Are *Epichloë* endophytes specific to *Elymus* grass hosts?

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ABSTRACT. *Epichloë* endophytes are widely distributed mutualists of cool-season grasses and can protect their hosts against biotic and abiotic stresses. Previous studies have shown that *Epichloë* endophytes are specific to their grass hosts in tall *Festuca* and *Lolium* species. However, no systematic analysis exists of host specificity of asexual *Epichloë* endophytes and Chinese *Elymus* species. We analyzed the phylogenetic relationships between Chinese *Elymus* species and their diploid donor *Hordeum* species, using their corresponding *Epichloë* endophyte sequences. We found that 1) the maternal donor of the Chinese *Elymus* species was the Chinese *Pseudoroegneria* (St genome) or *Hordeum* (H genome); and 2) Chinese *Hordeum* species probably contained two species of *Epichloë* endophytes. One *Epichloë* endophyte was also present in a North American *Elymus* species. The other *Epichloë* endophyte was found in a Chinese *Elymus* species. Our results indicate that *Epichloë* endophytes isolated from *Elymus* species did not show grass-host specificity. 3) Plant hybridization could probably transform endophyte-free plants (E-) to endophyte-infected plants (E+). Based on

our data, we formulate hypotheses about which *Epichloë* endophytes were spread via plant hybridization.

Key words: *Elymus* species; *Epichloë* endophyte; *Hordeum* species; Host specificity; Maternal donor; Phylogeny

INTRODUCTION

Forage grasses (Poaceae) often harbor endophytic fungi of the genus *Epichloë* (Clavicipitaceae, Ascomycota) (Clay and Schardl, 2002). Asexual *Epichloë* endophytes inhabit the intercellular spaces of their grass hosts and depend entirely on the host plant for propagation through its seeds or vegetative structures (Schardl, 2010). It is well documented that asexual *Epichloë* endophytes can spread vertically by infecting the seeds of the host plant without overt symptoms of disease (Clay and Schardl, 2002; Selosse and Schardl, 2007). Sexual *Epichloë* endophytes are horizontally transmitted and can develop a stroma from which contagious spores are released. Sexual *Epichloë* endophytes may induce symptoms of choke disease, in which the infected host grass aborts the development of reproductive structures (Johnson et al., 2013). However, not all sexual *Epichloë* endophytes induce choke symptoms; some sexual *Epichloë* endophytes are designated non-choke-inducing types (Clay and Schardl, 2002) and, under certain conditions, these species can have a positive effect on the grass host (Clay and Schardl, 2002; Schardl, 2010).

Many members of the grass family Poaceae have coevolved with *Epichloë* endophytes, including asexual morphs (Schardl et al., 1997). In most cases, *Epichloë* endophytes are only found in association with grasses of the subfamily Pooideae, including the tribes Aveneae, Brachypodieae, Bromeae, Meliceae, Poeae, Stipeae, and Triticeae, with the majority systematically colonizing their hosts and being transmitted vertically to the next generation (Schardl and Philips, 1997). *Epichloë* endophytes exhibit coevolutionary patterns at the tribe or genus level, but the significance of this evolutionary trend at the species or subspecies level of the grass host has not been fully investigated. A broad-range study on asexual *Epichloë* endophytes in grass hosts found that they did not coevolve or codiverge (Schardl et al., 2008). However, the relationships between Chinese *Elymus* species and related asexual *Epichloë* endophytes have not been analyzed.

