COST-STSM SCIENTIFIC REPORT

Short Term Scientific Mission

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"Determining Invasiveness and Risk of Dothistroma"

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STSM Topic:	Molecular identification of Italian fungi causing Red Band Needle Blight in pines

Introduction

Dothistroma Needle Blight is a plant disease that affects several species of conifer trees, mostly pines. Main agents of the Blight are represented by the pathogens *Dothistoma septosporum* (Dorog.) Morelet and *Dothistoma pini* Hulbary. They were recently distinguished as two species by means of molecular techniques, as they are identical on the basis of morphological analysis. *Dothistroma septosporum* has a widespread distribution while the latter was found in North Central America and in Ukraine even if it is acknowledged that both the fungi require the same environmental conditions to develop (EFSA Panel on Plant Health (PLH), 2013).

The infection process occurs during summer season, in places with high humidity rates, which offer a favourable condition for pathogen transmission. Initial symptoms occur during the last autumnal months as first- and second years needles start showing yellow and dark spots in the point where infection happened (Brown & Webber, 2008). Rapidly these early manifestations of the disease turn into the typical red or red-brown bands that quickly disappear after needle cast (EFSA Panel on Plant Health (PLH), 2013). Therefore at this moment bands are no longer clear and the fading colouration can easily be confused with other needle's pathogen. However (other) characteristic symptoms can be used to identify the disease. Developing of the infection continues on younger needles that, after casting of previously infected needles, give an ill aspect to trees, with a general decrease in crown size and typical needle tufts at branch tops (Brown & Webber, 2008).

Dothistroma Needle Blight origin is still unknown. Some authors infer it as endemic of high altitude South America rain forest, because of the presence of both teleomorph mating types. Others suggest it as native of Nepalese and Himalayan pine forest, where the pathogen was found in remote local forest hundreds of miles distant from any other disease outbreak in the same region (EFSA Panel on Plant Health (PLH), 2013). Whatever its place of origin red band needle blight has now a worldwide spread, becoming nowadays one the most important *Pinus* spp. diseases in the world (Watt, Kriticos et al. 2009).

In Europe it probably enters and spreads through several ways; however it is more likely that both *Dothistroma septosporum* and *D. pini* have access towards planting of infected host, coming from infested regions. Mycelia and spores carried by such material is subsequently scattered by natural means or human activity such as transport or trading of infected plant/material. Thanks to favourable environmental conditions the pathogens can establish in almost the whole of Europe; estimated boundaries for its diffusion are assessed to be some high altitude areas in the Swiss mountains, eastern regions of Spain and on the border with Russian federation (EFSA Panel on Plant Health (PLH), 2013).

Amongst the conifer species affected, 84 belong to *Pinus* genus, the rest to *Larix* spp. and various genera of spruce. Host susceptibility to pathogen attack changes considerably in relation to several environmental and species-specific factors. To the former category of notable importance are soil composition, average temperature, and relative moisture that influence directly spread of the pathogen and inoculum success; also stands' provenance results important in terms of host susceptibility, as native stands of a particular species show major resistance compared with exotic plantations of the same. Regarding species-specific factors, it was recently discovered that some susceptible pine species show a gene-regulated resistance to infection. However presently there is not a unique level of sensitivity for each species; as reported from the table below some species show different kind of susceptibility, due to external causes (M. Mullet, 2012; EFSA Panel on Plant Health (PLH), 2013; M. S. Watt et al., 2009).

Highly susceptible	Moderately susceptible	Slightly susceptible
Pinus attenuata	*Pinus bungeana*	*Larix decidua*
Pinus× attenuradiata	*P. canariensis*	*Picea abies*
P. brutia	*P. caribaea*	*P. omorika*
P. canariensis	*P. clausa*	*P. pungens*
P. caribaea var. *hondurensis*	*P. coulteri*	*P. shrenkiana*
P. caribaea var. *caribaea*	*P. cubensis*	*P. sitchensis*
P. caribaea var. *bahamensis*	*P. densiflora*	*Pinus aristata*
P. cembroides	*P. echinata*	*P. ayacahuite*
P. contorta	*P. echinata×taeda*	*P. contorta*
P. contorta var. *latifolia*	*P. elliottii*	*P. coulteri*
P. engelmannii	*P. elliottii var. densa*	*P. devoniana*
P. halepensis	*P. flexilis*	*P. elliottii*
P. jeffreyi	*P. jeffreyi*	*P. elliottii var. densa*
P. muricata	*P. kesiya*	*P. hartwegii*
P. nigra	*P. lambertiana*	*P. heldreichii*
P. nigra subsp. laricio	*P. massoniana*	*P. koraiensis*
P. pinea	*P. monticola*	*P. merkusii*
P. ponderosa	*P. mugo*	*P. montezumae*
P. radiata	*P. mugo subsp. mugo*	*P. monticola*
P. sabineana	*P. muricata*	*P. nigra subsp. nigra*
P. strobus	*P. occidentalis*	*P. oocarpa*
P. sylvestris	*P. palustris*	*P. patula*
P. thunbergii	*P. pinaster*	*P. pseudostrobus*
	P. ponderosa var. scopulorum	*P. rigida*
	P. pungens	*P. sabineana*
	P. radiata var. binata	*P. serotina*
	P. resinosa	*P. sibirica*
	P. roxburghii	*P. strobus*
	P. strobiformis	*P. strobus var. chiapensis*
	P. strobus	*P. sylvestris*
	P. taeda	*P. tabuliformis*
		P. taeda
		P. torreyana
		P. wallichiana
		Pseudotsuga menziessi

