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Non-systemic fungal endophytes in *Festuca rubra* plants infected by *Epichloë festucae* in subarctic habitats

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Abstract *Epichloë festucae* is an endophytic fungus that infects systemically the aerial tissues of the host grass *Festuca rubra*. This fungus is transmitted vertically from the mother plant to seeds. Hypothetically, the presence of *E. festucae* could affect the infection of a plant by other fungal species. This could occur if *E. festucae* metabolites produced *in planta* interfere negatively with other fungal infections; or alternatively, if the modulation of plant defenses by the endophyte favour further fungal infections. We have analyzed the presence of culturable non-systemic endophytes in plants of *F. rubra* infected (E+) and not infected (E−) by *E. festucae* in two subarctic habitats, meadows and riverbanks in Northern Finland. The observed non-systemic endophyte infection frequencies were similar among E+ and E− plants from riverbanks, and E+ plants from meadows. In contrast to these, the infection frequency was significantly lower in E− plants from meadows. This result suggests that the presence of *E. festucae* is not a main factor determining the presence of non-systemic endophytes in plants. Instead, plant genetic characteristics related to compatibility with *E. festucae* and other endophytes in the more stable meadow populations might play a role in these fungus–fungus–plant interactions. As a result of the survey, 18 different taxa of non-systemic endophytes were identified in plants of *F. rubra*. All were ascomycetes except for one basidiomycete. Three endophytic taxa could not be ascribed to a genus, but sequence data indicated that they were conspecific with other unidentified endophytes that have been isolated in cold biomes at different locations.

Keywords Grass · Fungi · Diversity · Finland

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Introduction

The majority of grass-endophyte literature has focused on *Neotyphodium* and *Epichloë* endophytes that form a life-long, systemic infection throughout the aerial organs of the host plant (Saikkonen et al. 1998, 2004, 2010a, b; Clay and Schardl 2002; Kuldau and Bacon 2008). However, surveys of fungal endophytes in grasses show that a relatively large number of other endophytic species can be found in association with particular grass species (Fisher and Petrini 1992; Sánchez et al. 2007, 2008, 2010, 2012; Pan et al. 2008; Ghimire et al. 2011; Mouhamadou et al. 2011; Purahong and Hyde 2011). A majority of these endophytic fungi are horizontally transmitted, form local infections, and are known as non-systemic or Class 3 endophytes (Saikkonen et al. 1998; Schulthess and Faeth 1998; Rodriguez et al. 2009; Sánchez et al. 2012). While the symbioses between systemic fungi and grasses are relatively well known, the function and effects of most non-systemic endophytes remain largely unknown. How the presence of systemic endophytes might affect other fungi capable of infecting the same host plants is a question that has been explored in relation to some plant pathogens (e.g. Wäli et al. 2006), but not to species of non-systemic endophytes.

Epichloë festucae Leuchtm., Schardl & M.R. Siegel is a fungal endophyte colonizing the intercellular space of leaf

sheaths, stems and seeds of the grass *Festuca rubra* L. (red fescue). The sexual reproduction of this fungus is rare, but its efficient vertical transmission from mother plant to offspring through seeds provides an important mechanism of asexual reproduction and dissemination (Leuchtmann et al. 1994). In natural populations from diverse habitats throughout Europe, *F. rubra* plants are often infected by *E. festucae* (Sampson 1933; White et al. 1993; Bazely et al. 1997; Zabalgogeazcoa et al. 1999, 2006; Saikkonen et al. 2000; Arroyo et al. 2002; Granath et al. 2007; Wäli et al. 2007). The association between *F. rubra* and *E. festucae* is under some circumstances mutualistic, for instance, alkaloids of fungal origin improve host plant resistance to herbivores (Bazely et al. 1997; Wilkinson et al. 2000). Also, plants infected by *E. festucae* have shown increased resistance to fungal diseases caused by the pathogens *Laetisaria fuciformis* and *Sclerotinia homeocarpa* (Bonos et al. 2005; Clarke et al. 2006). Because of this, *E. festucae* can be useful for the improvement of commercial cultivars of *F. rubra*, and endophyte-infected turfgrass cultivars are commercialized in several countries (Brilman 2005; Zabalgogeazcoa and Vazquez de Aldana 2007; Saikkonen et al. 2010a, b).

