Distribution and diversity of *Epichloë*/*Neotyphodium* fungal endophytes from different populations of *Achnatherum sibiricum* (*Poaceae*) in the Inner Mongolia Steppe, China

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There has been a recent surge in interest on grass-endophyte symbiosis as it exerts a strong influence on the agricultural ecosystem. Our understanding of incidence of infection and diversity of endophytic fungi in grasses is vital so as to discover new grass-endophyte associations and their beneficial effects. In this paper, we have documented the patterns of distribution and diversity of *Epichloë*/*Neotyphodium* fungal endophytes in a perennial bunchgrass, *Achnatherum sibiricum* (*Poaceae*) from the Inner Mongolia Steppe. We investigated yearly, seasonal and spatial variations in infection frequency of *Epichloë*/*Neotyphodium* endophytes in *A. sibiricum* from four study sites, i.e., Hulingol (HLG), West Ujimqin (WUM), Plot I and Plot II of Inner Mongolia Grassland Ecosystem Research Station of the Chinese Academy of Sciences (IMGERS). We also analysed correlations of infection frequency and diversity of endophytic fungi with selected soil factors and the growth characteristics of *A. sibiricum* in the four sampling sites. The infection frequencies of endophytic fungi were not significantly related to the soil factors or growth status of *A. sibiricum*, although variations in the infection frequencies among four *A. sibiricum* populations were observed during the summer. Twenty-seven isolates of *Neotyphodium* were obtained from the four *A. sibiricum* populations and categorized into 9 morphotypes based on colony morphology, growth rate, and conidial size on potato dextrose agar (PDA) medium. The greatest morphotypic diversity of *Neotyphodium* was observed in *A. sibiricum* population from IMGERS-Plot II. This observation was consistent with its highly heterogeneous habitat because the plot had been enclosed for more than 20 years. There was a significant ($P < 0.05$) trend for the conidial length of *Neotyphodium* to gradually increase from the eastern China to the western China populations of *A. sibiricum*. In addition, we observed *A. sibiricum* plants from HLG and WUM showing *Epichloë* stromata on their flowering culms. Our study suggests that the endophytic fungi in natural (wild) *A. sibiricum* populations are highly diverse and may have different life histories.

Key words: ecological factors, infection rate, mutualism, symbiosis

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Introduction

Endophytic fungi are an important group of ubiquitous and extremely diverse plant symbionts that live asymptomatically, and sometimes systematically within plant tissues (Carroll, 1988, 1991; Arnold *et al*., 2000; Kumar and Hyde, 2004; Suryanarayanan and Thennasaran, 2004; Wei and Xu, 2004; Promputtha *et al*., 2005). Many grasses in the subfamily *Pooideae* are infected by asexual (anamorphic), vertically transmitted endophytic fungi, *Neotyphodium* Glenn, C.W. Bacon & Hanlin*,* or their sexual (teleomorphic) counterparts of genus *Epichloë* (Fr.) Tul. & C. Tul. , which can be transmitted either vertically or horizontally (Schardl, 1996; Schardl *et al*., 1997). During the vegetative cycle of the plant host, endophytic fungal hyphae asymptomatically colonize the intercellular space of plant tissues/organs, such as leaf sheaths and blades. When the reproductive cycle of the host plant commences, the outcome of the grass-endophyte interaction can be asymptomatic or antagonistic, depending on the grass and endophytic fungal species involved (Faeth and Sullivan, 2003; Zabalgogeazcoa *et al*., 2003). The different outcomes of a host-endophyte interactions are attributed to the different life history of the individual symbiont, patterns of endophytic fungal infection, genotypic variation and ecological factors (Saikkonen *et al*., 1998; Photita *et al*., 2004, 2005; Müller and Krauss, 2005; Gonthier *et al*., 2005).

