Alkaloid Variation Among Epichloid Endophytes of Sleepygrass (*Achnatherum robustum*) and Consequences for Resistance to Insect Herbivores

By:Tatsiana Shymanovich, Susanna Saari, Mary E. Lovin, Alan K. Jarmusch, Scott A. Jarmusch, Ashleigh M. Musso, Nikki D. Charlton, Carolyn A. Young, Nadja B. Cech, Stanley H. Faeth

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Abstract:

Epichloid endophytes are well known symbionts of many cool-season grasses that may alleviate environmental stresses for their hosts. For example, endophytes produce alkaloid compounds that may be toxic to invertebrate or vertebrate herbivores. *Achnatherum robustum*, commonly called sleepygrass, was aptly named due to the presence of an endophyte that causes toxic effects to livestock and wildlife. Variation in alkaloid production observed in two *A. robustum* populations located near Weed and Cloudcroft in the Lincoln National Forest, New Mexico, suggests two different endophyte species are present in these populations. Genetic analyses of endophyte-infected samples revealed major differences in the endophyte alkaloid genetic profiles from the two populations, which were supported with chemical analyses. The endophyte present in the Weed population was shown to produce chanoclavine I, paspaline, and terpendoles, so thus resembles the previously described *Epichloë funkii*. The endophyte present in the Cloudcroft population produces chanoclavineI, ergonovine, lysergic acid amide, and paspaline, and is an undescribed endophyte species. We observed very low survival rates for aphids feeding on plants infected with the Cloudcroft endophyte, while aphid survival was better on endophyte infected plants in the Weed population. This observation led to the hypothesis that the alkaloid ergonovine is responsible for aphid mortality. Direct testing of aphid survival on oat leaves supplemented with ergonovine provided supporting evidence for this hypothesis. The results of this study suggest that alkaloids produced by the Cloudcroft endophyte, specifically ergonovine, have insecticidal properties.

Keywords: Alkaloid chemoprofiles | Epichloë | Ergonovine | Herbivores | Indole-diterpenes | Insecticide

Article:

Introduction

Wild grasses have evolved symbiotic relationships with endophytic fungi that cope with multiple abiotic and biotic stresses (Cheplick and Faeth *2009*; Kannadan and Rudgers *2008*). The most well studied of these fungal endophytes are *Epichloë* species that systemically infect many cool-season pooid grasses. The most pronounced and well known effect of these endophytes is the production of bioactive alkaloids that can protect their host from vertebrate and invertebrate herbivores and pathogens (Clay *1996*; Crawford et al.*2010*). Epichloid alkaloids are grouped into four classes: ergot alkaloids (e.g., chanoclavine, ergonovine, and ergovaline), lolines (e.g., *N*-acetylnorloline and *N*-formylloline), indole-diterpenes (e.g., terpendole C and lolitrem B), and peramine. Each has varying biological activity against vertebrate or invertebrate herbivores (Panaccione et al. *2014*). A given endophyte may produce alkaloids from one or more classes, and multiple alkaloids from within each class (Schardl et al. *2013a*, *c*). Genome sequencing of multiple *Epichloë* species has indicated that the source of variation for the type of alkaloid produced stems mainly from the remarkable variation in presence of alkaloid genes among *Epichloë* species and strains (Schardl et al. *2013c*). Hybrid*Epichloë* species, by the nature of arising from multiple progenitors, have the potential to increase genetic variation for alkaloid production (Schardl and Craven *2003*; Schardl et al. *2012*, *2013a, c*). However, environmental factors, such as soil nutrients or herbivore grazing, may modulate alkaloid levels (Bultman et al. *2004*; Hunt et al. *2005*). Accumulating evidence also indicates that *Epichloë* species and strains vary greatly not only among grass species but also within a single grass species. For example, *Hordelymus europaeus* (Oberhofer and Leuchtmann *2012*), *Festuca arizonica* (Sullivan and Faeth *2008*), and *Bromus laevipes* (Charlton et al. *2014*) can harbor both hybrid and nonhybrid *Epichloë* species.

Achnatherum robustum (formerly *Stipa robusta*) is native to mountainous areas of the southwestern USA, and is commonly known as sleepygrass because of its long-recognized toxic and narcotic effects on livestock (Jones et al. *2000*). Indeed, sleepygrass is one of the relatively few epichloid-infected native grasses known to be highly toxic to vertebrates (Faeth *2002*b). The toxic effects are due presumably to ergot alkaloids (ergonovine and lysergic acid amide) (Petroski et al. *1992*) produced by an asexual, seed-borne epichloid endophyte. Infected grasses with high levels of ergot alkaloids occur in a restricted range of *A. robustum* near Cloudcroft, NM in the Lincoln National Forest (Faeth et al. *2006*). Endophyte-infected *A. robustum* from this location show very high levels of the ergot alkaloids ergonovine (EN), lower levels of lysergic acid amide (LAA), isolysergic amide, and much lower levels of ergonovinine. The presence of these alkaloids could explain the toxic effects of sleepygrass on livestock, such as narcotized sleep, elevated body temperature, weakness, frequent urination, dizziness, hyper salivation, diarrhea, and potential death (Miles et al. *1996*; Petroski et al. *1992*). Cytotoxic effects to animal muscle tissue also have been described for ergonovine and ergonovinine (Zhang et al. *2014*). Although less well studied, ergot alkaloids also may have deterrent and toxic effects on

invertebrate herbivores (Panaccione et al. *2014*; Schardl et al. *2013a*). In contrast to the Cloudcroft population, endophyte-infected *A. robustum* from other nearby and distant populations do not produce ergot alkaloids such as those found in the Cloudcroft population. One of these populations is located within 22 km from Cloudcroft in the Lincoln National Forest near Weed, NM, USA (Faeth et al. *2006*).

