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FUNGAL DISEASES

A multiplex PCR assay for three pathogenic *Phytophthora* species related to kiwifruit diseases in China

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Abstract

Phytophthora cactorum, P. cinnamomi and *P. lateralis* were reported to be pathogenic on kiwifruit trees in the main planting areas of China. We attempted to simultaneously detect the three pathogens using a multiplex polymerase chain reaction (PCR) and to survey their occurrence in the main production areas. Because of the need to combine different primer pairs for the multiplex PCR and the low specificity of published specific primers for *P. cactorum, P. cinnamomi* and *P. lateralis*, new species-specific primers for the three species were designed based on the ras-related protein gene, *Ypt*1. The specificity of the designed primers was demonstrated using 52 isolates, including 44 *Phytophthora* species, three *Pythium* species, and three other soil-borne pathogens. A multiplex PCR method for the simultaneous detection of *P. cactorum, P. cinnamomi* and *P. lateralis* was established, and the three pathogens were detected in artificially and naturally infested soils, indicating that these markers can be used in the diagnosis of kiwifruit *Phytophthora* diseases. In a survey of these pathogens in the main kiwifruit planting areas of China, 99 soil samples were collected at different locations and in different seasons and subjected to the new method, and the distribution of the three pathogens in the main kiwifruit planting areas of China was determined.

Keywords *Phytophthora cactorum* · *P. cinnamomi* · *P. lateralis* · Ras-related protein gene *Ypt1* · Multiplex PCR

Introduction

The genus *Phytophthora*, which includes some of the most destructive plant pathogens, causes considerable economic losses to food crops and ornamentals (O'Brien et al. 2009). Several *Phytophthora* species, including *P. cryptogea, P. citrophthora, P. drechsleri, P. palmivora, P. cactorum, P. cinnamomi, P. megasperma, P. citricola* and *P. lateralis*, have been reported to be associated with kiwifruit diseases (Akilli et al. 2011; Baudry et al. 1991; Conn et al. 1991; Kurbetli and Ozan 2013; Latorre et al. 1991; Lee et al. 2001; Mahdavi 2013; Stewart and McCarrison 1991).

Over the past 30 years, kiwifruit (*Actinidia chinensis* and *A. deliciosa*) cultivation has dramatically increased in China, which has produced the most kiwis and has the largest area planted with kiwis in the world since 2013 (Ferguson 2014). Shaanxi Province alone produces at least 600,000 tons of kiwifruit each year, almost half of the total for China and more than any other country (Ferguson 2014).

Although *Phytophthora cactorum, P. cinnamomi*, and *P. lateralis* were first isolated from diseased kiwifruit roots in Henan Province in the 1990s (Huang and Qi 1998; Wang and Cao 1999), no other investigation of their distribution across China has been conducted in the last 20 years, even though root rot of kiwifruit trees is one of the most serious diseases of kiwifruit in China. Therefore, it is important to develop a simple and rapid method to detect and distinguish these pathogens.

It is often difficult to control diseases caused by *Phytophthora* spp. because the pathogens release resistant perennating oospores or chlamydospores into the soil. Early detection and diagnosis of the pathogen in plants, soil, or water is thus essential for effective disease control. Conventional and real-time PCR have emerged as important tools for the diagnosis and study of *Phytophthora* species and have solved some of the problems associated with their detection, control and containment (Kostov et al. 2016; Martin et al. 2012; O'Brien et al. 2009; Schena et al. 2008). Moreover, diagnostic PCR methods and specific primers have been developed

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for *Phytophthora* species, including *P. cactorum* (Bhat and Browne 2010; Causin et al. 2005; Li et al. 2011; Schena et al. 2008), *P. cinnamomi* (Engelbrecht et al. 2013; Kong et al. 2003; Langrell et al. 2011; O'Brien 2008; Williams et al. 2009), and *P. lateralis* (Schena et al. 2008; Schenck et al. 2016; Winton and Hansen 2001); however, most of these studies targeted the detection of a single pathogen. Moreover, because some closely related *Phytophthora* spp. were not distinguished in previous studies, it remains difficult to establish whether those primers are actually specific (Kunadiya et al. 2017; Li et al. 2011).

Multiplex PCR assays allow the simultaneous detection of several species and facilitate large-scale sample processing (Cooke et al. 2007; Martin et al. 2012; O'Brien et al. 2009); however, multiplex PCR has rarely been applied in plant pathology (Bilodeau et al. 2014; Li et al. 2011, 2013), partially because of difficulties in developing multiplex assays and the lower sensitivity of multiplex PCR compared with simplex PCR (Li et al. 2013; Schena et al. 2006). Recently, some new PCR detection techniques, such as the loop-mediated isothermal amplification (LAMP) assay (Feng et al. 2015, 2018; Hansen et al. 2016; Khan et al. 2017, Si Ammour et al. 2017) and the isothermal RPA (recombinase polymerase amplification) assay (Miles et al. 2014; Rojas et al. 2017) were developed for on-site detection of *Phytophthora* species. However, these reactions are easily contaminated, and the techniques are expensive and incapable of multi-target detection.

Most of these PCR-based diagnostics (PCRDs) are based on the internal transcribed spacer (ITS) regions of nuclear-encoded ribosomal DNA (rDNA) genes or sequence characterized amplified regions (SCAR) (O'Brien et al. 2009). However, ITS sequences are not always sufficiently variable to distinguish closely related taxa (Kroon et al. 2004; Li et al. 2011; Schena et al. 2006), and the development of SCAR primers is very laborious (Schena et al. 2004). Therefore, recently reported studies focused on the *cox* 1 and *cox* 2 genes of mitochondrial DNA, as well as the β-tubulin gene, elicitin gene, and ras-related protein gene *Ypt*1 (Chen and Roxby 1996) in some *Phytophthora* species (Martin et al. 2012; Meng and Wang 2010; Schena et al. 2006, 2008). Among alternative target genes proposed as the basis of PCRDs, the ras-related protein gene *Ypt*1 possesses conserved exons and highly variable introns suitable for the development of PCRDs for almost all *Phytophthora* species (Schena and Cooke 2006).

