Botanophila–Epichloë Interaction in a Wild Grass, *Puccinellia distans*, Lacks Dependence on the Fly Vector

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ABSTRACT In grass-infecting *Epichloë* (Ascomycetes: Clavicipitaceae) fungi, the transfer of spermatia for fungal fertilization depends on an insect vector: flies of the genus *Botanophila* (Diptera: Anthomyiidae). The flies use the fungal stroma, a spore-producing fungal structure surrounding the grass inflorescence, for laying eggs and as a food source for both adults and larvae. This fly–fungus interaction is generally regarded as obligatory and mutualistic. Two *Botanophila* taxa were noted among four populations of the nonagricultural grass *Puccinellia distans* (L.) Parl. that were infected with the fungus *Epichloë typhina* (Pers.) Tul. However, during the 7 yr of field observations, *Botanophila* flies were present every year in only one population of *P. distans*. The number of eggs per stroma ranged from zero to four and differed with year and site. Overall, eggs (or larvae) were observed on only 132 (19.2%) of the 687 stromata examined during the survey, with one (13.8%), two (4.5%), or more than two (0.9%) per stroma. However, 90.8% of the examined stromata were fertilized and produced perithecia, suggesting that other mechanisms or vectors of spermatia were responsible for fertilization.

KEY WORDS Anthomyiidae, *Botanophila* sp., *Epichloë* sp., fly–fungus interaction, *Puccinellia distans*

The genus *Botanophila* (Diptera: Anthomyiidae) contains ≈65 species (Michelsen 2004). Their larvae are predominantly phytophagous and feed on different parts of angiosperm plants (Hennig 1976, Komźaková and Rozkošný 2009). Some species of *Botanophila* have developed a close association with fungi of the genus *Epichloë* (Ascomycetes: Clavicipitaceae; Kohlmeyer and Kohlmeyer 1974, Bultman and Leuchtmann 2009). The *Epichloë* fungi infect grasses endophytically and form sexual fruiting structures (stromata) that enclose young inflorescences and prevent seed production (choke disease; Sampson 1933).

Because *Epichloë* fungi are heterothallic, their spermatia must be transferred between stromata of opposite mating types to sexually reproduce (Bultman et al. 1998). For the transfer of spermatia, *Epichloë* fungi depend on flies of the genus *Botanophila* (Bultman and White 1988), that are specifically attracted by fungal volatile compounds (Steinebrunner et al. 2008). During their visit to a stroma, flies feed on fungal material, including spermatia that pass through the digestive system intact (Bultman and Leuchtmann 2003); feces that are actively deposited on the subsequent stroma contain viable spermatia and are capable of cross-fertilization of the fungus. As a result, perithecia-containing asci with ascospores are formed on the stroma surface, and ejected ascospores may infect other grasses (Chung and Schardl 1997, Brem and Leuchtmann 1999).

The *Epichloë–Botanophila* interaction seems to be mutually beneficial (Parker and Bultman 1991; Bultman et al. 1995, 1998; Bultman and Leuchtmann 2009). The fungal stroma serves as a food source for the fly and is used as a place to lay eggs. As a result, the fungus becomes fertilized and can reproduce sexually. Moreover, through a specific visitation behavior *Botanophila* flies may have contributed to reproductive isolation of *Epichloë* species or host populations (Bultman et al. 2011). Until recently, *Botanophila* flies were thought to be the only vector for *Epichloë* spermatia (Bultman et al. 1995), and the interaction between the two organisms was regarded as obligatory mutualism. However, studies on cultivated hosts in Oregon indicate that the fungal sexual cycle can be completed without the participation of *Botanophila* spp. (Rao and Baumann 2004, Rao et al. 2005). A similar situation was observed in the wild grass *Dactylis glomerata* L. in Poland (Górzyńska et al. 2010).

The first record of a *Botanophila* fly associated with *Epichloë* in Poland was made on *Holcus lanatus* L. infected with *Epichloë clarkii* White (Chlebicki and Szkudlarz 2000). An intensive search for *Botanophila* flies was initiated after discovering that the fungus *Epichloë typhina* (Pers.) Tul. was commonly present in populations of the grass *Puccinellia distans* (L.) Parl. (weeping alkaligrass), a perennial Euro-Siberian halo-

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phyte found in marine and inland salines. Since the 1960s, *P. distans* has colonized artificial habitats of central Europe (Dettmar 1993, Jackowiak 1995). The sexual stage of *E. typhina* has been found only in those populations of *P. distans* that occur in habitats that have been anthropogenically altered (Lembicz 1998, Lembicz et al. 2009).

