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# Relationships among Non-*Acremonium* sp. Fungal Endophytes in Five Grass Species†

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**Many cool-season grasses (subfamily Pooideae) possess maternally transmitted fungal symbionts which cause no known pathology and often enhance the ecological fitness and biochemical capabilities of the grass hosts. The most commonly described endophytes are the *Acremonium* section *Albo-lanosa* spp. (*Acremonium* endophytes), which are conidial anamorphs (strictly asexual forms) of *Epichloë typhina*. Other endophytes which have been noted are a *Gliocladium*-like fungus in perennial ryegrass (*Lolium perenne* L.) and a *Phialophora*-like fungus in tall fescue (*Festuca arundinacea* Schreb.). Here, we report the identification of additional non-*Acremonium* sp. endophytes (herein designated p-endophytes) in three more grass species: *Festuca gigantea*, *Festuca arizonica*, and *Festuca pratensis*. In each grass species, the p-endophyte was cosymbiotic with an *Acremonium* endophyte. Serological analysis and sequence determinations of variable portions of their rRNA genes indicated that the two previously identified non-*Acremonium* endophytes are closely related to each other and to the newly identified p-endophytes. Therefore, the p-endophytes represent a second group of widely distributed grass symbionts.**

Nonpathogenic, seed-borne, fungal symbionts (endophytes) are a common feature of many grass species in the genera *Festuca* and *Lolium* (14, 28). The most commonly observed are classified as genus *Acremonium* Link, section *Albo-lanosa* Morgan-Jones et Gams, and are conidial anamorphs (strictly asexual forms) of the euascomycete *Epichloë typhina* (Persoon: Fries) Tulasne (20). Other seed-borne endophytes have also been described for the two grass species *Festuca arundinacea* Schreb. (tall fescue) and *Lolium perenne* L. (perennial ryegrass) but have never been formally classified. They have been designated *Phialophora*-like and *Gliocladium*-like fungi, respectively (13).

Because of their vertical transmission via seeds, grass endophytes behave as heritable components of symbiotic entities and as such, they provide important genetic, biochemical, and physiological capabilities to their hosts (24). The *Acremonium* endophytes are important for fitness and biological protection of their hosts (6, 24). Less is known about the ecological roles of non-*Acremonium* sp. endophytes, but observations that the *Phialophora*-like endophyte of tall fescue produces activity, in agar culture, against a wide spectrum of fungal pathogens of grasses (22) suggest that it may also have a positive effect on host fitness. Extensive microscopic studies of the *Gliocladium*-like endophyte in perennial ryegrass give no indication of a detrimental or pathogenic effect of the endophyte upon either the grass host or the cosymbiotic endophyte, *Acremonium lolii* Latch, Samuels, et Christensen (16–18).

The taxonomy and distribution of the non-*Acremonium* endophytes have received little attention. As yet, no formal binomial has been assigned to the *Phialophora*-like and *Gliocladium*-like endophytes, and their possible relationships to each other and to endophytes of other grasses have not been considered. Although there appear to be morphological similarities (such as the penicillate conidiophores), these two endophytes were thought to resemble organisms belonging to distinct genera (13). Both the *Phialophora*-like and *Gliocladium*-like endophytes were cosymbiotic with *Acremonium* endophytes in their host grasses. This article reports the identification of similar cosymbiotic associations in three additional grass species from Europe and North America and presents serological and DNA sequence data which indicate a close relationship between the *Phialophora*-like endophyte of tall fescue, the *Gliocladium*-like endophyte of perennial ryegrass, and the non-*Acremonium* endophytes of *Festuca arizonica* Vasey, *Festuca gigantea* L., and *Festuca pratensis* Huds.

## MATERIALS AND METHODS

**Biological materials.** Plants possessing non-*Acremonium* endophytes, *Acremonium* endophytes, or both are listed in Table 1. Endophytes were isolated from infected grass pseudostems by the method of Latch et al. (13). Each individual plant-fungus association is identified by a plant number, and the endophyte isolates from those associations are designated by the same number prefixed by either "e" for *Acremonium* endophytes (anamorphs of *E. typhina*) or "p" for non-*Acremonium* endophytes (p-endophytes, which, as will be shown, are related to the *Phialophora*-like endophyte of tall fescue). *E. typhina* E32 was previously identified in *Festuca rubra* Gaud. (21). To obtain pure cultures of either *Acremonium* or non-*Acremonium* endophytes from mixed cultures derived from plant tissues, mycelium was ground as previously described (4); then, dilutions were plated atop a sterile cellophane disk on potato dextrose agar (PDA; GIBCO BRL, Gaithersburg, Md.). Serial 10-fold dilutions were used to obtain individual colonies, which were then checked by tissue print immunoblot assay (9) (see below). The endophyte isolates and their

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TABLE 1. Serological reactivities of grass-fungus symbiotic entities and cultures derived from them

