

# 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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**Abstract** The Eurasian spruce bark beetle *Ips typographus* and their fungal associates can cause severe damage 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 outbreak and in two control areas. Sequencing of clone libraries of Internal Transcribed Spacer (ITS) identified 31 fungal Operational Taxonomic Units (OTUs), while fungal isolations yielded 55 OTUs. Only three OTUs were detected by both molecular and cultural methods indicating that both methods are necessary to adequately describe fungal richness. Fungal assemblages on insects from these four and from an additional 12 study sites differed among stands in response to varying ecological conditions and to the limited spreading ability of *I. typographus*. Ophiostomatoid fungi showed higher diversity in outbreak areas; the pathogenic *Ophiostoma polonicum* was relatively uncommon, while *O. bicolor* was the most abundant species. This result was not unexpected, as insects were trapped not at the peak but at the end of the outbreaks and supports the hypothesis of a temporal succession among *Ophiostoma*

species. Ubiquitous endophytes of trees or common airborne fungi were present both in outbreak and in control areas. Wood decaying basidiomycetes were almost never detected on beetles. Yeasts were detected only by molecular analysis, and the OTUs detected matched those reported elsewhere in Europe and in the world, suggesting a very long association between some yeasts and bark beetles.

**Keywords** Culture isolation · DNA cloning · Environmental sequencing · Fungal diversity · Ophiostomatoid fungi · *Picea abies*

## 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 damage (Sallé et al. 2005). In recent decades, numerous reports of significant damage 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

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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* Siemaszko, *O. bicolor* R.W. Davidson & D.E. 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 only as occasional associates (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, and is believed to be 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 2008; 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). 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: i) to compare fungal assemblages associated with *I. typographus* from mountain and hill stands of Norway spruce, ii) to compare fungi from stands experiencing an outbreak with those present in control areas characterized by endemic beetle populations, and iii) to compare the diagnostic efficacy of molecular and

cultural techniques in the detection of fungi on *I. typographus*. Based on previous studies (Solheim 1992a; Wichmann and Ravn 2001; Kirisits 2004; Lim et al. 2005; Persson et al. 2009; Kirisits 2010; Kautz et al. 2011), we expected to see diversity among fungal communities depending on environmental differences, levels of beetle populations, and detection approach. This is the first extensive study on fungi associated with *I. typographus* in the Alps using molecular and cultural methods.

## Materials and methods

### 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 areas of the Vigizzo, Antigorio, Soana and Susa Valleys (800 – 1150 m a.s.l.). Norway spruce was the dominant species in all stands.

In late July, *I. typographus* were trapped by baiting the insects 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 beetles per trap were randomly selected and pooled together in 1.5 ml microcentrifuge tubes. Fungal DNA extraction 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<sub>2</sub>, 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 study sites as follows: PCR products 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 deposited in the 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.

#### 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 (Table 1). 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 damage. Outbreak and control areas were about 4 and 2 km from each other, in the Antigorio and Soana Valleys, respectively. Pheromone traps were used to collect adult *I. typographus* beetles as previously described. Beetles were collected weekly and single individuals were transferred using sterile tweezers from the traps into sterile 1.5 ml microcentrifuge tubes. The tubes containing beetles for isolations were stored at 5 °C and those assigned for direct DNA extractions at –40 °C.

#### DNA-based 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; DNA was 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 2008). 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 et al. 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; von Arx 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 *Herbarium Patavinum* (PAD), University Museum Centre (CAM), University of Padua, Italy.

#### Data interpretation and analyses

The abundance of each OTU identified by sequencing or culturing was expressed as percentage of sequences/cultures of that OTU on the total number of sequences for each library or of isolates for each area, respectively. The Sorensen similarity index ( $S_s$ ) was used to compare fungal communities from different locations. The index was calculated as follows:

**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

$$S_s = 2c/(a + b),$$

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).

In order to determine whether the sampling size was appropriate to describe the total fungal richness, a rarefaction analysis was performed on data from the two outbreak and two control areas by pooling OTUs defined by culturing and DNA analysis. Rarefaction analysis was performed according to the methods for individual-based data as described by Colwell et al. (2012).