Elymus L. is the largest genus of grasses in the tribe Triticeae (Poaceae), with approximately 150 perennial species distributed across temperate zones throughout most of the worldwide (Dewey, 1984), except for Africa and Antarctica (Helfgott and Mason-Gamer, 2004). An analysis of the chromosomes of tetraploid, hexaploid, and octoploid *Elymus* species indicated that they have five potential diploid donors: *Pseudoroegneria* (St genome), *Hordeum* (H genome), *Agropyron* (P genome), *Australopyrum* (W genome), and an unknown species (Y genome) (Dewey, 1984; Jensen, 1990; Torabinejad and Mueller, 1993). A recent report indicated that *Pseudoroegneria* has no endophytes; however, many asexual *Epichloë* endophytes and *Epichloë bromicola* are found in *Hordeum* and *Elymus* species from China, respectively (Card et al., 2014; Song and Nan, 2015). For the present study, we collected polyploid *Elymus* and diploid *Hordeum* species in western China and isolated their *Epichloë* endophytes. Additionally, sequences of North American polyploid *Elymus* and diploid *Hordeum* species and their related *Epichloë* endophytes were obtained from GenBank. The goal of our study was to determine whether the same *Epichloë* endophytes were present in Chinese and American *Elymus* and *Hordeum* species.

MATERIAL AND METHODS

Ethics statement

No specific permissions were required for this study as we collected a limited number of seeds and stalks from native grassland that was not privately owned or protected. Our field study did not involve any endangered or protected species.

Plant materials

In total, 398 individuals from four diploid *Hordeum* species were sampled from four populations in western China. In addition, sequences of North American polyploid *Elymus* and diploid *Hordeum* species and their related *Epichloë* endophytes were obtained from GenBank. (Table 1).

Table 1. Samples and accessions used in the analysis.

Isolate	Host species	Host tribe	Accession No. of endophyte	Collection locality	*trn*L-F	*tubB*	*tefA*
Epichloë amarillas	*Elymus virginicus*	Triticeae	E1087	North America (USA)	AF519144	AF457478	AF457518
Epichloë clarkii	*Elymus hystrix*	Triticeae	ATCC201556	North America (USA)	AF519139	AF250744	AF457500
Epichloë elymi	*Elymus virginicus*	Triticeae	ATCC201553	North America (USA)	AF519145	AF062428	AF457498
Epichloë elymi	*Elymus hystrix*	Triticeae	ATCC201557	North America (USA)	EF396985	AF250745	AF457501
Epichloë elymi	*Elymus canadensis*	Triticeae	ATCC201551	North America (USA)	EU119363	L06962	AF231209
Epichloë elymi	*Elymus virginicus*	Triticeae	MP-III	North America (USA)	AF519143	L78273	AF231208
Epichloë bromicola	**Elymus excelsus**	**Triticeae**	**NI_201201**	**Asia (China)**	**KF905187**	**KJ585731**	**KJ585716**
Epichloë bromicola	**Elymus excelsus**	**Triticeae**	**NI_201203**	**Asia (China)**	**KF905189**	**KJ585732**	**KJ585717**
Epichloë bromicola	**Elymus tibeticus**	**Triticeae**	**NI_201206**	**Asia (China)**	**KF905192**	**KJ585733**	**KJ585718**
Epichloë bromicola	**Elymus dahuricus**	**Triticeae**	**NI_201208**	**Asia (China)**	**KF905194**	**KJ585734**	**KJ585720**
Epichloë bromicola	**Elymus tangutorum**	**Triticeae**	**NI_201210**	**Asia (China)**	**KF905196**	**KJ585736**	**KJ585721**
Epichloë bromicola	**Elymus tangutorum**	**Triticeae**	**NI_201213**	**Asia (China)**	**KF905199**	**KJ585737**	**KJ585722**
Epichloë bromicola	**Elymus excelsus**	**Triticeae**	**NI_201214**	**Asia (China)**	**KF905200**	**KJ585738**	**KJ585723**
Epichloë bromicola	**Elymus tangutorum**	**Triticeae**	**NI_201216**	**Asia (China)**	**KF905202**	**KJ585739**	**KJ585724**
Epichloë bromicola	**Elymus excelsus**	**Triticeae**	**NI_201218**	**Asia (China)**	**KF905204**	**KJ585740**	**KJ585725**
Epichloë bromicola	**Elymus tangutorum**	**Triticeae**	**NI_201221**	**Asia (China)**	**KF905207**	**KJ585741**	**KJ585726**
Epichloë bromicola	**Elymus dahuricus** var. **cylindricus**	**Triticeae**	**NI_201222**	**Asia (China)**	**KF905208**	**KJ585742**	**KJ585727**
Epichloë bromicola	**Elymus nutans**	**Triticeae**	**NI_201302**	**Asia (China)**	**KF905216**	**KJ585743**	**KJ585728**
Epichloë bromicola	**Elymus dahuricus**	**Triticeae**	**NI_201306**	**Asia (China)**	**KF905220**	**KJ585744**	**KJ585729**
Epichloë bromicola	**Elymus dahuricus** var. **cylindricus**	**Triticeae**	**NI_201308**	**Asia (China)**	**KF905222**	**KJ585745**	**KJ585730**
Epichloë sp	**Hordeum bogdanii**	**Triticeae**	**N100**	**Asia (China)**	**KP087958**	**KP087966**	**KP087962**
Epichloë sp	**Hordeum jubatum**	**Triticeae**	**N133**	**Asia (China)**	**KP087961**	**KP087968**	**KP087965**
Epichloë sp	**Hordeum jubatum**	**Triticeae**	**N13**	**Asia (China)**	**KP087960**	**KP087967**	**KP087964**
Epichloë sp	**Hordeum roshevitzii**	**Triticeae**	**N11**	**Asia (China)**	**KP087959**	**KP087969**	**KP087963**

