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Prevalence of an intraspecific *Neotyphodium* hybrid in natural populations of stout wood reed (*Cinna arundinacea* L.) from eastern North America

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Abstract: Members of genus *Neotyphodium* are asexual derivatives of sexual *Epichloë* species and maintain endophytic relationships with many cool-season grasses. Most *Neotyphodium* species analyzed so far are interspecific hybrids with combined or partial genomes of two or three ancestral species. In this study we characterized *Neotyphodium* isolates from *Cinna arundinacea*, a perennial cool-season grass from eastern North America. A total of 23 isolates grouping into two distinct morphotypes were obtained from five local populations of *C. arundinacea*. PCR amplification and cloning of translation-elongation factor 1-α (*tefA*) and β-tubulin (*tubB*) genes of 10 isolates comprising both morphotypes (two isolates per location) revealed that all 10 contain two copies of *tefA* and *tubB* genes. Surprisingly phylogenetic analysis of mainly non-coding sequence from these genes revealed that both copies in each isolate were inherited from *Epichloë typhina* ancestors, indicating that the *C. arundinacea* endophytes arose through intraspecific hybridization between two *E. typhina* progenitors with extant relatives infecting hosts *Poa nemoralis* and *Poa pratensis*. Furthermore the *tefA* sequences were identical between isolates, as were *tubB* sequences, despite obvious morphological differences. Profiling of alkaloid biosynthetic genes from these isolates indicated the presence of the peramine biosynthetic gene (*perA*) and the absence of genes required for biosynthesis of lolines, indole-diterpenes and ergot alkaloids. Thus this endophyte is potentially capable of producing peramine in planta and providing protection to its host from insect pests. The absence of genes for indole-diterpenes and ergot alkaloid biosynthesis makes this endophyte a candidate for agricultural applications. Based on our phylogenetic analysis, alkaloid profiling and description of morphological characteristics, we propose the name *Neotyphodium schardlii* for these isolates from *C. arundinacea*, a new member of genus *Neotyphodium* and the first described to have arisen through intraspecific hybridization.

Key words: endophyte, ergot alkaloids, indole-diterpenes, lolines, *Neotyphodium schardlii*, peramine

INTRODUCTION

Neotyphodium species are asexual derivatives of genus *Epichloë*, endophytic symbionts of cool-season grasses (White 1987). These fungi grow systemically between plant cells in the vegetative and reproductive tissues of the host without eliciting any obvious symptoms of infection (Schardl and Phillips 1997). As the grass matures and produces inflorescences the mycelium grows into developing ovules and colonizes the scutellum and embryo axis of the seed (Philipson and Christey 1986), thus enabling the fungus to transmit vertically to the next host generation. The relationships between *Neotyphodium* species and their grass hosts are often mutualistic, wherein the endophyte obtains nutrition and shelter from the host and in return confers on the host tolerance to a variety of biotic and abiotic stresses (Clay and Schardl 2002). Among the fitness benefits provided by these fungi to their hosts are improved drought tolerance and competitive abilities (Arechevaleta et al. 1989), enhanced tillering (Belesky et al. 1987) and potent chemical defense against a variety of insect and mammalian herbivores (Bacon et al. 1977, Fletcher and Harvey 1981, Kennedy and Bush 1983, Clay 1990).

Neotyphodium and *Epichloë* species produce a variety of bioprotective alkaloids including ergot alkaloids, pyrrolopyrazine (peramine), aminopyrrolizidines (lolines) and indole-diterpenes, including lolitrem B (Siegel 1990, Bush et al. 1997, Schardl et al. 2007a). Peramine and lolines exhibit insecticidal properties, whereas ergovaline (an ergot alkaloid) and lolitrem B

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TABLE I. Seed collection locations, GPS coordinates, dates of seed collection and seedling production

Location	GPS coordinates		Seed collection	Seedling production
	Latitude	Longitude		
Yellowwood by dam	N 39°10′36″	W 86°20′30″	Oct 2006	Nov 2008
Bayles Road by creek	N 39°13′10″	W 86°32′31″	Oct 2006	Nov 2008
Lilly-Dickey Woods	N 39°14′54″	W 86°13′05″	Oct 2006	Nov 2008
Scarce O'Fat Road	N 39°09′39″	W 86°20′51″	Sep 2006	Nov 2008
P.J.'s backyard	N 39°11′54″	W 86°29′09″	Sep 2006	Nov 2008

are poisonous to grazing livestock. *Neotyphodium* × *coenophialum* and *N. lolii* have been established as the causal agent of fescue toxicosis in cattle (Bacon et al. 1977, Schmidt et al. 1982) and ryegrass staggers in sheep (Fletcher and Harvey 1981, Latch et al. 1984) respectively. Therefore the relative importance of these endophytes in agriculture and natural ecosystems depend partly on their abilities to produce different types of alkaloids.

