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

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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 capitensis* 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, lef1, gapdh, LSU and rp02 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. capitensis* (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 *Phyllosticta* 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. capitensis* 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 (*P. ampelicida*) 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. musurum* 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. capitensis*) 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 *Laestadia* by *Viala* & *Ravaz* (1892), who applied the name only to *Sphaeria bidewelli* (= *G. bidewelli* = *P. ampelicida*), a species that is different from *Laestadia* (*Bissett* 1986).

*Petrik* (1957) included *G. bidewelli* 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 *Botryosphaeriaceae*. 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. (*Baeyer 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
data, morphology and culture characteristics (Crous et al. 2012, Su & Cai 2012, Wang et al. 2012, Wong et al. 2012, Zhang et al. 2015). Wicke 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 ascomata, often irregularly shaped, unilocular, and with a central ostiole. Ascii 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. Spermata 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, Wicke 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) (Kolzé 1981, Baldassari et al. 2008). The pathogen affects fruits and leaves of several citrus hosts causing various symptoms (Kiely 1948a, 1949, Kolzé 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, speckled or spotted 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 1987). 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, Rakoniansira 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, Amarim 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. citriasina, and also distinguished two subclades within P. citricarpa. Sequencing four partial regions of DNA, Glieneke et al. (2011) distinguished a new species, Phyllosticta citribrazilensis, 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. citribrazilensis 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 intra-specific variation (Okane et al. 2003, Rodrigues et al. 2004). Phyllosticta 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, Romao 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
non-legal movement of citrus plant propagating material. Therefore, leaf litter plays an important role and its removal or enhanced decomposition results in improved inoculum management (Kotzé 1963, McOnie 1964, Huang & Chang 1972, Reis et al. 2006, Fourie et al. 2013, Dummel et al. 2015). 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).

