New Zealand Journal of Agricultural Research

ISSN: 0028-8233 (Print) 1175-8775 (Online) Journal homepage: http://www.tandfonline.com/loi/tnza20

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To cite this article: Taixiang Chen, Wayne R. Simpson, Qiuyan Song, Shuihong Chen, Chunjie Li & Rana Z. Ahmad (2019) Identification of *Epichloë* endophytes associated with wild barley (*Hordeum brevisubulatum*) and characterisation of their alkaloid biosynthesis, New Zealand Journal of Agricultural Research, 62:2, 131-149, DOI: 10.1080/00288233.2018.1461658

To link to this article: https://doi.org/10.1080/00288233.2018.1461658

NEW ZEALAND JOURNAL OF AGRICULTURAL RESEARCH
2019, VOL. 62, NO. 2, 131–149
https://doi.org/10.1080/00288233.2018.1461658

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RESEARCH ARTICLE

Identification of *Epichloë* endophytes associated with wild barley (*Hordeum brevisubulatum*) and characterisation of their alkaloid biosynthesis

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ABSTRACT

Epichloë species are biotrophic symbionts of many cool-season grasses that can cause grazing animal toxicosis. We identified fungi from *Hordeum brevisubulatum* as *Epichloë bromicola* based on morphological characteristics and *tefA* and *actG* gene sequences. Three isolates were examined, they contained *perA* gene and 10 out of the 14 *EAS* cluster genes, previously identified in *Epichloë inebrians* E818. Five out of 11 *LTM/IDT* cluster genes previously identified in *Epichloë festucae* Fl1 were present, but no *idtG*, indicating an inability to produce even early intermediates in the lolitrem B pathway. Only one *lolC* gene out of 11 *LOL* cluster genes previously identified in *E. festucae* E2368 was present in the three isolates. Chemotype analyses revealed infected samples contained only peramine, and not ergine, ergonovine, ergovaline or lolitrem B. This study provides new information about the host range and phylogenetic definition of *E. bromicola* and reveal a novel endophyte–grass combination in China.

ARTICLE HISTORY
Received 27 September 2017
Accepted 4 April 2018
First published online
20 April 2018

KEYWORDS
Epichloë bromicola; *Hordeum brevisubulatum*; symbioses; phylogenetic analysis; PCR; alkaloids

Introduction

Fungal endophytes are organisms that live inside plants without causing any apparent damage to their hosts (Bongiorno et al. 2016). Many cool-season grasses (Poaceae, subfamily Poöideae) are infected with clavicipitaceous fungal endophytes, including both sexual and asexual taxa (Leuchtmann et al. 2014). The asexual (anamorphic) taxa were previously assigned to a separate genus (*Neotyphodium*), but have recently been combined with the sexual (teleomorphic) taxa within a single genus, *Epichloë* (Leuchtmann et al. 2014). The sexually reproducing *Epichloë* species are capable of forming stromata that engulf developing inflorescences and suppress seed production (choke disease) (White 1997), and they can be transmitted both vertically and horizontally with sexual or asexual life cycles (Saikkonen et al. 1998; Tadych et al. 2012). Asexually reproducing *Epichloë* species can colonise hosts asymptomatically. They colonise the intercellular spaces of

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Supplemental data for this article can be accessed at https://doi.org/10.1080/00288233.2018.1461658.

the above-ground tissues of the host grass including inflorescences. Vertical transmission in seeds from the mother plant to its offspring is the only described means of distribution (Schardl et al. 2012; Tadych et al. 2012; Saikkonen et al. 2016).

Many *Epichloë* endophytes can endow hosts with increased abiotic stress tolerance as a result of enhanced growth, tillering, reproduction and nutrient acquisition, particularly under drought, cold, salt and nutrient-deficiency conditions (Rodriguez et al. 2009; Zhang et al. 2015). However, the effects vary with species, strains and circumstances, and genetic factors modulate the compatibility between partners (Gundel et al. 2012). Some *Epichloë* species can also depress their host's fitness when their consumption of resources is greater than the benefits they provide (Cheplick 2007). In return, the host provides certain benefits to the endophyte such as shelter, nutrition and a platform for reproduction and/or dissemination through the host seed. Certain species *Epichloë* endophytes also confer biotic stress tolerance upon specific hosts by producing several classes of biologically active alkaloids. Lolines and peramine produced by the endophyte have anti-insect properties, while ergot alkaloids (ergovaline, ergonovine) and indole-diterpenes (lolitrem B) are toxic to mammalian herbivores, causing fescue toxicosis and ryegrass staggers, respectively (Wilkinson et al. 2000; Fleetwood et al. 2007; Schardl et al. 2012). Although ergot alkaloids and indole-diterpenes are considered to have some detrimental effects on livestock, their presence provides additional protection to the host plant (Rodriguez et al. 2009).

The occurrence of alkaloids has been extensively studied in many grasses infected by *Epichloë* endophytes, since endophyte infection has economic importance in many regions. Alkaloid production depends on specific interactions between the host and the fungus (Barker et al. 2015; He et al. 2017). The genes required for the biosynthesis of four classes of alkaloids have been identified; however, not all have been characterised, and many of their functions are still unknown (Spiering et al. 2005, 2013b; Tanaka et al. 2005; Young et al. 2005, 2006). A single gene located at the *PER* locus is required for peramine biosynthesis (Tanaka et al. 2005). The genes required for loline, indole-diterpene (lolitrem B) and ergot alkaloid biosynthesis are located in complex gene clusters containing large stretches of associated repetitive elements (Young et al. 2005, 2006; Fleetwood et al. 2007). The diversity of alkaloids produced by a metabolic pathway is dependent on the genes present in the genome, and the presence or absence of functional domains in the biosynthetic enzymes. Therefore, the presence or absence of certain genes and the sequences of the proteins they encode can be used to predict the capacity of an endophyte to produce an alkaloid or associated intermediates (Schardl et al. 2013a; Charlton et al. 2014; Takach and Young 2014). Such predictions are an inexpensive and rapid method to screen large endophyte–grass symbiosis populations for individuals likely to be useful in forage agriculture (Takach and Young 2014).

The genus *Hordeum* Linn. (family Poaceae, subfamily Pooideae, cereal tribe Triticeae or Hordeeae) includes several important forage and turf species (Wang et al. 2016). Wild barley (*Hordeum brevisubulatum*) is widely distributed in China and is extensively cultivated for pasture production globally. It is known for its high tolerance to several abiotic stresses including drought, salinity and alkalinity (Wang et al. 2016). Previous studies have demonstrated that *Hordeum* species are infected by asexual *Epichloë* endophytes (Moon et al. 2004; Wilson 2007; Leuchtmann et al. 2014), and Song et al. (2015b) reported that an *Epichloë* endophyte played an important role in maintaining the growth of

H. brevisubulatum by promoting nutrient absorption and maintaining the ionic balance under salt stress. Another study showed that endophyte infection of *H. brevisubulatum* increased its resistance to waterlogging (Song et al. 2015c). The endophytes of *H. brevisubulatum* in China have not yet been characterised, but *Hordeum* species from other countries have been reported to harbour hybrid and non-hybrid *Epichloë* endophytes (Moon et al. 2004; Wilson 2007; Leuchtmann et al. 2014).

The morphological and physiological characteristics of cultured endophytes and gene-based sequence analyses have been used to describe and distinguish sexual and asexual *Epichloë* species. The evolutionary relationships among *Epichloë* spp. endophytes have been explored by comparing the sequences of genes encoding highly conserved proteins such as translation elongation factor $1-\alpha$ (*tefA*) and actin (*actG*) (Moon et al. 2004). Genes involved in alkaloid biosynthesis have also been used in phylogenetic analyses of *Epichloë* endophytes (Hettiarachchige et al. 2015).

The purpose of this study was to characterise the *Epichloë* species found in *H. brevisubulatum* seeds collected from Linze County, Gansu Province, China, and to explore their capacity to produce various alkaloids by determining whether relevant genes were present in their genomes. The results of this study provide important information about the host range of *Epichloë*, and the risks of the *Epichloë–Hordeum* symbiosis to grazing animals and herbivorous insects.

Materials and methods

Plant materials

Seeds of *H. brevisubulatum* were collected from Linze county, Gansu province, China, in September 2014. Seed samples were taken from individual plants, and leaf sheaths were microscopically examined using the staining method described by Li (2005). Seed samples of endophyte-infected and endophyte-free plants were collected and stored at 4°C to maintain endophyte viability. For each sample, some seeds were used to isolate the endophyte, and some seeds were grown for 2 months in a constant temperature greenhouse (22°C) in vermiculite. The seedlings were irrigated with water and modified ½-strength Hoagland's nutrient solution as required. Endophyte infection was verified by PCR analyses of leaf material using the following *Epichloë*-specific PCR primer pair: (tub2-exon 1d-1: GAGAAAATGCGTGAGATTGT, tub2-exon 4u-2: GTTTCGTCC GAGTTCTCGAC) (Moon et al. 2002). The obtained seeds were stored at the Official Herbage and Turfgrass Seed Testing Centre, Ministry of Agriculture, China.