Twenty *Neotyphodium* spp. have been described so far based on morphology, host specificity and molecular characteristics (Moon et al. 2007, Chen et al. 2009, Ji et al. 2009, Kang et al. 2009). Most are interspecific hybrids (with hybrid status indicated by an × between genus and species epithets) with combined genomes or partial genomes of two or sometimes three ancestors (Tsai et al. 1994, Craven et al. 2001a) and originating from grass hosts belonging to tribes Poeae, Meliceae, Stipeae, Bromeae or Triticeae (Moon et al. 2004, 2007). Several *Neotyphodium* spp. are morphologically very similar and they all lack the sexual stage. Therefore the use of molecular information (primarily gene sequence data) has been a particularly powerful tool to discriminate among *Neotyphodium* species. The phylogenetic analysis of partial sequences of the translation elongation factor 1-α gene (*tefA*), β-tubulin gene (*tubB*) and actin gene (*act1*) are most commonly used to study the relationships among the members of *Epichloë* and *Neotyphodium* spp. (Craven et al. 2001a, b; Gentile et al. 2005).

In this study we used morphological and molecular phylogenetic approaches to characterize the endophytic inhabitants of stout wood reed (*Cinna arundinacea* L.), a perennial grass of eastern North America (Brandenburg et al. 1991). We describe the first *Neotyphodium* sp. resulting from an intraspecific hybridization between two genetically distinct *Epichloë typhina* populations originating from two hosts, wood bluegrass (*Poa nemoralis* L.) and Kentucky bluegrass (*Poa pratensis* L.). These findings expand on our current conception of *Epichloë/Neotyphodium* endophyte evolution and suggest the possibility of hybridization via sexual mating.

MATERIALS AND METHODS

Biological materials.—Plants (*Cinna arundinacea*) used to isolate endophytic fungi in this study were grown in the greenhouse from seeds collected from five locations near Bloomington, Indiana, USA. Information on the sampling locations, their GPS coordinates, and dates of seeds collection and seedling production are provided (TABLE I). A total of 10 endophyte isolates consisting of two representatives from each location were used for molecular characterization. Herbarium specimens were prepared as described by Pollack (1967) and deposited in the Cornell University Plant Pathology Herbarium (CUP).

Isolation of endophytes from plant tissues.—The youngest mature leaves and pseudo stems of endophyte-infected tillers were used to isolate the fungal endophyte. Leaves and tillers were cut into 2.5–3.0 cm pieces and surface sterilized (95% ethanol for 30 s, 70% ethanol for 60 s, 0.6% sodium hypochlorite for 10 min) followed by three washes with sterile distilled water. Plant materials were blot dried, cut into 2–3 mm pieces and placed on potato dextrose agar (PDA) plates amended with 100 μg mL^{-1} ampicillin, sodium salt (Agri-Bio, Bay Harbor, Florida). Plates were sealed and incubated in the dark at 22 C and examined regularly for endophyte growth for up to 2 mo. Endophytic fungi isolated in this manner were subjected to successive hyphal tipping to obtain a pure culture (Whitney and Parmeter 1963, Leslie et al. 2006).

Morphological examination.—Colony morphology was examined from cultures grown on PDA. Plates were inoculated with a 20 μL suspension of freshly ground mycelium isolated in an agar block (ca. 1.5 mm^3 in 100 μL sterile water) taken from the periphery of freshly grown colonies. Plates were incubated at 22 C in the dark, and measurements of radial growth were taken from 12 colonies for each isolate at 21 d post inoculation and averaged. Cultures were photographed and preserved as herbarium specimens.

Microscopic observations for fungal structures were performed after inoculating 1.5% water agar plates with a 20 μL suspension of freshly ground mycelium in an agar block as described above. Plates were incubated at 22 C in the dark for 10 d with regular monitoring for conidia production. Agar blocks from water agar cultures containing actively conidiating mycelium were placed on a slide, and cover slips were lightly pressed on the surface to remove air pockets. Fungal structures were examined with an Olympus BX41TF light microscope (Olympus Corp., Tokyo, Japan) at 1000× magnification with oil immersion optics.

Images were captured with an Olympus DP71 digital camera supported by DP-BSW application software (Olympus Corp., Tokyo). Images were used to measure length and width of mature conidia and length of conidiogenous cells as well as width at base and tip. The means and standard deviations were computed for each parameter based on 20 measurements for each isolate.

DNA isolation.—Hyphal material was taken from the edge of an actively growing colony in a PDA block (ca. 1.5 mm^3) and ground in a microcentrifuge tube containing 300 µL sterile water. The mycelial suspension was transferred to a sterile flask containing 50 mL potato dextrose broth. The culture was incubated at 22 C with shaking at 150 rpm 2–3 wk. The mycelium was harvested through filtration with Whatman filter paper, freeze dried at −80 C and lyophilized. Genomic DNA was extracted from 10 mg lyophilized mycelium with the DNeasy Plant Mini Kit (QIAGEN Inc., Valencia, California) as per manufacturer's instructions.

DNA amplification and sequencing of tefA *and* tubB *genes.*—DNA fragments representing partial sequences of *tefA* and *tubB* were amplified from 10 isolates by polymerase chain reaction (PCR) with 10–15 ng total genomic DNA template as described by Craven et al. (2001a, b). PCR products were purified with the QIAquick Spin Kit (QIAGEN Inc., Valencia, California) and bidirectional sequencing (with PCR primers for *tefA* and *tubB*) was performed with BigDye Terminator 3.1 standard procedure. Sequencing reactions were purified with Agencourt CleanSEQ dye-terminator removal system (Beckman Coulter Inc., Brea, California) following the manufacturer's protocol with Biomek FXP (Beckman Coulter Inc.). Purified sequencing samples were analyzed with the 3730 XL DNA analyzer (Applied Biosystems Inc., Foster City, California) following the manufacturer's protocol.

