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Diversity and phylogeny of *Neofusicoccum* species occurring in forest and urban environments in Portugal

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Abstract

A collection of *Neofusicoccum* isolates was obtained from a large number of plant species, showing dieback and canker symptoms, in forest and urban environments in Portugal. A total of 351 isolates was characterised by BOX-PCR fingerprinting to evaluate their overall genetic diversity. Representatives of each group identified in this analysis were selected for multilocus sequence analyses, using sequences of the ribosomal internal transcribed spacer region (ITS rDNA) and partial sequences of the translation elongation factor 1-alpha (*tef1*) and β-tubulin (*tub2*). Phylogenetic analysis of multilocus sequence data identified five species within the collection of isolates, namely *N. australe*, *N. eucalyptorum*, *N. kwambonambiense*, *N. luteum*, and *N. parvum*. Of these *N. australe* and *N. eucalyptorum* were the most frequent representing the vast majority of the isolates. Several new fungus-host associations were established for all of the *Neofusicoccum* species found. Here we report for the first time the occurrence of *N. eucalyptorum* on a host outside the family Myrtaceae. The results of this study show that the genus *Neofusicocccum* appears to be common and widespread on a broad range of hosts representing a potential threat to susceptible plants. Additionally, ornamental plants in urban environments are shown to be hosts of a diverse assemblage of *Neofusicoccum* species.

Key words – *Botryosphaeriaceae* **–** endophytic **–** host-association **–** ornamentals **–** pathogenic

Introduction

The genus *Neofusicoccum* is a member of the *Botryosphaeriaceae* (Botryosphaeriales, Dothideomycetes) comprising numerous species found on a wide range of plant hosts of agricultural, forestry, ecological and economic importance (Crous et al. 2006, Slippers &Wingfield 2007, Slippers et al. 2013). Host infections are thought to occur predominantly through horizontal transmission, i.e. individual infections *via* spores (ascospores or conidia) but also through the seeds (vertical transmission). Inside the plant, they have the ability to colonize without producing any external symptoms, remaining inside the host as endophytes (Slippers & Wingfield 2007). Endophytism could be, however, an important feature since these fungi can be moved easily and inconspicuously around the world in seeds, cuttings and even fruits, subsequently infecting both native and non-native trees in their new environments (Burgess et al. 2005, Slippers et al. 2013).

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The change from endophytic to pathogenic phase is often related to stress such as drought, extreme temperature fluctuations, nutrient deficiencies and mechanical injuries. Infected plants can exhibit a multiplicity of disease symptoms such as fruit rots, leaf spots, seedling damping-off and collar rot, cankers, blight of shoots and seedlings, gummosis, blue-stain of the sapwood, dieback and tree death (Slippers & Wingfield 2007).

Neofusicoccum is known to include a large number of phylogenetically closely related and morphologically similar cryptic species rendering phenotypic characteristics such as morphology, growth and culture appearance inadequate for species identification. Thus, species discrimination is based on a multilocus sequencing approach (Pavlic et al. 2009a, 2009b).

Within the 29 species currently accepted in the genus some are known to have very wide host and geographic ranges while others show some host preference. For example, *N. parvum* was reported from 90 hosts in 29 countries on six continents by Sakalidis et al. (2013). In contrast, *N. eucalypticola* and *N. mangiferae* were associated only with *Eucalyptus* spp. and *Mangifera indica* respectively and were geographically more restricted (Phillips et al. 2013). In general, species of *Neofusicoccum* are a constant presence in almost all kind of woody plants. For instance, they are frequently isolated from eucalypts (Iturritxa et al. 2011), almond (Inderbitzin et al. 2010), avocado (McDonald & Eskalen 2011), walnut (Yu et al. 2015), grapevine (Mondello et al. 2013, Berraf-Tebbal et al. 2014), olive (Triki et al. 2015), blueberry (Pérez et al. 2014), mango (Ismail et al. 2013), rubber tree (Ngobisa et al. 2013) and peach (Thomidis et al. 2011). Although these fungi have been relatively well studied on economically important crops, much less is known about their prevalence in others groups of plants namely the gymnosperms (Slippers et al. 2005, Golzar & Burgess 2011) and ornamental species (Zlatković et al. 2016). Although of low economic value, ornamental plant species should not be ignored as they provide a wide range of ecosystem services (Zlatković et al. 2016).

In Portugal the forest area occupies 35.4% of the total territory according to the National Forest Inventory 2013 (ICNF, 2013). *Eucalyptus globulus*, a non-native species, is the most abundant followed by *Quercus suber* and *Pinus pinaster*. Apart from the forest species Portugal has important crops such as grapevine and olive among others. In spite of this, knowledge about the diversity of the genus *Neofusicoccum* in Portugal is scarce. The few known studies were done on grapevines (Phillips 2002), conifers (Alves et al. 2013) and more recently on eucalypts (Barradas et al. 2016).

To gain further knowledge about the diversity of the genus *Neofusicoccum* in Portugal the main goal of this study was to identify the species associated with a wide diversity of plants. For this, a survey was performed on several species of forest and crop trees and also on ornamental species.

