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New or Unusual Disease Reports

Identification of *Neofusicoccum parvum* causing canker and twig blight on *Ficus carica* in Italy

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Summary. During June 2018, several symptomatic fig (*Ficus carica*) cuttings, showing twig blight, subcortical discolouration and apical dieback were collected from a nursery in Catania province, Sicily (Italy). Isolations from diseased tissue consistently showed the presence of the same fungal colony. Morphology of the fungal isolates together with sequence data of the nuclear rDNA internal transcriber spacer (ITS) region, translation elongation factor 1-alpha (*tef*1) gene and partial beta-tubulin (*tub2*) gene of representatives isolates revealed the presence of the fungus *Neofusicoccum parvum*. Pathogenicity tests were conducted by inoculating fig cuttings with mycelial plugs. After 10 days, the inoculated plants developed cankers similar to those observed in the greenhouse and after 26 days all inoculated plants were dead. To the best of our knowledge, this is the first report worldwide of *N. parvum* causing disease on this host.

Keywords. Fig cuttings, pathogenicity, molecular analysis, Botryosphaeriaceae.

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INTRODUCTION

Fig (*Ficus carica L.*) is one of the most cultivated crop of the Mediterranean basin Countries. Several diseased caused by bacteria, viruses, oomycetes and fungi are reported for this crop throughout the world (Ferguson *et al.*, 1990).

In the last years, there has been increased interest in "edible landscapes" and some woody ornamental plant genera or species are selected for attractive garden plants that also have edible fruit. Small edible ornamental figs obtained from cutting are produced in Sicily both for aesthetic value as well as consumption. During June 2018, examination of 1,500 fig cuttings in an ornamental nursery in Catania province, Sicily (Italy), revealed that up to 20% of the plants showed canker and twig blight during the rooting step of the propagation process. Therefore, the aim of this study was to identify the causal agent of canker and twig blight on fig in Sicily, Italy.

MATERIAL AND METHODS

Isolation and morphological characterization of the pathogen

Twenty symptomatic fig cuttings, showing canker and twig blight were collected from a greenhouse in Catania province, Sicily (Italy) during the rooting step of the propagation process. Small sections of diseased woody tissue were surface disinfected for 1 min in 1.5% sodium hypochlorite, rinsed in sterile water, placed on potato dextrose agar (3.9% PDA, Oxoid) amended with 100 mg/liter of streptomycin sulfate (Sigma-Aldrich) to prevent bacterial growth, and then incubated at 25 ± 1°C for three–four days. A total of 12 morphologically similar isolates were obtained from single conidium or hyphal tip of pure cultures on PDA at 25 ± 1°C. For the morphological characterization of the pathogen, the isolates were transferred on PDA and technical agar (TA, 1.2% agar, Oxoid) with sterilized pine needles deposited onto the surface, and then incubated in a growth chamber at 25 ± 1°C with a 12 h photoperiod. Size and shape of conidia were recorded from colonies grown on TA with sterilized pine needles after 3 weeks.

Pathogenicity tests

Pathogenicity tests were conducted on eighteen potted, healthy, 1-month-old cuttings of fig, with the same number for the control. A piece of bark was removed with a 6 mm diam. cork borer and 6-mm-diameter mycelial plugs taken from a 7-day-old culture of isolate Di3AFC1 (CBS 145622) were applied to two separate wounds on each twig. The wounds were covered with Parafilm® (Pechney Plastic Packaging Inc., Chicago, USA) to prevent desiccation. Controls consisted of sterile PDA plugs applied to wounds. All replicates were kept in a growth chamber with a 12 h photoperiod at 25 ± 1°C. The presence and length of the resulting lesions was recorded 10, 14 and 26 days after inoculation.