The sequence traces from all 10 isolates indicated the presence of multiple alleles for both genes evaluated. Therefore the individual alleles were isolated separately by cloning the PCR product with a TOPO TA cloning kit (Invitrogen Corp., Carlsbad, California). Clones selected (12 clones $isolate^{-1}$ $gene^{-1}$) by differential screening were grown in 96-well plates in liquid media (Terrific Broth) 20 h at 470 rpm in the HiGro microwell plate growth system (Genomic Solutions Inc., Ann Arbor, Michigan). Plasmid DNA isolation was performed by standard procedures with solutions I, II and III on a Biomek FXP (Beckman Coulter Inc.). DNA sequencing was performed as described above except that primers used for bidirectional sequencing were SP6 (5′-ATTTAGGTGACACTATAG-3′) and T7 (5′-TAATACGACTCACTATAGGG-3′). Gene sequences were manually inspected and appended into contigs with the bioinformatics software program Sequencher 4.9 (Gene Code Corp., Ann Arbor, Michigan). The unique *tefA* and *tubB* gene sequences were identified and deposited at GenBank (accession numbers HM138504–HM138507).

Phylogenetic analysis of tefA *and* tubB *genes.*—The sequences obtained from the *C. arundinacea* endophytes were aligned along with sequences from representative *Epichloë* species: *E. amarillans*, *E. baconii*, *E. brachyelytri*, *E. bromicola*, *E. clarkii*, *E. elymi*, *E. festucae*, *E. glyceriae*, *E. sylvatica* and *E. typhina* with Clustal W (Thompson et al. 1994). Parameters were adjusted empirically; a gap penalty of one and a gap extension penalty of zero resulted in a reasonable alignment of intron-exon junctions and introns. Alignments were checked manually for ambiguity and adjusted if needed. Alignments for both *tefA* and *tubB* genes are deposited at TreeBase (http://purl.org/phylo/treebase/phylows/study/TB2:S10445).

Maximum parsimony (MP) analysis employed the branch and bound option in PAUP* 4.0b10 (Swofford 1998) for exact solutions. For parsimony analysis the character changes were unweighted and unordered and gaps were treated as missing information. Robustness of inferred phylogenies was estimated by bootstrap replication, and branches receiving 70% or higher bootstrap values were considered well supported.

For likelihood (ML) analysis parameters including the proportion of invariable sites, nucleotide frequencies and substitution rates, and gamma shape parameter were estimated from the sequence dataset with ML implemented in ModelTest 3.06 (Posada 2006) and used as the starting parameters (AIC selected model) for the subsequent analysis. The ML tree was generated by 10 iterations of random sequence additions, followed by tree-bisection-reconnection. Starting branch lengths were obtained with the Rogers-Swofford approximation method implemented in PAUP.

Alkaloid gene profiling.—Oligonucleotide primers were used in PCR tests for genes associated with production of peramine, lolines, indole-diterpenes and ergot alkaloids from four test isolates (two isolates $morphotype^{-1}$) along with *Epichloë typhina* isolate E1022 and *Epichloë festucae* isolates E2368 and Fl1, known to contain gene clusters required for the biosynthesis of their respective alkaloidal compounds and therefore useful as positive controls. Primers used to test for presence of the peramine, loline, indole-diterpenes and ergot alkaloid biosynthesis genes are provided (TABLE II).

TAXONOMY

Neotyphodium × schardlii S.R. Ghimire, J.A. Rudgers et K.D. Craven, sp. nov. FIG. 1

MycoBank MB518750

Coloniae in PDA albae, gossypinae, 5–10 mm diam aetate 21 diarum ad 24 C, elevatae, leviter vel moderate convolutatae, saepe coactae margine irregulari vel laevi. Coloniae reversae eburnae vel spadiceae dilutae. Hyphae vegetativae hyalinae, septatae, 2.0–3.5 µm latae. Cellulae conidiogenae hyphis exorientiae solitariae, abundae, laterales, orthotropae, cylindraceae ad basim, gradatim angustiorae ad apicem, hyalinae, determinatae, plerumque nonsepatae, 9–24 µm longae, 1.3–2.8 µm latae in base, 0.8–1.3 µm ad apicem. Conidia asymmetra, navicularia vel reniformia, hyalina, levia, saepe transversaliter affixa in apice cellulae conidiogenae, 6.5–8.5 × 2.3–3.3 µm. Affinis genetice *Epichloës typhina* (Pers. : Fr.) Tul. ex hospitibus *Poa nemoralis* et *Poa pratensis*.

