Molecular and bioinformatic analysis of the *perA* locus in *Epichloë*

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Abstract

Fungal endophytes of the *Epichloë* genus form largely mutualistic symbioses with cool-season grasses, systemically colonising the intercellular spaces of the host in a strictly regulated fashion. The endophyte receives protection and sustenance from the host, and in return provides benefits such as increased growth, drought resistance and protection against herbivores. Protection against herbivory is mediated through the production of bio-protective fungal secondary metabolites (SM). Examples of these SMs include lolitrem B, the causative agent of 'ryegrass staggers' in stock, and the insect feeding deterrent peramine.

The genes responsible for the production of each of these SMs are usually found clustered together in the genome, and are often closely associated with a range of transposon relics. SM gene expression occurs only when the endophyte is growing *in planta*, indicating the presence of plant-fungal signalling. This study investigated the locus structure and organisation of the gene *perA* that encodes the non-ribosomal peptide synthetase PerA, which is both essential and sufficient for production of peramine. It was found that *perA* and its flanking intergenic sequences exhibit considerable transposon-mediated variability across *Epichloë*, and that this transposon activity is likely responsible for the taxonomically discontinuous production of peramine both within and across *Epichloë spp*.

The major facilitator superfamily transporter gene *EF102* is divergently transcribed from and co-regulated with *perA* (*EF103*). Transcriptome data were used to identify transcription start sites for both genes. Comparative analysis of the intergenic sequence separating *EF102/perA* from 10 *Epichloë* isolates covering six different species refined the *perA* translation start site, and identified conserved regions in the promoters of both genes proposed to be important for regulation. A motif search identified a conserved DNA motif present multiple times in the promoters of both genes.

Deletion analysis of *EF102* revealed the gene probably does not encode a peramine transporter, as was hypothesised; however the four independent Δ*EF102* mutants exhibited a reduction in peramine production relative to wild type, resulting in an alternative hypothesis that *EF102* encodes a transporter for a PerA substrate precursor molecule such as glutamate.

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Common Abbreviations

A1/2	NRPS adenylation domain from modules 1 or 2
Amp	Ampicillin
ASW	Argentine stem weevil
ATG	ATG translational start codon
bp	base pairs
C1	NRPS condensation domain from module 1
CDS	Coding sequence
d	Days
dATP	Deoxyadenine triphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra-acetic acid
EF	*Epichloë festucae*
g	Gravity
gDNA	Genomic DNA
h	Hours
HGT	Horizontal gene transfer
HPLC	High-performance liquid chromatography
Hyg	Hygromycin
Indel	Insertion or deletion
kb	Kilo base pairs
KO	Knock out
LB	Luria-Bertani medium
LB	Left border
LC	Liquid chromatography
M	Molar
M1	NRPS methylation domain from module 2
min	Minutes
MITE	Miniature inverted transposable element
mRNA	Messenger RNA
MFS	Major facilitator superfamily
MS	Mass spectrophotometer
MSA	Multiple sequence alignment
NCM	Nitrocellulose membrane
ND	Not detected
NRPS	Non-ribosomal peptide synthetase
NT	Not tested
P5C	1-pyrroline-5-carboxylate
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Potato dextrose medium
PKS	Polyketide synthase
R2/Rdom	NRPS reductase domain from module 2

RB	Right border
RCF	Relative centrifugal force
RE	Restriction enzyme
RG	Regeneration medium
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcription PCR
SDS	Sodium dodecyl sulfate
SSC	Saline sodium citrate
SM	Secondary metabolite
SNP	Single nucleotide polymorphism
T1/2	NRPS thiolation domain from module 1 or 2
TBE	Tris Borate EDTA buffer
UV	Ultra-violet
WT	Wild type

1. Introduction

1.1 Epichloë endophyte life cycle

Epichloë and *Neotyphodium spp*. are predominately mutualistic filamentous fungal endophytes of cool season grasses that systemically colonise the intercellular spaces of the aerial tissues of their host in a strictly regulated fashion (Scott, 2001). *Epichloë spp*. may transmit vertically (asexually) through colonisation of host seeds, as well as horizontally (sexually) through the formation of a fungal stromata over the host inflorescence, commonly known as 'choke', that prevents flowering (Fig. 1.1). *Botanophila* flies facilitate transfer of spermatia between fungal stroma of different mating types, allowing the stromata to mature and release ascospores that infect and colonise the ovary and ovule when they land on a floret, resulting in horizontal transfer to a new plant. In contrast *Neotyphodium spp*. are strictly asexual, being disseminated only by colonisation of the host seed (Clay & Schardl, 2002).

1.1.1 Endophyte growth *in planta*

The endophyte is transmitted vertically by colonising the host embryos, inside which the fungus lies dormant until the infected seed germinates. Hyphae colonise the shoot apical meristem, which then allows easy colonisation of the axillary buds and leaf primordia as they form. Grass blade growth is initiated at these axilliary buds through rapid division of the leaf primordial cells, followed by enlargement and extension of cells along the length of the expansion zone, which extends approximately 5 cm from the base of the leaf (Christensen *et al.*, 2008). This mode of extension is incompatible with the standard pattern of fungal growth, where growth occurs only at the hyphal tip, so a new hypothesis has been proposed that endophyte hyphae are able to grow by intercalary extension to avoid being sheared by the expansion of the plant cells to which they are attached, a novel growth mechanism in fungi (Christensen *et al.*, 2008). Strict control of fungal growth is essential to maintain the mutualism between endophyte and host. In a healthy, natural symbiosis endophyte hyphae grow between host cells parallel to the leaf axis. The presence of more than one fungal hypha per intercellular space is uncommon, horizontal hyphal branching is rare, and colonisation of the host cells and vascular bundles does not occur.

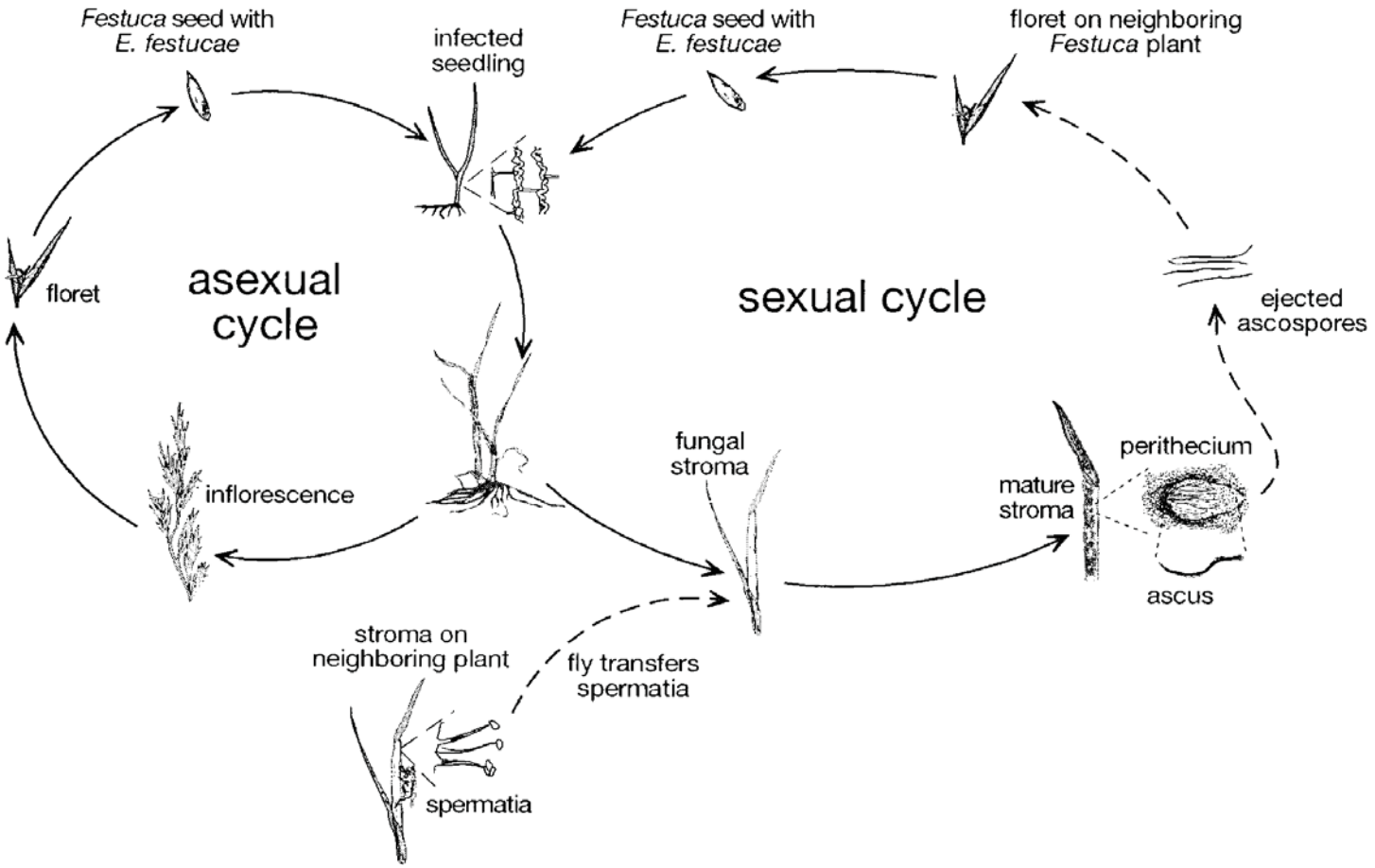


Figure 1.1 Lifecycle of epichloë endophytes

Life cycle of *Epichloë festucae* showing both sexual and asexual reproductive methods. Reproduced from Clay & Schardl., (2002). The American Naturalist 160(S4): 99-127.

A role for reactive oxygen species (ROS) in this regulation of the symbiosis was demonstrated by the deletion of the NADPH oxidase NoxA in *E. festucae* Fl1 (Tanaka *et al.*, 2006). This Δ*noxA* mutant exhibited a shift in the symbiosis from mutualism to antagonism, with infected plants visibly unhealthy and dying early, while fungal biomass increased dramatically following widespread host infection. Analysis of cytochemically-stained cells revealed that the accumulation of ROS in the fungal extracellular matrix (ECM) and at the interface between the ECM and host cell wall observed in plants infected with wild type Fl1 was lost in plants infected with the Δ*noxA* mutant. NoxR is a fungal protein that regulates NoxA, requiring binding to the small GTP binding protein RacA to do so. Δ*noxR* mutants have a very similar phenotype to Δ*noxA* mutants, with unhealthy plants containing highly branched prolific hyphae, but no obvious phenotype in culture (Takemoto *et al.*, 2006). While ROS have been previously shown to activate signal transduction pathways that regulate cell proliferation (Suh *et al.*, 1999) and modify the ECM (Lambeth *et al.*, 2000), the regulation of a plant-endophyte symbiosis is a novel function.

1.1.2 *E. festucae* and *L. perenne* as a model system

Perennial ryegrass (*Lolium perenne*) is an important pastoral grass species in New Zealand and many other parts of the world, and is naturally found in association with the asexual endophyte *Neotyphodium lolii*. *N. lolii* is very slow growing in culture and quite intractable towards scientific analysis, so the sexual endophyte *Epichloë festucae*, from which *N. lolii* is thought to descend (Schardl *et al.*, 1994), is used to model this symbiosis. *E. festucae* has occasionally been identified in a natural association with *L. perenne*, with which it forms a stable and reliable symbiosis ideal for a grass-endophyte model system (Scott *et al.*, 2007).

1.2 *Epichloë* taxonomy

Epichloë spp. (Clavicipitaceae, Ascomycota) are divided into two major clades (Fig. 1.2), with the main epichloë clade containing the species *E. festucae*, *E. amarillans*, *E. baconii*, *E. bromicola*, *E. elymi*, *E. glyceriae* and *E. brachyelytri*, and the second clade consisting of the species *E. typhina*, *E. clarkii* and *E. sylvatica*, commonly referred to as the '*E. typhina* complex' (Clay & Schardl, 2002). Each species from the first clade

infects a specific, distinct host tribe, whereas species from the *E. typhina* complex infect multiple host tribes. Asexual *Neotyphodium spp*. are commonly the result of interspecific hybridisation between two or more *Epichoë spp*., however some are derivatives of a single sexual species, such as *N. lolii*, which is thought to descent from a single *E. festucae* ancestor (Schardl *et al.*, 1994).

1.2.1 Hybrid origins are common for *Neotyphodium spp.*

A molecular phylogenetic analysis of 32 *Neotyphodium* isolates, chosen because of their lack of association with agriculture to avoid selection bias, revealed that 20 were heteroploid, with most of these approaching diploidy (Moon *et al.*, 2004). Loss of redundant genes following the hybridization event was demonstrated, and two of the *Neotyphodium* genotypes showed evidence of a second hybridization event, resulting in up to three copies of some of the markers tested. All *Epichloë* isolates tested were haploid.

Phylogenetic analyses of the β-tubulin (*tub2*) and translation elongation factor 1-α (*tef1*) genes from the large array of *Epichloë* and *Neotyphodium* species revealed that hybridization events were usually the result of inter-specific hybridization between different sexual and asexual species (Fig. 1.2; Moon *et al.*, 2004). A lack of vegetative incompatibility between *Epichloë spp.* allows the formation of interspecific heterokaryons, suggesting that the most likely mechanism for this hybridization is through somatic fusion (Chung and Schardl, 1997), even though this process is a rare result of interaction between *Epichloë* and *Neotyphodium spp*. (Christensen *et al*., 2000). In many cases hybridization appears to have followed loss of sexuality, suggesting a selective benefit for hybrids amongst asexual *Neotyphodium* species. These hybridization events are frequently associated with host species jumps, which may account for fusion events and loss of the sexual cycle.

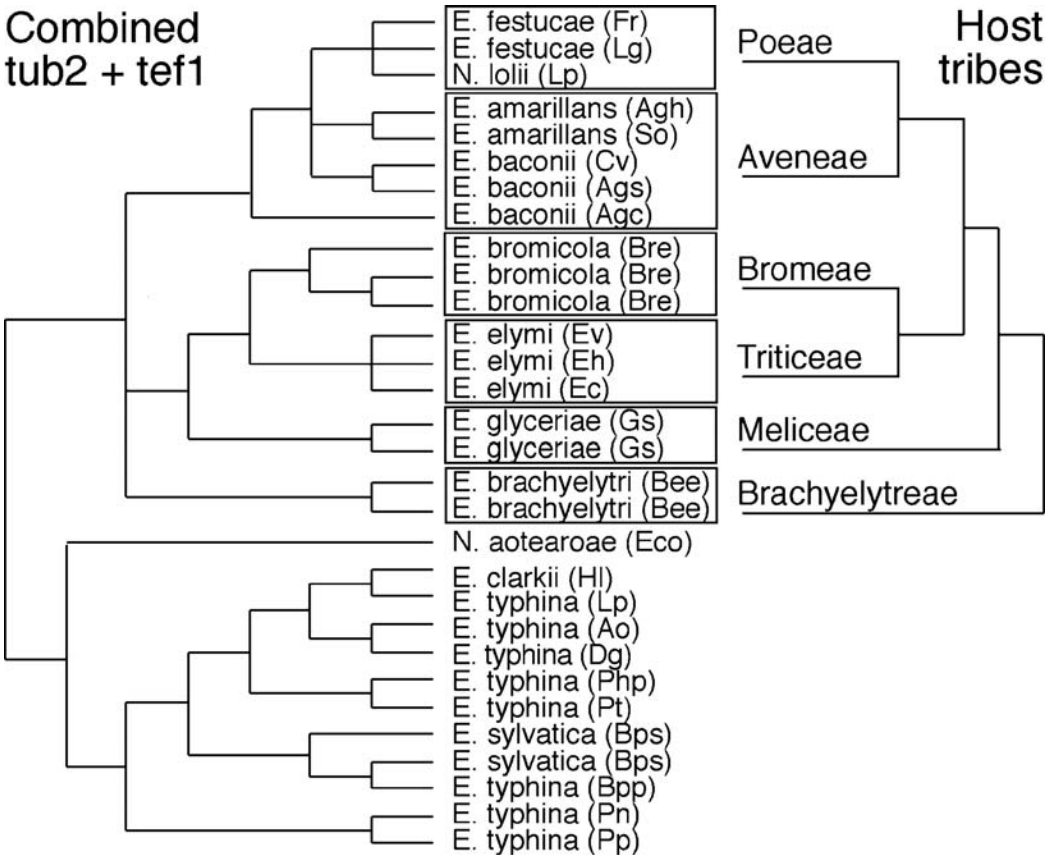


Figure 1.2 Epichloë phylogenetic tree
Phylogenetic relationship of *Epichloë* and non-hybrid *Neotyphodium spp*. shown relative to the phylogeny of their grass host tribe. The host phylogeny is not shown for the *E. typhina* complex because members of this group infect multiple host tribes. Host species are as follows: *Fr* (*Festuca rubra*); *Lg* (*Lolium gigantea*); *Lp* (*Lolium perenne*); *Agh* (*Agrostis hiemalis*); *So* (*Sphenopholis obtusata*); *Cv* (*Calamagrostis villosa*); *Ags* (*Agrostis stolonifera*); *Agc* (*Agrostis capillaris*); *Bre* (*Bromus erectus*); *Ev* (*Elymus virginicus*); *Eh* (*Elymus hystrix*); *Ec* (*Elymus canadensis*); *Gs* (*Glyceria striata*); *Bee* (*Brachyelytrum erectum*); *Eco* (*Echinopogon ovatus*); *Hl* (*Holcus lanatus*); *Ao* (*Anthoxanthum odoratum*); *Dg* (*Dactylis glomerata*); *Php* (*Phleum pratense*); *Pt* (*Poa trivialis*); *Bps* (*Brachypodium sylvaticum*); *Bpp* (*Brachypodium pinnatum*); *Pn* (*Poa nemoralis*); *Pp* (*Poa pratensis*) Reproduced from Clay & Schardl., (2002). The American Naturalist 160(S4): 99-127.

1.3 Benefits of the association

With the exception of occasional fungal stroma formation on the host by some *Epichloë spp*., the association between epichloë endophytes and their grass hosts is generally regarded as a mutualistic symbiosis, and is specifically proposed to be a defensive mutualism (Saikonnen *et al.*, 2010). The endophyte receives protection and sustenance in the intercellular spaces of the host, and a means of dissemination through the seed. In return the endophyte provides its host with a range of benefits, including drought tolerance (Hahn *et al.*, 2008), increased growth, and protection against both vertebrate and invertebrate herbivores (Clay & Schardl, 2002).

1.3.1 Bio-protective secondary metabolites are produced by epichloë endophytes *in planta*

A much-researched aspect of this plant-fungal interaction is the production of bio-protective secondary metabolites (SM) by the epichloë endophytes. The production or absence of production of a single secondary metabolite by an epichloë endophyte can dramatically change its agricultural usefulness as a pastoral symbiont. For example, the fungal indole-diterpene lolitrem B induces the stock toxicosis known as "ryegrass staggers" (Gallagher *et al.*, 1984), a reversible condition where mammalian herbivores grazing on endophyte infected pastures develop tremors, ataxia and sensitivity to external stimuli, a highly undesirable trait in a pastoral symbiont. Similarly the ergopeptine alkaloid ergovaline is implicated in stock toxicoses that cause considerable agricultural losses (Bacon *et al.*, 1986; Roberts & Andrae, 2004). In contrast the fungal secondary metabolite peramine is a potent insect feeding deterrent, shown to inhibit both larvae and adults of the perennial ryegrass pest *Listronotus bonariensis*, commonly known as the Argentine stem weevil (ASW; Rowan *et al.*, 1990). In addition some endophytes produce loline alkaloids that are potent broad-spectrum insecticides (Riedell *et al.*, 1991). The anti-insect activities of lolines and peramine are highly desirable in a pastoral symbiosis, significantly mitigating agricultural losses to invertebrate pests.

1.3.2 Fungal secondary metabolite gene clusters are plant-regulated

The production of a variety of secondary metabolites (SM) is a common feature amongst filamentous fungi, and genes for their biosynthesis tend to be clustered and co-regulated (Keller and Hohn, 1997; Fox & Howlett, 2008). Distribution of these clusters amongst related fungi is often highly discontinuous, with many examples where a particular fungus may not share production of a particular SM with a close relative, but does with one more distant (Schardl & Clay, 2002). While some SM genes show remarkable conservation between species, there is also much evidence that these clusters can evolve rapidly, driven by duplication, relocation and deletion events (Wong and Wolfe, 2005).

Horizontal gene transfer (HGT) of SM clusters between fungal species is a mechanism commonly proposed to explain instances of discontinuous SM distribution, however evidence is often lacking or inconclusive (Patron *et al.*, 2007). The distribution and relationships of polyketide synthase (PKS) genes in a range of ascomycotous fungi shows that while the distribution of PKS genes amongst the fungi analyzed are often discontinuous, in many cases this can be explained without HGT (Kroken *et al.*, 2003); however there is clear evidence showing that HGT of genes of bacterial or fungal origin into fungi does occur (Hall *et al.*, 2005; Khaldi *et al.*, 2008).

The epichloë SMs described in 1.3.1 are preferentially produced by the endophyte when growing *in planta*, and significant induction in culture has not been achieved to date, with the exception of lolines in *Neotyphodium uncinatum* (Blankenship *et al.*, 2001). This plant-based regulation occurs at the level of gene expression, with those genes involved in secondary metabolite production being expressed only *in planta* (Spiering *et al.*, 2005; Tanaka *et al.*, 2005; Young *et al.*, 2006; Fleetwood *et al.*, 2007). Lolitrem B synthesis depends on a cluster of 11 fungal genes, spread out over three sub-clusters in *E. festucae* Fl1 (Young *et al.*, 2006), and the production of lolines/ergovaline is similarly dependant on their respective fungal gene clusters (Spiering *et al.*, 2005; Fleetwood *et al.*, 2007). The lolitrem, ergovaline and loline gene clusters are located in what appear to be sub-telomeric regions of the endophyte genome (*Epichloë festucae* Genome Project, 2011), and are often found associated with transposon relics. An exception to this general rule is peramine, the production of which requires only a single

gene, *perA*, located amongst genes located distal to the telomere in related fungi (Tanaka *et al.*, 2005; Galagan *et al.*, 2005), which is not always associated with transposon relics (Fleetwood *et al.*, 2011).

1.3.3 Peramine: distribution and genotype effects

Peramine, a potent insect feeding deterrent, is a pyrrolopyrazine derived from the amino acids proline and arginine via a diketopiperazine intermediate (Rowan *et al.*, 1986; Rowan, 1993). Peramine consists of a lipophilic ring system linked to a hydrophilic guanidinium group, both of which are unique features amongst insect feeding deterrents (Fig. 1.3). The peramine content of an infected grass host is predominantly influenced by the fungal genotype, with a significant correlation observed between the genetic distance of *Epichloë* isolates and the peramine content of their grass hosts (Vázquez-de-Aldana *et al.*, 2009). This situation contrasts with that for the regulation of ergovaline, where plant genotype and environmental factors are more important. This correlation does not hold across different host species, with secondary metabolite production profiles varying greatly between symbioses of the same endophyte infecting different host species (Easton, 2007). The uniform distribution of peramine within the plant also differs to that of ergovaline, which is extremely heterogenous, indicating differences in *in planta* mobility and regulation of accumulation between the two secondary metabolites (Spiering *et al.*, 2002).

1.3.3.1 Peramine synthesis pathway and PerA protein structure

Peramine is produced by PerA, a two-module non-ribosomal peptide synthetase (NRPS) encoded by the gene *perA* (Tanaka *et al.*, 2005). The first module of this NRPS consists of an adenylation domain linked to thiolation and condensation domains, while the second module contains an adenylation domain linked to methylation, thiolation and reductase domains (Fig. 1.3). The proline precursor 1-pyrroline-5-carboxylate and arginine are the predicted substrates of modules one and two, respectively. The adenylation domain of each module provides substrate specificity and activation through adenylation, the thiolation domains anchor the substrate molecules through a thiol bond, and the condensation domain catalyses the formation of the peptide bond between the two substrates. The methylation domain methylates the α-NH_2 group of the arginine

substrate molecule, and the reductase domain is predicted to be required for reduction, cyclisation, and release. A final spontaneous oxidation step is proposed to produce the final pyrrolopyrazine product, peramine (Tanaka *et al*., 2005).

1.3.3.2 Conservation and distribution of *perA*

The gene *perA* has currently only been identified in the *Epichloë/Neotyphodium* genera, within which it appears to be present at the same genomic location in all instances, though it is not always functional (Scott *et al.*, 2009; Johnson *et al.*, 2007). The genes at the epichloë *perA* locus show high conservation of order and orientation when compared to other filamentous fungi (Fig. 1.4), with the exception of *perA* itself, which is often found flanked by repetitive sequence elements and transposon relics in epichloë, and is absent from the other fungi. This indicates that *perA* was transferred to this genomic locus at some point before the radiation of epichloë; however the origin of *perA* remains a mystery, as it is not known whether it originated in an epichloë ancestor before relocating to its current location, or was horizontally transferred from some other organism.

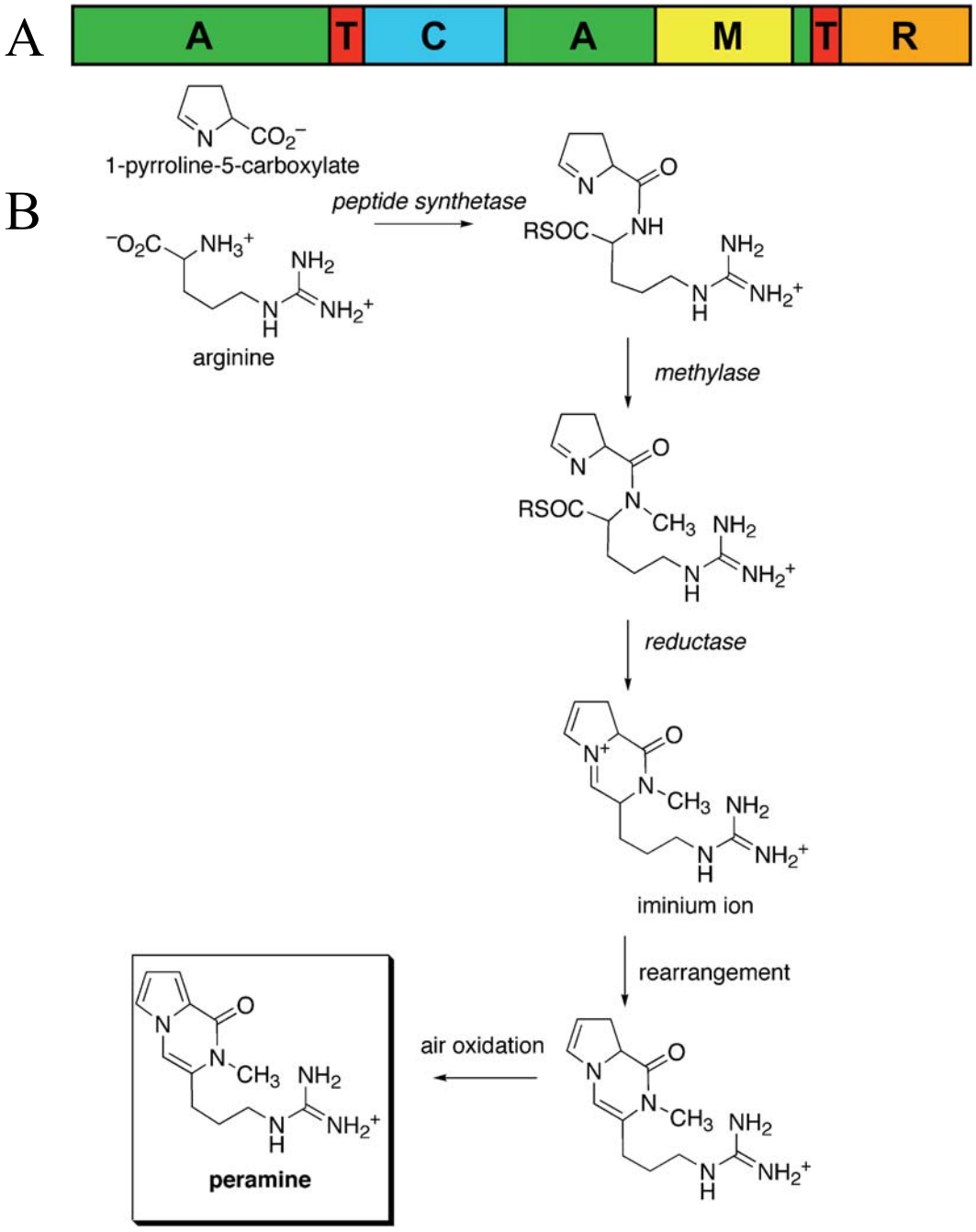


Figure 1.3 Domain structure of PerA and the peramine synthesis pathway
A. Domain structure of the two-module non-ribosomal peptide synthetase (NRPS) PerA. The first module consists of an adenylation (A), thiolation (T) and condensation (C) domain, and the second an adenylation, methylation (M), thiolation and reductase (R) domain. **B.** Proposed peramine biosynthesis pathway reproduced from Tanaka *et al.*, (2005). Mol. Microbiol 57: 1036-1050.

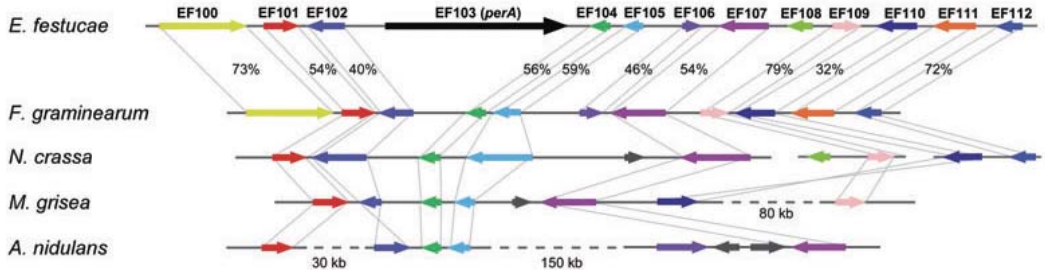


Figure 1.4 Synteny analysis of the *perA* locus with other filamentous fungi
Conserved synteny of the *E. festucae perA* locus with other filamentous fungi showing the order and orientation of genes *EF100-EF112*. Putative genes not found in *E. festucae* are coloured in grey, and the percentage identities between genes in *E. festucae* and *F. graminearum* are labelled. Reproduced from Tanaka *et al.*, (2005). Mol. Microbiol. 57: 1036-1050.

1.3.3.3 *PerA* locus in *E. festucae* E2368

The sequenced *E. festucae* strain E2368 was derived from progeny of an F1 cross of *E. festucae* E189 x *E. festucae* E434 backcrossed to *E. festucae* E189 (Wilkinson *et al.*, 2000). Strain E2368 contains a premature stop codon in *perA*, reducing the gene length from 8319 bp in Fl1 to 7116 bp in E2368. This premature stop codon, which occurs only 2 bp after homology to *perA* in Fl1 ends, likely resulted from insertion of the miniature inverted transposable element (MITE) EFT-3m into the 3' end of the *perA* gene resulting in the deletion of the entire reductase domain (Fleetwood *et al.*, 2011). Interestingly, the conservation between the truncated *perA* from E2368 and the same region in Fl1 is extremely high, with only a few SNPs and small indels present. In addition, analysis of the transcriptome data from E2368 shows that this truncated *perA* gene is still expressed (*Epichloë festucae* Genome Project, 2011). This indicates that this insertion event either occurred very recently, or that the truncated PerA protein may still produce a dipeptide that retains or provides some selective advantage to the symbiosis. A large uncharacterised AT-rich transposon relic named EFT-7 is found upstream of *perA* in strain E2368, and the identity of the genes upstream of this transposon is not known due to discontinuity in the genome assembly at this point. This is not the case for Fl1, which contains the predicated major facilitator superfamily transporter gene *EF102* 1.9 kb upstream of *perA*. Given that both *EF102* and *perA* (*EF103*) are located at the ends of their respective contigs in E2368, and the fact that both contigs terminate in an AT-rich transposon relic, linkage between these genes like in E2368 seems likely (*Epichloë festucae* Genome Project, 2011).

1.3.3.4 Detection of peramine in the host guttation fluid

Peramine concentrations in freeze-dried plant tissue samples (herbage) are routinely determined using high-performance liquid chromatography-UV spectroscopy (HPLC-UV; Spiering *et al.*, 2002); however this method is not sufficiently sensitive to detect the very low levels of peramine (< 0.1 ppm) found in cut leaf exudates or guttation fluid. The latter, produced from the tips of leaves during periods of high humidity, is driven by specialised tissues called hydathodes, and is utilised by the plant to excrete molecules (Taiz and Zeiger, 1991). Using an HPLC-mass spectroscopy (LC-MS) based method,

with a limit of detection of 0.002 ppm, very low levels of peramine can be measured in guttation fluid (Koulman *et al.*, 2007).

