available online at www.studiesinmycology.org

Studies in Mycology 87: 161–185 (2017).

First report of *Phyllosticta citricarpa* and description of two new species, *P. paracapitalensis* and *P. paracitricarpa*, from citrus in Europe

V. Guarnaccia[1*], J.Z. Groenewald[1], H. Li[2], C. Glienke[3], E. Carstens[4,5], V. Hattingh[4,6], P.H. Fourie[4,5], and P.W. Crous[1,7*]

[1]Westerdijk Fungal Biodiversity Institute, Uppsalalaan 8, 3584 CT, Utrecht, the Netherlands; [2]Institute of Biotechnology, Zhejiang University, Hangzhou, 310058, China; [3]Federal University of Paraná, Department of Genetics, Curitiba, Paraná, Brazil; [4]Citrus Research International, P.O. Box 28, Nelspruit, 1200, South Africa; [5]Department of Plant Pathology, Stellenbosch University, P. Bag X1, Stellenbosch, 7602, South Africa; [6]Department of Horticultural Science, Stellenbosch University, P. Bag X1, Stellenbosch, 7602, South Africa; [7]Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, P. Bag X20, Pretoria 0028, South Africa

*Correspondence: Vladimiro Guarnaccia, v.guarnaccia@westerdijkinstitute.nl; Pedro W. Crous, p.crous@westerdijkinstitute.nl

Abstract: The genus *Phyllosticta* occurs worldwide, and contains numerous plant pathogenic, endophytic and saprobic species. *Phyllosticta citricarpa* is the causal agent of Citrus Black Spot disease (CBS), affecting fruits and leaves of several citrus hosts (*Rutaceae*), and can also be isolated from asymptomatic citrus tissues. Citrus Black Spot occurs in citrus-growing regions with warm summer rainfall climates, but is absent in countries of the European Union (EU). *Phyllosticta capitalensis* is morphologically similar to *P. citricarpa*, but is a non-pathogenic endophyte, commonly isolated from citrus leaves and fruits and a wide range of other hosts, and is known to occur in Europe. To determine which *Phyllosticta* spp. occur within citrus growing regions of EU countries, several surveys were conducted (2015–2017) in the major citrus production areas of Greece, Italy, Malta, Portugal and Spain to collect both living plant material and leaf litter in commercial nurseries, orchards, gardens, backyards and plant collections. A total of 64 *Phyllosticta* isolates were obtained from citrus in Europe, of which 52 were included in a multi-locus (ITS, *actA*, *tef1*, *gapdh*, LSU and *rpb2* genes) DNA dataset. Two isolates from Florida (USA), three isolates from China, and several reference strains from Australia, South Africa and South America were included in the overall 99 isolate dataset. Based on the data obtained, two known species were identified, namely *P. capitalensis* (from asymptomatic living leaves of *Citrus* spp.) in Greece, Italy, Malta, Portugal and Spain, and *P. citricarpa* (from leaf litter of *C. sinensis* and *C. limon*) in Italy, Malta and Portugal. Moreover, two new species were described, namely *P. paracapitalensis* (from asymptomatic living leaves of *Citrus* spp.) in Italy and Spain, and *P. paracitricarpa* (from leaf litter of *C. limon*) in Greece. On a genotypic level, isolates of *P. citricarpa* populations from Italy and Malta (MAT1-2-1) represented a single clone, and those from Portugal (MAT1-1-1) another. Isolates of *P. citricarpa* and *P. paracitricarpa* were able to induce atypical lesions (necrosis) in artificially inoculated mature sweet orange fruit, while *P. capitalensis* and *P. paracapitalensis* induced no lesions. The *Phyllosticta* species recovered were not found to be widespread, and were not associated with disease symptoms, indicating that the fungi persisted over time, but did not cause disease.

Key words: *Citrus*, *Guignardia*, Multi-locus sequence typing, Systematics.
Taxonomic novelties: *Phyllosticta paracapitalensis* Guarnaccia & Crous, sp. nov., *P. paracitricarpa* Guarnaccia & Crous, sp. nov.

Available online 29 May 2017; http://dx.doi.org/10.1016/j.simyco.2017.05.003.

INTRODUCTION

The genus *Phyllosticta* was introduced by Persoon (1818), with *P. convallariae* (nom. cons.) (= *P. cruenta*) designated as the type species (Donk 1968). Species of *Phyllosticta* are known as plant pathogens of several hosts and responsible for various disease symptoms including leaf and fruit spots. Species included in the *P. ampelicida* species complex, which cause black rot disease on grapevines (Wicht *et al.* 2012, Zhou *et al.* 2015), and in the *P. musarum* species complex, which cause banana freckle disease, are economically important plant pathogens (Pu *et al.* 2008, Wong *et al.* 2012). Some species of *Phyllosticta* have also been isolated as endophytes from a wide range of hosts (e.g., *P. capitalensis*) and as saprobes (Glienke-Blanco *et al.* 2002, Huang *et al.* 2008, Thongkantha *et al.* 2008, Wikee *et al.* 2011, 2013b).

Sexual morphs have in the past been named in *Guignardia* (van der Aa 1973). The name *Guignardia* was introduced as a replacement for *Laestadia* by Viala & Ravaz (1892), who applied the name only to *Sphaeria bidwellii* (= *G. bidwellii* = *P. ampelicida*), a species that is different from *Laestadia* (Bissett 1986). Petrak (1957) included *G. bidwellii* and related species in *Botryosphaeria*, an opinion that was initially shared by Barr (1970, 1972). *Phyllosticta* was first monographed by van der Aa (1973) and more recently all species names described in *Phyllosticta* were re-described by van der Aa & Vanev (2002). Schoch *et al.* (2006) placed *Phyllosticta* in *Botryosphaeriales.* Several authors showed that *Botryosphaeriaceae* contained both *Botryosphaeria* and *Phyllosticta* spp., although this relationship remained poorly resolved (Crous *et al.* 2006, Schoch *et al.* 2006, Liu *et al.* 2012).

With the increasing use of molecular data to link asexual and sexual morphs, and the end of dual nomenclature for fungi (Hawksworth *et al.* 2011, Wingfield *et al.* 2012), the oldest and more commonly used name, *Phyllosticta*, was chosen over that of *Guignardia* (Glienke *et al.* 2011, Sultan *et al.* 2011, Wikee *et al.* 2011, 2013b, Wong *et al.* 2012). Moreover, several studies incorporated DNA sequence data to improve the identification and help resolve the taxonomy of *Phyllosticta* spp. (Baayen *et al.* 2002, Wulandari *et al.* 2009, Glienke *et al.* 2011, Wikee *et al.* 2011). Presently, new species of *Phyllosticta* are described based on a polyphasic approach, incorporating phylogenetic

Peer review under responsibility of Westerdijk Fungal Biodiversity Institute.

data, morphology and culture characteristics (Crous *et al.* 2012, Su & Cai 2012, Wang *et al.* 2012, Wong *et al.* 2012, Zhang *et al.* 2015). Wikee *et al.* (2013a) redefined *Phyllosticta*, showing that it clusters sister to the *Botryosphaeriaceae* for which the authors resurrected the family name *Phyllostictaceae*.

The main morphological characters used to recognise a species of *Phyllosticta* is the production of pycnidia containing aseptate, hyaline conidia that are covered by a mucoid layer and bearing a single apical appendage (van der Aa 1973). However, the mucoid layer and appendage are not always present. The sexual morph has erumpent, globose to pyriform ascomata, often irregularly shaped, unilocular, and with a central ostiole. Asci are 8-spored, bitunicate, clavate to broadly ellipsoid, with a wide, obtusely rounded or slightly square apex. Ascospores are ellipsoid to limoniform, sometimes slightly elongated, aseptate, hyaline, often with a large central guttule and a mucoid cap at each end. Spermatia produced in culture are hyaline, aseptate, cylindrical to dumbbell-shaped with guttules at both ends (van der Aa 1973).

Several *Phyllosticta* species have been associated with *Citrus* spp. worldwide (Baayen *et al.* 2002, Glienke-Blanco *et al.* 2002, Everett & Rees-George 2006, Baldassari *et al.* 2008, Wulandari *et al.* 2009, Glienke *et al.* 2011, Brentu *et al.* 2012, Wikee *et al.* 2013a, Er *et al.* 2014). Citrus black spot (CBS) is a foliar and fruit disease of *Citrus* spp. caused by *P. citricarpa* (sexual morph *Guignardia citricarpa*) (Kotzé 1981, Baldassari *et al.* 2008). The pathogen affects fruits and leaves of several citrus hosts causing various symptoms (Kiely 1948a, 1949, Kotzé 1981, 2000, Snowdon 1990) with lemons and 'Valencia' oranges being more susceptible (Kotzé 2000). Hard spot is the most common symptom characterised by sunken, pale brown necrotic lesions with a dark reddish brown raised border; lesions often containing the pycnidia (asexual sporocarps). Several other kinds of lesions are known: virulent spot, a sunken necrotic lesion without defined borders mostly on mature fruit; false melanose consisting of small black pustules usually in a tear stain pattern; and freckle, cracked or speckled spot. Leaf symptoms are seldom seen except on lemons. They appear as round, small, sunken necrotic spots with a yellow halo (Schubert *et al.* 2010). The infected leaves, when fallen on the orchard floor, represent a substrate for the development and maturation of pseudothecia from which the primary inoculum, ascospores, are released for new infections (McOnie 1967). *Phyllosticta citricarpa* has never been found on plant species outside of the *Rutaceae*, and can be isolated from asymptomatic citrus tissues (Baldassari *et al.* 2008).

Phyllosticta citricarpa is often associated with *P. capitalensis*, a morphologically similar but non-pathogenic species, previously incorrectly considered as the asexual morph of *Guignardia mangiferae* (Baayen *et al.* 2002, Everett & Rees-George 2006, Glienke *et al.* 2011). Based on a multi-locus phylogenetic analysis, however, Glienke *et al.* (2011) revealed that *P. capitalensis sensu lato* was genetically distinct from the reference isolate of *G. mangiferae*. *Phyllosticta capitalensis* was initially described on *Stanhopea* (*Orchidaceae*) from Brazil (Hennings 1908). Okane *et al.* (2001) attributed the name *P. capitalensis* to an endophytic species reported on ericaceous plants from Japan, and described the sexual morph as a new species, *G. endophyllicola*. Subsequently Baayen *et al.* (2002), based on DNA sequence data of the ITS nrDNA, considered a common endophytic species associated with several plants as morphologically similar to *G. endophyllicola*, but attributed this species to *G. mangiferae*, while the asexual morph was referred to as *P. capitalensis*. *Phyllosticta capitalensis* is a cosmopolitan fungus that has been reported from plants in 21 different families (Johnston 1998, Rodrigues & Samuels 1999, Okane *et al.* 2001, Baayen *et al.* 2002, Glienke-Blanco *et al.* 2002, Rodrigues *et al.* 2004, Everett & Rees-George 2006, Meyer *et al.* 2006, Rakotoniriana *et al.* 2008, Yuan *et al.* 2009, Bezerra *et al.* 2012) and has been found on citrus associated with both CBS affected and asymptomatic plants (Baayen *et al.* 2002, Everett & Rees-George 2006, Glienke *et al.* 2011). *Guignardia mangiferae sensu stricto* (not *P. capitalensis*) causes angular leaf spots on mango (Baldassari *et al.* 2008; Glienke *et al.* 2011).

The biology and ecology of *P. capitalensis* differs from that of *P. citricarpa*. *Phyllosticta capitalensis* is homothallic, whereas *P. citricarpa* is heterothallic (Zhang *et al.* 2015, Wang *et al.* 2016, Amorim *et al.* 2017). *Phyllosticta capitalensis* produces fertile pseudothecia on agar media and *P. citricarpa* produces them on leaf litter (Kiely 1948a). Moreover, *P. capitalensis* is an ubiquitous, cosmopolitan endophyte of woody plants (Baayen *et al.* 2002) and *P. citricarpa* is associated only with citrus plants (Glienke *et al.* 2011).

Significant progress in species differentiation was achieved with multi-locus phylogenetic analyses performed on a large number of *Phyllosticta* species, (Wulandari *et al.* 2009, Glienke *et al.* 2011, Wang *et al.* 2012). Using three partial DNA regions, Wulandari *et al.* (2009) revealed three *Phyllosticta* clades associated with citrus in Thailand, namely *P. capitalensis*, *P. citricarpa* and *P. citriasiana.* Wang *et al.* (2012) described one new species associated with citrus in China, namely *P. citrichinaensis*, and also distinguished two subclades within *P. citricarpa*. Sequencing four partial regions of DNA, Glienke *et al.* (2011) distinguished a new species, *Phyllosticta citribraziliensis*, associated with *Citrus* sp. in Brazil. *Phyllosticta citriasiana* causes Citrus Tan Spot disease on *Citrus maxima* in Asia (Wulandari *et al.* 2009). *Phyllosticta citrichinaensis* is a weak pathogen on various citrus species in Asia, and *P. citribraziliensis* is non-pathogenic endophyte on citrus in Brazil (Glienke *et al.* 2011, Wang *et al.* 2012). A recent study added a sixth *Phyllosticta* species associated with citrus, namely *P. citrimaxima*, which was isolated from Citrus Tan Spot on fruit of *C. maxima* in Thailand (Wikee *et al.* 2013a).

Based on sequences of the rDNA internal transcribed spacer (ITS) region, the *P. citricarpa* and *P. capitalensis* clades are clearly distinct, with each species showing low levels of intraspecific variation (Okane *et al.* 2003, Rodrigues *et al.* 2004). *Phyllostica citricarpa* and *P. capitalensis* have several morphological and physiological differences: colonies of *P. citricarpa* produce a yellow halo on oatmeal agar (OA), the growth rate is generally faster in *P. capitalensis*, conidia are coated with a thicker mucoid layer than observed in *P. citricarpa*, and there is a higher level of hydrolytic enzyme production in *P. citricarpa* than in *P. capitalensis* (Baayen *et al.* 2002, Glienke *et al.* 2011, Romão *et al.* 2011).

Windborne *P. citricarpa* ascospores produced in pseudothecia (ascocarps) and waterborne conidia produced in pycnidia may cause infection on citrus (Kiely 1948a, Kotzé 1963, 1996, 2000). The ascospores are considered the primary source of inoculum in the CBS disease cycle, while conidia may serve for short distance wash-down dispersal by rain (Kiely 1948a, Whiteside 1967, Spósito *et al.* 2011). Alternate wetting and sun drying of leaves and mild to warm temperature fluctuations are favourable conditions for maturation of pseudothecia and ascospore discharge (Kiely 1948a, Lee & Huang 1973, Truter

2010, Fourie *et al.* 2013, Hu *et al.* 2014). Subsequently, infection is dependent on the presence of long periods of free surface water and suitable microclimatic conditions (Kiely 1948a, b, 1949, Kotzé 1963, 1981, McOnie 1967). Leaf litter colonised by *P. citricarpa* serves as the primary inoculum source. Thus leaf litter plays an important role and its removal or enhanced decomposition results in improved inoculum management (Bellotte *et al.* 2009, Truter 2010, Sposito *et al.* 2011). Pseudothecia develop 40–180 d after leaf fall, releasing mature ascospores during rainfall that are dispersed by wind (Kotzé 1963, McOnie 1964, Huang & Chang 1972, Reis *et al.* 2006, Fourie *et al.* 2013, Dummel *et al.* 2015). Fruits are susceptible for 4–5 mo after petal fall (Kiely 1948b, Schutte *et al.* 2003, 2012, Miles *et al.* 2004). Therefore, the onset of rain, ascospore release and fruit susceptibility period are strongly correlated in summer rainfall regions resulting in fruit infection (Kotzé 1963, McOnie 1964, 1967, Whiteside 1967). Following a long latent period, the onset of symptom expression on fruit coincides with fruit ripening (Kiely 1948a, Whiteside 1967, Kotzé 1981, Spósito *et al.* 2008).

Phyllosticta citricarpa has been recorded in Australia since the late 19th century, causing CBS disease, specifically in coastal regions of New South Wales and Queensland (Benson 1895, Kotzé 1981, Miles *et al.* 2013), but not from the hot, dry inland citrus orchards, and not in the winter rainfall regions in Australia (Broadbent 1995). *Phyllosticta citricarpa* has also been recorded in summer rainfall citrus-growing regions in several areas: South America (Argentina, Brazil, Uruguay, Venezuela; Garran 1996, Kotzé 2000, European Union 2000, Paul *et al.* 2005), Central America (West Indies; Calavan 1960), North America (Dewdney *et al.* 2012, Schubert *et al.* 2012, Zavala *et al.* 2014), Asia (Bhutan, China, India, Indonesia, Philippines, Taiwan; Brodrick 1969, European Union 1998, Kotzé 2000, European Union 2000) and Africa (Ghana, Kenya, Mozambique, Nigeria, South Africa, Swaziland, Zambia, Zimbabwe; Doidge 1929, Kotzé 1981, 2000, European Union 1998, Baayen *et al.* 2002, Brentu *et al.* 2012). Several fruit and leaf diseases caused by different fungi such as *Colletotrichum* and *Alternaria* spp. (Vicent *et al.* 2007, Aiello *et al.* 2015) are present in the EU citrus-producing countries; however, the CBS disease has not been reported (Baker *et al.* 2014). In addition to the general phytosanitary regulations applicable to the import of citrus propagating plant material, the import of citrus fruit into the EU is subject to phytosanitary regulations relating to *P. citricarpa* (EC2000/29/EC, 2000).

Recent epidemiological studies (Paul *et al.* 2005, Yonow *et al.* 2013, Magarey *et al.* 2015) have shown that the climatic conditions in the citrus growing regions within the EU are unsuitable for establishment of *P. citricarpa* and development of CBS disease, with only small, restricted Mediterranean coastal areas where the climatic conditions have at most marginal potential suitability. Considering that citrus plants were moved from Asia, where CBS is endemic and also regarded as the centre of origin of citrus, to Northern Africa and other countries around the Mediterranean Sea by traders, as early as the 5th century BC (Ramón-Laca 2003, Mabberley 2004, Nicolosi 2007), it would be expected that *P. citricarpa* and/or other *Phyllosticta* spp. may have been introduced to these citrus-growing countries along with the hosts, especially since infected plant material is regarded as the means of long-distance spread of this pathogen (Kiely 1948b, Kotzé 1981). Likewise, there is always the possibility of illegal movement of citrus plant propagating material. Therefore, the potential occurrence of *Phyllosticta* spp. was included in a broad survey of fungal citrus pathogens undertaken in citrus growing regions within EU countries (Guarnaccia *et al.* 2017, Sandoval *et al.* 2018). During 2015–2017, several surveys were conducted in the major citrus production areas of the EU and included the following: (i) surveys of both fresh plant material and leaf litter in commercial nurseries and citrus orchards, gardens, backyards and plant collections, (ii) cultivation of as many *Phyllosticta* isolates as possible from this material, (iii) subject isolates to DNA sequence analyses combined with morphological characterisation, (iv) compare these results with data from other phylogenetic studies on *Phyllosticta*, (v) identification of genotypes and mating types of the *P. citricarpa* isolates found in this study and, (vi) to evaluate potential pathogenicity of the *Phyllosticta* spp. isolated.

MATERIALS AND METHODS

Sampling and isolation

The initial surveys were carried out in 2015 and 2016 covering a total of 95 sites located in some of the main citrus-producing regions of Europe (Table 1). Evaluations were conducted by sampling approx. 25 fruits, 25 twig portions, 50 living leaves and 50 leaves from the litter layer from each *Citrus* host present in each site investigated. Samples were collected from Andalusia, Mallorca, Valencia (Spain), Apulia, Calabria, Sicily (Italy), Algarve (Portugal), Crete, Mesolongi, Nafplio (Greece), Gozo and La Valletta (Malta) areas. Investigated citrus species included Australasian lime (*Citrus australasica*), citranges (*Citrus sinensis* × *Poncirus trifoliata*), citrons (*C. medica*, *C. medica* var. *sarcodactylis*), kumquat (*C. japonica*), limequats (*Citrus ×floridana*), calamondin (*×Citrofortunella microcarpa*), mandarins (*C. reticulata*), tangelo (*C. ×tangelo*), oranges (*C. ×aurantium*, *C. ×bergamia*, *C. ×sinensis*), pummelo (*C. maxima*), grapefruit (*C. paradisi*), limes (*C. ×aurantifolia*, *C. ×hystrix*, *C. ×latifolia*) and lemons (*C. ×limon*). New surveys were performed during December 2016 and January 2017 at the sites where *P. citricarpa* and *P. paracitricarpa* were found during the initial surveys (Table 1) to confirm these findings and to assay the presence of symptoms on fruit, leaves and twigs.