Table 1. Host susceptibility to *Dothistroma septosporum* and *Dothistroma pini* (EFSA Panel on Plant Health (PLH), 2013)

In Italy the first report of *Dothistroma* spp. dates back to 1977 in "Presence of *Dothistroma pini* on *Pinus radiata*." by Magnani. At a later stage no other study of the pathogen was carried out, resulting in a complete lack of knowledge about its spread in Italy. This project is intending to detect and assess the presence of *Dothistroma* species in Italy by using new technologies and methods for molecular and morphological detection.

Italian needle samples were collected and analysed considering its grade of genetic variability in a broad range of several pine species. The samples represent a collection of native species such as *Pinus sylvestris, P. nigra, P. mugo, P. pinea, P. pinaster, P. brutia*, and exotic ones like *P. banksiana, P. gerardiana, P. canariensis, P. wallichiana*. Symptomatic pine needles were collected both in the field and from botanic and private gardens, across Central and North Italy.

Included were also samples from Spain. Here the molecular detection of *Dothistroma* was included in a larger research project, aiming to test pathogen incidence on *Pinus nigra* in a tree species diversity gradient. It consists of different mixtures of tree species considering both quantitative and qualitative composition of the species in the area. *Dothistroma septosporum* is present in the area and it is likely that some of the needles are infected by the fungus.

Both Italian and Spanish samples were then processed in the same way to detect *D. septosporum* presence on them. Other potential pathogens on pine hosts were also detected. They include: *Lophodermium seditiosum* Minter, Staley a. Millar, *Cyclaneusma minus verum* (Butin) Di Cosmo, Peredo & Minter, *Cyclaneusma minus simile* (Butin) Di Cosmo, Peredo & Minter, *Sirococcus conigenus* (D.C.) P. Cannon & Minter, *Lophodermium pinastri* (Schrad. ex Hook.) Chev., *Dothistroma pini, Lecanosticta acicola* (Thüm.) Syd.

Molecular analysis on *D. septosporum* in fact did not lead to any sign of the pathogen's presence, so that an additional in-depth analysis on other pathogens was carried out This range of parasitic fungi was chosen considering pathogenic incidence on genus *Pinus* and their relative presence frequency on surveyed sites. Most of them are pine needles specific pathogens such as *Cyclaneusma minus verum, Cyclaneusma minus simile* and *Lecanosticta acicola*.

Specifically *Lecanosticta acicola* (teleomorph: *Mycosphaerella dearnessi*) is the main agent causing the brown spot needle blight disease. It is presented like purplish-brown spots which carry the conidial spores (Ioos, Fabre et al. 2010). In Europe first observation was reported in Czech Republic, in 2007. In Italy was discovered by La Porta N. and Capretti P. in 2000 in the Botanical Garden in Gardone, in the Northeast region (Janoušek J. & al 2014; Jankovský L. & al 2009).

Cyclaneusma minus verum and *Cyclaneusma minus simile* are no more assessed as varieties although as distinct species. In Europe they are frequently reported as endophytes. In fact their association with disease is complicated because they can be found in asymptomatic tissue, acting like endophytes or latent pathogens. Along with *Cyclaneusma niveum*, *Cyclaneusma minus verum* is the main agent of Cyclaneusma Needle Cast, a serious disease that affects many *Pinus* species, present in all continents where *Pinus* species are grown (Unpublished data; McDougal et al., 2012; Watt, Rolando et al. 2012).