1.4 Transcriptome analysis of a disrupted symbiosis *E. festucae* mutant

High-throughput whole-cell mRNA sequencing can be used to generate a 'transcriptome', a quantitative representation of gene expression in an organism or system (Wang *et al.*, 2009). A transcriptome is a powerful tool for analysing changes in gene expression between identical organisms grown under different conditions, or between wild type and mutant organisms. Transcriptome data can also be used to globally define transcription start sites and refine intron positions.

1.4.1 Transcriptome analysis of the *E. festucae* Fl1 Δ*sakA* mutant

The deletion of the stress-activated mitogen-activated protein kinase *sakA* from *E. festucae* Fl1 resulted in a switch from mutualistic to pathogenic growth of the endophyte *in planta* (Eaton *et al.*, 2010). Comparative analysis between transcriptome data of this mutant and wild type Fl1 growing under identical conditions *in planta* revealed that fungal genes associated with the maintenance of the mutualistic relationship, such as the lolitrem gene cluster and *perA*, were strongly downregulated in the mutant symbiosis. Fungal genes encoding hydrolytic enzymes and transporters and plant genes associated with increased stress and pathogen defence were upregulated in the mutant symbiosis, indicative of the change to pathogenic growth.

1.4.2 Transcriptome analysis identifies *EF102* as a potential peramine transporter

EF102 is a gene divergently transcribed from *perA* that encodes a major facilitator superfamily (MFS) transporter (Tanaka *et al.*, 2005). The core 12 trans-membrane domains characteristic of MFS transporters are highly conserved with related MFSs from other filamentous fungi, however the N- and C-terminal regions are highly variable (Appendix 7.3.2). *EF102* is downregulated in the mutant symbiosis, and is the only gene proximal to *perA* that shows any change in gene expression (Appendix 7.1.1). This indicates that, given the propensity of fungal SM genes to cluster, *EF102* and *perA*

may be components of a two gene co-regulated cluster, with *EF102* potentially encoding a peramine transporter (Eaton *et al.*, 2010).

1.5 Regulation of epichloë secondary metabolite genes

The regulation mechanisms of SM clusters in epichloë endophytes are not well understood. The 5' untranslated regions of the 11 genes involved in lolitrem B synthesis contain several conserved motifs, of which multiple copies are present in each putative promoter (Young *et al.*, 2006); however it is not known if any of these motifs are responsible for the plant specific regulation of the cluster genes. Conserved motifs are also present in the putative promoters of the loline (*lol*) genes; however an experiment using the *lolA* and *lolC* promoters to drive an ectopically inserted reporter gene did not show differential regulation, suggesting a position effect on genes in the loline cluster may be responsible for their condition-specific upregulation (Zhang *et al.*, 2009).

1.5.1 Comparative analysis as a tool for regulatory motif identification

Comparative analysis between sequences from related species is a powerful tool for the identification of genes and regulatory motifs. Because regulatory elements are functionally important to an organism, natural selection conserves these sequences during the evolution of species, resulting in regions of high sequence similarity between different species when compared to the less constrained flanking intergenic sequence. A whole-genome application of this principle using four *Saccharomyces spp.* demonstrated the power of comparative genomic comparison to refine the yeast gene catalogue and locate a number of novel and previously identified regulatory motifs (Kellis *et al.*, 2003). The effectiveness of a comparative genomics approach in identifying regulatory elements is dependent on the evolutionary distances between the species. The species must be sufficiently closely related to allow orthologous sequences to be easily aligned, yet distant enough that non-functional nucleotide sites will have undergone enough genetic drift to provide sufficient resolving power to identify conserved motifs. It is also important that the species compared be as taxonomically close as possible, as only biological aspects that the species share can be identified using this method.

1.6 Aims

This research focuses on the epichloë secondary metabolite gene *perA*, and addresses three biological questions: (i) How conserved is the structure and organisation of the *perA* locus across *Epichloë*? (ii) Are there conserved elements in the *perA* promoter that may be important for the plant-based regulation of *perA*? (iii) What is the relationship between *perA* and the divergently transcribed and co-regulated gene *EF102*? The known *perA* locus sequence from *E. festucae* strains Fl1 and E2368 (Tanaka *et al.*, 2005; *Epichloë festucae* Genome Project, 2011), and transcriptome data from plants infected with Fl1 and a pathogen-like Fl1 mutant (Eaton *et al.*, 2010) were used as foundations to initiate experiments that addressed each of these three questions.

The first aim of this research was to determine the peramine production profile and *Lolium perenne* infection phenotype of six *E. festucae* strains, as well as determine the structure and organisation of the *perA* locus in these *E. festucae* strains and a range of putative peramine-producing *Epichloë spp.* This aim was split into four main objectives:

1. Determine if the six *E. festucae* strains selected produce peramine *in planta*.

2. Determine if the *E. festucae* strains selected infect the non-natural host *Lolium perenne* as well as Fl1 does.

3. Determine the conservation of structure and organisation of the *perA* locus in a range of *Epichloë* isolates by PCR amplification of *perA*, surrounding genes, and connecting intergenic sequences.

4. Demonstrate linkage between *perA* and flanking genes *EF102* and *EF104* in a range of *Epichloë* isolates using Southern blotting.

The second aim of this research was to identify conserved elements in the *perA* promoter, and was split into two main objectives:

1. Determine if conserved elements can be identified in the *perA* promoter by comparing this region across a range of putative peramine-producing *Epichloë spp.*

2. Determine if the promoters of *perA* and *EF102* share any DNA motifs.

The third aim of this research was to investigate the relationship of the major facilitator superfamily transporter gene *EF102* to *perA*, which it is divergently transcribed from and co-regulated with. This aim had one main objective:

1. Determine if EF102 is a transporter for peramine using deletion analysis.

2. Materials & Methods

2.1 Biological material

Bacterial and fungal strains and plant material used in this study are listed in Table 2.1, plasmids and DNA used are listed in Table 2.2.

Table 2.1: Bacterial strains, fungal strains and plant material

Strain	Relevant Characteristics	Source/Reference
E. festucae		
PN2278	Wild type Fl1	Young *et al.*, (2005)
PN2134	Wild type Fg1	Leuchtmann *et al.*, (1994)
PN2241	Wild type E189	C. Schardl
PN2131	Wild type Frc5	Leuchtmann *et al.*, (1994)
PN2132	Wild type Frc7	Leuchtmann *et al.*, (1994)
PN2130	Wild type Fr1	Christensen *et al.*, (1993)
PN2133	Wild type Frr1	Leuchtmann *et al.*, (1994)
PN2802	Δ*EF102* #11; Fl1/Δ*EF102*::P*trpC-hph*; Hyg^R	This study
PN2803	Δ*EF102* #88; Fl1/Δ*EF102*::P*trpC-hph*; Hyg^R	This study
PN2804	Δ*EF102* #153; Fl1/Δ*EF102*::P*trpC-hph*; Hyg^R	This study
PN2806	Δ*EF102* #169; Fl1/Δ*EF102*::P*trpC-hph*; Hyg^R	This study
PN2734	Wild type E2368	C. Schardl
E. coli		
TOP10	F^-, *mcrA*, Δ(*mrr-hsdRMS-mcrBC*), ϕ80*lacZ*ΔM15, Δ*lacX74*, *deoR*, *recA1*, *araD139*, Δ(*ara-leu*)7697 *galU*, *galK*, *rpsL*(Str^R), *endA1*, *nupG*	Invitrogen
PN4137	TOP10/pDB01	This study
Plant material		
G4375-4379	*L. perenne* cv. Samson	This study
G4311; 4380-4384	*L. perenne* cv. Samson/PN2278; WT Fl1	This study
G4312, 4313	*L. perenne* cv. Samson/PN2134; WT Fg1	This study
G4314, 4315	*L. perenne* cv. Samson/PN2241; WT E189	This study
G4316	*L. perenne* cv. Samson/PN2131; WT Frc5	This study
G4305, 4317	*L. perenne* cv. Samson/PN2132; WT Frc7	This study
G4306, 4307	*L. perenne* cv. Samson/PN2130; WT Fr1	This study
G4308, 4309	*L. perenne* cv. Samson/PN2133; WT Frr1	This study
G4385-4389	*L. perenne* cv. Samson/PN2802; Δ*EF102*	This study
G4390-4394	*L. perenne* cv. Samson/PN2803; Δ*EF102*	This study
G4395-4399	*L. perenne* cv. Samson/PN2804; Δ*EF102*	This study
G4400-4403	*L. perenne* cv. Samson/PN2805; Δ*EF102*	This study

Table 2.2: Plasmids and DNA

Name	Relevant characteristics	Source/Reference
Plasmid		
pDB01	1.5 kb 102KOLBf/102KOLBr PCR fragment and 2 kb 102KORB2f/102KORB2r PCR fragments in pSF15.15	This study
pSF15.15	Amp^R, Hyg^R	Eaton *et al.*, (2008)
pCR4-TOPO	Kan^R	Invitrogen
DNA		
E8	Wild type *E. typhina*	Schardl *et al.*, (1997)
E425	Wild type *E. typhina*	C. Young
E57	Wild type *E. amarillans*	C. Young
E501	Wild type *E. bromicola*	C. Young
E799	Wild type *E. bromicola*	C. Young
E56	Wild type *E. elymi*	C. Young
WWG1	Wild type *E. elymi*	C. Young

2.2 Common stocks, growth media and conditions

2.2.1 Basic growth media

2.2.1.1 Potato Dextrose medium (PD)

PD medium contained 2.4% (w/v) potato dextrose in sterile Milli-Q H_2O, with 1.5% (w/v) agar added if plating.

2.2.1.2 Luria Bertani medium (LB)

LB medium (Miller, 1972) contained 85 mM NaCl, 1% (w/v) tryptone and 0.5% (w/v) yeast extract in sterile Milli-Q H_2O, with 1.5% (w/v) agar added if plating.

2.2.1.3 Regeneration medium (RG)

RG medium contained 2.4% (w/v) potato dextrose, 0.8 M sucrose and 1.5% (w/v) agar in sterile Milli-Q H_2O.

2.2.1.4 Sterilization conditions

Growth media and other solutions were autoclaved at 121°C for no less than 15 min. Pouring of growth media plates, subculturing and other contamination-sensitive activities were performed in a UV sterilized laminar flow unit.

2.2.2 Growth conditions

2.2.2.1 *Escherichia coli*

E. coli cultures were grown at 37°C overnight on LB agar plates, or in LB broth with shaking at 200 rpm. Ampicillin was added to a final concentration of 100 µg/mL when required for antibiotic selection. Cultures were maintained for short periods at 4°C, or stored at -80°C in 50% (v/v) glycerol.

2.2.2.2 *Epichloë festucae*

E. festucae cultures were grown at 22°C on PD plates for 6-9 d, or in PD broth with shaking at 150 rpm for 5-8 d. *E. festucae* protoplasts regenerating on RG plates were left until they grew through the antibiotic overlay (approx. 2 weeks). Hygromycin was added to a final concentration of 150 µg/mL when required for antibiotic selection. Cultures were stored at 4°C over periods of several months, or at -80°C for long-term storage.

2.2.3 Common stock solutions

2.2.3.1 SDS loading dye

SDS loading dye contained 20% (w/v) sucrose, 5 mM EDTA, 1% (w/v) SDS and 0.2% (w/v) bromophenol blue in sterile Milli-Q H_2O.

2.2.3.2 20x SSC Buffer

20x SSC buffer contained 3 M NaCl, 0.3 M sodium citrate in Milli-Q H_2O. 20x SSC was diluted in Milli-Q H_2O to obtain 2x SSC.

2.3 Standard *E. coli* cell methods

2.3.1 Cloning DNA fragments into an *E. coli* plasmid vector

Cloning of PCR products into an *E. coli* plasmid vector was performed using the TOPO TA Cloning® kit (Invitrogen), following the manufacturer's instructions.

2.3.2 *E. coli* transformations

One tube of One Shot® TOP10 cells (Invitrogen) per transformation was thawed on ice, then 2 µL of plasmid (from TOPO® cloning reaction or other) was added to the cells and incubated on ice for 5 min. Cells were then heat-shocked at 42°C for 30 sec and returned immediately to ice. 250 µL of room temperature SOC medium, 2% (w/v)

tryptone, 0.5% (w/v) yeast extract, 10mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added to each tube and incubated at 37°C with shaking (200 rpm) for 1 hour. 50 µL of this transformation mix was then spread onto a selective LB Agar plate with 0.1 mg/mL ampicillin, and 20 µL onto a second selective plate to ensure sufficient numbers of individual colonies would be obtained on at least one plate.

2.3.3 Screening *E. coli* transformants

Each *E. coli* colony growing on selective medium to be analyzed was picked using a yellow (2-200 µL) pipette tip and resuspended in 6 µL of LB. 3 µL of this suspension was then stored at 4°C for any future analyses, and the remaining 3 µL was added to 8 µL of cracking buffer (50 mM NaOH, 0.05% (w/v) SDS, 5 mM EDTA) and heated at 100°C for 1 minute, then cooled to room temperature. Once cooled 1 µL of restriction enzyme (whichever was selected for screening transformants) and 1 µL of RE 10x buffer (as appropriate for the selected RE) was added to the tube and incubated at 37°C (or other if required by RE) 10-30 min, depending on how many units of RE added. 2 µL of SDS loading dye was then added to the tube to stop the reaction and the results visualized via gel electrophoresis.

2.3.4 *E. coli* plasmid isolation

Plasmid isolation from *E. coli* was performed using the High Pure Plasmid Isolation Kit (Roche), as per the manufacturer's instructions.

2.4 Standard *E. festucae* cell methods

2.4.1 Fungal DNA extraction (Byrd *et al.*, 1990)

Fungal colonies were grown on PD agar plates at 22°C for ~7 d, 1 cm^2 of mycelia were then taken off the plate and ground in a 1.7 mL microfuge tube, mixed into 50 mL PD broth and grown at 22°C on a shaker at 200 rpm. After the liquid culture had grown sufficiently (~7 d) the mycelia were filtered from the PD broth, washed with sterile Milli-Q H_2O, blotted dry then frozen at -80°C. Once frozen, mycelia were freeze-dried overnight to remove all water, and the resulting product was stored at 4°C. 0.01 to 0.02

g of freeze-dried mycelia were ground under liquid nitrogen, dissolved into 800 µL of DNA extraction buffer (150 mM EDTA, 50 mM Tris, 1% (w/v) SDS, pH 8.0) and proteinase K added to 2 mg/mL followed by incubation at 37°C for 20 min. The resulting solution was centrifuged in a benchtop microcentrifuge at 16060 RCF and the supernatant transferred to a new tube. To this were added ½ volumes each of phenol and chloroform, followed by a brief vortex and spin for 10 min at 16060 RCF. The aqueous phase was again removed to a new tube and this phenol-chloroform extraction step was repeated two more times. Following this 1 volume of chloroform was added to the aqueous phase, mixed, and centrifuged for 10 min at 16060 RCF. The aqueous phase was then added to 1 volume of isopropanol, mixed and left for 10 min at room temperature to allow the DNA time to precipitate, followed by a 10 min spin at 16060 RCF. The supernatant was discarded and the DNA pellet washed with 1 mL of 70% (v/v) ethanol. The pellet was left to dry at 37°C for 10 min then resuspended in sterile Milli-Q H_2O.

2.4.2 Preparation of *E. festucae* fungal protoplasts (Yelton *et al.*, 1984)

1 cm^2 of *E. festucae* Fl1 mycelia, taken from a fresh culture plate, was ground in a 1.7 mL microfuge tube with LB medium using a micropestle, with the resulting homogenate used to inoculate 50 mL of PD medium that was then grown for 5 d at 22°C, with shaking (150 rpm). The PD was then poured through a coarse filter, which retains the mycelia, and the mycelia were washed three times with sterile Milli-Q H_2O, follwed by a final wash with OM buffer (1.2 M $MgSO_4$, 10 mM $NaHPO_4$, pH to 5.8 using NaH_2PO_4). To every 1 g of mycelia collected 10 mL of filter sterilized Glucanex (Sigma-Aldrich;10 mg/mL in OM buffer) was added followed by incubation at 30°C for 5 hours, with shaking (100 rpm). Samples were then examined by microscope to confirm protoplast formation (smooth, round cells with low osmotic resistance), and then filtered through a sterilized nappy liner into a sterile 200 mL Schott bottle. The filtrate was divided into Corex tubes (5 mL into each 15 mL tube) and overlaid with 2 mL ST buffer (0.6 M sorbitol, 100 mM Tris, pH 8.0) per tube. Tubes were then spun at 2375 RCF for 5 min at 4°C, and protoplasts, now at the interface between the Glucanex and ST buffer, were removed by removing the top layer with a 1 mL filter tip then placed in a fresh Corex tube. 5 mL of STC buffer (1 M sorbitol, 50 mM Tris, 50 mM $CaCl_2$, pH 8.0) was then added, mixed gently, and then spun at 2375 RCF. This step was

repeated 3 times and samples pooled until protoplasts were contained within a single tube. Protoplasts were then resuspended in 500 µL STC buffer, and a sample taken for dilution and concentration estimation using a haemocytometer. Protoplasts were then divided into 80 µL aliquots and 20 µL of 40% (v/v) polyethylene glycol was added to each. Tubes were then immersed in liquid nitrogen and stored at -80°C until required.

2.5 Standard plant methods

2.5.1 Plant inoculation (Latch & Christensen, 1985)

Perennial ryegrass seeds were surface sterilized by soaking in 50% (v/v) H_2SO_4 for 30 min, rinsing in tap water 3 times, soaking seeds in 50% (v/v) chlorine bleach for 30 min, rinsing in sterile water 3 times, then air drying on filter paper in a laminar flow cabinet. Seeds were then germinated on 3% (w/v) water agar in petri dishes (8 per plate), and the plates were placed on their side in the dark for 7 d at 22°C. Using a sterile scalpel and dissecting microscope in a laminar flow cabinet, a shallow 2-3 mm longitudinal slit was cut into the 7 day old seedlings between the mesocotyl and coleoptile, and a small piece of freshly grown fungal mycelia was inserted into the cut. Seedlings were then returned to the dark at 22°C for 7 d, checked for contamination after 2 d, then transferred to the light and incubated for another 7 d at 22°C. Seedlings were then planted into root trainers containing potting mix.

2.5.2 Plant immunoblotting to confirm fungal infection

Four to six weeks after seedlings were planted (provided sufficient tillers have grown) a tiller was cut using a scalpel as close to the base as possible from each plant to be tested, dead leaf sheaths were removed and the exposed cut was pressed onto a nitrocellulose membrane (NCM). The process was repeated for all plants to be tested, with each tiller being pressed into a separate cell on the NCM and labelled using pencil. The NCM was then immersed into freshly made blocking solution (20 mM Tris, 50 mM NaCl, 0.5% (w/v) non-fat milk powder) for 2 hours at room temperature, which was then decanted off and replaced by 5 mL of blocking solution with 5 µL of primary antibody (polyclonal rabbit antibodies raised against homogenised mycelium of *Neotyphodium lolii*, Christensen *et al.*, 1993) and incubated with slow (15 rpm) shaking overnight at

4°C. The following morning the blocking solution/primary antibody was decanted off and the NCM was washed 3 times in fresh blocking solution. 5 mL of fresh blocking solution and 2.5 µL of secondary antibody (goat anti-rabbit antibody with an alkaline phosphatase conjugate, Sigma) was added to the NCM and incubated on a shaker at room temperature for 2 hours before decanting off and washing the NCM three times in fresh blocking solution. Developing solution was prepared using SIGMAFAST™ Fast Red TR/Naphthol AS-MX tablets, as per the manufacturer's instructions, added to the NCM, and incubated on a shaker at room temperature for 15 min before decanting off and washing the NCM in Milli-Q H_2O. The NCM was then dried on blotting paper and the results visualized.

2.5.3 Preparation of infected ryegrass samples for confocal microscopy

Pseudostem samples were taken from endophyte infected perennial ryegrass plants by cutting near the base of tillers using a scalpel, removing any dead outer layers, then peeling off sections from the pseudostem, removing the bottom 1 cm, and then cutting this section in half longitudinally. Leaf samples were taken by cutting out the first 1 cm of leaf starting from the interface between leaf and pseudostem, and then cutting this section in half longitudinally. Samples were then placed in 95% (v/v) ethanol and kept overnight at 4°C, or until required. Samples were then treated in 10% (v/v) potassium hydroxide for 3 h, washed three times in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4), and incubated in staining solution (20 µL of 1% (w/v) aniline blue, 10 µL of 2% (v/v) Tween20, 10 µL of 1 mg/mL Alexa Fluor® 488 WGA (Invitrogen), made up to 1 mL in PBS) for 30 mins, including a 10 min vacuum infitration.

2.5.4 Confocal microscopy

Imaging of samples was done on the Leica SP5 DM6000B confocal microscope at the Manawatu Microscopy and Imaging Centre. A final magnification of 400x was used, and images were taken of hyphae just below the plant epidermal cells, with a stack of at least 8 images taken with a Z step size of 2 µm. Subsequent image modifications were done using the Fiji image processing package (http://fiji.sc/wiki/index.php/Fiji).

2.6 Standard DNA methods

2.6.1 DNA concentration measurement

The concentration of gDNA samples was measured on a DyNA Quant 200 Fluorometer (Hoefer), as per the manufacturer's instructions. PCR products and RNAse treated plasmid/gDNA samples were measured on a Implen NanoPhotometer™, as per the manufacturer's instructions.

2.6.2 Restriction enzyme digestion of DNA

2.6.2.1 Digestion of plasmid DNA

100 ng of plasmid DNA was added to 5 units of restriction enzyme, 2.5 µL of the appropriate commercial restriction enzyme buffer, and the volume was made up to 25 µL with sterile Milli-Q H_2O and mixed well. This reaction mix was then incubated at 37°C (or as appropriate for the restriction enzyme being used), followed by addition of 6 µL SDS loading dye to stop the reaction. Double digests of plasmid DNA were done in a similar manner, but with 5 units of each restriction enzyme, and a commercial buffer that was suitable for use with both enzymes. If no suitable buffer was available then sequential digests were performed, with purification of the digested DNA between reactions (2.6.2.6).

2.6.2.2 Digestion of genomic DNA for Southern blotting

1.1 µg of gDNA was added to 30 units of restriction enzyme, 5 µL of the appropriate commercial restriction enzyme, and the volume was made up to 50 µL using sterile Milli-Q H_2O and mixed gently to avoid shearing the gDNA. Reactions were then incubated overnight at 37°C (or as appropriate for restriction enzyme being used), followed by addition of 12 µL SDS loading dye to stop the reaction. 5.6 µL (100 ng) of this digest was then used to confirm complete digestion before the remaining 1 µg of digested gDNA was used for Southern blotting.

2.6.3 DNA Sequencing

DNA sequencing was performed by the Massey Genome Service using the BigDye™ Terminator (version 3.1) Ready Reaction Cycle Sequencing Kit (Applied Biosystems). Samples contained 500 ng plasmid and 6.4 pmol primer, and sequence analysis was done using MacVector® 10.0.2.

2.6.4 Polymerase Chain Reaction

Primers used in this study are listed in Table 2.3.

Table 2.3: Primers used in this study

Name	Sequence (5' – 3')	Used for
101f	TACAAGTGGCAGGATGCTGTGG	PCR/KO[a] Screening
101r	TCGTCGTCAATGGTGAGGAACC	PCR Screening
101-2f	CGTGCTGAGGAAATCTGTTGGAC	PCR Screening
101-2r	CGCCATCTTCGTCATCATTAGTG	PCR Screening
102f	GGAAGAGATGGAGTTGAAGAAGCC	PCR Screening
102r	TCGCCCAGCCTTTTATCCTG	PCR Screening
102fullf	TAATTCGTACGCCAGAAGGC	KO Screening
102fullr	GACAAGGACAGGGACAGGAA	KO Screening
102-3f	GGCTGGTTGGGTTGTTCTTACATC	PCR Screening
102-3r	TGTGATTTGCGGTCTTGACTGG	PCR Screening
102-3bf	CGCTCATGCTGCTCTCCTGTTC	PCR Screening
102-3br	TCCAGCTCCTTATAGGTCAATC	PCR Screening
102-3cf	GTGAGCCACTGAACGAGTGA	PCR Screening
102-3cr	ATTTGCGGTCTTGACTGGAC	PCR Screening
102-3df	GTGGCCGCTCATGATGCG	PCR Screening
102-3dr	CCGGGAGACTGGGATAGG	PCR/KO Screening
PN61f	ATCAACACTGTCAACAGCCG	PCR Screening
PN61r	GCCCTGATGAGCATGGATGT	PCR Screening
P9f	GCAAACGCCGTCTCTGCTCA	PCR Screening
P9r	GGATCCCCTTAACAACCACT	PCR Screening
Rdomf	GGTACTTCTGCAAGCAAGGC	PCR Screening
Rdomr	CCATCTGGCAAGGGCAAGACG	PCR Screening
103-4f	GGATGTTTTCATACACAACGGGG	PCR Screening
103-4r	TCATTTGCGAGAGGGACTTACG	PCR Screening
103-4bf	GTGGCAAAGGCGCTCGCCGACAG	PCR Screening
103-4br	GGACTGCGTCGAGGAGTGTACG	PCR Screening
103-4cf	CGCTGTCAGATATGCTCCAA	PCR Screening
103-4cr	TGTGTCTGCAGTCTTCCACC	PCR Screening
104f	GTCCACATCAAACAGAAACACCG	PCR Screening
104r	GAATAACCTTGGCAGGAGGAGC	PCR Screening
104-5f	AATGGAATCCCTTACCTTGGACTC	PCR Screening
104-5r	TGACGAGCCTTTACCCGCATAC	PCR Screening
105f	TTCGTATGCGGGTAAAGGCTC	PCR Screening
105r	CAGTGTAAAAGCGACAAGGGGAC	PCR Screening
YAP10	GATTTGTGTACGCCAGACAGTCC	KO Screening
YAP11	CTGAACTCACCGCGACGTCTGT	KO Screening
102-KO-LB-F	CTGCAGGTGGCCAGGATTCACATCTT	KO construct prep.
102-KO-LB-R	TCTAGACGGCCGAATCAAGTATCATT	KO construct prep.
102-KO-RB-F	ATCGATTGATGATGGAAGCAGAATGC	KO construct prep.
102-KO-RB-R	GTTAACGAATGAAGGAGCTGTCTGGC	KO construct prep.
102KORB2-F	ATCGATGGCTGGTTGGGTTGTTCTTACATC	KO construct prep.
102KORB2-R	GTTAACTGTGATTTGCGGTCTTGACTGG	KO construct prep.
102-103intseq	GTGCGTCTGTGCTTCATCAT	Sequencing

E56SeqR	CGCTGGACAAATGACAGAC	Sequencing
OtherSeqR	CTCCGCGTCCATTGGTGG	Sequencing
AllSeqF	AGCTACGAGTCGTATGGTG	Sequencing
E57SeqR	CTGACGAGGTCAGAGAC	Sequencing
E501FSeq	GTGGAGCTGTTAGGGATAC	Sequencing
E501RSeq	TGTTGCAGACACCCTGGCC	Sequencing
M13F	TGTAAAACGACGGCCAGT	Sequencing
M13R	GAGCGGATAACAATTTCACACAG	Sequencing

[a] KO = Knock out

2.6.4.1 Standard PCR

Standard PCR reactions were done using Roche™ Taq DNA Polymerase. Standard PCR reaction mixtures contained 35.8 µL sterile Milli-Q H_2O, 5 µL PCR buffer (supplied with polymerase), 1 µL primer 1 (10 pM), 1 µL primer 2 (10 pM), 2 µL dNTP mix (1.25 mM), 0.2 µL Taq DNA polymerase (5 U/µL) and 5 µL of template DNA (1 ng/µL) to a total of 50 µL. The reaction mixture was then gently mixed and run on an Eppendorf Mastercycler® Gradient using the following thermal cycling scheme: Lid 94°C (wait until reached); 1. 94°C 02:00; 2. 94°C 00:30; 3. 60°C[a] 01:00; 4. 72°C 02:00; Go to 2, repeat x30; 5. 72°C 07:00; Hold at 4°C.

[a] Actual annealing temperature dependent on T_m of primers used.

2.6.4.2 Difficult template PCR

Some regions were not possible to amplify using the standard PCR setup; in these cases 1 µL of H_2O was replaced with 1 µL (2% v/v) of dimethyl sulfoxide (DMSO), an inhibitor of DNA secondary structure.

2.6.4.3 Multiplex PCR

Method performed as per standard PCR (2.6.4.1), but with 1 µL of H_2O replaced with 1 µL of 10 pM primer stock for each additional primer.

2.6.4.4 High fidelity PCR (Expand Hi-Fi)

PCR products destined for sequence-critical applications were amplified using the DNA polymerase Expand High Fidelity (Roche). High Fidelity reaction mixtures contained 35.25 µL sterile Milli-Q H_2O, 5 µL PCR buffer (supplied with polymerase), 1 µL primer 1 (10 pM), 1 µL primer 2 (10 pM), 2 µL dNTP mix (1.25 mM), 0.75 µL Expand Hi-Fi DNA polymerase (3.4 U/µL) and 5 µL of template DNA (1 ng/µL gDNA, 0.2 ng/µL plasmid) to a total of 50 µL. 1 µL of H_2O was replaced with 1 µL DMSO when amplifying from a difficult template. The reaction mixture was then gently mixed and run on a Eppendorf Mastercycler® Gradient using the following thermal cycling scheme: Lid 94°C (wait until reached); 1. 94°C 03:00; 2. 94°C 00:15; 3. 60°C[a] 00:30; 4. 72°C[b] 04:00[c]; Go to 2, repeat x10; 5. 94°C 00:15; 6. 60°C[a] 00:30; 7. 72°C[b] 04:00[c] + 00:05/cycle; Go to 5, repeat x20; 8. 72°C 07:00; Hold at 4°C.

[a] Actual annealing temperature dependent on T_m of primers used.

[b] Elongation temperature 72°C for products < 3 kb, 68°C for products >3kb.

[c] Elongation duration dependant on product size: 45s < 0.75 kb, 1 min < 1.5 kb, 2 min < 3 kb, 4 min < 6 kb, 8 min < 10 kb.

2.6.4.5 High Fidelity PCR (Platinum® *Pfx*)

Platinum® *Pfx* (Invitrogen) reaction mixtures contained 19.7 µL H_2O, 5 µL PCR buffer (supplied with polymerase), 1 µL primer 1 (10 pM), 1 µL primer 2 (10 pM), 1.5 µL dNTP mix (1.25 mM), 15 µL PCR enhancer (supplied with polymerase), 1 µL 50 mM $MgSO_4$, 0.8 µL Platinum *Pfx* (2.5 U/µL) and 5 µL of template DNA (1 ng/µL) to a total of 50 µL. The reaction mixture was then gently mixed and run on a Eppendorf Mastercycler® Gradient using the following thermal cycling scheme: Lid 94°C (wait until reached); 1. 94°C 02:00; 2. 94°C 00:15; 3. 60°C[a] 00:30; 4. 68°C 02:00[b]; Go to 2, repeat x35; 5. 68°C 07:0; Hold at 4°C.

[a] Actual annealing temperature dependent on T_m of primers used.

[b] Extension time of 1 minute per kb

2.6.5 Gel Electrophoresis

Agarose gels were made by heating the desired amount of agarose (0.7% w/v for separation of large DNA fragments and up to 1.6% w/v for separation of smaller fragments) with 1x TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM Na_2EDTA) in the microwave until the agarose was fully dissolved. The liquid agarose mix was equilibrated to 56°C in a water bath, poured into the gel tray, had the well moulds inserted, and left to set. Once set the well moulds were removed and the gel was placed into a gel electrophoresis apparatus and immersed in 1x TBE buffer. DNA samples mixed with SDS loading dye were then inserted into the wells at the top of the gel, the gel apparatus was turned on, and the power pack was set to the desired voltage. Once the gel had been run it was removed from the gel apparatus and stained for 10 – 15 min in an ethidium bromide solution (1 μgmL^{-1} ethidium bromide in Milli-Q H_2O), and then destained for 10 min in tap water. Gels were visualized and photographed using a UV Transilluminator Gel Documentation System (Bio-Rad).

2.6.6 DNA purification following PCR or gel electrophoresis

Purification of DNA from PCR reactions and agarose gels were performed using the Wizard® SV Gel and PCR Clean-Up System (Promega), following the manufacturer's instructions.

2.6.7 A-tailing DNA fragments

A-tailing reactions were carried out using Taq DNA polymerase. 6 µL of DNA was mixed with 1 µL of Taq PCR Buffer (Roche), 2 µL of 1 mM dATP, 1 µL of Taq DNA polymerase (Roche) and 1 µL of sterile Milli-Q H_2O. The reaction was mixed well then heated for 30 min at 70°C in a thermal cycler. The A-tailed product was then purified (2.3.2.6) for use in DNA ligations.

