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ORIGINAL ARTICLE

Five new species of *Neopestalotiopsis* associated with diseased *Eucalyptus* spp. in Portugal

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Abstract

Eucalyptus globulus, an exotic species in Portugal, is one of the dominant and intensively managed forest species in the country. A disease syndrome characterised by leaf necrosis, stem girdling and cutting dieback in eucalyptus and associated with pestalotioid fungi has been detected in nurseries and young plantations in the last years. Twenty-seven isolates were recovered from diseased plants. Phylogenetic analysis based on internal transcribed spacers, partial translation elongation factor 1-α gene and partial β-tubulin gene sequence data grouped the isolates in five separate clades. Combining morphological, cultural and molecular data, five new species of *Neopestalotiopsis* are described, namely, *Neopestalotiopsis eucalyptorum*, *Neopestalotiopsis hispanica*, *Neopestalotiopsis iberica*, *Neopestalotiopsis longiappendiculata* and *Neopestalotiopsis lusitanica*.

Keywords Coelomycetes · *Eucalyptus globulus* · New species · Plant pathogens · Phylogeny · *Sporocadaceae* · Taxonomy

Introduction

Eucalyptus species are among the most widely planted hardwood timber trees in the world (FAO 2001). In Portugal, *Eucalyptus globulus* is the most important commercial species with a land usage of more than 845,000 ha (ICNF 2019), directed mostly at the papermaking industry (Pirralho et al. 2014).

Eucalyptus globulus was introduced into Portugal in the nineteenth century but only in the middle of the twentieth century did the great increase of plantations occur. In the following decades, eucalyptus plantations were relatively free of pests and diseases. However, since the 1970s, several pests appeared, firstly insects and then fungi (Branco et al. 2014). Leaf diseases caused by *Mycosphaerella*, *Teratosphaeria* and *Quambalaria* species and canker diseases caused by *Teratosphaeria gauchensis*, *Quambalaria eucalypti* and various species in Botryosphaeriaceae have been reported to cause important losses in eucalyptus plantations in the last years (Barradas et al. 2016; Bragança et al. 2016; Silva et al. 2015) . Grey mould caused by *Botrytis cinerea* and anthracnose caused by *Hendersonia eucaliptina* are also important diseases faced by Portuguese eucalypt nursery growers (Ferreira et al. 1994).

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Pestalotioid fungi have received considerable attention for their association with a wide range of cultivated plants, as well as for the production of economically important metabolites (Jayawardena et al. 2019; Maharachchikumbura et al. 2011; Metz et al. 2000; Mishra et al. 2014; Tejesvi et al. 2007; Xu et al. 2014). These fungi have ubiquitous distribution and are mostly regarded as secondary pathogens affecting weakened plants, or opportunistic saprobes found on dead material (Almeida et al. 2003; Bakry et al. 2011; Hopkins and McQuilken 2000; Old et al. 2000; Stone et al. 2004; Tejesvi et al. 2007). However, they have been found to cause damage on economically important plants, both in forestry and agriculture (Akinsanmi et al. 2017; Bakry et al. 2011; Chen et al. 2018; Espinoza et al. 2008; Hopkins and McQuilken 2000; Ismail et al. 2013; Lin et al. 2011; Morales-Rodríguez et al. 2019; Qi et al. 2021; Silva et al. 2020).

Since 2012, a disease causing stem girdling and dieback in young eucalyptus plants has been detected, and pestalotioid fungi are consistently associated with diseased plants. The aim of the work reported here was to identify the fungal species associated with this disease.

Material and methods

Sampling and isolation

In Portugal, the disease syndrome was first detected in November 2012 in a nursery where it caused stem girdling and dieback in young plants of *Eucalyptus globulus*. Since then, and until 2018, tissues of young eucalypts plants from recent plantations and nursery stocks have been collected and transported to the laboratory for observations and disease diagnosis.

Two methods were used to obtain pure cultures. When sporulation was observed on the plant material, as tendrils exuding from fruiting bodies, the spores were carefully removed with a fine needle avoiding any other sporulation from the host. They were transferred to a 2% water agar plate and streaked with a sterile loop. After 24 to 48 h, single germinating conidia were selected under a compound microscope and aseptically transferred to half strength potato dextrose agar (½PDA) plates prepared from 20 g/L Difco potato dextrose agar (Difco Laboratories, Detroit, MI, EUA), plus 10 g/L agar. When no sporulation could be observed, small fragments (2 mm^2) were cut from the margins of the lesions and treated as previously described (Diogo et al. 2010). Cultures resulting from single spores were deposited in the fungal collection of INIAV (formerly Micoteca da Estação Agronómica Nacional, MEAN). Representative isolates were also deposited in the CBS public fungal culture collection at the Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands. Dried specimens were deposited in the LISE herbarium of INIAV. Nomenclatural novelties and descriptions were deposited in MycoBank (www.MycoBank.org; Crous et al. 2004).

Morphology

Colony characteristics were registered after 7 days on PDA plates incubated at 25 °C with 12 h of near ultraviolet light per day. Colours were determined using the colour chart of Rayner (Rayner 1970). Growth rates were determined under the same conditions in the dark.

Fructifications and spores were mounted in water. Observations of micromorphological features were made with Leica MZ95 and Leica DMR microscopes, and digital images were recorded with Leica DC300 and Leica DFC320 cameras, respectively. Measurements were made with the measurement module of the Leica IM500 image management system (Leica Micro-systems GmbH, Wetzlar, Germany). Mean, standard deviation (SD) and 95% confidence intervals were calculated. Thirty spores and at least ten other structures were measured.

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 scraped from the surface of PDA cultures was disrupted by vortexing with approximately 200 µL glass beads (450–600 µm diameter) added to the extraction buffer according to Bragança et al. (2007). Three DNA regions, the internal transcribed spacer (ITS), translation elongation factor 1-α (*TEF1*) and partial β-tubulin gene (*TUB2*) were amplified with the primer pairs ITS5 and ITS4 (White et al. 1990), EF1-728F (Carbone and Kohn 1999) and EF2 (O'Donnell et al. 1998) and BT2A and BT2B (Glass and Donaldson 1995), respectively. All PCR reactions were performed in a 25 µL reaction containing DNA template (diluted 10×), 10×PCR reaction buffer, 3 mM $MgCL_2$, 0.5 mM of each deoxyribonucleotide triphosphate, 1 U of Taq DNA polymerase (BioTaqTM DNA polymerase—Bioline, London, UK) and 2 µM each primer. PCR reactions were performed in a Biometra TGradient thermo cycler (Biometra, Göttingen, Germany) as described by Silva et al. (2020). The products were resolved by electrophoresis at 5 Volts.cm^{-1} in agarose gel (1%) containing 0.5 µg/mL ethidium bromide and 1×TBE running buffer. Products were visualised by VersaDoc Gel Imaging System (BioRad, USA) and purified with the QIAquick PCR Purification Kit (Qiagen, USA) following the manufacturer's instructions. Sequencing was performed by STABVIDA (Caparica, Portugal) on an ABI PRISM 3730xl DNA analyser (Applied

Bio systems) with the same primers used for amplification. Sequences generated in this study were deposited in GenBank (Table 1) and alignments in TreeBase (study: TB2:S28369).

Phylogenetic analysis

Sequences of *Neopestalotiopsis* species referred to in recent studies (Table 1), including all sequences of type species available, were retrieved from GenBank. The individual loci were aligned separately with MAFFT V.7 (Katoh et al. 2019), checked and trimmed in MEGA v.7 (Kumar et al. 2016) and concatenated with sequence matrix (Vaidya et al. 2011). The combined dataset was analysed by maximum parsimony (MP), maximum likelihood (ML) and Bayesian Inference (BI). Sequences of *Pestalotiopsis diversiseta* MFLUCC 12–0287 were used as the outgroup.

MP analysis was performed using PAUP* v4.0a (Swofford 2002) implemented in the CIPRES science gateway (Miller et al. 2010) using the heuristic search option with random stepwise addition and tree bisection reconnection (TBR) as the branch swapping algorithm. Maxtrees were set to 1000, and branches of zero length were collapsed, and alignment gaps were treated as missing data. Clade

