Characterization of fungal communities associated with the bark beetle Ips typographus varies depending on detection method, location, and beetle population levels

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Characterization of fungal communities associated with the bark beetle *Ips typographus* varies depending on location and beetle population levels, and on applying direct DNA isolation or culturing

GIORDANO LUANA [(1)], GARBELOTTO MATTEO [(2)], NICOLOTTI GIOVANNI [(1†)], GONTHIER PAOLO [(1)]

[(1)] University of Torino, Department for the Exploitation and Protection of Agricultural and Forestry Resources (DI.VA.P.R.A.), Plant Pathology, Via L. da Vinci 44, I-10095 (TO), Italy.

[(2)] University of California, Department of Environmental Science, Policy and Management, Ecosystem Sciences Division (ESPM-ES), Berkeley, CA 94720, USA.

[†] Author deceased.

Corresponding author: P. Gonthier
E-mail: paolo.gonthier@unito.it
Tel: +390116708697
Fax: +390112368697

Abstract

The Eurasian spruce bark beetle *Ips typographus* and their fungal associates can cause severe damages to Norway spruce forests. In this paper, by using both molecular and cultural methods, we compared fungal assemblages on bark beetles from different locations, characterized by different beetle population levels. *Ips typographus* was trapped in the western Alps in two areas with outbreaks and in two control areas. By sequencing of clone libraries of Internal Transcribed Spacer (ITS) 31 fungal Operational Taxonomic Units (OTUs) were identified, while fungal isolations yielded 55 OTUs; only three OTUs were detected by both methods. Culturing and PCR-based assays provide different non-overlapping results. Fungal assemblages on insects varied from stand to stand in response to varying ecological conditions and to the short distance spreading ability of *I. typographus*, and this was supported by data from additional 12 stands. Ophiostomatoid fungi showed higher diversity in outbreak areas; the pathogenic *Ophiostoma polonicum* was relatively uncommon, while *O. bicolor* was the most abundant species, supporting the well-known hypothesis of a temporal succession. Ubiquitous endophytes of trees or common airborne fungi, like *Penicillium* spp., *Alternaria alternata*, *Epicoccum nigrum* and *Trichoderma* spp., were present both in outbreak and in control areas. Wood decaying basidiomycetes were almost never detected on beetles. Yeast assemblage, detected only by molecular analysis, was consistent with those reported elsewhere in Europe, suggesting a very long association between some yeasts and bark beetles.

Keywords: *Ips typographus, Picea abies, fungal diversity, cloning, culture isolation, ophiostomatoid*

Introduction

The Eurasian spruce bark beetle *Ips typographus* L. (Coleoptera, Curculionidae, Scolytinae) is one of the most destructive pests associated with Norway spruce [*Picea abies* (L.) Karst.] (Christiansen and Bakke 1988). This beetle normally breeds in weak or dead trees and in felled timber, but during outbreaks it may attack healthy trees causing severe damages (Sallé et al. 2005). In the last decades, numerous reports of significant damages caused by *I. typographus* have come from various regions of Europe (Solheim 1992a, b; Viiri and Lieutier 2004; Jankowiak 2005; Sallé et al. 2005; Kirisits 2010), including the Italian Alps (Frigimelica et al. 2000; Stergulc et al. 2001; Faccoli and Stergulc 2004).

Ips typographus is commonly associated with ophiostomatoid fungal genera, including *Ophiostoma*, *Grosmannia*, *Ceratocystiopsis*, *Ceratocystis*, *Leptographium* and *Pesotum* (Mathiesen-Käärik 1953; Christiansen and Solheim 1990; Wingfield et al. 1993; Viiri and Lieutier 2004; Jankowiak 2005). Propagules of these fungi are carried both on the exoskeleton of beetles (pronotum and elytra) and in their digestive tract (Furniss et al. 1990; Solheim 1993a; Viiri and Lieutier 2004). Spruce bark beetles introduce spores or conidia of ophiostomatoid fungi mostly into the phloem of Norway spruce while digging galleries and breeding chambers. Ophiostomatoid fungi develop in the walls of larval galleries and in adjacent sections of bark and sapwood, causing blue-staining, a condition that generally lowers the quality of wood and may also reduce tree vigour (Kirisits and Offenthaler 2002).

Three ophiostomatoid species are commonly vectored by *I. typographus*: *Ophiostoma polonicum* (Siem.) C. Moreau, *O. bicolor* Davids. and Wells, and *O. penicillatum* (Grosm.) Siem. (Furniss et al. 1990; Paine et al. 1997; Kirisits et al. 2002; Linnakoski et al. 2012). Other species have been reported occasionally (Solheim 1986; Solheim 1992b; Kirschner and Oberwinkler 1999; Viiri and Lieutier 2004; Jankowiak 2005; Jankowiak and Hilszczański 2005; Linnakoski et al. 2012). Among all ophiostomatoid species, *O. polonicum* is regarded as the most virulent vascular pathogen associated with mortality of Norway spruce trees (Kirisits and Offenthaler 2002).

In addition to ophiostomatoid fungi, several Zygomycota, Ascomycota, Basidiomycota, anamorphic fungi and yeasts have been reported as fungal associates of *I. typographus* (Siemaszko 1939; Leufvén and Nehls 1986; Solheim 1992a; Kirisits 2004; Jankowiak 2005; Persson et al. 2009), but very little is known about the overall fungal diversity of non-ophiostomatoid fungi present on this insect species.

Similarly, very little is known about the biogeographic diversity of fungal communities associated with bark beetles (Lieutier et al. 1991; Solheim 1993b; Roe et al. 2011).

Assemblages of fungi associated with bark beetles are usually investigated by isolations from bodies of individual insects and/or from gallery systems in the sapwood using various isolation methods (e.g., dilution plating, direct beetle streaking, indirect isolation through living beetle inoculation in sterilized logs, etc.) (Francke-Grosmann 1956; Juzwik and French 1983; Furniss et al. 1990; Klepzig et al. 1991; Six and Bentz 2003; Lee et al. 2006; Jankowiak and Rossa 2007; Kirisits 2010; Linnakoski et al. 2012). An array of DNA-based identification methods [e.g., amplified rDNA restriction and ribosomal DNA (rDNA) sequencing analyses]

applied directly on insects may avoid the problem posed by fungi that are difficult to culture (Smit et al. 1999; Allen et al. 2003), and at least in two cases, fungal diversity on beetles has been studied using both cultural and molecular techniques (Lim et al. 2005; Persson et al. 2009).

The main goals of this work were to compare i) fungal assemblages associated with *I. typographus* from mountain and hill Norway spruce stands, and ii) fungi from stands experiencing an outbreak with those present in control areas characterized by endemic beetle populations. From previous works on fungal assemblages associated with *I. typographus* (Solheim 1992a; Wichmann and Ravn 2001; Kirisits 2004, 2010; Kautz et al. 2011), we expected to see diversity among fungal communities depending on environmental differences and levels of beetle populations. An additional goal was to compare the diagnostic efficacy of molecular and cultural techniques in the detection of fungi on *I. typographus.* This is the first extensive study on fungi associated with *I. typographus* in the Alps using molecular and cultural methods.

Materials and Methods

1. Survey in mountain and hill stands

A total of 12 Norway spruce stands were surveyed in 2005 in northwestern Italy. A group of six stands was located in hill areas around Alessandria (400 – 600 m a.s.l.) while a second group of six stands was selected in the mountain alpine areas of the Vigezzo, Antigorio, Soana and Susa Valleys (800 – 1150 m a.s.l.). Norway spruce was the dominant species in all investigated stands.

In late July, traps were used to collect adult beetles of *I. typographus*. Traps were baited with the commercial pheromone Pheroprax® for 15 days. Beetles were collected weekly and individually transferred using sterile tweezers from the traps into sterile 1.5 ml microcentrifuge tubes and stored at -40°C before processing.