TABLE II. Primers used for alkaloid analysis and expected sizes of the PCR products

		Primer 1		Primer 2		
Alkaloid	Gene	Name	Sequence (5′ to 3′)	Name	Sequence (5′ to 3′)	Size (bp)
Peramine	*perA*	per5′-F	CATCCATGACGTGCCAAACAATC	per5′-R	TCGCAGCTGCAAGTCGAGCAC	697
		neo-per-F	CGTCGTGGTAACGCACGCAAACG	neo-per-R	CAGTCTGCCTTGCCGACCGGGGT	651
		per3′F	CGACGACTGGCTGTGGAGGATG	per3′-R	CTAGCCTCCAGATCTTGTGAAAG	519
Loline	*lolA*	lolA-F	GAGACACTAGAGAAATGGCAGCTGC	lolA-R	GGCATCCATGGTGGCGAAGATGTG	273
	lolC[a]	lolC-5′a	GTTGCCCACGGTGCGCGTCTTC	lolC-3′a	GGTCTAGTATTACGTTGCCAGGG	461
	locD	lolD-F	CTCGACGTTTCAACAGATTGCAG	lolD-R	GTCTTTGAAGACAAGCCAGTCC	430
	lolE	lolE-F	ACCAAGCCAACGGATATCTTCGC	lolE-R	ACGTCTTTGGTCCGTCTTGTTAG	587
	lolF	lolF-F	CTCTGATATGAAGACTCCTGAGC	lolF-R	GCCAAGCGGAGTTCAGATCATCC	635
	lolO	lolO-F	GTGAACTGGCAGTAGTCCGTATG	lolO-R	AATCCATGCCAGTGTCGGGAATG	719
	lolP	lolP-F	GTTCTAAACATCGTGACTGGGC	lolP-R	GGTAGGTCAGCATCTTGTCAACG	566
	lolT	lolT-F	CACTGACCTCCAAGTATACTTGC	lolT-R	CGTCATCCCGACCTCTTTCGGAT	545
	lolU	lolU-F	CGATGGTTGGATCAGTCGTTGC	lolU-R	GAGCTGATGCGGCATTGGCATC	658
Indole-Diterpene	*ltmB*[a]	ltmB-345	AACATCGCCTGGGAGCTCGTATA	ltmB-346	CGCAGGTCCTATTTCCATCGC	239
	ltmC[a]	ltmC-278	GAAACTGCCAATCGAGCATA	ltmC-279	TTCTTGCAATCATTTTGCAATTG	403
	ltmE[a]	ltmE-356	CCGAGTTTGATGACCTGCTG	ltmE-341	TTCCGCTTCCGAGTAGACTC	687
	ltmF[a]	ltmF-359	GAATTATGTTACTCTTGGGG	ltmF-360	AAGTTGGCACATAGGTCTTC	227
	ltmG[a]	ltmG-156	GCACAAACAATAAATTCGGCCAA	ltmG-157	AATTTGCCCTCTGTTAAATCCTC	383
	ltmJ[a]	ltmJ-205	CCAAGCATCGATTTGTCACC	ltmJ-206	AATCTGATCGCCATCTTTGC	242
	ltmK[a]	ltmK-160	ATATTGAATTGCTGCGTGAGGAG	ltmK-161	AGAGGCCAAGAAGCGGCCTGGACA	568
	ltmM[a]	ltmM-158	GTGATCGGTGCTGACGGGGTCCA	ltmM-159	TATCGCCATATTTGCTCCTTGCCC	669
	ltmP[a]	ltmP-280	ATGGCTGTCATTCATACAACAGCTATG	ltmP-281	AGCGTCCCGGACAGGCATATCTCCCA	508
	ltmQ[a]	ltmQ-313	CTACCAGGACAGGCGTGACGTCC	ltmQ-282	CAGAGGTTTAACCCTCTTGACGC	334
Ergot alkaloid	*easA*	easA-F	ATCTACCACAAGCTTGGCGGAC	easA-R	GCGGTTGCATTGAGAATCGCTC	349
	easE	easE-F	GTTCTGGTGTTCGTTGGCAATG	easE-R	CAGACTCGCTTCAGATTCACAC	387
	easF	easF-F	TCAAGCTGCGAGGCACTTACGTC	easF-R	GTTCTCGACATTCGTCTTGCTAC	415
	easG	easG-F	AAGAAAGGTGGACCTGCCATGG	easG-R	CTTCTCATCCGTTAATGTGCGG	316
	easH	easH-F	AGATATGGCATCGTGACCAGCC	easH-R	GGCATGTAGCATCAAATGGTGTC	332
	dmaW	neo-dmaWF4	GTGTACTTTACTGTGTTCGGCATG	dmaW6R	GTGGAGATACACACTTAAATATGGC	281
	lpsA	lpsA5′-F	ATGTCCGTGGATGGTGAGCAAAC	lpsA5′-R	ATGCAATGGGTGCCTGTCCGTTC	591
		lpsA-mid-F	CGCTGCTCTCTGTCATGCAGAAG	lpsA-mid-R	GTTCCTCATCCAACATCTCGATC	515
		lpsA3′-F	CGATCACCCAACTTTACATGATAC	lpsA3′-R	ACAAGTCATGGTCCGGATGTGTTG	604
	lpsB[a]	DamP9	GTATCGAAGAATGCTGAAGC	DamP16	CATTGTATGCTGCCTTGACC	186
		p12-F	CCGTCTTCCCGTATACCGAA	p12-R	TACCCACTGCCTCGAACTTG	597

[a] These genes were amplified using primers published in Spiering et al. 2002 (*lolC*); Young et al. 2009 (*ltm* genes); Tanaka et al. 2005 and Johnson et al. 2007 (*lpsB*).

