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Evolutionary diversification of fungal endophytes of tall fescue grass by hybridization with *Epichloë* species

(*Festuca arundinacea*/*Acremonium coenophialum*/Clavicipitaceae/mutualistic symbionts/β-tubulin genes)

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ABSTRACT **The mutualistic associations of tall fescue (*Festuca arundinacea*) with seed-borne fungal symbionts (endophytes) are important for fitness of the grass host and its survival under biotic and abiotic stress. The tall fescue endophytes are asexual relatives of biological species (mating populations) of genus *Epichloë* (Clavicipitaceae), sexual fungi that cause grass choke disease. Isozyme studies have suggested considerable genetic diversity among endophytes of tall fescue. Phylogenetic relationships among seven isolates from tall fescue, three from meadow fescue (a probable ancestor of tall fescue), and nine *Epichloë* isolates from other host species were investigated by comparing sequences of noncoding segments of the β-tubulin (*tub2*) and rRNA (*rrn*) genes. Whereas each *Epichloë* isolate and meadow fescue endophyte had only a single *tub2* gene, most tall fescue endophytes had two or three distinct *tub2* copies. Phylogenetic analysis of *tub2* sequences indicated that the presence of multiple copies in the tall fescue endophytes was a consequence of hybridization with *Epichloë* species. At least three hybridization events account for the distribution and relationships of *tub2* genes. These results suggest that interspecific hybridization is the major cause of genetic diversification of the tall fescue endophytes.**

Mutualistic fungal endophytes are important for the fitness of many grass hosts, such as hexaploid tall fescue (*Festuca arundinacea*), on which the endophytes can confer enhanced growth and fecundity, protection from herbivores and parasites, and a profound increase in drought tolerance (1). These endophytes spend their entire life cycles within host tissues, are maternally transmitted in seed, are completely nonpathogenic, and are asexual. They are evolutionarily derived from the sexual (teleomorphic) fungi of genus *Epichloë* (Ascomycotina, Clavicipitaceae), which cause grass choke disease (2). During the pathogenic stage of *Epichloë* (3, 4) the affected inflorescence of the host is sterilized and the fungus, if crossed with an opposite mating type of the same biological species (i.e., mating population), produces and disseminates ascospores (Fig. 1). *Epichloë* species vary widely in the relative importance of sporulation (choke disease) versus seed transmission during their life cycles. For example, the symbiont of *F. rubra* depicted in Fig. 1 typically exhibits a high level of seed transmission and provides biological protection to its host (5). More antagonistic *Epichloë* species completely sterilize infected plants (6). Thus, symbioses with *Epichloë* and endophytes span the spectrum from antagonism to mutualism, making them an ideal system for studying the evolution of fungal mutualists.

The endophytes contribute important and heritable genetic diversity to their symbiotic systems (7). Because they are maternally transmitted (8), produce several classes of antiherbivore alkaloids (9), and confer other fitness enhancements to their hosts (1), the diversity of the endophytes probably plays a major role in the long-term fitness and adaptability of the grass–fungus symbiotic entities. A recent survey assessing allozyme variation, morphological characteristics, and profiles of protective alkaloids indicated considerable diversity among tall fescue endophytes (10). Three taxa were described as *F. arundinacea* endophyte taxonomic grouping one (FaTG-1 = *Acremonium coenophialum*), FaTG-2, and FaTG-3. Endophytes of meadow fescue (*Festuca pratensis*) were also surveyed. This grass is the likely