Isolation of endophytes from seeds

The seeds were surface-sterilised with 75% ethanol for 3 min, then with 5% sodium hypochlorite solution for 3 min and then washed three times with sterile water. The seeds were blotted dry and placed on potato dextrose agar (PDA) containing 100 μg/ml ampicillin and 50 μg/ml streptomycin sulphate. The plates were sealed, incubated in the dark at 22°C and examined regularly for endophyte growth for up to 2 weeks. Fungal isolates were transferred onto fresh PDA plates to obtain a pure culture. All isolates have been deposited at the Mycological Herbarium of Lanzhou University, China.

Morphological examination

Colony morphology was examined in cultures grown on PDA plates. The plates were inoculated with 0.4-cm mycelial discs taken from the leading edge of an actively growing culture (six repeats for each isolate). Cultures were incubated at 22°C and 25°C in the dark. At 32 d, colonies were measured, the radial growth rate was recorded and averaged and the cultures were photographed. Microscopic observations of conidia and conidiogenous cells were performed after inoculating mycelial discs as described above, after incubation at 22°C in the dark for 2 weeks. Sterile cover slips were lightly pressed on the edge of the culture medium, and then the cultures were further incubated until the mycelium appeared on the surface of the cover slips. Measurements were conducted using an Olympus CX22LED light microscope (Olympus Corp., Tokyo, China). Images were captured with an Olympus BX51 camera supported by Cellsens Entry 1.8 software (Olympus Corp.). Images were used to measure the length and width of conidia and the length of conidiogenous cells (50 conidia and 30 conidiogenous cells were measured for each isolate). Data shown are mean ± standard error. The morphologies of isolates were compared with those of an *Epichloë* endophyte isolated from *Hordeum* spp. and other *Epichloë* species.

DNA isolation and PCR analyses

Isolates were grown in potato dextrose broth at 22°C with shaking at 120 rpm/min. After 2 weeks, the mycelia were harvested, washed three times with sterile water and ground into a fine powder in liquid nitrogen with a mortar and a pestle. Then, DNA was isolated from approximately 20 mg ground mycelium using a ComWin Dneasy Plant Mini Kit (ComWin Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. Intron-rich portions of the housekeeping genes used for phylogenetic analyses (*tefA* and *actG*) were amplified with the following primers: tef1-exon 1d-1: GGGTAAGGACGAAAAGACTCA, tef1-exon 5u-1: CGGCAGCGATAATCAGGATAG (Moon et al. 2002); and act1-exon 1d-1: TAATCAGTCACATGGAGGGT, act1-exon 6u-1: AACCACCGATCCAGACAGAGT (Moon et al. 2007). The PCR analyses were performed in a total volume of 25 µl (2 × Taq MasterMix mix containing 1.0 U Taq DNA polymerase, 1.5 mM $MgCl_2$, 200 µm each dNTP (ComWin Biotech Corp., Ltd., Beijing, China), 10 µL double-distilled H_2O, 5 ng DNA, 1 µM target-specific forward primer and 1 µM target-specific reverse primer). The cycling conditions used to amplify *tefA* and *actG* were an initial denaturation step for 5 min at 94°C, 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C (*tefA*), 50°C (*actG*) for 1 min, extension at 72°C for 1 min, followed by a final synthesis step at 72°C for 10 min. Products were analysed by gel electrophoresis on a 1.5% agarose gel in 1× Tris–acetic acid–EDTA (TAE) buffer. The DNA fragments were stained with Gold View (Solarbio Corp., Beijing, China) and viewed by UV transillumination.

Target bands of the expected size were excised from the agarose gel and purified with a DNA Purification Kit (Tiangen Corp., Ltd., Beijing, China). Then, each product was ligated into the pGEM-T vector (Promega Corp., Ltd., Beijing, China) and cloned into DH5α competent cells (Tiangen Corp. Ltd.) according to the manufacturer's instructions. The recombinant clones were screened by 'white–blue colony selection' and then cultured

overnight at 37°C in liquid LB growth medium. The products were screened by PCR, and the amplicons were analysed by gel electrophoresis on a 1.5% agarose gel. The target colonies were sequenced using Sanger dideoxy sequencing method by TsingKe Corp. Ltd on an ABI 3730xl DNA analyser (Applied Biosystems). Unique sequences have been submitted to GenBank under the accession numbers KU365146-148 (*tefA*) and KU365152-154 (*actG*).

Phylogenetic analyses

The obtained sequences were initially aligned with sequences from GenBank to predict if they belonged to *Epichloë* spp., and then sequences from representative *Epichloë* species were aligned with Clustal W. Phylogenetic analyses were performed using the maximum likelihood method using MEGA6 software as follows: the intron fragment of each sequence was determined using Gene Structure Display Server 2.0 (Hu et al. 2015), conserved regions were determined using Phylogeny.fr: Gblocks 0.91b (Dereeper et al. 2008) and substitution saturation was tested using DAMBE software. Maximum likelihood analysis parameters included substitution rates, nucleotide frequencies, proportion of invariable sites and gamma distribution shape. These parameters were selected by Akaike information criterion values in Modeltest 3.7 (Posada 2006). Phylogenetic trees were constructed using the best-fit model (Kimura 2 (*tefA*), Kimura 2 (*actG*)), using MEGA6 software, with 1000 bootstrap replicates. Alignments for *tefA* and *actG* were deposited in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S22291 (*tefA*); http://purl.org/phylo/treebase/phylows/study/TB2:S22110 (*actG*)).

Alkaloid gene profiling

To determine which alkaloid biosynthetic genes were present in their genomes, we used specific primers to amplify seven fragments of the *perA* gene, which is involved in peramine biosynthesis, 14 *EAS* cluster genes previously identified in *Epichloë inebrians* E818 that are required for the production of ergot alkaloids, 11 *LOL* cluster genes previously identified in *Epichloë festucae* E2368 that are required for the production of loline alkaloids and 11 *LTM*/*IDT* cluster genes previously identified in *E. festucae* Fl1 that are involved in the biosynthesis of indole-diterpenes. Fragments of genes involved in the biosynthesis of ergot alkaloids, indole-diterpenes, peramine and loline alkaloids were amplified from total genomic DNA extracted from fungal mycelia by PCR with the primer sets described by Charlton et al. (2012) and Berry et al. (2015). Each PCR mixture had a total volume of 25 µl as described above. The cycling conditions for PCR were as follows: initial denaturation for 1 min at 94°C, 30 cycles of denaturation at 94°C for 15 s, annealing at 56°C for 30 s, extension at 72°C for 1 min, then final synthesis at 72°C for 10 min. Amplicons were analysed by gel electrophoresis on a 1.5% agarose gel in 1× TAE buffer. DNA fragments were stained with Gold View (Solarbio Corp.) and viewed by UV transillumination. Markers to detect the mating-type genes *mtAC* and *mtBA* were used to determine the mating-type idiomorph. As positive controls, we used *E. inebrians* E818 for genes at the *EAS* locus (Chen 2015) and *E. festucae* var. *lolii* AR1 for the *perA* gene and some genes at the *IDT* locus (Young et al. 2009, 2013). *Epichloë* sp. FS001 isolated from *Festuca*

sinensis is known to produce lolitrem B and peramine (Kuang 2016) and served as the positive control for the genes related to these alkaloids.

Alkaloid extraction and detection

Endophyte-free (E−) and endophyte-infected (E+) plants were analysed to detect alkaloids. The vegetative shoot material was freeze-dried under dark conditions, and ground material was extracted in two stages with a two-phase solvent system. The alkaloids were detected using an Agilent 1100 HPLC using the protocol described by Wan et al. (2016) and Kuang (2016). Peramine, ergonovine, ergine, ergovaline and lolitrem B were analysed by reverse-phase HPLC and identified by comparison of their relative retention times with those of authentic standards. *Achnatherum inebrians* infected by *E. inebrians* E818, *Lolium perenne* infected by *Epichloë festucae* var. *lolii* AR1 and *Festuca sinensis* infected by *Epichloë* sp. FS001 were used as positive controls for ergot alkaloid, peramine and lolitrem B, respectively. The ergine used as a reference standard was a gift from Dr Miroslav Flieger (Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic). Ergonovine was purchased from Sigma-Aldrich (China). Ergovaline, lolitrem B and peramine used as reference standards were a gift from Dr Wade Mace (Forage Improvement, AgResearch Ltd., Grasslands Research Centre, Palmerston North, New Zealand).

