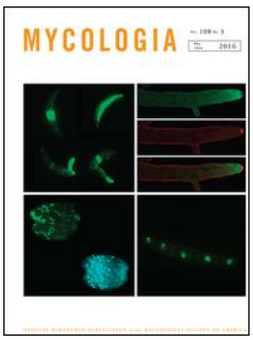

Mycologia

ISSN: 0027-5514 (Print) 1557-2536 (Online) Journal homepage: http://www.tandfonline.com/loi/umyc20

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To cite this article: Aaron S. David, Gina L. Quiram, Jennie I. Sirota & Eric W. Seabloom (2016) Quantifying the associations between fungal endophytes and biocontrol-induced herbivory of invasive purple loosestrife (Lythrum salicaria L.), Mycologia, 108:4, 625-637, DOI: 10.3852/15-207

To link to this article: http://dx.doi.org/10.3852/15-207

Mycologia, 108(4), 2016, pp. 625–637. DOI: 10.3852/15-207

Issued 2 September 2016

Quantifying the associations between fungal endophytes and biocontrol-induced herbivory of invasive purple loosestrife (*Lythrum salicaria* L.)

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Abstract: Fungal endophytes are one of several groups of heterotrophic organisms that associate with living plants. The net effects of these groups of organisms on each other and ultimately on their host plants depend in part on how they facilitate or antagonize one another. In this study we quantified the associations between endophyte communities and herbivory induced by a biological control in the invasive *Lythrum salicaria* at various spatial scales using a culture-based approach. We found positive associations between herbivory damage and endophyte isolation frequency and richness at the site level and weak, positive associations at the leaf level. Herbivory damage was more strongly influenced by processes at the site level than were endophyte isolation frequency and community structure, which were influenced by processes at the plant and leaf levels. Furthermore, endophytic taxa found in low herbivory sites were nested subsets of those taxa found at high herbivory sites. Our findings suggest that endophyte communities of *L. salicaria* are associated with, and potentially facilitated by, biocontrol-induced herbivory. Quantifying the associations between heterotrophic groups ultimately may lead to a clearer understanding of their complex interactions with plants.
Key words: community assembly, host-microbe interactions, microbial symbiont, nestedness, spatial structure

Introduction

Fungal endophytes, a diverse group of organisms found living within tissues of all major plant lineages, are one of several groups of heterotrophic taxa that use living plants for resources or reproduction (Petrini 1991, Bennett 2013, Borer et al. 2013). Endophytes may interact with other heterotrophic groups, such as mycorrhizal fungi, pathogens or herbivores, and these interactions ultimately may scale up to community or ecosystem-wide effects (van der Putten et al. 2001, Wardle et al. 2004, Bennett 2013, Busby et al. 2015). In particular, herbivores that consume plant material may interact positively or negatively with endophytes, and the net effects of these interactions have important consequences for herbivore and endophyte communities, as well as for the host plant (e.g. van der Putten et al. 2001, de la Peña et al. 2006, de Vos et al. 2006, Rudgers and Clay 2008, Humphrey et al. 2014).

Positive or negative associations between endophytes and herbivores observed in natural settings may be suggestive of facilitation or antagonism caused by one or more direct or indirect mechanisms. For instance, facilitation between herbivores and endophytes could arise if a plant is unable to defend against both, as has been reported between herbivores and bacteria inhabiting leaf surfaces (Humphrey et al. 2014). Herbivores may facilitate the entry of endophytes that require natural openings or wounds to colonize (Petrini 1991, Hatcher 1995, Quadt-Hallmann 1997) or may contribute to the dispersal of endophytes among host individuals (Devarajan and Suryanarayanan 2006). Antagonism of herbivores by endophytes is well documented and is often attributed to the production of secondary metabolites (Albrectsen et al. 2010, van Bael et al. 2012, Yang et al. 2012). For example, clavicipitaceous endophytes (e.g. *Epichlöe*) can alter herbivore communities and reduce herbivory through production of alkaloids (Schardl et al. 2004, Rudgers and Clay 2008). Conversely, herbivory can also induce plant defenses that reduce endophytes' abilities to infect plants (de Vos et al. 2006).

The spatiotemporal scale at which endophyte-herbivore interactions are considered also may be important for determining the strength and direction of the interaction. At the leaf level, facilitation or antagonism couldarise via direct contact between endophytes and herbivores (Schardl et al. 2004, de Vos et al. 2006, Rudgers and Clay 2008). At the plant level, either endophytes or herbavores might trigger host defenses that indirectly facilitate or antagonize the other (Humphrey et al. 2014). Herbivores and endophytes also may disperse easily between closely spaced leaves of

Submitted 22 Jul 2015; accepted for publication 4 Apr 2016.
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the same individual plant, and therefore individual plants could exhibit some degree of homogeneity with regards to herbivory and endophyte community (Grünig et al. 2002, Gripenberg and Roslin 2005, Barber and Marquis 2011, Cordier et al. 2012). At the site level, metapopulations or metacommunities of herbivores and endophytes may indicate habitat or climate suitability (Tack et al. 2010, Blaalid et al. 2014, Higgins et al. 2014). Finally, sites experiencing similar histories with respect to herbivory might contain similar endophyte taxa if local populations of these taxa have increased through time.