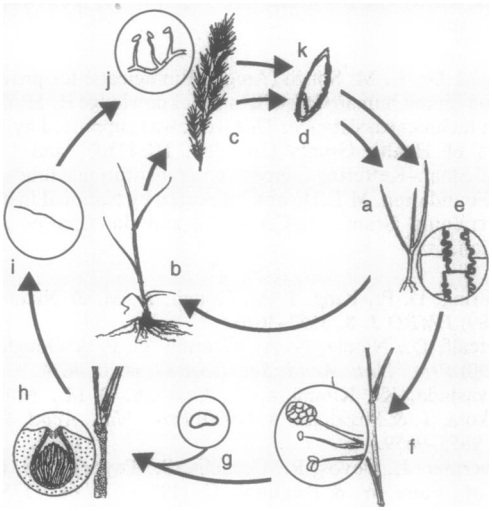

FIG. 1. Life cycles of *Epichloë* sp. MP-II in *Festuca rubra* (refs. 3 and 4; C.L.S. and M.R.S., unpublished data). Fungal structures are shown in circles. In the asexual cycle, highly convoluted hyphae grow intercellularly in leaf sheaths (*a*), floral meristems (*b*), and in the ovules of the florets (*c*) such that the fungus is transmitted in the next generation of seed (*d*). In the sexual cycle, the fungus also grows intercellularly in vegetative leaf sheaths without causing symptoms (*e*), but then emerges from the leaf sheath surrounding the immature host inflorescence, produces spermatia, and arrests inflorescence maturation (*f*). Fertilization occurs by transfer of spermatia of opposite mating type (*g*). If the parents are conspecific [same mating population (MP)], perithecia containing asci develop (*h*) and filamentous ascospores are ejected (*i*). Germinating ascospores initiate cycles of asexual sporulation (conidiation) and are postulated to cause infection of host florets (*j*) and ultimately of seed (*k*). Endophytes of tall fescue and meadow fescue are only known to undergo the asexual cycle.

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Abbreviations: FaTG, *Festuca arundinacea* endophyte taxonomic grouping; ITS, internal transcribed spacer; MP, mating population; IVS, intervening sequence.

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Table 1. Fungal isolates in this study

Species or taxon	Isolate	Host species	Ref.
A. coenophialum (=FaTG-1)	e19	*F. arundinacea*	2
A. coenophialum	Tf24	*F. arundinacea*	10
A. coenophialum	Tf28	*F. arundinacea*	10
FaTG-2	Tf13	*F. arundinacea*	10
FaTG-2	e41 (=Tf33)	*F. arundinacea*	2, 10
FaTG-2	Tf15	*F. arundinacea*	10
FaTG-3	Tf18	*F. arundinacea*	10
A. uncinatum	Fp1	*F. pratensis*	10
A. uncinatum	Fp3	*F. pratensis*	10
A. uncinatum	e166	*F. pratensis*	2
E. typhina MP-I	E8	*Lolium perenne*	2
E. typhina MP-I	E2461	*Dactylis glomerata*	12
Epichloë sp. MP-II	E28	*Festuca longifolia*	5, 9
Epichloë sp. MP-II	E32	*F. rubra* subsp. *commutata*	2
Epichloë sp. MP-II	E189	*F. rubra* subsp. *rubra*	5
Epichloë sp. MP-III	E56	*Elymus canadensis*	4, 13
Epichloë sp.	E52	*Sphenopholis obtusata*	3
Epichloë sp.	E57	*Agrostis hiemalis*	13
Epichloë sp.	E247	*Agrostis* sp.	13
Epichloë sp.	E248	*Agrostis stolonifera*	13

Taxonomic groups are as in ref. 10. Where determinations could be made of *Epichloë* MPs (14), they are designated accordingly. Only members of MP-I are named *Epichloë typhina* sensu stricto, according to ref. 12, whereas binomials have not been proposed for the other mating populations.

maternal ancestor of tall fescue (11), so it may have contained one or more ancestors of the tall fescue endophytes. However, unlike the tall fescue endophytes, those in meadow fescue exhibited little genetic diversity and clearly grouped in the single taxon, *Acremonium uncinatum*. These results raise three interesting evolutionary questions. First, why are tall fescue endophytes so diverse? Second, do they share any common ancestry with each other or with meadow fescue endophytes? Third, how are the tall fescue endophytes related evolutionary to *Epichloë* species?

This paper reports a molecular phylogenetic analysis of endophytes and *Epichloë* isolates based on sequences of nuclear genes encoding β-tubulin (*tub2*) and ribosomal RNA (*rrn*).¶

The results indicate the importance of interspecific hybridizations, probably between asexual endophytes and *Epichloë* species, in generating endophyte diversity in tall fescue.