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 (Kiyi 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; Brodick 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 Cellofloculicum and Alternaria spp. (Vincent 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 (Kiyi 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, Sandović 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), clementines (Citrus sinensis × Poncirus trifoliata), citrons (C. medica, C. medica var. sarcodactylis), kumquat (C. japonica), limequats (Citrus ×floridiana), 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. ×lattifolia) and lemons (C. ×limon). New surveys were performed during December 2016 and January 2017 at the sites where P. citricarpa and P. para-citricarpa 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 Fungal Biodiversity Institute, Utrecht, The Netherlands.
| City (country) | GPS coordinates | Site        | Plant age (years) | Condition |
|---------------|-----------------|-------------|-------------------|-----------|
| 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 |
| Alkianos (Greece) | 35.456657, 23.908632 | Orchard    | 15–25             | Cultivated |
| Alkianos (Greece) | 35.462384, 23.904367 | Orchard    | 10–15             | Unkept    |
| Alkianos (Greece) | 35.448440, 23.919798 | Orchard    | 10–15             | Unkept    |
| Alkianos (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 |
| Altira (Spain) | 39.156963, −0.490723 | Orchard    | 10–15             | Cultivated |
| Amfilochia (Greece) | 38.961381, 21.171635 | Orchard    | 10–15             | Cultivated |
| Argo (Greece) | 38.628645, 22.742179 | Orchard    | 10–15             | Cultivated |
| Argo (Spain) | 37.655558, 22.739309 | Orchard    | 10–15             | Cultivated |
| Argos (Spain) | 37.685387, 22.661719 | Orchard    | 10–15             | Cultivated |
| Arta (Greece) | 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    |
| Castelló (Spain) | 39.903922, −0.086197 | Orchard    | 10–15             | Cultivated |
| Castelló (Spain) | 39.883861, −0.088225 | Orchard    | 10–15             | Cultivated |
| Castelló (Spain) | 39.884013, −0.090945 | Orchard    | 10–15             | Cultivated |
| Cefalú (Italy) | 37.302481, 14.032267 | 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, 14.837159 | Orchard    | 15–25             | Unkept    |
| Conceição (Portugal) | 37.084841, −7.916927 | Orchard    | 15–25             | Cultivated |
| Curiglia (Italy) | 37.677729, 16.203763 | Orchard    | 20–30             | Unkept    |
| El Ejido (Spain) | 36.795207, −2.719992 | Orchard    | 20–30             | Cultivated |
| Estellenços (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) | 38.459777, 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) | 36.055138, 14.259907 | Backyard   | 60–70             | Unkept    |
| Gozo (Malta) | 36.058166, 14.284453 | Backyard   | 60–70             | Unkept    |
| Grotte (Italy) | 37.679295, 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 |
| Lerri (Italy) | 38.044422, 14.597517 | Backyard   | 30–40             | Cultivated |
| City (country) | GPS coordinates        | Site                | Plant age (years) | Condition  |
|---------------|------------------------|---------------------|-------------------|------------|
| 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 |
| 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, -8.492322 | Backyard            | 20–30             | Unkept     |
| Monchique (Portugal) | 37.326195, -8.526323 | 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, 22.695839   | Orchard             | 15–25             | Cultivated |
| Nafplio (Greece) | 37.582292, 22.696803   | Orchard             | 10–15             | Cultivated |
| Nafplio (Greece) | 37.588796, 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, 13.286953   | Backyard            | 15–25             | Unkept     |
| Pachino (Italy) | 36.722328, 13.089408   | Orchard             | 15–25             | Unkept     |
| Pedara (Italy) | 37.608768, 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, 15.100665  | 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) | 39.085993, 21.083398   | Orchard             | 10–15             | Cultivated |
| Silves (Portugal) | 37.164146, -8.390841  | Orchard             | 15–25             | Unkept     |
| Soller (Spain) | 39.764520, 2.709609    | Botanical garden    | 30–40             | Cultivated |
| Soller (Spain) | 39.770115, 2.726600    | Orchard             | 20–30             | Cultivated |
| Terme Vigliatore (Italy) | 38.145801, 15.163325  | Nursery             | 1–3               | Cultivated |
| Torremolinos (Spain) | 36.672722, -4.504134  | Orchard             | 30–40             | Cultivated |
| Trebisacce (Italy) | 39.910122, 16.564824   | Backyard            | 20–30             | Cultivated |
| Trebisacce (Italy) | 39.906711, 16.560634   | Orchard             | 3–6               | Cultivated |
| Zurrieq (Malta) | 35.823845, 14.505099   | Backyard            | 15–25             | Unkept     |

1 Site where *P. paracitricarpa* isolates were found associated with leaf litter sampled.
2 Site 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 *Phylllosticta* 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 *Phylllosticta* (incl. 64 European) isolates were included in the study (Table 2), of which 100 (incl. the outgroup, *Neo fusococcum 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 RP2B-5F2 (Sung et al. 2007) and RP2B-7CR (Liu et al. 1999). Glycerol-dehyde-3-phosphate dehydrogenase (*gapdh*) was amplified using primers Gpd1-LM and Gpd2-LM (Mylly et al. 2002). For *P. citricarpa* isolates the alternative primers Gpd1 (Guerber et al. 2003) and GPDHR2 (Gilenke 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 Gilenke et al. (2011). Due to the lack of available *rpb2* gene sequences of *Phylllosticta* isolates, we generated these sequences for all the strains used for this study (except for *P. citrinmaxima* 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 MgCl₂, 1.85 μM of each dNTP, 0.45 μM of each primer and 0.5 μL BioTaq Tag 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 Seq-Man 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](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 *Phylllosticta* species (Gilenke 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; Swoford 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 1000 replications. Sequences generated in this study were deposited in GenBank (Table 2) and alignments and phylogenetic trees in TreeBASE ([www.treebase.org](http://www.treebase.org)). Nomenclatural novelties were deposited in MycoBank (Crous et al. 2004).