It is likely that *A. robustum* is a host for more than one endophyte species, based upon dramatic differences in alkaloids produced among different endophyte-infected plants (Faeth et al. *2006*). Presently, only one endophyte species, *Epichloë funkii* (formerly *Neotyphodium funkii*) has been described from *A. robustum*(Leuchtmann et al. *2014*; Moon et al. *2007*). *Epichloë funkii*, a hybrid endophyte with *E. elymi* and *E. festucae* ancestral progenitors, was described based on a single plant collection in Colorado, USA (Moon et al. *2007*). Recent draft genome sequence of *E. funkii* indicates the presence of *EAS* biosynthesis genes required for production of chanoclavine I, an early ergot alkaloid pathway intermediate, *IDT*/*LTM* biosynthesis genes required for production of terpendoles from the indole-diterpene pathway, and the *perA* gene required for peramine production (Schardl et al. *2013c*). To date, however, only chanoclavine I has been detected from *E. funkii*-infected plant tissues, while peramine and indole-diterpenes have not been analyzed (Schardl et al. *2013a*, *c*). The alkaloid genetic profile of *E. funkii* does not support the production of ergonovine, yet ergonovine is found at high levels in endophyte-infected sleepygrass plants from the Cloudcroft population. Therefore, this evidence suggests that a different *Epichloë* species with the capability to produce ergonovine also infects *A. robustum*.

Our goal was to examine the variation in epichloid endophytes, their alkaloid genes and products, and the ecological consequences for herbivores, in two disjunct, but nearby *A. robustum* populations (Cloudcroft and Weed). We tested whether the endophytes and their associated alkaloids differentially affected herbivores via a standard insect bioassay with aphids. To test for a mechanism underlying the observed variation in aphid resistance, we tested the anti-herbivore properties of a specific ergot alkaloid, ergonovine, in controlled experiments.

Methods and Materials

Field Plants

To study endophyte infection status (E), variation in production of the alkaloids ergonovine and lysergic acid amide (A) and alkaloid levels, we sampled *A. robustum* plants established in 2005 in an experimental plot at the Arboretum of Flagstaff, Flagstaff, Arizona (Faeth et al. *2010*). The experimental plot included three groups: uninfected plants (E-A-), endophyte-infected not producing ergonovine (E+A-), and endophyte-infected producing ergonovine (E+A+). Seed used for this plot (Table 1), originated from Cloudcroft and Weed natural populations, New Mexico, USA collected during 2001–2004 (Faeth et al. *2006*). Each group was organized from multiple seedlings grown from 1 to 2 maternal plants. In September 2011, one tiller per plant was

collected, checked for endophyte infection, and stored at −20 °C for alkaloid analyses. In May 2013, we recollected several plant samples from each group and tested for endophyte infection, ergot alkaloid production, and endophyte alkaloid genotype. *Achnatherum robustum* is an obligate outcrossing species, and plants were allowed to naturally pollinate each other within the experimental plot to produce seed.

Table 1 Origin of *Achnatherum robustum* field plot plants

Group [a]	**Origin of population**	**Coordinates**	**Maternal plant ID**
E-A-	Weed, New Mexico	N:32° 47.7′ W:105° 35.7′	5–91 [b]
E+A-	Weed, New Mexico	N:32° 47.7′ W:105° 35.7′	5–110
E+A+	Cloudcroft, New Mexico	N:32°57.5′ W: 105° 43.1′	4–134
	Cloudcroft, New Mexico	N:32°57.5′ W: 105° 43.1′	4–136

[a]E- = *Epichloë* free plant, E+ = *Epichloë* infected plant; A- = no ergonovine production, A+ = ergonovine production [b]Subsequent analysis of these plants revealed endophyte-infected plants existed within this group

Maintenance of Greenhouse Plants

To establish plants for herbivory experiments, chemotyping, and genotyping, second generation seeds originating from the Cloudcroft and Weed populations (2010 collection from the experimental plot) were planted on January 5, 2011 in potting mix soil (Timberline, USA) in 300 ml pots. Seedlings were grown in the greenhouse at 25 °C/ 22 °C day/night temperatures and natural light conditions and fertilized with 20:20:20 soluble fertilizer with minor elements (Southern Agricultural Insecticides, Inc., Hendersonville, NC, USA) twice a month. One tiller was sampled prior to the herbivory experiment (April 2011) to determine endophyte infection status and alkaloid production. This sampling was repeated after the herbivory experiment (January 2012) to confirm endophyte infection. Samples for genetic studies were taken in December 2012.

Detection of Endophyte Infection Status

The Phytoscreen Immunoblot Kit "*Neotyphodium* Field Tiller" (Agrinostics, Ltd. Co, GA, USA) was used to determine the infection status of all plant samples. One tiller per plant was tested by imprinting the base of the tiller onto nitrocellulose paper to detect endophyte presence by immunoblot analysis, while the remainder of the tiller was retained for chemical analysis. Fresh samples were used from greenhouse plants and frozen samples from field plants.

Alkaloid Extraction

Leaf samples were freeze-dried and extracted with 95 % methanol (40 mg in 1 ml) at 5 °C for 48 h. The extract was filtered through a 0.22 μm spin filter (Corning Inc.), air dried, and re-dissolved in 17 % aqueous methanol. The resulting extracts were stored at 5 °C until time of analysis.

Lysergic Acid Amide and Ergonovine Analysis

To detect and quantify ergot alkaloids, HPLC-HESI-MS analyses were performed on a triple quadruple mass spectrometer (TSQ Quantum, Thermo, San Jose, CA, USA) interfaced to an HPLC system with photodiode array detector (monitored at 300 nm) and quaternary pump (Agilent HP1100 series). A binary solvent composition of aqueous 0.1 % formic acid (solvent A) and 0.1 % formic acid in methanol (solvent B) was employed with a flow rate of 0.20 ml/min on a C18 column (50 × 2.1 mm, 3 μm particle size, Prevail packing,Grace, Deerfield, IL, USA). Separation was achieved using a linear gradient that initiated at 95%A:5%B (v/v) and remained isocratic from 0 to 4 min; decreased linearly from 95%A:5%B to 90%A:10%B from 4 to 5 min; from 90 %A:10 %B to 70 %A:30 %B from 5 to 11.5 min; from 70 %A:30 %B to 10 %A:90 %B from 11.5 to 11.6 min; remained isocratic at 10 %A:90 %B from 11.6 to 16 min; increasing from 10 %A:90 %B to 95 %A:5 %B from 16 to 16.1 min; and remained isocratic at 95 %A:5 %B from 16.1 to 24 min.

