Characterization of Fungi Associated With Wood Decay of Tree Species and Grapevine in Greece

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Abstract

A two-year survey was conducted to identify fungi associated with wood decay in a range of tree species and grapevine. Fifty-eight fungal strains isolated from plants of 18 species showing typical wood decay symptoms were characterized by morphological, physiological, and molecular analyses. By 5.8S rRNA gene-ITS sequencing analysis, these isolates were classified into 25 distinct operational taxonomic units, including important phytopathogenic species of the phyla Pezizomycotina and Agaricomycotina, such as *Fomitiporia*, *Inonotus*, *Phellinus*, *Inocutis*, *Fuscoporia*, *Trametes*, *Fusarium*, *Eutypa*, *Phaeomoniella*, *Phaeoacremonium*, and *Pleurostomophora* spp. The white rot basidiomycetes *Fomitiporia mediterranea* (20 isolates, 34.5%) and *Inonotus hispidus* (6 isolates, 10.3%) were the most prevalent. Pathogenicity tests revealed for the first time that certain fungal species of the genera *Fomitiporia*,

Inonotus, *Phellinus*, *Pleurostomophora*, and *Fusarium* caused wood infection of various tree species in Greece and worldwide. To the best of our knowledge, this is the first report of *F. mediterranea* as the causal agent of wood decay in pear, pomegranate, kumquat, and silk tree. This is also the first record of *Inonotus hispidus*, *Phellinus pomaceus*, *Pleurostomophora richardsiae*, and *Fusarium solani* in apple, almond, avocado, and mulberry tree, respectively, whereas *P. richardsiae* was associated with wood infection of olive tree for the first time in Greece. Cross pathogenicity tests with *F. mediterranea* strains originated from grapevine applied on other woody hosts and from olive on grapevine demonstrated partial host specificity of the fungus. The potential of *F. mediterranea* to transinfect hosts other than those originated, along with the host range extension of the fungus, is discussed.

Wood decay diseases are caused by a wide range of fungi, affecting several tree species and grapevine worldwide (Fischer 2006; Roccotelli et al. 2014). Most wood-degrading microorganisms are white- or brown-rot basidiomycetes that can decompose the lignified cell walls of the woody plants (Blanchette 1991). These fungi can cause decay and discoloration of wood tissues, in particular in the trunk, branches, and roots. Although only a few of these fungi are directly responsible for tree mortality, wood-rot basidiomycetes lead to structural deterioration of woody tissues and economic damage due to the premature aging, decline, and substantially reduced crop productivity (Butin 1995; Lonsdale 1999). Apart from wood decay Basidiomycota, other fungi like *Fusarium*, *Phaeoacremonium*, *Phaeomoniella*, and *Pleurostomophora* strains have been reported as wood-penetrating agents of several plant species (Carlucci et al. 2013; Graham et al. 1985; Mugnai et al. 1999). Most of them penetrate through wounds, although several wood decaying species can invade the uninjured surface directly (Roccotelli et al. 2014).

A well-known basidiomycete associated with wood decay of grapevine (*Vitis vinifera* L.) in European grapevine-growing countries is *Fomitiporia mediterranea* M. Fisch. (Fischer 2002). This fungus has also been reported as the causal agent of heart wood rot in kiwifruit (*Actinidia deliciosa* L.) (Di Marco et al. 2004; Elena and Paplomatas 2002), olive (*Olea europaea* L.) (Fischer 2006; Paplomatas et al. 2006a, b), orange (*Citrus sinensis* L.), lemon (*C. limon* L.), mandarin (*C. reticulata* L.), and sour orange (*C. aurantium* L.) trees (Elena et al. 2006; Roccotelli et al. 2014). In addition, *F. mediterranea* has been detected in plane trees (*Platanus × acerifolia*) affected by canker diseases (Pilotti et al. 2005) and hazelnut trees (*Corylus avellana* L.) showing decay and decline (Pilotti et al. 2010). Moreover, the same species has been found on boxelder (*Acer negundo* L.), crape myrtle (*Lagerstroemia indica* L.), bay tree (*Laurus*

nobilis L.), common privet (*Ligustrum vulgare* L.), evergreen oak (*Quercus ilex* L.), and black locust (*Robinia pseudoacacia* L.) (Fischer 2006).

Pathogenicity tests revealed that citrus-originated *F. mediterranea* isolates were pathogenic on this particular host while isolates of this fungus from grapevine and kiwi were nonpathogenic to citrus trees (Elena et al. 2006; Roccotelli et al. 2014), suggesting either host specificity or involvement of host-specific cryptic species (Pilotti et al. 2010). On the other hand, *F. mediterranea* is not limited to grapevines, but also occurs in other tree species, indicating the potential role of other hosts (except grapevine) to serve as inoculum sources for the fungus to infect grapevines (Surico et al. 2006; 2008). However, the hypothesis that *F. mediterranea* isolates from non-*Vitis* hosts are pathogenic on grapevine has not been verified yet (Surico et al. 2006).

In the last decades, several tree species and grapevine with typical wood decay symptoms have been brought to phytopathological laboratories, demanding accurate disease diagnosis. Besides grapevine, olive, citrus, apple (*Malus domestica* L.), and pomegranate (*Punica granatum* L.) trees are plant species with high decline and/or dieback disease incidence, followed by other cultivated and ornamental species that are occasionally observed to exhibit such decay. For instance, the extensive damage of olive trees in Messinia and Arkadia prefectures in Greece and elsewhere, caused by *F. mediterranea* and various species primarily in the Botryosphaeriaceae, has been previously reported (Carlucci et al. 2013; Paplomatas et al. 2006a, b; Úrbez-Torres et al. 2013). Recently, serious damage in olive orchards has also been observed in Evros prefecture and in Crete, southern Greece (present study). Moreover, citrus wood rot is commonly found in old citrus trees throughout the globe and is due to the infection caused by endemic wood decay species (Bigelow et al. 1996; 1998; Elena et al. 2006; Mayorquin et al. 2016; Roccotelli et al. 2014). However, in most cases, the causal agents of these wood-decay-associated diseases remained unidentified. In addition, the reason for the significant increase of the disease incidence in woody hosts other than grapevine has not been extensively investigated.

Although wood decay fungi are considered as important primary decomposers in forest habitats, they can also lead to economic damage

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in cultivated fields, owing to tree decline and death (Butin 1995; Elena et al. 2006; Lonsdale 1999). Therefore, the main objectives of this work were: (i) to characterize fungi associated with wood decay of tree species and grapevine in Greece; (ii) to test the potential of *F. mediterranea* originated from grapevine to transinfect other hosts; and (iii) to investigate whether *F. mediterranea* from other plant species is also capable of infecting grapevine.

Materials and Methods

Sampling. Wood samples with typical decay symptoms as well as fruiting bodies of potential wood-decay fungi were collected over a 2-year period (2013–2014), during field surveys in orchards and boulevards located mostly in Crete (southern Greece). Some samples from olive orchards in Peloponnese (south-central Greece) and Thrace (northeastern Greece) were also collected and analyzed (Table 1). Woody samples were mainly received during the pruning period (November to February) and consisted of pieces of wood with decay symptoms from trunks and large branches of diseased trees.

Isolation of fungi. To obtain the potential causal agents of wood decay, samples were cut crosswise and lengthwise and wood fragments (~10 cm in length) were surface-disinfected by 95% ethyl alcohol for 2 min. For each sample, 10 xylem chips from the center of the white- or brown-colored spongy area and 10 additional chips from the surrounding brown, hard, necrotic wood zone were aseptically placed onto acidified potato dextrose agar (PDA). In the case of fruiting bodies, tissues were surface-disinfected by 95% ethyl alcohol and small pieces of internal tissues were transferred onto acidified PDA. Plates were incubated at 24°C in the dark for 3 weeks. The emerging fungal colonies that grew out of tissue excisions were examined visually and under a light microscope. The fungi isolated consistently and steadily from each sample were transferred onto new PDA plates. For short-term storage, all fungal isolates were maintained on PDA at 4°C.

