A Polymerase Chain Reaction-Based Procedure for Detection of *Acremonium coenophialum* in Tall Fescue

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ABSTRACT

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Inserts from clones of a genomic library constructed using DNA from the fungal endophyte *Acremonium coenophialum* were screened using dot blots and Southern blots. Several inserts that hybridized to DNA from *A. coenophialum* and to DNA from endophyte-infected (E+) tall fescue (*Festuca arundinacea*), but not to DNA from endophyte-free (E–) grass, were sequenced. Oligonucleotide primers were synthesized, and polymerase chain reaction (PCR) was carried out using DNA from E+ and E– tall fescue genotypes. PCR with one set of 21-mer primers yielded a prominent 1-kb product with DNA from *A. coenophialum*-infected plants but not from uninfected plants. This PCR-based procedure provides an accurate, rapid, and sensitive means of detecting *A. coenophialum* in tall fescue.

Additional keywords: *Epichloë*, fescue toxicosis, forage grass, turf grass.

Tall fescue (*Festuca arundinaceae* Schreb.) is an important forage and pasture grass grown on about 14 million hectares in the southeastern United States (3). It grows well during cool winters, survives warm summers, and tolerates soils with poor drainage and low pH. It produces a heavy turf, making it suitable for lawns and stabilization of road banks.

A fungal endophyte, *Acremonium coenophialum* Morgan-Jones and W. Gams (17), grows intercellularly and asymptomatically within the foliage of tall fescue (2). Natural transmission to uninfected plants has not been observed (24). (*Acremonium* endophytes are thought to be anamorphs of *Epichloë* sp. [32]). The host-fungus interaction is described as mutual-symbiosis, because both the plant and endophyte benefit from the relationship (25). Benefits to the host include enhanced rate of seed germination and increased seedling vigor, improved drought tolerance, greater persistence, increased resistance to insects and other pests, and reduced disease severity with some, but not all, pathogens (1,2,7, 8,12,25,29,31).

A. coenophialum produces alkaloids that are toxic to cattle and other livestock, and ingestion of endophyte-infected tall fescue by such animals can result in fescue toxicosis (2,11,23,26). Hence, depending on end-use, the presence of the endophyte can be a positive or negative factor in tall fescue production.

Methods of detecting endophyte hyphae in leaf sheaths, crowns, meristems, and seeds include tissue stains (5,20,28), immunological procedures (14,15,18), a combination of the two (10), and tissue culture of seed samples (6). Although all these methods are used, infection can be overlooked when hyphae are few or sparsely distributed in host tissue. Indeed, sometimes a plant considered as endophyte free (E–) may actually be endophyte infected (E+). Erratic infection of tillers and inflorescences have been noted for other species of *Acremonium* in other grasses (33) and for *A. coenophialum* in tall fescue (30).

Accurate determination of endophyte infection status is critical for experiments with tall fescue genotypes. Moreover, during the past 10 years commercial plant breeders have developed tall fescue populations with high percentages of E+ plants for use as turf cultivars and low percentages of E+ plants for use as hay and pasture cultivars. Therefore, knowledge of endophyte infection status is important for evaluating cultivars for these purposes and for plant breeding programs.

The purpose of this study was to develop and evaluate a method of detecting *A. coenophialum* in tall fescue based on polymerase chain reaction (PCR) (21). Such a method could provide an accurate, rapid, and sensitive means of determining the endophyte status of this important grass species.

MATERIAL AND METHODS

Fungal cultures and plant tissue. A culture of *A. coenophialum* (designated Ac011), originally isolated from tall fescue, was used as a source of DNA for construction of a genomic library. DNA was extracted from tall fescue genotypes thought to be E+ or E– on the basis of histological examination (5,28) or tissue culture assay (6). Leaf sheaths of tall fescue were obtained from field-grown plants, except for genotypes F-11 and D-11, which were greenhouse grown.

DNA also was extracted from leaf sheaths of *Acremonium* endophyte-infected creeping red fescue, *Festuca rubra* subsp. *rubra*; hard fescue, *F. ovina* var. *duriuscula*; Chewings fescue, *F. rubra*

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var. *commutata*; and perennial ryegrass, *Lolium perenne*; and seed of two wild barley species, *Hordeum bogdanii*; and *H. brevisubulatum* that also harbored *Acremonium* endophytes (34) (S. L. Clement, USDA-ARS, Pullman, WA, *unpublished data*). The barley seed was provided by S. L. Clement, Regional Plant Introduction Station, USDA-ARS, Pullman, WA. Leaf sheath tissue of creeping red fescue, hard fescue, Chewings fescue, and turf-type tall fescue (8030, 8300, 8400, and 'Titan') plants were provided by L. Brilman, Seed Research of Oregon, Inc., Corvallis, OR.