Aqueous solutions of the ergot alkaloids lysergic acid amide tartrate (98 % pure by LC-MS) and ergonovine maleate (Sigma-Aldrich, 100 % pure by TLC) were employed as standards for quantitation. The mass spectrometer was operated in the positive ion mode with a 0.1 s scan time and a scan width of 0.5 *m/z*.Quantification was performed using selected reaction monitoring (SRM) with a 268 to 208 transition for lysergic acid amide and a 326 to 223 transition for ergonovine. Alkaloid quantities were calculated by linear regression of the relevant calibration curves.

Analysis of N-acetylnorloline, Chanoclavine I, and Peramine

Loline alkaloids, chanoclavine I, and peramine were analyzed by using ultra performance liquid chromatography – high resolution mass spectrometry (UPLC-HRMS) on an Orbitrap mass spectrometer with electrospray ionization (ESI) source (LTQ Orbitrap XL, Thermo, San Jose, CA, USA) coupled to Acquity UPLC (Waters Corp., Milford, MA, USA). A hydrophilic interaction chromatography (HILIC) column (150 × 2.1 mm, 5 μm particle size, 120 Å pore size, Alltima packing, Grace, Deerfield, IL, USA) was utilized for the analysis of all extracts, with a 0.3 ml/min flow rate and a 3 μl injection volume. The samples were analyzed using the following gradient composition, where A = 0.1 % formic acid in (acetonitrile) and B = 0.1 % formic acid in (water), 95.1 % A from 0 to 8 min. Mass spectrometric detection was conducted in the positive ion mode with a scan range of 75–300 m/z. Capillary temperature was 275 °C, sheath gas pressure was 20 (arbitrary units), and spray, capillary, and tube lens voltages were 4.5 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 *N*-acetylnorloline, peramine, and chanoclavine I were all detected, consistent with previous literature (Charlton et al. *2012*; Clay and Schardl *2002*; Schardl et al. *2013c*). A synthetic standard of *N*-acetylnorloline also was analyzed as a positive control.

Indole-Diterpenes Chemical Analysis

Indole-diterpene analyses were performed by AgResearch in New Zealand using LC-MS/MS according to Rasmussen et al. (*2012*).

DNA Extraction and Chemoprofiling

Tillers from greenhouse and field plants were evaluated for the presence of associated *Epichloë* species, and the endophyte was characterized using PCR. DNA was isolated from plant material with MagAttract 96 DNA Plant Core Kit (QIAGEN Inc.) according to manufacturer's instructions. PCR with six multiplex primers sets were used to determine endophyte infection status, mating type, and genes present at each alkaloid loci as described in Charlton et al. (*2014*). In addition, the multiplex three primer set included primers, dmaW818(311 + 21)d (5′-AACCCATCAACGGAGCAACTG) and dmaW818(1068 + 21)u (5′-GCCAAACACTGTGAAATACACCTG), designed to the *E. gansuensis* var. *inebrians* e818 *dmaW* ^{EN} gene required for ergonovine production (L. Chen, C. L. Schardl unpublished).

Aphid Biological Assay

An aphid bioassay was employed to test the effects of endophytic alkaloids from different endophyte-infected *A. robustum* on herbivore resistance (e.g., Cheplick and Faeth *2009*). In total, 101 greenhouse grown plants originating from the Cloudcroft population and 54 plants from the Weed population were evaluated. Twenty seven plants from the Cloudcroft population with total ergonovine plus lysergic acid amide (EN+LAA) ergot alkaloid levels greater than 26.7 μg/kg (at the age of 3 months) were selected for one group, and 26 infected plants from the Weed population with no detectable ergonovine and lysergic acid amide alkaloids were selected for the other group. Two *Rhopalosiphum padi L.* aphid populations were used for this experiment: wild NC (North Carolina) origin (collected in Greensboro, NC) and NY (New York) origin (obtained from the UNC-Chapel Hill collection). The NY population has been observed to be more tolerant to endophytic alkaloids (M. Dekker, pers. communication). *Rhopalosiphum padi* has been used commonly to bioassay the effects of endophytic alkaloids on herbivores (Leuchtmann et al. *2000*; Saari et al. *2014*). Aphids were reared on oat (*Avena sativa*) plants, so they were naïve to fungal alkaloids (oats do not produce alkaloids). This experiment continued for 30 day in October-November 2011 when plants were 10 month old. Initially, three aphids were placed on *A. robustum* plants enclosed with clear plastic cups and thin fabric secured on top for air exchange. Every 3 days, wingless and winged aphid numbers were recorded, and an additional three aphids were added to each plant to maintain populations. Both wingless and winged forms

were recorded because aphids may produce winged forms when host plant quality deteriorates (Braendle et al. *2006*; De Barro *1992*).

Bioassay to Test Anti-Herbivore Activity of Ergonovine

To test the direct effects of the ergot alkaloid ergonovine on aphid herbivores, 20 one-wk-old oat (*Avena sativa*) seedlings (seed material from Nasco, Fort Atkinson, WI, USA) were cut at soil level and placed into an aqueous ergonovine solution (1.5 ppm, 1 ml) in a microcentrifuge tube covered with aluminum foil. Each leaf was secured in the tube with a small piece of sponge. Ergonovine was adsorbed naturally due to transpiration. A 15 ml clear plastic centrifuge tube with the end cut off was inverted to cover the leaf in the microfuge tube, and the hole was closed with a small roll of KimWipes to allow some gas exchange. Five *R. padi* (NY) aphids of 3rd and 4th instar were added to each leaf. For the control group, deionized water was used in place of the ergonovine solution. Plants were placed in a growth chamber at 25 °C with 16 h of L/D for 4 days. All aphids were counted, and leaves were freeze-dried to determine the ergonovine concentration. Extraction and LC-MS analysis of ergonovine levels in three control and 20 ergonovine treated leaves was performed as described above. We did not have sufficient lysergic acid amide, the second candidate for insecticidal properties, to test the direct effects on aphids.