Morphological and cultural characterization of isolates. Mycelial growth of the wood decay strains isolated was examined by transferring PDA agar plugs of actively growing mycelium (7 mm in diameter) into new PDA plates. Plates (three per isolate) were incubated at 24°C in the dark and the colonies' diameter was measured at day 3, 7, 10, 14, 17, and 21 or stopped earlier, when the plate surface was completely covered. The growth rate of fungal isolates was expressed in mm/day. At the end of the incubation period, colony characteristics (color, mycelium, colony type, and shape) were observed. Available conidia and hyphal features (color, shape, presence, or absence of septum and clamp) were also recorded. Pigmentation of *F. mediterranea* isolates was visually assessed by recording the appearance of pigment on the underside and upper side of PDA plates. All *F. mediterranea* cultures were scored in a five-point evaluation scale regarding the percentage of pigmented area with reference to the entire culture surface, where: 0 = white colony; 1 = 1 to 25%; 2 = 26 to 50%; 3 = 51 to 75%; and 4 = 76 to 100% of culture surface pigmented. Isolates with an average pigment grade of ≥ 2.0 on the underside of plates were classified into type S (staining of the culture medium), whereas the rest were fallen into type B (bleaching of the culture medium) (Fischer 2006). Moreover, mycelium abundance of *F. mediterranea* isolates was based on a visual scale from 0 to 4, where: 0 = yeast-like appearance; 1 = sparse development of aerial hyphae; 2 = cottony mycelial culture; 3 = modest woolly appearance; and 4 = intensive woolly mycelium. To evaluate and describe the microscopic features of the colonies, actively growing mycelia from culture's periphery were prepared and microscopic observations (30 readings per isolate) were carried out.

Sequencing of internal transcribed spacer regions. To molecularly characterize fungal isolates, the internal transcribed spacer (ITS) regions of ribosomal RNA gene were sequenced. Fungal DNA was extracted from 150 to 200 mg mycelium (fresh weight) by using the NucleoSpin Plant II genomic DNA extraction kit (Macherey-Nagel, Düren, Germany) and amplified with the use of universal primers ITS1 and ITS4 (White et al. 1990). All PCR assays were carried out in an MJ Research PTC-200 Gradient thermocycler, by the use of the KAPA Taq ReadyMix PCR kit (Kapa Biosystems, Wilmington, MA, USA). PCR performance included denaturation at 94°C for 3 min;

followed by 34 cycles of 1 min of denaturation at 94°C, 40 s of annealing at 54°C, and 40 s of extension at 72°C; and a final extension step at 72°C for 15 min. Amplified products were purified with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) and sequenced by both forward and reverse primers at MacroGen Europe (Amsterdam). The CAP3 Sequence Assembly Program was used to edit the raw sequencing data (Huang and Madan 1999) and the sequences assembled were deposited in GenBank as Accession Nos. KX881579 to KX881637. The latter were aligned by the Clustal Omega platform (Sievers et al. 2011) and estimation of sequence relatedness was calculated by the Tamura-Nei maximum-likelihood algorithm (Tamura and Nei 1993). Trees construction was carried out by MEGA (Tamura et al. 2013) through topology estimation by the neighbor-joining method (Saitou and Nei 1987) and bootstrap analysis of 1000 trees.

Identification and characterization of isolates. Morphological and physiological features along with ITS sequences were used to characterize the fungal isolates obtained from the diseased tissues. Molecular identification of the wood decay isolates was carried out using the BLASTn option at NCBI and comparing their ITS sequences with those from GenBank database. *Fomitiporia mediterranea* strain K.E. Fomit4, belonging to Benaki Phytopathological Institute Collection (BPIC), was provided by Benaki Phytopathological Institute, Kifissia, Athens, Greece, and served as the reference strain.

Pathogenicity and cross-pathogenicity tests. In order to confirm pathogenicity, *F. mediterranea* isolates A2LKP1, E3LK1, R1LK1, K1KP1, AXL1LK1, and AKKON1LK1 originated from grapevine, olive, pomegranate, kumquat (*Fortunella japonica* Swingle), pear (*Pyrus communis* L.), and silk tree (*Albizia julibrissin* Durazz), respectively, were used to inoculate healthy plants of the same species. In addition, the pathogenicity of *Pleurostomophora richardsiae* (Nannf.) L. Mostert, W. Gams & Crous isolates E1PK1 and AV1LK3 originated from olive and avocado (*Persea americana* Mill.), respectively, was tested on healthy plants of the same species. Moreover, the isolates A2LP1 (*Phaeomoniella chlamydospora* W. Gams, Crous, M.J. Wingf. & Mugnai), MOUR2P3 (*Fusarium solani* (Mart.) Sacc.), AM2XL1 (*Phellinus pomaceus* (Pers.) Maire), and MP1 (*Inonotus hispidus* (Bull.) P. Karst.) originated from grapevine, mulberry (*Morus platani-folia* L.), almond (*Prunus dulcis* Mill.), and apple tree, respectively, were used to inoculate healthy plants of the same species. Furthermore, cross-pathogenicity tests were conducted to evaluate the pathogenicity of *F. mediterranea* isolate A2LKP1 from grapevine on olive, pomegranate, kumquat, almond, and apple trees, as well as to test whether *F. mediterranea* isolate E3LK1 from olive is also pathogenic on grapevine.

Nine 2-year-old trees of each plant species (except the silk tree) were inoculated on April 2014. Artificial inoculation was performed by making a 5.5-mm $\times$ 10.0-mm hole in the trunk through the use of a Black & Decker EPC 12 drilling apparatus, inserting one 5.5-mm-diameter mycelial disc taken from the margin of a 15-day-old PDA culture, sealing the hole with vaseline and fastening the area with paper tape to protect the inoculum. Nine additional trees of each plant species were mock-inoculated with sterilized PDA and served as controls. Trees were placed under ambient conditions, in an adjacent experimental field of Institute of the Olive Tree, Subtropical Crops, and Viticulture in Chania, Crete, Greece. Each treatment consisted of three trees and the experiment was replicated three times (nine trees per treatment in total).

In April 2015, nine 8-month-old silk tree plants were artificially inoculated with *F. mediterranea* isolate AKKON1LK1 originated from a 20-year-old silk tree, as follows: two 5.5-mm-diameter mycelial discs taken from the margin of a 15-day-old PDA culture were placed in a 1.5-ml Eppendorf tube and the cover was removed; then, the apical end of the stem was cut with sterilized pruning scissors and the tube was adjusted onto stem, so as the fresh pruning wound to be in touch with the mycelial discs. Nine additional plants were mock-inoculated with sterilized PDA and served as controls. The tube together with the stem were fastened with cellophane membrane and covered with adhesive paper tape to protect the inoculum. At the same time, the abovementioned technique was also used to test the pathogenicity of *F. mediterranea* isolate E3LK1 from olive on nine

1-year-old grapevine plants. Due to the low extension of wood discoloration in pomegranate and kumquat trees inoculated by employing the trunk-drilling technique, 10 branches with a diameter of 6 to 8 mm from each pomegranate, kumquat, and olive tree were artificially inoculated with *F. mediterranea* isolates R1LK1, K1KP1, and E3LK1, respectively, to verify the efficiency of the pruning-

wound inoculation method. Branches mock-inoculated with sterilized PDA discs served as controls.

Disease assessment. Trunk-drilled inoculated trees were cut above soil level in November 2014 (7-months post inoculation) and longitudinal and transverse sections of trunks were performed to measure the extension of wood tissue symptoms above and below

Table 1. Morphological and physiological features of the fungal isolates (mean $\pm$ SE) analyzed in the present study, along with their host, geographic origin, and isolation year

