



Occurrence of *Phyllosticta citricarpa* and *Phyllosticta citriasiana* in EU imported *Citrus* spp. and its impact on diagnostics of citrus black spot on fruits

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Abstract Citrus black spot (CBS) is a fungal disease on *Citrus* spp. caused by *Phyllosticta citricarpa*, which is listed as a regulated pest in the European Union (EU). There are several closely related *Phyllosticta* species that also occur on citrus including *Phyllosticta citriasiana* which causes citrus tan spot and is not regulated by the European Union. A real-time PCR assay was developed to aid the detection of *P. citriasiana*. Subsequently an extensive sampling on the presence of *P. citricarpa* and *P. citriasiana* was performed on 273 citrus fruit consignments that were put on hold by the Dutch national plant protection organization (NPPO-NL) because of suspected presence of CBS. *P. citricarpa* was found on several *Citrus* spp. but never on *Citrus maxima* whilst *P. citriasiana* was only found on *C. maxima* and never on other *Citrus* spp. The unique fungus host associations observed after testing almost 1100 individual lesions, are somewhat obscured by previously reported (some unverified) observations of *C. maxima* fruits harbouring black spot. Nevertheless,

it was shown that a quick and simple direct morphological identification of *P. citricarpa*, based on the presence of CBS lesions harbouring pycnidia producing characteristic *Phyllosticta* conidia, is feasible on non-*Citrus maxima* fruits. It is proposed to adapt the European and Mediterranean Plant Protection Organisation (EPPO) and International Plant Protection Convention (IPPC) diagnostic protocols accordingly.

Keywords *Phyllosticta citriasiana* · *Phyllosticta citricarpa* · *Citrus* spp. · Citrus black spot · Citrus tan spot · Real-time PCR

Introduction

Phyllosticta citricarpa is an important pathogen on citrus causing citrus black spot (CBS). Symptoms of CBS are fruit-blemishing, premature fruit drop and fruit drop. Several CBS symptoms appear on fruit, such as hard spot, freckle spot, false melanose, virulent spot, lacy spot and cracked spot. Lesions with pycnidia are only found in hard spot and virulent spot (EPPO, 2020), these morphological features could justify a rapid detection of *P. citricarpa* as long as the pycnidia harbour the characteristic conidia. The current situation in Tunisia shows that *P. citricarpa* can, once introduced, spread rather rapidly and have devastating economic and social consequences for citrus producing regions, incurred by production losses (Benfradj et al., 2024; Ioos et al., 2024). Due to its

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economic impact the European Union (EU) has regulated the import of citrus fruit to prevent the introduction of *P. citricarpa* in EU citrus orchards (Regulation (EU) 2019/2072).

There are seven different *Phyllosticta* species associated with citrus to date. *Phyllosticta citricarpa* (Kiely 1948), *P. citriasiana* (Wulandari et al. 2009) and *P. citrimaxima* (Wikee et al. 2013a) are associated with distinct fruit lesions with pycnidia harbouring characteristic *Phyllosticta* conidia. *Phyllosticta citricarpa* is treated as a quarantine organism by the EU, but *P. citriasiana* and *P. citrimaxima*, both associated with citrus tan spot (CTS) on *Citrus maxima*, are not. Recently, *P. paracitricarpa* (Guarnaccia et al. 2017) was synonymised with *P. citricarpa* (van Ingen-Buijs et al. 2024) and is thus not treated separately in this paper. Although *P. citrichinaensis* is associated with small black to brown spots on citrus fruits, conidia bearing pycnidia were never reported in these spots (Wang et al., 2012). *Phyllosticta capitalensis* (Baayen et al. 2002) and the more recently described *P. citribraziliensis* (Glienke et al. 2011) and *P. paracapitalensis* (Guarnaccia et al. 2017) were not reported on symptomatic fruit. It is thus important to include all *Phyllosticta* species that are associated with citrus in validation studies to rule out false positive results in specific detection assays.

CBS was first diagnosed in the Netherlands on a number of consignments intercepted from the southern hemisphere in 1999 (Baayen, 2000), based on a direct *in planta* morphological identification on citrus fruit, i.e. the presence of lesions, pycnidia and *Phyllosticta* conidia. In the same year an *in vitro* morphological identification of a putative CBS isolate obtained from *Citrus x paradisi* fruit peel with minute lesions without pycnidia and without conidia resulted in the identification of ‘*Guignardia mangiferae*’ (later identified as *P. capitalensis*, a non-pathogenic *Phyllosticta* species for citrus; Baayen et al., 2002; Wikee et al., 2013b). From 2001 all putative CBS lesions, not bearing pycnidia and *Phyllosticta* conidia, were routinely tested by a conventional PCR (Bonants et al., 2003) followed by a more sensitive real-time PCR (van Gent-Pelzer et al., 2007) from 2005 onwards.

In 2005 the first case of putative CBS on *C. maxima* from Thailand was diagnosed, based on *in planta* morphological identification directly on the fruit (i.e. the presence of lesions, pycnidia and *Phyllosticta*

conidia). Interestingly, in this case there was no amplification in the CBS real-time PCR nor in any of the additional *C. maxima* lesions from Asia that followed. In 2009 it became clear that our initial CBS diagnose on *C. maxima* was incorrect when *P. citriasiana*, the putative causal agent of tan spot on *C. maxima* in Asia, was described (Wulandari et al., 2009).

Safeguarding EU citrus orchards from the regulated *P. citricarpa*, justifies strict phytosanitary inspections at import locations. However, these inspections should not affect citrus imports associated without, or with non-regulated *Phyllosticta* spp. because this would impose severe economic consequences on the exporters that cannot be justified. Therefore, the main objective of this study was to optimise the detection of *P. citricarpa*, particularly by understanding the occurrence of *P. citriasiana* and *P. citricarpa* on intercepted *Citrus* spp. fruits. A *P. citriasiana* species-specific real-time PCR assay was developed to enable the distinction between *P. citriasiana* and *P. citricarpa*. Based on the results of the initial *P. citriasiana* interceptions at European import locations and on the *Phyllosticta* resistance study by Miles et al. (2019) we hypothesised that *P. citriasiana* can only be detected on symptomatic *C. maxima* fruits and that *P. citricarpa* can be detected on several symptomatic *Citrus* spp. but not on *C. maxima*.

Materials and methods

Citrus fruit consignments

Fruit consignments of *Citrus* spp. of different origins (Table S1) with putative CBS or CTS symptoms were obtained from 273 interceptions between January 2005 and October 2024. Asymptomatic citrus fruits were obtained from a local supermarket.