Although a direct involvement of several fungal alkaloids has been shown to be a mechanism protecting *F. rubra* against micro and macro herbivores (Wilkinson et al. 2000; Kuldau and Bacon 2008; Vesterlund et al. 2011), the mechanisms involved in *Epichloë*-mediated plant responses to other stress factors than herbivores is not clearly known (e.g. Hamilton and Bauerle 2012; Hamilton et al. 2012). In plant-endophyte associations, several possible mechanisms against fungal pathogens have been proposed: production of antifungal compounds by endophytes, competition for space or resources with pathogens, or activation of plant defense mechanisms by endophytes (Arnold et al. 2003; Waller et al. 2005; Zabalgogeazcoa 2008; Tejesvi et al. 2011; Hamilton and Bauerle 2012; Hamilton et al. 2012). Compounds with antifungal activity have been detected in extracts from *E. festucae* cultures (Yue et al. 2000), and allelopathic substances are secreted by roots of *F. rubra* infected by *E. festucae* (Vázquez de Aldana et al. 2013). Such substances could perhaps limit fungal infections by horizontally transmitted endophytes and pathogenic fungi. In addition, the existence of nets of epiphyllous hyphae of *E. festucae* on leaf surfaces has been reported in some plant-endophyte combinations, and these nets may also limit the growth of other fungi (Tadych et al. 2007). In this work we studied if the infection by the systemic endophyte *E. festucae* affects the presence of non-systemic endophytes in plants of *F. rubra* L.. In addition, we identified non-systemic endophytes of *F. rubra* in a subarctic environment.

Materials and methods

Plant material and endophyte isolation

Plants were collected in Lapland, near Kevo Subarctic Research Institute, in the northern border of Finland. The thermal growing season (days having temperature >5 °C) in the area lasts 110–120 days from June to the beginning of September. *F. rubra* plants were sampled in two distinct habitats, meadows and riverbanks. The striking difference between the meadow and riverbank habitats is that the riverbank populations are disturbed nearly annually and destroyed regularly by the violent debacle in the spring. In contrast to these sandy riverbanks, meadows are more stable and fertile environments, and their grass populations are older and well established.

To estimate infection frequencies of plants infected by systemic fungal endophytes, we sampled 50 *F. rubra* individuals in three meadow (MS1K: N 69°38′5.6″, E 27°5′ 0.9″, 107 m.a.s.l.; MS2K: N 69°43′56.4″, E 27°1′11.6″, 91 m.a.s.l.; MS3K: N 69°45′32.4″, E 26°59′18.8″ 85 m.a.s.l.) and three riverbank populations (RB1S: N 69°54′35,1″, E 27°2′ 0,15″, 73 m.a.s.l.; RB2S: N 69°56′ 41,0″, E 26°43′21,9″ 85 m.a.s.l.; RB3S: N 69°56′10,5″, E 26°27′45,2″ 106 m.a.s.l.). The three populations of each type of habitat were at least 3 km apart. At each location, sampled plants were several meters apart, to avoid sampling the same individual. The plants were potted, kept in a greenhouse and later screened for their systemic endophyte infection. Three leaves from each plant were surface sterilized (30 s in 75 % ethanol; 3 min in 4 % sodium hypochlorite; 15 s in 75 % ethanol), cut into five segments and placed on Petri dishes containing potato dextrose agar (PDA). The presence of the systemic endophyte was detected when mycelium with characteristics of an *Epichloë* endophyte grew out of several leaf segments (Leuchtmann et al. 1994).