Table 1 Isolates used in this study

Species	Strain[a]	Host/Substrate	Origin	[b]GenBank Accession numbers			Reference
				ITS	*TUB2*	*TEF1*	
Neopestalotiopsis alpapicalis	MFLUCC 17-2544^T	*Rhizophora mucronata*	Thailand	MK357772	MK463545	MK463547	Kumar et al. 2019
N. alpapicalis	MFLUCC 17-2545	*Rhizophora apiculata*	Thailand	MK357773	MK463546	MK463548	Kumar et al. 2019
N. acrostichi	MFLUCC 17-1754^T	*Acrostichum aureum*	Thailand	MK764272	MK764338	MK764316	Norphanphour et al. 2019
N. acrostichi	MFLUCC 17-1755	*Acrostichum aureum*	Thailand	MK764273	MK764339	MK764317	Norphanphour et al. 2019
N. aotearoa	CBS 367.54^T	Canvas	New Zealand	KM199369	KM199454	KM199526	Maharachchikumbura et al. 2012
N. asiatica	MFLUCC 12-0286^T	Unidentified tree	China	JX398983	JX399018	JX399049	Maharachchikumbura et al. 2012
N. asiatica	SM11	*Castanea mollissima*	China, Sichuan Province	MW166227	MW218520	MW199746	Jiang et al. 2021
N. asiatica	SM7	*Castanea mollissima*	China, Sichuan Province	MW166225	MW218518	MW199744	Jiang et al. 2021
N. australis	CBS 114159^T	*Telopea* sp.	Australia, New South Wales	KM199348	KM199348	KM199537	Maharachchikumbura et al. 2014b
N. australis	KNU16-005	Soil	Brazil	KY549598	KY549633	KY549595	Conforto et al. 2019
N. australis	CMM1359	*Nopalea cochenillifera*	China	KU140695	KU140686	KU140677	Jayawardena et al. 2016
N. brachiata	MFLUCC 17-1555^T	*Rhizophora apiculata*	Thailand	MK764274	MK764340	MK764318	Norphanphour et al. 2019
N. brasiliensis	COAD 2166^T	*Psidium guajava*	Brazil	MG686469	MG692400	MG692402	Bezerra et al. 2018
N. brasiliensis	CFCC 54341	*Castanea mollissima*	China, Sichuan Province	MW166229	MW218522	MW199748	Jiang et al. 2021
N. brasiliensis	ZY4-2D	*Castanea mollissima*	China, Sichuan Province	MW166230	MW218523	MW199749	Jiang et al. 2021
N. chiangmaiensis	MFLUCC 18-0113^T	Dead leaves	Thailand	-	MH412725.	MH388404	Tibpromma et al. 2018
N. chrysea	MFLUCC 12-0261^T	*Pandanus* sp.	China	JX398985	JX399020	JX399051	Maharachchikumbura et al. 2012
N. chrysea	MFLUCC 12-0262	Dead plant	China	JX398986	JX399021	JX399052	Maharachchikumbura et al. 2012
N. clavispora	MFLUCC 12-0281^T	*Magnolia* sp.	China	JX398979	JX399014	JX399045	Maharachchikumbura et al. 2012
N. clavispora	MFLUCC 12-0280	*Magnolia* sp.	China	JX398978	JX399013	JX399044	Maharachchikumbura et al. 2012
N. cocoes	MFLUCC 15-0152^T	*Cocos nucifera*	Thailand	NR_156312	-	KX789689	Norphanphour et al. 2019
N. coffea. arabica	HGUP 4015	*Coffea arabica*	China	KF412647	KF412641	KF412644	Song et al. 2013
N. coffea. arabica	HGUP 4019^T	*Coffea arabica*	China	KF412649	KF412643	KF412646	Song et al. 2013
N. cubana	CBS 600.96^T	Leaf litter	Cuba	KM199347	KM199438	KM199521	Maharachchikumbura et al. 2014b
N. dendrobii	MFLUCC 14-0106^T	*Dendrobium cariniferum*	Chiang Rai, Thailand	MK993571	MK975835	MK975829	Ma et al. 2019
N. dendrobii	MFLUCC 14-0099	*Dendrobium cariniferum*	Chiang Rai, Thailand	MK993570	MK975834	MK975828	Ma et al. 2019
N. egyptiaca	CBS 140162^T	*Mangifera indica*	Egypt	KP943747	KP943746	KP943748	Crous et al. 2015
N. ellipsospora	MFLUCC 12-0283^T	Dead plant materials	China	JX398980	JX399016	JX399047	Maharachchikumbura et al. 2012
N. eucalypticola	CBS 264.37^T	*Eucalyptus globulus*	-	KM199376	KM199431	KM199551	Maharachchikumbura et al. 2014b
N. eucalyptorum	MEAN 1308 = CBS 147684^T	*Eucalyptus globulus*	Fundão, Portugal	MW794108	MW802841	MW805397	This study
N. eucalyptorum	MEAN 1309 = CBS 147685	*Eucalyptus globulus*	Guarda, Portugal	MW794098	MW802831	MW805398	This study
N. eucalyptorum.	MEAN 1322	*Eucalyptus globulus*	Pegões, Portugal	MW794096	MW802829	MW805411	This study
N. eucalyptorum	MEAN 1323	*Eucalyptus globulus*	Pegões, Portugal	MW794099	MW802832	MW805412	This study
N. eucalyptorum	MEAN 1324	*Eucalyptus globulus*	Tondela, Portugal	MW794100	MW802833	MW805413	This study
N. eucalyptorum	MEAN 1326	*Eucalyptus nitens*	Paredes de Coura, Portugal	MW794104	MW802837	MW805415	This study
N. foedans	CGMCC 3.9123^T	Mangrove plant	China	JX398987	JX399022	JX399053	Maharachchikumbura et al. 2012
N. foedans	CGMCC 3.9178	*Neodypsis decaryi*	China	JX398989	JX399024	JX399055	Maharachchikumbura et al. 2012
N. foedans	CGMCC 3.9202	*Calliandra haematocephala*	China, Xinglong, Hainan	JX398988	JX399023	JX399054	Maharachchikumbura et al. 2012
N. formicidarum	CBS 362.72^T	Dead *Formicidae* (ant)	Ghana	KM199358	KM199455	KM199517	Maharachchikumbura et al. 2014b
N. formicidarum	CBS 115.83	Plant debris	Cuba	KM199344	KM199444	KM199519	Maharachchikumbura et al. 2014b
N. hadrolaeliae	COAD 2637^T	*Hadrolaelia jongheana*	Minas Gerais, Brazil	MK454709	MK465120	MK465122	Freitas et al. 2019
N. hadrolaeliae	COAD 2638	*Hadrolaelia jongheana*	Minas Gerais, Brazil	MK454710	MK465121	MK465123	Freitas et al. 2019
N. hispanica	MEAN 1310 = CBS 147686^T	*Eucalyptus globulus*	Fundão, Portugal	MW794107	MW802840	MW805399	This study
N. hispanica	MEAN 1311	*Eucalyptus globulus*	Fundão, Portugal	MW794106	MW802839	MW805400	This study
N. hispanica	MEAN 1312 = CBS 147687	*Eucalyptus globulus*	Spain	MW794113	MW802846	MW805401	This study
N. honoluluana	CBS 114495^T	*Telopea* sp.	USA	KM199364	KM199457	KM199548	Maharachchikumbura et al. 2014b
N. hydeana	MFLUCC 20-0132	*Artocarpus heterophyllus*	Thailand	MW266069	MW251119	MW251129	Huanlauek et al. 2021
N. hydeana	MFLUCC 20-0129	*Garcinia mangostana*	Thailand	MW266072	MW251122	MW251132	Huanlauek et al. 2021

Table 1 (continued)

N. iberica	MEAN 1313 = CBS 147688^{T}	*Eucalyptus globulus*	Pegões, Portugal	MW794111	MW802844	MW805402	This study
N. iberica	MEAN 1314 = CBS 147689	*Eucalyptus globulus*	Spain	MW794114	MW802847	MW805403	This study
N. iranensis	CBS 137767	*Fragaria × ananassa*	Iran	KM074045	KM074056	KM074053	Ayoubi and Soleimani 2015
N. iranensis	CBS 137768^{T}	*Fragaria × ananassa*	Iran	KM074048	KM074057	KM074051	Ayoubi and Soleimani 2015
N. javaensis	CBS 257.31^{T}	*Cocos nucifera*	Indonesia	KM199357	KM199457	KM199548	Maharachchikumbura et al. 2014b
N. macadamiae	BRIP 63737c^{T}	*Macadamia integrifolia*	New South Wales, Australia	KX186604	KX186654	KX186627	Akinsanmi et al. 2017
N. macadamiae	BRIP 63737b	*Macadamia integrifolia*	New South Wales, Australia	KX186600	KX186653	KX186626	Akinsanmi et al. 2017
N. magna	MFLUCC 12-0652^{T}	*Pteridium* sp.	France	KF582795	KF582793	KF582791	Maharachchikumbura et al. 2014a
N. mesopotamica	CBS 299.74	*Eucalyptus* sp.	Turkey	KM199361	KM199435	KM199541	Maharachchikumbura et al. 2014b
N. mesopotamica	CBS 336.86^{T}	*Pinus brutia*	Turkey	KM199362	KM199441	KM199555	Maharachchikumbura et al. 2014b
N. musae	MFLUCC 15-0776^{T}	*Musa* sp.	Thailand	NR_156311	KX789686	KX789685	Norphanphour et al. 2019
N. natalensis	CBS 138.41^{T}	*Acacia mollissima*	South Africa	NR_156288	KM199466	KM199552	Maharachchikumbura et al. 2014a
N. nebuloides	BRIP 66617	*Sporobolus jacquemontii*	Australia, Queensland, Eton	MK966338	MK977632	MK977633	Crous et al. 2020
N. paeoniae	CBS 318.74	*Anacardium occidentale*	Nigeria	MH554031	MH554707	----	Liu et al. 2019
N. pandanicola	KUMCC 17-0175^{T}	*Pandanus* sp.	China	-	MH412720	MH388389	Tibpromma et al. 2018
N. pernambucana	URM 7148-01^{T}	*Vismia guianensis*	Brazil	KJ792466	----	KU306739	Silvério et al. 2016
N. pernambucana	URM 7148-02	*Vismia guianensis*	Brazil	KJ792467	----	KU306740	Silvério et al. 2016
N. petila	MFLUCC 17-1738^{T}	*Rhizophora apiculata*	Thailand	MK764276	MK764342	MK764320	Norphanphour et al. 2019
N. phangngaensis	MFLUCC 18-0119^{T}	*Pandanus* sp.	Thailand	MH388354	MH412721	MH388390	Tibpromma et al. 2018
N. piceana	CBS 394.48^{T}	*Picea* sp.	UK	KM199368	KM199453	KM199527	Maharachchikumbura et al. 2014b
N. protearum	CBS 114178	*Leucospermum cuneiforme*	Zimbabwe	JN712498	KM199463	KM199542	Crous et al. 2011
N. rhizophorae	MFLUCC 17-1550^{T}	*Rhizophora mucronata*	Thailand	MK764277	MK764343	MK764321	Norphanphour et al. 2019
N. rosae	CBS 101057^{T}	*Rosa* sp.	New Zealand	KM199359	KM199429	KM199523	Maharachchikumbura et al. 2014b
N. rosicola	CFCC 51992^{T}	*Rosa chinensis*	China	KY885239	KY885245	KY885243	Norphanphour et al. 2019
N. samarangensis	MFLUCC 12-0233^{T}	*Syzygium samarangense*	Thailand	JQ968609	JQ968610	JQ968611	Norphanphour et al. 2019
N. saprophytica	CBS 115452	*Litsea rotundifolia*	Hong Kong	KM199345	KM199433	KM199538	Maharachchikumbura et al. 2014b
N. saprophytica	MFLUCC 12-0282^{T}	*Magnolia* sp.	China	JX398982	JX399017	JX399048	Maharachchikumbura et al. 2014b
N. sichuanensis	CFCC 54338	*Castanea mollissima*	China, Sichuan Province	MW166231	MW218524	MW199750	Jiang et al. 2021
N. sichuanensis		*Castanea mollissima*	China, Sichuan Province	MW166232	MW218525	MW199751	Jiang et al. 2021
N_steyaertii	IMI 192475^{T}	*Eucalytpus viminalis*	Australia	KF582796	KF582794	KF582792	Maharachchikumbura et al. 2014a
N. sonneratae	MFLUCC 17-1745^{T}	*Sonneronata alba*	Thailand	MK764280	MK764346	MK764324	Norphanphour et al. 2019
Neopestalotiopsis sp.	MEAN 1325	*Eucalyptus globulus*	Aveiro, Portugal	MW794102	MW802835	MW805414	This study
Neopestalotiopsis sp.	MEAN 1327	*Eucalyptus globulus*	Paredes de Coura, Portugal	MW794105	MW802838	MW805416	This study
Neopestalotiopsis sp.	MEAN 1328	*Eucalyptus globulus*	Spain	MW794115	MW802848	MW805417	This study
N. surinamensis	CBS 450.74^{T}	Soil under *Elaeis guineensis*	Suriname	KM199351	KM199465	KM199518	Maharachchikumbura et al. 2014b
N. surinamensis	CBS 111494	*Protea eximia*	Zimbabwe	JX556232	KM199462	KM199530	Mahachchinkumbura et al. 2014b
N. thailandica	MFLUCC 17-1730^{T}	*Rhizophora mucronata*	Thailand	MK764281	MK764347	MK764325	Norphanphour et al. 2019
N. thailandica	MFLUCC 17-1731	*Rhizophora mucronata*	Thailand	MK764282	MK764348	MK764326	Norphanphour et al. 2019
N. umbrinospora	MFLUCC 12-0285^{T}	unidentified plant	China	JX398984	JX399019	JX399050	Maharachchikumbura et al. 2012
N. vitis	MFLUCC 15-1265^{T}	*Vitis vinifera*	China	KU140694	KU140685	KU140676	Jayawardena et al. 2016
N. vitis	MFLUCC 15-1266	*Vitis vinifera*	China	KU140695	KU140686	KU140677	Jayawardena et al. 2016
N. vitis	MFLUCC 15-1267	*Vitis vinifera*	China	KU140696	KU140687	KU140678	Jayawardena et al. 2016
N. zimbabwana	CBS 111495^{T}	*Leucospermum cunciforme*	Zimbabwe	MH554855	KM199456	KM199545	Maharachchikumbura et al. 2014b
N. zimbabwana	MEAN 1329	*Eucalyptus globulus*	Pegões, Portugal	MW794095	MW802828	MW805418	This study
N. zimbabwana	MEAN 1330	*Eucalyptus globulus*	Águeda, Portugal	MW794109	MW802842	MW805419	This study
Pestalotiopsis diversiteta	MFLUCC 12-0287^{T}	Dead plant material	China	NR_120187	JX399040	JX399073	Maharachchikumbura et al. 2012

