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**Article**

**Pestalotiopsis pini** sp. nov., an Emerging Pathogen on Stone Pine (*Pinus pinea* L.)

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**Abstract:** Research Highlights: *Pestalotiopsis pini* sp. nov. is an emerging pathogen on stone pine, *Pinus pinea* L., in Portugal. Background and Objectives: Stone pine is one of the most important forest tree species in Portugal and in the whole Mediterranean basin. *Pestalotiopsis* species are common endophytes, saprobes or pathogens in a variety of hosts and environments. The objective of the present study was to identify the *Pestalotiopsis* species associated with the symptomatic stone pine trees. Materials and Methods: Samples of stone pine trees showing shoot blight and stem necrosis were obtained from stone pine orchards and urban areas in Portugal, and the isolated *Pestalotiopsis* species were identified based on morphology and combined ITS, *TEF* and *TUB* DNA sequence data. Artificial inoculations on one-year-old stone pine seedlings were performed with the two species most frequently found in association with shoot blight disease. Results: Five *Pestalotiopsis* spp. were isolated. A taxonomic novelty, *Pestalotiopsis pini* is described, representing a new pathogen for stone pine. Conclusions: *Pestalotiopsis* species may represent a threat to the health of pine forests in the Mediterranean basin. Future research should be done in order to increase our knowledge about the potential impact of pestalotioid species in stone pine, in order to develop management strategies against these pathogens.

Keywords: dieback; Mediterranean forest; multi-locus phylogeny; pathogenicity; pestalotioid fungi

1. Introduction

Stone pine, *Pinus pinea* L., is one of the most important forestry species in Portugal and the Mediterranean basin. Stone pine forests play an important role in the economy of the areas where they are planted, especially due to the high value of edible pine nuts, which are the main resource of this industry [1]. *Pinus pinea* is broadly considered a robust species. In recent years, pine nut production has been decreasing due to several factors, including pests and diseases [1,2].

*Pestalotiopsis* is a widely distributed genus of appendage-bearing conidia belonging to the family Sporocadaceae [3]. Fungi within this genus are normally considered secondary pathogens that can be responsible for a variety of plant diseases, including cankers, dieback, leaf spots, needle blight, tip blight,
grey blight, severe chlorosis, fruit rots and various post-harvest diseases [4–11]. Species belonging to this genus are also commonly isolated as endophytes, and due to their ability to switch nutritional modes, many endophytic and plant pathogenic Pestalotiopsis species persist as saprobes [9,12].

Pestalotiopsis is distinguished from other pestalotioid genera in the family Sporocadaceae (Heterotruncatella, Neopestalotiopsis, Pseudopestalotiopsis and Truncatella) by the number of conidium cells and by the pigmentation of its median cells [9]. Pestalotiopsis can be easily identified based on its five-celled, fusoid conidia, with three brown concolourous median cells and hyaline end cells; Neopestalotiopsis can be distinguished from Pestalotiopsis by its five-celled, fusoid conidia, with versicolourous median cells; Pseudopestalotiopsis can be distinguished based on its five-celled, fusoid conidia, with three dark concolourous median cells; Truncatella and Heterotruncatella are easily identified based on their four-celled, fusoid conidia [3,9]. Nevertheless, identification to species level solely based on morphology is difficult, since the morphological characters used to differentiate species are limited, variable and may be influenced by different hosts and environments [10,13]. Combined phylogenetic analysis of the internal transcribed spacer of ribosomal DNA (ITS), partial β-tubulin (TUB) and partial translation elongation factor 1-alpha (TEF) DNA sequence data is often required for accurate species identification [3,7,9,10,12].

Few studies have been conducted regarding the pathogenicity of Pestalotiopsis species on pine tree species. Nonetheless, diverse studies obtained several Pestalotiopsis species as endophytes in Pinus and other conifers [9,14–18]. Hu et al. [16] reported the isolation of 19 different Pestalotiopsis species as endophytes from bark and needles of Pinus armandii Franch. in China. Botella and Diez [14] reported the isolation of a Pestalotiopsis sp. from Pinus halepensis Mill. in Spain, and Maharachchikumbura et al. [9] referred to a Pestalotiopsis sp. isolated from a Pinus sp. in China. Pestalotiopsis species have also been isolated as endophytes from pine seeds of Pinus armandii in China [17] and several other pine species across Europe and North America [15].

The objective of the present study was to identify the Pestalotiopsis species associated with stone pine diseases in pine orchards and urban areas across the mainland of Portugal, based on both morphological characters and multigene DNA phylogenetic inference.

2. Materials and Methods

2.1. Fungal Isolation

Isolates were obtained from samples of Pinus pinea showing shoot blight, trunk necrosis, needle blight and pine cone decay. A sample of Pinus pinaster Aiton with shoot blight was also analysed. After macro- and microscopic observation of the sampled material, small pieces from the leading edge of the lesions were surface sterilized for 1 min in 1% NaClO and plated onto potato dextrose agar (PDA) amended with 0.5 mg/mL of streptomycin sulphate in order to avoid bacterial growth. Materials were incubated for seven days with a 12 h light period at 23 ± 2 °C. The hyphal tips of fungi emerging from tissue pieces were transferred to PDA, and single-spore cultures were subsequently established. Fungal isolates were deposited in the culture collection of INIAV Institute (Micoteca da Estação Agronômica Nacional (MEAN)) (Table 1).
Table 1. Details of *Pestalotiopsis* isolates obtained in this study (bold) and of strains representing species of *Pestalotiopsis* and related genera retrieved from GenBank and used in phylogenetic analyses.

