Hybrid fungal endophytes symbiotic with the grass *Lolium pratense*

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Classification of endophytes inhabiting *Lolium pratense* (= *Festuca pratensis*) based on morphological and molecular phylogenetic characteristics is reported. From an extensive survey of *L. pratense* collected throughout Europe, we concluded that the overwhelmingly predominant endophyte was *Neotyphodium* x *uncinatum*. We constructed phylogenies based on three unlinked, nuclear genes and analyzed allozymes to evaluate the relationship of *N.* x *uncinatum* to *Epichloë* endophytes. Our results suggest that *N.* x *uncinatum* is an interspecific hybrid of two *Epichloë* species. A second, much rarer hybrid endophyte was isolated from *L. pratense* which may have shared one of its two *Epichloë* ancestors with *N.* x *uncinatum*. This second isolate is a previously undescribed species for which we propose the name *Neotyphodium* x *siegelii*.

Keywords: Clavicipitaceae, *Epichloë*, Hypocreales, *Neotyphodium*, *Festuca pratensis*.

Fungal symbionts in the genus *Neotyphodium* are mutualistic with their cool-season grass hosts. These fungi grow systemically between plant cells in the vegetative and reproductive tissues of the host grass without inducing disease (Schardl & Phillips, 1997). As the grass matures and flowers, the endophyte grows into the developing ovule of the host inflorescence and is transmitted asexually via the seed to the next generation. This close correspondence between host and symbiont life cycles likely has fostered many dramatic fitness enhancements that some grasses infected with *Neotyphodium* endophytes possess. Among the benefits provided by these fungi to their hosts are improved drought tolerance and competitive ability (Arechevaleta & al., 1989), enhanced tillering (Belesky & al., 1987), and potent chemical defense against a variety

of insect and mammalian herbivores (Kennedy & Bush, 1983; Clay, 1990). In fact, two such *Neotyphodium* species have been established as the causal agents of 'fescue toxicosis' in cattle (Bacon & al., 1977; Schmidt & al., 1982) and 'ryegrass staggers' in sheep (Fletcher & Harvey, 1981; Latch & al., 1984). The endophyte's ability to provide such dramatic defense against herbivory is attributable to a suite of novel secondary metabolites which are produced both *in planta* and in culture (Porter, 1994; Rowan & Latch, 1994).

Neotyphodium endophytes are asexual, and are derived from sexual relatives in the genus *Epichloë*, the causative agents of grass choke disease (Schardl & Clay, 1997). Also causing no symptoms for most of the grass life cycle, *Epichloë* species can initiate a pathogenic stage concurrently with the onset of host flowering. Some or all infected tillers are effectively sterilized and a fungal stroma emerges from which spermatia are transferred by a fly vector (genus *Botanophila*) to stromata on other grass plants (Bultman & al., 1998). Successful mating occurs if the spermatia and recipient stromata are of the opposite mating type (heterothallic) and of the same biological species (White & Bultman, 1987). Successful fertilization results in the production of mature perithecia containing ascospores which are forcibly ejected. Ascospores landing on nearby plants germinate and colonize vegetative tissues or developing inflorescences, giving rise to newly infected plants or seeds (Chung & Schardl, 1997; Brem & Leuchtmann, 1999). Some species of *Epichloë* choke every inflorescence and thus represent the antagonistic extreme in these interactions, whereas others have more balanced, and likely more co-evolved, relationships in which only some of the inflorescences are choked (Schardl & al., 1997). In the balanced interactions the rest of the flowering tillers develop normally and result in viable endophyte infected seeds, similar to vertical transmission characterizing the closely related *Neotyphodium* species.

Due to lack of an obvious sexual stage, identification and classification of *Neotyphodium* endophytes has been based primarily on morphological characteristics. Members of this genus are typically slow growing in culture (Morgan-Jones & Gams, 1982; Latch & al., 1984) and are seemingly restricted to endophytic, intercellular growth in their cool season grass hosts (Schardl & Phillips, 1997). Within the genus *Neotyphodium*, some useful morphological variation for discriminating distinct species can be detected in comparison of colony morphology and growth rate in culture, as well as shapes and dimensions of conidia and conidiophores (Morgan-Jones & Gams, 1982; Latch & al., 1984; White & Cole, 1986; White & Cole, 1987). However, little phenotypic variation exists between many of these *Neotyphodium* species, and

the utilization of gene sequence data has become increasingly important (Schardl & al., 1991; Tsai & al., 1994).

Obvious similarities in growth habits, morphological characteristics and serology have prompted investigation into the relationships between *Epichloë* and *Neotyphodium* species at the molecular level. Southern blot analysis has shown that some asexual *Neotyphodium* species contain multiple copies of the β-tubulin gene (*tub2*), whereas each *Epichloë* species contains a single copy (Schardl & al., 1994; Tsai & al., 1994). Surprisingly, for asexual species with multiple *tub2* copies, the phylogenetic pattern fails to group the copies together as would be characteristic of gene duplication and divergence. Instead, each of the multiple *tub2* copies aligns most closely with that of a different *Epichloë* species (Tsai & al., 1994; Schardl & al., 1994). Such a pattern is consistent with the notion that many *Neotyphodium* endophytes are interspecific hybrids of their sexual *Epichloë* relatives, and the list of such *Neotyphodium* hybrids (appropriately designated with a 'x' preceding the species name) continues to grow. For example, *Neotyphodium* x *coenophialum* (Morgan-Jones & Gams) Glenn, Bacon, & Hanlin, a common endophyte in *Lolium arundinaceum* (Schreber) S. J. Darbyshire (= *Festuca arundinacea* Schreb.; tall fescue; Darbyshire, 1993) and the causal agent of fescue toxicosis, is a hybrid of at least three *Epichloë* species: *Epichloë festucae* Leuchtmann, Schardl, & Siegel, *Epichloë baconii* J.F. White, and *Epichloë typhina* (Pers. : Fr.) Tul. (Tsai & al., 1994).

In this paper, we utilize morphological and molecular phylogenetic methods to characterize those endophyte inhabitants of *Lolium pratense* (Huds.) S. J. Darbyshire (= *Festuca pratensis* Huds.; meadow fescue; Darbyshire, 1993) that belong to the *Epichloë/Neotyphodium* group. Among these is *Neotyphodium* x *uncinatum* (W. Gams, Petrini & Schmidt) Glenn, Bacon, & Hanlin, the predominant asexual endophyte of *L. pratense* in our survey and among the highest known producers of loline alkaloids, which are potent neurotoxins effective against a variety of insect herbivores (Bush & al., 1997). Interestingly, *N.* x *uncinatum* contains a single *tub2* gene likely derived from an *E. typhina* genotype known only from the host, *Poa nemoralis* L. (Tsai & al., 1994; Moon & al., 2000). We examined two additional genes, the translation-elongation factor 1-α gene (*tef1*) and the actin gene (*act1*), to test whether there may be additional *Epichloë* ancestors in the evolution of *N.* x *uncinatum*. We also characterized an additional isolate from *L. pratense*; a new *Neotyphodium* hybrid with *E. festucae* and *Epichloë bromicola* Leuchtmann & Schardl ancestry.

Materials and methods

Biological materials

Tab. 1 lists the fungal isolates used in this study. An isolate of unknown identity [American Type Culture Collection (ATCC) 74483] was from *L. pratense* Plant Introduction (PI) accession 237707 (Western Regional PI Station; origin: Germany; cooperator: van Schmieder, M., Saatzuchtwirtschaft). Endophyte isolation was from infected plant material as described elsewhere (An & al., 1993; Schardl & An, 1993). Single conidiospore isolation was achieved through streaking conidia on potato dextrose agar (PDA; Difco, Detroit, Michigan) three times in succession. After each streak, plates were monitored daily by microscopic examination with colonies arising from germinating conidia chosen for the next round of streaking. Isolates were deposited in Centraalbureau Voor Schimmelcultures (CBS) and ATCC. Specimens were deposited at the Cornell University Plant Pathology Herbarium (CUP). Gene sequences were deposited in GenBank. Symbiotic associations were re-established between *L. pratense* and isolate ATCC74483 by seedling inoculation and serological screening, as previously described (An et al., 1993; Chung & al., 1997). Accessions of *L. pratense* were generously provided by I.D. Thomas (Grassland and Environmental Research, Aberystwyth, Wales).

Mating tests

Plants naturally infected with *E. festucae* strains of both mating types (Leuchtmann & al., 1994) were vernalized over winter in Lexington, Kentucky, to induce stroma formation. Similarly, plants with both mating types of *E. bromicola* (Leuchtmann & Schardl, 1998) were vernalized in Zürich, Switzerland. The following spring, the plants were moved into greenhouses where mating tests were performed on emergent stromata. Cultures containing conidia of ATCC74483 were applied onto the stromata, which were then monitored regularly for two months.

Morphological examination

Agar blocks from water agar cultures containing actively conidiating mycelium were placed on slides and cover slips were pressed on the surface to remove any air pockets, so that the fungal structures were immersed in the aqueous medium. The structures were then examined by light microscopy. Mean lengths and widths of mature conidia, including standard deviations, are reported based on 20 measurements for each isolate. A mature conidium was defined as fully detached from the conidiogenous cell (phialide), but prior to

Tab. 1.– Morphological characteristics of *Lolium pratense* endophytes compared with those of related hosts.