**Taxonomy**

A subset of isolates of the four *Phylllosticta* 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 ×1000 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/medium 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. | Host                  | Country     | Mating type idiormorph | GenBank no. |
|--------------------------|-------------|-----------------------|-------------|------------------------|-------------|
| Neofusidicocum mediterraneum | CBS 121718  | Eucalyptus sp.        | Greece      | –                      | GU251176, 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. capitatusensis        | CBS 226.77  | Paphiopedilum callosum, leaf spot | Germany     | –                      | FJ358336, FJ358452, FJ358394, JF343718, KF206289, KY855820 |
|                          | CBS 100175  | Citrus sp.           | Brazil      | –                      | FJ358320, FJ358436, FJ358378, JF343699, KF206327, KY855821 |
|                          | CBS 101228  | Nephelium lappaceum  | Hawaii      | –                      | FJ358319, FJ358435, FJ358377, KF289086, KF206325, KY855822 |
|                          | CBS 114751  | Vaccinium sp., leaf   | New Zealand | –                      | FJ358349, FJ358465, FJ358407, KF289088, EU167584, KY855823 |
|                          | CBS 123373  | Musa paradisiaca     | Thailand    | –                      | FJ358341, FJ358457, FJ358399, JF343703, JQ43604, KY855824 |
|                          | CBS 123374  | Citrus aurantium     | Thailand    | –                      | FJ358332, FJ358448, FJ358390, JF343702, KY855755, KY855825 |
| CBS 128856 = CPC 18848   | Staphylopora sp. | Brazil               | –                      | FJ261465, JF343647, JF261507, JF343776, KF206304, KY855826 |
| CPC 14609                | Zygzygium sp. | Madagascar           | –                      | –                      | KF206184, KF289064, KF289175, KF289081, KF206280, KY855837 |
| CPC 20592                | 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 mili | Thailand           | –                      | –                      | KC291347, KC342544, KC342567, KF289107, KF206230, KY855832 |
| CPC 20508                | Ixora chinensis | Thailand          | –                      | –                      | KF206198, KF289302, KF289185, KF289111, KF206225, KY855833 |
| CPC 25252                | Florida      | –                      | –                      | –                      | KY855585, KY855640, KY855914, KY855695, KY855756, KY855834 |
| CPC 27059                | Citrus limon, leaf | Italy            | –                      | –                      | KY855886, KY855644, KY855915, KY855696, KY855757, KY855835 |
| CPC 27060                | Citrus limon, leaf | Italy           | –                      | –                      | KY855597, KY855642, KY855916, KY855697, KY855764, KY855842 |
| CPC 27061                | Citrus limon, leaf | Italy           | –                      | –                      | KY855658, KY855643, KY855917, KY855698, KY855759, KY855837 |
| CPC 27062                | Citrus limon, leaf | Italy           | –                      | –                      | KY855659, 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, KY855649, 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.     | Italy         | –                      | –                      | KY855598, KY855653, KY855927, KY855708, KY855769, KY855847 |
| CPC 27826                | C. medica var.     | Italy         | –                      | –                      | KY855599, KY855654, KY855928, KY855709, KY855770, KY855848 |

