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Highly specific qPCR and amplicon sequencing method for detection of quarantine citrus pathogen *Phyllosticta citricarpa* applicable for air samples

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Abstract

The fungus *Phyllosticta citricarpa* is a quarantine pathogen in the EU and is of high economic importance in many parts of the world where favourable climate conditions drive the development of citrus black spot (CBS) disease. Disease symptoms include necrotic lesions on leaves and fruits. Low disease pressure can reduce crop marketability, while higher disease pressure can cause premature fruit drop, significantly increasing crop losses. The wind-dispersed spores of *P. citricarpa* are especially problematic for rapid pathogen dispersal, but also provide an opportunity for early detection of the disease spreading into a new area. In this study we have developed and validated a quantitative PCR (qPCR) assay based on the TEF1-α sequence. Specificity testing demonstrated that it is currently the only qPCR assay that does not cross-react with closely related *Phyllosticta* species. The assay is sensitive and can detect a single copy of the *TEF1* gene in a reaction, it is highly repeatable and reproducible and can be used for testing of the sticky tapes from spore traps as well as citrus fruit samples. High-throughput sequencing (HTS) of the DNA barcodes ITS1 and *TEF1* was also explored for the detection and discrimination of *P. citricarpa*. The limit of detection of the HTS was 1000 spores on a daily spore trap tape. This study makes an important improvement to the diagnostics of the CBS and the methods developed can also be applied to improve the surveillance and early detection of the pathogen when linked to spore samplers in the field.

KEYWORDS
detection, fungal spore sampling, internal transcribed region (ITS), translation elongation factor 1-α (*TEF1*)

[†]Deceased.

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1 | INTRODUCTION

The ascomycetous fungus *Phyllosticta citricarpa* (syn. *Guignardia citricarpa*) is an economically important fruit and foliar pathogen of *Citrus* species with increasing importance throughout the globe. It causes citrus black spot (CBS) disease with various symptoms such as hard spots, virulent spots, freckle spots and false melanose on fruit and lesions on leaves and twigs of almost all types of commercially produced citrus cultivars, especially lemons (*Citrus limon*) and sweet oranges (*C. sinensis*). Citrus fruits affected by CBS are unsuitable for the fresh market due to their cosmetic appearance. Severe infections cause premature fruit drop (Machado et al., 2022) and asymptomatic fruits at harvest may develop visible symptoms later during transportation or storage (Kotzé, 1981), when the commercial loss is even higher.

The species *P. citricarpa* is heterothallic: complementary mating types are needed for sexual reproduction (Tran et al., 2017). Sexual ascospores produced in pseudothecia on leaf litter may be wind-dispersed over large distances. Asexual conidia produced in pycnidia on fruit lesions, leaves, twigs and leaf litter are dispersed by rain splash over shorter distances (Perryman et al., 2014). In general, CBS epidemics are characterized by a long lag phase. The pathogen may be present in an area before symptoms are observed. Then, it may take years from the first symptoms being noticed until the disease reaches epidemic proportions, depending on host susceptibility and environmental conditions favourable for the infection (Kotzé, 1981). CBS disease is present in many citrus-growing regions with favourable climatic conditions in Asia, Africa, America and Australia. The suitability of the climatic conditions of the Mediterranean areas for the development of CBS has been debated (EFSA PLH Panel, 2014; Magarey et al., 2015; Martínez-Minaya et al., 2015, 2018; Yonow et al., 2013). Nevertheless, the disease has been recently confirmed to be widespread in the main citrus-growing region in Tunisia (Boughalleb-M'Hamdi et al., 2020).

P. citricarpa is a quarantine pathogen in the European Union (EU) under phytosanitary Regulations 2016/2031 and 2019/2072 (Anon, 2016, 2019). It is also included in the list of priority pests, for which annual surveys by Member States are mandatory, by Regulation 2019/1702. Guarnaccia et al. (2017) reported the presence of *P. citricarpa* from leaf litter of *C. sinensis* and *C. limon* from around trees in Italy, Malta and Portugal; none of the trees were reported to be displaying any CBS symptoms. However, official surveys conducted by the National Plant Protection Organizations (NPPOs) have not confirmed those findings (EFSA PLH Panel et al., 2018).

For quarantine pathogens such as *P. citricarpa* early detection is key to initiate effective outbreak response programmes. As introduced pathogens spread within a region, the scale of the problem increases and the potential for its eradication or containment is drastically diminished. The wind-dispersed ascospores of *P. citricarpa* that spread rapidly over relatively large geographical areas can be particularly problematic in this respect (EFSA, 2020). However, ascospores and conidia also present an opportunity for the early detection of *P. citricarpa* as they can be trapped and identified. One challenge is that spores of *P. citricarpa* are morphologically indistinguishable from other *Phyllosticta* species of nonquarantine relevance in the EU, such as *P. capitalensis*. Earlier works on airborne ascospore monitoring, based solely on morphological identification, were affected by this lack of specificity (Fourie et al., 2013). More recently, air samplers have been coupled with postsampling immunological or DNA-based methods to increase specificity (Tran et al., 2020; West & Kimber, 2015), and spore trapping followed by quantitative PCR (qPCR) was used to monitor the airborne inoculum of *P. citricarpa* in Queensland, Australia (Tran et al., 2020).

Hence, the aim of our study was to evaluate the performance of molecular methods for the detection of *P. citricarpa* in air samples collected in citrus orchards. First, two qPCR assays for the identification of *P. citricarpa* were used: targeting the rDNA internal transcribed spacer (ITS) sequence that discriminates *P. citricarpa* from the endophyte *P. capitalensis* (EPPO, 2020; Van Gent-Pelzer et al., 2007) and a qPCR assay that discriminates between *P. citricarpa*, *P. citriasiana* and *P. capitalensis* (Schirmacher et al., 2019). Importantly, both available qPCR assays do not differentiate between *P. citricarpa* and its closely related *P. paracitricarpa*, first described in China (Wang et al., 2012), which induces similar lesions to *P. citricarpa* when artificially inoculated onto citrus fruits (Guarnaccia et al., 2017). Therefore, a more specific qPCR assay for *P. citricarpa*, targeting the translation elongation factor 1-α gene (*TEF1*), was developed and evaluated. Furthermore, a nontargeted approach based on high-throughput sequencing (HTS) of fungal DNA barcodes was applied. HTS of the ITS has become a standard and reliable (Elbrecht et al., 2017) tool for profiling complex fungal communities across diverse habitat types, including air (Aguayo et al., 2021; Banchi et al., 2018, 2020; Nicolaisen et al., 2017), and has provided significant advances in our understanding of microbial diversity. However, use of ITS barcodes does not provide sufficient taxonomic resolution to capture the full range of microbial diversity present in a given sample (Aguayo et al., 2021; Bakker, 2018; Banchi et al., 2020). Thus, sequencing of two fungal DNA barcodes, ITS1 and *TEF1*, was performed on selected samples to assess the performance of HTS methods to be used for *P. citricarpa* detection, with the potential future benefit of being a broad-spectrum approach for surveillance of a range of pathogens in air samples.

2 | MATERIALS AND METHODS

2.1 | Fungal isolates, plant and environmental material

The performance of the qPCR assays was evaluated using different isolates of *P. citricarpa* and other pathogenic and nonpathogenic citrus-colonizing fungi (Table 1). DNA of the mock community of 19 fungal taxa from Ascomycota, Basidiomycota, Zygomycota, Glomeromycota and Chytridiomycota was received from M.G. Bakker (Mycotoxin Prevention and Applied Microbiology, USDA ARS) (Bakker, 2018).

TABLE 1 List of target and nontarget isolates used in testing the specificity of the quantitative PCR assays