Endophyte	Isolate	Host	Colony diameter at 21 days (cm)	Conidiogenous cell length (µm)	Conidiogenous cell width at base (µm)	Conidiogenous size at tip (µm)	Conidium (µm)
N. x *uncinatum*	CBS102646	*L. pratense*	1.2 ± 0.1	nd*	nd	nd	nd
N. x *siegelii*	ATCC74483	*L. pratense*	1.4 ± 0.1	18.0 ± 3.3	2.0 ± 0.4	1.0 ± 0.3	6.8 ± 0.5 × 3.0 ± 0.3
E. festucae	AL9436†	*F. rubra* subsp. *rubra*	5.2 ± 0.2	11.6 ± 2.6	1.4 ± 0.4	0.9 ± 0.2	5.0 ± 0.3 × 2.8 ± 0.3
E. festucae	ATCC90661	*F. rubra* subsp. *rubra*	3.8 ± 0.1	11.4 ± 1.9	1.8 ± 0.3	1.0 ± 0.2	5.0 ± 0.3 × 2.9 ± 0.3
E. festucae	CBS102474	*L. giganteum*	nd	14.3 ± 3.4	1.6 ± 0.3	1.0 ± 0.0	6.0 ± 0.4 × 3.0 ± 0.2

*nd = not determined.

† Maintained at Geobotanical Institute ETH.

germination. Mean lengths of conidiogenous cells, as well as width at base and tip, were determined from ten measurements for each isolate. Nuclear staining was performed using small agar pieces containing hyphae and conidia. Pieces were fixed 30 min in microcentrifuge tubes using 1 mL of para-formaldehyde / PIPES buffer [6% para-formaldehyde in 100 mM piperazine-N, N′-bis (2-ethanesulfonic acid), pH 6.5]. The fixative was removed and the agar washed with 1 mL TBS [10 mM Tris (hydroxymethyl) aminomethane, pH 7.5; 200 mM NaCl; 0.02% sodium azide]. Conidia and hyphae were then stained with 1 mL 4′,6-diamidino-2-phenylindole (DAPI) staining solution (0.7 μg mL^{-1} DAPI in TBS) for 5 min, followed by 3 washes with 1 mL TBS each and centrifuging between washes. The material was squashed on slides and visualized by fluorescence light microscopy (Osmani & al., 1987).

Growth assay

Plugs (0.5 cm diam) were taken from the edges of actively growing colonies of *N.* x *uncinatum* CBS102646, and *Neotyphodium* sp. ATCC74483, placed face down on PDA plates, and incubated in a 24 h dark regime at 24 °C for 21 days. Plugs were also taken from *E. festucae* isolates from hosts *Lolium giganteum* (L.) S. J. Darbyshire [= *Festuca gigantea* (L.) Villars] and *Festuca rubra* subsp. *rubra* (Gaud.) Hayek (fungal isolates CBS102474 and ATCC90661, respectively) in order to compare with the two *L. pratense* endophytes. Ten replicates were performed for each isolate.

Assessment of epiphyllous growth

To determine whether either *N.* x *uncinatum* or isolate ATCC74483 colonizes the exterior of the host leaf blade, individual tillers were taken from *L. pratense* plants artificially inoculated with *N.* x *uncinatum*, *L. pratense* inoculated with isolate ATCC74483, and uninfected *L. pratense* plants (5 tillers from different plants for each; 15 tillers total). Additionally, five tillers from *Lolium perenne* L. plants artificially inoculated with isolate ATCC74483 were also examined. Using the outermost leaf of each tiller, we obtained a peel from the sheath interior and a leaf blade segment (approx. 3 cm long) directly above the ligule. The sheath peel was stained for 10 min in tryphan blue and visualized by light microscopy. The abaxial surface of each blade segment was stained using the fluorescence indicator Calcofluor White M2R [0.02% 4,4′-bis-(4-anilino-6-diethyl-amino-s-triazin-2-ylamino)-2,2′-stilbene disulfonic acid; Polysciences, Inc., Warrington, Pennsylvania] for 20 min and visualized without a cover slip using fluorescence microscopy (Rohringer & al., 1977).

L. pratense survey

Plants tested for endophyte infection originated from seed collected from the following locations (followed by their ABY-BF accession numbers; Institute of Grassland and Environmental Research, Plas Gogerddan, Aberystwyth, Dyfed SY23 3EB, Wales): Ciuculi Depression, Romania (1201); Suceava Plateau, Romania (1203); North Carpathians, Romania (1208); Transylvanian Plateau, Romania (1211); Pre-Alps, Switzerland (957); Turkey (982); Monviso Alps, Italy (1055); Bergamo Alps, Italy (1097); Bergamo Alps, Italy (1099); Garessio, Italy (1056); Ligurian Alps, Italy (1057); Ardennes, Belgium (1083); Polders, Belgium (1084); Swiss Plateau, Switzerland (951); Vest Agder, Norway (1225); Rogaland, Norway (1226 & 1227); Oppoland, Norway (1241); Somerset Levels, Great Britain (1199); Thames Valley, Great Britain (1223); and Berkshire, Great Britain (1200). Geographic coordinates and habitat descriptions are in Thomas & al. (1996).

Loline alkaloid analysis

We utilized the method of Yates & al. (1990) with modification to determine levels of the loline alkaloids commonly found in grass-endophyte symbiota. Plants were grown in three parts Pro-mix BX (Premier Brands Inc., Red Hill, Pennsylvania) to one part top soil and fertilized twice monthly with 250 ppm 20-10-20 Peters Peatlite fertilizer (Marysville, Ohio). Single mature, vegetative plants (approx. 18 months old) infected with each endophyte and grown under greenhouse conditions with supplemental lighting were harvested in March 2000 on three separate occasions (designated days 1, 11, and 17). All living blade material above 8 cm from tillers of each plant was clipped, frozen at –80 °C, and finely ground with a Braun (Woburn, Massachusetts) coffee grinder. Samples (100 mg) of the ground material were placed in heat treated (110 °C for 48 h) 1.5 mL microcentrifuge tubes with 100µl saturated sodium bicarbonate solution. After allowing the ground tissue to become fully wetted, 1 mL of chloroform with quinoline (4.5 mg L^{-1}) as an internal standard was added. This mix was shaken 30 min and centrifuged at 16,000 g for 5 min, after which the supernatant was taken for analysis. All samples were analyzed with a Hewlett Packard (Avondale, Pennsylvania) 5890 Series II Plus gas chromatograph (GC) equipped with a fused silica capillary column (SE 30; J & W Scientific Inc., Rancho Cordova, California) with dimensions of 60 m length x 0.30 mm inner diameter, with 0.25µm film thickness of methyl polysiloxane. The GC was set up with an initial oven temperature of 100 °C, an injection temperature of 250 °C, and a detector temperature of 325 °C. The GC was

programmed to have a 2.2 min purge time and a 4 °C min^{-1} increase in column temperature.

Nitrogen was used as the carrier gas at a flow rate of 2.04 mL min^{-1}.

Isozyme electrophoresis

Sample preparation and electrophoretic technique were as previously described for isozyme analysis of *Neotyphodium/Epichloë* endophytes (Leuchtmann & Clay, 1990; Leuchtmann, 1994). Mycelium for enzyme extraction was grown axenically in liquid V-8 medium for 14-21 days. Mycelium was lyophilized and extracts from this were subjected to starch gel electrophoresis. Gel slices were stained for enzyme activity following published protocols (Soltis & al., 1983; Wendel & Weeden, 1989). The eight enzyme systems selected that were polymorphic for the isolates included: aldolase (ALD), acid phosphatase (ACP), diaphorase (DIA), glucose-6-phosphate dehydrogenase (G6PDH), leucine aminopeptidase (LAP), 6-phosphogluconate dehydrogenase (6PGD), phosphoglucomutase (PGM-1), and phosphoglucose isomerase (PGI). ALD, ACP, G6PDH, and 6PGD were run on citric acid/histidine-HCl buffer at pH 7.0; DIA, LAP, PGI, and PGM-1 on Tris citric acid/Tris citric acid buffer at pH 7.2.

DNA isolation

For each isolate, hyphal material was taken from a fresh, actively growing colony and ground in a microcentrifuge tube containing sterile water. Approximately 500 µL was then spread atop each of five cellophane disks on PDA, and cultured at room temperature for 1-2 weeks. Mycelium was then harvested and lyophilized. DNA was extracted as described elsewhere (Byrd & al., 1990; Schardl & al., 1997) from 250 mg of lyophilized mycelium.

