The occurrence and preference of Botanophila flies (Diptera: Anthomyiidae) for particular species of Epichloë fungi infecting wild grasses

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Abstract. Specific associations between species frequently occur in ecological interactions. The aim of this study was to determine the preferences of anthomyiid flies of the genus Botanophila for particular species of fungi as sites for laying eggs and as food for both larvae and adults. The associations of their eggs, larvae and flies with the stromata of different species of Epichloë fungi infecting 7 species of grass in Poland were analyzed. Scanning electron microscopy of the surface of their eggs and an analysis of the genetic sequences of their mitochondrial cytochrome oxidase (COII) were used to identify the taxa of the flies studied. Three types of eggs were distinguished based on their shape, colour and the presence of dorsal folds and sculpturing on the shells. The genetic sequences of their mitochondrial cytochrome oxidase (COII) were used to identify the taxa of the flies studied. Three types of eggs were distinguished based on their shape, colour and the presence of dorsal folds and sculpturing on the shells. Tentatively, these eggs were assigned to the following species: B. laterella, B. phrenione, B. dissecta and B. lobata. COII sequences obtained from larvae that hatched from two of the types of eggs formed three distinct clades associated with the reference sequences for Botanophila phrenione, B. lobata (new to the fauna of Poland) and a putative species, “Taxon 1”. Only one of these flies (B. lobata) was restricted to a single species of Epichloë (E. bromicola on Elymus repens); B. phrenione was recorded mainly from E. typhina infecting three different species of grass. The results of this study confirm that there is not a close species specific association between this fungus and this insect.

INTRODUCTION

The genus Botanophila currently includes approximately 70 species (Michelsen, 2011). Initially, only one of these species, B. phrenione (Séguy) [earlier described as Anthomyia spreta Meigen and Phorbia phrenione (Séguy)] was thought to be associated with Epichloë fungi (Kohlmeyer, 1956; Williams, 1971). At present, the relationship with Epichloë fungi is more or less confirmed for 6 European species of Botanophila [in addition to B. phrenione, these species include B. dissecta (Meigen), B. laterella (Collin), B. latifrons (Zetterstedt), B. lobata (Collin) and B. cuspidata (Collin)] (Collin, 1967; Ackland, 1972; Hennig, 1976). More recently the mitochondrial gene of subunit 2 of cytochrome oxidase (COII) of species of the genus Botanophila collected from different species of fungus in Switzerland, Poland and the USA was sequenced. These studies revealed that at least six distinct Botanophila taxa are associated with Epichloë fungi (Leuchtmann, 2007).

Flies of the genus Botanophila in transferring spermata between different mating types of heterothallic fungi behave like pollen-transferring insects (Bultman, 1995). In both cases, this process results in the fertilisation and production of dispersal structures. In Epichloë, these structures are meiotic ascospores, capable of infecting other plants (Chung & Schardl, 1997; Brem & Leuchtmann, 1999). Despite recent intensive research, the degree of specificity or preference shown by Botanophila for particular species of the fungus Epichloë has been little studied and the results ambiguous. Studies are needed to determine whether the associations are mutualistic, parasitic or neutral for one or both partners and whether it is a consequence of coevolution. Recently, new information has emerged that indicate that this fly-fungus interaction is not always a prerequisite for the initiation of the reproductive cycle of the fungus, as previously supposed (Rao & Baumann, 2004; Rao et al., 2005; Górzyńska et al., 2010, 2011).

The fungus-insect interaction, which resembles the interaction between angiosperms and pollinating insects, raises the question of possible species specificity between flies and their fungal hosts. However, molecular studies have not confirmed the co-speciation of the fly and the fungal host (Leuchtmann, 2007). All the species of Botanophila collected in Europe are recorded from more than one species of Epichloë and the most frequently recorded species are associated with as many as six fungal taxa. The only exception is the unidentified “Taxon 6”, a putative species that is recorded from only two localities in the USA and associated with only one host, E. glyceriae. However, the lack of specialisation

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stored in 95% ethanol before DNA extraction. DNA was
each combination of grass-fungus at each locality. They were
consisting of three larvae were collected using tweezers from
fungi infecting 7 species of grass in Poland (Table 1). Samples
collected from the stromata of different species of
molecular method.