MATERIALS AND METHODS

Biological Materials. Fungal strains are listed in Table 1. *Epichloë* isolate E189, from a *F. rubra* breeding line, was provided by C. R. Funk. *Epichloë* species or MPs were defined by tests of interfertility (12, 14). Fungi were isolated and grown and DNA was extracted as described (15). Isolates e19 and Tf18 were derived from single conidiospores. Because FaTG-3 isolates have been obtained only from uncultivated tall fescue plants, the hexaploid nature of these plants was confirmed cytologically (ref. 16; C.L.S. and K.-C. Chen, unpublished data).

Gene Library Construction and Screening. A library of e19 genomic DNA in λ phage vector FIX II (Stratagene Cloning Systems) was prepared and screened as described (15), and a clone of the *tub2* copy, *tub2-4*, was identified. The gene was on a 4.8-kb *Eco*RI to *Sal* I fragment, which was subcloned into pBluescript KS(−) (Stratagene Cloning Systems) to generate pKAES103.

¶The DNA sequences reported in this paper have been deposited in the GenBank data base (accession numbers L06946, L06951, L06952, L06955–L06959, L06961–L06964, L07131, L07132, L07138–L07142, L20305–L20308, X56847, and X60185).

Amplification of Genomic DNA Segments. Polymerase chain reactions (PCRs) were performed as described elsewhere (2) by using *Taq* DNA polymerase (Boehringer Mannheim or United States Biochemical) and a Perkin–Elmer thermal cycler. The temperature program was as follows: one incubation at 94°C for 105 sec; then 35 cycles of 94°C for 45 sec, 55°C for 45 sec, and 72°C for 75 sec; followed by one extension of 72°C for 5 min.

Single specific-primer PCR (17) was used to amplify the 5′ segments of the *tub2* copies from tall fescue endophytes. Genomic DNAs were cleaved with *Pst* I and *Bam*HI and ligated into similarly cleaved pBC KS(+) (Stratagene Cloning Systems). The ligation mixes were used as templates in PCR reactions employing primer 1215 (5′-TCGTTGAAGTAGACACTCAT-3′ complementary to *tub2* codons 53–47) and primer T3 [5′-ATTAACCCTCACTAAAG-3′ homologous to vector pBC KS(+) near the *Pst* I site]. The amplified fragments were purified by electrophoresis, reamplified by PCR, and then cloned (7).

The 5′ portions of *tub2*, including intervening sequences IVS1, IVS2, and IVS3, were amplified by using primers 1042 (5′-GAGAAAATGCGTGAGATTGT-3′) and 1214 (5′-TGGTCAACCAGCTCAGCACC-3′), which were homologous to conserved coding sequences (see Fig. 3). Sequence analysis of the products (see below) indicated ambiguities when they were derived from multiple divergent gene copies. In these instances, primers specific for gene copies and prior digestion of template DNA with restriction endonuclease allowed amplification and sequence determination of each copy. Digestion with *Ase* I prevented amplification of copies *tub2-2* and *tub2-4*. Digestion with *Mbo* I prevented amplification of *tub2-4*. PCR with primers 4414 (5′-CTGGTGCCTGAGATACCGC-3′) and 1214 (see Fig. 3) amplified the segments of *tub2-2* and *tub2-4* but not *tub2-3* or *tub2-5*.

The rRNA gene (*rrn*) segments containing internal transcribed spacers one (ITS1) and two (ITS2) were amplified and sequenced by using primers described by White *et al.* (18). Amplification of ITS2 used a primer (5′-GCTGCGTTCTTCATCGATGC-3′) homologous to the 5.8S rRNA coding sequence and a primer (5′-ATCCTCTGCAAATTACAACT-3′) complementary to the 26S rRNA D1 region.

DNA Sequence Determination. Cloned fragments were sequenced by the method of Toneguzzo *et al.* (19). PCR

products were sequenced using the *fmol* DNA sequencing system (Promega) with a modification of the manufacturer's protocol. Each of four termination reaction mixtures (6 μl) contained 50 mM tris(hydroxymethyl)aminomethane hydrochloride (pH 9.0); 2 mM $MgCl_2$; 200 nM sequencing primer (refs. 15 and 18, and see Fig. 3); 40–200 fmol of template DNA; 0.2 unit of modified *Taq* DNA polymerase (Promega) per μl; 2 μCi (1 Ci = 37 GBq) of [α-^{35}S]dATP (>1000 Ci/mmol); 6.7 μM each of dTTP, dCTP, dATP, and 7-deaza-dGTP; and a dideoxynucleotide (10 μM ddGTP, 117 μM ddATP, 200 μM ddTTP, or 67 μM ddCTP). The reactions were subjected to 30 cycles of 95°C for 30 sec, 58°C for 30 sec, and 70°C for 30 sec.

