DNA-Dependent Detection of the Grapevine Fungal Endophytes *Aureobasidium pullulans* and *Epicoccum nigrum*

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ABSTRACT

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Aureobasidium pullulans and *Epicoccum nigrum* are frequently reported as endophytes of various crops, including grapevine (*Vitis vinifera*). Because of their potential role as biological control agents against grapevine pathogens, we examined the occurrence of *A. pullulans* and *E. nigrum* in two grapevine varieties (Merlot and Prosecco) in Italian vineyards where spontaneous recovery from phytoplasma disease is recurrent. Species-specific primers for *A. pullulans* and two genetically distinct strains of *E. nigrum* were designed in variable regions of ITS1 and ITS2. Primer specificity was confirmed by polymerase chain reaction using purified DNA from other fungal endophytes that are usually encountered during isolation attempts from grapevine tissues and from several other strains of *A. pullulans* and *E. nigrum* isolated from other sources. In order to determine the occurrence of the two endophytes in grapevine plants, DNA was extracted from shoots of 44 grapevines collected in six vineyards from different localities of northeast Italy. Both endophytes were detected and their identity was confirmed by restriction fragment length polymorphism (RFLP) patterns obtained from reference strains. RFLP analyses confirmed the presence of two *E. nigrum* strains belonging to different RFLP groups in grapevine. The molecular methods described allowed a sensitive, specific, and reliable identification of the two endophytes in grapevine.

Aureobasidium pullulans (de Bary) Arnaud is a widespread epiphyte well adapted to the phyllosphere and carposphere. Some *A. pullulans* isolates exhibit antagonistic activity against a number of phytopathogenic fungi (33,34), competing for nutrients and space and producing numerous compounds such as pectolytic enzymes (28), antimicrobial metabolites (36), and high-molecular-weight polysaccharides (pullulan) (29). It is therefore used as a biocontrol agent against postharvest pathogens (e.g., *Botrytis cinerea* and *Penicillium expansum*) (27,33). *Epicoccum nigrum* Link is another component of the resident mycoflora on various plants and is a saprophyte on dying plant organs (22). *E. nigrum* produces a number of secondary metabolites such as epicoccone (10,17) and flavipin, which has antibiotic activity against *Monilinia laxa*, the causal agent of brown rot (17,24). Inhibition of the grape-

vine pathogens *Plasmopara viticola* and *B. cinerea* has been also reported by *E. nigrum* (11,13,20). Thus, *A. pullulans* and *E. nigrum* may potentially provide disease control of these important pathogens.

A. pullulans and *E. nigrum* have been isolated as endophytes from different crops, including grapevine (*Vitis vinifera* L.) (5,11,29,30,33). In particular, they have been found in leaves (mainly near the midribs; 1,30), berries (30,34), and dormant buds (9). It is recognized that endophytes are of great importance for the hosts, and their role in the protection of the plants against insects, nematodes, and pathogenic microorganisms has been well demonstrated (4,19,32). However, the role of endophytic microorganisms in relation to systemic, incurable diseases, such as those associated with phytoplasmas, has still to be investigated. Spontaneous recovery from phytoplasma-induced symptoms has been reported in grapevines and fruit crops, indicating that a type of resistance could occur in previously infected plants (26). It has been hypothesized that endophytic microorganisms may take part in this resistance phenomenon. In addition, fungi and yeasts associated with grapevines may play an important role in wine fermentation (12).

In order to determine the distribution and frequency of endophytes in host tissues, accurate identification of the organisms is required. Because endophytes do

not cause symptoms in their hosts, they are difficult to detect. Conventional methods for identifying fungal endophytes rely on isolation, culturing, and laboratory identification. However, some fungi develop slowly or do not readily grow in culture (25). Other methods used to detect endophytes include histochemical, immunological, and molecular methods (7).