To determine if the presence of *Epichlöe* affects the presence of culturable non-systemic endophytes in *F. rubra* plants, we collected several plants from two meadow (MS1K and MS2K) and two riverbank (RB1S, RB2S) populations. The plants were dug up and transported to the laboratory at Kevo Subarctic Research Institute, where *E. festucae* infection was determined by microscopy of stem pith scrapings stained with aniline blue (Bacon and White 1994). Five plants infected with *E. festucae* (E+) and five *Epichloë*-free (E−) from two meadow and two riverbank habitats were selected for the isolation of their non-systemic endophytes. Several 5 cm long fragments of leaf blades and sheaths from each plant were surface-disinfected by means of a 10 min immersion in a solution of sodium hypochlorite (1 % active chlorine), followed by a rinse in sterile water. This method of surface disinfection has been successfully used in numerous samples from several grass

species (i.e. Sánchez et al. 2008, 2010), where its efficiency was tested by leaf imprints in PDA (Schulz et al. 1998). The leaf samples were placed in individual dry sterile plastic tubes, and transported to a laboratory in Salamanca, Spain, where they were processed within 72 h. Twenty 5 mm long pieces of both leaf blade and leaf sheath tissue were placed in Petri plates containing PDA with 200 mg/L chloramphenicol and stored at room temperature (23–26 °C). When mycelium emerged from a plated leaf fragment, a small sample was transferred to a new PDA plate to obtain a fungal culture. Then, the leaf fragment and its surrounding agar were excised from the plate and discarded. When morphologically similar cultures were obtained from the same plant and tissue type, only one was maintained. The number of fragments from which mycelium emerged was counted during the first ten days after plating.

To estimate the infection load or amount of non-systemic endophyte infection in E+ and E− plants, the number of leaf fragments infected by endophytes on each plate was counted. Analysis of variance was used to analyse the effect of systemic *E. festucae* endophyte (E− or E+), type of tissue (leaf blade or leaf sheath) and the habitat (meadow or riverbank) on the number of tissue fragments infected with non-systemic endophytes. Statistica release 5.0 software (StatSoft, Inc. USA) was used to preform the statistical analyses.

Identification of non-systemic endophytes

To identify non-systemic fungal endophytes, isolates were first grouped into morphotypes according to macroscopic morphological characteristics of the cultures. Then, one or more cultures of each morphotype were identified using microscopic morphology in sporulating cultures, and the nucleotide sequence of the ITS1-5.8S-ITS2 region of rDNA. To obtain these sequences, DNA was extracted from mycelium of cultures growing on PDA medium, amplicons of the ITS region were obtained by means of a polymerase chain reaction using primers ITS4 and ITS5, and later the amplicons were sequenced (White et al. 1990; Sánchez et al. 2007). Nucleotide sequences representative of each morphotype were used to interrogate nucleotide databases using the BLAST algorithm, and the closest matches were considered appropriate identifications for genus and species if they had 99 % or greater similarity to several isolate sequences, including those of collection strains (Sánchez et al. 2007). Sequence similarities ranging from 97 % to less than 99 % were considered acceptable for identification to genus rank. When the similarity was less than 97 % the fungi were considered unidentified. As an aid to identification, a phylogenetic tree was build using sequences of the morphotypes. In this tree we included database sequences of the closest matches to our sequences, but choosing those corresponding to identified strains that had been deposited in fungal culture collections.

Results

Prevalence of systemic endophytes

The systemic endophytic fungus *E. festucae* was found in all the surveyed populations. However, the frequency of colonized plants was different between habitats. While an average (± standard error) of 24.0 %±7.7 % of the plants were colonized in the three riverbank populations, 53.0 %±4.0 % of the plants were colonized in meadow populations.

