

Comparative analysis of the biodiversity of fungal endophytes in insect-induced galls and surrounding foliar tissue

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Abstract Insect-induced galls are abnormal plant growths that can provide food and shelter to their inhabitants, resulting in stressed plant tissue that may alter the conditions for the colonization or proliferation of endophytic fungi. We investigated the effect gall formation has on fungal endophyte communities and diversity. Using three closely-related gall-forming aphid species that specialize on poplars, we characterized fungal endophyte diversity in galls and surrounding petiole and leaf lamina tissue. A total of 516 fungal endophyte samples were isolated from 272 tissue samples (32 leaves, 31 petioles, and 209 galls), resulting in 23 distinct morphotypes. Despite sharing a common host plant and often forming spatially contiguous galls, the endophyte profiles within the galls of each aphid species were distinct, not only from the galls of the other species, but also from surrounding plant tissue. These results suggest that insect galls can affect the composition of fungal endophyte species in plant tissues, by altering either the colonization or proliferation of their endophytic mycobiota. Likewise, fungal endophytes may be important in the ecology and evolution of insect galls.

Keywords Insect-induced galls · Fungal endophytes · Fungal endophyte-insect interactions · Pemphigus aphids · Poplar trees

Introduction

Over 13,000 species of herbivorous insects can induce galls on their host plants. Galls are tumor-like growths of tissue which are induced by the insect as it feeds and provide shelter,

nutrition, and protection from natural enemies (Stone and Schonrogge 2003). Galls often have conspicuous morphology, and in groups such as the nematine sawflies, gall midges, and cynipid wasps, gall formation is associated with exceptional phenotypic and evolutionary diversity. Much of the research on insect-induced galls has focused on their ecological and evolutionary functions, as well as the biochemical basis of gall induction (Raman 2011). One aspect of insect-induced galls that has received comparably less attention is how gall-forming insects interact with endophytic fungi embedded in surrounding plant tissues, and the consequences of these interactions for the fungal endophyte community.

Endophytic fungi compose a polyphyletic group of highly diverse fungi that are functionally defined by internal and asymptomatic occurrence in plant tissue (Saikkonen et al. 1998). In recent years, there has been a growing interest in how endophytic fungi affect patterns of insect herbivory, particularly with respect to the endophytic clavicipitalean fungi of grasses (Clay 1988; Clay and Schardl 2002; Rodriguez et al. 2009). Less is known about the diversity or functional roles of fungal endophytes in the foliar tissues of herbaceous plants and trees. Previous research has demonstrated that infection can be highly localized to distinct tissues in woody plants (Saikkonen et al. 1998; Porras-Alfaro and Bayman 2011; Albrechtsen et al. 2010; Botella and Diez 2011; Koukol et al. 2012; Li et al. 2012). These fungal endophytes typically remain quiescent within plant tissues until senescence or stress results in proliferation of fungal thalli (Stone et al. 2004; Sieber 2007). In contrast to fungal endophytes in grasses, it is less clear whether those which are dormant and localized in the leaf and vascular tissues of trees and shrubs act as mutualists or antagonists. Some evidence suggests, for example, that non-clavicipitalean species readily shift functional roles, depending on ecological or seasonal conditions (Sieber 2007; Purahong and Hyde 2011).

Galls may represent sites where either endophytic abundance or diversity persistently differs from that of surrounding tissues. Insect-induced galls can act as resource

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sinks, concentrating nutrients from surrounding plant tissues (Larson and Whitham 1997; Schonrogge et al. 2000). This concentration of nutrients likely affects the composition or proliferation of endophytes or other saprophytic or pathogenic fungi. Moreover, fungal endophytes may affect the performance or patterns of herbivory by gall-forming insects. The galling lifestyle represents an unusually intimate and persistent interaction between insects and plants. If fungal endophytes have effects that inhibit or promote the persistence of herbivores on plants, gall-forming insects may be acutely sensitive to their distribution in plant tissues or organs. Wilson and Carroll (1997), for example, found that a gall-forming cynipid wasp tends to avoid the area of oak leaves with greater densities of a common fungal endophyte, *Discula quercina* (Sordariomycetes: Diaporthales). Faeth and Hammon (1997) found positive associations between fungal endophyte infections and *Cameraria* sp. (Lepidoptera: Gracillariidae), leafminers, which form small tunnels on the leaves of their host plants while they feed.

