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A Fungal Endosymbiont of the Grass *Bromus setifolius*: Distribution in some Andean Populations, Identification, and Examination of Beneficial Properties

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Abstract

Endophyte infection levels were estimated in populations of the grass *Bromus setifolius* at several sites in the Andes Mountains in South America. The endophyte was identified as pertaining to the *Neotyphodium tembladerae* clade using sequence data. A high percentage of grass individuals infected by the endophyte was found in populations located in communities that contained leaf-cutting ants (*Acromyrmex* sp.). *N. tembladerae* was found to produce two mycotoxins (ergovaline and peramine) that are known to be toxic to insect herbivores. Feeding experiments demonstrated that fall armyworms preferred endophyte-free *B. setifolius* over endophyte-infected *B. setifolius* when given the option of both. The results of this investigation support the defensive mutualism hypothesis that defense of the host from herbivores is a basis for the mutualistic association between clavicipitaceous endosymbionts and host grasses.

Keywords: Endophyte, *Epichloë*, evolution, fungi, leaf-cutting ants, *Neotyphodium*

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1. Introduction

Endophytic microbes may colonize the interior of plants without eliciting defense responses from host plants or causing disease symptoms (Bacon and White, 2000). The benefits to plants of hosting such endosymbiotic microbes may be numerous. Diazotrophic bacterial endophytes in sugarcane have been shown to fix atmospheric nitrogen that aids hosts to grow in soils low in available nitrogen (Baldani et al., 1997). Seedlings of many plants that are infected by species of *Bacillus* have been shown to have an enhanced resistance to diseases (Hall et al., 1986). Tall fescue seedlings infected by the endophyte *Neotyphodium coenophialum* show enhanced resistance to damping-off disease caused by *Rhizoctonia solani;* while mature plants show increased drought tolerance and resistance to above ground and below ground insect and nematode pests (Gwinn and Gavin, 1992; West et al., 1988). Similarly, several grasses infected by the endophytes *Epichloë typhina, E. festucae,* and *E. clarkii* were found to deter the feeding of migratory locusts; while endophyte-free plants were readily consumed by the locusts (Lewis et al., 1993). It seems evident that plants benefit tremendously from the colonization of endosymbiotic microbes. The benefits to hosting mutualistic microbes likely outweigh losses in terms of nutrient use by the microbes.

The notable effects in increasing resistance of grass hosts to herbivores has resulted in the proposal of the 'defensive mutualism hypothesis' for many of the endophytes in the family Clavicipitaceae; Ascomycetes (including *Balansia, Epichloë,* and *Neotyphodium*) (Clay, 1988; Clay, 1990). The defensive mutualism hypothesis holds that a primary benefit to the host grass is defense against herbivory. Recently, Saikkonen et al. (1998) found that Arizona fescue (*Festuca arizonica*) infected by a *Neotyphodium* endophyte appears to lack insect deterrent properties. This suggests that the defensive mutualism hypothesis is not universal for all clavicipitaceous endophytes. It is apparent that a close scrutiny must be made of newly discovered endophyte associations to determine whether they represent cases of defensive mutualism.

Recently, we discovered and described an endophyte that is endemic to South American grasses (Cabral et al., 1999). In the study reported herein we: 1) examined distribution of an endophyte in the grass *Bromus setifolius* in several populations in the Andes Mountains; 2) examined correlation of the endophyte levels in populations to several community components; 3) examined phylogenetic affinities of the endophyte using rDNA sequence analysis; 4) conducted chemical analyses of infected plants to identify toxic or anti-insect compounds; and 5) conducted insect feeding experiments.

2. Materials and Methods

Isolation of endophytes

For this study endophytes were isolated from several Asian, North American, and South American grasses (Table 2). Seed from endophyte-infected plants was collected, surface disinfected for 30 min in 50% Clorox®, rinsed in sterile water, and plated onto Potato Dextrose Agar (Difco). The typical white cottony colonies of *Neotyphodium* emerged from germinating seed after about five weeks. Cultures were examined and observed to produce phialides and conidia characteristic of *Neotyphodium* (Morgan-Jones and Gams, 1982; White, 1987).

