THE DEVELOPMENT OF DIAGNOSTIC TOOLS FOR THE GRAPEVINE PATHOGEN *EUTYPA LATA*.

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ABSTRACT.

Eutypa lata is the causal agent of Eutypa dieback on grapevines. The fungus invades the vine and grows there unnoticed, possibly for several years, causing discolouration and deformation of the vine shoots and leaves. Most berries fail to establish on these shoots and the fungus eventually kills the vine. The damaging effects of this fungus have had a notable financial impact on the grape and wine industry world wide and *E. lata* is at present the primary constraint on vineyard longevity in many places including California and Australia. Little is known about the occurrence and distribution of Eutypa dieback within New Zealand. This is due mainly to difficulties associated with identification of the disease in grapevines.

To develop a molecular probe for the identification of *E. lata* from grapevine wood the Polymerase Chain Reaction (PCR) amplified the Internal Transcribed Spacers (ITS1 and ITS2) and the intervening 5.8S gene of ribosomal DNA (rDNA) from representative isolates. The sequences of the *E. lata* ITS regions were used to design two pairs of primers, each of which was subsequently shown to be specific for the amplification of predicted-size fragments from genomic DNA of *E. lata*. The primer pairs were further tested using template DNA extracted from healthy grapevines and from other fungi commonly isolated from dieback diseased grapevines but no PCR amplification was observed. Simple DNA extraction protocols, leading to the rapid release of DNA, were tested to enable identification of *E. lata* from pure culture and grapevine wood; however, a suitable DNA extraction method from these materials was not found.

Currently the only known source of inoculum is ascospores, which are released from perithecia during and immediately after rainfall. However, few perithecia have been found in New Zealand vineyards. This has prompted the study of the mating habits of *E. lata*. As the sexual stage of *E. lata* cannot be obtained in culture at present, the analysis of its mating system must be performed in natural populations. Molecular characterisation of the mating type at the outset of a mating project allows significant savings in time and effort as it drastically reduces the number of crosses that must be set up. So far, cloning of mating type (*MAT*) genes from fungi has been hampered by low conservation among them. Most ascomycete fungi have one mating type gene with two alternative forms or idiomorphs (*MAT1-1* and *MAT1-2*). One of the pair of *MAT* genes, *MAT1-2*, encodes a protein with a conserved DNA binding motif called the high mobility group (HMG) box. There is sufficient sequence conservation at the borders of the HMG box to allow PCR amplification. New Zealand isolates of *E. lata*, including sixteen single ascospore isolates from one perithecium, were tested for the presence of a *MAT1-2* idiomorph using this PCR based approach. Five different sets of primers were used which were designed to anneal at different target sites with different specificities. PCR products of the expected size were obtained and sequenced, but despite exhaustive attempts to optimise PCR specificity, none of these had convincing homology to fungal mating type genes.

Progress on the basic aspects of the genetics of *E. lata* will continue to be hampered until the organism is induced to complete its life cycle in culture. Molecular studies into the mating type genes which regulate sexual compatibility and sexual reproduction in

the fungus should lead to a deeper understanding of the life-cycle of *E. lata* and the critical influence of sex on population genetics. In addition, it will provide a scientific basis for a management program urgently needed to minimise the impact of this disease.

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To Robin Sage and the team at Erindale Vineyard Partnership I wish to express my gratitude for your cooperation and support. I'll catch up with you at the Rose and Crown one day.

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It was a thesis of two halves, full credit to all those involved. Thankfully, grapevines were the winner on the day.

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1. INTRODUCTION.

1.1 Winemaking- A rising star among New Zealand's industries.

1.1.1 From the vine to the bottle.

For many thousands of years, wine has been part of the human diet. It represents a safe and healthy beverage providing calories and vitamins and more importantly offering relaxation and relief from the stresses of everyday life (Amerine, Berg, Kunkee, Ough, Singleton & Webb, 1980). Species of the grapevine *Vitis* are grown all over the world, and the most important species for winemaking is *Vitis vinifera* (Linn).

The winemaking process begins in the vineyard where grapes are grown, harvested at an appropriate sugar level, destemmed and crushed. For white wines, the rapid separation of the juice from the skins and seeds is important as extended contact with the skins allows materials such as tannins to be extracted and remain in the finished wine (Ough, 1992). For red wines, the juice remains in contact with the skins to help extract colours and flavours from the skins (Surico, 2000). Sulphur dioxide may be added during or after the crush to prevent oxidation of the grape must and growth of wild yeast and bacteria that may be present on the grapes. After clarification of the juice, yeast is added to begin the alcoholic fermentation. In modern wineries, starter cultures of *Saccharomyces cerevisiae* (Meyen *ex* Hansen) or *Saccharomyces bayanus* (Saccardo) are usually used. They are selected for their ability to ferment vigorously, their contribution to flavour/style and their tolerance to ethanol and sulphur dioxide (Osborne, 2000). At the completion of alcoholic fermentation, the wine is racked off into another vessel leaving behind the yeasts and solids.

Some wines undergo a secondary fermentation, called malolactic fermentation, carried out by malolactic bacteria and may occur naturally or can be induced using starter cultures. It causes deacidification of high acid wines (such as those produced from cool climate regions, e.g. New Zealand) and adds flavour complexity (Osborne, 2000).

The wines may be filtered or fined, and then aged and blended depending on the types of wine produced. Finally the wines are bottled, packaged and sold for the consumers' enjoyment.

1.1.2 The New Zealand Grape and Wine Industry.

The New Zealand wine industry is building a prominent international profile through the sale of increasing volumes of premium quality, high value wines. High compound growth rates in value and volume have led to New Zealand wine exports in excess of NZD$100 million (Spense, 1998) per annum. This milestone was reached two years in advance of a 1992 prediction that annual wine exports would reach $100 million in the year 2000. It is a developing industry; the vineyard area has risen to 8,716 hectares (an increase of over 40% since 1993) with predictions that the producing area will grow to over 10,000 hectares by the year 2002 (Spense, 1998). This is producing rapid changes in the industry, forcing it to confront new challenges and rethink traditional strategies.

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The future for the industry appears bright but to build on the foundations that have been established so far, capital must be attracted to fund ongoing expansion of vineyards and wineries, investments in marketing and sales, research and technological developments in the industry. Major disease problems could jeopardise such investments.

1.2 Eutypa dieback.

Eutypa dieback, formerly known as "dead arm", is one of the most destructive diseases of woody tissues in commercially grown grapes. The disease is caused by the fungus *Eutypa lata* (Pers.: Fr.) Tulsane and Tulsane. (Syn. E. *armeniacae* Hansf. and Carter, anamorph *Libertella blepharis* A. L. Smith (Syn. *Cytosporina* sp.)). It invades the vine and grows there unnoticed, possibly for several years before killing the vines. The known distribution of the disease coincides with the distribution of the grapevine through out most countries of the world including France, the United States, Australia and New Zealand.

Carter (1957) made the first association between the vine dieback symptoms and the causal organism, *E. lata* (long known as a pathogen of the apricot) in Australia. However, it was Moller and Kasimatis (1981) who finally made the irrefutable connection between the symptoms and E. *lata*.

1.2.1 Epidemiology

1.2.1.1 Spore release.

Rain is a pre-requisite for the release of ascospores. Currently, this is the only known means of dispersal and infection. Vineyards that contain perithecia of E. *lata* have consistently higher rates of disease incidence than those that do not (Hughes, Munkvold & Samita, 1998; Munkvold, Duthie & Marois, 1993). This may reflect the contribution of these internal inoculum sources to disease incidence. However, other factors such as geographic location, cultivar, favourable environmental conditions, and vineyard age may also contribute to high disease levels. A disease gradient or edge effect has been reported in vineyards that do not contain perithecia (Munkvold *et al.*, 1993). One of these vineyards was found to be adjacent to a vineyard that contained inoculum sources. No evidence was found to indicate disease spread by any means other than airborne ascospores from distant sources, in any of the vineyards sampled in this study.

After dissemination, the ascospores of E. *lata* are able to survive long periods of freezing and in laboratory tests they have been shown to remain viable for several weeks after release (Ramos, Moller & English, 1975). In regions where winters are temperate, perithecia of E. *lata* reach maturity early in spring, and ascospores are disseminated with each rainfall of approximately 1 mm or more. Discharge of ascospores begins less than 3 h after rainfall has started and continues throughout the rainy period until rainfall has ceased and the stromata have dried (Pearson, 1980). Following a long dry period, perithecial stroma and the underlying wood tissues must be thoroughly wetted before ascospores can be released in abundance. This "conditioning" of the stroma enables the wood beneath to absorb water and act as a reservoir of moisture for the stroma.

A seasonal pattern of ascospore production and release has been revealed in Australia

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(Moller & Carter, 1965) and in California (Ramos *et al.*, 1975). The periods of very low ascospore output correspond to production of new perithecia in the stromata. By late autumn the perithecia are almost exhausted, nevertheless, sufficient ascospores are available to infect vines pruned during the following winter (Ramos *et al.*, 1975). In regions where temperatures below 0°C prevail in winter, dissemination of ascospores is greatest in late winter, Therefore they are in abundant supply at the time when grapevines are usually pruned.

1.2.1.2 Infection.

Ascospores deposited from the air on to plant surfaces are readily removed by water, implying that transport of the inoculum of *E. lata* from the perithecia to the open ends of vessels exposed by pruning wounds during intermittent rainfall is a two-fold process. This comprises deposition from spore laden air onto the plant surfaces when rain is not actually falling, followed by the redistribution of the deposited spores, during subsequent rain showers, by the splashing of rain drops or water flow along these surfaces (Carter, 1965).

Germination of the *E. lata* ascospores occurs within the vessels, usually 2 mm or more beneath the wound surface (Moller & Kasimatis, 1978). *In vitro*, ascospores germinate in 11-12 h at the optimal temperature of 20-25°C (Carter, 1957, reviewed 1991). The mycelia proliferate slowly, at first within the vessels, and later through associated elements of the functional wood. The fungus is present only in the lignified tissues. No symptoms are seen during the first one or two growing seasons after infection. By the third or fourth season a canker is usually apparent, often accompanied by foliage manifestations as described in Section 1.2.2. Several years may elapse before the affected arm or trunk is killed.

The susceptibility of wounds to infection has been shown to diminish markedly during the first two weeks following pruning, and after four weeks the wounds are unlikely to become infected (Munkvold & Marois, 1995). The maximum duration of grapevine wound susceptibility is unknown, but wounds that are more than a year old are not susceptible (Moller & Kasimatis, 1980). Therefore, infections are likely to be limited to the same dormant season in which the wounds are made. Another process affecting the susceptibility of pruning wounds is the physiological wound response that occurs in bark and wood (Bostock & Sterner, 1989). After pruning, the tissue begins to desiccate, and parenchyma cells become necrotic and accumulate higher levels of free and polymerised phenolic compounds. In the grapevine the accumulation of suberin and lignin is common in a wound response reaction. This accumulation of suberin and lignin has been linked to the decline in wound susceptibility during the first 28 days after pruning (Munkvold & Marois, 1995). The increase in lignin and suberin is also linked to the environmental conditions. At low temperatures, infection of the pruning wounds by *E. lata* is increased; whereas the growth of other microorganisms is reduced (Munkvold & Marois, 1995; Carter, 1991; Chapuis *et al.*, 1998) Moderate temperatures encourage growth of these other micro-organisms, notably the *Rhodotorula* yeast species. This natural non-pathogenic colonizer of grapevine pruning wounds is able to reduce the capacity of *E. lata* to infect pruning wounds, probably the result of competition (Chapuis *et al.*, 1998). The mechanisms that influence the decline in the susceptibility of pruned wood then, may include wound age, host wound response and

epiphytic populations. Temperature strongly affects the susceptibility of pruning wounds through these mechanisms

1.2.2 Symptom development.

The foliar symptoms are most evident during the first two months of the annual growth cycle, especially when the new seasons shoots are 25-30 cm long. *Eutypa* dieback causes deformation and discoloration of the shoots. The young leaves are smaller than normal, cupped, and chlorotic; they often develop small necrotic spots and tattered margins, sometimes with larger areas of necrosis as they age. A marked dwarfing of the internodes accompanies the development of these leaf symptoms. The disease induces ultra structural alterations in leaf cells of the grapevine such as cytoplasmic lysis with plasma membrane detachment and complete chloroplast disorganization as observed by electron microscopy (Philippe, Renaud, Tsoupras, De Angelis, Fallot & Tabacchi, 1992). Most berries that do establish on these shoots fail to mature. If the shoots are only mildly affected the tattered leaves appear on the first few nodes and subsequent growth is normal. These symptoms are readily seen until late spring, when affected shoots often become obscured from view by adjacent healthy growth.

The symptoms on foliage of diseased arms become more extensive each year; finally, part or the entire arm fails to produce shoots in the spring. It is common to find one side of the vine dead while the other side appears healthy hence the original name of the disease "dead arm". When the whole vine has been killed or is severely affected by Eutypa dieback, strong suckers (shoots) develop from the still healthy root system. Complete collapse and death of the vines or arms is uncommon once the shoots have emerged, they usually grow through the summer and die the next winter.

Figure 1.1 Typical foliar symptoms of grapevine dieback on Cabernet Sauvignon.
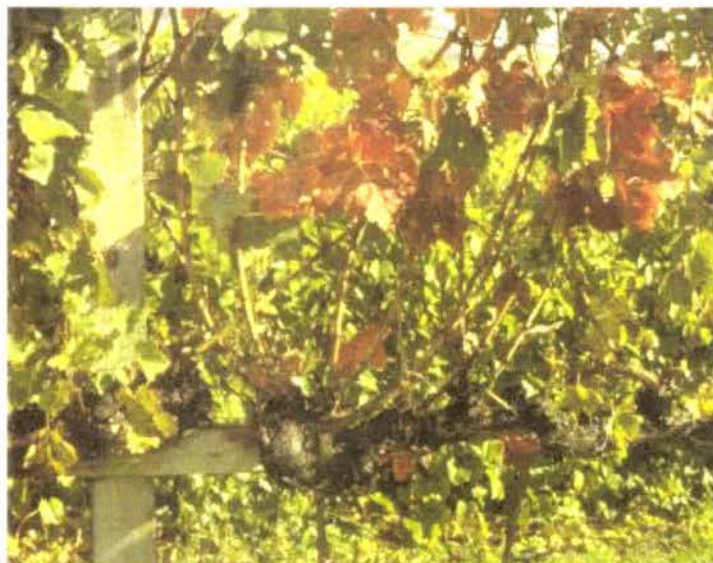
Close examination of an arm, cordon, or trunk with vascular connection to shoots bearing foliar symptoms usually reveals a canker surrounding a pruning wound made several years previously. Removal of the loose bark is necessary to show the magnitude of the canker. Trunk cankers can be extensive in length, and a cross section through the canker often reveals only a narrow strip of live wood. In its early stages, a canker (in

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cross section) appears as a wedge shaped zone of necrotic sapwood extending from the point of origin of the canker and coming to a point in the centre of the arm or trunk. This dead wood is brown, hard and brittle. Due to the slow progress of this disease, the canker is not apparent until the third or in some cases fourth season after infection. This makes identification of Eutypa dieback by visual inspection redundant; as, at this late stage the canker is usually too extensive for remedial surgery.

1.2.2.1 Role of Eutypine

The foliar symptoms associated with Eutypa dieback (Section 1.2.2) are believed to be induced by a translocatable toxin generated in the older wood invaded by the fungus (Tey-Rulh, Philippe, Renaud, Tsoupras, de Angelis, Fallot & Tabacchi, 1991). Fallot, Tey-Rulh, Coutouly, Pettiprez, Roustan, Philippe and Tabacchi (1989) successfully used plant tissue culture techniques to gain a better understanding of the interaction between *E. lata* and *V. vinifera*. An aldehyde compound named 'eutypine' (4-hydroxy-3- (3-methyl-3-butene-1-ynyl) benzyl aldehyde) was isolated and can be detected consistently in the tissues of vines invaded by the pathogen but not in the healthy vine wood.

The ultra structural alterations induced by eutypine in leaf cells and protoplasts isolated from plantlets of *V. vinifera* (cultivar (cv) Cabernet Sauvignon) were visualised by transmission electron microscopy (Deswarte, Rouquier, Roustan, Dargent & Fallot, 1994). Eutypine exhibits weak acid properties, a marked lipophilic character and induced alterations of the cellular ultrastructure that are similar to those observed *in vivo* in the leaves of diseased grapevines (Philippe *et al.*, 1992). It was also observed that the speed of symptom appearance and their intensity were proportional to the eutypine concentration. This confirmed that the eutypine synthesized by *E. lata* mycelium in the trunk or arms is involved in symptom expression of Eutypa dieback in the herbaceous parts of the grapevine.

In vitro studies have shown that the toxin rapidly penetrates grapevine cells through passive diffusion and tends to accumulate in the cytoplasm due to an ion trapping mechanism related to the ionisation state of the molecule (Deswarte, Canut, Klaebe, Roustan & Fallot, 1996). Eutypine then uncouples mitochondrial oxidative phosphorylation by increasing proton leakage through a cyclic protonophore mechanism (Deswarte, Eychenne, Davy de Virville, Roustan, Moreau & Fallot, 1996), thus modifying the rate of respiration and the energy balance of the grapevine cells. The level of sensitivity to Eutypa dieback depends upon the genotype of the host. The tolerance of some grape genotypes is related to their capacity to detoxify eutypine into its corresponding alcohol, eutypinol (4-hydroxy-3- (methyl-3-butene-1-ynyl) benzylic alcohol) that lacks phytotoxicity. This reaction has shown to be NADPH dependent (Fallot, Deswarte, Dalmayrac, Colrat & Roustan, 1997). Only limited effects are triggered by euptypine's biologically inactive derivative eutypinol (Amborabé, Fleurat-Lessard, Bonmort, Roustan & Roblin, 2001).

Metabolism of eutypine is not restricted to grapevine varieties but several plant species are also capable of converting eutypine into eutypinol, of which the mung bean (*Vigna radiata*) exhibits the highest activity (Fallot *et al.*, 1997). An NADPH-dependant aldehyde reductase has been purified from the mung bean that exhibits the ability to convert eutypine to eutypinol. Expression of this transgene in *Vitis vinifera* cells

cultured *in vitro* confers resistance to the eutypine toxin (Guillén, Guis, Martinez-Reina, Colrat, Dalmayrac, Deswarte, Bouzayen, Roustan, Fallot, Pech & Latché, 1998). This discovery opens up new biotechnological approaches for the generation of grapevines resistant to Eutypa dieback.

1.2.2.2 Formation of asexual and sexual stages.

Several years after the initial infection, *E. lata* produces perithecial stromata on diseased grapevine wood, at first in small patches surrounding the site of original infection, or sometimes at the wound stub that forms the point of entry. Later, as the vine is more extensively invaded, larger areas of stromatic tissue form on the surface of dead wood. The stromata are black and continuous and the perithecia are revealed when a shallow slice is removed from the surface with a blade. Once the perithecial stage has developed it may continue to discharge ascospores for five years or longer.

Figure 1.2 Typical *E. lata* perithecia on Cabernet Sauvignon grapevine wood.
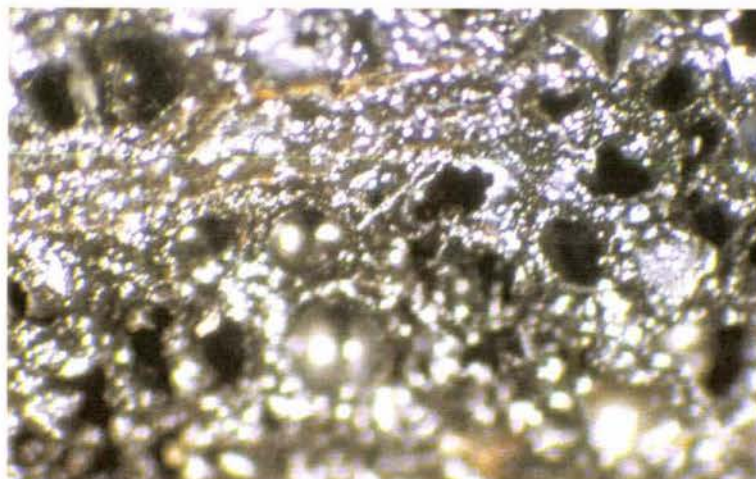
The perithecia (450 μm in diameter) of *E. lata* contain asci, which are borne on pedicels (60-130 μm long) and measure 30-60 x 5-7.5 μm, with an apical pore. The eight ascospores are pale yellow and allantoid and measure 6.5-11 x 1.8-2 μm (Carter & Talbot, 1957). Perithecia have not yet been produced in culture. The anamorph (*Libertella*) of *E. lata* produces conidiomata, which may develop in culture after six to eight weeks. They often exude characteristic single-celled conidia (18-45 x 0.8-1.5 μm) in orange cirri. On the grapevine, they are formed one to two years after infection.

On the vine the conidiomata are found on the inner bark covering the infected wood. The spores of the anamorph do not normally germinate on laboratory media and there is no evidence that they function as propagules. This is confirmed on the basis of spatial patterns studied by Munkvold *et al.* (1993), where no evidence of vine-to-vine spread of Eutypa dieback in vineyards without perithecia was detected. If Eutypa dieback were to be spread by conidia, one would expect clusters of diseased plants to develop more rapidly than if the disease were spread by ascospores, because conidia are produced more quickly after infection and are splash dispersed. The role of the asexual stage of *E. lata* is therefore unknown and more research needs to be undertaken to understand its function in the disease cycle.

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1.2.3 Host range and effect on yield.

1.2.3.1 Grape varieties.

No grape cultivars are known to be immune to Eutypa dieback infection (Carter, 1988). It was believed that the grapevine cultivars varied in their apparent susceptibility to Eutypa dieback in the field. For instance, disease monitoring in French vineyards indicated that Cabernet Sauvignon was susceptible while Merlot was tolerant (Carter, 1991). This is similar to the situation in Hawkes Bay vineyards (New Zealand) where growers report a higher incidence of infection in Cabernet Sauvignon vines, whereas, white varieties are relatively unaffected by the disease. However, current thought is that those cultivars reputed to be the most susceptible are likely to be those that exhibit spectacular foliage symptoms in response to the disease, rather than the rate at which their tissues are killed by the action of the pathogen (Carter, 1991). A laboratory study undertaken by Chapuis, Richard and Dubos (1998) concluded that there were no differences in susceptibility to infection between Cabernet Sauvignon (highly susceptible) and Merlot (tolerant) cultivars, although in the vineyard they differed significantly in symptom expression. These findings could possibly be a consequence of the high concentrations of ascospores used in the field studies, 1500 ascospores in 30 µL of sterile water. In defence of the work Chapuis *et al.* (1998) argued that this concentration gave detectable levels of infection even under unfavourable climatic conditions and postulated that the differences observed in the vineyard may be associated with the defence reactions of the plant cells toward fungal invasion in the sapwood, or, they may be explained by differences in susceptibility to the fungal toxin complex produced. In contrast, Munkvold and Marois' Californian study (1995) with an ascospore concentration of 1000 ascospores per 50 µL of sterile water showed significant differences in the susceptibility of nine different cultivars, although the relative susceptibility was not consistent between the two field trials performed. As no consistent experimental evidence for the differences in susceptibility of grapevine cultivars is available, it must be assumed that all cultivars are sensitive to infection by *E. lata*.

1.2.3.2 Other crops.

E. lata has a wide host range, 88 species distributed in 28 botanical families (Bolay & Carter, 1985; Carter, Bolay & Rappaz, 1983). Most of its hosts are tree species, including some that are components of natural forests. The most severely affected horticultural hosts are the grapevine and apricot. Eutypa dieback is known to occur on apricots in the Heathcote valley near Christchurch and throughout central Otago (Atkoman, 1971). Apples are the other horticultural crop of importance to New Zealand affected by *E. lata* (Pennycook, 1980). This could have implications on the management of the disease in some of our larger grape growing regions such as the Hawkes Bay, Gisborne, Marlborough and central Otago, where other fruit crops are grown in close proximity to the vineyards and may serve as reservoirs for infection.

From their own observations and those of others, Bolay and Carter (1985) noted that wherever the pathogen has been recorded on one or more of its numerous hosts, it is almost invariably associated with dieback of the grapevine in that region. However, the converse of this observation does not apply. As seen in Southern France, the pathogen can be found in nearly every mature aged vineyard but it is rare to find it in apricot orchards close to these vineyards, despite the abundance of inoculum produced on the

dead parts of the diseased vines. Similar observations have been made in Hungary and South Australia (Carter, 1991).

1.2.3.3 Effect on yield.

Yield losses of between 11% and 100% have been estimated on vines in Washington State, USA and Greece with symptoms ranging from 'moderate' to 'very severe' (Johnson & Lunden, 1985, Thanassoulopoulos, 1989). However, many complex factors must be addressed when quantitatively determining losses in vineyard productivity due to Eutypa dieback.

Eutypa dieback causes a significant reduction in the yield of infected grapevines when compared with healthy vines, even on vines with relatively low disease severity. Yield reduction is primarily due to a diminished number of clusters per vine, while the effect of the disease on mean cluster weight is smaller and not always significant (Munkvold, Duthie & Marois, 1994; Wicks & Davies, 1999). It could be expected that a loss of photosynthate in grapevines due to a reduced amount of vegetative growth may contribute to the reduction in yield. However, the effect of disease severity on vegetative growth is less pronounced than the effect on yield reduction and can only be considered a contributing factor in severely affected vines.

In areas where incidence of the disease is high, diseased vines become more numerous each year thereafter. In addition to this, Eutypa dieback usually does not appear until a vineyard is at least eight years old (Carter, 1988). Therefore, economic losses due to Eutypa dieback may be minor in early years but the most damaging effects are observed in older vines, which have large pruning wounds caused by drastic cutting to retrain (renovate) them. Grapevines are believed to compensate for the loss of fruiting buds (particularly due to severe pruning and in some cases as a result of a disease) by producing more fruit on shoots that arise from the remaining healthy buds (Lider, Kasimatis & Kliewer, 1975). Such compensation would mitigate the yield reduction that would result from a loss of spurs to Eutypa dieback. However, no evidence for yield compensation by healthy shoots on Eutypa dieback infected vines has been detected (Munkvold et al., 1994, Wicks & Davies, 1999).

The costs of reduced wine quality due to uneven berry maturity from infected vines as well as the costs associated with vine removal, replanting or reworking and the loss of productivity also need to be considered in addition to the direct yield losses attributed to E. lata. Therefore, the cumulative yield loss over the life of a vineyard represents a very substantial monetary loss.

At present Eutypa dieback is the primary constraint on vineyard longevity in northern California (Munkvold et al., 1994). Eutypa dieback appears to be increasing in severity in Australian vineyards and is expected to be a major limiting factor in the sustainability of Australian wine production (Pascoe & Cottral, 2000). With effects such as these on well-established wine industries elsewhere in the world, Eutypa dieback has the potential to become a major liability to New Zealand's burgeoning grape and wine industry. Hence, the urgent need for more research to provide a sound scientific basis for a management program to minimise the impact of this disease.

1.2.4 Management.

1.2.4.1 Sanitation methods.

The cultural practice of regular pruning of grapevines provides a multitude of entry points for the pathogen every year. Furthermore, the slow growth of the pathogen and the delayed manifestation of symptoms make recognition of the disease difficult until extensive invasion has occurred, by which time it is usually too late for remedial surgery. Hence, the disease is essentially uncontrolled. Because ascospores are dispersed after rain, pruning the grapevines late in the spring when rains are not as likely to occur can reduce the chance of infection. Late pruning is also important in reducing the susceptibility of the wounds (Chapuis *et al.*, 1998; Moller & Kasimatis, 1980). In regions where inoculum is abundantly produced on many alternative hosts, it is impossible to manage Eutypa dieback effectively by sanitation methods (eradication of disease sources) alone. However, in regions with vast plantings of grapes and few alternative hosts, sanitation is beneficial.

1.2.4.2 Fungicide control.

None of the chemicals used to routinely control other fungal diseases of the grapevine, provide adequate protection against *E. lata*, nor indeed is the timing of their application effective at preventing infection. However, the fungicides benomyl and flusilazole provide highly effective barriers against the invasion of pruning wounds by germinating ascospores if sufficient chemical is present in the tissue below the pruning wounds before the spores arrive (Munkvold & Kasimatis, 1980; Munkvold & Marois, 1993). To accomplish this, each wound must be flooded to saturation to ensure that the chemical is carried well into the exposed vesicles at the wound surfaces. Because of these conditions, applications of benomyl by conventional spraying machines have not been successful. Manual treatment of each pruning wound at the time of pruning, or the use of spraying secateurs, which facilitate the treatment of any selected wound with a saturating deposit of spray, are the only means of effective application (Carter, 1988).

1.2.4.3 Biological control.

Rapid colonization of the pruning wounds by biological control agents may be the key to effective control of large epidemics of *E. lata*. However, to be effective these organisms must have optimal temperatures for growth lower than those of *E. lata* and they must be able to grow under field conditions. Chapuis *et al.* (1998) investigated the possibility of using *Rhodotorula* yeast, a natural colonizer of grapevine pruning wounds, to reduce the infection capacity of *E. lata*. As *Rhodotorula* species grow rapidly it can be considered a good competitor against *E. lata*. However, the competition is temperature dependent. At the germination temperature zone for *E. lata* (-20°C-+10°C), the *Rhodotorula* population is unable to establish itself before *E. lata* germinates, as the germination temperature zone is unfavourable for *Rhodotorula* growth. The increase in epiphytic populations on grapevine wounds is positively correlated with a decrease in the ability of *E. lata* to infect the vines and with warmer temperatures. The reduction in the ability of *E. lata* to infect wounds at these warmer temperatures is probably due to competition. Ferreira, Matthee and Thomas (1991) found that an isolate of *Bacillus subtilus* ((Ehrenberg) Cohn) from grapevine wood inhibited growth of *E. lata in vitro*. The bacterium caused inhibition of mycelial growth and ascospore germination. Therefore, the biological control of *Eutypa* infection is possible but it is not always an economic alternative in the vineyard. This is partly due

to the cost of the agents themselves and the increase in vineyard labour cost because of the manual application of these agents to every wound made to the vines during pruning.

1.3 Application of molecular marker technology to *Eutypa lata*.

Recently, molecular markers such as isozymes and random amplified polymorphic DNA sequences (RAPDs) have been applied to *E. lata* in an attempt to explain differences in pathogenicity that have been observed between isolates of varying origin. RAPD is a PCR-based approach for the detection of polymorphisms between organisms for genetic mapping and strain identification (Welsh & McClelland, 1990; Williams, Kubelik, Livak, Rafalski & Tingey, 1990). It involves randomly amplifying short fragments of genomic DNA using a single short oligonucleotide primer, along with size fractionation by agarose gel electrophoresis, producing a specific pattern of products.

Variation in pathogenicity of *E. lata* isolates may be a factor in the variation of dieback incidence. In particular, it has been shown that some isolates do not cause the stem and foliar symptoms typical of the disease on grapevine cuttings (Munkvold & Marois, 1995). Péros, This, Confuron and Chacon (1996) set out to identify molecular markers suitable for studying the genetic variation and biology of *Eutypa*. They examined a variety of standard isozyme markers and a set of RAPD markers using a collection of 18 isolates. RAPDs appear to be more useful than isozymes to describe the genetic variation of the fungus, since RAPD analyses identify more polymorphism. However, they were unable to group the isolates according to their pathogenicity using RAPD and isozyme analysis. Genes controlling pathogenicity most likely represent a minute fraction of DNA in the genome; the markers identified in the study cover a limited part of the genome, possibly missing the genes controlling pathogenicity.

In a separate study the pathogenicity and cultural characteristics of 55 *E. lata* isolates, each collected from a different vine in a single vineyard, was examined (Péros *et al.*, 1997). The isolates showed a large variation in pathogenicity after inoculation on cuttings in the greenhouse. Variation was also observed in cultural traits and radial growth on potato dextrose agar, but no relationship was found between these characteristics and pathogenicity. Likewise, the pathogenicity of isolates was not influenced by the cultivar from which it originated (Péros, Jamaux-Despréaux, Berger & Gerba, 1999). Thirty-two RAPD markers were used to study the genetic relatedness of the isolates and the genetic structure of the population and 55 different RAPD patterns were observed, confirming the uniqueness of each isolate. It was also demonstrated that the subdivision of isolates based on the presence of shoot and foliar symptoms in the vineyard did not correspond to qualitative differences in pathogenicity. The existence of a high level of genetic variation is not surprising for a fungus propagated by ascospore dispersal.

RAPD markers have also confirmed random mating between *E. lata* populations in two vineyards located 390 km apart in Charente, France (Péros & Larignon, 1997; Péros & Larignon, 1998). The observed distribution of marker differences was such that random assortment of the RAPD markers was demonstrated. The frequency of RAPD markers in the two populations was found to be similar and indicated no genetic differentiation between the two populations, although they were separated by 390km. This suggests

gene flow between the two populations, the mechanism for this gene flow probably being the aerial transport of ascospores. The extent of diversity among single ascospore isolates of *E. lata* confirms *E. lata* as a random mating species with a high degree of genetic diversity (Péros & Berger, 1999).

Amplified Fragment Length Polymorphism (AFLP) analysis and sequence analysis of the Internal Transcribed Spacer (ITS) region of the ribosomal DNA of a large number of *Eutypa* isolates has elucidated two clades of *Eutypa* species and infers the presence of two species of *Eutypa* (*E. armeniacae* and *E. lata*) in California (DeScenzo, Engel, Gomez, Jackson, Munkvold, Weller & Irelan, 1999). Both species of *Eutypa* are capable of infecting native American and cultivated hosts. This suggests a possible explanation for the high incidence of disease reported in the Central Valley of California and the absence of mature stroma on grapevines in the region, in which native tree species potentially serve as inoculum sources for grape infection in California. Subsequently, molecular markers have proven to be useful tools in evaluating the genetic systems and structures of *Eutypa* populations.

1.4 A molecular approach to the identification of *Eutypa lata*.

1.4.1 Detection problems of *Eutypa lata*.

Little is known about the occurrence and distribution of the disease within New Zealand. This is due mainly to the difficulties associated with identification of the disease in grapevines. Therefore, this identification problem needs to be addressed before studies into the epidemiology and management of the disease are undertaken.

As *E. lata* infections in the vine are difficult to detect for a long period of time but then kill the vine quickly once the fungus begins to fruit, traditional methods of detecting the pathogen within the grapevine, such as visual inspection and culture indexing (an examination of diseased vines where identification is by isolation of the pathogen into pure culture), are time consuming and lack sensitivity. However, currently isolation of the fungus into axenic culture is necessary to confirm the presence of the pathogen in vines suspected of having Eutypa dieback. The reproducibility of this kind of diagnosis is poor.

Visual inspection of the vine is insensitive because the grapevine has already started to die once the visual symptoms of the fungus have become noticeable and the grapevine is then vulnerable to infection by other microbes, which may confuse the symptoms. At this time, viticulturists are unable to treat the dying vine without great expense and with no guarantee of success.

Serological identification of *E. lata* was investigated in the early 1970's. It was found that the ascospores and mycelium of *E. lata* differed antigenically (Francki & Carter, 1970). When antiserum was produced to cell wall free extracts of *E. lata* the anti-serum was highly specific and could be used to identify the fungus by gel diffusion assay (Price, 1973). However, this test was limited in its usefulness in that it still required *E. lata* to be cultured from the vine and cell wall free antigens from the mycelium prepared. Therefore, the results of serological identification tests have not been promising.

1.4.2 Culturing *Eutypa lata*.

Preparation of specimens and isolation of the pathogen in pure culture was described by Carter and Moller (1977). *E. lata* may be cultured onto common laboratory media (potato dextrose agar and malt agar) from small chips taken aseptically from the margin of discoloured sapwood in diseased arms or trunks. White mycelium grows from infected chips and is usually visible after two days. After five to six days at 20-25°C, the colonies are usually sufficiently well developed for recognition of the fungus. Exposing the plates to a 12/12 h light-dark regime or to near–ultraviolet radiation promotes sporulation. Not all isolates sporulate, and considerable variation in the amount of dark pigment produced in the medium after one and a half to two weeks is also observed. For these reasons, preliminary diagnosis is most readily accomplished by comparing the gross morphology of colonies six or seven days old with that of a reliable reference culture transferred at the same time.

Diagnosis of *E. lata* by culturing the pathogen from diseased wood may be complicated by the co-existence of other fungi in specimens selected for isolation. This is because once the grapevine has been injured by *E. lata* the tissue is then susceptible to invasion by a number of other saprophytic wood fungi including *Alternaria alternata* ((Fries) Keissler), *Botryosphaeria obtusa* (Schweinitz) and *Phomopsis viticola* ((Reddick) Goidanich). Difolotan and Captan have been used at low concentrations (3-5 µg/ml) to suppress the growth of some fungi (Carter, 1991). At low concentrations, these chemicals have little effect on the growth of *E. lata*. In addition, antibiotics such as oxytetracycline are commonly used to suppress the growth of bacteria (Carter 1991). In general, *E. lata* does not compete well *in vitro* and it may be difficult to confirm its presence by attempting to culture the pathogen from diseased wood.

1.4.3 The advantages of a PCR based pathogen identification system.

Since the first report of specific DNA amplification using the Polymerase Chain Reaction (PCR) in 1985, the number of different applications has grown steadily (Reviewed Saiki, 1989). PCR is the most rapid method for isolating a particular DNA sequence from the genome. A detection method based on a PCR protocol does not necessitate the growth of the fungi on artificial media, thus saving considerable time and money, and the procedure utilises small amounts of relatively crude preparations of total DNA.

One major advantage of a PCR based system is the speed of amplification. In a PCR based detection system for the *Ophiostoma* sap stain fungi, all PCR detection is completed in a period of less than 4 h (Kim, Uzunovic & Breuil, 1999).

The development of DNA-based PCR and taxon specific primers is making it increasingly feasible to detect and study fungi in their natural substrates. This includes the ability to characterise fungal symbionts by amplification of species-specific sequences. Further identification of these symbionts can be achieved by analysis of PCR amplified products using Restriction Fragment Length Polymorphisms (RFLP's), direct sequencing, or oligonucleotide probing techniques.

1.4.4 rDNA genes and their use as molecular markers.

Because of the ease of isolation and their relatively high gene copy number, ribosomal genes have become attractive targets for the construction of molecular probes. Although

the nucleotide sequences of the mature rRNAs are highly conserved, both non-transcribed and transcribed spacer sequences, which often make up approximately half of the rDNA repeating unit, are usually poorly conserved and may contain large sequence differences.

In filamentous fungi, the rDNA unit consists of highly conserved genes interspersed with variable DNA regions. The three largest rDNA genes are clustered and repeated in tandem arrays. Each repeat unit contains a copy of the 18S, 5.8S and 28S rDNA genes, in conserved order, which are separated by variable spacers (Figure 1.3). A fourth (5S) rDNA gene may also be contained within the repeat unit in some cases (Lockington, Taylor, Winther, Scazzocchio & Davies, 1982).

Figure 1.3 The ribosomal DNA unit.
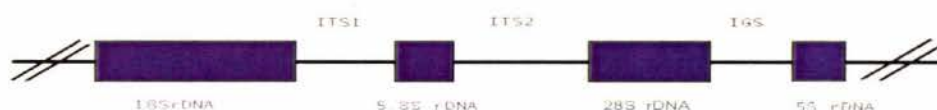
There are two types of spacer regions, the non-transcribed spacer (NTS) or intergenic spacer (IGS) flanks the repeat unit and is not transcribed. The other spacer region, the Internal Transcribed Sequence (ITS), flanks both sides of the 5.8S gene and is transcribed in the ribosomal primary transcript. The ITS spacers lack functional roles which could explain the high levels of sequence variation observed within them. Variation may be observed among species within a genus or among populations (White, Bruns, Lee & Taylor, 1990). However, conservation of the DNA sequence of ITS non-coding regions for correct rRNA folding, or for the processing of primary ribosomal transcripts, may account for the apparent clustering of variable residues (Kasuga, Woods, Woodward & Mitchelson, 1993).

Several features make the ITS region a convenient target for molecular identification of fungi: (i) in fungi, the entire ITS region is often between 600-800bp and can be readily amplified with universal primers that are complementary to sequences within the rRNA genes (White *et al.*, 1990), (ii) the multicopy nature of the rDNA repeat makes the ITS region easy to amplify from small, dilute, or highly degraded DNA samples, and (iii) several studies have demonstrated that the ITS region is often highly variable among morphologically distinct fungal species (Gardes *et al.*, 1991; Gardes & Bruns, 1991).

1.4.5 Detection of fungi using the ITS region.

Amplification of the ITS region by PCR using universal DNA primers specific for the conserved 18S and 28S elements (White *et al.*, 1990) followed by direct sequencing has been successfully used to detect fungal plant pathogens. Since it was first developed, this method has been applied to a number of fungi including *Armillaria* species, an important root pathogen of trees (Harrington & Wingfield, 1995), *Tuber* species and

host plant DNA (Paolocci, Angelini, Cristofari, Granetti & Arcioni, 1995), *Ophiostoma* sap stain fungi in wood (Kim *et al.*, 1999) and *Phaeoacremonium* species, responsible for the development of esca disease and black goo in grapevines (Tegli, Bertelli & Surico, 2000), among others.

