Origin of a Fungal Symbiont of Perennial Ryegrass by Interspecific Hybridization of a Mutualist with the Ryegrass Choke Pathogen, *Epichloë typhina*

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ABSTRACT

Seed-borne fungal symbionts (endophytes) provide many cool-season grass species with biological protection from biotic and abiotic stresses. The endophytes are asexual, whereas closely related sexual species of genus Epichloë (Clavicipitales) cause grass choke disease. Perennial ryegrass (*Lolium perenne*) is a host of two endophyte taxa, LpTG-1 (*L. perenne* endophyte taxonomic grouping one = *Acremonium lolii*) and LpTG-2, as well as the choke pathogen, *Epichloë typhina* (represented by isolate E8). Relationships among these fungi and other *Epichloë* species were investigated by analysis of gene sequences, DNA polymorphisms and allozymes. The results indicate that LpTG-2 is a heteroploid derived from an interspecific hybrid. The LpTG-2 isolates had two copies each of nine out of ten genes analyzed (the exception being the rRNA gene locus), and the profiles for seven of these were composites of those from *E. typhina* E8 and *A. lolii* isolate Lp5. Molecular phylogenetic analysis grouped the two β-tubulin genes of LpTG-2 into separate clades. One (tub2-1) was related to that of *E. typhina* E8, and the other (tub2-2) to that of *A. lolii.* The mitochondrial DNA profile of LpTG-2 was similar to that of *A. lolii,* but its rRNA gene sequence grouped it with *E. typhina* E8. A proposed model for the evolution of LpTG-2 involves infection of a *L. perenne-A. lolii* symbiont by *E. typhina,* followed by hybridization of the two fungi. Such interspecific hybridization may be a common and important mechanism for genetic variation in *Epichloë* endophytes.

**ASSOCIATIONS** of grasses with fungal species of genus *Epichloë* (Clavicipitales) and related asexual endophytes (classified in section Albo-lanosa of form genus *Acremonium* of the Fungi Imperfecti) comprise an evolutionary continuum from mutualism to antagonism. Among the associations of grasses with *Epichloë* species, the degree of mutualism or antagonism is related to the relative importance of the asexual or sexual life cycle of the fungus (*Clay* 1988). Asexual endophytes cause no disease and are apparently disseminated only by clonal propagation in host seed. Where fitness of the hosts has been assessed, the endophytes have conferred demonstrable benefits including protection from herbivory (mammalian and insect), disease (fungal and nematode) and drought, and increased production of biomass and seeds (*Clay* 1990; *Kimmons et al.* 1990; *Rice et al.* 1990). In contrast, the *Epichloë* species in their sexual life cycle, by suppressing host seed production, can be antagonistic to the life cycles of both the hosts and the asexual endophytes. The similarities of their endophytic growth habits, morphological characteristics and secondary metabolites (*Siegel et al.* 1990; *White et al.* 1991) suggest a close evolutionary relationship between sexual *Epichloë* species and asexual endophytes. These relationships have been confirmed by molecular genetic analyses based on allozyme profiles (*Christensen et al.* 1993; *Leuchtmann* and *Clay* 1990) and DNA sequence comparisons (*An et al.* 1992; *Schardl et al.* 1991), which support two significant conclusions. First, there appear to have been multiple evolutionary origins of the asexual endophytes from *Epichloë* species. Second, even endophyte isolates from the same grass species can show considerable genetic diversity.

*Epichloë* species and their asexual relatives persist and grow endophytically without causing disease in vegetative tissues of their hosts (*Bacon* and *De Battista* 1990) and are closely associated with meristematic tissues that give rise to leaf sheaths, tillers, rhizomes and inflorescences. The endophytes and many *Epichloë* species can infect host seed, also without discernible detriment to the host. As grass inflorescences develop, endophytic hyphae grow into the ovules and ultimately infect the endosperms and embryos in the seeds (*Phipson and Curstedt* 1986). Infected seed is very likely the only means of propagation of the asexual endophytes. Thus, clonal endophyte lineages are associated with the maternal lineages of their hosts. Such an extreme linkage by common descent should select for enhanced mutualism during evolution (*Siegel* and *Schardl* 1991).

