SYMBIOSIS (2008) 46, 21–31 ISSN 0334-5114

Diversity of clavicipitaceous fungal endophytes in *Achnatherum sibiricum* in natural grasslands of China

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(Received February 22, 2007; Accepted November 12, 2007)

Abstract

A total of 484 endophytic fungi isolates were isolated and purified from seven populations of *Achnatherum sibiricum* (L.) Keng collected at six geographical locations in Inner Mongolia, China. Based on growth rates as well as morphological characteristics including colony morphology, shape and size of conidia and conidiogenous cells, all the isolates were classified into five morphotypes, i.e. morphotypes A, B, C, D and E. Morphotype A, B and C were widely distributed in almost all populations tested. Among them morphotype A grew slowest; morphotype B and C were similar in growth rates but only morphotype C produced chlamydospores. Maximum parsimony tree showed that the three morphotypes A, B and C had high similarity and together with *Neotyphodium chisosum* they formed a cluster with a 99% bootstrap support. Based on morphological and rDNA-ITS sequence data, we ascribed morphotypes A, B and C to *N. chisosum*. Morphotypes D and E grew faster than A, B and C. Only morphotype D did not produce conidia even after inducement. Morphological characteristics of morphotype E, including colony texture and conidia, were almost the same as for *Epichloë amarillans*. Also, morphotype E only appeared in one plant population, where the endophyte showed both seed transmission and stroma formation on different culms of the same plants. Therefore, morphotype E might be *E. amarillans*. There was a slight tendency for morphotype diversity to decrease from the northeast to the southwest among the six sites studied.

Keywords: *Achnatherum sibiricum* (L.) Keng, endophyte, morphotype, growth rate, *Neotyphodium*

1. Introduction

Many grasses are infected by clavicipitaceous fungal endophytes that occur in aboveground plant tissues. Most infections are asymptomatic and the endophytes are transmitted maternally through the seeds of infected grasses (Clay, 1990). The benefits to grasses of hosting such endophytes may be numerous. Endophyte fungi in agronomic fescue and ryegrass decrease the palatability of the grass to insect and mammalian herbivores and can cause toxicosis in livestock due to production of alkaloids (Cheplick and Clay, 1988; Funk et al., 1983). The fungi can also enhance the growth and productivity of the host grasses, especially under abiotic stresses such as drought (Elmi and West, 1995; Hesse et al., 2003), high temperature (Marks and Clay, 1996), mineral stress (Arachevaleta et al., 1989; Bacon, 1989; White et al., 1992), and elevated radiation (Lewis, 2004; Newsham et al., 1998).

Endophyte-infected species occur in almost all habitats where grasses are common (Shelby and Dalrymple, 1987; White, 1987). The grasses include a large number of wild grasses, pasture grasses, lawn grasses, cultivated grains and their wild relatives, as well as weed grasses (Bacon et al., 1986; Fomba, 1984; White et al., 1993; Wilson et al., 1991). However, most previous work has focused on the two introduced and economically important grass species tall fescue (*Festuca arundinacea*) and perennial ryegrass (*Lolium perenne*) which represent but a small fraction of grass and endophyte diversity (Saikkonen et al., 2006). Research on endophyte-natural grass systems is relatively limited but shows that infection incidences among natural populations are highly variable (Lewis et al., 1997; Saikkonen et al., 2000; Schulthess and Faeth, 1998; Zabalgogeazcoa et al., 1998). There could be significant correlations between level of infection and climate variables, such as evapotranspiration, water supply deficit (Lewis et al., 1997) and elevation (White et al., 2001). Further studies by Faeth and collaborators suggest that most associations between *Neotyphodium* and grass species in natural systems are rarely defensive mutualistic (Faeth,

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2002; Feath et al., 2004; Faeth and Sullivan, 2003; Saikkonen et al., 2004). For those systems where defensive mutualism does exist, many grasses infected with endophytic fungi are toxic in only some parts of their range (Faeth et al., 2006; Jones et al., 2000; Miles et al., 1996).

One hypothesis is that this kind of variation results from geographic variation in endophyte species, strains or morphotypes (Saikkonen et al., 1998). To our knowledge, geographic variation in endophyte species of endophyte infected grasses has not yet been documented for any of the known wild grass hosts. Yet, this kind of population variation is not only essential to understand the outcome of interactions between host grass and systemic endophytes (Faeth et al., 2006; Sullivan and Faeth, 2004), but is also necessary to address the coevolution and ecology of species interactions (Thompson, 2005).

