GENETIC IDENTIFICATION AND EVOLUTION OF EPICHLOË ENDOPHYTES

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ABSTRACT

The Epichloë endophytes are a group of filamentous fungi that include both sexual (*Epichloë*) and asexual (*Neotyphodium*) species. As a group, they are genetically diverse and form mutualistic to antagonistic symbiotic associations with temperate grasses (subfamily Pooideae). In this study, a multi-locus microsatellite-based PCR fingerprinting assay was developed for the genetic identification of Epichloë endophytes, both in culture and in planta. Microsatellites were isolated from endophyte partial genomic libraries, or identified from existing endophyte DNA sequences, and PCR assays that amplify these loci were developed. Multiplex assays were optimised, and fluorescently labelled primers were employed to allow precise sizing and automatic analysis of the PCR products with a laser scanner and the appropriate software. A reference database of allele sizes has been established for the panel of endophytes examined, and it has been shown that this assay is able to resolve endophyte groupings to the level of known isozyme phenotype groups. In a blind test the assay was used successfully to identify a set of endophytes in planta. The segregation of microsatellite alleles from an *E. festucae* sexual mating was also examined.

This microsatellite assay, in addition to ß-tubulin and ribosomal RNA gene sequence analysis, was used to genetically characterise *Neotyphodium*-like endophytes from annual *Lolium* ryegrasses and *Hordeum* grasses. The endophytes examined were indeed found to be *Neotyphodium* with unique evolutionary origins. The common endophyte of annual ryegrasses was found to have a unique hybrid origin involving *E. baconii* and *E. bromicola* ancestry, and it is proposed that this taxonomic group is named LmTG-1. A second previously undescribed *Neotyphodium* endophyte was found in *L. canariense*, and this was shown to be an asexual derivative of *E. typhina*. The name proposed for this taxonomic group of endophytes is LcTG-1. The two *Hordeum* endophyte isolates, HaB and Hd1, were also both shown to have unique hybrid origins. HaB has *E. elymi* and *E. amarillans* ancestry, and Hd1 has *E. typhina* and *E. bromicola* ancestry. The proposed names for the *Neotyphodium* taxonomic groups that contain these isolates are HdTG-1 and HdTG-2 respectively. The revelation of interspecific hybrid origins for three more

Neotyphodium endophytes illustrates the prevalence of hybridisation in the evolution of the Epichloë endophytes, and further contributes to our understanding of the evolution of this group.

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CHAPTER ONE

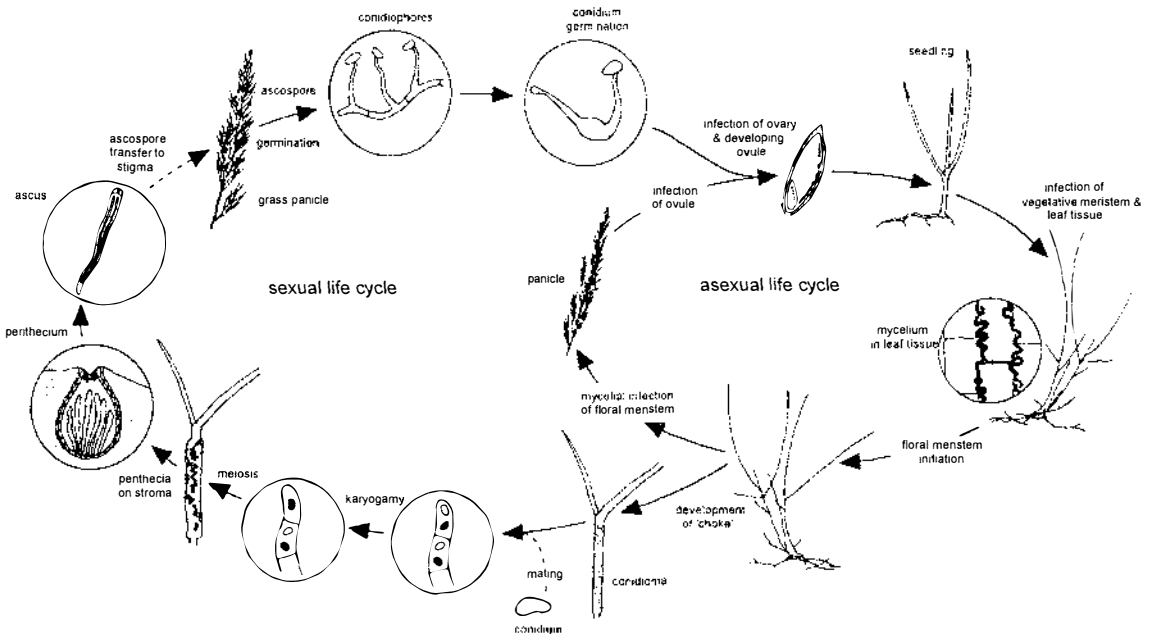
INTRODUCTION

1.1 THE LIFE CYCLES OF EPICHLOË ENDOPHYTES

The Epichloë endophytes are a group of filamentous fungi comprised of the sexual *Epichloë* species and their asexual derivatives the *Neotyphodium* species, which were formerly classified as *Acremonium* species (Glenn et al., 1996; Schardl, 1996a). This group of fungi, which hereafter may be referred to as 'endophytes', grow systemically in the vegetative tissues of cool season grasses (subfamily Pooideae), where they are confined to the apoplast (intercellular spaces). Endophytes can be identified by the presence of characteristic septate, infrequently branched hyphae, running longitudinally in the plant tissue (Christensen et al., 1991). In the apoplast the endophyte gains nutrition, but it is not known whether it actively promotes leakage of nutrients from the plant cells, or lives on normal apoplastic substituents (Schardl, 1996a), though the effect of infection on the host appears to be asymptomatic in naturally-occurring associations. The density of endophyte mycelium in the plant displays an annual cycle. In spring, mycelium grows into the leaf sheaths, leaves and inflorescences, reaching a maximum density in late summer and then declining. In the winter it is confined to the plant crown where it is most concentrated in the meristematic tissues (di Menna and Waller, 1986).

Dissemination of Epichloë endophytes to new hosts can be achieved both sexually and asexually, resulting in dramatically different effects on the host. Figure 1.1 illustrates these alternative life cycles of an *E. festucae* endophyte on *Festuca rubra*. The sexual cycle begins with growth of the fungus through the flowering tillers to the flag-leaf sheaths and immature inflorescences, where they emerge to form somatic reproductive structures called stromata. The stromata choke and sterilise the inflorescence. Spermatia, in the form of conidia, develop on the stroma and these are transferred to stroma of other infected hosts by symbiotic flies of the genus *Botanophila* (often referred to the genus *Phorbia*; Kohlmeyer and Kohlmeyer, 1974) (Bultman et al., 1995; Schardl and Leuchtmann, 1999). *Epichloë* species have a heterothallic mating system, and ascospores are formed following successful cross-fertilisation (White and Bultman, 1987). These are ejected, and initiate new infections which proceed into florets to ultimately give rise to infected embryos and seed (Chung and Schardl, 1997a).

Figure 1.1. The asexual and sexual life cycles of *Epichloë festucae* on *Festuca rubra*



In the asexual cycle, highly convoluted hyphae grow intercellularly in leaf sheaths, floral meristems, and in the ovules of the florets such that the fungus is transmitted in the next generation of seed. In the sexual cycle, the fungus also grows asymptomatically and intercellularly within the host, but then emerges from the leaf sheath surrounding the immature host inflorescence, produces spermatia, and arrests inflorescence maturation. Fertilisation occurs by transfer of spermatia of opposite mating type. If the parents are conspecific (same mating population), perithecia containing asci develop and filamentous ascospores are ejected. Germinating ascospores initiate cycles of asexual sporulation (conidiation) and are postulated to cause infection of host florets and ultimately of seed. Diagram adapted from Scott and Schardl (1993).

This horizontal mode of endophyte transmission is pathogenic to the host and is the causative process behind ryegrass choke by *E. typhina* (reviewed by Schardl, 1996b).

As illustrated in Figure 1.1, *E. festucae* is also able to undergo clonal (vertical) propagation within the host, which is the only mode of transmission for the asexual *Neotyphodium* endophytes. This is accomplished by vegetative growth of the endophyte through the floral tillers into the host ovules. The endophyte is incorporated into the embryo and aleurone of the seed, where it is disseminated (Philipson and Christey, 1986). This mode of transmission is highly efficient, where nearly 100% of seed from infected mother plants transmit the endophyte (Siegel et al., 1984). *Epichloë* species can undergo both horizontal and vertical transmission simultaneously, though the degree to which each species does this varies greatly and is discussed further in Section 1.3.

1.2 ANTIHERBIVORE ALKALOIDS

Endophyte-infected grasses are known to contain a number of biologically active alkaloids, which are responsible for many of the effects of endophyte presence on pasture growth and animal production. The main alkaloids responsible for these effects are classified into four chemical classes - ergopeptine alkaloids, lolines, lolitrems, and peramine. All of these have activities against insects and most, or all, are toxic to mammals at some level (reviewed by Scott and Schardl, 1993).

In 1977, it was demonstrated that the toxicity syndromes of tall fescue grasses (*Festuca arundinacea*) were directly related to the endophyte content of these pastures (Bacon et al., 1977). It was later shown that ergopeptine alkaloids, such as ergovaline and ergotamine, are synthesised by the fungus and occur in many endophyte-plant interactions (Bacon et al., 1986; Rowan and Shaw, 1987). These compounds cause the classical symptoms of fescue toxicosis - elevated temperatures, reduced feeding, reduced fertility, reduced lactation, to fat necrosis, vasoconstriction, stillbirth, gangrene and death, depending on dosage and environmental stresses (Raisbeck et al., 1991).

Similarly, the nervous disorder, ryegrass staggers, was found to be associated with the incidence of endophyte infected perennial ryegrass (*Lolium perenne*) (Fletcher and Harvey, 1981). It was later shown that lipophilic complex substituted-indole compounds, named lolitrems, produced by the endophyte-grass association produced these tremors in livestock (Gallagher et al., 1981). Lolitrem B is the main toxin implicated in ryegrass staggers (Gallagher et al., 1984; Raisbeck et al., 1991). Both ryegrass staggers and fescue toxicosis are responsible for large economic losses to the sheep industry in New Zealand and the beef and cattle industry in the United States. Consequently, there is considerable interest in maximising the beneficial aspects of this symbiotic association for pastoral agriculture.

Lolines, or saturated aminopyrrolizidines, are toxic to insects and may also play a direct role in fescue toxicosis of livestock. These compounds are the most abundant alkaloids in the associations between *Neotyphodium coenophialum* and tall fescue (*Festuca arundinacea*), with concentrations up to one thousand times greater than that of the other alkaloids (Dahlman et al., 1991). The production of lolines was only recently found to be of endophyte origin (pers. comm. Heather Wilkinson, University of Kentucky). Peramine, a pyrrolopyrazine, is a major feeding deterrent to insects and has been shown to play a key role in protection of perennial ryegrass from Argentine stem weevil (*Listronotis bonariensis*), a major introduced pest in New Zealand (Prestidge et al., 1985; Rowan and Gaynor, 1986).

The distribution of these alkaloid compounds within the host does not necessarily correlate with the location of endophyte infection. Peramine, ergovaline and the loline alkaloids (or a signal for their synthesis) appear to be translocated from the leaf sheath into the leaf blade where there is little fungal mycelium (Ball et al., 1995; Bush et al., 1982; Rowan and Shaw, 1987). However, lolitrem B tends to remain concentrated at the leaf sheaf near the base of the plant (Ball et al., 1995).

It is obvious that the effects of these alkaloids are highly beneficial to the endophyte-grass symbiosis, but are detrimental to livestock in agricultural grazing systems.

Therefore, 'elite' endophytes are highly sought after, which retain the benefits of the symbiosis without the detrimental effects to grazing livestock. The two main strategies of obtaining these 'elite' endophytes is by selection of existing endophyte strains, or by genetic manipulation. It has been shown that natural strains of *Neotyphodium* differ in the spectrum of alkaloids they produce, and that expression is also dependent on the genotype of the host (Christensen et al., 1993). Selection of endophytes which display desirable combinations of alkaloids may be used to artificially inoculate grasses and replace existing inferior grass-endophyte associations in pasture. Alternatively 'elite' endophytes may be obtained by the genetic modification of existing ones. Currently, an area of intense research is in the identification of genes involved in alkaloid biosynthesis, and notable progress has been made for lolitrem B, where a cluster of genes involved in the biosynthesis of a related indole diterpenoid, paxilline, have been cloned from *Penicillium paxilli* (Young et al., 1998; Young et al., 1999). Genes involved in ergot alkaloid biosynthesis have also been cloned from related Clavicipitaceous fungi (Wang et al., 1999), and progress has been made in identifying loline biosynthetic genes (pers. comm. Heather Wilkinson, University of Kentucky). Once these genes are isolated and characterised, modified endophytes can be engineered, disrupting synthesis of the detrimental alkaloids. Methods for the stable genetic transformation of *Neotyphodium* endophytes have already been successfully demonstrated, which include electroporation and polyethylene glycol (PEG) treatments (Murray et al., 1992; Tsai et al., 1992). Transformed endophytes can then be used to inoculate grass plants to form stable associations.

1.3 GRASS/ENDOPHYTE SYMBIOSES

The relationships between Epichloë endophytes and their hosts display extraordinary diversity, with symbioses between the two entities spanning the continuum from mutualism to antagonism, depending on the mode of dispersal of the endophyte (reviewed by Schardl, 1996a). At one extreme, the mutualistic asexual endophytes exist as asymptomatic fitness enhancing symbionts with benefits to the host involving

resistance to herbivores (Bacon et al., 1977; Gallagher et al., 1984; Kimmons et al., 1990; Prestidge et al., 1985; Siegel et al., 1990), and drought tolerance (Arachevaleta et al., 1989), resulting in enhanced persistence and fecundity (Latch et al., 1985; Siegel et al., 1985). Benefits to the endophyte include provision of nutrients by the hosts, and dispersal by seedborne dissemination (Siegel et al., 1984). The most intensely documented of these symbioses are those of the agriculturally important pasture grasses - tall fescue with *Neotyphodium coenophialum*, and perennial ryegrass with *N. lolii*, though *Epichloë* species may also form mutualistic associations. Strains of *E. bromicola* symbiotic with *Bromus ramosus* and *B. benekenii* never form stromata on their hosts, but their interfertility with *E. bromicola* strains that infect *B. erectus* have enabled these strains to be classified as *Epichloë* species (Leuchtmann and Schardl, 1998). The survival of mutualistic endophytes is dependent on selection at the level of the symbiotic association, rather than the endophyte alone.

At the other end of the spectrum, extreme cases of antagonism are displayed by obligately sexual endophytes. The relationship between *E. typhina* and perennial ryegrass illustrates this, where the sexual cycle results in choking of all inflorescences, rendering the host sterile. In a sense, these relationships are not strictly antagonistic as important mutualistic characteristics are also expressed before the sexual cycle of the endophyte is undertaken, such as antiherbivore activities and increased drought tolerance.

Several *Epichloë* endophytes exhibit pleiotropic symbioses with their hosts, whereby both asymptomatic vertical and pathogenic horizontal transmission of the endophyte are undertaken simultaneously on separate flowering tillers of an infected plant. Pleiotropism is illustrated in Figure 1.1 by *E. festucae* infected *F. rubra*. The degree of mutualism and antagonism displayed varies, and depends not only on the genotypes of the endophytes and hosts involved, but also temporal, developmental and environmental factors. Examples of more extreme pleiotropic symbioses include that of *E. typhina* infected *Poa nemoralis*, which is highly antagonistic but occasionally produces rare seedborne infections, and *E. brachyelytri* infected *Brachyelytrum erectum* which very rarely expresses stromata and almost always develops normal inflorescences giving rise

to infected seeds (Schardl and Leuchtmann, 1999). The continuum from mutualism to antagonism displayed by the Epichloë endophytes make them an ideal system for studying the evolution of fungal mutualists (Tsai et al., 1994).

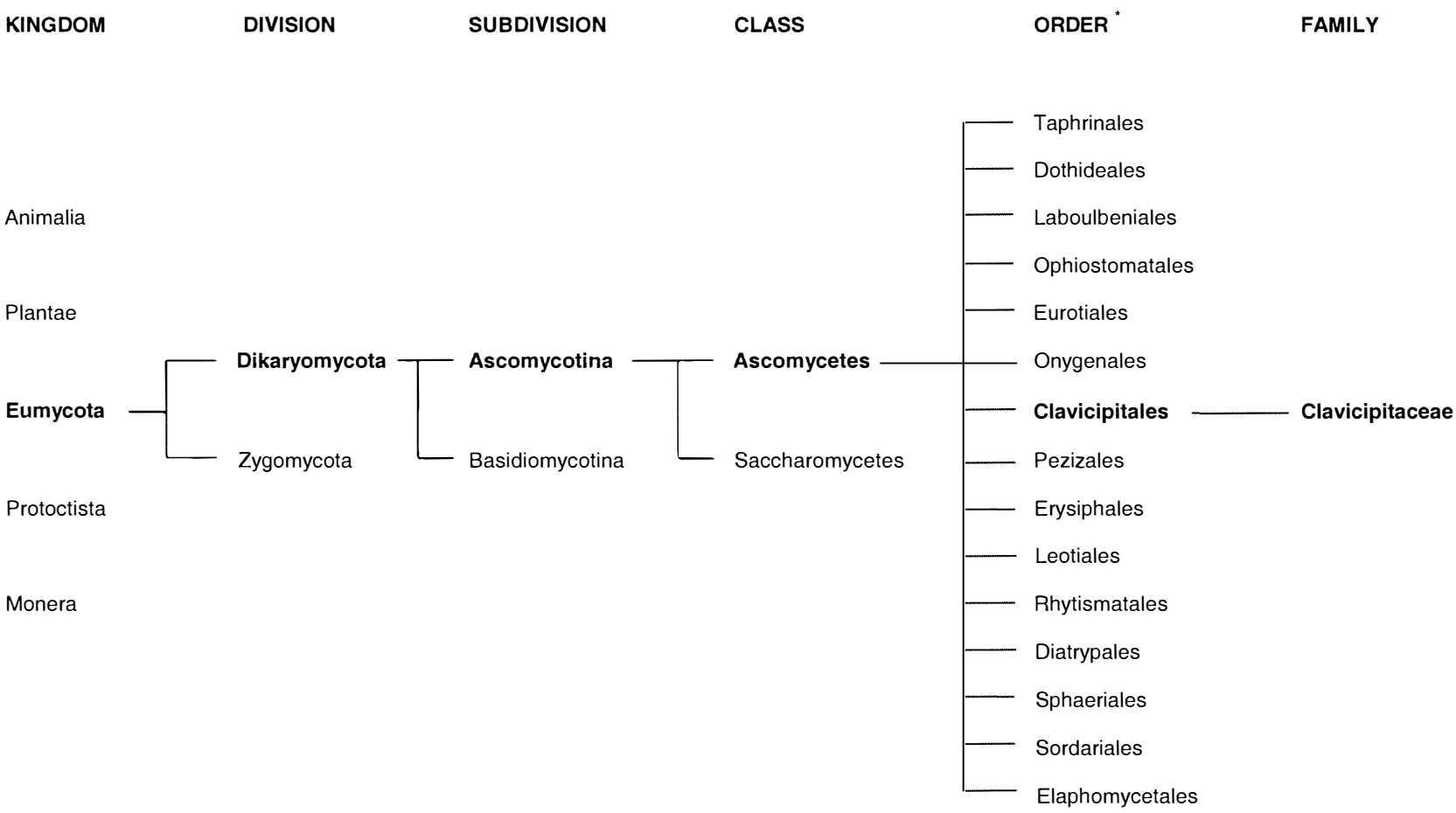
1.4 TAXONOMY AND EVOLUTION OF *EPICHLOË* ENDOPHYTES

1.4.1 EPICHLOË ENDOPHYTE TAXONOMY

The taxonomy of the Epichloë endophytes is an area which is complicated by taxonomic convention and changes in taxonomy over time. Sexual *Epichloë* species and their asexual derivatives, the *Neotyphodium* species, constitute a likely monophyletic group (Kuldau et al., 1997; White and Reddy, 1998) but traditional taxonomic conventions do not classify these groups together. Instead, they have been separated at the level of fungal subdivision (Ascomycotina for *Epichloë* and Deuteromycotina for *Neotyphodium*) on the basis of the ability to undergo a sexual life cycle (Ainsworth et al., 1973). Molecular approaches to fungal taxonomy have brought about advocation for the abandonment of this type of dual naming system, in favour of a more natural one which reflects the evolutionary relationships of the organisms. Such a system has been adopted by Kendrick (1992), which refers to fungal holomorphs that include both teleomorphic and anamorphic fungi. The current classification of the genus *Epichloë* from the level of Kingdom, according to Kendrick (1992), and Glenn et al. (1996) is given in Figures 1.2 and 1.3 respectively.

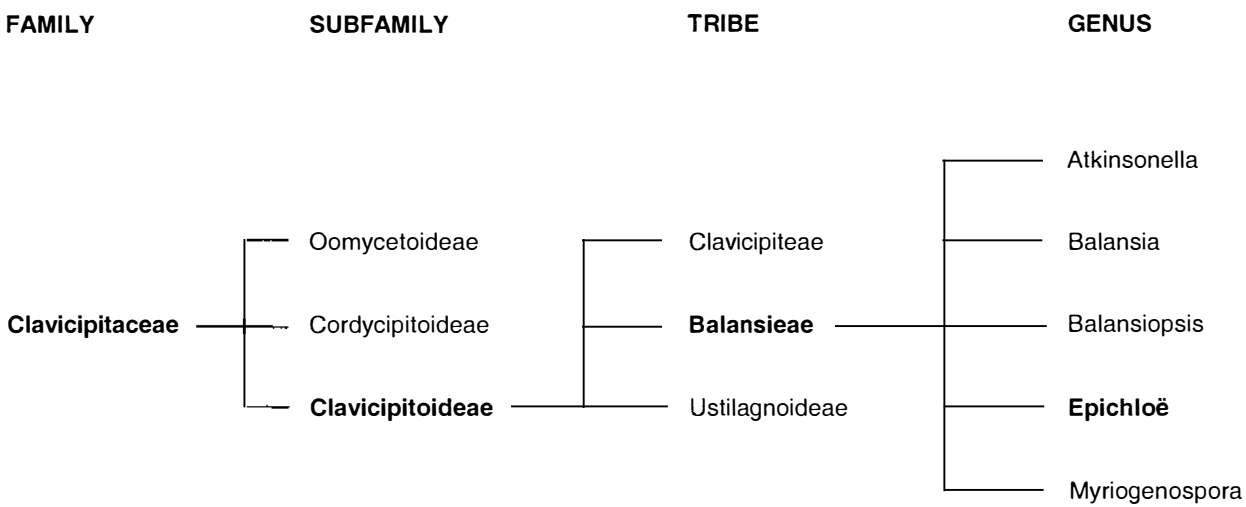
Over time the *Epichloë* endophytes have undergone several taxonomic, and hence nomenclature changes as evolutionary relationships are being continually elucidated, revised, and updated. The most recent example of a major nomenclature change is from a molecular study of the form genus *Acremonium* (Glenn et al., 1996). Here, the

Figure 1.2. Classification of the family Clavicipitaceae (Kendrick, 1992)



* 15 of the 44 orders of Ascomycetes are shown here (Kendrick, 1992)

Figure 1.3. Classification of the genus *Epichloë* (Glenn et al., 1996)



Epichloë anamorphs were classified alongside various saprobic fungal species, but placed in section *Albo-lanosa* (Morgan-Jones and Gams, 1982). Molecular data strongly supported the proposal of reclassification of the grass endophytes into the new form genus *Neotyphodium*, which enables the unique biological, morphological, and ecological characteristics of this group to be distinguished (Glenn et al., 1996). Until endophyte species were identified and described, the epithet *E. typhina* had been used to broadly describe all stroma-forming endophytes of pooid grasses (Groppe et al., 1995; Leuchtmann et al., 1994; White, 1993), and even to describe the asexual endophyte of tall fescue, which is now known as *N. coenophialum* (Bacon et al., 1977; Glenn et al., 1996). Thus, changes in taxonomy and nomenclature can be confusing, and one must be cautious and consider the date of publication of a piece of work and the state of the taxonomy of the organism at that time to determine which organism is actually being referred to. A summary of the different names used by the Epichloë endophytes is given in Table 1.1.

1.4.2 *EPICHLOË* SPECIES

To date, ten species of *Epichloë* have been described, primarily on the basis of interfertility groups and ascospore morphologies (White, 1993; White, 1994), but more recently with isozyme phenotype data and ß-tubulin (*tub2*) gene DNA sequence data (Leuchtmann and Schardl, 1998; Schardl and Leuchtmann, 1999). These include the native European species (mating populations, MP, in brackets): *E. typhina* sensu stricto (MP-I), *E. clarkii* White (MP-I), *E. festucae* Leuchtmann et al. (MP-II), *E. baconii* White (MP-V), *E. bromicola* Leuchtmann & Schardl (MP-VI), and *E. sylvatica* Leuchtmann & Schardl (MP-VII); and native North American species: *E. amarillans* White (MP-IV), *E. elymi* Schardl & Leuchtmann (MP-III), *E. glyceriae* Schardl & Leuchtmann (MP-VIII), and *E. brachyelytri* Schardl & Leuchtmann (MP-IX) (Leuchtmann and Schardl, 1998; Leuchtmann et al., 1994; Schardl and Leuchtmann, 1999; White, 1993; White, 1994). Each species constitutes a single mating population, except the morphospecies *E. typhina* and *E. clarkii*, which tend to be interfertile in mating tests.

Table 1.1. Summary of the main nomenclature changes within the Epichloë endophytes

Name	Reference	Previous names	Reference	Reclassification basis
Epichloë amarillans	White, 1994	*Epichloë typhina* *Epichloë* sp. MPIV	Schardl, 1996	mating tests size and density of perithecia size of asci and ascospores
Epichloë baconii	White, 1993	*Epichloë typhina* *Epichloë* sp. MPV	Schardl, 1996	mating tests disarticulation of ascospores to form 1-septum part spores
Epichloë brachyelytri	Schardl and Leuchtmann, 1999	*Epichloë typhina* *Epichloë* sp. MPIX	Schardl, 1996	mating tests *tub2* data
Epichloë bromicola	Leuchtmann and Schardl, 1998	*Epichloë typhina* *Epichloë* sp. MPVI	Schardl, 1996	mating tests isozyme data *tub2* data
Epichloë clarkii	White, 1993	*Epichloë typhina*		mating tests disarticulation of ascospores to form 3-6 septate part spores
Epichloë elymi	Schardl and Leuchtmann, 1999	*Epichloë typhina* *Epichloë* sp. MPIII	Schardl, 1996	mating tests *tub2* data
Epichloë festucae	Leuchtmann et al., 1994	*Epichloë typhina* *Epichloë* sp. MPII	Schardl, 1996	mating tests morphology of fruiting structures size and articulation of ejected ascospores

Table 1.1. ***continued***

Name	Reference	Previous names	Reference	Reclassification basis
Epichloe glyceriae	Schardl and Leuchtmann, 1999	*Epichloë typhina* *Epichloë* sp. MPVIII	Schardl, 1996	mating tests *tub2* data
Epichloë sylvatica	Leuchtmann and Schardl, 1998	*Epichloë typhina* *Epichloë* sp. MPVII	Schardl, 1996	mating tests isozyme data *tub2* data
Epichloë typhina (Pers.:Fr) Tul.	White, 1993	*Sphaeria typhina* *Dothidia typhina* *Stromatosphaeria typhina* *Hypocrea typhina* *Epichloë typhina,* MP I	Persoon, 1798 1823 1826 1860 Schardl, 1996	
Neotyphodium coenophialum	Glenn et al., 1996	*Acremonium coenophialum* FaTG-1	Morgan-Jones and Gams, 1982 Christensen et al., 1993	rDNA sequence data
Neotyphodium typhinum	Glenn et al., 1996	*Acremonium typhinum*	Morgan-Jones and Gams, 1982	rDNA sequence data
Neotyphodium lolii	Glenn et al., 1996	*Acremonium loliae* LpTG-1 *Acremonium lolii*	Latch et al., 1984 Christensen et al., 1993	rDNA sequence data
Neotyphodium chisosum	Glenn et al., 1996	*Acremonium chisosum*	White and Morgan-Jones, 1987a	rDNA sequence data

Table 1.1. ***continued***

Name	Reference	Previous names	Reference	Reclassification basis
Neotyphodium starrii	Glenn et al., 1996	*Acremonium starrii*	White and Morgan-Jones, 1987b	rDNA sequence data
Neotyphodium huerfanum	Glenn et al., 1996	*Acremonium huerfanum*	White et al., 1987	rDNA sequence data
Neotyphodium uncinatum	Glenn et al., 1996	*Acremonium uncinatum* FpTG-1	Gams et al., 1990 Christensen et al., 1993	rDNA sequence data
Neotyphodium chilense	Glenn et al., 1996	*Acremonium chilense*	Morgan-Jones et al., 1990	rDNA sequence data
LpTG-2	Christensen et al., 1993	*Acremonium lolii*	Latch et al., 1984	isozyme data, alkaloid production, morphological characters, and benomyl resistance
FaTG-2	Christensen et al., 1993	*Acremonium coenophialum*	Morgan-Jones and Gams, 1982	as above
FaTG-3	Christensen et al., 1993	*Acremonium coenophialum*	Morgan-Jones and Gams, 1982	as above

Epichloë clarkii differs from *E. typhina* only in its host specificity for *Holcus lanatus*, and disarticulation of the ejected ascospores (White, 1993).

All mating populations, except MP-I, tend to have restricted host ranges within the Pooideae, with *E. festucae* strains symbiotic with *Festuca* spp. (tribe Poeae), *E. elymi* with *Elymus* spp. (Triticeae), *E. amarillans* with North American spp. of *Sphenopholis* and probably *Agrostis* (Aveneae), *E. baconii* with Eurasian *Agrostis* and *Calamagrostis* spp. (Aveneae), *E. bromicola* with *Bromus* spp. (Bromeae), *E. sylvatica* with *Brachypodium sylvaticum* (Brachypodieae), *E. glyceriae* with *Glyceria striata* (Meliceae), and *E. brachyelytri* with *Brachyelytrum erectum* (Brachyelytreae) (Schardl and Leuchtmann, 1999). Mating population one has a rather broad host range which includes grasses of the tribes Poeae, Aveneae and Brachypodieae (Schardl et al., 1997). Host specificity is a likely contributing factor for the speciation of *Epichloë*, but host flowering times and the symbiotic fly are also likely to have played key roles (Schardl and Leuchtmann, 1999).

1.4.3 *NEOTYPHODIUM* SPECIES

The classification of asexual *Neotyphodium* species is not possible using a biological species concept, and depends on other criteria. Christensen et al. (1993) used a combination of cultural morphology, alkaloid production, grass host, and isozyme phenotypes to classify the *Neotyphodium* endophytes from perennial ryegrass, tall fescue, and meadow fescue. These endophytes were shown to have significant diversity and six taxonomic groups of endophytes were identified. These were later shown to correspond well with phylogenies derived from *tub2* and nuclear ribosomal DNA internal transcribed spacer region (rDNA-ITS) sequence data (Schardl et al., 1994; Tsai et al., 1994). Meadow fescue contained one taxon, *Festuca pratensis* taxonomic grouping one (FpTG-1) which met the original description of *N. uncinatum*. Perennial ryegrass contained two taxa, *Lolium perenne* taxonomic grouping one (LpTG-1), which met the original description of *N. lolii*, and LpTG-2. Tall fescue had three taxa associated with it:

Festuca arundinacea taxonomic grouping one (FaTG-1), FaTG-2, and FaTG-3. FaTG-1 met the definition of *N. coenophialum*, and so, was named this.

As a result of *Neotyphodium* endophytes never showing any symptoms or signs of a fungal infection on grasses, they are difficult to detect, but several species have been formally described, including: *N. chisosum* which infects *Stipa eminens* (White and Morgan-Jones, 1987a), *N. starrii* which infects *Bromus anomalus* and *Festuca* spp. (White and Morgan-Jones, 1987b), *N. huerfanum* which infects *F. arizonica* (White et al., 1987), and *N. chilense* which infects *Dactylis glomerata* pastures in Chile (Morgan-Jones et al., 1990). The placement of *N. chilense* within the genus *Neotyphodium* is tenuous though, and requires verification from genetic analysis (pers. comm. Mike Christensen, Grasslands AgResearch). Additional to the *Neotyphodium* spp. listed above, many grass endophytes have been detected that are also likely to be *Neotyphodium.* These include endophytes of the hosts: *Echinopogon ovatus*, a native of Australasia (Miles et al., 1998), annual *Lolium* ryegrasses (Latch et al., 1988), *Poa ampla* (Schardl, 1996a), *Hordeum* (pers. comm. Mike Christensen, Grasslands AgResearch). The apparent widespread distribution of *Neotyphodium* endophytes suggests that those listed above represent only a small fraction of this genus, and more species can be found with further systematic sampling of grasses.

1.4.4 INTERSPECIFIC HYBRIDISATION EVENTS

There appears to be at least two different pathways by which asexual *Neotyphodium* endophytes may evolve from their *Epichloë* predecessors (Schardl et al., 1994). The simple scenario whereby a pleiotropic *Epichloë* strain experiences a mutation that eliminates stroma expression, hence loss of sexual cycle, is postulated for *N. lolii* from *E. festucae* (Leuchtmann, 1994; Schardl, 1996a; Schardl et al., 1994). More commonly, asexual endophytes appear to have evolved by a interspecific somatic hybridisation mechanism (Schardl, 1996a). Interspecific hybrid endophytes have been identified by Tsai et al. (1994), where a phylogenetic study of the genetic diversity of tall fescue endophytes, revealed that whereas each *Epichloë* and meadow fescue isolate studied had

only a single *tub2* gene, most tall fescue endophytes had two to three distinct *tub2* copies. From sequence data of the non-coding region of the gene, it was proposed that the presence of the multiple copies of the gene is the consequence of multiple interspecific hybridisation events with *Epichloë* species (Tsai et al., 1994). Similarly *tub2*, rDNA-ITS, *pyr4* gene (which encodes orotidine-5'-monophosphate decarboxylase) restriction fragment length polymorphism, mitochondrial DNA, and isozyme analyses revealed that LpTG-2 endophytes of perennial ryegrass are the result of an interspecific hybridisation between *E. typhina* and an *N. lolii* mutualist (Schardl et al., 1994). Further findings suggest that *N. uncinatum* is also likely to be a hybrid endophyte of *E. typhina* and *E. bromicola* origins (pers. comm. Christopher Schardl, University of Kentucky). It therefore appears that interspecific hybridisation is a relatively common event in the origin of asexual endophytes. Table 1.2 shows a summary of the most likely ancestors of selected asexual *Neotyphodium* endophytes.

Table 1.2. Relationships of *Neotyphodium* grass endophytes to *Epichloë* species

Grass host	Anamorphic species	Likely ancestors or closest extant relatives
Festuca arundinacea	*N. coenophialum*	*N. uncinatum, E. baconii, E. festucae*
F. arundinacea	FaTG-2	*E. baconii, E. festucae*
F. arundinacea	FaTG-3	*E. baconii, E. typhina*
F. pratensis	*N. uncinatum*	*E. bromicola, E. typhina*
Lolium perenne	*N. lolii*	*E. festucae*
L. perenne	LpTG-2	*N. lolii, E. typhina*
Poa ampla	undescribed	*N. uncinatum, E. amarillans*

table adapted from Schardl (1996a)

Interspecific hybridisation is considered by some to be a mechanism to counteract Muller's ratchet, whereby in asexual species the accumulation of deleterious mutations will cause loss of fitness without the corrective influences of sexual recombination (Muller, 1964). The favoured hypothesis for the mechanism of interspecific hybridisation involves the infection of a mutualistic endophyte inhabited host by spermatia or ascospores of an *Epichloë* species via the stigma. Following duel infection of the host, it is assumed that anastomosis (hyphal fusion) takes place, with subsequent fusion of nuclei (Tsai et al., 1994). Thereafter, redundant chromosomes or chromosomal segments may be lost without detriment to the endophyte resulting in heteroploidy or aneuploidy of the hybrid (Schardl et al., 1994). The formation of interspecific hybrid endophytes is assumed to be relatively rare, and has not been observed under experimental conditions, though some of the processes implicated in hybrid formation have been demonstrated. Infection of single host plants by multiple endophyte isolates has been studied, both naturally-occurring and artificially induced symbiota, and it was observed that isolates tend to segregate at the tiller level (Meijer and Leuchtmann, 1999; pers. comm. Mike Christensen, Grasslands AgResearch). Anastomosis of pairs of *Epichloë* spp. has also been successfully demonstrated by complementation studies of nitrate non-utilising (*nit*) mutants, resulting in the production of heterokaryons (Chung and Schardl, 1997b).

1.5 THE GRASS SUBFAMILY POOIDEAE

The grass family Poaceae is the fourth largest of the flowering plant families, and includes all the major cereals such as wheat, maize, rice, barley and oats, as well sugar cane, sorghum, rye, bamboo, and millet (Kellogg, 1998). The Epichloë endophytes infect grasses of the Poaceae subfamily Pooideae, so a brief description of the organisation of this group is given, with particular detail to the grasses that host the endophytes species in this study.

Although the most recent classifications of Pooideae, based on morphological characters, describe seven to ten tribes, phylogenetic analyses using *ndh*F gene sequences suggest that the boundaries of this subfamily contain twelve tribes: Poeae, Aveneae, Triticeae, Bromeae, Brachypodieae, Meliceae, Stipeae, Lygeae, Nardeae, Diarrheneae, Brachyelytreae, and Phaenospermatae (Catalán et al., 1997). The *Epichloë* spp. that infect these tribes are described in Section 1.4.2. A principal core clade within Pooideae includes the closely related tribes: Poeae, Aveneae, Triticeae, and Bromeae. These four tribes have been referred to as the "core pooids", and their monophyly and relationship within the Pooideae has been well supported with independent chloroplast DNA (cpDNA) restriction site data and rDNA-ITS sequences (Davis and Soreng, 1993; Hsaio et al., 1995). Poeae and Aveneae form a sound monophyletic clade as does Triticeae and Bromeae, and these two clades are sisters. Within the Poeae/Aveneae clade, Aveneae appears to have given rise to Poeae (Aveneae is paraphyletic to Poeae), whilst within the Triticeae/Bromeae clade, each tribe is monophyletic and they are sister groups (Catalán et al., 1997).

The important forage grasses in this study *Festuca arundinacea* (tall fescue), *F. pratensis* (meadow fescue) and *Lolium perenne* (perennial ryegrass), are members of the Poeae tribe. The genus *Festuca* contains over 400 species that are separated into two subgenera: subg. *Schedonorus* (broad-leaved fescues, including tall and meadow fescues) and subg. *Festuca* (fine-leaved fescues) (Charmet et al., 1997). In contrast, *Lolium* contains only eight species (Charmet and Balfourier, 1994) and phylogenetic analyses show that they appear to have recently evolved from *Festuca* subg. *Schedonorus*. *Festuca pratensis* appears to be the *Festuca* spp. most closely related to this genus (Charmet et al., 1997). Elucidating the evolutionary relationships within the *Festuca-Lolium* complex can be difficult as many species are polyploid and have genomes that range from diploid, to decaploid states, and species divisions are largely based on morphological characters and may not reflect infertility between groups. The diploid genus *Lolium* exemplifies a group of species that include several freely interfertile 'species', *L. perenne*, *L. multiflorum*, and *L. rigidum*. These species are all outbreeding, and are even able to hybridise with *F. pratensis* to form sterile hybrids (Borrill, 1976).

The remaining *Lolium* spp., *L. canariense*, *L. persicum*, *L. remotum*, *L. subulatum*, and *L. temulentum*, are all inbreeding biological species, and together with *L. multiflorum* and *L. rigidum*, share annual or short-lived life cycles in contrast to the perennial nature of *L. perenne* (Borrill, 1976). The evolutionary relationships within the Pooideae are reasonably resolute at higher taxonomic levels, but the relationships between species are not fully resolved, and probably require data from additional phylogenetically informative loci.

1.6 EPICHLOË ENDOPHYTE GENOMES

Our current understanding of the basic organisation of Epichloë genomes is rather sparse, and hampered by the notorious lack of reliable methods to analyse fungal chromosomes. Classic cytological methods do not resolve the chromosomes very well due to their small size, the asynchronous movement of chromatids during nuclear division, and the lack of techniques for the preparation of chromosome spreads (Taga and Murata, 1994; Xu et al., 1995). Thus, contour-clamped homogeneous electric field (CHEF) gel-electrophoresis has commonly been used to analyse chromosomes from sexual and asexual-hybrid Epichloë endophytes (Murray et al., 1992). The conditions for CHEF separation can be difficult to optimise, and need to be sought for each species and taxonomic group of endophyte depending on the complexity of the genome and size range of its chromosomes (pers. comm. Austen Ganley, Massey University). Epichloë endophytes have also been shown to contain mitochondrial linear plasmids that exhibit mainly maternal transmission patterns (Chung et al., 1996), though the function and replicating mechanisms of these plasmids remain unknown (Schardl, 1996b).

Several genes have been characterised from the Epichloë endophytes, which have chiefly been targeted for phylogenetic purposes, or their role in the production of alkaloids. The ß-tubulin gene (*tub2*), which encodes ß-tubulin (a structural protein found in all eukaryotes which is a main component of the microtubule apparatus), has been isolated and completely sequenced from *E. typhina* (Byrd et al., 1990). The non-coding regions

of this gene have proved highly informative in phylogenetic studies (Schardl et al., 1994; Tsai et al., 1994). Another region of the genome which has been extensively used for Epichloë phylogenetic studies is the ribosomal RNA gene cluster (Glenn et al., 1996; Schardl et al., 1991; Schardl et al., 1994; Schardl and Tsai, 1992; Tsai et al., 1994; White and Reddy, 1998), and sets of 'universal' primers, based on sequences from various fungal species, have been designed for this region (White et al., 1990). The internal transcribed spacer region (rDNA-ITS) is a popular target for analysis from the population to the genera levels, due to its small size and relatively high rate of sequence change (Bruns et al., 1991). The rRNA genes themselves can also be used for such analyses (Glenn et al., 1996), but are more highly conserved and therefore less suited for resolving taxa that are very closely related. Interestingly, only a single rDNA-ITS sequence has been detected in all hybrid endophytes examined to date (Schardl et al., 1994; Tsai et al., 1994), though it has been shown that hybrid endophyte genomes contain several rDNA loci (pers. comm. Austen Ganley, Massey University). Concerted evolution, resulting from homogenisation of the rDNA repeat arrays, is likely to explain the absence of all parental rDNA-ITS sequences in the hybrid (Ganley and Scott, 1998). Phylogenies are currently being established using other informative genes that code for transcription elongation factor alpha (TEF), and actin, and these will further contribute to our understanding of how the endophytes have evolved (pers. comm. Christopher Schardl, University of Kentucky).

Other genes that have been characterised from the Epichloë endophytes include *pyr4*, which encodes orotidine-5'-monophosphate decarboxylase from an LpTG-2 isolate (Collett et al., 1995); *Hmg* from *N. lolii*, which encodes 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase (Dobson, 1997); and a *N. typhinum* proteinase, At1 (Reddy et al., 1996). The *pyr4* locus has been widely used as a selectable marker for gene manipulation in fungi, and was isolated to develop gene transfer and replacement systems in the *Neotyphodium* endophytes (Collett et al., 1995). HMG CoA reductase mediates the rate-determining step of cholesterol biosynthesis, and the product of this reaction, mevalonate, is a key intermediate in the isoprenoid pathway. The At1 gene was isolated as part of a characterisation of a highly abundant serine proteinase found in

N. typhinum/Poa ampla interactions. In culture this proteinase was found to be expressed by nutrient depletion (Lindstrom and Belanger, 1994), and is homologous to proteases suspected to be virulence factors in fungal pathogens of insects, nematodes and other fungi (Reddy et al., 1996). The characterisation of Epichloë genes is still very much in its infancy. From the focus of current research it is expected that many genes associated with the biosynthesis of lolitrems, ergot alkaloids, and lolines, will be found and characterised in the near future.

1.7 DNA FINGERPRINTING

1.7.1 FUNGAL IDENTIFICATION

The identification of fungal types, and the analysis of the relationships between or within different species, populations, and strains of fungi is a central task of many mycologists. Traditional taxonomic identification of fungi is based on micro- and macromorphological characteristics, such as cultural morphologies including colony and colour characteristics on specific culture media, the size, shape, and development of sexual and asexual spores and spore-forming structures. The life history and physiological characteristics, such as nitrogen and carbon source utilisation, are also analysed. These techniques have been increasingly complemented by more modern molecular methods, which include the analysis of chemical constituents (eg. secondary compounds), and characterisation of protein and DNA macromolecules. For some time, isozymes were the molecular markers of choice for studies of genetic diversity, but more recently attention has focused on DNA as a rich source of informative polymorphisms. As each individual's DNA sequence is unique, sequence information can be exploited for studies of genetic diversity and evolutionary relatedness between organisms.

1.7.2 DNA FINGERPRINTING STRATEGIES

In the past two decades, a wide variety of techniques have been developed to analyse DNA sequence polymorphisms. These include the direct sequencing of DNA (Sanger et al., 1977), restriction fragment length polymorphism (RFLP), classical 'hybridisation'-based fingerprinting (Jeffreys et al., 1985), and polymerase chain reaction (PCR; Saiki et al., 1985) based fingerprinting strategies (reviewed by Weising et al., 1995b). The advent of PCR in 1985 (Saiki et al., 1985) has opened up a new suite of rapid fingerprinting methodologies which require very little DNA. The polymerase chain reaction itself involves the in-vitro amplification of particular DNA sequences to high copy numbers using oligonucleotide primers (complementary to the ends of the sequence of interest), and thermostable DNA polymerases, in cycles of thermal denaturation, annealing and extension. The chosen primers direct the specific amplification of the target product, and for DNA fingerprinting strategies, these may be arbitrary, semispecific, or specific.

In 1990, several laboratories simultaneously introduced an amplification based fingerprinting strategy that made use of one or two short GC-rich primers of arbitrary sequence to generate PCR products from genomic DNA. This technique, which doesn't require any previous sequence information, was called randomly amplified polymorphic DNA (RAPD) analysis (Williams et al., 1990), arbitrarily primed polymerase chain reaction (AP-PCR; Welsh and McClelland, 1990), or DNA amplification fingerprinting (DAF; Caetano-Anollés et al., 1991). In general, the term RAPD has been adopted to describe the common characteristics of all these techniques. RAPD-PCR generates fingerprint-like patterns of variable complexity, and has been used by many research groups for identification and even phylogenetic purposes (Crowhurst et al., 1991; Jungehülsing and Tudzynski, 1997; Liu et al., 1995; Stammers et al., 1995). The widespread popularity of RAPD-PCR may be attributed to its rapidity and ease of use, though it has been criticised for poor reproducibility, and the homology of amplified bands of the same size can be questionable (Charmet et al., 1997). More recently, the amplified fragment length polymorphism (AFLP) system was devised, which combines

features of RFLP and PCR technologies by generating fingerprint patterns from the selected amplification of subsets of genomic restriction fragments (Lin and Kuo, 1995). This method boasts high reproducibility, no need for prior sequence information, and identification of large numbers of polymorphisms; though high quality DNA preparations is required, and the procedure is relatively slower than direct PCR methods. The popularity of AFLP markers for genetic mapping and identification purposes is steadily increasing (Gibson et al., 1998; Zhu et al., 1999), and they have been successfully applied to Epichloë endophytes for linkage analysis and strain identification purposes (pers. comm. Heather Wilkinson, University of Kentucky, and Andrew Griffiths, Grasslands AgResearch). While AFLP technology was shown to be superior to other fingerprinting methods in bacterial strain identification (Koeleman et al., 1998), the routine identification of large numbers of samples could be impractical as AFLP is relatively labour intensive.

Semispecific and specific-primer PCR-based fingerprinting techniques exploit the presence and polymorphic nature of repetitive DNA elements in the genome. Repetitive DNA is ubiquitously found in all eukaryotic genomes, and may be classified as either interspersed or tandemly repeated. Interspersed repeats occur at multiple sites throughout the genome, and tandem repeats consist of motifs arranged in a head-to-tail fashion, sometimes up to several thousand at a time. It is thought that most tandemly repeated DNA is noncoding. Tandem repeats may be classified according to the length and copy number of the basic repeated element, as well as their genomic location. Satellite DNA consists of very high repetitions (usually between 1000 and 100,000 copies) of a basic motif (usually of size 100 to 300 bp), and was originally described on the basis of this DNA fraction separating from bulk DNA in buoyant density gradient centrifugation. Satellites occur at few genomic loci only. Minisatellite DNA is a class of shorter motifs (usually 10 to 60 bp), and show a lower degree of tandem repetition at a given locus (Jeffreys et al., 1985). Minisatellite DNA occur at many loci in the genome. Microsatellite DNA, also known as simple tandem repeats (STR) or simple sequence repeats (SSR; Tautz and Renz, 1984) are very short motifs (between 1 and about 10 bp (Sia et al., 1997)) and have a comparatively low degree of repetition with tracts up to

100 bp. They are found evenly dispersed throughout eukaryotic genomes. Table 1.3 summarises the key features of these tandemly repeated DNA elements.

Table 1.3. Key features of tandemly repeated DNA classes

Repeat class	Motif size (bp)	Degree of repetition	Loci abundance
satellite	~ 100 to 300	very high	very few
minisatellite	~ 10 to 60	moderate	many
microsatellite	~ 1 to 10	low	highly dispersed

Polymorphism in the number of tandem repeats of microsatellite tracts are thought to arise by slippage-like events, where misalignment of motifs results in the expansion or contraction of the locus during synthesis or repair (Levinson and Gutman, 1987; Tautz, 1989). Slippage synthesis has been demonstrated *in vitro* (Schlötterer and Tautz, 1992), and rates appear to depend on a number of factors including motif length, GC composition and the processivity of the polymerase used. This contrasts with the mechanisms that influence the hypervariability of minisatellite repeats, which are thought to include unequal crossover events and gene conversion (Charlesworth et al., 1994).

PCR-based fingerprinting with semispecific primers utilises the interspersed nature of mini- and microsatellite DNA, with primers complementary to these repetitive elements. Amplification patterns are obtained if pairs of such loci are inversely facing, and within amplifiable distance of one another, thus the inter-repeat region is amplified. Like RAPD-PCR, these mini- and microsatellite-primed PCR assays (MP-PCR; Meyer et al., 1993) do not require prior sequence knowledge. Fingerprint variation is due to the presence, position, and sequence of these microsatellite loci, and this method has been used successfully in a growing number of applications, including the differentiation of

Cryptococcus neoformans strains (Meyer et al., 1993), and *Saccharomyces cerevisiae* strains (Baleiras Couto et al., 1996).

Variation in the number of repeating microsatellite motifs at a locus can be detected by amplification across the entire locus using primers specific to the unique sequences flanking the locus. This technique is referred to as microsatellite locus PCR (ML-PCR). Microsatellite loci represent almost optimal markers as they are polymorphic within populations, highly abundant and evenly distributed throughout eukaryotic genomes, inherited in a Mendelian co-dominant fashion, and are fast and easy to type with the option of automated analysis (Hearne et al., 1992; Weising et al., 1995a). Microsatellites also have very rapid evolutionary rates, but their utility in phylogenetic reconstruction has had limited success (Goldstein and Pollock, 1997). Microsatellite markers have been shown to be extremely useful in studies of population and conservation genetics including species as diverse as house sparrows (Neumann and Wetton, 1996) to sperm whales (Richard et al., 1996). Genome mapping has also benefited greatly from the identification of microsatellite markers (Broun and Tanksley, 1996; Morgante and Olivieri, 1993; Wu and Tanksley, 1993).

A major disadvantage in developing microsatellite markers is that prior sequence information is required to design flanking primer sets. Whereas informative primer pairs may be derived from sequence database entries (Akkaya et al., 1992), the usual strategy is to screen genomic libraries with appropriate probes, followed by the sequencing of positive clones (FitzSimmons et al., 1995; Menotti-Raymond and O'Brien, 1995). Suitable primer sequences need to be identified and synthesised before PCR-assay optimisation can be performed. The requirement for cloning and sequencing makes the generation of PCR-based microsatellite markers time-consuming and expensive, though increasingly more organisms are being studied in this manner. Several approaches have been developed to expedite microsatellite isolation, including the construction of libraries that are enriched for microsatellites (Edwards et al., 1996; Fisher et al., 1996; Karagyozov et al., 1993; Ostrander et al., 1992), and the screening of Southern blots of

RAPD profiles with microsatellite probes, thus omitting library construction altogether (Ender et al., 1996).

1.7.3 FUNGAL MICROSATELLITE LOCI

Very little information is known about the frequency and occurrence of microsatellite loci in fungal genomes. Extensive database research revealed that the relative abundance of different microsatellite motifs in plants and animals differ considerably. For example, the $(CA)_n$ repeat is one of the most frequently occurring microsatellites in humans and many mammals. In contrast, $(A)_n$ microsatellites are the most abundant in plants, while $(CA)_n$ is comparatively rare (Lagercrantz et al., 1993). Searches also indicated that microsatellites are five times less abundant in the genomes of plants than in mammals. In fungi, database searches revealed that microsatellites of many motif types are widespread, and that $(AT)_n$ motifs are by far the predominant type (Groppe et al., 1995).

Several features of microsatellite loci make them very attractive as markers for the identification of Epichloë endophytes. The high specificity of the primers used would enable endophyte identification in planta, otherwise the endophyte would first need to be cultured out of the grass to be used in techniques such as RAPD-PCR or MP-PCR. Also, interspecific hybrid endophytes would be readily identifiable by the presence of multiple allele bands. In 1995, a polymorphic $(AAG)_n$ microsatellite locus from *E. bromicola* was isolated after it had been identified from an analysis of RAPD patterns when assessing the genetic variability of a population of *Bromus erectus* endophytes (Groppe et al., 1995). This is thought to be the first microsatellite isolated from a fungal species, though the use of microsatellite loci as markers in fungal genomes has not yet become as widespread as in plants and animals.

1.8 OBJECTIVES

Given the importance of the Epichloë endophyte group, not only to agricultural pasture systems, but also to our understanding of the biology and evolution of grass-endophyte symbioses, it is imperative that endophyte isolates are able to be detected and accurately identified. As no single method was available to do this expeditiously, one of the objectives of this study was to develop a DNA-based fingerprinting assay for the rapid and accurate identification of Epichloë endophytes in planta. Initially a pilot study was carried out to evaluate several PCR-based techniques for their ability to distinguish between endophyte isolates, and for their ease of use. From this study, the most promising technique was chosen for further development into a working assay, and used in various applications to identify endophyte isolates.

In addition to developing the fingerprinting assay, the evolutionary origins of several putative *Neotyphodium* endophyte isolates from annual *Lolium* and *Hordeum* grasses were to be investigated. The objective here was to genetically characterise these poorly described isolates using the fingerprinting assay, as well as DNA sequences from the phylogenetically informative gene regions, *tub2* and rDNA-ITS.

CHAPTER TWO

MATERIALS AND METHODS

2.1 BIOLOGICAL MATERIALS

2.1.1 ENDOPHYTE ISOLATES

A list of all endophyte isolates and fungal strains used in this study is given in Table 2.1.

2.1.2 BACTERIAL STRAINS

Escherichia coli strain XL-1 Blue was used for propagating recombinant cloning vectors (Bullock et al., 1987). The genotype of this strain is: *sup*E44 *hsd*R17 *rec*A1 *end*A1 *gyr*A46 *thi rel*A1 *lac*$^-$ F' [*pro*AB$^+$ *lac*I^q *lac*ZΔM15 Tn10(Tet^R)].

2.1.3 CLONING VECTORS

The cloning vectors used in this study are listed below, and maps are given in Appendix 1.

Vector	Relevant characteristics	Source or reference
M13mp19	7.25 kb	Norrander et al., 1983
pUC118	3.2 kb Amp^R	Vieira and Messing, 1987
pGEM-T	3.0 kb Amp^R	Promega Corp.

Table 2.1. Endophyte and fungal isolates used in this study

Isolate	Host species	Source or reference
Epichloë amarillans		
E52	*Sphenopholis obtusata*	Schardl et al., 1997
E57	*Agrostis hiemalis*	Schardl et al., 1997
Epichloë baconii		
E421	*Agrostis capillaris*	Schardl et al., 1997
E424	*Agrostis tenuis*	Schardl et al., 1997
E1031	*Calamagrostis villosa*	Schardl et al., 1997
Epichloë brachyelytri		
E1040	*Brachyelytrum erectum*	Schardl and Leuchtmann, 1999
E1045	*Brachyelytrum erectum*	Schardl and Leuchtmann, 1999
E1046	*Brachyelytrum erectum*	Schardl and Leuchtmann, 1999
Epichloë bromicola		
E501	*Bromus erectus*	Leuchtmann and Schardl, 1998
E502	*Bromus erectus*	Leuchtmann and Schardl, 1998
E798	*Bromus benekenii*	Leuchtmann and Schardl, 1998
E799	*Bromus ramosus*	Leuchtmann and Schardl, 1998
Epichloë clarkii		
E422	*Holcus lanatus*	Schardl et al., 1997
E426	*Holcus lanatus*	Schardl et al., 1997
E427	*Holcus lanatus*	Schardl et al., 1997
Epichloë elymi		
E56	*Elymus canadensis*	Schardl and Leuchtmann, 1999
E184	*Elymus virginicus*	Schardl and Leuchtmann, 1999
Epichloë festucae		
Fr1	*Festuca rubra*	Leuchtmann, 1994
Frc5	*Festuca rubra* subsp. *commutata*	M. Christensen
Frc7	*Festuca rubra* subsp. *commutata*	M. Christensen
Frr1	*Festuca rubra* subsp. *rubra*	M. Christensen

Table 2.1. ***continued***

Isolate	Host species	Source or reference
Epichloë festucae		
Fg1	*Festuca glauca*	M. Christensen
Fl1	*Festuca longifolia*	Leuchtmann, 1994
E28	*Festuca longifolia*	Schardl et al., 1997
E32	*Festuca rubra* subsp. *commutata*	Schardl et al., 1997
E186	*Festuca rubra* subsp. *commutata*	Leuchtmann and Schardl, 1998
E189	*Festuca rubra* subsp. *rubra*	Leuchtmann and Schardl, 1998
E434	*Festuca gigantea*	Schardl et al., 1997
Epichloë glyceriae		
E277	*Glyceria striata*	Schardl and Leuchtmann, 1999
E2772	*Glyceria striata*	Schardl and Leuchtmann, 1999
Epichloë sylvatica		
E354	*Brachypodium sylvaticum*	Leuchtmann and Schardl, 1998
E503	*Brachypodium sylvaticum*	Leuchtmann and Schardl, 1998
Epichloë typhina		
E8	*Lolium perenne*	Schardl et al., 1997
E348	*Phleum pratense*	Schardl et al., 1997
E358	*Anthoxanthum odoratum*	Schardl et al., 1997
E425	*Phleum pratense*	Schardl et al., 1997
E428	*Poa trivialis*	Schardl et al., 1997
E431	*Poa silvicola*	Schardl et al., 1997
E469	*Dactylis glomerata*	Schardl et al., 1997
E470	*Anthoxanthum odoratum*	Schardl et al., 1997
E505	*Brachypodium pinnatum*	Schardl et al., 1997
E1021	*Poa nemoralis*	Leuchtmann and Schardl, 1998
E1022	*Poa nemoralis*	Leuchtmann and Schardl, 1998
E1032	*Poa pratensis*	C. Schardl
E1033	*Poa pratensis*	C. Schardl

Table 2.1. ***continued***

Isolate	Host species	Source or reference
Neotyphodium coenophialum (= FaTG-1)		
Tf5	*Festuca arundinacea*	Christensen et al., 1993
Tf27	*Festuca arundinacea*	Christensen et al., 1993
Tf28	*Festuca arundinacea*	Christensen et al., 1993
Tf35	*Festuca arundinacea*	M. Christensen
AR542	*Festuca arundinacea*	M. Christensen
Neotyphodium lolii (= LpTG-1)		
A9501	*Lolium perenne*	L. Mikkelsen
AR1	*Lolium perenne*	M. Christensen
AR1WF	*Lolium perenne*	M. Christensen
AR1CY	*Lolium perenne*	M. Christensen
CBS232.84	*Lolium perenne*	L. Mikkelsen
EndoSafe	*Lolium perenne*	M. Christensen
Lp5	*Lolium perenne*	Christensen et al., 1993
Lp6	*Lolium perenne*	Christensen et al., 1993
Lp7	*Lolium perenne*	Christensen et al., 1993
Lp9	*Lolium perenne*	Christensen et al., 1993
Lp13	*Lolium perenne*	Christensen et al., 1993
Lp14	*Lolium perenne*	Christensen et al., 1993
Lp19	*Lolium perenne*	Christensen et al., 1993
Lp20	*Lolium perenne*	Christensen et al., 1993
Lp21	*Lolium perenne*	Christensen et al., 1993
N. uncinatum (= FpTG-1)		
Fp1	*Festuca pratensis*	Christensen et al., 1993
Fp2	*Festuca pratensis*	Christensen et al., 1993
Fp4	*Festuca pratensis*	Christensen et al., 1993

Table 2.1. ***continued***

Isolate	Host species *	Source or reference
Neotyphodium sp. (FaTG-2)		
Tf13	*Festuca arundinacea*	Christensen et al., 1993
Tf15	*Festuca arundinacea*	Christensen et al., 1993
Tf20	*Festuca arundinacea*	Christensen et al., 1993
Neotyphodium sp. (FaTG-3)		
Tf16	*Festuca arundinacea*	Christensen et al., 1993
Tf18	*Festuca arundinacea*	Christensen et al., 1993
AR572	*Festuca arundinacea*	M. Christensen
AR577	*Festuca arundinacea*	M. Christensen
Neotyphodium sp. (LpTG-2)		
Lp1	*Lolium perenne*	Christensen et al., 1993
Lp2	*Lolium perenne*	Christensen et al., 1993
Neotyphodium spp.		
AR17	*Lolium perenne*	M. Christensen
HaB	*Hordeum bogdanii*	M. Christensen
Hd1	*Hordeum bogdanii*	M. Christensen
Lc1	*Lolium canariense* (020501)	M. Christensen
Lc2	*Lolium canariense* (020502)	M. Christensen
Lc3	*Lolium canariense* (020514)	M. Christensen
Lc4	*Lolium canariense* (020516)	M. Christensen
Lm1	*Lolium multiflorum* cv. Maverick	M. Christensen
Lm2	*Lolium multiflorum* cv. Maverick	M. Christensen
Lm3	*Lolium multiflorum* cv. Corvette (B3850)	M. Christensen
Lps1	*Lolium persicum* (39B)	M. Christensen
Lps2	*Lolium persicum* (630001)	M. Christensen

Table 2.1. ***continued***

Isolate	Host species *	Source or reference
Neotyphodium spp.		
Lre1	*Lolium remotum* (15-4)	M. Christensen
Lre2	*Lolium remotum* (40-C)	M. Christensen
Lre3	*Lolium remotum* (42B)	M. Christensen
Lrr1	*Lolium rigidum* var. *rigidum* (16.1)	M. Christensen
Lrr2	*Lolium rigidum* var. *rigidum* (9)	M. Christensen
Lrr3	*Lolium rigidum* var. *rigidum* (011460)	M. Christensen
Lrr4	*Lolium rigidum* var. *rigidum*	M. Christensen
Lro1	*Lolium rigidum* var. *rottboellioides* (36A)	M. Christensen
Ls1	*Lolium subulatum* (11-4)	M. Christensen
Lt1	*Lolium temulentum* (45B)	M. Christensen
Lt2	*Lolium temulentum* (61007)	M. Christensen
Claviceps purpurea		C. Schardl
Echinodothis tuberiformis		C. Schardl

* The host seedline, if known, is given in brackets (Margot Forge Forage Germplasm Centre, Grasslands AgResearch).

2.2 GROWTH OF CULTURES

Standard aseptic techniques were employed when managing cultures and sterile glass and plasticware was used throughout.

2.2.1 ISOLATION OF ENDOPHYTE FROM HOST TISSUE

Endophyte cultures were isolated from leaf sheath, nodal and immature inflorescence tissue by surface sterilising tissue briefly in 95% ethanol, 10% commercial bleach for 2 to 4 min, then rinsing in sterile water. Tissue was placed on sterile filter paper and leaf sheaths were transversely sectioned into 1 mm pieces. Tissue was transferred to PDA plates (Section 2.3.2) that were supplemented with tetracycline, or penicillin and streptomycin (Section 2.3.6). Plates were sealed with parafilm, incubated at 22°C, and examined regularly under a stereo dissecting microscope for endophyte growth and contamination.

2.2.2 MAINTENANCE OF ENDOPHYTE CULTURES

Endophyte cultures growing on PDA (Section 2.3.2) were maintained by subculturing small blocks of mycelia from the periphery of a growing colony on to fresh PDA. Plates were sealed and incubated inverted at 22°C.

2.2.3 ENDOPHYTE LIQUID CULTURES

Endophyte liquid cultures were obtained by grinding a 1 cm^2 area of mycelium from the periphery of a colony in 500 µl of PDB (Section 2.3.2) using a plastic grinder in a microcentrifuge tube. Flasks of 125 ml capacity containing 30 ml PDB were inoculated with 100 µl of ground mycelium and incubated at 22°C on a shaking platform at 200 rpm for 7 to 14 days.

2.2.4 *Escherichia coli* CULTURE CONDITIONS

E. coli XL-1 cultures were plated on LB agar plates (Section 2.3.1) with a glass spreader and incubated inverted overnight (16 to 20 hr) at 37°C. Liquid cultures were obtained by inoculation of 5 ml of LB (Section 2.3.1) with a single colony, and overnight incubation at 37°C with shaking at 200 rpm. LB medium was supplemented (Section 2.3.6) as required.