An UPGMA (Unweighted Pair Group Method with Arithmetic Mean) cluster analysis was performed on OTUs defined by culturing and DNA analysis pooled together, to visualize patterns and determine number of discrete fungal assemblages in outbreak and control beetle populations of the Antigorio and Soana Valley.

Tests were performed with PASW Statistics 18 (2009) and rarefaction analysis was performed by using EstimateS 8.2.0 (2009).

## Results

### 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 oeilensis* 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.

### 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 each of 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, three Basidiomycota; 17 ascomycetous, basidiomycetous, and unidentified yeasts; and two 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). The trends of rarefaction curves suggest that the sampling effort was adequate and comparable for all four sites (Fig. 1).

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

Fungal OTUs	GenBank accession n. <sup>a</sup>	Abundance (%)											
		Stand code <sup>b</sup>											
		1	2	3	4	5	6	7	8	9	10	11	12
<u>ASCOMYCOTA and ANAMORPHIC FUNGI</u>													
<i>Alternaria infectoria</i> E.G. Simmons	AY154691	-	-	-	-	19	-	-	-	-	-	-	-
<i>Alternaria tenuissima</i> (Kunze) Wiltshire	AY154712	-	-	-	14	-	-	22	-	-	-	25	-
<i>Aspergillus</i> sp.	U81264	-	-	-	-	-	26	-	-	-	-	-	-
<i>Aureobasidium pullulans</i> (de Bary) G. Arnaud	AM160630	-	-	-	-	19	9	-	-	-	-	-	-
<i>Beauveria bassiana</i> (Bals.-Criv.) Vuill.	AB027382	-	-	-	-	-	-	-	-	-	-	50	-
<i>Cladosporium cladosporioides</i> (Fresen.) G.A. de Vries	DQ008145	-	-	-	-	38	-	-	-	-	-	-	-
<i>Cladosporium herbarum</i> (Pers.) Link	DQ008149	13	-	-	-	-	-	-	-	-	-	-	-
<i>Cordyceps</i> sp.	AB044636	-	-	-	-	-	48	-	-	-	-	-	-
<i>Ophiostoma polonicum</i> Siemaszko	AF043601	-	-	-	-	-	-	-	-	4	25	-	-
<i>Taphrina</i> sp.	AY188378	-	-	-	-	8	-	-	-	-	-	-	-
<i>Valsa ceratosperma</i> (Tode) Maire	AF408387	-	-	-	-	-	-	-	9	7	-	-	-
<u>ASCOMYCOTA (YEASTS)</u>													
<i>Candida lassenensis</i> Kurtzman	AF017726	-	-	-	-	-	-	-	-	-	-	-	14
<i>Candida ontarioensis</i> Kurtzman & Robnett	DQ438183	-	-	-	-	-	-	-	-	7	25	-	-
<i>Cyberlindnera amyophila</i> (Kurtzman <i>et al.</i> ) Minter	DQ409158	-	-	-	-	4	-	-	-	-	-	-	-
<i>Lipomyces suomiensis</i> (M.T. Smith <i>et al.</i> ) Kurtzman, Albertyn & Basehoar-Powers	DQ519000	-	-	16	-	-	-	-	-	-	-	-	-
<i>Myxozyma melibiosi</i> (Shifrine & Phaff) V.D. Walt <i>et al.</i>	DQ518988	-	-	-	-	-	4	-	-	-	-	-	-
Saccharomycetaceae sp.	AY761152	-	-	-	-	-	-	-	18	57	25	-	-
<i>Wickerhamomyces bisporus</i> (O. Beck) Kurtzman, Robnett & Basehoar-Powers	U74589	-	-	16	-	-	-	-	-	10	-	-	58
<i>Yamadazyma scolyti</i> (Phaff & Yoney) Billon-Grand	AY761151	-	-	-	-	-	-	-	-	-	-	-	14
<i>Yarrowia</i> sp.	AJ616903	-	-	-	-	-	-	-	18	-	-	-	-
<u>BASIDIOMYCOTA</u>													
<i>Cylindrobasidium laeve</i> (Pers.) Chamuris	AY586651	-	-	-	-	-	-	-	-	-	-	-	14
<i>Phallus impudicus</i> L.	AY152404	-	-	-	-	-	-	-	28	-	-	-	-
<u>BASIDIOMYCOTA (YEASTS)</u>													
<i>Cryptococcus laurentii</i> (Kuff.) C.E. Skinner	AM160631	-	-	-	-	-	-	-	18	-	-	-	-
<i>Cryptococcus oeilensis</i> Fonseca, Scorzetti & Fell	AM160646	-	-	-	-	8	-	22	-	-	-	-	-
<i>Cryptococcus victoriae</i> Montes <i>et al.</i>	AM160647	40	-	-	-	-	-	-	-	-	-	-	-
<i>Rhodotorula aurantica</i> (Saito) Lodder	AY372178	-	-	-	-	-	-	11	-	4	-	-	-
<i>Sporobolomyces coprosmae</i> Hamam. & Nakase	AM160645	-	-	-	-	-	-	11	-	-	-	-	-
<u>ZYGOMYCOTA</u>													
<i>Mucor hiemalis</i> f. <i>hiemalis</i> Wehmer	AY706241	20	-	-	-	-	-	-	-	-	-	-	-
<u>OTHERS</u>													
<i>Phlyctochytrium planicorne</i> G.F. Atk.	AY439028	-	-	-	-	-	-	11	-	-	-	-	-

