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High prevalence of horizontal transmission of the fungal endophyte *Epichloë sylvatica*

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Summary

1 *Epichloë sylvatica* (Ascomycota, Clavicipitaceae) is a host-specific endophyte of the woodland grass *Brachypodium sylvaticum* with two alternative modes of reproduction. The predominant asexual strains are seed transmitted (vertical), whereas the sexual strains are capable of contagious transmission (horizontal), mediated by ascospores produced on stromata. However, transmission rate and mechanisms of infections are largely unknown in this system.

2 A transplant experiment using uninfected plants was designed to estimate the rate of horizontal transmission. At two sites where sexual stromata of *E. sylvatica* were present, 34% and 17% respectively, of the transplants became infected after two years, whereas at two other sites with only asexual strains all transplants remained uninfected. This suggests that contagious spread of the endophyte to established plants can occur frequently and that ascospores probably serve as inoculum.

3 Phenological observations were made in four natural populations to explore the possibility of infections via stigmata of florets. There was clear temporal coincidence of host flowering and availability of ascospores, indicating that infection through stigmata would be possible as mechanism for horizontal transmission.

4 However, in the transplant experiment, horizontal transmission via stigmata can be ruled out. Hence, stem or leaf tissues might provide an alternative route of entry, which have lead to new infections.

Keywords: *Brachypodium sylvaticum*, contagious spread, flowering phenology, grass endophyte, infection mechanisms, transplant experiment

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Introduction

Fungal endophytes represent a diverse group of unrelated, filamentous fungi. They occur in most plants and often stay within host tissues without being visible on the surface of the plant during their whole life cycle (Petrini *et al.* 1992).

The genus *Epichloë* (Ascomycotina, Clavicipitaceae) and its asexual descendants of the form genus *Neotyphodium* are endophytes of many grasses (subfamily Pooideae) (Leuchtmann 1992; Schardl 1996). The systemic and perennial interactions of these fungal endo-

phytes with host grasses are often mutualistic. An important factor affecting the nature of the symbiosis is the mode of reproduction of the endophyte. Obligatory sexual *Epichloë* species are more antagonistic, whereas asexual *Neotyphodium* endophytes tend to be highly beneficial to their hosts (Clay 1990a).

Sexually reproducing endophytes egress from their hosts during elongation of the culm and form fruiting structures (stromata) around aborted inflorescences of the grass ("choke disease") on which spermatia and ascospores are produced (Leuchtmann & Clay 1997). *Epichloë* species are heterothallic and therefore require cross-fertilization for sexual reproduction (White & Bultman 1987). Vectors for transferring spermatia are highly specialized flies of the genus *Botanophila* (Anthomyiidae) (Kohlmeyer & Kohlmeyer 1974; Bultman *et al.* 1995). Ascospores are believed to be propagules for contagious spread (horizontal transmission), but all experiments to demonstrate this role of the ascospores failed until recently (Diehl 1950; Bacon & Hinton 1991; Bucheli & Leuchtmann 1996). Chung & Schardl (1997) documented for the first time horizontal transmission of *Epichloë typhina* (Pers.: Fr.) Tul. via florets to the developing seeds in very low frequency.

Neotyphodium endophytes have no sexual stage and spend their entire life cycle within host tissues. Transmission is possible only through clonal growth of mycelium into host ovules and seeds (vertical transmission) (Sampson 1933; Philipson & Christey 1986; White *et al.* 1991). The relationship of these associations is considered mutualistic since inflorescences are not detrimentally affected and the host may benefit from infection in several ways. Endophytes produce antiherbivore metabolites in infected plants (Bush *et al.* 1997), enhance growth (Clay 1990b) and seed

yield (Rice *et al.* 1990), and confer resistance against drought stress (Malinowski *et al.* 1997) and different pests (Schuster *et al.* 1995). Because of these benefits and presumably insignificant costs inflicted on the host, the strictly asymptomatic associations have been considered clearly mutualistic (Clay 1988). However, a likely disadvantage for asexual endophytes is their clonal growth (Schardl *et al.* 1991), lacking the chance of reconstitution by recombination in a sexual cycle.