In natural settings the strength and direction of endophyte-herbivore interactions may leave their imprint on the direction of their correlation and spatial structure (sensu McIntire and Fajardo 2009). By observing patterns between endophytes and herbivores we may find evidence of facilitation or antagonism between these groups. Moreover, endophyte communities—often structured by environmental conditions or host preference (Arnold 2007, Saunders et al. 2010, Zimmerman and Vitousek 2012, David et al. 2015)—could be structured in the context of herbivory. We considered two ways in which this effect might be inferred with observational data. First, endophyte communities may exhibit patterns of non-randomness across leaves, individual plants or sites. Non-randomness patterns could suggest facilitative or antagonistic interactions among endophyte species (e.g. Pan and May 2009) or a common response to an external factor such as herbivory. Second, changes in endophyte community structure along a gradient of herbivory damage could arise either from the addition of new species to the existing community or from a compositional turnover in species. It therefore is useful to evaluate whether endophyte communities within the context of herbivory are nested—that is the extent to which the taxa inhabiting sites (or individual plants or leaves) of lower endophyte richness are composed of subsets of the taxa inhabiting sites of higher endophyte richness (Almeida-Neto and Guimaraes 2008). For instance, if herbivory were positively associated with endophyte diversity, would the taxa found in leaves with low herbivory (and hence low endophyte diversity) be distinct from those found in leaves with high herbivory (and high endophyte diversity), or would the taxa of the former be a subset of the latter? A more complete compositional turnover among leaves exhibiting low vs. high herbivory might suggest systemic effects of herbivores on endophyte communities. In contrast, if endophyte communities of leaves exhibiting low herbivory are perfectly nested within those exhibiting high herbivory, this finding might suggest there is a base community of endophytes found in all leaves and that the remaining endophytic taxa colonize after herbivory.

Systems with an invasive plant species and an introduced biological control agent are useful for investigating these questions because they contain a simplified food web consisting of a specialized plant-herbivore interaction. Furthermore, novel plant-herbivore-microbe interactions could promote or reduce the impact of herbivory on the invasive plant in the introduced range relative to the native range (Bennett 2013). However, it is not yet understood how biological control agents may interact with endophytes residing in the invasive plant they were introduced to control (Hayes et al. 2013). It has been proposed that endophytes may have an antagonistic relationship with herbivore biocontrol agents (Evans 2008, Newcombe et al. 2009) and that these interactions may vary over space and time (Tscharntke et al. 2008).

In this study we sampled endophytes from leaves of the invasive wetland perennial forb *Lythrum salicaria* L. (purple loosestrife) in sites experiencing varying degrees of herbivory caused by leaf-chewing beetles (*Galerucella pusilla* and *G. calmariensis*) introduced as biological control agents in Minnesota USA (Quiram 2013). Using a culture-based approach we characterized endophyte communities in six wetlands composed of near monocultures of *L. salicaria* and histories of high or low *Galerucella* spp. herbivory (Quiram 2013) to address the following questions: (i) What is the relationship between herbivory damage and endophyte abundance and richness at different spatiotemporal scales? (ii) At what spatiotemporal scale is each influenced? (iii) Do endophyte communities exhibit patterns of non-randomness suggestive of facilitation or antagonism among endophyte species? (iv) Are endophyte communities nested along a gradient of herbivory, suggestive of a base community of endophytes that accumulates species with herbivory? While this observational study cannot determine causality, identifying patterns between endophytes and herbivory does provide insights into their interactions.

MATERIALS AND METHODS

Study system.—Lythrum salicaria was first introduced to North America in the early 1800s and is considered an invasive species in wetlands throughout the United States and Canada (Malecki et al. 1993). *L. salicaria* typically grows in dense monocultures that degrade wetland structure and function, outcompete native plants and endanger wildlife (Malecki et al. 1993). Beginning in 1992 two leaf-chewing beetles, *Galerucella pusilla* and *G. calmariensis*, were introduced as biological control agents to consume *L. salicaria* in USA (Malecki et al. 1993). Since then, *Galerucella* introductions in Minnesota have had variable success in establishing and subsequently controlling *L. salicaria* populations (Quiram 2013). We characterized endophyte communities from six sites in southeastern Minnesota within a 75 km radius that ranged in their biological control efficacy (DATA SUPPLEMENT 1). Three sites—Circle Lake (*CL*), Pig's Eye (*EY*) and Winona

(*WI*)—were chosen for their historically high herbivory, and three sites—Dodge Center Nature Preserve (*DC*), Hall's Marsh (*HM*) and Pottery Pond (*PP*)—for their historically low herbivory (Quiram 2013). Historical herbivory was defined based on 10 y survey data of biocontrol agent presence and feeding damage to *L. salicaria* leaves completed 1998–2010 (Quiram 2013). Historically high herbivory sites were defined as wetlands with abundant biocontrol agents and 50% or greater leaf damage characteristic of *Galerucella* species feeding, and historically low herbivory sites were defined as wetlands with few or no biocontrol agents present and minimal leaf damage (Quiram 2013).

Sampling.—In Jun 2011 we sampled endophyte communities from leaves of 16 *L. salicaria* plants within each site. Of these 16 plants 10 were randomly selected along a 30 m transect, and six were haphazardly chosen along the transect, three of which exhibited relatively low herbivory and three of which exhibited relatively high herbivory for the site. This design let us sample across a broad range of plant herbivory within sites. Endophytes typically are cultured from healthy, undamaged leaves (e.g. Arnold et al. 2003, Zimmerman and Vitousek 2012, David et al. 2015), yet this was not possible at these sites because most leaves exhibited some degree of herbivory. We instead selected two leaves for culturing that exhibited the least amount of herbivory for each plant from the mid to upper stem. These leaves were photographed before culturing to measure the leaf-level herbivory. Two additional haphazardly chosen leaves from each plant were photographed in the field to measure plant-level herbivory.

Leaf damage.—Photographs of leaves from the field were analyzed for damage with ImageJ software (Abràmoff 2004). We measured the total leaf area and total area damaged to calculate percent herbivory for each plant. Where leaf margins had been removed by herbivores we approximated the previous tissue assuming a lanceolate cordate shape typical of *L. salicaria* leaves (Mal et al. 1992).

Culturing.—To characterize endophyte communities, we used a culture-based approach. While culture-based approaches may have biases toward certain groups of fungi, particularly fast-growers and against others such as obligate biotrophs, other methodologies such as next-generation sequencing have their own biases toward other groups of fungi (Arnold et al. 2007, Nguyen et al. 2014). Despite these biases, studies involving comparisons of endophyte diversity or community structure have found similar results with both culture-dependent and independent methods (Arnold et al. 2007, Pan and May 2009).

