Journal of General Plant Pathology
https://doi.org/10.1007/s10327-024-01179-z

DISEASE CONTROL

Multiplex PCR specific for genus *Phytophthora* and *P. nicotianae* with an internal plant DNA control for effective quarantine of *Phytophthora* species in Japan

Kayoko Otsubo[1] · Mingzhu Li[2] · Auliana Afandi[3] · Haruhisa Suga[4] · Koji Kageyama[1] · Ayaka Hieno[1]

Received: 10 February 2024 / Accepted: 12 April 2024

Abstract
To prevent threats from pathogens such as *Phytophthora* species from international plant trade, molecular identification techniques are needed for rapid, accurate quarantine inspection. Here, for quarantine control in Japan, we developed a simple DNA extraction for plants and a practical detection method that combines multiplexed PCR using primers specific for *Phytophthora* species, for *P. nicotianae*, which is the only non-quarantine *Phytophthora* species, and as internal controls, for plants. For the new genus-level primer set, we modified previously reported genus-specific primers to improve detectability. The new primers were able to detect mycelial DNA of 155 taxa among *Phytophthora* clades 1–10, with a sensitivity of 100 fg/μL for three representative species, *P. ramorum*, *P. kernoviae* and *P. nicotianae*. In the PCRs using DNA from non-target species, amplification was observed for only three taxa, and for some strains, four taxa in a closely related genus. Duplex and triplex PCR of the genus-specific primers combined with previously reported plant primers verified the success of DNA extraction and PCR detection from diseased plant samples, and in the triplex PCR, whether the pathogen was diagnosed as *P. nicotianae* or not by the species-specific primer. The new method detected the pathogen in naturally infected and inoculated plants. The amplicons using the genus-specific primer have enough variation to be sequenced to identify the species. This new method can be used immediately for detecting *Phytophthora* species and for quarantine control in Japan.

Keywords Multiplex PCR · *Phytophthora* · *Phytophthora nicotianae* · Internal control · Quarantine

Introduction

The oomycete genus *Phytophthora* includes many plant pathogens that cause destructive diseases and severe commercial losses, not only to agricultural crops, but also to forest trees and nursery plants. Many new species of *Phytophthora* have been identified from diseased nursery trees and plants in natural ecosystems, and new species continue to be found (Brasier 2009). With the expansion of global plant trade, *Phytophthora* species have also spread globally (Brasier 2009). These pathogens usually cause little damage to their host plants in their native habitats and have coevolved with their hosts, achieving a natural balance. However, when introduced by human activity into other regions with a favorable environment that lacks natural enemies, they can be highly virulent and cause serious damage on genetically susceptible host plants (Brasier 2008).

More precise phytosanitary measures are required to prevent economic loss of crops on a global scale and to conserve endemic biodiversity. In Japan, *P. ramorum* was isolated during quarantine inspection from discolored leaves of rhododendrons imported from the United Kingdom in 2015 (Sakoda et al. 2017). In Japan, *P. ramorum* and *P. kernoviae* are considered the most important quarantined species, and only *P. nicotianae* is exempt from quarantine, since it is already a major pathogen. Molecular identification techniques are required for simultaneous detection of

✉ Ayaka Hieno
hieno.ayaka.h9@f.gifu-u.ac.jp

[1] River Basin Research Center, Gifu University, 1-1 Yanagido, Gifu-city, Gifu 501-1193, Japan

[2] College of Life Sciences, Shaanxi Normal University, Xi'an, China

[3] Research Center for Estate Crops, National Research and Innovation Agency, Jl. Raya Jakarta- Bogor KM 46, Cibinong, Bogor, West Java 16911, Indonesia

[4] Life Science Research Center, Gifu University, 1-1 Yanagido, Gifu-city, Gifu 501-1193, Japan