In this study, an efficient DNA extraction method and a sensitive polymerase chain reaction (PCR) protocol were developed for the detection of the grapevine endophytes *A. pullulans* and *E. nigrum*. In addition, the relationship between the presence of endophytes and the spontaneous recovery from a phytoplasma disease was examined.

MATERIALS AND METHODS

Plant material. Tissue samples were collected from 10-year-old grapevines (*V. vinifera* cv. Prosecco) in a vineyard near Montebelluna in the Veneto region of northeastern Italy. Plants in this vineyard had been rated since 2000 for symptoms of phytoplasma diseases (26). Plants were categorized as Flavescente dorée (FD)-infected (showing phytoplasma symptoms), healthy (asymptomatic), or recovered (previously symptomatic but now asymptomatic). For the present work, five individual FD-symptomatic grapevines, five asymptomatic plants, and five recovered plants were sampled in October 2005. Five randomly sampled leaves and two 2-year-old shoots were collected from each of the plants, stored at 4°C in the dark, and processed within 24 h for fungal endophyte isolation.

Fungal isolates. To isolate endophytes, plant material was washed with water, surface-sterilized by immersion in 3% sodium hypochlorite for 4 min, and rinsed twice in sterile water for 1 min. Four tissue pieces (about 5 × 5 mm) per leaf or shoot were placed in 90-mm petri dishes containing potato dextrose agar (PDA; Difco, Plymouth, MN) and incubated in a growth chamber for 48 h at 25°C, 60% relative humidity, and cold UV light with a photoperiod of 12 h. Any fungal colonies recovered from tissue samples were subcultured five times on fresh PDA plates to obtain pure cultures. Cultures were then maintained on PDA at 4°C for short-term storage or stored in sterile H₂O at room temperature in the dark for longer-term storage.

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DNA extraction. High-molecular-weight DNA was extracted from fresh mycelia of the isolated endophytes and from other isolates of *A. pullulans* and *E. nigrum* (Table 1) following a modified procedure of Lecellier and Silar (23). After 7 days of growth, approximately 450 mg of mycelia was harvested from the surface of a sterilized cellophane membrane (BIORAD, Hercules, CA) on PDA with the aid of a sterile spatula. Mycelium was then placed in a sterile mortar with 3 ml of extraction buffer and homogenized with a sterile pestle. One milliliter of the homogenate was placed in a 2-ml microfuge tube, vortexed, frozen for 30 s in liquid nitrogen, and thawed at 70°C. The vortexing and freeze/thaw procedure was repeated three times. After centrifugation at 10,000 rpm for 10 min, the supernatant was transferred to another sterile microfuge tube and extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and with an equal volume of chloroform:isoamyl alcohol (24:1). Following centrifugation at 8,000 rpm for 10 min, the supernatant was transferred to another sterile microfuge tube, and 2/3 volume of cold isopropanol

was added and gently mixed by inverting several times. The tubes were then centrifuged at 12,000 rpm for 15 min at 4°C to pellet the DNA, which was washed with 0.5 ml of cold 70% ethanol after removal of the supernatant. Following centrifugation (12,000 rpm for 5 min at 4°C), the supernatant was discarded. The DNA pellets were then dried by a HetoVac vacuum and dissolved in 400 µl of TE buffer. Further DNA purification was carried out by DNA precipitation with 1/10 volume of 3 M sodium acetate at pH 5.2 and 2 volumes of cold 95% ethanol with gentle mixing. Following centrifugation (14,000 rpm for 5 min at 4°C), the supernatant was discarded and the DNA pellet was washed twice with 1 ml of 70% ethanol followed by a wash with 1 ml of 80% ethanol. After centrifugation, the DNA pellet was dried with a HetoVac vacuum, suspended in 100 µl of TE buffer, and treated with RNase A for 1 h at 37°C. DNA concentrations were determined with a spectrophotometer (Beckman Inc., Fullerton, CA).