Sirococcus conigenus instead is known as a shoot pathogen of a large range of conifers like pines, spruces and hemlocks. It is particularly damaging for current year needles, inducing premature cast. This species, as some others of the same genus, is not easily recognizable as it follow an endophytic scheme of infection so that disease is not evident until any kind of recover strategy becomes useless (Bahnweg, Schubert et al. 2000).

Lophodermium spp is one of the most widespread fungal genera, living in about 30 pine species and varieties. Species analysed present quite similar morphological characteristics even if they occupy different ecological niches inside the same individual. In particular *Lophodermium seditiosum* is a serious pathogen of pine seedlings in nurseries, causing heavy infections characterized by needle loss in young plantations and nurseries. On the other hand *Lophpodermium pinastri* is a common endophyte which can be found also in the same needle occupied by *Lophodermium seditiosum*. In a succession order *Lophodermium seditiosum* firstly infects younger needles, opening the access to endophyte infection caused by *Lophodermium pinastri* and other endophytic fungi (Stenstrom and Ihrmark, 2005; Lazarev et al., 2007).

The aim of the present investigation was to detect pathogen presence in different geographical areas in Italy and Spain where the presence was still not assessed.

Material and methods

Sampling method

Spanish samples were collected in natural reserve of Alto Tajo, on June 2013. Only plant material coming from trees of *P. nigra* was analysed. Italian needle samples were gathered from several sites, mainly belonging to Northern and Central macroregions in both anthropological and natural environments, in the period ranging from February 2014 and May 2014.

Following a North-Centre region gradient, assessment areas are located in the city centres and outskirts of Trento, Milan, Grosseto, Rome and Terracina.

Only needles with evident infection markings were collected. Characteristic symptoms are considered yellow and tan spots that begin at needle tips while the basis remains green. Red and brownish bands situated across the needle's length and brownish tips are also accounted as distinctive characters. Needles were collected from lower branches through manual harvesting or with the assistance of telescopic pruning scissors for the higher specimens. Samples were then preserved in paper bags as to reduce the growth of other fungi.

Pinus species analysed

The assessment of the Italian samples was aiming to investigate the presence of *Dothistoma* in a range of different pine species present in Italian territory. Samples analysed were representative of exotic and native pine species already lodged for a long time in sampling areas. On the basis of their geo-climatic provenance was possible to distinguish two groups within the native species. Needles coming exclusively from Northern regions (Sampling areas: Trentino, Lombardia) belong to pine species of: *Pinus mugo* Turra, *P. sylvestris* L., *P. nigra* Arnold. Common environment for this species are mountain plans on Alps and Apennines range, usually gathered in high-flying bush stands or pines stands. Samples of *P. halepensis* Miller, *P. pinea* L., *P. pinaster* Aiton were all collected from the Central Italian regions. They are representative species of the Mediterranean scrub, growing in dunes and arid slopes near the coast. *P. brutia* Ten. and *P. leucodermis* Antoine were gathered from the Botanical Garden of Rome; in nature they are characteristic examples spread in Southern Italian regions, developing their life cycle in mountain pines stands and rock slopes .

Exotic samples were represented by the following species: *P. banksiana* Lamb., *P. canariensis* C. Sm., *P. gerardiana* Wall., *P. strobus* L., *P. wallichiana* Jackson, *P. radiata* Don. Within this group the latter two samples came from Northern regions; in conjuction with *P. strobus* cultivated plantations of both species can be found throughout the country, especially *P. radiata*. Originating from California, it was largely used as main cultivar for Southern regions' reforestation. All others species were come from the Botanical Garden of Rome, as exemplary of their natural equivalents. Specifically *P. banksiana* and *P. strobus* are native of boreal forests in North America while *P. gerardiana* and *P. wallichiana* have wide distribution in Himalayan regions, the former also surrounding Afghan and Pakistani borders at 1600 and 3300 m of altitude. In fine *P. canariensis* is an endemism hailing from Canary archipelago which represents the only pine native species in the islands (Climent, Tapias et al. 2004; Pignatti, 1974; Despland and Holue, 1997; Kant et al., 2006).

Molecular investigation

From each sample group a representative part was selected as to estimate pathogen composition in each community.

23 Spanish samples were analysed, chosen randomly from the main stock. Only needles grown during 2013 were picked up, as to also point out a temporal benchmark.

From Italian samples 62 out of 112 *Pine* spp. individuals were used for molecular analysis, selected on the basis of *Pinus* species density and composition characteristic of each assessment area.

DNA was extracted with the following procedure: needles were freeze-dried for 3-5 days and later grinded by Pre-Cellys (Bertin Technologies). In a second batch of extractions grinding method was replaced by ball mills, more suited for milling thick pine needles. In order to break cell membrane the detergent cetyltrimethylammonium bromide (CTAB) was used; as organic solvent was added chloroform.