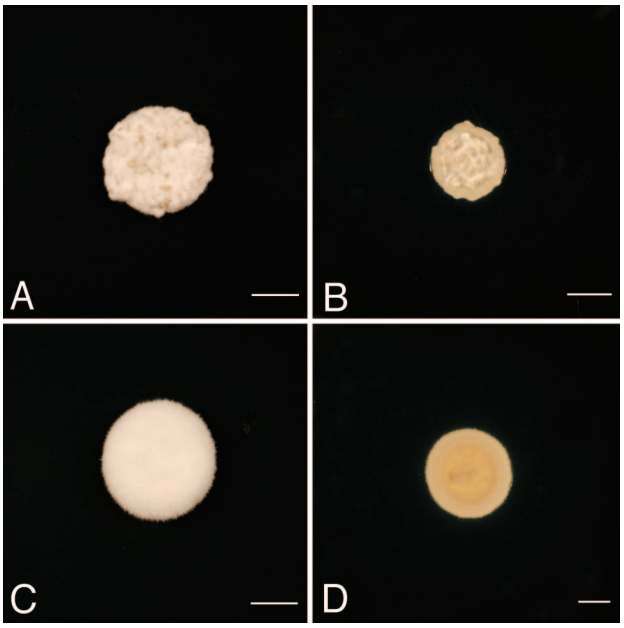

FIG. 1. Photographs of colony morphology of *N. schardlii* isolates from *C. arundinacea*. Cultures were grown 3 wk on PDA. Top represents morphotype I (A = surface, B = reverse) and bottom represents morphotype II (C = surface, D = bottom). Bars = 5 mm.

Colonies on PDA white, cottony, slow growing, attaining 5–10 mm diam in 21 d at 24 C. Colonies raised from agar surface, slightly or moderately convoluted, often felted and irregular or smooth margin, and colony reverse is tan to pale brown. Vegetative hyphae hyaline, septate, 2.0–3.5 µm wide. Conidiogenous cells arising solitarily from hyphae, produced abundantly, lateral, orthotropic, cylindrical at the base, tapering toward the apex, hyaline, determinate, usually lacking septa at or near the base, 9–24 µm long, 1.3–2.8 µm wide at the base, 0.8–1.3 µm wide at the apex. Conidia navicular asymmetric to reniform, hyaline, smooth, frequently oriented transversely across the conidiogenous cells after detachment, 6.5–8.5 × 2.3–3.3 µm. Genetic relationships to *Epichloë typhina* isolates from hosts *Poa nemoralis* and *Poa pratensis*.

Etymology. This endophyte is named in honor of Prof Christopher L. Schardl whose studies have greatly facilitated concepts of *Epichloë* and *Neotyphodium* taxonomy and evolution.

Holotype. USA, Near Bloomington, Indiana, infecting *Cinna arundinacea*, 2006, leg. K.D. Craven, CUP-067888 and CUP-067889.

Known host range. *Cinna arundinacea* L.

Known distribution. As endophytic fungi inhabiting aerial tissues and seeds of *Cinna arundinacea* grown near Bloomington, Indiana.

Specimen examined. ATCC MYA-4679 and ATCC MYA-4680 from the HOLOTYPE. CUP-067888 and CUP-067889 from seedlings of *Cinna arundinacea*, near Bloomington, Indiana, 2006, leg. K.D. Craven.

RESULTS

Characteristics of Cinna arundinacea *endophytes*.—Endophytes were isolated from surface-sterilized plant tissues of *Cinna arundinacea* grown in the greenhouse from seeds collected from five locations near Bloomington, Indiana. The endophyte infection frequency was variable among sampling locations (30–80%) with an average frequency of 46%. Altogether 23 isolates were recovered, and all grouped within one of two distinct morphologies henceforth referred as morphotype I or morphotype II (FIG. 1). Morphotype I and morphotype II constituted respectively 30% and 70% of the total endophyte isolations.

We summarized the results of our morphological examination along with those for other *Neotyphodium* and *Epichloë* species (TABLE III). Both morphotypes produced white, cottony and slow growing colonies on PDA but they differed in terms of the degree of felting, colony convolution, type of colony margin and colony growth rates (FIG. 1). Morphotype I formed smaller colonies than morphotype II (6.2 ± 0.2 mm diam vs. 8.7 ± 0.1 mm diam at 3 wk), and shorter conidiogenous cells (12.8 ± 0.5 µm vs. 18 ± 0.6 µm, FIG. 2). Similarly the colony margin of morphotype I was irregular but morphotype II had smooth margins (FIG. 1).

Phylogenetic relationships.—Irrespective of morphological differences the PCR amplification of all 10 test isolates from *C. arundinacea* yielded products from two copies of the *tefA* gene. One copy grouped with that of *E. typhina* isolates from *Poa nemoralis* and the other copy with *E. typhina* isolates from *Poa pratensis*. Copies from each isolate grouping with the same extant *Epichloë* species were identical in sequence. Phylogenetic analysis of these sequences with representative isolates from each *Epichloë* species resulted in 24 MP trees (one is shown in FIG 3). All branches inferring relationships of *tefA* copies from *C. arundinacea* endophytes were supported by bootstrap values of ≥ 88, and all MP trees supported these same inferred relationships (consensus tree not shown).

