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Endophyte-host Associations in Grasses. XXIV. Some Evidence to Support the Occurrence of Endophyte Microspecies Complexes Centered Around Sexually-Reproducing Species of *Epichloë*

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Abstract

Studies employing randomly amplified polymorphic DNA sequences are conducted on a range of grass endophytes of the genus *Epichloë* and anamorphs. Through the use of 96 bands we were able to consistently link anamorphs to teleomorphs. It was found that an isolate of *Acremonium coenophialum* from *Festuca arundinacea* and several isolates of *A. typhinum* from *F. ovina* cluster with *Epichloë festucae* while isolates from *Agrostis alba, Bromus ramosus, Poa ampla*, and *P. sylvestris* group with *E. typhina*. For comparative purposes, rDNA ITS1 and ITS2 sequences were obtained from GenBank and analyzed using phylogenetic procedures. The same pattern of clustering with sexually-reproducing species was observed. It is suggested that endophytes may form complexes of asexually-reproducing 'microspecies' or clones centered around the sexually-reproducing species of *Epichloë*.

Keywords: *Acremonium, Epichloë*, endophytes, Clavicipitaceae, speciation

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1. Introduction

Many grasses are known to bear infections by endophytic fungi of the tribe Balansieae (Clavicipitaceae; Ascomycotina). In warm-season grass species endophytes of the genus *Balansia* are commonly encountered (Diehl, 1950). These endophytes are limited to the Americas (White, 1994a). In cool-season grasses endophytes of the genus *Epichloë* and associated anamorphs are commonly encountered. The endophytes of this latter group are widespread in Europe and the Americas (White, 1987; White and Baldwin, 1992; Bertoni et al., 1993). In *Balansia*, anamorphs are generally associated with teleomorphs and taxonomic schemes have been constructed solely on the teleomorphs (Diehl, 1950). However, certain endophytes related to the genus *Epichloë* appear frequently to have lost the capacity for formation of stromata and consequently classification schemes have been devised to identify these endophytes using conidia produced on culture media (Morgan-Jones and Gams, 1982; White and Morgan-Jones, 1987). Conidial states of endophytic *Epichloë* have been classified in the deuteromycete genus *Acremonium* section *Albo-lanosa*. Recent work on field populations of *Epichloë* on different hosts has resulted in the establishment of several new species of *Epichloë* (White, 1993; White, 1994b; Leuchtmann et al., 1994). As a step toward the unification of these teleomorphic and anamorphic systems of classifying endophytes and to investigate species boundaries we have undertaken studies employing random amplified polymorphic DNA sequences (RAPDs).

2. Materials and Methods

Endophytes were isolated from grasses collected from North America and Europe (Table 1). DNA was extracted from four plugs (4 mm diameter) taken from margins of colonies grown on potato dextrose agar (PDA) overlaid with sterile cellophane disks (Flexel Inc., Covington, IN). Genomic DNA was extracted (Ashtorab and Cohen, 1992), and final DNA concentrations were standardized to 10 ng/µl DNA with a TKO-100 fluorometer (Hoeffer, San Francisco, CA).

Polymerase chain reaction (PCR) amplification of DNA sequences were performed with each of five oligonucleotide primers (OPA-1, OPA-9, OPA-18, OPA-19, and OPA-20) 10 bases in length, obtained from Operon Technologies, Inc. (Alameda, CA). Each 12-µl PCR reaction contained 1.2 units Stoffel fragment polymerase (Perkin-Elmer, Norwalk, CT) and 1X buffer (Perkin-Elmer), 0.2 mM of each dNTP (Perkin-Elmer), 1.3 pg primer, and 12 ng of template DNA. A Perkin-Elmer Thermocycler (model TC-1) was programmed

Table 1. Collection information and identification of isolates used in RAPD analysis.

