rolshausen *et al*. (2006) lead us to conclude that *E. lata* and *E. armeniacae* are synonymous. The molecular analyses also revealed the presence of a second species of *Eutypa*, namely *E. leptoplaca,* though our data suggest that isolates identified as *E. leptoplaca* may represent two species, and not one as previously thought. *Eutypa leptoplaca* was also recently reported from grapevines in California (Trouillas and Gubler, 2004), while the same study also concluded *E. lata* and *E. armeniacae* to be conspecific. The discovery of *E. leptoplaca* in California and the conclusions drawn on the conspecificity of *E. lata* and *E. armeniacae* are further supported by the results obtained in the present study from South African vineyards.

Eutypa lata proved to be distinct from *E. leptoplaca* as shown in the various analyses (Fig. 2-1, 2-2). The two trees had a similar topology with the taxa separating into five distinct groups, except where *E. vitis* was designated as group V in the ITS phylogenetic tree, and group IV in the β-tubulin phylogenetic tree. *Cryptovalsa ampelina* represented group V in the β-tubulin tree because this species shared greater sequence homology with *E. vitis* in the β-tubulin gene region. The *Eutypa* species in group III are clearly distinct form each other as evidenced by the phylogenies constructed using ITS and β-tubulin sequence data, though they are morphologically similar (Glawe and Rogers, 1982). The species resolved in this study concur with a previous study of the Diatrypaceae by Acero *et al*. (2004).

The *E. leptoplaca* isolate from South Africa, STE-U 5581, grouped with STE-U 5633/CBS 286.87 in a well-supported clade with a bootstrap support value of 94% (ITS) and 100% (β-tubulin). Based on molecular data, Trouillas and Gubler (2004) concluded that CBS 286.87 could represent *E. consobrina*. This would imply that STE-U 5581 could be *E. consobrina* and not *E. leptoplaca*. However, due to the lack of a reference isolate of *E. consobrina*, it is presently not possible to resolve the taxonomic placement of this isolate.

Eutypa lata and E. leptoplaca occurred together on the same vines, which suggested that they can coinfect the same niche, though *E. leptoplaca* was not isolated from all vineyards sampled. In culture, it was observed that *E. lata* grew faster than *E. leptoplaca*. Vines and shoots inoculated in the pathogenicity tests showed similar results, whereby the lesion lengths for *E. lata* were significantly larger than those for *E. leptoplaca*. This scenario could be the same in the environment, which would explain why *E. lata* was more dominant than *E. leptoplaca*. In Californian vineyards, *E. leptoplaca* was not isolated from the V-shaped cankers characteristic of Eutypa dieback, though it was isolated from severely affected grapevines.

While all the grapevines were infected with *E. lata*, none of the South African fruit trees were similarly infected with this fungus. The presence of *E. vitis* on grapevines,

including *C. ampelina*, together with the *Eutypa* species, complicates the rapid and accurate identification of these pathogens. *Cryptovalsa ampelina* causes symptoms similar to those produced by *E. lata* (Ferreira, 1987) though grapevines appear to be only weakly susceptible to the pathogen (Price, 1973; Mostert *et al*., 2004). *Eutypella vitis* has been identified as a possible pathogen of grapevines in Michigan (Jordan *et al*., 2005). Its presence in infected grapevines in South Africa would suggest the same. Pathogenicity testing revealed that the fungus was capable of causing stem lesions and although no foliar symptoms were observed, it would imply that *E. vitis* is pathogenic to grapevine, even though not highly virulent.

The presence of other diatrypaceous fungi has also been observed in California vineyards (Trouillas *et al.,* 2001), where ascospores of *Cryptovalsa, Diatrype* and *Diatrypella* species have been found on dead grapevine wood. These fungi were found to occur together with *E. lata* and *E. leptoplaca* in infected grapevines and on native California plant hosts like big leaf maple (*Acer macrophyllum* Pursh.), boxelder (*A. negundo* L.), California laurel (*Umbellularia californica* (Hook & Arn.) Nutt.), Oregon ash (*Fraxinus latifolia* Benth.) and oak (*Quercus* sp.). Molecular analyses of isolates collected from grapevines showing dieback symptoms in South Africa revealed the presence of *Cryptovalsa ampelina* and *Eutypella vitis*, but *Diatrypella* and *Diatrype* species were not identified. The stromata of *Cryptovalsa* and *Diatrypella* are similar in appearance and species within these genera have often been confused (Vasilyeva and Stephenson, 2005). Generic classification in the Diatrypaceae is primarily based on stromatal morphology which can be insufficient as it has been suggested that stromata development could be influenced by host species and humidity (Vasilyeva and Stephenson, 2004).

The occurrence of these diatrypaceous fungi alongside *E. lata* and *E. leptoplaca* in infected grapevines possibly contributes to the slow decline of vineyards. The toxin produced by *E. lata*, eutypine, certainly plays a large role in symptom development (Tey-Rulh *et al.,* 1991), but the presence of these other fungi may enhance the decline of the grapevines. *Eutypa lata* is considered to play a pioneering role in infecting pruning wounds which are the primary sites of infection (Larignon and Dubos, 1997; Carter,

1957). As a result, the grapevines physiology is compromised, and with it the ability to fight infection, which allows the entrance of other pathogens. This has enormous implications for any disease control strategies that could be implemented. Early detection, and the correct identification of organisms involved in the Eutypa dieback complex are important for establishing a disease control programme. Consequently, the aim of the work reported in the next chapter was to develop the reverse dot blot hybridisation technique to detect the pathogens responsible for Eutypa dieback. Also included in this work were several species of Botryosphaeriaceae known to cause canker diseases in grapevines, and also frequently co-occurring with *Eutypa* species.

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CHAPTER 3

A PCR-BASED ASSAY FOR THE DETECTION OF *EUTYPA LATA* AND SPECIES OF BOTRYOSPHAERIACEAE FROM GRAPEVINE

3.1 INTRODUCTION

Eutypa dieback of grapevines, caused by the fungus *Eutypa lata* (Pers.) Tul & C. Tul, is responsible for a slow decline of vineyards, thereby reducing growth and yield, eventually killing the grapevine. In 1998, this disease was estimated to have cost the US wine industry about US$260 million per annum in losses (Siebert, 2001). The losses for the South African Cabernet Sauvignon crop during the 2000/2001 season, was estimated to be R1.7 million (Van Niekerk *et al*., 2003).

The devastating impact of Eutypa dieback on grape-growing regions worldwide has fuelled the demand for efficient and reliable methods for the detection of the causal organisms. The causal organism initially implicated in Eutypa dieback was the ascomycetous fungus *E. lata* (Pers.:Fr.) Tul. & C. Tul. from the Diatrypaceae family. The recent discovery of a second *Eutypa* species namely, *E. leptoplaca* (Mont.) Rappaz, as well as other diatrypaceous fungi also capable of causing disease (Chapter 2) suggests that these organisms form part of the *Eutypa* disease complex which has further spurred the need for rapid and reliable detection methods. Traditional methods of identification are dependent on morphological and cultural characters. Several morphologically similar species are involved in the Eutypa disease complex and these methods have proven insufficient for rapid and accurate identification.

