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Diversity and abundance of culturable fungal endophytes in leaves of susceptible and resistant alternate hosts of *Cronartium pini* and *C. ribicola*

Juha Piispanen · Ulrich Bergmann · Jouni Karhu · Tuomas Kauppila · Johanna Witzell · Juha Kaitera

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Abstract *Cronartium pini* and *C. ribicola* are rust fungi that cause destructive diseases of pines (*Pinus* spp.). These rusts spread via alternate hosts, among which *Melampyrum* spp., *Veronica* spp. and *Impatiens* spp. are important for *C. pini* and *Ribes* spp. for *C. ribicola*. Congeneric alternate hosts vary in their susceptibility to *Cronartium* rusts, but the reasons for this variation are not clear. To clarify whether internal, endophytic fungi could explain these differences, we investigated the temporal and spatial variation in fungal endophyte composition of *C. pini*-resistant *M. pratense*, *V. chamaedrys* and *I. glandulifera*, *C. pini*-susceptible *M. sylvaticum*, *V. longifolia* and *I. balsamina*, *C. ribicola*-resistant *R. rubrum* and *C. ribicola*-susceptible *R. nigrum*. In total, 2695 fungal endophytic isolates were obtained and classified into 37 morphotypes, with 1373 cultures isolated in early summer and 1322 in late summer. Fifty-two isolates were identified to species or genus level. The most common morphotypes were identified as *Heterophoma* sp. Some variation in the abundance of morphotypes occurred between collection sites, but the same morphotypes dominated across the sites and species. The diversity of morphotypes was higher in early September than in late June in all species and the same morphotypes dominated in both early and late season. The diversity of fungal endophytes was higher in resistant *Veronica* and *Ribes* than in susceptible congeneric species, but the results suggest that the diversity or abundance of culturable fungal endophytes does not explain the differences in the congeneric species' susceptibility to rust fungi.

Keywords Alternate hosts · Endophytes · Rust resistance · Scots pine blister rust · White-pine blister rust

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J. Piispanen · J. Karhu · J. Kaitera (✉)
Natural Resources Institute Finland, Paavo Havaksen tie 3, FI-90570 Oulu, Finland
e-mail: juha.kaitera@luke.fi

U. Bergmann
Biocenter, University of Oulu, FI-90014 University of Oulu Oulu, Finland

T. Kauppila
Botanical Gardens, University of Oulu, FI-90014 University of Oulu Oulu, Finland

J. Witzell
Department of Forestry and Wood Technology, Linnaeus University, Georg Lückligs Väg 1, SE-35256 Växjö, Sweden

Introduction

Cronartium rusts cause severe damage in pine trees (*Pinus* spp.) in the northern hemisphere (Gäumann, 1959; Ziller, 1974), with *C. pini* (Willd.) Jørst damaging *P. sylvestris* L. in Europe and Asia (CABI, 2020;

Kim et al., 2022). In Scandinavia, this rust caused serious epidemics in the 2000s (Kaitera, 2000; Wulff et al., 2012). It can spread via over 50 alternate hosts (Kaitera et al., 2015). The main susceptible plant genera for *C. pini* are *Melampyrum*, *Pedicularis*, *Euphrasia*, *Veronica* and *Impatiens* (Kaitera, 1999; Kaitera et al., 1999, 2012, 2015, 2017, 2018). *Cronartium ribicola* Fisch. kills five-needle pines, especially in North America, but also in Europe and Asia (Kaitera & Nuorteva, 2006; Zambino, 2010). This rust spreads mainly via *Ribes* and *Pedicularis*. Previous research has shown that congeneric alternate hosts can differ in their susceptibility to *Cronartium* rusts. For instance, *Melampyrum sylvaticum* is more susceptible to *C. pini* than *M. pratense* (Kaitera et al., 1999, 2015; Kaitera, 1999). While the tight connection between the rust inoculum and the surrounding vegetation with alternate hosts is well characterized, we still know very little about the factors potentially influencing the susceptibility of these hosts to rusts. Better understanding of these interactions could help to develop nature-based solutions to suppress the negative effects of *Cronartium* rusts in Scots pine stands.

One biological factor potentially influencing the interactions between alternate host species and rusts is the plant's internal microbiome, in particular endophytic fungi. These fungi grow inside plants as taxonomically rich and spatially and temporally dynamic communities, generally without causing any harm to the host under normal conditions (Rajala et al., 2013; Terhonen et al., 2019). While the specific function of these communities and their members are largely unknown, there is increasing evidence that endophytes may positively affect tree resistance to pathogens (Ganley et al., 2008; Witzell et al., 2014; Terhonen et al., 2018). Endophytes may, e.g., directly antagonize pathogens or stimulate the host plant's defensive mechanisms so that pathogen colonization processes are impeded (Witzell & Martín, 2018).