Fungal isolates were obtained using two procedures. In the first, leaf and fruit sections (5 × 5 mm) were aseptically cut and surface-sterilised in a sodium hypochlorite solution (10 %) for 20 s, followed by 70 % ethanol for 30 s, and rinsed three times in autoclaved water. The sections were dried on autoclaved tissue paper, placed on malt extract agar (MEA; Crous *et al.* 2009) amended with 100 µg/mL penicillin and 100 µg/mL streptomycin (MEA-PS) and incubated at 25 °C until characteristic *Phyllosticta* colonies were observed. In the second procedure, leaf litter, living leaves, fruits and twig portions were incubated in moist chambers at room temperature (25 °C ± 3 °C) for up to 14 d and inspected daily for fungal sporulation. Sporulating pycnidia obtained through both procedures were collected and crushed in a drop of sterile water and then spread over the surface of MEA-PS plates. After 24–36 h germinating spores were individually transferred onto MEA plates. The isolates used in this study are maintained in the Westerdijk Fungal Biodiversity Institute (CBS culture collection), Utrecht, The Netherlands, and in the working collection of Pedro Crous (CPC), housed at the Westerdijk

Table 1. Location and characteristics of the investigated sites.

City (country)	GPS coordinates	Site	Plant age (years)	Condition[3]
Acitrezza (Italy)	37.561077, 15.161086	Backyard	20–30	Cultivated
Agia (Greece)	35.465979, 23.921240	Orchard	5–10	Cultivated
Algemesi (Spain)	39.207638, −0.449773	Orchard	5–10	Cultivated
Algemesi (Spain)	39.196895, −0.470823	Orchard	5–10	Cultivated
Alginet (Spain)	39.260069, −0.458032	Orchard	10–15	Cultivated
Alginet (Spain)	39.251407, −0.416424	Orchard	5–10	Cultivated
Alhaurin El Grande (Spain)	36.645374, −4.677086	Orchard	15–25	Unkept
Alhaurin El Grande (Spain)	36.664689, −4.698184	Orchard	15–25	Cultivated
Alikianos (Greece)	35.456657, 23.908632	Orchard	15–25	Cultivated
Alikianos (Greece)	35.462384, 23.904367	Orchard	10–15	Unkept
Alikianos (Greece)	35.446440, 23.919798	Orchard	10–15	Unkept
Alikianos (Greece)	35.466216, 23.945558	Orchard	10–15	Cultivated
Almeria (Spain)	36.834637, −2.402932	Experimental orchard	15–25	Cultivated
Almeria (Spain)	36.828832, −2.402892	Experimental orchard	15–25	Cultivated
Alzira (Spain)	39.156963, −0.490723	Orchard	10–15	Cultivated
Amfilochia (Greece)	38.961381, 21.171635	Orchard	10–15	Cultivated
Argo (Greece)	37.628645, 22.742179	Orchard	10–15	Cultivated
Argo (Greece)	37.655558, 22.739309	Orchard	10–15	Cultivated
Argos (Greece)	37.686587, 22.661719	Orchard	10–15	Cultivated
Arta (Greece)[1]	39.161719, 20.929585	Backyard	30–40	Unkept
Arta (Greece)	39.155661, 20.903791	Orchard	15–25	Cultivated
Arta (Greece)	39.160465, 20.918257	Orchard	5–10	Cultivated
Barcellona P.G. (Italy)	38.110560, 15.136794	Nursery	1–3	Cultivated
Brucoli (Italy)	37.294823, 15.110518	Orchard	15–25	Cultivated
Canicattì (Italy)	37.358434, 13.840898	Backyard	20–30	Cultivated
Carruba (Italy)	37.684625, 15.190943	Orchard	15–25	Unkept
Castellò (Spain)	39.903922, −0.086197	Orchard	10–15	Cultivated
Castellò (Spain)	39.883861, −0.088225	Orchard	10–15	Cultivated
Castellò (Spain)	39.884013, −0.090945	Orchard	10–15	Cultivated
Cefalù (Italy)	38.029481, 14.012267	Backyard	20–30	Unkept
Chania (Greece)	35.493153, 24.051141	Orchard	10–15	Cultivated
Chania (Greece)	35.477894, 23.948060	Orchard	10–15	Cultivated
Comiso (Italy)	36.943980, 14.637159	Orchard	15–25	Unkept
Conceicao (Portugal)	37.048481, −7.916927	Orchard	15–25	Cultivated
Curiglia (Italy)	38.767729, 16.203763	Orchard	20–30	Unkept
El Ejido (Spain)	36.795207, −2.719992	Orchard	20–30	Cultivated
Estellencs (Spain)	39.653504, 2.481876	Backyard	30–40	Unkept
Faro (Portugal)	37.108457, −7.916805	Orchard	20–30	Unkept
Faro (Portugal)	37.062641, −7.917432	Orchard	10–15	Unkept
Giarratana (Italy)	36.883438, 14.974420	Orchard	10–15	Cultivated
Gouria (Greece)	38.454977, 21.257646	Orchard	15–25	Cultivated
Gozo (Malta)	36.049069, 14.259299	Backyard	20–30	Unkept
Gozo (Malta)	36.037531, 14.260120	Orchard	10–15	Unkept
Gozo (Malta)	36.049646, 14.279360	Orchard	15–25	Cultivated
Gozo (Malta)[2]	36.055138, 14.259907	Backyard	60–70	Unkept
Gozo (Malta)	36.058166, 14.284453	Backyard	60–70	Unkept
Grotte (Italy)	37.679925, 15.177006	Orchard	15–25	Cultivated
Guardia (Italy)	37.662709, 15.175918	Orchard	15–25	Cultivated
Kirtomados (Greece)	35.478749, 23.916661	Orchard	15–25	Cultivated
Leni (Italy)	38.044422, 14.597517	Backyard	30–40	Cultivated

Table 1. (Continued).

City (country)	GPS coordinates	Site	Plant age (years)	Condition[3]
Leni (Italy)	38.552889, 14.827128	Backyard	30–40	Cultivated
Lentini (Italy)	37.320577, 15.020901	Orchard	15–25	Cultivated
Malaga (Spain)	36.761761, −4.427060	Botanical garden	40–50	Unkept
Mascali (Italy)	37.767684, 15.192503	Nursery	1–3	Cultivated
Mascali (Italy)	37.768258, 15.194639	Nursery	1–3	Cultivated
Massafra (Italy)	40.544756, 17.144112	Orchard	10–15	Cultivated
Mastro (Greece)	38.430287, 21.280539	Orchard	15–25	Cultivated
Mesquita (Portugal)	37.213673, −8.289493	Orchard	10–15	Cultivated
Mesquita (Portugal)	37.204525, −8.297812	Orchard	20–30	Unkept
Mineo (Italy)	37.350719, 14.690858	Orchard	15–25	Cultivated
Moncada (Spain)	39.588547, −0.394583	Experimental orchard	10–15	Cultivated
Monchique (Portugal)	37.332409, −8.514506	Backyard	20–30	Unkept
Monchique (Portugal)	37.336226, −8.503686	Backyard	20–30	Unkept
Monchique (Portugal)	37.332239, −8.492232	Backyard	20–30	Unkept
Monchique (Portugal)[2]	37.326195, −8.526232	Backyard	30–40	Unkept
Motta S. Anastasia (Italy)	37.482099, 14.886016	Orchard	15–25	Cultivated
Motta S. Anastasia (Italy)	37.469713, 14.954161	Orchard	15–25	Cultivated
Nafplio (Greece)	37.589312, 22.785267	Orchard	10–15	Unkept
Nafplio (Greece)	37.575095, 22.695589	Orchard	15–25	Cultivated
Nafplio (Greece)	37.582292, 22.696803	Orchard	10–15	Cultivated
Nafplio (Greece)	37.588798, 22.874844	Backyard	10–15	Cultivated
Nicolosi (Italy)	37.611273, 15.029477	Backyard	5–10	Cultivated
Niscemi (Italy)	37.139783, 14.393402	Backyard	15–25	Cultivated
Noto (Italy)	36.846497, 15.095445	Orchard	15–25	Unkept
Pachino (Italy)	36.720032, 15.086993	Backyard	15–25	Unkept
Pachino (Italy)	36.722328, 15.089408	Orchard	15–25	Unkept
Pedara (Italy)	37.608708, 15.066544	Backyard	30–40	Cultivated
Pizzo Calabro (Italy)	38.760390, 16.226005	Orchard	15–25	Cultivated
Ribera (Italy)	37.497113, 13.241850	Orchard	5–10	Cultivated
Ribera (Italy)	37.504423, 13.252070	Orchard	5–10	Cultivated
Riposto (Italy)	37.696470, 15.199345	Nursery	1–3	Cultivated
Rocca Imperiale (Italy)	40.108385, 16.617951	Orchard	10–15	Cultivated
San Gregorio (Italy)	37.562297, 15.100965	Backyard	60–70	Cultivated
Scordia (Italy)	37.281526, 14.869149	Orchard	15–25	Cultivated
Seville (Spain)	37.508538, −5.962815	Orchard	15–25	Cultivated
Seville (Spain)	37.482978, −5.954910	Orchard	15–25	Unkept
Sikoula (Greece)	39.085933, 21.083398	Orchard	10–15	Cultivated
Silves (Portugal)	37.164148, −8.390841	Orchard	15–25	Unkept
Soller (Spain)	39.764529, 2.709609	Botanical garden	30–40	Cultivated
Soller (Spain)	39.770115, 2.726600	Orchard	20–30	Cultivated
Terme Vigliatore (Italy)	38.145801, 15.163235	Nursery	1–3	Cultivated
Torremolinos (Spain)	36.672722, −4.504134	Orchard	30–40	Cultivated
Trebisacce (Italy)[2]	39.910122, 16.564824	Backyard	20–30	Cultivated
Trebisacce (Italy)	39.906711, 16.560634	Orchard	3–6	Cultivated
Zurrieq (Malta)[2]	35.823845, 14.505099	Backyard	15–25	Unkept

[1] Site where *P. paracitricarpa* isolates were found associated with leaf litter sampled.
[2] Sites where *P. citricarpa* isolates were found associated with leaf litter sampled.
[3] Cultivated: Plants kept under constant agronomical management. Unkept: Plants abandoned.

Institute. In addition, two *Phyllosticta* isolates collected in Florida, USA (CPC 25312, CPC 25327) and three from China (ZJUCC200933, ZJUCC200937, ZJUCC200952) were included in the phylogenetic analyses. Sequences from additional species were retrieved from NCBI's GenBank nucleotide database. A total of 111 *Phyllosticta* (incl. 64 European) isolates were included in the study (Table 2), of which 100 (incl. the outgroup, *Neofusicoccum mediterraneum* CBS 121718) were used in the phylogenetic analysis.

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted using a Wizard® Genomic DNA Purification Kit (Promega Corporation, WI, USA) following the manufacturer's instructions. Partial regions of six loci were amplified. The primers V9G (de Hoog & Gerrits van den Ende 1998) and ITS4 (White *et al.* 1990) were used to amplify the internal transcribed spacer region (ITS) of the nuclear ribosomal RNA operon, including the 3′ end of the 18S rRNA, the first internal transcribed spacer region, the 5.8S rRNA gene; the second internal transcribed spacer region and the 5′ end of the 28S rRNA gene. The primers EF1-728F (Carbone & Kohn 1999) and EF2 (O'Donnell *et al.* 1998) were used to amplify part of the translation elongation factor 1-α gene (*tef1*). The primers ACT-512F and ACT-783R (Carbone & Kohn 1999) were used to amplify part of the actin gene (*actA*). The 28S large subunit nrDNA (LSU) was amplified using primers LR0R (Moncalvo *et al.* 1995) and LR5 (Vilgalys & Hester 1990). The RNA polymerase II second largest subunit (*rpb2*) was amplified with RPB2-5F2 (Sung *et al.* 2007) and fRPB2-7cR (Liu *et al.* 1999). Glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) was amplified using primers Gpd1-LM and Gpd2-LM (Myllys *et al.* 2002). For *P. citricarpa* isolates the alternative primers Gpd1 (Guerber *et al.* 2003) and GPDHR2 (Glienke *et al.* 2011) were used to amplify *gapdh*. The PCR amplification mixtures and cycling conditions for ITS, *actA*, *tef1*, LSU and *gapdh* were followed as described by Glienke *et al.* (2011). Due to the lack of available *rpb2* gene sequences of *Phyllosticta* isolates, we generated these sequences for all the strains used for this study (except for *P. citrimaxima* CPC 20276 = CBS 136059, culture has been lost). The *rpb2* PCR was performed in a total volume of 25 μL and the mixture consisted of 1 μL genomic DNA, 1× PCR Buffer (Bioline GmbH, Luckenwalde, Germany), 0.75 μM $MgCl_2$, 1.85 μM of each dNTP, 0.45 μM of each primer and 0.5 μL BioTaq *Taq* DNA polymerase (Bioline GmbH, Luckenwalde, Germany). A touchdown PCR protocol was used for *rpb2*: initial denaturation (94 °C, 5 min), five amplification cycles (94 °C, 45 s; 60 °C, 45 s; 72 °C, 2 min), five amplification cycles (94 °C, 45 s; 58 °C, 45 s; 72 °C, 2 min), 30 amplification cycles (94 °C, 45 s; 54 °C, 45 s; 72 °C, 2 min) and a final extension step (72 °C, 8 min). The PCR products were sequenced in both directions using the BigDye® Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems Life Technologies, Carlsbad, CA, USA), after which amplicons were purified through Sephadex G-50 Fine columns (GE Healthcare, Freiburg, Germany) in MultiScreen HV plates (Millipore, Billerica, MA). Purified sequence reactions were analysed on an Applied Biosystems 3730xl DNA Analyzer (Life Technologies, Carlsbad, CA, USA). The DNA sequences generated were analysed and consensus sequences were computed using the program SeqMan Pro (DNASTAR, Madison, WI, USA).

Phylogenetic analyses

Novel sequences generated in this study were queried against the NCBI's GenBank nucleotide database to determine the closest relatives for a taxonomic framework of the studied isolates. Alignments of different gene regions, including sequences obtained from this study and sequences downloaded from GenBank, were initially performed by using the MAFFT v. 7 online server (http://mafft.cbrc.jp/alignment/server/index.html) (Katoh & Standley 2013), and then manually adjusted in MEGA v. 6.06 (Tamura *et al.* 2013). Additional reference sequences were selected based on recent studies on *Phyllosticta* species (Glienke *et al.* 2011, Wang *et al.* 2012, Wikee *et al.* 2013a).

Phylogenetic analyses were based on both Bayesian Inference (BI) and Maximum Parsimony (MP) analyses. For BI, the best evolutionary model for each partition was determined using MrModeltest v. 2.3 (Nylander 2004) and incorporated into the analysis. 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 100 generations. Analyses stopped once the average standard deviation of split frequencies was below 0.01. The MP analysis was done using PAUP (Phylogenetic Analysis Using Parsimony, v. 4.0b10; 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 bootstrap analysis (Hillis & Bull 1993) was based on 1 000 replications. Sequences generated in this study were deposited in GenBank (Table 2) and alignments and phylogenetic trees in TreeBASE (www.treebase.org). Nomenclatural novelties were deposited in MycoBank (Crous *et al.* 2004).

Taxonomy

A subset of isolates of the four *Phyllosticta* species collected in this study was morphologically characterised. After 14 d of incubation in the dark at 27 °C, the morphological characteristics were examined by mounting fungal structures in clear lactic acid and 30 measurements at ×1 000 magnification were determined for each isolate using a Zeiss Axioscope 2 microscope with interference contrast (DIC) optics. Colony colour and growth rate were established on MEA, potato dextrose agar (PDA) and OA according to Crous *et al.* (2009). Sporulation was induced on pine needle agar (PNA) (Smith *et al.* 1996) and synthetic nutrient-poor agar (SNA) under near UV-light. Colony colour was determined on MEA, OA and PDA using the colour charts of Rayner (1970). Colony growth rates were assessed on MEA, OA and PDA in 90 mm Petri plates at 9–39 °C at 3 °C intervals. Three plates were used for each culture/media and two measurements of colony diameter perpendicular to each were made after 3, 6, 9 and 12 d of incubation in the dark, after which averages were computed. For each species × growth medium × incubation time combination, data were normalised to the maximum growth observed for that combination. The combined dataset with relative growth values (0 = no growth,

Table 2. Collection details and GenBank accession numbers of isolates included in this study.

Species	Culture no.[1]	Host	Country	Mating type idiomorph	GenBank no.[2]					
					ITS	*actA*	*tef1*	*gapdh*	LSU	*rpb2*
Neofusicoccum mediterraneum	**CBS 121718**	*Eucalyptus* sp.	Greece	–	GU251176	KY855639	GU251308	KY855694	KY855754	KY855815
Phyllosticta aloeicola	**CPC 21020** = CBS 136058	*Aloe ferox*	South Africa	–	KF154280	KF289311	KF289193	KF289124	KF206214	KY855816
	CPC 21021	*Aloe ferox*	South Africa	–	KF154281	KF289312	KF289194	KF289125	KF206213	KY855817
P. bifrenariae	**CBS 128855** = VIC30556	*Bifrenaria harrisoniae*, leaf	Brazil	–	JF343565	JF343649	JF343586	JF343744	KF206209	KY855818
	CPC 17467	*Bifrenaria harrisoniae*, leaf	Brazil	–	KF170299	KF289283	KF289207	KF289138	KF206260	KY855819
P. capitalensis	CBS 226.77	*Paphiopedilum callosum*, leaf spot	Germany	–	FJ538336	FJ538452	FJ538394	JF343718	KF206289	KY855820
	CBS 100175	*Citrus* sp.	Brazil	–	FJ538320	FJ538436	FJ538378	JF343699	KF206327	KY855821
	CBS 101228	*Nephelium lappaceum*	Hawaii	–	FJ538319	FJ538435	FJ538377	KF289086	KF206325	KY855822
	CBS 114751	*Vaccinium* sp., leaf	New Zealand	–	FJ538349	FJ538465	FJ538407	KF289088	EU167584	KY855823
	CBS 123373	*Musa paradisiaca*	Thailand	–	FJ538341	FJ538457	FJ538399	JF343703	JQ743604	KY855824
	CBS 123374	*Citrus aurantium*	Thailand	–	FJ538332	FJ538448	FJ538390	JF343702	KY855755	KY855825
	CBS 128856 = CPC 18848	*Stanhopea* sp.	Brazil	–	JF261465	JF343647	JF261507	JF343776	KF206304	KY855826
	CPC 14609	*Zyzygium* sp.	Madagascar	–	KF206184	KF289264	KF289175	KF289081	KF206280	KY855827
	CPC 20259	*Orchidaceae*	Thailand	–	KC291340	KC342537	KC342560	KF289104	KF206244	KY855828
	CPC 20263	*Magnoliaceae*	Thailand	–	KC291341	KC342538	KC342561	KF289085	KF206241	KY855829
	CPC 20268	*Hibiscus syriacus*	Thailand	–	KC291343	KC342540	KC342563	KF289117	KF206236	KY855830
	CPC 20275	*Polyalthia longifolia*	Thailand	–	KC291347	KC342544	KC342567	KF289107	KF206230	KY855831
	CPC 20278	*Euphorbia milii*	Thailand	–	KC291347	KC342544	KC342567	KF289107	KF206230	KY855832
	CPC 20508	*Ixora chinensis*	Thailand	–	KF206198	KF289302	KF289185	KF289111	KF206225	KY855833
	CPC 25327	*Citrus sinensis*	Florida	–	KY855585	KY855640	KY855914	KY855695	KY855756	KY855834
	CPC 27059	*Citrus limon*, leaf	Italy	–	KY855586	KY855641	KY855915	KY855696	KY855757	KY855835
	CPC 27060	*Citrus limon*, leaf	Italy	–	KY855587	KY855642	KY855916	KY855697	KY855758	KY855836
	CPC 27061	*Citrus limon*, leaf	Italy	–	KY855588	KY855643	KY855917	KY855698	KY855759	KY855837
	CPC 27062	*Citrus limon*, leaf	Italy	–	KY855589	KY855644	KY855918	KY855699	KY855760	KY855838
	CPC 27084 = CBS 141345	*Citrus aurantifolia*, leaf	Italy	–	KY855590	KY855645	KY855919	KY855700	KY855761	KY855839
	CPC 27085	*Citrus aurantifolia*, leaf	Italy	–	KY855591	KY855646	KY855920	KY855701	KY855762	KY855840
	CPC 27086	*Citrus aurantifolia*, leaf	Italy	–	KY855592	KY855647	KY855921	KY855702	KY855763	KY855841
	CPC 27087	*Citrus aurantifolia*, leaf	Italy	–	KY855593	KY855648	KY855922	KY855703	KY855764	KY855842
	CPC 27786	*Citrus limon*, leaf	Greece	–	KY855594	KY855649	KY855923	KY855704	KY855765	KY855843
	CPC 27787	*Citrus limon*, leaf	Greece	–	KY855595	KY855650	KY855924	KY855705	KY855766	KY855844
	CPC 27788	*Citrus limon*, leaf	Greece	–	KY855596	KY855651	KY855925	KY855706	KY855767	KY855845
	CPC 27789	*Citrus limon*, leaf	Greece	–	KY855597	KY855652	KY855926	KY855707	KY855768	KY855846
	CPC 27825 = CBS 141346	*C. medica* var. *sarcodactylis*, leaf spot	Italy	–	KY855598	KY855653	KY855927	KY855708	KY855769	KY855847
	CPC 27826	*C. medica* var. *sarcodactylis*, leaf spot	Italy	–	KY855599	KY855654	KY855928	KY855709	KY855770	KY855848

(*continued on next page*)

Table 2. (Continued).