Fungal isolates

Isolates grown on cherry decoction agar (Crous et al., 2009) were used to determine the performance characteristics inclusivity and exclusivity of the real-time PCR assays for *P. citricarpa* and *P. citriasiana* (Table 1). Next to seven strains of *P. citriasiana* (originating from: China (3), Thailand (2) and Vietnam (2)) and 16 strains of *P. citricarpa* (originating from: Argentina (2), Australia (1),

Table 1 Details of isolates used in this study

Species	Culture number(s) ¹	Host	Isolated from	Symptom	Putative pathogenicity on Citrus fruit	Country	GenBank no. ITS ² /tef ³ /LSU ⁴	CTS real-time PCR	CBS real-time PCR
<i>Alternaria turkisafrica</i>	PD 014 04062292; CBS 121346	<i>Citrus paradisi</i> × <i>C. reticulata</i>	unknown	unknown	black rot	South Africa	KP124366//	negative	negative
<i>Alternaria citri</i>	PD 99/12272 ⁵	<i>Citrus medica</i>	unknown	unknown	black rot	unknown	MZ416900 //	negative	negative
<i>Aspergillus niger</i>	PD 014 04285154; CBS 118725	<i>Hyacinthus</i>	bulb	unknown	black mould rot	Netherlands	FJ629345//	negative	negative
<i>Aspergillus niveus</i>	PD 012 04764913; CBS 1115.27; ATCC 24486	unknown	unknown	unknown	Aspergillus rot	unknown	EF669615//	negative	negative
<i>Botrytis cinerea</i>	PD 012 04408644	<i>Asclepias</i>	sclerotia	sclerotia	grey mould rot	Netherlands	MZ416901 //	negative	negative
<i>Ceratocystis fimbriata</i>	PD 013 04266543	<i>Tectona grandis</i>	vascular tissue	bark blackening	Ceratocystis rot	Ghana	MZ416902 //	negative	negative
<i>Cladosporium subtitissimum</i>	PD 014 04062786; CBS 1113754	<i>Vitis</i>	grape berry	unknown	sooty mold	United States of America	EF679397//	negative	negative
<i>Colletotrichum coccodes</i>	PD 014 04062276; CBS 369.75	<i>Solanum tuberosum</i>	unknown	unknown	anthracnose	Netherlands	HM171679//	negative	negative
<i>Colletotrichum fructicola</i>	PD 95/7401—2; CBS 111.14	<i>Malus sylvestris</i>	fruit	unknown	anthracnose	Germany	KC566785//	negative	negative
<i>Diaporthe citri</i>	PD 016 06233948; CBS 135422	<i>Citrus</i>	twig	unknown	melanose	United States of America	KC843311//	negative	negative
<i>Didymella pisi</i>	PD 78/517; CBS 122785	<i>Pisum sativum</i>	pod	pod spots	freckle	Netherlands	GU37763//	negative	negative
<i>Elsinoë australis</i> *	PD 09 03282576; CBS 230.64	<i>Citrus × aurantium</i>	unknown	unknown	scab	Argentina	KX887197//	negative	negative
<i>Elsinoë fawcettii</i> *	PD 05 01895475; CBS 233.64	<i>Citrus × aurantium</i>	unknown	unknown	scab	Panama	KX887222//	negative	negative
<i>Fusarium oxysporum</i>	PD 87/91	<i>Capicum annuum</i>	base	stem base rot	Fusarium rot	Netherlands	MZ416903 //	negative	negative

Table 1 (continued)

Species	Culture number(s) ¹	Host	Isolated from	Symptom	Putative pathogenicity on Citrus fruit	Country	GenBank no. ITS ² /tef ³ /LSU ⁴	CTS real-time PCR	CBS real-time PCR
<i>Fusarium solani</i>	PD 89/682	<i>Cucumis sativus</i>	base	stem base dieback	Fusarium rot	Netherlands	MZ416904 //	negative	negative
<i>Geotrichum candidum</i>	PD 95/4032	<i>Daucus carota</i>	root	soft rot	sour rot	Netherlands	MZ416905 //	negative	negative
<i>Lasiodiplodia pseudothobromae</i>	PD 012 04761085; CBS 447.62	Citrus × aurantium	fruit	unknown	stem-end rot	Suriname	EF622081//	negative	negative
<i>Macrophoma phaeolina</i>	PD 93/1869	<i>Curcuma</i>	tuber	dry rot	charcol rot	Netherlands	MZ416906 //	negative	negative
<i>Penicillium digitatum</i>	PD 016 06233956; CBS 112082; IBT 13068	<i>Citrus limon</i>	unknown	unknown	green mold rot	Italy	KJ834506//	negative	negative
<i>Penicillium italicum</i>	PD 014 04062081; CBS 339.48	<i>Citrus</i>	fruit	unknown	blue mold rot	United States of America	JF772180//	negative	negative
<i>Phyllosticta bifrenariae</i>	PD 012 04761034; CBS 128855 ; VIC 30556	<i>Bifrenaria harrissoniae</i>	leaf	living	non host	Brazil	JF343565//	negative	negative
<i>Phyllosticta brazilianae</i>	PD 012 04764024; CBS 129060 ; LGMF 3303; CPC 17447	<i>Mangifera indica</i>	leaf	none	non host	Brazil	JF343572//	negative	negative
<i>Phyllosticta capitalensis</i>	PD 012 05428962; CBS 100176	<i>Citrus</i>	leaf	none	none (endophyte)	Brazil	FJ538321//	negative	negative
<i>Phyllosticta capitalensis</i>	PD 012 05428954; CBS 123374; NFW-220	<i>Citrus maxima</i>	unknown	unknown	none (endophyte)	Thailand	FJ538332//	negative	negative
<i>Phyllosticta capitalensis</i>	PD 016 06079565– 2 ^s	<i>Citrus maxima</i>	fruit	none	none (endophyte)	Vietnam	MZ416907 //	negative	negative
<i>Phyllosticta capitalensis</i>	PD 04 01844942 ^s ; CBS 120490; GC 14	<i>Citrus paradis</i>	fruit	none	none (endophyte)	United States of America	FJ538338//	negative	negative
<i>Phyllosticta citriasiana</i>	PD 013 05619152; LSVM 608	<i>Citrus maxima</i>	fruit	tan spot	tan spot	China	MZ416908 //	positive	negative

Table 1 (continued)

