Genetics

Isozyme Variation in the *Acremonium/Epichloë* Fungal Endophyte Complex

Adrian Leuchtmann and Keith Clay

Department of Biology, Indiana University, Bloomington 47405.
Present address of the first author: Geobotanisches Institut ETH, Zollikerstrasse 107, CH-8008 Zurich, Switzerland.
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ABSTRACT

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Isozyme variation of 219 isolates of *Acremonium* or *Epichloë* fungal endophytes from 17 host grass species was examined using starch gel electrophoresis. Ten enzyme systems selected for use out of 17 examined were variable, with three to nine distinct electromorphs per enzyme. Most isolates produced a single band per enzyme, consistent with a haploid fungus and a single isozyme locus. Double or triple bands were observed for several enzymes in certain isolates, particularly those from tall fescue. Isozyme variation was observed among isolates from 10 out of 12 different hosts where multiple isolates were sampled. Among the 52 isolates of *A. coenophialum* from tall fescue, 47 had the same isozyme phenotype, whereas five isolates from cultivar Triumph exhibited a different phenotype, which was most similar to *A. lolii* from perennial ryegrass (*Lolium perenne*). The relative uniformity of isolates of *A. coenophialum* from tall fescue probably reflects the limited number of endophyte genotypes present in the original plant material introduced from Europe. Isozyme banding patterns were not distinctive enough to distinguish between sexual *Epichloë* endophytes and asexual, seedborne *Acremonium* endophytes. There was no evidence that sexual endophytes were electrophoretically more variable within a host than asexual endophytes. Isozymes may provide useful genetic markers in future attempts to modify the grass/endophyte association.

Many grasses are infected by asymptomatic, systemic fungal endophytes with close resemblance to the asexual state of the Ascomycete fungus *Epichloë typhina* (Pers.:Fr.) Tul. (8,20). These endophytes, classified in the form genus *Acremonium* sect. *Albolanosa* (18), usually do not produce fruiting structures on their host plants and are transmitted maternally through the ovule of infected plants. The occasional production of ascospore-producing stromata on a few plants of some species suggests that they are anamorphs of *E. typhina* or related taxa (9,25). Endophytes of grasses are of applied significance given their role in toxic syndromes of domestic grazing animals, increased resistance of host grasses to pests, and their production of biologically active alkaloids (3,4,6).

There is interest in manipulating the grass/endophyte association through artificial inoculations of grasses with foreign endophytes or endophytes that have been genetically altered (8,20). It may be possible to produce endophyte-infected grasses with desired characteristics (such as vigor and insect resistance) while minimizing unwanted characteristics (such as livestock toxicity). However, there are few data available on genetic variation of endophytes to assist attempts to modify the relationship. Inoculation studies with grass seedlings suggest that endophytes may be transferred among host species, but that the potential host range may be constrained (13,21). In addition, alkaloid production by the tall fescue endophyte and other clavicipitaceous endophytes of grasses has been shown to vary considerably among isolates (1,4,19). To our knowledge, there have been no previous published studies of biochemical genetic variation in *Epichloë* or *Acremonium* endophytes of grasses. The *Acremonium*-like hyphomycetes from soil and other substrates examined in an electrophoretic study by Chesson et al (5) are not related to the *Acremonium* endophytes of grasses.

The purpose of this paper is to describe the patterns of biochemical variation, as determined by starch gel electrophoresis, of 219 isolates of *E. typhina* or related *Acremonium* anamorphs, including previously undescribed forms, isolated from 17 different grass species. Nearly one-quarter of the isolates were *A. coenophialum* Morgan-Jones & Gams from tall fescue. Our objectives were threefold: 1) to determine if endophytes isolated from a single host species were genetically variable; 2) to determine if asexual, seedborne *Acremonium* endophytes (as in tall fescue) could be distinguished from sexual *Epichloë* endophytes; and 3) to determine relationships of the endophytes from different hosts examined in this study based on similarity of their isozyme banding patterns. This information would enhance our knowledge of the biology of fungal endophytes that infect many important crop, pasture, and turf grasses.

TABLE 1. Collection sites and isolate numbers (source of isolation in parentheses) for endophyte-infected grass species