Species	Culture no.[1]	Host	Country	Mating type idiomorph	GenBank no.[2]					
					ITS	*actA*	*tef1*	*gapdh*	LSU	*rpb2*
	CPC 27827	*C. medica* var. *sarcodactylis*, leaf spot	Italy	–	KY855600	KY855655	KY855929	KY855710	KY855771	KY855849
	CPC 27828	*C. medica* var. *sarcodactylis*, leaf spot	Italy	–	KY855601	KY855656	KY855930	KY855711	KY855772	KY855850
	CPC 27917 = CBS 141347	*Citrus limon*, leaf	Malta	–	KY855602	KY855657	KY855931	KY855712	KY855773	KY855851
	CPC 27918	*Citrus limon*, leaf	Malta	–	KY855603	KY855658	KY855932	KY855713	KY855774	KY855852
	CPC 27919 = CBS 141348	*Citrus limon*, leaf	Portugal	–	KY855604	KY855659	KY855933	KY855714	KY855775	KY855853
	CPC 27920	*Citrus limon*, leaf	Portugal	–	KY855605	KY855660	KY855934	KY855715	KY855776	KY855854
	CPC 28124	*Citrus limon*, leaf	Spain	–	KY855606	KY855661	KY855935	KY855716	KY855777	KY855855
	CPC 28125	*Citrus limon*, leaf	Spain	–	KY855607	KY855662	KY855936	KY855717	KY855778	KY855856
	CPC 28126	*Citrus limon*, leaf	Spain	–	KY855608	KY855663	KY855937	KY855718	KY855779	KY855857
P. citriasiana	**CBS 120486**	*Citrus maxima*, fruit	Thailand	–	FJ538360	FJ538476	FJ538418	JF343686	KF206314	KY855858
	CBS 120487	*Citrus maxima*, fruit	China	–	FJ538361	FJ538477	FJ538419	JF343687	KF206313	KY855859
	CBS 123370	*Citrus maxima*, fruit	Vietnam	–	FJ538355	FJ538471	FJ538413	JF343689	KF206310	KY855860
P. citribraziliensis	**CBS 100098**	*Citrus* sp., leaf	Brazil	–	FJ538352	FJ538468	FJ538410	JF343691	KF206221	KY855861
	CPC 17464	*Citrus* sp., leaf	Brazil	–	KF170300	KF289280	KF289224	KF289159	KF206263	KY855862
	CPC 17465	*Citrus* sp., leaf	Brazil	–	KF170301	KF289281	KF289225	KF289160	KF206262	KY855863
P. citricarpa	CBS 122482	*Citrus sinensis*	Zimbabwe	MAT1-2-1	FJ538317	KF289265	FJ538375	KF289146	KF306230	KY855864
	CBS 127452	*Citrus reticulata*	Australia	MAT1-2-1	JF343581	JF343665	JF343602	JF343769	KF206307	KY855865
	CBS 127454	*Citrus limon*	Australia	MAT1-2-1	JF343583	JF343667	JF343604	JF343771	KF206306	KY855866
	CPC 16151	*Citrus* sp.	South Africa	MAT1-2-1	KF170291	KF289267	KF289221	KF289156	KF206276	KY855867
	CPC 16586	*Citrus limon*	Argentina	MAT1-1-1	KF170293	KF289269	KF289220	KF289155	KF206274	KY855868
	CPC 16603	*Citrus limon*	Uruguay	MAT1-1-1	KF170295	KF289274	KF289213	KF289147	KF206269	KY855869
	CPC 16609	*Citrus* sp.	Argentina	MAT1-1-1	KF170298	KF289277	KF289217	KF289152	KF206266	KY855870
	CPC 25312	*Citrus sinensis*	Florida	MAT1-2-1	KY855609	KY855664	KY855938	KY855719	KY855780	KY855871
	CPC 27909[3] = CBS 141349	*Citrus limon*, leaf litter	Italy	MAT1-2-1	KY855610	KY855665	KY855939	KY855720	KY855781	KY855872
	CPC 27910[3]	*Citrus limon*, leaf litter	Italy	MAT1-2-1	KY855611	KY855666	KY855940	KY855721	KY855782	KY855873
	CPC 27911[3]	*Citrus limon*, leaf litter	Italy	MAT1-2-1	KY855612	KY855667	KY855941	KY855722	KY855783	KY855874
	CPC 27912[3]	*Citrus limon*, leaf litter	Italy	MAT1-2-1	KY855613	KY855668	KY855942	KY855723	KY855784	KY855875
	CPC 27913[3] = CBS 141350	*Citrus sinensis*, leaf litter	Malta	MAT1-2-1	KY855614	KY855669	KY855943	KY855724	KY855785	KY855876
	CPC 27914[3]	*Citrus sinensis*, leaf litter	Malta	MAT1-2-1	KY855615	KY855670	KY855944	KY855725	KY855786	KY855877
	CPC 27915[3]	*Citrus sinensis*, leaf litter	Malta	MAT1-2-1	KY855616	KY855671	KY855945	KY855726	KY855787	KY855878
	CPC 27916[3]	*Citrus sinensis*, leaf litter	Malta	MAT1-2-1	KY855617	KY855672	KY855946	KY855727	KY855788	KY855879
	CPC 28104[3] = CBS 141351	*Citrus sinensis*, leaf litter	Portugal	MAT1-1-1	KY855618	KY855673	KY855947	KY855728	KY855789	KY855880
	CPC 28105[3] = CBS 141352	*Citrus sinensis*, leaf litter	Portugal	MAT1-1-1	KY855619	KY855674	KY855948	KY855729	KY855790	KY855881
	CPC 28106[3]	*Citrus sinensis*, leaf litter	Portugal	MAT1-1-1	KY855620	KY855675	KY855949	KY855730	KY855791	KY855882
	CPC 28107[3]	*Citrus sinensis*, leaf litter	Portugal	MAT1-1-1	KY855621	KY855676	KY855950	KY855731	KY855792	KY855883
	CPC 31171[3]	*Citrus sinensis*, leaf litter	Malta	MAT1-2-1	–	–	–	–	–	–
	CPC 31172[3]	*Citrus sinensis*, leaf litter	Malta	MAT1-2-1	–	–	–	–	–	–

	CPC 31173[3]	*Citrus sinensis*, leaf litter	Malta	MAT1-2-1	–	–	–	–	–	–
	CPC 31174[3]	*Citrus sinensis*, leaf litter	Malta	MAT1-2-1	–	–	–	–	–	–
	CPC 31279[3]	*Citrus sinensis*, leaf litter	Portugal	MAT1-1-1	–	–	–	–	–	–
	CPC 31280[3]	*Citrus sinensis*, leaf litter	Portugal	MAT1-1-1	–	–	–	–	–	–
	CPC 31281[3]	*Citrus sinensis*, leaf litter	Portugal	MAT1-1-1	–	–	–	–	–	–
	CPC 31282[3]	*Citrus sinensis*, leaf litter	Portugal	MAT1-1-1	–	–	–	–	–	–
	ZJUCC200952	*Citrus reticulata,* leaf	China	MAT1-2-1	JN791635	JN791556	JN791480	*KY855732*	*KY855793*	*KY855884*
P. citrichinaensis	CBS 129764 = ZJUCC2010100	*Citrus reticulata*, leaf	China	–	JN791598	JN791527	JN791453	*KY855733*	*KY855794*	*KY855885*
	CBS 130529 = ZJUCC201085 = CGMCC3.14302	*Citrus maxima*, leaf	China	–	JN791597	JN791526	JN791452	*KY855734*	*KY855795*	*KY855886*
P. citrimaxima	**CPC 20276** = CBS 136059 = MFLUCC10-0137	*Citrus maxima*, fruit	Thailand	–	KF170304	KF289300	KF289222	KF289157	KF206229	–
P. cordylinophila	**CPC 20261** = MFLUCC10-0166	*Cordyline fruticosa*	Thailand	–	KF170287	KF289295	KF289172	KF289076	KF206242	*KY855887*
	CPC 20277 = MFLUCC12-0014	*Cordyline fruticosa*	Thailand	–	KF170288	KF289301	KF289171	KF289075	KF206228	*KY855888*
P. cussonia	CPC 14873	*Cussonia* sp.	South Africa	–	JF343578	JF343662	JF343599	JF343764	KF206279	*KY855889*
	CPC 14875	*Cussonia* sp.	South Africa	–	JF343579	JF343663	JF343600	JF343765	KF206278	*KY855890*
P. eugeniae	**CBS 445.82**	*Eugenia aromatica*	Indonesia	–	AY042926	KF289246	KF289208	KF289139	KF206288	*KY855891*
P. hypoglossi	**CBS 434.92**	*Ruscus aculeatus*	Italy	–	FJ538367	FJ538483	FJ538425	JF343695	KF206299	*KY855892*
P. paracapitalensis	CBS 173.77	*Citrus aurantiifolia*	New Zealand	–	KF206179	KF289244	FJ538393	KF289100	KF306231	*KY855893*
	CPC 26517 = CBS 141353	*Citrus floridana*, leaf	Italy	–	*KY855622*	*KY855677*	*KY855951*	*KY855735*	*KY855796*	*KY855894*
	CPC 26518	*Citrus floridana*, leaf	Italy	–	*KY855623*	*KY855678*	*KY855952*	*KY855736*	*KY855797*	*KY855895*
	CPC 26700 = CBS 141354	*Citrus floridana*, leaf	Italy	–	*KY855624*	*KY855679*	*KY855953*	*KY855737*	*KY855798*	*KY855896*
	CPC 26701	*Citrus floridana*, leaf	Italy	–	*KY855625*	*KY855680*	*KY855954*	*KY855738*	*KY855799*	*KY855897*
	CPC 26805	*Citrus floridana*, leaf	Italy	–	*KY855626*	*KY855681*	*KY855955*	*KY855739*	*KY855800*	*KY855898*
	CPC 26806	*Citrus floridana*, leaf	Italy	–	*KY855627*	*KY855682*	*KY855956*	*KY855740*	*KY855801*	*KY855899*
	CPC 28120 = CBS 141355	*Citrus limon*, leaf	Spain	–	*KY855628*	*KY855683*	*KY855957*	*KY855741*	*KY855802*	*KY855900*
	CPC 28121	*Citrus limon*, leaf	Spain	–	*KY855629*	*KY855684*	*KY855958*	*KY855742*	*KY855803*	*KY855901*
	CPC 28122	*Citrus limon*, leaf	Spain	–	*KY855630*	*KY855685*	*KY855959*	*KY855743*	*KY855804*	*KY855902*
	CPC 28123	*Citrus limon*, leaf	Spain	–	*KY855631*	*KY855686*	*KY855960*	*KY855744*	*KY855805*	*KY855903*
	CPC 28127 = CBS 141356	*Citrus limon*, leaf	Spain	–	*KY855632*	*KY855687*	*KY855961*	*KY855745*	*KY855806*	*KY855904*
	CPC 28128	*Citrus limon*, leaf	Spain	–	*KY855633*	*KY855688*	*KY855962*	*KY855746*	*KY855807*	*KY855905*
	CPC 28129	*Citrus limon*, leaf	Spain	–	*KY855634*	*KY855689*	*KY855963*	*KY855747*	*KY855808*	*KY855906*
P. paracitricarpa	**CPC 27169** = CBS 141357	*Citrus limon*, leaf litter	Greece	–	*KY855635*	*KY855690*	*KY855964*	*KY855748*	*KY855809*	*KY855907*
	CPC 27170 = CBS 141358	*Citrus limon*, leaf litter	Greece	–	*KY855636*	*KY855691*	*KY855965*	*KY855749*	*KY855810*	*KY855908*
	CPC 27171 = CBS 141359	*Citrus limon*, leaf litter	Greece	–	*KY855637*	*KY855692*	*KY855966*	*KY855750*	*KY855811*	*KY855909*
	CPC 27172 = CBS 141360	*Citrus limon*, leaf litter	Greece	–	*KY855638*	*KY855693*	*KY855967*	*KY855751*	*KY855812*	*KY855910*
	CPC 31246	*Citrus limon*, leaf litter	Greece	–	–	–	–	–	–	–
	CPC 31247	*Citrus limon*, leaf litter	Greece	–	–	–	–	–	–	–

(*continued on next page*)

Table 2. (Continued).

Species	Culture no.[1]	Host	Country	Mating type idiomorph	GenBank no.[2]					
					ITS	*actA*	*tef1*	*gapdh*	LSU	*rpb2*
	CPC 31248	*Citrus limon*, leaf litter	Greece	–	–	–	–	–	–	–
	CPC 31249	*Citrus limon*, leaf litter	Greece	–	–	–	–	–	–	–
	ZJUCC200933	*Citrus sinensis*, fruit	China	–	JN791626	JN791544	JN791468	*KY855752*	*KY855813*	*KY855911*
	ZJUCC200937	*Citrus sinensis*, fruit	China	–	JN791627	JN791546	JN791470	*KY855753*	*KY855814*	*KY855912*
P. spinarum	**CBS 292.90**	*Chamaecyparis pisifera*	France	–	JF343585	JF343669	JF343606	JF343773	KF206301	*KY855913*

[1] CPC: Culture collection of P.W. Crous, housed at CBS; CBS: CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands; ZJUCC: Zhejiang University Culture Collection, China; MFLUCC: Mae Fah Luang University Culture Collection; CGMCC: China, General Microbiological Culture Collection, Beijing, China; VIC: Culture collection of Federal University of Viçosa, Viçosa, Brazil. Ex-type and ex-epitype cultures are indicated in **bold**.
[2] ITS: internal transcribed spacers 1 and 2 together with 5.8S nrDNA; *actA*: partial actin gene; *tef1*: partial translation elongation factor 1-α gene; *gapdh*: partial glyceraldehyde-3-phosphate dehydrogenase gene; LSU: partial 28S (large subunit) nrDNA; *rpb2*: partial RNA polymerase II second largest subunit gene. Sequences generated in this study indicated in *italics*.
[3] *P. citricarpa* isolates genotyped in this study.

1 = maximum growth) was subjected to non-linear regression using the BETE function: $Y = (a \times ((X - T_{min})/(T_{max} - T_{minx}))\hat{}\,b \times (1-((X - T_{min})/(T_{max} - T_{minx}))\hat{}\,c$ (Analytis 1977, Leggieri *et al.* 2017). Goodness of fit was determined through linear regression of the predicted against actual relative growth values.

Mating type identification

The mating types of *P. citricarpa* strains were determined based on PCR amplification of a diagnostic region from each mating type idiomorph by using four primers, MAT111F3 (5′-GCAATG TGGCAGCGCAATCC-3′) and MAT111R3 (5′-TCTGGACCA TCGGACTCATC-3′) for MAT1-1-1, and MAT121F6 (5′-GATC GTGGCAGGAGGCTTTG-3′) and MAT121R6 (5′-AACGAC-CAGCGATCGGTAAG-3′) for MAT1-2-1 (Amorim *et al.* 2017). The same reaction mixtures were used for the amplification of both primers sets. A total volume of 12.5 μL containing 1 μL genomic DNA, 1× PCR Buffer (Bioline GmbH, Luckenwalde, Germany), 0.63 μM $MgCl_2$, 0.7 μM of each dNTP, 0.25 μM of each primer and 0.5 μL BioTaq *Taq* DNA polymerase (Bioline GmbH, Luckenwalde, Germany), was used.

The PCR programme for the primers MAT111F3–MAT111R3 consisted of initial denaturation (94 °C, 3 min), 25 amplification cycles (94 °C, 30 s; 60 °C, 30 s; 72 °C, 1 min), and a final extension step (72 °C, 10 min). For the primers MAT121F6–MAT121R6, 30 amplification cycles (94 °C, 30 s; 55 °C, 30 s; 72 °C, 1 min) were used. The amplified fragments were separated by electrophoresis at 100 V for 25 min on a 1 % (w/v) agarose gel stained with GelRed™ (Biotium, Hayward, CA, USA), and viewed under ultra-violet light. Sizes of amplicons were determined against a HyperLadder™ I molecular marker (Bioline).

Genotyping of *P. citricarpa* isolates

Fifteen published polymorphic SSR markers (Wang *et al.* 2016, Carstens *et al.* 2017) were used to compare the genotypes of the *P. citricarpa* isolates found in this study with populations from Australia, Brazil, China, South Africa and the USA (Carstens *et al.* 2017). The primer labelling as well as the PCR reactions and cycling conditions were as previously described in Carstens *et al.* (2017). The SSR alleles were scored using Genemapper software v. 4 (Life Technologies). To determine the within-population genetic diversity the following were calculated in GenAlEx v. 6.5 (Peakall & Smouse, 2012): number of alleles (Na), number of effective alleles, number of private alleles, number of polymorphic loci and Nei's measure of gene diversity (Nei 1973). A zero value for Nei's gene diversity is an indication that there is no genetic diversity within the population. Isolates with identical alleles across all the loci were considered clones or multilocus genotypes (MLGs). For the allele-based genetic analyses, a per population clone-corrected dataset was used. To assess the genetic variation between the European populations and those from other continents, an analysis of molecular variance (AMOVA) was conducted. The statistical significance was tested using 999 permutations. In order to perform this analysis, the 12 *P. citricarpa* populations from Carstens *et al.* (2017) were included in the dataset. The AMOVA was performed in GenAlEx v. 6.5 (Peakall & Smouse 2012).

Pathogenicity

Two isolates of each of the four *Phyllosticta* species isolated from specimens collected in Europe (*P. capitalensis*: CPC 27825, CPC 27917; *P. paracapitalensis*: CPC 26517, CPC 26700; *P. citricarpa*: CPC 27909, CPC 27913; *P. paracitricarpa*: CPC 27169, CPC 27170), were inoculated into mature, untreated fruits of sweet orange (*Citrus sinensis* Osbeck), cultivar 'Valencia' (from Spain), following the method described by Perryman *et al.* (2014) to obtain indicative results about pathogenicity. Three fruits per replicate for each isolate were inoculated and were arranged in a randomised complete block design. Fruits were washed and surface disinfected by immersion for 10 min in 70 % ethanol, and rinsed twice in autoclaved water. A suspension of conidia (1.0 × 10^5 conidia/mL) was obtained from cultures grown on PDA for 15 d at 27 °C, and was injected, 100 mL at a time, into 12 inoculation points on the surface of oranges. The suspension was inoculated by inserting a hypodermic sterile needle into the albedo (the white pith area just below the peel), approx. 2 mm deep. Control fruits were inoculated with sterile water. The inoculation points on each fruit were labelled with a dot made with a permanent marker. The inoculated oranges were incubated in sterile plastic boxes at 20 °C, with 100 % relative humidity, under a lighting rig providing a 12 h photoperiod. Lesion development was evaluated 5, 10 and 25 d after inoculation. The inoculated fungi were re-isolated from any tissue showing lesions and the identity of the re-isolated fungi was confirmed by sequencing loci *tef1* and LSU.

RESULTS

Sampling and isolation

A total of 64 monosporic isolates resembling those of the genus *Phyllosticta* were collected. The *Phyllosticta* isolates were recovered from five species of *Citrus* at 11 different sites. Among them, 32 isolates were obtained from fresh leaves, 28 were associated with leaf litter and four with leaf spot symptoms (Table 2). During the surveys performed no CBS symptoms were observed.

Phylogenetic analyses

The combined species phylogeny of *Phyllosticta* consisted of 100 sequences, including the outgroup sequences of *Neofusicoccum mediterraneum* (culture CBS 121718). A total of 3 142 characters were included in the phylogenetic analyses; 693 characters were parsimony-informative, 315 were variable and parsimony-uninformative and 2 134 characters were constant. The maximum of 1 000 equally most parsimonious trees were saved (Tree length = 1 829, CI = 0.750, RI = 0.972 and RC = 0.729). Bootstrap support values from the parsimony analysis were plotted on the Bayesian phylogeny presented in Fig. 1. For the Bayesian analysis, MrModeltest suggested that the ITS partition should be analysed with a fixed state frequency distribution and all other loci with Dirichlet state frequency distributions. The following models were used in the Bayesian analysis: SYM+I+G (ITS), HKY+I (*actA*), GTR+G (*tef1*, *gapdh*, *rpb2*) and GTR+I (LSU).