No.	Host	Collection location	Endophyte identification
1	*Festuca rubra rubra*	New Jersey	*Epichloë festucae*
2	*Festuca rubra rubra*	New Jersey	*Epichloë festucae*
3	*Festuca rubra rubra*	New Jersey	*Epichloë festucae*
4	*Festuca ovina*	New Jersey	*Acremonium typhinum*
5	*Festuca rubra rubra*	New Jersey	*Epichloë festucae*
6	*Festuca rubra rubra*	Oregon	*Epichloë festucae*
7	*Festuca ovina*	New Jersey	*Acremonium typhinum*
8	*Festuca ovina*	New Jersey	*Acremonium typhinum*
9	*Festuca rubra commutata*	New Jersey	*Epichloë festucae*
10	*Agrostis stolonifera*	England	*Epichloë baconii*
11	*Poa sylvestris*	Missouri	*Acremonium typhinum*
12	*Poa ampla*	Alaska	*Acremonium typhinum*
13	*Festuca rubra commutata*	New Jersey	*Epichloë festucae*
14	*Festuca rubra commutata*	New Jersey	*Epichloë festucae*
15	*Festuca ovina*	New Jersey	*Acremonium typhinum*
16	*Festuca rubra rubra*	England	*Epichloë festucae*
17	*Festuca rubra commutata*	New Jersey	*Epichloë festucae*
18	*Festuca rubra*	Idaho	*Epichloë festucae*
19	*Festuca rubra commutata*	New Jersey	*Epichloë festucae*
20	*Festuca arundinacea*	New Jersey	*Acremonium coenophialum*
21	*Agrostis alba*	Arkansas	*Acremonium typhinum*
22	*Bromus ramosus*	England	*Acremonium typhinum*
23	*Dactylis glomerata*	England	*Epichloë typhina*

for an initial denaturation of 7 min at 94°C followed by 45 cycles. Each cycle consisted of a denaturation step at 94°C for 1 min, a primer annealing step at 36°C for 1 min, a primer extension step at 72°C for 2 min. The primer extension step of the final cycle was extended to 5 min. The ramp rate for heating between 36 and 72°C was 0.3°C/second; otherwise all heating and cooling rates were 1°C/second. Amplified fragments were resolved in a 7.5% acrylamide-bis gel (37.5:1; Fisher, Fairlawn, NJ) in 0.375 M Tris buffer (pH 8.8) at 200 V for 40 min with a Mini-Protean II (Bio-Rad, Richmond, CA). A modified protocol of the Bio-Rad silver stain kit (fixation step: 10% acetic acid for 30 min) was used to visualize the amplification products. RAPD patterns were produced for at least two replicate PCR reactions for each isolate. Data were collected from

Table 2. Data matrix showing euclidean metric distances (number of differences) using 96 RAPD bands.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	0	24	24	31	22	20	25	21	23	50	32	36	25	21	24	26	24	24	25	26	38	39	39
2		0	20	15	10	22	21	11	19	44	44	44	19	19	20	28	22	22	21	20	48	41	41
3			0	21	22	10	5	17	9	50	36	34	11	23	22	26	16	20	9	16	40	31	37
4				0	11	21	18	12	16	45	47	41	20	24	23	31	21	19	18	17	51	42	46
5					0	22	21	7	19	42	40	38	17	19	20	26	20	20	19	20	46	39	43
6						0	9	15	13	56	40	36	19	23	16	28	22	20	17	16	42	41	39
7							0	16	4	49	39	33	14	22	19	23	13	17	12	15	43	34	36
8								0	14	47	39	39	12	20	17	27	15	19	16	17	43	40	38
9									0	49	39	35	14	20	21	23	11	15	10	13	43	34	38
10										0	42	36	45	43	50	44	44	44	47	44	44	41	47
11											0	20	39	37	48	36	38	38	37	44	18	21	37
12												0	35	37	42	32	34	38	35	40	24	25	35
13													0	20	25	21	9	17	6	19	41	34	38
14														0	29	17	17	17	22	23	45	38	40
15															0	34	28	24	25	18	46	47	43
16																0	20	22	25	30	42	31	39
17																	0	16	11	20	46	35	37
18																		0	15	16	44	39	43
19																			0	15	41	32	40
20																				0	48	41	41
21																					0	29	41
22																						0	42
23																							0

replicated RAPD bands between fragment sizes 0.1 and 2.6 kb. Euclidean metric distances (Table 2) were calculated for all combinations of isolates following Huff, Peakall, and Smouse (1993).