| Species                      | Collection No. ¹ | Host/Source                                      | Country      | Collection Year | GenBank Accession Number ² |
|------------------------------|------------------|-------------------------------------------------|--------------|-----------------|----------------------------|
| Neopestalotiopsis australis  | CBS 114159       | *Telopea* sp.                                    | Australia    | 1999            | KM199348 KM199537 KM199432 |
| Neopestalotiopsis protarum   | CBS 114178       | *Leucocepernum cuneiforme*                      | Zimbabwe     | -               | LI853105 KM199542 KM199463 |
| Pestalotiopsis adusta        | ICMP 6088        | Refrigerator door PVC gasket                     | Fiji         | 1933            | KM199316 KM199489 KM199414 |
| Pestalotiopsis adusta        | CBS 263.33       | *Rhododendron ponticum*                         | Netherlands  | -               | KM199316 KM199489 KM199414 |
| Pestalotiopsis agarstorum    | LC6301           | *Camellia sinensis*                              | China        | -               | KX895015 KX895234 KX895348 |
| Pestalotiopsis anamarcaevarum| IFRDCC 2397      | *Mangifera indica*                               | USA          | -               | KC247154 KC247156 KC247155 |
| Pestalotiopsis arcuothii     | CBS 433.65       | *Arceuthobium campylopodum f. abietinum shoot*,  | USA          | -               | MH554046 MH554481 MH554722 |
|                             |                  | on *Abies amabilis*                              | USA          | -               | MH554046 MH554481 MH554722 |
| Pestalotiopsis arcuothii     | CBS 434.65       | *Arceuthobium campylopodum f. tsugense seed*, on | USA          | -               | MH554046 MH554481 MH554722 |
|                              |                  | *Tsuga heterophylla*                             | USA          | -               | MH554046 MH554481 MH554722 |
| Pestalotiopsis arengae       | CBS 331.92       | *Arenga undulatifolia*                           | Singapore    | 1991            | KM199340 KM199515 KM199426 |
| Pestalotiopsis australasiae  | CBS 114126       | *Knightia* sp.                                   | New Zealand  | 2002            | KM199297 KM199499 KM199409 |
| Pestalotiopsis australasiae  | CBS 114141       | *Protea cv. ‘Pink Ice’                          | Australia    | 1999            | KM199298 KM199501 KM199410 |
| Pestalotiopsis australasiae  | CBS 114193       | *Grevillea* sp.                                  | Australia    | 1999            | KM199332 KM199475 KM199383 |
| Pestalotiopsis australasiae  | CBS 119350       | *Brachyophyllum*                                 | South Africa | 2000            | KM199333 KM199476 KM199384 |
| Pestalotiopsis australasia   | MEAN 1096 = CPC  | *Pinus pinea*, blighted shoot                     | Portugal     | 2014            | MT374679 MT374692 MT374704 |
|                             | 36750 = CBS 146843|                                                 | Portugal     | 2014            | MT374679 MT374692 MT374704 |
| Pestalotiopsis australis     | MEAN 1109        | *Pinus pinea*, blighted shoot                     | Portugal     | 2017            | MT374683 - MT374708        |
|                             | MEAN 1110        | *Pinus pinea*, blighted shoot                     | Portugal     | 2017            | MT374684 MT374696 MT374709 |
| Pestalotiopsis australis     | MEAN 1111        | *Pinus pinea*, blighted shoot                     | Portugal     | 2017            | MT374685 MT374697 MT374710 |
|                             | MEAN 1112        | *Pinus pinea*, blighted shoot                     | Portugal     | 2017            | MT374686 MT374698 MT374711 |
| Pestalotiopsis biciliata     | CBS 124463       | *Platania x hispanica*                           | Slovakia     | -               | KM199308 KM199505 KM199399 |
| Pestalotiopsis biciliata     | CBS 236.38       | *Paeonia* sp.                                    | Italy        | 1938            | KM199309 KM199506 KM199401 |
| Pestalotiopsis biciliata     | MEAN 1168        | *Pinus pinea*, dry 1st-year conelet              | Portugal     | 2019            | MT374690 MT374702 MT374715 |
|                             | LC2988           | *Camellia* sp.                                   | China        | -               | KX894933 KX895150 KX895265 |
| Pestalotiopsis brassiaca     | CBS 170.26       | *Brassica napus*                                 | New Zealand  | 1926            | KM199379 KM199558 -        |
| Pestalotiopsis camelliae     | CBS 443.62       | *Camellia sinensis*                              | Turkey       | -               | KM199336 KM199512 KM199424 |
| Pestalotiopsis camelliae     | MFLUCC 12-0277   | *Camellia japonica*                              | China        | -               | JX399010 JX399074 JX399041 |
|                             | CBS 113607       | -                                                 | Italy        | 1971            | KM199326 KM199473 KM199391 |
| Pestalotiopsis chamaeropsis  | CBS 186.71       | *Chamaerops humilis*                             | China        | -               | KX894961 KX895178 KX895293 |
|                             | LC2322           | *Camellia sinensis*                              | China        | -               | KX894961 KX895178 KX895293 |
| Species | Collection No. | Host/Source | Country | Collection Year | GenBank Accession Number |
|---------|---------------|------------|---------|-----------------|--------------------------|
| Pestalotiopsis dilucida | LC8184 | Camellia sinensis | China | - | KY464138 KY464148 KY464158 |
| Pestalotiopsis diplodiae | CBS 115857 | Diplodia glaucescens | Hong Kong | 2001 | KM199320 KM199486 KM199419 |
| Pestalotiopsis disseminata | CBS 118552 | Eucalyptus botryoides | New Zealand | - | MH553986 MH554410 MH554652 |
| Pestalotiopsis disseminata | CBS 143904 | Persia americana | New Zealand | - | MH554132 MH554587 MH554685 |
| Pestalotiopsis disseminata | MEAN 1165 | Pinus pinea, blighted shoot | Portugal (Cascais) | 2018 | MT374687 MT374699 MT374712 |
| Pestalotiopsis disseminata | MEAN 1166 | Pinus pinea, blighted shoot | Portugal (Cascais) | 2018 | MT374688 MT374700 MT374713 |
| Pestalotiopsis diversiseta | MFLUCC 12-0054 | on dead grass | Taiwan | - | MH5809381 MH5809389 MH5809385 |
| Pestalotiopsis gaultheriae | IFRD 411-014 | Gaultheria forrestii | China | - | KCS73805 KCS73812 KCS73819 |
| Pestalotiopsis gibbosae | NOF 3175 | Gaultheria shallon | Canada | - | LC311589 LC311591 LC311590 |
| Pestalotiopsis gluviana | CBS 114491 | Leucocerpermus cv. 'Coral' | USA | 1999 | KM199339 KM199514 KM199428 |
| Pestalotiopsis hispanica | CBS 115,39 | Protea cv. 'Susara' | Spain | - | MH553981 MH554399 MH554640 |
| Pestalotiopsis hollandica | MEAN 1091 = CPC 36745 = CBS 146839 | Pinus pinea, blighted shoot | Portugal (Carregal do Sal) | 2014 | MT374678 MT374691 MT374703 |
| Pestalotiopsis humpicola | CBS 115450 | Ilex cinerea | Hong Kong | 2002 | KM199319 KM199487 KM199418 |
| Pestalotiopsis humpicola | CBS 336.97 | soil in tropical forest | Papua New Guinea | 1985 | KM199317 KM199484 KM199420 |
| Pestalotiopsis inflata | MFLUCC 12-0270 | unidentified tree | China | - | JX390008 JX390092 JX390039 |
| Pestalotiopsis intermedia | MFLUCC 12-0259 | unidentified tree | Italy | 2011 | KP781878 KP781881 KP781882 |
| Pestalotiopsis italiana | MFLUCC 12-0657 | Cupressus glabra | Papua New Guinea | - | KM199380 KM199554 KM199468 |
| Pestalotiopsis jesteri | CBS 109550 | Fragraea bodenii | Papua New Guinea | - | KM199302 KM199520 KM199395 |
| Pestalotiopsis jiangxensis | LC4999 | Camellia sp. | China | - | KX895009 KX895227 KX895341 |
| Pestalotiopsis jinjiangenensis | LC6056 | Camellia sinensis | China | - | KX895028 KX895247 KX895361 |
| Pestalotiopsis kengana | CBS 442.67 | Coffea sp. | Kenya | 1967 | KM199302 KM199502 KM199395 |
| Pestalotiopsis knightiae | CBS 114138 | Knightia sp. | New Zealand | - | KM199310 KM199497 KM199408 |
| Pestalotiopsis leucadendri | CBS 121417 | Leucadendron sp. | South Africa | - | MH553981 MH554412 MH554654 |
| Pestalotiopsis liculicalia | HGUP 4087 | Liculica grandis | China | 2012 | KC492509 KC481684 KC481683 |
| Pestalotiopsis linearis | MFLUCC 12-0271 | Trachelospermum sp. | China | - | JX390092 JX390085 JX390027 |
| Pestalotiopsis longipinnulata | LC3013 | Camellia sinensis | China | - | KX894939 KX895156 KX895271 |
| Pestalotiopsis luisianensis | LC4344 | Camellia sp. | China | - | KX895005 KX895223 KX895337 |
| Pestalotiopsis macadamiiae | BRIP 63738b | Macadamia integrifolia | Australia | - | KX186588 KX186621 KX186680 |
| Pestalotiopsis malayana | CBS 102220 | Maccarangia triobla | Malaysia | 1999 | KM199306 KM199482 KM199411 |
| Pestalotiopsis monochriata | CBS 144.97 | Quercus robur | Netherlands | 1996 | KM199337 KM199479 KM199486 |
| Pestalotiopsis nodulosa | NTUCC 17-011 | on leaf of Neolitsea villosa | Taiwan | - | MH5809381 MH5809391 MH5809387 |