All 10 endophyte isolates from *C. arundinacea* also yielded PCR products from two copies of the *tubB* gene. Similarly to the *tefA* gene one copy grouped with that of *E. typhina* isolates from *Poa nemoralis* and the other copy with *E. typhina* isolates from *Poa pratensis*. Phylogenetic analysis on the alignment of these sequences with representative isolates from each *Epichloë* species resulted in four MP trees (one

TABLE III. Morphological characteristics of some *Epichloë* and *Neotyphodium* species

Endophyte	Host	Growth on PDA (mm/wk)	Conidial shape	Conidia size (µm)	Conidiogenous cell (µm)		Reference
					Length	Width	
E. typhina	*Dactylis glomerata*	Nd[a]	Lunate to reniform	4.5–5.3 × 2.1–2.9	17–28	1.7–2.7	(White 1993)
E. clarkii	*Holcus lanatus*	Nd[a]	Lunate to reniform	4.0–4.8 × 1.8–2.0	28–40	1.8–2.2	(White 1993)
N. huerfanum	*Festuca arizonica*	7 at 20 C	Cylindrical to lunate	3.0–5.0 × 2.0–3.0	13–25	1.5–3.0	(White et al. 1987)
N. australiense	*Echinopogon ovatus*	2.6–3.8 at 22 C	Ellipsoidal to lunate	5.0–7.0 × 3.0–4.0	11–25	ca. 2.0	(Moon et al. 2002)
N. tembladerae	*Festuca argentina*	4.9–7.0 at 24 C	Lunate, reniform and sigmoid	4.0–10.0 × 2.0–4.0	10–31	1.5–3.5	(Cabral et al. 1999)
N. melicicola	*Melica racemosa*	2.0–3.8 at 22 C	Ellipsoidal to lunate	5.0–8.0 × 3.5–5.0	6–35	ca. 2.5	(Moon et al. 2002)
N. siegelii	*Lolium pratense*	4.7 at 24 C	Navicular asymmetrical to reniform	6.0–8.0 × 2.5–3.5	12–24	1.5–3.0	(Craven et al. 2001a)
N. sinofestucae	*Festuca parvigluma*	2.0–4.9 at 24 C	Ellipsoid to reniform	5.2–6.3 × 2.6–3.1	19–28	2.4–2.7	(Chen et al. 2009)
N. sinicum	*Roegneria* spp.	2.0–4.9 at 25 C	Reniform	4.8–5.9 × 2.1–3.4	16–24	1.8–2.5	(Kang et al. 2009)
N. schardlii (morphotype I)	*Cinna arundinacea*	1.6–2.7 at 24 C	Navicular asymmetrical to reniform	6.5–8.5 × 2.5–3.3	9–17	1.3–2.3	
N. schardlii (morphotype II)	*Cinna arundinacea*	2.7–3.3 at 24 C	Navicular asymmetrical to reniform	6.5–8.5 × 2.3–3.0	15–24	1.5–2.8	

[a] nd = not determined.

FIG. 2. Hyphae, conidiogenous cells and conidia of *N. schardlii* morphotypes I (A–B) and morphotypes II (C–D). Bars = 10 µm.

shown in FIG. 4). All branches inferring relationships of *tubB* copies from *C. arundinacea* endophytes were well supported by bootstrapping, and all MP trees were identical in their placement of *tefA* and *tubB* genes.

All relationships regarding placement of the gene copies from the *C. arundinacea* endophytes were identical between MP and ML analyses. Therefore ML trees are not shown.

Alkaloid gene profiling.—Four isolates of *C. arundinacea* endophytes (two from each morphotype) were tested by PCR for the presence of genes encoding proteins involved in the biosynthesis of the four major bioprotective alkaloids including peramine, lolines, indole-diterpenes and ergot alkaloids, using 33 different gene specific primer sets. A single gene involved in peramine biosynthesis was present in all four isolates (FIG. 5), whereas none of the genes involved in the biosynthesis of lolines, lolitrems and ergot alkaloids that we tested were detected in any isolate.

DISCUSSION

Cinna arundinacea is one of four species currently grouped into genus *Cinna*, all of which are restricted to moist habitats, such as stream banks and wetlands; *C. arundinacea* occurs in southeastern Canada and throughout the eastern USA at 0–900 m (Brandenburg 2007). In this study we report the first endophyte