Only a small number of studies have characterized the interaction between the fungal endophytes of trees and gall-forming or leafmining insects; a majority of those studies have described interactions on oak trees (Table 1). In this study, we asked how insect-induced galls affect the fungal endophytes of poplars. We characterized fungal endophyte diversity in the galls of three *Pemphigus* (Hemiptera: Pemphigidae). *Pemphigus* consists of 65 described species distributed throughout the northern hemisphere (Blackman and Eastop 1994). All species form galls on the leaves or petioles of poplars (*Populus* spp.). The three species, *P. populi-caulis*, *P. populi-transversus* and *P. obesinymphae* have overlapping ranges in eastern North America, and form galls on their primary host, *Populus deltoides* (Salicaceae). They differ, however, in the precise locations on the plants where they initiate galls, and in the seasonal timing and duration of the gall (Table 2; Abbot and Withgott 2004). The life history differences between these three species that share a common host plant allow for comparisons of how insect galls differ in fungal endophyte composition across plant tissues and seasons.

Materials and methods

Study system and field site

P. populi-caulis and *P. obesinymphae* form galls at the base of the leaf lamina, while *P. populi-transversus* forms galls on the leaf petiole. *P. populi-caulis* initiates galls in early spring, while *P. populi-transversus* and *P. obesinymphae* initiate galls later in the spring or early summer (Blackman and Eastop 1994). All plant tissues were collected from eastern cottonwoods (*Populus deltoides* W. Bartram ex. Marshall) in the greater Nashville, Tennessee area. The sites were in

disturbed areas near major roadways. The site coordinates were: Site 1: N 35.967552, W 086.778438; Site 2: N 36.08395, W 086.8882; Site 3: N 36.07786, W 086.91150; Site 4: N 36.13066, W 086.90326; Site 5: N 36.15406, W 086.95084, and Site 6: N 36.20914, W 086.88243. At each sampling date between May and August 2011, we collected between 30 and 40 galls with attached leaves and petioles. Standardized sections of leaves and petioles were obtained by cutting a 1 cm long section of the petiole proximal to the gall and a 1 cm×1 cm square of the leaf immediately distal to the gall. The leaf section included both midrib and leaf lamina.

Fungal isolations and identification

Within 24 h of collection, fungal endophytes were cultured from galls, and from a section of the leaf and petiole of approximately every 10th gall. Prior to plating, attached ungalled tissues were removed from galls using a sterile razor blade. Whole galls and samples of surrounding tissue were then plated and subcultured (described below).

Surface sterilization was performed on all gall, leaf, and petiole samples following a protocol from Deckert et al. (2001). Samples were agitated in 70 % ethanol for 1 min, then allowed to soak in the ethanol for 4 min. Samples were then soaked in 50 % bleach (6 % Sodium hypochlorite) for 5 min, sterile distilled H₂O for 5 min, and an additional wash in clean sterile distilled H₂O for 5 min. Under sterile conditions, gall segments were allowed to dry for 5 min before they were plated on Potato Dextrose Agar (PDA) plates with ampicillin at 1 ng/μL. A total of 210 galls, 32 leaves and 31 petioles were plated. There was no replication between tissue samples, because all the tissue was plated together on one plate at one time. All plant samples were then incubated on sealed plates at room temperature and checked daily under a microscope for signs of hyphal growth.

At the end of 4 weeks, fungi were subcultured from original inoculations. Under a sterile hood, agar plugs with one fungal morphotype were transferred from the original PDA plate to a fresh PDA plate and grown at room temperature for 2 weeks. Following the successful isolation a fungal morphotype, plugs from the subculture were removed and placed in 15 mL polypropylene tubes containing 5 mL sterile Potato Dextrose (PD) broth. Liquid cultures were grown for approximately 2 weeks at room temperature. Each morphotype was archived as a living voucher in 400 μL of an 80 % PDA, 20 % glycerol solution and stored at −80 °C in screw cap tubes (Hoffman and Arnold 2010).