The original detection technique has been extended to allow detection of the fungal pathogen from a diverse range of DNA sources including decayed wood (Harrington & Wingfield, 1995; Jasalavich, Ostrofsky & Jellison, 2000; Kim *et al.*, 1999; Tegli *et al.*, 2000), basidiomes (Harrington & Wingfield, 1995) or mycelia (Kim *et al.*, 1999). When universal ITS primers (White *et al.*, 1990) are used to amplify the ITS regions from ectomycorrhizae, both host plant and fungal PCR products are produced. The patterns produced are the sum of the patterns produced by the fungal symbiont and the host plant alone, therefore providing effective discrimination between ectomycorrhizal species, which are otherwise difficult to distinguish on the basis of morphological traits (Paolocci *et al.*, 1995).

In many natural situations, fungal DNA may be rare in comparison to plant host DNA, and thus specific or preferential amplification of fungal DNA would be desirable. Gardes and Bruns (1993) demonstrated the ability of fungal specific primers to amplify only fungal DNA by designing basidiomycete specific primers and demonstrating, from an artificial mixture of DNA, that even when the tree DNA was eleven times more concentrated than the fungal DNA, only the fungal DNA was visibly amplified.

Henson, Goins, Grey, Matthre and Elliot (1993) used PCR to specifically amplify DNA of *Gaeumannomyces graminis*, (Saccardo) a filamentous soil borne fungus that causes crown and root rot of cereals and turf grasses. Nested primers were used to amplify 188 bp fragments of mitochondrial DNA from fungal and infected plant samples, which were simply boiled, to release target DNA. This test used nested primers and two rounds of amplification to increase the specificity of the PCR reaction. In addition, the use of two rounds of amplification dilutes DNA polymerase inhibitors that may be present in plant samples.

1.4.6 Limitations of the ITS region for use in detection.

The use of an ITS size-related test for the detection of a fungus can limit the sensitivity of diagnosis. Some species can have the same length ITS region, for example studies of the ITS regions from rusts (Zambino & Szabo, 1993) and *Alternaria* (Jasalavich, Morales, Pelcher & Seguin-Swartz, 1995) have shown sequence variation at the interspecific level but generally low levels of intraspecific variation within well defined species. In a survey of the plant *Mimulus guttatus* (Wendl.) species complex (Ritalind, Ritalind & Straus, 1993) ITS1 showed significant length differences. Cooke and Duncan (1997) noted, "In *Phytophthora* less variation is evident in ITS2. Variation in sequence ranged from single base pair changes to multiple changes representing deletions and insertions. Most changes were present in several species but some single base pair changes were unique to a particular species". In the *Verticillium* wilt plant pathogens only five nucleotide differences were observed between the ITS sequence of *V. albo-atrum* (Reinke *et* Berthold) and *V. dahliae* (Klebahn). Three changes were found in ITS1 while two were observed in ITS2 (Nazar, Hu, Schmidt, Culham & Robb, 1991). However, as there is not enough ITS length variation to enable size-related detection of all fungi, the design and synthesis of fungal or species-specific primers

enables identification of the fungus on a presence–absence basis. Therefore, sidestepping the use of size as an identification criterion (Ward & Adams, 1998).

1.4.7 Random Amplification of Polymorphic DNA analysis.

An alternative way to detect *E. lata* infections is by using RAPD analysis. RAPD-PCR is a beneficial technique as it is generally faster and less expensive than other methods for detecting DNA sequence variation. However, RAPDs have a number of limitations, including poor reproducibility between laboratories (Davin-Regli, Abed, Charrel, Bollet & de Micco, 1995), a high level of intraspecific variability and the existence of primer derived non-specific amplification products in negative control reactions (Lanhham, Fennell, Moss, & Powell, 1992; Tingey, Ralalski, & Williams, 1992; Tingey and del Tufo, 1993; Williams *et al.*, 1990). Although RAPD analysis is an informative technique when studying genetic variation, it is not the most reliable method.

A modification of the RAPD PCR technique is microsatellite-based PCR finger printing. Microsatellites are a class of repetitive DNA that is a ubiquitous component of eukaryotic genomes. Microsatellites are composed of very short DNA motifs (1 to 10 nucleotides) that are found in tandem repeats. The number of tandem repeat units has been shown to be highly polymorphic between individuals and this is thought to be due to slippage of the DNA polymerase during the synthesis and repair of DNA. The variation in the number of tandem repeats can be detected by PCR with primers designed for the conserved DNA sequences flanking the locus. A multilocus microsatellite-based PCR finger printing assay has been successfully developed for the identification of Epichloë endophytes, both in culture and *in planta* (Moon, Tapper & Scott, 1999). However, this technique is also limited due to the high amount of intraspecific variation in some species (Ganley, 2000).

1.5 The mating system of *Eutypa lata*.

1.5.1 Mating types.

Mating type genes regulate sexual compatibility and sexual reproduction in fungi. Characterisation of the determinants of mating type in ascomycete mating systems have revealed many conserved components, such as gene regulatory polypeptides and pheromone/receptor signal transduction cascades, as well as conserved processes, like "self versus nonself" recognition and controlled nuclear migration (Kronstad & Staben, 1997; Nelson, 1996; Shiu & Glass, 2000). However, the components' structures and their genetic arrangements in the mating system vary greatly in different fungi. An understanding of mating type is important given the central role of sexual reproduction in the life cycle of *E. lata* and the critical influence of sex on population genetics.

Ascomycete fungi, such as *E. lata*, may self-fertilise, termed homothallism, or cross-fertilise, termed heterothallism. The lack of gametic disequilibrium between random amplified polymorphic DNA (RAPD) markers in populations from single vineyards (Péros et al, 1997; Péros & Larignon, 1998) suggests that *E. lata* is outcrossing.

All known heterothallic ascomycetes have a single-locus, two allele mating system. Therefore, the mating type limits sexual reproduction to crosses between strains of opposite specificity. In heterothallic ascomycetes, mating type controls initial fusion of the thalli (plasmogamy) and in some ascomycetes, the subsequent formation of

dikaryotic ascogenous hyphae necessary to form zygotes *eg. Neurospora crassa* (Shear & Dodge) (Nelson, 1996). In addition, some mating type genes have associated functions, such as vegetative incompatibility in *N. crassa*, that are not directly related to their sexual compatibility roles (Kronstad & Staben, 1997). Although *MAT* genes themselves may not be directly involved in pathogenesis, an understanding of mating may shed light on mechanisms of pathogenesis since both mating and disease development depend on cell-cell recognition for initiation of the process.

1.5.2 Mating type loci of filamentous ascomycetes.

The mating type loci (*MAT*) of filamentous fungi contain alternative DNA sequences termed idiomorphs to indicate that they may contain multiple genes, map to the same position on homologous chromosomes and that the genes of alternate mating types bear no obvious allelic relationship to one another.

Although the evolutionary origins of the dissimilar *MAT* idiomorphs of ascomycetes are unknown, it has been suggested the small pockets of identity in the *MAT* idiomorphs reflect a common ancestry (Shiu & Glass, 2001; Yun, Berbee, Yoder & Turgeon, 1999). These pockets may be remnants of a series of mutagenic events in a single ancestral gene. The mutations, coupled with recombination suppression, might have led to the highly divergent *MAT* genes that now encode different protein products. A similar process has been suggested for the evolution of the male Y chromosome (Rice, 1994).

The three best-characterised *MAT* loci from filamentous ascomycetes are those from *N. crassa*, *Podospora anserina* ((Cesati) Niessl.) and *Cochliobolus heterostrophus* (Drechsler). Gene replacement experiments in *N. crassa* (Chang & Staben, 1994), *P. anserina* (Picard, Debuchy & Coppin, 1991) and *C. heterostrophus* (Wirsel, Turgeon, & Yoder, 1996) suggest that the idiomorphic regions are necessary and sufficient to specify mating type in each system.

The *N. crassa* idiomorphs (*Mat a/Mat A*) were the first to be fully characterised (Glass, Vollmer, Staben, Grotelueschen, Metzenberg & Yanofsky, 1988). The 5.3 Kb *Mat A* idiomorph includes three mating type genes (*Mat A-1, Mat A-2, Mat A-3*) (Ferreira *et al.*, 1996). The *Mat a* idiomorph is 3.2 Kb with two transcription units, *Mat a-1* (Chang & Staben, 1994) and *Mat a-2* (Pöggeler & Kück, 2000). The *N. crassa* idiomorphs are flanked by DNA sequences common to both mating types. Similarly, the *P. anserina* *mat+* idiomorph has a single gene (*FPR1*) homologous to the *Mat a-1*. The *mat-* idiomorph encodes three genes (*FMR1, SMR1* and *SMR2*) similar to *Mat A-1, Mat A-2* and *Mat A-3*. Each idiomorph is flanked by a common DNA sequence. (Debuchy & Coppin, 1992; Debuchy, Arnaise & Lecellier, 1993).

C. heterostrophus is a typical loculoascomycete (as opposed to a pyrenomycete such as *P. anserina* and *N. crassa*) because the *MAT* idiomorphs each contain only a single gene. One, *MAT1-1*, containing a homologue of the *Saccharomyces cerevisiae Mat1α*, the second, *MAT1-2*, being a homologue of the *N. crassa Mat a-1* (Turgeon, Bohlmann, Ciuffetti, Christiansen, Yang, Schäfer & Yoder, 1993).

Comparisons between heterothallic and homothallic *Cochliobolus* revealed that the *MAT* organization is the same in all heterothallic species. Each *MAT* idiomorph carries one gene encoding a single *MAT*-specific DNA binding protein. In *MAT1-1* isolates it is

a protein with an alpha box motif, in *MAT1-2* it is a protein with a HMG motif. In contrast, each homothallic species carries both *MAT1-1* and *MAT1-2* genes, usually closely linked or fused. The organization of each homothallic *MAT* gene is unique to each homothallic species (Yun *et al.*, 1999). Structural analyses of these *MAT* sequences from homothallic and heterothallic *Cochliobolus* species suggest that homothallism is derived from heterothallism and the change in mating lifestyle is driven by a recombination event between short islands, as little as 8 bp, of identity within the *MAT* idiomorphs (Yun *et al.*, 1999). This hypothesis is confirmed by similar work in *Neurospora* and *Sordaria* (Pöggeler, 1999). Strong inter-relationships were found among homothallic and heterothallic members of both genera, indicating that in each genus a change from one reproductive strategy to another might result from one single event.

Similarly, mating type genes were cloned from three members of the *Gibberella*/*Fusarium* complex that differed in reproductive mode: heterothallic *Gibberella fujikuroi* ((Sawada) Ito.), homothallic *Gibberella zeae* and asexual *Fusarium oxysporum* (Schlechtendahl) (Yun, Arie, Kaneko, Yoder & Turgeon, 2000). The *G. fujikuroi MAT* locus was found to be typical of all other heterothallic pyrenomycetes characterised. *G. zeae* was found to have homologues of all four genes encoded by the two *G. fujikuroi MAT* idiomorphs, tightly linked on the same chromosome interspersed with sequences unique to *G. zeae*. Interestingly, field isolates of *F. oxysporum* (asexual) were found to have either the *MAT1-1* or the *MAT1-2* genes found in both sexual species. The data showed that *F. oxysporum* has *MAT* genes that are structurally indistinguishable from homologues in the heterothallic *G. fujikuroi* and that the genes were expressed in *F. oxysporum* itself. A recent report that an asexual ascomycete human pathogen, *Candida albicans*, with no known sexual cycle, also has mating type genes (Hull & Johnson, 1999) is consistent with a hypothesis that asexual fungi arise from sexual species.

The sequences of the genes within the *MAT* idiomorphs and the initial biochemical characterisation of the gene products suggest that the idiomorph products are gene regulatory polypeptides. The genes regulated by the *MAT* proteins and the interactions of the proteins with each other or with other cofactors are as yet unknown. The *MAT a-1* gene product and homologues contain a high mobility group (HMG) box domain and bind DNA *in vitro* (Philley & Staben, 1994). For uniformity, the idiomorph containing the HMG box is designated *MAT1-2* and the opposite idiomorph is designated *MAT1-1* (Kerényi, Zeller, Hornok & Leslie, 1999; Turgeon & Yoder, 2000).

1.5.3 Cloning of Mating Type genes.

Cloning of *MAT* genes from ascomycetes has been hampered by low conservation among the *MAT* proteins of different genera. Previously, cloning of the *MAT* genes required laborious strategies including chromosome walking for *N. crassa* (Glass *et al.*, 1988), complementation for *C. heterostrophus* (Turgeon *et al.*, 1993) and genomic subtraction for *Magnaporthe grisea* ((Herbert) Barr.) (Kang, Chumley & Valent, 1994). *MAT* gene sequences appear to evolve rapidly and the technique of heterologous hybridisation is largely unsuccessful because of the low level of sequence conservation. However, the *P. anserina FMR1* gene was cloned via heterologous hybridisation to it's close relative the *N. crassa MAT A-1 gene* (Picard *et al.*, 1991) and heterologous probing of cosmid libraries with *N. crassa* probes found the entire *Sordaria macrospora*

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mating type locus (Pöggeler, 1997).

Recently, PCR primers for specific amplification of the HMG region of the *MAT1-2* idiomorphs of both pyrenomycetes and loculoascomycetes have been designed (Arie, Christiansen, Yoder & Turgeon, 1997). The observation that sufficient conservation is found at the borders of the HMG DNA binding domain of the *C. heterostrophus MAT1-2* idiomorph and its homologues in other fungi, enabled the PCR primer design. These primers have led to the cloning of a large number of new *MAT* genes from a variety of ascomycete genera, including: *Cryphonectria* spp. (Arie *et al.*, 1997; Marra & Milgroom, 1999), *Gaeumannomyces* spp., *Mycosphaerella* spp., *Nectria* spp., *Pyrenophora* spp. and *Setosphaeria* spp. (Arie *et al.*, 1997), *Gibberella circinata* (Nirenberg et O'Donnell) (Covert, Briley, Wallace & McKinney, 1999), *Gibberella fujikuroi* (Kerényi *et al.*, 1999), a number of *Cochliobolus* spp. (Yun, Berbee, Yoder & Turgeon, 1999), *Sordaria* spp. (Pöggeler, 1999), *Neurospora* spp. (Pöggeler, 1999), *Ceratocystis* spp. (Witthuhn, Harrington, Wingfield, Steimel & Wingfield, 2000), *Fusarium oxysporum* (Arie, Kaneko, Yoshida, Noguchi, Nomura & Yamaguchi, 2000) and *Alternaria alternata* ((Fries) Keissler) (Arie *et al.*, 2000).

While most of the ascomycete mating systems currently determined have a single-locus, two idiomorph mating system there are already emerging exceptions to this generality, for example, *Glomerella graminicola* and its close relative *Glomerella cingulata* (Vaillancourt, Du, Wang, Rollins, Hanau, 2000). *Glomerella cingulata* has an unusual and complex mating system that is controlled by multiple, multiallelic loci. Cross fertility between different isolates occur via complementation of mutated fertility genes, a process known as unbalanced heterothallism. Although phenotypically heterothallic, they are clearly not analogous to other ascomycetes in which mating compatibility is regulated by mating type idiomorphs.

The traditional approach to determining mating type of any heterothallic fungal strain has been to attempt to cross it with each of two tester strains that are already known to differ at the *MAT* locus. The mating type of the individual being tested is thus the opposite of that with which it crosses successfully (i.e., produces ascospores). However, the ability to clone *MAT* genes has enabled mating type to be assigned on the basis of a PCR assay, initially relying on the amplification of the *MAT1-2* idiomorph. Because these tests do not assay the *MAT1-1* idiomorph directly, this approach assigns mating type unambiguously only when applied to established tester strains. This is because currently, the absence of the *MAT1-2* PCR product cannot be distinguished from a failed PCR reaction. In independent blind tests of the diagnostic ability of species specific *MAT1-2* PCR primers, Kerényi *et al.* (1999) found the results of PCR amplification of the *MAT1-2* idiomorph were identical to the results of sexual crosses with standard strains. Thus, the PCR results were predictive of the crossing results and the crossing results were predictive of the PCR results.

Recently, molecular tests of mating type have been developed to assay both idiomorphs concurrently in a multi-plex PCR (Steenkamp, Wingfield, Coutinho, Zeller, Wingfield, Marasas & Leslie, 2000; Wallace & Covert, 2000). Although advances in cloning of *MAT* loci has provided us with insight into the nature of fungal mating identity, much is still unclear about how mating-type polypeptides control complex programs of recognition, cellular specialisation, structural formation and cell-type determination.

1.6 Aims.

A major problem confronting pathologists and viticulturists is that of positive identification of the pathogen because the teleomorph of the pathogen has never yet been induced to form *in vitro*. Currently, the identification of cultures depends solely upon the recognition of the anamorph. This is a slow and laborious procedure, due to variability in the morphology and growth rates of *E. lata*. A new survey tool is needed to enable quick and accurate identification of the fungus, allowing the viticulturist to implement appropriate containment procedures.

In addition to addressing the problem of identification of *E. lata*, the project will also endeavour to reveal something of the epidemiology of the pathogen. Since the sexual stage of *E. lata* cannot be obtained in culture at present, the analysis of its mating system must be performed in natural populations. Molecular identification of the mating types of *E. lata* will determine how the mating types are distributed in the field, and identify compatible mating pairs for mating experiments. The ability to identify compatible pairs for mating experiments is especially valuable when first attempting to carry out sexual crosses in a species because it drastically reduces the number of crosses that must be set up. If working without any mating type characterisation, all potential members of a new mating population must be intercrossed, and thus the number of crosses that must be established increases as the square of the number of isolates being tested (Covert *et al.*, 1999). Therefore, molecular characterisation of the mating type at the outset of a mating project allows significant savings in time and effort.

- *Aim 1- To develop a PCR based test for the detection of* E. lata *within grapevine wood.*

The aim of this project is to develop a reliable technique for confirming the presence of *E. lata* in culture and grapevine wood. Initially, culture indexing of *E. lata* infected grapevines will be used to build up a picture of Eutypa dieback infections and other saprophytic fungi that are associated with the pathogen and decaying grapevines. This will be followed by the development of a molecular probe to the ITS region of *E. lata*. If this is unsuccessful, a robust fingerprinting method for *E. lata* will be developed using RAPD or microsatellite markers.

- *Aim 2- To develop a PCR assay to enable the identification of the mating type idiomorphs of* E. lata.

The aim of this project is to develop a tool to be used to assess the mating habits of the fungus in the natural environment. A PCR based approach (Arie *et al.*, 1997) will be used to isolate and clone the *MAT1-2* idiomorph of the *E. lata* mating type gene. These results could then be used to isolate and clone the *MAT1-1* idiomorph of the gene and develop a DNA based approach to characterise the different mating type.

2. MATERIALS AND METHODS.

2.1 Fungal strains, bacterial strains and plasmids.

The bacterial strains, fungal strains and plasmids used in this study are listed in Table 2.1.

Table 2.1 Fungal strains, bacterial strains and plasmids.

Strains and Plasmids.	Characteristics.	Source or reference.
Fungal strains.		
Botryosphaeria sp. Bot01.	Wild type New Zealand isolate.	Ex. W.F. Hartill.
Dothistroma pini NZE1.	Wild type New Zealand isolate.	Bradshaw, Ganley, Jones & Dyer (2000).
Eutypa lata E11787.	Wild type New Zealand isolate.	Eskdale, Hawkes Bay, 1993.
Eutypa lata E11788.	Wild type New Zealand isolate.	Eskdale, Hawkes Bay, 1993.
Eutypa lata EL01.	Wild type New Zealand isolate.	Ex W.F. Hartill.
Neurospora crassa 2961.	eas, Mat-a (*MAT1-2*).	Fungal Genetics Stock Centre, U.S.A.
Neurospora crassa 987.	Wild type isolate, *Mat-A* (*MAT1-1*).	Fungal Genetics Stock Centre, U.S.A.
Bacterial strain.		
Escherichia coli XL1-BLUE.	*SupE44, hsdR17, recA1, endA1, gyrA46, thi, relA1, lac', F' [proAB', lacZΔM15, Tn10 (tet')]*	Bullock, Fernandez & Short (1987).
Plasmids.		
PGEM®-T easy	*Amp', lacZ'*. (3.0 Kb).	Promega.
PGEM®-T	*Amp', lacZ'*. (3.0 Kb).	Promega.
PUC118	*Amp', lacZ'*. (3.2 Kb).	Messing (1987).

2.1.1 Infected grapevine samples.

Grapevine cordons, with symptoms of Eutypa dieback, to be used for isolation of fungal cultures (Section 2.3.1.5), were removed from the following vineyards on the 18th November 1998.

Vineyard 1
Der Ley Vineyard.
Mr Campbell.
Lawn Road, Havelock North, New Zealand.

Sauvignon Blanc, 8 years old.

Vineyard 2
Erindale Vineyard Partnership.
Mr Robin Sage.
Mt Erin Road, Havelock North, New Zealand.
Cabernet Sauvignon, 20 years old.
Cabernet Franc, 15 years old.

2.2 Media.

All media were prepared with milli-Q purified water and were sterilised by autoclaving at 15 p.s.i. (121°C) for 15 min. Liquid media were then cooled to room temperature before the addition of supplements. Solid media were cooled to 50°C before the addition of supplements and pouring. All uninoculated plates were stored at 4°C.

2.2.1 Luria-Bertani (LB) Media.

Luria-Bertani media contained (g/L): Tryptone (Difco), 10.0; NaCl, 5.0; yeast extract (Oxoid) 5.0. The ingredients were dissolved and the pH adjusted to 7.4 prior to autoclaving. For solid medium, agar (Davis) was added at the rate of 15.0 g/L. When required LB was supplemented after autoclaving to give final concentrations of: ampicillin, 100 μg/mL; tetracycline, 10 μg/mL; isopropylthio-β-D-galactoside (IPTG), 30 μg/mL; 3-indolyl-β-D-galactoside (X-gal) 60 μg/mL.

2.2.2 Potato Dextrose Agar (PDA).

Potato dextrose agar contained 39 g/L of Difco PDA dissolved in milli-Q water. For fungal isolations from wood the PDA agar was cooled to 50°C and then supplemented with oxytetracycline (5 mg/L) to suppress bacterial growth.

2.2.3 Malt Agar (MA).

Malt agar contained 15 g/L of agar powder (Davis) and 20 g/L of liquid malt extract (Maltexo). The medium was heated in a microwave on high power for 2 min to dissolve the Maltexo to prevent caramelisation in the flask, prior to autoclaving.

2.2.4 15% V8 Agar.

15%V8 agar contained 15 g of agar (Davis) and 150 mL of V8 vegetable juice and was made up to 1 L with water.

2.2.5 Water Agar (H_2O Agar).

Water agar contained 15 g of agar (Davis) and 1L of tap water.

2.2.6 Corn Meal Agar (CMA).

Corn meal agar contained 17 g/L of Corn meal agar (Difco). The agar was cooled to 50°C and supplemented with oxytetracycline (10 mg/L).

2.3 Growth and Maintenance of cultures.

2.3.1 Fungal cultures.

2.3.1.1 Subculturing.

Fungal cultures were sub-cultured by removing an 8 mm diameter plug of mycelium and agar from a culture, with a minimum of 5 days growth, and placing the plug on a new sterile agar plate. The culture was grown for 5 days at 20°C before storage at 4°C for up to 6 months. All fungal isolates used in this study were subcultured approximately once every two and a half months.

2.3.1.2 Long-term storage of cultures.

Some cultures were stored long term as silica gel stocks. The first step was to prepare spore suspensions in a 5% solution of non-fat milk powder. These were then poured into a cooled glass jar 2/3 full of silica gel (previously dried at 180°C for 90 min) and left to stand on ice for 15 min. The glass jars were placed at room temperature until the crystals were readily separated when shaken (~1 week). The silica gel stocks were then stored at 4°C. Silica gel stocks were reactivated, when necessary, by sprinkling a few crystals over PDA agar plates and incubating in the dark at 20°C for 10-14 days.

All fungal cultures used in this study were also stored by placing several 8 mm plugs of 5 day old PDA cultures into 5 mL sterile milli-Q water aliquots, for up to 2 years. These stocks were reactivated, when necessary, by aseptically removing a single mycelial plug and placing on a fresh PDA plate. These cultures were grown at 20°C for 10-14 days, in the dark.

2.3.1.3 Collection of fungal mycelium in preparation for DNA extraction.

To extract DNA from fungal mycelium, an 8 mm cube of mycelium was ground in 500 μl of milli-Q purified water in a 1.5 mL microcentrifuge tube using a sterile plastic grinder. A 200 μl aliquot of this crude mycelial suspension was then plated onto PDA plates overlaid with cellophane discs. The cultures were grown in the dark at 20°C for 10-14 days. Mycelium from the cultures was then collected into pre-weighed 1.5 mL microcentrifuge tubes and freeze dried overnight. The freeze-dried mycelia could then be stored at -20°C for up to 6 months before extraction of DNA.

2.3.1.4 Preparation of Single Spore Isolate (SSI) cultures.

2.3.1.4.1 *Eutypa lata* cultures.

E. lata SSI cultures were prepared by removing a single perithecium from a piece of stroma found on an infected grapevine and mascerating this with a sterile scalpel blade on a glass slide. Several drops of sterile water (~30 μL) were then added to the slide. The spore suspension was removed from the slide with a sterile pipette, leaving behind the pieces of mascerated stromal tissue, and the spores were further diluted in another 5 mL of sterile milli-Q water. A sample of the suspension was stained with cotton blue (Section 2.4.15) and checked under the microscope to confirm the presence of *E. lata* ascospores. The suspension was quantified using a haemocytometer and diluted again to obtain a suspension of approximately 80 spores/mL. An aliquot of the suspension (0.25 mL) was spread onto fresh PDA (Section 2.2.2, supplemented with oxytetracycline),

CMA (Section 2.2.6, supplemented with oxytetracycline) and H_2O agar (Section 2.2.5) medium and left to germinate, under cycled fluorescent light (12/12 h light/dark), overnight at 25°C. Alternatively, unsupplemented PDA and CMA agar was used for germination and oxytetracycline (final concentration 10 mg/L) was used to dilute the suspension to 80 spores/mL. Single germinated ascospores were then removed using a sterile hypodermic needle, placed onto fresh PDA medium, and left to grow at 25°C under cycled fluorescent light (12/12 h light/dark) for 10 days.

2.3.1.4.2 *Botryosphaeria obtusa* cultures.

Single spore isolates of 5 *Botryosphaeria obtusa* cultures isolated from the infected grapevine pieces sampled (Section 3.3) were made by placing 30 µL of sterile water onto a clean microscope slide and crushing a single pycnidium in the water using the blunt end of a sterile loop. The suspension was checked under a microscope to confirm the identity as *Botryosphaeria obtusa* and a single loopful was then streaked across a 2% water agar plate (Section 2.2.5) and left to germinate for 3 h at room temperature. After germination, the plates were examined under a stereomicroscope and single germinated spores were removed aseptically using a sterile hypodermic needle, replaced onto sterile PDA agar and incubated at 22°C for 10 days.

2.3.1.5 *Isolation of fungal cultures from infected grapevine pieces.*

Grapevine cordons suspected to be infected by *E. lata* were collected from Erindale and Der Ley vineyards on the 18 November 1998. The cordons were stored at approximately 4°C for 2 months over the summer before being sliced into smaller pieces using a chain saw. Each section was then cut longitudinally and examined for the characteristic brown lesions. A diagrammatic sketch of each cordon was drawn to try and determine the origin of each lesion. The information on the origin and direction of spread of the infection was used to assess the best places to sample and the variation within a disease lesion. After surface sterilisation by rapid flaming, three pieces of necrotic wood, 5 mm³ in size were taken from close to the margin of the lesion using a sterile scalpel blade (number 22). One piece was placed onto each of MA medium, PDA medium and 15% V8 agar medium and incubated at 25°C for 5 days. Plates were assessed visually for the presence of typical mycelia of *E. lata* and the results for each lesion were catalogued.

2.3.1.6 *Artificial inoculation of grapevine wood for PCR testing.*

Slices of grapevine wood (1 cm thick) were artificially inoculated with *E. lata* by applying several 8 mm agar plugs of mycelium face down on the cut surface of each slice of wood. This was incubated at 22°C for 12 months with periodic wetting of the wood surface to simulate rain.

The wood was infected with either a sample of strain E11787 or E11788; however, in some samples both E11787 and E11788 were used for infection to observe any interaction between the two strains.

The artificially inoculated grapevine wood was obtained from Miljana Vlazich and Dr. Peter Long (Institute of Natural Resources, Massey University). Portions of this wood were used for PCR amplification of the *E. lata* ITS region direct from infected wood samples (Section 2.13.3).

2.3.2 Bacterial cultures.

E. coli cultures were grown at 37°C overnight on LB agar plates, or in LB broth, with the appropriate selection supplements (Section 1.2.1). Plates were sealed with parafilm and stored at 4°C. All cultures were regularly subcultured by streaking onto fresh LB agar plates.

2.4 Common buffers and solutions.

All solutions were prepared with milli-Q water and sterilised by autoclaving at 121°C, 15 p.s.i. for 15 min and stored at room temperature, unless otherwise stated.

2.4.1 TE buffer.

TE buffer contained Tris-HCl at a final concentration of 10 mM and 1 mM Na_2EDTA (TE 10:1).

2.4.2 1x TAE Buffer.

TAE buffer contained Tris-HCl at a final concentration of 40 mM, with 2 mM Na_2EDTA and 20 mM acetic acid.

2.4.3 1x TBE buffer.

TBE buffer contained Tris-HCl at a final concentration of 89 mM, with 2.5 mM Na_2EDTA and 89 mM boric acid.

2.4.4 10x TNE buffer.

TNE buffer contained Tris, at a final concentration of 121 g/L, with 3.7 g/L Na_2EDTA and 58.4 g/L NaCl. This solution was filter purified.

2.4.5 20x SSC solution.

20x SSC solution contained NaCl at a final concentration of 3 M and 0.2 M tri-sodium citrate. 2x SSC was prepared by diluting 20x SSC with sterile milli-Q water.

2.4.6 Ethidium Bromide solution.

The ethidium bromide solution used for staining of agarose electrophoresis gels was prepared at a rate of 1 μL of a 10 mg/mL stock to 10 mL of milli-Q water to give a final concentration of 1 μg/mL.

2.4.7 RNase A (DNase free).

RNase A solution contained 10 mg/mL RNase A (Sigma) in 10 mM Tris-HCl (pH 7.5), 15 mM NaCl. The solution was boiled at 100°C for 15 min, and then allowed to cool slowly before being dispensed into aliquots and stored at -20°C.

2.4.8 10x Gel Loading Buffer

Gel loading buffer contained urea, glycerol, tris acetate, bromophenol blue and xylene cyanol at the following final concentrations: 2 M urea, 50% (v/v) glycerol, 50 mM Tris acetate, 0.4% (w/v) bromophenol blue and 0.4% (w/v) xylene cyanol. This buffer was stored at 4°C when in use, or -20°C long term.

2.4.9 Al-Samarrai DNA extraction lysis buffer.

The Al-Samarrai DNA extraction lysis buffer contained Tris-acetate at a final concentration of 40 mM (pH 7.8), with 20 mM Na$_2$EDTA and 1% (w/v) SDS.

2.4.10 Prehybridisation solution.

Prehybridisation solution contained the following: 3x SSC (Section 2.4.4), 0.2% (v/v) Denhardt's solution (Section 2.4.8), 0.5% (v/v) SDS and 2.5% (v/v) Blocking solution (Section 2.4.9). The solution was not autoclaved and was stored at 4°C for up to 4 months.

2.4.11 50x Denhardt's solution.

Denhardt's solution contained ficoll at a final concentration of 10g/L, 10 g/L polyvinylpyrolidone, 10 g/L bovine serum albumin. This solution was filter sterilised.

2.4.12 Blocking solution.

Blocking solution contained a final concentration of 5% (w/v) non-fat skim milk powder. This solution was autoclaved for 10 min in a pressure cooker and stored at 4°C for up to 3 weeks.

2.4.13 Southern Wash solution.

Southern Wash solution contained SSC (Section 2.4.4) at a final concentration of 3x and 0.2% (v/v) SDS. This solution was not autoclaved.

2.4.14 Membrane Stripping solution.

Membrane Stripping solution contained a final concentration of 0.2 M NaOH and 0.1% (v/v) SDS. This solution was not autoclaved.

2.4.15 Cotton blue dye.

Cotton blue dye contained 67.0 mL of anhydrous lactophenol, 20.0 mL of milli-Q water and 0.1 g of cotton blue.

2.4.16 Hoescht's fluorescent dye solution.

Hoescht's fluorescent dye solution contained 1x TNE (Section 2.4.4) and a final concentration of 0.1 µg/mL Hoeschst 33258 dye. This solution was not sterilised, it was necessary for this solution to be prepared fresh when needed.

2.5 DNA Isolation

2.5.1 DNA isolation from fungal cultures (Nucleon Phytopure™ kit).

The Nucleon Phytopure™ plant DNA extraction kit (Amersham) was used to isolate DNA from fungal cultures. The protocol was modified from the manufacturers instructions, by adding an extra phenol/chloroform extraction.

Freeze dried mycelium was ground in a mortar and pestle with liquid nitrogen. Lysis buffer, containing potassium and SDS, was added to lyse the cells. An RNase digestion to produce RNA-free DNA was done by adding RNase (Section 2.4.7) to a final concentration of 20 µg/mL and incubating at 37°C for 30 min. Chloroform and a resin were added. The resin complexes polysaccharides common in fungal extracts and forms

a physical barrier between the aqueous and chloroform phases. After the removal of the aqueous phase, an additional phenol/chloroform extraction (Section 2.6.1) was used to remove remaining protein before isopropanol precipitation. 70% ethanol was used to wash the DNA pellet before resuspension in TE buffer (10:1) (Section 2.4.1).

2.5.2 DNA isolation from fungal cultures (Al-Samarrai & Schmid, 2000).

This protocol for the extraction of high molecular weight DNA was based on the procedure of T. Al-Samarrai and J. Schmid (Al-Samarrai & Schmid, 2000). The preparation was simple, rapid and produced pure, easily digestible DNA.

First, 30 mg of freeze dried mycelium was ground to a fine powder with liquid nitrogen using a sterile mortar and pestle. The powder was then transferred to a clean 1.5 mL microcentrifuge tube using a dry, sterile spatula, was resuspended in 500 μl of lysis buffer (Section 1.4.9) and mixed by vigorous pipetting. Two μl of RNase A (10 mg/mL) (Section 2.4.7) was added, the mixture left to incubate at 37°C for 10 min. Then 164 μL of 5M NaCl was added to precipitate the cellular debris, mixed by inversion and then centrifuged at 4°C, 13 000 rpm for 20 min, in a microcentrifuge. The DNA was extracted in one volume of chloroform, centrifuged at 13 000 rpm for 6 min, precipitated in 2 volumes of cold (-20°C) 95% ethanol and centrifuged at 13 000 rpm for 5 min. After washing thee times in 500 μL of 70% ethanol and air drying the pellet was resuspended in 50 μL TE buffer (Section 1.4.1).

2.5.3 Qiagen® high quality column purification of plasmid DNA.

The QIAprep™ spin miniprep kit (Qiagen®) was used to isolate and purify up to 20 μg of high quality plasmid DNA to be used for automated sequencing. It was a modified protocol of Sambrook, Fritsch & Maniatis (1989) based on alkaline lysis of the bacterial cells followed by adsorption of the plasmid DNA to a silica gel membrane under high salt. After washing, the plasmid was resuspended in 50 μL of buffer EB as described in the protocol.

2.6 Purification of DNA.

2.6.1 Ethanol precipitation.

This method was based on that of Sambrook *et al.* (1989). One volume of DNA solution was added to 0.1 volumes of 3M sodium acetate (NaAc), and either 2.5 volumes of 95% ethanol or 0.6 volumes of isopropanol was added and mixed gently. For maximum recovery of DNA, the solution was incubated at -20°C for a minimum of 2 h to precipitate the DNA. The DNA was then pelleted by centrifugation for 30 min at 8000 x g at 4°C. The pellet was washed twice in 70% ethanol to remove salts, the ethanol removed with a sterile pipette and the pellet air dried before resuspension of the DNA in either TE buffer (Section 2.4.1) or milli-Q water.

2.6.2 Phenol/chloroform extraction.

This method was based on that of Sambrook *et al.* (1989). One volume of Tris-equilibrated phenol: chloroform (1:1 v/v) was added to one volume of aqueous DNA solution, mixed thoroughly and centrifuged at 15 000 x g for 10 min. The aqueous phase was re-extracted, as above, until a clear interface between the two phases was obtained.

Samples were then extracted once with 2 volumes of chloroform and the DNA precipitated (Section 2.6.2).

2.6.3 Qiagen® agarose gel purification of DNA fragments.

DNA was electrophoresed (Section 2.9.1) on a low melting point (Seaplaque) agarose gel made with 1x TAE buffer (Section 2.4.2). After staining, 30 min in ethidium bromide solution (Section 2.4.6), the band(s) of interest were excised from the gel under long wave UV using a sterile scalpel blade (number 9). The DNA was extracted from the agarose fragment using the Qiagen® QIAquick™ gel extraction kit, according to the protocol supplied by the manufacturer. It was eluted in 50 μL of buffer EB (as provided by the manufacturer) or in milli-Q water.

2.6.4 Qiagen® purification of DNA from a PCR reaction.

The QIAquick™ PCR purification Kit (Qiagen®) purifies PCR products from primers, nucleotides, polymerases and salts. The kit was used for direct purification of double- or single-stranded PCR products, 100 bp-10 Kb in size. A maximum of 50-100 μL of total PCR reaction (Section 2.13) was purified on a single column according to the manufacturers protocol and eluted in 30-50 μL of EB buffer (as supplied) or in milli-Q water.

2.7 Determination of DNA concentration.

2.7.1 Determination of DNA concentration by spectrophotometric assay.

Spectrophotometric quantification was used mostly for pure DNA samples of high concentration. Each sample was diluted appropriately and the absorbance measured at both 260 nm and 280 nm. The DNA concentration was calculated on the assumption that an absorbance of 1.0 at 260 nm is equivalent to 50 μg/mL. Purity was indicated by the 260/280 nm ratio; pure DNA has a ratio of 1.8.

2.7.2 Determination of DNA concentration by fluorometric assay.

DNA was quantified on a Hoefer Scientific TKO 100 Fluorometer according to the manufacturers protocol. The scale of the fluorometer was set to 100 using 2 μL of calf thymus DNA (100 ng/mL) added to 2 mL of Hoescht's fluorescent dye solution (Section 2.4.16). Once the scale was reliably set, 2 μL of sample DNA was added to 2 mL of the dye solution, and the resulting value recorded as the concentration of the DNA in ng/μL. Each sample was measured in triplicate.

2.7.3 Determination of DNA concentration by gel electrophoresis.

The concentration of DNA samples was determined by electrophoresing DNA samples on an agarose gel (usually 0.7-1.0% agarose) alongside a series of Lambda (λ) DNA or pUC118 DNA concentration standards. The concentration of the fragment of interest was estimated by comparing the intensity of the ethidium bromide fluorescence to that of the known DNA concentration standards.

2.8 Restriction endonuclease digestion of DNA.

2.8.1 Restriction endonuclease digestion of genomic DNA.

A total of 3-10 μg of genomic DNA was digested in a 30-60 μL volume. The commercial buffer, supplied with the enzyme, was used at a final concentration of 1x. The restriction enzyme was used at a concentration of 5 U/10 μL total reaction volume. In addition, 100 μg/mL acetylated BSA (Promega) and 500 μg/mL DNase free RNase (Section 2.4.7) were added. Digestions were performed overnight at 37°C. An aliquot of digest was then checked on a mini gel (Section 2.9.1) to ensure that the digestion was complete. If the process was incomplete, more enzyme was added and the reaction incubated further at 37°C until completion.

2.8.2 Restriction endonuclease digestion of plasmid DNA.

Plasmid DNA (200-500 ng) was typically digested in a total volume of 25 μL. Commercial buffer (1x) (matched to the restriction enzyme(s) used) and 2.5 U of enzyme were added. Digestion was performed for 1 h at 37°C and then checked for completion on a mini gel (Section 2.9.1).

2.9 Agarose gel electrophoresis.

2.9.1 Mini gels.

DNA fragments were size fractionated by electrophoresis though 0.7-2.5% (w/v) agarose gels, of various sizes, dissolved in 1x TAE (Section 2.4.2) or 1x TBE (Section 2.4.3) buffer at 5 V/cm. Gel loading buffer, (10x: Section 2.4.8) was diluted with milli-Q water and DNA sample to a final 1x concentration before loading the sample. After electrophoresis, the agarose gel was stained in ethidium bromide solution (Section 2.4.6) for 10-20 min before briefly destaining in milli-Q water. The DNA fragments were observed under short wave UV light on a transilluminator and photographed using the IS-1000 Digital Imaging System.

2.9.2 Overnight gels.

Agarose was dissolved in 1x TAE buffer (Section 2.4.2) to give a 0.8% (w/v) gel. This was cooled to 50°C in a water bath and then poured into a Horizon 11.14 (140 x 110 x 8 mm) gel apparatus. Once set the gel was covered in 1x TAE buffer. Loading dye (10x: Section 2.4.8) was added to the sample to give a final concentration of 1x and this mixture was loaded onto the gel. The DNA sample was run quickly out of the well and into the gel at 120 V for 10 min. The DNA was then separated slowly at 30 V for 16-20 h, stained in ethidium bromide solution (Section 2.4.6) 30-45 min and destained in milli-Q water before the DNA was visualised on a short wave UV transilluminator and photographed using an IS-1000 Digital Imaging System.

2.10 Determination of the molecular weights of DNA fragments.

DNA sizes were determined by comparison to ladder markers run alongside DNA samples on agarose gels (Section 2.9.1). The molecular weight of the unknown sample was determined by measuring the distance migrated (from well) by known fragments and comparing their relative mobility to that of the unknown DNA sample. The ladders

used in this study include: *HindIII-* digested lambda DNA, *HindIII-EcoRI* digested lambda DNA, 1Kb and 1Kb⁺ ladders (Gibco BRL).

