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The *Neotyphodium* Endophyte of Wild Barley (*Hordeum brevisubulatum* subsp. *violaceum*) Grows and Sporulates on Leaf Surfaces of the Host

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Abstract

The *Neotyphodium* endophyte of wild barley (*Hordeum brevisubulatum* subsp. *violaceum*) has been demonstrated via stained leaf impressions, SEM, and the plating to agar media of leaf washes from the host, to grow and sporulate on leaf surfaces under laboratory conditions. Superficial mycelium was usually sparse, and mycelial nets were not observed on the leaf epidermis. ITS sequence data were identical for *Neotyphodium* isolates derived from surface-disinfested leaf tissue and from leaf washes, and the isolates were indistinguishable by morphometric analysis when grown in culture. Conidia of the endophyte were capable of germination and growth on nutrient agar.

Keywords: Clavicipitaceae, endophytes, epiphyllous, *Hordeum*, *Neotyphodium*

1. Introduction

Recent studies have documented epiphyllous mycelial nets (Moy et al., 2000) or sparse superficial layers of mycelium (White et al., 1996) produced by endophytic clavicipitaceous fungi on the leaves of various grass hosts. These

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discoveries have altered previous concepts regarding the transmission of endophytes between hosts. Formerly, it was held that transmission was exclusively or mostly vertical, i.e., through seed or vegetative production of the infected host (Latch, 1998). The discovery of conidiogenous hyphae on plant surfaces has rendered horizontal transmission more probable. Also, in instances in which mycelial nets were formed, the possibility has been raised that infection by endophytes could deter or reduce infection by pathogenic fungi via niche exclusion. The subject was reviewed by Moy et al. (2000), who also reported instances in which external mycelium of endophytes appeared entirely lacking on endophyte-infected plants, thus raising the possibility that host-endophyte relationships in grasses may display a spectrum of external as well as internal colonization patterns and reproductive strategies.

Our primary objective was to determine if the *Neotyphodium* endophyte of *Hordeum brevisubulatum* subsp. *violaceum* (Boiss. & Hohen.) Tzvelev grows and sporulates on the epidermis of host leaves, and if so, to document the intensity of mycelial colonization and conidial production on the leaf surface relative to other instances in which *Neotyphodium* endophytes produce epiphyllous growth. We also wished to use sequence data to elucidate the phylogenetic position of the endophyte within the *Epichloë/Neotyphodium* complex.

2. Materials and Methods

Production of experimental plants, isolation of the endophyte and recording of events on leaf surfaces

Seeds of PI 440420 *Hordeum brevisubulatum* subsp. *violaceum*, a National Plant Germplasm System (NPGS) accession previously demonstrated to contain *Neotyphodium* sp. endophyte (Wilson et al., 1991; Youssef and Dugan, 2000), were surface-disinfested for 90 sec in 0.5% NaOCl (Youssef and Dugan, 2000), germinated on sterile filter paper moistened with sterile distilled water, and planted into moistened two-cm deep sterile potting mix placed inside sterile, clear plastic Magenta® GA-7 vessels (Magenta Corporation, Chicago, IL). After transfer to the vessels, plants were watered with modified sterile Hoagland's solution (1 ml/L KH_2PO_4, 5 ml/L KNO_3, 5 ml/L $Ca(NO_3)_2$, and 2 ml/L $MgSO_4 \cdot 7H_2O$). Plants were incubated in a growth chamber at 20°C with 8 hrs darkness / 16 hrs fluorescent light (1 standard 24 in tube, 1 Glolux® 24 in tube, 750 lux total illumination). Seeds planted on 14 February 2001 were disinfested with paleas and lemmas intact. Paleas and lemmas were removed prior to disinfestation on seeds planted 28 February; this practice reduced the number of non-*Neotyphodium* fungal species surviving disinfestation.