Total genomic DNA was extracted directly from pure, liquid cultures by grinding fungal samples in liquid nitrogen with a mortar and pestle, followed by application of a Qiagen DNeasy Plant Mini Kit[®]. The internal transcribed spacer sequence (ITS, using primers described in Bellemain et al. 2010) was amplified in a polymerase chain reaction (PCR)

Table 1 Summary of studies on the interaction between gall-forming or leafmining insects, fungal endophytes and their host plant

Plant	Fungus	Insect	Type of study	Effect of fungus on insect	Citation
<i>Quercus robur</i>	<i>Kabatiella apocrypta</i>	<i>Trioza remota</i> (psyllid)	Pair-wise	Negative	Butin 1992
	<i>Gloeosporium quercinum</i>	<i>Neuroterus numismalis</i> (gall wasp)		Negative	
	<i>Dichomera saubinetii</i>	<i>Polystepha panteli</i> (gall midge)		Negative	
<i>Pseudotsuga menziesii</i>	<i>Rhabdocline parkeri</i>	<i>Contarinia</i> sp. (gall midge)	Pair-wise	Negative	Carroll 1995
<i>Quercus emoryi</i>	<i>Ophiognomia cryptica</i>	<i>Cameraria</i> sp. (leafminer)	Community	Neutral	Faeth and Hammon 1997
	<i>Asteromella</i> sp.			Neutral	
	<i>Plectophomella</i> sp.			Neutral	
<i>Pinus densiflora</i>	<i>Phialocephala</i> sp.	<i>Thecodiplosis japonensis</i> (midge)	Community	Neutral	Hata and Futai 1994
<i>Picea glauca</i>	<i>Chladysporium sphaerospermum</i>	<i>Adelges abietis</i> (gall adelgid)	Pair-wise	Negative	Lasota et al. 1983
<i>Tilia cordata</i>	<i>Gloeosporium</i> sp.	Multiple sp.	Community	Negative	Pehl and Butin 1994
<i>Quercus robur</i>	<i>Gloeosporium</i> sp.	Multiple sp.		Negative	
<i>Fagus sylvatica</i>	<i>Gloeosporium</i> sp.	Multiple sp.		Negative	
<i>Acer pseudoplatanus</i>	<i>Diplodina acerina</i>	<i>Dasynerua vitrina</i>		Negative	
<i>Quercus gambelii</i>	<i>Gnomonia cerastis</i>	<i>Phyllonorycter</i> sp. (leaf mining moth)	Community	Positive	Preszler et al. 1996
<i>Quercus agrifolia</i>	<i>Discula quercina</i>	<i>Dryocosmus dubiosus</i> (gall wasp)	Community	Unresolved	Wilson 1995
	<i>Cryptosporiopsis quercina</i>				
	<i>Auerobasidium</i> sp.				
	<i>Phomopsis</i> sp.				
<i>Populus angustifolia</i>	<i>Verticillium lecanii</i>	<i>Pemphigus betae</i> (gall aphid)	Community	Negative	Wilson 1995
	<i>Cladosporium cladosporioides</i>				
	<i>Penicillium</i> sp.				
<i>Quercus garryana</i>	<i>Discula quercina</i>	<i>Besbicus mirabilis</i> (gall wasp)	Community	Negative	Wilson 1995
	<i>Apiognomonina</i> sp.				
	<i>Fusarium</i> sp.				
<i>Quercus garryana</i>	<i>Discula quercina</i>	<i>Besbicus mirabilis</i> (gall wasp)	Pair-wise	Neutral	Wilson and Carroll 1997
		<i>Bassettia ligni</i> (gall wasp)		Neutral	
<i>Quercus emoryi</i>	<i>Ophiognomonina cryptica</i>	<i>Cameraria</i> sp. (leafminer)	Pair-wise	Negative	Wilson and Faeth 2001
	<i>Plectophomella</i> sp.			Negative	
	<i>Asteromella</i> sp.			Negative	

The type of study indicates if the focus was on a specific interaction between one endophytic fungal species and one insect species (pair-wise) or examining the community of fungal endophytes in the gall tissue and subsequently, concentrating on the most common fungal species (community)

using the following protocol: 94 °C for 2.5 min; 30 cycles of 94 °C for 15 s, 56 °C for 30 s, 72 °C for 1.5 min; and 72 °C for 10 min. The PCR product was purified using ExoSAP-IT® (USB corporation, Cleveland, OH, USA). Purified samples were Sanger sequenced at GENEWIZ, Inc. (<http://www.genewiz.com>). Nearest species were determined using the Basic Local Alignment Search Tool (BLASTn) at the National

Center for Biotechnology Information (NCBI). Sequences that showed ≥ 98 % similarity to the best BLAST hit were taxonomically assigned to the same operational taxonomic unit (OTU). Those sequences with < 98 % similarity were assigned to the genus or family of the best BLASTn hits. The 98 % similarity cut-off is a conservative criterion and is based on studies suggesting the variability of the ITS region