Published online: 13 May 2024

Phytophthora species; therefore, we developed a simple identification technique using loop-mediated isothermal amplification (LAMP) with a quenching probe (QProbe) (Hieno et al. 2020) that was designed for a sequence specific to *P. nicotianae* in the amplified region of a LAMP primer specific for *Phytophthora*. The total time from sampling to detection is approximately 2 h; thus, it is a time-saving technique for import/export quarantine. Other advantages include high tolerance to inhibitors from biological sources, and thus, easy DNA extraction methods are applicable (Hieno et al. 2019). However, the multiplex reaction in the LAMP method presents difficulties in that the strong amplification of more abundant DNA may overwhelm the amplification of less abundant DNA, and the sensitivity of multiple targets may be reduced (Hieno et al. 2021). In contrast, PCR can achieve high sensitivity in a simultaneous detection method (Li et al. 2011). In this study, we aimed to develop a multiplex PCR method using primers specific for *Phytophthora* at the genus level and primers specific for *P. nicotianae* (Li et al. 2011) as an accurate and time-saving method to detect multiple targets. This multiplex PCR can be used to inspect samples for the broader *Phytophthora* genus and for *P. nicotianae* whether the pathogens are present in the sample or subject to quarantine or not. Furthermore, the genus-specific primers are expected to be used for sequencing to identify the *Phytophthora* species detected in the sample. In previous studies on PCR techniques using primers specific for *Phytophthora* species, most primers were designed for the ITS region, but identical ITS sequences have been identified for 16 pairings of species from molecular phylogenetic clades 1, 5, 6, 7 or 8, making identification of the species difficult (Yang and Hong 2018). The introns of the *Ypt1* gene are sufficiently polymorphic to discriminate all *Phytophthora* species and are located near the conserved coding regions, which is suitable for designing primers specific to the genus *Phytophthora* (Schena and Cooke 2006). In the present study, we designed genus-specific primers for *Ypt1* by modifying the primers reported by Schena et al. (2008). The modified forward and reverse primers have been used for multiplex PCR detection of the kiwifruit pathogens *Phytophthora cactorum*, *P. cinnamomi* and *P. lateralis* (Bi et al. 2019). The modified primers have the potential to be used for detecting additional species of *Phytophthora* but have not been tested with enough strains of *Phytophthora* and with other genera, including closely related *Pythium* and *Phytopythium*.

In this study, we reevaluated the applicability of our modified genus-specific primers by using a sufficient number of strains, then developed a duplex PCR using the *Phytophthora* genus-specific primers and plant primers (Martin et al. 2004) as an internal control. We also developed a triplex PCR using additional *P. nicotianae*-specific primers (Li et al. 2011) for effective quarantine control in Japan. By combining this multiplex PCR method with a simple method to extract DNA from diseased plants, we aimed to establish a more practicable and user-friendly protocol for quarantine inspections.

Materials and methods

Isolates and mycelial DNA extraction

Isolates of *Phytophthora* spp., *Phytopythium* spp., *Pythium* spp. and other pathogens used in this study are listed in Table 1. For the mycelial DNA extraction, isolates were grown on V8 juice agar plates [Miller (1955) with the following modifications: 1 l including 162 mL V8 juice (Campbell Japan), 20 g agar, pH modified with $CaCO_3$] at 25 °C until the mycelium reached the edge of the plates. The mycelium was scraped from the plates with inoculation needles into 1.5-ml Eppendorf tubes containing 100 µl of 50% PrepMan Ultra Reagent (Thermo Fischer Scientific, Waltham, MA, USA) and incubated at 100 °C for 10 min. After 3 min at room temperature, the sample was centrifuged at 15,000 rpm for 3 min. The supernatant was transferred to a new 1.5-ml tube. The DNA concentration was measured using the QuantiFluor dsDNA System (Promega, Madison, WI, USA) and adjusted to 100 pg/µl with Tris–EDTA buffer (TE buffer; 10 mM Tris–HCl, 1 mM EDTA, pH 8.0). The samples were stored at 4 °C until further use.