2.2.5 PHAGE M13mp19 CULTURE CONDITIONS

Plate cultures of M13mp19 were obtained by mixing (in order given) 100 µl of M13mp19 transformed XL-1 (Section 2.12.6), 200 µl of overnight culture of XL-1 (Section 2.2.4), 20 µl of X-gal (Section 2.3.6), and 20 µl of IPTG (Section 2.3.6) in a culture tube. Molten top agar (3 ml; Section 2.3.4), equilibrated to 50°C, was added to the tube, briefly vortexed and poured on to LB agar plates (Section 2.3.1) supplemented with tetracycline (Section 2.3.6). Top agar was allowed to set for 30 min, then plates were incubated inverted at 37°C for 16 to 20 hr.

Liquid cultures of M13mp19 were obtained by adding 250 µl of an overnight culture of XL-1 cells (Section 2.2.4) to 25 ml of 2 × YT medium (Section 2.3.5) and 1.2 ml aliquots were dispensed into culture tubes. Plaques of interest were picked with a sterile toothpick and used to inoculate culture tubes. Tubes were shaken at 300 rpm for 5 to 6 hr at 37°C.

2.3 MEDIA

All media were prepared with MilliQ water and sterilised at 121°C for 15 min prior to use. Liquid media was cooled to room temperature before addition of antibiotic(s) and inoculation. Solid media was cooled to approximately 50°C before addition of antibiotic(s) and pouring into sterile petri dishes.

2.3.1 LURIA BROTH MEDIA

Luria broth (LB) contained 10.0 g tryptone (Difco Laboratories), 5.0 g yeast extract (Difco Laboratories), 5.0 g NaCl per litre, and was adjusted to pH 7.0. LB agar was prepared by adding 15 g of agar (Life Technologies) per litre of LB media. Where required, LB media was supplemented (Section 2.3.6).

2.3.2 POTATO DEXTROSE MEDIA

Potato dextrose broth (PDB) contained 24.0 g of dehydrated potato dextrose broth (Difco Laboratories) reconstituted in 1 litre of MilliQ water and adjusted to pH 6.5. Potato dextrose agar (PDA) was prepared by addition of 15 g agar (Life Technologies) per litre of PDB. Where required, PD media was supplemented (Section 2.3.6).

2.3.3 SOC MEDIUM

SOC medium (Dower et al., 1988) contained 20.0 g tryptone (Difco Laboratories), 5.0 g yeast extract (Difco Laboratories), 0.6 g NaCl, 0.2 g KCl, 0.95 g $MgCl_2$, 2.5 g $MgSO_4.7H_2O$, and 3.6 g glucose per litre; and adjusted to pH 7.0.

2.3.4 TOP AGAR

Top agar contained 10.0 g tryptone (Difco Laboratories), 8 g NaCl, and 8 g agar (Life Technologies) per litre.

2.3.5 2 × YT MEDIUM

2 × YT medium contained 16 g tryptone (Difco Laboratories), 10 g yeast extract (Difco Laboratories), and 5.0 g NaCl per litre. The medium was adjusted to pH 7.4 and stored in 25 ml aliquots.

2.3.6 MEDIA SUPPLEMENTS

Media was supplemented at the following concentrations where required.

Supplement	Stock concentration	Final concentration
Ampicillin	100 mg/ml	100 μg/ml
Penicillin	100 mg/ml	100 μg/ml
Streptomycin	100 mg/ml	100 μg/ml
Tetracycline	5 mg/ml	7.5 μg/ml
IPTG	200 mg/ml	240 μg/ml
X-gal	20 mg/ml	24 μg/ml

All supplements were resuspended in MilliQ water and sterilised through a 0.22 μm filter, except tetracycline, which was dissolved in ethanol, and X-gal, which was dissolved in dimethylformamide.

2.4 COMMON BUFFERS, SOLUTIONS AND REAGENTS

All buffers, solutions and reagents were prepared with MilliQ water and stored at room temperature unless otherwise indicated.

2.4.1 COMMON STOCK SOLUTIONS

Common stock solutions are listed below.

Stock	Concentration	pH
Ethanol	70% (v/v)	
Ethidium bromide	10 mg/ml	
$MgCl_2$	0.5 M	
NaCl	1 M	
Disodium EDTA (Na_2EDTA)	0.5 M	8.0
Sodium acetate	3 M	4.2 and 7.0
Sodium dodecyl sulphate (SDS)	20% (w/v)	
Tris-HCl	1 M	7.6 and 8.0

2.4.2 ACRYLAMIDE MIX

Acrylamide mix for polyacrylamide gels contained 288 g of urea, 34.2 g of acrylamide and 1.8 g of bis-acrylamide made up to 500 ml with MilliQ water, and deionised with 5 g of amberlite MB-3 resin (Sigma) for at least 30 min with stirring. The solution was filtered through a porous sintered glass funnel and made up to a final volume of 600 ml with 60 ml of 10 × sequencing TBE (Section 2.4.11) and MilliQ water.

2.4.3 DIG HYBRIDISATION AND DETECTION BUFFERS

The following set of buffers were used for the chemiluminescent detection of hybridised DIG-labelled probes, as described in Sections 2.11.2 and 2.11.3. The washing and blocking buffers (Sections 2.4.3.3 and 2.4.3.4) were made on the day of use.

2.4.3.1 Standard Hybridisation Buffer contained 5 × SSC (Section 2.4.8.4), 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) SDS and 1.0% (w/v) blocking reagent (Boehringer Mannheim GmbH). This buffer was autoclaved before use.

2.4.3.2 Maleic Acid Buffer contained 0.1 M maleic acid and 0.15 M NaCl, adjusted to pH 7.5 with 10 M NaOH and autoclaved.

2.4.3.3 Washing Buffer consisted of Maleic Acid Buffer (Section 2.4.3.2) with addition of 0.3% (v/v) Tween 20.

2.4.3.4 Blocking Buffer consisted of Maleic Acid Buffer (Section 2.4.3.2) with addition of 1.0% (w/v) blocking reagent (Boehringer Mannheim GmbH) dissolved by gently heating in a microwave oven without boiling.

2.4.3.5 Detection Buffer contained 100 mM Tris-HCl (pH 9.5) and 100 mM NaCl.

2.4.4 LIGASE REACTION BUFFER

Ligase reaction buffer (King and Blakesley, 1986) at 5 × concentration contained 250 mM Tris-HCl (pH 7.6), 50 mM $MgCl_2$, 25% (w/v) polyethylene glycol 8000, 5 mM ATP, and 5 mM dithiothreitol. Buffer was stored at -20°C.

2.4.5 LYSOZYME

Lysozyme was resuspended to a concentration of 10 mg/ml in 10 mM Tris-HCl (pH 8.0), dispensed into small aliquots, and stored at -20°C.

2.4.6 RNaseA (DNase Free)

RNaseA was prepared at a concentration of 10 mg/ml in 10 mM Tris-HCl (pH 7.5) and 15 mM NaCl. The mixture was heated at 100°C for 15 min, allowed to cool slowly to room temperature, dispensed into small aliquots, and stored at -20°C. When used in an enzyme digestion reaction, RNase stock was diluted further to 0.2 mg/ml with MilliQ water.

2.4.7 SDS LOADING DYE

SDS loading dye contained 1% (w/v) SDS, 0.02% (w/v) bromophenol blue, 20% (w/v) sucrose and 5 mM Na_2EDTA (pH 8.0).

2.4.8 SOUTHERN BLOTTING SOLUTIONS

The following solutions were used in the Southern blotting procedure described in Section 2.10.

2.4.8.1 **Depurination Solution** consisted of 0.25 M HCl.

2.4.8.2 **Denaturation Solution** contained 0.5 M NaOH and 0.5 M NaCl.

2.4.8.3 **Neutralisation Solution** contained 2.0 M NaCl and 0.5 M Tris-HCl (pH 7.4).

2.4.8.4 **20 × SSC** contained 3 M NaCl and 0.3 M sodium citrate; pH 7.0.

2.4.8.5 **2 × SSC** contained 0.3 M NaCl and 0.03 M sodium citrate; pH 7.0.

2.4.9 TAE BUFFER

At 1 × concentration TAE buffer contained 40 mM Tris-acetate and 2 mM Na_2EDTA; pH 8.5.

2.4.10 TBE BUFFER

At 1 × concentration TBE buffer contained 89 mM Tris, 89 mM boric acid and 2.5 mM Na_2EDTA; pH 8.2.

2.4.11 TBE SEQUENCING BUFFER

At 1 × concentration TBE sequencing buffer contained 134 mM Tris, 45 mM boric acid and 2.5 mM Na_2EDTA; pH 8.8.

2.4.12 TE BUFFERS

TE (10:1) contained 10 mM Tris-HCl and 1 mM Na_2EDTA; pH 8.0.
TE (10:0.1) contained 10 mM Tris-HCl and 0.1 mM Na_2EDTA; pH 8.0.

2.4.13 TRIS-EQUILIBRATED PHENOL

Tris-equilibrated phenol was supplied by Amersham and supplemented with 0.1% (w/v) 8-hydroxyquinoline before storage at 4°C.

2.5 DNA ISOLATION AND PURIFICATION PROCEDURES

2.5.1 LARGE SCALE ISOLATION OF ENDOPHYTE TOTAL DNA

Large scale fungal genomic DNA was prepared by a modification of the method described by Byrd et al. (1990). Mycelia from liquid cultures (Section 2.2.3) was harvested by centrifugation, washed in MilliQ water and freeze dried. About 100 mg of freeze dried mycelia was ground to a fine powder under liquid nitrogen in a pre-chilled mortar and pestle, and resuspended in 10 ml of extraction buffer (150 mM Na_2EDTA, 50 mM Tris-HCl (pH 8.0), 1% (w/v) sodium lauroyl sarcosine, and 2 mg/ml proteinase K). This mixture was incubated at 37°C for 20 min, then centrifuged for 10 min at 20,200 × *g* and 4°C to pellet the cellular debris. All subsequent centrifugation steps were carried out at this speed and temperature. The supernatant was extracted successively with equal volumes of phenol, phenol/chloroform (1:1; v:v) and chloroform with mixing by inversion and centrifugation for 15 min between extractions. DNA was precipitated with an equal volume of isopropanol followed by chilling at -20°C for 15 min and centrifugation for 30 min. To remove polysaccharides the pelleted DNA was resuspended in 5 ml 1 M NaCl then centrifuged for 5 min. DNA was precipitated and pelleted as described, and the pellet was rinsed in 70% ethanol, air dried, and resuspended in 0.5 to 1.0 ml sterile MilliQ water.

2.5.2 MICROWAVE MINIPREP ISOLATION OF ENDOPHYTE TOTAL DNA

Total genomic DNA from very small amounts of fresh mycelium was extracted using a modified microwave miniprep method based on that of Goodwin and Lee (1993). Around 3 to 15 mg mycelium was ground in 100 µl of lysis buffer (50 mM Tris-HCl (pH 7.2), 50 mM Na_2EDTA, 3% (w/v) SDS, and 1% (v/v) 2-mercaptoethanol) in a microcentrifuge tube with a plastic grinder. Sample tubes were left open and loosely covered in plastic food wrap, and heated in a microwave oven (The Genius 650 W, National) on full power for a series of 15 sec, 10 sec and 5 sec bursts to prevent boiling. Immediately after heating, 300 µl lysis buffer was added to each sample, briefly mixed

and incubated at 80°C for 10 min. Sequential extractions with equal volumes of phenol, phenol:chloroform (1:1; v:v) and chloroform were performed with vortexing and centrifugation at 15,000 × *g* for 15 min between extractions. The aqueous phase was removed and DNA precipitated by addition of 16 µl 3 M sodium acetate (pH 7.0) and 0.54 volumes of isopropanol. DNA was pelleted by centrifugation at 15,000 × *g* for 15 min, briefly washed in 70% ethanol, then air dried. Pellets were resuspended in 30 to 50 µl of MilliQ water, depending on the initial mass of mycelium used.

2.5.3 TOTAL DNA ISOLATION USING A FASTDNA KIT

DNA suitable for PCR amplification was extracted from fungal culture and plant tissue samples using the FastDNA Kit (Bio 101 Inc.). To a lysing matrix tube containing a 6 mm ceramic sphere and garnet matrix, 10 to 200 mg of tissue was added followed by a 6 mm ceramic cylinder. To this, 800 µl CLS-VF and 200 µl PPS were added and the sample processed in a FastPrep shaker (Bio 101 Inc.) for 30 sec at speed 4.5. Samples were centrifuged for 5 min at 15,000 × *g* and the available supernatant was transferred to a new microcentrifuge tube. The supernatant was often contaminated with fine tissue debris, so tubes were spun for an additional 5 min at 15,000 × *g*, and 600 µl of the clear supernatant was transferred to a fresh tube containing 600 µl binding matrix. To allow DNA binding, samples were gently mixed and incubated at room temperature for 5 min, then spun for 1 min and the supernatant discarded. The pellet was washed by gently resuspending in 500 µl SEWS-M, then tubes were spun for 1 min and the supernatant discarded. A further 10 sec spin was performed to allow removal of residual liquid with a small bore tip. DNA was eluted from the binding matrix by addition of 100 µl DES followed by a 2 to 3 min incubation at room temperature. Samples were centrifuged for 1 min at 15,000 × *g* and the DNA containing supernatant transferred to a new tube.

2.5.4 PLANT DNA ISOLATION USING A CTAB METHOD

Total genomic DNA was isolated from endophyte-infected and endophyte-free plant tissue using the hexadecyltrimethylammonium bromide (CTAB) based method of Doyle

and Doyle (1990). Fresh grass pseudostem tissue, 0.5 to 1.0 g, was ground to a fine powder in a chilled mortar and pestle with liquid nitrogen, transferred to 5.0 to 7.5 ml CTAB isolation buffer (2% (w/v) CTAB, 1.4 M NaCl, 0.2% (v/v) 2-mercaptoethanol, 20 mM Na_2EDTA, and 100 mM Tris-HCl (pH 8.0)), that was preheated to 60°C, and swirled gently to mix. The sample was incubated at 60°C for 30 min, then extracted once with chloroform-isoamyl alcohol (24:1; v:v). Tubes were centrifuged at 1,600 × *g* for 5 min, the aqueous phase removed and a 0.67 volume of cold isopropanol was added to precipitate the DNA. Tubes were centrifuged at 500 × *g* for 1 to 2 min, the supernatant gently poured off, and the pellet washed for 20 min in 10 to 20 ml of wash buffer (76% (v/v) ethanol and 10 mM ammonium acetate). Samples were centrifuged at 1,600 × *g* for 10 min, the supernatant poured off and the DNA pellet air dried. DNA was resuspended in 1 ml TE (10:1; Section 2.4.12).

2.5.5 PLASMID DNA EXTRACTION USING A RAPID BOILING METHOD

Plasmid DNA from *E. coli* was isolated by the method of Holmes and Quigley (1981). Cells from 1.5 ml of an overnight culture (Section 2.2.4) were pelleted by centrifugation and thoroughly resuspended in 350 µl STET buffer (8% (w/v) sucrose, 5% (v/v) Triton X-100, 50 mM Na_2EDTA (pH 8.0), and 50 mM Tris-HCl (pH 8.0)). To this, 25 µl lysozyme (Section 2.4.5) was added, the solution vortexed and heated in a boiling water bath for 40 sec, immediately followed by centrifugation for 10 min at 15,000 × *g*. The gelatinous pellet was removed with a toothpick and DNA was precipitated by addition of an equal volume of isopropanol and cooling at -20°C for 30 min. DNA was pelleted by centrifugation for 10 min at 15,000 × g, washed in 70% ethanol, air dried and resuspended in 50 µl MilliQ water.

2.5.6 PLASMID DNA EXTRACTION USING AN ALKALINE LYSIS METHOD

Plasmid DNA for sequencing was prepared by the alkaline lysis miniprep method of Sambrook et al. (1989). Cells from 1.5 ml of an overnight culture of *E. coli*

(Section 2.2.4) were pelleted by centrifugation for 3 min at 15,000 × *g* (all centrifugation steps were performed at this speed) and resuspended in 100 µl of solution I (50 mM glucose, 25 mM Tris-HCl, and 10 mM Na_2EDTA; pH 8.0), followed by 200 µl of solution II (0.2 M NaOH and 1% (w/v) SDS). The contents of the tube were mixed thoroughly by inversion, then 150 µl solution III (29.44 g potassium acetate and 11.5 ml glacial acetic acid per 100 ml) was added and the mixture vortexed with the tube inverted. The solution was incubated on ice for 3 to 5 min and centrifuged for 5 min. To the supernatant, an equal volume of phenol (Section 2.4.13) was added, then vortexed and centrifuged for 5 min. The aqueous phase was extracted with an equal volume of phenol/chloroform (1:1; v:v), followed by an equal volume of chloroform. DNA was precipitated with two volumes of 95% ethanol, centrifuged for 5 min, washed with 70% ethanol, then air dried and resuspended in 50 µl of TE (10:0.1; Section 2.4.12).

2.5.7 PLASMID DNA EXTRACTION USING A QUANTUM MINIPREP KIT

Plasmid DNA required for sequencing or vector preparation was extracted using a Quantum Prep Plasmid Miniprep Kit (Bio-Rad). Cells from 1.5 to 3.0 ml of an overnight culture of *E. coli* (Section 2.2.4) were pelleted by centrifugation for 3 min at 15,000 × *g* (all centrifugation steps were all performed at this speed) and resuspended in 200 µl of cell resuspension solution. Cell lysis solution, 250 µl, was added and the tube inverted 10 times to lyse the cells, then 250 µl of neutralisation solution was added and the tube inverted a further 10 times to mix. Cellular debris was pelleted by centrifugation for 5 min and 200 µl matrix solution was added to the supernatant and mixed. The suspension was poured into a spin filter that had been inserted into a microcentrifuge tube, spun for 30 sec, and the filtrate discarded. The matrix was washed by addition of 500 µl wash solution to the spin filter, followed by centrifugation for 30 sec, and the supernatant discarded. A second wash was performed by addition of 500 µl wash solution followed by centrifugation for 2 min to remove all traces of ethanol. DNA was eluted by addition of 100 µl MilliQ water to the spin column in a new microcentrifuge tube and centrifuged for 30 sec.

2.5.8 SINGLE-STRANDED M13mp19 DNA ISOLATION

Single-stranded M13mp19 DNA suitable for sequencing was prepared from phage liquid cultures (Section 2.2.5). Cells were pelleted by centrifugation for 5 min at 15,000 × *g* and 0.8 ml of the supernatant was transferred to a new tube. The remainder of the liquid was aspirated off and the cells retained to extract the replicative form of the phage by the rapid boil method (Section 2.5.5). To the phage supernatant, 200 µl of a solution of 20% PEG 8000 in 2.5 M NaCl was added and incubated at room temperature for 15 min. Samples were centrifuged for 5 min at 15,000 × *g* and the supernatant aspirated off to leave the phage pellet. Addition of 100 µl TE (10:0.1; Section 2.4.12) and 50 µl phenol to the pellet was followed by vortexing for 10 sec, standing for 5 min and vortexing for 10 sec. Samples were centrifuged for 3 min at 15,000 × *g* and the aqueous phase removed and sequentially extracted with equal volumes of phenol:chloroform (1:1, v:v) and chloroform. DNA was precipitated by addition of 10 µl 3 M sodium acetate, 250 µl 95% ethanol and chilled overnight at –20°C. DNA was pelleted by centrifugation for 5 min at 15,000 × g, washed in 95% ethanol, air dried and resuspended in 30 µl TE (10:0.1; Section 2.4.12).

2.5.9 PCR PRODUCT PURIFICATION

Polymerase chain reaction (PCR) amplification products were purified from salts, free nucleotides, polymerases, and primers using the PCR Clean Up Kit (Boehringer Mannheim GmbH). PCR reactions were extracted with an equal volume of chloroform and centrifuged for 5 min at 15,000 × *g*. The aqueous phase was extracted and made up to 100 µl with TE (10:0.1; Section 2.4.12). Nucleic acid binding buffer, 400 µl, was added followed by 10 µl silica suspension. The mixture was incubated for 10 min at room temperature with vortexing every 2 to 3 min, then the matrix was pelleted by a 30 sec centrifugation and the supernatant discarded. The pellet was resuspended in 400 µl nucleic acid binding buffer, vortexed, pelleted, and the supernatant discarded. The pellet was washed by resuspension in 400 µl washing buffer, centrifugation and the supernatant discarded. This step was repeated once, then the pellet air dried at room

temperature for 15 min. DNA was eluted in 20 to 50 µl TE (10:0.1; Section 2.4.12) or MilliQ water at pH 8.0 to 8.5 for 10 min, then centrifuged for 30 sec and the DNA containing solution recovered.

2.5.10 ISOLATION OF DNA FROM AGAROSE

DNA was recovered from SeaPlaque agarose gels, 0.7% to 1.5% (w/v) in TAE electrophoresis buffer (Section 2.4.9), by phenol freeze extraction (Thuring et al., 1975). Following electrophoresis and ethidium bromide staining (Section 2.8.4), fragments of interest were excised under long wave UV light (wavelength 366 nm) and excess agarose was trimmed away. The agarose was melted at 65°C, mixed thoroughly with an equal volume of tris-equilibrated phenol (Section 2.4.13) and frozen at –20°C for at least 2 hr. The tube was centrifuged for 10 min at 15,000 × *g* at 4°C, the aqueous phase recovered and extracted with an equal volume of chloroform. DNA was precipitated by addition of 1/10 volume of 3 M sodium acetate, 2.5 volumes of 95% ethanol and chilled at –20°C for at least 2 hr. DNA was pelleted by centrifugation for 15 min at 15,000 × g, washed in 95% ethanol, air dried and resuspended in TE (10:0.1; Section 2.4.12) or MilliQ water.

2.6 DNA QUANTIFICATION

DNA preparations were quantified using one or both of the methods described.

2.6.1 FLUOROMETRIC QUANTIFICATION

DNA was quantified on a Hoefer Scientific TKO 100 Fluorometer using a dye solution containing 1 × TNE buffer (10 mM Tris-HCl, 1 mM Na_2EDTA, and 100 mM NaCl; pH 7.4) and 0.1 µg/ml Hoechst 33258 dye. The instrument was calibrated by adjusting the zero dial until a '0' reading was obtained from 2 ml dye only, and adjusting the scale dial until a '100' reading was consistently obtained when 2 µl of 100 ng/µl calf thymus DNA standard was added to the dye. Using these settings, the concentration of the DNA

sample to be quantified was determined by adding 2 µl of DNA (at a suitable dilution in water if necessary) to 2 ml of TNE dye. The resulting reading was recorded as the DNA concentration in ng/µl.

2.6.2 MINI GEL METHOD

A 1 or 2 µl aliquot of DNA to be quantified was electrophoresed (Section 2.8.2) on an agarose mini gel alongside a series of standard DNA solutions of known concentration. In general, λ DNA standards were used when quantifying genomic DNA, pUC118 DNA standards were used when quantifying plasmid DNA, and a low DNA mass ladder (Life Technologies) was used when quantifying PCR products. After staining with ethidium bromide and photographing (Section 2.8.4), the DNA concentration was estimated from the relative intensity of the sample of interest to those of the standard concentration samples.

2.7 RESTRICTION ENDONUCLEASE DIGESTION OF DNA

Restriction endonuclease digestion reactions were set up on ice using the commercial buffer supplied by the manufacturer and a 2 to 10-fold excess of restriction enzyme. Digestion reactions were incubated at the temperature recommended by the manufacturer, for 2 to 3 hr for plasmid DNA or 3 hr to overnight for genomic DNA. A small aliquot of digested DNA was checked on a minigel (Section 2.8.2) to ensure the digestion had gone to completion. If the reaction was incomplete, more enzyme was added and the reaction incubated for an additional hour or two. Once complete, a 1/5 volume of SDS loading dye (Section 2.4.7) was added to stop the reaction if the DNA was to be electrophoresed, otherwise the reaction was stored at –20°C until required.

2.8 AGAROSE GEL ELECTROPHORESIS

2.8.1 AGAROSE GELS AND DNA SIZE MARKERS

The concentration of agarose used was determined by the desired range of fragment separation as outlined in the table below. Agarose was added to 1 × TAE or 1 × TBE buffer (Sections 2.4.9 and 2.4.10) and melted by microwaving or pressure cooking. Molten agarose was equilibrated to 50°C before pouring. All gels were run in 1 × TBE buffer except when purifying DNA from agarose, a low melting point agarose in 1 × TAE buffer was used. DNA size markers were selected as outlined below depending on the range of fragment separation required, and estimates of band size were determined from the relative migration of the subject bands compared to the standard size markers. DIG-labelled DNA size markers (Boehringer Mannheim GmbH) were run if the gel was to be blotted and probed using the DIG system (Section 2.11).

Agarose grade	% (w/v) of agarose	DNA standard size markers	Size range of separation
Molecular Biology (BRL)	0.7	λ HindIII	2 to 25 kb
Molecular Biology (BRL)	1.0	λ HindIII	0.5 to 10 kb
Molecular Biology (BRL)	1.2	λ HindIII	0.4 to 5 kb
NuSieve (FMC)	2.0	1 kb ladder	0.3 to 1.5 kb
NuSieve (FMC)	3.0	50 or 100 bp ladder	0.1 to 1.0 kb
SeaPlaque (FMC)	0.7	λ HindIII	0.5 to 25 kb
SeaPlaque (FMC)	1.25	λ HindIII	0.15 to 6 kb

2.8.2 MINI GEL ELECTROPHORESIS

Horizontal agarose mini gels, of approximately 25 ml volume, were run at 60 to 100 V in 1 × TAE or 1 × TBE buffer (Sections 2.4.9 and 2.4.10). Gels were run until the SDS loading dye (Section 2.4.7) had migrated approximately ¾ of the way down the gel.

2.8.3 LARGE GEL ELECTROPHORESIS

Large gels, of approximately 150 ml volume, were run in a BioRad Sub-Cell apparatus at 30 V for about 18 hr, or until the SDS loading dye (Section 2.4.7) had migrated at least 150 mm from the wells.

2.8.4 STAINING AND PHOTOGRAPHING GELS

After electrophoresis, gels were stained in 1 μg/ml ethidium bromide for 10 to 20 min for a mini gel, or 30 min for a large gel. Gels were destained in water and bands were visualised on a UV transilluminator and photographed with either Polaroid 667 film (Polaroid Corp.) or an Alpha Innotech gel documentation system.

2.9 POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

Denaturing polyacrylamide gel electrophoresis (PAGE) was used to separate DNA fragments of small size including radio-labelled sequencing reactions (Section 2.15.1) and radio-labelled microsatellite locus PCR products (Section 2.13.5). PAGE gels (30 × 40 cm) were poured between glass plates with 0.4 mm spacers using 60 ml acrylamide mix (Section 2.4.2) containing 36 μl of N, N, N', N;-tetramethylethylenediamine (TEMED) and 360 μl of freshly prepared 10% (w/v) ammonium persulphate. Gels were run on a model S2 sequencing gel electrophoresis apparatus (Life Technologies), and were pre-run for 30 min at 1,500 V in 1 × sequencing TBE (Section 2.4.11). Samples with stop solution added were denatured at 95°C for 2 min and quenched on ice prior to

loading 2 to 3 µl onto the gel. Sequencing samples were run for either a short run of 2 hr (until the first blue dye front ran off) or a long run of about 6 hr (when 3 dye fronts had run off). The run time for microsatellite PCR products was adjusted to allow maximal separation of products and depended on the expected DNA fragment size for the microsatellite locus being run. Once run, gels were transferred to blotting paper, dried on a model 583 gel dryer (Bio-Rad) for 2 hr at 80°C, then exposed to X-ray film (Fuji Photo Film Co.) overnight or longer if necessary.

2.10 SOUTHERN BLOTTING

The blotting method used is based on that of Southern (1975). DNA to be transferred was separated on a large agarose gel (Section 2.8.3) alongside DIG-labelled DNA standard markers (Boehringer Mannheim GmbH), stained, destained, and photographed as described in Section 2.8.4. The gel was immersed and gently agitated in depurination solution (Section 2.4.8.1) for 15 min, denaturation solution (Section 2.4.8.2) for 30 min, and neutralisation solution (Section 2.4.8.3) for 30 min. A final wash was carried out in 2 × SSC (Section 2.4.8.5) for 2 min.

The blotting apparatus was assembled on a plastic trough with wells at each end. Two sheets of Whatman 3MM chromatography paper, cut to the width of the trough and just longer than the length of the apparatus, were soaked in 20 × SSC (Section 2.4.8.4) then placed in the trough so that the ends of the paper were submerged in the wells to act as a wick. The wells were filled with 20 × SSC. A piece of plastic food wrap was placed over the entire trough and a 'window' was cut from the centre that was 2 mm smaller on each side than the size of the gel. The treated gel was placed over the window and a piece of positively charged nylon membrane (Boehringer Mannheim GmbH), cut to 2 mm larger than the gel and presoaked in 2 × SSC, was placed on the gel. Four sheets of 3MM paper were cut to the size of the gel. Two of these were presoaked in 2 × SSC and laid on the nylon membrane followed by two dry sheets. A stack of paper towels, approximately 100 mm high, was laid on top of the 3MM paper followed by a large

plastic tray containing a weight of approximately 500 g to keep the stack flat. Following overnight DNA transfer, the apparatus was disassembled and the membrane washed in 2 × SSC for 5 min, then air dried. DNA was cross-linked by placing the membrane face down on a UV transilluminator and exposed to UV light for 2 min.

2.11 SOLUTION HYBRIDISATION USING DIGOXIGENIN (DIG)

The nonradioactive DIG system (Boehringer Mannheim GmbH) was used for all hybridisation work and procedures were based on The DIG System User's Guide for Filter Hybridisation manual (Boehringer Mannheim, 1995).

2.11.1 DIG 3'-END LABELLING OF OLIGONUCLEOTIDE PROBES

Oligonucleotide probes, listed in Table 2.2, were 3'-end labelled with DIG using the following protocol. In a microcentrifuge tube on ice, 4 µl of 5 × reaction buffer (1 M potassium cacodylate, 125 mM Tris-HCl, 1.25 mg/ml bovine serum albumin; pH 6.6), 4 µl of 25 mM cobalt chloride, 100 pmol of oligonucleotide, 1 µl of 1 mM digoxigenin-11-ddUTP, 1 U of terminal transferase, and MilliQ water to a total volume of 20 µl was added and gently mixed. The reaction was incubated at 37°C for 15 min and placed on ice where the reaction was stopped by addition of 1 µl 200 mM Na_2EDTA (pH 8.0). The labelled oligonucleotide probe was stored at –20°C. A control oligonucleotide, C1, was included in each batch of labelling reactions to monitor the labelling of sample oligonucleotides.

To check the yield of labelled probe, a side by side comparison of the DIG-labelled sample oligonucleotide with a commercially prepared DIG-labelled oligonucleotide control (Boehringer Mannheim GmbH) was performed. A five-fold dilution series in MilliQ water of the sample and control probes was made, including concentrations 50 fmol/µl, 10 fmol/µl, 2 fmol/µl, 0.4 fmol/µl, and 0.08 fmol/µl. Aliquots of 1 µl from each dilution were spotted in rows onto positively charged nylon membrane (Boehringer

Mannheim GmbH), allowed to air dry, and cross-linked (Section 2.10). Chemiluminescent detection was performed on the membrane as described in Section 2.11.3 and spot intensities were compared between the labelled control and sample dilutions to estimate the concentration of the sample probes.

2.11.2 HYBRIDISATION AND WASHING CONDITIONS

Hybridisations were carried out in roller bottles in a Bachofer hybridisation oven for Southern blots (Section 2.10), and in small plastic pots in a shaking waterbath for plaque filter lifts (Section 2.12.7). The membranes were pre-hybridised for an hour in DIG standard hybridisation buffer (Section 2.4.3.1) and the hybridisation temperature used was 60°C for $(CA)_{15}$ and $(GA)_{15}$ dinucleotide probes, or 50°C for $(CAA)_{10}$, $(GAA)_{10}$, and $(ATC)_{10}$ trinucleotide probes. Hybridisations were performed using 2 pmol/ml of each DIG 3'-end labelled oligonucleotide probe (Section 2.11.1) in DIG standard hybridisation buffer at the pre-hybridisation temperature for 2 to 6 hr. Following hybridisation, blots were washed twice in 0.1 × SSC for 10 min each at room temperature. A final stringency wash was performed for 2 min using a combination of temperature and salt concentration, which for low stringency conditions, was approximately 10 to 20°C below the T_m for the probe (calculated using the formula of Bolton and McCarthy, 1962) and for high stringency conditions was approximately 0 to 5°C below the T_m. Hybridised probes were then detected by chemiluminescent detection as described in the following section.

2.11.3 CHEMILUMINESCENT DETECTION OF DIG-LABELLED PROBES

Chemiluminescent detection was carried out at room temperature in clean plastic trays with gentle agitation to ensure the membranes were completely covered with solution. At no time were membranes allowed to air dry. After stringency washes the membranes were washed for 1 min in washing buffer (Section 2.4.3.3), then blocked in blocking buffer (Section 2.4.3.4) for 30 min. Anti-Digoxigenin-AP antibody (Boehringer Mannheim GmbH) was diluted 1:10,000 in blocking buffer and membranes were

incubated in this for 30 min, then washed twice in washing buffer for 15 min each. Membranes were equilibrated in detection buffer (Section 2.4.3.5) for 2 min, drained briefly and transferred face up to an opened out clear plastic bag. CSPD® (Boehringer Mannheim GmbH) was diluted 1:100 in detection buffer and pipetted over membranes. The bag was closed to create a liquid seal around the membranes and incubated for 5 min. The membranes were drained briefly, transferred to a clean plastic bag, sealed and incubated at 37°C for 15 min to activate the alkaline phosphatase. The membranes were then exposed to X-ray film (Fuji Photo Film Co.) for 30 to 120 min and developed.

2.12 LIBRARY CONSTRUCTION AND SCREENING

Partial libraries of size-selected DNA fragments from endophyte strains E8 (*E. typhina*) and Lp1 (LpTG-2) were constructed in the vector M13mp19 (Section 2.1.3) and screened using methods described by Sambrook et al. (1989).

2.12.1 CALF ALKALINE PHOSPHATASE (CAP) TREATMENT OF VECTOR

Digested vectors were treated with calf alkaline phosphatase (CAP) to prevent self ligation of vector in subsequent ligation reactions. Vector DNA, 5 µg, was digested to completion in an excess of the appropriate restriction enzyme (Section 2.7). The restriction enzyme was inactivated by heating for 10 min at 65°C and 0.5 U of CAP was added and incubated at 37°C for 30 min. Na_2EDTA (pH 8.0) to a final concentration of 5 mM, SDS to give a final concentration of 0.5%, and proteinase K to a final concentration of 50 µg/ml were added, and the mixture was incubated at 56°C for 30 min. The DNA was purified by sequential extractions with equal volumes of phenol, phenol/chloroform (1:1, v:v), and chloroform with centrifugation for 5 min at 15,000 × g between steps. DNA was then precipitated by addition of 1/10 volume of 3 M sodium acetate (pH 7.0) and 2.5 volumes of 95% ethanol, chilling at –20°C for 30 min and centrifugation for 10 min at 15,000 × g. The pellet was washed in 70% ethanol, air dried, resuspended in MilliQ water to a concentration of 20 ng/µl, and stored at 4°C.

2.12.2 PREPARATION OF LINKERS

*Bam*HI linkers were constructed from the self annealing of 5'-chemically phosphorylated 5'-pCGGGATCCCG oligonucleotides (Life Technologies) that were based on a sequence by Stratagene. In a microcentrifuge tube, 2 µl of 10 × annealing buffer (100 mM Tris-HCl (pH 7.6), 10 mM Na_2EDTA, and 1 M NaCl), 4 nmol of oligonucleotide, and MilliQ water to a total volume of 20 µl was mixed. The tube was incubated in a small waterbath at 65°C for 10 min, then the waterbath turned off and allowed to cool slowly to room temperature. The annealed linkers, at a final concentration of 100 pmol/µl, were stored at –20°C.

2.12.3 PREPARATION OF LINKERED INSERT DNA

Genomic inserts were prepared by the digestion of genomic DNA using combinations of the blunt end 4 bp cutters *Alu*I, *Hae*III, and *Tha*I, as well as *Bam*HI (Section 2.7). Digests were separated on SeaPlaque gels in TAE buffer (Section 2.8), and fragments in the size range 200 to 500 bp (E8 digests), or 100 to 1000 bp (Lp1 digests) were excised and purified from agarose (Section 2.5.10). Linkers were ligated to the blunt ends of the inserts to facilitate cloning, and the ligation reaction was set up on ice as follows: 100 to 500 ng of blunt end DNA fragments, 1 to 2 ug of phosphorylated linkers (Section 2.12.2), 4 µl of 5 × ligation buffer (Section 2.4.4), 0.8 U of T4 DNA ligase (New England Biolabs), and MilliQ water to a total volume of 20 µl. Ligation was carried out for 16 to 20 hr at 4°C and the ligase was inactivated by heating the reaction to 65°C for 15 min. Half microlitre aliquots of the ligation reaction were taken before addition of the ligase and after the ligation reaction and run on a mini gel (Section 2.8.2) to check for ligation. If fragments appeared ligated, 10 µl of the appropriate 10 × restriction enzyme buffer, 20 to 50 U of restriction enzyme and MilliQ water to a total volume of 100 µl was added to the inactivated ligation reaction and incubated for at least 4 hr at 37°C. A further 10 U of restriction enzyme was added and the reaction incubated for another hour, then Na_2EDTA (pH 8.0) was added to a final concentration of 10 mM to stop the reaction. DNA was extracted with an equal volume of phenol/chloroform (1:1, v:v) and

centrifuged for 2 min at 15,000 × g and the aqueous phase extracted. The linkered DNA fragments were purified from the digested linkers by gel purification in SeaPlaque agarose gels in TAE buffer as outlined in Section 2.5.10.

2.12.4 LIGATION REACTIONS

Ligation reactions were performed in 10 or 20 µl volumes. The quantities of reagents for a 20 µl reaction are as follows: 4 µl of 5 × ligation buffer (Section 2.4.4), 20 ng of vector, 40 U of T4 DNA ligase, and a 2 to 5 molar excess of insert:vector DNA. The ligation mixture was incubated for at least 16 hr at 4°C. To check that ligation had occurred, two 2 µl samples of the ligation mixture were removed, one before adding the ligase and the other after ligation, mixed with SDS loading dye (Section 2.4.7) and separated on a mini gel (Section 2.8.2).

2.12.5 PREPARATION OF ELECTROCOMPETENT XL-1 CELLS

One litre of LB (Section 2.3.1) was inoculated 1/100 with an overnight culture of XL-1 cells (Section 2.2.4) and grown at 37°C with vigorous shaking to mid-log phase (A_{600} of 0.5 to 1.0, approximately 3 hr). The cells were chilled on ice for 20 min and harvested by centrifugation at 4,000 × *g* for 10 min at 4°C. The cells were resuspended twice in ice-cold water (1 litre, then 500 ml,), followed by resuspension in 20 ml ice-cold 10% (v/v) glycerol, centrifuging as above between steps. The cells were resuspended in 4 ml ice-cold 10% (v/v) glycerol and stored in 40 µl aliquots at -70°C.

2.12.6 TRANSFORMATION BY ELECTROPORATION

Aliquots, 40 µl, of electrocompetent cells (Section 2.12.5) were thawed gently at room temperature, placed on ice and 1 to 2 µl of DNA to be transformed was added. The tube contents were gently mixed, and tubes were left on ice for 1 min. The Gene Pulser (Bio-Rad) was set to 25 µF and 2.5 kV, and the pulse controller set to 200 ohms resistance in parallel with the sample chamber. The mixture of DNA and cells was transferred to an

ice cold 0.2 cm electroporation cuvette and pulsed at the above settings. The time constant was checked and, if this was between 4 and 5, the cells were immediately resuspended in 1 ml of SOC medium (Section 2.3.3). Transformed cells were incubated at 37°C for an hour to aid the recovery of the *E. coli*. A positive control of 2 ng pUC118, and a negative control of water only was always employed with each set of transformations. Cells were plated (Sections 2.2.4 and 2.2.5) at suitable dilutions onto selective LB plates (Section 2.3.1).

2.12.7 FILTER LIFTS AND PRIMARY LIBRARY SCREENING

Recombinant M13mp19 phage were plated at approximately 10,000 plaques per plate to form the primary library. Filter lifts were taken from the library by marking nylon filters (Hybond N+, Amersham) asymmetrically and placing on the phage plates for 1 min. The marks were transferred to the plates for later alignment. Filters were placed on blotting paper to air dry and DNA was cross-linked by placing face down on a UV transilluminator and exposing for 2 min. To eliminate false positives, two lifts were taken from each plate where the second lift was left on the plate for 2 min. Hybridisation was performed as described in Section 2.11. Positive plaques, identified by alignment of the plate with positive hybridisation signals from the duplicate plaque lifts, were picked with a sterile cut-off 1 ml tip. The agar plug was ejected into 1 ml of LB medium (Section 2.3.1) and stored at 4°C overnight to elute the phage from the agar.

2.12.8 SECOND ROUND LIBRARY SCREENING

Phage from first round positive plaques (Section 2.12.7) were diluted to 10^{-6} to 10^{-8} in TE buffer (10:1; Section 2.4.12) and 100 µl plated as previously described (Section 2.2.5). Plates containing between 30 and 300 plaques were lifted and hybridised as previously described (Section 2.12.7). Positive plaques from the second round of library screening were picked with a toothpick and used to inoculate liquid cultures (Section 2.2.5).

2.12.9 SCREENING POSITIVE CLONES

Unique clones were identified by digesting plasmid DNA, isolated from positive plaques using a rapid boiling method (Section 2.5.5), with *Bam*HI (Section 2.7) to excise the insert fragments. Digests were separated on mini gels (Section 2.8.2) and unique clones were identified based on the size of their inserts. If many clones had inserts of the same size, they were analysed using a 'single tract sequencing' approach, where sequencing reactions were carried out using only a single terminating dideoxynucleotide. This was carried out as described in Section 2.15.1 but reactions contained the following quantities: 2 to 20 fmol of template DNA, 0.8 µl 10 × cycling mix, 4 pmol of sequencing primer, 0.2 µl of [α–^{33}P] dCTP (~2,500 Ci/mmol, Amersham), 2 µl termination mix and MilliQ water to bring the volume to 8 µl. Unique clones were then fully sequenced (Section 2.15).

2.13 POLYMERASE CHAIN REACTION AMPLIFICATION

PCR reactions were set up in a dedicated 'clean' area that was free of PCR product and genomic DNA handling. Reactions were typically performed in 25 µl volumes, though half and double volumes were sometimes used depending on the application. For n PCR reactions, a cocktail of n+1 reactions was prepared on ice using aerosol barrier tips to prevent contamination. The cocktail was dispensed into 0.2 ml tubes on ice and template DNA added. Positive and negative (water) control samples were always included with each set of reactions, and reactions were amplified in a Corbett PC-960 or FTS-960, or a GeneAmp 2400 (Perkin-Elmer Corp.) model thermocycler. Reactions were checked on a mini gel (Section 2.8.2).

2.13.1 PRIMERS

Oligonucleotide primers were manufactured by Life Technologies and rehydrated to a final concentration of 100 μM in autoclaved MilliQ water, except RAPD primers, which were kindly provided by Ross Crowhurst, HortResearch, Auckland, New Zealand. Primers were diluted to a working concentration of 5 μM and stored at –20ºC. The sequences of all primers and probes used in this study are listed in Table 2.2.

Table 2.2. Primer and probe sequences

Name	Sequence (5' – 3')	Application *
A1	$(GTG)_5$	MP-PCR
A2	$(GACA)_4$	MP-PCR
A3	GAG GGT GGY GGY TCT	MP-PCR
A4	$(TA)_{15}$	microsatellite probe
A5	$(TAA)_{10}$	microsatellite probe
A6	$(CA)_{15}$	microsatellite probe
A7	$(GA)_{15}$	microsatellite probe
A8	$(CAA)_{10}$	microsatellite probe
A9	$(GAA)_{10}$	microsatellite probe
A10	$(ATC)_{10}$	microsatellite probe
B1.1	CGC ACA ATA CGT CAG CTA GGA ATG	ML-PCR, B1
B1.1-FAM	fam - CGC ACA ATA CGT CAG CTA GGA ATG	ML-PCR, B1
B1.2	CCT GAA TCA ACT TTG CTA TCA GGC	ML-PCR, B1
B2.1-HEX	hex - TCC GTA ATG CTC TGC TTT GGC AGG	ML-PCR, B2
B2.1	TCC GTA ATG CTC TGC TTT GGC AGG	ML-PCR, B2
B2.2	CTG CTG ACA GTA GGT GGA TGA TGG	ML-PCR, B2
B3.1	TGA TGT CCG TTC TCG TGG CAT TCG	ML-PCR, B3
B3.2	CGG TGT AGA AGG ACC TGC AGT TTG	ML-PCR, B3
B4.1	TGG ACT CGA CTT GCC CTC TCT CAG	ML-PCR, B4
B4.1-FAM	fam - TGG ACT CGA CTT GCC CTC TCT CAG	ML-PCR, B4
B4.2	TGC GAG CAG CGT TTG CGT GTG CGT	ML-PCR, B4
B5.1-TET	tet - CAA CTC CAA CAA ACT CAA CCA GCG	ML-PCR, B5
B5.2	GGA CTC GTG CAA AGC TTC GGA TGG	ML-PCR, B5
B6.1	GGC ATG GTA TGG GCA ATG AGT GTC	ML-PCR, B6
B6.1-FAM	fam - GGC ATG GTA TGG GCA ATG AGT GTC	ML-PCR, B6
B6.2	CAT CAT CGA TGT TTT GTA CTG TGG	ML-PCR, B6
B7.1	AAG TTG AAT TAA CTA GGG GCT AGG	ML-PCR, B7
B7.2	TTA ATT CGC TAC CCT CTC TTT TGC	ML-PCR, B7

Table 2.2. ***continued***

Name	Sequence (5' – 3')	Application *
B8.1	CTA ACA CGC TCA CGG GAG ATT GCC	ML-PCR, B8
B8.2	GGC GAG GTG CAA CCC TGT TAA TGG	ML-PCR, B8
B9.1	AAT CGT TGT GCG AGC CAT TCT GGC	ML-PCR, B9
B9.1-TET	tet - AAT CGT TGT GCG AGC CAT TCT GGC	ML-PCR, B9
B9.2	TCC ATC TCC GCA ATC TGC ATG TCC	ML-PCR, B9
B9.4	GCC CCG TCA TGC ATT ATC TCC TTG	ML-PCR, B9
B10.1	CGC TCA GGG CTA CAT ACA CCA TGG	ML-PCR, B10
B10.1-TET	tet - CGC TCA GGG CTA CAT ACA CCA TGG	ML-PCR, B10
B10.2	CTC ATC GAG TAA CGC AGG CGA CG	ML-PCR, B10
B11.1-HEX	tet - CAT GGA TGG ACA AGA GAT TGC ACG	ML-PCR, B11
B11.2	CTG CTA CAA TTC TGT CAA GCT TCG	ML-PCR, B11
B11.4	TTC ACT GCT ACA ATT CTG TCC AGC	ML-PCR, B11
GTO2	TGG TGG GTC C	RAPD-PCR
RCO5	AGG AGA TAC C	RAPD-PCR
RCO8	GGA TGT CGA A	RAPD-PCR
RCO9	GAT AAC GCA C	RAPD-PCR
ITS4	TCC TCC GCT TAT TGA TAT GC	rDNA-ITS amplification
ITS5	GGA AGT AAA AGT CGT AAC AAG G	rDNA-ITS amplification
M13 fwd	GCC AGG GTT TTC CCA GTC ACG A	sequencing
M13 rev	GAG CGG ATA ACA ATT TCA CAC AGG	sequencing
T1.1	GAG AAA ATG CGT GAG ATT GT	*tub2* gene amplification
T1.2	TGG TCA ACC AGC TCA GCA CC	*tub2* gene amplification
T1.1-BamH1	CGG GAT CCG AGA AAA TGC GTG AGA TTG T	T1.1 primer with BamHI site at 5' end for cloning
T1.2-EcoR1	CGG AAT TCT GGT CAA CCA GCT CAG CAC C	T1.2 primer with EcoRI site at 5' end for cloning

* MP-PCR is micro/minisatellite primed PCR, ML-PCR is microsatellite locus PCR, and RAPD-PCR is randomly amplified polymorphic DNA PCR.

2.13.2 OPTIMISATION OF PCR REACTION CONDITIONS

In attempts to optimise PCR reaction conditions, annealing temperatures were varied between 50°C and 65°C, the Mg^{2+} concentration was varied between 1 and 5 mM, the polymerase concentration was varied between 5 and 80 mU/µl, and hotstart PCR reactions with AmpliWax beads (Perkin-Elmer Corp.) was tried. Products were evaluated on agarose mini gels (Section 2.8.2).

2.13.3 RANDOMLY AMPLIFIED POLYMORPHIC DNA PCR (RAPD-PCR)

The amplification conditions for RAPD-PCR reactions were based on those of Williams et al. (1990) using modifications as described by Crowhurst et al. (1991). Reactions contained 1 × *Taq* polymerase buffer, 50 µM of each dNTP, 0.04 U/µl of *Taq* polymerase, 200 nM of primer (GTO2, RCO5, RCO8, or RCO9; Table 2.2) and 40 pg/µl of fungal genomic DNA. Reactions were amplified using the following programme: 95°C for 3 min, then 40 cycles of 95°C for 1 min, 35°C for 1 min, and 72°C for 3 min.

2.13.4 MICRO/MINISATELLITE-PRIMED PCR (MP-PCR)

Primers of microsatellite sequence, or the M13 core minisatellite sequence (primer A3) were used to amplify sections of the genome that were flanked by the inverted primer sequences, producing characteristic fingerprint patterns. This method was based on the technique described by Meyer et al. (1993). MP-PCR reactions contained 1 × *Taq* polymerase buffer, 50 µM of each dNTP, 0.04 U/µl of *Taq* polymerase, 200 nM of primer (A1, A2, or A3; Table 2.2) and 40 pg/µl of fungal genomic DNA, and reactions were amplified using the following programme: 40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 10 min.

2.13.5 MICROSATELLITE LOCUS PCR (ML-PCR)

Microsatellite loci were amplified using primers designed to the unique sequences flanking the tandem repeat region. ML-PCR reactions contained 1 × *Taq* polymerase buffer, 50 μM of each dNTP, 0.04 U/μl of *Taq* polymerase, 200 nM of each microsatellite locus PCR primer (Table 2.2) and 40 pg/μl of fungal genomic DNA, and were amplified using the following programme: 30 cycles of 94°C for 1 min, 65°C for 2 min, and 72°C for 1 min, followed by a final extension at 72°C for 10 min. If products were to be radio-labelled for separation on PAGE gels (Section 2.9), [α–^{33}P] dCTP was added to the reaction mixture to a final concentration of 0.1 μCi/μl. For automated analysis of microsatellite products (Section 2.17), primers that were 5'-end labelled with the phosphoramidite dyes 6-carboxyfluorescein (FAM), 4, 7, 2', 7'-tetrachloro-6-carboxyfluorescein (TET), or 4, 7, 2', 4', 5', 7'-hexachloro-6-carboxyfluorescein (HEX) were used in the amplification. Specifically, primers B1.1, B4.1, and B6.1 were labelled with FAM; B5.1, B9.1, and B10.1 were labelled with TET; and B2.1 and B11.1 were labelled with HEX.

2.13.6 MULTIPLEX PCR

Conditions for the multiplex PCR of microsatellite loci were the same as those used for a single locus amplification (Section 2.13.5), but with additional primers added at a final concentration of 200 nM each. Microsatellite loci were grouped such that the size ranges of alleles from loci labelled with the same dyes did not overlap, thus allowing automated analysis of the reaction in a single run (Section 2.16). Two sets of multiplex primers were established including Primer Set I, which contained primers B4.1-FAM, B4.2, B6.1-FAM, B6.2, B9.1-TET, B9.4, B10.1-TET, B10.2, B11.1-HEX, and B11.4, and Primer Set II, which contained primers B1.1-FAM, B1.2, B2.1-HEX, B2.2, B5.1-TET, B5.2, B6.1-FAM, B6.2, B9.1-TET, and B9.2. Where amplifications were poor, a problem particularly noticeable for in planta amplification, the five locus multiplex reactions were partitioned into separate reactions by either pooling loci of the same dye-label, or dividing the reactions into their single locus constituents.

2.13.7 IN PLANTA PCR

Amplification of fungal microsatellites from the total genomic DNA of endophyte infected grass tissue (Sections 2.5.3 and 2.5.4) was carried out using the standard ML-PCR reactions conditions (Section 2.13.5), except the DNA concentration was increased to 2 ng/µl.

2.13.8 ß–TUBULIN GENE AMPLIFICATION

The 5' portion of the ß–tubulin gene, including IVS1, IVS2, and IVS3, were PCR amplified using primers (T1.1 and T1.2; Table 2.2) complementary to conserved coding sequences as previously described (Byrd et al., 1990). Reactions contained the following: 1 × Taq polymerase buffer, 50 µM of each dNTP, 0.04 U/µl of Taq polymerase, 200 nM of each primer and 40 pg/µl of fungal genomic DNA. The thermocycler was programmed as follows: an initial denaturation at 94°C for 105 sec; 35 cycles of 94°C for 45 sec, 60°C for 45 sec, and 72°C for 60 sec; followed by a final extension at 72°C for 10 min.

To facilitate the cloning of *tub2* PCR products, primers were redesigned to include *Bam*HI and *Eco*RI restriction enzyme recognition sequences at the 5'-ends. These primers, T1.1-BamHI and T1.2-EcoRI (Table 2.2) were used with the same amplification conditions as above.

2.13.9 rDNA-ITS AMPLIFICATION

PCR amplification of the nuclear ribosomal DNA region spanning the internal transcribed spacers (rDNA-ITS), and the 5.8S rRNA gene was performed using primers ITS5 and ITS4 (Table 2.2), which are complementary to the 16S and 28S rRNA genes near the *ITS1* and *ITS2* borders respectively (White et al., 1990). Initial reactions were performed in 25 µl volumes containing 1 × *Taq* polymerase buffer, 50 µM of each dNTP, 0.04 U/µl of *Taq* polymerase, 200 nM of each primer and 5 µl of 200 pg/µl fungal

genomic DNA. The thermocycler was programmed for an initial denaturation at 95°C for 3 min, then 30 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec, followed by a final extension at 72°C for 10 min. Products were evaluated on agarose mini gels (Section 2.8.2) and if the yield was too low to sequence, a second round of amplification was performed using similar conditions to those previously described with the following modifications. For increased fidelity of DNA synthesis, *Pwo* DNA polymerase (Boehringer Mannheim GmbH) was used instead of *Taq*, and the buffer system changed accordingly. Initial amplification products were diluted 1/10 to 1/1000 for use as templates and the number of cycles in the thermocycling programme was reduced to twenty.

2.14 CLONING OF PCR PRODUCTS

2.14.1 pGEM-T CLONING

PCR amplification by *Taq* polymerase often results in the addition of single deoxyadenosine residues to the 3' ends of the amplicon, therefore the pGEM-T vector (Promega Corp.; Section 2.1.3), which contains 3' thymidine overhangs at the insertion site, was be used to clone such fragments. PCR products were purified by standard phenol/chloroform and ethanol precipitation procedures, or the PCR clean up kit (Section 2.5.9). Ligation reactions were carried out in 10 µl volumes containing 25 ng of pGEM-T vector, 6 ng of PCR product, 1 × T4 DNA ligase buffer, 3 U of T4 DNA ligase, and MilliQ water, and were incubated and evaluated under the conditions described in Section 2.12.4. Recombinant products were transformed into *E. coli* XL-1 cells by electroporation (Section 2.12.6) and plated on to LB plates (Section 2.3.1) supplemented with ampicillin, IPTG, and X-gal (Section 2.3.6). White colonies were screened for unique clones as described in Section 2.12.9, though in this case *Sal*I was used to excise the insert fragments. Unique clones were fully sequenced using both M13 fwd and M13 rev primers that are complementary to sequences flanking the insertion site of the vector.

2.14.2 PREPARATION OF INSERTS FOR DIRECTIONAL CLONING

PCR *tub2* products amplified with T1.1-BamHI and T1.2-EcoRI (Section 2.13.8) were extracted with an equal volume of phenol, followed by an equal volume of chloroform to inactivate *Taq* polymerase, and digested with *Bam*HI and *Eco*RI in a 100 µl volume (Section 2.7). Products were then purified from the excised fragment ends using the PCR clean up kit (Section 2.5.9) and the concentrations were estimated using the mini gel method (Section 2.8.2).

2.14.3 DIRECTIONAL CLONING USING pUC118

The pUC118 vector (Section 2.1.3) was prepared for directional *Bam*HI and *Eco*RI cloning by restriction endonuclease digestion with these two enzymes (Section 2.7). The vector was purified on a SeaPlaque mini gel in TAE buffer (Section 2.8.2), extracted from agarose (Section 2.5.10), and resuspended at a final concentration of 10 ng/µl.

The prepared PCR insert fragments (Section 2.14.2) were ligated to the vector (Section 2.12.4), transformed into *E. coli* XL-1 cells by electroporation (Section 2.12.6) and plated on to LB plates (Section 2.3.1) supplemented with ampicillin, IPTG, and X-gal (Section 2.3.6). White colonies were screened for unique clones as described in Section 2.12.9, and by *Bam*HI/*Eco*RI double digests to confirm the presence of the insert. Unique clones were fully sequenced using both the T1.1-BamHI and T1.2-EcoRI primers.

2.15 DNA SEQUENCING

2.15.1 AMPLICYCLE SEQUENCING KIT

Purified DNA templates suitable for sequencing were sequenced using the AmpliCycle sequencing kit (Perkin-Elmer Corp.). A reaction was set up on ice containing 10 to 100 fmol of template DNA, 4 µl 10 × cycling mix, 20 pmol of sequencing primer, 1 µl of [α–^{33}P] dCTP (~2,500 Ci/mmol, Amersham), and MilliQ water to make a total volume of 30 µl. Six microlitre aliquots of this mixture was added to 2 µl each of the four dideoxy termination mixes in 0.2 ml microamp tubes and placed in a Corbett thermocycler set to 94°C. The following programme was used: 94°C for 60 sec, followed by 25 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 60 sec. Stop solution, 4 µl, was added to each reaction after thermocycling, and reactions were stored at –20°C. Reactions were separated on polyacrylamide gels as described in Section 2.9.

2.15.2 DYE TERMINATOR CYCLE SEQUENCING

DNA sequences to be analysed on an ABI Prism 377 DNA Sequencer or ABI Prism 310 Genetic Analyser (Section 2.17) were sequenced using the ABI Prism Dye, ABI Prism dRhodamine, or ABI Prism BigDye terminator cycle sequencing ready reaction kits (PE Applied Biosystems). Cycle sequencing reactions were set up on ice and contained: 8.0 µl of terminator ready reaction mix, 3.2 pmol of primer, 50 to 200 fmol of purified DNA template, and MilliQ water to a total volume of 20 µl. Reactions were placed in a thermocycler set to 96°C. The following programme was used: 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min for 25 cycles, with rapid thermal ramping between steps. Amplification products were precipitated by addition of 2.0 µl of 3 M sodium acetate (pH 4.6) and 50 µl of 95% ethanol, mixing, and chilling on ice for 10 min. Tubes were centrifuged for 30 min at 15,000 × *g* and the supernatant discarded. The pellet was rinsed in 70% ethanol, briefly vortexed, and centrifuged for 15 min at 15,000 × *g*. The supernatant was discarded and the pellet air dried at 37°C for 30 min. Samples to be run on an ABI Prism 377 DNA Sequencer were resuspended in 4 µl of formamide dye (83%

deionised formamide and 17% of 25 mM Na_2EDTA (pH 8.0) and 50 mg/ml of blue dextran) and stored at –20°C, and samples to be run on an ABI Prism 310 Genetic Analyser were resuspended in 12 to 25 µl of template suppression reagent (TSR; PE Applied Biosystems) and stored at 4°C until run (Section 2.17).

2.15.3 SEQUENCE ANALYSIS

DNA sequence data was analysed with the Wisconsin Package version 9.1, Genetics Computer Group (GCG), Madison, Wisc. The programs used were SEQED, GETSEQ, GAP, and PILEUP (Section 2.18.1). Basic local alignment search tool (BLAST) searches were performed using the Basic BLAST search option of BLAST 2.0 on the internet at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nih.gov). DNA sequences were deposited into GenBank using the BankIt option on the NCBI website.

2.16 MICROSATELLITE ANALYSIS

Microsatellite locus PCR products (Section 2.13.5) were analysed in several ways, depending on the requirement, and the stage of development of the microsatellite-based fingerprinting assay. To check the presence and concentration of PCR products, and to estimate the degree of polymorphism at a microsatellite locus, products were run on agarose mini gels (Section 2.8.2). High resolution separation of products was achieved by radio-labelling products (Section 2.13.5) followed by PAGE separation and autoradiography (Section 2.9). The control sequence from the AmpliCycle sequencing kit (Section 2.15.1) was loaded at regular intervals across the gel, and used as a DNA size marker. The sizes of microsatellite PCR products were precisely determined from the automated analysis of dye-labelled fragments (Section 2.13.5) using ABI Prism 377 or ABI Prism 310 analysers (PE Applied Biosystems; Section 2.17).

2.17 AUTOMATED GENETIC ANALYSIS

Automated analysis of dye-labelled sequencing reactions (Section 2.15.1) and dye-labelled microsatellite locus PCR products (Section 2.13.5) were performed on an ABI Prism 377 DNA Sequencer or an ABI Prism 310 Genetic Analyser (PE Applied Biosystems). The ABI Prism 377 is a slab gel based system where samples were separated on a 4.25% denaturing polyacrylamide gel. In contrast, the ABI Prism 310 is based on capillary electrophoresis in a performance optimised polymer (POP) matrix where samples were loaded by autoinjection and individually run.

Sequencing reactions prepared for analysis on an ABI Prism 377 were resuspended in formamide dye (Section 2.15.2), denatured at 95°C for 2 min, quenched on ice, and 0.75 to 1.5 µl were loaded onto the gel. Sequencing reactions to be analysed on an ABI Prism 310 were resuspended in TSR (Section 2.15.2), denatured at 95°C for 2 min, quenched on ice and autoinjected into a 61 cm capillary with POP-6 polymer (PE Applied Biosystems). Genetic analysers were run according to the manufacturers standard protocols and data was analysed using DNA Sequencing Analysis Software Version 3.3.

Dye-labelled microsatellite products (1.5 µl) to be analysed on an ABI Prism 377, were mixed with 2.5 µl of formamide, 0.5 µl of 5% blue dextran, and 0.5 µl of GS-500 TAMRA (Perkin-Elmer Corp.), an internal size standard, denatured at 95°C for 2 min, quenched on ice and 2 µl loaded onto the gel. If microsatellite products were to be analysed on an ABI Prism 310, 1 µl of diluted products was mixed with 12.5 µl of deionised formamide and 0.5 µl GS-500 TAMRA and samples were denatured for 5 min at 95°C, then quenched on ice. Samples were run through a 40 cm capillary with POP-4 polymer (PE Applied Biosystems). Genetic analysers were run according to the manufacturers standard protocols and data was analysed using GeneScan Analysis 2.1 software.

2.18 PHYLOGENETIC ANALYSIS

2.18.1 SEQUENCE ALIGNMENT

DNA sequences were aligned using the PILEUP program of the Wisconsin Package Version 9.1 (Section 2.15.3) and parameters were empirically adjusted to a gap penalty of one and gap extension penalty of zero, as described by Schardl and Leuchtmann (1999). Alignments were checked by eye for ambiguities and adjusted if necessary.

2.18.2 PHYLOGENETIC ANALYSIS BY MAXIMUM PARSIMONY

Maximum parsimony (MP) trees were inferred from aligned sequences (Section 2.18.1) using a branch-and-bound search algorithm for unordered and unweighted characters as implemented in PAUP* Version 4.64 (Swofford, 1998). If the computing time of the branch-and-bound search exceeded 48 hours, the heuristic search algorithm was used instead. Alignment gaps were treated as missing information, and all missing and unambiguous characters were excluded from analysis. Tree roots were inferred by mid-point rooting. One hundred bootstrap trees were generated under the maximum parsimony criteria, using the heuristic search option. Bootstrap values for the majority rule consensus tree have been shown.

2.18.3 PHYLOGENETIC ANALYSIS BY NEIGHBOR-JOINING

Neighbor-joining (NJ) trees were generated using the PAUP* Version 4.64 software package. Alignment gaps were treated as missing information, and all missing and unambiguous characters were excluded from analysis. Since the calculation of genetic distances between sequences should only be made on sites in the data that are free to vary, an estimate of the proportion of unvaried character sites in the data was made using the maximum likelihood approach (Lockhart et al., 1996). Trees were generated using both Jukes-Cantor (one-parameter) and Kimura two-parameter distance matrices, with the estimated proportion of unvaried sites removed in proportion to the empirical base

frequencies estimated from the constant sites. Bootstrap consensus trees of 100 replicates were performed using the NJ option.

CHAPTER THREE

DEVELOPMENT AND APPLICATION OF AN IN PLANTA EPICHLOË ENDOPHYTE FINGERPRINTING ASSAY

3.1 INTRODUCTION

As a group, the Epichloë endophytes show considerable variation in morphology and physiology in culture (Christensen and Latch, 1991; Christensen et al., 1991; Christensen et al., 1993). While these properties are useful in the identification of endophytes, used alone they are of limited value in defining distinct taxonomic groupings. However, when used in combination with molecular methods such as isozyme (Leuchtmann and Clay, 1990) and gene sequence (Schardl et al., 1991) analysis, distinct taxonomic groupings can be defined and the phylogeny established (Christensen et al., 1993; Schardl et al., 1994; Tsai et al., 1994). Both isozyme and DNA sequence analysis are lengthy processes for routine strain identification and require that the endophyte be first isolated from the grass host, a process which can take up to several weeks.

Several methods are available for the in planta detection of endophytes. These include histological staining (Latch et al., 1984), enzyme-linked immunosorbent assay (ELISA) (Reddick and Collins, 1988), and tissue print-immunoblot (Gwinn et al., 1991). More recently, PCR based methods, including both RAPD and microsatellite locus analysis, have been used for endophyte detection and quantification both in culture (Groppe et al., 1995) and in planta (Doss and Welty, 1995; Groppe and Boller, 1997). PCR methods provide fast, sensitive and specific amplification of target DNA sequences in complex DNA samples, and thus are particularly amenable to endophyte detection in the plant background.

