AEM
Journals.ASM.org

Fungal Endophytic Communities in Grapevines (*Vitis vinifera* L.) Respond to Crop Management

Michael Pancher, Marco Ceol, Paola Elisa Corneo, Claudia Maria Oliveira Longa, Sohail Yousaf, Ilaria Pertot, and Andrea Campisano

Sustainable Agro-Ecosystems and Bioresources Department, IASMA Research and Innovation Centre, Fondazione Edmund Mach, San Michele all'Adige, Italy

We studied the distribution of fungal endophytes of grapevine (*Vitis vinifera* L.) plants in a subalpine area of northern Italy, where viticulture is of high economic relevance. We adopted both cultivation-based and cultivation-independent approaches to address how various anthropic and nonanthropic factors shape microbial communities. Grapevine stems were harvested from several locations considering organic and integrated pest management (IPM) and from the cultivars Merlot and Chardonnay. Cultivable fungi were isolated and identified by internal-transcribed-spacer sequence analysis, using a novel colony-PCR method, to amplify DNA from fungal specimens. The composition of fungal communities was assessed using a cultivation-independent approach, automated ribosomal intergenic spacer analysis (ARISA). Multivariate statistical analysis of both culture-dependent and culture-independent data sets was convergent and indicated that fungal endophytic communities in grapevines from organically managed farms were different from those from farms utilizing IPM. Fungal communities in plants of cv. Merlot and cv. Chardonnay overlapped when analyzed using culture-dependent approaches but could be partially resolved using ARISA fingerprinting.

Microorganisms dwelling asymptomatically within plant tissues (endophytes) have been found in all studied plants. Endophytes can have either a mutualistic or a parasitic lifestyle; under some conditions, mutualists may switch to pathogens upon perception of plant-borne or environmental conditions (50). Known endophytes include viruses, phytoplasmas, bacteria, and fungi.

The study of plant-fungus interaction has long focused on pathogenic interaction. DNA-based approaches have been extensively used for fingerprinting, tracking, and identifying plant-pathogenic fungi (46, 61, 63), but fewer studies address nonpathogenic fungal communities. Fungal endophytes have been investigated for their role as plant growth promoters, biocontrol agents (43), enhancers of the plant's bioremediation potential (55), and producers of novel secondary metabolites (75) or enzymes (51). However, the relation between communities of endophytic fungi and host plants is still poorly studied, and as of yet, is far from being fully understood. In grapevines, recent studies have shed some light upon the bacterial endophytic communities (13, 14, 20, 47, 72), while investigations on fungal communities have been rare and often limited to culture-dependent methods (20, 30, 43, 71). Furthermore, research has mainly focused on subsoil plant-microbe associations (36, 65).

Several factors may affect plant-associated microbial communities, e.g., anthropic factors (52, 53), plant physiology (34), the environment (57, 74), and pathogen infections (4, 14, 15). A shift in the composition of microbial communities associated with plants can be driven by genetic and physiological diversities, e.g., between different cultivated varieties (1, 42, 47). In contrast to grasses and annual plants, fewer attempts have been made in woody plants to correlate fungal endophytic communities with cultivar (17, 30). To our knowledge, only a few studies attempt to link organic management or the use of antifungal treatments with modifications in the microbiota in woody plants (31, 54, 60).

When attempting to identify a high number of isolated fungi, as in environmental studies, isolation and purification of DNA is both time-consuming and expensive but required for the PCR amplification of taxonomically relevant DNA regions. Direct colony-PCR of fungal isolates is usually avoided since DNA availability and purity in heat-lysed fungi is frequently insufficient for the reaction (7). Furthermore, growth media often contain contaminants inhibiting PCR. Fungal metabolites inhibiting DNA polymerase and resilience of fungal spores or conidia to lysis are among the causes of unreliable or poor amplification when PCR is performed directly on fungal colonies (28, 35). Previous studies attempting to improve speed and quality of PCR amplification directly from fungi have several methodological limitations. Examples include dependence on DNA extraction (28, 41, 49, 56, 68), validation over a limited range of taxa (2, 39, 70), and the use of expensive, proprietary chemicals (2, 18).

Changes in the composition of plant-associated microbial communities have often been associated with plant physiology (9), health (14), and environmental perturbances (11). In the present study, we examined fungal endophytic communities in grapevines using both cultivation-based and cultivation-independent approaches. For the first time, we applied automated ribosomal intergenic spacer analysis (ARISA) to the study of fungal endophytic communities in grapevines comparing organic and integrated pest management and investigating the cultivar effect. Fungal ARISA is a community fingerprinting method based on the analysis of length polymorphism of the nuclear ribosomal DNA (rDNA) region containing the two internal transcribed spacers (ITS) and the 5.8S rRNA gene. It was chosen over terminal restriction fragment length polymorphism for its higher accuracy in describing the microbial community's diversity (22), as well as for its ease of use and the precision that capillary electrophoresis offers (21). Furthermore, we report a novel colony-PCR method

Received 29 December 2011 **Accepted** 28 March 2012
Published ahead of print 6 April 2012
Address correspondence to Andrea Campisano, andrea.campisano@iasma.it.

doi:10.1128/AEM.07655-11

TABLE 1 Sample names and their characteristics

Area (letter code)	Vineyard no.	Location	Cultivar	Type of pest management	Sample
Avio-Ala (A)	2	45°43′35.14″N, 10°56′55.99″E	Chardonnay	Organic	CO2A
	2	45°43′35.14″N, 10°56′55.99″E	Merlot	Organic	MO2A
	13	45°43′28.97″N, 10°56′44.06″E	Chardonnay	Integrated	CI13A
	13	45°43′28.97″N, 10°56′44.06″E	Merlot	Integrated	MI13A
Pergolese (B)	4	46°1′36.40″N, 10°57′38.33″E	Chardonnay	Organic	CO4B
	4	46°1′36.40″N, 10°57′38.33″E	Merlot	Organic	MO4B
	19	46°1′41.91″N, 10°57′26.97″E	Chardonnay	Integrated	CI19B
	19	46°1′41.91″N, 10°57′26.97″E	Merlot	Integrated	MI19B
Noarna (C)	8	45°54′48.86″N, 11°0′58.21″E	Chardonnay	Organic	CO8C
	8	45°54′48.86″N, 11°0′58.21″E	Merlot	Organic	MO8C
	17	45°54′13.14″N, 11°1′22.37″E	Chardonnay	Integrated	CI17C
	16	45°54′17.57″N, 11°0′52.56″E	Merlot	Integrated	MI16C
Isera (D)	9	45°53′16.49″N, 11°0′6.39″E	Chardonnay	Organic	CO9D
	9	45°53′16.49″N, 11°0′6.39″E	Merlot	Organic	MO9D
	14	45°53′3.96″N, 11°0′4.76″E	Chardonnay	Integrated	CI14D
	15	45°53′9.59″N, 11°0′13.42″E	Merlot	Integrated	MI15D
Pietramurata (E)	10	46°0′55.24″N, 10°57′10.51″E	Chardonnay	Organic	CO10E
	10	46°0′55.24″N, 10°57′10.51″E	Merlot	Organic	MO10E
	20	46°0′46.44″N, 10°57′13.61″E	Chardonnay	Integrated	CI20E
	20	46°0′46.44″N, 10°57′13.61″E	Merlot	Integrated	MI20E
Pressano-Sorni (F)	11	46°9′47.87″N, 11°6′49.36″E	Chardonnay	Organic	CO11F
	11	46°9′47.87″N, 11°6′49.36″E	Merlot	Organic	MO11F
	21	46°9′21.21″N, 11°6′43.20″E	Chardonnay	Integrated	CI21F
	22	46°10′47.74″N, 11°7′31.64″E	Merlot	Integrated	MI22F
Navicello (G)	12	45°52′46.30″N, 11°1′15.98″E	Chardonnay	Organic	CO12G
	12	45°52′46.30″N, 11°1′15.98″E	Merlot	Organic	MO12G
	18	45°52′33.36″N, 11°1′2.87″E	Chardonnay	Integrated	CI18G
	18	45°52′33.36″N, 11°1′2.87″E	Merlot	Integrated	MI18G

for the rapid identification of fungal isolates, which is both inexpensive and DNA isolation independent. This method was used to PCR amplify the ITS regions and identify all isolates obtained in the present study.