Statistical Analysis

RGui 32-bit software with R Commander Package was used for statistical analyses. For ergot alkaloid concentration measurements, averages and population standard deviations were determined. For the ergonovine testing bioassay, we used aphid means with SE counts; *one-way ANOVA* test was performed to determine the difference between the treatment groups, and a simple linear regression model was used to test the effect of ergonovine concentration on aphid numbers. Data from the aphid biological assay was non-normally distributed, so we used rank transformation and *Wilcoxon* nonparametric tests for comparing the differences at each of ten measurements between two plant and two aphid populations. Because of repeated measures, overall aphid numbers between populations also were compared with *Hotelling's T 2* test for ranked data. To test differences in the collective number of wingless and winged forms over all time periods, we used the *Pearson's Chi-square* test.

Results

Infection Status and Ergot Alkaloid Levels in Seedlings from Cloudcroft and Weed Populations

Differences were observed in alkaloid content and endophyte infection status between 3 month old seedlings originating from Cloudcroft and Weed populations. When the endophyte infection status was determined by immunoblot analysis for 155 greenhouse three-month old seedlings, only the Weed population tested positive for endophyte infection, while all Cloudcroft seedlings appeared to be endophyte free. However, chemical analysis revealed the presence of ergot alkaloids (ergonovine and lysergic acid amide) at varying levels in 74 out of 101 Cloudcroft

population seedlings (Table 2) despite negative immunoblot results. All 54 plants from the Weed population seedlings tested negative for the presence of ergot alkaloids, ergonovine, and lysergic acid amide.

Table 2 Ergonovine (EN) and Lysergic Acid Amide (LAA) levels from three-month-old *Achnatherum robustum*

Population # plants tested	**# of plants EN + LAA detected**	**# of plants EN + LAA Not detected**	**Highest concentration EN (ppb or µg/kg)[a]**	**Mean EN ± SD[b](ppb or µg/kg)**	**Highest concentration LAA (ppb or µg/kg)**	**Mean LAA ± SD (ppb or µg/kg)**
Cloudcroft 101 plants	74	27	248	25 ± 36	31	3.7 ± 5.1
Weed 54 plants	0	54	0	0	0	0

[a]Alkaloid concentrations were calculated as µg of alkaloid per kg of dry leaf material [b]Means and standard deviation (SD) were calculated for all plants tested in the group, including plants that produced no detectable alkaloids

Infection Status and Ergot Alkaloid Production in Field Plot Plants

Endophyte infection status and ergot alkaloid analysis of 105 adult plants originating from all four mother plants from the Cloudcroft and Weed populations were determined (Table 3). Endophyte infection was detected by the immunoblot method from the adult plants for both populations. We detected seven endophyte-free plants out of 59 Cloudcroft plants and six endophyte-free plants out of 46 plants from the Weed population. Surprisingly, the purported E-A- group (Faeth et al. *2006*) from Weed mother plant 5–91 had only four uninfected plants from the total of 21 plants (Table 3), suggesting that the original mother plant was mistakenly identified as uninfected. The original infection status of the majority (23 of 25) of the E+A- group plants was confirmed by immunoblot. As expected, the ergot alkaloids ergonovine and lysergic acid amide were detected only from plants that originated from Cloudcroft, E+A+ group. Ergonovine levels in dry plant tissues ranged from 0 to 2.67 µg/g, and lysergic acid amid levels ranged from 0 to 1.18 µg/g (Table 3).

Table 3 Endophyte infection status and Ergonovine (EN) and Lysergic Acid Amide (LAA) levels in *Achnatherum robustum* field plot plants in September 2011

Populatio	**Status in 2011**	**Range of**	**Mean EN**	**Range of**	**Mean**	**Total**

n /Status when planting/ # plants tested[a]	(immunoblotting and alkaloid testing)	EN[b](ppm or µg/g)[c]	± SD (ppm or µg/g)[d]	LAA[b](ppm or µg/g)[c]	LAA ± SD (ppm or µg/g)[d]	Mean EN+LAA ± SD (ppm or µg/g)[d]
Cloudcroft (E+A+) 59 plants	52 plants (E+A+)	0 to 2.67	1.023 ± 0.60	0 to 1.18	0.369 ± 0.28	1.392 ± 0.86
	7 plants (E-)	0	0	0	0	0
Weed (E+A-) 25 plants	23 plants (E+A-)	0	0	0	0	0
	2 plants (E-)	0	0	0	0	0
Weed (E-A-) 21 plants	4 plants (E-A-)	0	0	0	0	0
	17 plants (E+A-)[e]	0	0	0	0	0

[a]E- = *Epichloë* free plant, E+ = *Epichloë* infected plant; A- = no ergonovine production, A+ = ergonovine production [b] *EN* ergonovine, *LAA* lysergic acid amide [c]Alkaloid concentrations were calculated as µg of alkaloid per g of dry leaf material [d]Means and standard deviation (SD) were calculated for all plants tested in the group, including plants that produced no detectable alkaloids [e]Originally E-A- plants but changed to E+A- based upon positive immunoassay tests

Genetic and Chemical Variation of Endophytes from Two Populations

Infection status, mating type, and alkaloid gene profiles were determined for 26 Cloudcroft and nine Weed samples that originated from all mother plants used in our aphid experiments. Within each population, endophytes from all mother plants had the same genetic profiles represented in Fig. 1. However, the endophytes from the Weed and Cloudcroft populations are genetically distinct from each other (Fig. 1). The endophyte from the Weed population resembles *E. funkii* (Schardl et al. *2013c*), whereas the endophyte from the Cloudcroft population is distinct in mating type and alkaloid gene profiles.