2.11 DNA subcloning techniques.

Cloning of PCR products involved: ligation of the PCR product into a pGEM®-T or pGEM®-T easy vector (Promega) (Section 2.11.1) and transformation of the plasmid vector into *E. coli* (Section 2.11.2). Transformants were screened initially using blue/white selection and white colonies were further screened using colony PCR amplification (Section 2.13.4). Positive recombinants were streaked on LBAmp plates for pure cultures and plasmid DNA isolated from 3 mL of LBAmp liquid cultures using the QIAprep™ spin mini prep kit (Qiagen®) (Section 2.6.4). The plasmids were checked by restriction digest (Section 2.8.2), quantified fluorometrically (Section 2.7.2) and sent to the Massey University sequencing facility for sequencing (Section 2.14).

2.11.1 Ligation reactions.

Ligations were performed using the pGEM®-T or pGEM®-T easy vector kit (Promega) according to the protocol supplied by the manufacturer. The T-vector was pre-cut and partially filled in with thymidine. This prevented self ligation and allows efficient ligation of PCR products with adenosine overhangs created by *Taq* polymerase (Gibco BRL). Ligations direct from the PCR reaction mix were performed, except where PCR products were gel purified (Section 2.6.3) before incorporation into the T-vector as specified in the text.

Ligation mixtures contained 3 μL of PCR product (Section 2.13.3), 1x T4 ligase buffer, 50 ng of vector (pGEM®-T or pGEM®-T easy) and 3 U T4 ligase, in a final volume of 10 μL. Additional ligations included a control insert ligation and a vector only ligation. All ligations were incubated at room temperature for 30 min, then overnight at 4°C.

2.11.2 Transformation of *E. coli* by electroporation.

This method was a modification of the procedure developed by Dower, Miller and Ragsdale (1988).

2.11.2.1 *Preparation of electroporation competent* E. coli *cells.*

One litre of LB broth was inoculated with 10 mL of an overnight culture of the *E. coli* strain XL-1, and grown on a shaker at 37°C, to mid log phase (OD_{600} 0.5-1.0). The cells were chilled on ice for 20 min then harvested at 4°C by centrifugation for 10 min at 4000 g. The cells were washed (by resuspension, centrifugation (4000 g, 10 min, 4°C) and removal of the supernatant) repeatedly. Firstly in 1.0 L of ice cold sterile water, then in 0.5 L of the same, followed by a final wash in 20 mL of ice cold 10% (v/v) glycerol. The cells were then resuspended in 4 mL of ice cold 10% (v/v) glycerol and stored at -80°C in 40 μl aliquots.

2.11.2.2 *Electroporation.*

A 40 μL aliquot of electroporation competent *E. coli* cells (Section 2.11.2.1) was gently thawed at room temperature and placed on ice. Ligated DNA, in 2 μL ligation buffer, (Section 2.11.1) was added to the cells in a cold microcentrifuge tube, mixed well and

left on ice for 1 min. The Biorad Gene Pulser Transfection Apparatus was set to 2.5 V, 25 µF and 200 ohms resistance. The mixture was transferred to an ice cold 0.2 mL electroporation cuvette, pulsed and time constant checked. The time constant should be above 4.5 msec, any transformations with lower time constants were repeated in a new cuvette. Cells were immediately resuspended in 220 µL of LB broth (Section 2.2.1) and incubated at 37°C on a shaker for 30 min. A positive transformation control using 2 ng of pUC118 plasmid and a negative transformation control (2 µL of milli-Q water) were always transformed with each experiment. Cells were plated onto LB, Amp, IPTG, X-Gal plates (Section 2.2.1) for blue/white selection.

2.12 Southern Blotting and Hybridisation.

2.12.1 Southern Blotting (Capillary).

DNA to be transferred was first digested with *BamH1* or *EcoR1* restriction enzymes (Section 2.8.1), run on an 0.8% 1x TAE overnight gel (Section 2.9.2) and photographed with a ruler next to the gel. It was then transferred to Hybond-N$^+$ nylon membrane (Amersham), using a modification of the method described by Southern (1975) and Ausubel, Brent, Kingston, Moore, Smith, Seidman and Struhl (1994).

The gel was agitated in depurination solution (250 mM HCl) for 15 min, or until the bromophenol blue band on the gel became brown. The gel was then agitated in denaturation solution (500 mM NaOH, 1.5 M NaCl) for 45 min before being washed twice (2x 30 min) in neutralisation solution (500 mM Tris, 2 M NaCl). Finally the gel was washed in 20x SSC (Section 2.4.5) for 5 min and placed on the blotting apparatus overnight.

After blotting overnight, the nylon membrane was removed, washed in 2x SSC to remove excess salt and dried between 3MM filter paper. The DNA was fixed to the membrane by a 90 sec exposure to UV light on a short-wave UV transilluminator. Finally, the membrane was wrapped in plastic film and stored at 4°C until hybridisation of the probe (Section 2.12.3).

2.12.2 Preparation and labelling of probe.

Probe DNA was prepared using PCR amplification (Section 2.13), followed by gel purification (Section 2.6.3) and quantification (Section 2.7.3) before labelling.

2.12.2.1 *Preparation of DIG labelled DNA probe.*

DNA was random primer labelled with Digoxigenin-11-dUTP using DIG-High Prime (Roche), a 5x concentrated labelling mixture of random hexamers, dNTP mix containing alkali-labile Digoxigenin-11-dUTP, labelling grade Klenow enzyme and an optimised reaction buffer, according to the manufacturers instructions.

Alternatively, alkali-labile Digoxigenin-11-dUTP (Roche) was randomly incorporated into the probe DNA during PCR according to the protocol supplied by the manufacturer. The PCR reaction mix contained 1x PCR buffer, 2.0 mM MgCl$_2$, DIG synthesis mix (including the alkali-labile Digoxigenin-11-dUTP and dNTPs), 0.4 µM of each PCR primer, 1 U of Platinum *Taq* polymerase™ (Gibco BRL) and 20 ng of template DNA. PCR annealing temperatures used in the reaction were 53°C and 51°C.

The labelling efficiency of the DIG-labelling reactions was semi-quantitatively determined using the DIG Quantification test strips (Roche) according to the protocol supplied by the manufacturer. The quantification involves the detection of the labelled DNA by enzyme immunoassay, with anti-digoxigenin-AP and a colour substrate solution.

2.12.2.2 Preparation of [α-^{32}P] dCTP labelled DNA probe.

Probe DNA (25 ng-60 ng) was random primer labelled with 50 μCi of [α-^{32}P] dCTP using the High Prime labelling system (Roche) according to the protocol supplied by the manufacturer.

Unincorporated nucleotides were removed using ProbeQuant G-50 Micro Columns (Pharmacia) according to the protocol supplied by the manufacturer.

2.12.3 Southern blot hybridisation.

Nylon membranes (Section 2.11.1) were placed into glass hybridisation tubes and 20 mL of prehybridisation solution (Section 2.4.10) was added. The membranes were incubated at 58°C for 3 h in a rotating oven to prehybridise. The prehybridisation solution was then discarded and 7 mL of pre-warmed prehybridisation solution (58°C) and denatured radio labelled probe (Section 2.12.2.2) was added to the drained membrane. The membrane was then left to hybridise overnight at 58°C in a rotating oven.

After hybridisation, the solution was replaced with 30-50 mL of Southern wash solution (Section 2.4.13). The nylon membrane was washed three times (3x 25 min) at 58°C, and then wrapped in plastic cling film. This was then placed in an X-ray cassette with intensifying screens against either slow (Fuji Medical) or fast (Kodak Scientific Imaging) X-ray film. After exposure for an appropriate period of time at -80°C, the film was developed in a dark room by placing in developing solution for at least 3 min, fixed, then rinsed in water and dried.

2.12.4 Removal of [α-32P] dCTP labelled DNA from Southern blot membranes (Stripping).

Membranes to be stripped were washed in water for 1 min and then incubated twice in membrane stripping solution (Section 2.4.14) for 10 min at 37°C before rinsing thoroughly in 2x SSC (Section 2.4.5). Blots were then checked for activity before being stored in plastic film at 4°C.

2.13 Amplification of DNA by the Polymerase Chain Reaction (PCR).

PCR reactions were set up on ice using a cocktail which contained all common reagents used in the PCR in a ratio of n+1 PCR reactions (where n= the number of PCR reactions to be amplified, including positive and negative controls). Uncommon reagents were pipetted separately. Amplifications were performed in a Corbett FTS-960 thermocycler, Corbett PC-960 air cooled thermal cycler or a Corbett PC-960G gradient thermal cycler. Following amplification, the reactions were stored at 4°C and the products viewed using agarose gel electrophoresis (Section 2.9.1).

2.13.1 rDNA PCR.

2.13.1.1 *Universal ITS PCR.*

Universal fungal primers (Table 2.2) were used to amplify the Internal Transcribed Spacer (ITS) region between the 18S and 5.8S rDNA genes. Amplification reactions contained final concentrations of 1x PCR buffer (Roche), 1.5 mM $MgCl_2$, 100 µM dNTPs, 2 µM of ITS4 and ITS5 primers (Table 2.2), 1.0 U *Taq* DNA polymerase (Gibco BRL) and 20-50 ng of template DNA. These reactions were performed in a total reaction volume of 50 µL.

The cycling conditions for these primers were as follows: 94°C for 2 min, followed by 30 cycles of 94°C for 30 secs, 55°C for 30 sec, 72°C for 1 min. A final elongation of 72°C for 2 min was followed by soaking at 4°C.

Amplification products were analysed by electrophoresis of 2 µL of the reaction in a 1% agarose, 1x TBE gel, as described in Section 2.9.1.

2.13.1.2 **Eutypa lata** *specific PCR.*

The *E. lata* Internal Transcribed Spacer (ITS) region was selectively PCR amplified using the E.l.ITS primer series (Table 2.2) in a nested PCR amplification.

The first round of amplification was performed using primers E.l.ITS1 and E.l.ITS4. Amplification reactions contained final concentrations of 1x PCR buffer (Gibco BRL), 1.5 mM $MgCl_2$, 100 µM dNTPs, 2 µM of E.l.ITS1 and E.l.ITS4 primers, 1.0 U *Taq* DNA polymerase (Gibco BRL) and 20-50 ng template DNA. These reactions were performed in a total reaction volume of 50 µL.

The cycling conditions for these primers were as follows: 94°C for 2 min, followed by 35 cycles of 94°C for 30 secs, 62°C for 30 sec, 72°C for 1 min. A final elongation of 72°C for 2 min was followed by soaking at 4°C.

Amplification products were analysed by electrophoresis of 5 µL of the reaction in a 1% agarose, 1x TBE gel, as described in Section 2.9.1. The primers were expected to amplify only *E. lata* DNA and in the event of amplification, a PCR product of approximately 400 bp would be produced.

The second round of PCR amplification was completed using the primers E.l.ITS2 and E.l.ITS3. The 1st round PCR amplification product (0.4 µL) was added to 49.6 µl of PCR cocktail mix containing: 1x PCR buffer (Gibco BRL), 1.5 mM $MgCl_2$, 100 µM dNTPs, 2 µM of E.l.ITS2 and E.l.ITS3 primers and 1.0 U *Taq* DNA polymerase (Gibco BRL).

The cycling parameters for these primers (E.l.ITS2 and E.l.ITS3) were identical to the cycling conditions of primers E.l.ITS1 and E.l.ITS4, as described above.

Amplification products were analysed by electrophoresis of 5 µL of the reaction in a 1% agarose, 1x TBE gel, as described in Section 2.9.1. The primers were expected to amplify only *E. lata* DNA, giving a PCR product of approximately 370 bp.

2.13.2 PCR amplification direct from fungal cultures.

2.13.2.1 PCR amplification from boiled agar culture.

Blocks of growing hyphae (1 cm^2) were cut directly from agar plate cultures and transferred to a sterile microcentrifuge tube with 2 mL of 10 mM Tris-HCl (pH 8.0) buffer, before boiling for 20 min in a water bath. The negative control was a 1 cm^2 block of agar cut from a sterile agar plate. Aliquots (2 μL or 10 μL) of this boiled mixture were then used as a template for PCR amplification using the E.l.ITS primers (Section 2.13.1.2). This method was used successfully for the PCR amplification of *Gaeumannomyces graminis* by Henson, Goins, Grey, Matthre and Elliot (1993).

2.13.2.2 PCR amplification direct from growing mycelia.

PCR amplifications were performed directly from fungal hyphae by striking a yellow (p20) pipette tip across 1 cm of a 5 day old culture. The tip was then placed directly into the PCR reaction mix and combined thoroughly. No other alterations were made to the protocol described in section 2.13.1.2. This method was used successfully for the PCR amplification of *Armillaria* spp. by Harrington and Wingfield (1995).

2.13.2.3 PCR amplification using fungal spore extracts.

A 3 mm x 3 mm sample of mycelium was cut from a growing fungal culture. The sample was transferred to a labelled microcentrifuge tube and irradiated on high in a 800 W microwave oven for 5 min. Ice cold milli-Q water (30 μL) was added to the molten mycelia and agar before mixing thoroughly with a vortex for 30 secs. The sample was then spun in a desk top microcentrifuge at 13000 rpm for 5 min and 5 μL of supernatant was used as template for every 25 μL of PCR reaction mix. Triton-X-100 was also added to the PCR cocktail to a final concentration of 0.1% and the addition of glycerol (final concentration 5%) is optional. As most *Taq* polymerase preparations are suspended in glycerol, the addition of extra glycerol to the cocktail mix is not always necessary. The PCR cycling conditions remain the same as previously stated in section 2.13.1.2. This method is similar to the method of Kim, Uzunovic and Breuil (1999) used for the PCR amplification of *Ophiostoma* species. However, the authors do not add Triton X-100 or glycerol to the PCR cocktail.

2.13.2.4 PCR amplification from Eutypa lata *mycelia (Lecomté et al., 2000).*

After incubation for 1 week, 0.1 mg of fresh mycelium was scraped off the agar using a sterile loop, placed into 50 μL of sterile water and incubated at 95°C for 15 min. After incubation, the sample was immediately placed on ice before using 5 μL as the PCR template in an E.l.ITS primer amplification reaction.

2.13.3 PCR amplification direct from *Eutypa lata* infected grapevine wood.

A 3 mm^3 sample of infected wood added directly to an E.l.ITS PCR reaction mix (Section 2.13.1.2). In some cases this method was altered by adding 50 μg of non-fat skim milk powder to 50 μL of the PCR reaction mix, as a blocking agent to overcome inhibitors in PCR templates from plants, as described in De Boer (1995).

Tris-HCl (500 μL of 10 mM) at pH 8.0 was added to a 3 mm^3 sample of infected wood before irradiating in an 800 W microwave oven on high for 1 min. The samples were

then immediately placed on ice and 2 µl of the buffer solution was used as a template for *E. lata* specific PCR (Section 2.13.1.2).

2.13.3.1 PCR amplification of Eutypa lata *infected grapevine wood (Lecomté* et al., *2000).*

Thin wood shavings about 5 mm x 5 mm x 0.2 mm were cut using a sterile scalpel blade and placed individually into 1.5 mL microcentrifuge tubes. The tubes were then incubated at 95°C for 15 min with 50 µL of sterile milli-Q water. After incubation the samples were immediately stored on ice and 5 µL of a 1:10 and 1:100 dilution were used for PCR analysis using the protocol described in section 2.13.1.2.

2.13.4 Degenerate PCR.

Attempts were made to amplify the *E. lata MAT1-2* HMG box by PCR using the degenerate HMG primers (Table 2.2). The PCR amplification protocol and primers were based upon the protocol of Arie *et al.* (1997).

Amplification was performed using the primers NcHMG1, NcHMG2, GfHMG2, PaHMG2, SmHMG1, SmHMG2 or SmHMG3. Amplification reactions contained final concentrations of 1x PCR buffer (Gibco BRL), 2.0 mM $MgCl_2$, 0.3 mM dNTPs, 1 µM of each primer, 1.0 U Platinum *Taq* DNA polymerase™ (Gibco BRL) and 20-50 ng template DNA. These reactions were performed in a total reaction volume of 50 µL. In several reactions the 1x PCR buffer and 1.0 U of Platinum Taq DNA polymerase were replaced with 1x Fast start PCR buffer (Roche) and 1.0 U of Fast start Taq DNA polymerase (Roche).

The PCR cycling parameters were as follows: 95°C for 2 min, followed by 40 cycles of 94°C for 1 min, 48°C for 1 min and 72°C for 1 min. This was then followed by an extended elongation period of 72°C for 10 min and soaking at 25°C.

Amplification products were analysed by electrophoresis of 10 µL of the reaction in a 2-2.5% agarose, 1x TBE gel, as described in Section 2.9.1. The primers were expected to amplify a PCR product between 266-300 bp that contained an intron (average size 54-68 bp). The expected size of a fungal *MAT1-2* HMG box product is approximately 70 amino acids (not including the intron).

Other degenerate PCR programs.
Program 1:
Denaturing at 95°C for 2 min was followed by 35 cycles of 94°C for 15 sec, 51°C annealing for 30 sec and 1 min elongation at 72°C. This was then followed by an extended elongation period of 10 min at 72°C and soaking at 4°C.

Program 2:
Touch down PCR (Iqbal, Robinson, Deere, Saunders, Edwards & Porter (1997)).
The optimised annealing temperature for the degenerate HMG box primers had been found to be 48°C. After hot-start (3 min at 95°C), this initial annealing temperature was set at +10°C for the first PCR cycle (95°C 30 sec, 48°C + 10°C 30 sec, 72°C 1 min). The annealing temperature value was then decreased by 2°C for each subsequent cycle

for a total of 5 cycles. At this point the remaining 30 PCR cycles were completed with an annealing temperature of 48°C.

Program 3:
This program was designed to create a bias for producing short PCR products by reducing denaturing, annealing and extension times.

The program began with a hot start, 95°C for 3 mins and was followed by 40 cycles of 95°C for 15 sec, 48°C annealing for 30 sec and 20 sec elongation at 72°C. This was then followed by an extended elongation period of 10 min at 72°C and soaking at 25°C.

2.13.5 Colony screen PCR.

Positive transformants (white colonies in a blue/white colony screen) (Section 2.11.2.2) were screened using PCR and the T7/Sp6 primer combination (Table 2.2) that flank the insert site of the pGEM®-T/pGEM®-T easy vectors (Promega). A sample of the colony was taken with a sterile P20 tip and added directly to the PCR mixture. The only modification to the degenerate PCR cycle was an extended initial denaturation cycle at 95°C, which lysed the bacterial cells, releasing the plasmid as a template for PCR. In this way colonies with the correct sized insert could be identified.

Amplification products were analysed by electrophoresis of 10 μL of the reaction in a 2.5% agarose, 1x TBE gel, as described in Section 2.9.1.

Plasmid DNA was isolated from positive clones (Section 2.6.3) and digested (Section 2.8.2) with Restriction enzymes (*NcoI* and *PstI* when cloned into the pGEM®-T vector, or *EcoRI* in the case of pGEM®-T easy plasmids) to check the size of the insert DNA, before it was sent for sequencing (Section 2.14).

2.13.6 Randomly amplified polymorphic DNA (RAPD) PCR.

Genomic DNA was diluted to 3 ng/μL and quantified using fluorescence (Section 2.7.2) for RAPD amplification. PCR reactions were carried out using random 10-mer oligonucleotides obtained from Operon Technologies Inc. Each reaction was in a total volume of 25 μL and contained 1x *Taq* PCR buffer (Gibco BRL), 2.5 mM MgCl$_2$, 0.2 mM dNTPs (Roche), 0.4 μM primer (Table 2.2), 0.8 U of *Taq* polymerase (Gibco BRL) and 15 ng of template DNA. Components were added in the order listed and kept cold at all times.

The cycling conditions were based on a protocol devised by Hirst (1997), and involved an initial 3 min denaturation step at 94°C, followed by 1 min annealing at 37°C and 2 min elongation at 72°C. This was followed by 39 cycles of 95°C for 1 min, 37°C for 1 min and 72°C for 1.5 min. A final 72°C elongation step for eight min was followed by soaking at 10°C.

Amplification products were analysed by electrophoresis of 20 μL of the reaction in a 2% agarose 1x TBE gel, as described in Section 2.9.1.

2.13.7 5' Anchored Microsatellite PCR.

Degenerate 5' anchored primers (Table 2.2) were used to amplify microsatellite loci, based on the methods of Fisher, Gardner and Richardson (1996) and Ganley (2000).

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Amplification reactions contained 1x PCR buffer (Gibco BRL), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM primer, 3 U *Taq* DNA polymerase (Gibco BRL) and 30 ng of genomic DNA. These reactions were performed in a total reaction volume of 25 μL.

Cycling conditions involved an initial 3 min denaturation step at 94°C, followed by 5 cycles of 93°C for 30 sec, 59°C for 30 sec and 72°C for 30 sec. This was followed by 35 cycles of 93°C for 30 sec, 57°C for 30 sec and 72°C for 30 sec. A final 72°C elongation step, 2 min, was followed by soaking at 4°C.

Amplification products were analysed by electrophoresis of 15 μL of the reaction in a 1.5% agarose 1x TBE gel, as described in Section 2.9.1.

2.14 DNA sequencing.

Automated sequencing reactions were performed using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) with 300 ng of DNA template, 3.2 pmol primer and 8 μL of terminator ready reaction mix in a total volume of 20 μL. Samples were run on an ABI 377 automated sequencer. The T7 or Sp6 primers (Table 2.2) were used for sequencing the insert within the pGEM-T vector. Whereas, the *E. lata* ITS region was sequenced using the universal fungal ITS primers (Table 2.2) by directly sequencing Qiagen® purified PCR products (Section 2.6.4).

2.15 Sequence alignment.

DNA sequences were aligned using the CLUSTAL W algorithm (Thompson, Higgins & Gibson, 1994), which is available in the Gene-Jockey II® sequence handling program. The 18S and 28S ribosomal DNA sequences which flank the ITS regions were deleted prior to alignment. Where only the ITS1 region was available from the GenBank database a consensus 5.8S rDNA sequence, obtained from a smaller alignment of *E. lata* and *Botryosphaeria* sp. sequences (Fig. 3.10), was added to the sequence in order to anchor the ITS1 region. Matching of the central 5.8S rDNA region in each sequence was checked to ensure accuracy of the alignment. Protein sequences were also aligned in the Gene-Jockey II® sequence handling program using the CLUSTAL W algorithm.

Table 2.2 PCR and sequencing primers.

Primer.	Size (nt).	Tm (°C).	Sequence (5'-3').	Source.
rDNA				
ITS1	19	65	TCC GTA GGT GAA CCT GCG G	White et al. (1990).
ITS1F	22	66	CTT GGT CAT TTA GAG GAA GTA A	Gardes & Bruns. (1993).
ITS2	20	70	GCA TCG ATG AAG AAC GCA GC	White et al. (1990).
ITS3	20	62	GCA TCG ATG AAG AAC GCA GC	White et al. (1990).
ITS4	20	58	TCC TCC GCT TAT TGA TAT GC	White et al. (1990).
ITS5	22	63	GGA AGT AAA AGT CGT AAC AAG G	White et al. (1990).
E. lata specific				
E.l.ITS1	19	52	CGG GGA AGC CTA CCC GGT A	This study.
E.l.ITS2	21	55	GGG AGC GAG CTA CCC TGT AGC	This study.
E.l.ITS3	22	53	GGA GCT ATC CGG AGA TAG GCT C	This study.
E.l.ITS4	22	53	GCA AGA CGT CAG CCG TGA CAC A	This study
MAT1-2 HMG box				
NcHMG1	23	61	CCY CGY CCY CCY AAY GCN TAY AT	Arie et al. (1997).
NcHMG2	23	63	CGN GGR TTR TAR CGR TAR TNR GG	Arie et al. (1997).
PaHMG2	23	64	CGI GGI CGR TAY TGR TAR TCI GG	This study.
GfHMG2	23	62	CGI GGI ACR TAI CGR TAR TGI GG	This study.
SmHMG1	24	53	CTT ACG RTA NAR DAT RTA NGC RTT	This study.

PCR and sequencing primers continued.

Primer.	Size (nt).	Tm (°C).	Sequence (5'-3').	Source.
SmHMG2	24	59	ATT GGC ATC GTA GTT NAG NAG YTT	This study.
SmHMG3	20	59	AWH GAR YTN GGN CAY TGG AA	This study.
Sequencing.				
Sp6	22	58	TTT AGG TGA CAC TAT AGA ATA C	Promega.
T7	23	66	TAA TAC GAC TCA CTA TAG GGC GA	Promega.
Plant specific.				
RB138R	26	58	CAA GCT TCA CGA ATA ACT TCA TTA CC	L. Perrie (pers. comm.).
RB27F	24	60	GTC ACC ACA AAC AGA GAC TAA AGC	L. Perrie (pers. comm.).
Microsatellite.				
Anchored CT	19	68	KKV RVR VCT CTC TCT CTC T	Fisher et al. (1996). Ganley. (2000).
Anchored TG	19	48	KKV RVR VTG TGT GTG TGT G	Ganley. (2000).
Anchored AAG	22	47	KKY NSS HAA GAA GAA GAA GAA G	Ganley. (2000).
RAPD.				
OPD-03	10		GTC GCC GTC A	Hirst. (1997).
OPC-05	10		GAT GAC CGC C	Hirst. (1997).

* M=A+C, R=A+G, W=A+T, S=C+G, Y=C+T, K=G+T, V=A+C+G, H=A+G+T, B=C+G+T, N=A+C+G+T

3. VINE SURVEY OF EUTYPA DIEBACK AND DEVELOPMENT OF A MOLECULAR DIAGNOSTIC TOOL.

3.1 Introduction.

E. lata is usually identified by traditional methods of isolation and culturing (Carter, 1991). *E. lata* does not produce sexual structures in culture, so identification depends primarily on the morphology of the mycelium, although serological techniques have also been developed (Francki & Carter, 1970; Price, 1973). The use of traditional culturing techniques has many disadvantages. For example, Cabernet Sauvignon material collected from the Erindale vineyard had previously been tested for Eutypa dieback at the Mt Albert HortResearch station. Although the symptoms of the disease looked like Eutypa dieback, testing at HortResearch indicated that *Botryosphaeria* sp. was detected in the affected vines at most of the disease lesions. Such ambiguities are not uncommon, for example, in a survey of fungi associated with grapevine dieback in the Hunter Valley region, Australia, the fungus most frequently isolated was *B. obtusa* and *E. lata* was not isolated from any of the diseased grapevine arms (Castillo-Pando, Somers, Green, Priest & Sriskanthades, 2001).

Observation of conidia is often necessary to confirm the identification of *E. lata*, but their production requires at least one to two months and they are not produced in all isolates. The growth of *E. lata* on culture media may also be slower than that of other saprophytic microorganisms present in grapevine wood. Thus, the fungus may go unobserved on isolation plates, leading to an incorrect diagnosis.

PCR with species-specific primers is an easy way to detect fungal plant pathogens both in culture and in plant tissues. In fungi, the ITS region of nuclear ribosomal DNA shows notable interspecific variability while intraspecifically it exhibits significant conservation (White *et al.*, 1990). PCR amplification of the ITS region should provide detectable variation between *E. lata*, other wood rotting fungi and grapevine species, therefore, providing a more effective means of diagnosis of the pathogen.

3.1.1 Specific objectives.

This work has four objectives. To:

1) Isolate the fungus *E. lata* from grapevines affected with grapevine dieback and to determine the range of other saprophytic fungi that are commonly found with *E. lata* in infected vines.

2) Develop a molecular tool for the identification of the ITS region of *E. lata*.

3) Confirm that the probe developed in (2) does not detect the ITS regions of grapevines or other wood rotting fungi.

4) Develop an assay for the detection of *E. lata* directly from diseased grapevine wood.

3.2 Collection of samples and fungal isolations.

3.2.1 Materials and methods.

3.2.1.1 Collection of samples.

Vines suspected of grapevine dieback were collected from Der Ley and Erindale vineyards in the Hawkes Bay of New Zealand, on 18 November 1998. A Sauvignon Blanc vine cordon was collected from the Der Ley vineyard (Section 2.1.1). The vine collected was approximately eight years of age. Symptoms of grapevine dieback in the vineyard were first noticed in 1995 and since that time had spread to become quite severe in parts. Cabernet Sauvignon vine cordons (20 years of age) were collected from the Erindale vineyard (Section 2.1.1). The origin and cultivar of vines collected for sampling is summarised in Table 3.1.

Table 3.1 Origin and cultivar of infected grapevine cordons.

Cordon.	Cultivar.	Origin.	No. of sections.
V2	Sauvignon Blanc	Der Ley	5
V7	Cabernet Sauvignon	Erindale	7
V8	Cabernet Sauvignon	Erindale	3
V9	Cabernet Sauvignon	Erindale	2
V10	Cabernet Sauvignon	Erindale	5
V11	Cabernet Sauvignon	Erindale	3

3.2.1.2 Isolation of fungal colonies.

A total of 74 disease lesions were sampled on cordons with dieback symptoms from six grapevines. The number of isolations per vine depended upon the number and size of visible disease lesions. Isolations from each disease lesion (Section 2.3.1.5) were made on PDA (Section 2.2.2), 15%V8 (Section 2.2.4) and MA agar (Section 2.2.3). One 5 mm^3 piece of grapevine tissue was plated in each quarter of the agar plates (Fig. 3.1).

3.2.2 Results.

3.2.2.1 Distribution of symptoms.

No perithecia of *E. lata* were found in either vineyard, even though an examination of several rows of infected vines was made at each site.

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15%V8 agar

Malt agar   Potato Dextrose Agar
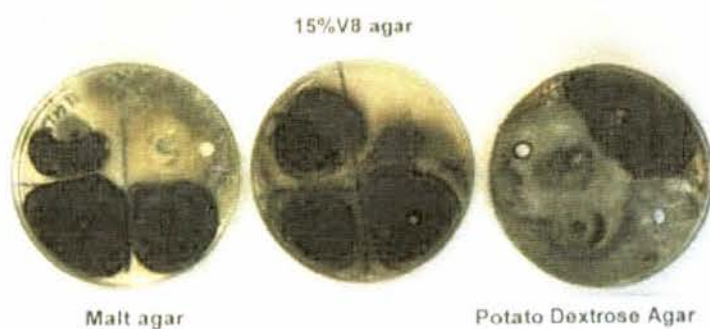
Figure 3.1 Original agar isolation plates from *Vine 7*. One piece of diseased tissue was plated in each quarter of the agar plate. This photograph was taken 2 weeks after inoculation with the grapevine pieces.

Longitudinal and cross sectioning of all vines sampled showed symptoms typical of Eutypa dieback, with dry brown wood necrosis extending away from the original pruning stub (Moller & Kasimatis, 1978). These internal symptoms were good indicators of Eutypa dieback within the vine. It was apparent that the infection was not uniformly distributed within the tissues of the vine (Fig. 3.2). Young cankers in cross section appeared as a wedge shape darkened area coming to a point in the centre of the arm or trunk. All vines displayed more than one independent disease lesion in the same cordon and each disease lesion within the vine was usually traceable back to an old pruning wound, the site of initial infection.
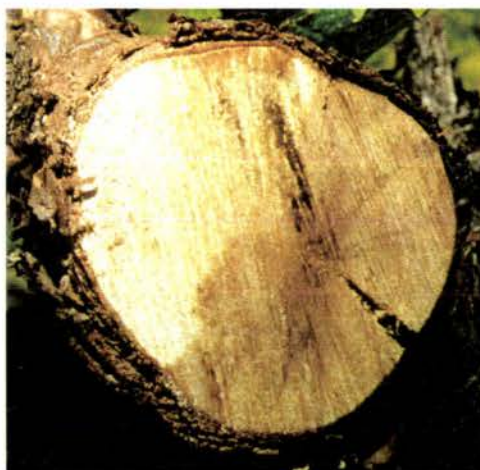
Figure 3.2 Cross section of a diseased cordon of *Vine 4* from Erindale vineyard.

3.2.2.2 Fungal isolations.

More than one fungus was frequently isolated from each piece of vine tissue and 314 fungal colonies were obtained from the 222 pieces of grapevine wood plated (Table 3.2).

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Table 3.2 Total number of fungal colonies, fungal genera and *E. lata* and *Botryosphaeria* sp. colonies obtained on three different agar media.

	PDA	15%V8	Malt	Total	X² value*	P
Total numbers of fungal colonies obtained.	112	92	110	314	2.319	0.50-0.20
Total numbers of fungal genera obtained.	15	19	16	50	0.521	0.80-0.50
Total numbers of *E. lata* colonies obtained.	15	11	9	35	1.600	0.50-0.20
Total number of *Botryosphaeria* spp. colonies obtained.	16	9	21	46	4.740	0.10-0.05

* In each case null hypothesis is of no difference between each of the 3 agar media. Df=2

A comparison of the efficiency of the three agar media for making isolations from diseased vine wood showed that fewer fungal colonies were obtained on the 15%V8 agar medium than on the other media tested, but a chi-squared test (Bishop, 1966) found that the total numbers of fungal colonies developing on each agar medium were not significantly different (X^2= 2.319 p=0.50-0.20). The null hypothesis, that there is no difference in the total numbers of fungal colonies obtained from the diseased cordons between the three agar media, is supported The mean number of total fungal colonies isolated per vine on each of the three different media is also not significant as illustrated in Fig. 3.3.

Nineteen fungal genera were isolated on the 15%V8 agar while the totals on PDA and Malt agars were fifteen and sixteen respectively (Table 3.2). However, once again the differences between the agars in numbers of fungal genera isolated were not statistically significant.

Although *E. lata* was isolated from each of the diseased vines, it comprised only 10% of the total number of isolations. It was the sole fungal isolate from 52% of samples. Forty eight percent of the *E. lata* isolates were associated with one, two or three other fungi, the most frequent association being with *Botryosphaeria* species isolates.

More isolates of *E. lata* were found on the PDA medium than on other media (Fig. 3.3), but a chi-squared test showed the differences were not significant (Table 3.2) and the null hypothesis is accepted.

Botryosphaeria species were isolated on more occasions than *E. lata* but were not found in vine *V9* (Fig. 3.4). *E. lata* was isolated from 30% of the disease lesions sampled while *Botryosphaeria* species were obtained from 40% of the disease lesions. The two fungi were found together in 40% of the lesions from which *E. lata* was obtained (10 versus 14 isolations respectively, Table 3.3).
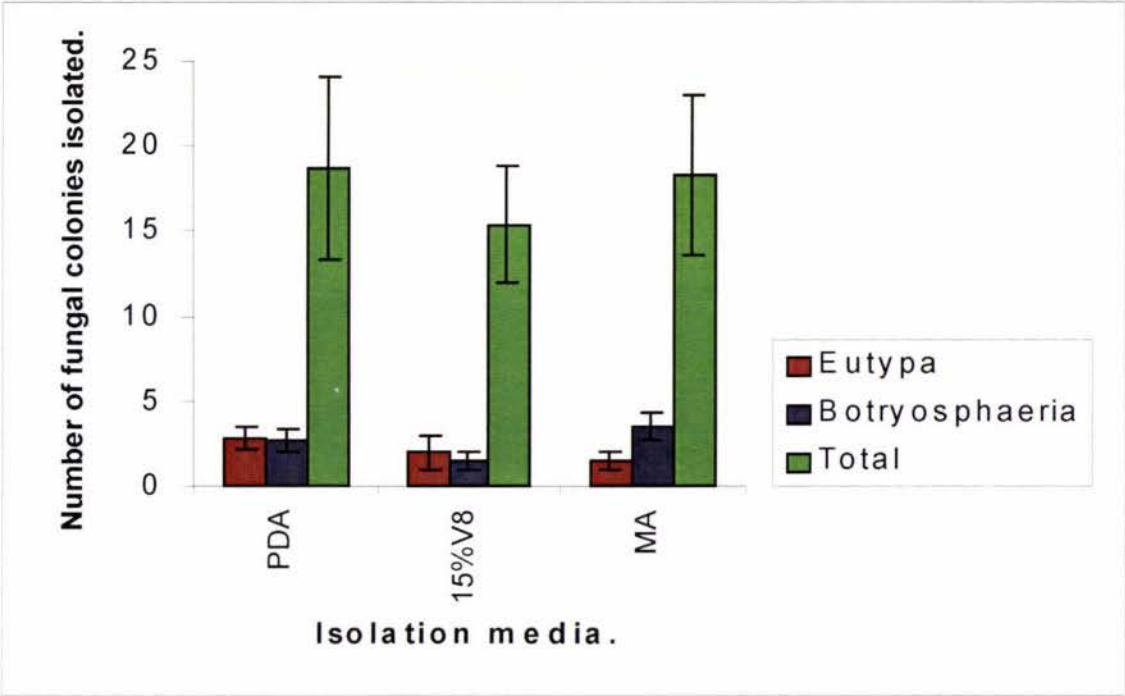
Figure 3.3 Mean number of fungal isolates per vine across three different agar isolation media.

Botryosphaeria was not isolated as frequently from 15%V8 medium as from other media (Fig. 3.3) and there was some indication that isolation of *Botryosphaeria* sp. depended upon the medium used for isolation (Table 3.2), since a chi-squared test gave a p value between 0.10 and 0.05, approaching the p=0.05 probability needed to discount the null hypothesis. However, there was stronger evidence to show that the isolation of *Botryosphaeria* sp. was dependent upon the vine from which the isolation took place. This is illustrated in Fig. 3.4 where the mean number of *Botryosphaeria* sp. isolated from vine *Erindale 9* is zero; the mean number of *Botryosphaeria* sp. isolated in the other vines is approximately three.

Table 3.3 Number of disease lesions from which *E. lata* and *Botryosphaeria* sp. were isolated together.

Vines	No. of lesions plated.	No. of lesions yielding *E. lata* only.	No. of lesions yielding *Botryosphaeria* sp. Only.	No. of lesions yielding both fungi.
V2	11	0	3	2
V7	18	4	3	1
V8	22	2	6	3
V9	3	2	0	0
V10	12	4	4	3
V11	8	2	5	1
Total	74	14	21	10
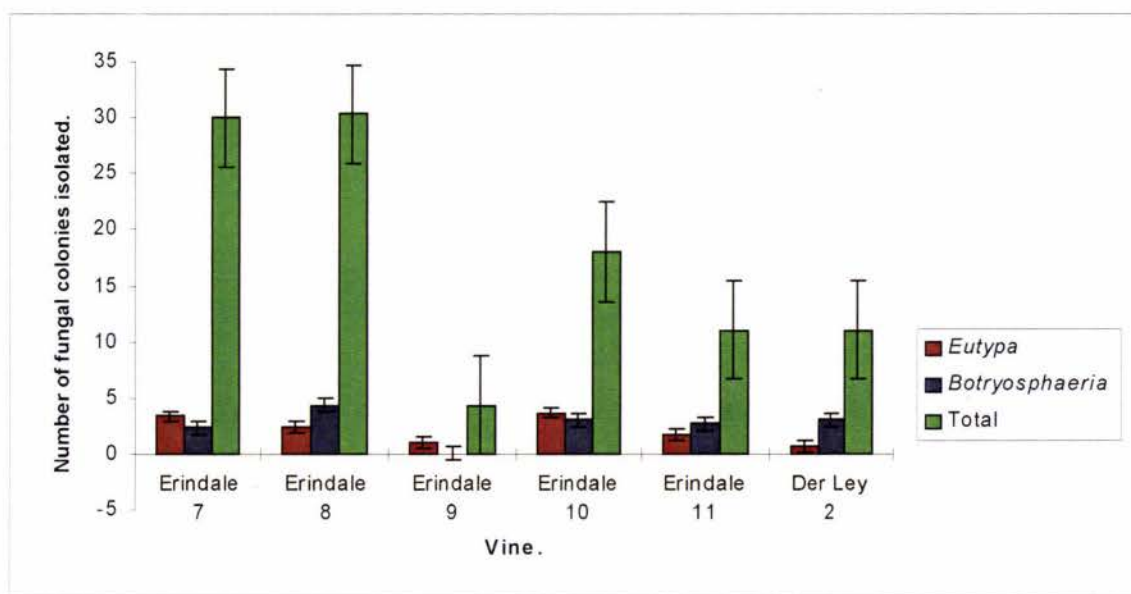
Figure 3.4 Mean number of fungi isolated from six diseased grapevines.

Not all pieces of vine tissue plated produced colonies of known pathogens. Nine unidentified fungal colonies were isolated. Eight percent of the 222 pieces of wood plated failed to produce any fungal colonies. However, none of the disease lesions were sterile and all lesions sampled produced at least one fungal colony on one or more of the three media tested. A total of 26 different fungal genera were isolated from the vines, however, not every genus was isolated on each of the media, or from every vine.

Bacterial colonies were also isolated from 5.5% of the 74 disease lesions sampled. However, they were isolated from vines *V7* and *V8* only. One bacterial colony was isolated on each of the three media tested from *V8* and a bacterial colony was also isolated on PDA agar from *V7*. The oxytetracycline antibiotic added to suppress bacterial growth in the PDA medium did not suppress all bacterial growth because 50% of bacterial colonies isolated were found on this medium. Oxytetracycline was not added to the 15%V8 or Malt agar media.

The three agar media yielded the same fungal species from 25% of the 74 disease lesions sampled. Nevertheless, two or more media were in agreement with at least one fungal species isolated from 77% of the disease lesions sampled.