<sup>a</sup> Accession number refers to closest match in BLAST

<sup>b</sup> 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

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 ( $S_{s, \text{outbreak}}=0.16$ ;  $S_{s, \text{control}}=0.26$ ).

In the Antigorio Valley, 41.8 % of OTUs were outbreak-specific while 40 % were only found in control areas; only

**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
<b>ASCOMYCOTA and ANAMORPHIC FUNGI</b>									
1. <i>Acremonium kiliense</i> Grütz	-	0.0	0.0	0.0	1.0	0.0	4.0	0.0	0.0
2. <i>Alternaria alternata</i> (Fr.) Keissl.	M000001	0.0	11.0	0.0	12.0	0.0	0.0	0.0	0.0
3. <i>Ampelomyces humuli</i> (Fautrey) Rudakov	AF455518	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.0
4. <i>Aspergillus gracilis</i> Bainier	EF652045	6.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5. <i>Aspergillus niger</i> Tiegh.	M000002	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0
6. <i>Aspergillus</i> sp.	EF652045	6.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
7. <i>Beauveria bassiana</i> (Bals.-Criv.) Vuill.	EU334677 / M000004	69.0	2.0	0.0	4.0	36.0	0.0	66.0	8.0
8. <i>Botrytis cinerea</i> Pers.	M000005	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0
9. <i>Capronia</i> sp.	EU139150	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0
10. <i>Ceratocystis tetropii</i> (Math.-Käärik) J. Hunt	AY934524 / M000006	0.0	0.0	0.0	0.0	0.7	4.0	0.0	0.0
11. <i>Cladosporium cladosporioides</i> (Fresen.) G.A. de Vries	M000007	0.0	8.0	0.0	14.0	0.0	0.0	0.0	0.0
12. <i>Cladosporium herbarum</i> (Pers.) Link	M000008	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0
13. <i>Cladosporium tenuissimum</i> Cooke	EU272531	0.0	0.0	2.0	0.0	0.0	0.0	0.0	0.0
14. <i>Clonostachys rosea</i> (Link) Schroers, Samuels, Seifert & W. Gams	M000010	0.0	0.0	0.0	2.0	0.0	0.0	0.0	0.0
15. <i>Epicoccum nigrum</i> Link	EU529998 / M000011	0.0	5.0	0.0	6.0	0.0	4.0	0.6	0.0
16. <i>Fusarium</i> spp.	-	0.0	0.6	0.0	6.0	0.0	0.0	0.0	15.0
17. <i>Graphiopsis chlorocephala</i> Trail.	-	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0
18. <i>Grosmannia cucullata</i> (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. <i>Grosmannia europhioides</i> (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. <i>Hypocrea pachybasioides</i> Yoshim. Doi	M000014	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0
21. <i>Isaria coleopterorum</i> (Samson & H.C. Evans) Samson & Hywel-Jones	-	0.0	0.6	0.0	1.0	0.0	0.0	0.0	0.0
22. <i>Lachnellula</i> sp.	U59145.1	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0
23. <i>Mycosphaerella</i> sp.	EF619925	0.0	0.0	2.0	0.0	0.0	0.0	0.0	0.0
24. <i>Neonectria fuckeliana</i> (C. Booth) Castl. & Rossman	-	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0
25. <i>Ophiostoma bicolor</i> R.W. Davidson & D.E. Wells	M000017	0.0	15.0	0.0	9.0	0.0	0.0	0.0	0.0
26. <i>Ophiostoma breviusculum</i> Chung, Yamaoka, Uzunovic & Kim	M000018	0.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0
27. <i>Ophiostoma piceae</i> (Münch) Syd. & P. Syd.	M000019	0.0	0.0	0.0	4.0	0.0	0.0	0.0	0.0
28. <i>Ophiostoma polonicum</i> Siemaszko	DQ318202	0.0	0.0	2.0	0.0	0.0	0.0	0.0	0.0
29. <i>Ophiostoma</i> sp. 1	-	0.0	0.0	0.0	0.0	0.0	11.5	0.0	0.0
30. <i>Ophiostoma</i> sp. 2	-	0.0	2.0	0.0	6.0	0.0	4.0	0.0	0.0
31. <i>Ophiostoma</i> sp. 3	-	0.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0
32. <i>Penicillium</i> spp.	-	0.0	11.0	0.0	12.0	0.0	69.0	0.0	23.0