Species	Culture number(s) ¹	Host	Isolated from	Symptom	Putative pathogenicity on Citrus fruit	Country	GenBank no. ITS ² /tef ³ /LSU ⁴	CTS real-time PCR	CBS real-time PCR
<i>Phyllosticta citriasiana</i>	PD 06 03125095 [§] ; CBS 120491	<i>Citrus maxima</i>	fruit	tan spot	tan spot	China	FJ538362//	positive	negative
<i>Phyllosticta citriasiana</i>	PD 07 03125829 [§]	<i>Citrus maxima</i>	fruit	tan spot	tan spot	China	MZ416909 //	positive	negative
<i>Phyllosticta citriasiana</i>	PD 05 01969753 [§] ; CBS 120486	<i>Citrus maxima</i>	fruit	tan spot	tan spot	Thailand	FJ538360//	positive	negative
<i>Phyllosticta citriasiana</i>	PD 05 02436019 [§] ; CBS 120488	<i>Citrus maxima</i>	fruit	tan spot	tan spot	Thailand	FJ538354//	positive	negative
<i>Phyllosticta citriasiana</i>	PD 08 04453728 [§] ; CBS 123393	<i>Citrus maxima</i>	fruit	tan spot	tan spot	Vietnam	FJ538358//	positive	negative
<i>Phyllosticta citriasiana</i>	PD 016 06079565–1 [§]	<i>Citrus maxima</i>	fruit	tan spot	tan spot	Vietnam	MZ416910 //	positive	negative
<i>Phyllosticta citribrazil-iensis</i>	PD 012 04761026; CBS 100098	<i>Citrus</i>	leaf	none	none (endo-phyte)	Brazil	FJ538352//	negative	negative
<i>Phyllosticta citricarpa</i> *	PD 010 04421662 ^{AS}	<i>Citrus</i>	fruit	black spot	black spot	Argentina	MZ416911/OK087470/OK087464	negative	positive
<i>Phyllosticta citricarpa</i> *	PD 04 01844862; CBS 111.20; DSM 3514; GC1 ^A	<i>Citrus</i>	fruit	black spot	black spot	China	AY042922/FJ538372/ FJ538314	negative	positive
<i>Phyllosticta citricarpa</i> *	PD 010 04427271 [§]	<i>Citrus</i>	fruit	black spot	black spot	South Africa	MZ416912 //	negative	positive
<i>Phyllosticta citricarpa</i> *	PD 014 04285121; CBS 127454 ; CPC 181176; BRIP 52614 a ^A	<i>Citrus limon</i>	fruit	black spot	black spot	Australia	JF343583/JF343604/ KF206306	negative	positive
<i>Phyllosticta citricarpa</i> *	PD 04 01844897 [§] ; CBS 120489 ^A	<i>Citrus limon</i>	fruit	black spot	black spot	Brazil	FJ538315/FJ38373/ KF206311	negative	positive
<i>Phyllosticta citricarpa</i> *	PD 015 05699420 ^{AS}	<i>Citrus sin-ensis</i>	fruit	black spot	black spot	South Africa	MZ416913/OK087473/OK087467	negative	positive
<i>Phyllosticta citricarpa</i> *	PD 014 04285111; CBS 122482; CPC 14848 ^A	<i>Citrus sin-ensis</i>	fruit	lesions	black spot	Zimbabwe	FJ538317/FJ538375/ KF306230	negative	positive

Table 1 (continued)

Species	Culture number(s) ¹	Host	Isolated from	Symptom	Putative pathogenicity on Citrus fruit	Country	GenBank no. ITS ² /tef ³ /LSU ⁴	CTS real-time PCR	CBS real-time PCR
<i>Phyllosticta citricarpa</i> *	PD 015 05577441 ^{AS}	<i>Citrus sinensis</i>	fruit	black spot	black spot	South Africa	MZ416917/OK087472/ OK087466	negative	positive
<i>Phyllosticta citricarpa</i> *	PD 015 04413400 ^{AS}	<i>Citrus sinensis</i>	fruit	black spot	black spot	South Africa	MZ416916/OK087471/ OK087465	negative	positive
<i>Phyllosticta citricarpa</i> *	PD 017 033508046 ^{AS}	<i>Citrus sinensis</i>	fruit	black spot	black spot	South Africa	MZ416919/OK087474/ OK087468	negative	positive
<i>Phyllosticta citricarpa</i> *	PD 017 033332650 ^{AS}	<i>Citrus limon</i>	fruit	black spot	black spot	Argentina	MZ416918/OK087475/ OK087469	negative	positive
<i>Phyllosticta citricarpa</i> *	PD 017 06232961; CBS 141350; CPC 27913 ^A	<i>Citrus sinensis</i>	leaf litter	none	black spot **	Malta	KY855614/KY855943/ KY855785	negative	positive
<i>Phyllosticta citricarpa</i> *	PD 017 06232971; CBS 141349; CPC 27909 ^A	<i>Citrus limon</i>	leaf litter	none	black spot **	Italy	KY855610/KY855939/ KY855781	negative	positive
<i>Phyllosticta citricarpa</i> *	PD 017 06233008; CBS 141351; CPC 28104 ^A	<i>Citrus sinensis</i>	leaf litter	none	black spot	Portugal	KY855618/KY855947/ KY855789	negative	positive
<i>Phyllosticta citricarpa</i> *	PD 017 06232988; CBS 141357; CPC 27169 ^A	<i>Citrus limon</i>	leaf litter	none	undefined **	Greece	KY855635/KY855964/ KY855809	negative	positive
<i>Phyllosticta citricarpa</i> *	PD 017 06232996; CBS 141358; CPC 27170 ^A	<i>Citrus limon</i>	leaf litter	none	undefined **	Greece	KY855636/KY855965/ KY855810	negative	positive
<i>Phyllosticta citrichinaensis</i>	PD 012 04764948; CBS 130529; CGMCCC3.14302	<i>Citrus maxima</i>	leaf	small brown spot	leaf spot	China	JN791597//	negative	negative
<i>Phyllosticta citrichinaensis</i>	PD 012 05428946; CBS 129764	<i>Citrus reticulata</i>	leaf	brown freckle	leaf spot	China	JN791660//	negative	negative
<i>Phyllosticta citrimaxima</i>	PD 014 04061556; CBS 136059; CPC 20276; MFLUCC 10–0137	<i>Citrus maxima</i>	fruit	tan spot	tan spot	Thailand	KF170304//	n/a	n/a

Table 1 (continued)

Species	Culture number(s) ¹	Host	Isolated from	Symptom	Putative pathogenicity on Citrus fruit	Country	GenBank no. ITS ² /tef ³ /LSU ⁴	CTS real-time PCR	CBS real-time PCR
<i>Phyllosticta concentrica</i>	PD 012 05428970; CBS 937.70	<i>Hedera helix</i>	leaf litter	unknown	non host	Italy	FJ538350//	negative	negative
<i>Phyllosticta hypoglossi</i>	PD 012 05428997; CBS 167.85	<i>Ruscus hypoglossum</i>	unknown	unknown	non host	Italy	FJ538366//	negative	negative
<i>Phyllosticta owaniana</i>	PD 012 05429009; CBS 776.97 ; CPC 1009–1010	<i>Brabejum stellatifolium</i>	leaf	leaf spot	non host	South Africa	FJ538368//	negative	negative
<i>Phyllosticta paracapitalensis</i>	PD 016 06233921; CBS 173.77	<i>Citrus aurantifolia</i>	fruit	unknown	none (endophyte)	New Zealand	KF206179//	negative	negative
<i>Phyllosticta paracapitalensis</i>	PD 017 06232945; CBS 141355; CPC 28120	<i>Citrus limon</i>	leaf	none	none (endophyte)	Spain	KY855628//	negative	negative
<i>Phytophthora citricola</i>	PD 09 03282551; CBS 221.88; IMI 021173	<i>Citrus sinensis</i>	fruit	unknown	brown rot	unknown	HQ665161//	negative	negative
<i>Phytophthora hibernalis</i>	PD 011 05011181; CBS 114104; ATTC 56353	<i>Citrus sinensis</i>	unknown	unknown	brown rot	Australia	HQ261576//	negative	negative
<i>Phytophthora syringae</i>	PD 07 03486501; CBS 364.52; IMI 045169	<i>Prunus armeniaca</i>	fruit	unknown	brown rot	New Zealand	MZ416914 //	negative	negative
<i>Stemphylium vesicarium</i>	PD 014 04062778; CBS 191.86	<i>Medicago sativa</i>	leaf	unknown	Stemphylium rot	India	KC584239//	negative	negative
<i>Pseudocercospora angolensis</i> *	PD 07 03205618; CBS 149.53; ATTC 11669	<i>Citrus sinensis</i>	leaf	unknown	Cercospora rot	Angola	JQ324975//	negative	negative
<i>Pseudomonas syringae</i> pv. <i>syringae</i>	PD BAC 2875 ⁵	<i>Citrus × limon</i>	unknown	unknown	black pit	United States of America	n/t ***	negative	negative
<i>Sclerotinia minor</i>	PD 014 04285138; CBS 339.39	<i>Lactuca sativa</i>	unknown	unknown	cottony rot	Italy	KF859929//	negative	negative
<i>Sclerotinia sclerotiorum</i>	PD 93/879	<i>Brassica oleracea</i>	unknown	unknown	cottony rot	Netherlands	MZ416915 //	negative	negative