Table 2 Time, duration and location of gall development of the investigated aphid species

Species	Month of gall initiation	Duration of gall	Location of gall
<i>P. populi-caulis</i>	March	2–3 months	Base of leaf lamina
<i>P. populi-transversus</i>	April	3–4 months	Middle of petiole
<i>P. obesinymphae</i>	May	5–6 months	Base of leaf lamina

across Kingdom Fungi is on average 2.51 % with a standard deviation of 4.57 (Nilsson et al. 2008). All the nucleotide sequences obtained in the study have been deposited in GenBank under accession numbers KF530731–KF530752.

Data analysis

Analyses were performed on gall, leaf and petiole samples (the total number of galls sampled exceeded that of the leaves and petioles). We assessed fungal endophyte diversity by counting the number of different OTUs isolated from the plate of a single gall, leaf or petiole sample. Based on these counts, colonization frequency (CF), isolation rate (IR), relative frequency (RF), and similarity coefficient (SC) were calculated. CF is the fraction of sampled tissue with at least one fungal endophyte and IR describes the average number of fungal endophytes per sample (Petrini et al. 1982). CF was compared using a contingency analysis. Indices of abundance or composition between the galls of aphid species and surrounding plant tissues were analyzed as nonparametric equivalents of one-way ANOVAs and, in the case of count data, general linear models with Poisson-distributed variances. Whole model tests were followed by pairwise contrasts (Sokal and Rohlf 1995). All statistical analyzes were performed in JMP v. 7.01 (SAS, Cary, NC, USA). All reported *p*-values are two-tailed. RF is the frequency of a specific fungal morphotype relative to the total number of fungal endophytes (Su et al. 2010; Yuan et al. 2010). We calculated the similarity coefficient (SC) as $2w/(a + b)$ where *w* equals the sum of the lowest RF of species in common between samples, *a* is the CF of the first sample, and *b* is the CF of the second sample (Carroll and Carroll 1978). The similarity coefficient measures the overall resemblance of the fungal endophyte communities between two samples.

Results

Fungal endophyte communities in galls

A total of 423 fungal endophytes were isolated from 209 gall samples (69 *P. populi-caulis* galls, 69 *P. populi-transversus* galls, and 70 *P. obesinymphae* galls). Colonization frequencies of galls were uniformly high, ranging from 95.7 to 98.6 % (Table 3). However, the average number of fungal endophytes isolated from each gall (IR) differed across aphid species (Wilcoxon test, *df*=2, *p*<0.001). The galls of the petiole-galler *P. populi-transversus* had a significantly lower isolation rate (1.70) than that of *P. populi-caulis* and *P. obesinymphae* galls (2.22 and 2.16, respectively; Wilcoxon tests on pairwise contrasts of IR, *P. obesinymphae* vs. *P. populi-caulis*, *p*=0.69; *P. obesinymphae* vs. *P. populi-transversus*, *p*<0.002; *P. populi-caulis* vs. *P. populi-transversus*, *p*<0.0001).

A total of 19 distinct morphotypes were isolated from all galls. Given the 98 % similarity threshold for name assignment using ITS, 16 of 19 morphotypes were successfully grouped to OTUs based on sequence similarity (Table 4). More fungal endophyte OTUs were identified in *P. obesinymphae* galls (18 OTUs) than in *P. populi-caulis* or *P. populi-transversus* galls (both with 13 OTUs). The frequency of particular OTUs also differed between the galls of the three aphid species. For example, *Cladosporium* (Dothideomycetes: Capnodiales) was the most commonly isolated fungal endophyte in *P. obesinymphae* galls, but detected only at low rates in the other two species. While further sampling may uncover more fungal diversity in the galls of each aphid species, some OTUs in our survey were unique to the galls of a particular aphid species. For example, OTUs with affiliation to *Alternaria* (Dothideomycetes: Pleosporales), *Neofusicoccum parvum* (Dothideomycetes: Botryosphaerales), *Nigrospora* (Sordariomycetes: Trichosphaerales), and *Xylaria* (Sordariomycetes: Xylariales) were only found in *P. obesinymphae* galls, while *Collectotrichum gloeosporioides* (Sordariomycetes: Glomerellales) was isolated only from *P. populi-transversus* galls. No unique OTUs were cultured from *P. populi-caulis* galls. Overall, the species composition was most similar between *P. populi-caulis* and *P. populi-transversus* galls (81.02 %), while the species composition of *P. obesinymphae* galls was markedly distinct from the other two species (60.14 % and 64.93 %, respectively).