MATERIALS AND METHODS

Study sites. To minimize environmental variability between samples from different areas, we followed strict criteria for selection. Communities were sampled only from lateral vine stocks of field grapevines in a restricted geographic region (Trentino, Northern Italy), with medium sandy, calcareous soils (48) characterized by humid, temperate, oceanic climate particularly in prealpine areas, with rainfalls maxima in the spring and autumn (16). Seven locations and a total of 28 vineyards were selected. In each location four vineyards were sampled, representing each of the four treatments: organic and integrated pest management (IPM) and cultivars Chardonnay and Merlot. Coordinates for sampling sites are listed in Table 1.

Sample collection and plant material. A total of 28 vineyards were sampled (Table 1). Samples were taken during the fall of 2010, from 27 October to 11 November. In each vineyard, four plants for each treatment were randomly selected. One lateral vine shoot was cut from each plant using pruning scissors. After the leaves were removed, the stems were transferred in a refrigerated basket for transportation for up to 6 h and stored at 4°C for up to 1 day.

Isolation of endophytic fungi. Lateral stems (1 to 2 m long) were cut to 20-cm-long fragments in the lab. These cuttings were surface disinfected by a succession of 2-min immersions, conducted under sterile laminar airflow, in 90% ethanol, 2.5% sodium hypochlorite solution, 70% ethanol, and sterile-distilled water. To test the efficacy of this method, random surface-disinfected stems were repeatedly rolled on nutrient broth (Oxoid, United Kingdom) or malt extract agar Vegitone (MEA-V; Fluka/Sigma-Aldrich, Switzerland) petri dishes, followed by incubation for 2 weeks at 20 to 25°C to confirm the absence of any microbial growth. After disinfection, the stems were cut into 0.5-cm sections and placed on MEA-V with the vascular vessels facing the medium. The plates were incubated for 7 to 15 days, and all morphologically different colonies were isolated. Mycelium from isolated colonies was freeze-dried and stored at room temperature.

Extraction-independent PCR amplification of DNA from cultivable isolates. Only a minor fraction of all of the morphologically different fungal isolates was identified, based on microscopic analysis of the hyphal and conidial morphology using available morphological keys (8). Therefore, we developed a new method for identification. The method was validated on a wide range of fungal taxa chosen from the culture collection at our institute (Fondazione Edmund Mach, San Michele all'Adige, Italy), from other collections, or from the isolates of the present study that were already identified based on morphological traits (Table 2). ITS sequences of all isolates were PCR amplified using either or both primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) (73) and either or both primers nu-SSU-0817-59 (TTAGCATGGAATAATRRAATAGGA) and nu-SSU-1196-39 (TCTGGACCTGGTGAGTTTCC) (10) primer pairs (Sigma-Aldrich) and the two protocols described below. Henceforth, we refer to these two protocols as freeze-dried mycelium (FDM) and actively growing mycelium (AGM). In FDM, freeze-dried fungal material was lysed mechanically using two sterile stainless steel 5/32-in. ball bearings and shaken using a tissue lyser (type MM200; Retsch, Germany) for 2 min at maximum frequency (25 Hz) to obtain a fine powder. We stored the pulverized mycelium without loss of PCR efficacy for at least 2 months (data not shown). For PCR, ~1 mg of this powder was suspended in 1 ml of sterile distilled water and mixed by vortexing for 20 s. In AGM, a 0.5-cm^2 plug, including fresh mycelium and the agar medium underneath, was frozen at −80°C. For method validation purposes, samples were harvested from either small fungal colonies

TABLE 2 Strains used for validation of the colony-PCR method and PCR results

Fungal isolate	Source	PCR amplification[a]					
		FDM		AGM			
				ITS1/ITS4		nu-SSU-0817-59/ nu-SSU-1196-39	
		ITS1/ITS4	nu-SSU-0817-59/ nu-SSU-1196-39	Early	Late	Early	Late
Absidia glauca 1B3C	Our collection	✓	✓	X	✓	X	✓
Alternaria sp. 2.1.Ca	Our collection	✓	✓	✓	✓	✓	✓
Alternaria sp. AL2	Our collection	X	X	X	✓	✓	✓
Aspergillus niger	Our collection	✓	X	X	X	✓	✓
Aspergillus niger	CBS 513.88	✓	✓	✓	✓	✓	✓
Botrytis cinerea	Our collection	✓	✓	✓	✓	✓	✓
Botrytis cinerea 9.4.Md	Our collection	✓	✓	X	✓	✓	✓
Cladosporium oxysporum	CBS 125.88	✓	✓	✓	✓	✓	✓
Cladosporium sp. 10.4.Mb	Our collection	✓	✓	✓	✓	✓	✓
Cladosporium sp. 4.2.Mb	Our collection	✓	✓	✓	✓	✓	✓
Epicoccum nigrum 2.1.Cb	Our collection	X	✓	✓	✓	✓	X
Fusarium graminearum PH1	ATCC MYA4620	✓	✓	✓	✓	✓	✓
Fusarium oxysporum	NRRL34936	✓	✓	X	✓	X	✓
Fusarium sp. 53F	Our collection	✓	✓	X	✓	✓	✓
Mortierella vertici lata F2(VR)	Our collection	✓	✓	✓	✓	✓	✓
Mucor hiemalis 1B2C	Our collection	✓	✓	✓	✓	✓	✓
Neurospora crassa OR74A	FGSC9013	✓	✓	X	✓	✓	✓
Penicillium chrysogenum	Our collection	X	X	X	✓	✓	✓
Penicillium chrysogenum 54-1255	NRRL1951	✓	✓	X	✓	✓	✓
Penicillium restrictum VR31	Our collection	✓	✓	X	✓	✓	✓
Penicillium spinulosum VR14	Our collection	✓	✓	X	X	✓	X
Phaeosphaeria nodorum SN15	FGSC10173	✓	✓	✓	X	✓	X
Pithomyces chartarum 9.2.Mb	Our collection	✓	✓	✓	✓	✓	✓
Podospora anserina	FGSC10383	✓	✓	X	✓	✓	✓
Rhizopus stolonifer 2948	Our collection	✓	✓	✓	✓	✓	✓
Sclerotinia sclerotiorum 1980	ATCC 18683	✓	✓	✓	✓	✓	✓
Trichoderma aggressivum	CBS 115901	X	X	✓	✓	✓	✓
Trichoderma atroviride MT8	Our collection	X	X	✓	X	✓	✓
Trichoderma reesei QMA	DSM 768	✓	✓	X	✓	✓	✓
Trichoderma virens	PGSC 10516	✓	✓	✓	X	✓	✓
Umbelopsis ramanniana F13	Our collection	✓	✓	✓	✓	✓	✓
Zygorhyncus moelleri F11(VR)	Our collection	✓	✓	✓	✓	✓	✓
Aureobasidium pullulans 4.3.Cc	Our collection	✓	✓	✓	✓	✓	✓
Debaryomyces hansenii	CBS 767	✓	✓	✓	✓	✓	✓
Hansenula polymorpha	CBS 4732	✓	✓	✓	✓	✓	✓
Pichia stipitis	CBS 6054	✓	✓	✓	✓	✓	✓
Saccharomyces cerevisiae S288C	ATCC 204508	✓	✓	✓	✓	✓	✓
Schizosaccaromyces pombe 972h	ATCC 24843	✓	✓	✓	✓	✓	✓
Yarrowia lipolytica	CBS 7504	✓	✓	✓	✓	✓	✓
Zygosaccaromyces rouxii	CBS 732	✓	✓	✓	✓	✓	✓

[a] PCR was performed using the primer pairs ITS1/ITS4 and nu-SSU-0817-59/nu-SSU-1196-3. Both FDM and AGM results are shown. AGM results are reported both for early and late stages. The symbols "✓" and "X" indicate successful and unsuccessful PCR amplifications, respectively. Yeast strains were tested in one stage only.