| Host | Code | Description | Reactivity[a] of pseudostems to antiserum against: | | Isolate(s)[b] | Reactivity[a] of culture to antiserum against: | | |
|---|---|---|---|---|---|---|---|---|
| | | | p180 | e19 | | p180 | p26 | e19 |
| *F. arizonica* Vasey | 1572 | Culture from a plant in New Mexico | NT | NT | p1572, e1572[c] | + | NT | + |
| *F. arundinacea* Schreb. | 19 | Plant artificially infected with *A. coenophialum* Morgan-Jones et Gams, isolated from *F. arundinacea* | − | + | e19 | − | − | + |
| | 26 | Plant artificially infected with the *Phialophora*-like sp. Latch, Samuels, et Christensen isolated from *F. arundinacea* | + | − | p26 | + | + | − |
| *F. pratensis* Huds. | 166–175, 179 | Plants from Swiss populations naturally infected with *Acremonium uncinatum* Gams, Petrini, et Schmidt | −[d] | +[d] | e166 | − | NT | + |
| | 176–178 | Plants from Swiss populations | +[d] | +[d] | e178 | − | NT | + |
| | | | | | p178 | + | NT | − |
| | 180 | Plant from a Swiss population | +[d] | −[d] | p180 | + | + | − |
| *F. gigantea* L. | 95 | Breeding stock, USDA[e] | NT | NT | p95, e95[c] | + | NT | + |
| | | | | | p95 | + | NT | − |
| *F. rubra commutata* Gaud. | 32 | Plant infected with *E. typhina* (Persoon: Fries) Tulasne | NT | NT | E32 | − | NT | + |
| *L. perenne* L. | 145 | Culture of *Gliocladium*-like sp. Latch, Samuels, et Christensen (ATCC 56212) | NT | NT | p145 | + | NT | − |

[a] Tissue print immunoblot. NT, not tested.
[b] Code number of the host from which the isolate was derived, prefixed by "e" for *Acremonium* endophytes, "E" for *E. typhina*, or "p" for p-endophytes.
[c] Mixed culture of two fungi from one symbiotic entity.
[d] Pseudostem sections from seed progeny.
[e] USDA, U.S. Department of Agriculture, Lexington, Ky.

serological reactivities are listed in Table 1. Other fungi used in this study are listed in Table 2.

**PCR and analysis of DNA.** Fungal DNA was extracted from fresh or freeze-dried mycelium by published methods (3, 4). The nuclear rRNA gene internal transcribed spacer 2 (*rrnITS2*) was amplified from genomic DNA of each isolate by symmetric polymerase chain reactions (PCR) using oligonucleotide primers and conditions described by White et al. (29). The 5′ portion of the 26S large-subunit rRNA (*rrnL*) was amplified by using an oligonucleotide primer homologous to a conserved 26S rRNA sequence near position 50 (5′-GCATATCAATAAGCGGAGGA-3′) and an oligonucleotide primer which was complementary to a conserved segment around position 660 (5′-GACTCCTTGGTCCGTGT TTCA-3′).

For restriction endonuclease cleavage of symmetric PCR products, 10 μl was mixed with an equal volume of digestion buffer (33 mM Tris-HCl [pH 7.9], 66 mM potassium acetate, 10 mM magnesium acetate, 4 mM spermidine-HCl, 0.5 mM dithiothreitol) containing 5 U of *Mlu*I or *Not*I (GIBCO BRL). The mixture was incubated at 37°C for 1 to 2 h and then was analyzed electrophoretically (2).

Single-stranded DNA templates were prepared for sequence determination by asymmetric PCR as described by White et al. (29). Alternatively, symmetric PCR products were cloned and sequenced as previously described (1). Sequences of both strands of each clone or PCR product were determined by the Sanger method, as described by Toneguzzo et al. (26). Sequencing primers included those used for PCR amplification and two additional oligonucleotides, one homologous to a conserved 26S rRNA sequence near position 375 (5′-GAAAAGCACTTTGAAAAGAGG GT-3′) and the other its complement (5′-ACCCTCTTTTCA AAGTGCTTTT-3′).

Sequences of rRNA gene segments have been deposited in the EMBL data base under accession numbers X60185 (*Acremonium coenophialum* Morgan-Jones et Gams isolated from *F. arundinacea*), X62978 (p241), X62979 (p180), X62980 (p1572), X62981 (e1572), X62986 (p178), X62987 (*E. typhina* from *F. rubra*), X62988 (*Emericella nidulans* [Eidam] Vuillemin), X62989 (p26), X62990 (*Gaeumannomyces graminis* Arx et Oliver), and X62991 (p26). Other sequences were described by Perasso et al. (15) and Gutell et al. (8).

**Phylogenetic analysis.** The most parsimonious phylogram was determined by using the branch-and-bound algorithm implemented in PAUP version 3.0q (25). Characters were treated as unordered, and all nucleotide substitution differences were weighted equally. Alignment gaps were considered equivalent to missing information. Segments that could not be aligned with confidence were not included in the analysis. For quasi-statistical evaluation of the cladogram, bootstrap replications (7) were performed by maximum parsimony with similar parameters and collapsing of zero-length branches.