The endophytic communities of alternate hosts of *Cronartium* rusts have not been systematically studied and thus their potential influence on the trajectories of conifer rust epidemics is unknown. The general aim of this study was to provide basic information about these fungal communities and to detect trends and patterns that could clarify whether the endophytic fungi have a role in the resistance of alternate host plants to *Cronartium* rusts of pines. A culture-based approach, accompanied by molecular identification of the most common morphotypes, was chosen as the method for this first step. The specific objectives were 1) to characterize the abundance and diversity of culturable fungal endophytes in the leaves of different alternate host plants, 2) to describe the temporal variation in fungal endophytic communities of alternate host plants in different parts of the growing season, 3) to assess site-specific variation in fungal endophytic diversity and abundance, and 4) to compare fungal endophytic communities in rust-susceptible and resistant alternate host species and to evaluate whether certain endophytes are positively or negatively connected to their resistance to rusts.

Material and methods

Collection of plants

Six alternate host species of *C. pini* and two of *C. ribicola* were collected for the study. *Cronartium pini*-resistant species were *Melampyrum pratense* L., *Veronica chamaedrys* L. and *Impatiens glandulifera* Royle, while the susceptible congeneric species were *M. sylvaticum* L., *V. longifolia* L. and *I. balsamina* L. The *C. ribicola*-resistant species was *R. rubrum* L., while susceptible species was *R. nigrum* L.

Five plants of each of seven of these species were collected at random from one to three locations in the city area of Oulu in early season (late June 2021) and late season (early September 2020); they were placed in paper bags and transported immediately to the laboratory. The early season collection in late June was performed at three locations (sites I-III) for the seven species. The late season collection in early September was performed in one area (site I for *M. sylvaticum*, *M. pratense* and *R. nigrum*), two areas (sites I and II for *V. chamaedrys* and *R. rubrum*) or three areas (sites I, II and III for *V. longifolia* and *I. glandulifera*). In addition, plants of the eight species, *I. balsamina*, were grown directly from seeds in a greenhouse and the leaves were collected as with the other plant species in late June and early September 2021. The geographic locations of the sites and the greenhouse are given in Table 1.

Isolation of fungal endophytes

In the laboratory, five healthy-looking, green leaves were separated aseptically from each plant in a

Table 1 Geographic sites of the plants

Plant species	Sites		
	I	II	III
M. sylvaticum and *M. pratense*	65º2,69N, 25º28,04E	65º1,29N, 25º25,38E	65º1,70N, 25º25,01E
V. chamaedrys	65º1,30N, 25º25,96E	65º1,70N, 25º25,01E	65º1,31N, 25º25,97E
V. longifolia	65º2,27N, 25º29,16E	65º2,26N, 25º29,12E	65º3,66N, 25º27,67E
I.glandulifera	65º3,03N, 25º25,20E	65º3,04N, 25º25,26E	65º3,02N, 25º25,16E
I.balsamina	65º3,86N, 25º27,79E		
R. nigrum	65º3,86N, 25º27,79E	65º2,26N, 25º29,12E	65º1,30N, 25º26,89E
R. rubrum	65º3,86N, 25º27,79E	65º3,85N, 25º27,80E	65º1,26N, 25º27,13E

laminar cabinet using a scalpel and tweezers. First, the leaves were surface sterilized as follows: rinsing with sterile water, 70 % ethanol (30 s), 1 % sodium hypochlorite (2 min), 70 % ethanol (30 s) and again with sterile water. Four pieces (about 0.5 × 0.5 cm) were cut from each leaf and placed on water agar (1.5 %) in Petri dishes. After an incubation period of 1-2 weeks at ca. 21.5 ºC, mycelia emerging from the pieces were transferred to fresh malt extract agar (MEA; 13.5 g Bacto agar, 15 g malt extract/1 L water, ø=9 cm Petri dishes), and when necessary, the cultures were transferred to new malt extract agar plates until morphologically homogeneous colonies were obtained. The water agar plates with the leaf pieces were also checked 2-4 weeks later to isolate possible slow-growing colonies. The rate (percentage of pieces yielding mycelial growth on each plate) was recorded.

Morphotyping of the endophytes

The pure cultures were incubated on MEA at 18 °C in a growth chamber (Binder GmbH, Tuttlingen, Germany). The isolates were grouped after one month into morphotypes based on colony characteristics, i.e. color, growth rate on MEA and the structure and form of the mycelia. The dominant color of the colony was described as hyaline, light, light brown, dark brown, red brown or green. The secondary (special) characteristics in color or form were described as: brownish, irregular, greenish, yellowish, reddish, with brownish spots, blotched, rhizoid, ring-shaped and filamentous. The growth rate was evaluated as either fast or slow, with fast growth rate indicating that the isolate filled the plate within one week whereas the isolates with slow growth rate filled the plate after several weeks to one month. A total of 2695 isolates were grouped into 37 morphotypes (Suppl. Table 1).