(0.5 to 1 cm in diameter) or the actively growing edge of larger colonies (3 to 6 cm in diameter). Henceforth, these two stages will be referred to as early and late, respectively. Lysis of the frozen samples was achieved using the same procedure described for FDM, after which 1 ml of sterile distilled water was added to the lysate and mixed by vortexing. The diluted AGM and FDM lysates were centrifuged 5 min at full speed (relative centrifugal force [RCF] of 16,000) on a tabletop centrifuge (5415R; Eppendorf, Germany) to sediment insoluble debris, including nonlysed cells, agar, and cell wall fragments. One microliter of the supernatant was used as a template in a 25-μl PCR, including 1×Dream *Taq* green PCR master mix (Fermentas, Lithuania) and 0.2 μM concentrations of each primer. PCR was performed for 35 cycles using the appropriate protocols to each primer pair (10, 73). PCR-amplified DNA was purified using Exo-SAP (Euroclone S.p.A., Italy) according to the manufacturer's instructions and sequenced using BigDye Terminator v3.1. Sequence analysis of the amplicons was performed by BLASTN comparison using the National Center for Biotechnology Information (NCBI) database's best hit (58) to confirm the identities of the selected strains. Whenever possible, sequences were identified to the species level. All fungal sequences were at least 98% identical to the best hit in the NCBI database. This value was considered sufficiently robust for species identification (66). For some isolates, when ITS sequence was not discriminant at the species level, the isolates were assigned to the corresponding genus. Once validated, the method was used to identify the unidentified isolates of the present study. The presence or absence of operational taxonomic unit(s) (OTU) was scored for each plant sample.

DNA extraction, PCR, and ARISA of total fungi. For cultivation-independent analysis of fungal communities, total DNA extraction was performed. Plant stems were surface disinfected as described above. Bark was carefully removed to avoid contamination with DNA from nonviable cells, which may persist on the surface after disinfection. Disc sections of lateral shoots used for microorganism isolation were frozen in liquid nitrogen and pulverized in sterile steel jars using a tissue lyser. DNA was isolated from 200 mg of ground specimens using the CTAB (cetyltrimethylammonium bromide) method as previously reported (24). Briefly, pulverized material was incubated 30 min at 65°C in prewarmed lysis buffer and extracted using chloroform-isoamyl alcohol (24:1). The genomic DNA was precipitated using isopropanol, and the pellet was washed with 70% ethanol.

The 18S-28S ITS of the fungal rDNA was amplified using the primer set carboxyfluorescein (FAM)-labeled 1406f (TGYACACACCGCCCGT) (27) and ITS2 (GCTGCGTTCTTCATCGATGC) (73). The 25-μl PCR mix contained 1×Dream *Taq* green PCR master mix, 1 μl of dimethyl sulfoxide, 25 μg of bovine serum albumin, and 0.2 μM concentrations of each primer. PCR was performed using an initial denaturation step at 95°C for 5 min, with 31 cycles as follows: denaturation at 95°C for 40 s, annealing at 54°C for 40 s, and elongation at 72°C for 1 min, followed by a final elongation at 72°C for 10 min. PCRs were performed in a PTC-200 thermal cycler (MJ Research, Inc., USA). The PCR product was checked on 1% agarose gel, and 1 μl of the product was mixed with 8.8 μl of Hi-Di formamide (Applied Biosystems, CA) and 0.2 μl of GeneScan 1200 LIZ size standard (Applied Biosystems), denatured for 5 min at 95°C, and then cooled in ice before loading.

Denatured amplicons were loaded on an ABI Prism 3130xl genetic analyzer (Applied Biosystems) equipped with 16 50-cm capillaries filled with POP 7 polymer (Applied Biosystems). Run conditions were set to 8.5 kV and 60°C, and the total run time was 6,700 s.

Electropherograms were analyzed using the Gene Mapper 4.0 software, using the normalization inside the experiment, and the fluorescence threshold was set at 50 relative fluorescence units (RFU). We found and scored for analysis fragments in the size range (length) of 100 to 800 bp. Peak binning was set to 1.5 bp, and manual correction was applied where peak shifts occurred. The tables for presence/absence and fluorescence associated with each peak were exported into spreadsheets for subsequent analysis.

Multivariate data analysis. ARISA electropherograms of individual plant-associated communities were transformed in a binary presence matrix (scoring 1 for presence or 0 for absence), and each peak was scored. Matrices generated both through identification of cultivable isolates and through ARISA peak scoring were then transformed by adding presence scores together for each of the four biological replicates. The frequency score obtained thus ranged from 0 to 4. The data matrices were analyzed by principal component analysis (PCA) and canonical correspondence analysis (CCA) (40) using the PAST software (32). PCA and CCA are similar procedures for finding variables (called components), which are linear combinations of all of the variation contained by the data set. The reduction of several variables to two provides the advantage of making a complex data set plottable, while preserving much of the variance in the data. In addition, it allows the generation of hypotheses regarding components and the controlled variables in the data set. As a correspondence analysis method, CCA is designed for counted data (integers). In CCA, the axes are linear combinations of the environmental variables. CCA is specifically suited to data where the gradient in environmental variables is known *a priori* and abundance (or presence/absences) is considered to be a response to this gradient (32).

A data set was obtained using linear combinations of the cultivated fungi and ARISA fingerprints matrices (henceforth referred to as the combined data set). Samples grouping according to treatments (organic/integrated management, cv. Merlot/Chardonnay) and across the seven areas of sampling was also studied by one-way ANOSIM (ANalysis Of SIMilarities) (19) and one-way NPMANOVA (Non-Parametric MANOVA) (3). One-way ANOSIM and NPMANOVA are nonparametric tests that analyze the significance of distance measures among multivariate groups (32).

RESULTS

Identification of fungi by colony-PCR. In the method validation, PCR amplification using FDM as a template was successful for 36 of 40 samples (90%), using either the ITS1/ITS4 primer pair or the nu-SSU-0817-59/nu-SSU-1196-39 primer pair. The four fungi recalcitrant to PCR amplification using both primer pairs were *Trichoderma atroviride*, *T. aggressivum*, *Penicillium chrysogenum*, and *Alternaria* sp. (Table 2). All yeasts lysates were successfully PCR amplified. Using AGM 38 of 40 samples were also successfully PCR amplified (exceptions were *P. spinulosum* and *Aspergillus niger*) using the ITS1/ITS4 primer pair, whereas all fungal isolates were successfully PCR amplified with the nu-SSU-0817-59/nu-SSU-1196-39 primer pair. However, PCR amplification of some isolates was partial or absent when these were harvested during either the early or late growth stage (Table 2). Strains more efficiently amplified when sampled during early growth include *P. spinulosum*, *T. atroviride*, *Epicoccum nigrum*, *T. virens*, and *Phaeosphaeria nodorum*. A larger number of strains were more efficiently amplified during late growth, including *Absidia glauca*, *P. restrictum*, *Alternaria* sp., *Botrytis cinerea*, *Fusarium* sp., *T. reesei*, *Fusarium oxysporum*, *Neurospora crassa*, *P. chrysogenum*, and *Podospora anserina*. After validation, either or both approaches were used to amplify the ITS region of the 377 fungi isolated here. Using the method described above, we were able to immediately amplify and sequence the vast majority (93%) of the ITS regions. Sequence analysis was successfully used to assign the isolate to a taxonomic group. For the identification of a minority (7%) of the isolates, a second PCR and subsequent sequencing reaction was required.

Isolation and identification of cultivable endophytic fungi. A total of 377 fungi were isolated from the 112 field samples analyzed. After identification, fungal isolates from the same sample that were assigned to the same OTU (by ITS sequence) were considered to be a unique isolate. From the 377 fungi, we identified 254 isolates. The total number of fungi did not significantly differ ($P \leq 0.05$) when considering the isolates from organic and IPM vineyards, as well as from the cultivars Merlot and Chardonnay. All isolates were placed in one of 14 OTU, according to the ITS DNA sequence (Fig. 1); of these, two OTU (*Alternaria* sp. and *Epicoccum nigrum*) were detected in all fields. Seven OTU (*Xylaria* sp., *Ampelomyces humuli*, *Gibberella pulicaris*, *Truncatella angustata*, *Neofusicoccum parvum*, *Phoma herbarum*, and *Davidiella tassiana*) were only isolated from a single vineyard, whereas the remaining seven OTU were present in at least three vineyards. Among the OTU found in multiple fields, *Leptosphaerulina chartarum* was only found in plants from organic farms and *Botryosphaeria* sp. was only found in plants from IPM farms (Fig. 1b). Thirteen of fourteen OTU could be isolated from vines of cv. Merlot (with the only exception of *N. parvum*), whereas only eight OTU were found in cv. Chardonnay vines (Fig. 1c).