The sexual cycles of pathogenic *Epichloë* species are...
TABLE 1

Fungal isolates in this study and their characteristics

<table>
<thead>
<tr>
<th>Species or taxon</th>
<th>Isolate</th>
<th>Mating type</th>
<th>Host species</th>
<th>Geographic origin of host</th>
<th>Sexual expression of fungus</th>
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<td>Lp1</td>
<td>mat-2</td>
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initiated by the production of stromata, sporogenous mycelial structures on host leaf sheaths and inflorescences that prevent maturation of the florets and thereby suppress seed production on the affected tillers (choke disease). The fungal stromata give rise to asexual spores (conidiospores) that serve as spermata in mating (WHITE and BULTMAN 1987). The sexual cycle culminates in the discharge of haploid ascospores derived from the meiotic products. In extreme cases of antagonism the hosts are completely sterilized. However, many Epichloe species are pleiotropic symbionts (MICHALAKIS et al. 1992) that can exhibit both the sexual life cycle of antagonists and the asexual life cycle of the mutualists. In these cases, some flowering tillers of an infected plant may exhibit chokes, whereas others on the same plant are unaffected, giving rise to seeds containing the fungus (SAHA et al. 1987).

Perennial ryegrass is a known host of three species or taxa in the Epichloe group. The two known taxa of asexual, seed-borne endophytes are Acremonium lolii (LATCH et al. 1984)—also classified as taxonomic grouping one from Lolium perenne (LpTG-1)—and LpTG-2 (CHRISTENSEN et al. 1993). Benefits to perennial ryegrass, particularly of _A. lolii_ mutualism, have been extensively documented (CLAY 1990). In contrast, _Epichloe typhina_ (i.e., _Epichloe_ mating population I = MP-I) represents the antagonistic extreme, completely sterilizing infected perennial ryegrass plants (SIEGEL et al. 1987). In this report, the genetic compositions and evolutionary relationships of the perennial ryegrass endophytes and _E. typhina_ are explored, and the results indicate that LpTG-2 is a heteroploid derived from a hybrid whose most likely ancestors are _A. lolii_ and _E. typhina_.

MATERIALS AND METHODS

Biological materials: Fungal isolates and their hosts and characteristics are listed in Table 1. Methods for their isolation from infected plants, culture, maintenance and characterization by serology are described elsewhere (AX et al. 1993; SCHRDL and AN 1993). Single conidiospore isolation of LpTG-2 isolate Lp1 was performed three times in succession. Each time, conidiospores were streaked, then monitored daily by microscope (50 ×) to ensure that colonies were chosen from germinated conidiospores and not from hyphal fragments. The method of WILSON (1992) was used to stain nuclei of conidiospores.

To identify biological species (i.e., mating populations or MP) of the _Epichloe_ isolates, mating tests were carried out as described previously (SCHRDL and TSAI 1992; WHITE 1993). _Epichloe_ completes its sexual cycle only after mating between individuals of the same biological species and of opposite mating type (mat-1 and mat-2). For example, isolate E8 from perennial ryegrass was identified as _E. typhina_ sensu stricto because it successfully mated with a known _E. typhina_ isolate (designated E2461 in Table 1) from another grass species (WHITE 1993). Test matings also indicated that isolates E32, E2461, E2466, E52, E56 or E248.