Molecular identification

Genomic DNA was extracted from isolates Di3AFC1, Di3AFC2 and Di3AFC3 (CBS 145622, CBS 145623 and CBS 145624) using the Wizard Genomic DNA Purification Kit (Promega Corporation, WI, USA). The internal transcriber spacer region (ITS) of the nuclear ribosomal RNA operon was amplified with primers ITS5 and ITS4 (White *et al.,* 1990), the primers EF1-728F and EF1-986R (Carbone and Kohn, 1999) were used to amplify part of the translation elongation factor 1alpha gene (*tef1*) while the primer set Bt-2a and Bt-2b (Glass and Donaldson 1995) were used for the partial beta-tubulin (*tub2*) gene. The PCR products were sequenced in both directions by Macrogen Inc. (South Korea). The DNA sequences generated were analysed and consensus sequences were computed using the program Geneious v. 11.1.5.

Phylogenetic analysis

The sequences obtained in this study were blasted against the NCBIs GenBank nucleotide database to determine the closest relatives to be included in the phylogenetic analysis. Blast analisys indicated that all the isolates belonged to the genus *Neofusicoccum*. Sequence alignments of the different gene regions, including sequences generated in this study and sequences downloaded from GenBank, were initially performed using the MAFFT v. 7 online server (http://mafft.cbrc.jp/alignment/server/index. html) (Katoh and Standley, 2013), and then manually adjusted in MEGA v. 7 (Kumar *et al*., 2016). To establish the identity of fungal isolates, phylogenetic analyses were conducted using individual locus (data not shown) as well as concatenated analyses of three loci (ITS, *tef1* and *tub2*). Additional reference sequences were selected based on recent studies on *Neofusicoccum* genus (Yang *et al*., 2017). Phylogenetic analyses were based on Maximum Parsimony (MP) for all the individual loci and on both MP and Bayesian Inference (BI) for the combined multilocus analyses. For BI, the best evolutionary model for each partition was selected based on MrModeltest v. 2.3 (Nylander 2004) and incorporated into the analyses. MrBayes v. 3.2.5 (Ronquist *et al*., 2012) was used to generate phylogenetic trees under optimal criteria per partition. The Markov Chain Monte Carlo (MCMC) analysis used four chains and started from a random tree topology. The heating parameter was set to 0.2 and trees were sampled every 1 000 generations. Analyses stopped once the average standard deviation of split frequencies was below 0.01. The MP analysis was done using PAUP (Swofford, 2003). Phylogenetic relationships were estimated by heuristic searches with 100 random addition sequences. Tree bisection- reconnection was used, with the branch swapping option set on 'best trees' only with all characters weighted equally and alignment gaps treated as fifth state. Tree length (TL), consistency index (CI), retention index (RI) and rescaled consistence index (RC) were calculated for parsimony and the bootstrap analyses (Hillis & Bull 1993) were based on 1 000 replicates. Sequences generated in this study were deposited in GenBank (Table 1).

Table 1. Collection details and GenBank accession numbers of isolates included in this study.