Results

Characteristics of Hordeum brevisubulatum *endophytes*

Endophytes were isolated from each plant generated from surface-sterilised seeds of *H. brevisubulatum*. Three isolates (WBE1, WBE3 and WBE4) with morphologies typical of *Epichloë* species were maintained. These three isolates showed different characteristics in culture (Figure 1). In culture on PDA, colonies of WBE1 were white, raised, slightly convoluted, cottony to fluffy, attaining 2.75–2.90 cm diameter in 32 d at 25°C, colony margins were tan, distinct to superficial (Figure 1A), reverse tan centrally to light tan at margin (Figure 1B). Colonies of WBE3 were white, flattened, lightly felted, attaining 2.30–3.75 cm diameter after 32 d at 25°C (Figure 1C), the reverse of colonies light brown centrally to cream at the margin (Figure 1D). Colonies of WBE4 reached 2.80–2.90 cm in diameter after 32 d at 25°C on PDA, aerial mycelium was white, arising from the agar surface, cottony to fluffy, slightly convoluted in the centre and flattening towards the perimeter (Figure 1E), and the reverse yellowish (Figure 1F). WBE3 showed the fastest growth rate among the three isolates (Table 1) ($p < .05$). WBE1 exhibited a more cottony phenotype than WBE4, while WBE3 had a more felt-like appearance with less aerial growth. Conidiogenous cells of the three isolates showed the same appearance, being phialidic, arising perpendicularly, solitarily from hyphae, produced abundantly, lateral, hyaline, 10–27.5 µm long and 1.25–3.75 µm wide at the base, less than 0.5 µm wide at the apex, usually without septa at or near the base (Figure 2). The conidia of the three isolates also showed the same characteristics, being reniform to ellipsoidal, smooth, hyaline, aseptate, 3–5.5 × 2.5–3.75 µm (Figure 2) with only one conidium produced by each conidiogenous cell. After nomenclatural realignment of *Neotyphodium*

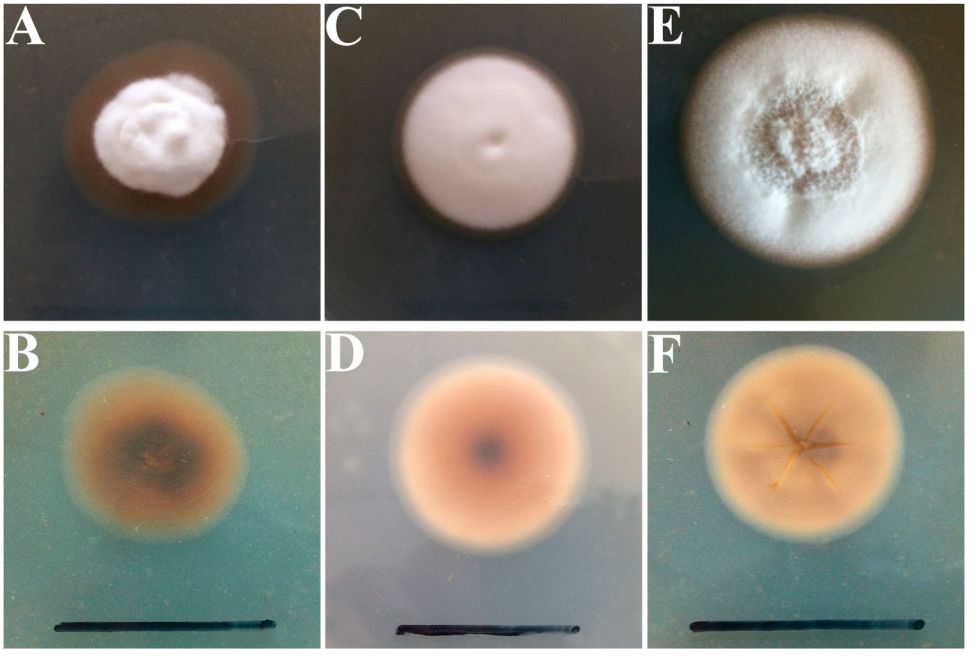

Figure 1. Colony morphology of *Epichloë* endophytes isolated from *Hordeum brevisubulatum* after incubation on PDA at 25°C for 32 d. **A,** surface of WBE1; **B,** reverse of WBE1; **C**, surface of WBE3; **D**, reverse of WBE3; **E**, surface of WBE4; **F**, reverse of WBE4. Bar = 3 cm.

species with the genus *Epichloë*, 34 unique taxa as well as several subspecies and varieties of *Epichloë* species were recognised (Leuchtmann et al. 2014). These species have been well characterised based on their morphology and molecular data. The three isolates in this study were distinguished from these *Epichloë* species by the following differences: the growth rates of our isolates appeared faster than those of the slow-growing species (*E. festucae* var. *lolii* (Li et al. 2004), *Epichloë typhina* var. *canariensis* (Moon et al. 2000), *Epichloë aotearoae* (Li et al. 2004), *Epichloë australiensis* (Li et al. 2004), *Epichloë chisosa* (Li et al. 2004), *Epichloë coenophiala* (Li et al. 2004), *Epichloë danica* (Leuchtmann and Oberhofer 2013), *Epichloë funkii* (Moon et al. 2007), *E. inebrians* (Moon et al. 2007), *Epichloë guerinii* (Moon et al. 2007), *Epichloë melicicola* (Li et al. 2004), *Epichloë occultans* (Li et al. 2004), *Epichloë pampeana* (Iannone et al. 2009), *Epichloë schardlii* (Ghimire et al. 2011), *Epichloë sibirica* (Zhang et al. 2009), *Epichloë siegelii* (Li et al. 2004), *Epichloë sinofestucae* (Chen et al. 2009) and *Epichloë uncinata* (Li et al. 2004)), and appeared slower than those of the fast-growing species (*Epichloë brachyelytri* (Schardl and Leuchtmann 1999), *Epichloë elymi* (Schardl and Leuchtmann 1999), *E. festucae* (Leuchtmann et al. 1994), *Epichloë glyceriae* (Schardl and Leuchtmann 1999), *Epichloë liyangensis* (Kang et al. 2011), *Epichloë sylvatica* (Leuchtmann and Schardl 1998), *E. sylvatica* subsp. *pollinensis* (Oberhofer et al. 2013), *E. typhina* subsp. *poae* (Tadych et al. 2012), *Epichloë canadensis* (Charlton et al. 2012), *Epichloë hordelymi* (Leuchtmann and Oberhofer 2013) and *Epichloë stromatolonga* (Ji et al. 2009)). In addition, the three isolates could be separated from *Epichloë* species with similar growth rates by other characteristics: their conidiogenous cells appeared shorter than those of *Epichloë amarillans* (White 1994), *Epichloë*

Table 1. Morphological characteristics of *Epichloë bromicola* isolated from *Hordelymus europaeus*, *Leymus chinensis*, *Elymus* spp., *Roegneria kamoji*, *Bromus* spp. and *Epichloë* endophytes isolated from *Hordeum* spp.

Endophyte	Host	Conidia size (μm)		Length of conidiogenous cell (μm)	Growth rate (mm/d)		Reference or source
		Length	Width		22°C	25°C	
WBE1 (ns)	*Hordeum brevisubulatum*	5.17 ± 0.06a	2.87 ± 0.17a	19.50 ± 1.06a	0.77 ± 0.02b	0.88 ± 0.01b	This study
WBE3 (ns)		5.08 ± 0.06a	2.75 ± 0.08a	15.75 ± 1.06a	1.17 ± 0.02a	1.02 ± 0.04a	This study
WBE4 (ns)		4.56 ± 0.22a	2.94 ± 0.16a	17.05 ± 1.57a	0.82 ± 0.01b	0.89 ± 0.01b	This study
Epichloë bromicola (ns)	*Hordelymus europaeus*	4.2 ± 0.4	2.1 ± 0.2	20.2 ± 4.7	1.43–1.67 (24°C, PDA)		Leuchtmann and Oberhofer 2013
Epichloë bromicola (s)	*Leymus chinensis*	5.3 ± 0.1	3.5 ± 0.1	29.0–31.0	1.7 ± 0.07 (25°C, PDA)		Zhu et al. 2013
Epichloë bromicola (ns)	*Elymus* spp.	nt			0.41–0.85 (25°C, PDA)		Song et al. 2015a
Epichloë bromicola (s)	*Roegneria kamoji*	4.7–5.2	2.0–2.9	16.5–25.8	0.83–2.58 (25°C, PDA)		Li et al. 2006
Epichloë bromicola (s)	*Bromus erectum*	3.7–4.4	1.9–2.1	nt	nt		Brem 2003
Epichloë bromicola (ns)	*Bromus ramosus* *Bromus benekenii*	3.7–4.8	1.8–2.3	nt	nt		Brem 2003
Epichloë bromicola (s)	*Bromus erectum*	3.8 ± 0.4	2.0 ± 0.3	8–23	2.29–2.48 (24°C, PDA)		Leuchtmann and Schardl 1998
Epichloë bromicola (ns)	*Bromus ramosus* *Bromus benekenii*	4.2 ± 0.5	2.0 ± 0.3	nt	0.90 (24°C, PDA)		Leuchtmann and Schardl 1998
Epichloë sp. (ns)	*Hordeum brevisubulatum* subsp. *violaceum*	3.9–7.1	2.2–3.4	11.2–28.6	nt		Dugan et al. 2002
Epichloë sp. (ns)	*Hordeum bogdanii*	3.1–6.5	nt	nt	nt		Clement et al. 1997
Epichloë sp. (ns)	*Hordeum brevisubulatum* ssp. *violaceum*	3.0–5.0	nt	nt	nt		Clement et al. 1997

Note: Different lower-case letters indicate significant differences among WBE1, WBE3 and WBE4 (Duncan's test, $p < 0.05$).
ns: Non-stromal strain; s: Stroma forming strain; nt: not tested.