Extracted DNA was purified using the DNeasy (Quiagen, Hilden, Germany) following the manufacturer's instruction. DNA concentration was assessed utilizing a UV spectrophotometer (NanoDrop, Thermo Scientific) and progressively diluted to working concentration of 0,5 $^{ng}/_{\mu l}$.

Both Italian and Spanish samples were initially assessed for the presence of fungal DNA using ITS specific primers.

The reagents applied for PCR Master Mix consist in molecular grade H_2O, Dream Buffer, dNTPs [200 µM], $MgCl_2$ [750 µM], Dream Taq Polymerase [0.01 µM].

PCR was conducted by using a GenAmp 9700 thermocycler (Applied Biosystems, Foster City, CA). The cycling conditions included an initial denaturation step at 95° C for 5 min; followed by 35 cycles of denaturation at 95° C for 30 s, annealing at 55° C for 30 s, and elongation at 72° C for 30 s; and a final extension at 72° C for 7 min.

After this preliminary survey *Dothistroma septosporum* specific primers were tested on both Italian and Spanish samples. β*tub*2 forward and reverse primers were used following subsequent conditions: an initial denaturation step at 95° C for 10 min was followed by 35 amplification cycles of denaturation at 95° C for 30 s, annealing at 60° C for 30 s and extension at 72° C for 1 min. The thermal cycling was ended by a final extension step at 72° C for 7 min (Ioos et al., 2010).

As this detection showed the absence of the pathogen in all samples analysed, other *Pinus* pathogens were analysed. Pathogens were chosen in accordance to frequency appearance in investigation areas and to the relative importance as *Pinus* parasitic association. PCR conditions and primer pairs reported in the table below were extrapolated by recent bibliography (Stenstrom and Ihrmark, 2005; Bahnweg, Schubert et al. 2000; Ioos et al., 2010; Unpublished data) .

All PCR products were separated by gel electrophoresis on 1% agarose gels. All PCR analyses were carried out using standard procedure to avoid cross-contamination DNA. A blank (sterile water) control was systematically processed for each reaction, in order to check the presence of contamination.

	Initial denaturation	Denaturation	Annealing	Elongation	Final elongation	Primer pairs (Forward/Reverse)
Lophodermium seditiosum	94° C for 5 min	94° C for 15 sec	64° C for 30 sec	72° C for 30 sec	72° C for 7 min	Ls 11/ Ls 12
Cyclaneusma minus verum	95° C for 3 min	95° C for 15 sec	63° C for 15 sec	72° C for 15 sec	72° C for 10 min	C. m. verum F/ C. m. verum R
Cyclaneusma minus simile	95° C for 3 min	95° C for 15 sec	63° C for 15 sec	72° C for 15 sec	72° C for 10 min	C. m. simile F/ C. m. simile R
Sirococcus conigenus	94° C for 4 min	94° C for 15 sec	66° C for 30 sec	72° C for 30 sec	72° C for 5 min	Sir 1/ Sir 6
Lophodermium pinastri	94° C for 5 min	94° C for 15 sec	68° C for 15 sec	72° C for 15 sec	72° C for 7 min	Lp 3/ Lp 6
Dothistroma pini	95° C for 10 min	95° C for 30 sec	60° C for 30 sec	72° C for 1 min	72° C for 10 min	Dp F/ Dp R
Lecanosticta acicola	95° C for 10 min	95° C for 30 sec	60° C for 30 sec	72° C for 1 min	72° C for 10 min	LA tef-F/ LA tef-R

Table 2. PCR cycle conditions and primer pairs.

Italian samples					
Macroregion	Assessment area	Host	*Lophodermium seditiosum*	*Cyclaneusma minus verum*	*Lophodermium pinastri*
North	Faedo pine stand	*P. mugo*	**Present**	**Present**	**Present**
	Giardino Fondazione Edmund Mach	*P. radiata*	**Present**	No present	**Present**
	Eremo S. Pancrazio, Sporminore	*P. sylvestris*	**Present**	No present	No present
Centre	Stand Uni. Tuscia, Viterbo	Exotic species	**Present**	No present	No present
	Stand Uni. Tuscia, Viterbo	Exotic species	**Present**	No present	No present
	Botanic Garden of Rome	*P. sylvestris*	No present	**Present**	No present
	Botanic Garden of Rome	*P. brutia*	No present	**Present**	No present
	Botanic Garden of Rome	*P. mugo*	No present	**Present**	No present
	Experimental Garden "La Sapienza", Rome	*P. halepensis*	No present	**Present**	No present
	Flacca street,Terracina	*P. pinastri*	**Present**	No present	No present

Table 3. Summary Italian samples results.

