Interspecific and intraspecific hybrid *Epichloë* species symbiotic with the North American native grass *Poa alsodes*

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Tatsiana Shymanovich, Nikki D. Charlton, Ashleigh M. Musso, Jonathan Scheerer, Nadja B. Cech, Stanley H. Faeth & Carolyn A. Young (2017) Interspecific and intraspecific hybrid *Epichloë* species symbiotic with the North American native grass *Poa alsodes*, *Mycologia*, 109:3, 459-474, DOI: 10.1080/00275514.2017.1340779

Abstract:

The endophyte presence and diversity in natural populations of *Poa alsodes* were evaluated along a latitudinal transect from the southern distribution range in North Carolina to New York. Two distinct *Epichloë* hybrid taxa were identified from 23 populations. Each taxon could easily be distinguished by polymerase chain reaction (PCR) genotyping with primers designed to mating type genes and alkaloid biosynthesis genes that encode key pathway steps for ergot alkaloids, indole-diterpenes, lolines, and peramine. The most commonly found *Epichloë* taxon, *Poa alsodes* Taxonomic Group-1 (PalTG-1), was detected in 22 populations at high infection frequencies (72–100%), with the exception of one population at high elevation (26% infection). The second taxon, PalTG-2, was observed only in five populations in Pennsylvania constituting 12% of infected samples. Phylogenetic analyses placed PalTG-1 as an interspecific hybrid of *E. amarillans* and *E. typhina* subsp. *poae* ancestors, and it is considered a new hybrid species, which the authors name *Epichloë alsodes*. PalTG-2 is an intraspecific hybrid of two *E. typhina* subsp. *poae* ancestors, similar to *E. schardlii* from the host *Cinna arundinacea*, which the authors propose as a new variety, *Epichloë schardlii* var. *pennsylvanica. Epichloë alsodes* isolates were all mating type *MTA MTB* and tested positive for *dmaW, easC, perA*, and some *LOL* genes, but only the alkaloid *N*-acetylnorloline was detected in *E. alsodes*–infected plant material. *Epichloë schardlii* var. *pennsylvanica* isolates were all mating type *MTB MTB* and tested positive for *perA*, but peramine was not produced. Both *E. alsodes* and *E. schardlii* var. *pennsylvanica* appeared to have complete *perA* genes, but point mutations were identified in *E. alsodes* that would render the encoded *perA* gene nonfunctional.

Keywords: Alkaloid genotype | Epichloë alsodes | Epichloë schardlii var. pennsylvanica | latitudinal transect | N-acetylnorloline | peramine

Article:

INTRODUCTION

In general, all plants have microbial endosymbionts, fungi and bacteria, that live inside their hosts and do not cause visible disease symptoms (Schulz and Boyle 2006; Rodriguez et al. 2009). Some endosymbionts appear to be localized non–host-specific infections that are transmitted horizontally, of which the functions and significance of these interactions are usually poorly understood. Contrary to that, *Epichloë* species are dominant systemic fungal endophytes of Pooid grasses that have been under intense scientific investigation for several decades, mostly due to their association with agronomically important grasses (Bacon et al. 1977; Siegel et al. 1987; Cheplick and Faeth 2009; Schardl et al. 2013a).

Many *Epichloë* species are asexual and only transmitted vertically into the developing seeds. However, some *Epichloë* species are sexual and can be transmitted by horizontal transmission, or can transmit via both horizontal and vertical transmission. Beneficial effects of *Epichloë* species on their hosts include enhanced nutrition and growth and anti-herbivory chemical defenses by production of bioactive alkaloids (Cheplick and Faeth 2009; Schardl and Chen 2010). Many studies have focused on *Epichloë* species from agronomically important grasses (e.g., tall fescue and perennial ryegrass), whereas the *Epichloë* species of wild grasses are often unknown and their ecological cost and benefits are only now being realized (Afkhami et al. 2014; Kazenel et al. 2015; Rudgers et al. 2016). In uncultivated environments, various stress factors may occur frequently and intensely such that *Epichloë* species may provide some benefit to their hosts by alleviating these environmental stresses. However, the cost of hosting an endophyte may overwhelm the benefits when resources are scarce (Faeth and Fagan 2002; Schulz and Boyle 2006; Cheplick and Faeth 2009; Schardl et al. 2013a; Panaccione et al. 2014).

Asexual species, of which many are interspecific hybrids, are considered more mutualistic than sexually reproducing species (Schardl and Craven 2003; Schardl and Chen 2010; Agrawal 2011). Interspecific hybrids typically contain multiple genome copies representing all contributing ancestors, yet some genes may only be present as single alleles or may be lost completely due to random gene loss after hybridization, or some ancestors may have lacked the genes. Thus, in comparison with haploid species, interspecific hybrids likely contain increased genetic variation that could enhance their adaptation potential for environmental stress (Moon et al. 2004; Saari and Faeth 2012; Schardl et al. 2012; Saikkonen et al. 2016). The majority of asexual *Epichloë* species are interspecific hybrids, with only one intraspecific hybrid, *E. schardlii*, described to date (Ghimire et al. 2011; Leuchtmann et al. 2014).

Epichloë species likely play an important role in natural grass populations, as their infection frequencies are often close to 100%. A single grass host species may be compatible with several *Epichloë* species but usually only supports one endophyte strain per individual plant (Clay and Schardl 2002; Cheplick and Faeth 2009; Oberhofer and Leuchtmann 2012; Charlton et al. 2014). Nevertheless, the success of these host-endophyte relationships can be complicated and may depend on specific environmental factors, such as water and nutrient availability, herbivore grazing pressure, and host-endophyte compatibility (Cheplick and Faeth 2009; Jani et al. 2010; Schardl et al. 2013a).

Endophytes that can produce insecticidal alkaloids such as lolines or the insect-feeding deterrent peramine can provide protection from insect herbivory and do not harm mammalian species, but some alkaloids such as the ergot alkaloid ergovaline and the indole-diterpene lolitrem B are considered toxic to livestock (Schardl et al. 2013a; Panaccione et al. 2014). Often, one endophyte species may produce several classes of alkaloids with a range of effects on mammalian and invertebrate herbivores. *Epichloë* species are widely used to increase host stress tolerance, persistence, and productivity without toxic effects to livestock for agronomic grasses such as tall fescue and perennial ryegrass (Siegel et al. 1987; Johnson et al. 2013; Young et al. 2013). From ecological and agronomic perspectives, it is important to understand the potential compounds that can be synthesized for each endophyte-host interaction. Recently, researchers have shifted attention to wild uncultivated grass species because they harbor remarkable variation in *Epichloë* species and genotypes within species and consequently a broad array of different alkaloid combinations (Charlton et al. 2012, 2014; Iannone et al. 2012; Takach et al. 2012; Leuchtmann and Oberhofer 2013; Chen et al. 2015; Kazenel et al. 2015; Shymanovich et al. 2015). New endophyte species may have commercial interest as material for artificial inoculations or a source of alternative alkaloid combinations. Also, for environmental restoration and conservation projects, the knowledge on endophytes is essential, as they may act as natural plant defense agents in a specific environment (Emery et al. 2015).

Poa alsodes (grove bluegrass) is a native cool-season woodland grass species that is widely distributed in eastern North America. This grass is known to harbor at least one *Epichloë* species (Clay 1996), but the identity of the endophyte species has remained unknown (Schardl et al. 2012), as has knowledge of the distribution and prevalence of the endophyte-infected host. Infection with *Epichloë* species was not included in a conservational assessment of this grass (Hill 2007). Furthermore, there have been no comprehensive studies on the distribution and potential variation of the *Epichloë* species that associate with *P. alsodes* across natural habitats that span the host latitudinal range. However, studies have shown that the endophyte in *P. alsodes* may ameliorate the negative effects of drought stress, may enhance competitive abilities against invasive species, and may increase host biomass in reduced-light conditions (Kannadan and Rudgers 2008; Davitt et al. 2010; Craig et al. 2011).

In this study, we determined the infection frequencies and discovered variability of *Epichloë* species inhabiting wild *Poa alsodes* populations across a latitudinal range of 1200 km. In addition, we evaluated the endophyte diversity on the basis of alkaloids produced by endophyte-infected *P. alsodes* by first genotyping the endophytes present in each population using multiplex polymerase chain reaction (PCR) to detect the presence of alkaloid biosynthesis genes and mating type and then testing endophyte-infected plant material for the levels of individual alkaloids in the populations. On the basis of these studies, one new *Epichloë* species, *E. alsodes*, is described and we propose a new variety, *E. schardlii* var. *pennsylvanica*.