In this study, we developed a multiplex PCR assay for the simultaneous detection of *P. cactorum, P. cinnamomi*, and *P. lateralis*, all of which infect kiwifruit plants in China. For the PCR, we designed primers specific for *P. cactorum, P. cinnamomi*, and *P. lateralis* based on the *Ypt*1 gene. We then successfully applied the novel multiplex PCR technique to samples from infested fields to investigate the distribution of the three pathogens in the main kiwifruit cultivation regions of China.

Materials and methods

Species and strain maintenance

A total of 44 *Phytophthora* spp. and six additional common soil-borne pathogens including *Pythium, Fusarium, Rhizoctonia*, and *Verticillium* spp. were used in this study (Table 1). Fifty-two isolates containing 17 type culture isolates were provided by several scientific resource institutions, including the CBS (Centraalbureau fur Schimmelcultures, The Netherlands), the WPC (World *Phytophthora* Genetic Resource Collection, USA), the MAFF (Ministry of Agriculture, Forestry and Fisheries, Japan), the NBRC (NITE Biological Resource Centre, Japan) and Gifu University of Japan. Other local isolates were collected from kiwifruit planting fields in Shaanxi Province, China. The *Phytophthora* spp. and all culturable isolates were maintained on corn meal agar or potato dextrose agar at 20 °C in the dark.

Primer design

The *Ypt*1 gene sequences from the 50 isolates of *Phytophthora* species and three of *Pythium* (Table 2) were aligned to develop specific primers for *P. cactorum, P. cinnamomi* and *P. lateralis* by BioEdit ver. 7.0.0 [Ionis (formerly Isis) Pharmaceuticals, Dublin, Ireland]. All *Ypt*1 gene sequences were collected from the National Center for Biotechnology Information (NCBI) DNA database. Candidate primers were analyzed for dimer and hairpin loop structures (Primer Premier Ver. 5.0; Premier Biosoft International, Palo Alto, CA, USA).

Sequencing

To amplify *Ypt*1 genes of *Phytophthora* spp., the *Phytophthora* universal primers Yph1F_mod2 and Yph2R_mod2 (Table 3) were designed and used in this study. The primer set (Yph1F_mod2/Yph2R_mod2) was a modified version based on the *Phytophthora* genus-specific primer set (Yph1F/Yph2R) reported by Schena et al. (2008). And the applicability of the modified primers was verified with 108 different *Phytophthora* species (data not shown). The reaction mixture contained 1 µM each primer, 1 U of Takara *Taq* DNA polymerase (Takara Bio, Shiga, Japan), 0.2 mM dNTP mixture, 1×PCR buffer [10 mM Tris–HCl (pH 8.3), 50 mM KCl, and 1.5 mM $MgCl_2$], 10 ng of bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA), and about 50 ng of DNA template in a total volume of 25 µl. PCR was conducted in a DNA thermal cycler (Gene Amp PCR System

Table 1 Species used in this study and specificity tests of reverse primers designed for *Phytophthora cactorum* (Yph_cac_R5), *P. cinnamomi* (Yph_cin_R2) and *P. lateralis* (Yph_lat_R4)

Clade[a]	Type isolate	Species	Isolate[b]	Original host/habitat	Region	Specificity tests[c]		
						Simplex PCR primer		
						Yph_cac_R5	Yph_cin_R2	Yph_lat_R4
1		*P. nicotianae*	GF468	Strawberry	Gifu, Japan	–	–	–
1a		***P. cactorum***	CH989A11	Strawberry	Gifu, Japan	+	–	–
			ZZ017	Kiwifruit	Shaanxi, China	+	–	–
	*	*P. hedraiandra*	CBS111725	*Viburnum* sp	Netherlands	–	–	–
	*	*P. idaei*	WPC6767	*Rubus idaeus*	UK	–	–	–
	*	*P. pseudotsugae*	WPC10339	*Pseudotsuga menziesii*	USA	–	N	N
1b		*P. clandestina*	WPC3942	*Trifolium subterraneum*	Australia	–	N	N
	*	*P. iranica*	CBS374.72	*Solanum melongena*	Iran	–	–	–
	*	*P. tentaculata*	CBS552.96	*Chrysanthemum leucanthemum*	Germany	–	N	N
1c		*P. infestans*	MAFF305586	Potato	Hokkaido, Japan	–	N	N
	*	*P. ipomoeae*	WPC10225	*Ipomoea longipedunculata*	Mexico	–	–	–
		P. mirabilis	WPC3005	*Mirabilis jalapa*	Mexico	–	N	N
		P. phaseoli	WPC10145	*Phaseolus lunatus*	USA	–	N	N
2		*P. citricola*	WPC1321	*Rubus* sp	California, USA	–	–	–
2a		*P. citrophthora*	CBS950.87	*Citrus* sp	California, USA	–	–	–
2b		*P. capsici*	WPC1319	Green bell pepper	California, USA	–	–	–
3		*P. pseudosyringae*	CBS111772	*Quercus robur*	Germany	–	–	–
5		*P. heveae*	WPC1102	Avocado	Guatemala	–	–	–
6		*P. humicola*	WPC6701	*Citrus* sp	Taiwan	–	–	–
		P. megasperma	NBRC32176	White trumpet lily	Yokohama, Japan	–	–	–
7a		*P. cambivora*	WPC0592	*Abies procera*	USA	–	–	–
	*	*P. europaea*	CBS109049	*Quercus* rhizosphere	France	–	–	–
	*	*P. fragariae*	CBS209.46	*Fragaria* sp	England	N	–	N
	*	*P. uliginosa*	CBS109054	*Quercus robur*	Poland	N	–	N
7b		*P. cajani*	WPC3105	*Cajanus cajan*	India	–	–	–
		P. cinnamomi	WPC2160	Grape	South Africa	–	+	–
			ZZ029	Kiwifruit	Shaanxi, China	–	+	–
	*	*P. parvispora*	CBS411.96	*Beaucamea* sp	Germany	–	–	–
	*	*P. melonis*	WPC6870	Cucumber	Japan	–	–	–
		P. sp. *niederhauserii*	CH96HE1	*Hedera helix*	Chiba, Japan	N	–	N
	*	*P. pistaciae*	CBS137185	*Pistachia vera*	Iran	N	–	N
		P. sojae	WPC7358	Soybean	NA	–	–	–
		P. vignae	Ph-9	Adzuki bean	Hokkaido, Japan	–	–	–
8a		*P. cryptogea*	WPC1088	*Callistephus chinensis*	California, USA	–	–	–
	*	*P. drechsleri*	WPC1087	Beet	California, USA	–	–	–
		P. medicaginis	WPC10138	*Medicago sativa*	California, USA	N	N	–
		P. sansomeana	WPC3163	*Silene latifolia* subsp. *alba*	New York, USA	N	N	–
8b		*P. brassicae*	CBS179.87	*Brassica oleracea*	Netherlands	–	–	–
		P. primulae	CBS620.97	*Primula acaulis*	Germany	N	N	–