Systemic endophytic fungi, such as *Neotyphodium*, in grasses are generally viewed as plant mutualists based on the action of endophyte-produced alkaloids in infected grasses. *Neotyphodium*-grass interactions can protect the host plant from herbivores and pathogens, and also enhance plant fitness and competitive abilities, such as greater drought tolerance (Latch, 1997; Malinowski *et al*., 1999). Most of the reported studies on grass-endophyte associations are limited to economically important grasses, such as perennial ryegrass (*Lolium perenne* L.) and tall fescue (*Festuca arundinacea* Schreb.). Infection frequencies of endophytic fungi in agronomic grasses often rapidly increase (within a few years) to near 100% because of the increase in general competitive abilities (Clay, 1998; Clay and Holah, 1999). Infection frequencies of *Neotyphodium* in natural (wild) grass populations are much more variable than those in domesticated grasses, ranging from 0 to 100%, even among populations of the same grass species (Saikkonen *et al*., 1998; Schulthess and Faeth, 1998; Vinton *et al*., 2001). However, there are few systematic studies on community structure of endophytic fungi in natural (wild) grass populations, though there have been a few surveys on infection of endophytic fungi in wild grasses (Saikkonen *et al*., 2000; Nan and Li, 2001; Li *et al*., 2004). The native grasses of Inner Mongolia Steppe, which is an

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important part of Steppe in Eurasia located in the north of China, have been scarcely studied for their associated endophytic fungi. There have been limited reports on endophyte-grass symbiosis from this important natural ecosystem. Thereby, a more comprehensive view of the ecology, diversity of endophytes and their interactions within host plants becomes essential.

In this paper, we made a survey on endophytic fungi associated with a perennial sparse bunchgrass, *Achnatherum sibiricum* from the Inner Mongolia Steppe, and discovered an endophyte-grass association of high infection and diversity. *Achnatherum sibiricum* is distributed over the moderately temperate steppe region and is native to the northeast and north of China. This grass can be found in different habitats such as meadow and typical steppes, in and out of forests, and can become dominant species in plant communities in semi-arid areas. Our hypotheses were that infection frequencies and diversity of endophytic fungi were correlated to soil factors and the growth status of *A. sibiricum* and grazing stress decreased the endophytic fungi diversity in *A. sibiricum* and had an effect on infection rates.

In the present study we 1) investigated the patterns of distribution of endophytic fungi in *A. sibiricum*; 2) examined morphological diversity of endophytic fungi associated with *A. sibiricm*, and 3) analyzed the correlations of infection frequency and diversity of endophytic fungi with selected soil factors and growth status of *A. sibiricum*.

Materials and methods

Climate of the study area

The climate of Inner Mongolia Steppe is of monsoon type, having some rains in the summer (from early July till late August) and being dry in the other seasons. In this area, there is an environmental gradient in aridity and air temperature from the northeast to the southwest, and this gradient provides an ideal model system for the study of ecological and genetic variations of symbiosis with climatic change.

Sampling

Achnatherum sibiricum from four different sites (Fig. 1) were examined. They included Hulingol (HLG) a typical Steppe, West Ujimqin (WUM) a meadow Steppe, Plot I of Inner Mongolia Grassland Ecosystem Research Station, Chinese Academy of Sciences (IMGERS-Plot I) a sandy Steppe and Plot II of IMGERS (IMGERS-Plot II) a typical Steppe.
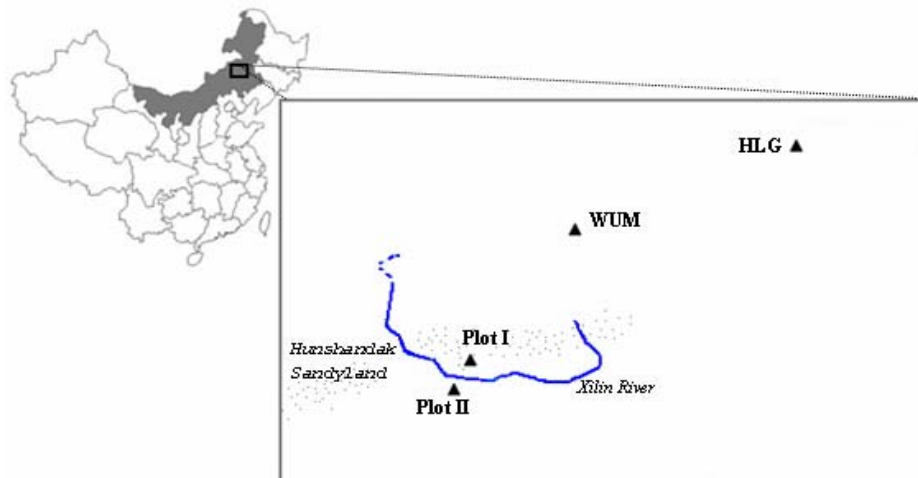
Fig. 1. Map of endophytic fungal populations used in the study. The four sampling sites are marked with solid triangles. Inner Mongolia Autonomous Region and its location within China (top left). Approximate distance between sites are: HLG to WUM, 190km; WUM to IMGERS-Plot I, 140km; IMGERS-Plot I to IMGERS-Plot II, 10km.