Species	Code	Host	Origin	18S assay (C_q)	FQ assay (C_q)	PC assay (C_q)	PC-2 assay (C_q)	Pc-TEF1 assay (C_q)
Phyllosticta citricarpa	CBS 828.97	*Citrus aurantium*	Brazil	26.0	n.t.	19.8	22.6	26.6
	CBS 102374	*C. aurantium*	Brazil	33.3	n.t.	24.0	28.7	32.8
	IVIA001	*Citrus sinensis*	Brazil	24.2	18.0	17.4	21.1	26.2
	IVIA003	*C. sinensis*	Brazil	22.8	19.8	20.1	23.0	28.0
	IVIA005	*C. sinensis*	Brazil	24.9	17.6	16.5	20.6	26.0
	IVIA007	*C. sinensis*	Brazil	20.6	17.7	16.6	19.7	25.4
	IVIA011	*C. sinensis*	Brazil	23.1	19.9	20.8	23.7	28.8
	IVIA012	*C. sinensis*	Brazil	20.5	18.0	17.2	20.1	25.8
	IVIA014	*C. sinensis*	Brazil	22.5	20.4	18.2	21.8	26.4
	IVIA023	*C. sinensis*	Brazil	23.9	22.9	20.6	22.9	28.4
	IVIA034	*Citrus limon*	Argentina	23.1	24.2	19.2	22.1	27.5
	IVIA038	*C. sinensis*	Brazil	20.4	20.9	16.6	19.1	24.4
	IVIA039	*C. sinensis*	Brazil	19.5	20.9	15.7	18.3	24.0
	IVIA040	*C. sinensis*	Brazil	20.9	21.8	16.8	19.5	24.9
	IVIA042	*C. sinensis*	Brazil	22.0	23.4	17.9	21.0	26.4
	IVIA043	*C. sinensis*	Brazil	20.1	20.8	16.3	18.9	24.4
	IVIA045	*C. sinensis*	Brazil	21.8	22.3	17.5	20.0	25.4
	IVIA047	*C. limon*	South Africa	20.6	21.8	16.9	19.1	24.6
	GC092	*C. limon*	South Africa	29.8	30.5	26.8	28.6	33.8
	IVIA081	*Citrus paradisi*	South Africa	22.0	22.6	19.0	21.4	26.8
	IVIA090	*C. sinensis*	Brazil	23.2	21.8	17.8	20.9	27.0
	IVIA091	*C. sinensis*	South Africa	23.9	23.8	18.9	22.1	28.0
	IVIA093	*C. sinensis*	Brazil	22.2	23.1	19.5	22.0	27.7
	IVIA099	*C. sinensis*	Brazil	24.5	23.0	19.4	21.9	27.0
	IVIA115	*C. limon*	Argentina	22.1	18.1	18.1	20.0	25.3
	IVIA116	*C. limon*	Argentina	23.0	19.2	19.2	21.0	26.6
	IVIA117	*C. limon*	Argentina	23.2	19.7	19.2	21.3	26.7
	IVIA125	*C. limon*	China	23.7	21.1	16.8	19.7	26.1
	CBS 127451	*Citrus reticulata*	Australia	22.0	n.t.	19.0	21.1	24.6
	IIA-GC003NA	*C. sinensis*	Angola	27.8	20.5	20.3	22.4	28.4
	CBS 127455	*C. sinensis*	Australia	25.8	n.t.	20.5	23.3	26.9
	CBS 122670	*C. sinensis*	South Africa	28.5	27.1	24.9	28.8	33.1
	ER 2012	*C. sinensis*	Zimbabwe	23.1	20.7	18.7	20.9	24.9
Phyllosticta paracitricarpa	CBS 141359	*C. limon*	Greece	25.4	25.0	22.9	24.8	neg
	CBS 141357	*C. limon*	Greece	21.5	21.2	20.1	19.2	neg
Phyllosticta capitalensis	CBS 120490	*C. paradisi*	USA	20.8	n.t.	neg	neg	neg
	IVIA004	*C. sinensis*	Brazil	22.8	19.7	neg	neg	neg
	IVIA031	*C. sinensis*	Argentina	19.3	19.2	neg	neg	neg
	IVIA095	*C. sinensis*	South Africa	23.7	23.2	36.1	39.0	neg
	IVIA098	*C. sinensis*	Brazil	23.4	19.0	neg	neg	neg
	IVIA118	*C. sinensis*	Brazil	24.3	18.8	35.0	38.4	neg
	IVIA122	*C. sinensis*	Argentina	24.2	18.3	36.0	>40	neg
	IVIA123	*C. sinensis*	Argentina	35.3	17.8	39.5	neg	neg
	IVIA124	*C. sinensis*	Argentina	29.1	18.3	37.8[a]	neg	neg
	ER 1897	*Citrus* sp.	ILVO	21.4	n.t.	35.2[a]	neg	neg

TABLE 1 (Continued)

Species	Code	Host	Origin	18S assay (C_q)	FQ assay (C_q)	PC assay (C_q)	PC-2 assay (C_q)	Pc-TEF1 assay (C_q)
Phyllosticta citriasiana	IVIA120	*Citrus maxima*	China	27.2	19.5	36.3	>40	neg
	IVIA121	*C. maxima*	China	22.8	19.3	>40	38.8[a]	neg
	ER 1891	*Citrus* sp.	China	22.1	n.t.	39.4	neg	neg
Botrytis cinerea	KIS20-1005	*Malus domestica*	Slovenia	25.6	18.3	neg	neg	neg
Colletotrichum gloeosporioides	ER 1063	*C. sinensis*	Italy	28.5	19.0	neg	neg	neg
Cercospora sp.	ER 1867	*C. limon*	Italy	22.0	18.8	neg	neg	neg
Colletotrichum sp.	ER 2100	*C. maxima*	Italy	32.3	24.7	neg	neg	neg
Colletotrichum sp.	KIS20-794	*M. domestica*	Slovenia	21.2	17.3	neg	neg	neg
Diaporthe citri	ER 1356	*C. limon*	Italy	27.1	19.6	neg	neg	neg
Mycosphaerella sp.	ER 1800	*C. limon*	Italy	21.6	20.8	neg	neg	neg
Penicillium sp.	ER 891	*C. sinensis*	Italy	23.7	23.8	neg	neg	neg
Phytophthora sp.	KIS20-1040	*Citrus* sp.	Slovenia	20.5	25.2	neg	neg	neg
Plenodomus tracheiphilus	ER 2221	*C. sinensis*	Italy	29.3	17.2	neg	neg	neg
Mock community	Bakker (2018)	na	na	n.t.	n.t.	neg	n.t.	neg

Note: Performance of the new Pc-TEF1 assay was compared to other qPCR assays for *Phyllosticta* detection (PC, Van Gent-Pelzer et al., 2007; PC-2, Schirmacher et al., 2019). Control assays (18S, 18S rDNA assay; FQ, FungiQuant) were used for confirmation of DNA presence in the sample. Isolates labelled CBS were obtained from the culture collection CBS-KNAW, isolates labelled KIS were obtained from culture collection at Agricultural Institute of Slovenia, and isolates labelled with ER from Italy were from CREA-DC culture collection. Isolates labelled IVIA/GC and ER from Brazil, Argentina, South Africa, China and Zimbabwe were obtained from fruit imported to Spain (IVIA/GC) or Italy (ER) and intercepted during phytosanitary inspections. The isolate from Angola was described by Bassimba et al. (2018). The host from which the original isolate was obtained is indicated. Results of qPCR analysis are given as average C_q values calculated from three technical replications or >40 when the C_q values were above 40.

Abbreviations: n.t., not tested; neg, no amplification signal.

[a]Indicates that only one replicate was positive.

Samples used in this validation study consisted of citrus fruit, mycelium and spores trapped onto Melinex tape as used in Hirst-type volumetric spore traps (Burkard Scientific Ltd). Healthy (asymptomatic) sweet orange fruits were purchased in Slovenia. Symptomatic lemon and pomelo fruits originating from Tunisia and China, respectively, were imported into Italy and intercepted during phytosanitary inspections. A spore trap was deployed in 2018 in a sweet orange orchard at IVIA, where *P. citricarpa* is absent, to provide background spore tapes. The spore trap was placed in the centre of the orchards with the orifice 0.5 m above the floor and a flow rate of 10 L/min. Airborne particles in the orchard air were trapped onto a rotating drum with a Melinex plastic tape coated with silicone oil (CAS no. 63148-62-9; Merck) with a viscosity of 100 mPa/s. The drum completed one rotation per week and the plastic tape exposed to the orchard air was replaced each week with a clean unexposed plastic tape. The spore traps operated continuously to provide background spore tapes (i.e., exposed), that were stored at 4°C before further processing.

2.2 | DNA extraction

Optimization and evaluation of the spore trap tape testing protocol, from DNA extraction to qPCR analysis, was done on daily sections (48 mm long) of Melinex tapes. Tapes were cut and transferred with sterile, DNase- and RNase-free disposable forceps into a 2 ml screw cap tube filled with 1 g of 0.5 mm zirconia silica beads (Next Advance) and one 5 mm ceramic sphere (MP Biomedicals). To each tube, 800 µl CTAB lysis buffer (120 mM sodium phosphate buffer pH 8, 2% CTAB, 1.5 M NaCl, 2% antifoam B emulsion) and 16 µl (1 mg/ml) of RNase A was added.

Samples were vortexed for 2 min at maximum speed and incubated at 65°C for 20 min. They were transferred to a FastPrep homogenizer (24/2; MP Biomedicals) and shaken at 6 m/s for 60. After centrifugation at 8000 × *g* for 3 min, the supernatant was transferred to the filters of a Nucleospin Plant 2 Kit (Macherey-Nagel). From the filtration of the supernatant onwards, the DNA extraction was performed according to the manufacturer's instructions. Finally, the extract was eluted in 50 µl of prewarmed (65°C) PE buffer. The same protocol was used for the extraction of DNA from pure culture as follows. Isolates were grown for 14 days at room temperature on potato dextrose agar (PDA), 100–200 mg of fresh mycelium was scraped from the culture surface and from symptomatic and asymptomatic citrus peel and processed as described above.