Fungal endophyte communities in poplars

Next, we asked how fungal endophytes vary across tissue (gall, petiole and leaf) in the three aphid species. In all, we isolated 155 fungal endophytes from 95 samples across the three aphid species (31 petioles, 32 galls, and 32 leaves; Table 5). First, we considered the joint effects of aphid species and tissue type on fungal endophyte numbers. No transformation of the fungal endophyte count data satisfied the requirements for a two-way ANOVA, thus we analyzed the data with a general linear model and Poisson-distributed variances. The overall model was not significant ($\chi^2=9.87$; *df*=8; *p*=0.28) and there was no significant interaction between aphid species and tissue type on fungal endophyte numbers. However, although the overall model was not significant, there was a significant effect of tissue type on fungal endophyte numbers ($\chi^2=6.89$; *df*=2; *p*=0.032). We, therefore, analyzed the relationship between tissue type and fungal endophyte numbers for each aphid species separately. For two of the three species, there were significant differences across tissues in the number of isolated fungal endophyte OTUs (*P. populi-caulis*: Wilcoxon test, $\chi^2=7.0$; *df*=2; *p*=0.03; *P. populi-transversus*, $\chi^2=11.02$; *df*=2; *p*<0.01). As above, the overall pattern seems to be driven by relatively

Table 3 Colonization frequency (CF) and isolation rate (IR) of endophytic fungi (EF) in galls of three different aphid species

Species	Tissue	No. of samples plated	No. colonized by EF	CF (%)	Total # of EF	IR
<i>P. populi-caulis</i>	Gall	69	68	98.6	153	2.22
<i>P. populi-transversus</i>	Gall	70	67	95.7	119	1.70*
<i>P. obesinymphae</i>	Gall	70	69	98.6	151	2.16

Asterisks indicate significant differences ($p < 0.05$)

impoverished state of fungal endophytes in the petiole tissue compared to the leaf or gall. In petiole tissue, not only was the number of unique endophytic OTUs recovered significantly lower, but the CF index was also significantly lower. The CF index measures the percentage of plant tissue colonized by fungal endophytes, and ranged from 80.6 % in the petiole, to 96.9 % in the gall and in the 100 % in the leaf (Table 5; $\chi^2=10.62$, $df=2$, $p < 0.001$). When all tissues were analyzed together, regardless of the aphid species, the number of fungal endophytes in the petiole was significantly lower than in the gall or leaf (Wilcoxon multiple comparisons test; leaf vs. gall, Z score = -1.06, $p = 0.29$; petiole vs. leaf, Z score = -2.92,

$p = 0.004$; petiole vs. gall, Z score = -3.53, $p = 0.0004$). Independent contrasts of the number of fungal endophytes between tissues in each species revealed the same pattern: petiole tissue in poplars harbors fewer fungal endophytes than leaf tissue or gall tissue.

However, while petioles may be impoverished, it is the gall that is most distinctive in terms of fungal endophyte composition. In particular, there is a notable contrast between the gall and the plant tissue from which it was formed. For each aphid species, the similarity coefficients were quite small: *P. populi-caulis* gall vs. leaf 47.09 %; *P. obesinymphae* gall vs. leaf 46.76 %; *P. populi-transversus* gall vs. petiole 35.29 %. However, regardless of species, leaf and gall tissues were the most different, with a similarity coefficient of 66.35 % (Table 6). Moreover, while the most common OTUs in all plant tissue types were *Peniophora cinerea* (Agaricomycetes: Russulales), *Phoma putaminum* (Dothideomycetes: Pleosporales), *Phlebia* (Agaricomycetes: Corticiales) and *Glomerella acutata* (Sordariomycetes: Glomerellales), many OTUs were found to be unique to a specific tissue type. *Coniochaetaceae* (Sordariomycetes: Coniochaetales) and *Neofusicoccum parvum* (Dothideomycetes: Botryosphaerales) were only isolated from petiole tissue. *Xylaria* and a not namable OTU were found exclusively in the gall, and *Blakeslea trispora* (Mucorales),