(continued on next page)
| Species | Culture no. | Host | Country | Mating type | GenBank no. | 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 27917 | 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 27919 | 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 |
| CBS 120487 | Citrus maxima, fruit | China | – | – | FJ538361 FJ538477 FJ538419 JF343687 KF206313 |
| CBS 123370 | Citrus maxima, fruit | Vietnam | – | – | FJ538355 FJ538471 FJ538413 JF343689 KF206310 |
| P. citribraziliensis CBS 100098 | Citrus sp., leaf | Brazil | – | – | FJ538352 FJ538468 FJ538410 JF343691 KF206221 |
| CPC 17464 | Citrus sp., leaf | Brazil | – | – | KF170300 KF289280 KF289224 KF289159 KF206263 |
| CPC 17465 | Citrus sp., leaf | Brazil | – | – | KF170301 KF289281 KF289225 KF289160 KF206262 |
| P. citricarpa CBS 122482 | Citrus sinensis | Zimbabwe | MAT1-2-1 | | KY855609 KY855664 KY855938 KY855719 KY855780 KY855870 |
| CBS 127452 | Citrus reticulata | Australia | MAT1-2-1 | | JF343581 JF343665 JF343602 JF343769 KF206307 |
| CBS 127454 | Citrus limon | Australia | MAT1-2-1 | | JF343583 JF343667 JF343604 JF343771 KF206306 |
| CPC 16586 | Citrus limon | South Africa | MAT1-1-1 | | KF170293 KF289269 KF289220 KF289155 KF206274 |
| CPC 16603 | Citrus limon | Uruguay | MAT1-1-1 | | KF170295 KF289274 KF289213 KF289147 KF206269 |
| CPC 16609 | Citrus sp. | Argentina | MAT1-1-1 | | KF170298 KF289277 KF289217 KF289152 KF206266 |
| CPC 25312 | Citrus sinensis | Florida | MAT1-2-1 | | KY855609 KY855664 KY855938 KY855719 KY855780 KY855871 |
| CPC 27909 = CBS 141349 | Citrus limon, leaf litter | Italy | MAT1-2-1 | | KY855610 KY855665 KY855939 KY855720 KY855781 KY855872 |
| CPC 27910 | Citrus limon, leaf litter | Italy | MAT1-2-1 | | KY855611 KY855666 KY855940 KY855721 KY855782 KY855873 |
| CPC 27911 | Citrus limon, leaf litter | Italy | MAT1-2-1 | | KY855612 KY855667 KY855941 KY855722 KY855783 KY855874 |
| CPC 27912 | Citrus limon, leaf litter | Italy | MAT1-2-1 | | KY855613 KY855668 KY855942 KY855723 KY855784 KY855875 |
| CPC 27913 = CBS 141350 | Citrus sinensis, leaf litter | Malta | MAT1-2-1 | | KY855614 KY855669 KY855943 KY855724 KY855785 KY855876 |
| CPC 27914 | Citrus sinensis, leaf litter | Malta | MAT1-2-1 | | KY855615 KY855670 KY855944 KY855725 KY855786 KY855877 |
| CPC 27915 | Citrus sinensis, leaf litter | Malta | MAT1-2-1 | | KY855616 KY855671 KY855945 KY855726 KY855787 KY855878 |
| CPC 27916 | Citrus sinensis, leaf litter | Malta | MAT1-2-1 | | KY855617 KY855672 KY855946 KY855727 KY855788 KY855879 |
| CPC 28104 = CBS 141351 | Citrus sinensis, leaf litter | Portugal | MAT1-1-1 | | KY855618 KY855673 KY855947 KY855728 KY855789 KY855880 |
| CPC 28105 = CBS 141352 | Citrus sinensis, leaf litter | Portugal | MAT1-1-1 | | KY855619 KY855674 KY855948 KY855729 KY855790 KY855881 |
| CPC 28106 | Citrus sinensis, leaf litter | Portugal | MAT1-1-1 | | KY855620 KY855675 KY855950 KY855730 KY855791 KY855882 |
| CPC 28107 | Citrus sinensis, leaf litter | Portugal | MAT1-1-1 | | KY855621 KY855676 KY855950 KY855731 KY855792 KY855883 |
| CPC 31171 | Citrus sinensis, leaf litter | Malta | MAT1-2-1 | | – – – – – – |
| CPC 31172 | Citrus sinensis, leaf litter | Malta | MAT1-2-1 | | – – – – – – |
| Strain Number | Description | Location | Accession Numbers |