The preference of a particular species of Botanophila
for a given species of fungus may result in two conse-
quences. First, it may maximise the efficiency of fungal
cross-fertilisation and thus ensure sufficient food for their
developing larvae. Second, it may result in the prezygotic
reproductive isolation between certain species of fungi
that co-occur in a given area. For instance, crosses
between the species E. typhina and E. clarkii may occur
(Leuchtmann & Schardl, 1998), although such hybrids
have not been recorded in nature (Steinebrunner &
Leuchtmann, unpubl., after Bultman et al., 2011).

The aim of this study was to investigate the possible
preferences and specificity of flies for the species of fungi
they lay their eggs on and on which both their larvae and
adults feed. For this we had to identify the flies collected
from the different fungus-grass systems studied. How-
ever, currently there is no single method that can be used
to identify the flies. Identification based on morpho-
logical traits is dependent on male flies being available,
however, they are very difficult to collect in the field, and
molecular identification does not provide unambiguous
taxonomic results in the form of specific species names.
Thus, we used both techniques and, in addition, checked
the usefulness of morphological traits of eggs for identi-
fying the species. A quick method for identifying the flies
would greatly facilitate future studies on the ecology of
this fly-fungus system.

**MATERIAL AND METHODS**

**Morphological identification of flies**

The only morphological characters that can currently be used
to identify Botanophila phrenione and related species associated
with the mycelium of Epichloë fungi are features of the termi-
nalia of adult males. Unfortunately attempts to recover male
flies from soil samples collected in the field that might have
contained Botanophila puparia were unsuccessful. Similarly, we
did not succeed in collecting any male specimens in the field.
Hence, we investigated the differences in the surface structures
of eggs and used these differences to make a preliminary dis-
tinction between the different fly taxa. A detailed SEM exami-
nation of the different egg morphotypes was conducted in the
Electron and Confocal Microscope Laboratory at A. Mickiewicz
University, Poznań, Poland. Subsequently, an attempt was made
to assign these morphotypes to the taxa distinguished using a
molecular method.

**Molecular identification of flies**

These analyses were conducted in June 2009 on fly larvae
collected from the stromata of different species of Epichloë
fungi infecting 7 species of grass in Poland (Table 1). Samples
consisting of three larvae were collected using tweezers from
each combination of grass-fungus at each locality. They were
stored in 95% ethanol before DNA extraction. DNA was
extracted using the DNeasy Tissue Kit (Qiagen Inc., Valencia,
CA, USA) and following the manufacturer’s protocol. Next, the
mitochondrial gene of subunit 2 of cytochrome c oxidase (COII)
was amplified using a modification of primer TL2-J-3037 (5’-
ATATATGGCAGATTAGTGCA-3’) and primer TD-N-3885
(5’-CAATATATGGCAGATTAGTGCA-3’). These primers bind to the conserved
regions of the genes (tRNA-Leu or tRNA-Asp, respectively)
flanking the COII gene, and are expected to amplify an 860 bp
fragment. The PCR reaction was conducted in a 25 µl volume
containing 2.5 µl of 10X buffer, 2.5 mM MgCl₂, 0.1 mM of
each dNTP, 0.4 µM of each primer, 1.25 U DNA Taq
polymerase and 0.5-1 ng template DNA. Amplification was
conducted in a thermocycler (PTC-200, MJ Research) using a
program with the following parameters: 7 min 94°C; 45 s 94°C,
45 s 50°C, 2 min 70°C × 25; 5 min 70°C. The products of the
reaction were separated on a 1% agarose gel stained with
ethidium bromide for confirmation. Sequencing reactions were
then performed in 10 µl volumes using BigDye Terminator v2.1
(Applied Biosystems, Foster City, CA, USA) and the products
cleaned with a Purification Kit (Qiagen, Hilden, Germany), fol-
lowing the manufacturer’s protocol, before obtaining the
sequences using a 3130 Genetic Analyzer (Applied Bio-
systems). The sequences were edited and assembled using
Sequencher 4.8 (Gene Codes Corp., Ann Arbor, MI, USA) and
compared with aligned reference sequences of the six taxa of
Botanophila distinguished by Leuchtmann (2007).