Genetic identification of the fungal endophytes

From each plant species, the isolates representing the dominant morphotypes were selected for DNA sequencing. The isolates, in total 65 and representing 9 morphotypes, were grown on 1.5% malt extract (see above) on a thin cellophane film. DNA was isolated using PrepManTM Ultra Sample Preparation Reagent (Thermo Fisher, Catalog number 4318930). First, 50 µl PrepMan solution was pipetted into 1.5 ml Eppendorf vials and a small amount of mycelium was transferred into the tubes and ground using a glass rod. The samples were then placed in a thermal block at 95 ºC for 10 min. After that, the vials were left to cool at room temperature for ca. 5 minutes and centrifuged at 12000 rpm for 10 min. The supernatant was transferred to a new 1.5 ml Eppendorf vial. The sample was diluted to 1:50 for PCR.

The PCR was performed on the samples using ThermoFisher DreamTag Green Master Mix (Catalog number K1081). The mixture was as follows: DreamTag Green Master mix x 2 12.5 µl, primers ITS1F 25 µM 0.125 µl and ITS4R 25 µM 0.125 µl, sterilized water 11.25 µl and template 1 µl (diluted 1:50). The PCR program included 35 cycles at 95 ºC for 3 min, 95 ºC for 30 s, 55 ºC for 30 s, 72 ºC for 1 min, 72 ºC for 10 min and 4 ºC for ∞. The selected primers, ITS1F and ITS4R, produce PCR product of the fungi that covers ITS1 and ITS2 regions and the 5.8S region between them, which contains high variation between fungi and thus is very suitable for identification purposes. The primers were:

ITS1F Forward CTTGGTCATTTAGAGGAA GTAA and
ITS4R Reverse TAAACTTCAGGGTGACCA AAAAATCA.

The PCR products were processed in 1% agarose gel. One clear PCR product was obtained from each sample and purified using an Exo-Sap reagent (Applied Biosystems™)[5 µl EXO (20 U/ µl), 100 µl SAP (1 U/ µl), 895 µl water]. For 5 µl of the PCR product, 2 µl of Exo-Sap reagent was used. The purification was performed by incubating at 37 ºC for 15 min and heating at 80 ºC for 15 min.

The purified PCR products were sequenced by Macrogen (Amsterdam, the Netherlands) with the primer ITS1F. Poor quality parts of the sequences from the beginning and the end were removed using the Geneious Prime® 2021.0.1 program (https://www.geneious.com/prime). A search for the purified sequences was performed using Blast with NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and Unite [UNITE (ut.ee)], with over 98% similarity criterion.

Results

Morphotype abundance and diversity in the alternate host species

The colonization rate was 74% for all the leaf material. Among plant species, mycelia emerged from 93% (*M. sylvaticum* and *M. pratense*), 80% (*V. longifolia*), 79% (*V. chamaedrys*), 75% (*R. rubrum*), 63% (*I. glandulifera*), 52% (*R. nigrum*) and 47% (*I. balsamina*) of the leaf pieces. The highest total number of fungal endophyte isolates was recovered from *V. longifolia* (Table 2). The isolation frequency was relatively high in samples collected from both *Melampyrym* species, *I. glandulifera*, *V. chamaedrys* and *R. rubrum,* whereas clearly fewer isolates were recovered from *R. nigrum* and the lowest number of fungal endophytes was isolated from the greenhouse-grown *I. balsamina* (Table 2).

The plant species exhibited distinct morphotype abundance profiles, but overall, morphotypes 13 and 19 were found to be common in all alternate hosts, along with morphotypes 2, 3, 31 and 35 (Fig. 1). The abundance of each morphotype was lowest in *I. balsamina,* but in this species, morphotypes 13, 19, 31 and 35 were also the most common ones (for the data, see Suppl. Table 1). The diversity of fungal endophytes, estimated as the number of distinct morphotypes found per plant species across all sites and time points, was highest in *V. chamaedrys* and *R. rubrum* and lowest in *R. nigrum* and *I. balsamina* (Table 2).

Fifty-two isolates were identified at least to genus-level and represented mainly Ascomycota (Table 3). The most common isolates from *Melampyrum* species, classified to morphotypes 13 and 19, were identified as *Heterophoma* species. However, isolates from *R. rubrum* that were classified to morphotype 19 were identified as several other species. Several common fungi, including *Alternaria alternata* (Fr.) Keissl. and *Cladosporium* spp. were identified among isolates classified to other frequently detected morphotypes (2, 31 and 35).