Here, we present data on the incidence of *Botanophila* spp. in the populations of *P. distans* infected with *E. typhina* that were collected during 7 yr of field observations. This report includes 1) determination of fly species connected with *E. typhina* that infects *P. distans*, 2) inventory of the *P. distans* localities in which the fly was observed over the study period, and 3) data on the incidence of fly eggs and larvae on fungal stromata for each year and locality. We also discuss and compare observed patterns with patterns found in populations of other cultivated and wild grasses infected with *Epichloë* fungi.

Materials and Methods

Site Descriptions. Observations of *Botanophila* flies were carried out between 2000 and 2009, excluding 2004, 2005, and 2007. The presence of insects was monitored in four *E. typhina*-infected populations of *P. distans*, located in central Poland in Pakość (52° 47.531′ N, 18° 06.118′ E), Węgierce (52° 45.493′ N, 18° 08.276′ E), Giebnia (52° 46.544′ N, 18° 06.190′ E) and Janikowo (52° 46.384′ N, 18° 08.032′ E). These populations showed various levels of fungal infection: 91.2% (Pakość), 89.3% (Węgierce), 81.0% (Giebnia), and 74.0% (Janikowo) (Lembicz and Olejniczak 2009). The populations were found in habitats under strong pressure from human activities. They are affected by leaking brine, salty water and emission of calcium dust and carbon dioxide—by-products of the nearby soda-producing plant. All habitats had a high level of salinity, ranging from 3.11 to 7.61 mS/cm (Lembicz and Olejniczak 2009).

P. distans plants were found in these habitats coexisting with characteristic species of natural inland salines. The habitat that was the most natural and had the highest proportion and diversity of halophytes was a meadow at the Pakość site. Other habitats included a pasture (in Węgierce) and salinated, formerly arable wastelands (Giebnia and Janikowo).

Fungus and Fly Identification. Presence of fungal intercellular mycelium was confirmed using a microscopic method (Clark et al. 1983). The epidermal tissue was mounted in a drop of aniline blue stain (1 g of aniline blue +100 ml of water + 200 ml of lactic acid), on a microscope slide and covered with a coverslip, heated over flame and examined at 100–400× under a light microscope. The presence of mycelium was monitored in 30 individuals from each population, in each year of the study. Polymerase chain reaction (PCR) using two specific fungal primers designed for the β-tubulin gene of *Epichloë* endophytes (IS-1, 5′-GGTGTTGAGCCCCCCTGATTT-3′ and IS-3, 5′-GTCTCATCTCCGGGGCGGTAT-3′; Doss et al. 1998) was used to confirm identity of the fungus. Amplifications were performed with the following parameters: 95°C for 3 min and then 35 cycles at 94°C for 15 s and 60°C for 1 min, followed by 72°C for 10 min. Sequences of the amplified DNA fragments were then compared with data from GenBank.

The identification of fly species was based on the sequence of the mitochondrial cytochrome oxidase gene (*COII*) as described in Leuchtmann (2007). Genomic DNA was isolated from fly larvae collected from the fungal stromata, the *COII* gene was amplified using primers TL2-J-3037 (5′-TAATATGGCAGATTAGTGCA-3′) and TD-N-3885 (5′-TTTAGTTTGACATACTAATGTTAT-3′) (Simon et al. 1994), and PCR products were sequenced using a BigDye Terminator cycle sequencing kit (Applied Biosystems, Austin, TX). Reference sequences are deposited in GenBank under accessions EF064346 (taxon 1) and EF064349 (*B. phrenione*).

Sampling and Measurements. All studied sites were monitored each year in June to determine the presence of fungal stromata and *Botanophila* flies. From each site, 30 tillers from different clumps with symptoms of choke disease were collected. Stromata were examined under a stereomicroscope to record 1) perithecial development and 2) the number of *Botanophila* eggs. We assumed that the number of deposited eggs corresponded to the number of visible eggs plus the number of empty brood chambers (those without eggs on them). Perithecial development observed under a stereomicroscope provided evidence for fungal cross-fertilization. In 2000, we observed on stromata two types of eggs that differed in shape and color (white and gray). After 2000–2003, the numbers of eggs were recorded separately for each type.

The frequency of eggs on fungal stromata, expressed as fractions of stromata possessing zero, one, two or more than two eggs, was calculated for each year and site. Similarly, the incidence of *Botanophila*-infested stromata that were fertilized was estimated for each year and site.

Results

Fungus and Fly Identification. Sequences of the amplified fungal DNA fragments confirmed their origin from the *E. typhina* genome (GenBank accession DQ267692). Furthermore, species identity was verified by phylogenetic analysis of sequences obtained from isolates of the sexual stage (unpublished data).