Isolates of *E. lata* that produced the anamorph stage *(Libertella blepharis)*, in culture were isolated on three occasions. The *Libertella* stage was not formed on 15%V8 agar and was isolated from vines *V7* and *V8* only. *Libertella blepharis* (previously *Cytosporina*) was identified by a speckled appearance created by distinctive black pycnidia scattered irregularly throughout the white mycelia (Fig. 3.5). The pycnidia were sometimes as large as 2 mm in diameter.

Cytosporina

Figure 3.5 Typical morphology of the anamorph of *E. lata*, (*Libertella blepharis* stage (previously *Cytosporina*)). The isolate was photographed on PDA medium sixteen days after inoculation.

Other fungal species that were frequently isolated with *E. lata* include *Botrytis cinerea, Gliocladium roseum* and *Alternaria alternata. Alternaria alternata* and *Penicillium* sp. were isolated more frequently (72 and 50 isolations respectively) than *E. lata* or the *Botryosphaeria* species and are common saprophytes in dead plant material. Species of *Gliocladium, Epicoccum, Fusarium, Mucor, Botrytis* and *Cladosporium* with 30, 12, 12, 10, 10 and 7 isolations respectively, were the next most commonly encountered fungi within the vines.

A complete listing of fungal genera isolated from the vines and raw data from vine isolations can be found in Appendix 1.

3.2.2.3 Colony characteristics of Eutypa lata and Botryosphaeria spp. isolates.

Large variations were observed in the morphology of the *E. lata* isolates, especially after ageing. The colour of the cultures varied from white to grey and some isolates had a cottony appearance. Confirmation of identification by spore production was rarely possible in less than four weeks and some cultures had not produced spores after more than two months. Those that did produce conidiomata extruded the characteristic spores in pale yellow to orange coloured tendrils. Certain isolates exuded various quantities of brown pigment into the growth medium after one or two weeks and the amount of pigment found in the medium increased with age. Occasionally the dark pigment would discolour the colony mycelium. Isolates of *E. lata* were generally slower growing than most of the other fungi isolated from the vines.

Botryosphaeria spp. isolates were identified by fluffy grey to black mycelia. However, not all isolates were fluffy and the mycelium of some isolates was smooth and velvety. A dark brown to black pigment was exuded into the growth media by some isolates. *Botryosphaeria obtusa* and *Botryosphaeria ribis* were the most frequently isolated species of *Botryosphaeria* and they both grew faster than isolates of *E. lata*. *Botryosphaeria* species other than *B. obtusa* or *B. ribis* were not identified to the species level.

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Typical colonies of *E. lata* were readily distinguishable from *Botryosphaeria* species after several days growth on the agar but the *B. obtusa* and *B. ribis* species required microscopic analysis for species identification. Colonies intermediate in morphology between *E. lata* and *Botryosphaeria* spp. were often found (Fig. 3.6), these colonies with intermediate morphology were not readily distinguishable from one another as most did not produce conidia.

Figure 3.6 Variability in cultures of *E. lata* and *Botryosphaeria* spp. sixteen days after inoculation on PDA agar. The top three cultures are isolates of typical *Botryosphaeria* morphology. The bottom row of cultures, are *E. lata* isolates of typical morphology. The middle rows are *E. lata* isolates that demonstrate the variation found in morphology from the grapevines.

Fungi such as *Botrytis cinerea*, *Mucor* sp., *Glomerella cingulata*, *Epicoccum* sp. *Pestalotia* sp., *Phomopsis* sp., *Fusarium* sp. and *Gliocladium roseum* were readily identifiable on the agar media (Fig. 3.7).

The traditional method of detecting *E. lata* in grapevine wood by selective media identification is tedious and time consuming and the identifying characteristics of the fungus in culture are ambiguous. There is a need to develop a PCR based assay system for the detection of *E. lata*. This will present researchers and growers with an efficient tool with which to measure the incidence of the disease in a vineyard, to enhance the eradication process, and enable in depth study of the fungus.

Figure 3.7 Typical features of colonies of *Botrytis cinerea*, *Mucor* sp., *Glomerella cingulata*, *Pestalotia* sp., *Phomopsis* sp., *Fusarium* sp. and *Gliocladium*. The isolates were grown on PDA media at 22°C for 14 days.

3.3 Development of a molecular tool for the detection of *Eutypa lata*.

3.3.1 Database searching.

The international database GenBank (URL http://www.ncbi.nlm.nih.gov/) was searched for the Internal Transcribed Spacer (ITS) sequences of fungi, which have been, or could be, retrieved from infected vine tissue. Table 3.4 indicates sequences resulting from this search.

A wide range of ITS sequences from fungal pathogens and fungi isolated from grapevine wood in the vine survey (Section 3.2) were found in the database, including an *E. lata* sequence (AF099911) from an isolate originating in France. However, it was

important to obtain at least one ITS sequence from New Zealand isolates of *E. lata* to ensure that the primers (to be designed to the ITS regions) would not be inhibited by sequence polymorphisms unique to New Zealand isolates at the primer annealing sites.

Table 3.4 Summary of ITS sequences of fungal plant pathogens obtained from GenBank database.

Species	Accession number	Sequence (bp)	Comments*
Alternaria alternata AA6	U05195	550	ITS1,5.8S,ITS2
Alternaria alternata	AF071346	507	(18S),ITS1,5.8S,ITS2,(28S)
Botryosphaeria dothidea	AF027752	527	(18S),ITS1,5.8S,ITS2,(28S)
Botryosphaeria obtusa	AF027759	517	(18S),ITS1,5.8S,ITS2,(28S)
Botrytis cinerea	Z73765	481	ITS1,5.8S,ITS2
Colletotrichum gleosporiodes	Y16203	171	ITS1
Colletotrichum graminicola	AF059676	581	(18S),ITS1,5.8S,ITS2,(28S)
Dothidea insculpta	AF027764	530	(18S),ITS1,5.8S,ITS2,(28S)
Eutypa lata	AF099911	574	ITS1,5.8S,ITS2
Fusarium sp.	X73808	151	ITS1
Gliocladium sp. *FI-360*	AF139857	516	(18S),ITS1,5.8S,ITS2,(28S)
Gliocladium sp. *FI-442*	AF139858	517	(18S),ITS1,5.8S,ITS2,(28S)
Lasiodiplodia theobromae	AF027762	479	(18S),ITS1,5.8S,ITS2,(28S)
Penicillium sp. *WSF 420*	AF178528	545	ITS1,5.8S,ITS2,(28S)
Penicillium sp. *RMF8832*	AF178525	545	ITS1,5.8S,ITS2(28S)
Pestalotia palmarum	AF009818	535	ITS1,5.8S,ITS2
Phaeoacremonium aleophilum	AF017651	626	(18S),ITS1,5.8S,ITS2,(28S)
Phaeoacremonium chlamydosporum	AF017652	650	(18S),ITS1,5.8S,ITS2,(28S)
Phaeoacremonium sp. *LCP93 3886*	AF118137	538	(18S),ITS1,5.8S,ITS2,(28S)
Phomopsis longicolla	AF132796	492	ITS1,5.8S,ITS2
Phomopsis sp. *00W7*	AJ246145	631	(18S),ITS1,5.8S,ITS2
Phomopsis sp. *00044*	AJ246146	596	(18S),ITS1,5.8S,ITS2

*Sequences underlined indicate the availability of partial sequence only from the GenBank database. Sequences in parentheses were removed before alignment (Section 2.15).

3.3.2 DNA extraction of grapevine and fungal DNA.

DNA extractions yielded approximately 250 ng of DNA from 0.1 g of wet weight mycelium of each fungal isolate. The efficiency of DNA extraction was not affected by the method used (Sections 2.5.1 & 2.5.2). The efficiency of DNA extraction from grapevine tissue was considerably lower than that from the fungi. This could have been due to degradation of DNA by DNases in the leaf tissue since the sample material was not immediately frozen after removal from the vine.

3.3.3 PCR amplification of the ITS region.

The ITS1, ITS4 and ITS5 primers (Table 2.2) specifically amplified the entire length of the ITS region including the 5.8S rDNA, plus small portions of the 3' end of 18S and the 5' end of 28S rDNA (Fig. 3.8).
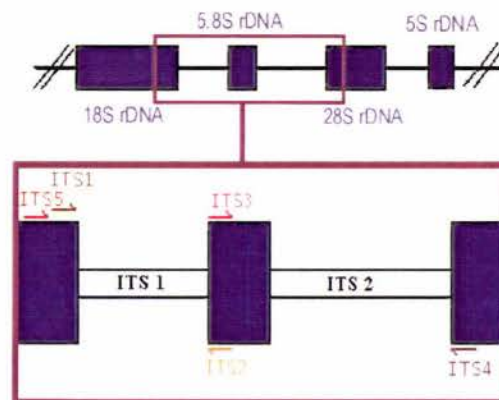
Figure 3.8 Location on nuclear rDNA of PCR primers designed to PCR amplify the ITS regions. The arrowheads represent the 3' end of each primer.

Gel electrophoresis of PCR products from *E. lata* with the ITS5 and ITS4 primers always yielded a single band approximately 580 bp in length (Fig. 3.9). The size of this fragment was approximately the same in all *E. lata* isolates examined when estimated on agarose gels. No PCR product was obtained in the negative control. The *Botryosphaeria* sp. isolate also produced a PCR product with the ITS4 and ITS5 primers. This product was marginally smaller than the *E. lata* product, approximately 560 bp in length. The *Botryosphaeria* sp. and *E. lata* PCR amplification products were compared with the fungus *Dothistroma pini*. The *D. pini* ITS region had previously been PCR amplified and sequenced within the laboratory. The *D. pini* ITS5 and ITS4 PCR fragment is 560 bp in size.
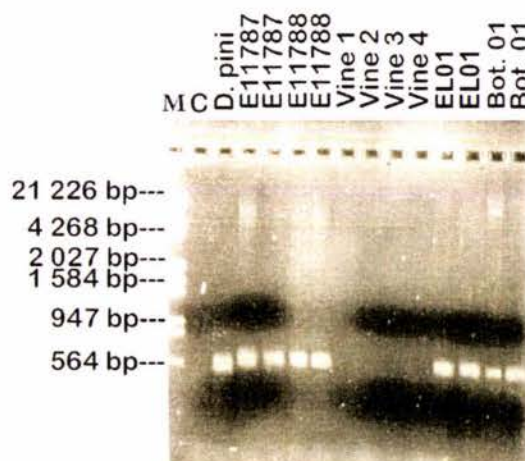
Figure 3.9 Agarose gel (1%) with PCR amplification products of the ITS1-5.8s-ITS2 region of rDNA from 3 *E. lata* isolates (E11787, E11788 and EL01) and 1 *Botryosphaeria* sp., using ITS4 and ITS5 as primers. Also included are four independent tests of grapevine DNA (variety Chardonnay), the lambda *Eco Hind* size marker (M), a positive control of *Dothistroma pini* DNA (isolate NZE1) and C a negative (no DNA) control.

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3.3.3.1 PCR amplification of the ITS region of grapevine DNA

The DNA from the grapevine tissue did not produce a PCR product with the universal ITS primers, even though positive and negative controls in the experiments were successful. The suitability of the grapevine DNA for use as a DNA template was checked with the RB1381R and RB27F primers (Table 2.2). These primers are plant specific PCR primers positioned at the 5' and 3' ends of the *rbcL* gene, an ancient conserved plant gene. The primers were expected to amplify a 1300 bp fragment. Once again, the grapevine DNA failed to produce any PCR product with valid positive (Shield fern DNA) and negative controls (results not shown).

Attempts to remove possible PCR inhibitors from the grapevine template DNA using phenol/chloroform precipitations and fresh DNA extractions did not alter these results. It must also be noted that attempts to check the integrity of the grapevine DNA by visualisation on agarose gel, showed low molecular weight DNA smears suggesting that the template was deteriorating. This indicates that DNAses in the leaf tissue were not removed or inactivated during the extraction process. The low DNA extraction efficiency could also imply that the DNA had been partially degraded in the leaf tissue prior to extraction since the sample material was not immediately frozen after removal from the vine. Alternatively, the DNA could have been intact when extracted but the subsequent degradation of DNA could indicate that TE buffer (10:1) is not an appropriate buffering system for the grapevine DNA, resulting in rapid degradation.

3.3.4 Sequencing of the ITS region.

The ITS regions of three isolates of *E. lata* and one isolate of *Botryosphaeria* sp. were directly sequenced on both strands from PCR products obtained using the ITS5 and ITS4 primers (Table 3.5). The region was initially sequenced using the ITS1, ITS5 and ITS4 primers. However, where errors were encountered the primers ITS2 (Table 2.2) and ITS3 (Table 2.2) were used to sequence the ITS1 and ITS2 rDNA regions separately (Fig. 3.8).

Table 3.5 Fungal species and strains for which the ITS region (ITS1-5.8S-ITS2 rDNA) has been sequenced in this project.

Fungal species.	Strain.	Origin.	Sequence (bp)*.
E. lata	E11787	Eskdale, Hawkes Bay 1993.	504
E. lata	E11788	Eskdale, Hawkes Bay 1993.	504
E. lata	EL01	ex. W. F. Hartill.	505
Botryosphaeria sp.	Bot01	ex. W. F. Hartill.	477

*18S and 28S rDNA priming sites are not included in sequence.

The sequences obtained for these New Zealand isolates were in agreement with the ITS sequence subsequently obtained for *E. lata* from France (Lecomté *et al.*, 2000) (GenBank accession number AF099911). The aligned sequences are illustrated in Fig. 3.10. The conserved 5.8S rDNA is found at the centre of the alignment (bp 211-369). No base pair changes were found in the *E. lata* 5.8S rDNA sequences.

Sequencing resolved the exact lengths of the ITS region. There was very little variation in sequence length between the three New Zealand *E. lata* isolates (Table 3.5). The *E. lata* sequences were not identical and the four isolates (E11787, E11788, EL01 and AF099911) exhibited four sequence haplotypes. In total there are eight nucleotide changes between the four *E. lata* ITS sequences.

Variations in the ITS sequence between the four isolates ranged from single nucleotide substitutions (Fig. 3.10, *E. lata* E11788 bp 425, C to G; *E. lata* EL01 bp 483, G to A) to a single nucleotide deletion (C) which is subsequently corrected by an insertion (A) (Fig. 3.10, *E. lata* AF099911 bp 98-100). The ITS1 region (bp 1-210) of the three New Zealand *E. lata* isolates was completely conserved. However, the ITS2 sequences (bp 370-579) contain five nucleotide differences between the four isolates, including two nucleotide changes (bp 425 and bp 483), mentioned previously, found among the three New Zealand isolates.

These changes are likely to be real sequence polymorphisms and not PCR or sequencing errors, as the ITS regions from each New Zealand isolate were PCR amplified and sequenced independently on multiple occasions. Thus, it is not likely that these changes are PCR artefacts or sequencing errors, as (a) the changes were found on both the non-coding and coding strands of DNA that were sequenced independently and (b) the sequencing electropherograms do not indicate mixed signals (as would be expected with a PCR error directly sequenced) at either nucleotide change.

The web databases were searched to identify sequences related to the *E. lata* and *Botryosphaeria* sp. ITS regions, using the NCBI Basic Local Alignment Search Tool (BLASTn) website (URL http://www.ncbi.nlm.nih.gov/blast). Not surprisingly the closest related database sequence to the E11787, E11788 and EL01 sequences was the *E. lata* ITS1, 5.8s rDNA, ITS2 sequence (AF099911). This sequence showed 96% sequence identity with the E11787 sequence and 95% identity with the EL01 and E11788 sequences. This confirmed the identity of the DNA fragments sequenced as ITS1-5.8S-ITS2 sequences. Other closely related database sequences to the *E. lata* sequences (E11787, E11788, E01) include the *Xylaria cubensis* ribosomal ITS region (AF163032) with 94% identity and the *Diatrypella frostii* ribosomal ITS region (AF192322) with 92% identity between the sequences.

The *Botryosphaeria* sp. sequence (Bot01) was most similar to other *Botryosphaeria* sp. sequences (AB034821, AB034820, AB034819) found in the databases. There was 99% sequence identity between the sequences of these isolates. The Bot01 sequence also showed identity with *Botryosphaeria parva*, *Botryosphaeria ribis* and *Botryosphaeria dothidea* sequences.

3.3.5 Sequence alignment.

The DNA sequences for the species listed in Table 3.4 were aligned as described in Section 2.15. The three *E. lata* sequences and the *Botryosphaeria* sp. sequence obtained in this project were also included in the alignment (Fig.3.12).

The 5.8S rDNA region can be clearly seen as a highly conserved block between nucleotides 219 and 379. The alignment shows once again there is good conservation of both the ITS1 and the ITS2 sequences for the four *E. lata* sequences. No other species tested have ITS sequences identical or even very similar to those of *E. lata*.

Figure 3.10 Multiple alignment (Gene-Jockey II®) of ITS1-5.8S-ITS2 sequences of four isolates of *E. lata* and one isolate of *Botryosphaeria* species.

The start (>) and end (<) of the 5.8S rDNA are indicated. In the aligned sequences a gap in the alignment is indicated by a hyphen. The contig sequence at the top of the alignment indicates nucleotides that are common to all five sequences. *E. lata* isolates EL01, E11787 and E11788 and the *Botryosphaeria* species 01 isolate AF099911 isolate is from the GenBank database.

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                     10        20        30        40        50        60        70        80        90       100       110
                      |         |         |         |         |         |         |         |         |         |         |
Contig# 1            NNNNNNNNGAGNNNNNNNNTNCAGAGTTACCTAA CTCCAAACCCATGTGAACTTACCTATGTTGCCTCGG CGGGGAAGCCTACCCCGGTACCTACCCTGTAGCTACCCGGG
Eutypa lata EL01     --------------------CAGAGTTACCTAA CTCCAAACCCATGTGAACTTACCTATGTTGCCTCGG CGGGGAAGCCTHCCC GGTACCTACCCTGTAGCTACCCGGG
Eutypa lata EL11787  ----------------CAGAGTTACCTAA CTCCAAACCCATGTGAACTTACCTATGTTGCCTCGG CGGGGAAGCCTHCCC GGTACCTACCCTGTAGCTACCCGGG
Eutypa lata EL11788  ----------------CAGAGTTACCTAA CTCCAAACCCATGTGAACTTACCTATGTTGCCTCGG CGGGGAAGCCTACCC GGTACCTACCCTGTAGCTACCCGGG
Eutypa lata AF099911 ACTTGCGGGAGGGATCATTACGGAGTTACCTAAACTCCAAACCCATGTGAACTTAC TATGTTGCCTTGGGCGGGGAAGCTTACCCCGGTHCTTACCTGATAGCTACCCGGG
Botryosphaeria sp. 01  GAG   CTCCGGC TCGNCT  CTCCCHCCCAATGTGTACCTHCCTCTGTTGCTTTGG CGGG        CCGCGGT CCTCCGCACCGGCGCCCTTCG

                     120       130       140       150       160       170       180       190       200       210       220
                      |         |         |         |         |         |         |         |         |         |      > |
Contig# 1            AGCGAGCTACCCTGTAGCCCGCTGCAGGCCTACCCGCCGGTGGACACTTAAACTCTTGTTTTTTTAGTGATTATCTGAGTGTTTATACTTAATAAGTTAAAACTTTCAACAA
Eutypa lata EL01     AGCGAGCTACCCTGTAGCCCGCTGCAGGCCTACCCGCCGGTGGACACTTAAACTCTTGTTTTTTTAGTGATTATCTGAGTGTTTATACTTAATAAGTTAAAACTTTCAACAA
Eutypa lata EL11787  AGCGAGCTACCCTGTAGCCCGCTGCAGGCCTACCCGCCGGTGGACACTTAAACTCTTGTTTTTTTAGTGATTATCTGAGTGTTTATACTTAATAAGTTAAAACTTTCAACAA
Eutypa lata EL11788  AGCGAGCTACCCTGTAGCCCGCTGCAGGCCTACCCGCCGGTGGACACTTAAACTCTTGTTTTTTTAGTGATTATCTGAGTGTTTATACTTAATAAGTTAAAACTTTCAACAA
Eutypa lata AF099911 AGCGAGCTACCCTGTAGCCCGCTGCAGGCCTACCCGCCGGTGGACACTTAAACTCTTGTTTTTT AGTGATTATCTGAGTGTTTATACTTAATAAGTTAAAACTTTCAACAA
Botryosphaeria sp. 01 AG GGGCTGGCCAGC GCCCGCCAGAGGACCA      TAAA ACTCCAGTCAGTGAACTTC  GCA    GTCTGAAAAACAAG  TTAATAAACTAAAACTTTCAACAA

                     230       240       250       260       270       280       290       300       310       320       330
                      |         |         |         |         |         |         |         |         |         |         |
Contig# 1            CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGT
Eutypa lata EL01     CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGT
Eutypa lata EL11787  CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGT
Eutypa lata EL11788  CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGT
Eutypa lata AF099911 CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGT
Botryosphaeria sp. 01 CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGT

                     340       350       360       370       380       390       400       410       420       430       440
                      |         |         |         |         |         |         |         |         |         |         |
Contig# 1            ATTCTAGTGGGCATGCCTGTTCGAGCGTCATTTCGACCTTCAAGCCCTAGCTGCTTGGTGTTGGGAGCCTATCTCCGGATA GCTCCTCAAAAGCATTGGCGGAGTCGCGGT
Eutypa lata EL01     ATTCTAGTGGGCATGCCTGTTCGAGCGTCATTTCGACCTTCAAGCCCTAGCTGCTTGGTGTTGGGAGCCTATCTCCGGATA GCTCCTCAAAAGCATTGGCGGAGTCGCGGT
Eutypa lata EL11787  ATTCTAGTGGGCATGCCTGTTCGAGCGTCATTTCGACCTTCAAGCCCTAGCTGCTTGGTGTTGGGAGCCTATCTCCGGATA GCTCCTCAAAAGCATTGGCGGAGTCGCGGT
Eutypa lata EL11788  ATTCTAGTGGGCATGCCTGTTCGAGCGTCATTTCGACCTTCAAGCCCTAGCTGCTTGGTGTTGGGAGCCTATCTCCGGATA GCTCCTGAAAAGCATTGGCGGAGTCGCGGT
Eutypa lata AF099911 ATTCTAGTGGGCATGCCTGTTCGAGCGTCATTTCGACCTTCAAGCCCTAGCTGCTTGGTGTTGGGAGCCTATCTCCGGATA GCTCCTCAAAAGCATTGGCGGAGTCGCGGT
Botryosphaeria sp. 01 ATTCCGAGGGGCATGCCTGTTCGAGCGTCATTTCHACCCTCAAGCTC  TGCTTGGTATTGGGCCCCGTCCTCCACGGACGCGCCTTAAAGACCTCGCCGGTGGCGTCTT

                    450       460       470       480       490       500       510       520       530       540       550       560
                     |         |         |         |         |         |         |         |         |         |         |         |
Contig# 1            GACCCCAAGCGTAGTA ATTCTTCTCGCTTTAGGTGTGTCACGGCTGACGTCTTGCCGTTAAACCCCCAATTTTTTAAATGGTTGACC CGGA AGGTAGGAATACC  T
Eutypa lata EL01     GACCCCAAGCGTAGTA ATTCTTCTCGCTTTHGATGTGTCACGGCTGACGTCTTGCCGTTAAACCCCCAATTTTTTAAATG
Eutypa lata EL11787  GACCCCAAGCGTAGTA ATTCTTCTCGCTTTAGGTGTGTCACGGCTGACGTCTTGCCGTTAAACCCCCAATTTTTTAAATG
Eutypa lata EL11788  GACCCCAAGCGTAGTA ATTCTTCTCGCTTTAGGTGTGTCACGGCTGACGTCTTGCCGTTAAACCCCCAATTTTTTAAATG
Eutypa lata AF099911 GGCCCCAAGCGTAGTA ATTCTTCGCGCTTTHGGTGTGTCACGGCTGACGTCTTGCCGTTAAACCCCCAATTTTTTAAATGGTTGACCTCGGATAAGGTAGGAATACCGCT
Botryosphaeria sp. 01 G CCTCAAGCGTAGTAGAAAACACCTCGCTTTGGA GCG CACGGC   GTCG    CCCG            CCGGACGA    ACCTTT