From an evolutionary point of view, associations with a mixed strategy of reproduction of the endophyte have probably the most benefits in the long term. One such association is formed by *Brachypodium sylvaticum* (Huds.) P. B. and its specific endophyte *Epichloë sylvatica* Leuchtmann & Schardl (Leuchtmann & Schardl 1998). This association is characterized by very high infection rates with virtually all plants being infected in natural populations (Bucheli & Leuchtmann 1996; Meijer & Leuchtmann 1999). In the majority of plants the association is asymptomatic and the endophyte is seed-transmitted, while few populations include stroma-bearing plants occurring as small clusters within the population. To maintain the observed high prevalence of infected plants, horizontal transmission by contagious spread of ascospores is assumed to be necessary (Bucheli & Leuchtmann 1996; Ravel *et al.* 1997). Moreover, isozyme analysis of *E. sylvatica* isolates revealed that multistrain infections in *B. sylvaticum* are common (Meijer & Leuchtmann 1999), which are most likely the result of contagious spread. The close relationship of *Epichloë* to the genus *Claviceps* suggests, that a homologous floret infection (Tudzynski *et al.* 1995) of the host is the most likely mechanism of new infections. However, other routes of infection such as wounds in vegetative tissues are also conceivable.

In this study, endophyte-free plants of *B. sylvaticum* were transplanted to natural populations of infected grasses and monitored over two years to demonstrate horizontal transmission. Furthermore, we investigated whether or not flowering of the grass and mature stromata with ejecting ascospores coincide in nature, which would be a precondition for horizontal transmission via the florets of the host grass.

Materials and methods

Brachypodium sylvaticum (Huds.) P. B. is a perennial, clump-forming grass without stolones of the tribe Triticeae. The distribution of the species includes temperate Eurasia, but it has also been introduced as ornamental grass elsewhere. It is a typical element of the central European beech forests and very common in Switzerland, especially on rather basic and moist soils. The grass is most abundant on clearings and along forest roads (Ellenberg 1996).

SOURCE OF SEEDS

Seeds of *B. sylvaticum* were collected in autumn 1996 from one-year old plants cultivated at the Botanical Garden, Zürich. All plants originated from natural sites in northern Switzerland, near Zürich. Two locations were in the Sihlwald area, "Risleten" (with seed lines S_1 and S_5) and "Langrain" (with seed line LR_1), and one close to Zürich, "Albisgüetli" (with seed line AG_5). Plants were treated with the fungicide Benlate® (DuPont) to obtain endophyte-free seed lines.

HEAT TREATMENT AND GERMINATION

To certify that no viable endophyte remained in the seed, all seeds were subjected to heat treatment. Seeds were surface-sterilized (Leuchtmann & Clay 1990) and incubated at

37 °C in the dark at 100% humidity for three weeks (Nott & Latch 1993). To break dormancy, heat-treated seeds were incubated for another three weeks in petri dishes at 3 °C. For germination, the dishes were transferred to an incubator with continuous light at 22 °C.

TRANSPLANTING OF ENDOPHYTE-FREE SEEDLINGS INTO NATURAL POPULATIONS

Seedlings derived from endophyte-free seed lines (S_1 , S_5 , LR_1 and AG_5) were grown on water agar for three weeks under sterile conditions. Then seedlings were routinely checked under the light microscope for out-growing hyphae at a magnification of 400x. Previous research indicated that this method is reliable to eventually detect infected seeds which would have been removed (Leuchtmann & Clay 1993; Meijer & Leuchtmann 1999). Prior to transplanting, plants were kept for another two weeks in seedling trays at a shady place and then put in pots (5 x 5 x 7 cm³) made of pressed peat.

Plants from each seed line were transplanted to four natural populations of *B. sylvaticum* in beech forests around Zürich. They were arranged in a line with equal distances between plants within natural stands of *B. sylvaticum*. Two of the selected populations had stroma-forming plants present in high frequency (Albisgüetli and Risleten), whereas in two other populations no stromata were observed ("Galgenbüel", Zollikerberg; "Isleren", Zumikon). Transplants were labelled and connected with a tagging twine to facilitate locating of the plants later. Their position was mapped exactly and the plants were revisited every one to three weeks. Three and 15 months after transplanting, leaves of two tillers including leaf sheaths were taken from each plant and checked for infection following standard techniques (Clark *et al.* 1983).