Phylogenetic Analysis. Gene trees were generated by Wagner parsimony implemented in PAUP (20). The branch-and-bound algorithm was applied to sequence data to obtain exact solutions. All nucleotide substitutions were equally weighted and unordered. Alignment gaps were treated as missing information. Bootstrap replications (21) were also by maximum parsimony. Neighbor-joining with one parameter and two parameter distances (22) gave the same tree structures as did parsimony.

Miscellaneous Procedures. Nuclear staining was by the method of Wilson (23). Southern blot hybridization was as described (24), but the digoxigenin-labeled hybridization probe was generated by PCR (25).

RESULTS

Multiple *tub2* Genes in Tall Fescue Endophytes. Multiple *tub2* copies were identified in most tall fescue endophytes (Table 2). For example, hybridization to Southern blot DNA transfers indicated three copies in FaTG-1 isolate e19 and two copies in FaTG-2 isolate e41, whereas each *Epichloë* isolate gave only a single hybridizing band (Fig. 2). One *tub2* copy, designated *tub2-4*, was subcloned from a genomic library of isolate e19. The 5′ regions of copies *tub2-2* and *tub2-3* were amplified by PCR and cloned. Physical maps of the clones (Fig. 3) indicated that the differences observed in Fig. 2 were due to the positions of *Pst* I cleavage sites in the 5′ regions of the *tub2* copies.

Giemsa staining indicated that e19 conidiospores were uninucleate (H.-F.T. and C.L.S., unpublished data). Therefore, the identification of *tub2-2*, *-3*, and *-4* in four independent, single conidiospore isolates (H.-F.T., unpublished data) indicated that all three copies occurred in a single nuclear genotype and that the *tub2* polymorphism was not due to heterokaryosis (multiple, genetically distinct nuclei in a hypha).

Cloned segments of the three *tub2* copies from isolate e19 and the two copies from e41 were sequenced and compared (Fig. 4). These segments included 358–388 bp from the first three introns, within which most of the sequence variation occurred. In keeping with their similar restriction patterns (Fig. 3), the two copies in e41 had very similar (though not identical) sequences to *tub2-2* and *tub2-4*, so they have been designated accordingly. However, there was considerable divergence between the copies in each isolate. The 560-bp 5′ regions of *tub2-2*, *tub2-3*, and *tub2-4* differed by 14–26 nucleotide substitutions, a level comparable to their 17–35 differences with *tub2* of *E. typhina* isolate E8 (15). For this reason, the hypothesis is proposed that the multiple copies in the tall fescue endophytes have been acquired, not by gene duplication but by hybridization with distantly related *Epichloë* strains.

Table 2. *tub2* gene copies in endophytes

Isolate	*tub2* copies 2	3	4	5
e19	+	+	+	−
Tf24	+	+	+	−
Tf28	+	+	+	−
Tf13	−	−	+	−
e41	+	−	+	−
Tf15	+	−	+	−
Tf18	−	−	+	+
Fp1	−	+	−	−
Fp3	−	+	−	−
e166	−	+	−	−

+, Present; −, not detected.