Non-systemic endophyte infections in plants

In the first week after plating plant fragments on culture media, and before the emergence of *E. festucae* colonies, non-systemic fungi emerged from 84.5 % of plant fragments. The percentage of infected leaf fragments was greater in plants colonized by *E. festucae* than in those free of the fungus (E+: 87.4 %±2.9 %; E−: 80.9 %±4.1 %), but the difference was not statistically significant (Table 1). The non-systemic endophyte infection rate was significantly greater in riverbanks (88.7 %±3.0 %) than in meadows (80.0 %±3.8 %) and interacted with habitat being lower in E− plants in meadows (Table 1, Fig. 1). Regarding the type of tissue, the amount of infections by non-systemic endophytes was significantly greater in leaf blades (91.3 %± 2.8 %) than in leaf sheaths (77.6 %±3.7 %). Although the difference was not significant, the amount of infected fragments was greater in E+ plants in both leaf blades (E+= 95.9 %; E−=85.6 %) and leaf sheaths (E+=78.8 %; E−= 75.9 %). The above results suggest that the presence of *Epichloe* endophytes do not have an inhibitory effect upon the presence of other non-systemic endophytes in E+ plants.

Table 1 Analysis of variance of the effect of *Epichloë festucae* infection, type of plant tissue (leaf blades or sheaths) and habitat (meadow or riverbank) on the number of plant fragments of *Festuca rubra* infected by non-systemic endophytes

Effect	df	*F*	*p*-level
Epichloë infection (E)	1	2.598	0.111
Habitat (H)	1	6.103	0.016*
Type of tissue (T)	1	8.279	0.005**
E*H	1	6.616	0.012*
E*T	1	1.417	0.238
T*H	1	0.153	0.697
E*T*H	1	0.637	0.428
Error	67		

*Significant with $p<0.05$; **$p<0.01$

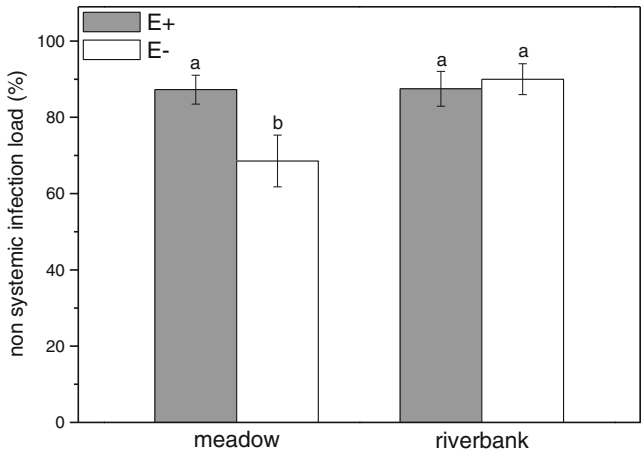

Fig. 1 Non-systemic endophyte infections in leaf blades and leaf sheaths of 40 *Festuca rubra* plants sampled at meadows and riverbanks. *Bars* indicate mean percentage±standard error. Differences between means with different letters are statistically significant (least significant difference, $p<0.01$)

Identification of non-systemic endophytic fungi

When several morphologically similar isolates were obtained from the same plant, only one was further analysed. As a result, 85 isolates were obtained from the 40 *F. rubra* plants, and all those were classified into 64 different morphotypes. Each morphotype was identified by sequencing the ITS1-5.8S rRNA-ITS2 region of one or more of its isolates. Using the molecular data, the 64 morphotypes could be grouped into 18 different taxa (Table 2). Six of these taxa could not be identified because their nucleotide sequences were less than 96 % similar to the closest match from the nucleotide database. Nevertheless, a phylogenetic analysis allowed to ascribe five of the unknown fungi to Ascomycota, and one to Basidiomycota (Fig. 2). The most common taxon was *Phaeosphaeria*, found in 16 plants, followed by *Davidiella*, *Aureobasidium* and *Glomerella*. Eleven fungal taxa were identified in E+ plants and 12 in E− plants. This result suggests that regarding non-systemic species richness, E+ plants can support a non-systemic endophytic assemblage as diverse as that of E− plants. With respect to habitat of origin, 14 taxa were identified in plants from meadows and 12 taxa in plants from riverbanks. Except for *Fusarium*, which was isolated only from plants at riverbanks, all other taxa represented by more than one isolate were found at both habitats.