ARISA fingerprinting of total fungal endophytic communities. Using ARISA fingerprinting, 66 distinct markers (electrophoretic peaks) were observed. A total of 943 peaks were scored, with individual samples showing from 4 to 18 (average, 8.6) peaks. Grapevine-associated fungi did not show a significant difference in number of ARISA markers between organic and IPM farms or between cv. Chardonnay and cv. Merlot (chi-square, $P \leq 0.05$).

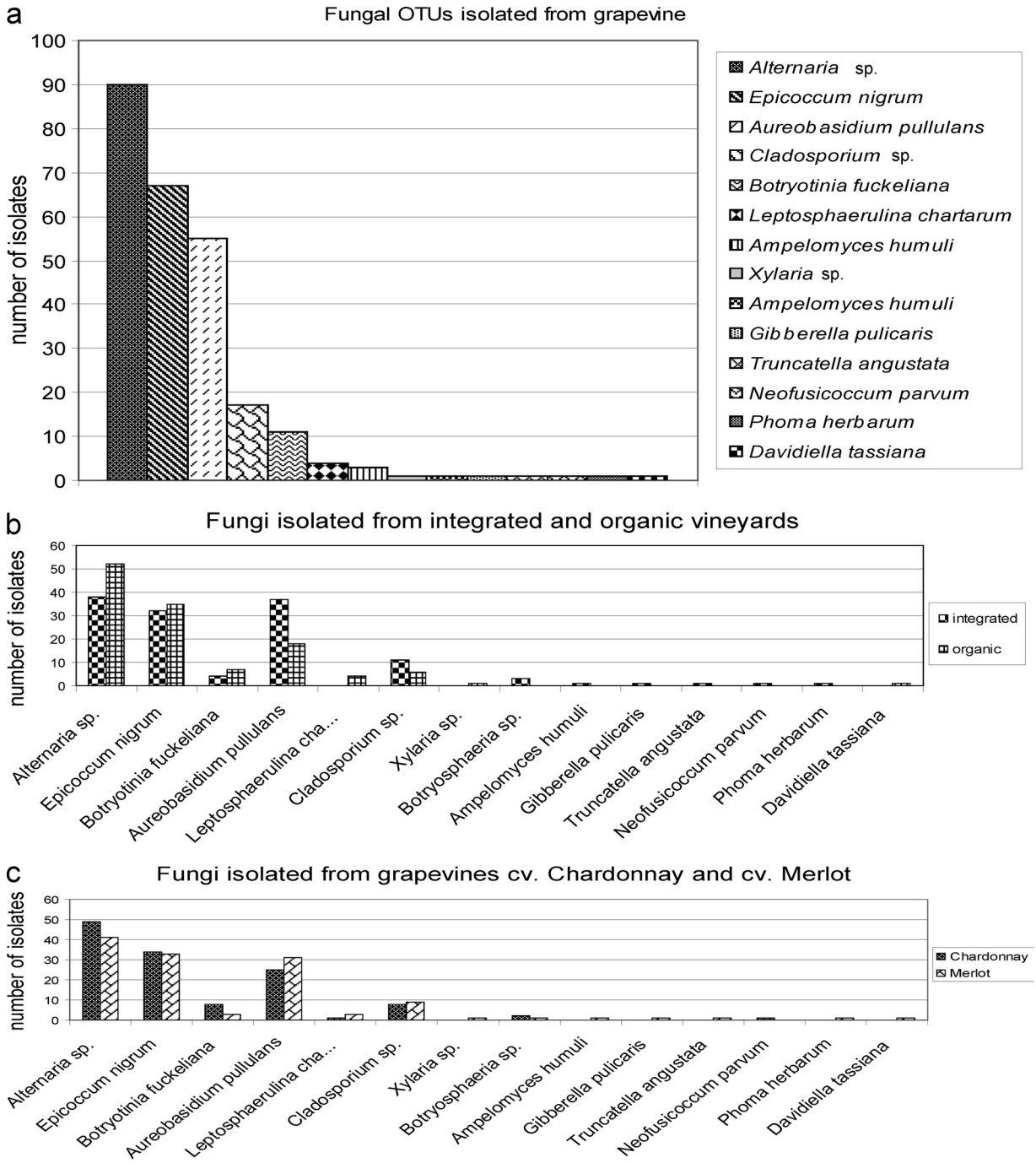

FIG 1 (a) Distribution of fungi isolated in the present study by OTU. (b and c) Distribution of isolated fungi according to the source of isolation, considering the phytosanitary regime (b) and cultivar (c).

Multivariate data analysis. The scatter plots obtained by multivariate analysis showed samples from organic and IPM to be partially or completely separated according to the two main components, using either CCA (Fig. 2) and PCA (Fig. 3). The separation can be observed using the data from both isolated fungi and ARISA fingerprinting. Similarly, the scatter plot based on the combination of ARISA fingerprints and culturable fungi showed partial separation of microbial communities in grapevines of cv. Merlot and cv. Chardonnay (Fig. 2d). The same does not apply for the cultivable fungal community. CCA of this matrix produced results similar to those obtained using the ARISA-derived data matrix.

Multivariate analysis (either one-way ANOSIM or one-way NPMANOVA) of either ARISA and combined data grouped according to treatments also indicated that fungal communities from organic agriculture were quantitatively different from those obtained from IPM vineyards (Table 3). No statistically significant difference was found between communities from grapevines of cv.