Materials & Methods

Fungal isolation and morphological characterization

Isolates were obtained from samples collected from plants in natural forest ecosystems and ornamentals planted in urban environments (e.g. parks, gardens, streetscapes). The following hosts were sampled: *Acacia longifolia*, *Aesculus hippocastanum*, *Castanea sativa*, *Ferula communis*, *Fraxinus angustifolia*, *Fraxinus excelsior*, *Fraxinus ornus*, *Hydrangea macrophylla*, *Malus domestica*, *Melia azedarach*, *Olea europaea*, *Populus alba*, *Populus tremula*, *Pyracantha coccinea*, *Quercus robur*, *Rosa* sp., *Tilia platyphyllos* and *Ulmus minor*. This study also included isolates from diseased and healthy *Eucalyptus globulus* that were previously obtained by Barradas et al. (2016). The remaining isolates were obtained from hosts showing disease symptoms on stems, trunks and branches such as canker and dieback (Fig. 1). Samples from diseased plants were initially screened for the presence of fruiting bodies (ascomata and/or conidiomata) and when present single spore isolations were made as described previously (Phillips et al. 2013). In the absence of fruiting bodies isolations were made by plating pieces of diseased tissues following

Fig. 1 – **a**. *Aesculus hippocastanum* with trunk canker. **b**. Detailed view of trunk canker. **c,d**. *Tilia platyphyllos* with symptoms of dieback of twigs and branches associated with *N. luteum* and *N. australe*. **e**. Ascus and ascospores of *N. australe* on *Ferula communis*. **f**. Developing conidia of *N. luteum* on *Melia azedarach.* **g**. Conidia of *N. australe* from *Acacia longifolia*.

surface sterilization as described by Alves et al. (2013). Cultures were routinely grown and maintained on half-strength potato-dextrose agar (PDA) (HIMEDIA, India).

To assign isolates to the genus *Neofusicoccum* conidial micromorphological characteristics and mode of conidiogenesis were observed with a Nikon 80i microscope and images captured with a Nikon DS-Ri1 camera. Isolates were induced to sporulate by plating them on ¼ strength PDA (Merck, Germany) containing sterilised pine needles and incubating at room temperature (about 20–25ºC) under diffused daylight until pycnidia developed. For microscopy, pycnidia were mounted in 100% lactic acid.

Molecular characterization

Genomic DNA was extracted from fresh mycelium grown on half-strength PDA plates for 5 d at approximately 23°C, according to Alves et al. (2004). All PCR reactions were carried out in 25 µL reaction mixtures with NZYTaq 2× Green Master Mix (2.5 mM MgCl2; 200 µM dNTPs; 0.2 U/µL DNA polymerase) (Lisbon, Portugal) in a Bio-Rad C-1000 Touch™ Thermal Cycler (Hercules, CA, USA). Negative controls with sterile water instead of the template DNA were used in every PCR reaction.

BOX-PCR fingerprinting was done as described previously (Alves et al. 2007) using primer BOXA1R. Representatives of each group identified in this analysis were selected for multilocus sequence analyses (Table 1). Primers ITS1 and ITS4 (White et al. 1990) were used for amplification and sequencing of the ITS region of the ribosomal RNA as described by Alves et al. (2004). Part of the translation elongation factor 1-alpha (*tef1*) was amplified with the primers EF1-688F and EF1-1251R (Alves et al. 2008) with the conditions described by Phillips et al. (2005). Part of the β-tubulin gene (*tub2*) was amplified with primers T1 (O'Donnell & Cigelnik 1997) and Bt2b (Glass & Donaldson 1995) using the following conditions: 95°C for 3 min; 35 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min; final extension at 72°C for 10 min.

PCR amplicons were purified with the DNA Clean & Concentrator™-5 kit (Zymo Research, CA, USA) before DNA sequencing. Both strands of the PCR products were sequenced at GATC Biotech (Cologne, Germany). The nucleotide sequences were read with FinchTV v.1.4.0 (Geospiza Inc.). All sequences were checked manually, and nucleotide arrangements at ambiguous positions were clarified using both primer direction sequences. Sequences were deposited in GenBank (Table 1) and the alignment in TreeBase (S19901).

Available ITS, *tef1* and *tub2* sequences from known and well-characterized *Neofusicoccum* species were retrieved from GenBank and also included in the phylogenetic analyses. Sequences of *Dothiorella sarmentorum* and *Do. iberica* were used as outgroup (Table 1).

Sequences were aligned with ClustalX v. 2.1 (Thompson et al. 1997), using the following parameters: pairwise alignment parameters (gap opening = 10, gap extension = 0.1) and multiple alignment parameters (gap opening = 10, gap extension = 0.2, transition weight = 0.5, delay divergent sequences = 25 %). Alignments were checked and edited with BioEdit Alignment Editor v. 7.2.5 (Hall 1999). Phylogenetic analyses of sequence data were done with MEGA6 v. 6.06 (Tamura et al. 2013). All gaps were included in the analyses. The model of DNA sequence evolution used for each dataset was determined by the software. Maximum likelihood (ML) analyses were performed on a neighbour-joining (NJ) starting tree automatically generated by the software. Bootstrap analyses with 1000 replicates were used to estimate the consistency of each node of the trees. Trees were visualized and edited with Interactive tree of life (iTOL) v3 (Letunic & Bork 2016).