Table 1 (continued)

Clade[a]	Type isolate	Species	Isolate[b]	Original host/habitat	Region	Specificity tests[c]		
						Simplex PCR primer		
						Yph_cac_R5	Yph_cin_R2	Yph_lat_R4
8c		*P. syringae*	MAFF645010	*Malus pumila*	Aomori, Japan	–	–	–
	*	*P. foliorum*	WPC10974	Azalea	Tennessee, USA	N	N	–
		P. hibernalis	CBS114104	*Citrus sinensis*	Australia	–	–	–
	*	***P. lateralis***	WPC3361	*Chamaecyparis lawsoniana*	Oregon, USA	–	–	+
	*	*P. ramorum*	CBS101553	*Rhododendron catawbiense*	Germany	–	–	–
9	*	*P. insolita*	WPC6195	Soil	Taiwan	–	–	–
10		*P. kernoviae*	NA	NA	NA	–	–	–
		Pythium vexans	MS6-10-8V	Forest soil	Gifu, Japan	–	–	–
		Py. helicoides	NBRC100107	Rose	Gifu, Japan	–	–	–
		Py. irregulare	CBS263.30	*Nicotiana tabacum*	USA	–	–	–
		Fusarium oxysporum	MAFF727510	NA	NA	–	–	–
		Rhizoctonia solani	S02	NA	NA	–	–	–
		Verticillium albo-atrum	Vaal 130,308	NA	NA	–	–	–

*Type isolate of species

[a]Molecular phylogenetic clade according to Martin et al. (2014)

[b]International identification abbreviations: CBS, Centraalbureau fur Schimmelcultures, The Netherlands; WPC, World *Phytophthora* Genetic Resource Collection, USA; MAFF, Ministry of Agriculture, Forestry and Fisheries, Japan; NBRC, NITE Biological Resource Centre, Japan

[c]Yph1F_mod2 was used as the forward primer for all 3 species

NA not available, + amplified, − not amplified, *N* not tested

2700; Applied Biosystems, Foster City, CA, USA) under the following conditions: 95 °C for 2 min; 40 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 45 s, and extension at 72 °C for 30 s; and a final extension at 72 °C for 10 min. After purification of the PCR products, a BigDye Terminator ver. 3.1 Cycle Sequencing kit (Thermo Fisher Scientific, Waltham, MA, USA) was used for cycle sequencing on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Consensus sequences were generated based on the forward and reverse sequences using Chromas Pro (ver. 1.33; Technelysium Pty. Ltd., Tewantin, Australia).

Collection of soil samples

A total of 99 soil samples in two main kiwifruit production areas (Zhouzhi and Meixian Prefectures) in Shaanxi Province of China were collected in April, July and October of 2017 to survey the pathogen distribution (Table 4). In Zhouzhi Prefecture, 51 soil samples were collected from 10 orchards. In Meixian Prefecture, 48 soil samples were collected from 9 orchards. In each orchard, 5–6 soil samples were collected. For each soil sample, four subsamples (each about 100 g) were randomly collected and mixed thoroughly. From the soil mixture, approximately 200 g was removed and stored at 5 °C. The soil pH was then measured with a FieldScout pH 400 m (Spectrum Technologies, Aurora, IL, USA). Soil texture was determined according to the criterion of the International Society of Soil Science (Li et al. 2011). Disease histories of the kiwifruit planting fields were provided by local agricultural research centers.

DNA extraction from mycelia and soil

Total genomic DNA from mycelia was extracted as described by Kageyama et al. (2003). Mycelia grown on V8 juice broth were used for DNA extraction from culturable species. For soil DNA extraction, the method refined by Kageyama et al. (2003) was modified by incorporating a magnetic bead purification step (MagExtractor-Plant Genome; Toyobo Co., Osaka, Japan) to purify soil DNA extracts as described by Li et al. (2010). Briefly, 0.2 g of soil was added to autoclaved 2.0-ml Eppendorf tubes containing 0.2 g of 1-mm-diameter glass beads. The soil was then suspended in 250 µl of extraction buffer [100 mM Tris HCl (pH 9.0), 40 mM EDTA, 2% (wt/vol) sodium dodecyl sulfate, 0.8% [wt/vol] skim milk; Difco Laboratories,

Table 2 Accession information for *Ypt*1 sequences in the National Center for Biotechnology Information (NCBI) DNA database