Collection site	Isolate number(s) and source[a]
Cedar Bluff, Monroe Co., IN	8730(E), 8732(A), 8733(A), 8830(A), 8851(A), 8862(S), 8864(S)
Griffy Lake, Monroe Co., IN	8724(A), 8805(A), 8806(A), 8858(A)
Lake Lemon, Monroe Co., IN	8859(A)
Crooked Creek, Lake Monroe, Monroe Co., IN	8828(A), 8829(A)
Shawnee Bluffs, Monroe Co., IN	8860(A)
Bloomington, Monroe Co., IN	8838(E), 8867(A), 8868(A), 8869(A), 8873(A)
Clear Creek, Monroe Co., IN	8902(A)
Highway 446 S, Monroe Co., IN	8870(A), 8871(A)
Nashville, Brown Co., IN	8865(A)
Brown Co. State Park (SP), Brown Co., IN	8837(E), 8857(A), 8863(S)
Harrison-Crawford SP, Harrison Co., IN	8749(E), 8750(E)
McCormick Creek SP, Owen Co., IN	8809(A), 8810(A), 8811(A), 8832(A)
Spring Mill SP, Lawrence Co., IN	8807(A), 8808(A)
Indiana Dunes SP, Porter Co., IN	8861(A)
Highlands, Macon Co., NC	8842(E)
Blue Ridge Pkwy., Haywood Co., NC	8835(E)
Canastota, Madison Co., NY	8735(E)
Otero Co., NM[b]	8854(S)
Brazoria Co., TX[b]	8710(S)
Osgoode, Ottawa, Canada	8734(E)
Hannover, West Germany	8612(E)
Experimental seed, tall fescue 'Johnstone'[c]	8906(S)
Experimental seed, tall fescue 'Triumph'[c]	8907(S)
Commercial seed, tall fescue 'Ky-31'	8911(S)
Experimental seed, Auburn University, tall fescue line 7-464[d]	8913(S), 8914(S), 8915(S)
Commercial seed, perennial ryegrass 'Repell'	8912(S)

[a]Isolated from symptomless, infected plants as *Acremonium* (A); from stromata of *Epichloë* (E); from seeds or seedlings grown from infected seed (S).
[b]Seed provided by J. White, Auburn University-Montgomery, AL.
[c]Seed provided M. Siegel, University of Kentucky.
[d]Seed provided by N. Hill, University of Georgia.

MATERIALS AND METHODS

Source of isolates. Isolates were obtained from endophyte-infected plants originating from either plants or seeds collected in the field in 1987 and 1988 or from infected seeds provided by other researchers. Where several isolates were obtained from a single host population, different plants were collected at least 5 m apart but no more than 50 m apart within a single contiguous population. Where several isolates were obtained from seeds, the seeds were bulk collected from 10–20 scattered plants from throughout the entire population at the site, and a random subset of seeds was germinated. Where two or more host species were sampled at the same site (e.g., *Elymus* spp. and *Hystrix*) the different host species typically occurred in different microhabitats. Neighboring plants of different species were not collected together.

In symptomless plants, endophytes were isolated from surface sterilized tissues as described previously (14), whereas in stromata-producing plants isolates were obtained from single ascospores (one to six per stroma). Seeds were surface sterilized by soaking in 50% sulfuric acid for 20 min, rinsing in sterile water, immersing in 50% commercial bleach (2.63% Na-hypochlorite) solution for 20 min, transferring to 95% EtOH, and rinsing again in sterile water. All isolations were made at room temperature on cornmeal-malt extract-agar (17 g of cornmeal agar, 20 g of malt extract, 2 g of yeast extract, 1 L of distilled water) supplemented with oxytetracycline (50 mg/L of medium).

Collection sites, isolate numbers, and source of isolation are listed in Table 1. Most plants and seeds were collected in southern Indiana around Indiana University. The host species, identity of endophytes, and sample size (number of isolates) are given in Table 2. For most hosts where the endophyte is identified as *Epichloë*, only a very small fraction of the infected plants bore stromata (9). The number of isolates per host species ranged from one (for five species) up to 52 (for tall fescue). Stock cultures of each isolate were grown on cornmeal-malt extract-agar slants before processing. A representative number of strains is maintained at Indiana University.

Sample preparation. Mycelium for enzyme extraction was grown in 50 ml of liquid medium (30 g of sucrose, 20 g of malt extract, 2 g of Bacto peptone, 1 g of yeast extract, 0.5 g of magnesium sulfate, 0.5 g of potassium chloride, 1 g of potassium phosphate monobasic, 0.05 g of chloramphenicol, 1 L of distilled water) (2), that was inoculated with pieces of the stock cultures. Liquid cultures were maintained in 125-ml Erlenmeyer flasks on a rotary shaker (140 rpm) at 25 C. The sample preparation followed the method described by Micales et al (16,17) and utilized by Leuchtmann and Clay (15). In brief, samples were harvested in the second half of the log growth phase, mycelium was vacuum filtered from the medium, lyophilized, crushed into a powder, and stored at −20 C over desiccant. Samples for electrophoresis were prepared by suspending 25 mg of lyophilized mycelium in 0.3 ml of 0.1 M Tris-HCl-PVP extraction buffer (pH 7.5) of Soltis et al (23). Samples were stored overnight at 4 C, then centrifuged, and the supernatant was absorbed onto 2- × 13-mm filter-paper wicks. Cultures of all isolates were treated similarly (e.g., number of transfers, culture conditions, stage of harvest) to prevent variation in electromorphs resulting from variable laboratory procedures. In this study dried mycelium from the same liquid culture was used in replicate runs of the same isolate. Previous research has shown that different cultures of the same isolate consistently produce the same isozyme phenotype (Leuchtmann and Clay, *unpublished*).