2.6.8 DNA ligation

Ligation reactions were performed by mixing 20 ng of linearised vector (dephosphorylated by alkaline phosphatase), 2 µL of 10x ligation buffer (New England Biolabs), x ng of insert DNA (where the value of x (in ng) was determined by the equation "[amount of vector DNA] x [length of insert (kb)] / [length of vector (kb)]"), with Milli-Q H_2O added to a total of 20 µL. 5 µL was then removed to 5 µL SDS loading dye for a before-ligation sample, and 1 µL of T4 ligase (40 units, New England Biolabs) was added to the remaining 15 µL and mixed well, followed by overnight incubation at 4°C. The following morning 5 µL was removed to 5 µL SDS loading dye for an after ligation sample, which was then analyzed with the before ligation sample via gel electrophoresis. If the ligation had gone to completion then the ligated product was used to transform *E. coli* cells (2.4.2).

2.6.9 Southern Blotting (Southern, 1975)

1 µg of digested gDNA from each sample plus SDS loading dye was loaded into each well of a large format 0.7% (w/v) agarose gel along with a DNA ladder, and in some cases an uncut gDNA control. Gels were run overnight at 30 V, stained with ethidium bromide, destained in water and imaged next to a reference measure using the Gel Doc equipment. Gels were depurinated in 0.25 M HCl for 15 min, denaturated in 0.5 M NaOH/0.5 M NaCl for 40 min and then neutralized in 0.5 M Tris, pH 7.4, 2 M NaCl for 40 min before being washed in 2x SSC for 2 min and placed on a blotting apparatus for overnight DNA transfer to a nylon membrane. The blotting apparatus consisted of 2 "wicks" of 320 x 156 mm Whatman 3MM paper soaked in 20x SSC, the ends of which were submerged in wells of 20x SSC, with the gel placed on top protected by a plastic cling-film layer with a square hole cut in the centre slightly smaller than the size of the gel to prevent short-circuiting of the capillary action which drives the blotting process. A positively charged nylon transfer membrane soaked in 2x SSC cut slightly larger (~4 mm) than the gel it was to transfer from was then placed on the gel, followed by 2 sheets of Whatman 3MM paper the same size as the gel soaked in 2x SSC, and two more identical dry sheets. A pile of paper towels was then placed on top of this stack, weighted down evenly and left to blot overnight. The following morning the nylon

membrane was removed and washed in 2x SSC before being cross-linked to any transferred DNA using the Ultraviolet Crosslinker Cex-800 at 120,000 μjoules/cm^2.

2.6.10 Radioactive hybridization and visualization (Southern, 1975)

30 ng of the designated probe DNA (plasmid or PCR product) in 11 μL of H_2O was radiolabelled by boiling for 3 min to denature the DNA strands, placing immediately on ice for 5 min, then adding 4 μL of Roche™ High Prime solution mix, 5 μL of [α-^{32}P]dCTP (50 μCi total) and incubating at 37°C for 1 hour. 35 μL TES buffer (10 mM Tris, 1 mM NaEDTA, 100 mM NaCl, pH 8) was then added to each reaction mix before being transferred to GE Healthcare G-50 micro-columns and centrifuged for 2 min at 735 RCF, and a further 50 μL TES buffer added to the flow-through, which was used for hybridization. Southern blots were prepared for hybridization by placing in a glass hybridization tube with 30 mL of 10x Denhardt's solution (0.4 M Hepes buffer (pH 7), 3x SSC, 0.02 mg/mL *E. coli* tRNA, 0.1% (w/v) SDS, 0.2% (w/v) ficoll, 0.2% (w/v) BSA, 0.2% (w/v) PVP, 18 μg/mL phenol-extracted herring DNA) and pre-hybridized in a rotisserie oven at 65°C for at least 2 hours. Most of the Denhardt's solution was then drained, leaving only 5 mL, and the radioactive probe added. The hybridization tube was then left overnight in the rotisserie oven at 65°C. In the morning the fluid was decanted off the Southern blot, and the blot was removed from the hybridization tube and washed 4 times in 50°C 2x SSC containing 0.1% (w/v) SDS, blotted dry, wrapped in gladwrap then exposed to X-ray film at -20°C. X-ray film was then developed using the 100Plus automatic X-ray processor (All-Pro Imaging), with length of exposure dependant on the strength of radioactivity on the blot.

2.7 Peramine analysis

Methods 2.7.1 and 2.7.2 were obtained from and performed by Mace Wade, AgResearch Grasslands Research Centre, Palmerston North.

2.7.1 Peramine HPLC-UV analysis

Peramine was extracted from plant material using a method modified from Spiering *et al*., (2002) and Hunt *et al.*, (2005). A 50 mg sample of freeze-dried grass tissue was

extracted for 1 hour with 1 mL of the prepared extraction solvent (50% (v/v) methanol with 2.064 ng/mL homoperamine nitrate (AgResearch Grasslands) as internal standard). The sample was then centrifuged for 5 min at 8000 RCF, and a 500 μL aliquot of the supernatant was transferred to a high performance liquid chromatography (HPLC) vial for separation on a Synergi Polar-RP 100 x 2.00mm (2.5um) column (Phenomenex) and analysed by ultraviolet (UV) spectroscopy at 280 nm. The limit of detection of this technique was 0.1 μg/g for herbage.

2.7.2 Peramine LC-MS analysis

Peramine was extracted as in 2.7.1, and the extract was analysed on a triple-quad liquid chromatography-mass spectrometer (LC-MS) using the Synergi Polar-RP 100 x 2.00mm (2.5um) column (Phenomenex). Multiple reaction monitoring focused on the following molecular weight ranges: Peramine 248.1–175.1, 248.1– 206.1; Homoperamine 262.1 – 203.1, 262.1 – 245.1. The limit of detection of this technique was 0.02 μg/g for herbage. Gutation fluid samples were analysed by diluting 50/50 with extraction solution prior to running through the instrument. This improves the limit of detection ten-fold (e.g. 0.01 μg/mL for HPLC, and 0.002 μg/mL for LC-MS). The limit of quantification was 5 times limit of detection.

2.8 *EF102* deletion mutant creation

2.8.1 Creation of *EF102* deletion construct

The left border fragment desired for the *EF102* deletion construct was amplified using the proof-reading DNA polymerase Expand High-Fidelity (Roche) (2.3.2.4) with PCR primer pair 102KOLBf/102KOLBr from an *E. festucae* Fl1 gDNA template. The right border was similarly amplified using the proof-reading DNA polymerase Platinum Pfx (Invitrogen) (2.3.2.5) with primer pair 102KORB2f/102KORB2r. Each primer contained a 5' overhang designed to introduce a restriction enzyme site into the PCR amplification product (102KO_LBf with PstI site, 102KO_LBr with XbaI site, 102KO_RB2f with ClaI site, 102KO_RB2r with HpaI site). PCR amplification products for the left and right borders were then cloned into the Invitrogen TOPO TA® plasmid

vector (2.4.1), with the right border fragment requiring prior A-tailing (2.3.4) as it was amplified using Platinum *Pfx*, and transformed into *E. coli* (2.4.2). Transformants were then screened (2.4.3), and *E. coli* strains displaying the correct banding pattern were grown in LB with ampicillin overnight at 37°C. The next day plasmid DNA was extracted from the *E. coli* cultures (2.4.4) and used to sequence the insert. Sequencing results were compared to the Fl1 *perA* locus sequence (Tanaka *et al.*, 2005) and plasmids containing the left and right border fragments without sequence errors were selected to provide the final products for generation of the *EF102* deletion construct. The left border fragment was cut out of the TOPO vector by double digestion of the plasmid (2.3.1.1) with the restriction enzymes *Pst*I and *Xba*I using Roche restriction enzyme Buffer H at 37°C for 1 hour. The resulting digest was gel purified (2.3.2.6) and then ligated (2.3.5) into the ampicillin/hygromycin resistance vector pSF15.15, which had been similarly digested and purified. The sticky ends on the left border and plasmid fragments from restriction enzyme digestion allowed for selective, directional cloning of the left border fragment into the pSF15.15 plasmid, with the ligation reaction being transformed into *E. coli*, followed by selection and screening to identify correct transformants from which pSF15.15+LB plasmid DNA was isolated. The right border fragment was then introduced into the pSF15.15+LB plasmid through a similar process, using the restriction enzymes *Cla*I and *Hpa*I with Roche Buffer A to create the final *EF102* deletion construct pDB01.

2.8.2 Transformation of *Epichloë festucae* (Oliver *et al.*, 1987)

Three 100 µL tubes of protoplasts were thawed on ice, then 2 µL of spermidine and 5 µL of heparin was added to each tube. Tube 1 had 2 µg of deletion construct DNA added (a purified PCR product (2.6.6) amplified with the primers 102KO_LBf/102KO_RB2r by the DNA polymerase Expand High-Fidelity (2.6.4.4)), while tube 2 was a protoplast only (negative) control, and tube 3 a positive control, with 5 µg of the hygromycin resistance plasmid pAN7-1 being added. All tubes were vortexed gently then left on ice for 30 min, then 900 µL 40% (v/v) polyethylene glycol was added to each tube, vortexed gently and maintained on ice. Twelve x 5 mL tubes were then prepared with 3.5 mL RG agar in each (2.4% (w/v) potato dextrose, 0.8 M sucrose, 0.8% (w/v) agar, pH 6.5) and maintained on a hot block at 50°C. The following was added to each tube of RG agar: 1 x 100 µL undiluted protoplast only solution

(negative control); 1 x 100 µL undiluted protoplast only solution (protoplast viability control); 1 x 100 µL protoplast only solution diluted 10^1 in STC buffer (protoplast viability control); 1 x 100 µL protoplast only solution diluted 10^2 in STC buffer (protoplast viability control); 1 x 100 µL protoplast only solution diluted 10^3 in STC buffer (protoplast viability control); 3 x 330 µL protoplasts + 1.5 µg pAN7-1 Hyg^R plasmid (positive control); 3 x 330 µL protoplasts + 2 µg 102KO construct; 1 x 100 µL protoplasts + 102KO construct diluted 10^2 in STC buffer (burst control). The contents of each 5 mL tube was then immediately spread onto pre-poured RG plates (16 mL RG per plate) and incubated at 22°C overnight. The following day plates from groups 2, 3, 4, 5 and 8 were overlaid with 5 mL 0.8% (w/v) agar RG media (no selection), while plates from groups 1, 6 and 7 were overlaid with 5 mL 0.8% (w/v) agar RG media containing 735 µg/mL hygromycin (selection to a final concentration of 150 μgmL^{-1} hygromycin when the final volume of the plate (24.5 mL) was factored in). All plates were then incubated at 22°C for 2 weeks then analyzed. Positive, negative, burst and protoplast viability controls all worked as expected, so 200 individual transformants (colonies which had grown through the selective RG layer) were picked off plates from group 7 and grown on fresh PD + Hyg (150 μgmL^{-1}) plates for further analysis.

2.8.3 Screening for Δ*102* mutants

The 200 transformants selected following transformation with the *EF102* deletion construct were grown at 22°C for 1 week then subcultured to a new PD + Hyg plates. This process of subculturing was repeated a further 3 times to ensure the nuclear purity of selected transformants. Following the final round of subculturing and a week of growth, 1 cm^2 of mycelia was taken from each transformant to be analyzed and ground in a 1.7 mL microfuge tube containing 150 µL PD broth (with 150 µg/mL hygromycin) using a micropestle. Tubes were then incubated at 22°C with shaking (150 rpm) for 3 d. Tubes were then spun for 10 min at 16060 RCF and the supernatant discarded. 150 µL of lysis buffer (100 mM tris, 100 mM EDTA, 1% (w/v) SDS, pH 8.0) was added to each tube and the pellets were ground with micropestles to break up and resuspend the mycelia in each tube, followed by incubation on a hot block at 70°C for 30 min. 150 µL of 5M potassium acetate was then added to each tube, mixed in by inverting tubes 8 times, then incubated on ice for 10 min. Tubes were then centrifuged for 20 min at 16060 RCF and the supernatant transferred to a fresh tube along with 0.7 volumes of

isopropanol and inverted 6 times to mix. Tubes were spun again at 16060 RCF for 15 min and the supernatant discarded. 300 µL of 70% (v/v) ethanol was added to the pellet then the tube was spun for 5 min at 16060 RCF and the supernatant discarded. After air-drying the pellet for 10 min at 37°C it was resuspended in 20 µL of sterile Milli-Q H_2O. 2 µL of this suspension was then used from each of the samples to perform a multiplex PCR reaction (2.6.4.3) with primers 101-2f, 102f, 102r, and 102FULLr, and the resulting products were visualized via gel electrophoresis to identify correct Δ*102* deletion mutants. DNA from putative deletion mutants was then extracted and used to confirm the mutant genotype by Southern blotting *Bcl*I and *Nde*I digests of mutant gDNA and probing with the EF102 deletion construct.

2.9 Bioinformatic methods

2.9.1 Multiple sequence alignments

Initial sequence editing was done using MacVector®10.0.2, and an initial alignment of the 10 different sequences was done using Clustal W (Thompson *et al.*, 1994). The output was manually refined and annotated using the MSA editor "Jalview" (Waterhouse *et al.*, 2009).

2.9.2 DNA motif identification

Comparison of the *perA* and *EF102* promoter query sequences was done using the web-based tool for promoter analysis "Melina II" (Okumura *et al.*, 2007) using the criteria that any motif had to be present in the promoters of both *EF102* and *perA*, highly conserved between all epichloë isolates, and detected at a significantly higher level than expected based on the mononucleotide composition of the tested sequences. The web interfaces for the MEME (Bailey & Elkan, 1994) and Gibbs (Lawrence *et al.*, 1993) search algorithms were then used to confirm results.

3. Epichloë *perA* Locus Comparison

3.1 PCR analysis of the *perA* locus in epichloë

The *perA* locus structure from a range of epichloë isolates was analysed by PCR amplification of genomic DNA (gDNA) using primers designed to the published *E. festucae* Fl1 *perA* locus sequence (Tanaka *et al.*, 2005). All intergenic sequences between *EF101* and *EF105* were amplified, as well as a representative set of intragenic sequences for the genes *EF101* through *EF105* inclusive (Fig. 3.1).

3.1.1 Genes surrounding *perA* are highly conserved within epichloë

Identical sized PCR amplification products were generated for all isolates using primer pairs that amplified within *EF101*, *EF102*, *EF104* and *EF105* (Fig. 3.2), with the exception of the *E. amarillans* E57 amplification product from within *EF102*, which was ~10 bp larger than in all other isolates. The intergenic regions separating *EF101/EF102* and *EF104/EF105* were also highly conserved, with all *E. festucae* strains generating amplification products of identical length, and only minor differences observed among epichloë species including a slightly smaller product for the *EF104-EF105* intergenic region in the two *E. bromicola* strains. No amplification product was produced to the *EF101/EF102* intergenic sequence in *E. typhina* E425.

3.1.2 *PerA* and flanking intergenic sequences are highly variable within epichloë

Amplification products of the same size from the A1-C1 and A2 domains of *perA* were generated for most isolates (Fig. 3.2), except no products were amplified from *E. bromicola* E799 and *E. elymi* E56 for A1-C1. Amplification products from within the R2 domain of *perA* showed considerable variation within *E. festucae*. Strains Fl1, Frc7 and Frr1 produced products approximately 1000 bp long, whereas products of strains E189, Frc5, and Fr1 were 850 bp in size. Fg1 produced both the 850 and 1000-bp products. No product could be amplified from Frc7 with primer pair p9f/103-4Cr.

PCR products from *Epichloë spp.* other than *E. festucae* showed significant variations in the size of the *perA-EF104* intergenic sequence when compared to Fl1 (Fig. 3.2), but regions amplified within *perA* were of the same size. Exceptions to this result were *E.*

elymi WWG1, which did not amplify across the *perA*-EF104 intergenic region, and *E. bromicola* E799, which failed to amplify with all primer combinations containing binding sites within *perA*, except p9f/p9r. The intergenic region between *EF102* and *perA* was similarly variable. *E. festucae* strains Fl1, Frc7 and Frr1 produced products of 1.9 kb, while Fg1, E189, Frc5 and Frr1 failed to amplify. All other epichloë isolates produced a variety of products larger than that observed for Fl1.

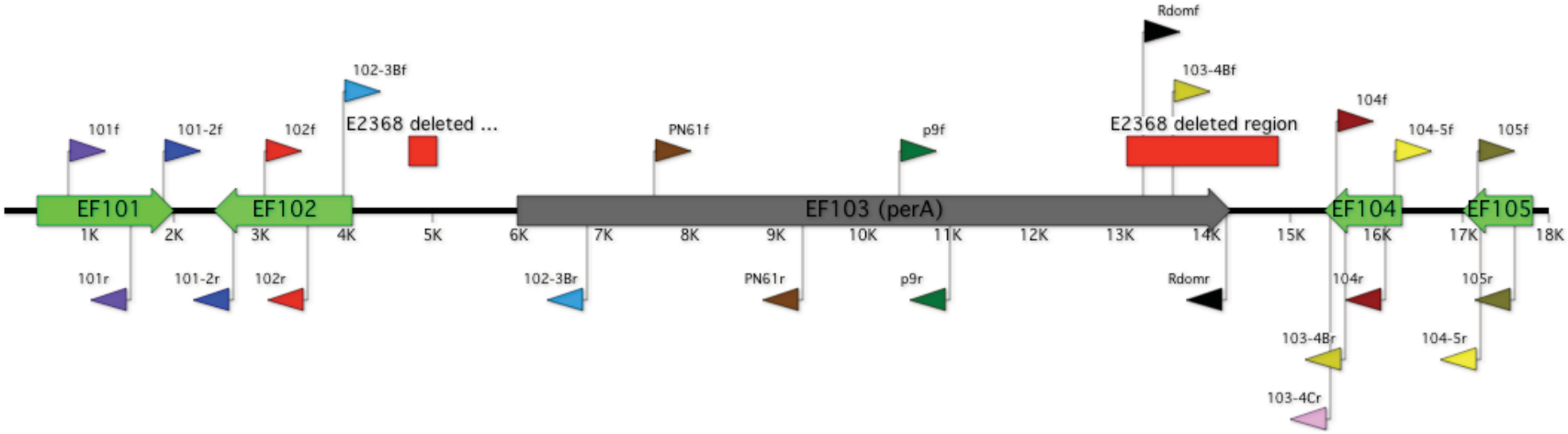
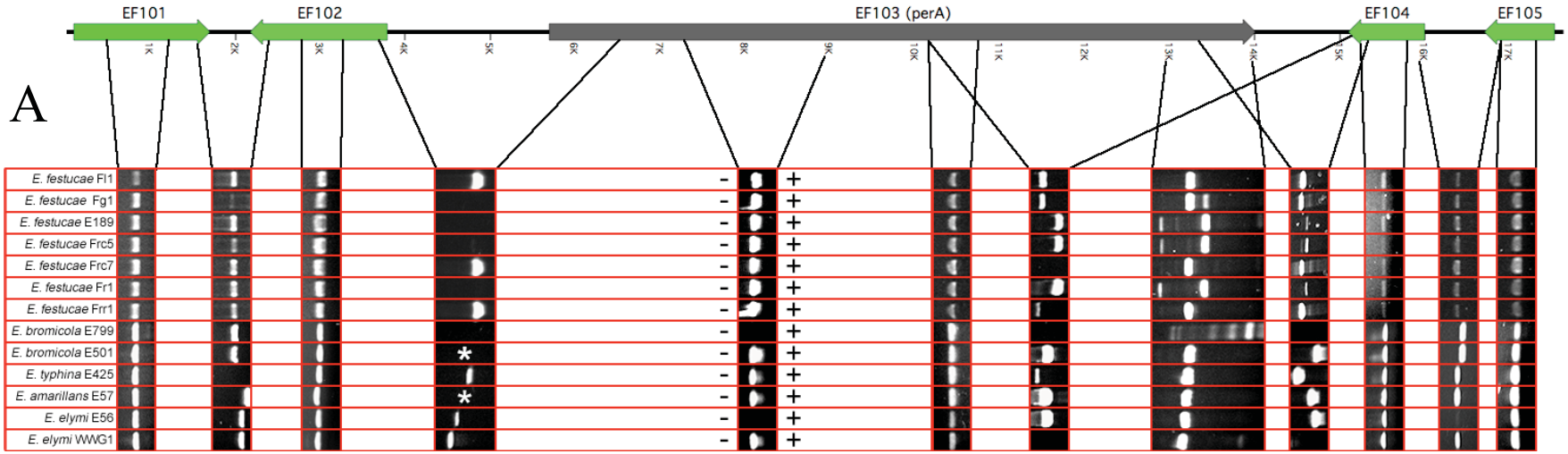


Figure 3.1 Gene map of the *perA* locus with PCR primers annotated

Gene map of the *E. festucae* Fl1 *perA* locus annotated with the primers used for PCR screening. Also annotated in red are the two regions in Fl1 sequence which are not present in *E. festucae* strain E2368.



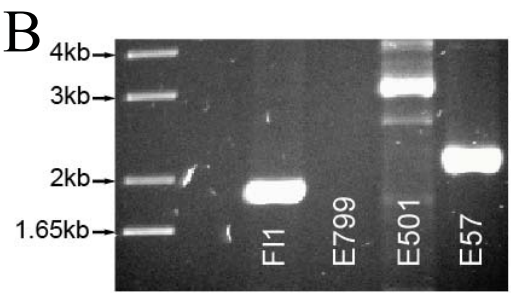


Figure 3.2 PCR based comparative analysis of the *perA* locus within epichloë
A. Composite image of the PCR products obtained when gDNA from each of the labelled epichloë isolates was amplified using primers designed to the indicated regions in *E. festucae* Fl1. Gels are orientated with cathode to the left (-) and anode to the right (+). Lanes marked by a white asterisk indicate that amplification of this region was successful using primers other than those shown here (see **B**). PCR primer pairs used are (from left to right): 101f/r, 101-2f/r, 102f/r, 102-3Bf/r, PN61f/r, p9f/r, p9f/103-4Cr, Rdomf/r, 103-4Bf/r, 104f/r, 104-5f/r, 105f/r. **B.** PCR products amplified from the intergenic region between *EF102* and *perA* in *E. bromicola* E501 and *E. amarillans* E57 using primer pair 102-3Df/102-3Dr.

3.2 Southern blot analysis of the *perA* locus in epichloë

Four Southern blots (Figs 3.3-3.6) were generated using gDNA from the different *E. festucae* wild-type isolates (excluding E2368) digested by one of four different restriction enzymes, and a fifth blot was made using gDNA from *E. festucae* Fl1, *E. bromicola* E799, *E bromicola* E501, *E. typhina* E425 and *E. elymi* WWG1 (Fig. 3.7). Each of these Southern blots was then sequentially hybridised to a *perA*-, *EF102*- and *EF104*-containing probe and developed. A sixth blot was made using gDNA from *E. festucae* Fl1 and E2368, which was probed with the *perA*- and *EF102*-containing probes (Fig. 3.8).

3.2.1 Southern blot analysis of the *perA* locus reveals considerable variation within *E. festucae*

Analysis of the four Southern blots (Figs. 3.3-3.6) showed *E. festucae* strains Fl1, Frc7 and Frr1 produced identical banding patterns in all cases, with the exception of the *Nde*I blot (Fig. 3.4), in which the Fl1 banding pattern does not match that predicted from the known Fl1 sequence of this region. These results demonstrate linkage between *perA* and both *EF102*/*EF104* for these strains. *E. festucae* strains E189 and Fr1 produced identical banding patterns on all blots which differ significantly to those observed for Fl1, including a reduction in the size of bands spanning the 3' end of *perA* and/or the intergenic region between *perA* and *EF104*, and a large increase in the size of bands spanning the *EF102*/*perA* intergenic region. E189 was also shown to have an identical banding pattern to E2368 (Fig. 3.8). These results demonstrate linkage between *perA* and *EF104*, but not *EF102* and *perA*, for these strains. Frc5 showed a very similar banding pattern to E189/Fr1, however in the *Nde*I digest blot (Fig. 3.4) there was an increase in size of the *EF102*-containing fragment, and in the *Bcl*I digest blot (Fig. 3.6) the smallest fragment observed in E189/Fr1 was missing. An Frc5 sample was not present in the *Bam*HI digest blot, and instead was replaced by a sample of unknown identity that mirrors the Fl1 banding pattern. Fg1 showed banding patterns between the two extremes, with an increase in the size of bands representing the *EF102-perA* intergenic region, but no difference in the 3' end of *perA*. These results demonstrate linkage between *perA* and *EF104,* but not *EF102* and *perA*, for Fg1.

3.2.2 Southern blot analysis shows variation in the intergenic regions flanking *perA* within the ephichloë species tested

Each of the different epichloë isolates tested showed a different banding pattern to *E. festcuae* Fl1 for the fragments spanning the intergenic regions flanking *perA* (Fig. 3.7), however all except *E. bromicola* E799 showed an identical *perA* internal fragment to Fl1. Cross-hybridisation to the same bands between the different probes demonstrates linkage between *perA* and both *EF102/EF104* for all isolates tested.

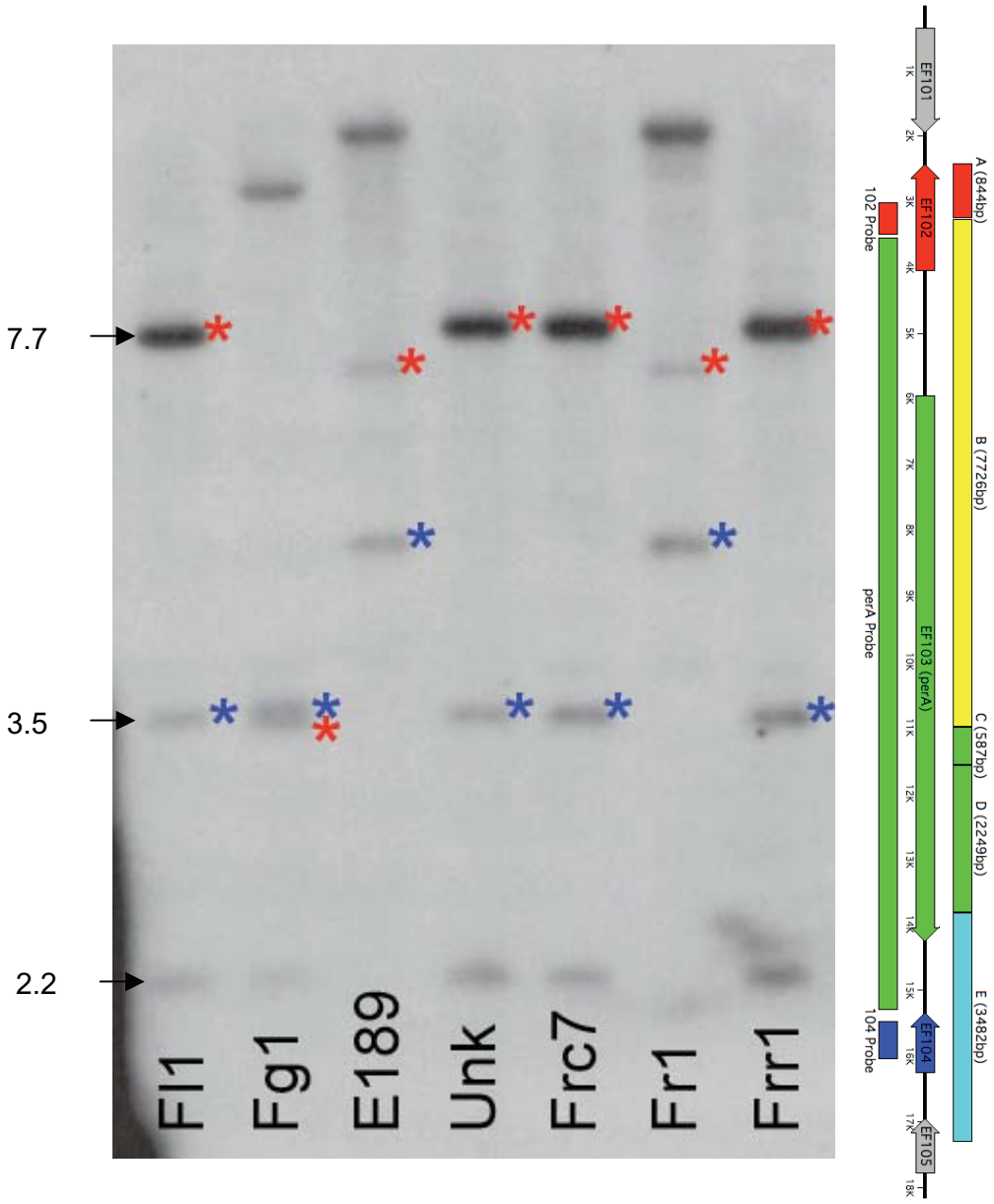


Figure 3.3 Southern hybridisation of *Bam*HI digested gDNA from various *E. festucae* isolates

Autoradiograph made by hybridizing a Southern blot of *Bam*HI digested gDNA from six different *E. festucae* strains (Fl1, Fg1, E189, Frc7, Fr1, Frr1) and one unknown *E. festucae* sample (Unk) with a [^{32}P]-dCTP labelled plasmid containing *perA* in a 12-kb *E. festucae* Fl1 fragment (Tanaka *et al.*, 2005). Any band marked with an asterisk indicates that band also lit up when probed with a PCR-amplified product from inside the *EF102* (red) or *EF104* (blue) genes. An annotated map of the *perA* locus in *E. festucae* Fl1 is shown to the right. The bands above the gene map show the expected fragment sizes when Fl1 DNA is cut with *Bam*HI. The colour of each band indicates whether it is expected to hybridise to a *perA* probe (green), *EF102* probe (red), or *EF104* probe (blue). Yellow/cyan bands indicate that the fragment is expected to hybridise to both *EF102*/*perA* probes or *perA*/*EF104* probes, respectively. The probe used is annotated beneath the gene map.

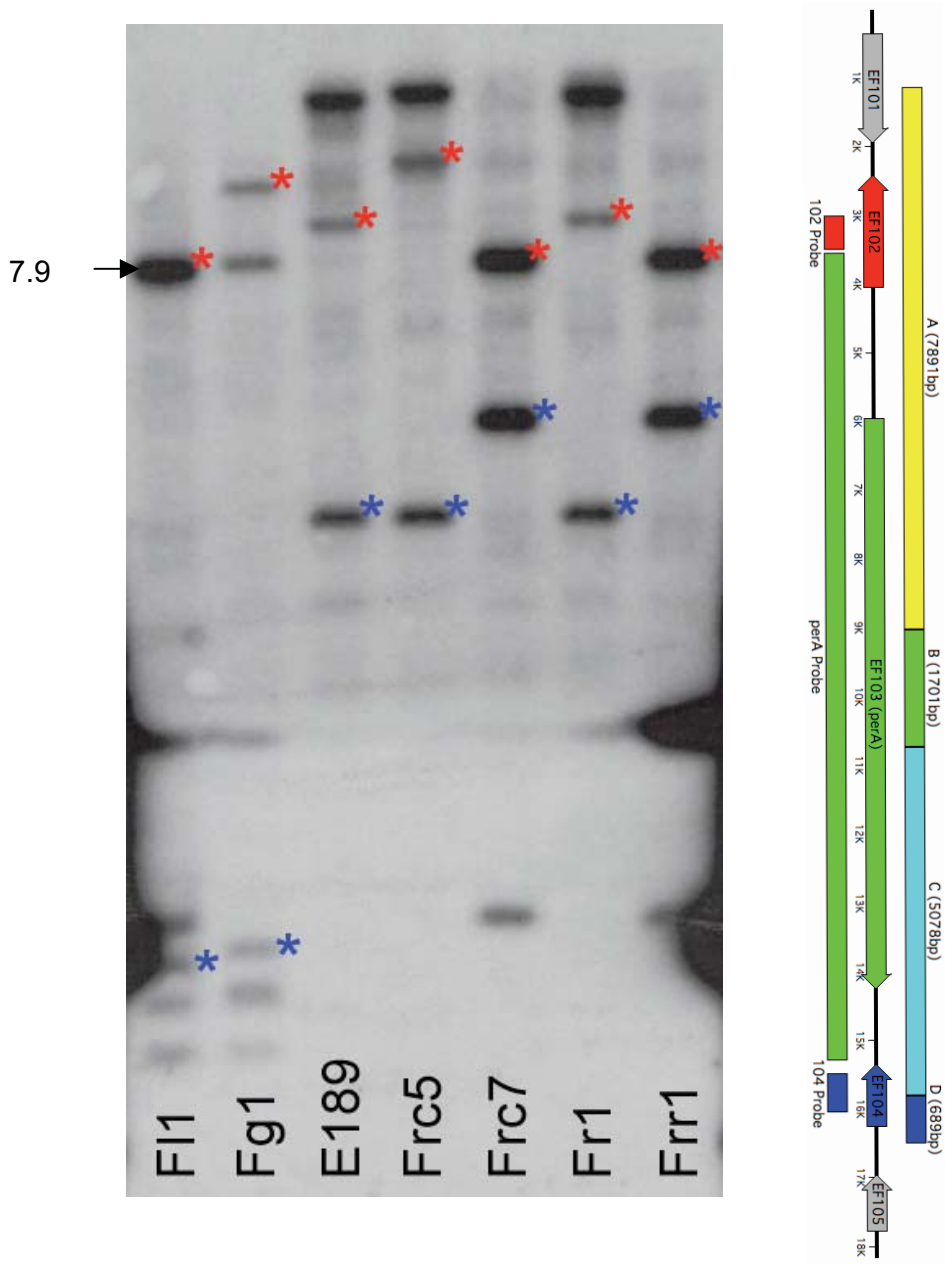


Figure 3.4 Southern hybridisation of *Nde*I digested gDNA from various *E. festucae* isolates

Autoradiograph made by hybridizing a Southern blot of *Nde*I digested gDNA from seven different *E. festucae* strains (Fl1, Fg1, E189, Frc5, Frc7, Fr1, Frr1) with a [^{32}P]-dCTP labelled plasmid containing *perA* in a 12-kb *E. festucae* Fl1 fragment (Tanaka *et al.*, 2005). Any band marked with an asterisk indicates that band also lit up when probed with a PCR amplified product from inside the *EF102* (red) or *EF104* (blue) genes. An annotated map of the *perA* locus in *E. festucae* Fl1 is shown to the right. The bands above the gene map show the expected fragment sizes when Fl1 DNA is cut with *Nde*I. The colour of each band indicates whether it is expected to hybridise to a *perA* probe (green), *EF102* probe (red), or *EF104* probe (blue). Yellow/cyan bands indicate that the fragment is expected to hybridise to both *EF102*/*perA* probes or *perA*/*EF104* probes, respectively. The probe used is annotated beneath the gene map.