Eutypa lata and *E. leptoplaca* have only recently been reported in association with other members of the Diatrypaceae on infected grapevine material (Trouillas and Gubler, 2004). These latter fungi have been identified as *Cryptovalsa ampelina* (Nitschke) Fuckel and *Eutypella vitis* (Schwein.) Ellis & Everh. by employing molecular analysis

based on ITS, β-tubulin and large subunit sequence data (Chapter 2). Other fungi that are also frequently isolated from diseased vines with V-shaped cankers characteristic of Eutypa dieback, are species of Botryosphaeriaceae (Crous *et al.,* 2000). Several species of *Botryosphaeria* are known from grapevines, of which three species, *Diplodia seriata* De Not, *B. dothidea* (Moug.: Fr.) Ces. & De Not. and *Neofusicoccum ribis* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips, have been commonly isolated from grapevines in South Africa (Crous *et al*., 2000). However, a study by Van Niekerk *et al*. (2004) using molecular methods and morphological characteristics to compare South African *Botryosphaeria* isolates failed to confirm the presence of *B. dothidea* and *N. ribis* on grapevines in South Africa. Other common species known from grapevines are *N. luteum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips and *N. parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips, which also occur on this host in South Africa. Common disease symptoms include canker formation, shoot and branch dieback, decline and brown wood streaking. These disease symptoms are easily confused with those produced by the *Eutypa* disease complex. Consequently, it was imperative that a detection method be employed that could correctly identify the presence of one or more of these pathogens. In previous studies a reverse dot blot hybridisation (RDBH) method was successfully developed to identify unknown isolates of *Pythium* Pringsh. and *Phytophthora* de Bary (Levesque *et al.,* 1998), and therefore, one aim of the present study was to investigate the usability of RDBH in the grapevine pathosystem. A further aim was to screen infected grapevine material for the presence of the *Eutypa* species involved in the Eutypa disease complex and those Botryosphaeriaceae species known to cause disease on this host.

3.2 MATERIALS AND METHODS

3.2.1 Fungal isolates and DNA isolation

The fungal isolates used were obtained from infected grapevine material and grown for 2-4 wks on potato-dextrose agar (PDA; 39g/l, Biolab, Merck) at 25^{o}C. Fresh mycelium was harvested by scraping the surface of the agar with a scalpel and transferring it to a

microcentrifuge tube containing extraction buffer (50mM Tris-HCl, pH 8.0, 150 mM NaCl, 100mM EDTA, pH 8.0, 2% w/v SDS). Total DNA was isolated according to the method of Lee and Taylor (1990). The DNA was resuspended in sterile HPLC water (BDH, Merck) and examined on a 0.8% agarose gel by electrophoresis. For PCR reactions the DNA samples were diluted 1:10 or 1:50 using sterile HPLC water.

3.2.2 Detection of Eutypa dieback by reverse dot blot hybridisation

PCR amplification and labelling. All PCR amplifications were performed in 50 µl reactions on a MJ Research PTC 200 thermal cycler. Each DNA sample was amplified using universal ITS primers ITS4 and ITS5 (White *et al.,* 1990), β-tubulin primers Bt2b and T1 (Glass and Donaldson, 1995), and large subunit (LSU) RNA primers LROR and LR7 (Vilgalys and Hester, 1990; Rehner and Samuels, 1994). The cycling programs for the ITS PCR consisted of 35 cycles with a 45 s denaturation at 94^{o}C, a 30 s annealing at 53^{o}C, a 1 min extension at 72^{o}C and a final extension period of 10 min at 72^{o}C. The β-tubulin PCR program consisted of 36 cycles with a 30 s denaturation at 94^{o}C, a 30 s annealing at 50^{o}C, a 90 s extension at 72^{o}C and a final extension period of 7 min at 72^{o}C. The large subunit PCR program consisted of 35 cycles with an initial denaturation of 10 min at 95^{o}C, followed by 30 s denaturation at 94^{o}C, a 30 s annealing at 55^{o}C, a 1 min extension at 72^{o}C and a 10 min final extension period at 72^{o}C. For the labelling reactions, the PCR conditions were as described above, except that the universal primers were used with 10x DIG-dNTPs [100 µM dATP, 100 µM dGTP, 100 µM dCTP, 65 µM dTTP and 35 µM alkaline labile DIG-dUTP (digoxigenin-11-dUTP; Roche Diagnostics, South Africa Pty. Ltd.)]. The final PCR products were purified with a PCR purification kit (GFX^{TM} PCR DNA and Gel Band Purification Kit, Amersham Pharmacia Biotech Inc, NJ). These labelled and purified PCR products would be used in the subsequent hybridisation experiments as probes.

Blotting oligonucleotides. Species-specific oligonucleotides for *E. lata* and *E. leptoplaca* were synthesised from internal transcribed spacer, β-tubulin and nuclear large subunit

ribosomal DNA sequence data. Each oligonucleotide (200 µM) was poly(dT)-tailed at the 3' end according to the manufacturer's protocol (Roche Diagnostics, South Africa Pty. Ltd.). Reactions were incubated at 37°C for 2 h and then placed on ice. The reactions were stopped with the addition of 1 µl EDTA (200mM, pH 8.0). The oligonucleotides were blotted onto the respective positively charged nylon membranes (Roche Diagnostics, South Africa Pty. Ltd.). The ITS I and ITS II regions of a *Phoma* sp. (Sacc.) and a *Colletotrichum* sp. (Corda) were amplified using ITS4 and ITS5. An equal mix of this amplified DNA, 5 ng in total, was added to the membranes as a control. Similarly, controls of the β-tubulin region amplified with Bt2b and T1 and the large subunit region amplified with LROR and LR7 were added to the respective membranes by using 5 ng in total of an equal mix of amplified DNA of *Phoma* sp. and *Colletotrichum* sp. The detection control dot contained DNA labelled with alkaline stable DIG-dUTP provided in the labelling kits (Roche Diagnostics, South Africa Pty. Ltd). A negative control of an unrelated species, *Botrytis cinerea* Pers., was also included. These controls were blotted onto the respective nylon membranes after heat denaturation. The membranes were then irradiated for 7 min by UV illumination to bind the DNA.

Hybridisation with immobilised oligonucleotides. Membranes were placed in hybridisation bottles and prehybridised in 10 ml DIG Easy Hyb buffer (Roche Diagnostics) for 2 h at hybridisation temperatures of 50-55°C. From 30 to 80 ng of DIG-labelled probe was boiled for 10 min and added to 10 ml of fresh DIG Easy Hyb buffer. This solution of probe and buffer was filtered through a 0.45 µm filter (Cameo 25AS, Osmonics) and added to the hybridisation bottles after the prehybridisation solution was poured off. Following overnight hybridisation the hybridisation solution containing the probe was decanted and stored at -20°C. The membranes were washed at hybridisation temperature in 2.0X SSC. Subsequent washes were done in either 0.5X SSC / 0.1% SDS or 2X SSC / 0.1% SDS. Digoxigenin was detected using a three-step chemiluminescent procedure. In step one, the membranes were treated with a blocking reagent. In step two, the membranes were incubated with antibody, anti-DIG alkaline phosphatase (Roche

Diagnostics. South Africa Pty. Ltd). In step three, the membranes were allowed to react with the chemiluminescent substrate, CSPD, and exposed to X-ray film.

3.2.3 Detection of Botryosphaeriaceae species by reverse dot blot hybridisation

PCR amplification and labelling. All PCR amplifications were performed in 50 µl reactions on a MJ Research PTC 200 thermal cycler. Each DNA sample was amplified using universal elongation factor-1α primers, EF1-728f and EF1-986r (Carbone and Kohn, 1999). The elongation factor PCR program consisted of 35 cycles with an initial denaturation of 7 min at 94°C, followed by 45 s denaturation at 95°C, a 60 s annealing at 55°C, a 2 min extension at 72°C and a 2 min final extension period at 72°C. For the labelling reactions, the PCR conditions were as described, except that the universal primers were used with 10x DIG-dNTPs [100 µM dATP, 100 µM dGTP, 100 µM dCTP, 65 µM dTTP and 35 µM alkaline labile DIG-dUTP (digoxigenin-11-dUTP; Roche Diagnostics, South Africa Pty. Ltd.)]. The final PCR products were purified with a PCR purification kit (GFX™ PCR DNA and Gel Band Purification Kit, Amersham Pharmacia Biotech Inc, NJ). These labelled and purified PCR products would be used in the subsequent hybridisation experiments as probes.