Sequence data

Fresh mycelium of one of several *Epichloë* and *Neotyphodium* isolates was lifted off cellulose acetate sheets on PDA and ground in liquid nitrogen. Genomic DNA was extracted using DNeasy™ Plant Protocol (Quiagen, Inc., Valencia, California). The 5.8S rDNA and flanking internal transcribed spacer regions (ITS1&2) were amplified from 2 µl of undiluted genomic DNA in a 100 µl reaction using the primers ITS5 and ITS4 (White et al., 1990). Each reaction contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 12.5 pmol each dNTP, 50 pmol each primer, and 2 U Taq polymerase (Desai and Pfaffle, 1995). PCR (25 cycles) was carried out in a GeneAmp 9600 thermocycler (Perkin Elmer Corporation, Foster City, California) set to 95°C for 10 s, 56°C for 30 s, and 72°C for 1 min. Initial denaturation was conducted at 95°C for 1 min with a final extension for 10 min at 72°C. Successful PCR products were cleaned of primers and salts, using the QIAquick PCR Purification Kit (Quiagen, Inc., Valencia, California). AmpliTaq® FS cycle sequencing reactions (Perkin Elmer Corporation, Foster City, California) were prepared according to the manufacturer's protocol, using primers ITS5 and ITS4 and the PCR product as template (White et al., 1990). Reactions were analyzed on an ABI 373A Automated DNA Sequencer (Perkin Elmer Corporation, Foster City, California). Sequences from previous studies obtained from GenBank as well as sequences obtained for this study (deposited in GenBank) are listed in Table 2.

Phylogenetic analysis

The GCG programs Gap, Pileup and the SeqLab interface for the Wisconsin Package Version 9.1 (Genetics Computer Group, Madison, WI) were used to

analyse sequences, generate alignments and make manual adjustments. PAUP version 4b7 for Unix (Swofford, 1999) was used for phylogenetic analysis. Base frequencies across taxa were compared using PAUP. MODELTEST (Posada et al., 1998) was used to establish the model of DNA evolution that best fit the data model (Akaike Information Criterion) and maximum likelihood analysis was performed (using PAUP) with the resulting model. Taxa were added randomly in ten replicates with a random starting seed. One tree was held at each step during stepwise addition. The tree-bisection-reconnection branch-swapping algorithm was used. Branches were collapsed (creating polytomies) if branch length was less than or equal to 1^{-10}. A successive approximation approach was used to refine the model parameters (Swofford et al., 1996). Substitution parameters from a Jukes-Cantor tree (Jukes and Cantor, 1969) were used as a starting point. If a tree of higher likelihood was found during likelihood analysis, the substitution parameters were estimated for the new tree (using the same model) and used for a new round of likelihood searches. Parameter estimation and tree searching continued until the same tree was found in successive iterations. MrBayes 1.11, a Bayesian phylogenetic inference program (Huelsenbeck, 2000) was used to derive branch support. Likelihood parameters selected by MODELTEST were used for the Markov Chain Monte Carlo (four chain) analysis run for 420,000 generations, sampling every 100 generations to yield 4200 trees. The first 200 trees were discarded. A majority rule consensus tree was produced with PAUP using the remaining 4000 trees, calculating the frequency of each bipartition.

Ecological survey

A survey was conducted of endophyte infection at 13 sites in the Andes Mountains near the resort town of Las Leñas (Province Mendoza, Argentina). At each of the sites approximately 20 culms of *B. setifolius* were sampled by arbitrary selection; and concentration of *B. setifolius* was estimated by walking three transects of 50 m each and counting plants found within two meters of either side of the transect. Sites were evaluated for elevation, dominant vegetation, number of colonies of leaf cutting ants (*Acromyrmex* sp.) in the immediate vicinity of the population (an area of approximately 2500 m^2 was searched) (Table 1). Each of the culms was assessed for presence of endophytic mycelium by examination of culm tissues using a light microscope. Endophytic mycelium was visualized by staining tissue scraped from within culms with aniline blue (0.1% aqueous). Culms were identified as endophyte-infected if typical non-branching intercellular mycelium was evident among plant parenchyma tissues (White, 1987).

The correlation of presence of endophyte infection level and elevation,

Table 1. Endophyte levels in populations (% infection) and other ecological and site data.

Site	Alt., m	Loc. coordin.(S/W)	Ave. den./50 m[x]	Dom. veg.	Ant mounds	% Inf.
34	2867	35 06 34/70 08 45	29.0	grasses	absent	0
52	2481	35 06 44/70 05 11	28.7	grasses	absent	0
35	2193	35 10 45/70 04 15	9.3	small shrubs[y]	absent	0
36	2128	35 11 54/70 03 20	2.3	small shrubs	absent	0
37	2068	35 11 28/70 00 03	<1	small shrubs	absent	0
39	1711	35 12 04/69 45 57	5.7	large shrubs[t]	4	100
40	1657	35 12 45/69 44 17	5.3	large shrubs	4	95
41	1700	35 12 56/69 43 20	6.3	large shrubs	2	85
42	1546	35 13 08/69 40 48	4.7	large shrubs	2	100
45	1634	35 03 24/69 36 25	<1	leafless plants[*]	0	15
47	1650	35 01 29/69 37 31	<1	leafless plants	0	15
48	1724	34 59 53/69 38 35	5.3	large shrubs	1	70
50	1790	34 56 27/69 44 08	4.0	large shrub	1	70