Spanish samples			
Sample Code	Assessment area	Host	*Lophodermium seditiosum*
SPA 2-50	Natural Reserve Alto Tajo,Castilla-La Mancha	*P. nigra*	Present
SPA 08-1	Natural Reserve Alto Tajo,Castilla-La Mancha	*P. nigra*	Present
SPA 08-2	Natural Reserve Alto Tajo,Castilla-La Mancha	*P. nigra*	Present

Table 4. Summary Spanish samples results.

Results

None of the assessed samples were found positive for infection caused by *Dothistroma septosporum*.

In order to estimate the presence of pathogenic fungi in the needles, some of the most common *Pinus* needles pathogen previously recognized in sampling areas were investigated. Different assortments of primers were analysed for each group. Specifically, for Spanish samples only main pathogens already found in sites analysed were evaluated. They were: *Dothistroma septosporum*, *Lophodemium seditiosum*, *Sirococcus conigenus*, *Cyclaneusma minus verum*, *Cyclaneusma minus simile*, *Lophodermium pinastri*. For the Italian needles, in addition to the above-mentioned species, was also tested the presence of *Lecanosticta acicola* and *Dothistroma pini*.

Most of tested primers were treated with the appropriate positive control; only in these cases bands on electrophoresis gel were a secure illustration of pathogen occurrence in needles. Primer pairs detected without positive control were: *Lecanosticta acicola*, *Cyclaneusma minus simile*, *Lophodermium pinastri* (only for Spanish samples). In this conditions pathogen presence in analysed samples cannot be confirmed. Because of the lack of positive control, part of Northern Italian samples were not investigated for the presence of *Cyclaneusma minus simile* (see Annex 1).

For the Italian samples 10 out of 62 samples presented positive results relative to 3 primer pairs. No strict association between sampling sites and discovered pathogen was noticed. Electrophoresis bands relative to following fungi were found: *Lophodermium seditiosum*, *Cyclaneusma minus verum*, *Lophodermium pinastri*. As noted from Table 3, single samples of *Pinus mugo* from Faedo and *Pinus radiata* from Giardino FEM show evidence of the presence of three and two multiple pathogens respectively. Both of them came from the same macro region but they are collected from different sites, even though they were close each other. No bands related to the other pathogens were shown (see Annex 1).

Three positive results from Spanish needles relative to *Lophodermium seditiosum* were found (Table 4). No bands related to the other pathogens were shown (see Annex 2).

Discussion and conclusions

Dothistroma septosporum is a genus-specific pathogen with a cosmopolitan distribution. In the last decades it has been evaluated as a pest also in Europe and its identification is important to prevent pathogen dispersal.

Fungus infection presents an extremely broad range of suitable environmental conditions: this can occur from tropical to subartic climate (Watt, Kriticos et al. 2009). Specifically, moisture level and temperature are key factors influencing infection results; they are determinant during the phases of inoculum and spore germination. High humidity is necessary for a good infection outcome as well as optimal temperatures that range from a minimum optimal of 5°C/ 12°C to 20°C/24°C as maximum optimal temperature. Also of importance is the dry period after inoculum: the longer the drought, the lower pathogen impact and further spread possibilities (EFSA Panel on Plant Health (PLH), 2013; Watt et al., 2011).

Throughout the course of this research no signs of *Dothistroma* spp were found. A possible reason of this lack is due to the incompatible environmental conditions characterizing several geographical regions analysed.

In Spain, Natural Reserve of Alto Tajo is characterized by Mediterranean climate, with a consistent continental character and moderate rainfalls. A wet and cold winter followed by a dry summer are main features of this area (Aguilar et al., 2012).

Those features do not match to environmental suitable condition for *D. septosporum* spreading. Therefore is credible to suppose pathogen's absence in surveyed area.

Northern Italian regions instead have a predominant continental climate, characterized by frigid winters with frequent snowfalls and mild-hot summers; temperatures range during the year covers values from -2°C to 25°C, in Trentino and from -1.6°C to 39°C in Milan. Moreover, frequent rainfalls are not so common during spring months when spore dispersal starts and develops.