Excised leaf segments ca. 5–15 mm in length were used to generate leaf impressions with double-sided sticky tape (Endo, 1966), Rhoplex® (Rohm and Haas Company) (Dhingra and Sinclair, 1994), or cellulose acetate (Sigma) (Rowell, 1967). Impressions were mounted in 1% aniline blue in 85% lactic acid. Fungal structures were photographed under bright field or differential interference contrast (DIC) with an Olympus DP11 digital camera mounted on an Olympus BH-2 compound microscope. Leaves were excised for examination from the material planted 14 February at 27–50 days. Leaves were excised for examination from the material planted 28 February at 37–40 days. On each harvest date, a different plant was picked for examination. From the same leaf and/or plant on each date, two additional sets of leaf segments were harvested, one set being surface-disinfested as above and the other not disinfested prior to being plated onto potato dextrose agar amended with streptomycin and penicillin (StrPen PDA) for suppression of bacteria (Youssef and Dugan, 2000).

On 18 April, leaves from 5 plants were excised from material planted 28 February, their cut ends dipped into 70% ethanol (to kill any fungal growth immediately interior to the cut) and placed into 1 ml sterile 0.1% water agar, and vortexed. The resulting suspensions were streaked onto StrPen PDA and the leaves themselves subsequently cut into segments ca 1 cm long, some of which were surface-disinfested and others not disinfested, and plated onto StrPen PDA. Two other plates of StrPen PDA were streaked with washings from each leaf examined. All plates were incubated at 20°C ca. 50 cm from two Sylvania Cool White fluorescent bulbs (16 hrs darkness and 8 hrs light). Portions of leaves from one of the five plants, plus leaf segments from three other identically grown plants, were processed for scanning electron microscopy (SEM) according to Clement et al. (1997). On 28 April, the washing of leaves and plating of leaf segments as above were repeated for an additional seven plants.

On 19 May, a subculture of a typical colony recovered from leaf washings on 18 April was labeled NexHbv1 and transferred to 1/2 strength V8 agar (Stevens, 1981), as was a subculture of an isolate labeled NexHbv2 recovered from one of the surface-disinfested leaves harvested on 22 March. These cultures were preserved in liquid nitrogen (Smith and Onions, 1994), revived, and each strain plated to 2 plates of 1/2 V8, and incubated for 27 days as previously described, after which colony diameters were averaged (3 per plate) and other colony characters recorded. Portions of each strain were harvested, mounted in 85% lactic acid, examined under DIC at 1000X, and lengths and widths recorded for 50 conidia and 25 conidiophores. The process was replicated once for each strain. Results were analyzed by ANOVA and least significant differences (LSD) with Systat® 9 (SPSS Inc, Chicago, IL).

Table 1. DNA sequences used for phylogenetic analysis

Endophyte species	GenBank accession no.	Host
Epichloë amarillans	L07129	*Agrostis hiemalis* 1
Epichloë amarillans	AF385206	*Agrostis hiemalis* 2
Epichloë amarillans	U57665	*Agrostis hiemalis* 3
Epichloë baconii	L07138	*Agrostis stolonifera*
Epichloë brachyelytri	L78296	*Brachyelytrum erectum*
Epichloë sylvatica	L78304	*Brachypodium sylvaticum*
Epichloë bromicola	L78295	*Bromus erectus*
Neotyphodium tembladerae	AF385205	*Bromus setifolius*
Epichloë elymi	L07131	*Elymus canadensis*
Neotyphodium sp.	AF385202	*Elymus* sp.
Neotyphodium tembladerae	AF385207	*Festuca argentina*
Neotyphodium sp.	L07140	*Festuca arundinacea*
Epichloë festucae	AF059731	*Festuca brevipila*
Neotyphodium sp.	AF385209	*Festuca hieromymi* 1
Neotyphodium sp.	AF385208	*Festuca hieromymi* 2
Epichloë festucae	L07139	*Festuca longifolia*
Epichloë festucae	AF385213	*Festuca rubra* 1
Epichloë festucae	AF059730	*Festuca rubra* 2
Epichloë festucae	AF385214	*Festuca rubra* 3
Epichloë glyceriae	L07136	*Glyceria striata* 1
Epichloë glyceriae	L78302	*Glyceria striata* 2
Neotyphodium sp.		*Hordeum brevisubulatum*
Epichloë clarkii	U57666	*Holcus lanatus*
Epichloë typhina	L07132	*Lolium perenne*
Neotyphodium tembladerae	AF385210	*Poa huecu* 1
Neotyphodium tembladerae	AF385211	*Poa huecu* 2
Neotyphodium sp.	AF385212	*Poa rigidifolia*
Epichloë typhina	L78293	*Poa silvicola*
Epichloë amarillans	U57664	*Sphenopholis obtusata*