For each DNA extraction a negative control of extraction, that is, buffer without Melinex tape, fungal mycelium or citrus fruit tissue, was subjected to the same procedure.

Each DNA sample was diluted 10-fold in molecular-grade water and tested in triplicate using qPCR. In case of inhibition observed in qPCR, a 100-fold dilution of the DNA was prepared and tested.

2.3 | Design of new qPCR assay to differentiate *Phyllosticta* species

Sequences of ITS, *actA*, *TEF1*, *gapdh*, LSU and *rpb2* of *P. citricarpa*, *P. paracitricarpa*, *P. citriasiana*, *P. capitalensis* and *P. paracapitalensis* available in GenBank were aligned using Clustal Omega and inspected for regions suitable for differentiation among *P. citricarpa* and *P. paracitricarpa*. The *tef1* sequence of *P. citricarpa* (acc. no. JF343604) was used for primer design at a location where three single-nucleotide polymorphisms (SNPs) and two point deletions/insertions were found to differentiate the sequence of *P. citricarpa* from *P. paracitricarpa*. Primers and a probe were designed using Primer Express software (Applied Biosystems). The secondary structure formation of the *TEF1* amplicon was tested using the OligoAnalyzer tool (Integrated DNA Technologies, Inc.), and the specificity of binding was tested using BLAST searches of the nonredundant nucleotide database. The assay was referred to as the Pc-TEF1 assay and used primer pair Pc-TEF1-F, Pc-TEF1-R and specific TaqMan Pc-TEF1-Probe (Table 2). The probe was labelled with a 5′ VIC reporter and a 3′ nonfluorescent minor groove binder (MGB) quencher (Kutyavin et al., 2000) to increase the specificity of the probe and expand the options of multiplexing with other assays.

2.4 | Validation of the Pc-TEF1 qPCR assay

2.4.1 | Specificity of the Pc-TEF1 qPCR assay

The specificity of the Pc-TEF1 qPCR assay was evaluated by (a) in-silico analysis of the *TEF1* amplicon sequence similarities to *Phyllosticta* species and other non-*Phyllosticta* sequences available in the nonredundant nucleotide database (81,750,643 sequences, database posted on 1 May 2022) using the MEGABLASTN algorithm; (b) qPCR analysis of DNA from 34 *P. citricarpa*, 2 *P. paracitricarpa*, 11 *P. capitalensis*, 3 *P. citriasiana* isolates, other citrus-associated fungi and common air-dispersed fungi (Table 1); (c) qPCR analysis of the DNA from spore trap plastic tapes, sampled at IVIA citrus orchard and spiked with *P. citricarpa* conidial suspensions (see Section 2.4.2); (d) local BLASTN analysis of the ITS1 and *TEF1* metagenomic reads of the tested spore trap plastic tapes (described in 2.1), and a mock community (Bakker, 2018) with a known fungal composition (data used for identification of fungi present in real samples).

All DNA samples listed above were tested using the Pc-TEF1 assay and in parallel with two previously published qPCR assays targeting the ITS1 of *P. citricarpa*: (a) the PC assay developed by Van Gent-Pelzer et al. (2007); and (b) the PC-2 assay published by Schirmacher et al. (2019). In addition, general amplicons for detection of eukaryotic DNA (Eukaryotic 18S rRNA Endogenous Control, 4319413E; Applied Biosystems) and all fungi (Liu et al., 2012; FQ assay), were used to confirm the presence of fungal DNA in the sample (Table 2) and thus exclude false negative results.

2.4.2 | Analytical and diagnostic sensitivity of qPCR assays

The analytical sensitivity of the Pc-TEF1 assay was determined based on a dilution series of DNA extracted from *P. citricarpa* (CBS 127451) mycelium tested in three replicates. The dynamic range, where the method performs in a linear manner, was determined, and in this range, the slope of linear regression line (k) and squared regression (R^2) between the logarithmic values of the relative DNA concentrations and the C_q values was calculated. Known numbers of spores applied to exposed (run within a spore sampler in a known-healthy orchard at IVIA) and unexposed Melinex tapes were used to determine the diagnostic sensitivity of the qPCR assays. Tapes were divided into seven pieces, each of 48 mm in length, corresponding to one sampling day. For spiking the tapes, a conidial suspension of *P. citricarpa* was used instead of ascospores, as conidia can be produced in culture in large amounts. A conidial suspension was prepared by gently scraping the surface of 1-month-old cultures of *P. citricarpa* (GC092) grown on PDA with a slightly moistened brush. The extracted conidia and mucilage were placed in an Eppendorf tube filled with 1 ml of sterile distilled water. A drop of Tween 80 was added to this base solution to facilitate additional conidia desegregation. The concentration of the conidial suspension was determined using a haemocytometer under the microscope at 400× magnification and serially diluted concentrations of 10^6, 10^5, 10^4, 10^3, 10^2 and 10 conidia/ml were prepared. A 100 µl drop of each concentration was pipetted onto exposed (see Section 2.1) and unexposed plastic tapes and then allowed to dry in the laminar flow hood. As described above, the plastic tapes were further subjected to homogenization, DNA extraction and qPCR analysis.

2.4.3 | Repeatability and reproducibility of the Pc-TEF1 qPCR assay

The repeatability of the Pc-TEF1 assay was evaluated by analysing three replicates of DNA samples containing high (380–3100 copies/reaction), medium (40–80 copies/reaction) and low (3–15 copies/reaction) concentrations of the *TEF1* target gene.

The within-laboratory reproducibility was analysed for samples with medium (c.500 *TEF1* copies/reaction) and low (c.50 *TEF1* copies/reaction) amounts of target DNA in 32 different qPCR runs on 28 different days, with two different devices (ABI 7900 HT Fast and ViiA7; Applied Biosystems) and different lots of chemicals. In run number 20 the probe manufacturer was changed (from Applied Biosystems to Microsynth AG).

The assay performance was further evaluated by testing the inhibitory effect of field samples of DNA extracted from air sampled in

TABLE 2 List of quantitative PCR assays used in the study, targeting *Phyllosticta citricarpa* (and other related species with some assays), all fungi (broad range fungi assay FQ) and eukaryotic DNA (18S rRNA assay)

Target organisms	Assay code	Target sequence	Primer/probe designation	Primer/probe sequence (5′–3′)	Reference
Phyllosticta citricarpa (including *P. paracitricarpa*, *P. citriasiana*)	PC	ITS	GcF	GGTGATGGAAGGGAGGCCT	Van Gent-Pelzer et al. (2007); EPPO (2020)
			GcR	GCAACATGGTAGATACACAAGGGT	
			GcP	FAM-AAAAAGCCGCCCGACCTACCTTCA-TAMRA	
P. citricarpa (including *P. paracitricarpa*)	PC-2	ITS	Gc-F2	AGGTGATGGAAGGGAGGCCTT	Schirmacher et al. (2019)
			Gc-R2	CAGGCGTCCTGGCCTAGAG	
			Gc-Probe	FAM-AAAAAGCCGCCCGACCTACCTTCA-BHQ	
P. citricarpa	Pc-TEF1	TEF1	Pc-TEF1-F	GAAGGTCAGTTGCCTCACACTTT	This study
			Pc-TEF1-R	GTCATATAACCGAGCGCCAAA	
			Pc-TEF1-Probe	VIC-TTGCGCCTCCACTTG-MGBNFQ	
Fungi (broad range)	FQ	18S rRNA	Fungi Quant F	GGRAAACTCACCAGGTCCAG	Liu et al. (2012)
			Fungi Quant R	GSWCTATCCCCAKCACGA	
			Fungi Quant P	FAM-TGGTGCATGGCCGTT-NFQ-MGB	
18S rRNA	18S			Commercial kit, data not available	

Note: Broad range fungi assay FQ and eukaryotic DNA assays were used for control of DNA extraction.

Malta and Italy spiked with *P. citricarpa* DNA and the effect of sweet orange peel, by testing the dilution series of *P. citricarpa* mycelium (100–200 mg) mixed with sweet orange peel pieces (2 cm^2). The latter mixture was homogenized either in sterile distilled water or in CTAB extraction buffer and DNA was extracted as stated in Section 2.2. The applicability of the assay was additionally tested on DNA extracted from symptomatic fruits in parallel with PC and PC-2 assays.

2.4.4 | qPCR assays and reaction conditions

Each qPCR, except for FungiQuant (FQ), consisted of 1× Universal Master Mix (Applied Biosystems), 300 nM of each primer, 200 nM TaqMan probe and 2 µl of a DNA sample in a total volume of 10 µl. For the FQ assay the reaction consisted of Quantabio PerfeCTa qPCR ToughMix Low ROX and 900 µM of each forward and reverse primer and 250 nM of the TaqMan probe.