|---------------|-------------|----------|------------------|
| CPC 31173     | Citrus sinensis, leaf litter | Malta | MAT1-2-1 |
| CPC 31174     | Citrus sinensis, leaf litter | Malta | MAT1-2-1 |
| CPC 31279     | Citrus sinensis, leaf litter | Portugal | MAT1-1-1 |
| CPC 31280     | Citrus sinensis, leaf litter | Portugal | MAT1-1-1 |
| CPC 31281     | Citrus sinensis, leaf litter | Portugal | MAT1-1-1 |
| CPC 31282     | Citrus sinensis, leaf litter | Portugal | MAT1-1-1 |
| ZJUCC200952   | Citrus reticulata, leaf | China | MAT1-2-1 |
|                |             |          | JN791635 JN791556 JN791480 KY855732 KY855793 KY855844 |
| P. citrichinaensis | Citrus reticulata, leaf | China | – |
| CBS 129764    | –           |          | JN791598 JN791527 JN791453 KY855733 KY855794 KY855885 |
| CBS 130529    | Citrus maxima, leaf | Portugal | MAT1-1-1 |
|                |             |          | JN791597 JN791526 JN791452 KY855734 KY855795 KY855866 |
| P. citrichinaensis | Citrus maxima, fruit | Thailand | – |
| CPC 20276     | –           |          | KF170304 KF289300 KF289222 KF289157 KF206229 |
| P. citrimaxima | Citrus maxima, fruit | –         | KF170287 KF289295 KF289172 KF289076 KF206242 KY855887 |
| CPC 20261     | Cordyline fruticosa | Thailand | – |
| CPC 20277     | –           |          | KF170288 KF289301 KF289171 KF289075 KF206228 KY855888 |
| P. cordylinophila | Cordyline fruticosa | Thailand | – |
| CPC 14873     | Cussonia sp. | South Africa | – |
| CPC 14875     | Cussonia sp. | South Africa | – |
| P. cussonia    | –           |          | JF343578 JF343662 JF343599 JF343764 |
| P. eugeniaiae  | Eugenia aromatica | Indonesia | – |
| CBS 445.82    | –           |          | AY042926 KF289246 KF289208 KF289139 |
| P. hypoglossi | Ruscus aculeatus | Italy | – |
| CBS 434.92    | –           |          | FJ538367 FJ538483 FJ538425 |
| P. paracapitans | –           |          | JF343695 |
| CBS 173.77    | Citrus aurantifolia | New Zealand | – |
| CPC 26517     | –           |          | KF206179 KF289244 FJ538303 KF289100 |
| CPC 26518     | Citrus floridana, leaf | Italy | – |
| CPC 26700     | –           |          | KY855622 KY855677 KY855961 |
| CPC 26701     | Citrus floridana, leaf | Italy | – |
| CPC 28005     | –           |          | KY855626 KY855679 KY855962 KY855736 KY855797 |
| CPC 26806     | Citrus floridana, leaf | Italy | – |
| CPC 28121     | Citrus limon, leaf | Spain | – |
| CPC 28122     | Citrus limon, leaf | Spain | – |
| CPC 28123     | –           |          | KY855630 KY855685 |
| CPC 28127     | –           |          | KY855631 |
| CPC 28128     | Citrus limon, leaf | Spain | – |
| CPC 28129     | –           |          | KY855634 |
| P. paracarcipera | –           |          | KY855635 KY855690 KY855694 KY855748 KY855809 KY855907 |
| CPC 27169     | Citrus limon, leaf litter | Greece | – |
| CPC 27170     | –           |          | KY855636 KY855691 KY855965 |
| CPC 27171     | –           |          | KY855637 |
| CPC 27172     | Citrus limon, leaf litter | Greece | – |
| CPC 31246     | –           |          | KY855638 KY855693 KY85567 KY855751 KY855812 |
| CPC 31247     | Citrus limon, leaf litter | Greece | – |