The objective of this study was to develop a PCR-based fingerprinting assay for the rapid and accurate identification of Epichloë endophytes in planta. Initially a pilot study was carried out to evaluate several PCR-based techniques for their ability to distinguish between endophyte isolates, and for their ease of use. The most promising technique was further developed into a working assay and used in various applications to identify endophyte isolates.

3.2 RESULTS

3.2.1 PILOT STUDY TO EVALUATE PCR-BASED FINGERPRINTING METHODS

A pilot study was conducted to evaluate three PCR-based fingerprinting methods, RAPD-PCR (Section 2.13.3), MP-PCR (Section 2.13.4) and ML-PCR (Section 2.13.5), for their ease of use and ability to discriminate between isolates. A panel of twelve Epichloë endophyte isolates (Section 2.1.1), representing LpTG-2 (Lp1), *Neotyphodium lolii* (Lp5, Lp7, Lp13, and Lp19), FaTG-2 (Tf13), FaTG-3 (Tf18), *N. coenophialum* (Tf27 and Tf28), *N. uncinatum* (Fp2), *Epichloë typhina* (E8), and *E. festucae* (Fr1), were typed using each of the fingerprinting methods and comparisons of the methods were made.

3.2.1.1 RAPD-PCR

DNA samples from the panel of twelve Epichloë endophyte isolates (Section 3.2.1) were amplified by RAPD-PCR (Section 2.13.3) using primers RCO5, RCO8, and RCO9 (Table 2.2) and separated on 1.75% agarose gels. These results are shown in Fig. 3.1. Overall, the fingerprints had very few diagnostic bands in order to identify endophyte isolates, even at the species or taxonomic group level. The non-amplification of samples, such as in lanes 2, 3, and 11 of Fig. 3.1B, was frequently observed and was inconsistent between repeated runs. Similarly, fingerprint patterns were not consistently reproducible between runs, and contamination of the PCR negative control was occasionally experienced.

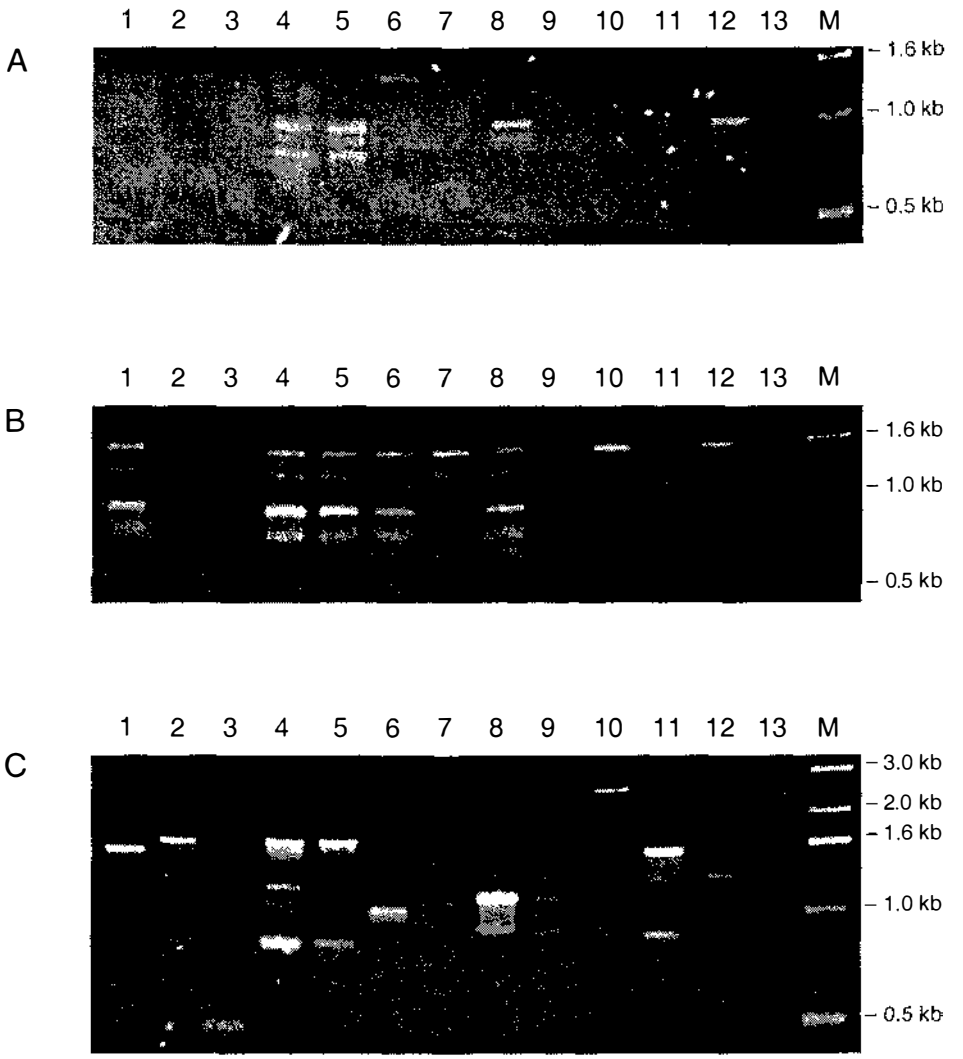


Fig. 3.1. RAPD-PCR profiles from a range of Epichloë endophyte isolates

RAPD profiles following amplification with primers RCO5 (panel A), RCO8 (panel B), and RCO9 (panel C), and separated on 1.75% agarose gels. Reactions contained genomic DNA from isolates Lp1 (lane 1), Lp5 (lane 2), Lp7 (lane 3), Lp13 (lane 4), Lp19 (lane 5), Tf13 (lane 6), Tf18 (lane 7), Tf27 (lane 8), Tf28 (lane 9), Fp2 (lane 10), E8 (lane 11), and Fr1 (lane 12). Lane 13 is a PCR negative control without DNA and lane M contains 1 kb ladder.

3.2.1.2 MP-PCR

Fingerprint patterns from the panel of twelve endophyte isolates (Section 3.2.1), generated by MP-PCR (Section 2.13.4) using primers A1, A2, and A3 (Table 2.2), are shown in Fig. 3.2. Fingerprints amplified using primer A1 (Fig. 3.2A) generated patterns of 2 to 9 bands between 0.5 and 1.5 kb in size. High background smearing, and the tight clustering of bands made these fingerprints difficult to analyse. In contrast, amplification using primers A2 and A3 produced clear banding patterns of 7 to 20 bands in the size range 0.2 to 3.5 kb. Species and taxonomic groups of Epichloë endophytes were able to be clearly distinguished, and intraspecific differences were detectable within *N. lolii* and *N. coenophialum*. As MP-PCR fingerprinting with the A2 and A3 primers looked promising for strain differentiation, a more extensive range of endophyte isolates were fingerprinted using the A2 (Fig. 3.3) and the A3 (Fig. 3.4) primers. Common banding patterns were clearly identified for each endophyte species, or taxonomic group, and minor intraspecific differences were detected as observed previously. The reproducibility of fingerprints generated using this method was good.

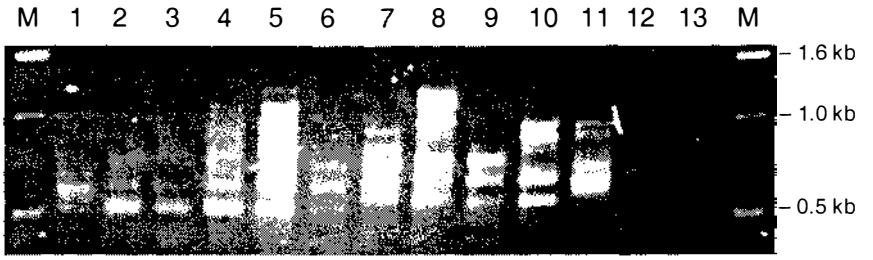
3.2.1.3 ML-PCR

Primers B1.1 and B1.2 (Table 2.2), which amplify an $(AAG)_n$ microsatellite (Groppe et al., 1995), were used in ML-PCR amplification (Section 2.13.5) of DNA samples from the panel of twelve endophyte isolates (Section 3.2.1). Amplification products, separated on a 3% NuSieve agarose gel (Fig 3.5A), were obtained for all isolates except Tf18 and E8, and were of the expected fragment size of around 300 bp (Groppe et al., 1995). As the variation in fragment length was expected to be as little as 3 bp, products were radiolabelled with [α-^{33}P] dCTP (Section 2.13.15) and separated on a denaturing polyacrylamide gel followed by autoradiography (Section 2.9; Fig. 3.5B). Alleles were detected from all samples, and multiple bands were detected from Lp1, Tf13, Tf27, Tf28, and Fp2, which are endophytes known to have interspecific hybrid origins. Each allele occurred as a doublet of two bands, and this was presumed to be each labelled strand of the product which had separated under the denaturing conditions.

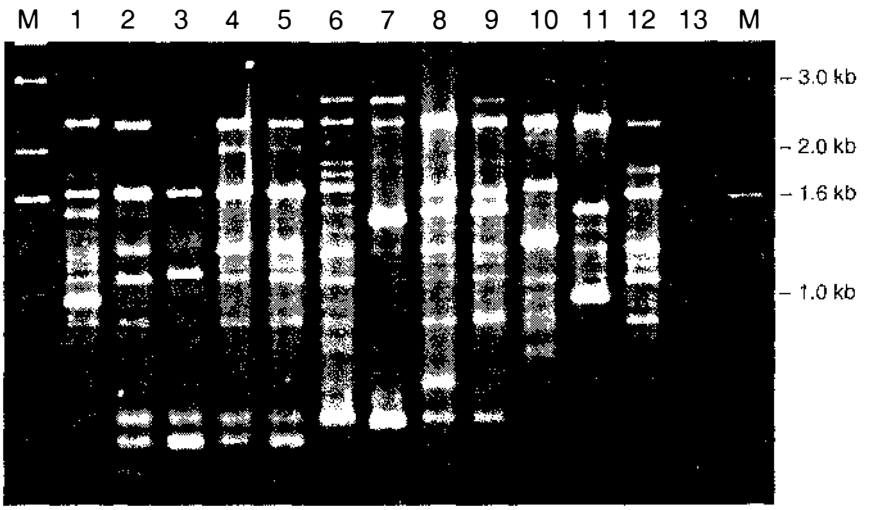
Fig. 3.2. MP-PCR profiles from a range of Epichloë endophyte isolates

MP-PCR profiles following amplification with primers A1 (panel A), A2 (panel B), and A3 (panel C), and separated on 1.75% agarose gels. Reactions contained genomic DNA from isolates Lp1 (lane 1), Lp5 (lane 2), Lp7 (lane 3), Lp13 (lane 4), Lp19 (lane 5), Tf13 (lane 6), Tf18 (lane 7), Tf27 (lane 8), Tf28 (lane 9), Fp2 (lane 10), E8 (lane 11), and Fr1 (lane 12). Lane 13 is a PCR negative control without DNA and lane M contains 1 kb ladder.

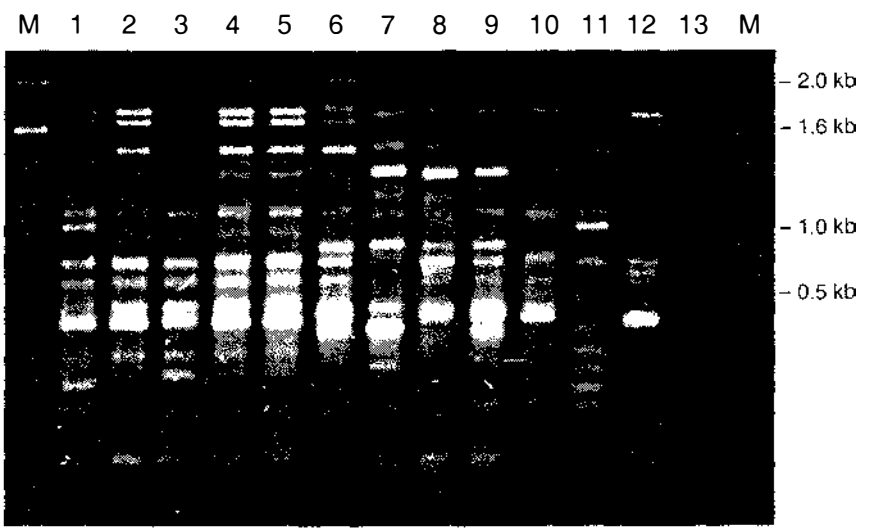
A


M 1 2 3 4 5 6 7 8 9 10 11 12 13 M
1.6 kb
1.0 kb
0.5 kb

B


M 1 2 3 4 5 6 7 8 9 10 11 12 13 M
3.0 kb
2.0 kb
1.6 kb
1.0 kb

C


M 1 2 3 4 5 6 7 8 9 10 11 12 13 M
2.0 kb
1.6 kb
1.0 kb
0.5 kb

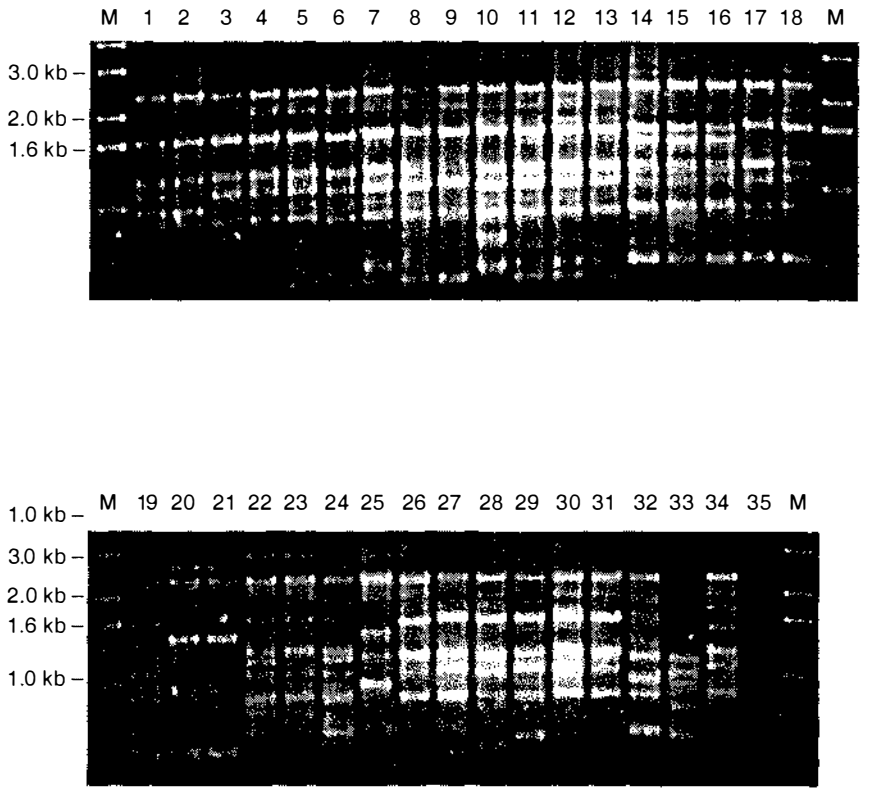


Fig. 3.3. MP-PCR profiles of isolates using the A2 primer

Lanes (bold) contain PCR reactions with genomic DNA from isolates: **1**, Lp1; **2**, Lp2; **3**, Lp5; **4**, Lp6; **5**, Lp13; **6**, Lp19; **7**, Lp20; **8**, Lp21; **9**, Lp7; **10**, Lp14; **11**, AR1; **12**, AR1WF; **13**, AR1CY; **14**, Tf5; **15**, Tf27; **16**, Tf28; **17**, Tf13; **18**, Tf15; **19**, Tf20; **20**, Tf16; **21**, Tf18; **22**, Fp1; **23**, Fp2; **24**, Fp4; **25**, E8; **26**, Fr1; **27**, Frc5; **28**, Frc7; **29**, Frr1; **30**, Fg1; **31**, Fl1; **32**, HaB; **33**, Hd1; and **34**, EndoSafe. Lane **35** contains PCR negative control, and **M** contains 1 kb ladder. MP-PCR profiles were resolved on 1.25% agarose gels.

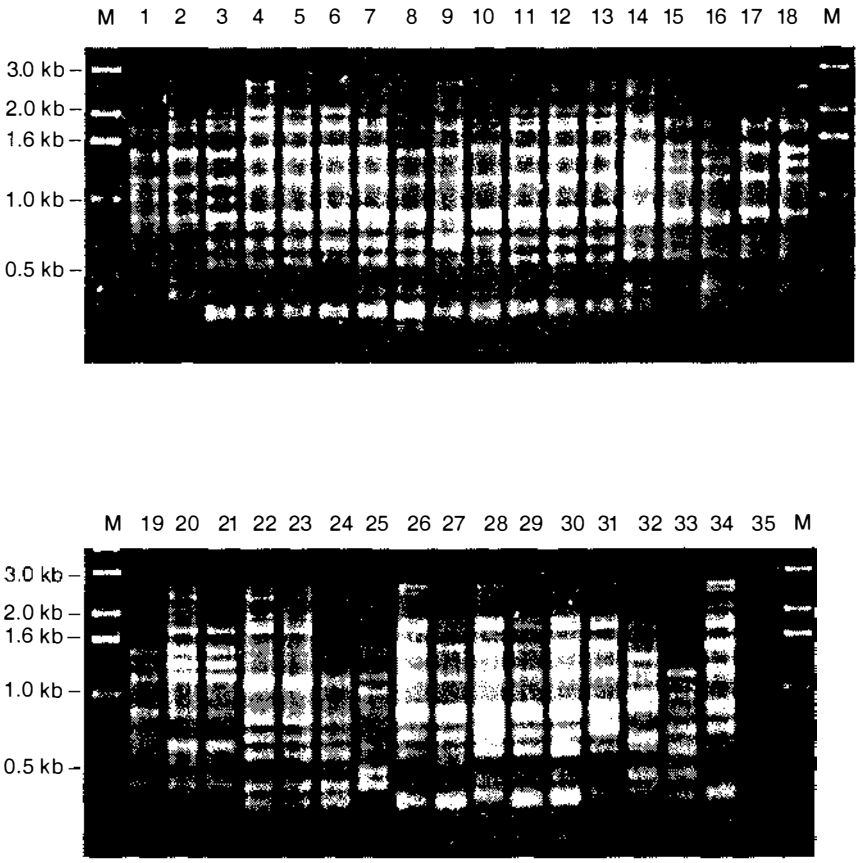
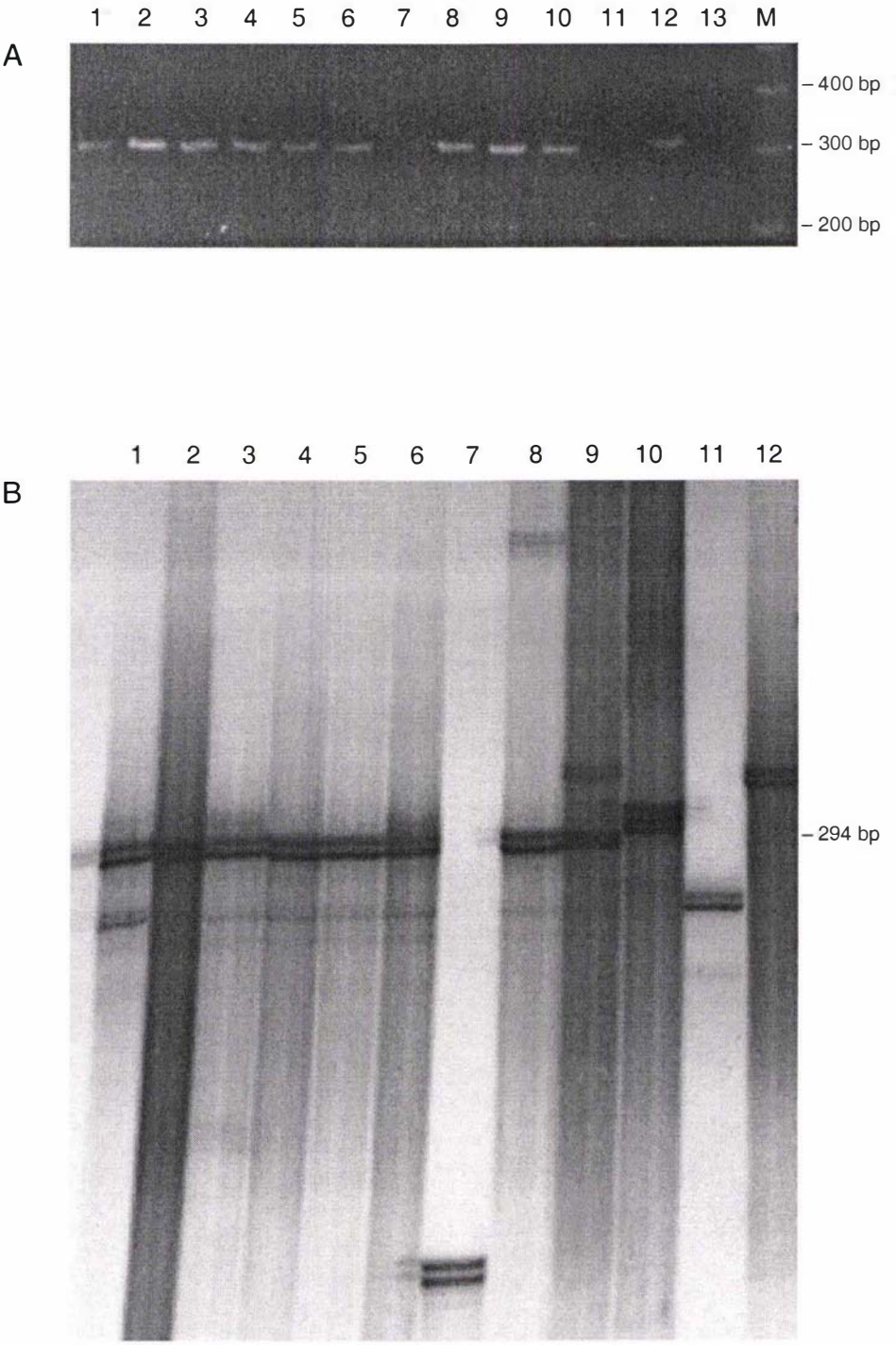


Fig. 3.4. MP-PCR profiles of isolates using the A3 primer

Lanes (bold) contain PCR reactions with genomic DNA from isolates: **1**, Lp1; **2**, Lp2; **3**, Lp5; **4**, Lp6; **5**, Lp13; **6**, Lp19; **7**, Lp20; **8**, Lp21; **9**, Lp7; **10**, Lp14; **11**, AR1; **12**, AR1WF; **13**, AR1CY; **14**, Tf5; **15**, Tf27; **16**, Tf28; **17**, Tf13; **18**, Tf15; **19**, Tf20; **20**, Tf16; **21**, Tf18; **22**, Fp1; **23**, Fp2; **24**, Fp4; **25**, E8; **26**, Fr1; **27**, Frc5; **28**, Frc7; **29**, Frr1; **30**, Fg1; **31**, Fl1; **32**, HaB; **33**, Hd1; and **34**, EndoSafe. Lane **35** is a PCR negative control without DNA and **M** contains 1 kb ladder. MP-PCR profiles were resolved on 1.25% agarose gels.

Fig. 3.5. ML-PCR amplification of a range of Epichloë endophyte isolates

Products were amplified with primers B1.1 and B1.2, and resolved on a 3% NuSieve agarose gel (panel A), and a denaturing 6% polyacrylamide gel (panel B). Reactions contained genomic DNA from isolates Lp1 (lane 1), Lp5 (lane 2), Lp7 (lane 3), Lp13 (lane 4), Lp19 (lane 5), Tf13 (lane 6), Tf18 (lane 7), Tf27 (lane 8), Tf28 (lane 9), Fp2 (lane 10), E8 (lane 11), and Fr1 (lane 12). On panel A, lane 13 is a PCR negative control without DNA and lane M contains 100 bp ladder.


A
1 2 3 4 5 6 7 8 9 10 11 12 13 M
400 bp
300 bp
200 bp
B
1 2 3 4 5 6 7 8 9 10 11 12
294 bp

3.2.1.4 Evaluation of Fingerprinting Methods

On the basis of the above results, ML-PCR was chosen over RAPD and MP-PCR for further development of an endophyte identification assay. This is further discussed in Section 3.3.1.

3.2.2 DEVELOPMENT OF A PCR-BASED MICROSATELLITE FINGERPRINTING ASSAY

3.2.2.1 Detection of Microsatellites within Epichloë Endophyte Genomes

To determine the presence and relative abundance of microsatellites in the genomes of endophyte isolates E8 and Lp1 (Table 2.1), genomic DNA was digested to completion with combinations of the 4 bp blunt-end cutting restriction enzymes *Alu*I, *Hae*III, and *Tha*I, as well as *Bam*HI (Section 2.7), and Southern blots (Section 2.10) of these digests were probed for the presence of microsatellite sequences (Section 2.11). These digests resulted in large populations of small DNA fragments of a size suitable for the construction of small insert libraries (Fig. 3.6A). Hybridisation of Lp1 digests with DIG-labelled microsatellite probes, A6 to A10 (Table 2.2), under conditions of low stringency (Section 2.11) revealed up to 25 bands for dinucleotide repeat probes (Fig. 3.6B), and up to 20 bands for the trinucleotide repeat probes (Fig. 3.6C). This represents an average frequency of one $(CA)_n$ or $(GA)_n$ microsatellite per 2 Mb of DNA, or one $(CAA)_n$, $(GAA)_n$, or $(ATG)_n$ microsatellite per 2.5 Mb of DNA, assuming a genome size of 50 Mb for Lp1 (Murray et al., 1992). Genomic digests of strain E8 were also screened with di- and trinucleotide repeat probes and similar results were obtained (results not shown).

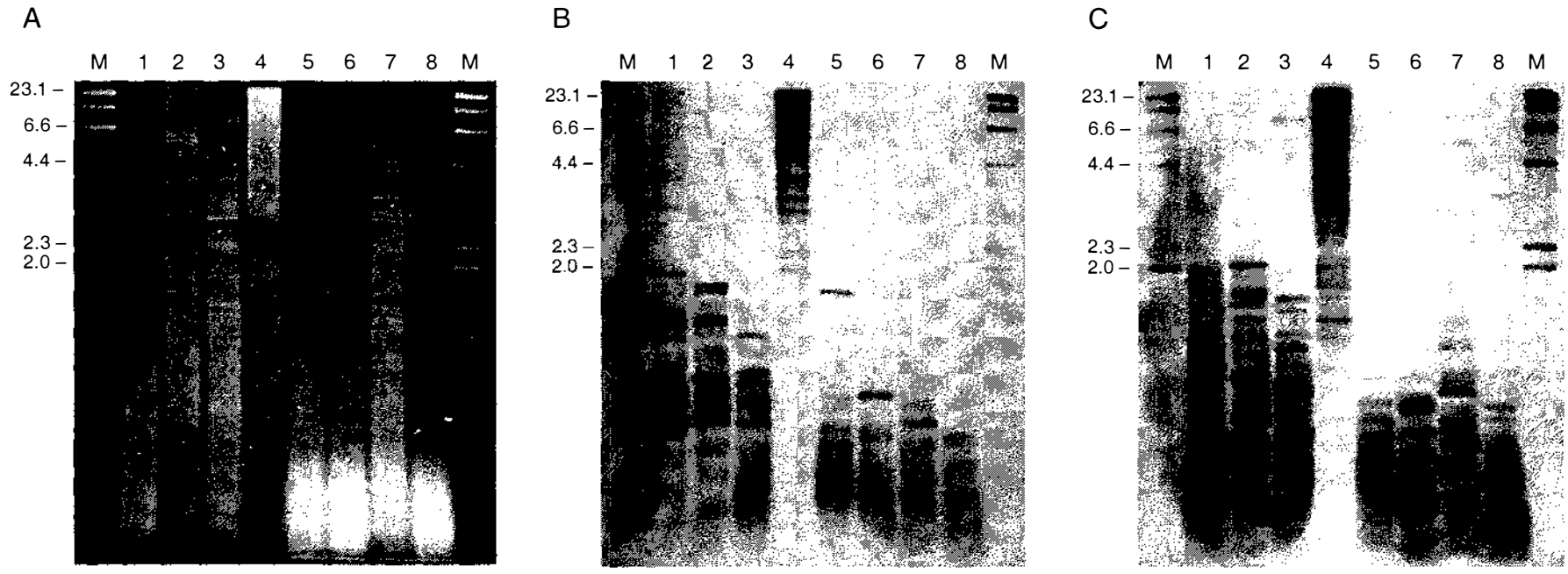


Fig. 3.6. Hybridisation of microsatellite probes to the Lp1 genome

(A) Agarose (1.25%) gel of Lp1 genomic DNA (1 µg/lane) digested with *Alu*I (lane 1); *Tha*I (lane 2); *Hae*III (lane 3); *Bam*HI (lane 4); *Alu*I, *Tha*I, and *Bam*HI (lane 5); *Alu*I, *Hae*III, and *Bam*HI (lane 6); *Tha*I, *Hae*III, and *Bam*HI (lane 7); and *Alu*I, *Tha*I, *Hae*III, and *Bam*HI (lane 8). Lane M contains *Hin*dIII digested lambda DNA, and band sizes are given in kb. (B) and (C) show autoradiographs of a Southern blot of the gel of panel A that has been probed at low stringency with pools of DIG-labeled oligonucleotides $(CA)_{15}$ and $(GA)_{15}$ (panel B), and $(CAA)_{10}$, $(GAA)_{10}$, and $(ATC)_{10}$ (panel C), followed by chemiluminescent detection.

3.2.2.2 Library Construction and Screening

Partial genomic libraries were constructed in the vector M13mp19 using size-fractionated DNA inserts from strains E8 and Lp1. Genomic DNA was digested to completion with the same combinations of 4-bp cutting restriction enzymes and *Bam*HI as described previously (Section 3.2.2.1), and fragments were separated on 1.25% SeaPlaque agarose gels in TAE buffer (Section 2.8). Fragments of 200 to 500 bp from E8, and 100 to 1000 bp from Lp1, were excised and purified from the agarose (Section 2.5.10). Insert DNA was prepared using *Bam*HI linkers (Section 2.12.3), and inserts were ligated to CAP-treated *Bam*HI digested M13mp19 (Section 2.12.1). Libraries were transformed into *E. coli* strain XL-1 Blue (Section 2.12.6), titred, then plated at a density of approximately 10,000 white plaques per plate.

3.2.2.2.1 E8 Library

Approximately 110,000 plaques of the E8 library were screened by plaque hybridisation with a mixture of DIG-labelled dinucleotide probes, at a low stringency. Under these conditions, 1% of the plaques hybridised and 51 of these were taken through to the second round of screening. When the same filters were screened with a mixture of DIG-labelled trinucleotide probes under conditions of high stringency, 0.1% of the plaques hybridised and 30 of these were taken through a second round of screening. Second round screening confirmed 35 plaques identified with the dinucleotide probe mixture as positive, and 22 with the trinucleotide probe mixture.

3.2.2.2.2 Lp1 Library

Screening of 120,000 plaques of the Lp1 library was carried out under conditions of low stringency for both pools of di- and tri- nucleotide probes. Using these conditions 0.4% of the plaques hybridised with each set of probes, and 24 and 12 plaques respectively were taken through to second round of screening. Second round screening confirmed 22

plaques identified with the dinucleotide probe mixture as positive, and nine with the trinucleotide probe mixture.

3.2.2.3 Analysis of Positive Clones

The second round positive plaques were selected and amplified in *E. coli* and both single and double-stranded M13 DNA was isolated from each clone (Section 2.5). The double-stranded DNA was digested with *Bam*HI and separated by agarose gel electrophoresis to determine the insert size. Many of the inserts appeared to be the same size, and therefore were possibly clones of one another. This was confirmed by single dideoxy/deoxynucleotide sequencing reactions of single-stranded DNA templates (Section 2.12.9). Unique clones identified by this method were then completely sequenced using the M13 forward primer (Section 2.15). Ten different microsatellite types were identified; six from E8 (B2 to B6, and B12) and four from Lp1 (B7 to B10). The microsatellite sequences identified are listed in Fig. 3.7, and with the exception of B12, have been deposited into GenBank with accession numbers AF063085 to AF063094. Of these microsatellites, five (B2, B3, B5, B8, and B10) contained perfect repeats, and the remaining loci (B4, B6, B7, B9, and B12) contained imperfect repeats. Additional single nucleotide microsatellite loci were identified in clones B3, B8, and B9. For B8, the single nucleotide locus was immediately adjacent to the dinucleotide repeat locus as a compound microsatellite. For B5 and B9 the repeat motifs identified were only partially complementary to the probes used (tri- and di- nucleotide mixtures respectively), suggesting that they were identified as positives because of the low stringency hybridisation conditions employed in the screening process.

Fig. 3.7. DNA sequences of the microsatellite loci used in this study

The DNA sequences of the microsatellite loci used in this study are given below with microsatellite sequences highlighted in green, and primer binding sites indicated in red. For loci which were isolated by library screening, sequence data from the entire insert, excluding linkers, is given.

Microsatellite locus B1 (370 bp) isolated from *Epichloë bromicola* by Groppe et al. (1995). GenBank accession number X91791.

```
1       AATGCCGCAGCACTTTCCCAAGCCCGCACAATACGTCAGCTAGGAATGTTGCATACTTGA
                                  B1.1 =>
61      TTTCCATCCATCTGGCTCTAGACCTGATGACAGATTCCATGGTCGAACAAACGCGATCGC

121     CCCAAGCTCGTTTTATGTCGACGAAAAGAAGAAAAGAAACTATGCAACGTCTGAAGAGTT

181     AAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAAGA

241     GACGGCCCAACATCAAGATTCATTCGTCAACCTATAGCCCTGGTCCAGACCAAATGGGTT

301     CTCTCTCATATACCTACTAACATCCGCCTGATAGCAAAGTTGATTCAGGTAATCATGGAC
                                        <= B1.2
361     CTGCGGCATT
```

Microsatellite locus B2 clone (215 bp) isolated from E8 library with dinucleotide probe mixture. GenBank accession number AF063085.

```
1       TGGCAATAATACATAATGTAATCCGTAATGCTCTGCTTTGGCAGGATGCGAATCGTCTTG
                                  B2.1 =>
61      CCTGAAGCTGCCTTCTGCGGACCGTTGCTCCCTACATATTACGTGCACACACACACACAC

121     ACACACACACACACACACACACGTAATATGTCAGCATCCACCATCATCCACCTACTGTCA
                                                          <= B2.2
181     GCAGCCAGAAGATAAGATAGACGCTGCTCATTGCG
```

Microsatellite locus B3 clone (184 bp) isolated from E8 library with dinucleotide probe mixture. GenBank accession number AF063086.

```
1       CGGTTCGGTACGCCCCCCCCCCCCGGTCTTTTTCTGCCTCGCTTGCCAAACCCAAACATG

61      TTGAAAATATGCTGTCCGTTCTCGTGGCATTCGGTCGGGGGGTCTTTTTTCGAGAGAGAG
                          B3.1 =>

121     AGAGAGCCGGAACGAGCCGGGGATACACGATTCTACAAATCAAACTGCAGGTCCTTCTAC
                                                          <= B3.2

181     ACCG
```

Microsatellite locus B4 clone (127 bp) isolated from E8 library with dinucleotide probe mixture. GenBank accession number AF063087.

```
1       GTGGACTCGACTTGCCCTCTCTCAGCCTCAGCAATCATTGTTGCCATCATTCGAACTCAC
              B4.1 =>

61      AAAACGCACACAGCGCACACAACGCACACAACACACACAAACGCACACGCAAACGCTGC
                                                      <= B4.2

121     TCGCACG
```

Microsatellite locus B5 clone (221 bp) isolated from E8 library with trinucleotide probe mixture. GenBank accession number AF063088.

```
1       TTCCAACTCCAACAAACTCAACCAGCGCATCTCATCACATCTCATCACATCTCATCACAT
              B5.1 =>

61      CTCATCACATCTCATCACAATGCTTTCCATCCGAAGCTTTGCACGAGTCCGCACCCCGGG
                                              <= B5.2

121     CTGTATCTCGCTCCACCGCTTCAATGCGATCTAGCGTCGCCGCGCCGGCACCCTCATCAA

181     GACATCAGCCGTGAGCGCGCTGCGGCAATCCACCGGTGCAG
```

Microsatellite locus B6 clone (206 bp) isolated from E8 library with trinucleotide probe mixture. GenBank accession number AF063089.

```
1      GTGGAATATTCATCTGCATAATTTTTGGCATGGTATGGGCAATGAGTGTCTTTGAAATCT
                                                 B6.1 =>
61     AGACGGCGCTGGCTGCGACTGCCGGTGTTGACCTGGCAAAGTATGCAATGACAAACGCAA

121    GATCCACAGCAACCCATCACATCAACCACCATCTCTTCATCATCACCATCATCATCCACA

181    GTACAAAACATCGCAGCAGTAAGAGG
         <= B6.2
```

Microsatellite locus B7 clone (178 bp) isolated from Lp1 library with dinucleotide probe mixture. GenBank accession number AF063090.

```
1      CTATCGATCTGCATGATATTAATGATAATCTTGTAGTTAAATTCGTGGTTATCTTTGATT

61     ATAAACTTGAATTAACTAGGGGCTAGGAAAGAGAGAGAGAAGAGAGGGAAAGTCTAGATG
              B7.1 =>
121    CTAGGAAAGTAGGCGAAACCCGTATATAAGGCAAAAGAGAGGGTAGCGAATTAATATA
                                                 <= B7.2
```

Microsatellite locus B8 clone (235 bp) isolated from Lp1 library with dinucleotide probe mixture. GenBank accession number AF063091.

```
1      CTCTAACACGCTCAAGGGAGATTGCCTAAAGCATCCCATCCACAACGACACGGGCATTCA
              B8.1 =>
61     CACACACACACACACTTTTTTCATCGTCACTCCAACTCCCCGTCCACGCATCCTTCCCGC

121    CCATTAACAGGGTTGCACCTCGCCACCTTGCCCCCCCCCAGCTCAACGACGTACTTGCAC
         <= B8.2
181    CCCCCGTACCGCTCGCTCGCACCCTCGACAAAGAAGTCGCTCGACACAATCTGCG
```

Microsatellite locus B9 clone (501 bp) isolated from Lp1 library with dinucleotide probe mixture. GenBank accession number AF063092.

```
1      CTTCATGGAAGCAAAAAAATGATTTCCTAGAATCAGAGTGGCTAGCAGGGTTACACACAA

61     TCCACAATCGTTGTGCGAGCCATTCTGGCATTCCAAGTCTACCCGACAGACTGGGGGAGG
                  B9.1 =>

121    GGGGGGGTCAACAAGGGATGCATCTGTGACTCGCGTGCGTGAGAAGATTGATGCGAGAGG

181    AGAGCGAGGAGAGGACATGGACATGCAGATTGCGGAGATGGACGGGGTCAAGCTGATGTA
                                    <= B9.2

241    CTGTAACATGCATCACGCGAGCAACCACCCACGAGCTTTAACACGCTCAAGGAGATAATG
                                                          <= B9.4

301    CATGACGGGGCGCCGAAGCAGCGCCATTGCGGGAGTGAATTGTGGCTGGTTGGCTGCATT

361    GTGCGGCATTGTCAGCCTGGCTTCTGTGAGCAGACACGTCTGATGGTGTTGTAAACATTC

421    CCCAGACCATCATTTGTATTTATCATTTCATGCAACATGCAACATGCAACATGCAACATG

481    CAACAACCACCTACAAGTTGG
```

Microsatellite locus B10 clone (180 bp) isolated from Lp1 library with trinucleotide probe mixture. GenBank accession number AF063093.

```
1      CCGCTCAGGGCTACATACACCATGGTCAACATCACCAGCAACAGCCGCAGCAGCCGCAAC
             B10.1 =>

61     AGCAGCAGCCGCATCATCATCAACAACAACAACAACCACGACAGCGACCCCAAAACTGGA

121    ACATGTATCCTGGCATGTACTATGACAGTAACCAAAACGTCGCCTGCGTTACTCGATGAG
                                              <= B10.2
```

Microsatellite locus B11 (179 bp) from Lp19 HMG CoA 3' untranscribed sequence. GenBank accession number AF063094. The primer binding site for B11.4 is underlined.

```
1       TTCCATGGATGGACAAGAGATTGCACGAGTCAAGGCGCTGGTGTGAGGGACAGTTACAGA
                  B11.1 =>
61      TGGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGA

121     CAGACAGACAGACATGTACAGGAGATATCGAAGCTGGACAGAATTGTAGCAGTGAAACA
                                    <= B11.2      <= B11.4
```

Microsatellite locus B12 clone (171 bp) isolated from E8 library with dinucleotide probe mixture.

```
1       CCTGTGGCGGGATTTATTTCCTCTCGTCATCAGGTTGTTTGCTTTGTGCTTGGATTTCGG

61      CGGGCTTTGTTTGCTTTGCGAACCTGTAGAATGTTTGCTATCAGGTTGGGTAGTCGAGAA

121     TGAAGGGGCGGCGAGAACTGATGAGAGATGAGAGAGATGAGAGAGCGAACG
```

3.2.2.4 Identification of Additional Epichloë Endophyte Microsatellite Loci

Two additional endophyte microsatellite sequences have been used in this study and are included in Fig. 3.7. An $(AAG)_n$ microsatellite, referred to here as B1, was identified by Groppe et al. (1995) in an *Epichloë bromicola* isolate from *Bromus erectus.* A second locus, B11, was serendipitously identified 3' of the untranscribed region of the 3-hydroxyl-3-methylglutaryl Coenzyme A (HMG CoA) reductase gene from *N. lolii* strain Lp19 (Dobson, 1997).

3.2.2.5 ML-PCR Assay Design and Evaluation

3.2.2.5.1 Primer Design and Preliminary Amplifications

To establish PCR assays which amplify the microsatellite loci identified, primers were designed by eye to the sequences flanking each of the microsatellites (Fig. 3.7 and Table 2.2). A particular consideration in the design of the primers was for all to have a similar length and GC content to facilitate potential multiplexing of all loci. Primer sequences to the B1 locus were those used previously by Groppe et al. (1995). Unfortunately primers could not be designed to locus B12 as insufficient flanking sequence to one side of the microsatellite was isolated, therefore further analysis of this locus was abandoned. Initial evaluation of primer pairs (Bx.1/Bx.2 for each locus, Bx) was performed by screening a panel of twelve isolates, representative of seven taxonomic groups of endophytes, using a standard set of conditions (Figs. 3.8 and 3.9). All loci, apart from, B3, B7 and B11 gave specific products with high yield using these primers.

No products were amplified from the B3 locus using standard conditions (Fig. 3.8B), so the annealing temperature was reduced to 61°C, 58°C, and 55°C. This resulted in the amplification of both specific and nonspecific bands of increasing yield (results not shown). Despite attempts to further optimise the amplification conditions (Section 2.13.2), the nonspecific bands could not be eliminated. Given the difficulty of

Fig. 3.8. ML-PCR amplification with primers to microsatellite loci from the E8 library

Products were amplified from loci B2 (panel A), B3 (panel B), B4 (panel C), B5 (panel D), and B6 (panel E) and resolved on 3% NuSieve gels. PCR reactions contained genomic DNA from isolates: Lp1 (lane 1), Lp5 (lane 2), Lp7 (lane 3), Lp13 (lane 4), Lp19 (lane 5), Tf13 (lane 6), Tf18 (lane 7), Tf27 (lane 8), Tf28 (lane 9), Fp2 (lane 10), E8 (lane 11), and Fr1 (lane 12). Lane 13 is a PCR negative control without DNA and lane M contains 1 kb ladder (panels A, B, C, and E) or 50 bp ladder (panel D).

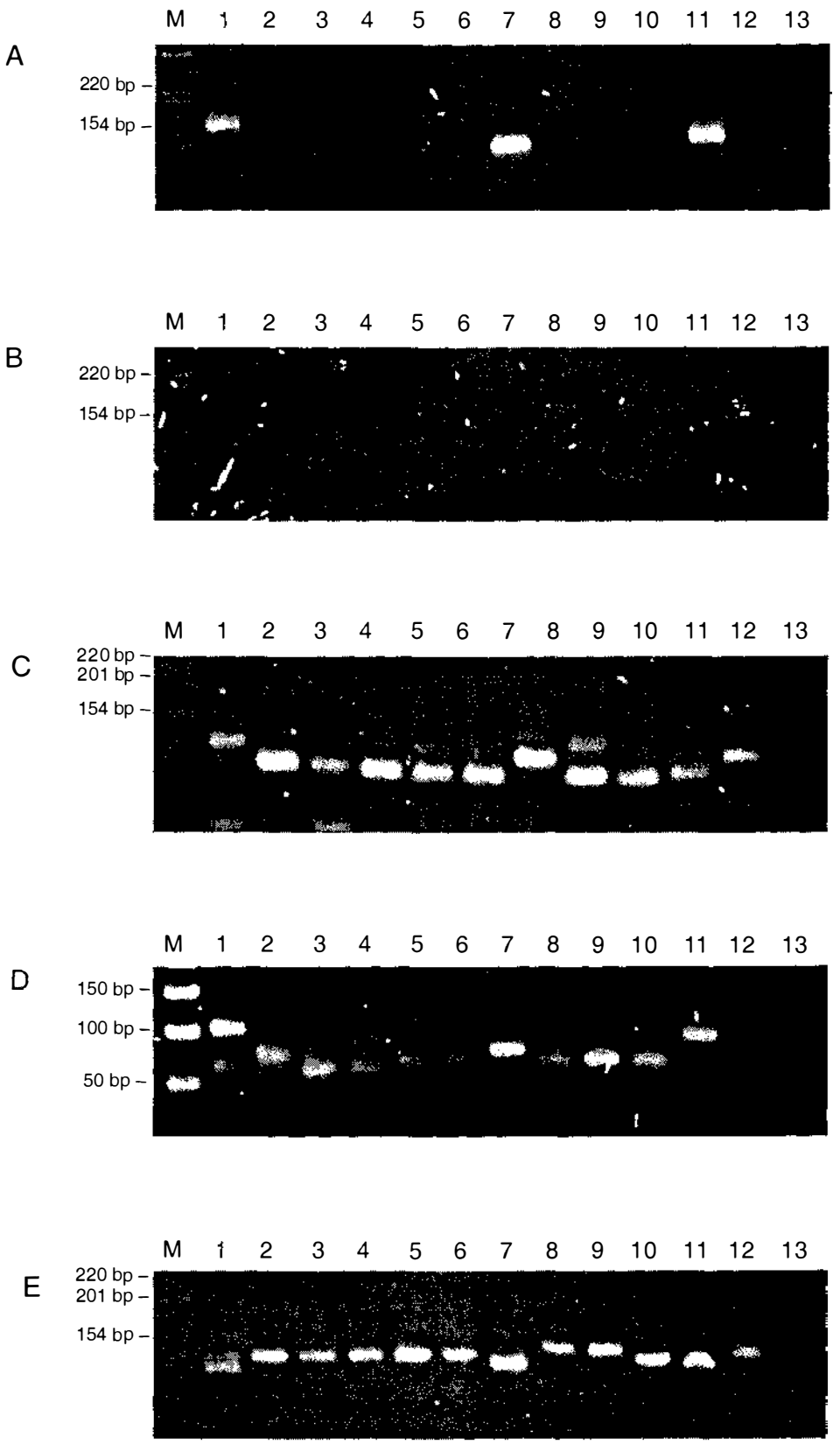
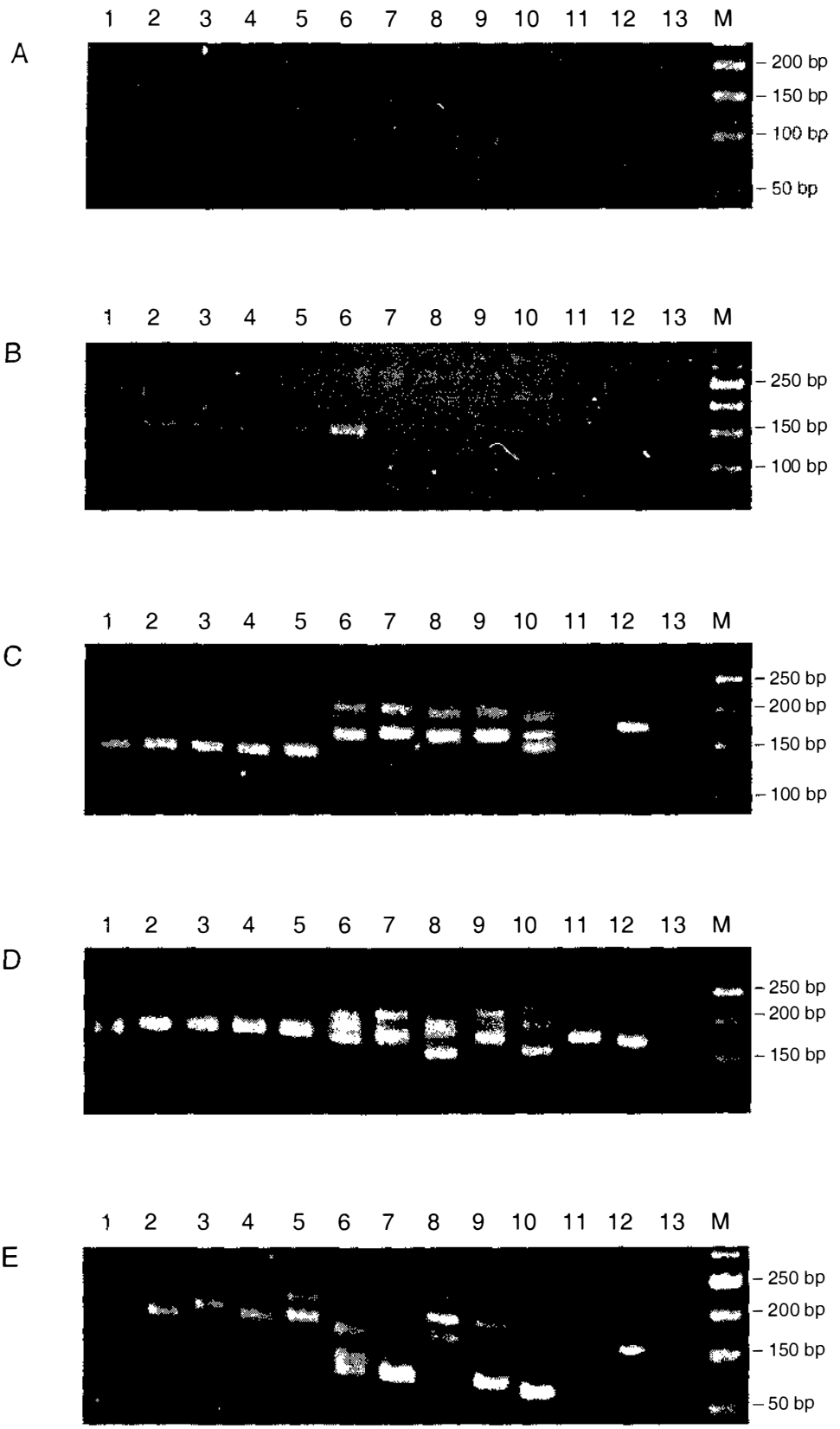

A
M 1 2 3 4 5 6 7 8 9 10 11 12 13
220 bp
154 bp
B
M 1 2 3 4 5 6 7 8 9 10 11 12 13
220 bp
154 bp
C
M 1 2 3 4 5 6 7 8 9 10 11 12 13
220 bp
201 bp
154 bp
D
M 1 2 3 4 5 6 7 8 9 10 11 12 13
150 bp
100 bp
50 bp
E
M 1 2 3 4 5 6 7 8 9 10 11 12 13
220 bp
201 bp
154 bp

Fig. 3.9. ML-PCR amplification with primers to microsatellite loci from the Lp1 library and HMG CoA reductase DNA sequence

Products were amplified from loci B7 (panel A), B8 (panel B), B9 (panel C), B10 (panel D), and B11 (using primers B11.1 and B11.4; panel E) and resolved on 3% NuSieve gels. PCR reactions contained genomic DNA from isolates: Lp1 (lane 1), Lp5 (lane 2), Lp7 (lane 3), Lp13 (lane 4), Lp19 (lane 5), Tf13 (lane 6), Tf18 (lane 7), Tf27 (lane 8), Tf28 (lane 9), Fp2 (lane 10), E8 (lane 11), and Fr1 (lane 12). Lane 13 is a PCR negative control without DNA and lane M contains 50 bp ladder.


A
1 2 3 4 5 6 7 8 9 10 11 12 13 M
200 bp
150 bp
100 bp
50 bp
B
1 2 3 4 5 6 7 8 9 10 11 12 13 M
250 bp
150 bp
100 bp
C
1 2 3 4 5 6 7 8 9 10 11 12 13 M
250 bp
200 bp
150 bp
100 bp
D
1 2 3 4 5 6 7 8 9 10 11 12 13 M
250 bp
200 bp
150 bp
E
1 2 3 4 5 6 7 8 9 10 11 12 13 M
250 bp
200 bp
150 bp
50 bp

redesigning primers to this locus because of the lack of available flanking sequences, further analysis of locus B3 was discontinued.

Amplification of locus B7 only yielded a faint product from Tf18 (Fig. 3.9A). Attempts to optimise the yield were not successful so any further work that was performed using this locus was under standard conditions.

Amplification of the B11 locus with primer pair B11.1 and B11.2 produced weak specific bands and high yields of primer dimers (results not shown). Further inspection of the primer sequences revealed that there was 3'-end complementarity. Consequently primer B11.4 was designed 4 bp downstream from the primer B11.2. The new primer combination of B11.1 coupled with B11.4 yielded specific products of good concentration under standard amplification conditions (Fig. 3.9E).

3.2.2.5.2 Amplification of Fungal Outgroups

To test the specificity of the ML-PCR assays for Epichloë endophytes, total DNA from two outgroups from the family Clavicipitaceae, including *Echinodothis tuberiformis* and *Claviceps purpurea* were also amplified under the standard conditions for each ML-PCR assay, but no products were obtained with any of these primer sets (results not shown).

3.2.2.5.3 In Planta Amplification

To determine the specificity of the microsatellite locus primers for detecting *Epichloë* endophyte DNA in planta, total DNA extracted from endophyte-infected grass tissue using the CTAB method (Section 2.5.4) was used as a template in PCR reactions. The results for AR1-infected samples using primers to locus B1 are shown in Fig. 3.10A. A product of approximately 298 bp was amplified from genomic DNA isolated from endophyte-infected plant material (lanes 2, 4, 6, and 8) but no product was detected in endophyte-free material (lanes 1, 3, 5, and 7). The DNA sequence of this product was identical to that from AR1 alone confirming the specificity of the assay for detecting

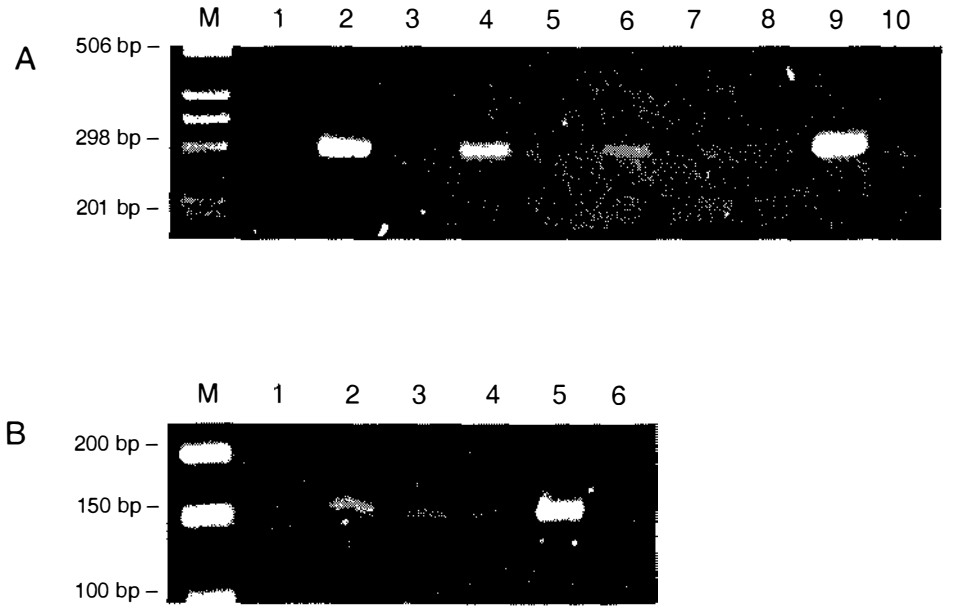


Fig. 3.10. Optimising the template DNA concentration for amplification of Epichloë microsatellite loci from endophyte-infected plant material

Agarose (3% NuSieve) gels of PCR products amplified from genomic DNA isolated from endophyte-infected and endophyte-free grass tissue by using primers to microsatellite loci B1 (panel A) and B10 (panel B). (A) PCR products generated with DNA prepared from AR1-infected *L. perenne* × *L. multiflorum* hybrid ryegrass (lanes 2, 4, 6, and 8), and endophyte-free hybrid ryegrass (lanes 1, 3, 5, and 7) by the CTAB method at total DNA concentrations of 2 ng/µl (lanes 1 and 2), 400 pg/µl (lanes 3 and 4), 200 pg/µl (lanes 5 and 6), 40 pg/µl (lanes 7 and 8); and 40 pg/µl of AR1 fungal genomic DNA (lane 9), and with no DNA (lane 10). Lane M, 1 kb ladder. (B) PCR products generated with DNA prepared from AR1-infected *L. perenne* × *L. multiflorum* hybrid ryegrass by the FastDNA Kit H minipreparation method at total DNA concentrations of 2 ng/µl (lane 1), 400 pg/µl (lane 2), 80 pg/µl (lane 3), 16 pg/µl (lane 4); and 40 pg/µl of AR1 fungal genomic DNA (lane 5), and with no DNA (lane 6). Lane M, 50 bp ladder.

endophyte sequences in a template mixture that is predominantly plant in origin. The best yield of product (lane 2) was obtained using a template concentration (2 ng/μl) that was fifty-fold higher than that required to amplify the corresponding fungal DNA (lane 9); presumably reflecting the relative abundance of fungal DNA to plant DNA in this particular association.

To improve the rapidity with which in planta detection of endophyte strains could be carried out, microsatellite loci were amplified using DNA extracted from plant material using the FastDNA Kit H miniprep method (Section 2.5.3). The results for AR1-infected *L. perenne* × *L. multiflorum* hybrid ryegrass material amplified with primers to locus B10 are shown in Fig. 3.10B. A product of the size (178 bp) characteristic of this locus was obtained, but only when DNA concentrations in the range of 16 to 400 pg/μl were used (lanes 2 to 4). At a DNA concentration of 2 ng/μl (lane 1), which was shown to be optimal for DNA prepared by the CTAB method (Fig. 3.10A), no product was generated. This was presumably a result of the presence of inhibitors in DNA prepared by the FastDNA method.

To test that endophyte amplification in planta occurred at each microsatellite locus (B1, B4 to B6, and B8 to B11), genomic DNA from AR1- infected *L. perenne* × *L. multiflorum* hybrid ryegrass, Fl1-infected tall fescue cv. Grasslands Roa, and Tf15-infected tall fescue cv. Kentucky 31 (results not shown), was amplified for each locus under standard conditions. DNA from the corresponding uninfected grasses along with fungal genomic DNA were also amplified. The results of amplification for the B4 and B6 loci are shown in Fig. 3.11. For all microsatellite loci, products of the sizes characteristic of the corresponding endophyte alleles were amplified from the total DNA of in planta samples and fungal genomic DNA, and no products were detected from plant DNA only.

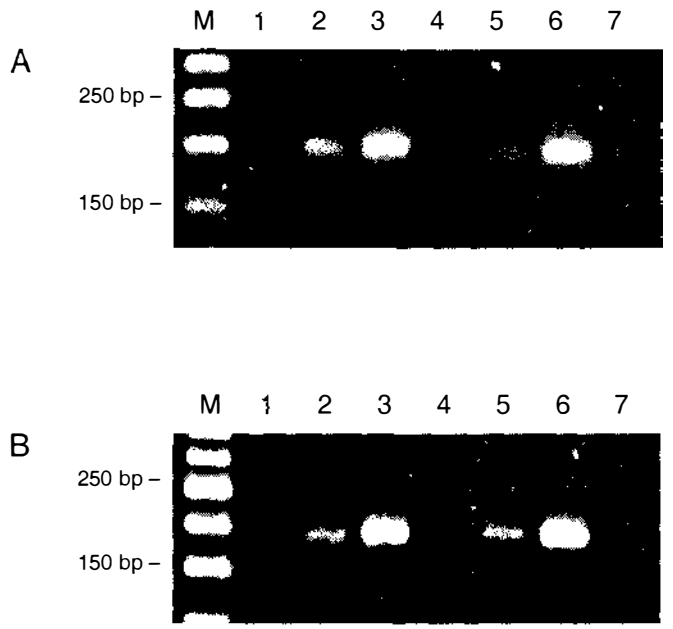


Fig. 3.11. Amplification of Epichloë microsatellite loci from endophyte-infected plant material.

Agarose (3% NuSieve) gels show microsatellite PCR products amplified using primers to microsatellite loci B4 (panel A) and B6 (panel B) with template DNA from: endophyte-free hybrid ryegrass, 2 ng/µl (lane 1), AR1-infected *L. perenne* × *L. multiflorum* hybrid ryegrass, 2 ng/µl (lane 2), AR1 fungal genomic DNA, 40 pg/µl (lane 3), endophyte-free tall fescue, 2 ng/µl (lane 4), Fl1-infected tall fescue, 2 ng/µl (lane 5), and Fl1 fungal genomic DNA, 40 pg/µl (lane 6). Lane M, 50 bp ladder.

3.2.2.5.4 Screening Epichloë Endophyte Isolates Through ML-PCR Assays with High Resolution Separation of Alleles

Having established standard conditions for PCR amplification of *Epichloë* endophyte microsatellite loci, 35 endophyte isolates were screened with primers to loci B1, B2 and B4 to B11. The isolates included representatives of the asexual taxonomic groups LpTG-2, *N. lolii*, *N. coenophialum*, FaTG-2, FaTG-3, and *N. uncinatum*, though sexual *E. typhina* and *E. festucae* isolates and two undescribed endophytes from *Hordeum bogdanii* were also screened (Table 2.1). Products were labelled by [α–^{33}P] dCTP incorporation (Section 2.13.5), and separated on denaturing polyacrylamide gels to determine the size and size variation of the single stranded products generated at each locus (Section 2.9). Figures 3.12 to 3.21 show autoradiographs of the gels separating the endophyte isolate alleles amplified in each ML-PCR assay.

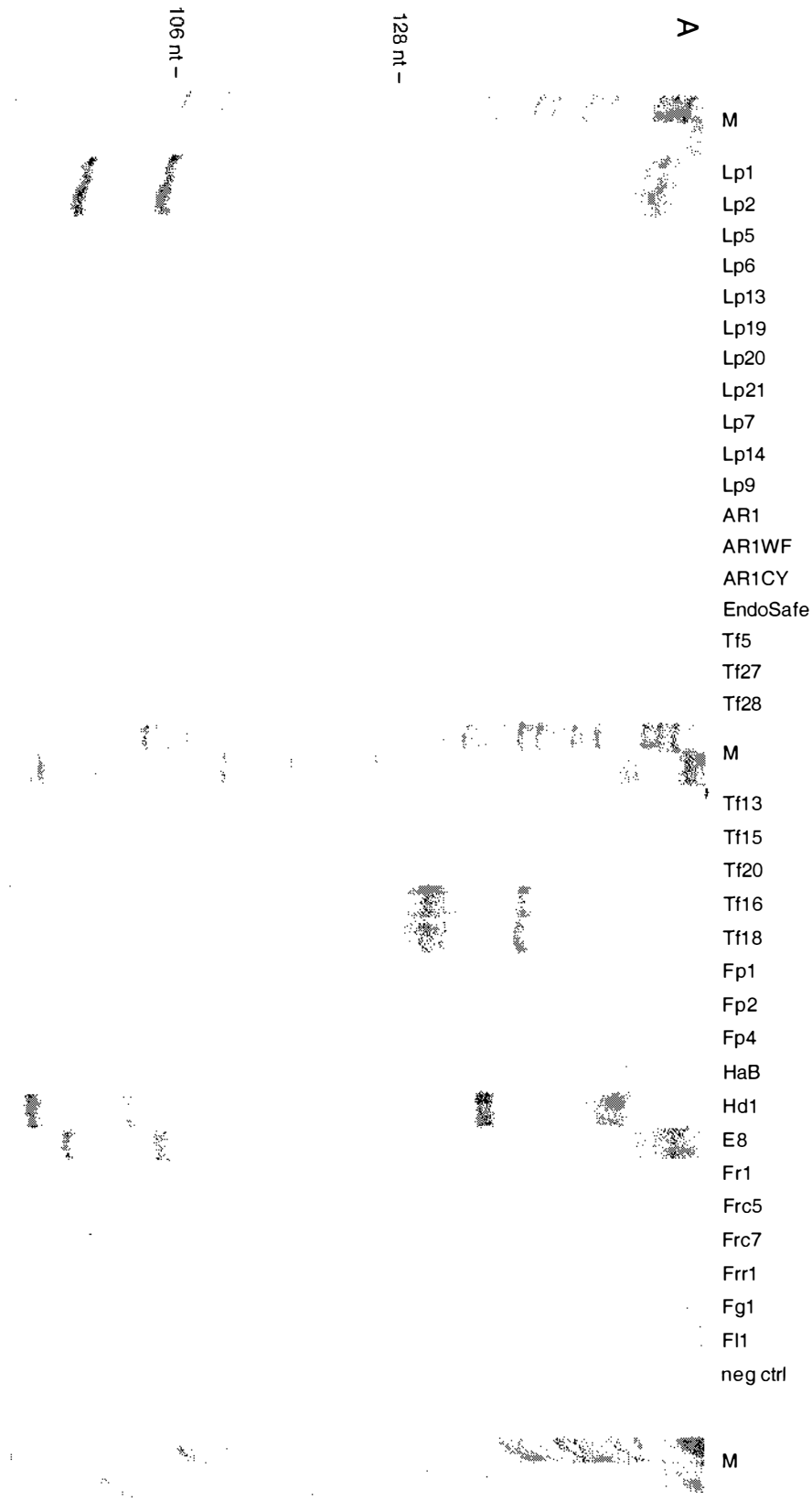
Isolates sharing like-banding patterns were readily identifiable and fingerprinting trends between different taxonomic groups were readily recognised. The absolute size of individual alleles was difficult to determine as: (i) in most cases, each microsatellite allele was represented by several bands, and (ii) it was difficult to size bands run some distance from the sequencing ladder, due to the variation in electrophoretic mobility across the gel. Multiple bands can be attributed to a combination of separation of the product into single strands under denaturing electrophoretic conditions, slippage products generated during amplification, and the ability of *Taq* polymerase to add an additional deoxyadenylate to the end of products. Typically, alleles were observed to migrate in a cluster of four bands and this was interpreted as the presence of the two single strands, both with and without the extra deoxyadenylate added. Despite these limitations it was often possible to establish allele groupings, including the identification of hybrids which contain multiple alleles.