Generation of sequence data and phylogenetic analysis

Fresh mycelium of the *Neotyphodium* isolates was lifted off cellulose acetate sheets on PDA and ground in liquid nitrogen. Genomic DNA was extracted using DNeasy™ Plant Protocol (Qiagen Inc., Valencia, CA). 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

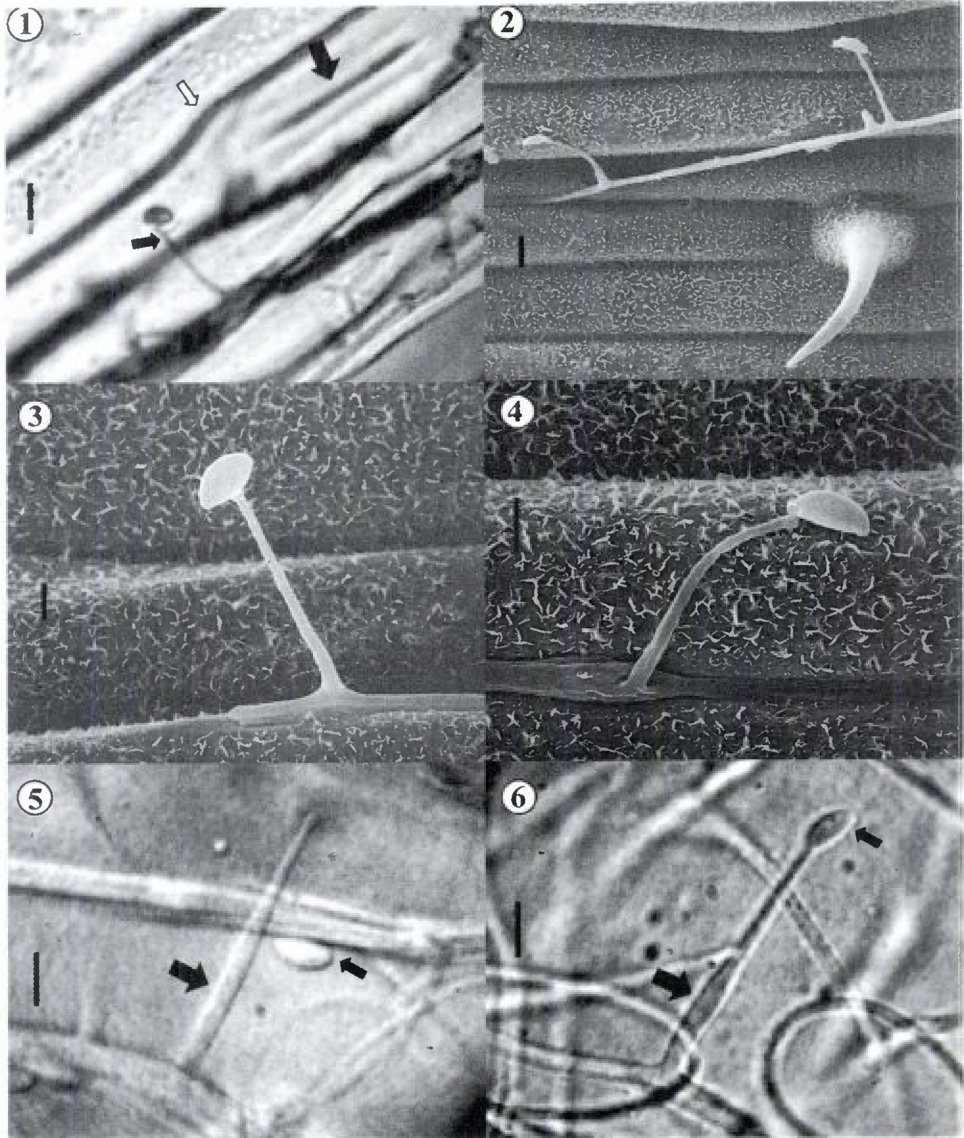
Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 12.5 pmol each dNTP, 50 pmol each primer, and 2 U Taq polymerase (Desai and Pfaffle, 1995).