Table 1 (continued)

Species	Culture number(s) ¹	Host	Isolated from	Symptom	Putative pathogenicity on Citrus fruit	Country	GenBank no. ITS ² /tef ³ /LSU ⁴	CTS real-time PCR	CBS real-time PCR
<i>Septoria citricola</i>	PD 012 04761069; CBS 356.36	<i>Citrus sinensis</i>	fruit	unknown	Septoria rot	Italy	KF251382//	negative	negative
<i>Septoria limonum</i>	PD 014 04285146; CBS 419.51	<i>Citrus limonum</i>	unknown	unknown	Septoria rot	Italy	KF251459//	negative	negative
<i>Trichoderma viride</i>	PD 014 04062761; CBS 101526	<i>cellulosic tissue</i>	undefined	undefined	Trichoderma rot	Netherlands	AY380908//	negative	negative
<i>Trichothecium roseum</i>	PD 012 04764921; CBS 567.50; ATCC 13422	Unknown	unknown	unknown	pink mold rot	unknown	MH856758//	negative	negative
<i>Xanthomonas citri</i> pv. <i>citri</i> *	PD BAC 990 ⁵	<i>Citrus limon</i>	unknown	unknown	bacterial canker	New Zealand	n/t ***	negative	negative
<i>Zasmidium citri</i>	PD 012 04761042; CBS 122455	<i>Citrus</i>	unknown	unknown	greasy spot rind blotch	United States of America	EU514227//	negative	negative

¹ATCC: American Type Culture Collection, Virginia, U.S.A.; BRIP: Plant Pathology Herbarium, Biosecurity Queensland, Dutton Park, Queensland, Australia; CBS: CBS-KNAW Westerdijk Fungal Biodiversity Institute, Utrecht, Netherlands; CPC: Culture Collection of P.W. Crous, housed at CBS, Utrecht, Netherlands; CGMCC: China General Microbiological Culture Collection, Beijing, China; DSM: Leibniz Institute DSMZ, Braunschweig, Germany; IMI: International Mycological Institute CABI-Bioscience, Egham, United Kingdom; IBT: Culture collection Department of Biotechnology Technical University of Denmark, Lyngby, Denmark; GC: Wageningen University and Research –business unit biointeractions and plant health, Wageningen, Netherlands; LGMF: Culture collection of Laboratory of Genetics of Microorganisms, Federal University of Panama, Curibita, Brazil; LSMV: Anses, Laboratoire de la Santé des Végétaux, Malzeville, France; MFLUCC: Mae Fah Luang University Culture Collection, Muang, Thailand; NFW: Culture collection of N.F. Wulandari, Chiang Mai, Thailand; P: Culture collection of M.D. Coffey, University of California, Riverside, United States of America; PD: Dutch National Plant Protection Organization, Wageningen, Netherlands; VIC: Culture collection of Federal University of Viçosa, Viçosa, Brazil

²ITS: Internal transcribed spacer region, including 5.8S gene

³tef1: Translation elongation factor 1 α

⁴LSU: Ribosomal large subunit

Ex-type *Phyllosticta* spp. cultures are in **bold**

The newly generated sequences in this study are in **bold**

* indicates species with EU status (i.e. IIAI)

** atypical lesions (necrosis) observed in artificially inoculated *C. sinensis* fruit (Guarnaccia et al. 2017)

*** not tested; no sequencing performed on bacterial specimen

^A indicates specimens which were multi-locus analysed (i.e. ITS, *tef1*, and LSU) for identification

⁵ indicates specimens that were isolated from intercepted fruits

Brazil (1), China (1), Greece (2), Italy (1), Malta (1), Portugal (1), South Africa (5) and Zimbabwe (1)) other fungal (42) and bacterial (2) species known to occur on citrus fruit were used (Snowdon, 1990; Table 1). Additionally, closely related non-*Citrus Phyllosticta* species (5) were included (Table 1).

DNA extraction

Individual lesions or pure fungal cultures grown on cherry decoction agar (1 cm² aerial mycelium) were added to a tube containing 300 µL extraction buffer (0.02 M PBS buffer, 0.5% Tween 20, 2% polyvinylpyrrolidone, 0.2% bovine serum albumin) and a 3.2 mm stainless steel bead. Material was homogenized using a MM300 Mixer Mill (Retsch, Haan, Germany) for 80 s on 30,000 bpm and the homogenized material (75 µL) was processed using the DNA QuickPick™ SML Plant DNA Kit (Bio-Nobile, Pargas, Finland) on the KingFisher 96 magnetic particle processor (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Positive isolation controls (pooled homogenised black spot or tan spot lesions from resampled positive consignments) and negative isolation controls (*C. sinensis* fruit peels without symptoms) were used for quality assurance during each isolation.

Sanger sequencing

To verify the strain identity of the obtained genomic DNA, sequencing of the internal transcribed spacer (ITS) barcode region (ITS = partial 18S, ITS1, 5.8S, ITS2 and partial 28S) was performed on all fungal specimens listed in Table 1. Amplification was performed with primers ITS1 and ITS4 (Table 2). PCR was performed in a volume of 25 µL containing 300 nM of each primer, 0.08 mM dNTPs, 1.5 mM MgCl₂, 0.8 U Taq DNA polymerase (Roche, Basel, Switzerland) and 2 µL template DNA using the following cycle conditions: 94 °C for 30 s, then 40 cycles of 94 °C for 15 s, 55 °C for 30 s, 72 °C for 90 s, with a final 72 °C for 10 min. In case low quality amplicons were obtained with the ITS1 and ITS4 primers, ITS5 and ITS4 primers (Table 2) were used as an alternative to amplify the ITS barcode. Using a volume of 25 µL, containing 200 nM of each primer, 1X Bio-ACT Short mix form Bio-line (GC biotech, Waddinxveen, Netherlands) and 2 µL template DNA using the following cycle conditions: 95 °C for 5 min, then 40 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 90 s, with a final 72 °C for 10 min.

PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Cycle sequence products were produced for both strands using BigDye Terminator v1.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA) using the amplification primers according to the manufacturer's instructions. Cycle sequence

Table 2 Primers and probes used in this study

Name	Sequence (5'–3')	reference
GcF1	GGTGATGGAAGGGAGGCCT	van Gent-Pelzer et al. (2007)
GcR1	GCAACATGGTAGATACACAAGGGT	van Gent-Pelzer et al. (2007)
GcP1	FAM-AAAAAGCCGCCCGACCTACCTTCA-TAMRA	van Gent-Pelzer et al. (2007)
GpF	GGTGATGAGGGGAGGCCG	this study
GpRv2	CCGGGTCGGCCCGCCAAAGCAACG	this study
GpP	FAM-AAAAAGCCGCCCAACCTACCTTCAC-TAMRA	this study
ITS5	GGAAGTAAAGTCGTAACAAGG	White et al. (1990)
ITS4	TCCTCCGCTTATTGATATGC	White et al. (1990)
ITS1	TTCGTAGGTGAACCTGCGG	White et al. (1990)
EF1-728 F	CATCGAGAAGTTCGAGAAGG	Carbone and Kohn (1999)
EF2	GGARGTACCAGTSATCATGTT	O'Donnell et al. (1998)
LROR	ACCCGCTGAACTTAAGC	Moncalvo et al. (1995)
LR5	TCCTGAGGGAACTTCG	Vilgalys and Hester (1990)

products were purified using the DyeEx 2.0 Spin Kit (Qiagen, Hilden, Germany) prior to sequencing using a 3500 Genetic Analyzer (Life Technologies, Carlsbad, CA, USA).

To confirm species identity, additional sequencing was performed on selected strains of *P. citricarpa*. Multi locus DNA datasets (i.e. *tefl* and LSU) were generated as described by Guarnaccia et al. (2017), with primers EF1-728 F and EF2, and LROR and LR5 respectively (Table 2).

Consensus sequences were generated from both trace files in Geneious R10 (Biomatters, Auckland, New Zealand; Kearse et al., 2012). Amplification primers were trimmed from the consensus sequence, and when needed additional trimming was performed to obtain high quality (PHRED >30) consensus sequence data.

Generated sequences were aligned with MAFFT algorithm (Katoh et al., 2002) in Geneious R10 and queried against the NCBI's GenBank nucleotide database. Newly generated sequences were deposited in GenBank (Table 1).

Real-time PCRs

Based on the alignment of ITS1 locus sequences of *P. citriasiana*, *P. citricarpa* and *P. capitalensis* available from the National Center of Biotechnology Information (NCBI) non-redundant nucleotide database (GenBank) in 2007, primers (GpF and GpRv2) and a probe (GpP) specific for *P. citriasiana* were designed (Table 2). For the detection of *P. citricarpa* the primers (GcF1 and GcR1) and probe (GcP1) described by van Gent-Pelzer et al. (2007) were used.

Molecular detection and identification of *P. citricarpa* and *P. citriasiana* was done by real-time PCR in 30 µL volumes consisting of 250 nM of each primer, 100 nM probe, 1× TaqMan Universal PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and 1 µL template DNA. Real-time PCRs were performed in duplicate on a 7900HT (Thermo Fisher Scientific, Waltham, MA, USA) or on a CFX96 (Bio-Rad, Hercules, CA, USA) using the following cycle conditions: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Real-time PCR reactions were regarded positive for samples producing Ct values <40 and exponential amplification curves. Validation of the real-time PCR test showed that no pre-determined Ct cut-off value was needed.

Positive amplification controls (DNA purified from PD 04 01844862 *P. citricarpa* and PD 06 03125095 *P. citriasiana*) and negative amplification controls (molecular grade water) were used in each real-time PCR run to monitor the performance of the real-time PCR test and to detect potential contamination.

Performance characteristics

The performance of the newly developed *P. citriasiana* real-time PCR test was evaluated by determining the performance characteristics: analytical sensitivity, analytical specificity, selectivity, repeatability and reproducibility following EPPO pm7/98 (4) (EPPO, 2019).

The analytical sensitivity was assessed by determining the limit of detection (LOD). The LOD is defined as the lowest DNA concentration that can be reliably detected with a certain level of confidence. The initial concentration of pure culture *P. citriasiana* genomic DNA was determined with the Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) in combination with the dsDNA High Sensitivity Kit (Life Technologies, Carlsbad, CA, USA), and eight individual dilution series were prepared (range 10 ng to 0.195 pg). The LOD was estimated using the fitted logistic regression model in RStudio v2023.03.1 (RStudio Team, 2020). The proportion of detection was determined per amount of DNA (ng), and the LOD was calculated at a 95% probability level.

Analytical specificity was determined by testing a range of *P. citriasiana* culture strains from varying geographical origin and genetic diversity (i.e. inclusivity) and by testing potential cross-reactions with non-target organisms using DNA extracted from 65 specimens covering 48 different species (i.e. exclusivity; Table 1), the identity of these specimens was verified by sequence analysis of the ITS, *tefl* and/or LSU barcode (as detailed in the section Sanger sequencing).

The real-time PCR test for *P. citriasiana* was performed with DNA in eight individual dilution series in both elution buffer and homogenized citrus skin (range 10 ng to 0.195 pg), to assess the analytical selectivity.

The reproducibility and repeatability were determined by analysing a dilution series of *P. citriasiana* DNA (range 10 ng to 0.195 pg) by three technicians using the newly developed real-time PCR test. In addition, the results of a standardized positive amplification

control (PAC), consisting of *P. citriasiana* DNA, that was included in all 18 routine diagnostic runs between 2017 and 2024, were assessed to determine repeatability.

Citrus spp. consignments

A citrus fruit consignment could consist of one or multiple fruits and each fruit could contain one or more lesions. Using the species specific real-time PCR by van Gent-Pelzer et al. (2007) and the newly developed *P. citriasiana* assay here, the presence of *P. citricarpa* and *P. citriasiana* was tested in a total of 1082 lesions from intercepted citrus fruit consignments. Four-hundred-fifty-four of these individual lesions were taken from 140 individual *C. maxima* consignments. The remaining 628 lesions were obtained from 133 individual non-*C. maxima* consignments (i.e. *C. sinensis*, *C. reticulata*, *C. limon* and *C. paradisi*).

Review of *P. citricarpa* and *P. citriasiana* hosts

The *P. citricarpa* and *P. citriasiana* host distribution was reviewed by compiling reports of nearly 500 *Phyllosticta* isolates (Everett & Rees-George, 2006; Glienke et al., 2011; Guarnaccia et al., 2017; Hu et al., 2014; Miles et al., 2013, 2019; Su & Cai, 2012; Wang et al., 2012; Wikee et al., 2013a, b; Wong et al., 2012; Wulandari et al., 2009; Zhang et al., 2013). The reports were grouped according to their respective host(s), substrate(s) and possible symptom(s). An emphasis was put on the reports of *P. citricarpa* on *C. maxima* and on reports of *P. citriasiana* on non-*C. maxima* species. Europhyt (https://food.ec.europa.eu/plants/plant-health-and-biosecurity/europhyt_en) notifications of CBS on *C. maxima* and CTS on non-*C. maxima* were also examined (reports before the description of *P. citriasiana* were ignored).