                    570
                     |
Contig# 1            GAA TTAAA ATHTCAAGG
Eutypa lata AF099911 GAACTTAAGCATATCAAGG
Botryosphaeria sp. 01 GAATT   ATTTCTCAAG
```

Variations in the sequences range from single base pair changes to multiple changes representing deletions and insertions. Most changes were present in several species but some single base pair changes were unique to a particular species. More sequence variation was evident in ITS1 with only a few nucleotides showing complete homology across all species examined, whereas, ITS2 sequences were more conserved with many short regions of complete identity interspersed with more variable regions.

3.3.6 Primer design.

On the basis of sequence data for the ITS regions two pairs of *E. lata* specific PCR primers were designed. A nested design was chosen for the species-specific primers as this significantly increases the sensitivity and specificity of the PCR. The nested design involves the use of primers E.l.ITS1 and E.l.ITS4 in the first round of PCR, and primers E.l.ITS2 and E.l.ITS3 in the second (Fig.3.11).
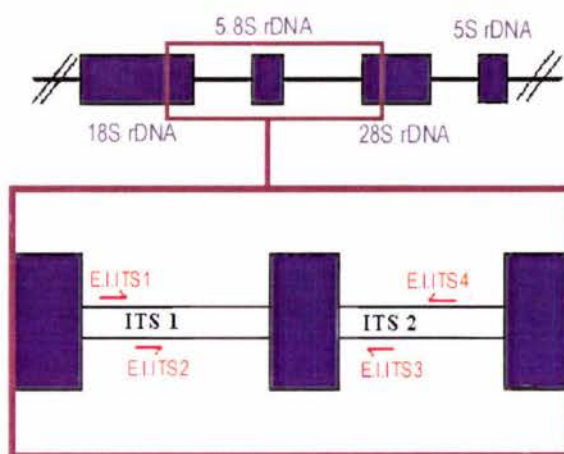
Figure 3.11 Design of the *E. lata* specific nested PCR reactions.

Primers targeting the *E. lata* ITS regions were designed so that the last four bases at the 3' termini hybridised with regions specific to the *E. lata* rDNA sequence. The positions of the *E. lata* specific primers are indicated on the alignment of fungal ITS regions (Fig. 3.12). The primer E.l.ITS1 (Table 2.2) was located at 74-93 on the alignment, E.l.ITS2 (Table 2.2) at 112-132, E.l.ITS3 (Table 2.2) at 410-445 and E.l.ITS4 (Table 2.2) at 509-530. Therefore, E.l.ITS1 and E.l.ITS2 are specific to the *E. lata* ITS1 region and E.l.ITS3 and E.l.ITS4 are specific to the *E. lata* ITS2 region. Primer lengths were 19 nucleotides for E.l.ITS1, 21 nucleotides for E.l.ITS2 and 22 nucleotides for E.l.ITS3 and E.l.ITS4. The positions of the primers on the alignment does not match the length of the primers as in some cases the primers span gaps in the alignment sequence. The G+C content for each primer varied from 68% for E.l.ITS1 to 66% for E.l.ITS2 and 59% each for E.l.ITS3 and E.l.ITS4. The melting temperatures for each primer were matched as follows: E.l.ITS1 52°C, E.l.ITS2 55°C, E.l.ITS3 53°C and E.l.ITS4 53°C.

Figure 3.12 Multiple sequence alignment (Gene-Jockey II®) ITS1-5.8S-ITS2 of plant pathogenic fungi. The start (>) and end (<) of the 5.8S rDNA gene are indicated. Blocks in the alignment indicate the locations of the primers pairs E.l.ITS1-E.l.ITS4 and E.l.ITS2-E.l.ITS3, designed for the detection of *E. lata*. The sense of each primer is in accordance to the arrow reported above the sequences.

E.I.ITS1> E.I.ITS2>

Contig# 1
E. lata EL01
E. lata EL11788
E. lata EL11787
E. lata AF099911
A. alternata
A. alternata AA6
B. dothidea
B. obtusa
Botryosphaeria sp. 01
B. cinerea
C. gloeosporioides
C. graminicola
D. insculpta
Fusarium sp.
Gliocladium sp. FI-360
Gliocladium sp. FI-442
L. theobromae
Penicillium sp. RMF8832
Penicillium sp. WSF420
P. palmarum
P. aleophilum
P. chlamydosporum
Phaeoacremonium sp.
P. longicolla
Phomopsis sp. 00044
Phomopsis sp. 00047

Contig# 1
E. lata EL01
E. lata EL11788
E. lata EL11787
E. lata AF099911
A. alternata
A. alternata AA6
B. dothidea
B. obtusa
Botryosphaeria sp. 01
B. cinerea
C. gloeosporioides
C. graminicola
D. insculpta
Fusarium sp.
Gliocladium sp. FI-360
Gliocladium sp. FI-442
L. theobromae
Penicillium sp. RMF8832
Penicillium sp. WSF420
P. palmarum
P. aleophilum
P. chlamydosporum
Phaeoacremonium sp.
P. longicolla
Phomopsis sp. 00044
Phomopsis sp. 00047

PCR amplification with the primers E.l.ITS1 and E.l.ITS4 was expected to produce a PCR product approximately 430 bp in size, while PCR amplification with the inner E.l.ITS2 and E.l.ITS3 primers was expected to produce a PCR product approximately 315 bp in length.

Pair wise matching of the primers, to each of the fungal ITS sequences (Table 3.4) was carried out in a computer simulation using the Gene-Jockey II® sequence handling programme, with a minimum alignment of 6 nucleotides with gaps. Two other primers not shown above showed a gapped alignment to a *Pestalotia palmarum* sequence (AF009818) as well as perfect alignment to the *E. lata* sequences. These primers were discarded and primers designed to new regions of the alignment. This result confirmed the sensitivity of the computer simulation for determining whether or not the primer is specific for *E. lata*.

3.3.7 PCR amplification with the *Eutypa lata* specific primers.

PCR experiments with the specific primer pairs, E.l.ITS1 and E.l.ITS4, E.l.ITS1 and E.l.ITS3, E.l.ITS2 and E.l.ITS3, and E.l.ITS2 and E.l.ITS4, with genomic DNA template produced bands in accordance with those predicted from the sequence analysis for all *E. lata* isolates (results not shown).

3.3.7.1 Primers E.l.ITS1 and E.l.ITS4

Initially, PCR amplification with the primers E.l.ITS1 and E.l.ITS4 was performed with an annealing temperature of 55°C, however, the annealing temperature was increased to 62°C to raise the specificity of the reaction, in order to eliminate PCR products produced by *Botryosphaeria* species templates (subsequently found to be contaminated with *E. lata* DNA). The primer pair yielded a single amplicon of approximately 450 bp when they were used for PCR with DNAs extracted from the four laboratory *E. lata* stock cultures (E11787, E11788, EL01and E11PDA2). This product is close to the predicted size of 430 bp indicating the primers were indeed amplifying the *E. lata* ITS region (Fig. 3.13).

The identity of this PCR product as *E. lata* ITS rDNA was confirmed by sequence analysis. The fragments amplified from E11787 and E11788 DNA templates with the primers E.l.ITS1 and E.l.ITS4 were co-purified (the sequences differ at nucleotides 321 and 401 only) and used as a template for direct sequencing using the E.l.ITS1 primer. Pair wise alignments of the resulting sequence with the ITS sequences from isolates E11788 and E11787 established that the primers had indeed amplified the ITS region.

3.3.7.2 Primers E.l.ITS2 and E.l.ITS3.

An initial attempt at nested *E. lata* specific PCR with the primers E.l.ITS2 and E.l.ITS3 using the PCR product from *E. lata* E11788 with primers E.l.ITS1 and E.l.ITS4 as a template, failed to produce PCR products of the expected size. This initial attempt at PCR contained 1 μL of the 1ˢᵗ round PCR product, 1x PCR buffer (Gibco BRL), 1.5 mM Mg^{2+}, 100 μM dNTPs, 1 U of *Taq* polymerase (Gibco BRL) and 2 μM of each primer. The cycling conditions were 94°C for 2 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min. A final elongation period of 72°C for 2 min was followed by soaking at 4°C.
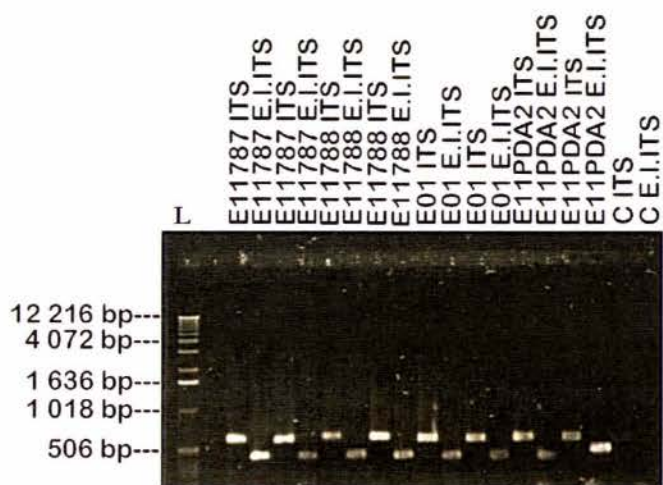
Figure 3.13 **Agarose gel (1%) with PCR amplification products of the ITS1-5.8s-ITS2 region of the rDNA from 4 *E. lata* isolates in duplicate, using the primers E.l.ITS1 and E.l.ITS4 (E.l.ITS) and ITS1 and ITS4 (ITS). C ITS and C E.l.ITS indicate negative (no DNA) controls for each of the primer sets; L- 1 Kb ladder. The numbers at the side of the figure indicate the fragment sizes of the 1 Kb ladder.**

In order to achieve specific nested amplification of the *E. lata* ITS region, further adjustments to the PCR protocol were needed. A titration of the amount of first round PCR product to be added to the PCR reaction demonstrated that 0.4 µl of PCR product effected the clearest band as viewed by gel electrophoresis. Additionally, the number of complete PCR cycles was increased from 30 to 35. The use of 0.4 µL of PCR template and 35 PCR reaction cycles with an annealing temperature of 62°C (as for primers E.l.ITS1 and E.l.ITS4) was adopted as the standard PCR amplification procedure for PCR when using primers E.l.ITS2 and E.l.ITS3. These conditions are described in the materials and methods (Section 2.13.1.2).

All *E. lata* PCR templates tested produced a single PCR product approximately 350 bp in size (Fig. 3.14). The estimated PCR product size for the primers is 330 bp.

3.4 Confirmation of primer specificities for *Eutypa lata* using fungi isolated from diseased grapevines.

3.4.1 *Botryosphaeria* sp. PCR amplification.

Initially, 5 *Botryosphaeria* sp. DNA samples were PCR amplified with the *E. lata* specific primers E.l.ITS1 and E.l.ITS4. Four of the isolates were isolated from infected grapevine cordons (Section 3.3) and the other was the laboratory stock culture Bot. 01, previously used for sequencing of the ITS region using universal rDNA primers (Section 3.3.4).

Surprisingly primers E.l.ITS1 and E.l.ITS4 produced a single PCR product of approximately 450 bp with each of the *Botryosphaeria* templates. The internal *E. lata* specific primers E.l.ITS2 and E.l.ITS3 also amplified a single PCR product of the size
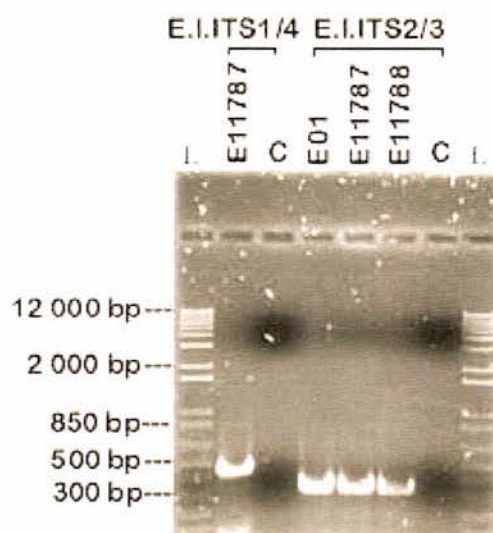
Figure 3.14 Agarose gel (1%) with PCR amplification products of the ITS1-5.8s-ITS2 region of the rDNA from 3 *E. lata* isolates, using the primers E.l.ITS2 and E.l.ITS3. The size of the PCR products obtained with the E.l.ITS2 and E.l.ITS3 were compared to PCR products with the first round PCR primers E.l.ITS1 and E.l.ITS4. C indicates a negative (no DNA) control. The numbers at the side of the figure indicate the fragment sizes in L, the 1 Kb+ ladder (Gibco BRL).

expected for *E. lata* when the product from the first round of PCR amplification was used as DNA template. The intensity of the PCR product produced varied greatly between repetitions, and in some cases, the templates failed to produce a PCR product at all. However, increasing the annealing temperature from 55°C to 62°C markedly reduced the amount of PCR product produced with the *Botryosphaeria* samples. Hence, 62°C was adopted as the standard annealing temperature for the *E. lata* specific primers (Section 3.4.7).

The PCR product produced by the *Botryosphaeria* sp. stock culture Bot. 01 and primers E.l.ITS1 and E.l.ITS4 was purified (Section 2.6.4) and directly sequenced (Section 2.14) with the primer E.l.ITS1. The sequence results conclusively showed the PCR product was due to contamination of the *Botryosphaeria* sp. isolates, or the DNA extracted from these isolates, with *E. lata* DNA (results not shown). The *Botryosphaeria* sp. E.l.ITS1 and E.l.ITS4 PCR product exhibited the same ITS sequence haplotype as the *E. lata* E11787 sequence.

As the contaminated DNA sample of *Botryosphaeria* sp. Bot 01 was different from the DNA sample used for sequencing the *Botryosphaeria* sp. ITS region, the contamination was traced back to a DNA extraction procedure in which DNA was extracted from the *Botryosphaeria* sp. Bot 01 and *E. lata* stock cultures E11787, E11788 and EL01 on the same day. However, this could not explain the contamination of the 4 *Botryosphaeria* sp. strains isolated from the grapevine cordons, the DNA of which was extracted on a different day. Sequencing of the E.l.ITS1 and E.l.ITS4 PCR products from these isolates was not performed. The origin of the contamination in these *Botryosphaeria* isolates cannot be traced. The isolates from which the DNA was extracted were possibly mixed cultures, alternatively, the DNA could have been resuspended in contaminated buffer.

Since the existing batches of *Botryosphaeria* DNA were contaminated with *E. lata* fresh single spore isolates of *Botryosphaeria obtusa* (Section 2.3.1.4.2) were prepared from *Botryosphaeria obtusa* E10 PDA 10 (Erindale vineyard, vine 10, PDA isolation agar, disease lesion 10), *Botryosphaeria obtusa* D2 15%V8 1 (Der Ley vineyard, vine 2, 15%V8 isolation agar, disease lesion 1), *Botryosphaeria obtusa* E8 PDA 18, *Botryosphaeria obtusa* E7 PDA 21 and *Botryosphaeria obtusa* E10 malt 1. PCR amplification of DNA from these cultures with the *E. lata* specific primers E.l.ITS1 and E.l.ITS4 failed to produce any PCR products (Fig. 3.15). PCR amplification was also performed for the inner *E. lata* specific primers E.l.ITS2 and E.l.ITS3 (results not shown). Valid *E. lata* controls were PCR amplified in each case and the experiments were repeated twice. PCR products were not produced in the negative (no DNA) controls.

To confirm that the quality of the newly isolated *Botryosphaeria* DNA was appropriate for PCR amplification a separate positive control PCR reaction was performed using the universal ITS primers ITS1 and ITS4. A single PCR product of approximately 550 bp in size was produced in each case.

3.4.2 PCR amplification with other fungal species.

The DNA from a wide range of fungal species isolated from infected grapevine cordons (Section 2.3.1.5) gave no PCR amplification products with the *E. lata* specific primers E.l.ITS1 and E.l.ITS4 (Fig. 3.15). The DNA was checked against the Universal ITS primers ITS1 and ITS4 as positive controls: All products were of the expected size (between 450 bp and 800 bp). In all, eight different non *E. lata* fungal species were tested with the Eutypa specific primers and all (excluding the *Botryosphaeria* species) are listed in Table 3.6. This showed the DNA templates were capable of PCR amplification and any negative PCR results with the E.l.ITS primers were due to primer mismatch and not to poor quality DNA. Hence the primers appear to be specific for *E. lata*.

3.5 Comparison of *Eutypa lata* detection by specific PCR amplification and selective media isolation from infected wood.

The sensitivity of the new DNA based *E. lata* detection procedure was compared with that of the traditional selective media identification assay. *E. lata* cultures, isolated from grapevine cordons (Section 3.3), that displayed considerable variation in morphology (Fig. 3.6) were tested with the *E. lata* specific primers to determine whether their identification as *E. lata* by cultural characteristics was correct. The PCR products produced by the nine *E. lata* cultures (bottom 3 rows from Fig. 3.6) were compared to *E. lata* culture E11787 as a positive control and a single *Botryosphaeria* culture (E11 PDA 1, middle culture top row of Fig. 3.6). Of the nine *E. lata* cultures, eight produced a single PCR product of the expected size (by comparison with the positive control) with the *E. lata* specific primers E.l.ITS1 and E.l.ITS4, confirming their identity as *E. lata* (Fig. 3.16). The culture E10 15%V8 10 (middle culture 2nd row from the top of Fig. 3.6) failed to produce a PCR product with the E.l.ITS1 and E.l.ITS4 primer pair. The *Botryosphaeria* culture control (E11 PDA 1) also failed to produce PCR products with the *E. lata* specific primers.

The experiment was repeated using the nested E.l.ITS2 and E.l.ITS3 primers (results not shown). Once again only eight of the *E. lata* cultures produced a single PCR amplicon of the expected size for *E. lata*. The E10 15%V8 10 (middle culture 2nd row from the top of Fig. 3.6) culture did not produce any PCR products with the *E. lata* specific primers. Both the positive (E11787) and negative (no DNA and *Botryosphaeria* sp. E11 PDA 1) PCR controls were as expected. The E10 15%V8 10 culture produced a PCR product approximately 530 bp with the universal ITS primers ITS1 and ITS4 (results not shown). This band is smaller than expected for both *E. lata* and *Botryosphaeria* species. This indicated the E10 15%V8 10 culture was not *E. lata*.

Of the nine *E. lata* cultures (Fig. 3.6), the morphology of the E10 15%V8 10 culture, (flat, grey mycelia) is closer to that of *Botryosphaeria* sp. than the other eight *E. lata* cultures.

Table 3.6 *E. lata* specific PCR analysis of fungi found in diseased grapevine wood.

Fungal sp.	Size of PCR products obtained with primers ITS1 and ITS4 (bp)	Size of PCR products produced with the *E. lata* specific E.l.ITS1 and E.l.ITS4 primers (bp)
Alternaria alternata E9 malt 5	550	No PCR product
Epicoccum sp. 1 E7 PDA 5	600	No PCR product
Epicoccum sp. 2 E8 malt 16	600	No PCR product
Fusarium sp. 1 E8 malt 8	580	No PCR product
Fusarium sp. 2 E8 malt 9	580	No PCR product
Gliocladium sp. 1 E8 malt 7	550	No PCR product
Gliocladium sp. 2 E10 malt 8	550	No PCR product
Pestalotia sp. E7 malt 2	600	No PCR product
Rhizopus sp. 1 E8 PDA 7	560	No PCR product
Rhizopus sp. 2 E10 PDA 9	560	No PCR product

Origin of isolate names, E indicates Erindale vineyard and the following number indicates the vine from which it was isolated, then the agar on which it was isolated and last the number of the disease lesion from which it was isolated.

Figure 3.15 Amplified ITS1-5.8s-ITS2 products fractionated by 1% agarose gel electrophoresis.

A) Agarose gel (1%) of PCR products amplified from the genomic DNA of 5 *Botryosphaeria obtusa* single ascospore isolates with the ITS primers (ITS1-ITS4) and *E. lata* specific (E.l.ITS) primers (E.l.ITS1-E.l.ITS4). The reaction products from a positive control, *E. lata* E11787 genomic DNA, are shown for both sets of primers. C ITS and C E.l.ITS indicate negative (no DNA) controls; L indicates the 1 Kb+ ladder (Gibco BRL).

B) Agarose gels (1%) of PCR products amplified from the genomic DNA of a variety of fungal grapevine pathogens with the *E. lata* specific (E.l.ITS) primers, E.l.ITS1-E.l.ITS4. Four *E. lata* isolates are included as positive controls. C indicates a negative (no DNA) control; L indicates the 1 Kb+ ladder (Gibco BRL).

Numbers at the sides of the figures indicate the fragment sizes of the 1 Kb+ ladder (Gibco BRL).

A)

B)

Epicoccum sp. 1
Epicoccum sp. 2
Fusarium sp. 1
Fusarium sp. 2
Pestalotia sp.
Rhizopus sp. 1
Rhizopus sp. 2
Gliocladium sp. 1
Gliocladium sp. 2
Alternaria sp.

E. lata E01
E. lata E11787
E. lata E11788
E. lata E11PDA2
B. obtusa E10PDA10
B. obtusa D215%V81
B. obtusa E8PDA18
B. obtusa E7PDA21
B. obtusa E10malt1

12 000 bp
2 000 bp
850 bp
500 bp
300 bp
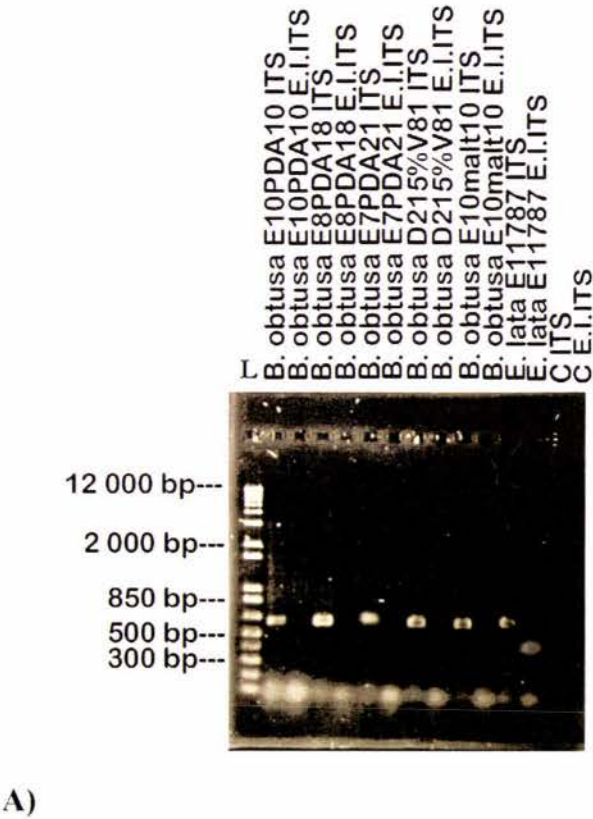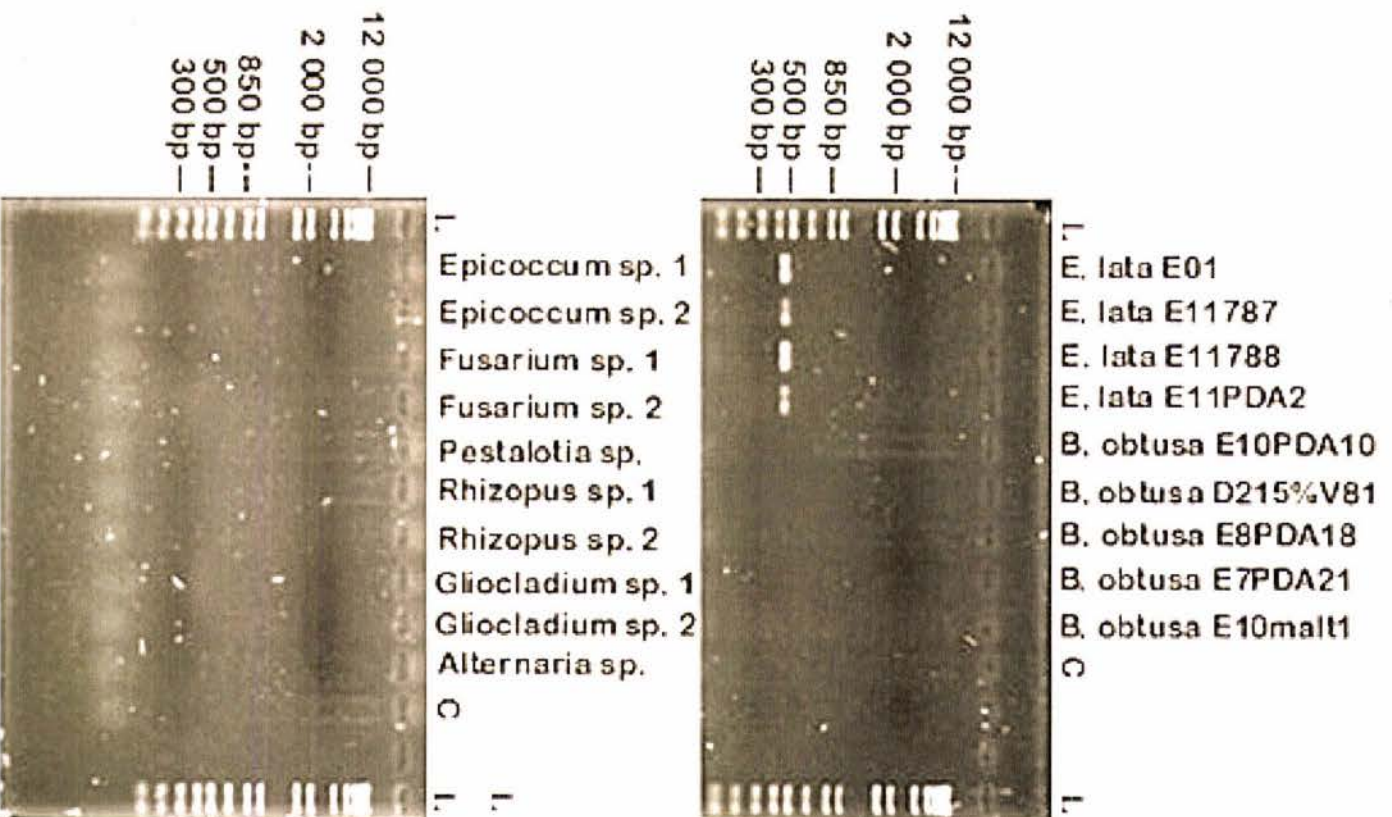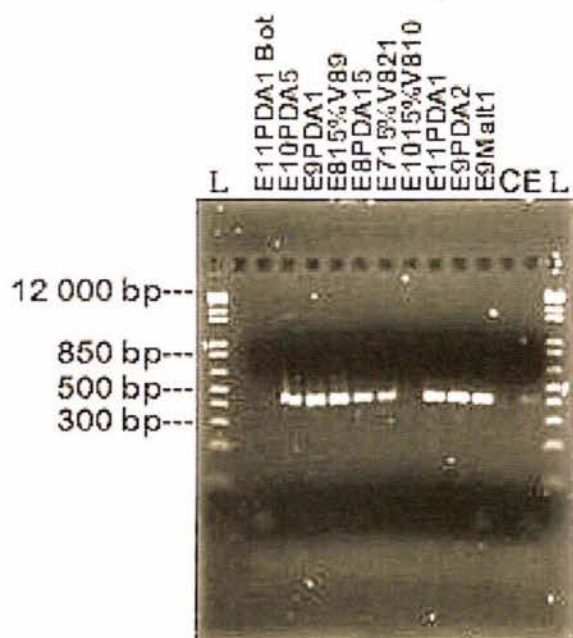
Figure 3.16 Agarose gel (1%) of PCR products amplified from the genomic DNA of 9 *E. lata* isolates and a *Botryosphaeria* sp. isolate (E11PDA1 Bot) with the *E. lata* specific ITS primers (E.l.ITS1-E.l.ITS4). The reaction products from a positive control (E), *E. lata* E11787 genomic DNA, are shown, however, due to the reproduction of the photograph the band is faint. C indicates a negative (no DNA) control; L indicates the 1 Kb+ ladder (Gibco BRL). Numbers at the side of the figure indicate the fragment sizes of the 1 Kb+ ladder.

3.6 Development of an assay to detect *Eutypa lata* in grapevine wood.

The use of a PCR based detection system avoids the visual comparison of isolates with reference cultures and the need to wait for the uncertain formation of characteristic conidia, which can take one to two months to complete. Additionally, PCR assisted identification of *E. lata* cultures can be performed as soon as sufficient fungal material is produced on agar medium, thus a substantial time saving is created.

However, additional timesavings can be made by minimising the time taken for DNA extraction, by detecting the fungus directly from crude mycelial extracts. Additionally, to develop an efficient and rapid detection system, it is desirable to directly extract fungal DNA from diseased samples, thus completely avoiding the isolation and growth of the fungus from the diseased wood. The feasibility of detecting *E. lata* directly from mycelial extracts or from total DNA isolated from diseased grapevine cordons was assessed.

3.6.1 Detection of *Eutypa lata* direct from mycelial extracts.

The use of crude *E. lata* DNA from mycelial extracts as a PCR template gave mixed results with the *E. lata* specific primers E.l.ITS1 and E.l.ITS4. PCR amplification of the *E. lata* ITS region using a boiled agar culture template (Section 2.13.2.1) and primers E.l.ITS1 and E.l.ITS4 did not result in any PCR products. The addition of non-fat skim

milk powder, to act as a blocking agent to overcome inhibitors in the PCR template, also failed to obtain any PCR products.

PCR amplification direct from 5 day old cultures (Section 2.13.2.2) using the same primers resulted in two PCR products with the *E. lata* cultures. The major PCR product was produced with all isolates and was approximately 500 bp in size. However, a band of the same size was also produced in the PCR negative control reaction, in which a yellow pipette tip was struck across a sterile PDA agar plate. A second PCR product was produced only in PCR reactions containing the *E. lata* cultures. This product was approximately 100 bp in size and was not expected to be the *E. lata* ITS rDNA product. In a repeat of this experiment, the *E. lata* cultures did not produce any PCR products and the negative control produced two PCR products (450 bp and > 650 bp) while the positive control of *E. lata* DNA (isolate E11787) produced a single PCR product approximately 450 bp and similar to that produced in the negative control reaction.

PCR amplification using a method originally developed for fungal spore extracts (Section 2.13.2.3) resulted in limited success with *E. lata* mycelium and the E.l.ITS1 and E.l.ITS4 primers. The *E. lata* cultures failed to produce enough spores for use as a PCR template so the protocol was adapted to use mycelia. The standard PCR procedure failed to produce any PCR products, however, when glycerol (final concentration 5%) was added a single, faint PCR product of the expected size (430 bp) was obtained from the mycelium of *E. lata* (isolate E11787) (Fig. 3.17). However, the addition of Triton X-100 as well as glycerol to the PCR reaction inhibited the positive control of *E. lata* DNA (isolate E11787) under these conditions, PCR products were not obtained in the negative PCR control or with the *E. lata* isolates EL01 or E11788.
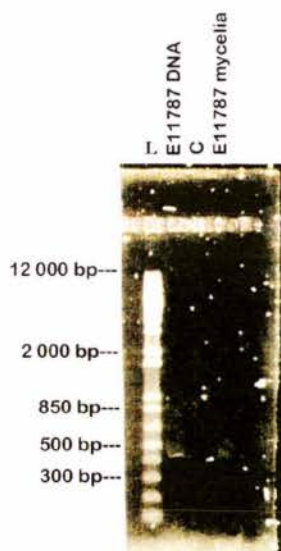
Figure 3.17Agarose gel (1%) of PCR products amplified from the mycelial extract of the *E. lata* isolate E11787 with the *E. lata* specific ITS primers (E.l.ITS1-E.l.ITS4). The reaction products from a positive control, *E. lata* E11787 DNA and a negative (no DNA) control (C) are included. Due to a poor reproduction of the gel photograph the bands are faint. L= 1 KB+ ladder (Gibco BRL). The numbers at the side of the figure refer to the fragment sizes in the 1 KB+ ladder.

A crude mycelial extract prepared according to the protocol of Lecomté *et al.* (2000) (Section 2.13.2.4) did not consistently PCR amplify a single PCR product of the expected size with the primers E.l.ITS1 and E.l.ITS4. The experiment was repeated in triplicate with varying results (Fig. 3.18). *E. lata* extracts from isolates E11787 and E11788 produced a single PCR product of the expected size (as compared with the positive control of *E. lata* EL01 DNA) in one of the replicates. *E. lata* mycelial extract from isolate EL01 failed to produce any PCR products with the E.l.ITS1 and E.l.ITS4 primers. The results from two of the replicates are shown in Fig. 3.18.
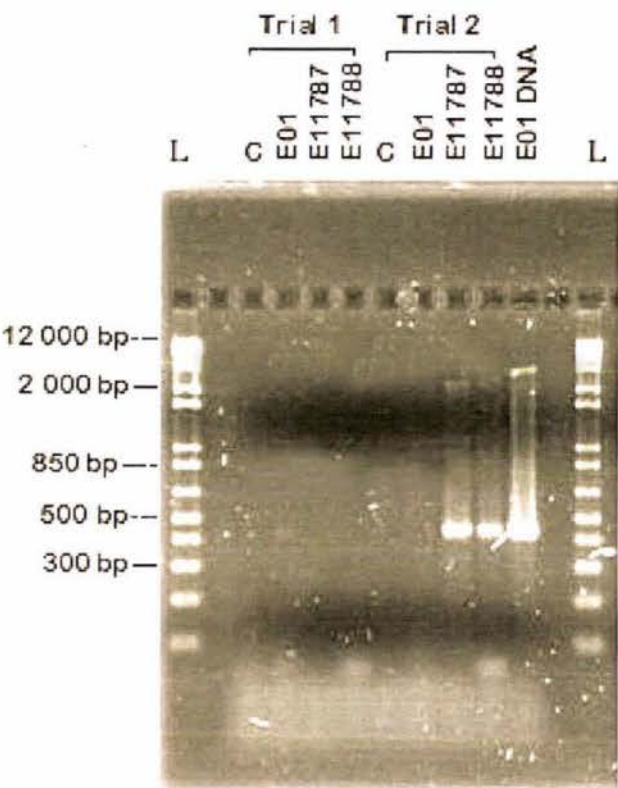
Figure 3.18 Agarose gel (1%) of PCR products amplified from the mycelial extracts of three *E. lata* isolates (E01, E11787 and E11788) with the *E. lata* specific ITS primers (E.l.ITS1-E.l.ITS4). The reaction products from a single positive control, *E. lata* EL01 DNA and a negative (no DNA) control (C) for each trial are included. L indicates the 1 Kb+ ladder (Gibco BRL) and the numbers to the left of the figure refer to the fragment sizes of the 1 Kb+ ladder.

3.6.2 Detection of *Eutypa lata* in grapevine wood.

Inoculated wood pieces were used to determine whether *E. lata* could be detected in grapevine wood by PCR amplification using total DNA isolated from the colonised wood as the DNA template. To this end wood chips from grapevine cordons were artificially inoculated with *E. lata* cultures (Section 2.3.1.6).

After twelve months of colonisation, the wood pieces had become dry and brittle. Although dry, the interior wood had a relatively unchanged appearance and the exterior surfaces were covered with a dry mat of fungal mycelia, which could be removed with a

scalpel blade. The exterior mycelial mat was removed and discarded. Wood samples used in the PCR reactions were taken from the interior wood below.

Primers E.l.ITS1 and E.l.ITS4 were used to attempt PCR amplification of *E. lata* DNA from wood inoculated with isolates E11787 and E11788, following a range of total DNA extraction procedures. Each experiment was repeated in triplicate with 1.0-5.0 mg of inoculated wood.

Direct PCR analysis of thin wood shavings did not indicate the presence of *E. lata* in the wood pieces examined. The addition of 50 μg of non-fat skim milk powder as a blocking agent to overcome plant derived PCR inhibitors did not enhance the specificity of the PCR reaction (results not shown).

After irradiating the wood sample in Tris-HCl for 1 min in the microwave, the colour of the buffer changed from colourless to brown. The intensity of the brown colour was directly proportional to the amount of wood added to the solution. However, no *E. lata* ITS rDNA PCR products were obtained with this method of crude DNA extraction using the E.l.ITS1 and E.l.ITS4 PCR primers. The PCR reaction positive control (*E. lata* E11787 DNA template) produced a single PCR product approximately 430 bp in size as expected and the negative control, containing 2 μL of irradiated sterile Tris-HCl, failed to produce any PCR products (results not shown).

Incubation of wood shavings at 95°C in sterile milli-Q water, as described in Lecomté *et al.* (2000) (Section 2.13.3.1) also failed to produce any PCR products with the E.l.ITS1 and E.l.ITS4 primers (results not shown). Positive controls of E11787 and E11788 DNA produced a single band of approximately 430 bp.

To ascertain whether *E. lata* had indeed established itself within the inoculated wood pieces, an attempt was made to re-isolate *E. lata* from the infected wood (Section 2.3.1.5). After two weeks incubation, characteristic white mycelial growth was not observed from any of the surface sterilised wood chips placed on PDA medium.

3.7 Discussion.

3.7.1 Collection of grapevine wood.

Although searches were made on several vines in each vineyard, no perithecia were found. This could indicate that the infections within the vineyards were relatively new and perithecia had not formed as it takes several years for them to appear. This hypothesis is supported by the fact that the Eutypa dieback infection was first noticed in 1995 in the Der Ley vineyard, only three years earlier. However, the infection since then had become very severe and widespread within the vineyard, indicating rapid dissemination. A source of ascospores would be expected for such a rapid spread of *E. lata* within the vineyard.

However, since no perithecia could be found within the vineyards, the source of ascospores for the initial and subsequent infections must have come from another vineyard in the Hawkes Bay area or an alternative host species, such as the many apricot or apple orchards in the surrounding area (Carter, 1991).

3.7.2 Analysis of *Eutypa lata* fungal community.

The objective of this work was to analyse the fungal community in vines displaying symptoms of grapevine dieback. Although *E. lata* was recovered from all the vines sampled, other fungi were often associated with it in the vines, as observed in France (Larignon & Dubos, 1997; Péros *et al.*, 1999). These associations may be frequent and this would explain the earlier difficulties in confirming the cause of dead arm disease as *E. lata* not *Phomopsis viticola* (Moller & Kasimatis, 1981).

The most frequent fungal association with *E. lata* in the current work was *B. obtusa*. This is hardly surprising in the vines taken from Erindale vineyard (*V7-V11*) as the Mt Albert HortResearch station had already diagnosed infection by *B. obtusa* in the vines. However, *V2* from Der Ley was also found to be heavily infected with *B. obtusa*. Interestingly, recent attempts to associate *E. lata* with Semillon decline in the Hunter Valley region, Australia, were unsuccessful (Castillo-Pando, Somers, Green, Priest & Sriskanthades, 2001). *B. obtusa* was detected there in affected vines at eleven sites in six vineyards. The frequency with which *B. obtusa* was isolated from wood of Semillon vines showing symptoms of dieback and the results of limited pathogenicity testing suggest that *B. obtusa* has a role in the cause of grapevine decline, at least in the Hunter valley region (Castillo-Pando *et al.*, 2001).

It is evident that vines showing Eutypa dieback symptoms harbour a fungal community similar to that described for vines with esca disease (Chiarappa, 1959; Larignon & Dubos, 1997) and in other woody species showing decay symptoms. The differences in the composition, structure and functioning of the fungal community probably cause large variations in disease and symptom development. Carter (1991) has already postulated that grapevine cultivars reputed to be highly susceptible to Eutypa dieback are likely to be those that exhibit spectacular foliage symptoms in response to the disease. It is then plausible that pseudo tolerance to Eutypa dieback infection, those vines exhibiting only mild dieback symptoms, may also be due to the fungal community associated with *E. lata* in the vines.

It is likely that fast growing fungal isolates, such as *Rhizopus*, *Mucor* and to a certain extent *Penicillium* sp., isolated from the vines out competed *E. lata* in the isolations. If this is in fact the case, the incidence of *E. lata* would be under reported in this study.

No attempt was made to correlate the type of lesion with the isolation of *E. lata* or other fungi as in similar studies (Larignon & Dubos, 1997; Péros *et al.*, 1999) and the sample size was too small to make any valid inferences on differences in co-colonising fungi between cultivars. However, sectorial brown lesions (Lesion E. Péros *et al.*, 1999) the typical internal symptoms of Eutypa dieback were prevalent in the vines. This sectorial brown lesion has also been associated with esca disease (Larignon & Dubos, 1997).

It has been suggested that *E. lata* may play a pioneer role in wood colonisation (Larignon & Dubos, 1997), paving the way for secondary infection by *Phellinus ignarius*, responsible for esca disease in grapevines. *Phaeoacremonium chlamydosporum* and *Phaeoacremonium aleophilum*, also involved in esca decay are thought to be able to colonise grapevines independently of *E. lata* (Pascoe & Cottral, 2000). *P. ignarius*, *P. chlamydosporum* and *P. aleophilum* were not isolated in this study indicating no link between the two diseases in the sampled vineyards. The

isolations of *P. ignarius*, *P. chlamydosporum* and *P. aleophilum* may also have been under reported in the current study, due to unsuitable growth conditions in culture and the fact they are difficult fungi to identify (Larignon & Dubos, 1997). Identification of these three fungi was not a primary objective in this study; however, few isolates remained unidentified.

Antagonism of *Gliocladium roseum* towards *E. lata* has been observed *in vitro* (Ricard, Grosclaude & Ale-Agha, 1974). However, *G. roseum* and *E. lata* were often isolated from the same disease lesion in the grapevines and occasionally from the same vine tissue sample. This is in agreement with Péros *et al.* (1999) who describe frequent simultaneous isolations of *G. roseum* and *E. lata*.

The presence of the *Libertella* stage of *E. lata* in the vines may suggest a role for the anamorph as spermatia in the fertilisation of perithecia. This is supported by the lack of evidence to implicate the anamorph as a propagule of Eutypa dieback (Carter, 1991; Munkvold *et al.*, 1993).

The results presented here emphasise the complex nature of grapevine dieback. The presence of other fungi may explain the wide variation in symptom development observed but other factors are undoubtedly functioning. Some of the other fungi associated with *E. lata* may be genetically diverse. This would determine if they are antagonistic to, or synergistic with *E. lata*, as well as deciding their degree of pathogenicity to grapevines. Another source of variation would be the effectiveness of host response. This could vary from vine to vine even on the same cultivar depending on vine age, architecture, cultural practices, soil and climatic conditions. A better knowledge of the functioning of the fungal community in relation to the host response and overall environment could be useful in determining more effective disease management strategies.

3.7.3 Sequence analysis of the ITS ribosomal DNA in three *Eutypa lata* isolates and development of a PCR based assay for the detection of *Eutypa lata*.

3.7.3.1 The Eutypa lata ITS sequence.

The sequences obtained for the New Zealand isolates of *E. lata* were in agreement with the ITS sequence already obtained for *E. lata* from France (Lecomté *et al.*, 2000) (GenBank accession number AF099911). However, the *E. lata* sequences were not identical and the four isolates (E11787, E11788, EL01 and AF099911) exhibited four sequence haplotypes. Variations in the ITS sequences between the *E. lata* isolates ranged from single base pair changes to multiple changes in sequence including single nucleotide insertions and deletions. Two nucleotide changes (Fig. 3.10, bp 425 and bp 483 on alignment) were found between the three New Zealand isolates. Although the PCR products sequenced were amplified with *Taq* DNA polymerase which lacks 3' to 5' exonuclease (Proof reading) activity, it is not likely that these nucleotide changes are PCR artefacts or sequencing errors, as the changes were found on both the non-coding and coding strands of DNA and the sequencing electropherograms did not indicate mixed signals (as would be expected with a PCR error directly sequenced) at either change.

Enzymes with 3' exonuclease activity, such as *Pwo* polymerase have better fidelity than *Taq* DNA polymerase and are a convenient way to ensure error proof sequence. Alternatively, decreasing the concentration of dNTPs and ensuring the concentration of all four nucleotides is the same (Bogetto *et al.*, 2000), or performing fewer cycles of PCR, improves PCR fidelity, since the product of each cycle becomes a template for subsequent cycles.

3.7.3.2 Primer design.

Two ITS-derived primer pairs, E.l.ITS1-E.l.ITS4 and E.l.ITS2-E.l.ITS3, which are specific for and detect the fungus *E. lata* have been developed in this work. The two pairs of primers, designed and used with PCR conditions established here show the potential of this method as a diagnostic test for *E. lata* in grapevine wood and rooted vine cuttings.

The results showed that each of the PCR primer pairs E.l.ITS1-E.l.ITS4 and E.l.ITS2-E.l.ITS3 amplified a specific fragment from the ITS region of *E. lata*. No amplification was observed with these primers when DNA from other fungi that commonly colonise grapevine wood concomitantly with *E. lata* was used. Thirty-five cycles of PCR amplification using the *E. lata* specific primers produced a sufficient amount of fragments of the predicted sizes (430 bp for E.l.ITS1-E.l.ITS4 and 330 bp for E.l.ITS2-E.l.ITS3) to visualise them on an ethidium bromide-stained agarose gel, when 2 µL of the PCR reaction was loaded.

During the development of the *E. lata* identification system outlined in this project, two other systems, developed in parallel, were published (Irelan, 1999; Lecomté *et al.*, 2000). The details concerning the *E. lata* detection system developed by Irelan are contained within a patent. The identification system is "a molecular detection tool that recognises the ITS region of *E. lata*", "using the detection tool the authors can detect the presence of the fungus in plant tissue".

Similarly, the PCR assay developed by Lecomté *et al.* (2000) enables "the unambiguous identification of *E. lata* in less than 5 h, from among the whitish mycelia obtained from wood chips deposited on agar plates. Of their six published *E. lata* specific primers designed to the ITS region, Lata 1 anneals to fourteen of the same nucleotides as the primer E.l.ITS2 (this study; Table 2.2) and Lata 2-1 and Lata 2-2 anneal to eighteen of the same nucleotides as primers E.l.ITS3 and E.l.ITS4 (this study; Table 2.2) respectively.

3.7.3.3 Primer specificity.

The primers developed by Lecomté *et al.* (2000) were tested against DNA from 60 isolates of *E. lata* from France, Spain and Italy. The primers consistently and specifically isolated the ribosomal ITS region from each isolate. The primers were also extensively tested against DNA from 96 isolates of fungi and bacteria representing more than 50 different species of microorganisms that are commonly associated with grapevine. No false positive signals were produced with any of the associated DNAs tested.

The inability to PCR amplify the grapevine DNA with the universal ITS primers suggests that DNAses in the leaf tissue were not removed or inactivated during the

extraction process, resulting in rapid degradation of the DNA and low DNA extraction efficiencies. The low DNA extraction efficiency could also imply that the DNA had been partially degraded in the leaf tissue prior to extraction since the sample material was not immediately frozen after removal from the vine. Alternatively, the DNA was intact when extracted but the subsequent degradation of DNA indicates that TE buffer (10:1) was not an appropriate buffering system for the grapevine DNA and resulted in rapid degradation of the DNA.

The specificity of primers developed to *Phaeoacremonium* sp. ITS sequences (Tegli *et al.*, 2000) was tested against grapevine DNA. The DNA was extracted from the interveinal mesophyll and petioles of expanded leaves of healthy looking grapevine plants (cv. Sangiovese), according to a grapevine DNA protocol developed by Lodhi *et al.* (1994). Grapevine DNA used in this project was extracted from freeze-dried whole leaf tissue (cv Chardonnay), using standard DNA extraction protocols developed by Amersham (Nucleon phytopure™ kit, Section 2.5.1), and Al-Samarrai and Schmid (2000, Section 2.5.2). Further DNA extractions attempted from grapevine leaf tissue should employ the protocol described by Lodhi *et al.* (1994). Alternatively, DNA extraction from fresh leaf tissue may give a higher extraction efficiency and yield clean DNA for PCR amplification.

3.7.3.4 Primer sensitivity.

Irelan (1999) compared the sensitivity of the DNA based *E. lata* detection procedure to that of the traditional selective media identification assay, by analysing approximately 60 infected spurs and pruning wounds. The PCR detection test was found to be more efficient and sensitive in all cases. Of 26 pruning wounds tested that yielded double positive results using the PCR test, *E. lata* mycelia were obtained from only six. A double blind test was performed by Lecomté *et al.* (2000), once again the PCR test was found to be more sensitive than the traditional selective media approach. Although, both PCR and subculturing identified two false positives out of 18 samples suspected to be *E. lata*, the test sensitivities differed when 15 samples classified as other mycelia were examined. These samples were not identified as *E. lata* by subculturing, however, *E. lata* was detected by PCR in two of these samples. Subsequent culturing confirmed that these two samples were indeed mixed cultures containing minute amounts of *E. lata*, These findings were mirrored in this study, as demonstrated by the specific and consistent amplification of *E. lata* specific ITS products from the DNA of *Botryosphaeria* sp. cultures contaminated with minute amounts of *E. lata*.

Primers derived from RAPD DNA fragments and specific to *E. lata* were also designed by Lecomté *et al.* (2000). However, the primer pairs derived from the ribosomal DNA internal transcribed spacer sequences were found to be more efficient in detecting *E. lata* from wood samples that were less heavily colonised. This difference is probably due to the high copy number of rDNA targeted by the primers. The use of a nested series of PCR primers also increased the reliability and resolution of the *E. lata* specific primers described in this thesis. Tegli *et al.* (2000) showed that under PCR conditions similar to those described in this thesis, the detection threshold of *Phaeoacremonium* sp. using species-specific primers designed to ITS ribosomal DNA was found to be 10 pg of fungal genomic DNA. The detection threshold for the *E. lata* system outlined here, and analogous to that of Tegli *et al.* (2000), is expected to be similar.

3.7.3.5 General discussion of method.

As demonstrated, avoiding cross-contamination of samples is crucial. Nevertheless, the cross contamination of isolates during the sampling procedure is less critical when species-specific primers are used for detection, as opposed to a broad spectrum based detection procedure. Jasalavich *et al.* (2000) developed a DNA based method to reliably detect brown and white rot fungi in spruce wood using the primers ITS1-F (higher fungus specific) and ITS4B (basidiomycete specific), designed by Gardes and Bruns (1993), to amplify the ITS region. Fungi are then identified using restriction enzyme digestion patterns of the PCR amplicon. While the primer pair ITS1-F and ITS4-B will detect only basidiomycetes, it will detect any basidiomycete present. Therefore, cross contamination of samples could obscure the true identity of the main wood rotting fungi sampled in any one test.

However, the very lack of sequence specificity that limits the direct identification of the fungus to species level in the Jasalavich *et al.* (2000) test can be an advantage. Although their PCR method is broad based for basidiomycetes, if used in conjunction with species-specific primers, it would be possible to detect a particular wood decay species of interest and also be alerted to the presence of other basidiomycetes, i.e., other potential decay fungi, in the wood sample. Such a system could be employed for analysis of ascomycete and basidiomycete decay within the grapevine. The only limitation at present is the lack of ascomycete and specific primers for the remaining fungal species responsible for grapevine rot, although primers have recently been published for the detection of *Phaeoacremonium* sp., responsible for the development of esca disease (Tegli *et al.*, 2000).

3.7.4 Development of a PCR based assay.

3.7.4.1 PCR amplification direct from fungal cultures or crude mycelial extracts.

PCR amplification direct from fungal cultures or crude mycelial extracts produced varying results. Not surprisingly the protocol described by Lecomté *et al.* (Section 2.13.2.4), developed for the PCR amplification of the ITS region of *E. lata*, produced the most promising results. However, the success rate of the procedure was not very high and produced PCR amplification products in only one out of the three repetitions. This indicates that *E. lata* may have some PCR inhibitory substances inherent in the mycelia. However, this is not inferred in either the Lecomté *et al.* (2000) or the Irelan (1999) papers. Other methods of PCR amplification from mycelial extracts (Sections 2.13.2.1-Section 2.13.2.3) were all unsuccessful.

3.7.4.2 PCR amplification direct from grapevine wood.

Lecomté *et al.* (2000) developed a PCR assay where *E. lata* was detected in wood samples using 1:10 and 1:100 dilutions of supernatants obtained by boiling thin wood shavings. The success was attributed to the relative lack of PCR inhibitors released from necrotic wood tissues and/or to the dilution of extraction material. This protocol for PCR amplification was tested three times during this research project and failed to produce any PCR products with wood artificially inoculated with *E. lata*. There are several explanations for this observation, the most likely of which is that the artificial inoculation of the wood was unsuccessful and that *E. lata* had failed to colonise the internal wood used for the PCR tests. Alternatively, *E. lata* had colonised the wood pieces but during the long incubation period (12 months) had died as the wood became

progressively drier and over time the DNA disintegrated. Jasalavich *et al.* (2000) describes a situation where some late stage brown-rot samples appeared to have weaker PCR amplification signals than less decayed samples. This could be due to carry over of by-products of wood decay inhibitory to PCR, degradation of DNA in the late stages of wood decay, or a combination of the two.

Such a scenario would also explain why PCR amplification directly from thin wood shavings, with or without non-fat skim milk powder as a blocking agent to overcome plant derived PCR inhibitors, or irradiating a wood sample in Tris-HCl also failed to produced PCR products with the Eutypa specific primers, even though Henson *et al.* (1993) used these techniques successfully for PCR amplification of *Gaeumannomyces graminis* DNA from plant material.

Further work is required to ensure that the Eutypa primers developed here are effective at specifically amplifying *E. lata* DNA from infected grapevine species. New artificial inoculations of grapevine wood could be made to provide wood samples with which PCR optimisation could be attempted. Alternatively, *E. lata* DNA could be artificially mixed with grapevine wood in the PCR reaction. This would show the presence of PCR inhibitors in grapevine wood. A double blind test such as the one described by Lecomté *et al.* (2000) would reveal whether the primers are effective at detection of the fungus within the vine. Similarly, further work is required to verify that the PCR detection assay can also reliably detect the fungus in grapevine tissue that is not necrotic.

For extraction of total DNA from diseased vines, Irelan (1999) washed all vine tissue with distilled water. Thin cross sections (<1 mm) were then excised from the vine with a scalpel and placed in sterile micro-centrifuge tubes containing extraction buffer and ground for 30 seconds with a disposable pestle powered by a hand-held device. Immediately after grinding, an aliquot of the supernatant was diluted with buffer and used as a template with the DNA-based diagnostic primers. It would be useful to try a similar method of DNA extraction. Although the procedure for DNA isolation and purification may be longer than desired to routinely screen large numbers of wood samples, it may be best to continue the process of assay development with a method highly likely to yield DNA amplifiable by PCR, since many by-products of wood decay, if present at too high a concentration in the reaction, would inhibit amplification of the DNA template. A similar DNA extraction procedure was undertaken by Jasalavich *et al.* (2000) using a CTAB lysis buffer for preparation of DNA from spruce wood affected by basidiomycete decay. Total DNA extracted from endophyte-infected rye grass tissue, using a CTAB lysis buffer and similar extraction procedure, was also successfully used to detect Epichloë endophytes *in planta* (Moon, Tapper & Scott, 1999).

Detection of *Phaeoacremonium aleophilum* and *P. chlamydosporum* in grapevine plants by Tegli *et al.* (2000) involved the inoculation of Potato Dextrose Broth cultures with samples of infected wood, incubation from 1-7 days, and the subsequent centrifugation of the culture. The pellet was then utilised for fungal DNA extraction. This approach to *in situ* PCR amplification may prove to be a useful alternative.

In order to achieve specific amplification of DNA isolated from decayed wood, further adjustments to the PCR protocol may be necessary. The addition of nonacetylated

bovine serum albumin (BSA) to PCR reactions, which is known to relieve inhibition of amplification by humic acids, fulvic acids, and organic components of soils and manure, allowed some amplification to occur from samples containing inhibitory wood decay by-products prepared by Jasalavich *et al.* (2000); but this amplification was often non-specific. A hot-start protocol either by the traditional method or a *Taq* polymerase antibody system, was required to obtain specific amplification of DNA isolated from wood decay samples.

3.7.5 Conclusions.

Once a quick, sensitive, reproducible method for detecting the presence of *E. lata* in woody tissue has been developed, this can be used to investigate host pathogen interactions requiring localisation and identification of the fungus. PCR identification of *E. lata* could be used to monitor the development and trace the movement of the fungus in grapevine tissues, to assess infections *in situ*, or to compare the pathogenicity of isolates or the resistance of grapevine cultivars. Rapid methods to detect *E. lata* from grapevine wood material will also be useful in the study of the epidemiology of the disease, such as wound receptivity.

The Eutypa DNA-based diagnostic system has been shown to be effective, highly specific and sensitive, at detecting the presence of the fungus from DNA samples. Unlike traditional identification procedures that require several days from start to finish, this method has the potential to directly detect the presence of the fungus in woody tissue, even if there are no visual symptoms.

4. ISOLATION AND CLONING OF THE *EUTYPA LATA* MATING TYPE GENE.

4.1 Introduction.

Little is known about the life cycle of *E. lata*. The fungus is thought to be heterothallic on the basis of genetic studies with perithecial isolates, which have shown considerable diversity. However, heterothallism has never been confirmed because *E. lata* has so far not been induced to produce the sexual stage in culture. The aim of this study was to clone the mating type gene so that mating types can be identified at the molecular level. This would confirm whether *E. lata* is homothallic or heterothallic and thus facilitate the development of techniques for inducing mating in axenic culture and could also be used to monitor mating behaviour in the field.

Most ascomycete fungi have one mating type gene with two idiomorphs (*MAT1-1* and *MAT1-2*). The *MAT1-2* High Mobility Group box (HMG box) is sufficiently conserved between species, such that, this region can often be cloned using PCR with degenerate primers (Arie *et al.*, 1997). Mating type genes have been cloned from a large number of fungal species using the degenerate PCR approach. A partial list is shown in Table 4.1. In this method, primers are designed to the HMG box of the *MAT1-2* gene and PCR products of approximately 200-350 bp (depending on the size of the intron present) are obtained. DNA sequence analysis of these products confirms their similarity to the HMG box motif found in all fungal *MAT1-2* genes studied so far.

4.1.1 Specific objectives.

This work has three objectives. To:

1) PCR amplify and sequence the *MAT1-2* HMG box from a range of *E. lata* isolates.

2) Design a set of *E. lata* specific *MAT1-2* primers to directly identify the *MAT1-2* idiomorph from *E. lata* genomic DNA.

3) Isolate the full length *MAT1-2* idiomorph from *E. lata* using a mixture of inverse and TAIL-PCR.

4.2 *MAT* gene sequence analysis.

4.2.1 Database searching.

The international database GenBank (URL http://www.ncbi.nlm.nih.gov/) was searched for *MAT* protein sequences from fungi of both *MAT1-1* and *MAT1-2* mating types. Table 4.2 indicates *MAT1-1* sequences and Table 4.3 indicates *MAT1-2* sequences resulting from this search.

MAT sequences of a wide range of fungal plant pathogens were found in the database. Arie *et al.* (1997) found it was necessary to use *MAT1-2* primers with a bias towards pyrenomycete sequences when PCR amplifying the HMG box from pyrenomycete templates. Therefore, the bulk of the sequences retrieved from the database were

Table 4.1 Ascomycete genera from which *MAT1-2* **genes have been obtained using degenerate PCR methods based on Arie** *et al.* **(1997).**

Genus.	References.
Pyrenomycetes.	
Cryphonectria	Arie *et al.*, 1997.
Gaeumannomyces	Arie *et al.*, 1997.
Nectria	Arie *et al.*, 1997.
Neurospora	Arie *et al.*, 1997; *Pöggeler*, 1999.
Podospora	Arie *et al.*, 1997.
Sordaria	*Pöggeler*, 1999.
Gibberella/Fusarium	Yun *et al.*, 2000; Arie *et al.*, 2000.
Glommerella	Valliancourt *et al.*, 2000.
Loculoascomycetes.	
Cochliobolus	Arie *et al.*, 1997; Yun *et al.*, 1999.
Mycosphaerella	Arie *et al.*, 1997.
Pyrenophora	Arie *et al.*, 1997.
Setosphaeria	Arie *et al.*, 1997.
Alternaria	Arie *et al.*, 2000.
Discomycetes.	
Pyrenopeziza	Singh *et al.*, 1997.
Tapesia	Singh *et al.*, 1997.
Plectomycetes.	
Ceratocystis	Witthuhn *et al.*, 2000.

pyrenomycete sequences as *E. lata* is a pyrenomycete of the order Sphaeriales. Other sequences collected that were of the order Sphaeriales, and likely to be closely related, were from the genera *Neurospora*, *Sordaria*, *Gaeumannomyces* and *Podospora*. Other pyrenomycete sequences resulting from the database search include sequences from the orders Hypocreales (*Nectria* and *Gibberella*) and Diaporthales (*Glomerella* and *Cryphonectria*), the *Ceratocystis eucalypti MAT1-2* sequence, which is a Plectomycete, was also retrieved from the database.

4.2.2 *MAT1-1* Amino acid sequence alignment.

The protein sequences for the species listed in Table 4.2 were aligned as described in section 2.15 (Fig. 4.1).

The alignment contains five full-length *MAT1-1* sequences; *Fusarium oxysporum*, *Gibberella fujikuroi*, *Neurospora crassa*, *Podospora anserina* and *Sordaria macrospora*. Eleven partial sequences have also been included in the alignment: four from the genus *Sordaria*, six from the genus *Neurospora* and a partial *Gibberella fujikuroi* sequence. The alignment shows that there is very little sequence conservation between all the species aligned. Amino acids common to all sequences are indicated by a black dot in the top row of the alignment and are found mainly between amino acids 90-130 on the alignment. From the alignment it can be seen that although it would be possible to design a set a of degenerate primers to the *MAT1-1* idiomorph, this would be difficult, as there is very little conservation between *MAT1-1* proteins and thus the design of the primers would be very degenerate.

Table 4.2 Summary of *MAT1-1* sequences obtained from the GenBank database.

Species	Accession number	Sequence (AA)	Comments
Fusarium Oxysporum	AB011379	357	Complete cds* for *MAT-1* protein.
Gibberella fujikuroi	AB015641	109	Partial cds for *MAT* protein containing alpha-box.
Neurospora africana	AJ133139	132	Partial cds for Mat A-1 protein exons 1-2.
Neurospora crassa	M33876	293	Complete cds for *Mat A-1* protein.
Neurospora dodgei	AJ133140	132	Partial cds for *Mat A-1* protein, exons 1-2.
Neurospora galapagosensis	AJ133141	132	Partial cds for *Mat A-1* protein, exons 1-2.
Neurospora lineolata	AJ133142	132	Partial cds for *Mat A-1* protein, exons 1-2.
Neurospora pannonica	AJ133143	132	Partial cds for *Mat A-1* protein, exons 1-2.
Neurospora terricola	AJ133144	132	Partial cds for *Mat A-1* protein, exons 1-2.
Podospora anserina	X64194	305	*FMR1* gene exons 1 and 2.
Sordaria brevicollis	AJ133137	134	Partial cds for *Mat A-1* protein, exons 1-2.
Sordaria fimicola DSM63038	AJ133136	138	Partial cds for *Mat A-1* protein, exons 1-2.
Sordaria fimicola FGSC2918	AJ133135	138	Partial cds for *Mat A-1* protein, exons 1-2.
Sordaria macrospora	Y10616	306	Complete cds for *SmtA-1* protein.
Sordaria sclerogenia	AJ133138	134	Partial cds for *Mat A-1* protein, exons 1-2.

* cds = coding sequence.

Table 4.3 Summary of *MAT1-2* sequences obtained from the GenBank database.

Species.	Accession number.	Sequence (AA).	Comments.
Ceratocystis eucalypti	AF182425	122	Mating type protein *MAT2-1*.
Fusarium monoliforme	AJ131527	72	Partial cds* of *MAT-2*.
Fusarium oxysporum	AB011378	126	*MAT-2*.
Fusarium subglutinans	AF025888	72	Partial cds of putative HMG box protein (*MAT-2*).
Gibberella circinata	AF123501	99	Partial cds of *MAT-2*.
Gibberella fujikuroi	AF100926	223	Complete cds *MAT-2*.
Neurospora crassa	M54787	381	Complete cds of *mat a-1*. exons 1,2 and 3.
Neurospora discreta	AJ133040	104	Partial cds of *mat a-1* gene, exons 2-3.
Neurospora intermedia	AJ133047	104	Partial cds of *mat a-1* gene, exons 2-3.
Neurospora pannonica	AJ133044	104	Partial cds of *mat a-1* gene, exons 2-3.
Neurospora sitophila	AJ133048	105	Partial cds of *mat a-1* gene, exons 2-3.
Neurospora terricola	AJ133045	105	Partial cds of *mat a-1* gene, exons 2-3.
Neurospora tetrasperma	AJ133046	105	Partial cds of *mat a-1* gene, exons 2-3.
Podospora anserina	X64195	402	Complete cds of *FPR1*, exons 1,2 and 3.
Sordaria brevicollis	AJ133042	104	Partial cds of *mat a-1* gene, exons 2-3.
Sordaria fimicola DSM63038	AJ133039	105	Partial cds of *mat a-1* gene, exons 2-3.
Sordaria fimicola FGSC2918	AJ133041	105	Partial cds of *mat a-1* gene, exons 2-3.
Sordaria macrospora	Y10616	105	Partial cds of *Smta-1* gene, exons 2-3.
Sordaria sclerogenia	AJ133043	103	Partial cds of *mat a-1* gene, exons 2-3.

* cds = coding sequence.

Figure 4.1 Alignment (Gene-Jockey II©) of the *MAT1-1* amino acid sequences from pyrenomycete fungi. Amino acids common to all sequences are indicated by a black dot in the top row.