We sampled endophytes from two leaves of each plant. Leaves were surface-sterilized with a rinse in sterile, deionized water, followed by successive baths in 70% ethanol (1 min), 70% bleach (3.675% NaOCl; 2 min), 70% ethanol (1 min) and a second rinse in sterile DI water (David et al. 2015). Following surface-sterilization, leaves were cut into five segments (total area ~ 11mm^2) and were placed in Petri dishes containing one of three types of nutrient media. For the 10 randomly sampled plants, leaf segments were placed on 2% potato dextrose agar, 2% malt extract agar or 10% malt extract agar (2 leaves × 3 media types × 5 segments per plate = 30 total segments per plant). Three media types were used for this subset of samples to determine whether fungal richness depended on the media used, but we found no evidence for this (d.f. = 2, χ^2 = 1.14, p = 0.565). For the other six plants collected (representative of low and high site herbivory) segments were plated on only 2% malt extract agar (2 leaves × 1 media type × 5 segments per plate = 10 total segments per plant). We plated tissue from the interior sections of plant leaves away from chewed sections and leaf margins to avoid sampling fungi that may not have established within the leaf. We verified surface-sterilization effectiveness with leaf prints of sterilized tissue pressed upon 2% malt extract agar plates (Fröhlich et al. 2000). Cultures were monitored 2 mo after plating, and emergent fungi were subcultured onto separate Petri dishes with 2% malt extract agar.

Endophyte classification.—We sequenced fungal isolates and classified them into operational taxonomic units (OTUs). DNA was extracted from isolates with the REDExtract N' Amp Kit (Sigma Aldrich Corp., Saint Louis, Missouri). We amplified the internal spacer region and a portion of the large subunit as a single fragment with the ITS1-F (Gardes and Bruns 1993) and LR3 primers (Vilgalys and Hester 1990) with 20 μL reactions (10 μL REDExtract N' Amp Mix, 0.8 μL (10 μM) each of forward and reverse primers, 4.4 μL water, and 4 μL DNA template; PCR conditions consisted of an initial denaturation step at 95 C for 3 min, 35 cycles of 95 C for 30 s, 54 C for 30 s, 72 C for 1 min and an additional 10 min extension at 72 C). We sequenced the amplified DNA with ITS1-F primer, which resulted in ~700 bp fragments. Sequences were trimmed with Geneious Pro 5.5.7 (Kearse et al. 2012) to exclude regions with >5% error per base and to allow a maximum of 10 low quality bases and six ambiguities. We clustered sequences with a workflow (Monacell and Carbone 2014) that extracted the ITS1 and ITS2 regions using ITS extractor (Nilsson et al. 2010), checked for chimeras with UChime (Edgar et al. 2011) and clustered sequences based on alignment-free similarity with ESPRIT (Sun et al. 2009) and MOTHUR (Schloss et al. 2009). We used the 97% similarity for all subsequent analyses but obtained similar results when OTUs were clustered at the 95% and 90% similarity. We assigned taxa to our OTUs with the naïve Bayes classifier in MOTHUR (minimum confidence allowed = 0.60) implemented through QIIME (Caporaso et al. 2010). We classified taxa based on the accessions within a combined database of the UNITE (Abarenkov et al. 2010) and the Emerencia databases (Ryberg et al. 2009). We calculated species accumulation curves to evaluate how well our sampling captured overall endophyte OTU richness and richness within each of the six sites.

Data analysis.—Leaf- and plant-level herbivory were highly correlated (Pearson's r = 0.825, t = 20.1, d.f. = 190, $P < 0.001$), and therefore we only included leaf-level herbivory in subsequent analyses. Site-level herbivory was calculated as the average herbivory of the 10 randomly selected plants (excluding those plants specifically chosen for low or high

TABLE I. Operational taxonomic units (OTUs) with their overall isolation frequencies and taxonomic assignments

OTU	No. isolations	Phylum	Class	Order	Family	Taxon assignment	Confidence
OTU28	4	Ascomycota	Dothideomycetes	Capnodiales	Mycosphaerellaceae	*Cercospora* sp.	1.00
OTU7	4	Ascomycota	Dothideomycetes	Capnodiales	Davidiellaceae	*Cladosporium pseudocladosporioides*	0.97
OTU26	2	Ascomycota	Dothideomycetes	Capnodiales	Mycosphaerellaceae	*Stenella lythri*	1.00
OTU3	1	Ascomycota	Dothideomycetes	Dothideales	Dothioraceae	*Aureobasidium pullulans*	0.99
OTU12	1	Ascomycota	Dothideomycetes	Myriangiales	Elsinoaceae	*Sphaceloma bidentis*	0.65
OTU1	74	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	*Alternaria* sp.	1.00
OTU22	2	Ascomycota	Dothideomycetes	Pleosporales	Leptosphaeriaceae	*Leptosphaeria microscopica*	1.00
OTU9	5	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	*Lewia infectoria*	0.88
OTU2	2	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	*Nimbya* sp.	0.93
OTU31	1	Ascomycota	Dothideomycetes	Pleosporales	Incertae sedis	*Periconia* sp.	1.00
OTU19	1	Ascomycota	Dothideomycetes	Pleosporales	Incertae sedis	*Peyronellaea* sp.	0.82
OTU15	1	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	*Phaeosphaeria pontiformis*	0.73
OTU10	3	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	*Phaeosphaeria* sp.	0.99
OTU21	6	Ascomycota	Dothideomycetes	Pleosporales	unidentified	*Pleosporales* sp.	0.99
OTU6	1	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	*Pyrenophora triticirepentis*	1.00
OTU32	2	Ascomycota	Dothideomycetes	Pleosporales	Massarinaceae	*Saccharicola bicolor*	0.99
OTU35	1	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	*Stagonospora pseudocaricis*	0.94
OTU20	6	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	*Aspergillus insuetus*	0.99
OTU34	4	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	*Penicillium funiculosum*	0.98
OTU14	23	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	*Penicillium* sp.	1.00
OTU30	1	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	*Penicillium steckii*	1.00
OTU23	2	Ascomycota	Leotiomycetes	Helotiales	Incertae sedis	*Cadophora luteoolivacea*	0.77
OTU33	1	Ascomycota	Leotiomycetes	Helotiales	Sclerotiniaceae	Sclerotiniaceae sp.	1.00
OTU24	1	Ascomycota	Sordariomycetes	Diaporthales	Diaporthaceae	*Phomopsis* sp.	0.92
OTU11	2	Ascomycota	Sordariomycetes	Microascales	Halosphaeriaceae	Halosphaeriaceae sp.	1.00
OTU27	1	Ascomycota	Sordariomycetes	Microascales	Halosphaeriaceae	Halosphaeriaceae sp.	0.82
OTU36	1	Ascomycota	Sordariomycetes	Sordariales	Unidentified	Sordariales sp.	1.00
OTU13	2	Ascomycota	Sordariomycetes	Xylariales	Xylariaceae	*Biscogniauxia mediterranea*	1.00
OTU5	5	Ascomycota	Sordariomycetes	Xylariales	Xylariaceae	*Hypoxylon submonticulosum*	1.00
OTU17	1	Ascomycota	Sordariomycetes	Xylariales	Xylariaceae	*Nemania* sp.	0.67
OTU8	1	Ascomycota	Sordariomycetes	Xylariales	Amphisphaeriaceae	*Seimatosporium discosioides*	0.91
OTU29	1	Ascomycota	Sordariomycetes	Xylariales	Unidentified	Xylariales sp.	1.00
OTU16	1	Ascomycota	Unidentified	Unidentified	Unidentified	Ascomycota sp.	1.00
OTU25	1	Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	*Coprinellus micaceus*	1.00