**Antiserum production and immunodetection.** Antisera were prepared by a modification of the methods of Johnson

TABLE 2. Fungal cultures and their serological reactivities

| Fungus | Code | Source | Reactivity[a] to antiserum against: | |
|---|---|---|---|---|
| | | | p180 | e19 |
| *Cladosporium cucumerinum* Ellist et Arthur | TP-20 | J. Kuc, University of Kentucky | – | – |
| *Cochliobolus sativus* (Ito et Kuribayashi) Drechsler ex Dasture | TP-17 | L. Trevathan, Mississippi State University | – | – |
| *Colletotrichum graminicola* (Ces) Wilson | TP-01 | N. Jackson, University of Rhode Island | – | – |
| *Drechslera erythrospila* (Drechs.) Shoemaker | TP-02 | N. Jackson | – | – |
| *Drechslera siccans* (Drechs.) Shoemaker | TP-03 | N. Jackson | – | – |
| *Emericella nidulans* (Eidam) Vuillemin | FGSC A237 | Fungal Genetics Stock Center, Manhattan, Kans. | NT | NT |
| *Gaeumannomyces graminis* Arx et Oliver var. *avenae* Turner | TP-04 | N. Jackson | – | – |
| *Gaeumannomyces graminis* var. *graminis* Arx et Oliver | TP-05 | N. Jackson | – | – |
| *Gloeotinia granigena* (Quél.) T. Schumacher | TP-18 | S. Alderman, Oregon State University | – | – |
| *Laetisaria fuciformis* (McAlpine) Burdsall | TP-06 | N. Jackson | – | – |
| *Leptosphaeria korrae* Walke et Smith | TP-07 | N. Jackson | – | – |
| *Leptosphaerulina australis* McAlpine | TP-08 | N. Jackson | – | – |
| *Limnomyces roseipellis* Stalpers et Lorakker | TP-09 | N. Jackson | – | – |
| *Magnaporthe poae* Landschoot et Jackson | TP-10 | N. Jackson | – | – |
| *Microdochium bolleyi* (R. Sprague) De Hoog et Hermanides-Nijhof | TP-11 | N. Jackson | – | – |
| *Microdochium nivale* (Fr.) Samuels et Hallett | TP-12 | N. Jackson | – | – |
| *Neurospora crassa* Sheer et Dodge | FGSC 2489 | Fungal Genetics Stock Center | NT | NT |
| *Ophiosphaerella herpotrichus* (Fr.: Fr.) Walker | TP-13 | N. Jackson | – | – |
| *Penicillium chrysogenum* Thom. | ATCC 9480 | American Type Culture Collection | ± | ± |
| *Rhizoctonia cerealis* van der Hoeven | TP-15 | N. Jackson | – | – |
| *Rhizoctonia solani* Kuhn | TP-19 | K. Gwinn, University of Tennessee | – | – |
| *Sclerotinia homeocarpa* Bennet | TP-16 | N. Jackson | – | – |

[a] Tissue print immunoblot. NT, not tested; ±, atypical reaction (see text).

et al. (10) and Reddick and Collins (19). Mycelium of isolate e19, p26, or p180 was initially cultured for 7 to 14 days on a cellophane disk atop PDA. Subcultures were prepared by grinding the mycelium in 10 to 15 ml of sterile water in an Omni Mixer Homogenizer (R. J. Rauch & Assoc., Holland, Ohio) and then inoculating 1 to 2 ml of the slurry into 50 ml of potato dextrose broth (GIBCO BRL) in 300-ml flasks. The mycelial contents of six culture flasks were required for each rabbit to be inoculated. The cultures were grown at 21°C in an orbital incubator (130 to 200 rpm) for 2 to 3 weeks. Mycelia were collected by vacuum filtration and washed with ca. 2 liters of phosphate-buffered saline minus potassium (PBNa) (20 mM sodium phosphate buffer [pH 7.3]–150 mM NaCl) and were resuspended in 75 ml of PBNa and homogenized in a Polytron PT20 (Brinkmann, Westbury, N.Y.) for 45 s at setting 7. The homogenate was centrifuged for 10 min at 8,000 × *g*. The supernatant was decanted into a 250-ml beaker and cooled to 4°C. Solid polyethylene glycol (molecular weight, 8,000) was slowly added, with stirring, to a concentration of 10% (wt/vol). The protein was precipitated overnight at 4°C, collected by a 20-min centrifugation at 12,000 × *g*, resuspended in 5.0 ml of PBNa containing 0.1% sodium dodecyl sulfate, and then warmed to 60°C and incubated for 5 min. Insoluble material was repelleted and discarded. The supernatant was cooled on ice, and 4 volumes of cold (−20°C) acetone was added. Protein was precipitated overnight at −20°C, repelleted, resuspended in 5.0 ml of PBNa, and divided into 1-ml aliquots. The concentration of protein was estimated by $A_{280}$ (2). The aliquots were freeze-dried for storage. For rabbit inoculations, protein extracts were dissolved in 0.5 ml of water, thoroughly emulsified with 0.5 ml of Freund's incomplete adjuvant, and injected subcutaneously or intramuscularly. The rabbits were injected three times at 2-week intervals and then bled 2 weeks following the last injection. Two rabbits were injected with extracts from *A. coenophialum* e19, and four rabbits each were injected with extracts from p26 and p180.

Tissue print immunoblots were performed by the method of Gwinn et al. (9). Duplicate pseudostem sections (length, 1 mm) and half-seeds were placed between a nitrocellulose filter (pore size, 0.45 μm; Bio-Rad, Richmond, Calif.) and waxed paper and then crushed onto the nitrocellulose. Positive controls were sections of pseudostems or half-seeds known to be infected with previously described endophytes (11, 12) (Table 1). Negative controls were the corresponding tissues from plants with no endophyte. Appropriate dilutions (typically 1:500 to 1:2,500) were determined empirically for each antiserum to obtain the most unambiguous and specific identifications of positive reactions against endophytes. Colorimetric detection of bound antibody was performed as described by Gwinn et al. (9). To avoid false-positive results, it was important to check the color reactions on damp filters which were well illuminated and magnified with a dissecting microscope. Positive reactions were typified by a deep red color or deep red spots, whereas a light pink or tan color was often observed with the negative controls.

Tissue prints of fungal mycelia were performed in similar fashion. Duplicate samples of fresh mycelium were removed from agar plates with a 4-mm-diameter cork bore. The agar

was cut away, and the mycelium was blotted. Each filter also had blots of p26, p180, and e19 mycelia as controls.

Indirect enzyme-linked immunosorbent assays (ELISA) utilized anti-p180 or anti-e19 antisera (diluted 1:2,000), anti-rabbit (whole molecule)–alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, Mo.), and the method of Clark and Bar-Joseph (5). Optical densities at 405 nm were read with a Titertek Multiskan ELISA plate reader (ICN-Flow Laboratories, Costa Mesa, Calif.).

