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Diversity and origins of endophytic fungal symbionts of the North American grass *Festuca arizonica*

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Summary. *Acremonium* spp. endophytes are mutualistic fungal symbionts of many C3 grasses. They are anamorphs of *Epichloë typhina* (Clavicipitaceae) that have become strictly seedborne, heritable components of symbiotic units ("symbiota"). In order to test the possibility that endophytes may contribute to the genetic diversity of symbiota, a survey was conducted of plants from nine populations of *Festuca arizonica* in the southern Rocky Mountains. Sequence analysis of rRNA gene segments distinguished three *Acremonium* endophyte types. Parsimony analysis indicated at least two distinct evolutionary origins of the *Acremonium* endophytes from *E. typhina*. Either or both of these evolutionary lineages may have involved cospeciation with the host.

Key words: rRNA gene – *Epichloë typhina* (*Acremonium typhinum*) – Evolution – Population – Parsimony analysis

Introduction

Festuca species and other cool season grasses (subfamily Pooideae) often harbor seedborne fungal symbionts that protect them from various biotic and abiotic stresses (Bacon and De Battista 1990; Clay 1990; Siegel and Schardl 1991). The most common of the mycosymbionts are the *Acremonium* endophytes, which have evolved from biotypes of the grass choke pathogen *Epichloë typhina* (anamorph = *Acremonium typhinum*) (Schardl et al. 1991). Considerable morphological (Christensen and Latch 1991; Christensen et al. 1991), biochemical (Siegel et al. 1990), physiological (White et al. 1991), and phylogenetic (Schardl et al. 1991) diversity has been observed among grass endophytes. A wide range of ecological and physiological benefits have been associated with endophyte-grass interactions, and the high incidence of endophytes in many grass species indicates that the symbioses are mutualistic (Clay 1990). Because they are vertically transmitted to successive host generations the endophytes contribute to the overall, heritable diversity of the symbiota which, in some cases, may represent coevolved systems (Clay 1991; Schardl et al. 1991).

Phylogenetic relationships between grass mycosymbionts have been investigated by sequence analysis of variable portions of the nuclear rRNA gene (*rrn*) repeats (Schardl and Siegel 1992). These genes have become widely used for molecular phylogenetic analysis of taxa within several kingdoms of the eukarya (Qu et al. 1988; Sogin et al. 1989; Zimmer et al. 1989; Bruns et al. 1991) because of their universal occurrence, conserved organization, and functional constancy, and because different segments are known to fix mutations at different rates (Jorgensen and Cluster 1988). Active gene conversion by processes perhaps unique to the rRNA repeats (Dvorak 1990; Hillis et al. 1991) generally eliminates ambiguities that might arise in the analysis of other duplicated genes. The *rrn* repeats of *E. typhina* and anamorphs are present as tandem copies at a single locus as determined by restriction endonuclease mapping (Schardl and Siegel 1992) and Mendelian segregation (unpublished data). The variable internal transcribed spacers, *rrnITS1* (between the 18S and 5.8S rRNA coding sequences) and *rrnITS2* (between the 5.8S and 26S rRNA sequences), have been used in phylogenetic studies to determine the interrelationships of *E. typhina* and anamorphs from different grass species (Schardl et al. 1991). However, there is little detailed information on the origins and diversity of endophytes of a single grass species.

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This paper describes a survey of *rrnITS*1-2 sequence diversity in endophytes from populations of *Festuca arizonica*, a North American grass restricted to high elevations in the southern Rocky Mountains. Discrete populations in the mountain ranges ("land islands") are separated by the hot and dry low altitude conditions. The results of this survey indicated significant diversity of *F. arizonica* endophytes, which was established before the separation of the land islands.

Materials and methods

Endophytic fungi were isolated from 13 *F. arizonica* plants from nine sites in New Mexico, southern Colorado and western Texas (Table 1). Culture and maintenance conditions were as described previously (Byrd et al. 1990). Fungal DNA was extracted from fresh of freeze-dried mycelium (Bruns et al. 1990; Byrd et al. 1990). Segments of the rRNA genes including the two internal transcribed spacers (*rrnITS*1 and *rrnITS*2) were amplified by symmetric polymerase chain reactions (PCR) (Bruns et al. 1990; White et al. 1990). Single-stranded DNA (ssDNA) templates for sequence determination were subsequently prepared by asymmetric PCR (White et al. 1990). In a previous study (Schardl et al. 1991) no ambiguity was evident in sequences obtained directly by this method, indicating that most or all copies of the *rrn* repeat units were identical in each isolate. On the basis of this observation, and to improve and facilitate the analysis, many of the sequences described in this paper were determined from clones. The symmetric PCR products were ligated to pBSKS(+) (Stratagene Cloning Systems, La Jolla, Calif.) (Marchuk et al. 1991), and ssDNA was obtained by co-infection with helper phage M13K07 (Vieira and Messing 1987). Sequences were determined as in Toneguzzo et al. (1988).