Species	Isolates	Accession	Species	Isolates	Accession
Phytophthora alni subsp. *alni*	SCRP2	DQ162953	*P. lateralis*	IMI040503	DQ162991
P. boehmeriae	SCRP23	DQ270324		US1-2	KM975317
P. cactorum	IMI296524	DQ162960	*P. medicaginis*	SCRP407	DQ162990
	CH03OKTYPE1	HQ850000	*P. megakarya*	P8517	HQ850008
P. cambivora	SCRP82	DQ162956	*P. megasperma*	IMI133317	DQ162986
P. capsici	IMI352321	DQ162972	*P. melonis*	PMNJHG1	EF649778
P. cinnamomi	CBS270.55	DQ162959	*P. mirabilis*	CBS678.85	HQ850009
	SCRP118	DQ270317	*P. multivesiculata*	CBS545.96	HQ850010
P. citricola	SCRP143	DQ162971	*P. nemorosa*	SCRP910	DQ162965
P. citrophthora	IMI332632	DQ162973	*P. nicotianae*	CH02FPK3	HQ849999
P. clandestina	CBS347.86	HQ850002	*P. obscura*	BBA 2/94-II-B	KJ755158
P. cryptogea	IMI045168	DQ162987	*P. palmivora*	IPPc3	HQ850011
P. drechsleri	ATCC46724	DQ162989	*P. phaseoli*	CBS120373	HQ850012
P. erythroseptica	SCRP240	DQ162988	*P. pistaciae*	IMI386658	DQ162957
P. europaea	SCRP622	DQ162952	*P. pseudosyringae*	SCRP734	DQ162967
P. foliorum	CBS121655	KJ755148	*P. pseudotsugae*	CBS444.84	HQ850013
P. fragariae	SCRP245	DQ162950	*P. psychrophila*	SCRP630	DQ162964
P. hedraiandra	CBS111725	HQ850003	*P. quercina*	SCRP550	DQ162979
P. hibernalis	JKI906242	KJ755160	*P. ramorum*	SCRP911	DQ162992
P. idaei	CBS971.95	HQ850004	*P. sansomeana*	— [a]	FJ966876
P. ilicis	SCRP379	DQ162963	*P. sojae*	SCRP555	DQ162958
P. infestans	CBS368.51	HQ850005	*P. syringae*	CBS110161	KF882681
P. insolita	IMI288805	DQ162974	*P. tentaculata*	C45	HQ850014
P. inundata	SCRP649	DQ162985	*P. uliginosa*	BBA 12/02 – 1	KJ755139
P. ipomoeae	CBS122203	HQ850006	*P. vignae*	BBA P3017	KJ755145
P. iranica	CBS374.72	HQ850007	*Pythium oedochilum*	CBS597.68	HQ850015
P. katsurae	SCRP388	DQ162980	*P. helicoides*	TCG3	HQ850016
P. kernoviae	SCRP722	DQ162975	*P. ostracodes*	CBS768.73	HQ850017

[a]Not given

Table 3 Primers used in this study

Target species	Universal primer	Primer type	Gene locus	Sequence (5′–3′)	Target size (bp)	Reference
Fungi	18S69F	Forward	18S rDNA	CTGCGAATGGCTCATTAAATCAGT	Variable	Asano et al. (2010)
	18S1118R	Reverse		GGTGGTGCCCTTCCGTCAA		
Phytophthora spp	Yph1F_mod2	Forward	*Ypt*1	CGACCATKGGTGTGGACTTTG	Variable ≈ 470	This study
	Yph2R_mod2	Reverse		ACGTTCTCRCAGGCGTATCTG		
P. cactorum	Yph_cac_R5	Reverse	*Ypt1*	CTGGGCACAACCGCAATAAAGA	112	This study
P. cinnamomi	Yph_cin_R1	Reverse	*Ypt1*	CACTACAGCAGCACCATTTATTT	229	This study
	Yph_cin_R2	Reverse		AGGCGAATAGGACCACGAAGG	176	
P. lateralis	Yph_lat_F1	Forward	*Ypt*1	GAGATTTTTTCCCGCTTTCCTT	307	This study
	Yph_lat_R2	Reverse		GGAAAAAATCTCCCGCAGACA	189	
	Yph_lat_R4	Reverse		CGTCGTTGCTACAGGAAACTT	225	

Detroit, MI, USA), and RNase A at 200 µg/ml (Nippongene, Toyama, Japan), then vigorously vortexed at 4200 rpm for 1 min. Next, benzyl chloride (150 µl) was added to the mixture, and the tube was again vigorously vortexed for 2 min. The samples were then incubated at 60 °C for 15 min, after which 150 µl of 3 M sodium acetate was added to the suspension, and the mixture was lightly vortexed. After 15 min of incubation on ice, this suspension was cleared by two

Table 4 Detection of *Phytophthora cactorum, P. cinnamomi* and *P. lateralis* in the main kiwifruit production areas of China in 2017 using multiplex PCR

Prefecture	Month sampled	No. samples with *Phytophthora* / No. samples collected	Soil texture	pH	Symptoms in field	Detected pathogens
Zhouzhi	April	0/15	L, SL, CL	6.3 ~ 7.1	Phytophthora rot, leaves yellowing	No
	July	3/20	N	5.9 ~ 6.9	Root rot, bacterial canker, Phytophthora rot	*P. cactorum, P. cinnamomi*
	October	2/16	N	6.2 ~ 6.8	Leaves yellowing, gray mold	*P. cactorum*
Meixian	April	0/15	L, SL, CL	7.1 ~ 7.6	Bacterial canker, leaves yellowing	No
	July	3/18	Nt	6.8 ~ 7.7	Root rot, leaves yellowing	*P. cactorum*
	October	0/15	N	7.0 ~ 7.5	Bacterial canker, leaves yellowing	No

L loam, *SL* sandy loam, *CL* clay loam, *Nt* not tested

rounds of centrifugation at 18,000×*g* for 10 min, and the upper layer was transferred to a clean tube. Purification of the extracted DNA was subsequently performed according to the manufacturer's instructions in the purification step of the MagExtractor-Plant Genome kit (Toyobo Co., Osaka, Japan). Finally, 50 µl of the purified DNA was obtained.

