

## Research Article

## Dothistroma Needle Blight in protected pine forests in Italy

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## Abstract

Widespread and locally severe foliar symptoms resembling Dothistroma Needle Blight (DNB), one of the most important infectious diseases of forest trees worldwide, were recently observed in La Sila Massif, a mountain plateau covered with native forests of *Pinus nigra* subsp. *laricio* in La Sila National Park, Southern Italy. At the same time, DNB symptoms were observed in *Pinus cembra* and *Pinus mugo* forests in the Paneveggio-Pale di San Martino Nature Park and in Val Sarentino, Northeastern Italy. Defoliation was extensive at all sites and severe on the majority of plants of affected species, both adult trees and renovation. In particular on *Pinus cembra*, the disease was so serious as to locally threaten the species' reproduction and survival, an unusually heavy damage on this host. Species-specific real time PCR diagnostics, recommended by EPPO, was applied to needle samples from these sites and the presence of *Dothistroma septosporum* was ascertained, while *Dothistroma pini*, the morphologically identical congeneric species causing the same disease, was not detected. The pathogen was isolated from conidiomata and species attribution was confirmed by sequencing of the ITS region. In addition, a culture independent survey based on the same molecular assays was carried out in other areas of Northern, Central and Southern Italy, where pines of various species were affected by similar symptoms, and gave negative results for both *Dothistroma* species. Results show that *D. septosporum* currently has in Italy a much larger distribution and host range than reported and is associated to life-threatening damage to native pine species growing in established populations for *in situ* conservation of genetic resources, which would require an update of specific conservation actions.

**Key words:** emerging diseases, pest surveillance, *Pinus nigra* subsp. *laricio*, *Pinus cembra*, *Pinus mugo*, red band needle blight, *Dothistroma septosporum*, TaqMan diagnostics

## Introduction

Dothistroma needle blight (DNB) is one of the most important infectious diseases of forest trees in the family Pinaceae. The disease is caused by two morphologically indistinguishable Ascomycete species separated using

DNA sequence differences (Barnes et al. 2004): *Dothistroma septosporum* (Dorogin) M. Morelet and *Dothistroma pini* Hulbary (Barnes, Crous, Wingfield & Wingfield, 2004). Both species, which have been long regulated in Europe under the collective name *Scirrhia pini* Funk and Parker (EU 2000), are currently included in the list of Union regulated non-quarantine pests (“RNQPs”) (EU 2016). These two *Dothistroma* species generally colonize older needles causing premature defoliation that results in growth reduction, and mortality after repeated attacks especially in young plants (Gibson 1972; Ivory 1972; Rodas et al. 2016). Infected needles develop yellow to red colored spots and bands where dark conidiomata form. The most susceptible species are in genus *Pinus*, but more than 100 species in the family Pinaceae are hosts with varying susceptibility (Watt et al. 2009; Drenkhan et al. 2016; Mullett et al. 2018).

*Dothistroma septosporum* has become infamous as an invasive species mostly on *Pinus* plantations in the Southern Hemisphere, but both species, *D. septosporum* and *D. pini*, recently caused unexpected epidemics on pines in the Northern Hemisphere (Mullett et al. 2018; Drenkhan et al. 2016). According to current niche models, the potential geographic range of DNB pathogens in Europe is larger than their current known distribution (Möykkynen et al. 2017), which includes several countries where the pathogens have few occurrences or a restricted distribution (Mullett et al. 2018; EPPO 2020). In Italy the only published report of DNB dates back to 1977 in a young plantation of the non-native species *Pinus radiata* D. Don at San Pietro di Caridà, on the Southern Apennines ridge connecting the Serre Mountain range to the Aspromonte Massif, Region Calabria (Magnani 1977). The only other mention of DNB fungi in Italy is in the inventory of forest pests and pathogens of Region Friuli Venezia Giulia, which reports the detection of *Mycosphaerella pini* Rostr. Ex Munk (the former name for *D. septosporum*) on *Pinus nigra* J.F. Arnold and *Pinus sylvestris* L. at a single location in Moggio Udinese (Udine, Italy) (Bernardinelli 2016). To our knowledge, these reports have not been confirmed through specific molecular diagnostics and the actual species involved remain, at least for the report in Southern Italy, uncertain. To date, in the scientific literature as in the global database of pest-specific information maintained by the Secretariat of the European and Mediterranean Plant Protection Organization (EPPO) there are no other reports of *Dothistroma* species in Italy than the one by Magnani (1977).