Electrophoresis. Techniques of horizontal starch gel electrophoresis are based on those of Soltis et al (23), and details are described in Leuchtmann and Clay (15). In brief, gels were prepared in trays with 12.8% hydrolized starch (Sigma) and loaded with 32 wicks. One of three buffer systems listed in Table 3 was used (23). Gels were run at 4 C and wicks were removed after 10–12 min of electrophoresis.

Gel slices were stained for enzyme activity following published recipes (23,24). The enzyme names with enzyme commission (EC) number (11), abbreviations, number of electromorphs determined, and buffer system used are given in Table 3. Initially, 17 enzymes were tested for activity and clear resolution of bands. Ten enzymes were selected that had well-resolved bands in all isolates. The other enzymes showed inconsistent activity among isolates, or bands that were difficult to resolve because of streaking or smearing, and are not included in this study. Enzymes examined but not used included alanine aminotransferase (EC 2.6.1.2), diaphorase (EC 1.6.4.3), esterase (EC 3.1.1.-), fumarase (EC 4.2.1.2), B-glucosidase (EC 3.2.1.21), shikimate dehydrogenase (EC 1.1.1.25), and superoxide dismutase (EC 1.15.1.1). The latter enzyme was the only enzyme tested that showed no activity at all.

Electromorphs for each enzyme were determined by repeated side by side comparisons. The most cathodal electromorph was designated A, and the other electromorphs of the same enzyme were assigned letters B, C, D, etc., according to their increasing mobility relative to A. The positions of the bands in Figure 1 are based on the absolute migration distance on the same gel. In five enzymes both single- and multiple-banded patterns were found among isolates, while in the other enzymes only either single-banded (four enzymes) or double-banded (one enzyme) patterns were observed in all isolates.

The genetic basis of the electromorphs examined here is not known, and crosses were not made to test the Mendelian inheritance of the allozymes. Whereas crosses would have been possible with *E. typhina*, they would be impossible for the asexual *Acremonium* endophytes, the largest group in this study. In the four enzymes that produced only single-banded phenotypes, simple genetic control by a single locus seems a likely explanation. However, in the other enzymes where complex multibanded patterns were evident, it is impossible to assign bands to particular loci and to interpret the genetic basis of their variation without crossing experiments. Therefore, the isozyme data were interpreted conservatively, considering only the banding