Results

Fungal Isolation

A collection of 351 isolates from different hosts was established. These isolates were initially selected based on culture characteristics typical of the *Botryosphaeriaceae*, namely fast-growing fluffy white aerial mycelium becoming grey to black with grey to indigo-grey or black pigment visible from the reverse side of Petri dishes. Most isolates sporulated within 2–3 weeks on ¼ strength PDA supplemented with pine needles. Micromorphology of conidia and conidiogenesis assigned them to *Neofusicoccum*. Host ranges of the species were determined from the SMML Fungus-Host Distribution Database as well as available literature. Several new fungus-host associations were established (Table 2).

Table 1 Identity of the isolates studied and GenBank accession numbers of sequences used in phylogenetic analyses.

				GenBank Accession[b]		
Species	**Isolate[a]**	**Origin**	**Host**	**ITS**	***tef1***	***tub2***
Dothiorella iberica	**CBS115041**	Spain	*Quercus ilex*	*AY573202*	*AY573222*	*EU673096*
D. sarmentorum	**IMI63581b**	United Kingdom	*Ulmus* sp.	*AY573212*	*AY573235*	*EU673102*
N. algeriense	**CBS137504**	Algeria	*Vitis vinifera*	*KJ657702*	*KX505893*	*KX505915*
	CAA322	Portugal	*Malus domestica*	*KX505906*	*KX505894*	*KX505916*
	CAA366	Portugal	*Eucalyptus globulus*	*KT440951*	*KT441011*	KX871764
	PE32	Portugal	*Eucalyptus globulus*	*KT440952*	*KT441012*	KX871765
N. andinum	**CBS117453**	Venezuela	*Eucalyptus* sp.	*GU251155*	*GU251287*	*GU251815*
N. arbuti	**CBS116131**	USA	*Arbutus menziesii*	*AY819720*	*KF531792*	*KF531793*
	CBS117090	USA	*Arbutus menziesii*	*AY819724*	*KF531791*	*KF531794*
N. australe	**CMW6837**	Australia	*Acacia* sp.	*AY339262*	*AY339270*	*AY339254*
	CMW6853	Australia	*Sequoiadendron giganteum*	*AY339263*	*AY339271*	*AY339255*
	CAA178	Portugal	*Ferula communis*	KX871844	KX871800	KX871709
	CAA184	Portugal	*Ferula communis*	KX871845	KX871801	KX871710
	CAA191	Portugal	*Ferula communis*	KX871846	KX871802	KX871711
	CAA195	Portugal	*Ferula communis*	KX871847	KX871803	KX871712
	CAA197	Portugal	*Ferula communis*	KX871848	KX871804	KX871713
	CAA202	Portugal	*Melia azedarach*	KX871849	KX871805	KX871714
	CAA231	Portugal	*Hydrangea macrophylla*	KX871850	KX871806	KX871715
	CAA233	Portugal	*Hydrangea macrophylla*	KX871851	KX871807	KX871716
	CAA242	Portugal	*Hydrangea macrophylla*	KX871852	KX871808	KX871717
	CAA319	Portugal	*Eucalyptus globulus*	*KT440900*	*KT440960*	KX871718
	CAA320	Portugal	*Eucalyptus globulus*	*KT440901*	*KT440961*	KX871719
	CAA326	Portugal	*Pyracantha coccinea*	KX871853	KX871809	KX871720
	CAA327	Portugal	*Pyracantha coccinea*	KX871854	KX871810	KX871721
	CAA332	Portugal	*Eucalyptus globulus*	*KT440902*	*KT440962*	KX871722
	CAA341	Portugal	*Eucalyptus globulus*	*KT440903*	*KT440963*	KX871723
	CAA344	Portugal	*Eucalyptus globulus*	*KT440904*	*KT440964*	KX871724
	CAA351	Portugal	*Eucalyptus globulus*	*KT440905*	*KT440965*	KX871725
	CAA357	Portugal	*Eucalyptus globulus*	*KT440906*	*KT440966*	KX871726
	CAA359	Portugal	*Eucalyptus globulus*	*KT440907*	*KT440967*	KX871727
	CAA392	Portugal	*Quercus robur*	KX871855	KX871811	KX871728
	CAA398	Portugal	*Eucalyptus globulus*	KX871856	KX871812	KX871729
	CAA400	Portugal	*Eucalyptus globulus*	*KT440908*	*KT440968*	KX871730
	CAA401	Portugal	*Eucalyptus globulus*	*KT440909*	*KT440969*	KX871731
	CAA406	Portugal	*Eucalyptus globulus*	*KT440910*	*KT440970*	KX871732
	CAA420	Portugal	*Eucalyptus globulus*	*KT440911*	*KT440971*	KX871733
	CAA427	Portugal	*Eucalyptus globulus*	*KT440912*	*KT440972*	KX871734