DNA isolation: Total fungal DNA was prepared as previously described (BIRD et al. 1990; MURRAY et al. 1992). Mitochondrial DNA (mtDNA) was isolated as described by MOGEN et al. (1991) with modifications. Isolate Lp1 was grown for 1 week on five 155-mm diameter cellophane disks on potato-dextrose agar (Difco, Detroit, Michigan) (SCHRDL and AN 1993) and ground in cold buffer containing 15% sucrose, 10 mM Tricine-KOH pH 7.5, 0.2 mM ethylenediaminetetraacetic acid (EDTA).
The cellular debris and nuclei were removed by centrifugation at 10,000 x g for 10 min, then reextracted once. The supernatants were pooled and centrifuged at 15,000 x g for 15 min, then the pelleted mitochondria were resuspended in 20% sucrose, 10 mM Tricine-KOH, pH 7.5, 0.1 mM EDTA and repelleted. Mitochondria were resuspended in buffer C (1.75 mM sucrose, 10 mM Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.5, 5 mM EDTA), brought to 12 mM MgCl₂ and 100 µg ml⁻¹ deoxyribonuclease I, incubated 90 min at 4°C, repelleted at 20,000 x g for 10 min, washed in buffer C, and lysed in 0.44 M sucrose, 1% sodium dodecyl sulfate (SDS). Proteinase K (Sigma Chemical Co., St. Louis, Missouri) was added to 55 µg ml⁻¹, and the extract was incubated 45 min at 37°C, then extracted with Tris-HCl-saturated phenol (pH 8.0), phenolchloroform-isooamyl alcohol (25:24:1) and chloroform-isooamyl alcohol (24:1). The DNA was concentrated by centrifugal dialysis in Centricon-30 cells (Amicon Div., Beverly, Massachusetts), precipitated by addition of sodium acetate to 300 mM and two volumes of ethanol, rinsed in 70% ethanol, and redissolved in 10 mM Tris-HCl pH 8, 1 mM EDTA.

DNA amplification and sequence determination: Most oligonucleotide primers for amplification and for sequence analysis were described previously (Bryd et al. 1990; Schardl et al. 1991). The 5′ portions of tub2, inclusive of introns IVS1, IVS2 and IVS3, were amplified using primers 1042 (5′-GAGAAATTGGCAGAGATTG-3′) and 1214 (5′-TGTC-AACGGCTGAGACCC-3′), homologous to conserved, protein coding sequences. In some cases the 5′ segments of tub2 genes could be amplified using primer 4414 (5′-CGTTGCTGAGATACCG-3′) in place of primer 1042. Primer 4414 was homologous to sequence approximately 320 bp upstream of the start codon of some, but not all, tub2 genes in the study. Amplification of rnr ITS1 and ITS2 regions (the internal transcribed spacers of the rRNA genes) employed primers described by White et al. (1990). Polymerase chain reactions (PCR) were performed as described previously (Schardl et al. 1991) using either Taq DNA polymerase (Boehringer-Mannheim Biochemical, Indianapolis, Indiana) or AmpliTaq™ (U.S. Biochemical Corp., Cleveland, Ohio). A Perkin-Elmer (Norwalk, Connecticut) thermal cycler was programmed for the following temperature regimes: one incubation at 94°C for 105 sec; 35 cycles of 94°C 45 sec, 55°C 45 sec, 72°C 75 sec; one 5-min incubation at 72°C; then cooling to 4°C. Products were purified using Magic Prep™ resin cartridges (Promega Corp., Madison, Wisconsin).

Sequences were obtained directly from both strands of each PCR product using the fmol™ kit from Promega Corp. The purpose for sequencing the PCR products directly, rather than cloning them first, was to virtually eliminate the potential for sequencing artifacts due to occasional misincorporations by Taq DNA polymerase (Thomas and Kocher 1993). Reactions were as recommended by the manufacturer, with modifications. Each of four termination reactions (6 µl) contained 50 mM Tris-HCl, pH 9.0, 2 mM MgCl₂, 200 µM sequencing primer (Bryd et al. 1990; White et al. 1990), 40–200 fmol template DNA, 0.2 unit µl⁻¹ modified Taq DNA polymerase (sequencing grade from Promega), 2 µM [α-³²P]dATP (>1000 Ci mmol⁻¹; DuPont-NEN, Boston, Massachusetts), 6.7 µM each of dTTP, dCTP, dATP and dGTP, plus a deoxynucleotide triphosphate (10 µM dGTP, 117 µM ddATP, 200 µM ddTTP or 67 µM ddCTP). The reactions were performed with 30 temperature cycles of 95°C 30 sec, 58°C 30 sec, 70°C 30 sec. The products were sequenced by acrylamide gel electrophoresis (Ausubel et al. 1987–1993).