Areas correspondent to Italian central regions should be distinguished in sites with Mediterranean climate and others also characterized by marks of continental climate features. This is the case of samples coming from Viterbo[1]; those areas are influenced by their peculiar position so that temperatures are warmer than in the Northern regions but still characterized by frequent rainfalls throughout the year and quite low winter temperature. On the other hand Rome, Grosseto and Terracina[1] present an ordinary Mediterranean climate, with winter rainfalls profuse enough to allow an uniform plant covering throughout the year; also temperature must be below 15°C but never decreases under freezing (0°C), granting a mild winter (Aschmann, 1973). A typical dry summer is common to all Centre Italy sites evaluated.

Comparing these environmental features with the (environmental) limits of *Dothistroma* spreading is definitively clear that those conditions do not match with areas analysed.

In order to discover if other common pine pathogens were affecting the samples, additional surveys were conducted on several fungal needle pathogens specific for *Pinus* species.

This investigation registered only few results, regarding 3 individuals out of 23 Spanish samples and 9 individuals out of 62 Italian samples. Spanish results coming from *Lophodermium pinastri* analysis were not considered reliable because of lack of positive control.

Spanish needles affected showed the presence of European widespread pathogen *Lophodermium seditiosum*, already observed in the area. *Pinus nigra* is an highly susceptible host. However, as

Notes: [1] Data coming from http://www.meteopiateda.it/stazioni-meteo/centro-italia/lazio.html website

favourable conditions were observed specifically in countries from North and central Europe, it can be hypothesized that risk of further diffusion in the area is low. Also *Lophodermium seditiosum* is a serious (problem) usually found in nurseries because of the proximity of great amount of seedlings with environmental transmission vehicles like high moisture (due to irrigation) that facilitate its spread in a closed place (Lazarev et al., 2007).

Five out of nine Italian infected samples were positive to *Cyclaneusma minus verum* specific primers. This fungus is the main agent of Cyclaneusma Needle Cast, an important disease involving many *Pinus* species worldwide. Most affected species are *Pinus radiata* and *Pinus sylvestris* where infection phases involve an endophytic stage that hinder disease identification. In this study *C. minus verum* was detected on the following species: *P. sylvestris*, *P. brutia*, *P. halepensis* and 2 specimen of *P. mugo*, coming respectively from Faedo pine stand, in Trentino and Botanic garden of Rome; the former 3 specimens are also coming from Roman sites. As in Europe it is often reported as fungal endophyte or saprophyte it could be inferred that role in this present survey (McDougal et al., 2012; Unpublished data). In Italy it was firstly reported as asymptomatic endophyte on needles of *Pinus uncinata* and *Pinus sylvestris* by Gonthier and Giordano (2010).

Remaining needles pointed out presence of *Lophodermium seditiosum* and *Lophodermium pinastri*. In particular they were detected in the same specimen of *Pinus mugo*, coming from Northern regions and *P. radiata* also coming from Trentino, in S. Michele all'Adige. Occurrence of more species on the same samples is an evidence of endophytism widely registered in recent bibliography (Schulz B., Boyle C.; 2005; Lazarev et al., 2007). Samples of *P. pinaster*, *P. sylvester* and one of the undertemined species hailing respectively from Terracina and the Botanical Garden of Rome showed presence of *Lophodermium seditiosum* as only pathogen detected. With the exception of *P. sylvestris* all other species are uncommon hosts for *Lophodermium seditiosum* (Lazarev et al., 2007).

In count of future perspectives a wider collection of pine species is required, in order to outline a broader and detailed framework of pathogens distribution in the macroregion assessed .

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Annex 1

Code	Tube number	ITS Primers	*Lophodermium seditiosum*	*Cyclaneusma minus verum*	*Lophodermium pinastri*	*Dothistroma septosporum* *Sirococcus conigenum* *Dothistroma pini* *Lecanosticta acicola*	*Cyclaneusma minus simile*
01 NIG	1 y	yes	no	no	no	no	no
02 WAL	2 y	yes	no	no	no	no	no
03 NIG	3 y	yes	no	no	no	no	no
04 WAL	4 yx	yes (very dim band)	no	no	no	no	no
11 BAN a	5 y	yes	no	no	no	no	no
11 BAN b	6 y	yes	no	no	no	no	no
11 BAN c	7 yx	yes (dim band)	no	no	no	no	no
11 SYL b	8 y	yes	no	yes (bright band)	no	no	no
11 WAL	9 yx	yes (bright band)	no	no	no	no	no
11 STR	10 yx	yes (bright band)	no	no	no	no	no
11 BRU	11 yx	yes (bright band)	no	yes (bright band)	no	no	no
11 MUG	12 yx	yes (bright band)	no	yes (bright multiple band)	no	no	no
11 CAN	13 yx	yes (bright band)	no	no	no	no	no
11 LEU	14 y	dim band	no	no	no	no	no
11 GER	15 yx	yes (bright band)	no	no	no	no	no
11 CEM	16 yx	yes (bright band)	no	no	no	no	no
11 SYL a	17 y	yes (dim band)	no	no	no	no	no