1. Collection No.
2. GenBank Accession Number
| Species                          | Collection No. | Host/Source        | Country          | Collection Year | GenBank Accession Number |
|---------------------------------|----------------|--------------------|------------------|----------------|--------------------------|
| **Pestalotiopsis novae-hollandiae** CBS 130973 | Bankia grandis | Australia          | 2010             | KM199337        | KM199511 KM199425       |
| Pestalotiopsis orae CBS 353.69 | Orgia sativa   | Denmark            | -                | KM199299        | KM199496 KM199398       |
| Pestalotiopsis pallidoloeae MAFF 240993 | Pieris japonica | Japan              | -                | NR11022         | LC31585 LC31584         |
| Pestalotiopsis pippuna CBS 531.96 | soil along the coast | Papua New Guinea | 1995            | KM199321        | KM199491 KM199413       |
| Pestalotiopsis pura CBS 114972 | Leaf           | Hong Kong          | -                | MH553980        | MH554397 MH704625       |
| Pestalotiopsis pura CBS 278.35  | Lecotoehe fontanesiana | China              | 1935            | KM199313        | KM199509 KM199405       |
| Pestalotiopsis photinicolor GZCC 16-0028* | Photinia serrulata | China              | 2015            | KY092404        | KY047662 KY047663       |
| **Pestalotiopsis pinisp. nov.** MEAN 1092 = CPC 36746 = CBS 146840 | Pinus pinea, blighted shoot | Portugal (Salvaterra de Magos) | 2016 | MT374680 | MT374693 MT374705 |
| **Pestalotiopsis pinisp. nov.** MEAN 1094 = CPC 36748 = CBS 146841 | Pinus pinea, trunk of declining tree (necrosis and salmon- pinkish discoloration of wood) | Portugal (Lisbon) | 2017 | MT374681 | MT374694 MT374706 |
| **Pestalotiopsis pinisp. nov.** MEAN 1095 = CPC 36749 = CBS 146842 | Pinus pinea, blighted shoot | Portugal | 2018 | MT374682 | MT374695 MT374707 |
| Pestalotiopsis portugalllica CBS 684.85 | Camellia japonica | New Zealand        | 1948             | KM199335        | KM199510 KM199422       |
| Pestalotiopsis portugalllica CBS 393.48 | -               | Portugal           | 1948             | KM199335        | KM199510 KM199422       |
| Pestalotiopsis rhizophorae MFLUCC 17-0416 | Rizosphora apiculata | Thailand          | 2011             | KM764283        | KM764327 KM764349       |
| Pestalotiopsis rhododendri IRFDC 2399 | Rhododendron sinogrande | China          | -                | KC337804        | KC337811 KC337818       |
| Pestalotiopsis rhododendri CBS 144024 | Pinus sp. | Zimbabwe           | -                | MH554109        | MH554543 MH554782       |
| Pestalotiopsis rhodomyrtus HGUP 4230 | Rhodomyrtus tomentosa | China          | 2011             | KF112648        | KF112645 KF112642       |
| Pestalotiopsis rhodomyrtus LC3413 | Camellia sinensis | China            | -                | KX854981        | KX854982 KX854983       |
| Pestalotiopsis rosea MFLUCC 12-0258 | Pinus sp. | Portugal           | 1925             | KM199330        | KM199478 KM199393       |
| Pestalotiopsis scoparia CBS 176.25 | Chamaecyparis sp. | -                | 1925             | KM199330        | KM199478 KM199393       |
| Pestalotiopsis sequoiae MFLUCC 13-0399 | Sequoia sempervirens | Italy          | 2011             | KX572339        | - -                   |
| Pestalotiopsis sp. 7_FL_2019 CBS 110326 | Pinus sp. | USA               | -                | MH553957        | MH554375 MH554616       |
| Pestalotiopsis sp. 7_FL_2019 CBS 127.80 | Pinus radiata | Chile            | -                | MH553995        | MH554422 MH554664       |
| Pestalotiopsis spathulata CBS 356.86 | Guevina avellana | Chile            | 1961             | KM199338        | KM199515 KM199423       |
| Pestalotiopsis spathulappendiculata CBS 144035 | Phoenix canariensis | Australia        | -                | MH554172        | MH554607 MH554845       |
| Pestalotiopsis telopae CBS 114137 | Protea cv. 'Pink Ice' | Australia       | 1999             | KM199301        | KM199539 KM199466       |
| Pestalotiopsis telopae CBS 114161 | Telopae sp. | Australia          | 1999             | KM199296        | KM199580 KM199403       |
| Pestalotiopsis terricola CBS 141.69 | Soil           | Pacific Islands   | -                | MH554004        | MH554438 MH554680       |
| Pestalotiopsis thailandica MFLUCC 17-1616 | Rizosphora apiculata | Thailand         | 2016             | MK764285        | MK764329 MK764351       |
| Pestalotiopsis trachicarpica MFLUCC 2440 | Trachycarpus fortunii | China    | -                | JQ845947        | JQ845946 JQ845945       |
| Pestalotiopsis uniclor MFLUCC 12-0275 | unidentified tree | China            | -                | JX399098        | JX399063 JX399029       |
| Pestalotiopsis uniclor MFLUCC 12-0276 | Rhododendron sp. | China            | -                | JX399099        | JX399030               |
| Pestalotiopsis verruculosae MFLUCC 12-0274 | Rhododendron sp. | China            | -                | JX399096        | JX399061               |
| Pestalotiopsis cf. verruculosae CBS 365.54 | Chamaecyparis lawsoniana | Netherlands     | -                | MH554037        | MH554472 MH554713       |
Table 1. Cont.