**Table 3** (continued)

Fungal OTUs	GenBank accession n. /Voucher	Antigorio Valley – Abundance (%)				Soana Valley – Abundance (%)			
		Outbreak		Control		Outbreak		Control	
		Clones	Isolates	Clones	Isolates	Clones	Isolates	Clones	Isolates
33. <i>Phoma herbarum</i> Westend.	AY337712	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0
34. <i>Phoma pomorum</i> Thüm.	M000020	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0
35. <i>Rhizosphaera kalkhoffii</i> Bubák	AY183366	0.0	0.0	2.0	0.0	0.0	0.0	0.0	0.0
36. <i>Sporothrix</i> sp.	M000021	0.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0
37. <i>Talaromyces emersonii</i> Stolk	-	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0
38. <i>Talaromyces trachyspermus</i> (Shear) Stolk & Samson	-	0.0	0.0	0.0	0.0	0.0	0.0	0.0	15.0
39. <i>Trichoderma</i> spp.	-	0.0	21.0	0.0	2.0	0.0	0.0	0.0	0.0
<u>ASCOMYCOTA (YEAST)</u>									
40. <i>Candida</i> sp.	EU484318	0.0	0.0	5.0	0.0	0.0	0.0	0.0	0.0
41. <i>Candida fructus</i> (Nakase) S.A. Mey. & Yarrow	EU484318	0.0	0.0	2.0	0.0	0.0	0.0	0.0	0.0
42. <i>Candida ontarioensis</i> Kurtzman & Robnett	EU343818	12.5	0.0	0.0	0.0	2.0	0.0	0.6	0.0
43. <i>Kuraishia capsulata</i> (Wick.) Y. Yamada, K. Maeda & Mikata	EF568066	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0
44. <i>Nakazawaea holstii</i> (Wick.) Y. Yamada, K. Maeda & Mikata	AB449811	0.0	0.0	2.0	0.0	0.7	0.0	0.0	0.0
45. <i>Ogataea zsolatii</i> (G. Péter, Tornai-Leh., Fülöp & Dlačny) Nagats., S. Saito & Sugiy	AB440285	0.0	0.0	2.0	0.0	0.7	0.0	0.0	0.0
46. <i>Yamadazyma mexicanum</i> (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. <i>Yamadazyma scolyti</i> (Phaff & Yoney) Billon-Grand	EU343807	0.0	0.0	0.0	0.0	4.0	0.0	2.5	0.0
48. <i>Yamadazyma</i> sp.	EU343807	0.0	0.0	0.0	0.0	0.0	0.0	2.0	0.0
<u>BASIDIOMYCOTA</u>									
49. Basidiomycete with clamp connections	M000003	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0
50. <i>Coprinus</i> sp.	M000001	0.0	0.0	0.0	0.0	0.0	0.0	0.0	8.0
51. <i>Polyporus</i> sp.	AY523813	0.0	0.0	2.0	0.0	0.0	0.0	0.0	0.0
<u>BASIDIOMYCOTA (YEAST)</u>									
52. <i>Cryptococcus</i> sp.	FM246505	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0
53. <i>Rhodotorula fujisanensis</i> (Soneda) E.A. Johnson & Phaff	AF444574	6.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0
54. <i>Rhodotorula mucilaginosa</i> (A. Jörg.) F.C. Harrison	AF444635	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0
55. <i>Rhodotorula pinicola</i> 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
<u>ZYGOMYCOTA</u>									
56. <i>Mucor hiemalis</i> Wehmer	M000015	0.0	1.0	0.0	3.0	0.0	0.0	0.0	31.0
57. <i>Mucor plumbeus</i> Bonord.	M000016	0.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0
<u>OTHERS</u>									
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

