Genetics of Host Specificity in *Epichloë typhina*

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ABSTRACT

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Epichloë typhina perennially and systemically infects grass plants, causing choke disease in which maturation of host inflorescences is suppressed. In seedling-inoculation tests, isolate E8 from perennial ryegrass established and maintained infection in this host but not in orchardgrass. In contrast, isolates E469, E2466, and E2467 from orchardgrass varied in infection frequency and stability in orchardgrass, but all were unable to establish stable infections in perennial ryegrass. To investigate the genetics of host specificity, isolate E8 was crossed with each of the isolates from orchardgrass. Seedlings of parental host species were inoculated with F₁ progeny, and the frequencies of seedling infection and stability in adult plants were assessed. In the E8 × E2466 cross, the F₁ progeny exhibited a wide range of infection frequency and stability in each parental host. In crosses E8 × E469 and E8 × E2467, where the orchardgrass-derived parents infected 5 to 13% of inoculated perennial ryegrass seedlings, the distributions of infection frequencies for the F₁ progeny were skewed toward levels comparable to that of the parent from perennial ryegrass. In all crosses, most progeny had low frequencies of infection in

orchardgrass. However, transgression was evident in a cross of E8 with E469, an isolate that infected orchardgrass seedlings at a low frequency (2 to 3%). The E8 × E469 cross had a few F₁ progeny that infected orchardgrass at high efficiency (up to 81%). A Spearman rank correlation applied to the E8 × E2466 progeny indicated a significant negative correlation between infection frequencies in perennial ryegrass and orchardgrass. Also, there was a significant correlation of infection frequency and stability in perennial ryegrass but not in orchardgrass. To test whether only a few genes governed infection frequency in perennial ryegrass, an E8 × E2466 F₁ progeny (designated E386.04), which had intermediate compatibility with this host, was backcrossed to E8. The progeny of this backcross exhibited a distribution of infection frequencies in perennial ryegrass between that of E386.04 and the backcross parent, suggesting that multiple genes may determine compatibility at the seedling infection stage. The results of these experiments indicated multiple genetic determinants of compatibility or incompatibility with each host, with intermediate or high heritability.

Additional keywords: endophytes, genetic segregation, host compatibility, oligogenic traits, symbiosis.

Epichloë typhina (Pers.:Fr.) Tul. (Ascomycotina: Clavicipitaceae), other *Epichloë* species, and closely related asexual species of form genus *Neotyphodium* (= *Acremonium* section *Albo-lanosa*) are symbionts ("endophytes") of C3 grasses (subfamily Pooideae) (20). These fungi infect host embryos and seeds either vertically via the infected mother plant (19) or horizontally (contagiously) via ascospores (6) and grow in plant apoplasts primarily in shoot apical meristems and leaf sheaths. *Epichloë* and *Neotyphodium* spp. infections persist for the life of the plant and have little or no negative effects on the vegetative tissues of their hosts (8). However, initiation of inflorescence development can trigger the fungal sexual cycle and contagious spread. This developmental stage is termed "choke disease" because it arrests development of the affected host inflorescence. Often only a minority of the inflorescences exhibit choke disease, and the host still produces numerous seeds in which the symbiont is transmitted vertically. However, in some associations, the fungus affects all host inflorescences and is not transmitted vertically in the seeds of rare fertile florets (6).

The *Epichloë* sexual cycle commences with an ectophytic growth stage (stroma), surrounding the host flag-leaf sheath and immature inflorescence. *Epichloë* species are heterothallic, with a bipolar mating system (28). In natural ecosystems, a symbiotic fly, *Phorbia phrenione* Ségué, mediates transfer of spermatia between stromata of opposite mating types. When ascospores are mature, they are ejected through the ostioles of the perithecia, initiating infections of florets of nearby plants and, ultimately, their seeds (6). All crosses between opposite mating types cause perithecia to

form but not all result in production of viable ascospores. The sexual cycle can be mimicked with conidia from fungal cultures as spermatia (16). Furthermore, the natural infection route can be bypassed by inoculation of wounded seedlings (13). These techniques permit a Mendelian analysis of *Epichloë* interactions with host species.