MATERIALS AND METHODS

Sampling host grass populations along the latitudinal gradient

A latitudinal collection of natural *P. alsodes* populations was collected along the Appalachian Mountains from the southern distribution range to the US-Canadian border in 2011–2014 (SUPPLEMENTARY TABLE 1). The population names include an abbreviation for the state and numerical order of collection; for example, NC-3 stands for North Carolina, the third population collected. All sampling times were when the plants were flowering or had seed heads, as this was needed for species identification. Fifty individual plants growing at least 0.5 m apart were sampled from each population. Exceptions are the populations sampled in 2011 (populations NC-1A to NC-1D) and also one very small population in Virginia (VA-7) with only five plants identified. The majority of populations were sampled from several areas with plants located at a distance of several hundred meters and up to 5 km apart. *Poa alsodes* seeds have been shown to germinate after deer consumption (Hill 2007), and this likely represents the mechanism of dispersal that affects (or explains relatively large) population areas. Only aboveground material was collected from the plants. Tillers were kept on ice or refrigerated until the endophyte was isolated and then frozen at −20 C. Seeds were harvested separately, dried, and stored at −20 C.

Table 1. *Poa alsodes* populations (north to south).

State-Order collected	Park name	*Epichloë* positive/ Total samples	% Endophyte-infected
MI-20	Waterloo recreation area	50/50	100
NY-12	Higlew Flow State Park	49/50	98
NY-13	Verona Beach State Park	49/50	98
NY-14	Clark Reservation State Park	50/50	100
NY-11	Allegany State Park	50/50	100
PA-16	Kinzua Bridge State Park	48/50	96
PA-10	Chapman State Park	37/50	74
PA-17	Elk State Park	46/50	92
PA-18	Allegheny National Forest	49/50	98
PA-15	Bendigo State Park	48/50	96
PA-9	Oil Creek State Park	50/50	100
PA-8	Cook Forest State Park	48/50	96
PA-19	Clear Creek State Park	49/50	98
WV-5	Blackwater Falls State Park	50/50	100
WV-6	Seneca Forest State Park	45/50	90
VA-7	Grayson Highlands State Park	5/5	100
NC-2	Great Smoky Mountains National Park	48/50	96
TN-3	Great Smoky Mountains National Park	50/50	100
NC-4	Great Smoky Mountains National Park	13/50	26
NC-1B	Pisgah National Forest	7/7	100
NC-1C	Pisgah National Forest	7/7	100
NC-1D	Pisgah National Forest	10/10	100
NC-1A	Pisgah National Forest	18/18	100

Epichloë infection frequency and species variation in natural populations

Infection status for each individual plant (two to three tillers per plant) was initially determined by an immunoblot assay that utilizes monoclonal antibodies to detect *Epichloë* endophytes (Phytoscreen Immunoblot Kit; Agrinostics, Watkinsville, Georgia). The infection status was reconfirmed using a PCR-based method that could also detect endophyte genotypic variation. DNA was isolated from freeze-dried tillers with the MagAttract 96 DNA Plant Core Kit (Qiagen Inc., Germantown, Maryland) according to manufacturer's instructions. Five multiplex primer

combinations (SUPPLEMENTARY TABLE 2) were used for PCR to genetically characterize the endophyte with respect to infection status (*tefA*), mating type (*MTA*and *MTB*), and presence of peramine (*PER*), ergot (*EAS*), loline (*LOL*), indole-diterpene (*IDT/LTM*) alkaloid genes, as described in Charlton et al. (2014). The endophytes were grouped on the basis of presence or absence of these PCR markers, and the alkaloid potential of the endophyte was predicted.

Table 2. Genotype analysis and predicted alkaloids of *Poa alsodes* endophytes.

Marker	**Gene**[a]	**PalTG-1 *E. alsodes***	**PalTG-2 *E. schardlii* var. *pennsylvanica***
Mating type	*mtAC*	+	–
	mtBA	+	++
Peramine	*perA*-5′	++	+
	perA-T2	++	+
	perA-R	++	+
Lolines	*lolC*	+	–
	lolA	+	–
	lolO	+	–
	lolN	–	–
	lolM	–	–
	lolP	–	–
Ergot alkaloids	*dmaW*	+[b]	–
	easF	–	–
	easC	+	–
	easE	–	–
	easA	–	–
	cloA	–	–
	lpsB	–	–
Indole-diterpenes	*idtG*	–	–
	ltmQ	–	–
	ltmJ	–	–
Mating type		*MTA MTB*	*MTB MTB*
Predicted chemotype		Peramine/NANL[c]	Peramine

[a] Tested for presence of the gene or partial gene fragment.
[b] *damW* = pseudogene.
[c] NANL = *N*-acetylnorloline

PCR conditions

PCR was performed in a total volume of 25 µL (detection only) or 50 µL (for amplicons that required sequencing) containing 3 ng DNA, 1.0 U GoTaq DNA polymerase (Promega Corp., Madison, Wisconsin), 1× Green GoTaq reaction buffer containing 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (Promega), and 1 µM target-specific primers (SUPPLEMENTARY TABLE 2). The cycling parameters were 94 C for 1 min, then 30 cycles of 94 C for 15 s, 56 C for 30 s, and 72 C for 45 s (or 1 min per kb for larger products), followed by 72 C for 10 min. Multiplex and single-target PCR used the same reaction and cycling conditions. Amplicons were analyzed by gel electrophoresis with a 1.5% agarose gel in 1× Tris-boric-EDTA (TBE) buffer. DNA fragments were visualized with ethidium bromide by ultraviolet (UV) transillumination.

Endophyte isolation

Fungal isolations were performed from fresh tillers of 20 individual plants per population (except for NC-1 and MI-20). Pseudostems (3–5 cm) of three tillers were surface-sterilized (70% ethanol for 1 min, 5% sodium hypochlorite for 4 min, 70% ethanol for 30 s, sterile water for 1 min), cut into three 5-mm pieces, and placed on potato dextrose agar (PDA) plate with 100 µg/mL ampicillin. Plates were kept in the dark at room temperature until fungal growth occurred. Single-spore isolations were performed three times to obtain pure cultures. These cultures were preserved in sterile tubes under a mineral oil for long-term storage. From each population two representative individuals for each endophyte were selected and were used for more detailed studies. DNA was isolated from representative cultures by harvesting mycelia grown for about 10 d on sterilized cellophane above PDA medium (Cassago et al. 2002).

Endophyte species identification

DNA from mycelia was extracted with the Quick-DNA Fungal/Bacterial DNA MiniPrep Kit (Zymo Research, Irvine, California, USA), and the housekeeping genes that encode translation elongation factor 1-α (*tefA*), β-tubulin (*tubB*), and calmodulin (*calM*) were PCR-amplified and directly sequenced. Primers used for amplification are listed in SUPPLEMENTARY TABLE 2. When polymorphic peaks were observed, these PCR products were cloned using the pGEM-T Easy Vector System I (Promega). Ligations were used to transform *Escherichia coli* One Shot TOP10 chemically competent cells (Invitrogen, Carlsbad, California) via manufacturer's instructions. Twelve white colonies were selected with X-gal/IPTG screen on LB agar amended with ampicillin and used for sequencing with Big Dye Terminator Chemistry 3.1 (Applied Biosystems, Foster City, California). Sequences were analyzed with Geneious 6.1.6 (Biomatters Ltd., Auckland, New Zealand) or Sequencher 5.0 (Gene Codes Corp., Ann Arbor, Michigan) software, and individual alleles were distinguished.

