A Multiplex PCR for the Detection of *Phytophthora nicotianae* and *P. cactorum*, and a Survey of Their Occurrence in Strawberry Production Areas of Japan

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Abstract

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We aimed to simultaneously detect two pathogens causing strawberry diseases, *Phytophthora nicotianae* and *P. cactorum*, by multiplex polymerase chain reaction (PCR), and to survey their occurrence in the main strawberry production areas of Japan. Due to the need to combine different primer pairs for multiplex PCR and the low specificity of published specific primers for *P. nicotianae* and *P. cactorum*, new species-specific primers for *P. nicotianae* and *P. cactorum* were designed based on the internal transcribed spacer regions of ribosomal DNA and the ras-related protein gene *Ypt*1, respectively. Specificity of the designed primers was demonstrated using 68 isolates, including *Phytophthora* spp., *Pythium* spp., and other soilborne pathogens. Multiplex PCR discriminated between *P. nicotianae* and *P. cactorum* in DNA mixtures of mycelia of the two species. Moreover, both species were detected in artificially and naturally infested soils, indicating that these markers can be used in diagnosis of strawberry diseases. For investigation of the geographic distribution of the two pathogens in Japan, soil samples were collected in 89 strawberry fields from eight prefectures (Gifu, Saga, Nara, Tochigi, Chiba, Shizuoka, Yamanashi, and Hokkaido) of Japan. The method that was developed was successfully applied to survey *P. nicotianae* and *P. cactorum*, and distribution of the two pathogens in strawberry plantings in Japan was determined.

In Japan, new cultivars of strawberry have been bred in order to satisfy consumer tastes. However, most of the cultivars are highly susceptible to diseases. The most serious strawberry diseases, such as anthracnose, Phytophthora rot and Fusarium wilt, were responsible for losses of almost $50 million over the last 4 years in Japan.

In 1978, an outbreak of Phytophthora rot of strawberry was recorded in Shizuoka Prefecture, Japan (26,27). The species commonly causing Phytophthora rot of strawberry are *Phytophthora nicotianae* and *P. cactorum* (12,26). Although these pathogens are not widespread in Japan, the crown rot and wilt symptoms are similar to those caused by the anthracnose pathogen *Colletotrichum gloeosporioides*. Consequently, incorrect diagnoses may occur, which complicate pathogen control and transplant storage. Moreover, no investigations into their distribution across Japan have been conducted. Therefore, it is important to develop a simple and fast 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 essential for development of an effective disease control strategy. Traditional detection methods such as baiting and soil dilution plating methods are difficult and time consuming, and require extensive experience. With the development of DNA-based techniques, polymerase chain reaction (PCR) became a primary method of plant pathogen identification and detection (7,8,19). Diagnostic PCR methods and primers have been devised for *Phytophthora* spp., including *P. nicotianae* (9,16,18,21) and *P. cactorum* (2,4,17,23). However, because some closely related *Phytophthora* spp. were not distinguished in previous research, it remains difficult to establish that those primers actually are specific. Furthermore, there is no report on simultaneous detection of the two species.

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The internal transcribed spacer (ITS) regions of nuclear-encoded ribosomal DNA (rDNA) genes are widely used to identify and detect *Phytophthora* spp. (6). However, they are not always sufficiently diverse to allow separation of closely related taxa (16,22,24). The elicitin gene *parA*1 and the putative storage protein gene family, *Lpv*, proved to be suitable targets for the specific detection of *P. nicotianae* and *P. cinnamomi*, respectively (14,15). Unfortunately, these genes do not contain introns and, therefore, are unlikely to be sufficiently diverse to enable the distinction of a broad range of species (22). The ras-related protein gene *Ypt*1 (5) appears to be a more promising target because it contains sufficient variation to allow for the development of molecular markers for almost all *Phytophthora* spp. (22). However, available sequence data are limited.

In this study, we developed a multiplex PCR assay for the simultaneous detection of *P. nicotianae* and *P. cactorum* that infect strawberry plants in Japan. We designed primers specific for *P. nicotianae* and *P. cactorum* based on ITS regions and the *Ypt*1 gene, respectively. We applied the novel multiplex PCR technique to samples from infested fields, which enabled us to investigate the distribution of the two pathogens in the main strawberry cultivation regions of Japan.

Materials and Methods

Species and strain maintenance. In all, 31 *Phytophthora* spp., 11 additional oomycetes (genera *Pythium* and *Saprolegnia*), and 5 other soilborne pathogens, including *Plasmodiophora*, *Pyrenochaeta*, *Rhizoctonia*, and *Verticillium* spp., were used (Table 1). The *P. nicotianae* isolates with different hosts were provided by Centraalbureau für Schimmelcultures and the local agriculture research centers of Japan. The *P. cactorum* isolates from strawberry were collected from different prefectures of Japan. Other *Phytophthora* spp., *Pythium* spp., and pathogens were collected from several scientific resource institutions and Gifu University Cultures Collection. The *Phytophthora* spp. and all culturable isolates were maintained on corn meal agar or potato dextrose agar at 20°C in the dark.

Collection of soil samples. In total, 89 soil samples from 89 sampling strawberry fields in eight strawberry production areas (Gifu, Nara, Chiba, Saga, Shizuoka, Tochigi, Yamanashi, and

Table 1. Specificity of the designed primer pairs to *Phytophthora nicotianae* and *P. cactorum* using simplex polymerase chain reaction[a]