**Table 3** (continued)

Fungal OTUs	GenBank accession n. /Voucher	Antigorio Valley – Abundance (%)				Soana Valley – Abundance (%)			
		Outbreak		Control		Outbreak		Control	
		Clones	Isolates	Clones	Isolates	Clones	Isolates	Clones	Isolates
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

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 ( $S_s=0.43$ ).

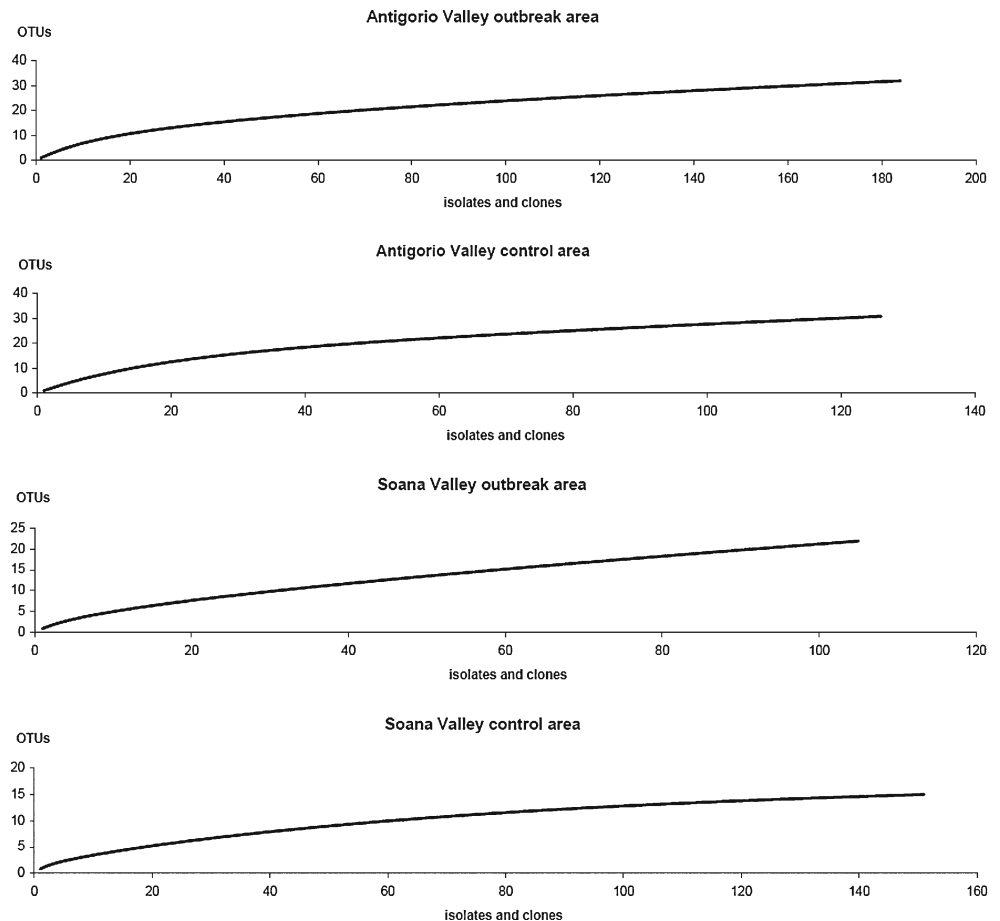
*Beauveria bassiana*, an entomopathogenic fungus, was the most abundant OTU and the only one with *Epicoccum nigrum* and *Penicillium* spp. to be detected in all four areas.