Table 3. Morphological characteristics of *Epichloë alsodes* (PalTG-1) and *Epichloë. Schardlii* var. *pennsylvanica* (PalTG-2), endophytes of *Poa alsodes.*

Endophyte[a] (morphotype)	Samples examined	Growth mm/wk	Conidiophore dimensions[b] (µm)	Conidia size[c] (µm)	Conidial shape
Epichloë alsodes (I)	30	5.7–7.5	22.2±4.8 × 1.9±0.2	7.9±0.6 × 3.1±0.2	Obovate to reniform
E. alsodes (II)	2	4.9–6.3	18.0±5.3 × 1.8±0.6	8.1±0.6 × 3.2±0.1	Obovate to oblong
E. alsodes (III)	2	7.1–7.7	27.4±9.1 × 1.8±0.1	7.7±0.3 × 3.1±0.2	Obovate to allantoid
Epichloë schardlii var. *pennsylvanica* (I)	4	4.5–5.4	27.1±6.2 × 1.9±0.1	8.1±0.6 × 3.1±0.2	Obovate to allantoid
E. schardlii var. *pennsylvanica* (II)	6	4.6–5.5	25.9±7.6 × 1.8±0.1	7.8±0.6 × 3.1±0.2	Obovate to allanatoid

[a] Isolates used for morphotype analysis. *E. alsodes* (I): NC-2-17, NC-2-42, TN-3-9, TN-3-40, NC-4-35, NC-4-46, WV-5-20, WV-5-41, WV-6-6, WV-6-47, VA-7-2, VA-7- 5, PA-8-2, PA-8-20, PA-9-16, PA-9-42, NY-11-1, NY-11-48, NY-13-15, NY-13-48, NY-14-1, NY-14-44, PA-15-3, PA-15-42, PA-17-7, PA-17-24, PA-18-9, PA-18-35, PA-19-7, PA-19-39; *E. alsodes* (II): NY-12-14, NY-12-47; *E alsodes* (III): PA-16-07, PA-16-50; *E. schardlii* var. *pennsylvanica* (I): PA-8-27, PA-8-45, PA-10-42, PA-18- 45; *E. schardlii* var. *pennsylvanica* (II): PA-10-10, PA-17-33, PA-17-44, PA-18-42, PA-19-28, PA-19-34.
[b] Length by width of base.
[c] Length by width.

Phylogenetic trees were inferred by maximum likelihood (ML) and maximum parsimony (MP). Sequences from the *P. alsodes* endophytes and representative *Epichloë* species were aligned with MUSCLE 3.8.1 (Edgar 2004) without Gblocks curation via the Phylogeny.fr Web site (http://www.phylogeny.fr). ML analyses were performed with PhyML 3.1/3.0 (Guindon et al. 2010) and branch support estimated by the approximate likelihood-ratio test (aLRT) (Dereeper et

al. 2008, 2010). MP analyses were performed with MEGA7 (Kumar et al. 2016) with the random addition of sequences and SPR (subtree-pruning-regrafting) (Nei and Kumar 2000). Alignment and trees are available in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2; S20421, S20416, and S20425). GenBank accession numbers are provided in SUPPLEMENTARY TABLES 3 and 4.

Genetic characterization of endophytes across populations

To characterize genetic variation of endophytes across populations and detect the ancestry of several genes, 50 representative isolates were examined by sequencing the PCR products of *mtAC* (785 bp), *mtBA* (619 bp), *perA*-R* (600 bp), *dmaW* (1450 bp), and the *lolC* (1630 bp) gene fragments if they were present (Charlton et al. 2014). For these sequencing reactions, total DNA from the plant material was used, as mycelia was not available for the 2011 and 2013 collections. For the 2012 collections, DNA was extracted from freeze-dried fungal cultures with the ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research). PCR with additional primer sets were used to screen for the presence of additional ergot alkaloid and loline genes (SUPPLEMENTARY TABLE 2). Each domain of the *perA* gene was also amplified and sequenced as described in Berry et al. (2015). Accession numbers and isolate information for *tefA, calM, tubB*, and alkaloid genes used in this study are listed in SUPPLEMENTARY TABLE 4.

Morphological examination

Morphological examinations were performed on representative samples from 18 populations collected in 2012–2013. To study colony growth and morphology, PDA plates (3 plates/isolate) were inoculated with 20 µL of an isolate suspension (1.5 mm^3 of culture macerated in 100 µL of sterile water) and grown in the dark at 24 C. After 21 d, the colony diameter, color, texture, and back side were examined, recorded, and photographed. On the basis of these analyses, isolates were grouped by morphotype. For microscopic examinations of conidia and conidophores, 10 µL from the same culture suspensions were placed on 1.5% water agar plates. Plates were kept in a dark growth chamber at 24 C for 10–11 d when conidiation started. An agar block of one colony was cut, placed on a microscopic slide with a cover slip pressed gently on the culture surface, and a drop of emersion oil applied. Examination was performed with Nikon Eclipse 50i microscope (Nikon, Melville, New York) by taking multiple photographs with Nikon Digital Sight Fi1 camera at 1000× magnification with a 10 µm scale bar imbedded. Measurements of conidiogenous cells (length and width at tip and base) and conidia (length and width) were taken from 15–20 structures for each isolate. Twenty hyphal width measurements were taken from several isolates for each endophyte.

Extraction and analysis of alkaloids in planta

Five individual plants per each population (all populations except NC-1, VA-7, and MI-20 were analyzed) per endophyte species were selected for alkaloid detection, resulting in 105 samples. Leaf samples were freeze-dried and extracted with 95% methanol from 5-mm grass pieces (40 mg plant material in 1 mL methanol) at 5 C for 48 h. The extract was filtered through a 0.22-µm spin filter (Corning Inc., Corning, New York) and stored at 5 C. A 2-propanol–lactic acid

method was also used to extract peramine (Spiering et al. 2002) from 10 *P. alsodes* samples representing each *Epichloë* species and the positive-control samples *Festuca arizonica* infected with either *E. tembladerae* or *E. typhina* subsp. *poae* var. *huerfana* (Faeth et al. 2002; Faeth and Fagan 2002; Leuchtmann et al. 2014). Independent peramine analyses were performed by AgResearch (Palmerston North, New Zealand) (Berry et al. 2015) testing two independent endophyte-infected *P. alsodes* samples representing each *Epichloë* species, where each sample was mixed from leaf clippings of multiple plants with the same endophyte.

N-acetylnorloline (NANL), chanoclavine I, and peramine were analyzed by ultra-performance liquid chromatography–high resolution mass spectrometry (UPLC-HRMS) with an Orbitrap mass spectrometer with an electrospray ionization (ESI) source (LTQ Orbitrap XL; Thermo, San Jose, California) coupled to Acquity UPLC (Waters Corp., Milford, Massachusetts), with a slight modification to previously described methods (Shymanovich et al. 2015). Mass spectrometric detection was conducted in the positive ion mode with a scan range of 75–600 *m*/*z*. Capillary temperature was 300 C, sheath gas pressure was 5 (arbitrary units), and spray, capillary, and tube lens voltages were 4.0 kV, 20 V, and 100 V, respectively. For comparison, this method was applied to the analysis of endophyte-infected *Elymus canadensis* (strain NFe746) and the alkaloids NANL, chanoclavine I, and peramine were all detected, consistent with previous literature (Clay and Schardl 2002; Charlton et al. 2012; Schardl et al. 2013b, 2013c). In addition, a sample of endophyte-infected sleepygrass (*Achnatherum robustum*) previously shown to contain chanoclavine (Shymanovich et al. 2015; Jarmusch et al. 2016) was also analyzed, and this compound was detected in the control sample, as expected. A synthetic standard of NANL was included as a positive control and for the purpose of estimating NANL concentration in plant samples. NANL concentrations were extrapolated from a calibration curve plotted as the peak area for the selected ion trace for the NANL $[M+H]^+$ ion (*m*/*z* 183.1128) versus concentration. The calibration curve was prepared by 2-fold serial dilutions at a range of concentrations from 0.625 to 20 µg/mL.

RESULTS

Host population sampling and *Epichloë* infection

During 2011, four natural *P. alsodes* populations were sampled (NC-1A to NC-1D, each with 7–18 plants) from the Pisgah National Forest, North Carolina. From this pilot study, infection of *Epichloë* species was detected in all *P. alsodes* samples. In 2012, Kings Mountain State Park in South Carolina, which is known as the historical southern distribution edge for this species (Hill 2007), was checked for the presence of *P. alsodes*, but no plants were observed. Thus, the southern distribution range of the host grass in this study was considered the location at Pisgah National Forest, North Carolina. In June 2012, 19 locations were visited from North Carolina to New York, with 13 populations sampled. A more detailed collection in Pennsylvania performed in June 2013 resulted in sampling five additional populations. In June 2014, *P. alsodes* was collected from a single population in Michigan. A total of 23 populations were sampled along a latitudinal gradient over a distance of 1200 km (TABLE 1, SUPPLEMENTARY FIG. 1, SUPPLEMENTARY TABLE 1). All plants (n = 947) were tested for endophyte infection by immunoblot analysis, and 92.5% of the samples were endophyte infected. Only four populations,

PA-10, PA-17, WV-6, and NC-4, had endophyte infection frequencies less than 96%, with the lowest infection rate of 26% in population NC-4.