Table 1. Proportion of infected plants, originating from four seed-lines (S_1 , S_5 , LR_1 , AG_5), transplanted into four different populations of *B. sylvaticum* with or without stromata present, observed in October 1997 and 1998

Population	Year	Proportion of infected plants				
		S_1	S_5	LR_1	AG_5	Total
Without stromata						
Galgenbüel	1997	0/13	0/14	0/14	0/3	0/44
	1998	0/6	0/5	0/5	0/2	0/18
Isleren	1997	0/12	0/15	0/13	0/4	0/44
	1998	0/2	0/4	0/3	0/1	0/10
With stromata						
Risleten	1997	0/15	0/14	0/13	0/1	0/43
	1998	1/10	1/8	2/10	1/1	5/29
Denzlerweg	1997	1/15	2/14	0/15	0/1	3/45
	1998	7/13	3/13	3/11	0/1	13/38

PHENOLOGICAL OBSERVATIONS WITH TRANSECT METHOD

Populations selected for this part of the study exhibited a high incidence of stroma-forming plants; two were located in the Sihlwald area (Risleten and "Eichbach") and two near Zürich (Albisgüetli and "Denzlerweg"). A string of 10 m length with a knot every 40 cm was used as flexible device. Transects were placed randomly within the populations of *B. sylvaticum*, and for the nearest plant to each knot we counted the number of tillers and classified their developmental stage. Classes used were: (1) no tiller with inflorescence visible, (2) tiller with inflorescence visible but not flowering, (3) tiller with flowering inflorescence (stigmata visible), and (4) tiller after flowering. Stromata-bearing tillers were classified into three developmental stages: (1) immature stromata, (2) mature stromata (with released ascospores visible as transparent filaments at the perithecial ostiolum), and (3) stromata with empty perithecia. At each date of phenological observation, one transect consisting of 26 plants per population was ex-

amined. Recording was done weekly during the vegetation period when tillers were emerging (June–September).

Results

HORIZONTAL TRANSMISSION

The survival rate of the endophyte-free transplants was remarkably high. At the end of the first year 96 % out of 183 transplants survived; at the end of the second year, 52 % were still alive (Table 1). However, size of the surviving plants varied widely within the populations (1–20 tillers per plant) and among the populations (mean number of tillers per plant \pm SE: Denzlerweg 6.1 ± 2.9 ; Galgenbüel 2.9 ± 1.7 ; Risleten 2.5 ± 1.7 ; Isleren 2.3 ± 1.4). Three months after transplanting, three of the transplants were infected by *E. sylvatica* based on the routine check of two tillers per plant, while the remaining plants were uninfected (Table 1). The three infected plants were derived from seeds of two different seed lines and were found at Denzlerweg, a population where stroma-forming plants were present.

No new infections were evident at the other populations in 1997. At the end of the second year (1998), the number of infected plants increased to 13 (34%) at Denzlerweg and to 5 (17%) at Risleten, another stromata-forming population. Infected plants were distributed among all four seed lines used in the study, thus the origin of the seed line did not seem to have an effect on successful infections (Table 1). The three infected plants found in 1997 at Denzlerweg were still infected in 1998. Transplants at Galgenbüel and Isleren, where no stromata-forming plants were present, remained uninfected. In all cases, both tissue samples taken per plant for checking infection were consistently either infected or endophyte-free.

COINCIDENCE OF FLOWERING AND ASCOSPORE RELEASE

Phenological observations of flowering time of *B. sylvaticum* and ascospore release of mature stromata of *E. sylvatica* are shown in Figs. 1 and 2. The number of mature stromata with ejecting ascospores were at (or near) its peak, when the largest percentage of inflorescences had open stigmata in most populations. In 1996, two of the three populations (Eichbach and Risleten) showed an almost perfect coincidence, whereas at Denzlerweg fungal stromata formation was somewhat behind host flowering (Fig. 1). In 1997 all populations showed coincidence except for the additional population Albisgüetli (Fig. 2). However, in all populations and both years there was still considerable overlap of host grass flowering and presence of mature stromata.