Species	Isolate[b]	Host	Location	ITS region, Nic-F1/R1[c]	*Ypt*1 gene, Cac-F3/ R3-2[d]
P. nicotianae	CH02FPK3	Strawberry	Chiba, Japan	+	–
	GF465	Strawberry	Gifu, Japan	+	–
	GF101	Karankoe	Gifu, Japan	+	–
	CBS305.29	Tobacco	Taiwan	+	–
	CBS101655	Alstromerea	Netherland	+	–
	C08	Ardisia crispa	Chiba, Japan	+	–
	C15	*Echium wildpretii*	Chiba, Japan	+	–
	C23	*Abutilon* sp.	Chiba, Japan	+	–
	C26	*Lavandula angustifokia*	Chiba, Japan	+	–
	C58	Gerbera	Chiba, Japan	+	–
	C83	*Strelitzia reginae*	Okinawa, Japan	+	–
	F03	*Nerine sarniensis*	Fukuoka, Japan	+	–
	Ch03OKTYPE3	Strawberry	Okayama, Japan	+	–
P. cactorum	GF654	Strawberry	Gifu, Japan	–	+
	CH03 OKTYPE1	Strawberry	Okayama, Japan	–	+
	CH99PFT4	Strawberry	Chiba, Japan	–	+
	CH01FPA1	Strawberry	Chiba, Japan	–	+
	CH07INBA1-2	Strawberry	Chiba, Japan	–	+
	CH99FV2	Strawberry	Miyagi, Japan	–	+
	CH08N4-2	Strawberry	Shimane, Japan	–	+
	CH02PMN001	Strawberry	Tokushima, Japan	–	+
	EID2	Strawberry	Chiba, Japan	–	+
P. cajani	WPC3105	*Cajanus cajan*	India	–	–
P. cambivora	WPC6358	Almond	Australia	–	–
P. capsici	WPC0253	Cacao	Mexico	–	–
	NBRC30696	*Cucurbita* sp.	Japan	–	–
P. cinnamomi	NBRC33182	*Hypericum androsaemum*	Japan	–	–
P. citropthora	WPC1200	*Cacao*	Brazil	–	–
P. clandestina	CBS347.86	*Trifolium subterraneum*	Australia	–	–
P. erythroseptica	WPC0340	Potato	Australia	–	–
P. hedraiandra	CBS111725	*Viburnum* sp.	Netherlands	–	–
P. heveae	WPC1102	Avocado	Guatemala	–	–
P. humicola	WPC3826	NA	Taiwan	–	–
P. idaei	CBS971.95	*Rubus idaeus*	UK	–	–
P. infestans	CBS368.51	*Solanum tuberosum*	Netherlands	–	–
P. insolita	WPC6159	NA	NA	–	–
P. ipomoeae	CBS122203	*Ingolfiella longipes*	Mexico	–	–
P. iranica	CBS374.72	*Solanum melongena*	Iran	–	–
P. meadii	WPC3500	NA	NA	–	–
P. medicaginis	WPC7029	Alfalfa	United States	–	–
P. megasperma	WPC3163	NA	United States	–	–
P. melonis	WPC1371	Cucumber	NA	–	–
P. mirabilis	CBS678.85	*Mirabilis jalapa*	Mexico	–	–
P. multivesiculata	CBS545.96	*Cymbidium* sp.	Netherlands	–	–
P. palmivora	WPC0113	Papaya	United States	–	–
P. phaseoli	CBS120373	*Phaseolus lunatus*	United States	–	–
P. pseudotsugae	CBS444.84	*Pseudotsuga menziesii*	United States	–	–
P. richardiae	WPC7788	Carrot	United Kingdom	–	–
P. sojae	NBRC31016	*Glycine max*	Japan	–	–
P. tentaculata	C45	*Calendula arvensis*	Chiba, Japan	–	–
P. undulata	WPC7505	NA	NA	–	–
P. vignae	HoAz1	Azki bean	Hokkaido, Japan	–	–
Plasmodiophora brassicae	An	Chinese cabbage	Mie, Japan	–	–
Pythium helicoides	CBS286.31	*Phaseolus vulgaris*	United States	–	–
P. irregulare	NBRC100108	Carrot	Gifu, Japan	–	–
P. myriotylum	NBRC100113	Kidney bean	Hokkaido, Japan	–	–
P. ostracodes	CBS768.73	Soil	Spain	–	–
P. paddicum	NBRC31993	*Hordeum vulgare*	Japan	–	–
P. pyrilobum	NBRC32560	*Agrostis palustris*	NA	–	–
P. spinosum	NBRC100116	Soil	Gifu, Japan	–	–
P. sylvaticum	NBRC100119	Soil	Gifu, Japan	–	–
P. ultimum	NBRC100123	Soil	Gifu, Japan	–	–
P. vexans	MS6-10-8V	Soil	Gifu, Japan	–	–
Pyrenochaeta lycopercisi	Type1	Tomato	Japan	–	–
Rhizoctonia solani	RGR38	NA	Japan	–	–
Saprolegnia sp.	NBRC32708	*Salmo trutta*	NA	–	–
Verticillium albo-atrum	Vaal 130308	NA	NA	–	–
V. dahliae	Vd84034	NA	NA	–	–

[a] Symbols: + = amplified, – = no amplification, and NA = not available.
[b] Isolates were collected from Centraalbureau fur Schimmelcultures (CBS), NITE Biological Research Centre (NBRC), World *Phytophthora* Genetic Resource Collection WPC), and Gifu University Cultures Collection.
[c] Specific primers Nic-F1 and Nic-R1 were designed based on the internal transcribed spacer region for *P. nicotianae*.
[d] Specific primers Cac-F3 and Cac-R3-2 were designed based on *Ypt*1 gene for *P. cactorum*.

Hokkaido Prefectures) throughout Japan were collected for the survey of pathogen distribution in the summer of 2010 after strawberry cultivation. In each field, five subsamples (each about 100 g) were randomly collected and mixed thoroughly. From the soil mixture, approximately 200 g were recovered and stored at 5°C. Soil pH was measured in 1 M potassium chloride. Soil groups and textures were determined according to the classification of the Japanese Ministry of Land, Infrastructure, Transport and Tourism, and the criterion of the International Society of Soil Science, respectively. Disease histories of the fields were provided by local agriculture research centers.

DNA extraction from mycelia and soil. Total genomic DNA from mycelia was extracted according to the procedure of Kageyama et al. (13). Mycelia grown on V8 juice broth medium were used for DNA extraction from culturable species. For soil DNA extraction, the method refined by Kageyama et al. (13) was modified by incorporating a magnetic bead purification step (MagExtractor-Plant Genome; Toyobo Co., Ltd., Osaka, Japan) to purify soil DNA extracts as described by Li et al. (20). 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 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, Detroit), and RNase A at 200 µg/ml (Nippongene, Toyama, Japan), and then vigorously vortexed at 4,200 rpm for 1 min. Benzyl chloride (150 µl) was added to the mixture, and the tube was vigorously vortexed for 2 min. After 15 min of incubation at 60°C, 150 µl of 3 M NaOAc was added to the suspension and the mixture was lightly vortexed. After 15 min of incubation on ice, this suspension was cleared by two 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 performed according to the manufacturer's instructions in the purification step of the MagExtractor-Plant Genome kit. Finally, 50 µl of the purified DNA was obtained.