The following cycling conditions were applied for all reactions: 2 min at 50°C for uracil N-glycosylase (UNG) treatment, 10 min at 95°C for *Taq* DNA polymerase activation, and 45 cycles at 95°C for 15 s for denaturation and at 65°C for 1 min. The qPCR was carried out in an ABI PRISM 7900 Sequence Detector or ABI PRISM ViiA7 (Applied Biosystems). The fluorescence reading occurred after each annealing/elongation step. The threshold value was manually adjusted. All sigmoid amplification curves were considered as valid (positive signal) and average C_q values were calculated and given for each sample, except for average C_q value above 40, where >40 is given. In each qPCR analysis, results of the positive and negative controls were checked before individual analysis continued. The success of DNA extraction and absence of inhibition of qPCR amplification was checked using the 18S rDNA and FungiQuant (FQ) qPCR assays.

2.4.5 | Droplet digital PCR for *TEF1* copy number determination

To determine the absolute number of *TEF1* gene copies, droplet digital PCR (ddPCR) was used. Reactions were prepared using ddPCR Supermix for Probes (No dUTP) (Bio-Rad), 900 nM of each primer, 250 nM TaqMan probe and 5.5 µl of DNA. Droplets were generated using the AutoDG droplet generator (Bio-Rad). PCR cycling conditions were 95°C for 10 min; 40 cycles of 94°C for 30 s and 60°C for 1 min 30 s; with a final incubation step of 98°C for 10 min. The ramp rate was set at 1.5°C/s. Droplets were read using the QX100 (Bio-Rad). QuantaSoft software v. 1.7.4 (Bio-Rad) was used to manually determine the thresholds. The raw data were then exported and analysed in Excel (Microsoft). A dilution series of CBS 127451 DNA was tested in triplicate, and the experiment was repeated twice. The copy number of the *TEF1* gene was calculated as the amplicon copy number determined by ddPCR, adjusted for the dilution factor. Copy number determined in each dilution, within the range of quantification, and in both repetitions was calculated. The logarithmic values of copy number were plotted against C_q values determined by qPCR on the same sample dilutions and used as calibration curve.

2.5 | Fungal ITS and *TEF1* amplicon sequencing of air samples

Nontargeted fungal diversity analysis by HTS of amplicons was performed twice with two primer sets targeting two distinct barcodes. Forward primers were composed of 33 nucleotide (nt) Illumina tails on the 5′ end followed by five wobble bases and a

barcode-specific primer sequence. Reverse primers were composed in a similar way starting with 34 nt Illumina tail on the 5′ end and followed by a target-specific primer sequence. All primer pairs (Table 3) were first tested for their amplification performance on DNA extracted from exposed spore trap Melinex tapes without *P. citricarpa* or spiked with various amounts (1, 10, 10^2, 10^3, 10^4, 10^5) of *P. citricarpa* conidia, as well as with negative DNA extraction controls (see Section 2.2). The samples for HTS were subjected to the first step PCR using Phusion High-Fidelity (Thermo Scientific) DNA polymerase.

In the first metagenome experiment only ITS1 was amplified and sequenced (data not presented). In the second experiment both ITS1 and *TEF1* were amplified in separate reactions by using primer pairs ITS1f/58A2R and EF1_728F/EF2, respectively. In both experiments, negative controls, fungal mock communities and DNA samples of previously exposed Melinex tapes with various amounts of spiked *P. citricarpa* conidia (0, 1, 10, 10^2, 10^3, 10^4, 10^5) were analysed. All samples except the fungal mock communities were sequenced in duplicates. Fungal mock communities A and B (Bakker, 2018) comprising 19 different fungal taxa were used as positive controls for the experiment. Additionally, an in-house *Phyllosticta* DNA control (mock *Phyllosticta*) was prepared by combining 1 ng DNA of each of *P. citricarpa* (CBS 127451), *P. paracitricarpa* (CBS 141357), *P. capitalensis* (CBS 120490) and *P. citriasiana* (ER1891) extracted from 100 mg of pure cultures grown on PDA. One microlitre of DNA sample was used for PCR amplification.

Amplification of ITS1 was carried out in a PCR Mastercycler Ep Gradient (Eppendorf) with initial denaturation of 2 min at 98°C; followed by 30 or 35 cycles of 10 s at 98°C, 25 s at 54°C and 25 s at 72°C; with a final elongation of 7 min at 72°C using primer pair ITS1f/58A2R. For amplification of *TEF1*, 35 amplification cycles were used with annealing at 52°C and final elongation of 5 min. All amplifications were performed in a T100 thermal cycler (Bio-Rad) with Phusion Hot Start II high-fidelity DNA polymerase (Thermo Fisher Scientific) and enclosed HF buffer.

Finally, the samples were sequenced on Illumina Miseq using Nextera second-step PCR including pooling library preparation kit and sequencing of 300 bp paired end reads (2 ×300). The reads were quality checked, trimmed (minimum quality score 20) and analysed with the QIIME2 2018.8 (Quantitative Insights Into Microbial Ecology) software package (Caporaso et al., 2010). The forward read data were denoised using the DADA2 (Callahan et al., 2016) algorithm, which implements filtering, dereplication and chimera identification using the default parameters.

For determination of the limit of detection (LOD) of reads belonging to *P. citricarpa* the procedure was as follows: the representative sequences after DADA2 denoising and clustering were transformed into a local nucleotide BLAST database using BLAST+ 2.11.0 (Camacho et al., 2009). Then, representative ITS1 (NR_147332) and *TEF1* (JF343604) sequences of strain *P. citricarpa* (CBS 127454) were used as queries for search using BLASTN with the default parameters. The output table revealed feature IDs (amplicon sequence variants, ASVs) belonging to *P. citricarpa*. ASVs belonging to a specific sample were determined from denoisetable.qzv. The samples with lowest concentrations of spiked *P. citricarpa* spores in which representative reads belonging to *P. citricarpa* were still identified represented the LOD of the metagenomics analysis.

Amplicon sequencing reads corresponding to the best BLASTN matches of *P. citricarpa* ITS1 (NR_147332) and *TEF1* (JF343604) queries were retrieved from corresponding metagenome sequence databases and aligned together with *P. paracitricarpa* (CPC 27172) and *P. citriasiana* (CBS 120486) ITS1 (KY855638, NR_145217) and *TEF1* (KY855967, FJ538418) sequences. The evolutionary relatedness of the sequences was inferred by using the maximum-likelihood method and Tamura–Nei model by employing the default parameters (Tamura & Nei, 1993). Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

3 | RESULTS

3.1 | Design of new specific *P. citricarpa* qPCR assay

Among the available barcoding markers of *P. citricarpa* and *P. paracitricarpa* including ITS, *actA*, *TEF1*, *gapdh*, LSU and *rpb2*, only *TEF1* revealed three mismatches and two point insertions (shadowed in Figure 1) that could be exploited for primer design. The rest of the sequences (ITS, *actA*, *gapdh*, LSU and *rpb2*) were identical for *P. citricarpa* and *P. paracitricarpa* (data not shown).

BLASTN search of the *TEF1* amplicon among available sequences in the nonredundant NCBI database excluding taxon *P. citricarpa* revealed 55 BLAST records with BLAST best hit presented by *P. paracitricarpa* CPC 27169 possessing 97.78% identity (corresponding to the two mismatches and one point deletion difference in the *TEF1* sequences between the two species). Furthermore, a BLASTN search excluding the whole *Phyllosticta* genus revealed

TABLE 3 Primers used for high-throughput sequencing of fungal rDNA internal transcribed spacer region (ITS) and *TEF1* amplicons in air samples

Barcode	Primer name	Primer sequence (5′–3′) without Illumina tails and wobble bases	Reference
ITS1	ITS1F	CTTGGTCATTTAGAGGAAGTAA	Gardes and Bruns (1993)
	58A2R	CTGCGTTCTTCATCGAT	Martin and Rygiewicz (2005)
TEF1	EF1_728F	CATCGAGAAGTTCGAGAAGG	Carbone and Kohn (1999)
	EF2	GGARGTACCAGTSATCATGTT	O'Donnell et al. (1998)

