

Botanophila flies associated with Epichloë host species: no evidence for coevolution

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Abstract

Epichloë species usually depend on flies of genus *Botanophila* which transfer gametes for sexual reproduction. Sequence analysis of *COII* detected six distinct *Botanophila* taxa that were associated with *Epichloë* in Europe and North America. Three were restricted to samples from Europe, two to samples from North America, and one was present on both continents. In North America the common taxon (identified as *B. lobata*) was found on *Epichloë* hosts of native grasses as well as on *E. typhina* and *E. festucae* of introduced grasses. In Switzerland, up to four different *Botanophila* taxa occurred at a single location. There was no strict specificity of flies for *Epichloë* host species, although several taxa appeared to prefer some hosts over others. Comparison of the *Botanophila* phylogeny with that of the associated *Epichloë* hosts did not suggest co-evolution of fungus and fly. Thus, associations between *Botanophila* flies and *Epichloë* hosts may have arisen independently more than once.

Keywords: Anthomyiidae, Diptera, Clavicipitaceae, choke disease, cytochrome oxidase, grass endophytes, molecular phylogeny, mutualism

Introduction

Species of *Epichloë* maintain an obligate symbiotic relationship with flies of the genus *Botanophila* (Diptera: Anthomyiidae). Sexual reproduction of the fungus usually requires a visit by *Botanophila*, which serves to “pollinate” the fungus with spermatia of the opposite mating type; the flies in turn deposit their eggs on fungal tissues upon which the larvae feed. Thus, the *Botanophila* - *Epichloë* relationship is generally viewed as an obligatory mutualism (Bultman *et al.* 1998).

Early researchers believed that only a single fly species, *B. phrenione* (previously *Phorbia phrenione*), was associated with *Epichloë* hosts (Kohlmeyer 1956; Kohlmeyer *et al.* 1974). However, taxonomic work done in Britain indicated that several morphologically similar species may occur on *Epichloë* including *B. dissecta*, *B. laterella* and *B. lobata* (Ackland 1972; Collin 1967). There are currently six *Botanophila* species in Europe for which *Epichloë* is a confirmed or suspected host (Hennig 1976). Observations from experimental field plots suggest that some fly species may predominantly visit one host, while visiting other hosts less frequently (Bultman *et al.* 2003). Species-specific visiting patterns of flies would maximise cross-fertilisation, which is required to provide the nutritional basis for *Botanophila* larvae. The mechanism by which *Botanophila* flies locate *Epichloë* fungi is not precisely known, but it is likely that volatiles play a role in attracting flies. Most recently, a volatile compound, named chokol K, was identified from *Epichloë* stromata, which is detected by the fly’s antenna and which attracted *Botanophila* flies in the field (Schiestl *et al.* 2006).

In this study, we examined a large sample of *Botanophila* flies collected as larvae from various *Epichloë* hosts in Europe and North America. Our goals were to determine the geographic distribution of *Botanophila* taxa and their associations with different *Epichloë* hosts. We used sequences of the mitochondrial cytochrome oxidase gene (*COII*) as molecular markers.

Morphological identification of female flies visiting *Epichloë* stromata is not possible. Therefore, only two of the six taxa characterised molecularly could so far be matched with described morphological species of *Botanophila*.

Methods

Two areas in Switzerland, one in the north-eastern and one in the western part, were intensively sampled (Table 1). Additional specimens originated from Poland and the USA. *Botanophila* was collected as larvae from parasitised *Epichloë* stromata. Larvae were either preserved in 95% ethanol or frozen before DNA extraction. Male flies of *B. lobata* and *B. phrenione* were caught in grassland in the vicinity of *Epichloë* populations, or were reared from a larva. Outgroup taxa, *Egle rhinoimeta* and *E. parva*, were collected on their natural host *Salix* spp.