Simplex PCR with species-specific primers

The specificity of each developed primer pair was confirmed by simplex PCR with the isolates listed in Table 1. The reaction mixture contained 0.5 µM developed primers, 0.675 U of Takara *Taq* Hot Start Ver. DNA polymerase (Takara Bio,), 0.2 mM dNTP mixture, 1×PCR buffer [10 mM Tris–HCl (pH 8.3), 50 mM KCl, and 2.0 mM $MgCl_2$], 10 ng of bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA), and about 10 ng of DNA template in a total volume of 25 µl. PCR was conducted in a DNA thermal cycler (Gene Amp PCR System 2700; Applied Biosystems) by subjecting the samples to 95 °C for 2 min; 40 cycles of denaturation at 94 °C for 30 s, annealing at 63 °C for 35 s, and extension at 72 °C for 30 s; and final extension at 72 °C for 10 min. Amplification was confirmed by electrophoresis in 2% certified agarose S (Nippon Gene Co., Tokyo, Japan). Gels were stained with GelRed™ Nucleic Acid Gel Stain (Biotium, Fremont, CA, USA) and photographed under ultraviolet light.

Multiplex PCR

As a positive control to ensure the success of DNA extraction, two fungal universal primers, 18S-69F and 18S-1118R (Table 3), were added to the multiplex PCR reaction mixture. The multiplex PCR reaction mixture was prepared as described for simplex PCR, except that the primers were used at different concentrations as follows: 0.4 µM for primer pairs 18S-69F/18S-1118R, 0.2 µM for *P. cactorum* specific Yph_cac_R5, 1 µM for *P. cinnamomi* specific Yph_cin_R2 and *P. lateralis* specific Yph_lat_R4, and 2.2 µM for commonly shared forward *Phytophthora* universal primer Yph1F_mod2. Reaction conditions were the same as in the simplex PCR, except that the annealing temperature was 66 °C.

The applicability of the multiplex PCR was tested using soil samples from four kiwifruit-planting fields with *Phytophthora* disease histories and two artificially infested soil samples. Pure culture DNA mixtures of the three species served as a positive control.

Sensitivity of detection

Sensitivity to *P. cactorum, P. cinnamomi*, and *P. lateralis* DNA was tested in both simplex PCR and multiplex PCR. DNA from two *P. cactorum* isolates (CH989A11 and ZZ017), two *P. cinnamomi* isolates (WPC2160 and ZZ029) and one *P. lateralis* isolate (WPC3361) was tested at 10-fold dilutions (250 pg to 25 fg) in the simplex PCR. Two DNA mixtures (CH989A11 × WPC2160 × WPC3361 and ZZ017 × ZZ029 × WPC3361) were diluted from 250 pg to 250 fg for use in the multiplex PCR.

Inoculum addition to soil samples

For producing *Phytophthora* mycelia to mix with soil samples, a block (about 7 mm in diameter) of a 5-day-old culture of *Phytophthora* from V8 juice agar was transferred to V8 broth and cultured at 20 °C for 7 days. Mycelia were collected by centrifugation at 4000 rpm for 10 min. The supernatant was discarded, and 100 ml of sterilized distilled water was added to the tube for homogenization at 3000 rpm for

5 min. This mycelial suspension was then mixed thoroughly into 500 g sterilized soil.

Comparison of different DNA polymerases

DNA polymerase is an important factor affecting sensitivity of the PCR detection system. In this study, 10 DNA polymerases including Takara Taq Hot Start Ver., Takara Taq, Takara Ex Taq Hot start Ver., Takara Mighty Amp, Tks Gflex, PrimeSTAR GXL (6 different DNA polymerases from Takara Bio, Inc., Japan), Platinu SuperFi (Thermo Fisher Scientific, USA), FastStart Taq (Roche Applied Science, Germany), KOD FX Neo (Toyobo, Osaka, Japan) and Kaneka High-Speed (Kaneka Corp., Japan) were tested and compared for amplification efficiencies. Takara Taq Hot Start Ver. yielded the highest amplification efficiency (data not shown).

Results

Primer design and specificity tests

The species-specific DNA regions in the *Ypt*1 gene were sought for *P. cactorum, P. cinnamomi*, and *P. lateralis*. Based on variations in *Ypt*1, species-specific primers (Table 3) for each pathogen were designed. Two universal primers for the genus *Phytophthora*, Yph1F_mod2 and Yph2R_mod2, were also designed (Table 3). For the reverse species-specific primers (Yph_cac_R5, Yph_cin_R1, Yph_cin_R2, Yph_lat_R2, Yph_lat_R4), the forward *Phytophthora* universal primer, Yph1F_mod2, was used as the forward primer in PCR. Similarly, for the forward species-specific primer (Yph_lat_F1), the reverse *Phytophthora* universal primer, Yph2R_mod2, was used as the reverse primer in PCR.

Overall, 52 isolates including 44 *Phytophthora* spp. together with six soil-borne pathogens (Table 1) were used to test the specificity of the designed primers for *P. cactorum, P. cinnamomi*, and *P. lateralis*. The presence of amplified DNA from all isolates was confirmed using the fungal universal primers 18S-69F and 18S-1118R. Primers Yph1F_mod2 and Yph_cac_R5 only amplified a specific band of 112 bp from *P. cactorum* isolates (Table 1; Fig. 1a), primers Yph1F_mod2 and Yph_cin_R2 amplified a unique band of 176 bp from *P. cinnamomi* isolates (Table 1; Fig. 2b) and primers Yph1F_mod2 and Yph_lat_R4 amplified unique band of 225 bp from *P. lateralis* isolates (Table 1; Fig. 2c). Primer pair Yph1F_mod2/Yph_cin_R1 amplified a unique band of 229 bp from *P. cinnamomi* isolates (data not shown), and for *P. lateralis* isolates, primer pair Yph_lat_F1/Yph2R_mod2 amplified a 307 bp band and Yph1F_mod2/Yph_lat_R2 amplified a 189 bp band (data not shown).