In 2017 widespread and locally severe foliar symptoms resembling DNB (orangey-red brown distal needle ends, dark red bands, and green bases, with or without black fruiting bodies within the band) were observed in La Sila Massif, a mountain plateau at about 1200 meters above sea level (MASL), covered with forests of the native *Pinus nigra* subsp. *laricio* (Poir.) Maire in La Sila National Park, a protected area for biodiversity conservation in the southernmost continental Italy, about 150 kilometers north-west of

the site of first report of *Dothistroma* (Magnani 1977). In 2017, sporadic and moderate symptoms similar to DNB were also observed on *Pinus cembra* L. growing in natural forests of Paneveggio-Pale di San Martino Nature Park, Region Trentino Alto-Adige, a protected area at the opposite end of Italy, towards the border with Austria. Defoliation became more severe and widespread during 2018 on *P. cembra* and also on *Pinus mugo* Turra subsp. *mugo* growing naturally in the same area. Finally, in late summer 2018 similar symptoms accompanied by heavy defoliation were observed in Val Sarentino, Region Trentino Alto-Adige, on native *P. mugo* and *P. cembra* forests.

Here we report about detection, by species specific PCR assays and fungal isolation, of DNB fungi from native pine species in these areas. We also include the results of a culture independent survey that we performed in other areas of Italy, where similar symptoms were observed, but no conidiomata alike those produced by *Dothistroma* species were found on pine needles.

## Materials and methods

### *Plant material*

Symptomatic pine needles with or without visible conidiomata from individual trees (104 samples in total, 3–10 trees per site) growing at several sites in Italy in 2017, 2018 and 2019 (Table 1) were analyzed. Each sample was individually packed in hermetically sealed bag and was kept refrigerated at  $4 \pm 2$  °C until further processing.

### *DNA extraction from needles and Dothistroma detection by real-time PCR*

DNA was extracted from 5-mm-long needle pieces (about 70 mg) with red band symptoms with or without visible conidiomata. Needle pieces were transferred into 2-ml microcentrifuge tube, frozen at  $-20$  °C and ground for 1 min at 30 Hz with two 3-mm sterile steel beads in a Retsch GmbH Retsch mixer mill MM400 (Haan, Germany). Total DNA was extracted using the Nucleospin Plant II kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. Total DNA concentrations were estimated using a Thermo Scientific NanoDrop® ND-1000 spectrophotometer (Wilmington, DE, USA). Eluted DNA samples were kept at  $-20$  °C until analysis. Each DNA extract was tested by real-time PCR using the TaqMan probe assays for *D. septosporum* and *D. pini* from Ioos et al. (2010). Both *Dothistroma* probes (DStub2-P1 for *D. septosporum* and Dptef-P1 for *D. pini*, Ioos et al. 2010) were dual-labelled with FAM-TAMRA and each species-specific assay was performed separately. Prior to testing with the specific assay, amplificability of samples (as such and 1/10 dilution) was checked with the real-time 18S rDNA assay developed by Ioos et al. (2010). Real-time PCR reactions were performed with an ABI 7300 Real-Time PCR system

**Table 1.** Geographic location of sites where symptomatic needles from various Pinaceae species were tested by real-time PCR assays (loos et al. 2010) for *Dothistroma pini* and *Dothistroma septosporum*. # ID as in Figure 1.