PCR (25 cycles) was carried out in a GeneAmp 9600 thermocycler (Perkin Elmer Corporation, Foster City, CA) 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. The PCR products were cleaned of primers and salts, using the QIAaquick PCR Purification Kit (Qiagen Inc., Valencia, CA). AmpliTaq® FS cycle sequencing reactions (Perkin Elmer Corporation, Foster City, CA) 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, CA). Both strands were sequenced. Sequences from previous studies obtained from GenBank as well as sequences obtained for this study (deposited in GenBank) are listed in Table 1.

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 Solaris (unix) (Swofford, 1999) was used for phylogenetic analysis. Base frequencies across taxa were compared using PAUP. MODELTEST (Posada and Crandall, 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 520,000 generations, sampling every 500 generations to yield 1040 trees. The first 40 trees were considered "burnin" and discarded. A majority rule consensus tree was produced with PAUP with the remaining 1000 trees, calculating the frequency of each bipartition.



Figs. 1–6.

Figure 1. *Neotyphodium* sporulating on surface of wild barley. Conidiophore with attached conidium (small arrow), stoma (large arrow) and interface of adjacent epidermal cell walls (open arrow). Cellulose acetate impression, bright field, oil immersion. Bar = 5 µm.

Figure 2. Hypha adjacent to trichome and bearing two conidiophores, each with a single conidium at the apex. SEM. Bar = 7 µm.

Figure 3. Conidiophore with conidium at apex in 'T' configuration typical for *Neotyphodium*. SEM. Bar = 3 µm.

Viability of conidia

Six colonies of NexHbv2 were established by subculture to a single plate of 1/2 V8, grown for 30 days, and washed with sterile distilled H_2O to remove conidia. Drops of the suspension were placed onto slide cultures of Difco PDA and examined 48 hours later to see if conidia were capable of germination.

3. Results

Recovery of endophyte and events on leaf surfaces

Neotyphodium conidiophores and attached conidia were observed and photographed in leaf impressions from seven of nine plants examined (Fig. 1). No analogous sporulation was observed on the remaining two plants. Hyphae, often bearing *Neotyphodium* conidiophores with attached or closely adjacent conidia, were present only at low densities. Hyphae were usually growing parallel to longitudinal periclinal walls of host epidermal cells, and often in the longitudinal valley between epidermal cells. No particular association was noted between the fungus and stomata or trichomes, although both plant structures were distinct in the impressions. The density of hyphae was sufficiently low that most fields of view at 200× revealed no hyphae. There was no indication of a reticulated hyphal net over the leaf surface.

When leaf segments of the four plants were examined with SEM, conidiophores and conidia of *Neotyphodium* were observed on segments from three of four plants, and conidia on segments from the other plant. As with results from leaf impressions, hyphae were very sparsely distributed and usually aligned with the longitudinal junctions between epidermal cell walls (Figs. 2–4).

At least one and usually all leaf segments of any given harvested plant produced *Neotyphodium* growth from one or both cut ends of the segments. Resultant colonies were slow growing (ca. 6 mm radial growth in 40 days, counting the time taken to emerge from cut ends), off-white to cream, smooth to slightly (almost imperceptibly) velutinous, later (approx. 30 days) with

Figure 4. Conidiophore with conidium displaying typical flattening on one side. SEM. Bar = 3 µm.

Figure 5. NexHbv1, isolated from leaf surface, on 1/2 V8 agar. Conidiophore (big arrow) and adjacent, detached conidium (little arrow). DIC, oil immersion. Bar = 5 µm.