[x]The mean of the plant count in three 50 m transects. [y]Shrubs of this zone (sites 52, 35, 36 and 37) included *Adesmia pinifolia*. [t]Shrubs of this zone (sites 39, 40, 41, 42, 48 and 50) included *Colliguaya integerrima, Larrea nitida, Prosopis ruizleali, Psila spartioides,* and *Schinus polygamus*. [*]Small leafless shrubs.

number of ant mounds, and average plant density was examined through use of the JMP 3.1.5 Statistical Analysis Program Package (SAS Institute, Inc., Cary, North Carolina). Linear and second-degree polynomial fit regression analyses were conducted on each of the measured parameters. Fig. 2 shows the fit of the second-degree polynomial curve to the number of ant mounds.

Mycotoxin analysis

Plant material used in this study included endophyte-infected plants: *Bromus setifolius* (from site 40), *Poa* sp. from the Province of Salta, Argentina (Cabral et al., 1999), and several selections of *Lolium perenne* and *Festuca arundinacea* from East Europe and North America, respectively. All plants were maintained in a green house at Rutgers University (New Brunswick, New Jersey). Plants were fertilized every two weeks using Peter's Professional Water Soluble Fertilizer (20:20:20) following the label protocol.

Peramine, ergovaline and lolitrem B were extracted and analyzed by the methods described in detail in Yue et al. (2000a). Fresh plant tissues (leaf blades and sheaths) were homogenized in liquid nitrogen and stored at –20°C. For peramine analysis, 100 mg of homogenized plant samples were extracted

Table 2. DNA sequences used for phylogenetic analysis.

Endophyte species	GenBank accession no.	Host	Collection location
Neotyphodium sp.	AF385198	*Achnatherum robustum*	Cloudcroft, New Mexico, USA[5]
Neotyphodium sp.	AF385204	*Achnatherum sibericum* 1	Baiyinkulum, China[5]
Neotyphodium sp.	AF385203	*Achnatherum sibericum* 2	Inner Mongolia, China[5]
Epichloë amarillans	AF385200	*Achnatherum sibericum* 3	Saihatela, China[5]
Epichloë amarillans	L07129	*Agrostis hiemalis* 1	Alabama, USA[1]
Epichloë amarillans	AF385206	*Agrostis hiemalis* 2	Alabama, USA[5]
Epichloë amarillans	U57665	*Agrostis hiemalis* 3	Alabama, USA[3]
Epichloë baconii	L07138	*Agrostis stolonifera*	England[1]
Epichloë brachyelytri	L78296	*Brachyelytrum erectum*	Switzerland[1]
Epichloë sylvatica	L78304	*Brachyelytrum sylvaticum*	Switzerland[1]
Epichloë bromicola	L78295	*Bromus erectus*	Switzerland[1]
Neotyphodium tembladerae	AF385205	*Bromus setifolius*	Mendoza (site 40), Argentina[5]
Epichloë elymi	L07131	*Elymus canadensis*	Austin, Texas, USA[1]
Epichloë sp.	AF385202	*Elymus* sp.	Hohhot, China
Neotyphodium tembladerae	AF385207	*Festuca argentina*	Neuguen, Argentina[5]
Neotyphodium sp.	L07140	*Festuca arundinacea*	North Africa[2]
Epichloë festucae	AF059731	*Festuca brevipila*	Europe[4]
Neotyphodium sp.	AF385209	*Festuca hieromymi* 1	Tucuman, Argentina[5]
Neotyphodium sp.	AF385208	*Festuca hieromymi* 2	Tucuman, Argentina[5]
Epichloë festucae	L07139	*Festuca longifolia*	Switzerland[1]
Epichloë festucae	AF385213	*Festuca rubra* 1	England[5]
Epichloë festucae	AF059730	*Festuca rubra* 2	Atlantic City[4]
Epichloë festucae	AF385214	*Festuca rubra* 3	England[5]
Epichloë glyceriae	L78301	*Glyceria striata* 1	New York, USA[1]
Epichloë glyceriae	L07136	*Glyceria striata* 2	New York, USA[1]
Epichloë glyceriae	L78302	*Glyceria striata* 3	New York, USA[1]
Epichloë clarkii	U57666	*Holcus lanatus*	Yorkshire, England[3]
Epichloë typhina	L07132	*Lolium perenne*	Europe[1]
Neotyphodium tembladerae	AF385210	*Poa huecu* 1	Neuguen, Argentina[5]
Neotyphodium tembladerae	AF385211	*Poa huecu* 2	Neuguen, Argentina[5]

Table 2. Continued.