ID#	Site (Area)	Region	Lat	Long	altitude (MASL)	Sampling time (+ conidiomata)	Tree species	<i>D. pini</i>	<i>D. septosporum</i> [* isolates]
1	Reggio Calabria	Calabria	38.10173	15.63816	18	2017/06 (-)	<i>Pinus halepensis</i>	negative	negative
1	Loisa	Calabria	38.14299	15.76107	880	2017/06 (-)	<i>Pinus nigra</i> subsp. <i>laricio</i>	negative	negative
1	Gambarie	Calabria	38.17468	15.81612	1132	2017/06 (-)	<i>Pinus nigra</i> subsp. <i>laricio</i> , <i>Abies alba</i>	all negative	all negative
2	Copanello	Calabria	38.76745	16.56328	18	2017/06 (-)	<i>Pinus pinea</i>	negative	negative
3	Lago Arvo (La Sila Massif)	Calabria	39.23694	16.47111	1460	2017/06 (-)	<i>Pinus nigra</i> subsp. <i>laricio</i>	negative	positive
3	Rovale (La Sila Massif)	Calabria	39.24250	16.54472	1310	2017/06 (-)	<i>Pinus nigra</i> subsp. <i>laricio</i>	negative	positive
3	Lago di Cecita (La Sila Massif)	Calabria	39.39243	16.52822	1160	2017/06 (-)	<i>Cedrus atlantica</i> , <i>Pinus nigra</i> subsp. <i>laricio</i> , <i>Pinus pinaster</i> , <i>Pinus radiata</i> , <i>Pseudotsuga menziesii</i>	all negative	all positive except <i>P. pinaster</i>
3	Fossiata (La Sila Massif)	Calabria	39.39562	16.58892	1300	2017/06 (-)	<i>Pinus nigra</i> subsp. <i>laricio</i> , <i>Pinus sylvestris</i>	all negative	all positive
3	Bonolegno (La Sila Massif)	Calabria	39.26596	16.66053	1160	2017/01 (+) 2019/11 (+)	<i>Pinus nigra</i> subsp. <i>laricio</i>	negative	positive [*]
3	Moriani (La Sila Massif)	Calabria	39.459165	16.460938	1113	2017/06 (-)	<i>Pinus nigra</i> subsp. <i>laricio</i>	negative	positive
3	Scanciamoneta (La Sila Massif)	Calabria	39.504973	16.543383	1293	2017/06 (-)	<i>Pinus nigra</i> subsp. <i>laricio</i>	negative	positive
4	Cosenza	Calabria	39.33120	16.23973	210	2018/06 (-)	<i>Pinus pinea</i>	negative	negative
5	Bocchigliero	Calabria	39.36320	16.71917	1270	2018/06 (-)	<i>Pinus nigra</i> subsp. <i>laricio</i>	negative	negative
6	Cerenzia	Calabria	39.24875	16.77955	700	2018/06 (-)	<i>Pinus pinea</i>	negative	negative
7	Campo di Giove	Abruzzo	42.00349	14.05602	1070	2019/08 (-)	<i>Pinus nigra</i>	negative	negative
8	Vallombrosa	Toscana	43.73145	11.55415	990	2019/06 (-)	<i>Pinus strobus</i> , <i>Pinus contorta</i> subsp. <i>murrayana</i> , <i>Pinus nigra</i> subsp. <i>laricio</i>	all negative	all negative
9	Maresca	Toscana	44.049191	10.838300	850	2019/06 (-)	<i>Pinus mugo</i>	negative	negative
10	Gardone Riviera	Lombardia	45.620430	10.562160	100	2018/06 (-)	<i>Pinus brutia</i> , <i>Pinus halepensis</i> , <i>Pinus mugo</i> , <i>Pinus nigra</i> , <i>Pinus wallichiana</i>	all negative	all negative
11	Pai	Veneto	45.650294	10.721644	110	2018/06 (-)	<i>Pinus brutia</i> , <i>Pinus pinea</i>	all negative	all negative
12	Lagolo	Trentino-Alto Adige	46.040814	11.008251	990	2018/09 (-)	<i>Pinus mugo</i> , <i>Pinus sylvestris</i>	all negative	all negative
13	Paluzza	Friuli Venezia Giulia	46.524836	13.002392	560	2019/09 (-)	<i>Pinus mugo</i>	negative	negative
14	Pietrabec	Friuli Venezia Giulia	46.619224	12.757934	1610	2019/09 (-)	<i>Picea abies</i>	negative	negative
15	San Martino di Castrozza (Paneveggio Pale di San Martino)	Trentino-Alto Adige	46.26077	11.79903	1450	2018/09 (+)	<i>Pinus mugo</i>	negative	positive
15	Colbricon (Paneveggio Pale di San Martino)	Trentino-Alto Adige	46.282650	11.766221	1920	2018/09 (+) 2019/10 (+)	<i>Pinus cembra</i> , <i>Pinus mugo</i>	all negative	all positive [*]
16	Sarentino San Martino	Trentino-Alto Adige	46.681405	11.434400	1640	2018/09 (+) 2019/11 (+)	<i>Pinus cembra</i> , <i>Pinus mugo</i>	all negative	all positive [*]
16	Sarentino Valdurna	Trentino-Alto Adige	46.740827	11.443171	1560	2018/09 (+) 2019/11 (+)	<i>Pinus cembra</i>	all negative	all positive [*]

(Applied Biosystems). The reactions were carried out in a final volume of 20  $\mu$ l using: 1  $\mu$ l of template DNA, 10  $\mu$ l of 2X GoTaq Probe qPCR Master Mix (Promega), primers/probe sets (Ioos et al. 2010): DStub2-F1/Dstub2-R1/DStub2-P1 (*D. septosporum*); DPtef-F1/DPtef-R1/DPtef-P1 (*D. pini*); 18S uni-F/18S uni-R/18S uni-P at a final concentration of 0.4  $\mu$ M each, and DNase free water to final volume. Positive controls (external DNA extracts from *D. septosporum* and *D. pini* cultures) and no-template controls were included in all reactions to ensure optimal PCR reaction conditions and verify the absence of contamination. The real-time PCR cycling conditions included an initial denaturation step at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s and annealing and elongation at 60 °C for 55 s. The cycle threshold (Ct) value for each reaction was determined using the instrument's software, with automatic setting of the threshold line above the mean baseline fluorescence level.