Blotting oligonucleotides. *S*pecies-specific oligonucleotides for *D. seriata*, *B. dothidea, N. luteum, N. parvum* and *N. ribis* were synthesised from elongation factor-1α sequence data. Each oligonucleotide (200 µM) was poly(dT)-tailed at the 3' end according to the manufacturer's protocol (Roche Diagnostics, South Africa Pty. Ltd.). Reactions were incubated at 37°C for 2 h and then placed on ice. The reactions were stopped with the addition of 1 µl EDTA (200 mM, pH 8.0). The oligonucleotides were blotted onto the respective nylon membranes. The elongation factor region of a *Phoma* sp. and a *Colletotrichum* sp. was amplified with the primer pair EF1-728f and EF1-986r. An equal mix of this amplified DNA, 5 ng in total, was added to the membranes as a control. The detection control dot contained DNA labelled with alkaline stable DIG-dUTP provided in the labelling kits (Roche Diagnostics, South Africa Pty. Ltd). The same negative control

as explained above was included in the experiments. These controls were blotted to the respective nylon membranes after heat denaturation and the membranes irradiated as described above. The procedure for the hybridisation and detection of the *Botryosphaeria* species was also performed as described above for the *Eutypa* species.

3.2.4 Direct detection of *E. lata, E. leptoplaca* and Botryosphaeriaceae species from grapevine wood

One-year-old rooted vines of Sauvignon Blanc were inoculated in a pathogenicity study with mycelium from actively growing *E. lata* grown on PDA plates (Chapter 2). The vines were inoculated in the same manner with isolates of *E. leptoplaca*, *C. ampelina* and *E. vitis* collected from grapevines with dieback symptoms. *Cryptovalsa ampelina* and *E. vitis* were included in the detection method as negative controls. The presence of these fungi was confirmed by culturing from vines with necrotic lesions. These infected vines were used in attempts to detect *E. lata* and *E. leptoplaca* species directly from the woody material.

Infected woody material was surface sterilised in 20% (w/v) hypochlorite solution. Wood shavings were cut from the necrotic region of each isolate using a sterile scalpel and placed in a mortar. The wood shavings (0.5 g) were crushed with a pestle in liquid nitrogen. Total DNA was extracted following a CTAB extraction protocol. CTAB extraction buffer was added and the mixture transferred to a 50 ml centrifuge tube. The samples were incubated at 65^{o}C with occasional shaking for 2 h. Samples were centrifuged at 12,000 ×g for 30 min and the supernatant transferred to a 30 ml Corex tube. The samples were washed with an equal volume of chloroform:isoamyalcohol (24:1) and centrifuged at 10,000 ×g for 30 min. The aqueous phase was subsequently divided between two new Corex tubes to which two volumes of precipitation buffer was added. After an incubation period of 2 h at room temperature the samples were centrifuged at 10,000 ×g for 30 min. The supernatant was discarded and the pellet dissolved in 1 ml 1M CsCl. Two volumes of ice-cold ethanol were added to precipitate the DNA overnight at -20^{o}C. The samples were centrifuged at 10,000 ×g for 30 min.

The pellet was washed in ice-cold 70% (v/v) ethanol, centrifuged and dried. The DNA was suspended in sterile HPLC grade water (BDH, Merck) and used at 1:10, 1:50 and 1:100 dilutions in the PCR amplification and labeling reactions. These extraction protocols were also used to obtain DNA from wood shavings to which pure preparations of *E. lata* DNA was added.

For the detection of the Botryosphaeriaceae species grapevine wood shavings were incubated with individual 2 ml suspensions of *D. seriata*, *B. dothidea, N. luteum, N. parvum* and *N. ribis* at room temperature for 30 min to 2 h. A 50 µl aliquot of each sample was transferred to 1.5 ml microcentrifuge tubes and incubated at 95^{o}C for 15 min, and immediately placed on ice. Five microlitres of 1:10, 1:50 and 1:100 dilutions of the supernatant were used for PCR amplification and labeling in the reverse dot blot hybridisation method. Similarly, the CTAB method of obtaining DNA as described above was also used.

In a second experiment, the rapid DNA extraction method published by Lecomte *et al.* (2000) was also tested. Wood shavings were cut from the necrotic region of each isolate using a sterile scalpel and placed in 1.5 ml microcentrifuge tubes containing 50 µl of sterile, distilled water. The tubes were then incubated at 95^{o}C for 15 min, and immediately placed on ice. Five microlitres of 1:10, 1:50 and 1:100 dilutions of the supernatant were used for PCR amplification in preparation for the reverse dot blot hybridisation method.

3.2.5 Detection of *E. lata* by PCR based on primers designed by Lecomte *et al.* (2000)

In addition to the reverse dot blot hybridisation, a method developed by Lecomte *et al.* (2000) for the detection of *E. lata* by PCR directly from infected grapevine wood material was tested. These authors designed primers for the detection of *E. lata* from ITS sequence data and from randomly amplified polymorphic DNA (RAPD) fragments. In

particular, the primer pair Lata 1 and Lata 2.2 derived from ITS sequences, was tested because Lecomte *et al.* (2000) obtained positive results using these primers.

3.3 RESULTS

3.3.1 Sequencing and oligonucleotide design

Sequences from the four molecular markers used in this study (ITS, β-tubulin, large subunit and elongation factor-1α) were each aligned using a manual alignment program (Se-Al 2.0a8) and potential species-specific primers were designed using Primer 3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3). Selected oligonucleotides for the ITS and LSU regions were synthesized by IDT Technologies (Whitehead Scientific, SA) and were tested by reverse dot blot. Although oligonucleotides were developed from β-tubulin sequence data, they were not tested in the reverse dot blot hybridisation method. This was due to problems encountered during a DNA phylogeny study using this marker (Chapter 2). The universal primers used, Bt2b/T1, did not consistently amplify a region during PCR. The PCR had to be repeated, sometimes more than once, before a PCR fragment was obtained.

3.3.2 Reverse dot blot with immobilised oligonucleotides

The optimum conditions selected for hybridisation and washes were found to be 52^{o}C with subsequent washes using 2.0X SSC / 0.1% SDS. These conditions were optimal for the detection of both *Eutypa* and Botryosphaeriaceae species. In all cases the hybridisation results were consistent and highly reproducible.

DNA from *E. lata* isolates that was labelled and purified to be used as probes, hybridised to the *E. lata* ITS oligonucleotides, Ela1 and Ela2, (Fig. 3-1A). None of the other members of Diatrypaceae tested (Fig. 3-1B – G) hybridised to these oligonucleotides. Probes from *E. leptoplaca* hybridised to *E. leptoplaca* ITS oligonucleotides, Elep1 and Elep2, (Fig. 3-1B), but probes from any of the other fungal genera tested (Fig. 3-1A; C – G) did not.

From the large subunit sequence data, oligonucleotides were only designed for the detection of *E. lata* (LS1 and LS2) by reverse dot blot hybridisation. The amplification of a partial region of the large subunit did not show much sequence variation between *E. lata* and *E. leptoplaca* (Chapter 2; Fig. 2-3*)*. This made the design of species-specific primers difficult. Nevertheless, many of the isolates of *E. lata* used as probes hybridised to the oligonucleotides LS1 and LS2. The result of one probe, *E. lata* STE-U 5519, is shown (Fig. 3-2A). *Eutypa leptoplaca* probes hybridised to oligonucleotide LS1, but after washes at increasing stringencies the pathogen did not hybridise to the oligonucleotide (result not shown). None of the probes from the other related species hybridised to the two oligonucleotides (Fig. 3-2B – F).