| Species                  | Collection No. ¹ | Host/Source | Country | Collection Year | GenBank Accession Number ² |
|--------------------------|------------------|-------------|---------|-----------------|----------------------------|
| Pestalotiopsis yanglingensis | LC3412           | Camellia sinensis | China   | -               | KX894980 KX895197 KX895312 |
| Pestalotiopsis yanglingensis | LC4553           | Camellia sinensis | China   | -               | KX895012 KX895231 KX895345 |

¹ Culture collections—BRIP: Queensland Plant Pathology Herbarium, Australia; CBS: Culture collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CPC: Working collection of Pedro W. Crous, housed at the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; GZCC: Guizhou Academy of Agricultural Sciences Culture Collection, GuiZhou, China; HGUP: Plant Pathology Herbarium of Guizhou University, GuiZhou, China; ICMP: International Collection of Micro-organisms from Plants, Landcare Research, Auckland, New Zealand; IFRDCC: International Fungal Research and Development Culture Collection, Yunnan, China; LC: working collection of Lei Cai, housed at the Institute of Microbiology, Chinese Academy of Sciences, Beijing, China; MEAN: culture collection of INIAV Institute, Oeiras, Portugal; MFLUCC—Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; NOF: The Fungus Culture Collection of the Northern Forestry Centre, Alberta, Canada; NTUCC: National Taiwan University Culture Collection, Taiwan; ² ITS: internal transcribed spacer-rDNA; TEF: translation elongation factor 1-α; TUB: β-tubulin.
2.2. Morphology

Colony morphology was observed after 7 days of cultivation on PDA at 23 ± 2 °C at 12 h daylight. Conidiomatal development was observed on Synthetic Nutrient-poor Agar (SNA) by cultivating the isolates on autoclaved pine needles placed on the surface of SNA. Colony colour was determined on PDA using the colour charts of Rayner [19]. Conidia and conidiogenous cells were mounted in distilled water, and at least 30 measurements per structure were recorded at 400× magnification under a compound light microscope (Olympus BX51, Olympus Corporation, Tokyo, Japan) using the program Olympus DP-Soft, or under a Nikon Eclipse 80i compound microscope with differential interference contrast (DIC) illumination, equipped with a Nikon DS-Ri2 high definition colour digital camera.

2.3. DNA Extraction, PCR Amplification and Sequencing

Genomic DNA was extracted using the “DNA, RNA and Protein Purification—NucleoSpin Plant II” (Macherey-Nagel—MN) following the manufacturer’s instructions. Fresh mycelium was disrupted by vortexing with approximately 200 μL glass beads (450–600 μm diameter) added to the extraction buffer [20].