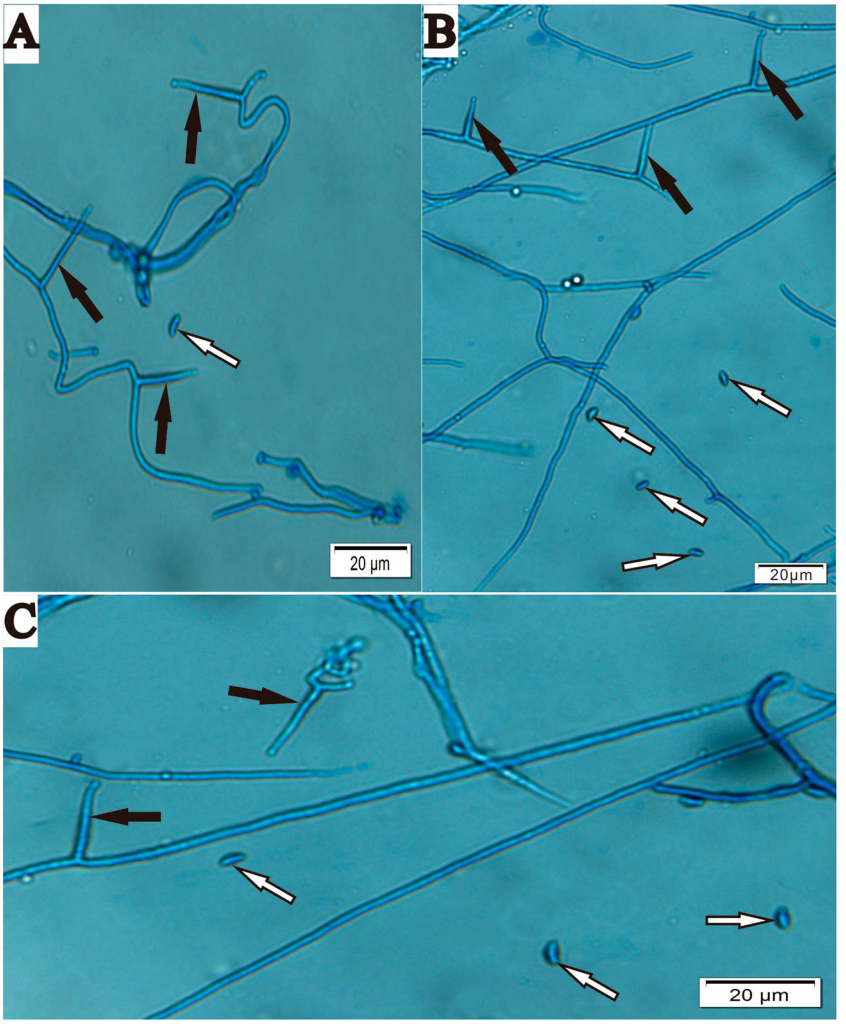

Figure 2. Conidiogenous cells and conidia of *Epichloë* endophyte isolated from *Hordeum brevisubulatum* after incubation on PDA at 22°C. **A**, Conidia (white arrow) and conidiogenous cells (black arrow) of WBE1; **B**, Conidia (white arrow) and conidiogenous cells (black arrow) of WBE3; **C**, Conidia (white arrow) and conidiogenous cells (black arrow) of WBE4. Bar = 20 µm.

baconii (White 1993), *E. typhina* subsp. *clarkii* (White 1993), *E. typhina* var. *aonikenkana* (Mc Cargo et al. 2014), *Epichloë cabralii* (Mc Cargo et al. 2014) and *Epichloë disjuncta* (Leuchtmann and Oberhofer 2013). They also differed from *E. typhina* (Li et al. 2004), *E. typhina* var. *huerfana* (Li et al. 2004), *Epichloë gansuensis* (Li et al. 2004), *Epichloë mollis* (Morgan-Jones and Gams 1982), *Epichloë sinica* (Kang et al. 2009) and *Epichloë tembladerae* (Li et al. 2004), which have septa near the base of their conidiogenous cells. In addition, the appearance of the colonies of the three isolates differed from those of *E. typhina* var. *ammophilae* (White et al. 1992), which become yellow-golden to orange with age. The morphological characteristics of the three isolates in culture were consistent with the original description of *Epichloë bromicola* (Leuchtmann and Schardl 1998). Table 1

summarises the morphology of *E. bromicola* in different hosts and *Epichloë* species in different host plants in the *Hordeum* genus. The growth rates of *E. bromicola* from *Hordelymus europaeus*, *Leymus chinensis* and *Bromus erectum* reported in previous studies appeared higher than those of the three isolates determined in this study. In addition, the conidia of the three isolates in this study appeared larger than those of *E. bromicola* from *H. europaeus*, *B. erectum*, *Bromus ramosus* and *Bromus benekenii*, and appeared smaller than those of *E. bromicola* from *L. chinensis*. The conidiogenous cells of the three isolates in this study appeared shorter than those of *E. bromicola* isolates from *L. chinensis*.

Phylogenetic relationships

The PCR amplification of *tefA* and *actG* from endophyte genomic DNA of the three test isolates from *H. brevisubulatum* yielded products of about 860 bp and 1300 bp, respectively. A single gene was amplified from each isolate, indicating that these *Epichloë* species have not hybridised. Phylogenetic analyses were carried out based on the *tefA* and *actG* intron sequences to determine the origin. All three isolates grouped together in each gene phylogram. The *tefA* phylogenetic tree grouped the sequences from the three isolates in the same clade as *E. bromicola*, which constituted a well-supported clade including two sister clades, one of its sister clades included only sequences of *E. bromicola* from Eurasia *H. brevisubulatum* and our three isolates, the other sister clades included *E. bromicola* from *H. europaeus*, *Bromus* spp. and *Roegneria kamoji* (Figure 3). In the *actG* phylogenetic tree, the three isolates in this study grouped with a well-supported *E. bromicola* clade that included *E. bromicola* from *L. chinensis* and *Elymus* spp. in China (Figure 4).

From the above results, the three isolates most closely resembled *E. bromicola* from European *H. brevisubulatum*, Chinese *L. chinensis* and Chinese *Elymus* spp. Therefore, the three isolates obtained from *H. brevisubulatum* were considered to be conspecific with *E. bromicola*. Their morphological features in culture were consistent with the original description of *E. bromicola* (Leuchtmann and Schardl 1998).