Bolded entries represent sequences generated in this study.

DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing

Genomic DNAs of diploid *Hordeum* species were extracted from fresh leaves using a plant DNA isolation kit (TaKaRa, Dalian, China). Endophyte genomic DNAs from mature plants (Schardl et al., 2013) were extracted using the HP fungal DNA kit (Omega, Beijing, China).

The chloroplast non-coding region *trn*L-F was amplified from diploid *Hordeum* species using primers described previously (Taberlet et al., 1991; Zhu et al., 2013). Intron-rich portions of the housekeeping genes β-tubulin (*tubB*) and translation elongation factor 1-α (*tefA*) were amplified from the endophytes using the PCR protocols described by Moon et al. (2002).

PCR products were cloned into the pMD18-T vector (TaKaRa, Dalian, China) following the manufacturer instructions. Three to five positive clones per accession were sequenced by TaKaRa Biotechnology Co. Ltd (Dalian, China). Sequences were deposited in GenBank (Table 1) under accession Nos. KP087958-KP087961 (*trn*L-F), KP087966-KP087969 (*tubB*), and KP087962-KP087965 (*tefA*).

Phylogenetic analyses

Sequences were aligned using the MAFFT 7.0 software (Katoh and Standley, 2013) and adjusted using the Genedoc software (Nicholas and Nicholas, 1997). Phylogenetic relationships were estimated using maximum parsimony (MP) and maximum likelihood (ML) methods in the PAUP 4.0b10 package (Swofford, 2002) with 1000 replications for each. Endophyte phylogenetic trees were constructed using *tubB* and *tefA* intron sequences after they were concatenated using the SequenceMatrix software (Vaidya et al., 2011). MP trees were estimated using a heuristic search based on tree bisection-reconnection (TBR) branch swapping and 100 random-addition replicates. The ML estimate used the best-fitting model of sequence evolution in MrModeltest (Nylander, 2004). This was estimated to be K80+G for the *tubB* + *tefA* endophyte dataset and HKY for the grass *trn*L-F dataset. Alignment gaps were treated as missing information. Nucleotide substitutions were unordered and unweighted.