Results

Performance characteristics real-time PCRs

ITS barcode sequences generated for all fungal isolates used in this study were 100% identical with specimen or species entries available in NCBI database, except for one isolate (Table 1). The ITS barcode sequence of the type specimen and the only

available isolate of *P. citrimaxima* (CPC 20276) (Wikee et al., 2013a) was 100% identical with ITS barcode sequences of *P. citriasiana*. This finding was confirmed by subsequent multi-locus analyses by the Westerdijk Fungal Biodiversity Institute (Utrecht, The Netherlands), who kindly provided the strain (Personal communication E. Groenewald, Westerdijk Fungal Biodiversity Institute). Therefore this specimen was deemed to be misidentified and thus excluded in the specificity assessment of both CBS and CTS real-time PCRs. Additional *tef1* and LSU sequences that were generated for selected *P. citricarpa* strains did also not show divergence from the sequences in the NCBI database according to the current definition of this species.

Sixty eight fungal and two bacterial isolates confirmed the analytical specificity of both real-time PCR assays, including 48 isolates obtained from citrus material (mainly fruit). There were 37 *Phyllosticta* isolates covering 11 different *Phyllosticta* species including *P. citricarpa* ($n = 16$) and *P. citriasiana* ($n = 7$) from several locations (Table 1). The *P. citriasiana* real-time PCR only detected *P. citriasiana* and generated no false positive results for any of the other species tested which are all known to occur on symptomatic or asymptomatic citrus fruits as described by (Snowdon, 1990).

The standard curve for the *P. citriasiana* real-time PCR assay shows consistent results for eight individually tested dilution series and a detection limit of 2.92 pg DNA was determined at a 95% detection probability (Table S2; Figure S1).

The assay was further validated through the detection of *P. citriasiana* DNA diluted in both elution buffer and homogenized citrus skin. Small differences between the dilution series in host tissue homogenate and elution buffer were observed, with increasing differences when DNA concentrations decrease. These are attributed to differences in pipetting rather than a true substrate effect (Table S3; Figure S2).

Consistent detection results between three individual laboratory technicians, with standard deviations ranging from 0.1 to 1.8 cycles, confirmed the reproducibility of the real-time PCR test (Table S4). The PAC results after implementation of the test for routine diagnostics also demonstrates reproducibility. Positive results were obtained in all 18 runs, with a mean Cq value of 25.9 ± 0.5 , Cq values never exceeded the mean Cq $\pm 2SD$ level.

The *P. citricarpa* real-time PCR by van Gent-Pelzer et al. (2007) did not generate false positive results for any of the other species tested (Table 1).

Presence of *P. citricarpa* and *P. citriasiana* in EU imported fruits

Using two simplex real-time PCRs the presence of *P. citriasiana* was only found on *C. maxima* (413 individual lesions obtained from 135 consignments; Fig. 1) and *P. citricarpa* was only found on *Citrus* spp. other than *C. maxima* (466 individual lesions from 107 consignments; Fig. 2).

The remaining consignments/lesions of *C. maxima* (5 consignments; 41 lesions) and non-*C. maxima* (26 consignments; 162 lesions) were negative for both target organisms. Although lesions were present, in all of these cases pycnidia and *Phyllosticta* conidia were absent. Both organisms were never detected simultaneously (Table S5). Consignments with *P. citricarpa* came from South Africa, Brazil, Argentina, Uruguay, Swaziland, China, Cuba and Zimbabwe, whilst consignments with *P. citriasiana* were restricted to China, Vietnam and Thailand. The origin of one additional CTS positive consignment remains unknown (Table S1).

Phyllosticta citriasiana host distribution in literature

The host associations of *P. citricarpa* in literature, published after 2009 (year of publication of *P. citriasiana*), only report *C. maxima* in a few cases ($n = 14$; Table S6). In these cases, an attempt was made to check both the fungal and host species. In all cases ($n = 6$) that we could review, either the host or the fungal species were corrected, some cases were not resolved ($n = 8$).

Discussion

An exclusive occurrence of *P. citricarpa* on non-*C. maxima* citrus fruits and *P. citriasiana* on *C. maxima* fruits was observed in this study. Overall, the reported combinations of CBS and CTS on their respective hosts followed our host occurrence hypothesis, however, a few reports of *P. citricarpa* on *C. maxima* were found (Table S6). In some of these cases authors and/or diagnostic laboratories could be contacted and diagnostic results could be checked, in all these cases there is evidence for a misidentification of either the fungus or the host (Table S6). In other cases it proved difficult to confirm or refute the host or fungal species identity (Glienke et al., 2011; Wikee et al., 2013a).

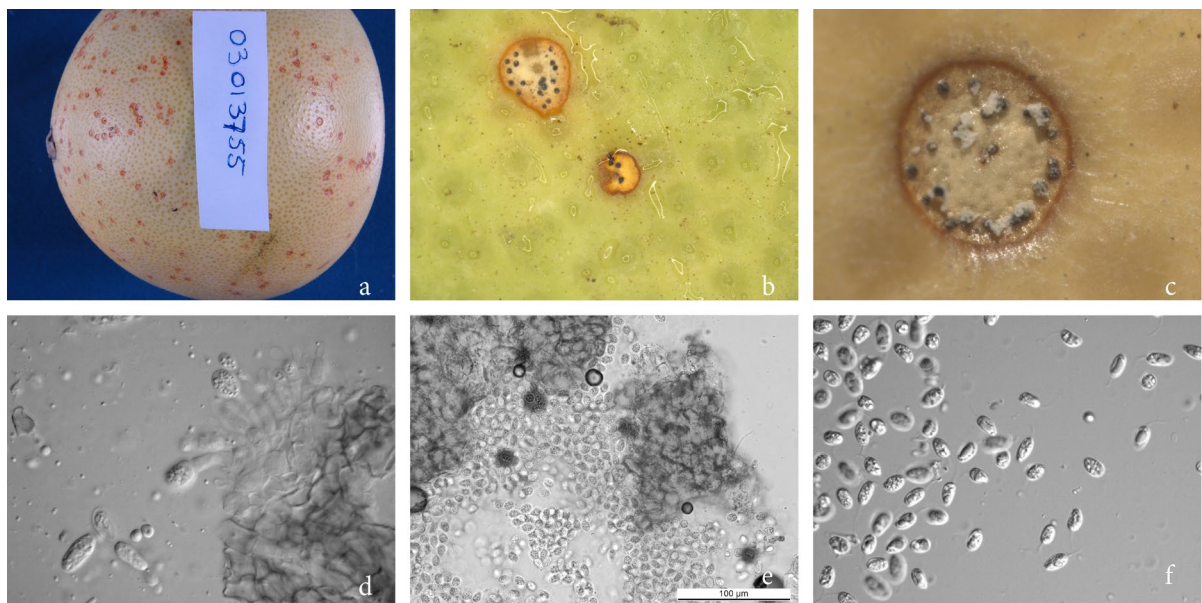


Fig. 1 *Phyllosticta citriasiana* on *Citrus maxima*. **a.** CTS symptoms on a *C. maxima* fruit. **b.** Tan spot lesions with pycnidia. **c.** Tan spot lesion with pycnidia oozing conidial masses.