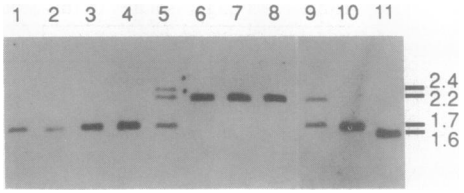

FIG. 2. Southern blot analysis of *tub2* genes from *Epichloë* (numbers prefaced by E) and endophytes (numbers prefaced by e). Total DNA preparations were from isolates E2461 (lane 1), E247 (lane 2), E56 (lane 3), E57 (lane 4), e19 (lane 5), E189 (lane 6), E28 (lane 7), E32 (lane 8), e41 (lane 9), E8 (lane 10), and e166 (lane 11). DNA samples were digested with *Pst* I and *Bam*HI and probed with a digoxigenin-labeled DNA fragment extending from codon 1 to codon 199 of the e19 *tub2-4* gene, inclusive of the first three introns. Multiple *tub2* genes were indicated in tall fescue endophytes e19 (lane 5) and e41 (lane 9). The 1.7-kb and 2.2-kb fragments were observed in both, and the corresponding gene copies were designated *tub2-4* and *tub2-2*, respectively. A third copy in e19, designated *tub2-3*, yielded the 2.4-kb fragment.

Phylogenetic Evidence for Hybridization in Tall Fescue Endophytes. To determine the sequence relationships and the possible sources of the endophyte *tub2* copies, sequences were obtained from three *A. uncinatum* isolates, nine *Epichloë* isolates, and five additional tall fescue endophytes (Table 1). The endophytes represented the range of genetic variation previously inferred by allozyme comparisons (10). To determine whether each isolate had multiple forms of *tub2*, the 5′ regions were amplified by PCR and sequenced. Ambiguities in the sequences indicated multiple copies in all tall fescue endophytes except Tf13 (Table 2), but only a single gene copy in each *Epichloë* and *A. uncinatum* isolate.

Individual gene copies were separately amplified and sequenced (see *Materials and Methods*). Their aligned sequences are shown in Fig. 4. The three FaTG-1 isolates all possessed a *tub2-3* copy, and the sequence from e19 *tub2-3* was identical to that of the meadow fescue endophyte *A.*

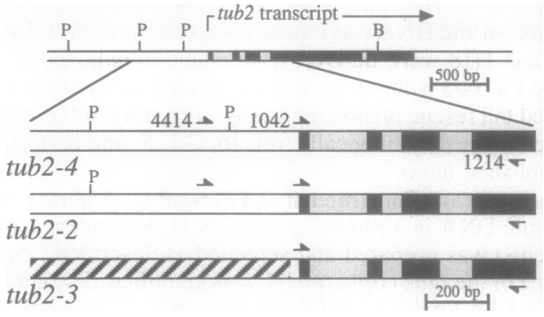

FIG. 3. Maps of the β-tubulin gene copies *tub2-4*, *tub2-2*, and *tub2-3* in the tall fescue endophyte isolate e19. Stippled boxes (▨) indicate introns, and black boxes (■) indicate coding regions. The hatched box (▨) in the *tub2-3* map indicates the portion not homologous to the other *tub2* copies. Numbered half arrows (◭) indicate the primers used in PCR amplification and DNA sequencing. Restriction endonuclease cleavage sites are *Pst* I (P) and *Bam*HI (B).