In the Bayesian analysis, the ITS partition had 189 unique site patterns, the *actA* partition had 116 unique site patterns, the *tef1* partition had 158 unique site patterns, the *gapdh* partition had 105 unique site patterns, the LSU partition had 76 unique site patterns, the *rpb2* partition had 245 unique site patterns and the analysis ran for 1 900 000 generations, resulting in 38 002 trees of which 28 502 trees were used to calculate the posterior probabilities (Fig. 1). The main difference between the Bayesian and MP trees was the position of *P. bifrenariae*; in the Bayesian tree this species clustered basal to *P. citricarpa* whereas it was basal to the broader lineage containing the species clades of *P. citricarpa* to *P. citribraziliensis* in the parsimony analysis (data not shown). All other species clades were identical between the two analyses. The tree resolved 15 *Phyllosticta* species, two of which (*P. paracapitalensis* and *P. paracitricarpa*) are described as new in the Results – Taxonomy section.

Nucleotide variations were observed in 49 base positions within the alignment of *P. capitalensis* isolates and those of the new species, *P. paracapitalensis*, included in this study (Table 3), and in 14 positions for *P. citricarpa* and the new species *P. paracitricarpa* (Table 4). Between the *P. capitalensis* and *P. paracapitalensis* clades, differences were present in all regions sequenced except for ITS. Specifically, 20 fixed nucleotide changes were observed over 3 142 nucleotides (one for *actA*, four for *tef1*, one for *gapdh* and 14 for *rpb2*). Moreover, seven fixed nucleotide changes were observed between *P. citricarpa* and *P. paracitricarpa* clades (five for *tef1* and two for LSU). ITS, LSU and *tef1* were sequenced to identify a further eight isolates of *P. citricarpa* (CPC 31171, CPC 31172, CPC 31173, CPC 31174, from Malta and CPC 31179, CPC 31180, CPC 31181, CPC 31182 from Portugal) and four isolates of *P. paracitricarpa* (CPC 31246, CPC 31247, CPC 31248, CPC 31249 from Greece) (data not shown).

Taxonomy

Morphological observations, supported by phylogenetic inference, were used to distinguish two known species (*P. capitalensis* and *P. citricarpa*) from two novel species. Culture characteristics were noted as dissimilar. The colour of upper and lower surfaces of Petri dishes were determined (Fig. 2). The BETE function fitted the relative growth data very well (R^2 values 0.81 to 0.87) and predicted cardinal and optimal temperatures of 12.5–27.2–34.0 °C for *P. citricarpa*, 10.7–26.4–33.2 °C for *P. paracitricarpa*, 9.4–27.3–33.3 °C for *P. capitalensis*, and 11.8–28.6–33.3 °C for *P. paracapitalensis* (Fig. 3). After 9 d of incubation at 27 °C, *P. capitalensis* and *P. paracapitalensis* grew significantly faster (8.6–8.7 mm/d) on PDA and OA than *P. citricarpa* (4.8 and 6.6 mm/d, respectively) and *P. paracitricarpa* (4.0 and 5.4 mm/d, respectively), while growth of these species were significantly slower on MEA (5.7, 4.4, 4.5 and 3.3 mm/d, respectively). The isolates also differed morphologically from the other *Phyllosticta* species associated with citrus worldwide (Table 5). Based on the results of both the phylogenetic and morphological analyses, the two new species are described below.

Phyllosticta paracapitalensis Guarnaccia & Crous, **sp. nov.** MycoBank MB817204; Fig. 4.

Etymology: Named after its close morphological resemblance and phylogenetic relationship to *P. capitalensis*.

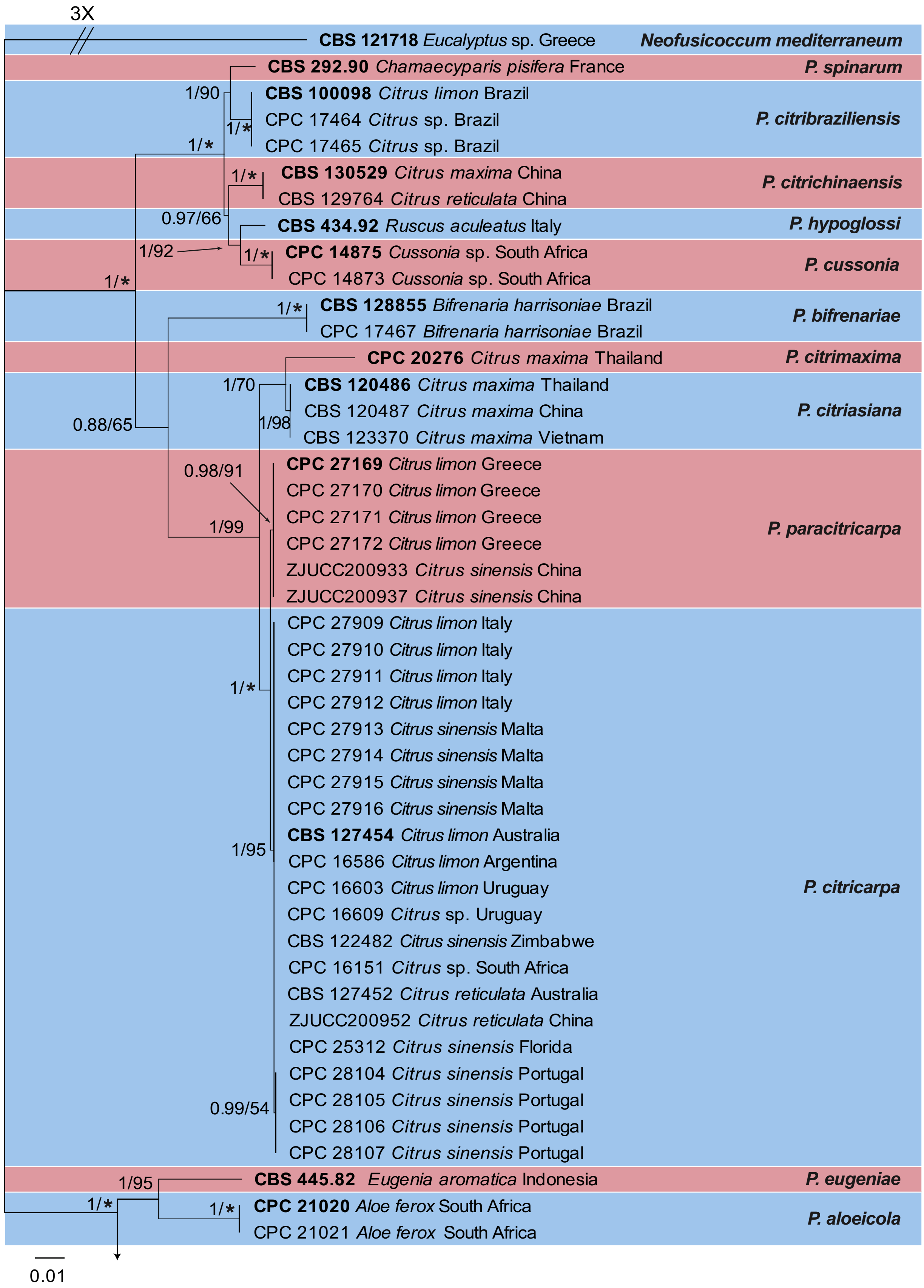

Fig. 1. Consensus phylogram resulting from a Bayesian analysis of the combined ITS, *actA*, *tef1*, *gapdh*, LSU and *rpb2* sequence alignments. Bootstrap support values and Bayesian posterior probability values are indicated at the nodes. Substrate and country of origin, where known, are indicated next to the strain numbers. The tree was rooted to *Neofusicoccum mediterraneum* (CBS 121718).

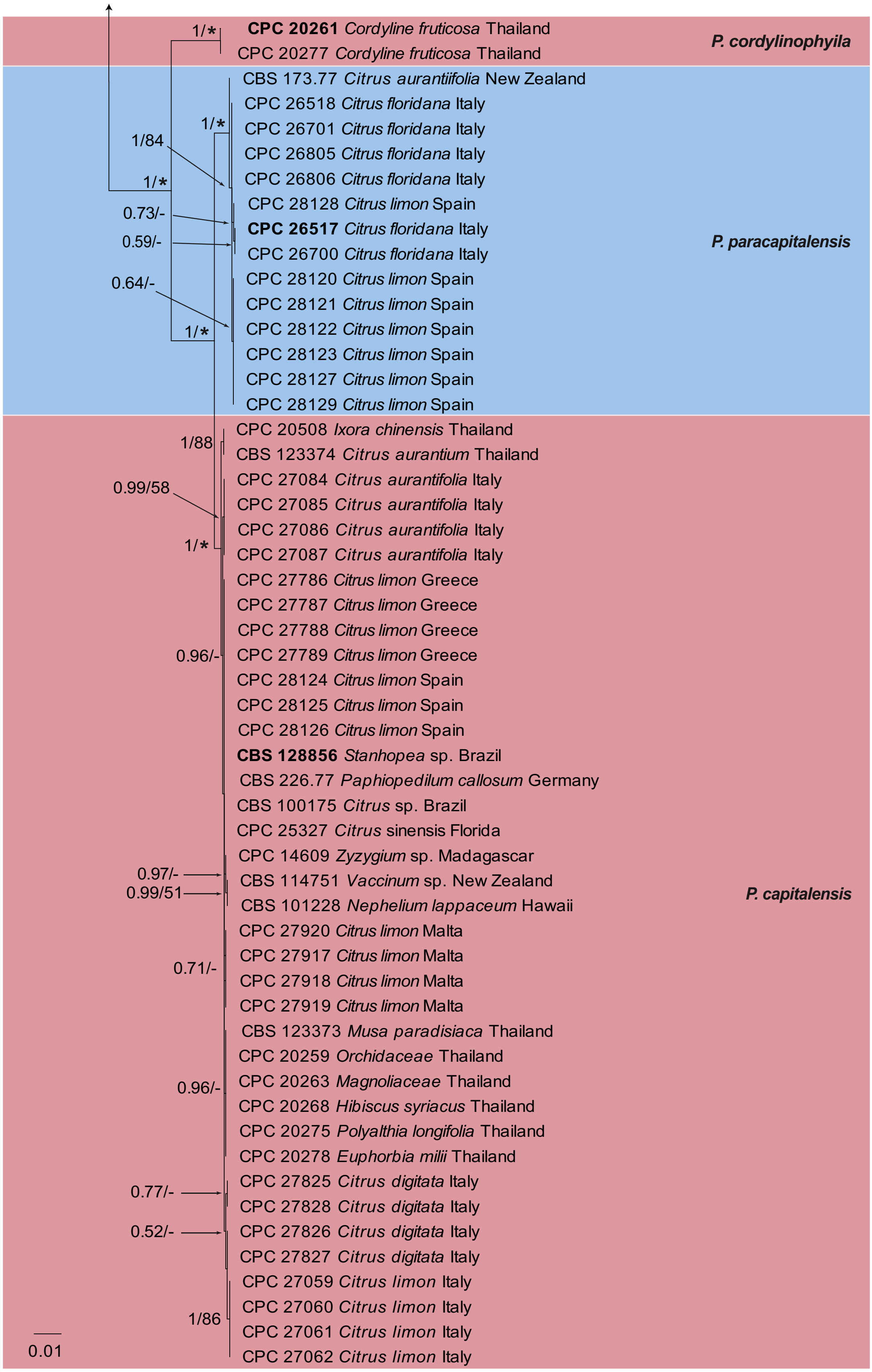

Fig. 1. (Continued).

Table 3. Nucleotide differences observed among *P. paracapitalensis* and *P. capitalensis* isolates used in this study. Base positions include spaces caused by alignment gaps and refer to the position in the alignment deposited in TreeBASE. Base positions representing fixed nucleotide differences between the two species are in **bold**.

	ITS								*actA*								*tef1*					*gapdh*									LSU	*rpb2*																	
	113	129	184	205	245	260	278	425	**628**	682	702	714	718	803	812	825	**909**	1070	**1073**	**1074**	**1078**	1240	**1266**	1316	1354	1610	1618	1694	1706	1739	1836	**2738**	**2759**	2804	**2822**	**2834**	**2918**	**2940**	**2948**	**2999**	3002	**3144**	3147	**3176**	**3179**	**3191**	**3266**	3326	**3335**
Phyllosticta paracapitalensis																																																	
CPC 26517 *Citrus floridana* Italy	T	T	G	C	T	T	-	C	G	C	T	T	C	C	G	A	G	C	A	T	T	C	A	G	C	T	G	C	C	C	A	T	C	C	T	G	T	T	T	T	G	C	G	C	T	T	C	C	G
CPC 26700 *Citrus floridana* Italy	T	T	G	C	T	T	-	C	G	C	T	T	C	C	G	A	G	C	A	T	T	C	A	G	C	T	G	C	C	C	A	T	C	C	T	G	T	T	T	T	G	C	G	C	T	T	C	C	G
CPC 26518 *Citrus floridana* Italy	T	T	G	C	T	T	-	C	G	C	T	T	C	C	G	A	G	C	A	T	T	C	A	G	C	T	C	C	C	C	A	T	C	C	T	G	T	T	T	T	G	C	G	C	T	T	C	C	G
CPC 26701 *Citrus floridana* Italy	T	T	G	C	T	T	-	C	G	C	T	T	C	C	G	A	G	C	A	T	T	C	A	G	C	T	C	C	C	C	A	T	C	C	T	G	T	T	T	T	G	C	G	C	T	T	C	C	G
CPC 26805 *Citrus floridana* Italy	T	T	G	C	T	T	-	C	G	C	T	T	C	C	G	A	G	C	A	T	T	C	A	G	C	T	C	C	C	C	A	T	C	C	T	G	T	T	T	T	G	C	G	C	T	T	C	C	G
CPC 26806 *Citrus floridana* Italy	T	T	G	C	T	T	-	C	G	C	T	T	C	C	G	A	G	C	A	T	T	C	A	G	C	T	C	C	C	C	A	T	C	C	T	G	T	T	T	T	G	C	G	C	T	T	C	C	G
CPC 28120 *Citrus limon* Spain	T	T	G	C	T	C	-	C	G	C	T	T	C	C	G	A	G	C	A	T	T	C	A	G	C	T	C	C	C	C	A	T	C	C	T	G	T	T	T	T	G	C	G	C	T	T	C	C	G
CPC 28121 *Citrus limon* Spain	T	T	G	C	T	C	-	C	G	C	T	T	C	C	G	A	G	C	A	T	T	C	A	G	C	T	C	C	C	C	A	T	C	C	T	G	T	T	T	T	G	C	G	C	T	T	C	C	G
CPC 28122 *Citrus limon* Spain	T	T	G	C	T	C	-	C	G	C	T	T	C	C	G	A	G	C	A	T	T	C	A	G	C	T	C	C	C	C	A	T	C	C	T	G	T	T	T	T	G	C	G	C	T	T	C	C	G
CPC 28123 *Citrus limon* Spain	T	T	G	C	T	C	-	C	G	C	T	T	C	C	G	A	G	C	A	T	T	C	A	G	C	T	C	C	C	C	A	T	C	C	T	G	T	T	T	T	G	C	G	C	T	T	C	C	G
CPC 28127 *Citrus limon* Spain	T	T	G	C	T	C	-	C	G	C	T	T	C	C	G	A	G	C	A	T	T	C	A	G	C	T	C	C	C	C	A	T	C	C	T	G	T	T	T	T	G	C	G	C	T	T	C	C	G
CPC 28128 *Citrus limon* Spain	T	T	G	C	T	C	-	C	G	C	T	T	C	C	G	A	G	C	A	T	T	G	A	G	C	T	G	C	C	C	A	T	C	C	T	G	T	T	T	T	G	C	G	C	T	T	C	C	G
CPC 28129 *Citrus limon* Spain	T	T	G	C	T	C	-	C	G	C	T	T	C	C	G	A	G	C	A	T	T	C	A	G	C	T	C	C	C	C	A	T	C	C	T	G	T	T	T	T	G	C	G	C	T	T	C	C	G
CBS 173.77 *Citrus aurantiifolia* New Zealand	T	C	T	C	T	T	-	T	G	C	T	T	C	C	G	A	G	C	A	T	T	C	A	G	C	T	C	C	C	C	A	T	C	C	T	G	T	T	T	T	G	C	G	C	T	T	C	C	G
Phyllosticta capitalensis																																																	
CPC 27059 *Citrus limon* Italy	T	T	T	C	T	T	-	T	C	T	T	T	A	C	G	C	A	T	G	C	C	C	G	G	C	T	C	A	T	G	A	C	T	C	C	A	C	C	C	C	G	T	A	T	C	C	T	C	A
CPC 27060 *Citrus limon* Italy	T	T	T	C	T	T	-	T	C	T	T	T	A	C	G	C	A	T	G	C	C	C	G	G	C	T	C	A	T	C	A	C	T	C	C	A	C	C	C	C	G	T	A	T	C	C	T	C	A
CPC 27061 *Citrus limon* Italy	T	T	T	C	T	T	-	T	C	T	T	T	A	C	G	C	A	T	G	C	C	C	G	G	C	T	C	A	T	G	A	C	T	C	C	A	C	C	C	C	G	T	A	T	C	C	T	C	A
CPC 27062 *Citrus limon* Italy	T	T	T	C	T	T	-	T	C	T	T	T	A	C	G	C	A	T	G	C	C	C	G	G	C	T	C	A	T	G	A	C	T	C	C	A	C	C	C	C	G	T	A	T	C	C	T	C	A
CPC 27084 *Citrus aurantifolia* Italy	T	T	T	C	T	T	-	T	G	T	T	T	A	C	A	A	A	T	G	C	C	C	G	G	C	T	C	C	C	C	A	C	T	C	C	A	C	C	C	C	G	T	G	T	C	C	T	C	A
CPC 27085 *Citrus aurantifolia* Italy	T	T	T	C	T	T	-	T	G	T	T	T	A	C	A	A	A	T	G	C	C	C	G	G	C	T	C	C	C	G	A	C	T	C	C	A	C	C	C	C	G	T	G	T	C	C	T	C	A
CPC 27086 *Citrus aurantifolia* Italy	T	T	T	C	T	T	-	T	G	T	T	T	A	C	A	A	A	T	G	C	C	C	G	G	G	T	C	C	C	C	A	C	T	C	C	A	C	C	C	C	G	T	G	T	C	C	T	C	A
CPC 27087 *Citrus aurantifolia* Italy	T	T	T	C	T	T	-	T	G	T	T	T	A	C	A	A	A	T	G	C	C	C	G	G	C	T	C	C	C	C	A	C	T	C	C	A	C	C	C	C	G	T	G	T	C	C	T	C	A
CPC 27786 *Citrus limon* Greece	T	T	T	C	T	T	-	T	G	T	T	T	A	C	G	C	A	T	G	C	C	C	G	G	C	T	C	C	T	C	A	C	T	C	C	A	C	C	C	C	G	T	G	T	C	C	T	C	A
CPC 27787 *Citrus limon* Greece	T	T	T	C	T	T	-	T	G	T	T	T	A	C	G	C	A	T	G	C	C	C	G	G	C	T	C	C	T	G	A	C	T	C	C	A	C	C	C	C	G	T	G	T	C	C	T	C	A
CPC 27788 *Citrus limon* Greece	T	T	T	C	T	T	-	T	G	T	T	T	A	C	G	C	A	T	G	C	C	C	G	G	G	T	C	C	T	C	A	C	T	C	C	A	C	C	C	C	G	T	G	T	C	C	T	C	A
CPC 27789 *Citrus limon* Greece	T	T	T	C	T	T	-	T	G	T	T	T	A	C	G	C	A	T	G	C	C	C	G	G	C	T	C	C	C	C	A	C	T	C	C	A	C	C	C	C	G	T	G	T	C	C	T	C	A
CPC 27825 *Citrus digitata* Italy	T	T	T	C	T	T	-	T	C	T	T	T	A	C	G	C	A	T	G	C	C	C	G	G	C	T	C	C	C	C	A	C	T	C	C	A	C	C	C	C	G	T	G	T	C	C	T	C	A
CPC 27826 *Citrus digitata* Italy	T	T	T	C	T	T	-	T	C	T	T	T	A	C	G	C	A	T	G	C	C	C	G	G	C	T	C	C	T	G	A	C	T	C	C	A	C	C	C	C	G	T	G	T	C	C	T	C	A
CPC 27827 *Citrus digitata* Italy	T	T	T	C	T	T	-	T	C	T	T	T	A	C	G	C	A	T	G	C	C	C	G	G	C	T	C	C	T	C	A	C	T	C	C	A	C	C	C	C	G	T	G	T	C	C	T	C	A
CPC 27828 *Citrus digitata* Italy	T	T	T	C	T	T	-	T	C	T	T	T	A	C	G	C	A	T	G	C	C	C	G	G	C	T	C	C	C	C	A	C	T	C	C	A	C	C	C	C	G	T	G	T	C	C	T	C	A
CPC 27917 *Citrus limon* Malta	T	T	T	C	T	T	-	T	G	T	T	T	A	C	G	C	A	T	G	C	C	C	G	G	C	T	C	C	T	C	G	C	T	C	C	A	C	C	C	C	G	T	G	T	C	C	T	C	A
CPC 27918 *Citrus limon* Malta	T	T	T	C	T	T	-	T	G	T	T	T	A	C	G	C	A	T	G	C	C	C	G	G	C	T	C	C	T	G	G	C	T	C	C	A	C	C	C	C	G	T	G	T	C	C	T	C	A
CPC 27919 *Citrus limon* Malta	T	T	T	C	T	T	-	T	G	T	T	T	A	C	G	C	A	T	G	C	C	C	G	G	C	T	C	C	T	G	G	C	T	C	C	A	C	C	C	C	G	T	G	T	C	C	T	C	A
CPC 27920 *Citrus limon* Malta	T	T	T	C	T	T	-	T	G	T	T	T	A	C	G	C	A	T	G	C	C	C	G	G	C	T	C	C	C	C	G	C	T	C	C	A	C	C	C	C	G	T	G	T	C	C	T	C	A
CPC 28124 *Citrus limon* Spain	T	T	T	C	T	T	-	T	G	T	T	T	A	C	G	C	A	T	G	C	C	C	G	G	C	T	C	C	T	G	A	C	T	C	C	A	C	C	C	C	G	T	G	T	C	C	T	C	A
CPC 28125 *Citrus limon* Spain	T	T	T	C	T	T	-	T	G	T	T	T	A	C	G	C	A	T	G	C	C	C	G	G	C	T	C	C	T	G	A	C	T	C	C	A	C	C	C	C	G	T	G	T	C	C	T	C	A
CPC 28126 *Citrus limon* Spain	T	T	T	C	T	T	-	T	G	T	T	T	A	C	G	C	A	T	G	C	C	C	G	G	C	T	C	C	T	G	A	C	T	C	C	A	C	C	C	C	G	T	G	T	C	C	T	G	A
CBS 128856 *Stanhopea* sp. Brazil	T	T	T	C	T	T	-	T	G	T	T	T	A	C	G	C	A	T	G	C	C	C	G	G	C	T	C	C	C	C	A	C	T	C	C	A	C	C	C	C	G	T	G	T	C	C	T	C	A
CBS 114751 *Vaccinum* sp. New Zealand	T	T	T	C	T	T	-	T	G	T	T	T	A	C	G	C	A	C	G	C	C	C	G	G	C	C	C	C	C	C	A	C	T	C	C	A	C	C	C	C	G	T	G	T	C	C	T	C	A
CBS 101228 *Nephelium lappaceum* Hawaii	T	T	T	C	T	T	-	T	G	T	T	T	A	C	G	C	A	C	G	C	C	C	G	G	C	C	C	C	C	C	A	C	T	C	C	A	C	C	C	C	G	T	G	T	C	C	T	C	A
CBS 123373 *Musa paradisiaca* Thailand	T	T	T	C	A	T	T	T	G	T	C	T	A	C	G	C	A	T	G	C	C	C	G	G	C	C	C	C	C	C	A	C	T	C	C	A	C	C	C	C	T	T	G	T	C	C	T	C	A
CPC 20259 *Orchidaceae* Thailand	T	T	T	C	A	T	T	T	G	T	T	T	A	C	G	C	A	T	G	C	C	C	G	G	C	T	C	C	C	C	A	C	T	C	C	A	C	C	C	C	G	T	G	T	C	C	T	C	A
CPC 20263 *Magnoliaceae* Thailand	T	T	T	C	A	T	T	T	G	T	T	T	A	C	G	C	A	T	G	C	C	C	G	G	C	T	C	C	C	C	A	C	T	C	C	A	C	C	C	C	G	T	G	T	C	C	T	C	A
CPC 20268 *Hibiscus syriacus* Thailand	T	T	T	C	A	T	T	T	G	T	T	T	A	C	G	C	A	T	G	C	C	C	G	G	C	T	C	C	C	C	A	C	T	C	C	A	C	C	C	C	G	T	G	T	C	C	T	C	A
CPC 20275 *Polyalthia longifolia* Thailand	T	T	T	C	A	T	T	T	G	T	T	T	A	C	G	C	A	T	G	C	C	C	G	G	C	T	C	C	C	C	A	C	T	T	C	A	C	C	C	C	G	T	G	T	C	C	T	C	A
CPC 20278 *Euphorbia milii* Thailand	C	T	T	C	A	T	T	T	G	T	T	T	A	C	G	C	A	T	G	C	C	C	G	G	C	T	C	C	C	C	A	C	T	C	C	A	C	C	C	C	G	T	G	T	C	C	T	C	A
CPC 20508 *Ixora chinensis* Thailand	T	T	T	C	A	T	T	T	G	T	T	A	C	C	G	A	A	T	G	C	C	C	G	G	C	T	C	C	C	C	A	C	T	C	C	A	C	C	C	C	G	T	G	T	C	C	T	C	A
CBS 123374 *Citrus aurantium* Thailand	T	T	T	C	A	T	T	T	G	T	T	A	C	C	G	A	A	T	G	C	C	C	G	G	C	T	C	C	C	C	A	C	T	C	C	A	C	C	C	C	G	T	G	T	C	C	T	C	A
CBS 100175 *Citrus* sp. Brazil	T	T	T	C	T	T	-	T	G	T	T	T	A	C	G	C	A	T	G	C	C	C	G	G	C	T	C	C	C	C	A	C	T	C	C	A	C	C	C	C	G	T	G	T	C	C	T	C	A
CPC 14609 *Zyzygium* sp. Madagascar	T	T	T	C	T	T	-	T	G	T	T	T	A	C	G	C	A	C	G	C	C	C	G	G	C	T	C	C	C	C	A	C	T	C	C	A	C	C	C	C	G	T	G	T	C	C	T	C	A
CPC 25327 *Citrus sinensis* Florida	T	T	T	C	T	T	-	T	G	T	T	T	A	C	G	C	A	T	G	C	C	C	G	G	C	T	C	C	C	C	A	C	T	C	C	A	C	C	C	C	G	T	G	T	C	C	T	C	A
CBS 226.77 *Paphiopedilum callosum* Germany	T	T	T	T	T	T	-	T	G	T	T	T	A	G	G	C	A	T	G	C	C	C	G	A	C	T	C	C	C	C	A	C	T	C	C	A	C	C	C	C	G	T	G	T	C	C	T	C	A