				GenBank Accession[b]		
Species	**Isolate[a]**	**Origin**	**Host**	**ITS**	***tef1***	***tub2***
	CAA434	Portugal	*Eucalyptus globulus*	*KT440913*	*KT440973*	*KX505927*
	CAA441	Portugal	*Eucalyptus globulus*	*KT440914*	*KT440974*	KX871735
	CAA455	Portugal	*Eucalyptus globulus*	*KT440915*	*KT440975*	*KX505928*
	CAA464	Portugal	*Eucalyptus globulus*	*KT440916*	*KT440976*	KX871736
	CAA466	Portugal	*Eucalyptus globulus*	*KT440917*	*KT440977*	KX871737
	CAA468	Portugal	*Olea europaea*	KX871857	KX871813	KX871738
	CAA475	Portugal	*Olea europaea*	KX871858	KX871814	KX871739
	CAA546	Portugal	*Eucalyptus globulus*	*KT440918*	*KT440978*	KX871740
	CAA549	Portugal	*Eucalyptus globulus*	*KT440919*	*KT440979*	KX871741
	CAA550	Portugal	*Eucalyptus globulus*	KX871859	KX871815	KX871742
	CAA571	Portugal	*Eucalyptus globulus*	KX871860	KX871816	KX871743
	CAA647	Portugal	*Eucalyptus globulus*	*KT440920*	*KT440980*	KX871744
	CAA648	Portugal	*Eucalyptus globulus*	*KT440921*	*KT440981*	KX871745
	CAA649	Portugal	*Eucalyptus globulus*	KX871861	KX871817	KX871746
	CAA723	Portugal	*Tilia platyphyllos*	KX871862	KX871818	KX871747
	CAA741	Portugal	*Acacia longifolia*	KX871863	KX871819	KX871748
	CAA743	Portugal	*Acacia longifolia*	KX871864	KX871820	KX871749
	CAA747	Portugal	*Acacia longifolia*	KX871865	KX871821	KX871750
	CAA749	Portugal	*Acacia longifolia*	KX871866	KX871822	KX871751
	CAA750	Portugal	*Acacia longifolia*	KX871867	KX871823	KX871752
	CAA751	Portugal	*Acacia longifolia*	KX871868	KX871824	KX871753
N. batangarum	**CBS124924**	Cameroon	*Terminalia catappa*	*FJ900607*	*FJ900653*	*FJ900634*
	CBS124923	Cameroon	*Terminalia catappa*	*FJ900608*	*FJ900654*	*FJ900635*
N. brasiliense	**CMM1338**	Brazil	*Mangifera indica*	*JX513630*	*JX513610*	*KC794031*
	CMM1285	Brazil	*Mangifera indica*	*JX513628*	*JX513608*	*KC794030*
N. cordaticola	**CBS123634**	South Africa	*Syzygium cordatum*	*EU821898*	*EU821868*	*EU821838*
	CBS123635	South Africa	*Syzygium cordatum*	*EU821903*	*EU821873*	*EU821843*
N. cryptoaustrale	CMW23785	South Africa	*Eucalyptus* sp.	*FJ752742*	*FJ752713*	*FJ752756*
	CMW20738	South Africa	*Eucalyptus citriodora*	*FJ752740*	*FJ752710*	*FJ752754*
N. eucalypticola	**CBS115679**	Australia	*Eucalyptus grandis*	*AY615141*	*AY615133*	*AY615125*
	CBS115766	Australia	*Eucalyptus rossi*	*AY615143*	*AY615135*	*AY615127*
N. eucalyptorum	**CBS115791**	South Africa	*Eucalyptus grandis*	*AF283686*	*AY236891*	*AY236920*
	CAA369	Portugal	*Eucalyptus globulus*	*KT440922*	*KT440982*	KX871773
	CAA450	Portugal	*Eucalyptus globulus*	*KT440923*	*KT440983*	KX871774
	CAA517	Portugal	*Eucalyptus globulus*	*KT440924*	*KT440984*	KX871775
	CAA518	Portugal	*Eucalyptus globulus*	KX871883	KX871839	KX871776
	CAA520	Portugal	*Eucalyptus globulus*	*KT440925*	*KT440985*	KX871777
	CAA522	Portugal	*Eucalyptus globulus*	*KT440926*	*KT440986*	KX871778
	CAA528	Portugal	*Eucalyptus globulus*	*KT440927*	*KT440987*	KX871779