Grass species have not been reported to exhibit resistance to their natural *Epichloë* or *Neotyphodium* symbionts, but artificial infections by symbionts of related species often fail or elicit host responses and are unstable (14,15). Even when novel symbioses can be established, ultrastructural changes in the host suggest a defensive response that is not characteristic of natural symbiont-host combinations (4,12). Recent identification of *Epichloë* mating populations (biological species) and characterization of their host ranges (16), together with the application of Mendelian genetics (5), provide the opportunity to elucidate the genetics of *Epichloë* host compatibility and incompatibility. This paper reports a genetic analysis of *E. typhina* genotypes specific for perennial ryegrass and orchardgrass and provides evidence for multiple genetic determinants of compatibility and incompatibility with the host species.

MATERIALS AND METHODS

Biological materials and their maintenance. *E. typhina*-infected grass plants were maintained indefinitely in the greenhouse by regular repotting of ramets. Plants were grown in a 3:1 mixture of commercial potting mix (Pro-mix BX, Premier Brands, Inc., Red Hill, PA) and soil and were trimmed and fertilized regularly. To prevent their release, imported strains were handled under the conditions specified by a permit from the U.S. Department of Agriculture, Animal and Plant Health Inspection Service, requiring

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that access to the greenhouse and growth facilities was restricted, all discarded plant material was steamed, and greenhouse tools were dipped in 70% ethanol after each use.

E. typhina isolate E8 (mating type *mat-1*) (3) was cultured from systemically infected perennial ryegrass. Although the infected grass was identified in the United States, all known *E. typhina* hosts, including perennial ryegrass, are Eurasian (22). Therefore, isolate E8 probably represents an accidental introduction from Europe. Isolates E2466, E2467, and E469 (all of mating type *mat-2*) were from natural ecotypes of orchardgrass and were collected in Switzerland by D. Schmidt (Federal Agricultural Research Station, Nyon, Switzerland). These isolates were from surface-sterilized stems and leaf sheaths of infected plants and were subsequently single-spore isolated (21). Genetic crosses utilized the E8-infected perennial ryegrass plants from earlier studies (24). Although isolate E8 had not been single-spore purified beforehand, analysis of alleles at 15 loci indicates it represents a single genotype (16,23).

Crosses. Almost all crosses were conducted on stromata of *E. typhina* strain E8 on perennial ryegrass plants. Infected plants were vernalized to initiate development of inflorescences and the associated stromata. A Percival (Boone, IA) model PT-80 growth chamber, with maximum fluorescent and incandescent lighting, was programmed for the following regime: 14 days of 18.3 and 7.2°C day and night temperatures, respectively, and an 11-h photoperiod; 7 days of 12.8 and 1.6°C day and night temperatures, respectively, and a 10-h photoperiod; 12 days of 7.2 and 1°C day and night temperatures, respectively, and a 9.5-h photoperiod; 21 days of 3 and -1°C day and night temperatures, respectively, and a 9-h photoperiod; 7 days of 7.2 and 1°C day and night temperatures, respectively, and a 9.5-h photoperiod; 7 days of 12.8 and 1.6°C day and night temperatures, respectively, and a 10-h photoperiod; and 7 days of 18.3 and 7.2°C day and night temperatures, respectively, and a 12-h photoperiod. Four equally spaced 25-min defrost cycles were included throughout. Plants vernalized in this regime were repotted in the greenhouse and grown untrimmed until inflorescences or fungal stromata emerged.

To initiate crosses, stromata were rubbed with cotton swabs soaked in suspensions of cultured conidia and hyphae of the spermatial parent (16). Plants with fertilized stromata were placed in growth chambers (21°C, 12-h photoperiod) with positive air flow through HEPA filters to prevent environmental release of ascospores. Beginning 3 weeks after mating, perithecia were squashed in aniline blue solution (7) and examined by light microscopy for ascospores. Once mature ascospores were evident, the stromata were mounted on the lids of overturned water agar plates. Ascospores ejected onto the water agar germinated in 1 to 3 days, directly producing conidiogenous cells (2). Conidiospores from individual germinated ascospores were visualized with a dissecting microscope and transferred on the edge of a scalpel blade to potato dextrose agar plates. All ascospore-derived cultures subsequently were streaked three times to ensure their purity, and mycelium and spores were suspended in 50% glycerol and stored at -80°C

TABLE 1. Infection frequency and stability of *Epichloë typhina* parental isolates in perennial ryegrass and orchardgrass

Isolate	Perennial ryegrass			Orchardgrass		
	<i>n</i> ^x	% ^y	Stability ^z	<i>n</i> ^x	% ^y	Stability ^z
E8	261	58	H	108	0	nd
E2466	112	0	nd	96	60	H
E2467	58	5	U	70	59	M
E469	44	13	U	51	2	H

^x Number of inoculated seedlings examined for infection.