In China, endophytic fungi have been found in at least 13 genera and 25 species of native grasses growing in natural grasslands (Nan and Li, 2004). For most grasses, however, nothing but endophyte infection rates is documented. Much research on diversity of endophyte fungi species and the interaction between endophytes and the host grasses is still required. *Achnatherum sibiricum* (L.) Keng is a caespitose perennial grass that is widely distributed in the north of China. After four years of surveys, we found that *A. sibiricum* is highly infected by clavicipitaceous fungi, and there is little difference among infection rates in different geographic populations. Within the genus *Achnatherum*, there are five sections. *A. sibiricum* belongs to Sect. Achnatheropsis (Tzvel.) N. S. Probatova, the highest evolved section. There are 9 species in this section, including 7 Asian species and 2 American species (Wu and Lu, 1995). To date, only two species, *Achnatherum inebrians* and *Achnatherum robustum*, are reported to be infected by *Neotyphodium* endophytes. Both are notorious for the narcotic properties to livestock, and hence, named as "drunken horse grasses" and "sleepy grass", respectively (Bruehl et al., 1994; Petroski et al., 1992). The toxicity of *A. inebrians* is a result of infection by the endophyte, *Neotyphodium inebrians* (Bruehl et al., 1994; Miles et al., 1996). For *A. robustum*, the *Neotyphodium* endophyte has not yet been identified to species. According to Kaiser et al. (1996), it appears intermediate in morphological characteristics between *Neotyphodium starii* and *Neotyphodium chisosum* and also different from *N. inebrians*. In contrast to *A. inebrians* and *A. robustum*, *A. sibiricum* has no obvious herbivore deterrence according to local records and our own observations.

To ascertain geographic variation in endophyte diversity, we sampled seven populations of *A. sibiricum* along the Inner Mongolian steppe, which represents the main distribution of this species in China. We assessed the morphological diversity of endophytic fungi in all populations. We also compared the similarities of these endophytes with published *Epichloë/Neotyphodium* species in order to identify them to species.

2. Materials and Methods

General introduction to sampling sites

The Inner Mongolian steppe is an important part of Eurasian steppe. The climate in this region varies from semi-arid type to arid type. The annual mean air temperature is 0–8°C. Mean temperature is between –12 and –24°C in January, and from 20 to 24°C in July. The number of frost-free days is 100–120 d. From the east to the west, three types of steppe are found, i.e. meadow steppe, typical steppe and desert steppe. Meadow steppe is a transitional type between forest and steppe, usually formed under moist weather conditions, with annual precipitation averaging 450 mm. The meadow steppe is rich in plant species, about 35 species per square meter. The canopy height of the community is 40–50 cm. Mesophytic or xerophytically-mesophytic perennial grasses are dominant, and there are also non-grass perennials. Most area of meadow steppe is used for grazing. The typical steppe takes the largest area of the Inner Mongolia steppe and is characteristic of the steppe communities where species of xerophytes or mesophytic xerophytes dominate. The annual precipitation in this area averages 350 mm. The species richness is about 15 species per square meter and the average canopy height is about 30 cm. Xerophytic grasses are dominant species, accompanied by some non-grass perennials. Desert steppe is a transitional type from steppe to desert, and is the most drought-tolerant type of the steppe, found in the western part of Inner Mongolia. The annual precipitation averages 200 mm.

Populations and sampling

During the summers of 2003 and 2004, as a pilot study, we examined endophyte infections of 41 grass species, 56 sites and 172 local populations throughout the Inner Mongolian Steppe, China, to study frequencies of endophyte infected grasses in wild populations. For details of collection sites see Wei et al. (2006). *Achnatherum sibiricum* was found only at eight geographical locations studied (Fig. 1), and their endophyte infection rates were almost equally high (86–100%). In the summer of 2005, we focused more closely on morphological diversity of endophytes associating with different populations of *A. sibiricum*. We sampled seven populations (Table 1) in six locations. One population was sampled in each geographic location except that two populations were chosen within (*Leymus* plot 1) and outside of (*Leymus* plot 2) the herbivore enclosure in the *Leymus chinensis* plot population. The *L. chinensis* plot has been fenced-off since

Table 1. Location, soil type and habitat characters of *Achnatherum sibiricum* populations in Inner Mongolia.

Population	Code	Geographic position	Altitude (m)	Annual precipit. (mm)	Annual mean temp. (°C)	Degree days ≥10°C in a year*	Soil type (Wang, 1994)	Vegetation	Type of farming
Hailar	POP1	49.06°N, 119.40°E	629	350	–2.0	1992	Dark Chestnut	Meadow steppe	Producing grass
Arshan	POP2	47.12°N, 119.55°E	1122	460	–3.1	1440	Dark	Meadow steppe	No use
Huolingol	POP3	45.25°N, 119.45°E	990	340	1.5	2238	Dark Chestnut	Meadow steppe	Grazing
Xiwuqi	POP4	44.58°N, 117.60°E	996	340	1.5	2256	Dark Chestnut	Edge of typical steppe	Producing grass
Dingweizhan	POP5	43.38°N, 116.42°E	983	350	2.4	2400	Chestnut	Typical steppe	Producing grass
Leymus plot 1	POP6	43.58°N, 115.04°E	1156	350	1.6	2496	Chestnut	Typical steppe	Producing grass
Leymus plot 2	POP7	43.58°N, 115.04°E	1156	350	1.6	2496	Chestnut	Typical steppe	Grazing

*The average daily temperature × the number of days when the temperature is ≥10°C.