[a] BRIP; Queensland Plant Pathology Herbarium, Brisbane, Australia; CBS: Culture Collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CFCC: China Forestry Culture Collection Center, Research Institute of Forest Ecology, Environment and Protection, Beijing, China; CGMCC: China General Microbiological Culture Collection Center, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China; COAD: Coleção Octávio Almeida Drummond, Universidade Federal de Viçosa, Brazil; HGUP: Plant Pathology Herbarium of Guizhou University, China; IMI: Culture Collection of CABI Europe UK Centre, Egham, UK; KUMCC: Kunming Institute of Botany Culture Collection, Yunnan, China; MEAN – Instituto Nacional de Investigação Agrária e Veterinária I. P. Fungal Culture Collection, Oeiras, Portugal; MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; URM: Culture Collection of the Universidade Federal de Pernambuco, Brazil

[a]ITS: internal transcribed spacers and intervening 5.8S nrDNA; *TEF1*: partial translation elongation factor 1-α gene; *TUB2*: partial β-tubulin gene

^{T} = ex-type culture. Strains sequenced in this study are in bold type

stability and robustness of the most parsimonious trees were assessed using bootstrap analysis with 1000 pseudoreplicates, each with 10 replicates of random stepwise addition of taxa (Felsenstein 1985). Tree length (TL), consistency index (CI), retention index (RI) and homoplasy index (HI) were calculated.

For BI and ML, a partitioned analysis was performed with three partitions: ITS, *TEF1* and *TUB2*. MrModeltest v2.4 (Nylander 2004) was used to select the best-fit nucleotide substitution model for each partition using the Akaike information criterion (AIC) (Akaike 1974) implemented in PAUP* v. 4.0b10. The HKY + G (Hasegawa et al. 1985) model was selected as the most suitable for all partitions. BI was performed with the Markov chain Monte Carlo method (MCMC) with MrBayes 3.2.7a (Ronquist et al. 2012) implemented in the CIPRES portal. Four MCMC chains were run simultaneously, starting from random trees for 1,000,000 generations. Trees were sampled every 100th generation for a total of 10,000 trees. The first 1000 trees were discarded as the burn-in phase of each analysis. Posterior probabilities (Rannala and Yang 1996) were determined from a majority-rule consensus tree generated with the remaining 9000 trees. This analysis was repeated three times starting from different random trees to ensure trees from the same tree space were sampled during each analysis.

The ML analysis was performed in the CIPRES portal (Miller et al. 2010) using RAxML-HPC2 on XSEDE (8.2.12) (Stamatakis 2014). The optimal ML tree search was conducted with 1000 rapid bootstrap inferences. The final tree was selected among suboptimal trees from each run by comparing likelihood scores under the GTR + GAMMA substitution model. The resulting trees were visualised with MEGA v. 70.026 (Kumar et al. 2016) and edited in Microsoft PowerPoint.

Results

Symptoms, fungal isolation and identification

Most eucalypt plants with symptoms consistent with pestalotioid fungi that were analysed were very young plants of *E. globulus* (Fig. 1). Symptoms were typically found in one of three situations: (1) in the nursery, sometimes associated with intense mortality in early stages of plant production; (2) in forest yards, where young eucalypts awaited plantation; or (3) shortly after plantation, usually within a few months. In forest yards and plantations, mortality was variable, ranging from a few plants to 30–40%. In most cases, sporulation was observed on the lesions especially on the nursery plants. In total, 27 isolates were identified from their morphology as belonging to the genus *Neopestalotiopsis*. From these, only 23 were included in the phylogenetic analysis due to the failure of amplification of the β-tubulin gene.

Phylogenetic analysis

The concatenated dataset of 100 ingroup taxa (representing 56 species) and 1 outgroup taxon consisted of 1512 characters including alignment gaps (527 for ITS, 550 for *TEF1* and 435 for *TUB2*). Of these characters, 1037 were constant, and 242 variable characters were parsimony uninformative. MP analysis of the remaining 233 parsimony informative characters resulted in 1000 equally most parsimonious trees of 940 steps (CI = 0.661, RI = 0.742, HI = 0.339). Topology of the trees generated by MP and BI (Treebase study TB2: S28369) did not differ significantly from the tree generated by ML (Fig. 2, final ML optimization likelihood = −7379.899056).

Of the 22 isolates from *E. globulus* and one from *E. nitens*, isolates MEAN 1328 and MEAN 1329 grouped with *N. zimbabwana*, in a clade containing *N. hadrolaeliae* and *N. honoluluana* (Fig. 2). Isolates MEAN 1325, MEAN 1327 and MEAN 1228 grouped close to *N. rosae* and *N. javensis* in an unresolved clade. The remaining eighteen isolates grouped in five well-resolved clades and are here described as new species.

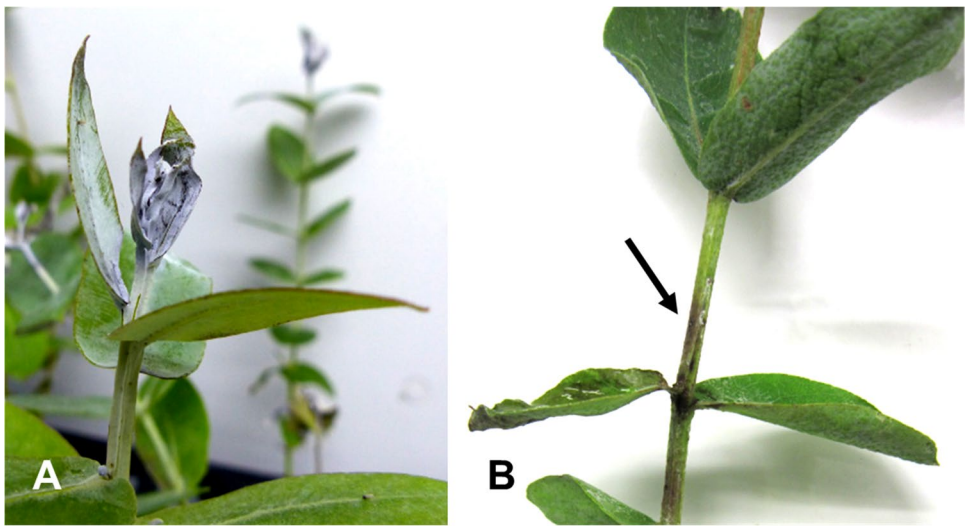

Fig. 1 Symptoms caused by species of *Neopestalotiopsis* on *Eucalytpus* spp. **A** young plant with necrosis on the apical meristem, **B** young plant with necrosis on the stem

