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New insights into scabby canker of *Opuntia ficus-indica*, caused by *Neofusicoccum batangarum*

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Summary. This study characterizes a fungal disease of cactus pear (*Opuntia ficus-indica*, *Cactaceae*), reported from the minor islands of Sicily. The disease, originally named 'gummy canker', was first reported in 1973 from Linosa, a small island of the Pelagian archipelago, south of Sicily. The causal agent was identified as *Dothiorella ribis* (currently *Neofusicoccum ribis*, *Botryosphaeriaceae*). In a recent survey the disease has been found to be widespread in minor islands around Sicily, including Lampedusa, Linosa, Favignana and Ustica. The causal agent was identified in *Botryosphaeriaceae* as *Neofusicoccum batangarum* on the basis of the phylogenetic analysis of the DNA sequences from ITS, *tef1* and *tub2* sequences, and the disease was renamed 'scabby canker', which describes the typical symptoms on cactus pear cladodes. In artificial inoculations, *N. batangarum* induced symptoms on cactus pear cladodes identical to those observed in naturally infected plants. The fungus also induced cankers on artificially wound-inoculated stems of several common Mediterranean plants including Aleppo pine (*Pinus halepensis*), almond (*Prunus dulcis*), sweet orange (*Citrus* × *sinensis*), citrange (*Citrus sinensis* × *Poncirus trifoliata*) and holm oak (*Quercus ilex*), indicating that the pathogen has a wide potential host range. Isolates of *N. batangarum* from cactus pear from several small islands around Sicily were genetically uniform, as inferred from microsatellite primed (MSP)-PCR electrophoretic profiles, suggesting the pathogen populations in these islands have a common origin. A preliminary report of the identity of the causal agent of this disease has been published as the first record of *N. batangarum* in Europe and on cactus pear worldwide.

Keywords. *Botryosphaeriaceae*, cactus pear, phylogenetic analysis, marker genes, host range.

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INTRODUCTION

Cactus pear [*Opuntia ficus-indica* (L.) Mill.] is probably native to Mexico (Kiesling and Metzing, 2017), and, after the discovery of America, this plant was introduced into the Mediterranean basin where it is naturalized (Ochoa and Barbera, 2017). In Sicily, cactus pear has become an economically important fruit crop and is a characteristic feature of the landscape. It is also cultivated in the Sardinia, Apulia, Calabria, and Basilicata regions of Italy, and this country is the second cactus pear fruit producer, after Mexico. Sicily produces approx. 90% of cactus pear fruit in the European Union. Cactus pear was introduced into the small islands around Sicily, where it is mainly grown as productive living fences. According to Pretto *et al.* (2012), *O. ficus-indica* was introduced into the small Mediterranean islands in the nineteenth century, as fodder or to fence fields.

Somma *et al.* (1973) reported a severe and unusual disease of *O. ficus-indica* on Linosa, a small island of the Pelagian archipelago, south of Sicily. The disease was named 'gummy canker', referring to the typical symptoms on cladodes, and the causal agent was identified as *Dothiorella ribis*, currently *Neofusicoccum ribis* (*Botryosphaeriaceae*). In a subsequent review of cactus pear diseases (Granata *et al*., 2017), the 'gummy canker' described by Somma *et al.* (1973) was equated to a disease named 'cladode and fruit rot', and the causal agent was considered to be the cosmopolitan fungus *Lasiodiplodia theobromae* (Pat.) Giff. & Maubl. This fungus is in the same family as *N. ribis*. Recently, 'gummy canker' was seen to be destroying the cactus pear stands in Lampedusa, the southernmost island of the Pelagian archipelago, and the disease was also present in other minor islands near Sicily, including Favignana of the Aegadian archipelago, and Ustica, a small island around 67 km northwest of Palermo in the Tyrrhenian sea (Schena *et al*., 2018). The name of the disease was changed to 'scabby canker', as this was more appropriate to describe the characteristic symptoms on cladodes. The fungus responsible for the disease was identified as *Neofusicoccum batangarum* Begoude, Jol. Roux & Slippers (Schena *et al*., 2018), not previously reported in Europe (Phillips *et al*., 2013; Dissanayake *et al*., 2016a). This was the first report of *N. batangarum* on cactus pear, but there are records of this fungus in Brazil as a pathogen of cochineal cactus [*Nopalea cochenillifera* (L.) Salm-Dyck, syn. *Opuntia cochenillifera* (L.) Mill.], which is a close relative of cactus pear (Conforto *et al*., 2016; Garcete-Gómez *et al*., 2017).

The disease of cochineal cactus, named 'cladode brown spot', has some traits in common with 'scabby canker' occurring in the minor Sicilian islands. However, other fungi, besides *N. batangarum*, are responsible for 'cladode brown spot' in Brazil (Conforto *et al*., 2019; Feijo *et al*., 2019). *Neofusicoccum batangarum* was first described as an endophyte of Indian almond (*Terminalia catappa* L., *Combretaceae*) in Africa (Begoude *et al*., 2010, 2011). This fungus was also reported in Florida (USA) as a contaminant of seeds of *Schinus terebinthifolius*, the Brazilian pepper tree (Shetty *et al*., 2011), and more recently as an aggressive pathogen causing stem cankers of fruit trees in the tropics (Netto *et al*., 2017; Serrato-Diaz *et al*., 2020). The distribution of *N. batangarum* includes Africa, Brazil, Puerto Rico and the United States of America (Phillips *et al*., 2013; Dissanayake *et al*., 2016a; Conforto *et al*., 2019; Serrato-Diaz *et al*., 2020).

The present study aimed to gain insights into the aetiology and epidemiology of 'scabby canker' that is destroying cactus pear on the minor islands of Sicily, and also poses a serious threat to cactus pear crops in Sicily. Specific objectives included: i) to determine the present distribution of 'scabby canker'; ii) to characterize *N. batangarum* isolates from different minor islands of Sicily; and iii) to investigate if the potential host range of this fungus includes other Mediterranean plants which could act as alternative hosts or inoculum reservoirs for this pathogen.

MATERIALS AND METHODS

Fungus isolates, distribution and incidence of the disease

Samples were collected from 2013 to 2018, from the minor islands of Sicily, and a survey was carried out in the islands of Favignana, Lampedusa, Linosa and Ustica (Figure 1) to determine the distribution and the incidence of the disease. Since the extent of these islands is limited, all prickly pear hedges and plantations in each island were examined systematically. Isolations were made from the margins of active cankers developing on cladodes of the host plants. Pieces (5 mm) of diseased tissue were plated onto potato dextrose agar (PDA, Oxoid Limited) supplemented with 1 mg mL^{-1} of streptomycin, and were incubated at 22°C. Isolates were also obtained as single conidium isolations from conidiomata emerging from cankers of diseased plants, as described by Phillips *et al.* (2013). A total of 26 representative isolates of *N. batangarum* from cactus pear were characterized in this study. Table 1 lists these isolates and their origins. Cultures were routinely grown and maintained on PDA in the collection of the Molecular Plant Pathology laboratory of the Di3A, University of Catania.