Plant material was sampled in September 2003 and 2004, and July and September 2005. To examine the infection rates of the endophytes within a plot, thirty plants were randomly collected at a distance of more than 5 meters.

Detection of Epichloë/Neotyphodium endophytic fungi

Five tillers were collected randomly from each plant and the outermost non-senescent leaf sheath of each tiller was used in this assay. A strip of epidermis was peeled from the inner surface of the leaf sheath close to the stem base. The strip was placed on a slide, mounted in aniline blue stain (Latch *et al*., 1987) and the slide was heated over a flame until the stain reached boiling point. It was then examined for hyphae under ×400 magnification.

Plants infected by antagonistic endophytic fungi were easily recognized by the presence of stromata in the stems.

Isolation of endophytic fungi

Endophytic fungi were isolated from the four *Achnatherum sibiricum* populations and a total of 80 endophyte-infected plants (20 plants per population) were used for isolation. Plant seeds were surface sterilized for 20

minutes in 50% sulfuric acid, rinsed in axenic water and sterilized for 20 minutes in 3% sodium hypochlorite. They were finally rinsed in axenic water three times and placed on Petri dishes (9 cm diam) containing potato dextrose agar (PDA). The plates were incubated in darkness at a constant temperature $(25 \pm 1ºC)$, and were checked every two days. Typical white and cottony colonies of *Neotyphodium* emerged from seeds after about three weeks and were transferred to new plates immediately. Classification of the endophytic fungi was based on phialide and conidial morphology, and growth on PDA medium (Marshall *et al*., 1999). The *Neotyphodium* fungal endophytes were identified based on solitary phialides arising from aerial hyphae, without basal septum. The isolates that did not sporulate were exposed to UV light or transferred to malt extract agar (MEA) medium to induce sporulation (Schulthess and Faeth, 1998). All isolates were distinguished by morphology of the colonies on PDA medium and similar isolates were grouped into one morphotype (Lacap *et al*., 2003; Wang *et al*., 2005). The radial growth was measured per week and the size of conidia was measured in twenty replicates.

Soil analysis and host plant growth measurement

Ten soil samples (15-30 cm layer) were taken from each of the four sites in September 2005, for determination of soil nutrients: carbon (C), total nitrogen (N), available phosphorus (P), available potassium (K) and water contents. These soil samples were air-dried at room temperature for 15 days, and 50 g of dried soil was used for nutrient analysis. The water content of the soil was determined by subtracting dry mass (drying at 80ºC) from the fresh weight of the sample, and dividing by the dry mass of the soil.

Plant growth was measured for host plants at four sites in 2005. In each of these sites, 30 individual plants were sampled and each of them had derived from a single genotype unit. Plant height was recorded in mid-July and late September of the growing season, and number of tillers and above-ground biomass were recorded at the end of growing season.

Statistical analyses

One-way ANOVA and Duncan's SSR tests were employed to determine the significance of differences among the four sites for the following factors: soil nutrient and water content, height of plant, number of tillers and above-ground biomass of *Achnatherum sibiricum*. Spearman's rho correlation coefficients were calculated to test whether or not the rates of infection by

endophytes correlated with soil nutrients and water content, or plant growth. Statistical analyses were performed with the statistical package SPSS 10.0.