The nucleotide sequence of Unidentified ascomycete 1 was similar to those of other unidentified fungi isolated from snow covered soils in Austria (Genbank accession number EU516923), from house dust in Finland (AM901892), and from a plant in Tibet (JX401949). The closest match (99 %) to the nucleotide sequence of Unidentified ascomycete 2 was a fungus isolated from oilseed rape roots in Sweden (EU754982). The sequence of Unidentified ascomycete 3 was identical to that of a fungus (FJ378725) isolated from plant roots in Himalayan alpine meadow. The fact that the sequences of unidentified fungi from this study resembled those of other unidentified strains obtained in cold regions might indicate that these species are common in cold biomes.

Table 2 Number of plants of *Festuca rubra* infected (E+) or not (E−) by *Epichloë festucae* where non-systemic endophytes were isolated in meadow and riverbank habitats in a subarctic environment in Lapland, Northern Finland

Non-systemic fungal taxa	Meadow		Riverbank		Total
	E−	E+	E−	E+	
Phaeosphaeria spp.	4	5	4	3	16
Davidiella sp.	1	0	6	0	7
Aureobasidium pullulans	0	2	1	3	6
Glomerella graminicola	3	1	0	2	6
Microdochium nivale	1	1	0	1	3
Fusarium sp.	0	0	1	1	2
Oculimacula sp.	1	0	1	0	2
Unidentified ascomycete1	0	1	0	1	2
Phoma spp.	1	0	1	0	2
Podospora sp.	0	1	0	0	1
Drechslera sp.	0	0	1	0	1
Coniothyrium sp.	0	1	0	0	1
Lecythophora sp.	0	1	0	0	1
Unidentified basidiomycete 1	1	0	0	0	1
Unidentified ascomycete 2	1	0	0	0	1
Unidentified ascomycete 3	0	0	0	1	1
Unidentified ascomycete 4	0	0	0	1	1
Unidentified ascomycete 5	1	0	0	0	1

Discussion

Previous works have shown that *E. festucae* can affect the outcome of fungal diseases in *F. rubra* (Bonos et al. 2005; Clarke et al. 2006; Wäli et al. 2006). Thus, we investigated whether the symbiosis with *E. festucae* would inhibit the infection of plants by horizontally transmitted endophytes. In contrast to studies on grass pathogens, our results do not support the hypothesis of *Epichloë* inhibiting horizontally transmitted, non-systemic fungi. The non-systemic endophyte infection rate, however, was lower in meadows where prevailing selection pressures favour systemic *E. festucae* endophyte colonisations (Saikkonen et al. 2000; Wäli et al. 2007). Furthermore, non-systemic endophyte infection rate was significantly lower in E− plants in meadows. The number of non-systemic endophyte infections in E+ and E− leaf blade and leaf sheath tissues was not significantly different, and the number of fungal taxa was comparable in E+ and E− plants. These results suggest that non-systemic endophyte