DNA was isolated from larvae (or pupae) frozen with liquid nitrogen following the method of Garnery *et al.* (1991). DNA of the protein coding mitochondrial gene *COII* was amplified by using a modification of primer TL2-J-3037 (5'-TAATATGGCAGATTAGTGCA-3') and primer TD-N-3885 (Simon *et al.* 1994) and sequenced using a BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems). Sequence products were separated on a capillary 3100-Avant Genetic Analyzer and analysed with Sequencing Analysis version 3.7 software (PE Applied Biosystems).

Sequences were aligned with Sequence Navigator version 1.0.2b3 (PE, Applied Biosystems) and manually adjusted. Maximum-parsimony (MP) analyses were performed by heuristic search in PAUP* 4.0b10 (Swofford 2003). Robustness of the topology was estimated by 1000 bootstrap replications (Swofford *et al.* 1996). Maximum-likelihood (ML) trees were identified in PAUP* 4.0b10, using a general time reversible model and maximum likelihood settings from best-fit model (TrN+G) selected by hierarchical likelihood ratio tests in MODELTEST 3.6 (Posada *et al.* 1998).

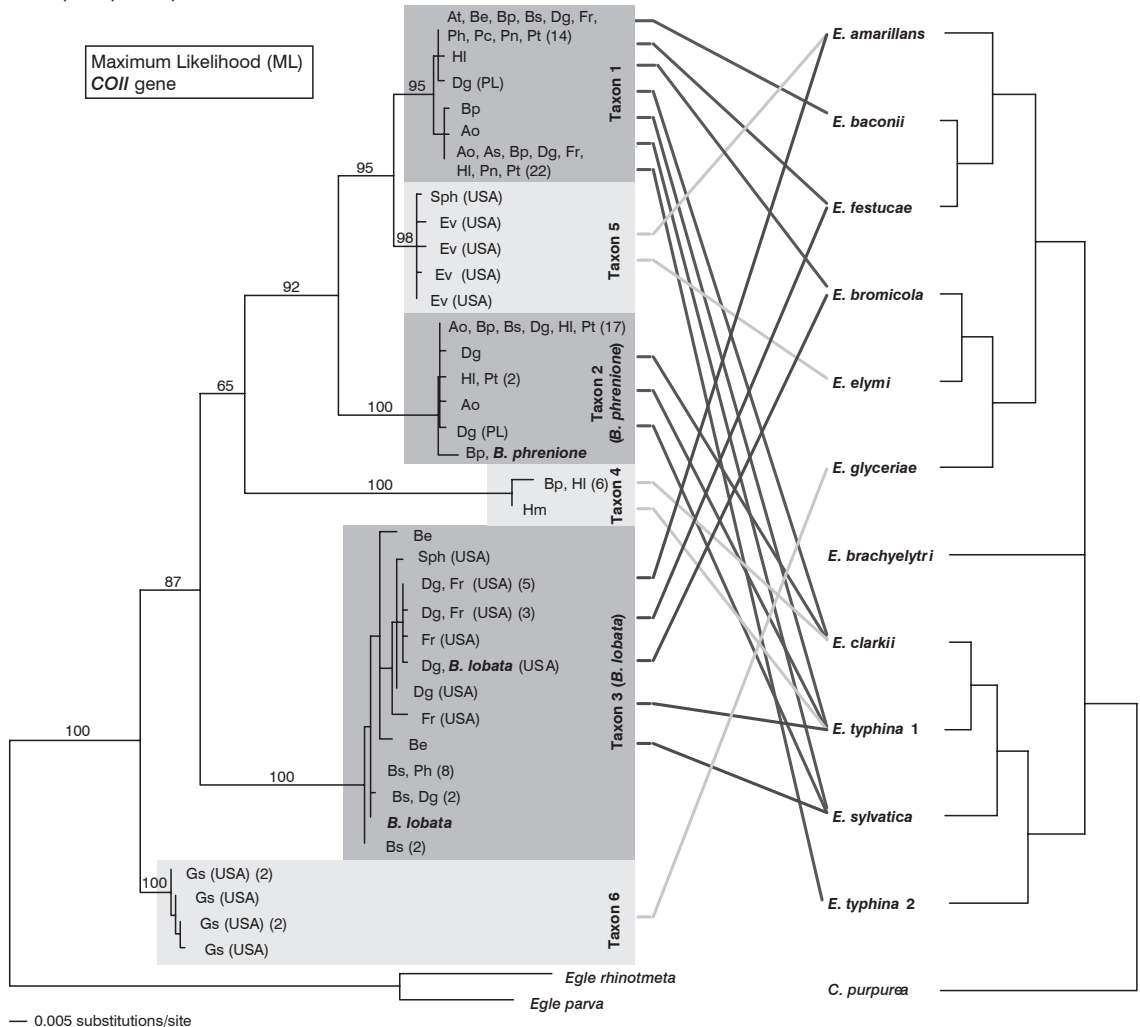
Results and Discussion

Molecular analysis based on sequences of the mitochondrial cytochrome oxidase gene (*COII*) revealed that six putative fly taxa may be associated with *Epichloë* in the study area (Table 1, Fig. 1). Taxon 2 refers to *B. phrenione* and taxon 3 to *B. lobata*. These two species were confirmed based on sequences derived from identified male specimens. Three taxa were found in samples from Europe only, two were restricted to hosts in North America, and one was present on hosts of both continents. The common taxon (identified as *B. lobata*) was found in North America on the introduced European hosts, *E. typhina* infecting *Dactylis glomerata* and on *E. festucae* infecting *Festuca rubra*, in addition to the native host *E. amarillans*. Besides Europe and America, *B. lobata* has been reported from Japan (Suwa 1999) implying that the species has a holarctic distribution.