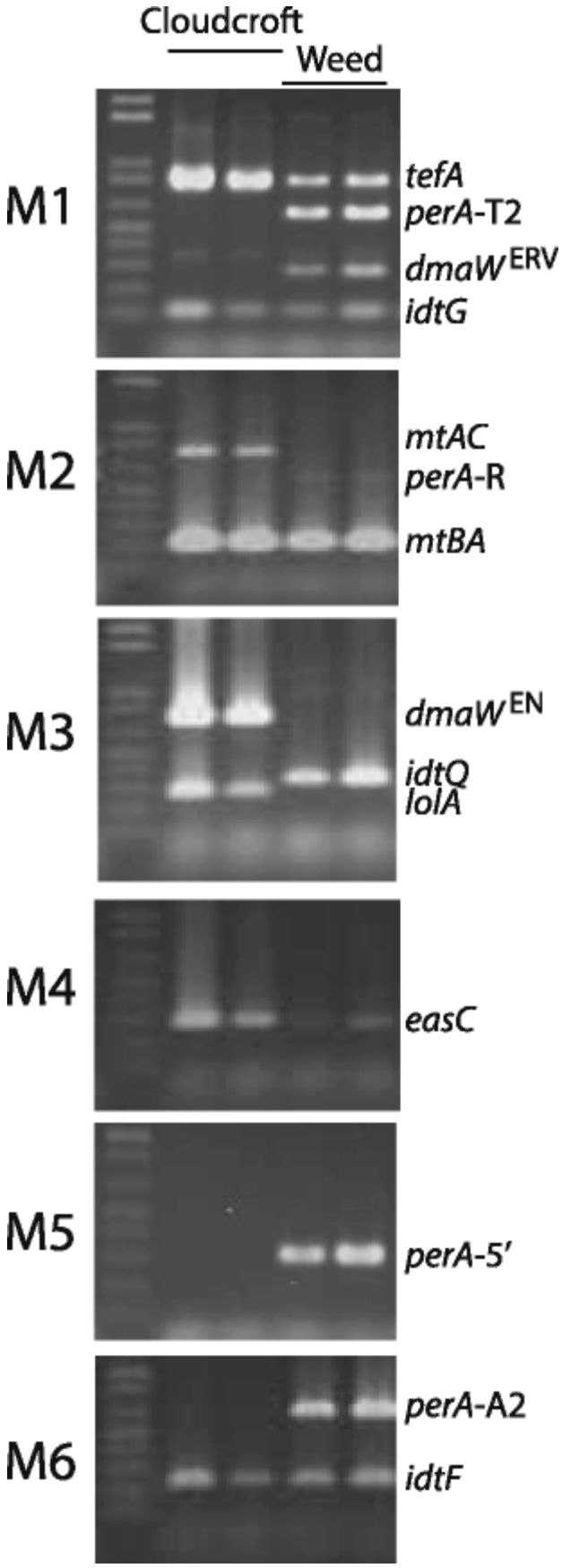
Cloudcroft
Weed
M1
tefA
perA-T2
dmaW ERV
idtG
M2
mtAC
perA-R
mtBA
M3
dmaW EN
idtQ
lolA
M4
easC
M5
perA-5′
M6
perA-A2
idtF

Fig. 1 Genetic analysis of endophyte-infected *Achnatherum robustum* from Cloudcroft and Weed populations. Each column represents analysis of DNA from individual plants from Cloudcroft and Weed populations amplified with markers to determine endophyte genetic diversity across the two populations

The endophytes from each of the locations have different mating types (Fig. 1, Table 4). The Cloudcroft endophyte contains both mating type idiomorphs, *MTA* and *MTB*, which indicates a hybrid origin. The endophyte from the Weed population has one mating type *MTB*, but probably is also a hybrid where both ancestral progenitors were *MTB*. In addition, the endophytes from each location contained different alkaloid gene profiles that suggest they are capable of producing different alkaloids (Fig. 1). The presence or absence of key pathway genes allowed us to predict the likelihood of an alkaloid being produced based on our knowledge of the associated biosynthetic pathways (Schardl et al. *2013b*).

Table 4 Endophyte genetic profiles from maternal plants originating from Cloudcroft and Weed populations

Detection	**Genes**	**Multiplex**	**Fragment size, bp**	**Cloudcroft**		**Weed**	
				Mother plant		**Mother plant**	
				4–134	**4–136**	**5–91**	**5–110**
Mating type	*mtAC*	M2	785	+	+	–	–
	mtBA	M2	213	+	+	+	+
Mating type genotype				*MTA MTB*		*MTB MTB*	
Peramine	*perA*-5′	M5	309	–	–	+	+
	perA-A2	M6	652	–	–	+	+
	perA-T2	M1	600	–	–	+	+
	perA-R	M2	589	–	–	+ faint	+ faint
Predicted *PER* chemotype[a]				nonproducer		unknown	
Ergots	*dmaW*^{ERV}	M1	282	–	–	+	+
	dmaW^{EN}	M3	758	+	+	–	–
	easC	M4	278	+	+	+	+

	easA	M4	350	–	–	–	–
	cloA	M5	383	–	–	–	–
	lpsB	M3	598	–	–	–	–
Predicted *EAS* chemotype[b]				CC, EN, LAA		CC	
Indole-diterpenes	*idtG*	M1	113	+	+	+	+
	ltmQ	M3	334	–	–	+	+
	ltmF	M6	277	+	+	+	+
	ltmJ	M5	242	–	–	–	–
Predicted *IDT/LTM* chemotype [c]				PAS		PAS, PAX, TER	
Lolines	*lolC*	M1	442	–	–	–	–
	lolA	M3	270	+	+	–	–
	lolO	M4	719	–	–	–	–
	lolP	M5	566	–	–	–	–
Predicted *LOL* chemotype[d]				nonproducer		nonproducer	