Isolate ^w	Host	Substrate ^x	Location	Year	Growth rate (mm/day) ^y	Colony color	Conidial dimensions (μ m) ^z	Hyphae width (μ m)
E1KP1	Olive tree	Wood	Nileas, Kalamata	2013	3.92 $\pm$ 0.05	yellow to pale brown	nf	2.43 $\pm$ 0.11
E1PK1	Olive tree	Wood	Nileas, Kalamata	2013	1.81 $\pm$ 0.16	light to dark brown	4.79 ($\pm$ 0.25) $\times$ 2.41 ($\pm$ 0.07)	1.98 $\pm$ 0.10
E2mXL1	Olive tree	Fruiting body	Kissamos, Chania	2014	4.51 $\pm$ 0.07	white to pale yellow	nf	2.67 $\pm$ 0.22
E2LK3	Olive tree	Wood	Kissamos, Chania	2014	4.22 $\pm$ 0.16	white to pale yellow	nf	2.76 $\pm$ 0.21
E3LK1	Olive tree	Wood	Kissamos, Chania	2014	3.44 $\pm$ 0.05	white to yellow	nf	2.54 $\pm$ 0.13
E5LK1	Olive tree	Fruiting body	Platanias, Chania	2014	1.89 $\pm$ 0.13	pale brown to golden	nf	2.85 $\pm$ 0.10
E6LK1	Olive tree	Wood	Apokoronas, Chania	2014	4.39 $\pm$ 0.04	white to golden	nf	2.56 $\pm$ 0.12
E7LK1	Olive tree	Wood	Apokoronas, Chania	2014	4.39 $\pm$ 0.04	white to pale yellow	nf	2.72 $\pm$ 0.16
E9mLK1	Olive tree	Fruiting body	Makri, Alexandroupolis	2014	6.23 $\pm$ 0.16	pale brown	nf	2.47 $\pm$ 0.14
E10mLK1	Olive tree	Fruiting body	Makri, Alexandroupolis	2014	4.29 $\pm$ 0.03	white to pale golden	nf	2.68 $\pm$ 0.14
E11mLK1	Olive tree	Fruiting body	Makri, Alexandroupolis	2014	3.10 $\pm$ 0.05	white to golden	nf	2.35 $\pm$ 0.08
A2LKP1	Grapevine	Wood	Episkopi, Iraklio	2014	3.41 $\pm$ 0.11	white to golden	nf	2.78 $\pm$ 0.15
A2LP1	Grapevine	Wood	Episkopi, Iraklio	2014	1.14 $\pm$ 0.03	olive-green	4.53 ($\pm$ 0.20) $\times$ 1.88 ($\pm$ 0.08)	2.64 $\pm$ 0.11
A3LKP2	Grapevine	Wood	Agios Thomas, Iraklio	2014	4.60 $\pm$ 0.17	white to pale golden	nf	2.84 $\pm$ 0.19
A4LKP1	Grapevine	Wood	Garipa, Iraklio	2014	4.08 $\pm$ 0.13	white to golden	nf	2.75 $\pm$ 0.20
A5LP1	Grapevine	Wood	Kolimpari, Chania	2014	1.24 $\pm$ 0.01	dark olive-green	4.36 ($\pm$ 0.20) $\times$ 2.04 ($\pm$ 0.08)	2.71 $\pm$ 0.10
A5LKK1	Grapevine	Wood	Kolimpari, Chania	2014	0.84 $\pm$ 0.04	pale orange	5.50 ($\pm$ 0.25) $\times$ 2.59 ($\pm$ 0.13)	2.65 $\pm$ 0.09
A5LV1	Grapevine	Wood	Kolimpari, Chania	2014	3.50 $\pm$ 0.08	white to pale olive-green	nf	2.70 $\pm$ 0.13
P1KP1	Orange tree	Wood	Platanias, Chania	2014	4.67 $\pm$ 0.08	white to pale golden	nf	3.03 $\pm$ 0.20
P2KP1	Orange tree	Wood	Apokoronas, Chania	2014	4.38 $\pm$ 0.05	white to pale golden	nf	2.75 $\pm$ 0.12
R1LK1	Pomegranate tree	Wood	Agios Thomas, Iraklio	2014	3.82 $\pm$ 0.14	white to pale golden	nf	2.63 $\pm$ 0.19
R1K1	Pomegranate tree	Wood	Agios Thomas, Iraklio	2014	3.25 $\pm$ 0.04	pale yellow	6.32 ($\pm$ 0.30) $\times$ 2.24 ($\pm$ 0.09)	2.49 $\pm$ 0.09
R1LKP1	Pomegranate tree	Wood	Agios Thomas, Iraklio	2014	3.95 $\pm$ 0.03	white to pale golden	nf	2.28 $\pm$ 0.10
R1YKL1	Pomegranate tree	Wood	Agios Thomas, Iraklio	2014	3.63 $\pm$ 0.13	beige to olive-green	9.23 ($\pm$ 0.66) $\times$ 4.98 ($\pm$ 0.14)	4.88 $\pm$ 0.34
R2LK1	Pomegranate tree	Wood	Kalykas, Chania	2014	4.78 $\pm$ 0.02	white to yellow	nf	3.04 $\pm$ 0.20
K1KP1	Kumquat tree	Wood	Agrokipio, Chania	2014	3.71 $\pm$ 0.11	white to pale golden	nf	2.69 $\pm$ 0.16
K1LVK1	Kumquat tree	Wood	Agrokipio, Chania	2014	5.20 $\pm$ 0.02	white to light olive-green	nf	3.88 $\pm$ 0.32
K1LK1	Kumquat tree	Wood	Agrokipio, Chania	2014	2.77 $\pm$ 0.21	white to olive-green	4.78 ($\pm$ 0.16) $\times$ 2.06 ($\pm$ 0.09)	2.67 $\pm$ 0.07
K1AL1	Kumquat tree	Wood	Agrokipio, Chania	2014	10.70 $\pm$ 0.11	white	nf	4.90 $\pm$ 0.40
AKKON1LK1	Silk tree	Wood	Iraklio	2014	4.08 $\pm$ 0.04	white to pale golden	nf	2.74 $\pm$ 0.13
MP1	Apple tree	Wood	Lasithi plateau	2013	3.32 $\pm$ 0.20	white to golden	nf	2.99 $\pm$ 0.16
MA2	Apple tree	Wood	Lasithi plateau	2013	2.06 $\pm$ 0.08	white to pale pink	6.41 ($\pm$ 0.25) $\times$ 2.35 ($\pm$ 0.11)	2.99 $\pm$ 0.10
MK1	Apple tree	Wood	Lasithi plateau	2013	1.95 $\pm$ 0.29	white to golden	nf	3.29 $\pm$ 0.16
MSP1	Apple tree	Wood	Lasithi plateau	2013	2.94 $\pm$ 0.02	white to pale pink	6.53 ($\pm$ 0.28) $\times$ 2.68 ($\pm$ 0.10)	2.72 $\pm$ 0.08
MIL2mLK1	Apple tree	Fruiting body	Lasithi plateau	2014	5.10 $\pm$ 0.11	white to pale yellow	nf	4.55 $\pm$ 0.23
MIL2LKP1	Apple tree	Wood	Lasithi plateau	2014	3.04 $\pm$ 0.20	white to golden	nf	4.48 $\pm$ 0.23
MOUR1P1	Mulberry tree	Wood	Lasithi plateau	2013	2.91 $\pm$ 0.21	white to pink	6.78 ($\pm$ 0.25) $\times$ 3.63 ($\pm$ 0.12)	3.96 $\pm$ 0.20
MOUR2P3	Mulberry tree	Wood	Agrokipio, Chania	2014	5.15 $\pm$ 0.05	white to pink	7.32 ($\pm$ 0.45) $\times$ 4.30 ($\pm$ 0.17)	4.24 $\pm$ 0.17
MOUR2A1	Mulberry tree	Wood	Agrokipio, Chania	2014	7.77 $\pm$ 0.06	pale pink	11.78 ($\pm$ 1.53) $\times$ 4.73 ($\pm$ 0.17)	3.08 $\pm$ 0.14
MOUR3mKL1	Mulberry tree	Fruiting body	Iraklio	2014	5.29 $\pm$ 0.04	white to pale golden	nf	4.11 $\pm$ 0.21
MOUR4mLK1	Mulberry tree	Fruiting body	Therissos, Chania	2014	4.57 $\pm$ 0.13	white to yellow	nf	4.36 $\pm$ 0.26
AM1KL1	Almond tree	Wood	Apokoronas, Chania	2014	10.98 $\pm$ 0.02	light olive-green	4.78 ($\pm$ 0.17) $\times$ 2.48 ($\pm$ 0.10)	3.49 $\pm$ 0.20
AM1mXL1	Almond tree	Fruiting body	Apokoronas, Chania	2014	4.48 $\pm$ 0.28	dark golden	nf	2.61 $\pm$ 0.13
AM2XL1	Almond tree	Wood	Apokoronas, Chania	2014	4.51 $\pm$ 0.19	dark golden	nf	2.67 $\pm$ 0.10
AL1mLK1	Tamarisk tree	Fruiting body	Nea hora, Chania	2014	3.08 $\pm$ 0.10	pale pink to yellow	nf	3.13 $\pm$ 0.12
MEL1LK1	Chinaberry tree	Wood	Nea hora, Chania	2014	3.16 $\pm$ 0.02	pale yellow	6.08 ($\pm$ 0.23) $\times$ 2.23 ($\pm$ 0.05)	2.57 $\pm$ 0.10
D1LV1	Loquat tree	Wood	Agios Thomas, Iraklio	2014	16.74 $\pm$ 0.02	white	nf	3.48 $\pm$ 0.19
AV1LK3	Avocado tree	Wood	Nea hora, Chania	2014	2.29 $\pm$ 0.27	light to dark brown	4.28 ($\pm$ 0.17) $\times$ 2.83 ($\pm$ 0.08)	2.40 $\pm$ 0.09
AGK2L1	Jerusalem sage	Wood	Apokoronas, Chania	2014	6.37 $\pm$ 0.21	white	nf	2.70 $\pm$ 0.12
AGK2mL1	Jerusalem sage	Fruiting body	Apokoronas, Chania	2014	6.01 $\pm$ 0.42	white	nf	2.64 $\pm$ 0.12
AXL1LK1	Pear tree	Wood	Lasithi plateau	2014	4.05 $\pm$ 0.29	white to golden	nf	2.46 $\pm$ 0.15
AXL1SP1	Pear tree	Wood	Lasithi plateau	2014	2.43 $\pm$ 0.09	dark olive-green to gray	nf	3.11 $\pm$ 0.16
KAR1mLP1	Walnut tree	Fruiting body	Lasithi plateau	2014	3.42 $\pm$ 0.58	white to golden	nf	3.58 $\pm$ 0.20
KAR1LEN1	Walnut tree	Wood	Lasithi plateau	2014	4.11 $\pm$ 0.10	white	nf	2.63 $\pm$ 0.14
KAR1LKON1	Walnut tree	Wood	Lasithi plateau	2014	5.11 $\pm$ 0.15	white	nf	3.22 $\pm$ 0.20
PLAT1mL1	Plane tree	Fruiting body	Chania	2014	8.35 $\pm$ 0.11	white	nf	3.10 $\pm$ 0.17
GKFR1V3	Grapefruit tree	Wood	Agrokipio, Chania	2014	7.48 $\pm$ 0.07	white to light pink	nf	3.64 $\pm$ 0.26
ROMP1mLP1	Black locust tree	Fruiting body	Chania	2014	2.06 $\pm$ 0.05	white to golden	nf	3.35 $\pm$ 0.21
K.E. Fomit4	Olive tree	Wood	Messinia, Kalamata	2001	5.13 $\pm$ 0.02	white to golden	nf	3.06 $\pm$ 0.28