## RESULTS

**Identification of non-*Acremonium* endophytes.** Microscopic examination of the *Gliocladium*-like endophyte in perennial ryegrass has indicated growth characteristics which are readily distinguishable from those of the *A. lolii* cosymbiont (16). Characteristic morphologies of the *Phialophora*-like endophyte and *A. coenophialum* in tall fescue were examined, and similar differences were observed. Leaf sheaths were prepared and examined in a fashion which is routinely and widely used to screen for endophyte infections in grasses. The *Phialophora*-like endophyte could be distinguished from *A. coenophialum* in stained leaf sheaths by its distinct hyphal morphology, as shown in Fig. 1. Similar characteristics were observed for *F. pratensis* plants listed in Table 1 which harbored non-*Acremonium* endophytes (data not shown). Also, the *Phialophora*-like endophyte was detected in tall fescue seed endosperm, though mycelia were sparse (Fig. 1). Later tissue print immunoblot assays of half-seeds suggested that the hyphae of this non-*Acremonium* endophyte were mainly concentrated in the embryo (data not shown).

The non-*Acremonium* endophytes were isolated from surface sterilized pseudostems of seed progeny from *F. pratensis* plants 176, 178, and 180. They were typically slow-growing, white in color, and difficult to distinguish from *Acremonium* endophyte cultures at the macroscopic level. They were found to be distinct from *Acremonium* endophytes by serology (tissue print immunoblot and ELISA) and by *rrnITS2* sequence (see below). The non-*Acremonium* endophyte in progeny of plant 180 was consistently obtained in pure culture, but those from progeny of plants 176 and 178 were isolated as mixed cultures with *Acremonium* endophytes. By plating serial dilutions of ground mycelia, several non-*Acremonium* endophytes which exhibited no significant reaction to antiserum against *A. coenophialum* were obtained in pure culture. Non-*Acremonium* endophytes were also obtained from *F. arizonica* plant 1572 and *F. gigantea* plant 95 (Table 1), and in both cases, *Acremonium* cosymbionts were also present. Together with the previous identifications of endophytes termed *Phialophora*-like and *Gliocladium*-like in *F. arundinacea* and *L. perenne*, respectively (13), this amounted to five plant species in which cosymbioses of *Acremonium* and non-*Acremonium* endophytes were observed.

**Serological relationships of non-*Acremonium* p-endophytes.** Serological analyses indicated a relationship between the non-*Acremonium* endophytes of all five grass species and also suggested that they were not closely related to *Acremonium* endophytes. Because of this relationship, the non-*Acremonium* endophytes identified in these five grass species are hereafter referred to collectively as p-endophytes. Antiserum was raised against isolate p180, the p-endophyte from seed progeny of *F. pratensis* plant 180. The reactivity of the anti-p180 antiserum was tested by indirect ELISA against *A. coenophialum* e19 and the *Phialophora*-like p-endophyte isolate p26 from tall fescue (Table 3). Although these tests are not quantitative, they consistently indicated an intense reaction of anti-p180 antiserum against both p180 and p26 and a weak reaction against e19. This difference was most apparent at the lower concentration of mycelia (1 μg/ml). Conversely, antiserum raised against isolate e19 reacted most strongly with e19 and very weakly with p180 and p26. Neither of these two antisera showed appreciable reaction to another ascomycetous fungus, *Penicillium chrysogenum* Thom.

TABLE 3. ELISA results for endophyte mycelia

| Fungus | Concn (μg/ml) | $OD_{405}$[a] | | |
|---|---|---|---|---|
| | | Anti-e19 | Anti-p180 | |
| | | | Plate 1 | Plate 2 |
| *A. coenophialum* e19 | 10 | 1.92 | NT | 0.38 |
| | 1 | 1.39 | NT | 0.14 |
| p180 | 10 | NT | 1.93 | NT |
| | 1 | NT | 0.89 | NT |
| *Phialophora*-like sp. p26 | 10 | 0.06 | 1.71 | 1.71 |
| | 1 | 0.00 | 1.23 | 0.97 |
| *P. chrysogenum* | 10 | 0.08 | 0.23 | NT |
| | 1 | 0.04 | 0.18 | NT |

[a] $OD_{405}$, optical density at 405 nm; NT, not tested.

The specificities of the antisera for either p-endophytes or *Acremonium* endophytes allowed discrimination of the endophyte types in tissue print immunoblot assays (Table 1). Non-*Acremonium* p-endophytes p180 and p26 both reacted with anti-p180 and anti-p26 but not with anti-e19. Conversely, anti-e19 showed reactivity against *A. coenophialum* e19 but not against p180 or p26. Antiserum from each of the inoculated rabbits showed specificity for either p-endophytes or *Acremonium* endophytes strictly in accordance with the type of endophyte with which the rabbit had been inoculated (data not shown).

Pure cultures of p95 and p178 and the *Gliocladium*-like endophyte from perennial ryegrass were also tested by tissue print immunoblot with both anti-p180 and anti-e19 (Table 1). They all reacted strongly with the anti-p180 antiserum but not with anti-e19. Conversely, pure cultures of *Acremonium* endophytes e166 and e178 from *F. pratensis* reacted with anti-e19 but not with anti-p180.

The antisera were also sufficiently specific to discriminate the two endophyte types in pseudostem sections from *F. pratensis* and *F. arundinacea* plants (Table 1). Those plants from which mixed cultures had been obtained reacted with both antisera, those from which pure p-endophyte cultures were obtained reacted with anti-p180 but not anti-e19, and plants from which pure *Acremonium* cultures were obtained reacted with anti-e19 but not with anti-p180. Tall fescue plants 26 and 19, which had specific endophytes introduced into them (p26 and e19, respectively) reacted as expected. In addition, 30 half-seeds from plant p26 were analyzed by the same method; 86% were serologically positive for p-endophytes, and as expected, all were negative for *Acremonium* endophytes. In contrast, seeds from *F. arundinacea* cv. Kentucky 31 were consistently positive for *Acremonium* endophytes and negative for p-endophytes.