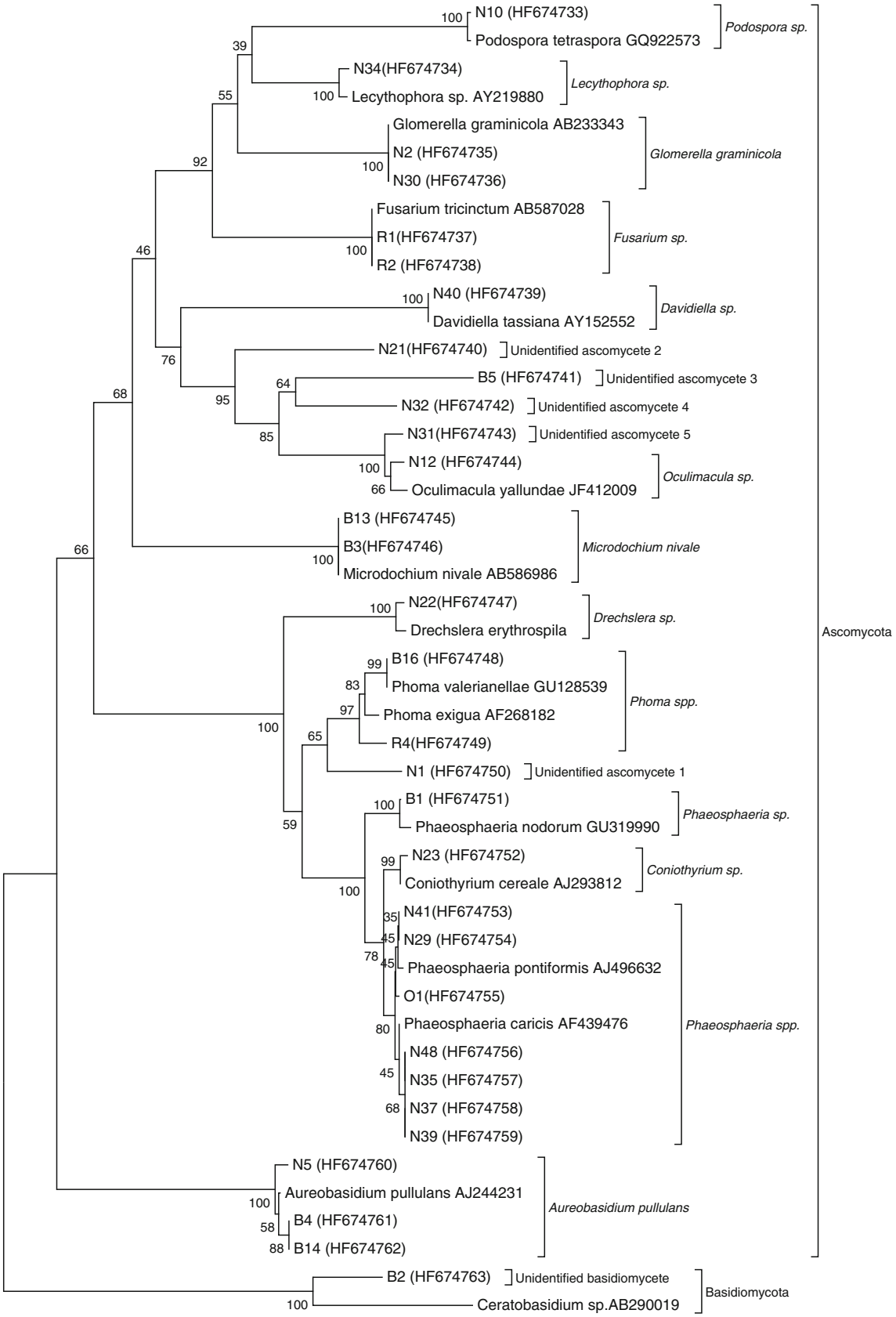

Fig. 2 Neighbor joining phylogenetic tree made with ITS1-5.8SrRNA-ITS2 sequences of endophytes isolated from *Festuca rubra* in Lapland, Northern Finland, indicated by isolate and sequence accession numbers. Reference sequences indicating taxa are followed by their accession numbers. Bootstrap values based on 1000 replications, are showed in the branches

infections are driven by environmental and/or other factors than direct fungus–fungus interactions between systemic and non-systemic fungi.

In this work we used a culture-dependent technique to isolate *F. rubra* endophytes. It should be noted that in endophyte surveys the techniques used for isolation can have an important impact in the results obtained (Hyde and Soytong 2008). For instance, if a culture independent technique (i.e. DNA based; Martin and Rygiewicz 2005; O'Brien et al. 2005; Martinson et al. 2012) would have been used, non culturable species and other culturable taxa different from the ones we observed might have been detected.