Amplification and sequencing. The nuclear rDNA internal transcribed spacer regions (ITS1 and ITS2) and the 5.8S rRNA gene from all the endophytes, in-

cluding one *A. pullulans* and the two *E. nigrum* isolates, were amplified by PCR with the universal primers ITS1 and ITS4 (38). The extracted DNA was diluted 1:50 in sterile water to obtain a concentration of about 10 ng of DNA/µl. Amplifications were performed with an automated thermal cycler (MJ Research DNA Thermal Cycler PTC-100) in 25-µl reactions containing 200 µM each of the four dNTPs, 0.4 µM of each primer, 1.5 mM MgCl₂, 0.625 units of DNA polymerase, POLYTAQ (Polymed, Florence, Italy), and 1 µl of 1:50 diluted DNA. The PCR program consisted of 38 cycles: denaturation at 94°C for 1 min (2 min for the first cycle), annealing at 54°C for 1 min, and extension at 72°C for 1 min (10 min for the last cycle). Five microliters of the amplified products were visualized by electrophoresis through a 1% agarose gel stained in ethidium bromide.

The ITS1/ITS4 PCR products (about 500 to 600 bp) were purified using a Wizard SV Gel and the PCR Clean-Up System Kit (Promega, Madison, WI, USA). Sequencing was performed with an automated DNA sequencer (ABI Prism Model 3730) at the Genelab (ENEA Casaccia, Rome, Italy) using the ITS4 primer. The

Table 1. Source of fungal isolates included in this study

Fungal species ^a	Isolate code	GenBank accession no.	Host plant	Location (Italian province)
<i>Aureobasidium pullulans</i> ^b	R6T4	EU529999	<i>Vitis vinifera</i>	Montebelluna (TV)
<i>Epicoccum nigrum</i> ^b	H2F1	EU529998	<i>Vitis vinifera</i>	Montebelluna (TV)
<i>Epicoccum nigrum</i> ^b	R11T3	EU530001	<i>Vitis vinifera</i>	Montebelluna (TV)
<i>Alternaria alternata</i> ^b	T65A	EU530004	<i>Vitis vinifera</i>	Montebelluna (TV)
<i>Arthrinium sacchari</i> ^b	H5T3	EU529997	<i>Vitis vinifera</i>	Montebelluna (TV)
<i>Leptosphaerulina chartarum</i> ^b	R4F1	EU529996	<i>Vitis vinifera</i>	Montebelluna (TV)
<i>Nigrospora oryzae</i> ^b	H5T4	EU529995	<i>Vitis vinifera</i>	Montebelluna (TV)
<i>Nigrospora oryzae</i> ^b	R11F1	EU529994	<i>Vitis vinifera</i>	Montebelluna (TV)
<i>Nigrospora oryzae</i> ^b	R4F3	EU529993	<i>Vitis vinifera</i>	Montebelluna (TV)
<i>Phoma</i> sp. ^b	P45A	EU530003	<i>Vitis vinifera</i>	Montebelluna (TV)
<i>Phomopsis</i> sp. ^b	R42	—	<i>Vitis vinifera</i>	Montebelluna (TV)
<i>Schizophyllum commune</i> ^b	R6T2	EU530002	<i>Vitis vinifera</i>	Montebelluna (TV)
<i>Sclerotinia sclerotiorum</i> ^b	H3F2	EU530000	<i>Vitis vinifera</i>	Montebelluna (TV)
<i>Aureobasidium pullulans</i> ^c	VIF1a	—	<i>Vitis vinifera</i>	Monopoli (BA)
<i>Aureobasidium pullulans</i> ^c	VIFg1a	—	<i>Vitis vinifera</i>	Monopoli (BA)
<i>Aureobasidium pullulans</i> ^c	PF1a	—	<i>Prunus domestica</i>	Senigallia (AN)
<i>Aureobasidium pullulans</i> ^c	Nf1a	—	<i>Juglans regia</i>	Ancona (AN)
<i>Aureobasidium pullulans</i> ^c	NF1a	—	<i>Juglans regia</i>	Ancona (AN)
<i>Aureobasidium pullulans</i> ^c	PEF1a	—	<i>Pyrus communis</i>	Monopoli (BA)
<i>Aureobasidium pullulans</i> ^c	PTfg1a	—	<i>Pittosporum</i> spp.	Ancona (AN)
<i>Aureobasidium pullulans</i> ^c	AF1a	—	<i>Prunus armeniaca</i> L.	Senigallia (AN)
<i>Aureobasidium pullulans</i> ^c	MF1a	—	<i>Morus</i> spp.	Ancona (AN)
<i>Aureobasidium pullulans</i> ^c	POMF1a	—	<i>Lycopersicum esculentum</i>	Monopoli (BA)
<i>Epicoccum nigrum</i> ^d	E (IPV)	—	<i>Zea mays</i>	Milano (MI)
<i>Epicoccum nigrum</i> ^e	E1	—	<i>Quercus cerris</i>	Firenze (FI)
<i>Epicoccum nigrum</i> ^e	E3	—	<i>Quercus cerris</i>	Firenze (FI)
<i>Epicoccum nigrum</i> ^f	EPV1	—	<i>Oryza sativa</i>	Pavia (PV)
<i>Epicoccum nigrum</i> ^f	EPV2	—	<i>Phragmites australis</i>	Pavia (PV)
<i>Epicoccum nigrum</i> ^g	IPOL1B1	—	<i>Malus mali</i>	Polcenigo (PN)
<i>Epicoccum nigrum</i> ^g	DC1Pa	—	<i>Malus mali</i>	Pantianicco (UD)
<i>Epicoccum nigrum</i> ^g	DD20.1En	—	<i>Malus mali</i>	Enemonzo (UD)
<i>Epicoccum nigrum</i> ^g	DD8En	—	<i>Malus mali</i>	Enemonzo (UD)
<i>Epicoccum nigrum</i> ^g	DDFannaS04	—	<i>Malus mali</i>	Fanna (PN)