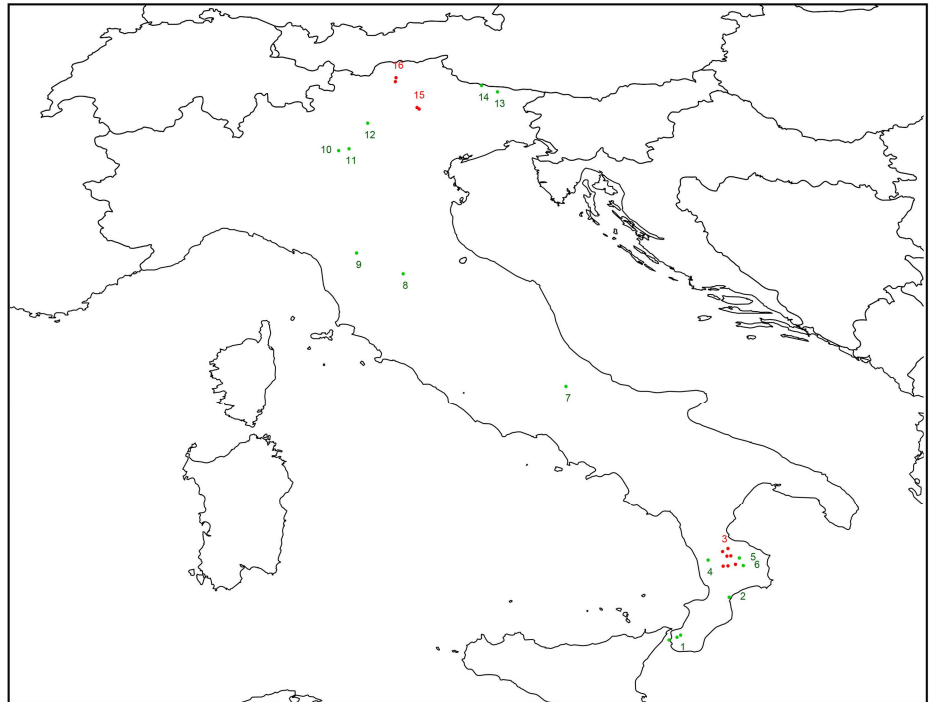
Conventional PCR was applied on a subsample of positive needle DNA extracts using primers DStub2-F and DStub2-R as described in Ioos et al. (2010) and recommended by EPPO (2015). PCRs were run on a Biometra Trio thermocycler (Analytik Jena, Jena, Germany) with the following thermal cycling conditions: 1 cycle at 95 °C for 10 min, 35 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, followed by a final extension cycle at 72 °C for 10 min. For each sample, 2  $\mu$ l of PCR amplicon was visualized after electrophoresis in 1% agarose gel (Sigma-Aldrich) in 1 $\times$  Tris-acetate-EDTA (TAE) buffer and staining with ethidium bromide (0.5  $\mu$ g mL<sup>-1</sup>). DNA fragments were purified from the agarose gel by NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany) and sent for Sanger sequencing to StarSEQ® GmbH (Mainz, Germany). Nucleotide sequences were visualized using CHROMAS LITE v. 2.01 (Technelysium, South Brisbane, Australia) and aligned using MUSCLE (Edgar 2004) as implemented in MEGA 7 (Kumar et al. 2016). Sequences were blasted against the GenBank database (National Center for Biotechnology Information (NCBI), Bethesda, MD) to check the correspondence to the expected target.

### *Conidial morphology*

Conidia from conidiomata found on needle samples from La Sila Massif (Bonolegno), Paneveggio-Pale di San Martino (Colbricon) and Val Sarentino (site IDs 3, 15 and 16, respectively, Table 1, Figure 1) were mounted in lactophenol cotton blue and examined under a light microscope (Axiophot, Carl Zeiss) at up to  $\times$  40 magnification. At least 30 conidia were measured per sample.

### *Isolation of fungi, DNA extraction from micelia and sequencing*

Isolations on 2% malt-extract agar (MEA) + streptomycin from surface sterilized (by spraying with 96% ethanol and wiping with a tissue) needles



**Figure 1.** Map of locations (ID numbers as in Table 1) where symptomatic needles from Pinaceae species tested negative (green) or positive (red) to the specific real-time PCR assay for *Dothistroma septosporum* by Ioos et al. (2010). All of the samples tested negative for *Dothistroma pini*. Map was done using R 3.5.0 (R Core Team, 2019) and rworldmap v1.3-6 (South 2016) package.

bearing fruiting bodies were performed with the technique of acervuli rolling as described in Mullet and Barnes (2012). Petri dishes were incubated at 20 °C and single germinating colonies showing the distinctive zig-zagging hyphae typical of *Dothistroma* were transferred on 2% MEA with yeast extract (5 g/L) after a few days. Axenic colonies on MEA were incubated at 20 °C for 4 weeks in the dark and conserved in Petri dishes at 4 °C for short term use. For DNA extractions, mycelia obtained from cultures growing on a cellophane disc placed on MEA in Petri dishes were frozen and ground into a powder using the Retsch GmbH Retsch mixer mill MM400 (Haan, Germany). Total DNA was extracted from about 70 mg of each mycelium using the Nucleospin Plant II kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. DNA concentrations were measured using a Thermo Scientific NanoDrop® ND-1000 spectrophotometer (Wilmington, DE, USA).