B

	×								Lp1
	×								Lp2
	×								Lp5
	×								Lp6
	×								Lp13
	×								Lp19
	×								Lp20
	×								Lp21
	×								Lp7
					×				Lp14
	×								AR1
	×								AR1WF
	×								AR1CY
	×								Tf5
	×								Tf27
	×								Tf28
	×								Tf13
	×								Tf15
	×								Tf20
									Tf16
									Tf18
			×						Fp1
			×						Fp2
		×							Fp4
									E8
						×			Fr1
					×				Frc5
					×				Frc7
							×		Frr1
		×							Fg1
					×				Fl1
×				×					HaB
								×	Hd1
	×								EndoSafe

Fig. 3.12. PAGE separation of microsatellite alleles amplified from endophyte isolates with locus B1

(A) Autoradiograph of [α-^{33}P] radiolabelled ML-PCR products amplified from fungal genomic DNA and separated on a denaturing 6% polyacrylamide gel. (B) Schematic diagram showing an interpretation of the position of bands observed in panel A. Band estimates are represented by **x.**


A
128 nt –
106 nt –
M
Lp1
Lp2
Lp5
Lp6
Lp13
Lp19
Lp20
Lp21
Lp7
Lp14
Lp9
AR1
AR1WF
AR1CY
EndoSafe
Tf5
Tf27
Tf28
M
Tf13
Tf15
Tf20
Tf16
Tf18
Fp1
Fp2
Fp4
HaB
Hd1
E8
Fr1
Frc5
Frc7
Frr1
Fg1
Fl1
neg ctrl
M

B

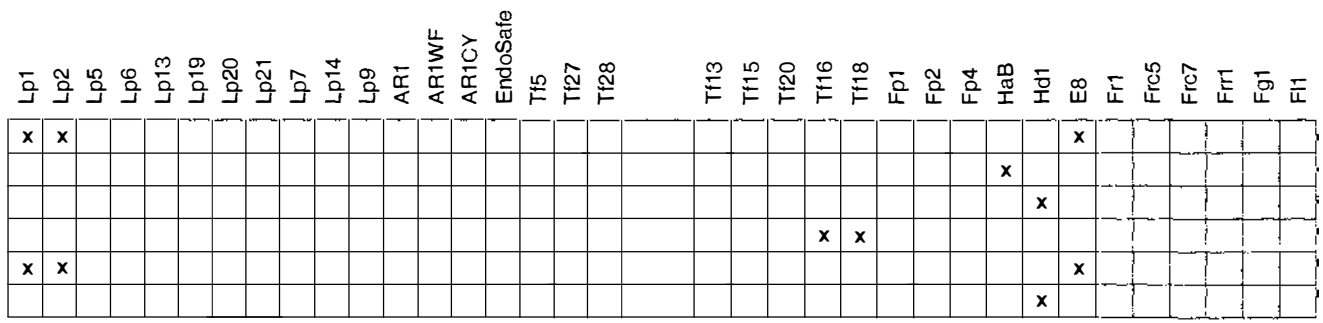


Fig. 3.13. PAGE separation of microsatellite alleles amplified from endophyte isolates with locus B2

(A) Autoradiograph of radiolabelled [α-^{33}P] ML-PCR products amplified from fungal genomic DNA and separated on a denaturing 6% polyacrylamide gel. (B) Schematic diagram showing an interpretation of the position of bands observed in panel A. Band estimates are represented by **x.**

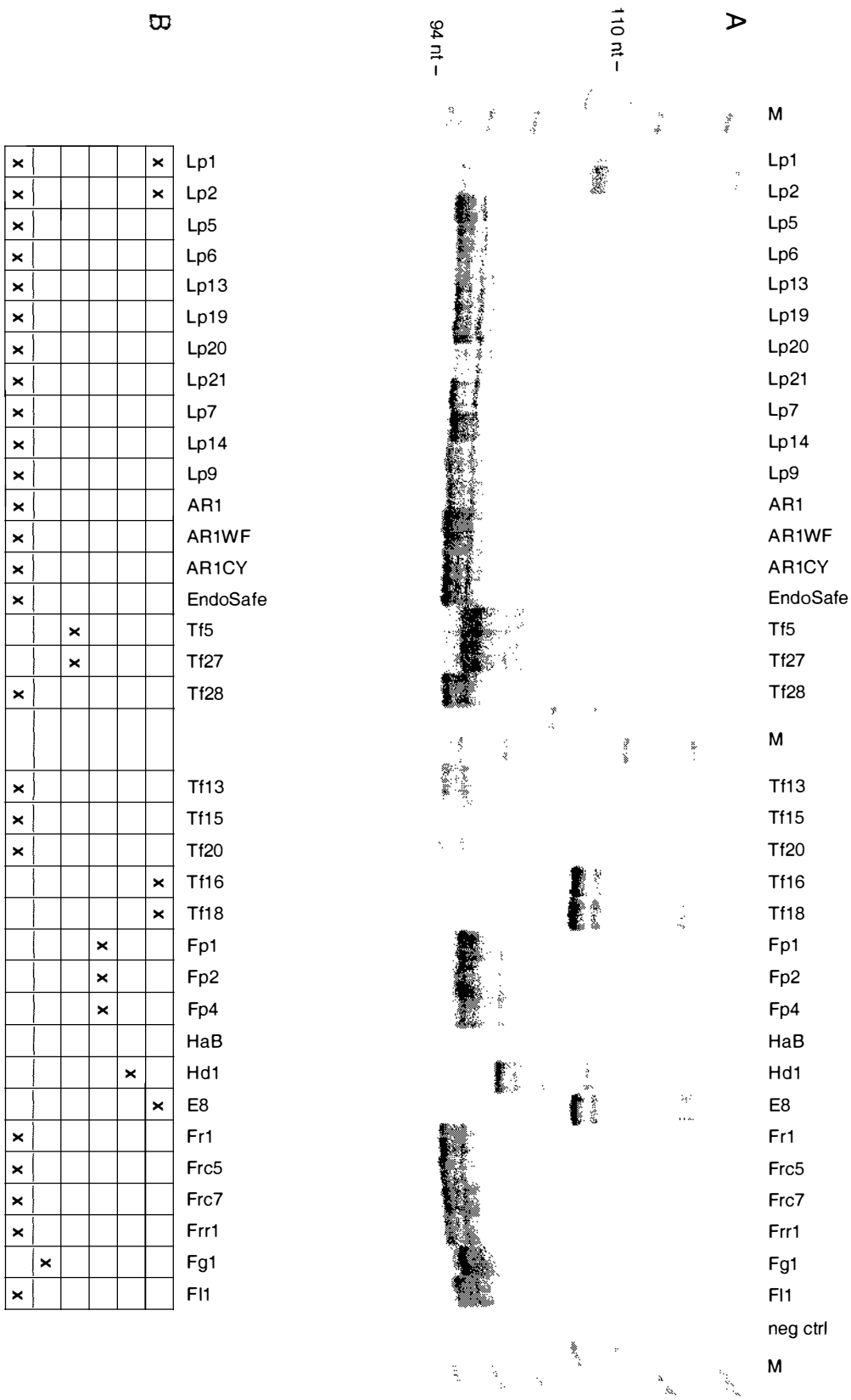

A
110 nt –
94 nt –
M
Lp1
Lp2
Lp5
Lp6
Lp13
Lp19
Lp20
Lp21
Lp7
Lp14
Lp9
AR1
AR1WF
AR1CY
EndoSafe
Tf5
Tf27
Tf28
M
Tf13
Tf15
Tf20
Tf16
Tf18
Fp1
Fp2
Fp4
HaB
Hd1
E8
Fr1
Frc5
Frc7
Frr1
Fg1
Fl1
neg ctrl
M
B
Lp1
Lp2
Lp5
Lp6
Lp13
Lp19
Lp20
Lp21
Lp7
Lp14
Lp9
AR1
AR1WF
AR1CY
EndoSafe
Tf5
Tf27
Tf28
Tf13
Tf15
Tf20
Tf16
Tf18
Fp1
Fp2
Fp4
HaB
Hd1
E8
Fr1
Frc5
Frc7
Frr1
Fg1
Fl1

Fig. 3.14. PAGE separation of microsatellite alleles amplified from endophyte isolates with locus B4

(A) Autoradiograph of [α-^{33}P] radiolabelled ML-PCR products amplified from fungal genomic DNA and separated on a denaturing 6% polyacrylamide gel. (B) Schematic diagram showing an interpretation of the position of bands observed in panel A. Band estimates are represented by **x.**

A

90 nt –

M

Lp1
Lp2
Lp5
Lp6
Lp13
Lp19
Lp20
Lp21
Lp7
Lp14
Lp9
AR1
AR1WF
AR1CY
EndoSafe
Tf5
Tf27
Tf28
Tf13
Tf15
Tf20
Tf16
Tf18

M

Fp1
Fp2
Fp4
HaB
Hd1
E8
Fr1
Frc5
Frc7
Frr1
Fg1
Fl1

M

B

	× Lp1
	× Lp2
×	Lp5
×	Lp6
	Lp13
	Lp19
	Lp20
	Lp21
	Lp7
	Lp14
	Lp9
	AR1
	AR1WF
	AR1CY
	EndoSafe
×	Tf5
×	Tf27
×	Tf28
	Tf13
	Tf15
	Tf20
	Tf16
	Tf18
×	Fp1
×	Fp2
×	Fp4
	HaB
×	Hd1
	× E8
	Fr1
	Frc5
	Frc7
	Frr1
	Fg1
	Fl1

Fig. 3.15. PAGE separation of microsatellite alleles amplified from endophyte isolates with locus B5

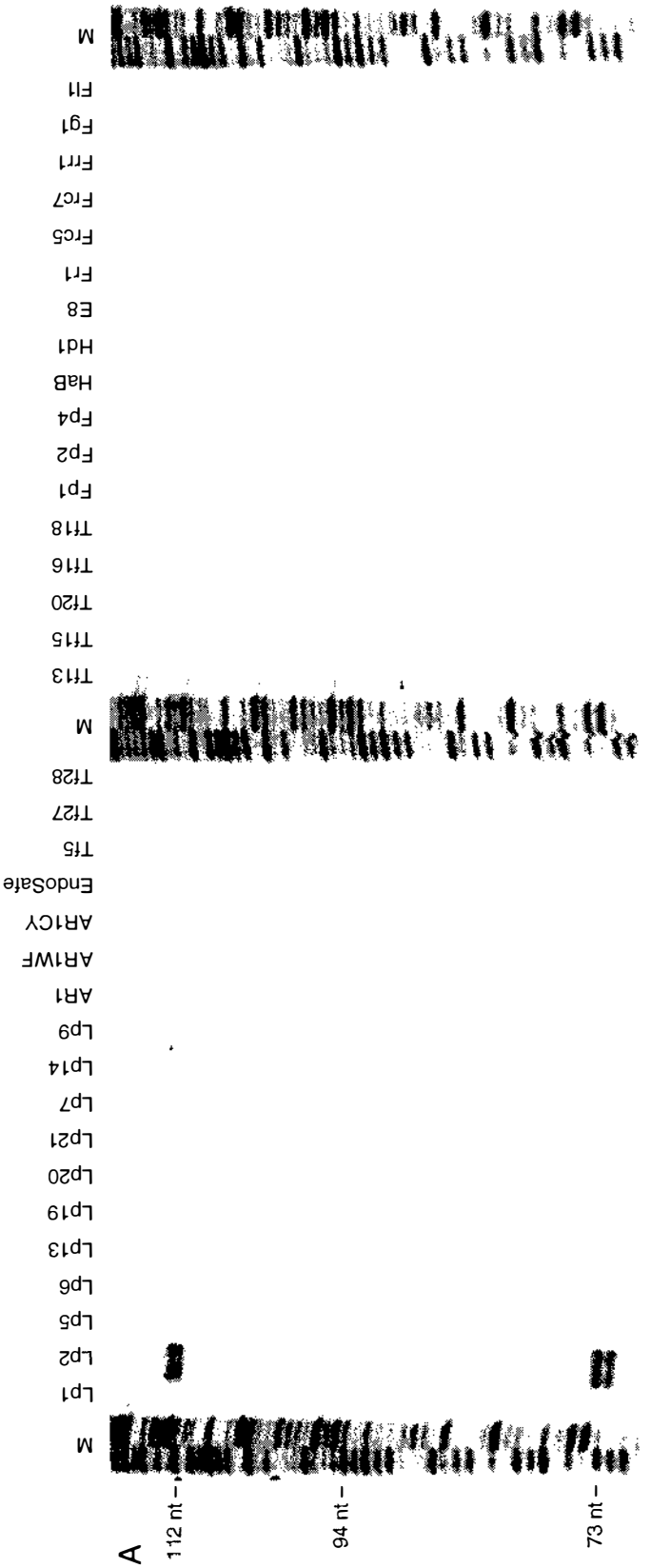
(A) Autoradiograph of [α-^{33}P] radiolabelled ML-PCR products amplified from fungal genomic DNA and separated on a denaturing 6% polyacrylamide gel. (B) Schematic diagram showing an interpretation of the position of bands observed in panel A. Band estimates are represented by **x.**

B

x					x				Lp1
x					x				Lp2
					x				Lp5
					x				Lp6
					x				Lp13
					x				Lp19
					x				Lp20
					x				Lp21
					x				Lp7
					x				Lp14
						x			Lp9
					x				AR1
					x				AR1WF
					x				AR1CY
					x				EndoSafe
							x		Tf5
								x	Tf27
							x		Tf28
						x			Tf13
						x			Tf15
						x			Tf20
x									Tf16
x									Tf18
			x						Fp1
			x						Fp2
			x						Fp4
				x					HaB
	x								Hd1
x									E8
						x			Fr1
							x		Frc5
						x			Frc7
		x							Frr1
						x			Fg1
						x			Fl1

Fig. 3.16. PAGE separation of microsatellite alleles amplified from endophyte isolates with locus B6

(A) Autoradiograph of [α-^{33}P] radiolabelled ML-PCR products amplified from fungal genomic DNA and separated on a denaturing 6% polyacrylamide gel. (B) Schematic diagram showing an interpretation of the position of bands observed in panel A. Band estimates are represented by **x.**


A
112 nt
94 nt
73 nt
M
Lp1
Lp2
Lp5
Lp6
Lp13
Lp19
Lp20
Lp21
Lp7
Lp14
Lp9
AR1
AR1WF
AR1CY
EndoSafe
Tf5
Tf27
Tf28
M
Tf13
Tf15
Tf20
Tf16
Tf18
Fp1
Fp2
Fp4
HaB
Hd1
E8
Fr1
Frc5
Frc7
Frr1
Fg1
Fl1
M

B

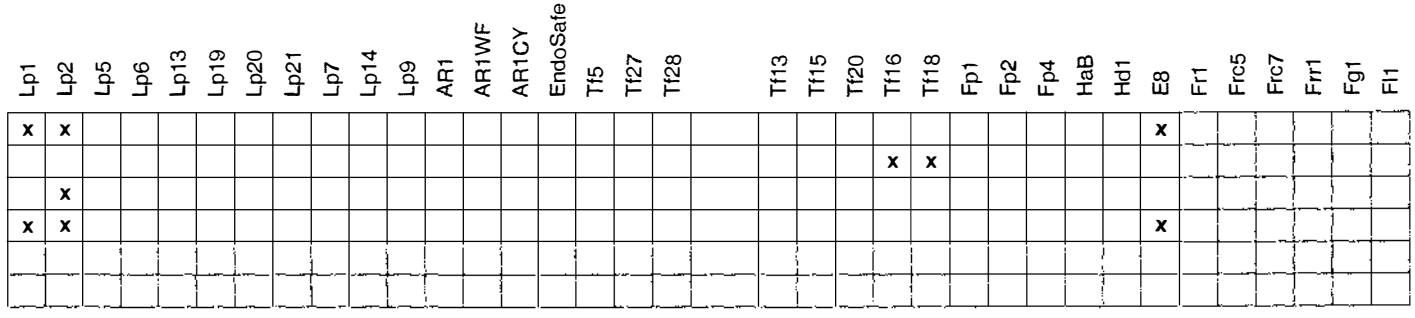


Fig. 3.17. PAGE separation of microsatellite alleles amplified from endophyte isolates with locus B7

(A) Autoradiograph of [α-^{33}P] radiolabelled ML-PCR products amplified from fungal genomic DNA and separated on a denaturing 6% polyacrylamide gel. (B) Schematic diagram showing an interpretation of the position of bands observed in panel A. Band estimates are represented by **x.**

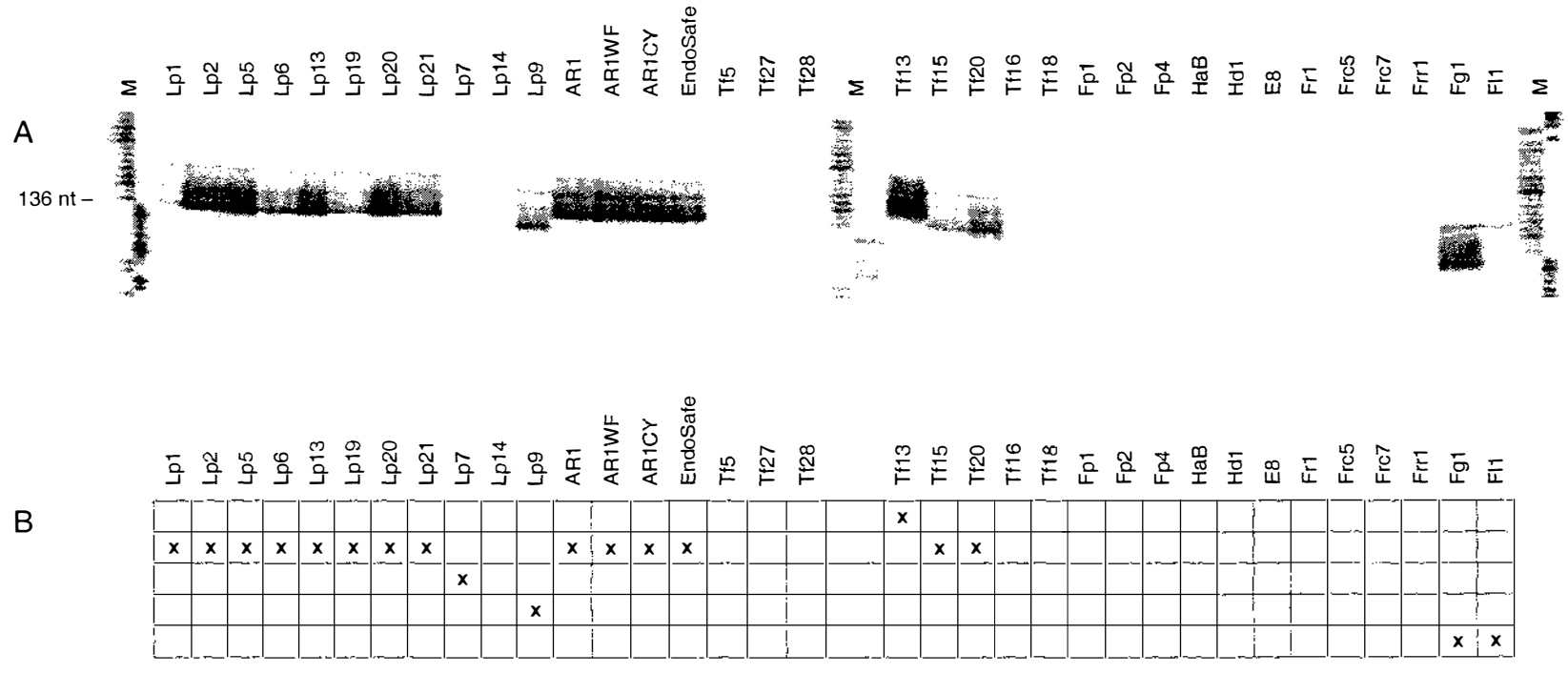
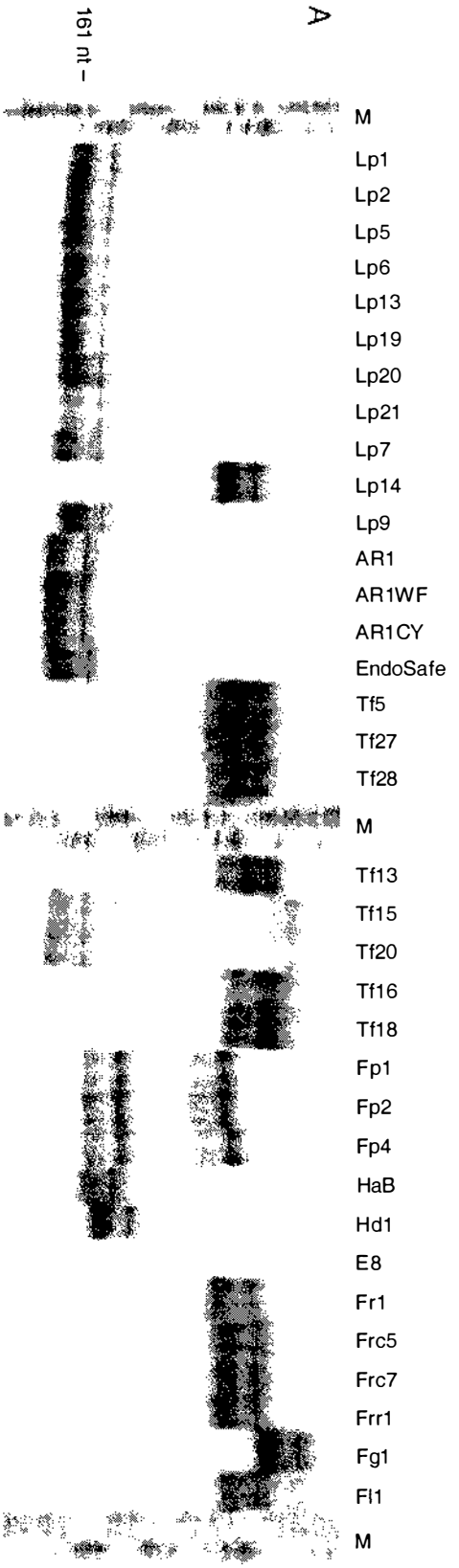


Fig. 3.18. PAGE separation of microsatellite alleles amplified from endophyte isolates with locus B8

(A) Autoradiograph of [α-^{33}P] radiolabelled ML-PCR products amplified from fungal genomic DNA and separated on a denaturing 6% polyacrylamide gel. (B) Schematic diagram showing an interpretation of the position of bands observed in panel A. Band estimates are represented by **x.**


A
161 nt –
M
Lp1
Lp2
Lp5
Lp6
Lp13
Lp19
Lp20
Lp21
Lp7
Lp14
Lp9
AR1
AR1WF
AR1CY
EndoSafe
Tf5
Tf27
Tf28
M
Tf13
Tf15
Tf20
Tf16
Tf18
Fp1
Fp2
Fp4
HaB
Hd1
E8
Fr1
Frc5
Frc7
Frr1
Fg1
Fl1
M

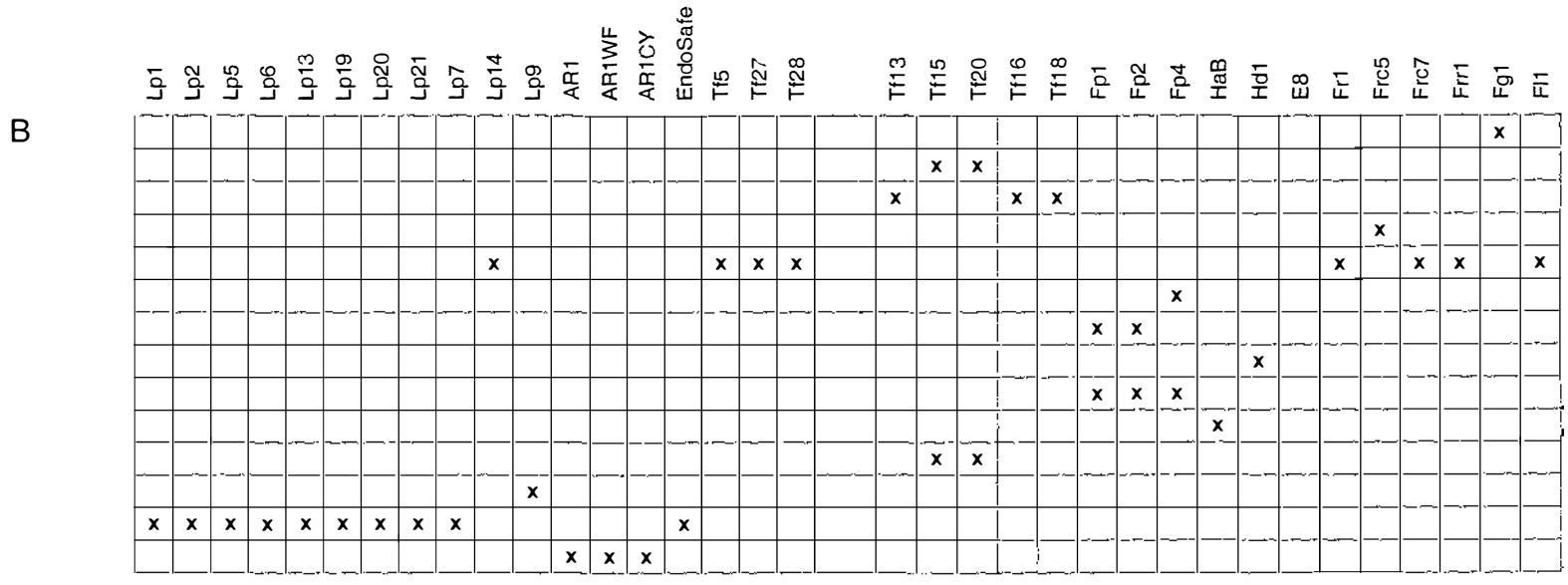
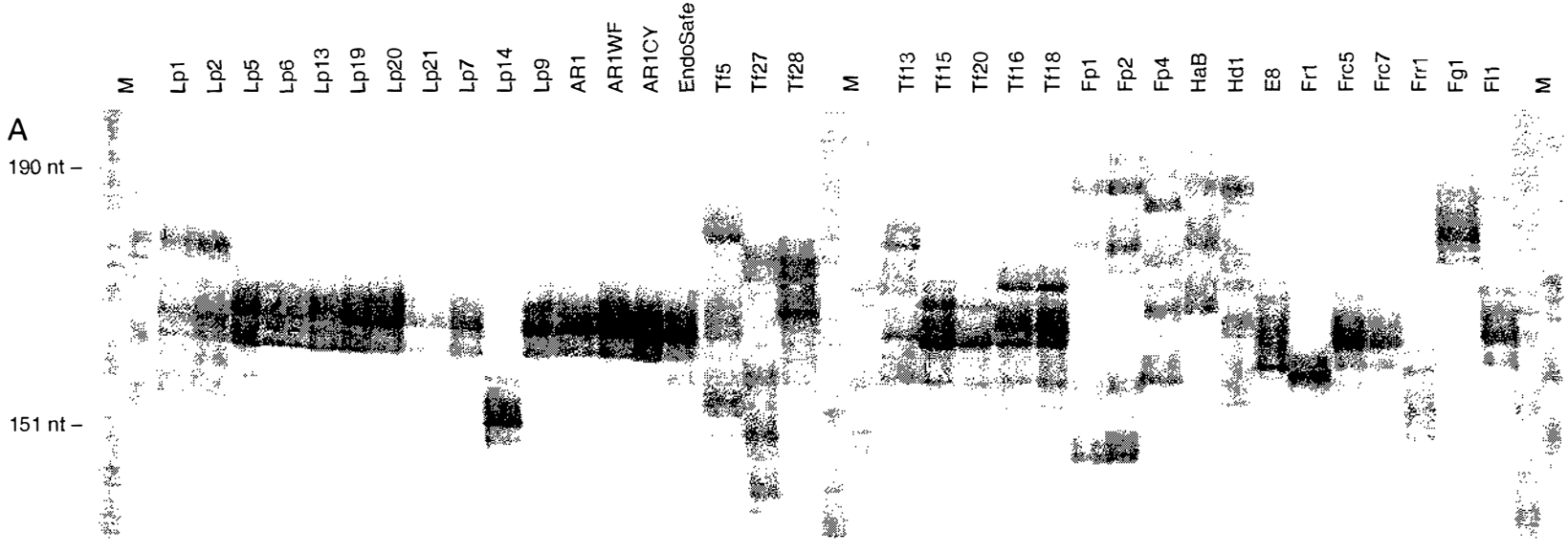


Fig. 3.19. PAGE separation of microsatellite alleles amplified from endophyte isolates with locus B9

(A) Autoradiograph of radiolabelled [α-^{33}P] ML-PCR products amplified from fungal genomic DNA and separated on a denaturing 6% polyacrylamide gel. (B) Schematic diagram showing an interpretation of the position of bands observed in panel A. Band estimates are represented by **x.**

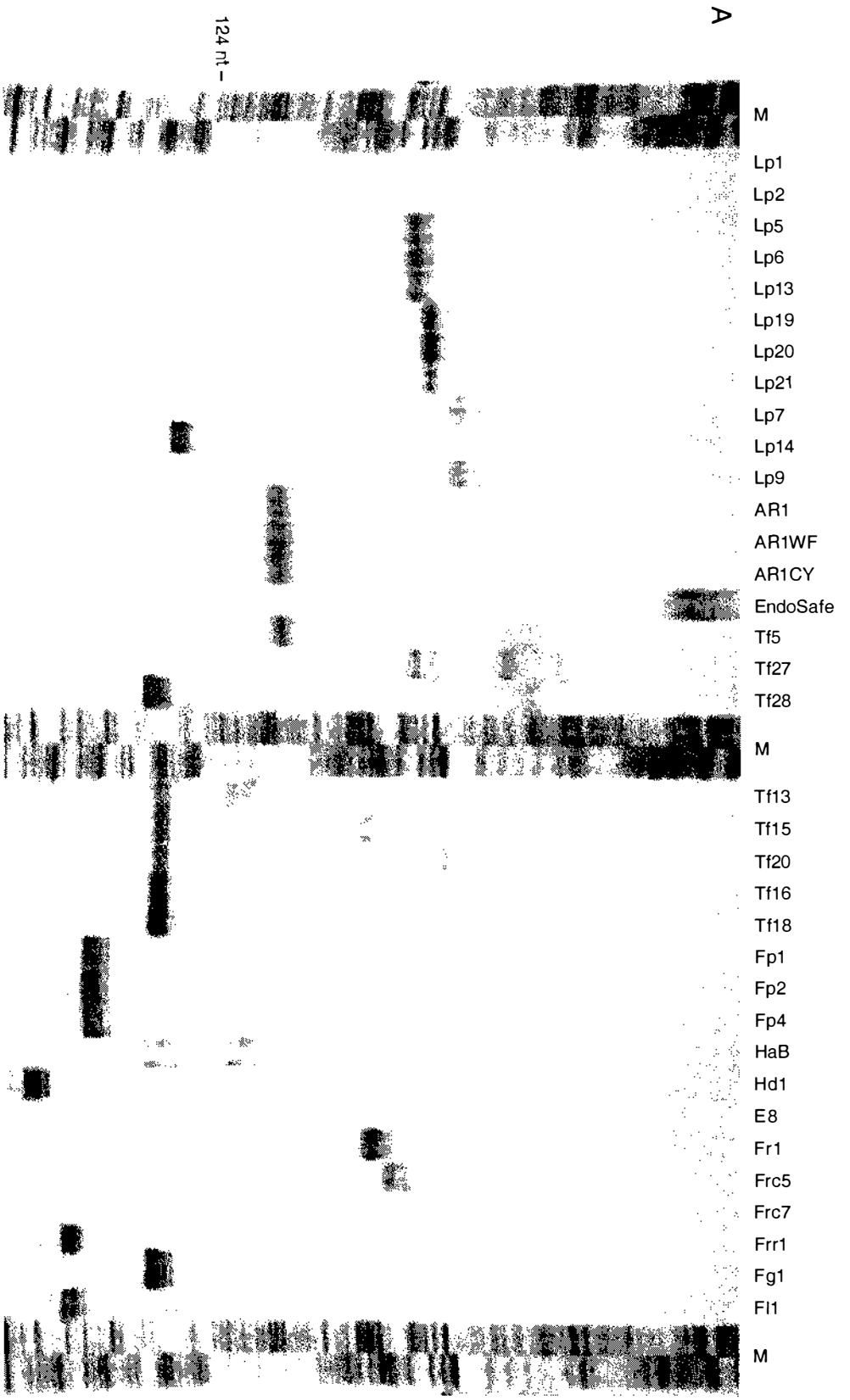
Fig. 3.20. PAGE separation of microsatellite alleles amplified from endophyte isolates with locus B10

(A) Autoradiograph of [α-^{33}P] radiolabelled ML-PCR products amplified from fungal genomic DNA and separated on a denaturing 6% polyacrylamide gel. (B) Schematic diagram showing an interpretation of the position of bands observed in panel A. Band estimates are represented by **x.**



B

							x						x						Lp1
							x						x						Lp2
										x									Lp5
										x									Lp6
										x									Lp13
										x									Lp19
										x									Lp20
										x									Lp21
										x									Lp7
		x																	Lp14
										x									Lp9
										x									AR1
										x									AR1WF
										x									AR1CY
										x									EndoSafe
						x								x					Tf5
x												x							Tf27
								x					x						Tf28
								x						x					Tf13
									x										Tf15
									x										Tf20
					x						x								Tf16
					x						x								Tf18
	x																	x	Fp1
	x																	x	Fp2
								x									x		Fp4
													x					x	HaB
							x									x			Hd1
									x										E8
			x																Fr1
									x										Frc5
									x										Frc7
				x															Frr1
															x				Fg1
									x										Fl1


A
124 nt –
M
Lp1
Lp2
Lp5
Lp6
Lp13
Lp19
Lp20
Lp21
Lp7
Lp14
Lp9
AR1
AR1WF
AR1CY
EndoSafe
Tf5
Tf27
Tf28
M
Tf13
Tf15
Tf20
Tf16
Tf18
Fp1
Fp2
Fp4
HaB
Hd1
E8
Fr1
Frc5
Frc7
Frr1
Fg1
Fl1
M

B

Lp1	
Lp2	
Lp5	
Lp6	
Lp13	
Lp19	
Lp20	
Lp21	
Lp7	
Lp14	
Lp9	
AR1	
AR1WF	
AR1CY	
EndoSafe	
Tf5	
Tf27	
Tf28	
Tf13	
Tf15	
Tf20	
Tf16	
Tf18	
Fp1	
Fp2	
Fp4	
HaB	
Hd1	
E8	
Fr1	
Frc5	
Frc7	
Frr1	
Fg1	
Fl1	

Fig. 3.21. PAGE separation of microsatellite alleles amplified from endophyte isolates with locus B11

(A) Autoradiograph of [α-^{33}P] radiolabelled ML-PCR products amplified from fungal genomic DNA and separated on a denaturing 6% polyacrylamide gel. (B) Schematic diagram showing an interpretation of the position of bands observed in panel A. Band estimates are represented by **x.**

3.2.2.5.5 Summary of Screening Epichloë Endophyte Isolates

For polyacrylamide gels (Figs. 3.12A to 3.21A), an interpretation of how the different allele groupings were made are compiled in schematic diagrams (Figs. 3.12B to 3.21B). A summary of these is given in Table 3.1.

Screening with microsatellite locus B2 (Fig. 3.13), only LpTG-2, FaTG-3, HaB, Hd1, and *E. typhina* amplified. Banding patterns were difficult to interpret as the usual tight cluster of four bands were not present, but the different taxonomic groups within this group were distinguishable from one another. Primers to the B5 locus amplified LpTG-2, *N. uncinatum*, Hd1, and *E. typhina* (Fig. 3.15). Very faint amplification of *N. lolii* (loA) and *N. coenophialum* samples was visible. Of these samples, only two alleles were identified, so it was not a very informative locus. The B7 locus was amplified in LpTG-2, FaTG-3, and *E. typhina* (Fig. 3.17). Only two different fingerprints were seen, which grouped LpTG-2 with *E. typhina.* The B8 locus was amplified in LpTG-2, *N. lolii*, FaTG-2 and *E. festucae* isolates Fg1 and Fl1, though the different taxonomic groups were not completely distinguishable from one another.

Primers to the remaining six loci (B1, B4, B6, B9, B10, and B11) amplified almost all of the isolates tested and, between them, were able to distinguish isolates to at least their isozyme phenotype grouping (as described by Christensen et al., 1993). Locus B11 was particularly informative, with 23 different fingerprints distinguishable across the 35 isolates surveyed. This was the only locus that was able to distinguish most of the different isozyme phenotypes within the *N. lolii* group. Banding patterns from the B10 locus were difficult to interpret as there were multiple overlapping allele clusters present and it was difficult to determine whether alleles were homologous. Nonetheless, loci B9 and B10 proved to be particularly informative in identifying known hybrid endophytes, such as the tall fescue endophytes, that are characterised by the presence of multiple alleles. Multiple products were amplified from these same loci in *N. uncinatum,* providing further evidence for a hybrid origin for this group.

Table 3.1. Summary of polymorphism and size range of ML-PCR products amplified from Epichloë endophyte isolates with PAGE separation

ML-PCR assay	Expected product size (bp)	Isolate of original microsatellite	Size range of products (bp)	No. of fingerprint patterns [a]
B1	325	NF 62A	300 – 340	9 [b]
B2	163	E8	95 – 170	4
B4	124	E8	105 – 125	7
B5	107	E8	70 – 110	2
B6	173	E8	150 – 200	10
B7	111	Lp1	70 – 120	4
B8	142	Lp1	130 – 150	5
B9 [c]	157	Lp1	145 – 190	12
B9 [d]	246	Lp1	235 – 280	7
B10	179	Lp1	145 – 200	16
B11	173	Lp19	115 – 240	23

[a] For 35 endophyte isolates screened

[b] Lp9 was not included in this screen

[c] With primer pair B9.1 and B9.2

[d] With primer pair B9.1 and B9.4

3.2.2.6 Development of Primer Set I

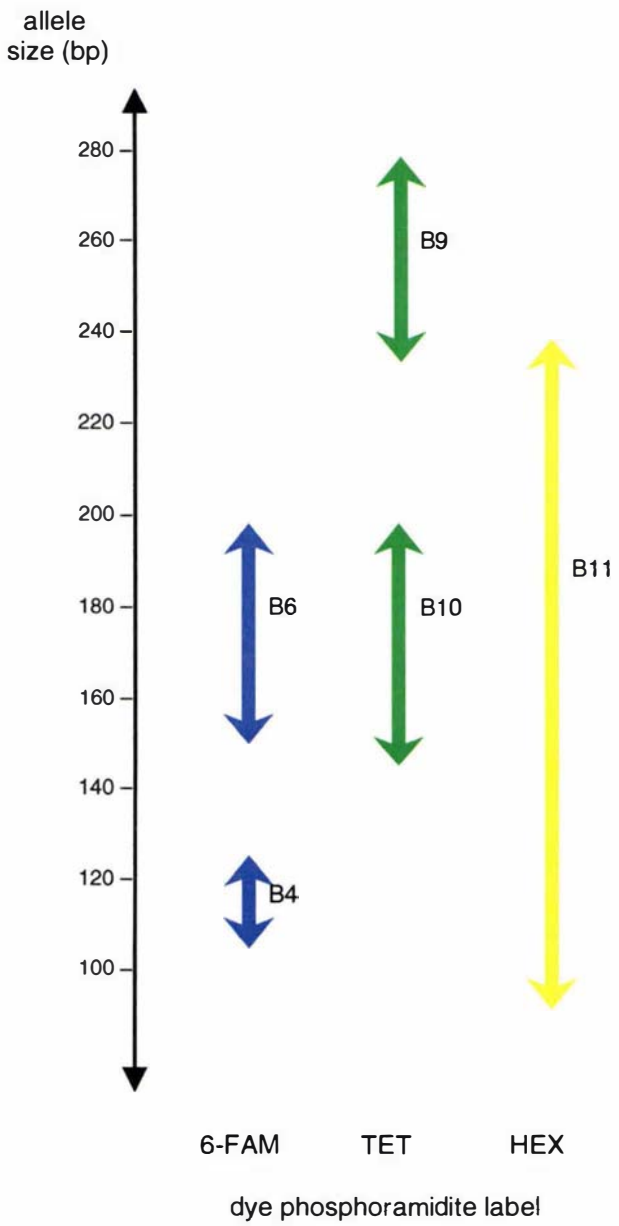
Given that primers to microsatellite loci B4, B6, B9, B10, and B11 amplified almost all of the 35 isolates tested, these primer sets were selected for the development of an automated DNA fingerprinting system utilising fluorescently labelled primers and the laser detection technology associated with the ABI automatic sequencer. The size range of alleles generated for each of these microsatellite loci are summarised in Table 3.1. With reference to these size ranges, a primer dye-labelling strategy that utilises the three available dye phosphoramidites: 6-FAM (blue), TET (green), and HEX (yellow), was formulated to allow the simultaneous size analysis of the products of all five microsatellite loci on a single lane of the automatic sequencer (Fig. 3.22). The Bx.1 primer of each primer pair was arbitrarily chosen to be 5'-end labelled with the dye phosphoramidite. As microsatellite B11 had such a wide range of PCR product sizes, it was assigned its own dye, HEX. Primers to loci B4 and B6 were both labelled with 6-FAM. As the allele size ranges from both B9 (145 to 190 bp) and B10 (145 to 200 bp) overlapped, both loci could not be labelled with the remaining dye, TET. Therefore a new primer to the B9 locus (B9.4) was designed to pair with B9.1, to increase the size range of alleles to 235 to 280 bp. A consequence of this change was a reduction in the level of polymorphism at the B9 locus (described in Section 3.2.2.6.1), but sufficiently informative fingerprints were generated at the remaining four loci to resolve the endophyte groups. The primers: Bx.1/Bx.2 for B4, B6, and B10, and Bx.1/Bx.4 for B9 and B11, were therefore grouped together, and are hereby referred to as Primer Set I.

3.2.2.6.1 Redesigning Primers to Microsatellite Locus B9

A new primer, B9.4, was designed 89 bp downstream of B9.2, to pair with B9.1 (Fig. 3.7). Standard amplification conditions were used to amplify genomic DNA from the original panel of 12 endophyte isolates (Section 3.2.1) using the B9.1/B9.4 primer combination. Good amplification was obtained (Fig. 3.23) though Tf18, Fp2, and E8 failed to amplify, and multiple alleles were not detected from the tall fescue endophytes, contrary to amplification with the B9.1/B9.2 pair (Fig. 3.9C). The panel of 35 endophyte

Fig. 3.22. Strategy for the dye-labelling of primers to microsatellite loci for Primer Set I

The strategy shows how the products for microsatellite loci B4, B6, B9, B10, and B11 can be analysed in a single lane of an automatic genetic analyser. Double headed arrows indicate the observed size range of microsatellite PCR products, as determined by separation on polyacrylamide gels, for the endophyte isolates analysed. For each locus, Bx, primers Bx.1 and Bx.2 were used, except loci B9 and B11, where B9.1 and B9.4, and B11.1 and B11.4 were used, respectively.


allele
size (bp)
280
260
240
220
200
180
160
140
120
100
B9
B6
B10
B11
B4
6-FAM
TET
HEX
dye phosphoramidite label

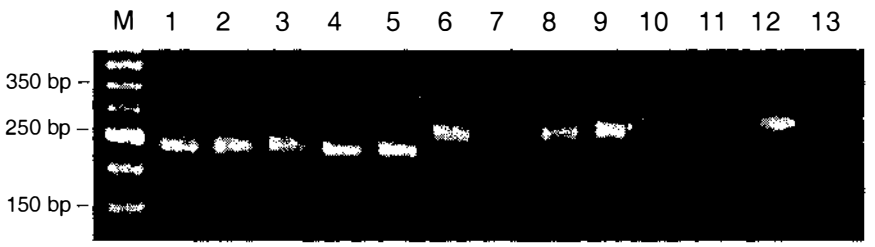


Fig. 3.23. ML-PCR amplification of locus B9 using a new primer pair, B9.1 and B9.4

PCR reactions contained genomic DNA from isolates: Lp1 (lane 1), Lp5 (lane 2), Lp7 (lane 3), Lp13 (lane 4), Lp19 (lane 5), Tf13 (lane 6), Tf18 (lane 7), Tf27 (lane 8), Tf28 (lane 9), Fp2 (lane 10), E8 (lane 11), and Fr1 (lane 12). Lane 13 is a PCR negative control without DNA and lane M contains 50 bp ladder. Products were separated on a 3% NuSieve gel.

isolates were screened through the B9.1/B9.4 assay, with polyacrylamide gel separation as previously described (Section 3.2.2.5.4) and an autoradiograph of this gel is shown in Figure 3.24. In this screening *N. uncinatum* and isolates Tf16, HaB, and E8 failed to amplify, and very weak amplification was detected from Hd1 and Tf18. Also, multiple alleles were not amplified from hybrid endophytes, though very good amplification was obtained from all other isolates.

3.2.2.6.2 Multiplex PCR of Primer Set I

Primers to the five informative microsatellite loci identified above (B4, B6, B9, B10, and B11) were tested in a single multiplex reaction (Section 2.13.6) for their ability to amplify all loci, thus economising on reagents, time, and labour. A comparison of the reaction products generated for the five-locus multiplex reaction and for individual locus amplifications, using template DNA from endophytes Tf13 and Fr1, is shown in Figure 3.25. All products amplified in single-locus reactions were present in the multiplex reaction. This was confirmed by carrying out the same reactions in the presence of [$\alpha-^{33}$P] dCTP followed by separation of the products on polyacrylamide gels and detection by autoradiography. The products of the single-locus amplifications matched exactly the size of the products of the multiplex reaction (results not shown). The success of the multiplex reaction was however dependent of the quality of the template DNA used. Multiplex PCR of DNA prepared from plant material using the CTAB method (Section 2.5.4) gave consistently stronger signals than that prepared using the FastDNA Kit (Section 2.5.3); presumably a consequence of the presence of inhibitors in the latter.

To increase the signal strength of the weaker products from sub-optimal amplifications, alternative PCR strategies were undertaken including single-dye, and single-locus PCR reactions with subsequent pooling of products. Single-dye multiplex PCR reactions amplified loci, whose primers were labelled with the same dye, in a single reaction. Thus three reactions, one for each dye, were performed for each sample. This approach reduced the complexity of the multiplex reaction and allowed good amplification of

B

		×					Lp1
		×					Lp2
		×					Lp5
		×					Lp6
		×					Lp13
		×					Lp19
		×					Lp20
		×					Lp21
		×					Lp7
				×			Lp14
			×				Lp9
	×						AR1
	×						AR1WF
	×						AR1CY
		×					EndoSafe
					×		Tf5
					×		Tf27
					×		Tf28
					×		Tf13
		×					Tf15
		×					Tf20
							Tf16
					×		Tf18
							Fp1
							Fp2
							Fp4
							HaB
×							Hd1
							E8
				×			Fr1
					×		Frc5
				×			Frc7
				×			Frr1
						×	Fg1
				×			Fl1

A

Fig. 3.24. PAGE separation of microsatellite alleles amplified from endophyte isolates with locus B9, using primers B9.1 and B9.4

(A) Autoradiograph of [α-^{33}P] radiolabelled ML-PCR products amplified from fungal genomic DNA and separated on a denaturing 6% polyacrylamide gel. (B) Schematic diagram showing an interpretation of the position of bands observed in panel A. Band estimates are represented by **x.**

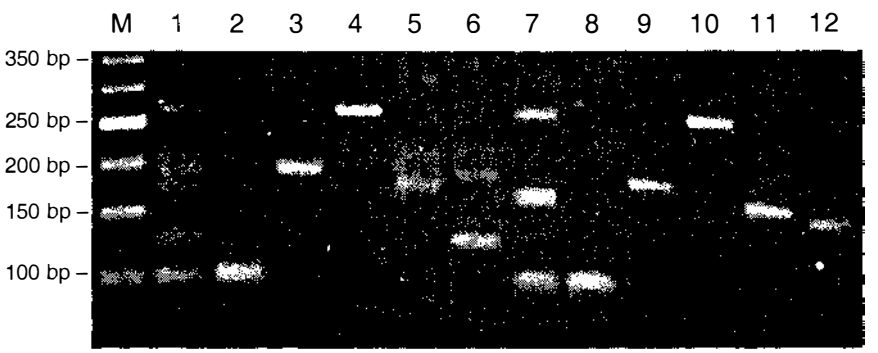


Fig. 3.25. Multiplex amplification of microsatellite loci using Primer Set I

Products were amplified by either single locus or multiplex PCR for endophyte isolates Tf13 (lanes 1–6), and Fr1 (lanes 7–12). PCR reactions contained the primer combinations: Primer Set I (lanes 1 and 7), B4.1 and B4.2 (lanes 2 and 8), B6.1 and B6.2 (lanes 3 and 9), B9.1 and B9.4 (lanes 4 and 10), B10.1 and B10.2 (lanes 5 and 11), and B11.1 and B11.4 (lanes 6 and 12). Lane M contained 50 bp ladder. Products were separated on a 3% NuSieve agarose gel.

samples which did not amplify optimally in the five locus multiplex. In extreme cases single loci could be amplified separately, then pooled for subsequent analysis.

3.2.2.7 Development of Primer Set II

On the basis of the high amount of allele length polymorphism observed when representative isolates of each of the ten *Epichloë* spp. were screened through ML-PCR assays that were not included in Primer Set I, a second set of dye-labelled primers, Primer Set II, was developed. Primer Set II further extends the analysis of microsatellite genotypes in Epichloë endophytes and amplifies microsatellite loci B1, B2, B5, B6, and B9.

3.2.2.7.1 Screening Epichloë Species with ML-PCR Assays

Following Primer Set I development, fungal genomic DNA from 41 isolates representing each Epichloë species, were made available (C. L. Schardl). These DNA samples were prepared using the method of Byrd et al. (1990). Isolates were screened through ML-PCR assays using standard amplification conditions (Section 2.13.5) which amplified the loci excluded from Primer Set I, ie. B1, B2, B5, B7, B8, and locus B9 using primers B9.1 and B9.2. Amplification products were separated on agarose gels (Section 2.8), these are shown in Figure 3.26. No products were detected from *Epichloë* spp. when screened with microsatellite locus B8 (Fig. 3.26E), and only *E. clarkii* and several *E. typhina* isolates (E8, E348, E425, E428, E431, and E470) amplified weakly with primers to the B7 locus (Fig. 3.26D). The remaining four loci amplified a considerable number of the isolates tested, and the results of these are summarised in Table 3.2. Locus B1 amplified all isolates except *E. typhina*, *E. clarkii* and *E. baconii* isolates E421 and E424 (Fig. 3.26A); locus B2 amplified *E. typhina*, *E. clarkii*, *E. elymi*, *E. bromicola*, and *E. sylvatica* (Fig. 3.26B); locus B5 amplified *E. typhina*, *E. clarkii*, and *E. sylvatica* (Fig. 3.26C); and B9 amplified all isolates except *E. clarkii*, *E. amarillans*, *E. glyceriae* and *E. typhina* isolates E8, E348, E358, E425, E428, E431, E469, E470, E505, and *E. sylvatica* isolate E503 (Fig. 3.26B). Although the

Fig. 3.26. ML-PCR screening of representative isolates of *Epichloë* spp. using primer pairs excluded from Primer Set I

Agarose (3%) gels showing PCR products amplified with primers to microsatellite loci B1 (Fig. 3.26A), B2 (Fig. 3.26B), B5 (Fig 3.26C), B7 (Fig 3.26D), B8 (Fig 3.26E), and B9 (Fig 3.26F). Lanes contain reactions with genomic DNA from isolates: (1) E8, (2) E348, (3) E358, (4) E425, (5) E428, (6) E431, (7) E469, (8) E470, (9) E505, (10) E1021, (11) E1022, (12) E1032, (13) E1033, (14) E2466, (15) E422, (16) E426, (17) E427, (18) E28, (19) E32, (20) E186, (21) E189, (22) E434, (25) E56, (26) E184, (27) E52, (28) E57, (29) E421, (30) E424, (31) E1031, (32) E501, (33) E502, (34) E798, (35) E799, (36) E354, (37) E503, (38) E277, (39) E2772, (40) E1040, (41) E1045, (42) E1046, (43) E1035.30, (23 and 44) PCR positive control. Lanes 24 and 45 are PCR negative controls without DNA and lane M contains 123 bp ladder. The positive control genomic DNA used in amplifications of loci B1, B8, and B9 is from isolate Lp6, and for amplifications B2, B5, and B7 is from isolate E8.

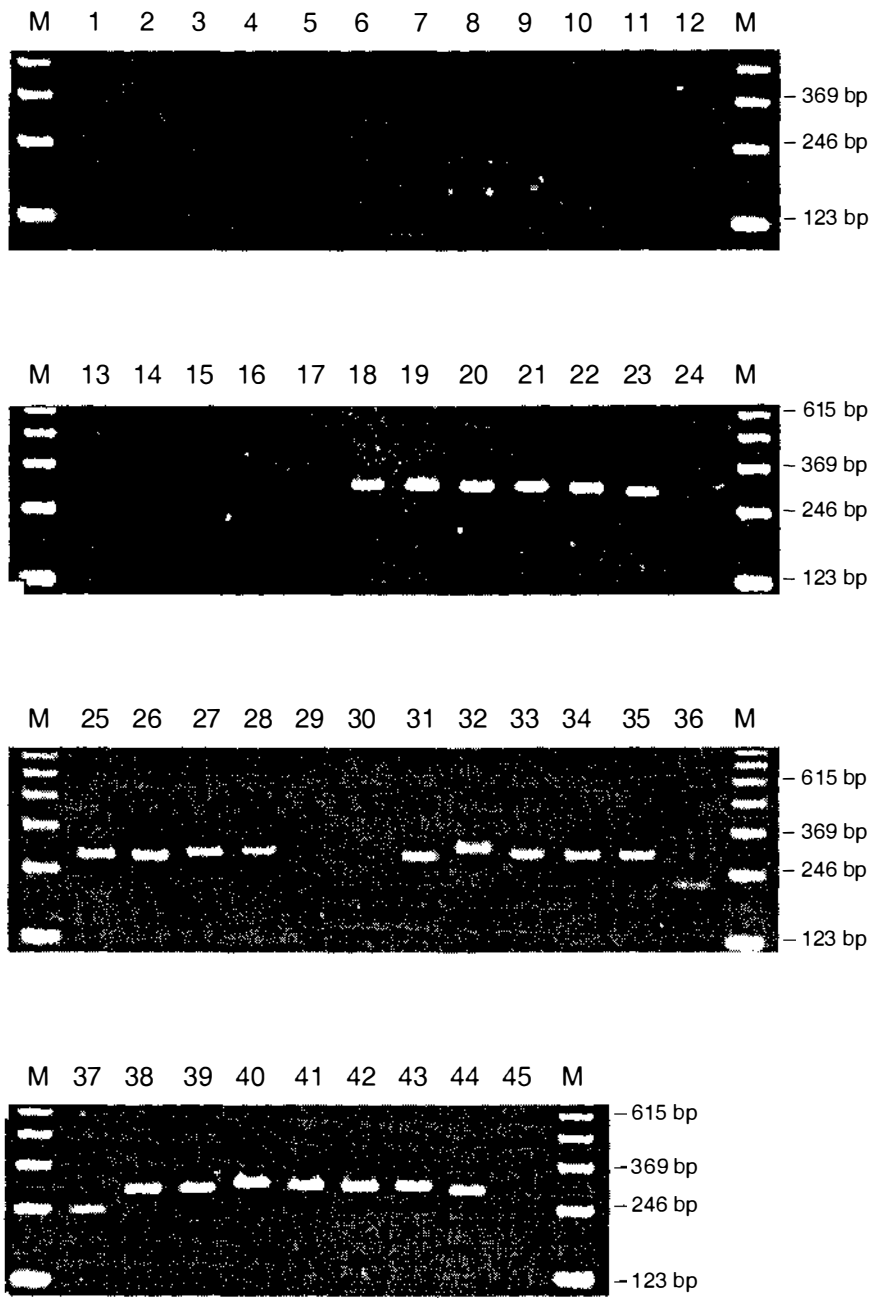


Fig. 3.26A. ML-PCR screening of representative isolates of *Epichloë* spp. with primers to the B1 locus.

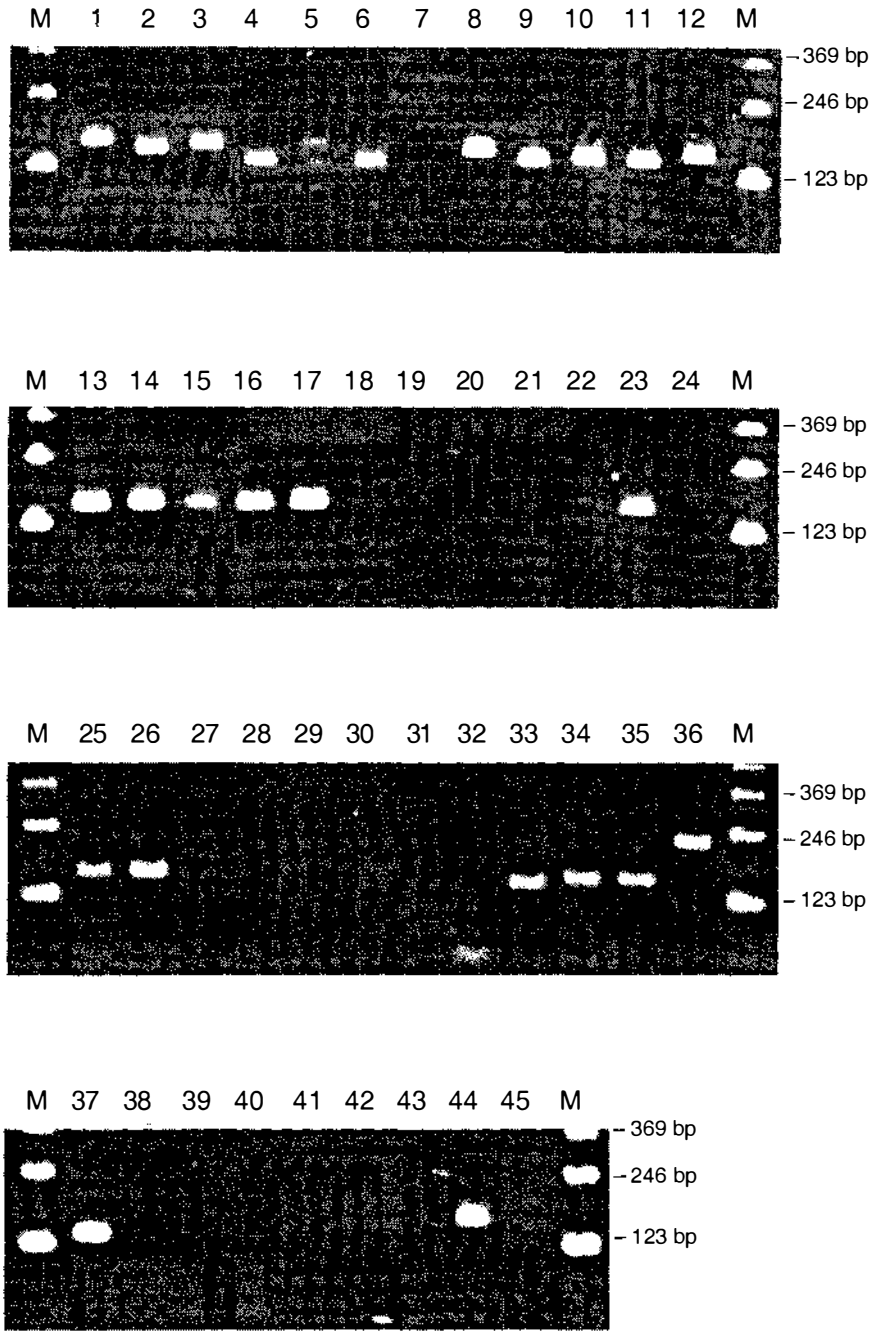


Fig. 3.26B. ML-PCR screening of representative isolates of *Epichloë* spp. with primers to the B2 locus.

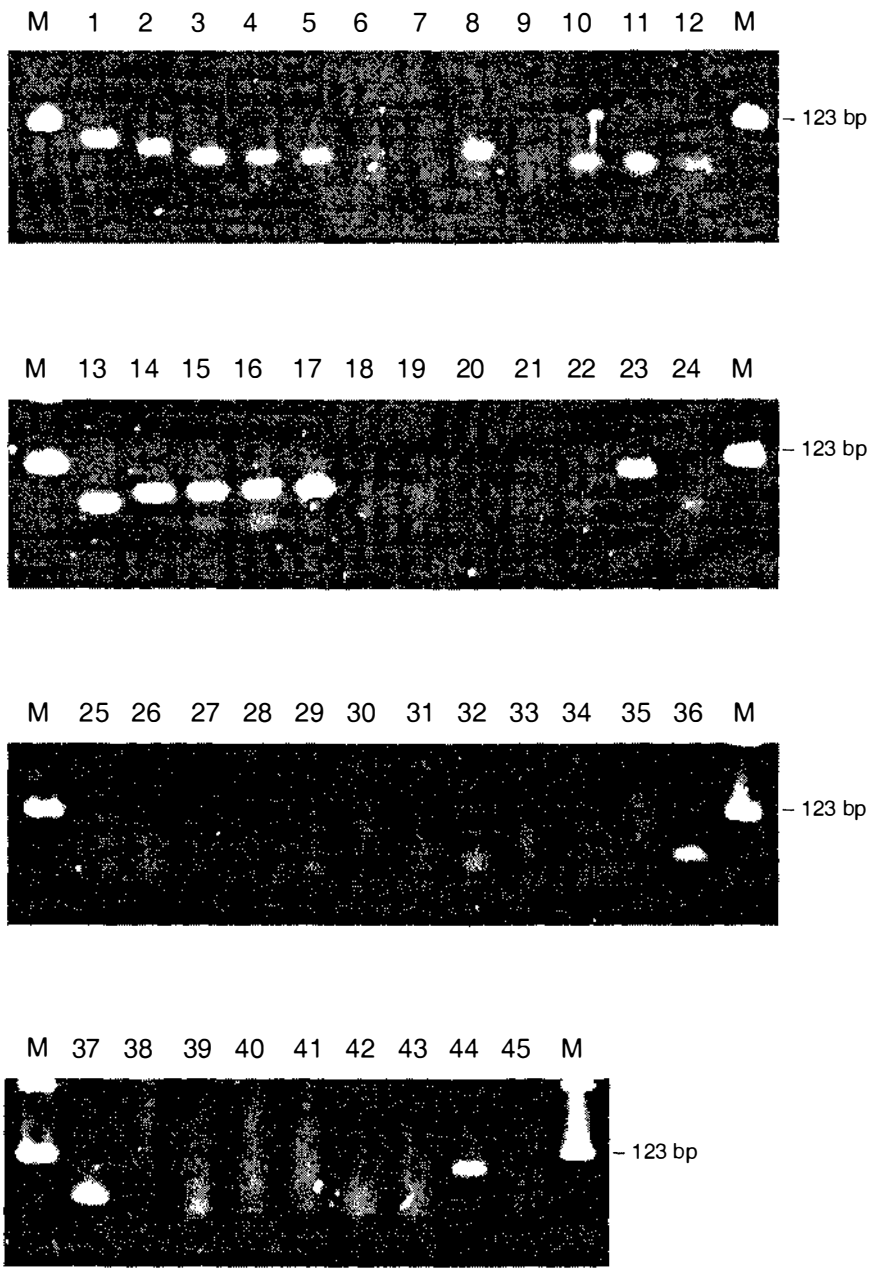


Fig. 3.26C. ML-PCR screening of representative isolates of *Epichloë* spp. with primers to the B5 locus.