DNA amplification and sequence determination

Gene segments containing *tub2* introns 1-3, *tef1* introns 1-5, and *act1* introns 1-5 were sequenced for *N.* x *uncinatum* and *Neotyphodium* sp. ATCC74483. Accession numbers for *N.* x *uncinatum* are: L06946 (*tub2*), AF308134 and AF308135 (*act1*), and AF308131 (*tef1*). Accession numbers for isolate ATCC74483 are: AF308138 and AF308139 (*tub2*), AF308136 and AF308137 (*act1*), and AF308132 and AF308133 (*tef1*). Accession numbers for all *Epichloë* spp. are listed elsewhere (Craven & al., 2001). DNAs were amplified as described previously (Schardl & al., 1997; Schardl & al., 1994) using AmpliTaq Gold (PE Biosystems, Foster City, California). Products were sequenced by the Sanger method (Thomas & Kocher, 1993) with

a GeneAmp PCR System 2400 using rhodamine-labeled dideoxynucleotide triphosphates or BigDye Terminator Cycle sequencing kit (PE Biosystems). Sequence reaction products were analyzed using an ABI model 310 capillary electrophoresis genetic analyzer (PE Biosystems). Both DNA strands were sequenced.

Phylogenetic analysis

Sequences were aligned using the PILEUP program in the Wisconsin Package (GCG, 1996). PILEUP parameters were adjusted empirically; a gap penalty of one and a gap extension penalty of zero resulted in accurate alignment of intron-exon junctions and in a reasonable alignment of the introns. Alignments were checked by eye. Maximum parsimony (MP) employed the branch and bound option in PAUP* (Swofford, 1998) for exact solutions. For parsimony analysis, character changes were unweighted and unordered; gaps were treated as missing information. Robustness of the inferred phylogenies was estimated by bootstrap replications. All branches receiving 70% or higher bootstrap values were considered well supported. For distance based analysis several methods were employed to derive pairwise distances. These included Jukes-Cantor and Kimura two-parameter distances [assuming transition/transversion (ts/tv) = 2], and various gamma values to adjust for between-site variation. Distance trees were inferred by neighbor-joining (NJ) implemented in PAUP* (Swofford, 1998) with random taxon additions. Maximum likelihood (ML) trees were constructed using PUZZLE in PAUP*. A Hasegawa-Kishino-Yano model was employed assuming ts/tv = 2.0. All such trees were similar to the MP trees, and are therefore not shown.

Results

Morphological characteristics

The results of our morphological examination are summarized in Tab. 1. *Neotyphodium* x *uncinatum* CBS102646 exhibited the slowest rate of colony growth among all isolates examined. The inability of *N.* x *uncinatum* conidia to readily detach from the phialide, and a tendency for these spores to precociously germinate while on the phialide made accurate measurements of these conidia and phialides impractical. However, the characteristic sickle-shape of *N.* x *uncinatum* conidia was evident (Fig. 1 A-B), and rough size estimates were within the range previously published (Gams & al., 1990). Isolate ATCC74483 had a slightly faster colony growth rate and sporulated abundantly (Fig. 1D). The calculated average conidium size was larger than that commonly observed for *Epichloë*

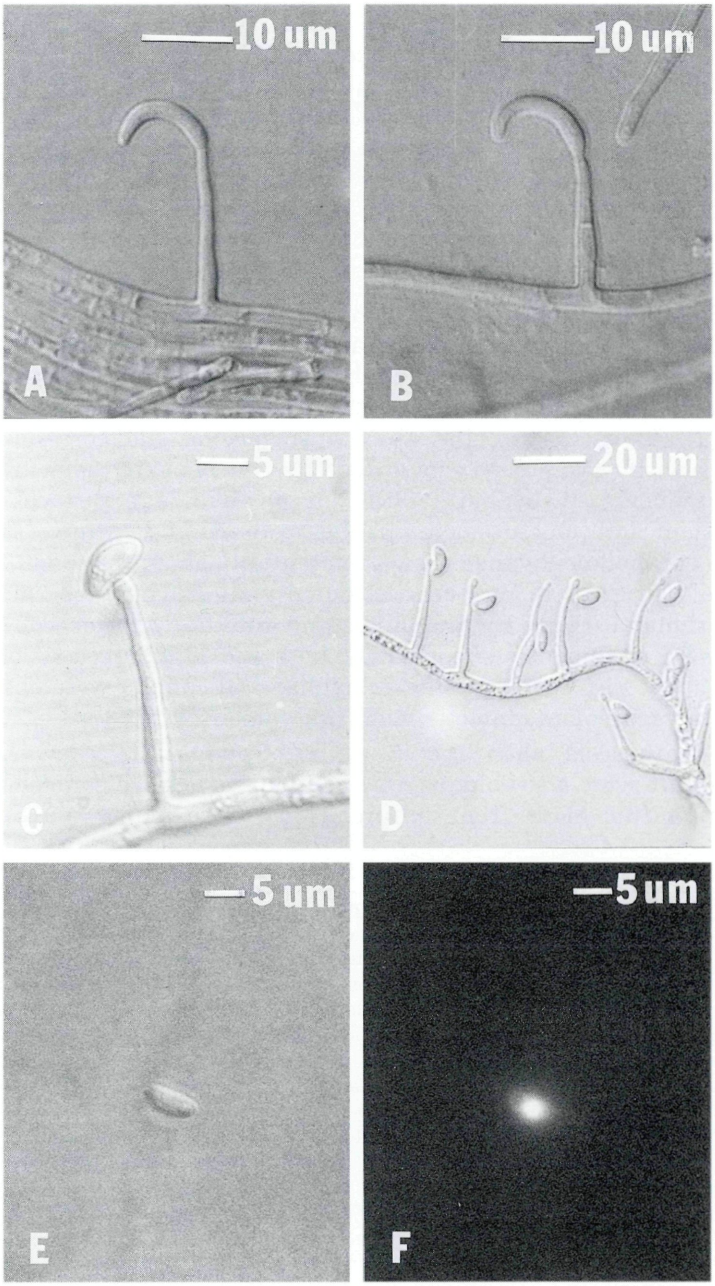


Fig. 1. – Conidiogenous cells and conidia; A-B, of *N.* x *uncinatum* from *L. pratense*; C-F of *N.* x *siegelii* from *L. pratense*. – A-E by light microscopy. – F by fluorescence microscopy (DAPI stain).

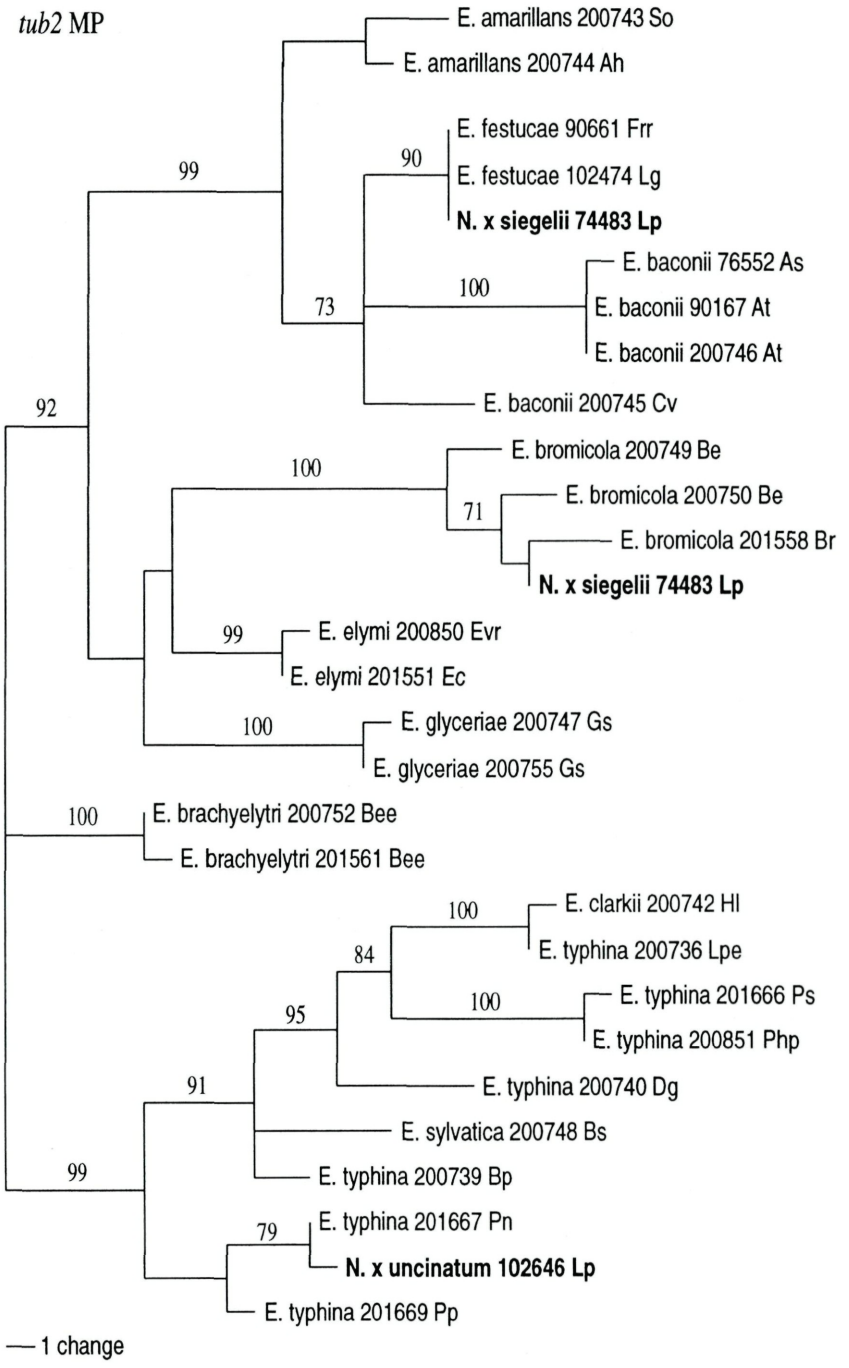
species. Results of DAPI staining indicated that these conidia were uninucleate (Fig. 1F). Both isolates from *L. pratense* grew considerably slower than *E. festucae* isolates from *L. giganteum* and *F. rubra* subsp. *rubra* (Tab. 1). Conidia from isolate ATCC74483 were not interfertile with stromata of *E. festucae* on *F. rubra* subsp. *rubra* or with stromata of *E. bromicola* on *Bromus erectus* Huds. In the matings with *mat-2* strains they caused production of structures superficially similar to perithecia, but no ascospores were produced.