DNA analysis

Ten specimens of *I. typographus* per trap were randomly selected and pooled together as an individual sample in 1.5 ml microcentrifuge tubes. Fungal DNA extraction from pooled beetles was carried out according to method described by Schweigkofler et al. (2005). PCR amplification of the Internal Transcribed Spacer (ITS2) was performed with primers ITS3 (White et al. 1990) and TW13 (O'Donnell 1993). The PCR mix consisted of 6.25 µL template DNA, 10X PCR Buffer, 2 mM dNTPs, 5 U/µM Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA), 50 mM $MgCl_2$, and 10 µM of forward and reverse primers in a 25 µL reaction volume. Amplifications were run in an iCycler thermocycler (Bio-Rad, Hercules, CA) with the following cycling parameters: initial denaturation at 95°C for 1.25 min, then 34 cycles of denaturation at 93°C for 35 s, annealing at 58°C for 55 s, extension at 72°C for 50 s, with an increase of 5 s after each cycle and a final extension at 72°C for 10 min.

One clone library was generated for each of the 12 stands studied as follows: PCR products from the ten pooled insects were cloned into plasmids and transformed into *Escherichia coli* using the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Positive colonies from each reaction were amplified using T7 forward and M13 reverse primers (Sambrook et al. 1989),

visualized on 1.5% agarose gel and subsequently sequenced on an ABI 3100 sequencer.

All sequences were compared with those of known fungi, using National Center for Biotechnology Information's (NCBI) GenBank nucleotide BLAST search. The sequence homology for delimiting fungal species and genera was set at 98 – 100% and 95 – 97%, respectively (Persson et al. 2009).

Sequences that were of insect origin were excluded from the analysis.

2. Survey in stands with outbreak presence of *I. typographus* and control areas

Four areas, two in the Antigorio Valley and two in the Soana Valley, were selected for the study. In each valley, one outbreak (i.e. epidemic) and one control (i.e. endemic) population of the beetle were identified on the basis of the extent of visible tree damages. Outbreak and control areas were about four and two kilometers from each other, in the Antigorio and Soana Valleys, respectively (Table 1). Pheromone traps were used to collect adult *I. typographus* beetles as previously described. Beetles were collected weekly and individually transferred using sterile tweezers from the traps into sterile 1.5 ml microcentrifuge tubes. The tubes containing beetles assigned for fungal isolations were stored at 5°C and the tubes assigned for direct DNA extractions at -40°C.

DNA- and culture-based fungal diagnoses

For DNA-based diagnosis, ten specimens per trap of *I. typographus* were randomly selected and pooled together as an individual sample in 1.5 ml microcentrifuge tubes, DNAs were extracted as described above. One clone library was constructed

for each area, for a total of four libraries. Operational Taxonomic Units (OTUs) were designated using sequence similarity with fungal sequences deposited in GenBank, as described above.

For culture-based diagnosis, 15 insects per trap were randomly selected and used for isolations. Isolations were performed by rolling insects, without surface sterilization, across the medium with sterile tweezers (Aukema et al. 2005; Jankowiak and Rossa 2007). To minimize the selective bias linked to the use of a specific culture media, isolations were made on three different media: i) 2% Malt Extract Agar (2% MEA; 20 g malt extract, 20 g agar, 1000 mL distilled water) supplemented with 200 mg tetracycline to isolate the general fungal flora (Jankowiak 2005); ii) 2% MEA supplemented with 4 mg benomyl and 100 mg ampicillin to isolate preferentially the Basidiomycota (Kim et al. 2005); iii) selective medium (CH-MA; 20 g malt, 16 g agar, 1000 mL distilled water) supplemented with 100 mg streptomycin sulphate and 200 mg cycloheximide for the isolation of *Ophiostoma* spp. and their anamorphs (Harrington 1981; Kirisits et al. 2002).

Petri dishes were incubated in the light at room temperature for four weeks and were daily inspected for fungal growth and the occurrence of sexual and asexual fungal structures. Pure fungal cultures were obtained by transferring small pieces of agar containing mycelium onto fresh 2% MEA plates for subsequent morphotyping. Fungal isolates were grouped into mycelial morphotypes based on growth morphology and macroscopic features. OTUs identification via traditional methods was achieved by macro- and micro-morphological analysis using taxonomic guides and standard procedures (Domsch et al. 1980; Arx et al. 1981; Kiffer and Morelet 1997). Non-sporulating fungi were grouped as sterile mycelia (*sensu* Lacap et al. 2003) and divided into different morphotypes based on similar

cultural characters. Among these, sterile basidiomycetes were identified by scoring mycelia for presence of clamp connections. DNA sequence information was used to assist in identification of OTUs that were unresolved by morphological analysis or were sterile.

A representative sample of identified OTUs has been deposited as dried cultures at the culture collection of the *Herbarium Patavinum* (PAD), University Museum Centre (CAM), University of Padua, Italy.

3. Data interpretation and analyses

The abundance of each OTU identified following either sequencing or culturing techniques was expressed as percentage of sequences of that OTU on total number of sequences for each library and as percentage of isolates of that OTU on the total number of isolates for each area. The Sorensen similarity index (S_s) was used to compare fungal communities from different locations. This index is calculated as follows: $2c/(a+b)$, where a is the number of OTUs in one sampling, b is the number of OTUs in a second sampling and c is the number of OTUs shared by the two samplings (Magurran 2004).

An UPGMA (Unweighted Pair Group Method with Arithmetic Mean) cluster analysis was performed to visualize patterns and determine number of discrete fungal assemblages in outbreak and control beetle populations of the Antigorio and Soana Valley. For the cluster analysis, according to S_s (see results), data from libraries and culture isolations were analysed together.

All tests were performed with PASW Statistics 18 (2009).

Results

1. Survey in mountain and hill stands

A total of 114 ITS sequences representing 29 fungal OTUs using a 95% similarity threshold were obtained from the libraries, including 55 from the mountain stands and 59 from the hill stands. The mycobiota was dominated by Ascomycota and anamorphic fungi (20 OTUs, 69%), followed by Basidiomycota (7 OTUs, 24%) and Zygomycota (1 OTU, 3%). Fourteen OTUs of Ascomycota and Basidiomycota yeasts were identified (Table 2). In mountain stands, the number of OTUs per stand ranged from 2 to 6, while in hill stands that number ranged from 0 to 6.

The two groups of stands (i.e., mountain *vs* hill stands) were characterized by very different fungal assemblages (S_s = 0.18), with only three OTUs (*Alternaria tenuissima*, *Cryptococcus oeirensis* and *Wickerhamomyces bisporus*) found in both. Additionally, in both stand groups, the composition of fungal assemblages differed from stand to stand, and few fungal OTUs were found in at least two sites. A single ophiostomatoid fungus, *Ophiostoma polonicum*, was identified in two mountain stands of the Soana Valley.

2. Survey in stands with outbreak presence of *I. typographus* and control areas

To increase our ability to identify rare fungal OTUs, our second study was performed using a more intensive sampling approach in one outbreak and one control area in two valleys (Antigorio and Soana).

A total of 367 ITS sequences representing 31 fungal OTUs (using 95% sequence similarity as a threshold) were obtained from the libraries, including 151 from outbreak and 216 from control areas.

A total of 313 fungal isolates, representing 55 fungal OTUs, were obtained from isolations. 69% of 205 plated insects generated at least one fungal colony, while the remaining 31% were either colonized by bacteria or appeared to be uncolonized. There was no effect of the three different culture media on number of OTUs recovered, but about 90% of ophiostomatoid isolates were obtained from selective medium CH-MA (data not shown).