TABLE 2. Isozyme phenotypes of endophyte isolates from 17 host species

		ACP	ACO	ALD	G6P	LAP	MDH	PGI	PGM	6PG	TPI
Agrostis hiemalis, infected by *Epichloë typhina*											
8733[a]	(1)[b]	E[c]	G	E	C	E	A	A	D	I	A
8842	(2)	E	G	E	C	C	A	A	E	D	B
	(1)	E	G	E	C	C	A	A	I	D	A
	(1)	E	G	E	C	D	A	A	E	D	A
	(2)	E	G	E	D	C	A	A	E	I	B
	(2)	E	G	E	D	C	A	A	I	I	A
	(2)	E	G	E	D	D	A	A	E	I	A
	(1)	E	G	E	D	D	A	A	E	I	B
	(1)	E	G	E	D	D	A	A	I	I	A
Agrostis perennans, infected by *Epichloë typhina*											
8837	(1)	E	G	E	C	E	A	A	E	D	A
8857	(1)	E	H	E	C	E	A	A	D	D	A
8858	(1)	E	G	E	B	E	A	A	D	D	A
8859	(1)	E	G	E	B	F	A	A	I	D	A
8860	(1)	E	G	E	C	E	A	A	E	I	A
8861	(1)	E	G	E	C	E	A	A	D	D	A
Brachyelytrum erectum, infected by *Epichloë typhina*											
8749	(1)	E	A	D	C	D	G	A	A	D	C
8835	(2)	C	A	D	C	D	E	A	A	D	C
8863	(15)	E	A	D	B	D	E	A	A	D	C
Elymus villosus, infected *Epichloë typhina*											
8851	(1)	E	A	D	C	E	A	C	D	D	B
Elymus virginicus, infected by *Epichloë typhina*											
8832	(3)	E	H	E	C	E	A	A	I	D	A
8862	(32)	D	A	D	C	F	A	C	I	D	B
	(4)	E	A	D	B	F	F	C	I	D	B
8838	(1)	E	A	D	C	F	F	C	I	D	B
Festuca arundinacea, infected by *Acremonium coenophialum*											
8865	(1)	B	C	B	B	F	A	B	C	C	C
8867	(1)	B	C	B	B	F	A	B	C	C	C
8868	(1)	B	C	B	B	F	A	B	C	C	C
8869	(2)	B	C	B	B	F	A	B	C	C	C
8870	(2)	B	C	B	B	F	A	B	C	C	C
8871	(2)	B	C	B	B	F	A	B	C	C	C
8873	(2)	B	C	B	B	F	A	B	C	C	C
8902	(1)	B	C	B	B	F	A	B	C	C	C
8906	(18)	B	C	B	B	F	A	B	C	C	C
8907	(5)	D	A	B	A	B	C	D	E	D	C
8911	(8)	B	C	B	B	F	A	B	C	C	C
8913	(3)	B	C	B	B	F	A	B	C	C	C
8914	(3)	B	C	B	B	F	A	B	C	C	C
8915	(3)	B	C	B	B	F	A	B	C	C	C
Festuca obtusa, infected by cf. *Acremonium starrii*											
8732	(1)	A	F	D	C	B	F	A	G	D	C
8805	(3)	C	F	D	C	B	F	A	G	A	C
	(2)	E	A	A	B	E	A	C	E	D	C
8808	(5)	C	F	D	C	B	F	A	G	A	C
8811	(2)	A	B	D	B	B	B	B	C	E	C
8828	(1)	C	E	D	C	B	F	A	G	A	C
Glyceria striata, infected by *Epichloë typhina*											
8734	(3)	E	G	G	B	F	A	C	I	C	C
8735	(4)	E	G	G	B	F	A	C	I	C	C
Holcus lanatus, infected by *Epichloë typhina*											
8612	(1)	D	A	F	A	B	A	C	H	C	C
Hystrix patula, infected by *Epichloë typhina*											
8730	(2)	C	A	D	B	E	A	C	D	D	B
	(1)	C	A	D	B	E	A	C	I	H	B
	(1)	C	A	D	B	E	A	C	I	D	B
	(1)	D	A	D	B	E	A	C	D	H	B
	(1)	D	A	D	C	F	A	C	I	D	B
8864	(34)	D	B	D	C	F	A	C	D	D	B
Lolium perenne, infected by *Acremonium lolii*											
8912	(6)	D	A	A	C	B	A	C	E	F	C
Poa autumnalis, infected by *Acremonium* sp.											
8710	(1)	D	A	D	C	E	A	A	F	B	B
Poa sylvestris, infected by cf. *Acremonium starrii*											
8806	(6)	A	F	D	C	B	F	A	G	A	C
8807	(5)	A	F	D	C	B	H	A	G	D	C
8810	(2)	B	D	B	C	···	A	B	E	D	C
Poa wulfii, infected by cf. *Acremonium starrii*											
8809	(1)	B	F	C	C	A	A	B	G	G	C
Sphenopholis nitida, infected by cf. *Acremonium starrii*											
8724	(1)	A	F	D	C	B	F	A	C	D	C
8750	(1)	E	G	E	C	F	A	A	G	D	A
Sphenopholis pallens, infected by cf. *Acremonium huerfanum*											
8829	(2)	E	H	E	C	E	A	A	E	D	A
8830	(1)	E	···	E	C	E	A	A	E	D	A
Stipa robusta, infected by *Acremonium* sp.											
8854	(1)	D	A	B	B	D	D	C	B	D	B

[a] Isolate number.
[b] Sample size.
[c] Identity of electromorph shown in Figure 1.

TABLE 3. List of enzymes with enzyme commission (EC) number, abbreviations, number of electromorphs determined, and buffer systems used in this study

Enzyme[a]	EC no.	Abbreviation	Electromorphs	Buffer system[b]
Acid phosphatase	3.1.3.2	ACP	5	II
Aconitase	4.2.1.3	ACO	8	III
Aldolase	4.1.2.13	ALD	7	III
Glucose-6-phosphate dehydrogenase	1.1.1.49	G6P	4	I
Leucine aminopeptidase	3.4.11.1	LAP	6	III
Malate dehydrogenase	1.1.1.37	MDH	8	II
Phosphoglucose isomerase	5.3.1.9	PGI	4	II
Phosphoglucomutase	5.4.2.2	PGM	9	II
6-Phosphogluconate dehydrogenase	1.1.1.44	6PG	9	I
Triosephosphate isomerase	5.3.1.1	TPI	3	II

[a] All stain recipes from (23).

[b] Buffer systems (from 23) and electrical requirements: I = pH 7.0 citric acid/histidine HCl system using 75 mA constant current until the marker dye (bromophenol blue) migrated 6 cm; II = pH 7.2 Tris citric acid/Tris citric acid system using 50 mA constant current until the marker dye migrated 9 cm; III = pH 8.0 Tris citric acid/Tris citric acid system using 50 mA constant current until the marker dye migrated 9 cm.

phenotype, where each banding pattern (per enzyme and isolate) was considered as a different electromorph.

Data analysis. The Pearson product-moment correlation coefficient *r* (22) between the pooled isolate samples from each host species was calculated based on the frequencies of electromorphs. This measure of phenotypic resemblance has been widely used on data where characters are present in more than one state (22) and is an appropriate similarity coefficient for our data. A dendrogram was constructed by cluster analysis (average linkage, UPGMA) applied to the coefficient matrix. Calculations were performed with the statistical package SYSTAT, Release 3.2 (26).