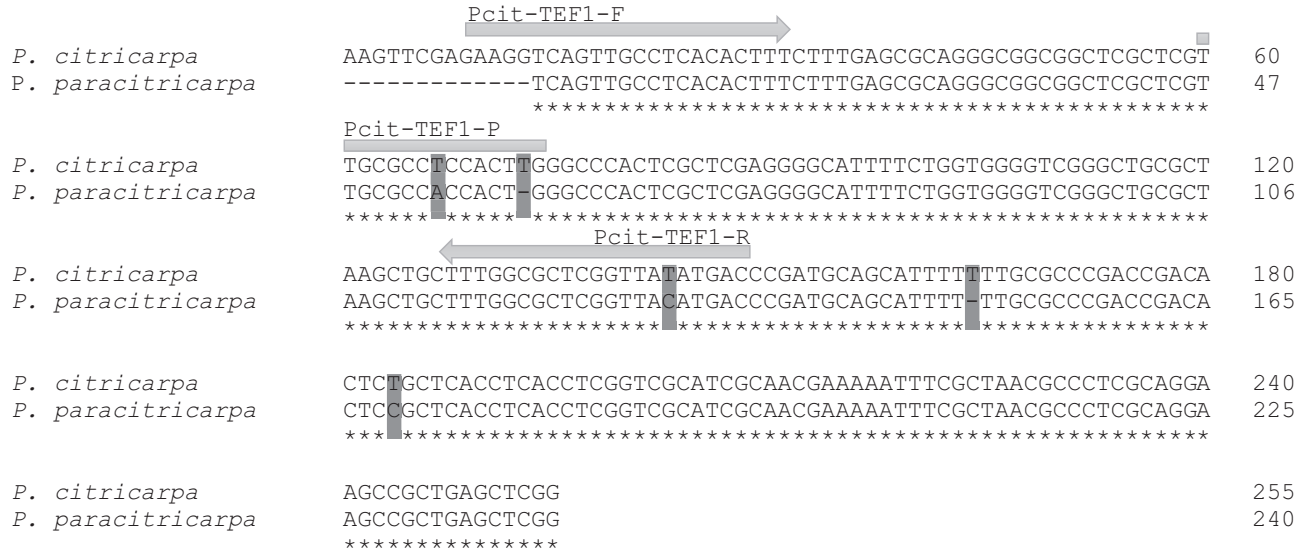

FIGURE 1 Alignment of consensus sequences of the transcription elongation factor 1-α (*TEF1*) of *Phyllosticta citricarpa* (strains CBS 122482, CBS 127452, CBS 127454, CPC16151, CPC16586, CPC 16586, CPC27913) and *P. paracitricarpa* (strains CPC 27169, CPC 27170, CPC 27171, CPC 27172, ZJUCC200937, ZJUCC200933) (Clustal Omega v. 1.2.4 multiple sequence alignment) and binding sites of primers and probe. Mismatches between the consensus sequences are shadowed.

only three BLAST records showing as low as 83% identities (with the taxa *Sphaerulina cornicola*, *Guignardia psidii* and *G. aesculi*). Thus, the in-silico analysis did not reveal any likely cross-reaction of the *TEF1* amplicon with the *TEF1* gene of any other fungi where sequence is available.

The secondary structures of the *TEF1* amplicon of the qPCR assay analysed by using OligoAnalyzer Tool (IDT technologies) showed that all the hairpin structures had lower Tm than the annealing temperature conditions of the qPCRs and thus were not predicted to affect the assay performance. The analysis uncovered potentially problematic homodimeric structures with up to eight base pairs and strong Δ*G* values (−19.32 kcal/mol).

3.2 | Specificity of the Pc-TEF1 qPCR assay

The newly designed Pc-TEF1 assay was positive when DNA of all *P. citricarpa* isolates was tested and negative when DNA of all other species was tested (Table 1). The PC and PC-2 assays gave a positive signal when DNA isolated from all *P. paracitricarpa* cultures and some *P. capitalensis* and *P. citriasiana* cultures was tested. Isolates came from different hosts and originated from different locations, which did not impact the specificity of the assays. None of the assays gave positive results when DNA isolated from other pathogenic fungi found on citrus or from other commonly encountered fungi was tested. Control assays for 18S rDNA and FQ showed sigmoidal amplification profiles and the resulting C_q values were in the expected range for all the samples (between 20.5 and 35.5 for 18S rDNA and 17.0 and 30.5 for FQ). The experimental findings were in concordance with the in-silico analysis, which did not reveal any cross-reaction of the *TEF1* amplicon with the *TEF1* gene of any other fungi.

3.3 | Sensitivity of the Pc-TEF1 qPCR assay

To test the sensitivity of the Pc-TEF1 assay, serial dilutions of DNA extracted from *P. citricarpa* mycelium were made. DNA of *P. citricarpa* (CBS 127451) was diluted in nuclease-free water at concentrations of 10^{-1}, 10^{-2}, 5×10^{-2}, 10^{-3}, 2×10^{-3}, 10^{-4}, 5×10^{-4}, 10^{-5}, 2×10^{-5} and 5×10^{-5}. Samples were also tested using the PC and PC-2 assays side by side. The range of detection of the Pc-TEF1 assay was lower compared to PC and PC-2 assays (Table 4). The dynamic range, where all three replicates were positive, was set and the observed limit of quantification for Pc-TEF1 assay was 10 times lower compared to the PC and PC-2 assays (Figure 2). With ddPCR we determined 17,500 copies of the *TEF1* gene per microlitre of undiluted sample. Using the calibration curve, we set the quantification limit of Pc-TEF1 qPCR assay to 3.5 copies of target DNA per reaction and a limit of detection as low as 1 copy of target DNA per qPCR.

To evaluate the sensitivity of the assays on fruit peel we tested the performance of the assays on simulated samples using *P. citricarpa* mycelium mixed with sweet orange peel and DNA was extracted. The addition of sweet orange peel did not affect the sensitivity of Pc-TEF1, PC or PC-2 assays (Figure 2).

3.4 | Performance of the Pc-TEF1 qPCR assay

The repeatability of the Pc-TEF1 qPCR assay was evaluated by analysing *P. citricarpa* (CBS 127451) DNA in three replicates. Under the same conditions the assay showed high repeatability when samples with more than 40 *TEF1* copies/reaction were tested, and the standard deviation (*SD*) between C_q values was always lower than 0.3. As expected, the *SD* of C_q values was higher in samples with 3–15 *TEF1* copies/reaction (Table S2).

TABLE 4 Performance of PC assay (Van Gent-Pelzer et al., 2007), PC-2 assay (Schirmacher et al., 2019) and Pc-TEF1 assay determined by testing *Phyllosticta citricarpa* (CBS 127451) DNA diluted in nuclease-free water

Assay	Range of detection	Dynamic range	*k*	R^2
PC	0.1–3500	0.1–3500	−3.4	0.994
PC-2	0.1–3500	0.2–3500	−3.7	0.998
Pc-TEF1	0.2–3500	0.4–3500	−3.6	0.998

Note: Range of detection (between lowest and highest detected copy number) and dynamic range (linear part of detection range, with lowest to highest quantifiable copy number) are given as range of number of copies of *TEF1* gene in quantitative PCR that can be detected with each of the assays. *k*, slope of the linear regression line in the plot of C_q against log[copy number]; R^2, mean square regression coefficient.

The reproducibility was analysed by testing two DNA samples with 10-fold difference in the amount of target (*P. citricarpa*, CBS 127451). The sample with approximately 500 *TEF1* copies/reaction was tested in the first 14 qPCR runs and the sample with approximately 50 *TEF1* copies/reaction in the next 18 runs. In run 20 we changed the Pc-TEF1 probe provider, which had an impact on C_q value (Figure 3). Each run was made on a different day and through repeatability tests, different lots of qPCR chemicals were used. Most runs (28) were done using the 7900 HT Fast (Applied Biosystems) instrument and four using the ViiA7 (Applied Biosystems). C_q values were always in the expected range with low standard deviation from the original value, that is, 27.9 ±0.5 for samples with 500 *TEF1* copies/reaction, and 32.2 ±1.5 (old probe) and 34.2 ±0.6 (new probe) for samples with 50 *TEF1* copies/reaction (Figure 3).

By testing samples from two locations (air samples from Italy and Malta), asymptomatic sweet orange peel and samples prepared using different buffers, all spiked with *P. citricarpa* DNA or mycelium, we evaluated the effect of the matrix to the Pc-TEF1 assay. The C_q values obtained did not differ considerably (*SD* <1) between samples, showing good performance of the assay in various matrices (Figure 2 for sweet orange peel). We also tested DNA extracted from symptomatic fruits imported from Tunisia and China. Out of 21 samples of diseased fruits, 10 were positive with our specific Pc-TEF1 assay, which confirmed the presence of *P. citricarpa*. One fruit had a high C_q (37.0) with PC assay, but negative with PC-2 and Pc-TEF1, and with additional testing (EPPO, 2020) we confirmed the presence of *P. citriasiana*. The remaining eight samples were positive with the PC and PC-2 assays but negative with Pc-TEF1, however, because of low concentration of target DNA (C_q >32) and lower sensitivity of Pc-TEF1 assay, we could not reliably confirm the presence of *P. citricarpa* in those samples (data not shown).

3.5 | Evaluation of qPCR assays for spore trap tape testing

Exposed and unexposed spore trap Melinex tapes with spiked *P. citricarpa* conidia were analysed to verify that the whole testing protocol, from DNA extraction to qPCR analysis, is suitable for detection of fungal DNA in those samples. Both PC and PC-2 qPCR assays showed high diagnostic sensitivity and detected as little as 1 conidium per unexposed spore tape, while the Pc-TEF1 assay showed lower sensitivity (Table 5). An effect of exposure of tapes to the orchard air was observed, as higher C_q values were acquired in the samples of DNA extracted from exposed spore tapes compared to the DNA samples extracted from the unexposed spore tapes.