The ITS barcoding region was amplified using the primers ITS4 and ITS5 (White et al. 1990). Each PCR mix included 2.5 µl of 10× DreamTaq Green Buffer (Thermo Scientific), 0.4 µl of DreamTaq DNA Polymerase (Thermo Fischer Scientific, USA) (5 U µl<sup>-1</sup>), 0.8 µl dNTPs (µM 6.25), 0.25 µl of forward and reverse primer (50 µM), 2 µl of DNA and dsH<sub>2</sub>O to a total volume of 25 µl. PCRs were run on a Biometra Trio thermocycler (Analytik Jena, Jena, Germany) with the following thermal cycling conditions: 1 cycle at 95 °C for 5 min, 40 cycles of 95 °C for 45 s, 50 °C for 30 s, 72 °C for 30 s,



**Figure 2.** Details of symptomatic needles bearing *D. septosporum* conidiomata (A, B) and symptomatic young plants (C,D) of *Pinus nigra* subsp. *laricio* in naturally regenerating forests in Bonolegno (ID3 Figure 1 and Table 1, La Sila National Park, Calabria, Southern Italy). Photographs by Luisa Ghelardini (A, B) and Francesco Loria (C, D).

followed by a final extension cycle at 72 °C for 10 min. For each sample, 2 µl of PCR amplicon was visualized after electrophoresis in 1% agarose gel (Sigma-Aldrich) in 1× Tris-acetate-EDTA (TAE) buffer and staining with ethidium bromide (0.5 µg mL<sup>-1</sup>). DNA fragments were purified from the agarose gel by NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany) and sent for Sanger sequencing to StarSEQ® GmbH (Mainz, Germany). Nucleotide sequences were visualized using CHROMAS LITE v. 2.01 (Technelysium, South Brisbane, Australia) and aligned using MUSCLE (Edgar 2004) as implemented in MEGA 7 (Kumar et al. 2016). Identity searches of the resulting consensus sequences were performed in the GenBank database.

## Results

### *Molecular testing for DNB in La Sila Massif, Region Calabria*

Real-time PCR analysis showed that all DNA extracts from pine needle samples collected in La Sila Massif were amplifiable (Ct values for the 18S assay 15.4–27.1) and positive to *Dothistroma septosporum*, which had thus spread in this mountain plateau in natural forests of *P. nigra* subsp. *laricio* growing at elevation around 1200 MASL (Ct values for *D. septosporum* assay 25.9–32.7). Defoliation was widespread on *P. nigra* subsp. *laricio* and locally heavy (complete loss of needles other than the current season's ones) in the proximity of lakes, especially the Arvo Lake (Figure 2). In La Sila Massif, besides *P. nigra* subsp. *laricio*, which comprised the majority of tested individuals, a few plants belonging to other Pinaceae species, i.e. *P. sylvestris*, *P. radiata*, *Pseudotsuga menziesi* (Mirb.) Franco, and *Cedrus*

*atlantica* (Endl.) Manetti ex Carrière, although not severely damaged, showed red needle bands or dots and resulted positive to *D. septosporum* by real-time PCR. However no fruiting bodies were observed on these species. All samples were negative to *D. pini*, while positive controls, which were included in all real-time PCR reactions, resulted positive.

The  $\beta$ -tub2 gene region fragment (231 bp) amplified by conventional PCR and sequenced from a subsample of positive needle DNA extracts following the procedure by Ioos et al. (2010) showed 100% identity to *D. septosporum* reference strain Genbank ID: KX364411.1.