**Phylogenetic analysis**

The analysis of the 33 new sequences included three refer-
ceence sequences and two out-group sequences (taken from a pre-

<table>
<thead>
<tr>
<th>Fungal host</th>
<th>Grass host</th>
<th>Site</th>
<th>Location</th>
<th>Fly species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dactylis glomerata</td>
<td>Morasko</td>
<td>52°27.857’N 16°55.868’E</td>
<td>B. phrenione</td>
<td>Taxon 1</td>
</tr>
<tr>
<td>Ppakő 1</td>
<td>52°47.531’N 18°06.118’E</td>
<td>B. phrenione</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poa trivialis</td>
<td>Rogalin</td>
<td>52°14.072’N 16°55.229’E</td>
<td>B. phrenione</td>
<td></td>
</tr>
<tr>
<td>E. typhina</td>
<td>Janikowo</td>
<td>52°46.384’N 18°08.032’E</td>
<td>B. phrenione</td>
<td></td>
</tr>
<tr>
<td>Puccinellia distans</td>
<td>Giebnią</td>
<td>52°46.544’N 18°06.190’E</td>
<td>B. phrenione</td>
<td></td>
</tr>
<tr>
<td>E. boronicola</td>
<td>Paksi 2</td>
<td>52°47.397’N 18°06.064’E</td>
<td>B. phrenione</td>
<td></td>
</tr>
<tr>
<td>E. clarkii</td>
<td>Moskoro</td>
<td>52°27.910’N 16°55.370’E</td>
<td>B. phrenione</td>
<td></td>
</tr>
<tr>
<td>E. festucae</td>
<td>Festuca rubra</td>
<td>52°27.854’N 16°55.774’E</td>
<td>B. phrenione</td>
<td></td>
</tr>
<tr>
<td>E. baconii</td>
<td>Agrostis alba</td>
<td>52°47.356’N 18°06.662’E</td>
<td>B. phrenione</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.** The association of the larvae of Botanophila with Epichloë fungi infecting particular species of grass, their geo-
graphic origin and identity of those species included in this study.
vious study, Leuchtmann, 2007). The maximum-likelihood (ML) tree was identified in PAUP* 4.0B10 using a general time-reversible (GTR) model and maximum likelihood settings from the best-fit model (TrN + G) selected by hierarchical likelihood ratio tests in MODELTEST 3.7 (Posada & Crandall, 1998). The tree was inferred from 100 iterations with a random order of taxon addition and different values of the random number of seed. Confidence levels for the clades were assessed using ML bootstrap analysis (Efron & Tibshirani, 1993) based on 100 heuristic replicates in PAUP.

RESULTS

Morphological identification of eggs and their shells

Three types of eggs were laid by female Botanophila on stromata of Epichloë fungi. They differed in shape, colour, presence or absence of dorsal folds and the sculpturing on the egg shells: (1) grey eggs (Fig. 1a, b), “slender”; without dorsal folds, the shell surface with a reticulated polygonal pattern with no pits within the poly-

Fig. 1. Eggs of Botanophila flies collected from the stromata of Epichloë sp. infecting the grasses Dactylis glomerata (a, b, e, f) and Puccinellia distans (c, d); “grey eggs” (a, b), “cream eggs” (c, d), “white eggs” (e, f); (b, d, f) – magnified parts of the egg shells.
gons; (2) cream coloured eggs (Figs 1c, d, 2), with a double dorsal fold, the shell surface with a reticulated polygonal pattern with numerous pits and distinct spike-like projections between the pits; and (3) pure white eggs (Figs 1e, f, 2), bulky, with a double dorsal fold and the shell surface with a reticulated polygonal pattern and numerous pits, but no spike-like projections between pits. These differences in the egg shells were not correlated with the species of fungus.