Endophyte variation among *P. alsodes* samples

Two genotype patterns were identified by multiplex PCR that determined the presence or absence of mating type (*mtAC* and *mtBA*), ergot alkaloid (*EAS*), loline (*LOL*), indole-diterpene (*IDT*), and peramine (*PER*) genes (TABLE 2, SUPPLEMENTARY FIG. 2A). The majority of infected samples (88%) contain *MTA* and *MTB* and are also positive for *EAS, LOL*, and *PER* markers. This endophyte genotype was considered *P. alsodes* Taxonomic Group 1, PalTG-1. The presence of genes from both mating types suggests that PalTG-1 is a hybrid species. The remaining endophyte-infected samples (12%), indicated as PalTG-2, were positive for the *MTB* and *PER* markers.

Alkaloid potential based on genetic analyses

The presence or absence of genes can be used to make predictions on the likelihood of a functional alkaloid pathway being present. Both PalTG-1 and PalTG-2 isolates contained all the markers used to predict the likely production of peramine. PalTG-2 did not test positive for any other alkaloid biosynthesis gene markers and, therefore, would be unlikely to produce ergot alkaloids, lolines, or indole-diterpenes.

The PalTG-1 isolates were more complex and, in addition to the *PER* markers, contained markers for *EAS* and *LOL* genes. Data from the original multiplex PCR show the presence of *dmaW* and *easC*. However, the other *EAS* genes (*easF* and *easE*) that encode early pathway steps required for the biosynthesis of chanoclavine were absent (SUPPLEMENTARY FIG. 2B); therefore, it is highly unlikely that PalTG-1 isolates have the capability to produce even simple ergot alkaloids. *LOL* genes that encode early to mid-pathway steps were present in PalTG-1 isolates, but these isolates lacked the late pathway genes, *lolM, lolN*, and *lolP* (SUPPLEMENTARY FIG. 2C). Therefore, the PalTG-1 isolates are predicted to produce the loline intermediate NANL (TABLE 2), which is consistent with other NANL-producing *Epichloë* species (Schardl et al. 2013b).

Infection frequency of each endophyte genotype in each population

The majority of *P. alsodes* populations had high endophyte infection frequencies (96–100%) with PalTG-1, which is widely distributed along all collection sites (FIG. 1). The lowest infection rate (26%) for PalTG-1 was observed at a high elevation in the Great Smoky Mountains National Park, NC-4 population, in which in one area all the plants (n = 19) were endophyte free, and in the second area the infection rate was 38% (n = 31 plants). Interestingly, the PalTG-2 distribution range was limited to the state of Pennsylvania. Four populations (PA-8, PA-17, PA-18, and PA-19) had plants infected with either PalTG-1 or PalTG-2, where PalTG-2 infection ranged from 14% to 48% of sampled plants in these collections. PA-10 was the only population that contained only PalTG-2–infected plants (74% infection rate).

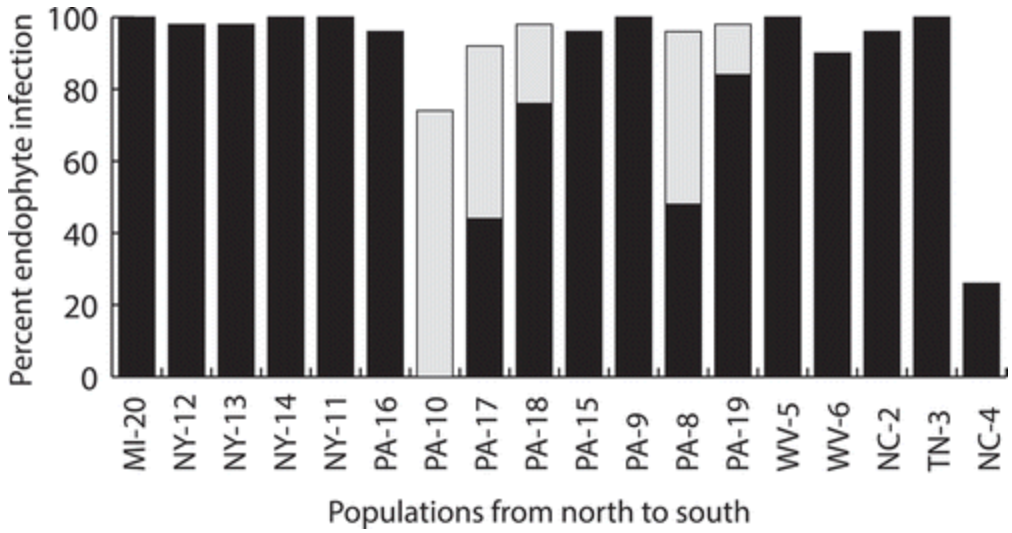

Figure 1. *Epichloë* infection frequencies and genetic variation in natural populations of the woodland grass, *Poa alsodes*, along a latitudinal gradient. Black represents PalTG-1; gray represents PalTG-2. Population IDs are composed of the state abbreviation and collection number.

Phylogenetic analyses for species identification and hybrid status

Sequence data generated from the partial *tefA, calM*, and *tubB* genes of representative PalTG-1 isolates showed that two alleles were present. Maximum likelihood trees inferred from the alignment of *tefA, calM*, and *tubB* partial gene sequences indicated two ancestral progenitor origins. Each allele from PalTG-1 consistently grouped into one of two separate clades with strong bootstrap support. Based on the *tefA* phylogenetic tree, PalTG-1 was identified as an *E. amarillians* × *E. typhina* subsp. *poae* hybrid, which is distinctly different from *E. cabralii*, a hybrid with the closest phylogenetic placements (FIG. 2). The phylogenetic tree inferred from the partial *calM* and *tubB* (FIG. 3) sequences supported the ancestral origins deduced from the *tefA* gene. We propose the name *Epichloë alsodes* for the PalTG-1 taxon (see Taxonomy below).

Sequencing of the partial *tefA* and *tubB* genes revealed that isolates associated with the PalTG-2 genotype are also hybrids (FIGS. 2, 3). In contrast to PalTG-1, the PalTG-2 alleles had very few polymorphisms between each gene copy, with seven for *tefA* and three for *tubB*. Phylogenetic analysis of the partial *tefA* allele sequences from the representative isolate PA-10-10 shows that each allele is distinctly different, but they both group within the *E. typhina*subsp. *poae* clade (FIG. 2). One of the PalTG-2 *tefA* alleles clusters with the PalTG-1 allele from the *E. typhina* subsp. *poae* ancestor (FIG. 2). In addition, each allele was highly similar to the intraspecific hybrid *E. schardlii* (*E. typhina* subsp. *poae* × *E. typhina* subsp. *poae*) symbiotic with *Cinna arundinacea* (Ghimire et al. 2011). However, *E. schardlii* and *E. schardlii* var. *pennsylvanica* could be distinguished by two single-nucleotide polymorphisms (SNPs) in the *tefA-p2* alleles.