#### *Molecular testing for DNB in Paneveggio-Pale di San Martino and Val Sarentino, Region Trentino Alto-Adige*

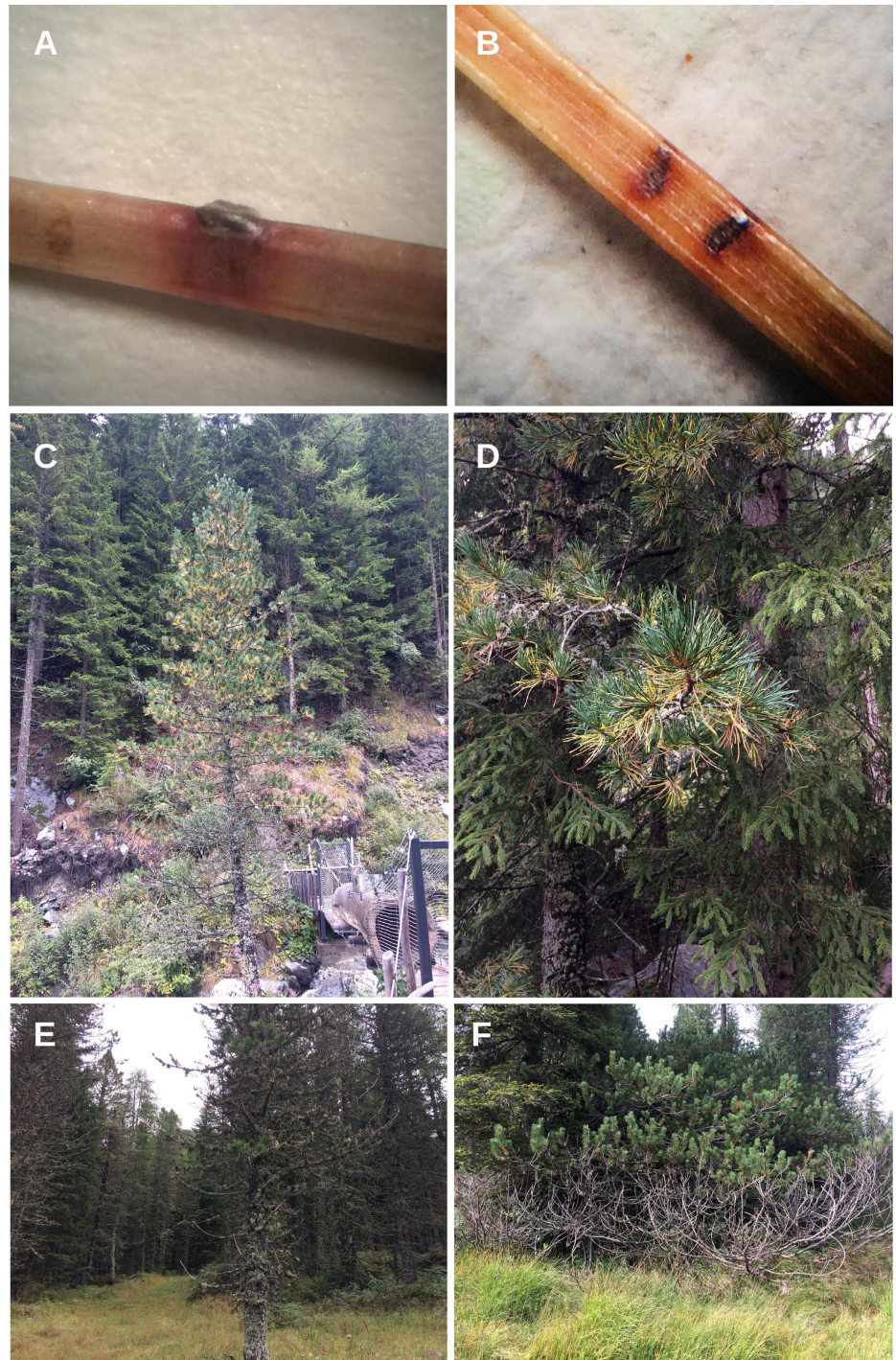
All pine needle extracts from Region Trentino Alto-Adige were amplifiable according to the internal control (Ct values for the 18S assay 15.3–23.3) and real-time PCR results showed that *D. septosporum* infection was widespread on *P. cembra* and *P. mugo* trees growing in natural forests both in the area of Colbricon (Paneveggio-Pale di San Martino, Trento) and at San Martino Reinswald and Valdurna, the two symptomatic sites in Val Sarentino (Bolzano) (Ct values for *D. septosporum* assay 25.9–32.7) (Table 1, Figures 1, 3). Defoliation was extensive at both sites and severe on the majority of plants of the two affected species, both adult trees and renovation. Around the Colbricon Ponds and the Valdurna Lake, and along streams in Val Sarentino, defoliation commonly reached life-threatening degrees on *P. cembra* individuals, which appeared almost needleless. The  $\beta$ -tub2 gene region fragment (231 bp) amplified by conventional PCR and sequenced from a subsample of positive needle DNA extracts following the procedure by Ioos et al. (2010) showed 100% identity to *D. septosporum* reference strain Genbank Accession number KX364411.1.

#### *Conidial morphology, isolation of fungi, DNA extraction from micelia and sequencing from samples from La Sila Massif, Paneveggio-Pale di San Martino and Val Sarentino*

Conidia from typical conidiomata on needle samples collected in La Sila Massif (site ID 3, Table 1) Paneveggio-Pale di San Martino (site ID 15, Table 1), and Val Sarentino (site ID 16, Table 1) were thin-walled, hyaline, smooth, fusiform to short-clavate, straight or more frequently curved, (1)2–3(5) septate,  $12\text{--}40 \times 2\text{--}3 \mu\text{m}$ , hence showing morphological features consistent with attribution to *D. septosporum* or *D. pini* (EPPO 2015).