Fly sequence analysis detected two distinct *Botanophila* taxa that were associated with *E. typhina* on *P. distans*. One taxon was identified as *Botanophila phrenione* (Séguy, 1937) according to a sequence that was derived from an identified male specimen (Leuchtmann 2007). For the other taxon (designated taxon 1), no reference specimen was available despite intensive surveys and several attempts to rear male flies from larvae; for this reason, taxon 1 is not assigned to one of the several described species associated with *Epichloë*, but it clearly represents a distinct species of *Botanophila* (Leuchtmann 2007).

Table 1. Presence of *Botanophila* eggs and larvae and stroma fertilization in habitats of four *P. distans* populations

Yr	Meadow			Salinated wastelands, formerly arable						Pasture		
	Pakość			Janikowo			Giebnia			Węgierce		
	Infestation %	Larvae	Fertilization %	Infestation %	Larvae	Fertilization %	Infestation %	Larvae	Fertilization %	Infestation %	Larvae	Fertilization %
2000[a]	10.0	+	100	73.3	+	100	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
2001	3.3	−	100	0.0	−	100	0.0	−	100	0.0	−	100
2002	6.7	+	100	10.0	−	100	0.0	−	100	10.0	−	100
2003	66.7	+	100	60.0	+	100	26.7	+	100	76.7	−	100
2006	10.0	−	100	N.D.	N.D.	N.D.	13.3	−	100	N.D.	N.D.	N.D.
2008	20.0	+	100	16.7	+	40.0	3.3	−	10.0	10.0	+	80
2009	13.3	+	90.0	10.0	+	93.3	10.0	−	76.7	N.D.	N.D.	N.D.
Overall	18.5	+	98.6	28.3	+	88.9	7.2	+	81.1	24.8	+	94.9

Infestation is the fraction of stromata infested by the fly and fertilization includes fractions of fertilized stromata. The column "Larvae" shows presence of a larva, concluded from the presence of its brood chamber (+, present; −, absent; N.D., no data).

[a] In 2000, no stromata were found in two localities (N.D.); in other cases, the collection of stromata was hampered by events independent of the researchers, e.g., a mowed meadow.

Presence of *Botanophila* Flies. *Botanophila* flies were found in each of the four studied *E. typhina*-infected populations of *P. distans* (Table 1). Both insect eggs and larvae also were observed in all populations. Two types of eggs, that differed in color and shape, were observed (Fig. 1). Gray eggs are narrower, more oval and rounded at their ends. Their upper surface lacks two longitudinal folds. During 2000–2003, 85 eggs in total were observed in all examined stromata, including 56 (65.9%) white and 29 (34.1%) gray eggs. No larvae hatched from the gray eggs. During the 7 yr of the study, *Botanophila* flies were consistently present at only one site, Pakość (Table 1). However, no larvae (or brood chambers) were recorded at this site for two growing seasons (2001 and 2006). The greatest variation in number of eggs on *E. typhina* stromata was observed in Giebnia and Węgierce (Table 1). At all studied sites, fertilized stromata with perithecia were found, irrespective of the presence of *Botanophila* eggs and larvae. Most striking was 2001, when a small number of fly-infested stromata was observed only at the Pakość site, but 100% of the stromata collected at all four sites were fertilized.

Frequency of *Botanophila* Eggs on Fungal Stromata. In total, eggs were recorded on 132 (19.2%) stromata out of 687 examined. The number of eggs per stroma ranged from zero to four and differed depending on the year and site. Overall, 80.8% of all examined stromata had no eggs or brood chambers; the rest had one (13.8%), two (4.5%), or more than two (0.9%). Similar results were obtained across each year of the experiment and at different localities (Fig. 2A and B). An exception was in 2003, when the number of fly-infested stromata (57.5%) was higher than uninfested stromata (42.5%).

The percentage of fertilized stromata ranged from 10 to 100%, whereas the percentage of fly-infested stromata ranged from 0 to 76.7%, depending on the year and site (Table 1; Fig. 2). Among all collected stromata, 90.8% possessed perithecia, but only 20.8% showed signs of fly visitation.

Discussion

There are no long-term studies that show the pattern of *Botanophila* fly distribution over time. Our study that involved monitoring of four populations of *P. distans* for 7yr is the first long-term study of the *Epichloë–Botanophila* association.