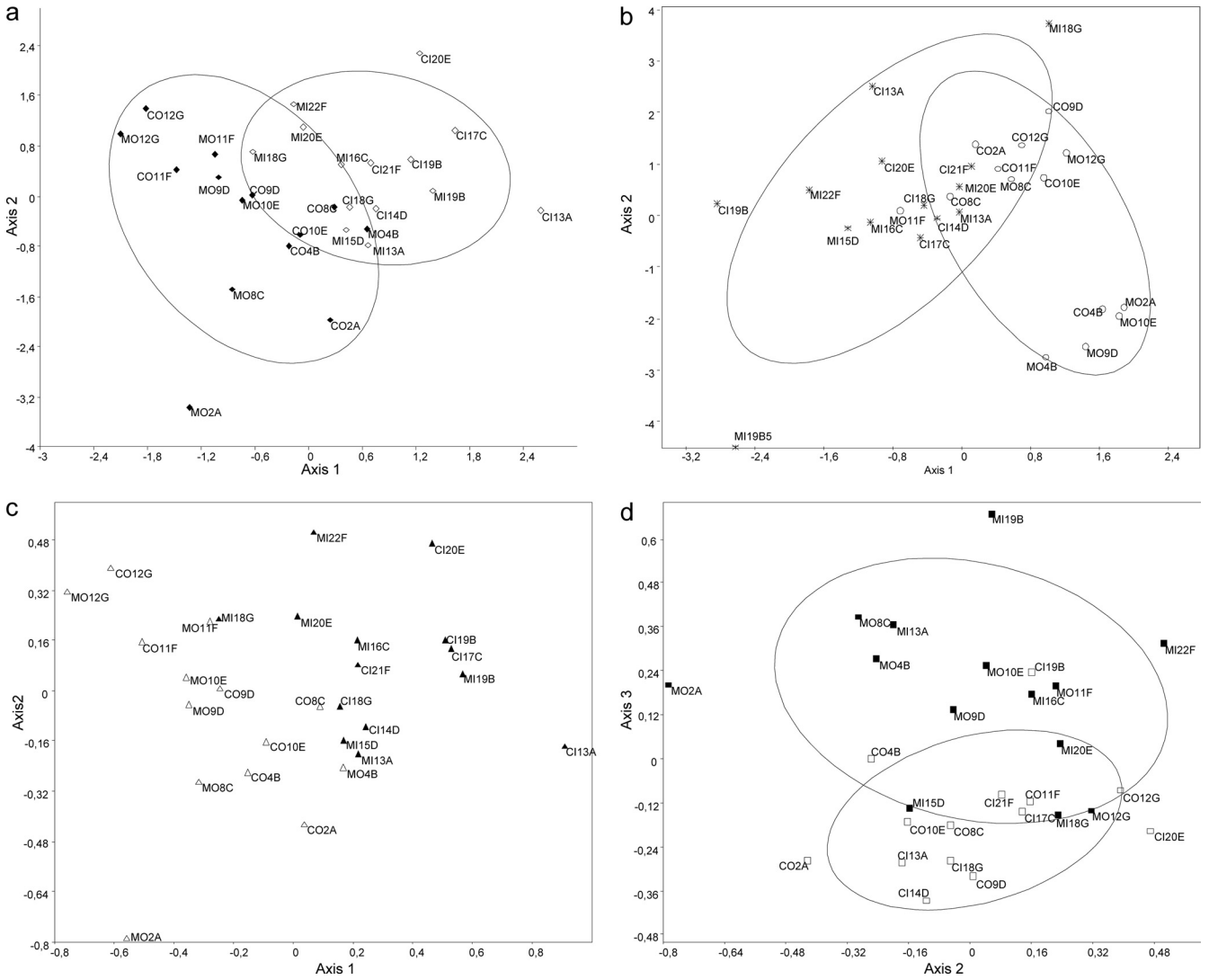

FIG 2 CCA scatter plots. (a) ARISA data, organic (◆) and IPM (◇) farms. (b) Cultured fungi data, organic (○) and IPM (✳) farms. (c) Combined data, organic (△) and IPM (▲) farms. (d) Combined data, cv. Chardonnay (□) and cv. Merlot (■). Ellipses are designed using a confidence threshold of 75%.

Merlot and cv. Chardonnay (data not shown). The same data sets were used for comparison of fungal communities grouped according to the sampling area indicated that there is no significant difference among most areas (Table 4). Significant pairwise differences (using a value of $P < 0.05$) were observed between the areas G (Navicello) and C (Noarna), G and E (Pietramurata), and E and F (Pressano-Sorni).

DISCUSSION

In this study, we have completed a broad comparison of the endophytic fungal communities of grapevines in vineyards under IPM or organic management and between cv. Merlot and cv. Chardonnay. We approached the study of grapevine endophytic fungal community composition and its biomarkers across seven locations in Trentino (Italy) with similar characteristics regarding soil and climate. Our results indicate that mycota in grapevines from organic farms form communities that are significantly different from those in grapevines from IPM farms. We also found the DNA-dependent approach to be more powerful compared to the analysis of culturable fungi. To accomplish this, we established novel experimental protocols, which, after initial validation, allowed us to efficiently identify fungal isolates from the communities analyzed without the need for DNA extraction.

The need to analyze a large collection of fungal isolates has led to the development of a method for colony-PCR using nonpurified fungal mycelial lysates. The method developed here requires little hands-on labor, does not require separation of fungal mycelium from the agar medium, and is validated for a diverse array of fungal taxa. To the best of our knowledge, no previously known protocol combines all three of these highly desirable features, and our protocol is thus a significant improvement. We achieved PCR amplification of ITS regions for all 377 fungi in the collection by either or both of the methods described here, and the sequence of PCR products placed each isolate in 1 of the 14 OTU identified in the study. ITS PCR was highly effective both against the test panel and the collection of isolates (with success rates ranging from 67.5 to 95%, Table 2). Overall, taxonomically relevant sequences could be PCR amplified from all tested fungi prepared using either FDM

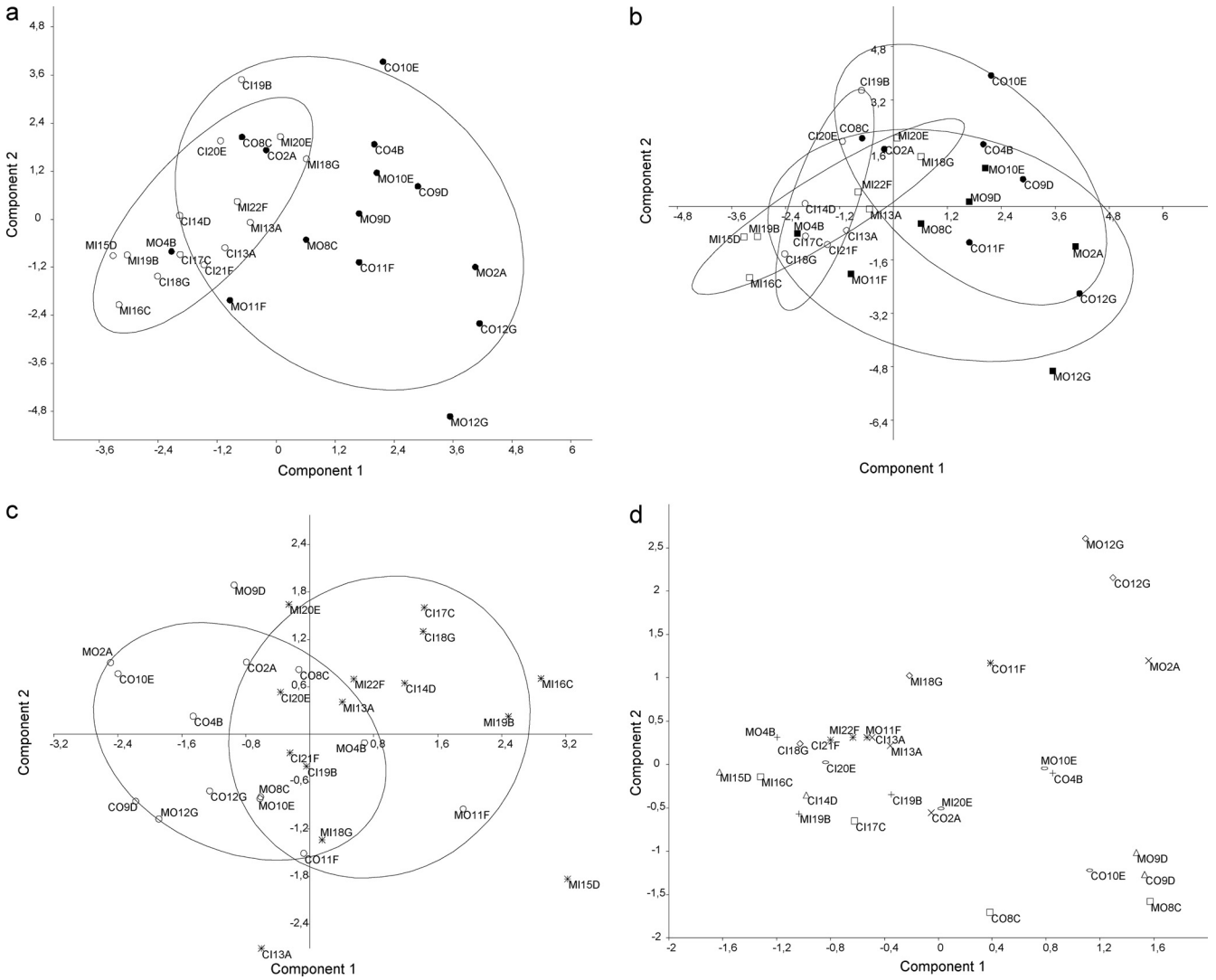

FIG 3 PCA scatter plots. (a) Combined data, organic (●) and IPM (○) farms. (b) Combined data, organic Merlot (■), IPM Merlot (□), organic Chardonnay (●), IPM Chardonnay (○). (c) Cultured fungi data, organic (○) and IPM (✳) farms. (d) Combined data by area, A (×), B (+), C (□), D (△), E (⬭), F (✳), and G (◇). Areas are referred to by letters as defined in Table 1. Ellipses are designed using a confidence threshold of 75%.

or AGM and using at least one of the two primer pairs tested here. The colony-PCR approach described here enables rapid screening of numerous fungal isolates and can be easily applicable to further studies of fungal communities that use a culture-dependent approach. Furthermore, AGM can be applied directly to early-stage fungal colonies from any kind of environmental monitoring (be it air, plant-associated microflora, or food processing surfaces), even prior to isolation of their pure cultures.