Figure 6. NexHbv2, isolated from leaf interior, on 1/2 V8 agar. Conidiophore (big arrow) with attached conidium (little arrow). DIC, oil immersion. Bar = 5 µm.

patches of white, low flocculent mycelium, dense and compact, with a cream-colored reverse. Occasionally segments that had not been surface-disinfested were overgrown with *Cladosporium* or *Penicillium* colonies. Leaf segments not disinfested frequently produced *Neotyphodium* colonies from areas along the segment length in addition to colonies originating from cut ends. One disinfested segment produced a *Penicillium* colony.

When the first set of leaves was washed on 18 April and the resulting suspensions plated to StrPen PDA, washings from two of five plants produced only *Neotyphodium* colonies. One such plant produced one to three such colonies per plate, while the other produced ca. 30–50 such per plate. One remaining plant also produced one to four *Neotyphodium* colonies on three of four 'wash' plates, all of which produced mostly *Cladosporium* spp. and/or *Penicillium* spp. 'Wash' plates from the other two plants were overgrown with *Cladosporium* sp. and/or *Penicillium* spp. before any of the slow-growing *Neotyphodium* colonies were detected.

When the second set of leaves was washed on 28 April and the resulting suspension plated to StrPen PDA, washings from four plants were overgrown with *Penicillium* or *Cladosporium* colonies before any *Neotyphodium* colonies could be seen. Washings from three other plants developed ca. 10–60 *Neotyphodium* colonies per 'wash' plate. In all cases, *Neotyphodium* colonies grew from disinfested leaf segments, but non-disinfested segments were sometimes overgrown with *Penicillium* or *Cladosporium* prior to appearance of *Neotyphodium*.

No consistent differences were noticeable in amount of mycelium visible on primary, secondary or tertiary leaves, nor between leaves of various ages, although leaf washings from one tertiary leaf produced colonies (ca. 30–50, above) far in excess of colonies produced from other leaves. On the second set of washings, washings from one plant produced more colonies (ca. 50–60 per plate) than those of other plants (ca. 10 per plate).

Endophyte identification and phylogenetic analysis

When revived from liquid nitrogen and plated to 1/2 V8, NexHbv1 and NexHbv2 both produced cream-colored, finely velutinous, slightly mounded colonies, pale dirty cream in reverse, with radial growth averaging 5 mm in 27 days and with glabrous margins of approximately 1–1.5 mm. NexHbv1 produced conidia 3.2–7.3 × 2.2–3.5 µm (mean of 5.6 × 2.9 µm) from awl-shaped conidiophores 11.2–41.4 × 1.3–2.4 µm (mean of 21.0 × 1.9 µm) (Fig. 5). NexHbv2 produced conidia 3.9–7.1 × 2.2–3.4 µm (mean of 5.7 × 2.8 µm) from awl-shaped conidiophores 11.2–28.6 × 1.1–2.8 (mean of 19.5 × 1.8 µm) (Fig. 6). In each strain, conidia displayed a tendency toward flattening on one side, and

conidiophores tapered to a conidiogenous locus approximately 1 µm in diam (Figs. 5 and 6). ANOVA detected no significant differences between the strains with regard to length and width of conidia or conidiophores.

The 550 bp sequenced for ITS1, 5.8S and ITS2 of NexHbv1 and NexHbv2 were 100% identical. The data matrix contained 29 taxa, each with 583 characters (including gaps and missing data); 482 characters were constant. Base frequencies were homogeneous across taxa (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 rounds of successive approximations, the most likely, equilibrated tree was found with a -ln likelihood = 1607.1448 and the following parameters: a transition/ transversion ratio equal to 2.407115; base frequencies A:0.221448 C:0.300989 G:0.257578 T:0.219985; gamma shape parameter: 0.352019. Of the 1000 trees sampled from the Bayesian analysis, 31 trees were identical to the most likely tree and no tree had a lower likelihood score. The 1000 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. 7 with branch support above nodes. The *Hordeum* endophyte grouped with *Epichloë glyceriae* (Fig. 7).

Viability of conidia

Conidia of NexHbv2 produced on 1/2 V8 proved capable of nearly 100% germination and growth after 48 hrs on Difco PDA.