Epiphyllous growth

Sheath peels taken from *L. pratense* plants infected with isolate ATCC74483 were all colonized by intercellular hyphae characteristic of *Epichloë/Neotyphodium* endophytes and of comparable width to hyphae of this isolate grown under culture conditions (approx. 2 µm; Tab. 1). In each case, the abaxial surface of the corresponding leaf segment was also colonized by fairly extensive mycelia. Measurements of hyphal widths on all five leaf surfaces were consistent with measurements in sheath tissue and culture (Tab. 1). These epiphyllous hyphae grew preferentially along the longitudinal axis of the grass blade and occasionally appeared to be emerging from stomata. Many conidiophores and conidia were also observed and were of dimensions consistent with those produced in culture (see Tab. 1; Fig. 1C). Very similar hyphae and conidia were observed on *L. perenne* plants infected with this same isolate.

Sheath peels taken from *L. pratense* plants infected with *N.* x *uncinatum* were also colonized by endophytic hyphae conforming to measurements made from culture. However, in contrast to plants

Fig. 2. – *Epichloë/Neotyphodium tub2* gene tree based on maximum parsimony (MP) analysis of introns 1-3. Shown is the single MP tree obtained by branch and bound search. Number of parsimony informative characters = 74; uninformative characters = 19; tree length = 127 steps; consistency index = 0.8346; retention index = 0.9335; rescaled consistency index = 0.7792; midpoint root is at the left edge. Bar represents one inferred nucleotide substitution. Numbers at branches are the percentage of trees containing the corresponding clade in 1000 bootstrap replications. Bootstrap values greater than 70% are considered supportive of the clades indicated by the branches. Numbers following endophyte isolate refer to ATCC numbers except *E. festucae* 102474 and *N. uncinatum* 102646 which refer to CBS numbers. Letters following these numbers refer to host designations as follows: So (*Sphenopholis obtusata*); Ah (*Agrostis hiemalis*); As (*Agrostis stolonifera*); At (*Agrostis tenuis*); Frr (*Festuca rubra* subsp. *rubra*); Lg (*Lolium giganteum*); Lp (*Lolium pratense*); Cv (*Calamagrostis villosa*); Be (*Bromus erectus*); Br (*Bromus ramosus*); Evr (*Elymus virginicus*); Ec (*Elymus canadensis*); Gs (*Glyceria striata*); Bee (*Brachyelytrum erectum*); Hl (*Holcus lanatus*); Lpe (*Lolium perenne*); Ps (*Poa sylvicola*); Php (*Phleum pratense*); Dg (*Dactylis glomerata*); Bs (*Brachypodium sylvaticum*); Bp (*Brachypodium pinnatum*); Pn (*Poa nemoralis*); Pp (*Poa pratensis*).



infected with isolate ATCC74483, plants infected with *N.* x *uncinatum* exhibited no observable epiphyllous hyphae or conidia. Plants uninoculated with either endophyte (uninfected) were devoid of observable hyphae in both sheath peel and on the abaxial blade surface.

Lolium pratense endophyte survey

To estimate the frequencies of occurrence in *L. pratense* of *N.* x *uncinatum* and isolates similar to ATCC74483, we grew out endophyte from plants arising from seed collected throughout Europe (Thomas & al., 1996) as well as *L. pratense* PI 237707 from which isolate ATCC74483 had been obtained. From the Europe-wide survey we obtained a 58% infection frequency (221 plants, 129 infections) for *N.* x *uncinatum*, while no other *Neotyphodium* species was obtained. Likewise, in 29 plants from PI237707 only *N.* x *uncinatum* was identified.

Loline alkaloids

We generated *L. pratense* plants symbiotic with *N.* x *uncinatum* and *L. pratense* plants symbiotic with ATCC74483, then analyzed the plants for three loline alkaloids: N-acetylnorloline, N-formylloline, and N-acetylloline. The levels recovered for both endophytes are presented in Tab. 2.

Allozyme analysis

Out of 13 enzymes analyzed, polymorphisms were evident for eight in comparisons of *E. festucae*, *E. bromicola*, and the endophytes of *L. pratense*. Of these eight enzymes, four indicated multiple loci in isolate ATCC74483 (Tab. 3). For three of these heterozygous loci (PGM-1, PGI, and ACP), migration distances matched those of both *E. bromicola* and *E. festucae*. The middle segregating band observed in PGI and ACP loci were likely heterodimers. *Neotyphodium* x *uncinatum* was polymorphic only for the allozyme 6PGD, with migration distances corresponding to those of *E. bromicola* and *E. typhina*.

tub2 phylogeny

PCR amplification yielded a single *tub2* gene copy for *N.* x *uncinatum*. This copy grouped with that of an *E. typhina* isolate from *Poa nemoralis*. Isolate ATCC74483 possessed two *tub2* gene copies, for which segments could be separately amplified using copy-specific primers based on unique polymorphisms. Sequence from one copy was identical to *tub2* in all *E. festucae* isolates analyzed, and the other copy had very similar sequence to those of *E. bromicola*

Tab. 2. – Loline alkaloid levels in *L. pratense* with endophytes.*

Endophyte	Isolate	N-formylloline			N-acetylloline			N-acetylnorloline		
		day 1†	day 11	day 17	day 1	day 11	day 17	day 1	day 11	day 17
N. x *uncinatum*	CBS102646	3020	6069	11036	1317	1409	919	481	711	7766
N. x *siegelii*	ATCC74483	852	17810	15687	153	945	915	61	572	675

*Alkaloid amounts are in µg/g dry wt.
† Days of sampling are indicated, whereby mature potted plants (one plant for each endophyte) were first sampled on day 1, then re-growth sampled 11 days later and again 17 days later.

Tab. 3. – Allozyme electrophoresis results.*

Endophyte	Isolate	Grass host	PGM-1	PGI	DIA	ALD	6PGD	G6PDH	ACP	LAP
N. x *siegelii*	ATCC74483	*L. pratense*	30/34	31/33/35	21	24/25	16	18	6/9/12	21
N. x *uncinatum*	AL9093	*L. pratense*	32	27	17	28	16/18	19	7	24
E. festucae	ATCC90660	*F. rubra* subsp. *commutata*	34	31	22	22	18	19	12	24
E. festucae	ATCC90661	*F. rubra* subsp. *rubra*	34	31	22	22	18	19	10	24
E. festucae	CBS102474	*L. giganteum*	33	31	22	22	18	19	12	24
E. festucae	ATCC201552	*F. valesiaca*	33	24	22	22	18	19	12	23
E. bromicola	ATCC200750	*B. erectus*	30	31	23	26	16	19	6	25
E. bromicola	ATCC201558	*B. ramosus*	32	31	23	24	16	19	6	24
E. bromicola	AL8918	*B. benekenii*	30	31	23	24	16	19	6	23
E. bromicola	AL8923/1	*B. benekenii*	30	35	23	24	16	19	6	24
E. bromicola	AL8920/1	*B. benekenii*	30	31	23	24	16	20	10	24
E. bromicola	AL9514	*P. nemoralis*	32/35	28	19	26	18	19	9	22

* Values represent mm migrated. Enzyme abbreviations are given in text.