Sequencing. The *Ypt*1 gene of was sequenced in 19 *Phytophthora* isolates (Table 2). To amplify the *Ypt*1 genes of *Phytophthora* spp., the *Phytophthora* genus-specific primers Yph1F and Yph2R (Table 3) were used in PCR. The reaction mixture contained 1 µM each primer, 1 unit of r*Taq* DNA polymerase (Takara Bio Inc., 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), 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 2700; Applied Biosystems, Foster City, CA), under the following conditions: 95°C for 2 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 45 s, and extension at 72°C for 30 s; followed by a final extension step at 72°C for 10 min.

After purification of the PCR products, a BigDye Terminator ver. 3.1 Cycle Sequencing kit (Applied Biosystems) was used for cycle sequencing and run 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).

Primer design. A collection of the ITS region sequences, including 52 *Phytophthora* spp. and three *Pythium* spp., were aligned (BioEdit ver. 7.0.0; Isis Pharmaceuticals Inc., Dublin) to design specific primers for *P. nicotianae*. Similarly, an alignment of the *Ypt*1 gene sequences from 42 *Phytophthora* spp. and three *Pythium* spp. was produced to define specific primers for *P. cactorum*. All of the ITS sequences and 29 *Ypt*1 gene sequences were collected from the DNA database, and another 19 *Ypt*1 gene sequences were sequenced in this study (Table 2). Candidate primers were analyzed for dimer and hairpin loop structures (Primer Premier ver. 5.0; Premier Biosoft International, Inc., CA).

Simplex PCR with species-specific primers. Specificity of each developed primer pair was confirmed in simplex PCR with all the isolates listed in Table 1. The reaction mixture contained 1 µM developed primers, 1 unit of FastStart *Taq* DNA polymerase (Roche Applied Science, Mannheim, Germany), 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), 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 2700) at 95°C for 5 min; followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 66°C for 30 s, and extension at 72°C for 1 min; with a final extension at 72°C for 10 min.

Specificity was compared among five published primer pairs (Pn5B/Pn6, $P_{NIC}1/P_{NIC}2$, PN-F/PN-R, IL7/IL8, and Pn1/Pn2) designed for *P. nicotianae* and three primer pairs (Ycac1F/Ycac2R, BPhycacL87FRG/BPhycacR87RRG, and BPhycacL89FRG/BPhycacR176RRG) for *P. cactorum*. PCR was performed according to the protocols provided in the original article reporting the primer pairs. Thirteen closely related *Phytophthora* spp., including *P. nicotianae*, *P. cactorum*, *P. hedraiandra*, *P. idea*, *P. pseudotsugae*, *P. clandestine*, *P. iranica*, *P. tentaculata*, *P. infestans*, *P. ipomoeae*, *P. mirabilis*, *P. phaseoli*, and *P. multivesiculata*, were used for specificity test. Amplification was confirmed by electrophoresis in 3% certified low-range ultra agarose (Bio-Rad Laboratories, Hercules, CA). Gels were stained with ethidium bromide and photographed under ultraviolet light.

Multiplex PCR. As a positive control to ensure the success of DNA extraction, two universal primers, 18S-69F and 18S-1118R (Table 3), were added to the multiplex PCR reaction mixture. Magnesium chloride was added to improve the amplification of multiloci. The multiplex PCR reaction mixture was prepared as described for simplex PCR, except that the final concentrations of $MgCl_2$ and the primer pairs 18S-69F/18S-1118R, *P. nicotianae*-specific primer pair Nic-F1/Nic-R1, and *P. cactorum*-specific primer pair Cac-F3/Cac-R3-2 were 3 mM, 0.25 µM, 1 µM, and 2 µM, respectively. Reaction conditions were the same as in simplex PCR. PCR products were electrophoresed in 3% certified low-range ultra agarose.

The applicability of the multiplex PCR was tested using soil samples from six strawberry-growing locations with *P. nicotianae* disease histories and two artificially infested soil samples. Two samples from locations with *P. cactorum* disease histories were analyzed as well. Pure culture DNA mixtures of the two species served as a positive control.

Sensitivity tests. Sensitivity to *P. nicotianae* and *P. cactorum* DNA was tested in both simplex PCR and multiplex PCR. Ten-fold dilution series (1 ng to 10 fg) of DNA from three *P. nicotianae* isolates (CH03OKTYPE3, CH02FPK3, and GF465) and three *P. cactorum* isolates (GF654, CH07INBA1-2, and EID2) were used in simplex PCR. Three DNA mixtures (CH03OKTYPE3 × GF654, CH02FPK3 × CH07INBA1-2, and GF465 × EID2), diluted from 1 ng to 10 fg, were used in multiplex PCR.

Results

Primer design. Previously, unknown sequences of the *Ypt*1 gene were determined in the present study (Table 2). The primers Nic-F1and Nic-R1 were designed based on the ITS region for specific amplification of *P. nicotianae*. Similarly, the primers Cac-F3 and Cac-R3-2 were designed based on the *Ypt*1 gene of *P. cactorum*. The primers were designed for an annealing temperature of over 56°C.

Specificity confirmation of designed primers and comparison with other primers. In all, 13 and 9 isolates of *P. nicotianae* and *P. cactorum*, respectively, from different hosts and geographic locations in Japan were used together with 45 non-target species (Table 1) to test the specificity of the designed primers for each species. The presence of amplified DNA from all isolates was confirmed using the primers 18S-69F and 18S-1118R. The primers Nic-F1 and Nic-R1 only amplified the *P. nicotianae* isolates with a specific band of 267 bp evaluated by sequence analysis, and Cac-F3 and Cac-R3-2 exclusively amplified the *P. cactorum* isolates with a unique band of 223 bp evaluated by sequence analysis. The two target bands were clearly distinguished in electrophoresis.