^a Isolates of *Epicoccum nigrum* were further characterized into restriction fragment length polymorphism (RFLP) groups: isolates H2F1, E1, EPV1, IPOL1B1, DD20.1En, DD8En, and DDFannaS04 were designated group A; and R11T3, E (IPV), E3, EPV2, and DC1Pa were designated group B.

^b Isolated from grapevine in this study and maintained at the Department of "Biologia e Protezione delle Pianta", University of Udine, Italy.

^c Provided by G. Romanazzi, University of Ancona, Italy (18).

^d Provided by G. Assante, University of Milano, Italy.

^e Provided by S. Moricca, University of Firenze, Italy (37).

^f Provided by M. Rodolfi, University of Pavia, Italy.

^g Provided by S. Moruzzi and maintained at the Department of "Biologia e Protezione delle Pianta", University of Udine, Italy.

obtained nucleotide sequences were compared with those present in GenBank. The nucleotide sequences were deposited in GenBank (Table 1).

Primer design. DNA sequences of *A. pullulans* and *E. nigrum* strains obtained in this study were aligned with sequences of *A. pullulans* and *E. nigrum* strains and other closely related fungal species deposited in GenBank (2,39,40) using the NCBI *BLASTn* 2.2.20 program. The multiple alignments were generated with the output option "flat query-anchored with identities". Primers specific for *A. pullulans* were designed in ITS2 regions that were conserved among *A. pullulans* strains. Primers specific for *E. nigrum* were designed in ITS regions. Species-specific PCR primers were developed using software Primer3 (31). Primers for *A. pullulans* were named Apu(R6T4)F2 (5'-GCT ATG CTT GGT ATT GGG CGT-3')/Apu(R6T4)R2 (5'-AAA GGT TTC AGT CGG CAG AGT T-3'), and primers specific for *E. nigrum* were named Eni(H2F1)F1 (5'-CCT AGA GTT TGT AGA CTT CGG T-3')/Eni(H2F1)R1 (5'-GAC GTC GTC GTT ATG AGT G-3').