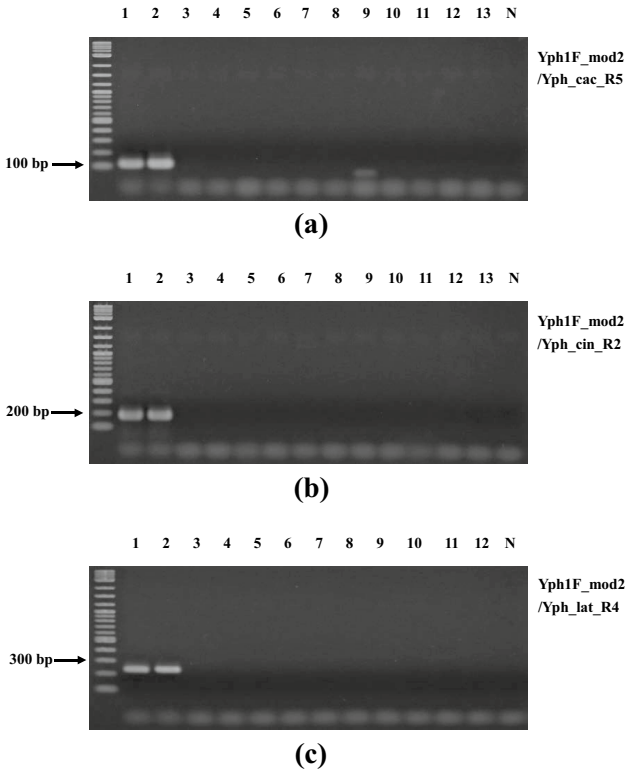

Fig. 1 Specificity tests of specific primers designed for three *Phytophthora* species based on closely related species. **a** *P. cactorum* specific primer pair Yph1F_mod2/Yph_cac_R5; lanes: 1, *P. cactorum* CH989A11; 2, *P. cactorum* ZZ017; 3, *P. hedraiandra* CBS111725; 4, *P. idaei* WPC6767; 5, *P. pseudotsugae* WPC10339; 6, *P. clandestina* WPC3942; 7, *P. iranica* CBS374.72; 8, *P. tentaculata* CBS552.96), 9, *P. infestans* MAFF305586; 10, *P. ipomoeae* WPC10225; 11 *P. mirabilis* WPC3005; 12, *P. phaseoli* WPC10145; 13, *P. nicotianae* GF468. **b** *P. cinnamomi* specific primer pair Yph1F_mod2/Yph_cin_R2; lanes: 1, *P. cinnamomi* WPC2160; 2, *P. cinnamomi* ZZ029; 3, *P. parvispora* CBS411.96; 4, *P.* sp. *niederhauserii* CH96HE1; 5, *P. sojae* WPC7358; 6, *P. pistaciae* CBS137185; 7, *P. melonis* WPC6870; 8, *P. cajani* WPC3105; 9, *P. vignae* Ph-9; 10, *P. uliginosa* CBS109054; 11, *P. europaea* CBS109049; 12, *P. fragariae* CBS209.46; 13, *P. cambivora* WPC0592. **c** *P. lateralis* specific primer pair Yph1F_mod2/Yph_lat_R4; lanes: 1 and 2, *P. lateralis* WPC3361; 3, *P. ramorum* CBS101553; 4, *P. hibernalis* CBS114104; 5, *P. foliorum* WPC10974; 6, *P. syringae* MAFF645010; 7, *P. primulae* CBS620.97; 8, *P. brassicae* CBS179.87; 9, *P. sansomeana* WPC3163; 10, *P. medicaginis* WPC10138; 11, *P. drechsleri* WPC1087; 12, *P. cryptogea* WPC1088. *N* sterile distilled water. Size marker: 100-bp DNA ladder

Sensitivity tests

In the simplex PCR, primers Yph1F_mod2 and Yph_cac_R5 detected as little as 250 fg of DNA of *P. cactorum* isolate CH989A11 (Fig. 2a). The same sensitivity was also obtained for *P. cactorum* isolate ZZ017. Specific primer pairs Yph1F_mod2/Yph_cin_R2 and Yph1F_mod2/Yph_lat_R4 were sensitive to 250 fg of DNA of *P. cinnamomi* isolate WPC2160 and *P. lateralis* isolate WPC3361, respectively