Figure 1. Sicily and the minor surrounding islands.

Morphological characteristics and cardinal temperatures for growth of the isolates

The isolates were induced to sporulate by plating them on PDA containing sterilized pine needles (Smith *et al.*, 1996), and incubating at room temperature (approx. 20 to 25°C) under diffused day light or near-UV light, until pycnidia developed. For microscopy, pycnidia and conidia were mounted in sterile distilled water or 100% lactic acid and observed microscopically at ×40 and ×100 magnifications, with an Axioskop (Zeiss) microscope. Images were captured with an AxioCam MRc5 camera (Zeiss), and measurements were made with the software AxioVision. For each isolate, 50 conidia were randomly selected and their lengths, widths and shape were recorded. For pycnidium dimensions, 20 measurements were made. Colony characters and pigment production were noted after 4 to 6 d of growth on PDA or malt extract agar (MEA) at 25°C, in the dark. Colony colours (upper and lower surfaces) were rated according to Rayner (1970).

Four isolates, one from each island, were deposited at Westerdijk Fungal Biodiversity Institute, with strain code numbers CBS 143023, CBS 143024, CBS 143025, and CBS 143026 (Schena *et al.*, 2018).

Radial growth rate and cardinal temperatures for radial growth were determined by growing the isolates on PDA in Petri dishes (9 cm diam.), and incubating at 5, 10, 15, 20, 25, 30 35°C, in the dark. Means of radial growth at the different temperatures were adjusted to a regression curve using Statgraphics Plus 5.1 software (Manugistics Inc.), and the best polynomial model was chosen based on parameter significance ($P < 0.05$) and coefficient of determination (R^2) to estimate the optimum growth temperature for each isolate. Four replicates of each isolate were evaluated and each experiment was repeated twice.

Amplification and sequencing of target genes

Genomic DNA was isolated from 1-week-old cultures grown on PDA at 25°C in the dark using the procedure of Schena and Cooke (2006). The internal transcribed spacer (ITS) region of the ribosomal DNA was amplified and sequenced with primers ITS5/ITS4 (White *et al.* 1990), part of the translation elongation factor 1 alpha gene (*tef1*) was sequenced and amplified with primers EF1-728F/EF1-986R (Carbone and Kohn, 1999), and the β-tubulin gene (*tub2*) was sequenced and amplified with Bt2a and Bt2b (Glass and Donaldson, 1995).

Amplified products with both forward and reverse primers were sequenced by Macrogen Europe. CHROMASPRO v. 1.5 (http://www.technelysium.com.au/) was used to evaluate reliability of sequences and to create consensus sequences. Unreliable sequences were re-sequenced.

Molecular identification and phylogenetic analyses

The preliminary identification of isolates and their association to *N. batangarum* were carried by BLAST analyses. For the accurate identification, sequences of ITS, *tef1* and *tub2* loci from the isolates obtained in the present study were phylogenetically analyzed along with validated sequences representative of *N. batangarum* and closely related species as defined by comprehensive phylogenetic studies (Slippers *et al.*, 2013; Lopes *et al.*, 2017; Yang *et al.*, 2017). Two additional isolates of *N. batangarum* for which ITS, *tef1* and *tub2* sequences were available in GenBank were included in the analysis. Table 2 lists the analyzed isolates. For all isolates, ITS, *tef1* and *tub2* sequences were trimmed to a common length and concatenated using the Sequence Matrix software (Vaidya *et al.*, 2011). Concatenated sequences were aligned with MUSCLE (Edgar, 2004) as implemented in Mega Version 7.0 (Kumar *et al.*, 2016), and edited manually for check-

Table 1. Identity of the *Neofusicoccum batangarum* isolates studied, and GenBank accession numbers, for isolates recovered from the small islands of Sicily.

Isolate	Species	Island origin	ITS	β -tubulin	EF1-α
FIF G	*N. batangarum*	Favignana	MF414731	MF414750	MF414769
FIF A	*N. batangarum*	Favignana	MF414732	MF414751	MF414770
FIF D	*N. batangarum*	Favignana	MF414730	MF414749	MF414768
FIF F1	*N. batangarum*	Favignana	MF414733	MF414752	MF414771
FIF E	*N. batangarum*	Favignana	MF414734	MF414753	MF414772
FIF F	*N. batangarum*	Favignana	MF414735	MF414754	MF414773
FIF I	*N. batangarum*	Favignana	MF414736	MF414755	MF414774
FIF H	*N. batangarum*	Favignana	MF414737	MF414756	MF414775
FILI F1	*N. batangarum*	Linosa	MF414747	MF414766	MF414785
OB43	*N. batangarum*	Linosa	MG609040	MG609057	MG609074
OB44	*N. batangarum*	Linosa	MG609041	MG609058	MG609075
OB46	*N. batangarum*	Linosa	MG609043	MG609060	MG609077
FIU 1	*N. batangarum*	Ustica	MF414739	MF414758	MF414777
FIU 1B	*N. batangarum*	Ustica	MF414738	MF414757	MF414776
FIU 2	*N. batangarum*	Ustica	MF414740	MF414759	MF414778
FIU 3	*N. batangarum*	Ustica	MF414741	MF414760	MF414779
FIU 3A	*N. batangarum*	Ustica	MF414742	MF414761	MF414780
FIU 3B	*N. batangarum*	Ustica	MF414743	MF414762	MF414781
FIU 4	*N. batangarum*	Ustica	MF414744	MF414763	MF414782
FIU 5	*N. batangarum*	Ustica	MF414745	MF414764	MF414783
FIU 6	*N. batangarum*	Ustica	MF414746	MF414765	MF414784
FILA 4	*N. batangarum*	Lampedusa	MF414748	MF414767	MF414786
OP5	*N. batangarum*	Lampedusa	MG609050	MG609067	MG609084
OP6	*N. batangarum*	Lampedusa	MG609051	MG609068	MG609085
OP9	*N. batangarum*	Lampedusa	MG609052	MG609069	MG609086
OB47	*N. batangarum*	Lampedusa	MG609053	MG609070	MG609087

ing indels and single nucleotide polymorphisms. Phylogenetic analyses were performed in Mega with the maximum likelihood method using the Tamura–Nei model and 1000 bootstrap replications (Tamura and Nei, 1993; Tamura *et al*., 2013).