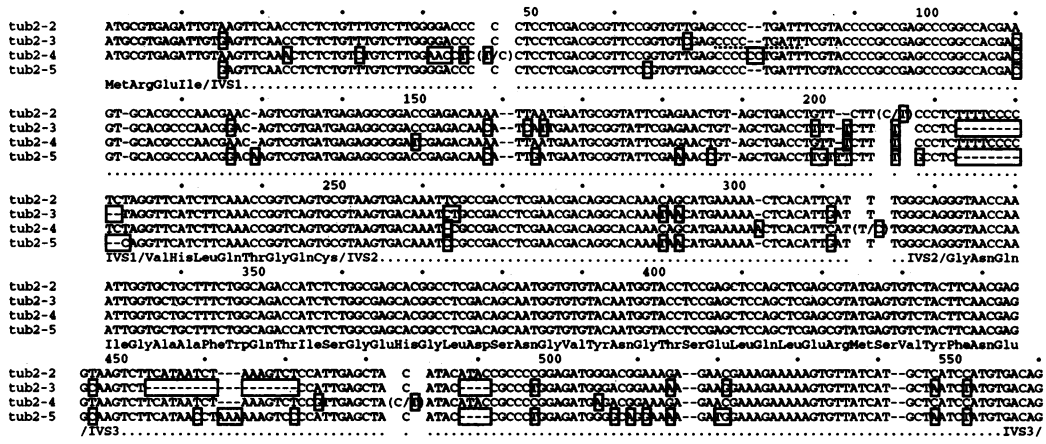

FIG. 4. Sequence alignment of 5′ regions of *tub2* copies from tall fescue endophytes. Boxed nucleotides differ from the reference sequence, *tub2-2* of e19. Boxed dashes (-) indicate alignment gaps. Variations of *tub2-2* (position 207) or *tub2-4* (positions 47, 314, and 483) are indicated in parentheses as (*x*/*y*), where *x* is observed in the FaTG-1 isolates and *y* in FaTG-2 and FaTG-3. A *tub2-3* sequence identical to that of e19 was identified in FaTG-1 isolate Tf24 and in all *A. uncinatum* isolates. The *tub2-3* sequence in isolate Tf28 had a deletion, which is indicated by the dotted line (positions 73–83).

uncinatum. The *tub2-3* copy was not detected in isolates of FaTG-2 and FaTG-3. A sequence designated *tub2-5* was found only in the FaTG-3 isolate Tf18. Each FaTG-1 isolate and two of the three FaTG-2 isolates had *tub2-2* sequences. This form was not detected in FaTG-2 isolate Tf13 or in FaTG-3 isolate Tf18. The *tub2-4* forms were present in every isolate from all three taxa of tall fescue endophytes. The *tub2-2* and *tub2-4* copies showed sequence variation between taxa but not between isolates within a taxon (Fig. 4).

The divergence of *tub2* intron sequences suggested that different copies had different evolutionary origins. To identify their probable sources, gene trees were inferred for all *tub2* sequences identified (Fig. 5). The *tub2-4* sequences grouped with a very high level of confidence (96% of the bootstrap trees) with *tub2* of *Epichloë* E248 from *Agrostis stolonifera*. The *tub2-2* copies strongly grouped with *tub2* from the *Epichloë* MP-II isolates. Likewise, separation of clades containing *tub2-2* and *tub2-4* from those containing *tub2-3* and the *A. uncinatum* gene was strongly supported. Finally, the *tub2-5* sequence unique to FaTG-3 grouped strongly with the *E. typhina* sequences. These phylogenetic relationships imply that different *tub2* copies were from different *Epichloë* ancestors.

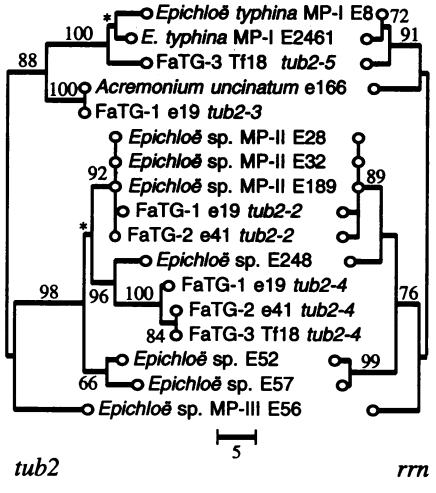

FIG. 5. Most parsimonious, unrooted gene trees on sequences of variable portions of *tub2* and *rrn*. The *tub2-2*, *-3*, *-4*, and *-5* gene copies from the tall fescue endophytes are indicated. *Epichloë* MPs (where known) and endophyte taxa are indicated, along with isolate designations. The *tub2* tree shown is the most highly resolved, and equally parsimonious trees lack one or both of the edges indicated by asterisks (*). The bar indicates a scale of five nucleotide substitutions. Numbers on each edge indicate the percentage of trees in which they were represented in 100 bootstrap replications.

Relationships of rRNA Genes. In contrast to *tub2*, only a single *rrn* ITS2 sequence was detected in each endophyte isolate (ref. 2; data not shown). Because the differences within taxa were not informative for parsimony, they did not contradict the taxonomic grouping or the conclusions drawn from phylogenetic analysis of *tub2*.

Convergence of the *rrn* and *tub2* trees (Fig. 5) further supported the proposed evolutionary relationships among *Epichloë* species and endophytes. For every endophyte isolate except Tf13, the *rrn* phylogeny converged with that of one of its *tub2* gene copies. Isolate Tf13, the only one not exhibiting convergence of *tub2* and *rrn* phylogenies, lacked *tub2-2* but had an identical ITS2 sequence with isolate e41.