^w Codes of fungal isolates obtained from various hosts with wood decay symptoms. *Fomitiporia mediterranea* strain K.E. Fomit4, belonging to Benaki Phytopathological Institute Collection (BPIC), was provided by Benaki Phytopathological Institute, Kifissia, Athens, Greece, and served as the reference strain.

^x Substrate (wood or fruiting body) where fungal strains were isolated.

^y Isolates were grown on PDA, at 24°C in the dark and the colonies' diameter was measured at day 3, 7, 10, 14, 17, and 21 or stopped earlier, when Petri dish was completely covered.

^z nf indicates that isolates did not form conidia within the 21-day incubation period.

the inoculation point. Likewise, pruning-wound inoculated stems and branches were cut in November 2015 (7-months post inoculation), the plastic tube was removed, and longitudinal sections through the pruning-wound surface were carried out to measure the extension of symptomatic wood below the inoculation point. To verify the presence of the applied causal agent in diseased wood tissues and confirm pathogenicity in each plant-pathogen pathosystem, reisolations of the pathogens were performed by following the procedure described previously. An isolate was considered pathogenic on a given host when the wood discoloration length above and below the inoculation point in artificially inoculated plants was significantly higher compared with the mock-inoculated ones (control) and the applied fungus was successfully reisolated from artificially inoculated plants, but not from controls.

Statistical analysis. In trunk-drilling inoculated plants, analysis of variance (ANOVA) was employed to evaluate the pathogenic effects of the fungal strains on wood discoloration length above and below the inoculation point. When a significant *F* test was obtained for

treatments ($P < 0.05$), the data were subjected to means separation by Tukey's test and standard errors of means were also calculated. Morphological and physiological characteristics of *F. mediterranea* isolates were also analyzed by Tukey's test ($P < 0.05$) and by determining the Pearson's *r* correlation coefficients ($P < 0.05$ or 0.01). Moreover, in pruning-wood inoculated plants, data on wood discoloration length below the inoculation point were analyzed by carrying out a two-sample *t* test ($P < 0.05$).

Results

Morphological, physiological and molecular identification of fungal isolates. Morphological and physiological characteristics of 58 fungal strains isolated from 18 plant species showing typical wood decay symptoms are presented in Table 1. Among Ascomycota isolates, colony color was indicative of their holotypes. All isolated ascomycetes formed conidia of various sizes within the 21-day incubation period examined, apart from *Disyosphaeria*, *Eutypa*, and *Phialocephala* strains. Among Ascomycota members, *Phialophora*

Table 2. Phylogenetic relatives of the 58 fungal isolates obtained from 18 plant species and exhibited typical wood decay symptoms

OTU	Isolate	Host (substrate)	Phylogenetic relative	Similarity (%)	Isolation source	Reference
Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Hypocreales; Nectriaceae						
1	R1K1, MEL1LK1	Pomegranate tree (Wood), Chinaberry tree (Wood)	strain 212d/ <i>Xenocremonium falcatum</i> CBS 400.85/X. <i>recifei</i> CBS 541.89	98.8-100/99.0-99.4/99.0-99.4	endophyte from roots of eastern cottonwood/ <i>Pinus radiata</i> /soil, Brazil	JX244031/Lombard et al. (2015)/Lombard et al. (2015)
2	MA2, MSP1	Apple tree (Wood)	<i>Cosmospora</i> sp. OT1A-312b/ <i>Cosmospora</i> sp. IMI 362240/ <i>Pseudocosmospora rogersonii</i> G.J.S. 09-1384	99.9/95.2/95.2	leaves of Verdeal-Transmontana olive cultivar/stem tissue of <i>Vitis/Eutypella</i> sp.	KT804138/KJ676169/Herrera et al. (2013)
3	MOUR1P1, MOUR2P3, MOUR2A1	Mulberry tree (Wood)	<i>Fusarium solani</i> UOC/PTS/RDWW-22	99.6-100	endophyte- <i>Calamus thwaitesii</i>	KT150278
Ascomycota; Pezizomycotina; Sordariomycetes; Sordariomycetidae; Diaporthales; Togniniaceae						
4	A5LKK1	Grapevine (Wood)	<i>Phaeoacremonium minimum</i> CBS 100548	100	<i>Olea europaea</i> , Italy	Groenewald et al. (2001)
Ascomycota; Pezizomycotina; Sordariomycetes; Sordariomycetidae; Calosphaeriales; Pleurostomataceae						
5	E1PK1, AV1LK3	Olive tree (Wood), Avocado tree (Wood)	<i>Pleurostomophora richardsiae</i> IFM41579/ <i>Pleurostomophora richardsiae</i> CBS 117177	99.6/99.4	clinical/grapevine	AB364695/EF042106
Ascomycota; Pezizomycotina; Sordariomycetes; Sordariomycetidae; Cephalothecaceae						
6	K1LK1	Kumquat tree (Wood)	strain 695_ICIPE/ <i>Phialemonium dimorphosporum</i> E9430h	99.8/99.6	seed-born fungal endophyte/endophytes-rainforest plant	KM463111/JN559402
Ascomycota; Pezizomycotina; Sordariomycetes; Xylariomycetidae; Xylariales; Diatrypaceae						
7	GKFRLV3	Grapefruit tree (Wood)	<i>Eutypa leptoplaca</i> CBS 286.87	99.8	grapevine pathogen	Trouillas and Gubler (2004)
Ascomycota; Pezizomycotina; Dothideomycetes; Dothideomycetidae; Dothideales; Saccoteliaceae						
8	R1YKL1	Pomegranate tree (Wood)	<i>Aureobasidium pullulans</i> CBS 584.75	100	<i>Vitis vinifera</i>	KT693733
Ascomycota; Pezizomycotina; Dothideomycetes; Pleosporomycetidae; Pleosporales; Didymosphaeriaceae						
9	K1LVK1, A5LV1	Kumquat tree (Wood), Grapevine (Wood)	<i>Didymosphaeria variabile</i> STE-U 6311	99.8	stone fruit trees and woody hosts	Damm et al. (2008)
Ascomycota; Pezizomycotina; Eurotiomycetes; Chaetothyriomycetidae; Chaetothyriales; Herpotrichiellaceae						
10	AM1KL1	Almond tree (Wood)	<i>Phialophora alba</i> ICMP: 17034	100	forests and kiwifruit orchards	Johnston et al. (2010)
Ascomycota; Pezizomycotina; Eurotiomycetes; Chaetothyriomycetidae; Phaeomoniellales; Phaeomoniellaceae						
11	A2LP1, A5LP1	Grapevine (Wood)	<i>Phaeomoniella chlamydospora</i> ICMP: 17117	99.6-100	<i>Vitis vinifera</i>	HM116754
Ascomycota; Pezizomycotina; Leotiomyces; Leotiomycetidae; Helotiales; Vibrissaceae						
12	AXL1SP1	Pear tree (Wood)	<i>Phialocephala dimorphospora</i> CBS 300.62	99.0	slime in pulp mill	Grünig et al. (2002)
Basidiomycota; Agaricomycotina; Agaricomycetes; Polyporales; Polyporaceae						
13	D1LV1	Loquat tree (Wood)	<i>Trametes versicolor</i> X-29	99.8	saprophyte from soil	Thorn et al. (1996)
14	PLAT1mL1	Plane tree (Fruiting body)	<i>Trametes ljubarskyi</i> PRM 622107	99.5	<i>Prunus cerasus</i>	Tomšovský et al. (2006)
Basidiomycota; Agaricomycotina; Agaricomycetes; Polyporales; Polyporaceae						
15	AGK2L1, AGK2mL1	Jerusalem sage (Wood), Jerusalem sage (Fruiting body)	<i>Perenniporia truncatospora</i> Cui 6987/ <i>P. tenuis</i> Cui 5523	98.0/98.0	fruiting body/fruiting body	Zhao and Cui (2013)/Zhao and Cui (2012)
Basidiomycota; Agaricomycotina; Agaricomycetes; Auriculariales; Auriculariaceae						
16	KAR1LEN1	Walnut tree (Wood)	<i>Auricularia auricula-judae</i> LYBR 5404	99.3	leaves (Feuillu)	KP776003