^y Percentage of seedlings examined that were infected 8 to 10 weeks postinoculation.

^z Assessed 8 months postinoculation for five plants of each association; H = high stability (associations maintained in all plants); M = moderate stability (loss of associations in some plants); U = unstable (loss of associations in all plants); nd = not determined.

(21). Each cross is designated by the formula stromal (maternal) parent × spermatial (paternal) parent. Crosses between stromata of *E. typhina* E8 and spermatia of isolates from orchardgrass produced numerous viable ascospores, but reciprocal crosses almost always failed. In 21 attempts on six *E. typhina*-infected orchardgrass plants, only one progeny was obtained. This progeny was from the E469 × E8 cross. The F₁ progeny E386.04, from E8 × E2466, initiated stromata in orchardgrass and was crossed with spermatia of E8. Progeny E386.04 also was used as a spermatial parent backcrossed to E8.

Isozyme analysis. To confirm parentages of the F₁ progeny, four enzymes representing five isozyme loci were analyzed by horizontal starch gel electrophoresis: diaphorase (EC 1.6.4.3), phosphoglucose isomerase (EC 5.3.1.9), phosphoglucomutase (EC 5.4.2.2), and leucine aminopeptidase (LAP, EC 3.4.11.1). Methods and locus designations were described by Leuchtman (14). Segregation ratios were compared to expected ratios (1:1 for a single locus in a haploid organism) by chi-square with Yates' correction for small sample size (25).

Inoculations. As a measure of compatibility of parental isolates and ascospore progeny with seedlings and young plants of each host species, seedlings were inoculated by the procedure of Latch and Christensen (13) with modifications. Seeds of perennial ryegrass cv. Gator and orchardgrass cv. Rapidio, from International Seeds, Inc. (Halsey, OR), were stored at 4°C until needed. Seeds were soaked in 50% sulfuric acid for 10 to 20 min, rinsed three times with sterile water, soaked in 50% commercial bleach (2.6% sodium hypochlorite) for 10 to 20 min, and rinsed again. Treated seeds were germinated in the dark on 1.5% Bacto agar (Difco Laboratories, Detroit) for 1 week at 21°C. The shoot meristem was visualized (using a dissecting microscope) as a slight swelling

TABLE 2. Segregation of isozyme alleles in progeny of *Epichloë typhina* E8 × E2466^x

Isolate ^y	PGM-1	PGM-2	PGI	DIA	LAP
E8	a	a	a	a	a
E2466	b	b	b	b	b
E386.01	b	b	b	a	a
E386.03	b	b	b	a	b
E386.04	a	a	a	a	a
E386.13	b	a	a	b	a
E386.14	a	b	b	b	b
E386.15	b	a	b	b	a
E386.16	a	a	b	a	b
E386.17	b	a	b	b	a
E386.23	a	a	a	b	b
E386.27	a	b	b	a	a
E386.38	b	b	b	a	a
E386.46	b	b	b	b	a
E386.49	a	b	a	b	b
E386.50	b	a	a	b	a
E386.51	a	b	a	a	a
E386.52	b	b	a	a	b
E386.53	a	a	a	a	a
E386.54	b	a	b	a	a
E386.55	a	b	a	a	b
E386.62	b	a	a	b	a
E386.65	b	b	b	b	a
E386.66	b	b	b	b	a
E386.67	b	b	a	b	a
E386.70	b	b	b	a	a
E386.71	a	b	a	b	b
E386.73	a	b	b	b	a
E386.75	a	b	a	b	a
Segregation ratio	12:15	10:17	13:14	12:15	19:8
Probability ^z	0.56	0.18	0.85	0.56	0.03

^x Alleles designated based on isozyme migration in starch gel relative to those of parent strain E8 (a) or E2466 (b).

^y Parental isolates are E8 (female) and E2466 (male); progeny are designated E386.01 through E386.75.