TABLE I. Continued.

OTU	No. isolations	Phylum	Class	Order	Family	Taxon assignment	Confidence
OTU4	5	Basidiomycota	Agaricomycetes	Polyporales	Meruliaceae	*Irpex lacteus*	1.00
OTU18	1	Basidiomycota	Agaricomycetes	Russulales	Peniophoraceae	*Peniophora cinerea*	0.89

OTUs were clustered based on 97% similarity of the ITS1 and ITS2 regions. Isolations were measured as the number of emergent fungal cultures that clustered into that OTU. Taxonomy was assigned with the naïve Bayes classifier in MOTHUR using the combined UNITE and Emerencia databases. Confidence is the bootstrap confidence score (minimum allowed = 0.60).

herbivory) within a site. We analyzed differences in site-level herbivory with ANOVA, and we used a *t*-test to assess whether measured site-level herbivory differed between sites with low and high historical herbivory.

We calculated endophyte isolation frequency (a measure of abundance) as the number of emergent fungi isolated from each leaf or site divided by the total number of surface-sterilized leaf segments analyzed for that leaf or site. We used generalized linear mixed-effects models with binomial error to investigate the relationships between isolation frequency and site- and leaf-level herbivory damage using the nested random effects of historical herbivory, site and (for the leaf-level analyses) plant. Significance was determined by removing the herbivory damage term and comparing models with likelihood ratio tests, and we report χ^2 test statistics and p values from these model comparisons in the results. For those leaves containing at least one OTU, OTU richness was analyzed with similarly nested linear mixed-effects models with maximum likelihood for the site and leaf levels. OTU richness was scaled by the sampling effort and log-transformed. For both isolation frequency and OTU richness we conducted separate analyses for each site to determine whether among-site patterns also were detected within sites. We also investigated the effect of leaf herbivory on the probability of isolating the two most common endophytes (OTU1,OTU14) using logistic generalized linear mixed-effects models with analysis of deviance to test for significance.

To investigate the influence of varying spatiotemporal scales, we used variance components analysis to quantify the variation of leaf herbivory, isolation frequency and endophyte community explained at the hierarchical levels of historical site herbivory (high or low), site, plant and residuals. We interpreted the residuals as the variance explained by leaves because each leaf represented one sampling unit. Herbivory damage was analyzed with a nested mixed-effects model with restricted maximum likelihood. Isolation frequency was analyzed as a nested generalized linear mixed-effects model with binomial error. Endophyte communities were analyzed with permutational analysis of variance (100 000 permutations) using historical herbivory, site and plant as fixed-effects, and the sum of squares were used to determine proportion of variance explained.

We investigated endophyte community structure in two ways. First, we considered whether endophyte communities exhibited non-randomness indicative of facilitation or antagonism among endophyte OTUs. We analyzed the number of checkerboard units (i.e. the number of times an OTU was only present in a first plant but not a second, and a second OTU was present in a second plant but not the first) and compared it to a fixed column-fixed row null model with the swap algorithm (Stone and Roberts 1990). We tested for non-randomness at the levels of the leaf, plant, site and plants within a site. Second, we investigated nestedness of endophyte communities along the herbivory damage gradient and evaluated the nestedness metric based on overlap and decreasing fill (NODF) of the endophyte community matrix at the levels of the leaf, plant, site and plants within sites (Almeida-Neto and Guimaraes 2008). NODF has significant advantages over other nestedness indices in that it is a more conservative test less prone to Type I error (Ulrich

and Gotelli 2007, Almeida-Neto and Guimaraes 2008). To test for significance of nestedness, we compared the NODF index to simulated random communities with the *r1* null hypothesis, which uses the species marginal frequencies as probabilities (Wright 1998) and, unlike fixed-total null hypotheses, avoids Type II error (Ulrich and Gotelli 2013).

All statistical analyses were conducted with R 3.0.2 (R Development Core Team 2013) and the vegan (Oksanen et al. 2013) and lme4 packages (Bates et al. 2015). Data, including GenBank accession numbers (KT290958-KT291128) of all sequenced isolates, metadata and R script, are provided (DATA SUPPLEMENTS 2–6).