Specificity of the primers for *A. pullulans* and *E. nigrum* was assessed by PCR using 10 ng of purified DNA from *A. pullulans* (R6T4 isolate), *E. nigrum* (both H2F1 and R11T3 isolates), and 10 other grapevine endophytes. The specificity of the primers was also tested against other *A. pullulans* strains provided by G. Romanazzi (University of Marche, Italy) (18) and other *E. nigrum* strains maintained in our collection, or provided by M. Rodolfi (University of Pavia, Italy), G. Assante (University of Milano, Italy), and S. Moricca (University of Firenze, Italy) (37). These strains were isolated from several hosts with different geographic origins in Italy (Table 1). PCR reactions were identical to those described above for *A. pullulans*, but slightly adjusted for *E. nigrum* (0.3 μ M of forward primer and 1.3 mM $MgCl_2$). Amplification conditions included an initial denaturation of 2 min at 94°C followed by 40 cycles of 40 s at 94°C, 20 s at 60°C (for *E. nigrum*) or 30 s at 57°C (for *A. pullulans*), and 40 s at 72°C with a final extension of 72°C for 8 min. Five microliters of the amplicons were visualized after electrophoresis through a 1 or 1.5% agarose gel as previously described.

The sensitivity of the species-specific PCR assays was determined by testing 10-fold serial dilutions (50 ng/ μ l to 5 fg/ μ l) of genomic DNA preparations of *A. pullulans* isolate R6T4 and *E. nigrum* isolate H2F1. Serial dilutions were prepared either in molecular grade water or in 20 ng/ μ l of total DNA from endophyte-free micropropagated grapevine tissue (cv. Chardonnay) to confirm that the presence of plant DNA would not interfere with the PCR reactions. The micropropagated grapevines were provided by Massimo Gardiman

from Centro di Ricerca per la Viticoltura (CRA-VIT, Conegliano TV, Italy).

Detection of *A. pullulans* and *E. nigrum* in plant tissue. Reliability of the primers was verified on 44 grapevine samples (cultivars Prosecco and Merlot) collected in October 2007 and 2008 from six different vineyards located in Treviso (Montebelluna) and Pordenone (Caneva, Fiaschetti, Vigonovo, Nave, Fontanafredda) provinces in Italy, where recovery from Flavescence dorée is recurrent. Two 2-year-old shoots were collected for each grapevine that was either symptomatic for phytoplasma infection, healthy, or recovered. DNA was extracted from branch internodes following a modified version of the procedure described by Doyle and Doyle (8). Shoots were sterilized as described above, and the outer layer was aseptically removed to avoid contamination of the samples (14,35). One gram of vascular tissue from each shoot sample was ground in a sterile mortar with liquid nitrogen. Five milliliters of 2.5% CTAB buffer (100 mM Tris pH 8.0; 1.4 M NaCl; 50 mM EDTA pH 8.0; 2.5% CTAB; 1% PVP-40; 0.2% 2-mercaptoethanol added before use), previously heated in a water bath at 65°C, was added to the pulverized tissue. One milliliter of the homogenate was transferred to a sterile 2-ml tube, incubated at 65°C for 30 min, and extracted with an equal volume of chloroform:isoamyl alcohol (24:1). Following centrifugation at 13,000 rpm for 15 min at 4°C, the supernatant was transferred to another sterile microfuge tube, and 2/3 volume of cold isopropanol was added and gently mixed by inverting several times. Tubes were centrifuged at 13,000 rpm for

10 min at 4°C to pellet the DNA. The final steps in the procedure were conducted as previously described.