When comparing the two identification approaches, there were 28 OTUs detected exclusively by clone libraries, 52 OTUs detected exclusively by culturing, and only three OTUs were detected by both methods (Table 3). Community compositions revealed by each method were almost totally different ($S_s = 0.07$). To account for the potential effects of geographic location, the same analysis was performed independently for each valley, with identical results of insignificant overlap between diagnostic approaches ($S_s = 0.03$ for Antigorio Valley; $S_s = 0.18$ for Soana Valley).

Cultures of non-ophiostomatoid OTUs showing abundance > 10% in at least one area were: *Alternaria alternata, Cladosporium cladosporioides, Fusarium* spp., *Mucor hiemalis*, *Penicillium* spp., *Talaromyces trachyspermus* and *Trichoderma* spp. OTUs defined by DNA sequencing showing abundance > 10% in at least one area were: *Beauveria bassiana*, *Candida ontarioensis*, unidentified yeast BAF5 and unidentified yeast BAF22. Based on DNA analysis, *Beauveria bassiana* was the most abundant OTU (0-69% of OTUs by site). All ascomycetous and basidiomycetous yeasts were detected only by the molecular method.

Except for *Ophiostoma polonicum,* all ophiostomatoid fungi were detected by culture isolations. *Ceratocystis tetropii* was the only one detected by both methods. Ophiostomatoid OTUs showing abundance > 10% in at least one area included *Ophiostoma bicolor* and *Ophiostoma* sp. 1.

Combining the data of clone libraries with those obtained by culturing, the overall fungal community associated with *I. typographus* included 11 ophiostomatoid fungi, 28 other Ascomycota and anamorphic fungi, 3 Basidiomycota, 17 ascomycetous, basidiomycetous and unidentified yeasts, and 2 Zygomycota. Twenty-one morphological types of fungi were dark, hyaline and pinkish sterile mycelia whose sterility persisted in colonies grown on 2% MEA incubated at 20 – 25°C for several months (Table 3).

There were 54 OTUs exclusive to the Antigorio Valley and 16 OTUs exclusive to the Soana Valley. Fungal communities from the two valleys were largely different from one another ($S_s = 0.27$). When pooling the two control and the two outbreak populations irrespective of the valley of origin, the resulting S_s was even lower than that obtained by comparing the overall fungal community between the two sites ($S_{s\ outbreak} = 0.16$; $S_{s\ control} = 0.26$).

In the Antigorio Valley, 41.8% of OTUs were outbreak-specific while 40% were only found in control areas; only 17.9% were detected in both populations ($S_s = 0.30$). In the Soana Valley 48% of OTUs were outbreak-specific, 24% control-specific and 27.6% were shared by two populations, thus resulting in a higher similarity value between outbreak and control areas ($S_s = 0.43$).

Beauveria bassiana, an entomopathogenic fungus, was the most abundant OTU and the only one with *Epicoccum nigrum* and *Penicillium* spp. to be detected in all four areas.

The assemblage of ophiostomatoid fungi consisted of *Ceratocystis tetropii, Grosmannia cucullata*, *G. europhioides*, *Ophiostoma bicolor*, *O. breviusculum*, *O. piceae, O. polonicum*, three other *Ophiostoma* OTUs and one *Sporothrix* OTU. *Grosmannia europhioides*, *O. polonicum* and *O. piceae*, were detected exclusively in control areas, all other species were present in outbreak areas. Finally, *G.*

cucullata, *O. bicolor* and *Ophiostoma* sp. 2 were detected in both outbreak and control areas. Among them, *O. bicolor* was the most common OTU.

Cluster analyses based on S_s divided the fungal community of each valley into three clusters (Figures 1 and 2). Two of these clusters are exclusively associated with outbreak or control beetle populations respectively, while the third includes taxa shared by both types of beetle populations.

Discussion

This is the first study using both molecular and cultural methods on fungi associated with *I. typographus* in mountain and hill stands of the Alps characterized both by outbreak and endemic populations of this beetle.

On the whole, the spectrum of fungi identified was consistent with that reported for *I. typographus* elsewhere in Europe (Mathiesen-Käärik 1953; Solheim 1986, 1992a, b, 1993a; Viiri and Lieutier 2004; Jankowiak 2005; Sallé et al. 2005; Persson et al. 2009; Kirisits 2010; Linnakoski et al. 2012).

The first survey identified two distinct and mostly non-overlapping assemblages of fungi in mountain and hill stands. The composition of fungal assemblages was different even when comparing sites within the same altitudinal range. Only *Taphrina* sp. and *Phallus impudicus* cannot be considered directly associated with *I. typographus* and they were deemed to be contaminant.

Environmental and climatic factors are likely to affect the composition of the fungal flora associated with *I. typographus* (Kirisits 2004), however both ecological-environmental conditions and spatial segregation may have resulted in the differences reported in this study. The minimal distance among study sites in fact was 2 – 5 km, a distance that may exceed the normal range of dispersal of this

beetle. On average in fact, 95% of new outbreaks are reported to occur within 500 m from infestations occurred in the previous year (Wichmann and Ravn 2001; Kautz et al. 2011). Additionally, the fact that buffer zones between 100 m and 1500 m have prevented significant attacks in managed forests (Wermelinger 2004) points to a limited dispersal range of this beetle species.

In the second survey, our data clearly shows that cultural method allow to identify a greater number of fungal OTUs compared to molecular method, and thus appears to be superior with respect to the detected species diversity. This finding would support the belief that investigations on fungal diversity in environmental samples are probably incomplete when identification rely exclusively on DNA isolation. Nevertheless, it should be noted that many fungal clones in our studies remained unidentified due to small length of the sequences or the low similarity with ITS sequences in the GenBank database, suggesting that our inability to identify these OTUs are related to technical issues (e.g. quality of DNA, unavailability of sequences in GenBank database), rather than a per se limit of the molecular method. It appears that for a complete description of the fungal community vectored by *I. typographus* both molecular and cultural methodologies are necessary. This finding is supported by previous studies (Lim et al. 2005; Persson et al. 2009) showing that different methods tend to provide information on different groups of fungi; thus, the methods complemented each other.

Several fungi detected by molecular method were never isolated in pure culture, indicating that some might be unculturable from environmental samples. In general, ophiostomatoid fungi and fast-growing fungi were more frequently detected by pure culture isolations. By contrast, ascomycetous and basidiomycetous yeasts could be detected exclusively by using the molecular method as previously reported (Lim et al. 2005; Persson et al. 2009). Since every method or medium of isolation

may be selective for some species but not appropriate for others, we cannot exclude yeasts could have been isolated by using other isolation techniques (e.g. dilution plating) or media, and the same is true for other groups of fungi. Three of the yeasts detected in this study on *I. typographus* had identical ITS sequences to those detected by Lim et al. (2005) on bark beetles in Canada, one on *Dendroctonus ponderosae* (BAF22), and two on *Ips pini* (BAF5 and BAF15). Recently, BAF22 and BAF5 were also detected on *I. typographus* by the direct molecular analysis (T-RFLP) (Persson et al. 2009). Whether the sequence identity of yeasts between this and other studies is the result of a true association of the same yeast species with different insect species from different continents, or whether it is the result of ITS transfers among species, remains to be determined. In either case, the data are suggestive of a very long association between some yeasts and bark beetles. Leufvén and Nehls (1986) have in the past successfully isolated other yeasts reported to be associates of *I. typographus*, e.g. *Kuraishia capsulata*, *Nakazawaea holstii*, and species in the genera *Cryptococcus* and *Ogataea*. The role of yeasts in the life cycle of *I. typographus* is yet unknown but the common association with the bark beetle suggests that they can play an important ecological role. There is experimental evidence that some yeasts and bacteria, living in the digestive tract of insects, might be involved in digestion, detoxification processes of plant chemicals to which the insects are exposed, and pheromone production (Vega and Dowd 2005; Rivera et al. 2007).