Sequences have been deposited in GenBank and EMBL databases under accession numbers L06956, L06958, L07130–L07133, L07138, L07139, L07141, L07142, L06955, L06957 and L06950–L06962.

Phylogenetic analysis: Because of the close relationships among the sequences, all alignments were made by eye.

Most parsimonious trees were inferred from the aligned sequences using the branch-and-bound search, implemented in PAUP (Swofford 1993), to obtain exact solutions. Nucleotide substitutions were unordered and weighted. Alignment gaps were either treated as missing information or recoded for parsimony. In the latter case, only gaps identical in all sequences possessing them were recoded, and each recoded gap was treated as one nucleotide substitution regardless of its length (see Figures 1 and 3). Structures of the trees were similar whether or not gaps were recoded, though there was a modest increase in bootstrap support of some branches. Bootstrap analysis (Felsenstein 1985) used 100 replications by branch-and-bound.

Neighbor-joining (Saitou and Nei 1987) trees were calculated using PHYLIP (Felsenstein 1993). Alignment gaps were treated as missing information. The tree structures were the same whether or not two-parameter distance corrections were employed.

Analysis of DNA polymorphisms: Restriction endonuclease digestion, electrophoretic separation and Southern transfers were as previously described (Murray et al. 1992; Tsai et al. 1992). For detection of pyr4 sequences a 32P-labeled probe was generated from the 0.65-kb StuI fragment of clone pRS4 (Smit and Tudosny 1992). This probe includes the pyr4 gene of Claviceps purpurea. DNA hybridizations were carried out at 65°C in 5× SSC (Whitfield et al. 1982), and washes were at 65°C first in 3× SSC, and finally in 1× SSC containing 0.1% SDS. Clones of pyr4 genes were from an EMBL A plasmid library prepared and screened by standard protocols (Sambrook et al. 1989).

For detection of tub2 sequences a digoxigenin-labeled probe was generated by PCR (Lion and Haas 1990) using as template a cloned tub2 gene from the tall fescue endophyte FaTG-1 isolate e19 (Tsai et al. 1994). The probe was 980 bp extending from codon 1–199 of the gene, and including the first three introns. Hybridization, wash and detection procedures were as previously described (Tsai et al. 1992).

Allozymes: Allozyme analyses were by horizontal starch gel electrophoresis, as described previously (Christensen et al. 1992; Leuchtman and Clay 1990).

RESULTS

Detection of two β-tubulin genes in LpTG-2 isolates: Sequences of the 5′ regions of tub2 genes, including introns IVS1, IVS2 and IVS3, are aligned in Figure 1. All sequence determinations were initially attempted by analyzing products of PCR reactions that employed genomic DNA templates and primers homologous to the conserved coding regions. In nearly all cases the sequences were unambiguous, indicating only a single form of the gene in most isolates. The only exceptions were the tub2 sequences of the LpTG-2 isolates.

After the tub25′ regions were amplified from isolates Lp1 and Lp2, numerous ambiguities in the sequences of the PCR products suggested that these isolates had two tub2 genes. These ambiguities were observed only with products amplified using primers 1042 (exon 1) and 1214 (exon 4). When an upstream primer, 4414, was used in place of primer 1042 only one sequence was
which it was derived from the LpTG2 isolates, and the gene copy from one LpTG2 isolate, Lp2, was identical to that of the Epichloë isolate Lp5, and to those of 159-166 in Fig. 1), so in order to prevent amplification of tub2-2, Lp1 and Lp2 genomic DNAs were first digested with AseI and subsequently used as templates in

obtained from the LpTG2 isolates. This sequence was identical to that of A. lolii isolate Lp5, and to those of the Epichloë MP-II isolates, and the gene copy from which it was derived was designated tub2-2. An AseI recognition site was present in a variable region (positions 159-166 in Fig. 1), so in order to prevent amplification of tub2-2, Lp1 and Lp2 genomic DNAs were first digested with AseI and subsequently used as templates in

Figure 1.—Sequence alignment of tub2 5' regions. The Lp1 tub2-1 sequence shown here was identical to that of the other LpTG2 isolate, Lp2. Also, the Lp1 tub2-2 sequence was identical to those of Lp2, Lp5, E28, E32 and E189. Dashes (-) indicate alignment gaps, and those dashes enclosed in boxes indicate alignment gaps sometimes recorded as characters in parsimony analysis.