For the detection of *P. nicotianae* and *P. cactorum*, several specific primers were developed previously (Table 3). We compared their specificity using 13 closely related *Phytophthora* spp. belonging to clade 1 according to Blair et al. (3). First DNA amplification was performed using the two universal primers pairs ITS1/ITS4 and 18S-69F/18S-1118R (Fig. 1). The Nic-F1/Nic-R1 primers amplified the expected 267-bp fragment only from *P. nicotianae*. The Pn5B/Pn6 primers amplified the same size of DNAs from four species: *P. nicotianae*, *P. cactorum*, *P. hedraiandra*, and *P. idaei*. The PN-F/PN-R primers amplified the same size of segments from *P. nicotianae* and *P. cactorum*. The primers $P_{NIC}1/P_{NIC}2$, IL7/IL8, and Pn1/Pn2 produced a signal with DNA from *P. nicotianae*; a slight amplicon of the same size from *P. cactorum* was found as well (Fig. 1).

With *P. cactorum* DNA, Cac-F3/Cac-R3-2 amplified a segment of 223 bp but Ycac1F/Ycac2R and BPhycacL87FRG/

Table 2. Accession number of the sequences of the internal transcribed spacer region and *Ypt*1 gene in DNA database[a]

	ITS region		*Ypt*1 gene	
Species	**Isolates**	**Accession**	**Isolates**	**Accession**
Phytophthora nicotianae	P1452	FJ801769	IMI268688	DQ162981
	P7146	FJ801963	CH02FPK3[b]	HQ849999
	P11000	FJ801542		
P. cactorum	CH98PEC1	AB367364	IMI296524	DQ162960
	CH03 OKTYPE1	AB367366	CH03OKTYPE1[b]	HQ850000
	CH02MKPY001	AB367365	EID2[b]	HQ850001
P. alni subsp. *alni*	P16203	GU259292	SCRP2	DQ162953
P. bisheria	Cg.2.3.3	AY241924	N	N
P. botryosa	P6945	FJ801954	N	N
P. cambivora	P0592	GU259025	SCRP82	DQ162956
P. capsici	P1091	GU259193	IMI352321	DQ162972
P. chrysanthemi	GF749	AB437135	N	N
P. cinnamomi	P3232	GU594781	CBS270.55	DQ162959
P. citricola	P7902	GU259136	SCRP143	DQ162971
P. citrophthora	P6310	FJ801913	IMI332632	DQ162973
P. clandestina	P3942	FJ801888	CBS347.86[b]	HQ850002
P. colocasiae	P6318	GU258989	N	N
P. cryptogea	CBS290.35	AF228099	IMI045168	DQ162987
P. drechsleri	P10331	FJ801387	ATCC46724	DQ162989
P. erythroseptica	CBS 956.87	AF228082	SCRP240	DQ162988
P. europaea	CBS109049	DQ275190	SCRP622	DQ162952
P. fragariae var. *fragariae*		AF266762	SCRP245	DQ162950
P. hedraiandra	P11056	EU080072	CBS111725[b]	HQ850003
P. idaei	P6767	FJ801946	CBS971.95[b]	HQ850004
P. ilicis	P2159	AY302164	SCRP379	DQ162963
P. infestans	P10650	FJ801470	CBS368.51[b]	HQ850005
P. inflate	IMI342898	AF266789	N	N
P. insolita	IMI288805	AF271222	IMI288805	DQ162974
P. inundata	P8478	FJ802005	SCRP649	DQ162985
P. ipomoeae	P10225	FJ801323	CBS122203[b]	HQ850006
P. iranica	CBS374.72	L41378	CBS374.72[b]	HQ850007
P. katsurae	P10187	GU259517	SCRP388	DQ162980
P. kernoviae	P1571	AY940661	SCRP722	DQ162975
P. lateralis	P3888	FJ802093	IMI040503	DQ162991
P. meadii	P6128	GU259180	N	N
P. medicaginis	P10683	GU259090	SCRP407	DQ162990
P. megakarya	P8516	FJ802010	P8517[b]	HQ850008
P. megasperma	P3136	GU258789	IMI133317	DQ162986
P. melonis	P10994	FJ801540	PMNJHG1	EF649778
P. mexicana	P0646	FJ801253	N	N
P. mirabilis	P3005	FJ802098	CBS678.85[b]	HQ850009
P. multivesiculata	CBS545.96	DQ988192	CBS545.96[b]	HQ850010
P. nemorosa	P10288	FJ801359	SCRP910	DQ162965
P. palmivora	P0255	FJ801246	IPPc3[b]	HQ850011
P. parsiana	C25	AY659739	N	N
P. phaseoli	P10145	FJ802106	CBS120373[b]	HQ850012
P. pistaciae	P6197	FJ801904	IMI386658	DQ162957
P. polonica	P131445	AB511828	N	N
P. pseudosyringae	P10437	FJ801438	SCRP734	DQ162967
P. pseudotsugae	IMI331662	AF266774	CBS444.84[b]	HQ850013
P. psychrophila	P10433	FJ801435	SCRP630	DQ162964
P. quercina	CBS 115973	AY853200	SCRP550	DQ162979
P. ramorum	P10301	FJ801362	SCRP911	DQ162992
P. richardiae	RICH-P7789	AB367498	N	N
P. sojae	P3114	FJ801828	SCRP555	DQ162958
P. tentaculata	CBS552.96	AF266775	C45[b]	HQ850014
Pythium oedochilum	N	N	CBS597.68[b]	HQ850015
P. helicoides	H5sz1C14	AB108025	TCG3[b]	HQ850016
P. ostracodes	N	N	CBS768.73[b]	HQ850017
P. undulatum		AF271230	N	N
P. vexans	CBS 119.80	AY598713	N	N

[a] N = species not used for this DNA region.
[b] Isolates sequenced in this study.

BPhycacR87RRG amplified DNA sequences from *P. hedraiandra*, *P. idaei*, and *P. pseudotsugae* as well. No amplification by BPhycacL89FRG/BPhycacR176RRG was found (Fig. 2).