The assemblage of ophiostomatoid fungi consisted of *Ceratocystis tetropii*, *Grosmannia cucullata*, *G. europhioides*, *Ophiostoma bicolor*, *O. brevisculum*, *O. piceae*,

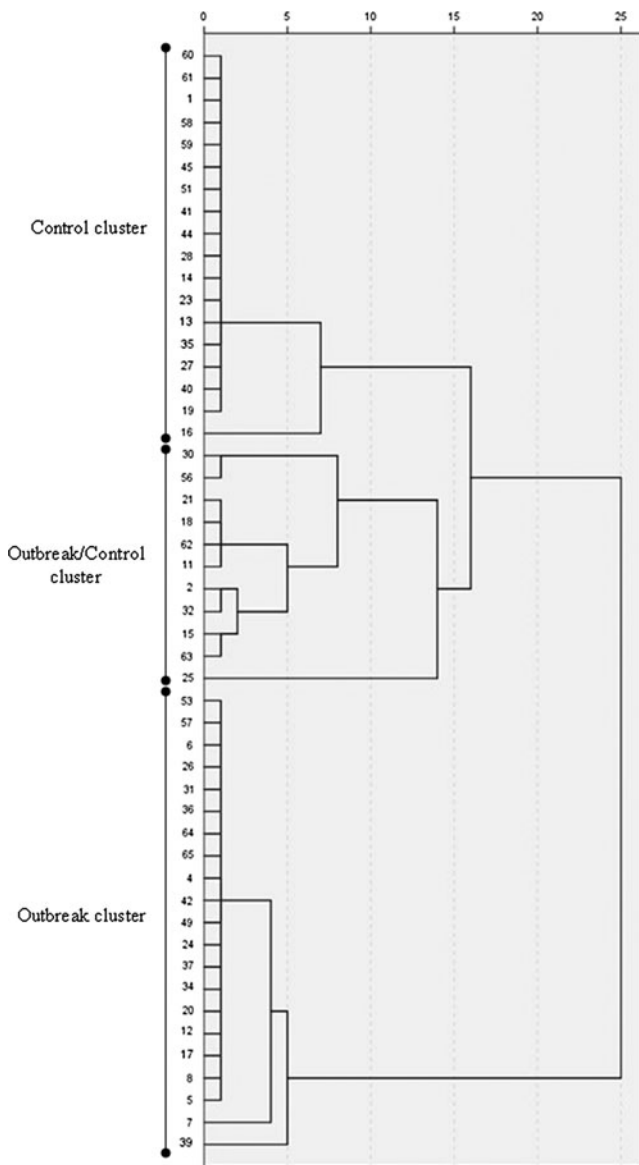
*O. polonicum*, three other *Ophiostoma* OTUs and one *Sporothrix* OTU. *Grosmannia europhioides*, *O. polonicum* and *O. piceae*, were detected exclusively in control areas, all other species were present in outbreak areas. Finally, *G. cucullata*, *O. bicolor* and *Ophiostoma* sp. 2 were detected in 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 (Figs. 2 and 3). Two of these clusters are exclusively associated with outbreak or control beetle populations respectively, while

**Fig. 1** Individual-based rarefaction curves of the two outbreak and two control areas sampled in Antigorio and Soana Valleys