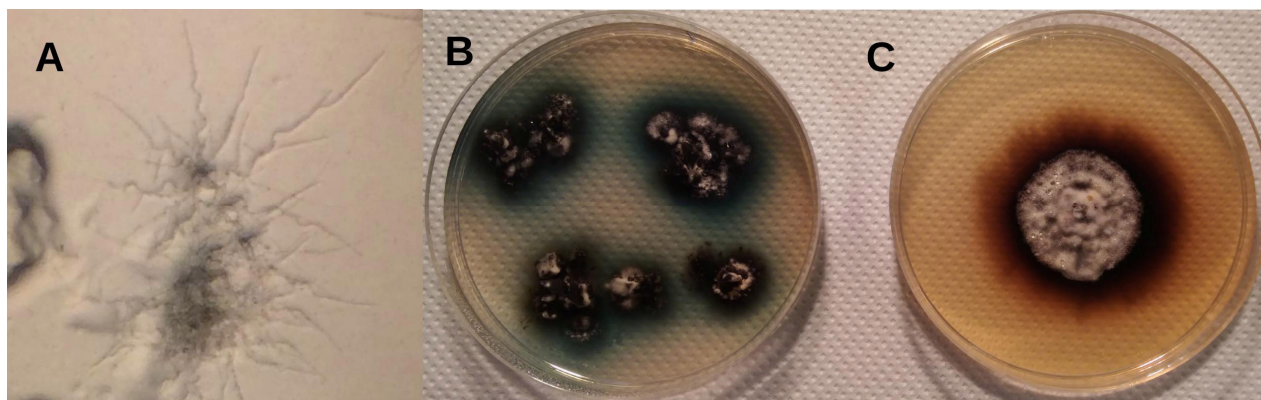
Dark brown colonies resembling those of *Dothistroma* species were isolated from typical conidiomata (Figure 4) from the same samples. Cultures were typically slow growing, had distinctive zig-zagging hyphae short after germination, and showed visible production of dothistromin, the red/blue colored toxin typically produced by *Dothistroma* species, after 1–2 weeks growth in culture (Mullett and Barnes 2012, Figure 4). The sequences of





**Figure 3.** Details of symptomatic *Pinus cembra* needles bearing *D. septosporum* conidiomata (top), symptomatic young and heavily defoliated adult *P. cembra* plants in Val Sarentino (A–E) (ID16 Figure 1 and Table 1, Alto Adige, Northern Italy), and defoliated *Pinus mugo* plants (F) in Colbricon (ID 15 Figure 1 and Table 1, Paneveggio Nature Park, Trentino, Northern Italy). Photographs by Luisa Ghelardini.

the ITS region obtained from three of these isolates (one per site: 494, bp, site ID 15; 479 bp, site ID 16; and 498 bp, site ID 3), when blasted against the GenBank database, showed 99–100% identity to strains of *D. septosporum*: Best hit Accession number MH865094.1 (sequences from sites IDs 15 and 16) and Accession number MG720608.1 (sequence from site ID3).



**Figure 4.** Colonies of *D. septosporum* isolated (A) in Valdurna (ID 16 Figure 1, Val Sarentino, Alto Adige, Northern Italy) showing the typical zig-zagging hyphae, and (B–C) in Bonolegno (ID 3 Figure 1, La Sila National Park, Calabria, Southern Italy) producing blue or red dothistromin pigmentation on PDA. Photographs by Luisa Ghelardini.

#### *Molecular testing for DNB at other sites in Italy*

At all other sites in Northern, Central and Southern Italy, where a culture independent survey was carried out using the same real-time PCR assays on needles of various host species (Table 1), displaying symptoms similar to DNB but bearing no conidiomata, all DNA samples resulted amplifiable (Ct values for the 18S assay 21.1–25.9) and negative to *D. septosporum* and *D. pini*, while positive controls for both *Dothistroma* species, which were included in all PCR reactions, resulted positive.

#### **Discussion**

In this work we have ascertained the presence of *Dothistroma septosporum* at several sites in Northeastern and Southern Italy, while *D. pini*, the morphologically identical congeneric species causing the same disease in neighboring European countries (Piškur et al. 2013; Piou and Ioos 2014; Queloz et al. 2014; Ondrušková et al. 2018) was not detected. In Italy the only published report of DNB pathogens was about nursery plants of *P. radiata* in the Region Calabria (Magnani 1977) and dates 40 years back, when morphological diagnostics did not enable *Dothistroma* species discrimination (Barnes et al. 2004). The presence of DNB in Region Friuli Venezia Giulia (Northeastern Italy) results from the regional forest inventory of the year 2015 (Bernardinelli 2016), but there is no specification about diagnostic methods applied. During the EU COST Action FP1102 DIAROD, specific real-time PCR assays carried out at a few locations in Northern and Central Italy, gave negative results (Dello Jacovo 2014). To date, according to the literature cited by EPPO (2020), DNB in Italy is restricted to *P. radiata* at the only reported location in Calabria.