C. L. Schardl et al.
PCR reactions with primers 1042 and 1214. Sequences of the amplification products obtained in this manner were unambiguous, and nearly identical to the sequence from E. typhina isolate E8 (Figure 1). Thus, a second gene copy, designated tub2-1, was identified in the two LpTG2 isolates.

The presence of two tub2 copies in isolate Lp1 was confirmed by analysis of total DNA, cleaved with PstI and BamHI and hybridized to a 980-bp digoxigenin-labeled probe which included the first three introns and codons from tub2 (Figure 2). Cleavage of genomic DNA from E. typhina E8 (lane 5) released a 1.7-kb hybridizing fragment extending from 235 bp upstream of the start codon to a BamHI site at codons 344–346 (Bao et al. 1990). A fragment of similar size was obtained from the other E. typhina isolates E2466 (lane 2) and E2461 (H.-F. Tsai unpublished data). However, a larger fragment (2.2 kb) was obtained from A. lolii and from Epichloë MP-II isolates E32 and E189 (lanes 5–8), which lacked the PstI site at position –235 bp. The LpTG2 isolate Lp1 (lane 4) had tub2-homologous fragments of both sizes, the larger arising from tub2-2 and the smaller from tub2-1.

Phylogenetic analysis of rRNA and β-tubulin genes:
To determine the origin of the two tub2 genes in LpTG-2 a phylogenetic analysis was conducted on sequences of the tub2 introns, as well as the rRNA ITS1 and ITS2 regions. The sequences of ITS1 and ITS2 are aligned in Figure 3. As in the analysis of tub2, PCR amplification and subsequent sequence analysis of the rRNA segments were performed to identify ambiguities, but these were rarely observed. The only ambiguity detected, at position 98 (Figure 3) of ITS1 from E2461, was confirmed by analysis of both DNA strands. This indicated a polymorphism possibly due either to a mixture of two related genotypes (E2461 was not single-spore isolated) or to variation among the tandem rRNA gene copies [the typical arrangement in rRNA loci in fungi (Free et al. 1979)]. The ambiguity represented only a minor difference not affecting phylogenetic inferences. Only a single sequence of this region was detected in the LpTG-2 isolates; this was identical to ITS1 and ITS2 of E. typhina E8. Southern blot analysis of the rRNA structural gene also identified only a single type in isolate Lp1, similar to that of E. typhina E8, but distinct from those of A. lolii, Epichloë MP-II and other E. typhina isolates (H.-F. Tsai and D. M. Watt unpublished data).

The phylogenetic relationships of the rRNA and tub2 sequences were inferred by parsimony and by the distance-based method, neighbor-joining (Figure 4). The known sexual (Epichloë) isolates showed convergence of the tub2 and rRNA phylogenies, strongly suggesting that the gene trees accurately reflected the evolutionary relationships of the organisms (Penny et al. 1982) and that most of the observed sequence divergence occurred after speciation. Furthermore, both trees linked the A. lolii isolate Lp5 with the isolates of Epichloë MP-II.

The sequences of the two tub2 copies in the LpTG-2 endophytes were related much more closely to the single tub2 genes of other isolates than to each other (Figure 4). The tub2-1 and tub2-2 sequences differed by 54% of the variable sites (28 nucleotide substitutions). In contrast, tub2-1 differed from tub2 of E. typhina E8 by only one substitution (2%), and the tub2-2 sequence was identical to that of A. lolii isolate Lp5 and the Epichloë MP-II isolates. Bootstrap analysis strongly supported the separation of tub2-1 and tub2-2 into separate clades. Hence, phylogenetic inference for the tub2 copies strongly suggests gene transfer or interspecific hybridization in the evolution of the endophyte taxon LpTG-2.