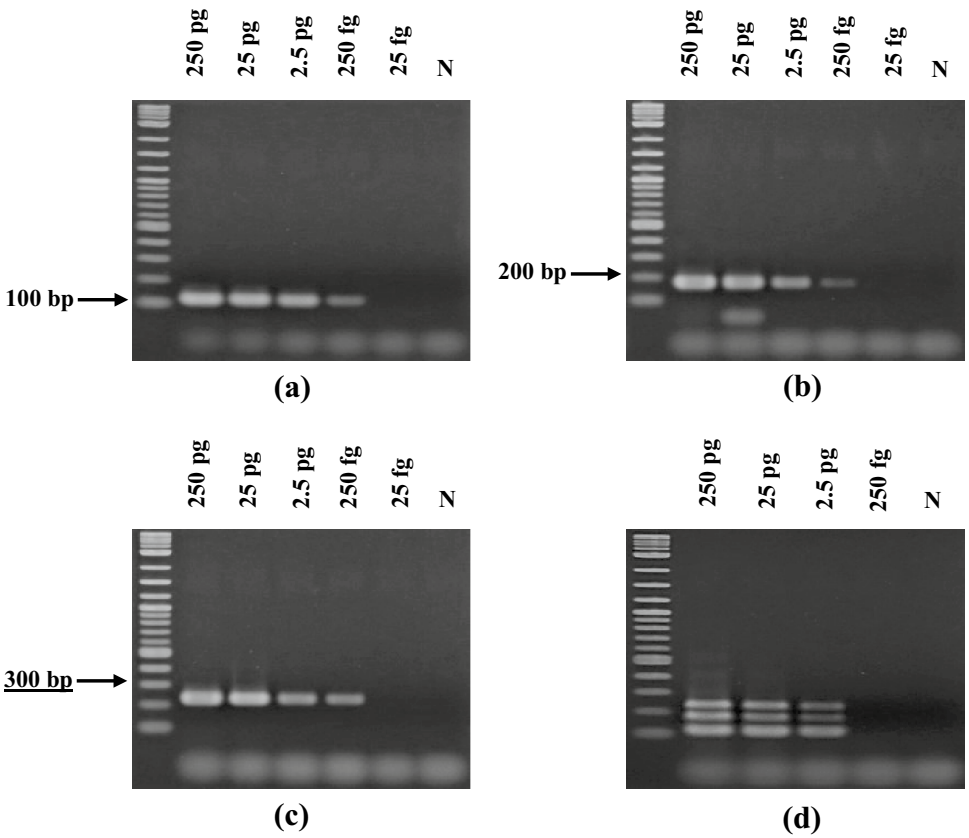

Fig. 2 Sensitivity tests using specific primers designed for *Phytophthora cactorum, P. cinnamomi* and *P. lateralis* in single-plex PCR with 250 pg to 25 fg DNA from pure cultures of tested isolates or 250 pg to 250 fg in multiplex PCR. Amplification with species-specific primer pair **a** Yph1F_mod2/Yph_cac_R5 for *P. cactorum* isolate CH989A11, **b** Yph1F_mod2/Yph_cin_R2 for *P. cinnamomi* isolate WPC2160, **c** Yph1F_mod2/Yph_lat_R4 for *P. lateralis* isolate WPC3361. **d** Multiplex PCR with four primers (Yph1F_mod2, Yph_cac_R5, Yph_cin_R2 and Yph_lat_R4). *N* sterile distilled water. Size marker: 100-bp DNA ladder

(Fig. 2b, c). The same sensitivity was also obtained for *P. cinnamomi* isolate ZZ029 using the primer pair Yph1F_mod2/Yph_cin_R2. For multiplex PCR, the sensitivity was 2.5 pg of DNA for each species from two DNA mixtures: CH989A11 × WPC2160 × WPC3361 (Fig. 2d) and ZZ017 × ZZ029 × WPC3361.

Application of multiplex PCR in naturally and artificially infested soils

In the multiplex PCR, four species-specific primers (Yph1F_mod2, Yph_cac_R5, Yph_cin_R2 and Yph_lat_R4) together with two fungal universal primers (18S-69F and 18S-1118R) were used. Multiplex PCR analysis of naturally and artificially infested soils amplified fragments obtained from all samples using the fungal universal primers 18S-69F/18S-1118R (Fig. 3), and a specific fragment was amplified, respectively, from samples infested with *P. cactorum* and *P. cinnamomi*.

Detection of *P. cactorum, P. cinnamomi* and *P. lateralis* in the main kiwifruit planting areas of China

Although the soil samples showed different soil textures (Table 4), the fungal universal primers 18S-69F and

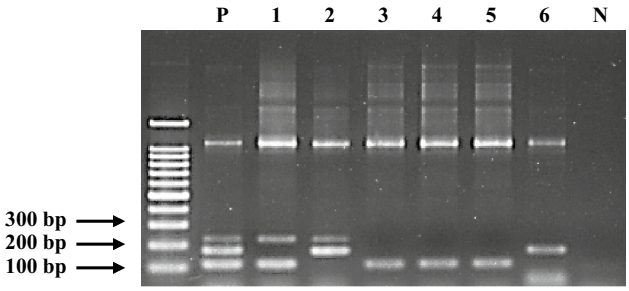

Fig. 3 Multiplex PCR from artificially and naturally infested soils to detect *Phytophthora cactorum, P. cinnamomi* and *P. lateralis*. Soil sample 1 was artificially infested with *P. cactorum* and *P. lateralis*. Soil sample 2 was artificially infested with *P. cinnamomi* and *P. lateralis*. Soil samples 3–6 were collected from the main kiwifruit areas in Shaanxi Province of China. *P* mixture of mycelial DNA from *P. cactorum, P. cinnamomi*, and *P. lateralis* as a positive control, *N* sterile distilled water as a negative control. Size marker: 100-bp DNA ladder

18S-1118R amplified DNA from all samples, indicating successful extraction and amplification. The *Phytophthora* universal primers were used before the multiplex PCR to detect *Phytophthora* spp. with the same sensitivity level to the simplex PCR. No pathogens were detected in the 15 samples collected from Zhouzhi Prefecture in April. Among the 20 samples collected in July, *P. cactorum* was detected in two samples from a field with *Phytophthora* rot history, and *P.*

cinnamomi was detected in one sample with root rot history. Among the 16 samples collected in October, *P. cactorum* was detected in two with leaf yellowing history. In Meixian Prefecture, no pathogens were detected in samples collected in April and October, only *P. cactorum* was detected in three samples with root rot history in July, and *P. lateralis* was not found in any samples.

In summary, *P. cactorum* and *P. cinnamomi* were found in Zhouzhi Prefecture, only *P. cactorum* was found in Meixian Prefecture, and *P. lateralis* was not found in either prefecture.

Discussion

In this study, we developed a reliable method to identify and detect *P. cactorum, P. cinnamomi* and *P. lateralis* simultaneously. New species-specific primers were designed based on the *Ypt*1 gene for the three species, then the multiplex PCR was optimized and successfully applied to survey all three pathogens in soils.

Primer specificity is crucial for PCR-based diagnosis. In preliminary tests, we examined nine DNA loci, including the rDNA ITS region, 28S rDNA, 60S ribosomal protein L10 gene, ß-tubulin gene, elongation factor 1 α gene, enolase gene, heat shock protein 90 gene, *tigA* gene fusion protein sequence, and the *Ypt*1 gene. When the sequences of species belonging to *Phytophthora* clade 1 (described by Blair et al. 2008), which is closely related to *P. cactorum*, were compared to identify interspecies variations suitable for the definition of specific primers, only the *Ypt*1 gene emerged as a promising candidate for *P. cactorum*. Li et al. (2011) also designed two specific primers for *P. cactorum* based on the *Ypt*1 and compared it with most of the reported specific primers for *P. cactorum*; it was the most reliable for distinguishing *P. cactorum* from other closely related species (*P. hedraiandra, P. idaei*, and *P. pseudotsugae*).