[a] *PER* peramine [b] *EAS* ergot alkaloid. *dmaW* ^{ERV} is associated with *EAS* clusters from endophytes that produce only CC or ergovaline (ERV) [e.g. *E. funkii* and *E. festucae*; (Schardl et al. *2013c*)] while *dmaW* ^{EN} is associated with*EAS* clusters from endophytes that produce EN and LAA [e.g. *E. gansusensis* var. *inebrians* (Schardl et al.*2013b*)]: *CC* chanoclavine-I, *EN* ergonovine, *LAA* lysergic acid amide [c] *IDT*/*LTM* indole-diterpenes/lolitrems, *PAS* paspaline, *PAX* paxiline, *TER* terpendoles [d] *LOL* lolines

Two *dmaW* markers were used to identify variation at the *EAS* locus. The *dmaW* ^{ERV} and *dmaW* ^{EN} markers were designed to different *dmaW* alleles identified within *Epichloë* species. The *dmaW* ^{ERV} marker was designed for species that are able to produce chanoclavine (e.g., *E. elymi* E56) or ergovaline (e.g., *E. festucae* Fl1); while *dmaW* ^{EN} is specific for ergonovine producers such as *E. gansuensis* var. *inebrians* from*Achnatherum inebrians* (Schardl et al. *2013b*). The presence of only *dmaW* ^{ERV} and *easC* in the Weed population endophyte is suggestive of a chanoclavine producer, as markers to the later *EAS* pathway genes were not detected. The endophyte present in the Cloudcroft population has the *dmaW* ^{EN} and *easC* markers (Fig. 1, Table 4), and this profile has been associated with ergonovine and lysergic acid amide producers (L. Chen, C. L. Schardl unpublished). It is likely

that other ergonovine pathway specific genes exist in the Cloudcroft endophyte but these were not tested for in our study.

Markers for the *perA* gene encoding peramine synthetase were detected only in endophyte-infected plants from the Weed population. Three of the four *perA* markers produced PCR bands, while the expected band for the *perA* reductase domain was faint, so it is unclear if this gene is likely to encode a functional protein, and if peramine would be produced (Fig. 1). The complete absence of all *PER* markers in the Cloudcroft population endophyte indicates this endophyte likely lacks the *perA* gene and would be unable to make peramine (Table 4).

Variation also was identified within the *IDT*/*LTM* locus of the endophytes from each population (Fig. 1). The endophyte from the Weed population contained the markers for *idtG, idtF, and idtQ*. Based on this genetic profile, we would predict this endophyte could produce early indole-diterpene products, such as paspaline, paxiline, and some terpendoles. The endophyte present in the Cloudcroft population contained the markers*idtG* and *ltmF*. The absence of a product for *idtQ* suggests that the indole-diterpene pathway for this endophyte may be blocked early, which would result in the biosynthesis of paspaline (Fig. 1, Table 4).

Neither the Cloudcroft nor the Weed population endophytes have the potential for loline alkaloid production. PCR products for the *LOL* markers were not detected from samples of endophyte-infected material from the Weed population. Although the endophyte in the Cloudcroft population contained *lolA*, a gene associated with the *LOL* gene cluster, the presence of this gene alone will not support synthesis of any loline compounds (Fig. 1, Table 4) (Schardl et al. *2013b*).

Genetic analysis to detect the presence of key genes from each alkaloid locus provides knowledge of alkaloids that could be produced within the different endophyte-infected populations (Table 5). To date, ergot alkaloids were expected only from endophyte-infected plants from the Cloudcroft population, as ergonovine and lysergic acid amide have previously been detected (Faeth et al. *2006*). However, the genetic profile of the endophyte from the Weed population indicated the capacity of this endophyte to produce chanoclavine, which was confirmed by chemical analysis. Peramine production was not detected in the Weed population, thus supporting the likelihood that the *perA* gene is not functional. Chemical analysis for indole-diterpenes confirmed their presence in plant tissues from both populations. Paspaline was detected in infected plants from both populations. Furthermore, as predicted by genetic analysis, terpendoles E, I, J, and C were detected only from Weed population plants. As expected based upon genetic profiles, no lolines, which are well known insecticides (e.g. Panaccione et al. *2013*; Siegel et al. *1990*), were detected in infected plants from either population.

Table 5 Alkaloids detected by chemical analysis compared to predictions based on genetic analyses

		Cloudcroft population	**Weed population**

Alkaloid class	Alkaloid	Predicted	Detected	Predicted	Detected
Peramine	Peramine	No	No	Unsure [a]	No
Ergot alkaloids	ChanoclavineI	Yes	Yes	Yes	Yes
	Ergonovine	Yes	Yes	No	No
	Lysergic acid amide	Yes	Yes	No	No
Lolines	NANL	No	No	No	No
Indole-diterpenes	Paspaline	Unsure	Yes	Yes	Yes
	Paxilline	No	No	Yes	Unsure
	Terpendoles E, I, J, C	No	No	Yes	Yes

[a]Only three out of four gene regions could be amplified

Response of Aphids to Endophyte-Infected Plants

Aphid numbers were significantly lower on the Cloudcroft population than Weed population plants at each of the 10 sampling periods ($P < 0.001$) and across all dates (*Hotelling'sT 2* $P < 0.001$). Aphids on most of the Cloudcroft plants did not survive at all during sample periods, so it was necessary to add more aphids to all plants after each aphid count. In contrast, aphid numbers increased over time on the Weed population plants (Fig. 2). The two aphid strains did not differ (*Hotelling's T 2* $P = 0.39$) in overall numbers on plants from each of the Weed and Cloudcroft populations, although the NY aphid strain had higher mean population sizes, especially on Weed plants. Aphids reared on the endophyte-infected Weed population plants produced more winged forms (20 and 11 % for NY and NC aphids, respectively), than on Cloudcroft plants (13 and 4 % for NY and NC aphids, respectively). However, increased proportion of winged forms likely stems from denser aphids populations on Weed plants due to decreased host plant quality (Braendle et al. *2006*; De Barro *1992*) rather than host toxicity, since very few aphids survived on any Cloudcroft plants.