3.6 | Fungal ITS1 and *TEF1* amplicon sequencing of air samples

A total of 1,610,922 and 990,220 single-end raw reads were obtained through ITS1 and *TEF1* amplicon sequencing. Reads belonging to the ITS1 and *TEF1* amplicon sequencing had average lengths of 279 and 258 bp, respectively. Both datasets produced high-quality data with a mean phred score of 37 (Table 6).

For the LOD analysis of ITS1 metagenomes 805,461 forward raw reads were used, of these 414,478 passed the quality filter and 363,623 were left after denoising. Of these, 95% were nonchimeric, resulting in 347,658 reads, ranging from 14,132 to 33,737 per sample (without mock and negative control; Table 7), corresponding to 1028 ASVs (Table 6). For the *TEF1* metagenome, 495,110 forward raw reads were analysed: 493,635 passed quality filtering (99.7%), 463,552 were left after denoising and 98% of these were nonchimeric. The final 454,212 reads ranging from 11,494 to 31,000 per sample (Table 7) represented the final dataset corresponding to 616 ASVs (Table 6).

The estimated maximum-likelihood phylogeny of the best BLASTN hits revealed that *TEF1* amplicon sequencing reads of *P. citricarpa* and *P. paracitricarpa* could be distinguished, whereas ITS reads of *P. citricarpa* and *P. paracitricarpa* were indistinguishable (Figure 4).

The LOD of the amplicon analysis of ITS1 and *TEF1* was determined as the lowest number of spiked spores that resulted in the detection of reads belonging to *P. citricarpa* in the sequenced sample as identified by BLASTN (Table 8). For both ITS1 and *TEF1* the LOD was the same: 1000 spores present on the tape sample exposed to the air for 24 h (100 µl of spiked spore suspension of concentration 10^4 spores/ml). However, the read count corresponding to *P. citricarpa* in these samples was greater in the case of *TEF1* amplicon sequencing (520 and 1186 reads) than for ITS1 (73 and 98 reads).

No reads corresponding to *P. citricarpa* were determined either for negative controls, unspiked air spore trap plastic tape samples (air tape 0_1 and air tape 0_2) or for mock samples that did not contain *Phyllosticta* spp. (Bakker, 2018). Conversely, 916 ITS1 and 4369 *TEF1* reads were sequenced in our positive control, that is, a mock sample composed of DNA of *P. citricarpa*, *P. paracitricarpa*, *P. citriasiana* and *P. capitalensis*.

4 | DISCUSSION

The fungus *P. citricarpa* is included in the EPPO A1 list (EPPO, 2022) and it is a quarantine pest not present in the EU

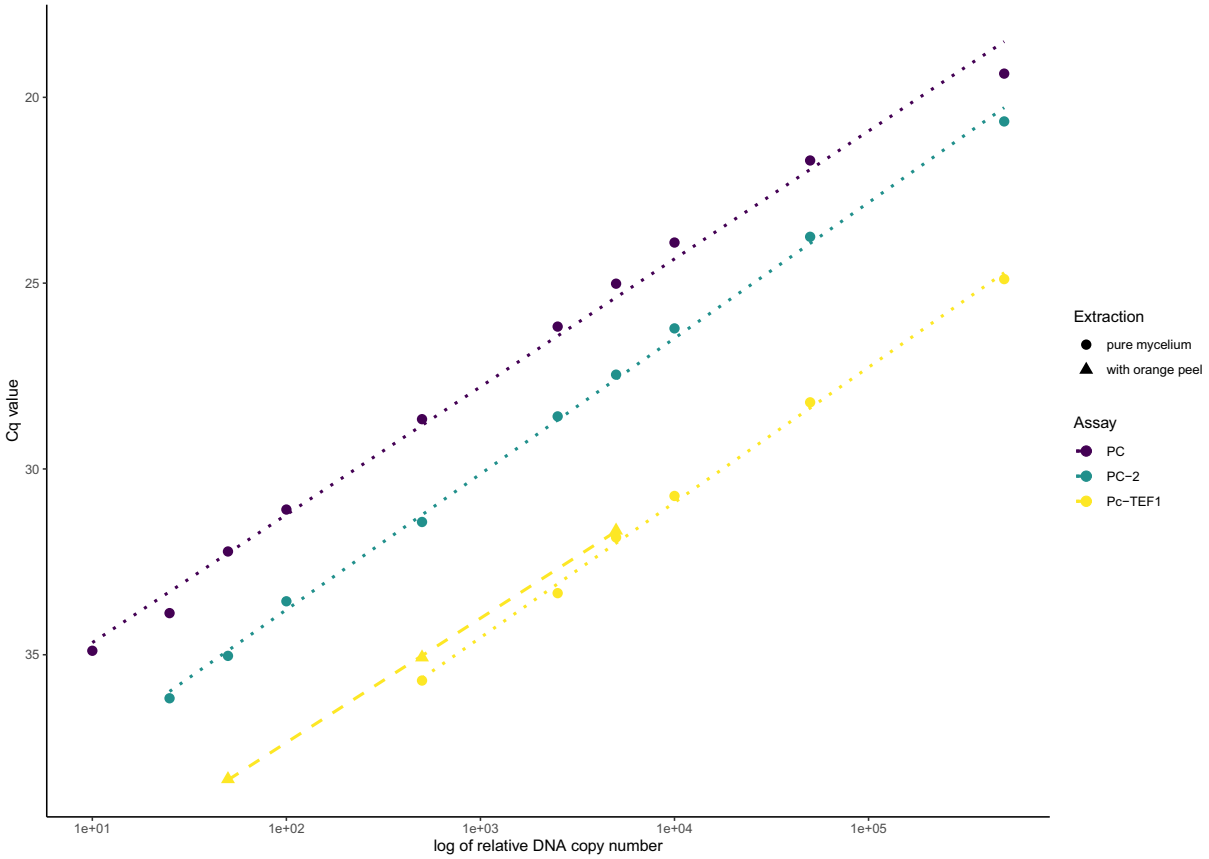

FIGURE 2 Dilution series of the DNA extracted from mycelium of *Phyllosticta citricarpa* in nuclease-free water tested with PC (Van Gent-Pelzer et al., 2007), PC-2 (Schirmacher et al., 2019) and Pc-TEF1 assays and serial dilutions of *P. citricarpa* mycelium in sweet orange peel tested with Pc-TEF1 assay; C_q values are an average of three replicates. Data on dynamic range and linearity of all assays are given in Table 4. [Colour figure can be viewed at wileyonlinelibrary.com]

territory (Regulation 2019/2072; Anon, 2019). Once introduced into a new area, early detection is key to initiate adequate outbreak response programmes. In light of the current Regulation EU 2016/2031 enforcing annual surveillance over large geographic areas in the EU (EFSA, 2020), we focused our research on development and evaluation of targeted (qPCR) and untargeted (HTS) molecular methods applicable for the detection and identification of *P. citricarpa* spores in air samples collected by volumetric spore traps sited in citrus orchards.

Our study focused on *P. citricarpa* conidia, as they are produced in culture and suspensions at high concentrations and so can be readily obtained. Conidia can be airborne as well, with wind-driven rains (Perryman et al., 2014), and are responsible for major epidemics (Hendricks et al., 2017; Serra et al., 2022; Spósito et al., 2008; Wang et al., 2016). In addition, conidia are known to be relevant during the early stages of disease establishment in new areas (Garran, 1996; Whiteside, 1967). Although the performance of the qPCR and HTS methods applied is not likely to be negatively affected by the presence of ascospores, deemed as the main airborne inoculum of *P. citricarpa* (Tran et al., 2020), our method would benefit from further testing with *P. citricarpa* ascospores. However, production of ascospores to the concentrations and volumes needed for the evaluation of the DNA extraction and molecular methods remains a challenge (Tran et al., 2017).

The most widely used ITS region is not always informative enough to provide sufficient taxonomic resolution to enable species identification of fungi; this is also the case for *Phyllosticta* spp. Specific detection of *P. citricarpa* in plant samples is currently based on qPCR assays targeting the ITS region, whereas its discrimination from the closely related species *P. paracitricarpa* is subsequently done by inefficient and time-consuming isolation of *P. citricarpa* from citrus peels or leaves in pure culture and subsequent sequencing of the *TEF1* region (EPPO, 2020). The economic importance of *P. paracitricarpa* is not yet known; however, it induces similar symptoms to *P. citricarpa* when artificially inoculated on citrus fruits (Guarnaccia et al., 2017). As a result, an accurate and simple assay for the detection and discrimination of the two closely related species is needed. The specificity of the qPCR assay Pc-TEF1 reported here, targeting the *TEF1* gene, was evaluated by testing two available *P. paracitricarpa* isolates, several isolates of other *Phyllosticta* spp., and fungi associated with citrus or commonly present in the environment. In each case the Pc-TEF1 qPCR assay only gave positive results with *P. citricarpa*, indicating its high specificity.