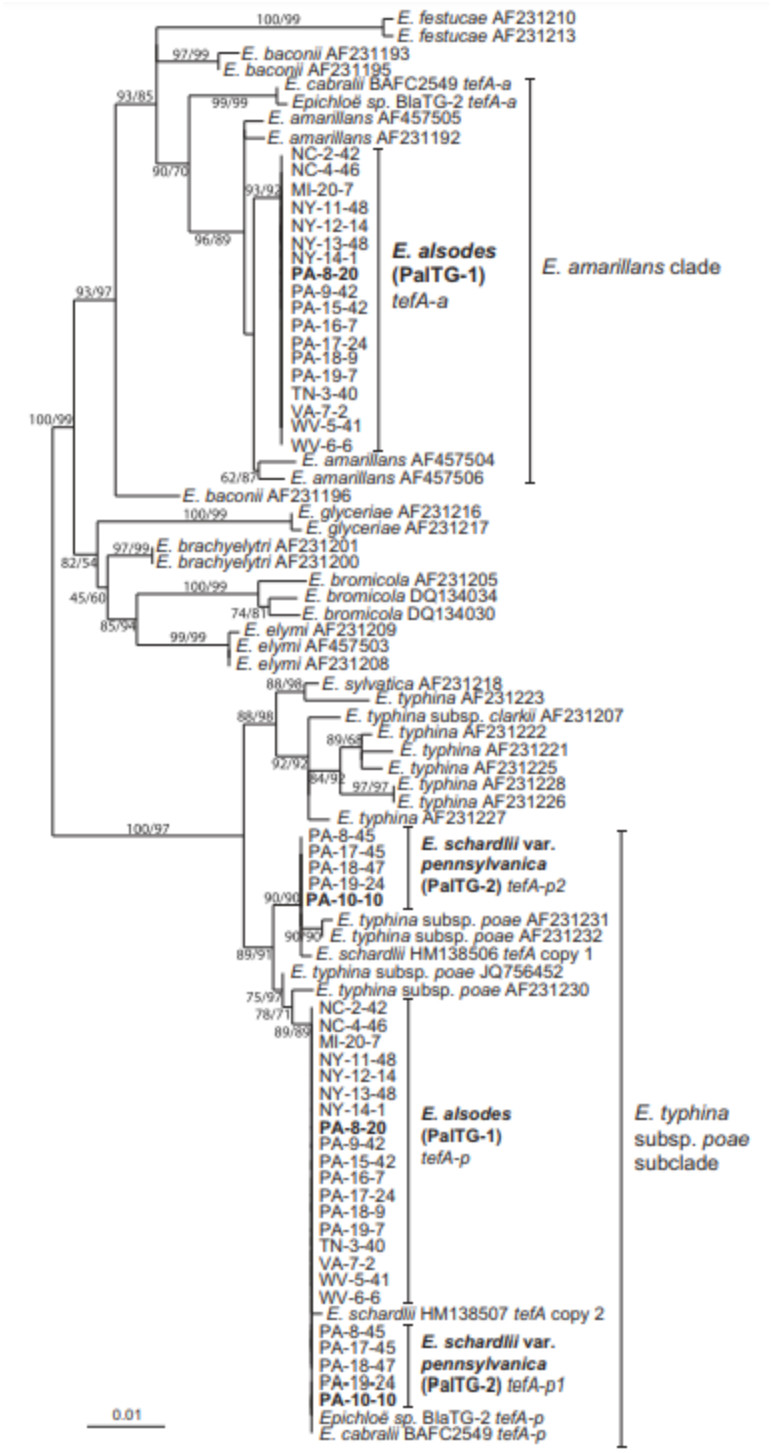

Figure 2. Phylogenetic tree of partial *tefA* gene sequences from representative *Epichloë* species and alleles obtained from PalTG-1 = *Epichloë alsodes* and PalTG-2 = *Epichloë schardlii* var. *pennsylvanica*. Numbers on branches are aLRT support (ML) followed by bootstrap support (MP) where they are >50%. Different alleles of *tefA* are indicated by *a* = *Epichloë amarillans* or *p, p1*, or *p2* = *Epichloë typhina* subsp. *poae*. The likelihood substitution model was HKY85 with a likelihood value of −ln L = 2654.97.

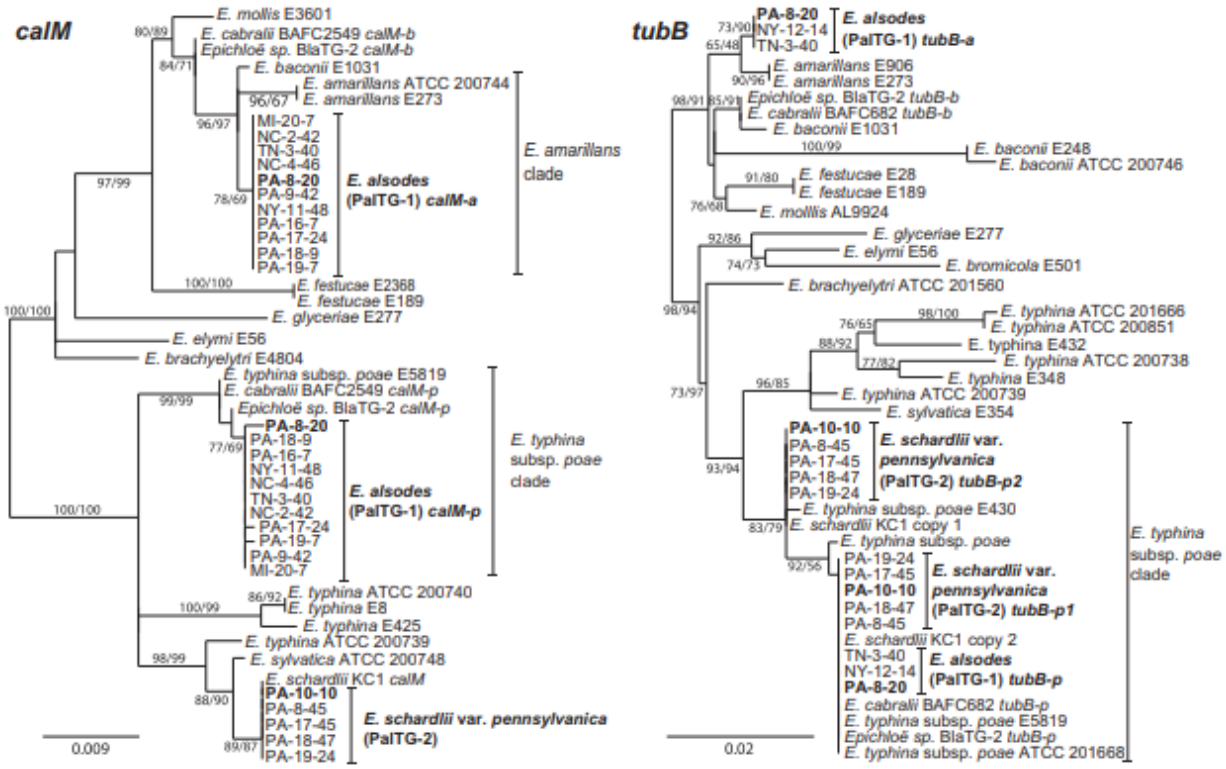

Figure 3. Phylogenetic analyses of partial *calM* and *tubB* gene sequences from representative *Epichloë* species and alleles obtained from PalTG-1 = *Epichloë alsodes* and PalTG-2 = *Epichloë schardlii* var. *pennsylvanica*. Numbers on branches are aLRT support (ML) followed by bootstrap support (MP) where they are >50%. Different alleles of *calM* and *tubB* are indicated by *a = Epichloë amarillans; b = E. baconii*; and *p, p1*, or *p2 = Epichloë typhina* subsp. *poae*. The likelihood substitution model was HKY85 with a likelihood value of −ln L = 3473.91 (calM) and 3473.91.

The partial *calM* direct sequences obtained from PalTG-2 showed no evidence of polymorphic peaks, which indicates that there may only be one allele of this gene or two identical alleles from the two *E. typhina* progenitors. However, we can't discount the possibility that the primers for *calM* were only specific to one allele. The *calM* gene from the *E. schardlii* isolates KC1 and KC2 were also present as a single allele and were identical to *calM* from the PalTG-2 PA-10-10 isolate (FIG. 3). Interestingly, *calM* from PalTG-2 and *E. schardlii* did not group with the *E. typhina* subsp. *poae* clade.

Inheritance of mating type and alkaloid genes

PalTG-1 isolates received a single copy of each mating type idiomorph, wherein *MTA* was from *E. typhina* subsp. *poae* and *MTB* from the *E. amarillans* ancestors (FIG. 4A, B). Sequence analysis of *dmaW* and *lolC* from PalTG-1 isolates shows that each gene was present as a single copy. However, *dmaW* was considered a pseudogene due to a single base deletion in exon 1. The phylogenetic analysis of *dmaW* revealed that the *dmaW* allele is likely from an *E. typhina* subsp. *poae* ancestor and has similarity to *dmaW* from the hybrid *Epichloë* sp. BlaTG-2 from the host *Bromus laevipes* (FIG. 4C). The *lolC* gene grouped in the *E. amarillans* clade (FIG. 4D).

Sequence analysis of the PalTG-1 *perA*-T2 and *perA*-R* domains showed polymorphic peaks, which is indicative of two copies of *perA*, one from each ancestor (data not shown).