Sensitivity test. In simplex, the primers Nic-F1/Nic-R1 detected as little as 100 fg of DNA of *P. nicotianae* isolate CH03OKTYPE3 (Fig. 3A). The same sensitivity was also obtained in the other two *P. nicotianae* isolates, CH02FPK3 and GF465. The primers Cac-F3/Cac-R3-2 detected down to 1 pg of DNA of *P. cactorum* isolate GF654 (Fig. 3B). In the other two *P. cactorum* isolates, CH07INBA1-2 and EID2, the same sensitivity was obtained as well. In multiplex PCR, the same sensitivity as in simplex PCR was obtained from all DNA mixtures: CH03OKTYPE3 × GF654 (Fig. 3C), CH02FPK3 × CH07INBA1-2, and GF465 × EID2.

Application of the multiplex PCR in naturally and artificially infested soils. Using the naturally and artificially infested soils, the multiplex PCR with primer pairs Nic-F1/Nic-R1, Cac-F3/Cac-R3-2, and 18S-69F/18S-1118R amplified the segments obtained by the universal primers 18S-69F/18S-1118R in all samples (Fig. 4). In the samples infested with *P. nicotianae*, Nic-F1/Nic-R1 amplified a specific DNA segment. In the samples infested with *P. cactorum*, Cac-F3/Cac-R3-2 amplified a specific DNA segment as well.

Distribution of *P. nicotianae* and *P. cactorum* in the main strawberry production areas of Japan. Although the soil samples showed different soil textures and soil groups (Table 4), the universal primers 18S-69F and 18S-1118R amplified DNA in all samples, indicating that DNA extraction had been performed successfully in all soil samples from 89 fields. In all, 10 samples had an anthracnose history and 6 had a *Fusarium* wilt history; neither *P. nicotianae* nor *P. cactorum* were detected in these 16 samples. Two samples (Chiba 1 and Saga 1) had a Phytophthora rot history. *P. nicotianae* as well as *P. cactorum* were detected in Saga 1 but neither was detected in Chiba 1. Moreover, among the samples without disease history, *P. nicotianae* was detected in two samples from Nara Prefecture and two from Saga, while *P. cactorum* was detected in one sample from Nara and one from Yamanashi.

In summary, neither of the two species was found in the Gifu, Chiba, Shizuoka, Tochigi, and Hokkaido Prefectures, while both were found in Nara and Saga Prefectures; only *P. cactorum* was found in Yamanashi Prefecture.

Discussion

In this study, we developed a reliable method to identify and detect *P. nicotianae* and *P. cactorum* simultaneously. Species-specific primer pairs were designed in the ITS region and the *Ypt*1 gene for *P. nicotianae* and *P. cactorum*, respectively. Multiplex PCR was optimized and successfully applied to survey both species in soil.

Primer designing is crucial for PCR-based diagnosis. In preliminary tests, we examined nine DNA loci, including the rDNA ITS region, 28S rDNA, the 60S ribosomal protein L10 gene, the β-tubulin gene, the elongation factor 1 α gene, the enolase gene, the heat shock protein 90 gene, the *tig*A gene fusion protein sequence, and *Ypt*1. When the sequences of species of the *Phytophthora* clade 1 (described by Blair et al. [3]), which is closely related to *P. nicotianae* and *P. cactorum*, were compared to identify interspecies variations suitable for the definition of specific primers, the ITS region and the *Ypt*1 gene emerged as promising candidates. We successfully designed primers based on the ITS region for *P. nicotianae*. Only the *Ypt*1 gene proved to be a suitable target for primers specific to *P. cactorum*.

The ITS regions of rDNA are useful targets for fungal species-specific primers due to their high copy number, sequence variability, and fidelity among pathogen species or subspecies. Therefore, in recent years, they were widely used to identify and detect *Phytophthora* spp. However, sequence variations in this region between closely related species are not always sufficient to define highly specific primers. Two primer pairs (Pn5B/Pn6 and $P_{NIC}1/P_{NIC}2$) were designed based on the ITS regions of *P. nicotianae* by Ippolito et al. (11) and Grote et al. (9), respectively. However, Pn5B and Pn6 could not discriminate *P. cactorum*, *P. hedraiandra*, and *P. idaei* in the specificity test (Fig. 1). $P_{NIC}1$ and $P_{NIC}2$ amplified a sequence from *P. nicotianae* but also from *P. cactorum* isolate GF654. We designed the two primers Nic-F1 and Nic-R1 that target ITS regions of *P. nicotianae* with high specificity. Moreover, the two primer pairs PN-F/PN-R and IL7/IL8, targeting the elicitin gene ParA1, and the primer pair Pn1/Pn2, targeting the *Ypt*1 gene, were used for the comparison of specificities. PN-F and PN-R could not separate *P. nicotianae* and *P. cactorum*. The primers IL7/IL8 and Pn1/Pn2 reacted to *P. cactorum* isolate GF654. To overcome the low specificity of the primers of