Analysis of genetic variability of isolates

Six representative isolates (FIU 1B, OP6, FIF D, FILA 4, OB43, and FILI F1) collected from four different islands were characterized according to their microsatellite-primed PCR (MSP-PCR) profiles using primer M13 (Meyer *et al*., 1993; Santos and Phillips, 2009), (CAG)5 (Freeman and Shabi, 1996), and (GGA)5 (Uddin *et al*., 1997). Each amplification was carried out in a total volume of 25 µL, containing 1 µL (50 ng) of fungal DNA, 1 µM of primer, 2 mM [(primer (CAG)5 and (GGA)5] or 4 mM (primer M13) of $MgCl_2$ and 1U of GoTaq DNA Polymerase (Promega Corporation). Reactions were incubated for 2 min at 95°C, followed by 35 cycles of 30s at 95°C, 30 s at 49°C [primers (GGA5)5 and M13] or 52°C [primer CAG)5] and 1 min at 72°C. All reactions ended with a final extension of 5 min at 72°C. PCR profiles were visualized on 2% agarose electrophoresis gels (Merck) in 1× TBE buffer stained with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific).

Pathogenicity tests

Four *N. batangarum* isolates obtained from cactus pear cankers (isolates FIF D, FIU 1B, OP6 and OB43 from, respectively, Favignana, Ustica, Lampedusa and Linosa) were used in pathogenicity tests on cactus pear plants. These isolates were used to inoculate mature cladodes (cladodes of the previous year) and the stems of field-grown cactus pear plants (three plants per isolate and two cladodes per plant). On each cladode, two holes (5 mm diam.) were made 20 cm apart with a cork-borer, while only one hole was made on the stem. An agar plug from a 5-d-old colony grow-

Table 2. GenBank accession numbers of sequences of the *Neofusicoccum* spp. isolates of different country and host origins used as references in phylogenetic analyses

Species	Isolate	Country	Host	Source	GenBank accession number		
					ITS	tef1	β-tubulin
N. algeriense	CAA322	Portugal	*Malus domestica*	Lopes *et al.*, 2017	KX505906	KX505894	KX505916
N. algeriense	CBS137504	Algeria	*Vitis vinifera*	Lopes *et al.*, 2017	KJ657702	KX505893	KX505915
N. batangarum	CBS124922	Cameroon	*Terminalia catappa*	Yang *et al.*, 2017	FJ900606	FJ900652	FJ900633
N. batangarum	CBS127348	USA: Florida	*Schinus terebinthifolius*	Yang *et al.*, 2017	HM357636	KX464674	KX464952
N. batangarum	CMM4553	Brasil	*Anacardium* sp.	Unpublished	KT728917	KT728921	KT728913
N. batangarum	CBS124924 (ex-type)	Cameroon	*Terminalia catappa*	Lopes *et al.*, 2016	FJ900607	FJ900653	FJ900634
N. batangarum	CBS124923	Cameroon	*Terminalia catappa*	Lopes *et al.*, 2016	FJ900608	FJ900654	FJ900635
N. brasiliense	CMM1285	Brazil	*Mangifera indica*	Lopes *et al.*, 2016	JX513628	JX513608	KC794030
N. brasiliense	CMM1338	Brazil	*Mangifera indica*	Lopes *et al.*, 2016	JX513630	JX513610	KC794031
N. cordaticola	CBS123634	South Africa	*Syzygium cordatum*	Lopes *et al.*, 2016	EU821898	EU821868	EU821838
N. cordaticola	CBS123635	South Africa	*Syzygium cordatum*	Lopes *et al.*, 2016	EU821903	EU821873	EU821843
N. kwambonambiense	CBS123639	South Africa	*Syzygium cordatum*	Lopes *et al.*, 2016	EU821900	EU821870	EU821840
N. kwambonambiense	CBS123641	South Africa	*Syzygium cordatum*	Lopes *et al.*, 2016	EU821919	EU821889	EU821859
N. macroclavatum	CBS118223	Australia	*Eucalyptus globulus*	Lopes *et al.*, 2016	DQ093196	DQ093217	DQ093206
N. macroclavatum	WAC12445	Australia	*Eucalyptus globulus*	Lopes *et al.*, 2016	DQ093197	DQ093218	DQ093208
N. occulatum	CBS128008	Australia	*Eucalyptus grandis hybrid*	Lopes *et al.*, 2016	EU301030	EU339509	EU339472
N. occulatum	MUCC286	Australia	*Eucalyptus pellita*	Lopes *et al.*, 2016	EU736947	EU339511	EU339474
N. parvum	CBS110301	Portugal	*Vitis vinifera*	Lopes *et al.*, 2017	AY259098	AY573221	EU673095
N. parvum	CMW 9081	New Zealand	*Populus nigra*	Lopes *et al.*, 2017	AY236943	AY236888	AY236917
N. ribis	CBS115475	USA	*Ribes* sp.	Lopes *et al.*, 2016	AY236935	AY236877	AY236906
N. umdonicola	CBS123645	South Africa	*Syzygium cordatum*	Lopes *et al.*, 2016	EU821904	EU821874	EU821844
N. umdonicola	CBS123646	South Africa	*Syzygium cordatum*	Lopes *et al.*, 2016	EU821905	EU821875	EU821845
Neofusicoccum sp. 5	CBS157.26	Sri Lanka	*Camellia sinensis*	Yang *et al.*, 2017	KX464214	KX464744	KX465034

ing on PDA was inserted into each hole. Three plants inoculated with sterile agar served as controls. Wounds were sealed with the excised tissues and inspected daily for 20 d after inoculation (a.i.). Lesion diameters was recorded 30 d a.i., and the size of the cankers on cladodes was calculated as the circle area. Inoculations were first performed in June 2014 in an experimental field at the University of Catania (Sicily), and these were repeated each year in June for three consecutive years on different healthy cactus pear plants. Commencing from 20 d a.i., inoculated plants were inspected each month to observe development of symptoms. Trials were also repeated in an experimental field at the University of Palermo (Sicily).