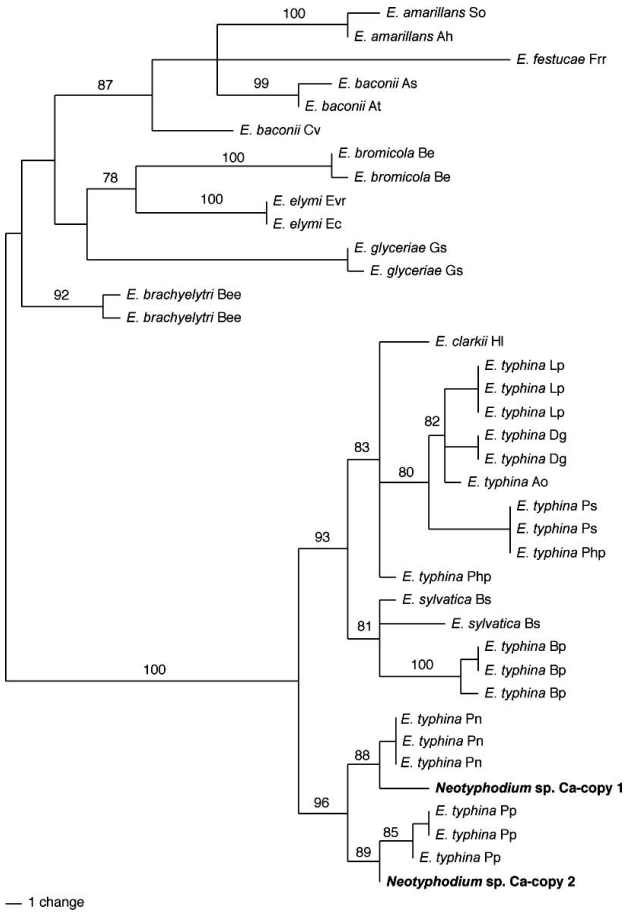

FIG. 3. Phylogeny derived from maximum parsimony (MP) analysis of introns 1–4 of the *tef1*-alpha gene from representative haploid *Epichloë* species and two copies obtained from an unknown endophyte isolated from *Cinna arundinacea*. Nine additional isolates were examined and sequences obtained from each were identical to one shown. Tree shown is one of 24 MP trees obtained by branch and bound search. Number of parsimony-informative characters = 111; uninformative characters = 28; tree length = 172 steps; consistency index = 0.8663; retention index = 0.9586; rescaled consistency index = 0.8304; midpoint root is at the left edge. Numbers at branches are the percentage of trees containing the corresponding clade in 1000 bootstrap replications. Letters after each endophyte refer to host designations as follows: So (*Sphenopholis obtusata*); Ah (*Agrostis hiemalis*); Cv (*Calamagrostis villosa*); As (*Agrostis stolonifera*); At (*Agrostis tenuis*); Frr (*Festuca rubra* subsp. *rubra*); Fl (*Festuca longifolia*); Be (*Bromus erectus*); Evr (*Elymus virginicus*); Ec (*Elymus canadensis*); Gs (*Glyceria striata*); Bee (*Brachyelytrum erectum*); Hl (*Holcus lanatus*); Lp (*Lolium perenne*); Ao (*Anthoxanthum odoratum*); Ps (*Poa sylvicola*); Pt (*Poa trivialis*); Dg (*Dactylis glomerata*); Ae (*Arrenatherum elatius*); Bs (*Brachypodium sylvaticum*); Bp (*Brachypodium pinnatum*); Pn (*Poa nemoralis*); Ca (*Cinna arundinacea*); Pp (*Poa pratensis*).

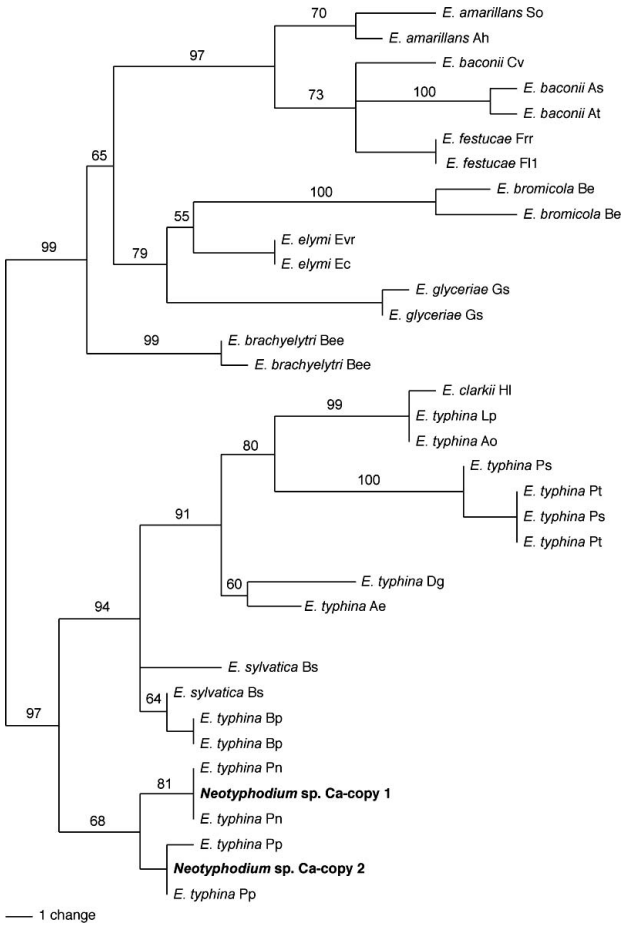

FIG. 4. Phylogeny derived from maximum parsimony (MP) analysis of introns 1–3 of the *tub2* gene from representative haploid *Epichloe* species and two copies obtained from an unknown endophyte isolated from *Cinna arundinacea*. Nine additional isolates were examined and sequences obtained from each were identical to one shown. Tree is one of four MP trees obtained by branch and bound search. Number of parsimony informative characters = 80; uninformative characters = 14; tree length = 145 steps; consistency index = 0.7379; retention index = 0.9031; rescaled consistency index = 0.6664; midpoint root is at the left edge. Numbers at branches are the percentage of trees containing the corresponding clade in 1000 bootstrap replications. (Letters following each endophyte refer to host designations as listed in FIG. 3.)

to be characterized from *C. arundinacea* and describe it as a new species of *Neotyphodium*, *N. schardlii*.