Table 4. Nucleotide differences observed among *P. paracitricarpa* and *P. citricarpa* isolates used in this study. Base positions include spaces caused by alignment gaps and refer to the position in the alignment deposited in TreeBASE. Base positions representing fixed nucleotide differences between the two species are in **bold**.

	actA				*tef1*					*gapdh*			LSU	
	635	638	641	822	**925**	**931**	**1012**	**1035**	**1054**	1689	1705	1706	**2191**	**2418**
Phyllosticta paracitricarpa														
CPC 27169 *Citrus limon* Greece	G	G	T	A	**A**	**-**	**C**	**-**	**C**	G	T	C	**C**	**T**
CPC 27170 *Citrus limon* Greece	G	G	T	A	**A**	**-**	**C**	**-**	**C**	G	T	C	**C**	**T**
CPC 27171 *Citrus limon* Greece	G	G	T	A	**A**	**-**	**C**	**-**	**C**	G	T	C	**C**	**T**
CPC 27172 *Citrus limon* Greece	G	G	T	A	**A**	**-**	**C**	**-**	**C**	G	T	C	**C**	**T**
ZJUCC200933 *Citrus sinensis* China	G	G	T	A	**A**	**-**	**C**	**-**	**C**	G	T	C	**C**	**T**
ZJUCC200937 *Citrus sinensis* China	G	G	T	A	**A**	**-**	**C**	**-**	**C**	G	T	C	**C**	**T**
Phyllosticta citricarpa														
CPC 27909 *Citrus limon* Italy	G	G	T	A	**T**	**T**	**T**	**T**	**T**	G	T	C	**T**	**C**
CPC 27910 *Citrus limon* Italy	G	G	T	A	**T**	**T**	**T**	**T**	**T**	G	T	C	**T**	**C**
CPC 27911 *Citrus limon* Italy	G	G	T	A	**T**	**T**	**T**	**T**	**T**	G	T	C	**T**	**C**
CPC 27912 *Citrus limon* Italy	G	G	T	A	**T**	**T**	**T**	**T**	**T**	G	T	C	**T**	**C**
CPC 27913 *Citrus sinensis* Malta	G	G	T	A	**T**	**T**	**T**	**T**	**T**	G	T	C	**T**	**C**
CPC 27914 *Citrus sinensis* Malta	G	G	T	A	**T**	**T**	**T**	**T**	**T**	G	T	C	**T**	**C**
CPC 27915 *Citrus sinensis* Malta	G	G	T	A	**T**	**T**	**T**	**T**	**T**	G	T	C	**T**	**C**
CPC 27916 *Citrus sinensis* Malta	G	G	T	A	**T**	**T**	**T**	**T**	**T**	G	T	C	**T**	**C**
CPC 28104 *Citrus sinensis* Portugal	G	G	T	G	**T**	**T**	**T**	**T**	**T**	G	T	C	**T**	**C**
CPC 28105 *Citrus sinensis* Portugal	G	G	T	G	**T**	**T**	**T**	**T**	**T**	G	T	C	**T**	**C**
CPC 28106 *Citrus sinensis* Portugal	G	G	T	G	**T**	**T**	**T**	**T**	**T**	G	T	C	**T**	**C**
CPC 28107 *Citrus sinensis* Portugal	G	G	T	G	**T**	**T**	**T**	**T**	**T**	G	T	C	**T**	**C**
CBS 127454 *Citrus limon* Australia	G	G	T	A	**T**	**T**	**T**	**T**	**T**	G	T	C	**T**	**C**
CPC 16586 *Citrus limon* Argentina	G	G	T	A	**T**	**T**	**T**	**T**	**T**	C	T	C	**T**	**C**
CPC 16603 *Citrus limon* Uruguay	G	G	T	A	**T**	**T**	**T**	**T**	**T**	G	T	C	**T**	**C**
CPC 16609 *Citrus* sp. Uruguay	G	G	T	A	**T**	**T**	**T**	**T**	**T**	G	T	C	**T**	**C**
CBS 122482 *Citrus sinensis* Zimbabwe	G	G	T	A	**T**	**T**	**T**	**T**	**T**	G	T	C	**T**	**C**
CPC 16151 *Citrus* sp. South Africa	G	G	C	A	**T**	**T**	**T**	**T**	**T**	G	T	C	**T**	**C**
CBS 127452 *Citrus reticulata* Australia	G	G	T	A	**T**	**T**	**T**	**T**	**T**	G	T	C	**T**	**C**
ZJUCC200952 *Citrus reticulata* China	G	G	T	A	**T**	**T**	**T**	**T**	**T**	G	C	G	**T**	**C**
CPC 25312 *Citrus sinensis* Florida	C	C	T	A	**T**	**T**	**T**	**T**	**T**	G	T	C	**T**	**C**

Conidiomata (on PNA) pycnidial, solitary, black, erumpent, globose, exuding colourless conidial masses; pycnidia up to 250 µm diam, elongated in culture on PNA; pycnidial wall of several layers of *textura angularis*, to 30 µm thick; inner wall of hyaline *textura angularis*. *Ostiole* central, to 20 µm diam. *Conidiophores* subcylindrical to ampulliform, reduced to conidiogenous cells, or with 1–2 supporting cell, that can be branched at the base, 7–20 × 4–6 µm. *Conidiogenous cells* terminal, subcylindrical, hyaline, smooth, coated in a mucoid layer, 7–15 × 3–4 µm; proliferating several times percurrently near apex. *Conidia* (9–)12–13(–14) × (6–)7 µm, solitary, hyaline, aseptate, thin and smooth-walled, granular, or with a single large central guttule, fusoid-ellipsoid, tapering towards a narrow truncate base, 3–4 µm diam, enclosed in a persistent mucoid sheath, 2–3 µm thick, and bearing a hyaline, apical mucoid appendage, (4–)5–7(–8) × 1.5(–2) µm, flexible, unbranched, tapering towards an acutely rounded tip. *Ascomata* solitary or in clusters of 2–3, erumpent, globose, up to 300 µm diam, with elongated neck to 500 µm long, with central ostiole; wall of 3–6 layers of brown *textura angularis*. *Asci* bitunicate, 8-spored, stipitate, with small pedicel and well developed apical chamber, hyaline, subcylindrical to clavate, 40–75 × 10–12 µm. *Ascospores* bi- to multiseriate, hyaline, smooth, granular with large central guttule, aseptate, straight, rarely curved, widest in the middle, limoniform with mucoid caps at obtuse ends, (15–)16–17(–18) × 6(–7) µm.

Culture characteristics: On MEA, colonies appear woolly, flat, irregular, initially white with abundant mycelium, gradually becoming greenish to dark green after 2–3 d with white hyphae on the undulate margin; reverse dark green to black. On OA, colonies appear flat with a regular margin, initially hyaline with abundant mycelium, gradually becoming dark greenish after 3–4 d; reverse dark green to black. On PDA, colonies appear irregular, woolly, initially white, gradually becoming greenish to dark green after 2–3 d with white hyphae on the undulate margin; reverse black. After 12 d in the dark at 27 °C, mycelium reached the edge of the Petri dish. The optimum growth rate was observed at 27 °C. No growth was observed at 12 °C and 39 °C.

Specimen examined: **Italy**, Sicily, on living leaf of *Citrus* × *floridana*, 4 Mar. 2015, V. Guarnaccia (**holotype** CBS H-22663, culture ex-type CPC 26517 = CBS 141353).

Notes: *Phyllosticta paracapitalensis* was isolated from leaves of *Citrus limon* and *C.* ×*floridana* as an endophyte. This species is similar to *P. capitalensis*, its sister species, but represents a distinct taxon, supported by molecular and morphological differences. *Phyllosticta paracapitalensis* differs from *P. capitalensis* in having longer ascomatal necks, narrower asci, and slightly larger ascospores. The asexual morph presents solitary and globose conidiomata that differ from those of *P. capitalensis* (aggregated and globose to ampuliform). Furthermore, the

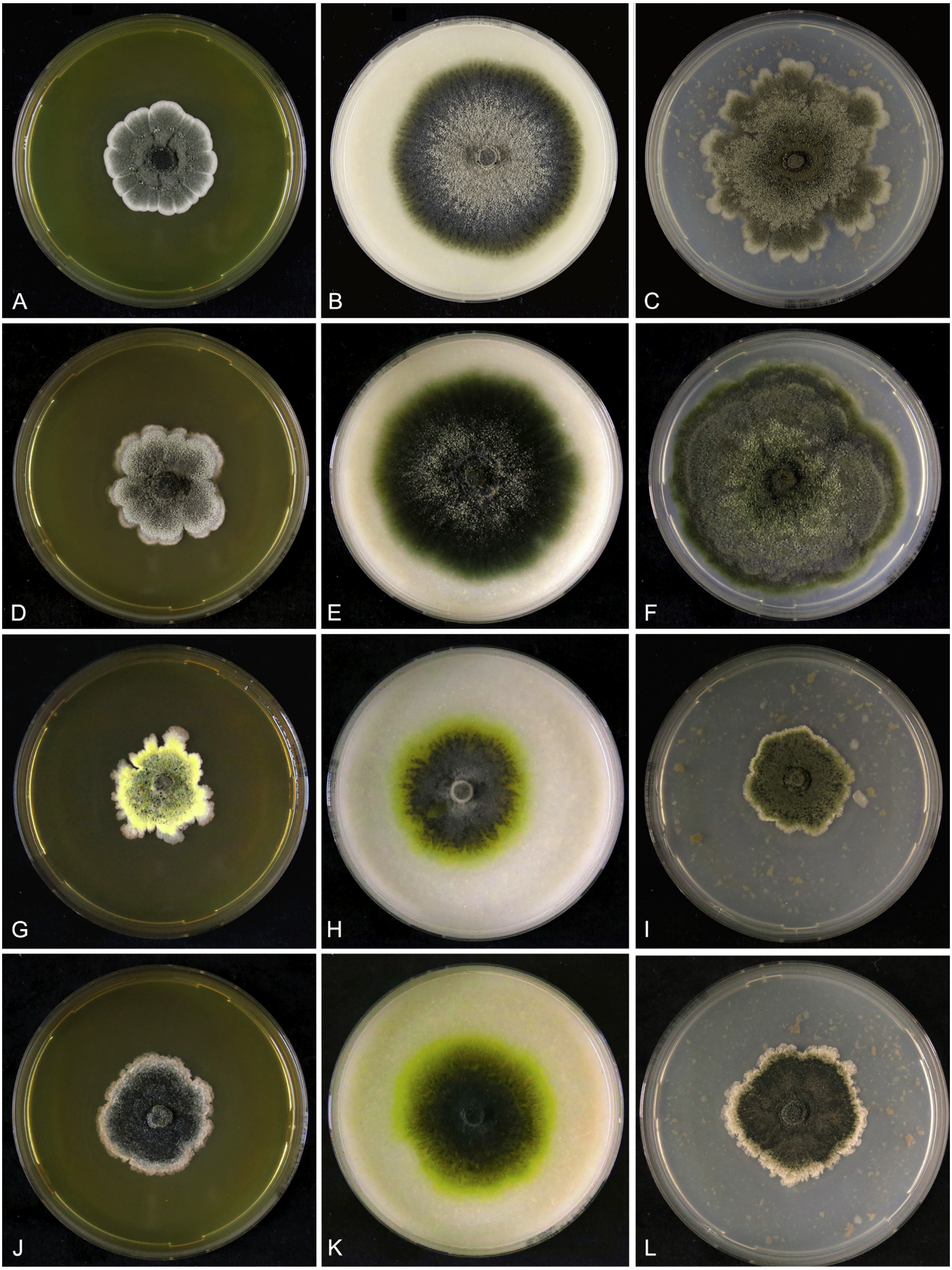

Fig. 2. Cultural characteristics of *Phyllosticta* species collected from citrus in Europe after 7 d at 27 °C on MEA, OA and PDA (respectively in 1st, 2nd and 3rd column). **A–C.** *P. paracapitalensis*. **D–F.** *P. capitalensis*. **G–I.** *P. paracitricarpa*. **J–L.** *P. citricarpa*.