Table 3. Primers used in this study

Target species	Primers[a]	Gene locus[b]	Sequence (5′–3′)	Target size (bp)	Reference
Fungi	ITS1	ITS region	TCCGTAGGTGAACCTGCGG	Variable	28
	ITS4		TCCTCCGCTTATTGATATGC		
	18S69F	18S rDNA	CTGCGAATGGCTCATTAAATCAGT	Variable	1
	18S1118R		GGTGGTGCCCTTCCGTCAA		
Phytophthora spp.	YPh1F	*Ypt*1 gene	CGACCATKGGTGTGGACTTT	Variable	23
	YPh2R		ACGTTCTCMCAGGCGTATCT	≈470	
Phytophthora nicotianae	**Nic-F1**	ITS region	CCTATCAAAAACAAGGCGAACG	267	This study
	Nic-R1		TGGCATACTTCCAGGACTAACC		
	Pn5B	ITS region	GAACAATGCAACTTATTGGACGTTT	120	11
	Pn6		AACCGAAGCTGCCACCCTAC		
	$P_{NIC}1$	ITS region	CAATAGTTGGGGGTCTTATT	737	9
	$P_{NIC}2$		GTATACCGAAGTACACATTAAG		
	PN-F	Elicitin gene *parA1*	CCACCACGCAGCAAACTGCGGC	230	16
	PN-R		TTGAGTACCAGGCCGCTCGTAG		
	IL7	Elicitin gene *parA1*	CTCCAGCTAGTCAAGCCTAGT	378	18
	IL8		GCACCGTCAACTCGCAGTC		
	Pn1	*Ypt*1 gene	GACTTTGTAAGTGCCACCATAC	389	21
	Pn2		CTCAGCTCTTTTCCTTGGATCT		
P. cactorum	**Cac-F3**	*Ypt*1 gene	CGTGGCGTGTTTCCTATTC	223	This study
	Cac-R3-2		TTCCGTCGGCTCTTTCAG		
	Ycac1F	*Ypt*1 gene	CCATACAAAATTCTGCGCTAGG	194	23
	Ycac2R		AGACACACAAGTGGACCGTTAG		
	BPhycacL87FRG	ITS region	CTTCGGCCTGAGCTAGTAGCT	340 and 480	2
	BPhycacR87RRG		CAGTCGGTCCGAAAACCAG		
	BPhycacL89FRG	ITS region	GGCTTCGGCCTGAGCTAGTAGCT	431	2
	BPhycacR176RRG		CAACTTGCTACAATA		

[a] Primers in bold are designed in this study.
[b] ITS = internal transcribed spacer and rDNA = ribosomal DNA.

both *P. nicotianae* and *P. cactorum*, optimization of DNA polymerase and dNTP concentration will be needed rather than PCR condition, because the PCR conditions were followed as in the original articles.

Primer design was more complicated for *P. cactorum.* As described by Blair et al. (3), *P. cactorum* is phylogenetically closely related to *P. hedraiandra*, *P. idaei*, and *P. pseudotsugae*. For these species, little interspecies variation was present in the nine loci that we scrutinized. In fact, the two primer pairs BPhycacL87FRG/BPhycacR87RRG and BPhycacL89FRG/BPhycacR176RRG that Bhat et al. (2) had designed based on the ITS regions could not differentiate the four species (Fig. 2). The primers BPhycacL89FRG/BPhycacR176RRG did not amplify. Possibly because the two primers are the second-round primers designed for a nested PCR, direct PCR under high annealing temperature would affect amplification. Even in the *Ypt*1 gene, which was found to exhibit

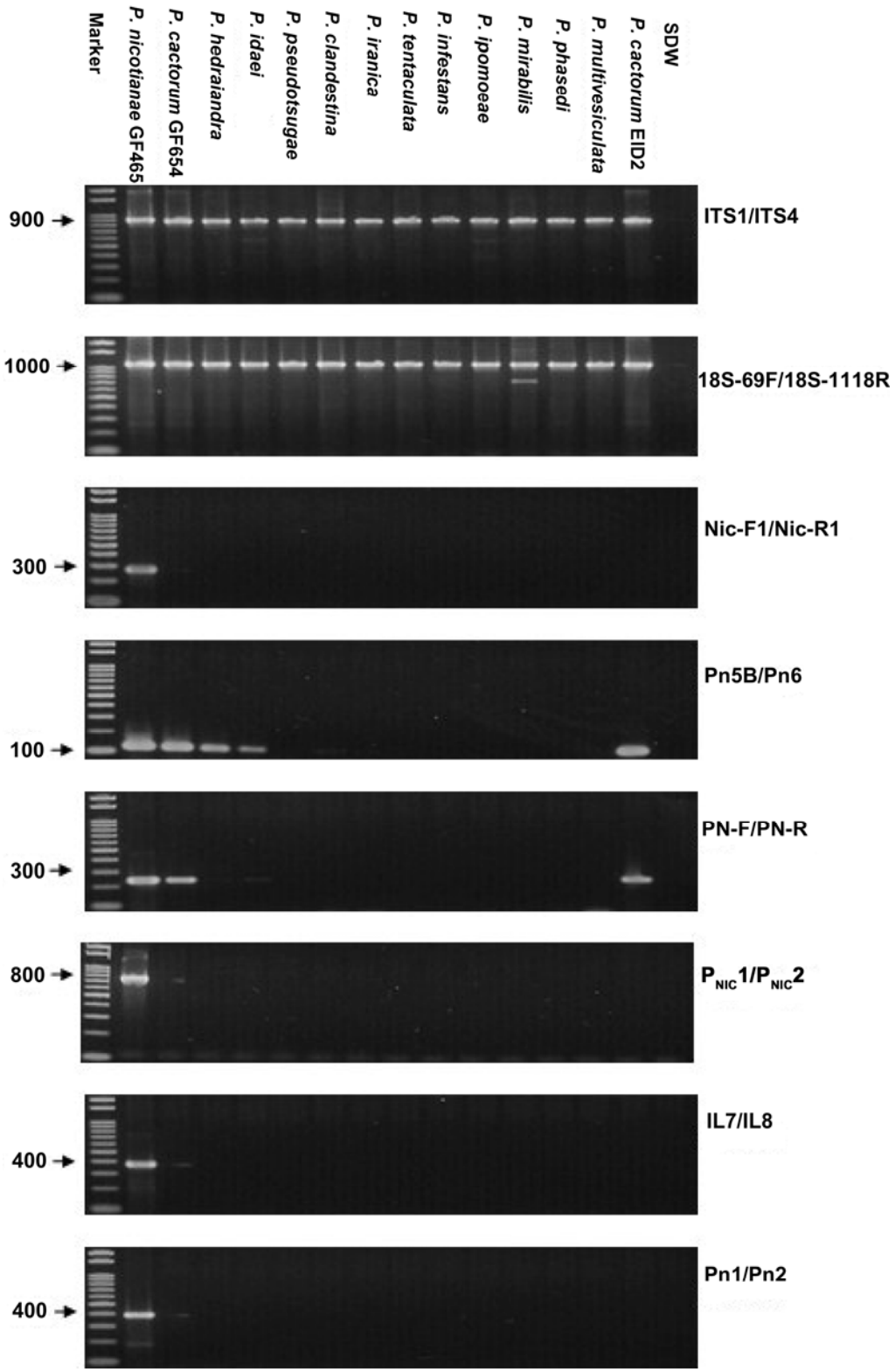

Fig. 1. Specificity of the designed and the published primer pairs for *Phytophthora nicotianae*. Thirteen closely related *Phytophthora* spp. were used to test the specificity of the primer pairs. SDW = sterile distilled water. Size marker: 100-bp DNA ladder.