(Fig. 2). Phylogenetic analysis on the alignment of *N.* x *uncinatum* and ATCC74483 *tub2* copies with representatives from each *Epichloë* species resulted in the single MP tree shown in Fig. 2. All branches inferring relationships of *tub2* copies from the *L. pratense* endophytes were strongly supported by bootstrapping. Both NJ and ML trees were consistent with the MP tree and are therefore not shown.

tef1 gene phylogeny

MP analysis resulted in 288 trees differing only in the placement of *Epichloë glyceriae* Schardl & Leuchtmann. One tree is shown to provide branch lengths (Fig. 3), while the strict consensus tree is provided with bootstrap values (Fig. 4). Similarly to analysis of *tub2*, we were able to isolate a single *N.* x *uncinatum tef1* gene. However, while the *N.* x *uncinatum tub2* gene was most similar to that of an *E. typhina* isolate, its *tef1* gene grouped within the *E. bromicola* clade (Figs. 3 & 4). Also grouping in this clade was one of the two *tef1* genes possessed by isolate ATCC74483. The other *tef1* copy from ATCC74483 grouped with *E. festucae*. Interestingly, the *E. festucae*-like *tef1* copy in ATCC74483 carried four mutational differences (within intron regions) from the other *E. festucae tef1* genes. Those of *E. festucae* were all identical except for two indels (insertions/deletions) involving homopolymer stretches, and one indel involving a tandem repeat. Such indels create gaps in the alignment, but were not used in evaluating phylogenetic relationships. All clades involving *N.* x *uncinatum* and ATCC74483 were strongly supported by bootstrapping. Both NJ and ML trees were consistent with the MP consensus tree and are not shown.

act1 gene phylogeny

MP analysis yielded 3336 trees (an example is shown in Fig. 5), and the consensus was less resolved than for either *tef1* or *tub2* alignments. The *act1* strict consensus tree is shown in Fig. 6. *E. festucae* isolate 028 from *Festuca longifolia* was substituted for *E. festucae* CBS102474 from *L. giganteum*, for which the *act1* gene was not available. Despite its lack of resolution, the *act1* gene tree was consistent with the other gene trees both for *Epichloë* species and the *L. pratense* endophytes. Both ATCC74483 and *N.* x *uncinatum* have an *act1* gene copy that grouped within the *E. bromicola* clade. The second *act1* copy from ATCC74483 grouped in the *E. festucae* clade. *Neotyphodium* x *uncinatum* also had a second *act1* gene, and this grouped with that of the *E. typhina* isolate from *P. nemoralis*. Although bootstrapping was infeasible for this data set, PUZZLE analysis suggested that all nodes grouping the isolates in question were well supported (Fig. 7).

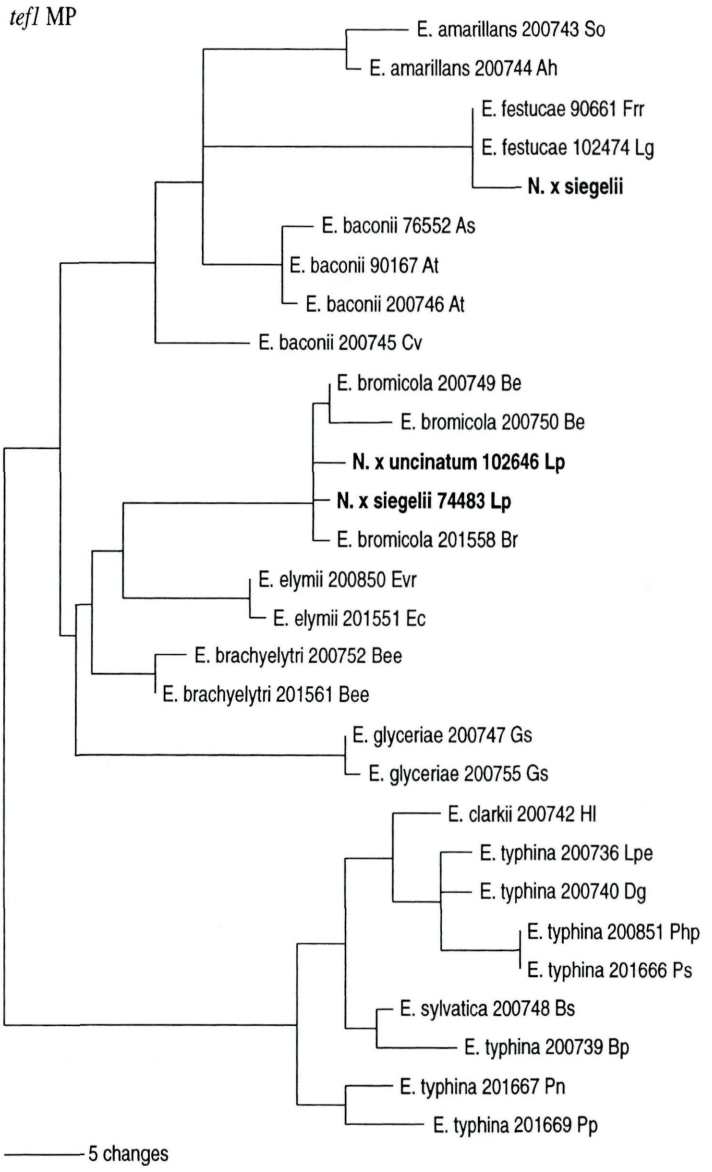


Fig. 3. – *Epichloë/Neotyphodium tef1* gene tree based on MP analysis of introns 1–4. Branch and bound search resulted in 288 MP trees one of which is shown: number of parsimony informative characters = 115; uninformative characters = 28; tree length = 177 steps; consistency index = 0.8644; retention index = 0.9483; rescaled consistency index = 0.8197; midpoint root is at the left edge. Bar represents 5 inferred nucleotide substitutions. Some sequences separated on zero-length branches differ only in indels, which were treated as missing information in this analysis. Isolate numbers and key to hosts are as in Fig. 2.

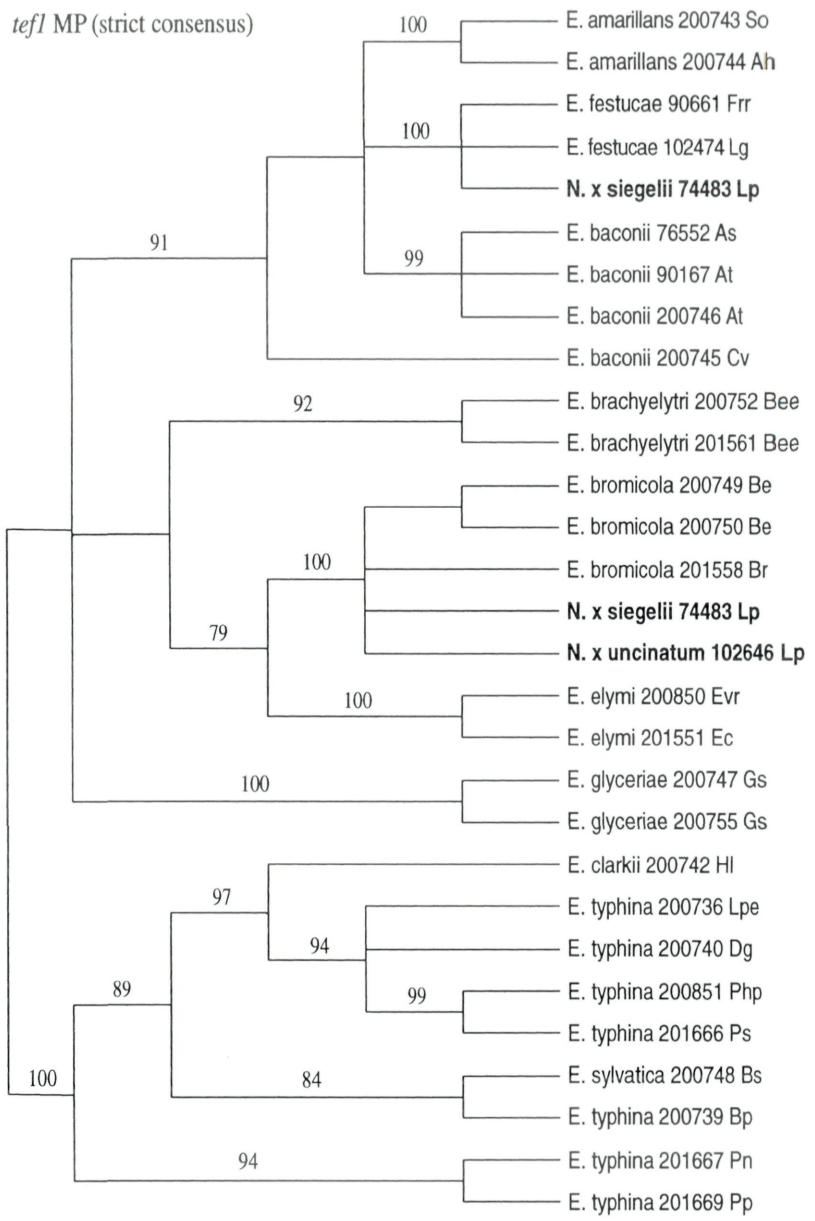


Fig. 4. – Strict consensus cladogram from the 288 MP trees derived from a branch and bound search on *tef1* sequence alignment. Numbers at branches are the percentages of trees containing the corresponding clade in 500 bootstrap replications. Bootstrap values greater than 70% are considered supportive of the clades indicated by the branches. Isolate numbers and key to hosts are as in Fig. 2.