Polymerase Chain Reactions (PCR) were performed to amplify three distinct DNA regions—the internal transcribed spacer of the ribosomal DNA (ITS), the partial translation elongation factor 1-alpha (TEF) and partial β-tubulin (TUB). The ITS, TEF and TUB genes were amplified using the primer pairs ITS5/ITS4 [21], EF1-728F/EF1-986R [22], and T1/Bt-2b [23,24].

All PCR reactions were performed in a 25 μL reaction containing DNA template (diluted 10x), 10x PCR reaction buffer, 3 mM MgCl2, 0.5 mM of each deoxyribonucleotide triphosphate, 1 U of Taq DNA Polymerase, (BioTaqTM DNA Polymerase—Bioline, London, UK) and 2 μM of each primer, for ITS and TUB amplification, or 6 μM of each primer, for TEF amplification.

PCR reactions were performed in a Biometra TGradient thermo cycler (Biometra, Göttingen, Germany) with the following thermal cycling conditions, for ITS: initial denaturation at 94 °C for 3 min, followed by 30 cycles consisting of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min; for TEF: initial denaturation at 94 °C for 8 min, followed by 35 cycles consisting of denaturation at 94 °C for 15 s, annealing at 55 °C for 20 s and extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min; and for TUB: initial denaturation at 94 °C for 1 min, followed by 30 cycles consisting of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min.

PCR products were sequenced in both directions at STABVida Sequencing Laboratory (Caparica, Portugal) on an ABI PRISM 3730xl DNA analyser (Applied Bio systems) using the same primers as those used for the amplification reactions. The resulting nucleotide sequences were edited using the programs FinchTV version 1.4.0 (Geospisa Inc.) and BioEdit version 7.2.6 [25], and a consensus sequence was made from the forward and reverse sequences. Sequences obtained in this study were deposited in GenBank (see Table 1).

2.4. Phylogenetic Analyses

A BLAST engine search was used for sequence similarity searching on GenBank (NCBI—National Centre for Biotechnology Information). Based on blast search results and the literature, additional sequences were selected from GenBank and incorporated in the analyses (Table 1). Sequence alignments of the three individual loci (ITS, TEF, TUB) were made using MAFFT v. 7 (http://mafft.cbrc.jp/alignment/server/index.html), and were then manually edited using BioEdit version 7.2.6. Single gene datasets were combined using SequenceMatrix [26].

Phylogenetic analyses of the combined three-locus sequence dataset comprised Maximum Likelihood (ML), Maximum Parsimony (MP) and Bayesian Inference (BI).
ML were implemented on the CIPRES Science Gateway portal (https://www.phylo.org) [27] using RAxML-HPC2 on XSEDE v. 8.2.12 [28]. For ML analyses, a GTR+CAT substitution model with 1000 bootstrap iterations was set.

MP analysis was performed using Phylogenetic Analysis Using Parsimony (PAUP) v. 4.0b10 [29]. Gaps were treated as missing data. Trees were inferred using heuristic search with random stepwise addition and tree-bisection reconnection (TBR). Maxtrees were set to 10,000 and branches of zero length were collapsed. Bootstrap support values with 1000 replications [30] were calculated for tree branches. Tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC) and homoplasy index (HI) were calculated for trees generated under different optimality criteria.

BI was performed by using the Markov Chain Monte Carlo method (MCMC) with MrBayes v. 3.2.6 [31]. JModelTest2 on XSEDE [32], implemented via the CIPRES portal, was used to determine the best-fit nucleotide substitution model for each partition using the Akaike Information Criterion (AIC) [33]. The GTR + I + G model was selected as the most suitable for ITS and TUB data partitions, and the GTR + G model was selected for TEF data partition. Four MCMC chains were run simultaneously, starting from random trees for 1,000,000 generations. Trees were sampled every 100 generations for a total of 10,000 trees. The burn-in fraction was set to 0.25, after which posterior probabilities were determined from a majority-rule consensus tree [34].

2.5. Pathogenicity Tests

Two isolates representing the most common Pestalotiopsis species isolated from stone pine trees with shoot blight disease in this study were selected to perform the pathogenicity tests: MEAN1095—Pestalotiopsis pini sp. nov. and MEAN1096—Pestalotiopsis australis Maharachch., K.D. Hyde & Crous.

To carry out the pathogenicity tests, 93 one-year-old stone pine seedlings were sourced from a nursery, where they were cultivated from seeds of a certified orchard. For each isolate and for the control treatment, 31 seedlings were randomly chosen and distributed along a plastic cell pack (6 × 11 plastic cells container). Each plastic cell pack with plants was randomly located in the greenhouse test area. The plants were then acclimatized during one month under greenhouse conditions, with temperatures varying from 18 to 28 °C, watered as needed (circa 2 L per plastic cell pack container, twice a week).

Spore suspensions of each isolate were prepared from cultures on PDA, grown at 25 ± 1 °C for 14 days (four plates/isolate). Sterile deionized water was added to the cultures and spores were dislodged by a sterile glass rod. The spore suspensions were resuspended in sterile deionized water and concentration adjusted to 1 × 10^5 conidia mL^-1 with a haemocytometer.

The inoculations were performed by two combined methods. First, the stems were damaged by gently piercing them with a dissection needle that was previously dipped into the spore solution, while, in the control, the stems were pierced with a sterile needle. Five to six wounds were made per plant, approximately 3 cm apart from each other, in the upper third of the stem. Secondly, based on Talgø et al. [35], some needles were removed from plants, and the injured area subsequently brushed with the spore suspension. Sterile water was used in the control. Each container was covered with a plastic bag and maintained for one week to enhance fungal development.