The presence of *A. pullulans* and *E. nigrum* was assessed by direct-PCR with species-specific primers following the PCR conditions previously described and using about 20 ng of total genomic DNA. In case of negative results, a nested-PCR procedure was adopted in which the first PCR run was performed with the universal fungal primer pair ITS1/ITS4 (15) followed by nested-PCR with specific primer pairs after 1:30 dilution. Direct-PCR with ITS1/ITS4 was performed with the reaction conditions described above with the following cycling parameters: an initial denaturation (2 min at 94°C) followed by 30 cycles of 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C, with a final extension of 72°C for 8 min. Nested-PCR reactions of 36 cycles with specific primer pairs were conducted following the conditions previously described. Purified DNA from *A. pullulans* R6T4 and *E. nigrum* H2F1 and R11T3 were used as reference strains.

To confirm the identity of the PCR products amplified with Apu(R6T4)F2/R2 and Eni(H2F1)F1/R1 primer pairs, restriction fragment length polymorphism (RFLP) analysis was performed. About 6 μ l of Apu(R6T4)F2/R2 products was digested individually with *Tru*II and *Bsu*RI (Fermentas, Lithuania) restriction enzymes, and 6 μ l of the Eni(H2F1)F1/R1 products was digested individually with *Tru*II and *Hin*6I (Fermentas, Lithuania). The digested products were then separated by electrophoresis through an 8% polyacrylamide gel in 1 $\times$ TBE buffer, stained, and visualized as above. The RFLP pro-

Fig. 1. Agarose gel of polymerase chain reaction (PCR) products obtained with species-specific primers **A**, Apu(R6T4)F2/R2 and **B**, Eni(H2F1)F1/R1. Aal: *Alternaria alternata*; Apu: *Aureobasidium pullulans*; Asa: *Arthrinium sacchari*; Eni: *Epicoccum nigrum*; Nor: *Nigrospora oryzae*; Pho: *Phoma* sp.; Phom: *Phomopsis* sp.; Sco: *Schizophyllum commune*; Ssc: *Sclerotinia sclerotiorum*; Lch: *Lepidosphaerulina chartarum*. M: 1 kb DNA ladder (bottom to top: 250 to 10,000 bp).

files obtained from grapevine samples were compared with those from the *A. pullulans* R6T4 and *E. nigrum* H2F1 and R11T3 reference strains. The same RFLP analysis was performed on PCR products obtained with species-specific primers from all *A. pullulans* and *E. nigrum* isolates.

RESULTS

Fungal isolates. Ninety-four fungal endophytes, representing 26 morphotypes, were obtained from leaf and shoot materials sampled from 15 grapevine plants. Thirteen endophytes isolated exclusively from grapevines that had shown phytoplasma symptoms, but then became asymptomatic (recovered plants), were retained (Table 1). All fungal genera were identified based on colony morphology on PDA medium and microscopic characteristics. The identifications were confirmed by the Centraalbureau voor Schimmelcultures Institute (the Netherlands). The 13 fungal endophytes belonged to the following genera: *Alternaria*, *Aureobasidium*, *Arthrinium*, *Epicoccum*, *Leptosphaerulina*, *Nigrospora*, *Phoma*, *Phomopsis*, and *Schizophyllum*. One isolate of *A. pullulans* (R6T4) and two isolates of *E. nigrum* (H2F1 and R11T3) were included. Voucher specimens have been deposited and stored in the culture collection of the Department of "Biologia e Protezione delle Piante", University of Udine, under codes reported in Table 1.

