Identification of NRPS gene families from *Neotyphodium/Epichloë* endophytic fungi that form mutualistic associations with cool season grasses.

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Abstract

Neotyphodium and *Epichloë* species are closely related asexual and sexual endophytic fungi, respectively, that form symbiotic associations with cool season grasses of the sub family Pooideae, including several important forage and turf grass species. The endophytes confer a number of advantages to their hosts, but also can cause animal toxicosis. These positive and negative effects are, in many cases, due to the production of fungal secondary metabolites. In filamentous fungi secondary metabolite genes are commonly clustered and are well documented to often contain non-ribosomal peptide synthetases (NRPSs) at their core. Members of this gene family encode large multifunctional proteins that synthesise a diverse range of bioactive compounds, many of which have been shown to serve as pathogenicity or virulence factors, in addition to suggested roles in niche adaptation. We have used a degenerate PCR approach to identify members of the NRPS gene family from *Neotyphodium* and *Epichloë* species, and have shown that at least 13 NRPS genes exist among these genomes. The distribution of these genes among different *Neotyphodium/Epichloë* lineages suggests that a common ancestor contributed most of the complement of NRPS genes, which have been either retained or lost during the evolution of these fungi.

Keywords: *Neotyphodium*, *Epichloë*, non-ribosomal peptide synthetase, NRPS

Introduction

Temperate grasses belonging to the family Poodieae commonly harbour fungal endophytes of the *Neotyphodium/Epichloë* genus. For example, *N. lolii* and *N. coenophialum* are fungal endophytes that live entirely within the intercellular spaces of perennial ryegrass and tall fescue, respectively. Infection is symptomless and the endophytes rely entirely on the host plant for dissemination via seed or through vegetative structures (see review by Schardl *et al*. 2004). The association is mutually beneficial since the endophyte confers a number of biotic and abiotic advantages to the host (Scott 2001; Schardl *et al*. 2004). Some of these host benefits are due to the production of fungal secondary metabolites such as peramine (pyrrolopyrazine), loline (aminopyrrolizidine), ergovaline (ergopeptine) and lolitrem (indole diterpene) alkaloids (Tanaka *et al*. 2005; Spiering *et al.* 2005; Panaccione *et al.* 2001; Young *et al.* 2006), which can also cause mammalian toxicoses in some cases.

In filamentous fungi secondary metabolite genes are commonly clustered (Keller & Hohn 1996) and are well documented to often contain non-ribosomal peptide synthetases (NRPSs) at their core (Walton 2006; Gardiner *et al.* 2004). Members of this gene family encode large multifunctional proteins (Finking & Marahiel 2004) that synthesise a diverse range of bioactive compounds, some of which serve as pathogenicity or virulence factors (Walton 2006; Johnson *et al*. 2000; Gardiner *et al.* 2004; Lee *et al*. 2005), in addition to suggested roles in niche adaption (Lee *et al*. 2005). In this study we sought to survey the numbers of NRPS genes from endophytes of the *Neotyphodium/Epichloë* genus. Two NRPS genes have already been shown to play important roles in this symbiosis; peramine and ergovaline are synthesised wholly or in part by NRPSs (Panaccione *et al.* 2001; Tanaka *et al.* 2005). In addition, we carried out a comprehensive strain distribution study across different taxonomic groupings of endophytes (Tsai *et al*. 1994; Moon *et al.* 2004), including those of non-hybrid, di-hybrid or tri-hybrid origin to gain an insight into the evolution of these genes within the *Neotyphodium/Epichloë* complex.

Degenerate PCR with primers designed to conserved motifs within NRPS genes, have been shown to be useful for identifiying members of this gene family from a number of filamentous fungi (Pannaccione 1996; Johnson *et al.* 2000). We used this approach in the present study to identify both conserved adenylation domains, characteristic of all classes of NRPSs, as well as methyl transferase domains, which are restricted to certain classes of NRPSs (Finking & Marahiel 2004) and are responsible for N-methylation of the peptide product.

Materials and Methods

Endophyte strains

Endophyte strains used in this study are listed in Table 2.

Genomic DNA

Genomic DNA was kindly provided by Andrew Griffiths (AgResearch Grasslands, New Zealand) and Professor Christopher Schardl (University of Kentucky, USA). DNAs were subsequently amplified using Genomiphi (Amersham) as per the manufacturer's instructions, and diluted to 100 µg per ml prior to use in PCR.