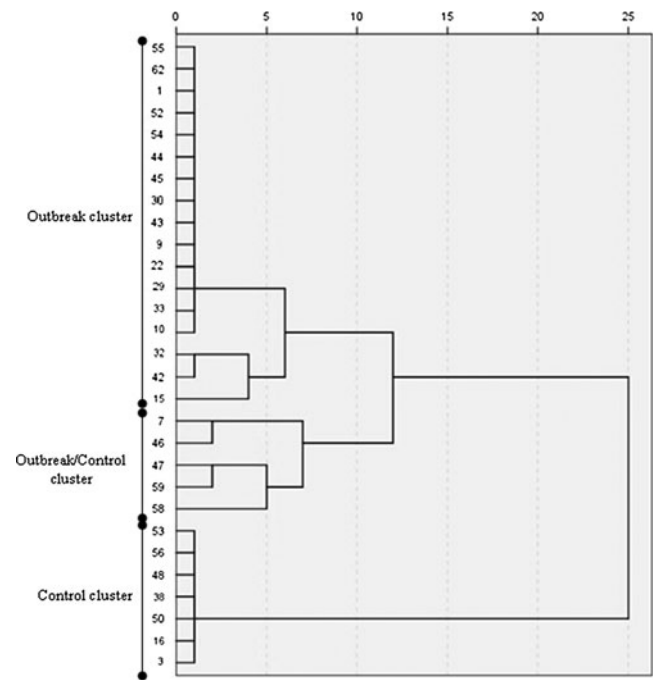
**Fig. 2** Dendrogram of cluster analysis using UPGMA for Antigorio Valley. Numbers refer to taxa code, Table 3

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).



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

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 that occurred in the previous year (Wichmann and Ravn 2001; Kautz et al. 2011). The fact that buffer zones between 100 m and 1500 m have prevented significant attacks in managed forests (Wermelinger 2004) also points to a limited dispersal range of this beetle species.

Data from our second survey clearly show that the cultural method identifies a greater number of fungal OTUs compared to the molecular method. This finding would support the belief that investigations on fungal diversity in environmental samples are probably incomplete when identifications rely exclusively on DNA analyses. Nevertheless, it should be noted that many fungal clones in our studies remained unidentified due to the small length of the sequences or to the low similarity with ITS sequences in the GenBank database, suggesting that our inability to identify

these OTUs may be related to technical issues (e.g. quality of DNA, unavailability of sequences in GenBank database), rather than to an intrinsic 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 complementary information on fungal diversity.

Several fungi detected by the molecular method were never isolated in pure culture, indicating that some might be unculturable directly from environmental samples. In general, ophiostomatoid fungi and fast-growing fungi were more frequently detected by pure culture isolations. By contrast, and in agreement with previous reports (Lim et al. 2005; Persson et al. 2009), ascomycetous and basidiomycetous yeasts could be detected exclusively by using the molecular method. Since every method or medium of isolation may be selective for some species but not appropriate for others, we cannot exclude that 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 by the direct molecular analysis (T-RFLP) on *I. typographus* beetles hibernated under the bark of standing trees and in forest litter (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 how different factors may affect this variation (Linnakoski et al. 2012).

*Penicillium* spp. were among the most abundant OTUs ranging between 11 % and 12 % in the Antigorio Valley and between 23 % and 69 % in the Soana Valley. This finding is in accordance with reports of Jankowiak (2006) and Jankowiak and Rossa (2008) on fungi associated with two bark beetles *Tomicus piniperda* (L.) and *Pityogenes bidentatus* (Herbst) from 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 were 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 traditional isolation techniques are likely to underestimate the actual occurrence of these fungi (Linnakoski et al. 2012). There is emerging evidence that Basidiomycota are more common associates of bark beetles than previously thought, and a clear association between a few taxa and *I. typographus* has been reported (Kirschner et al. 2001a, b; Kolařík et al. 2006; Oberwinkler et al. 2006; Persson et al. 2009).

More than 25 ophiostomatoid fungi have been previously 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. brevisculum* 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 sporadic 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 beetle 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 mycobionts associated with *I. typographus*. As a result of the combined approach used in this study, our data may be among the first to provide a list of fungi inclusive of taxa from all of the most important functional groups associated with this bark beetle. Different fungal assemblages between hill and mountain stands were likely the consequence of different ecological features. Differences between outbreak and control areas, instead, were likely the result of different stages in the dynamics of beetle populations. Finally, based on results of rarefaction analysis, we surmise that 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 rather than by an inadequate sampling resulting in the underestimation of the total fungal richness. 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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