Temporal variation in morphotype abundance and diversity

The abundance and diversity of culturable fungal endophytes showed some temporal variation (Fig. 2).

Table 2 Isolation frequency, percentage of isolates from all isolates, number of distinct morphotypes (MTs) and their percentage in eight alternate hosts of *Cronartium* rusts

Species	Nr isol.	% of all isolates	Nr of distinct MTs	% of all MTs
Melampyrum pratense	372	13.8	20	57
Melampyrum sylvaticum	372	13.8	23	62
Veronica chamaedrys	396	14.7	26	70
Veronica longifolia	485	18.0	21	57
Ribes nigrum	210	7.8	18	49
Ribes rubrum	385	14.3	26	70
Impatiens balsamina	94	3.5	10	27
Impatiens glandulifera	381	14.1	22	59
Sum	**2695**	**100**		

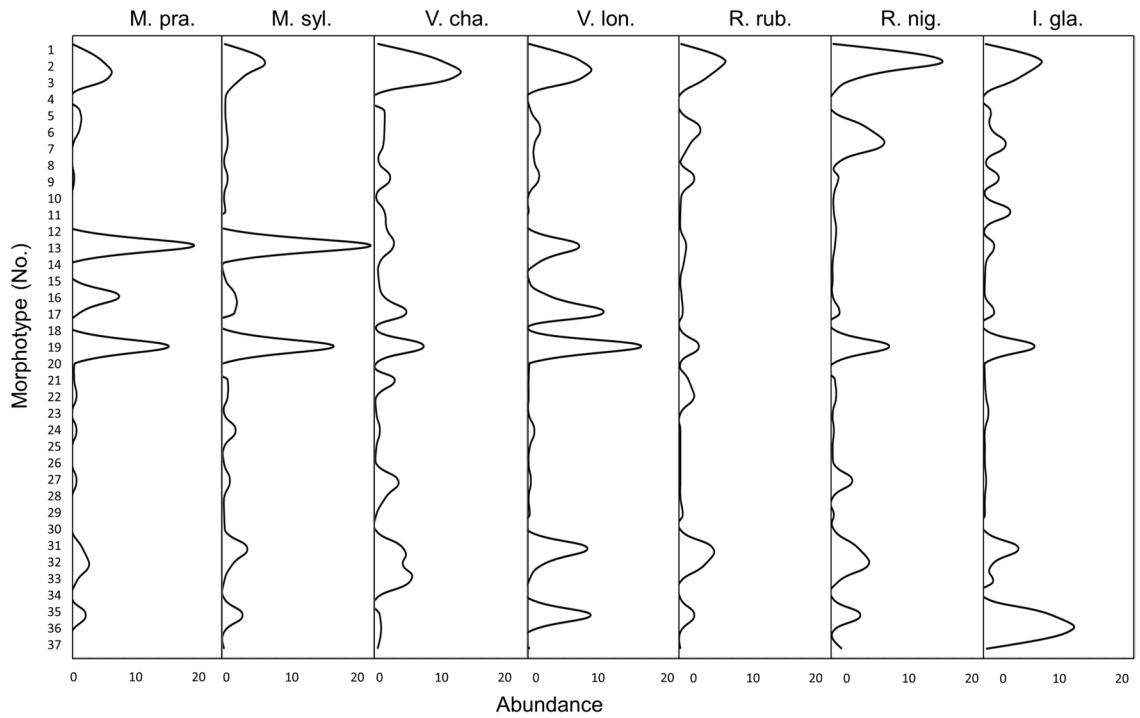

Fig. 1 Abundance-profiles of fungal morphotypes in seven plant species (M.pra. = *Melampyrum pratense*; M. syl. = *M. sylvaticum*; V. cha. = *Veronica chamaedrys*; V. lon. = *V. longifolia*; I. gla. = *Impatiens glandulifera*; R. nig. = *Ribes nigrum* and R. rub. = *R. rubrum*) that are alternate hosts to *Cronartium* rusts. Shown is the sum abundance across three sites and two time points

The pooled morphotype abundance, shown as violin plots that depict the distributions of the data points (including outliers) for the different morphotypes, indicated higher frequencies for several of the dominant morphotypes (13 and 19) in samples collected in June 2021, compared to those collected in September 2020 (Fig. 2). All 37 morphotypes were found in the samples collected in September 2020, whereas only 26 morphotypes were represented in samples collected in June 2021 (Suppl. Table 1). In the pooled data, several morphotypes (e.g., 10, 14, 16, 18, 20) that were present in samples collected in September 2020 were missing from the samples collected in June 2021 (Fig 2).