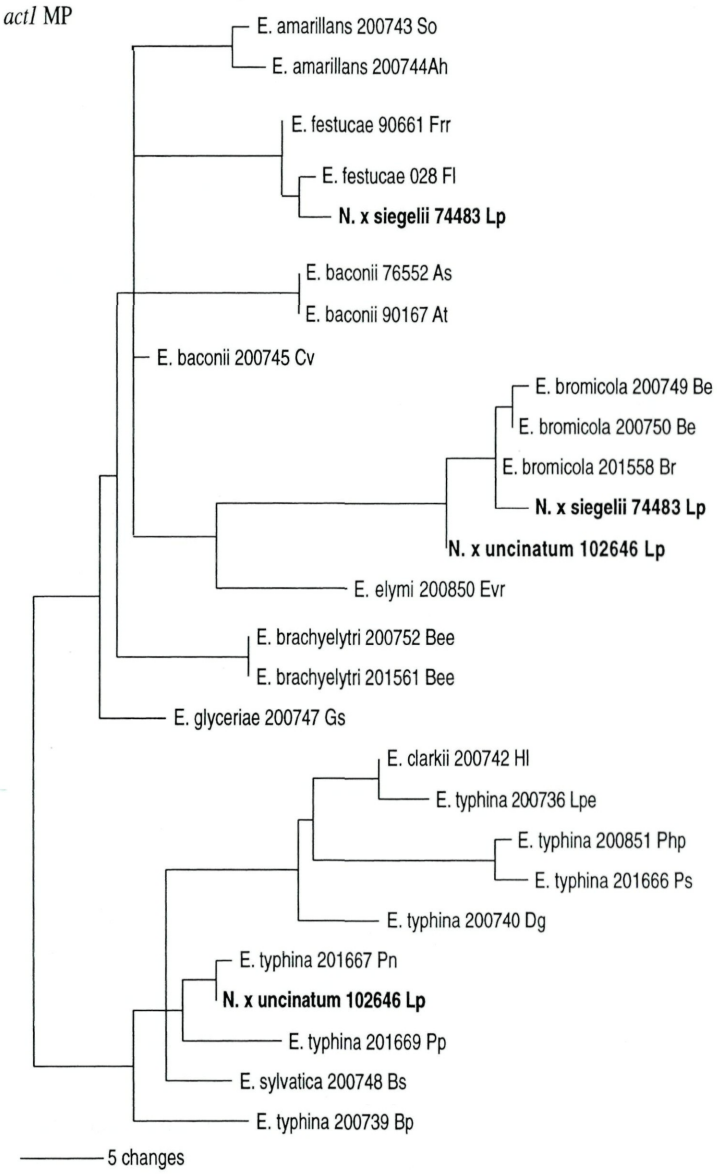


Fig. 5. – *Epichloë/Neotyphodium act1* gene tree based on MP analysis of introns 1–4. Branch and bound search resulted in 3336 MP trees one of which is shown: number of parsimony informative characters = 87; uninformative characters = 38; tree length = 149 steps; consistency index = 0.8926; retention index = 0.9492; rescaled consistency index = 0.8473; midpoint root is at the left edge. Bar represents 5 inferred nucleotide substitutions. Isolate numbers and key to hosts are as in Fig. 2 with the substitution of *E. festucae* 028 Fl (028 = laboratory designation; Fl = *Festuca longifolia*) for *E. festucae* CBS102474 Lg.

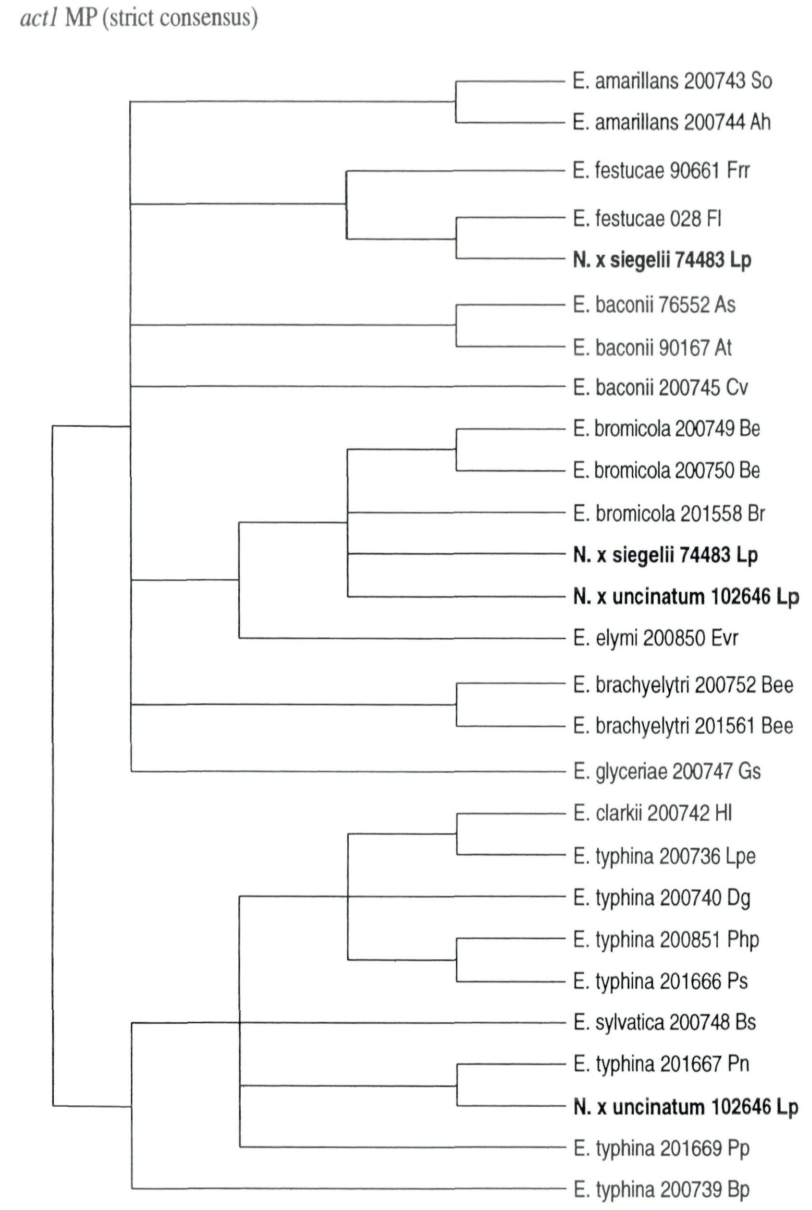


Fig. 6. – Strict consensus cladogram from the 3336 MP trees derived from a branch and bound search on *act1* sequence alignment. Number of parsimony informative characters = 87; uninformative characters = 38; tree length = 149 steps; consistency index = 0.8926; retention index = 0.9492; rescaled consistency index = 0.8473; midpoint root is at the left edge. Isolate numbers and key to hosts are as in Figs. 2 and 5.

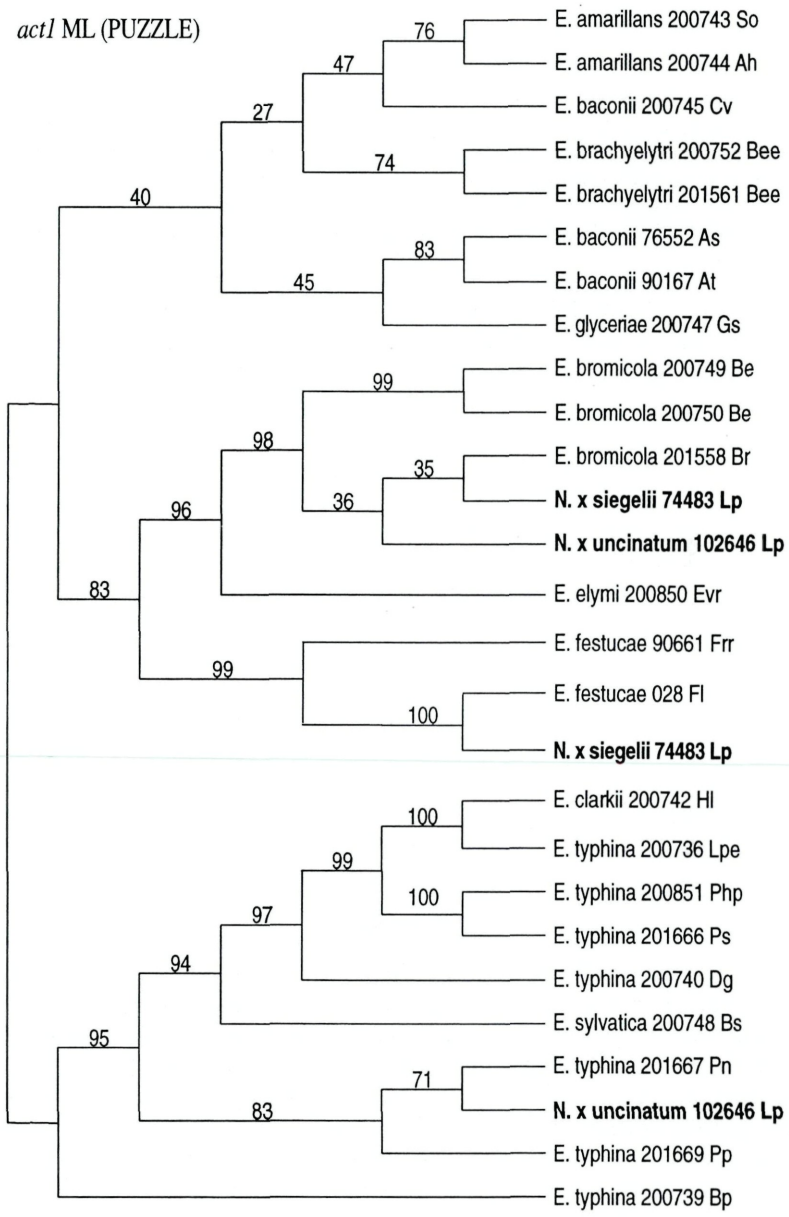


Fig. 7. – *Epichloë/Neotyphodium act1* gene tree based on ML PUZZLE analysis of introns 1-4. Tree is based on a Hasegawa-Kishino-Yano model with ts/tv = 2 (kappa = 3.98314); number of puzzling steps = 1000. Numbers at branches are estimations of support for each branch. Isolate numbers and key to hosts are as in Figs. 2 and 5.