Alkaloid gene profiling

The genomic DNA of the three isolates was tested by PCR to detect key genes required for alkaloid production and to define their mating-type idiomorphs. The three isolates showed the same gene profiles (Supplementary Figure). The genomes of the three isolates contained *mtAC*, but not *mtBA*, indicating that they are mating-type 1 (MTA) (Table 2). All three isolates tested positive for the peramine markers (seven segments of *perA*: *perA*-A1, *perA*-T1, *perA*-C, *perA*-A2, *perA*-M, *perA*-T2 and *perA*-R*) but negative for *perA*-△R* alleles (Table 2), which contain a transposon-associated deletion of the *perA* region encoding the C-terminal reductase domain (Berry et al. 2015), indicating that they can produce peramine. The *dmaW*, *easF*, *easE* and *easC* genes at the *EAS* locus were detected (Table 2), suggesting that they could produce chanoclavine I, but the genes *easD*, *lpsC*, *easO* and *easP* were absent, indicating that they could not produce ergot alkaloids beyond chanoclavine I. Of the 11 *E. festucae* E2368 *LOL* cluster genes, only *lolC* was detected in the three isolates, indicating that they could not produce loline alkaloids *in planta* (Table 2). Of the 11 *E. festucae* Fl1 *LTM/IDT* cluster genes,

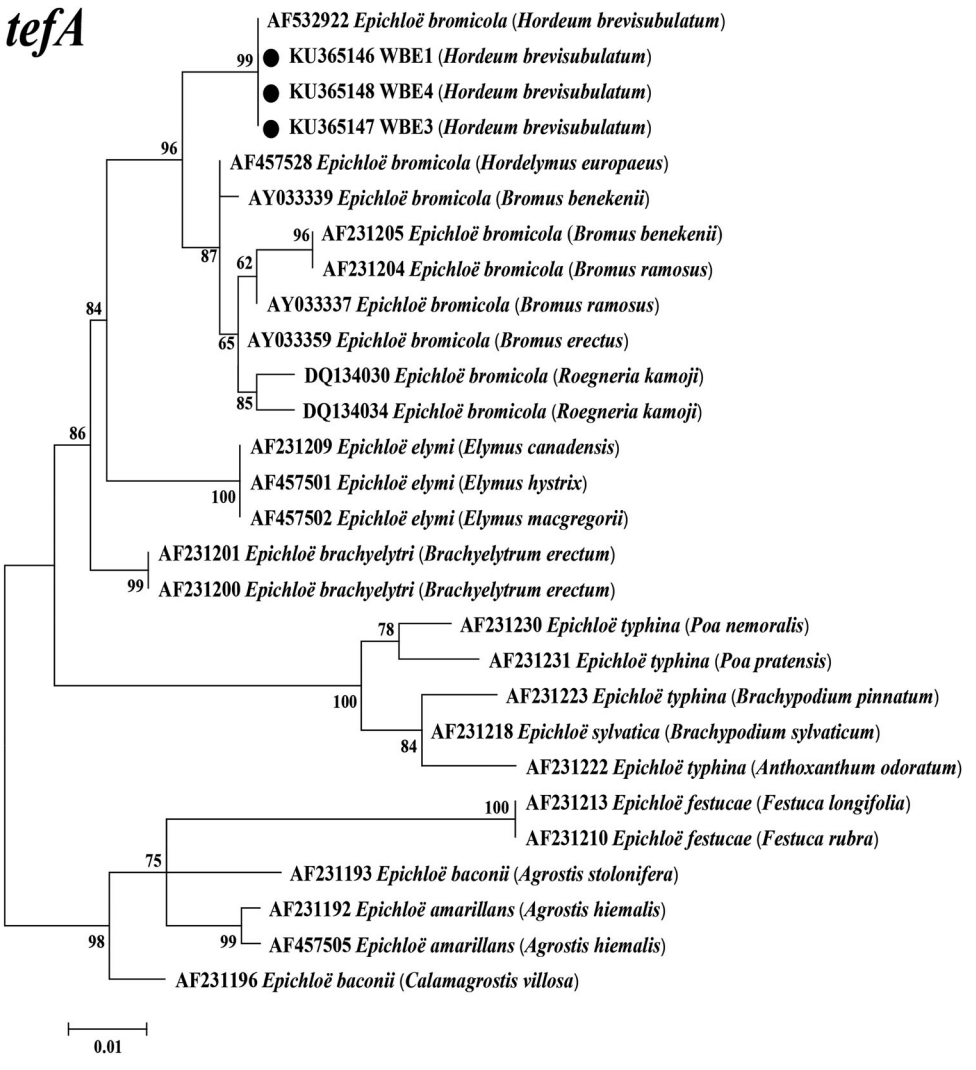

Figure 3. Molecular phylogeny derived from maximum likelihood (ML) analysis of introns of *tefA* gene from representative *Epichloë* species and endophyte WBE1,3,4 isolated from *Hordeum brevisubulatum*. Evolutionary history was inferred using ML method based on the Kimura 2-parameter model. The tree with highest log likelihood (−1293.4036) is shown. The percentage of trees in which associated taxa clustered together is shown next to branches. Initial tree(s) for heuristic search were obtained by applying the neighbour-joining method to a matrix of pairwise distances estimated using the maximum composite likelihood approach. The tree is drawn to scale, with branch lengths measured in number of substitutions per site. The analysis involved 28 nucleotide sequences, and the final dataset had 421 positions. Host designations are shown in parentheses after endophyte.

idtB, idtC, idtK, i*dtS* and *idtM* were detected (Table 2); however, the three isolates appeared to lack a functional *idtG* gene, which has been shown to be required for the biosynthesis of early intermediates in the lolitrem B pathway (Young et al. 2009). These results indicated that the three isolates could not produce even early intermediates in the lolitrem B pathway in symbiotic plants.

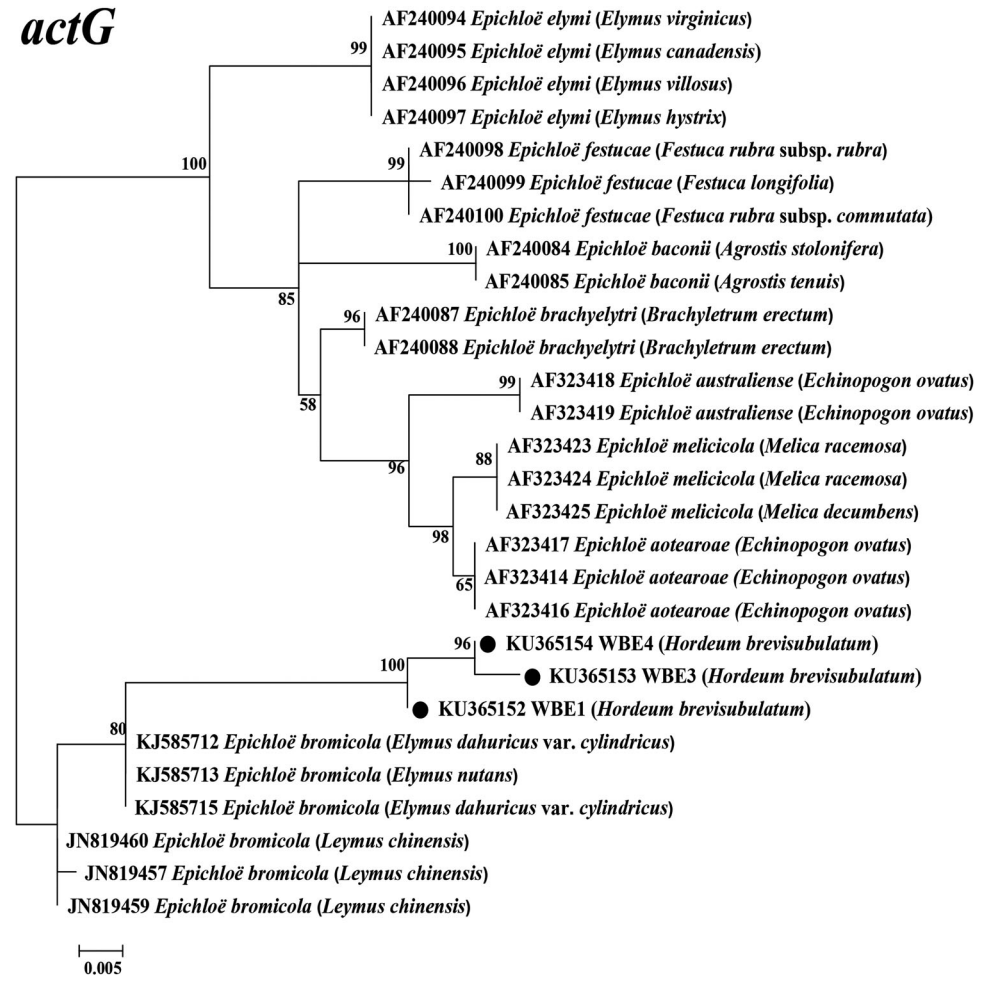

Figure 4. Molecular phylogeny derived from maximum likelihood (ML) analysis of introns of *actG* gene from representative *Epichloë* species and endophyte WBE1,3,4 isolated from *Hordeum brevisubulatum*. Evolutionary history was inferred by using the ML method based on Kimura 2-parameter model. The tree with highest log likelihood (−997.5261) is shown. The percentage of trees in which associated taxa clustered together is shown next to branches. Initial tree(s) for heuristic search were obtained by applying the neighbour-joining method to a matrix of pairwise distances estimated using the maximum composite likelihood approach. The tree is drawn to scale, with branch lengths measured in number of substitutions per site. The analysis involved 28 nucleotide sequences, and the final dataset had 399 positions. Host designations are shown in parentheses after endophyte.