The sensitivity of the Pc-TEF1 assay was compared to the sensitivity of two published assays, PC (Van Gent-Pelzer et al., 2007) and PC-2 (Schirmacher et al., 2019), and was considerably lower. The difference in the sensitivity is the consequence of the multiple copies of the ITS region in *Phyllosticta* spp. genomes (Urbina

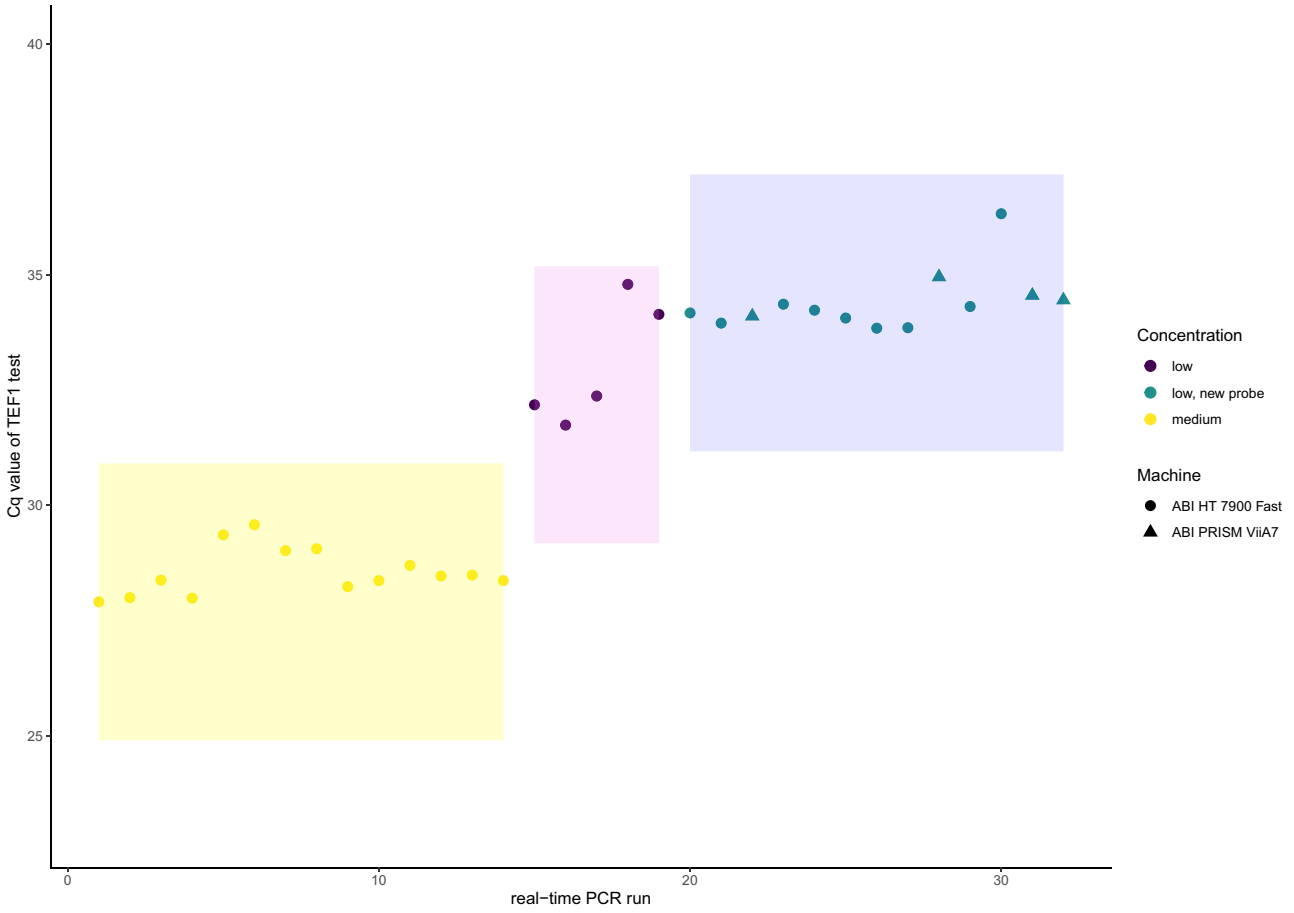

FIGURE 3 Reproducibility of the Pc-TEF1 assay shown as C_q values in 32 independent runs. Samples with medium (100× dilution) and low (1000× dilution) concentration of *Phyllosticta citricarpa* DNA (ER 1761) were tested. From run number 20 onwards, a probe from different provider was used. Two different qPCR machines were used. Boxes show the accepted difference between C_q values (±3 C_q). [Colour figure can be viewed at wileyonlinelibrary.com]

et al., 2021), targeted by PC and PC-2 assays, in contrast to a single copy *TEF1* gene (O'Donnell, 2000). When analysing a dilution series of the conidia on tapes, the PC assay detected as few as 10 conidia on a daily section of the exposed spore trap Melinex tape, while at least 100 conidia were needed to get a single positive signal with the Pc-TEF1 assay (C_q values in Table 5). Nevertheless, the sensitivity of the Pc-TEF1 assay would allow the detection of *P. citricarpa* spores in real-world samples in CBS-affected orchards. In Queensland, for instance, the *P. citricarpa* inoculum dynamics ranged from <5 ascospores/m^3 up to 98.7 ascospores/m^3, which corresponds to between 72 ascospores/day and 1421 ascospores/day (Tran et al., 2020). However, here optimized testing protocols incorporating the PC or PC-2 assay should be used for reliable and accurate epidemiological studies in endemic areas where possible cross-reactions with other *Phyllosticta* spp. would not have biosecurity implications. The Pc-TEF1 assay should be used for confirmation of *P. citricarpa* to avoid misidentification with other *Phyllosticta* spp. and thus limit consequential socioeconomic impacts. Testing of conidia on tapes produced stochastic C_q values in all qPCR assays that could not be linked to the effect of background (silicon oil, other particles caught on spore trap plastic tapes), because it was excluded with comparative analysis (data not shown). Most probably the preparation of dilutions of the conidia solution (aggregates, mucilage), the inoculation of the tapes and tape storage conditions contributed to the differences in extracted DNA.

Furthermore, DNA dilution series and dilution series of *P. citricarpa* mycelium in sweet orange fruit peel were tested to evaluate the diagnostic sensitivity and wider applicability of the Pc-TEF1 assay. The latter was also assessed on symptomatic fruits. The Pc-TEF1 assay enables the detection of less than one copy of *P. citricarpa* DNA in a qPCR and it can be used for absolute quantification with ddPCR as well, which is not straightforward with the PC and PC-2 assays. Here, DNA digestion is required to separate copies of the target DNA and thus avoid introducing multiple consecutive copies into a single droplet (Jouanin et al., 2020), leading to underestimation of the total number of DNA copies. The Pc-TEF1 assay can be applied to citrus asymptomatic fruit testing because no decline in C_q values and sensitivity was observed. More importantly, it can be used for testing of symptomatic fruits as well, however, a precise and efficient sampling and extraction is needed to get sufficient fungal DNA.

As the *TEF1* barcode currently offers the possibility of identifying *P. citricarpa*, we explored the analysis of ITS1 and *TEF1* barcodes

TABLE 5 Data of serial dilution of *Phyllosticta citricarpa* conidia (GC092) suspension on spore trap plastic tapes exposed and unexposed (clean) to the orchard air

		PC			PC-2			Pc-TEF1		
Tape	No. of conidia	C_q	*SD*	No. of positive replicates	C_q	*SD*	No. of positive replicates	C_q	*SD*	No. of positive replicates
Exposed	1	Undet.	n.a.	0	Undet.	n.a.	0	Undet.	n.a.	0
	10	36.7	0.0	1	Undet.	n.a.	0	Undet.	n.a.	0
	100	33.5	0.9	3	36.0	0.6	3	36.9	0.0	1
	1000	34.7	0.4	3	Undet.	n.a.	0	Undet.	n.a.	0
	10,000	30.1	0.2	3	32.9	0.2	3	37.5	0.7	3
	100,000	29.7	0.2	3	32.6	0.2	3	38.9	0.0	1
Unexposed	1	35.4	0.00	1	38.4	0.3	2	Undet.	n.a.	0
	10	Undet.	n.a.	0	Undet.	n.a.	0	Undet.	n.a.	0
	100	33.5	0.0	1	Undet.	n.a.	0	Undet.	n.a.	0
	1000	25.0	0.3	3	30.7	0.4	3	36.9	0.5	3
	10,000	29.0	1.0	3	28.0	1.8	3	35.1	0.8	2
	100,000	26.8	0.7	3	28.6	1.1	3	33.3	1.3	3

Note: Extracted DNA was tested in triplicate with PC, PC-2 and Pc-TEF1 quantitative PCR assays. Mean C_q values, their standard deviation (*SD*) and number of positive replicates is given. Undet., undetermined/no amplification.