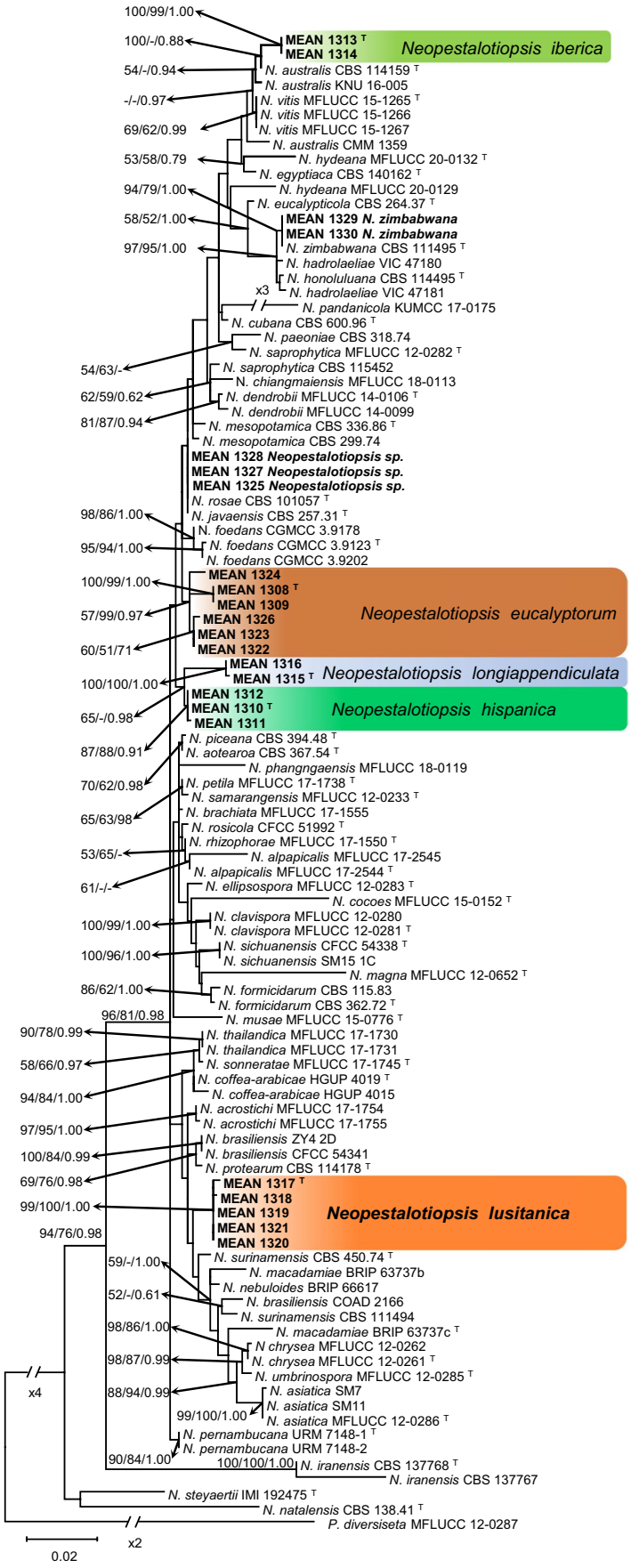

Fig. 2 Consensus phylogram of 1000 trees resulting from a RAxML analysis of the (ITS + *TUB2* + *TEF1*) alignment of the analysed *Neopestalotiopsis* sequences. RAxML bootstrap support values (MLB), maximum parsimony bootstrap supports (MPB) and Bayesian posterior probabilities are given at the nodes (MLB/MPB/BPP). Strains sequenced in this study are in bold and T denotes ex-type strains. Scale bar corresponds to 0.02 substitutions per sites. The tree is rooted with *Pestalotiopsis diversiseta* MFLUCC 12–0287

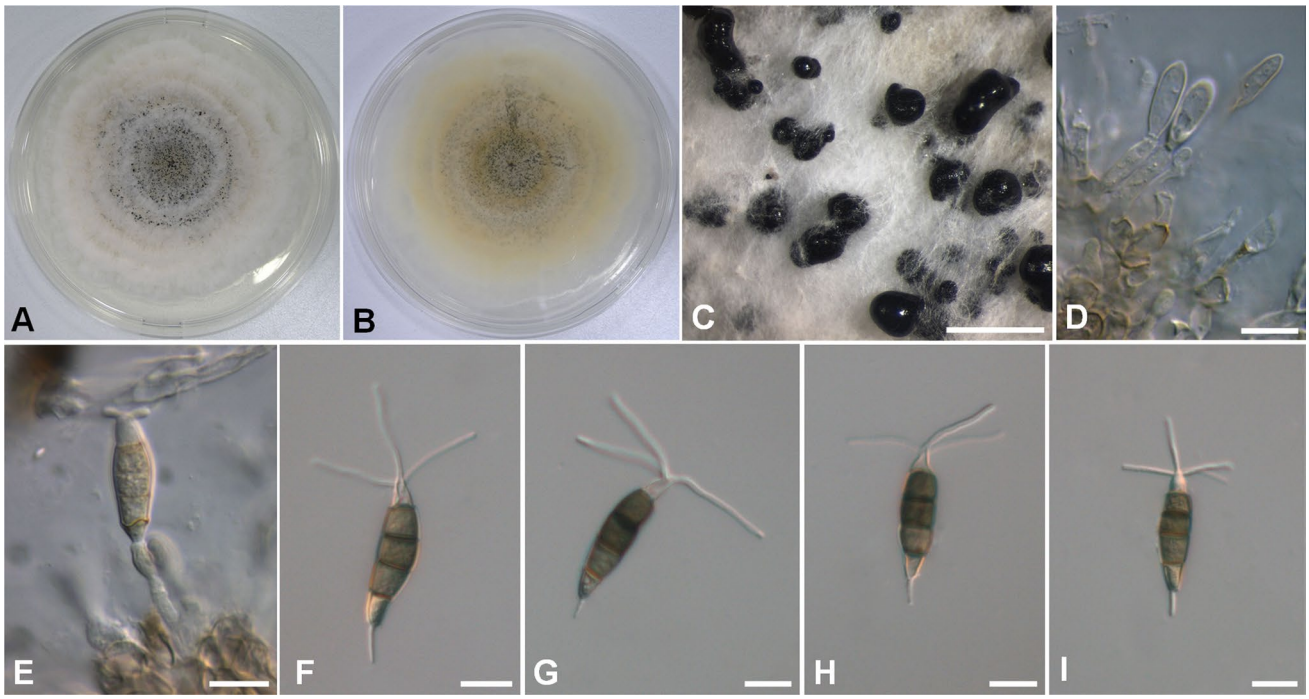

Fig. 3 *Neopestalotiopsis eucalyptorum* (Holotype LISE 96318, ex-type culture CBS 147684 = MEAN 1308). **A**–**B** Colony on PDA (above and reverse), **C** Conidiomata on PDA, **D**–**E** Conidiogenous cells, **F**–**I** Conidia. Scale bars **C** = 1 mm, **D**–**I** = 10 µm

Taxonomy

Neopestalotiopsis eucalyptorum E. Diogo, M. H. Bragança & A. J. L. Phillips sp. nov., Figure 3

MycoBank: MB839457.

Holotype: LISE 96318, (dried culture), ex-type culture MEAN 1308 = CBS 147684.

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

Associated with leaf necrosis and stem basal cankers of *Eucalyptus globulus* young plants. Sexual state not observed. Asexual state: *Conidiomata* in culture on PDA pycnidial, globose, black, up to 470 µm diam., covered with white mycelium, aggregated or discrete and scattered, semi-immersed, exuding black globose, glistening, conidial masses. *Conidiophores* indistinct and reduced to conidiogenous cells. *Conidiogenous cells* discrete, ampulliform, mostly cylindrical to subcylindrical, smooth, thin-walled, simple, 7.1–20 × 2.4–5.8 µm, apex 1.7–2.6 µm diameter. *Conidia* fusoid to ellipsoid, straight or slightly curved, 4 septate, (22.6) 27.5–29.2 (33.2) × (6.4) 7.6–8.1(9.5) µm, $\bar{x}$ ± SD = 28.3 ± 2.4 × 7.9 ± 0.7 µm; basal cell obconic to subcylindrical with a truncate base, hyaline to pale brown, smooth, and thin-walled, 3.2–7.3 µm long, three median cells doliiform to subcylindrical, (15.1) 17.6–18.6 (22.1) µm long, $\bar{x}$ ± SD = 18.1 ± 1.5 µm, smooth-walled, versicoloured, septa darker than the rest of cell, second cell from the base pale brown, 5.1–8.6 µm long, third cell honey brown 4.6–6.8 µm long, fourth cell honey brown to brown, 4.6–7.7 µm long, apical cell 3.9–7.2 µm long, hyaline, subcylindrical to conical, smooth, thin-walled, with 2–4 apical appendages (mostly 3) arising from the apical crest, unbranched, filiform, flexuous, (12.7)16.2–18.8(27.7) µm long $\bar{x}$ ± SD = 17.5 ± 3.7 µm; basal appendage single, filiform, unbranched, centric, 3.4–8.1 µm long.

Culture characteristics: Colony on PDA attaining 76 mm diameter after 7 days at 25 °C with 12 h of near UV light per day, with undulate edge, pale rosy buff with fluffy white aerial mycelium, conidiomata scattered, isolated. Reverse pale buff.

Habitat: On leaves and stems of *Eucalyptus globulus.*

Distribution: Fundão, Guarda, Portugal.

Specimens examined: PORTUGAL, Fundão, on *Eucalyptus globulus*, March 2016, leg. C. Valente, holotype LISE 96318, culture ex-type MEAN 1308 = CBS 147684; Guarda, Rochoso, on *Eucalyptus globulus,* September 2013, leg. E. Diogo, culture MEAN 1309 = CBS 147685; Pegões, on *Eucalyptus globulus*, April 2013, leg. C. Valente, cultures MEAN 1322, MEAN 1323; Tondela, on *Eucalyptus globulus*, June 2014, leg. C. Valente, culture MEAN 1324; Paredes de Coura, on *Eucalyptus globulus*, March 2016, leg. C. Valente, culture MEAN 1326.