Endophyte species	GenBank accession no.	Host	Collection location
Neotyphodium sp.	AF385212	*Poa rigidifolia*	Tiera del Fuego, Argentina[5]
Epichloë typhina	L78293	*Poa silvicola*	Switzerland[1]
Neotyphodium chisosum	AF385201	*Stipa eminens*	Texas, USA[5]
Neotyphodium sp.	AF385199	*Stipa lobata*	Texas, USA[5]
Epichloë amarillans	U57664	*Sphenopholus obtusata*	Georgia, USA[3]

[1]Schardl et al., 1997; [2]Schardl et al., 1991; [3]Unpublished; [4]Tredway et al., 1999; [5]This study.

with 3 ml of 30% isopropanol for 45 min at 90°C. After cooling and centrifuging, a 1-ml sample of the supernatant was transferred to a preconditioned 1-ml Varian Bond Elut carboxylic acid column packed with 100 mg of adsorbent. Peramine was eluted with 1 ml of 5% formic acid in 80% aqueous methanol. Peramine was further purified by reverse phase HPLC with an isocratic mobile phase of 20% (v/v) acetonitrile in a guanidine carbonate (10 mM)-formic acid (1.6 ml/l) buffer, and quantified using a UV detector at 280 nm. Extraction efficiency (89±3%) was estimated using the results obtained by spiking an endophyte free sample with a standard solution of peramine. For ergovaline analysis, a 0.5 g sample was extracted in 20 ml of carbon tetrachloride-0.001 M sodium hydroxide (40:1) for 1 hour on an orbital shaker. Twenty micro-liters of 30 µg/ml of ergotamine ditartarate internal standard solution were added to the sample prior to extraction. The mixture was filtered and 10 ml of the filtrate was passed through a chloroform preconditioned Ergosil solid phase column. Pigments were removed by addition of 2 ml of chloroform-acetone (75:25). The ergot alkaloids were eluted with 3 ml of methanol. Ergovaline was further purified by reverse phase HPLC with a step-gradient of acetonitrile in 0.1 M ammonium acetate buffer pH 7.6, and quantified using a florescence detector with an excitation wavelength 250 nm and an emission wavelength of 420 nm. For lolitrem B, samples (500 mg) were extracted in 10 ml of $CHCl_3$: methanol (2:1) for 1 hr on an orbital shaker, centrifuged for 5 min, and 5-ml aliquots of the supernatant were transferred to small vials and dried under a stream of air. The dried samples were dissolved in four 0.5-ml aliquots of dichloro-methane, and loaded on a conditioned (wetted with 2 ml dichloro-methane) Waters Sep-Pak silica cartridge (500 mg). The Sep-Pak cartridge

was washed with 2 ml of dichloro-methane and lolitrem B was eluted with two 1-ml volumes of dichloro-methane: acetonitrile (4:1). Lolitrem B (found in the second 1-ml volume) was purified by isocratic normal phase HPLC with a silica column and a mobile phase of 15% (v/v) acetonitrile in dichloro-methane, and quantified using a fluorescence detector with an excitation wavelength of 268 nm and an emission wavelength of 440 nm. Extraction efficiency was estimated at 78% using the results obtained by spiking an endophyte free sample with a standard solution of lolitrem B. The limit of detection for each of the alkaloids was: 5 ng/g for ergovaline, 500 ng/g for peramine and 2 ng/g for lolitrem B.

Fall armyworm choice studies

Feeding studies were conducted using a single batch of fall armyworms (*Spodoptera frugiperda*) at 1 week after germination that had been reared on corn meal at 23 °C. Culms from endophyte-infected and endophyte-free plants (derived from seed gathered from sites 35 (E–), 36 (E–), 37 (E–), 47(E–), 40 (E+), 41 (E+), 48 (E+)) were cut and placed in plastic petri dishes with the bases in moist paper towels to prevent drying (see Table 1 for site locations). One endophyte-infected (E+) and one endophyte-free (E–) culm at approximately equal stages of maturation bearing 3 leaves cut to approximately 10 cm in length (from culm base to cut leaf end) was placed in each petri dish. The bases of all tillers were wrapped in moistened tissue to prevent drying. At least one replicate of each combination of E+ and E– plants from the above sites was made. Two fall armyworms were placed into each plate. The plates were incubated at room temperature for approximately 10 hours after which feeding was assessed. A culm was assessed as having been consumed if: 1) fecal material was evident around culm, and 2) observable portions of leaf or leaf sheath material had been removed through consumption. A culm was assessed as not having been consumed if none of the above were observed.