DNA isolation. DNA was isolated from fungal mycelia of isolate Ac011 or from leaf sheaths or seed using the procedure of Rogers and Bendich (19). DNA then was treated with RNase A (50 g/ml in 10 mm of Tris HCl [pH 8], 1 mM EDTA, 1 M NaCl), extracted with phenol/chloroform/isoamyl alcohol (25:24:1, vol/vol/vol), and precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol. The concentration was estimated using the ethidium bromide fluorescence method (22) after suspension in 10 mM Tris HCl (pH 8) containing 1 mM EDTA.

To determine whether a less-complicated DNA extraction procedure could be used, the method of Stewart and Via (27) was employed with the turf-type tall fescues and when making the comparison between endophyte detection procedures (described in the results). This method, which takes 10 min or less per sample, yields DNA suitable for PCR applications (27).

Genomic library construction and screening. Standard procedures were used for construction of a genomic library (22). Fungal DNA was digested with *Sau*3AI and ligated into pSportI (Life Technologies, Inc., Grand Island, NY) that had been digested with *Bam*HI and treated with calf alkaline intestinal phosphatase.

Transformation was carried out using electrocompetent *Escherichia coli* strain DH12S (Life Technologies) and a Cell-Porator electroporation system with voltage booster (Life Technologies). Colonies of transformed bacteria that contained fungal DNA inserts were identified on LB-amp plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside and isopropylthio-β-galactoside (white/blue screening) (22).

Plasmids were isolated from recombinant clones using standard methods (22), and inserts were excised by double digestion with the restriction endonucleases *Xba*I and *Hin*dIII. Inserts were purified after electrophoresis through low melting temperature agarose (FMC Corp., Philadelphia) using Spin-Bind units (FMC), and labeled with α-^{32}P-dCTP using a random primer labeling kit (Life Technologies) (9).

Dot blotting was carried out with a Minifold apparatus (Schleicher & Schuell, Inc., Keene, NH) with either nitrocellulose or Nytran membranes (Schleicher & Schuell) and procedures suggested by the vendor. Southern blotting was done using Nytran membrane and standard methods (22), except that a semidry capillary blotting procedure was used (13).

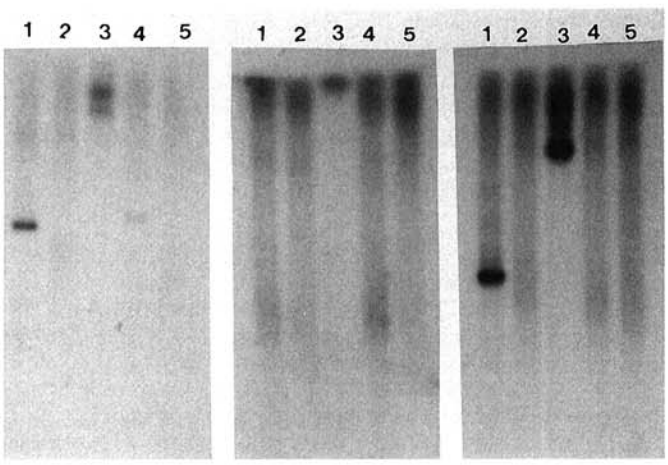

Fig. 1. Southern blots of *Acremonium coenophialum* isolate Ac011 DNA probed with α-^{32}P-dCTP–labeled inserts from plasmid clones 11 (left panel), 16 (middle panel), and 25 (right panel). Fungal DNA (0.3 μg per lane) was digested with lane 1, *Eco*RI; lane 2, *Sal*I; lane 3, *Sau*3AI; lane 4, *Xba*I; and lane 5, *Hin*dIII and subjected to agarose gel electrophoresis (1% gel).

Sequencing of inserts and primer synthesis. Sequencing of inserts was carried out by the Central Service Laboratory of Oregon State University's Center for Gene Research and Biotechnology. SP6 and T7 primers (Applied Biosystems [Foster City, CA] dye primers; SP6, 5′-ATT TAG GTG ACA GTA TAG-3′; T7, TAA TAC GAC TCA CTA TAG GG) were used with plasmid prepared using Magic Minipreps (Promega, Madison, WI).