				GenBank Accession[b]		
Species	**Isolate[a]**	**Origin**	**Host**	**ITS**	***tef1***	***tub2***
	CAA532	Portugal	*Eucalyptus globulus*	*KT440928*	*KT440988*	KX871780
	CAA535	Portugal	*Eucalyptus globulus*	*KT440929*	*KT440989*	KX871781
	CAA536	Portugal	*Eucalyptus globulus*	*KT440930*	*KT440990*	KX871782
	CAA539	Portugal	*Eucalyptus globulus*	KX871884	KX871840	KX871783
	CAA542	Portugal	*Eucalyptus globulus*	*KT440931*	*KT440991*	KX871784
	CAA558	Portugal	*Eucalyptus globulus*	*KT440932*	*KT440992*	KX871785
	CAA561	Portugal	*Fraxinus excelsior*	KX871885	KX871841	KX871786
	CAA601	Portugal	*Eucalyptus globulus*	*KT440933*	*KT440993*	KX871787
	CAA604	Portugal	*Eucalyptus globulus*	*KT440934*	*KT440994*	KX871788
	CAA618	Portugal	*Eucalyptus globulus*	*KT440935*	*KT440995*	KX871789
	CAA624	Portugal	*Eucalyptus globulus*	*KT440936*	*KT440996*	KX871790
	CAA651	Portugal	*Eucalyptus globulus*	*KT440937*	*KT440997*	KX871791
	CAA680	Portugal	*Eucalyptus globulus*	*KT440938*	*KT440998*	KX871792
	CAA683	Portugal	*Eucalyptus globulus*	*KT440939*	*KT440999*	KX871793
	CAA695	Portugal	*Eucalyptus globulus*	*KT440940*	*KT441000*	KX871794
	CAA709	Portugal	*Eucalyptus globulus*	*KT440941*	*KT441001*	*KX505920*
	CAA712	Portugal	*Eucalyptus globulus*	*KT440942*	*KT441002*	KX871795
	CAA713	Portugal	*Eucalyptus globulus*	*KT440943*	*KT441003*	*KX505921*
	CAA714	Portugal	*Eucalyptus globulus*	KX871886	KX871842	KX871796
	PE20	Portugal	*Eucalyptus globulus*	*KT440944*	*KT441004*	KX871797
	PE21	Portugal	*Eucalyptus globulus*	*KT440945*	*KT441005*	KX871798
	PE23	Portugal	*Eucalyptus globulus*	KX871887	KX871843	KX871799
N. hellenicum	**CERC1947**	Greece	*Pistacia vera*	*KP217053*	*KP217061*	*KP217069*
	CERC1948	Greece	*Pistacia vera*	*KP217054*	*KP217062*	*KP217070*
N. kwambonambiense	**CBS123639**	South Africa	*Syzygium cordatum*	*EU821900*	*EU821870*	*EU821840*
	CBS123641	South Africa	*Syzygium cordatum*	*EU821919*	*EU821889*	*EU821859*
	CAA755	Portugal	*Eucalyptus globulus*	*KT440946*	*KT441006*	*KX505917*
N. luteum	**CBS110299**	Portugal	*Vitis vinifera*	*AY259091*	*AY573217*	*DQ458848*
	CBS110497	Portugal	*Vitis vinifera*	*EU673311*	*EU673277*	*EU673092*
	CAA200	Portugal	*Melia azedarach*	KX871869	KX871825	KX871754
	CAA203	Portugal	*Melia azedarach*	KX871870	KX871826	KX871755
	CAA352	Portugal	*Quercus robur*	KX871871	KX871827	KX871756
	CAA360	Portugal	*Fraxinus ornus*	KX871872	KX871828	KX871757
	CAA362	Portugal	*Fraxinus ornus*	KX871873	KX871829	KX871758
	CAA365	Portugal	*Quercus robur*	KX871874	KX871830	KX871759
	CAA379	Portugal	*Melia azedarach*	KX871875	KX871831	KX871760
	CAA412	Portugal	*Populus alba*	KX871876	KX871832	KX871761
	CAA505	Portugal	*Fraxinus ornus*	KX871877	KX871833	KX871762
	CAA628	Portugal	*Fraxinus excelsior*	*KX505911*	*KX505902*	*KX505929*

				GenBank Accession[b]		
Species	**Isolate[a]**	**Origin**	**Host**	**ITS**	***tef1***	***tub2***
	CAA720	Portugal	*Tilia platyphyllos*	KX871878	KX871834	KX871763
N. macroclavatum	**CBS118223**	Australia	*Eucalyptus globulus*	*DQ093196*	*DQ093217*	*DQ093206*
	WAC12445	Australia	*Eucalyptus globulus*	*DQ093197*	*DQ093218*	*DQ093208*
N. mangiferae	CBS118531	Australia	*Mangifera indica*	*AY615185*	*DQ093221*	*AY615172*
	CBS118532	Australia	*Mangifera indica*	*AY615186*	*DQ093220*	*AY615173*
N. mediterraneum	**CBS121718**	Greece	*Eucalyptus* sp.	*GU251176*	*GU251308*	*GU251836*
	CBS121558	USA	*Vitis vinifera*	*GU799463*	*GU799462*	*GU799461*
N. nonquaesitum	**CBS126655**	USA	*Umbellularia californica*	*GU251163*	*GU251295*	*GU251823*
N. nonquaesitum	PD301	Chile	*Vaccinium corymbosum*	*GU251164*	*GU251296*	*GU251824*
N. occulatum	**CBS128008**	Australia	*Eucalyptus grandis* hybrid	*EU301030*	*EU339509*	*EU339472*
	MUCC286	Australia	*Eucalyptus pellita*	*EU736947*	*EU339511*	*EU339474*
N. parvum	**CMW9081**	New Zealand	*Populus nigra*	*AY236943*	*AY236888*	*AY236917*
	UCR-NP2	USA	*Vitis vinifera*	*AORE01001444*	*AORE01000046*	*AORE01001255*
	CBS110301	Portugal	*Vitis vinifera*	*AY259098*	*AY573221*	*EU673095*
	CAA189	Portugal	*Ferula communis*	KX871879	KX871835	KX871766
	CAA192	Portugal	*Ferula communis*	*KX505905*	*KX505892*	*KX505913*
	CAA384	Portugal	*Rosa* sp.	KX871880	KX871836	KX871767
	CAA386	Portugal	*Rosa* sp.	KX871881	KX871837	KX871768
	CAA608	Portugal	*Aesculus hippocastanum*	KX871882	KX871838	KX871769
	CAA692	Portugal	*Eucalyptus globulus*	*KT440950*	*KT441010*	KX871770
	CAA704	Portugal	*Eucalyptus globulus*	*KT440947*	*KT441007*	*KX505914*
	PE17	Portugal	*Eucalyptus globulus*	*KT440948*	*KT441008*	KX871771
	PE18	Portugal	*Eucalyptus globulus*	*KT440949*	*KT441009*	KX871772
N. pennatisporum	**MUCC510**	Australia	*Allocasuarina fraseriana*	*EF591925*	*EF591976*	*EF591959*
N. protearum	MUCC497	Australia	*Santalum acuminatum*	*EF591912*	*EF591965*	*EF591948*
N. ribis	**CBS115475**	USA	*Ribes* sp.	*AY236935*	*AY236877*	*AY236906*
	CBS121.26	Unknown	*Ribes rubrum*	*AF241177*	*AY236879*	*AY236908*
N. umdonicola	**CBS123645**	South Africa	*Syzygium cordatum*	*EU821904*	*EU821874*	*EU821844*
	CBS123646	South Africa	*Syzygium cordatum*	*EU821905*	*EU821875*	*EU821845*
N. vitifusiforme	5H022	California	*Juglans regia*	*KF778869*	*KF779059*	*KF778964*
	B8	Italy	*Vitis vinifera*	*KC469638*	*KX505897*	*KX505922*
	B9	Italy	*Vitis vinifera*	*KX505908*	*KX505898*	*KX505923*