A UPGMA tree (Fig. 1) was then produced from the distance matrix employing program options available in MEGA (Kumar, Tamura, and Nei, 1993). For comparison to rDNA ITS sequences, ITS1 and ITS2 sequences were obtained from GenBank (Table 3), aligned using DNASTAR® program options, and a tree produced (Fig. 2) using the neighbor-joining procedure following Kimura's 2-parameter model with complete deletion (MEGA). The ITS sequence data was developed by Chris Schardl and coworkers (Schardl et al., 1991).

Table 3. Sources of rDNA ITS1 and ITS2 sequences used in clustering analysis.

Endophyte	Source	Host	Location
Epichloë festucae	GB L07139	*Festuca longifolia*	Europe
Epichloë festucae	GB X62987	*Festuca rubra rubra*	Europe
Acremonium starrii	Schardl et al., 1991	*Festuca arizonica*	Texas
Acremonium lolii	GB L07130	*Lolium perenne*	Europe
Acremonium coenophialum	Schardl et al., 1991	*Festuca arundinacea*	Europe
Epichloë baconii	GB L07138	*Agrostis stolonifera*	England
Epichloë amarillans	GB L07141	*Sphenopholis obtusata*	Georgia
Epichloë amarillans	GB L07142	*Agrostis hiemalis*	Alabama
Acremonium typhinum	GB L07134	*Poa ampla*	Canada
Epichloë typhina	GB L07136	*Glyceria striata*	New York
Epichloë typhina	GB L07133	*Dactylis glomerata*	England

3. Results and Discussion

The analysis of diversity seen in RAPDs (Fig. 1) suggests that endophytes from fescues tall fescue (*Festuca arundinaceae*), sheeps fescue (*F. ovina*), and red fescues (*F. rubra* ssp. *rubra*, and *F. rubra* ssp. *commutata*) possess numerous band similarities, forming a single clade. This clade may be identified as the *Epichloë festucae* clade due to the numerous isolates of this species from red fescues represented within the clade. It is interesting that within this clade isolates of nonstroma-forming endophytes identifiable as *Acremonium typhinum* from sheeps fescue group together with *A. coenophialum* from tall fescue. Examination of the neighbor-joining tree (Fig. 2) made by analysis of rDNA ITS sequences shows a similar clade where *E. festucae* isolates are grouped with anamorphs *A. lolii*, *A. coenophialum*, and *A. starrii*. It seems reasonable to conclude that all of these anamorphic endophytes have evolved from sexually-reproducing *E. festucae*. Similarly, RAPD analysis (Fig. 1) indicates that nonstroma-forming isolates of *A. typhinum* from *Agrostis alba*, *Bromus ramosus*, *Poa ampla*, and *P. sylvestris* group with *Epichloë typhina*. This grouping seems to be supported by rDNA data (Fig. 2) where the *P. ampla* endophyte is seen to group with two isolates of *E. typhina*. The results of both rDNA and RAPD analysis make it clear that the anamorphic species category *A. typhinum* is artificial in that it does not reflect a single natural species, since these isolates were shown to belong to multiple distinct biological species