```
                10        20        30        40        50        60        70        80        90       100       110       120
                 |         |         |         |         |         |         |         |         |         |         |         |
Contig# 1                                                                                                  ||•|••  |•|   •• • ||• •• •• |  • ||
Fusarium oxysporum       M-----------------------RA  O  NR AEA   HM KEM  FGHE   EGE K NG  G    RAKR  NA MA R    K   OQKNA  G  Q  GG  HANK A
Gibberella fujikuroi     ------------------------------------------GHE   FE Q K RGA G A   RAKR  NA MA R    K   OQKNA  G  Q  GG  HANK A
Gibberella fujikuroi MAT1-1 ME  NAAK EOK AA N  K A MRA  O  NR AEA   HM EM  FGHE   FE Q K HGA G A   KAKR  NA MA R    K   OQKNA  G  Q  GG  HR K A
Neurospora africana      ------------------------------------------------------------------------------ R     F Q  QKER  FMT  QH  HNE NFM
Neurospora crassa MAT A-1 M G  Q  K ---A  RE  REAMRA  RM------------------MRRG E  RR  ------RAKKK NG MG R     F Q  QKER  FMT  QH  HNE FM
Neurospora dodgei        ------------------------------------------------------------------------------ R     F Q  QKER  FMT  QH  HNE NFM
Neurospora galapagosensis ------------------------------------------------------------------------------ R     F Q  QKER  FMT  QH  HNE NFM
Neurospora lineolata     ------------------------------------------------------------------------------ R     F Q  QKER  FMT  QH  HNE NFM
Neurospora pannonica     ------------------------------------------------------------------------------G R     F Q  QKER  FMT  QH  HNE FM
Neurospora terricola     ------------------------------------------------------------------------------G R     F Q  QKER  FMT  QH  HNE FM
Podospora anserina       MRG N  Q ---EG GEG RAE  K   M----------------MREG  R-----------Q AKKK NG MG R     MR Q  QKER   T  QQ  HKE FM
Sordaria brevicollis     ------------------------------------------------------------------------------G R     F Q  QKMR  FMT  QH  HAE FM
Sordaria fimicola        ------------------------------------------------------------------------------G R     F Q  QKAR  FMT  QH  HNE FM
Sordaria fimicola FGSC#2918 ------------------------------------------------------------------------------ R     F Q  QKAR  FMT  QH  HNE FM
Sordaria macrospora Smat-A1 M  Q  K ---AK  EGERNAA NA  AM------------------M  G G RQ  E   QA A KKK NG MG R     F Q  QKAR  FMT  QH  HNE FM
Sordaria sclerogenia     ------------------------------------------------------------------------------G R     F F  QKMR  FMT  QQ  HTE FM

               130       140       150       160       170       180       190       200       210       220       230       240       250
                |         |         |         |         |         |         |         |         |         |         |         |         |
Contig# 1       ••• |• •          | •   |   •   | |   •| |••   |•    | |     | |•   | •|    |•    | |      | || • | |
Fusarium oxysporum       AK  F R Q GKG ------ M  A  G AC  MMM E    E  G EQ A  QG   QQH  MKAN AA  M R  E       AQ   F Q  MRM ACQNG M   A AQNN
Gibberella fujikuroi     AK  F R Q GK  ------M  A  G AC  MMM E
Gibberella fujikuroi MAT1-1 AK  F R Q GK ------ M  A  G AC  MMM E    E  G QQ A  QG   QQH  MKAN AC  NAQ  E       A  AQ   F Q  MRM ACQNG M   A AQN
Neurospora africana      C M   R M EQ------EK  Q  H A RH G   R N MA  G N Q  NG H  ER A    QH------N Q MNG C  KC E G --  AN--------- H  A
Neurospora crassa MAT A-1 C M   R M EQ------EK  Q  H A RGH G   R M MA  G N R  NG H  ER A    QH-----N Q MNG C  KC E G --  AN--------- H  AK   M
Neurospora dodgei        C M   R M EQ------EK  Q  H A RH G   R N MA  G K Q  NG H  ER A    QH-----N Q MNG C  KC E G --  AN--------- H  A
Neurospora galapagosensis C M   R M EQ------EK  Q  H A RH G   R N MA  G N Q  NG H  ER A    QH-----N Q MNG C  KC E G --  AN--------- H  A
Neurospora lineolata     C M   R M EQ------EK  Q  H A RH G   R N MA  G N Q  NG H  ER A    QH-----N Q MNG C  KC E G --  AN--------- H  A
Neurospora pannonica     C M   R M EE------EK  Q  H A RGH G   R N MA  G N Q  NG H  ER A    QH-----N Q MNG C  KC E G --  AN--------- H  A
Neurospora terricola     C M   R M EQ------EK  Q  H A RGH G   R N MA  G N Q  NG H  ER A    QH-----N Q MNG C  KC E G --  AN--------- H  A
Podospora anserina       CA M  A R Q A------EQM  Q  Q AH  G A R G MEA G  R   G H  QRM    R-----H Q MNG G F  C NGG --   --------- QN  Q    A
Sordaria brevicollis     C M   RA  EE------QK  Q  H A AQMG   R H MA  G R G RNG   R QQ M RR-----N Q MNG C  KC Q G NKH H--------- H  A
Sordaria fimicola        C M   RN  EQ NAQREKK  Q  H A  MG  CREN   G   M NG   MR AM  RK-----N Q M G C  KCQEGG Q-- N---------QH  A
Sordaria fimicola FGSC#2918 C M   RN  EQ NAQREKK  Q  H A  MG  CREN   G   M NG   MR AM  RK-----N Q MNG C  KCQEGG Q-- N---------QH  A
Sordaria macrospora Smat-A1 C M   RN  EQ NAQREKK  Q  H A  MG  CREN   G   M NG   MR AM  RK-----N Q MNG C  KC Q G N---------QH  RK   M
Sordaria sclerogenia     C M   RT  EE------QK  Q  H A GQMG   R N MA  G R GQ RNG   R A  M RR----- Q MNG C  KC Q G NKH H--------- H  A

               260       270       280       290       300       310       320       330       340       350       360       370
                |         |         |         |•   |||     | ||  |   |         |         |         |         |         |
Contig# 1       |         |         |         |•   |||     | ||  |   |         |         |         |         |         |    |  |
Fusarium oxysporum       MAT QH  E   T AEK  NA RE RN AAH   G E  AA  GNR H  E O   ON Q  A   ME N  NFRM  QC  Q E        A   Q  HEKQ KMQ
Gibberella fujikuroi MAT1-1 MAT QH  E   T EK  NA RE RN AAQ  GAD  AA  GNR H  E O  A  QH Q  A   ME N  MHM R  R QA E   G   A   Q  HEKQ KM
Neurospora crassa MAT A-1 H NKR HRQQ--------- R Q  E E G  AM --  RN  AA-------E  G N     H  QQG-------EGG E G  AQ E  -   ENGHA  N  NMA A  MM--
Podospora anserina       C N O  K  G  M G RQ AKQN A AM   Q    A------QG GM E H  O  Q  O---------MRQM   ME H  RE NM AK NERE  A   M M--
Sordaria macrospora Smat-A1 U NKR H QQR--------- R QA   E G A  -- RN A AA-------ER  R Q  MQQG---------  GE G  QE  - G   ENGHA N  NMA AM  MM--

               380
                |
Contig# 1       |
Fusarium oxysporum       QQCK VF FRN  C
Gibberella fujikuroi MAT1-1 QQCQ VF  KN  V
Neurospora crassa MAT A-1 ---G NO-----QA
Podospora anserina       ---G   NK DG
Sordaria macrospora Smat-A1 ---G MQ-----QA
```

However, the alignment shows there is good sequence conservation between two groups of homologous sequences. The first containing the *Gibberella* and *Fusarium* sequences and the second group containing the *Neurospora*, *Podospora* and *Sordaria* sequences, with the *Neurospora* and *Sordaria* sequences being most alike. Variations in the sequences range from single amino acid changes to multiple changes. Most changes were present in several species but some single amino acid changes were unique to a particular species, such as the alanine at position 120 in the *Sordaria brevicollis* sequence. Thus, it may be possible to isolate and clone other *Eutypa* species *MAT1-1* sequences using degenerate primers to the *E. lata MAT1-1* sequence, once it has been obtained.

4.2.3 *MAT1-2* Amino acid sequence alignment.

The *MAT1-2* HMG box protein sequences for the species listed in Table 4.3 were aligned as described in section 2.15. *MAT1-2* HMG box sequences for *Nectria haematococca*, *Cryphonectria parasitica* and *Gaeumannomyces graminis* not in the GenBank database but published in Arie *et al.* (1997) were also added to the alignment.

The alignment shows the *Neurospora* and *Sordaria* sequences group closely together and the *Gibberella/Fusarium* and *Nectria* sequences also group together. Although, *Gaeumannomyces* species are related to species of *Gibberella/Fusarium* and *Nectria* the *Gaeumannomyces graminis* HMG box does not group with the *Gibberella/Fusarium* sequences showing considerable divergence. The *Podospora*, *Cryphonectria* and *Ceratocystis* sequences also show considerable divergence from the other pyrenomycete sequences.

The positions of the degenerate primers designed to the *Neurospora crassa* sequence by Arie *et al.* (1997) are indicated on the alignment. The upstream (5') region (primer NcHMG1) is highly conserved between all the pyrenomycetes for which sequence information is available. However, the downstream (3') region (primer NcHMG2) is variable in amino acid sequence between the different species. Although *Eutypa* species are most closely related to species of *Sordaria* and *Neurospora*, additional primers were designed for use with *E. lata* based on the sequences of two species of *Gibberella* (primer GfHMG2) and *Podospora anserina* (primer PaHMG2) to maximise the probability of successful PCR amplification (Table 4.4).

The degenerate primers were designed so that the 3' ends were anchored down with at least 2 identical bases. Where amino acids with four codons, such as valine, were found in the primer region inosine was used in the 3rd position to reduce the degeneracy of the primers.

Table 4.4 PCR primers designed to the *MAT1-2* HMG box region.

Primer.	Nucleotide sequence (5'-3').	Amino acid sequence.
NcHMG1	CCY CGY CCY CCY AAY GCN TAY AT	PRPPNAYI
NcHMG2*	CGN GGR TTR TAR CGR TAR TNR GG	PD#YRYNPR
GfHMG2*	CGI GGI CGR TAY TGR TAR TCI GG	PDYQYRPR
PaHMG2*	CGI GGI ACR TAI CGR TAR TGI GG	PHYRYVPR

* Primers are designed to anneal to the complementary (non-coding) DNA strand.
# D: D, Y, N or H.

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Figure 4.2 Alignment (Gene-Jockey ®II) of pyrenomycete *MAT1-2* HMG box polypeptides. Amino acids common to all sequences are indicated by a black dot in the top row. Regions to which primers were designed are underlined. The NcHMG1 primer was designed to amino acids 21-26. The primer NcHMG2 was designed to the amino acids 84-89.