RESULTS

Ten enzymes were selected and routinely resolved for each of the 219 isolates examined (Table 3). From three to nine distinct electromorphs occurred per enzyme and a total of 63 for all enzymes (Fig. 1). However, not all isolates formed the same number of bands in each enzyme system. In four enzyme systems (ACP, G6P, LAP, TPI), only a single band per isolate was resolved (Fig. 1), consistent with a haploid organism with a single isozyme locus. In the remaining six enzymes a few isolates exhibited one or two additional bands, although most exhibited a single band. Since no crosses have been made, the interpretation of these

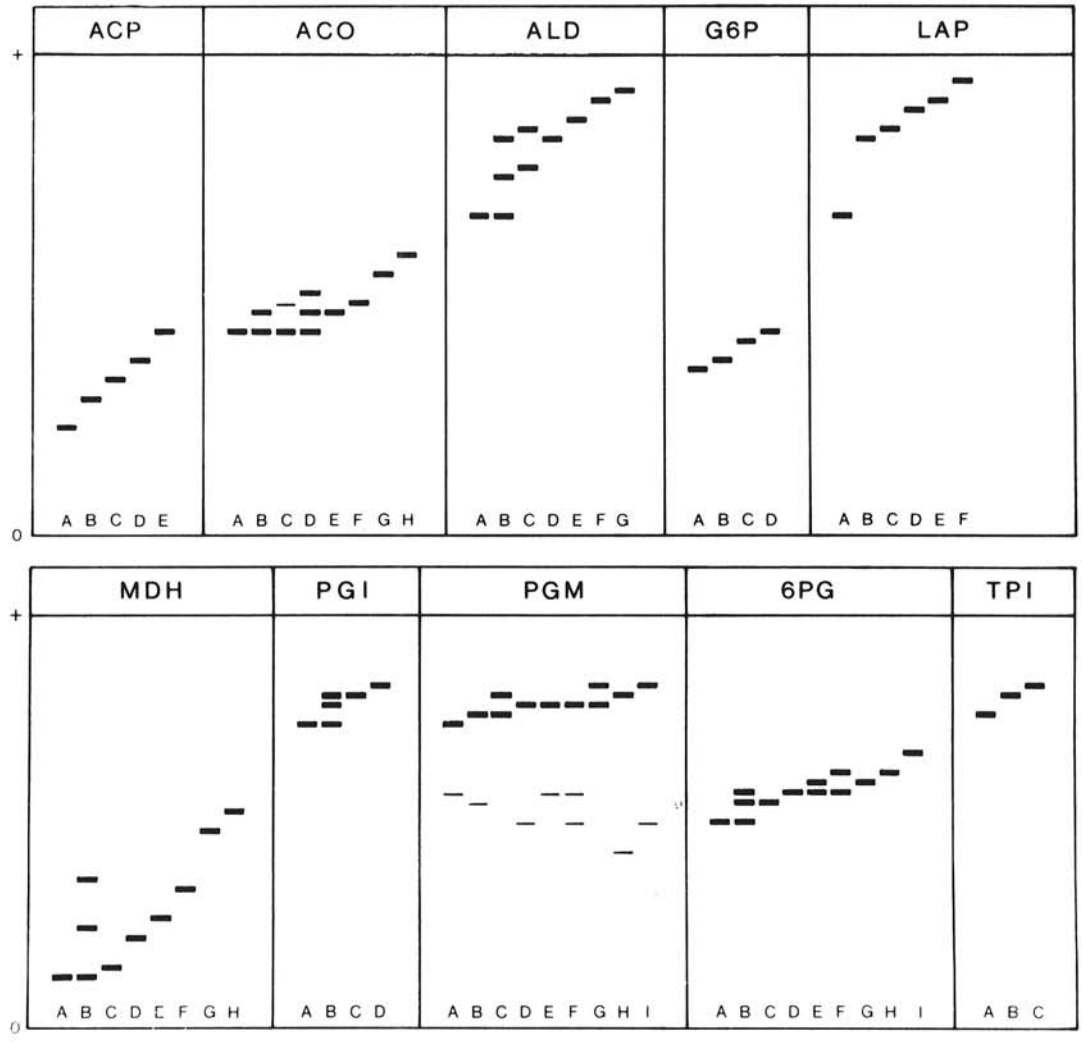

Fig. 1. Electromorphs of 10 enzyme systems found in 219 isolates of *Acremonium* or *Epichloë*. Electromorphs indicated by figures A-I. ACP = acid phosphotase, ACO = aconitase, ALD = aldolase, G6P glucose-6-phosphate dehydrogenase, LAP = leucine aminopeptidase, MDH = malate dehydrogenase, PGI = phosphoglucose isomerase, PCM = phosphoglucomutase, 6PG = 6-phosphogluconate dehydrogenase, TPI = triosephosphate isomerase.

multiple bands remains doubtful. If each band represents the product of a single locus, isolates exhibiting multibanded electromorphs may possess multiple copies of the gene with different allozymes through heterokaryosis and/or aneuploidy. Multibanded electromorphs were found primarily in asexual *Acremonium* endophytes, where gross chromosomal abnormalities interfering with meiosis would be of no consequence. For example, two isolates of sample 8811 from *Festuca obtusa* Spreng. exhibited electromorphs with three bands for malate dehydrogenase (MDH, electromorph B) and phosphoglucose isomerase (PGI, electromorph B). All isolates of *A. coenophialum* from tall fescue except those from cultivar Triumph exhibited electromorph B for PGI along with isolates from *F. obtusa*, *Poa sylvestris* A. Gray, and *P. wulfii* Scribn. (Table 2). Certain electromorphs of the enzymes ACO, ALD, and 6PG exhibited a more complicated pattern where both two and three bands were found (Fig. 1), but only in a subset of all isolates. Electromorph B of aldolase (ALD) was observed in all isolates of *A. coenophialum* from tall fescue but in very few other isolates.