d. Conidiogenous cells giving rise to conidia. **e.** Pycnidial wall and conidia. **f.** Conidia with mucoid sheaths and apical mucilaginous appendages

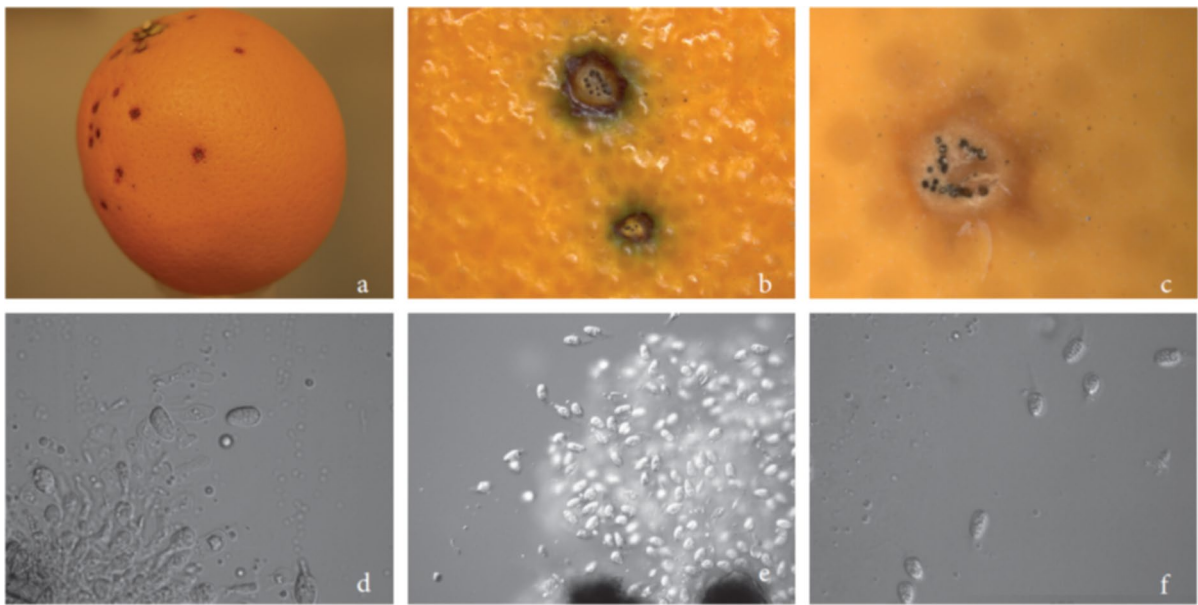


Fig. 2 *Phyllosticta citricarpa* on *Citrus sinensis*. **A.** CBS symptoms on a *C. sinensis* fruit. **B.** Black spot lesions with pycnidia. **C.** Black spot lesion with pycnidia oozing conidial

masses. **D.** Conidiogenous cells giving rise to conidia. **E.** Pycnidial wall and conidia. **F.** Conidia with mucoid sheaths and apical mucilaginous appendages

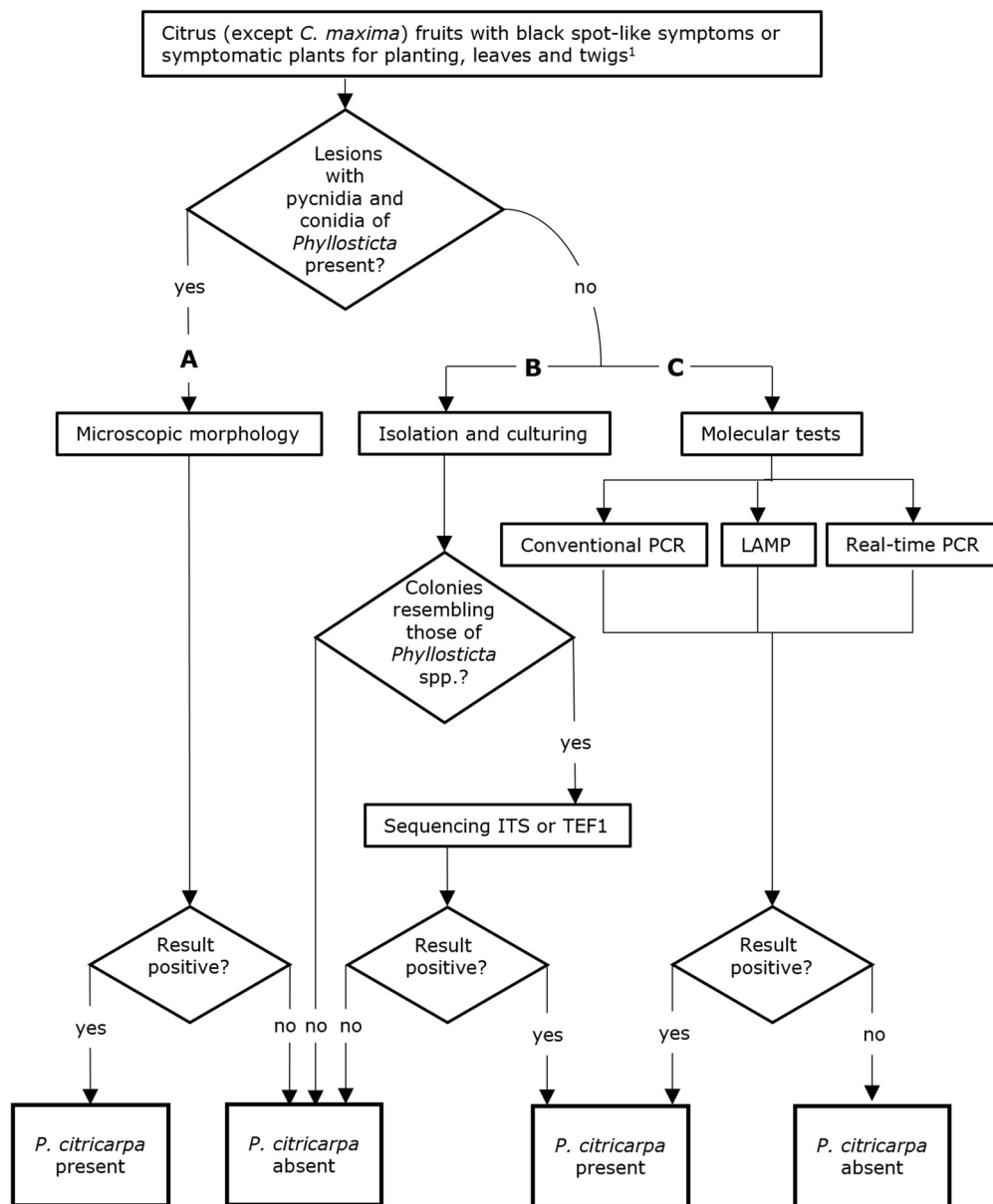
Seven different *Phyllosticta* species are presently known to occur on *Citrus* spp., namely *P. citricarpa*, *P. citriasiana*, *P. citrimaxima*, *P. capitalensis*, *P. paracapitalensis* and *P. citrichinaensis* which are all reported from fruit, whereas *P. citribraziliensis* is only found on leaves. *P. capitalensis*, *P. paracapitalensis* and *P. citribraziliensis* are known as asymptomatic endophytes on *Citrus* spp. (Everett & Rees-George, 2006; Glienke et al., 2011; Guarnaccia et al., 2017; Hu et al., 2014; Miles et al., 2013; Wang et al., 2012; Wikee et al., 2013a; Wulandari et al., 2009),