Table 4 Relative frequency of fungal OTUs in galls of three aphid species

Class	Fungal OTU	Relative frequency (%)			
		<i>P. populi-caulis</i>	<i>P. populi-transversus</i>	<i>P. obesinymphae</i>	Total
Dothideomycetes	<i>Phoma putaminum</i>	25.49	19.33	12.58	19.15
Sordariomycetes	<i>Glomerella acutata</i>	18.95	9.24	11.92	13.71
Agaricomycetes	<i>Peniophora cinerea</i>	14.38	20.17	16.56	16.79
Agaricomycetes	<i>Phlebia</i>	11.76	15.97	5.30	10.64
Dothideomycetes	<i>Cladosporium cladosporioides</i>	6.54	4.20	3.31	4.73
Dothideomycetes	<i>Cladosporium</i>	5.88	6.72	19.88	11.11
	Unknown morphotype 1	5.23	8.40	0.66	4.49
Dothideomycetes	<i>Aureobasidium pullulans</i>	5.23	4.20	0.66	3.31
Dothideomycetes	<i>Epicoccum nigrum</i>	2.61	2.52	0.66	1.89
Sordariomycetes	<i>Plectosphaerella</i>	1.31	1.68	5.30	2.84
Exobasidiomycetes	<i>Malassezia restricta</i>	1.31	0.00	2.65	1.42
Agaricomycetes	<i>Schizophyllum commune</i>	0.65	5.04	6.62	4.02
	Unknown morphotype 2	0.65	0.00	0.66	0.47
Sordariomycetes	<i>Colletotrichum gloeosporioides</i>	0.00	1.68	0.00	0.47
Sordariomycetes	<i>Xylariaceae</i>	0.00	0.84	0.66	0.47
Sordariomycetes	<i>Xylaria</i>	0.00	0.00	5.96	2.13
Dothideomycetes	<i>Alternaria</i>	0.00	0.00	5.30	1.89
Dothideomycetes	<i>Neofusicoccum parvum</i>	0.00	0.00	0.66	0.24
Sordariomycetes	<i>Nigrospora</i>	0.00	0.00	0.66	0.24
	Total no. of distinct morphotypes	13	13	18	19

OTUs are named on the basis of the names under which the best matching sequences were deposited

Table 5 Colonization frequency (CF) and isolation rate (IR) of endophytic fungi (EF) in different plant tissue types; combined for all aphid species

Species	Tissue	No. of samples plated	No. colonized by EF	CF (%)	Total # of EF	IR
Combined	Leaf	32	32	100.0	56	1.75
Combined	Gall	32	31	96.9	62	1.94
Combined	Petiole	31	25	80.6*	37	1.19*

Asterisks indicate significant differences ($p < 0.05$)

Colletotrichum gloeosporioides, *Nigrospora*, *Phomopsis* (Sordariomycetes: Diaporthales) and another not namable OTU were present only in leaf tissue (Table 7). However, the fungal endophytes isolated exclusively from a specific tissue type were isolated at a low frequency and could represent an artifact of sampling.

Discussion

Plant galls are abnormal growths that may represent sites of altered proliferation or colonization of fungal endophytes. Some endophytes can provide various beneficial services to woody plants. In particular, foliar fungal endophytes have been shown to have adverse effects on insect herbivores, either by deterring herbivory, slowing larval development, or reducing survivorship and fecundity of adults (Hartley and Gange 2009; Saikkonen et al. 2010). We surveyed the fungal endophyte diversity associated with the galls of three species of gall-forming aphids on poplars. We found that the fungal endophyte composition differed between the galls of aphid species, and that the site of gall induction is important in determining the IR and composition of fungal endophytes.

Fungal endophyte communities in galls

It is not known to what degree, if any, fungal endophytes alter the success of gall formation by aphids, or whether these aphids choose galling sites based on the fungal endophyte composition in leaves. Sedentary insects like gall-formers or leafminers may avoid high fungal endophyte space if endophytes negatively affect fitness. Wilson and Carroll (1997) found that the cynipid gall wasp, *Besbicus mirabilis* (Hymenoptera: Cynipidae), seems to avoid high fungal endophyte space on the leaf. It is likely, however, that all galling insects encounter fungal endophytes in their galls during feeding. Even if galling sites are chosen that are comparatively free of fungal endophytes, galling itself may promote fungal growth (Butin 1992; Faeth and Hammon 1997). In this study, we found that the colonization frequency of fungal endophytes in gall tissue was extremely high for all aphid species, ranging from 95.7 to 98.6 % (Table 3). This implies that the insects are likely encountering many fungal endophytes, though we do not know what effects the fungal endophytes of poplars may have on *Pemphigus*. Comparisons

to other studies of related species may be useful as guides, but the tripartite relationship between an insect, a fungus and a vascular plant is complex and can depend on the particular species involved (Shorthouse and Rohfritsch 1992; Wilson 1995; Raman 2012; Raman et al. 2012).