The ordination of fungal taxa identified cumulatively by two methods shows that there are three different fungal assemblages in both valleys: two of these are exclusively associated with outbreak or control beetle populations respectively, while the third includes taxa shared by both types of beetle populations. The variation in the spectrum of fungi can depend on various factors, including the

levels of beetle populations (Kirisits 2004, 2010), the ecological differences among stands (Kirisits 2004), the stage of the infestation and tree damage (Solheim 1992a), and the distance among study sites in relation to the ability of the insects to spread (Wichmann and Ravn 2001; Kautz et al. 2011). However, very little is known on the extend to which and how the different factors affect this variation (Linnakoski et al. 2012).

Penicillium spp. were among the most abundant OTUs ranging from 11% to 12% in the Antigorio Valley and from 23% to 69% in the Soana Valley. This finding is in accordance with reports of Jankowiak (2006) and Jankowiak and Rossa (2008) about fungi associated with two bark beetles *Tomicus piniperda* (L.) and *Pityogenes bidentatus* (Herbst) affecting Scots pine (*Pinus sylvestris* L.). Other *Penicillium* species were isolated from *Dendroctonus* sp. (Whitney 1982), *Crypturgus cinereus* (Hrbst.) and *Ips sexdentatus* (Börn.) (Kirschner 2001). *Penicillium* species are widely distributed in nature and are not associated with any specific species of bark beetles on coniferous trees (Jankowiak 2006); they are primarily wind dispersed and do not depend on beetle vectors for dissemination. Some other OTUs identified in this study (e.g. *Ampelomyces humuli* and *Graphiopsis chlorocephala* which are known to be obligate parasites of fungi and plants, respectively) cannot be considered associated with *I. typographus*, and were thus deemed to be contaminant.

Other frequent OTUs, such as *Alternaria alternata, Cladosporium cladosporioides, Epicoccum nigrum* and *Trichoderma* spp., were reported as ubiquitous fungi and endophytes of trees (Fisher et al. 1991; Lumley et al. 2001; Ragazzi et al. 2003; Lygis et al. 2005; Menkis et al. 2006; Wang and Guo 2007) and often associated with different bark beetle species (Lieutier et al. 1989; Peverieri et al. 2006; Bueno et al. 2010). Noteworthy is also the common occurrence of some insect-pathogenic

fungi, such as *Beauveria bassiana* and *Isaria coleopterorum*, and of one mycoparasite, *Clonostachys rosea*.

The ubiquitous nature of species that are commonly air-dispersed, including those that are endophytic or pathogenic to insects, may justify the presence of these OTUs both in outbreak and control areas. In the Antigorio Valley, with the exception of *B. bassiana* and *Trichoderma* spp. that were only found in the "outbreak cluster", all other OTUs (i.e. *A. alternata*, *C. cladosporioides*, *E. nigrum*, *I. coleopterorum* and *Penicillium* spp.) were found in the "outbreak/control cluster". In the Soana Valley, *E. nigrum* and *Penicillium* spp. belong to the "outbreak cluster", while *B. bassiana* to the "outbreak/control cluster", while all other OTUs are absent. However, it must be emphasized that *B. bassiana* and *Trichoderma* spp. were also present in the control area (4% and 2%, respectively) of Antigorio Valley and *E. nigrum* and *Penicillium* spp. were also present in the control area (0.6% and 23%, respectively) of Soana Valley, thus further confirming that the species mentioned in this section can all be considered ubiquitous associates of *I. typographus*.

A limited number of Basidiomycota was isolated, despite the use of a selective medium, and only *Polyporus* sp. was detected in clone libraries. Historically, Basidiomycota have only occasionally been found in association with bark beetles because of traditional isolation techniques applied in the bark beetle investigations underestimate the actual occurrence of these fungi (Linnakoski et al. 2012). There is emerging evidence that Basidiomycota are more common associates of bark beetles that previously thought and sometimes a clear association with *I. typographus* has been found (Kirschner et al. 2001a, b; Oberwinkler et al. 2006; Persson et al. 2009).

More than 25 ophiostomatoid fungi have so far been reported as fungal associates of *I. typographus* in various parts of Europe (Kirisits 2004). Many of these taxa

were encountered in only a few investigations or generally occurred at low frequency. In this study, ophiostomatoid fungi represented 13% of the total number of taxa detected in all four areas. *Ophiostoma penicillatum* and *O. ainoae* H. Solheim, were previously reported to be consistently associated with *I. typographus* across Europe (Viiri and Lieutier 2004; Jankowiak 2005; Sallé et al. 2005; Persson et al. 2009; Kirisits 2010), but were not recorded in this study. This is the first report of *O. breviusculum* associated with *I. typographus* in Europe. Previously, this species within the *O. picea*-complex was isolated only from *Ips subelongatus* Motschulsky and *Dryocoetes baikalicus* Reitter from *Larix kaempferi* (Lamb.) Carr. in Japan (Chung et al. 2006; Yamaoka et al. 2009).

Ophiostomatoid fungi occurred with varying frequencies in the two investigated sites but, on the whole, outbreak areas showed higher diversity than control ones. However, the most pathogenic species, *Ophiostoma polonicum*, was unexpectedly overall rare. Reports on the abundance of *O. polonicum* vary, but it has often been reported as a dominant element of the mycobiota of *I. typographus* (Solheim 1986, 1992a, b; Krokene and Solheim 1996; Kirisits 2004). The sporadical presence of *O. polonicum* and the high frequency of *O. bicolor* in this study may be related to the population dynamics of *I. typographus.* Solheim (1993a) suggested that *O. polonicum* occurs at low frequencies during endemic periods, but its frequency increases during the outbreak phase when vigorous trees are attacked; this pathogen, in fact, tolerates oxygen-deficient conditions of wood of healthy trees (Solheim 1992a). Evidence for this hypothesis is, however, not conclusive, as *O. polonicum* has either not been reported at all (Grosmann 1931) or was rarely found also in areas with high damage levels of *I. typographus* (Mathiesen-Käärik 1953; Kirisits 2004; Jankowiak 2005; Jankowiak and Hilszczański 2005; Sallé et al. 2005). Solheim (1992a, b), Jankowiak and Hilszczański (2005) and Jankowiak (2005) have

demonstrated that a temporal succession of fungi into phloem and sapwood could be responsible for the varying frequencies reported for ophiostomatoid fungi in Norway spruce. In this successional scenario, *O. polonicum* is the primary invader, occurring most frequently in the sapwood of Norway spruce trees during the early stages of brood development of *I. typographus*. Within two or three weeks though, new *Ophiostoma* species substitute *O. polonicum*. *Ophiostoma bicolor* is known to be one of the first species to follow *O. polonicum*, and its frequency increases rapidly during the first weeks after attack. In our study, the fact that *I. typographus* specimens were collected when Norway spruce trees were already dying could have greatly accounted for the low frequency of occurrence of *O. polonicum.* This interpretation is also supported by the occurrence in our study of *G. europhioides* [in many investigations referred to as *Grosmannia piceaperda* (Rumbold) Goid.] and *C. tetropii*, previously reported as tertiary and quaternary invaders, respectively, of Norway spruce (Solheim 1992a). Besides and maybe in addition to the successional theory, vectoring of pathogenic *Ophiostoma* spp. may be explained by threshold effects, and infection may be associated with *I. typographus* outbreaks only when insect populations reach a certain size. In that case, even if frequency of detection is low for any given fungal species, that low frequency may be counterbalanced by large numbers of insect vectors. It is likely that our sampling occurred when beetles populations were already crashing, and thus it is possible our sampling occurred when populations of the beetle had gone under the threshold levels necessary for effective vectoring of *O. polonicum* (Wermelinger 2004).