There was only one discernible rRNA form in the LpTG-2 isolates, and their ITS1 and ITS2 sequences were identical to those of E. typhina E8. Therefore, the rRNA phylogeny of the LpTG-2 isolates converged with the tub2-1 phylogeny.

Isozymes and pyr4 polymorphisms: Two pyr4 genes were cloned from the Lp1 genome (Figure 5). Following BamHI digestion one of the genes, pyr4-1 (lane 1), appeared similar to that of E. typhina E8 (lane 2); the other, pyr4-2 (lane 6), appeared similar to those of A. lolii isolates Lp5 and Lp9 (lanes 4 and 5).

Allozyme profiles (Table 2) also indicated multiple gene copies in the LpTG-2 isolates. Seven isozyme loci were identified which, because they exhibited variation among A. lolii and E. typhina isolates, could be used to test the hypothesis that LpTG-2 had a hybrid origin. For each locus, two allozymes were observed from Lp1 and Lp2. Five of the seven patterns could be explained as combinations of allozyme profiles that typify E. typhina isolate E8 and A. lolii isolate Lp5. In the cases of phosphoglucomutase-1, one allozyme could have been derived from the E8 genotype, but the origin of the other was not apparent. Possibly the specific ancestral A. lolii
genotype was not among those sampled (Table 2; and see Christensen et al. [1995]). Alternatively, mutations affecting allozyme mobility may have occurred in LpTG-2 after hybridization.

Mitochondrial DNA profiles: Due to their high copy number relative to single-copy nuclear DNA, mtDNA fragments from restriction endonuclease-digested total DNAs were observed as intense bands in electropherograms (Figure 6). The mtDNA profile from Lp1 exactly matched that of A. lotii isolates 44 and 46, but differed substantially from those of E. typhina and Epiclado MP-II isolates (Figure 6). Lp1 also had a high copy-number Psbl fragment of 6.4 kb, not derived from the mtDNA. Blot-hybridization analysis (not shown) assigned this fragment and a co-migrating fragment from E. typhina E8 to the rnr repeats.

Evidence for a heteroploid nuclear genotype: Three single-conidiospore isolates from Lp1 were analyzed for tub2 polymorphism by sequence analysis of PCR products, and both gene copies were present in each (C. L. Schardl, unpublished data). Likewise, Southern-blot hybridization identified both pyr4 genes in each of six isolates from single conidiospores (M. A. Collett, unpublished data). To establish whether the two copies of each gene were characteristic of a single nuclear genotype, it was necessary to determine the number of nuclei in the conidiospores. Therefore, conidiospores were stained with giemsa and visualized (Figure 7). Of over 100

**Figure 3**.—Sequence alignment of rnr gene segments including ITS1 (positions 1–205) and ITS2 (positions 351–542). Sequences listed as LpTG-2 were from isolates Lp1 and Lp2. Sequences listed as MP-II were from isolates E28, E32 and E189. The sequences from isolates E8, Lp5 (=e2), E32 and E56 were reported earlier (Schardl et al. 1991). Hyphens enclosed in boxes indicate the alignment gaps recoded as equivalent to a nucleotide substitution for the purpose of parsimony analysis.
Hybrid Origin of an Endophytic Fungus

The presence in isolates Lp1 and Lp2 of two copies of nine of the ten genes investigated, but of only single copies in A. lolii and Epichloë isolates, indicated that endophyte taxon LpTG-2 was heteroploid. In the LpTG-2 isolates, two loci were detected for seven isozymes, and restriction fragment length polymorphisms indicated two copies each of pyr4 and tub2. Molecular phylogenetic analysis of the tub2 genes indicated that LpTG-2 was derived from an interspecific hybrid. Each tub2 copy was linked by sequence similarity to a different clade and to a different Epichloë species. The tub2-1 gene and rrn sequence indicated a particularly close relationship with an E. typhina isolate from perennial ryegrass. The tub2-2 sequence was identical to those obtained from A. lolii and Epichloë MP-II, and could therefore distinguish neither as a likely ancestor.