(continued on next page)

^z The reference strain K.E. Fomit4 was also included.

and *Phaeoacremonium* exhibited the highest and lowest growth rate, respectively. The hyphal width of the isolated ascomycetes varied between 1.98 and 4.88 μm . All Basidiomycota isolates also had the typical colony color of their holotypes. Their growth rate varied widely from 1.89 to 16.74 mm/day, while their hyphal width ranged between 2.28 and 4.90 μm (Table 1).

By 5.8S rRNA gene-ITS sequencing analysis, the fungal isolates were classified into 25 operational taxonomic units (OTUs), which were equally split into the phyla Ascomycota and Basidiomycota (Table 2). In particular, OTUs nos. 1 to 12 were ascomycetes belonging to the genera *Xenoacremonium*, *Cosmospora*, *Fusarium*, *Phaeoacremonium*, *Pleurostomophora*, *Phialemonium*, *Eutypa*, *Aureobasidium*, *Didymosphaeria*, *Phialophora*, *Phaeoconiella*, and *Phialocephala*, whereas OTUs nos. 13 to 25 were basidiomycetes affiliated with *Trametes*, *Perenniporia*, *Auricularia*, *Aphanobasidium*, *Coniophora*, *Fuscoporia*, *Inonotus*, *Phellinus*, *Inocutis*, and *Fomitiporia* spp. (Table 2; Figs. 1 and 2). Apart from isolate E1PK1, which was related to *Pleurostomophora richardsiae*, no other ascomycetes were found to cause wood tissue discoloration of olive tree in the present study. *Fomitiporia mediterranea* was the major causal agent of grapevine wood decay, with the exception of the Kolimpari specimen where the infection was attributed only to a mixture of ascomycetes strains. Considering further host

specificity, chinaberry (*Melia azedarach* L.), avocado, and grapefruit (*Citrus paradisi* L.) trees were infected by ascomycetes, whereas locust (*Eriobotrya japonica* L.), plane, walnut (*Juglans regia* L.), black locust, tamarisk (*Tamarix parviflora* DC.), and orange trees as well as Jerusalem sage (*Phlomis fruticosa* L.) were susceptible to Agaricomycetes rot pathogens; however, the number of these plant samples infected was marginal to permit reliable conclusions. Among these isolates, important phytopathogenic strains of the genera *Fomitiporia*, *Inonotus*, *Phellinus*, *Inocutis*, *Fuscoporia*, *Trametes*, *Fusarium*, *Eutypa*, *Phaeoconiella*, *Phaeoacremonium*, and *Pleurostomophora* were included. The white rot basidiomycetes *F. mediterranea* (20 isolates out of 58, 34.5%) and *I. hispidus* (6 isolates out of 58, 10.3%) were the most predominant (Table 2). In particular, *F. mediterranea* and *I. hispidus* strains affected 19 and 5 of the samples examined, respectively (Tables 1 and 2). In most cases, only a single fungal taxon was found in each sample; however, two or three fungal species were recovered from 14 out of 38 samples collected. More rarely, two fungal taxa were observed to emerge simultaneously from the same wood chip, i.e., *F. mediterranea*/*P. chlamydospora* and *P. chlamydospora*/*Phaeoacremonium minimum* W. Gams, Crous, M.J. Wingf. & Mugnai in grapevine tissues, and *F. mediterranea*/*P. richardsiae* in olive tree tissues. Among the 21 *F. mediterranea* isolates sequenced (including the reference strain K.E.

Table 2. (continued from preceding page)

OTU	Isolate	Host (substrate)	Phylogenetic relative	Similarity (%)	Isolation source	Reference
Basidiomycota; Agaricomycotina; Agaricomycetes; Agaricomycetidae; Agaricales; Pterulaceae						
17	KAR1LKON1	Walnut tree (Wood)	<i>Aphanobasidium pseudotsugae</i> CFMR: HHB-822	82.9	Quercus log	Binder et al. (2010)
Basidiomycota; Agaricomycotina; Agaricomycetes; Agaricomycetidae; Boletales; Coniophoraceae						
18	K1AL1	Kumquat tree (Wood)	strain C1/ <i>Coniophora marmorata</i> P158/C. olivacea P151	99.8/89.1/88.7	wood rot of citrus/mining timber 1952/brown-rot fungi-decay in buildings woodwork	Roccatelli et al. (2014)/ Schmidt et al. (2002)/ Schmidt et al. (2002)
Basidiomycota; Agaricomycotina; Agaricomycetes; Hymenochaetales; Hymenochaetaceae						
19	E11mLK1	Olive tree (Fruiting body)	<i>Fuscoporia torulosa</i> 759	100	wood rot fungus of standing trees	Guglielmo et al. (2007)
20	MP1, MOUR4mLK1, MK1, MIL2mLK1, MIL2LK1, KAR1mLP1	Apple tree (Wood), Mulberry tree (Fruiting body), Apple tree (Wood), Apple tree (Fruiting body), Apple tree (Wood), Walnut tree (Fruiting body)	<i>Inonotus hispidus</i> 15	98.3-99.1	wood decay fungus of <i>Malus</i> sp.	Schmidt et al. (2012)
21	MOUR3mKL1	Mulberry tree (Fruiting body)	<i>Inonotus rickii</i> IFP 10359	99.0	canker and decay of <i>Hevea brasiliensis</i>	Dai et al. (2010)
22	ROMP1mLP1	Black locust tree (Fruiting body)	<i>Phellinus rimosus</i> / <i>Fulvifomes</i> (ex <i>Phellinus</i>) <i>merrillii</i> PM950703-1	95.3/88.3	NR/NR	JX484016/EU035311
23	AM1mXL1, AM2XL1	Almond tree (Fruiting body), Almond tree (Wood)	<i>Phellinus</i> sp. 1 DE-2016/ <i>Phellinus pomaceus</i> TN-449	98.6-98.8/98.3-98.5	fungus from deciduous-peach/white trunk rot of hardwoods- <i>Prunus domestica</i>	KU863540/Brazee (2015)
24	AL1mLK1	Tamarisk tree (Fruiting body)	<i>Inocutis tamaricis</i> HAI 1291/ <i>Inocutis tamaricis</i> 6-27	99.0/95.8	NR/pathogen of <i>Tamarix chinensis</i>	GQ253453/HM050416
25	K1KP1, R1LK1, R1LK1P1, E2mXL1, E2LK3, A3LK1P2, A4LK1P1, E7LK1, P1KP1, P2KP1, AKKON1LK1, E9mLK1, E10mLK1, AXL1LK1, A2LK1P1, E6LK1, E5LK1, R2LK1, E1KP1, K.E. Fomit4 ^z , E3LK1	Kumquat tree (Wood), Pomegranate tree (Wood), Olive tree (Fruiting body), Olive tree (Wood), Grapevine (Wood), Grapevine (Wood), Olive tree (Wood), Orange tree (Wood), Orange tree (Wood), Silk tree (Wood), Olive tree (Fruiting body), Olive tree (Fruiting body), Pear tree (Wood), Grapevine (Wood), Olive tree (Wood), Olive tree (Fruiting body), Pomegranate tree (Wood), Olive tree (Wood), Olive tree (Wood)	<i>Fomitiporia mediterranea</i> 45/23.3	98.7-99.6	wood decay fungus-grapevine	Fischer (2002)