In conclusion, molecular and cultural methods provided a different picture of the fungal communities on beetles, emphasizing the need to use both approaches to provide a conclusive picture of the mycoflora associated with *I. typographus*. In light of this finding, we suggest this study may be one of the first to provide a

relatively complete analysis of fungi that may be associated with this bark beetle. Different fungal assemblages occurred in hill and mountain stands as a consequence of different ecological features of stands or of different stages in the dynamics of beetle populations. Additionally, the lack of overlap of fungal OTUs reported in this study may be explained by the limited ability of the insect to move at distances greater than 500 m. Sampling of sites less than 500 m apart and when beetles populations are peeking during the outbreak should result in a significant larger overlap of fungal assemblages, because of the ability of beetles to move between sites at that distance and because of a greater match between the population dynamics of the beetles in nearby sites.

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References

Allen TR, Millar T, Berch SM, Berbee ML (2003) Culturing and direct DNA extraction find different fungi from the same ericoid mycorrhizal roots. New Phytol 160: 255–272

Arx JA von (1981) The genera of fungi sporulating in pure culture. J. Cramer, Vaduz, Germany

Aukema BH, Werner RA, Haberkern KE, Illman BL, Clayton MK, Raffa KF (2005) Quantifying sources of variation in the frequency of fungi associated with spruce beetles: implications for hypothesis testing and sampling methodology in bark beetle-symbiont relationships. Forest Ecol Manag 217: 187–202

Bueno A, Diez JJ, Fernández MM (2010) Ophiostomatoid fungi transported by *Ips sexdentatus* (*Coleoptera; Scolytidae*) in *Pinus pinaster* in NW Spain. Silva Fenn 44: 387–397

Christiansen E, Bakke A (1988) The spruce bark beetle of Eurasia. In: Berryman AA (ed) Dynamics of Forest Insect Populations. Pattern, Causes, Implications. Plenum Press, New York and London, pp 479–503
Christiansen E, Solheim H (1990) The bark beetle-associated blue-stain fungus *Ophiostoma polonicum* can kill various spruces and Douglas fir. Eur J Forest Pathol 20: 436–446
Chung WH, Kim JJ, Yamaoka Y, Uzunovic A, Masuya H, Breuil C (2006) *Ophiostoma breviusculum* sp. nov. (*Ophiostomatales, Ascomycota*) is a new species in the *Ophiostoma piceae* complex associated with bark beetles infesting larch in Japan. Mycologia 98: 801–814
Domsch KH, Gams W, Anderson TH (1980) Compendium of soil fungi. Academic Press, London, England
Faccoli M, Stergulc F (2004) *Ips typographus* (L.) pheromone trapping in south Alps: spring catches determine damage thresholds. J Appl Entomol 128: 307–311
Fisher PJ, Petrini O, Petrini LE (1991) Endophytic ascomycetes and deuteromycetes in roots of *Pinus sylvestris*. Nova Hedwigia 52: 11–15
Francke-Grosmann H (1956) Hautdrüsen als Trager der Pilzsymbiose bei Ambrosiakäfern. Z Morph u Ökol Tiere 45: 275–308
Frigimelica G, Stergulc F, Faccoli M, Battisti A (2000) A comparison of damage caused by fungi and insects in pure and mixed stands of *Picea abies* in the south-eastern Italian Alps. In: Hasenauer H. (ed) Proceedings of the International Conference “Forest ecosystem restoration. ecological and economical impacts of restoration processes in secondary coniferous forests”. Vienna, Austria, pp 85–91
Furniss MM, Solheim H, Christiansen E (1990) Transmission of blue-stain fungi by *Ips typographus* (*Coleoptera: Scolytidae*) in Norway spruce. Ann Entomol Soc Am 83: 712–716
Grosmann H (1931) Beiträge zur Kenntnis der Lebensgemeinschaft zwischen Borkenkäfern und Pilzen. Z Parasitenk 3: 56–102
Harrington TC, Furniss MM, Shaw CG (1981) Dissemination of *Hymenomycetes* by *Dendroctonus pseudotsugae* (*Coleoptera: Scolytidae*). Phytopathology 71: 551–554
Jankowiak R (2005) Fungi associated with *Ips typographus* on *Picea abies* in southern Poland and their succession into the phloem and sapwood of beetle-infested trees and logs. Forest Pathol 35: 37–55
Jankowiak R (2006) Fungi associated with *Tomicus piniperda* in Poland and assessment of their virulence using Scots pine seedlings. Ann For Sci 63: 801–808
Jankowiak R, Hilszczański J (2005) Ophiostomatoid fungi associated with *Ips typographus* (L.) on *Picea abies* (L.) H. Karst. and *Pinus sylvestris* L. in North-Eastern Poland. Acta Soc Bot Pol 74: 345–350
Jankowiak R, Rossa R (2008) Associations between *Pityogenes bidentatus* and fungi in young managed Scots pine stands in Poland. Forest Pathol 38: 169–177
Juzwik J, French DW (1983) *Ceratocystis fagacearum* and *Ceratocystis piceae* on the surface of free-flying and fungus-mat-inhabiting nitidulids. Phytopathology 73: 1164–1168

Kautz M, Dworschak K, Gruppe A, Schopf R (2011) Quantifying spatio-temporal dispersion of bark beetle infestations in epidemic and non-epidemic conditions. Forest Ecol Manag 262: 598–608

Klepzig KD, Raffa KF, Smalley EB (1991) Association of an insect-fungal complex with red pine decline in Wisconsin. Forest Sci 37: 1119–1139

Kiffer E, Morelet M (1997) Les deutéromycètes. Classification et clés d'identification générique. INRA Editions, Paris, France

Kim JJ, Allen EA, Humble LM, Breuil C (2005) Ophiostomatoid and basidiomycetous fungi associated with green, red and grey lodgepole pines after mountain pine beetle (*Dendroctonus ponderosae*) infestation. Can J Forest Res 35: 274–284

Kirisits T (2004) Fungal associates of European bark beetles with special emphasis on the ophiostomatoid fungi. In: Lieutier F, Day KR, Battisti A, Grégoire J-C, Evans HF (eds) Bark and wood boring insects in living trees in Europe, a synthesis. Springer, Dordrecht, The Netherlands, pp 181–236

Kirisits T (2010) Fungi isolated from *Picea abies* infested by the bark beetle *Ips typographus* in the Bialowieza forest in north-eastern Poland. Forest Pathol 40:100–110

Kirisits T, Offenthaler I (2002) Xylem sap flow of Norway spruce after inoculation with the blue-stain fungus *Ceratocystis polonica*. Plant Pathol 51: 359–364

Kirisits T, Wingfield MJ, Chettri DB (2002) Studies on the association of blue-stain fungi with the Eastern Himalayan spruce bark beetle (*Ips schmutzenhoferi*) and with other bark beetles in Buthan. Yusipang Report, RNR RC Yusipang

Kirschner R (2001) Diversity of filamentous fungi in bark beetle galleries in central Europe. In: Misra JK, Horn BW, Robert W (eds) Trichomycetes and other fungal groups. Science Publishers, Inc., pp 175–196

Kirschner R, Oberwinkler F (1999) A new *Ophiostoma* species associated with bark beetles infesting Norway spruce. Can J Botany 77: 247–252

Kirschner R, Begerow D, Oberwinkler F (2001a) A new *Chionosphaera* species associated with conifer inhabiting bark beetles. Mycol Res 105: 1403–1408

Kirschner R, Sampaio JP, Gadanho M, Weiss M, Oberwinkler F (2001b) *Cuniculitrema polymorpha* (Tremellales, gen. nov. and sp. nov.), a heterobasidiomycete vectored by bark beetles, which is the teleomorph of *Sterigmatosporidium polymorphum*. Antoine van Leeuwenhoek 80: 149–161

Krokene P, Solheim H (1996) Fungal associates of five bark beetle species colonizing Norway spruce. Can J Forest Res 26: 2115–2122

Lacap DC, Hyde KD, Liew ECY (2003) An evaluation of the fungal "morphotypes" concept based on ribosomial DNA sequences. Fungal Divers 12: 53–66