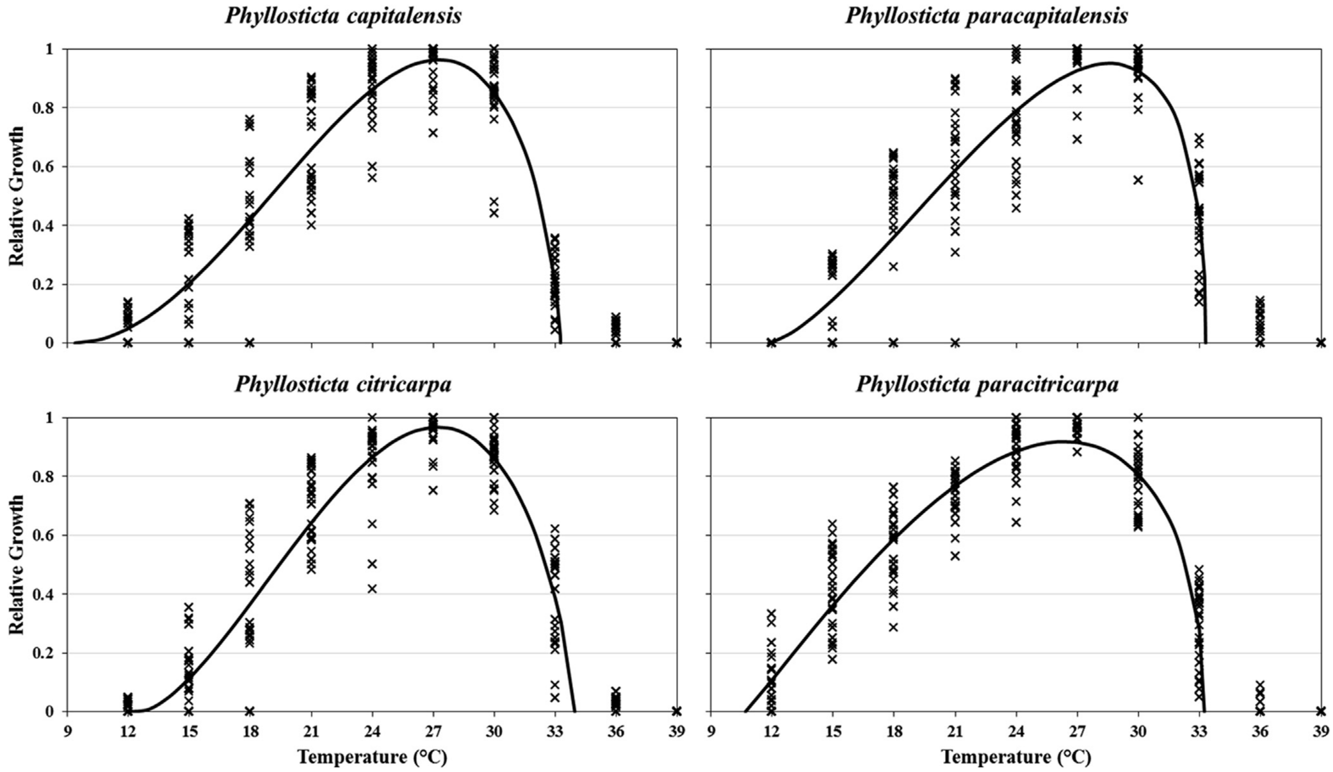

Fig. 3. Relative growth (0 to 1 scale) values on MEA, OA and PDA of *Phyllosticta* species collected in this study as influenced by incubation temperatures of 9–39 °C as fitted to a BETE function [$Y = (a \times ((X - T_{min})/(T_{max} - T_{minx}))^b \times (1-((X - T_{min})/(T_{max} - T_{minx}))^c]$ with parameter values of a, T_{min}, T_{max}, b, c, and goodness of fit for *P. capitalensis* (8.942, 9.357, 33.261, 2.988, 0.665, R^2 = 0.835), *P. paracapitalensis* (9.715, 11.820, 33.310, 3.551, 0.408, R^2 = 0.806), *P. citricarpa* (6.932, 12.541, 33.962, 2.179, 0.749, R^2 = 0.866) and *P. paracitricarpa* (6.281, 10.687, 33.247, 2.283, 0.471, R^2 = 0.873).

ostioles are larger and the conidiogenous cells are longer than *P. paracapitalensis*.

Phyllosticta paracitricarpa Guarnaccia & Crous, **sp. nov.** MycoBank MB817205. Fig. 5.

Etymology: Named after its close morphological resemblance and phylogenetic relationship to *P. citricarpa*.

Conidiomata (on PNA) pycnidial, solitary, black, erumpent, globose, exuding colourless conidial masses; pycnidia up to 250 µm diam, elongated in culture on PNA; pycnidial wall of several layers of *textura angularis*, 20–30 µm thick; inner wall of hyaline *textura angularis*. *Ostiole* central, up to 10 µm diam. *Conidiophores* subcylindrical to ampulliform, reduced to conidiogenous cells, or with 1–2 supporting cell, that can be branched at the base, 15–25 × 4–5 µm. *Conidiogenous cells* terminal, subcylindrical, hyaline, smooth, coated in a mucoid layer, 12–17 × 3–4 µm; proliferating several times percurrently near apex. *Conidia* (9–)11–13(–15) × 7–8(–9) µm, solitary, hyaline, aseptate, thin and smooth-walled, granular, or with a single large central guttule, ellipsoid to obovoid, tapering towards a narrow truncate base, 3–4 µm diam, enclosed in a thin persistent mucoid sheath, 1–1.5 µm thick, and bearing a hyaline, apical mucoid appendage, (8–)10–12(–15) × 1.5(–2) µm, flexible, unbranched, tapering towards an acutely rounded tip.

Culture characteristics: Colonies on MEA flat, with irregular edge; surface initially yellow becoming leaden grey in the centre, yellow at margin, and leaden grey underneath. On PDA colonies were flat, rather regular and slow growing, initially white-grey mycelium, gradually becoming greenish to dark green, with white hyphae at the margin; reverse black. On OA flat, spreading, olivaceous grey, becoming pale dark grey towards the margin, with sparse to moderate aerial mycelium; surrounded by a diffuse yellow pigment in the agar medium. After 12 d in the dark the optimum growth was observed at 27 °C on MEA, OA and PDA (33, 53 and 41 mm). No growth was observed at 9 °C and 39 °C.

Specimen examined: **Greece**, Mastro, on leaf litter of *Citrus limon*, 6 May 2015, V. Guarnaccia (**holotype** CBS H-22664, culture ex-type CPC 27169 = CBS 141357).

Notes: *Phyllosticta paracitricarpa* was isolated from *Citrus limon* leaf litter in Europe (this study) and from lesions on *C. sinensis* fruits in China (Wang *et al.* 2012). This species is similar to *P. citricarpa*, its sister species, but represents a distinct taxon, based on phylogenetic analyses and morphological differences. *Phyllosticta paracitricarpa* differs from *P. citricarpa* in having longer and slightly narrower conidiophores, larger conidiogenous cells and conidia. *Phyllosticta paracitricarpa* colonies on MEA appear yellow becoming leaden-grey in the centre, and yellow at the margin, different from *P. citricarpa* colonies that are olivaceous grey.

Mating type identification of *P. citricarpa*

The *Phyllosticta* mating type primer sets were successful in amplifying the respective portions of the MAT1-1-1 or the MAT1-2-1 idiomorphs of the 21 *P. citricarpa* isolates tested (Table 2). The primer pair MAT111F3–MAT111R3 amplified a fragment of approximately 606 bp in eight isolates, and the primer pair MAT121F6–MAT121R6 amplified 692-bp-fragments in the remaining 13 isolates.

Table 5. Morphological characteristics of *Phyllosticta* spp. associated with citrus.

Species	Ascomata		Asci		Ascospores		Conidiomata		Conidiogenous cells		Conidia		Spermatia		Reference
	Size (μm)	Shape	Size (μm)	Shape	Size (μm)	Shape	Size (μm)	Shape	Size (μm)	Shape	Size (μm)	Shape	Size (μm)	Shape	
P. capitalensis	250	globose to pyriform	58–80 × 11–15	clavate	15–17 × 5–6	limoniform	300 × 250	globose to ampulliform	7–10 × 3–5	subcylindrical to ampulliform to doliiform	(10–)11–12(–14) × (5–)6–7	ellipsoid to obovoid	–	–	Hennings (1908)
P. citriasiana	–	–	–	–	–	–	120–240 × 125–225	globose, subglobose to ellipsoidal	7–17 × 3–5	subcylindrical to ampulliform or doliiform	(10–)12–14(–16) × (5–)6–7(–8)	ellipsoid to obovoid	3–5 × 1–2	bacilliform to ellipsoid	Wulandari et al. (2009)
P. citribraziliensis	–	–	–	–	–	–	250	globose	7–20 × 3–4	subcylindrical to doliiform	10–12 × 6–7	ellipsoid to obovoid	–	–	Glienke et al. (2011)
P. citricarpa	–	–	–	–	–	–	250	globose to ampulliform	7–12 × 3–4	subcylindrical to doliiform	(10–)11–12(–14) × (–)7(–8)	ellipsoid to obovoid	–	–	Van der Aa (1973)
P. citrichinaensis	100–300 × 100–200	subglobose to pyriform	42–81 × 10–14	subclavate to cylindrical	14–20 × 7–8	fusiform to ellipsoidal	100–200 × 100–200	globose or subglobose	6–12 × 2–5	lageniform	(7–)8–12(–13) × 6–9	ellipsoid to obovoid	7–9 × 1–2	bacilliform	Wang et al. (2012)
P. citrimaxima	–	–	–	–	–	–	150–160 × 120–130	globose	3–5 × 1–2	cylindrical	5(–8) × (3–)4(–7)	ellipsoid	–	–	Wikee et al. (2013a, b)
P. paracapitalensis	up to 300	globose	40–75 × 10–12	subcylindrical to clavate	16–17 × 6 (–7)	limoniform	up to 250	globose	7–15 × 3–4	subcylindrical	(9–)12–13(–14) × (6–)7	ellipsoid to obovoid	–	–	This study
P. paracitricarpa	–	–	–	–	–	–	250	globose	12–17 × 3–4	subcylindrical	(9–)11–13(–15) × 7–8(–9)	ellipsoid to obovoid	–	–	This study

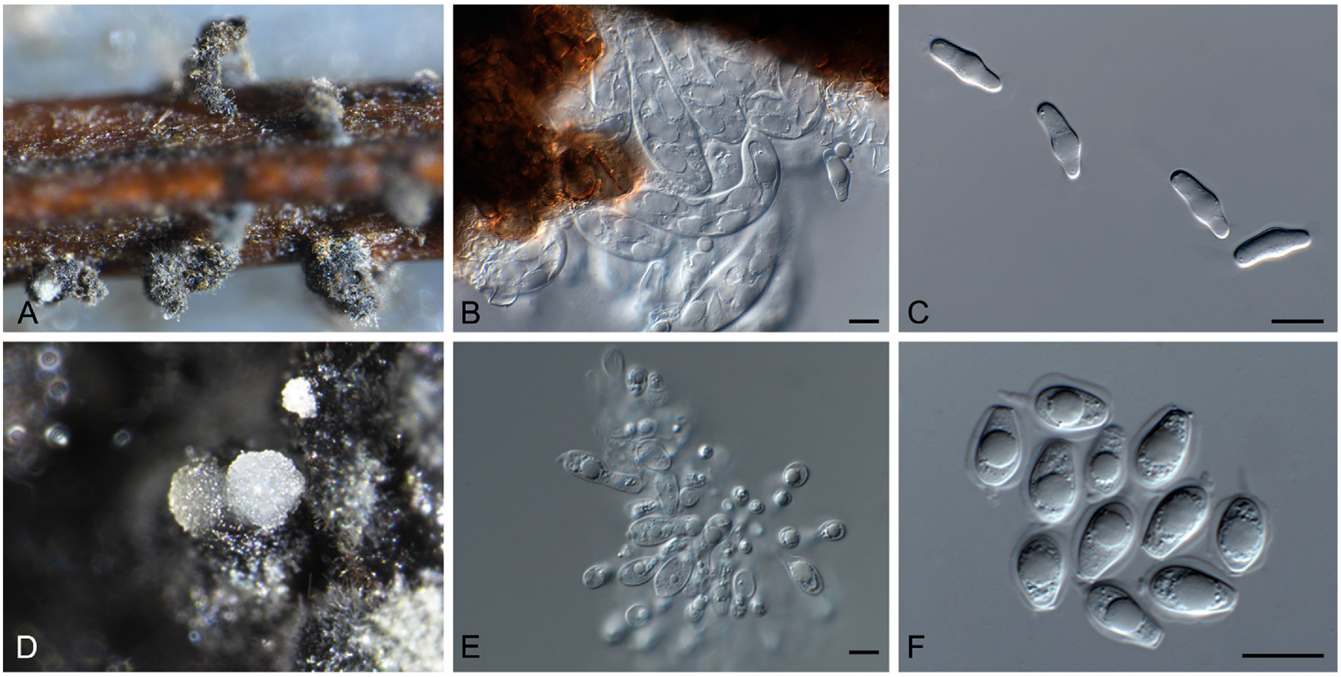

Fig. 4. *Phyllosticta paracapitalensis* (CBS 141353). **A.** Ascomata forming on PNA. **B.** Asci with ascospores. **C.** Ascospores. **D.** Conidiomata forming on SNA. **E.** Conidiogenous cells giving rise to conidia. **F.** Conidia with mucoid sheaths and apical appendages. Scale bars = 10 µm.

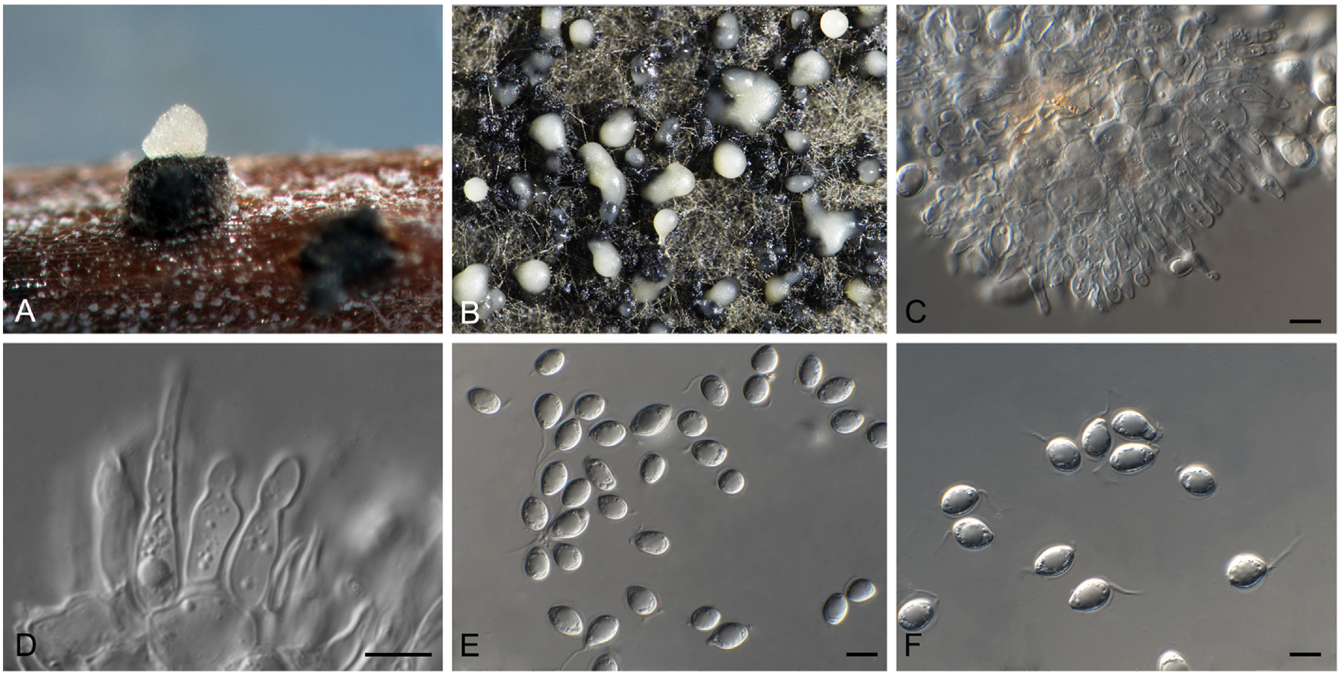

Fig. 5. *Phyllosticta paracitricarpa* (CBS 141357). **A, B.** Conidiomata forming on PNA. **C, D.** Conidiogenous cells giving rise to conidia. **E, F.** Conidia with mucoid sheaths and apical appendages. Scale bars = 10 µm.

Genotyping of *P. citricarpa* isolates

The 20 *P. citricarpa* isolates from four localities in three countries (Malta, Italy and Portugal) were regarded as four "putative" populations (due to the low number of isolates obtained and the sampling strategy employed) and were genotyped with the 15 SSR markers. Among the 20 isolates that were analysed, only two MLGs were identified. The two populations from Malta and the population from Italy shared a single MLG; the other MLG was identified in the population from Portugal. None of the 15 SSR markers were polymorphic in the populations from Italy, Malta and Portugal and therefore indicated very low gene diversity in the populations (0.000; results not shown). The population from Portugal shared its single MLG with all three populations from Australia, while the populations from Italy and Malta shared one MLG, which was not shared with any of the populations from Australia, Brazil, China, Portugal, South Africa or the USA. For the AMOVA analyses, the data from the three populations from Italy and Malta were combined as one population (Italy+Malta) as these three populations shared one MLG. Pairwise *PhiPT* values (Table 6) indicated that the Portugal population was genetically significantly ($P \leq 0.05$) differentiated from the China (*PhiPT* = 0.634; $P = 0.001$), Italy+Malta (*PhiPT* = 1.000; $P = 0.001$), South Africa

Table 6. Pairwise *PhiPT* values (below the diagonal) averaged over 15 microsatellite loci of *Phyllosticta citricarpa* populations from Australia, Brazil, China, Italy+Malta, Portugal, South Africa and the United States. Significance *P*-values are indicated above the diagonal.

	Australia	Brazil	China	Italy + Malta	Portugal	South Africa	USA
Australia	–	0.011	0.001	0.001	0.418	0.001	0.422
Brazil	0.097	–	0.001	0.043	0.155	0.313	0.342
China	0.649	0.659	–	0.002	0.001	0.001	0.001
Italy + Malta	0.258	0.483	0.651	–	0.001	0.002	0.001
Portugal	0.000	0.322	0.634	1.000	–	0.002	0.001
South Africa	0.165	0.013	0.700	0.365	0.311	–	0.452
USA	0.000	0.013	0.674	1.000	1.000	0.000	–

(*PhiPT* = 0.311; *P* = 0.002), and the USA (*PhiPT* = 1.000; *P* = 0.001) populations. The Portugal population was not significantly differentiated from the Australia population (*PhiPT* = 0.000; *P* = 0.418), and also not from the Brazil population (*PhiPT* = 0.322; *P* = 0.155). The Italy+Malta population was significantly ($P \leq 0.05$) differentiated from the Australia (*PhiPT* = 0.258; *P* = 0.001), China (*PhiPT* = 0.651; *P* = 0.002), South Africa (*PhiPT* = 0.365; *P* = 0.002), Brazil (*PhiPT* = 0.483; *P* = 0.043), the USA (*PhiPT* = 1.000; *P* = 0.001) and Portugal (*PhiPT* = 1.000; *P* = 0.001) populations.

Pathogenicity

After 25 d, some inoculation points (approx. 75 %) showed atypical lesions. The lesions developed only on fruits inoculated with *P. citricarpa* (CPC 27909, CPC 27913) and *P. paracitricarpa* isolates (CPC 27169, CPC 27170). No lesions were observed on fruits inoculated with *P. capitalensis* (CPC 27825, CPC 27917), *P. paracapitalensis* (CPC 26517, CPC 26700) (Fig. 6), or on control fruits (not shown). The lesions caused by *P. citricarpa* and *P. paracitricarpa* were similar (Fig. 6). The latter species were consistently re-isolated from the fruit lesions, albeit from lesions atypical of the CBS disease, and identified by sequencing and comparing the loci *tef1* and LSU.

DISCUSSION

Phylogenetic studies published on the genus *Phyllosticta* in recent years have substantially reshaped its taxonomy (Glienke *et al.* 2011, Wang *et al.* 2012, Wikee *et al.* 2013a). The present study represents the first results of fresh collections of several *Phyllosticta* isolates and species associated with citrus in Europe, and the first DNA sequence analyses of strains from almost all continents.

Phyllosticta capitalensis has been recorded worldwide as a common endophyte of diverse host plants (Baayen *et al.* 2002). *Phyllosticta citricarpa* is confined to *Citrus* species on which it causes CBS in summer rainfall citrus growing areas in several countries. Despite the fact that these two species are morphologically distinct, their identification has often been confused (Everett & Rees-George 2006). Conidia of *P. citricarpa* (11–12 × 7 µm) are similar to those of *P. capitalensis* (11–12 × 6–7 µm), but have a thinner mucoid sheath. Moreover, *P. citricarpa* strains produce a distinct yellow pigment on OA, and are slower growing than *P. capitalensis*. The most recent studies focussing on the taxonomy of *Phyllosticta* species showed the occurrence of additional species associated with *Citrus*. Glienke *et al.* (2011) described *P. citribraziliensis* from healthy leaves. An additional three species were reported as *Citrus* pathogens in Asia: *P. citriasiana* and *P. citrimaxima* cause Citrus Tan Spot on pomelo fruits (Wulandari *et al.* 2009, Wikee *et al.* 2013a) and *P. citrichinaensis* causes a brown spot and red-brown protuberant freckle on citrus leaves and fruits (Wang *et al.* 2012).

Citrus Black Spot and symptoms similar to that caused by *P. citricarpa*, *P. citriasiana*, *P. citrimaxima* and *P. citrichinaensis* have never been reported in citrus-producing European countries (European Union 1998, Kotzé 2000). Climatic conditions play a primary role in the ability of *P. citricarpa* to establish and to cause CBS disease, most notably warm summer rainfall conditions that would allow spore production, dissemination and infection during periods of fruit susceptibility (Kiely 1948a, b, Kotzé 1963, 1981, McOnie 1967, 1964, Huang & Chang 1972, Lee & Huang 1973, Noronha 2002, Fourie *et al.* 2013, Yonow *et al.* 2013, Magarey *et al.* 2015).