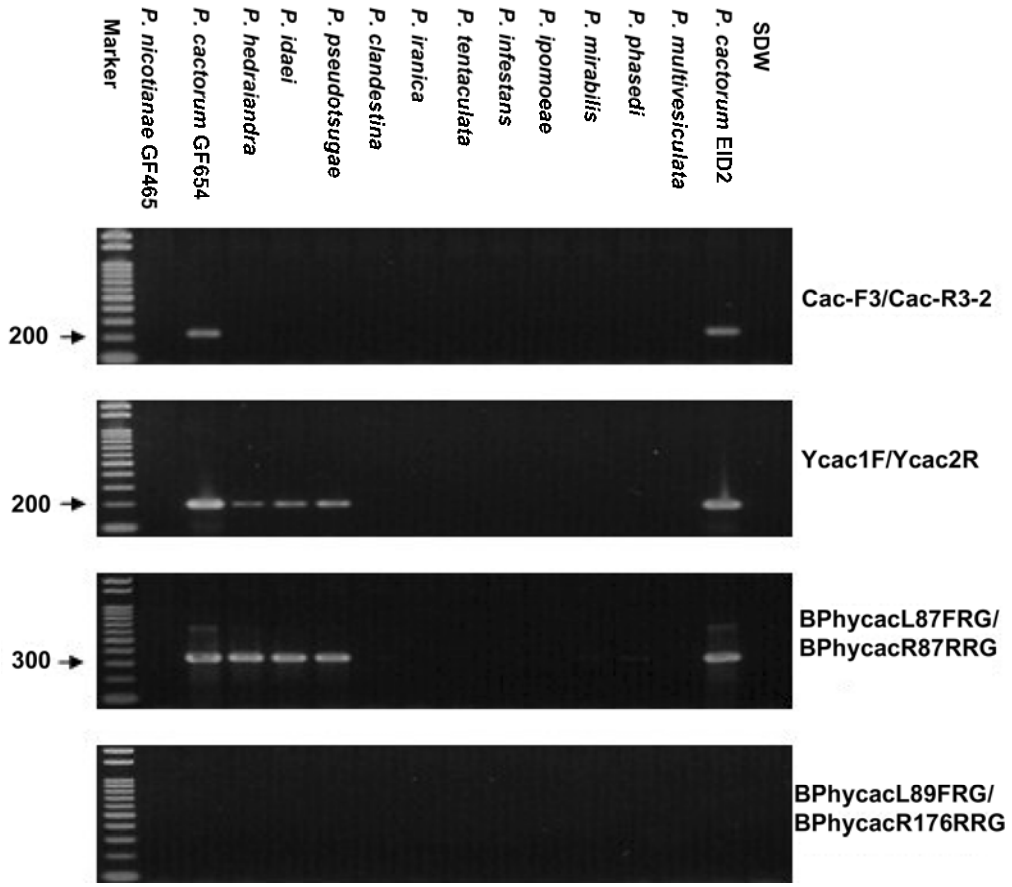

Fig. 2. Specificity of the designed and the published primer pairs for *Phytophthora cactorum*. Thirteen closely related *Phytophthora* spp. were used. SDW = sterile distilled water. Size marker: 100-bp DNA ladder.

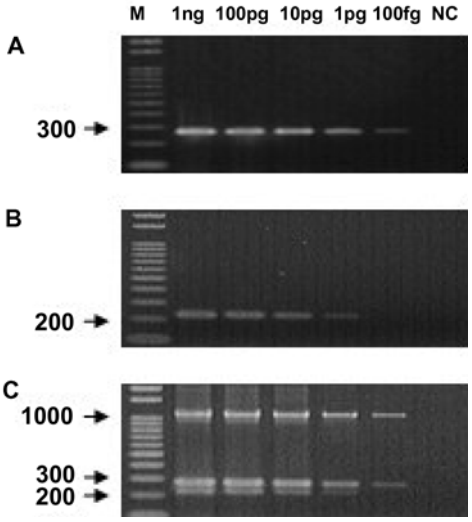

Fig. 3. Detection limits for *Phytophthora nicotianae* and *P. cactorum*. **A,** Amplification with species-specific primer pair Nic-F1/Nic-R1 for *P. nicotianae* isolate CH03OKTYPE3. **B,** Amplification with species-specific primer pair Cac-F3/Cac-R3-2 for *P. cactorum* isolate GF654. **C,** Multiplex polymerase chain reaction (PCR) with three primer pairs (18S-69F/18S-1118R, Nic-F1/Nic-R1, and Cac-F3/Cac-R3-2). DNA from pure cultures was diluted from 1 ng to 100 fg and used to test the sensitivity of the primer pairs. For multiplex PCR, the DNA mixtures of *P. nicotianae* isolate CH03OKTYPE3 and *P. cactorum* isolate GF654 were diluted from 1 ng to 100 fg. NC = sterile distilled water as negative control and M = 100-bp DNA ladder.

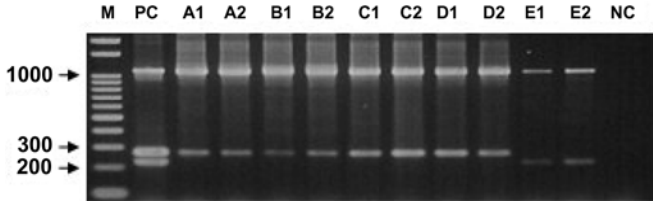

Fig. 4. Multiplex polymerase chain reaction from different soils for the detection of *Phytophthora nicotianae* and *P. cactorum*. Soil samples A1/A2, B1/B2, C1/C2, D1/D2, and E1/E2 were collected from the Chiba, Nara, Saga, Saga, and Nara Prefectures of Japan, respectively. PC = the mixture of mycelial DNA of *P. nicotianae* and *P. cactorum* as positive control, NC = sterile distilled water as negative control, and M = 100-bp DNA ladder.

sufficient variation to enable the development of molecular markers for almost all *Phytophthora* spp., possibilities to design specific primers for *P. cactorum* were limited. Schena et al. (23) developed two primers, Ycac1F and Ycac2R, for *P. cactorum* using the *Ypt*1 gene. However, in our tests, these primers could not distinguish *P. cactorum* from *P. hedraiandra*, *P. idaei*, and *P. pseudotsugae* (Fig. 2). Only one of the *Ypt*1-based primer pairs that we tested in preliminary experiments, Cac-F3 and Cac-R3-2, was found to reliably identify *P. cactorum*.