Code	Tube number	ITS Primers	*Lophodermium seditiosum*	*Cyclaneusma minus verum*	*Lophodermium pinastri*	*Dothistroma septosporum* *Sirococcus conigenum* *Dothistroma pini* *Lecanosticta acicola*	*Cyclaneusma minus simile*
12 HAL	18 y	yes (dim band)	no	yes (dim multiple band)	no	no	no
13 HAL	19 yx	yes (dim band)	no	no	no	no	no
13 PIN.A b	20 yx	yes (dim band)	no	no	no	no	no
13 PIN.A d	21 yx	yes (dim band)	no	no	no	no	no
13 PIN.A a	22 yx	yes (dim band)	no	no	no	no	no
13 PIN.A c	23 yx	yes (dim band)	no	no	no	no	no
13 PIN.A e	24 y	yes	no	no	no	no	no
14 PIN.Ab	25 yx	yes (bright band)	no	no	no	no	no
14 PIN.A a	26 yx	yes (bright band)	no	no	no	no	no
20 HAL a	27 yx	yes (bright band)	no	no	no	no	no
20 HAL b	28 y	yes (bright band)	no	no	no	no	no
31 PIN.A	29 yx	yes (bright band)	no	no	no	no	no
31 PIN.T	30 yx	yes (bright band)	no	no	no	no	no
31 HAL	31 y	yes (bright band)	no	no	no	no	no
32 NIG a	32 yx	yes (bright band)	no	no	no	no	no
32 NIG b	33 y	yes	no	no	no	no	no

Code	Tube number	ITS Primers	*Lophodermium seditiosum*	*Cyclaneusma minus verum*	*Lophodermium pinastri*	*Dothistroma septosporum* *Sirococcus conigenum* *Dothistroma pini* *Lecanosticta acicola*	*Cyclaneusma minus simile*
32 NIG c	34 y	yes (bright band)	no	no	no	no	no
32 HAL a	35 y	yes (bright band)	no	no	no	no	no
32 HAL b	36 y	yes (bright band)	no	no	no	no	no
32 PIN.A	37 y	yes (bright band)	no	no	no	no	no
33 PIN.A a	38 y	yes (bright band)	no	no	no	no	no
33 PIN.A b	39 y	yes (bright band)	no	no	no	no	no
33 PIN.A c	40 y	yes (bright band)	no	no	no	no	no
34 PIN.A	41 y	yes (bright band)	no	no	no	no	no
34 PIN.T a	42 y	yes (bright band)	no	no	no	no	no
34 PIN.T b	43 y	yes (bright band)	no	no	no	no	no
35 HAL a	44 yx	yes (bright band)	no	no	no	no	no
35 HAL b	45 y	yes (bright band)	no	no	no	no	no
35 PIN.T a	46 y	yes (bright band)	no	no	no	no	no
35 PIN.T b	47 y	yes (bright band)	yes (bright band)	no	no	no	no
35 PIN.A	48 y	yes (bright band)	no	no	no	no	no

Code	Tube number	ITS Primers	*Lophodermium seditiosum*	*Cyclaneusma minus verum*	*Lophodermium pinastri*	*Dothistroma septosporum* *Sirococcus conigenum* *Dothistroma pini* *Lecanosticta acicola*	*Cyclaneusma minus simile*
41 MUG	49 y	yes	yes (dim band)	yes (dim band)	yes (bright band)	no	not detected
42 MUG	50 y	yes (bright band)	no	no	no	no	not detected
41 RAD	51 y	yes (bright band)	no	no	no	no	not detected
42 RAD	52 y	yes (bright band)	yes	no	yes (bright band)	no	not detected
41 WAL	53 y	yes	no	no	no	no	not detected
42 WAL	54 y	yes	no	no	no	no	not detected
40 SYL	55 y	yes (bright band)	no	no	no	no	not detected
50 SYL	56 y	yes	yes	no	no	no	not detected
51 Species undetermined	57 y	yes (bright band)	other length	no	no	no	not detected
52 Species undetermined	58 y	yes (bright band)	no	no	no	no	not detected
53 Species undetermined	59 y	yes (bright band)	no	no	no	no	not detected
54 Species undetermined	60 y	yes (bright band)	yes (dim band)	no	no	no	not detected
55 Species undetermined	61 y	yes (bright band)	no	no	no	no	not detected
56 PIN.T	62 y	yes (bright band)	no	no	no	no	not detected