All studied populations of the nonagricultural halophytic grass *P. distans* occur in artificial saline habitats. Fly eggs and larvae were found in each of these populations. However, our long-term observations

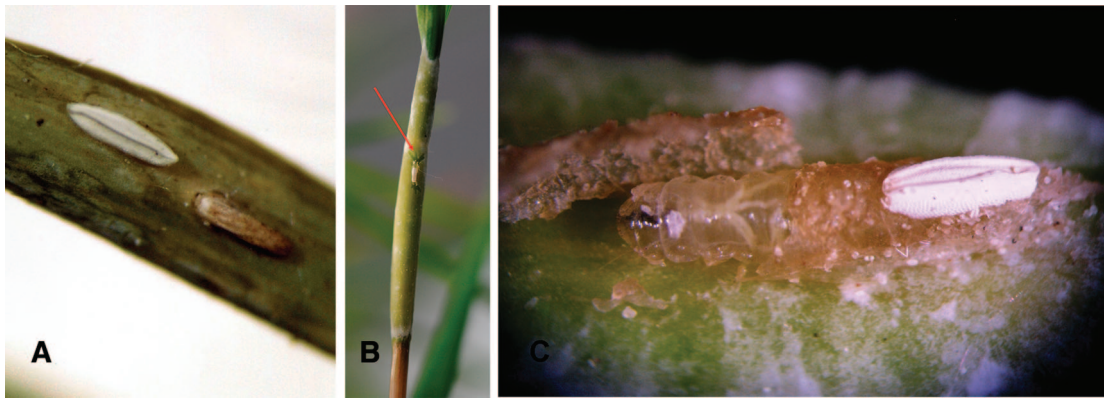

Fig. 1. *Botanophila* sp. on *E. typhina* infecting *P. distans* in Poland. (A) Two types of egg observed on the stroma surface. (B) Traces of *Botanophila* feeding on a newly formed stroma, indicated by arrow. (C) Larva of the *Botanophila* fly emerging from the brood chamber. (Online figure in color.)

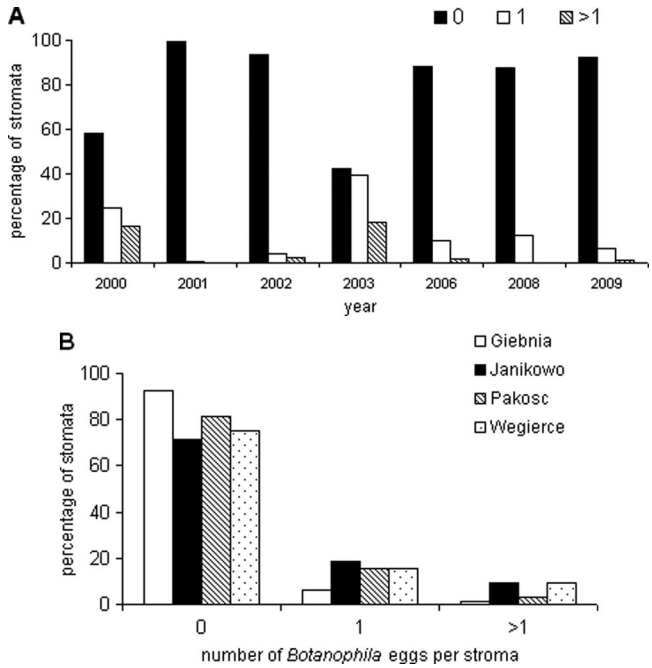

Fig. 2. Incidence of *Botanophila* eggs on *E. typhina* stromata in each year of the study (A) and in each of four populations in Poland (B).

showed that the fly may not appear at each locality each year (Table 1). The flies were consistently present throughout all 7yr of the study in only one population (Pakość), in which *E. typhina* was found as early as in 1992. This was a meadow population, characterized by the highest level of fungal infection. The proportion of clumps with choke disease was 91.2% (unpublished data).

For the majority of all analyzed stromata (80.8%), we saw no indication of *Botanophila* visitation and two or more eggs were observed only in 5.4% of them. This finding is similar to that reported by Rao et al. (2005), for cultivated *Festuca* species in the United States, where >80% of stromata were not infested with *Botanophila* flies, and only 3.6% had two or more fly larvae. However, compared with *Festuca*, fungal stromata on *P. distans* were smaller in size (mean length, 13.8 ± 3.5 mm on *P. distans* and 20.0 ± 0.2 mm on *Festuca*). Low frequencies of *Botanophila*-infested stromata also were found on cultivated orchardgrass (*D. glomerata*) in the United States, with no eggs or larvae on 61.9% and two or more eggs on 5.9% of examined stromata (Rao and Baumann 2004). Stromata on *D. glomerata* are typically much longer (reaching up to 125 mm in length) than those on *P. distans* and may carry as many as 10 *Botanophila* larvae on a single stroma, whereas a maximum of four were observed on *P. distans.* In other wild grasses, the proportion of stromata with no indication of fly visitation was much lower (33–39%) for the *Elymus* sp.–*Epichloë elymi* association in the United States (Bultman and White 1988; Parker and Bultman 1991; Bultman et al. 1995, 1998) and 24.8% for the *Dactylis glomerata*–*Epichloë typhina* association in Poland (Górzyńska et al. 2010). Our results, with 80.8% of stromata without eggs, represent a unique case, unrecorded in populations of wild grasses thus far.