RESULTS AND DISCUSSION

Grass chloroplast *trn*L-F phylogeny

The parsimony analysis of *trn*L-F sequences yielded a tree with a length of 95 (consistency index [CI] = 0.968, rescaled consistency index [RI] = 0.967). Phylogenetic relationships among North American and Chinese *Elymus* species are shown in Figure 1. Only the data from the MP tree are shown here. The *trn*L-F phylogenetic tree of Chinese and North American *Elymus* and Chinese *Hordeum* species identified three clades (Figure 1). Clade I contained two sequences from the North American *Elymus hystrix* and Chinese *E. tangutorum*. Clade II was weakly supported (60/69) and contained two collections from the North American *E. canadensis* and Chinese *E. dahuricus* var. *cylindricus*. The species constituting clades I and II support the hypothesis that *Elymus* specieshas had multiple dispersal events. The rich diversity of *Elymus* species in central Asia suggests that it could be the center of origin for *Elymus* species (Lu and Salömon, 1992). Therefore, it is not surprising that North American and Chinese *Elymus* species may have a common ancestor.

As described above, five basic genomes are found in *Elymus* and are derived from *Pseudoroegneria* (St genome), *Hordeum* (H genome), *Agropyron* (P genome), *Australopyrum* (W genome), and an unknown species (Y genome) (Torabinejad and Mueller, 1993; Sun, 2014). The St genome is a fundamental genome that originated in *Elymus* and is found in all *Elymus* species (Sun, 2014). North American *Elymus* species contain the St and H genomes (Mason-Gamer, 2013), whereas Asian *Elymus* species contain the Y genome (Lu, 1993). Here we found that *Elymus* species from clades I and II contained the St and H genomes: *E. hystrix*, StH; *E. tangutorum*, StStH; *E. canadensis*, StH; *E. dahuricus* var. *cylindricus*, StHY (Mason-Gamer et al., 2010; Mason-Gamer, 2013). The chloroplast genome typically represents the maternal parent in angiosperms, and previous studies have found that North American *Pseudoroegneria* species (St

genome) are the maternal donor to North American *Elymus* species (Mason-Gamer and Orme, 2002; Sun, 2002, 2014). We found that the maternal parent of Chinese *E. tangutorum* (KF905207) and *E. dahuricus* var. *cylindricus* (KF905208) is also a North American *Pseudoroegneria* species (St genome). Interestingly, other Chinese collections of *E. tangutorum* (KF905199, KF905196 and KF905202) and *E. dahuricus* var. *cylindricus* (KF905222) did not cluster with North American *Elymus* species, suggesting that they probably had different maternal donors.
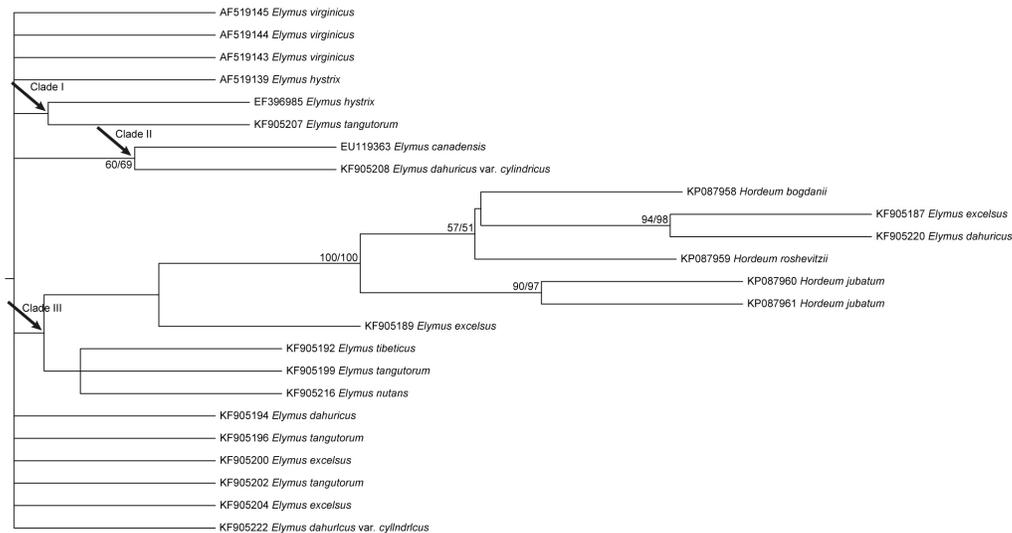
Figure 1. Chloroplast *trn*L-F maximum-parsimony tree. Bootstrap support values were calculated from 1000 replications. Branches with bootstrap values >50% are shown. MP bootstrap values are listed first, followed by ML bootstrap values. Arrows indicate the clades described in the text.