Molecular identification and phylogenetic relationships

COII sequences of the 33 fly larvae collected from the stromata of different species of Epichloë formed three distinct clades (Fig. 3). Each clade was closely associated with the reference sequence of one of three Botanophila taxa. Eighteen larvae grouped with B. phrenione, five with B. lobata and 10 with unidentified “Taxon 1”. No species name could be assigned to this taxon, because the absence of adult male specimens made it impossible to identify this species. Although there was some genetic variability among sequences assigned to particular taxa, clades were well-separated and had 97–100% bootstrap support.

Fly preferences

Only one of the three fly taxa, identified as B. lobata, was restricted to a single species of Epichloë (E. bromicola on Elymus repens) (Table 1). This species of Botanophila was recorded at all three localities at which this association occurred. In each case, however, B. lobata was accompanied by another fly taxon, denoted “Taxon 1”. The second species, B. phrenione, was recorded mainly from E. typhina infecting three different grass species (Table 1) but also from the stromata of E. clarkii infecting Holcus lanatus. For the latter, it was the only fly taxon recorded from this species of Epichloë. The third fly taxon, “Taxon 1”, was the most frequent. It was recorded from all the fungal species studied except the stromata of E. clarkii on H. lanatus (Table 1). Only on two species of fungi, E. clarkii and E. festucaceae, were the larvae of a single taxon recorded (B. phrenione and “Taxon 1”, respectively).

DISCUSSION

The molecular techniques used in this study revealed that there are three different taxa of flies, belonging to the genus Botanophila, in Poland. One of them is B. phrenione, a species recorded before from two grasses, Dactylis glomerata and Puccinellia distans, infected with the fungus Epichloë typhina (Gorzyńska et al., 2010, 2011). The second is B. lobata, which is a new record for Poland. The third denoted “Taxon 1”, remains unidentiﬁed because of the absence of adult male specimens. However, based on the characteristics of its association with a particular host fungus, it is considered to be a new species for Poland. These three taxa of Botanophila have...
not yet been listed in taxonomic and faunistic works that include Poland (Komzaková & Rozkošný, 2009; Michelsen, 2011).

The results of our studies confirm the hypothesis that there is not a close species specific association between fungi and this insect (Leuchtmann, 2007). Of the three fly taxa recorded in Poland only B. lobata was recorded from a single fungal species, Epichloë bromicola infecting Elymus repens. This species of Botanophila was observed at all the localities where E. bromicola was present. The other two taxa, B. phrenione and “Taxon 1”, do not have a specific association with a particular species of fungus. A previous study (Leuchtmann, 2007) records that all the European taxa are associated with more than one species of Epichloë and the most frequent taxon, the unidentified “Taxon 1”, is recorded from as many as six species. The only exception is a taxon designated “Taxon 6”, recorded at two localities in the USA, which is associated with just one fungal species, E. glyceriae.

The analysis of the associations of the different taxa of flies with particular species of grass infected with these fungi, revealed certain differences not recorded by Leuchtmann (2007). First, regarding the E. typhina – P. distans interaction, there are two fly taxa (B. phrenione and “Taxon 1”), not just one (“Taxon 1”). This is possibly due to sample size. In this study eight samples were collected from three localities, while Leuchtmann (2007) analyzed only one sample, which originated from Giebniowice in Poland. Second, B. lobata was recorded in Poland only from the stromata of E. bromicola, whereas this species is recorded by Leuchtmann (2007) as being associated with the interaction E. typhina – D. glomerata in Switzerland and with E. festucae – F. rubra in the USA. In addition, this author records B. lobata from stromata on three other host grasses not included in the present study. A probable reason for this discrepancy, besides the limited number of samples examined, may be the local preference of B. lobata for E. bromicola rather than E. typhina or E. festucae in Poland. Previous studies also suggest that certain species of this fly visit one host more frequently than other hosts (Bultman & Leuchtmann, 2003; Bultman et al., 2011).