At some localities and in some years (Table 1), very few or no *Botanophila* flies were observed, but all or nearly all stromata collected at these localities were still fertilized. This may suggest that fertilization is not dependent on the spermatia-carrying *Botanophila* flies and that other vectors or mechanisms may be involved. The most straightforward explanation—wind transfer—was excluded (Bultmann and White 1988). Rao and Baumann (2004) suggested that in the case of grasses raised for seed, which grow in a specific type of habitat, characterized by a very high density of plants bearing fungal stromata, fertilization through direct contact can occur. Such explanation has no foundation in the Polish populations of *D. glomerata* that grow naturally, with individuals considerably distant one from the other (Górzyńska et al. 2010). Although our research on *P. distans* was performed on a wild grass species that was naturally occurring in artificial habitats, this grass shows a different distribution pattern in comparison to wild *D. glomerata.* It grows in dense stands in the studied sites and this may diminish the role of the fly in cross-fertilization in favor of the direct contact of stromata. Alternatively, it is possible that ascospore-mediated fertilization takes place, as recently described by Alderman and Rao (2008) for *E. typhina* in Oregon. These authors have experimentally shown that ejected ascospores can serve as spermatia and fertilize stromata. If this were the case, *Botanophila* flies may account for the fertilization of early

emerging stromata, which then provides ascospores for subsequent fertilization of neighboring, egg-free stromata. Ascosporic fertilization can occur only if stromata are produced over a considerably long time period, so that ascospores are released when late-egressing stromata are present. In the studied populations, fungal stromata appear from May to June, thus, ascosporic fertilization may play an important role here.

Rao et al. (2010) reported recently that slugs (molluscs) forage on *Epichloë* stromata infecting *D. glomerata* and that spermatia are transmitted through the gut of a slug, which may result in cross fertilization of the fungus. Such an explanation of the presence of fertilized stomata without visible signs of fly visitation may be valid also for our study. We observed the grove snail (*Cepaea nemoralis* L.) on infected *P. distans* but only in one of the studied sites (Czarnoleski et al. 2010). The snails mainly forage on plant tissues but some of them were located on fungal stromata. Further studies are needed to confirm that *C. nemoralis* can be a vector of *Epichloë* spermatia.

Finally, it is possible that the lack of eggs on a stroma does not mean that a stroma was not visited by the fly. A female could simply visit stromata without laying eggs but still fertilize stromata during these visits. Spermatia occasionally cling to external body parts of *Botanophila* (Bultman and White 1988), and flies may possibly cross fertilize some stromata through this mode of transfer. Alternatively, the fly could appear on stromata only to forage. Although this explanation is highly possible, it is very difficult to confirm.

Although our results suggest the occurrence of other mechanisms of cross-fertilization in *E. typhina*-infected *P. distans*, they do not exclude *Botanophila* flies as one of the vectors. In our study, the effectiveness of *Botanophila* flies as a spermatial vector was high; 98.5% of the stromata that possessed fly eggs produced perithecia, whereas in Bultman et al. (1998) only 70.6% produced perithecia.

Both *Botanophila* egg types were found at only two sites, Janikowo and Węgierce, with gray eggs comprising 44.9% (Janikowo) and 36.4% (Węgierce) of the total eggs in each location. At the other sites, Giebnia and Pakość, only white eggs were present throughout the study. We are not certain whether both types of eggs represent different species of *Botanophila*, although morphological differences between them seem to favor this option. However, no larvae hatched from the gray eggs. Thus, it cannot be excluded that gray eggs formed in an oviduct are the result of parasite infection. It is also possible that these eggs get fertilized but embryonic development is retarded during the early stages of ontogenesis. Some further investigation on the relation between the egg type and a fly species is needed.

Our results do not support the previously proposed concept of an obligatory mutualism between *Epichloë* fungi and *Botanophila* flies. Both the fungus and fly benefit from this interaction; but, we provided evidence that the fly is not the only vector of fungal spermatia. Further research is required to check what additional mechanisms can be responsible for the fertilization of the fungus *E. typhina* in populations of the wild grass *P. distans* in Poland.

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