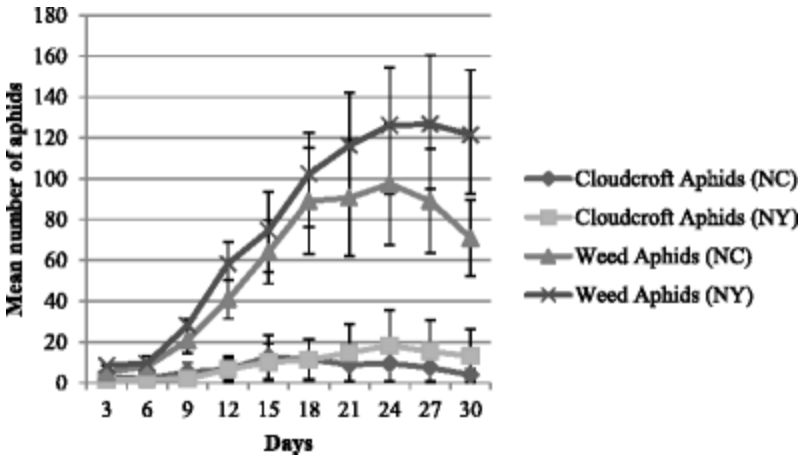

Fig. 2 Change in mean numbers of NY and NC aphid strains on Weed and Cloudcroft endophyte-infected plants during the recording days. *Error bars* represent SE

Aphid Performance From the Ergonovine Insecticidal Bioassay

Aphids reared on oat leaves supplemented with ergonovine had reduced mean numbers (8.4 ± 1.0 SE) when compared to aphids reared on control plants (12.2 ± 1.3 SE) (*ANOVA* $F_{1,38} = 5.348$, $P = 0.026$). The mean concentration of ergonovine in the oat leaves after treatment was 0.123 ± 0.05 μg/kg, approximately 8.3 times lower than the ergonovine concentration measured from field plants (Table 3). However, in a linear regression model of actual concentrations vs. aphid numbers, ergonovine concentration measurements at the end of the treatment had marginally negative relationship on aphid numbers ($P = 0.087$). Because experimental levels of ergonovine were low relative to naturally-infected plants, our assay suggests that even very low levels of ergonovine may reduce aphid numbers, presumably via reduced aphid performance and survival.

Discussion

It is well established that systemic *Epichloë* species infecting native grasses are genetically diverse among grass species (Leuchtmann et al. *2014*; Schardl et al. *1997*; Schardl and Phillips *1997*). More recent evidence suggests that endophytes also can be highly variable within wild grass species, whereby endophyte diversity identified within a single host species can be due to different endophyte species or different strains of the same endophyte species representing different alkaloid potential (Charlton et al. *2012*,*2014*; Iannone et al. *2012*; Kang et al. *2011*; Moon et al. *2004*; Oberhofer and Leuchtmann *2012*; Takach et al. *2012*; Takach and Young *2014*; Wali et al. *2007*). Genetic variation among endophytes can lead to phenotypic changes in host grasses that may be greater than that caused by endophyte infection per se (Morse et al. *2007*; Oberhofer et al. *2014*). These phenotypic changes caused by endophyte genetic variation, especially in alkaloid production, can then profoundly affect competing plant species, herbivores, and natural enemies of herbivores (Cheplick and Faeth *2009*).

Our results indicate two genetically distinct endophytes inhabit two *A. robustum* populations in close proximity (22 km apart). Based on genotype and chemotype, the endophyte from the Weed population resembles *E. funkii*, described by Moon et al. (*2007*) from an *A. robustum* Colorado population and analyzed for alkaloid gene diversity by Schardl et al. (*2013a, c*). Phylogenetic data are needed to confirm that the Weed endophyte is indeed *E. funkii*. Interestingly, the Cloudcroft endophyte that likely has been responsible for the name "sleepygrass" due to its well-renowned toxicity to livestock is an undescribed new *Epichloë*species. A forthcoming paper (M. Oberhofer, T. Shymanovich, C. Young, and S. Faeth, unpublished data) will include detailed genetic and morphological data that will describe this endophyte species. Notably, this endophyte appears to be restricted to the Cloudcroft region in the distribution range of *A. robustum*, whereas the endophyte identified from the Weed population is more widespread based upon absence of ergonovine production (Faeth et al. *2006*; Jones et al. *2000*) and endophyte phylogeny (Moon et al. *2004*, *2007*). Similarity can be seen between the *A. robustum* Cloudcroft population endophyte with *E. gansusensis* var.*inebrians* from *A. inebrians* hosts in China. Each is known to produce ergonovine and lysergic acid amide, although *E. gansusensis* var. *inebrians* also can produce lysergic acid α-hydroxyethylamide (Schardl et al.*2013b*). Similarly, *A. inebrians* also is known to be a host for two different endophytes, *E. gansusensis* var.*inebrians*, the likely causal agent of "drunken horse grass", and *E. gansuensis* that is unable to produce ergot alkaloids (Moon et al. *2007*; Schardl et al. *2013b*).

As we predicted, *A. robustum* hosts two different endophytes, which have different effects on insect herbivore performance and survival. Aphid survival and abundances were reduced on infected plants from the Cloudcroft population in comparison to endophyte-infected plants from the Weed population. The main difference observed in the endophyte-infected Cloudcroft population as compared to the endophyte-infected Weed population was the presence of the ergot alkaloids ergonovine and lysergic acid amide. Ergot alkaloids are thought to be effective mainly against vertebrate herbivores (Jackson et al. *1987*; Zavos et al. *1987*; Zhang et al. *2014*). Nonetheless, it seems that ergonovine and possibly lysergic acid amide, produced in the Cloudcroft population, are likely candidates for anti-herbivore effects against aphids. Moreover, there is growing evidence that some ergot alkaloids from different groups, ergopeptines, clavines, and simple amides of lysergic acid possess insecticidal and nematicidal activities (Panaccione et al. *2014*; Potter et al. *2008*). Ergonovine causes feeding inhibition of Japanese beetle, *Popillia japonica*, grubs (Patterson et al. *1991*). The adult black lawn beetle, *Heteronychus arator*, showed a moderate reduction of artificial feed consumption in the presence of ergonovine but not lysergic acid amide (Ball et al. *1997*). Likewise, ergonovine caused weight reduction in fall armyworm, *Spodoptera frugiperda*, larvae but not reduction in leaf area consumed (Clay and Cheplick *1989*). Consistent with these findings, our bioassays with ergonovine-treated *A. sativa* leaves confirmed that the ergot alkaloid ergonovine reduced aphid survival and reproduction. Our study is the first to indicate that ergonovine has insecticidal activity against sucking insects such as aphids. Moreover, reduction in aphid number occurs even when ergonovine is at very low levels. Because endophyte-infected *A. robustum* plants had