[a]Acronyms of culture collections: **CAA** – Personal culture collection Artur Alves, Universidade de Aveiro, Portugal; **CBS** – Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; **CERC** – China Eucalypt Research Center, Beijing, China; **CMM** – Coleção de culturas de fungos fitopatogénicos Prof. Maria Menezes, Universidade Federal Rural de Pernambuco, Brazil; **CMW** – Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; **IMI** - International Mycological Institute, CBI-Bioscience, Egham, Bakeham Lane, UK; **MUCC** – Murdoch University Culture Collection, Perth, Australia; **PD** - University of California, Davis, USA; **UCR** – College of Natural and Agricultural Sciences, Riverside, California, USA; **WAC** - Department of Agriculture, Western Australia Plant Pathogen Collection, South Perth, Western Australia.
[b]Sequence numbers in italics were retrieved from GenBank. All others were determined in the present study.
Isolates in bold are ex-type cultures.

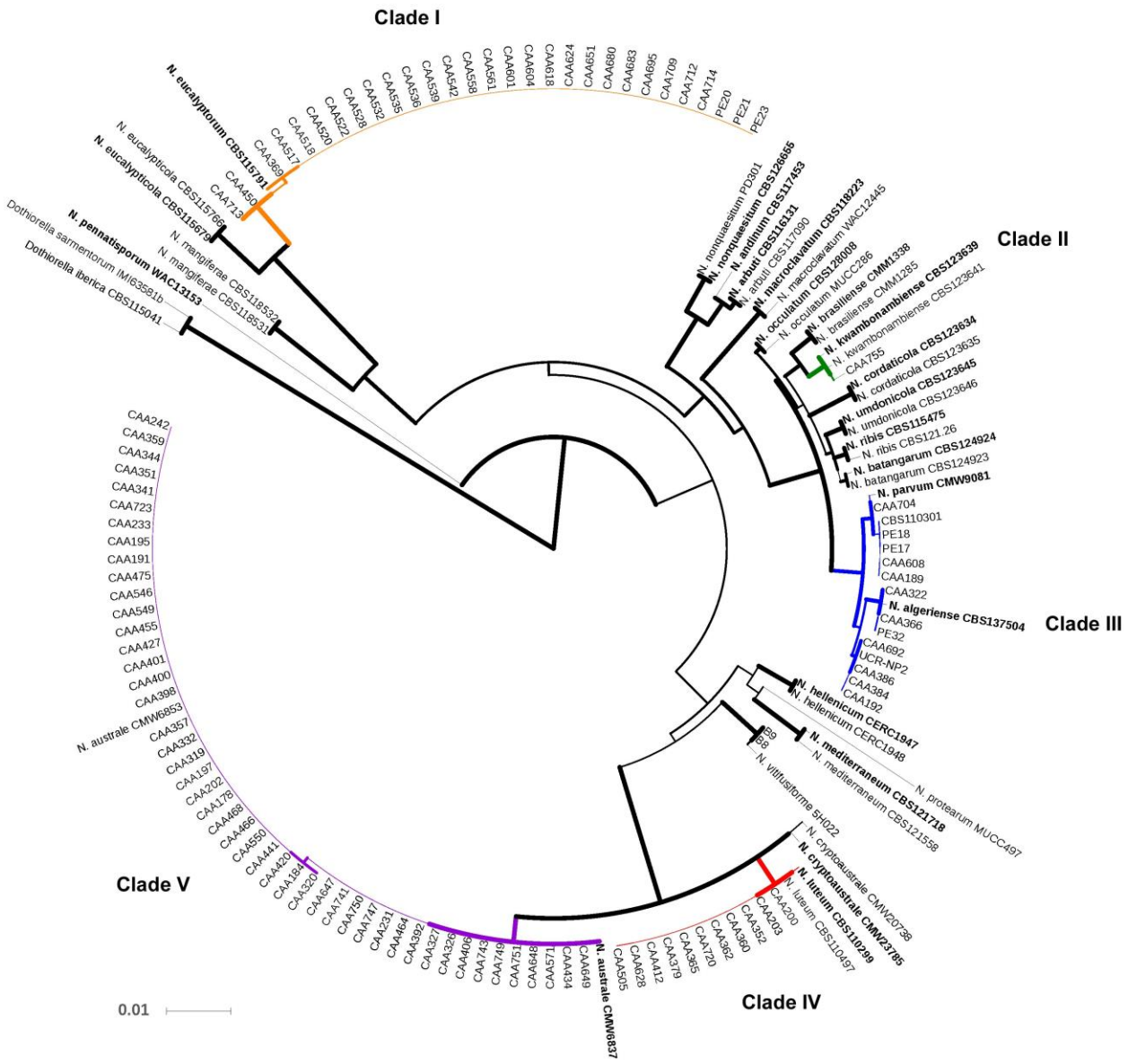

Fig. 2 – Combined ITS, *tef1* and *tub2* maximum likelihood tree based on the Tamura 3-parameter model. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The thickness of branches is proportional to bootstrap support values.

Molecular Characterization

BOX-PCR fingerprinting analysis divided the 351 isolates into 7 distinct clusters, which were presumed to represent distinct species. A total of 99 isolates representative of each group were selected for further molecular characterization (Table 1). The 7 clusters formed by the BOX-PCR fingerprinting analysis were resolved into 5 clades by multilocus (ITS, *tef1* and *tub2*) phylogenetic analysis (Fig. 2).

Clades I, II, IV and V were clearly resolved and represent the species *N. eucalyptorum*, *N. kwambonambiense*, *N. luteum* and *N. australe* respectively. Clade III contained isolates belonging to two species (*N. parvum* and *N. algeriense*) and was further divided into 3 subclades. However, these showed incongruence between phylogenetic analyses results obtained from each individual locus (data

not shown) and from the combined dataset (Fig. 2). Moreover, there were no fixed alleles between the different subclades.