Maternal-line transmission of p-endophytes and *Acremo-*

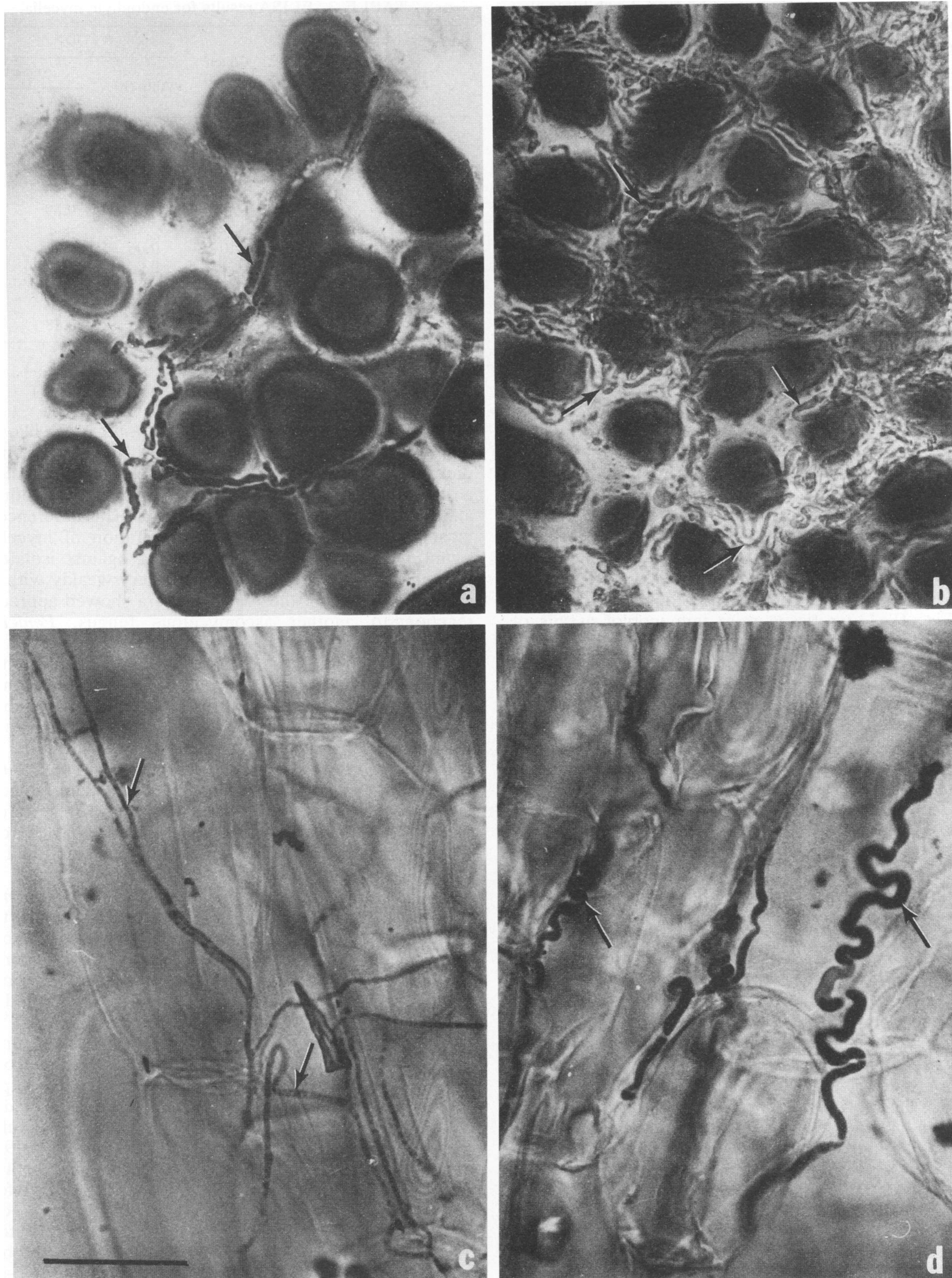


FIG. 1. Photomicrographs of p-endophytes (a and c) and *A. coenophialum* (b and d) in the seed aleurone tissue (a and b) and leaf sheaths (c and d) of *F. arundinacea*, stained by the method of Latch et al. (14). Fungal hyphae are indicated by arrows. Note that, in seed tissue (a), the p-endophyte is highly septated. The *A. coenophialum* mycelium (b) is much more densely packed around the darkly stained aleurone cells. In leaf sheaths (c), the p-endophyte produces straight, branched, intercellular hyphae, which are thinner than those of *A. coenophialum* (d). The latter has highly convoluted hyphae which typically follow the longitudinal axis of the host cells. Bar, 10 μm (magnification, ×400).