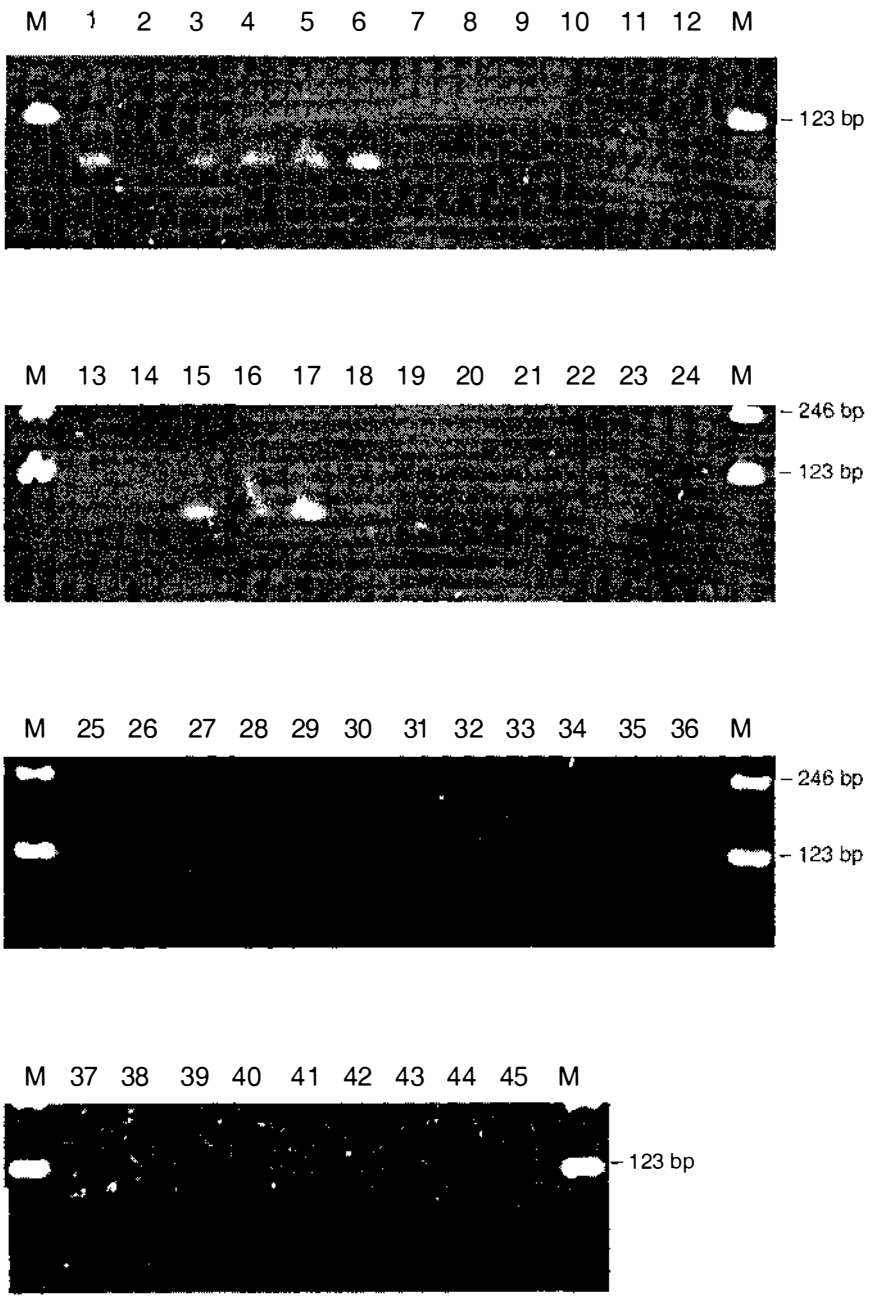


Fig. 3.26D. ML-PCR screening of representative isolates of *Epichloë* spp. with primers to the B7 locus.

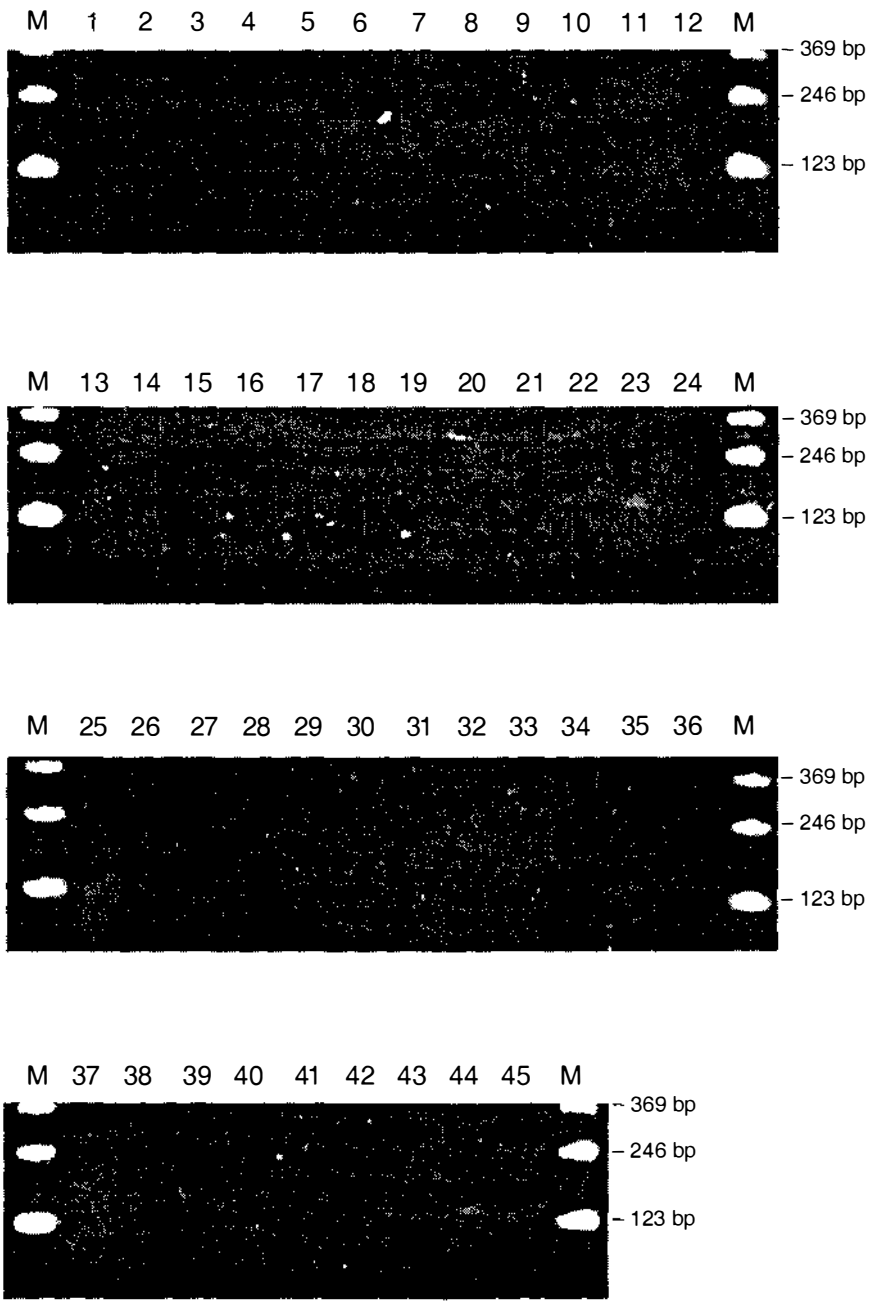


Fig. 3.26E. ML-PCR screening of representative isolates of *Epichloë* spp. with primers to the B8 locus.

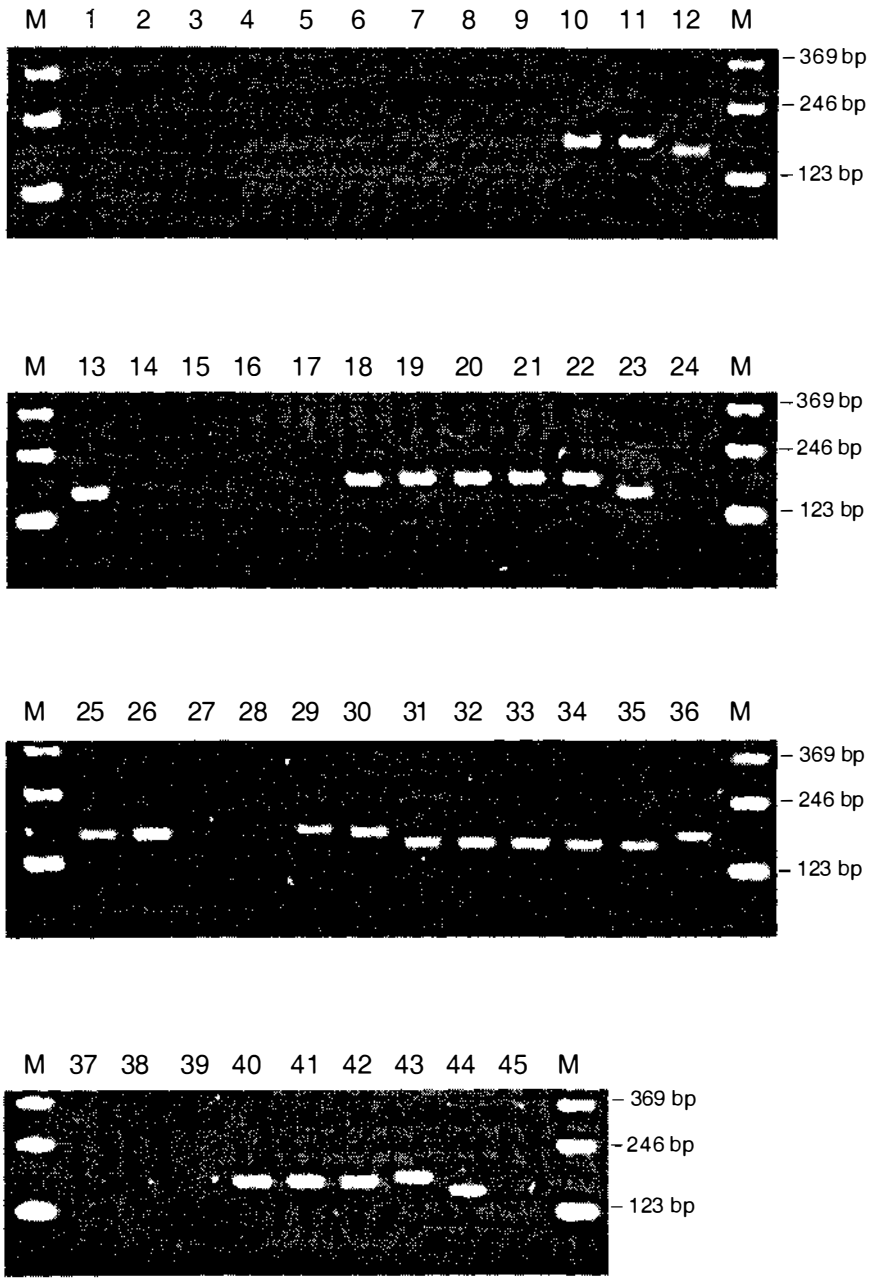


Fig. 3.26F. ML-PCR screening of representative isolates of *Epichloë* spp. with primers to the B9 locus, B9.1 and B9.2

Table 3.2. Summary of amplification of representative isolates of *Epichloë* spp. in B1, B2, B5, B7, B8, and B9 ML-PCR assays

Epichloë spp.	Mating population [a]	Isolates amplified					
		B1	B2	B5	B7	B8	B9
E. typhina	I	x	√	√	some	x	some
E. clarkii	I	x	√	√	√	x	x
E. festucae	II	√	x	x	x	x	√
E. elymi	III	√	√	x	x	x	√
E. amarillans	IV	√	x	x	x	x	x
E. baconii	V	some	x	x	x	x	√
E. bromicola	VI	√	√	x	unsure	x	√
E. sylvatica	VII	√	√	√	unsure	x	some
E. glyceriae	VIII	√	x	x	unsure	x	x
E. brachyelytri	IX	√	x	x	unsure	x	√

[a] Mating population designations as defined by Schardl et al. (1997).

PCR products in these screenings were not resolved on polyacrylamide gels, allele size polymorphism was clearly observed. An unexpected result was the presence of two bands when the B2 locus of *E. typhina* isolate E428 was amplified (lane 5, Fig. 3.26B). This may be the result of gene duplication, or the remnant of an interspecific hybridisation event.

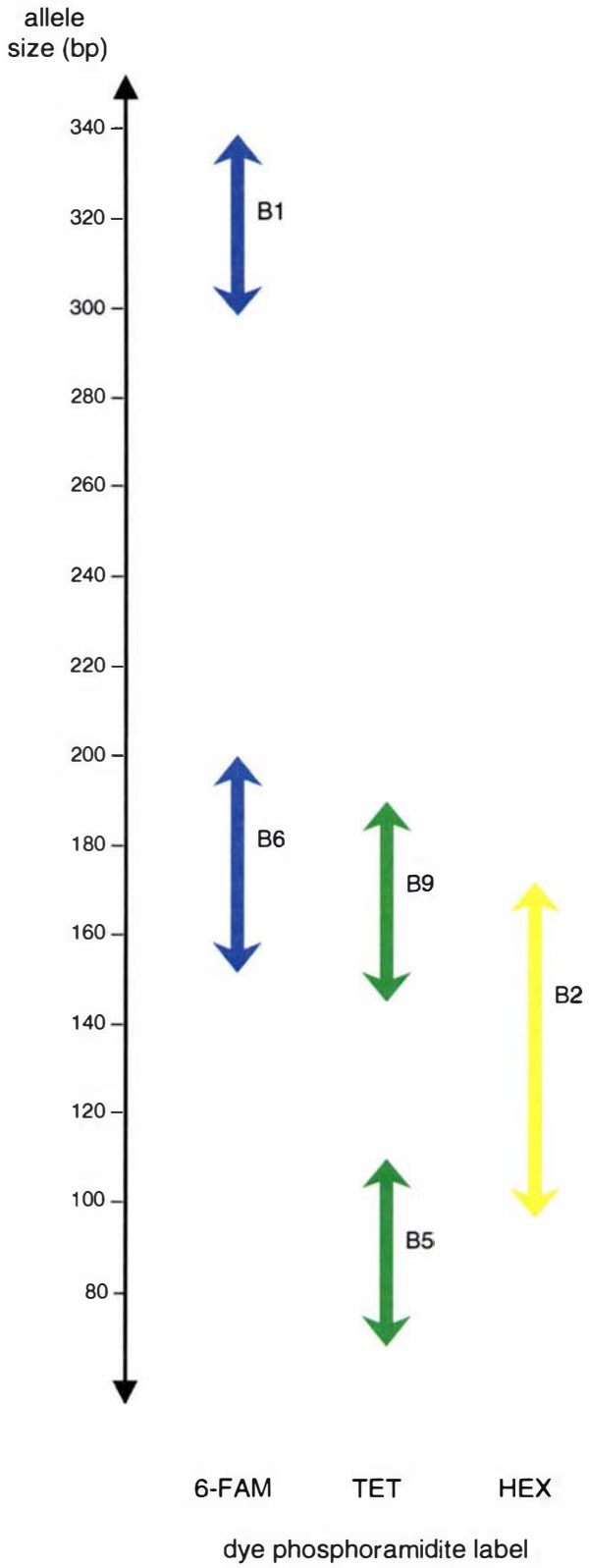
3.2.2.7.2 Multiplex PCR of Primer Set II

As primers to microsatellite loci B1, B2, B5, and B9 amplified a wide range of *Epichloë* isolates, these primer sets were selected for the development of a second automated DNA fingerprinting system. Given the size ranges of alleles, which are summarised for each of these microsatellite loci in Table 3.1, a second primer dye-labelling strategy was developed to allow the simultaneous size analysis of the products of these loci on a single lane of the automatic sequencer (Fig. 3.27). As microsatellite B2 generated the greatest size range of PCR products, it was assigned its own dye, HEX. Primer B9.1 was already labelled with TET from Primer Set I, and this primer coupled with B9.2, generated products in the size range 145 to 190 bp. The size range of products of B5 (70 to 110 bp) do not overlap with those of B9, so this locus was also labelled with TET. B1 was labelled with 6-FAM, and 6-FAM labelled B6 was also included as a control between Primer Set I and Primer Set II.

As with Primer Set I (Section 3.2.2.6.2), the ability of the primers in Primer Set II to amplify all microsatellite products in a single multiplex PCR reaction was tested. A comparison of the reaction products generated for the five-locus multiplex reaction and for individual locus amplifications, using template DNA from endophytes E8, E1021, and E354, is shown in Figure 3.28. All products amplified in single-locus reactions were present in the multiplex reaction. Further research with regard to the multiplex reaction of Primer Set II was not performed as it was expected that any problems would be overcome as discussed in Section 3.2.2.6.2.

Fig. 3.27. Strategy for the dye-labelling of primers to microsatellite loci for Primer Set II

The strategy shows how the products for microsatellite loci B1, B2, B5, B6, and B9 can be analysed in a single lane of an automatic genetic analyser. Double headed arrows indicate the observed size range of microsatellite PCR products, as determined by separation on polyacrylamide gels, for the endophyte isolates analysed. For each locus, Bx, primers Bx.1 and Bx.2 were used.


allele
size (bp)
340
320
300
280
260
240
220
200
180
160
140
120
100
80
B1
B6
B9
B2
B5
6-FAM
TET
HEX
dye phosphoramidite label

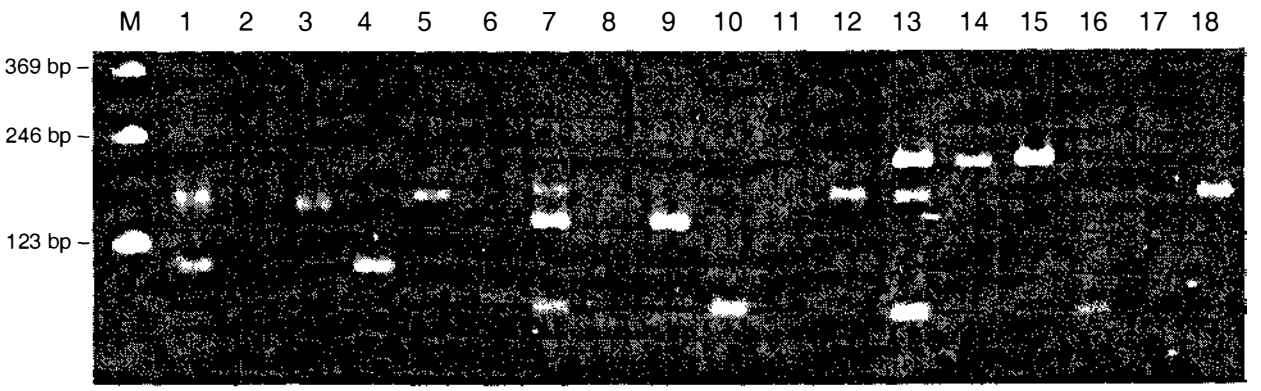


Fig. 3.28. Multiplex amplification of microsatellite loci using Primer Set II

Products were amplified by either single locus or multiplex PCR for endophyte isolates E8 (lanes 1–6), E1021 (lanes 7–12), and E354 (lanes 13–18). PCR reactions contained the primer combinations: Primer Set II (lanes 1, 7, and 13), B1.1 and B1.2 (lanes 2, 8, and 14), B2.1 and B2.2 (lanes 3, 9, and 15), B5.1 and B5.2 (lane 4, 10, and 16), B6.1 and B6.2 (lanes 5, 11, and 17), and B9.1 and B9.2 (lanes 6, 12, and 18). Lane M contains 123 bp ladder. Products were resolved on a 3% NuSieve agarose gel.

3.2.2.8 Automated Analysis of Fingerprints

A limitation of the radiolabelling method described in Section 3.2.2.5.4 is the inability to accurately determine both the size and the number of alleles present in a given sample. This problem was particularly acute in samples from hybrid endophytes with multiple alleles. To overcome these problems, samples were amplified using fluorescently labelled primers and the products analysed on ABI Prism 377 and 310 analysers using GeneScan Analysis 2.1 software (Section 2.17). The electropherogram for one such analysis, that for Tf28 amplified with Primer Set I and analysed on an ABI Prism 377 DNA Sequencer, is shown in Figure 3.29 and the allele sizes, in nucleotide units (ntu), for this sample are shown in Table 3.3.

The peaks associated with each microsatellite locus were identified by reference to the dye label and the expected size range of products at each locus (Figs. 3.24 and 3.27). Many peaks occurred as a doublet, one nucleotide apart, corresponding to the labelled strand of the PCR with and without the extra deoxyadenylate added at the 3'-end by *Taq* polymerase. In constructing a database of allele sizes for each endophyte strain, we have entered the size of the product without the additional deoxyadenylate. Where only a single peak was observed, the value for this was used. The allele sizes determined by this method were generally in good agreement with those generated by radiolabelled PCR (Table 3.1), though the ease of size determination was far greater for the automated multi-dye colour system. In addition, automated analysis allows precise size determination of multiple products, including similar sized products from different loci, in a single run. To check the reproducibility of allele size-calling by the automatic sequencer, dilutions of various fluorescently-labelled PCR products of Tf13 were submitted for analysis. Allele sizes were found to be most consistent when the peak heights of the samples were less than 2000 fluorescent units (fu). Working within this range resulted in allele size variation of up to 0.4 nucleotide units.

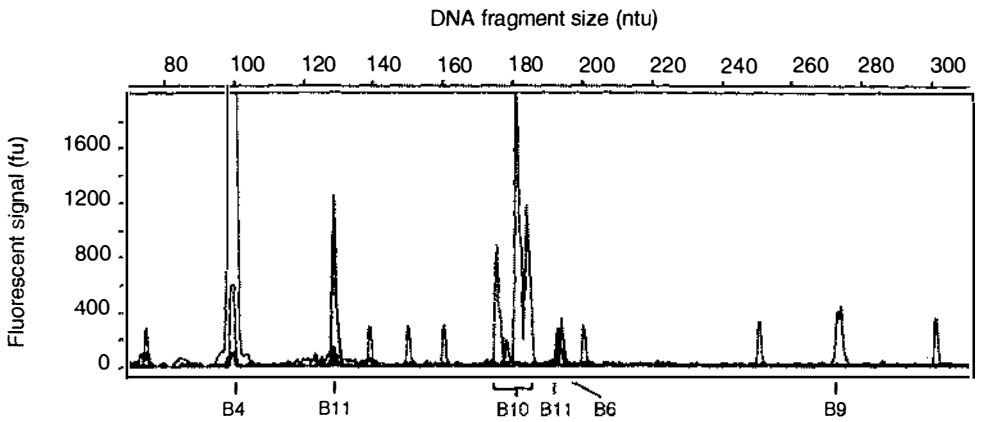


Fig. 3.29. Electropherogram of an endophyte microsatellite fingerprint.

Genomic DNA from Tf28 was amplified with fluorescently-labelled Primer Set I, and the products were separated by PAGE. GeneScan 2.1 software was used to generate the electropherogram, and the sizes of the products were determined by reference to GS-500 TAMRA internal size standards. Peaks in red, blue, green, and black correspond to the size standards, 6-FAM (B4 and B6), TET (B9 and B10), and HEX (B11)-labelled products, respectively.

Table 3.3. Allele sizes for microsatellite loci of Tf28 from electropherogram of Fig. 3.29

Colour	Dye label	Peak size (ntu)	Peak height (fu)	Deduced microsatellite locus
blue	6-FAM	100.00	4726	B4
blue	6-FAM	193.75	348	B6
green	TET	175.14	909	B10
green	TET	176.15	382	B10
green	TET	180.93	2054	B10
green	TET	181.93	990	B10
green	TET	183.81	1191	B10
green	TET	184.81	632	B10
green	TET	272.23	407	B9
green	TET	273.22	447	B9
yellow	HEX	128.65	1275	B11
yellow	HEX	192.65	273	B11
yellow	HEX	193.64	127	B11
red	TAMRA	75.00	292	GS-500 TAMRA
red	TAMRA	100.00	113	GS-500 TAMRA
red	TAMRA	139.00	298	GS-500 TAMRA
red	TAMRA	150.00	296	GS-500 TAMRA
red	TAMRA	160.00	301	GS-500 TAMRA
red	TAMRA	200.00	304	GS-500 TAMRA
red	TAMRA	250.00	337	GS-500 TAMRA
red	TAMRA	300.00	366	GS-500 TAMRA

3.2.2.8.1 Comparison of Fingerprints Analysed on an ABI Prism 377 DNA Sequencer and an ABI Prism 310 Genetic Analyser

DNA fragment size estimation using ABI Prism analysers depends on the electrophoretic mobility of the single-stranded DNA fragment relative to the electrophoretic mobilities of a set of standard fragments of known sizes. The use of different separation matrices can alter the electrophoretic mobility of a DNA molecule in a sequence-specific manner, thus resulting in different size-calling (Rosenblum et al., 1997). Indeed, discrepancies in size-calling of up to 6 ntu were observed between identical samples which had been analysed on both the slab gel based ABI Prism 377 DNA Sequencer and the capillary polymer based ABI Prism 310 Genetic Analyser (Section 2.17). To quantify the extent of the deviation precisely at each microsatellite locus, the sizing of fingerprint patterns determined on both models of ABI Prism analyser was compared. Specifically, the fingerprints analysed were from isolates Lp6, Fp1, Tf15, E348, E32, E56, and E798 amplified with Primer Set I; and isolates E1021, E422, E32, E354, and E1040 amplified with Primer Set II (Section 2.13). These results and the calculated offsets are shown in Tables 3.4 and 3.5. Generally the sizes determined on the ABI Prism 377 were larger than those on the ABI Prism 310 for all loci except B1. The offsets ranged between -0.8 to +5.9 ntu, with loci B4 and B5 showing the greatest differences with average offsets of 4.3 ntu and 5.0 ntu respectively.

It was hoped that precise universal offsets might be calculated at each locus, so that data generated on either model of ABI Prism analyser could be readily compared in a common database, but the range of offset sizes at many of the loci were wider than expected. For all loci except B4, the offset range was less than ±0.9 ntu from the average offset, but for locus B4 the offset range was ±1.8 ntu. Despite these wide ranges, the calculated average offsets have still been used in this study to make comparisons between the genotypes analysed by either ABI system. In the analysis of human and livestock microsatellite genotyping, it is common practise to compare data generated on either model of ABI Prism by correcting the data by a locus specific offset, but in these cases only single species are compared, and these are assumed to have conserved sequences

Table 3.4. Calculation of the average size differences of ML-PCR Primer Set I products when automatically analysed on an ABI Prism 377 slab gel system compared to an ABI Prism 310 capillary electrophoresis system.

Isolate	Allele size (ntu)														
	B4			B6			B9			B10			B11		
	377	310	offset	377	310	offset	377	310	offset	377	310	offset	377	310	offset
Lp6	100.1	95.0	5.1	187.6	185.4	2.2	247.3	245.0	2.2	178.1	175.8	2.3	177.0	176.4	0.6
Fp1	84.8	87.1	2.3	178.7	177.1	1.6	-	-	-	160.7	157.8	2.9	120.7	118.5	2.2
	104.4	98.9	5.5							195.8	192.9	2.9			
Tf15	101.0	95.1	5.9	186.6	185.5	1.1	247.5	245.1	2.4	172.1	169.8	2.3	128.8	126.6	2.2
										178.0	175.7	2.3	165.2	164.4	0.8
E348	128.3	124.6	3.7	171.8	170.5	1.3	-	-	-	192.4	190.8	1.6	128.2	127.0	1.2
E32	99.4	95.5	3.9	187.6	186.1	1.5	271.9	269.6	2.3	171.8	170.5	1.3	165.3	164.5	0.8
E56	83.0	79.1	3.9	178.8	177.4	1.4	-	-	-	195.0	192.8	2.2	128.3	126.1	2.2
E798	84.3	80.0	4.3	178.7	177.2	1.5	-	-	-	186.3	184.0	2.3	112.7	110.5	2.2
average offset			4.3			1.5			2.3			2.2			1.5
offset range			3.6			1.1			0.2			1.6			1.4
n			8			7			3			9			8

Table 3.5. Calculation of the average size differences of ML-PCR Primer Set II products when automatically analysed on an ABI Prism 377 slab gel system compared to an ABI Prism 310 capillary electrophoresis system

Isolate	Allele size (ntu)											
	B1			B2			B5			B9		
	377	310	offset	377	310	offset	377	310	offset	377	310	offset
E1021	-	-	-	147.5	143.8	3.7	75.1	70.7	4.4	180.1	179.1	1.0
E422	-	-	-	160.9	158.5	2.4	85.8	80.1	5.7	-	-	-
E32	304.7	305.2	-0.5	-	-	-	-	-	-	182.9	182.2	0.7
E354	302.5	-	-	225.9	221.8	4.1	75.1	70.2	4.9	180.2	179.3	0.9
E1040	314.1	314.9	-0.8	-	-	-	-	-	-	174.1	173.1	1.0
average offset			-0.7			3.4			5.0			0.9
offset range			0.3			1.7			1.3			0.3
n			2			3			3			4

flanking the microsatellite. As the Epichloë endophytes represent a wide assemblage of species, the sequence differences in the flanking regions affect the migration and hence allele sizing on both systems, thus resulting in the wide range of offsets observed. For more accurate offset calculation, sequence specific offsets would need to be determined at each locus for each species individually (pers. comm. Mark Holloway, PE Applied Biosystems).

3.2.3 APPLICATION OF THE PCR-BASED MICROSATELLITE FINGERPRINTING ASSAYS

3.2.3.1 Microsatellite Fingerprinting of Epichloë Endophyte Isolates

Having established optimum conditions for automated analysis (Section 3.2.2.8), fungal genomic DNA from 80 Epichloë endophyte isolates were amplified with both Primer Sets I and II in separate multiplex reactions. The dye-labelled PCR products were automatically analysed on either ABI Prism model 377 or 310 genetic analysers (Section 2.17), and the sizes of the products were entered into a database. A summary of the genotypes from these isolates is shown in Table 3.6. Microsatellite electropherogram files from all samples are available on the CD-ROM with details in Appendix 3.

3.2.3.2 Application of Microsatellite Fingerprinting Assay in a Blind Test

To test the robustness of the microsatellite fingerprinting assay for identifying endophyte strains in planta, twelve samples of plant material from endophyte-infected perennial ryegrass, tall fescue and meadow fescue were submitted blind by an independent researcher at AgResearch (M. J. Christensen). DNA was isolated from these samples using the FastDNA Kit H (Section 2.5.3) and amplified with Primer Set I in single-dye PCR reactions (Section 2.13.6). The products from each reaction were pooled and submitted for automatic analysis on an ABI Prism 377 genetic analyser (Section 2.17). Alleles for each sample were identified, and genotypes were compared with those entered

Table 3.6. Allele sizes of Epichloë endophyte microsatellite loci determined by ABI Prism automatic analysis.

Taxonomic group	Isolate	Allele size (ntu)								
		Primer Set I					Primer Set II			
		B4	B6	B9	B10	B11	B1	B2	B5	B9
LpTG-2	Lp1	100.0 119.8	172.4 187.6+	247.4+	169.3+ 178.1+	119.8	293.7	160.8	105.2	172.2 187.6
	Lp2	100.0 120.0	172.4 187.5	247.3+	169.3 178.1+	119.6	293.7	160.8	105.4	172.2 187.4
LpTG-1	Lp5	100.0	187.7	247.3+	178.1+	177.0+	293.7	-	-	158.4
	Lp6	100.1	187.6	247.3+	178.0+	176.9+	293.7	-	-	158.2
	Lp13	100.0	187.7	247.4+	178.0+	177.0+	293.7	-	-	158.2
	Lp19	100.0	187.6	247.4+	178.0	180.8	293.7	-	-	158.3
	Lp20	100.0	187.6	247.4+	178.1+	180.9	293.7	-	-	158.3
	Lp21	100.0	187.6	247.4+	178.1	180.9	293.7	-	-	158.2
	Lp7	100.0	187.6+	247.4+	178.1	188.9	293.7	-	-	158.2
	Lp14	100.0	186.6+	271.2+	163.5	132.8	293.7	-	-	181.9
	Lp9	100.1	187.6	248.3+	178.0+	188.8+	293.7	-	-	159.2
	AR1	100.0	187.6	246.4+	178.0+	149.8	293.7	-	-	157.3
	AR1WF	100.0	187.6	246.4+	178.1+	149.8+	293.7	-	-	157.3
	AR1CY	100.0	187.5	246.3+	178.0+	149.8	293.7	-	-	157.4
	EndoSafe	100.0	187.7	247.4+	178.0+	241.4+	293.7	-	-	158.0+

Table 3.6. ***continued***

Taxonomic group	Isolate	Allele size (ntu)								
		Primer Set I					Primer Set II			
		B4	B6	B9	B10	B11	B1	B2	B5	B9
LpTG-1 cont.	A9501	100.3	187.7+	247.5+	178.1+	177.0+	293.7	-	-	158.2
	CBS232.84	100.2	187.6	247.3+	178.1+	176.9+	293.7	-	-	158.3
FaTG-1	Tf5	100.0 103.9	193.7	272.1+	160.7+ 172.2+ 186.7+	149.9 192.7+	293.6	-	74.8+	180.0 182.9
	Tf27	100.0 103.2	196.8	272.2+	155.0+ 163.5+ 180.9+	172.9+ 197.0+	293.6	-	75.0+	180.0 183.0
	Tf28	100.0	193.8	272.2+	175.1+ 180.9+ 183.8+	128.7 192.7+	293.7	-	74.8+	180.0 182.9
	Tf35	99.9 103.7	196.9	272.2	155.0+ 163.5+ 181.0+	173.0+ 197.0+	293.6	-	75.1	180.0 182.9
	AR542	100.3 104.0	193.8	272.2+	163.5+ 172.3+	181.1 192.7+	293.6	-	75.0	180.0 183.0
FaTG-2	Tf13	100.5	187.6	271.3+	172.1+ 186.8+	128.7+ 141.0	293.7	-	-	181.8 186.0
	Tf15	101.0	186.6+	247.5+	172.1+ 178.0+	128.8+ 165.2+	293.6	-	-	158.3 187.1

Table 3.6. ***continued***

Taxonomic group	Isolate	Allele size (ntu)								
		Primer Set I					Primer Set II			
		B4	B6	B9	B10	B11	B1	B2	B5	B9
FaTG-2 cont.	Tf20	100.3	187.6	247.4+	172.1+ 178.0+	128.6 184.8+	293.7	-	-	158.2 187.1
FaTG-3	Tf16	120.4	171.6+	-	172.2+ 180.9+	128.6	295.6	144.3	-	186.2
	Tf18	120.3	172.6	-	172.2+ 180.9+	128.6	291.8	144.4	-	186.0
	AR572	120.3	172.6+	-	172.2 181.0+	128.6	291.7	144.4	-	186.1
	AR577	119.9	171.8+	-	172.1+ 180.9+	128.6	295.6	144.4	-	186.0
FpTG-1	Fp1	84.8 104.4	178.7	-	160.7+ 195.8+	120.7	298.5	-	75.1	162.8+ 179.3+
	Fp2	84.7 103.7	178.7	-	160.6 195.7+	120.7	298.4	-	75.1	163.0 178.9
	Fp4	84.7 103.9	178.7	-	172.1+ 192.7	120.7	295.6	-	74.4+	163.2+ 179.7
E. typhina	E8	120.5	172.5+	-	178.1	-	291.8	162.7	104.4	-
	E348*	124.6	170.5	-	190.8	127.0	-	147.9+	90.7	-
	E358*	116.0	170.5	-	182.0	106.8	-	154.3+	80.2+	-
	E425*	115.8	-	-	179.0	-	-	130.6+	80.2+	-

Table 3.6. ***continued***

Taxonomic group	Isolate	Allele size (ntu)								
		Primer Set I					Primer Set II			
		B4	B6	B9	B10	B11	B1	B2	B5	B9
E. typhina cont.	E428*	115.9	-	-	178.3 184.6	-	-	160.6+	80.0+	-
	E431*	115.9	-	-	195.9	-	-	130.6+	80.2+	-
	E470*	116.0	170.2	-	182.0	-	-	152.1+	80.1+	-
	E505*	104.2	-	-	175.4	-	-	139.0+	-	-
	E1021*	99.6	-	-	179.0	-	-	143.8+	70.7+	179.1+
	E1022*	99.5	-	-	178.5	-	-	143.8+	70.7+	179.1+
	E1032*	102.3	-	-	184.0	-	-	152.0+	70.8+	165.0+
	E1033*	102.4	-	-	183.9	-	-	152.0+	70.7+	164.9+
	E2466*	116.0	170.2	-	175.9	-	-	156.5+	80.3+	-
E. clarkii	E422*	116.0	-	-	184.6	-	-	158.5+	80.0+	-
	E426*	116.1	-	-	184.5	-	-	158.5+	81.0+	-
	E427*	116.0	-	-	185.3	-	-	160.5+	80.0+	-
E. festucae	E28*	95.4	186.0	269.6	176.3	114.4	301.4+	-	-	182.4
	E32*	95.5	186.0	269.6	170.5	164.5	305.2	-	-	182.2+
	E186*	95.5	185.8	269.8	176.1	147.2	301.4+	-	-	182.3
	E189*	95.4	186.0	269.7	170.2	164.4	304.3+	-	-	182.1
	E434*	95.4	185.7	270.0	176.0	147.2	304.4+	-	-	182.2
	Fr1	100.0	186.6	272.1+	172.1+	165.3	304.9	-	-	182.9
	Frc5	100.5	192.5+	273.2+	178.0+	169.1+	301.8	-	-	184.0

Table 3.6. ***continued***

Taxonomic group	Isolate	Allele size (ntu)								
		Primer Set I					Primer Set II			
		B4	B6	B9	B10	B11	B1	B2	B5	B9
E. festucae cont.	Frc7	100.0	187.7	272.1+	178.0+	149.9	301.8	-	-	181.9
	Frr1	100.0	175.7	272.1+	166.3+	116.8	307.7	-	-	182.6
	Fg1	100.0	187.7	279.0+	195.7+	128.7	296.4	-	-	189.7
	Fl1	100.0	187.5	272.2+	178.0+	116.8+	296.4 302.0	-	-	182.9 190.1
E. elymi	E56*	79.1	177.4	-	192.8	126.1	286.7	-	-	170.2
	E184*	79.3	177.1	-	192.6	126.5	285.7+	-	-	170.1
E. amarillans	E52*	-	177.2	-	167.6	138.3	297.7	-	-	-
	E57*	-	180.2	-	181.3	138.2	298.6	-	-	-
E. baconii	E421*	-	-	-	169.5	131.0	-	-	-	182.9+
	E424*	-	-	-	170.0	131.0	-	-	-	178.8+
	E1031*	-	-	209.1	167.5	147.1	288.7+	-	-	161.8+
E. bromicola	E501*	-	177.2	-	189.7	110.8	310.2	-	-	162.7+
	E502*	-	177.3	-	189.6	110.7	292.4	-	-	162.7+
	E798*	80.0	177.2	-	184.0	110.5	291.5+	-	-	161.6+
	E799*	79.9	177.3	-	184.0	110.5	292.4	-	-	161.4+
E. sylvatica	E354*	107.9	177.0	-	170.3	-	-	221.8+	70.2+	179.3+
	E503*	99.2	-	-	172.9	-	-	137.1+	70.2+	-

Table 3.6. ***continued***

Taxonomic group	Isolate	Allele size (ntu)								
		Primer Set I					Primer Set II			
		B4	B6	B9	B10	B11	B1	B2	B5	B9
E. glyceriae	E277*	78.1	-	-	154.7	118.4	294.9+	-	-	-
	E2772*	78.2	-	-	154.6	118.4	294.9+	-	-	-
E. brachyelytri	E1040*	90.5	-	-	166.8	134.2	314.9	-	-	173.1
	E1045*	90.4	-	-	166.7	134.1	305.9	-	-	173.2+
	E1046*	90.5	-	-	166.8	134.2	305.8	-	-	173.2+
Neotyphodium spp.	HaB	83.8	178.8	-	184.0 195.7	128.6 141.0	287.2 293.6	-	73.1	162.1 178.0
	Hd1	111.5	161.0 173.7	-	172.1 192.7	112.9	309.4	155.3	74.8	-
	AR17	100.1	186.6+	278.9+	183.8+	153.8	296.4	-	-	189.9

+ indicates that a peak, approximately 1 ntu larger than that given in the table, was also detected

* indicates that the automatic analysis of this fingerprint was performed on an ABI Prism 310 genetic analyser, whereas all other fingerprints were analysed on an ABI Prism 377

- indicates that no product was detected at this locus

in the database. In all cases a correct match was found with a taxonomic grouping and isozyme phenotype (if available) that had been previously determined for that isolate (Lp1, Lp5, Lp14, AR1, Tf13, Tf15, Tf27, AR542, Tf16, Fl1, Fp1, and AR17) using a combination of morphological, biochemical, and genetic criteria (results not shown).

3.2.3.3 Analysis of a DNA Deletion in B1 Amplified Alleles

As reported in Section 3.2.2.7.1, representative isolates of all ten *Epichloë* spp. were screened through ML-PCR assays of microsatellite loci B1, B2, B5, B7, B8, and B9. For a representative sample of isolates that did not amplify using the standard ML-PCR conditions, further work was done by adjusting annealing temperature from 65°C, to 60°C and 55°C (Section 2.13.2), to lower the annealing stringency. Products were only obtained from isolates when the annealing temperature was reduced to 55°C for the B1 assay. Therefore, all isolates that did not amplify at 65°C for locus B1 (Fig. 3.26A), *E. typhina*, *E. clarkii*, *E. baconii*, as well as *E. sylvatica*, were screened with the annealing temperature at 55°C, and these products are shown in Figure 3.30A. Several isolates (E358, E425, E428, E431, E470, E505, E2466, E354, and E503) had B1 alleles of around 240 bp, a result that was also observed for the amplification of *E. sylvatica* isolates under standard PCR conditions (Fig. 3.26A). This is significantly smaller than the product size expected at the B1 locus (around 300 bp), and the difference is greater than one would expect solely from variation in the number of microsatellite repeats. Therefore, the B1 products of E354, E503, E358, E502, E8, and Lp6 were fully sequenced (Section 2.15), and aligned (Section 2.18.1) with the original B1 sequence from *E. bromicola* (Groppe et al., 1995), to characterise the size reduction. The sequence alignment, shown in Figure 3.31, reveals a 73 bp deletion relative to the original *E. bromicola* isolate NF62A, for isolates E354, E503, and E358. It was therefore assumed that all B1 alleles around 240 bp long contained the 73 bp deletion.

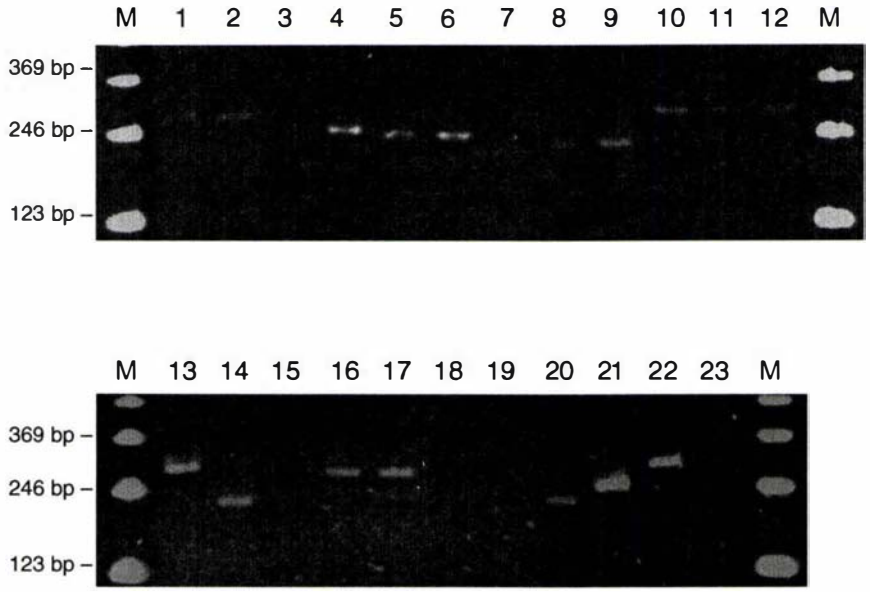
The origin and evolutionary distribution of the deletion product was analysed by mapping the occurrence of deletion to a phylogeny of European *Epichloë* spp. which was based on *tub2* sequence data (Figure 3.30B). The deletion was confined to a clade

Fig. 3.30. Distribution of a 73 bp B1 deletion product across *Epichloë* species

(A) Screening ML-PCR B1 products from *Epichloë typhina*, *E. clarkii*, *E. baconii*, and *E. sylvatica* isolates for deletion product. Agarose gel (3%) separation of *Epichloë* isolates amplified in the B1 ML-PCR assay with the annealing temperature reduced to 55C. Reactions contained genomic DNA from isolates: E8 (lane 1), E348 (lane 2), E358 (lane 3), E425 (lane 4), E428 (lane 5), E431 (lane 6), E469 (lane 7), E470 (lane 8), E505 (lane 9), E1021 (lane 10), E1022 (lane 11), E1032 (lane 12), E1033 (lane 13), E2466 (lane 14), E422 (lane 15), E426 (lane 16), E427 (lane 17), E421 (lane 18), E424 (lane 19), E354 (lane 20), E503 (lane 21), and Lp6 (lane 22). Lane 23 contained PCR negative control and M contains 123 bp ladder.

(B) Distribution of the 73 bp B1 deletion product with respect to the evolution of European *Epichloë* species. A strict consensus cladogram from the six most parsimonious trees derived from a branch-and-bound search on *tub2* intron sequences (Leuchtmann and Schardl, 1998) is shown, with numbers on branches indicating the percentage of trees possessing the branch in 450 bootstrap replications. Isolates have been marked with a d if it contains the 73 bp deletion, f if it does not, and n if no B1 product was amplified. All other isolates were not tested.

A


M 1 2 3 4 5 6 7 8 9 10 11 12 M
369 bp –
246 bp –
123 bp –
M 13 14 15 16 17 18 19 20 21 22 23 M
369 bp –
246 bp –
123 bp –

B

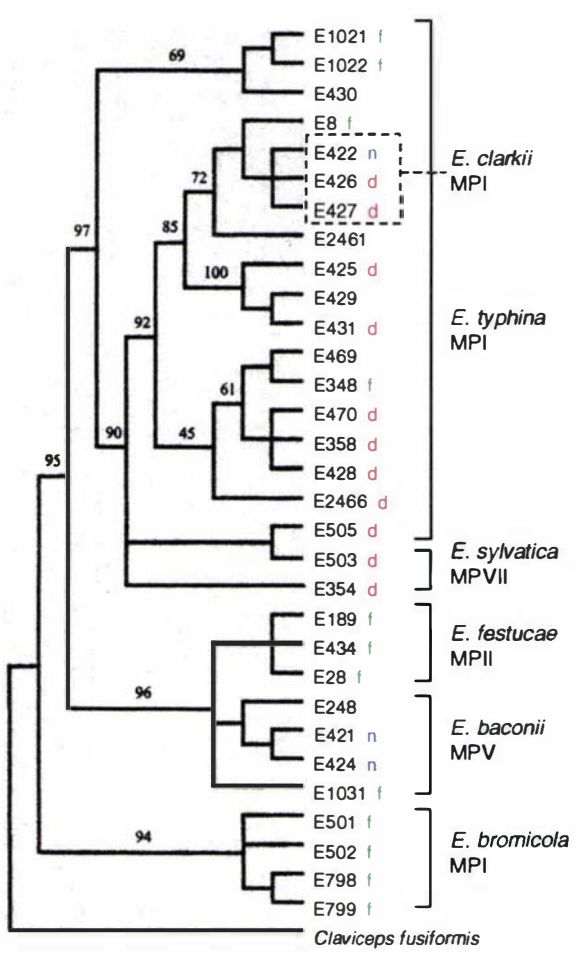

E1021 f
E1022 f
E430
E8 f
E422 n
E426 d
E427 d
E2461
E425 d
E429
E431 d
E469
E348 f
E470 d
E358 d
E428 d
E2466 d
E505 d
E503 d
E354 d
E189 f
E434 f
E28 f
E248
E421 n
E424 n
E1031 f
E501 f
E502 f
E798 f
E799 f
Claviceps fusiformis
69
72
97
85
100
92
61
90
45
95
96
94
E. clarkii MPI
E. typhina MPI
E. sylvatica MPVII
E. festucae MPII
E. baconii MPV
E. bromicola MPI

Fig. 3.31. Sequence alignment of B1-amplified ML-PCR products of Epichloë endophyte isolates

The DNA sequences of B1 amplified products from *E. typhina* isolates E8 and E358, *E. bromicola* isolates E502 and NF62A, *E. sylvatica* isolates E354 and E503, and *N. lolii* isolate Lp6 were aligned using the PILEUP programme of the Wisconsin Package with the gap penalty set to one and the gap extension penalty set to zero. Gaps (.) were introduced to facilitate sequence alignment. The $(AAG)_n$ microsatellite sequence is highlighted in blue.

```
        1                                                        50
 E354   CAATA.CGTC AGCTAGGAAT GTTGCATACT TGA.......  ..........
 E503   CAATA.CGTC AGCTAGGAAT GTTGCATACT TGA.......  ..........
 E358   CAATA.CGTC AGCTAGGAAT GTCGCGTACT TGA.......  ..........
 E502   CAATA.CGTC AGCTAGGAAT GTTGCATACT TGAT.TT..C CATCCATCTG
NF62A   CAATA.CGTC AGCTAGGAAT GTTGCATACT TGAT.TT..C CATCCATCTG
  Lp6   CAATATCGTC AGCTAGGAAT GTTGCATACT TGAT.TT..C CATCCGTCTG
   E8   CAATA.CGTC AGCTAGGAAT GTCGCATACT TGATATTATC CATCG...TG

        51                                                      100
 E354   .......... .......... .......... .......... ..........
 E503   .......... .......... .......... .......... ..........
 E358   .......... .......... .......... .......... ..........
 E502   GCTCTAGACC TGATGACAGA TTCCATGGTC GAACCAACGC AATCGCCCCA
NF62A   GCTCTAGACC TGATGACAGA TTCCATGGTC GAACAAACGC GATCGCCCCA
  Lp6   GCTCTTTATC TGATGACAGA TTCCATCGTC GAACCAACGC GATCGCCCCA
   E8   GCTCTTGATC TGATGACAGA TTCCATCGTC GAACCAACGC AATCTCCCCA

        101                                                     150
 E354   .........T ATGTCCACGA AAAGAAGAAA AGACAATATG CAACGTCTGA
 E503   .........T ATGTCCACGA AAAGAAGAAA AGACACTATG CAACGTCTGA
 E358   .........T ATGTCCGCGA AAAGAAGGAA AGAAACTATG CATCATCTGA
 E502   AGCTCGTTTT ATGTCGACGA AAAGAAGAAA AGAAACTATG CAACGTCTGA
NF62A   AGCTCGTTTT ATGTCGACGA AAAGAAGAAA AGAAACTATG CAACGTCTGA
  Lp6   AGCTCGTTTT ATGTCCACGA AAAGAAGAAG AGAAACTATG CAACGTCTGA
   E8   AGCTCGTTTT ATGTCCATGA AAAGAAAAAA AGAAACTATG CAACGTCTGA

        151                                                     200
 E354   AGAGTAAACC AAGAACAAGA AGAAGAAGAA G.........  ..........
 E503   AGAGTAAAAC AAGAAGAAGA AGAAGAAGAA GAAGAAGAAG AAGAAGAAGA
 E358   AGAGTGAAAC AAGAAGAAGA A.........  ..........  .AGAAAAATG
 E502   AGAGTTAAAG AAGAAGAAGA AGAAGAAGAA G.........  ..........
NF62A   AGAGTTAAAG AAGAAGAAGA AGAAGAAGAA GAAGAAGAAG AAGAAGAAGA
  Lp6   AGAGTAA...  ..........  ..........  ....TAAAAA AGGAAGAAGA
   E8   AGAGTAA...  ..........  ..........  ..........  ......ACCA
```

```
       201                                                  250
 E354  .......... ..AAAAGACG GCCCAACATC AAGATTCATT CGTCAACCTA
 E503  AGAAGAAG.. ..AAAAGACG GCCCAACATC AAGATTCATT CGTCAACCTA
 E358  AGAAGAAG.. ..AAAAGACG GCCCAACATC AAGATTCATT CGTCAACCTA
 E502  .......... .AAAGAGACG GCCCAACATC AAGATTCATT CGTCAACCTA
NF62A  AGAAGAAGAA GAAAGAGACG GCCCAACATC AAGATTCATT CGTCAACCTA
  Lp6  AGAAGAAGAA ..AAAAGACG GCCCAACATC AAGATTCATT CGCCAACCTA
   E8  AGAAGAAGAA GAAA.AGACG GCCCAACATC AAGATTCATT CGTCAACCTA

       251                                                  300
 E354  TAGCCTTGAT CCAGGCCAAA TGGGTTCTCT CTCATATACC TACTAACATC
 E503  TAGCCTTGAT CCAGGCCAAA TGGGTTCTCT CTCATATACC TACTAACATC
 E358  TAGCCTTGAT CTAGGGCAAA TGGGTTCTCT CTCATATACC TACTAACATC
 E502  TAGCCCTGGT CCAGACCAAA TGGGTTCTCT CTCATATACC TACTAACATC
NF62A  TAGCCCTGGT CCAGACCAAA TGGGTTCTCT CTCATATACC TACTAACATC
  Lp6  TAGCCTTGAT CCAGACCAAA TGGGTTCTCT CTCATATACC TACTAACATC
   E8  TAGCCTTGAT CCAGGGCAAA TGGGTTCTCT CTCATATACC TACTAACATC

       301                   325
 E354  GGCCTGATAG CAAAGTTGAT TCAGG
 E503  GGCCTGATAG CAANNNTGAT TCAGG
 E358  GGCCTGATAG CAAAGTTGAT TCAGG
 E502  CGCCTGATAG CAAAGTTGAT TCAGG
NF62A  CGCCTGATAG CAAAGTTGAT TCAGG
  Lp6  CGCCTGATAG CAAAGTTGAT TCAGG
   E8  GGCCTGATAG CAAAGTTGAT TCAGG
```

containing *E. typhina*, *E. clarkii*, and *E. sylvatica,* and more specifically, was found in both of the *E. sylvatica* isolates tested, two of the three *E. clarkii* isolates tested, and dispersed throughout *E. typhina.*

3.2.3.4 Inheritance of Microsatellite Alleles in an *E. festucae* Sexual Cross

Fungal genomic DNA from 42 randomly selected progeny of a sexual cross between *E. festucae* isolates E189 and E434, were made available for the analysis of the segregation of microsatellite alleles (H. H. Wilkinson). In this cross, E189 (Primer Set I genotype: 95.4 (B4), 186.0 (B6), 269.7 (B9), 170.2 (B10), and 164.4 ntu (B11)) was used as the stromal parent, and E434 (Primer Set I genotype: 95.4 (B4), 185.7 (B6), 270.0 (B9), 176.0 (B10), and 147.2 ntu (B11)) was used as the spermatial parent in an artificially induced mating. Genomic DNA from each progeny, prepared using the method of Byrd et al. (1990), was amplified with Primer Set I and analysed on an ABI Prism 310 Genetic Analyser.

As expected, the progeny contained B4, B6, and B9 alleles identical to the parental types. However, the genotypes at loci B10 and B11 were variable, and all four expected genotypes at these loci were observed, as summarised in Figure 3.32. Nine progeny had identical genotypes to parental isolate E189, and 19 progeny had identical genotypes to parental isolate E434. Both possible recombinant genotypes were represented by seven progeny each.

To show that the inheritance of the microsatellite genotypes conformed to Mendel's laws of segregation and independent assortment, data was tested with the chi-square (χ^2) test for goodness-of-fit between the observed and expected results.

Mendel's first law, the principle of segregation, states that the two members of a gene pair (alleles) segregate from each other in the formation of gametes (Russell, 1990). Under the null hypothesis that microsatellite alleles segregate at the B10 and B11 loci, it is expected that the allele frequencies at each locus conform to a 1:1 ratio in the progeny,

P generation

	E189	×	E434
genotypes	170 164		176 147

F_1 generation

frequencies of genotypes

×	170	176	subtotal
147	7	19	26
164	9	7	16
subtotal	16	26	**42**

Fig. 3.32. Segregation of B10 and B11 microsatellite alleles in a sexual *E. festucae* cross

E. festucae isolate E189 (stromal parent) was crossed with E434 (spermatial parent) and 42 progeny were randomly selected. Parental and progeny isolates were microsatellite fingerprinted using Primer Set I, and allele polymorphisms were observed at loci B10 and B11. Genotypes are shown with B10 allele sizes are indicated in blue and B11 allele sizes are indicated in green. Allele sizes are given in nucleotide units (ntu). Frequency of progeny are given in box below.

which represents frequencies of 21 each. The observed allele frequencies at each locus are 16 and 26 (Fig. 3.32), but this difference is not considered statistically significant at the 5% level using the chi-square test (see Appendix 4). Thus the null hypothesis that the microsatellite alleles are segregating in accordance to Mendel's first law is supported.

Mendel's second law, the principle of independent assortment, states that the genes for different traits assort independently of one another during gametogenesis (Russell, 1990). Under the null hypothesis that microsatellite alleles of loci B10 and B11 assort independently of one another, the expected frequencies of the four possible progeny genotypes in the haploid dihybrid cross should fit a 1:1:1:1 ratio, which is a frequency of 10.5 each. The observed data (Fig. 3.32) clearly differ from these expected values, and this difference is statistically significant at the 5% level using the chi-square test (see Appendix 4). Thus the null hypothesis is rejected. The likely explanation for non-independent assortment is that loci B10 and B11 are linked in *E. festucae*. If this is indeed the case, the genetic map distance between these loci is equal to the percentage of recombinant genotypes, which is 33.3 map units (mu).

3.3 DISCUSSION

The molecular cloning of a set of microsatellite loci from Epichloë grass endophytes and the development of an automated PCR fingerprinting method based on sequences at those loci, that distinguishes the different groups of isolates to the level of their isozyme phenotype grouping is described. Surprisingly, there are few reports to date on the cloning of microsatellites from fungi (Geistlinger et al., 1997; Groppe et al., 1995; Longato and Bonfante, 1997) despite their widespread use for kinship, population structure and mapping studies in plants and animals, though their popularity has been gaining in the past year (Bart-Delabesse et al., 1998; McKay et al., 1999). We are aware of only one other such study that utilises microsatellite markers as the basis of a fungal typing method with automated analysis, which is for *Aspergillus fumigatus* isolate identification (Bart-Delabesse et al., 1998).

The assay described in this study has been used in several applications, including the identification of endophytes in infected grass tissue, and tracking the inheritance of microsatellite alleles in an *E. festucae* genetic cross. The genotype data generated from the assay has also been shown to support evolutionary relationships between Epichloë endophyte species and taxonomic groups generated by molecular methods, and therefore may be used to supplement such studies.

3.3.1 AN ASSESSMENT OF THE PCR-BASED FINGERPRINTING METHODS

The ML-PCR method offers several advantages over RAPD and MP-PCR for endophyte identification, and it is the method of choice for further assay development. The main benefit of ML-PCR is that the high specificity of the primers gives the assay potential to be used on in planta samples, therefore eliminating the need to initially isolate the endophyte from the grass, as required with the other two methods. Another appeal of the ML-PCR method is that the identity of the amplified bands is known, and the

microsatellite locus copy number can be readily determined. This information is particularly useful for Epichloë endophyte isolates where many of the asexual endophytes are of interspecific hybrid origin, and the relationship between the hybrid and the parents can be seen. A prime example is illustrated by ML-PCR amplification of the B1 microsatellite locus in Fig. 3.5B, where Lp1 of the taxonomic group LpTG-2 shows two bands, whose sizes correspond to those of its parents, *N. lolii* and *E. typhina.* In contrast, RAPD-PCR and MP-PCR generates fingerprint patterns where the identity of the bands are unknown. Analysis of fingerprints of this type is laborious, and requires that standard samples are included in each run to make scoring consistent between runs.

A concern in developing the ML-PCR assay is whether there would be enough polymorphism at microsatellite loci to identify isolates to the required level. Fingerprints produced by MP-PCR were able to distinguish isolates intraspecifically, and the assay was reasonably reproducible. By contrast, RAPD-PCR did not show such high variability in fingerprint patterns, the reproducibility between runs was not reliable, and contamination of runs was occasionally experienced which is probably a consequence of using arbitrary primers at such low annealing specificity. Overall, RAPD-PCR did not appear to be a reliable method for fingerprinting endophytes so further development of this method was discontinued.

Consequently, it was decided to further develop the ML-PCR assay to include several informative polymorphic microsatellite loci. It was hoped that these loci could be amplified in a multiplex reaction using dye-labelled primers so that fingerprints could be analysed precisely using laser detection technology, such as that associated with the Applied Biosystems automatic sequencers.

3.3.2 DEVELOPMENT OF A PCR-BASED MICROSATELLITE FINGERPRINTING ASSAY

3.3.2.1 The Frequency of Microsatellites in Fungi

Based on Southern analysis, an average frequency of one $(CA)_n$ or $(GA)_n$ locus per 2 Mb of DNA, and one $(CAA)_n$, $(GAA)_n$, or $(ATC)_n$ locus per 2.5 Mb of the Lp1 genome was found. By comparison with similar estimates for plants, $(CA)_n$ and $(GA)_n$ frequencies range from 1/270 kb in wheat (Röder et al., 1995), to 1/1.2 Mb in tomato (Broun and Tanksley, 1996). The frequency of the same classes of microsatellites in mammals is even greater (Lagercrantz et al., 1993). However, these figures should be regarded as a minimum, since a single hybridising band in a Southern may correspond to more than one locus and, like the alternative approach of determining the number of positives in a library screen, they will also vary depending on the stringency of the hybridisation used. The effect of the latter was evident here in that not all of the positive clones identified had microsatellite sequences that were perfect matches with the probes used. For example, the B5 locus $(CATCTCATCA)_5$ and the B9 locus $(GAGAG)_2C(GAGGA)_2$ only partially match the sequences of the tri- and di- nucleotide repeat probes that were used for the isolation of these sequences.

It would also appear that the partial libraries made in M13mp19 were not as representative of the diversity of loci as the Southern blots had indicated. For example, the Southern analysis of Lp1 genomic DNA indicated that there were at least 20 $(CA)_n$ and $(GA)_n$ microsatellites in the size range of 100 – 1000 bp, used to construct the library, yet only three different microsatellite clone types were identified from the 24 clones screened. This was probably a consequence of preferential ligation of the smaller inserts into the M13mp19 vector, as all clones that were analysed had inserts of between 100 and 300 bp in size. In retrospect, small insert library construction using a random DNA fractionation method, such as shearing or sonication, would be favoured to generate inserts. Therefore, most, if not all of the genome would be represented in the small fragment size range. More accurate determinations of both the frequency and

diversity of microsatellites in fungi and other organisms will emerge from analysis of DNA sequence data generated from either partial or complete genome sequencing initiatives, such as that for *Saccharomyces cerevisiae* (Field and Wills, 1998; Richard and Dujon, 1996).

A search by Groppe et al. (1995) of fungal DNA sequences held in the GenBank and EMBL databases for all possible mono-, di-, and trinucleotide motifs (>20 bp) showed that the predominant microsatellite type found in fungi is $(AT)_n$; representing 42% of the microsatellites identified. The other most frequently occurring microsatellite types were in decreasing order: $(A)_n$, $(AAT)_n$, $(AAC)_n$, $(AAG)_n$, $(ATC)_n$, $(AC)_n$, and $(AG)_n$. Screening for the abundant $(AT)_n$ repeat class was not carried out in this study since these sequences are self- complementary, making it very difficult to isolate such sequences by solution hybridisation methodology (Kostia et al., 1995; Lagercrantz et al., 1993).

3.3.2.2 An Overview of the ML-PCR Assays

As shown in Figure 3.7 the ten microsatellite loci isolated contain a variety of sequence motifs ranging from repeats of dinucleotides to decanucleotides. To determine the degree of length polymorphism associated with these microsatellites, and those isolated by Groppe et al. (1995) and Dobson (1997), PCR assays were initially carried out with DNA from a collection of Epichloë endophytes, predominantly representative of asexual isolates from the forage grasses: perennial ryegrass, tall fescue, and meadow fescue. Further screening of representative isolates of the ten *Epichloë* spp. were carried out, and in total, fingerprinting assays utilising eight of the loci in two sets of primers, designated Primer Sets I and II, were devised.

The degree of length polymorphism at each locus was estimated by the number of alleles observed, and varied between eight and 25 alleles at each locus for the 80 isolates listed in Table 3.6. Allele numbers were recorded after the approximate standardisation of alleles for the ABI 377 DNA Sequencer, using the average offsets calculated in Tables 3.4 and 3.5 to convert data that was analysed on an ABI 310. The most

polymorphic loci were B10 and B11 with 25 alleles each, followed by B1 and B9 (using primer pair B9.1 and B9.2) with 20 alleles each, B2 and B6 with 14 alleles each, B4 with 12 alleles, and lastly B5 and B9 (using primer pair B9.1 and B9.4) with eight alleles each. The size ranges of alleles observed in the 80 isolates varied between 29 ntu (B1) and 133 ntu (B11) with an average range of 56 ntu. No products were amplified from isolates in several cases and this may be due to either: the absence of the microsatellite locus or at least one of its priming sites, or sufficient sequence divergence at the priming site so that the primer cannot anneal at the given stringency. Nonetheless, the non-amplification of alleles provides useful insights into the evolutionary relationships between groups of endophytes, and this is discussed further in Section 3.3.3.1.

Locus B11, serendipitously identified downstream of the HMG CoA reductase gene from strain Lp19 (Dobson, 1997), together with locus B10, are the most polymorphic microsatellite loci in this study. As HMG CoA reductase is an essential enzyme in the isoprenoid metabolism pathway, the transcription level of the gene encoding it is relatively high. Simple repeat sequence tracts are destabilised by high rates of transcription (Wierdl et al., 1996), so this could partially explain the high degree of polymorphism observed at the B11 locus. There is also good evidence to support the notion that B10 and B11 are linked loci (Section 3.2.3.4), thus B10 may also be found in an area of high transcriptional activity. The proximity to highly transcribed regions of the genome of the remaining microsatellite loci in this study are not known, though tract stability is also dependent on its length and 'purity'. This is presumably because the opportunity for a stable misalignment is lower in shorter tracts, and tracts interspersed with non-microsatellite sequences (Wierdl et al., 1996). Apart from locus B11 ($(GACA)_{18}$ in Lp19), which is both relatively long and continuous, there are no obvious relationships between the length and continuity of the microsatellite tract, and the degree of polymorphism at the other loci. In fact, most loci contain imperfect repeats (B4, B6, B7, B9, and B10) or are composed of compound microsatellites (B8, B9, and B10) in the isolates they were identified from.

An interesting observation that was made while screening with locus B11 is that alleles amplified from *Epichloë* spp. were generally shorter than those amplified from *Neotyphodium* isolates. The relevance of this is not known, and such a pattern was not seen in the other loci.

No microsatellite PCR products were obtained with any of the primer pairs with the outgroups *Claviceps purpurea* and *Echinodothis tuberiformis*, which are both from the same family as the Epichloë endophytes (Clavicipitaceae). This shows that these primers are highly specific for the Epichloë endophytes, demonstrating that the assay is not influenced by the presence of unrelated fungal endophytes or contaminating fungal species in the plant tissue.