3. Results

Endophyte identification and phylogenetic analysis

The data matrix contained 35 taxa, each with 582 characters; 467 characters were constant. Using PAUP base frequencies were found to be homogeneous across taxa even when constant characters were excluded (P=0.99). MODELTEST selected a Hasegawa-Kishino-Yano (HKY85) model (two substitution types with unequal base frequencies) (Hasegawa et al., 1985), with rate heterogeneity distributed according to a gamma distribution. After two

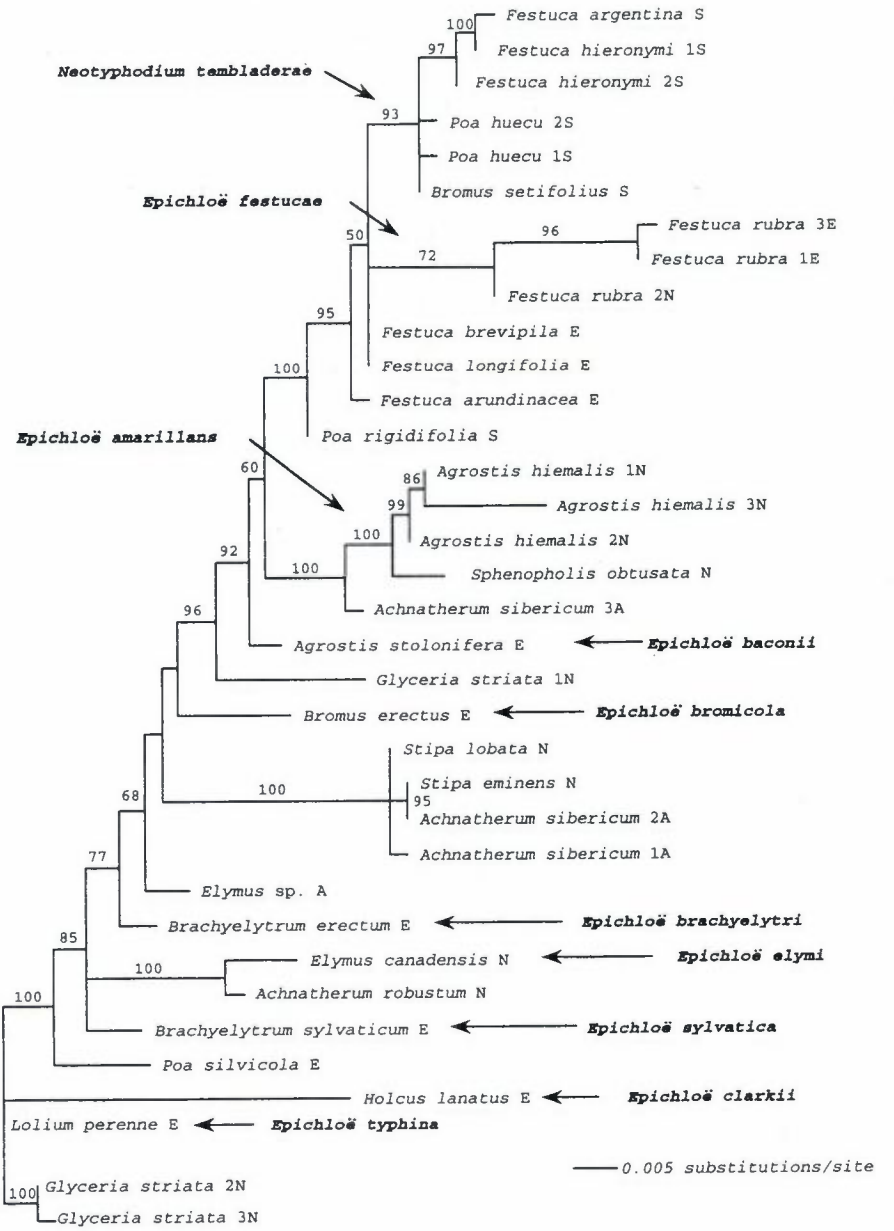

Figure 1. Maximum likelihood tree using the HKY85 evolutionary model. Branch support indicated above nodes results from partition frequencies of a majority rule consensus tree constructed from 4,000 trees samples from a 420,000 generation Bayesian analysis. Terminal taxon labels are of the host species. Corresponding endophyte taxa are indicated at relevant nodes. The letter following the host name indicates the continent of origin (A = Asia; E = Europe; N = North America; S = South America).

rounds of successive approximations, the most likely, equilibrated tree was found with a -ln likelihood= 1760.93068 and the following parameters: a transition/transversion ratio equal to 2.510529; base frequencies: A:0.212797 C:0.296752 G:0.264828 T:0.225623; gamma shape parameter: 0.359476. Of the 4000 trees sampled from the Bayesian analysis, 83 trees were identical to the most likely tree and no tree had a lower likelihood score. The 4000 Bayesian trees were used to construct a majority-rule consensus tree with frequencies of each bipartition inferring branch support. The maximum likelihood tree is depicted in Fig. 1 with branch support above nodes.