Results

Distribution and frequency of Epichloë/Neotyphodium endophytic fungi

Hyaline, sparsely branched septate vegetative hyphae were observed, growing parallel to the long axis of plant cells in intercellular spaces of leaf sheaths, and twisted hyphae in the innermost seed coat were observed in asymptomatic plants from the four populations (Figs 2a,b). Plants in HLG and WUM were also found to be infected with *Epichloë*, having stromata on their flowering culms (Fig. 2c).
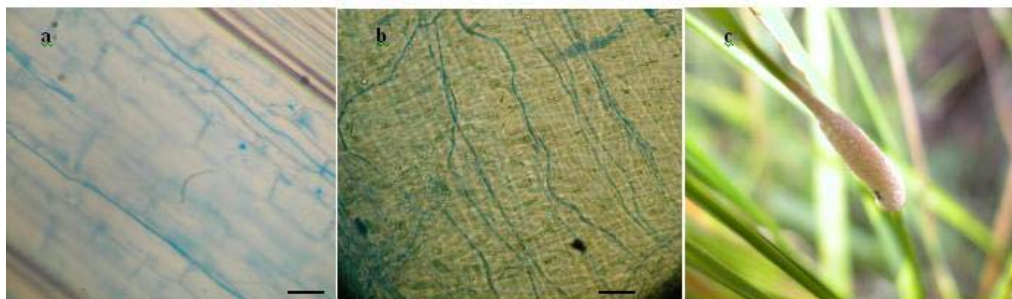

Fig. 2. Photographs of fungal endophyte of *Achnatherum sibiricum* grass (bar = 10μm). (a) Endophytic hyphae in intercellular spaces within leaf sheath of *Achnatherum sibiricum.* (b) Mycelium of *Neotyphodium* in innermost seed coat of *Achnatherum sibiricum.* (c) Stromata of *Epichoë* on aborted flowering culm of *Achnatherum sibiricum.*

The four sites were examined for endophytic fungal infection over the experimental period and the results showed that there was seasonal variations in the frequency of endophytic fungal infection. No statistically significant variations however were found between years or locations except for HLG population (Fig. 3). In September 2003, the infection rates of endophytic fungi at all four sites reached 100%. We collected plants from the same four sites in September 2004 and found that the infection rate had decreased to 40% in the HLG population, while the infection rates of the other three sites stayed at 100%. In July 2005, the infection rates in the four *A. sibiricum* populations ranged from 36.7% to 50% (with an average of 38%), though the differences were not statistically significant. The frequencies of endophyte infection of the above collections were similar in September, 2005.
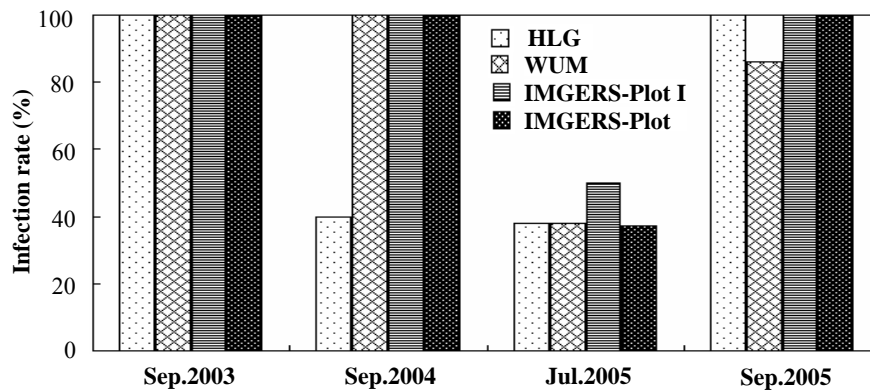
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Fig. 3. Infection frequencies of endophytic fungi in *Achnatherum sibiricum* at four sites

The IMGERS-Plot II had been enclosed for more than 20 years while the other plots were under grazing. Unexpectedly, there was no significant difference in infection rate between IMGERS-Plot II and the other three sites.

Differences in soil nutrients, water content and plant growth parameters among the four sites using Duncan's SSR test were significant ($P<0.05$) except for phosphorus (Fig. 4). The infection rates of endophytic fungi however did not show any significant correlations with soil nutrients (carbon, nitrogen, phosphorus or potassium), or water content, either in July or in September 2005 using Spearman's rho test. Similarly, the infection rates of endophytic fungi in each of the four sites did not correlate significantly with plant growth parameters either in July or in September data (Table 1).

Identification and diversity of Epichloë/Neotyphodium endophytic fungi

The strains isolated from host seeds of the four populations (HLG, WUM, IMGERS-Plot I and Plot II) were identified as *Neotyphodium* based on colonies and conidia on agar (Morgan-Jones and Gams, 1982; Latch *et al.*, 1984; White and Morgan-Jones, 1987). Colony and conidial shapes were given in Table 2 and Table 3. Conidiogenous cells (phialides) were very sparse on PDA, stemming solitarily from aerial hyphae and were discrete, orthotropic, unbranched, and hyaline. *Epichloë* species was identified by the typical stromata formed in flowering culms of *A. sibiricum*.