Lee S, Kim JJ, Breuil C (2006) Diversity of fungi associated with mountain pine beetle, *Dendroctonus ponderosae*, and infested lodgepole pines in British Columbia. Fungal Divers 22: 91–105

Leufvén A, Nehls L (1986) Quantification of different yeasts associated with the bark beetle, *Ips typographus*, during its attack on a spruce tree. Microb Ecol 12: 237–243

Lieutier F, Yart A, Garcia J, Ham MC, Morelet M, Levieux J (1989) Champignons phytopathogènes associés à deux coléoptères scolytidae du pin sylvestres (*Pinus sylvestris* L.) et étude préliminaire de leur agressivité envers l'hôte. Ann For Sci 46:201–216

Lieutier F, Garcia J, Yart A, Vouland G, Pettinetti M, Morelet M (1991) Ophiostomatales (Ascomycètes) associées à *Ips acuminatus* Gyll (*Coleoptera: Scolytitidae*) sur le pin sylvestre (*Pinus sylvestris* L.) dans le Sud-Est de la France et comparison avec *Ips sexdentatus* Boern. Agronomie 11: 807–817

Lim WY, Kim JJ, Lu M, Breuil C (2005) Determining fungal diversity on *Dendroctonus ponderosae* and *Ips pini* affecting lodgepole pine using cultural and molecular methods. Fungal Divers 19: 79–94

Linnakoski R, Wilhelm de Beer Z, Niemelä P, Wingfield MJ (2012) Associations of conifer-infesting bark beetles and fungi in Fennoscandia. Insects 3: 200–227

Lumley TC, Gignac LD, Currah RS (2001) Microfungus communities of white spruce and trembling aspen logs at different stages of decay in disturbed and undisturbed sites in the boreal mixedwood region of Alberta. Can J Botany 79: 76–92

Lygis V, Vasiliauskas R, Larsson KH, Stenlid J (2005) Wood-inhabiting fungi in stems of *Fraxinus excelsior* in declining ash stands of northern Lithuania, with particular reference to *Armillaria cepistipes*. Scand J Forest Res 20: 337–346

Magurran A (2004) Measuring biological diversity. Blackwell Publishing, Oxford, UK

Mathiesen-Käärik A (1953) Eine Übersicht uber die gewöhnlichsten mit Borkenkäfern assoziierten Bläuepilze in Schweden einige für Sweden neue Bläuepilze. Med Stat Skogsf Inst Stockholm 43: 1–74

Menkis A, Vasiliauskas R, Taylor AFS, Stenström E, Stenlid J, Finlay R (2006) Fungi in decayed roots of conifer seedlings in forest nurseries, afforested clear-cuts and abandoned farmland. Plant Pathol 55: 117–129

Oberwinkler F, Kirschner R, Arenal F, Villarreal M, Rubio V, Begerow D, Bauer R (2006) Two new pycnidial members of the Atractiellales: *Basidiopycnis hyaline* and *Proceropycnis pinicola*. Mycologia 98: 637–649

O'Donnell K (1993) *Fusarium* and its near relatives. In: Reynolds DR, Taylor JW (eds) The fungal holomorph: mitotic, meiotic and pleomorphic speciation in fungal systematics. CAB International, Wallingford, UK, pp 225–233

Paine TD, Raffa KF, Harrington TC (1997) Interactions among scolytid bark beetles, their associated fungi, and live host conifers. Annu Rev Entomol 42: 179–206

PASW Statistics 18 (2009) Software version 18.0.0, SPSS Inc., Chicago, Illinois

Persson Y, Vasaitis R, Långström B, Öhrn P, Ihrmark K, Stenlid J (2009) Fungi vectored by the bark beetle *Ips typographus* following hibernation under the bark of standing trees and in the litter. Microb Ecol 58: 651–659

Peverieri GS, Capretti P, Tiberi R (2006) Associations between *Tomicus destruens* and *Leptographium* spp. in *Pinus pinea* and *Pinus pinaster* stands in Tuscany, central Italy. Forest Pathol 36: 14–20

Ragazzi A, Moricca S, Capretti P, Dellavalle I, Turco E (2003) Differences in composition of endophytic mycobiota in twigs and leaves of healthy and declining *Quercus* species in Italy. Forest Pathol 33: 31–38

Rivera FN, Gómez Z, González E, López N, Hernández Rodríguez CH, Zúñiga G (2007) Yeasts associated with bark beetles of the genus *Dendroctonus* Erichson (*Coleoptera, Curculionidae, Scolytinae*): molecular identification and biochemical characterization. In: Bentz B, Cognato A, Raffa K (eds) Proceedings from the Third Workshop on genetics of bark beetles and associated microorganisms. Fort Collins, U.S., pp 45–48

Roe AD, James PMA, Rice AV, Cooke JEK, Sperling FAH (2011) Spatial community structure of mountain pine beetle fungal symbionts across a latitudinal gradient. Microb Ecol 62: 347–360.

Sallé A, Monclus R, Yart A, Garcia J, Romary P, Lieutier F (2005) Fungal flora associated with *Ips typographus*: frequency, virulence, and ability to stimulate the host defence reaction in relation to insect population levels. Can J Forest Res 35: 365–373.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Springs Harbor Laboratory Press, New York

Schweigkofler W, Otrosina WJ, Smith SL, Cluck DR, Maeda K, Peay KG, Garbelotto M (2005) Detection and quantification of *Leptographium wageneri*, the cause of black-stain root disease, from bark beetles (*Coleoptera: Scolytidae*) in Northern California using regular and real-time PCR. Can J Forest Res 35: 1798–1808

Siemaszko W (1939) Zespoly grzbow towarzyszacych kornikom polskim. Planta Pol 7: 1–54

Six DL, Bentz BJ (2003) Fungi associated with the North American spruce beetle, *Dendroctonus rufipennis.* Can J Forest Res 33: 1815–1820

Smit E, Leeflang P, Glandorf B, van Elsas JD, Wernars K (1999) Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes encoding 18S rRNA and temperature gradient gel electrophoresis. Appl Environ Microb 65: 2614–2621

Solheim H (1986) Species of *Ophiostomataceae* isolated from *Picea abies* infested by the bark beetle *Ips typographus*. Nord J Bot 6: 199–207

Solheim H (1992a) Fungal succession in sapwood of Norway spruce infested by the bark beetle *Ips typographus*. Eur J Forest Pathol 22: 136–148

Solheim H (1992b) The early stages of fungal invasion in Norway spruce infested by the bark beetle *Ips typographus*. Can J Botany 70: 1–5

Solheim H (1993a) Ecological aspects of fungi associated with the spruce bark beetle *Ips typographus* in Norway. In: Wingfield MJ, Seifert KA, Webber JF (eds) *Ceratocystis* and *Ophiostoma*. Taxonomy, ecology and pathogenicity. American Phytopathological Society, St. Paul, MN, pp 235–242

Solheim H (1993b) Fungi associated with the spruce bark beetle *Ips typographus* in an endemic area in Norway. Scand J Forest Res 8: 118–122.

Stergulc F, Frigimelica G, Faccoli M, Battisti A (2001) Pest and disease damage in natural and secondary conifer stands of the Friuli – Venezia Giulia (North-eastern Italy). In: Forster B, Knízek M, Grodzki W (eds) Methodology of forest insect and disease survey in central Europe.