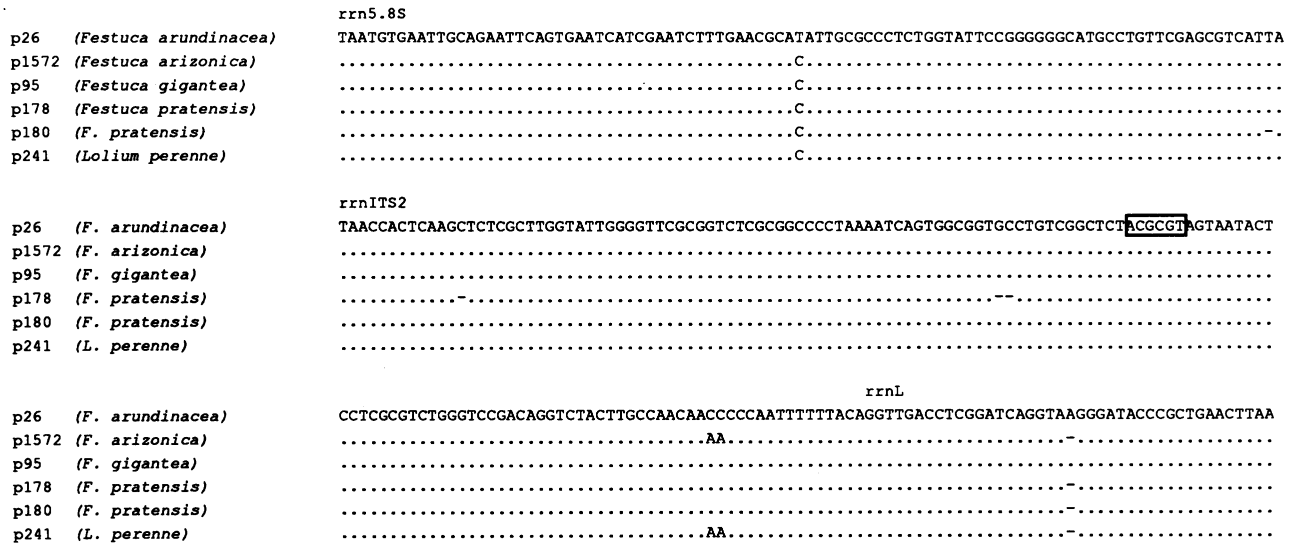


FIG. 2. Sequence alignments of a segment of internal transcribed spacer 2 (*rrnITS2*) and flanking portions of rRNA structural genes from the indicated p-endophytes. Identity to the reference sequence at the top is indicated by dots; alignment gaps (insertion or deletion differences) are indicated by dashes. The *Mlu*I site common to the p-endophyte *rrnITS2* sequences is boxed.

*nium* endophytes in *F. pratensis* was confirmed by tissue print immunoblots. Of five progeny of plants 176 and 177, all were positive for both p-endophytes and *Acremonium* endophytes. Of five progeny of plant 178, four were positive for p-endophytes and all were positive for *Acremonium* endophytes. None of the progeny of plant 180 appeared to have *Acremonium* endophytes, but four of the five were positive for p-endophytes. Seeds from other plants gave rise to progeny which consistently possessed *Acremonium* endophytes. Three to five progeny each from plants 166 to 175 and 179 were screened, and all but one (from plant 169) were positive for *Acremonium* endophytes and negative for p-endophytes.

In order to further assess the usefulness of the antisera in distinguishing the endophytes from several possible grass pathogens and saprophytes which may occur in seed or vegetative tissues, the serological reactivities of 20 other fungi were tested by tissue print immunoblot (Table 2). In each case, mycelia from p180 and e19 were included as controls. No strong positive reactions against any nonendophytic fungi were observed. Only *P. chrysogenum* gave an apparent weak reaction, but examination under a dissecting microscope indicated that the color was of a light shade (pink rather than deep red), as was sometimes also observed with the negative controls and uninfected plant tissues. A similar specificity was observed for the anti-e19 antiserum in tests in which e19 and p26 mycelia served as controls. Again, among the nonendophytic fungi listed in Table 2, only *P. chrysogenum* gave a reaction that may have been interpreted as positive, but on microscopic examination it was judged atypical of the positive control.

**DNA sequence relationships of the p-endophytes.** The possible relationships of the p-endophytes with each other and with the *Acremonium* endophytes were investigated by sequence analysis of *rrnITS2* (Fig. 2 and 3). The p-endophyte sequences were closely related to each other, differing by 0 to 3 nucleotide substitutions and 1 to 4 positions with alignment gaps, in a total of 148 positions (Fig. 2). Although none of the sequences were identical, the similarity between the p-endophyte *rrnITS2* sequences was much greater than that between the *rrnITS2* sequences of *Acremonium* endophytes and those of *E. typhina* isolates, which differed by up to 16 nucleotide substitutions (1, 20). Thus, the *Phialophora*-like endophyte, the *Gliocladium*-like endophyte, and other p-endophytes exhibited sequence similarities suggestive of congeneric or even conspecific relationships.

Inspection of the *rrnITS2* sequence indicated the possibility that PCR, followed by restriction endonuclease cleavage, could be a rapid, nonserological method to distinguish p-endophytes from *Acremonium* endophytes. The p-endophyte *rrnITS2* fragments were cleaved with *Mlu*I but not with *Not*I, whereas *Acremonium* endophyte *rrnITS2* contained a *Not*I site but no *Mlu*I site. Thus, amplification and cleavage of this DNA segment resulted in a pattern characteristic of the type of endophyte from which the DNA was obtained (Fig. 4). Both patterns were obtained from mixed cultures (data not shown).