^z Based on Yates' correction of chi-square with 1 df.

at the junction of the mesocotyl and coleoptile. An insect pin was used to introduce wounds just above and below the meristem, and a piece of actively growing fungal mycelium was placed over each wound. Inoculated seedlings were incubated for an additional week in the dark to allow the fungus to grow into the wound sites. The seedlings were kept under fluorescent light (12-h photoperiod) at 21°C for 2 to 3 days, after which they were gently lifted out of the agar, planted in 3:1 potting mix/soil, replaced in the lighted incubator for 2 weeks, and transferred and maintained in the greenhouse for 8 to 10 weeks until multiple tillers were produced. The bases of two vegetative tillers from each plant were assayed for *E. typhina* infection by tissue-print immunoblot (10), using antiserum prepared by An et al. (1). Up to five plants infected with each isolate or progeny were repotted and maintained in the greenhouse for an additional 6 months and then retested to assess stability of the fungal infections.

At least 10 seedlings were inoculated with each parent or progeny isolate. Although some seedlings died before transplantation, at least seven of the inoculated seedlings survived in almost every case. Parental strains were included as controls in each infection trial and care was taken that cultures used as inoculum were not waxy variants; such variants arise spontaneously, produce no aerial hyphae or conidia, and have reduced infectivity (4). Infection frequency was determined as the number of infected plants over the total number of inoculated plants tested for infection. If all plants with an isolate maintained their infections up to 8 months postinoculation, the isolate-host species association was considered highly stable; loss of infections from some but not all previously infected plants was considered moderately stable; and if all infections with an isolate were lost, the association was considered unstable. Assessments of stability in vegetative and vernalized plants were controlled with perennial ryegrass cv. Gator and orchardgrass cv. Rapidio plants infected with parental isolates.

Alkaloids and mating types. Production of peramine in planta was determined by thin-layer chromatography (9). Mating types were determined by test crosses as in Leuchtmann (14).

RESULTS

Host specificity of parental isolates. Tests of seedling infection frequency and stability indicated various degrees of host specificity among an *E. typhina* isolate from perennial ryegrass (isolate E8) and three isolates from orchardgrass (E469, E2466, and E2467). Isolate E8 infected perennial ryegrass seedlings with high frequency but failed to infect orchardgrass, and two of the three isolates from orchardgrass infected orchardgrass much more frequently than they infected perennial ryegrass (Table 1). Two isolates from orchardgrass (E469 and E2467) infected perennial ryegrass at low frequency and were unstable in this host, whereas a third (E2466) never infected perennial ryegrass. The isolates from orchardgrass differed in infection frequency and stability in their host of origin, although there was no apparent relationship between the infection frequency and stability of each isolate in orchardgrass.

Infection frequency and stability of F₁ progeny in parental host species. Parental isolates differing in host specificity were crossed, and ascospore progeny were collected. To confirm that the progeny were derived meiotically, segregation of alleles for five unlinked isozyme loci was tested for 27 progeny from the E8 × E2466 cross (Table 2). Segregation at four loci was not significantly different from 1:1. A fifth polymorphic locus, *LAP*, segregated with a ratio (19:8) significantly different from 1:1 ($P = 0.03$). These results demonstrated Mendelian segregation of alleles in F₁ progeny, although the *LAP* result demonstrated the possibility of biased transmission of some genes.

In total, 80 progeny from the E8 × E2466 cross were used to inoculate perennial ryegrass and orchardgrass seedlings (Table 3; Fig. 1A and B). Infection frequencies among the progeny ranged from 0 to 100% in perennial ryegrass and from 0 to 71% in or-

TABLE 3. Infection frequency and stability in perennial ryegrass and orchardgrass of progeny from the cross of *Epichloë typhina* strains E8 and E2466