Variation in morphotype diversity and abundance between sampling sites

Our results suggest that while the same morphotypes tended to dominate the fungal endophytic communities at the different sites, some differences existed (Suppl. Table 1; Fig. 3). Based on the pooled data, site I had the richest diversity: 33 of the 37 morphotypes were found in samples collected from site I, whereas only 26 of the morphotypes were found in samples collected from site III (Suppl. Table 1; Fig. 3).

Fungal endophyte abundance and diversity in congeneric plants showing different resistance and susceptibility to rusts

There were no clear patterns in the abundance of fungal endophytes between the congeneric species differing in resistance to *Cronartium* rusts. However, comparison of pooled data on diversity, estimated as the number of distinct morphotypes present on a species, showed that diversity was higher in the resistant *V. chamaedrys* and *R. rubrum* compared to their susceptible congeneric species *V. longifolia* and *R. nigrum* (Table 2).

Table 3 Identification of endophytes isolated from leaves of plant species that are alternative hosts for *Cronartium* rusts of pines (*Melampyrum pratense*; *M. sylvaticum*; *Veronica chamaedrys*; *V. longifolia*; *Impatiens glandulifera*; *I. balsamina*; *Ribes nigrum* and *R. rubrum*). Sequences of >98% match to GenBank were listed. Time: 1=Late June 2021, 2=Early September 2020 (for *I. balsamina* September 2021). The plants were growing on three sites, I-III. MO=Morphotype

Plant species	Time	Site	MO	*Taxa*	Match (%)
M. sylvaticum	2	I	13	*Heterophoma* sp.	100
M. sylvaticum	2	I	13	*Heterophoma* sp.	100
M. sylvaticum	2	I	13	*Heterophoma* sp.	100
M. sylvaticum	1	I	13	*Heterophoma* sp.	100
M. sylvaticum	1	II	13	*Heterophoma* sp.	100
M. sylvaticum	1	III	13	*Heterophoma* sp.	100
M. sylvaticum	2	I	19	*Heterophoma* sp.	100
M. sylvaticum	2	I	19	*Heterophoma* sp.	100
M. sylvaticum	1	I	19	*Heterophoma* sp.	100
M. sylvaticum	1	I	19	*Heterophoma* sp.	100
M. sylvaticum	1	II	19	*Heterophoma* sp.	100
M. sylvaticum	1	III	19	*Heterophoma* sp.	100
M. pratense	2	I	13	*Heterophoma* sp.	100
M. pratense	2	I	13	*Heterophoma* sp.	100
M. pratense	2	I	13	*Heterophoma* sp.	100
M. pratense	1	II	13	*Heterophoma* sp.	100
M. pratense	1	III	13	*Heterophoma* sp.	99,8
M. pratense	1	III	13	*Heterophoma* sp.	100
M. pratense	2	I	19	*Heterophoma* sp.	100
M. pratense	1	II	19	*Heterophoma* sp.	100
M. pratense	1	III	19	*Heterophoma* sp.	100
M. pratense	1	III	19	*Heterophoma* sp.	100
V. chamaedrys	1	I	3	*Diaporthe padi var. padi*	99,0
V. chamaedrys	2	I	2	*Trichoderma* sp.	99,8
V. chamaedrys	2	II	2	*Seimatosporium lichenicola*	99.6
V. chamaedrys	1	I	2	*Sistotrema brinkmannii*	100
V. longifolia	2	I	19	*Penicillium* sp.	100
V. longifolia	2	III	19	*Pleosporaceae* sp.	99,8
V. longifolia	1	I	19	*Phaeosphaeria* sp.	100
V. longifolia	1	I	19	*Pleosporaceae* sp.	100
I. glandulifera	2	I	36	*Mycocentrospora acerina*	100
I. glandulifera	1	III	35	*Rhexocercosporidium* sp.	98,9
I. balsamina	2	I	35	*Alternaria alternata*	100
I. balsamina	2	I	35	*Alternaria alternata*	100
I. balsamina	2	I	35	*Alternaria alternata*	100
I. balsamina	2	I	35	*Alternaria alternata*	100
I. balsamina	2	I	31	*Cladosporium sp.*	100
I. balsamina	2	I	31	*Cladosporium cladosporioides*	100
I. balsamina	2	I	31	*Penicillium* sp.	99,8
R. nigrum	2	I	2	*Phlebia tremellosa*	99,1
R. nigrum	1	III	2	*Phlebiopsis gigantea*	100
R. nigrum	1	III	2	*Phlebiopsis gigantea*	100
R. nigrum	1	I	31	*Dothiora ribesia*	100
R. nigrum	1	I	31	*Cladosporium cladosporioides*	100
R. nigrum	2	I	7	*Jackrogersella multiformis*	99,0
R. nigrum	1	III	7	*Microsphaeropsis olivacea* or *Didymellaceae* sp.	99,8
R. nigrum	1	III	7	*Microsphaeropsis olivacea* or *Didymellaceae* sp.	100