ergonovine levels more than eight times higher than our experimental assay, we would expect even much stronger effects of ergonovine in plants in natural populations. Although ergonovine seems like a probable candidate for the reduced aphid numbers on Cloudcroft plants, other alkaloidal and non-alkaloidal differences (e.g., nutritional or water content) cannot be ruled out.

There have been two previous studies (Faeth et al. *2010*, Jani et al. *2010*) on the effects of infection in sleepygrass on herbivory or herbivore abundances and species richness. Most relevant to the current study, Faeth et al. (*2010*) showed that infected plants from the Weed population reduced seed dry biomass and reproductive effort under ambient herbivory treatments compared to conditions of greatly reduced herbivory. In contrast, seed production and reproductive effort of infected plants from the Cloudcroft population were equivalent under ambient and reduced herbivory, suggesting a protective effect of infection and alkaloids in this population. However, Jani et al. (*2010*) found that natural enemies of herbivores also may be affected by alkaloids in infected plants in the Cloudcroft population. They found that abundances and species richness of herbivores and natural enemies was greater and lower, respectively, on sleepygrass plants with high ergot alkaloids compared to plants with low or no ergot alkaloids. They concluded that high alkaloid plants may provide "enemy-reduced" space for specialist herbivores and, thus, herbivory could be greater on infected grasses with high alkaloid levels. Therefore, whether endophytes that produce ergonovine or other alkaloids in sleepygrass reduce, increase, or have no effect on herbivory in nature likely depends on the herbivore species and the presence of natural enemies.

Alkaloid synthesis is energetically and nutritionally costly because alkaloids contain nitrogen that is often limiting in southwestern USA soils (Faeth *2002*a; Faeth and Sullivan *2003*). From this perspective, production of alkaloids that are diverse yet part of the same biosynthetic pathway and effective against both vertebrate and invertebrate herbivores may be more efficient at protecting the host than alkaloids produced from multiple pathways. The ergot alkaloids ergonovine and lysergic acid amide are known to have toxic effects on vertebrates (Oliver et al. *1993*; Schiff *2006*), and ergonovine, according to our study and other sources (Ball et al. *1997*; Clay and Cheplick *1989*; Patterson et al. *1991*), has insecticidal or insect deterring properties. The Cloudcroft endophyte is devoid of *LOL* and *PER* genes required for loline and peramine production, and although it is capable of producing indole-diterpenes, the *IDT* pathway is greatly reduced. Thus, the endophyte from the Cloudcroft population may provide host protection against both vertebrate and invertebrate herbivores through the production of alkaloids from a single biosynthetic pathway.

Although the endophyte present in the Weed population also is capable of producing alkaloids from the *EAS*and *IDT* pathways, the compounds produced were different from the Cloudcroft endophyte and were not efficient at providing protection against aphids. Moreover, the *perA* gene encoding peramine synthetase for the insect feeding deterrent peramine (Tanaka et al. *2005*) is present in the Weed endophyte, but it appears to be non-functional, and peramine was not detected in endophyte-infected samples from the Weed population. We predict that the

endophyte in the Weed population affords less protection against both invertebrate and vertebrate herbivores based upon its alkaloid potential (chanoclavine and terpendole production). The environment could influence host fitness benefits provided by the endophyte. If herbivory is reduced or resources are more limiting within an environment, reduction of alkaloid pathways may lower the metabolic cost of maintaining alkaloid defenses. Interestingly, the Weed and Cloudcroft populations are only a short distance apart, yet they vary in rainfall and soil nutrients. The Weed location has lower rainfall and fewer nutrients than Cloudcroft (Tong Jia et al. unpublished). Thus, it is possible that the Weed environment selected for persistence of an endophyte that produced fewer alkaloids due to higher costs and lower benefits, or that this endophyte provides another yet to be discovered benefit.

Epichloid endophytes that associate with *A. robustum* are challenging to work with compared to other infected grass species. Traditional detection methods such as microscopic examination of leaf tissue and seeds or culturing the endophyte for identification have been unreliable. For example, the endophyte within the Cloudcroft population is slow growing and has only been isolated successfully from the seeds after a 5 month growth period (M. Oberhofer et al. unpublished). Similarly, endophyte infection in 3-month-old Cloudcroft plants could not be detected by the more reliable and commonly used tissue-print immunoblot method. Additionally, vertical transmission rates appear much lower (T. Shymanovich, personal obs.) than other asexual endophytes (Afkhami and Rudgers *2008*). However, unlike detection of the endophyte itself, we could reliably detect ergot alkaloids in endophyte-infected Cloudcroft plant material in 3-month-old seedlings. In contrast, the Weed endophyte was easily detected by the immunoblot method. By using multiple detection methods, genotypic profiling, and alkaloid analyses, we are confident of the endophyte infection status and genotype of endophyte-infected material used in this study.

Our study shows that natural populations of cool-season grasses can harbor genetically different endophyte species or genotypic variants. In turn, these endophytes can have different effects on host plant phenotypes through the production of bioactive alkaloids. In our study, two genetically distinct endophytes from *A. robustum* produced different alkaloid compounds, resulting in varying resistance to aphid herbivores. Although ergot alkaloids are traditionally viewed as active against vertebrates, at least one ergot alkaloid, ergonovine, from the Cloudcroft population has insecticidal properties against aphids, even at low levels.

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