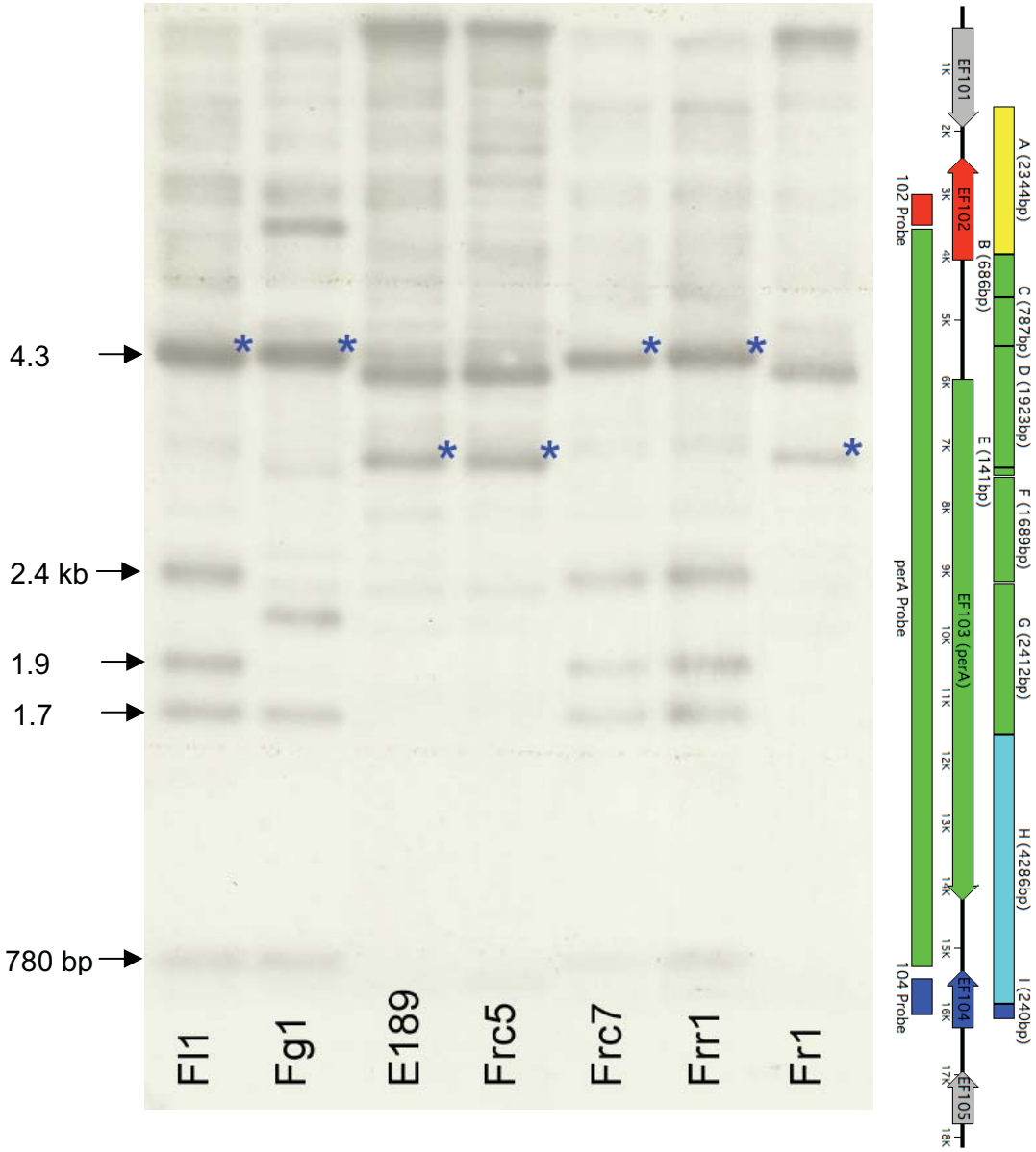


Figure 3.5 Southern hybridisation of *Sal*I digested gDNA from various *E. festucae* isolates

Autoradiograph made by hybridizing a Southern blot of *Sal*I digested gDNA from seven different *E. festucae* strains (Fl1, Fg1, E189, Frc5, Frc7, Fr1, Frr1) with a [^{32}P]-dCTP labelled plasmid containing *perA* in a 12-kb *E. festucae* Fl1 fragment (Tanaka *et al.*, 2005). Any band marked with an asterisk indicates that band also lit up when probed with a PCR amplified product from inside the *EF102* (red) or *EF104* (blue) genes. An annotated map of the *perA* locus in *E. festucae* Fl1 is shown to the right. The bands above the gene map show the expected fragment sizes when Fl1 DNA is cut with *Sal*I. The colour of each band indicates whether it is expected to hybridise to a *perA* probe (green), *EF102* probe (red), or *EF104* probe (blue). Yellow/cyan bands indicate that the fragment is expected to hybridise to both *EF102*/*perA* probes or *perA*/*EF104* probes, respectively. The probe used is annotated beneath the gene map.

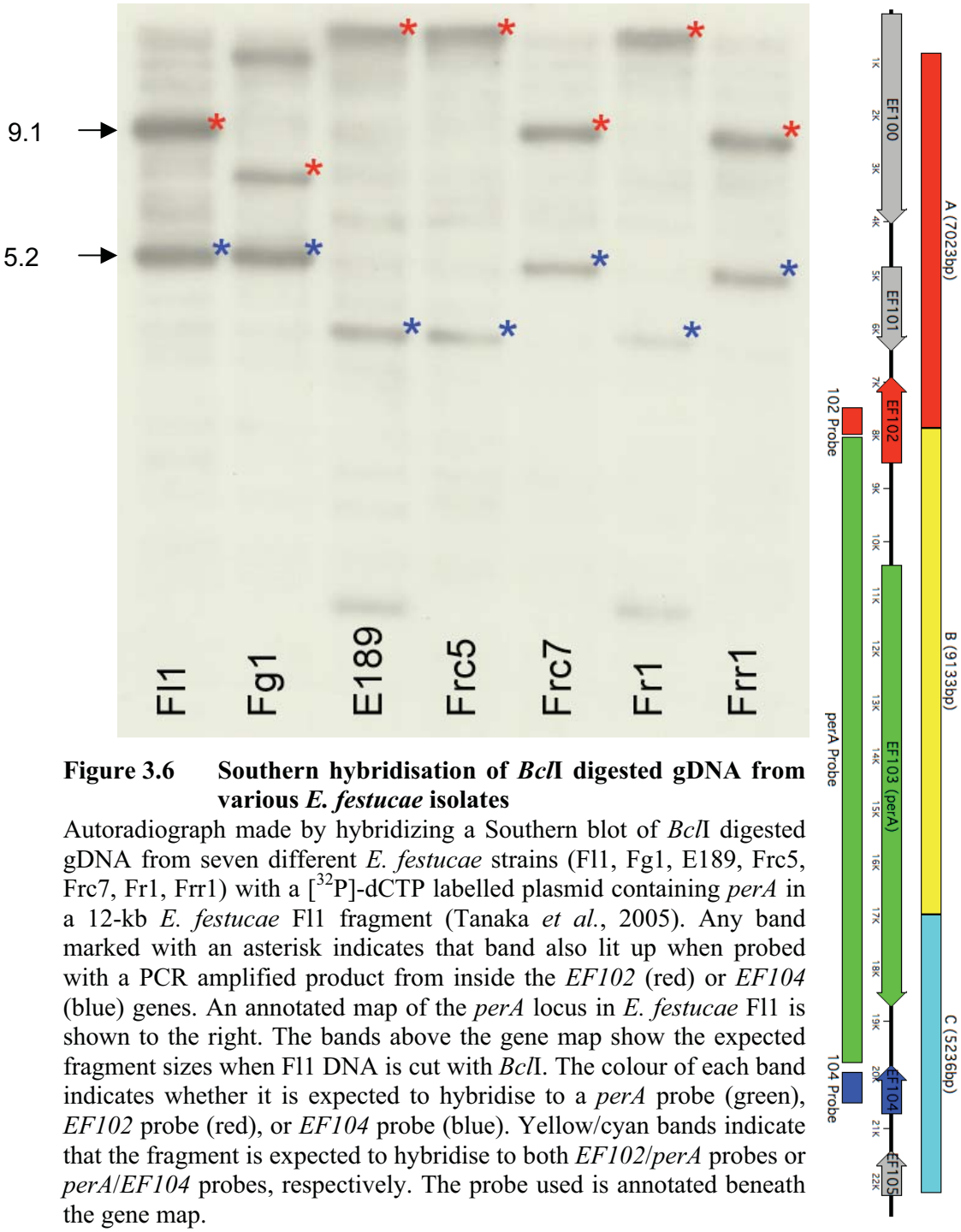


Figure 3.6 Southern hybridisation of *Bcl*I digested gDNA from various *E. festucae* isolates

Autoradiograph made by hybridizing a Southern blot of *Bcl*I digested gDNA from seven different *E. festucae* strains (Fl1, Fg1, E189, Frc5, Frc7, Fr1, Frr1) with a [^{32}P]-dCTP labelled plasmid containing *perA* in a 12-kb *E. festucae* Fl1 fragment (Tanaka *et al.*, 2005). Any band marked with an asterisk indicates that band also lit up when probed with a PCR amplified product from inside the *EF102* (red) or *EF104* (blue) genes. An annotated map of the *perA* locus in *E. festucae* Fl1 is shown to the right. The bands above the gene map show the expected fragment sizes when Fl1 DNA is cut with *Bcl*I. The colour of each band indicates whether it is expected to hybridise to a *perA* probe (green), *EF102* probe (red), or *EF104* probe (blue). Yellow/cyan bands indicate that the fragment is expected to hybridise to both *EF102*/*perA* probes or *perA*/*EF104* probes, respectively. The probe used is annotated beneath the gene map.

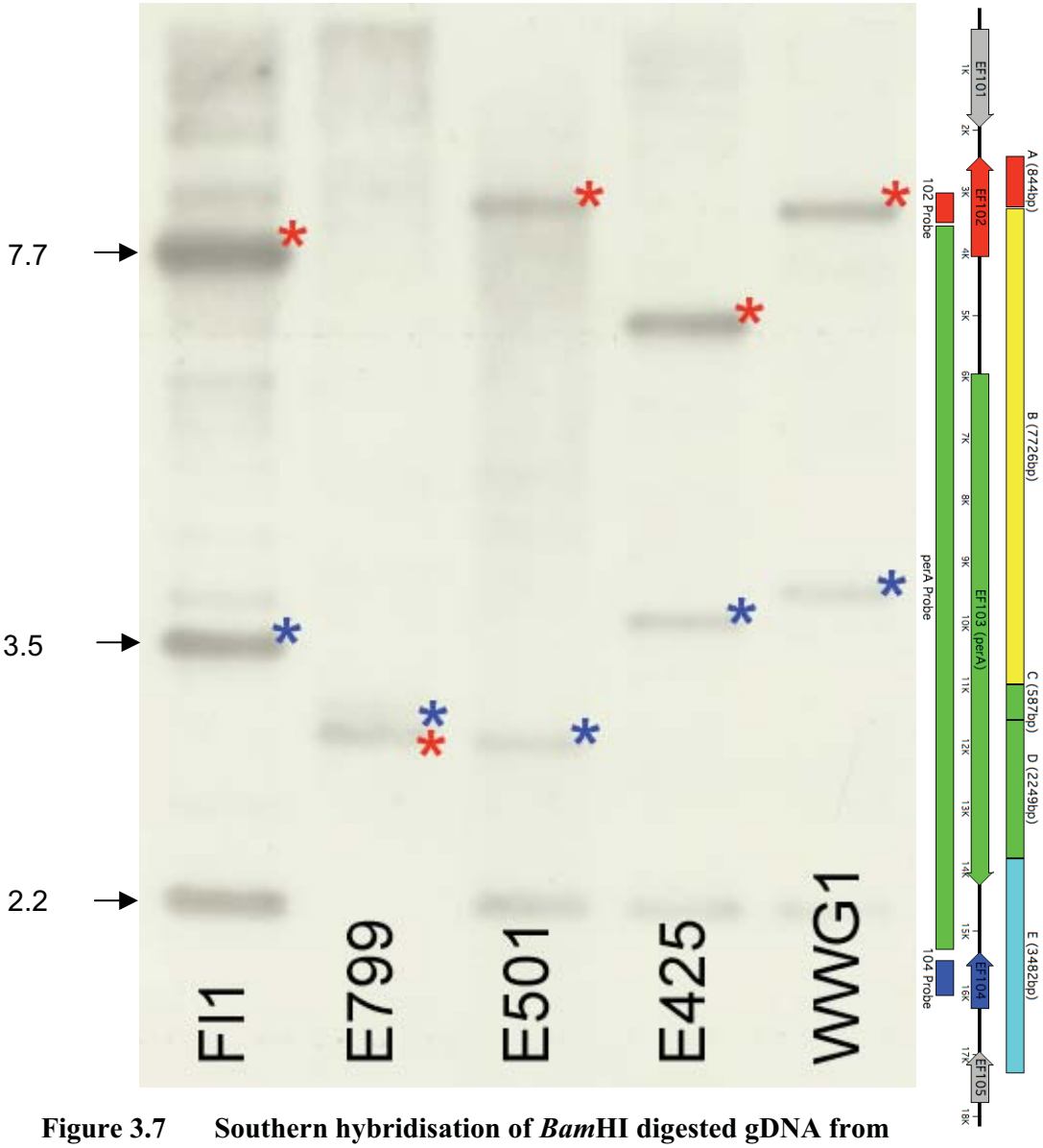


Figure 3.7 Southern hybridisation of *Bam*HI digested gDNA from various epichloë isolates

Autoradiograph made by hybridizing a Southern blot of BamHI digested gDNA from six different epichloë species (*E. festucae* Fl1, *E. bromicola* E799/E501, *E. typhina* E425 and *E. elymi* WWG1) to a [^{32}P]-dCTP labelled plasmid containing *perA* in a 12-kb *E. festucae* Fl1 fragment (Tanaka *et al.*, 2005). Any band marked with an asterisk indicates that band also lit up when probed with a PCR amplified product from inside the *EF102* (red) or *EF104* (blue) genes. An annotated map of the *perA* locus in *E. festucae* Fl1 is shown to the right. The bands above the gene map show the expected fragment sizes when Fl1 DNA is cut with *Bcl*I. The colour of each band indicates whether it is expected to hybridise to a *perA* probe (green), *EF102* probe (red), or *EF104* probe (blue). Yellow/cyan bands indicate that the fragment is expected to hybridise to both *EF102*/*perA* probes or *perA*/*EF104* probes, respectively. The probe used is annotated beneath the gene map.

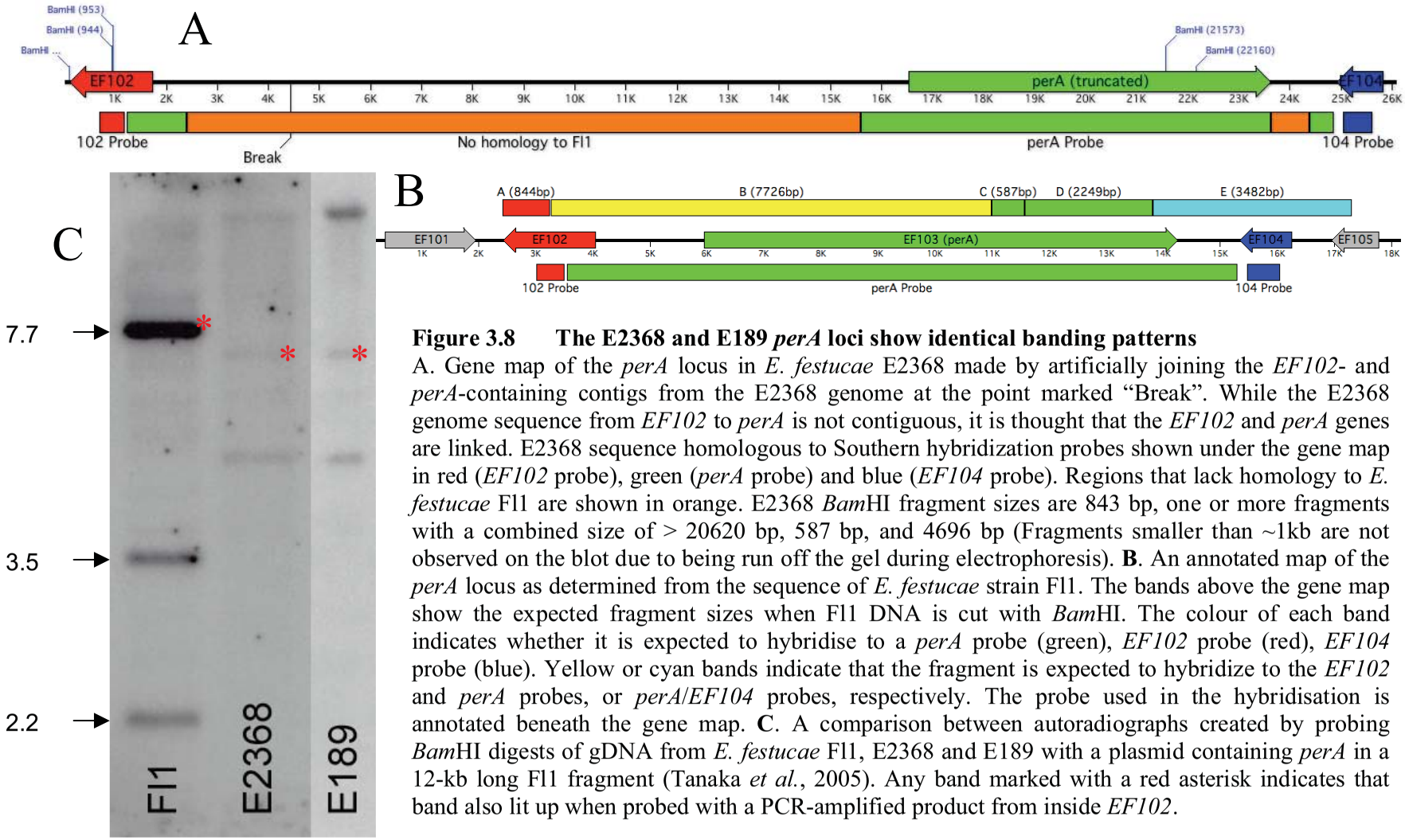


Figure 3.8 The E2368 and E189 *perA* loci show identical banding patterns

A. Gene map of the *perA* locus in *E. festucae* E2368 made by artificially joining the *EF102*- and *perA*-containing contigs from the E2368 genome at the point marked "Break". While the E2368 genome sequence from *EF102* to *perA* is not contiguous, it is thought that the *EF102* and *perA* genes are linked. E2368 sequence homologous to Southern hybridization probes shown under the gene map in red (*EF102* probe), green (*perA* probe) and blue (*EF104* probe). Regions that lack homology to *E. festucae* Fl1 are shown in orange. E2368 *Bam*HI fragment sizes are 843 bp, one or more fragments with a combined size of > 20620 bp, 587 bp, and 4696 bp (Fragments smaller than ~1kb are not observed on the blot due to being run off the gel during electrophoresis). **B**. An annotated map of the *perA* locus as determined from the sequence of *E. festucae* strain Fl1. The bands above the gene map show the expected fragment sizes when Fl1 DNA is cut with *Bam*HI. The colour of each band indicates whether it is expected to hybridise to a *perA* probe (green), *EF102* probe (red), *EF104* probe (blue). Yellow or cyan bands indicate that the fragment is expected to hybridize to the *EF102* and *perA* probes, or *perA*/*EF104* probes, respectively. The probe used in the hybridisation is annotated beneath the gene map. **C**. A comparison between autoradiographs created by probing *Bam*HI digests of gDNA from *E. festucae* Fl1, E2368 and E189 with a plasmid containing *perA* in a 12-kb long Fl1 fragment (Tanaka *et al.*, 2005). Any band marked with a red asterisk indicates that band also lit up when probed with a PCR-amplified product from inside *EF102*.

3.3 Peramine production analysis of *E. festucae* isolates

Of the seven *E. festucae* strains analysed only Fl1 has been tested for peramine production. Endophyte-free perennial ryegrass (*Lolium perenne)* seedlings were infected with strains Fg1, E189, Frc5, Frc7, Fr1 and Frr1, and infection was confirmed by immunoblotting. Infected plants were repotted and grown in a greenhouse until many tillers were visible. Several tillers were excised from each plant and sent to AgResearch for peramine analysis. The results (Table 3.1) showed that of the six strains tested only Frc7 and Frr1 were peramine producers. All tested plants were reconfirmed for infection by immunoblotting post-testing.

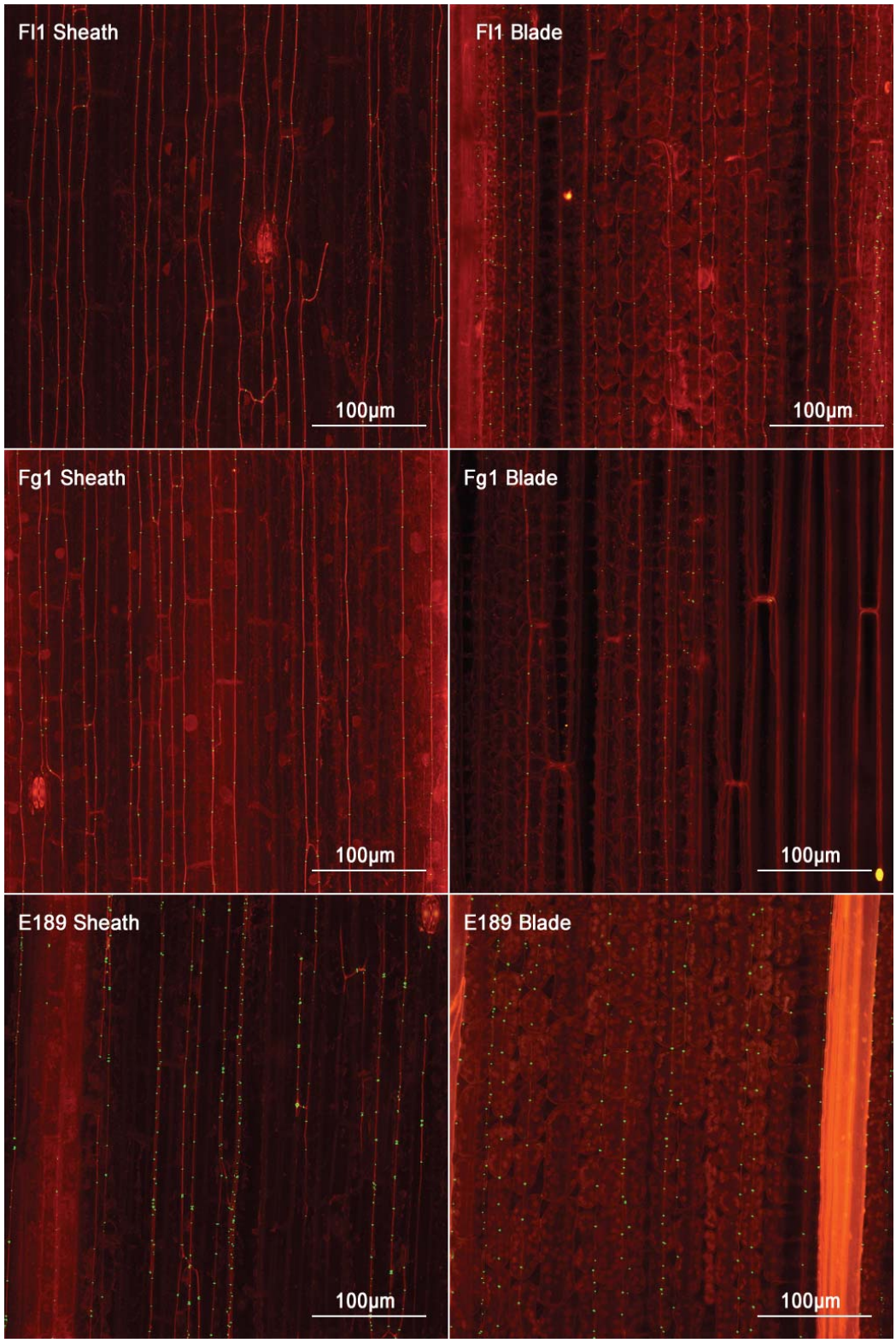
3.4 Confocal analysis of *E. festucae* infected ryegrass samples

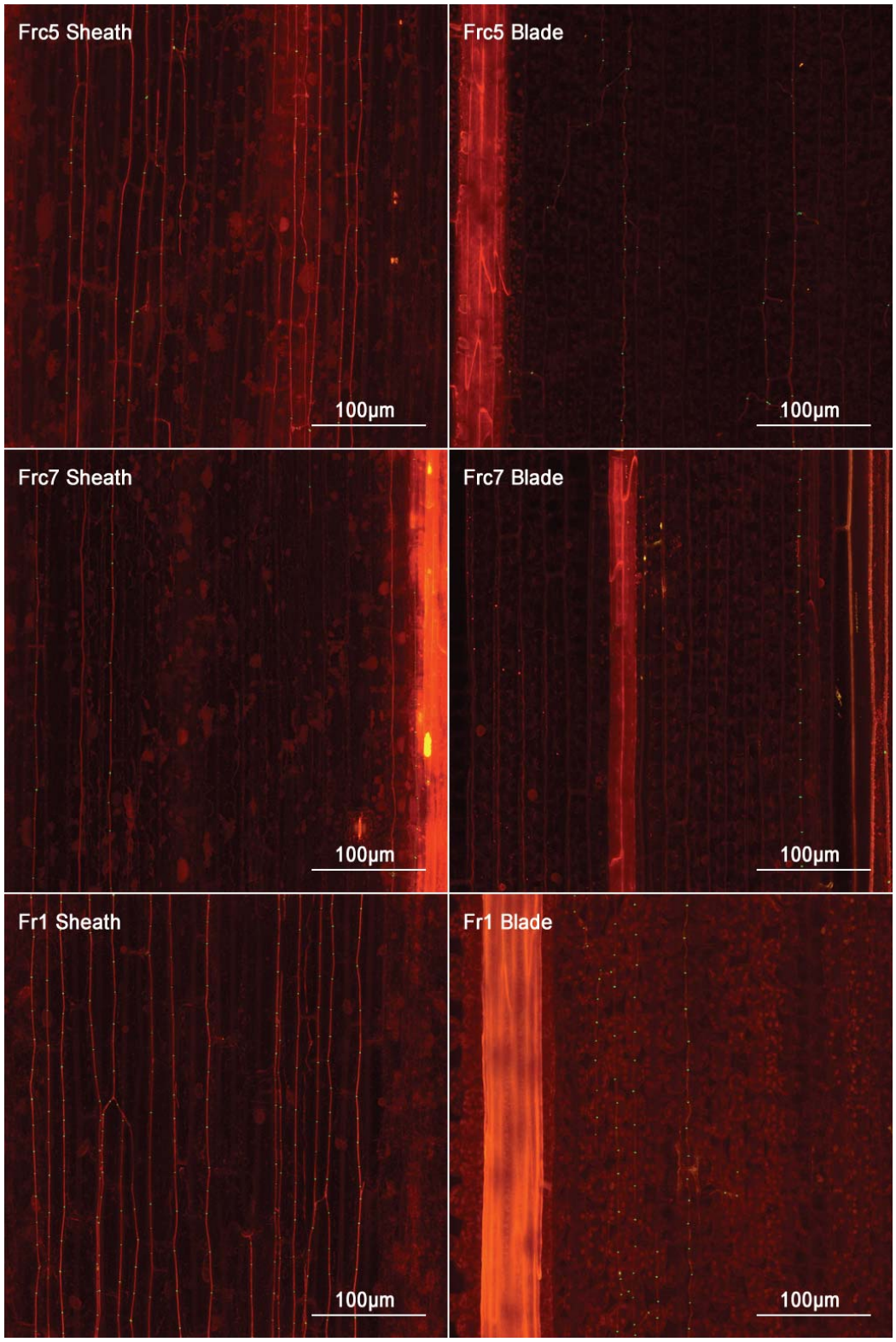
Lolium perenne is not the natural host of the *E. festucae* isolates used in this study, which normally inhabit specific species of the *Festuca* genus, so confocal microscopy was used to investigate the stability of the plant-fungal interaction. Samples were taken from the leaf sheath and blade of perennial ryegrass plants infected with the *E. festucae* strains Fl1, Fg1, E189, Frc5, Frc7, Fr1 and Frr1, stained with a mixture of Alexafluor and aniline blue, and examined by confocal microscopy to examine fungal colonisation of the leaf tissue. Confocal microscope images were taken of hyphae located just below the epidermal cells, with a stack of at least 8 images taken with a Z step size of 2 μm. The images show that fungal hyphae were detected in both the leaf sheath and blade for all samples (Fig. 3.9). The concentration of fungal hyphae appeared to be reduced in both sheath and blade tissues of plants infected with Frc5 and Frc7 when compared with other isolates. Hyphae in Frc7, in particular, proved very difficult to locate. Conversely instances of multiple hypha located in a single intercellular space appeared to be a common occurrence in E189-infected samples.

Table 3.1 Whole tiller peramine concentration in plants infected by different *E. festucae* isolates

***Epichloë festucae* strain**	**Peramine conc. (ppm) [a, b, c]**
Fg1	ND
E189	ND
Frc5	ND
Frc7	19.5
Fr1	ND
Frr1	137.2

[a] As determined by combined liquid chromatography/UV spectroscopy
[b] Limit of detection = 0.1 ppm, Limit of quantification = 0.5 ppm
[c] ND = Not Detected


Fl1 Sheath
100μm
Fl1 Blade
100μm
Fg1 Sheath
100μm
Fg1 Blade
100μm
E189 Sheath
100μm
E189 Blade
100μm


Frc5 Sheath
100μm
Frc5 Blade
100μm
Frc7 Sheath
100μm
Frc7 Blade
100μm
Fr1 Sheath
100μm
Fr1 Blade
100μm

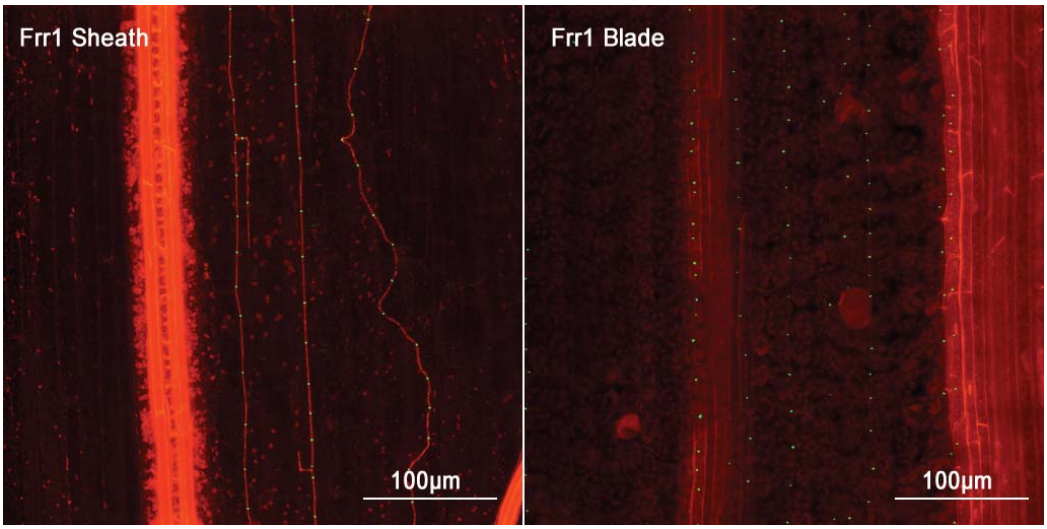


Figure 3.9 Confocal microscopy of *E. festucae* infected perennial ryegrass samples

Representative confocal microscope images of perennial ryegrass infected with different *E. festucae* isolates. Samples were taken from the leaf sheath and blade tissue of 7 month old plants grown in the greenhouse and stained with Alexafluor and aniline blue to identify fungal hyphae (red) and septa (green). The large, bright red structures observed in some images are host vascular bundles. Images taken are of hyphae residing immediately below the host epidermal cells at x40 magnification. Each image is a composite of 8 images, each taken 2 µm apart, resulting in a final image depth of 14 µm.