1979, preventing grazing by large animals. During three years (2003–05) of surveys, we did not find any difference between endophyte infection rate in populations within and outside the herbivore enclosure. In each plot 20 adult plants, more than 5 m apart, were selected randomly. One reproductive tiller of each plant was cut and the seeds were collected for endophyte isolation.

Endophyte isolation

Endophyte isolation was performed in accordance with procedures in Bacon (1989). Seeds from different populations were surface sterilized, placed on potato dextrose agar (PDA) and then incubated at 25°C in darkness. Two weeks after fungal colonies appeared, small agar blocks (ca. 1 mm^2) were cut from the margins of actively growing colonies and used to incubate PDA plates. Single morphological colony types were achieved through inoculating small agar blocks on PDA media three times in succession. Endophyte isolation was performed on 6 seeds from each of 20 plants chosen at random from each population. Fungi were isolated from almost all seeds (120 seeds in each population) tested, but some of them did not look like clavicipitaceous fungi in appearance and some became contaminated during purifying process. The final isolation rate of clavicipitaceous fungi was around 65% in each population. We chose 70 isolates in POP1 to POP5, and 67 isolates in POP 6 to POP7, so a total of 484 isolates were used for observation and classification.

Macroscopic examination of endophyte morphology in culture

For the macroscopic examination of endophyte morphology, small agar blocks (ca. 1 mm^2) were cut from the margins of purified mycelium colonies and used to incubate PDA plates. Each isolate incubated five plates. Morphological observations and colony diameter measurements were taken after 7 d for the fastest growing isolates, 14 d for the second fastest growing isolates and 28 d for other isolates. The growth rate was calculated according to the following formula.

Growth rate = (Maximum diameter + minimum diameter) / (2 × incubation days).

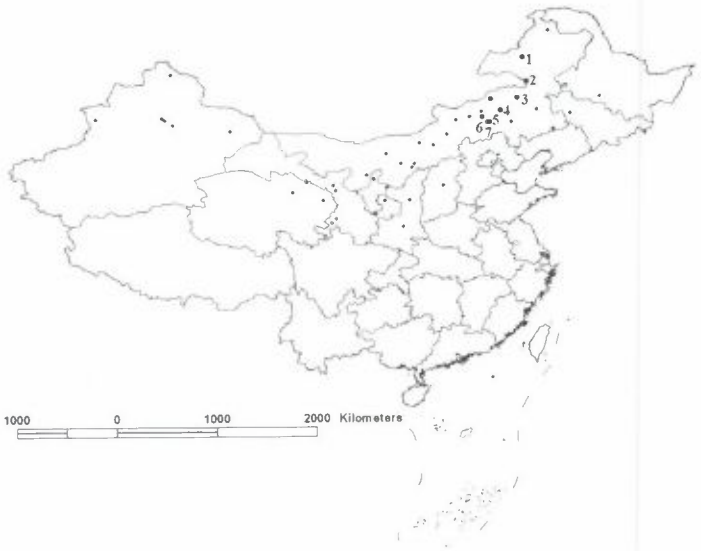

Figure 1. Sampling sites of grasses for endophyte detection and *Achnatherum sibirium* populations for endophytic diversity. Dots indicate the locations where grasses were sampled for endophyte detection. Larger dots denote sites where *A. sibirium* species occur. Numbers indicate the seven sites with *A. sibirium* from which endophytes were studied.

Table 2. DNA sequences used for phylogenetic analysis. A, B and C denote endophytic morphotype, and the following numbers represent isolate number.

Endophyte species	GenBank accession no.	Host	*A. sibiricum* population
A1-1	DQ675587	*Achnatherum sibiricum*	POP1
A1-2	DQ675588	*Achnatherum sibiricum*	POP1
A2	DQ675584	*Achnatherum sibiricum*	POP2
A5-1	DQ675574	*Achnatherum sibiricum*	POP5
A5-2	DQ884462	*Achnatherum sibiricum*	POP5
A6	DQ855269	*Achnatherum sibiricum*	POP6
B2-1	DQ675582	*Achnatherum sibiricum*	POP2
B2-2	DQ675583	*Achnatherum sibiricum*	POP2
B4-1	DQ675577	*Achnatherum sibiricum*	POP4
B4-2	DQ675578	*Achnatherum sibiricum*	POP4
C1	DQ675586	*Achnatherum sibiricum*	POP1
C3-1	DQ855270	*Achnatherum sibiricum*	POP3
C3-2	DQ675576	*Achnatherum sibiricum*	POP3
C4	DQ675579	*Achnatherum sibiricum*	POP4
Epichloë brachyelytri	L78296	*Brachyelytrum erectum*	Switzerland
Epichloë glyceriae	L07136	*Glyceria striata*	New York, USA
Epichloë sylvatica	L78304	*Brachypodium sylvaticum*	Switzerland
Epichloë baconii	L07138	*Agrostis stolonifera*	England
Epichloë amarillans (U)	U57665	*Agrostis hiemalis*	Alabama, USA
Epichloë amarillans (AF)	AF385200	*Achnatherum sibiricum*	Inner Mongolia, China
Epichloë elymi	L07131	*Elymus canadensis*	Texas, USA
Neotyphodium chisosum (201)	AF385201	*Stipa eminens*	Texas, USA
Neotyphodium chisosum (203)	AF385203	*Achnatherum sibiricum*	Inner Mongolia, China
Neotyphodium sp. ATCC	AF385199	*Stipa lobata*	Texas, USA