Taxonomy

Neotyphodium* x *siegelii K.D. Craven, A. Leuchtmann & C.L. Schardl, **sp. nov.** - Fig. 1 C–F.

Coloniae albae, gossypinae, lente crescentes, 14 mm diametro aetate 21 dierum ad 22 C in PDA. Hyphae vegetativae hyalinae, 1–2.5 µm latae. Cellulae conidiogenae singulae, plerumque non septatae ad basim, cylindraceae, 12–24 µm longae, 1.5–3.0 µm latae ad basim, ad 0.5–1.5 µm gradatim angustatae. Conidia navicularia asymmetrica vel reniformia, hyalina, laevia, saepe transversaliter affixa, 6.0–8.0 × 2.5–3.5 µm. Affinitas genetica ad *Epichloë bromicolam* et *E. festucae*.

Colonies on PDA white, cottony, slow growing, attaining a diameter of 14 mm in 21 days at 24 C. Colony reverse tan to pale brown. – Vegetative hyphae hyaline, septate, 1–2.5 µm wide. – Conidiogenous cells arising solitarily from hyphae, produced abundantly, lateral, orthotropic, cylindrical at the base, tapering towards the apex, hyaline, determinate, usually lacking septa at or near the base, 12–24 µm long, 1.5–3.0 µm wide at the base, 0.5–1.5 µm wide at the apex. – Conidia navicular asymmetric to reniform, hyaline, smooth, frequently oriented transversely across the conidiogenous cell following detachment, 6.0–8.0 × 2.5–3.5 µm. – Genetic relationships to *Epichloë bromicola* and *Epichloë festucae*.

Holotype. – GERMANY, from plant grown from seed of *Lolium pratense* PI 237707, originally collected 08 March 1957, by Advanta Seeds Pacific, Inc., 33725 Columbus St. SE, Albany, Oregon 97321-7246; CUP 64960.

Etymology. – This endophyte is named in honor of Dr. Malcolm R. Siegel.

Discussion

We used a multiple gene phylogenetic approach to describe the endophytic community of *L. pratense* belonging to the *Epichloë/ Neotyphodium* group. One of these inhabitants, *N.* x *uncinatum* CBS102646, has been studied in some detail due to its extraordinarily high loline alkaloid production (Bush & al., 1997) and highly efficient seed transmission (Gams & al., 1990). Classification in the genus *Neotyphodium* is restricted to those endophytes with close genetic and morphological ties to *Epichloë* species, but lacking a sexual stage altogether (Glenn & al., 1996). Most such *Neotyphodium* spp. exhibit evidence of hybrid origins, likely originating through parasexual anastomosis between *Epichloë* species or between an *Epichloë* and a *Neotyphodium* species (Tsai & al., 1994; Schardl & al., 1994). However, initial analysis revealed a

single *N.* x *uncinatum tub2* gene that is closely related to that of an *E. typhina* isolate from *P. nemoralis* (Tsai & al., 1994; Moon & al., 2000). As aneuploidy often occurs in the fungi (Tolmsoff, 1983), it is possible that *N.* x *uncinatum* is truly a hybrid that had lost one or more of its *tub2* genes at some point following hybridization. Our analysis of both *tef1* and *act1* genes lends support for this notion; *N.* x *uncinatum* has a single *tef1* gene, but apparently from *E. bromicola* rather than *E. typhina* (Fig. 3). Furthermore, *N.* x *uncinatum* has retained two *act1* genes, one from *E. bromicola* and the other from *E. typhina* (Fig. 5). Interestingly, allozyme analysis of *N.* x *uncinatum* reveals a double-banded phenotype only for 6-phosphogluconate dehydrogenase (Tab. 3). Such phenotypes are common in endophytes with hybrid origins, as gene copies from different *Epichloë* ancestors give rise to divergent allozymes with distinct migration patterns. The single-banded phenotypes for most isozymes taken together with the *tub2* and *tef1* results, suggest that *N.* x *uncinatum* has lost much of the gene redundancy observed for other hybrids such as *N.* x *coenophialum* (Leuchtmann & Clay, 1990; unpublished results) and *N.* x *siegelii* (this study). Whether or not such genome reduction represents a more ancient hybrid simply losing redundant genetic information in a more or less clockwise manner or some sort of instability between certain *Epichloë* genotypic combinations cannot be ascertained from this study. Regardless, we have firmly established *N.* x *uncinatum* as a hybrid with at least two *Epichloë* species as ancestors.

We have also utilized phylogenetic analysis to characterize a rare new species, *N.* x *siegelii*, infecting *L. pratense*. Phylogenies derived from *tub2*, *tef1*, and *act1* all support the hybrid origin of *N.* x *siegelii* ATCC74483 with *E. festucae* and *E. bromicola* ancestry (Figs. 2, 3 & 5). Results obtained from allozymes (Tab. 3) also indicate hybrid origins of *N.* x *siegelii*, and are consistent with the above proposed *Epichloë* spp. ancestors.

Conidium sizes observed for *N.* x *siegelii* fall within the ranges seen for other hybrid *Neotyphodium* species including: *N.* x *coenophialum* (8.2 ± 1.4 µm $\times$ 2.2 ± 0.5 µm; Kuldau & al., 1999), *N.* x *uncinatum* (5–13 µm $\times$ 1–2 µm; Gams & al., 1990), and a currently unnamed isolate from *L. perenne*, Lp1 (5.5 ± 0.5 µm $\times$ 2.4 ± 0.3 µm; Kuldau & al., 1999). Interestingly, a different endophyte of *L. perenne*, *Neotyphodium lolii* Latch, Christensen & Samuels, also produces conidia within this size range (4.7–7.3 $\times$ 1.9–2.5; Latch & al., 1984) but shows no evidence of hybridization (Schardl & al., 1994; Kuldau & al., 1999). A correlation has been noted between spore size and genome content for *Epichloë* and *Neotyphodium* endophytes (Kuldau & al., 1999), so that hybrid *Neotyphodium*

species tend to have larger spore sizes than their *Epichloë* relatives (again, *N. lolii* may represent an exception).

In our survey of plants grown from seed collected at various locations across Europe, we were unable to isolate any *N.* x *siegelii*, but *N.* x *uncinatum* was fairly common. In fact, this new hybrid was only found during attempts to isolate *N.* x *uncinatum* from *L. pratense* PI 237707 for introduction into *Lolium perenne*. It is not uncommon for plant introduction centers to lose endophytes in their accessions, as material is stored to maximize seed, not endophyte, viability. Still, the viability of *N.* x *uncinatum* within these collections did not appear severely compromised. Therefore, it is likely that *N.* x *siegelii* is much rarer than *N.* x *uncinatum* in *L. pratense*. We note that we also failed to detect a second *N.* x *uncinatum* phenotype which is distinguishable by its straight conidia (Christensen & al., 1993). Our inability to recover either *N.* x *siegelii* or the straight conidium phenotype of *N.* x *uncinatum* in our available European *L. pratense* populations may simply reflect a low incidence in those populations. The straight conidium phenotype is reported only in Spain (Christensen & al., 1993), suggesting it may be geographically restricted. Likewise, it is possible that substantial populations of *N.* x *siegelii* occur in unsampled locales.

The apparent ability of *N.* x *siegelii* to colonize the phylloplane of its *L. pratense* host is fascinating. The presence of epiphyllous hyphae and conidia has been noted for at least one *Epichloë* species (White & al., 1996). This analysis illustrates the potential for at least this hybrid *Neotyphodium* species to likewise colonize the host exterior. It is not known whether the conidia produced there are infectious. However, as with other endophytes (Chung & al., 1997), invasive and difficult manipulations were needed for artificial inoculations to result in infection. Additionally, *Neotyphodium* infections do not appear to spread from infected to uninfected plants under greenhouse conditions, even though such plants are often in direct contact and their frequent clipping creates wound sites for potential infections (unpublished results). Such was the case here, as *N.* x *siegelii* infected *L. pratense* plants were in very close proximity to the uninfected plants, yet the endophyte was never observed in the originally uninfected plants. However, another possible role for the external conidia might be as spermatia in a sexual cycle. *Epichloë* conidia produced in culture can be used to fertilize conspecific stromata of opposite mating type (Schardl & An, 1993). Our attempts to utilize conidia from *N.* x *siegelii* culture to fertilize stromata of *E. festucae* or *E. bromicola* (the two likely ancestral species) were unsuccessful. Therefore, it seems unlikely that the external *N.* x *siegelii* conidia can serve as gametes or cause contagious spread, though these remain as possibilities.