Species	Culture no.[1]	Host	Country	GenBank no.[2]		
				ITS	*tef1*	*tub2*
Diplodia seriata	CBS 110875	*Vitis vinifera*	South Africa	AY343456	KX464592	KX464827
Neofusicoccum arbuti	CBS 116131^T	*Arbutus menziesii*	USA: Washington	AY819720	KF531792	KF531793
N. australe	CBS 139662^T	*Acacia* sp.	Australia	AY339262	AY339270	AY339254
	CBS 121115	*Prunus persica*	South Africa	EF445355	EF445386	KX464948
N. batangarum	CBS 124924^T	*Terminalia catappa*	Cameroon	FJ900607	FJ900653	FJ900634
N. cryptoaustrale	CBS 122813^T	*Eucalyptus* sp.	South Africa	FJ752742	FJ752713	FJ752756
N. kwambonambiense	CBS 102.17^T	*Carya illinoensis*	USA: Florida	KX464169	KX464686	KX464964
N. luteum	CBS 562.92^T	*Actinidia deliciosa*	New Zealand	KX464170	KX464690	KX464968
N. mangiferae	CBS 118532	*Mangifera indica*	Australia	AY615186	DQ093220	AY615173
N. mediterraneum	CBS 121718^T	*Eucalyptus* sp.	Greece	GU251176	GU251308	-
N. parvum	CBS 123650	*Syzygium cordatum*	South Africa	KX464182	KX464708	KX464994
	CMW 9081^T	*Populus nigra*	New Zealand	AY236943	AY236888	AY236917
	Di3AFC1	*Ficus carica*	Italy	*MN611179*	*MN623346*	*MN623343*
	Di3AFC2	*Ficus carica*	Italy	*MN611180*	*MN623347*	*MN623344*
	Di3AFC3	*Ficus carica*	Italy	*MN611181*	*MN623348*	*MN623345*
N. pistaciarum	CBS 113083^T	*Pistacia vera*	USA: California	KX464186	KX464712	KX464998
N. protearum	CBS 114176	*Leucadendron laureolum*	South Africa	AF452539	KX464720	KX465006
N. stellenboschiana	CBS 110864^T	*Vitis vinifera*	South Africa	AY343407	AY343348	KX465047
	CBS 121116	*Prunus armeniaca*	South Africa	EF445356	EF445387	KX465049
N. terminaliae	CBS 125264	*Terminalia sericea*	South Africa	GQ471804	GQ471782	KX465053
N. vitifusiforme	CBS 110887^T	*Vitis vinifera*	South Africa	AY343383	AY343343	KX465061

[1] CBS: Westerdijk Fungal Biodiversity Institute, Utrecht; CMW: Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; Di3A: Dipartimento di Agricoltura Alimentazione e Ambiente. Ex-type and ex-epitype cultures are indicated with ^{T}.

[2] ITS: internal transcribed spacers 1 and 2 together with 5.8S nrDNA; tef1: translation elongation factor 1-alpha; tub2: partial beta-tubulin gene. Sequences generated in this study are indicated in italics.

RESULTS AND DISCUSSION

Symptomatic plants showed cankers, shoot blight and subcortical discolouration (Figure 1a, b). One type of colony was consistently obtained from these symptomatic tissues. A total of 12 fungal isolates were established from single conidium or hyphal tip cultures on PDA. The isolates developed abundant, aerial mycelium that became grey after 2–3 days and then black with age. On pine needles the isolates formed black, globose pycnidia after 3 weeks. Conidia were hyaline, non-septate, ellipsoid, thin walled and measured 14–20 × 4.5–7 µm. After 10 days from inoculation cankers, characterized by discolouration and necrotic internal tissue, developed at both inoculation points with an average lesion length of 14.5 cm. Pycnidia developed above and near each lesion. Infection also resulted in shoot blight and apical dieback of the inoculated plants. After 14 days, 44% of the inoculated plants were dead and after 26 days, all the inoculated plants had died (Figure 1c, d, e). These symptoms on the inoculated plants were identical to those observed in diseased fig cuttings in the greenhouse. All the control plants, inoculated only with agar plugs, did not develop any symptoms. Some of the diseased tested plants were used to re-isolate the fungus in order to fulfill the Koch's postulates. These results confirm that the isolated fungus was the causal agent of the disease.

The three MP trees derived from the single gene sequence alignments (ITS, *tef1* and *tub2*) produced topologically similar trees. The combined phylogeny of *Neofusicoccum* species consisted of 21 sequences, including the outgroup sequences of *Diplodia seriata* (culture CBS 110875). A total of 1 262 characters (ITS: 1–507, *tef1*: 514–821, *tub2*: 828–1262) were included in the phylogenetic analysis. The results showed 114 characters were parsimony-informative, 185 were variable and parsimony-uninformative and 951 characters were constant. A maximum of 1 000 equally most parsimonious trees were saved (Tree length = 438, CI = 0.811, RI = 0.842 and RC = 0.682). Bootstrap support values from the parsimony analysis are plotted on the Bayes-