The endophyte from the South American grass *Bromus setifolius* grouped (93% branch support) with other endophytes from South American grasses, including *Festuca argentina, F. hieronymi,* and *Poa huecu* (Fig. 1). These formed the *Neotyphodium tembladerae* clade. The endophyte from South American *Poa rigidifolia* did not group with this clade, instead it appeared basal in the *E. festucae* clade.

Distribution studies

Examination and analyses of the ecological data (Table 1) revealed some correlations. The correlation between endophyte concentration and average density of *B. setifolius* at each site was very poor (linear model r-square = 0.10; non-linear second-degree polynomial r-square = 0.40). Endophytes were largely absent or in low levels in populations occurring at and above 2068 m elevation. There was a weak correlation (r-square = 0.46) between elevation and endophyte level in the grass using the linear fit model. The non-linear second-degree polynomial model gave a slightly better fit (r-square = 0.55). Endophyte levels tended to be high in populations in communities that included certain large shrubs such as *Colliguaya integerrima, Larrea nitida,* and *Schinus polygamus*. The level of endophyte detected in populations correlated closely with the number of colonies of leaf-cutting ants detected in the community. For this correlation the correlation coefficient in the linear fit model was moderate (r-square = 0.78); in the second-degree polynomial fit the correlation was high (r-square = 0.97). The polynomial correlation of endophyte level and number of ant colonies was highly significant according to ANOVA F- and Student t-tests ($P<0.0001$) (Fig. 2).

Mycotoxin analysis

Endophyte-infected plants of *Bromus setifolius* and *Poa* sp. were both found to contain ergovaline and peramine. However, lolitrem B was not detected in *N. tembladerae*-infected grasses (Table 3).

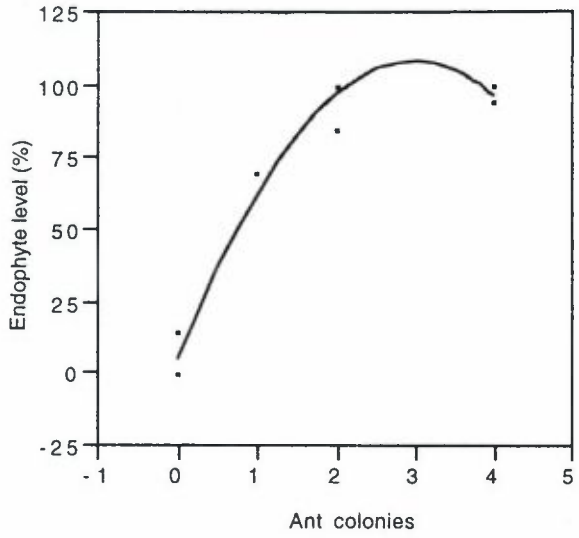

Figure 2. Graph of number of leaf-cutting ant (*Acromyrmex* sp.) colonies (x-axis = ant colonies) located in community vs level of infection by endophyte (y-axis = endophyte level). Curve is a non-linear second-degree polynomial fit of the data points (r-square = 0.97).

Table 3. Comparison of mycotoxins in selected endophyte-infected grasses.

Grass	Origin	Ergovaline (ng/g)	Peramine (μg/g)	Lolitrem B (μg/g)
Poa sp.	South America	18.33±3.08	21.44±1.44	n.d.
Bromus setifolius	South America	16.64±2.98	10.00±0.77	n.d.
Lolium perenne (876)	East Europe	52.71±7.83	36.00±4.81	1.82±0.37
Lolium perenne (9574)	East Europe	n.d.	5.2±0.56	2.02±0.73
Lolium perenne (9576)	East Europe	n.d.	3.24±1.05	0.03±0.01
Lolium perenne (9677)	East Europe	n.d.	6.06±1.56	1.30±0.57
Lolium perenne (9739)	East Europe	88.49±7.83	11.18±1.75	0.22±0.10
Lolium perenne (9820)	East Europe	108.34±22.57	1.50±0.22	0.07±0.03
Festuca arundinacea (171)	North America	1500±110.00	3.08±0.19	n.d.
Festuca arundinacea (230)	North America	2030±150.00	1.38±0.41	n.d.
Festuca arundinacea (246)	North America	1110±90.00	1.63±0.05	n.d.