Table 3 (continued)

Plant species	Time	Site	MO	*Taxa*	Match (%)
R. rubrum	1	III	2	*Calophoma sandfjordenica*	99,8
R. rubrum	1	I	19	*Alternaria anthropophila*	100
R. rubrum	1	I	19	*Didymella tanaceti*	99,4
R. rubrum	1	III	19	*Colletotrichum dematium*	100
R. rubrum	1	III	19	*Phaeosphaeria lycopodina*	99,4

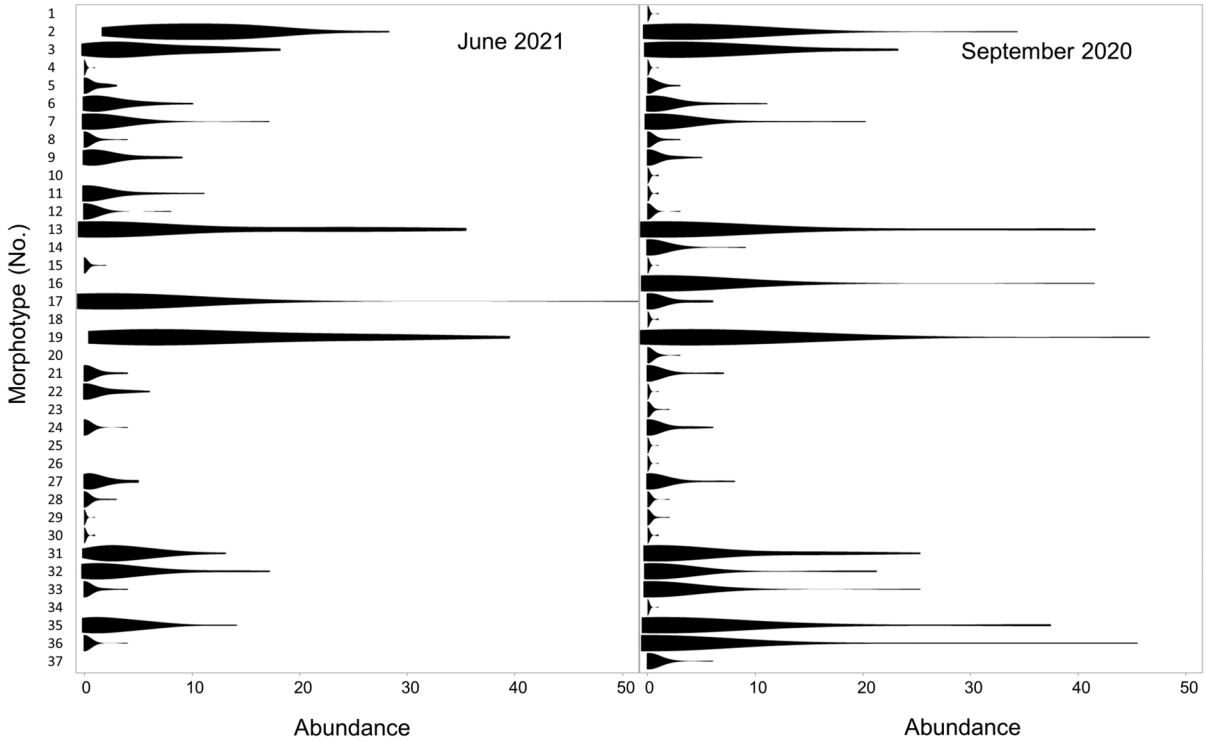

Fig. 2 Violin plots visualizing the distribution of the abundance of fungal morphotypes in June 2021 and in September 2020. The fungi were isolated from leaves of seven plant species (*Melampyrum pratense*, *M. sylvaticum*, *Veronica chamaedrys*, *V. longifolia*, *Impatiens glandulifera*, *Ribes nigrum* and *R. rubrum*) that are alternate hosts of *Cronartium* rusts and were growing at three sites