Several species-specific primers for *P. cinnamomi* and *P. lateralis* were published in recent studies (Engelbrecht et al. 2013; Kong et al. 2003; Langrell et al. 2011; Miles et al. 2014; O'Brien 2008; Schena et al. 2008; Schenck et al. 2016; Williams et al. 2009; Winton and Hansen 2001). However, no specificity tests against closely related species have been conducted for most of the species-specific primers that have been published. In our experience, specificity of these published species-specific primers should be reconfirmed against closely related species in the same clade before use. Our opinion is supported by the results of a study by Kunadiya et al. (2017), who tested eight sets of different species-specific primers reported by different researchers for *P. cinnamomi* against closely related species from clade 7 and found that only three sets were truly specific to *P. cinnamomi*. The number of known *Phytophthora* species has doubled in the past 20 years; therefore, it is difficult to obtain cultures or DNA for all species. Nevertheless, researchers should at least attempt to obtain and test species belonging to same phylogenetic clade or sub-clade.

The ITS regions of rDNA are useful targets for fungal species-specific primers because of their high copy number, sequence variability, and fidelity among pathogen species or subspecies. Therefore, they have been widely applied to identify and detect *Phytophthora* spp. in recent years. However, sequence variations in this region between closely related species are not always sufficient to define highly specific primers. Another disadvantage for rDNA genes is the intraspecific variation of copy number (Meng and Wang 2010; Spies et al. 2011), which may have an effect on the accuracy of pathogen quantification. Moreover, variations in a target locus at an intraspecific level may also limit the utility for analysis of community structure (Lamour 2013).

Unlike rDNA genes, which are generally present in multiple copies, *Ypt*1 is present as a single copy. In sensitivity tests, DNA of *P. cactorum, P. cinnamomi*, and *P. lateralis* was detected at levels as low as 250 fg by simplex PCR analysis (Fig. 1). Although the *Ypt*1 gene is inferior to rDNA genes with respect to sensitivity, this level of sensitivity appears sufficient for detection and quantification. Schena et al. (2008) used a nested approach based on a first-round amplification with *Phytophthora*-genus-specific primers and a second amplification with *Phytophthora*-species-specific multiplex real-time PCR and found that sensitivity increased to 100 fg. However, in the present study, a comparable level of sensitivity was obtained in a single round of amplification.

For most practical applications, the lower level of sensitivity achieved with *Ypt*1 might be a minor problem; however, the fact that the gene exists in a single copy suggests that single propagules of target species could be detected by a single multiplex real-time PCR. Methods based on single-copy genes are not affected by the number of repeats as in multi-copy genes, and there is the potential to correlate C_t values accurately with the pathogen biomass and/or the number of propagules (Li et al. 2013). Therefore, for application in pathogen quantification, primers designed for *Ypt*1 should be more suitable than those from multi-copy genes.

Multiplex PCR was successfully performed using the fungal universal primers (18S-69F and 18S-1118R) and species-specific primers (Yph1F_mod2, Yph_cac_R5, Yph_cin_R2, and Yph_lat_R4). According to the critical parameters in multiplex PCR discussed by Henegariu et al. (1997), we optimized four factors: annealing temperature (AT), buffer concentration, primer amounts, and the balance of dNTP and magnesium chloride. When we designed the species-specific primers for the three pathogens based on *Ypt*1, we tested the *Phytophthora* universal primers as common forward or reverse primers for the three species to reduce primer amounts as far as possible. Therefore,

we re-designed new species-specific primers, rather than using published primers. Moreover, to balance the competitive reaction for *Ypt*1 for the simultaneous amplification of three different targets, we adjusted the concentrations of different species-specific primers.

For investigation of the distribution of *P. cactorum, P. cinnamomi*, and *P. lateralis* in two main kiwifruit planting prefectures of China, samples with different soil properties and disease histories were collected. Using the DNA extraction method refined by Kageyama et al. (2003) and modified by incorporating a magnetic bead purification step (Li et al. 2010), we ensured that the DNA extracted was of high quality and sufficient quantity, as further corroborated by pre-amplification with the 18S gene fungal universal primers (Table 3). We found that more kiwifruit trees suffered from bacterial canker, leaf yellowing, or root rot, rather than the diseases caused by *Phytophthora* spp. in Zhouzhi and Meixian prefectures. A few samples had a known *Phytophthora* rot history, but *P. cactorum* was detected in some samples without *Phytophthora* rot history, indicating that several kiwifruit fields could potentially be infected with *Phytophthora* spp. In a recent investigation of kiwifruit *Phytophthora* diseases around the world, major pathogenic species included *P. cryptogea, P. citrophthora, P. drechsleri, P. palmivora, P. cactorum, P. cinnamomi, P. megasperma, P. citricola* and *P. lateralis*. However, in the present investigation, only *P. cactorum* and *P. cinnamomi* were detected in Zhouzhi and Meixian prefectures of China.

We note that it is difficult to judge contamination in a field using the multiplex PCR alone in practical application because whether the disease will develop or not depends on the amount of *Phytophthora* spp. and environmental conditions. In addition, even if other *Phytophthora* spp. are present, it cannot detect them. Therefore, we suggest doing a preliminary selection by using the *Phytophthora* genus-specific primers (Yph1F_mod2 and Yph2R_mod2), which proved to be equally sensitive as the specific primers (data not shown). Besides, because of the lower detection limit of the multiplex PCR, simplex PCR is suggested when a soil sample yields positive result with the genus-specific primers but is negative in the multiplex PCR.

Kiwifruit vine is commonly infected by pathogenic bacteria, fungi, and viruses. Some symptoms of *Phytophthora* diseases and diseases caused by other pathogens are very similar, but fungicides used for their control are completely different. Therefore, incorrect diagnoses will cause avoidable problems and financial loss. Although *Phytophthora* rot has not severely threatened the main kiwifruit production areas in China thus far, it is still helpful to differentiate *Phytophthora* disease from those caused by other pathogens to prevent serious economic losses. The technique introduced here was useful and effective in discriminating these pathogens and will be helpful in the early diagnosis of seedling infection and disease control.

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Compliance with ethical standards

Research involving human or animal participants This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

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