RESULTS

We found significant differences in leaf-level herbivory damage across sites ($F_{5,186} = 17.6$, $P < 0.001$). However, site-level herbivory damage differences did not match our a priori predictions based on consistent historical herbivory observed at the study sites 1998–2010 (Quiram 2013); there was no effect of historical herbivory on measured site-level herbivory damage ($t = 0.422$, d.f. = 4, $P = 0.695$). In particular, site *EY* had the least herbivory damage despite having historically high herbivory, while the historically low herbivory site *DC* had herbivory higher than expected. While the sites used had been observed to have consistent historical herbivory over 12 y, *Galerucella* populations can vary temporally and the impacts of biological control programs on *L. salicaria* populations can continue to change 10–20 y after the initial release of the agent (Blossey et al. 2001, Lindgren 2003).

In total we isolated 235 emergent fungi (10.9% isolation frequency). Of these we successfully sequenced the ITS1 and ITS2 regions of 171 isolates and classified them into 36 OTUs. The species accumulation curve did not plateau for most sites (DATA SUPPLEMENT 7), suggesting our sampling may not have fully captured the total richness in the system. Of the 36 OTUs, 18 were isolated only once and seven were isolated two times. The most common taxa were *Alternaria* and *Penicillium*, and the orders Pleosporales (Dothideomycetes) and Eurotiales (Eurotiomycetes) were particularly dominant (TABLE I). All OTUs were from the phylum Ascomycota with the exception of three OTUs from the order Agaricales in the phylum Basidiomycota.

We found positive relationships between herbivory damage and endophyte isolation frequency at site level (d.f. = 1, $\chi^2 = 37.9$, $P < 0.001$) and leaf level (d.f. = 1, $\chi^2 = 15.3$, $P < 0.001$; FIG. 1a–b). However, site *CL* had much higher herbivory damage than the other sites; we therefore re-analyzed the data without this site and we still found a significantly positive relationship at the site level (d.f. = 1, $\chi^2 = 33.2$, $P < 0.001$) but not at the leaf level (d.f. = 1, $\chi^2 = 2.43$, $P = 0.119$). Within sites there was a strong positive relationship between leaf-level herbivory damage and endophyte isolation frequency at the site *CL* (d.f. = 1, $\chi^2 = 7.16$, $P = 0.007$), but no significant relationships within any other site level ($P > 0.05$ for all other sites; FIG. 1b). Similarly we found a significant positive relationship between herbivory damage and OTU richness at the site level (d.f. = 1, $\chi^2 = 6.49$, $P = 0.011$) and a marginally-significant positive effect at the leaf level (d.f. = 1, $\chi^2 = 3.44$, $P = 0.064$; FIG. 1c–d). The site-level analysis was robust to removal of the high herbivory *CL* site (d.f. = 1, $\chi^2 = 5.53$, $P = 0.019$). There were no significant differences between leaf-level herbivory damage and OTU richness within sites (*PP* had insufficient data to run analysis, all other sites $P > 0.05$) (FIG. 1d). The probability of isolating the most common OTU, OTU1 (*Alternaria* sp.) increased with herbivory damage (d.f. = 1, $\chi^2 = 8.56$, $P = 0.003$), but we found no relationship for the second-most common OTU, OTU14 (*Penicillium sp.*) and herbivory damage (d.f. = 1, $\chi^2 = 1.72$, $P = 0.190$).

The variance components analysis revealed that herbivory damage and endophyte communities were mostly explained at the plant level, while endophyte isolation frequency was mostly explained at the leaf level (residuals) (FIG. 2). A substantial amount of variation for herbivory damage also was explained at the site level, while the site level did not explain much of the endophyte isolation frequency or community variation. Historical herbivory explained little variance for any of the response variables.

Endophyte communities did not show clear evidence of non-randomness at the site or plant levels, in that the *C*-score from the checkerboard analyses did not significantly differ from that of the null models (TABLE II). At the leaf level we found significantly fewer checkerboard units than would be expected from random, which is indicative of positive associations among endophyte taxa (TABLE II).

Endophyte communities were nested along the herbivory damage gradient at both the site and plant levels, such that sites or plants with less herbivory damage contained endophyte communities that were subsets of those in sites or plants with high herbivory damage (TABLE II, FIG. 3). This finding was robust to the removal of the common OTU1 at the site level (Null NODF = 30.0 NODF = 20.9, z = 3.43, $P < 0.001$) but not at the plant level (Null NODF = 4.38 NODF = 3.87, z = 0.816, $P = 0.405$). Both the site-level NODF (Null NODF = 30.0 NODF = 21.0, z = 3.54, $P < 0.001$) and the plant-level NODF analyses (Null NODF = 12.5 NODF = 9.14, z = 2.21, $P = 0.040$) were robust to the removal of the second-most common OTU14. We did not find evidence of nestedness among leaves, potentially due to low matrix fill caused by low endophyte richness within individual leaves (TABLE II). There was evidence of within-site nestedness for the