Alkaloid detection

Plants infected with the three isolates showed the same alkaloid chemotype. Comparative studies using E+ and E− *H. brevisubulatum* showed that none of the E− samples contained peramine, ergonovine, ergine, ergovaline or lolitrem B at detectable concentrations. The HPLC analysis showed that peramine was present in E+ *H. brevisubulatum*. Based on comparisons with authentic standards and the positive control, the unknown compounds were not ergonovine (also known as ergometrine or ergobasine), ergine (also known as

Table 2. Mating-type, alkaloid biosynthesis genes in the genome of *Epichloë* endophyte WBE1, WBE3 and WBE4 isolated from *Hordeum brevisubulatum*.

Gene	Present or absent in endophyte genome	Gene	Present or absent in endophyte genome
Mating-type genes		Indole-diterpene (*IDT/LTM*) genes	
mtAC	+		
mtBA	–	*idtG*	–
Segments of *perA* gene		*idtB*	+
perA-A1	+	*idtM*	+
perA-T1	+	*idtC*	+
perA-C	+	*idtP*	–
perA-A2	+	*idtQ*	–
perA-M	+	*idtF*	–
perA-T2	+	*idtE*	–
perA-R*	+	*idtK*	+
perA-△R*	–	*idtS*	+
Ergot alkaloid (*EAS*) genes		*idtJ*	–
dmaW	+	Loline (*LOL*) genes	
easF	+		
easE	+	*lolA*	–
easC	+	*lolC*	+
easD	–	*lolD*	–
easA	+	*lolE*	–
easG	+	*lolF*	–
cloA	+	*lolO*	–
lpsA	+	*lolP*	–
lpsB	+	*lolT*	–
easH	+	*lolU*	–
lpsC	–	*lolM*	–
easO	–	*lolN*	–
easP	–		

+: gene present in endophyte genome; –: gene absent from endophyte genome.

lysergic acid amide), ergovaline (also known as ergopeptine) or lolitrem B. We did not try to detect loline, because all of the isolates lacked the genes for loline biosynthesis.

Discussion

The endophytes isolated from *H. brevisubulatum* were identified as *E. bromicola* based on their morphological characteristics and phylogenetic analyses. The three isolates showed minor phenotypic differences in their morphology, but they grouped together in the phylogenetic analyses. *Epichloë bromicola* has a broad host range and infects grasses including *Bromus* spp., *Elymus* spp., *H. europaeus* and *H. brevisubulatum* from Europe, and *L. chinensis*, *R. kamoji* and *Elymus* spp. from Asia. *Epichloë bromicola* is capable of vertical transmission in some of its hosts (*B. benekenii, B. ramosus*, *H. europaeus* and *H. brevisubulata* (Europe), and *L. chinensis* and *R. kamoji*) but produces the sexual stromata structure ('choke') in other host species (*B. erectus*, *E. repens* and *R. kamoji*) (Leuchtmann et al. 2014). To our knowledge, there are no reports on whether the teleomorph is expressed in the *E. bromicola–H. brevisubulata* symbiosis, and it is yet to be determined whether the sexual stromata stage is present in field-grown plants. The incidence of the endophyte in *Hordeum* was first reported in 1991 (Wilson et al. 1991), when it was found in the seeds of *Hordeum bogdani* (seed infection percentage, 47%–88%), *H. brevisubulatum* ssp. *violaceum* (68%–98%) and *Hordeum comosum* (74%–92%).

Accessions of *Hordeum brachyantherum*, *Hordeum bulbosum*, *Hordeum chilense*, *Hordeum jubatum*, *Hordeum murinum* and *Hordeum stenostachys* were endophyte-free (Wilson et al. 1991). Wilson et al. (1992) further reported the infection frequency according to the origin of these endophyte-infected *Hordeum* species. The infection frequency varied widely in *H. bogdani* from Afghanistan (47%), USSR (62%–80%), and China (0%–99%), in *H. brevisubulatum* ssp. *violaceum* from Iran (0%–68%) and the USSR (0%–98%), and in *H. comosum* originating from Argentina (0%–92%). However, these endophytes were simply identified as *Acremonium* species (Leuchtmann et al. 2014), and their morphological characteristics were not established. Clement et al. (1997) and Dugan et al. (2002) preliminarily described the dimensions of their conidiophores and conidia, and called these endophytes '*Neotyphodium*-like isolates' because their taxonomic identity had not been established at that time. Moon et al. (2004) first identified endophytes from *Hordeum* to the species level from phylogenetic relationships based on the sequences of *tefA* and *tubB*. They were identified as asexual *E. bromicola* from Eurasian *H. brevisubulatum,* but their morphological characteristics were not described. Our identification of *Epichloë* from *H. brevisubulatum* based on morphological characteristics and phylogenetic analyses has broadened the known host range of *E. bromicola*. In addition, this is a new endophyte–grass combination from China. It is yet to be determined whether this association is widespread.

Determining the presence or absence of biosynthetic genes in *Epichloë* isolates can define whether they are capable of producing certain alkaloids in their hosts. If the gene cluster for a specific alkaloid is missing from the *Epichloë* species, then the species cannot synthesise that compound because it lacks the proteins or enzymes required for alkaloid biosynthesis. Furthermore, if key pathway genes are missing (or non-functional), then critical pathway steps cannot proceed and the resulting metabolites will not be produced. Alternatively, if genes involved in later stages of a pathway or are missing or non-functional, biologically active pathway intermediates may still be produced (Young et al. 2010). In this study, alkaloid gene profiling and HPLC analyses revealed that the three isolates contain *perA*, and therefore, are capable of producing peramine *in planta*. Further PCR analyses detected 10 out of 14 *EAS* cluster genes previously identified in *E. inebrians* E818 and 5 out of 11 *LTM/IDT* cluster genes previously identified in *E. festucae* Fl1; yet, they did not produce ergot alkaloids beyond chanoclavine I or even early intermediate products of lolitrem B *in planta*. Further HPLC analyses confirmed that ergine, ergonovine, ergovaline and lolitrem B were not present in the endophyte-infected plants. These results were consistent with the finding that the genomes of the three isolates lacked the genes essential for the synthesis of the three ergot alkaloids (ergine, ergonovine, ergovaline) and lolitrem B. In *Epichloë,* four genes (*dmaW*, *easC*, *easE* and *easF*) are required for the biosynthesis of chanoclavine I, the ergot alkaloid intermediate that is thought to be required for the production of more complex ergot alkaloids (Schardl et al. 2013b). *Epichloë* strains lacking *idtE* and *idtJ* but containing at least the core set of genes (*idtB*, *idtC*, *idtG* and *idtM*) are capable of producing indole-diterpene intermediates (Young et al. 2009). The absence of some of early pathway genes (*easD*, *lpsC* and *idtG*) rendered these endophytes unable to synthesise complex ergot alkaloids such as lolitrem B and loline *in planta*. *Epichloë bromicola* is known to be one of the ancestors of several *Epichloë* species, including *E. liyangensis, E. chisosa, E. danica, E. hordelymi, E. occultans, E. siegelii, E. sinica, E. sinofestucae* and *E. uncinata* (Leuchtmann et al.

2014). Therefore, these species with an *E. bromicola* progenitor are likely to harbour some of the alkaloid genes from *E. bromicola* that contribute to alkaloid variations.

Epichloë-infected *Hordeum bogdanii* samples (PI269406, Afghanistan) and *H. brevisubulatum* ssp. *violaceum* samples (PI440420, USSR) were found to contain N-formylloline (Tepaske et al. 1993). However, neither the seeds nor forage samples of *H. bogdanii* and *H. brevisubulatum* ssp. *violaceum* contained loline (Tepaske et al. 1993). Analyses of ergot-type alkaloids showed that ergovaline, ergosine and ergotamine were present in *H. brevisubulatum* ssp. violaceum forage samples (PI440420), and ergovaline was present in *H. bogdanii* forage samples (PI269406). However, neither seeds nor forage materials of *H. bogdanii* or *H. brevisubulatum* contained ergonovine, lysergic acid amide, ergovaline, ergosine, ergotamine or ergocryptine (Tepaske et al. 1993). Although some ergot alkaloids are produced in *Hordeum* species, little is known about their toxicity to livestock. Our results suggest that not all *Epichloë* endophyte-infected *Hordeum* grasses produce the same alkaloids, as demonstrated by the fact that our isolates did not produce complex ergot alkaloids as a product of the relationship with their symbionts.

Most studies on the *Epichloë* endophyte–grass symbiosis have focused on the selection of symbioses that produce high levels of beneficial alkaloids but no detrimental alkaloids, to balance the risk of mammalian toxicosis against the need for effective control of crop insects and nematodes. However, this target can be difficult to achieve because the host plant genotype and environmental conditions affect the relative abundance and amounts of alkaloids produced in the *Epichloë* endophyte symbiosis (Young et al. 2009). The gene profiling and chemotype analyses described in this study provided information about the types of alkaloids that could be produced, and those that were produced, in the *E. bromicola–H. brevisubulatum* association. This association may represent a mammalian-friendly symbiosis that retains the positive benefits of insect pest resistance. Further field persistence and animal safety analyses of this symbiosis are required.