Microscopic examination of endophyte morphology in culture

Each colony was examined by light microscopy. For fungi colonies lacking conidia, some measures, such as incubation on malt extract agar (Guo et al., 1998) or sweeping mycelia with a brush (Guo et al., 2003), were taken to induce conidia production. Once conidia appeared, the mycelia were processed according to the following procedure. Firstly, 10 ml 20% glycerite was added in a Petri dish (Φ = 9 cm) with filter paper in the bottom. Then a U-shape glass rod was put into the Petri dish, and biconcave slides were placed on top of the glass rod. Next PDA medium was dripped into the concave depression of the slide. Finally agar blocks containing actively conidiating mycelium were placed on the PDA media and covered with cover slips. The Petri dishes were maintained at 25°C in darkness. After two weeks, mean lengths and widths of 15 mature conidia and 5 conidiogenous cells were recorded for each block, five blocks for each colony.

DNA extraction

All isolates were classified into five morphotypes based on colony morphology, growth rate, texture and color before being subjected to DNA extraction. In each morphotype four to six isolates were chosen for total DNA extraction. DNA was extracted by the CTAB method (Guo et al., 2000), with slight modification. Approximately 50 mg mycelium and 0.2 g sterile quartz sand were transferred into a 1.5 ml Eppendorf microcentrifuge tube with 700 µl of pre-warmed (60°C) extraction buffers, i.e. 2 × CTAB buffer (2% CTAB, 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, 1% β-mercaptoethanol, 1% PVP-30, pH 8.0).

Then a sharply pointed glass stick was used to grind mycelium for 5–10 min until a homogenous fungal suspension was formed. Next the tube was incubated in a 60°C water bath for 60 min with occasional gentle swirling. Then 500 µl of phenol:chloroform (1:1) was added to each tube and mixed thoroughly to form an emulsion. The mixture was spun at 13,000 rpm for 15 min at room temperature and the aqueous phase (ca. 500–600 µl) was then transferred into a new 1.5 ml tube. This aqueous solution containing the total DNA of fungi was re-extracted with equal volumes of chloroform: isoamyl alcohol (24:1) until no interface was visible. 50 µl of 5 M KOAc (10% of the total aqueous volume) was added followed by 400 µl of isopropanol, and the tube was inverted gently to mix and stored overnight at –20°C. The total DNA was then precipitated at 10,000 rpm for 60 min at 4°C. The DNA pellet was washed with 70% ethanol twice and dried. The DNA pellet was then mixed with 200 µl TE buffer and RNAase (1:100 in volume) and incubated in a 30°C water bath for 60 min. Next, the same volume of phenol:

chloroform: isopropanol (25:24:1) was added and mixed. After 10 min, the total DNA was precipitated at 8,000 rpm for 10 min at 4°C. 5 M KOAc (10% of the total aqueous volume) was added followed by ethanol and stored at –20°C for 30 min. The total DNA was precipitated at 1,000 rpm for 10 min at 4°C. The DNA pellet was washed with 70% ethanol twice and dried. The pellet was finally re-suspended in 100 µl TE buffer and stored at –20°C for subsequent PCR amplification.

rDNA-ITS sequence amplification and analysis

PCR amplifications were performed with two primers described by White et al. (1990). They were ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). PCR reactions were carried out in 50 µl reaction volume containing: 10 mM PCR buffer, 1.5 mM $MgCl_2$, 200 µM each dNTP, 150 nM primers, 100 ng template DNA, and 2.5 U of *Taq* DNA polymerase. The thermal cycling was programmed for one cycle of 3 min at 95°C followed by 35 cycles of 40 s at 95°C, 50 s at 52°C, and finished by one cycle of 10 min at 72°C. Amplified products were resolved by electrophoresis in 1% agarose gel and visualized after staining with ethidium bromide.

The target band of the correct size (about 600 bp) was excised from a 1% agarose gel and purified with a DNA Purification Kit. Then the products were ligated into *p*UCm-T vectors and cloned in DH5α competent cells using the manufacturer's protocols (Shanghai Sangon Biological Engineering Technology & Services Co. Ltd., China). Transformed colonies were selected with LB growth media including ampicillin and then agitated. Plasmid DNA was extracted and excised with enzyme Pst I. The products were further detected using 1% agarose gel. The target colonies were cultured overnight in liquid media and then sequenced by Biochip Co. Ltd., China.