For the detection of species of Botryosphaeriaceae from grapevine, oligonucleotides were designed from the elongation factor-1α sequence data. Data generated from amplification of the ITS region could not be used for the design of oligonucleotides because of the close-relatedness between taxa. Oligonucleotides, Bob-EF1, Bob-EF2, Bdo-EF1, Bdo-EF2, Blu-EF1, Blu-EF2, Bpa-EF1 Bpa-EF2 and Bri-EF were designed for *D. seriata*, *B. dothidea*, *N. luteum*, *N. parvum* and *N. ribis*, respectively.

Probes prepared from isolates of *N. ribis* hybridised to the *N. ribis* oligonucleotide, Bri-EF (Fig. 3-3A), while probes from *B. dothidea* isolates hybridised to both *B. dothidea* oligonucleotides, Bdo-EF1 and Bdo-EF2 (Fig. 3-3B). Probes from *D. seriata* hybridised to one of the *B. dothidea* oligonucleotides, Bdo-EF2 (Fig. 3.3C). Probes from *N. luteum* hybridised to both oligonucleotides, Blu-EF1 and Blu-EF2 (Fig. 3-3D), and *N. parvum* probes hybridised to both oligonucleotides, Bpa-EF1 and Bpa-EF2 (Fig. 3-3E). With the exception of the oligonucleotides Blu-EF1 and Blu-EF2 which respectively detected isolates of *E. lata* (Fig. 3-3F) and *C. ampelina* (Fig. 3-3I), the rest of the oligonucleotides did not detect other species which occur together with a particular Botryosphaeriaceae species in infected wood tissue (Fig. 3-3F – I). These immobilised oligonucleotides were, therefore, mostly specific for the detection of the species they were designed for. Unfortunately, the oligonucleotides designed for the detection of *B. obtusa* were less

successful. They were not specific and hybridised to probes of other diatrypaceous fungi as well (Fig. 3-4A - C).

3.3.3 Direct detection of *E. lata, E. leptoplaca* and Botryosphaeriaceae species from grapevine wood

PCR amplification of the ITS region from DNA obtained from infected grapevine material using the CTAB extraction protocol was inconsistent. The addition of polyvinyl-pyrrolidone (PVP) did not improve the quality of DNA for PCR. Similarly, the inclusion of β-mercaptoethanol to the PCR reactions did not improve the PCR results.

PCR amplification of the DNA recovered by the method described by Lecomte *et al.* (2000) with the universal primers did not produce a single PCR fragment (Fig. 3-5; lanes 5- 8). Successful amplification of the fungal species did not occur regardless of the DNA template dilutions used. The wood shaving samples were incubated at room temperature to encourage the release of the fungi into the solution. This did not improve the amplification of DNA and positive PCR fragments were not obtained. Positive controls using genomic DNA from pure isolates yielded PCR products of the expected size amplified with the universal ITS primers (Fig. 3-5; lanes 1 – 4).

3.3.4 Detection of *E. lata* by PCR based on primers designed by Lecomte *et al.* (2000)

Eutypa lata could be detected by PCR with the primers Lata 1/Lata 2.2, but only when pure preparations of DNA were used (Fig. 3-6; lanes 1 - 4). Included with *E. lata* in the analysis were pure preparations of DNA from the following isolates: *Botryosphaeria dothidea, N. luteum, N. parvum, N. ribis, E. leptoplaca, Eutypella vitis* and *C. ampelina*.

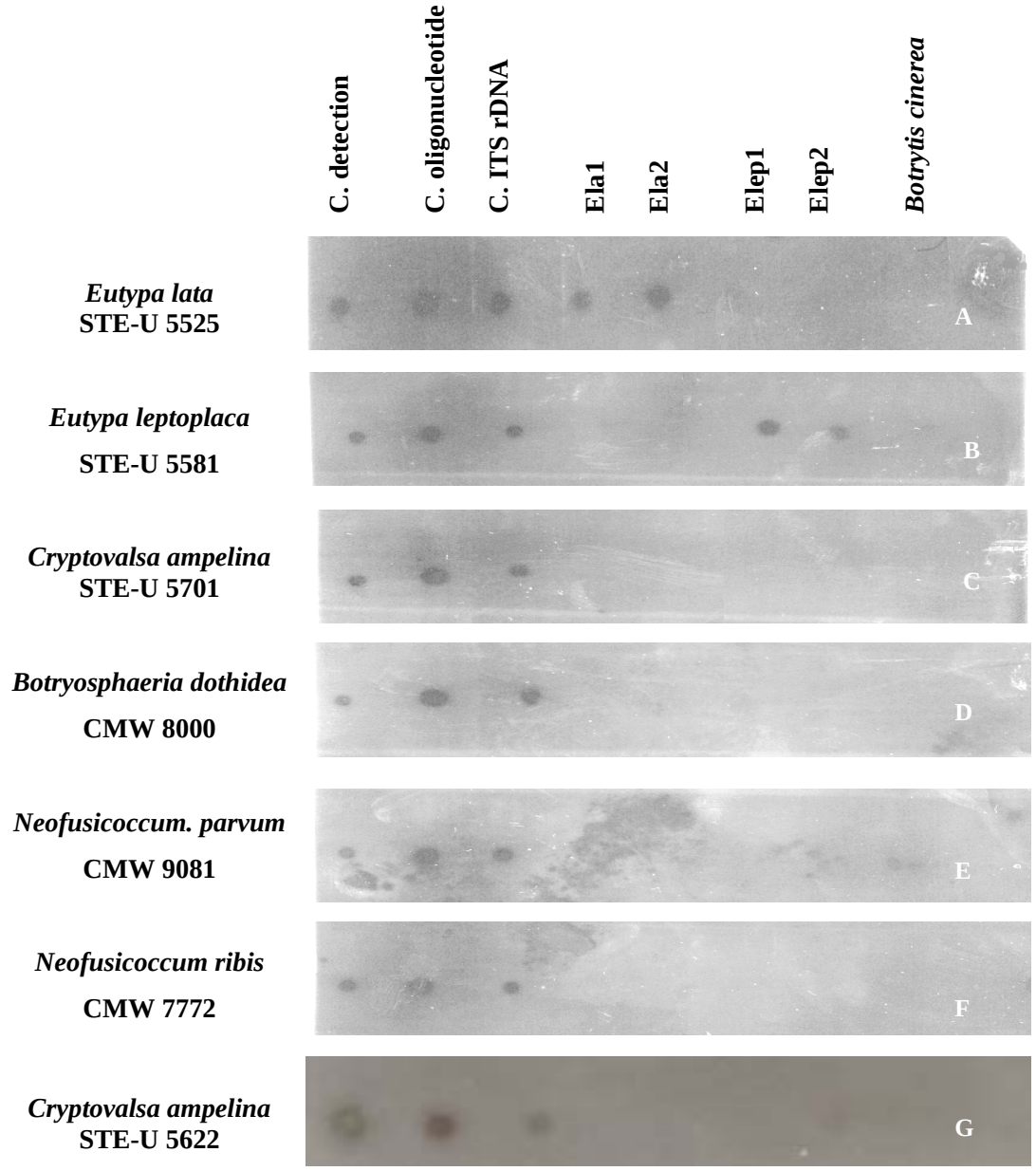

Fig. 3-1 A – G. Reverse dot blot hybridisation with immobilised specific oligonucleotides to demonstrate specificity of the *Eutypa lata* oligonucleotides (Ela1 and Ela2) and the *Eutypa leptoplaca* oligonucleotides (Elep1 and Elep2). The strains listed on the left were used to prepare the probes for each hybridisation. The first three dots on the left are controls (C.): detection = control DNA amplified and labelled with alkaline stable digoxigenin-11-dUTP; oligonucleoide = universal ITS 2; and ITS rDNA = amplified and mixed ITS I and ITS II from wide range of genera. A negative control, *Botrytis cinerea*, was added, as no hybridisation should occur at this site.