Conclusions

(1) *Epichloë* endophytes isolated from *H. brevisubulatum* plants from Gansu, China, were identified as *E. bromicola*.
(2) The three isolates contained genes encoding enzymes to produce peramine and chanoclavine I; however, they lacked genes encoding enzymes to produce lolitrem B, loline and complex ergot alkaloids beyond chanoclavine I.
(3) These *E. bromicola* strains were unable to synthesise ergine, ergonovine, ergovaline or lolitrem B in *H. brevisubulatum*.
(4) *E. bromicola* may protect *H. brevisubulatum* from herbivorous insects.

Acknowledgements

The authors thank Zhiwen Wan and Xiuzhang Li for assistance with alkaloid analyses and sample collection, Jennifer Smith (PhD) for editing the English text of the manuscript and Professor Cory Matthew, Massey University, for helpful comments.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This study was supported by National Basic Research Program of China (2014CB138702), the Natural Science Foundation of China (31372366), Program for Changjiang Scholars and Innovative Research Team in University, China (IRT17R50), Fundamental Research Funds for the Central Universities (LZUJBKY–2016–180, 2016-bt10, 2017-kb10) and 111Project (B12002).

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References

Barker GM, Patchett BJ, Cameron NF. 2015. *Epichloë uncinata* infection and loline content afford *Festulolium* grasses protection from black beetle (*Heteronychus arator*). New Zealand Journal of Agricultural Research. 58(1):35–56.

Berry D, Takach JE, Schardl CL, Charlton ND, Scott B, Young CA. 2015. Disparate independent genetic events disrupt the secondary metabolism gene *perA* in certain symbiotic *Epichloë* species. Applied and Environmental Microbiology. 81:2797–2807.

Bongiorno VA, Rhoden SA, Garcia A, Polonio JC, Azevedo JL, Pereira JO, Pamphile JA. 2016. Genetic diversity of endophytic fungi from *Coffea arabica* cv. IAPAR–59 in organic crops. Annals of Microbiology. 66:855–865.

Brem DLA. 2003. Molecular evidence for host–adapted races of the fungal endophyte *Epichloë bromicola* after presumed host shifts. Evolution. 57:37–51.

Charlton ND, Craven KD, Afkhami ME, Hall BA, Ghimire SR, Young CA. 2014. Interspecific hybridization and bioactive alkaloid variation increases diversity in endophytic *Epichloë* species of *Bromus laevipes*. FEMS Microbiology Ecology. 90:276–289.

Charlton ND, Craven KD, Mittal S, Hopkins AA, Young CA. 2012. *Epichloë canadensis*, a new interspecific epichloid hybrid symbiotic with Canada wildrye (*Elymus canadensis*). Mycologia. 104:1187–1199.

Chen L. 2015. Molecular detection, genotypes and chemotypes of *Epichloë* endophytes in *Achnatherum inebrians* [Dissertation]. Lanzhou University.

Chen YG, Ji YL, Yu HS, Wang ZW. 2009. A new *Neotyphodium* species from *Festuca parvigluma* Steud. grown in China. Mycologia. 101:681–685.

Cheplick GP. 2007. Costs of fungal endophyte infection in *Lolium perenne* genotypes from Eurasia and North Africa under extreme resource limitation. Environmental and Experimental Botany. 60:202–210.

Clement S, Wilson AD, Lester D, Davitt C. 1997. Fungal endophytes of wild barley and their effects on *Diuraphis noxia* population development. Entomologia Experimentalis Et Applicata. 82:275–281.

Dereeper A, Guignon V, Blanc G, Audic S, Buffet S, Chevenet F, Dufayard JF, Guindon S, Lefort V, Lescot M, et al. 2008. Phylogeny.fr: robust phylogenetic analysis for the non-specialist. Nucleic Acids Research. 36:W465–W469.

Dugan F, Sitton J, Sullivan R, White J. 2002. The *Neotyphodium* endophyte of wild barley (*Hordeum brevisubulatum* subsp. *violaceum*) grows and sporulates on leaf surfaces of the host. Symbiosis. 32:147–159.

Fleetwood DJ, Scott B, Lane GA, Tanaka A, Johnson RD. 2007. A complex ergovaline gene cluster in epichloe endophytes of grasses. Applied and Environmental Microbiology. 73:2571–2579.

Ghimire SR, Rudgers JA, Charlton ND, Young C, Craven KD. 2011. Prevalence of an intraspecific *Neotyphodium* hybrid in natural populations of stout wood reed (*Cinna arundinacea* L.) from eastern North America. Mycologia. 103:75–84.

Gundel PE, Martínez-Ghersa MA, Omacini M, Cuyeu R, Pagano E, Ríos R, Ghersa CM. 2012. Mutualism effectiveness and vertical transmission of symbiotic fungal endophytes in response to host genetic background. Evolutionary Applications. 5:838–849.

He L, Hatier JHB, Matthew C. 2017. Drought tolerance of two perennial ryegrass cultivars with and without AR37 endophyte. New Zealand Journal of Agricultural Research. 60(2):173–188.

Hettiarachchige IK, Ekanayake PN, Mann RC, Guthridge KM, Sawbridge TI, Spangenberg GC, Forster JW. 2015. Phylogenomics of asexual *Epichloë* fungal endophytes forming associations with perennial ryegrass. BMC Evolutionary Biology. 15:72–85.

Hu B, Jin J, Guo AY, Zhang H, Luo J, Gao G. 2015. GSDS 2.0: an upgraded gene feature visualization server. Bioinformatics. 31:1296–1297.

Iannone LJ, Cabral D, Schardl CL, Rossi MS. 2009. Phylogenetic divergence, morphological and physiological differences distinguish a new *Neotyphodium* endophyte species in the grass *Bromus auleticus* from South America. Mycologia. 101:340–351.

Ji YL, Zhan LH, Kang Y, Sun XH, Yu HS, Wang ZW. 2009. A new stromata-producing *Neotyphodium* species symbiotic with clonal grass *Calamagrostis epigeios* (L.) Roth. grown in China. Mycologia. 101:200–205.

Kang Y, Ji YL, Sun XH, Zhan LH, Li W, Yu HS, Wang ZW. 2009. Taxonomy of *Neotyphodium* endophytes of Chinese native *Roegneria* plants. Mycologia. 101:211–219.

Kang Y, Ji YL, Zhu KR, Wang H, Miao HM, Wang ZW. 2011. A new *Epichloë* species with interspecific hybrid origins from *Poa pratensis* ssp. pratensis in Liyang, China. Mycologia. 103:1341–1350.

Kuang Y. 2016. Characteristics of *Epichloë* endophyte–festuca sinensis symbiote [Dissertation]. Lanzhou University.

Leuchtmann A, Schardl CL, Siegel MR. 1994. Sexual compatibility and taxonomy of a new species of epichloe symbiotic with fine fescue grasses. Mycologia. 86:802–812.

Leuchtmann A, Schardl CL. 1998. Mating compatibility and phylogenetic relationships among two new species of *Epichloë* and other congeneric European species. Mycological Research. 102:1169–1182.

Leuchtmann A, Oberhofer M. 2013. The *Epichloë* endophytes associated with the woodland grass *Hordelymus europaeus* including four new taxa. Mycologia. 105:1315–1324.

Leuchtmann A, Bacon CW, Schardl CL, White Jr JF, Tadych M. 2014. Nomenclatural realignment of *Neotyphodium* species with genus *Epichloë*. Mycologia. 106:202–215.

Li CJ. 2005. Biological and ecological characteristics of *Achnatherum inebrians*/*Neotyphodium* endophyte symbiont [Dissertation]. Lanzhou University.

Li CJ, Nan ZB, Paul VH, Dapprich PD, Liu Y. 2004. A new *Neotyphodium* species symbiotic with drunken horse grass (*Achnatherum inebrians*) in China. Mycotaxon. 90:141–147.

Li W, Ji YL, Yu HS, Wang ZW. 2006. A new species of *Epichloë* symbiotic with Chinese grasses. Mycologia. 98:560–570.

Mc Cargo PD, Iannone LJ, Vignale MV, Schardl CL, Rossi MS. 2014. Species diversity of *Epichloë* symbiotic with two grasses from southern Argentinean Patagonia. Mycologia. 106:339–352.

Morgan-Jones G, Gams W. 1982. Notes on hyphomycetes. XLI. An endophyte of *Festuca arundinacea* and the anamorph of *Epichloe typhina*, new taxa in one of two new sections of *Acremonium*. Mycotaxon. 15:311–318.

Moon CD, Scott B, Schardl CL, Christensen MJ. 2000. The evolutionary origins of *Epichloë* endophytes from annual ryegrasses. Mycologia. 92:1103–1118.