Phylogenetic analysis revealed that *N. schardlii* ATCC MYA-4679 and ATCC MYA-4680 carry two copies of *tefA* and *tubB* genes and both surprisingly were inherited from *E. typhina* ancestors. Our analysis indicates that *N. schardlii* is an intraspecific hybrid between two host-associated *E. typhina* populations, one from the grass *Poa nemoralis* and the other from the closely related *Poa pratensis*. These findings

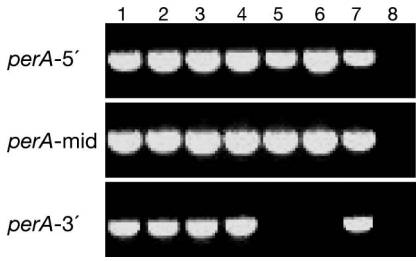

FIG. 5. PCR analysis of the peramine biosynthesis gene *perA*. Samples were loaded in this order: Lanes 1 to 8 are *N. schardlii* morphotype I isolate Mt1-1; *N. schardlii* morphotype I isolate Mt1-2; *N. schardlii* morphotype II isolate Mt2-1; *N. schardlii* morphotype II isolate Mt2-2; *E. typhina* isolate 1022; *E. festucae* isolate 2368; *E. festucae* isolate Fl1 and negative control respectively.

extend the current understanding of the origins of *Neotyphodium* spp. because all other *Neotyphodium* hybrids analyzed to date are interspecific hybrids with genomes derived from *Epichloë/Neotyphodium* ancestors whose extant members are considered to be distinct species (Tsai et al. 1994, Schardl and Craven 2003). Both *Poa* species are native to Eurasia but introduced to North America. Unless hybridization was recent, this suggests the possibility that the ancestral lineage of *N. schardlii* in *C. arundinacea* is an intraspecific hybrid endophyte present in European species of *Cinna*.

Alkaloid profiling of *N. schardlii* isolates indicated the presence of the *perA* gene, thought to be the only gene required for peramine biosynthesis. Thus these isolates are potentially capable of producing peramine in planta. Peramine is an effective insect-feeding deterrent exclusively synthesized by the *Epichloë* group of fungal endophytes, including *E. typhina*, in association with their grass hosts (Rowan et al. 1986, Lane et al. 2000, Clay and Schardl 2002). In fact recent work showed that this endophyte does indeed provide protection from feeding by several species of phytophagous insects (Crawford pers comm). Of interest the isolate of *E. typhina* from *Poa nemoralis* we tested (E1022) apparently lacks the 3′ region of the peramine gene as does *E. festucae* isolate E2368 (FIG. 5, lanes 5, 6). While E1022 has not been tested for peramine production, E2368 does not produce peramine. Furthermore only one isolate of *E. typhina* from *Poa nemoralis* has been checked for alkaloid production (Leuchtmann et al. 2000) and was negative for peramine. These results along with the presence of the 5′ and middle regions of the *perA* gene suggest that these particular strains (E1022 and E2368) likely had intact gene copies at one time but subsequently have experienced gene deletions. In any case the presence of an intact peramine biosynthesis gene in *N. schardlii* suggests that one or both of its *E. typhina* progenitors had a functional gene copy at the time of hybridization. The absence of genes responsible for ergot alkaloid and indole-diterpene biosynthesis in *N. schardlii* isolates makes them interesting candidates for further testing for agricultural applications.

Epichloë typhina is a predominantly sexual, horizontally transmitted endophyte species and traditionally has been considered as a collection of interfertile populations having a wide host range, referred to as the ''*E. typhina* complex'' (Craven et al. 2001b, Leuchtmann 2003). However recent phylogenetic evidence indicates that, although these *E. typhina* populations often retain the ability to be mated in an experimental setting, they remain largely segregated in nature (Schardl et al. 2007b). These data, combined with other work showing that most genotypes of *E. typhina* exhibit strong host specificity (Chung et al. 1997), have been used to support the notion that there is likely cryptic speciation within the *E. typhina* complex. A possible explanation for these results is that the offspring generated by matings between host-associated *E. typhina* isolates might be incapable of successfully colonizing the host species of either parental genotype.

Hybridization that give rise to *Neotyphodium* spp. are believed to result from vegetative fusion (anastomosis) because all hybrids described before this work involve progenitors from distinct *Epichloë* spp. and mating barriers between species are strong (Schardl et al. 1997). However an intriguing possibility in this instance is that, given the potential for sexual crosses between *E. typhina* populations noted above, *N. schardlii* arose from a failure to reduce chromosome number (via nondisjunction) after meiosis. Indeed this endophyte may represent true gene flow between these otherwise isolated *E. typhina* populations. Following the line of reasoning mentioned above the resulting hybrid offspring might have been incapable of colonizing the *Poa* host of either of its parents but fortuitously landed on and was compatible with a sympatrically occurring *C. arundinacea* plant. While this is pure speculation at this point, this new hybrid species poses interesting challenges to current ideas regarding evolution of these unique grass symbionts.

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