Twenty-seven isolates of *Neotyphodium* were obtained from the four sites. The 27 isolates were categorized into nine morphotypes on PDA medium (Fig. 5). Of nine morphotypes categorized, isolates HLG d, Plot II d, Plot II i, Plot II l and Plot II m had unique morphological characters, and were regarded as individual morphotypes. The greatest morphotypic diversity occurred in the
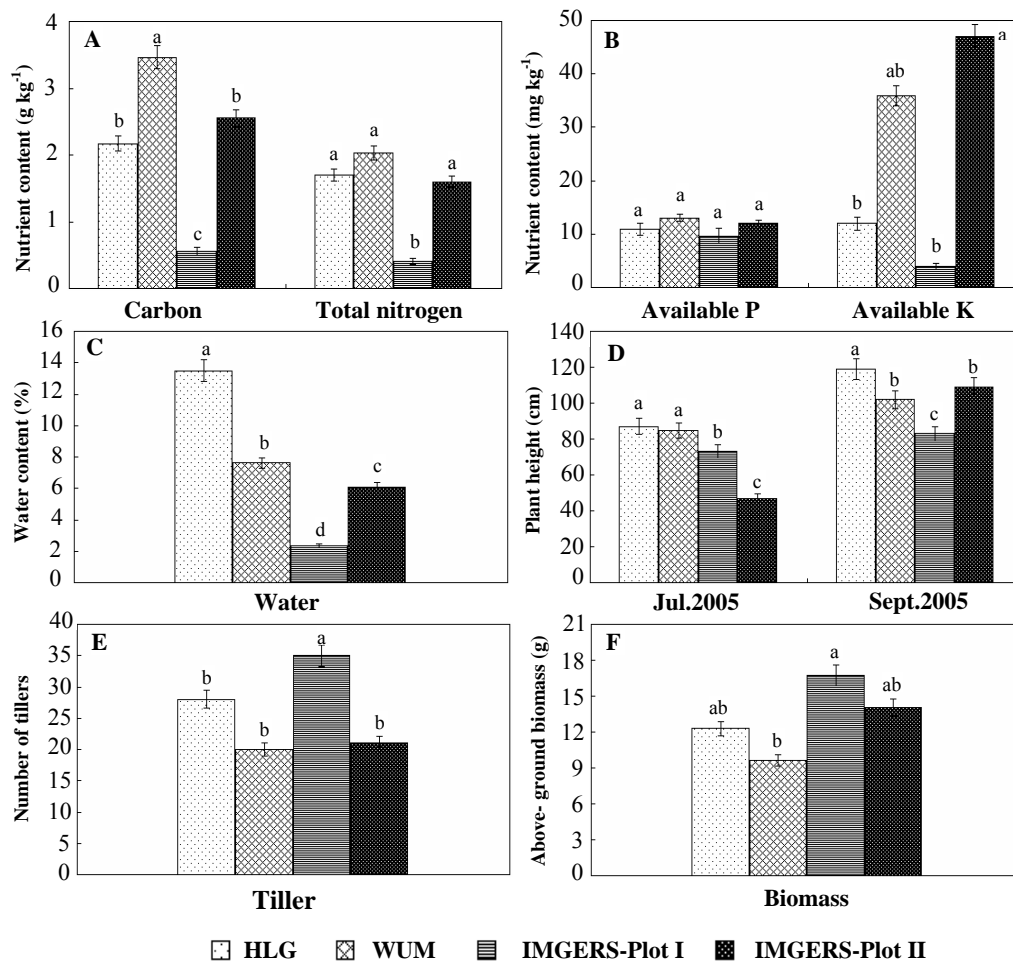
Fig. 4. Soil nutrient, water contents and growth of *Achnatherum sibiricum* in the four investigation sites (mean ± SE). Common letters in column indicate no statistically significant difference in certain parameter ($P < 0.05$).

endophytes of the IMGERS-Plot II population (six morphotypes), followed by those of the HLG population (two morphotypes), and those of the WUM and IMGERS-Plot I populations had the lowest diversity (each had one morphotype) (Table 2).