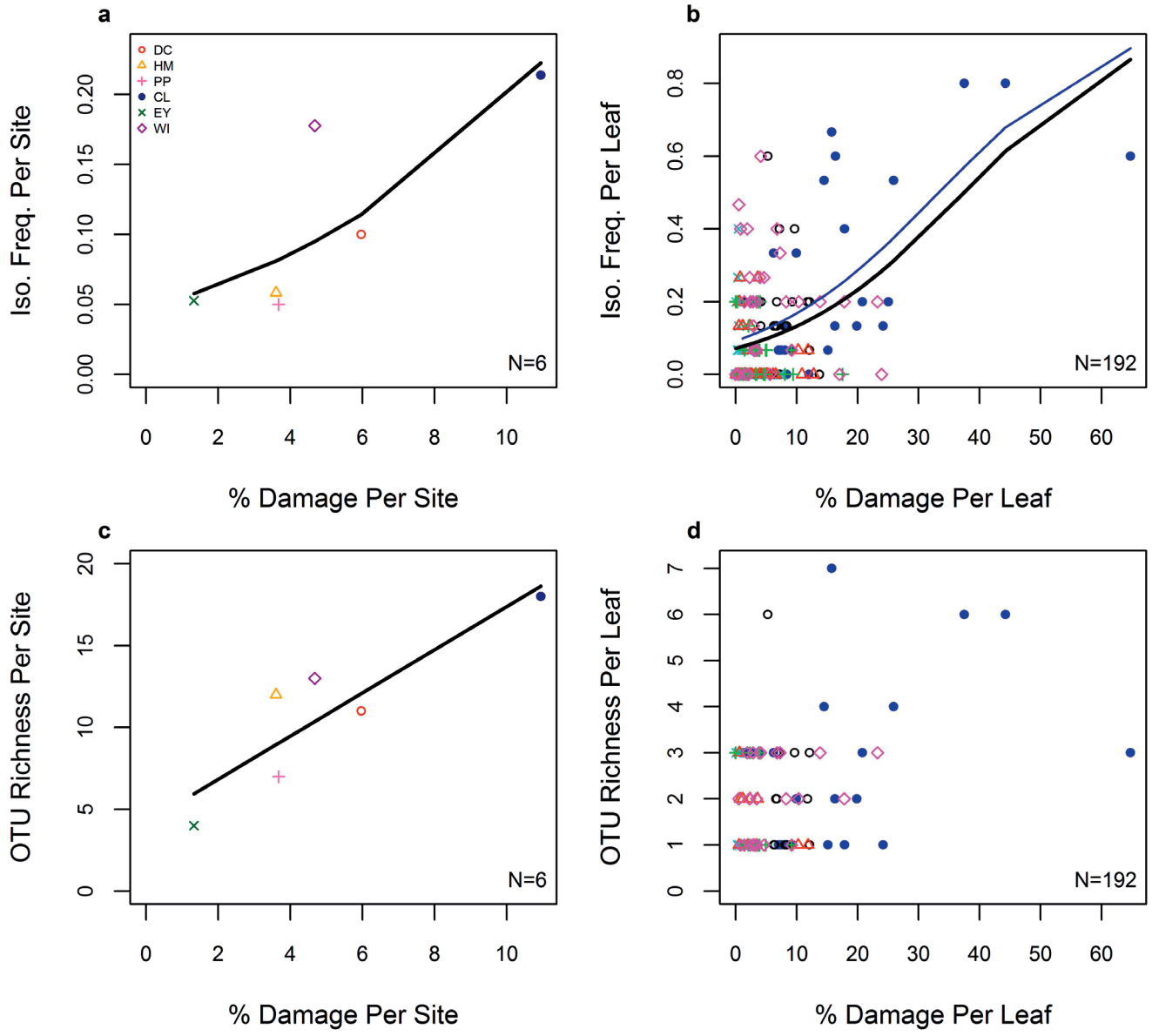

FIG. 1. Associations between endophytes and herbivory damage. Total endophyte isolation frequency at (a) the site level and (b) the leaf level and OTU richness shown at (c) the site level and (d) the leaf level. Thick solid black line shows overall significant trends using the predicted, back-transformed values of univariate generalized linear (a, b) or linear models (c). Significant within-site associations are in color. Because individual leaves were sampled with either five or 15 surface-sterilized tissue segments and thus were not directly comparable, the points (d) are scaled to be the expected richness per leaf for 15 segments.

sites exhibiting the highest herbivory damage (*CL* and *DC*), indicating that endophyte communities in low herbivory plants were subsets of those in high herbivory plants within these two sites (TABLE II).

DISCUSSION

In this study we investigated the associations between two heterotrophic groups—fungal endophytes and herbivores—in a natural setting at varying spatiotemporal scales. We report four key findings. First, herbivory damage and endophyte abundance, as measured by isolation frequency, were positively associated at the site and leaf levels. There were generally no associations between isolation frequency and herbivory damage within sites, with the exception of a positive association found at the site (*CL*) experiencing the greatest herbivory damage. The endophyte-herbivory relationship likely was influenced by the most common OTU, OTU1 *Alternaria* sp., whose probability of occurrence increased with herbivory. Second, we found evidence that herbivory damage occurred at a larger spatiotemporal scale than did endophyte isolation frequency or

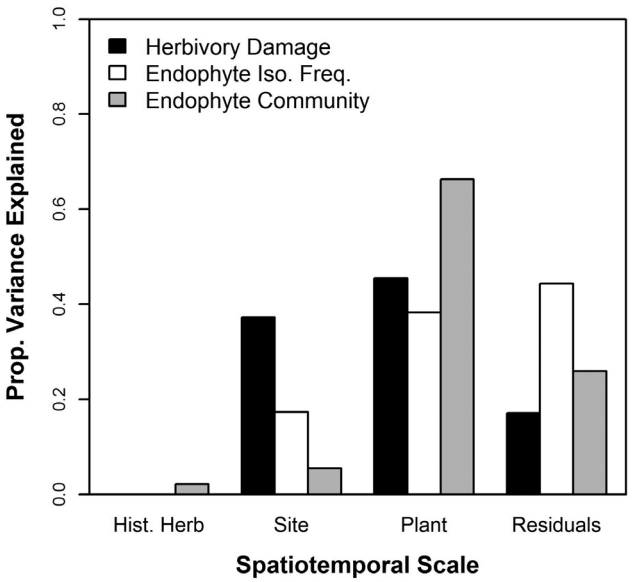

FIG. 2. Proportion of variance explained for leaf-level herbivory, endophyte isolation frequency and endophyte community at the spatiotemporal scales of historical herbivory, site, plant and leaf (residuals). Variance components were calculated from a linear mixed-effect model with restricted maximum likelihood for leaf herbivory, a generalized mixed-effects model with binomial error for endophyte isolation frequency and permutational ANOVA for endophyte community.

community structure. Herbivory damage was structured at the site and plant levels, while endophytes (isolation frequency and community) were structured at the plant and leaf levels. Historical herbivory of the site accounted for little of the variation for either herbivory or endophytes. Third, endophyte communities revealed patterns of non-randomness in the checkerboard analysis at the leaf level, indicative of overall positive associations among taxa but no evidence of non-randomness at the site of plant levels. Fourth, endophyte communities were nested along a gradient of herbivory damage, with taxa in low herbivory sites and plants representing nested subsets of those taxa found in high herbivory sites and plants. Taken together, we find no support for antagonism between endophytes and herbivory and instead find support for facilitation. However, while support for facilitation was evident across sites, there was less evidence of facilitation within sites, which we discuss below.