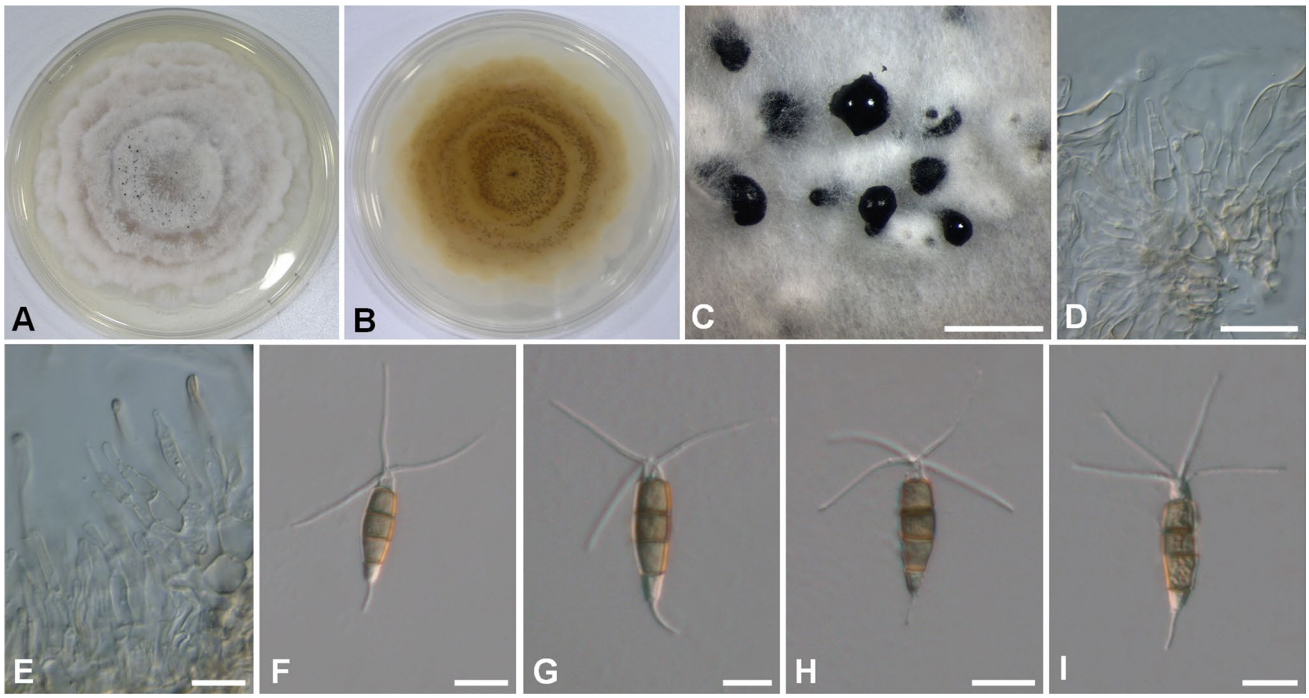

Fig. 4 *Neopestalotiopsis hispanica* (Holotype LISE 96319, ex-type culture CBS 147686 = MEAN 1310). **A**–**B** Colony on PDA (above and reverse), **C** Conidiomata on PDA, **D**–**E** Conidiogenous cells, **F**–**I** Conidia. Scale bars **C** = 1 mm, **D**–**I** = 10 µm

Notes: *Neopestalotiopsis eucalyptorum* is phylogenetically closely related to, but distinct from *N. foedans* and can be distinguished by its larger conidia (*N. eucalyptorum* (22.6)27.5–29.2(33.2) × (6.4)7.6–8.1(9.5) and *N. foedans* 19–24 × 5.7–6.9 ($\bar{x}$ = 20.7 × 6.4). *Neopestalotiopsis foedans* has a wide host range (Maharachchikumbura et al. 2012) but to the best or our knowledge was never isolated from *Eucalyptus* species. Blast searches in GenBank restricted to type species showed that *N. eucalyptorum* differs from *N. foedans* in ITS (NR_111785; identities 463/467 (99%), no gaps); *TUB2* (JX399022; identities 414/417 (99%), no gaps) and *TEF1* (JX399053; identities 467/482 (97%), 5 gaps (1%)).

Neopestalotiopsis hispanica E. Diogo, M. H. Bragança & A.J.L.Phillips sp. nov., Fig. 4

MycoBank MB839458.

Holotype: LISE 96319 (dried culture), ex-type culture MEAN 1310 = CBS 147686.

Etymology: Named after Hispania, the old roman name for the Iberian Peninsula.

Associated with leaves and stem necrosis of *Eucalyptus globulus* young plants and nursery plants for planting. Sexual state unknown. Asexual state: *Conidiomata* in culture on PDA pycnidial, globose, black, up to 690 µm, aggregated or discrete and scattered, semi-immersed, exuding black globose, glistening, conidial masses. *Conidiophores* indistinct and reduced to conidiogenous cells. *Conidiogenous cells* discrete, proliferating 1–2 times percurrently, collarete inconspicuous, cylindrical to subcylindrical, smooth, thin-walled, simple, 7–14.7 × 2.4–4.1 µm, apex 1.5–2.1 µm diameter. *Conidia* fusoid to ellipsoid, straight, 4 septate, (21.5)24.4–25.3(27.1) × (5.9)7.2–7.8(8.7) µm, $\bar{x}$ ± SD = 24.9 ± 1.3 × 7.5 ± 0.8 µm; basal cell obconic to subcylindrical with a truncate base, hyaline, smooth, and thin-walled, 3.3–5.5 µm long, three median cells doliiform, (13.5)15.8–16.5(17.8) µm long, $\bar{x}$ ± SD = 16.2 ± 1.0, wall smooth, versicoloured, septa darker than the cell, second cell from the base pale brown, 4.8–6.8 µm long, third cell honey brown 4.2–6.1 µm long, fourth cell brown to pale brown, 3.7–6.5 µm long, apical cell 2.8–6.2 µm long, hyaline, conical, smooth and thin-walled with 3–4 apical appendages arising from the apical crest, unbranched, filiform, flexuous, (30.7)19.5–22.6(30.7) µm long $\bar{x}$ ± SD = 21.0 ± 4.4 µm; basal appendage single, filiform, unbranched, centric, 5.1–15.5 µm long.

Culture characteristics: Colony on PDA attaining 74 mm diameter after 7 days at 25 °C, with smooth to slightly undulate edge, whitish with sparse aerial mycelium. Conidiomata scattered, isolated with concentric rings of more abundant conidiomata. Reverse pale buff to ochreous.

Habitat: On leaves and stems of *Eucalyptus globulus.*

Distribution: Fundão, Portugal and an unknown location in Spain.

Table 2 Morphological comparison of *Neopestalotiopsis* species related to this study

Species	Strain[a]	Conidial size (µm)	Apical appendages		Basal appendage
			Number	**Length (µm)**	**Length (µm)**
Neopestalotiopsis lusitanica	MEAN 1317	(27)30.6–32.5(38.7)×(8.2)9.6–10.3(12.5)	3–4(3)	(8.7)12.5–14.7(20.3)	(3.8)9.1–11(15)
N. brasiliense	COAD 2166	20–26.5×5–8.5	1–3	8.5–15	2–5
N. macadamiae	BRIP 63737c	(23)24–28(29)×(6)6.5–7.5(8)	2–3(3)	(24)25–30(32)	3–7
N. asiatica	MFLUCC 12–0286	20–26×5–7 $\bar{x}$ = 22.6×6.25	2–4(3)	20–30 ($\bar{x}$ = 25.6)	4–8($\bar{x}$ = 5.65)
N. umbrinospora	MFLUCC 12–0285	19–25×6–8 ($\bar{x}$= 21.3×6.5)	1–3(3)	22–35 ($\bar{x}$= 27.7)	5–7($\bar{x}$ = 5.9)
N. chrysea	MFLUCC 12–0261	20–24×5.5–7 ($\bar{x}$ = 22.3×6.1)	3	22–30 ($\bar{x}$= 26)	3–6($\bar{x}$= 4.4)
N. surinamensis	CBS 450.74	(23)24–28(29)×(7)7.5–9(9.5) $\bar{x}$ ± SD = 27.7 ± 1 × 8.1 ± 0.4	2–3	(15–)18–27(–28)	5–7
N. eucalyptorum	MEAN 1308	(22.6)27.5–29.2(33.2)×(6.4)7.6–8.1(9.5)	3–4(3)	(12.7)16.2–18.8(27.7)	(3.4)5.4–6.2(8.1)
N. foedans	CGMCC 3.9123	19–24×5.7–6.9 ($\bar{x}$= 20.7×6.4)	2–3(3)	6–18 ($\bar{x}$ = 13.3)	3–6($\bar{x}$ = 4.3)
N. hispanica	MEAN 1313	(21.4) 22.9–24.1 (29.4)×(7.2)8.2–8.7(9.8)	2–3(3)	(13)18.2–20.3(24.6)	(3.1)5.2–6.1(8.8)
N. longiappendiculata	MEAN 1315	(20.5) 22.4–23.4 (26.1)×(5.7)7–7.8(9.6)	2–4	(17.1)25.7–30.2(42.7)	(3.3)6.2–7.7(11.6)
N. iberica	MEAN 1310	(21.5) 24.4–25.3 (27.1)×(5.9)7.2–7.8(8.7)	3–4	(11)19.5–22.6(30.7)	(5.1)7.5–9.1(14.5)
N. australis	CBS 114159	(19)21–27(28)×(7)7.5–9(9.5) $\bar{x}$ ± SD = 24.6 ± 1.8 × 8 ± 0.4	3–4(4)	(19)21–32(34) $\bar{x}$ ± SD = 26.6 ± 3	3–7
N. vitis	MFLUCC 15–1265	(20)22–28(29)×(5)6–9.8(10.7) $\bar{x}$ ±SD 24.7 ± 1.4 × 7.7 ± 0.9	2–4(3)	(14)19–38.6(43) $\bar{x}$ ±SD = 24.2 ± 4.5	2.2–7.2

[a] References: see Table 1; strains in this study are in bold type

Specimens examined: Portugal, Fundão, on one year old plants, March 2016, leg. C. Valente, holotype LISE 96319, culture ex-type MEAN 1310 = CBS 147686; *idem* MEAN 1311; Spain, unknown location, on imported plants for plantation, May 2018, leg. C. Valente, culture MEAN 1312 = CBS 147687.