Legends

yes = presence of band in gel after PCR
no= no band present
x = second attempt of extraction due to negative result in ITS primers detection

Acronym	Species
BAN	*P. banksiana*
BRU	*P. brutia*
CAN	*P. canariensis*
CEM	*P. cembra*
GER	*P. gerardiana*
HAL	*P. halepensis*
LEU	*P. leucodermis*
MUG	*P. mugo*
NIG	*P. nigra*
PIN.A	*P. pinea*
PIN.T	*P. pinaster*
RAD	*P. radiata*
STR	*P. strobus*
SYL	*P. sylvestris*
WAL	*P. wallichiana*

Area	Location	Code
Milan	Vesuvio Square	01
Milan	Po Square	02
Milan	Baggio Park	03
Milan	Parco Sempione	04
Rome	Botanic garden "La Sapienza	11
Rome	Experimental garden "La Sapienza"	12
Rome	Villa Doria Pamphili	13
Rome	University La Sapienza	14
Sperlonga	Statal street Sperlonga-Itri	20
Terracina	"La Fossata"	31
Terracina	S. Silviano	32
Terracina	"La pineta"	33
Terracina	Streets near my home	34
Terracina	Flacca street	35

Macroregion	Detailed area	Species	Code
Trentino	Edmund Mach Garden	*Wallichiana*	41
Trentino	Edmund Mach Garden	*Wallichiana*	42
Trentino	Edmund Mach Garden	*Radiata*	41
Trentino	Edmund Mach Garden	*Radiata*	42
Trentino	Faedo pine stand	*Mugo*	41
Trentino	Faedo pine stand	*Mugo*	42
Trentino	Faedo pine stand	*Sylvestris*	40
Trentino	Eremo S. Pancrazio (Sporminore)	*Sylvestris*	50
Grosseto-Viterbo	Tuscia University pine stand	Undetermined species	51
Grosseto-Viterbo	Tuscia University pine stand	Undetermined species	52
Grosseto-Viterbo	Tuscia University pine stand	Undetermined species	53
Grosseto-Viterbo	Tuscia University pine stand	Undetermined species	54
Grosseto-Viterbo	Tuscia University pine stand	Undetermined species	55
Grosseto-Viterbo	Albinia-Talamone	*Pinastri*	56

Annex 2

Code	Tube number	ITS Primers	*Lophodermium seditiosum*	*Dothistroma septosporum* *Sirococcus conigenus* *Cyclaneusma minus verum* *Cyclaneusma minus simile*	*Lophodermium pinastri*
SPA 15-38	1	yes (bright band)	no	no	yes (low band)
SPA 24-53	2	yes (bright band)	no	no	yes (multiple band)
SPA 26-8	3	yes (dim band)	no	no	yes (multiple band)
SPA 2-50	4	yes (dim band)	yes (dim band)	no	yes (low band)
SPA 23-33	5	yes (bright band)	no	no	yes (multiple band)
SPA 35-37	6	yes (bright band)	no	no	yes (multiple band)
SPA 13-NN5	7 x	yes (bright band)	no	no	yes (multiple band)
SPA 12-44	8 x	yes (bright band)	no	no	yes (multiple band)
SPA 12-41	9 x	yes (dim band)	no	no	yes (multiple band)
SPA 7-23	10	yes (dim band)	no	no	yes (multiple band)
SPA 26-NN1	12	yes (dim band)	no	no	yes (multiple band)
SPA 25-5	13	yes (bright band)	no	no	yes (bright band)
SPA 22-51	14	yes (bright band)	no	no	yes (multiple band)
SPA 36-26	15	yes (bright band)	no	no	yes (multiple band)
SPA 25-9	16	yes (dim band)	no	no	yes (multiple band)
SPA 14-25	17 x	yes (bright band)	no	no	yes (multiple band)
SPA 12-24	18	yes (dim band)	no	no	yes (multiple band)
SPA 06-30	19 x	yes (bright band)	no	no	yes (multiple band)
SPA 06-37	20	yes (bright band)	no	no	yes (multiple band)
SPA 08-1	21 x	yes (smerring bright band)	yes	no	yes (multiple band)
SPA 03-18	22	yes (bright band)	no	no	yes
SPA 08-2	23 x	yes (smerring bright band)	yes (dim band)	no	yes
SPA 03-33	24	yes (bright band)	no	no	yes (multiple band)

Legend

yes = presence of band in gel after PCR
no= no band present
x = second attempt of extraction due to needle thickness