The density of stromata forming tillers differed significantly among the four populations (Pearson chi-square test: $\chi^2 = 49.0$, $P < 0.001$). At the time when the largest percentage of inflorescences was at the flowering stage, 2.3 tillers per plant were choked at Denzlerweg,

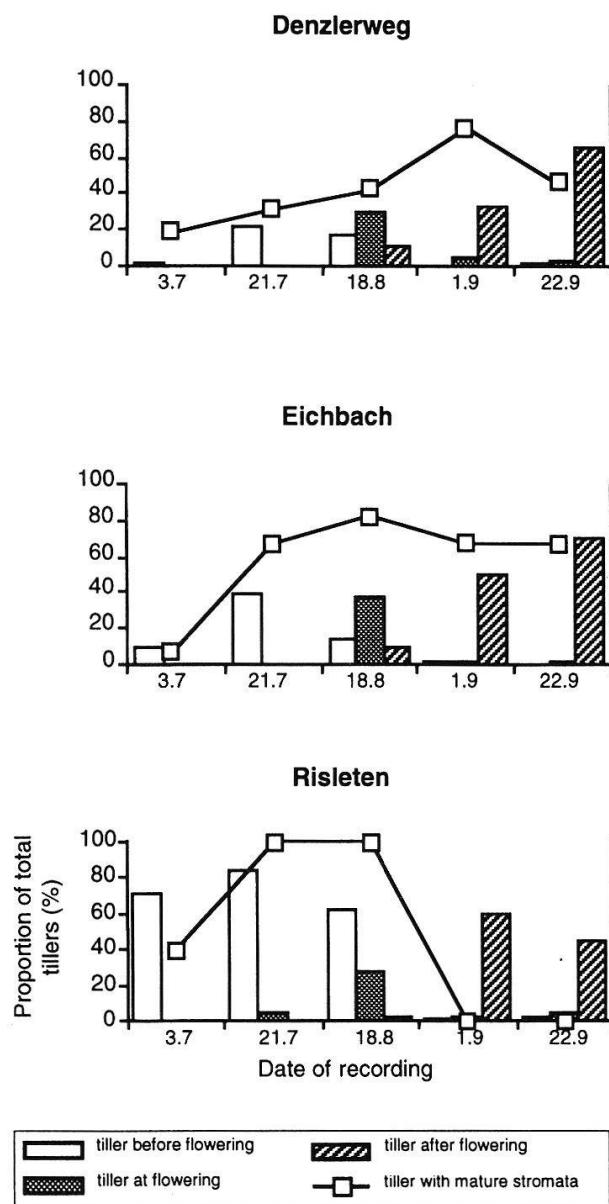


Fig. 1. Coincidence of flowering time of *B. sylvaticum* and ascospore release of *E. sylvatica* in 1996 in four different populations. Bars indicate the percentages of the total number of flowering tillers recorded at each date with different developmental stages of the inflorescences. Line (with open squares) indicate tillers with mature stromata (ejecting ascospores).

compared to only 0.2 at Eichbach (Table 2). The other two populations showed intermediate levels of choking. The number of new infections was positively correlated with the amount of stromata observed within each population.