DNA preparations from inoculated and from naturally infected plants

The host–pathogen combinations used in the inoculation tests are shown in supplementary Table S1. *P. nicotianae* (GK10Eg1 and 13ASP1-1), *P. capsici* (CH01CUCU10 and CH02UE0202), *P. hedraiandra* (TGTA1-1) and *P. melonis* (CH00ME21-21) were grown on V8 juice agar plates at 25 °C until mycelial growth reached the edge of the plate, and 6- or 10-mm-diameter mycelial disks were taken from actively growing colonies. For tomato, eggplant, pumpkin and cucumber, the smaller mycelial discs were placed on the fruit. Inoculated materials were placed on wet paper towels in plastic trays, which were covered with polyethylene bags to maintain high humidity and incubated in a growth chamber (12 h light/12 h dark) at 25 °C for 5–7 days until symptoms were obvious. For hydrangea in pots, the larger mycelial disks were placed on a needle wound on the basal stem, then wrapped in parafilm. The whole plant was covered with a polyethylene bag to maintain humidity and kept at room temperature (approximately 25 °C) in the laboratory for 10 days until symptoms were seen. For ivy, *P. citrophthora* (CH94HE11) was cultured in V8 juice broth in a 6 cm Petri dish until the entire

Table 1 PCR with *Phytophthra* genus-specific primers

Species		Clade	Isolate	Host/source	Location	PCR	
Phytophthora							
	Ph. nicotianae	1	CBS 305.29	Tobacco	Taiwan	+	
			CBS 535.92	Soil	Unknown	+	*
			GF468	Strawberry	Gifu, Japan	+	*
			MAFF 235784	*Vanda* sp.	Chiba, Japan	+	*
			MAFF 235786	*Bougainvillea spectabilis*	Chiba, Japan	+	*
			MAFF 235794	*Abutilon*×*hybridum*	Chiba, Japan	+	*
	Ph. cactorum	1a	MAFF 242859	*Eriobotrya japonica*	Chiba, Japan	+	*
			MAFF 242860	*Eriobotrya japonica*	Chiba, Japan	+	
			MAFF 242861	*Pyrus pyrifolia* var. *culta*	Chiba, Japan	+	
			MAFF 242862	*Aralia elata*	Tokushima, Japan	+	*
			MAFF 242863	*Fragaria grandiflora*	Okayama, Japan	+	*
			MAFF 245799	*Paeonia albiflora*	Hokkaido, Japan	+	*
			MAFF 731066	Strawberry	Iwate, Japan	+	*
	Ph. hedraiandra	1a	CBS 111725	*Viburnum* sp.	Netherlands	+	
			NBRC 32194	Tulip cv. Monte Carlo	Niigata, Japan	+	*
			P11056	*Rhododendron* sp.	USA	+	*
			TGTA1-1	Hydrangea macrophylla	Tochigi, Japan	+	*
	Ph. idaei	1a	P6767	*Rubus idaeus*	Scotland, UK	+	
	Ph. pseudotsugae	1a	P10339	*Pseudotsuga menziesii*	Oregon, USA	+	
	Ph. clandestina	1b	P3942	*Trifolium subterraneum*	Australia	+	
			CBS 347.86	*Trifolium subterraneum*	Australia	+	
	Ph. iranica	1b	CBS 374.72	*Solanum melongena*	Teheran, Iran	+	
	Ph. tentaculata	1b	CBS 552.96	*Chrysanthemum leucanthemum*	Germany	+	
			MAFF 245861	*Gazania* sp.	Chiba, Japan	+	
	Ph. andina	1c	CBS 115547	*Solanum brevifolium*	Tungurahua Province, Ecuador	+	
	Ph. infestans	1c	MAFF 236324	Tomato	Ibaraki, Japan	+	
			MAFF 305586	*Solanum tuberosum*	Hokkaido, Japan	+	*
	Ph. ipomoeae	1c	P10225	*Ipomoea longipedunculata*	México, Mexico	+	
	Ph. mirabilis	1c	P3005	*Mirabilis jalapa*	Mexico	+	
	Ph. phaseoli	1c	P10145	*Phaseolus lunatus*	Delaware, USA	+	
	Ph. mengei	2	ATCC MYA-4554	Avocado	California, USA	+	
	Ph. multivesiculata	2	P10410	*Cymbidium* sp.	Mijdrecht, Netherlands	+	
	Ph. siskiyouensis	2	CBS 122779	Seasonal tributary	Oregon, USA	+	
	Ph. tropicalis	2	CBS 434.91	*Macadamia integrifolia*	Hawaii, USA	+	
	Ph. botryosa	2a	CBS 581.69	*Hevea brasiliensis*	Perlis, Malaysia	+	
	Ph. citrophthora	2a	CBS 950.87	*Citrus* sp.	California, USA	+	
			MAFF 242875	*Citrus unshiu*	Chiba, Japan	+	*
			MAFF 245812	*Actinidia deliciosa*	Chiba, Japan	+	
			MAFF 245813	*Citrus unshiu*	Chiba, Japan	+	*
			MAFF 245816	*Ficus carica*	Chiba, Japan	+	
			P1200	*Theobroma cacao*	Brazil	+	*
			P3693	*Citrus* sp.	Brazil	+	
	Ph. colocasiae	2a	P6317	Taro	Indonesia	+	
	Ph. himalsilva	2a	CBS 128767	Soil	Bajura, Nepal	+	
	Ph. meadii	2a	CBS 219.88	*Hevea brasiliensis*	Kerala, India	+	
			P3500	*Hevea brasiliensis*	Sri Lanka	+	