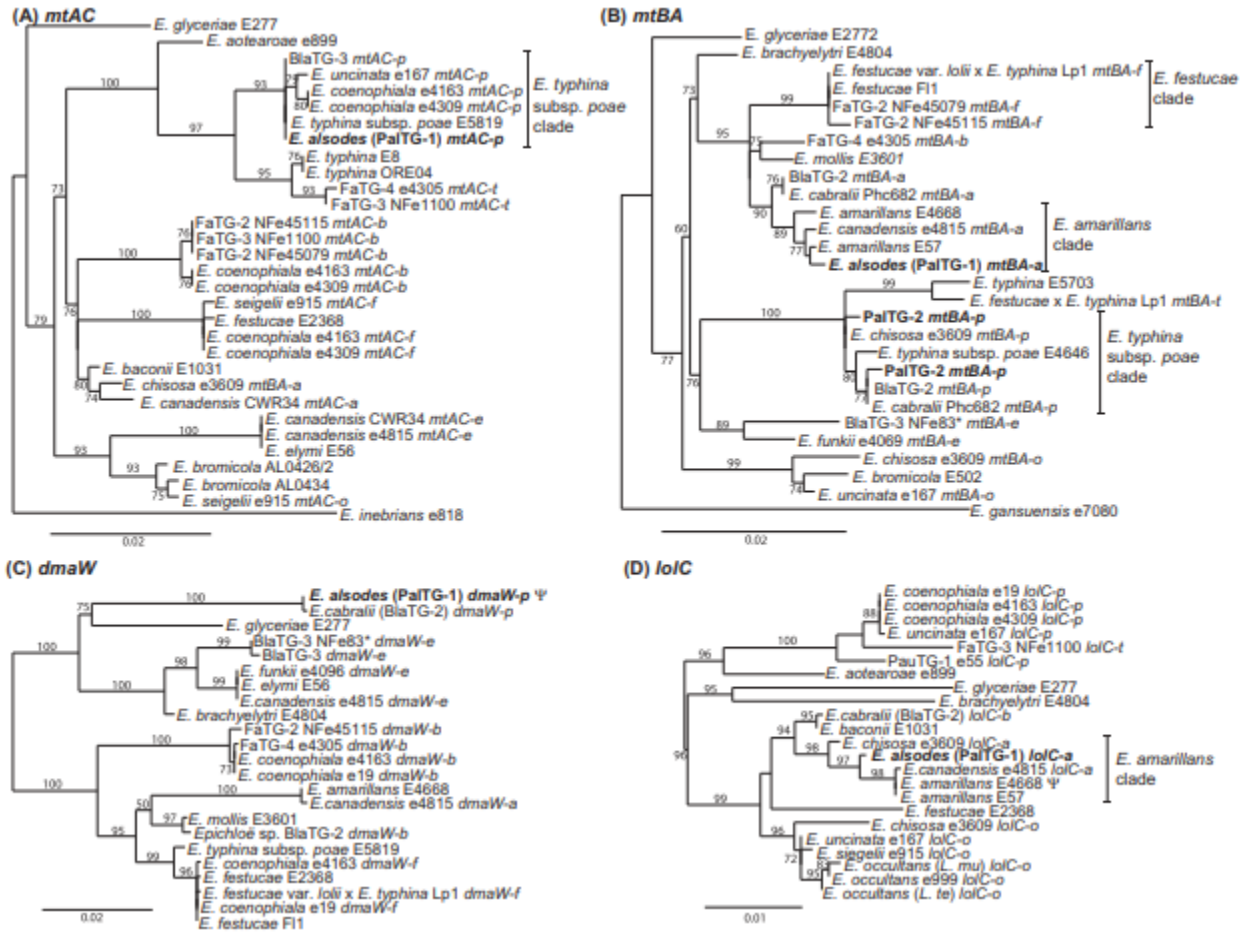

Figure 4. Inheritance of mating type and alkaloid genes from *Poa alsodes* endophytes; PalTG-1 = *Epichloë alsodes* and PalTG-2 = *Epichloë schardlii* var. *pennsylvanica*. A. *mtAC*. B. *mtBA*. C. *dmaW*. D. *lolC*. Numbers on branches are aLRT support (ML) where they are >50%. The ancestral progenitor of hybrids possessing more than one allele is indicated by the letters *a* = *E. amarillans, b* = *E. baconii* and *Lolium*–associated *Epichloë* subclade, *e* = *E. elymi, f* = *E. festucae, o* = *E. bromicola, p* = *E. typhina* subsp. *poae, t* = *E. typhina*. ψ = pseudogene.

The *mtBA* sequences from PalTG-2 isolates are present as two copies, in which two polymorphic bases were identified. Each allele was almost identical, as expected for an intraspecific hybrid, and grouped within the *E. typhina* clade (FIG. 4B). Sequence of multiple *perA* domains displayed no polymorphic peaks, which may indicate that *perA* is a single copy or that both copies are identical. However, the complete gene was not sequenced, so we cannot discount that polymorphisms do exist but are yet to be identified.

Confirmation of alkaloid production

On the basis of the presence of alkaloid gene markers, the PalTG-1 isolates are predicted to produce peramine and NANL, whereas the PalTG-2 isolates are predicted to produce only

peramine (TABLE 2, SUPPLEMENTARY FIG. 2). Although *EAS* genes were present in PalTG-1, the *dmaW* gene was not functional due to a frameshift, and additional genes (*easE* and *easF*) encoding the early pathway steps were absent (TABLE 2). The frameshift was confirmed in 16 independent isolates representing eight populations, which indicates that the frameshift mutation in *dmaW* is widespread in PalTG-1. To confirm our alkaloid predictions, we tested endophyte-infected *P. alsodes* for the presence of ergot alkaloids, lolines, and peramine.

Only NANL was detected in the plant material infected with PalTG-1 isolates. As expected, chanoclavine I was not produced, but more surprising was that peramine was not detected. NANL was detected from all 16 populations analyzed and was found to be present above the limit of detection in 74 of the 78 individual plants tested. The mean (± SD) NANL levels estimated for each population ranged from 0.48 ± 0.5 mg/g in the NC-4 population (the lowest) to 3.48 ± 0.6 mg/g in the TN-3 (the highest) of dry leaf material (FIG. 5). The highest level of NANL detected from an individual plant, TN-3-22, was 4.36 mg/g.

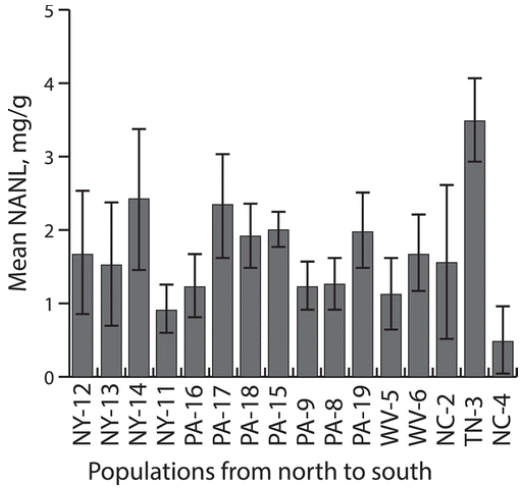

Figure 5. Mean (± SD) *N*-acetylnorloline (NANL) concentrations estimated in vegetative freeze-dried tissues from five plants per population infected with the PalTG-1 = *Epichloë alsodes*.

Peramine was not detected in PalTG-1– or PalTG-2–infected plant tissue. An additional extraction method using 2-propanol–lactic acid was also tested, but peramine was not observed. Independent analyses for peramine production performed by AgResearch further supported the absence of peramine in plants infected with each taxon. The lack of peramine production by both taxa was surprising. An almost complete sequence of the PalTG-1 *perA*alleles revealed one frameshift mutation in the *perA*-M domain and a second frameshift mutation in the *perA*-T1 domain. PCR with allele-specific primers confirmed each mutation across multiple isolates. It is still unclear whether *perA* is functional in PalTG-2 isolates. Only a single copy of *perA* was identified, and no mutations were detected, but we have not been able to complete the whole gene sequence.

Morphological examination

The two endophyte taxa symbiotic with *P. alsodes* were very distinct by colony morphology and growth rate. PalTG-1 has a faster growth rate than PalTG-2, and the colonies are less dense,

often with long aerial hyphae (FIG. 6, TABLE 3). The majority of PalTG-1 isolates (n = 30) showed consistent morphology and is represented by PA-8-20 (FIG. 6A–C). However, slight variation was observed in the populations NY-12 (FIG. 6D–E) and PA-16 (FIG. 6G–H) on the basis of growth rate and aerial hyphae. Two morphotypes of PalTG-2 were observed: morphotype I is convoluted and morphotype II is cottony (TABLE 3, FIG. 6J–M).