Figure 1. Natural symptoms of disease (a, b) and disease symptoms reproduced from artificial inoculation of fig cuttings (c–e). a, shoot blight; b, internal discolouration; c, shoot blight and apical dieback 26 days after pathogen inoculation; d, dark pycnidia above the inoculation point; e, internal lesion and discolouration under the inoculation point after 10 days on artificially inoculated fig cuttings.

ian phylogenies in Figure 2. For the Bayesian analyses, MrModeltest suggested that all partitions should be analysed with dirichlet state frequency distributions. The following models were recommended by MrModeltest and used: K80+I+G for ITS, HKY+G for *tef1* and *tub2*. In the Bayesian analysis, the ITS partition had 74 unique site patterns, the *tef1* partition had 118 unique site patterns and the *tub2* partition had 62 unique site patterns, and the analysis ran for 1100000 generations, resulting in 2022 trees of which 1320 trees were used to calculate the posterior probabilities.

In the combined analyses, the three representative isolates clustered with one reference strain and the ex-type strain of *N. parvum* (CMW9081). The individual alignments and trees of the single loci used in the analyses were compared with respect to their performance in species recognition. According with morphological and molecular analyses, the isolates were identified as *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips.

Fungi belonging to the Botryosphaeriaceae family are reported worldwide as pathogens of several *Ficus* species (Al-Bedak, 2018; Mohali *et al*., 2017; El-Atta and Aref, 2013). The cultivated fig (*F. carica*), is reported to be attacked by different species in the Botryosphaeriaceae, including *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. (Çeliker and Michailides, 2012) and *Neoscytalidium dimidiatum* (Penz.) Crous & Slippers (Elshafie and Ba-Omar, 2002; Ray *et al*., 2010). Until now, *N. parvum* has been reported only on the ornamental *Ficus microcarpa* L. in association with other Botryosphaeriaceae species causing branch canker and dieback (Mayorquin *et al*., 2012).

Neofusicoccum parvum, characterized by the proven ability to induce disease, has a very wide geographical and host distribution (Sakalidis *et al.,* 2013), and in Italy was already reported to cause several diseases on different woody hosts, like olive (Carlucci *et al.,* 2013), mango (Ismail *et al.,* 2013), avocado (Guarnaccia *et al.,* 2016;), loquat (Giambra *et al.,* 2016), pomegranate (Riccioni *et al.,* 2017) grapevine (Mondello *et al.,* 2013; Carlucci *et al.,* 2015) and *Rhaphiolepis indica* (Gusella *et al*., 2020). Since *N. parvum* has been reported on the genus *Ficus*, although on a different species (Mayorquin *et al*., 2012), it is no surprise to find it on another species of the same genus. Nevertheless, we need to point out that in this

case *N. parvum* was found causing serious disease on an important crop for the southern regions of in Italy, and for several other countries of the Mediterranean Basin, like Turkey, Morocco, Egypt and Algeria. In addition, we need to highlight that this report refers to the propagation process of fig cuttings that represents a crucial step to avoid severe infections before the commercialization of this species in Italy and abroad. The recovery of this fungus from fig cuttings confirms its high diffusion in Italy and the high risk for other susceptible crops. To the best of our knowledge, this is the first report worldwide of the fungus *N. parvum* attacking *F. carica*.