Degenerate PCR and cloning of NRPS fragments

Primers were designed to both conserved adenylation and methyl transferase domains as follows. The forward and reverse primers for the adenylation domain were designed to the conserved motifs Y G P T E and Y K T G D L, respectively as described in Johnson *et al.* (2000). For the methyl transferase primers, core motifs N S V A/V Q Y F P and I/V K/E/Q H V E V/I L/I P K were used to design forward and reverse primers respectively. Primer pairs were used in degenerate PCR experiments, with genomic DNA of *N. lolii* strains Lp19 and AR66, *E. typhina* strain E8, and taxonomic group FATG-3 strain AR501, as described in Johnson *et al.* (2000). PCR products were ligated into the pCRTOPO2.1 TA cloning vector (Invitrogen) and clones were sequenced using an M13R universal primer and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequences were run on an Applied Biosystems automated ABI3100 sequencer.

Bioinfomatics

Sequences were manually trimmed of vector sequence and degenerate primer binding sites were identified and removed. Sequence alignments were perfomed using AlignX from the vector NTI suite 9.0 (Invitrogen) and a dendrogram was constructed showing the relatedness between NRPS sequences.

Table 1 Classification of NRPS genes identified from endophytes of the *Neotyphodium/Epichloë* complex.

NRPS	e-value[2]	Top hit accession	Proposed function	Reference
1	2e-38	AAA74270	Toxin -like	This Study
2	2e-39	BAE06841	Siderophore	This Study
3	7e-16	BAE06838	Unknown	This Study
4	2e-29	BAE06845	Unknown	This Study
5	2e-22	BAE06845	Unknown	This Study
6	1e-16	XP_682495	Methylated peptide	This Study
7	2e-25	AAX09985	Methylated peptide	This Study
8 (PerA)	8e-49	BAE06845	Peramine synthesis	This Study; Tanaka et al. (2005)
9	2e-33	XP_385548	Siderophore	This Study
CPS1	3e-41	AAP12366	Virulence (CPS1-like)	This Study
LpsA	0	AAL26315	Ergovaline synthesis	Panaccione et al. (2002)
LpsB	0	CAD28788	Ergovaline synthesis	Fleetwood et al.(2007)
AC202	0	AAA74265	Unknown	Panaccione (1996)
AC203	0	AAA74268	Unknown	Panaccione (1996)

[1] Based on BLASTX analysis

Table 2 Pattern of distribution of NRPS genes across the *Neotyphodium/Epichloë* complex.

Strain	Species[1]	Hybrid components	---------------------- NRPS ----------------------									CPS	AC20		Lps		
			1	2	3	4	5	6	7	8	9	1	3	2	A	B	Tef
AR512	*N.coen.*	Ef x Et x Eb	+	+	-	-	+	-	-	+	+	+	+	+	+	+	+
AR535	*N.coen.*	Ef x Et x Eb	+	+	-	-	+	-	-	+	+	+	+	+	+	+	+
AR514	*N.coen.*	Ef x Et x Eb	+	+	-	-	+	-	-	+	+	+	+	+	+	+	+
AR525	*N.coen.*	Ef x Et x Eb	+	+	-	-	+	-	-	+	+	+	+	+	+	+	+
AR546	*N.coen.*	Ef x Et x Eb	+	+	+	-	+	-	-	+	+	+	+	+	+	+	+
AR567	*N.coen.*	Ef x Et x Eb	+	+	+	-	+	-	-	+	+	+	+	+	+	+	+
RS2	*N.coen.*	Ef x Et x Eb	+	+	+	-	+	-	-	+	+	+	+	+	+	+	+
RS6	*N.coen.*	Ef x Et x Eb	+	+	+	-	+	-	-	+	+	+	+	+	+	+	+
AR604	*N.coen.*	Ef x Et x Eb	+	+	+	-	+	-	-	+	+	+	+	+	+	+	+
AR542	*N.coen.*	Ef x Et x Eb	+	+	+	-	+	-	-	+	+	+	+	+	-	-	+
AR584	*N.coen.*	Ef x Et x Eb	+	+	+	-	+	-	-	+	+	+	+	+	-	-	+
AR593	*N.coen.*	Ef x Et x Eb	+	+	+	-	+	-	-	+	+	+	+	+	-	-	+
AR565	*N.coen*	Ef x Et x Eb	+	+	+	-	+	-	-	+	+	+	+	+	+	+	+
AR553	FaTG-2	Ef x Eb	+	+	-	-	+	-	-	+	+	+	+	-	+	+	+
AR555	FaTG-2	Ef x Eb	+	+	-	-	+	+	-	+	+	+	+	-	+	+	+
AR501	FaTG-3	Et x Eb	+	+	-	-	+	+	+	+	+	+	+	+	-	-	+
AR502	FaTG-3	Et x Eb	+	+	-	+	+	+	+	+	+	+	+	+	-	-	+
AR506	FaTG-3	Et x Eb	+	+	-	-	+	+	+	+	+	+	+	-	-	-	+
AR507	FaTG-3	Et x Eb	+	+	-	-	+	+	+	+	+	+	+	+	-	-	+
AR510	FaTG-3	Et x Eb	+	+	-	-	+	+	+	+	+	+	+	+	-	-	+
AR26	LpTG-2	Et x Nl	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
Lp1	LpTG-2	Et x Nl	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
E938	*N.aust.*	Et x Ef	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
E1169	*N.tem.*	Et x Ef	+	+	-	+	+	+	+	+	+	+	+	+	-	-	+
Fp1	*N.unc.*	Et x Ebr	+	+	-	+	+	+	-	+	+	+	+	+	-	-	+
AR1002	*N.unc.*	Et x Ebr	+	+	-	+	+	+	-	+	+	+	+	+	-	-	+
Fp3	*N.unc.*	Et x Ebr	+	+	-	+	+	+	-	+	+	+	+	+	-	-	+
Fp5	*N.unc.*	Et x Ebr	+	+	-	+	+	+	-	+	+	+	+	+	-	-	+
AR1007	*N sieg.*	Ef x Ebr	+	+	-	+	+	+	-	+	+	+	+	+	-	-	+
E915	*N.sieg.*	Ef x Ebr	+	+	+	+	+	+	-	+	+	+	+	+	-	-	+
E822	*N. mel.*	Ef x Na	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
AR1501	*E. fes.*	Non-hybrid	+	+	-	-	+	-	-	+	+	+	+	-	+	+	+
Frr1	*E. fes.*	Non-hybrid	+	+	+	-	+	-	+	+	+	+	+	-	+	+	+
AR40	*N. lolii*	Non-hybrid	+	+	-	-	+	-	-	+	+	+	+	-	-	-	+