The seedlings were kept in the greenhouse for four months (18 July to 17 November 2017). At the end of the experiment, the number of affected plants was noted, and in order to attest Koch’s postulates, re-isolations of fungi were carried out from the disease margins of three symptomatic seedlings, following the methodology described in Section 2.1.

3. Results

3.1. Fungal Isolation and Identification

Among other fungi, a total of 18 pestalotiopsis-like colonies were observed. After morphological observation and ITS sequence analyses, five isolates were identified as belonging to Heterotruncatella
and 13 to *Pestalotiopsis*. Further molecular studies were performed to identify the *Pestalotiopsis* species isolated.

### 3.2. Phylogenetic Analyses of Combined ITS, TEF and TUB Sequences

To determine the phylogenetic position of the *Pestalotiopsis* isolates, phylogenetic analyses were performed based on the combined ITS, TEF, and TUB sequence data. The combined alignment contained sequences from 104 strains (including two outgroups) with 1427 characters (including alignment gaps), divided in three partitions with 494 (ITS), 491 (TEF) and 442 (TUB) characters; 417 of these were parsimony-informative, 151 were variable and parsimony-uninformative, and 859 were constant. The combined *Pestalotiopsis* dataset was analysed using ML, MP and BI (Figure 1). The phylograms from the three analyses showed similar results in topology, and hence the best scoring tree resulting from ML analyses, with a final likelihood value of –10,646.254559, is shown in Figure 1. Maximum likelihood, MP bootstrap support values, and BI posterior probabilities (MLBS/MPBS/BIPP) are shown at common branches.

Isolates MEAN 1092, MEAN 1094, MEAN 1095 and MEAN 1167 were identical in our primary observations and formed a distinct clade, separate from previously described species within the genus. These isolates are well supported by all three phylogenetic analyses, and hence they are described as a new species of *Pestalotiopsis*.

Phylogenetic analyses allowed to identify the remaining isolates obtained in this study as belonging to four different species of *Pestalotiopsis*: *Pe. australis* (five isolates), *Pestalotiopsis disseminata* (Thüm.) Steyaert (two isolates), *Pestalotiopsis biciliata* Maharachch., K.D. Hyde & Crous (one isolate) and *Pestalotiopsis hollandica* Maharachch., K.D. Hyde & Crous (one isolate). Isolates MEAN 1109, MEAN 1110, MEAN 1096, MEAN 1111 and MEAN 1112 formed a clade along with reference strains of *Pe. australis*. MEAN 1165 and MEAN 1166 clustered with strains of *Pe. disseminata*. Isolate MEAN 1168 grouped with *Pe. biciliata*, while isolate MEAN 1091 was closely related to *Pe. hollandica*.

### 3.3. Morphology and Taxonomy

*Pestalotiopsis pini* A.C. Silva, E. Diogo & H. Bragança, sp. nov. (Figure 2)

**MycoBank:** MB 835952

**Holotype:** LISE 96316

**Etymology:** Named after the host genus from which it was isolated, *Pinus*.

**Host/Distribution:** On needles, shoots and trunks of *Pinus pinea* and on *Pinus pinaster* in Portugal (this study). Seen on *Pinus radiata* in Chile and on *Pinus* sp. in the USA also [3].

**Description:** Colonies on PDA attaining 82–85 mm diam after 7 d at 25 °C, with smooth edge, whitish to pale salmon coloured, with cottony aerial mycelium, forming abundant acervuli exuding black spore masses after two weeks. Reverse pale peach to salmon coloured. Conidiomata acervular on PDA, globose, aggregated or scattered, semi-immersed or partly erumpent, exuding black conidial masses. Conidiophores septate near base, simple or rarely branched at base, subcylindrical with a swollen base, hyaline, up to 28 μm long. Conidiogenous cells discrete, cylindrical, hyaline, smooth, 12–25 × 2–4 μm. Conidial sori to ellipsoid, straight to slightly curved, 4-septate, occasionally slightly constricted at septa (20.0–)23.3–24.6(–27.6) × (4.7–)7.4–7.8(–8.2) μm, av. ± S.D. = 24.0 ± 1.8 × 7.6 ± 0.6 μm; basal cell obconic, hyaline, smooth and thin-walled, 3.9–7.3 μm long; three median cells doliform, (12.2–)14.8–15.6(–17.3) μm long, av. ± S.D. = 15.2 ± 1.3 μm, smooth and thin-walled, concolourous, but occasionally the two upper median cells are slightly darker than the lower median cell, olivaceous to brown, septa darker than the rest of the cell (second cell from the base 3.8–6.0 μm long; third cell 3.2–6.6 μm long; fourth cell 3.4–6.1 μm long); apical cell 2.4–4.8 μm long, hyaline, conical to subcylindrical, thin- and smooth-walled; with 3–4 tubular apical appendages (mostly 3), arising from the apical crest, unbranched, filiform, (9.7–)18.4–19.8(–27.8) μm long, av. ± S.D. = 19.1 ± 3.5 μm; basal appendage single, filiform, unbranched, centric, 1.4–7.6 μm long.
Figure 1. Phylogram generated from maximum likelihood (ML) analysis based on combined ITS, TUB and TEF sequence alignment for species of Pestalotiopsis. The best scoring ML tree with a final likelihood value of -10646.254559 is presented. The tree was rooted to Neopestalotiopsis australis (CBS 114159) and N. protearum (CBS 114178). Maximum likelihood and maximum parsimony bootstrap support values $\geq 50\%$ and Bayesian Inference posterior probabilities $\geq 0.90$ (MLBS/MPBS/BIPP) are given at the nodes in common branches. The isolates obtained in this study are in bold. The scale bar represents the expected number of changes per site.
Figure 1. Phylogram generated from maximum likelihood (ML) analysis based on combined ITS, TUB and TEF sequence alignment for species of *Pestalotiopsis*. The best scoring ML tree with a final likelihood value of $-10,646.254559$ is presented. The tree was rooted to *Neopestalotiopsis australis* (CBS 114159) and *N. protearum* (CBS 114178). Maximum likelihood and maximum parsimony bootstrap support values $\geq 50\%$ and Bayesian Inference posterior probabilities $\geq 0.90$ (MLBS/MPBS/BIPP) are given at the nodes in common branches. The isolates obtained in this study are in bold. The scale bar represents the expected number of changes per site.
Figure 2. Pestalotiopsis pini (MEAN 1094). (a,b) Colony on PDA after 10 days at 23±2°C—surface view and reverse, respectively. (c–f) Conidiophores, conidiogenous cells and attached conidia. (g–l) Conidia. Scale bars: 10 μm.