Table 1 (continued)

Species		Clade	Isolate	Host/source	Location	PCR	
	Ph. mekongensis	2a	CBS 135136	*Citrus grandis*	Vinh Long Province, Vietnam	+	
	Ph. occultans	2a	P19955	*Buxus sempervirens*	Netherlands	+	*
	Ph. terminalis	2a	P19956	*Pachysandra terminalis*	Netherlands	+	
	Ph. amaranthi	2b	P20892	*Amaranthus tricolor*	Yunlin County, Taiwan	+	
	Ph. capsici	2b	MAFF 242866	*Citrullus vulgaris*	Chiba, Japan	+	*
			MAFF 245800	*Cucumis melo*	Chiba, Japan	+	*
			MAFF 245803	*Cucumis sativus*	Saitama, Japan	+	
			MAFF 305920	Watermelon	Shizuoka, Japan	+	*
			P0253	*Theobroma cacao*	Mexico	+	
			P1319	*Capsicum annuum*	California, USA	+	*
	Ph. glovera	2b	CBS 121969	*Nicotiana tabacum*	Santa Catarina, Brazil	+	
	Ph. mexicana	2b	CBS 554.88	*Solanum lycopersicum*	Mexico	+	
	Ph. caryae	2c	ATCC TSD-54	Water	Massachusetts, USA	+	*
	Ph. capensis	2c	CBS 128319	*Curtisia dentata*	Western Cape Province, South Africa	+	
	Ph. acerina	2c	CBS 133931	*Acer pseudoplatanus*	Lombardy, Italy	+	
	Ph. citricola	2c	CBS 221.88	*Citrus sinensis*	Unknown	+	*
			MAFF 245808	*Eustoma grandiflorum*	Chiba, Japan	+	*
			P0713	Citrus	Argentina	+	*
			P1321	Citrus	California, USA	+	*
			P1817	*Medicago sativa*	South Africa	+	*
	Ph. multivora	2c	NBRC 31016	Soybean	Shizuoka, Japan	+	
	Ph. pachypleura	2c	WPC P19987	*Aucuba japonica*	England, UK	+	
	Ph. pini	2c	CBS 181.25	*Pinus resinosa*	Minnesota, USA	+	
	Ph. plurivora	2c	CBS 124093	*Fagus sylvatica*	Upper Bavaria, Bavaria, Germany	+	
	Ph. bishii	2d	CBS 122081	Strawberry	North Carolina, USA	+	
	Ph. elongata	2d	CBS 125799	Soil	Peel, Western Australia, Australia	+	
	Ph. frigida	2d	CBS 121941	*Eucalyptus smithii*	KwaZulu-Natal, South Africa	+	
	Ph. ilicis	3a	P3939	Holly	British Columbia, Canada	+	*
	Ph. nemorosa	3a	C71	*Eustoma grandiflorum*	Chiba, Japan	+	
	Ph. pluvialis	3a	ATCC MYA-4930	Water	Oregon, USA	+	
	Ph. pseudosyringae	3a	CBS 111772	Soil	Lower Franconia, Bavaria, Germany	+	
	Ph. psychrophila	3a	CBS 803.95	Soil	Südbayern, Bavaria, Germany	+	
	Ph. castanetorum	3b	CBS 142299	Soil	Algarve, Portugal	+	
	Ph. tubulina	3b	CBS 141212	Soil	Lower Austria, Austria	+	*
	Ph. quercina	3b	CBS 784.95	*Quercus robur*	Südbayern, Bavaria, Germany	+	
	Ph. alticola	4	CBS 121939	*Eucalyptus dunnii*	KwaZulu-Natal, South Africa	+	
	Ph. arenaria	4	CBS 127950	Soil	Wheatbelt, Western Australia, Australia	+	
	Ph. boodjera	4	CBS 138637	Soil	Wheatbelt, Western Australia, Australia	+	
	Ph. megakarya	4	P8517	Unknown	Unknown	+	*
			CBS 238.83	*Theobroma cacao*	Cameroon	+	