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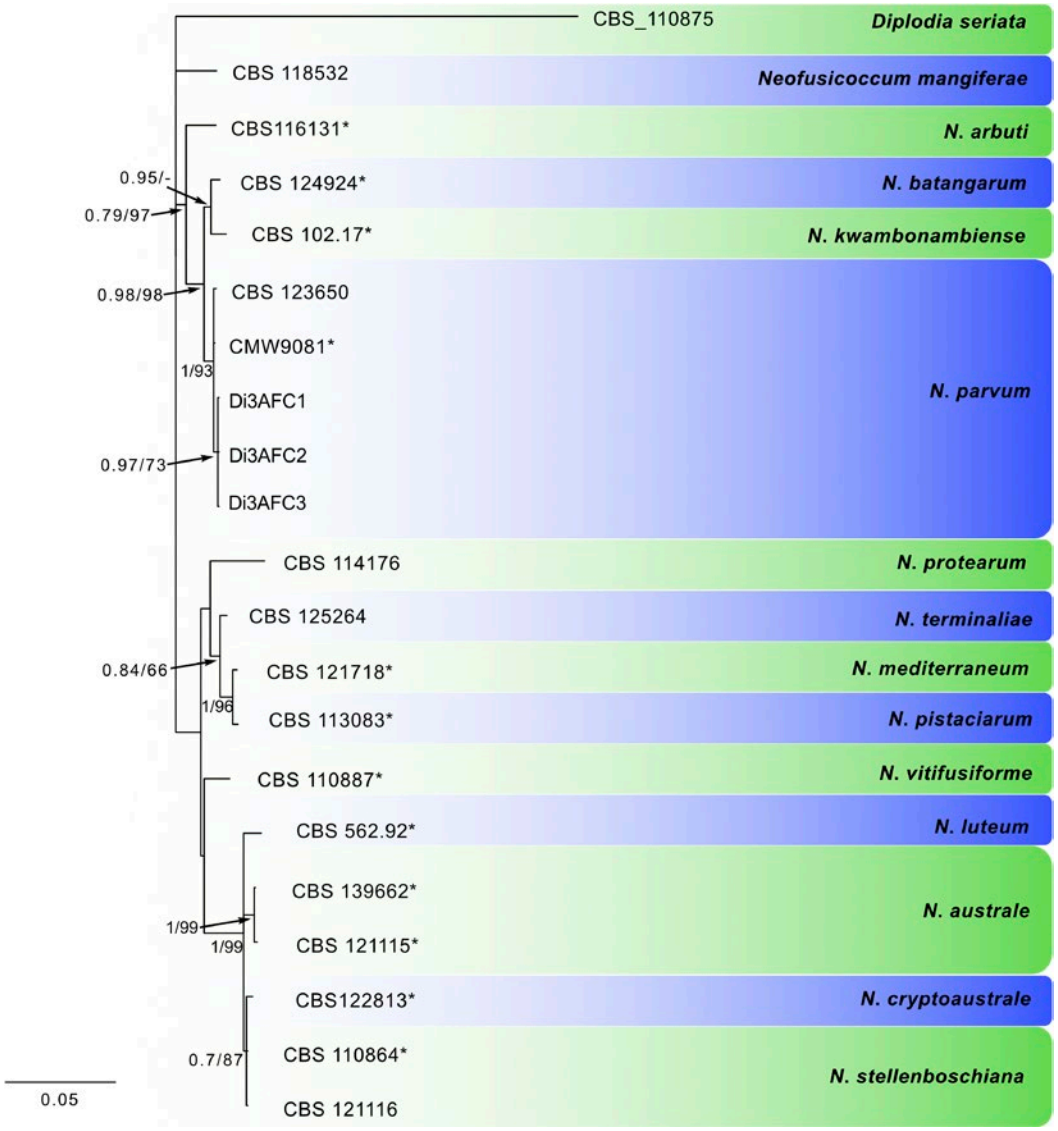

Figure 2. Consensus phylogram of 1320 trees resulting from a Bayesian analysis of the combined ITS, *tef1* and *tub2* sequences. Bootstrap support values and Bayesian posterior probability values are indicated at the nodes. *Neofusicoccum* species are listed next to the strain numbers. *indicates ex-type strains. The tree was rooted to *Diplodia seriata* (CBS 110875).

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