Primers (21-mers) to be used for PCR were designed to amplify as large a fragment of DNA as possible and to possess a GC content of about 50%. Synthesis was carried out at the Central Services Laboratory, and the primers were purified using Nensorb (NEN, Boston) cartridges.

PCR. PCR (21) was carried out in 500-μl microcentrifuge tubes using 1.25 units of AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, CT) per 50-μl reaction. Reactant concentrations suggested by Perkin-Elmer were used with 2 mM $MgCl_2$ and 0.5 μg of DNA per reaction. After formulation (on ice), reactions were held at 94°C for 1 min and then subjected to 30 cycles of the following schedule: 94°C for 1 min, 60°C for 2 min, 72°C for 2 min. After cycling, a 10-min incubation at 72°C was used to complete elongations.

PCR products (10 μl of the 50-μl reactions) were subjected to agarose gel electrophoresis 0.5% (wt/vol) agarose (Sigma A-9539; Sigma Chemical Co., St. Louis) and 0.75% (wt/vol) Synergel, (Diversified Biotech, Newton Centre, MA) (equivalent to 2% agarose) and examined after soaking the gel in 0.5 μg of ethidium bromide per ml for 30 min and destaining in water for 30 min.

RESULTS

DNA yield from fungal tissue (mycelium was blotted dry with filter paper) was about 12 μg of DNA per g wet weight. Grass yielded from 25 to 80 μg of DNA per g fresh weight (leaf sheaths) or about 100 μg of DNA per g dry weight (wild barley seeds).

In a preliminary screening of eight inserts of various sizes (150 to 1,000 bp) using dot blots, seven hybridized to endophyte DNA, and five of these also hybridized to DNA from a tall fescue genotype (D-11) that was colonized by the endophyte. Using these five inserts to probe Southern blots of endophyte DNA indicated two (from clones 11 and 25) were complementary to low or single copy sequence (Fig. 1, panels 1 and 3). Two other inserts (from clones 1 and 16) yielded smears when used to probe Southern blots, indicating they were complementary to high copy number sequence. The fifth insert (from clone 3) failed to give a signal when used to probe Southern blots.

Inserts from clones 11, 16, and 25 were examined further using dot blots (Fig. 2) with DNA from *A. coenophialum* isolate Ac011 and from seven tall fescue genotypes. Hybridization signals with DNA from three E+ genotypes (D-11, 213-8, and 213-10) were stronger than signals seen with DNA from two E– genotypes (F-11 and 104-10). The signal with DNA from genotype 407-3 (plant 28 [30]) was not visibly stronger than that seen with the negative controls. This genotype was included because in earlier studies (30) it had exhibited low incidence of seed infection (2%) and low ergovaline content (27 ng/mg). Interestingly, DNA from genotype 205-9 (plant 27 [30]), which in earlier studies had exhibited no seed infection but an ergovaline content of 33 ng/mg (30), gave a positive hybridization signal. Both of these genotypes exhibited the PCR product typical of endophyte-infected (E+) tall fescue plants (described below).

Nucleotide sequence information was obtained from both ends of the insert from clone 11 (insert size about 1,000 bp), and the entire sequence of inserts from clones 25 (573 bp) and 16 (181 bp) was ascertained. PCR primers (21-mers) were chosen to allow

amplification of as large a fragment as possible. (Primer 11-1, 5′-TCA TAC CGG CTA ACC GGC AAT-3′; Primer 11-2, 5′-TGT TAC AGG ATT GGT AGA GGC-3′; primer 16-1, 5′-TCC AAG CTA AGG TCT CTT-3′; primer 16-2, 5′-TCC CTA GTC ATG TGA CCT TCT-3′; primer 25-1, 5′-TTA TCC GAA GGA GAT GGA CAT-3′; and primer 25-2, 5′-ATC TGC GCA AGG TAG TCG GCA-3′) Preliminary tests indicated that PCR would be most selective with the primer pair complementary to the ends of the insert from clone 11 (primers 11-1 and 11-2).

When the tall fescue genotypes used for dot blots (Fig. 2) were examined using PCR, a 1-kb amplification product was obtained only when DNA from infected plants was used (Fig. 3A). With four of the five infected genotypes, this 1-kb band was quite prominent. With genotype 407-3, one of the tall fescue plants with a low incidence of endophyte mycelia, the band was clearly present but faint. This genotype failed to give a positive signal with the dot blot. The 1-kb band was not seen with DNA from either of the E– plants.