Table 2 *Neofusicoccum* species isolated in this study and their respective hosts

Species	Host
N. australe	*Acacia longifolia*[a,b]
	Castanea sativa[a,b]
	Eucalyptus globulus
	Ferula communis[a,b]
	Fraxinus excelsior[a,b]
	Hydrangea macrophylla[a,b]
	Melia azedarach[a,b]
	Olea europaea[b]
	Populus alba[a,b]
	Pyracantha coccinea[a,b]
	Quercus robur
	Tilia platyphyllos[a,b]
	Ulmus minor[a,b]
N. eucalyptorum	*Eucalyptus globulus*
	Fraxinus excelsior[a,b]
N. kwambonambiense	*Eucalyptus globulus*
N. luteum	*Fraxinus excelsior*[a,b]
	Fraxinus ornus[a,b]
	Melia azedarach[a,b]
	Populus alba[a,b]
	Populus tremula[a,b]
	Quercus robur
	Tilia platyphyllos[a,b]
N. parvum	*Aesculus hippocastanum*[b]
	Eucalyptus globulus
	Ferula communis[a,b]
	Malus domestica
	Melia azedarach[a,b]
	Rosa sp.[a,b]

[a]new host reported for the species
[b]first report from Portugal

Within the clades formed by *N. eucalyptorum* and *N. australe* two subclades were also noticeable. However, a comparison of sequences of the three loci from members of each subclade showed minor differences between them. Thus, only 1 bp difference in the *tef1* of *N. eucalyptorum* isolates and 1 bp in the *tub2* sequence of *N. australe* isolates.

Discussion

In this study a collection of 351 isolates retrieved from a large diversity of plant hosts was characterised by morphological and PCR typing analysis. Selected representative isolates of each PCR

typing group were further characterised by multilocus phylogenetic analyses. The isolates studied grouped into five clades, four of which clearly represented distinct species (Fig. 2).

The clade containing *N. parvum* and *N. algeriense* (Clade III) was not clearly resolved, exhibiting incongruence between phylogenetic analysis results obtained from each individual locus and the combined dataset. A similar inconsistency was seen in phylogenetic analyses based on *MAT* genes (Lopes et al. 2016). By applying the principle of Phylogenetic Species Recognition (Taylor et al. 2000) where the transition from concordance to conflict determines the limits of species Lopes et al. (2016) considered that this clade represented a single species and synonymized *N. algeriense* with *N. parvum*. This study is in agreement with this previous finding.