Fomit4), eight distinct genotypes were identified, with half of them being identical in ITS-5.8S rRNA gene with previously deposited sequences (Fig. 2). The other four genotypes were distinct and reported for the first time, showing phylogenetic relatedness to known phytopathogenic strains of the species *F. mediterranea* (Fischer 2002; Roccotelli et al. 2014). All *F. mediterranea* strains isolated in the present study showed 98.7 to 99.6% similarity with strain 45/23, which is the holotype of *F. mediterranea* (Fischer 2002).

Physiological and morphological characterization of *Fomitiporia mediterranea* isolates. Analysis of variance revealed that *F. mediterranea* isolates differed significantly in terms of growth rate (df = 20, $F = 51.99$, $P < 0.001$), pigmentation on the upper side (df = 20, $F = 15.56$, $P < 0.001$) and underside (df = 20, $F = 37.84$, $P < 0.001$) of PDA plates, and mycelium abundance (df = 20, $F = 30.14$, $P < 0.001$) (Fig. 3).

However, no significant differences among isolates were observed in hyphae width (df = 20, $F = 1.48$, $P = 0.083$). The average growth rate values of *F. mediterranea* isolates ranged from 1.89 to 6.23 mm/day, while pigmentation values on the upper side and the underside of PDA plates ranged from 0.5 to 4.0 and 0.0 to 3.5, respectively. Among the 21 *F. mediterranea* isolates assessed (the reference strain K.E. Fomit4 was also included), eight were designated as S- and 13 as B-type, according to Fischer (2006). In addition, mycelium abundance of the isolates varied within 1.0 (sparse development of aerial hyphae) and 4.0 (intensive woolly mycelium), whereas isolates' hyphae width ranged between 2.28 and 3.06 μm . Moreover, the growth rate of isolate E9mLK1 was the greatest, followed by strain K.E. Fomit4, while the respective rates of isolates E3LK1, E5LK2, and A2LKP1 were the lowest ($P < 0.05$). Interestingly, these three isolates exhibiting the slowest

Continued on following page

Fig. 1. Phylogenetic allocation of ascomycetes **A**, and basidiomycetes (other than *Fomitiporia*) **B**, isolated from plant species, showing typical wood decay symptoms.

growth rates were placed in distinct genotypes. Apart from the growth rate, no other relationships among morphological and physiological traits, host specificity, and phylogeny were found. Moreover, hyphae width and growth rate were negatively correlated with pigmentation on the upper side ($r = -0.459$, $P < 0.05$) and underside ($r = -0.473$, $P < 0.05$), respectively. A positive correlation between pigmentation on the upper side and underside was also identified ($r = 0.467$, $P < 0.05$). This indicates that pigment formation was less in the fast-growing fungal strains isolated. In addition, mycelium abundance was highly correlated with the pigmentation on the upper side and underside ($r > 0.600$, $P < 0.01$).

Pathogenicity of fungal isolates. After a 7-month incubation period, longitudinal and transverse sections of sampled trunks revealed statistically significant wood tissue discoloration in all trunk-drilling inoculated trees treated with isolates originated from the same plant species compared with the controls (Table 3). Typical internal

symptoms were developed as dark brown lesions around the inoculation point (Fig. 4A, E, F, and G). In the cases of the olive-*F. mediterranea* and the grapevine-*F. mediterranea* pathosystems, a light-colored rotten area around the infection hole, similar to those observed under natural infection conditions, was occasionally noticed. On the other hand, control plants exhibited a brown wood discoloration limited mostly around the inoculation site. All fungal isolates tested were pathogenic on the plant species that had been obtained as the extension of wood tissue discoloration above and below the inoculation point in grapevine, olive, pomegranate, kumquat, pear, apple, almond, avocado, and mulberry tree was significantly higher compared with their mock-inoculated applications (Table 4). Moreover, the applied fungus in each plant-pathogen pathosystem was successfully reisolated from the artificially inoculated trees, but not from controls, thus confirming pathogenicity. In addition, *F. mediterranea* isolate A2LKP1 from grapevine was also pathogenic on olive, but

Fig. 1. Continued from previous page.

nonpathogenic on pomegranate, kumquat, apple, and almond tree. No significant differences in wood discoloration length above and below the inoculation point were observed within each plant species (Table 4).

Fig. 2. Phylogenetic position of 21 *Fomitiporia mediterranea* strains, including the reference strain K.E. Fomit4, isolated from 18 plant species, which exhibited extended pathogenicity to their hosts.

In the case of the pruning wound-inoculated trees, a typical light-colored rotten wood zone adjacent to the inoculation point in stems and branches was commonly observed 7 months after inoculation, followed by a brown-hard-necrotic wood area extending downward (Fig. 4B-D). At this period, a light-yellow mycelium was visible onto the wound surface and inside the wood due to fungal colonization. In control plants, a less-extended necrotic area without either wood rot or colonizing mycelium was observed. The length of wood discoloration below the inoculation site in stems and branches of silk tree, olive, pomegranate, kumquat, and grapevine inoculated with *F. mediterranea* isolates AKKON1LK1, E3LK1, R1LK1, K1KP1, and E3LK1, respectively, was significantly higher than in control plants (Fig. 5). Furthermore, *F. mediterranea* was steadily reisolated from all inoculated plants, but not from controls; therefore, pathogenicity of isolates was verified.

Discussion

In recent years, increased incidence of wood decay diseases in cultivated and ornamental tree species has been observed worldwide. This increase could be attributed to the more thorough study of the wood-decay pathogens in the recent years than earlier. Among plant species affected frequently are grapevine, olive, citrus, apple, and pomegranate trees. To a lesser extent, other plant species are also noticed to exhibit typical wood rot and decline symptoms, leading occasionally to tree death. Although the causal agents of these diseases have been sufficiently determined in several hosts (Di Marco et al. 2004; Elena et al. 2006; Elena and Paplomatas 2002; Fischer 2006; Paplomatas et al. 2006a, b; Pilotti et al. 2005; 2010; Roccotelli et al. 2014), the etiology of such decay on others still remains unknown. In the present study, a two-year survey was conducted to identify fungi associated with wood decay of tree species.

The isolation method followed here was efficient enough to obtain the three most prominent fungal species associated with esca disease in grapevine, i.e., *F. mediterranea*, *P. chlamydospora*, and *P. minimum* (Sparapano et al. 2000), and the main causal agent (*F. mediterranea*) of heart wood rot in olive (Paplomatas et al. 2006a, b) and orange trees (Elena et al. 2006) in Greece. *Fomitiporia mediterranea* was also isolated from diseased pear, kumquat, pomegranate, and silk

Fig. 3. Differential morphological characteristics of *Fomitiporia mediterranea* isolates grown on PDA cultures. Pigmentation was visually assessed in a five-point evaluation system regarding the percentage of pigmented area with reference to the entire culture surface on the underside **A**, and upper side **B**, of PDA cultures (0 = white colony, 1 = 1 to 25%, 2 = 26 to 50%, 3 = 51 to 75%, and 4 = 76 to 100% of culture surface pigmented). **C**, Mycelium abundance of fungal cultures was based on a 0 to 4 visual scale (0 = yeast-like appearance, 1 = sparse development of aerial hyphae, 2 = cottony mycelial culture, 3 = modest woolly appearance, and 4 = intensive woolly mycelium).

trees for the first time worldwide. Artificial inoculation experiments with *F. mediterranea* derived from these hosts verified the pathogenicity of isolates on the plant species that were originated, indicating a further extension of *F. mediterranea* host range list, including hereafter pear, pomegranate, kumquat, and silk tree.