Sequences were deposited in GenBank (Table 2). Sequences obtained were initially blasted in GenBank to predict the family and/or order for each isolate. For closer phylogenetic placement, additional exemplar sequences were included from *Epichloë*/*Neotyphodium* genera (Table 2) to which blast analyses indicated the majority of endophytes had taxonomic affinities. Sequences were aligned using Clustal X1.83 (Chenna et al., 2003). The assembled data were analyzed with maximum parsimony. Maximum parsimony analyses were conducted in PAUP 4.0b10 (Swofford, 1998). Bootstrap support values were estimated by 1000 replications using heuristic searches.

Statistical analyses

All data were analyzed with SPSS10.0 software package. One-way ANOVA was performed for each of the traits. Euclidean distance coefficients were estimated for morphotype distribution of each pair of populations. The resulting Euclidean distance matrix was used for cluster analysis.

3. Results

Morphological characteristics and growth rates of different endophytic isolates

Most colonies on PDA were cottony, white to gray, many with elevated central areas. Based on growth rates as well as morphological characteristics, all isolates were separated into five morphotypes, i.e. morphotype A, B, C, D and E (Table 3, Fig. 2). Morphotype A, B and C were widely distributed in almost all populations tested. Morphotype A grew extremely slowly on PDA media and with no discernible aerial mycelium. Morphotype B was similar in growth rate to morphotype C, but the colonies of morphotype C had yellowish central areas and produced chlamydospores. Morphotype D grew faster than morphotype A, B and C, and conidia did not appear even after two types of inducement. Morphotype E grew fastest of all morphological types, up to 10.6 mm/d. The conidia of this morphotype were binate instead of solitary. Morphotype D and E were found in POP2 and POP3. Both of these populations contained tillers with choke symptoms.

Endophyte identification and phylogenetic analysis

In order to examine how well species were determined based on morphotypes, we undertook rDNA-ITS sequence analysis to morphotype A, B and C. The sequence of all isolates tested had high similarity, and there were only five informative sites altogether. Maximum parsimony tree showed that the three morphotypes and *Neotyphodium chisosum*, isolated from *A. sibiricum* and from *Stipa eminens*, formed a cluster with a 99% bootstrap support (Fig. 3). The sequence of all morphotype A isolates tested was almost the same, except A1-2 with only one base difference. They clustered together with a 91% bootstrap support. B2-2 had the same sequence as *N. chisosum* (201) from *S. eminens* and had a one base difference compared to *N. chisosum* (203) from *A. sibiricum*. B2-1 and B4-2 had a one base difference compared to *N. chisosum* (201). B4-1 had two bases different from *N. chisosum* (201). Morphotype B and *N. chisosum* formed a cluster with a 63% bootstrap support. C1 had the same sequence with innominated *Neotyphodium* sp. ATCC. C3-1 and C3-2 clustered together with a 68% bootstrap support.

Relationship between morphotype diversity and geographic environment

Different morphotypes occurred in the same

Table 3. Morphological characters and growth rates of different endophytic colonies isolated from *Achnatherum sibiricum* in Inner Mongolia.

Morpho-type	Population	Colony	Growth rate (mm/d)	Conidia			Conidiogenous cell			Conidial insertion
				Shape	Length (μm)	Width (μm)	Length (μm)	Top width (μm)	Base width (μm)	
A	POP1 POP2 POP3 POP4 POP5 POP6 POP7	White or off-white, cottony, pulvinate, aerial mycelium absent	0.5±0.1	Ellipsoid	3.8±0.5	1.9±0.1	15.5±2.8	1.1±0.1	2.4±0.3	Conidia solitary
B	POP1 POP2 POP3 POP4 POP5 POP6 POP7	White to off-white, cottony to felty, mycelium discernible	1.4±0.2	Ellipsoid to obovate	4.3±0.4	2.0±0.2	13.4±1.1	1.1±0.1	1.4±0.1	Conidia solitary
C	POP1 POP3 POP4 POP5	White to milky, cottony, centre yellowish	1.4±0.2	Ellipsoid to obovate	3.3±0.3	1.8±0.1	15.7±3.4	0.7±0.1	1.5±0.1	Conidia solitary, with chlamydospores
D	POP1 POP2	Off-white, cottony to felty, centre raised	5.7±0.2							
E	POP3	White to yellowish, cottony, aerial mycelium abundant	10.6±0.1	Lunate to reniform	4.6±0.2	1.9±0.1	–	–	–	Conidia binate

Data are presented as mean values ± SD. "–" Data was not recorded.

A. sibiricum population. Morphotype diversity tended to decrease from the northeast to the southwest (Fig. 4). In POP1 and POP3, there were four different morphotypes; in POP4 and POP5 there were three morphotypes while in POP6 and POP7, there were only two morphotypes. Morphotypes A and B made up 59–100% in all seven populations. Morphotype C was less abundant, and it accounted for 10–41% in POP1, POP3, POP4 and POP5. Morphotype D was occasionally found in POP1 and POP2 and morphotype E only appeared in POP3. The herbivore enclosure did not have any influence on diversity of endophyte morphotypes. Both morphotype diversity and the ratio of different morphotypes were completely identical within and outside of the herbivore enclosure.