Notes: *N. hispanica* formed a sister clade with *N. longiappendiculata* sp. nov. These two species are easily distinguished because *N. longiappendiculata* has very long apical appendages (Table 2). Alignments of the sequences of these two species showed that ITS are identical, in *TUB2* differs in 2 b.p. and in *TEF1* differs in 15 b.p. with 5 gaps.

Neopestalotiopsis iberica E. Diogo, M. H. Bragança & A. J. L. Phillips sp. nov., Figure 5

MycoBank: MB839459.

Holotype: LISE 96320 (dried culture), ex-type culture MEAN 1313 = CBS 147688.

Etymology: Named after the region where it was found, the Iberian Peninsula.

Associated with leaves and stem necrosis of *Eucalyptus globulus* seedlings. Sexual state unknown. Asexual state: *Conidiomata* in culture on PDA picnidial, globose, black, up to 540 µm, aggregated or scattered, semi-immersed, exuding black, globose, glistening, conidial masses. *Conidiophores* indistinct and reduced to the conidiogenous cell. *Conidiogenous* cells discrete, ampulliform to lageniform, smooth, thin-walled, simple, 6.9–10.9 × 1.9–5.6 µm, apex 1.7–2.5 µm diameter. *Conidia* ellipsoid to obovoid, straight, 4 septate, (21.4) 22.9–24.1 (29.4) × (7.2) 8.2–8.7(9.8) µm, $\bar{x}$ ± SD = 23.5 ± 1.6 × 8.4 ± 0.7 µm; basal cell obconic to subcylindrical with a truncate base, hyaline to pale brown, smooth, and thin-walled, 3.4–5.6 µm long, three median cells doliiform, (13)14.8–15.5(17.7) µm long, $\bar{x}$ ± SD = 15.1 ± 0.9, smooth-walled, versicoloured, third septa from the base darker than the cell, second cell from the base pale brown, 3.9–6.7 µm long, third cell honey brown 3.7–6.2 µm long, fourth cell honey brown to brown, 4.5–6.6 µm long, apical cell 3.4–6.2 µm long, hyaline, subcylindrical to conical, smooth and thin-walled; with 3, rarely 2 apical appendages arising from the apical crest, unbranched, filiform, flexuous, (13)18.2–20.3(24.6) µm long $\bar{x}$ ± SD = 19.3 ± 3 µm; basal appendage single, filiform, unbranched, centric, 3.1–8.8 µm long.

Culture characteristics: Colony on PDA attaining 78 mm diameter after 7 days at 25 °C, with smooth to slightly undulate edge, pale rosy-buff with fluffy white aerial mycelium; conidiomata scattered, isolated with concentric rings of more abundant conidiomata. Reverse pale ochreous.

Habitat: On leaves and stems of *Eucalyptus globulus.*

Distribution: Pegões, Portugal and an unknown location in Spain.

Specimens examined: PORTUGAL, Pegões, on nursery plants, June 2017, leg. C. Valente, holotype LISE 96320, ex-type culture MEAN 1313 = CBS 147688; SPAIN, unknown

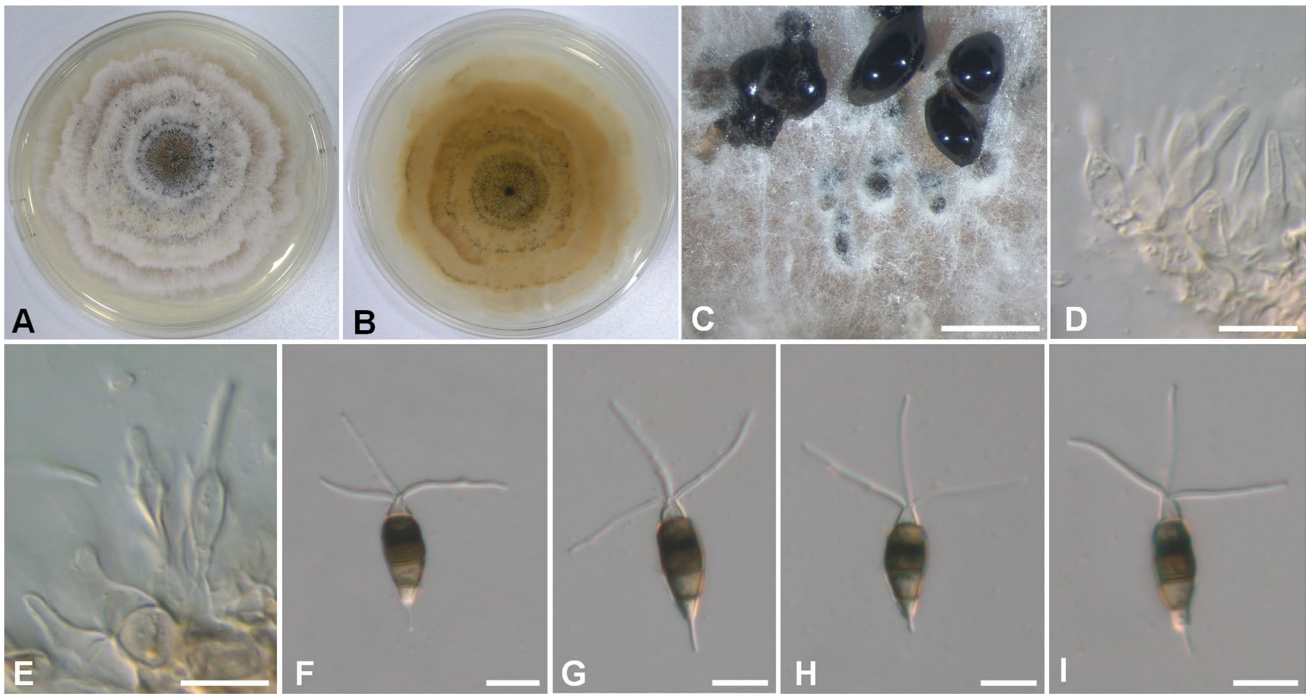

Fig. 5 *Neopestalotiopsis iberica* (Holotype LISE 96320, ex-type culture MEAN 1313 = CBS CBS 147688). **A**–**B** Colony on PDA (above and reverse), **C** Conidiomata on PDA, **D**–**E** Conidiogenous cells, **F**–**I** Conidia. Scale bars **C** = 1 mm, **D**–**I** = 10 µm

location, on nursery plants, May 2018, leg. C. Valente, culture MEAN 1314 = CBS 147689.

Notes: *Neopestalotiopsis iberica* is phylogenetically related to *N. autralis* and *N. vitis*. These three species are morphologically similar, but *N. iberica* has shorter apical appendages (*N. iberica* (11)19.5–22.6(30.7); *N. australis* (19)21–32(34); *N. vitis* (14)19–38.6(43)) and a longer single basal appendage (*N. iberica* 7.5–9.1; *N. australis* 3–7; *N. vitis* 2.2–7.2), while *N. vitis* has one or two basal appendages (Jayawardena et al. 2016, Table 2). Blast searches in GenBank restricted to type species showed that *N. iberica* differs from *N. australis* in ITS (KM199348; identities 494/497(99%), 2 gaps (0%)); *TUB2* (KM199432; identities 401/405 (99%), no gaps) and *TEF1* (KM199537; identities 458/466 (98%), no gaps) and differs from *N. vitis* ITS (KU140694; identities 419/421(99%), 1 gap (0%)); *TUB2* (KU140685; identities 316/319 (99%), no gaps) and *TEF1* (KU1406767; identities 370/373 (99%), no gaps).

Neopestalotiopsis longiappendiculata E. Diogo, M. H. Bragança & A.J.L.Phillips sp. nov., Figure 6

MycoBank: MB839460.

Holotype: LISE 96321 (dried culture), ex-type culture MEAN 1315 = CBS 147690.

Etymology: The name reflects the long apical appendages.

Associated with leaves and stem necrosis of *Eucalyptus globulus* plantlets. Sexual state unknown. Asexual state: *Conidiomata* in water agar with sterile eucalyptus leaf pieces pycnidial, globose, black, up to 490 µm, scattered, semi-immersed, exuding black globose, glistening, conidial masses. *Conidiophores* indistinct and reduced to conidiogenous cells. *Conidiogenous cells* discrete, ampulliform or subcylindrical, smooth, thin-walled, simple, 5.5–16.7 × 2.3–8.4 µm, apex 1.7–3.0 µm diameter. *Conidia* fusoid to ellipsoid, straight or slightly curved, 4 septate, (20.5)22.4–23.4(26.1) × (5.7)7–7.8(9.6) µm, $\bar{x}$ ± SD = 22.9 ± 1.5 × 7.4 ± 1.0 µm; basal cell obconic to subcylindrical with a truncate base, hyaline to pale brown, smooth, and thin-walled, 2.8–4.8 µm long, three median cells doliiform, (13.1)14.5–15.2(17.8) µm long, $\bar{x}$ ± SD = 14.8 ± 1, wall smooth, versicoloured, third septa from the base darker than the cell, second cell from the base pale brown, 4.1–5.9 µm long, third cell honey brown 4.4–6.0 µm long, fourth cell honey brown to brown, 4.2–6.6 µm long, apical cell 3.0–5.0 µm long, hyaline, subcylindrical to conical, smooth and thin-walled; with 2–4, mostly 3 apical appendages arising from the apical crest, unbranched, filiform, flexuous, (17.1)25.7–30.2(42.7) µm long $\bar{x}$ ± SD = 28 ± 6.3 µm; basal appendage single, filiform, unbranched, centric, 3.3–11.6 µm long.