(continued on next page)
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′-GCATG TGCCAGCGCAATCC-3′) and MAT111R3 (5′-TCTTGGACA TCGGACTCATC-3′) for MAT1-1, and MAT121F6 (5′-GATC GTGSCAGGGCTTTG-3′) and MAT121R6 (5′-AACGAC- CAGCGATCGGTAAG-3′) for MAT1-2 (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 μL MgCl₂, 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 × 10⁵ 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, changes were observed over 3142 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).

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 3142 characters were included in the phylogenetic analyses; 693 characters were parsimony-informative, 315 were variable and parsimony-uninformative and 2134 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 28 902 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. citribrasilienis 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. Cultivar 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 (R² values 0.81 to 0.87) and predicted cardinal and optimal temperatures of 12.5–27.2–34.0 °C for P. capitalensis, 10.7–26.4–33.2 °C for P. paracapitalensis, 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. paracapitana*sis and *P. capitans* isolates used in this study. Base positions representing fixed nucleotide differences between the two species are in **bold**.

| Species            | **Phytophthora paracapitana** | **Phytophthora capitans** |
|--------------------|--------------------------------|--------------------------|
| CPC 28117 Citrus *sp.* Italy | GACGGTTCGCTGCCCA | **TCGTTTTGAC** |
| CPC 28500 Citrus *sp.* Italy | GACGGTTCGCTGCCCA | **TCGTTTTGAC** |
| CPC 28518 Citrus *sp.* Italy | GACGGTTCGCTGCCCA | **TCGTTTTGAC** |
| CPC 28571 Citrus *sp.* Italy | GACGGTTCGCTGCCCA | **TCGTTTTGAC** |
| CPC 28604 Citrus *sp.* Italy | GACGGTTCGCTGCCCA | **TCGTTTTGAC** |
| CPC 33149 Citrus *sp.* Spain | GACGGTTCGCTGCCCA | **TCGTTTTGAC** |
| CPC 33150 Citrus *sp.* Spain | GACGGTTCGCTGCCCA | **TCGTTTTGAC** |
| CPC 33151 Citrus *sp.* Spain | GACGGTTCGCTGCCCA | **TCGTTTTGAC** |
| CPC 33152 Citrus *sp.* Spain | GACGGTTCGCTGCCCA | **TCGTTTTGAC** |
| CPC 33153 Citrus *sp.* Spain | GACGGTTCGCTGCCCA | **TCGTTTTGAC** |
| CBS 173.77 Citrus *sp.* New Zealand | GACGGTTCGCTGCCCA | **TCGTTTTGAC** |

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**Note:** The table continues with similar entries for other species and isolates, but these are not fully transcribed here due to the size of the table. Each entry represents a nucleotide variation observed between the two species, with the differences highlighted in **bold**.

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**Additional Information:**

- Italy
- Spain
- Greece
- Malta
- Thailand
**Table 4.** Nucleotide differences observed among *P. paracapitalensis* 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 |
|-----------------------|------|------|-------|-----|
| **Phyllosticta paracapitalensis** |      |      |       |     |
| 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 cells, that can be branched at the base, 7–20 × 4–6 μm. *Conidiogenous cells* terminal, subcylindrical, hyaline, smooth, coated in a mucoid sheath, 7–15 × 3–4 μm; proliferating several times percurrently near apex. *Conidia* (9–)12–13–(14) × (6–)7 μm, solitary, hyaline, asperate, thin and smooth-walled, granular, or with a single large central guttule, fusoid-ellipsoid, tapering towards a narrow truncate base, 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 trinucleate, hyaline, smooth, granular with large central guttule, asperate, 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 ascii, and slightly larger ascospores. The asexual morph presents solitary and globose conidiomata that differ from those of *P. capitalensis* (aggregated and globose to ampulliform). 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. capitans*.  
**G–I.** *P. paracitrarpa*.  
**J–L.** *P. citrarpa*.  