In order to understand the relationship between morphotype diversity and geographic environment, cluster analysis of seven populations of *A. sibiricum* was conducted based on percentages of different morphotypes.

The result showed that morphotype diversity was related to geographic location to some degree (Fig. 5). For example, POP6 and POP7, and POP4 and POP5 clustered together most closely, which corresponded to their geographic distance. However, endophytic morphology did not completely correlate with the geographic distance. For example, POP5 was close to POP6 and POP7 in geographic location, but not in morphotype similarity.

4. Discussion

Morphological characteristics and identification of endophytic fungi

The appropriate criteria to use for the definition of species among the endophytic clavicipitaceous fungi remain an unsettled question. Especially for *Neotyphodium*

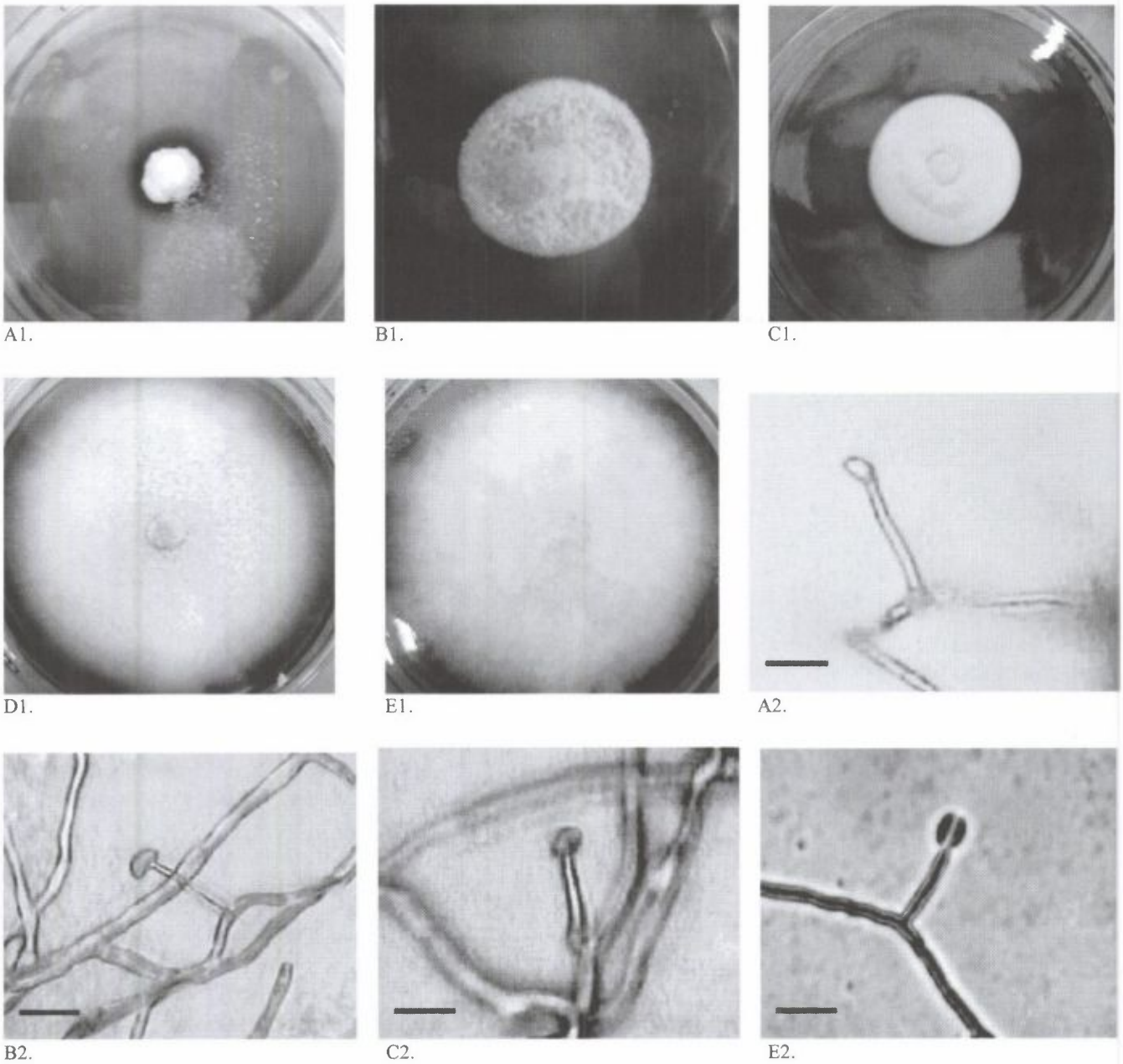

Figure 2. Colonies and conidial morphologies of different endophytic morphotypes isolated from *Achnatherum sibirium*. Capital letters denote morphotypes. 1 and 2 represent colony and conidial morphologies, respectively. Cultures were grown on PDA and were 28 d old for A, B and C, 14 d old for D and 7 d old for E. Scale bar in conidia pictures is 10 μm.

endophytes, identification and classification were previously based primarily on morphological characteristics due to lack of an obvious sexual stage (Morgan-Jones and Gams, 1982; White and Morgan Jones, 1987a,b). To reduce reliance on morphological characteristics, endophyte taxonomists later introduced isozyme analysis (Leuchtmann and Clay, 1990), randomly amplified polymorphic DNA sequence analysis (White and Huff, 1996), ribosomal DNA sequence analysis (Reddy et al., 1998), and sequence analysis of intron regions of the genes encoding β-tubulin (*tub2*), translation elongation factor 1-α (*tef1*), and actin (*act1*) (Moon et al., 2002; Schardl et al., 1997; Spatafora et al., 2007).