TABLE 3 ANOSIM and NPMANOVA *P* values for comparisons of populations from organic and IPM farms using the combined data set

Analysis	Farms	P^a	
		Organic farms	IPM farms
ANOSIM	Organic farms		0.0012
	IPM farms	0.0012	
NPMANOVA	Organic farms		0.0005
	IPM farms	0.0005	

[a] Note that all values are <0.05.

Most fungi isolated in the course of the present study are previously known grapevine endophytes (30, 43), but, to our knowledge, this is the first report of the isolation of *Ampelomyces humuli* and *Gibberella pulicaris* (*Fusarium sambucinum*) from the grapevine endosphere. Isolates identified as *Alternaria* sp., *Epicoccum nigrum*, and *Aureobasidium pullulans* were found frequently in plants from both organic and IPM farms. Fungi belonging to the genus *Alternaria* are among the most common fungal endophytes in grapevines, and some strains may play a role in biocontrol of *Plasmopara viticola* (44). *E. nigrum* is commonly considered either a saprophyte or a biocontrol agent of important grapevine pathogens (26, 37). *A. pullulans* is often found both as an epiphyte and as an endophyte and is considered an antagonist of grapevine disease agents (59).

Interestingly, we also consistently isolated *Botrytis cinerea*. Although the common occurrence of this species as a grapevine

TABLE 4 ANOSIM and NPMANOVA *P* values for comparisons of populations from seven locations in this study using the combined data set

Analysis and area[a]	*P*[b]						
	A	B	C	D	E	F	G
ANOSIM							
A		0.7974	0.3788	0.9156	0.6802	0.4903	0.3717
B	0.7974		0.1979	0.2844	0.3158	0.0555	0.2903
C	0.3788	0.1979		0.4277	0.107	0.3432	0.0246
D	0.9156	0.2844	0.4277		0.1977	0.6229	0.2516
E	0.6802	0.3158	0.107	0.1977		0.0273	0.0281
F	0.4903	0.0555	0.3432	0.6229	0.0273		0.1775
G	0.3717	0.2903	0.0246	0.2516	0.0281	0.1775	
NPMANOVA							
A		0.652	0.2854	0.9454	0.3057	0.6313	0.4561
B	0.652		0.2016	0.2055	0.2013	0.0842	0.1987
C	0.2854	0.2016		0.4046	0.057	0.1988	0.0283
D	0.9454	0.2055	0.4046		0.2965	0.6341	0.2846
E	0.3057	0.2013	0.057	0.2965		0.0284	0.0304
F	0.6313	0.0842	0.1988	0.6341	0.0284		0.1711
G	0.4561	0.1987	0.0283	0.2846	0.0304	0.1711	

[a] See Table 1, column 1, for the area letter definitions.
[b] Shaded values are <0.05.

endophyte was previously reported (17, 30), it must be noted that it is considered an important grapevine pathogen. *B. cinerea* could be latent (25), therefore behaving as an asymptomatic plant endophyte and turn pathogenic only under specific physiological or environmental conditions.

The species *Botryosphaeria obtusa*, *Botryosphaeria dothidea*, *Truncatella angustata*, *Neofusicoccum parvum*, *Phoma herbarum*, and *Davidiella tassiana* were isolated from apparently healthy vines but are also sometimes regarded as grapevine pathogens (23, 38, 45, 64, 67, 69). However, the majority of these fungi (with the exception of *D. tassiana*) were isolated from IPM vineyards (Fig. 1b) and may thus represent potential pathogens not detected in grapevines from organic farms. Fungicides used in IPM may be a driving force in shaping the composition of the fungal communities observed, but the level of tolerance to these fungicides among the fungi we isolated is unknown. Differences between fungi isolated from organic or IPM plants in the response to the applied fungicides will be tested in future experiments.

Several studies have investigated microbial communities in soil and their shifts under different land management practices (12, 29). Comparatively fewer attempts have been made to assess the effect of agricultural management on the endophytic microbial communities present in crops (60, 62). In the present study, both multivariate analysis of cultivable fungi and a DNA-based approach concur to indicate that IPM has an impact on the composition of endophytic fungal communities. A likely factor behind this could be the long-term use of synthetic fungicides in IPM or the use of organic fertilizers in organic farming.

Multivariate analysis indicated that fungal community composition differed between the organic and IPM vineyards, with partially distinct areas in CCA scatter plots (canonical correspondence analysis) when considering data sets from cultivable fungi, total fungal DNA analysis, or both (Fig. 2a and b). A combination of the two data sets under the same analysis showed a more marked separation of the samples from organic and IPM farms (Fig. 2c). Interestingly, when the same combined data were projected across the second and third axes, communities from Merlot and Chardonnay grapevines were partially distinct (Fig. 2d). This suggests that differences between grapevine cultivars may drive a minor shift in endophyte composition, as seen for other plant species (42), but that the extent of this diversity is secondary, compared to the effect of crop management. Previous literature has shown that the composition of the culturable fungal endophytic community may be influenced by the cultivar. Casieri et al. (17) investigated the fungi in the endosphere of five grapevine cultivars in Switzerland and found that the community composition across cultivars differed both when considering the phyla and the species of isolated fungi. Others researchers have analyzed the endophytic mycota associated with grapevine in Spain, finding that the composition across cultivars differed when the order of isolated fungal taxa was considered (30). Finally, some information is available on the cultivar influence on microbial communities associated with the roots (47). Unfortunately, none of these previous reports included a statistical analysis of their interesting findings, making it difficult to compare the studies regarding the extent of these differences in microbial communities.

PCA analysis of the ARISA fingerprinting data set (i.e., cultivation-independent communities) allows identification of the loadings of the principal components. The two main components' contributors are the 371-bp (peak 43) and 354-bp (peak 29) fragments for the main axis and 358-bp (peak 34) and 360-bp (peak 35) fragments for the secondary axis (Fig. 3a). Linking ARISA peaks to fungal species or OTU is a ticklish operation. As pointed out previously (5), different species may have ITS regions of identical size (33), while some species may display multiple and different (i.e., polymorphic) ITS copies (6). For this reason, the taxonomically ambiguous entities (sometimes referred to as ribotypes) produced by ARISA fingerprinting are not reliable indicators of species richness. We thus refrained from formulating any hypotheses on the correspondence of relevant ARISA peaks to taxonomic units.

PCA of the cultured fungi data set (Fig. 3c) indicated that the variance of the main axis, roughly dividing samples from organic and integrated management, is mainly due to several OTU: *A. pullulans*, *Alternaria* sp., and to a lesser extent by *Epicoccum nigrum*. In contrast with previous findings (60), we found *A. pullulans* more frequently in vineyards using IPM than in organic vineyards. Schmid et al. in 2011 (60) found *A. pullulans* to be more abundant in plants from organic vineyards, whereas these researchers observed the opposite for the yeast *Sporidiobolus pararoseus*, which was never isolated from grapevines in our study. Possible factors, related to these differences, are the different cultivars, the *terroir* differences, the environmental dissimilarities and the methodology used. In the former study, the abundance of *A. pullulans* in the samples was measured by quantitative amplification of ITS sequences. while we estimate the distribution of each OTU making no assumptions regarding the quantitative assessment of each microorganism. Remarkably, both studies indicate that *A. pullulans* is significantly affected by plant protection strategies.

Noticeably, when PCA is applied to the combined data set, data points representing microbial communities from IPM vineyards (with both cv. Merlot and cv. Chardonnay) span a smaller range on the principal component than those from organic vineyards (Fig. 3b). This observation suggests that the variability across fun-

gal endophytic communities from IPM farms may be smaller than that from organic farms.

With respect to marker counts (ARISA peaks or isolated fungi) across the seven sampled areas, we noted that in most cases data points relative to fungal communities from cv. Merlot and cv. Chardonnay grapevines in the same area were grouped together (Fig. 3d). The same could not be observed when we compared organic and integrated vineyards. This suggests that the difference between fungi from organic vineyards and IPM vineyards is larger than the difference between the plant cultivars.