```
                        10        20        30        40        50        60        70        80        90       100
                        |         |         |         |         |         |         |         |         |         |
Contig# 1                                   ●●●●●●●|●●    |   |      ●    ●   ●        ●     ●    ● ●         ●      ●|●|● ●●||  | |●●
Ceratocystis eucalypti          -------------------K  R  NA    FK R QA K  F N  NNE  K  GKR REE A  RE  REQAEA KK MEM     R K RKA EKKRRRRN
Cryphonectria parasitica        -------------------R  NA    FAA HR   EA  A N E  KK GRQCQ E EE R A RKKAA  KAA M A  H R N
Fusarium moniliforme            -------------------R  NA    FKER HH   KAQR   NNE  Q  GR  N E RE RA  KQMA QKKAEHRRQ  H R N R
Fusarium oxysporum              -------------------K  R  NA    FKER HQ  KAQR   NNE  Q  GR  N E RE RA  KQMA QKKAEHRRQ   Q R RR   ERRRRNNA
Fusarium subglutinans           -------------------R  NA    FKER HH   KAQR   NNE  Q  GR  N E RE RA  KQMA QKKAEHRRQ  H R N R
Gaeumannomyces graminis         -------------------R  NA    FKE HA  KANN G HNNM  A GKQ ANE  MR K KKMA  KRK HEK H R N
Gibberella circinata            -------------------R  NA    FKER HH  KAQR   NNE  Q  GR  N E RE RA  KQMA QKKAEHRRQ   Q R RR   ER
Gibberella fujikuroi            -------------------K  R  NA    FKER HH  KAQR   NNE  Q  GR  N E RE RA  KQME QKKAEHRRQ   Q R RR   ERRRRNNA
Nectria haematococca            -------------------R  NA    FKER HH  K M G  NNE  Q  GR  NMENRA TRAE K RA E KR H EK H R N
Neurospora crassa               -------------------K  R  NA   RK  HHRE REQN G HNNEM-   GNM R EQ H REK FNM NE K R   EN  R N RR Q  RRR   K
Neurospora discreta             KG HA A KEQQKK-AK   R  NA   RK  HHRE REQN G HNNE   N GNM R EQ H REK FNM NE K R   EH  R N RR Q  RRR   K
Neurospora intermedia           KG HA A KEQQKK-AK   R  NA   RK  HHRE REQN G HNNE   N GNM R EQ H REK FNM NE K R   EN  R N RR Q  RRR   K
Neurospora pannonica            KG HA A KEQQKK- K   R  NA   RK  HHRE RERN G HNNE   N GNM R EQ H REK FNMANE K RM  EH  R N RR Q  RRR   K
Neurospora sitophila            KG HA A KEQQKK- K   R  NA   RK  HHRE RERN G HNNE   N GNM R EQ H REK FNM NE K RM  EH  R N RR Q  RRR   K
Neurospora terricola            KG HA A KEQQKK-AK   R  NA   FKNH RE RERN G HNNE   N GNM R EQ H REK FNMANE K RM  EH  R N RR Q  RRR   K
Neurospora tetrasperma          KG HA A KEQQKK-AK   R  NA   RK  HHRE REQN G HNNE   N GNM R EQ H REK FNM NE K RM  EH  R N RR Q  RRR   K
Podospora anserina              -------------------K  R  NA   RK  QQHA KHRN G  NN   M GGM KKE  E RRE QRRA E KAK M A  H R R RR   E RRRA RRNR
Sordaria brevicollis            KG HA A KEQEKK- K   R  NA   RK  HHRE R RN G HNNE   N GNM R EQ H REK FNMAKE KRR  EH  R N RR Q  RRR   H K
Sordaria fimicola               KG HAE  KQQQKKKAK   R  NA   RK  HHRQ REQN G HNNE   N GNM R EQ H R K F MANE KAR   N  R N RR Q  RRR   K
Sordaria fimicola AJ133039      KG HAEA KQQQKKKAK   R  NA   RK  HHRQ REQN G HNNE   N GNM R EQ H R K F MANE KAR   N  R N RR Q  RRR   K
Sordaria macrospora Smta-1      KG HAEA KQQQKKKAK   R  NA   RK  HHRQ REQN G HNNE   N GNM R EQ H R K F MANE KAR   N  R N RR Q  RRR   K
Sordaria sclerogenia            KG HA A KEQQKK- K   R  NA   RK  HHRE R RN G-HNNE   N GNM R EQ H RER FNMAKE KRR  EH  R N RR Q  RRR   H K
                                             NcHMG1                                                            NcHMG2
```

4.3 PCR amplification of the *MAT1-2* HMG box.

4.3.1 Establishment of *Neurospora crassa* as a positive control for PCR.

Isolates of both mating types of *N. crassa* (*mat a* = *MAT1-2* and *mat A* = *MAT1-1*) used by Arie *et al.* (1997) were obtained for use as PCR controls from the Fungal Genetics Stock Centre, Kentucky, U.S.A. (Table 2.1). The optimal PCR amplification conditions for use with the NcHMG1 and NcHMG2 primers were found by varying the concentration of Mg^{2+} ions, dNTPs and DNA template in independent PCR reactions. The optimal PCR amplification conditions were found to be 35 PCR cycles at an annealing temperature of 53°C, 2.0 mM Mg^{2+}, 0.2 mM dNTPs 2 μM of each primer and 20 ng of template DNA per 50 μL PCR reaction. PCR amplification from genomic DNA of the *N. crassa* strains with primers NcHMG1 & NcHMG2 under these conditions yielded a single 300 bp product from the *mat a* strain (*N. crassa* #2961) as expected. Direct DNA sequence analysis of this product confirmed its identity as part of the *MAT1-2* (*mat a*) gene. Lowering the stringency of the PCR reaction by decreasing the annealing temperature from 53°C to 47°C enabled a PCR product of approximately 250 bp to be produced with *Mat A* (*MAT1-1*, *N. crassa* #987) DNA (results not shown).

The use of the new primer combinations NcHMG1 and GfHMG2, and NcHMG1 with PaHMG2, with *N. crassa mat a* (*MAT1-2*) genomic template at an annealing temperature of 53°C gave an amplified product of the expected size as well as additional products (results not shown). Additional PCR products were also obtained with *N. crassa Mat A* (*MAT1-1*) genomic DNA and the NcHMG1 and GfHMG2 and NcHMG1 and PaHMG2 primer combinations.

4.3.2 Polymorphism analysis of *Eutypa lata* isolates EL01, E11PDA2, E11787 and E11788

Four NZ isolates of *E. lata* were initially available in the laboratory. It was not known whether these were heterothallic (mating type 1 or 2), homothallic or even whether the isolates were genetically identical. However, in order to increase the probability that at least one of the four isolates carried the *MAT1-2* gene it was important to dismiss the possibility that they were genetically identical to one another (clonal), as found with other fungal pathogens (eg. *Dothistroma pini*) in New Zealand (Hirst, 1997).

4.3.2.1 Randomly Amplified Polymorphic DNA (RAPD) analysis.

RAPD markers have been used extensively to analyse the genetic structure of several populations of *E. lata* in France (Péros *et al.*, 1996; Péros *et al.*, 1997; Péros & Larignon, 1998; Péros *et al.*, 1999; Péros & Berger, 1999). These studies indicated a high degree of genetic diversity between isolates of *E. lata*. The four laboratory isolates of *E. lata* were analysed by RAPD testing (Section 2.13.6) with two different decamer primers OPD-03 and OPC-05 (Table 2.3) and two different annealing temperatures (37°C and 42°C). However, RAPD analysis did not generate clear PCR products, rather, a smear of PCR products were produced with each isolate (results not shown). PCR products were not produced in the negative control reactions. Therefore, confirmation that the four isolates were not genetically identical was not possible using the RAPD primers available.

4.3.2.2 Randomly amplified microsatellite DNA (RAMS) analysis.

Microsatellite profiles produced using the 5' anchored microsatellite PCR technique (Section 2.13.7) and the anchored CT primer (Table 2.3) showed reproducible polymorphic differences between each of the four *E. lata* isolates (Fig. 4.3) in two independent tests. Although this did not imply anything about mating types of the four isolates, it did indicate that the isolates were not genetically identical to one another.

4.3.3 Attempts to isolate the *Eutypa lata MAT1-2* gene via heterologous probing.

Although heterologous probing between the *Neurospora crassa* and *E. lata* genomes was not expected to isolate the *E. lata MAT1-2* HMG box, because of a lack of overall nucleotide sequence identity, heterologous probing had been successfully used to isolate the *Podospora anserina MAT1-1* idiomorph using a *MAT1-1* probe from *N. crassa* (Picard *et al.*, 1991).

4.3.3.1 Preparation of a DIG labelled DNA probe.

Probe DNA was prepared by PCR amplification of 300 bp of the *N. crassa MAT1-2* HMG box using the NcHMG1 and NcHMG2 primers (Arie *et al.*, 1997; Table 2.3) as previously described (Section 4.2.5). The DNA was then Digoxygenin-11-dUTP labelled using the DIG-high prime kit (Roche) (Section 2.12.2.1). However, the procedure produced only 10 pg/μL of labelled DNA from 800 ng of *N. crassa* PCR product. Therefore, exhibiting a very low labelling efficiency.
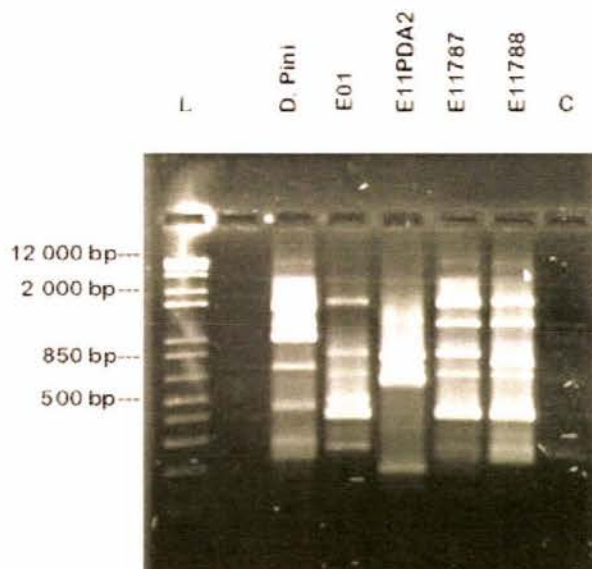
Figure 4.3 Agarose gel (1.5%) with 5' anchored PCR amplification products from the 4 laboratory *E. lata* isolates obtained with the anchored CT primer. A positive control (*D. pini*) and a negative (no DNA) control (C) have been included in the PCR reaction. The numbers to the left of the figure indicate the fragment sizes (bp) of the 1 Kb+ ladder (L).

To increase the labelling efficiency of the reaction, directly incorporating the DIG label into the probe DNA during PCR was attempted. However, no PCR product was produced with the *N. crassa mat a* (*MAT1-2*) DNA and the DIG labelled dNTPS, even

though a positive control PCR with *N. crassa mat a* DNA and unlabelled dNTPs produced the desired 300 bp fragment with the NcHMG1 and NcHMG2 primers.

4.3.3.2 *Preparation of a [•³²P]dCTP labelled DNA probe and Southern hybridisation.*

As the *N. crassa* HMG box PCR product did not label efficiently with Digoxygenin-11-dUTP, the probe DNA was random primer labelled with [•³²P] dCTP using the High prime labelling system (Section 2.12.2.2). Genomic DNA from *mat a* (*MAT1-2*) and *mat A* (*MAT1-1*) *N. crassa* isolates and the four laboratory strains of *E. lata* (unknown mating types) were digested with the *BamH1* and *EcoR1* restriction enzymes, before Southern blotting (Section 2.12.1) and hybridisation at 58°C.

In the resulting Southern blot the *N. crassa MAT1-2* fragment hybridised to *N. crassa mat a* genomic DNA but not to *N. crassa mat A* or *E. lata* DNA (results not shown). This indicated the four NZ *E. lata* isolates were *MAT1-1*, or, the overall nucleotide sequence identity between the *N. crassa* and *E. lata MAT1-2* HMG boxes was not adequate to allow identification of the *E. lata MAT1-2* by hybridisation.

4.3.4 Optimisation of PCR amplification conditions.

The three sets of *MAT*-specific primers (NcHMG1 with NcHMG2 or GfHMG2 or PaHMG2) were tested with the four isolates of *E. lata*. It was not known what size intron (if any) would be present in the *E. lata MAT1-2* HMG box, for this reason, the PCR products were expected to be between 200 and 350 bp.

In several cases PCR products within the expected size range were seen (Fig.4.5). However, these were not distinct such as those published by Arie *et al.* (1997) and were accompanied in most cases by additional products of different sizes. Exhaustive attempts were made to improve the specificity and efficiency of amplification of fragments in the expected size range. The following parameters were varied in independent experiments, with DNA from the *N. crassa Mat-a* strain as a control template in each case.

Variation of dNTP concentration.
The dNTP concentration was varied from 0.1-2.5 mM. Decreasing the concentration of dNTPS in the PCR reaction, such that the final concentration was below 0.2 mM, eliminated all PCR product obtained with *N. crassa Mat a* (*MAT1-2*) and *E. lata* genomic DNA templates (results not shown). Similarly, PCR reactions with dNTP concentrations greater than or equal to 0.8 mM did not produce any PCR products with either the *N. crassa Mat a* positive control or the four laboratory *E. lata* isolates (results not shown).

PCR products of similar size and specificity were obtained with DNA from the *E. lata* isolates when the dNTP concentration was between 0.2 mM and 0.3 mM (Fig. 4.4). However, a final dNTP concentration of 0.3 mM yielded more PCR product. Fewer PCR products of the expected size range were obtained when PCR amplification was performed with *E. lata* DNA and dNTP concentrations between 0.4 and 0.6 mM (Fig. 4.4). Additional PCR products were obtained with *N. crassa Mat a* DNA when the dNTP concentration was increased beyond 0.3 mM (until PCR products were no longer obtained at 0.8 mM).

Variation of magnesium concentration.

The magnesium concentration was varied from 0.8-2.5 mM with a dNTP concentration of 0.3 mM. Concentrations of magnesium less than or equal to 1.2 mM totally inhibited PCR amplification. A single PCR product of the expected size was obtained with the *N. crassa Mat a* DNA at magnesium concentrations between 1.5 mM and 2.0 mM. Higher concentrations of magnesium resulted in greater yields of PCR product but the number of non-specific PCR products also increased. PCR products were not obtained with *E. lata* genomic DNA templates at magnesium concentrations below 2.0 mM (results not shown).
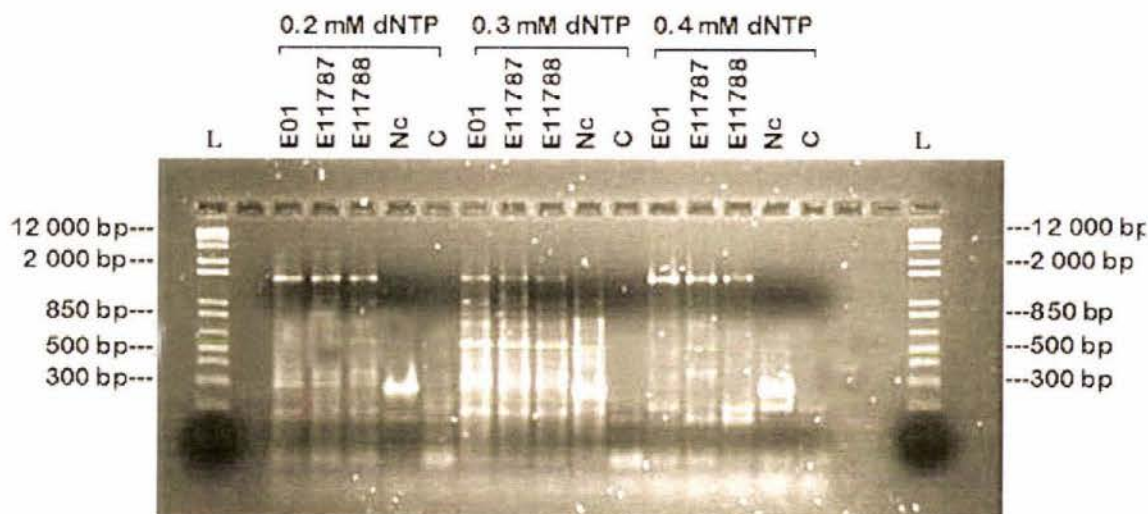
Figure 4.4 Agarose gel (2%) with PCR amplification products obtained over a variety of dNTP concentrations with the NcHMG1-NcHMG2 PCR primers. Nc indicates the PCR products obtained with *N. crassa Mat a* (*MAT1-2*) genomic DNA templates. C indicates a negative control (no DNA). The numbers at the sides of the figure indicate the fragment sizes of the 1 Kb+ ladder (L) (Gibco BRL).

Variation of template concentration.

The amount of genomic DNA template in each 50 μL PCR reaction was varied from 10-50 ng. The amount of DNA template did not affect the number of PCR products produced with the degenerate PCR primers, however, the yield of each PCR product obtained was increased as the amount of genomic template increased (results not shown).

Variation of primer concentration.

No effect was observed when the concentration of degenerate primers in the PCR reactions was varied in 0.25 μM increments between 1-2 μM. This is not a large range of primer concentration and it is very likely that variation can be observed outside this range, however, PCR with specific primers require concentrations around 0.2 μM, hence, the primer concentrations used here ensure an excess of degenerate primers and variation has little consequence.

Variation of Taq *polymerase concentration.*

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The amount of *Taq* polymerase in each 50 μL reaction was varied from 1-2 U. The number and size of PCR products obtained with the degenerate primers and genomic templates were not affected by the amount of *Taq* polymerase present. However, the amount of PCR product varied with the amount of *Taq* polymerase present, such that, increasing the concentration of *Taq* polymerase, increased the concentration of PCR products obtained (results not shown).

Type of Taq *DNA polymerase.*
Singh *et al.* (1999) found that PCR amplification of the *Pyrenopeziza brassicae* and *Tapesia yallundae MAT1-2* HMG boxes only occurred when hot start PCR procedures were employed. Platinum *Taq* polymerase (Gibco BRL) was used as a convenient hot start method. The use of the Platinum *Taq* polymerase decreased the amount of background smear produced during the degenerate PCR reaction amplifying clear bands of varying intensity within the desired size range. Figure 4.5 illustrates PCR amplification with the Platinum *Taq* polymerase and the clear PCR products obtained. However, for a direct comparison between the amplification products obtained with Platinum *Taq* polymerase and *Taq* polymerase, refer to the CD-Rom accompanying this thesis.

Template DNA extraction method.
Template DNA was prepared using the Nucleon Phytopure™ DNA extraction Kit (Section 2.5.1) or the Al-Samarrai and Schmid (2000) method (Section 2.5.2). The method of template preparation did not alter the yield or specificity of the degenerate PCR reaction (results not shown).

Annealing temperature.
The PCR annealing temperature was varied from 44-55°C using a Corbett PC-960 gradient thermal cycler. A single PCR product of 300 bp was obtained with the *N. crassa Mat a* DNA in PCR reactions with an annealing temperature greater than or equal to 52°C. Decreasing the annealing temperature below 52°C produced additional PCR fragments, however, the yield of PCR product was increased. Similarly, a greater yield of PCR products was obtained with *E. lata* genomic DNA templates and the degenerate primers when the annealing temperature was below 50°C although, they were more likely to contain additional PCR products of high molecular weight. The number of PCR products obtained decreased as the annealing temperature increased. For an example gel from one of the gradient PCR reactions described here, refer to the CD-Rom accompanying this thesis.

The optimised PCR conditions are those given in section 2.13.4. Despite exhaustive testing with different PCR conditions, clear and specific amplification of a single DNA fragment in the size range 200-350 bp was not seen for any of the strains with any of the primer combinations. However, as seen in Figure 4.5, some fragments were within the expected size range. Amplification with single primers (e.g. GfHMG2 only) was also carried out to identify whether these products were obtained only in the presence of both primers rather than one primer priming both ends non-specifically. Those products obtained with both PCR primers were more likely to be genuine *MAT1-2* gene products.
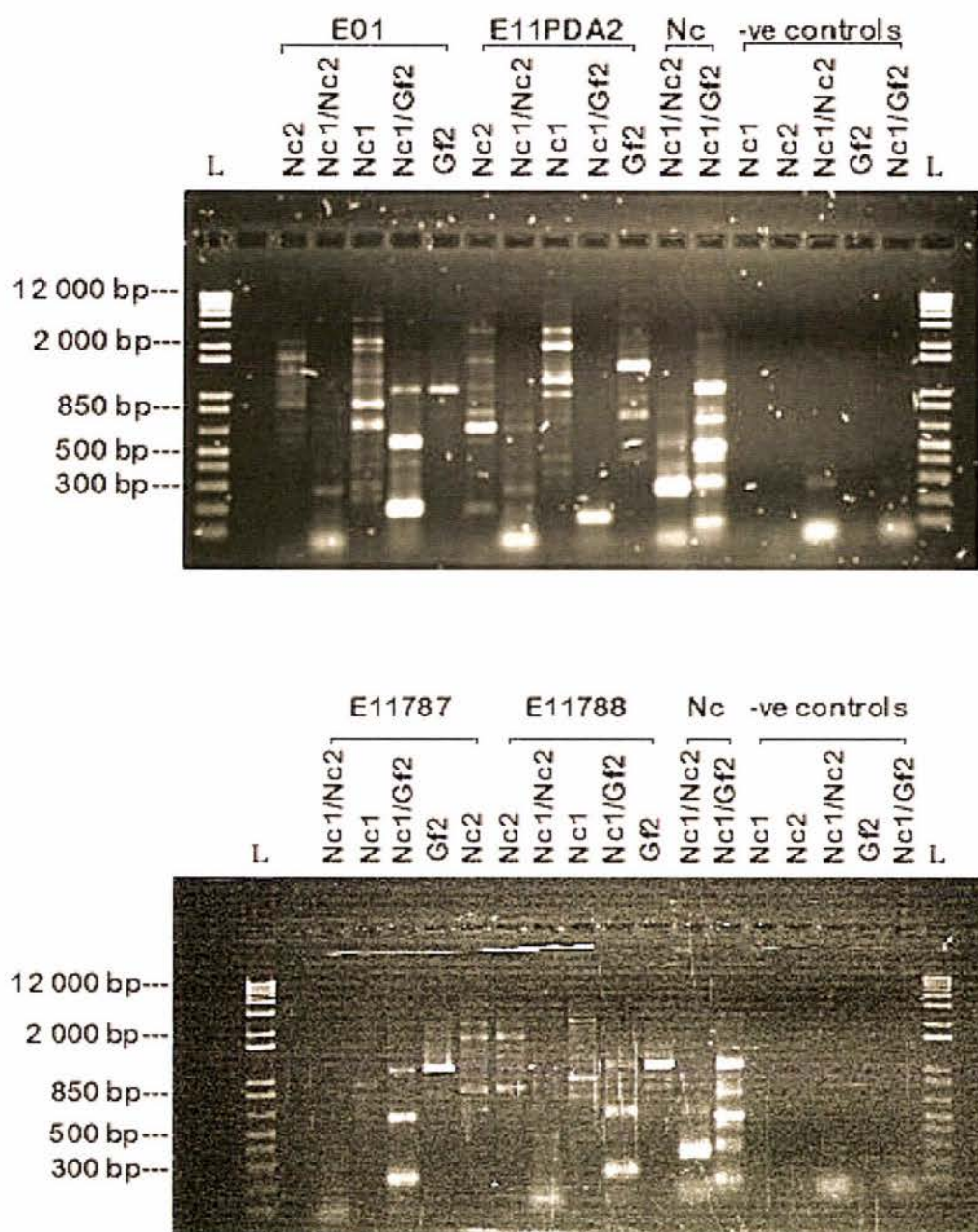
Figure 4.5 Agarose gels (2%) with PCR amplification products obtained from four *E. lata* isolates (EL01, E11PDA2, E11787and E11788). Nc1 indicates the primer NcHMG1 was used in the PCR reaction. Similarly, Nc2 and Gf2 indicate the primers NcHMG2 and GfHMG2 were used in the PCR reaction. Nc reactions are *N. crassa* genomic DNA positive controls. Negative controls contain no DNA in the PCR reactions. The numbers at the side of the agarose gels indicate the fragment sizes of the 1 Kb+ (L) ladder (Gibco BRL).

4.3.5 Sequence analysis of products from four New Zealand isolates.

As the PCR products obtained with the four *E. lata* isolates were similar (Fig. 4.5), PCR products between 200-350 bp in size were selected from amplifications of isolates EL01

and E11PDA2 with the primer combinations NcHMG1/NcHMG2 and NcHMG1/GfHMG2. Four products were purified from an agarose gel (Section 2.6.3) and sequenced using the NcHMG1 primer (Section 2.14). These initial direct sequencing results suggested a mixed template or multiple priming sites within the template.

The PCR products were therefore ligated into a pGEM-T vector (Table 2.1) and cloned in *E. coli* (Table 2.1), under permit GMO 00/MU/03 from ERMA New Zealand. This was initially achieved by gel purification (Section 2.6.3) of PCR products prior to ligation (Section 2.11), but subsequently a shotgun approach (in which all PCR products were included in the ligation reaction) was found to be more efficient. One hundred and sixty plasmids with inserts of the required size range (200-350 bp) were purified from recombinant transformants and 17 of these were sequenced using the SP6 and/or T7 primers (Table 2.3) that anneal to regions flanking the cloning site of the pGEM-T vector. None of the sequences showed any matches to fungal mating type genes when analysed using the BLAST (Basic Local Alignment Search Tool) suite of programs in Bionavigator. In fact, the plasmid inserts commonly showed no significant similarity to any of the database sequences. However, weak matches were found between some database sequences and plasmid inserts, in such cases the score was never above 35. For example, clones were found that showed some similarity to a putative non-LTR retro element transcriptase (Genbank accession number AC007018) in *Arabidopsis* (Score 30.4, E-value 3.4) and a melanocortin 1 receptor (Genbank accession number AF362606) from *Tangara cucullata* (Score 33.9, E-value 1.1).

4.4 Obtaining and testing new *Eutypa lata* isolates.

4.4.1 Isolation of sixteen single ascospore *Eutypa lata* isolates.

A major limitation of the work up to this point was the small number of isolates examined. It seemed very likely that none of them contained a *MAT1-2* gene, therefore, further isolates were required.

In the expectation that *E. lata* would be heterothallic, attempts were made to isolate ascospore progeny from a single ascus (which should contain four progeny of each mating type). It proved difficult to isolate intact asci for micromanipulation due to the perithecia being buried deep within the infected wood that was available and because the asci were very ripe and burst open easily. An alternative method tested was the collection of groups of eight ascospores onto a rotating petri plate of PDA suspended over moistened perithecia but this approach was also abandoned as it was not possible to induce the release of ascospores.

A revised plan was to isolate ascospores from an individual perithecium. From this the probability of obtaining at least one *MAT1-2* isolate out of n isolates would be $1 - (0.5)^n$, if *E. lata* is heterothallic and assuming a 1:1 ratio of *MAT1-1:MAT1-2* progeny. Only one piece of grapevine with perithecia was found in a search of heavily infected vineyards in New Zealand. This piece was taken to the laboratory where a perithecium was crushed, the ascospores diluted in water and plated out (Section 2.3.1.4). Sixteen single ascospore isolates were obtained.

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Confirmation that these sixteen isolates were indeed *E. lata* was achieved using *E. lata*-specific primers designed to the ITS rDNA region (Section 3.4.6), along with the universal ITS primers, ITS1 and ITS4 (Table 2.2), as a positive control. Each of the isolates gave a PCR product of the size expected for *E. lata* with the E.l.ITS1 and E.l.ITS4 primers. The PCR products obtained by the *E. lata* single ascospore isolates 1-6 are illustrated in Fig.4.6. Thus, the probability that one of the sixteen *E. lata* single ascospore isolates would be *MAT1-2* is $1-(0.5)^{16}$ or p=0.999 assuming the perithecia from which they were isolated is heterothallic and a 1:1 ratio of *MAT1-1*: *MAT1-2* progeny exists naturally.
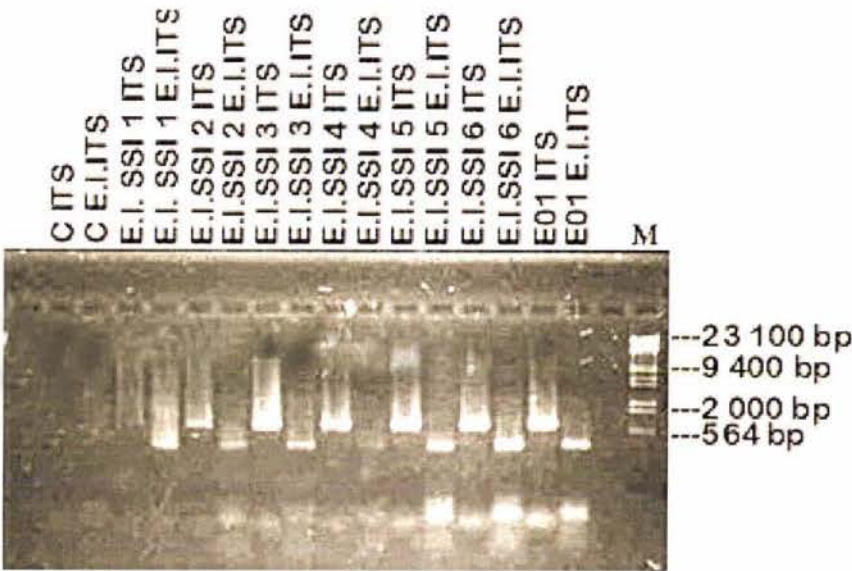
Figure 4.6 Agarose gel (1%) of PCR products amplified from the genomic DNA of 6 *E. lata* single ascospore isolates with the ITS primers (ITS1-ITS4) and *E. lata* specific (E.l.ITS) primers (E.l.ITS1-E.l.ITS4). The reaction products from a positive control, *E. lata* EL01, are shown for both sets of primers. C ITS and C E.l.ITS indicate negative (no DNA) controls; M indicates the lambda *Hind III* size marker (Gibco BRL).

Attempts were made to verify that the perithecium was heterothallic by RAMS DNA fingerprinting of the sixteen single ascospore isolates (Section 2.13.7). Three 5' anchored microsatellite primers, anchored CT, anchored AAG and anchored TG (Table 2.3), were used and the experiments were carried out in duplicate. Figure 4.7 shows results obtained with the anchored CT primer. Figure 4.7A and Figure 4.7B are from one of the replicates of the anchored CT microsatellite analysis. Figure 4.7A showing duplicates of single ascospore isolates 1-5 and Figure 4.7B showing the anchored CT microsatellite profiles for single ascospore isolates 6-16. Figure 4.7C shows an independent RAMS analysis with the sixteen single ascospore isolates.

The microsatellite profiles produced between the two replicates (Figure 4.7A/B and Figure 4.7C) look to be identical at first glance, however, the polymorphisms shown in Figures 4.7A and Figures 4.7B are not conserved in Figure 4.7C. For example, the red arrow in Figure 4.7B indicates a 100 bp amplicon produced only in single ascospore 8, however, in Figure 4.7C the 100 bp amplicon (indicated by the red arrow) is produced

only in single ascospore 7. The gold arrow highlights the differing presence and absence of a 400 bp amplicon in single ascospore 15. The duplicates in Figure 4.7A show that the templates were consistently amplifying the same bands in the first replicate and the positive control, *E. lata* E11787 genomic DNA (E), produced identical banding patterns in the replicates. The change in position of the 100 bp amplicon from single ascospore 8 to 7 could possibly be explained by the addition of the wrong template to the reactions in Figure 4.7C, however, this does not explain the absence of the 400 bp band in single ascospore 15 and similar trends of non-consistent amplification shown with the other anchored microsatellite primers.

Figure 4.8 shows the results obtained with the anchored AAG primer. Although convincing polymorphisms were seen in the first test with this primer (Figure 4.8A), the differences seen were not reproducible (Figure 4.8B). For example, in A the strong band approximately 650 bp in size (indicated by a green arrow) was not PCR amplified in all sixteen single ascospore isolates. However, in B it is present in all single ascospore isolates except E.l.SSI 10. E.l.SSI 10 previously produced the amplicon in A. The positive control, *E. lata* E11787 genomic DNA, produces the same banding pattern in both replicates, however, the intensity in the bands differs between Figure 4.8A and Figure 4.8B.

Figure 4.9 shows the results obtained with the anchored TG primer. Once again convincing polymorphisms were seen in the first test with this primer (Figure 4.9A) but the differences seen were not reproducible, as seen in Figure 4.9B. In Figure 4.9A, an amplicon approximately 550 bp in size (indicated by a purple arrow) is present in eight single ascospore isolates (E.l.SSI 1, E.l.SSI 2, E.l.SSI 4, E.l.SSI 7, E.l.SSI 9, E.l.SSI 10, E.l.SSI 12 and E.l.SSI 13) and absent in eight of the sixteen single ascospore isolates (E.l.SSI 3, E.l.SSI 5, E.l.SSI 6, E.l.SSI 8, E.l.SSI 11, E.l.SSI 14, E.l.SSI 15, E.l.SSI 16). However, the 550 bp amplicon (purple arrow) is present in all sixteen single ascospore isolates in Figure 4.9B. Indeed the anchored TG microsatellite profiles in Figure 4.9B look identical for all sixteen single ascospore isolates. The *E. lata* E11787 positive control reaction produces the same banding pattern in both Figure 4.9A and Figure 4.9B.

Each of Figures 4.7, 4.8 and 4.9 show two runs of an experiment which differe from one another. It is impossible to tell which run is correct. Normally at least one further run of the experiment is necessary to be confident in determining which is more accurate, however, due to time constraints in this project this was unable to be undertaken. Therefore, this technique did not dismiss the possibility that the sixteen isolates were genetically identical to one another (clonal).

4.4.2 PCR amplification of single ascospore isolates.

The three sets of *MAT*-specific primers (NcHMG1 with NcHMG2 or GfHMG2 or PaHMG2) were tested with the sixteen single ascospore isolates of *E. lata*. Again there were no clear distinct single bands indicative of a *MAT1-2* gene fragment so optimisation was carried out as before (Section 4.3.4). The following parameters were also altered:

Type of hot start Taq *polymerase.*
FastStart *Taq* polymerase (Roche), an alternative hot start *Taq* polymerase, was

Figure 4.7 Anchored CT microsatellite analysis of 16 single ascospore *E. lata* isolates (E.l.SSI 1-E.l.SSI 16). Figures 4.7A and B illustrate microsatellite profiles from a single replicate while Figure 4.7C illustrates the profiles obtained in independent reactions. The red arrows highlight an amplicon, approximately 100 bp in size, showing polymorphism, while the gold arrows highlight a 400 bp amplicon present in E.l.SSI 15 in the first experiment but absent in the repeat experiment. E indicates an *E. lata* E11787 positive control reaction. C indicates a negative (no DNA) control reaction. The numbers to the left of the gels indicate the sized of DNA fragments in L the 1 Kb+ ladder (Gibco BRL).

A.) Agarose gel (1.5%) of 5' anchored microsatellite PCR products obtained with the anchored CT primer and replicates of *E. lata* single ascospore isolates 1-5.

B.) Agarose gel (1.5%) of 5' anchored microsatellite PCR products obtained with the anchored CT primer and genomic DNA of *E. lata* single ascospore isolates 6-16.

C.) Agarose gel (1.5%) of 5' anchored microsatellite PCR products obtained with the anchored CT primer and genomic DNA from 16 *E. lata* single ascospore isolates.

Figure 4.8 Anchored AAG microsatellite analysis of 16 single ascospore *E. lata* isolates. The photographs of the gels are aligned to match the positions of DNA profiles of the 16 single ascospore isolates (E.l.SSI1-16). E indicates an *E. lata* E11787 positive control. C indicates a negative (no DNA) control reaction. The numbers to the left of the figure indicate the size of DNA fragments in L, the 1 Kb+ ladder (Gibco BRL). The green arrows highlight an amplicon, approximately 650 bp in size, showing polymorphism.

A.) Agarose gel (1.5%) of 5' anchored microsatellite profiles obtained with the anchored AAG primer and genomic DNA from sixteen single ascospore isolates.

B.) Agarose gel (1.5%) of 5' anchored microsatellite profiles with the anchored AAG primer and genomic DNA from sixteen single ascospore isolates obtained in an independent experiment.

Figure 4.9 Anchored TG microsatellite analysis of 16 single ascospore *E. lata* isolates. The photographs of the gels are aligned to match the positions of DNA profiles of the 16 single ascospore isolates (E.l.SSI 1-E.l.SSI 16). E indicates an *E. lata* E11787 positive control. C indicates a negative (no DNA) control reaction. The numbers to the left of the figure indicate the size of DNA fragments in L, the 1 Kb+ ladder (Gibco BRL). The purple arrows highlight an amplicon, approximately 550 bp in size, showing polymorphism.

A.) Agarose gel (1.5%) of 5' anchored microsatellite profiles obtained with the anchored TG primer and genomic DNA from sixteen single ascospore isolates.
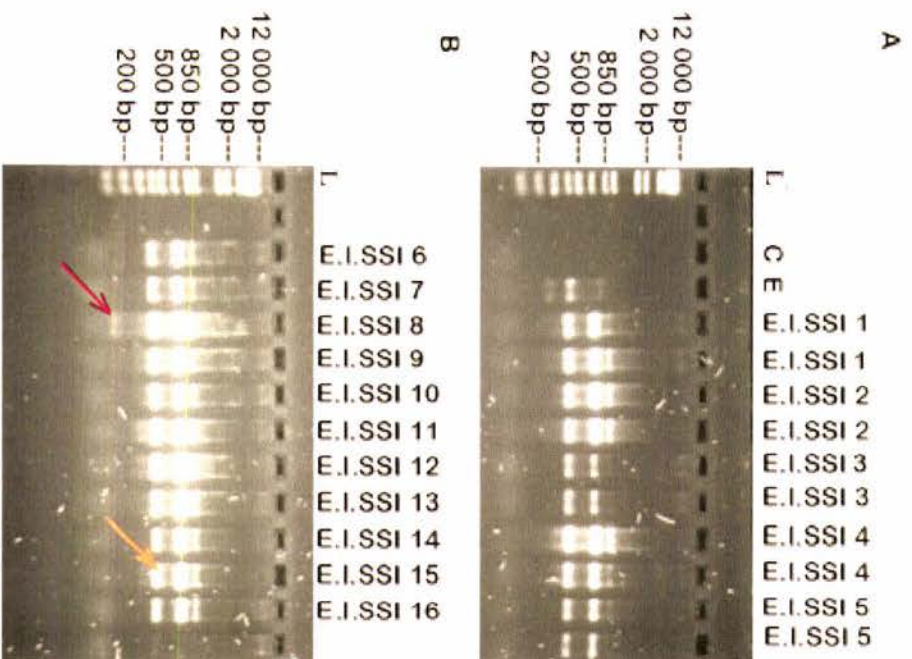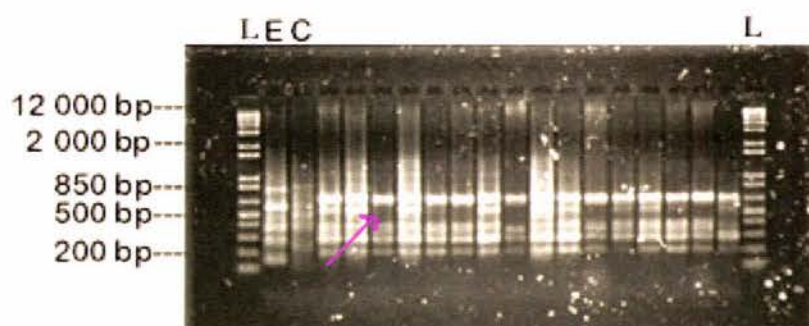
B.) Agarose gel (1.5%) of 5' anchored microsatellite profiles with the anchored TG primer and genomic DNA from sixteen single ascospore isolates obtained in an independent experiment.
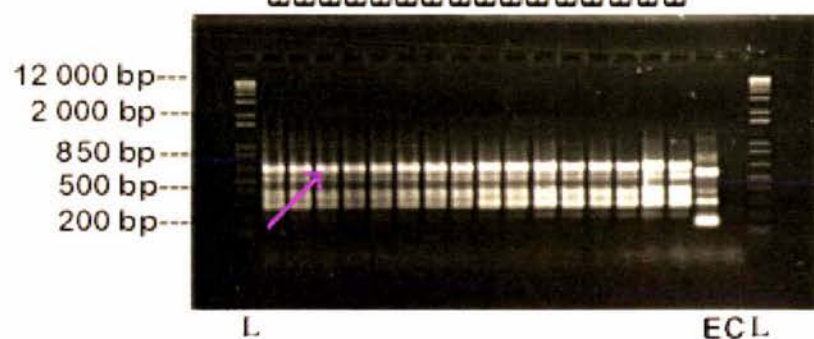
compared with the Platinum *Taq* polymerase (Gibco BRL). FastStart Taq polymerase did not increase the specificity of the PCR reaction.

Two main PCR products (200 bp and 300 bp) were amplified by the two *Taq* polymerases, with DNA from the single spore isolates E.l.SSI7, E.l.SSI8 and E.l.SSI9, at an annealing temperature of 48°C. However, FastStart *Taq* PCR reactions contained unwanted additional smears of PCR amplification (results not shown).

Thermal cycling parameters.
The duration and number of PCR cycles were varied as described in section 2.13.4.

Program 1 - The denaturing time was reduced from 1 min to 15 sec and the annealing temp increased to 51°C in program 1. Two weak PCR products, 300 bp and 350 bp, were produced with the *E. lata* single spore isolates E.l.SSI2, E.l.SSI3, E.l.SSI4, E.l.SSI5, E.l.SSI6, E.l.SSI8 and E.l.SSI9 (Fig. 4.10).

Program 2 - No PCR products were obtained with NcHMG1 and NcHMG2 PCR primers, the Touchdown PCR technique and *E. lata* E.l.SSI7, E.l.SSI8, E.l.SSI9 DNA templates, however, a single 300 bp PCR product was produced with the *N. crassa Mat a* DNA (results not shown). PCR products were not produced in the negative no DNA control.

Program 3 - Decreasing the denaturing, annealing and elongation times and increasing the number of PCR cycles to 40 obtained a faint but single PCR product of 300 bp. Initially this program was tested on isolates E.l.SSI7, E.l.SSI8 and E.l.SSI9, with only isolate E.l.SSI8 producing the PCR product (results not shown). However, when tested with all sixteen single spore isolates, the 300 bp product was obtained with isolates E.l.SSI7, E.l.SSI8, E.l.SSI9 and E.l.SSI10 (Fig. 4.11). *N. crassa Mat a* DNA also produced a single 300 bp product; this reaction yielded more PCR product than the *E. lata* reactions.

4.4.3 Sequence analysis of PCR products from single ascospore isolates.

PCR products were found in the expected size range for isolates E.l.SSI2, E.l.SSI3, E.l.SSI4, E.l.SSI5, E.l.SSI6, E.l.SSI7, E.l.SSI8, E.l.SSI9, E.l.SSI10, E.l.SSI15 and E.l.SSI16 with primers NcHMG1/NcHMG2 and NcHMG1/GfHMG2, along with products of other sizes. PCR products from reactions containing template DNA from E.l.SSI7, E.l.SSI8, E.l.SSI9, E.l.SSI10, E.l.SSI15 and E.l.SSI16 were shotgun cloned into pGEM-T, followed by sequence analysis as before (Section 4.3.5).

In total 150 recombinant plasmids were obtained with insert sizes ranging from 200-350 bp. Sequence analysis of 18 representative samples yielded sequences with no significant matches to any fungal mating type genes in the DNA or protein databases.

However, closer inspection of one of the sequences, clone 88.32, revealed the presence of some conserved amino acids and a putative intron (Fig. 4.12). Clone 88.32 was obtained from the isolate E.l.SSI9 with primers NcHMG1 and NcHMG2 using the PCR program given in Section 2.13.4. Two other clones with sequences identical to 88.32 were obtained from the same original PCR reaction. Two independent ligation reactions, one from a PCR containing E.l.SSI8 DNA template and the other with PCR products

100
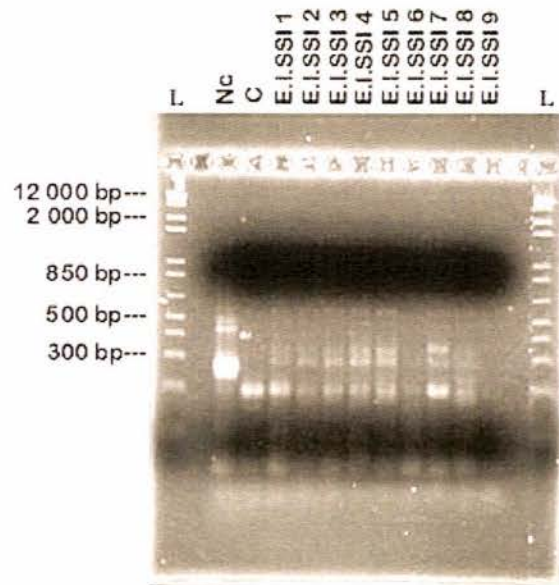
Figure 4.10 Agarose gel (2%) of PCR products amplified from the genomic DNA of 9 *E. lata* single ascospore isolates (E.l.SSI) using the NcHMG1-NcHMG2 primers. Nc indicates the *N. crassa Mat a* (*MAT1-2*) positive control and C indicates the negative (no DNA) control. The numbers at the left of the figure refer to the fragment sizes of the 1Kb + ladder (L) (Gibco BRL).
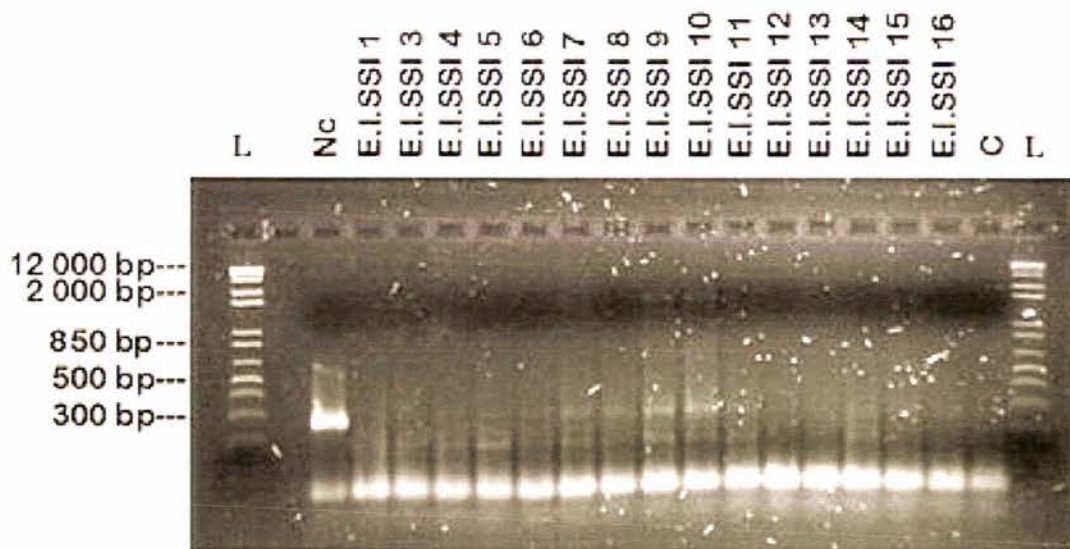
Figure 4.11 Agarose gel (2%) showing the PCR products obtained from PCR amplification of genomic DNA from 15 *E. lata* single ascospore isolates E.l.SSI 1-16) and the PCR primers NcHMG1-NcHMG2. Nc is a positive control reaction containing genomic DNA from *N. crassa Mat a* (*MAT1-2*). C is a negative (no DNA) control. The numbers at the left of the figure refer to the fragment sizes of the 1Kb + ladder (L) (Gibco BRL).

pooled from E.l.SSI7, E.l.SSI8, E.l.SSI9 and E.l.SSI10 DNA templates, also produced two additional clones with the 88.32 sequence.

The putative intron illustrated in blue (Fig. 4.12) is positioned at 93-133 bp within the clone and was found by searching all clone sequences for the fungal consensus intron splice sites. The 5' intron splice sequence is 5'-GTAGGTA-3', which fits the fungal consensus intron splice site sequence (5'-GT(A/T/G)NGTY-3') well (Ballance, 1986), although the 3' adenosine should be a cytosine or thymidine. At the 3' end the intron is bounded by 5'-TAG-3' a perfect match to the 5'-YAG-3' consensus sequence. The size of this intron, 40 bp, is within the 30-60 bp range expected for the conserved intron found in fungal *MAT1-2* HMG boxes when PCR amplified from genomic DNA. However, the predicted amino acid identity between clone 88.32 and other fungal mating type gene products was considerably lower than expected. Indeed, the closest related database sequence to clone 88.32 was a possible carnobacteriocin B2 immunity protein (Genbank accession number P38582) with 24% identity found along a short stretch of the clone (Score 31.6, E-value 6.3). No mating type gene sequences were found to have any significant identity to the sequence of clone 88.32.

Six out of eight conserved amino acids found within the *MAT1-2* HMG box could be found in clone 88.32. However, in order to place the conserved amino acids in the same reading frame it would be necessary to propose a frame-shift and the presence of a second intron with poor consensus splice sequences. The frame shift is required to place the serine-37 and asparagine-46 into the correct reading frame along with the other conserved amino acids, this is necessary somewhere between 50-56 bp in the clone. There is a stop codon found in one of the reading frames nearby the proposed frame shift, however, careful sequence analysis from all of the available clone 88.32 sequences did not support the proposed frame shift. In addition a putative second intron with poor splice sequences would be positioned 207-258 bp in the clone sequence in order to place the 3' primer sequence back into the open reading frame. The 5' splice sequence of this intron, 5'-GGTGCTC-3', differs from the consensus splice sequence 5'-GT(A/G/T)NGTY-3', where a thymidine and guanine in the consensus sequence are replaced with a guanine in the clone and a cytosine in the clone sequence. Hence clone 88.32 is unlikely to be a divergent *MAT1-2* HMG box from *E. lata*.

4.5 Designing primers to a new area of the *MAT1-2* gene.

To account for the possibility that an intron in the putative *E. lata MAT1-2* gene was positioned within one of the priming sites used for degenerate PCR, alternative primers were designed. Based partly on the priming sites used by Pöggeler (1999), the primers were designed to an alignment of full-length *MAT1-2* sequences. The positions of primers SmHMG1,2,3 in the *MAT1-2* alignment are shown in Figure 4.14. A combination of primers SmHMG1/SmHMG3 was expected to give a PCR product of 330 bp, whilst primers SmHMG2/SmHMG3 were expected to yield 580 bp (plus possible intron sequence of up to 60 bp). Faint, but distinct PCR products of the expected sizes were seen in two of four isolates initially tested (Fig. 4.13).

However, when all sixteen ascospore isolates were tested simultaneously, with the primers SmHMG1 and SmHMG3 all of them produced a fragment of the size predicted for a *MAT1-2* amplification product, including the isolate that gave a negative result in the initial test (results not shown).

Figure 4.12 Nucleotide sequence (292 bases, 5'-3') and predicted translation sequence (three forward frames) of clone 88.32 from *E. lata*. The primer sequences are underlined and a putative intron is shown in blue. Amino acids conserved in pyrenomycete *MAT1-2* HMG regions are shown in red. The putative second intron with poor splice sequences is shown in green.
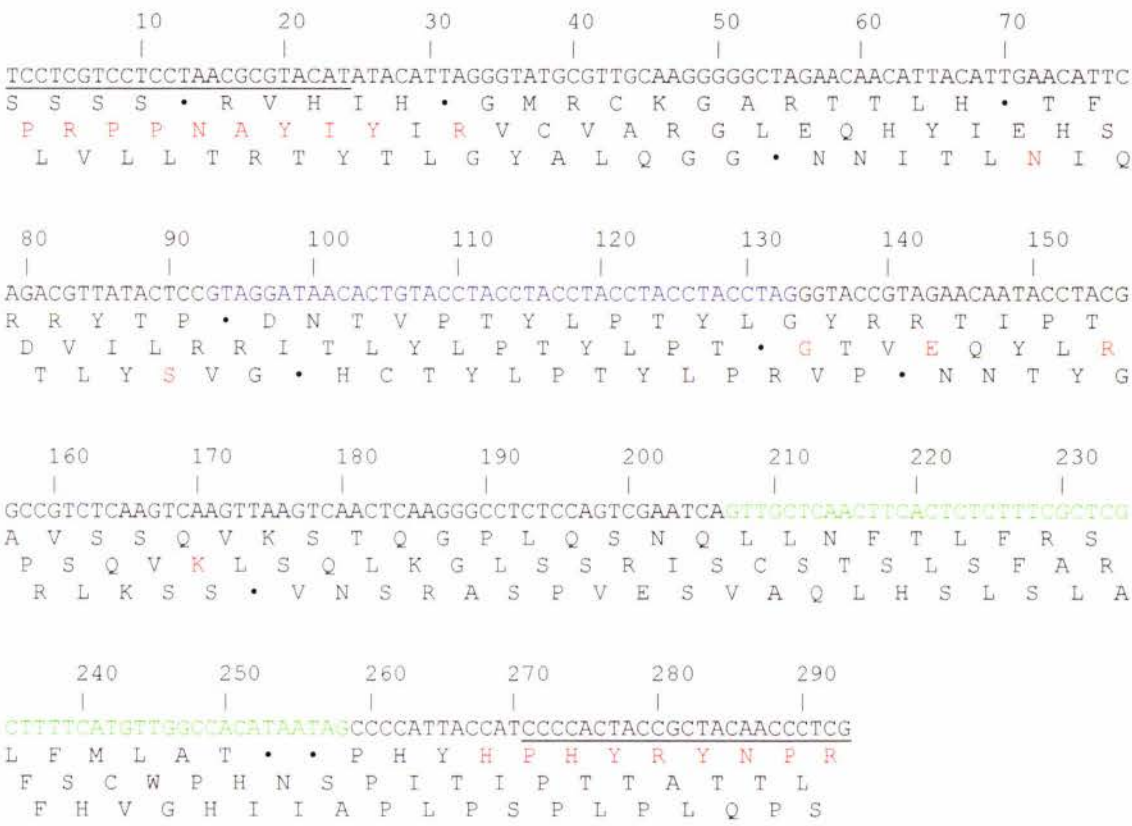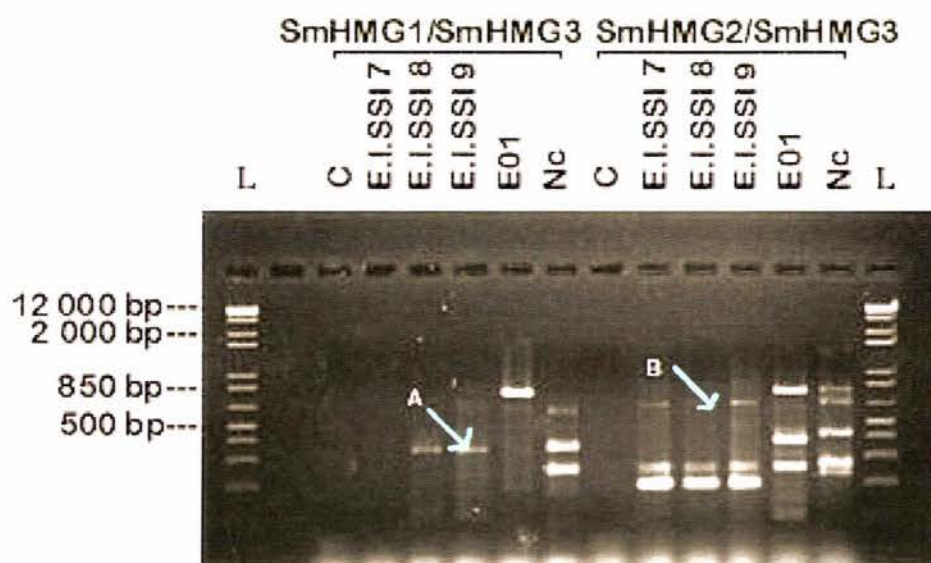
Figure 4.13 PCR amplification with the *MAT1-2* primers SmHMG1 and SmHMG3 and SmHMG2 and SmHMG3 primers. PCR templates (Genomic DNA) were: C-negative (no DNA), E.l.SSI7-9 - *E. lata* single ascospore isolates 7-9, E01 – *E. lata* EL01, Nc – *N. crassa mat a* (*MAT1-2*). L contains a 1 KB+ ladder (Gibco BRL) and the numbers to the left of the figure indicate the sizes of the 1 Kb+ ladder fragments. Arrows indicate potential *MAT1-2* gene fragments of approx. 325 bp (A) and 640 bp (B).

The 325 bp SmHMG1/SmHMG3 PCR product (Fig. 4.13 (A)) was cloned and sequenced using the shotgun cloning method. In total 70 recombinant plasmids were obtained with insert sizes ranging from 300-400 bp. Out of eleven clones sequenced, only three shared identical sequence and none of the clones showed any sequence similarities to fungal mating type genes. Moreover, none of them encoded the conserved amino acid sequence 'ARD' found in the pyrenomycete *MAT1-2* alignment in Figure 4.14 (positions 124-126) from which the SmHMG primers were designed.

Due to time constraints, the 640 bp product (Fig. 4.13 (B)) obtained with SmHMG2/SmHMG3 primers (expected to yield 580 bp + intron) was not cloned and sequenced.

4.6 Discussion.

4.6.1 Attempts to isolate the *MAT1-2* HMG box by heterologous probing.

The low level of sequence conservation among mating type genes from ascomycetes has generally precluded cloning of these genes from fungi via heterologous hybridisation. Nevertheless, the *P. anserina FMR1* gene was cloned via hybridisation to the *Mat A-1* gene of its close relative *N. crassa* (Picard *et al.*, 1991). Like *E. lata, P. anserina* is a pyrenomycete from the order Sphaeriales and the amino acid sequence identity between the *N. crassa MAT1-2* HMG box and *P. anserina* sequence is very low, as seen in the *MAT1-2* amino acid alignment (Fig. 4.2).

Figure 4.14 Alignment (Gene-Jockey II[®]) of the full-length *MAT1-2* amino acid sequences of four pyrenomycete species. The positions of the primers SmHMG1, SmHMG2 and SmHMG3 are indicated by a block and the sense of each primer by the direction of the arrowhead reported beneath the alignment.

Contig# 1

Gibberella fujikuroi
Neuropora crassa
Podospora anserina
Sordaria macrospora

```
            10        20        30        40        50        60        70        80        90       100
             |         |         |         |         |         |         |         |         |         |
Contig# 1                                                                                  |  •||       |
Gibberella fujikuroi  MANF-----  M  G-----A    F  MJ  EH -------  R  Q-------C H  K  --------------------K  K  ---   YNE
Neuropora crassa      M G------N TH A ------------------------------------------------N K TMA---  R  NQ GH N RK    F N H
Podospora anserina    MAAFNFEAF  OG   AA R AA A  R  QQQCG  G GNRAFQ  A  E   E AN G TE  TAK NHF  Q GH NT K    AQMF  N
Sordaria macrospora   ME ------N MH A  ------------------------------------------------ AE RA MA---  R  NQ GH N RK R      F  AH

                                                                                          SmHMG3
           110       120       130       140       150       160       170       180       190       200
             |         |         |         |         |         |         |         |         |         |
Contig# 1     |   •|        •     ||•••|       |  |•  |     |        |         |         ••••••••••••••••|||   ||   •|| •
Gibberella fujikuroi  GAKAGFAN  M H GM -   AR  QNNC  GA R FFAKGGM  N  GA E --F HR EK AF -----AK  R  NAV  VRKERHH  KAQR   IN
Neuropora crassa       Q G  AEFKKA G-EEGMFAR  E  G M  G K FK     HA ---GN FI  KG HA A KEQQKK-AK  R  NAV  VRK HHRE REQN G HN
Podospora anserina    HTKKG  N MK   GGA AN AR A NGQ    A RR ME N---   G  I U  KKRH QA AE-----AK  R  NAV  VRK OQAA KAAN G  N
Sordaria macrospora    HAG AE KKA G-EEGMFAR  EA E M  G K K    E---GN FI  KG HAEA KQQQKKKAK  R  NAV  VRK HHRQ REQN G HN

                                                                                      SmHMG1
           210       220       230       240       250       260       270       280       290       300
             |         |         |         |         |         |         |         |         |         |
Contig# 1    •||   |  •  |•  •    ||•  •     ||  •|      •|•|• •••|  |  •••        |         |         |
Gibberella fujikuroi  NE  Q  GR  N E RE RA  KQME QKKAEHRRO    O R RR  ERRRRN---------------------------------------------NA   R
Neuropora crassa      NEM-  FGNM R EQ H REK  NM NE K R   EN  VR N RR Q  RRR     K K  N  NGN   G  NAE AA  RTHFHG VR  EEM  GCR
Podospora anserina    N   M GGM KKE  E RAE QRRA E KAK M AH H R   FRR  E RRRA R--------------------------------RNRAQE ANA   GEN G
Sordaria macrospora   NE   M GNM R EQ H R K  MANE KAR    N   R N RR Q  RRR  EY K  N  GN N   G  NAE AA  RTHFHG VR EET  EGCR

                                                                                  SmHMG2
           310       320       330       340       350       360       370       380       390       400
             |         |         |         |         |         |         |         |         |         |
Contig# 1       |  |  | |     |  |•  ||| |        •|  |  ||•       ||  ||   | | •  |•      |•  ••       ||  |  •|   •
Gibberella fujikuroi  A--- A  QQM A
Neuropora crassa       CR  AG RK RAA    NM R   TT FIE   AQAFNFN   GGA F  NEH    NQN  FNA   N --N H    H  GMEA  HN ONM AO
Podospora anserina    A---   GN   MEQQO     A NQE  K N  H      H ------F GQ  E M  ANF   M REG   H FRA  NGH GNNG   --CA
Sordaria macrospora    CR  AG RK RAAN  NM R    A   EE EAA