PCR also was carried out in a blind test using primers 11-1 and 11-2 and DNA isolated from 18 additional tall fescue genotypes. Prominent amplification of a 1-kb fragment again occurred only with DNA from genotypes known to be endophyte infected (Fig. 3B). Endophyte-free genotypes did not yield the 1-kb product, although a faint 0.7-kb band, presumably a PCR artifact, could be seen in some cases with DNA from both E+ and E– plants. The results discussed above were obtained with forage-type tall fescue genotypes. Because these forage genotypes have a common origin (discussed below), four endophyte turf genotypes also were examined. They too yielded DNA that supported amplification of the diagnostic 1-kb product (Fig. 3C). Finally, when leaf sheaths from 45 tall fescue seedlings, grown from E+ and E– seed, were examined using both a histological method (28) and the PCR-based procedure, 31 were rated E+ on the basis of both techniques, 10 were rated E– on the basis of both techniques, and 4 were rated E– by the PCR technique and E+ after histological examination.

The amplification product diagnostic for E+ tall fescue was not obtained using DNA from several other endophyte-infected *Festuca* species (Fig. 4). Similarly, DNA from E+ perennial ryegrass and E+ wild barley did not serve as a template for amplification of the 1-kb fragment. If DNA from *A. coenophialum* was added to DNA from these grasses the typical 1-kb fragment was amplified. This indicated that the failure to obtain a signal with the unspiked samples was not a result of inhibition of the reaction by the grass extracts.

DISCUSSION

PCR can be used to detect *A. coenophialum* in tall fescue tissue. A diagnostic procedure based on this technique provides an accurate, rapid, and sensitive method to ascertain the endophyte status of tall fescue genotypes.

With the PCR method described here, it is possible to screen several dozen tall fescue lines in a day, and, with modification (e.g., rapid DNA extraction, microtiter plate PCR format, etc.), screen-

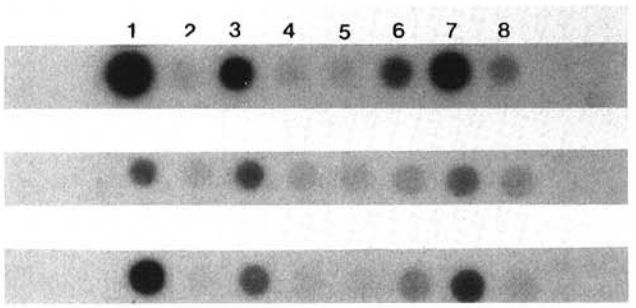

Fig. 2. Dot blots of *Acremonium coenophialum* and tall fescue DNA probed with α-^{32}P-dCTP–labeled inserts from clones 11 (top row), 16 (middle row), or 25 (bottom row). Blotted DNA from the left in each row was from lane 1, *A. coenophialum* isolate Ac011 (10 ng per dot) and tall fescue genotypes (1 μg per dot); lane 2, F-11, E–; lane 3, D-11, E+; lane 4, 407-3, E+; lane 5, 104-10, E–; lane 6, 213-8, E+; lane 7, 213-10, E+; and lane 8, 205-9, E+.