3.3.2.3 Automated Fingerprint Analysis

While the microsatellite allele data generated by manual analysis of autoradiographs of ^{33}P labelled products was largely concordant with that generated by automated analysis of fluorescently labelled products, the latter method was superior in many respects. Automated analysis allows precise PCR product size estimation over a wide size range in a single run, with a sensitivity that reduces the amount of product required for analysis. This becomes particularly important in the detection of endophytes in planta, where the fungal biomass can be very low. Dye-labelled primers and products are able to be stored for long periods of time without signal decay and are safer to use than radioisotopes. Automated analysis is also faster since data is collected while the products are being electophoresed, thus eliminating the need for gel drying, film exposure, and development. The complexity of the banding pattern from a single product is reduced since only one strand of the product is labelled; this greatly simplifies the analysis of hybrid endophytes which have multiple alleles of similar size for some loci. The three-colour dye system allows products from different loci to be identified simultaneously, even if their peaks overlap, as shown in Figure 3.29. This cannot be done with manual methods, and separate gels are often required to analyse individual loci. Finally, the data are generated as the estimated size of the DNA fragment, enabling comparisons to be

readily made between laboratories, a task which is difficult to achieve with other typing methods, such as restriction fragment length polymorphism (RFLP) or RAPD-PCR analysis. Thus, microsatellite fingerprints may be generated at independent laboratories and submitted into a common database. However, as the estimated size of the fragment is dependent on its electrophoretic mobility, using a different separating matrix may alter the mobility of the DNA fragment, thus resulting in different size calling. Indeed this was the case when identical endophyte fingerprints were analysed with separation in both a non-crosslinked network polymer and a denaturing polyacrylamide gel. Unfortunately universal offsets could not be precisely determined for the endophytes as a group as they are such a diverse assemblage, though the average offsets calculated in this study may be used as an approximate guideline. Thus, when analysing fingerprints it is important to specify which model of genetic analyser was used when reporting its genotype.

A problem that was encountered when analysing fingerprints from the automated system was the inconsistent presence of split peak signals, 1 nt apart; this was a consequence of the inherent property of *Taq* polymerase to occasionally add a deoxyadenylate to the 3' terminus of the PCR product. The primer dependence of this property of *Taq* polymerase (Brownstein et al., 1996) was reflected in the frequent occurrence of split peaks for amplification at loci B2, B5, B9, B10, and B11. Single peaks predominated at loci B1, B4, and B6 and were assumed to be the products without the addition of a deoxyadenylate. Methods that would potentially eliminate this problem would be the use of a DNA polymerase such as *Pwo* or *Tfl* (which amplify only fragments with blunt ends), enzymatic removal of the overhang by T4 DNA polymerase (Ginot et al., 1996), or use of the 'PIGtailing' method (Brownstein et al., 1996).

Another difficulty when amplifying loci using a multiplex assay is that alleles can be amplified with widely different efficiencies. When examined on an automatic analyser, particularly strong signal peaks would often appear with false subpeaks of different colours directly beneath the main peak; this situation was often observed when analysing products from multiplex amplification with Primer Set I, B4 allele(s) amplified strongly and green and yellow subpeaks were often displayed under the main blue peak

(Fig. 3.29). The subpeaks, which are an artefact of the dye-detection system and the algorithms in the analytical software, were ignored when analysing the endophyte genotypes. A more serious problem was that strong signals were occasionally displayed as having flat peaks, thus fragment sizing would be imprecisely determined on the basis of the midpoint of the plateau. These issues can be resolved by amplifying products at each locus individually, followed by pooling products in proportions which would give them approximately the same amounts, or rerunning dilutions of the multiplexed sample so that the concentrated product is within the peak height range for accurate size calling.

3.3.3 APPLICATIONS OF THE MICROSATELLITE BASED FINGERPRINTING ASSAY FOR EPICHLOË ENDOPHYTES

3.3.3.1 Evolutionary Relationships Between Endophyte Groups are Generally Supported by Microsatellite Genotype Data

As outlined in the introduction, the evolutionary relationships between Epichloë endophyte species and taxonomic groups are complex, and there is very good evidence from molecular phylogenetic studies that the asexual *Neotyphodium spp.* have evolved from the sexual *Epichloë spp.* (Schardl, 1996a). The microsatellite genotype data shown in Table 3.6 shows support for many of these relationships.

3.3.3.1.1 The Evolutionary Relationships of Asexual Endophytes

The asexual endophytes from perennial ryegrass studied by Christensen et al. (1993) fall into two taxonomic groups; LpTG-1 (= *N. lolii*) and LpTG-2, with the former being further resolved by isozyme analysis into six different phenotype groupings. While most of the microsatellite loci were unable to resolve the *N. lolii* isolates to the level of the isozyme phenotype, locus B11, was particularly informative, with six different alleles being amplified. The other loci were less informative in resolving this relatively homogeneous group, though isolates Lp14 (loE) and Lp9 (loF), were frequently shown to

be different from the other *N. lolii* isolates. An interesting finding was that the endophyte isolate used in Grasslands Pacific EndoSafe, hereafter referred to as EndoSafe, shares the same alleles as *N. lolii* isolates of loA, loB and loD phenotypes at all loci except B11. This provides overwhelming support for the classification of EndoSafe as an *N. lolii* isolate, whereas originally Grasslands Pacific EndoSafe was thought to have been inoculated with the LpTG-2 isolate, Lp1. From molecular phylogenetic studies, the most closely related sexual ancestors of the *N. lolii* group are the *E. festucae* (Leuchtmann, 1994; Schardl et al., 1994). Locus B4 is particularly informative in demonstrating this evolutionary connection with both groups sharing a common allele.

LpTG-2 is represented in this study by just two isolates, Lp1 and Lp2 (Christensen et al., 1993) and both are known to be interspecific hybrids between *E. typhina* and *N. lolii* (Schardl et al., 1994). The hybrid nature of these isolates is supported here in that the two different alleles found in *E. typhina* isolate E8 and *N. lolii*, for loci B4 and B6 were both amplified from the LpTG-2 isolates. Alleles amplified in *N. lolii* isolates at loci B1, B9, and B10 were also present in LpTG-2.

A third type of asexual endophyte found within the perennial ryegrass, is represented by isolate AR17, has *E. festucae*-like morphological characteristics, though stroma production on infected plants has not been observed (pers. comm. Mike Christensen, Grasslands AgResearch). Mating attempts using AR17 (conidiating) cultures as spermatial material to inoculate stroma of *E. festucae* isolates have also been unsuccessful (pers. comm. Christopher Schardl, University of Kentucky). However, the microsatellite genotype of AR17 is highly similar to those of *E. festucae* isolates, particularly Fg1, where six of the nine alleles analysed are shared between these isolates (see Table 3.6). This level of variability is smaller than that observed between *E. festucae* isolates themselves.

The endophyte isolates from tall fescue have been resolved into three taxonomic groups (Christensen et al., 1993) and all are known to be interspecific hybrids (Tsai et al., 1994). The proposed evolutionary origins of these hybrids is complex in that multiple

hybridisation events with *Epichloë* species would be required to account for the diversity of *tub2* and other genes that are found in this group (Tsai et al., 1994). Loci B9, B10, and B11 are particularly supportive of a hybrid origin for this group of isolates as multiple alleles are present for all strains at locus B10, and for FaTG-1 and FaTG-2 at loci B11 and B9 (using primers B9.1 and B9.2). Typically, sexual species and the haploid *N. lolii* group have single alleles at these loci. Although the high degree of polymorphism observed at these loci obscures clear evolutionary origins of these hybrid groups, many common alleles were found between the tall fescue isolates and their proposed ancestors (Schardl, 1996a), which include *N. uncinatum, E. festucae* and *E. baconii* for FaTG-1; *E. festucae* and *E. baconii* for FaTG-2; and *E. typhina* and *E. baconii* for FaTG-3.

Only single alleles were amplified from *N. coenophialum* isolates at the B1 locus, though in a study by Groppe and Boller, DNA samples from *N. coenophialum* isolates were amplified using a reduced annealing temperature (50°C) and two bands were amplified from each isolate (Groppe and Boller, 1997). Of these bands, one was of constant size (approximately 300 bp), but the other was highly polymorphic and its size ranged between 400 and 600 bp. It will be interesting to sequence these larger alleles to determine whether the size increase is due solely to microsatellite expansion.

The isolates from meadow fescue used in this study were readily identified from the other endophytes by their distinctive microsatellite genotypes. While *N. uncinatum* has only a single *E. typhina*-like copy of the β–tubulin gene (*tub2*), the phylogeny generated from sequences for this gene contradicts that for the rDNA-ITS, suggesting that *N. uncinatum* is also a hybrid (Schardl, 1996a), a concept which is also supported by the presence of multiple bands from isozyme analysis (Leuchtmann, 1994). Further evidence from transcription elongation factor alpha (TEF) sequence data suggests that *N. uncinatum* is the product of interspecific hybridisation between *E. bromicola* and *E. typhina*, the *E. typhina* isolates being closely related to those from the grass host *Poa nemoralis* (pers. comm. Christopher Schardl, University of Kentucky). This relationship is strongly supported by the sharing of many microsatellite alleles between these groups,

particularly at loci B4 and B9, where the alleles found in *E. bromicola* and two *E. typhina* isolates E1021 and E1022, from *P. nemoralis*, are both observed in the *N. uncinatum* isolates. Furthermore, alleles amplified in *E. bromicola* at loci B6 and B10, and in *E. typhina* (isolates E1021 and E1022) at locus B5, are all found in *N. uncinatum.*

The final group of asexual endophytes used in the development of this microsatellite assay were two rather poorly described isolates, HaB and Hd1, from *Hordeum* grasses. The microsatellite genotypes of these isolates enables them to be readily distinguished from the other endophytes and from each other. From phylogenetic analysis of *tub2* and rDNA-ITS sequences (Section 4.2.7), HaB and Hd1 are thought to have interspecific hybrid origins involving *E. amarillans* and *E. elymi*, and *E. typhina* and *E. bromicola* respectively. The presence of multiple alleles at microsatellite loci B1, B9, B10, and B11 for HaB, and B6 and B10 for Hd1, supports the hybrid origins of these isolates, but direct correlation of these alleles to those of their putative ancestral species were not apparent. Common alleles were observed between Hd1 and *E. bromicola* at loci B11, and Hd1 and *E. typhina* at loci B2 and B5, but no common alleles were observed between HaB and its putative ancestors. A number of common alleles were observed between HaB and Hd1 and many of the asexual endophytes (particularly HaB with *N. uncinatum*) but it is unclear whether these have common ancestry.

3.3.3.1.2 The Evolutionary Relationships of Sexual Endophytes

Epichloë typhina and *E. festucae* were the only species of sexual endophytes where a substantial number of isolates were available to study. These isolates, on average, showed a higher degree of polymorphism than the groups of asexual isolates, which would be expected for an outcrossing sexual species. However, it is interesting to note that the stability of microsatellite repeat tracts is similar both during meiosis and mitosis (Strand et al., 1993), therefore the observed variation in polymorphism is likely to be due to non-random sampling of the isolates, rather than a genuine difference in amount of microsatellite polymorphisms generated between sexual and asexual groups.

An unexpected result was the amplification of multiple alleles at the B2 and B10 loci of *E. typhina* isolate E428. The multiple alleles at the B2 locus were observed when E428 DNA was amplified with primers B2.1 and B2.2 followed by separation on agarose gels (Fig. 3.26B), though only one allele was detected when amplified with Primer Set II followed by automated analysis. Two alleles were also amplified at the B1 locus of *E. festucae* isolate Fl1. These multiple alleles may be due to gene duplication events or aberrant chromosome segregation. Alternatively, these isolates may be the product of a hybridisation event, resulting in heteroploidy or aneuploidy, but this is highly unlikely as multiple alleles were observed at relatively few microsatellite loci. Leuchtmann has also observed occasional double-banded electromorphs during allozyme analysis of *Epichloë* species, and he considers that these are due to gene duplication events (Leuchtmann and Clay, 1990; Leuchtmann et al., 1994).

Fundamental relationships between *Epichloë* spp. were supported by microsatellite data, particularly with regard to the actual amplification of alleles from isolates. A *tub2* gene phylogeny of isolates representing all ten *Epichloë* spp. (Schardl et al., 1997) separates the species into two main clades containing *E. typhina*, *E. clarkii*, and *E. sylvatica* in one, and the remaining species in the other. This elementary division is supported by amplification of loci B1, B2, B5, and B11 (with the occasional exception), where out of all *Epichloë* spp. alleles were only amplified from isolates of *E. typhina*, *E. clarkii*, and *E. sylvatica* with loci B2 and B5. In contrast, alleles from loci B1 and B11 were amplified in all isolates except *E. typhina*, *E. clarkii*, and *E. sylvatica* (though *E. baconii* isolates, E421 and E424, did not amplify with the B1 primers either).

The major differences between *E. typhina* and *E. clarkii* are that the ascospores of the latter species undergo disarticulation within the ascus to form uniquely shaped part-spores, and *E. clarkii* also appears to be confined to the host genus *Holcus* (White, 1993). Despite their species status, *E. typhina* and *E. clarkii* isolates tend to be interfertile in artificial matings, and are grouped together in the same mating population (MP I; Leuchtmann and Schardl, 1998). Their close evolutionary relationship is reflected in the

similarities of their microsatellite genotypes, in particular the genotypes of *E. typhina* isolate E428 and *E. clarkii* isolate E422 are almost identical (Table 3.6).

Isolates of *E. brachyelytri* form a discrete basal clade in *Epichloë* phylogenies based on *tub2* sequence data (Schardl and Leuchtmann, 1999). Morphologically they are also distinguished by a flattened ascus cap which is unique to this species of *Epichloë*. So far, all microsatellite alleles amplified from *E. brachyelytri* isolates are unique to this species.

3.3.3.1.3 The Distribution of a Deletion among B1 Alleles – Implications for Endophyte Evolution

A 73 bp deletion was discovered in the flanking regions of B1 alleles amplified from *E. typhina*, *E. clarkii*, and *E. sylvatica* isolates. Specifically, the deletion was found in both of the *E. sylvatica* isolates tested, two of the three *E. clarkii* isolates tested, and dispersed throughout *E. typhina* (Section 3.2.3.3). This finding raises questions of how such a conserved deletion has become so widespread throughout the three species. Three scenarios for the distribution of the deletion are considered. The deletion may have arose long ago in a progenitor to the three *Epichloë* species, and was passed on to each one by vertical transmission. Alternatively, it may have arisen in one species after speciation, and has transferred to the other species by interspecific mating or hybridisation events. A final possibility is that the deleted segment is some transposable genetic element (TGE) which has excised from some isolates.

By process of elimination, the first scenario appears to be the most likely of the three to explain the widespread distribution of the deletion. If the deletion was a recent event and originated in one species with subsequent spreading to the others by interspecific mating or hybridisation, it would be expected that the sequences flanking the deletion would be quite conserved, but a reasonable degree of sequence variation was observed in these regions. Also, if the deletion had spread via interspecific hybridisation, it would be expected that multiple alleles would be apparent at other genetic loci, though this is rarely observed in *Epichloë* species. It is therefore unlikely that the deletion has spread

this way. The possible scenario that the deleted region is some TGE which has excised also appears unlikely. Integration of TGEs often results in short direct repeats of the target sites flanking the elements and these duplications may be retained after the TGE has excised. Short direct repeats are not apparent in the deleted or non deleted B1 alleles sequenced. Moreover, the sizes of TGEs are often two to three times the order of magnitude of the deletion reported in this study. The deletion is therefore likely to have originated some time ago in a progenitor to the three *Epichloë* species, and has distributed to each species by vertical transmission. This hypothesis is supported by the numerous sequence differences in the flanking regions of deleted alleles. If the deletion event occurred a long time ago, this provide the opportunity to accumulate a reasonable number of mutations in these regions. The fact that the deletion is not found in all B1 alleles of *E. typhina*, *E. clarkii*, and *E. sylvatica* isolates may reflect that the deletion has not been fixed in these populations.

In an independent study by Groppe and Boller where a range of Epichloë isolates were amplified with the B1.1 and B1.2 primers, only alleles of approximately 300 bp in size were amplified from isolates of *E. typhina* and *E. clarkii* at an annealing temperature of 50°C (Groppe and Boller, 1997). However, smaller alleles of approximately 250 bp were amplified with these primers from the *Tridens flavus* endophyte *Balansia epichloë* (Groppe and Boller, 1997). It will be interesting to sequence these alleles to find whether they contain the same 73 bp deletion as found in this study.

3.3.3.1.4 Phylogenetic Inference using Microsatellite Data

Although many evolutionary relationships between endophyte species and taxonomic groups are supported by the sharing of common microsatellite alleles (Sections 3.3.3.1.1 and 3.3.3.1.2), phylogenetic inference using microsatellite allele length data has not been approached in this study. Though several genetic distance statistics have been formulated for microsatellite data to infer phylogenies, these have had mixed success in resolving evolutionary relationships between groups in various applications (Goldstein et al., 1995; Kimmel et al., 1996; Paetkau et al., 1997). The mutational models used in

computer simulations are generally based on stepwise mutation models as expected for DNA slippage synthesis, though these tend to oversimplify the actual dynamics of mutations at microsatellite loci (Paetkau et al., 1997). For example, these models do not necessarily account for: non-'perfectness' of microsatellite tracts; compound microsatellite loci; and mutation of the flanking sequences, particularly insertions and deletions which affect allele length. There is also debate as to whether the rate and direction of mutation can vary between closely related species (Ellegren et al., 1995; Rubinsztein et al., 1995), and it has been suggested that the usefulness of microsatellite data for inferring phylogeny may be limited to a short time perspective and to relatively small populations (Nauta and Weissing, 1996). Given the doubts regarding the reliability of these methods, in this study evolutionary analysis has been restricted to basic observations of common allele sharing between groups.

3.3.3.2 Endophyte Identification In Planta – A Trial Application

This assay has been designed for the rapid, large-scale identification of endophytes in the field situation, and this has been accomplished by: *in planta* endophyte detection, the use of rapid miniprep DNA extraction methods, PCR based microsatellite amplification for fingerprint generation, and the automated analysis of fingerprints. To test this assay in the field situation, a blind test was carried out to identify the endophyte isolates infecting twelve samples of grass tissues (Section 3.2.3.2). In all cases a correct match was made with a taxonomic grouping and isozyme phenotype (if available) that had previously been determined for that isolate. The simple and robust procedures employed in the assay enables results to be obtained in as little as 24 hours. Although identification cannot be resolved to the level of a specific isolate, it can be deduced to a group of closely related isolates that share the same microsatellite genotype. Moreover, isolates in the reference database that have different genotypes can be eliminated as a positive match.

Plant DNA extracts notoriously contain compounds which are inhibitory to PCR, thus a safeguard against false-negative PCR results (Section 3.2.2.5.3) could be employed in

conjunction with this assay. Such an approach was taken by Groppe and Boller (1997) who developed a multiplex reaction which amplified both plant and fungal sequences to ensure that reaction conditions were suitable for amplification.

3.3.3.3 Microsatellite Loci as Markers in Endophyte Genomes

Microsatellite loci are rapidly becoming popular genetic markers in the mapping of animal and plant genomes (Broun and Tanksley, 1996; Buchanan et al., 1994; Röder et al., 1995; Wu and Tanksley, 1993), though their application to fungal genomes has not yet been approached. As physical markers in Epichloë endophyte genomes, microsatellite loci could prove useful in documenting the chromosomal changes which have occurred during the evolution of this group, particularly in the case of interspecific hybrid endophytes. These groups, which are often heteroploid or aneuploid (Leuchtmann and Clay, 1990), are thought to have undergone nuclear fusion followed by a subsequent loss of redundant chromosomes or chromosomal segments (Tsai et al., 1994). Microsatellite markers may also be genetically linked to loci for traits such as alkaloid production and sexual mating types.

The microsatellite loci from this study were to be physically mapped to the endophyte genomes, but this was not achieved due to time and financial constraints. Currently, the only information regarding the genomic location of microsatellite loci used in this study, is from B10 and B11, whose alleles both show 1:1 segregation in *E. festucae* crosses (Section 3.2.3.4). Since this is the segregation ratio expected for nuclear genes, these loci are probably located in the nucleus, not the mitochondria. Locus B11 is linked to the gene encoding HMG CoA in Lp19 (Dobson, 1997), and it is also linked to B10 in *E. festucae* (Section 3.2.3.4). It is possible that these three loci are linked in many Epichloë endophyte isolates.

3.4 CONCLUSION

A multilocus microsatellite-based PCR fingerprinting assay for the identification of Epichloë endophytes, both in culture and in planta, has been developed and successfully used to identify endophyte isolates from infected grass tissues. This assay should therefore be a useful tool for grass breeders to rapidly determine both the identity of known endophytes in pasture to the isozyme phenotype level, and also identify novel endophytes which are potential sources for new low alkaloid phenotypes. This assay should also be of value to other endophyte researchers as a general fingerprinting tool, and as an adjunct to the study of evolution of the endophytes, particularly with regard to the many hybrid asexual Epichloë endophytes.

CHAPTER FOUR

THE EVOLUTIONARY ORIGINS OF EPICHLOË ENDOPHYTES FROM ANNUAL *LOLIUM* AND *HORDEUM* GRASSES

4.1 INTRODUCTION

The genus *Lolium* contains eight species of ryegrasses (Charmet et al., 1997), including the agriculturally important perennial ryegrass (*L. perenne*) and Italian ryegrass (*L. multiflorum*). Perennial ryegrass is the most widely used of these grasses for forage purposes, and it has been extensively surveyed for endophytes where it is found to host several taxonomic groups of Epichloë endophyte. These include *Neotyphodium lolii* (Latch et al., 1984), *Epichloë typhina* (Schardl et al., 1991), an asexual hybrid of these two endophytes that has been designated as LpTG-2 (Christensen et al., 1993), and an *E. festucae*-like endophyte (pers. comm. Mike Christensen, Grasslands AgResearch). In contrast, little research on endophytes of annual ryegrasses has been undertaken. Latch et al. (1988) had surveyed six species of annual and one species of hybrid ryegrass for endophytes and reported the occurrence of a seedborne endophyte that is basally confined in the vegetative tillers of these grasses. The growth habit of this endophyte, both in planta and in culture, and the results of serological tests (Carter et al., 1997) suggest these endophytes are related to *Epichloë* and *Neotyphodium* species, and as such, have so far been described as *Neotyphodium*-like endophytes (Latch et al., 1988). Very little is known about the taxonomy and evolution of these endophytes, and this is possibly due to the difficulties in isolating and obtaining sustainable cultures for analysis, as reported by Latch et al. (1988). Also, these grasses are not as widely used in pasture systems as perennial ryegrass and are not associated with serious livestock disorders. However, loline alkaloids have been detected in endophyte-infected annual ryegrasses (TePaske et al., 1993), and the presence of endophyte does deter Argentine stem weevil (*Listronotus bonariensis*) feeding on young seedlings (Latch et al., 1988). The aim of this study was to genetically characterise the *Neotyphodium*-like endophytes from all seven species of annual ryegrasses, and determine their evolutionary origins from *tub2* and rDNA-ITS gene sequence data and microsatellite fingerprint profiles.

Additionally, two endophyte isolates, denoted HaB and Hd1, from the grass genus *Hordeum* were available in the Scott Laboratory fungal culture collection. *Hordeum*, a member of the grass tribe Triticeae, comprises about 45 species and subspecies

distributed across temperate regions of the world (De Bustos et al., 1999), and includes cultivated barley, *H. vulgare* subsp. *vulgare*. Extensive studies of endophytic fungi within the genus *Hordeum* have not been conducted, but barley is not known to contain Epichloë endophytes. Little background information is available regarding HaB and Hd1, though it is believed they were found in seed from *Hordeum* spp., in particular Hd1 is probably from *H. bogdanii*, collected on an expedition in China in the 1980's. The seed have since been kept in the Margot Forde Forage Germplasm Centre at Grasslands AgResearch (pers. comm. Mike Christensen, Grasslands AgResearch). The evolutionary origins of HaB and Hd1 were also to be investigated, using an approach similar to that for the genetic characterisation of the endophytes of annual ryegrasses.

4.2 RESULTS

4.2.1 ENDOPHYTE ISOLATION FROM PLANT TISSUE

Isolations were attempted from plants of four populations of *L. canariense* Steud., two populations of *L. multiflorum* Lam. cv. Maverick, one population of *L. persicum* Boiss & Hoh., two populations of *L. remotum* Schrank., three populations of *L. rigidum* Gaud. var. *rigidum* sens. Terrell, one population of *L. rigidum* Gaud. var. *rottboellioides* Heldr. ex Boiss, two populations of *L. subulatum* Vis., and four populations of *L. temulentum* L. All plants used in these isolations had been confirmed as containing hyphae similar in appearance to that of the *Neotyphodium*-like endophyte by examining aniline blue-stained tissue, removed from the basal region of leaves, under a compound microscope. Isolations were carried out in early and late spring following the initiation of elongation of reproductive tillers. The use of elevated nodes and associated basal leaf sheaths, and in the case of the uppermost nodes, immature inflorescences, was to reduce fungal and bacterial contamination that frequently occurs when isolations are attempted from the basal leaf sheath of vegetative tillers. Endophytes were isolated as described in Section 2.2.1.

Colonies consistent with those of the *Neotyphodium*-like endophyte previously described by Latch et al. (1988) were obtained from a few tissue pieces from two populations of *L. multiflorum* cv. Maverick (Lm1 and Lm2), three of *L. rigidum* var. *rigidum* (Lrr1, Lrr2 and Lrr4), and one of *L. rigidum* var. *rottboellioides* (Lro1). These colonies were very slow to emerge from excised incubated tissue with slow subsequent growth, waxy, convoluted, white to tan coloured and lacking conidia. Examples of such colonies isolated from *L. multiflorum* cv. Maverick (Lm1) and *L. rigidum* var. *rigidum* (Lrr1) are shown in Figs. 4.1A and 4.1B.

From one population of *L. canariense* (Lc4), colonies developed after just one to two weeks incubation. These sterile colonies differed from those previously described for the endophyte of annual ryegrasses as they were dark and felted in appearance, and they

Fig. 4.1. Cultures of *Neotyphodium* endophytes isolated from annual ryegrass tissues

Endophytes were isolated from plant tissue and grown on ABPDA media. (A) Colony of Lm1 endophyte isolated from *L. multiflorum* cv. Maverick seed material after 14 weeks incubation. (B) Colony of Lrr1 isolated from *L. rigidum* var. *rigidum* immature inflorescence tissue after 16 weeks incubation. (C) The variable growth forms of a 4 week old colony of Lc4 from *L. canariense* which had been subcultured. Scale bars are 1 mm long.

A

B

C

were able to be repeatedly subcultured (Fig. 4.1C). Re-examination of plants from this population revealed hyphae throughout the leaf sheath, rather than being confined to the most basal tissue. Further isolations from the sheath and blades of vegetative leaves gave rise to similar colonies.

Surface sterilised tissue pieces from the remainder of plants failed to give rise to colonies, although trace hyphal growth was observed from plants of two *L. temulentum* populations. Many of the excised tissue pieces became dark and mushy after a few weeks incubation. Fungal contaminants, including *Acremonium* spp., arose from a number of tissue pieces, but these were not included in this study.

4.2.2 FUNGAL AND IN PLANTA DNA EXTRACTION

Total genomic DNA was isolated from fresh fungal endophyte cultures Lc4, Lm1, Lm2, Lrr1, Lrr2, and Lro1 using a microwave miniprep method (Section 2.5.2). The DNA yields from the pure cultures were too low to visualise on agarose gels, and fluorometer readings were too low to give accurate readings, however when extracted DNA was used directly in PCR reactions, amplification yields were generally satisfactory. The small amount of biomass and consequent low yields of DNA from each annual ryegrass endophyte limited the number of PCR reactions that could be done.

Total genomic DNA from fresh nodal tissue of endophyte-infected grasses, where endophyte is known to be highly abundant (Latch et al., 1988), was isolated using the FastDNA Kit (Section 2.5.3). High yields of DNA were obtained from endophyte infected grasses, about 3 to 10 μg per preparation, which was more than sufficient for microsatellite fingerprinting.

4.2.3 rDNA-ITS AMPLIFICATION AND SEQUENCING

PCR amplification of the nuclear ribosomal DNA region spanning the internal transcribed spacers (rDNA-ITS), and the 5.8S rRNA gene (Section 2.13.9) was amplified

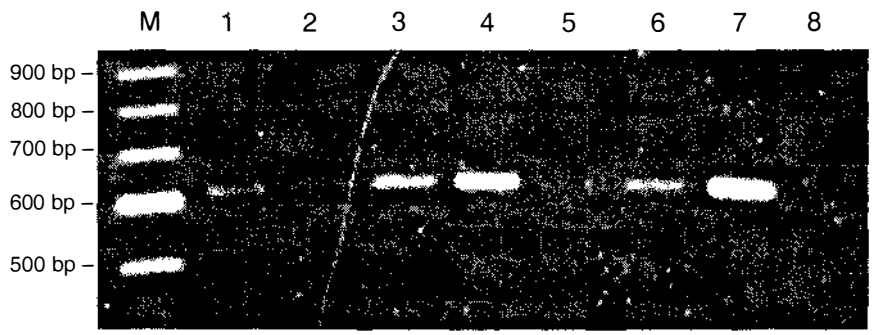
with primers ITS5 and ITS4, using DNA prepared from annual ryegrass endophyte cultures (Section 4.2.2). As shown in Figure 4.2, the amplification products were resolved as single bands of approximately 600 bp when separated on 2% agarose minigels (Section 2.8). If the PCR product yields were very low, as in lanes 1, 2, 5, and 6, products were diluted and re-amplified with *Pwo* DNA polymerase (Section 2.13.9) to obtain quantities sufficient for sequencing. PCR products were excised from the gel and purified (Section 2.5.10), and both strands were sequenced using each of the ITS5 and ITS4 primers (Section 2.15.2).

The complete sequences of the rDNA-ITS regions were obtained from the endophyte isolates: Lc4, Lm1, Lm2, Lrr1, Lrr2, and Lro1. Figure 4.3 shows an alignment of these sequences against that of *N. coenophialum* isolate e19 (Schardl et al., 1991), generated using the PILEUP program of the Wisconsin Package (Section 2.18.1). The alignment revealed that the rDNA-ITS sequences of Lm1, Lm2, Lrr1, and Lro1 were identical, and that Lrr2 differed from this by a 2 nt indel (AT) in the *ITS2* region (positions 445 and 446). The rDNA-ITS sequence of Lc4 had 97% identity to those of the other aligned annual ryegrass endophyte isolates, and all sequence differences were located in the *ITS1* and *ITS2* regions. Database similarity searching of these sequences by BLAST (Section 2.15.3) identified only sequences from the *Epichloë* and *Neotyphodium* genera.

4.2.4 *tub2* AMPLIFICATION AND SEQUENCING

The 5' portions of the ß-tubulin (*tub2*) genes, including introns IVS1, IVS2, and IVS3, were PCR amplified with primers T1.1 and T1.2 (Section 2.13.8) using DNA prepared from Lc4, Lm1, Lm2, Lrr1, Lrr2, and Lro1 endophyte cultures (Section 4.2.2). An agarose gel of the *tub2* amplification products from various annual ryegrass endophyte isolates is shown in Figure 4.4. Amplification of Lc4 (lane 1) yielded a single product that gave unambiguous results when sequenced directly (Section 2.15). BLAST similarity searching of this sequence revealed that it had high similarity to the *tub2* gene from *N. uncinatum* and the *tub2*-3 copy from *N. coenophialum* (Tsai et al., 1994).

Fig. 4.2. rDNA- ITS amplification products from annual ryegrass endophyte isolates



Products were amplified with primers ITS5 and ITS4 and resolved on a 2% agarose gel. Reactions contained genomic DNA from endophyte isolates Lrr1 (lane 1), Lrr2 (lane 2), Lro1 (lane 3), Lro1 (lane 4), Lrr1 (lane 5), Lm2 (lane 6), and positive control isolate Frr1 (lane 7). Lane 8 contains a PCR negative control without DNA, and the numbers on the left of the figure indicates the sizes in bp of the 100 bp ladder in lane M.

Fig. 4.3. Alignment of rDNA-ITS sequences from annual ryegrass endophyte isolates.

The DNA sequences of the rDNA-ITS regions from annual ryegrass endophyte isolates Lc4, Lm1, Lm2, Lrr1, Lrr2, and Lro1, and *N. coenophialum* isolate e19 were aligned using the PILEUP programme of the Wisconsin Package with the gap penalty set to one and the gap extension penalty set to zero. Gaps (.) were introduced to facilitate sequence alignment. The sequenced region from e19 encodes ITS1 (positions 1 – 169), 5.8S rRNA (positions 170 – 337, shown italicised), and ITS2 (positions 338 – 509) (Schardl et al., 1991). Nucleotides that differ from the reference sequence, e19, are highlighted in green.

```
      1                                                   50
 e19  CGAGTTTACA CTCCCAAACC CCTGTGAACC T.TACCTTTA CTGTTGCCTC
 Lc4  CGAGTTTACA CTCCCAAACC CCTGTGGACC TATACCCTCA CTGTTGCCTC
Lrr2  CGAGTTTTCA CTCCCAAACC CCTGTGGACT TATACCTTTA CCGTTGCCTC
Lrr1  CGAGTTTTCA CTCCCAAACC CCTGTGGACT TATACCTTTA CCGTTGCCTC
Lro1  CGAGTTTTCA CTCCCAAACC CCTGTGGACT TATACCTTTA CCGTTGCCTC
 Lm2  CGAGTTTTCA CTCCCAAACC CCTGTGGACT TATACCTTTA CCGTTGCCTC
 Lm1  CGAGTTTTCA CTCCCAAACC CCTGTGGACT TATACCTTTA CCGTTGCCTC

      51                                                  100
 e19  GGCGGGCACG GCCGCGGACG CCCCCTCGCG GGGGCACCGG GGCCGGGCGC
 Lc4  GGCGGGCACG GCCGCGGACG CCCCCTCGCG GGGGCGCCGG GGCCAGGCGC
Lrr2  GGCGGGCACG GCCGCGGACG CCCCCTCGCG GGGGCACCGG GGCCAGGCGC
Lrr1  GGCGGGCACG GCCGCGGACG CCCCCTCGCG GGGGCACCGG GGCCAGGCGC
Lro1  GGCGGGCACG GCCGCGGACG CCCCCTCGCG GGGGCACCGG GGCCAGGCGC
 Lm2  GGCGGGCACG GCCGCGGACG CCCCCTCGCG GGGGCACCGG GGCCAGGCGC
 Lm1  GGCGGGCACG GCCGCGGACG CCCCCTCGCG GGGGCACCGG GGCCAGGCGC

      101                                                 150
 e19  CCGCCGGAGG ACCCAAACCC TTCTGTATTT TTCTTACGCA TGTCTGAGTG
 Lc4  CCGCCGGAGG ACCCAAACCC TTCTGTA.TT TTCTTGCGCA TGTCTGAGTG
Lrr2  CCGCCGGAGG ACCCAAACCC TTCTGTA.TT TTCTTACGCA TGTCTGAGTG
Lrr1  CCGCCGGAGG ACCCAAACCC TTCTGTA.TT TTCTTACGCA TGTCTGAGTG
Lro1  CCGCCGGAGG ACCCAAACCC TTCTGTA.TT TTCTTACGCA TGTCTGAGTG
 Lm2  CCGCCGGAGG ACCCAAACCC TTCTGTA.TT TTCTTACGCA TGTCTGAGTG
 Lm1  CCGCCGGAGG ACCCAAACCC TTCTGTA.TT TTCTTACGCA TGTCTGAGTG
```

```
      151                                                        200
 e19  GATTTAATAT CAAATGAATC AAAACTTTCA ACAACGGATC TCTTGGTTCT
 Lc4  GATTTA.... CAAATGAATC AAAACTTTCA ACAACGGATC TCTTGGTTCT
Lrr2  GATTTAATAT CAAATGAATC AAAACTTTCA ACAACGGATC TCTTGGTTCT
Lrr1  GATTTAATAT CAAATGAATC AAAACTTTCA ACAACGGATC TCTTGGTTCT
Lro1  GATTTAATAT CAAATGAATC AAAACTTTCA ACAACGGATC TCTTGGTTCT
 Lm2  GATTTAATAT CAAATGAATC AAAACTTTCA ACAACGGATC TCTTGGTTCT
 Lm1  GATTTAATAT CAAATGAATC AAAACTTTCA ACAACGGATC TCTTGGTTCT

      201                                                        250
 e19  GGCATCGATG AAGAACGCAG CGAAATGCGA TAAGTAATGT GAATTGCAGA
 Lc4  GGCATCGATG AAGAACGCAG CGAAATGCGA TAAGTAATGT GAATTGCAGA
Lrr2  GGCATCGATG AAGAACGCAG CGAAATGCGA TAAGTAATGT GAATTGCAGA
Lrr1  GGCATCGATG AAGAACGCAG CGAAATGCGA TAAGTAATGT GAATTGCAGA
Lro1  GGCATCGATG AAGAACGCAG CGAAATGCGA TAAGTAATGT GAATTGCAGA
 Lm2  GGCATCGATG AAGAACGCAG CGAAATGCGA TAAGTAATGT GAATTGCAGA
 Lm1  GGCATCGATG AAGAACGCAG CGAAATGCGA TAAGTAATGT GAATTGCAGA

      251                                                        300
 e19  ATTCAGTGAA TCATCGAATC TTTGAACGCA CATTGCGCCC GCCAGTATTC
 Lc4  ATTCAGTGAA TCATCGAATC TTTGAACGCA CATTGCGCCC GCCAGTATTC
Lrr2  ATTCAGTGAA TCATCGAATC TTTGAACGCA CATTGCGCCC GCCAGTATTC
Lrr1  ATTCAGTGAA TCATCGAATC TTTGAACGCA CATTGCGCCC GCCAGTATTC
Lro1  ATTCAGTGAA TCATCGAATC TTTGAACGCA CATTGCGCCC GCCAGTATTC
 Lm2  ATTCAGTGAA TCATCGAATC TTTGAACGCA CATTGCGCCC GCCAGTATTC
 Lm1  ATTCAGTGAA TCATCGAATC TTTGAACGCA CATTGCGCCC GCCAGTATTC

      301                                                        350
 e19  TGGCGGGCAT GCCTGTTCGA GCGTCATTTC AACCCTCAAG CCCGCTGCGC
 Lc4  TGGCGGGCAT GCCTGTTCGA GCGTCATTTC AACCCTCAAG CCCGCTGCGT
Lrr2  TGGCGGGCAT GCCTGTTCGA GCGTCATTTC AACCCTCAAG CCCGCTGCGC
Lrr1  TGGCGGGCAT GCCTGTTCGA GCGTCATTTC AACCCTCAAG CCCGCTGCGC
Lro1  TGGCGGGCAT GCCTGTTCGA GCGTCATTTC AACCCTCAAG CCCGCTGCGC
 Lm2  TGGCGGGCAT GCCTGTTCGA GCGTCATTTC AACCCTCAAG CCCGCTGCGC
 Lm1  TGGCGGGCAT GCCTGTTCGA GCGTCATTTC AACCCTCAAG CCCGCTGCGC

      351                                                        400
 e19  GCTTGCTGTT GGGGACCGGC TCACCCGCCT CGCGGCGGCG GCCGCCCCCG
 Lc4  GCTTGGTGTT GGGGACCGGC CCGCCCGCCT CGCGGCGGCG GCCGCCCCTG
Lrr2  GCTTGGTGTT GGGGACCGGC CCGCCCGCCT CGCGGCGGCG GCCGCCCCCG
Lrr1  GCTTGGTGTT GGGGACCGGC CCGCCCGCCT CGCGGCGGCG GCCGCCCCCG
Lro1  GCTTGGTGTT GGGGACCGGC CCGCCCGCCT CGCGGCGGCG GCCGCCCCCG
 Lm2  GCTTGGTGTT GGGGACCGGC CCGCCCGCCT CGCGGCGGCG GCCGCCCCCG
 Lm1  GCTTGGTGTT GGGGACCGGC CCGCCCGCCT CGCGGCGGCG GCCGCCCCCG
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      401                                                        450
 e19  AAATGAATCG GCGGTCTCGT CGCAAGCCTC CTTTGCGTAG TAGC..ACAC
 Lc4  AAATGAATTG GCGGTCTCGT CGC.AGCGTC CTTTGCGTAG TAGC..ACAC
Lrr2  AAATGAATTG GCGGTCTCGT CGC.GGCCTC CTTTGCGTAG TAACATATAC
Lrr1  AAATGAATTG GCGGTCTCGT CGC.GGCCTC CTTTGCGTAG TAAC..ATAC
Lro1  AAATGAATTG GCGGTCTCGT CGC.GGCCTC CTTTGCGTAG TAAC..ATAC
 Lm2  AAATGAATTG GCGGTCTCGT CGC.GGCCTC CTTTGCGTAG TAAC..ATAC
 Lm1  AAATGAATTG GCGGTCTCGT CGC.GGCCTC CTTTGCGTAG TAAC..ATAC

      451                                                        500
 e19  CACCTCGCAA CCGGGAGCGC GGCGCGGCCA CAGCCGTAAA ACGCCCAACT
 Lc4  CACCTCGCAA CCGGGAGCGC GGCGCGGCCA CTGCCGTAAA ACGCCCAAC.
Lrr2  CACCTCGCAA CCGGGAGCGC GGCGCGGCCA CTGCCGTAAA ACGCCCAAC.
Lrr1  CACCTCGCAA CCGGGAGCGC GGCGCGGCCA CTGCCGTAAA ACGCCCAAC.
Lro1  CACCTCGCAA CCGGGAGCGC GGCGCGGCCA CTGCCGTAAA ACGCCCAAC.
 Lm2  CACCTCGCAA CCGGGAGCGC GGCGCGGCCA CTGCCGTAAA ACGCCCAAC.
 Lm1  CACCTCGCAA CCGGGAGCGC GGCGCGGCCA CTGCCGTAAA ACGCCCAAC.

      501
 e19  TTCTCCAAG
 Lc4  TTCTCCAAG
Lrr2  TTCTCCAAG
Lrr1  TTCTCCAAG
Lro1  TTCTCCAAG
 Lm2  TTCTCCAAG
 Lm1  TTCTCCAAG
```
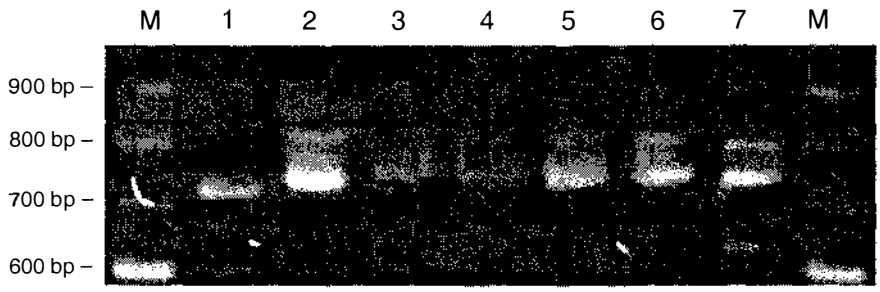

Fig. 4.4. *tub*2 amplification products from annual ryegrass and *Hordeum* endophyte isolates



Products were amplified with primers T1.1-BamHI and T1.2-EcoRI and resolved on a 2% agarose gel. Reactions contained genomic DNA from endophyte isolates Lc4 (lane 1), Lrr1 (lane 2), Lrr4 (lane 3), Lro1 (lane 4), Lm2 (lane 5), HaB (lane 6) and Hd1 (lane 7). The numbers on the left of the figure indicates the sizes in bp of the 100 bp ladder in lane M.

In contrast, the *tub2* PCR products from the remaining annual ryegrass endophytes yielded multiple bands between approximately 700 and 800 bp, examples of these are shown in Figure 4.4, lanes 2 to 5. These bands could not be separated, so had to be cloned for sequencing. The *tub2* PCR products from Lm1 were cloned into the pGEM-T vector (Section 2.14.1), but extremely low numbers of white colonies, five per transformation, were produced using this method despite several attempts to optimise the ligation conditions. To increase the cloning efficiency, a new strategy involving sticky-end ligation reactions was undertaken. Primers to the *tub2* gene were redesigned to affix *Bam*HI and *Eco*RI restriction enzyme recognition sequences to the 5'-ends of T1.1 and T1.2 respectively. These primers, T1.1-BamHI and T1.2-EcoRI (Table 2), were used with the same amplification conditions (Section 2.13.8), and the resulting products were prepared for cloning by cleaving with *Bam*HI and *Eco*RI to produce sticky ends (Section 2.14.2). Products were then directionally cloned into *Eco*RI and *Bam*HI cleaved pUC118, and about 24 white clones from each transformation were screened as described previously (Section 2.12.9). All unique clone types were fully sequenced (Section 2.15).

Three to five unique clone types were identified from each endophyte isolate but analysis of sequence alignments showed that many of these were recombinant products, which were probably generated during PCR. The 'true' *tub2* genes were identified based on both their similarity to extant *Epichloë* and *Neotyphodium tub2* genes, and the relative frequencies of the different clone types as recombinant genes typically accounted for about one sixth of the clones screened. Overall it was found that each endophyte isolate: Lm1, Lm2, Lrr1, Lrr2, and Lro1 had two distinct copies of *tub2* genes, and copies were labelled *tub2-a* and *tub2-b*. BLAST similarity searches (Section 2.15.3) revealed that these copies showed high similarity to *tub2* genes of *E. baconii* and *E. bromicola* respectively.

A PILEUP generated alignment (Section 2.18.1) of the *tub2* DNA sequences from each of the annual ryegrass endophyte isolates analysed, and *N. coenophialum* isolate e19 *tub2-2* copy (Tsai et al., 1994) is shown in Figure 4.5. The *E. baconii*-like genes, Lm1

Fig. 4.5. Alignment of the *tub2* gene sequences from annual ryegrass endophyte isolates

The DNA sequences of the *tub2* genes from annual ryegrass endophyte isolates Lc4, Lm1, Lm2, Lrr1, Lrr2, and Lro1, and *N. coenophialum* isolate e19 (*tub2-2* copy) were aligned using the PILEUP programme of the Wisconsin Package, with the gap penalty set to one and the gap extension penalty set to zero. Gaps (.) were introduced to facilitate sequence alignment. The sequenced region from e19 encodes IVS1 (positions 1 – 206), exon 2 (positions 207 – 231), IVS2 (positions 232 – 304), exon 3 (positions 305 – 428), IVS3 (positions 429 – 536), and exon 4 (positions 537 – 542) (Tsai et al., 1994). Exon positions are shown italicised, and nucleotides that differ from the reference sequence, e19, are highlighted in green.

```
                1                                                    50
 e19 tub2-2     AAGTTCAACC TCTCTGTTTG TCTTGGGGAC CCCCTCCTCG ACGCGTTCCG
   Lc4 tub2     GAGTTCAACC TCTCTGTTTG TCTTGGGGAC CCCCTCCTCG ACGCGTTCCG
 Lm1 tub2-a     AAGTTCAAAC TCTCTGTCTG TCTTGGAACC CCTCTCCTCG ACGCGTTCCG
 Lm2 tub2-a     AAGTTCAAAC TCTCTGTCTG TCTTGGAACC CCTCTCCTCG ACGCGTTCCG
Lro1 tub2-a     AAGTTCAAAC TCTCTGTCTG TCTTGGAACC CCTCTCCTCG ACGCGTTCCG
Lrr2 tub2-a     AAGTTCAAA. .CTCTGTCTG TCTTGGAACC CCTCTCCTCG ACGCGTTCCG
Lrr1 tub2-a     AAGTTCAAAC TCTCTGTCTG TCTTGGAACC CCTCTCCTCG ACGCGTTCCG
 Lm1 tub2-b     AAGTTCAACC TCTCTGTTTG TCTTGGGGAC CCCCTCCTCG ACGCGTTCCG
 Lm2 tub2-b     AAGTTCAACC TCTCTGTTTG TCTTGGGGAC CCCCTCCTCG ACGCGTTCCG
Lro1 tub2-b     AAGTTCAACC TCTCTGTTTG TCTTGGGGAC CCCCTCCTCG ACGCGTTCCG
Lrr1 tub2-b     AAGTTCAACC TCTCTGTTTG TCTTGGGGAC CCCCTCCTCG ACGCGTTCCG
Lrr2 tub2-b     AAGTTCAACC TCTCTGTTTG TCTTGGGGAC CCCCTCCTCG ACGCGTTCCG

                51                                                   100
 e19 tub2-2     GTGTTGAGCC CCTGATTTCG TACCCCGCCG AGCCCGGCCA CGAAGTGCAC
   Lc4 tub2     GTGTCGAGCC CCTGATTTCG TACCCCGCCG AGCCCGGCCA CGACGTGCAC
 Lm1 tub2-a     GTGTTGAGCC CCTGATTTCG TACCCCCCCG AGCCCGGCCA CGACGTGCAC
 Lm2 tub2-a     GTGTTGAGCC CCTGATTTCG TACCCCCCCG AGCCCGGCCA CGACGTGCAC
Lro1 tub2-a     GTGTTGAGCC CCTGATTTCG TACCCCCCCG AGCCCGGCCA CGACGTGCAC
Lrr2 tub2-a     GTGTTGAGCC CCTGATTTCG TACCCCCCCG AGCCCGGCCA CGACGTGCAC
Lrr1 tub2-a     GTGTTGAGCC CCTGATTTCG TACCCCCCCG AGCCCGGCCA CGACGTGCAC
 Lm1 tub2-b     GTGTTGAGCC CCTGATTTCG TACCCCGCCG AGCCCGGCCA CGACGTGCAC
 Lm2 tub2-b     GTGTTGAGCC CCTGATTTCG TACCCCGCCG AGCCCGGCCA CGACGTGCAC
Lro1 tub2-b     GTGTTGAGCC CCTGATTTCG TACCCCGCCG AGCCCGGCCA CGACGTGCAC
Lrr1 tub2-b     GTGTTGAGCC CCTGATTTCG TACCCCGCCG AACCCGGCCA CGACGTGCAC
Lrr2 tub2-b     GTGTTGAGCC CCTGATTTCG TACCCCGCCG AACCCGGCCA CGACGTGCAC
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                101                                                 150
 e19 tub2-2     GCCCAACGAA CAGTCGTGAT GAGAGGCGGA CCGAGAC... AAAATTAATG
   Lc4 tub2     GCCCAACGGA CAGTCGTGAT GAGAGGCGGA CCGAGAC... AACATCATTG
 Lm1 tub2-a     GCCCAACGAA CAGTCGTGAT GAGAGGCGGA TCGAGAC... AAAATTAATG
 Lm2 tub2-a     GCCCAACGAA CAGTCGTGAT GAGAGGCGGA TCGAGAC... AAAATTAATG
Lro1 tub2-a     GCCCAACGAA CAGTCGTGAT GAGAGGCGGA TCGAGAC... AAAATTAATG
Lrr2 tub2-a     GCCCAACGAA CAGTCGTGAT GAGAGGCGGA TCGAGAC... AAAATTAATG
Lrr1 tub2-a     GCCCAACGAA CAGTCGTGAT GAGAGGCGGA TCGAGAC... AAAATTAATG
 Lm1 tub2-b     GCCCAATGAA CAGTCGTGAT GAGAGGCGGA CCGAGACAAA AAAAATAATG
 Lm2 tub2-b     GCCCAATGAA CAGTCGTGAT GAGAGGCGGA CCGAGACAAA AAAAATAATG
Lro1 tub2-b     GCCCAATGAA CAGTCGTGAT GAGAGGCGGA CCGAGACAAA AAAAATAATG
Lrr1 tub2-b     GCCCAATGAA CAGTCGTGAT GAGAGGCGGA CCGAGACAAA AAAAATAATG
Lrr2 tub2-b     GCCCAATGAA CAGTCGTGAT GAGAGGCGGA CCGAGACAAA AAAAATAATG

                151                                                 200
 e19 tub2-2     AATGCGGTAT TCGAGAACTG TAGCTGACCT G.TTCTTCCC CTCTTTTCCC
   Lc4 tub2     AATGCGGTAT TCGAGAACTG TAGCTGACCT TTTTCTTT.. ........CC
 Lm1 tub2-a     AATGCGGTAT TCGAGAACTG TAGCTGACCT GTTTCTTTCC CTCTTTTCCC
 Lm2 tub2-a     AATGCGGTAT TCGAGAACTG TAGCTGACCT GTTTCTTTCC CTCTTTTCCC
Lro1 tub2-a     AATGCGGTAT TCGAGAACTG TAGCTGACCT GTTTCTTTCC CTCTTTTCCC
Lrr2 tub2-a     AATGCGGTAT TCGAGAACTG TAGCTGACCT GTTTCTTTCC CTCTTTTCCC
Lrr1 tub2-a     AATGCGGTAT TCGAGAACTG TAGCTGACCT GTTTCTTTCC CTCTTTTCCC
 Lm1 tub2-b     ATTGCTGTAT TCGAGAACTG TAGCTGACCT .TTTC..... ..CTTTT..C
 Lm2 tub2-b     ATTGCTGTAT TCGAGAACTG TAGCTGACCT .TTTC.......CTTTT..C
Lro1 tub2-b     ATTGCTGTAT TCGAGAACTG TAGCTGACCT .TTTC.......CTTTT..C
Lrr1 tub2-b     ATTGCTGTAT TCGAGAACTG TAGCTGACCT .TTTC.......CTTTT..C
Lrr2 tub2-b     ATTGCTGTAT TCGAGAACTG TAGCTGACCT .TTTC.......CTTTT..C

                201                                                 250
 e19 tub2-2     CTCTAGGTTC ATCTTCAAAC CGGTCAGTGC GTAAGTGACA AATTCGCCGA
   Lc4 tub2     CTCTAGGTTC ATCTTCAAAC CGGTCAGTGC GTAAGTGACA AATCTGCCGA
 Lm1 tub2-a     CTCTAGGTTC ATCTTCAAAC CGGTCAGTGC GTAAGTGACA AA.TCGCCGA
 Lm2 tub2-a     CTCTAGGTTC ATCTTCAAAC CGGTCAGTGC GTAAGTGACA AA.TCGCCGA
Lro1 tub2-a     CTCTAGGTTC ATCTTCAAAC CGGTCAGTGC GTAAGTGACA AA.TCGCCGA
Lrr2 tub2-a     CTCTAGGTTC ATCTTCAAAC CGGTCAGTGC GTAAGTGACA AA.TCGCCGA
Lrr1 tub2-a     CTCTAGGTTC ATCTTCAAAC CGGTCAGTGC GTAAGTGACA AA.TCGCCGA
 Lm1 tub2-b     CTCTAGGTTC ATCTTCAAAC CGGTCAGTGC GTAAGTGACA AATCCGCCGA
 Lm2 tub2-b     CTCTAGGTTC ATCTTCAAAC CGGTCAGTGC GTAAGTGACA AATCCGCCGA
Lro1 tub2-b     CTCTAGGTTC ATCTTCAAAC CGGTCAGTGC GTAAGTGACA AATCCGCCGA
Lrr1 tub2-b     CTCTAGGTTC ATCTTCAAAC CGGTCAGTGC GTAAGTGACA AATCCGCCGA
Lrr2 tub2-b     CTCTAGGTTC ATCTTCAAAC CGGTCAGTGC GTAAGTGACA AATCCGCCGA

                251                                                 300
 e19 tub2-2     CCTCGAACGA CAGGCACAAA CA..GCATGA AAAA.CTCAC ATTCATTTGG
   Lc4 tub2     CCTCGAACGA CAGGCACAAA TA..ACATGA AAAA.CTCAC ATTGATTTGG
 Lm1 tub2-a     CCTCGAACAA CAGGCACAAA CA..GCATGA AAAAACTCAC ATTCATTTGA
 Lm2 tub2-a     CCTCGAACAA CAGGCACAAA CA..GCATGA AAAAACTCAC ATTCATTTGA
Lro1 tub2-a     CCTCGAACAA CAGGCACAAA CA..GCATGA AAAAACTCAC ATTCATTTGA
Lrr2 tub2-a     CCTCGAACAA CAGGCACAAA CA..GCATGA AAAAACTCAC ATTCATTTGG
Lrr1 tub2-a     CCTCGAACAA CAGGCACAAA CA..GCATGA AAAATCTCAC ATTCATTTGG
 Lm1 tub2-b     CCTCGAACGA CAGGCACAAA CAACACATGA AAAA.CTCAC ATTTCTTTGG
 Lm2 tub2-b     CCTCGAACGA CAGGCACAAA CAACACATGA AAAA.CTCAC ATTTCTTTGG
Lro1 tub2-b     CCTCGAACGA CAGGCACAAA CAACACATGA AAAA.CTCAC ATTTCTTTGG
Lrr1 tub2-b     CCTCGAACGA CAGGCACAAA CAACACATGA AAAA.CTCAC ATTTCTTTGG
Lrr2 tub2-b     CCTCGAACGA CAGGCACAAA CAACACATGA AAAA.CTCAC ATTTCTTTGG
```

```
                301                                                 350
 e19 tub2-2  GCAGGGTAAC CAAATTGGTG CTGCTTTCTG GCAGACCATC TCTGGCGAGC
   Lc4 tub2  GCAGGGTAAC CAAATTGGTG CTGCTTTCTG GCAGACCATC TCTGGCGAGC
 Lm1 tub2-a  GCAGGGTAAC CAAATTGGTG CTGCTTTCTG GCAGACCATC TCTGGCGAGC
 Lm2 tub2-a  GCAGGGTAAC CAAATTGGTG CTGCTTTCTG GCAGACCATC TCTGGCGAGC
Lro1 tub2-a  GCAGGGTAAC CTAATTGGTG CTGCTTTCTG GCAGACCATC TCTGGCGAGC
Lrr2 tub2-a  GCAGGGTAAC CAAATTGGTG CTGCTTTCTG GCAGACCATC TCTGGCGAGC
Lrr1 tub2-a  GCAGGGTAAC CAAATTGGTG CTGCTTTCTG GCAGACCATC TCTGGCGAGC
 Lm1 tub2-b  GCAGGGTAAC CAAATTGGTG CTGCTTTCTG GCAGACCATC TCTGGCGAGC
 Lm2 tub2-b  GCAGGGTAAC CAAATTGGTG CTGCTTTCTG GCAGACCATC TCTGGCGAGC
Lro1 tub2-b  GCAGGGTAAC CAAATTGGTG CTGCTTTCTG GCAGACCATC TCTGGCGAGC
Lrr1 tub2-b  GCAGGGTAAC CAAATTGGTG CTGCTTTCTG GCAGACCATC TCTGGCGAGC
Lrr2 tub2-b  GCAGGGTAAC CAAATTGGTG CTGCTTTCTG GCAGACCATC TCTGGCGAGC

                351                                                 400
 e19 tub2-2  ACGGCCTCGA CAGCAATGGT GTGTACAATG GTACCTCCGA GCTCCAGCTC
   Lc4 tub2  ACGGCCTCGA CAGCAATGGT GTGTACAATG GTACCTCCGA GCTCCAGCTC
 Lm1 tub2-a  ACGGCCTCGA CAGCAATGGT GTGTACAATG GTACCTCCGA GCTCCAGCTC
 Lm2 tub2-a  ACGGCCTCGA CAGCAATGGT GTGTACAATG GTACCTCCGA GCTCCAGCTC
Lro1 tub2-a  ACGGCCTCGA CAGCAATGGT GTGTACAATG GTACCTCCGA GCTCCAGCTC
Lrr2 tub2-a  ACGGCCTCGA CAGCAATGGT GTGTACAATG GTACCTCCGA GCTCCAGCTC
Lrr1 tub2-a  ACGGCCTCGA CAGCAATGGT GTGTACAATG GTACCTCCGA GCTCCAGCTC
 Lm1 tub2-b  ACGGTCTCGA CAGCAATGGT GTGTACAACG GTACCTCCGA GCTCCAGCTG
 Lm2 tub2-b  ACGGTCTCGA CAGCAATGGT GTGTACAACG GTACCTCCGA GCTCCAGCTG
Lro1 tub2-b  ACGGTCTCGA CAGCAATGGT GTGTACAACG GTACCTCCGA GCTCCAGCTG
Lrr1 tub2-b  ACGGTCTCGA CAGCAATGGT GTGTACAACG GTACCTCCGA GCTCCAGCTG
Lrr2 tub2-b  GCGGTCTCGA CAGCAATGGT GTGTACAACG GTACCTCCGA GCTCCAGCTG

                401                                                 450
 e19 tub2-2  GAGCGTATGA GTGTCTACTT CAACGAGGTA AGTCTTCATA ATCTAAAGTC
   Lc4 tub2  GAGCGTATGA GTGTCTACTT CAACGAGG.. .......... ....CAAGTC
 Lm1 tub2-a  GAGCGTATGA GTGTCTACTT CAACGAGGTA AGTCTTCATA ATCTAAAGTC
 Lm2 tub2-a  GAGCGTATGA GTGTCTACTT CAACGAGGTA AGTCTTCATA ATCTAAAGTC
Lro1 tub2-a  GAGCGTATGA GTGTCTACTT CAACGAGGTA AGTCTTCATA ATCTAAAGTC
Lrr2 tub2-a  GAGCGTATGA GTGTCTACTT CAACGAGGTA AGTCTTCATA ATCTAAAGTC
Lrr1 tub2-a  GAGCGTATGA GTGTCTACTT CAACGAGGTA AGTCTTCATA ATCTAAAGTC
 Lm1 tub2-b  GAGCGTATGA GTGTCTACTT CAACGAGGTA AGTCTTCATA ATCTAAAGTC
 Lm2 tub2-b  GAGCGTATGA GTGTCTACTT CAACGAGGTA AGTCTTCATA ATCTAAAGTC
Lro1 tub2-b  GAGCGTATGA GTGTCTACTT CAACGAGGTA AGTCTTCATA ATCTAAAGTC
Lrr1 tub2-b  GAGCGTATGA GTGTCTACTT CAACGAGGTA AGTCTTCATA ATCTAAAGTC
Lrr2 tub2-b  GAGCGTATGA GTGTCTACTT CAACGAGGTA AGTCTTCATA ATCTAAAGTC

                451                                                 500
 e19 tub2-2  TCCATTGAGC TACATACATA CCGCCCCGGA GATGGGACGG AAAGAGAACG
   Lc4 tub2  TCCATTGAGC ....TACATA CCGCCCTGAA GATGGGACGG AAAAAGAAGG
 Lm1 tub2-a  TCCGTTGAGC TACATACATA CCGCCCCGGA GATGAGACGG AAAGAGAACG
 Lm2 tub2-a  TCCGTTGAGC TACATACATA CCGCCCCGGA GATGAGACGG AAAGAGAACG
Lro1 tub2-a  TCCGTTGAGC TACATACATA CCGCCCCGGA GATGAGACGG AAAGAGAACG
Lrr2 tub2-a  TCCGTTGAGC TACATACATA CCGCCCCGGA GATGAGACGG AAAGAGAACG
Lrr1 tub2-a  TCCGTTGAGC TACATACATA CCGCCCCGGA GATGAAACGG AAAGAGAACG
 Lm1 tub2-b  TCCATTGAGC ....TACATA CCGCCCTGGA GATGAGACGG AAAGAGATCG
 Lm2 tub2-b  TCCATTGAGC ....TACATA CCGCCCTGGA GATGAGACGG AAAGAGATCG
Lro1 tub2-b  TCCATTGAGC ....TACATA CCGCCCTGGA GATGAGACGG AAAGAGATCG
Lrr1 tub2-b  TCCATTGAGC ....TACATA CCGCCCTGGA GATGAGACGG AAAGAGATCG
Lrr2 tub2-b  TCCATTGAGC ....TACATA CCGCCCTGGA GATGAGACGG AAAGAGATCG
```

```
                501                                                 542
 e19 tub2-2     AAAGAAAAAG TGTTATCATG CTCATCCATG TGACAGGCTT CT
   Lc4 tub2     AAAGAAAAAG TGTTATCATG CTAATCTATG TGACAGGCTT CT
 Lm1 tub2-a     AAAGAAAAAG TGTTATCATG CTCATCCATG TGACAGGCTT CT
 Lm2 tub2-a     AAAGAAAAAG TGTTATCATG CTCATCCATG TGACAGGCTT CT
Lro1 tub2-a     AAAGAAAAAG TGTTATCATG CTCATCCATG TGACAGGCTT CT
Lrr2 tub2-a     AAAGAAAAAG TGTTATCATG CTCATCCATG TGACAGGCTT CT
Lrr1 tub2-a     AAAGAAAAAG TGTTATCATG CTCATCCATG TGACAGGCTT CT
 Lm1 tub2-b     ..ATAAAAAG TGTGAACATG CTAATCTATG TGACAGGCTT CT
 Lm2 tub2-b     ..ATAAAAAG TGTGAACATG CTAATCTATG TGACAGGCTT CT
Lro1 tub2-b     ..ATAAAAAG TGTGAACATG CTAATCTATG TGACAGGCTT CT
Lrr1 tub2-b     ..ATAAAAAG TGTGAACATG CTAATCTATG TGACAGGCTT CT
Lrr2 tub2-b     ..ATAAAAAG TGTGAACATG CTAATCTATG TGACAGGCTT CT
```

tub2-a and Lm2 tub2-*a* were identical, as were the *E. bromicola*-like genes Lm1 *tub2-b*, Lm2 *tub2-b*, and Lro1 *tub2-b*.