Strain	Species[1]	Hybrid components	NRPS									CPS	AC20		Lps		
			1	2	3	4	5	6	7	8	9	1	3	2	A	B	Tef
AR1	*N. lolii*	Non-hybrid	+	+	-	-	+	+	-	+	+	+	+	-	+	+	+
AR12	*N. lolii*	Non-hybrid	+	+	-	-	+	+	-	+	+	+	+	-	+	+	+
AR56	*N. lolii*	Non-hybrid	+	+	-	-	+	+	-	+	+	+	+	-	+	+	+
AR59	*N. lolii*	Non-hybrid	+	+	-	-	+	+	-	+	+	+	+	-	+	+	+
AR29	*N. lolii*	Non-hybrid	+	+	-	-	+	+	-	+	+	+	+	-	+	+	+
AR30	*N. lolii*	Non-hybrid	+	+	-	-	+	+	-	+	+	+	+	-	+	+	+
AR42	*N. lolii*	Non-hybrid	+	+	+	-	+	+	-	+	+	+	+	-	+	+	+
AR47	*N. lolii*	Non-hybrid	+	+	+	-	+	+	+	+	+	+	+	-	-	-	+
AR48	*N. lolii*	Non-hybrid	+	+	+	-	+	+	+	+	+	+	+	-	-	-	+
AR49	*N. lolii*	Non-hybrid	+	+	+	-	+	+	+	+	+	+	+	-	-	-	+
AR64	*N. lolii*	Non-hybrid	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+
AR17	*N. lolii*	Non-hybrid	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
AR66	*N. lolii*	Non-hybrid	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
E8	*E. typh.*	Non-hybrid	+	+	-	+	+	+	-	+	+	+	+	+	-	-	+
E505	*E. typh.*	Non-hybrid	+	+	-	+	+	+	-	+	+	+	+	+	-	-	+
-	*N. aot.*	Non-hybrid	+	+	-	+	+	+	-	+	+	+	-	+	-	-	+
E354	*E. sylv.*	Non-hybrid	+	+	-	+	+	+	-	+	+	+	+	+	-	-	+
E503	*E. sylv.*	Non-hybrid	+	+	-	+	+	+	-	+	+	+	+	+	-	-	+
E422	*E. clark.*	Non-hybrid	+	+	-	+	+	-	-	+	+	+	+	+	+	+	+
E2772	*E.gl.*	Non-hybrid	+	+	-	+	+	-	-	+	+	+	+	-	+	+	+
E799	*E.br.*	Non-hybrid	+	+	-	+	+	+	-	+	+	+	+	+	-	-	+
E501	*E. br.*	Non-hybrid	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+
E248	*E.ba*	Non-hybrid	+	+	-	+	+	+	-	+	+	+	+	-	+	+	+
E1031	*E.ba.*	Non-hybrid	+	+	-	+	+	+	-	+	+	+	+	-	-	-	+
E57	*E.am.*	Non-hybrid	+	+	-	+	+	+	-	+	+	+	+	-	+	+	+
E52	*E.am.*	Non-hybrid	+	+	-	+	+	+	+	+	+	+	+	-	-	-	+
E1040	*E.b.*	Non-hybrid	+	+	-	+	+	+	-	+	+	+	+	-	+	+	+