The conidia from isolates at the four sites were solitary, hyaline and smooth-walled, but their shape and size varied (Table 3). The HLG population was characterized by obovate or semi-globular conidia with mean length 3.07 μm; the WUM's conidia were obovate, semi-globular, oblong and reniform ($\bar{x}$ = 3.99 μm); The IMGERS-Plot I's conidia were reniform or navicular ($\bar{x}$ =

Table 1. Spearman's rho correlation coefficients between endophyte infection rate and different parameters

Variable	Infection rate July 2005	September 2005
Carbon	−0.400	−0.775
Nitrogen	−0.200	−0.775
Phosphorus	−0.400	−0.775
Potassium	−0.800	−0.258
Soil water content	−0.400	−0.258
Plant height	0.200	0.258
Number of tillers	–	0.775
Above-ground biomass	–	0.775

4.27 μm); The IMGERS- Plot II's conidia were navicular and reniform ($\bar{x}$ = 4.36 μm). The mean length of conidia between the four populations showed significant differences except that between IMGERS-Plot I and Plot II, and there was a trend that the conidium length gradually increased from the east to the west populations (Fig. 6).

Discussion

Endophytic fungal infection

Although fluctuations in the infection rates during the growing season, they reached 100% at the end of the season (September) in almost all *Achntherum sibiricum* populations tested. Such a high infection rate has been reported on other grasses and in other regions. Saikkonen *et al.* (2000) found that populations of tall fescue in Finland had 96-100% infection rates, and Vinton *et al.* (2001) reported that virtually all populations of *Elymus canadensis*, a native prairie grass, had 100% infection. However, most of former studies showed that there was variability in the frequency of natural plant populations.

Lewis *et al.* (1997) detected wild populations of *Lolium* spp. from 15 of 20 European countries. Of 523 populations examined, 38% contained no infection, 48% contained 1-50% infection and 14% contained 51-100% infection. Several other studies showed variable infection frequencies in host endophyte populations (Bazely *et al.*, 1997; Schulthess and Faeth, 1998; Zabalgogeazcoa *et al.*, 1999; Clement, 2001; Jensen and Roulund, 2004). This suggests that, unlike agronomic grasses, infected grasses in natural habitats did not necessarily become competitive or dominant, because endophyte-host plant

Table 2. The nine morphotypes of *Neotyphodium* isolated from *Achnatherum sibiricum* at four sites.

Morphotype	Isolates	Colony color	Colony texture	Colony form	Aerial mycelium	Growth rate (mm·d⁻¹)
I	HLGa,HLGb, HLGc, HLGf	yellowish in center, white in margin	cottony, slightly loose	depressed in center with margin raised	abundant	1.24–1.63
II	WUMa, WUMb, WUMc, WUMd, WUMe, WUMf,WUMg	white	lanose, loose	depressed in center with margin raised	abundant	1.45–2.38
III	Plot I b, Plot I c, Plot I d, Plot I g, Plot II b, Plot II e, Plot II h, Plot II j, Plot II k	white	cottony, compact and strongly aggregated	raised	absent	0.22–0.92
IV	Plot II a, Plot II c	off-white	felted, radial draped, tight	elevated to appressed	scarce	1.83–1.23
V	HLG d	off-white	lanose, loose	appressed	abundant	2.86
VI	Plot II d	white	cottony, loose	semi-globular riased	abundant	1.81
VII	Plot II i	white	cottony, compact	centre raised, coral-like in margin	absent	0.48
VIII	Plot II l	black green	cottony to pulvinate, radial draped, compact	elevated to appressed	absent	1.06
IX	Plot II m	buff	waxy	irregular piled up	absent	– [a]

[a] Growth rate of isolate Plot II m could not be measured because of its abnormal growth.

interactions are variable and range from antagonistic to mutualistic (Saikkonen *et al*., 1998).

 Our results suggest that there were two possible reasons for the high infection rates at the end of growing season and seasonal variation of endophytic fungal frequencies in *A. sibiricum* populations. One possibility is

Table 3. The characteristics of conidia of *Neotyphodium* on PDA from four sites.