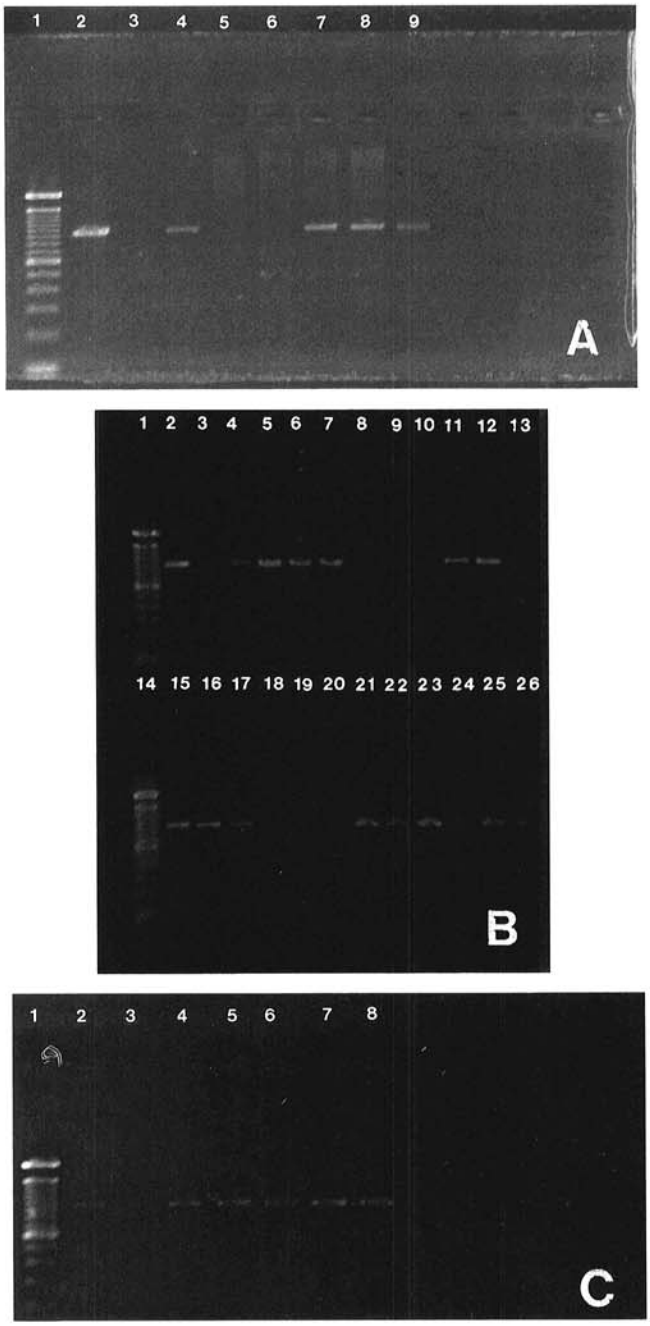

Fig. 3. Amplification products from polymerase chain reaction (PCR) using DNA from **A,** *Acremonium coenophialum* isolate Ac011 (lane 2) and the tall fescue genotypes examined using dot blots (Fig. 2). Lane 1 was loaded with a 100-bp sizing ladder. Lanes 3–9 were loaded with PCR products obtained using DNA from the following tall fescue genotypes: F-11, E–; D-11, E+; 407-3, E+; 104-10, E–; 213-8, E+; 213-10, E+; and 205-9, E+. **B,** *A. coenophialum* isolate Ac011 and 23 tall fescue genotypes. Lanes 1 and 14 were loaded with a 100-bp sizing ladder. Lane 2 was loaded with PCR products obtained using DNA from *A. coenophialum* isolate Ac011. Lanes 3–13 and 15–26 were loaded with PCR products obtained using DNA from the following tall fescue genotypes: F-11, E–; D-11, E+; 106, E+; 111, E+; 116, E+; 121, E–; 124, E–; 131, E–; 305, E+; 317, E+; 322, E–; 327, E+; 333, E+; 337, E+; 509, E–; 513, E–; 524, E–; 528, E+; 534, E+; 539, E+; 213-8, E+; 213-10, E+; and 205-9, E+. **C,** *A. coenophialum* isolate Ac011, 3 forage-type tall fescues, and 4 turf-type fescues. Lanes 1–4 were loaded as in **B**. Lanes 5–8 were loaded with PCR products obtained from the following turf-type tall fescues genotypes (all E+): 8030, 8300, 8400, 'Titan.'

ing of several hundred lines per day is feasible. Some, but not all, of the existing techniques take up to 28 days for screening (6). Another advantage of this method is that only small amounts of tissue are required, and an assay based on amplification of DNA from a sample of individual seeds could provide information about the percentage of endophyte infection in a seed lot.

In developing the PCR technique, two tall fescue genotypes 205-9 and 407-3 (plants 27 and 28 [30]) that exhibited 0 and 2% incidence of seed infection, respectively, yielded DNA that supported amplification of the 1-kb product diagnostic for infection with *A. coenophialum.* These genotypes previously had been judged to be E–; however, after detecting low levels of ergovaline in the seed (30), careful reexamination revealed a few widely scattered hyphae typical of the fungus. Similarly, genotype 513 had been considered E+ on the basis of a tissue culture assay. The negative result obtained in the PCR analysis prompted reexamination of this genotype at which time it was discovered that 513 harbors an endophyte morphologically distinct from *A. coenophialum.*

Four false negatives were obtained when the PCR-based technique was compared to a histological method (28). However, with three of these four, infection was extremely light. Because the plants in question were grown from E+ seed, it was expected that the endophyte would be present, and protracted inspection involving extensive amounts of tissue was undertaken. Under the usual diagnostic conditions, these plants probably would have been given a less thorough examination and would have been scored E–. Hence, it is likely that the PCR method will provide as sensitive a means of detecting *A. coenophialum* in tall fescue as those used currently. Moreover, given clear-cut protocols, the PCR test will be easier to perform and require less experience for interpretation than any of the tests currently in use.