Culture characteristics: Colony on PDA attaining 80 mm diameter after 7 days at 25 °C, with smooth to slightly undulate edge, pale rosy-buff with sparse aerial mycelium. Conidiomata very rare, scattered. Reverse pale buff.

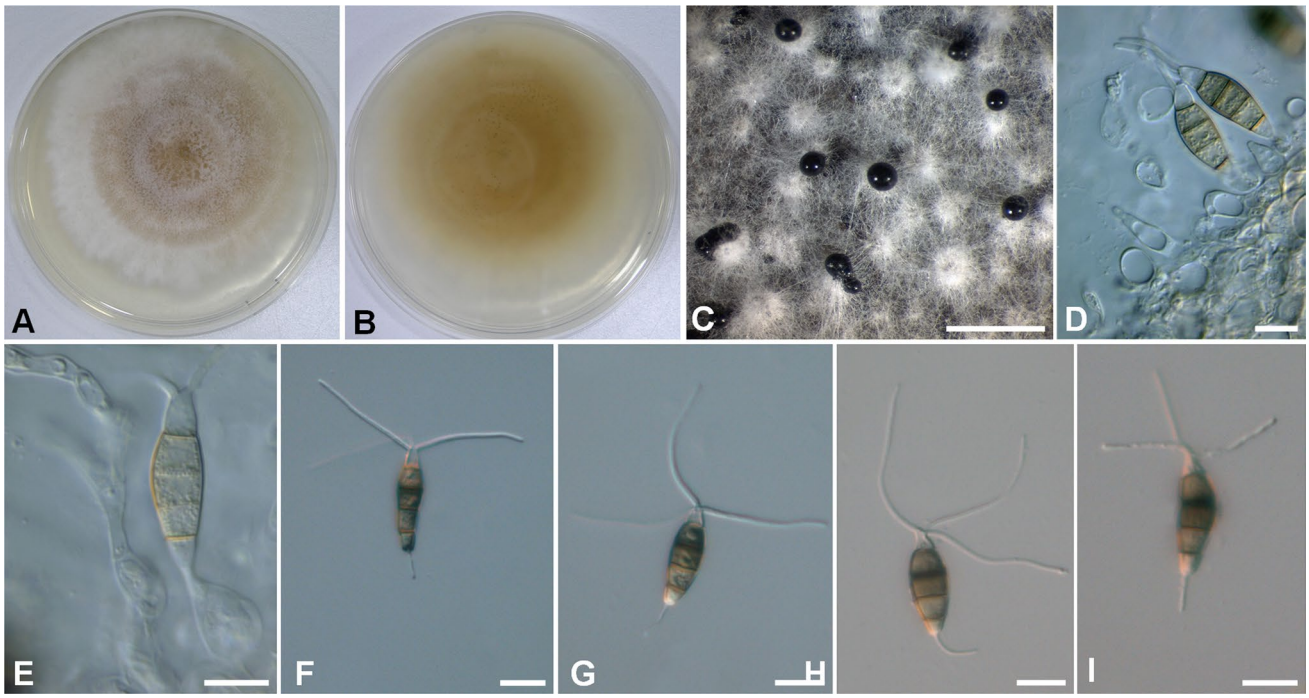

Fig. 6 *Neopestalotiopsis longiappendiculata* (Holotype LISE 96321, ex-type culture CBS 147690 = MEAN 1315). **A**–**B** Colony on PDA (above and reverse), **C** Conidiomata on PDA, **D**–**E** Conidiogenous cells, **F**–**I** Conidia. Scale bars **C** = 1 mm, **D**–**I** = 10 µm

Habitat: On leaves and stems of *Eucalyptus globulus* and *E. nitens*.

Distribution: Furadouro, Paredes de Coura, Portugal.

Specimens examined: PORTUGAL, Furadouro, on *Eucalyptus globulus* nursery plants, November 2017, leg. A. Reis, holotype LISE 96321, ex-type culture MEAN 1315 = CBS 147690; Paredes de Coura, on young plants of *Eucalyptus nitens*, March 2016, leg. C. Valente, culture MEAN 1316 = CBS 147691.

Notes: See notes under *N. hispanica*.

Neopestalotiopsis lusitanica E. Diogo, M. H. Bragança & A. J. L. Phillips sp. nov., Figure 7

MycoBank: MB839461.

Holotype: LISE 96322 (dried culture), ex-type culture MEAN 1317 = CBS 147692. *Etymology*: Named after the ancient Roman province of Lusitania that later became Portugal, the country where it was first detected.

Associated with leaf and stem necrosis of *Eucalyptus globulus* seedlings. Sexual state not observed. Asexual state: *Conidiomata* in culture on PDA pycnidial, globose, black, covered by white mycelia, up to 580 µm, aggregated or scattered, semi-immersed, exuding black globose, glistening, conidial masses. *Conidiophores* indistinct and reduced to conidiogenous cells. *Conidiogenous cells* discrete, ampulliform to lageniform, rarely cylindrical to subcylindrical, smooth, thin-walled, simple, 5.2–13.7×2.5 5.7 µm, apex 1.6–2.9 µm diameter. *Conidia* fusoid to ellipsoid, straight, rarely slightly curved, 4 septate, (27.5)30.6–32.5(38.7)×(8.2)9.6–10.3(12.5) µm, $\bar{x}$ ± SD = 31.6 ± 2.6 × 9.9 ± 1.0 µm; basal cell obconic with a truncate base, hyaline to very pale brown, smooth, and thin-walled, 4.5–7.9 µm long, three median cells doliiform, (17)18.9–20.4(24.1) µm long, $\bar{x}$ ± SD = 19.6 ± 2.1, wall smooth and slightly thick-walled, sometimes very slightly constricted at the septa, versicoloured, septa darker than the cell, second cell from the base pale brown, 5.4–9.6 µm long, third cell honey brown 4.7–7.6 µm long, fourth cell honey brown to brown, 4.7–7.6 µm long, apical cell 3.2–7.6 µm long, hyaline, conical to subcylindrical, rugose and thin-walled; with 3–4 apical appendages, rarely 2, arising from the apical crest, unbranched, filiform, flexuous, (8.2)12.5–14.7(19.7) µm long $\bar{x}$ ± SD = 13.6 ± 3.1 µm; basal appendage single, filiform, unbranched, centric, 3.8–15.0 µm long.

Culture characteristics: Colony on PDA attaining 85 mm diameter after 7 days at 25 °C with 12 h of near UV light, with smooth to slightly undulate edge, pale rosy-buff with sparse white aerial mycelium. Conidiomata scattered, isolated or aggregated with concentric rings of more abundant conidiomata. Reverse pale buff.

Habitat: Associated with leaves and stem necrosis of *Eucalyptus globulus*.

Distribution: Pegões, Portugal.

Specimens examined: PORTUGAL, Pegões, on *Eucalyptus globulus* nursery plants, June 2017, leg. C. Valente, holotype LISE 96322, ex-type culture MEAN 1317 = CBS 147692; Pegões, on *Eucalyptus globulus* nursery plants, December 2012, leg. C. Valente, culture MEAN 1318 = CBS

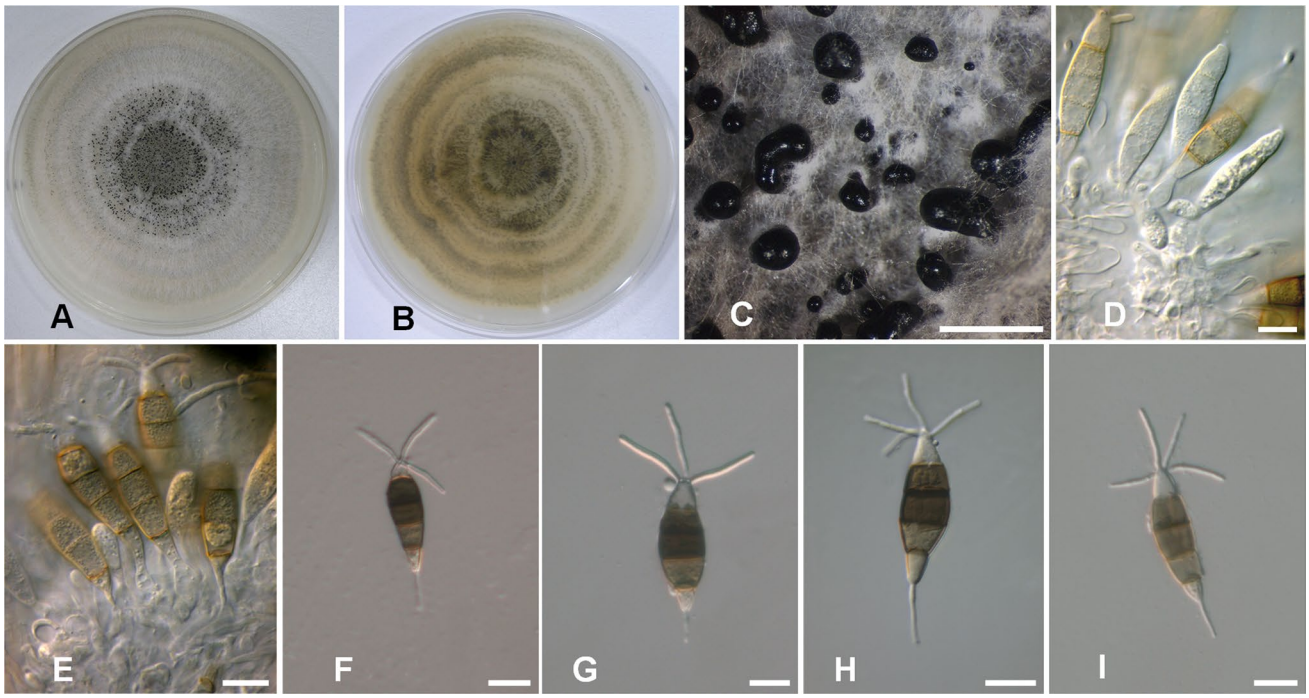

Fig. 7 *Neopestalotiopsis lusitanica* (Holotype LISE 96322, ex-type culture CBS 147692 = MEAN 1317). **A**–**B** Colony on PDA (above and reverse), **C** Conidiomata on PDA, **D**–**E** Conidiogenous cells, **F**–**I** Conidia. Scale bars **C** = 1 mm, **D**–**I** = 10 µm

147692, MEAN 1319; Pegões, on *Eucalyptus globulus* nursery plants, March 2013, leg. C. Valente, culture MEAN 1321.