Discussion

Our results indicate that fungal endophytic communities vary between the plant species that act as alternate hosts to *Cronartium* rusts. This result is in agreement with previous studies that have shown the importance of host species as a determinant of fungal endophytic communities (Leopold & Busby, 2020; Romeralo et al., 2022), which may reflect the varying quality of the different hosts as a substrate for fungi. The high colonization rate of *Melampyrum* and *Veronica* species indicates that they are suitable hosts for endophytic fungi. The quality of leaves as a habitat for endophytic fungi could not be analyzed in this study, but it has been suggested that secondary metabolites, such as potentially antifungal phenolics could be a factor directing the microbiome composition (Witzell et al., 2022). In previous studies, some of these compounds have been found to be high in rust-resistant alternate host species (Kaitera & Witzell, 2016; Piispanen et al., 2023). In the plants of this study, chlorogenic acid is found at high levels in *M. pratense*, *R. rubrum* and *R. nigrum* (Piispanen et al., 2023) and quercitrin in *I. glandulifera*, *I.*

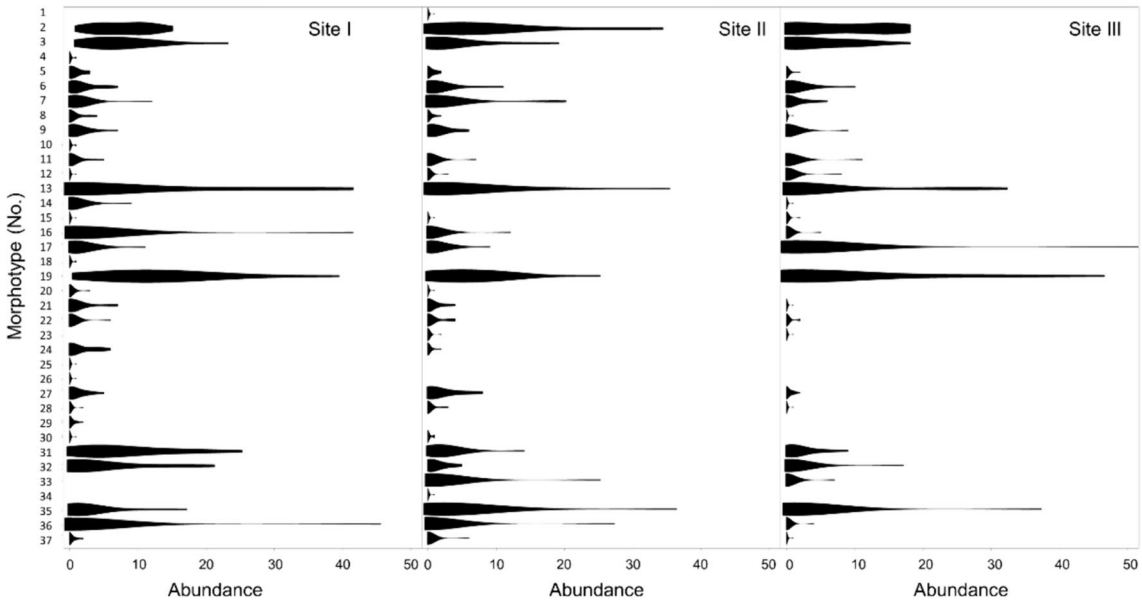

Fig. 3 Violin plots visualizing the distribution of the abundance of fungal morphotypes in three sites (I-III). Show is the sum abundance of each morphotype in seven plant species (*Melampyrum pratense*, *M. sylvaticum*, *Veronica chamaedrys*, *V. longifolia*, *Impatiens glandulifera*, *Ribes nigrum* and *R. rubrum*) that are alternate hosts of *Cronartium* rusts and were sampled at two time points (September 2020 and June 2021)

balsamina, *R.rubrum* and *R. nigrum* (Piispanen et al., 2023). Quercitrin is also present at higher levels in rust-resistant *V. chamaedrys* than rust-susceptible *V. longifolia* (Piispanen et al., 2023), while quercitrin and apigenin derivates are higher in rust-resistant *M. pratense* than rust-susceptible *M. sylvaticum* (Kaitera & Witzell, 2016; Piispanen et al., 2023). However, the relationship between endophytic fungi and phenolic metabolites may not be straightforward (Witzell et al., 2022), and some fungal endophytes are known to be able to use phenolics as a carbon source (Blumenstein et al., 2015).