Material examined: PORTUGAL, Lisbon, on rotten trunk of Pinus pinea, Ana C. Silva and Helena Bragança, March 2017 (LISE 96316 holotype; ex-type culture, MEAN 1094 = CPC 36748 = CBS 146841); PORTUGAL, Santarém, on blighted shoots of Pinus pinea, Ana C. Silva and Helena Bragança, March 2016 (living culture, MEAN 1092 = CPC 36746 = CBS 146840). PORTUGAL, Santarém, on blighted shoots of Pinus pinea, Ana C. Silva and Helena Bragança, March 2017 (living culture, MEAN 1095 = CPC 36749 = CBS 146842). PORTUGAL, unknown district, on blighted shoots of Pinus pinaster, Ana C. Silva, Eugénio Diogo and Helena Bragança, November 2018 (living culture, MEAN 1167).

Notes: Pestalotiopsis pini has similar-sized conidia to Pestalotiopsis clavata Maharachch., K.D. Hyde & Crous and Pestalotiopsis lushanensis F. Liu & L. Cai (20.0–27.6×4.7–8.2 μm in Pe. pini vs. 20–27×6.5–8 μm in Pe. clavata and 20–27×7.5–10 μm in Pe. lushanensis), but they are different in the number of appendages (Pe. pini has 3–4 appendages while Pe. clavata and Pe. lushanensis have 2–3 apical appendages) [12,36]. They are clearly separated in the phylogram based on combined ITS, TEF, and TUB sequence data, Pe. pini isolates formed a separate clade with strong support values on the three analyses performed (ML, MP and BI), (see Figure 1).

3.4. Pathogenicity

Two isolates, representing the most common Pestalotiopsis species isolated from pine trees with shoot blight disease in the present study, were submitted to pathogenicity tests by artificial inoculation on stone pine seedlings: MEAN1095—Pestalotiopsis pini sp. nov. and MEAN1096—Pestalotiopsis australis.

The development of disease symptoms was observed during a four-month period. Initial symptoms started after four weeks on seedlings inoculated with the Pe. pini isolate. Seedlings started to show yellowish and wilted needles in the apical third of the trunk. By the end of the experiment, symptomatic plants exhibited a dried apex in the inoculated branch/trunk (Figure 3). In total, 19.4% (6/31) of the plants inoculated with Pe. pini isolate MEAN 1095 were symptomatic. No symptoms were observed on the
control treatment, nor in plants inoculated with *Pe. australis* isolate MEAN 1096. *Pestalotiopsis pini* was successfully re-isolated from the three symptomatic plants sampled, thus fulfilling Koch’s postulates and confirming its pathogenicity to stone pine.

![Figure 3. Aspect of inoculated seedlings four months after the inoculations. (a) Asymptomatic plant. (b,c) Symptomatic plants inoculated with *Pestalotiopsis pini* sp. nov. (d,e) Detail of dead apical shoots on symptomatic plants.](image)

### 4. Discussion

In the present study *Pestalotiopsis pini* is described as a new species causing shoot blight and stem necrosis on *Pinus pinea*. Based on the morphology and molecular phylogenetic analyses of combined ITS, TEF and TUB sequence data, this taxon proved distinct from other species known from pine, or from DNA sequence data. Four other species of *Pestalotiopsis* were identified in association with symptomatic stone pines, namely, *Pe. australis, Pe. biciliata, Pe. disseminata* and *Pe. hollandica*.

*Pestalotiopsis pini* isolates obtained in this study (MEAN 1095, MEAN 1092, MEAN 1094, MEAN 1167) were grouped along with two unclassified *Pestalotiopsis* sp. strains included in the revision of Sporocadaceae, performed by Liu et al. [3], namely CBS 110326 and CBS 127.80. In the latter study, the authors retained these two isolates as an “informal species” “*Pestalotiopsis* sp.7 FL-2019”, due to the lack of more isolates and limited phylogenetic support. In our phylogenetic analyses (Figure 1), these two strains were grouped with the four isolates obtained in this study, forming a separate clade with strong support values in all the phylogenetic analyses performed (MLBS = 100%, MPBS = 99%, BIPP = 1.00).

In the present study, *Pe. pini* was isolated from blighted shoots of *P. pinea* and *P. pinaster* trees in pine plantations, and from the necrotic wood of a decayed stone pine trunk located in Monsanto Forest Park in Lisbon. Pathogenicity tests performed confirmed that *Pe. pini* is pathogenic to stone pine. Furthermore, in the Monsanto Forest Park, various stone pine trees exhibited the same symptoms, and no other potential pathogens were isolated along with *Pe. pini*, suggesting that this could be a primary pathogen for this host. Interestingly, despite *Pestalotiopsis* species generally not being regarded as host-specific and normally being found on a wide range of plants and substrates [9], the two *Pe. pini* strains included in the study of Liu et al. [3] were also isolated in pines—*Pinus* sp. in the USA (CBS 110326) and *Pinus radiata* D. Don. in Chile (CBS 127.80)—although no information about the health of these pine trees is available.
In this study, Pestalotiopsis australis was isolated from blighted stone pine shoots in *P. pinea* orchards. This is the first report of *Pe. australis* isolated from conifers and in Europe. Under the conditions of the trials, no symptom development occurred in any of the inoculated seedlings, suggesting that *Pe. australis* may behave as an endophyte on stone pine. *Pestalotiopsis australis* has been reported from *Proteaceae* hosts, it was isolated from *Grevillea* sp. in Australia and South Africa, and from *Protea neriifolia × susannae* cv. 'Pink Ice' and dead leaves of *Brabejum stellatifolium* L. in South Africa [3,9].