Progeny number	Perennial ryegrass			Orchardgrass		
	<i>n</i> ^x	Infection freq. (%) ^y	Stability ^z	<i>n</i> ^x	Infection freq. (%) ^y	Stability ^z
E386.01	61	41	5/5	50	0	nd
E386.02	53	15	5/5	54	0	nd
E386.03	50	16	5/5	23	4	0/1
E386.04	53	43	0/5	34	3	1/1
E386.05	16	81	3/3	13	0	nd
E386.06	20	65	3/3	13	0	nd
E386.07	19	26	2/3	20	0	nd
E386.08	22	0	nd	22	14	2/3
E386.09	15	80	3/3	23	0	nd
E386.10	20	70	3/3	13	0	nd
E386.11	24	0	nd	14	0	nd
E386.12	23	22	2/5	15	40	4/5
E386.13	13	92	5/5	22	9	2/2
E386.14	29	10	0/3	28	0	nd
E386.15	22	59	5/5	27	0	nd
E386.16	22	18	0/4	22	59	4/5
E386.17	13	92	3/3	18	6	1/1
E386.18	10	90	3/3	18	0	nd
E386.19	15	40	3/3	14	29	2/4
E386.20	11	64	3/3	17	12	2/2
E386.21	11	27	3/3	17	0	nd
E386.22	11	18	2/2	19	58	3/4
E386.23	16	63	0/3	21	5	1/1
E386.24	12	8	0/1	24	21	0/3
E386.25	13	85	3/3	20	0	nd
E386.26	10	10	nd	22	23	3/3
E386.27	19	47	5/5	16	31	2/4
E386.28	7	86	3/5	23	0	nd
E386.29	10	40	2/2	26	23	1/3
E386.30	13	54	3/3	19	68	1/4
E386.31	8	13	0/1	13	46	3/3
E386.32	14	14	1/2	18	67	3/3
E386.33	8	0	nd	18	0	nd
E386.34	11	36	0/2	17	6	0/1
E386.35	11	18	0/2	21	24	3/3
E386.36	13	54	3/3	18	22	nd
E386.37	12	50	3/3	17	71	4/7
E386.38	13	77	3/3	15	13	2/2
E386.39	10	50	5/5	14	14	2/2
E386.40	15	13	2/2	16	0	nd
E386.41	9	78	2/2	18	0	nd
E386.42	7	0	nd	22	0	nd
E386.43	10	40	2/3	15	13	0/2
E386.44	12	58	1/2	17	0	nd
E386.45	10	80	2/2	20	0	nd
E386.46	14	21	1/3	17	6	0/1
E386.47	10	40	3/3	15	0	nd
E386.48	12	42	3/3	19	5	0/1
E386.49	10	50	0/5	24	0	nd
E386.50	22	41	5/5	22	0	nd
E386.51	23	35	5/5	25	4	0/1
E386.52	24	50	0/5	33	39	2/5
E386.53	14	14	0/2	18	22	0/4
E386.54	14	43	5/5	18	0	nd
E386.55	16	38	2/5	12	0	nd
E386.56	17	47	5/5	19	11	nd
E386.57	8	100	3/3	18	0	nd
E386.58	8	0	nd	17	71	2/4
E386.59	11	18	0/2	19	0	nd
E386.60	10	10	0/1	14	43	2/3
E386.61	12	75	2/2	18	0	nd
E386.62	10	90	2/2	19	0	nd
E386.63	12	0	nd	19	58	2/5
E386.64	12	8	0/1	16	0	nd
E386.65	52	19	0/5	49	10	0/5
E386.66	9	44	2/2	22	0	nd
E386.67	19	47	3/3	16	0	nd
E386.68	12	0	nd	16	6	0/1
E386.69	22	59	2/5	22	0	nd
E386.70	13	0	nd	18	6	0/1
E386.71	23	52	5/5	24	0	nd
E386.72	24	8	0/2	19	16	0/3
E386.73	17	24	0/4	13	8	1/1
E386.74	11	18	0/2	21	10	2/2
E386.75	19	32	0/5	24	13	2/3
E386.76	9	56	3/3	21	14	0/3
E386.77	11	18	2/2	20	5	0/3
E386.78	11	9	1/1	18	61	3/3
E386.79	11	91	3/3	22	9	2/2
E386.80	14	71	5/5	18	0	nd

^x Number of inoculated seedlings surviving to test.

^y Percentage of plants infected 8 to 10 weeks postinoculation.