4. Discussion

Because *Neotyphodium*-like isolates recovered from the cut ends of surface disinfested leaf segments were virtually identical in colony morphology and microscopic characters to those recovered from leaf washings, and because representative strains from leaf washings (NexHbv1) and disinfested leaf segments (NexHbv2) were indistinguishable morphologically and by ITS sequence data, we assume that the *Neotyphodium*-like fungi seen in leaf impressions and SEM are conspecific. Placement in the phylogenetic tree (Fig. 7) confirms that they are members of the genus *Neotyphodium*. Verification of close relatedness to *E. glyceriae* is contingent on sequence analyses of additional genes and congruence of gene topologies (Schardl, 2001).

The epiphyllous growth habit of the *Neotyphodium* endophyte of wild barley appears intermediate between the reticulate hyphal nets formed on the leaf surfaces of various hosts (*Bromus setifolius* Presl., *Festuca ovina* var.

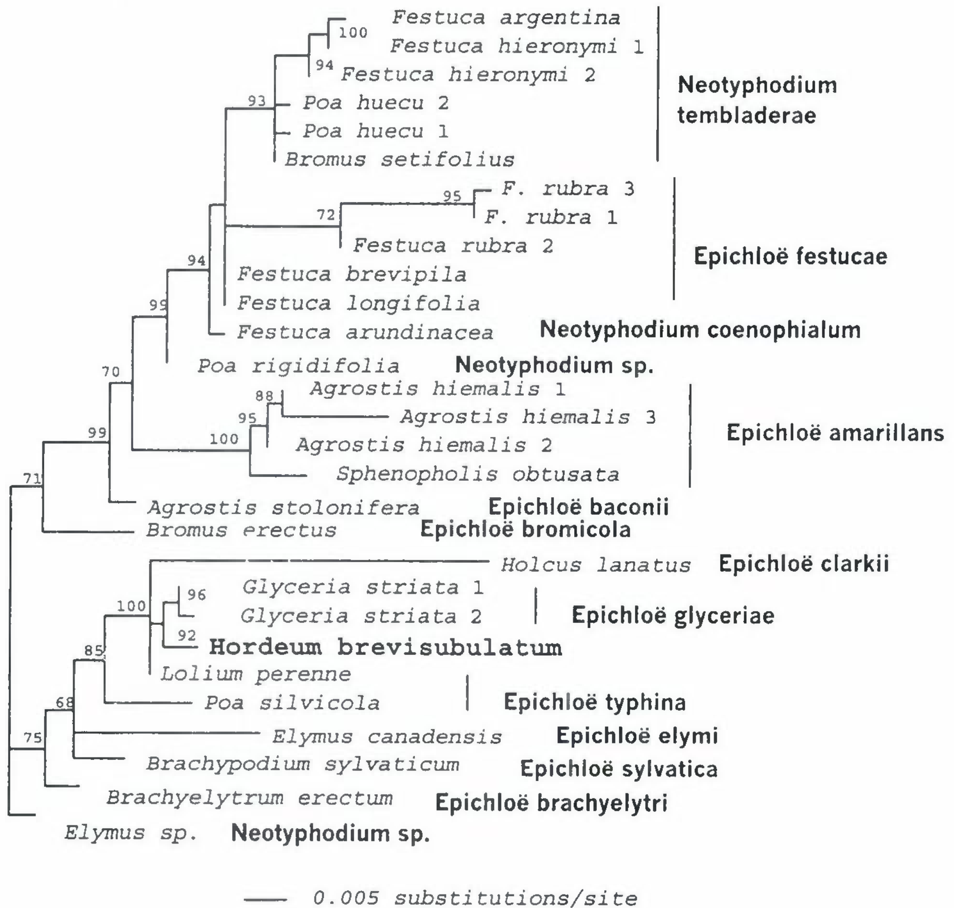


Figure 7. Maximum likelihood tree using the HKY85 evolutionary model. Branch support indicated above nodes results from partition frequencies of a majority rule consensus tree constructed from 1000 trees samples from a 520,000 generation Bayesian analysis. Nodal taxon labels are of the endophyte host, instead of endophyte itself.