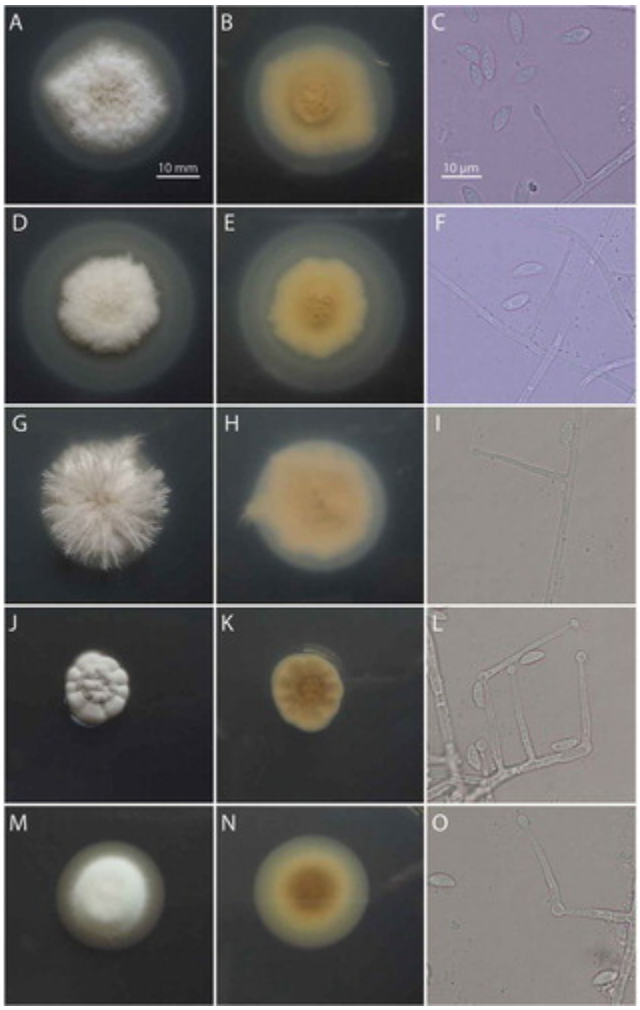

Figure 6. Colony morphology, conidiogeneous cells and conidia of PalTG-1 = *Epichloë alsodes* and PalTG-2 = *Epichloë schardlii* var. *pennsylvanica* isolates from *Poa alsodes*. Colonies were grown for 3 wk on PDA. Colony surface, reverse, and conidiogenous cells with conidia photos. A–C. *E. alsodes* morphotype I isolate PA-8-20. D–F. *E. alsodes* morphotype II isolate NY-12-47. G–I. *E. alsodes* morphotype III isolate PA-16-7. J–L. *Epichloë schardlii* var. *pennsylvanica* morphotype I isolate PA-8-45. M–O. *Epichloë schardlii* var. *pennsylvanica* morphotype II isolate PA-10-10. All microscopic pictures are from the isolates from the same morphotype group.

Conidiogenous cells appear more uniform in PalTG-1 than in PalTG-2 isolates (FIG. 6). The PalTG-1 isolates form solitary conidiogenous cells. In comparison with PalTG-1 morphotype I, morphotype II has shorter and thinner conidiophores and morphotype III has more variation in conidiophore length. Conidia shapes vary, even within one sample; in general, they are obovate

to reniform or allantoid (TABLE 3, FIG. 6 C, F, I, L, and O). The most interesting observation is that PalTG-2 can produce various conidiophores: solitary, apical elongated, and sympodial double or triple (FIG. 6 L, O). Young mycelia of PalTG-2 produce mainly single conidiophores, but after 10 d sympodial conidiophores can be observed.

Due to differences between *E. schardlii* from *C. arundinacea* and PalTG-2 associated with host relationships, sequence variation in *tefA*, and growth rates, we propose that PalTG-2 represents a new variety and recommend the name *Epichloë schardlii* var. *pennsylvanica*.

TAXONOMY

Epichloë alsodes T. Shymanovich, C.A. Young, N.D. Charlton & S.H. Faeth, sp. nov. (FIG. 6A–C, TABLE 3) MycoBank MB821286

Typification: UNITED STATES. PENNSYLVANIA: Cook Forest State Park, culture of strain PA-8-20 from *Poa alsodes* pseudostem, N41°19.488′, W79°11.555′, elevation 366 m, 28 June 2012, T. Shymanovich, GenBank *tefA* alleles KT749543, KT749544 (**holotype** CUP-068243).

Colonies on PDA white, reaching 22–33 mm diam after 21 d at 24 C (FIG. 6). Colonies raised with aerial hyphae from 2 to 10 mm long. Colony reverse tan center to cream at margin. Vegetative hyphae hyaline, septate, 1.3–2.3 µm wide. Sporulation moderately abundant; conidiogenous cells arising solitarily from hyphae, 12.5–36.7 µm long, 1.7–2.1 µm at the base and 0.78–1.12 µm wide at a tip, usually lacking basal septum. Conidia obovate to reniform, oblong or allantiod, hyaline, aseptate, smooth, 7.1–8.7 × 2.8–3.4 µm. Interspecific hybrid, genetic relationships to *Epichloë typhina* subsp. *poae* (Tadych, K.V. Ambrose, F.C. Belanger & J.F. White) Tadych and *Epichloë amarillians* J.F. White

Etymology: In reference to the host *Poa alsodes*.

Holotype: PA-8-20 USA, Pennsylvania, infecting *Poa alsodes*, deposited in the Cornell University Plant Pathology Herbarium as CUP-068243. Ex-type PA-8-20 maintained in laboratory of S.H.F. as PA-8-20, and at the Noble Research Institute as NFe90810.

Habitat: Endophyte of *Poa alsodes*.

Known distribution: Widely distributed across the host populations along the Appalachian Mountains from North Carolina to the Canadian border, and in Michigan.

Specimens examined: Two representative mycelia isolates were examined per population, resulting in 34 isolates total. Isolates numbers are NC-2-17, NC-2-42, TN-3-9, TN-3-40, NC-4-35, NC-4-46, WV-5-20, WV-5-41, WV-6-6, WV-6-47, VA-7-2, VA-7-5, PA-8-2, PA-8-20, PA-9-16, PA-9-42, NY-11-1, NY-11-48, NY-12-14, NY-12-47, NY-13-15, NY-13-48, NY-14-1, NY-14-44, PA-15-3, PA-15-42, PA-16-07, PA-16-50, PA-17-7, PA-17-24, PA-18-9, PA-18-35, PA-19-7, and PA-19-39. Locations of isolates are listed in SUPPLEMENTARY TABLE 1. Collector: Tatsiana Shymanovich.

Epichloë schardlii* var. *pennsylvanica T. Shymanovich, C.A. Young, N.D. Charlton & S.H. Faeth, var. nov.(FIG. 6M–O, TABLE 3) MycoBank MB821287

Typification: UNITED STATES. PENNSYLVANIA: Chapman State Park, culture of strain PA-10-10 from *Poa alsodes* pseudostem, N41°44.915′, W79°10.368′, elevation 456 m, 29 June 2012, T. Shymanovich, GenBank *tefA* alleles KT749529, KT749530 (**holotype** CUP-068244).

Colonies on PDA white, cottony, reaching 13–16 mm diam after 21 d at 24 C (FIG. 6). Colonies raised, slight or moderate convolution, felted with irregular or smooth margin. Colony reverse tan center to cream at margin. Vegetative hyphae hyaline, septate, 1.3–2.6 µm wide. Sporulation moderately abundant; conidiogenous cells arising solitarily from hyphae, 18.3–33.5 µm long, 1.7–2.1 µm at the base and 0.8–1.1 µm wide at apex, usually lacking basal septum, but also sympodial conidiation was observed. Conidia obovate to allantoid, hyaline, aseptate, smooth, 7.2–8.6 × 2.9–3.3 µm. Intraspecific hybrid, genetic relationships to *Epichloë typhina* subsp. *Poae* (Tadych, K.V. Ambrose, F.C. Belanger & J.F. White) Tadych.

Etymology: Varietal form of *Epichloë schardlii* referring to the geographic origin of the state of Pennsylvania.

Holotype: PA-10-10 USA, Pennsylvania, infecting *Poa alsodes*, deposited in the Cornell University Plant Pathology Herbarium as CUP-068244. Ex-type PA-10-10 maintained in the laboratory of S.H.F. as PA-10-10, and at the Noble Research Institute as NFe91010.

Habitat: Endophyte of *Poa alsodes*.

Known distribution: This species is distributed as an endophytic fungus in the host *Poa alodes*in Chapman State Park, Elk State Park, Allegheny National Forest, Cook Forest State Park, and Clear Creek State Park in Pennsylvania, USA.

Specimens examined: Two representative mycelial isolates were examined per population resulting in 10 isolates total. Isolates examined were PA-8-27, PA-8-45, PA-10-10, PA-10-42, PA-17-33, PA-17-44, PA-18-42, PA-18-45, PA-19-28, and PA-19-34. Locations of isolates are listed in SUPPLEMENTARY TABLE 1. Collector: Tatsiana Shymanovich.

DISCUSSION

Our study aimed to describe *Epichloë* spp. from a latitudinal transect collection of *P. alsodes* natural populations starting from the southern edge of its distribution to the Canadian border. In 1993, South Carolina was identified as the southern edge of distribution for *P. alsodes* (Hill 2007). However, 19 years later, we did not observe this plant species at the described location, and the southern distribution edge was considered the mountains of North Carolina. The *P. alsodes* populations showed high infection frequency (92.5%) with endophytic *Epichloë* species across the majority of collection sites. The lowest infection rate (26%) was observed at a single location, NC-4 population, in the Great Smoky Mountains National Park with an elevation above 1669 m.