Clade III contained 10 samples from Chinese *Elymus* and *Hordeum* species, indicating that a Chinese *Pseudoroegneria* species (St genome) was the maternal donor to some of the Chinese *Elymus* species. In addition, six Chinese *Elymus* species were unresolved in the *trn*L-F phylogeny. The maternal donor of these *Elymus* species may have been a Chinese *Pseudoroegneria* (St genome) or *Hordeum* (H genome). However, this result needs further study. Nevertheless, it is certain that Chinese *Elymus* species did not have a single maternal donor, as do North American *Elymus* species. Instead, Chinese *Elymus* species might have had a number of maternal donors, including *Pseudoroegneria* (St genome) or *Hordeum* (H genome).

Endophytic *tubB* + *tefA* tree

The MP analysis of *tub*B + *tef*A yielded a tree with a length of 136 (Figure 2; CI = 0.9485; RI = 0.9663). Topology of the ML tree was slightly different from that of the MP tree, but both methods recovered one clade (Figure 2, shaded branches).

Both MP and ML phylogenetic trees identified a clade including sexual *Epichloë* endophytes from North American *E. canadensis*, *E. hystrix,* and *E. virginicus*, and asexual *Epichloë* endophytes from Chinese *Hordeum jubatum* and *H. roshevitzii* (Figure 2), suggesting that the endophytes in these *Elymus* and *Hordeum* species have the same origin. Previous studies have

shown that grass-endophyte interactions are highly specific (Ekanayake et al., 2012; Gundel et al., 2012; Karimi et al., 2012), and that each designated endophyte grouping generally has a high degree of host specificity (Schardl et al., 1997). As anticipated, *Epichloë coenophiala* was found in Continental hexaploid tall fescue host plants, including the tetraploid maternal progenitor of Continental hexaploid tall fescue (Ekanayake et al., 2012). Karimi et al. (2012) found that the host specificity of asexual *Epichloë* endophytes from *Festuca arundinacea*, *F. pratensis*, and *Lolium perenne* was consistent in geographically distant host grasses of the same species. However, Card et al. (2014) analyzed endophytes isolated from *Hordeum* and *Elymus* species and found that most endophytes cluster according to geographical regions rather than host species. Our results were not consistent with either of the above findings. The host specificity of endophytes from Chinese *Hordeum* species was not necessarily consistent between multiple samples from a particular host species, and they were not consistent according to geographical location of the collection (Figure 2). Although there are differences between the MP and ML tree topologies, we noted that an asexual *Epichloë* endophyte isolated from Chinese *H. bogdanii* had the same origin as asexual isolates from some of the Chinese *Elymus* species, indicating that Chinese *Hordeum* species carry a variety of *Epichloë* endophytes. Asexual *Epichloë* endophytes from Chinese *Hordeum* species have a common ancestor with sexual *Epichloë* sexual endophytes from North American *Elymus* species. Additionally, asexual *Epichloë* endophytes from Chinese *Hordeum* and *Elymus* species have the same origin. Isoenzyme analyses suggest that Eurasian and American StH-genome tetraploid *Elymus* species may have originated independently from different H-genome donors (Jaaska, 1992). Genomic (Wang and Hsiao, 1986), and karyotypic analyses (Linde-Laursen et al., 1994) suggest that Old and New World taxa may be of different origin, and therefore, Chinese and North American *Elymus* species may carry different endophytes because they have different *Hordeum* species donors.
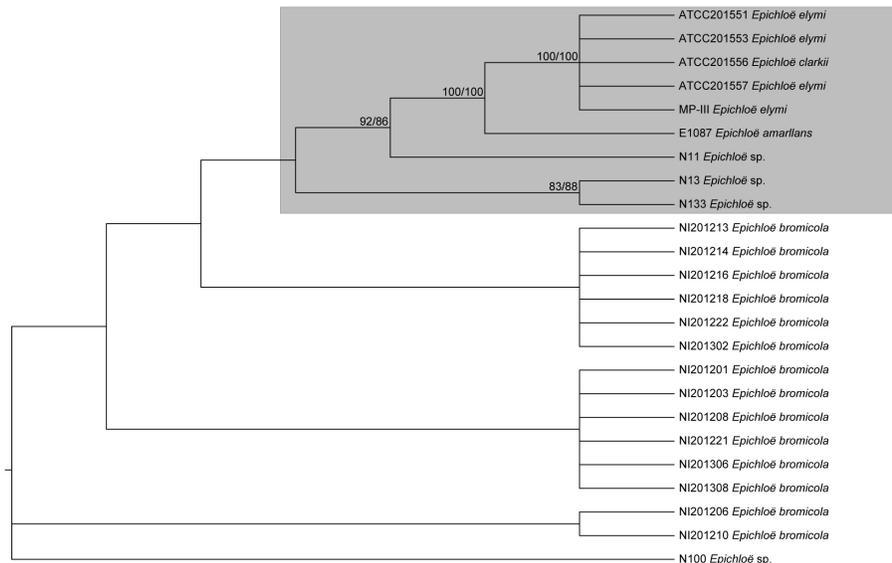
Figure 2. Maximum-parsimony tree of *Epichloë* endophytes based on *tubB+tefA* intron sequences. Bootstrap support values were calculated from 1000 replications. Branches with bootstrap values >50% are shown. MP bootstrap values are listed first on each branch, followed by ML bootstrap values. The gray box contains a clade recovered in MP and ML analyses.