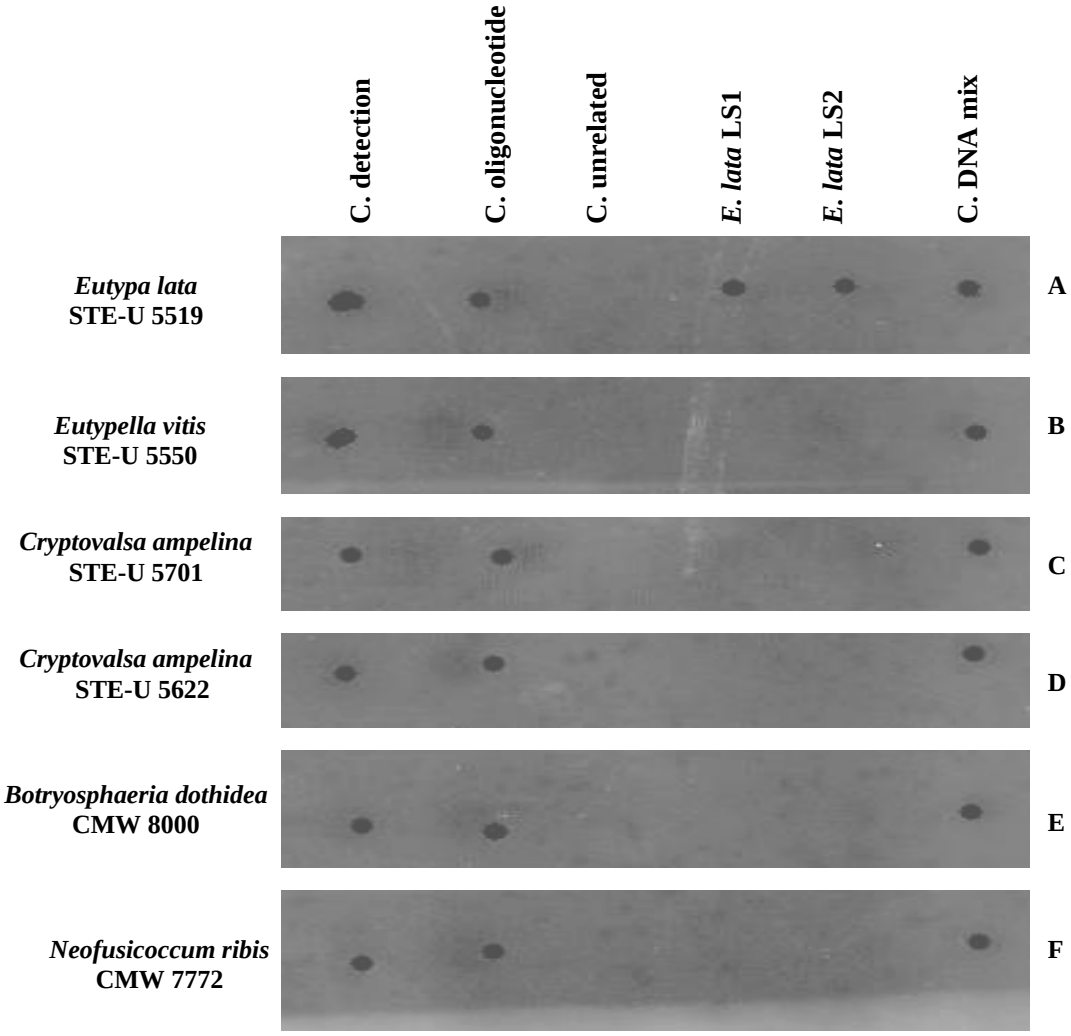

Fig. 3-2 A – F. Reverse dot blot hybridisation with immobilised specific oligonucleotides to demonstrate specificity of the *Eutypa lata* oligonucleotides (LS1 and LS2). The strains listed on the left were used to prepare the probes for each hybridisation. The first three dots on the left are controls (C.): detection = control DNA labelled with alkaline stable digoxigenin-11-dUTP; oligonucleoide = universal LROR; DNA mix = amplified and mixed large subunit region from wide range of genera, including related species, and unrelated = a negative control, *Phoma sp.*, was added, as no hybridisation should occur at this site.