It has been previously hypothesized that *F. mediterranea* inocula reach vineyards from external sources such as old vineyards nearby or carpophores that have been developed on non-*Vitis* hosts (Surico et al. 2006; 2008). However, no experimental work has been conducted to support such hypothesis (Surico et al. 2006). Cross-pathogenicity tests with *F. mediterranea* conducted here revealed that isolate E3LK1 originated from olive was pathogenic on olive and grapevine. Moreover, *F. mediterranea* isolate A2LKP1 from grapevine was pathogenic on grapevine and olive, but not on pomegranate, kumquat, apple and

almond trees, suggesting a partial host range specificity of the fungus. Therefore, the potential efficiency of *F. mediterranea* to be dispersed and transfect hosts other than that originated in nature should not be excluded. This hypothesis is strengthened by farmers' reports that olive and citrus orchards with high disease incidence are either neighbored with esca-infected vineyards or they have been established in discontinued vineyards shown severe esca symptoms (Elena et al. 2006; present study). Other researchers have likewise proposed host specificity or host-specific cryptic species within *F. mediterranea*, on the basis of pathogenicity tests on citrus trees with *F. mediterranea* isolates from citrus and noncitrus hosts (Elena et al. 2006; Pilotti et al. 2010; Roccotelli et al. 2014).

In contrast to other plant-pathogen pathosystems, artificial inoculation experiments with *F. mediterranea* on tree species and grapevine

Table 3. Analysis of variance for wood discoloration length above and below the inoculation point for grapevine, olive, pomegranate, kumquat, pear, apple, almond, avocado, mulberry and silk tree, artificially inoculated with the isolates A2LKP1-A2LP1, E3LK1-E1PK1-A2LKP1, R1LK1-A2LKP1, K1KP1-A2LKP1, AXL1LK1, MP1-A2LKP1, AM2XL1-A2LKP1, AV1LK3, MOYR2P3, and AKKON1LK1 or mock inoculated controls, respectively

Source	df, <i>F</i> values ^z									
	Grapevine	Olive tree	Pomegranate tree	Kumquat tree	Pear tree	Apple tree	Almond tree	Avocado tree	Mulberry tree	Silk tree
Wood discoloration length above the inoculation point										
Replication	2, 2.05	2, 2.53	2, 1.17	2, 0.05	2, 0.30	2, 0.04	2, 0.13	2, 0.11	2, 1.54	-
Treatment	2, 58.16***	3, 19.58***	2, 16.84***	2, 9.46**	1, 24.74***	2, 11.54**	2, 21.54***	1, 27.83***	1, 647.78***	-
Replication × treatment	4, 0.89	6, 0.88	4, 0.64	4, 0.39	2, 0.30	4, 0.24	4, 0.07	2, 0.11	2, 1.54	-
Wood discoloration length below the inoculation point										
Replication	2, 0.02	2, 0.33	2, 1.33	2, 0.29	2, 0.22	2, 0.83	2, 0.01	2, 0.07	2, 1.03	2, 0.93
Treatment	2, 26.04***	3, 7.57***	2, 10.80**	2, 11.22**	1, 26.62***	2, 18.35***	2, 45.37***	1, 29.78***	1, 34.83***	1, 27.23***
Replication × treatment	4, 0.08	6, 0.53	4, 0.52	4, 0.77	2, 0.22	4, 1.32	4, 0.07	2, 0.07	2, 1.03	2, 0.93

^y Degrees of freedom among groups.

^z Symbols ** and *** indicate significance at $P < 0.01$ and 0.001 , respectively, according to the *F* test.

Fig. 4. Wood discoloration and decay symptoms in olive tree **A**, pomegranate tree **B**, kumquat tree **C**, and silk tree **D**, artificially infested with *Fomitiporia mediterranea*, and in almond tree **E**, apple tree **F**, and avocado tree **G**, artificially infested with *Phellinus pomaceus*, *Inonotus hispidus*, and *Pleurostomophora richardsiae*, respectively.

are laborious and time-consuming. In some cases, no significant differences in disease development between control and artificially inoculated plants were observed, even after a long incubation period (Di Marco et al. 2004; Laveau et al. 2009; Paplomatas et al. 2006a). In other cases,

the observed differences were marginally significant (Rocchetti et al. 2014), suggesting the extremely slow progress in disease development. In the present study, by employing the trunk-drilling method, all plant species inoculated with *F. mediterranea* strains that were isolated from

Table 4. Pathogenicity of fungal isolates on their hosts and cross-pathogenicity of the *F. mediterranea* isolate A2LKP1 from grapevine on olive, pomegranate, kumquat, apple, and almond trees

Host inoculated	Fungal species	Isolate	Host of origin	Discoloration length from the inoculation point (mm) ^y		Reisolation ^z
				Above	Below	
Grapevine	Control			5.1 ± 0.9 a	6.1 ± 1.0 a	–
	<i>F. mediterranea</i>	A2LKP1	Grapevine	42.0 ± 4.2 b	52.3 ± 5.1 b	+
	<i>Ph. chlamydospora</i>	A2LP1	Grapevine	79.6 ± 7.6 c	90.2 ± 11.4 c	+
Olive	Control			4.9 ± 0.2 a	5.1 ± 0.6 a	–
	<i>F. mediterranea</i>	E3LK1	Olive tree	23.2 ± 2.6 b	21.6 ± 3.4 b	+
	<i>Pl. richardsiae</i>	E1PK1	Olive tree	16.5 ± 2.3 b	16.5 ± 2.3 b	+
	<i>F. mediterranea</i>	A2LKP1	Grapevine	18.7 ± 1.3 b	17.9 ± 2.4 b	+
Pomegranate	Control			5.7 ± 0.2 a	6.5 ± 0.3 a	–
	<i>F. mediterranea</i>	R1LK1	Pomegranate tree	13.0 ± 1.4 b	15.9 ± 2.7 b	+
	<i>F. mediterranea</i>	A2LKP1	Grapevine	7.1 ± 0.9 a	6.0 ± 1.1 a	–
Kumquat	Control			2.5 ± 0.2 a	2.6 ± 0.3 a	–
	<i>F. mediterranea</i>	K1KP1	Kumquat tree	8.4 ± 1.5 b	8.9 ± 1.7 b	+
	<i>F. mediterranea</i>	A2LKP1	Grapevine	2.7 ± 0.2 a	2.8 ± 0.2 a	–
Pear	Control			3.2 ± 0.1 a	5.7 ± 0.7 a	–
	<i>F. mediterranea</i>	AXL1LK1	Pear tree	23.3 ± 3.7 b	19.6 ± 2.3 b	+
Apple	Control			14.0 ± 0.6 a	12.8 ± 0.8 a	–
	<i>I. hispidus</i>	MP1	Apple tree	72.6 ± 13.5 b	55.3 ± 9.1 b	+
	<i>F. mediterranea</i>	A2LKP1	Grapevine	20.5 ± 1.9 a	16.4 ± 1.4 a	–
Almond	Control			3.2 ± 0.2 a	2.7 ± 0.1 a	–
	<i>Ph. pomaceus</i>	AM2XL1	Almond tree	47.1 ± 8.3 b	50.0 ± 6.3 b	+
	<i>F. mediterranea</i>	A2LKP1	Grapevine	5.0 ± 1.1 a	7.1 ± 1.2 a	–
Avocado	Control			9.3 ± 0.9 a	13.0 ± 1.7 a	–
	<i>Pl. richardsiae</i>	AV1LK3	Avocado tree	63.9 ± 9.6 b	49.0 ± 6.8 b	+
Mulberry	Control			24.0 ± 1.0 a	22.7 ± 0.9 a	–
	<i>F. solani</i>	MOYR2P3	Mulberry tree	74.7 ± 1.9 b	83.9 ± 10.0 b	+

^y The pathogenicity of *Fomitiporia mediterranea*, *Phaeomoniella chlamydospora*, *Pleurostomophora richardsiae*, *Inonotus hispidus*, *Phellinus pomaceus* and *Fusarium solani* isolates was evaluated on the basis of the extension of wood tissue discoloration above and below the inoculation point after a 7-month incubation period. Artificial inoculations were conducted by employing the trunk-drilling method and inserting a 5.5-mm-diameter mycelial disc taken from the margin of a 15-day-old PDA culture. Control plants were mock-inoculated with a 5.5-mm-diameter sterilized PDA plug. According to Tukey's HSD test at $P < 0.05$, values followed by the same letter did not differ significantly within each plant species inoculated.