           410       420       430       440
             |         |         |         |
Contig# 1     •||   |   •||   |   •    •|     |•  •  |
Neuropora crassa      QEANEAAA           R    R  M   Q  --
Podospora anserina    E E Q  F G    M  N AF  W  GTEE  AQ  GQ
```

Heterologous probing between the *Neurospora crassa* and *E. lata* genomes was not expected to isolate the *E. lata MAT1-2* HMG box, because of a lack of overall nucleotide sequence identity and in the resulting Southern blot the *N. crassa MAT1-2* fragment hybridised to *N. crassa mat a* genomic DNA but not to *N. crassa Mat A* or *E. lata* DNA. This indicated the four NZ *E. lata* isolates were *MAT1-1*, or, the overall nucleotide sequence identity between the *N. crassa* and *E. lata MAT1-2* HMG boxes was not adequate to allow identification of the *E. lata MAT1-2* by hybridisation with the *N. crassa Mat a* idiomorph. It may be possible to isolate the *E. lata MAT1-2* HMG box by hybridisation with the *P. anserina MAT1-2* homologue. Alternatively an end labelled NcHMG1 primer could be used to isolate the *E. lata MAT1-2* idiomorph, as the NcHMG1 primer position is highly conserved among pryenomycetes (Fig. 4.2).

It is likely that the low labelling efficiency exhibited when the probe DNA was Digoxygenin-11-dUTP labelled using the DIG-high prime kit (Roche), was due to the age of the labelling kits used in the reaction.

4.6.2 Genetic diversity analysis.

Random Amplification of Polymorphic DNA (RAPD) analysis has been extensively used to examine the genetic diversity between isolates of *E. lata* (Péros *et al.*, 1996; Péros *et al.*, 1997; Péros & Larignon, 1998; Péros *et al.*, 1999; Péros & Berger, 1999). A high level of genetic variation has been found between isolates originating from different geographic locations, isolates from a single vineyard and single ascospore isolates from the same stroma, using this technique. However, in this project RAPD analysis failed to generate any amplified products and hence no polymorphism was observed between the four laboratory *E. lata* cultures (EL01, E11787, E11788 and E11PDA2). Two different decamer primers were used in the analysis and amplification was performed under high (42°C) and low (37°C) stringency conditions.

Although the thermal cycling conditions used by Péros *et al.* (1996) are identical to those used in this study, there are several differences in the PCR reaction mix that may be responsible for the lack of amplification seen in this project. Péros *et al.* (1996) added 0.01% (w/v) gelatin and 0.1% Triton X-100 to the PCR reaction and the amount of *Taq* polymerase, primer and DNA template was also decreased. However, the protocol and primers used in this project (Section 2.13.6) had previously been successfully used to examine diversity within the New Zealand population of *Dothistroma pini* DNA (Hirst, 1997) and PCR amplification of the positive control *D. pini* DNA was not possible, indicating that the PCR reagents were not all functioning.

4.6.2.1 RAMS analysis.

The diversity within the 20 *E. lata* isolates was investigated using Random Amplification of Microsatellites (RAMS) analysis. Three degenerate anchored primers were used; Anchored AAG, anchored TG and Anchored CT (Table2.2), as these microsatellite sequences are abundant in fungal genomic sequences deposited in the Genbank databases (Groppe *et al.*, 1995). Amplification of the four laboratory *E. lata* cultures (E01, E11787, E11788 and E11PDA2) with the anchored CT primer generated distinct banding profiles and identified each as a unique genotype. However, RAMS profiling of the 16 single ascospore isolates did not produce reproducible polymorphism between the isolates.

PCR amplification was repeated twice on the single ascospore isolates for each of the 5' anchored primers and although the protocol used by Fisher *et al.* (1996) optimised to reproducibly PCR amplify microsatellite loci using the 5' anchored primers was adopted, reproducible banding patterns were not obtained. Figure 4.8 illustrates the variation in the banding patterns produced with the anchored AAG primer, in addition to the complete absence of any amplification with some of the templates, the number, size and intensity of the bands produced varied. Similar trends were also seen with the other primers (Fig. 4.7 and Fig. 4.9).

The 5' anchored procedure is designed to consistently anchor the PCR primers at the 5' ends of the microsatellite, amplifying two close and inverted simple sequence repeats and the region between them (Fisher *et al.*, 1996). The primers consisted of a 3' component designed to anneal to a microsatellite repeat and a redundant 5' anchor complementary to one in six possible random sequences adjacent to the repeat. In practice this approach has proven difficult to optimise for complex genomes and there has been little evidence that variation in microsatellite repeat length at the primer binding sites has been preserved (Zietkeiwicz *et al.*, 1994).

Fisher *et al.* (1996) describe the determination of PCR conditions, such that consistently anchored amplicons are produced, as crucial. Excessive stringency should produce no amplicons, whereas low stringency will permit slippage of the primer to the 3' end of the targeted microsatellite loci resulting in loss of repeat length variation. To prevent this slippage, the annealing stringency had to be high enough so that amplification occurred only when one or more of the five anchoring bases on the primer paired with the template. In experiments designed to determine the optimum annealing temperature for consistent anchoring with the anchored CT primer (Fisher et al., 1996), the anchoring window (in which reproducible banding patterns were produced) was approximately $\pm4°C$ for a sequence that was perfectly complementary to the five degenerate anchoring bases. However, when the sequence matched at only two of the five anchoring bases, this window was reduced to $\pm1°C$, and a high proportion of the amplicons resulted from unanchored amplification.

The difficulties presented in the present study may have arisen because the PCR primers failed to anchor at the 5' end of microsatellites, instead slipping to the 3' end of the microsatellites during PCR. Each amplified fragment therefore contained exactly the number of repeats found in the primers and any repeat length polymorphism is lost. Alternatively, the degeneracy of the anchored regions in the primers may have allowed amplification in two unrelated regions of the genome.

Banding or smearing in the negative control was observed for all three 5' anchored primers. In all cases, this banding was faint, not reproducible and did not correlate with the bands observed in the amplification of the isolates. Amplification of products in the negative control reaction is common in RAPD amplification and is believed to be a result of primer extension caused by primer dimer formation. Due to the repetitive microsatellite sequence and the degenerate nature of the 5' anchored primers used in these reactions this seems a likely explanation for the presence of these products in the negative control. The availability of a DNA template would out-compete primer dimer extension. In addition, an excessive amount of 5' anchored primer was required for each reaction, which would increase the probability of this occurring. An excess of primer

and possibly primer dimers was observed in most reactions as a smear below the 100 bp mark.

4.6.3 PCR amplification of the *MAT1-2* HMG box.

The objective of this work was to PCR amplify and sequence the *MAT1-2* HMG box from a range of *E. lata* isolates. A range of New Zealand isolates, including sixteen single ascospore isolates from one perithecium, were tested for the presence of a *MAT1-2* idiomorph by PCR amplification using five different sets of primers, designed to anneal at different target sites with different specificities. PCR products of the expected size were obtained and sequenced, but despite exhaustive attempts to optimise PCR specificity, none of these had convincing homology to fungal mating type genes.

Optimisation of PCR specificity.
The variation of annealing temperature, dNTP and magnesium concentration had the greatest effect on the PCR amplification of products from genomic DNA using degenerate primers designed to the *MAT1-2* HMG box.

Decreasing the PCR annealing temperature lowers the specificity of the reaction and increases the yield of PCR products. It was found that the PCR reaction had a range of dNTP and magnesium concentrations at which PCR could occur. However, decreasing or increasing the concentration of dNTPs (or magnesium) in the PCR reaction outside this range eliminates all PCR products. When the final magnesium or dNTP concentration is within this range, increasing the concentration increases the yield of PCR product. However, fewer PCR products in the size range (200-350 bp) are obtained and the number of non-specific PCR products is also increased.

In addition to the optimisation of the magnesium and dNTP concentration, varying the following conditions may also useful when optimising a degenerate PCR reaction:

- The use of nested primers for subsequent rounds of PCR amplification increases the specificity of the PCR reaction. Nested PCR may be performed directly from the previous PCR reaction, without the need to excise the product from an agarose gel and purify, when more than one PCR product is obtained.
- As the primers are degenerate it is important to ensure an excess of primers.
- If a gradient thermal cycler is unavailable, vary the annealing temperature by starting the PCR reactions at a low annealing temp (47°C) and move up in 2°C increments.
- The use of a hot start PCR procedure. This can be achieved manually by only adding *Taq* polymerase after the template has reached the denaturing temperature, or through the use of a modified *Taq* polymerase.

A survey of the literature in which the PCR method has been used to clone *MAT1-2* gene fragments from a wide variety of ascomycete fungi (Table 4.1) suggests that in most cases a single (or predominant) PCR product is obtained corresponding to the HMG domain. There are several explanations for why we have been unable to find a convincing candidate for a *MAT1-2* gene from *E. lata* thus far, despite using the same methods:

PCR conditions were sub-optimal to achieve amplification of the MAT1-2 gene fragment.

This possibility seems unlikely as the positive control (genomic DNA from a *Neurospora crassa MAT1-2* strain) confirmed that all PCR reagents were functional. Moreover, attempts to optimise conditions for *E. lata* by varying parameters of the reaction were exhaustive. Arie *et al.* (1997) also reported lack of success when attempting to PCR amplify the equivalent fragment from 13 fungal genera, of which eight were pyrenomycetes. Their negative results were tentative as only two or three trials had been carried out with a single set of PCR conditions in each case. However, the mating type gene sequences of some of these fungi have since been reported and the sequences cloned using the PCR method (e.g. *Fusarium oxysporum*, Arie *et al.* 2000).

All isolates tested were of mating type 1 (MAT1-1) by chance.
A total of 20 New Zealand isolates were tested for the presence of the *MAT1-2* idiomorph by degenerate PCR. Sixteen of these were taken from a single perithecium, which was expected to be heterothallic. The probability of 16 single ascospore progeny derived from a hybrid perithecium all being *MAT1-1* isolates by chance is only $(0.5)^{16} = 1.53 \times 10^{5}$ (assuming equal segregation and equal viability of the two mating types), hence seems unlikely. However, the possibility remains that the *E. lata* isolates tested may all have been of the opposite mating type (*MAT1-1*) and more isolates from different geographical regions (and countries) need to be tested for the presence of the *MAT1-2* HMG box.

All isolates tested were of mating type 1 (MAT1-1) because they were progeny of a MAT1-1 isolate capable of self-fertilisation.
Although initial DNA profiling of the 16 single ascospore isolates revealed polymorphism (thereby supporting the expectation of heterothallism) the results were not reproducible (Fig. 4.7- Fig. 4.9). This cast doubt on whether the observed polymorphisms were real or PCR artefacts and whether sexual outcrossing had occurred. Therefore, it is possible that the sixteen single ascospore isolates are the progeny of a *MAT1-1* isolate capable of selfing, or put another way the *E. lata* perithecium examined was homothallic (and all perithecial isolates tested were *MAT1-1*). Since the genetic evidence for heterothallism is convincing (Péros and Berger, 1999), this would imply that some *E. lata* strains could be heterothallic while others are homothallic.

Milgroom (1995) demonstrated that population structures in a fungal pathogen may vary among geographic location, where asexual reproduction, outcrossing and self-fertilisation were shown to intervene at different rates in populations of *Cryphonectria parasitica*. Therefore, explaining how perithecia examined in France can display outcrossing (Péros and Berger, 1999) while perithecia examined in New Zealand are capable of selfing (this study). In addition, the genus *Fusarium* displays evidence that both homothallic and heterothallic strains can occur in the same species (Booth 1971).

The amino acid sequence of the E. lata *MAT1-2 idiomorph is not conserved at the sites to which PCR primers were designed.*
The amino acid sequence of the *MAT1-2* idiomorph in *E. lata* may not be conserved in the regions used for priming. The consequence of this would be low specificity of primer annealing to the *MAT1-2* gene. However, the regions of *MAT1-2* to which the PCR primers were designed are conserved across a wide variety of fungal genera (Fig.

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4.2), hence it seems likely that *E. lata* will contain the conserved sequences, if the *MAT1-2* idiomorph is present.

However, PCR products of the expected size were not obtained in thirteen fungal genera tested by Arie *et al.* (1997). The authors cite several likely explanations including the fact that the primers may not be a suitable match. It is significant that primers designed by Arie *et al.* (1997) for the amplification of loculoascomycetes did not amplify pyrenomycete DNA and vice versa. This suggests that as *MAT* sequence data become available for a wide variety of taxa, refinements in primer design may be necessary to amplify *MAT1-2* specific HMG boxes from recalcitrant fungi such as *E. lata*. The inability by Arie *et al.* (1997) to amplify the HMG type protein from *Ustilago maydis* demonstrates this. *U. maydis*, a basidiomycete, does not encode the HMG proteins at the *MAT* loci. However, another gene encoding an HMG protein that is a key regulator of the mating response pathway was cloned using a similar PCR procedure (Hartmann *et al.*, 1996). The primers described by Arie *et al.* (1997) fail to amplify the HMG box region of this protein because of key differences between the primer sets. It is not yet known whether all the fungi that gave negative results in the PCR amplifications of Arie *et al.* (1997) contain conserved *MAT1-2* idiomorphs, divergent forms of *MAT1-2* such as *U. maydis*, or have an alternative mating control system.

Five different sets of degenerate primers with different amino acid specificities and designed to anneal to different target sites within the *MAT1-2* idiomorph were used for PCR amplification with genomic DNA from *E. lata*. Hence, it is unlikely that the *MAT1-2* idiomorph in *E. lata* is not homologous in all of the regions used for priming with all of the primer sets.

The E. lata *MAT1-2 gene is different in sequence to most other fungal MAT1-2 genes and has already been cloned.*
It is possible the mating type genes of *E. lata* are unrecognisable by comparison with those of other ascomycetes. The amino acid sequences for *Podospora anserina* and *Cryphonectria parasitica MAT1-2* HMG box regions are quite different from those of other pyrenomycetes (Fig. 4.2). In between the conserved areas used for PCR primer design there are only seven well-spaced conserved amino acids. Clone 88.32 (Fig. 4.12) also contains some of these conserved amino acids, although not all in the same reading frame. Because independent clones were found with the same sequence it is unlikely that frame-shift errors were introduced during PCR amplification and the sequence shown is probably correct. Although clone 88.32 is very unlikely to be part of a mating-type gene, it has the interesting feature of a $(TACC)_6$ repeat in the putative intron, and thus has potential as a microsatellite marker for genetic diversity studies.

*E. lata *is heterothallic but has a different type of mating system control to that of most other pyrenomycetes.*
There are some fungi in which mating is controlled by alternative systems. For example, the pyrenomycete *Glomerella cingulata* is thought to have a multi-allelic system of 'unbalanced heterothallism' (Cisar and DeBeest, 1999) whilst *Glomerella graminicola* has two mating type loci that are independent of the *MAT1* locus (Vaillancourt, 2000). In the latter, all mating types carried the *MAT1-2* idiomorph, which appeared to be non-functional in terms of controlling mating compatibility. It is possible that an alternative mating control system exists to determine heterothallism in

E. lata. In this case the isolates tested may contain non-functional *MAT1-1* idiomorphs only.

4.6.4 Conclusions.

Twenty New Zealand isolates of *E. lata*, including sixteen single ascospore isolates from one perithecium, were tested for the presence of a *MAT1-2* idiomorph by PCR amplification. Five different sets of primers were used which were designed to anneal at different target sites with different specificities. PCR products of the expected size were obtained and sequenced, but despite exhaustive attempts to optimise PCR specificity, none of these had convincing homology to fungal mating type genes.

5. DISCUSSION AND FUTURE DIRECTIONS.

5.1 *E. lata* detection.

Grapevine dieback has had a notable financial impact on the grape and wine industry world-wide (Johnson & Lunden, 1985; Thanassoulopoulos, 1989). The fungus *E. lata* has been identified as a major cause of grapevine dieback but obtaining confirmation of the cause of dieback, whether it be *E. lata* or a range of other fungi such as *Botryopshaeria* species, is not simple (Carter, 1957, Castillo-Pando *et al.*, 2001). This project aimed to determine whether molecular detection techniques would be a viable alternative to the conventional selective media plating technique.

Selective media isolations from grapevine cordons with dieback symptoms yielded a wide range of fungi, in which *E. lata* and *Botryosphaeria* species were predominantly isolated. None of the agar media used for sampling were superior to the others for isolation and culturing of the pathogen or of other fungi associated with dead and dying grapevine wood. While typical cultures were readily identified in the main, a considerable number required incubation over several weeks to obtain spore production and confirmation of identification. This would be a major drawback in any large-scale vineyard survey because of the large numbers of cultures that would be held in incubators for long periods of time, the amount of labour required to maintain and examine them and the length of time before results are available to the viticulturalist.

An *E. lata* DNA based diagnostic system, consisting of two specific PCR primer pairs designed to the ribosomal Internal Transcribed Spacer region (ITS) of the fungus, was developed. It was shown to be highly specific and sensitive for detecting the presence of the fungus. The results showed that that each of the PCR primer pairs amplified a specific fragment from the ITS region of *E. lata*. No amplification was observed with these primers when DNA from vines or from other fungi that commonly colonise grapevine wood concomitantly with *E. lata* was used.

Unlike traditional identification procedures that require several weeks from start to finish, the results here show the DNA based approach has the potential to directly detect the presence of the fungus in woody tissue, even if there are no visual symptoms. As the data showed, the PCR based detection of *E. lata* would markedly speed up the identification process and indicate in a short time whether specific precautions are required to prevent the spread of grapevine dieback. In addition, this PCR assay would serve to ensure that the sanitation procedures employed had been effective. Another advantage of the PCR based assay is that no specialised mycological training is required and the reagents and equipment needed are common in most molecular biology laboratories. Identification of *E. lata* by selective media isolation requires sub-culturing unknown fungi to purity and subsequent taxonomic identification by a trained mycologist. *E. lata* does not produce sexual structures in artificial media, so it is usually characterised by "white and fluffy" mycelia. Hence, the molecular detection of *E. lata* using a ITS based PCR assay is a fast, effective alternative to the conventional selective media plating technique, with the detection of *E. lata* from DNA samples able to be completed in a single day.

5.1.1 Future work.

The *E. lata* specific primers presented here are effective for detecting *E. lata* from genomic DNA template, however, further work is required to improve the sensitivity of the PCR based assay when diseased grapevine wood is used as a template.

In particular, the DNA extraction and PCR technique from diseased grapevines must be examined further, to ensure reliable, consistent results. Many by-products of wood decay, if present at too high a concentration in the reaction, would inhibit PCR amplification of the DNA template. Removal of PCR inhibitors from wood extract using ß-mercaptoethanol incubation (Jasalavich *et al*, 2000) and a CTAB lysis buffer (Irelan, 1999; Moon *et al*., 1998), during the DNA extraction process should provide DNA clean enough to PCR amplify. However, such procedures for DNA isolation and purification are longer than desired to routinely screen large numbers of wood samples. Alternatively, methods in which the fungus is cultured out of the diseased wood in liquid culture and the DNA subsequently extracted from culture pellets could be investigated (Tegli *et al*., 2000).

In addition to this PCR procedures, such as the addition of Bovine Serum Albumin (BSA), (known to relieve inhibition of amplification by humic acids, fulvic acids, and organic components of soils and manure) to the PCR reaction (Jasalavich *et al*., 2000), dilution of the total DNA extract PCR template (thus diluting any PCR inhibitors present) (Lecomté *et al*., 1999), and hot-start PCR procedures (to circumvent non-specific priming during the set up and start of PCR) (Bogetto *et al*., 2000), are likely to allow amplification to occur from diseased grapevine wood.

To increase the detection power of the assay more work must be completed. The ability to detect *E. lata* in other host wood species (eg Apricot trees) should also be examined. The differing chemical compositions of both the un-decayed and decayed forms of these substrates could introduce new kinds of PCR-inhibitory compounds that may or may not be eliminated or neutralised by current methodologies. Currently, the assay is only qualitative; more work needs to be done to make it a quantitative assay.

Once a quick, sensitive, reproducible method for detecting the presence of *E. lata* in woody tissue is developed, this can be used to investigate host pathogen interactions requiring localisation and identification of the fungus. PCR identification of *E. lata* could be used to monitor the development and trace the movement of the fungus in grapevine tissues and to assess infections *in situ*. The system can also be used to identify strains of *E. lata* to be used in pathogenicity studies or to assess the resistance of grapevine cultivars. Similar procedures may also be used to detect and/or study micro-organisms associated with wood cankers or diseases in other perennials.

The opportunity now exists to assess the presence and severity of grapevine dieback long before disease symptoms are visually apparent. Rapid methods to detect *E. lata* from grapevine wood material will also be useful in the study of the epidemiology of the disease, such as wound receptivity.

The PCR assay may also be adapted to study the nature and the complexity of the fungal community present in the decaying grapevine wood. The use of broad based primers, such as ITS4-B (Gardes *et al*., 1993) which efficiently amplifies basidiomycete DNA,

and species-specific primers such as those developed here can identify a fungus of interest and alert investigators to the presence of other fungi in the decaying wood. In the broader context, taxon selective amplification of the ITS region is likely to become a common approach in molecular identification strategies. Taxon-selective ITS amplification has already been used for the detection of decay fungi in spruce wood (Jasalavich *et al.*, 2000) and the fungal pathogen *Verticillium* (Nazar *et al.*, 1991).

If the natural substrate contains a single dominant fungus, the species-specific identification method is probably the fastest way to analyse it. However, in more complex communities (e.g. decaying wood and soil), specific taxa could be assayed by oligonucleotide probing when such probes become available. By designing a series of strain-specific non-radioactively labeled probes (Bugwan *et al.*, 1988; Saiki *et al.*, 1989), it should be feasible to rapidly identify strains and to follow their survival in the field. Additionally, fluorescent species-specific primers could be designed for identification of strains using *in situ* PCR. However, perhaps the most exciting future direction is the use of DNA microarrays to identify plant-fungal interactions. Here fungal species-specific oligonucleotide probes are attached to a glass chip to which total plant fungal DNA extracts are applied. Hybridisation of the DNA extract with any of the oligonucleotide probes results in a change in the fluorescence of the probe. A single DNA microarray can be used to store oligonucleotide probes to identify all the major grapevine fungal diseases, such as grapevine dieback and esca, and bacterial and viral infections commonly found in grapevines. At present the major limitation to such work is the lack of suitable oligonucleotide probes.

5.2 *E. lata* mating type identification.

Very little is known about the life cycle or mating habits of *E. lata*, except that it is thought to be heterothallic on the basis of genetic studies with perithecial isolates that have shown considerable diversity (Péros & Larignon, 1998). As the sexual stage of *E. lata* cannot be obtained in culture at present, the analysis of its mating system must be performed in natural populations. The aim was to develop a tool to be used to assess the mating types of the fungus in the natural environment.

Cloning of mating type (*MAT*) genes from ascomycetes has been hampered by low conservation among them. Most ascomycete fungi have one mating type gene with two alternative forms or idiomorphs (*MAT1-1* and *MAT1-2*). One of the pair of *MAT* idiomorphs, *MAT1-2*, encodes a protein with a conserved DNA binding motif called the high mobility group (HMG) box. There is sufficient sequence conservation at the borders of the HMG box to allow PCR amplification (Arie *et al.*, 1997). New Zealand isolates of *E. lata*, including sixteen single ascospore isolates from one perithecium, were tested for the presence of a *MAT1-2* idiomorph using this PCR based approach. Five different sets of primers were used which were designed to anneal at different target sites with different specificities. PCR products of the expected size were obtained and sequenced, but despite exhaustive attempts to optimise PCR specificity, none of these had convincing homology to fungal mating type genes.

As neither *E. lata* mating type idiomorph was isolated or cloned in this project, it was not possible to develop a rapid and reliable assay for determining which mating type idiomorph is present in any given *E. lata* isolate.

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5.2.1 Future work.

Because it has not been possible to determine mating types of *E. lata* by inducing the sexual cycle in culture so far, it is worth pursuing the molecular approach. Although it is possible that *E. lata* does not have a conserved *MAT1-2* gene, there are several experiments which should be carried out to determine whether the lack of success so far was due to the limited number of isolates tested, or to intrinsic differences in the mating type genes of *E. lata* compared to most other fungi:

The examination of different *E. lata* isolates from overseas and around New Zealand for the presence of the *MAT1-2* idiomorph will compensate for the limited and geographically restricted sample size used so far. The use of all sets of *MAT1-2* specific primers developed, optimised PCR parameters and the *N. crassa Mat a* DNA as a positive control will quickly identify any putative *MAT1-2* PCR products. In addition, at least two more perithecia from New Zealand should be examined for reproducible polymorphic differences between single ascospore isolates using RAPD (Péros & Berger, 1998; Péros & Larignon, 1999) and RAMS analysis (Zietkiewicz *et al.*, 1994; Ganely & Bradshaw, 2001). If polymorphism is found in these perithecia, these isolates should be screened for amplification of a *MAT1-2* gene fragment testing, since population structures in fungal pathogens may vary among locations (Milgroom, 1996).

Once a *MAT1-2* clone is obtained, a sequence correct copy of the HMG box should be independently PCR amplified using the degenerate primers and a DNA polymerase with proof reading capability, such as *Pwo*. The primers used to obtain the clone are degenerate, hence, the sequences in the primer binding regions will probably be different. The full length *MAT1-2* idiomorph can then be isolated from genomic DNA using Thermal Asymmetric Inter-Laced (TAIL) PCR (Lui & Whittier, 1995), Inverse PCR or probing a Southern blot with the *MAT1-2* product and the subsequent cloning of large (approximately 1-5 Kb) positively hybridising fragments into *E. coli* to make a sub-genomic library. The transformants can be screened by probing with the *MAT1-2* product in a colony hybridisation assay or PCR.

In addition to examining different *E. lata* isolates, analysis of the existing clones obtained so far should be continued. Nested PCR can be performed with the as yet uncharacterised 640 bp SmHMG2/SmHMG3 PCR product and internal *MAT1-2* primers (NcHMG1 and NcHMG2). Cloning and sequencing the 640 bp product will also identify any putative *MAT1-2* gene fragments. However, as the 325 bp fragment has already been sequenced and the resulting sequences did not display any similarity to other fungal *MAT1-2* sequences, the likelihood of this fragment being an *E. lata MAT1-2* HMG box is very low. However, there were only seven conserved amino acid residues with which to identify any putative *MAT1-2* sequence in the SmHMG1/SmHMG3 PCR product. The SmHMG2/SmHMG3 PCR product should contain those seven amino acids plus the entire *MAT1-2* HMG box (an additional thirty three conserved amino acids) if it is indeed of *MAT1-2* origin.

Once a putative *MAT1-2* fragment has been identified, this fragment can be used to design *MAT1-2* idiomorph specific PCR primers to test for the presence or absence of these sequences in overseas and single ascospore isolates using high stringency PCR. However, the use of a single set of idiomorph specific primers to assign mating type is not ideal because a *MAT1-1* isolate, indicated by the absence of a PCR product with the

MAT1-2 primers, cannot be distinguished from a failed PCR reaction. In addition, contamination of a *MAT1-1* sample with *MAT1-2* DNA gives a false-positive signal. Therefore, to obtain an unambiguous result in a PCR based mating type assay it is essential that the *MAT1-1* and *MAT1-2* idiomorphs are assayed simultaneously, as in the multiplex PCR identification systems developed for *Gibberella fujikuroi* and *Fusarium circinata* (Steenkamp *et al.*, 2000; Wallace & Covert, 2000).

The isolation and cloning of the *MAT1-1* idiomorph, necessary for a multiplex PCR reaction (as described by Steenkamp et al., 2000 and Wallace & Covert, 2000) can be achieved by attempting to PCR amplify a region of the idiomorph using the existing New Zealand isolates with primers designed to the α1 domain (Pöggeler 1999). This region is not so conserved as the *MAT1-2* HMG domain and Pöggeler had to use nested PCR to achieve success with this method.

Alternatively, the *MAT1-1* idiomorph can be isolated and cloned using PCR once the full length *MAT1-2* idiomorph has been obtained from *E. lata*. This is because the regions flanking the *MAT1-2* idiomorph are also common to the *MAT1-1* idiomorph. The converse, in which the *MAT1-2* idiomorph is obtained using PCR primers designed to the flanking regions of the *MAT1-1* idiomorph (should it be possible to isolate and clone the *MAT1-1* idiomorph using nested PCR to the •1 domain), is also true.

A DNA based approach to characterising the different mating types, such as the multiplex PCR reactions developed by Wallace and Covert (2000) and Steenkamp *et al.* (2000), can be used in attempts to complete the sexual life cycle of *E. lata* in culture, as well as to determine how the mating types are distributed in the field. The ability to identify compatible pairs for mating experiments is especially valuable when first attempting to carry out sexual crosses in a new species because it drastically reduces the number of crosses that must be set up. If working without any mating type characterisation, all potential members of a new mating population must be intercrossed, and thus the number of crosses that must be established increases as the square of the number of isolates being tested (Covert *et al.*, 1999). Therefore, molecular characterisation of mating type at the outset of a mating project allows significant savings in time and effort.

5.3 Concluding remarks.

5.3.1 Aim 1- To develop a PCR based test for the detection of *Eutypa lata* within grapevine wood.

5.3.1.1 Progress.
An *E. lata* DNA based diagnostic system, consisting of two specific PCR primer pairs designed to the ribosomal Internal Transcribed Spacer region (ITS) of the fungus, was developed. It was shown to be highly specific and sensitive for detecting the presence of the fungus from genomic DNA extracted from *E. lata* cultures. The results showed that each of the PCR primer pairs amplified a specific fragment from the ITS region of *E. lata*. No amplification was observed with these primers when DNA from vines of from other fungi that commonly colonise grapevine wood concomitantly with *E. lata* was used. In this study it has not been possible to reproducibly amplify the *E. lata* specific

fragments, directly from *E. lata* mycelial extracts or from total genomic DNA extracted from diseased grapevines.

5.3.1.2 Future work.

Further work is required to optimise the PCR based assay for use with diseased grapevine wood templates. This should lead to the development of a rapid, sensitive and reproducible method for detecting the presence of *E. lata* in woody tissue, which will have many applications in the vineyard.

5.3.2 Aim 2- To develop a PCR assay to enable the identification of the mating type idiomorphs of *Eutypa lata*.

5.3.2.1 Progress.

Twenty New Zealand isolates of *E. lata*, including sixteen single ascospore isolates from one perithecium, were tested for the presence of a *MAT1-2* idiomorph using a PCR based approach. Five different sets of primers were used which were designed to anneal at different target sites with different specificities. Three hundred and seventy plasmids containing PCR products of the expected size were obtained by shotgun cloning, directly from the degenerate PCR reactions. The inserts from 46 of these plasmids were sequenced, but despite exhaustive attempts to optimise PCR specificity, none of the inserts sequenced had convincing homology to fungal mating type genes. As neither *E. lata* mating type idiomorph was cloned in this project, it was not possible to develop an assay for determining which mating type idiomorph is present in any given *E. lata* isolate.

5.3.2.2 Future work.

In order to determine whether the lack of PCR amplification of the *MAT1-2* gene fragment so far was due to the limited number of isolates tested, or to intrinsic differences in the mating type genes of *E. lata* compared to most other fungi, four experiments are necessary:

a) Test different *E. lata* isolates from overseas for the *MAT1-2* HMG box, to compensate for the limited and geographically restricted sample size used in this study.

b) Test at least two more perithecia from New Zealand for reproducible polymorphic differences using RAMS profiling and RAPD analysis, this will eliminate the possibility that New Zealand isolates of *E. lata* are *MAT1-1* homothallic.

c) Continue the analysis of the uncharacterised 640 bp SmHMG2/SmHMG3 PCR clone. Examination of this clone should reveal whether the amino acid sequences to which the *MAT1-2* HMG box primers are designed are not conserved in *E. lata*.

d) Attempt to isolate the *MAT1-1* idiomorph by PCR amplification of •1 domain using nested primers and DNA from the existing New Zealand *E. lata* isolates.

5.3.3 Conclusion.

Grapevine dieback appears to be increasing in severity in Australian vineyards and is expected to be a major limiting factor in the sustainability of Australian wine production (Pascoe & Cottral, 2000). Here in New Zealand, *E. lata* will continue to be a major

problem in vineyards until a scientifically based management program designed to minimise the impact of the disease is implemented. Progress with work on identification of the fungus should lead to the development of an effective and rapid method for detecting *E. lata* within grapevines. This will provide information on the occurrence and distribution of the disease within New Zealand, essential for the development of management practices. In addition. rapid detection of *E. lata* will also be useful in evaluating the success of any management strategies implemented.

Progress on the basic aspects of the genetics of *E. lata* will continue to be hampered because the organism has not yet been induced to complete its life cycle in culture. Molecular studies into the mating type genes which regulate sexual compatibility and sexual reproduction in the fungus should lead to the development of an assay for the characterisation of the mating type. Molecular characterisation of the mating type before attempting to complete the *E. lata* life-cycle in culture will enable significant savings in time and effort.

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APPENDIX.

1. VINE ISOLATION RAW MATERIAL.

The *Alternaria* species most frequently found was *Alternaria alternata*.
The *Botryosphaeria* species most frequently found were *Botryosphaeria obtusa*, *Botryosphaeria ribis*.
The *Gliocladium* species most frequently found was *Gliocladium roseum*.

1.1 *Vine 2.*

Table A Raw data from *Vine 2* agar isolations.

Disease lesion	PDA	15%V8	Malt
1	*Botryosphaeria*	*Botryosphaeria*	*Botryosphaeria*
2	*Eutypa lata*	*Botryosphaeria*	*Penicillium, Botryosphaeria*
3	*Eutypa lata*	Sterile site	*Penicillium, Botryosphaeria*
4	Sterile site	*Penicillium*	*Mucor*
5	*Penicillium*	*Penicillium, Botryosphaeria*	*Penicillium, Botryosphaeria*
6	Sterile site	*Penicillium*	*Penicillium*
7	*Penicillium*	*Penicillium*	Sterile site
8	*Gliocladium*	*Penicillium*	*Penicillium*
9	*Penicillium, Botryosphaeria*	*Penicillium*	*Penicillium*
10	Sterile site	*Trychaderma*	*Cladosporium*
11	*Glomerella cingulata*	*Penicillium*	*Penicillium*
Summary	**9 fungal isolates obtained**	**11 fungal isolates obtained**	**13 fungal isolates obtained**
	5 fungal genera	**3 fungal genera**	**4 fungal genera**
	3 Sterile sites	**1 Sterile site**	**1 Sterile site**
	0 bacterial colonies	**0 bacterial colonies**	**0 bacterial colonies**

1.2 *Vine 7.*

Table B Raw data from *Vine 7* agar isolations.

Disease lesion	PDA	15%V8	MA
1	*Botrytis cinerea*	*Alternaria*	*Alternaria*
2	*Eutypa lata, Ruby Fusarium*	*Alternaria*	*Pestalotia*
3	*Eutypa lata*	Sterile site	*Eutypa lata, Botrytis cinerea*
4	*Libertella*	*Alternaria, Cladosporium, Gliocladium*	*Alternaria*
5	*Epicoccum*	*Alternaria*	*Botryosphaeria*
6	*Botrytis cinerea, Epicoccum, Gliocladium, Penicillium*	Unknown *D*	Unknown *D*
7	*Penicillium*	Unknown *F*	*Botryosphaeria, Penicillium*
8	*Alternaria, Penicillium,*	*Alternaria*	*Alternaria, Penicillium*

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		Gliocladium	
9	*Alternaria*	*Alternaria*	*Penicillium, Cladosporium, Penicillium*
10	*Alternaria*	Sterile site	Sterile site
11	*Penicillium, Gliocladium*	Unknown *G*, *Penicillium*	*Gliocladium*
12	*Gliocladium, Epicoccum, Alternaria*	*Alternaria, Penicillium*	*Eutypa lata, Botrytis cinerea*
13	*Gliocladium, Epicoccum, Alternaria*	*Alternaria, Botrytis cinerea*	*Alternaria*
14	*Eutypa lata*	*Phomopsis*	*Phomopsis*
15	*Botryosphaeria, Epicoccum, Gliocladium*	*Penicillium, Epicoccum*	*Botryosphaeria*
16	*Bacterial isolate*	*Alternaria*	*Alternaria*
17	*Gliocladium*	*Gliocladium*	*Gliocladium*
18	*Cladosporium*	Unknown *F*	*Alternaria*
19	*Libertella*	*Fusarium*	*Fusarium*
20	*Cladosporium*	*Alternaria*	Unknown *E*
21	*Eutypa lata, Botryosphaeria*	*Eutypa lata, Botryosphaeria*	*Eutypa lata, Botryosphaeria*
22	*Alternaria*	*Alternaria*	*Alternaria*
Summary	**35 fungal isolates obtained**	**27 fungal isolates obtained**	**28 fungal isolates obtained**
	11 fungal genera	**13 fungal genera**	**12 fungal genera**
	0 Sterile sites	**2 Sterile sites**	**1 Sterile site**
	0 Bacterial colonies	**0 Bacterial colonies**	**0 Bacterial colonies**

1.3 *Vine 8.*

Table C Raw data *Vine 8* agar isolations.

Disease lesion	PDA	15%V8	MA
1	*Alternaria,* Unknown *I*	*Ruby Fusarium, Epicoccum,* Unknown *G*	Sterile site
2	*Bacteria*	Sterile site	*Botryosphaeria, Penicillium*
3	*Botrytis cinerea, Eutypa lata*	*Botryosphaeria*	*Botrytis cinerea, Penicillium*
4	Unknown *I, Alternaria, Penicillium*	*Alternaria*	*Epicoccum, Alternaria, Gliocladium*
5	*Botryosphaeria*	*Alternaria, Gliocladium*	*Botryosphaeria, Cladosporium*
6	*Cladosporium,* Unknown *I*	Sterile site	*Fusarium A, Alternaria*
7	*Mucor*	*Eutypa lata,* Unknown *F,* Unknown *H*	*Fusarium A, Bacteria, Fusarium B, Gliocladium*
8	*Mucor*	Unknown *B*	*Fusarium A, Epicoccum,* Unknown *G*
9	*Penicillium, Gliocladium,* Unknown *G*	*Eutypa lata, Alternaria*	*Fusarium C, Eutypa lata, Botrytis cinerea*
10	*Mucor*	*Gliocladium*	*Gliocladium*
11	*Ruby Fusarium*	*Penicillium*	*Botrytis cinerea*
12	*Alternaria, Mucor*	*Libertella*	*Botrytis cinerea*

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13	*Alternaria*, Unknown *I*	*Alternaria*	*Alternaria, Botryosphaeria*
14	*Ruby Fusarium, Botryosphaeria, Penicillium*	*Bacteria, Botryosphaeria, Penicillium*	*Botryosphaeria,* Penicillium
15	*Eutypa lata, Alternaria*	*Botryosphaeria*	Penicillium, *Botryosphaeria*
16	*Botryosphaeria, Gliocladium*	*Eutypa lata*	Alternaria, Epicoccum
17	*Alternaria, Botryosphaeria*	Unknown *A*	Alternaria, Penicillium
18	*Alternaria, Gliocladium, Botryosphaeria*	*Ruby Fusarium, Epicoccum*	Alternaria
Summary	**33 fungal isolates obtained** **11 fungal genera** **0 sterile sites** **1 bacterial isolate**	**24 fungal isolates obtained** **13 fungal genera** **2 sterile sites** **1 bacterial isolate**	**34 fungal isolates obtained** **10 fungal genera** **2 sterile sites** **1 bacterial isolate**

1.4 *Vine 9.*

Table D Raw data *Vine 9* agar isolations.

Disease lesion	PDA	15%V8	MA
1	*Eutypa lata*	*Penicillium, Trychaderma, Alternaria*	*Eutypa lata*
2	*Eutypa lata*	*Penicillium, Alternaria*	*Alternaria*
3	Sterile site	*Alternaria, Penicillium, Cladosporium*	*Alternaria*
Summary	**2 fungal isolates obtained** **1 fungal genera** **1 sterile site** **0 bacterial colonies**	**8 fungal isolates obtained** **4 fungal genera** **0 sterile sites** **0 bacterial colonies**	**3 fungal isolates obtained** **2 fungal genera** **0 sterile sites** **0 bacterial colonies**

1.5 *Vine 10.*

Table E Raw data *Vine 10* agar isolations.

Disease lesion	PDA	15%V8	MA
1	*Alternaria, Botryosphaeria*	Mucor, Alternaria	*Alternaria, Botryosphaeria, Penicillium*
2	Alternaria, Penicillium	*Alternaria*	Alternaria, Epicoccum
3	*Alternaria, Botryosphaeria*	*Mucor*	*Botryosphaeria*
4	Sterile site	Sterile site	*Botryosphaeria, Alternaria, Gliocladium*
5	*Eutypa lata*	*Eutypa lata*	*Eutypa lata*
6	*Gliocladium*	*Eutypa lata*	*Eutypa lata*
7	*Alternaria, Botryosphaeria*	*Alternaria*	*Eutypa lata*
8	*Gliocladium, Phomopsis*	*Gliocladium, Eutypa lata*	Gliocladium, Alternaria
9	*Mucor*	*Eutypa lata*	Unknown *C*
10	*Gliocladium, Alternaria, Eutypa lata*	*Gliocladium, Alternaria, Eutypa lata*	*Mucor, Botryosphaeria*

11	*Mucor*	*Botryosphaeria*	*Botryosphaeria*, Unknown *C*
12	*Alternaria*	*Eutypa lata*	*Alternaria, Penicillium*
Summary	**18 fungal isolates obtained**	**15 fungal isolates obtained**	**21 fungal isolates obtained**
	7 fungal genera	**5 fungal genera**	**8 fungal genera**
	1 sterile site	**1 sterile site**	**0 sterile sites**
	0 bacterial colonies	**0 bacterial colonies**	**0 bacterial colonies**

1.6 *Vine 11.*

Table F Raw data *Vine 11* agar isolations.

Disease lesion	PDA	15%V8	MA
1	*Eutypa lata, Alternaria, Botryosphaeria*	Sterile site	*Botryosphaeria, Alternaria*
2	*Eutypa lata, Gliocladium*	*Eutypa lata*	*Eutypa lata*
3	*Botryosphaeria, Gliocladium*	*Penicillium*	*Botryosphaeria, Gliocladium*
4	*Penicillium, Botryosphaeria*	Sterile site	*Penicillium*
5	*Botryosphaeria, Penicillium, Alternaria*	*Alternaria, Penicillium*	*Alternaria*
6	Sterile site	Sterile site	*Botryosphaeria*
7	*Alternaria, Eutypa lata*	*Alternaria*	*Alternaria*
8	*Alternaria*	*Alternaria, Botryosphaeria*	*Alternaria, Penicillium*
Summary	**15 fungal isolates obtained**	**7 fungal isolates obtained**	**11 fungal isolates obtained**
	5 fungal genera	**4 fungal genera**	**5 fungal genera**
	1 sterile site	**3 sterile sites**	**0 sterile sites**
	0 bacterial colonies	**0 bacterial colonies**	**0 bacterial colonies**

1.7 Description of unidentified isolates.

Unknown *A* -   Slow growing slimy white mycelium.
Unknown *B* -   Thin, ropey, white mycelium.
Unknown *C* -   Thin pink mycelium.
Unknown *D* -   Grey mycelium, colonies have white margins.
Unknown *E* -   Slow growing white mycelium.
Unknown *F* -   Fast growing powdery white mycelium.
Unknown *G* -   Slow growing white grey mycelium.
Unknown *H* -   Dense, slimy white mycelium.
Unknown *I* -   Arial, grey, fluffy mycelium.

1.8 Total fungal genera obtained.

Table G Numbers of fungal isolates found in diseased vines.

Fungal genera found in diseased vines	Number of isolates
Alternaria	72
Penicillium	50
Botryosphaeria	46
Eutypa lata	35
Gliocladium	30
Sterile site	18
Epicoccum	12
Fusarium	12
Botrytis	10
Mucor	10
Cladosporium	7
Bacterial isolates	4
Unknown *G*	4
Unknown *I*	4
Phomopsis	3
Unknown *F*	3
Trychaderma	2
Unknown *C*	2
Unknown *D*	2
Glomerella	1
Pestalotia	1
Unknown *A*	1
Unknown *B*	1
Unknown *E*	1
Unknown *H*	1

2. PLASMID MAPS.

2.1.1 pGem-T vector.

T7 ↓	1 start
Apa I	14
Aat II	20
Sph I	26
BstZ I	31
Nco I	37
Sac II	46
Spe I	55
Not I	62
BstZ I	62
Pst I	73
Sal I	75
Nde I	82
Sac I	94
BstX I	103
Nsi I	112
	126
↑ SP6	

Xmn I 1994
Sca I 1875
Nae I 2692
f1 ori
Amp'
pGEM®-T Vector (3000bp)
lacZ
ori

2.1.2 pGem-T easy vector.

T7 ↓	1 start
Apa I	14
Aat II	20
Sph I	26
BstZ I	31
Nco I	37
BstZ I	43
Not I	43
Sac II	49
EcoR I	52
Spe I	64
EcoR I	70
Not I	77
BstZ I	77
Pst I	88
Sal I	90
Nde I	97
Sac I	109
BstX I	118
Nsi I	127
	141
↑ SP6	

Xmn I 2009
Sca I 1890
Nae I 2707
f1 ori
Amp'
pGEM®-T Easy Vector (3015bp)
lacZ
ori