*Pestalotiopsis hollandica* was isolated from the blighted shoots of stone pine trees in stone pine orchards. *Pestalotiopsis hollandica* was first described from Sciadopityaceaee (Sciadopitys verticillata (Thunb.) Siebold & Zucc.) in the Netherlands [9] and it has already been isolated from conifers in Spain, namely from *Cupressus sempervirens* L. (*Cupressaceae*) [37]. Isolate MEAN 1091 was closely related to the reference strain of *Pe. hollandica*. However, *Pe. hollandica* was not well resolved from *Pestalotiopsis brassicae* Maharachch., K.D. Hyde & Crous, *Pestalotiopsis Italiana* Maharachch., Camporesi & K.D. Hyde, *Pestalotiopsis Monochaeta* Maharachch., K.D. Hyde & Crous, *Pestalotiopsis sequoiae* W.J. Li, Camporesi & K.D. Hyde and *Pestalotiopsis Verruculosa* Maharachch. & K.D. Hyde, suggesting that these isolates may represent a single species, as suggested by Liu et al. [3]. Some of those species’ names have also been associated with conifers in the past [9,38].

*Pestalotiopsis biciliata* was isolated from a dry conelet (1st year) from a stone pine orchard. This species was first described by Maharachchikumbura et al. [9], isolated from dry needles of *Taxus baccata* L. in the Netherlands, from *Paenia* sp. in Italy and from *Platanus × hispanica* in Slovakia. *Pe. biciliata* was also isolated from dry needles of *Taxus baccata* in the UK [3]. The fungus was referred to as the causal agent of fruit rot on withered grapes in Italy [8], and is associated with grapevine trunk diseases in France [10]. Recently *Pe. biciliata* was also reported as a foliar pathogen of *Eucalyptus* spp. [11].

*Pestalotiopsis disseminata* was isolated from blighted shoots of stone pine trees in a stone pine orchard. *Pe. disseminata* was first described from *Eucalyptus botryoides* Sm. in Portugal [39], and has already been isolated from a wide range of hosts and locations worldwide [3,15,18,40], including the genus *Pinus* [15,16,18]. It was isolated as an endophyte from *Pinus armandii* in China, along with 18 other pestalotioid species [16]; from *Pinus parviflora* Siebold & Zucc. var. *pentaphylla* (Mayr) in Japan [18] and from seeds of *P. pinea* in Turkey, *Pinus elliottii* Engel., *Pinus patula* Schltdl & Cham, *P. radiata*, *Pinus taeda* L. in the USA and *P. pinaster* in Portugal [15].

Isolates identified in this study were associated with symptomatic stone pine trees with shoot blight, trunk necrosis and pinecone decay in Portugal. At least one of the five identified species, *Pestalotiopsis pini* sp. nov., is pathogenic to stone pine. In recent years, various species of *Pestalotiopsis* have been described [3,4,7,9,10], with many being associated with plant diseases and shown to be pathogenic to their host under certain biotic and abiotic conditions [4,5,8,11,41,42].

The symptoms observed in stone pine orchards in Portugal, in particular shoot blight disease, might be of special concern to the forest industry, since dry shoots in the tree canopy could lead to a decrease in pinecone development and pine nut production, which is the most profitable resource of this industry [1,2].

Shoot blight disease on stone pine and other pine species is normally associated with *Diplodia sapinea* (Fr.) Fuckel [43,44], and has recently also been associated with *Sydowiopsis polyspora* (Bref. & Tavel) E. Müller [45]. In the present study, various *Pestalotiopsis* species were isolated from stone pine samples with similar symptoms, moreover, *Pe. pini* proved to be pathogenic on stone pine, causing dry shoots on artificially inoculated seedlings, thus suggesting that *Pe. pini* should also have an active role in the expression of shoot blight disease on stone pine. The fact that in the pathogenicity tests, *Pe. pini* only caused disease symptoms in approximately 20% of the inoculated seedlings may indicate relative host resistance due to genetic differences among the seedlings. Alternatively, the development of shoot blight disease is due to more than one factor, biotic or abiotic. In fact, *D. sapinea*, *S. polyspora* and other fungi were also present in some of the sampled symptomatic material (data not shown). Diverse authors also report more than one species involved in dieback and blight diseases, including pestalotioid species and other fungi [8,45-47] and observed that some abiotic factors also have a major
role in disease development, namely water stress and air temperature [41,42,47]. In this case, a synergic effect among Pe. pini and other pathogenic or endophytic fungi found in stone pine shoots may also trigger the development of shoot blight disease symptoms. Future research should be performed to evaluate shoot blight disease prevalence on P. pinea orchards in Portugal and other Mediterranean areas and the diverse biotic and abiotic agents that can be involved in disease development.

The present study represents a preliminary contribution of the Pestalotiopsis species diversity associated with shoot blight disease of stone pine in Portugal. Knowledge of Pestalotiopsis species associated with shoot blight and other pine diseases will provide a basis to better understand disease development and help to develop management strategies against these pathogens.

5. Conclusions

A novel fungal species, Pestalotiopsis pini was described. This study proves that Pe. pini is an emerging pathogen causing shoot blight and trunk necrosis on Pinus pinea in the Mediterranean area.

To our knowledge, this is also the first report of Pe. australis on conifers and in Europe, and of Pe. hollandica and Pe. biciliata on Pinus spp. and in Portugal. Information about Pestalotiopsis species associated with shoot blight and other diseases on pine species will help to provide a basis for a better understanding of disease development, and the development of management strategies against these pathogens.

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