The most parsimonious phylogram was determined using PAUP version 3.0q (Swofford 1991). Characters were treated as unordered, and all nucleotide substitution differences were weighted equally. Alignment gaps were considered to be equivalent to missing information.

Results

Three distinct sequences were obtained from the *F. arizonica* endophytes. That of isolate e1491 (designated eI) was shared by 9 other isolates spanning the entire survey area; second sequence (eII) was shared by 2 sympatric isolates, e1481 and e1482; the third sequence (eIII) was obtained only from isolate e1572. The sequence of the *rrnITS*1 region was obtained from 5 of the isolates (Fig. 1). The eI types (e1461, e1491, and e1483) had identical sequences. Also, no differences were observed between sequences from e1481 and e1482 (eII), which differed from e1572 (eIII) by 4 nucleotide substitutions. The complete eI sequence differed from eII and eIII by 19 and 16 nucleotide substitutions, respectively (not including insertions and deletions) (Fig. 1).

The data did not suggest a biogeographical pattern to *rrn* sequence polymorphisms. Type eI extended throughout the survey area from Colorado to Texas. In the Jemez range, an endophyte with type eI sequence (e1483) was sympatric with those possessing type eII sequences (e1481 and e1482). Sequence types eII and eIII were relatively rare and so distinct from eI that they were likely to have had a distinct evolutionary origin. This was confirmed by phylogenetic analysis of their *rrnITS*1–*rrnITS*2 sequences and sequences of *E. typhina* isolates from other grass species. In addition to isolates analyzed

Table 1. Fungal rRNA gene sequences compared in this study

Isolate[a]	Host	Origin of host	*rrnITS*1	*rrnITS*2
e40	*Festuca arizonica*	Guadalupe range, Tex.		√
e61	*F. arizonica*	Sangre de Cristos range, Colo.		√
e1461	*F. arizonica*	Mogollon range, N.M.	√	√
e1471	*F. arizonica*	Sacramento range, N.M.		√
e1481	*F. arizonica*	Jemez range, N.M.	√	√
e1482	*F. arizonica*	Jemez range, N.M.	√	√
e1483	*F. arizonica*	Jemez range, N.M.		√
e1491	*F. arizonica*	Sangre de Cristos range, N.M.	√	√
e1501	*F. arizonica*	San Mateo range, N.M.		√
e1511	*F. arizonica*	Zuni range, N.M.		√
e1521	*F. arizonica*	Guadalupe range, N.M.		√
e1531	*F. arizonica*	Elk range, N.M.	√	√
e1572	*F. arizonica*	Manzano range, N.M.	√	√
e132	*Stipa robusta*	Sacramento range, N.M.	√	√
Epichloë typhina E114	*Agrostis hiemalis*	Montgomery, Ala.	√	√
E2462	*Dactylis glomerata*	Europe	√	√
E56	*Elymus canadensis*	Austin, Tex.	√	√
E32	*Festuca rubra*	Europe	√	√
E187	*Poa ampula*	Yukon valley, Alaska	√	√
Atkinsonella hypoxylon 112	*Danthonia spicata*	New Brunswick, N.J.	√	√

[a] Isolates are designated: E, *Epichloë typhina*; e, nonpathogenic clavicipitaceous endophyte