Isolates FIFD and FIU1B were also used to inoculate twigs and stems of 2-year-old trees of sweet orange 'Navelina' (*Citrus* × *sinensis*) grafted on citrange 'Carrizo' (*Citrus sinensis* × *Poncirus trifoliata*) rootstock, grown in a greenhouse maintained at 20 to 26°C. These isolates were also used to inoculate the stems of field-grown trees of woody plants typical of the Mediterranean region, in an experimental field at the University of Catania. These included 3-year-old trees of almond [*Prunus dulcis* (Mill.) D.A. Webb], 5-year-old trees of holm oak (*Quercus ilex* L.) and 6-year old trees of Aleppo pine (*Pinus halepensis* Mill.). Inoculations were performed in June 2016. On sweet orange (two twigs per tree) were inoculated (four trees per isolate). A hole in each twig was made with a 3 mm cork-borer. A 3 mm diam. mycelium plug from 5-d-old PDA culture was placed on the freshly wounded surface, the wound was covered with the excised bark disk and sealed with Parafilm®. The stem of each tree (the 'Carrizo' citrange rootstock) was inoculated 10 cm above soil level (a single hole per stem) using the same method. Four trees inoculated with sterile agar served as controls. The length and breadth of each resulting lesion were recorded 30 d a.i., and the outer surface areas of the bark cankers on twigs and stems were calculated as ellipses. Almond, holm oak and Aleppo pine trees were wound inoculated on the stems using a cork borer (5 mm diam.). Four trees per host species were inoculated (three holes per tree 60 cm apart), and the wounds were each covered with the excised bark disk. Four trees inoculated with sterile agar served as controls. The lengths and breadths of the lesions were recorded at 30 and 70 d a.i.

In all pathogenicity tests, re-isolations were made from lesions, and resulting fungal colonies were confirmed morphologically and by sequencing part of the ITS, *tef1* and *tub2* genes, as described above, to fulfill Koch's postulates.

Statistical analyses of data

Data from pathogenicity tests were analyzed using RStudio v.1.2.5 (R). When comparing the means of multiple groups, a one-way ANOVA followed by Tukey's HSD *post hoc* test was performed. Significant differences between groups ($P < 0.05$) were denoted with different letters. When comparing independent groups, Student's t-test was used. The significance level was reported as follows: * = $P < 0.05$, ** = $P < 0.01$, or *** = $P < 0.001$.

RESULTS

Symptoms, distribution and incidence of the disease

Symptoms were visible on cladodes and included radially expanding, crusty, concentric, silvery, perennial cankers, each with a leathery, brown halo (Figure 2A–C). Pycnidia were erumpent from the host epidermis, visible to the naked eye as minute, black dots, formed on the silver-coloured internal area of each canker (Figure 2C) The cankers had radial and tangential cracks (Figure 2B–C). A milky to buff-coloured abundant and viscous exudate of polysaccharide nature, caking on contact with air, oozed from active cankers and formed strips or cerebriform masses. The exudate, being water soluble, was partially washed away by rain, while masses of exudates that remained on the cankers became black due to the growth of sooty molds, giving the cankers an appearance of carbonaceous crusts. The cankers ceased to expand in the coldest season of each year. An individual canker rarely reached a maximum diameter of more than 25 cm, but cankers often coalesced and formed larger lesions extending to the whole cladode or up to its edge, causing wilting. The cladodes collapsed when the bases were girdled by cankers. Infections on thicker cladodes and stems gave rise to very prominent cankers and eruptions of solid exudates at scattered points far from the lesions. Cladodes and stems of heavily infected plants became senescent and the whole plants collapsed, appearing gray and ghostly. In a systematic survey of prickly pear hedges and plantations, symptoms were observed in all hedges and plantings with 80% of plants symptomatic in the island of Lampedusa (20.2 km^2) and 40% of plants symptomatic plants on Linosa (5.43 km^2). Conversely, on Favignana (19.8 km^2), symptoms were observed at two sites three km apart, with incidences of 40% and 100% of plants with symptoms. On the island of Ustica (8.24 km^2), symptoms were observed only at one site on the northern coast, overlooking the sea (Figure 2A). However, this disease out-

Figure 2. A. Cankers on cladodes incited by *Neofusicoccum batangarum* in a cactus pear hedge on the Island of Ustica, May 2014. B. Coalescing, concentric cankers incited by *Neofusicoccum batangarum* on a cactus pear cladode. C. Concentric expanding canker incited by *Neofusicoccum batangarum* on a cactus pear cladode. Note pycnidia, as small dark spots, and cracking on the silvery, intermediate area of the canker, the dark colour and the sooty appearance of the exudate after rain and the tan colour of the edge, indicating that the canker is still active. D. Mycelium emerging from conidiomata of *Neofusicoccum batangarum* formed on cankers (photograph taken using a stereomicroscope).

break was severe, with approx. 400 m of hedges containing 100% of plants with symptoms.

Morphological and molecular identification of the pathogen

A fungus with white aerial mycelium that turned gray with age was consistently recovered from canker tissues, with 100% of positive isolations. The same fungus was obtained by plating single conidia taken from conidiomata emerging individually or in groups from cankers (Figure 2D). Colonies on MEA formed concentric rings. On PDA mycelium was white and became smoky gray to gray-olivaceous after 5 d (Figure 3A). The mycelium was fast-growing (Table 3) and covered the 9 cm diam. Petri dishes after 5 d incubation at 25° C in the dark. Optimum temperature for radial colony growth was between 25 and 30°C for all the isolates tested. Little growth was observed at 10 or 35°C. Stromatic conidiomata were produced in pine needle cultures within 14 d. The condidiomata were solitary, covered by mycelium, obpyriform to ampulliform, and each had