From our analysis, we conclude that both *N.* x *siegelii* and *N.* x *uncinatum* possess genes likely derived from different *Epichloë* species inhabiting grasses belonging to the tribe Poeae. One of the ancestors of *N.* x *siegelii*, *E. festucae*, infects several grasses in this tribe and exhibits little genetic diversity. Isolates of *E. festucae* can easily be introduced into various *Lolium* and *Festuca* species (Christensen & al., 1997). Therefore, in nature *E. festucae* might readily move into other grasses within genus *Lolium* and tribe Poeae. *Neotyphodium* x *uncinatum* also has *tub2* and *act1* genes grouping with *E. typhina*. Although members of this genetically diverse sexual species actually infect grasses in three grass tribes (Poeae, Aveneae, and Brachypodieae), *N.* x *uncinatum* has retained genes most closely related to an *E. typhina* isolate from *Poa nemoralis*, a member of the Poeae. This particular *E. typhina* genotype appears to have contributed to the genomes of several other *Neotyphodium* endophytes infecting grasses in the genus *Lolium* (Tsai & al., 1994, Moon & al., 2000). Whether or not this represents the predominance of this *E. typhina* genotype in nature or an increased tendency to hybridize is unknown. Regardless, it is reasonable to assume that one of the *Epichloë* ancestors of both *N.* x *siegelii* and *N.* x *uncinatum* (*E. festucae* and *E. typhina* from *Poa nemoralis*, respectively) could have infected *L. pratense*.

Also interesting is the second apparent ancestor to both hybrid endophytes, namely *E. bromicola*, which infects grasses in the tribe Bromeae. Phylogenetic trees of both *act1* and *tef1* are consistent with the possibility that both *N.* x *uncinatum* and *N.* x *siegelii* have a common *E. bromicola* ancestor (Figs. 3 & 7). Within this tribe, *Bromus benekenii* (Lange) Trimen and *Bromus ramosus* Huds. are infected with *E. bromicola* isolates seemingly restricted to vertical seed transmission. In contrast, the *E. bromicola* isolates infecting *B. erectus* are strong chokers and rarely allow the host grass to set seed (Leuchtmann & Schardl, 1998). If we assume that the hybridization events giving rise to *N.* x *uncinatum* and *N.* x *siegelii* occurred within *L. pratense*, we must also assume that *E. bromicola*, or some recent ancestor to it, was also able to infect *L. pratense*. This would necessitate sexual expression by *E. bromicola*. However, our analysis indicates that *N.* x *siegelii* and *N.* x *uncinatum* may group closer to the isolates from *B. ramosus* and *B. benekenii*, although the distinction is far from clear-cut. It remains entirely possible that an *E. bromicola* genotype infecting these or another closely related host was one of the ancestors to the *L. pratense* endophytes.

We propose two of the more parsimonious scenarios by which these *Neotyphodium* hybrids may have evolved. The first alternative involves two separate events in which single hybridizations created two distinct *Neotyphodium* species. Under this scenario, *E. bromicola*

would have twice infected *L. pratense* and hybridized; once with *E. typhina*, and the other time with *E. festucae*. The second alternative involves a single, twice hybridized *Neotyphodium* analogous to *N.* x *coenophialum*, which has at least three *Epichloë* ancestors (Tsai & al., 1994). If an ancestral *L. pratense* endophyte had *E. festucae*, *E. typhina* and *E. bromicola* ancestors, this hybrid may have lost some or all *E. typhina* genes in one lineage (giving rise to *N.* x *siegelii*), and some or all *E. festucae* genes in the other (giving rise to *N.* x *uncinatum*). Indeed, the allozyme results obtained for 6PGD in this study implicate both *E. bromicola* and *E. typhina* as likely ancestors to *N.* x *uncinatum*, but do not exclude *E. festucae* as a third potential progenitor (Tab. 3). A similar scenario was hypothesized by Tsai & al. (1994) to explain the origins of two other tall fescue endophytes besides *N.* x *coenophialum* (currently designated FaTG-2 and FaTG-3), each having genes apparently derived from some of the same *Epichloë* ancestors as *N.* x *coenophialum*. Our fairly thorough screening of plants derived from seed collected at various locations across Europe did not uncover a hybrid *Neotyphodium* species with three ancestors. However, we also failed to recover any additional *N.* x *siegelii* isolates, so the double hybrid may exist and be rare. Alternatively, it may have been maladapted to *L. pratense* and only stabilized the interaction by eliminating gene copies or entire chromosomes.

Neotyphodium x *uncinatum* may reflect a successful example of increased compatibility between host and symbiont, manifested as high levels of seed transmission and natural infection. Such stability may have driven the evolution of an increasingly mutualistic relationship mediated, in part, through the production of novel insecticidal chemicals (loline alkaloids). Lolines provide extremely effective defense (Siegel & al., 1990) and are produced quite abundantly in endophyte infected *L. pratense*. We detected total loline alkaloids constituting up to 2.0% of plant dry weight. Surprisingly, *N.* x *siegelii* is also an adept producer of loline alkaloids, with total loline alkaloid levels up to 1.9% of plant dry weight. Loline levels of this magnitude make *N.* x *siegelii* a new candidate for turf development programs. *Neotyphodium* x *siegelii* grows faster than *N. uncinatum* thus reducing time requirements for culture and possibly improving inoculation efficiency.

Another exciting finding was the effect of plant clipping on loline alkaloid production by both *N.* x *uncinatum* and *N.* x *siegelii*. Levels detected following the second and third clippings increased by up to 20-fold over the previous sampling. This is consistent with previous findings of increased alkaloid levels in regrowth tissue (Bush & al., 1993), and may indicate induction of defensive chemical production in response to host damage. Such induction might

alleviate any fitness costs to the host associated with constitutive alkaloid production.

It is interesting to note that among the described *Epichloë* species, only *E. festucae* from *Lolium giganteum* is capable of producing lolines (Siegel & al., 1990; Leuchtmann & al., 2000). As neither of the present day *Epichloë* species proposed as ancestors to *N.* x *uncinatum* have any known loline production capabilities, it is tempting to favor the notion of both *L. pratense* endophytes being derived from the same double hybrid. Although *N.* x *uncinatum* has not retained *tub2*, *tef1*, or *act1* genes from a potential *E. festucae* ancestor, it may have retained the capacity for loline production. Since both *L. giganteum* and *L. pratense* lie within the subgenus *Schedonorus*, it may be that an *E. festucae* lineage in an early member of subg. *Schedonorus* evolved the capacity for loline production, which was subsequently passed down to endophytes of more derived species including *L. pratense*. Endophyte species with mixed transmission strategies like *E. festucae* have been proposed as more likely co-evolving with their hosts (Schardl & al., 1997). It is hypothesized that this strategy affords the benefits of sexuality to both partners in the symbiosis, such that neither succumbs to the evolutionary consequences of strict asexuality (Muller, 1964).

If indeed endophytes like *E. festucae* have tracked the evolution of their host grasses, it is not unreasonable to assume that this symbiont was present during the speciation leading to *L. pratense*. Tall fescue (*L. arundinaceum*) is also classified in the subgenus *Schedonorus* (Darbyshire, 1993), and one of it endophytes, *N.* x *coenophialum*, is also a hybrid known to produce lolines (Siegel & al., 1990; Tsai & al., 1994). Analysis of *N.* x *coenophialum tub2* sequences suggests relationships to *E. festucae, N.* x *uncinatum* (or *E. typhina*) and a relative of *E. baconii*. Of the two other tall fescue endophytes mentioned above (FaTG-2 and FaTG-3), only FaTG-3 is known to produce lolines; yet *tub2* sequences for FaTG-3 do not indicate an *E. festucae* ancestor, whereas FaTG-2 is not known to produce lolines but does have a *tub2* gene copy from *E. festucae*. However, both have *E. baconii*-like sequences suggestive of a relationship to, or derivation from, *N. coenophialum* (Tsai & al., 1994). Again, it is possible that loss of redundant gene copies obscures some of the apparently complex pedigrees for hybrid endophytes. The widespread (but not universal) distribution of loline alkaloid biosynthesis capability in hybrid endophytes of *Lolium* subg. *Schedonorus* may be a consequence of numerous hybridization events, the details of which remain to be fully elucidated.

Our development of sequence databases from multiple genes of *Epichloë/Neotyphodium* endophytes has enabled us to extract much new information regarding the evolutionary origins and relation-

ships among this group of fungi. Importantly, this approach also can be used to address questions regarding evolutionary processes such as gene flow and speciation. Here, we have used multiple gene phylogenetics to describe the *L. pratense* endophytes. This research characterizes both as *Neotyphodium* species and indicates their likely *Epichloë* ancestors. As more *Neotyphodium* and *Epichloë* species are isolated and subjected to such analysis, we will be able to gain deeper insights into the processes promoting speciation and mutualism within this unique group of symbionts. The synthesis of this approach with information obtained from more traditional methods provides a much more comprehensive picture that can only strengthen the resolving power in future analyses of fungal evolution.

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