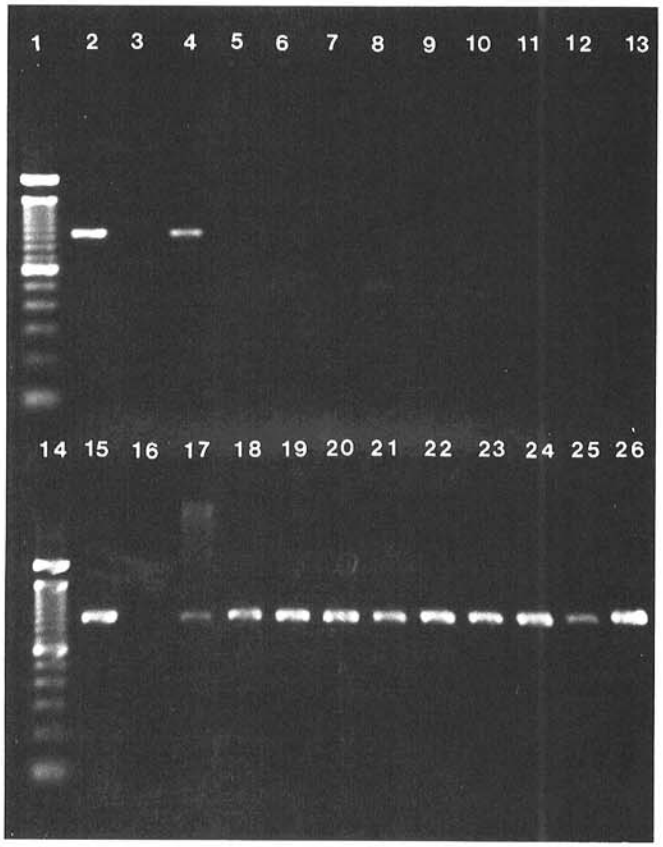

Fig. 4. Amplification products from polymerase chain reaction (PCR) carried out with DNA from *Acremonium coenophialum,* with DNA from an endophyte-free (F-11), an endophyte-infected (D-11) tall fescue genotype, and DNA from several other endophyte-infected grass species. Lane 1 was loaded with a 100-bp sizing ladder. Lane 2 was loaded with PCR products obtained using DNA from *A. coenophialum* isolate Ac011. Lanes 3–13 were loaded with PCR products obtained using DNA from the following grasses: tall fescue genotypes F-11, E–, and D-11, E+; Chewings fescue, E+; hard fescue, E+; creeping red fescue, E+; blue fescue; *Hordeum bogdanii,* PI 314696, E+; *H. brevisubulatum,* PI 401386, E+; *H. bogdanii,* PI 296406, E+; perennial ryegrass, R12P19, E+, and R9P4, E+. Lanes 14–17 were loaded exactly as lanes 1–4. Lanes 18–26 were loaded with PCR products obtained using DNA from the grasses described above to which had been added 0.25 µg of DNA from *A. coenophialum* isolate Ac011.

Leuchtmann and Clay (16) reported that 47 of 52 isolates of *A. coenophialum* from tall fescue exhibited identical isozyme phenotypes, and concluded that the fungus was "...genetically depauparate compared to most other endophytes...." Similarly, cluster analysis, also based on isozymes, carried out by Christensen et al. (4), placed all of the *A. coenophialum* isolates examined into a single taxonomic grouping in which no less than 7 of the 11 enzymes examined exhibited identical electrophoretic behavior. Several other endophytes of tall fescue, as well as endophytes of other grasses, were placed in different taxonomic groupings. Christensen et al. (4) speculated that *A. coenophialum* found in a taxonomic grouping of isolates of tall fescue plants derived from a northern European population are geographically and genetically isolated from Spanish and North African taxonomic groupings. This inferred genetic similarity of *A. coenophialum* isolates from a number of tall fescue genotypes, and dissimilarity with respect to other *Acremonium* sp., is consistent with the finding of the present study that the 1-kb amplification product was obtained only with DNA from tall fescue infected with *A. coenophialum* and not with DNA from several other E+ grasses. All of the forage-type tall fescue genotypes used in this study, indeed nearly all of the genotypes grown in the United States, originated from one or a few populations (4,16). The turf-type genotypes examined are not derived from these populations, suggesting that the PCR method is capable of detecting *A. coenophialum* in other sources of tall fescue germ plasm.

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