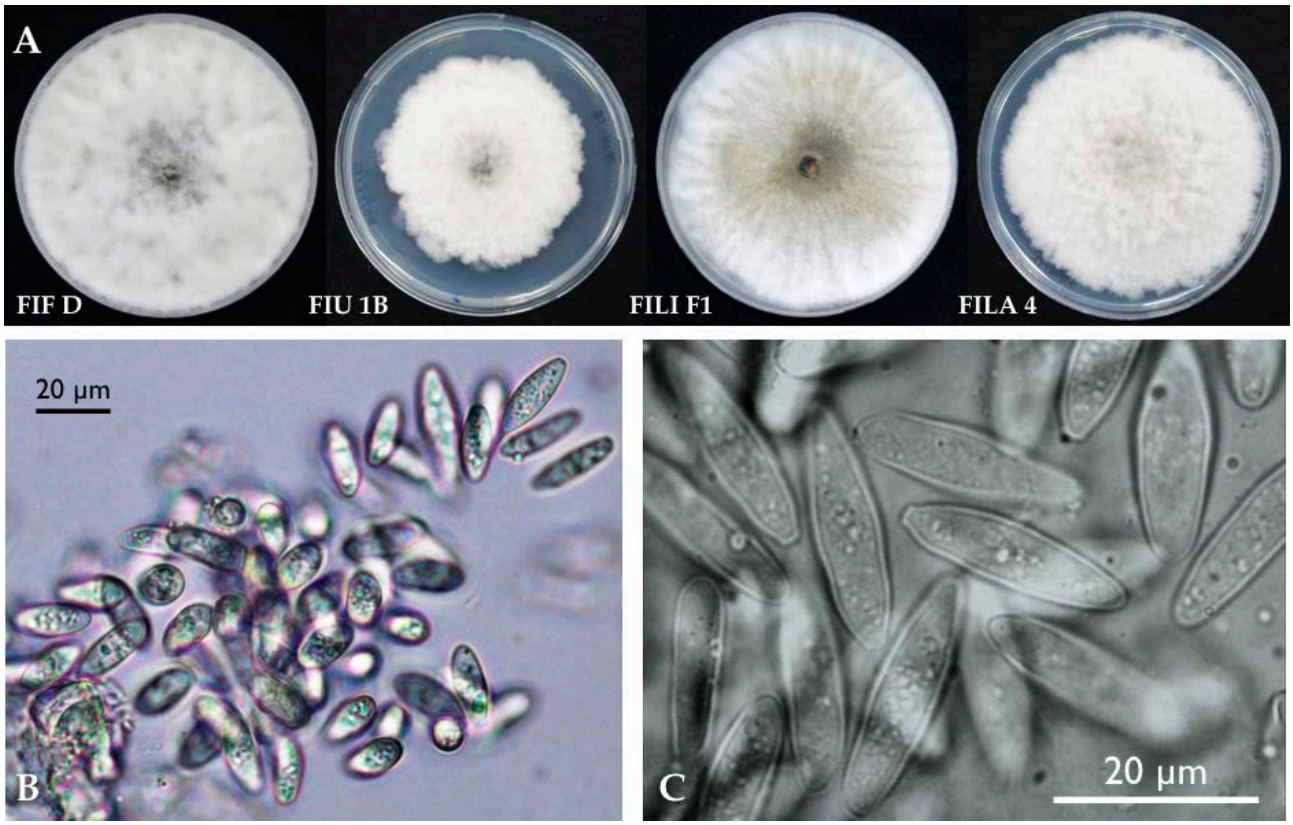

Figure 3. A. Four representative isolates of *Neofusicoccum batangarum* after 5 d of incubation on PDA at 25°C; CBS143023 (FIF D), CBS143024 (FIU 1B), CBS143025 (FILI F1), and CBS143026 (FILA 4). B and C. Unicellular, fusiform, thin-walled hyaline conidia of *Neofusicoccum batangarum*.

Table 3. Mean radial growth rates of colonies of *Neofusicoccum batangarum* isolates on PDA at three different temperatures, as determined after 3 d of incubation.

Isolates of *N. batangarum*	Island origin	15°C (mm d^{-1}) mean ± S.D.[a]	25°C (mm d^{-1}) mean ± S.D.	30°C (mm d^{-1}) mean ± S.D.
FILI-F1	Linosa	3.83 ± 0.35	7.50 ± 0.00	5.90 ± 0.26
FILA 4	Lampedusa	3.97 ± 0.34	7.50 ± 0.00	7.50 ± 0.00
FIU-1B	Ustica	3.00 ± 0.58	6.53 ± 0.63	6.26 ± 0.42
FIF-D	Favignana	3.40 ± 0.41	7.50 ± 0.00	7.50 ± 0.00
OP5	Lampedusa	4.32 ± 0.05	9.77 ± 0.09	9.83 ± 0.06
OP6	Lampedusa	4.20 ± 0.21	12.94 ± 0.23	9.75 ± 0.13
OP9	Lampedusa	4.31 ± 0.09	13.00 ± 0.19	9.33 ± 0.24
OB44	Linosa	3.73 ± 0.81	12.83 ± 0.10	9.69 ± 0.06
OB46	Linosa	3.44 ± 0.44	13.03 ± 0.18	9.23 ± 0.42
OB47	Lampedusa	4.16 ± 0.10	12.94 ± 0.16	9.77 ± 0.11
OB43	Linosa	2.40 ± 0.22	7.82 ± 0.07	6.47 ± 0.06

[a] Mean of four replicate Petri dishes.

a central and circular unilocular ostiole, and measured 250-300 µm in diameter. Conidia were non-septate (bicellular conidia were observed only very occasionally), hyaline, smooth, fusoid to ovoid, thin-walled, and measured 17.1-21.8 × 4.6-8.9 µm, with a mean length to width ratio = 2.9 (Figure 3B–C).

The isolates obtained had identical ITS, *tef1* and *tub2* sequences. Preliminary BLAST analyses of these three genes yielded several identical sequences of *Neofusicoccum* spp., deposited with different taxa names. Consequently, this analysis enabled the identification at the genus level, but did not provide reliable information on the species. The phylogenetic analysis of the combined data set of sequences from ITS, *tef1* and *tub2* sequences (Figure 4) produced trees with a high concordance with those reported by Lopes *et al.* (2017) and Yang *et al.* (2017). According to this analysis, isolates from cactus pear were identified as *N. batangarum*, since they clearly clustered with the ex-type (CBS 124924 from *Terminalia catappa*; Lopes *et al.*, 2016) and other reference isolates of this species, and were differentiated from other *Neofusicoccum* species, including