P. citrimaxima, described from Thailand as a second *Phyllosticta* species causing tan spot on *C. maxima* fruit (Wikee et al., 2013a) could not be included in our analytical specificity tests because the only available isolate turned out to be misidentified and could not be distinguished from *P. citriasiana* by multi-locus sequencing. The only specimen on which Wikee et al. (2013a) based their species description was reported lost by Guarnaccia et al. (2017). To date no data are available that claim the occurrence of *P. citrimaxima* on non-*C. maxima* fruits. Attempts to isolate this species from *C. maxima* fruits exhibiting tan spot symptoms proved unsuccessful thus far. *P. citrimaxima* is consequently considered a species

inquirenda. Given the results of the in silico BLAST test it is not expected that *P. citrimaxima* interferes with the real-time PCRs for either *P. citriasiana* or *P. citricarpa*.

Several *C. maxima* and non-*C. maxima* consignments/lesions proved negative for both target organisms, suggesting a non-specific symptom was observed in the consignment.

A species-specific real-time PCR assay was successfully developed for *P. citriasiana*. The assay proved to be specific and highly sensitive for detection of *P. citriasiana*. The real-time PCR assay for *P. citricarpa*, developed by van Gent-Pelzer et al. (2007), was also highly specific. These assays facilitate quick diagnosis of both *P. citricarpa* and *P. citriasiana* respectively. The van Gent-Pelzer et al. (2007) real-time PCR assay, which is based on the multicopy ribosomal DNA internal transcribed spacer (ITS), is sensitive and is well suited for phytosanitary diagnostics. Subsequently several other real-time PCR assays have been developed either also based on multicopy (Hu et al., 2014; Schirmacher et al., 2019) or single copy loci (Ioos et al., 2023; Zajc et al., 2023). These assays were to improve specificity and distinction between closely related species (e.g. the now



¹Paths A, B and C have been validated on fruits not on other plant material (see: EPPO, 2020)

Fig. 3 Proposed diagnostic flow diagram for the identification of *P. citricarpa* on citrus fruits. For fruits of *Citrus* spp. with pycnidia and conidia a direct morphological path (A) is available. For non-*C. maxima* citrus fruits without pycnidia and

conidia a morphological (*in vitro*) path (B) and molecular path (C) is available. On non-*Citrus maxima* fruits a direct morphological (*in planta*) identification of *P. citricarpa* is only possible if lesions, pycnidia and conidia are present

synonymised *P. paracitricarpa*). For phytosanitary diagnostics at citrus import where only symptomatic citrus fruits are tested, sensitivity is the key performance characteristic, thus an assay based on a multi-copy gene is preferred. Since there were no quality

assurance issues with the assay by van Gent-Pelzer et al. (2007) there was no reason to change to another assay.

Although *P. citriasiana* is not regulated in the EU (or elsewhere), this new real-time assay fulfils a

phytosanitary purpose by providing a quick, sensitive and specific diagnostic tool for official surveys or in case of new outbreak situations. Moreover, this assay provides a means to establish a better understanding of the global distribution of this citrus pathogen and this may aid effective citrus production and risk based phytosanitary measures.

The exclusive occurrence of *P. citricarpa* on fruits of non-*C. maxima* citrus species observed in this study, supports re-inclusion of direct morphological *in planta* identification of *P. citricarpa* on all *Citrus* spp. It also questions the need to inspect *C. maxima* for *P. citricarpa*. Given the unresolved reports of *P. citricarpa* on *C. maxima* (Table S6) targeted inspections of *C. maxima* for CBS are recommended. Attention could be given to specific countries in Asia (i.e. Philippines and China), Africa (i.e. Benin, Cameroon and South Africa) and South America (Brazil) (Table S6). The few known reports of CBS on *C. maxima* obscure a straightforward CTS identification on *C. maxima*. Therefore, our suggested diagnostic flow diagram (Fig. 3) separates *C. maxima* from non-*C. maxima* fruits at the beginning of the diagnosis to facilitate a direct morphological identification of CBS on non-*C. maxima*.

Morphological *in planta* diagnosis of CBS on citrus fruit –based on distinct symptoms and the presence of both pycnidia and *Phyllosticta* conidia– was considered cheap, fast and effective until *P. citriasiana* (Wulandari et al. 2009) was described and detailed information concerning host specificity of *P. citricarpa* and *P. citriasiana* was still lacking. Currently, both EPPO and IPPC diagnostic standards on CBS propose isolation and culturing or molecular tests (i.e. conventional PCR, real-time PCR (*in planta* or *in vitro*) and or sequencing (EPPO, 2020; FAO, 2016). An additional third molecular test (i.e. LAMP; Tomlinson et al., 2013) is included in the latest version of the EPPO standard on CBS.

Given the speed and relatively low costs of specific *in planta* real-time or conventional PCRs are preferred by National Plant Protection Organisations (NPPOs) over culturing and sequence based techniques.

The present results suggest that the re-implementation of a morphological identification of CBS on non-*C. maxima* fruits –based on distinct symptoms and the presence of both pycnidia and *Phyllosticta*

conidia– is a valid diagnostic procedure. This would be the fastest reliable method available and therefore it would be justified to adapt the EPPO and IPPC diagnostic protocols accordingly.

Only *P. citricarpa*, *P. citriasiana* and *P. citrimaxima* are found to cause distinct lesions with pycnidia and conidia on citrus fruit. *P. citrimaxima* was not taken into account because *in silico* analyses have shown that the real-time PCR assays for *P. citricarpa* will not detect *P. citrimaxima*. Furthermore *P. citrimaxima* is reported to be distinguishable via morphological identification.

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Data Availability All sequences in this study are available at <https://www.ncbi.nlm.nih.gov/genbank/>, including the newly generated sequences with GenBank numbers: MZ416900-MZ416919 and OK087464-OK087475. All other data generated or analysed during this study are included in this published article and its supplementary files.

Declarations

Competing interests The authors state they have no competing interests.

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