Data presented as mean±standard deviation; n.d. = not detectable.

Fall armyworm feeding studies

The fall armyworms were observed to sample both E+ and E– tillers but minimal evidence of feeding was found on E+ tillers. However, the endophyte-free plants of *B. setifolius* showed evidence of extensive consumption in 22 of 26 trials (84.6% of the feeding trials). According to the chi-squared test, this is a significant ($P<0.05$) preference for endophyte-free plant material.

4. Discussion

Identification and evolutionary considerations

Endophytes of *Festuca argentina*, *F. hieronymi*, and *Poa huecu* have previously been identified as *N. tembladerae* (Cabral et al., 1999). Due to the 93% branch support for the grouping of the *B. setifolius* endophyte with endophytes previously identified to *N. tembladerae*, it is probable that the endophyte from *B. setifolius* bears affinity to that species (Fig. 1). It is notable that *P. huecu* is the type host of *N. tembladerae* (Cabral et al., 1999); and the placement of the endophytes from *B. setifolius* and *P. huecu* were especially close in our analysis (Fig. 1). The *N. tembladerae* clade was placed near *E. festucae* (Fig. 1), suggesting a close evolutionary relationship. *Epichloë festucae* employs both asexual and sexual reproduction. We have not found sexual stages in the life cycles of South American grass endophytes, and thus we believe that they have been lost; and reproduction of these endophytes in South America is exclusively asexual. Given that asexual endophytes such as *N. tembladerae* are derived from sexual endophytes; and that the phylogenetically closest sexually-reproducing endophyte to *N. tembladerae* is *Epichloë festucae*, it seems reasonable to propose that *N. tembladerae* may be derived from *E. festucae*. Since *E. festucae* is common in the Northern Hemisphere but absent in the Southern Hemisphere it logically follows that populations from the Northern Hemisphere gave rise to the South American populations. Some uncertainty must be expressed in that it is possible that asexual endophytes such as these have undergone asexual genetic recombination and may be polyploidal or chimeric and parentage may be more complicated (Tsai et al., 1994; Schardl et al., 1997). The endophyte from the South American grass *Poa rigidifolia* does not appear to group with these endophytes. This is consistent with a previous analysis where this endophyte was placed outside the *N. tembladerae* clade (Cabral et al., 1999). This may be an indication that multiple lineages of endophytes are evident in South American grasses; and may reflect independent colonizations of South American grasses.

Distribution studies

N. tembladerae infection was high in *B. setifolius* populations that occurred in communities that contained leaf-cutting ants and low in populations that did not show evidence of the ants (Table 1; Fig. 2). A direct correlation between presence of the ants and endophyte is suggested by the very high (97%) correlation coefficient of the number of ant colonies and the level of endophyte infection in the *B. setifolius* populations. In desert communities leaf-cutting ants are frequently the dominant herbivores (Fowler et al., 1990). Sites 39, 40, 41, 42, 48, and 50 were all below 1711 m elevation and contained desert communities, including leaf-cutting ants, various shrubs, and grasses (Table 1). Sites 45 and 47, also below 1711 m elevation, demonstrated a low 15% level of infection by the endophyte. Evidence of the leaf-cutting ants at these two sites was not found. The leafy plants (*Colliguaya integerrima, Larrea nitida,* and *Schinus polygamus*) were absent, instead an unidentified leafless shrub dominated (Table 1). We postulate that the leafy plants represent the food base of the herbivore populations. In high elevation communities (sites 34, 35, 36, 37, and 52) the cooler, harsher, environmental conditions may have been limiting to the survival of the ants and other herbivores. In these communities fewer shrubs were evident and they tended to be smaller. At the highest elevations (2481 m and above) grasses became the dominant vegetation as is evidenced by the increased *B. setifolius* density measurements at sites 34 and 52 (Table 1).

The correlation between ant abundance and endophyte infection can be explained if the leaf-cutting ants avoid endophyte-infected plants. We have no direct experimental evidence to support that hypothesis. However, Knoch et al. (1993) demonstrated that some species of leaf-cutting ants selectively avoid or cull seeds of tall fescue grass (*Festuca arundinacea*) when it is infected with the endophyte *Neotyphodium coenophialum*. Culling the infected seed places them in refuse piles where nutrients are high and their survival is enhanced. When Knoch et al. (1993) provided ants with endophyte-infected tall fescue as their sole food source, over a period of time, health of the colony declined: queen life span decreased, worker productivity declined, and the fungus gardens decreased in size. The reason for the negative effects on the colonies is a matter for speculation. Because plant material is provided to a symbiotic fungus to process prior to consumption by leaf-cutting ants, it is possible that endophyte mycotoxins have inhibitory effects on the fungus colony. Yue et al. (2000b) demonstrated that many of the toxins produced by *Neotyphodium* endophytes are fungal inhibitors. However, because worker ants masticate plant material for a period of time prior to providing it to the symbiotic fungus, direct toxicosis of worker ants is also possible. Tibbets and Faeth (1999) found that leaf cutting ants provided with exclusively