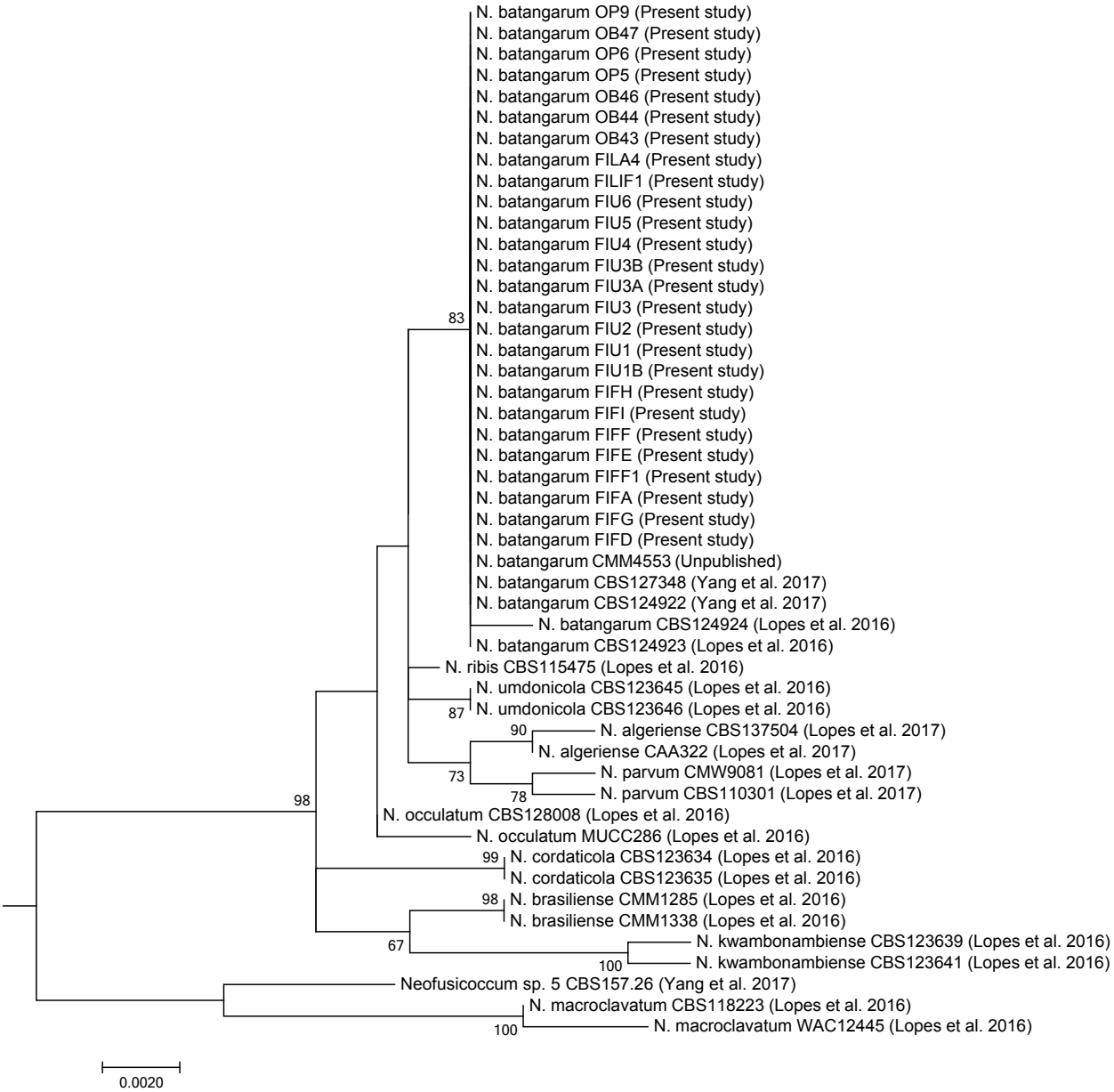

Figure 4. Phylogenetic tree of isolates of *Neofusicoccum* collected in the present study from *Opuntia ficus-indica*, and representative isolates of *Neofusicoccum batangarum* and closely related species as defined by in comprehensive phylogenetic studies (Tables 1 and 2). The tree was built using concatenated sequences of ITS-5.8S-ITS2 region, tef1-α gene and β-tubulin gene. Numbers on nodes indicate the posterior probabilities from the maximum likelihood method.

the closely related species *N. ribis, N. umdonicola,* and *N. occulatum* (Annex 1).

Analysis of the genetic variability of isolates

The MSP-PCR characterization of the six representative isolates of *N. batangarum* revealed high genetic uniformity, since isolates showed identical banding patterns with the three tested primers (Annex 2).

Pathogenicity tests

Four isolates, one from each island, were deposited at Westerdijk Fungal Biodiversity Institute [CBS 143023

Table 4. Mean lesion areas on cladodes of cactus pear (*Opuntia ficus-indica*) 30 d after wound inoculation with representative isolates of *Neofusicoccum batangarum* obtained from cactus pear from four minor islands of Sicily.

Isolate	Island origin	Mean lesion area (cm^2) ± S.D. [a,b]
FIF D	Favignana	4.4 ± 1.2
OP6	Lampedusa	4.4 ± 1.8
OB43	Linosa	4.5 ± 2.3
FIU 1B	Ustica	4.6 ± 0.6

[a] Means of 12 replicate values.
[b] ANOVA, $F_{(3,44)} = 0.372$, $P > 0.05$

Table 5. Mean lesion areas on stems of sweet orange 'Navelina' (*Citrus × sinensis*) trees grafted on 'Carrizo' Citrange (*C. sinensis × Poncirus trifoliata*) rootstock, 30 d after wound inoculation with representative isolates of *Neofusicoccum batangarum* from four minor islands of Sicily

Isolate [f]	Island origin	Mean lesion area (cm^2) ± S.D.[a]	
		rootstock [b,d]	scion[c,e]
FIF D***	Favignana	1.4 ± 0.18	1.8 ± 0.1
FIU 1B**	Ustica	1.4 ± 0.12	1.8 ± 0.2
FILI F1*	Linosa	1.4 ± 0.15	1.7 ± 0.2
FILA 4**	Lampedusa	1.5 ± 0.26	1.9 ± 0.4

[a] Rootstock: means of four replicate values. Scion: means of eight replicate values.
[b] Symptoms included barely noticeable gummy exudate.
[c] Symptoms included abundant gummosis.
[d] ANOVA rootstock, $F_{(3,12)} = 0$, $P > 0.05$.
[e] ANOVA scion, $F_{(3,28)} = 0$, $P > 0.05$.
[f] Comparing rootstock and scion according to Student's t-test.
(* = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$).

(FIF D), CBS 143024 (FIU 1B), CBS 143025 (FILI F1), and CBS 143026 (FILA 4)], and these were tested for their pathogenicity on cactus pear and other species of plants. Additional isolates from Lampedusa and Linosa were also included in the pathogenicity tests. Since the results of four inoculation series performed, respectively, in 2014, 2015, 2016 and 2017 were very similar, only the results of inoculations performed in 2014 are reported here in detail. All isolates were pathogenic on the inoculated plant species (Tables 4, 5, 6 and 7). The isolates induced cankers in all inoculated plants while no symptoms were observed on the controls.

On cactus pear plants, symptoms appeared after 4 d, as brown circular halos around the inoculation wounds with viscous exudates oozing from the lesions that consolidated in contact with the air to form long strips

Table 6. Mean lesion areas on stems of holm oak (*Quercus ilex*) trees 30 d and 70 d after wound inoculation with representative isolates of *Neofusicoccum batangarum* from two minor islands of Sicily.

Isolate	Island origin	Mean lesion area (cm^2) ± S.D.[a]	
		30 d[b]	70 d[c]
FIF D	Favignana	5.2 ± 1.3	8.2 ± 1.1
FIU 1B	Ustica	5.8 ± 2.4	10.0 ± 3.0

[a] Means of six replicate values.
[b] ANOVA 30 d, $F_{(1,10)} = 0.274$, $P > 0.05$.
[c] ANOVA 70 d, $F_{(1,10)} = 1.517$, $P > 0$ .05.

Table 7. Mean lesion areas on stems of Aleppo pine (*Pinus halepensis*) or almond (*Prunus dulcis*) trees 70 d after wound inoculations with representative isolates of *Neofusicoccum batangarum* from four minor islands of Sicily.