Allozyme profiles suggested that the other ancestor was A. lolii, a possibility strongly supported by the close similarity of the mtDNA profiles from A. lolii and LpTG-2.

A proposed scenario for the origin of the two endophyte taxa, A. lolii and LpTG-2, suggests two different pathways by which asexual endophytes may evolve (Figure 8). The simple scenario, whereby a pleiotropic Epichloë strain experiences a mutation that eliminates stroma expression, is omitted even though A. lolii could conceivably have arisen in this manner. Alternatively, a host species or genotype may suppress the stromatal stage of the fungus. For example, Figure 8 illustrates a host species transfer of MP-II as a possible origin of A. lolii in perennial ryegrass. Such a new host-fungus combination may not produce stromata or may do so only rarely. A different scenario, involving interspecific hybridization of co-symbiotic fungi, is proposed to account for the origin of taxon LpTG-2 (Figure 8). In general, an Epichloë spore infecting a plant that already possesses an asexual endophyte or another Epichloë strain may yield a simultaneous association of one plant with two related fungi. Because of their similar growth habits and host tissue specificity (Siegel et al. 1987), the two fungi should have ample opportunity to interact. If they are vegetatively compatible, anastomosis (hyphal fusion) may eventually be followed by karyogamy, a fusion of the dissimilar nuclei to give rise to an allopolyploid hybrid. This may be followed by loss of some chromosomes or chromosome segments whose genes are redundant.
1.2.1.3) with italic text, and
5) (\(\text{EC no.} 5.3.1.9\)) (PGI), phosphoglucomutase (EC no. 5.4.2.2) (PGM-1, PGM-2). Only homomeric bands are indicated.

TABLE 2

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</tbody>
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Numbers indicate allozyme migration relative to that of a reference strain, as in CHRISTENSEN et al. (1995). Enzymes are aconitase (EC no. 4.2.1.3) (ACO), aldolase (EC no. 4.1.2.13) (ALD), leucine aminopeptidase (EC no. 3.4.11.1) (LAP), malate dehydrogenase (EC no. 1.1.1.37) (MDH-I), phosphoglucone isomerase (EC no. 5.3.1.9) (PGI), phosphoglucomutase (EC no. 5.4.2.2) (PGM-1, PGM-2). Only homomeric bands are indicated.

FIGURE 6.—Electrophoretograms of fungal DNA cleaved with PstI. Samples are mtDNA from isolate Lp1 (lane 1), and total DNAs from E. typhina isolates E2466 (lane 2) and E8 (lane 3). LpTG-2 isolate Lp1 (lane 4). A. loli isolates e44 (lane 5) and e46 (lane 6), and Epichloë MP-II isolates E32 (lane 7) and E189 (lane 8). Size markers are DNA from bacteriophage lambda cleaved with HindIII (lane m1) or HindIII and EcoRI (lane m2). Lines at left mark mtDNA fragments of Lp1. The black arrow head marks the 6.4-kb fragment from the rDNA repeats in Lp1 and E8. Hybridization to probes for tub2 (Figure 2) and nuclear rDNA (H.-F. Tsai, unpublished data) indicated that the mtDNA in lane 1 was not significantly contaminated by nuclear DNA.

(Peberdy 1991). For the most part, LpTG-2 appears to have retained the genes of its two ancestors, except that only one form each of ribosomal DNA and mtDNA remain.