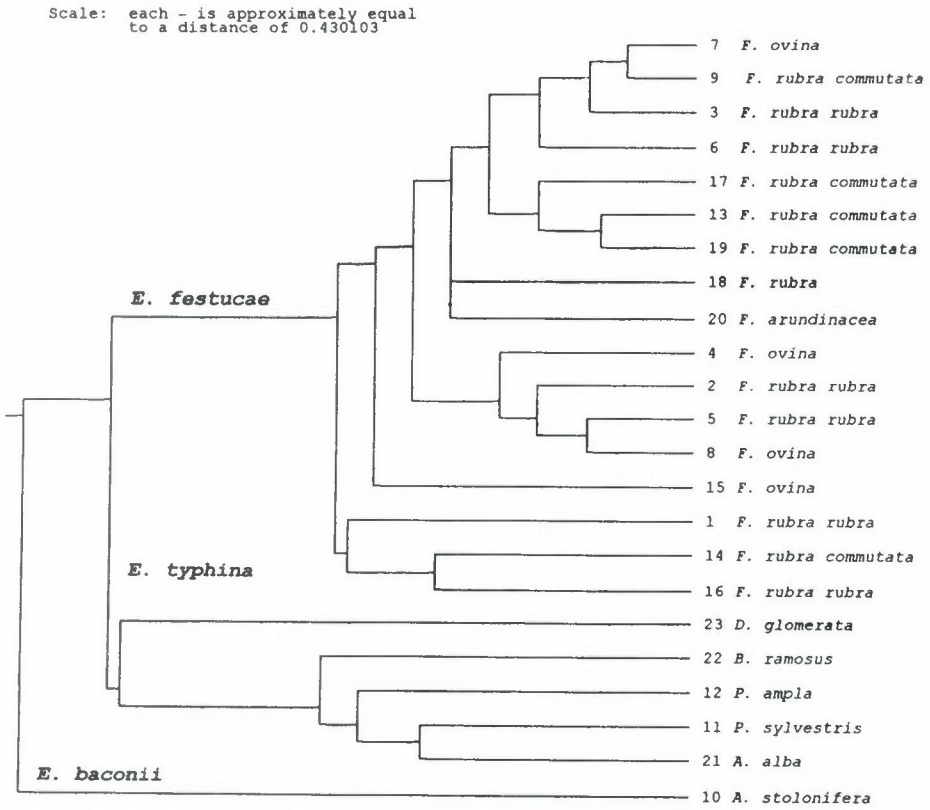

Figure 1. UPGMA tree (MEGA) compiled from euclidean metric distances obtained by examination of 96 polymorphic RAPD markers produced by subjecting DNA of 23 isolates of endophytes to five primers. In this phylogram 3 clades are evident and identified as *Epichloë festucae, E. typhina,* and *E. baconii.* All endophytes possess anamorphs classified as *A. typhinum* except that of *Festuca arundinacea* (number 20) which is classified as *A. coenophialum.*

groups. If the classification of anamorphs into distinct species categories is to have biological meaning, additional features will need to be identified that may be used to separate biological species.

While this study is not exhaustive, it does show that through examination of both RAPD and rDNA ITS1 and ITS2 sequences anamorphic nonstroma-forming endophytes can be linked to teleomorphs with reasonable consistency. This is unexpected since it has been proposed that the asexual nonstroma-forming endophytes may have originated through hybridizations between distinct species of *Epichloë* (Tsai et al., 1994). This 'hybridization hypothesis'