^z Persistence in greenhouse conditions over 8 months; number of infected plants over number of plants tested.

chardgrass. Seventy-two of the eighty progeny were able to infect perennial ryegrass seedlings at various frequencies. Forty-five progeny infected orchardgrass, five of which (numbers 8, 58, 63, 68, and 70) failed to infect perennial ryegrass. Thirty-five of the eighty progeny failed to infect orchardgrass, and of these, three (numbers 11, 33, and 42) also failed to infect perennial ryegrass. Only 13 progeny infected orchardgrass with efficiencies greater than 35%. Because *E. typhina* is haploid, the fact that most progeny infected parental hosts at frequencies intermediate to those of the parents indicated that in *E. typhina* multiple genes are likely to be involved in this measure of host compatibility.

A total of 49 progeny from the crosses $E8 \times E469$, $E469 \times E8$, and $E8 \times E2467$ also were assessed for compatibility with the two host species. $E8 \times E469$ and $E8 \times E2467$ progeny infected per-

ennial ryegrass at various frequencies, with distributions skewed toward that of E8 (Fig. 1C and E). Most progeny had low infection frequencies in orchardgrass. However, the $E8 \times E469$ cross resulted in transgression, whereby a minority of the progeny infected orchardgrass seedlings at a much higher frequency than did either parent (Fig. 1F). The single progeny from $E469 \times E8$ (this cross was nearly infertile) behaved similarly to most $E8 \times E469$ progeny, being highly compatible with perennial ryegrass but unable to infect orchardgrass. Overall, the progeny from the $E8 \times E2466$ cross, in which the parents represented the extremes of compatibility and incompatibility with the two hosts, tended to have the most even distribution of infection frequencies in perennial ryegrass.

The stability of the $E8 \times E2466$ progeny in adult plants of both host species was tested (Table 3). Spearman rank correlations were

Fig. 1. Distribution of infection frequencies of progeny from three *Epichloë typhina* crosses: **A and B**, $E8 \times E2466$, **C and D**, $E8 \times E2467$, and **E and F**, $E8 \times E469$ in perennial ryegrass (**A**, **C**, and **E**) and orchardgrass (**B**, **D**, and **F**). At least 10 seedlings of each host were inoculated near the meristems with parental strains and each progeny and were examined after 2 months by tissue-print immunoblot for successful colonization. The frequencies are indicated by percent, in 10% increments. Infection frequencies of parental strains in each experiment are indicated by the downward arrows, and ranges of infection frequencies in two or more trials are indicated by double-headed arrows.

determined to assess whether there was any genetic relationship between infection frequency and stability or between compatibility with the two host species. There was a significant positive correlation between infection frequency and stability of the F_1 progeny in perennial ryegrass ($P < 0.01$, Spearman rank correlation for 71 observations). However, no significant correlation was observed for infection frequency and stability in orchardgrass. There was also a significant negative correlation ($P < 0.05$, Spearman rank correlation for 80 observations) between frequencies with which progeny infected the two host species. However, stability in the different hosts showed no significant positive or negative correlation.

Variations among F_1 progeny in infection frequency of and stability in each parental host suggest oligogenic or polygenic control of these phenotypes. However, because of the complex experimental process used to establish infection and because stability could not be quantified readily for large numbers of progeny, the results did not preclude the possibility that very few genes are involved in these phenotypes. Additional indications were sought by analyzing backcross progeny.

Compatibility of backcross progeny. If few genes govern infection frequency or stability in either host and if these genes are inherited in a Mendelian fashion, discrete phenotypic classes, including both parental types, should be evident in a backcross involving an F_1 progeny with intermediate compatibility. To test this possibility, reciprocal backcrosses were conducted between parental isolate E8 and an F_1 progeny (E386.04) from the E8 $\times$ E2466

cross. Isolate E386.04 infected perennial ryegrass seedlings with an efficiency intermediate to those of its parents, although it was unstable in this grass (Table 3). In total, 43 backcross (BC_1) progeny from E386.04 $\times$ E8 and 12 from E8 $\times$ E386.04 were tested for infection frequency and stability in each grass species (Fig. 2). The BC_1 progeny infected perennial ryegrass with frequencies distributed from intermediate to high, with a skew toward infection frequencies comparable to E8 (Fig. 2A and C). Within this distribution, there were very few BC_1 progeny with infection frequencies similar to those of E386.04. The results were similar for both reciprocal crosses (Fig. 2, comparison of panels A and C), indicating there was no dramatic maternal or paternal bias in inheritance of genes affecting infection frequency. All of the BC_1 progeny had low infection frequencies in orchardgrass, similar to their parents. However, unlike E386.04, none was stable in orchardgrass.