glauca (Lam.) Koch., and *F. rubra* L.) by their respective symbionts, and those in which hyphae of symbionts were not detected on leaf surfaces (*F. arundinacea* Schreb. and *Lolium perenne* L.) (Moy et al., 2000). Results here more closely resemble the growth habit of *Neotyphodium typhinum* (Morgan-Jones & W. Gams) Glenn, Bacon & Hanlin on *Agrostis hiemalis* (Walt.) B.S.P. and *Poa rigidifolia* Steudel, characterized as a "sparse, superficial layer of mycelium – parallel to the long axis of the leaf and – located within the surface grooves at the interface of adjacent epidermal cells" (White et al.,

1996). Observations on *Lolium pratense* (Huds.) S.J. Darbyshire (= *Festuca pratensis* Huds.) were similar with regard to intensity of colonization and growth habit (Craven et al., 2001). However, in our studies the extent of mycelial growth was even more restricted. Moy et al. (2000) reported some progression in mycelium development with increasing leaf age. Although we could discern no analogous progression, it should be borne in mind that our experiments were conducted in an artificial environment over a relatively short time frame. The internal distribution of endophytic hyphae in wild barley PI 440420 and instances of egression of hyphae to the exterior have been reported (Youssef and Dugan, 2000).

It seems unlikely that the very sparsely distributed external hyphae of the *Neotyphodium* of wild barley would exclude pathogenic fungi from the epidermal surface via 'niche exclusion' as was proposed as a possible function of mycelial nets by Moy et al. (2000). No reports of protection against pathogenic fungi by endophyte infection of *H. brevisubulatum* are known to us and published reports for protection against phytopathogens in other endophyte-grass relationships are relatively few (Moy et al., 2000). Although we never witnessed spores or conidiophores of other (non-*Neotyphodium*) fungi on leaf impressions, contaminating fungi were sometimes present on leaf surfaces as indicated by results of leaf washings and plating of non-disinfested leaves to agar. Saprophytic fungi commonly grow from surface-disinfested, asymptomatic grass seed, even when disinfestation is considerably more stringent than employed here (Dugan, unpublished results), hence we anticipated contamination of a portion of our plants. We suspect the single instance of *Penicillium* from a disinfested leaf segment arose from a conidium lodged inside a stoma or covered by cuticular waxes.

We also believe that such sparsely distributed external hyphae seem unlikely to deter herbivory by arthropods feeding at the leaf surface. Clement et al. (1997) have demonstrated that protection of another species of wild barley, *Hordeum bogdanii* Wilensky, from aphids is dependent on endophyte genotype and correlated with in vivo alkaloid production. In the same set of experiments, endophyte-infected PI 440420 strongly deterred herbivory compared to germplasm of the same clone freed of endophyte by treatment with propiconazole, and deterrence versus non-deterrence was again correlated with in vivo alkaloid production (Clement et al., 1997). Although hyphal densities in or on the leaves subjected to aphid feeding were not measured by Clement et al. (1997), their results, together with our own, strongly suggest that high external or internal hyphal densities are not a prerequisite for such protection. Studies with endophyte-infected *F. arundinacea* and *L. perenne*, both found free of epiphyllous mycelial nets by Moy et al. (2000), have demonstrated effective insect resistance due to endophytic infection (Clement et al., 1994; Clement et al., 1996; Clement et al., 2001). Because deterrence has

been found in instances in which epiphyllous growth is apparently lacking or highly restricted, and because such deterrence has also been linked to alkaloid production, it is probable that alkaloid-producing internal hyphae are sufficient to explain many instances of resistance to herbivory.

For the above reasons, the external growth and sporulation of the *Neotyphodium* on epidermal surfaces of *H. brevisubulatum* seems unlikely to significantly contribute to deterrence of pathogens or herbivory, although such factors may contribute to deterrence in other symbioses. But the production of even limited numbers of conidia on the leaf surface poses the possibility of horizontal transfer in nature. It remains to be determined whether such conidial production is a survival strategy, or merely a fortuitous event or artifact without adaptive value.

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