Quantitative analysis by one-way ANOSIM and one-way NPMANOVA supported the conclusions deduced by visual analysis of PCA and CCA scatter plots, pointing out that crop management (IPM and organic) modifies the structure of fungal endophytic communities (Table 3). One can hypothesize that the use of synthetic systemic fungicides may have a role in these differences. Organic fertilizers may also be a source of microorganisms, which may establish them as endophytes. Further research on the role of systemic fungicide or introduction by the application of organic fertilizers will be crucial to prove these two hypotheses. The analysis tools used in the present study suggest that the grapevine cultivar and cultivar-dependent plant physiology may also play a role in shaping endophytic communities, but to a lesser extent compared to crop management or sampling sites.

ACKNOWLEDGMENTS

This research was funded by Provincia Autonoma di Trento (project PAT-Call 2 Team 2009–Incoming–Mecagrafic).

We acknowledge Enzo Mescalchin, Matteo Secchi, and Roberto Zanzotti for identification of the sampling areas, Valerio Mazzoni and Ron Wehrens for useful insights on statistical analysis, and Jonas Bengtsson for help with critically reviewing the manuscript.

REFERENCES

1. **Adams PD, Kloepper JW.** 2002. Effect of host genotype on indigenous bacterial endophytes of cotton (*Gossypium hirsutum* L.). Plant Soil **240**: 181–189.
2. **AlShahni MM, et al.** 2009. Direct colony PCR of several medically important fungi using Ampdirect® Plus. Jpn. J. Infect. Dis. **62**:164–167.
3. **Anderson MJ.** 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecol. **26**:32–46.
4. **Araujo WL, et al.** 2002. Diversity of endophytic bacterial populations and their interaction with *Xylella fastidiosa* in citrus plants. Appl. Environ. Microbiol. **68**:4906–4914.
5. **Avis PG, Branco S, Tang Y, Mueller GM.** 2010. Pooled samples bias fungal community descriptions. Mol. Ecol. Resour. **10**:135–141.
6. **Avis PG, Dickie IA, Mueller GM.** 2006. A "dirty" business: testing the limitations of terminal restriction fragment length polymorphism (TRFLP) analysis of soil fungi. Mol. Ecol. **15**:873–882.
7. **Bainbridge BW, Spreadbury CL, Scalise FG, Cohen J.** 1990. Improved methods for the preparation of high molecular-weight DNA from large and small-scale cultures of filamentous fungi. FEMS Microbiol. Lett. **66**: 113–117.
8. **Barnett HL, Hunter BB.** 1998. Illustrated genera of imperfect fungi, 4th ed. APS Press, St. Paul, MN.
9. **Bittleston LS, Brockmann F, Wcislo W, Van Bael SA.** 2011. Endophytic fungi reduce leaf-cutting ant damage to seedlings. Biol. Lett. **7**:30–32.
10. **Borneman J, Hartin RJ.** 2000. PCR primers that amplify fungal rRNA genes from environmental samples. Appl. Environ. Microbiol. **66**:4356–4360.
11. **Brosi GB, et al.** 2011. Effects of multiple climate change factors on the tall fescue-fungal endophyte symbiosis: infection frequency and tissue chemistry. New Phytol. **189**:797–805.
12. **Bucher AE, Lanyon LE.** 2005. Evaluating soil management with community-level physiological microbial profiles. Appl. Soil Ecol. **29**:59–71.
13. **Bulgari D, et al.** 2009. Endophytic bacterial diversity in grapevine (*Vitis vinifera* L.) leaves described by 16S rRNA gene sequence analysis and length heterogeneity-PCR. J. Microbiol. **47**:393–401.
14. **Bulgari D, et al.** 2011. Restructuring of endophytic bacterial communities in grapevine yellows-diseased and recovered *Vitis vinifera* L. plants. Appl. Environ. Microbiol. **77**:5018–5022.
15. **Buyer JS, Zuberer DA, Nichols KA, Franzluebbers AJ.** 2011. Soil microbial community function, structure, and glomalin in response to tall fescue endophyte infection. Plant Soil **339**:401–412.
16. **Caffarra A, Eccel E.** 2011. Projecting the impacts of climate change on the phenology of grapevine in a mountain area. Aust. J. Grape Wine Res. **17**:52–61.
17. **Casieri L, Hofstetter V, Viret O, Gindro K.** 2009. Fungal communities living in the wood of different cultivars of young *Vitis vinifera* plants. Phytopathol. Mediterr. **48**:73–83.
18. **Chen HR, Hsu MT, Cheng SC.** 1995. Spheroplast preparation facilitates PCR screening of yeast sequence. Biotechniques **19**:744–748.
19. **Clarke KR.** 1993. Nonparametric multivariate analyses of changes in community structure. Aust. J. Ecol. **18**:117–143.
20. **Compant S, Mitter B, Colli-Mull JG, Gangl H, Sessitsch A.** 2011. Endophytes of grapevine flowers, berries, and seeds: identification of cultivable bacteria, comparison with other plant parts, and visualization of niches of colonization. Microb. Ecol. **62**:188–197.
21. **Crosby LD, Criddle CS.** 2003. Understanding bias in microbial community analysis techniques due to rrn operon copy number heterogeneity. Biotechniques **34**:790–794.
22. **Danovaro R, Luna GM, Dell'Anno A, Pietrangeli B.** 2006. Comparison of two fingerprinting techniques, terminal restriction fragment length polymorphism and automated ribosomal intergenic spacer analysis, for determination of bacterial diversity in aquatic environments. Appl. Environ. Microbiol. **72**:5982–5989.
23. **Diaz GA, Prehn D, Besoain X, Chavez ER, Latorre BA.** 2011. *Neofusicoccum parvum* associated with grapevine trunk diseases in Chile. Plant Dis. **95**:1032–1033.
24. **Doyle JJ, Doyle JL.** 1990. Isolation of plant DNA from fresh tissue. Focus **12**:13–15.
25. **Elmer PAG, et al.** 2007. Epidemiology of *Botrytis cinerea* in orchard and vine crops, p 243–272. *In* Botrytis: biology, pathology, and control. Springer, Amsterdam, Netherlands.
26. **Elmer PAG, Reglinski T.** 2006. Biosuppression of *Botrytis cinerea* in grapes. Plant Pathol. **55**:155–177.
27. **Fisher MM, Triplett EW.** 1999. Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. Appl. Environ. Microbiol. **65**:4630–4636.
28. **Fredricks DN, Smith C, Meier A.** 2005. Comparison of six DNA extraction methods for recovery of fungal DNA as assessed by quantitative PCR. J. Clin. Microbiol. **43**:5122–5128.
29. **Gomez E, Ferreras L, Toresani S.** 2006. Soil bacterial functional diversity as influenced by organic amendment application. Bioresour. Technol. **97**: 1484–1489.
30. **Gonzalez V, Tello ML.** 2011. The endophytic mycota associated with *Vitis vinifera* in central Spain. Fungal Divers. **47**:29–42.
31. **Granado J, et al.** 2008. Culturable fungi of stored "golden delicious" apple fruits: a one-season comparison study of organic and integrated production systems in Switzerland. Microb. Ecol. **56**:720–732.
32. **Hammer Ø, Harper DAT, Ryan PD.** 2001. PAST: paleontological statistics software for education and data analysis. Palaeontol. Electronica **4**:1–9.
33. **Horton TR.** 2002. Molecular approaches to ectomycorrhizal diversity studies: variation in ITS at a local scale. Plant Soil **244**:29–39.
34. **Islam SMA, et al.** 2010. Effect of plant age on endophytic bacterial diversity of balloon flower (*Platycodon grandiflorum*) root and their antimicrobial activities. Curr. Microbiol. **61**:346–356.
35. **Khot PD, Fredricks DN.** 2009. PCR-based diagnosis of human fungal infections. Expert Rev. Anti-Infect. Ther. **7**:1201–1221.
36. **Koranda M, et al.** 2011. Microbial processes and community composition in the rhizosphere of European beech: the influence of plant C exudates. Soil Biol. Biochem. **43**:551–558.
37. **Kortekamp A.** 1997. *Epicoccum nigrum* link: a biological control agent of *Plasmopara viticola* (Berk. et Curt.) Berl. et De Toni? Vitis **36**:215–216.
38. **Krol E.** 2006. Fungi inhibiting decaying grapevine (*Vitis* spp.) cuttings. J. Plant Prot. Res. **46**:353–358.
39. **Lau A, Sorrell TC, Lee O, Stanley K, Halliday C.** 2008. Colony multi-