4. Analysis of the *perA* Promoter

4. Analysis of the *EF102-perA* intergenic sequence in epichloë

The 5' coding region of both *EF102* and *perA*, along with the intergenic sequence separating them, was PCR amplified using a high-fidelity DNA polymerase and sequenced for the isolates *E. festucae* Fl1/Frc7/Frr1, *E. bromicola* E501, *E. typhina* E425, *E amarillans* E57 and *E. elymi* E56/WWG1. PCR amplification of this region required the addition of DMSO (a secondary structure inhibitor) and an extended initial denaturation period. C. Young provided sequence of this region from the genomes of *E. typhina* E8 and *E. brachyelytri* E4804, and sequencing a PCR amplification product from this region completed the missing E8 sequence. An initial alignment of the 10 different sequences was done using Clustal W (Thompson *et al.*, 1994), and the output was manually refined and annotated using the multiple sequence alignment (MSA) editor "Jalview" (Waterhouse *et al.*, 2009).

4.1 Refining the *perA* translation start site

The multiple sequence alignment (MSA) showed that *E. brachyelytri* E4804 and *E. typhina* strains E8 and E425 had large deletions immediately following the annotated *perA* start codon (Tanaka et al. 2005), with the ATG itself being disrupted in the *E. typhina* strains (Fig. 4.1). The MSA also showed that that there were frameshift mutations downstream of the annotated start codon in *E. festucae* strains Frc7 and Frr1, which are known to produce peramine (Table 3.1). On the basis of this multiple species alignment a new start codon was proposed, 183 bp downstream from the original. There were no sequence changes downstream of this new start site that alter the reading frame of PerA, and moving the start site downstream did not disrupt any conserved domains of PerA. The sequence around the new start site (ACCA**AUG**GUC) also fits reasonably well with the *Neurospora crassa* Kozak consensus sequence of CAMM**AUG**GCU (Edelmann & Staben, 1994), and the draft epichloë Kozak sequence of CAMM**AUG**RMS obtained by aligning the sequences surrounding 41 *E. festucae/N. lolii* start codons (Fig. 4.2). The entire MSA is included in the Appendix (7.3.1).

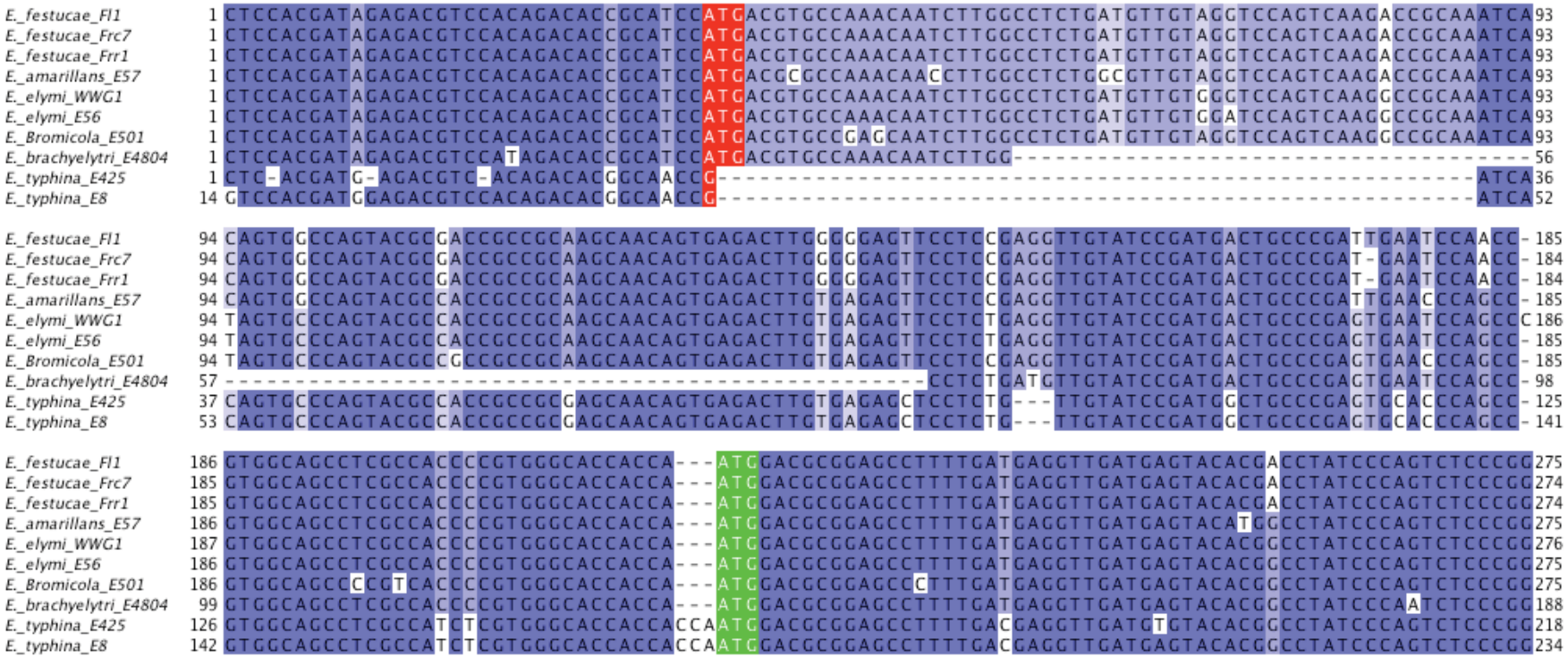


Figure 4.1 Refining the location of the *perA* start codon

DNA multiple sequence alignment of the region surrounding the *perA* start codon in 10 different *Epichloë* isolates. The original start codon (Tanaka *et al.*, 2005) is shaded red, and the refined start codon in green. Note the large deletions present in/after the original start codon in isolates *E. brachyelytri* E4804 and *E. typhina* E425 and E8, and the frameshift mutations present at position 173 in the known peramine-producing *E. festucae* strains Frc7 and Frr1.

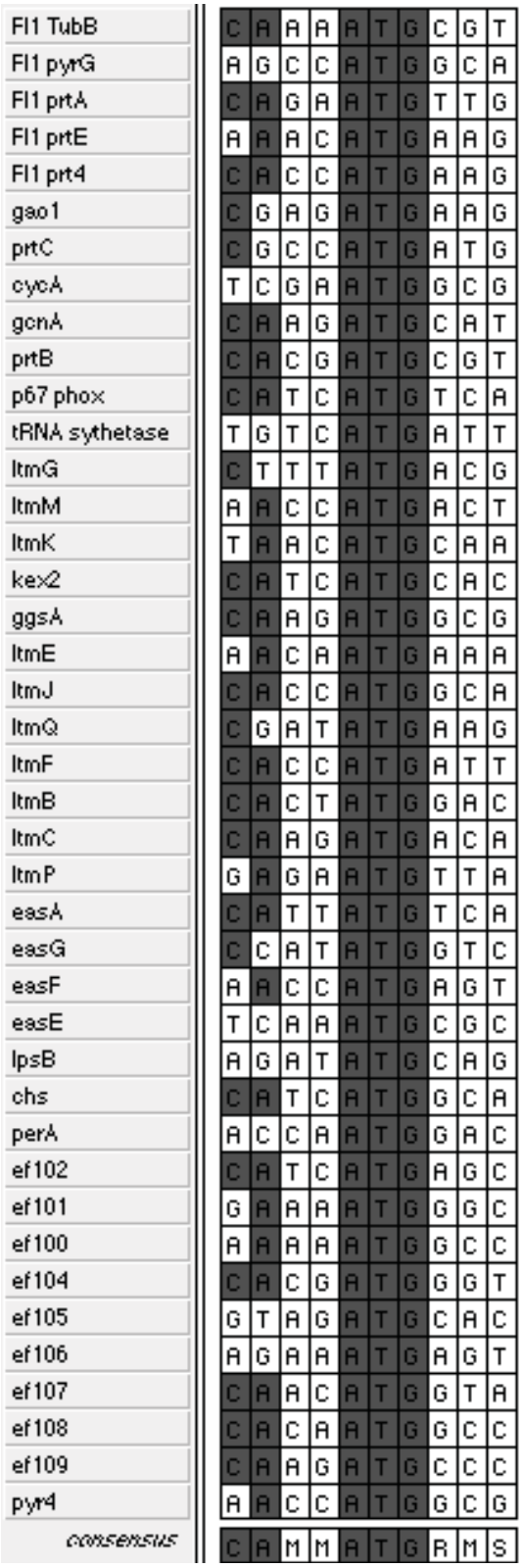


Figure 4.2 Consensus Kozak sequence for epichloë

The sequences surrounding 41 start codons from *E. festucae* and *N. lolii* genes were aligned to determine a draft consensus Kozak sequence for epichloë.

4.2 Establishing the transcription start sites of *EF102* and *perA*

An analysis of the transcriptome data set for *E. festucae* Fl1 growing *in planta* (Eaton *et al.*, 2010) showed that *perA* mRNA reads started 534 bp upstream of the start codon in Fl1 (Fig. 4.3), and *EF102* mRNA reads started 121 bp upstream of the start codon in Fl1. The sequence between the transcription start sites and start codons of *perA/EF102* was relatively highly conserved between the different isolates (Fig. 4.4), with the exception of several indels shared only by a subset of genetically similar isolates. This conservation was maintained to a lesser extent for a further 496 bp upstream of the transcription initiation site of *EF102* in Fl1, and for 423 bp in *perA* in Fl1, after which conservation between the strains breaks down completely, with the presence of large insertions, shared only by a subset of phylogenetically close isolates. These blocks of conserved but non-transcribed sequence in the *EF102/perA* promoters were used for subsequent motif searches.

4.3 *EF102* and *perA* promoters share a common DNA motif

As *EF102* encodes a potential peramine transporter, and is co-regulated with *perA*, a comparison of the *perA* and *EF102* promoter query sequences (Fig. 4.4) was done using the web-based tool for promoter analysis "Melina II" (Okumura *et al.*, 2007). The high conservation of the *perA* and *EF102* queries between isolates meant that motif search algorithms could not differentiate between actual functional sites and conservation due to the evolutionary proximity of the isolates. To compensate for this limitation strict criteria were applied to remove spurious results. Any motif had to be present in the promoters of both *EF102* and *perA*, highly conserved between all epichloë isolates, and detected at a significantly higher level than expected based on the mononucleotide composition of the tested sequences. Motifs that occurred multiple times in each promoter sequence were further analysed. Many of the motifs identified when these criteria were applied were examples of short tandem repeats that were present in both promoters, and the application of the further criterium of proximity to the transcription start site eliminated many of these matches. Application of all these criteria narrowed the list of potential regulatory candidates to a single 7 bp long DNA motif (Fig. 4.5) originally identified using both the MEME (Bailey & Elkan, 1994) and Gibbs (Lawrence *et al.*, 1993) search algorithms. This motif was present 3 times in the *EF102*

promoter, at 112, 135 and 149 bp upstream of the *EF102* transcription start site in Fl1 (Fig. 4.7), and twice in the *perA* promoter, at 73 and 148 bp upstream of the *perA* transcription start site in Fl1. The central 5 bp of this motif was very strongly conserved between these 5 sites, with only a single SNP change observed in the element located 148 bp upstream of *perA* in *E. amarillans* E57. There are two additional copies of this 5 bp motif in the *EF102* promoter, at 60 and 106 bp upstream of the transcription start site in Fl1 (Fig. 4.7). Given the query sequence background letter frequency of: A = 0.2536, G = 0.2770, T = 0.2107 and C = 0.2587, the expected frequency of this motif in the combined query length of 9350 bp was 0.6049. The observed frequency of 50 was significantly higher than this, with a probability of only 1.214×10^{-11} of occurring by chance. Conservation between the two motif sites in the *perA* promoter was higher when compared to the three sites in the *EF102* promoter, with sequence conservation retained when the motif search length was extended up to 10 bp long, as opposed to only 7 bp when the *EF102* sequences are included (Fig. 4.5).

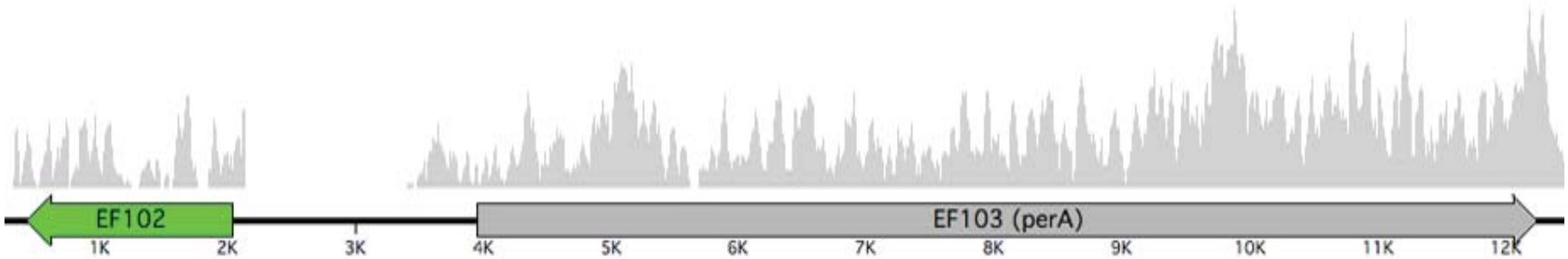


Figure 4.3 Transcriptome reads showing the transcription start sites of *EF102* and *perA*

Histogram representing the number of wild type *E. festucae* Fl1 transcriptome reads mapped against *EF102*, *perA* and the intervening sequence, showing that the approximate transcription start site for *EF102* and *perA* is 121 and 534 bp upstream of the translation start site, respectively (Eaton *et al.*, 2010).

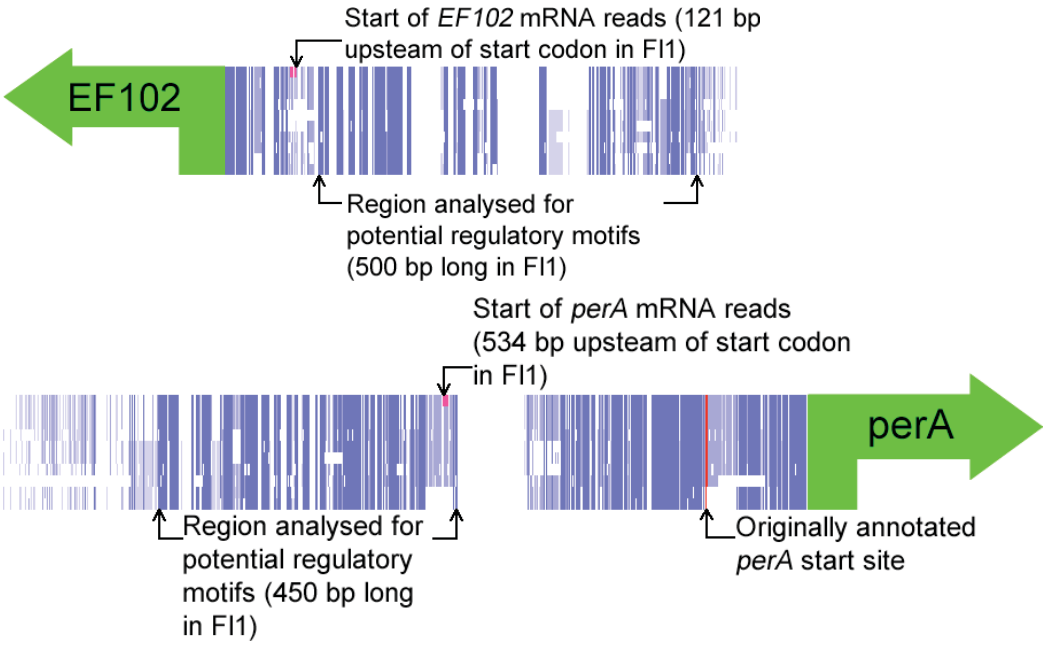


Figure 4.4 Sequence conservation in the promoters of *perA* and *EF102*
Graphical representation of a sequence alignment of the *perA* and *EF102* putative promoter regions from 10 different epichloë isolates. The intensity of the blue colouring indicates the strength of sequence conservation, with conservation between at least five isolates required for colouring. The regions of the *perA* and *EF102* promoters used to search for conserved DNA motifs are annotated, along with the location at which the first mRNA reads for each gene appear (approximate transcription start site).

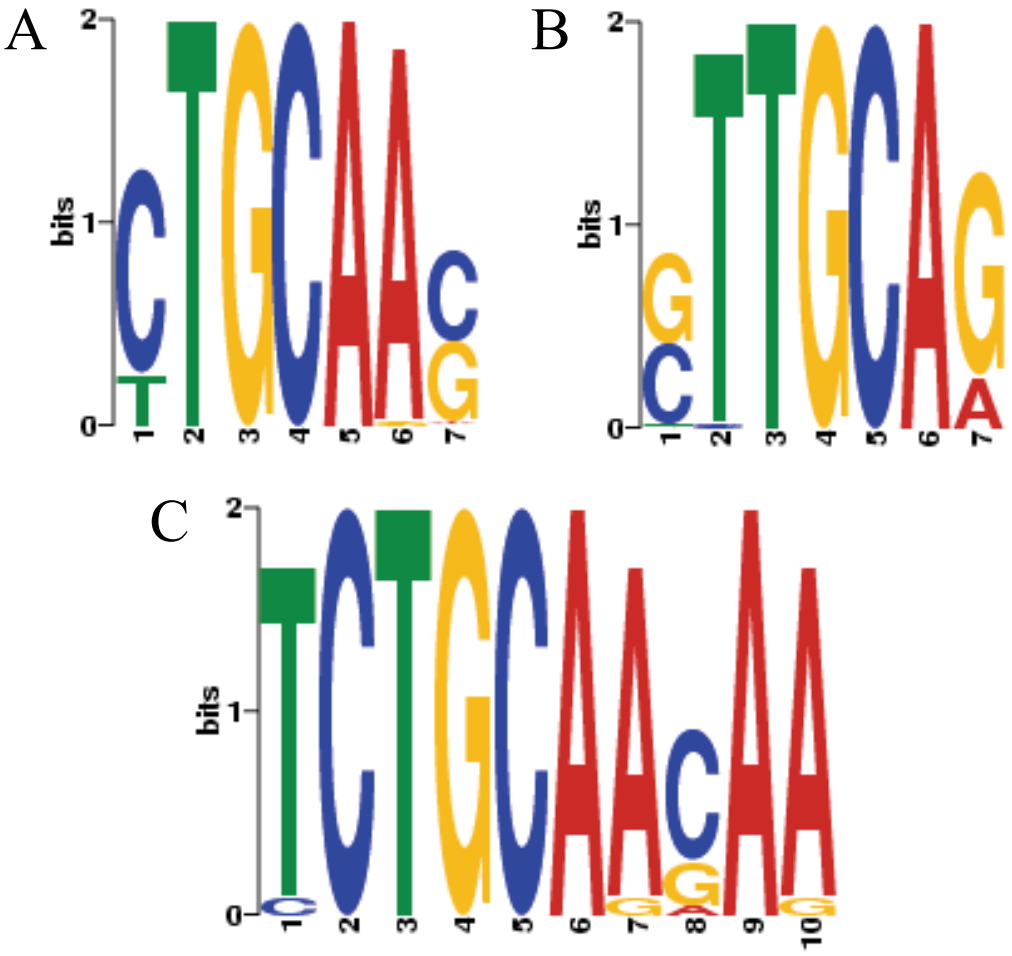


Figure 4.5 A conserved DNA motif identified in the promoters of *EF102/perA*
A. Consensus sequence of the 7-bp DNA motif identified in the promoters of *EF102* and *perA*, as it appears on the coding DNA strand. **B**. The same consensus DNA motif as it appears on the anti-sense DNA strand relative to the CDS of *EF102* or *perA*. **C**. Alignment of the two DNA motifs in the *perA* promoter extended to 10 bp to show the high sequence conservation between these two motifs.

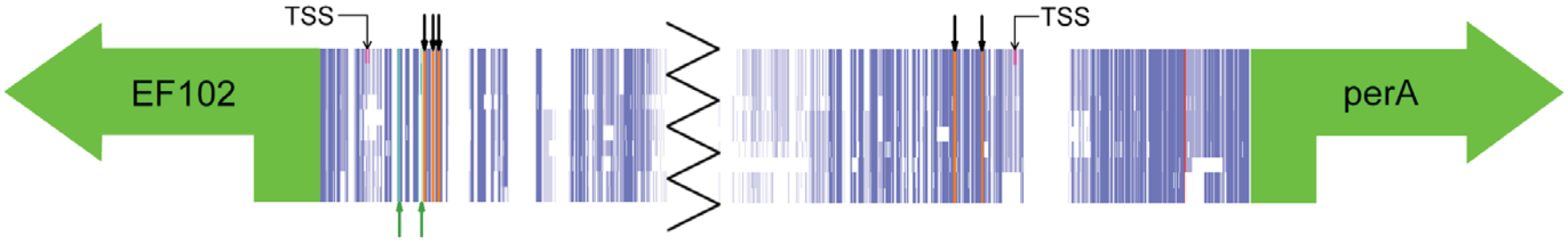


Figure 4.6 Potential regulatory motifs in the promoters of *EF102* and *perA*

The location of the DNA motifs identified in the promoters of *EF102* and *perA* are shown against a graphical representation of sequence conservation between 10 different epichloë isolates. The intensity of the blue colouring represents the level of sequence conservation between isolates (darker = more conserved), with conservation by at least 5 of the 10 isolates required for the lightest shade of colouring. Black arrows indicate the locations of the 7-bp motif (vertical orange lines on the MSA), and green arrows show additional locations where the 5-bp core of this motif is found (vertical cyan lines). The transcription start sites for *EF102* and *perA* are also annotated, and a vertical red line represents the original proposed PerA translation start site. The break in the middle represents an extended region of low conservation between the different isolates.

5. Deletion Analysis of the Major Facilitator Superfamily Transporter EF102

5.1 Deletion of *EF102*

EF102 was deleted by transforming *E. festucae* Fl1 with an *EF102* deletion construct. This construct contained the DNA sequences immediately up- and down-stream from *EF102*, including the first 234 bp of the *EF102* cds, incorporated into the hygromycin resistance plasmid vector pSF15.15 (Fig. 5.1). 48 of the resulting hygromycin resistant transformants were screened by PCR (Fig. 5.2), identifying 4 putative Δ*EF102* mutants. Southern blot analysis of digested gDNA from these four Δ*EF102* mutants probed with the *EF102* deletion construct confirmed deletion of *EF102* in all cases (Fig. 5.3). This Southern blot analysis also showed that tandem insertions of the deletion construct had occurred at the target locus in all 4 mutants, with the order from highest to lowest copy number being Δ*EF102* #11, Δ*EF102* #153, Δ*EF102* #169 then Δ*EF102* #88.

5.2 Δ*EF102* chemotype analysis

Guttation fluid was used as a way to investigate peramine transport, as if peramine transport was disrupted by the deletion of *EF102*, confining peramine within the fungal cells, it would be expected that the levels of peramine found in the plant guttation fluid would drop dramatically or disappear altogether. Perennial ryegrass seedlings were infected with each of the four Δ*EF102* mutants. After 8 weeks, infection was confirmed by immunoblotting, and infected plants were re-potted and cut back. Four weeks post-immunoblotting, guttation fluid and herbage samples were taken from a single plant infected with each Δ*EF102* mutant, as well as from an Fl1 infected plant as a positive control, and an uninfected plant as a negative control. Samples were sent to AgResearch and analysed by HPLC-UV spectroscopy (all samples; Spiering *et al.*, (2002)), and LC-MS (guttation samples only; Koulman *et al.*, (2007)).

The results from these analyses (Table 5.1) show that peramine was detected in the whole tiller samples from all of the mutant- and Fl1-infected plants, but not the uninfected negative control. Higher peramine levels were detected in the wild-type Fl1 sample than in all mutants, however as no replicates of these assays were performed it is not clear if these differences are statistically significant. No peramine was detected in any of the guttation samples using the HPLC-UV method, due to detection limits. LC-MS detected peramine in all samples except the uninfected control. Higher levels of

peramine were detected in guttation fluid from the wild type Fl1 infected plant than those infected with Δ*102* mutants #88, 153 and 169. Guttation fluid from the plant infected with mutant #11 contained more peramine than wild type. The Fl1 herbage sample was also analysed by LC-MS, however as the peramine concentration in this sample was well above the linear range of this method the result is prone to large potential error, and as such the HPLC-UV value for this sample is considered more reliable. A second LC-MS analysis of the guttation samples was done using a less peramine-sensitive separation column (Appendix 7.1.2), the results of which approximately mirror those in Table 5.1.

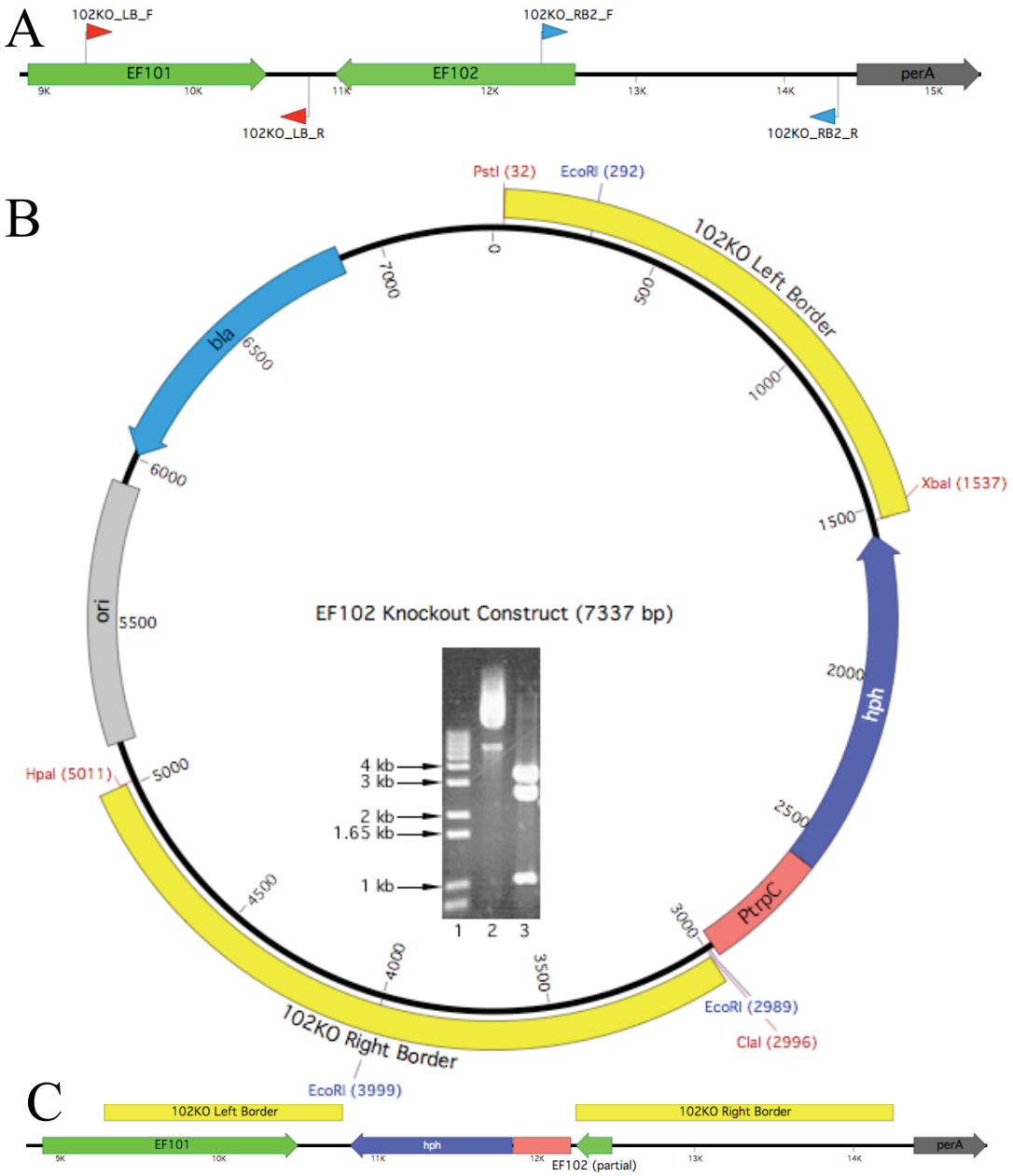


Figure 5.1 *EF102* deletion construct

A. The 102KO left border fragment was amplified from *E. festucae* Fl1 gDNA by PCR using the proofreading DNA polymerase "Expand High-Fidelity". The primers used for this were 102KO_LB_F, which introduces a *Pst*I site into the 5' end of the amplification product, and 102KO_LB_R, which introduces an *Xba*I site into the 3' end. Similarly the 102KO right border fragment (amplified using the proof-reading polymerase "Platinum PFX") was amplified using primers 102KO_RB2_F/102KO_RB2_R, which incorporate *Cla*I/*Hpa*I cut sites into the amplification product, respectively. **B.** After confirming sequence fidelity of the 102KO left and right border fragments by sequencing, these fragments were cloned into the hygromycin resistance vector pSF15.15 using the RE sites introduced during PCR amplification to ensure correct configuration of the inserts in the resulting deletion vector. **Inset:** lane 1 = 1kb+ ladder, lane 2 = uncut *EF102* deletion construct, lane 3 = *EF102* deletion construct digested with *Eco*RI. Expected band sizes are 3.6 kb, 2.7 kb and 1 kb. **C.** Expected physical map of the deletion mutant.