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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 cells, 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 obvoid, 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.
| Species                 | Ascomata Size (μm) | Ascomata Shape | Asci Size (μm) | Asci Shape | Ascospores Size (μm) | Ascospores Shape | Conidiomata Size (μm) | Conidiomata Shape | Conidiogenous cells Shape | Conidia Size (μm) | Conidia Shape | Spermatia Size (μm) | Spermatia Shape | Reference     |
|-------------------------|---------------------|----------------|--------------|------------|----------------------|-----------------|----------------------|---------------------|-----------------------|-----------------|---------------|-------------------|----------------|--------------|
| *P. capitalensis*       | 250                 | globose        | 58–80        | to pyriform| 15–17 × 5–6         | limoniform      | 300 × 250            | globose to ampulliform| 7–10 × 3–5          | subcylindrical to ampulliform or doliiform | (10–)11–12(–14) | (5–)(6–7) | ellipsoid         | to obovoid | Hennings (1908) |
| *P. citriasiana*        | –                   | –              | –            | –          | –                   | –               | –                   | –                   | –                     | –               | –             | –                 | –             | 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             | 7–12 × 3–4          | subcylindrical to doliiform | (10–)11–12(–14) | (6–)7 | ellipsoid         | to obovoid | Van der Aa (1973) |
| *P. citrichinaensis*    | 100–300             | subglobose     | 42–81        | to pyriform| 14–20 × 7–8         | fusiform        | 100–200             | globose or subglobose| 6–12 × 2–5          | lageniform           | (7–)8–12(–13) | 6–9       | ellipsoid         | to obovoid | Wang et al. (2012) |
| *P. citrimaxima*        | –                   | –              | –            | –          | –                   | –               | 150–160             | 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        | to pyriform| 16–17 × 6 (–7) limoniform | to clavate | up to 250            | globose             | 7–15 × 3–4          | subcylindrical       | (9–)12–13(–14) | (6–)7       | ellipsoid         | to obovoid | This study    |
| *P. paracitr carp*a     | –                   | –              | –            | –          | 250                 | globose         | 12–17 × 3–4         | subcylindrical      | 9–11–13(–15)        | (7–)8(–9)           | –                 | –             | –                 | –             | This study    |
|                        |                     |                |              |            |                     |                 |                     |                     |                       |                  |               |                   |               |              |
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* ≤ 0.05) differentiated from the China (*PhiPT* = 0.634; *P* = 0.001), Italy+Malta (*PhiPT* = 1.000; *P* = 0.001), South Africa.
Comparing the loci atypical of the CBS disease, and identically consistently re-isolated from the fruit lesions, albeit from lesions.

**DISCUSSION**

*P. paracapitalensis* fruits inoculated with isolates (CPC 27169, CPC 27170). No lesions were observed on with *P. paracitricarpa* (CPC 27909, CPC 27913) and *P. capitalensis* (CPC 26517, CPC 26700) (*P. citricarpa* = 0.365; *P. paracitricarpa* = 0.001), and the USA (*P. citricarpa* = 1.000; *P. paracitricarpa* = 0.002) populations. The Portugal population was not significantly differentiated from the Australia population (*P. citricarpa* = 0.000; *P. paracitricarpa* = 0.418), and also not from the Brazil population (*P. citricarpa* = 0.322; *P. paracitricarpa* = 0.155). The Italy+Malta population was significantly (*P. citricarpa* = 0.258; *P. paracitricarpa* = 0.001), China (*P. citricarpa* = 0.651; *P. paracitricarpa* = 0.002), South Africa (*P. citricarpa* = 0.365; *P. paracitricarpa* = 0.002), Brazil (*P. citricarpa* = 0.483; *P. paracitricarpa* = 0.043), the USA (*P. citricarpa* = 1.000; *P. paracitricarpa* = 0.001) and Portugal (*P. citricarpa* = 1.000; *P. paracitricarpa* = 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 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 capitataensis* 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. capitataensis* (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 capitataensis* 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 capitataensis* was associated with *P. paracapitalensis* in the same specimens.

**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        | –   |
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 CBS.
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. aurantifolia 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 BTEF 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. citricinhaensis, P. citriasana and P. citrimaxima are present only in Asia, and the endophyte P. citribrazilensis 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 Phyllosticta 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 Ariens 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.

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