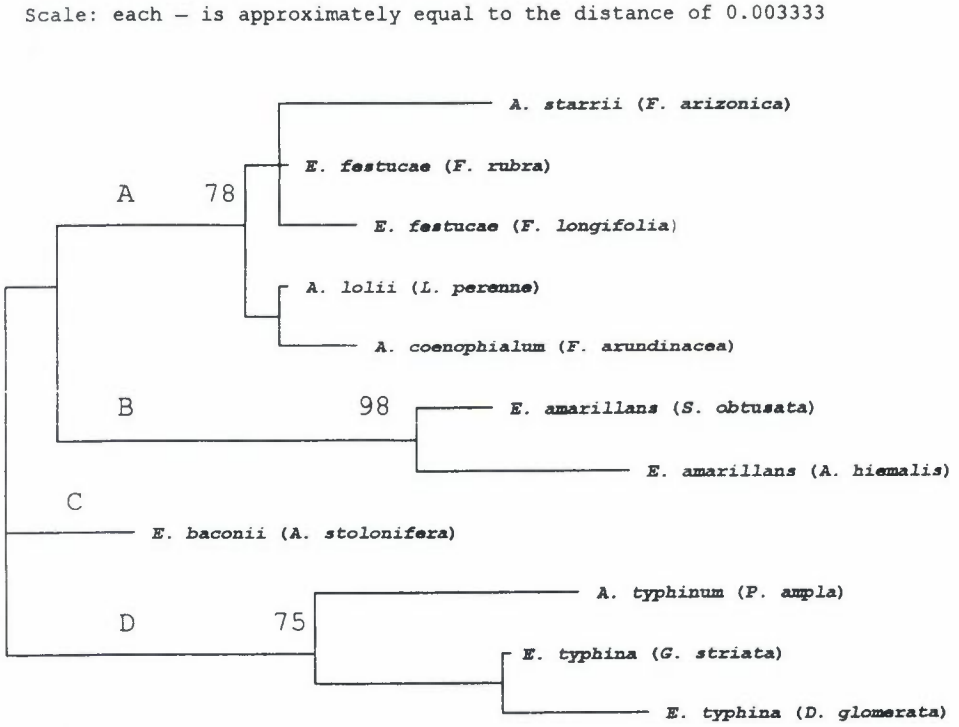

Figure 2. Neighbor-joining tree compiled from ITS1 and ITS2 sequence data available in GenBank made using Kimura's 2-parameter model with complete deletion (MEGA). Selected bootstrap confidence levels are indicated (500 reps). Four clades are indicated as follows: A) *Epichloë festucae;* B) *E. amarillans;* C) *E. baconii;* and D) *E. typhina.*

is supported by the discovery of duplicates of several genes where individual copies seem to be derived from distinct species of *Epichloë*. The *A. coenophialum* endophyte of tall fescue was proposed to be produced through hybridization of three distinct species of *Epichloë* (Tsai et al., 1994) and the *A. lolii* endophyte of perennial ryegrass was proposed to be the result of hybridization between two distinct species of *Epichloë* (Schardl et al., 1994). It seems reasonable that such hybridization would result in the obliteration of species boundaries. However, cladistic analysis of RAPDs (Fig. 1) and ITS data (Fig. 2) do not show this to be the case. This is especially notable for *A. coenophialum* where three distinct genomes are believed to have hybridized. This endophyte groups consistently within the *E. festucae* clade in both RAPD and rDNA analysis (Figs. 1 and 2).

It is difficult to explain why results of cladistic analysis of RAPDs and rDNA (Figs. 1 and 2) are not congruous with the 'hybridization hypothesis'. Perhaps hybridization events are followed by introgression or some comparable process to restabilize the genome. Such a process may result in the maintenance of the majority of the genes of the 'host endophyte species' and elimination of the majority of the genes of the 'invading genome'. Introgression has long been known to occur in grasses and other groups of organisms (Stebbins, 1971). However, in these groups of organisms after the initial hybridization between sexually-reproducing individuals has occurred, back crossing to one parent occurs to stabilize the genome. In fungi the parasexual cycle could also account for hybridization events and stabilization of genomes through loss of the majority of the genes of the 'invading genome' (Webster, 1970). It is also possible that duplicate genes might originate and diverge within a lineage without interspecific hybridization having occurred. Whether any of these processes are occurring in the endophytes is unknown.

The results of our analysis suggest that populations of endophytes in grasses may form complexes of 'microspecies' or clones centered around sexually-reproducing species of *Epichloë*. This population structure may be comparable to that seen in the North American plant *Crepis occidentalis*, where some 27 distinct asexual microspecies have developed from the sexually-reproducing populations (Grant, 1977). In fungi anamorphic genera *Penicillium*, *Paecilomyces*, and *Aspergillus* appear to represent comparable complexes of microspecies (Berbee et al., 1995). Whether, the asexually-reproducing *Acremonium* endophytes are distinct enough from their sexually-reproducing parent populations to warrant distinct taxonomic status is yet to be determined.

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