plex-tandem PCR for rapid, accurate identification of fungal cultures. J. Clin. Microbiol. **46**:4058–4060.
40. **Legendre P, Legendre L.** 1998. Numerical ecology, p 594–604. Elsevier Science BV, Amsterdam, Netherlands.
41. **Liu D, Coloe S, Baird R, Pedersen J.** 2000. Rapid mini-preparation of fungal DNA for PCR. J. Clin. Microbiol. **38**:471.
42. **Manter DK, Delgado JA, Holm DG, Stong RA.** 2010. Pyrosequencing reveals a highly diverse and cultivar-specific bacterial endophyte community in potato roots. Microb. Ecol. **60**:157–166.
43. **Martini M, et al.** 2009. DNA-dependent detection of the grapevine fungal endophytes *Aureobasidium pullulans* and *Epicoccum nigrum*. Plant Dis. **93**:993–998.
44. **Musetti R, et al.** 2006. Inhibition of sporulation and ultrastructural alterations of grapevine downy mildew by the endophytic fungus *Alternaria alternata*. Phytopathology **96**:689–698.
45. **Niekerk JM, Fourie PH, Hallenn F, Crous P.** 2006. *Botryosphaeria* spp. as grapevine trunk disease pathogens. Phytopathol. Mediterr. **46**:S43–S54.
46. **Oliveri C, Campisano A, Catara A, Cirvilleri G.** 2007. Characterization and fAFLP genotyping of *Penicillium* strains from postharvest samples and packinghouse environments. J. Plant Pathol. **89**:29–40.
47. **Parker SR, Kluepfel DA.** 2009. Effect of rootstock genotype on functional and taxonomic diversity of rhizosphere communities and endophyte communities of grapevine in California. Phytopathology **99**:S100–S100.
48. **Pinamonti F, Stringari G, Gasperi F, Zorzi G.** 1997. The use of compost: its effects on heavy metal levels in soil and plants. Resour. Conserv. Recycling **21**:129–143.
49. **Plaza GA, Upchurch R, Brigmon RL, Whitman WB, Ulfig K.** 2004. Rapid DNA extraction for screening soil filamentous fungi using PCR amplification. Pol. J. Environ. Stud. **13**:315–318.
50. **Porras-Alfaro A, Bayman P.** 2011. Hidden fungi, emergent properties: endophytes and microbiomes. Annu. Rev. Phytopathol. **49**:291–315.
51. **Rajulu MBG, et al.** 2011. Chitinolytic enzymes from endophytic fungi. Fungal Divers. **47**:43–53.
52. **Rasche F, et al.** 2006. Rhizosphere bacteria affected by transgenic potatoes with antibacterial activities compared with the effects of soil, wild-type potatoes, vegetation stage and pathogen exposure. FEMS Microbiol. Ecol. **56**:219–235.
53. **Rasche F, et al.** 2006. Impact of transgenic potatoes expressing antibacterial agents on bacterial endophytes is comparable with the effects of plant genotype, soil type and pathogen infection. J. Appl. Ecol. **43**:555–566.
54. **Ruimy R, et al.** 2010. Organic and conventional fruits and vegetables contain equivalent counts of Gram-negative bacteria expressing resistance to antibacterial agents. Environ. Microbiol. **12**:608–615.
55. **Russell JR, et al.** 2011. Biodegradation of polyester polyurethane by endophytic fungi. Appl. Environ. Microbiol. **77**:6076–6084.
56. **Saitoh K-I, Togashi K, Arie T, Teraoka T.** 2006. A simple method for a mini-preparation of fungal DNA. J. Gen. Plant Pathol. **72**:348–350.
57. **Saona NM, Albrectsen BR, Ericson L, Bazely DR.** 2010. Environmental stresses mediate endophyte-grass interactions in a boreal archipelago. J. Ecol. **98**:470–479.
58. **Sayers EW, et al.** 2010. Database resources of the National Center for Biotechnology Information. Nucleic Acids Res. **38**:D5–D16.
59. **Schena L, et al.** 1999. Genetic diversity and biocontrol activity of *Aureobasidium pullulans* isolates against postharvest rots. Postharvest Biol. Technol. **17**:189–199.
60. **Schmid F, Moser G, Muller H, Berg G.** 2011. Functional and structural microbial diversity in organic and conventional viticulture: organic farming benefits natural biocontrol agents. Appl. Environ. Microbiol. **77**:2188–2191.
61. **Schmidt H, Ehrmann M, Vogel RE, Taniwaki MH, Niessen L.** 2003. Molecular typing of *Aspergillus ochraceus* and construction of species specific SCAR-primers based on AFLP. Syst. Appl. Microbiol. **26**:138–146.
62. **Seghers D, Wittebolle L, Top EM, Verstraete W, Siciliano SD.** 2004. Impact of agricultural practices on the *Zea mays* L. endophytic community. Appl. Environ. Microbiol. **70**:1475–1482.
63. **Singh PK, Hughes GR.** 2006. Genetic similarity among isolates of *Pyrenophora tritici-repentis*, causal agent of tan spot of wheat. J. Phytopathol. **154**:178–184.
64. **Slippers B, Wingfield MJ.** 2007. *Botryosphaeriaceae* as endophytes and latent pathogens of woody plants: diversity, ecology, and impact. Fungal Biol. Rev. **21**:90–106.
65. **Sundram S, Meon S, Abu Seman I, Othman R.** 2011. Symbiotic interaction of endophytic bacteria with arbuscular mycorrhizal fungi and its antagonistic effect on *Ganoderma boninense*. J. Microbiol. **49**:551–557.
66. **Taylor DL, Houston S.** 2011. A bioinformatics pipeline for sequence-based analyses of fungal biodiversity. Methods Mol. Biol. **722**:141–155.
67. **Urbez-Torres JR, Adams P, Kamas J, Gubler WD.** 2009. Identification, incidence, and pathogenicity of fungal species associated with grapevine dieback in Texas. Am. J. Enol. Vitic. **60**:497–507.
68. **Van Burik JAH, Schreckhise RW, White TC, Bowden RA, Myerson D.** 1998. Comparison of six extraction techniques for isolation of DNA from filamentous fungi. Med. Mycol. **36**:299–303.
69. **van Niekerk JM, Crous PW, Groenewald JZ, Fourie PH, Halleen F.** 2004. DNA phylogeny, morphology, and pathogenicity of *Botryosphaeria* species on grapevines. Mycologia **96**:781–798.
70. **van Zeijl CMJ, et al.** 1998. An improved colony-PCR method for filamentous fungi for amplification of PCR fragments of several kilobases. J. Biotechnol. **59**:221–224.
71. **Vontiedemann S, Brendel G, Fehrmann H.** 1988. Investigations on endophytic fungi of grapevine with special emphasis on the vascular system of rootstocks. J. Phytopathol. **122**:147–165.
72. **West ER, Cother EJ, Steel CC, Ash GJ.** 2010. The characterization and diversity of bacterial endophytes of grapevine. Can. J. Microbiol. **56**:209–216.
73. **White TJ, Bruns T, Lee SW, Taylor JW.** 1990. Amplification and direct sequencing of fungal rRNA genes for phylogenetics, p 315–322. *In* Innis MA, Gelfand DH, Sninsky JJ, White TJ (ed), PCR protocols: a guide to methods and applications. Academic Press, Inc, New York, NY.
74. **Yousaf S, Andria V, Reichenauer TG, Smalla K, Sessitsch A.** 2010. Phylogenetic and functional diversity of alkane degrading bacteria associated with Italian ryegrass (*Lolium multiflorum*) and Birdsfoot trefoil (*Lotus corniculatus*) in a petroleum oil-contaminated environment. J. Hazard. Mater. **184**:523–532.
75. **Zhao J, Shan T, Mou Y, Zhou L.** 2011. Plant-derived bioactive compounds produced by endophytic fungi. Mini Rev. Med. Chem. **11**:159–168.