Two distinct *Epichloë* taxa were identified, where *E. alsodes* (PalTG-1) was present in 22 of the 23 populations sampled along the latitudinal transect and *E. schardlii* var. *pennsylvanica* (PalTG-2) was detected in five populations, all from Pennsylvania. Four of the five populations with *E. schardlii* var. *pennsylvanica* also had *E. alsodes*, with only one population, PA-10, present as a single infection with just *E. schardlii* var. *pennsylvanica* present. Such a localized distribution pattern of *E. schardlii* var. *pennsylvanica* may reflect the initial location of the *P. alsodes–E. schardlii* var. *pennsylvanica* symbiosis origin or effects of natural environmental control mechanisms. In the first case, radial expansion of *E. schardlii* var. *pennsylvanica*–infected plants may be expected over time. In the second case, the future distribution of *E. schardlii* var. *pennsylvanica* may be affected by changing environmental factors. More work is needed to find support for each of these hypotheses.

Both *Epichloë* species identified as symbionts of *P. alsodes* most likely originated via parasexual hybridization, where *E. alsodes* (hybrid of *E. amarillans* × *E. typhina* subsp. *poae*) represents an interspecific hybridization, whereas *E. schardlii* var. *pennsylvanica* is considered intraspecific (hybrid of *E. typhina* subsp. *poae* × *E. typhina* subsp. *poae*). *Epichloë alsodes* represents a new taxon with ancestors from North America (*E. amarillans*) and Europe (*E. typhina* subsp. *poae*), which suggests hybridization occurred after introduction of European hosts to America. *Epichloë schardlii* var. *pennsylvanica* resembles the first and only other known intraspecific hybrid, *E. schardlii*, identified as a symbiont of *Cinna arundinacea* (Ghimire et al. 2011).

Very little variation was detected across *E. alsodes*. This is reflected in the genotype data that show that all plants infected with *E. alsodes* had the same alkaloid genotype and mating type (*MTA, MTB*). No significant variation was observed from *tefA* and *calM* sequences, and mutations identified in *dmaW* and *perA* were consistent across all representative isolates from multiple populations. These data provide support for a single hybridization event hypothesis with radiation of *E. alsodes* being distributed with its host.

Phylogenetic analysis of *tefA, calM*, and *tubB* shows that *E. schardlii* var. *pennsylvanica* has strong similarity with *E. schardlii* from the host *Cinna arundinacea*. At this stage, it is unclear whether the symbiosis of *P. alsodes* with *E. schardlii* var. *pennsylvanica* represents a host jump between *C. arundinacea* and *P. alsodes* (direction unknown) or *E. schardlii* var. *pennsylvanica* and *E. schardlii* may have originated from different hybridization events. Both *C. arundinacea* and *P. alsodes* are native to the USA and have overlapping distributions, but *C. arundinacea* is more widespread than *P. alsodes* (Gilliam et al. 2014; USDA Plants Database POAL3, CIAR2). Unfortunately, during our collections we did not survey other grasses in the area to determine whether *C. arundinacea* infected with *E. schardlii* was present, which may have provided support of a host jump.

Previously, endophyte-infected *P. alsodes* plant samples from North Carolina were analyzed for alkaloids, where the lolines *N*-formylloline and *N*-acetylloline and the ergot alkaloids ergosine and ergocryptine were detected (TePaske et al. 1993). This is not consistent with our findings, as we detected *N*-acetylnorloline only in *E. alsodes*–infected plants, and no alkaloids were detected in plants infected with *E. schardlii* var. *pennsylvanica*. The sampling regime used for our study spanned 1200 km latitude and included multiple sampling locations in North Carolina. Of the 876 plants that tested positive for endophyte infection, only two *Epichloë* taxa were identified,

and within these taxa no diversity was seen. Neither taxon has the genetic capability to produce *N*-formylloline, *N*-acetylloline, ergosine, or ergocryptine. Ergosine and ergocryptine are ergot alkaloids typically associated with *Claviceps pupurea*, and *Epichloë* species are not known to produce these compounds (Guerre 2015; Young et al. 2015). Although some genes that encode steps for ergot alkaloid biosynthesis were detected in *E. alsodes*, the first step in the pathway encoded by *dmaW* was not functional due to a frameshift within the gene. The remaining genes required for ergosine and ergocryptine production are not present in either *E. alsodes* or *E. schardlii* var. *pennsylvanica* isolates, so our genetic analysis supports our chemical analyses. It is possible that chemical analysis in the TePaske study (TePaske et al. 1993) detected alkaloids from a *Claviceps*-contaminated sample, as ergopeptines are more common and diverse in the *Claviceps* genus than in *Epichloë* (Robinson and Panaccione 2015). To address the disparity with the lolines, the potential to continue the loline pathway after NANL production was determined. The genes *lolN, lolM*, and *lolP*, encoding the last steps of the pathway to generate *N*-formylloline and *N*-acetylloline, were absent, confirming that the loline pathway would be truncated at NANL and consistent with contribution from an *E. amarillans* ancestor (Schardl et al. 2013c). Interestingly, neither *Epichloë* taxon was able to produce peramine, yet it appeared that both taxa contained full-length *perA* genes. Nonfunctional *perA* genes are known to have independent mutations that would render the encoded gene nonfunctional (Berry et al. 2015). Through sequence analysis, at least two independent mutations were detected in *perA* from *E. alsodes*, but given the large size of the gene (8.3 kb) and that each ancestor contributes a copy, we were unable to identify all the likely mutations. Thus, the only alkaloid detected in endophyte-infected *P. alsodes* was NANL from *E. alsodes*–infected plants, which may provide protection from insect herbivory and should not affect mammalian grazers (Schardl et al. 2009).

The two *Epichloë* taxa from *P. alsodes* are easy to differentiate by colony morphology and growth rate. *Epichloë alsodes* colonies often produce abundant aerial hypha and grow about twice as fast as the dense cottony colonies of *E. schardlii* var. *pennsylvanica*. For *E. schardlii* var. *pennsylvanica* isolates, two morphotypes were observed, which are similar to the *E. schardlii* morphotypes from *C. arundinacea*. At the microscopic level, the two *P. alsodes* endophytic species were also distinct: *E. alsodes* produces single conidiophores common to many *Epichloë* species, whereas *E. schardlii* var. *pennsylvanica* has both single and sympodial conidiophores. This feature was not described for the closely related *E. schardlii* from the *C. arundinacea* hosts (Ghimire et al. 2011). Differences in sympodial conidiophore production might be explained by different sampling regimes between Ghimire et al. (2011), which checked daily for conidiation and studied early-emerged conidiophores, and our study, where sympodial conidiophores were observed mostly after 10 d. However, colony growth rates of *E. schardlii* var. *pennsylvanica* appear significantly faster (4.5–5.5 mm/wk at 24 C) than *E. schardlii* (1.6–3.3 mm/wk at 24 C).

In summary, endophytic *Epichloë* species from the *P. alsodes* hosts were sampled along a significant latitudinal transect, and two taxa were observed. A widely distributed new interspecific hybrid species, *E. alsodes*, was described in this study. The second taxon, *E. schardlii* var. *pennsylvanica*, which resembles the intraspecific hybrid *E. schardlii*, was only found in northwestern Pennsylvania. Potential for endophyte chemical defenses for both species were analyzed with genetic markers designed to key alkaloid biosynthesis genes and

confirmation by chemical detection. Future research will address the effects of these two endophyte species on host fitness and protection against insect herbivores.

ACKNOWLEDGMENTS

We gratefully thank the Great Smoky National Park, Allegheny National Forest, Pisgah National Forest, and state parks in South Carolina, Virginia, West Virginia, Pennsylvania, and New York for assistance with collections; University of North Carolina at Greensboro Department of Biology; Faeth's lab members Dr. Susanna Saari, Dr. Martina Oberhofer, and Danielle Heyes; Shymanovich family for help with collections; Noble Research Institute Genomic Core Facility and Forage Analysis Core Facility; Ginger Swoboda and Mihwa Yi (Noble Research Institute) for research support; and Wade Mace (AgResearch, New Zealand) for analysis of samples for peramine.

FUNDING

C.A.Y. is supported by the Noble Research Institute, LLC. S.H.F. and N.B.C. are supported by NSF grant DEB 0917741.

Supplemental data
Supplemental data for this article can be accessed on the publisher's Web site.

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