Arnold et al. (2001) used a morphospecies approach to characterize diversity and spatial heterogeneity in endophytic fungi from broadleaf forest trees at a tropical

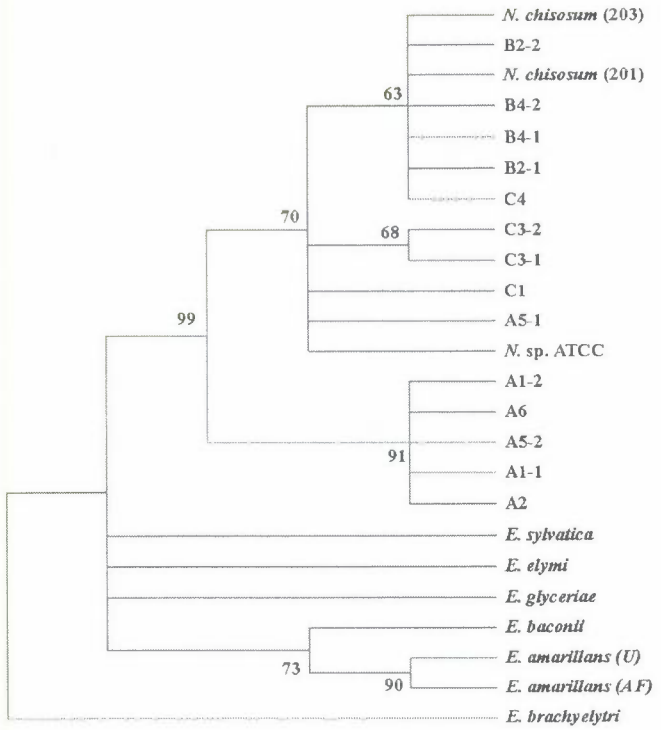

Figure 3. Maximum parsimony tree based on ITS and 5.8 S sequences, representing phylogenetic relationships of different morphotypes isolated from *Achnatherum sibiricum* and additional isolates. Numbers associated with branches are bootstrap support percentages (≥50%) assessed with 1000 replications. A, B and C denote endophytic morphotype, and the numbers followed represent population code - isolate number.

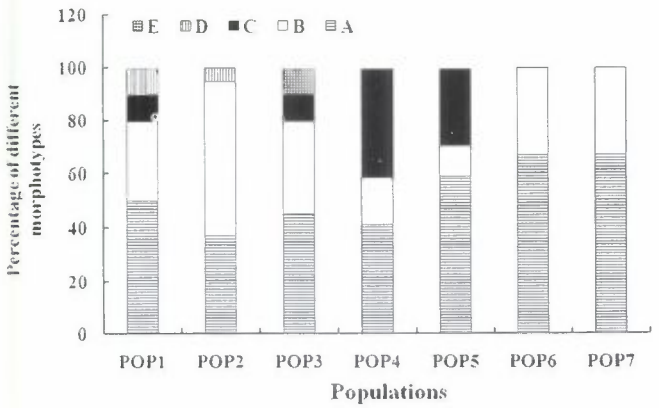

Figure 4. Ratio of different morphotypes of clavicipitaceous endophytes in seven populations of *Achnatherum sibiricum* in Inner Mongolia.

site. The use of morphotypes to group *Neotyphodium* isolates from a single host plant species was also used by Schulthess and Faeth (1998).

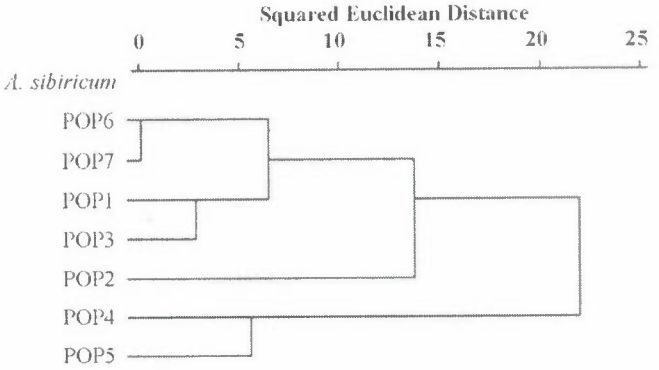

Figure 5. Dendrogram based on Euclidean distances among morphotype percentages of endophytes in seven different populations of *Achnatherum sibiricum* in Inner Mongolia.