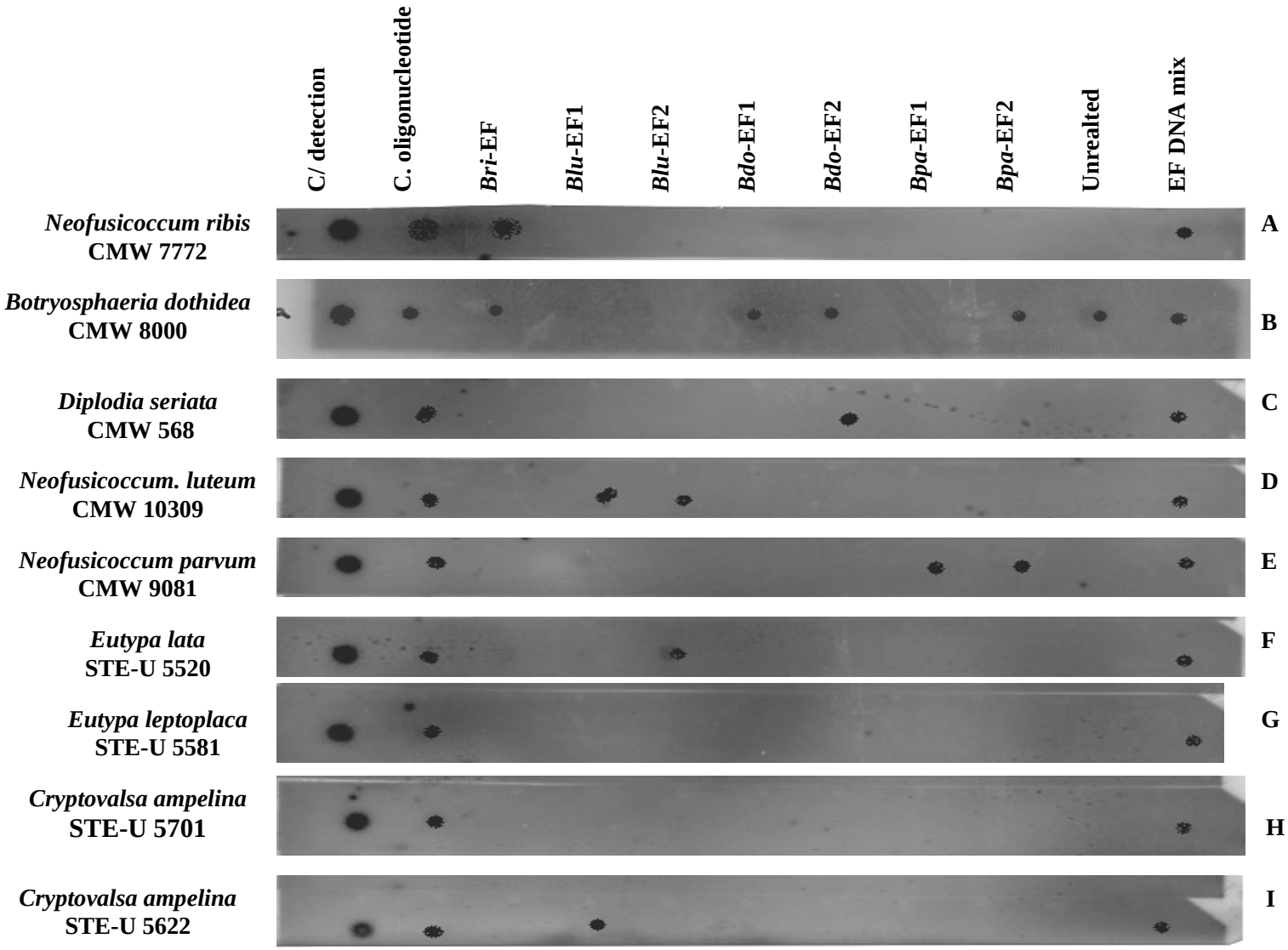

Fig. 3-3 A – I. Reverse dot blot hybridisation with immobilised specific oligonucleotides to demonstrate specificity of the *Botryosphaeria spp.* oligonucleotides. The strains listed on the left were used to prepare the probes for each hybridisation. The controls are (C.): detection = control DNA labelled with alkaline stable digoxigenin-11-dUTP; oligonucleoide = universal EF 986R; DNA mix = amplified and mixed elongation factor region from wide range of genera including related species, and unrelated = a negative control, *Phoma sp.*, was added, as no hybridisation should occur at this site.

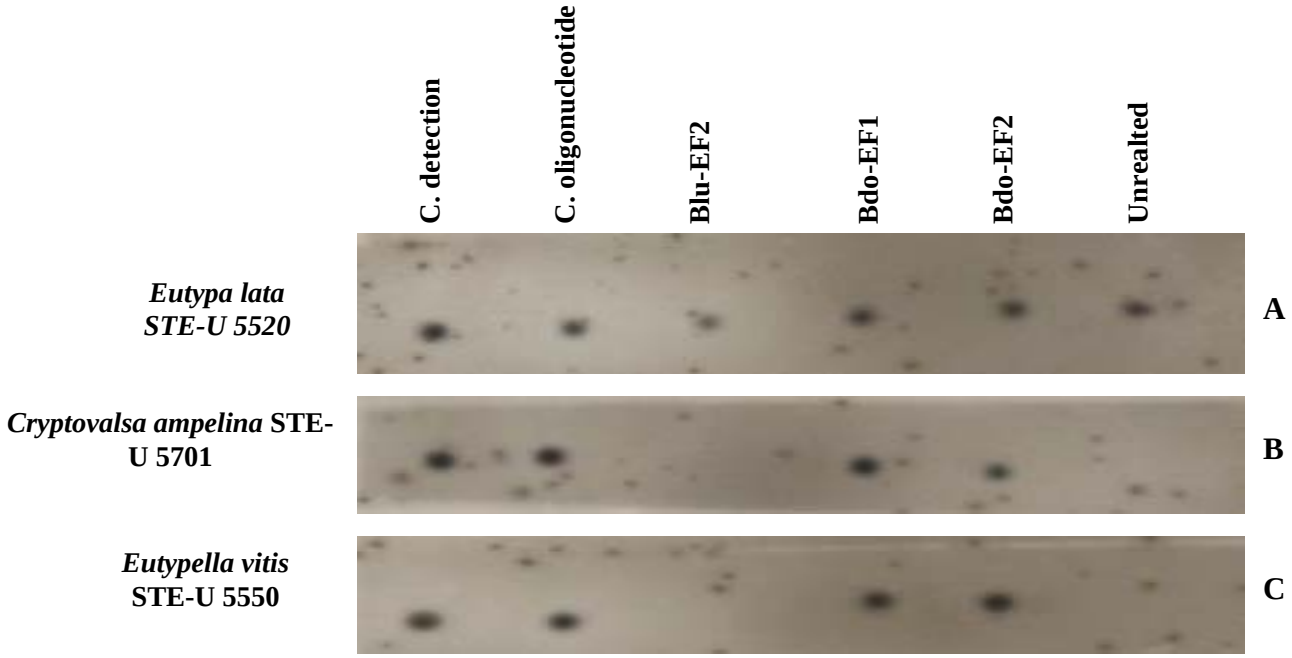

Fig. 3-4 A – C. Reverse dot blot hybridisation with immobilised specific oligonucleotides to demonstrate specificity of the *Botryosphaeria spp.* oligonucleotides. The strains listed on the left were used to prepare the probes for each hybridisation. The controls are (C.): detection = control DNA labelled with alkaline stable digoxigenin-11-dUTP; oligonucleoide = universal EF1-986r; and unrelated = a negative control, *Phoma sp.*, was added, as no hybridisation should occur at this site.

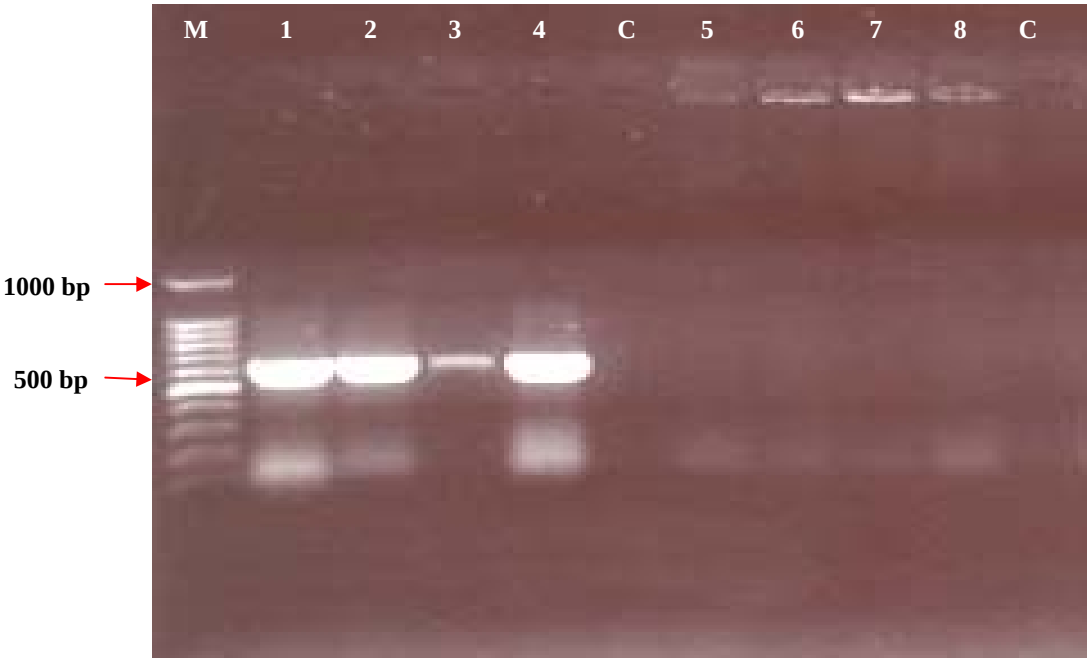

Fig. 3-5. PCR amplification of the DNA recovered by the method described by Lecomte *et al.* (2000) with ITS primers ITS4/ITS5. Lanes: M = 100bp ladder, (1) *E. lata* STE-U 5525; (2) *E. leptoplaca* STE-U 5581; (3) *Cryptovalsa ampelina* STE-U 5701; (4) *Cryptovalsa ampelina* STE- U 5622; (5) *E. lata* STE-U 5525; (6) *E. leptoplaca* STE-U 5581; (7) *Cryptovalsa ampelina* STE-U 5701; (8) *Cryptovalsa ampelina* STE-U 5622; C = negative control. The positive controls, lanes 1 to 4, are genomic DNA obtained from pure isolates.