Most of the approximately 65 known *Botanophila* species develop on angiosperms and their larvae feed on different plant parts, including rosettes, stems and fruits (Hennig 1976;

Figure 1 Comparative trees from molecular phylogenetic analysis of *Botanophila* flies and the associated *Epichloë* host species. Association of fly taxa (represented by major clades) with *Epichloë* hosts is indicated by connecting lines. The phylogram of *Botanophila* (at left) is based on sequences of the mitochondrial cytochrome oxidase gene (*COII*) including 108 samples collected as larvae, and three collected as adult males. It is inferred from a Maximum-Likelihood (ML) analysis with general time reversible model of character evolution and gamma distribution of rate substitutions (TrN+G). Bootstrap percentages indicated above the supporting branches are taken from Maximum-Parsimony (MP) analysis with 1000 replications. Sequences are designated by abbreviations of grass host species followed by the country code of origin (PL = Poland, USA = United States of America) if outside of Switzerland, and (in parenthesis) the total number of flies from these hosts exhibiting identical sequences. The *Epichloë* host phylogram is derived from *tub2* sequences using Maximum-Parsimony and is taken with modification from Craven *et al.* (2001). Both phylograms are rooted with outgroup species listed at the bottom end of the phylograms.

Grass host abbreviations: Ao, *Anthoxanthum odoratum*; As, *Agrostis stolonifera*; At, *A. tenuis*; Be, *Bromus erectus*; Bp, *Brachypodium pinnatum*; Bs, *B. sylvaticum*; Dg, *Dactylis glomerata*; Ev, *Elymus virginicus*; Fr, *Festuca rubra*; Gs, *Glyceria striata*; Hl, *Holcus lanatus*; Hm, *H. mollis*; Ph, *Phleum pratense*; Pc, *Puccinellia distans*; Pn, *Poa nemoralis*; Pt, *P. trivialis*; and Sph, *Sphenopholis obtusata*.