DISCUSSION

The results of this study indicate the evolution and diversification of tall fescue endophytes by interspecific hybridization with *Epichloë* species. This conclusion is based principally on the presence in most of the endophytes of multiple *tub2* genes related to those of different *Epichloë* clades. The identification of four different *tub2* gene copies among endophytes and of each copy with at least one other in single-conidium isolates indicates at least three hybridizations in the evolution of the tall fescue endophytes.

Two hypotheses for the origin of hybrid endophytes are (*i*) fusion of vegetative hyphae and subsequent fusion of nuclei or (*ii*) aberrant segregations (such as chromosome nondisjunction) from matings between *Epichloë* species. Either hypothesis is consistent with the two *tub2* genes in FaTG-2 and FaTG-3, but the three *tub2* copies in FaTG-1 indicate that an ancestral hybrid (most likely asexual) underwent a second hybridization. This supports the hypothesis of vegetative hybridization. Furthermore, the *tub2-3* sequence suggests that FaTG-1 descended from an *A. uncinatum* endophyte that was transmitted maternally in the hybrid origin of tall fescue (Fig. 6). If so, then both hybridizations in the FaTG-1 ancestry would have been vegetative. Since the asexual endophytes are confined to host tissues throughout their life cycles, vegetative hybridization must have required *Epichloë* infection of the plant or developing seed of the grass–endophyte symbiosis. Thus, despite their asexual nature,

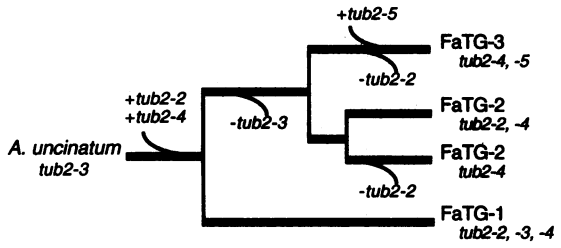

FIG. 6. Possible scenario for the origins of multiple *tub2* copies during evolution of the tall fescue endophyte taxa FaTG-1, FaTG-2, and FaTG-3 from an ancestral meadow fescue endophyte, *A. uncinatum*. Plus (+) or minus (−) indicates the gain or loss, respectively, of a *tub2* copy.

endophytes are not necessarily in genetic isolation from their sexual relatives.

One possible evolutionary scenario is presented in Fig. 6; the scenario is based in part on *tub2-4* sequence relationships suggesting that FaTG-2 and FaTG-3 are sister groups (Figs. 4 and 5). Other evolutionary scenarios are possible. For example, different taxa may have originated by independent hybridizations. Additional hybridizations not indicated by *tub2* or *rrn* profiles may also have occurred. Finally, hybridizations may have actually triggered the divergence of major endophyte taxa by genomic disruption. So, for example, the FaTG-2 and FaTG-3 genotypes may have arisen from a single hybrid nucleus whose descendants tended to lose redundant chromosomes or chromosome segments (hence, losses of *tub2-3* and *tub2-2* proposed in Fig. 6).

Allozyme analysis (10, 26) also indicated multiple gene copies in asexual endophytes but not in sexual *Epichloë* species, so *tub2* genes do not represent a special case. However, no more than one *rrn* form in any asexual or sexual isolate was detected in this and previous investigations (2, 7). The biological basis for this observation is unknown, but it brings into question the use of only one gene, particularly *rrn*, for molecular phylogenetic analysis of asexual fungi.

Asexual endophytes are probably susceptible to Muller's rachet (27), the relentless accumulation of marginally deleterious mutations, causing loss of fitness without the corrective influences of sexual recombination. Endophyte instability due to Muller's ratchet (2) would also be deleterious for the grass host, to which the endophytes provide important biological protection (1). The ability of endophytes to obtain new genetic information from close sexual relatives—namely, the *Epichloë* species—may counteract the ratchet. Whether or not Muller's ratchet caused selection for hybrids, interspecific hybridization was apparently the major cause of diversification of tall fescue endophytes.

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