The profiles of the fungal endophyte communities differ between the galls of the three aphid species. The leaf-gallers *P. populi-caulis* and *P. obesinymphae* both have a significantly higher IR than the petiole-galler, *P. populi-transversus*, which is consistent with the lower IR of petiole tissue itself. Fungal endophytes may be ecologically less dense or abundant in both the petiole tissue and petiole galls. Although IR is much higher in the galls of the leaf-galling aphid species, we found that they do not share the most similar fungal endophyte communities. *P. obesinymphae* galls contain a larger number of distinct fungal morphotypes (Table 4), while the fungal endophyte profile of *P. populi-caulis* galls is more similar to that of the petiole galls of *P. populi-transversus*. Possibly, the aphids themselves may be infecting the gall with different fungal endophytes. Aphids are notorious vectors of plant

Table 6 Similarity of the endophyte community among different plant tissues for all aphids and separated by aphid species

Tissue comparison	SF (%)
Leaf vs. gall	66.35
Leaf vs. petiole	77.66
Gall vs. petiole	74.66
Tissues divided by species	SF (%)
<i>P. populi-caulis</i> leaf vs. petiole	67.23
<i>P. populi-caulis</i> leaf vs. gall	47.09
<i>P. populi-caulis</i> gall vs. petiole	55.27
<i>P. populi-transversus</i> leaf vs. petiole	39.22
<i>P. populi-transversus</i> gall vs. petiole	35.39
<i>P. populi-transversus</i> leaf vs. gall	69.29
<i>P. obesinymphae</i> leaf vs. petiole	55.73
<i>P. obesinymphae</i> gall vs. leaf	46.76
<i>P. obesinymphae</i> gall vs. petiole	60.68

Highlighted is the similarity between communities in the galls and the corresponding uninfected tissue

Table 7 Relative frequency of fungal OTUs in the three tissue types

Class	Fungal OTU	Relative frequency (%)		
		Leaf	Gall	Petiole
Agaricomycetes	<i>Peniophora cinerea</i>	26.79	16.13	21.62
Dothideomycetes	<i>Phoma putaminum</i>	19.64	24.19	13.51
Sordariomycetes	<i>Glomerella acutata</i>	12.50	9.68	10.81
Dothideomycetes	<i>Cladosporium</i>	8.93	4.84	2.70
Dothideomycetes	<i>Alternaria</i>	7.14	3.23	5.41
Dothideomycetes	<i>Epicoccum nigrum</i>	5.36	1.61	5.41
Agaricomycetes	<i>Phlebia</i>	3.57	16.13	5.41
Sordariomycetes	<i>Plectosphaerella</i>	3.57	3.23	5.41
Agaricomycetes	<i>Schizophyllum commune</i>	1.79	6.45	13.51
	Unknown morphotype 1	1.79	1.61	5.41
Dothideomycetes	<i>Aureobasidium pullulans</i>	1.79	0.00	0.00
Sordariomycetes	<i>Nigrospora</i>	1.79	0.00	0.00
Sordariomycetes	<i>Colletotrichum gloeosporioides</i>	1.79	0.00	0.00
Sordariomycetes	<i>Phomopsis</i>	1.79	0.00	0.00
	Unknown morphotype 3	1.79	0.00	0.00
Dothideomycetes	<i>Cladosporium cladosporioides</i>	0.00	8.06	2.70
Sordariomycetes	<i>Xylaria</i>	0.00	3.23	0.00
	Unknown morphotype 2	0.00	1.61	0.00
Dothideomycetes	<i>Neofusicoccum parvum</i>	0.00	0.00	2.70
Not assigned	<i>Blakeslea trispora</i>	0.00	0.00	2.70
Sordariomycetes	<i>Coniochaetaceae</i>	0.00	0.00	2.70
	Total no. of distinct morphotypes	15	13	14

OTUs are named on the basis of the names of the best BLAST hits

viruses (Nault 1997; Andret-Link and Fuchs 2005), but it is not known if fungal pathogens are also transmitted by aphids.