Other *E. typhina* phenotypes. Among the genetic and phenotypic differences between parental isolates E8 and E2466, their mating types (14), expression of the alkaloid, peramine (9), and mitochondrial plasmid (5) profiles were all unrelated to host specificity.

DISCUSSION

Few genetic studies have been undertaken of host specificity of plant-associated fungi. The phenomenon of host specificity of *E. typhina* with grasses is particularly intriguing because of the very

Fig. 2. Distributions of infection frequencies in **A** and **C**, perennial ryegrass and **B** and **D**, orchardgrass of progeny from reciprocal *Epichloë typhina* backcrosses E8 $\times$ E386.04 (**A** and **B**) and E386.04 $\times$ E8 (**C** and **D**). The parent, E386.04, was one of the progeny from the E8 $\times$ E2466 cross and exhibited an intermediate ability to infect perennial ryegrass and a low infection frequency in orchardgrass. Infection frequencies of parental strains in either host are indicated by downward arrows.

long-term nature of the symbioses and the coordination of host and fungal life cycles. Also, ultrastructural studies indicate that in most natural symbioses there is no discernible host response to symbiotic *Epichloë* species or anamorphs; however, host responses are evident in artificially generated associations between different fungal isolates and host species (12). In some of these “alien” associations, osmophilic materials (perhaps phenolics) accumulate in host cell walls and matrices adjacent to fungal hyphae, and the hyphae vesiculate their endoplasmic reticula and die. The high degree of specificity of *Epichloë*-grass interactions and the host response in novel combinations indicate genetic determinants of compatibility. This study provides evidence that host specificity of *E. typhina* genotypes is heritable and that either oligogenic or polygenic determinants with some likely epistatic interactions govern compatibility with inoculated seedlings.

Parental isolates E8 and E2466 represent phenotypic extremes, in that E8 infected only perennial ryegrass and E2466 infected only orchardgrass. The wide distribution among their progeny of the frequencies with which they infected perennial ryegrass seedlings suggested that at least two genes govern this aspect of host specificity. The histogram (Fig. 1A) suggests a possible trimodal distribution that could indicate the effect of two major genes. A backcross was conducted to test this possibility. The F_1 progeny, E386.04, gave an intermediate infection frequency, so if two loci controlled this trait, then E386.04 most likely inherited the E8 allele of one locus and the E2466 allele of the other locus. If this were the case, only one major gene controlling this phenotype should segregate in the BC_1 generation. Provided that segregation was not heavily biased, the BC_1 progeny would fall into two classes based on the frequencies with which they infect perennial ryegrass seedlings: one class having frequencies similar to those of E386.04 and the other having frequencies similar to those of E8. If three genes segregated in the F_1 generation, the BC_1 progeny could have grouped into three classes. Again, assuming no bias of gene transmission, approximately one quarter of the BC_1 progeny would have been similar to E386.04 in infection frequencies, one quarter would have been similar to E8, and half would have had intermediate infection frequencies. The results were not as expected under either of these models and were particularly inconsistent with the possibility of two genes controlling this phenotype (Fig. 2A and C). There was a distribution of infection frequencies approximately bounded by the frequencies obtained for the parents of the backcross. Particularly under-represented were BC_1 progeny with infection frequencies in perennial ryegrass comparable to those of E386.04.

Possible explanations for the results from the $E8 \times E2466$ cross and the backcross include: (i) the involvement of more than two genes, each with additive or epistatic effects on infection frequencies; (ii) environmental effects masking genetic effects; or (iii) skewing of inheritance patterns for genes controlling this trait. If environment effectively masked genetic effects on this phenotype, then the BC_1 generation likely would have exhibited a broader distribution, with some progeny having lower infection frequencies than E386.04. The possibility of skewed inheritance is suggested by the observation that *LAP* (one of five isozyme loci tested) showed a skewed inheritance in $E8 \times E2466$ progeny. Even so, it would be surprising to have such a skew in infection frequencies among BC_1 progeny with no such tendency apparent in the F_1 generation. Thus, the explanation we favor is that multiple genes control seedling infection frequencies, possibly with epistatic interactions among some or all of them.