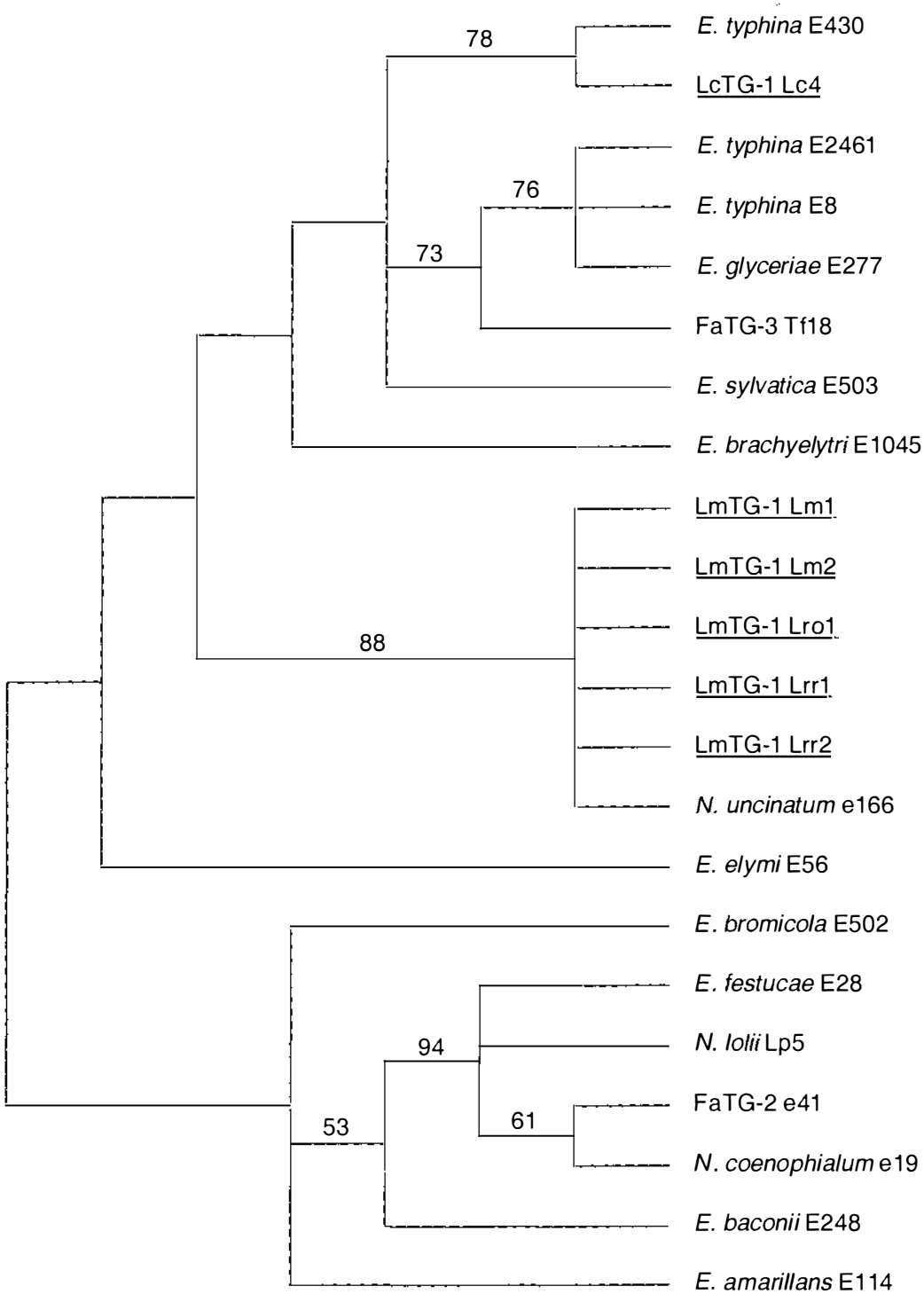
4.2.5 PHYLOGENETIC ANALYSIS OF DNA SEQUENCES

To determine the most closely related genes to those found in the endophytes of annual ryegrasses, phylogenetic analyses were carried out using *tub2* and rDNA-ITS sequences from these isolates, as well as representative isolates from *Epichloë* species and closely related *Neotyphodium* isolates from perennial ryegrass, tall fescue and meadow fescue (GenBank accession numbers given in Appendix 5; Leuchtmann and Schardl, 1998; Schardl et al., 1991; Schardl and Leuchtmann, 1999; Schardl et al., 1997; Schardl et al., 1994; Tsai et al., 1994). Sequences were aligned using PILEUP (Section 2.18.1), and gene trees using both distance and maximum parsimony (MP) criteria were generated from sequence alignments using PAUP* Version 4.64 (Sections 2.18.2 to 2.18.3). Sequence alignment files are given in Appendix 6. For neighbor-joining (NJ) analysis of the rDNA-ITS sequences, 89.33% constant sites were observed in the data, and 81.63% of the sites were estimated as invariable, and excluded from analysis. Similarly, 79.73% constant sites were observed in the *tub2* alignment, and 64.47% of the sites were estimated as invariable and excluded from neighbor joining analyses (Section 2.18.3). The support for clades within the generated trees was verified by bootstrap analysis as described (Section 2.18).

Four most parsimonious trees, of length 55 steps, were obtained from MP analysis of the rDNA-ITS sequence alignment (Section 2.18.2), and the strict consensus of these is shown in Figure 4.6. Bootstrap analysis, using 100 replicates, was performed to determine the support for clades within this topology. The basic topology of this tree was similar to the one by Schardl et al. (1997), and likewise, bootstrap analysis indicated a lack of support for many of the internal branches. This is likely to be due to homoplasy and/or the smaller number of informative characters compared to *tub2* sequences (Schardl et al., 1997). However, bootstrap replication values indicate there is good

Fig. 4.6. rDNA-ITS gene phylogeny based on Epichloë and annual ryegrass endophyte sequences

The strict consensus tree of the four most parsimonious gene trees identified by a branch-and-bound search is shown. The total length of each of the maximum parsimony trees was 55 steps. The rDNA-ITS sequences are from representative Epichloë endophytes (GenBank accession numbers given in Appendix 5) and annual ryegrass endophyte isolates (shown underlined). The percentages (>50%) that each clade was represented in 100 bootstrap replications are indicated on their respective branches. The left-hand edge of the tree was inferred by midpoint rooting.


78
76
73
88
94
53
61
E. typhina E430
LcTG-1 Lc4
E. typhina E2461
E. typhina E8
E. glyceriae E277
FaTG-3 Tf18
E. sylvatica E503
E. brachyelytri E1045
LmTG-1 Lm1
LmTG-1 Lm2
LmTG-1 Lro1
LmTG-1 Lrr1
LmTG-1 Lrr2
N. uncinatum e166
E. elymi E56
E. bromicola E502
E. festucae E28
N. lolii Lp5
FaTG-2 e41
N. coenophialum e19
E. baconii E248
E. amarillans E114

support for the clusters of interest within the tree. Specifically, there was 78% support for the clade grouping Lc4 with *E. typhina* isolate E430, and 88% support for the clustering of Lm1, Lm2, Lrr1, Lrr2, and Lro1 with *N. uncinatum* isolate e166. The topologies of the neighbor-joining trees, constructed using both the Kimura 2-parameter and Jukes-Cantor distance matrices (Section 2.18.3), were very similar to each other, but differed from the MP consensus tree in the branching order of E56 and the annual ryegrass endophyte clade (results not shown).

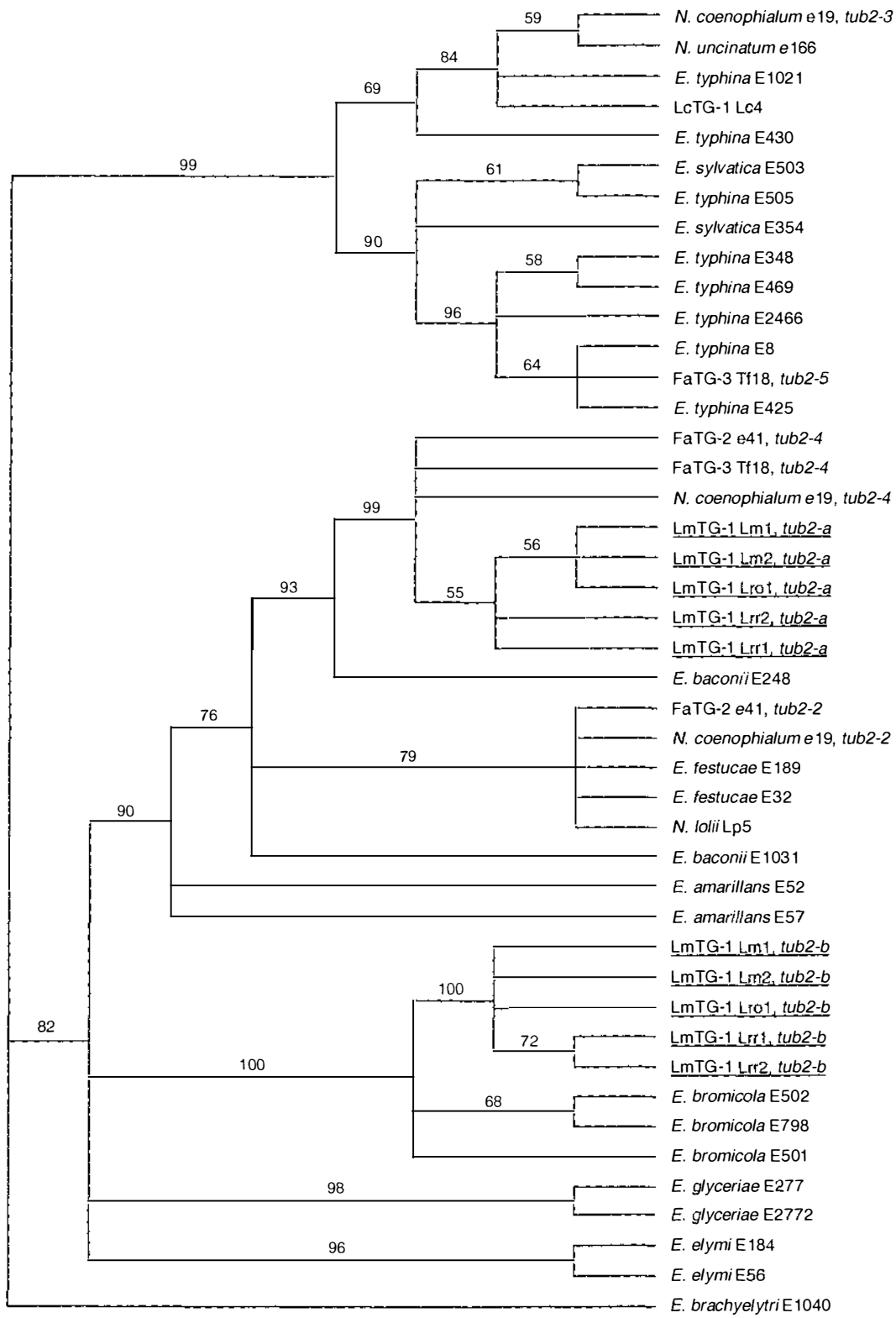
Forty most parsimonious trees of length 131 steps were obtained from MP analysis of the *tub2* sequence alignment (Section 2.18.2) by a heuristic search, and the strict consensus of these is shown in Figure 4.7. The basic topology of this tree conforms closely to that of all Epichloë mating populations generated by Schardl et al. (1997). One hundred bootstrap replicates were performed and high bootstrap replication percentages were obtained for many of the tree's internal nodes, indicating that many patterns in the data supported these clades. Furthermore, the tree topologies obtained from both NJ methods employed (Section 2.18.3) were almost identical to the MP trees (results not shown). The *tub2-a* copies from Lm1, Lm2, Lrr1, Lrr2, and Lro1 grouped with the *E. baconii*-like *tub2-4* genes from all tall fescue endophytes. The *tub2-b* copies grouped with the *E. bromicola* isolates; the bootstrap percentages for these clades were 99% and 100% respectively. The *tub2* gene from Lc4 grouped with that of *E. typhina* isolate E1021, the *tub2*-3 gene copy from *N. coenophialum* isolate e19, and the single *tub2* copy of *N. uncinatum* isolate e166. The bootstrap value for this clade is 84%, and pairwise comparisons of the *tub2* sequences within the clade show that there is only one nucleotide substitution difference between E1021 and each of the other isolates.

4.2.6 MICROSATELLITE ANALYSIS

To rapidly determine the genetic diversity of endophyte isolates from all seven annual ryegrass species, including those that cultures were not available from, microsatellite fingerprints using Primer Set I (Section 3.2.2.6) were amplified either from fungal genomic DNA, or from in planta DNA preparations. In planta amplification often

Fig. 4.7. *tub2* gene phylogeny based on Epichloë and annual ryegrass endophyte sequences

The strict consensus tree of the 40 most parsimonious trees identified by a heuristic search is shown. The total length of each of the maximum parsimony trees was 131 steps. The *tub2* sequences of introns 1 – 3 are from representative Epichloë endophytes (GenBank accession numbers given in Appendix 5) and annual ryegrass endophyte isolates (shown underlined). The percentages (>50%) that each clade was represented in 100 bootstrap replications are indicated on their respective branches. The left-hand edge of the tree was inferred by midpoint rooting. The topologies of the distance-based neighbor-joining trees, using both Jukes-Cantor and Kimura 2-parameter distance matrices, closely matched the MP trees.


N. coenophialum e19, tub2-3
N. uncinatum e166
E. typhina E1021
LcTG-1 Lc4
E. typhina E430
E. sylvatica E503
E. typhina E505
E. sylvatica E354
E. typhina E348
E. typhina E469
E. typhina E2466
E. typhina E8
FaTG-3 Tf18, tub2-5
E. typhina E425
FaTG-2 e41, tub2-4
FaTG-3 Tf18, tub2-4
N. coenophialum e19, tub2-4
LmTG-1 Lm1, tub2-a
LmTG-1 Lm2, tub2-a
LmTG-1 Lro1, tub2-a
LmTG-1 Lr2, tub2-a
LmTG-1 Lr1, tub2-a
E. baconii E248
FaTG-2 e41, tub2-2
N. coenophialum e19, tub2-2
E. festucae E189
E. festucae E32
N. lolii Lp5
E. baconii E1031
E. amarillans E52
E. amarillans E57
LmTG-1 Lm1, tub2-b
LmTG-1 Lm2, tub2-b
LmTG-1 Lro1, tub2-b
LmTG-1 Lr1, tub2-b
LmTG-1 Lr2, tub2-b
E. bromicola E502
E. bromicola E798
E. bromicola E501
E. glyceriae E277
E. glyceriae E2772
E. elymi E184
E. elymi E56
E. brachyelytri E1040
59
84
69
99
61
90
58
96
64
99
56
55
93
76
79
90
82
100
72
100
68
98
96

yielded very low amounts of product, so a second round of amplification was performed on a 1/100 dilution of the PCR reaction before automated analysis on an ABI Prism 377 DNA Sequencer (Section 2.16).

The microsatellite genotypes of the annual ryegrass endophyte isolates are listed in Table 4.1. All isolates, except Lc4, have very similar genotypes, with amplification at four of the five microsatellite loci. No amplification products were obtained from locus B9. Alleles of size 84/85, 179, and 109 ntu were amplified at loci B4, B6, and B11 respectively, and a second allele was occasionally amplified at locus B4, which was about 130 ntu. Microsatellite locus B10 was highly polymorphic with a total of five alleles observed at this locus (152, 155, 158, 161 and 166 ntu). Two alleles were consistently amplified at B10, suggestive of a hybrid origin for these endophytes. Several of the B10 alleles were also observed in other closely related endophytes. For example, *N. uncinatum* also has a B4 allele of 85 ntu, B6 of 179 ntu, B10 of 161 ntu and no amplification at locus B9. *E. bromicola* shared the same B4, B6 and B9 alleles with the common endophyte, but there were no *E. baconii* alleles in common. Additionally, isolates of *N. coenophialum* have been observed to contain B10 of 161 ntu and *E. festucae* B10 had 166 ntu.

Only two microsatellite loci amplified from isolate Lc4. These were B4 and B10, whose allele sizes were 104 and 181 ntu respectively. This genotype is identical to that of *E. typhina* strains E1021 and E1022 from *Poa nemoralis* (Table 3.4). Lc4 also shares alleles with *N. uncinatum* (locus B4 contains 104 ntu allele) and FaTG-3 (locus B10 contains 181 ntu allele). Three other seedlines of endophyte infected L. canariense tissue were also examined and it is interesting to note that the microsatellite profiles of these endophytes, Lc1, Lc2, and Lc3, were similar to those of the rest of the annual ryegrass endophytes (Table 4.1).

Table 4.1. Microsatellite genotypes of annual ryegrass endophyte isolates

Isolate	Tissue †	Allele size (ntu)			
		B4	B6	B10	B11
Lc1	IP	84.3	178.8	151.6* 166.2*	108.8*
Lc2	IP	84.7*	178.8*	151.9* *167.4	*109.9
Lc3	IP	84.2* 130.4*	178.8	151.6* 166.2*	108.9*
Lc4	C	104.4	-	180.8*	-
Lm1	C	85.0	178.8	151.8 166.4	109.0
Lm2	C	***86.5**	178.7	151.9* 166.4*	108.8*
Lm3	IP	84.3*	178.7	151.6* 166.2*	109.0*
Lps1	IP	84.1 130.5	178.8	151.6* 166.2*	108.9*
Lps2	IP	84.4	178.8	151.6* 166.2*	108.9*
Lre1	IP	**85.4***	178.6*	154.6* *161.7	*109.9
Lre2	IP	84.2	178.8	151.6* 166.2*	108.9*
Lre3	IP	84.3* 130.3*	178.8	151.7* 166.3*	108.9*
Lrr1	C	84.8	178.7	154.7* 160.6*	108.9*
Lrr1	IP	**86.0**	178.7*	*155.7 *161.7	*109.9
Lrr2	IP	84.4* 130.8*	178.7	151.7* 157.7*	108.9*
Lrr3	IP	84.3* 131.2	178.8	151.7* 166.2*	108.8*
Lrr4	C	85.7	178.8*	151.8* 166.4*	108.9*

Table 4.1. ***continued***

Isolate	Tissue †	Allele size (ntu)			
		B4	B6	B10	B11
Lro1	C	**86.8**	178.8*	*152.9 *167.5	*109.8
Ls1	IP	84.3* 131.3	178.7	151.6* 157.5*	108.9*
Lt1	IP	*85.3 130.6	179.0*	151.9* 166.6*	*110.1
Lt2	IP	84.7 130.8*	*179.9	151.8* 166.6*	*109.9

† Fingerprints were amplified from DNA prepared from in planta (IP), or fungal culture (C) tissue

* an asterisk shown after the allele indicates the presence of a band ~1 ntu larger than that shown, and an asterisk shown before the allele indicates that it is assumed that this is the +A band only present

alleles in boldface indicate that an extremely large peak height was observed when this allele was automatically analysed, and size-calling may not be accurate

4.2.7 ANALYSIS OF ENDOPHYTE ISOLATES FROM *HORDEUM* GRASSES

Two poorly described endophyte isolates, HaB and Hd1, from *Hordeum* grasses were available in the Scott Laboratory fungal culture collection. These isolates were recovered from –70°C storage as agar blocks in 50% glycerol, and maintained on PDA plates (Section 2.2.2) for further study.

4.2.7.1 Culture Descriptions

Figure 4.8 shows four week old colonies of HaB (Fig. 4.8A) and Hd1 (Fig. 4.8B) growing on PDA medium (Section 2.2.2). HaB formed white raised colonies with abundant cottony mycelia, which were aggregated into erect tufts at the centres. Colonies have superficial margins. HaB is a fast growing isolate, with an average radial growth rate of 0.57 mm d^{-1} over a four week period. Moderate conidia production was observed. In contrast, Hd1 formed flat white felted colonies with superficial margins. The average radial growth rate was 0.64 mm d^{-1} over a four week period, and conidia production was abundant.

For all genetic analyses that follow, DNA from these isolates was prepared from liquid cultures of fungal mycelia, using the large scale isolation procedure (Section 2.5.1).

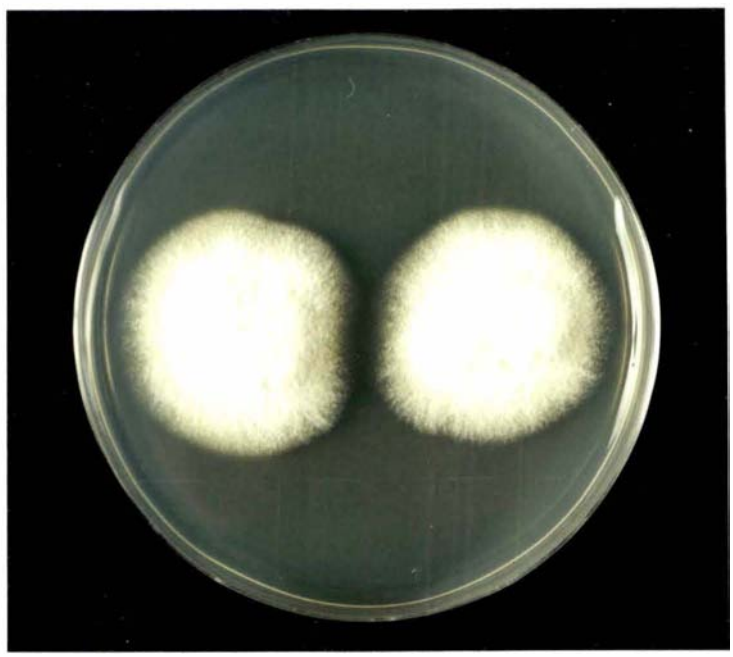
4.2.7.2 rDNA-ITS Analysis

The rDNA-ITS regions of HaB and Hd1 were amplified from fungal genomic DNA (Section 2.5.1) as described in Section 4.2.3. Amplification products were resolved as single bands of around 600 bp, similar to those shown in Figure 4.2, when separated on 2% agarose gels. Products were purified and both strands sequenced (Section 2.15), the sequences for these and the rDNA-ITS region from *N. coenophialum* isolate e19 are shown in an alignment in Figure 4.9. The HaB and Hd1 rDNA-ITS sequences have 96.7% identity to one another. BLAST database similarity searches (Section 2.15.3) were conducted and the HaB sequence identified rDNA-ITS sequences from

Fig. 4.8. Cultures of *Hordeum* endophyte isolates, HaB and Hd1

Four week old cultures of HaB and Hd1 that were grown on PDA at 22°C are shown. Scale bar is 10 mm long.

A



B

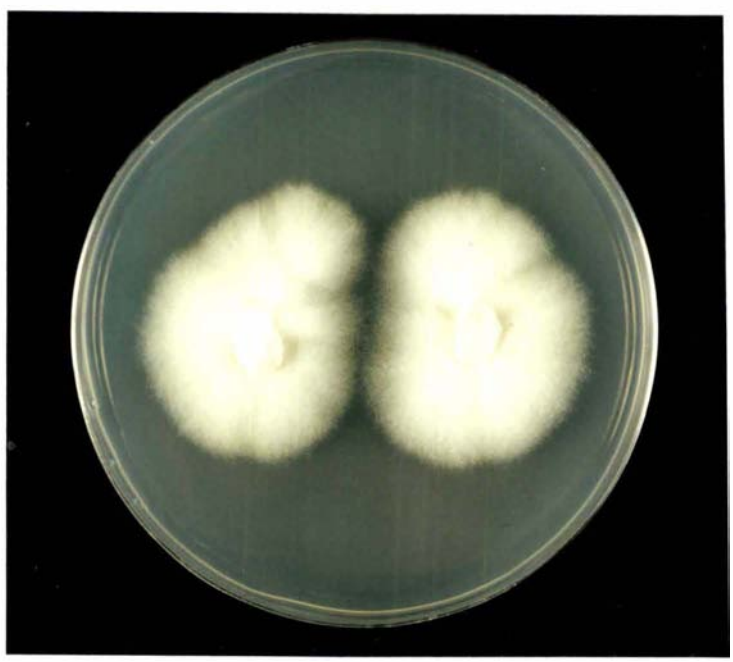


Fig. 4.9. Alignment of rDNA-ITS sequences from *Hordeum* endophyte isolates

The DNA sequences of the rDNA-ITS regions from *Hordeum* endophyte isolates HaB and Hd1, and *N. coenophialum* isolate e19 were aligned using the PILEUP programme of the Wisconsin Package with the gap penalty set to one and the gap extension penalty set to zero. Gaps (.) were introduced to facilitate sequence alignment. The sequenced region from e19 encodes ITS1 (positions 1 – 170), 5.8S rRNA (positions 171 – 338, shown italicised), and ITS2 (positions 339 – 508) (Schardl et al., 1991). Nucleotides that differ from the reference sequence, e19, are highlighted in green.

```
     1                                                            50
e19  CGAGTTTACA CTCCC.AAAC CCCTGTGAAC CT.TACCTTT ACTGTTGCCT
Hd1  CGAGTTTACA CTCCC.AAAC CCCTGTGGAC CTATACCTTT ACTGTTGCCT
HaB  CGAGTTTACA CTCCCAAAAC CCCTGTGCAC CTATACCTCT ACTGTTGCCT

     51                                                          100
e19  CGGCGGGCAC GGCCGCGGAC GCCCCCTCGC GGGGGCACCG GGGCCGGGCG
Hd1  CGGCGGGCAC GGCCGCGGAC GCCCCCTCGC GGGGGCACCG GGGCCAGGCG
HaB  CGGCGGGCAC GGCCGCGGAC GCCCCCTCGC GGGGGCGCCG GGGCTAGGCG

     101                                                         150
e19  CCCGCCGGAG GACCCAAACC CTTCTGTATT TTTCTTACGC ATGTCTGAGT
Hd1  CCCGCCGGAG GACCCAAACC CTTCTGTA.T TTTCTTACGC ATGTCTGAGT
HaB  CCCGCCGGGG GACCCAAACC CTTCTGTA.T TTTCTTACGC ATGTCTGAGT

     151                                                         200
e19  GGATTTAATA TCAAATGAAT CAAAACTTTC AACAACGGAT CTCTTGGTTC
Hd1  GGATT...TA .CAAATGAAT CAAAACTTTC AACAACGGAT CTCTTGGTTC
HaB  GGATTGAATA TCGAATGAAT CAAAACTTTC AACAACGGAT CTCTTGGTTC

     201                                                         250
e19  TGGCATCGAT GAAGAACGCA GCGAAATGCG ATAAGTAATG TGAATTGCAG
Hd1  TGGCATCGAT GAAGAACGCA GCGAAATGCG ATAAGTAATG TGAATTGCAG
HaB  TGGCATCGAT GAAGAACGCA GCGAAATGCG ATAAGTAATG TGAATTGCAG

     251                                                         300
e19  AATTCAGTGA ATCATCGAAT CTTTGAACGC ACATTGCGCC CGCCAGTATT
Hd1  AATTCAGTGA ATCATCGAAT CTTTGAACGC ACATTGCGCC CGCCAGTATT
HaB  AATTCAGTGA ATCATCGAAT CTTTGAACGC ACATTGCGCC CGCCAGTATT
```

```
     301                                                        350
e19  CTGGCGGGCA TGCCTGTTCG AGCGTCATTT CAACCCTCAA GCCCGCTGCG
Hd1  CTGGCGGGCA TGCCTGTTCG AGCGTCATTT CAACCCTCAA GCCCGCTGCG
HaB  CTGGCGGGCA TGCCTGTTCG AGCGTCATTT CAACCCTCAA GCCCGCTGCG

     351                                                        400
e19  CGCTTGCTGT TGGGGACCGG CTCACCCGCC TCGCGGCGGC GGCCGCCCCC
Hd1  TGCTTGGTGT TGGGGACCGG CCAGCCCGCC TCGCGGCGGC GGCCGCCCCT
HaB  CGCTTGGTGT TGGGGACCGG CTCGCCCGCC TCGCGGCGGC GGCCGCCCCT

     401                                                        450
e19  GAAATGAATC GGCGGTCTCG TCGCAAGCCT CCTTTGCGTA GTAGCACACC
Hd1  GAAATGAATT GGCGGTCTCG CCGC.AGCCT TCTTTGCGTA GTAACACACC
HaB  GAAATGAATC GGCGGTCTCG TCGC.AGCCT CCTTTGCGTA GTAACACACC

     451                                                        500
e19  ACCTCGCAAC CGGGAGCGCG GCGCGGCCAC AGCCGTAAAA CGCCCAACTT
Hd1  ACCTCGCAAC CAGGAGCGCG GCGCGGCCAC TGCCGTAAAA CGCCCAAC.T
HaB  ACCTCGCAAC CGGGAGCGCG GCGCGG.CAC TGCCGTAAAA CGCCCAAC.T

     501
e19  TCTCCAAG
Hd1  TCTCCAAG
HaB  TCTCCAAG
```

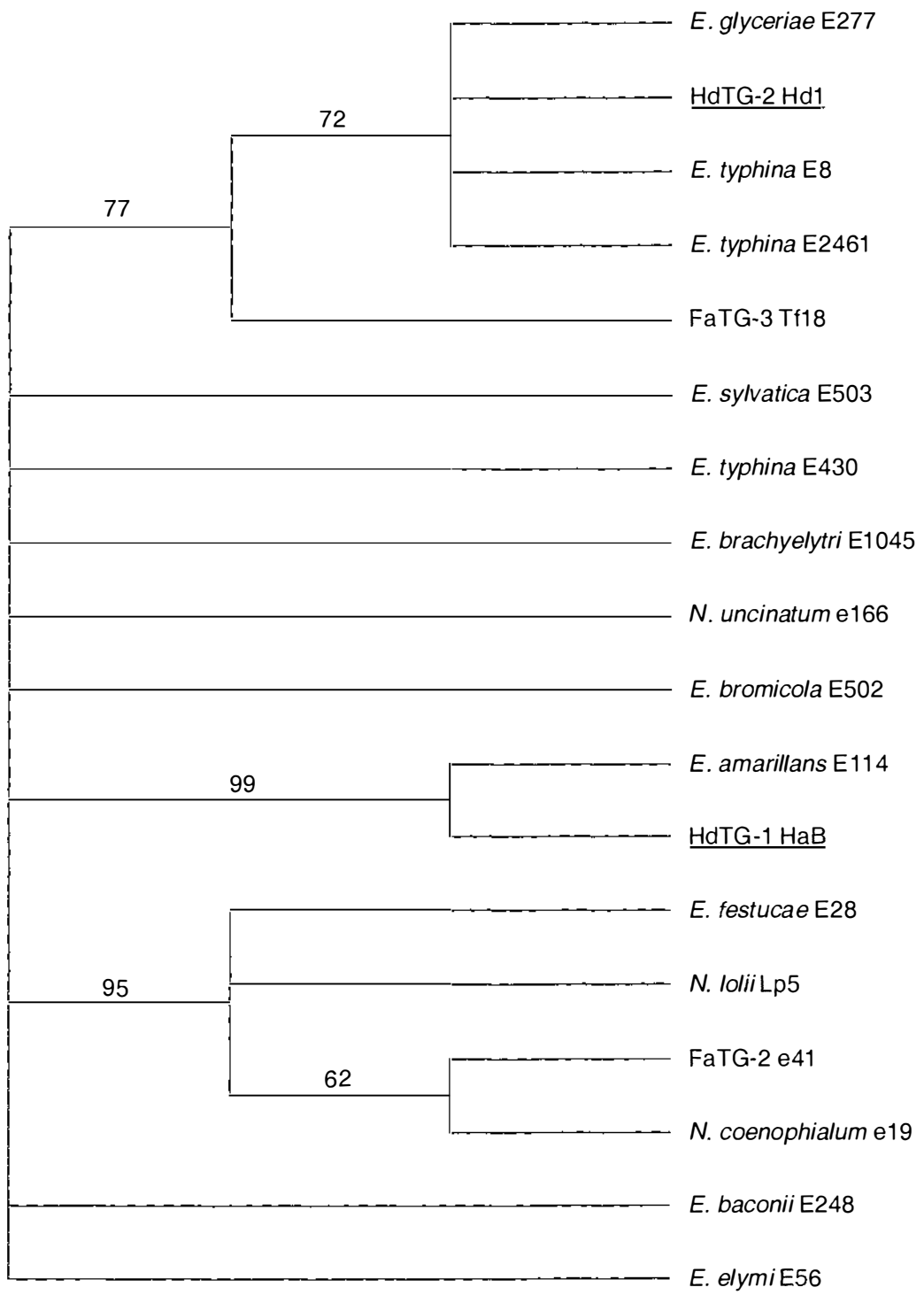
E. amarillans, and Hd1 identified rDNA-ITS sequences from *E. typhina* and *E. glyceriae* in the first five entries of each search.

As with the rDNA-ITS phylogenetic analysis of the annual ryegrass endophytes (Section 4.2.5), to determine the most closely related genes to those of the *Hordeum* endophyte isolates, phylogenetic analyses were performed using rDNA-ITS sequences from HaB and Hd1, as well as the same representative isolates from *Epichloë* and *Neotyphodium* isolates that were used in Figure 4.6. Sequences were aligned (Appendix 6), and gene trees were constructed using both distance and MP criteria. For NJ analyses, 89.83% constant sites were observed in the data, and 83.72% of the sites were estimated as invariable and excluded (Section 2.18.3) . The support for clades within the generated trees was determined by bootstrap analysis (Section 2.18).

Sixty-nine most parsimonious trees of length 54 steps were obtained from MP analysis of the rDNA-ITS sequence alignment using a branch-and-bound search, and the strict consensus of these is shown in Figure 4.10. The basic topology of this tree is unresolved, unlike the one by Schardl et al. (1997). Bootstrap analysis indicated a lack of support for many of the branches, and hence the overall topology of the tree. Again, this is likely to be due to homoplasy and/or the smaller number of informative characters compared to *tub2* sequences, however bootstrap replication values indicate there is reasonable support for the clusters of interest within the tree. Specifically, there was 72% support for the clade grouping Hd1 with *E. typhina* and *E. glyceriae* isolates, and 99% support for the clustering of HaB with *E. amarillans* isolate E114. The topologies of the NJ trees, constructed using both the Kimura 2-parameter and Jukes-Cantor distance matrices (Section 2.18.3), were almost identical, and closely matched the NJ trees generated from the analysis of annual ryegrass endophyte rDNA-ITS data (Section 4.2.5), though minor differences in the branching order of E1045 and E56 were apparent (results not shown).

Fig. 4.10. rDNA-ITS gene phylogeny based on Epichloë and *Hordeum* endophyte isolate sequences

The strict consensus tree of the 69 most parsimonious trees identified by a branch-and-bound search is shown. The total length of each of the maximum parsimony trees was 54 steps. The rDNA-ITS sequences are from representative Epichloë endophytes (GenBank accession numbers given in Appendix 5) and *Hordeum* endophyte isolates (shown underlined). The percentages (>50%) that each clade was represented in 100 bootstrap replications are indicated on their respective branches. The left-hand edge of the tree was inferred by midpoint rooting.


E. glyceriae E277
HdTG-2 Hd1
72
E. typhina E8
77
E. typhina E2461
FaTG-3 Tf18
E. sylvatica E503
E. typhina E430
E. brachyelytri E1045
N. uncinatum e166
E. bromicola E502
E. amarillans E114
99
HdTG-1 HaB
E. festucae E28
95
N. lolii Lp5
FaTG-2 e41
62
N. coenophialum e19
E. baconii E248
E. elymi E56

4.2.7.3 *tub2* Gene Analysis

The *tub2* genes of HaB and Hd1 were amplified from fungal genomic DNA as described in Section 4.2.4. Like the annual ryegrass endophytes, the *Hordeum* endophytes also yielded multiple bands, as shown in lanes 6 and 7 of Figure 4.4. These products were cloned and sequenced using the pUC118 cloning method implemented for the annual ryegrass endophyte *tub2* analysis (Section 4.2.4). Sequence analysis of the various clones identified two 'true' tub2 genes from each Hordeum endophyte isolate, and these gene copies were designated *tub2-c*, *tub2-d* from HaB and *tub2-e* and *tub2-f* from Hd1. The sequence of the 620 bp fragment from Hd1 (lane 7, Fig. 4.4) showed no significant similarity to entries in the database by BLAST searches (Section 2.15.3), so it was presumed that this was a spurious amplification product and was not included in further analysis. BLAST database searches of the *tub2-c* copy from HaB identified *tub2* genes from *E. elymi* (the first three entries), the *tub2-d* copy from HaB identified *tub2* genes from *E. amarillans* (the first three entries), the *tub2-e* copy from Hd1 identified *tub2* genes from *E. typhina* (the first six entries), the *tub2-f* copy from Hd1 identified *tub2* genes from *E. bromicola and E. elymi* (the first four entries). The *tub2* gene copies from HaB and Hd1 were aligned with the *tub2-2* gene copy from *N. coenophialum* isolate e19 and this alignment is shown Figure 4.11

The most closely related *tub2* genes to those of the *Hordeum* endophyte isolates were determined by phylogenetic analyses using the *tub2* sequences from HaB and Hd1, as well as the same representative isolates from *Epichloë* and *Neotyphodium* isolates that were used in the *tub2* analysis of Figure 4.7. Sequences were aligned and gene trees, using both distance and MP, were generated. For NJ analyses, 79.81% constant sites were observed in the data, and 62.89% of the sites were estimated as invariable and excluded (Section 2.18.3). The support for clades within the generated trees was verified by bootstrap analysis (Section 2.18).

Fig. 4.11. Alignment of the *tub2* gene sequences from *Hordeum* endophyte isolates

The DNA sequences of the *tub2* genes from the *Hordeum* endophyte isolates HaB and Hd1, and *N. coenophialum* isolate e19 (*tub2-2* copy) were aligned using the PILEUP programme of the Wisconsin Package, with the gap penalty set to one and the gap extension penalty set to zero. Gaps (.) were introduced to facilitate sequence alignment. The sequenced region from e19 encodes IVS1 (positions 1 – 209), exon 2 (positions 210 – 234), IVS2 (positions 235 – 306), exon 3 (positions 307 – 430), IVS3 (positions 431 – 543), and exon 4 (positions 544 – 549) (Tsai et al., 1994). Exon positions are shown italicised, and nucleotides that differ from the reference sequence, e19, are highlighted in green. Ambiguous (G, A, T, or C) nucleotide positions are denoted by N.

```
                1                                                    50
e19 tub2-2      AAGTTCAACC TCTCTGTTTG TCTTGGGGAC CCCCTCCTCG ACGCGTTCCG
HaB tub2-d      NNNNNNNNCC TCTCTGTTTG TCTTGGGGAC CCCCTCCTCG ACGCGTTCCG
HaB tub2-c      NCGTTTAACC GCTCTGTTTG TCTTGGGGAC CCCCTCCTCG ACGCGTTCCG
Hd1 tub2-f      NNNNNNNNNN NNNNNNNNTG TCTTGGGGAC CCCCTCCTCG ACGCGTTCCA
Hd1 tub2-e      NNNNNNNNNN NNTCTG.TTG TCTTGGGGAC CCCCTTCTCG ACGCGTTCCA

                51                                                  100
e19 tub2-2      GTGTTGAGCC CCTGATTTCG TACCCCGCCG AGCCCGGCCA CGAAGTGCAC
HaB tub2-d      GTGTTGAGCC CCTGATTTCG TACCCCGCCG AGCCCGGCCA CGACGTGCAC
HaB tub2-c      GTGTTGAACC CCTGATTTCG TACCCCGCCG AGCCCGGCCA CGACGTGCAC
Hd1 tub2-f      GTGTTGAGCC CCTGATTTCG TACCCCGCCG AGCCCGGCCA CGACGTGCAC
Hd1 tub2-e      GTGTTGAGCC CCTGATTTCG TACCCCGNCG AGCCCGGNCA CGACGTGCAC

                101                                                 150
e19 tub2-2      GCCCAACGAA C.AGTCGTGA TGAGAGGCGG ACCGAGAC.. ..AAAATTAA
HaB tub2-d      GCCCAACGAA C.AGTCGTGA TGAGAGGCGG ACCGAGAC.. ..AAAATGAA
HaB tub2-c      GCCCAATGGA CAAGTCGTGA TGAGAGGCGG ACCGAGAC.. AAAAAATTAA
Hd1 tub2-f      GCCCAANGGA C.AGTCGTGA TGAGAGGCGG ACCGAGACAA AAAAAATTAA
Hd1 tub2-e      GCCCAACGGA CNAGT GTGA TGAGAGGCGG ACCGAGAC.. ..AACATTGA

                151                                                 200
e19 tub2-2      TGAATGCGGT ATTCGAGAAC TG.TAGCTGA CCTG.TTCTT CCCCTCTTTT
HaB tub2-d      CGAATGCGGT ATTCGAGAAC TG.TAGCTGA CCTGCTTCTT TCCCTCTTTT
HaB tub2-c      TGATTGCGGT ATTCGAGAAC TGTTAGCTGA CCTTTTTC.. ........TT
Hd1 tub2-f      TGATTGCGGT ATTCGAGAAC TG.TCGCTGA CATTTTAC.. ........TT
Hd1 tub2-e      TGAATGCGGT ATTCGAAAAC CG.TAGCTGA CCTTTTTT.. ......TCTT

                201                                                 250
e19 tub2-2      CCCCTCTAGG TTCATCTTCA AACCGGTCAG TGCGTAA..G TGACAAATTC
HaB tub2-d      CCCCTCTAGG TTCATCTTCA AACCGGTCAG TGCGTAA..G TGACAAATCC
HaB tub2-c      CCCCTCTAGG TTCATCTTCA AACCGGTCAG TGCGTAA..G TGACAAATCC
Hd1 tub2-f      CCCCTCTAGG TTCATCTTCA AACCGGTCAG TGCGTAAGTG TGACAAATCC
Hd1 tub2-e      TCCCTCTAGG TTCATCTTCA AACCGGCCAG TGCGTAA..G TGAAAAATCC
```

```
                251                                                 300
e19 tub2-2      GCCGACCTCG AACGACAGGC ACAAACAGCA TGAAAAACTC ACATTCATTT
HaB tub2-d      GCCGACCTCG AACGACAGGC ACAAACAGCA TGAAAAACTC ACATTCATTT
HaB tub2-c      GCCGACCTAG AACGACGGGC ACAAATAACA TGAAAAACTC ACATTTATTT
Hd1 tub2-f      GCCGACCTCG AACGACAGGC ACAGATAACA TGAAAAACTC ACATTTCTTT
Hd1 tub2-e      GCCGACCTCG AACGACAGGC ACAGATAACA TGAAAAACTC ACATTGATTT

                301                                                 350
e19 tub2-2      GGGCAGGGTA ACCAAATTGG TGCTGCTTTC TGGCAGACCA TCTCTGGCGA
HaB tub2-d      GGGCAGGGTA ACCAAATTGG TGCTGCTTTC TGGCAGACCA TCTCTGGCGA
HaB tub2-c      GGGCAGGGTA ACCAAATTGG TGCTGCTTTC TGGCAGACCA TCTCTGGCGA
Hd1 tub2-f      GGGCAGGGTA ACCAAATTGG TGCTGCTTTC TGGCAGACCA TCTCTGGCGA
Hd1 tub2-e      GGGCAGGGTA ACCAAATTGG TGCTGCTTTC TGGCAGACCA TCTCTGGCGA

                351                                                 400
e19 tub2-2      GCACGGCCTC GACAGCAATG GTGTGTACAA TGGTACCTCC GAGCTCCAGC
HaB tub2-d      GCACGGCCTC GACAGCAATG GTGTGTACAA TGGTACCTCC GAGCTCCAGC
HaB tub2-c      GCACGGCCTC GACAGCAATG GTGTGTACAA TGGTACCTCC GAGCTCCAGC
Hd1 tub2-f      GCACGGTCTC GACAGCAATG GTGTGTACAA CGGTACCTCC GAGCTCCAGC
Hd1 tub2-e      GCACGGCCTC GACAGCAATG GTGTGTACAA TGGTACCTTC GAGCTCCAGC

                401                                                 450
e19 tub2-2      TCGAGCGTAT GAGTGTCTAC TTCAACGAGG TAAGTCTTCA TAATCT...A
HaB tub2-d      TTGAGCGTAT GAGTGTCTAC TTCAACGAGG TAAGTCTTCA TAATCT...A
HaB tub2-c      TGGAGCGTAT GAGTGTCTAC TTCAACGAGG TATGTCTTCA TAATCT...A
Hd1 tub2-f      TGGAGCGTAT GAGTGTCTAC TTCAACGAGG TAAGTCTTCA TAATTT...A
Hd1 tub2-e      TCGAGCGTAT GAGTGTCTAC TTCAACGAGG CAAGTCTTCA TAATCTAAAA

                451                                                 500
e19 tub2-2      AAGTCTCCAT TGAGCTACAT ACATACCGCC CCGGAGATGG GACGGAAAGA
HaB tub2-d      AAGTCTCCAT TGAGCTACAT ACATACCGCC CCGGAGATGA GACGGAAAGA
HaB tub2-c      AAGTCTCCAT TGAGC....T ACATACCGCC CTGGAGATGA GACGGAAAGA
Hd1 tub2-f      AAGTCTCCAT TGAGC....T ACATACCGCC CTGGAGATGA GACGGAAAGA
Hd1 tub2-e      AAGTCTCCAT TGAGC....T ACATACCGCC CTGGAGATGG GACGGGAAAA

                501                                                 549
e19 tub2-2      GAACGAAAGA AAAAGTGTTA T..CATGCTC ATCCATGTGA CAGGCTTCT
HaB tub2-d      GAACGAGAGA AAAAGTGTCA T..CATGCTC ATCCATGTGA CAGGCTTCT
HaB tub2-c      GAACGAAAGA AAAAGTGTCA TCATATGCTA ATCTATGTGA CAGGCTTCT
Hd1 tub2-f      GAACG..ATG AAAAGTGT.. TAACATGCTA ATCTATGTGA CAGGCTTCT
Hd1 tub2-e      GAAGCGGAGA AAAAGTATTA T..CATGCTA ATCTATGTGA CAGGCTTCT
```

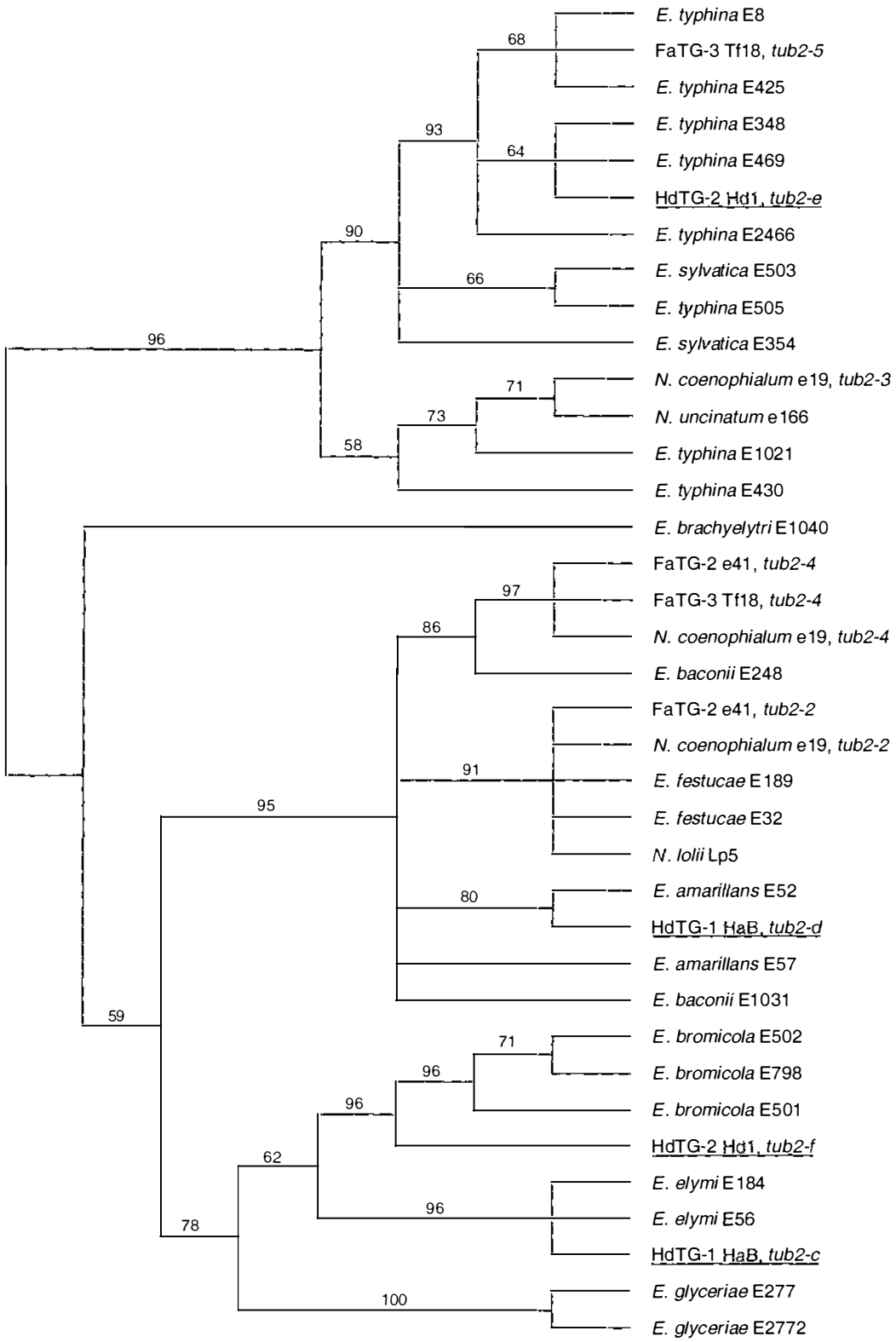
Twenty-four most parsimonious trees of length 123 steps were obtained from MP analysis of the *tub2* gene sequence alignment (Section 2.18.2) using a branch-and-bound search, and the strict consensus of these is shown in Figure 4.12. The basic topology of this tree conforms closely to that of all Epichloë mating populations generated by Schardl et al. (1997). One hundred bootstrap replications were performed (Section 2.18) and high bootstrap replication percentages were obtained for most of the tree's internal nodes, indicating that many patterns in the data supported these clades. Furthermore, the tree topologies obtained from both NJ methods employed (Section 2.18.3) were highly similar to the MP trees (results not shown). The *tub2-c* gene copy from HaB grouped with high confidence with the *E. elymi* isolates E184 and E56 (96% of the bootstrap trees), and the *tub2-d* gene copy from HaB grouped together with the *E. amarillans* isolate E52 in 80% of the bootstrap trees. The Hd1 *tub2-e* copy grouped with *E. typhina* gene from isolates E348 and E469 (64% of bootstrap trees), and the *tub2-f* copy grouped with the three *E. bromicola* isolates in 96% of the bootstrap trees.

4.2.7.4 Microsatellite Analysis

Microsatellite fingerprints of HaB and Hd1 were generated from fungal genomic DNA with Primer Sets I and II (Sections 2.13 and 2.16), and their genotypes are listed in Table 3.6. Multiple alleles were observed at several loci, which supports the hybrid origins of both of these isolates, and common alleles shared with other endophytes are discussed in Section 3.3.3.1.1.

Fig. 4.12. *tub2* gene phylogeny based on Epichloë and *Hordeum* endophyte isolate sequences

The strict consensus tree of the 24 most parsimonious trees identified by a branch-and-bound search is shown. The total length of each of the maximum parsimony trees was 123 steps. The *tub2* sequences of introns 1 – 3 are from representative Epichloë endophytes (GenBank accession numbers given in Appendix 5) and *Hordeum* endophyte isolates (shown underlined). The percentages (>50%) that each clade was represented in 100 bootstrap replications are indicated on their respective branches. The left-hand edge of the tree was inferred by midpoint rooting. The topologies of the distance-based neighbor-joining trees, using both Jukes-Cantor and Kimura 2-parameter distance matrices, closely matched the MP trees.


E. typhina E8
FaTG-3 Tf18, tub2-5
E. typhina E425
E. typhina E348
E. typhina E469
HdTG-2 Hd1, tub2-e
E. typhina E2466
E. sylvatica E503
E. typhina E505
E. sylvatica E354
N. coenophialum e19, tub2-3
N. uncinatum e166
E. typhina E1021
E. typhina E430
E. brachyelytri E1040
FaTG-2 e41, tub2-4
FaTG-3 Tf18, tub2-4
N. coenophialum e19, tub2-4
E. baconii E248
FaTG-2 e41, tub2-2
N. coenophialum e19, tub2-2
E. festucae E189
E. festucae E32
N. lolii Lp5
E. amarillans E52
HdTG-1 HaB, tub2-d
E. amarillans E57
E. baconii E1031
E. bromicola E502
E. bromicola E798
E. bromicola E501
HdTG-2 Hd1, tub2-f
E. elymi E184
E. elymi E56
HdTG-1 HaB, tub2-c
E. glyceriae E277
E. glyceriae E2772
68
93
64
90
66
96
71
73
58
97
86
91
95
80
59
71
96
96
62
96
78
100

4.3 DISCUSSION

4.3.1 TWO NEW ENDOPHYTE TAXONOMIC GROUPS ARE IDENTIFIED FROM ANNUAL RYEGRASSES

This study has confirmed through genetic analysis that the seed-borne endophytic fungus of *Lolium* annual ryegrass species, which was isolated and described by Latch et al. (1988), is a single distinct *Neotyphodium* species. We have surveyed all seven species of annual ryegrasses and have found this common endophyte in each. A second previously undescribed *Neotyphodium* endophyte was found in *L. canariense.*

The distinctive in planta distribution of the common endophyte (Latch et al., 1988) was conserved across all seven host grass species, where mycelia was confined to the basal regions of vegetative tillers, and was more extensively distributed in flowering tillers. Cultures were isolated from *L. multiflorum*, *L. rigidum* var. *rigidum* and *L. rigidum* var. *rottboellioides*, but the extremely slow growth rate of this fungus made isolation difficult, so despite attempts, cultures were not obtained from the other annual ryegrass species. Nonetheless, these strains were genetically characterised by in planta microsatellite fingerprinting, and the results show that the degree of fingerprint variability is of a level similar or less than that displayed by established taxonomic grouping, or species of *Neotyphodium* (Moon et al., 1999). Phylogenetic analysis of the rDNA-ITS and *tub2* gene sequences reveal unique evolutionary origins for this group of endophytes, and it is proposed that this group be named *Lolium multiflorum* taxonomic group one (LmTG-1), after the convention used by Christensen et al. (1993), and by reference to the endophyte of *L. multiflorum*, as this was the first grass to be isolated from in this study.

The second distinctive type of endophyte found in this study, isolate Lc4, was readily isolated from one of the four seedlines of *L. canariense* examined, and to our knowledge, represents the first report of such an endophyte.. The in planta distribution of Lc4 was more extensive than LmTG-1, and the cultural characteristics were clearly different, both in the rate of growth and the colony appearance. The genetic profile of Lc4 for

rDNA-ITS, *tub2*, and microsatellite data is unique, suggesting that this is a new species of an asexual Epichloë endophyte. It is therefore proposed that this endophyte be named *Lolium canariense* taxonomic group one (LcTG-1). It should be noted that neither of the two endophyte taxonomic groups described in this study, LmTG-1 or LcTG-1, have been found in other grasses, including closely related perennial ryegrass, meadow fescue or tall fescue.

In addition to Epichloë endophytes, annual ryegrasses have been reported to contain *Acremonium*-like seedborne fungal endophytes, which are also basally confined in the plant, and produce rod shaped conidia (Naffaa et al., 1998). These fast growing endophytes are morphologically similar to *A. chilense*, though we did not observe such endophytes in this study. More specifically, we did not have evidence to suggest the *Acremonium* endophytes encountered in this study were growing as seedborne endophytes as they were not isolated from immature inflorescences of any of the annual ryegrass species. *A. chilense* is a seed transmitted endophyte of *Dactylis glomerata* (Morgan-Jones et al., 1990), and though it was renamed *Neotyphodium chilense* by Glenn et al. (1996), genetic and morphological data suggest that it is a true *Acremonium* species. The position of this endophyte within the *Neotyphodium* genus is currently under review (pers. comm. Mike Christensen, Grasslands AgResearch).

4.3.2 THE EVOLUTIONARY ORIGIN OF LmTG-1

LmTG-1 contained multiple copies of *tub2* genes and multiple alleles at microsatellite loci, a result suggestive of an interspecific hybrid origin. From phylogenetic analysis of the *tub2* genes, it is proposed that the parental endophytes include *E. baconii* and *E. bromicola*, or close relatives. This result is also supported by the LmTG-1 rDNA-ITS sequence similarities to *N. uncinatum* (which has *E. typhina* and *E. bromicola* ancestry), and the common microsatellite alleles of LmTG-1 endophytes with extant *E. bromicola* strains. This genetic data confirms that LmTG-1 is a *Neotyphodium* of unique hybrid origin, and does not group with *N. lolii*, LpTG-2 or other currently recognised endophyte species.

An interesting question regarding the evolution of LmTG-1 endophytes is how an endophyte with ancestors from *E. baconii* and *E. bromicola* origins, could have arisen in a Poeae host, given that the parents inhabit grasses of host tribes Aveneae and Bromeae respectively. Higher order grass phylogeny shows that within the subfamily Pooideae, four tribes constitute a core monophyletic group – Bromeae, Triticeae, Aveneae and Poeae, and these have been referred to as the "core" pooids (Davis and Soreng, 1993), where Poeae and Aveneae form a monophyletic group, the Aveneae being paraphyletic to Poeae (Catalán et al., 1997), and Bromeae and Triticeae form sister tribes within a monophyletic group (Catalán et al., 1997; Hsaio et al., 1995). The most likely scenario for the evolution of LmTG-1 is that an ancestral *E. baconii* existed before the Poeae diverged from Aveneae, and was passed on to both tribes. If an *E. baconii* infected Poeae host, ancestral to the *Lolium* ryegrasses, was infected by an *E. bromicola* spore and hybridisation followed, this could have given rise to LmTG-1. Support for this scenario comes from the fact that *E baconii*-like *tub2* gene copies (*tub2-4*) have also been found in all three taxonomic groups of *Neotyphodium* from tall fescue (Tsai et al., 1994), a Poeae grass. These *tub2-4* genes group closely with the *E. baconii*-like *tub2* copies from the LmTG-1 endophytes, and they are somewhat diverged from extant *E. baconii* sequences, so the sequence differences may reflect changes that have accumulated since the Poeae/Aveneae divergence. If the proposed evolutionary scenario was true, it would be likely that *E. baconii*-like species are present in Poeae grasses, but possibly have not been sampled yet.

An alternative hypothesis for the evolution of LmTG-1 is that if *E. baconii* is actually confined to Aveneae hosts, an ascospore infection of this endophyte into the *Lolium* ancestor may have established a rare stable symbiosis long enough for subsequent infection by an *E. bromicola* spore. Tall fescue endophyte may have arisen through infection and hybridisation of similar genotypes of *E. baconii*, hence the similarity in extant *Neotyphodium* endophyte *E. baconii*-like *tub2* sequences. Another option to this hypothesis is that the initial *E. baconii* infection could have infected a *Festuca/Lolium* ancestor, and was thus passed on to both tall fescue and ryegrass endophytes. This scenario would explain the apparent absence of *E. baconii*-like strains throughout the

Poeae. However, it is doubtful whether the same endophyte isolate could be compatible with such diverged hosts, as would be required with this scenario (Christensen et al., 1997).

4.3.3 THE EVOLUTIONARY ORIGIN OF LcTG-1

On the basis of genetic similarity, it is proposed that LcTG-1 is an asexual derivative of an *E. typhina* genotype closely related to the extant E1021 from *Poa nemoralis*. The microsatellite genotypes of these two endophytes are identical, and there is only an single nucleotide difference between the *tub2* sequences. There was no rDNA-ITS sequence available from E1021, but the sequence from a closely related *E. typhina* isolate, E430, showed high similarity to that of LcTG-1. Only single copies of microsatellite alleles, *tub2* genes, and rDNA-ITS sequences were found in the LcTG-1 endophyte, thus giving no evidence for interspecific hybridisation. *Neotyphodium lolii* is also thought to have evolved directly from a sexual *Epichloë* sp. (*E. festucae*; Leuchtmann, 1994; Schardl, 1996a; Schardl et al., 1994), and there are interesting morphological similarities between *N. lolii* and LcTG-1. The colony morphologies of both endophytes are often highly unstable and heterogeneous when isolated directly from plant tissue, and both have rare or apparent lack of conidia production (Christensen and Latch, 1991; Christensen et al., 1993).

It is interesting to note that unlike the majority of *E. typhina* isolates examined, which choke all infected flowering tillers, the *E. typhina* isolates from *Poa nemoralis* such as E1021, have been observed to from rare seed-borne infections, though they also normally choke all infected flowering tillers (pers. comm. Christopher Schardl, University of Kentucky). E1021-like *tub2* genes have now been found in three *Neotyphodium* taxonomic groups, including *N. coenophialum*, *N. uncinatum* and LcTG-1. Whether there is a relationship between the ability of *E. typhina* to become seed-borne, and the contribution of this or related isolates to taxonomic groups of *Neotyphodium* is not known at this time.

4.3.4 THE EVOLUTION OF *LOLIUM* ENDOPHYTES

Assuming a general mode of coevolution by common descent of grass hosts and their endophytes (Schardl et al., 1997), a possible scenario for the evolution of Epichloë endophytes within *Lolium* ryegrasses is proposed. Several *Lolium* and *Festuca* phylogenies have been established based on RAPD profiles, rDNA-ITS sequences and chloroplast (cpDNA) restriction site data (Charmet et al., 1997; Stammers et al., 1995), and it is estimated that the diploid genus *Lolium* is of very recent origin, and diverged from the *F. pratensis* lineage about 2 Myr ago. Differentiation into several *Lolium* species is estimated to have started only 1 Myr ago (Charmet et al., 1997). Because of such recent evolution, the order of speciation is not resolved with certainty, and differs depending on the type of data used. It is generally observed that the interfertile outcrossing species, *L. perenne*, *L. multiflorum*, and *L. rigidum*, group together and are differentiated from the self-fertilising species (Bennett, 1997; Charmet et al., 1997). The *Lolium* ryegrasses are very closely related to diploid *F. pratensis*, and this is reflected in the ability of the outbreeding *Lolium* species to readily hybridise with this grass (Borrill, 1976). The reproductive cycle of *L. canariense* is unusual as this grass is generally self-fertilising, but it also displays moderate levels of outbreeding (Charmet and Balfourier, 1994). *L. canariense* also has unique genetic affinities as rDNA-ITS and isozyme data group it together with *F. pratensis*, though cpDNA analysis groups it with annual *Lolium* ryegrasses (Charmet and Balfourier, 1994; Charmet et al., 1997). This suggests *L. canariense* could be a hybrid grass of a maternal annual *Lolium* with *F. pratensis* parentage. Hexaploid tall fescue, *F. arundinacea*, is also closely related to the *Lolium* grasses, and from analysis of cpDNA and rDNA-ITS data it appears to be the result of hybridisation between a maternal tetraploid *F. glaucesens* with *F. pratensis* (Charmet et al., 1997). A proposed scenario for the evolution of *Lolium* endophytes within the *Lolium* genus is shown in Figure 4.13. This is based on a UPGMA dendrogram of *Festuca* and *Lolium* species generated from cpDNA restriction site data (Charmet et al., 1997), where all extant *Lolium* species form a monophyletic group that has descended from a common ancestor.

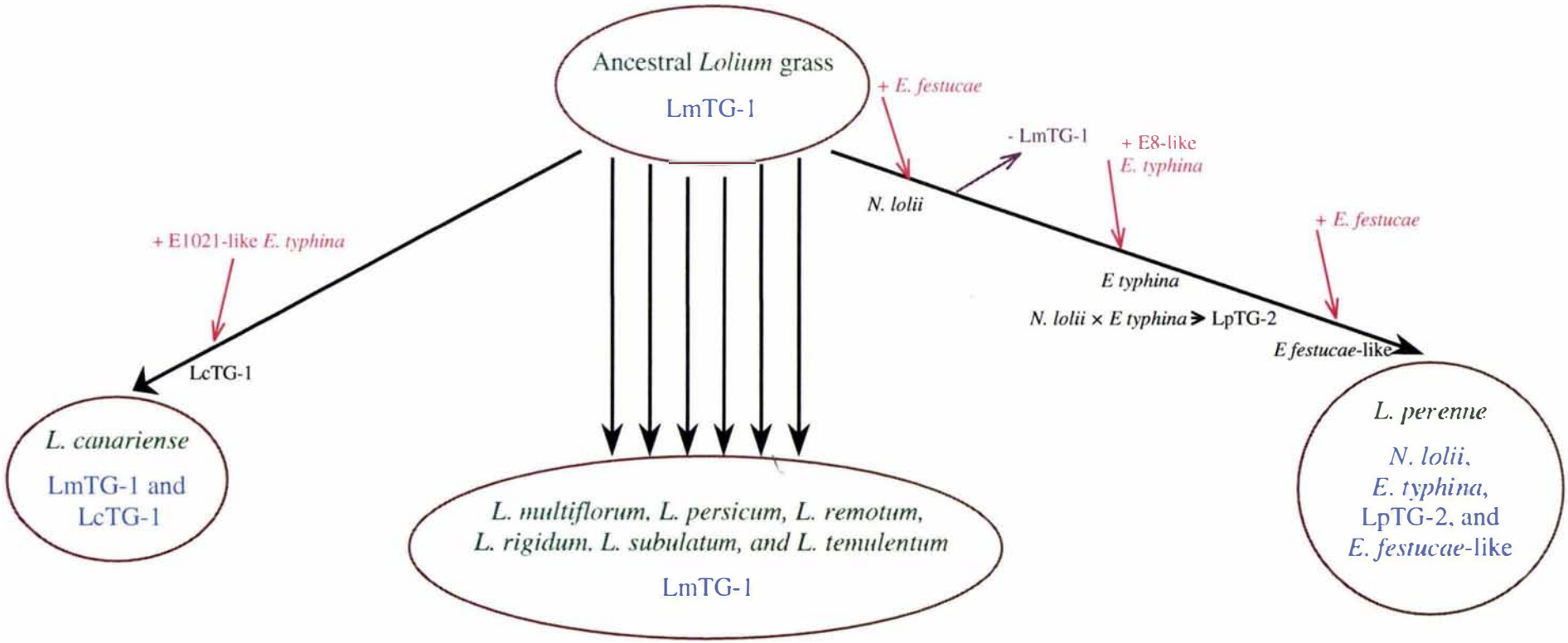


Fig. 4.13. Possible scenario for the distribution of Epichloë endophytes during the evolution of *Lolium* spp.

A schematic diagram of *Lolium* speciation is shown, with a scenario proposed to explain the distribution of Epichloë endophytes (blue) within this host genus (green). Plus (+, red) or minus (-, purple) indicates the gain or loss, respectively, of Epichloë endophytes from the host lineage. The branch lengths and branching order shown have been simplified for diagrammatic purposes.