Amplification and sequencing. PCR with the universal primers ITS1 and ITS4 using high-molecular-weight DNA prepared from the 13 grapevine endophytes produced an amplicon of approximately 500 to 600 bp. The PCR products were sequenced and the sequence data were used to support identification by database

comparison. Isolate H2F1 had a sequence similarity of 99% with *E. nigrum* strain CBS 318.83 (GenBank accession no. AJ279448), and R11T3 had a sequence similarity of 100% with *E. nigrum* strains ATCC 62191 (GenBank accession no. FJ424241) and ATCC 32948 (GenBank accession no. FJ424240). The two *E. nigrum* strains H2F1 and R11T3 shared an ITS sequence similarity of 98%. Isolate R6T4 had a 100% sequence similarity with *A. pullulans* var. *pullulans* strains CBS 584.75 (GenBank accession no. FJ150906), CBS 100524 (GenBank accession no. FJ150905), CBS 146.30 (GenBank accession no. FJ150902), and CBS 100280 (GenBank accession no. FJ150910).

Primer specificity and sensitivity. Primer pair Apu(R6T4)F2/R2 yielded an amplicon of the predicted size (135 bp) from extracted DNA of *A. pullulans* R6T4 isolated from grapevine and other *A. pullulans* strains from different sources (Table 1, Fig. 1A). Primer pair Eni(H2F1)F1/R1 yielded an amplicon of the predicted size (435 bp) from extracted DNA of *E. nigrum* H2F1 and R11T3 strains isolated from grapevine and other *E. nigrum* strains obtained from different sources (Table 1, Fig. 1B). DNA from other grapevine fungal endophytes did not amplify.

PCR amplification of the dilution series (ranging from 50 ng/μl to 5 fg/μl) of purified DNA from *A. pullulans* R6T4 produced a visible product with 50 pg/μl when dilutions were prepared either with 20 ng/μl of total DNA from endophyte-free grapevine or with molecular grade water (Fig. 2A and B). PCR amplification of DNA from *E. nigrum* H2F1 produced a visible product with 5 pg/μl when dilutions were prepared with 20 ng/μl of total DNA from endophyte-free grapevine (Fig. 2C) and with 500 fg/μl when dilutions were prepared with molecular grade water (Fig. 2D).

Detection of *A. pullulans* and *E. nigrum* in plant tissue. PCR of all of the DNA samples extracted from grapevine

tissue yielded the expected PCR product for *A. pullulans*, and 86.4% of the samples yielded the expected amplicon for *E. nigrum* (Fig. 3A and B). The two endophytes were obtained from grapevines that were symptomatic or asymptomatic for FD or from plants that had recovered from the disease. In direct-PCR, almost 100% of shoot samples (29/30) collected from different vineyards in Pordenone Province tested positive for the presence of *A. pullulans* (the remaining sample was positive in nested-PCR); whereas 20 out of the 30 samples tested positive for *E. nigrum*. Six out of 30 samples remained negative after nested-PCR with primers specific for *E. nigrum*. Eight out of 14 and nine out of 14 of the shoot samples from Montebelluna grapevines were positive for *A. pullulans* and *E. nigrum*, respectively, using direct-PCR. Nested-PCR successfully detected the fungi in the negative reactions by direct PCR.

RFLP analyses of specific PCR products were conducted to verify amplicon identity. RFLP profiles obtained with *Tru1I* and *Bsu1I* on Apu(R6T4)F2/R2 PCR products from grapevine samples were identical to each other and to the *A. pullulans* R6T4 profile (Fig. 4A and B). RFLP analyses with *Tru1I* and *Hin6I* on Eni(H2F1)F1/R1 PCR products differentiated the two *E. nigrum* isolates from grapevines and all other isolates (Fig. 4C and D). The *E. nigrum* isolates were separated into two different groups (A and B) (Table 1). Group A was detected in 26 of 38 grapevine samples, group B was detected in 1 of 38 samples, and both groups were detected in 11 samples (Fig. 4C and D). The majority (78%) of healthy and recovered plants hosted *E. nigrum* strains of RFLP group A, while the remaining plants hosted both groups. Group B isolates were not detected in any of the healthy or recovered plants. In diseased plants, *E. nigrum* RFLP groups A, A+B, and B were detected in 53, 40, and 7% of the samples, respectively.