Plants have a variety of structural and pathogen-induced defense mechanisms to prevent or control fungal infections (Agrios 2005), but in spite of this, the presence of fungal endophytes is ubiquitous in the plant kingdom (Rodriguez et al. 2009). This might indicate that many fungal species can modulate plant defense responses to a point where after penetration, these fungi can remain alive inside plant tissues as endophytes. Endophytic infection has been postulated as the result of a balanced antagonism between the plant defensive mechanisms and the endophyte capability to penetrate and grow in plant tissues (Schulz and Boyle 2005). In this sense, in plants of *Lolium perenne* the endophyte *Neotyphodium lolii* overproduces a superoxide dismutase that may protect it from reactive oxygen species produced by the plant as a defense mechanism (Zhang et al. 2011). Such interplays between reactive oxygen species and antioxidants produced by both plants and endophytes could be involved in the modulation of plant defense, allowing endophytic infection, as well as in the limitation of damage that endophytes could cause (White and Torres 2010; Hamilton and Bauerle 2012; Hamilton et al. 2012; Gundel et al. 2012). In this context, an endophyte like *E. festucae*, capable of colonizing systemically the intercellular space of leaf blades, sheaths, reproductive stems and seeds, is suggested to perturb plant defense responses and thereby could open a window for other fungi. Our results, however, do not support this other hypothesis. The rates of non-systemic endophyte infections were comparable in E+ plants in meadows to those in both E+ and E− plants in riverbanks. Instead, the infection rate of non-systemic endophytes was markedly lower in E− plants in meadows.

We propose that genetically determined resistance properties of the host grass drive non-systemic endophyte infections in grasses similarly to horizontally transmitted fungi in woody plants (Ahlholm et al. 2002a; b; Saikkonen 2007; Saikkonen et al. 2010a). The higher proportion of E+ plants detected in meadows suggests that *Epichloë* endophytes provide a selective advantage to the host in meadows (see also Wäli et al. 2007; Saikkonen et al. 2010b) which are more stable and fertile environments. In meadows grass populations are older and plant competition is higher compared to riverbanks, which suffer almost annual disturbance due to spring flooding (see also Wäli et al. 2007; Saikkonen et al. 2010b). However, *Epichloë* endophyte infections appear to be fragile, genetic mismatches constrain compatible combinations between the *Epichloë* endophyte and the out-crossing host, thereby leading to loss of less fit fungus–plant genotype combinations in established grass populations (Saikkonen et al. 2010b). Genetic divergence of E− plants in our study could be explained if genetic mismatch between the *Epichloë* endophytes genetically correlates with higher resistance to fungi in plants in general.

Dominant non-systemic endophytic species identified in *F. rubra* plants, e.g. *Phaeosphaeria*, *Aureobasidium* and *Glomerella*, have also been described as dominant taxa in surveys of endophytes in other grass species (Stone et al. 2004; Sánchez et al. 2012). It is interesting that *Cladosporium* and *Alternaria*, the two most prominent taxa of non-systemic grass endophytes found in many grass species throughout temperate areas (Sánchez et al. 2012), were absent in *F. rubra* plants collected from meadow and riverbank habitats in Northern Finland. This might be an indication of a geographical distribution of *Alternaria* and *Cladosporium*, which might not be common grass endophytes in arctic latitudes. These two taxa have been isolated as endophytes from plants of *F. rubra* in Spain (Martín et al. 2008), and could be considered latent saprobes that sporulate on the surfaces of their plant hosts after tissue senescence or death (Sánchez et al. 2012).

Several fungal taxa isolated in this survey (Table 2) seem to be common to cold biomes, for example the pathogen *Microdochium nivale*, a snow mold, was isolated from several plants in this study. In addition, three different taxa of ascomycetes that were not identified (unidentified ascomycetes 1, 2, and 3) had nucleotide sequences identical or very similar to unidentified fungi isolated from cold environments: alpine Himalayan meadows, Swedish crop fields, and house dust samples from Finland (Kauserud et al. 2005; Pitkäranta et al. 2008; Gao and Yang 2010). In contrast, the taxa of other non-systemic endophytes that were identified (Table 2), are similar to those reported in surveys of endophytes from grasses occurring in Southern Europe (Sánchez et al. 2012).

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