The most obvious alternative scenario for the origin of LpTG-2 involves a rare ascospore arising from an interspecific mating of E. typhina and Epichloë MP-II. However, in numerous attempts at interspecific mating of Epichloë species, only barren perithecia have been obtained (C. L. Schardl and M. R. Siegel, unpublished data). Even if interspecific sexual hybrids form very rarely, they must also infect susceptible host tissue, so the likelihood of sexual hybrids producing new plant-fungus combinations is probably small. Yet, apparently, hybrid endophytes are not rare. In a survey of asexual Epichloë-type endophytes of tall fescue, no fewer than three hybridization events account for the distribution of multiple, divergent tub2 gene copies. One endophyte taxon, FaTG-1 (= Acremonium coenophialum), had three tub2 genes, each exhibiting a sequence relationship to tub2 of a different Epichloë species. This strongly suggests that FaTG-1 originated as a hybrid of an Epichloë species with a fungal ancestor that was, itself, a hybrid. So far, no known sexual strain of Epichloë exhibits the indications of heteroploidy, such as multiband allozyme profiles (CHRISTENSEN et al. 1993; LEUCHTMANN and CLAY 1995) and multiple tub2 gene copies (Tsai et al. 1994). Thus, the existence of a triple hybrid like FaTG-1 suggests that vegetative hybridization can occur in this group of fungi.

A potential barrier to interspecific hybridization of fungi in planta is vegetative incompatibility (Leslie 1993). At present, there is little information regarding vegetative incompatibility in Epichloë species, their asexual relatives, or any other Clavicipitales. However, fungal behavior in mating tests suggests that Epichloë does not exhibit vegetative incompatibility. The first change observed when a stroma of one mating type is inoculated with spermata of the other mating type is a thick, spreading mycelium (Schardl and Tsai 1992). This is probably heterokaryotic because, within several days it gives rise to raised bumps which, in interspecific mating, mature into fertile perithecia. The initial my-
Hybrid Origin of an Endophytic Fungus

cepelial growth has been consistently observed in attempted matings between opposite mating types, regardless of whether the stromatal and spermatidal parents are of the same mating population (SCHARDL and TSAI 1992). This observation suggests that there is no genetic system in Epichloë suppressing proliferation of heterokaryotic mycelia.

Heterokaryotic euascomycetes have been generated in the laboratory by anastomosis of vegetatively compatible hyphae or by fusion of protoplasts (PERERDY 1991). Nuclei in heterokaryons fuse on rare occasions to produce diploids (or polyploids), which may lose chromosomes to give aneuploids and, eventually, haploids. This process of generating new genetic combinations without an intermediate meiotic stage is termed parasexuality. Whether hybridization or parasexual cycles play any significant role in nature has remained unknown either because they occur very rarely (GLASS and KULDAU 1992) or because natural hybrids have escaped notice. The genotypes of LpTG-2 and of certain endophyte isolates from Festuca arundinacea (TSAI et al. 1994) indicate such hybrids exist, at least in this group of euascomycetes. Previous studies of allozyme patterns (CHRISTENSEN et al. 1993; LEUCHTMANN and CUY 1990) have also suggested that many asexual endophytes are heteroploid (or perhaps sometimes heterokaryotic) in contrast to their haploid, sexual relatives, suggesting that interspecific hybridization between Epichloë and endophytes may be common.

It is interesting to consider the consequences to the host of hybridization between A. lolii and E. typhina. The choke pathogen isolate E8, introduced into a number of L. perenne plants from various cultivars, has consistently exhibited the ability to sterilize these plants (M. R. SIEGEL, personal communication). If this fungus infected plants containing A. lolii, the choke stage of E. typhina would be antagonistic both to the plant and to the seed-disseminated endophyte. Sexual recombination of the host plant would be eliminated, and the plant (with endophyte and pathogen) would be restricted to localized and vegetative propagation via tillers. At this point, some genetic change in the symbiotum—either
within the plant or fungal genome—that releases the restriction on production of seed will be of obvious selective advantage to the plant. In the origin of LpTG-2, this genetic change involved hybridization of the two fungal species, and yielded either immediately or eventually a new, seed-borne and non-pathogenic endophyte. This scenario adds a twist to the concept of evolution of mutualists from antagonists (Loeschcke and Christiansen 1990; Michaelakis et al. 1992). If the mutualists tend to lose the sexual cycle (Law and Lewis 1985), they may exhibit mechanisms for evolution and diversification very different from those of their sexual cousins. Significantly, A. lolii (and perhaps other grass endophytes) can apparently disarm a related antagonist by genetic hybridization.

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