Abbreviation: n.a., not available.

using HTS analysis. This approach has been employed previously, for example the assessment of *Fusarium* spp. diversity in soil by *TEF1* metagenome sequencing (Karlsson et al., 2016). The study demonstrated that this is a promising approach for more targeted monitoring of certain fungal taxa. However, our experimental setup was not focused on the taxa composition in air samples, but rather aimed to specifically detect airborne *P. citricarpa*. The *TEF1* HTS protocol employed here provides sufficient resolution for the detection and identification of *P. citricarpa* in air samples. The protocol offers separation of sequences on very few SNPs, whereas ITS1 sequencing enables detection but not discrimination of the closely related species. On the other hand, ITS1 HTS of spore trap tape samples resulted in a significantly higher number of ASVs, 1028 compared to 616 for the *TEF1* barcode, covering a wider taxonomic view. Indeed, this suggests that ITS HTS is more useful for general screening of taxa diversity in surveillance activities, whereas *TEF1* HTS is more appropriate when specific fungi, such as *P. citricarpa*, are targeted in an aerobiology network.

The usefulness of the HTS approach was evaluated by determination of the lowest amount of target, that is, limit of detection (LOD), that can be detected, for instance the lowest number of conidia or spores of a fungus as in our case. By spiking the air trap tapes with known amounts of *P. citricarpa* conidia, we were able to determine the HTS LOD of *P. citricarpa* for both barcodes. The LOD was the same for ITS1 and *TEF1*, 1000 conidia per exposed spore trap plastic tape. Surprisingly, the number of reads obtained in the sample at the LOD was up to 100 times higher for the *TEF1* amplicon than for ITS1, albeit this coding gene is, in contrast to the noncoding ITS region, present in single copy. The reason for this was not further investigated in the study, but probably lies in the efficiency or even stochasticity of the PCR amplifications used in the metagenomic library preparation (Elbrecht et al., 2017; Schmidt et al., 2013) or perhaps lower formation of chimeric reads. Nevertheless, the sequencing results are at best semiquantitative, so read numbers

TABLE 6 Sequencing summary data

	ITS1 metagenome	*TEF1* metagenome
Total reads	1,610,922	990,220
Average read length	279	258
Average read Q	37	37
Readcount	805,461	495,110
Readcount passed filter	414,478	493,635
Readcount passed filter (%)	51.5	99.7
Denoised readcount	363,623	463,522
Nonchimeric readcount	347,658	454,212
Nonchimeric readcount (%)	95.6	98.0
ASVs	1028	616

TABLE 7 Sequence counts of rDNA internal transcribed spacer (ITS1) and *TEF1* metagenome samples

Sample type	No. of spiked conidia	Sequencing sample ID	Sequence read counts	
			ITS1	*TEF1*
Spore trap tape 0_1	0	ITS1-SEST-1-0_S20	14,132	22,700
Spore trap tape 0_2	0	ITS2-SEST-1-0_S21	28,080	18,967
Spore trap tape 10_1	1	ITS3-SEST-1-10_S22	23,602	11,494
Spore trap tape 10_2	1	ITS4-SEST-1-10_S23	25,368	12,501
Spore trap tape 10^2_1	10	ITS5-SEST-1-100_S24	33,737	22,101
Spore trap tape 10^2_2	10	ITS6-SEST-1-100_S25	25,293	25,776
Spore trap tape 10^3_1	100	ITS7-SEST-1-103_S26	20,831	28,890
Spore trap tape 10^3_2	100	ITS8-SEST-1-103_S27	19,986	29,060
Spore trap tape 10^4_1	1000	ITS10-SEST-1-104_S29	29,057	23,654
Spore trap tape 10^4_2	1000	ITS9-SEST-1-104_S28	20,229	24,915
Spore trap tape 10^5_1	10,000	ITS11-SEST-1-105_S30	20,118	31,000
Spore trap tape 10^5_2	10,000	ITS12-SEST-1-105_S31	23,443	17,352
Spore trap tape 10^6_1	100,000	ITS13-SEST-1-106_S32	22,749	19,795
Spore trap tape 10^6_2	100,000	ITS14-SEST-1-106_S33	18,861	19,478
Mock (Bakker, 2018)_1	0	ITS15-Mock-A_S34	14,289	44,789
Mock (Bakker, 2018)_2	0	ITS16-Mock-B_S35	3432	64,826
Mock Phyllosticta (inhouse)_1	1 ng DNA[a]	ITS17-Mock-C_S36	4449	16,105
Negative control_1	0	ITS18-NTC_S37	1	5243
Negative control_2	0	ITS19-NTC_S38	1	15,566
Total			347,658	454,212

[a]Instead of conidia, DNA was extracted from pure culture mycelia of individual *Phyllosticta* spp. and combined as described in Section 2.5.

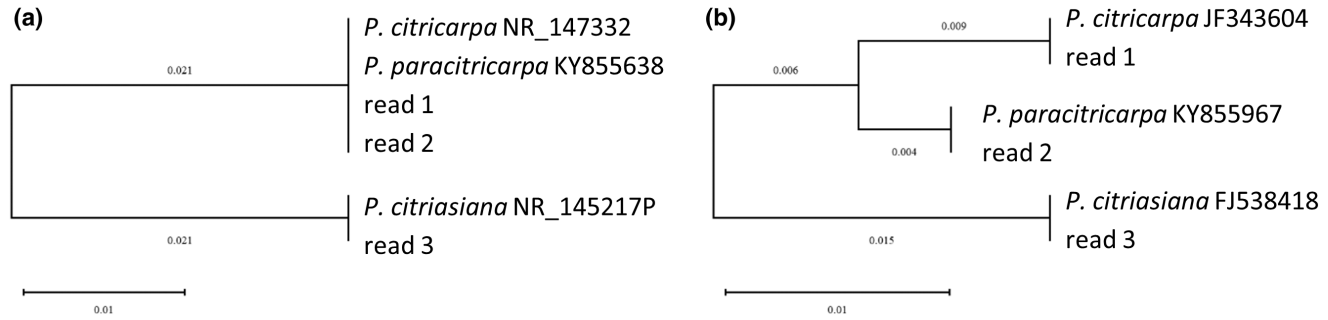

FIGURE 4 Maximum-likelihood trees estimated from alignments of ITS1 (a) and *TEF1* (b) amplicon sequencing reads. Reads identified by BLASTN as corresponding to *Phyllosticta* spp. were aligned with corresponding GenBank sequences of *P. citricarpa* (CBS 127454), *P. paracitricarpa* (CPC 27172) and *P. citriasiana* (CBS 120486) and the phylogenies were estimated with MEGA X. Branch lengths expressed as the number of substitutions per site are shown.

TABLE 8 Number of reads corresponding to *Phyllosticta citricarpa* in rDNA internal transcribed spacer (ITS1) and *TEF1* amplicon sequencing of DNA isolated from spore trap plastic tape samples of air

Sample	No. of spiked conidia	No. of ITS1 reads corresponding to *P. (para)citricarpa*		No. of *TEF1* reads corresponding to *P. citricarpa*	
		1	2	1	2
Spore trap tape 0	**0**	0	0	0	0
Spore trap tape 10	1	0	0	0	0
Spore trap tape 10^2	10	0	0	0	0
Spore trap tape 10^3	100	0	0	0	0
Spore trap tape 10^4	**1000**	**73**	**98**	**1186**	**520**
Spore trap tape 10^5	10,000	317	448	2083	1484
Spore trap tape 10^6	100,000	4723	4075	14,153	12,691
Mock (Bakker, 2018)_1	0	0	nd	0	nd
Mock (Bakker, 2018)_2	0	0	nd	0	nd
Mock Phyllosticta	1 ng DNA *P. citricarpa*[a]	915	nd	4369	nd
Negative control_1	0	0	0	0	0
Negative control_2	0	0	0	0	0

Note: Bold text: Limit of detection (LOD) determined as the sample with the lowest number of spiked *P. citricarpa* spores.

[a]Instead of conidia, DNA was extracted from pure culture mycelia of individual *Phyllosticta* spp. and combined as described in Section 2.5.

among the samples and various barcodes cannot accurately be compared. Given the low concentrations of ascospores in the air of infected orchards (for instance <72 ascospores/day to 1421 ascospores/day according to the study of Tran et al., 2020), the HTS approach would allow the detection of *P. citricarpa* in the ascospore peak production periods of the pathogen cycle.

The study demonstrates that spore trap tapes used in aerobiology networks to monitor pollen and fungi via morphological examinations under the microscope can also be used for the detection and identification of fungal plant pathogens using molecular methods. The more sensitive PC assay is recommended for use in surveillance and the newly developed assay Pc-TEF1 should be used for specific confirmation of *P. citricarpa* presence. The Pc-TEF1 assay was validated using artificially inoculated spore trap samples and its applicability for diagnostics in citrus fruits was demonstrated. The suitability of the HTS approach for monitoring *P. citricarpa* in the air was also demonstrated.

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passed away during the finalization of this manuscript. We will keep him in memory as a great researcher, diagnostician and colleague and follow his innovative ideas on plant protection.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Additional supporting information can be found online in the Supporting Information section at the end of this article.

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