In this study, five morphotypes of clavicipitaceous endophytes were achieved based on growth rates as well as morphological characteristics, i.e. morphotype A, B, C, D and E.

Morphotype A, B and C were similar to *N. chisosum* in color and texture, but were not identical. The growth rate of *N. chisosum* on PDA is 0.28 mm/d at 20°C. Conidia are ellipsoid to obovate, 5–9 μm long and 2.5–4 μm wide. Conidiogenous cells are 10–30 μm long, 2–5 μm wide at the base, and gradually tapering to 1–2 μm wide at the apex (White and Morgan Jones, 1987a). Morphotype A grew basically as slowly as *N. chisosum* but had smaller conidia. Morphotype B and C grew faster than *N. chisosum*. Furthermore, chlamydospores appeared in morphotype C but not in *N. chisosum*. Maximum parsimony tree showed that the three morphotypes had high similarity. Together with *N. chisosum* they formed a cluster with a 99% bootstrap support. Based on both morphological and molecular data, we ascribed morphotype A, B and C to *N. chisosum*. Certainly, there exist some morphological differences within the three morphotypes, but minor morphological differences may not be sufficient reason to create new species (Cabral et al., 1999). Also, Christensen et al. (1991) suggested that the species category might have been defined too narrowly and that many variants should be recognized as the same species even though possessing differences in growth rate, colony appearance, and secondary compound production.

Both morphotype D and E grew fast on PDA media, but they were completely different in conidia production. Morphotype D produced no conidia even after inducement. Morphotype E formed large numbers of conidia *in vitro*. Regretfully, we did not achieve ITS sequence of the two morphotypes successfully. So we can only make some inference based on our morphological data. The morphological characteristics of morphotype E, including

colony texture and conidia, were almost the same as for *Epichloë amarillans*, except that morphotype E grew faster. According to Kaiser et al. (1996), growth rate may have limited applicability in distinguishing taxa, since variability among isolates of the same species is considerable. However, conidia shape and size, especially for *Epichloë* species, are relatively constant. *E. amarillans* was first identified in *Agrostis hiemalis* by White (1994). In this type of infection, termed a type II association, the endophyte shows both seed transmission and stroma formation, often on different culms of the same plants. The same phenomenon was observed during our field experiments although we did not get stromata isolates successfully because occurrence of stroma was infrequent. Also, White et al. (2001) identified *E. amarillans* in *A. sibiricum* in Inner Mongolia, China, based on ITS sequence analysis. Consequently, we supposed that morphotype E might be *E. amarillans*.

Morphotype diversity and its relationship with geographical environment

It is supposed that the fungal endophyte communities of perennial grasses may be as diverse as fungal endophyte communities of woody shrubs and trees (Schulthess and Faeth, 1998). In the present study, we did find high morphological diversity of *Neotyphodium/Epichloë* fungi in *A. sibiricum* accessions based on both macroscopic and microscopic observations. But species diversity did not seem to be high based on rDNA-ITS sequence analysis. Low level of genotypic variation in contrast to the distinctive cultural variants was also observed in *Neotyphodium aotearoae* (Moon et al., 2002), a foliar endophyte in spruce (Stefani and Bérubé, 2006) and in the root endophyte *Phialocephala fortinii* (Addy et al., 2000).

For interaction of grass-endophyte symbionts, not only endophyte species but also endophyte strain/morphotype can play a decisive role (Müller and Krauss, 2005; Saikkonen et al., 1998). For example, Bultman et al. (2004) found that the profile of alkaloids produced by endophytes differed between fungal strains, and such variation was crucial for the performance of herbivores. The cultural morphotypes may reflect physiological and genetic differences which are not detected with analyses used thus far (Addy et al., 2000). In the present study, morphotype A, B and C belonged to the same species, but the same morphotype tended to cluster together, which indicated that different morphotypes might represent different genotypes. Clarification of the possible interaction in the endophyte-*A. sibiricum* symbiosis might be gained by examining the morphotypes obtained in this study in terms of effect on the grass host through inoculation studies.

Morphotype diversity tended to decrease from the northeast to the southwest in the studied *A. sibiricum* populations, but there existed the same dominant type in all populations, i.e. morphotype A. However, only seven *A. sibiricum* populations were studied, two of them from the same geographical site. Based on *rrn* sequence polymorphisms, An et al. (1992) also documented that the dominant type occurred throughout the survey area. Faeth et al. (2006) studied spatial variation in alkaloid production in *A. robustum*. According to their report, alkaloid production varied considerably across the geographical range but did not seem to be closely related to geographical location. Such a similarity may indicate very recent geographic distribution of infected grasses, or possibly horizontal transmission of isolates.

Acknowledgments

This research was funded by National Key Basic Research Special Foundation (No. 2007CB106802), National Natural Science Foundation (30370239) and Tianjin Natural Science Foundation (No. BE020071) in China. We thank Dr. Keith Clay at Indiana University for helpful comments on the manuscript.

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