LmTG-1 was found to be distributed throughout the annual *Lolium* species, and it is likely that this endophyte was formed, or at least was present, in the ancestral *Lolium* grass. *L. canariense*, which is endemic to the Canary Islands, was found to host both LmTG-1 and LcTG-1 endophytes, so it appears that after the *L. canariense* lineage had diverged from the other ryegrass species, it became infected with LcTG-1. A possible mechanism for infection is transfer through the stigma of a spore from an *E. typhina* genotype related to E1021, and subsequent infection of the seed. On the contrary, *L. perenne* is not known to contain LmTG-1 or LcTG-1 endophytes. Rather, the most common endophyte is *N. lolii*, though *L. perenne* occasionally hosts other endophytes including *E. typhina*, LpTG-2, and *E. festucae*-like endophytes. A plausible explanation for the origin of *N. lolii* is that the newly speciated *L. perenne* contained LmTG-1, but it subsequently became infected with *E. festucae*, which evolved into *N. lolii*. It is thought that the *Lolium* genus originated from a ryegrass with close affinity to the extant annual *L. rigidum* (Charmet and Balfourier, 1994). Therefore, assuming the life cycle of *L. perenne* evolved from an annual to a perennial cycle, the more extensive in planta distribution of *N. lolii* and subsequently higher production of protective alkaloid compounds gave the *N. lolii* symbioses a selective advantage over the LmTG-1 symbioses, and LmTG-1 was subsequently lost. In addition to *N. lolii*, *E. typhina* is also found in *L. perenne*, and this has probably come from a successful spore infection from an E8-like *E. typhina* isolate. Subsequent hybridisation between these two groups formed LpTG-2 (Schardl et al., 1994). The order of events along the lineage of *L. perenne* shown in Figure 4.13 are not certain, but it is accepted that *N. lolii* and *E. typhina* were established before LpTG-2 was formed (Schardl et al., 1994). Recently, a rare *E. festucae*-like endophyte was found in *L. perenne*, which is thought to be a recent introduction as its morphology is very similar to *E. festucae*, lending credence to the possibility that *E. festucae* infection gave rise to *N. lolii*.

The commonality of genotypes shared between LmTG-1, LcTG-1 and *N. uncinatum*, which appears to have hybrid origins involving E1021-like *E. typhina* and *E. bromicola* strains (pers. comm. Christopher Schardl, University of Kentucky); and the close relationships between their hosts suggest that these endophytes may have arose in related

events. A possibility is that the common ancestor of *F. pratensis* and *Lolium* contained a hybrid endophyte involving an E1021-like *E. typhina*, *E. baconii*, and *E. bromicola* parentage. The loss of the appropriate genes in each host lineage could explain the distribution of genes that are seen in these endophytes today. This scenario would appear unlikely though as it would involve the apparent non random loss of genes from the original hybrid. For example, in the extreme case of LcTG-1, affinities to only one of the three parents is evident. The simplest explanation for the evolution of these endophytes is the independent evolution of each endophyte type within its host lineage. Direct evolutionary relationships between the endophyte of ryegrass, meadow fescue and tall fescue are not obvious from *tub2* data and many scenarios could be postulated to explain the distributions of these genotypes. Further sequence data from other phylogenetically informative loci, such as the TEF genes, may help to elucidate these relationships.

4.3.5 THE EVOLUTIONARY ORIGINS OF TWO ENDOPHYTE ISOLATES FROM *HORDEUM* GRASSES

The results of this study strongly support the unique hybrid origins of each of the two previously undescribed *Hordeum* endophyte isolates, HaB and Hd1. This conclusion is based principally on data from phylogenetic analysis of rDNA-ITS and *tub2* DNA sequences, but also from microsatellite genotypes. Two copies of *tub2* genes are present in HaB, and their sequences suggest that HaB has descended from *E. amarillans* and *E. elymi* ancestry. The rDNA-ITS sequence from HaB shows high similarity to those of *E. amarillans* isolates, providing further support for this hypothesis. As *E. elymi* is presently found in *Elymus* grasses, which are also of the tribe Triticeae, it is most likely that *E. elymi*, or an *E. elymi*-like endophyte is the maternal parent of HaB. *E. amarillans* infects grasses of the more distantly related tribe, Aveneae, so presumably a spore from *E. amarillans* was transmitted to an *E. elymi* infected *Hordeum* host for hybridisation to ensue. Analysis of microsatellite genotypes reveal that there are no common alleles between HaB and its putative parents, and this presumably reflects changes accumulated over a considerable length of time since the hybridisation event.

Two copies of *tub2* genes are also found in Hd1, and these identify closely to the *tub2* genes of extant endophyte strains from *E. typhina* and *E. bromicola.* Interestingly, it is this combination of species that are presumed to be the progenitors of *N. uncinatum* (pers. comm. Christopher Schardl, University of Kentucky), though the hybridisation event that gave rise to Hd1 is probably independent of the one that produced *N. uncinatum,* since the *E. typhina*-like *tub2* genes of Hd1 identify more closely to those of *E. typhina* strains from *Phleum* and *Dactylis* hosts, rather than that from *N. uncinatum* (Fig. 12). It is again assumed that the maternal parent is the one that is currently found infecting hosts most closely related to *Hordeum*, so this is likely to be *E. bromicola. E. bromicola* is fairly closely related to *E. elymi*, and the tribes of their host grasses, Bromeae and Triticeae respectively, share a close evolutionary relationship (Catalán et al., 1997). Hd1 was therefore probably derived from a hybridisation event involving an *E. typhina* spore infecting an *E. bromicola*-infected *Hordeum* grass. There are a few common microsatellite alleles shared between Hd1 and extant *E. bromicola* and *E. typhina* isolates to provide further support for this relationship.

On the basis of their unique genetic identities, and hence, unique evolutionary origins, it is proposed that the *Neotyphodium* taxonomic groups that HaB and Hd1 belong, are named *Hordeum* taxonomic groups one and two (HdTG-1 and HdTG-2) respectively.

4.3.6 INTERSPECIFIC HYBRIDISATION IN THE EPICHLOË ENDOPHYTE GROUP

Hybridisation events, as speculated for the evolution of LmTG-1, HaB, and Hd1 are relatively common within the *Neotyphodium* endophytes eg. LpTG-2 (Schardl et al., 1994), all tall fescue endophytes (Tsai et al., 1994), and *N. uncinatum* (pers. comm. Christopher Schardl, University of Kentucky); and might counteract Muller's ratchet, which states that within an asexual species the inevitable accumulation of deleterious mutations would cause a loss of fitness without the correcting influences of sexual recombination (Muller, 1964). In the case of seedborne asexual grass endophytes,

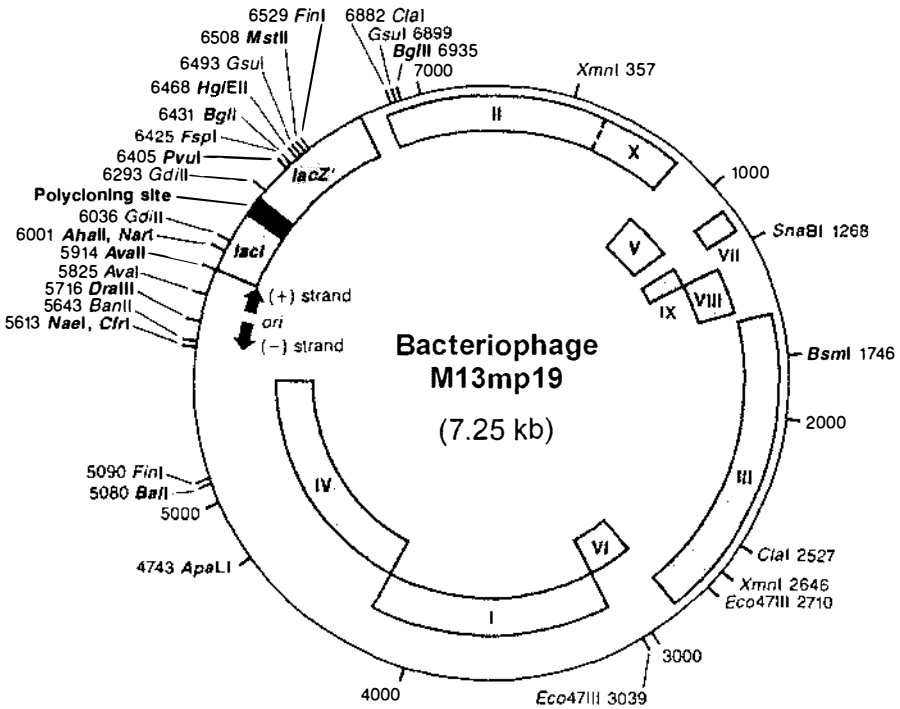
interspecific hybridisation can increase the fitness of the endophyte in several ways. The resulting heteroploid genome carries duplicate copies of genes, therefore to disrupt the function of a particular gene, multiple mutations would be required to disable each of its gene copies. Hybridisation may also introduce or augment protective characteristics of the endophyte such as the protective alkaloids produced by endophytes. New alkaloid types may be produced in the hybrid, and/or the production levels of existing alkaloids may be increased.

4.4 CONCLUSION

The *Neotyphodium*-like endophytes of annual ryegrasses, described by Latch et al. (1988), were found to have a unique hybrid origin involving *Epichloë baconii* and *E. bromicola* ancestry. These endophytes form a new *Neotyphodium* taxonomic group and it is proposed that this is named LmTG-1. A second previously undescribed *Neotyphodium* endophyte was found in *Lolium canariense*, and this was shown to be an asexual derivative of *E. typhina.* The name proposed for this taxonomic group of *Neotyphodium* endophytes is LcTG-1. The two *Hordeum* endophyte isolates, HaB and Hd1, were also both shown to have unique hybrid origins. HaB has *E. elymi* and *E. amarillans* ancestry, and Hd1 has *E. typhina* and *E. bromicola* ancestry. The proposed names for the *Neotyphodium* taxonomic groups that contain these isolates are HdTG-1 and HdTG-2 respectively. The revelation of interspecific hybrid origins for three more *Neotyphodium* endophytes illustrates the prevalence of hybridisation in the evolution of the Epichloë endophytes, and further contributes to our understanding of the evolution of this group.

APPENDICES

APPENDIX 1 VECTOR MAPS

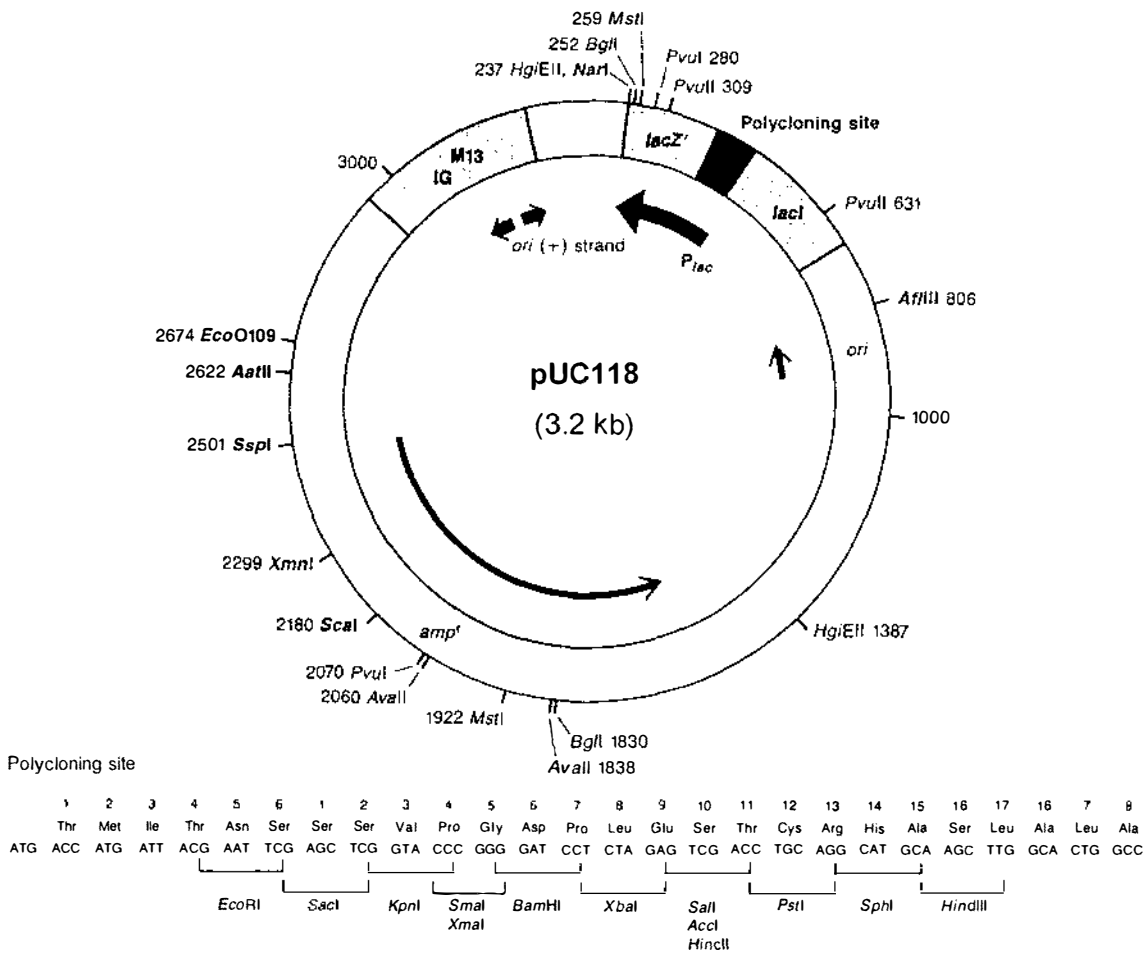


Polycloning site

1	2	3	4	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	5	6	7	8
	Thr	Met	Ile	Thr	Pro	Ser	Leu	His	Ala	Cys	Arg	Ser	Thr	Leu	Glu	Asp	Pro	Arg	Val	Pro	Ser	Ser	Asn	Ser	Leu	Ala
ATG	ACC	ATG	ATT	ACG	CCA	AGC	TTG	CAT	GCC	TGC	AGG	TCG	ACT	CTA	GAG	GAT	CCC	CGG	GTA	CCG	AGC	TCG	AAT	TCA	CTG	GCC
						HindIII		SphI		PstI		SalI AccI HincII		XbaI		BamHI	SmaI XmaI		KpnI		SacI		EcoRI		

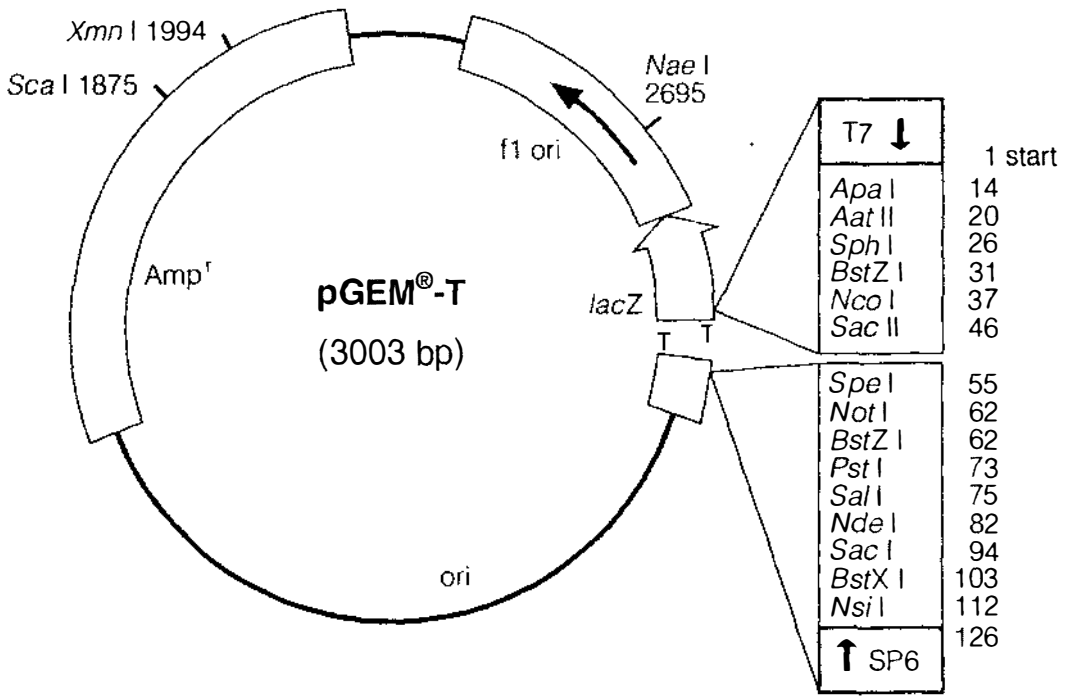
A1.1 M13mp19

Restriction map of the bacteriophage vector M13mp19 (Norrander et al., 1983). The figure shows the sites at which a number of restriction enzymes cleave the double-stranded replicative form of M13mp19. The *Hin*dIII site of the polycloning site lies immediately downstream from P_{lac}. Figure adapted from Sambrook et al. (1989).



A1.2 pUC118

Restriction map of the plasmid vector pUC118 (Vieira and Messing, 1987). The figure shows the sites at which a number of restriction enzymes cleave this vector. The *Eco*RI site of the polycloning site lies immediately downstream from P_{lac}. Figure adapted from Sambrook et al. (1989).



A1.3 pGEM-T

Map of the PCR product cloning vector, pGEM-T (Promega Corp.). The figure shows the sites at which a number of restriction enzymes cleave this vector. The binding sites of the M13 forward sequencing primer is 2944 to 2960 bp, and the M13 reverse sequencing primer is 161 to 177 bp. Figure adapted from Promega Corp.

APPENDIX 2 MANUFACTURER'S REFERENCES

References for the manufacturers used in this study are listed below.

Company	Address
Alpha Innotech Corporation	San Leandro, CA., USA
Amersham	Buckinghamshire, UK
Bio101 Inc.	La Jolla, CA., USA
Bio-Rad	Richmond, CA., USA
Boehringer Mannheim GmbH	Mannheim, Germany
Claris Corp.	Santa Clara, CA., USA
Corbett Research	Mortlake, Australia
Difco Laboratories	Detroit, MI., USA
FMC Bioproducts	Rockland, MA., USA
Fuji Photo Film Co.	Tokyo, Japan
Hoeffer Scientific Instruments	San Francisco, CA., USA
Life Technologies	Gaithersberg, MD., USA
New England Biolabs	Beverly, MA., USA
PE Applied Biosystems	Foster City, CA., USA
Perkin-Elmer Corp.	Foster City, CA., USA
Polaroid Corp.	Cambridge, MA., USA
Promega Corp.	Madison, WI., USA
Savant Instruments Inc.	Farmingdale, NY., USA
Sigma Chemical Co.	St Louis, MO., USA
Stratagene	La Jolla, CA., USA

APPENDIX 3 MICROSATELLITE ELECTROPHEROGRAMS

Electropherogram files of the microsatellite fingerprints, analysed on ABI Prism model 310 or 377 genetic analysers with Genescan 2.1 software, are supplied on CD-ROM accompanying this thesis. These files require GeneScan software to be opened.

For file identification, the file name format includes:

- the endophyte isolate name, followed by
- the primer set used in the amplification: "1" or "2", for Primers Sets I or II, respectively, and
- the model of ABI genetic analyser used: "310" or "377"

For example, the file "Tf27_2.310" refers to the analysis of DNA from endophyte isolate Tf27, amplified by Primer Set II, and analysed on an ABI Prism 310 genetic analyser.

APPENDIX 4 CHI-SQUARE (χ^2) TESTS

Chi-square (χ^2) tests to examine for goodness-of-fit between observed and expected frequency data were used to test the hypotheses in Section 3.2.3.4.

A4.1 MENDEL'S FIRST LAW

To test Mendel's first law, the principle of segregation, the potential of the observed data to fit the expected Mendelian ratio of 1:1 was examined.

A4.1.1 Test One

Hypotheses:

H_o = Microsatellite alleles at the B10 locus segregate at a ratio of 1:1

H_a = Microsatellite alleles at the B10 locus do not segregate at a ratio of 1:1

Chi-square test:

B10 genotypes	Observed frequency (O)	Expected frequency (E)	$(O - E)^2/E$
170	16	21	1.190
176	26	21	1.190
Sum	42	42	$X^2 = 2.380$

Conclusion:

For $X^2 = 2.380$ at 1 degree of freedom (df), $(0.10 < P < 0.20)$, so H_o is accepted at the 5% significance level.

A4.1.2 Test Two

Hypotheses:

H_o = Microsatellite alleles at the B11 locus segregate at a ratio of 1:1

H_a = Microsatellite alleles at the B11 locus do not segregate at a ratio of 1:1

Chi-square test:

B11 genotypes	Observed frequency (O)	Expected frequency (E)	$(O - E)^2/E$
147	26	21	1.190
164	16	21	1.190
Sum	42	42	$X^2 = 2.380$

Conclusion:

For $X^2 = 2.380$ at 1 degree of freedom (df), $(0.10 < P < 0.20)$, so H_o is accepted at the 5% significance level.

A4.2 MENDEL'S SECOND LAW

To test Mendel's second law, the principle of independent assortment, the potential of the observed data to fit the expected Mendelian ratio of 1:1:1:1 for haploid organisms was examined.

Hypotheses:

H_o = Microsatellite alleles at B10 and B11 loci assort independently of one another

H_a = Microsatellite alleles at B10 and B11 loci do not assort independently of one another

Chi-square test:

Genotypes (B10/B11)	Observed frequency (O)	Expected frequency (E)	$(O - E)^2/E$
170/147	7	10.5	1.167
170/164	9	10.5	0.214
176/147	19	10.5	6.881
176/164	7	10.5	1.167
Sum	42	42	$X^2 = 9.429$

Conclusion:

For $X^2 = 9.429$ at 1 degree of freedom (df), $(0.01 < P < 0.001)$, so H_o is rejected at the 5% significance level.

APPENDIX 5 GENBANK ACCESSION NUMBERS

The GenBank accession numbers for all sequences used for phylogenetic analyses in this thesis (Sections 4.2.5, 4.2.7.2, and 4.2.7.3) are listed below.

Epichloë species	Isolate	Gene	GenBank accession
E. amarillans	E52	*tub2*	L06958
E. amarillans	E57	*tub2*	L06959
E. baconii	E248	*tub2*	L06961
E. baconii	E1031	*tub2*	L78270
E. brachyelytri	E1040	*tub2*	L78271
E. bromicola	E501	*tub2*	L78289
E. bromicola	E502	*tub2*	L78290
E. bromicola	E798	*tub2*	AF062430
E. elymi	E56	*tub2*	L06962
E. elymi	E184	*tub2*	L78273
E. festucae	E32	*tub2*	L06957
E. festucae	E189	*tub2*	L06955
E. glyceriae	E277	*tub2*	L78275
E. glyceriae	E2772	*tub2*	L78276
E. sylvatica	E354	*tub2*	L78278
E. sylvatica	E503	*tub2*	L78291
E. typhina	E8	*tub2*	X52616
E. typhina	E348	*tub2*	L78277
E. typhina	E425	*tub2*	L78280
E. typhina	E430	*tub2*	L78284
E. typhina	E469	*tub2*	L78287
E. typhina	E505	*tub2*	L78292
E. typhina	E1021	*tub2*	AF062429
E. typhina	E2466	*tub2*	L78274

APPENDIX 5 ***continued***

Epichloë species	Isolate	Gene	GenBank accession
N. coenophialum	e19	*tub2 (tub2-2)*	L06951
N. coenophialum	e19	*tub2 (tub2-3)*	L06964
N. coenophialum	e19	*tub2 (tub2-4)*	X56847
N. lolii	Lp5	*tub2*	sequence identical to E32 and E189 (Schardl et al., 1994)
N. uncinatum	e166	*tub2 (tub2-3)*	sequence identical to e19 *tub2-3* (Tsai et al., 1994)
FaTG-2	e41	*tub2 (tub2-2)*	L06952
FaTG-2	e41	*tub2 (tub2-4)*	L06963
FaTG-3	Tf18	*tub2 (tub2-4)*	sequence identical to e41 *tub2-4* (Tsai et al., 1994)
FaTG-3	Tf18	*tub2 (tub2-5)*	L020308
E. amarillans	E114	rDNA-ITS	L07129
E. baconii	E248	rDNA-ITS	L07138
E. brachyelytri	E1045	rDNA-ITS	L78296
E. bromicola	E502	rDNA-ITS	L78295
E. elymi	E56	rDNA-ITS	L07131
E. festucae	E28	rDNA-ITS	L07139
E. glyceriae	E277	rDNA-ITS	L78302
E. sylvatica	E503	rDNA-ITS	L78304
E. typhina	E8	rDNA-ITS	L07132
E. typhina	E430	rDNA-ITS	L78298
E. typhina	E2461	rDNA-ITS	L20306
N. coenophialum	e19	rDNA-ITS	U68115
N. lolii	Lp5	rDNA-ITS	L07130
N. uncinatum	e166	rDNA-ITS	L07135
FaTG-2	e41	rDNA-ITS	L07140

APPENDIX 6 DNA SEQUENCE ALIGNMENTS

The DNA sequence alignment files, used for phylogenetic analysis with PAUP* software (Sections 4.2.5, 4.2.7.2, and 4.2.7.3), are supplied on the CD-ROM accompanying this thesis. "ITS Lolium align" and "tub2 Lolium align" are alignments of the annual ryegrass endophytes with representative Epichloë endophyte rDNA-ITS and *tub2* gene sequences respectively. Similarly, "ITS Hordeum align" and "tub2 Hordeum align" show the gene sequences from the *Hordeum* isolates, HaB and Hd1, aligned with the same representative Epichloe isolates.

APPENDIX 7 PUBLICATION

The following publication was based on work from this thesis:

Moon, C. D., Tapper, B. A., and Scott, B. (1999). Identification of Epichloë endophytes in planta by a microsatellite-based PCR fingerprinting assay with automated analysis. *Applied and Environmental Microbiology* **65**(3)**:** 1268-1279.

REFERENCES

REFERENCES

Ainsworth, G. C., Sparrow, F. K., and Sussman, A. S. (1973). "The Fungi - An Advanced Treatise." first ed., IVA Academic Press, New York.

Akkaya, M. S., Bhagwat, A. A., and Cregan, P. B. (1992). Length polymorphisms of simple sequence repeat DNA in soybean. *Genetics* **132:** 1131-1139.

Arachevaleta, M., Bacon, C. W., Hoveland, C. S., and Radcliffe, D. E. (1989). Effect of the tall fescue endophyte on plant response to environmental stress. *Agronomy Journal* **81:** 83-90.

Bacon, C. W., Lyons, P. C., Porter, J. K., and Robbins, J. D. (1986). Ergot toxicity from endophyte-infected grasses: A review. *Agronomy Journal* **78:** 106-116.

Bacon, C. W., Porter, J. K., Robbins, J. D., and Luttrell, E. S. (1977). *Epichloë typhina* from toxic tall fescue grasses. *Applied and Environmental Microbiology* **34**(5)**:** 576-581.

Baleiras Couto, M. M., Eijsma, B., Hofstra, H., Huis in't Veld, J. H. J., and Van Der Vossen, J. M. B. M. (1996). Evaluation of molecular typing techniques to assign genetic diversity among *Saccharomyces cerevisiae* strains. *Applied and Environmental Microbiology* **62**(1)**:** 41-46.

Ball, O. J.-P., Prestidge, R. A., and Sprosen, J. M. (1995). Interrelationships between *Acremonium lolii*, peramine, and lolitrem B in perennial ryegrass. *Applied and Environmental Microbiology* **61**(4)**:** 1527-1533.

Bart-Delabesse, E., Humbert, H.-F., Delabesse, É., and Bretagne, S. (1998). Microsatellite markers for typing *Aspergillus fumigatus* isolates. *Journal of Clinical Microbiology* **36**(9)**:** 2413-2418.

Bennett, S. J. (1997). A phenetic analysis and lateral key of the genus *Lolium* (Gramineae). *Genetic Resources and Crop Evolution* **44:** 63-72.

Boehringer Mannheim. (1995). "The DIG system user's guide for filter hybridisation.". Biochemica (R. van Miltenburg, B. Rüger, S. Grünewald-Janho, M. Leons, and C. Schröder, Eds.), Mannheim.

Bolton, E. T., and McCarthy, B. J. (1962). A general method for the isolation of RNA complementary to DNA. *Proceedings of the National Academy of Science of the United States of America* **48:** 1390.

Borrill, M. (1976). Temperate grasses. 1st ed. *In* "Evolution of Crop Plants" (N. W. Simmonds, Ed.), pp. 137-142. Longman Scientific & Technical, Edinburgh.

Broun, P., and Tanksley, S. D. (1996). Characterization and genetic mapping of simple repeat sequences in the tomato genome. *Molecular and General Genetics* **250:** 39-49.

Brownstein, M. J., Carpten, J. D., and Smith, J. R. (1996). Modulation of non-templated nucleotide addition by Taq DNA polymerase: Primer modifications that facilitate genotyping. *BioTechniques* **20:** 1004-1010.

Bruns, T. D., White, T. J., and Taylor, J. W. (1991). Fungal Molecular Systematics. *Annual Review of Ecology and Systematics* **22:** 525-564.

Buchanan, F. C., Adams, L. J., Littlejohn, R. P., Maddox, J. F., and Crawford, A. M. (1994). Determination of evolutionary relationships among sheep breeds using microsatellites. *Genomics* **22:** 397-403.

Bullock, W. O., Fernandez, J. M., and Short, J. M. (1987). XL1-Blue: a high efficiency plasmid transforming *rec*A *Escherichia coli* strain with beta-galactosidase selection. *Biotechniques* **5:** 376-378.

Bultman, T. L., White, J. F., Jr., Bowdish, T. I., Welch, A. M., and Johnston, J. (1995). Mutualistic transfer of *Epichloë* spermatia by *Phorbia* flies. *Mycologia* **87**(2)**:** 182-189.

Bush, L. P., Cornelius, P. L., Buckner, R. C., Varney, D. R., Chapman, R. A., Burrus II, P. B., Kennedy, C. W., Jones, T. A., and Saunders, M. J. (1982). Association of N-acetyl loline and N-formyl loline with *Epichloë typhina* in tall fescue. *Crop Science* **22:** 941-942.

Byrd, A. D., Schardl, C. L., Songlin, P. J., Mogen, K. L., and Siegel, M. R. (1990). The ß-tubulin gene of *Epichloë typhina* from perennial ryegrass (*Lolium perenne*). *Current Genetics* **18:** 347-354.

Caetano-Anollés, G., Bassam, B. J., and Gresshoff, P. M. (1991). DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. *Biotechnology* **9:** 553-557.

Carter, J. D., Ball, O. J.-P., Gwinn, K. D., and Fribourg, H. A. (1997). Immunological detection of the *Neotyphodium*-like endophyte of annual ryegrass. *In* "*Neotyphodium*/Grass Interactions" (C. W. Bacon, and N. S. Hill, Eds.), pp. 247-249. Plenum Press, New York.

Catalán, P., Kellogg, E. A., and Olmstead, R. G. (1997). Phylogeny of Poaceae subfamily Pooideae based on chloroplast *ndh*F gene sequences. *Molecular Phylogenetics and Evolution* **8**(2)**:** 150-166.

Charlesworth, B., Sniegowski, P., and Stephan, W. (1994). The evolutionary dynamics of repetitive DNA in eukaryotes. *Nature* **371:** 215-220.

Charmet, G., Ravel, C., and Balfourier, F. (1997). Phylogenetic analysis in the *Festuca-Lolium* complex using molecular markers and ITS rDNA. *Theoretical and Applied Genetics* **94:** 1038-1046.

Charmet, G., and Balfourier, F. (1994). Isozyme variation and species relationships in the genus *Lolium* L. (ryegrasses, Graminaceae). *Theoretical and Applied Genetics* **87:** 641-649.

Christensen, M. J., Ball, O. J.-P., Bennett, R. J., and Schardl, C. L. (1997). Fungal and host genotype effects on compatibility and vascular colonisation by *Epichloë festucae*. *Mycological Research* **101**(4)**:** 493-501.

Christensen, M. J., Leuchtmann, A., Rowan, D. D., and Tapper, B. A. (1993). Taxonomy of *Acremonium* endophytes of tall fescue (*Festuca arundinacea*), meadow fescue (*F. pratensis*) and perennial rye-grass (*Lolium perenne*). *Mycological Research* **97**(9)**:** 1083-1092.

Christensen, M. J., and Latch, G. C. M. (1991). Variation among isolates of *Acremonium* endophytes (*A. coenophialum* and possibly *A. typhinum*) from tall fescue (*Festuca arundinacea*). *Mycological Research* **95**(9)**:** 1123-1126.

Christensen, M. J., Latch, G. C. M., and Tapper, B. A. (1991). Variation within isolates of *Acremonium* endophytes from perennial rye-grasses. *Mycological Research* **95**(8)**:** 918-923.

Chung, K.-R., and Schardl, C. L. (1997a). Sexual cycle and horizontal transmission of the grass symbiont, *Epichloë typhina*. *Mycological Research* **101**(3)**:** 295-301.

Chung, K.-R., and Schardl, C. L. (1997b). Vegetative compatibility between and within *Epichloë* species. *Mycologia* **89**(4)**:** 558-565.

Chung, K.-R., Leuchtmann, A., and Schardl, C. L. (1996). Inheritance of mitochondrial DNA and plasmids in the Ascomycetous fungus, *Epichloë typhina*. *Genetics* **142:** 259-265.

Collett, M. A., Bradshaw, R. E., and Scott, D. B. (1995). A mutualistic fungal symbiont of perennial ryegrass contains two different *pyr4* genes, both expressing orotidine-5'-monophosphate decarboxylase. *Gene* **158:** 31-39.

Crowhurst, R. N., Hawthorne, B. T., Rikkerink, E. H. A., and Templeton, M. D. (1991). Differentiation of *Fusarium solani* f. sp. *curcurbitae* races 1 and 2 by random amplification of polymorphic DNA. *Current Genetics* **20:** 391-396.

Dahlman, D. L., Eichenseer, H., and Siegel, M. R. (1991). Chemical perspectives on endophyte-grass interactions and their implications to insect herbivory. *In* "Microbial Mediation of Plant-Herbivore Interactions" (P. Barbosa, V. A. Krischik, and C. G. Jones, Eds.), pp. 227-252. John Wiley & Sons, Inc, New York.

Davis, J. I., and Soreng, R. J. (1993). Phylogenetic structure in the grass family (Poaceae) as inferred from chloroplast DNA restriction site variation. *American Journal of Botany* **80**(12)**:** 1444-1454.

De Bustos, A., Soler, C., and Jouve, N. (1999). Analysis by PCR-based markers using designed primers to study relationships between species of *Hordeum* (Poaceae). *Genome* **42:** 129-138.

di Menna, M. E., and Waller, J. E. (1986). Visual assessment of seasonal changes in amount of mycelium of *Acremonium loliae* in leaf sheaths of perennial ryegrass. *New Zealand Journal of Agricultural Research* **29:** 111-116.

Dobson, J. M. (1997). Masters thesis. Massey University, Palmerston North.

Doss, R. P., and Welty, R. E. (1995). A polymerase chain reaction-based procedure for detection of *Acremonium coenophialum* in tall fescue. *Phytopathology* **85**(8)**:** 913-917.

Dower, W. J., Miller, J. F., and Ragsdale, C. W. (1988). High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Research* **16:** 6127-6145.

Doyle, J. J., and Doyle, J. L. (1990). Isolation of plant DNA from fresh tissue. *Focus* **12:** 13-15.

Edwards, K. J., Barker, J. H. A., Daly, A., Jones, C., and Karp, A. (1996). Microsatellite libraries enriched for several microsatellite sequences in plants. *Biotechniques* **20:** 758-760.

Ellegren, H., Primmer, C. R., and Sheldon, B. C. (1995). Microsatellite 'evolution': directionality or bias? *Nature Genetics* **11:** 360-362.

Ender, A., Schwenk, K., Städler, T., Streit, B., and Schierwater, B. (1996). RAPD identification of microsatellites in *Daphnia. Molecular Ecology* **5:** 437-441.

Field, D., and Wills, C. (1998). Abundant microsatellite polymorphism in *Saccharomyces cerevisiae*, and the different distributions of microsatellites in eight prokaryotes and *S. cerevisiae*, result from strong mutation pressures and a variety of selective forces. *Proceedings of the National Academy of Sciences (USA)* **95**(4)**:** 1647-1652.

Fisher, P. J., Gardner, R. C., and Richardson, T. E. (1996). Single locus microsatellites isolated using 5' anchored PCR. *Nucleic Acids Research* **24**(21)**:** 4369-4371.

FitzSimmons, N. N., Moritz, C., and Moore, S. S. (1995). Conservation and dynamics of microsatellite loci over 300 million years of marine turtle evolution. *Molecular Biology and Evolution* **12**(3)**:** 432-440.

Fletcher, L. R., and Harvey, I. C. (1981). An association of a *Lolium* endophyte with ryegrass staggers. *New Zealand Veterinary Journal* **29:** 185-186.

Gallagher, R. T., Hawkes, A. D., Steyn, P. S., and Vleggaar, R. (1984). Tremorgenic neurotoxins from perennial ryegrass causing ryegrass staggers disorder of livestock: Structure elucidation of lolitrem B. *J. Chem. Soc., Chem. Commun.***:** 614-616.

Gallagher, R. T., White, E. P., and Mortimer, P. H. (1981). Ryegrass staggers: Isolation of potent neurotoxins lolitem A and lolitrem B from staggers-producing pastures. *New Zealand Veterinary Journal* **29:** 189-190.

Gams, W., Petrini, O., and Schmidt, D. (1990). *Acremonium uncinatum*, a new endophyte in *Festuca pratensis. Mycotaxon* **37:** 67-71.

Ganley, A. R. D., and Scott, B. (1998). Extraordinary ribosomal spacer length heterogeneity in a Neotyphodium endophyte hybrid: Implications for concerted evolution. *Genetics* **150:** 1625-1637.

Geistlinger, J., Maqbool, S., Kaiser, W. J., and Kahl, G. (1997). Detection of microsatellite fingerprint markers and their Mendelian inheritance in *Ascochyta rabiei*. *Mycological Research* **101**(9): 1113-1121.

Gibson, J. R., Slater, E., Xerry, J., Tompkins, D. S., and Owen, R. J. (1998). Use of an amplified-fragment length polymorphism technique to fingerprint and differentiate isolates of *Helicobacter pylori*. *Journal of Clinical Microbiology* **36**(9): 2580-2585.

Ginot, F., Bordelais, I., Nguyen, S., and Gyapay, G. (1996). Correction of some genotyping errors in automated fluorescent microsatellite analysis by enzymatic removal of one base overhangs. *Nucleic Acids Research* **24**(3): 540-541.

Glenn, A. E., Bacon, C. W., Price, R., and Hanlin, R. (1996). Molecular phylogeny of *Acremonium* and its taxonomic implications. *Mycologia* **88**(3): 369-383.

Goldstein, D. B., and Pollock, D. D. (1997). Launching microsatellites: A review of mutation processes and methods of phylogenetic inference. *Journal of Heredity* **88:** 335-342.

Goldstein, D. B., Linares, A. R., Cavalli-Sforza, L. L., and Feldman, M. W. (1995). An evaluation of genetic distances for use with microsatellite loci. *Genetics* **139:** 463-471.

Goodwin, D. C., and Lee, S. B. (1993). Microwave miniprep of total genomic DNA from fungi, plants, protists and animals for PCR. *Biotechniques* **15**(3): 438-444.

Groppe, K., and Boller, T. (1997). PCR assay based on a microsatellite-containing locus for detection and quantification of *Epichloë* endophytes in grass tissue. *Applied and Environmental Microbiology* **63**(4): 1543-1550.

Groppe, K., Sanders, I., Weimken, A., and Boller, T. (1995). A microsatellite marker for studying the ecology and diversity of fungal endophytes (*Epichloë* spp.) in grasses. *Applied and Environmental Microbiology* **61**(11): 3943-3949.

Gwinn, K. D., Collins-Shepard, M. H., and Reddick, B. B. (1991). Tissue print-immunoblot, an accurate method for the detection of *Acremonium coenophialum* in tall fescue. *Phytopathology* **81**(7): 747-748.

Hearne, C. M., Ghosh, S., and Todd, J. A. (1992). Microsatellites for linkage analysis of genetic traits. *Trends In Genetics* **8**(8): 288-294.

Hsaio, C., Chatterton, N. J., Asay, K. H., and Jensen, K. B. (1995). Molecualr phylogeny of the Pooideae (Poaceae) based on nuclear rDNA (ITS) sequences. *Theoretical and Applied Genetics* **90:** 389-398.

Holmes, D. S., and Quigley, M. (1981). A rapid boiling method for the preparation of bacterial plasmids. *Analytical Biochemistry* **114:** 193-197.

Jeffreys, A. J., Wilson, V., and Thein, S. L. (1985). Individual-specific 'fingerprints' of human DNA. *Nature* **316:** 76-79.

Jungehülsing, U., and Tudzynski, P. (1997). Analysis of genetic diversity in *Claviceps purpurea* by RAPD markers. *Mycological Research* **101**(1)**:** 1-6.

Karagyozov, L., Kalcheva, I. D., and Chapman, V. M. (1993). Construction of random small-insert genomic libraries highly enriched for simple sequence repeats. *Nucleic Acids Research* **21**(16)**:** 3911-3912.

Kellogg, E. A. (1998). Relationships of cereal crops and other grasses. *Proceedings of the National Academy of Sciences (USA)* **95:** 2005-2010.

Kendrick, B. (1992). "The Fifth Kingdon." 2nd ed. Focus Information Group, Newburyport MA.

Kimmel, M., Chakraborty, R., Stivers, D. N., and Deka, R. (1996). Dynamics of repeat polymorphisms under a forward-backward mutation model: Within- and between-population variability at microsatellite loci. *Genetics* **143:** 549-555.

Kimmons, C. A., Gwinn, K. D., and Bernard, E. C. (1990). Nemotode reproduction on endophyte-infected and endophyte-free tall fescue. *Plant Disease* **74**(10)**:** 757-761.

King, P. V., and Blakesley, R. W. (1986). Optimizing DNA ligations for transformation. *Focus* **8**(1)**:** 1-3.

Koeleman, J. G. M., Stoof, J., Biesmans, D. J., Savelkoul, P. H. M., and Vandenbroucke-Grauls, C. M. J. E. (1998). Comparison of amplified ribosomal DNA restriction analysis, random amplified polymorphic DNA analysis, and amplified fragment length polymorphism fingerprinting for identification of *Acinetobacter* genomic species and typing of *Acinetobacter baumannii. Journal of Clinical Microbiology* **36**(9)**:** 2522-2529.

Kohlmeyer, J., and Kohlmeyer, E. (1974). Distribution of *Epichloë typhina* (Ascomycetes) and its parasitic fly. *Mycologia* **66:** 77-86.

Kostia, S., Varvio, S.-L., Vakkari, P., and Pulkkinen, P. (1995). Microsatellite sequences in a conifer, *Pinus sylvestris*. *Genome* **38:** 1244-1248.

Kuldau, G. A., Liu, J.-S., White, J. F., Jr., Siegel, M. R., and Schardl, C. L. (1997). Molecular systematics of Clavicipitaceae supporting monophyly of genus *Epichloë* and form genus *Ephelis*. *Mycologia* **89**(3)**:** 431-441.

Lagercrantz, U., Ellegren, H., and Andersson, L. (1993). The abundance of various polymorphic microsatellite motifs differs between plants and vertebrates. *Nucleic Acids Research* **21**(5)**:** 1111-1115.

Latch, G. C. M., Christensen, M. J., and Hickson, R. E. (1988). Endophytes of annual and hybrid ryegrasses. *New Zealand Journal of Agricultural Research* **31:** 57-63.

Latch, G. C. M., Hunt, W. F., and Musgrave, D. R. (1985). Endophytic fungi affect growth of perennial ryegrass. *New Zealand Journal of Agricultural Research* **28:** 165-168.

Latch, G. C. M., Christensen, M. J., and Samuels, G. J. (1984). Five endophytes of *Lolium* and *Festuca* in New Zealand. *Mycotaxon* **20**(2)**:** 535-550.

Leuchtmann, A., and Schardl, C. L. (1998). Mating compatibility and phylogenetic relationships among two new species of *Epichloë* and other congeneric European species. *Mycological Research* **102**(10)**:** 1169-1182.

Leuchtmann, A. (1994). Isozyme relationships of *Acremonium* endophytes from twelve *Festuca* species. *Mycological Research* **98**(1)**:** 25-33.

Leuchtmann, A., Schardl, C. L., and Siegel, M. R. (1994). Sexual compatibility and taxonomy of a new species of *Epichloë* symbiotic with fine fescue grasses. *Mycologia* **86**(6)**:** 802-812.

Leuchtmann, A., and Clay, K. (1990). Isozyme variation in the *Acremonium/Epichloë* fungal endophyte complex. *Phytopathology* **80**(10)**:** 1133-1139.

Levinson, G., and Gutman, G. A. (1987). Slipped-strand mispairing: A major mechanism for DNA sequence evolution. *Molecular Biology and Evolution* **4**(3)**:** 203-221.

Lin, J.-J., and Kuo, J. (1995). AFLP: A novel PCR-based assay for plant and bacterial DNA fingerprinting. *Focus* **17**(2)**:** 66-70.

Lindstrom, J. T., and Belanger, F. C. (1994). Purification and characterization of an endophytic fungal proteinase that is abundantly expressed in the infected host grass. *Plant Physiology* **106:** 7-16.

Liu, D., van Heeswijck, R., Latch, G., Leonforte, T., Panaccio, M., Langford, C., Cunningham, P., and Reed, K. (1995). Rapid identification of *Acremonium lolii* and *Acremonium coenophialum* endophyte through arbitrarily primed PCR. *FEMS Microbiology Letters* **133:** 95-98.

Lockhart, P. J., Larkum, A. W. D., Steel, M. A., Waddell, P. J., and Penny, D. (1996). Evolution of chlorophyll and bacteriochlorophyll: The problem of invariant sites in sequence analysis. *Proceedings of the National Academy of Sciences (USA)* **93:** 1930-1934.

Longato, S., and Bonfante, P. (1997). Molecular identification of mycorrhizal fungi by direct amplification of microsatellite regions. *Mycological Research* **101**(4)**:** 425-432.

McKay, G. J., Egan, D., Morris, E., Scott, C., and Brown, A. (1999). Genetic and morpholgical characterization of *Cladobotryum* species causing cobweb disease of mushrooms. *Applied and Environmental Microbiology* **65**(2)**:** 606-610.

Meijer, G., and Leuchtmann, A. (1999). Multistrain infections of the grass *Brachypodium sylvaticum* by its fungal endophyte *Epichloë sylvatica. New Phytologist* **141:** 355-368.

Menotti-Raymond, M. A., and O'Brien, S. J. (1995). Evolutionary conservation of ten microsatellite loci in four species of Felidae. *Journal of Heredity* **86**(4)**:** 319-322.

Meyer, W., Mitchell, T. G., Freedman, E. Z., and Vilgalys, R. (1993). Hybridisation probes for conventional DNA fingerprinting used as single primers in the polymerase chain reaction to distinguish strains of *Cryptococcus neoformans. Journal of Clinical Microbiology* **31**(9)**:** 2274-2280.

Miles, C. O., Di Menna, M. E., Jacobs, S. W. L., Garthwaite, I., Lane, G. A., Prestidge, R. A., Marshall, S. L., Wilkinson, H. H., Schardl, C. L., Ball, O. J.-P., and Latch, G. C. M. (1998). Endophytic fungi in indigenous Australasian grasses associated with toxicity to livestock. *Applied and Environmental Microbiology* **64**(2)**:** 601-606.

Moon, C. D., Tapper, B. A., and Scott, B. (1999). Identification of Epichloë endophytes in planta by a microsatellite-based PCR fingerprinting assay with automated analysis. *Applied and Environmental Microbiology* **65**(3)**:** 1268-1279.

Morgan-Jones, G., White, J. F., Jr., and Piontelli, E. L. (1990). Endophyte-Host Associations in Forage Grasses. XIII. *Acremonium chilense*, an undescribed endophyte occurring in *Dactylis glomerata* in Chile. *Mycotaxon* **39:** 441-454.

Morgan-Jones, G., and Gams, W. (1982). Notes on Hyphomycetes. XLI. An endophyte of *Festuca Arundinacea* and the anamorph of *Epichloë typhina*, new taxa in one of two new sections of *Acremonium*. *Mycotaxon* **15:** 311-318.

Morgante, M., and Olivieri, A. M. (1993). PCR-amplified microsatellites as markers in plant genetics. *Plant Journal* **3**(1)**:** 175-182.

Muller, H. J. (1964). The relation of recombination to mutational advance. *Mutation Research* **1:** 2-9.

Murray, F. R., Latch, G. C. M., and Scott, D. B. (1992). Surrogate transformation of perennial ryegrass, *Lolium perenne*, using genetically modified *Acremonium* endophyte. *Molecular and General Genetics* **233:** 1-9.

Naffaa, W., Ravel, C., and Guillaumin, J. J. (1998). A new group of endophytes in European grasses. *Annals of Applied Biology* **132:** 211-226.

Nauta, M. J., and Weissing, F. J. (1996). Constraints on allele size at microsatellite loci: implications for genetic differentiation. *Genetics* **143**(1021-1032).

Neumann, K., and Wetton, J. H. (1996). Highly polymorphic microsatellites in the house sparrow *Passer domesticus*. *Molecular Ecology* **5**(2)**:** 307-309.

Norrander, J., Kempe, T., and Messing, J. (1983). Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. *Gene* **26:** 101-106.

Ostrander, E. A., Jong, P. M., Rine, J., and Duyk, G. (1992). Construction of small-insert genomic DNA libraries highly enriched for microsatellite repeat sequences. *Proceedings of the National Academy of Science of the United States of America* **89:** 3419-3423.

Paetkau, D., Waits, L. P., Clarkson, P. L., Craighead, L., and Strobeck, C. (1997). An Empirical Evaluation of Genetic Distance Statistics Using Microsatellite Data From Bear (Ursidae) Populations. *Genetics* **147:** 1943-1957.

Philipson, M. N., and Christey, M. C. (1986). The relationship of host and endophyte during flowering, seed formation, and germination of *Lolium perenne*. *New Zealand Journal of Botany* **24:** 125-134.

Prestidge, R. A., Lauren, D. R., Van Der Zijpp, S. G., and di Menna, M. E. (1985). Isolation of feeding deterrents to Argentine stem weevil in cultures of endophytes of perennial ryegrass and tall fescue. *New Zealand Journal of Agricultural Research* **28:** 87-92.

Raisbeck, M. F., Rottinghaus, G. E., and Kendall, J. D. (1991). Effects of naturally occurring mycotoxins on ruminants. *In* "Mycotoxins and Animal Foods" (J. E. Smith, and R. S. Henderson, Eds.), pp. 647-677. CRC Press, Boca Raton.

Reddick, B. B., and Collins, M. H. (1988). An improved method for detection of *Acremonium coenophialum* in tall fescue plants. *Phytopathology* **78**(4)**:** 418-420.

Reddy, P. V., Lam, C. K., and Belanger, F. C. (1996). Mutualistic fungal endophytes express a proteinase that is homologous to proteases suspected to be important in fungal pathogenicity. *Plant Physiology* **111:** 1209-1218.

Richard, G. F., and Dujon, B. (1996). Distribution and variability of trinucleotide repeats in the genome of the yeast Saccharomyces cerevisiae. *Gene* **174**(1)**:** 165-174.

Richard, K. R., Whitehead, H., and Wright, J. M. (1996). Polymorphic microsatellites from sperm whales and their use in the genetic identification of individuals from naturally sloughed pieces of skin. *Molecular Ecology* **5**(2)**:** 313-315.

Röder, M. S., Plaschke, J., König, S. U., Börner, A., Sorrells, M. E., Tanksley, S. D., and Ganal, M. W. (1995). Abundance, variability and chromosomal location of microsatellites in wheat. *Molecular and General Genetics* **246:** 327-333.

Rosenblum, B. B., Oaks, F., Menchen, S., and Johnson, B. (1997). Improved single-strand DNA sizing accuracy in capillary electrophoresis. *Nucleic Acids Research* **25**(19)**:** 3925-3929.

Rowan, D. D., and Shaw, G. J. (1987). Detection of ergopeptine alkaloids in endophyte-infected perennial ryegrass by tandem mass spectrometry. *New Zealand Veterinary Journal* **35:** 197-198.

Rowan, D. D., and Gaynor, D. L. (1986). Isolation of feeding deterrents against argentine stem weevil from ryegrass infected with the endophyte *Acremonium loliae. Journal of Chemical Ecology* **12**(3)**:** 647-658.

Rubinsztein, D. C., Amos, W., Leggo, J., Goodburn, S., and Jain, S. (1995). Microsatellite evolution - evidence for directionality and variation in rate between species. *Nature Genetics* **10:** 337-343.

Russell, P. J. (1990). "Genetics." Scott, Foresman and Company, Glenview, Illinois.

Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A., and Arnheim, N. (1985). Enzymatic amplification of ß-globin genomic sequences and restriction site analysis for diagnosis of Sickle Cell Anemia. *Science* **230:** 1350-1354.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular cloning: a laboratory manual." 2 ed. Cold Spring Harbor Laboratory Press, New York.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* **74**(12)**:** 5463-5467.

Schardl, C. L., and Leuchtmann, A. (1999). Three new species of *Epichloë* symbiotic with North American grasses. *Mycologia* **91:** 95-107.

Schardl, C. L., Leuchtmann, A., Chung, K.-R., Penny, D., and Siegel, M. R. (1997). Coevolution by common descent of fungal symbionts (*Epichloë* spp.) and grass hosts. *Molecular Biology and Evolution* **14**(2)**:** 133-143.

Schardl, C. L. (1996a). *Epichloë* species: Fungal symbionts of grasses. *Annual Review of Phytopathology* **34:** 109-130.

Schardl, C. L. (1996b). Interactions of grasses with endophytic *Epichloë* species and hybrids. *In* "Plant-Microbe Interactions" (G. Stacey, and N. T. Keen, Eds.), Vol. One, pp. 105-140. Chapman and Hall, New York.

Schardl, C. L., Leuchtmann, A., Tsai, H.-F., Collett, M. A., Watt, D. M., and Scott, D. B. (1994). Origin of a fungal symbiont of perennial ryegrass by interspecific hybridization of a mutualist with the ryegrass choke pathogen, *Epichloë typhina*. *Genetics* **136:** 1307-1317.

Schardl, C. L., and Tsai, H.-F. (1992). Molecular biology and evolution of the grass endophytes. *Natural Toxins* **1:** 171-184.

Schardl, C. L., Liu, J.-S., White, J. F., Jr., Finkel, R. A., An, Z., and Siegel, M. R. (1991). Molecular phylogenetic relationships of nonpathogenic grass mycosymbionts and clavicipitaceous plant pathogens. *Plant Systematics and Evolution* **178:** 27-41.

Schlötterer, C., and Tautz, D. (1992). Slippage synthesis of simple sequence DNA. *Nucleic Acids Research* **20**(2)**:** 211-215.

Scott, B., and Schardl, C. (1993). Fungal symbionts of grasses: evolutionary insights and agricultural potential. *Trends in Microbiology* **1**(5)**:** 196-200.

Sia, E. A., Jinks-Robertson, S., and Petes, T. D. (1997). Genetic control of microsatellite stability. *Mutation Research* **383:** 61-70.

Siegel, M. R., Latch, G. C. M., Bush, L. P., Fannin, F. F., Rowan, D. D., Tapper, B. A., Bacon, C. W., and Johnson, M. C. (1990). Fungal endophyte-infected grasses: Alkaloid accumulation and aphid response. *Journal of Chemical Ecology* **16**(12)**:** 3301-3315.

Siegel, M. R., Latch, G. C. M., and Johnson, M. C. (1985). *Acremonium* fungal endophytes of tall fescue and perennial ryegrass: Significance and control. *Plant Disease* **69**(2)**:** 179-183.

Siegel, M. R., Johnson, M. C., Varney, D. R., Nesmith, W. C., Buckner, R. C., Bush, L. P., Burrus II, P. B., Jones, T. A., and Boling, J. A. (1984). A fungal endophyte in tall fescue: Incidence and dissemination. *Phytopathology* **74**(8)**:** 932-937.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98:** 503-517.

Stammers, M., Harris, J., Evans, G. M., Hayward, M. D., and Forster, J. W. (1995). Use of random PCR (RAPD) technology to analyse phylogenetic relationships in the *Lolium/Festuca* complex. *Heredity* **74**(19-27).

Strand, M., Prolla, T. A., Liskay, R. M., and Petes, T. D. (1993). Destabilization of tracts of simple repetitve DNA in yeast by mutations affecting DNA mismatch repair. *Nature* **365:** 274-276.

Taga, M., and Murata, M. (1994). Visualization of mitotic chromosomes in filamentous fungi by fluorescence staining and fluorescence in situ hybridisation. *Chromosoma (Berlin)* **103:** 408-413.

Tautz, D. (1989). Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acids Research* **17**(16)**:** 6463-6471.

Tautz, D., and Renz, M. (1984). Simple sequences are ubiquitous repetitive components of eukaryotic genomes. *Nucleic Acids Research* **12:** 4127-4138.

TePaske, M. R., Powell, R. G., and Clement, S. L. (1993). Analyses of selected endophyte-infected grasses for the presence of loline-type and ergot-type alkaloids. *Journal of Agricultural and Food Chemistry* **41:** 2299-2303.

Thuring, R. W. J., Sanders, J. P. M., and Borst, P. (1975). A freeze-squeeze method for recovering long DNA from agarose gels. *Analytical Biochemistry* **66:** 213-220.

Tsai, H.-F., Liu, J.-S., Staben, C., Christensen, M. J., Latch, G. C. M., Siegel, M. R., and Schardl, C. L. (1994). Evolutionary diversification of fungal endophytes of tall fescue grass by hybridization with *Epichloë* species. *Proceedings of the National Academy of Sciences (USA)* **91:** 2542-2546.

Tsai, H.-F., Siegel, M. R., and Schardl, C. L. (1992). Transformation of *Acremonium coenophialum*, a protective fungal symbiont of the grass *Festuca arundinacea*. *Current Genetics* **22:** 399-406.

Vieira, J., and Messing, J. (1987). Production of single-stranded plasmid DNA. *Methods in Enzymology* **153:** 3-11.

Wang, J., Machado, C., Panaccione, D., and Schardl, C. (1999). *Fungal Genetics Conference, Asilomar, CA.*

Weising, K., Atkinson, R. G., and Gardner, R. C. (1995a). Genomic fingerprinting by microsatellite-primed PCR: A critical evaluation. *PCR Methods and Applications* **4:** 249-255.

Weising, K., Nybom, H., Wolff, K., and Meyer, W. (1995b). "DNA Fingerprinting in Plants and Fungi." CRC Press, Boca Raton.

Welsh, J., and McClelland, M. (1990). Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research* **18**(24)**:** 7213-7218.

White, J. F., Jr., and Reddy, P. V. (1998). Examination of structure and molecular phylogenetic relationships of some graminicolous symbionts in genera *Epichloë* and *Parepichloë*. *Mycologia* **90**(2)**:** 226-234.

White, J. F., Jr. (1994). Endophyte-Host Associations in Grasses. XX. Structural and reproductive studies of *Epichloë amarillans sp. nov.* and comparisons to *E. typhina*. *Mycologia* **86**(4)**:** 571-580.

White, J. F., Jr. (1993). Endophyte-Host Associations in Grasses. XIX. A systematic study of some sympatric species of *Epichloë* in England. *Mycologia* **85**(3)**:** 444-455.

White, T. J., Bruns, T., Lee, S., and Taylor, J. (1990). Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. *In* "PCR Protocols - A Guide to Methods and Applications" (M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, Eds.). Academic Press, Inc., San Diego.

White, J. F., Jr., and Bultman, T. L. (1987). Endophyte Host Associations in Forage Grasses. VIII. Heterothallism in *Epichloë typhina. American Journal of Botany* **74**(11)**:** 1716-1721.

White, J. F., Jr., Cole, G. T., and Morgan-Jones, G. (1987). Endophyte-Host Associations In Forage Grasses. VI. A new species of *Acremonium* isolated from *Festuca Arizonica. Mycologia* **79**(1)**:** 148-152.

White, J. F., Jr., and Morgan-Jones, G. (1987a). Endophyte-Host Associations in Forage Grasses. VII. *Acremonium chisosum*, a new species isolated from *Stipa eminens* in Texas. *Mycotaxon* **28:** 179-189.

White, J. F., Jr., and Morgan-Jones, G. (1987b). Endophyte-Host Associations in Forage Grasses. X. Cultural studies on some species of *Acremonium* sect. *Albo-lanosa*, including a new species, *A. starrii. Mycotaxon* **30:** 87-95.

Wierdl, M., Greene, C. N., Datta, A., Jinks-Robertson, S., and Petes, T. D. (1996). Destabilization of simple repetitive DNA sequences by transcription in yeast. *Genetics* **143**(713-721).

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A., and Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* **18**(22)**:** 6531-6535.

Wu, K.-S., and Tanksley, S. D. (1993). Abundance, polymorphism and genetic mapping of microsatellites in rice. *Molecular and General Genetics* **241:** 225-235.

Xu, J.-R., Yan, K., Dickman, M. B., and Leslie, J. F. (1995). Electrophoretic karyotypes distinguish the biological species of *Gibberella fujikuroi* (*Fusarium* Section Liseola). *Molecular Plant - Microbe Interactions* **8**(1)**:** 74-84.

Young, C., McMillan, L., and Scott, B. (1999). *Fungal Genetics Conference, Asilomar, CA.*

Young, C., Itoh, Y., Johnson, R., Garthwaite, I., Miles, C. O., Munday-Finch, S. C., and Scott, B. (1998). Paxilline-negative mutants of *Penicillium paxilli* generated by heterologous and homologous plasmid integration. *Current Genetics* **33:** 368-377.

Zhu, J. H., Stephenson, P., Laurie, D. A., Li, W., Tang, D., and Gale, M. D. (1999). Towards rice genome scanning by map-based AFLP fingerprinting. *Molecular and General Genetics* **261:** 184-195.

In Erratum

CHAPTER ONE

pg 2	line 10	replace "apoplastic substituents" with "apoplastic constituents"
	line 24	replace "to the genus" with "to as the genus"
pg 4	line 24	replace ", to fat necrosis" with ", fat necrosis"
pg 5	line 10	replace "for pastoral agriculture." with "for pastoral agriculture, refer to Section 1.3."
pg 6	line 12	replace "have been cloned" with "has been cloned"
pg 11	line 13	replace "used by the Epichloë" with "used to describe the Epichloë"
pg 14	ins. line 7	"*Neotyphodium tembladerae* Cabral et al., 1999 *Endoconidium tembladerae* Rivas and Zanolli, 1909 rDNA and protease gene sequence"
pg 15	line 27	replace "and LpTG-2." with "and *Neotyphodium* sp. LpTG-2"
pg 16	line 8	replace "1987), and" with "1987), and *N. tembladerae* which infects *Festuca argentina*, *F. hieronymi*, *Poa huecu*, and an unidentified *Poa* sp. (Cabral et al., 1999), and"
pg 16	line 25	replace "by a interspecific" with "by an interspecific"
pg 21	line 24	replace "At1" with "*At1*"
	line 29	replace "At1" with "*At1*"
pg 24	line 5	replace "is required" to "are required"
	line 27	replace "Minisatellite DNA occur" with "Minisatellite DNA motifs occur"
pg 25	line 3	replace "tracts are thought" with "tracts is thought"
pg 28	line 7	replace "ability to distinguish" with "utility for distinguishing"

CHAPTER TWO

pg 33	Table 2.1	replace "EndoSafe" with "EndoSafe‡"
pg 35	* footnote	replace "AgResearch)." with "AgResearch, Palmerston North, New Zealand)."
	add footnote	"‡ EndoSafe refers to the endophyte isolate used to inoculate Grasslands Pacific EndoSafe, a commercially available low lolitrem B producing perennial ryegrass developed by Grasslands AgResearch."
pg 42	line 2	replace "Lysozyme was" with "Lysozyme (Boehringer Mannheim GmbH) was"
	line 5	replace "RNaseA was" with "RNaseA (Sigma Chemical Co.) was"
pg 44	line 8	replace "2 mg/ml proteinase K" with "2 mg/ml proteinase K (Boehringer Mannheim GmbH)"
pg 54	line 14	replace "terminal transferase" with "terminal transferase (Boehringer Mannheim GmbH)"

pg 64	line 10	replace "*Taq* polymerase," with "*Taq* polymerase (Boehringer Mannheim GmbH),"
pg 57	line 13	replace "SeaPlaque gels" with "SeaPlaque agarose gels"
pg 67	line 16	replace "was be used to" with "was used to"

CHAPTER THREE

pg 76	line 16	replace "in order to identify" with "for the identification of"
pg 78	line 15	replace "fingerprints generated using this method was good." with "fingerprints generated using this method, relative to RAPD-PCR, was generally good."
pg 94	line 20	replace "B11" with "B8"
pg 130	line 22	replace "latter" with "latter."
pg 142	Table 3.2	replace "unsure" with "unsure[b]"
	add footnote	"[b] extremely faint amplification product(s) detected on agarose gel"
pg 148	line 7	add "Allele sizes are listed in Table 3.3, but the subpeaks beneath the blue B4 allele are not shown. Refer to pages 174 and 175 for discussion."
pg 160	line 4	replace "genetic criteria (results not shown)." with "genetic criteria (Christensen et al., 1993; pers. comm. Mike Christensen, Grasslands AgResearch)."
pg 161	line 4	replace "55C" with "55°C"
	line 16	green "d" should be printed in red, and red "f" should be printed in green
pg 181	line 10	replace "this provide" with "this would provide"

CHAPTER FOUR

pg 209	line 22	replace "L. canariense" with "*L. canariense*"
pg 226	line 22	replace "first grass to be isolated from in this study." with "first grass from which this species was isolated."
pg 227	line 26	replace "This genetic data confirms" with "These genetic data confirm"
pg 232	line 30	replace "may have arose" with "may have arisen"
pg 233	line 26	replace "genotypes reveal" with "genotypes reveals"
pg 234	line 8	replace "(Fig. 12)" with "(Fig. 4.12)"

REFERENCES

pg 252	ins. line 30	"Cabral, D., Cafaro, M. J., Saidman, B., Lugo, M., Reddy, P. V., White, J. F., Jr. (1999). Evidence supporting the occurrence of a new species of endophyte in some South American grasses. *Mycologia* **91**(2):315-324"