^z Pathogen reisolation was performed by making longitudinal sections of the trunk and placing 10 wood chips taken from the symptomatic wood, above and below the inoculation point, onto acidified PDA. Plates were incubated at 24°C in the dark for 3 weeks. In each plant-pathogen pathosystem, at least 3 plants were used for pathogen reisolation. Symbols '+' and '-' represent successful and unsuccessful pathogen reisolation, respectively.

Fig. 5. Length of wood tissue discoloration below the inoculation point of silk, olive, pomegranate, kumquat, and grapevine stems or branches, 7 months after adjusting mycelial discs of *Fomitiporia mediterranea* isolates onto fresh pruning wounds. Control plants were mock inoculated with sterilized PDA discs. Within each plant species, columns followed by different letters differ significantly, according to the t test at $P < 0.001$. Vertical bars indicate standard errors. Above each column, symbols '+' and '-' into brackets represent successful and unsuccessful pathogen reisolation, respectively.

the same host, exhibited significantly higher disease reaction compared with their mock-inoculated controls. Moreover, the fungus was successfully reisolated from diseased tissues, but not from controls, thus confirming its pathogenicity. However, the extension of wood discoloration was much lower in pomegranate and kumquat trees than in other hosts inoculated. Given that *F. mediterranea* penetrates through wounds (Elena et al. 2006; Mugnai et al. 1999; Roccotelli et al. 2014), we attempted to simulate the natural infection process and prompt disease development by using the pruning-wound method to inoculate grapevine, olive, pomegranate, kumquat, and silk tree. The pruning-wound inoculation approach was proved to be a robust method since most inoculated plants exhibited typical wood decay symptoms, similar to those observed under natural infection conditions. Moreover, the extension of wood tissue lesions in olive and pomegranate was greater compared with those observed in trunk-drilling inoculated plants, whereas lesions' length in kumquat remained low. Other researchers have likewise employed the pruning-wound instead of the trunk-drilling inoculation method to evaluate pathogenicity (Gonzalez et al. 2009; Sparapano et al. 2000). In addition, other citrus species have also shown a very narrow disease reaction in pathogenicity tests with *F. mediterranea* (Elena et al. 2006; Roccotelli et al. 2014).

It has often been documented that *F. mediterranea* isolates vary in morphology and physiology onto solid culture media (Fischer 2002; 2006; Pilotti et al. 2005; 2010; Roccotelli et al. 2014). Therefore, Fischer (1987) divided *F. mediterranea* isolates into either bleaching (B) or staining (S) type: in type B, the mycelium in solid media is cottony to woolly, forming yellow to brown hyphae, and exhibiting weak or no pigmentation of the medium and fast growth rates; whereas in type S, the mycelium is characterized by sparse aerial hyphae, stronger pigmentation of the medium, and slower growth rate. Indeed, *F. mediterranea* isolates analyzed in the present study differed significantly in growth rate, culture pigmentation on the under- and upper- side, and mycelium abundance. However, classification system proposed by Fischer (1987) was not completely feasible for the *F. mediterranea* isolates examined here. Several isolates exhibited features that were contradictory, thus they could not be placed into either B- or S- type. For instance, isolate E5LKE exhibited the lowest growth rate and intense pigmentation on both sides of cultures, suggesting type S, but also woolly mycelium, indicating type B. In addition, isolate E6LKL1 showed the lowest pigmentation on the undersides of cultures, indicating type B; however, its very sparse aerial hyphae indicated type S. Therefore, in the present study, classification of isolates into either B- or S- type was carried out by assessing the appearance of pigment on the underside of culture plates, solely, according to Fischer (2006): isolates exhibiting modest to strong pigmentation of the culture medium were designated as type S, whereas those with weak or no pigmentation, as type B. Hence, analysis of cultures' morphology revealed the presence of both B and S types within 21 *F. mediterranea* isolates tested (including the reference strain K.E. Fomit 4), where most of them were type B, as has been reported in other studies (Fischer 2006; Roccotelli et al. 2014). On the other hand, the growth rates and phylogenetic position of the isolates E3LK1, E5LK2, and A2LKP1 are distinct to other *F. mediterranea* isolates, indicating intraspecies variation possibly linked to the severity of disease symptoms caused by *F. mediterranea*.

Besides *F. mediterranea*, several other important phytopathogenic species such as *I. hispidus*, *P. pomaceus*, *P. richardsiae*, and *F. solani* are presented for the first time worldwide as the causal agents of wood infection in apple, almond, avocado, and mulberry trees, respectively. Although *I. hispidus* commonly occurs on ash, apple, and walnut in Europe (Fischer and Gonzalez-Garcia 2015; Schwarze et al. 2000), this is the first experimental evidence of *I. hispidus* on apple trees by means of pathogenicity tests. Likewise, Gonzalez et al. (2009) reported the presence of *I. hispidus* carpophores on esca-infected grapevines in Spain, revealing also the pathogenicity of this fungus on healthy vines by in vitro bioassays. Moreover, although *P. pomaceus* is considered as a common plant pathogen of various *Prunus* species (Lonsdale 1999; Rayner and Boddy 1988), it has never been reported as primary pathogen on almond trees. In addition, *P. richardsiae* has been associated with wood streaking of olive and almond trees in Italy and Spain (Carlucci et al. 2013;

Olmo et al. 2015) and with grapevine trunk diseases in Italy, California, and South Africa (Carlucci et al. 2015; Eskalen et al. 2004; Rolshausen et al. 2010; White et al. 2011). However, this is the first record of *P. richardsiae* as a wood-infecting microorganism in avocado, while the same fungus is associated with wood streaking of olive trees for the first time in Greece. The species *F. solani* is a well-described cosmopolitan plant pathogen, which includes endophytic strains that can act as tissue decomposers (Schroers et al. 2017), although this is the first report of *F. solani* as a wood deterioration inducer in mulberry trees.

Despite the lack of pathogenicity tests, *Cosmospora* from apple tree, *Auricularia aurica-judae* (Bull.) J.Schröt. and *Aphanobasidium*-like fungi from walnut tree, *Phialophora* from almond tree, *Fusicoporia* from olive tree, *Didymosphaeria* from kumquat tree and grapevine, *Phialemonium* and *Coniophoraceae* from kumquat tree, *Eutypa* from grapefruit tree, *Inonotus rickii* (Pat.) D.A. Reid from mulberry tree, *Penniporia* from Jerusalem sage, *Xenoacremonium* from pomegranate and chinaberry tree, *Trametes* from loquat, and *T. ljubarskyi* Pilát from plane tree were also isolated for the first time from diseased hosts. In addition, several other fungi—namely, the well-described wood decay pathogens *Inonotus hispidus* on walnut and mulberry (Schwarze et al. 2000) and *Phellinus rimosus* (Berk.) Pilát on black locust tree (Riffle et al. 1985)—were detected in the present study for the first time in Greece. However, no pathogenicity tests were conducted for the above-mentioned pathosystems in the present study, thus the phytopathogenic potential of these fungi on their hosts remains uncertain.

Moreover, certain basidiomycetes from the present study, which were associated with wood decay of various tree species, were distantly related to their known taxonomic relatives, representing novel phylogenetic linkages among the class Agaricomycetes (Table 2). In particular, isolate KARILKON1 possessed a distinct phylogenetic position within the order Agaricales, showing only 82.9% similarity to an *Aphanobasidium* sp. Apart from the high similarity to its non-taxonomically described strain C1, isolate K1AL1 showed 88.7 to 89.1% phylogenetic relatedness to known *Coniophora* species, indicating that this strain is a new member of the family Coniophoraceae. Moreover, isolate ROMP1mLP1 obtained from black locust tree was distantly related to *Phellinus/Fulvifomes* spp., therefore being a novel *Phellinus*-like representative.

In view of all the above, wood decay diseases in tree species could be attributed to diverse fungal pathogens. Identification of wood-decay fungi and investigation of their phytopathogenic role is essential to provide successful disease diagnosis and management. Moreover, the further extension of *F. mediterranea* host range along with the potential efficiency of the fungus to transinfect hosts other than its original ones in practice should be taken into account to estimate disease risk and plan future control strategies.

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