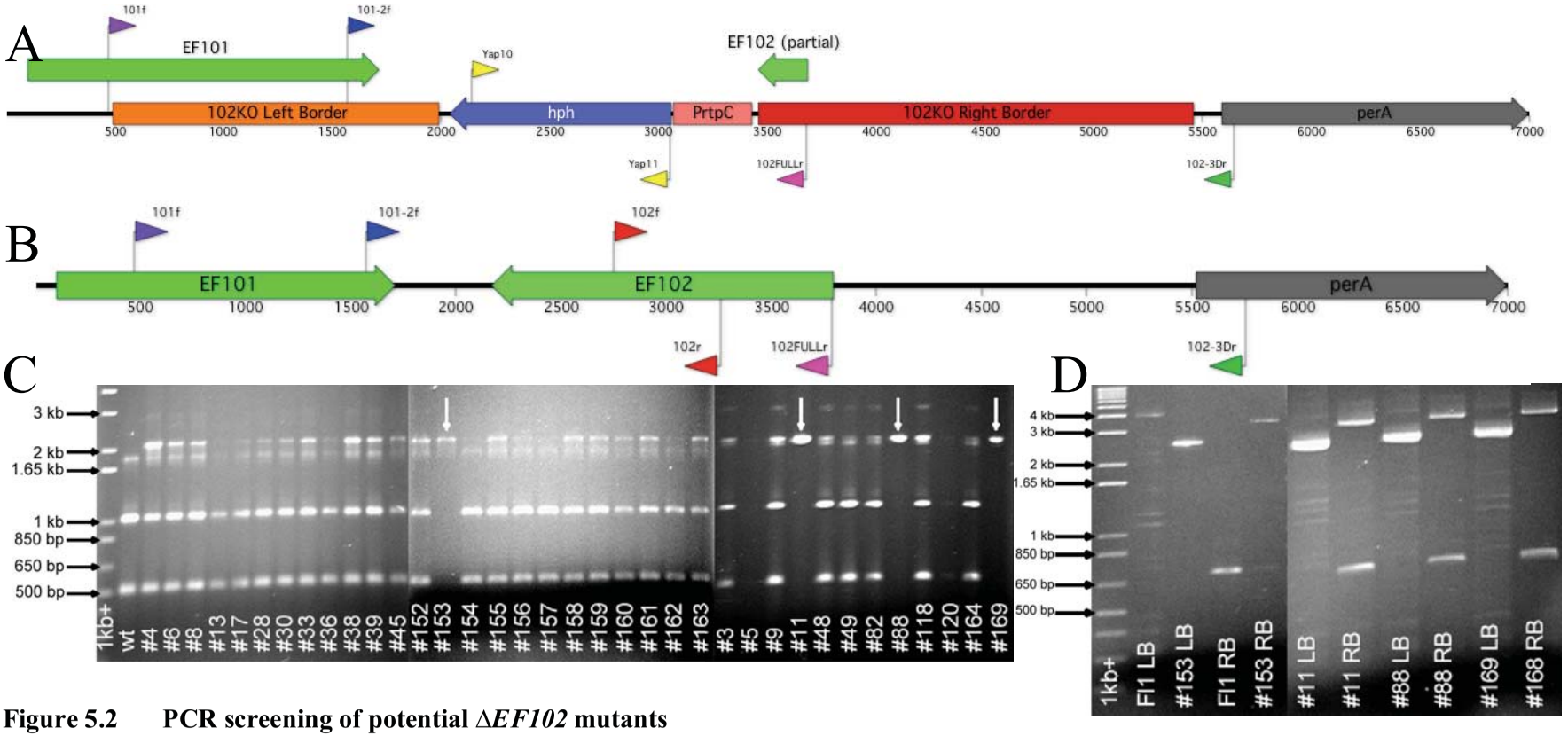


Figure 5.2 PCR screening of potential Δ*EF102* mutants

A. Physical map of a single copy EF102 deletion mutant showing primers used for screening. **B.** Physical map of the wild-type Fl1 EF102 locus showing primers used for screening. **C.** E. *festucae* Fl1 transformants were initially screened using primers 101-2f, 102f, 102r and 102FULLr which produce 4 amplification products (2217, 1689, 1035 and 507 bp long in transformants with a wt *EF102* locus, but only one in transformants in which the *EF102* gene had been deleted (2107bp). **D.** Subsequent PCR screen of mutants #11, 88, 153 and 169, where the correct placement of the 102KO construct is confirmed by amplification over the left and right borders using primer pairs 101f/Yap11 and Yap10/102-3Dr respectively. Expected mutant band sizes for left and right border fragments are 2582 bp and 1539 bp respectively. No bands are expected for wt Fl1, however both LB and RB amplifications are observed to produce non-specific products, one of which is also observed in all of the mutants.

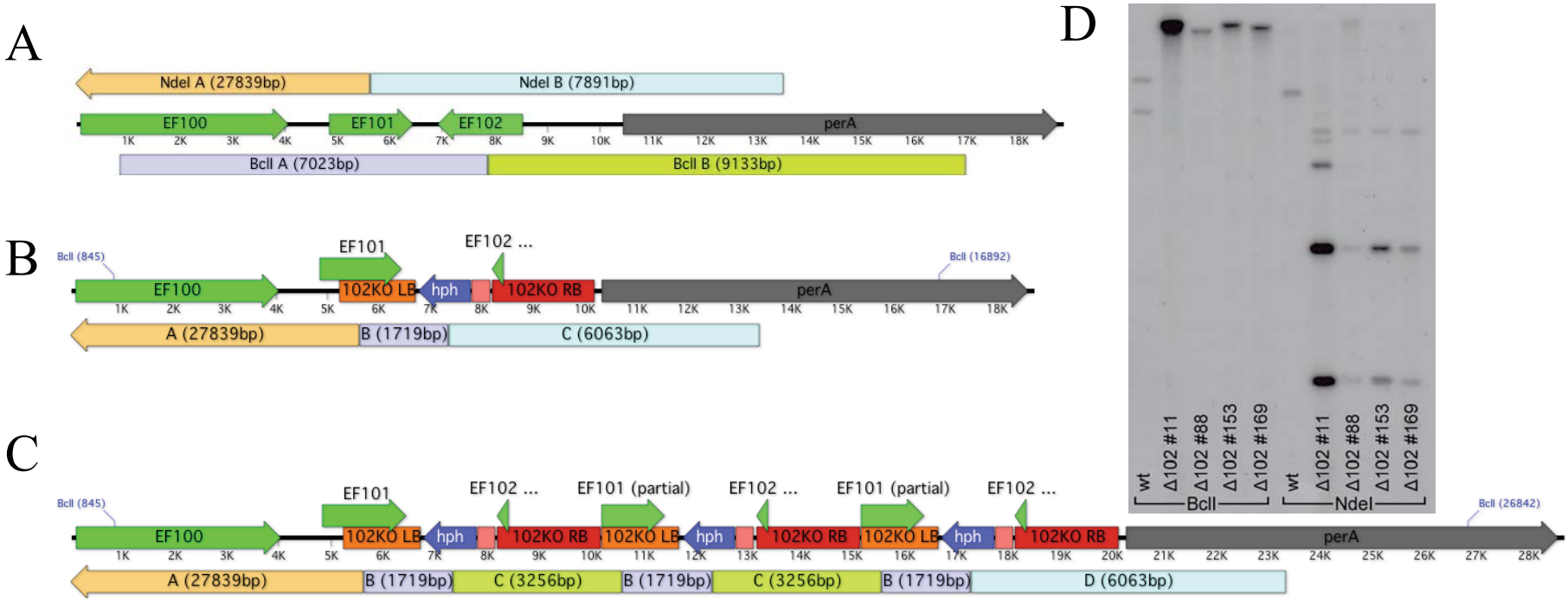


Figure 5.3 Southern blot analysis of ΔEF102 mutants

A. Physical map of the *EF102* wild type locus in *E. festucae* Fl1 showing bands expected to hybridize when Fl1 gDNA is cut with *Nde*I (top) or *Bcl*I (bottom) and probed with the [^{32}P]-labelled *EF102* deletion construct. **B.** Physical map of the *EF102* mutant locus showing bands expected to hybridize when gDNA is cut with *Nde*I and probed with the [^{32}P]-labelled *EF102* deletion construct. Also shown are the *Bcl*I restriction enzyme sites, with a single band of 16047 bp expected. **C.** Gene map of a ΔEF*102* mutant with a tandem insertion of the deletion construct showing the bands expected when the Southern blot of *Nde*I digests is probed with the deletion construct, *Bcl*I sites are also annotated. **D.** Autoradiograph of Southern blot of gDNA from wt *E. festucae* Fl1 and four independent ΔEF*102* mutants digested with *Bcl*I (left) and *Nde*I (right), probed with the [^{32}P]-labelled *EF102* deletion construct.

Table 5.1 Peramine concentrations in whole tiller and guttation fluid samples for wild type *E. festucae* Fl1 and Δ*EF102* mutant infected plants

Endophyte Strain	Sample Type [d]	HPLC-UV Peramine conc. (ppm) [a, c]	LC-MS Peramine conc. (ppm) [b, c]
102KO #11	Herbage	106.9	NT
102KO #88	Herbage	93.2	NT
102KO #153	Herbage	85.8	NT
102KO #169	Herbage	55.3	NT
Fl1	Herbage	>200	116.2
uninfected	Herbage	ND	NT
102KO #11	Guttation	ND	0.22
102KO #88	Guttation	ND	0.04
102KO #153	Guttation	ND	0.05
102KO #169	Guttation	ND	0.04
Fl1	Guttation	ND	0.09
uninfected	Guttation	ND	ND

[a] LOD HPLC-UV = 0.1 ppm, LOQ = 0.5 ppm
[b] LOD LC-MS = 0.002 ppm, LOQ = 0.01 ppm
[c] ND = Not Detected, NT = Not Tested
[d] The values for the whole tiller and guttation samples cannot be directly compared, as whole tiller analyses were done on freeze-dried samples, while guttation samples are aqueous.

6. Discussion

6.1 The *perA* locus structure differs significantly within *E. festucae*

The secondary metabolite (SM) gene *perA* is widely distributed across *Epichloë*, though not always in a functional form, making it an attractive locus to study the organisation and structure of a SM gene across a wide range of *Epichloë spp.* Analysis of the *perA* locus structure in the seven *E. festucae* strains Fl1, Fg1, E189, Frc5, Frc7, Fr1 and Frr1 revealed that these strains can be sorted into four sub-groups (Fig. 6.1). The first group consists of strains Fl1, Frc7 and Frr1, and peramine production is exclusive to these strains. The second group consists of strains E189 and Fr1, which exhibit identical banding patterns to the sequenced strain E2368. In E2368 the 3' end of *perA* has been replaced by the MITE EFT-3m, and the large uncharacterised retrotransposon relic EFT-7 has been inserted upstream of *perA*, resulting in discontinuity of the genome sequence for the putative *EF102/perA* intergenic region. E2368 was derived from progeny of an F1 cross of E189 x *E. festucae* E434 backcrossed to E189 (Wilkinson *et al.*, 2000), so it is likely that the *perA* locus in E2368 originates from E189. Linkage between *perA* and *EF104*, but not *EF102*, is shown for group two strains. Frc5 and Fg1 are the sole members of groups three and four, respectively. Frc5 is very similar to group two strains, however the insertion between *EF102* and *perA* differs, as linkage between these two genes is shown for this strain. Unlike group two and three strains, the *perA* reductase domain is not deleted in Fg1, however a large insertion is present upstream of *perA*, thereby thwarting attempts to show linkage between *EF102* and *perA*.

A region of extended conservation, thought to be important for *perA* expression, was identified in the putative *perA* promoter by aligning the sequences from a range of different epichloë isolates that are thought or known to produce peramine. Surprisingly, this region is also conserved between Fl1 and E2368, and conservation is maintained for nearly the entire region predicted to be important for regulation of *perA* expression even though the reductase domain of *perA* is deleted in E2368, and the truncated *perA* gene in E2368 is still expressed (*Epichloë festucae* Genome Project, 2011). This result raises the possibility that while E189, Frc5 and Fr1 are unable to produce peramine due to the deletion of the *perA* reductase domain, the truncated PerA may still be produced in these isolates, and may still produce a dipeptide that retains or provides some selective advantage to the symbiosis. It is therefore interesting that Fg1, in which the reductase domain is not deleted, does not produce peramine. Fg1 contains a large insertion in the

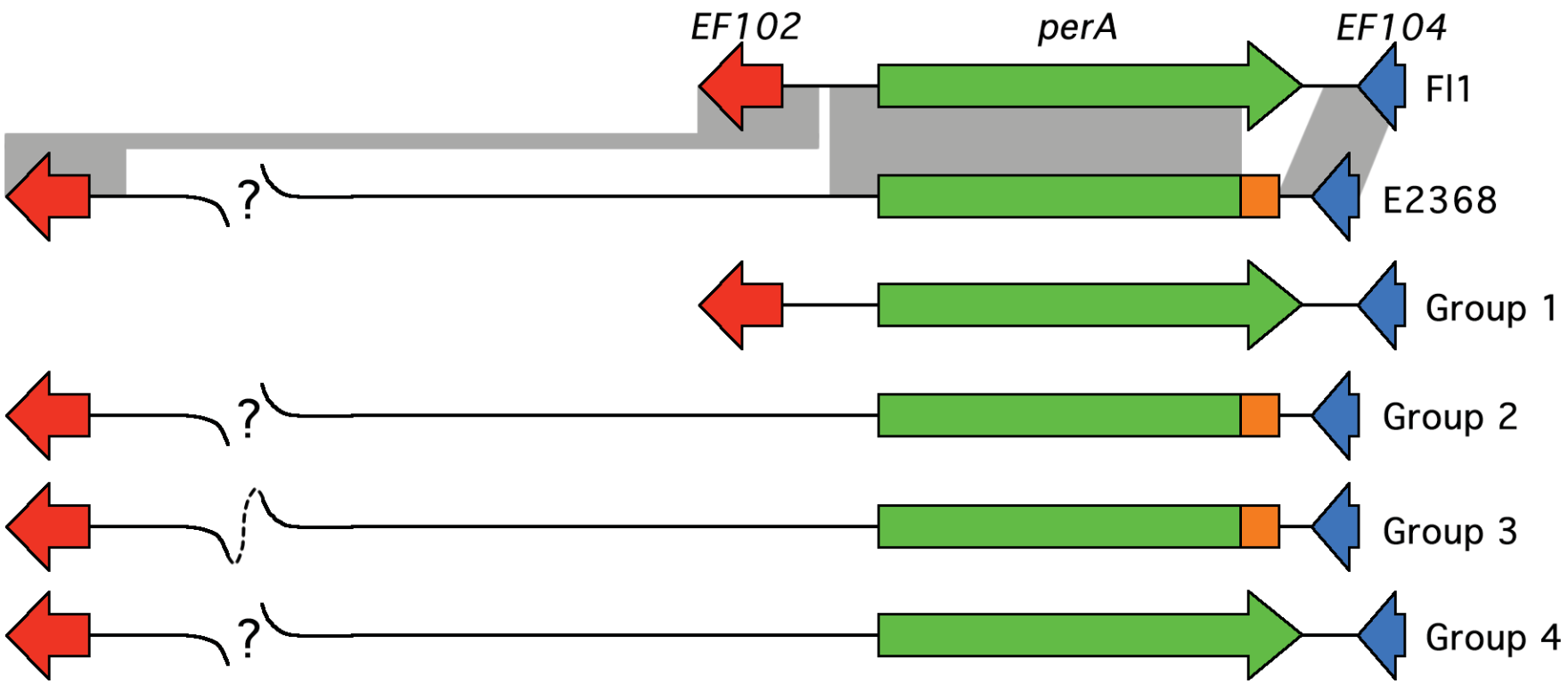


Figure 6.1 Known and proposed physical map of the *perA* locus in different *E. festucae* strains
The physical maps of *E. festucae* strains Fl1 and E2368 for the *perA* locus shown were determined by sequencing data, with homologous regions linked in grey, and the MITE EFT-3m shown in orange at the end of the truncated *perA* gene in E2368. Discontinuity between *perA* and *EF102* in E2368 is proposed to be caused by the transposon relic EFT-7. The proposed physical maps for Group 1 strains (Fl1, Frc7, Frr1), Group 2 (E189, Fr1), Group 3 (Frc5), and Group 4 (Fg1) are show below based on comparitive data from PCR and Southern blot experiments.

intergenic region proposed to separate *EF102* and *perA* that differs from the insertion identified in the other isolates, so the lack of peramine production in Fg1 may be due to disruption of the 5' regulatory sequences by this large insertion resulting in the loss of *perA* gene expression, or alternatively that there is a nonsense or frameshift mutation in *perA* that cannot be detected by PCR or Southern analysis.

In contrast to the large transposon-mediated variability observed around the *perA* locus within *E. festucae*, there is absolute conservation of PCR product sizes for the genes *EF101*, *EF102*, *EF104*, *EF105* and the intergenic sequences separating these genes in all strains. This indicates that the order and structure of the genes surrounding *perA* is conserved within *E. festucae*, and that transposon activity in this region is exclusive to *perA* and its flanking intergenic sequences, an association often observed for epichloë secondary metabolite genes (Young *et al.*, 2006; Fleetwood *et al.*, 2007).

6.2 The *perA* locus structure is conserved between putative peramine producing *Epichloë spp.*

The PCR and Southern blot analyses of *E. typhina* E425, *E. amarillans* E57*, E. bromicola* E501 and E799, and *E. elymi* E56 and WWG1 showed that the sizes of internal *perA* bands of all these isolates, with one exception, were identical to Fl1. The flanking intergenic sequences vary in size between isolates, however multiple sequence alignment of the *EF102-perA* intergenic sequence from these isolates showed that the intergenic sequence proximal to *perA* and *EF102* remains highly conserved, and that the differences in size of this region among isolates is due to insertion of a variety of sequence elements, none of which approach the size of the insertions in E2368, in the central region where conservation between isolates breaks down. The one exception to this conservation is *E. bromicola* E799, which contains the *perA* A2 domain, but otherwise shows complete sequence disruption within *perA* and its flanking intergenic regions.

The genes surrounding *perA* and intergenic sequences separating them show high conservation within epichloë, with only minor differences in PCR product sizes being observed. This shows that, as in *E. festucae*, the order and structure of genes

surrounding *perA* are highly conserved between *Epichloë spp.*, and that transposon activity is limited to *perA* and its flanking intergenic sequences.

6.3 All *E. festucae* isolates are able to infect *Lolium perenne*

Confocal analysis of samples taken from the leaf sheath and blade of *Lolium perenne* plants infected with *E. festucae* strains Fl1, Fg1, E189, Frc5, Frc7, Fr1 and Frr1 showed that all isolates were able to infect both the pseudostem and blade tissue, despite each only naturally infecting a specific species of the *Festuca* genus. An apparent reduction in the endophyte concentration of plants infected with strains Frc5 and Frc7 was noticed. The statistical significance of this reduction was not determined due to the limited sample size of the analysis, however reduced endophyte levels would explain the reduced peramine concentration observed in the Frc7 infected plant material relative to Fl1, as the *perA* locus is near identical between Fl1 and Frc7, suggesting that genetic differences are unlikely to be the cause. E189 infected plants appeared to contain multiple hypha in single intercellular spaces, indicating that this association may not be as compatible as Fl1, however the small sample size precluded a statistical analysis.

6.4 A new translation start site was identified for *perA*

Multiple sequence alignment (MSA) of 10 different epichloë endophytes revealed that the annotated ATG codon for perA from strain Fl1 was probably incorrect (Tanaka *et al.*, 2005), as this codon was followed by large deletion and frameshift mutations in other peramine producing isolates. By analysing this MSA, a new in-frame start codon was identified 183 bp downstream of the originally annotated start site. This proposed new translation start site maintains the reading frame for PerA, shows reasonable similarity to the predicted epichloë Kozak consensus sequence, and is still located well upstream of the cds for the first predicted functional domain of perA. This result demonstrates the power of comparative genomics, as has been previously shown for *Saccharomyces spp.* (Kellis *et al*., 2003), to refine gene models. As additional *Epichloë/Neotyphodium* genome sequences become available this approach will be a powerful tool for annotating the correct translation start sites for genes from *Epichloë spp*.

6.5 Transcription start sites were identified for *EF102* and *perA*

Using transcriptome data from wild type *E. festucae* Fl1 grown *in planta* (Eaton *et al.*, 2010) the transcription start of *EF102* was located 121 bp upstream of the translation start site, and the transcription start site of *perA* was located 534 bp upstream of the new translation start site. This *perA* transcription start site is relatively far upstream of the translation start site compared to most fungal transcription start locations, and the number of mRNA reads increase gradually, contrasting other genes such as *EF102*, in which the number of reads increase sharply after initiation of transcription. This result indicates that transcription initiation in *perA* does not always occur at a single position, but rather that -534 bp represents the maximum distance upstream of the translation start site from which transcription is initiated. Experimental analysis will be required to confirm whether there are multiple transcription start sites for *perA*.

6.6 Identification of an extended region of sequence conservation upstream of the *perA* transcription start site

Because of the evolutionary proximity of the *Epichloë spp.* used in this study the rate of sequence change in the *perA* promoter was not sufficient to provide the resolving power required to distinguish functional motifs from a background of non-functional sequence. In contrast genomic comparison of only four *Saccharomyces spp.* was sufficient to identify many novel and previously identified regulatory motifs, as these were conserved against a background of considerable genetic change (Kellis *et al.*, 2003). Comparison of the *perA* promoter from 10 different isolates encompassing 6 different *Epichloë* species was able to identify an extended region of conservation upstream of the transcription start site, but was not able to resolve which motifs within this region that may be crucial for perA regulation. The level of conservation in this MSA is too high for standard motif identification algorithms like MEME (Bailey & Elkan, 1994) or Gibbs (Lawrence *at al.*, 1993) to further refine the motif search, and indeed seems to be too high even for search algorithms designed to take phylogenetic proximity into account. Despite these limitations the MSA has identified a 450-bp long region to focus on for future promoter analysis using experimental approaches such as deletion or site-directed mutagenesis of these sequences fused to a reporter gene. As conservation in this region is somewhat discontinuous, the relevance of any motifs identified through

deletion or comparative analysis can be assayed by referring to this MSA to determine if they are conserved between peramine producing species. Alternatively high conservation of this 450-bp long region as a single unit, as opposed to its constitutive parts, may be crucial for *perA* regulation.

6.7 Potential limitations of comparative analysis within epichloë

The high sequence conservation observed in the *perA* promoter may reflect an epichloë-wide issue for the identification of regulatory motifs of biological aspects specific to epichloë, based solely on sequence conservation between the promoters of orthologous genes. This should not pose a restriction to the identification of regulatory motifs shared between the promoters of different genes within epichloë, such as the lolitrem or loline cluster genes. Likewise the recent sequencing of a multitude of fungi, including the morning glory epiphyte *Periglandula ipomea*, that are phylogenetically close to *Epichloë* has exciting potential for comparative genomics to identify regulatory motifs for genes with shared biological functions (*Epichloë festucae* Genome Project, 2011).

6.8 *EF102* and *perA* share a common promoter motif

MSA of the intergenic sequence separating divergently transcribed and co-regulated genes *EF102* and *perA* between 10 different epichloë isolates identified extended regions of conservation in both genes upstream of the transcription start sites. These regions are proposed to contain regulatory elements that may affect expression of *EF102* and *perA*. A comparison of the sequence from these regions for 10 different epichloë isolates identified a 7-bp long DNA motif significantly overrepresented in the promoters of both genes. This motif was present twice in the *perA* promoter and four times in the *EF102* promoter at a comparable distance upstream of the transcription start site of the respective genes. The 5-bp core of this motif was observed a further two times in the promoter of *EF102*. Interestingly the two motifs in the *perA* promoter share extended conservation, indicating the sequence recognised by any DNA binding factor may be larger than the 7-bp motif observed. This may explain why *perA* is downregulated to a greater degree than *EF102* in the Δ*sakA* transcriptome data (Eaton *et al.*, 2010). Whether these regulatory motifs are involved in regulating *perA* and *EF102* remains to be experimentally confirmed.

6.9 A peramine transport role for *EF102* is not supported: alternative substrate transport hypothesis proposed

EF102 is a predicted major facilitator superfamily (MFS) transporter that was hypothesised to transport peramine due to the co-regulation of *EF102* and *perA* in *E. festucae* Fl1 (Tanaka *et al.*, 2005; Eaton *et al.*, 2010). Four independent *EF102* deletion mutants were generated to test this hypothesis, with guttation fluid samples taken from plants infected with each mutant and wild type Fl1, all grown under identical conditions. Peramine was detected in all guttation samples except the uninfected control using an LC-MS-based method, which showed that guttation fluid from plants infected with Δ*EF102* mutants #88, 153 and 169 all contained less peramine than the wild type infected plant, while mutant #11 contained more. The increase in peramine for mutant #11 was unexpected, however this mutant contains an ectopic insertion of the *EF102* deletion construct that may have resulted in this anomalous result. The peramine reduction in the other three mutants was not to the extent expected for a peramine transport mutant, and as such the single data points obtained preclude determination of the significance of this reduction; however peramine content in the wild type Fl1 infected herbage sample was 2-4 times higher than observed in the mutant infected samples, indicating that the insertion of the *EF102* deletion construct in these strains may have caused a reduction in peramine production. The *EF102* deletion construct contains the putative *perA* promoter, and has inserted in tandem in all four independent Δ*EF102* mutants. These additional promoters may be competing with the native *perA* promoter for transcription factors, resulting in reduced peramine production. If this were the case it would be expected that the mutant strains with the highest *EF102* deletion construct copy number would produce the least peramine. This is not the case, as the mutant with the highest copy number (#11) actually produces the most peramine, however insufficient replication makes the statistical significance of these results unclear.

Another potential explanation for this drop in peramine levels is a reduced availability of the PerA substrates arginine and 1-pyrroline-5-carboxylate (P5C) in the deletion mutants. A re-examination of the functional role of MFS transporters revealed that two MFS subfamilies contain amino acid transporters with representatives in plants, fungi and mammals (Wipf *et al.*, 2002). One subfamily contains known transporters for

aromatic amino acids, and the other transports glutamate. Both arginine and P5C can be synthesized from glutamate via the arginine and proline metabolism pathways (Fig. 6.2). Analysis of the Fl1 genome sequence (*Epichloë festucae* Genome Project, 2011) revealed that all of the enzymes required for these metabolic inter-conversions are present in Fl1 (Table 6.1). Transcriptome analysis shows that none of these genes are symbiotically regulated (Eaton *et al.*, 2010), indicating that any symbiosis-based regulation of PerA substrate production would occur at a metabolic level by controlling the availability of precursors such as glutamate. Disruption of plant-fungal signalling results in a relatively modest reduction in expression of *perA* and *EF102* (3.4- and 2.4-fold respectively) compared to other secondary metabolite genes, such as in the lolitrem genes, which are downregulated to a much greater extent (Eaton *et al.*, 2010). These results correspond well with reverse transcription PCR data obtained from Fl1 culture and *in planta* samples, which demonstrated that while *perA* was downregulated in culture, the change was not nearly as dramatic as observed for the lolitrem synthesis gene *ltmG* (Tanaka *et al.*, 2005). This result indicates that the reduction of peramine production to the very low levels observed in culture (Rowan, 1993) is unlikely to be achieved through gene regulation alone, making regulation through restriction of a substrate precursor such as glutamate an attractive hypothesis.

Blast search analysis using the EF102 protein sequence does not identify any matches where the transporter target molecule has been identified, and a literature search indicates that MFS amino acid transporters are poorly characterised in fungi. To test this substrate transporter hypothesis a sufficient number of independent plants will be analysed for peramine to provide robust statistics on whether the reduction in peramine production observed in plants infected with the Δ*EF102* mutants is significant. In addition a complementation construct should be made and introduced into a Δ*EF102* mutant to see if this restores wild type levels of peramine, as was previously shown for a Δ*perA* mutant strain using an ectopic complementation construct (Tanaka *et al*., 2005).

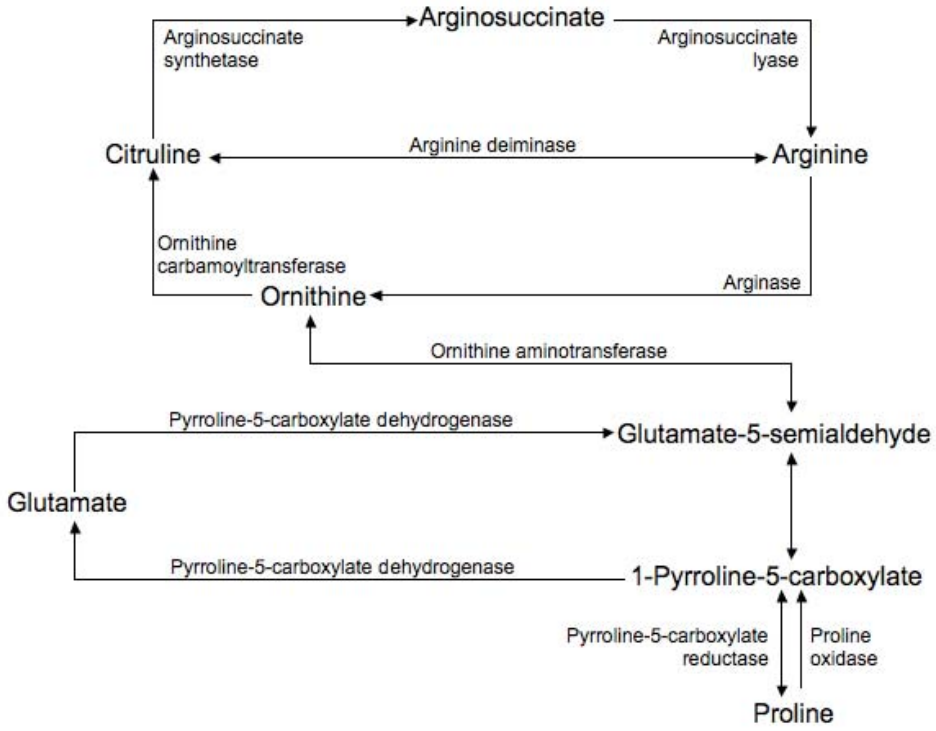


Figure 6.2 Simplified arginine and proline metabolism pathways

Simplified arginine and proline metabolism pathways showing connection to glutamate. Includes common names of enzymes involved, but excludes alternate pathways and other substrates or products involved in these reactions. Pathway data obtained from Kyoto Encyclopedia of Genes and Genomes, 2011.

Table 6.1 Arginine and proline metabolism genes are not differentially regulated in *E. festucae*[a]

Common enzyme name	*E. festucae* gene model[b]	Fold difference[c]	Q-value	Significant?[d]
Proline oxidase	EfM2.108120_A061106_1097_3938_6463_+_Alt0_PolyA	-1.4	<0.0001	no
Pyrroline-5-carboxylate reductase	EfM2.005060_A061106_22_12423_14100_+_Alt0_PolyA	+1.1	0.6556	no
Pyrroline-5-carboxylate dehydrogenase	EfM2.062450_A061106_442_9341_11679_+_Alt0_PolyA	+1.7	<0.0001	no
Arginine deiminase	EfM2.049200_A061106_330_2456_4400_-_Alt0_PolyA	+2.4	0.0879	no
Ornithine aminotransferase	EfM2.083220_A061106_682_7154_9522_+_Alt0_PolyA	+1.2	0.2179	no
Ornithine carbamoyltransferase	EfM2.112430_A061106_1254_4524_5837_+_Alt0_PolyA	-1.2	0.5133	no
Argininosuccinate synthase	EfM2.093400_A061106_816_24534_27970_+_Alt0_PolyA	+1.2	0.0624	no
Argininosuccinate lyase	EfM2.027310_A061106_173_36529_38308_-_Alt0_PolyA	+1.5	<0.0001	no
Arginase	EfM2.111390_A061106_1224_1018_3248_-_Alt0_PolyA	-1.3	0.0810	no

[a] Eaton *et al.*, 2010
[b] *Epichloë festucae* Genome Project, 2011
[c] Where 1-fold represents no change in expression, shown as change in expression in Δ*sakA* mutant relative to wild type
[d] Results were considered significant if fold change was > 2 and Q-value was > 0.05

6.10 Conclusions

The association between transposon DNA and secondary metabolite (SM) genes is well established in epichloë. This study adds to this body of research by demonstrating that the SM gene *perA* and immediate flanking intergenic sequences are often found associated with both larger and smaller transposon relics that appear to be directly responsible for the taxonomic discontinuity of strains that produce peramine across *Epichloë*. In contrast the genes surrounding *perA* and their associated intergenic sequences are highly conserved across epichloë.

Analysis of *Lolium perenne* plants infected with seven *E. festucae* isolates showed that all isolates were able to infect the stem and blade tissue of their host, despite only naturally infecting plants of the *Festuca* genus.

Transcription start sites were identified for the co-regulated genes *perA* and *EF102*, and the translation start site of *perA* was refined. Conserved regions were identified in the promoters of both genes, and comparison between these two regions identified a shared DNA motif. Deletion of *EF102* did not appear to affect peramine transport, however it did cause a 2- to 4-fold reduction in peramine production, resulting in an alternative hypothesis that EF102 is a transporter of glutamate, a known precursor for the PerA substrates arginine and 1-pyrroline-5-carboxylate. The lack of replication limited the conclusions that can be drawn based on peramine levels in mutant versus wild type samples. The decision to only obtain single samples was based on cost and the assumption that deletion of a peramine transporter would result in a dramatic guttation fluid peramine chemotype. In hindsight this decision impacted the significance of conclusions reached, however sufficient evidence was gathered to reject the initial hypothesis and develop an alternative hypothesis.