Notes: *Neopestalotiopsis lusitanica* forms a well-resolved and well-supported clade (Fig. 2), sister to a large clade containing *N. brasiliense, N. macadamiae, N. asiatica, N. umbrinospora, N. chrysea* and *N. surimanemsis.* However, it can be distinguished from all these species by the larger conidia and longer basal appendage (Table 2). Blast searches in GenBank restricted to type species showed that *N. lusitanica* differs from *N. brasiliensis* in ITS (MG686469; identities 449/452(99%), no gaps); *TUB2* (MG692400; identities 383/385(99%), no gaps) and *TEF1* (MG692402; identities 455/474(96%), 2 gaps(0%)); from *N. asiatica* in ITS (NR_120181; identities 482/484 (99%), 2 gaps (0%)); *TUB2* (JX399018; identities 399/403 (99%)), no gaps) and *TEF1* (JX399049; identities 457/477 (96%), 7 gaps(1%)); from *N. umbrinospora* in ITS (NR_111783; identities 482/484 (99%), 2 gaps (0%)); *TUB2* (JX399019; identities 398/403 (99%)), no gaps) and *TEF1* (JX399050; identities 458/474 (97%), 2 gaps (0%)); from *N. chrysea* in ITS (NR_111784; identities 482/484 (99%), 2 gaps (0%)); *TUB2* (JX399020.; identities 398/403 (99%)), no gaps) and *TEF1* (JX399051; identities 456/474 (96%), 2 gaps(0%)) and from *N. surinamensis TUB2* (KM199465; identities 388/390 (99%), no gaps) and *TEF1* (KM199518; identities 442/455 (97%), 2 gaps (0%)); from *N. macadamiae* in ITS (NR_161002; identities 457/463 (99%), no gaps); *TUB2* (KX186654; identities 390/395 (99%), no gaps) and *TEF1* (9 pb difference and two gaps, verified in an 213 pb alignment with MEAN 1317, since the *TEF1* sequence of the type species of *N. macadamiae* is very short and is not available for blast searches in Genbank).

Discussion

In this study, twenty-three isolates of *Neopestalotiopsis* species derived from diseased young plants of *E. globulus* and *E. nitens* were characterised in terms of morphology and phylogeny based on combined ITS, *TEF1* and *TUB2* sequence data. Two isolates clustered with the *N. hadrolaeliae/N. honoluluana/N. zimbabwana* complex, and four clustered in a poorly resolved clade sister to *N. eucalyptorum*. Fourteen isolates were resolved into five well-supported clades separate from all other species in *Neopestalotiopsis* and were described and introduced as new species. These are the first reports of *Neopestalotiopsis* species associated with *Eucalyptus* in Portugal.

Morphological identification of pestalotioid species is unreliable due to overlapping morphological characteristics used to define species and the plasticity of these characters that change with culture conditions, age of cultures and subculturing (Hu et al. 2007). In a phylogenetic study of diversity of endophytic *Pestalotiopsis* species

obtained from *Pinus armandii* and *Ribes* sp., Hu et al. (2007) found that ITS was not sufficiently informative to segregate species in this genus and proposed β-tubulin as an additional gene. Later, Maharachchikumbura et al. (2012) tested ten different genes (*act*, *cal*, *gs*, *gapdh*, ITS, LSU, 18 S nrDNA, *rpb1*, *TEF1* and *TUB2*) and selected ITS, *TEF1* and *TUB2* as the most suitable to resolve species in *Pestalotiopsis*.

Neopestalotiopsis was introduced by Maharachchikumbura et al. (2014b) who segregated *Pestalotiopsis* into three genera, namely, *Pestalotiopsis*, *Neopestalotiopsis* and *Pseudopestalotiopsis*, based on morphology and phylogenetic analysis. Accordingly, *Neopestalotiopsis* can be distinguished from *Pestalotiopsis* s.s. by versicoloured conidial cells and indistinct conidiophores that are often reduced to the conidiogenous cells; *Pestalotiopsis* species have concolourous conidial cells and conspicuous conidiophores, while *Pseudopestalotiopsis* species have darker concolourous median cells and indistinct conidiophores. Morphologically, all the strains isolated in this study fit within *Neopestalotiopsis*, having versicoloured conidial cells and indistinct conidiophores.

Following the work of Maharachchikumbura et al. (2014b), phylogenetic analyses of *Neopestalotiopsis* have been based on combined ITS, *TEF1* and *TUB2*, and numerous species have been introduced in the genus (see Table 1). In our analysis, not all strains could be identified, but five clades received good support and were considered to represent previously unknown species that are introduced here as new species. Two isolates clustered in a clade with three known species that are not well-resolved. For this reason, we decided not to give them a name until that clade has been resolved. This is in accordance with recent studies where poor resolution was obtained for some species in *Neopestalotiopsis* (Belisário et al. 2020; Gerardo-Lugo et al. 2020; Liu et al. 2017).

Pestalotiopsis s. l. is generally considered to be endophytes or weak pathogens causing disease only when the plants are under stress (Maharachchikumbura et al. 2011). However, a new *Pestalotiopsis* species, *P. pini*, was reported recently in Portugal causing shoot blight and trunk necrosis on *Pinus pinea* (Silva et al. 2020). Furthermore, a common disease in nurseries in Brazil has been attributed to an unidentified species of *Pestalotiopsis*. It was regarded as a minor disease associated with the conditions required for the propagation process. This includes wounds, high humidity, and long periods of leaf wetness (Alfenas et al. 2009; Santos et al. 2020). Therefore, this species has been regarded as a secondary and opportunistic pathogen. Recently, Belisário et al. (2020) demonstrated that *N. rosae* and *N. protearum* as well as other unidentified species can infect unwounded leaves of *Eucalyptus* under long periods of leaf wetness. They also tested the pathogenicity of six other species from different hosts and found that all tested species caused similar symptoms. Santos et al. (2020) also identified *N. rosae*, *N. australis* and *N. eucalypti* causing disease in old cuttings in conditions less favourable to infection. On the pathogenicity tests, in addition to the injury on the cutting stem at the inoculation point, Santos et al. (2020) observed the presence of the fungus in the leaf petiole above the inoculation point, suggesting that the fungus may migrate through the vascular system of the plant and cause lesions at points distant from the site of inoculation. In the present study, we also found lesions in plants recently transplanted into the field suggesting that the fungus may remain latent in the nursery plants but then cause symptoms during the transplantation stress. It is well-known that some fungi that are considered endophytic may became pathogenic when their hosts became stressed (Stergiopoulos and Gordon 2014). Therefore, it is important to monitor and control the presence of these fungi in nurseries.

Although pathogenicity of the species described in this study has not been determined, according to pathogenicity tests previously done on *Eucalyptus* (Belisário et al. 2020; Santos et al. 2020), several species of *Neopestalotiopsis* proved to be pathogenic and cannot be regarded simply as endophytes or opportunistic pathogens. Since all isolates obtained in this study were obtained from diseases plants, it likely that they are pathogenic. In addition, several recently described *Neopestalotiopsis* species have been associated with plant diseases. Solarte el al. (2018) showed that guava scab in Colombia is caused by several species of *Pestalotiopsis* and *Neopestalotiopsis*. *Neopestalotiopsis rosicola* causes rose stem canker in China (Jiang et al. 2018), and *Neopestalotiopsis vitis* is responsible for leaf spots of *Vitis vinifera* also in China (Jayawardena et al. 2016). Various *Neopestalotiopsis* species were also isolated in association with grapevine trunk diseases in France (Maharachchikumbura et al. 2016). *Neopestalotiopsis macadamiae* causes dry flower disease of Macadamia in Australia (Akinsanmi et al. 2017). Chen et al. (2018) reported that *Neopestalotiopsis clavispora* and other pestalotioid species cause grey blight disease on *Camellia sinensis* in China. Furthermore, other pestalotioid fungi (e.g., *Pestalotiopsis biciliata*) have already been proven to be pathogenic on *Eucalyptus* species (Morales-Rodríguez et al. 2019).

Pathogenicity, host specificity and geographic distribution of the new species remain unknown, and these are issues that should be considered in future studies. To our knowledge this is the first report of *Neopestalotiopsis* species causing leaf and stem necrosis on *Eucalyptus globulus* and *E. nitens* in Portugal.

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Author contribution H.B., E.D., C.I.G. and C.V. designed the experiments; E.D. and A.C.S. performed morphological and phylogenetic analysis; C.I.B and E.D. wrote the first draft of the manuscript; H.B and A.J.L.P. reviewed and edited the manuscript. HB and A.J.L.P. supervised the study. All authors have read, edited and approved the final manuscript.

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Data availability Strains are available at MEAN (https://www.iniav.pt/recursos-geneticos-microbianos) and CBS (https://wi.knaw.nl/page/Collection) culture collections. Sequences are available at GenBank (https://www.ncbi.nlm.nih.gov/genbank) and alignments and trees are deposited in TreeBase (https://treebase.org).

Declarations

Conflict of interest The authors declare no competing interests.

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