Michelsen 2004). Thus, it is likely that ancestral *Botanophila* has originally evolved in association with plant hosts. In the course of evolution, host range may have been expanded to *Epichloë* infected grasses, where fungus tissues could be exploited as an additional food source. Some of today's species associated with *Epichloë* do in fact consume, not only fungal material from the stroma surface, but also plant tissues when larvae burrow inside

the grass stem and feed on both plant and fungal tissues (Ackland 1972). Although it is speculative, the substantial phylogenetic divergence observed among *Epichloë*-associated *Botanophila* species and the fact that closely related flies are indigenous to different continents suggest that associations of *Botanophila* with *Epichloë* fungi have arisen more than once. It is unclear which of the six taxa is the most ancestral, because results were

Table 1 *Botanophila* taxa found in Switzerland, Poland, and North America.

Sampling area	Sample size	Taxon (no. of specimens)					
		1 (40)	2 (23)	3 (28)	4 (7)	5 (5)	6 (6)
Switzerland, north-east	48	+	+	+	+		
Switzerland, west	33	+	+	+	+		
Poland	3	+	+				
USA (IN, KY, MO, OR)	24			+		+	+

ambiguous. In the Maximum Likelihood phylogram (ML) one of the American taxa (taxon 6) was most basal (Fig. 1), whereas in Maximum-Parsimony analysis (MP) taxon 4 came out as the most basal (data not shown). Interestingly, larvae of taxon 4 live inside the grass stem very early on, a behaviour which may represent the transitional stage between a purely plant-associated to fungus-associated species. Considering the wide distribution and host range of *B. lobata*, it is also possible that this species represents the oldest association, which may have later given rise to other taxa on both continents. Further research including sequencing of additional genes will be necessary to confirm which of the hypotheses is correct.

Available data do not suggest that fly taxa are specific to a particular *Epichloë* host, with the exception of *E. glyceriae* (Fig. 1). However, some preference of *Botanophila* flies may exist on the level of individual host species. Two *Epichloë* species in Europe (*E. baconii* and *E. festucae*) and two in North America (*E. elymi* and *E. glyceriae*) attracted a single fly taxon. Previous observations on the spermatial composition found in faeces of flies from experimental field plots, suggested that individual flies may predominantly visit one host while visiting other hosts less frequently (Bultman & Leuchtman 2003). Such selective fly behaviour could favour intraspecific matings of *Epichloë* and thus maintain or promote reproductive isolation of fungal species.

Comparison of molecular phylogenetic relationships of *Botanophila* species with those of the associated *Epichloë* hosts (Craven *et al.* 2001) did not reveal congruence between the major clades of the two phylogenies (Fig. 1), and thus co-evolution of fly and fungal species is not indicated. Co-evolution would be possible if flies were host specific and interactions with particular fungal species persisted over evolutionary time scales. Such conditions of long-term persistence on the level of species probably were not met, but rather flies may have interacted loosely with different clades of *Epichloë* in the course of evolution. In co-evolved systems of insects and flowering plants, such as fig wasps and figs, pollinating parasites are highly specialised with almost every fig species being pollinated by a different wasp species (Bronstein 1992). As wasps can only reproduce in the inflorescences of that fig and their larvae feed on the developing seeds, species interactions represent obligate mutualisms. While sharing many similarities with these co-evolved interactions, the *Epichloë*-*Botanophila* system is obviously not mutualistic under all circumstances. In cultivated fields in Oregon, USA, fly larvae completed their development on unfertilised stromata of *E. festucae*, and on *D. glomerata* infected by *E. typhina*, perithecia and ascospores developed in the absence of *Botanophila* flies (Rao *et al.* 2004; Rao *et al.* 2005). If *Botanophila* species can develop on *Epichloë* without providing the service of fertilisation to the fungus, selection for specialisation of flies will be weak. This may explain the limited or absent species-specific interactions observed in the *Epichloë*-*Botanophila* system. However, the close coordination of the life cycles of flies and fungi and the

typically mutual dependence of the two partners suggest that the interaction as a whole is co-evolved.

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