B. phrenione occurs in the Western Palaearctic (Komzaková & Rozkošný, 2009). This species was recorded by Leuchtmann (2007) only from grass – fungus systems in Europe (from E. clarkii, E. sylvatica and E. typhina infecting six different species of grass). In contrast, B. lobata occurs in Eurasia, but previously noted only in Europe and Japan (Collin, 1967; Suwa, 1999). In Europe, this species is most commonly recorded from E. bromicola infecting E. repens (this study) and Bromus erectus, and from E. sylvatica infecting Brachypodium sylvaticum and is only recorded once from E. typhina infecting D. glomerata (Leuchtmann, 2007). In the USA, however, larvae of B. lobata are frequently recorded from the stromata of the introduced E. typhina infecting D. glomerata and E. festucae infecting F. rubra. Presumably, B. lobata is not a native of the USA but was brought to the USA with the fungus (Rao et al., 2005). The absence of natural enemies (e.g., parasitoids) may have facilitated the acclimatisation of this taxon, which is the most frequently recorded representative of the Epichloë-associated Botanophila species in the USA (Rao & Baumann, 2004; Rao et al., 2005). Two additional putative species (“Taxon 5” and “Taxon 6”) distinguished by Leuchtmann (2007) are only found in the USA and not so far recorded in Poland.

Among the six Botanophila taxa previously identified from Epichloë hosts by Leuchtmann (2007), three are European and two North American. One taxon occurs on both continents. However, the phylogeny of these taxa does not reflect their geographic origin. B. phrenione and “Taxon 1” form a common clade with the American “Taxon 5”, whereas B. lobata is more distantly related to this group. B. phrenione and “Taxon 1” records are collected in the field (Leuchtmann, 2007). The third species (denoted “Taxon 1”) is designated putative, because this species has not been confirmed. Since COII genes are maternally inherited and no other nuclear genes were analyzed, it is not possible to exclude the false interpretation of the clade denoted “Taxon 1”. However, all the COII sequences of larvae of “Taxon 1” from different sites in Switzerland (Leuchtmann, 2007) and four in Poland (this study) form a distinct and uniform clade that is well-separated from B. phrenione and B. lobata. Furthermore, in the taxonomic literature there are at least six Botanophila species associated with Epichloë fungi in Europe (Collin, 1967; Ackland, 1972; Hennig, 1976) and it is likely that “Taxon 1” is one of them.

The molecular analysis needs to be associated with specific morphological identifications. However, the adult males required for identification are not recorded from fungal stromata and are difficult to find in the nearby vegetation. Therefore, attempts were made to distinguish fly taxa based on egg morphology. Scanning electron microscopy of the shells of eggs revealed that there are three morphologically distinct types of eggs, which differ in shape, colour, presence or absence of dorsal folds and sculpturing of the surface (Figs 1, 2). It is likely that these differences will be used in the future for identifying these flies. Tentatively, the three colours of eggs are associated with the following species of flies: “grey” B. laterella, “white” B. phrenione and B. dissecta and “cream” B.
lobata. However, the different eggs have yet to be associated with particular species of *Botanophila* based on molecular identification. As the “grey” eggs did not hatch it was not possible to use molecular sequences to identify them. Larvae referred to as “Taxon 1” most likely represent *B. dissecta*, because all the larvae assigned to “Taxon 1” based on molecular sequences hatched from “white” eggs. Results and observations on egg morphology will be used in the future to identify the flies associated with particular species of *Epichloë*.

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REFERENCES

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