[1]*N.coen.- N. coenophialum; N.aust.-N. australiense; N.tem.-N. tembledera e; N.unc.- N. uncinatum; N.sieg. – N. siegelii; N.mel.- N. melicola; E. fes.-E. festucae; E.typh.–E. typhina; N. aot.-N. aotearoae; E. sylv.-E.sylvatica; E.clark.-E. clarkii; E.gl. – E. glyceriae; E.br.-E. bromicola; E.ba.- E. baconii; E.a- E. amirillans; E.b.- E. brachyeltri*

Strain distribution studies

Gene specific primers were designed to NRPS related sequences identified in this study, to a further NRPS gene (here designated NRPS9) identified from an *N. lolii* EST library (Johnson *et al.* 2007), to additional sequences previously published (Panaccione 1996; Panaccione *et al.* 2001) and to *LpsB*, a second NRPS identified in the ergovaline biosynthetic gene cluster (Fleetwood *et al.* 2007). These primers were used in PCR experiments with different endophyte strains to determine the distribution of the different NRPS sequences across the *Neotyphodium/Epichloë* complex. Primers designed to the conserved translation elongation factor gene were used to verify the integrity of the DNA in PCR experiments. Primer pairs were used in PCR experiments using standard conditions.

Results and Discussion

Cloning, sequencing and bioinfomatics of NRPS fragments

Degenerate PCR, using primers designed to either adenylation (A) or methyl transferase (MT) domain motifs, both yielded products of the target size, which ranged between 250 bp and 350 bp (data not shown). After cloning, 18 A domain and 6 MT domain NRPS fragments were sequenced for each of the four strains used in the degenerate PCR experiments. Sequence alignment revealed that the NRPS sequences fell into eight distinct clades (Figs. 1A & 1B). NRPS sequences that fell within the same clade differed by the presence of species specific SNPs, which in some instances corresponded to multiple copies of the gene within hybrid genomes. A further unique sequence was also identified from the degenerate PCR screen which had homology to a related adenylate forming enzyme, but is not strictly an NRPS. Due to the multi-modular nature of NRPS genes, probes corresponding to NRPSs representative of the different clades were used to screen a *N. lolii* BAC library. For NRPSs represented in this library, hybridisation to different BAC clones indicated that the NRPS fragments most likely represented independent NRPS genes (data not shown) rather than components of the same gene. Because some NRPS sequences were specific to strains other than Lp19, not all of them were represented in the BAC library. However, classification based on homology to other reported NRPS sequences and their strain distribution in the present study provided evidence that they were likely to be independent of each other.

NRPS classification

Based on BLASTX analysis against the non redundant GenBank databases and the classifications of Lee *et al*. (2005), we determined that the NRPS gene fragments identified in this study corresponded to several different classes (Table 1). These include NRPSs involved in the biosynthesis of siderophores, toxins involved in pathogenesis, N-methylated peptides, and several that could not be classified. In addition we also identified a degenerate PCR sequence with homology to *CPS1* (Lu *et al.* 2003), that

Figure 1A Relationship between NRPS adenylation domains cloned by degenerate PCR

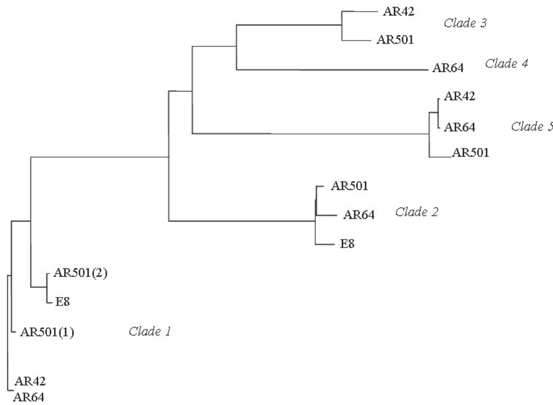
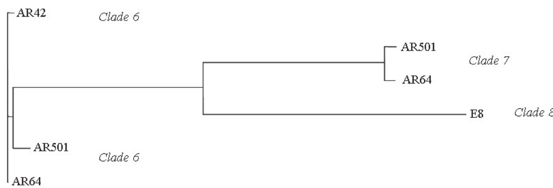