Fig. 2. Sensitivity of polymerase chain reaction (PCR) assays with primer pair Apu(R6T4)F2/R2 at 10-fold serial dilutions **A**, from 50 ng/μl to 5 fg/μl in sterile water and **B**, in 20 ng/μl of endophyte-free grapevine total genomic DNA; and with primers Eni(H2F1)F1/R1 at 10-fold serial dilutions **C**, from 50 ng/μl to 5 fg/μl in sterile water and **D**, in 20 ng/μl of endophyte-free grapevine total genomic DNA. M: 1 kb DNA ladder.

Fig. 3. Polymerase chain reaction (PCR) with species-specific primers **A**, Apu(R6T4)F2/R2 and **B**, Eni(H2F1)F1/R1 with DNA extracted from grapevine shoot tissue. Reference strains H6T4 for *Aureobasidium pullulans* and H2F1 and R11T3 for *Epicoccum nigrum*. Lanes 1 to 12: grapevine shoot samples collected in different locations in northeastern Italy. Lane M: 1 kb DNA ladder.

DISCUSSION

Specific primers were developed here for the detection of *A. pullulans* and *E. nigrum* in grapevine tissues. These endophytes were present in high frequency in grapevines in northeastern Italy. The occurrence of both *A. pullulans* and *E. nigrum* in the same tissue has been reported (21,30), indicating that these endophytes may coexist in a host (16). The specific primers amplified DNA from all tested *E. nigrum* and *A. pullulans* strains, including isolates from other sources. The primers did not amplify DNA from other fungal endophytes that are usually encountered during isolation attempts from grapevine tissues. PCR assays of both targets were highly sensitive; picogram quantities of DNA were suitable templates for *A. pullulans*, while femtogram quantities of DNA could be amplified for *E. nigrum*. The efficiency of the two species-specific PCR assays was not influenced by the presence of host plant DNA. Where the species-specific primers did not amplify target DNA, nested PCR was successfully used.

Two strains of *E. nigrum* were distinguished in this study. High genetic variability in *E. nigrum* populations unrelated to geographic origin has been well documented (2,3). In grapevines in Italy, one group was much more common, but both genotypes occasionally occurred in the same plant. This is noteworthy because the different distribution of *E. nigrum* RFLP groups was apparently associated with a particular disease status. However, the role of endophytic microorganisms in relation to phytoplasma infection and to the phenomena of spontaneous recovery from disease symptoms has still to be investigated.

Endophytic fungi may influence the microbial community within and on grapevines. For example, they may beneficially retard the establishment of wound pathogens such as *B. cinerea* (9), and some strains of *A. pullulans* are able to reduce ochratoxin accumulation in wine grapes by inhibiting *Aspergillus* spp. (6). Since microorganisms in grapes may influence wine quality, the association of endophytes such as *A. pullulans* and *E. nigrum* may play an important role in wine processing.

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Fig. 4. Restriction fragment length polymorphism (RFLP) analyses with restriction enzymes **A**, *Tru1I* and **B**, *BsuRI* on polymerase chain reaction (PCR) products Apu(R6T4)F2/R2; **C**, *Tru1I* and **D**, *Hin6I* on PCR products Eni(H2F1)F1/R1. RFLP products were separated by electrophoresis through an 8% polyacrylamide gel. Apu: *Aureobasidium pullulans*, Eni: *Epicoccum nigrum*. Reference strains are H6T4 for *A. pullulans* and H2F1 (RFLP group A) and R11T3 (RFLP group B) for *E. nigrum*. Lanes 1 to 11: grapevine shoot samples collected in different locations in northeastern Italy. Lane S: Φ 174 *HaeIII* digested (New England BioLabs, USA; fragment sizes [bp] from top to bottom: 1,353, 1,078, 872, 603, 310, 281, 271, 234, 194, 118, 72).

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