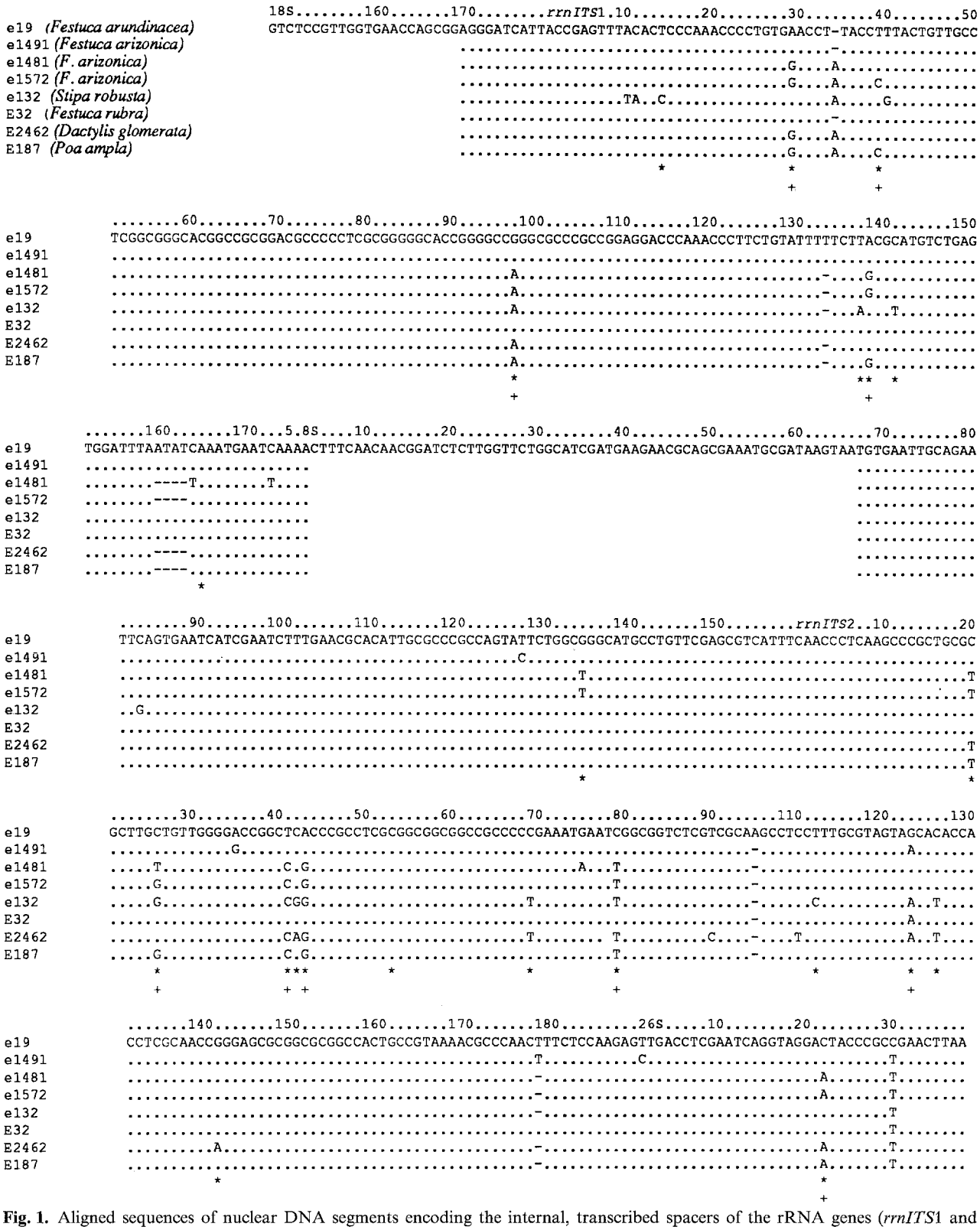

Fig. 1. Aligned sequences of nuclear DNA segments encoding the internal, transcribed spacers of the rRNA genes (*rrnITS1* and *rrnITS2*) from *Epichloë typhina* from *Festuca rubra* (*E32*), *Poa ampla* (*E187*), and *Dactylis glomerata* (*E2462*), and from *Acremonium* endophytes of *Festuca arizonica* (*e1491*, *e1481*, and *e1572*), *Stipa robusta* (*e132*) and *Festuca arundinacea* (*e19*; EMBL accession number X62981). Portions of the flanking structural genes for 18S, 5.8S, and 26S rRNAs are also shown. *Periods* (.) indicate identity with the top reference sequence (*e19*); *asterisks* (*) indicate positions that are informative for parsimony, *plus signs* (+) indicate winning sites favoring the most parsimonious tree (Fig. 2) over a constrained tree that groups the *F. arizonica* isolate sequences (see text). Host species are indicated in *parentheses*