Given the long history of trade in citrus propagation material between Europe and Asia, where CBS is endemic and also regarded as the centre of origin of citrus, (Ramón-Laca 2003, Mabberley 2004, Nicolosi 2007), and the potential for illegal movement of plant propagating material, the likely coincidental spread of citrus-specific *Phyllosticta* species to Europe could reasonably be expected. To investigate this possibility, several surveys were carried out during this study, resulting in the collection of 64 *Phyllosticta* isolates. A subset of 52 European isolates were compared to several reference isolates using partial gene sequences of six different loci, as well as morphological characteristics. Based on a comparison with sequences retrieved from GenBank of an additional 43 strains (Glienke *et al.* 2011, Wang *et al.* 2012, Wikee *et al.* 2013a), four distinct *Phyllosticta* species, including two new species, were delineated from several *Citrus* species growing in five European countries.

The distribution of the *Phyllosticta* species isolated in this study varied in terms of host and tissue type from which they were recovered. *Phyllosticta capitalensis* was recovered in all countries sampled and *P. paracapitalensis* in Italy and Spain only. Both species were isolated from asymptomatic leaves. *Phyllosticta citricarpa* and *P. paracitricarpa* were isolated from leaf litter only. *Phyllosticta citricarpa* was found in Italy, Malta and Portugal, whereas *P. paracitricarpa* was isolated only from samples collected in Greece. *Phyllosticta capitalensis* was associated with *P. paracapitalensis* in the same specimens

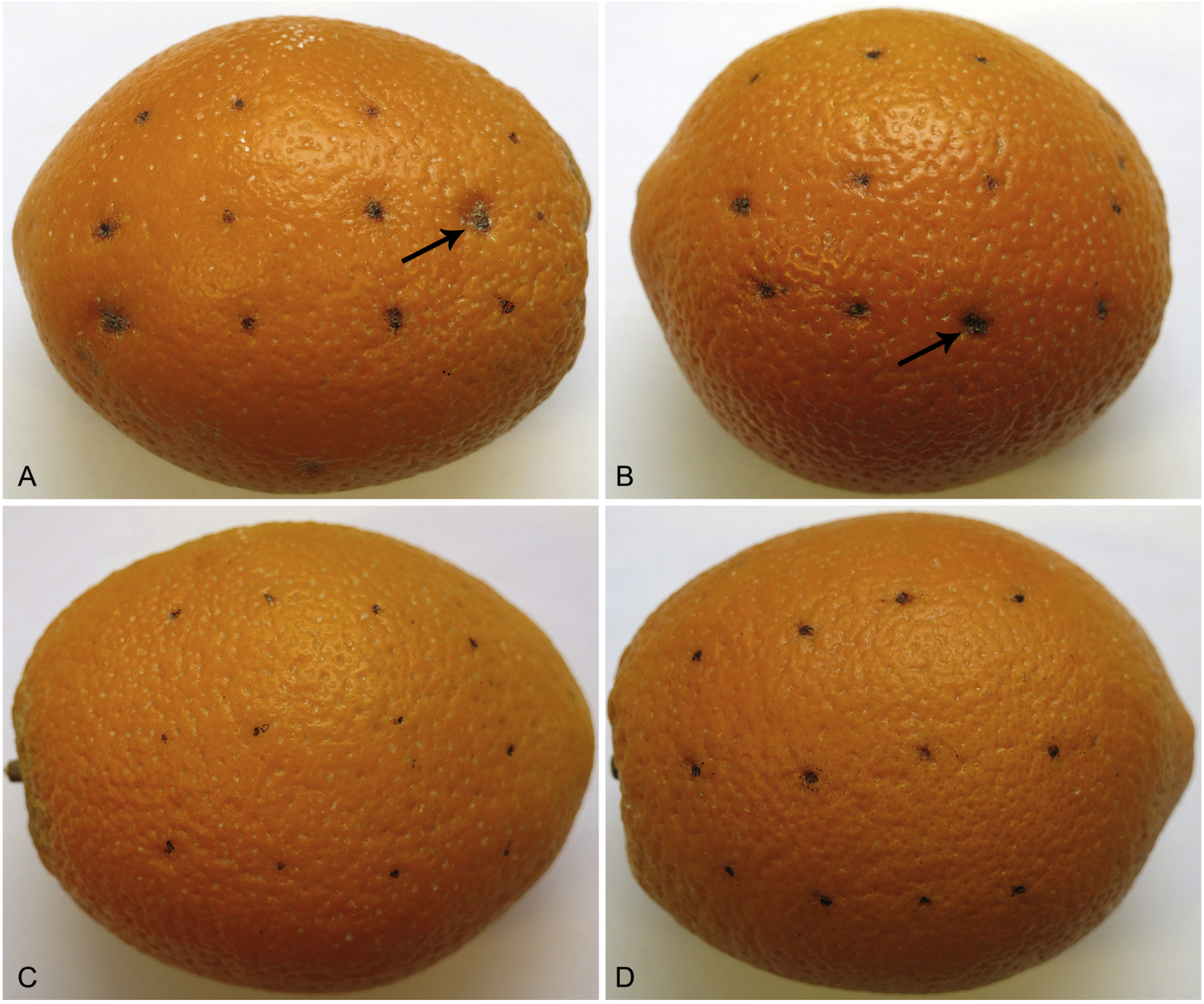

Fig. 6. Fruit of *Citrus sinensis* ('Valencia') artificially inoculated with *Phyllosticta* spp. **A.** Lesions caused by *P. citricarpa*. **B.** Lesions caused by *P. paracitricarpa*. **C, D.** No symptoms were observed on fruits inoculated with *P. capitalensis* and *P. paracapitalensis*.

collected in Spain, but in this survey *P. citricarpa* and *P. paracitricarpa* were not found associated with *P. capitalensis*.

Wang *et al.* (2012) reported two sub-clades (I and II) of *P. citricarpa* associated with *Citrus* spp. in China by comparison of ITS, *actA* and *tef1* sequences data. In this study, we used partial regions of an additional three loci, and fixed nucleotide differences were observed within the *tef1* and LSU regions, supporting the splitting of the "*P. citricarpa*" clade in two taxa: *P. citricarpa s.str*. and the new species *P. paracitricarpa*. Moreover, this study establishes the presence of *P. paracitricarpa* only in Asia and Europe and represents the first report of *P. citricarpa* in Europe. *Phyllosticta paracitricarpa* was isolated from fruit lesions in China and caused lesions on citrus fruit in the pathogenicity test performed in this study. Further surveys and research is required to determine the importance of *P. paracitricarpa* as a citrus pathogen.

The origin of *P. citricarpa* in Europe is not clear at present. On a genotypic level, the *P. citricarpa* populations from Italy+Malta and Portugal represented two respective clones, differing from each other in both their MLGs and mating types. These populations further differed from one another in their degree of connectivity and differentiation from the other populations from Australia, Brazil, China, South Africa and the USA. Analysis of molecular variance showed that populations from Portugal and Australia are more strongly connected to each other than to other populations. Interestingly, "Lisbon" lemon was introduced into Australia from Portugal in 1824 (Morton 1987), while CBS was first described in Australia in 1895 (Benson 1895). Very little connectivity was evident between the Portuguese population and those from the other continents, including the population from Italy+Malta. Also, the Italy+Malta population seemed to be distinct from the other populations. These findings suggest two separate introductions into Europe. However, in order to determine whether there were other introductions of *P. citricarpa* into Europe and to infer the origin of these introductions, additional populations from Europe, Asia and the Oceania countries need to be studied. The description of *P. paracitricarpa* from Greece and China suggests connectivity in this species with Asia.

No evidence of CBS disease in European citrus trees was observed in this study. The *P. citricarpa* isolates were found in leaf litter of old *C. limon* and *C. sinensis* trees (20 to 60 years old) that were situated in gardens, and not found in any of the commercial orchards or nurseries surveyed. Fruit is not considered a pathway for spread (USDA APHIS 2010) and evidence that might suggest a fruit pathway (such as nearby compost heap, waste disposal or processing plants; Baker *et al.* 2014) was not observed. Movement of infected plant material is regarded as the most likely means of long-distance spread of

P. citricarpa (Kiely 1948b, Kotzé 1981). Whilst import of citrus plants for planting is presently not permitted, unless it is plant propagation material that is handled through appropriate quarantine procedures, the introduction of *P. citricarpa* found in Portugal, Malta and Italy therefore most likely occurred via the introduction of plants many years ago or via illegal movement of such plants.

Phyllosticta citricarpa was found at very low frequency only in a few of the sites investigated, while *P. paracitricarpa* was found only at one site in Greece. CBS disease symptoms were never observed. Our results indicate that the presence of *P. citricarpa* and *P. paracitricarpa* is not associated with disease under European climatic conditions.

Twenty-three *P. capitalensis* strains were isolated as endophyte from leaves of four *Citrus* species collected. This taxon can occur in fruit or leaf lesions caused by other fungi or insects (Wikee *et al.* 2013b). Indeed, in this study, *P. capitalensis* was found associated with leaf lesions (caused by insects) of the ornamental *C. medica* var. *sarcodactylis.* Wikee *et al.* (2013a) indicated that the phylogeny of *Phyllosticta* derived from the ITS and *actA* genomic loci is sufficiently robust to differentiate most taxa, except those closely related to *P. capitalensis*. In our study, sequences of a partial region of *rpb2* of *Phyllosticta* spp. helped to resolve differences in nucleotides within *P. capitalensis*. Moreover, fixed nucleotide differences were observed in *tef1*, demonstrating the separation of the new species *P. paracapitalensis* with highly supported independent lineages in the phylogenetic tree. *Phyllosticta paracapitalensis* was isolated as endophyte from commercial orchards of *C. limon* in Spain and from *C. floridana* cultivated in ornamental plant nurseries in Italy. One strain (CBS 173.77) isolated from *C. aurantiifolia* in New Zealand during February 1974, previously identified as *P. capitalensis*, grouped with the European isolates of *P. paracapitalensis* in the present phylogenetic analyses. Further studies must be conducted on a wider global selection of strains to clarify its host association and distribution.

Morphological characteristics of isolates grown on several media were consistent with those already reported in literature (Baayen *et al.* 2002, Glienke *et al.* 2011, Wikee *et al.* 2013a). Optimal temperatures for *P. citricarpa* (27.2 °C) and *P. capitalensis* (27.3 °C) predicted from the BETE function fitted to the relative growth data were similar to those reported by previous studies (Kotzé 1981, Er *et al.* 2014), but cardinal temperatures were more contracted with T_{min} of (12.5 and 9.4 °C, respectively). Optimal temperatures for *P. paracitricarpa* and *P. paracapitalensis* were lower (26.4 °C) and higher (28.6 °C), respectively. Growth rates of *P. capitalensis* and *P. paracapitalensis* were similar and significantly faster than those of *P. citricarpa* and *P. paracitricarpa*.

Results of this study showed that two (*P. citricarpa* and *P. paracitricarpa*) of the four species isolated from specimens collected in Europe induced atypical lesions (necrosis) in artificially inoculated mature sweet orange fruit and could be re-isolated from these lesions, while *P. capitalensis* and *P. paracapitalensis* induced no lesions. From this assay, it appears that *P. paracapitalensis* is similar to *P. capitalensis*, demonstrating them to have similar ecologies, occurring as asymptomatic endophytes in citrus tissue. Considering that mature citrus fruit are resistant to *P. citricarpa* infection under field conditions (Kiely 1948b, Schutte *et al.* 2003, 2012, Miles *et al.* 2004), and since the harsh artificial inoculation technique used in the pathogenicity assay did not resemble natural field infection (*i.e.* direct penetration of unwounded tissue following long wetness periods; Kotzé 1963, McOnie 1967, Noronha 2002) these findings should be regarded as preliminary. *Phyllosticta paracitricarpa* caused similar lesions to those caused by *P. citricarpa* in this assay and appears to be pathogenic, which is supported by its isolation from lesions on fruit in China, but further surveys are required to elucidate.

Including the two taxa newly described in this study, eight *Phyllosticta* species are now associated with citrus: *P. citricarpa* and *P. capitalensis* are present on all continents where citrus is cultivated, *P. paracapitalensis* is reported in Europe and New Zealand, while *P. paracitricarpa* is present in Asia and Europe. As previously published by several authors, the pathogenic *P. citrichinaensis*, *P. citriasiana* and *P. citrimaxima* are present only in Asia, and the endophyte *P. citribraziliensis* has been isolated only in South America (Wulandari *et al.* 2009, Glienke *et al.* 2011, Wang *et al.* 2012, Wikee *et al.* 2013a). The presence in Europe of both *P. citricarpa* and *P. paracitricarpa* was not associated with any visible signs of infection; indeed, neither CBS or Citrus Tan Spot have ever been reported or observed during the extensive surveys performed in the present study.

Recent studies performed in Florida, USA (Zhang *et al.* 2015, Wang *et al.* 2016), supported the heterotallism of *P. citricarpa*, finding only MAT1-2-1 isolates present in Florida (based on 113 isolates) while 26 strains from Australia displayed an equal ratio of the two mating types. Amorim *et al.* (2017) recently showed that in Brazil the two idiomorphs occur in a 1:1 ratio. Furthermore, Tran *et al.* (2017) reported for the first time the successful mating *in vitro* of *P. citricarpa*, confirming that this species is heterothallic and requires isolates of different MAT idiomorphs to be in direct physical contact for mating and production of sexual fruiting bodies. We found both MAT1-1-1 and MAT1-2-1 isolates present in Europe, but both mating types were not recovered together in the same country, indicating separate introductions that have not spread and remained isolated. A broader sampling is required, however, to determine whether this holds up when a larger population per area is sampled.

This study contributed significantly towards our understanding of the genotypic variation in *P. capitalensis* and *P. citricarpa*, splitting both groups into different taxa, and clearly showing that a multi-locus approach works well for distinguishing these species. The use of a three-gene analysis (ITS, *actA*, *tef1*) performed in a previous study (Wang *et al.* 2012) showed two poorly supported subclades within *P. citricarpa*. We used a further three genomic loci (*gapdh*, LSU and *rpb2*) to confirm that the two subclades actually represent two distinct species.

In this study we established the presence of *P. citricarpa* and the similar new species, *P. paracitricarpa*, for the first time in Europe. In spite of the occurrence of these species, neither was associated with disease symptoms, evidently because of unfavourable climatic conditions (Yonow *et al.* 2013, Magarey *et al.* 2015). Whilst it appears that these fungi were introduced with plant material many years ago, they apparently persist under these unfavourable conditions, most likely endophytically, and possibly through asexual reproduction. The latter hypothesis is supported by the finding that only one mating type was found per locality, and that some *P. citricarpa* pycnidiospore infection events were predicted to occur in these regions (Magarey *et al.* 2015). The number of suitable infection periods was, however, markedly fewer than those for regions where *P. citricarpa* causes CBS disease. Magarey *et al.* (2015) doubted the ability of *P. citricarpa* to persist and cause disease at a location where there is a low frequency of suitable seasons. Likewise, the climate suitability modelling conducted by Paul *et al.* (2005) and Yonow *et al.* (2013), indicated climatic unsuitability across the EU, with the exception of a few isolated areas around the Mediterranean Sea, where marginally suitable climatic conditions can be found. All these climate modelling studies were calibrated for climate suitability according to the presence, absence, distribution and severity of CBS disease, and not the potential presence of the fungus in the absence of disease. The findings from our study indicate that *P. citricarpa* was able to persist but did not induce CBS symptoms or spread, considering that it was found in only a few of the sites investigated and at very low frequency.

ACKNOWLEDGEMENTS

The authors are grateful to Arien van Iperen (cultures), Marjan Vermaas (photo plates) and Mieke Starink-Willemse (DNA isolation, amplification, and sequencing) for their technical assistance, to Ariena van Bruggen, (University of Florida) for sharing some strains, and to Tian Schutte for sharing his experience in surveying for CBS disease symptoms.

REFERENCES

Aa HA van der (1973). Studies in *Phyllosticta* I. *Studies in Mycology* **5**: 1–110.
Aa HA van der, Vanev S (2002). *A revision of the species described in Phyllosticta*. Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands.
Aiello D, Carrieri R, Guarnaccia V, *et al.* (2015). Characterization and pathogenicity of *Colletotrichum gloeosporioides* and *C. karstii* causing preharvest disease on *Citrus sinensis* in Italy. *Journal of Phytopathology* **163**: 168–177.
Amorim R, Savi DC, Ferreira-Maba L, *et al.* (2017). *MAT* gene idiomorphs suggest a heterothallic sexual cycle in the citrus pathogen *Phyllosticta citricarpa*. *European Journal of Plant Pathology* **147**: 325–337.
Analytis S (1977). Uber die relation zwischen biologischer entwicklung und temperatur bei phytopathogenen pilzen. *Phytopathologische Zeitschrift* **90**: 64–76.
Baayen R, Bonants P, Verkley GJM, *et al.* (2002). Nonpathogenic isolates of the citrus black spot fungus, *Guignardia citricarpa*, identified as a cosmopolitan endophyte of woody plants, *G. mangiferae* (*Phyllosticta capitalensis*). *Phytopathology* **92**: 464–477.
Baker R, Bragard C, Candresse T, *et al.* (2014). Scientific Opinion on the risk of *Phyllosticta citricarpa* (*Guignardia citricarpa*) for the EU territory with identification and evaluation of risk reduction options. *EFSA Journal* **12**: 3557.
Baldassari RB, Wickert E, de Goes A (2008). Pathogenicity, colony morphology and diversity of isolates of *Guignardia citricarpa* and *G. mangiferae* isolated from *Citrus* spp. *European Journal of Plant Pathology* **120**: 103–110.
Barr ME (1970). Some amerosporous ascomycetes on *Ericaceae* and *Empetraceae*. *Mycologia* **62**: 377–394.
Barr ME (1972). Preliminary studies on the *Dothideales* in temperate North America. *Contributions from the University of Michigan Herbarium* **9**: 523–638.
Bellotte JAM, Kupper KC, Rinaldo D, *et al.* (2009). Acceleration of the decomposition of Sicilian lemon leaves as an auxiliary measure in the control of citrus black spot. *Tropical Plant Pathology* **34**: 71–76.
Benson AH (1895). Black spot of the orange. *The Agricultural Gazette of New South Wales* **4**: 249–252.
Bezerra JPD, Santos MGS, Svedese VM, *et al.* (2012). Richness of endophytic fungi isolated from *Opuntia ficus-indica* Mill. (*Cactaceae*) and preliminary screening for enzyme production. *World Journal Microbiology Biotechnology* **28**: 1989–1995.
Bissett J (1986). *Discochora yuccae* sp. nov. with *Phyllosticta* and *Leptodothiorella* synanamorphs. *Canadian Journal of Botany* **64**: 1720–1726.
Brentu FC, Oduro KA, Offei SK, *et al.* (2012). Crop loss, etiology, and epidemiology of citrus black spot in Ghana. *European Journal of Plant Pathology* **133**: 657–670.
Broadbent P (1995). Quarantine in relation to Australian citrus imports and exports. *Australasian Plant Pathology* **24**: 145–156.
Brodrick HT (1969). *Physiological studies with Guignardia citricarpa Kiely*. D.Sc. Thesis. Department of Microbiology and Plant Pathology, University of Pretoria.
Calavan EC (1960). Black spot of citrus. *Citrus Grower* **323**: 11–15.
Carbone I, Kohn LM (1999). A method for designing primer sets for the speciation studies in filamentous ascomycetes. *Mycologia* **91**: 553–556.
Carstens E, Linde CC, Slabbert R, *et al.* (2017). A global perspective on the population structure and reproductive system of *Phyllosticta citricarpa*. *Phytopathology*. http://dx.doi.org/10.1094/PHYTO-08-16-0292-R.
Crous PW, Gams W, Stalpers JA, *et al.* (2004). MycoBank: an online initiative to launch mycology into the 21st century. *Studies in Mycology* **50**: 19–22.
Crous PW, Slippers B, Wingfield MJ, *et al.* (2006). Phylogenetic lineages in the *Botryosphaeriaceae*. *Studies in Mycology* **55**: 235–253.
Crous PW, Summerell BA, Shivas RG, *et al.* (2012). Fungal Planet description sheets: 107–127. *Persoonia* **28**: 138–182.
Crous PW, Verkley GJM, Groenewald JZ, *et al.* (eds) (2009). *Fungal Biodiversity. CBS Laboratory Manual Series 1*. Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands.
De Hoog GS, Gerrits van den Ende AHG (1998). Molecular diagnostics of clinical strains of filamentous basidiomycetes. *Mycoses* **41**: 183–189.
Dewdney MM, Schubert TS, Estes MR, *et al.* (2012). *Florida citrus pest management guide: Citrus Black Spot*. University of Florida. IFAS Extension PP279.
Doidge EM (1929). Some diseases of *Citrus* prevalent in South Africa. *South African Journal of Science* **26**: 324.
Donk MA (1968). Report of the committee for Fungi and Lichen 1964–1968. *Taxon* **17**: 578–581.
Dummel DM, Agostini JP, Moschini R (2015). Predictive model for ascospore release of *Guignardia citricarpa* using climatological data. Proceedings of the XIIth International Citrus Congress, Valencia, Spain, B. Sabater-Munoz *et al. Acta Horticulturae* **1065**: 953–963.
EC 2000/29/EC (2000). Council Directive on protective measures against the introduction into the Community of organisms harmful to plants and plant products and against their spread within the Community. *Official Journal of the European Communities L* **169**: 1–112.
Er HL, Hendricks K, Goss EM, *et al.* (2014). Isolation and biological characterization of *Guignardia* species from citrus in Florida. *Journal of Plant Pathology* **96**: 43–55.
European Union (1998). Commission Decision of 8 January 1998 recognizing certain third countries and certain areas of third countries as being free of *Xanthomonas campestris* (all strains pathogenic to Citrus), *Cercospora angolensis* Carv. et Mendes and *Guignardia citricarpa* Kiely (all strains pathogenic to Citrus). *Official Journal of the European Communities* **15**: 41–42.
European Union (2000). *Final report of a mission carried out in Brazil from 3 to 6 July 2000 in order to evaluate the pre-export inspections on citrus fruit originating in Brazil and exported to the European Union.* http://ec.europa.eu/food/fs/inspections/pi/reports/brazil/pi_rep_braz_1180-2000_en.pdf.
Everett KR, Rees-George J (2006). Reclassification of an isolate of *Guignardia citricarpa* from New Zealand as *Guignardia mangiferae* by sequence analysis. *Plant Pathology* **55**: 194–199.
Fourie P, Schutte T, Serfontein S, *et al.* (2013). Modeling the effect of temperature and wetness on *Guignardia* pseudothecium maturation and ascospore release in citrus orchards. *Phytopathology* **103**: 281–292.
Garran SM (1996). Citrus Black spot in the North East of Entre Rios: etiology epidemiology and control. *Proceedings of the International Society of Citriculture*, 466–471.
Glienke C, Pereira O, Stringari D, *et al.* (2011). Endophytic and pathogenic *Phyllosticta* species, with reference to those associated with Citrus Black Spot. *Persoonia* **26**: 47–56.
Glienke-Blanco C, Aguilar-Vildoso CI, Vieira MLC, *et al.* (2002). Genetic variability in the endophytic fungus *Guignardia citricarpa* isolated from citrus plants. *Genetics and Molecular Biology* **25**: 251–255.
Guarnaccia V, Groenewald JZ, Polizzi G, *et al.* (2017). High species diversity in Colletotrichum associated with citrus diseases in Europe. *Persoonia* **39**: 32–50.
Guerber JC, Liu B, Correll JC, *et al.* (2003). Characterization of diversity in *Colletotrichum acutatum sensu lato* by sequence analysis of two gene introns, mtDNA and intron RFLPs, and mating compatibility. *Mycologia* **95**: 872–895.
Hawksworth DL, Crous PW, Redhead SA, *et al.* (2011). The Amsterdam declaration on fungal nomenclature. *IMA Fungus* **2**: 105–112.
Hennings P (1908). Fungi S. Paulenses IV a cl. Puttemans collecti. *Hedwigia* **48**: 1–20.