Isolate	Origin	Mean lesion area (cm^2) ± S.D.[a,f]	
		Aleppo pine[b,d]	Almond[c,e]
FIF D	Favignana	7.2 ± 0.6 a	4.9 ± 1.0 a
FIU 1B	Ustica	7.1 ± 0.9 ab	5.1 ± 1.4 a
FILA 4	Lampedusa	7.6 ± 2.8 ab	5.1 ± 1.7 a
FILI F1	Linosa	6.6 ± 0.6 b	5.8 ± 1.7 a

[a] Means of six replicates.
[b] Symptoms included resinous exudates.
[c] Symptoms included abundant gummous exudates.
[d] ANOVA Aleppo pine, $F_{(3,20)} = 6.015$, $P = 0.004$.
[e] ANOVA Almond, $F_{(3,20)} = 2.783$, $P = 0.06$.
[f] Means accompanied by the same letters are not statistically different ($P < 0.05$, Tukey's HSD test).

(Figure 5A–B). Cankers expanded progressively and concentrically (Figure 5D). They were roughly circular, with irregular margins, identical or very similar to those observed on plants with natural infections, and the canker expansion reduced during the coldest months (late December to early February). Some cankers stopped growing permanently and healed, but most cankers resumed growth and the production of exudate when temperatures increased after winter. Table 4 presents the mean lesion areas induced on cactus pear cladodes by the four tested isolates 30 d after wound inoculation. Analysis of variance revealed no statistically significant differences in pathogenicity between the isolates. Five years after the first inoculation, most cankers were still active, and they continued to expand and produce abundant exudates (Figure 5D). In some cases, the cankers expanded more rapidly in one direction and became asymmetrical and irregular (Figure 5D). After rain, the cankers became dark, with a carbonaceous appearance.

Figure 5. A. Brown, circular lesions and waxy exudate oozing from lesions on a cactus pear cladode wound-inoculated with *Neofusicoccum batangarum*, 7 d after inoculation (a.i.) B. Brown lesion and exudate oozing from a lesion on a cactus pear cladode wound-inoculated with *N. batangarum*, 4 d a.i. C. A fusiform dry canker induced by wound-inoculation of *N. batangarum* on the stem of a holm oak tree 70 d a.i. D. A still active canker on a cactus pear cladode artificially inoculated with *Neofusicoccum batangarum* in the field, September 2019, 5 years a.i. E. A resinous canker induced by wound-inoculation with *N. batangarum* on the stem of an Aleppo pine tree, 70 d a.i. F. A gummy canker induced by wound-inoculation with *N. batangarum* on the stem of a young almond tree, 14 d a.i. G. A gummy canker induced by wound-inoculation with *N. batangarum* on the stem of a young 'Navelina' sweet orange tree, 14 d a.i. H. Pycnidia on a canker induced by wound-inoculation with *N. batangarum* on the stem of a young 'Navelina' sweet orange tree, 14 d a.i.

Pycnidia were visible on cankers 14-20 d a.i. From six months to 5 years a.i., small cankers and scattered eruptions of exudate, similar to runny wax, appeared on artificially inoculated cladodes approx. 10-20 cm from the inoculation points.

All four tested *N. batangarum* isolates induced necrotic lesions on the citrange rootstock and the sweet orange scion at 7 d a.i. Symptoms were more severe on the sweet orange scions (Table 5) and included abundant gummosis (Figure 5G), while gummosis was much less abundant on citrange. Pycnidia emerged from the necrotic lesions on sweet orange stems from 10 to 14 d a.i. (Figure 5H). Differences in mean lesion size between the sweet orange scion and the citrange rootstock were significant ($P < 0.05$), according to Student's *t*-test. However, no statistically significant differences in patho-

genicity ($P > 0.05$) were observed among the fungal isolates on both citrus symbionts. No symptoms were observed on the controls.

On holm oak (Table 6), no gummy reaction was observed (Figure 5 C), but cankers expanded progressively along the stems and were still active 3 years after inoculation. No symptoms were observed on the controls. There were no significant differences ($P > 0.05$) among isolates in the pathogenicity test on holm oak at 30 d a.i. or 70 d a.i.

On almond and Aleppo pine (Table 7), *N. batangarum* isolates incited necrotic bark lesions with gummy exudates on almond (Figure 5F) and resinous exudates on Aleppo pine (Figure 5E). No symptoms were observed on the controls. Also on almond and Aleppo pine, cankers were still active 3 years a.i.

In the pathogenicity tests on almond and Aleppo pine there were small but statistically significant differences between the isolates on these two hosts (almond, $P = 0.06$; Aleppo pine, $P = 0.004$). All the *N. batangarum* isolates were re-isolated from inoculated plants, while no fungal pathogens were isolated from control plants, thus fulfilling Koch's postulates for the *N. batangarum* isolates.

DISCUSSION

Identification of the *Botryosphaeriaceae* prior to the application of DNA sequencing and phylogenetic inference should be considered with caution. The classification and nomenclature of these fungi have evolved rapidly and have been substantially revised, as previous classification based on morphological characters was confusing and most species were actually complexes of different taxa (Slippers *et al*., 2004, 2013; Crous *et al*., 2006; Phillips *et al*., 2008, 2013; Crous *et al*., 2017). According to the molecular taxonomy of the *Botryosphaeraceae*, *N. batangarum* is the appropriate name of the fungus responsible for the chronic disease observed on the cladodes of cactus pear in the minor islands of Sicily. *Neofusicoccum batangarum* was confirmed to be the sole causal agent of the 'scabby canker' disease. The fungus was consistently associated with symptomatic cactus pear plants and, when artificially inoculated onto this host, induced the same type of cankers as natural infections. From an etiological and ecological perspective, *N. batangarum* was the only fungus in the family *Botryosphaeriaceae* recovered from infected cactus pear in these small islands belonging to different archipelagos.

Several species of this family frequently occur together on the same host (Jami *et al*., 2017), although not all are able to cause disease (Lawrence *et al*., 2017). In Brazil, *N. batangarum*, alone or in association with other fungi including several species of *Botryosphaeriaceae*, was reported to be responsible for 'brown spot' of cladodes, a severe disease of cochineal cactus that is grown as fodder for livestock in the semi-arid region of the north-east of that country (Conforto *et al*., 2016, 2019). Although the syndromes of 'cladode brown spot' in north-eastern Brazil and 'scabby canker' in minor islands of Sicily have some traits in common, they are distinct. Differences between the symptoms of these diseases include the presence of crusty, silvery, perennial cankers, and exudates oozing from the cankers in the 'scabby canker' disease. However, differences might be due to environmental conditions, host plant and/or cultivation systems. In Brazil, cochineal cactus is pruned repeatedly for the production of fresh forage. The cactus pear fences in the minor islands near Sicily are pruned only occasionally, thus allowing the disease to become chronic on mature cladodes. The cladode brown spot in Brazil is also a complex disease and the causal agent may vary according to the season and the geographical region (Santana *et al*., 2020).