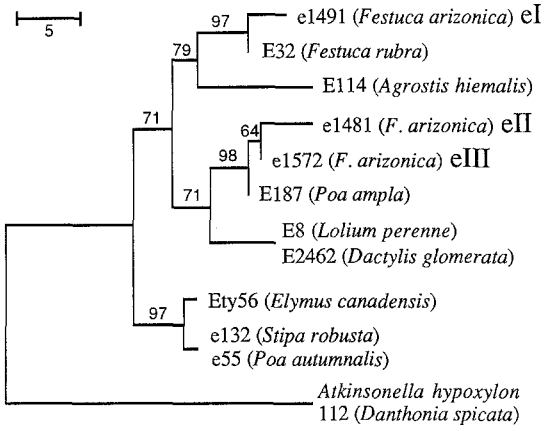

Fig. 2. The single most parsimonious phylogram based on the aligned sequences of endophytes and *Epichloë typhina* shown in Fig. 1 and in Schardl et al. (1991). The *bar* indicates five nucleotide substitution differences. The frequencies with which the clades were represented in 100 bootstrap replications (Felsenstein 1985) are indicated on their respective branches. The total length of the tree was 77, and the consistency index, excluding autapomorphies, was 0.882 (Swofford 1991)

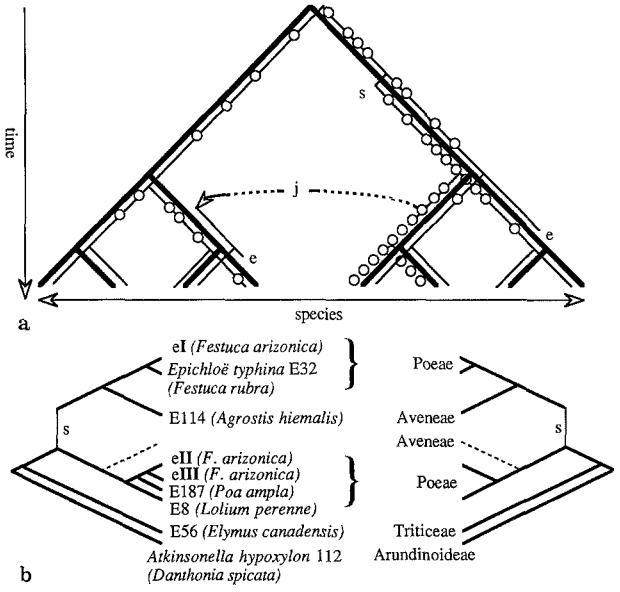

Fig. 3. a Model of evolutionary relationships between grass hosts and their associated *Epichloë typhina* strains and endophytes. Host phylogeny is indicated by *thick lines*. Vertical transmission by seed dissemination of the mycosymbionts is indicated by *thin lines in parallel*. *Circles* indicate horizontal transmission by ascospore infection of host plants. Two possible scenarios are indicated that can give rise to diverse symbionts of individual grass species: speciation (*s*) of the mycosymbionts on an ancestral host; or an ascospore-mediated jump (*j*) across host species lines. Occasionally lines of *E. typhina* or the endophyte may become extinct (*e*) and unavailable for analysis. **b** *rrn* gene tree of *E. typhina* and endophytes of *F. arizonica* (*left*) juxtaposed with the host tribes (*right*). Outgroups are *Atkinsonella hypoxylon* and its grass host. Phylogeny of the host tribes was based on chloroplast DNA analysis (Soreng et al. 1990). Redundancy, indicated by the *vertical lines*, reflects the possibility of a speciation event (*s*) resulting in two coevolved fungal clades. The *dashed line* indicates an hypothetical clade predicted to occur in association with the Aveneae

previously (Schardl et al. 1991), endophytes from three additional grass species were included (Table 1, Fig. 1): Strain E187 from *Poa ampla,* collected in Alaska, very rarely exhibits the formation of stromata (associated with sterilization of inflorescences) in association with its host; in contrast, the association of E2462 with *Dactylis glomerata* generally manifests choke on most or all of the flowering panicles; strain e132 is a seed-transmitted endophyte of *Stipa robusta* collected in the Sacramento range near Cloudcroft, New Mexico.

The phylogram (Fig. 2) indicated that type eI was closely related to an *E. typhina* isolate from the European grass species, *Festuca rubra,* and also to strictly seedborne *Acremonium* endophytes of *Festuca arundinacea* (e19) and *Lolium perenne* (e2) (Schardl et al. 1991). Types eII and eIII were both related to the *P. ampla* isolate E187. The three sequences, eI, eII, and eIII, did not represent a continuum of polymorphism within one group. To demonstrate this, the phylogram was recalculated with a constraint grouping all three sequences from *F. arizonica* endophytes. The resulting seven trees required 89 mutations, as opposed to the most parsimonious, unconstrained tree that required only 77 changes. The constraint also decreased the consistency index from 0.89 to 0.65. Of the 21 informative sites were 11 winning sites (Prager and Wilson 1988). None favored the constrained trees, so support for the most parsimonious tree was highly significant ($P < 0.0005$). It is concluded that the isolates with the type eI sequence evolved from a different *E. typhina* ancestor than did those with types eII and eIII.