Site	Isolate	Conidium form	Conidium length x width (μm)	Mean length of conidia (μm)
HLG	HLGa	reniform	2.3-3.7×1.2-2.1	3.07
	HLGb	obovate, oblong, semi-globular	2.6-3.4×1.8-2.1	
	HLGc	semi-globular	2.6-3.5×1.4-2	
	HLGf	obovate, semi-globular	2.7-3.8×1.5-2	
WUM	WUMa	obovate, semi-globular	3.3-5×2.3-3.9	3.99
	WUMb	oblong, reniform	3.2-4.9×1.8-2.9	
IMGERS-Plot I	Plot I b	reniform	3.8-6×1.5-3.8	4.27
	Plot I c	reniform, navicular	3.8-5.1×1.7-2	
	Plot I d	reniform, navicular	2.6-4.6×1.4-2.2	
IMGERS-Plot II	Plot II b	navicular	3.2-5.0×1.5-2	4.36
	Plot II d	reniform	3.1-4.3×1.4-2.1	
	Plot II e	reniform	4.1-5.2×1.8-2.3	
	Plot II i	navicular	3-3.2×1.1-1.4	
	Plot II j	navicular	2.6-4.3×1.4-1.6	
	Plot II k	navicular, lunate	3.0-3.8×1-1.5	

that horizontal transmission increased the infection rates because the stromata of *Epichloë* spp. were found on flowering culms of host plants. Schardl (1996) described life cycles of *Epichloë festucae* in pleiotropic symbiosis with *Festuca rubra*. In the sexual cycle, the fungus also grows intercellularly in vegetative leaf sheaths without causing symptoms, but then overgrows the leaf sheath into a stroma surrounding the immature host inflorescence, produces spermatia, and arrests inflorescence maturation. Fertilization occurs by transfer of spermatia of opposite mating type. If the parents are conspecific (same mating population), perithecia containing asci develop and filamentous ascospores are ejected. Germinating ascospores on host florets cause new infections of developing seeds. Contagious (horizontal) spread should not be ruled out even in *Neotyphodium* endophytes because they produce asexual conidia on growth media and on living plants, and recent evidence indicates horizontal transmission in natural grass populations (Saikkonen *et al*., 2004).

Another possibility is that there might have existed an obligate insect, which efficiently spreaded spermatia in *A. sibiricum* populations. *Epichloë* is transmitted horizontally via conidia whose transportation to another stroma requires an insect vector (Müller and Krauss, 2005). For example, stromata of *Epichloë typhina* forming on aborted flowering culms of *Elymus virginicus*,
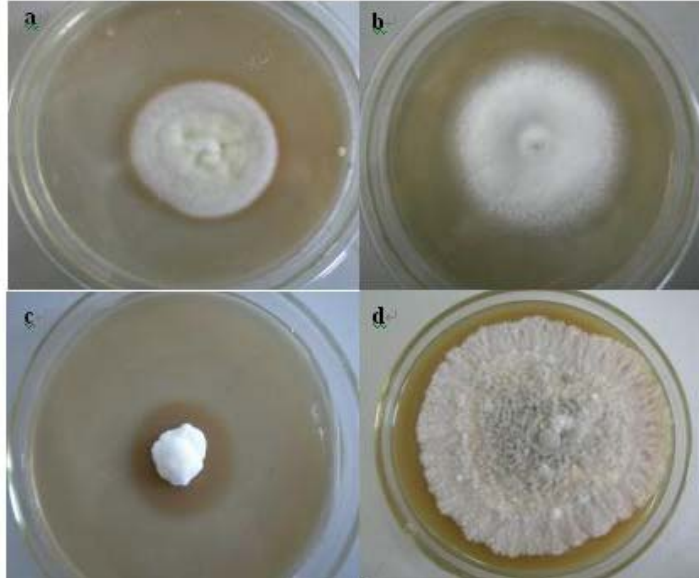
Fig. 5 . Photographs of endophytic fungal cultures showing four typical colonies of four morphotypes (bar=10mm) : (a) morphotype I, HLG b; (b) morphotype II, WUM f; (c) morphotype III, IMGERS-Plot I e; (d) morphotype IV, IMGERS-Plot II c.

larvae of fly vectors conidia and effects fungal fertilization (Clay and Schardl, 2002).

Whether this high infection frequency had a correlation with symbiosis of *Neotyphodium* endophytes of different life histories or whether there existed *Epichloë* Type II associations (White *et al*., 1993) in *A. sibiricum* needs to be tested further in future.

The plants however underwent drought regularly which made it difficult for the hyphae to propagate into plant texture or made the hyphae unviable in seeds in early period of growing season. This conclusion can also be drawn from the results that the correlation coefficient of endophyte infection with plant growth was higher than with environmental factors (Table 1). There were no obvious connections between infection rate and environment factors; On the contrary, infection rates may just be determined by biological characters of endophyte itself.