Table 1 (continued)

Species	Clade	Isolate	Host/source	Location	PCR	
Ph. palmivora	4	P0113	*Carica papaya*	Hawaii, USA	+	*
		GF534	*Ficus carica*	Gifu, Japan	+	
		MAFF 235787	*Oncidium* sp.	Chiba, Japan	+	
		C88-1	*Oncidium* sp.	Chiba, Japan	+	*
		P0633	*Areca catechu*	India	+	*
Ph. quercetorum	4	CBS 121119	Soil	Maryland, USA	+	
Ph. agathidicida	5	ICMP 17027	*Agathis australis*	Great Barrier Island, New Zealand	+	*
Ph. castaneae	5	NBRC 9753	*Castanea crenata*	Japan	+	
Ph. cocois	5	ICMP 16948	*Cocos nucifera*	Hawaii, USA	+	*
Ph. heveae	5	P1102	Avocado	Guatemala	+	
Ph. novae-guineae	5	ICMP 19637	*Auracaria* sp.	Papua New Guinea	+	
Ph. lacustris	6	P10337	*Salix matsudana*	Greater London, UK	+	
Ph. gemini	6a	CBS 123381	*Zostea marina*	Lake Grevelingen, Netherlands	+	
Ph. humicola	6a	P3826	Soil	Taiwan	+	
		P6701	*Citrus* sp.	Taiwan	+	*
Ph. inundata	6a	P8478	*Aesculus hippocastanum*	England, UK	+	
Ph. rosacearum	6a	CBS 124696	Apple	California, USA	+	
Ph. amnicola	6b	CBS 131652	Water	Perth, Western Australia, Australia	+	
Ph. bilorbang	6b	CBS 161653	Soil	Warren River, Western Australia, Australia	+	
Ph. borealis	6b	ATCC MYA-4881	Forest streams	Alaska, USA	+	
Ph. chlamydospora	6b	P6133	*Prunus* sp.	Gloucestershire, England, UK	+	*
Ph. fluvialis	6b	CBS 129424	Water	Wheatbelt, Western Australia, Australia	+	
Ph. gibbosa	6b	CBS 127951	Soil	Scott River, Western Australia, Australia	+	
Ph. gonapodyides	6b	P7050	*Vegetable debris*	England, UK	+	*
Ph. gregata	6b	CH97TUL2	*Tulipa gesneriana*	Chiba, Japan	+	
Ph. litoralis	6b	CBS 127953	Soil	Great Southern, Western Australia, Australia	+	
Ph. megasperma	6b	NBRC 32176	White trumpet lily	Kagosyima, Japan	+	*
		CBS 402.72	*Althaea rosea*	USA	+	
Ph. mississippiae	6b	ATCC MYA-4946	Water	Mississippi, USA	+	
Ph. moyootj	6b	CBS 138759	Soil	South West, Western Australia, Australia	+	
Ph. pinifolia	6b	CBS 122924	*Pinus radiata*	Bío Bío, Chile	+	*
Ph. riparia	6b	ATCC MYA-4882	Forest streams	Oregon, USA	+	
Ph. × *stagnum*	6b	ATCC MYA-4926	Water	Virginia, USA	+	*
Ph. thermophila	6b	CBS 127954	Soil	Peel, Western Australia, Australia	+	
Ph. asparagi	6d	CBS 132095	*Asparagus* sp.	Michigan, USA	+	
Ph. attenuata	7a	CBS 141199	Soil	Hsinchu County, Taiwan	+	
Ph. × *cambivora*	7a	P0592	*Abies procera*	Oregon, USA	+	
		MAFF 305918	Apple	Hokkaido, Japan	+	*
		CBS 141218	Soil	Sicily, Italy	+	
Ph. cinnamomi	7a	P2160	*Vitis vinifera*	South Africa	+	
		NBRC 33180	*Hypericum androsaemum*	Kochi, Japan	+	
Ph. europaea	7a	CBS 109049	Soil	France	+	