7. Appendices

7.1 Supplemental tables

7.1.1 Gene expression changes around the *perA* locus in the Δ*sakA* mutant relative to wild type Fl1 (Eaton *et al.*, 2010)

Gene Name	Direction of Change	Fold Difference[a]	Q-value	Significant?[b]
EF96	+	2.0	0.0049	no
EF97	-	1.2	0.4259	no
EF98	+	1.8	<0.0001	no
EF99	+	1.3	0.0449	no
EF100	-	1.2	0.0005	no
EF101	+	1.1	0.9094	no
EF102	-	2.4	<0.0001	yes
perA (*EF103*)	-	3.4	<0.0001	yes
EF104	+	1.0	0.9738	no
EF105	-	1.8	0.0002	no
EF106	-	1.1	0.7576	no
EF107	-	1.1	0.8770	no
EF108	+	1.9	0.0001	no
EF109	+	1.2	0.2861	no

[a] Where 1.0 = no change.
[b] Results were considered significant if fold change was > 2 and Q-value was > 0.05

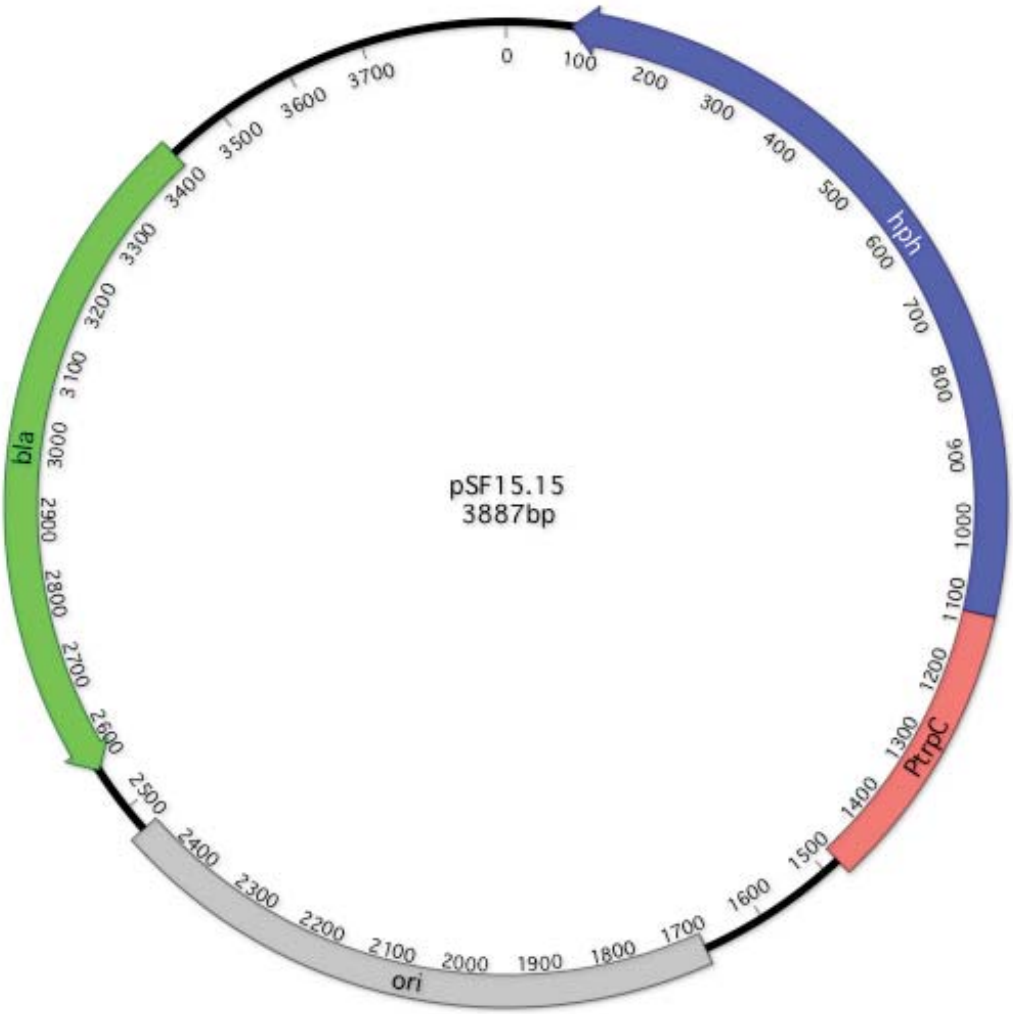
7.1.2 Peramine concentrations guttation fluid samples for wild type *E. festucae* Fl1 and Δ*EF102* mutant infected plants

Endophyte Strain	Sample Type	LC-MS Peramine conc. (ppm) [a, b]
102KO #11	Guttation	0.124
102KO #88	Guttation	0.008
102KO #153	Guttation	0.008
102KO #169	Guttation	0.002
Fl1	Guttation	0.026
uninfected	Guttation	ND

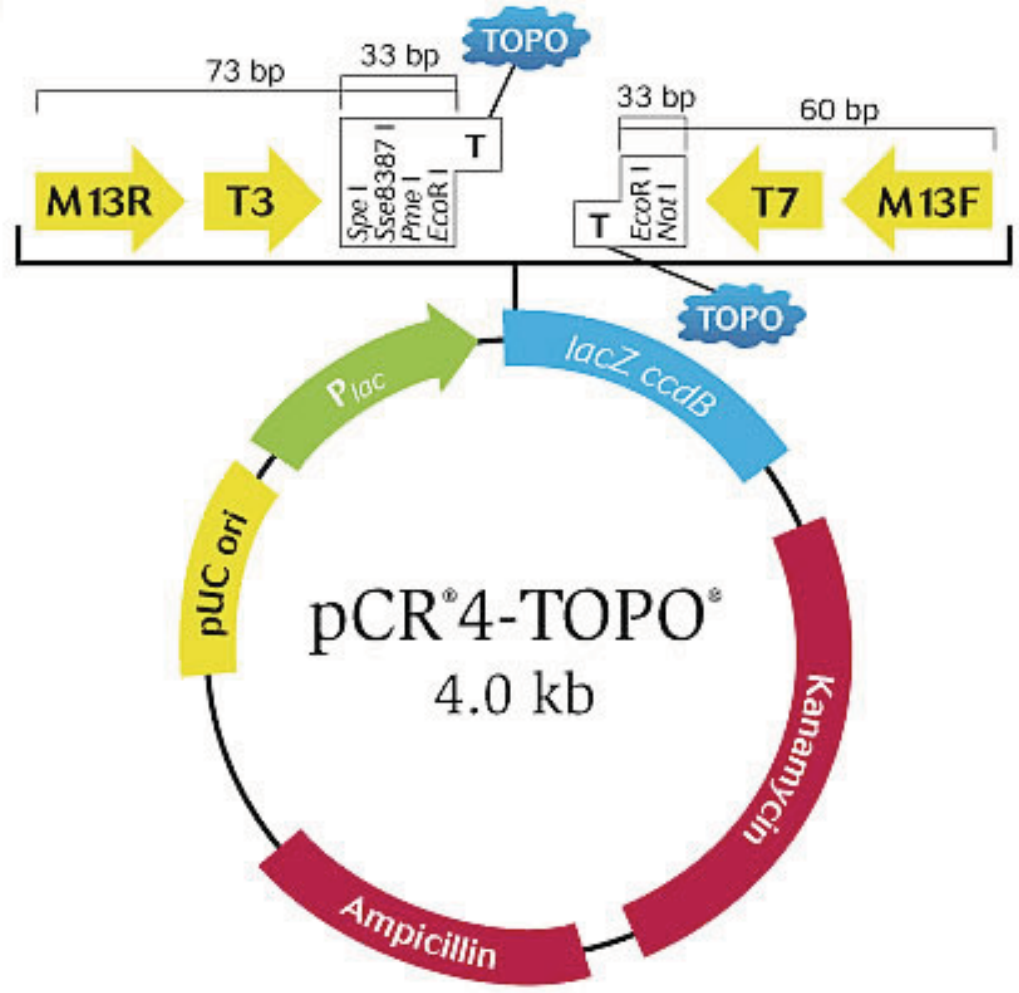
[a] LOD = 0.002 ppm, LOQ = 0.01 ppm
[b] ND = Not Detected

7.2 Vector maps

7.2.1 pSF15.15 (Eaton *et al.*, 2008)



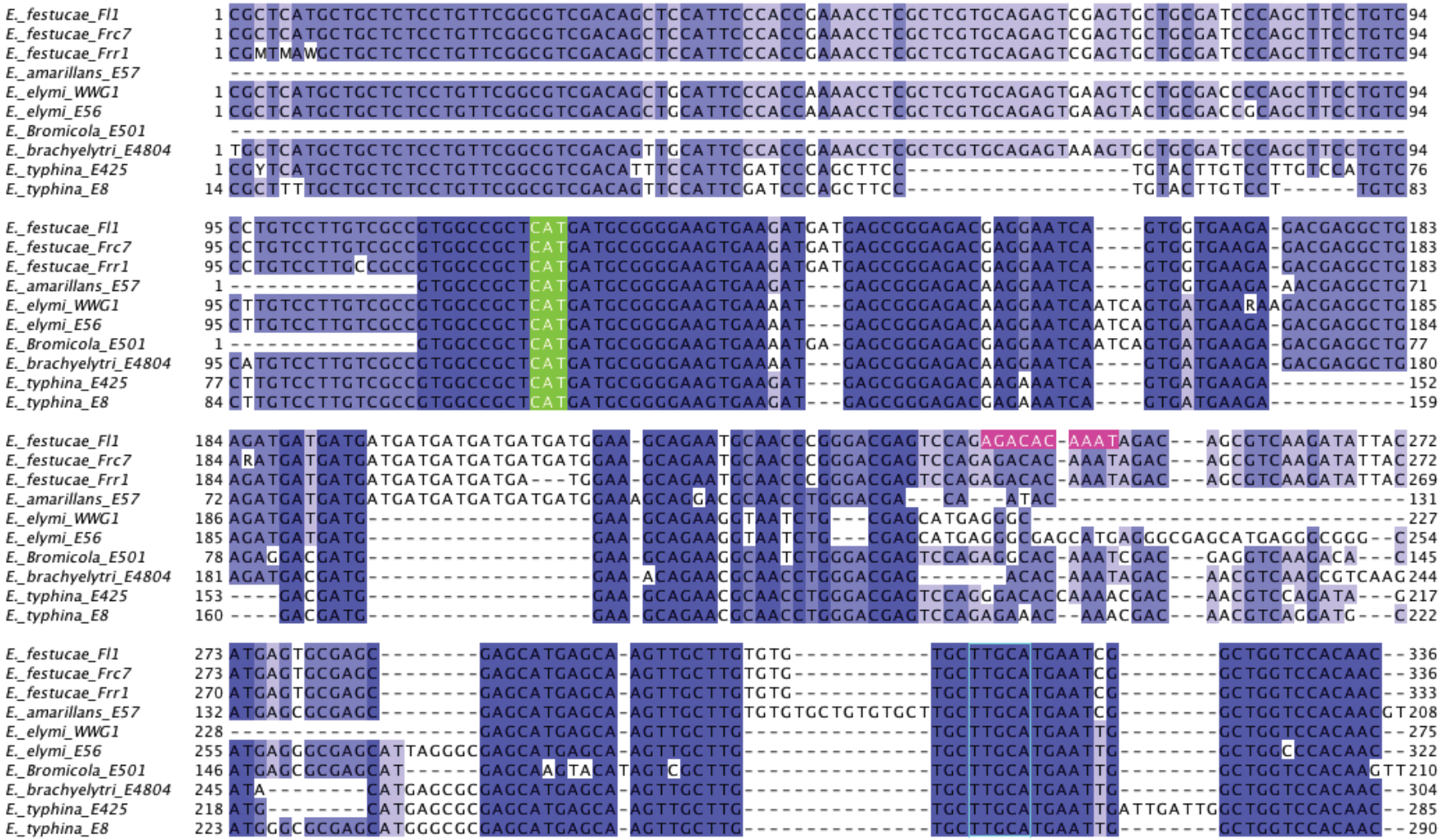
7.2.2 pCR4-TOPO (Invitrogen)



7.3 Multiple sequence alignments

7.3.1 MSA of the *EF102-perA* intergenic sequence from 10 epichloë isolates

MSA of the *EF102-perA* intergenic sequnce from 10 epichloë isolates is shown over the next 14 pages, with the beginning of both genes included. Intensity of colouring indicates the level of conservation, with conservation between at least 50% of the isolates required for the lightest shade. ATG codons for *EF102* and *perA* are coloured bright green, and the originally annotated *perA* ATG codon is coloured red. Transcription start sites for both genes are coloured pink in the Fl1 sequence, 7 bp motifs are bordered in red, and 5 bp motifs in cyan. Sections of extended conservation between smaller sub-sets of isolates have been re-coloured to aid comprehension, with the intensity of colour representing conservation within this sub-set of isolates. Sequence missing from some isolates at both extremes of the MSA due to different primers being used to amplify this region in these isolates.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E._festucae_Frr1
E._amarillans_E57
E._elymi_WWG1
E._elymi_E56
E._Bromicola_E501
E._brachyelytri_E4804
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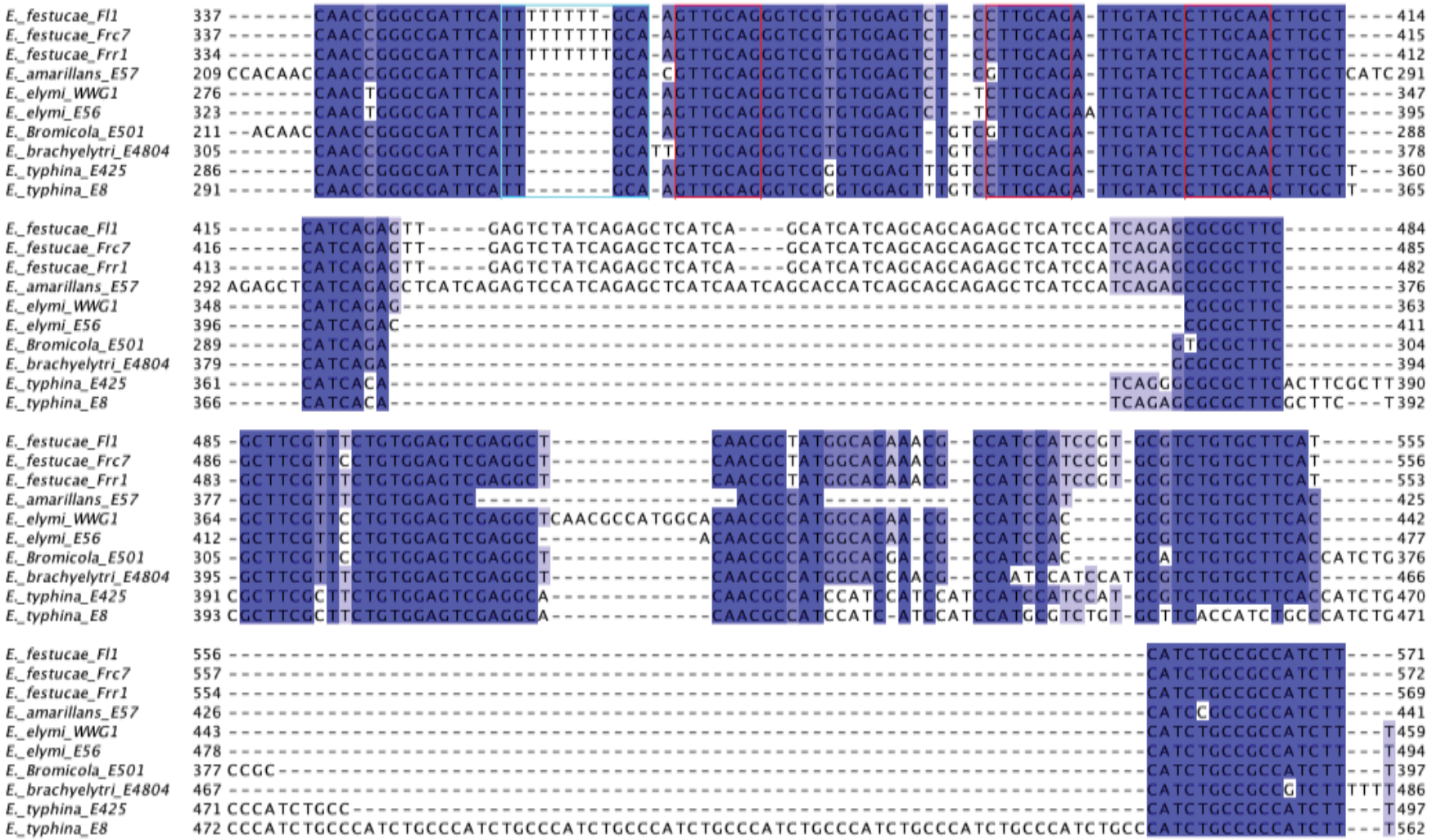
E._typhina_E8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E._festucae_Frr1
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E._typhina_E425
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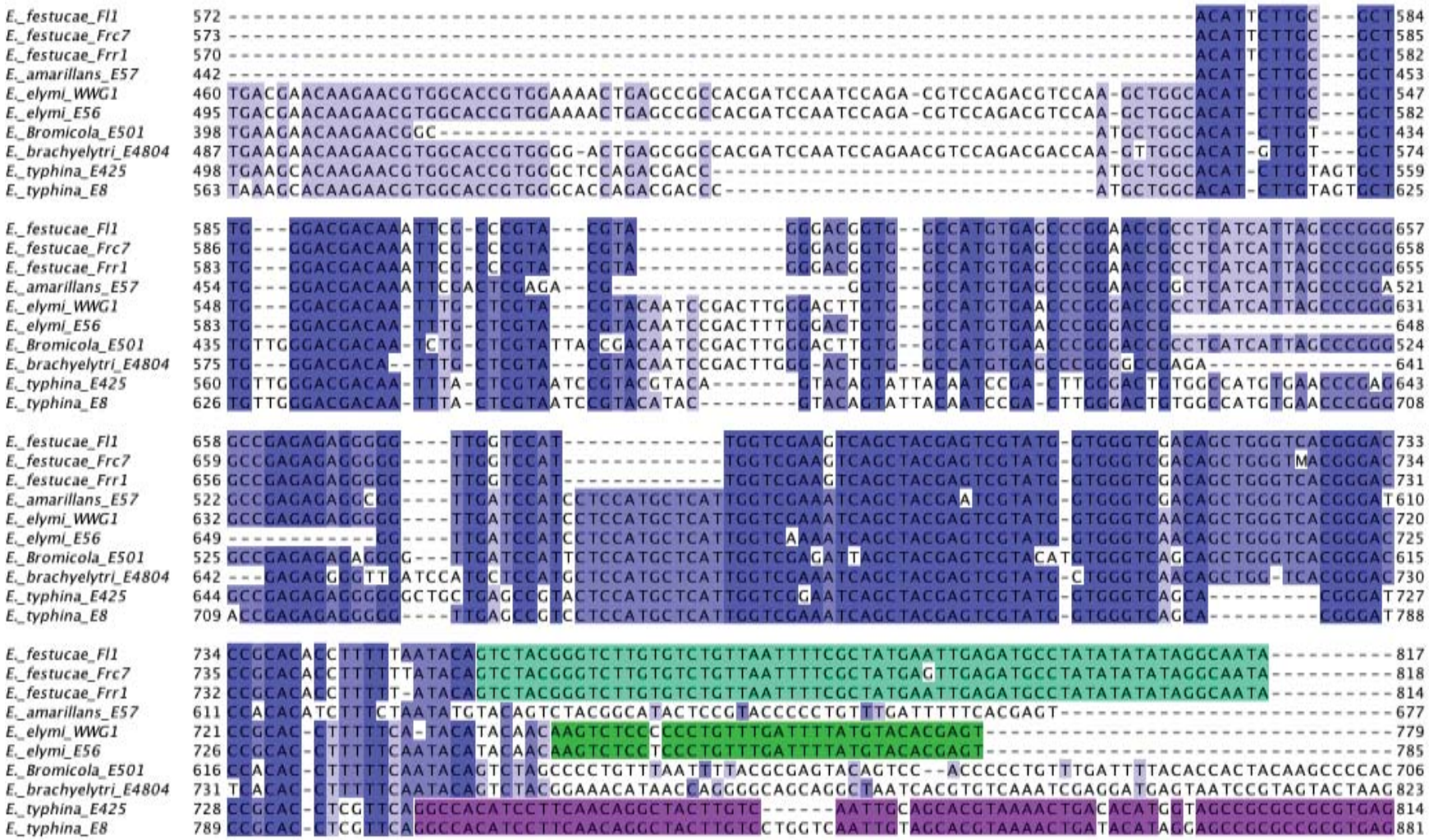
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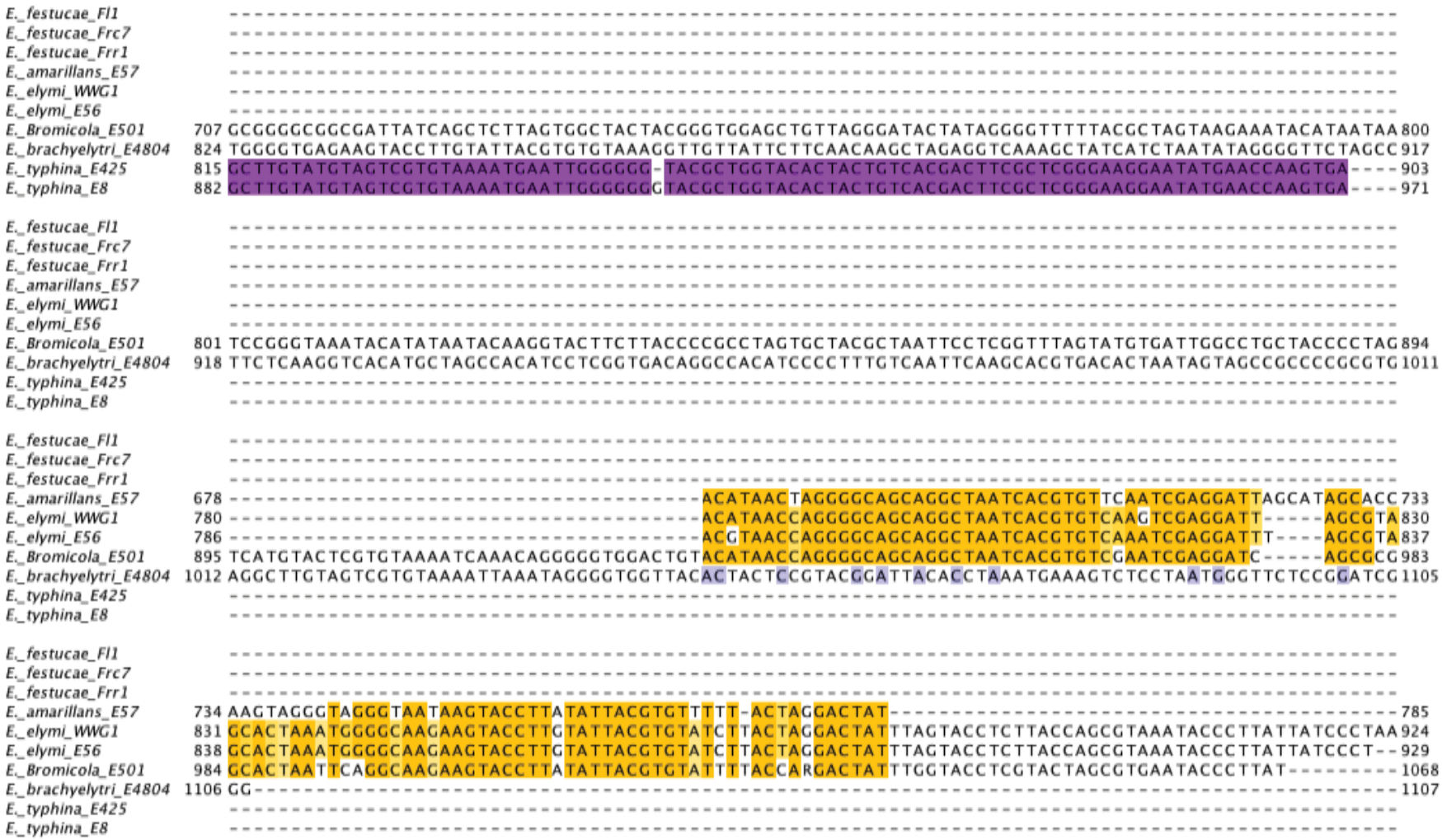
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E._brachyelytri_E4804 824 TGGGGTGAGAAGTACCTTGTATTACGTGTGTAAAGGTTGTTATTCTTCAACAAGCTAGAGGTCAAAGCTATCATCTAATATAGGGGTTCTAGCC 917
E._typhina_E425 815 GCTTGTATGTAGTCGTGTAAAATGAATTGGGGGG-TACGCTGGTACACTACTGTCACGACTTCGCTCGGGAAGGAATATGAACCAAGTGA---- 903
E._typhina_E8 882 GCTTGTATGTAGTCGTGTAAAATGAATTGGGGGGGTACGCTGGTACACTACTGTCACGACTTCGCTCGGGAAGGAATATGAACCAAGTGA---- 971
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E._festucae_Frr1
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E._elymi_E56
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E._festucae_Frc7
E._festucae_Frr1
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E._festucae_Frr1
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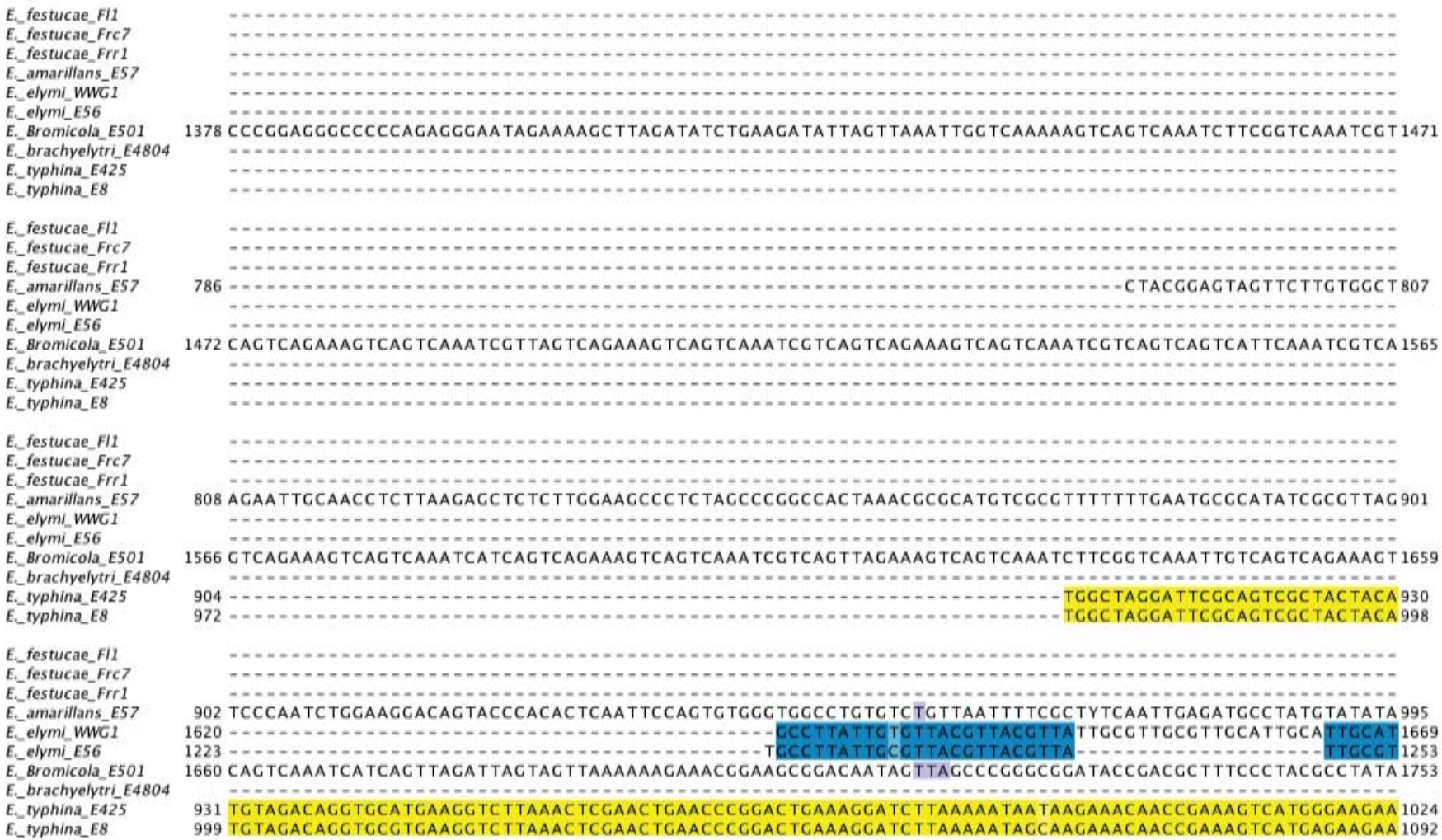
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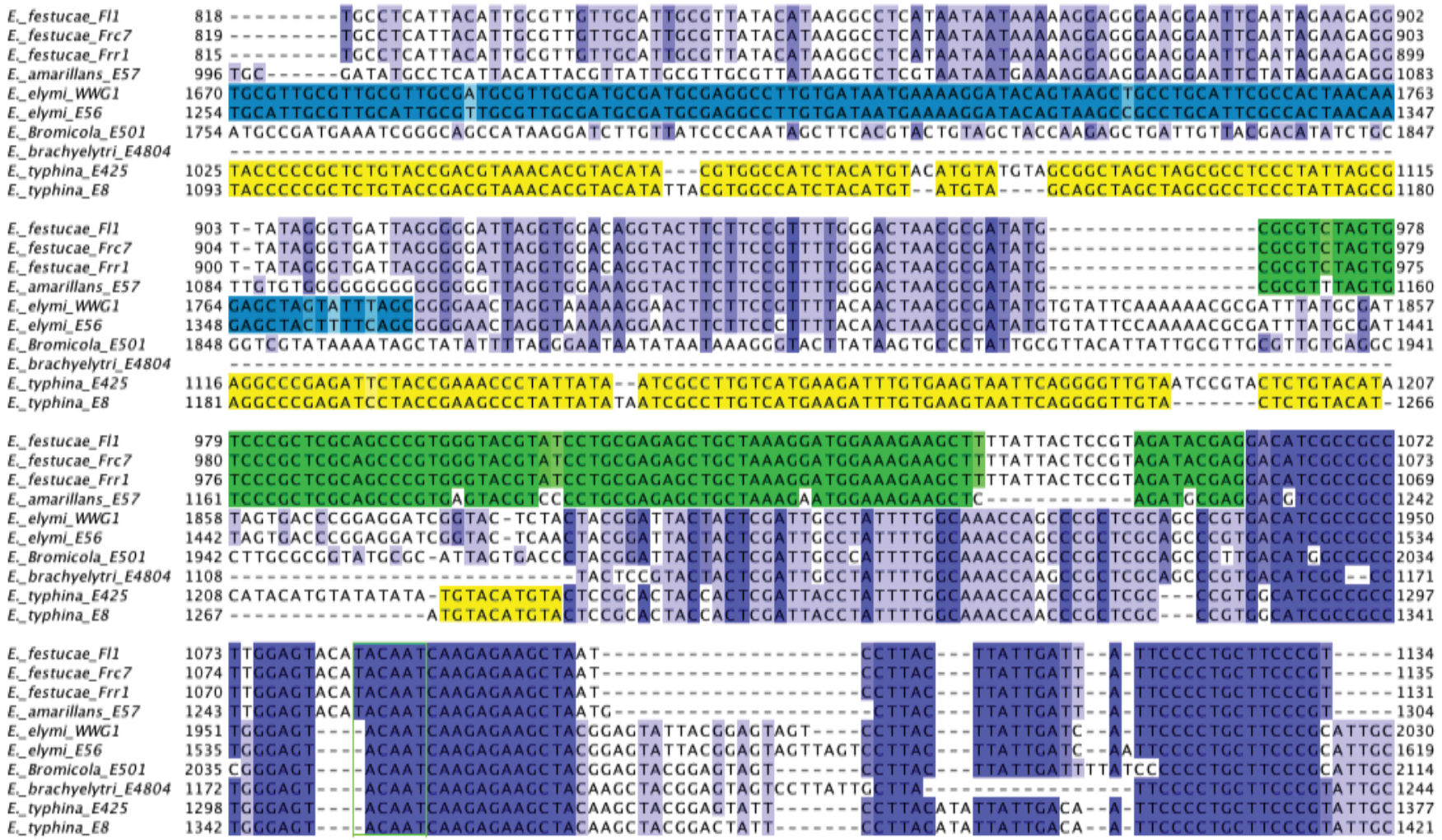
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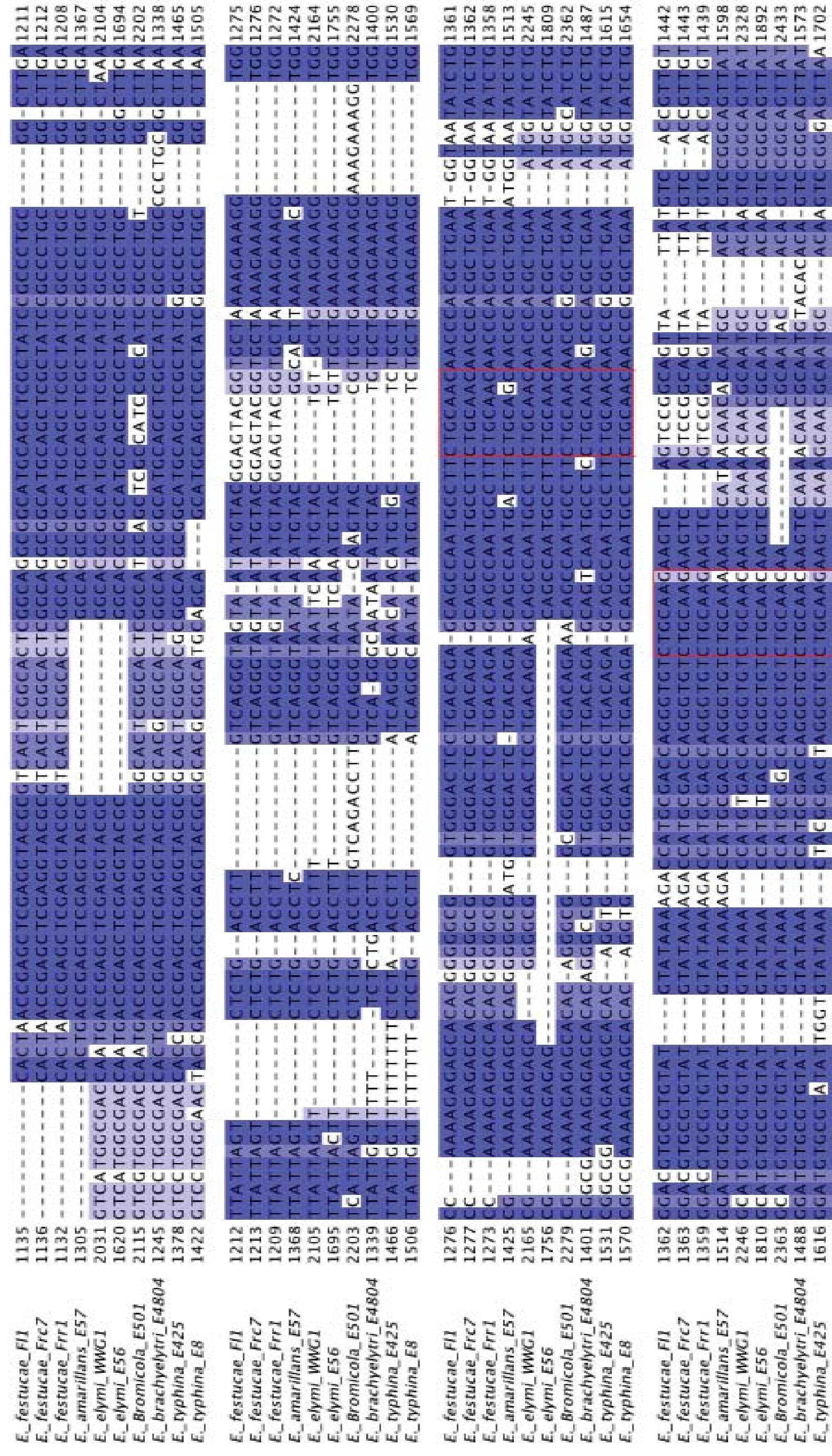