Discussion

Among the *Acremonium* endophytes in various grass plants and species, some may have coevolved with their hosts, whereas others may have been derived from ascospore-mediated transfers of *E. typhina* strains from other grass species ("jumps") (Fig. 3). A history of coevolution is expected for endophytes which, having lost any external means of transmission, have become strictly seedborne. It is clearly possible that these relationships extend back to a host ancestral to the tribe Poeae and that subsequent cospeciation has occurred. Even the *E. typhina* isolates from *E. rubra* and *P. ampla* very rarely sporulate on their hosts and probably depend upon vertical transmission as their primary means of propagation. It is noteworthy that the *F. arizonica* endophytes were most closely related to these two *E. typhina* types (Fig. 2) and more distantly related to the more pathogenic types.

This result suggests that evolution of the *F. arizonica* endophytes as well as of related endophytes of *F. arundinacea* and *L. perenne* (Schardl et al. 1991) involved gradual progression from antagonism to mutualism.

The fact that the endophytes of *F. arizonica* group into two distinct clades does not preclude coevolutionary histories for all of them. They may simply represent two coevolved clades – i.e., cryptic but distinct biological species – as has been postulated for other systems (Page 1990) (Fig. 3a). From a comparison of the endophyte phylogenies inferred from *rrn* sequences with the phylogenetic relationships among the host tribes as inferred by restriction endonuclease mapping of plastid genomes (Soreng et al. 1990) it may be hypothesized that the speciation event that gave rise to two *E. typhina* clades occurred in association with a common ancestor of the sister grass tribes Aveneae and Poeae (Fig. 3b). An alternative explanation of the data is that an jump occurred between hosts early in the evolution of the Poeae. Of course, there are more complex possibilities. In order to determine whether there are significant coevolutionary patterns in this system, orthologous sequence data from the host plants and fungal isolates must be obtained, then inferred cladograms (Simberloff 1987) as well as corresponding distance matrices (Schnell et al. 1985; Hafner and Nadler 1990) must be compared statistically. The use of grass *rrn* sequences for such a purpose is being explored.

At present, two observations suggest that the *F. arizonica* endophytes arose by cospeciation. One is the wide geographical range of the clades to which they belong. The related *E. typhina* isolates E32 and E187 were collected well outside of the present range of *F. arizonica*. The other observation is that, so far, other members of the clades that include the *F. arizonica* endophytes are from related grass species (tribe Poeae). The majority of isolates from the Poeae fall into these two clades. An exception is the endophyte from *Poa autumnalis*, which has a sequence identical to those of the *S. robusta* endophyte and *E. typhina* from *Elymus canadensis* (Fig. 2). Since the three hosts are not closely related and are placed in different tribes (Gould and Shaw 1983; Soreng et al. 1990), the evolution of this clade of *E. typhina* and endophytes must have involved horizontal transfers. However, no such jumps need be postulated for the origin of the *F. arizonica* endophytes.

Coevolutionary relationships of *Festuca* spp., *Lolium* spp., and other grasses of tribe Poeae with *Acremonium* endophytes may explain the wide spectrum of fitness enhancements attributable to these endophytes (Clay 1990; Siegel and Schardl 1991). Among the known benefits conferred by *Acremonium* spp. to *F. arundinacea* (the most extensively studied of the interactions) are activity against mammalian herbivores (Lacey 1991), insects (Dahlman et al. 1991), and nematodes (Kimmons et al. 1990; West et al. 1988), enhanced resistance to seedling disease caused by *Rhizoctonia zeae* (Gwinn and Gavin 1992), allelopathy against other plant species (Petroski et al. 1990), and enhancement of drought tolerance (Arachevaleta et al. 1989), root growth, tillering, biomass production (De Battista et al. 1990; Hill et al. 1990) and fecundity (seed production) (Rice et al. 1990). Many of these same benefits have also been observed in *L. perenne-Acremonium* interactions and others (Clay 1990; Siegel and Schardl 1991). Thus, these grasses appear to be both physiologically adapted to their *Acremonium* endophytes and dependent upon them under certain biotic or abiotic stress conditions. The ecological fitness and adaptability of *F. arizonica* and other pooid grasses may be enhanced by the diversity of the endophytes they possess.

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