Neofusicoccum australe and *N. eucalyptorum* were the most common species found. *Neofusicoccum australe* was originally regarded as native to Australia but since then it has been shown to have a widespread distribution occurring on a broad range of hosts (Sakalidis et al. 2011, Phillips et al. 2013). In Portugal, *N. australe* was found on *Rubus* sp. (Phillips et al. 2006), *Quercus robur* (Barradas et al. 2013), *Eucalyptus globulus* (Barradas et al. 2016), *Robinia pseudoacacia* (van Niekerk et al. 2004) and several species of conifers (Alves et al. 2013). To our knowledge, this study is the first to report *N. australe* occurring on *A. longifolia, C. sativa, F. communis, F. excelsior, H. macrophylla, M. azedarach, P. alba, P. coccinea, T. platyphyllos* and *U. minor*. It is also the first time that this species is found on *O. europaea* in Portugal. Another interesting finding was the isolation of *N. australe* from *A. longifolia*, being the first report of this species colonizing *Acacia* spp. outside of Australia. This could have serious repercussions on the dissemination of *N. australe* in Portugal since *Acacia* spp. are introduced exotic species that have spread rapidly to several new areas, from the coast to inland forests. Colonization of the invasive species *A. longifolia* will allow *N. australe* to be rapidly introduced into new geographic areas, possibly infecting new hosts.

Neofusicoccum eucalyptorum was first found on diseased *Eucalyptus grandis* and *E. nitens* in South Africa (Smith et al. 2001). Later, the species was isolated from cankers on native and planted eucalypts in eastern Australia (Slippers et al. 2004). Based on the dominance and wide distribution in eastern Australia, the authors suggested that the pathogen is probably native to this area (Slippers et al. 2004). Meanwhile, the presence of *N. eucalyptorum* was also detected on eucalypt species in other countries including Portugal (Barradas et al. 2016). Several authors suggested that the occurrence of the species on *Eucalyptus* in others parts of the world is a consequence of anthropogenic actions due to the large amounts of germplasm traded (Pérez et al. 2009, Barradas et al. 2016). Although this species is apparently specialized in the infection of *Eucalyptus* spp., it has also been associated with other genera in the Myrtaceae (Pérez et al. 2009, Pérez et al. 2010). In our study we report the occurrence of *N. eucalyptorum* in *Fraxinus excelsior* (Oleaceae) planted as ornamental. This is the first time that *N. eucalyptorum* is associated with a host outside of the family Myrtaceae. However, it is important to note that the *F. excelsior* tree from which the fungus was isolated was surrounded by a large number of eucalypts. Thus, it is possible that *F. excelsior* was colonized due to the high pressure of the surrounding inoculum or the fungus used it as a transition host. Further studies should be carried out to test the pathogenicity of *N. eucalyptorum* to this host and evaluate the impact that host jumps may have on the fungus host expansion and pathogenicity.

The species *N. luteum* and *N. parvum* were also found in this study although the number of isolates was lower. Both species are known to occur on a wide range of hosts worldwide (Phillips et al. 2013). *Neofusicoccum luteum* has been associated with dieback and canker mostly on crops (e.g. Phillips 2002, Úrbez-Torres et al. 2013) but also on ornamentals (Marincowitz et al. 2008, Varela et al. 2011). In Portugal, *N. luteum* has been found to infect conifers (Alves et al. 2013), *Quercus robur* (Barradas et al. 2013), grapevines, *Fraxinus angustifolia* and *Sophora japonica* (Phillips et al. 2002). In our study we found new host associations namely with *M. azedarach*, *F. ornus*, *F. excelsior*, *P. alba*, *P. tremula* and *T. platyphyllos*, all of them planted as ornamentals.

Neofusicoccum parvum is probably the species within the genus with the widest geographic distribution, host range and proven ability to cause disease (Phillips et al. 2013, Sakalidis et al. 2013). It has been found associated with many forest species (Iturritxa et al. 2011), fruit trees (Ismail et al. 2013) and ornamental plants (Marincowitz et al. 2008, Zlatković et al. 2016). In Portugal, *N. parvum* was found associated with *Protea cynaroides* and *P. repens* (Crous et al. 2013), grapevines (Phillips 2002), conifers (Alves et al. 2013) and *E. globulus* (Barradas et al. 2016). To our knowledge, this study is the first to report the association of *N. parvum* with *Rosa* spp., *F. communis*, *M. azedarach* and also the first occurrence of *N. parvum* on *A. hippocastanum* in Portugal. The fungus was only recently associated for the first time with *A. hippocastanum* in the Western Balkans, showing symptoms of canker and dieback (Zlatković et al. 2016). In our study it was isolated from severely affected trees with trunk cankers (Fig. 1) and planted as ornamentals on streetscapes. However, since no pathogenicity tests were carried out we cannot conclude that *N. parvum* was the cause of the observed symptoms. This aspect should be addressed in future studies.

The presence, in this study, of species in such a wide diversity of hosts confirms that *Neofusicoccum* species are opportunistic fungi that can potentially colonize most plant hosts that it comes into contact with and represents a threat to vulnerable plants. This study reinforces the urgent need to understand the routes of introduction and dissemination of these fungi, not only in natural environments but also in the less studied urban environments where many potential hosts are planted as ornamentals.

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