Multiplex PCR was successfully performed with three primer pairs: 18S-69F/18S-1118R, Nic-F1/Nic-R1, and Cac-F3/Cac-R3-2. According to the critical parameters in multiplex PCR as discussed by Henegariu et al. (10), we optimized mainly four factors: annealing temperature (AT), buffer concentration, primer amounts, and the balance of dNTP and magnesium chloride. Because co-amplification targeted three different loci in our case, the reactions performed satisfactorily even at high AT. Raising the buffer concentration may improve the efficiency of the multiplex reaction but, when we raised the buffer concentration to 1.6×, the bands amplified by the universal primers (18S-69F/18S-1118R) disappeared (*data not shown*). Possibly, this occurred because primers with long amplification products would work better at lower salt concentration, whereas primers with a short amplicon would am-

Table 4. Detection of *Phytophthora nicotianae* and *P. cactorum* in the main strawberry production areas of Japan using multiplex polymerase chain reaction

Prefectures	Field number	Soil group[a]	Soil texture[b]	pH	Disease history[c]	Detected pathogens
Gifu	1	N	N	N	Fusarium wilt	No
	2	N	N	N	Anthracnose	No
	3	N	N	N	Anthracnose, Fusarium wilt	No
	4–14	N	N	N	U	No
Nara	1	N	N	N	U	*P. nicotianae*
	2	N	N	N	U	*P. cactorum*
	3	1	L	5.8	U	*P. nicotianae*
	4–12	1	L	5.4–6.8	U	No
Chiba	1	2	L	5.5	Phytophthora rot	No
	2	4	L	6.6	Fusarium wilt	No
	3–10	2, 4, 5, 6	L, SL	5.5–7.7	U	No
Saga	1	1	CL	5.8	Phytophthora rot	Both
	2	N	N	N	U	*P. nicotianae*
	3	N	N	N	U	*P. nicotianae*
	4–12	1, 3	CL	4.4–6.2	U	No
Shizuoka	1	N	N	5.3	Anthracnose	No
	2	N	N	7.1	Anthracnose	No
	3	9	LS	5.5	Anthracnose	No
	4	4	LS	5.5	Anthracnose	No
	5–20	5, 9	L, LS	5.3–7.1	U	No
Tochigi	1	7	L	4.9	Anthracnose	No
	2	2	CL	5.2	Fusarium wilt	No
	3	8	L	5.2	Anthracnose, Fusarium wilt	No
	4	1	SiL	5.7	Anthracnose, Fusarium wilt	No
	5	7	CL	6.6	Anthracnose	No
	6–11	1, 7, 8	L, SL, SiC, CL	5.1–6.6	U	No
Yamanashi	1	N	N	6.6	U	*P. cactorum*
	2–8	N	N	5.0–7.2	U	No
Hokkaido	1, 2	N	N	N	U	No

[a] Soil groups included N = not tested; 1, gray lowland soils; 2, gray lowland soils (fine-textured); 3, yellow soils; 4, gley soils (fine-textured); 5, brown lowland soils; 6, brown forest soils; 7, ando soils; 8, gleyed ando soils; and 9, sand-dune regosols.
[b] Soil textures included N = not tested;. L, loam; SL, sandy loam; CL, clay loam; LS, loamy sand; SiC, silty clay
[c] U = unknown.

plify better at higher salt concentrations which inhibit the denaturation of longer products. In multiplex PCR, adjusting the concentrations of the primer pairs is very important due to the varying copy numbers of the target loci. To obtain similar target signals, the concentrations of different primer pairs were balanced in this research.

Nested PCR is used frequently to improve sensitivity. Meng and Wang (21) applied nested PCR to increase the sensitivity of primers specific for *P. nicotianae* from 1 ng to 10 pg. Similarly, Schena et al. (23) were able to raise the sensitivity of primers for 15 *Phytophthora* spp. from 100 pg to 100 fg. We found detection limits for *P. nicotianae* and *P. cactorum* of 100 fg and 1 pg of DNA, respectively, in single as well as multiplex PCR, suggesting very high amplification efficiencies of our primers. Unlike rDNA genes which generally are present in multiple copies, the *Ypt*1 gene exists as a single copy only (5). This explains why the sensitivity for *P. nicotianae* was always higher than the sensitivity for *P. cactorum* in our tests.

For our investigation into the distribution of *P. nicotianae* and *P. cactorum* in eight strawberry production prefectures of Japan, samples with quite different soil properties were collected. DNA extraction from andosols usually is difficult (25) but our effective extraction method allowed DNA extraction from all soil types. We found that more strawberry production areas were invaded by anthracnose or Fusarium wilt than by *Phytophthora* spp. in Japan. A few samples had a known Phytophthora rot history but *P. nicotianae* and *P. cactorum* were detected in some samples independent of their Phytophthora rot history, indicating that several strawberry fields would be potentially infected with *Phytophthora* spp. The disease caused by *P. cactorum* is often more common in cooler temperatures. However, *P. cactorum* was found in Saga, a warm region of Japan.

The symptoms of anthracnose and Phytophthora diseases are very similar but fungicides for their control are completely different. Therefore, incorrect diagnoses will cause avoidable problems and financial loss. Although Phytophthora rot has not severely threatened strawberry production in Japan thus far, it is helpful in differentiating Phytophthora disease from anthracnose, which causes serious economic losses in Japan. The technique here introduced proved useful and effective in the discrimination of the pathogens, and will be helpful in the early diagnosis of seedling infection and disease control.

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