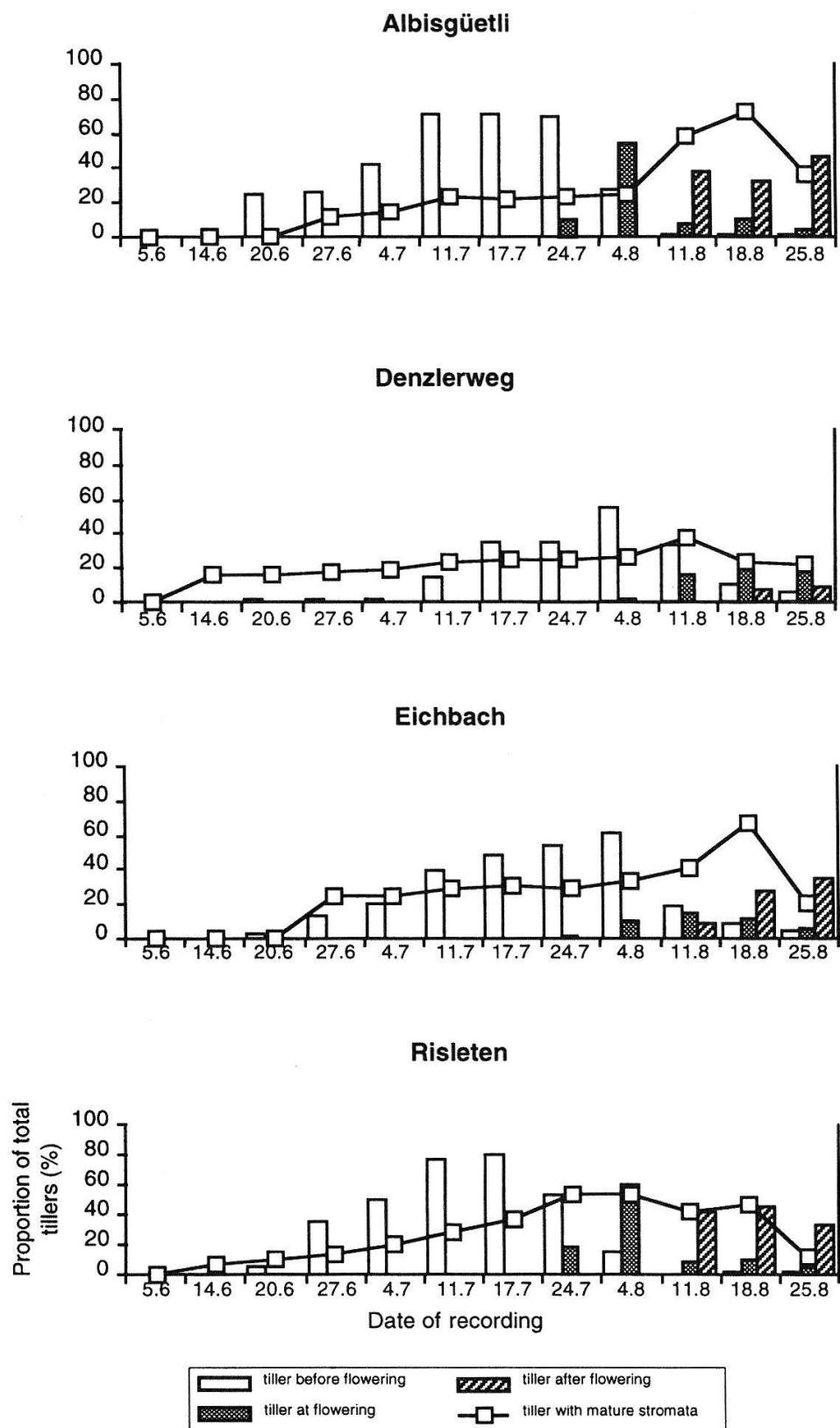


Fig. 2. Coincidence of flowering time of *B. sylvaticum* and ascospore release of *E. sylvatica* in 1997 in four populations. Bars indicate the percentages of the total number of flowering tillers recorded at each date with different developmental stages of the inflorescences. Line (with open squares) indicate tillers with mature stromata (ejecting ascospores).

Table 2. Numbers and proportions of stromata bearing tillers at the time when most inflorescences were flowering. Each record (transect) consisted of 26 examined plants. Number of stromata differs significantly among populations (Pearson chi-square: $\chi^2 = 49.0$, $P < 0.001$)

Population	Total tillers per plant	Stromata per plant	Choked tillers in populations (%)	Date of recording 1997
Risleten	30.5 ± 8.3	1.5	5	04.08
Denzlerweg	14.6 ± 5.2	2.3	16	18.08
Eichbach	10.7 ± 3.0	0.2	2	11.08
Albisgüetli	34.2 ± 10.5	2.3	7	04.08