Figure 1B Relationship between NRPS methyl transferase domains cloned by degenerate PCR



whilst not strictly an NRPS has a proven role in pathogenicity across a wide range of fungal genera. Using primers designed specifically to methyl transferase domains, a feature present only in some NRPS systems, we were able to identify three NRPSs related to this class. One of these, NRPS8, matched the sequence for the methyl transferase domain of *perA*, a NRPS shown to be required for peramine biosynthesis in *Epichloë festucae* (Tanaka *et al.* 2005). A further NRPS sequence (NRPS9) was independently identified from an *N. lolii* EST library (Johnson *et al*. 2007). Additional endophyte NRPS genes that have been previously reported but not identified in the present study were *lpsA* (Panaccione *et al*. 2001), and *LpsB* (Fleetwood *et al*. 2007) which are both involved in ergovaline biosynthesis, as well as AC203 and AC202 (Panaccione 1996), NRPSs with unknown function. Some of the NRPS gene fragments reported here matched degenerate PCR products cloned in other studies, such as NRPS2 which matches ps4 (Tanaka *et al.* 2005), NRPS1 which matches AC205 and ps7 (Panaccione 1996; Tanaka *et al*. 2005), and NRPS8 which matches AC406 and *perA* (Panaccione 1996; Tanaka *et al.* 2005).

Strain distribution study

Results of a PCR based strain distribution study are presented in Table 2. Some NRPS genes (1, 2, 5, 8, 9, AC203), as well as a sequence related to *CPS1*, showed a global pattern of distribution indicating they have been strongly conserved during the evolution of these fungi. Apart from NRPS8 (*perA*) which has been shown to be required for the synthesis of the anti-insect feeding deterrent peramine (Tanaka *et al*. 2005) the functions of the other conserved NRPSs identified in this study have not previously been assigned. However, their global retention within the genomes of *Neotyphodium/Epichloë* species suggests they have been strongly selected for during symbiosis of these endophytes with their grass hosts and presumably play a role in these associations. We have now shown that NRPS2 and NRPS4 are involved in the biosynthesis of siderophores – essential, low molecular weight, iron-chelating agents (Johnson *et al*. 2007). For the remainder of the NRPS genes (3, 6, 7, 4, AC202, *lpsB* and *lpsA*), their distribution across the strain collection was inconsistent. These NRPS genes do not therefore have essential roles in these fungi. The absence of some NRPS genes from certain endophyte species is most likely to be due to gene loss through speciation or hybridisation. It is likely that NRPS genes with essential functions such as NRPS2, involved in siderophore biosynthesis, have been globally retained, whilst others with non-critical function have been lost from some strains. Interestingly, the *N. coenophialum* strains have equal or lower numbers of NRPS genes compared to non-hybrid species, despite the fact that they are tri-hybrid in origin. For example, NRPS4, NRPS6 and NRPS7 are all absent in *N. coenophialum* despite these genes being present in their component genomes. This strongly suggests that NRPS loss is commonly associated with hybridisation events between different endophyte lineages. An alternative hypothesis for the discontinuous distribution of some NRPS genes is that they were not present in a common ancestor and so instead of being lost during the evolution of these fungi they were introduced into multiple species through horizontal gene transfer (Walton 2000). However, given the numerous instances that this would need to occur, and the lack of strong evidence for such events in filamentous fungi, this scenario seems unlikely.

Conclusions

Our results indicate that *Neotyphodium/Epichloë* species contain at least 13 NRPS genes, including members (for example, NRPS2) that are conserved within the ascomycetes, as well as some that appear to be unique to these grass endophytes. This suggests that *Neotyphodium/Epichloë* species are likely to synthesise a much greater number of small peptide secondary metabolites than has currently been identified. Further studies are currently in progress to functionally characterise the additional novel NRPS genes identified in this study with the aim to identify possible roles during *Neotyphodium/Epichloë* symbioses with their grass hosts, as well as to discover the potential secondary metabolite end products.

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