To investigate the relationship between the p-endophytes and euascomycetes, particularly *E. typhina* and the *Acremonium* endophytes, sequence comparisons of more conserved portions of the rRNA genes were made (Fig. 3). Sequences which could not be aligned unambiguously (lowercase letters in Fig. 3) were omitted from the phylogenetic analysis. The remaining regions exhibited enough usable variation for parsimony analysis. The *E. typhina* and *A. coenophialum* sequences were identical in the aligned regions and were, therefore, treated as a single taxonomic unit. The single most parsimonious tree (Fig. 5) placed the tall fescue p-endophyte (p26) within the euascomycetes but closer to the plectomycetes than to the pyrenomycetes. In contrast, *A. coenophialum* was placed with the pyrenomycetes (represented by *E. typhina*, *G. graminis*, and *Neurospora crassa* Sheer et Dodge). Bootstrap replications suggested a high confidence for separating the pyrenomycete clade from the other fungi, including the p-endophyte, *E. nidulans*, and *P. chrysogenum*. Although these results do not identify the specific taxonomic position of the p-endophytes within the euascomycetes, they do indicate distinct evolutionary origins of the two endophyte types.

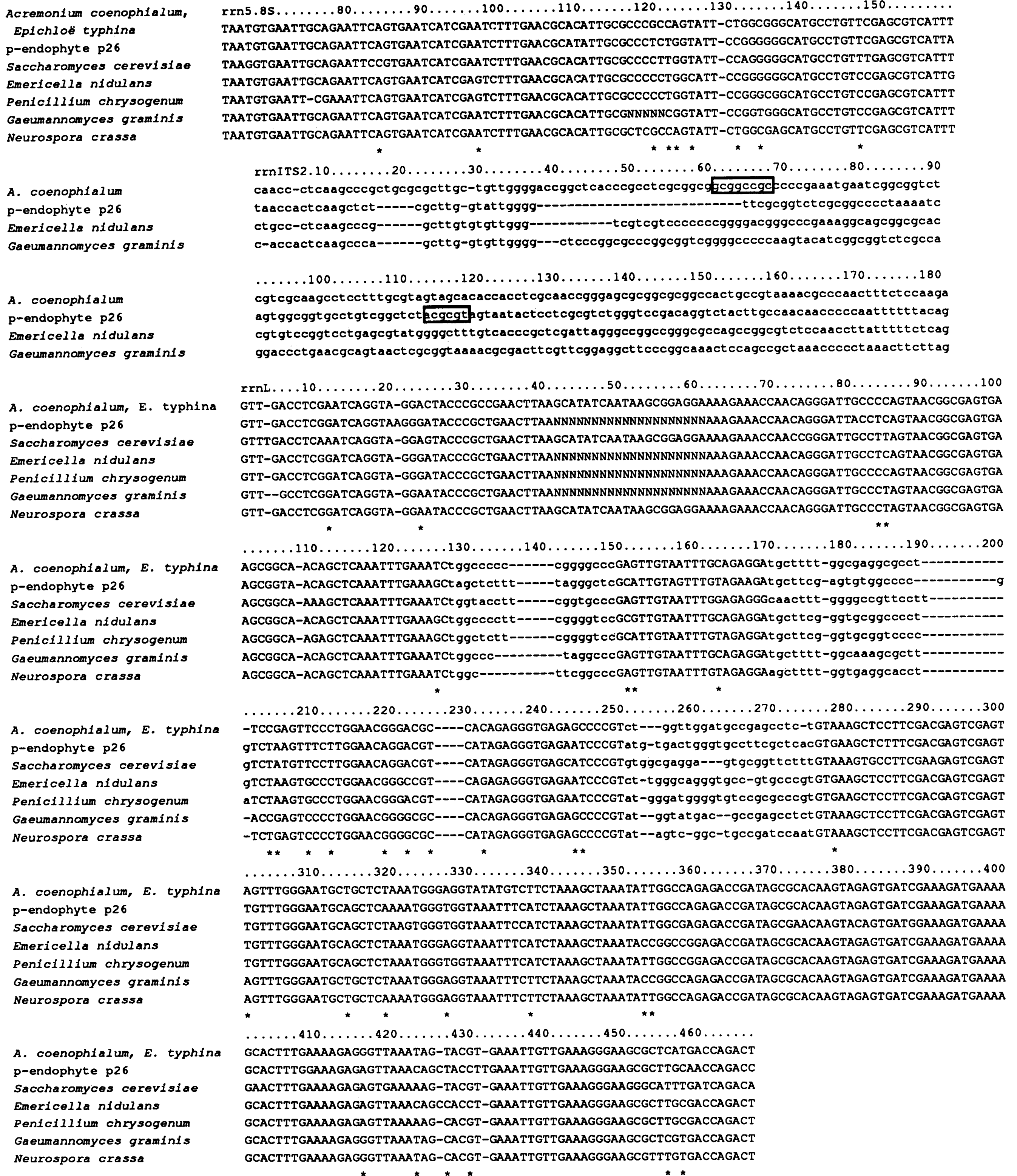

FIG. 3. Sequence alignments of a segment of the rRNA gene repeats encoding the 3′ portion of the 5.8S rRNA (*rrn5.8*), internal transcribed spacer 2 (*rrnITS2*), and the 5′-terminal portion of the 26S large-subunit rRNA (*rrnL*). Positions in the *rrnL* segment were aligned and numbered according to the system of Perasso et al. (15). Regions which were too divergent for unambiguous alignment and, therefore, were not used in phylogenetic analysis are indicated by lowercase letters; positions that were informative and used for parsimony (Fig. 5) are indicated by asterisks. The *Not*I site in *rrnITS2* of *A. coenophialum* and the *Mlu*I site in *rrnITS2* of p-endophyte p26, which were used for restriction analysis of PCR products, are boxed.