For many years, *Botryosphaeriaceae*, which are widespread in tropical and temperate regions, were considered to be opportunistic pathogens infecting hosts exclusively through wounds or natural openings in their periderms. Since the late 1980s, however, these fungi have been recognized as endophytes that remain latent in woody host plants for long periods. With the onset of abiotic stress conditions (drought, physical damage, water-logging, frost and unsuitable environments for the growth) the latent pathogens cause disease (Slippers and Wingfield, 2007; Pavlic-Zupanc *et al*., 2015; Marsberg *et al*., 2017). The prolonged latent infection or endophytic phase implies that these fungi can easily pass undetected through phytosanitary controls or during the selection of propagation material.

The genus *Neofusicoccum* comprises species with widespread geographical and host distributions, and these fungi are typically endophytes, which in stressful environments can cause symptoms such as dieback, cankers and gummosis (Crous *et al*., 2006; Lopes *et al*., 2017; Zhang *et al*., 2017; Burgess *et al*., 2018). Wounds caused by hailstorms may have been the factor triggering the epidemic outbreak of *N. batangarum* on cactus pear in the small islands of Sicily. The climate of these islands is affected by the proximity to the sea, and this may have favoured the development of the disease and the survival of the inoculum. In *in vitro* tests, *N. batangarum* showed an optimum temperature for growth around 25°C, a minimum of approx. 10°C and a maxi-

mum of approx. 35°C. On artificially inoculated cladodes, the fungus formed pycnidia between 14 and 20 d a.i. In winter, conidia collected from pycnidia formed on cladodes artificially inoculated in the spring or autumn of the previous year were viable.

Most species of *Botryosphaeriaceae* have broad host ranges, and only very few have been described from a limited number of host species or are host specific (Slippers and Wingfield, 2007; Marsberg *et al*., 2017). The ability to infect multiple hosts and to move among unrelated hosts facilitates the establishment and spread of species and genotypes of this family into new areas.

Neofusicoccum batangarum has been reported as an endophyte as well as a pathogen of several host plants in the tropics (Begoude *et al*., 2010, 2011; Shetty *et al*., 2011; Conforto *et al*., 2016; Netto *et al*., 2017). A very recent report has further expanded the known hosts (Serrato-Diaz *et al*., 2020). Pathogenicity tests in the present study showed that this fungus has an even wider potential host range, encompassing woody forest and cultivated plants typical of the Mediterranean macro-region. These hosts include Aleppo pine, almond, citrus and holm oak. Like other *Botryosphaeriaceae*, *N. batangarum* can be regarded as a generalist pathogen, although in natural conditions the host affinity of polyphagous *Botryosphaeriaceae* species is strongly influenced by the environment (Slippers and Wingfield, 2007). On artificially inoculated sweet orange stems, *N. batangarum* induced the typical symptoms of 'gummy cankers' or 'bot gummosis', already known as 'Dothiorella gummosis'. These are minor, but widespread, diseases caused by diverse species of *Botryosphaeriaceae*, and they commonly occur in citrus groves in California and in the Mediterranean basin (Adesemoye *et al*., 2014; Guarnaccia and Crous, 2017). Consistently with the typical symptoms of 'gummy canker' of citrus, in artificially inoculated symbiont citrus plant, symptoms were more severe on the sweet orange scion than on rootstock.

The polyphagy of *N. batangarum* may be related to its ability to produce non-host specific phytotoxins (Masi *et al*., 2020). These toxins may have roles in pathogenicity as virulence factors and may also enhance the ecological fitness of the fungus by inhibiting other microorganisms competing in plant biospheres. Production of diffusible phytotoxins could also explain the systemic spread of symptoms on cactus pear cladodes and their appearance far from inoculation points (Masi *et al*. 2020).

Reports of *Botryosphaeriaceae* associated with various hosts have increased worldwide in recent years. In Italy, this family is common and widespread on a broad range of hosts, and is an increasing concern for agricultural crops and urban and natural forest ecosystems (Burruano *et al*., 2008; Linaldeddu *et al*., 2014, 2016; Dissanayake *et al*., 2016b;). The disease of cactus pear caused by *N. batangarum* was noticed for the first time in Linosa more than 45 years ago (Somma *et al*., 1973), and at that time it was widespread and had been established for many years. Similarly, the chronic nature of symptoms observed recently in Favignana, Lampedusa and Ustica and the widespread occurrence of the disease in Lampedusa, clearly indicate it has not emerged recently. During the last 50 years the disease has probably been favoured by the low frequency of cactus pear pruning as a consequence of the reduced importance of this plant as a crop, and the drastic reduction in the use of cladodes as fodder. Although Sicily is the main cactus pear fruit producer in Italy, with more than 3,500 ha of specialized culture (Ochoa and Barbera, 2017), this disease has not been reported in cactus pear cultivations in Sicily. The present study has shown that *N. batangarum* populations from Favignana, Lampedusa, Linosa and Ustica were genetically uniform, despite their geographical isolation.

It can be assumed that conidia and ascospores of *Botryosphaeriaceae* are dispersed by wind and rain only over short distances. The occurrence of *N. batangarum* only on cactus pear plantations of the minor islands, and the genetic uniformity of the fungus populations, may indicate that these populations have a common origin, and that the widespread distribution of a single genotype of the pathogen has resulted from anthropogenic activity. *Neofusicoccum batangarum*, as an endophytic or latent pathogen, may have been introduced with cactus pear cladodes collected in other geographical areas and used as propagation material. This hypothesis is consistent with cactus pear being introduced on a large scale into small islands of the Mediterranean Sea as fodder for livestock, for field fences or for edible fruit production, at one recent time (Pretto *et al*., 2012). This was when more intensive colonization and exploitation of agriculture in these islands were promoted by the public authorities.

From an ecological perspective, the emergence of this disease in such an aggressive form, which has become a limiting factor for the cultivation of cactus pear in these small islands, may be partly due to the failure of the acclimatization of a non-native plant species. However, the occurrence of a serious disease of a crop of economic and landscaping relevance for Sicily in a restricted geographical area, but very close and frequently connected to the main island by tourist traffic, may have phytosanitary implications. Appropriate actions should be taken to prevent further spread and introduction of *N. batan-*

garum into areas where the non-native cactus pear host is naturalized and intensively cultivated.

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