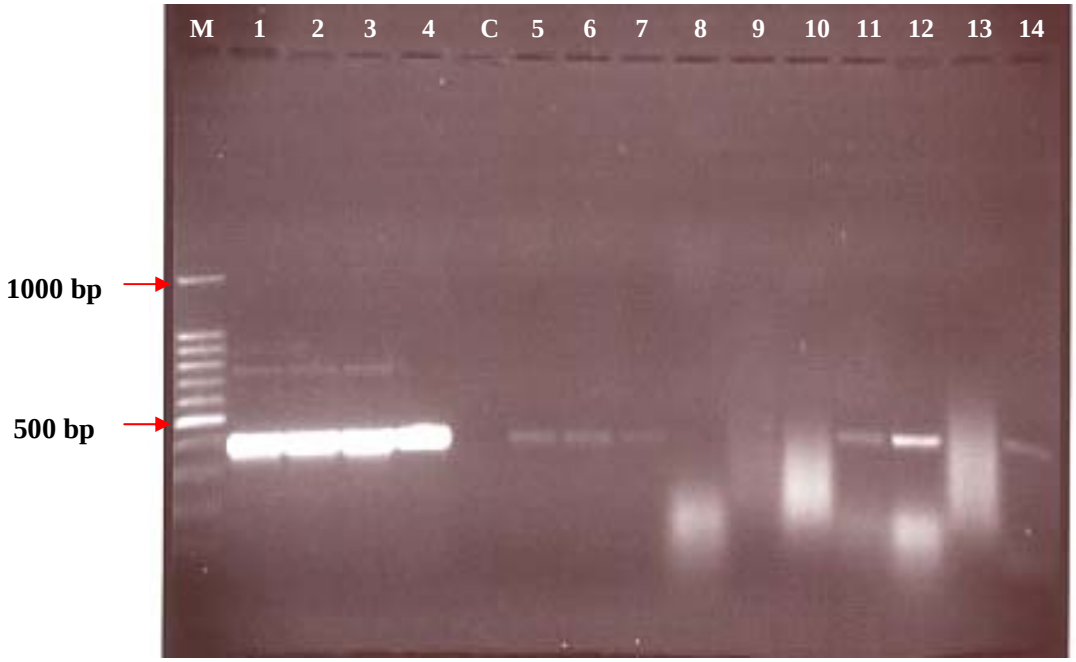

Fig. 3-6. PCR amplification of pure DNA preparations of *Eutypa* and related isolates with primers Lata 1/Lata 2.2 (Lecomte *et al*., 2000). Lanes: M = 100 bp ladder; (1) *E. lata* STE-U 5519; (2) *E. lata* STE-U 5520; (3) *E. lata* STE-U 5525; (4) *E. lata* STE-U 5527; (5) *E. lata* STE-U 5533; (6) *E. leptoplaca* STE-U 5581; (7) *C. ampelina*. STE-U 5701; (8) *C. ampelina* STE-U 5622; (9) *N. ribis* CMW 7772; (10) *N. luteum* CMW 10309; (11) *D. seriata* CMW 568; (12) *B. dothidea* CMW 8000; (13) *N. parvum* CMW 9081; (14) *Eutypella vitis* STE-U 5551. C = negative control.

The primers amplified a PCR fragment in each of these isolates, as well. The primers Lata 1/Lata 2.2 amplified a PCR fragment in *E. leptoplaca* (Fig. 3.6; lane 6), in one isolate of *C. ampelina* (Fig. 3.6; lane 7) but not in the other (Fig. 3.6; lane 8). These primers also amplified a PCR fragment in DNA preparations from *B. dothidea* (Fig. 3.6; lanes 11 and 12) and in *E. vitis* (Fig. 3.6; lane 14). Smears were obtained for those isolates which did not produce a PCR fragment, namely, *C. ampelina* (Fig. 3.6; lane 8), *N. ribis* (Fig. 3.6; lane 9), *N. luteum* (Fig. 3.6; lane 10) and *N. parvum* (Fig. 3.6; lane 13).

3.4 DISCUSSION

The objective of this study was to apply the RDBH method for the screening of grapevine material infected with species of *Eutypa* and Botryosphaeriaceae implicated as grapevine pathogens. Lardner *et al*. (2005), Rolshausen *et al*. (2004) and Lecomte *et al.* (2000) developed molecular methods that can identify and detect *E. lata*. Lardner *et al.* (2005) developed sequence characterised amplified regions (SCARs) to identify *E. lata* directly from grapevine wood and in mixed cultures. Rolshausen *et al*. (2004) developed a PCR-RFLP method for the identification of *E. lata* while Lecomte *et al*. (2000) designed PCR primers from ribosomal DNA ITS sequences and from randomly amplified polymorphic DNA fragments for the detection of *E. lata* in grapevine wood samples. This, however, is the first study in which RDBH has been used to detect *E. lata* and *E. leptoplaca* in a single assay. This is also the first study in which *B. dothidea, N. luteum, N. parvum* and *N. ribis* have been detected, in a single assay, using reverse dot blot hybridisation.

Reverse dot blot hybridisation was developed for the detection of mutations related to human disorders (Saiki *et al*., 1989) and was subsequently developed for the detection of bacteria (Voordouw *et al*., 1993, McManus and Jones, 1995). The efficacy of the technique was then applied for the identification of oomycetes (Levesque *et al*., 1998).

In the present study RDBH was successfully applied for the detection of the pathogens involved in Eutypa dieback and selected members of Botryosphaeriaceae responsible for diseases in grapevines. The species-specific oligonucleotides designed from the ITS sequence data for the positive identification of *E. lata* and *E. leptoplaca* could consistently detect these pathogens during the RDBH method. For the detection of *E. lata* and *E. leptoplaca* involved in the *Eutypa* disease complex, the ITS region proved the most useful for the design of the species-specific oligonucleotides and as several copies of the ITS rDNA region can be found in a genome, ITS-based detection can be more sensitive than detection for a single copy amplicon. The ITS region also provided the most consistent PCR amplification rates of the fungal DNA used in the RDBH method.

The β-tubulin region is well able to distinguish between *Eutypa* species (Chapter 2; Fig. 2-2). However, the use of its universal primers Bt2b/T1 in RDBH did not result in consistent amplification. Successful amplification of the partial region of the 5'-end of the gene was unpredictable, i.e. a PCR product was inconsistenly obtained. This disadvantage would account for too many delays in trying to obtain a PCR product for it to be useful in RDBH.

In the case of the large subunit, only a partial region was amplified and sequenced. The sequence variation between *E. lata* and *E. leptoplaca* was minimal and made the design of species-specific oligonucleotides difficult. Also, the lack of comparative large subunit sequence data from other diatrypaceous fungi in the GenBank database would not result in the design of species-specific oligonucleotides with high confidence. As a case in point, Lecomte *et al.* (2000) based the design of their six primers said to detect *E. lata* on one isolate, i.e. isolate BX1-10. At the time when these primers were designed, there were no other representative diatrypaceous fungi in GenBank. Since then, several sequences of Diatrypaceae have been deposited, and these primers were shown to lack specificity (Rolshausen *et al.,* 2004, Lardner *et al.,* 2005).