Hillis DM, Bull JJ (1993). An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Systematic Biology* **42**: 182–192.

Hu J, Johnson EG, Wang NY, *et al*. (2014). qPCR quantification of pathogenic *Guignardia citricarpa* and nonpathogenic *G. mangiferae* in Citrus. *Plant Disease* **98**: 112–120.

Huang CS, Chang SL (1972). Leaf infection with citrus black spot and perithecial development in relation to ascospore discharge of *Guignardia citricarpa* Kiely. *Journal of Taiwan Agricultural Research* **21**: 256–263.

Huang WY, Cai YZ, Hyde KD, *et al*. (2008). Biodiversity of endophytic fungi associated with 29 traditional Chinese medicinal plants. *Fungal Diversity* **33**: 61–75.

Johnston PR (1998). Leaf endophytes of manuka (*Leptospermum scoparium*). *Mycological Research* **102**: 1009–1016.

Katoh K, Standley DM (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution* **30**: 772–780.

Kiely TB (1948a). Preliminary studies on *Guignardia citricarpa* (n. sp.), the ascigerous stage of *Phoma citricarpa* McAlp., and its relation to black spot of citrus. *Proceedings of the Linnean Society of New South Wales* **73**: 249–292.

Kiely TB (1948b). *Guignardia citricarpa* (n.sp.) and its relationship to the black spot disease of Citrus in coastal orchards of New South Wales. *Journal of the Australian Institute of Agriculture Science* **14**: 81–83.

Kiely TB (1949). Black spot of citrus in New South Wales coastal orchards. *The Agricultural Gazette of New South Wales* **60**: 17–20.

Kotzé JM (1963). *Studies on the black spot disease of citrus caused by Guignardia citricarpa Kiely, with particular reference to its epiphytology and control at Letaba*. D.Sc. (Agric.) thesis. University of Pretoria, South Africa.

Kotzé JM (1981). Epidemiology and control of citrus black spot in South Africa. *Plant Disease Reporter* **65**: 945–950.

Kotzé JM (1996). History and epidemiology of citrus black spot in South Africa. *Proceedings of the International Society of Citriculture* **2**: 1296–1299.

Kotzé JM (2000). Black spot. In: *Compendium of Citrus Diseases* (Timmer LW, Garnsey SM, Graham JH, eds), 2nd edn. The American Phytopathological Society, St. Paul, MN, USA: 23–25.

Lee YS, Huang CS (1973). Effect of climatic factors on the development and discharge of ascospores of the citrus black spot fungus. *Journal of Taiwan Agricultural Research* **22**: 135–144.

Leggieri MC, Decontardi S, Bertuzzi T, *et al*. (2017). Modeling growth and toxin production of toxigenic fungi signaled in cheese under different temperature and water activity regimes. *Toxins* **9**: 1–17.

Liu JK, Phookamsak R, Doilom M, *et al*. (2012). Towards a natural classification of *Botryosphaeriales*. *Fungal Diversity* **57**: 149–210.

Liu YJ, Whelen S, Hall BD (1999). Phylogenetic relationships among ascomycetes: evidence from an RNA polymerse II subunit. *Molecular Biology and Evolution* **16**: 1799–1808.

Mabberley DJ (2004). Citrus *(Rutaceae)*: a review of recent advances in etymology, systematics and medical applications. *Blumea* **49**: 481–498.

Magarey RD, Hong SC, Fourie PH, *et al*. (2015). Prediction of *Phyllosticta citricarpa* using an hourly infection model and validation with prevalence data from South Africa and Australia. *Crop Protection* **75**: 104–114.

McOnie KC (1964). Source of inoculum of *Guignardia citricarpa*, the Citrus Black Spot pathogen. *Phytopathology* **54**: 64–67.

McOnie KC (1967). Germination and infection of citrus by ascospores of *Guignardia citricarpa* in relation to control of black spot. *Phytopathology* **57**: 743–746.

Meyer L, Sanders GM, Jacobs R, *et al*. (2006). A one-day sensitive method to detect and distinguish between the citrus black spot pathogen *Guignardia citricarpa* and the endophyte *Guignardia mangiferae*. *Plant Disease* **90**: 97–101.

Miles AK, Tan YP, Tan MK, *et al*. (2013). *Phyllosticta* spp. on cultivated *Citrus* in Australia. *Australasian Plant Pathology* **42**(4): 461–467.

Miles AK, Willingham SL, Cooke AW (2004). Field evaluation of strobilurins and a plant activator for the control of citrus black spot. *Australasian Plant Pathology* **33**: 371–378.

Moncalvo JM, Wang HH, Hseu RS (1995). Phylogenetic relationships in *Ganoderma* inferred from the internal transcribed spacer and 25S ribosomal DNA sequences. *Mycologia* **87**: 223–238.

Morton JF (1987). *Fruits of warm climates*. Creative Resource Systems, Inc., Miami, Florida, USA: 160–168.

Myllys L, Stenroos S, Thell A (2002). New genes for phylogenetic studies of lichenized fungi: glyceraldehyde-3-phosphate dehydrogenase and beta-tubulin genes. *Lichenologist* **34**: 237–246.

Nei M (1973). Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences* **70**: 3321–3323.

Nicolosi E (2007). Origin and taxonomy. In: *Citrus genetics, breeding and biotechnology* (Khan IA, ed). CAB International, Oxfordshire UK: 19–43.

Noronha MDA (2002). *Escala diagramatica para avaliacao da mancha preta em folhas de citros efeito da temperatura e da duracao do molhamento na prepenetracao de conidios de Guignardia citricarpa Kiely [Phyllosticta citricarpa (McAlp.) Van der Aa]*. Agronomy: Concentration of Plant Pathology Masters Dissertation. Universidade de Sao Paulo.

Nylander JAA (2004). *MrModeltest v. 2*. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.

O'Donnell K, Kistler HC, Cigelnik E, *et al*. (1998). Multiple evolutionary origins of the fungus causing Panama disease of banana: concordant evidence from nuclear and mitochondrial gene genealogies. *Proceedings of the National Academy of Sciences of the United States of America* **95**: 2044–2049.

Okane I, Lumyong S, Nakagiri A, *et al*. (2003). Extensive host range of an endophytic fungus, *Guignardia endophyllicola* (anamorph: *Phyllosticta capitalensis*). *Mycoscience* **44**: 353–363.

Okane I, Nakagiri A, Ito T (2001). Identity of *Guignardia* sp. inhabiting ericaceous plants. *Canadian Journal of Botany* **79**: 101–109.

Paul I, van Jaarsveld AS, Korsten L, *et al*. (2005). The potential global geographical distribution of Citrus Black Spot caused by *Guignardia citricarpa* (Kiely): likelihood of disease establishment in the European Union. *Crop Protection* **24**: 297–308.

Peakall R, Smouse PE (2012). GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics* **28**: 2537–2539.

Perryman SAM, Clark SJ, West JS (2014). Splash dispersal of *Phyllosticta citricarpa* conidia from infected citrus fruit. *Scientific Reports* **4**: 6568.

Persoon CH (1818). *Traité sur les champignons comestibles, contenant l'indication des espèces nuisibles; a l'histoire des champignons*. Belin-Leprieur, Paris, France.

Petrak F (1957). Über die Gattungen *Guignardia* Viala & Ravaz und *Discosphaerina* v. Höhnel. *Sydowia* **11**: 435–445.

Pu J, Xie Y, Zhang X, *et al*. (2008). Preinfection behaviour of *Phyllosticta musarum* on banana leaves. *Australasian Plant Pathology* **37**: 60–64.

Rakotoniriana EF, Munaut F, Decock C, *et al*. (2008). Endophytic fungi from leaves of *Centella asiatica*: occurrence and potential interactions within leaves. *Antonie van Leeuwenhoek* **93**: 27–36.

Ramón-Laca L (2003). The introduction of cultivated Citrus to Europe via northern Africa and the Iberian Peninsula. *Economic Botany* **57**: 502–514.

Rayner RW (1970). *A mycological colour chart*. CMI and British Mycological Society, Kew, Surrey, England.

Reis RF, Timmer LW, de Goes A (2006). Effect of temperature, leaf wetness and rainfall on the production of *Guignardia citricarpa* ascospores and on black spot severity on sweet orange. *Fitopatologia Brasileira* **31**: 29–34.

Rodrigues KF, Samuels GJ (1999). Fungal endophytes of *Spondias mombin* leaves in Brazil. *Journal of Basic Microbiology* **39**: 131–135.

Rodrigues KF, Sieber TN, Grünig CR, *et al*. (2004). Characterization of *Guignardia mangiferae* isolated from tropical plants based on morphology, ISSR-PCR amplifications and ITS1-5.8S-ITS2 sequences. *Mycological Research* **108**: 45–52.

Romão AS, Spósito MB, Andreote FD, *et al*. (2011). Enzymatic differences between the endophyte *Guignardia mangiferae* (*Botryosphaeriaceae*) and the citrus pathogen *G. citricarpa*. *Genetics and Molecular Research* **10**: 243–252.

Ronquist F, Teslenko M, van der Mark P, *et al*. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* **61**: 539–542.

Sandoval-Denis M, Guarnaccia V, Polizzi G, *et al*. (2018). Symptomatic Citrus trees reveal a new pathogenic lineage in *Fusarium* and two new *Neocosmospora* species. *Persoonia* **40**: 1–25.

Schoch CL, Shoemaker RA, Seifert KA, *et al*. (2006). A multigene phylogeny of the *Dothideomycetes* using four nuclear loci. *Mycologia* **98**: 1041–1052.

Schubert TS, Dewdney MM, Peres NA, *et al*. (2012). First report of *Guignardia citricarpa* associated with citrus black spot on sweet orange [*Citrus sinensis* (L.) Osbeck] in North America. *Plant Disease* **96**: 1225.

Schubert TS, Sutton B, Jeyaprakash A (2010). Citrus black spot (*Guignardia citricarpa*) discovered in Florida. In: *Pest Alert DACS-P-01723*. Florida Department of Agriculture and Consumer Services, Division of Plant Industry, Gainesville, FL, USA.

Schutte GC, Kotze C, van Zyl JG, *et al*. (2012). Assessment of retention and persistence of copper fungicides on orange fruit and leaves using fluorometry and copper residue analyses. *Crop Protection* **42**: 1–9.

Schutte GC, Mansfield RI, Smith H, *et al*. (2003). Application of azoxystrobin for control of benomyl-resistant *Guignardia citricarpa* on 'Valencia' oranges in South Africa. *Plant Disease* **87**: 784–788.

Smith H, Wingfield MJ, Crous PW, *et al*. (1996). *Sphaeropsis sapinea* and *Botryosphaeria dothidea* endophytic in *Pinus* spp. and *Eucalyptus* spp. in South Africa. *South African Journal of Botany* **62**: 86–88.

Snowdon AL (1990). *A colour atlas of post-harvest diseases and disorders of fruits and vegetables, vol. 1: general introduction and fruits*. Wolfe Scientific Ltd., London, UK: 62–63.

Spósito MB, Amorim L, Bassanezi RB, *et al*. (2008). Spatial pattern of black spot incidence within citrus trees related to disease severity and pathogen dispersal. *Plant Pathology* **57**: 103–108.

Sposito MB, Amorim L, Bassanezi RB, *et al*. (2011). Relative importance of inoculum sources of *Guignardia citricarpa* on the citrus black spot epidemic in Brazil. *Crop Protection* **30**: 1546–1552.

Su YY, Cai L (2012). Polyphasic characterisation of three new *Phyllosticta* spp. *Persoonia* **28**: 76–84.

Sultan A, Johnston PR, Park D, *et al*. (2011). Two new pathogenic ascomycetes in *Guignardia* and *Rosenscheldiella* on New Zealand's pygmy mistletoes (*Korthalsella*: *Viscaceae*). *Studies in Mycology* **68**: 237–247.

Sung GH, Sung JM, Hywel-Jones NL, *et al*. (2007). A multi-gene phylogeny of *Clavicipitaceae* (Ascomycota, Fungi): identification of localized incongruence using a combinational bootstrap approach. *Molecular Phylogenetics and Evolution* **44**: 1204–1223.

Swofford DL (2003). *PAUP*. Phylogenetic analysis using parsimony (*and other methods), v. 4.0b10*. Sinauer Associates, Sunderland, Massachusetts.

Tamura K, Stecher G, Peterson D, *et al*. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* **30**: 2725–2729.

Thongkantha S, Lumyong S, McKenzie EHC, *et al*. (2008). Fungal saprobes and pathogens occurrence on tissues of *Dracaena loureiri* and *Pandanus* spp. *Fungal Diversity* **30**: 149–179.

Tran NT, Miles A, Dietzgen RG, *et al*. (2017). Sexual reproduction in the Citrus Black Spot pathogen, *Phyllosticta citricarpa*. *Phytopathology*. http://dx.doi.org/10.1094/PHYTO-11-16-0419-R.

Truter M (2010). *Epidemiology of citrus black spot disease in South Africa and its impact on phytosanitary trade restrictions*. PhD thesis. University of Pretoria, Pretoria, South Africa.

USDA APHIS (United States Department of Agriculture Animal and Plant Health Inspection Service) (2010). *Risk assessment of* Citrus *spp. fruit as a pathway for the introduction of* Guignardia citricarpa *Kiely, the organism that causes Citrus Black Spot disease*. Center for Plant Health Science and Technology, Plant Epidemiology and Risk Analysis Laboratory, Raleigh, NC, USA.

Viala P, Ravaz L (1892). Sur la dénomination botanique (*Guignardia bidwellii*) du black-rot. *Bulletin de la Société Mycologique de France* **8**: 63.

Vicent A, Armengol J, García-Jiménez J (2007). Rain fastness and persistence of fungicides for control of Alternaria brown spot of citrus. *Plant Disease* **91**: 393–399.

Vilgalys R, Hester M (1990). Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology* **172**: 4238–4246.

Wang X, Chen G, Huang F, *et al*. (2012). *Phyllosticta* species associated with citrus diseases in China. *Fungal Diversity* **52**: 209–224.

Wang NY, Zhang K, Huguet-Tapia JC, *et al*. (2016). Mating type and simple sequence repeat markers indicate a clonal population of *Phyllosticta citricarpa* in Florida. *Phytopathology* **106**: 1300–1310.

White TJ, Bruns T, Lee S, *et al*. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR protocols: a guide to methods and applications* (Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds). Academic Press, San Diego, California: 315–322.

Whiteside JO (1967). Sources of inoculum of the black spot fungus, *Guignardia citricarpa*, in infected Rhodesian orchards. *Rhodesia, Zambia and Malawi Journal of Agricultural Research* **5**: 171–177.

Wicht B, Petrini O, Jermini M, *et al*. (2012). Molecular, proteomic and morphological characterization of the ascomycete *Guignardia bidwellii*, a phylogenetic re-evaluation of *Phyllosticta* (*Botryosphaeriales*) agent of grape black rot: a polyphasic approach to fungal identification. *Mycologia* **104**: 1036–1045.

Wikee S, Lombard L, Nakashima C, *et al*. (2013a). A phylogenetic re-evaluation of *Phyllosticta* (*Botryosphaeriales*). *Studies in Mycology* **76**: 1–29.

Wikee S, Lombard L, Crous PW, *et al*. (2013b). *Phyllosticta capitalensis*, a widespread endophyte of plants. *Fungal Diversity* **60**: 91–105.

Wikee S, Udayanga D, Crous PW, *et al*. (2011). *Phyllosticta*: an overview of current status of species recognition. *Fungal Diversity* **51**: 43–61.

Wingfield MJ, de Beer ZW, Slippers B, *et al*. (2012). One fungus, one name promotes progressive plant pathology. *Molecular Plant Pathology* **13**: 604–613.

Wong MH, Crous PW, Henderson J, *et al*. (2012). *Phyllosticta* species associated with freckle disease of banana. *Fungal Diversity* **56**: 173–187.

Wulandari NF, To-anun C, Hyde KD, *et al*. (2009). *Phyllosticta citriasiana* sp. nov., the cause of Citrus tan spot of *Citrus maxima* in Asia. *Fungal Diversity* **34**: 23–39.

Yonow T, Hattingh V, de Villiers M (2013). CLIMEX modelling of the potential global distribution of the citrus black spot disease caused by *Guignardia citricarpa* and the risk posed to Europe. *Crop Protection* **44**: 18–28.

Yuan Z, Chen Y, Yang Y (2009). Diverse non-mycorrhizal fungal endophytes inhabiting an epiphytic, medicinal orchid (*Dendrobium nobile*): estimation and characterization. *World Journal of Microbiology and Biotechnology* **25**: 295–303.

Zavala MGM, Er HL, Goss EM, *et al*. (2014). Genetic variation among *Phyllosticta* strains isolated from citrus in Florida that are pathogenic or nonpathogenic to citrus. *Tropical Plant Pathology* **39**: 119–128.

Zhang K, Wang NY, Dewdney MM, *et al*. (2015). Lonely peninsula: the mating-type and population of *Phyllosticta citricarpa* in Florida. In: *APS Annual Meeting, August 1–5, Pasadena, California, USA*.

Zhou N, Chen Q, Carroll G, *et al*. (2015). Polyphasic characterization of four new plant pathogenic *Phyllosticta* species from China, Japan, and the United States. *Fungal Biology* **119**: 433–446.