endophyte-infected tall fescue frequently showed symptoms of "nervous system breakdown, including shaking, tremors, and failure to tend their fungus gardens." These symptoms are comparable to those seen in vertebrates experiencing the 'staggers' toxicosis caused by mycotoxins in endophyte-infected perennial ryegrass (*Lolium perenne*). Whether the Andean populations of leaf-cutting ants would show a comparable toxicosis if forced to consume infected *B. setifolius* is unknown.

Cost of endophyte to host

The tendency of the levels of endophyte to be low or absent in populations of *B. setifolius* not under herbivore pressure implies that there is a cost to hosting the endophyte. It is apparent that in the absence of strong herbivore pressure, endophyte-free plants have an evolutionary advantage. There is no evidence that *N. tembladerae* is pathogenic to its grass hosts, since stromata that sterilize plants have not been observed. Perhaps, the presence of the endophyte represents a nutritional drain on plants that reduces their fitness. Endophytes also may produce plant growth regulators such as auxin (Yue et al., 1999b). These may alter the physiology of infected plants to the extent that their fitness is reduced. Experimental work is needed to evaluate the possible costs of endophyte infection.

Mycotoxin production

The presence of relatively high levels of peramine in *N. tembladerae*-infected plants of *B. setifolius* and *Poa* sp. (Table 3) suggests a basis for insect deterrence in grasses infected with this endophyte. The alkaloid peramine is frequently associated with insect deterrence (Prestidge et al., 1985). The ergot alkaloid ergovaline was low in endophyte-infected *B. setifolius* and *Poa* sp. compared to the levels observed in tall fescue (Table 3). However, it is likely that other ergot alkaloids that were not measured in this study are also present in grasses infected by *N. tembladerae*. In a previous study of alkaloids produced by *Epichloë festucae*, Yue et al. (2000b) found 4 ergot alkaloids that were major components of culture filtrates and 6 ergot alkaloids that were minor components. We did not detect lolitrem B in either grass infected by *N. tembladerae*. However, using an antibody-based assay, Miles et al. (1995) reported that *N. tembladerae*-infected *Poa huecu* and *Festuca argentina* contained indole-diterpenoid tremorgens other than lolitrem B. Pomilio et al. (1989) identified toxic glycoproteins as lethal components of the *N. tembladerae*-infected grass *P. huecu*. Thus it seems probable that *N. tembladerae* is producing a more diverse assemblage of mycotoxins than we

have measured. In Argentina, a staggers toxicosis frequently develops when animals consume *N. tembladerae*-infected grasses of the species *F. argentina, F. heronymi,* and *P. huecu* (Cabral et al., 1999), and in some localities consumption of endophyte-infected grasses may result in death. Farmers in regions of the Andes where *N. tembladerae*-infected grasses are dominant components of the vegetation have reported that cattle will avoid consumption of the grass (White and Cabral, unpublished).

Protection from insect herbivory

As a way to evaluate the potential for insect deterrence of *N. tembladerae*-infected grasses, we conducted fall armyworm choice trials. The fall armyworms demonstrated a statistically significant preference for endophyte-free plant material. This is a confirmation that presence of the endophyte *N. tembladerae* provides grass hosts with enhanced protection from insect herbivory. Grasshoppers and other insects are present in the communities where *B. setifolius* contains endophytes and it is possible that they contribute to the selection for endophytes in the *B. setifolius* populations in those communities.

5. Conclusions

The results presented in this study are consistent with the defensive mutualism hypothesis as articulated by Clay (1988). *N. tembladerae* infection in *B. setifolius* populations showed a correlation with an important desert herbivore, leaf cutting ants; suggesting that herbivore pressure selected for higher levels of the endosymbiont in grass populations. *N. tembladerae* was found to produce two mycotoxins (ergovaline and peramine) that are known to be toxic to insect and other herbivores. Feeding studies demonstrated that fall armyworms showed a preference for endophyte-free *B. setifolius* over endophyte-infected *B. setifolius;* thus confirming insect deterrence effects due to *N. tembladerae* infection. Based on our observations and experiments it must be concluded that the association of *N. tembladerae* with its grass hosts may represent a case defensive mutualism.

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