The fungal endophyte composition of the galls of *P. obesinymphae* is distinct from the other two aphid species, even though both *P. obesinymphae* and *P. populi-caulis* share similar sites for gall induction at the base of the leaf lamina. We suspect that seasonal differences in the life histories of these aphids may also contribute to these differences. Gall-forming insects often have complex life histories that are closely matched to the seasonal schedules of their host plants. Some aphid species, like *P. populi-caulis* and *P. populi-transversus*, alternate between woody and herbaceous host plants. In these, aphids return to their woody hosts in the autumn, where a sexually-produced egg is deposited and persists over the winter months until the following spring. *P. obesinymphae*, by contrast, overwinters as adults, and the sexual generation is therefore delayed until the spring. Thus, while *P. obesinymphae* and *P. populi-caulis* are both leaf-gallers, they are forming galls on seasonally and developmentally distinct foliar tissue. Seasonal variation in fungal endophyte communities is well-described (Pehl and Butin 1994; Faeth and Hammon 1997; Wei et al. 2007). It is possible that the distinct *P. obesinymphae* profile is due to either seasonal differences (fungal endophytes of poplars

differ from spring to summer), or that the summer flush leaves themselves actively recruit distinct fungal endophytes because of intrinsic differences from spring flush leaves.

The similarity coefficients suggest, however, that the distinctiveness of fungal communities inhabiting galls of different aphid species is not solely explained by seasonal or developmental traits of the poplar leaves they attack. The leaves associated with the galls of the three species are equally distinct in terms of similarity coefficients, regardless of the season in which they flush. The IR from *P. populi-transversus* galls is significantly lower than either of the other two species, even that of the seasonally synchronous *P. populi-caulis*. All three species exhibit fungal endophyte profiles that are more distinct from their associated plant tissue (*P. obesinymphae* and *P. populi-caulis* vs. leaves; *P. populi-transversus* vs. petioles) than different tissue types are to each other (e.g., between leaves and petioles). Thus, galls hold a different fungal endophyte community compared to the surrounding plant tissue.

Fungal endophyte communities in plant tissue

Because of the economic importance of poplars, there has been some study of the endophytic community of their foliar

tissues (Bailey et al. 2005; Santamaria and Diez 2005; Doty et al. 2009; Albrechtsen et al. 2010; Martín-García et al. 2011). We isolated many OTUs with affiliation to fungal species previously described to occur in poplars, such as *Alternaria*, *Aureobasidium pullulans* (Dothideomycetes: Dothideales), *Cladosporium*, *Cladosporium cladosporioides*, and *Epicoccum nigrum* (Dothideomycetes). Most of the common fungal OTUs were shared across plant tissues, indicating the cosmopolitan nature of many fungal endophytes (Table 7).

However, we found differences in CF, IR and number of distinct fungal endophytes in petiole tissue compared to that of the leaf or gall (Table 5). Previous work has found similar disparities in the fungal endophyte CFs of leaves and petioles (Mishra et al. 2012), but the pattern appears to be specific to the plant species (Suryanarayanan and Vijaykrishna 2001; Kumar and Hyde 2004). Variation in the fungal endophyte composition of plant tissues is common (Rodríguez et al. 2009).

In conclusion, our study constitutes the first comparative description of the natural communities of fungal endophytes in poplar galls. In galls of each of the aphid species, which are closely-related and share a common host plant and many ecological and life history characteristics, pair-wise comparisons between leaf, petiole and gall tissue indicated that galls were distinct. Gall-forming insects typically exhibit highly specialized, tissue-specific preferences for gall formation. Our results suggest that insect galls provide distinct opportunities for colonization or proliferation of non-overlapping sets of fungal endophytes on plants (Table 7). It has been suggested that insect-induced plant modifications, like galls, can affect biodiversity at higher trophic levels by adding habitat complexity and facilitating opportunities for finer niche partitioning. For example, Waltz and Whitham (1997) showed that the presence of the galls of another *Pemphigus* species, *P. betae*, corresponded to an increase in arthropod diversity. Similar results have been described in leafrollers and sawflies (Martinsen et al. 2000; Bailey and Whitham 2003). Our results suggest that the effects of galls on diversity extend not only to higher trophic levels, but downward to the fungal endophyte communities as well. The degree to which galls represent more than small-scale features amidst a large set of factors governing tree fungal endophyte ecology, and rather act as persistent drivers of fungal community structure and co-evolutionary change, would benefit from further study.

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