Moon CD, Miles CO, Järlfors U, Schardl CL. 2002. The evolutionary origins of three new *Neotyphodium* endophyte species from grasses indigenous to the Southern Hemisphere. Mycologia. 94:694–711.

Moon CD, Craven KD, Leuchtmann A, Clement SL, Schardl CL. 2004. Prevalence of interspecific hybrids amongst asexual fungal endophytes of grasses. Molecular Ecology. 13:1455–1467.

Moon C, Guillaumin J, Ravel C, Li C, Craven K, Schardl C. 2007. New *Neotyphodium* endophyte species from the grass tribes Stipeae and Meliceae. Mycologia. 99:895–905.
Oberhofer M, Güsewell S, Leuchtmann A. 2013. Effects of natural hybrid and non-hybrid *Epichloë* endophytes on the response of *Hordelymus europaeus* to drought stress. New Phytologist. 201:242–253.
Posada D. 2006. Modeltest server: a web–based tool for the statistical selection of models of nucleotide substitution online. Nucleic Acids Research. 34:W700–W703.
Rodriguez RJ, White Jr JF, Arnold AE, Redman RS. 2009. Fungal endophytes: diversity and functional roles. New Phytologist. 182:314–330.
Saikkonen K, Faeth SH, Helander M, Sullivan TJ. 1998. Fungal endophytes: A continuum of interactions with host plants. Annual Review of Ecology and Systematics. 29:319–343.
Saikkonen K, Young CA, Helander M, Schardl CL. 2016. Endophytic *Epichloë* species and their grass hosts: from evolution to applications. Plant Molecular Biology. 90:665–675.
Schardl CL, Leuchtmann A. 1999. Three new species of *Epichloë* symbiotic with North American grasses. Mycologia. 91:95–107.
Schardl CL, Young CA, Faulkner JR, Florea S, Pan J. 2012. Chemotypic diversity of epichloae, fungal symbionts of grasses. Fungal Ecology. 5:331–344.
Schardl CL, Young CA, Hesse U, Amyotte SG, Andreeva K, Calie PJ, Fleetwood DJ, Haws DC, Moore N, Oeser B, et al. 2013a. Plant-symbiotic fungi as chemical engineers: multi-genome analysis of the Clavicipitaceae reveals dynamics of alkaloid loci. PLoS Genetics. 9:e1003323.
Schardl CL, Young CA, Pan J, Florea S, Takach JE, Panaccione DG, Farman ML, Webb JS, Jaromczyk J, Charlton ND, et al. 2013b. Currencies of mutualisms: sources of alkaloid genes in vertically transmitted epichloae. Toxins. 5:1064–1088.
Song H, Nan ZB, Tian P. 2015a. Characteristic of asexual endophytes isolated from species in northwest China. Acta Prataculturae Sinica. 24:89–95. Chinese, with English abstract.
Song ML, Chai Q, Li XZ, Yao X, Li CJ, Christensen MJ, Nan ZB. 2015b. An asexual *Epichloë* endophyte modifies the nutrient stoichiometry of wild barley (*Hordeum brevisubulatum*) under salt stress. Plant and Soil. 387:153–165.
Song ML, Li XZ, Saikkonen K, Li CJ, Nan ZB. 2015c. An asexual *Epichloë* endophyte enhances waterlogging tolerance of *Hordeum brevisubulatum*. Fungal Ecology. 13:44–52.
Spiering MJ, Moon CD, Wilkinson HH, Schardl CL. 2005. Gene clusters for insecticidal loline alkaloids in the grass-endophytic fungus *Neotyphodium uncinatum*. Genetics. 169:1403–1414.
Tadych M, Ambrose KV, Bergen MS, Belanger FC, White J. 2012. Taxonomic placement of *Epichloë poae* sp. nov. and horizontal dissemination to seedlings via conidia. Fungal Diversity. 54:117–131.
Takach JE, Young CA. 2014. Alkaloid genotype diversity of tall fescue endophytes. Crop Science. 54:667–678.
Tanaka A, Tapper BA, Popay A, Parker EJ, Scott B. 2005. A symbiosis expressed non–ribosomal peptide synthetase from a mutualistic fungal endophyte of perennial ryegrass confers protection to the symbiotum from insect herbivory. Molecular Microbiology. 57:1036–1050.
Tepaske MR, Powell RG, Clement SL. 1993. Analyses of selected endophyte–infected grasses for the presence of loline–type and ergot–type alkaloids. Journal of Agricultural and Food Chemistry. 41:2299–2303.
Wan ZW, Feng JR, Chen ZJ, Cao Y, Li CJ. 2016. Effects of different pH on growth and ergot alkaloids concentrations of symbiont of *Epichloë gansuensis–Achnatherum inebrians* seedling. Acta Bot Boreali–Occidentalia Sinica. 36:715–720. Chinese, with English abstract.
Wang CM, Xia ZR, Wu GQ, Yuan HJ, Wang XR, Li JH, Tian FP, Zhang Q, Zhu XQ, He JJ, et al. 2016. The coordinated regulation of Na^+ and K^+ in *Hordeum brevisubulatum* responding to time of salt stress. Plant Science. 252:358–366.
Wilkinson HH, Siegel MR, Blankenship JD, Mallory AC, Bush LP, Schardl CL. 2000. Contribution of fungal loline alkaloids to protection from aphids in a grass–endophyte mutualism. Molecular Plant–Microbe Interactions. 13:1027–1033.
Wilson A, Clement S, Kaiser W, Lester D. 1991. First report of clavicipitaceous anamorphic endophytes in *Hordeum* species. Plant Disease. 75:215.

Wilson AD. 2007. Clavicipitaceous anamorphic endophytes in *Hordeum* germplasm. Plant Pathology Journal. 6:1–13.
Wilson AD, Clement SL, Kaiser WJ. 1992. Endophytic fungi in a *Hordeum* germplasm collection. Plant Genetic Resources Newsletter. 87:1–4.
White JF. 1993. Endophyte-host associations in grasses. XIX. A systematic study of some sympatric species of *Epichloë* in England. Mycologia. 85:444–455.
White JF. 1994. Endophyte-host associations in grasses. XX. Structural and reproductive studies of *Epichloë amarillans* sp. nov. and comparisons to *E. typhina*. Mycologia. 86:571–580.
White JF. 1997. Perithecial ontogeny in the fungal genus *Epichloë*: An examination of the clavicipitalean centrum. American Journal of Botany. 84:170–178.
White JF, Halisky PM, Sun S, Morgan-Jones G, Funk CR. 1992. Endophyte-host associations in grasses. XVI. Patterns of endophyte distribution in species of the tribe Agrostideae. American Journal of Botany. 79:472–477.
Young CA, Bryant MK, Christensen MJ, Tapper BA, Bryan GT, Scott B. 2005. Molecular cloning and genetic analysis of a symbiosis–expressed gene cluster for lolitrem biosynthesis from a mutualistic endophyte of perennial ryegrass. Molecular Genetics and Genomics. 274:13–29.
Young CA, Felitti S, Shields K, Spangenberg G, Johnson RD, Bryan GT, Saikia S, Scott B. 2006. A complex gene cluster for indole–diterpene biosynthesis in the grass endophyte *Neotyphodium lolii*. Fungal Genetics and Biology. 43:679–693.
Young CA, Tapper BA, May K, Moon CD, Schardl CL, Scott B. 2009. Indole–diterpene biosynthetic capability of *Epichloë* endophytes as predicted by *ltm* gene analysis. Applied and Environmental Microbiology. 75:2200–2211.
Young CA, Mittal S, Takach JE. 2010. Chemical diversity of bioprotective alkaloids of endophytic fungi in cool season grasses. Poster session presented at: International House Student Club; Queensland, Australia.
Young CA, Hume DE, Mcculley RL. 2013. Forages and pastures symposium: fungal endophytes of tall fescue and perennial ryegrass: pasture friend or foe? Journal of Animal Science. 91:2379–2394.
Zhang X, Ren AZ, Wei YK, Lin F, Li C, Liu ZJ, Gao YB. 2009. Taxonomy, diversity and origins of symbiotic endophytes of *Achnatherum sibiricum* in the Inner Mongolia Steppe of China. FEMS Microbiology Letters. 301:12–20.
Zhang XX, Xia C, Nan ZB. 2015. Effects of symbiotic *Epichloë gansuensis* endophyte on drunken horse grass (*Achnatherum inebrians*) growth and seed production. New Zealand Journal of Agricultural Research. 58(2):234–240.
Zhu MJ, Ren AZ, Wen W, Gao YB. 2013. Diversity and taxonomy of endophytes from *Leymus chinensis* in the Inner Mongolia steppe of China. FEMS Microbiology Letters. 340:135–145.