Every isolate examined in this study exhibited a two-banded electromorph of phosphoglucomutase (PGM), typically consisting of a dark-staining anodal band and a fainter, cathodal band (Fig. 1). The fainter bands were well separated from the more intense bands and were found in the majority of isolates. Two PGM isozymes were also found in *Atkinsonella hypoxylon* (Peck) Diehl, a fungus closely related to *E. typhina* (15). A faint band was also observed in electromorph C of aconitase (ACO), which was found only in one of two phenotypes of *A. coenophialum*.

Summed over all isolates and all enzymes, there were 63 distinct electromorphs detected in this study. They were found in 47 different combinations or phenotypes. The phenotypes are presented in Table 2 by host species and isolate number. Multiple isolates from a single host species always exhibited electrophoretic variation with the exceptions of *Lolium perenne* L., where six isolates of *Acremonium lolii* Latch et al were obtained from one commercial cultivar, and *Glyceria striata* (Lam.) Hitchc., where two samples of *Epichloë typhina* from Canada and New York State were identical (Table 2). Isolates of *A. coenophialum* from tall fescue were noteworthy in that 47 of 52 isolates collected from a large number of different populations and from commercial and experimental seed lines exhibited the same isozyme phenotype (Table 2). A second phenotype was found in five isolates from cultivar Triumph. This phenotype (isolate number 8907) differed from the other phenotype from tall fescue at eight of 10 enzymes (Table 2) but differed from the phenotype of *A. lolii* from perennial ryegrass at only five of 10 enzymes.

The low level of variation in *A. coenophialum* contrasts with the variation observed in seedborne *Acremonium* endophytes from wild grasses. For example, six phenotypes were detected among 14 isolates, including multiple isolates from the same site, from *F. obtusa*, a congener of tall fescue (Table 2). Similarly, multiple isozyme phenotypes were detected in isolates from *Poa*