Proceeding of the IUFRO working party Busteni, Romania: Forest Research and Management Institute (ICAS), pp 167–169

Vega EF, Dowd PF (2005) The role of yeast as insect endosymbionts. In: Vega FE, Blackwell M (eds) Insect-fungal associations: ecology and evolution. Oxford University Press, New York, pp. 211–243

Viiri H, Lieutier F (2004) Ophiostomatoid fungi associated with the spruce bark beetle, *Ips typographus*, in three areas in France. Ann Sci Forest 61: 215–219

Wang Y, Guo LD (2007) A comparative study of endophytic fungi in needles, bark, and xylem of *Pinus tabulaeformis*. Can J Botany 85: 911–917

Wermelinger B (2004) Ecology and management of the spruce bark beetle *Ips typographus* – a review of recent research. Forest Ecol Manag 202: 67–82

White TJ, Bruns TD, Lee S, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press, San Diego, CA, pp 315–322

Whitney HS (1982) Relationships between bark beetles and symbiotic organisms. In: Mitton JB, Sturgeon KB (eds) Bark beetles in North American Conifers: a system for study of evolutionary biology. Austin, University of Texas Press, pp 183–211

Wichmann L, Ravn HP (2001) The spread of *Ips typographus* (L.) (*Coleoptera*, *Scolytidae*) attacks following heavy windthrow in Denmark, analysed using GIS. Forest Ecol Manag 148: 31–39

Wingfield MJ, Seifert KA, Webber JF (1993) *Ceratocystis* and *Ophiostoma*. Taxonomy, ecology, and pathogenicity. American Phytopathological Society, St. Paul, MN

Yamaoka Y, Chung WH, Masuya H (2009) Constant association of ophiostomatoid fungi with the bark beetle *Ips subelongatus* invading Japanese larch logs. Mycoscience 50: 165–172

Table titles

Table 1 Main features of forest sites investigated

Site	Location	Coordinates (Lat-Long)	Elevation (m a.s.l.)	Area	Trapped insects (n.)
Antigorio Valley	Crodo (VB)	46°14’56.82”N 8°18’41.97”E	820-880	Outbreak	1057
Antigorio Valley	Baceno (VB)	46°16’19.38”N 8°17’27.70”E	1000-1130	Control	400
Soana Valley	Ronco Canavese (TO)	45°29’32.96”N 7°32’45.30”E	1000-1127	Outbreak	304
Soana Valley	Ronco Canavese (TO)	45°29’05.33”N 7°32’36.68”E	816-905	Control	78

Table 2 Abundance (%) of fungal OTUs obtained from *I. typographus* in mountain and hill Norway spruce stands

Fungal OTUs	GenBank accession n.[a]	Abundance (%)											
		Stand code[b]											
		1	2	3	4	5	6	7	8	9	10	11	12
ASCOMYCOTA and ANAMORPHIC FUNGI													
Alternaria infectoria E.G. Simmons	AY154691	-	-	-	-	19	-	-	-	-	-	-	-
Alternaria tenuissima (Kunze) Wiltshire	AY154712	-	-	-	14	-	-	22	-	-	-	25	-
Aspergillus sp.	U81264	-	-	-	-	-	26	-	-	-	-	-	-
Aureobasidium pullulans (de Bary) G. Arnaud	AM160630	-	-	-	-	19	9	-	-	-	-	-	-
Beauveria bassiana (Bals.-Criv.) Vuill.	AB027382	-	-	-	-	-	-	-	-	-	-	50	-
Cladosporium cladosporioides (Fresen.) G.A. de Vries	DQ008145	-	-	-	-	38	-	-	-	-	-	-	-
Cladosporium herbarum (Pers.) Link	DQ008149	13	-	-	-	-	-	-	-	-	-	-	-
Cordyceps sp.	AB044636	-	-	-	-	-	48	-	-	-	-	-	-
Ophiostoma polonicum Siemaszko	AF043601	-	-	-	-	-	-	-	-	4	25	-	-
Taphrina sp.	AY188378	-	-	-	-	8	-	-	-	-	-	-	-
Valsa ceratosperma (Tode) Maire	AF408387	-	-	-	-	-	-	-	9	7	-	-	-
ASCOMYCOTA (YEASTS)													
Candida lassenensis Kurtzman	AF017726	-	-	-	-	-	-	-	-	-	-	-	14
Candida ontarioensis Kurtzman & Robnett	DQ438183	-	-	-	-	-	-	-	-	7	25	-	-
Cyberlindnera amylophila (Kurtzman *et al.*) Minter	DQ409158	-	-	-	-	4	-	-	-	-	-	-	-
Lipomyces suomiensis (M.T. Smith *et al.*) Kurtzman, Albertyn & Basehoar-Powers	DQ519000	-	-	16	-	-	-	-	-	-	-	-	-
Myxozyma melibiosi (Shifrine & Phaff) V.D. Walt *et al.*	DQ518988	-	-	-	-	-	4	-	-	-	-	-	-
Saccharomycetaceae sp.	AY761152	-	-	-	-	-	-	-	18	57	25	-	-
Wickerhamomyces bisporus (O. Beck) Kurtzman, Robnett & Basehoar-Powers	U74589	-	-	16	-	-	-	-	-	10	-	-	58
Yamadazyma scolyti (Phaff & Yoney) Billon-Grand	AY761151	-	-	-	-	-	-	-	-	-	-	-	14
Yarrowia sp.	AJ616903	-	-	-	-	-	-	-	18	-	-	-	-
BASIDIOMYCOTA													
Cylindrobasidium laeve (Pers.) Chamuris	AY586651	-	-	-	-	-	-	-	-	-	-	-	14
Phallus impudicus L.	AY152404	-	-	-	-	-	-	-	28	-	-	-	-
BASIDIOMYCOTA (YEASTS)													

Cryptococcus laurentii (Kuff.) C.E. Skinner	AM160631	-	-	-	-	-	-	-	18	-	-	-	-
Cryptococcus oeirensis Fonseca, Scorzetti & Fell	AM160646	-	-	-	-	8	-	22	-	-	-	-	-
Cryptococcus victoriae Montes *et al.*	AM160647	40	-	-	-	-	-	-	-	-	-	-	-
Rhodotorula aurantica (Saito) Lodder	AY372178	-	-	-	-	-	-	11	-	4	-	-	-
Sporobolomyces coprosmae Hamam. & Nakase	AM160645	-	-	-	-	-	-	11	-	-	-	-	-
ZIGOMYCOTA													
Mucor hiemalis f. hiemalis Wehmer	AY706241	20	-	-	-	-	-	-	-	-	-	-	-
OTHERS													
Phlyctochytrium planicorne G.F. Atk.	AY439028	-	-	-	-	-	-	11	-	-	-	-	-

[a] Accession number refers to closest match in BLAST

[b] Stand code: *hill stands* from 1 to 6 – Alessandria; *mountain stands*: 7 – Vigezzo Valley; 8 – Antigorio Valley; 9 to 11 – Soana Valley; 12 – Susa Valley

Table 3 Abundance (%) of fungal OTUs from *I. typographus* in outbreak and control areas of two forest sites. GenBank accession number of the closest match in BLAST and voucher number (*Herbarium Patavinum* (PAD), University Museum Centre, University of Padua, Italy) of representative OTUs are reported.