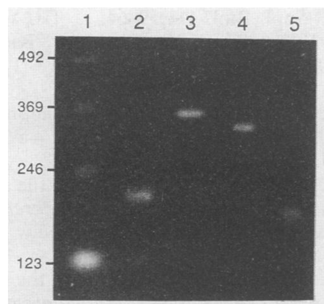


FIG. 4. Analysis of amplified DNA spanning *rrnITS2* by restriction endonuclease cleavage and electrophoresis. Lane 2, the amplified product from *A. coenophialum* e19, subsequently cleaved with *Not*I; lane 3, the e19 product treated with *Mlu*I, which did not cleave it; lane 4, the amplified product from p-endophyte p26, treated with *Not*I and remaining uncleaved; lane 5, the p26 product cleaved with *Mlu*I. The sizes (in base pairs) of DNA markers (lane 1) are indicated on the left.

## DISCUSSION

The results reported herein indicate that the non-*Acremonium* p-endophytes can occur cosymbiotically with *Acremonium* endophytes in several species of the related grass genera *Festuca* and *Lolium*. They further indicate that the p-endophytes, including those previously distinguished as *Phialophora*-like and *Gliocladium*-like (13), are closely related. Thus, they constitute a second group of endophytes, which is more widely distributed than previously suspected. Serological and PCR techniques were adapted for their identification in host vegetative tissues and seeds and in culture.

Although the two p-endophytes previously reported were not originally considered congeneric (13), their *rrnITS2* sequence homology is actually much higher than that exhibited by different isolates of the single teleomorphic species *E. typhina* and much higher than that between *Acremonium* endophytes of the genera *Festuca* and *Lolium* (20). Two possibilities, which are not mutually exclusive, may explain this relatively close sequence similarity of the p-endophytes. First, rates of evolution of the *rrnITS2* sequences in these fungi are not known, and it is possible that the *Acremonium* sequences evolve faster than those of the p-endophytes. Second, it is also possible that the p-endophytes are more rapidly disseminated than the *Acremonium* endophytes and *E. typhina*. The p-endophyte of perennial ryegrass sporulates in nature (16), and unlike the *Acremonium* endophytes, it can be introduced into host seedlings without prior wounding of the plant (12). In contrast, *Acremonium* endophytes are exclusively transmitted via host seeds, rhizomes, and tillers (23). Spore dissemination may lead to a more rapid spread of the p-endophytes geographically and among different hosts. For this reason, it is possible that the p-endophytes in this study have a more recent common ancestry than do the *Acremonium* endophytes from the same host grasses.

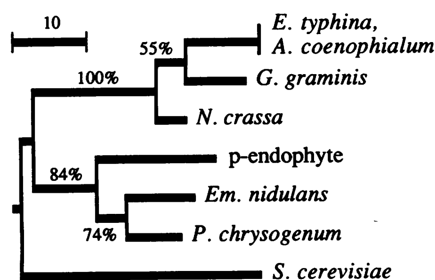


FIG. 5. The most parsimonious gene tree based on aligned 5.8S and *rrnL* sequences shown in Fig. 3. A distance of 10 nucleotide substitution differences is indicated by a bar at the upper left. The frequencies with which each branch was represented in 1,000 bootstrap replications (7) are indicated. The total length of each tree was 122, and the consistency index, excluding autapomorphies, was 0.710. The root of the tree was established by using orthologous sequences from *Mus musculus* and *L. perenne*.

In addition to taxonomy and phylogeny, there are other interesting differences between p-endophytes and *Acremonium* endophytes. The most thoroughly studied p-endophyte is that of perennial ryegrass, which (like the tall fescue p-endophyte) is found in virtually all plant tissues (13, 16). In contrast, *Acremonium* endophytes are not observed in roots and can have restricted distribution in leaf blades (23). Also, the *L. perenne* p-endophyte has been observed to invade host cells and to sporulate, but only in senescent host tissues (18). For this reason, the p-endophyte produced spores without causing any apparent disease, unlike *E. typhina*, which sterilizes host panicles during its sporogenous stage.

In contrast to the *Acremonium* endophytes, the possible benefits of the p-endophytes remain relatively unexplored. The wide spectrum of antifungal activity exhibited in agar culture by the p-endophyte of tall fescue suggests that it may enhance resistance to fungal diseases (22). Since p-endophytes often occurred together with *Acremonium* endophytes in *Festuca* and *Lolium* spp., it is also possible that the two mycosymbionts have synergistic activities in biological protection and other aspects of host fitness. However, the possibility that some or all of the p-endophytes are commensal symbionts or are even antagonistic to their hosts must also be considered. Their apparent antifungal activities and the lack of obvious disease symptoms associated with p-endophyte infection do not constitute adequate evidence of mutualism. Extensive fitness studies comparing two-part symbiotic entities (plants with one endophyte) and three-part symbiotic entities (plants with cosymbiotic *Acremonium* endophytes and p-endophytes) with host grasses lacking endophytic fungi need to be undertaken. If the p-endophytes are found to be deleterious to their hosts or otherwise undesirable (e.g., toxic to livestock), then the serological and PCR-based detection techniques described here may be used to screen seed lots and thereby reduce their presence in commercial cultivars. If the p-endophytes are mutualists, then these tools can be used in a program to introduce them into cultivars.

The ability of *Festuca* and *Lolium* spp. to harbor nonpathogenic, seed-borne mycosymbionts effectively increases their biological complexity and heritable genetic diversity. Under certain conditions of biotic or abiotic stress, these grasses appear to be ecologically dependent upon their *Acremonium* endophytes (6, 23, 27). However, it is possible that, in many tests of the benefits of *Acremonium* endophytes, it was unknown whether the *Acremonium* endophytes were present alone or in cosymbiosis with p-endophytes. In the future, it will be important to determine the status of experimental plants with regard to the presence of both endophyte types and to investigate the ecological importance of the p-endophyte–grass interactions as well as the possible interactions of cosymbiotic endophytes.

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