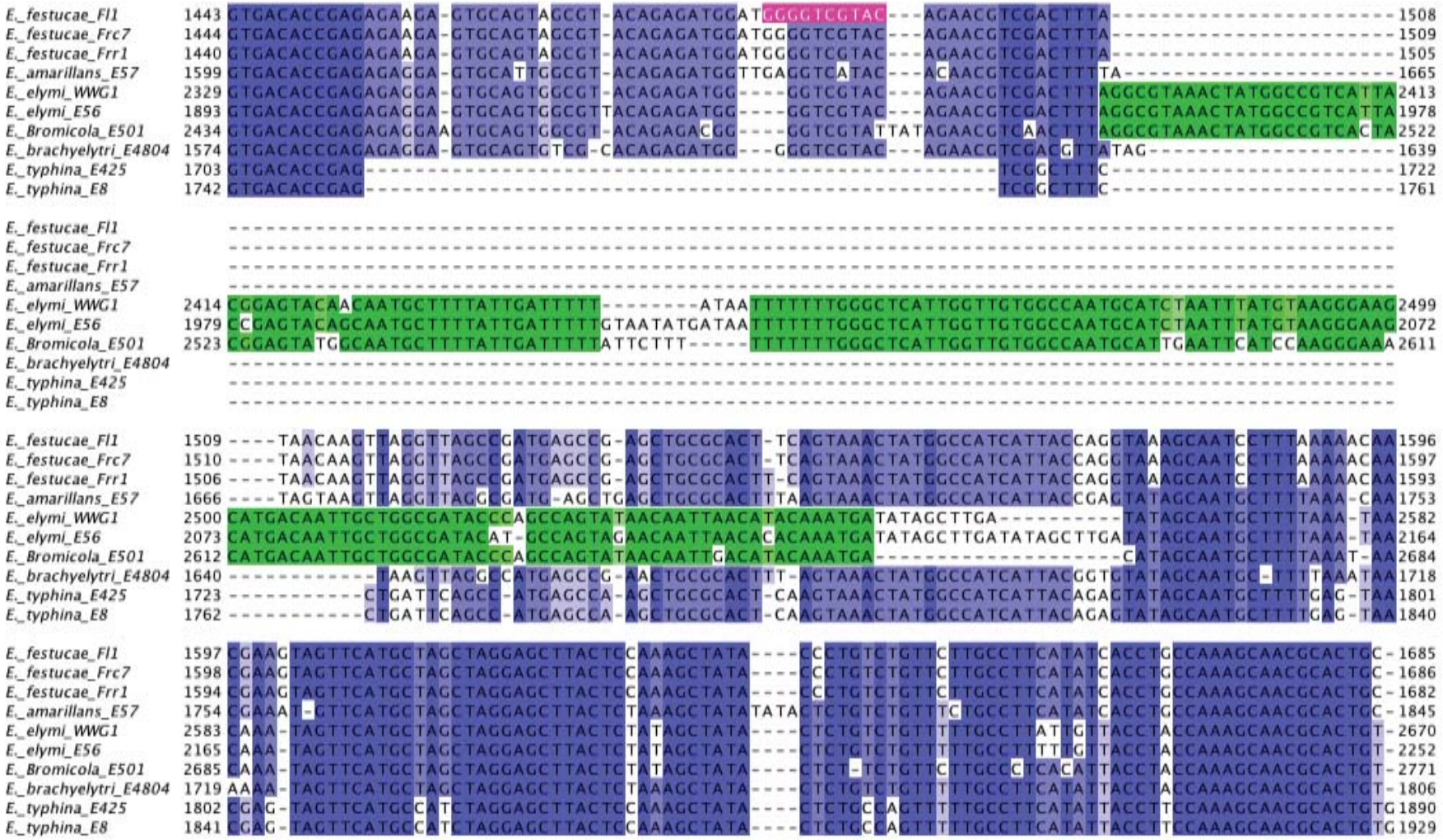
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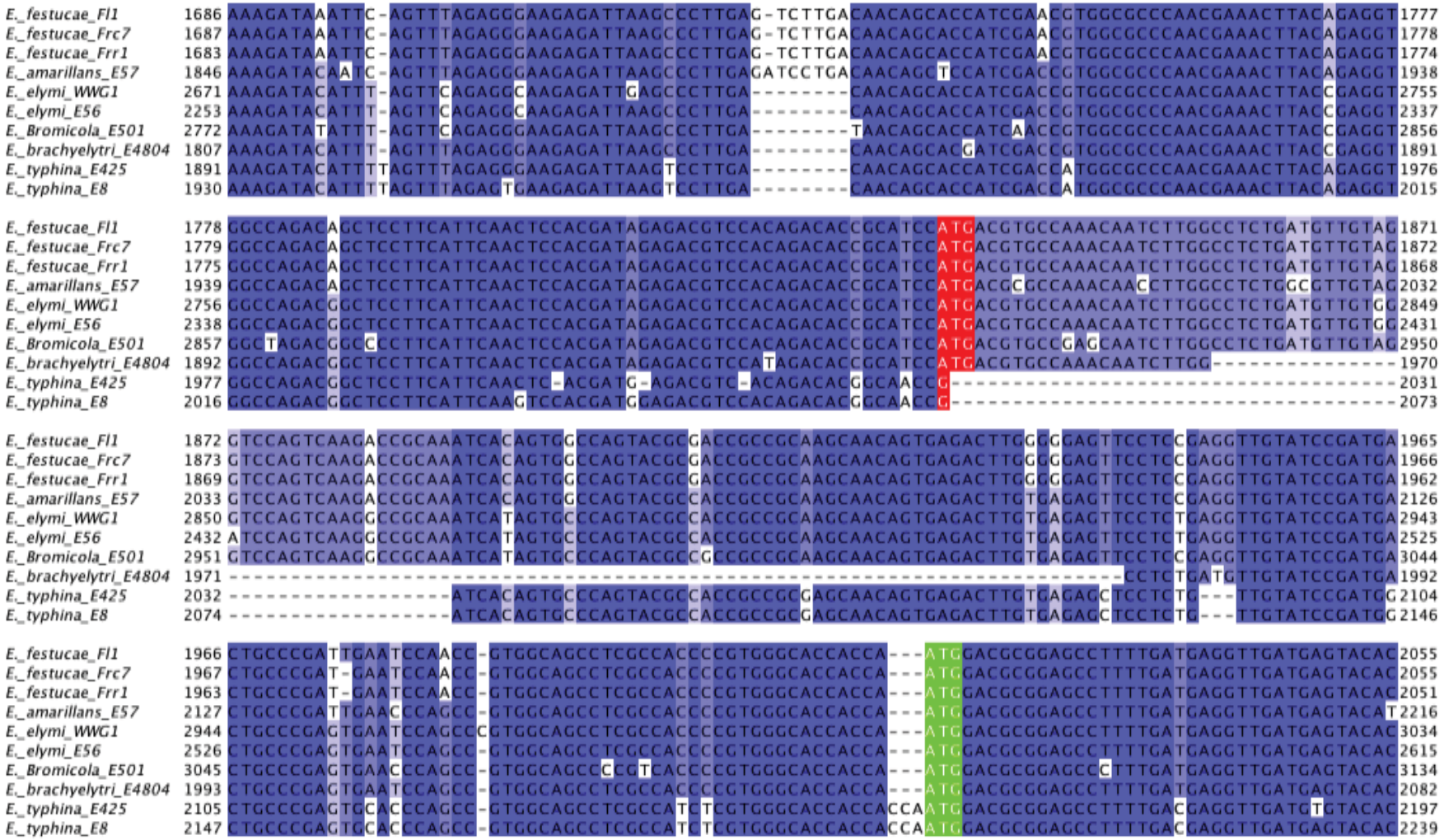
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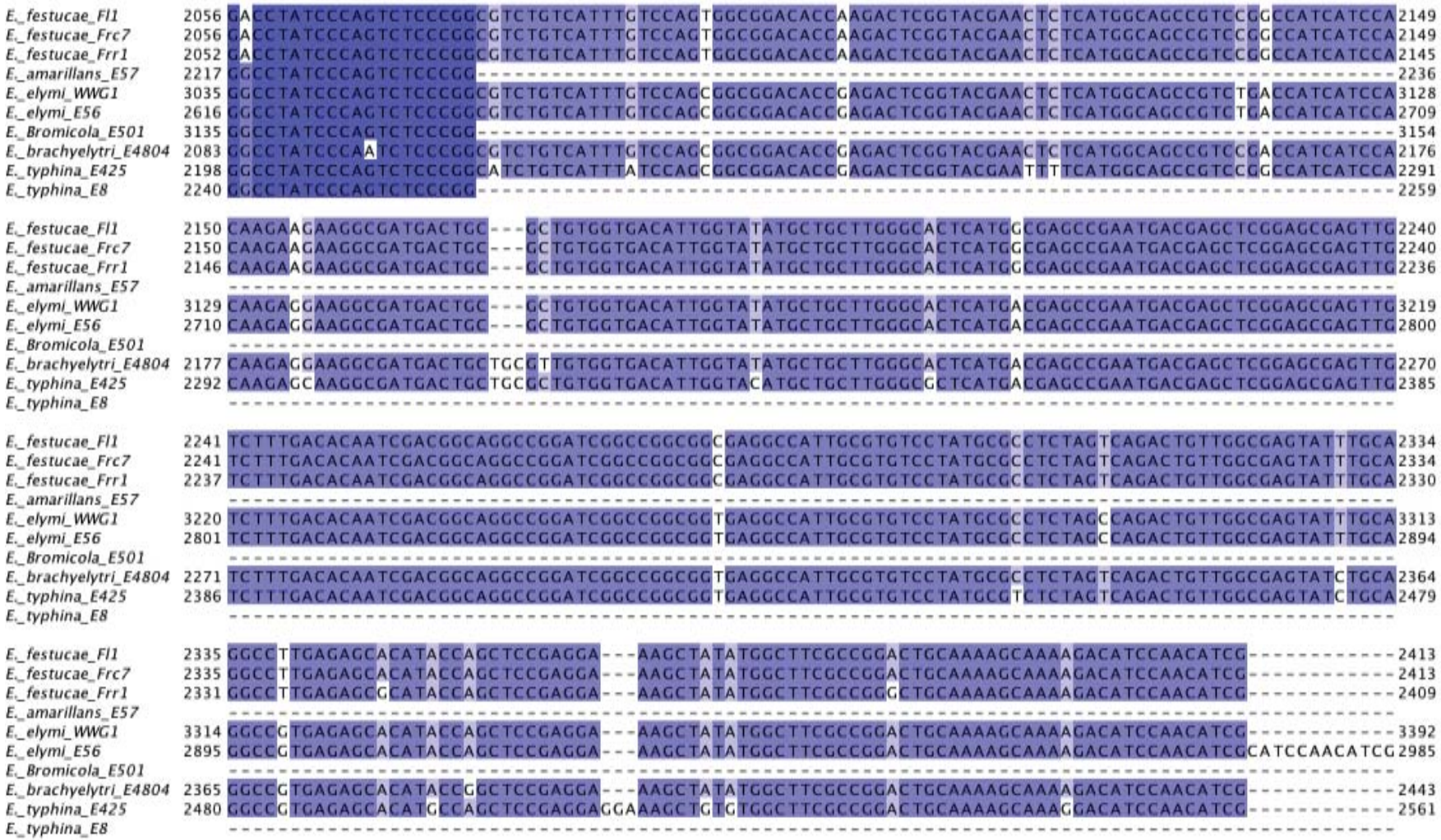
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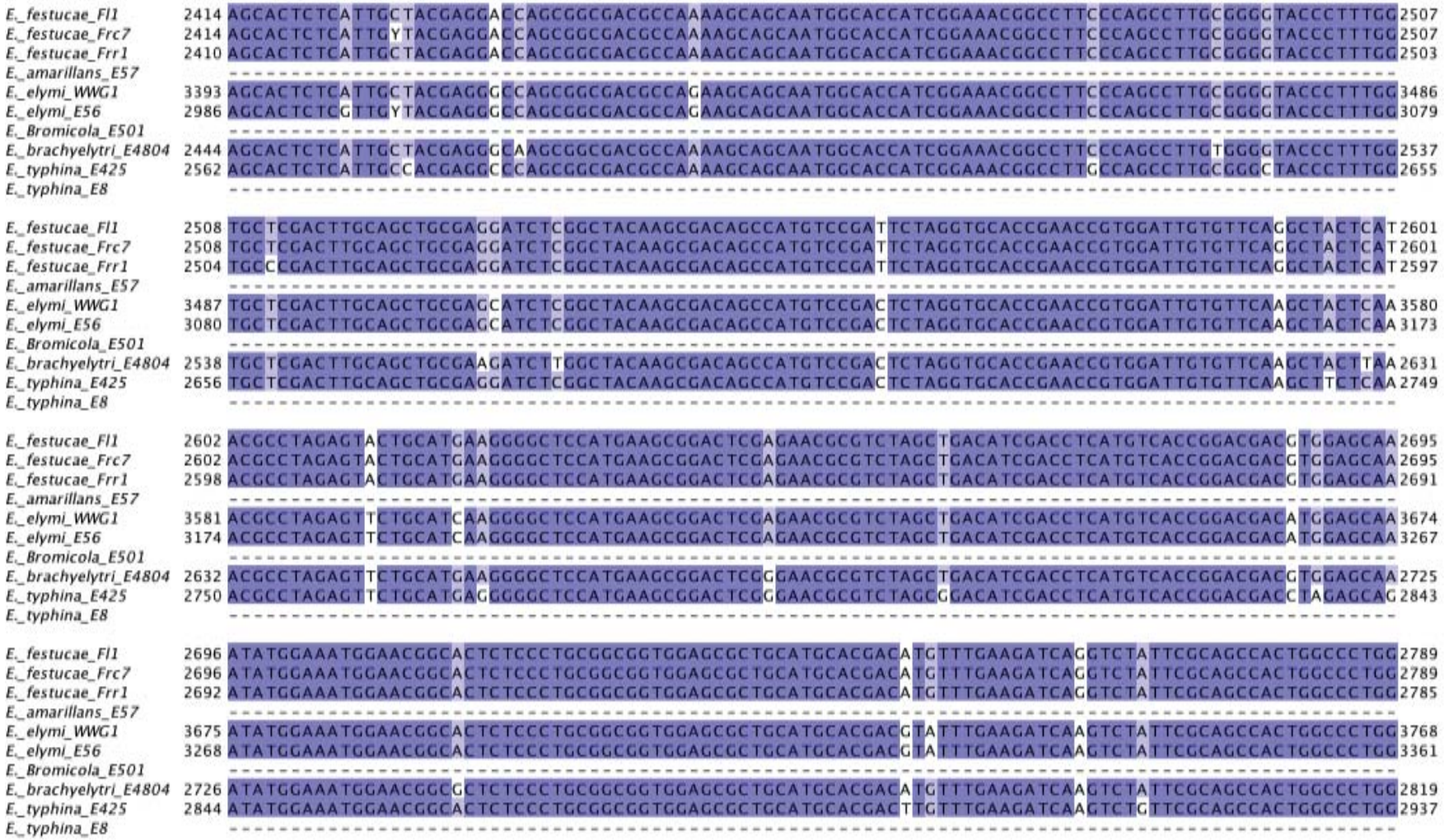
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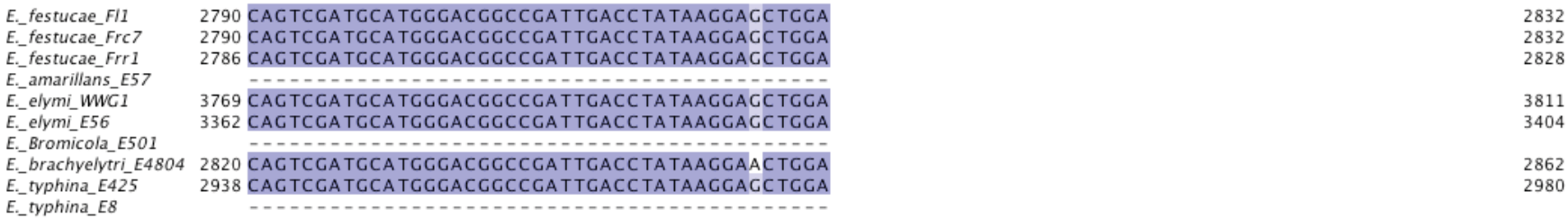
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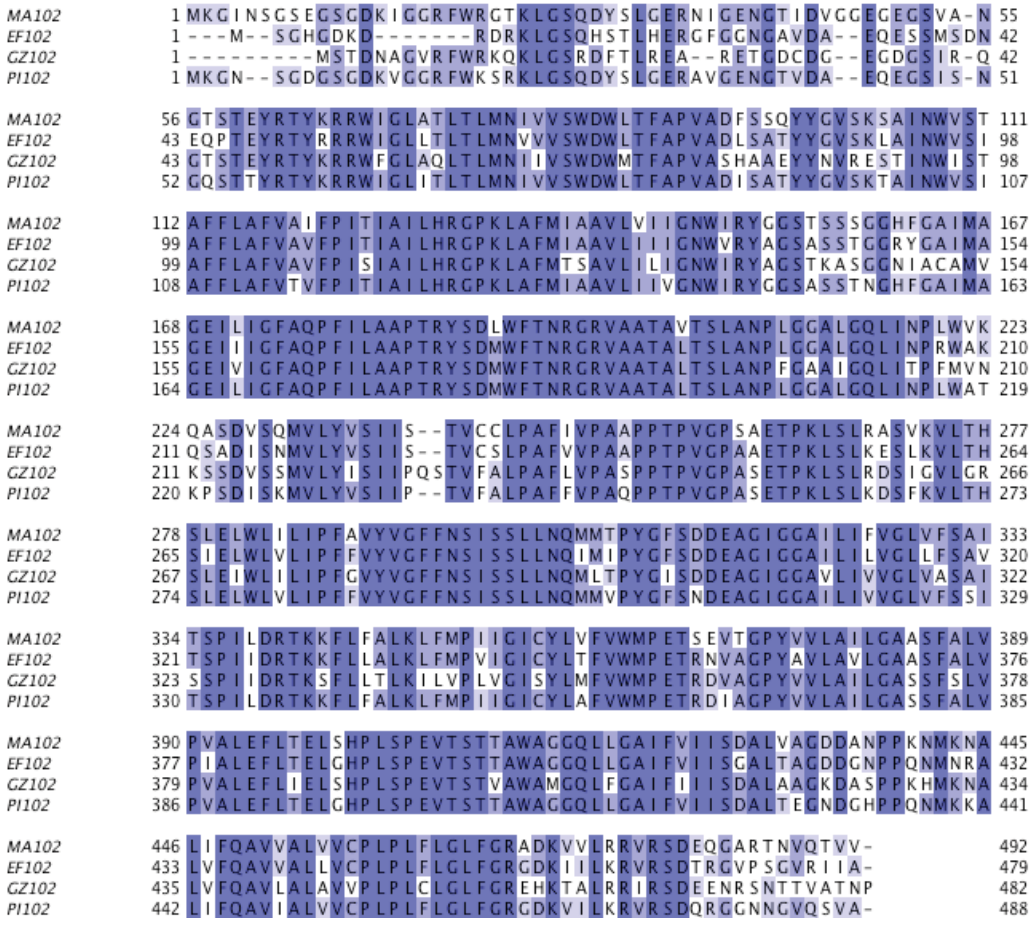
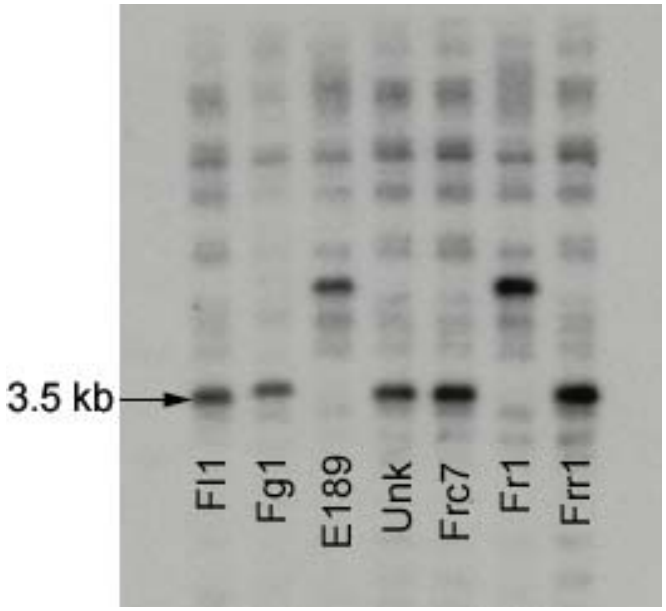
7.3.2 MSA of EF102 and homologues from *Periglandula Ipomea*, *Gibberella zeae* and *Metarhizium anisopliae*: N- and C-terminal domains show significant sequence variation.



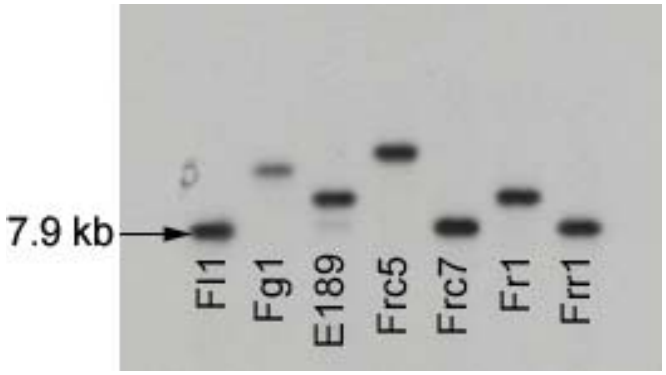
7.4 Supplemental Southern blots

7.4.1 Southern blot from Fig. 3.3 probed with *EF102*

7.4.2 Southern blot from Fig. 3.3 probed with *EF104*

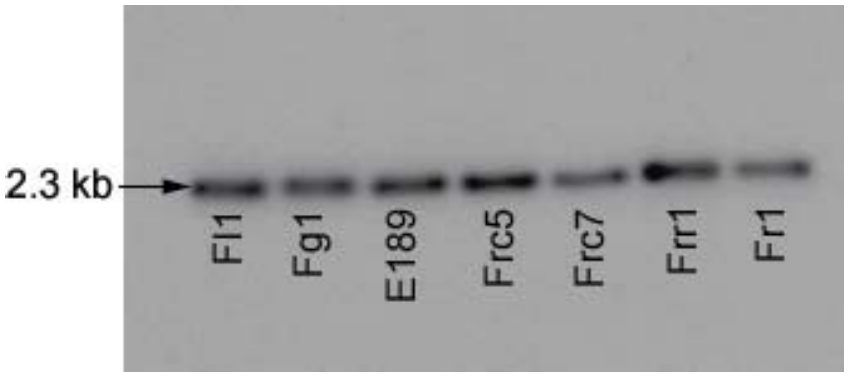


7.4.3 Southern blot from Fig. 3.4 probed with *EF102*

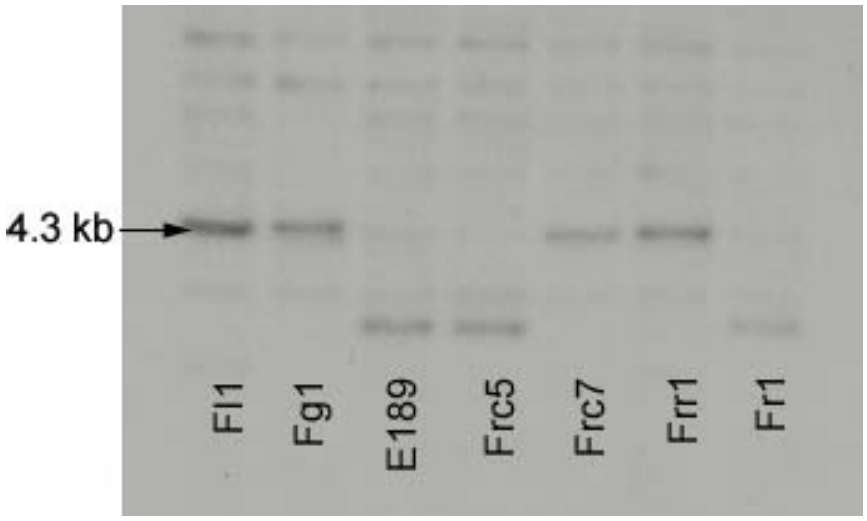


7.4.4 Southern blot from Fig. 3.4 probed with *EF104*

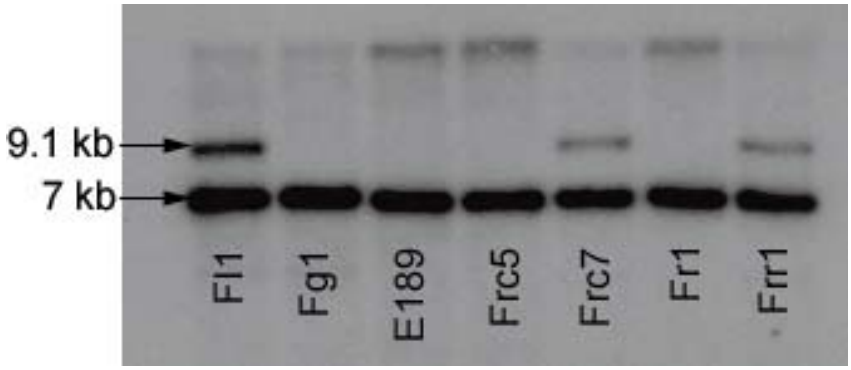
7.4.5 Southern blot from Fig. 3.5 probed with *EF102*



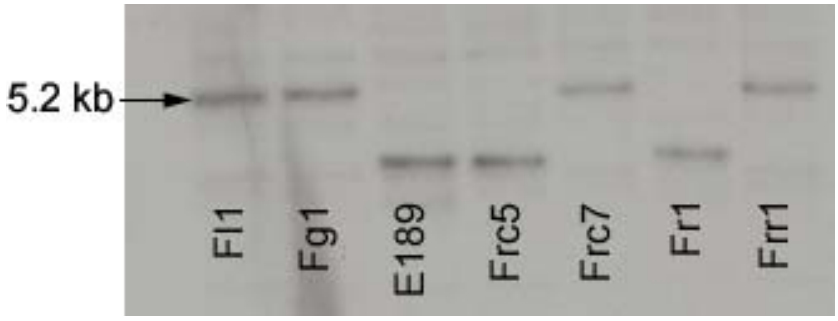
7.4.6 Southern blot from Fig. 3.5 probed with *EF104*



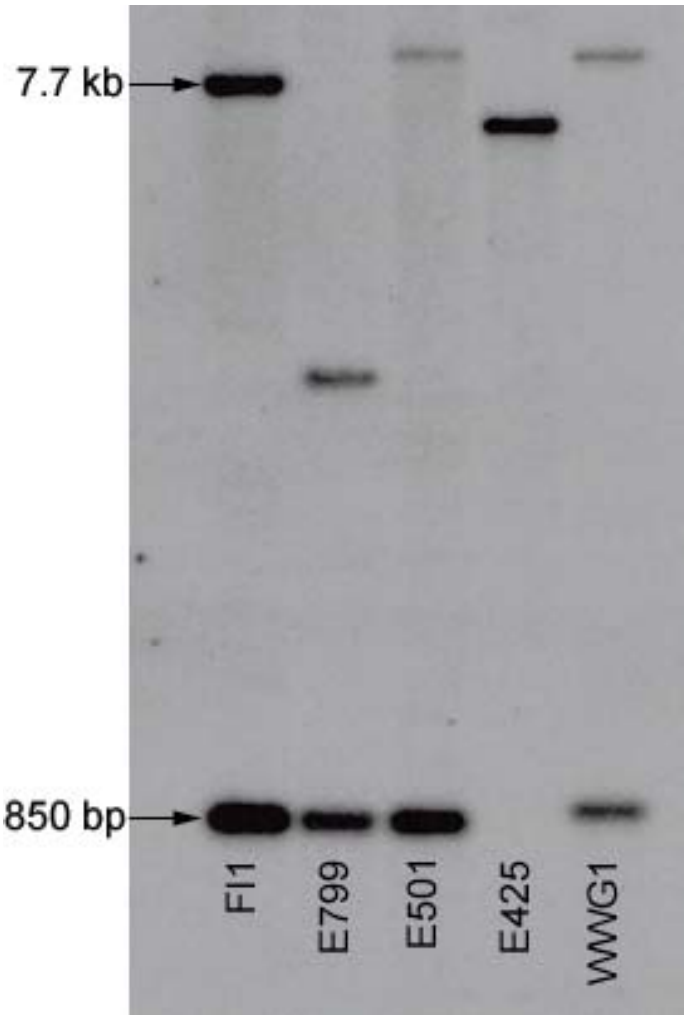
7.4.7 Southern blot from Fig. 3.6 probed with *EF102*



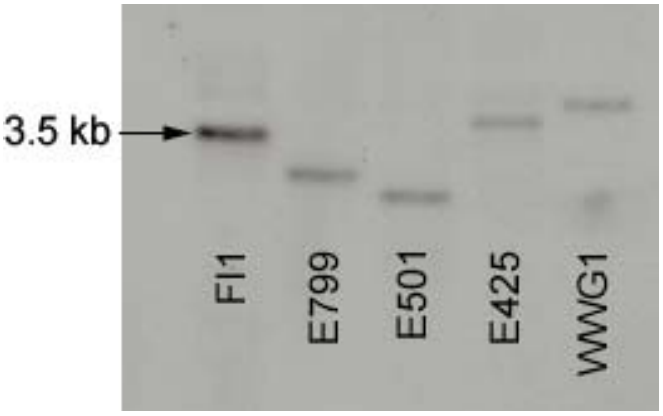
7.4.8 Southern blot from Fig. 3.6 probed with *EF104*



7.4.9 Southern blot from Fig. 3.7 probed with *EF102*



7.4.10 Southern blot from Fig. 3.7 probed with *EF104*



7.4.11 Southern blot from Fig 3.8 probed with *EF102*

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