Discussion

HORIZONTAL TRANSMISSION

Contagious spread of *E. sylvatica* appears to be more frequent in nature than previously assumed. During the first three months of our experiment, three of the endophyte-free transplants became newly infected by *E. sylvatica*. One year later, 15 additional plants were infected, resulting in an infection rate of 34% at one site and 17% at another site of the surviving transplants. This is probably a conservative estimate, since infection status of plants was determined in two tillers per plant only. Individual tillers of additional transplants could have been infected which were not scored. However, the majority of plants had no more than two tillers in the second season. This strongly suggests that *E. sylvatica* can spread contagiously under natural conditions as hypothesized by Bucheli & Leuchtmann (1996) and Meijer & Leuchtmann (1999). Newly infected plants were restricted to populations with stromata-forming plants, whereas in asymptomatic populations all transplants remained uninfected. Therefore, ascospores produced on the stromata were most likely the propagules for horizontal transmission and mediated infection of new host plants.

Successful horizontal transmission is dependent on several parameters influencing dispersal and viability of ascospores. Ascospore ejection requires a relative high humidity of the air, sufficient water supply of the host plant, and occurs predominantly during the night (Raynal 1991; Welch & Bultman 1993). Since filamentous ascospores are wind-dispersed, the highest inoculum density is near the source of ejection and should decrease exponentially with distance from the source (Webster 1980). Different environmental conditions could explain the different transmission rates found in our study. The population at Denzlerweg showed the highest rate of new infections, probably because the microclimate in the very dense plant population was relatively humid. In contrast, population Risleten with lower rate of infection was more scattered and appeared to be less humid. Furthermore, the number of stromata and therefore the potential inoculum was much higher at Denzlerweg compared to Risleten. In addition, transplants produced more tillers at Denzlerweg, providing more leaf surface area for potential infection.

Newly infected plants occurred in all four seed lines (Table 1). This may indicate that there is no local adaptation in susceptibility of host plants to certain fungal genotypes.

ROUTE OF INFECTION

The coincidence of flowering time of the grass and ascospore release indicates that infection through stigmata with subsequent seed transmission is a possible route of new infections. Infection of ovaries mediated by germinating ascospores or conidia on the stigmata is the only mechanism of infection in the related genus *Claviceps* and has also been observed under controlled conditions in *E. typhina* (Chung & Schardl 1997). These authors tested seed progeny of uninfected plants, which have been surrounded by plants with mature stromata at the time of flowering, and found 13 out of 1034 screened plants to be newly infected. However, it is not clear whether germinating ascospores can directly infect host tissues. Most ascospores germinate by iterative conidiation on agar media (Bacon & Hinton 1988, 1991). Therefore, conidia produced after germination may serve as inoculum. This process would parallel the infection mechanism of *Claviceps* (Tudzynski *et al.* 1995).

If the fungus would enter through the stigmata it would only infect the developing seed, but not the established mother plant. Therefore, this infection mechanism cannot explain our observations in the transplant experiment. Stem or leaf tissues must provide an alternative route of entry. Possible routes of infection are wounds (Diehl 1950; Western & Cavett 1959), stomata or the meristematic region of the host grass which could be reached by germinating ascospores or conidia. The existence of these alternative routes of infection are consistent with the high percentage of multistain infections of single plants documented recently for *B. sylvaticum* (Meijer & Leuchtmann 1999).

Vertical transmission of endophytes into seeds may not always be 100% successful and loss of endophyte viability is possible during

resting of the seeds in the soil before germination (Ravel *et al.* 1997; Meijer & Leuchtmann 1999). This would lead to uninfected seedlings and plants which should be even more competitive according to recent experiments (D. Brem, unpubl. data). However, virtually no uninfected plants of *B. sylvaticum* can be found in natural populations in Switzerland. Thus, a high rate of contagious transmission is necessary to maintain the high level of infection. In asexual populations where no stromata are produced, vegetatively formed conidia on the host surface could serve as inoculum (White *et al.* 1996; Meijer & Leuchtmann 1999). However, transmission rate of conidia is obviously much lower, since no new infections were observed among the transplants at the two populations without stromata after two years.

The results of this study indicate, that contagious spread occurs frequently in nature and that ascospores probably serve as inoculum. Further studies are necessary to evaluate the relative importance of infection via stigmata and the suggested alternative routes of infection in the *Brachypodium* system.

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