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Diversity of Neotyphodium fungal endophytes

Our results showed that at least one fungal species had infected all *A. sibiricum* plants, and variations in morphology of the cultures on PDA indicated high genetic diversity among endophyte populations.

The most diversified isolates of IMGERS-Plot II may be explained by higher habitat heterogeneity and diversity of community. The heterogeneity of the IMGERS-Plot II habitat was greater than that of the IMGERS-Plot I because the former was within the enclosure for more than 20 years while the habitat of the IMGERS-Plot I was characterized by sandy chestnut soil and sparse vegetation under heavy-grazing. In addition, the distance was 10 km from the IMGERS-Plot I to the IMGERS-Plot II populations, so their growth and morphological characteristics such as colony shape, growth rate and conidia size were more similar than two other sites.

Among the four endophytic fungal sites, to a certain extent, there was a difference in isolate growth rate. The HLG and WUM populations were similar in isolate growth rate, and in the same way the IMGERS-Plot I and Plot II populations were similar in isolate growth rate, but isolates from the HLG and WUM populations grew faster than those from the IMGERS-Plot I and Plot II populations (Fig. 6). This indicated that there was biological diversity in different endophyte populations.

The mean length of conidia between the four populations showed significant differences except between IMGERS-Plot I and Plot II, and there was a trend that the conidium length gradually increased from the east to the west populations (Fig. 6). Clement *et al*. (2001) found that the genetic diversity can be reflected by the variation in mean conidial length of isolates from tall fescue from each of three countries, where lengths averaged 6.27 μm (Sardinia), 5.69 μm (Morocco) and 4.93 μμm (Tunisia). The present study indicated that the genetic diversity of *Neotyphodium* fungi was rich in *Achantherum sibiricum*.

While studying distribution, abundance and associations of the endophytic fungal community of Arizona fescue (*Festuca arizonica*), Schulthess and Faeth (1998) noted that whereas the focus on grass endophytes has been traditionally on specialized, systemic endophytes, these same grasses typically harbor hundreds of more generalized, horizontally transmitted endophytic species. Our results was in agreement with the generalization of Schulthess and Faeth (1998) that the fungal endophyte communities of *Achantherum sibiricum* may be as diverse as fungal endophyte communities of woody shrubs and trees.
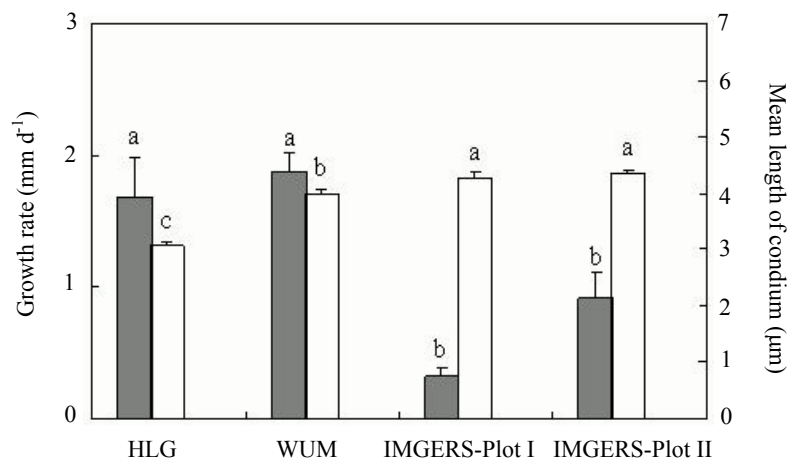
Fig. 6 . Growth rate (gray column) of *Neotyphodium* isolates and mean length of conidia (white column) of four populations (mean ±SE). Common letters above column indicate no statistically significant difference (*P*<0.05) in each item.

The diversity of the fungal endophyte available can offer opportunities for developing new grass-endophytic fungal symbiont to obtain the fully beneficial aspects of the association. So related studies about endophyte diversity seem to be more and more important. Although the origin of endophyte diversity in *Achantherum sibiricum* populations discussed in this paper has not been confirmed, the results obtained from our experiment suggested that several fungal populations may have derived from different ancestors, or spatial distance baffled the genetic flow and resulted in the morphological diversity.

Acknowledgements

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