Phylogenetic analyses of β-tubulin (*tubB*) and rDNA-ITS sequences (Moon et al., 2000) support the hypothesis that asexual *Epichloë* species evolved from sexually reproducing species that lost the sexual state. However, our results do not entirely support this sequence of events. *Hordeum* species are the potential diploid donors, and they may have formed earlier than *Elymus* species. Our results also suggest that isolates from Chinese *Hordeum* and North American *Elymus* species have the same origin, suggesting that sexual *Epichloë* endophytes from North American *Elymus* species are derived from asexual *Epichloë* endophytes from Chinese *Hordeum* species. Therefore, the most plausible explanation based on our analyses is that sexual *Epichloë* endophytes evolved from asexual *Epichloë* endophytes.

Hypothesis of endophyte distribution based on host hybridization

The artificial infection of endophyte-free plants (E-) with isolates from endophyte-infected plants (E+) is used to improve biotic and abiotic resistance of plants (Leuchtmann and Clay, 1993). However, the success rate of this procedure is low. Based on our results, we hypothesize that endophytes may spread between grass hosts via plant hybridization. Our study provides preliminary evidence for this viewpoint in that the trees of grass hosts and endophytes suggested that sexual and asexual endophytes from North American and western Chinese *Elymus* species are most closely related to endophytes from Chinese *Hordeum* species. A previous study (Torabinejad and Mueller, 1993) found that the polyploid *Elymus* species in this study contain genomes of the St, H and Y types, potentially from *Pseudoroegneria*, *Hordeum* and an unknown species. *Pseudoroegneria* species do not contain endophytes (Card et al., 2014). The transmission of asexual *Epichloë* endophytes to the next generation generally occurs via seeds (White et al., 1993). Here, we hypothesize that *Epichloë* endophytes spread from *Hordeum* to *Elymus* via hybridization between *Elymus* and *Hordeum* species. We hope to find additional evidence for our hypothesis in a future study.

Conflicts of interest

The authors declare no conflict of interest.

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