Table 1 (continued)

Species		Clade	Isolate	Host/source	Location	PCR	
	Ph. flexuosa	7a	CBS 141201	Soil	Yilan County, Taiwan	+	
	Ph. formosa	7a	CBS 141203	Soil	Nantou County, Taiwan	+	
	Ph. fragariae	7a	CBS 209.46	*Fragaria* sp.	England, UK	+	
	Ph. × *heterohybrida*	7a	CBS 141207	Water	New Taipei City, Taiwan	+	
	Ph. × *incrassata*	7a	CBS 141209	Water	New Taipei City, Taiwan	+	
	Ph. intricata	7a	CBS 141211	Soil	New Taipei City, Taiwan	+	
	Ph. parvispora	7a	CBS 411.96	*Beaucamea* sp.	Hesse, Germany	+	
	Ph. rubi	7a	CBS 967.95	Raspberry	Scotland, UK	+	
	Ph. tyrrhenica	7a	CBS 142301	Soil	Sardinia, Italy	+	*
	Ph. uliginosa	7a	CBS 109054	Soil	Lesser Poland, Poland	+	
	Ph. vulcanica	7a	CBS 141216	Soil	Tempa Rossa, Italy	+	*
	Ph. asiatica	7b	CBS 133347	*Pueraria lobata*	Toyama, Japan	+	
	Ph. cajani	7b	P3105	*Cajanus cajan*	India	+	
	Ph. cinnamomi var. *robiniae*	7b	P16351	*Robinia pseudoacacia*	Jiangsu, China	+	
	Ph. melonis	7b	P6870	*Cucumis sativus*	Japan	+	
	Ph. niederhauserii	7b	CH96HE1	*Hedera helix*	Chiba, Japan	+	
			MAFF 245825	*Hedera rhombea*	Chiba, Japan	+	*
	Ph. pisi	7b	CBS 130350	*Pisum sativum*	Scania, Sweden	+	
	Ph. pistaciae	7b	CBS 137185	*Pistacia vera*	Kerman, Iran	+	
	Ph. sojae	7b	P7358	Soybean	Indiana, USA	+	*
			TosB3	Soybean	Toyama, Japan	+	*
			TosB5	Soybean	Toyama, Japan	+	*
			Pm-1	Soybean	Hokkaido, Japan	+	
	Ph. vignae	7b	Ph-9	*Vigna angularis*	Hokkaido, Japan	+	
	Ph. fragariaefolia	7c	CBS 135747	*Fragaria* × *ananassa*	Hokkaido, Japan	+	
	Ph. nagaii	7c	CBS 133248	*Rosa* sp.	Chiba, Japan	+	
	Ph. cryptogea	8a	CBS 113.19	Tomato or Petunia	Ireland	+	
			P1088	*Callistephus chinensis*	California, USA	+	*
	Ph. drechsleri	8a	P1087	*Beta vulgaris* var. *altissima*	California, USA	+	
	Ph. erythroseptica	8a	CBS 129.23	*Solanum tuberosum*	Ireland	+	
			P0340	*Solanum tuberosum*	Australia	+	
	Ph. kelmanii	8a	GF433	Gerbera	Gifu, Japan	+	*
			GF543	Gerbera	Gifu, Japan	+	*
			MAFF 247472	*Gerbera* x *hybrida*	Gifu, Japan	+	
	Ph. medicaginis	8a	P10138	*Medicago sativa*	California, USA	+	
			P7029	*Medicago sativa*	California, USA	+	*
	Ph. pseudocryptogea	8a	CBS 139749	*Isopogon buxifolius*	Great Southern, Western Australia, Australia	+	
	Ph. sansomeana	8a	P3163	White cockle	New York, USA	+	
			CH95PHG8	Gerbera	Chiba, Japan	+	*
			MAFF 245828	*Gerbera* x *hybrida*	Chiba, Japan	+	*
			NBRC 31624	Soil	Hokkaido, Japan	+	*
	Ph. trifolii	8a	P6980	Clover	Mississippi, USA	+	
	Ph. austrocedri	8d	P16040	*Austrocedrus chilensis*	Argentina	+	
	Ph. brassicae	8b	CBS 179.87	*Brassica oleracea*	Netherlands	+	
	Ph. cichorii	8b	CBS 115029	*Cichorium intybus* var. *foliosum*	Netherlands	+	
	Ph. dauci	8b	P19845	*Daucus carota*	France	+	