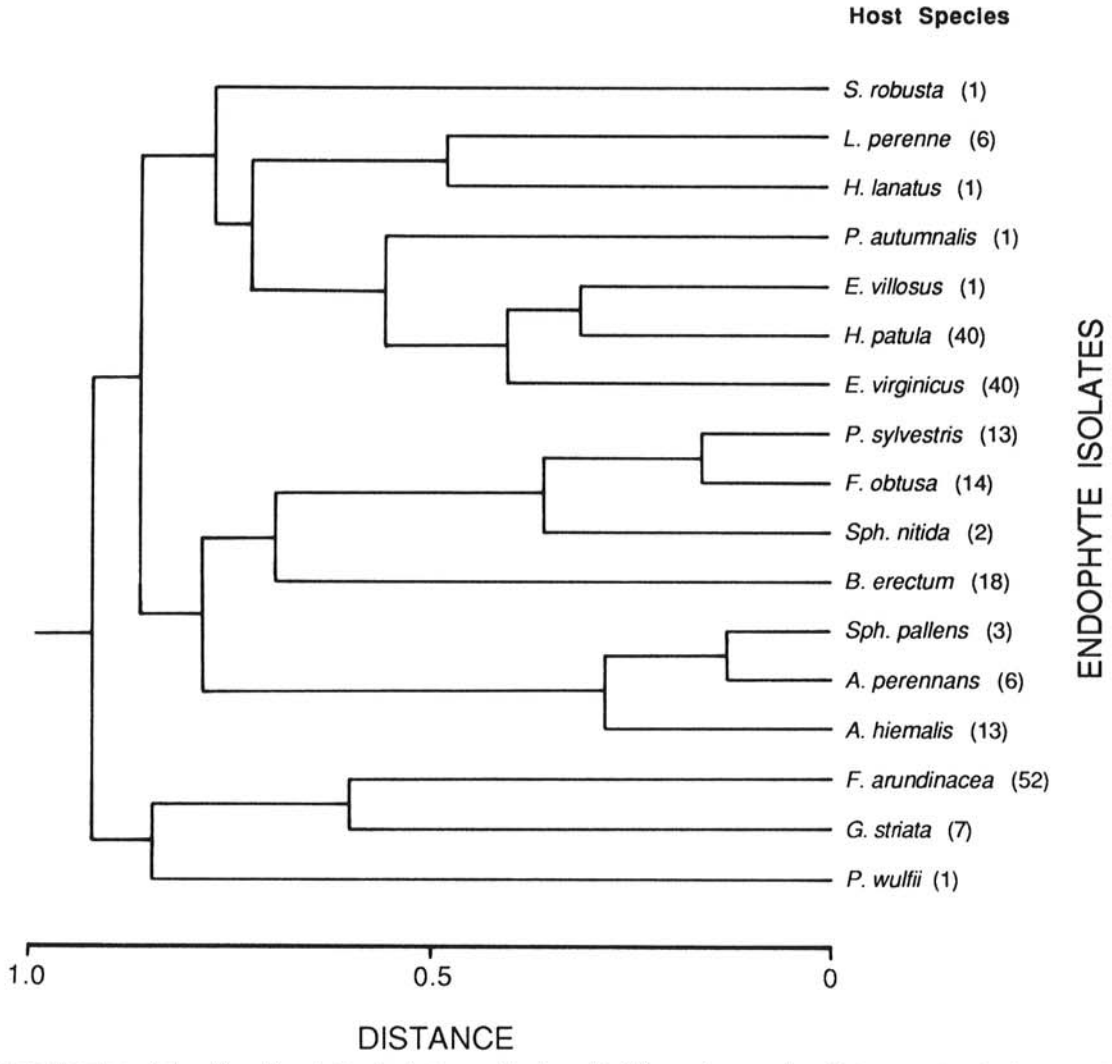

Fig. 2. Dendrogram showing relationships of pooled endophyte samples from 17 different host species of *Agrostis, Brachyelytrum, Elymus, Festuca, Glyceria, Holcus, Hystrix, Lolium, Poa, Sphenopholis,* and *Stipa* (number of endophyte isolates in parentheses). The average linkage method of clustering was used based on Pearson correlation coefficients.

sylvestris, *Sphenopholis nitida* (Spreng.) Scribn., and *S. pallens* (Spreng.) Scribn. infected by symptomless *Acremonium* endophytes (Table 2).

Isolates of *Epichloë* (from grasses bearing characteristic stromata) also varied within a host species except for two isolates from *Glyceria striata* (Table 2). Multiple phenotypes were found in isolates from *Agrostis hiemalis* (Walt.) BSP., *A. perennans* (Walt.) Tuckerm., *Elymus virginicus* L., and *Hystrix patula* Moench collected from individual sites (Table 2). A different phenotype was found in isolates from *Brachyelytrum erectum* (Schreb.) Beauv. from each of three sites; however, all isolates from a site were identical (Table 2).

A cluster analysis based on the Pearson correlation coefficient was performed to provide insights into the relationships among *Acremonium* and *Epichloë* endophytes from different host grasses. Results are presented in Figure 2. Three clusters with distances of less than 0.5 linking isolates from different host species were evident. One included the *Agrostis* species and *Sphenopholis pallens*, the second included *S. nitida*, *F. obtusa*, and *P. sylvestris*, and the third included the two *Elymus* species and *H. patula* (Fig. 2). Isolates from different hosts that exhibited distances greater than 0.5 from isolates from the most closely linked host included the endophytes of *Stipa robusta* Scribn., *L. perenne*, *Holcus lanatus* L., *Brachyelytrum erectum*, *F. arundinacea* Schreb., *Glyceria striata*, and *P. wulfii* (Fig. 2). Isolates from *Stipa robusta* and *P. wulfii* were the most divergent endophytes examined.

There was no tendency for isolates identified as *E. typhina* to cluster together, separately from *Acremonium* spp. In many cases, an *Epichloë* isolate clustered more closely with an *Acremonium* isolate than another *Epichloë* isolate, and vice versa. Considering congeneric hosts, sometimes isolates clustered closely (e.g., *Agrostis, Elymus*), whereas in other congeneric hosts the isolates were quite different (e.g., *Festuca, Poa*).

DISCUSSION

Several significant conclusions arise from the analysis of isozyme variation in fungal endophytes of the *Acremonium/Epichloë* complex. Endophytes exhibit biochemical variation among isolates within a single host population, among isolates from different populations of the same host species, and among isolates from different host species. Moreover, variation of endophytes within hosts and similarity among hosts were often unrelated to their taxonomy and reproductive system. The endophyte of tall fescue, *A. coenophialum*, appears genetically depauparate compared to most other endophytes based on the 10 selected enzymes.

Only two isozyme phenotypes were observed in 52 isolates of *A. coenophialum*. One was found in the majority of isolates from wild populations and several cultivars, and the second was found in the five isolates from cultivar Triumph. The divergent phenotype from cultivar Triumph had strong similarities to *A. lolii* from perennial ryegrass, although this cultivar does not have perennial ryegrass in its breeding history (M. Siegel, *personal communication*). In a cluster analysis (not shown) this phenotype was grouped together with isolates of *A. lolii* in the same cluster. Although only six isolates of *A. lolii* from perennial ryegrass were examined, they too exhibited only a single phenotype. Commercial endophyte-infected grasses subject to strong artificial selection may contain only a limited number of endophyte genotypes as a result. New endophyte strains introduced into grasses may be more likely to exhibit variation in their characteristics if the endophytes are isolated from other wild grasses, rather than domesticated tall fescue (or perennial ryegrass) plants, thus providing a diversity of germ plasm for breeding and plant improvement.