Our results contribute to a developing understanding of how endophyte communities are structured in natural settings. Recent work has revealed strong influences of the surrounding environment and dispersal (Saunders et al. 2010, Zimmerman and Vitousek 2012, Higgins et al. 2014, David et al. 2015), yet less attention has been directed toward the ways in which herbivores, in conjunction with environment and dispersal, might shape communities. While vertically transmitted clavicipitaceous endophytes are known to impede herbivory through the production of compounds such as alkaloids (Schardl et al. 2004) and alter herbivore communities (Rudgers and Clay 2008), such antagonism could be favored because of the close ecological and evolutionary relationship those particular endophytes have with their plant hosts. Most endophyte taxa, however, are horizontally transmitted and

TABLE II. Non-randomness and nestedness of endophyte communities along an herbivory gradient at different spatial scales

	N	Null C-score	C-score	z-statistic	*P*	Null NODF	NODF	z-statistic	*P*
Among sites									
Site	6	0.981	0.989	−0.335	0.836	33.836	23.609	3.964	0.001
Plant	96	7.005	6.570	1.997	0.081	15.116	11.173	2.463	0.031
Leaf	192	10.084	9.395	2.800	0.020	9.272	8.622	0.554	0.560
Within sites									
DC	16	0.168	0.174	−0.625	0.652	15.227	9.385	2.175	0.049
HM	16	0.108	0.099	1.545	0.186	6.738	5.875	0.381	0.760
PP	16	0.035	0.038	−0.610	1.000	0.000	3.546	−1.848	0.195
CL	16	0.479	0.453	0.842	0.333	20.93	15.449	2.167	0.045
EY	16	0.143	0.144	−0.123	1.000	5.556	10.182	−1.473	0.171
WI	16	0.224	0.220	0.314	0.783	17.949	15.684	0.744	0.451

Non-randomness was tested with checkerboard analysis and the fixed-fixed null hypothesis generated by the swap algorithm. Nestedness was tested with the nestedness metric based on overlap and decreasing fill (NODF) index and the *r1* null hypothesis. Binary matrices were sorted by rows from high herbivory to low herbivory and by columns from most abundant to least abundant OTU. For the three among-site analyses, all samples were grouped by either site, plant or leaf. For the six within-site analyses samples were grouped by plant within each site. *P* values in boldface where significant.