The distributions of infection frequencies in perennial ryegrass for $E8 \times E2467$ and $E8 \times E469$ progeny also suggest oligogenic or polygenic determination of this phenotype, probably with epistasis. A skewed distribution of seedling infection frequencies was a characteristic of both crosses (Fig. 1C and E). This skew may have been related to the fact that, unlike E2466, parents E2467 and E469 infected perennial ryegrass seedlings, albeit at a low

frequency. As with the reciprocal $E8 \times E386.04$ backcross, the skewed distributions may indicate bias in transmission of the genes controlling this phenotype, or they may simply be due to the fact that the parents in the crosses exhibited infection frequencies close to the bounds of possible values (0 and 100%).

The genetic complexity of host specificity and likely epistatic interactions are underscored by transgression in the crosses of E8 with E469. Although both parents had low infection frequencies in orchardgrass, occasional F_1 progeny had high infection frequencies in this host. This observation strongly suggests epistatic gene effects. For example, there may have been an unmasking of some genes from isolate E8 that enhance compatibility with orchardgrass, even though this parent apparently was unable to infect orchardgrass. Thus, gene interactions may give rise to host specificities that are not characteristic of the parents. The possible role of gene interactions may explain why individual isolates can be host specific even though *E. typhina* as a biological species has a broad host range encompassing at least six grass genera (22).

For any *E. typhina* genotype to complete its life cycle, it must establish symbiosis, maintain infection throughout the host plant's life, and be able to coordinate its own sexual expression with that of its host. One progeny, E386.04, was successfully induced to develop its sexual stage on orchardgrass, and the $E386.04 \times E8$ progeny were viable and survived in perennial ryegrass. This observation demonstrates the potential for *E. typhina* lineages to arise from hybrids between genotypes with different host specificities. However, a test of the heritability of stroma formation and ability to complete the sexual cycle was impractical, because resources were inadequate to vernalize enough symbiots and controls (orchardgrass with parental strains E469, E2466, and E2467) did not display stromata. The likely reason that stromata rarely were produced on orchardgrass was that the artificial vernalization conditions did not adequately mimic natural conditions, although the possibility was not excluded that orchardgrass cv. Rapidio largely suppresses stroma expression.

These studies did not address another important step in the *E. typhina* life cycle: the natural route of horizontal infection that is ascospore mediated and results in infected seeds (6). Seedling-inoculation experiments may be irrelevant to this initial stage of natural infection but are probably a better reflection of compatibility with host seedlings and young plants (a possibility strongly suggested by the positive correlation of seedling infection frequency with stability in perennial ryegrass). If additional genetic interactions govern natural ascospore-mediated infections, crosses between strains specialized to different hosts should yield a much smaller proportion of ecologically fit progeny than indicated in this study. In a previous investigation, rare infected seeds were obtained by transmission of $E8 \times E2466$ ascospores to florets of perennial ryegrass (6), but it was not feasible to determine how many sibling ascospores failed to initiate floret infections due to genetic incompatibility. Nevertheless, nature likely affords far more opportunities for novel *E. typhina* genotypes to infect various host species, and it also is conceivable that novel genetic combinations may permit the host range of *E. typhina* to expand.

Gene-for-gene interactions often control cultivar specificity within a host species in many host-fungus interactions and have been shown to confer host specificity in some bacterium-host systems (11,17). It has been suggested that gene-for-gene relationships also might control host, as opposed to cultivar, specificity in fungal-plant interactions (18). Recently, both monogenic and polygenic inheritance of host species specificity have been demonstrated in the fungus *Magnaporthe grisea* (27,29), causal agent of blast disease in rice and other grasses. A gene (*PWL2*) conferring host specificity in *M. grisea* has been cloned and shown to act similarly to an avirulence gene, preventing this fungus from infecting weeping lovegrass (*Eragrostis curvula*) (26). Gene-for-gene interactions usually involve localized necrosis of the host tissue at the infection court (the hypersensitive response). In the *E. typhina*-

grass interactions, necrosis of host crown tissue sometimes was observed (data not shown) but was not indicative of host resistance to infection. For example, E2466 and E2467, but not E469, caused necrosis in young, infected orchardgrass; however, all three of these associations were stable. This observation and the lack of discrete segregation classes suggest that although gene-for-gene effects cannot be ruled out they are not principally responsible for host specificity of *E. typhina* genotypes.

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