Fungal OTUs	GenBank accession n. /*Voucher*	Antigorio Valley – Abundance (%)				Soana Valley – Abundance (%)			
		Outbreak		Control		Outbreak		Control	
		Clones	Isolates	Clones	Isolates	Clones	Isolates	Clones	Isolates
ASCOMYCOTA and ANAMORPHIC FUNGI									
1. *Acremonium kiliense* Grütz	-	0.0	0.0	0.0	1.0	0.0	4.0	0.0	0.0
2. *Alternaria alternata* (Fr.) Keissl.	*M000001*	0.0	11.0	0.0	12.0	0.0	0.0	0.0	0.0
3. *Ampelomyces humuli* (Fautrey) Rudakov	AF455518	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.0
4. *Aspergillus gracilis* Bainier	EF652045	6.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5. *Aspergillus niger* Tiegh.	*M000002*	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0
6. *Aspergillus* sp.	EF652045	6.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
7. *Beauveria bassiana* (Bals.-Criv.) Vuill.	EU334677 / *M000004*	69.0	2.0	0.0	4.0	36.0	0.0	66.0	8.0
8. *Botrytis cinerea* Pers.	*M000005*	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0
9. *Capronia* sp.	EU139150	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0
10. *Ceratocystis tetropii* (Math.-Käärik) J. Hunt	AY934524 / *M000006*	0.0	0.0	0.0	0.0	0.7	4.0	0.0	0.0
11. *Cladosporium cladosporioides* (Fresen.) G.A. de Vries	*M000007*	0.0	8.0	0.0	14.0	0.0	0.0	0.0	0.0
12. *Cladosporium herbarum* (Pers.) Link	*M000008*	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0
13. *Cladosporium tenuissimum* Cooke	EU272531	0.0	0.0	2.0	0.0	0.0	0.0	0.0	0.0
14. *Clonostachys rosea* (Link) Schroers, Samuels, Seifert & W. Gams	*M000010*	0.0	0.0	0.0	2.0	0.0	0.0	0.0	0.0
15. *Epicoccum nigrum* Link	EU529998 / *M000011*	0.0	5.0	0.0	6.0	0.0	4.0	0.6	0.0
16. *Fusarium* spp.	-	0.0	0.6	0.0	6.0	0.0	0.0	0.0	15.0
17. *Graphiopsis chlorocephala* Trail.	-	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0
18. *Grosmannia cucullata* (H. Sol.) Zipfel, Z.W. de Beer & M.J. Wingf.	*M000012*	0.0	0.6	0.0	1.0	0.0	0.0	0.0	0.0

19. *Grosmannia europhioides* (E.F. Wri. & Cain) Zip., Z.W. de Beer & M.J. Wingf.	*M000013*	0.0	0.0	0.0	5.0	0.0	0.0	0.0	0.0
20. *Hypocrea pachybasioides* Yoshim. Doi	*M000014*	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0
21. *Isaria coleopterorum* (Samson & H.C. Evans) Samson & Hywel-Jones	-	0.0	0.6	0.0	1.0	0.0	0.0	0.0	0.0
22. *Lachnellula* sp.	U59145.1	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0
23. *Mycosphaerella* sp.	EF619925	0.0	0.0	2.0	0.0	0.0	0.0	0.0	0.0
24. *Neonectria fuckeliana* (C. Booth) Castl. & Rossman	-	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0
25. *Ophiostoma bicolor* R.W. Davidson & D.E. Wells	*M000017*	0.0	15.0	0.0	9.0	0.0	0.0	0.0	0.0
26. *Ophiostoma breviusculum* Chung, Yamaoka, Uzunovic & Kim	*M000018*	0.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0
27. *Ophiostoma piceae* (Münch) Syd. & P. Syd.	*M000019*	0.0	0.0	0.0	4.0	0.0	0.0	0.0	0.0
28. *Ophiostoma polonicum* Siemaszko	DQ318202	0.0	0.0	2.0	0.0	0.0	0.0	0.0	0.0
29. *Ophiostoma* sp. 1	-	0.0	0.0	0.0	0.0	0.0	11.5	0.0	0.0
30. *Ophiostoma* sp. 2	-	0.0	2.0	0.0	6.0	0.0	4.0	0.0	0.0
31. *Ophiostoma* sp. 3	-	0.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0
32. *Penicillium* spp.	-	0.0	11.0	0.0	12.0	0.0	69.0	0.0	23.0
33. *Phoma herbarum* Westend.	AY337712	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0
34. *Phoma pomorum* Thüm.	*M000020*	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0
35. *Rhizosphaera kalkhoffii* Bubák	AY183366	0.0	0.0	2.0	0.0	0.0	0.0	0.0	0.0
36. *Sporothrix* sp.	*M000021*	0.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0
37. *Talaromyces emersonii* Stolk	-	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0
38. *Talaromyces trachyspermus* (Shear) Stolk & Samson	-	0.0	0.0	0.0	0.0	0.0	0.0	0.0	15.0
39. *Trichoderma* spp.	-	0.0	21.0	0.0	2.0	0.0	0.0	0.0	0.0
ASCOMYCOTA (YEAST)									
40. *Candida* sp.	EU484318	0.0	0.0	5.0	0.0	0.0	0.0	0.0	0.0
41. *Candida fructus* (Nakase) S.A. Mey. & Yarrow	EU484318	0.0	0.0	2.0	0.0	0.0	0.0	0.0	0.0
42. *Candida ontarioensis* Kurtzman & Robnett	EU343818	12.5	0.0	0.0	0.0	2.0	0.0	0.6	0.0
43. *Kuraishia capsulata* (Wick.) Y. Yamada, K. Maeda & Mikata	EF568066	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0
44. *Nakazawaea holstii* (Wick.) Y. Yamada, K. Maeda & Mikata	AB449811	0.0	0.0	2.0	0.0	0.7	0.0	0.0	0.0

45. *Ogataea zsoltii* (G. Péter, Tornai-Leh., Fülöp & Dlauchy) Nagats., S. Saito & Sugiy	AB440285	0.0	0.0	2.0	0.0	0.7	0.0	0.0	0.0
46. *Yamadazyma mexicanum* (M. Miranda, Holzschu, Phaff & Starmer) Billon-Grand	AB365477	0.0	0.0	0.0	0.0	0.7	0.0	2.0	0.0
47. *Yamadazyma scolyti* (Phaff & Yoney) Billon-Grand	EU343807	0.0	0.0	0.0	0.0	4.0	0.0	2.5	0.0
48. *Yamadazyma* sp.	EU343807	0.0	0.0	0.0	0.0	0.0	0.0	2.0	0.0
BASIDIOMYCOTA									
49. Basidiomycete with clamp connections	*M000003*	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0
50. *Coprinus* sp.	FJ791132 / *M000001*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	8.0
51. *Polyporus* sp.	AY523813	0.0	0.0	2.0	0.0	0.0	0.0	0.0	0.0
BASIDIOMYCOTA (YEAST)									
52. *Cryptococcus* sp.	FM246505	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0
53. *Rhodotorula fujisanensis* (Soneda) E.A. Johnson & Phaff	AF444574	6.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0
54. *Rhodotorula mucilaginosa* (A. Jörg.) F.C. Harrison	AF444635	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0
55. *Rhodotorula pinicola* F.Y. Bai, L.D. Guo & J.H. Zhao	AF444292	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0
ZYGOMYCOTA									
56. *Mucor hiemalis* Wehmer	*M000015*	0.0	1.0	0.0	3.0	0.0	0.0	0.0	31.0
57. *Mucor plumbeus* Bonord.	*M000016*	0.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0
OTHERS									
58. BAF 5 – unidentified yeast	AY761164	0.0	0.0	2.0	0.0	8.0	0.0	11.0	0.0
59. BAF 15 – unidentified yeast	AY761172	0.0	0.0	2.0	0.0	0.7	0.0	0.6	0.0
60. BAF 22 – unidentified yeast	AY761174	0.0	0.0	24.0	0.0	0.0	0.0	0.0	0.0
61. F3110A – uncultured fungus	AM999722	0.0	0.0	2.0	0.0	0.0	0.0	0.0	0.0
62. Dark sterile mycelia (10 morphotypes)	-	0.0	3.5	0.0	6.0	0.0	4.0	0.0	0.0
63. Hyaline sterile mycelia (10 morphotypes)	-	0.0	3.5	0.0	4.0	0.0	0.0	0.0	0.0

64. Pinkish sterile mycelium (1 morphotype)	-	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0
65. Unidentified dark mycelium	-	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0
Unidentified fungi	-	0.0	0.0	49.0	0.0	41.0	0.0	13.0	0.0

Figure legend

Fig. 1 Dendrogram of cluster analysis using UPGMA for Antigorio Valley. Numbers refer to taxa code, Table 3

Fig. 2 Dendrogram of cluster analysis using UPGMA for Soana Valley. Numbers refer to taxa code, Table 3