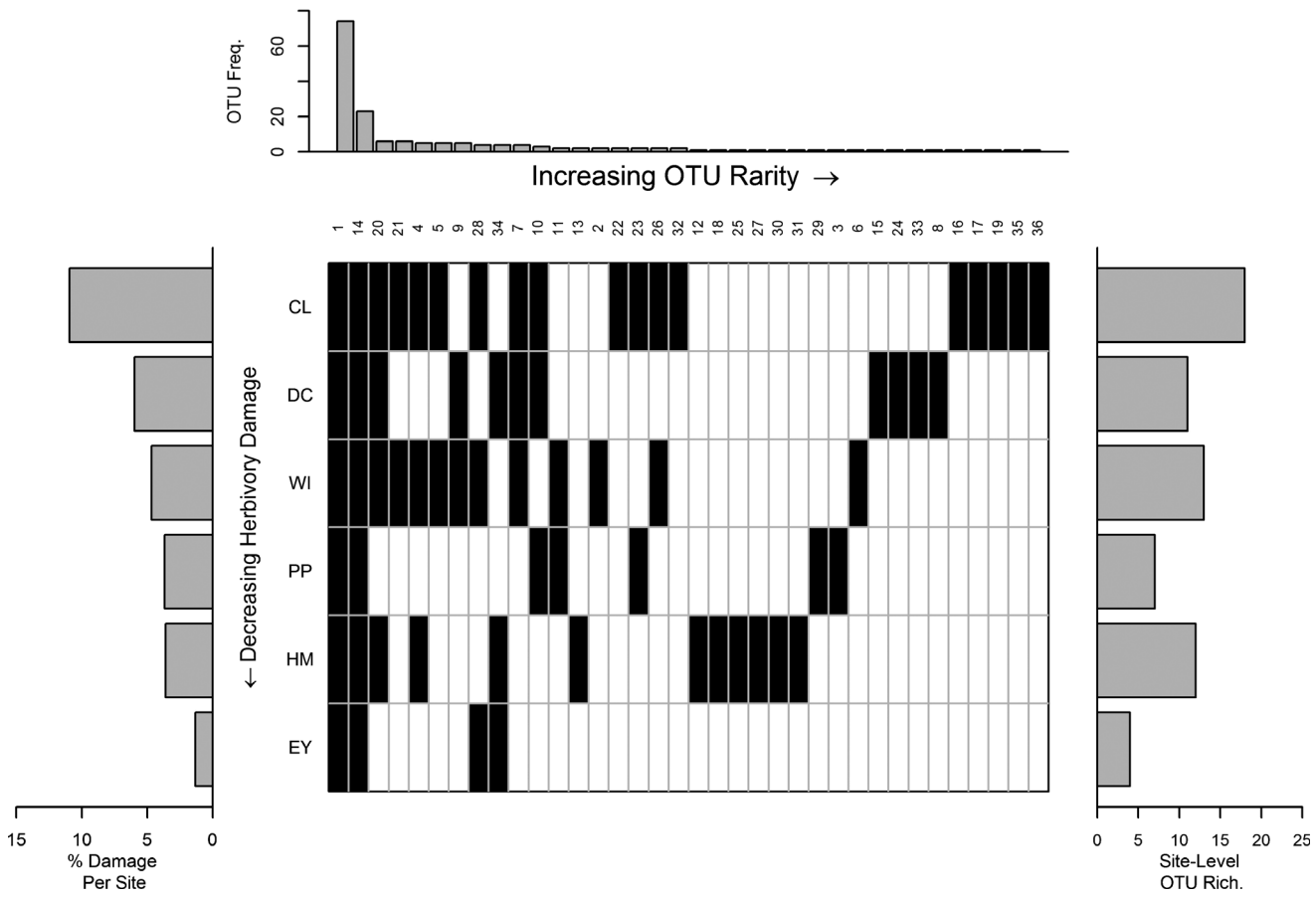

FIG. 3. Nestedness of endophyte communities along a gradient of plant herbivory using the nestedness metric based on overlap and decreasing fill (NODF). Plot shows the presence of individual OTUs (top x-axis) in each site (y-axis). OTUs are sorted by increasing rarity from left to right, with the frequency of each OTU in the top panel. Sites are sorted by decreasing herbivory from top to bottom in left panel, and the OTU richness of each site is shown in right panel.

consist of local infections within their host (i.e. Type III endophytes, Rodriguez et al. 2009), which could suggest there is less selective advantage toward antagonizing herbivores. Many studies considering endophyte diversity focus on leaves exhibiting no herbivory to avoid sampling potentially non-endophytic fungi that have opportunistically colonized a wounded leaf (e.g. Arnold et al. 2003). However, our results suggest that herbivores could play important roles in structuring endophyte communities, and studies that avoid sampling leaves exhibiting herbivory might underestimate endophyte diversity.

Although our observational study does not resolve whether it is endophytes or herbivores that drive this positive association, our results do lend credence to the idea that endophyte communities do not influence herbivory in this system. Herbivory was structured at the site and plant levels, while endophyte isolation frequency and communities were structured at the plant and leaf levels (residual). Given this spatial structuring and the finding that herbivory and endophytes were positively correlated with one another, it seems unlikely that endophytes are inhibiting biocontrol agents from feeding on *L. salicaria* and therefore are an unlikely cause for biocontrol failure at historically low herbivory sites.

An alternative hypothesis that endophytes colonize or increase leaf abundance where herbivory has already occurred is supported by our findings, although this would need to be confirmed experimentally. If herbivores facilitated endophytes, then we might have expected a positive relationship to hold both across and within sites. Instead our results revealed that the relationships between herbivory and endophyte isolation frequency, richness and nestedness were most strongly supported across sites rather than within sites. This could be interpreted as evidence that herbivores and endophytes do not interact but are both influenced by the same factors among sites. For instance, frequency of inundation can reduce *Galerucella* (Yeates et al. 2011) and endophyte abundance (Dolinar and Gaberscik 2010). However, this result

also could be attributed to at least two alternative causes that are still consistent with the hypothesis that herbivores cause changes in endophyte assemblages. First, low variation in herbivory within the sites for which we found no herbivory-endophyte relationship could obscure detection of a within-site relationship. Further experimental studies that directly manipulate herbivory could determine whether this is the case. Second, there may be a threshold at which herbivory begins to facilitate endophyte taxa. For instance, if herbivory facilitates endophytes through the creation of wounds in the plant, sufficient wounding might be necessary before endophytes are able to capitalize and increase their abundance.

Our study also contributes to a growing understanding of the communities of endophytes residing within invasive plants (Shipunov et al. 2008, Aschehoug et al. 2012, David et al. 2015). Although these endophyte communities often are overlooked, invasive plants are known to harbor distinct endophyte communities in their introduced habitat as compared to their native habitat (Shipunov et al. 2008), suggesting they interact with endophytes with which they have not necessarily evolved. In *L. salicaria* we found endophyte communities to be generally sparse but the communities typically contained *Alternaria* and *Penicillium*. Both are high spore-producing, cosmopolitan genera, whose members may be pathogenic, saprobic or endophytic and produce a variety of mycotoxins (Pitt 2002, Thomma 2003, Sandberg et al. 2014). Endophytes acquired in the introduced range of other invasive plant species have been shown to increase competitive ability or reduce seed predation (Newcombe et al. 2009, Aschehoug et al. 2012). While neither of the two common OTUs in our study exhibited the negative isolation frequency-herbivory damage relationship that would suggest they defend *L. salicaria* from herbivory, congeners of these OTUS are known to increase competitive ability of invasive forbs (Aschehoug et al. 2012) or reduce disease severity (de Cal et al. 1997).

The work we present here is to our knowledge the first to examine the interactions between a biological control agent of an invasive plant and its endophyte communities. The intentional introduction of a biological control agent may have important short- and long-term effects on ecological systems, particularly for those species with which the agent directly and indirectly interacts (David et al. 2013). In our system there may be short-term alterations in endophyte community composition, but there could also be long-term effects if ecosystem functions such as leaf decomposition are altered (e.g. Purahong and Hyde 2010, but see Osono 2006). The roles of most endophytes and other plant-associated symbiotic microbes in ecological systems are still poorly understood, yet their communities are susceptible to anthropogenic changes (Olsrud et al. 2009, Kivlin et al. 2013, Rudgers et al. 2014). Future work should focus on the ways in which endophyte communities may be altered by anthropogenic changes, and how such changes alter interactions between other heterotrophic groups and host plants.

ACKNOWLEDGMENTS

We thank G. May for providing laboratory resources, as well as M. Howard and P. Lenz for their assistance in the field and laboratory. We thank G. May and D. Tilman for thoughtful feedback on the manuscript, as well as the executive editor L. Taylor and two anonymous reviewers for their comments. Financial support provided by the NSF-IGERT Introduced Species and Genotypes program (DGE-0653827) to A.S.D and G.L.Q, the NSF Graduate Research Fellowship program (NSF 00039202) and the University of Minnesota Bell Museum Dayton Award to A.S.D and the University of Minnesota Life Sciences Summer Undergraduate Research Program to J.I.S.

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