Our results show that *D. septosporum* currently has in Italy a much larger distribution than reported, comprising several forest sites both in the South and in the North-East of the country. These new locations lie in some of the areas predicted at the highest risk of infection by *D. septosporum* on

the base of climatic suitability (Möykkynen et al. 2017), validating current predictive models and strengthening the concern that the pathogens' actual range in Italy be still underestimated. At all infected sites, the pathogen spread to native pine species (*P. nigra* subsp. *laricio* in the south and *P. mugo* and *P. cembra* in the north of the country), in naturally regenerating forests as in plantations. All tree species found positive to *D. septosporum* in this work are known hosts of the pathogen (Drenkhan et al. 2016; Mullett et al. 2018). However, while *P. mugo* and *P. nigra* subsp. *laricio*, which were found extensively and severely damaged in this study, are classified as sensitive species, *P. cembra* has rarely been reported as a host and it is believed to suffer minor damage (Bednářová et al. 2005). With this study, *P. cembra* individuals, both adult and young trees, are reported as severely affected by DNB and the DNB infection locally threatens the species' reproduction and survival. Unusually heavy damage on *P. cembra* forests might be due to repeated occurrence of favorable weather conditions in the study areas, to occurrence of locally conducive climatic conditions because of small-scale changes in topography, to intra-specific variation in host susceptibility (Dvorak et al. 2012; Perry et al. 2016; Woods et al. 2016; Ghelardini et al. 2017), or to coinfection by multiple pathogens (Johnson and Hoverman 2012); finally it might be the sign of a recent introduction, since *D. septosporum*, may become less virulent over a relatively short period of time in a new environment (Bradshaw et al. 2019).

At some locations, symptoms resembling those caused by *Dothistroma* sp. infection were observed but conidiomata were not found and real time PCR did not confirm the presence of *Dothistroma* species. All samples included in this study, except for those from Friuli Venezia Giulia, were previously tested, and resulted negative (Capretti et al. 2019), to species-specific real-time PCR (Ioos et al. 2010) for *Lecanosticta acicola* (von Thümen) Sydow, a threatful pine pathogen which is currently spreading in Europe, produces similar symptoms on infected pine branches as those caused by DNB and which may co-occur with *Dothistroma* species (van der Nest et al. 2019). Historically, *L. acicola* has been reported in Italy at a single location in a botanical garden on the shores of the Garda Lake (La Porta and Capretti 2000). It is known that sap-sucker insect species, such as for instance *Haematoloma dorsatum* (Ahrens), may produce patterns of red discoloration on pine needles similar to initial stages of DNB (Covassi et al. 1989; Sallé and Battisti 2016), which makes it necessary to apply molecular assays for fast, easy and reliable diagnosis of early stages of *Dothistroma* sp. infection. Surveillance could benefit from availability of specific and rapid molecular tools for *in-situ* detection of *Dothistroma* species, such as a Loop Mediated Isothermal Amplification (LAMP) assay, which we recently developed and are currently optimizing (Aglietti et al. 2019).

Infected forests by *D. septosporum* identified in this study are located within special areas of conservation (La Sila National Park and

Paneveggio-Pale di San Martino Nature Park), and comprise protected sites under Natura2000 Habitats directive (Pal. 42.65 Calabrian laricio pine forests, Pal. 42.32 Eastern Alpine calcicolous larch and arolla forests). They also include tree populations nationally designated for *in situ* conservation of forest genetic resources (Genetic Conservation Units GCUs) for *P. cembra* (GCU ITA00193, ITA00201) and *P. nigra* subsp. *laricio* (GCU ITA00032, ITA00034, 00151, ITA00156), which are conserved under the European Forest Genetic Resources Programme EUFORGEN. As regards to *P. nigra* subsp. *laricio*, the global population of this subspecies in the *P. nigra* complex is naturally separated into disjunct subpopulations, of which, the most important outside the island of Corsica (France) are in La Sila Massif. In all, including small scattered populations in Sicily and in the Southern Apennines, there are only 7–10 locations, where the specie's genetic diversity is preserved (Farjon 2013). The presence of *D. septosporum* at these sites is impairing, if not compromising, conservation measures for both of these species in Italy and would make it appropriate to revise their conservation status and requirements for conservation action.

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