In the case of the several species of Botryosphaeriaceae found on grapevines, all capable of causing disease (Phillips, 1998 and 2002; Van Niekerk *et al*., 2004; Taylor *et al.,*

2005), the ITS region, although able to assess variability between species (Dupont *et al*., 2000), was insufficient in distinguishing between closely related species (Tooley *et al*., 1996; Taylor and Fischer, 2003; Crous *et al*., 2004). Single gene phylogenetic studies, although valuable to Botryosphaeriaceae taxonomy (Jacobs and Rehner, 1998; Denman *et al*., 2000; Ogata *et al*., 2000; Taylor *et al.,* 2005) have not differentiated between closely related sequences, and therefore, multiple gene phylogenies are necessary to resolve the species (Taylor *et al.,* 2000). Many studies have used sequence data derived from multiple molecular markers to resolve the Botryosphaeriaceae (Slippers *et al*., 2004a; 2004b; Van Niekerk *et al*., 2004). Consequently, species-specific oligonucleotides were synthesised from elongation factor-1α sequence data of the Botryosphaeriaceae found on grapevines in South Africa (Van Niekerk *et al*., 2004) for the use in the RDBH assays.

Species-specific oligonucleotides designed from the elongation factor-1α sequence data for the positive identification of *B. dothidea*, *N. luteum*, *N. parvum* and *N. ribis* could consistently detect these pathogens during the RDBH technique, with the exception of oligonucleotides Bdo-EF2, Blu-EF1, Blu-EF2, Bob-EF1 and Bob-EF2 that detected isolates other than for which they were specifically designed. From the species reported as pathogens of grapevines in South Africa, namely *N. ribis*, *B. dothidea* and *D. seriata*, (Crous *et al*., 2000), only *D. seriata* was consistently isolated. No isolates of *B. dothidea* or *N. ribis* were isolated from grapevines in South Africa (Van Niekerk *et al*., 2004). The RDBH technique employed here was, therefore, unsuccessful for the detection of *D. seriata*, the Botryosphaeriaceae species most prevalent on grapevines in South Africa. However, the availability of oligonucleotides that can detect for *B. dothidea*, *N. luteum*, *N. parvum* and *N. ribis* would contribute to diagnostic tests screening for the presence of these and other members of Botryosphaeriaceae from grapevine material. Van Niekerk *et al*. (2004) also isolated *B. rhodina*, *B. australis*, *N. parvum* from grapevines, in addition to two new *Fusicoccum* species, namely *F. viticlavatum* Van Niekerk & Crous and *F. vitifusiforme* Van Niekerk & Crous.

In addition to this lack of success, the RDBH method was not amenable to the detection of pathogens directly from field or environmental diagnostic samples but required pure

cultures. The oligonucleotides designed in this study could not detect the target pathogens directly from infected wood tissue, which reduces the value of this method.

The lack of success in the direct detection of the fungal pathogens targeted in this study could be explained by (1) the use of universal primers in RDBH allowed for the potential amplification of several other fungi inhabiting the infected grapevine, (2) the presence of other species in the Diatrypaceae (*Cryptovalsa ampelina* and *Eutypella vitis*) complicates the positive identification of the target pathogens, and (3) the presence of phenolics, the most important PCR-inhibiting compounds present in plant tissue (Nielsen *et al*., 2002), prevents positive amplification of the target pathogens.

The use of universal primers from the molecular regions mentioned could potentially amplify several fungi on infected plant material, but this was perhaps not the greatest drawback in the direct detection of the target pathogens. The greatest drawback encountered in the direct detection of the target pathogens was the PCR-inhibiting compounds present in the plant tissue. Lardner *et al.* (2005) found that the addition of PVP enhanced amplification but not in all reactions. PCR bands were either faint or not visible on the gels. The addition of PVP would thus not necessarily overcome the inhibitory effect the presence of phenolic compounds has on PCR. The lack of success in detecting the target pathogens directly from the infected wood tissue is likely due to the continued presence of phenolic compounds despite the addition of PVP and even organic solvents added to a PCR.

Organic solvents shown to enhance PCR could affect the melting temperature of oligonucleotides and thereby influence strand separation in a PCR reaction (Pomp and Medrano, 1991). Levesque *et al.* (1998) stated that the standardisation of the melting temperatures of the oligonucleotides is a critical consideration in RDBH. If organic solvents affect the melting temperature it would be difficult to standardise the conditions for a successful RDBH assay in the direct detection of pathogens. Optimal hybridisation conditions need to be accurately determined, particularly if several pathogens are to be integrated in a single assay.

In addition, the DNA isolation method used in this chapter was optimised for the isolation of PCR-competent DNA from leaf material, and not woody tissue. Lardner *et al*. (2005) tested several DNA extraction protocols to obtain PCR-competent DNA. The only protocol which met the requirements was obtained with a DNA isolation kit, the Bio-101 soil DNA extraction kit, which proved too expensive for the routine detection of *E. lata.*

The rapid DNA method described by Lecomte *et al.* (2000) failed to produce a single PCR fragment, primarily because heating the infected material to 95^{o}C apparently does not remove the phenolic compounds. The primers Lata 1/Lata 2.2 were not specific for *E. lata* as previously thought but could amplify a fragment in *E. leptoplaca* and in related diatrypaceous fungi, viz., *C. ampelina* and *E. vitis*.

Reverse dot blot hybridisation can be used for the identification and detection of the pathogens involved in Eutypa dieback and Botryosphaeriaceae species responsible for diseases on grapevines. The method as described here, though, could not be used for detection directly from infected material. Despite this disadvantage, the RDBH method is useful because it allows for the identification of multiple pathogens in a single assay. From pure preparations of DNA, oligonucleotides designed for the detection of the two *Eutypa* species and four Botryosphaeriaceae species, although not tested against a wide range of fungi, did not detect the diatrypaceous fungi *C. ampelina* and *E. vitis* which occur together with the target pathogens in infected wood tissue. As DNA extraction methods are amended, improved and honed, so would it increase the usefulness of RDBH. With the potential use of RDBH as a macroarray with many different oligonucleotides bound to a single membrane, the opportunities for the routine identification of plant pathogens cannot be discounted. However, alternative methods could be developed for the detection of these pathogens.

Research done on the use of nested primers has shown to increase the sensitivity of detection (Henson and French, 1993; McManus and Jones, 1995; Clair *et al*., 2003; Martin *et al.,* 2004). PCR assays that employ species-specific primers for the detection and identification of pathogens are not new, but have their limitations in that these assays

only detect one specific pathogen and if another is present it will not generate a positive PCR response. Also, many of these primer pairs can not be applied *in situ*. The species-specific primers developed by Lecomte *et al*. (2000) developed for the detection of *E. lata* were not tested on other Diatrypaceous fungi. Rolshausen *et al*. (2004) tested these primers on *E. lata* isolates collected from grapevines in California and reported a lack of specificity. This is consistent with our findings presented here. Rolshausen *et al*. (2004) developed a technique for the detection of *E. lata* by PCR-RFLP of the rDNA ITS region but the method required pure cultures. Lardner *et al*. (2005) was more successful with the SCAR markers but this was not cost effective because of the expense of the DNA isolation using the Bio-101 soil DNA extraction kit. The presence of polyphenols that were isolated with the genomic DNA in other protocols resulted in PCR inhibition.

These technical problems could be resolved with the development of nested primers to use in a second round of amplification, i.e. a set of universal primers is used in a first round of amplification followed by a second round of amplification using species-specific nested primers. Species-specific nested primers would be used to increase the sensitivity of the PCR and reduce the "incidence of false negatives" (Martin *et al.*, 2000).

Molecular methods can not be discounted as a valuable tool in diagnostics because it ensures reliability and accuracy of the results. With the advances made in disease diagnosis, it is required that protocols and methods be revised on a regular basis to ensure that the demands of the industries for quick and accurate diagnostic tools are met. The development of new methods also ensure that costs of new tests are evaluated and that the most efficient and cost effective methods are employed.

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