Although isolates of *A. coenophialum* showed little variation, they exhibited several unusual biochemical features. In phosphoglucose isomerase (PGI) there was always a triple band evident, suggesting that there are multiple copies of the locus with different allozymes. Multiple copies of the gene may have resulted from heterokaryosis or aneuploidy. As *A. coenophialum* has probably dispensed with sexual reproduction, such nuclear or chromosomal abnormalities would not interfere with meiosis. Triple-banded PGI electromorphs were found only in a few other isolates of different, but also asexual, endophytes. Similarly, a triple-banded electromorph in aldolase (ALD) was present in the tall fescue endophytes and two other *Acremonium* isolates. An additional faintly staining band for aconitase (ACO) occurred only in isolates from tall fescue cultivar Ky-31. A thorough genetic and molecular analysis of the tall fescue endophyte may reveal more unusual features.

The relative uniformity of isolates of *A. coenophialum* contrasts strongly with the amount of isozyme variation observed in the congeneric host *F. obtusa* collected from several wild, woodland populations. Among 14 isolates from five different sites, six distinct phenotypes were observed. Isolates from different sites were always different, and there was variation among isolates from one of the sites. Like *A. coenophialum*, the endophyte of *F. obtusa* has not been observed to produce stromata and so is presumed to be completely asexual and seedborne.

Considering all endophytes examined in this study, it is apparent that there is an abundance of isozyme variation present in most enzymes. Variation in isozyme phenotype was observed among isolates collected from within single host populations for four host species. It should be recognized, however, that detection of variation at this level will depend strongly on the number of isolates sampled and the particular enzymes used. For wild grasses, with two exceptions, isolates from different host populations always differed from each other. The exceptions were isolates of *E. typhina* from *G. striata* and two populations of *P. sylvestris*. In contrast, endophyte isolates from tall fescue and perennial ryegrass were generally identical. Most significantly, the same isozyme phenotypes were never detected in endophytes from different hosts, with the exception of *E. virginicus* and *H. patula*, two closely related species collected from the same site. Sexual reproduction in *Epichloë* would result in recombination of specific genotypes so that it is perhaps surprising to find that the same isozyme phenotypes often occurred in different isolates from the same host population and in different host populations.

The cluster analysis provided an indication of relationships among endophytes from different hosts. Three clusters each with three host species exhibited greater similarity of isozyme phenotypes than other combinations of host specific endophytes (Fig. 2). Within each cluster, the host grasses were often sympatric in southern Indiana habitats and were often closely related taxonomically (e.g., two *Agrostis* and *Elymus* species). *H. patula* has sometimes been considered congeneric with *Elymus* (10). However, isolates from some hosts that clustered closely together were from more distantly related host genera (e.g., *Festuca* and *Poa*).

Similarity of endophytes from sympatric species might be expected if ascospores of *Epichloë* spread contagiously among co-occurring populations. This could happen among the *Elymus* and *Hystrix* populations, and among the *Agrostis* and *Sphenopholis* populations, where all six species have been observed to bear *Epichloë* stromata. Cross compatibility among *Epichloë* strains from the two *Elymus* species and *Hysterix* with all three host plants has been recently demonstrated in inoculation experiments (Leuchtmann, *unpublished*). However, this explanation is less tenable for *F. obtusa* and *P. sylvestris*, where stromata have never been observed on *F. obtusa* (although reported by Kohlmeyer and Kohlmeyer [12]), and on only one plant of *P. sylvestris* (9). However, stroma-producing endophytes of *P. sylvestris* and *F. obtusa* may be different from the host-specific asexual endophytes.

The results presented here indicate that often *Acremonium* isolates were more similar to *Epichloë* isolates than other *Acremonium* isolates, and vice versa. These findings support the idea that asexual, seedborne *Acremonium* species are anamorphs of *Epichloë* spp. with lost or unknown teleomorphs (7,25). Consequently, these *Epichloë* teleomorphs of known *Acremonium* species could be named differently from *E. typhina*. The lack

of stroma formation by *A. coenophialum* infecting tall fescue (and *A. lolii* infecting perennial ryegrass) may reflect the limited number of endophyte genotypes present in each host, resulting from their artificial breeding and selection. Stromata might be produced in their native range where a greater diversity of endophyte genotypes could occur.

Wild, endophyte-infected grass populations represent repositories of genetic diversity of endophyte strains for grass breeding and improvement. In addition, while isozyme loci may be unrelated to other genes controlling important endophyte functions, such as the regulation of alkaloid production, they are thought to represent a random cross section of structural gene loci. As such, the isozyme differences illustrated in this study may provide useful markers for grass breeding efforts and for inferring phylogenetic relationships among fungal endophytes of grasses.

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