Although practical biocontrol applications relying on endophytes are still pending, there is a growing body of evidence demonstrating this potential (Busby et al., 2016). Ganley et al. (2008) found that endophyte-inoculated *Pinus monticola* Dougl. ex D. Don seedlings survived longer than their endophyte-free counterparts after infection by *Cronartium ribicola*, and they also exhibited a notable reduction in the severity of white pine blister rust disease. Bullington et al. (2018) provided evidence that endophytes and terpenes simultaneously contribute to *C. ribicola* resistance in *Pinus albicaulis* Engelm. and concluded that endophytes have potential as biocontrol agents to protect the trees from *C. ribicola* infection. Raghavendra and Newcombe (2013) found that four foliar endophytes accounted for 54% of the variation in quantitative resistance among six poplar genotypes exhibiting diverse genetic resistance to virulent isolates of *Melampsora*, suggesting that foliar endophytes serve as a secondary line of defense, complementing major genes in the resistance against leaf rust. Based on this evidence, we expected that the fungal endophyte diversity or abundance between the congeneric plant species pairs studied would show a distinct pattern, indicating that fungal endophytic communities could contribute to the different patterns of susceptibility to *Cronartium* pathogens. Indeed, we found that the diversity of fungal endophytes, estimated as the pooled number of distinct morphotypes found per plant species across all sites and time points, was higher in the resistant *V. chamaedrys* and *R. rubrum* compared to their susceptible congeneric species, *V. longifolia* and *R. nigrum*. However, whether these differences have functional and biological consequences for the rusts in forest ecosystems remains to be studied. The lack of clear patterns in the abundance of morphotypes suggests that the role of fungal endophytes as determinants of rust resistance in alternate

host species may not be critical. Similar results were reported by Moler et al. (2022), who inoculated *Pinus albicaulis* seedlings with foliar endophytic fungi but found no evidence of a biocontrol effect against *C. ribicola*. In a recent study, Ata et al. (2023) characterized fungal endophytic communities in the needles of both *C. ribicola*-resistant and susceptible *Pinus flexilis* E. James trees. However, they did not find significant differences in the diversity or richness of the mycobiota and suggested that factors such as host size or site elevation could be more crucial determinants of the fungal endophytic communities.

When the data for all species were pooled across the three sites, the diversity of the culturable fungal endophytes in leaves of alternate host species seemed to be rather stable, i.e., the same morphotypes tended to dominate the endophyte profiles of the fungal species at different sites. Endophytes infect the plants from the surrounding environment (Gomes et al., 2018) and the dominance of certain fungi in the culturable fraction may thus reflect the high frequency of these fungi in the environmental inoculum in the region of the study area. The fact that the same morphotypes dominated in the samples from the greenhouse-grown *I. balsamina* supports this view – in greenhouses, the environmental inoculum is limited to spores that are most abundant in the incoming air (Witzell et al., 2022).

The morphotype diversity tended to be higher in the late season sampling compared to the early sampling, suggesting accumulation of infections during the growing season. Seasonal changes in environmental conditions have been found to change the environmental pool of microbe propagules (Collado et al., 1999). However, the age and developmental stage of the plants or plant parts may also influence the endophyte community composition (Nascimento et al., 2015; Oono et al., 2015).

It should be noted that because culture-dependent methods like the one we used can only capture a small fraction of the total diversity in fungal communities (Fan et al., 2020; Dos Reis et al., 2022), further studies with metabarcoding and metagenomics should be conducted to provide more detailed information about the diversity of fungal communities in these plants. The ITS1 and ITS2 regions typically offer resolution at a within-genus level and often within-species level (Nilsson et al., 2008). While it is used as the fungal DNA barcode, the ITS region may lack the required resolution in certain fungal groups and the use of other or additional markers could thus have improved the resolution of our analyses. Several of the fungi identified to species or genus level in our study have also been commonly reported among fungal endophytes in other studies. For instance, *Alternaria alternata* (Fr.) Keissl. is a cosmopolitan species that has been reported as an endophyte and a pathogen from a wide range of plant hosts (Woudenberg et al., 2013; DeMers, 2022). We found consistent evidence for the association between both *Melampyrum* species and *Heterophoma* species. Little is known about the ecology of *Heterophoma* species in northern forests, but *Heterophoma sylvatica* (Sacc.) Qian Chen & L. Cai has been reported as a part of the biodiversity of *Vaccinium myrtillus* L., a common heath species (Gomzhina et al., 2022). In general, the functional roles of individual endophyte species in hosts are still poorly known. In future, it may be possible to explore the functions using synthetic microbial communities (SynCom), an emerging approach that involves co-culturing multiple taxa under well-defined conditions to mimic the structure and function of a microbiome (de Souza et al., 2020).

In conclusion, our results indicate that the culturable endophyte communities do not have a straightforward role in the *Cronartium* rust resistance or susceptibility of alternate host plants. However, there is generally strong evidence supporting the view that fungal endophytes can influence plant health and productivity (Witzell & Martin, 2018; Terhonen et al., 2018), and the community structure captured in cultures is known to be limited. Therefore, culture-independent approaches that allow comparisons of whole communities of fungal endophytes could be used in future studies to further explore the possible role of endophytic fungi in the interaction between heteroecious rusts and their hosts.

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations The authors bear all the ethical responsibilities of this manuscript. They declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest and that it does not include any animal and/or human trials.

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