Table 1 (continued)

Species	Clade	Isolate	Host/source	Location	PCR	
Ph. lactucae	8b	P19872	*Lactuca sativa*	Greece	+	
Ph. porri	8b	CBS 140.87	*Allium cepa*	Fukuoka, Japan	+	
		CBS 688.79	*Daucus carota*	Alberta, Canada	+	*
		MAFF 237664	*Allium victorialis* var. *platyphyllum*	Toyama, Japan	+	*
		MAFF 237666	*Allium cepa*	Toyama, Japan	+	*
		NBRC 30416	*Allium cepa*	Fukuoka, Japan	+	*
		NBRC 30417	*Allium grayi*	Fukuoka, Japan	+	*
Ph. primulae	8b	CBS 620.97	*Primula acaulis*	Germany	+	
Ph. pseudolactucae	8b	CBS 137103	*Lactuca sativa*	Kagawa, Japan	+	
Ph. foliorum	8c	P10974	*Rhododendron* sp.	Tennessee, USA	+	
Ph. hibernalis	8c	CBS 114104	*Citrus sinensis*	Western Australia, Australia	+	
Ph. lateralis	8c	P3361	*Chamaecyparis lawsoniana*	Oregon, USA	+	
Ph. ramorum	8c	CBS 101553	*Rhododendron catawbiense*	Germany	+	
		Pr-1	*Quercus agrifolia*	California, USA	+	
Ph. obscura	8d	P19796	Soil	Germany	+	
Ph. syringae	8d	FIUm1	Japanese apricot	Fukui, Japan	+	*
		FIUm3	Japanese apricot	Fukui, Japan	+	
Ph. aquimorbida	9a	ATCC MYA-4578	Water	Virginia, USA	+	
Ph. chrysanthemi	9a	NBRC 104918	*Chrysanthemum* sp.	Toyama, Japan	+	*
		CBS123163	*Chrysanthemum* sp.	Gifu, Japan	+	
Ph. sp. cuyabensis	9a	P8213	Unknown	Ecuador	+	*
Ph. hydrogena	9a	ATCC MYA-4919	Water	Virginia, USA	+	
Ph. hydropathica	9a	ATCC MYA-4460	Water	Virginia, USA	+	
Ph. insolita	9b	P6195	Soil	Taiwan	+	
Ph. irrigata	9a	ATCC MYA-4457	Water	Virginia, USA	+	
Ph. sp. lagoariana	9a	P8217	Water	Ecuador	+	
Ph. macilentosa	9a	ATCC MYA-4945	Water	Mississippi, USA	+	*
Ph. parsiana	9a	C25	*Ficus carica*	Bushehr Province, Iran	+	
Ph. virginiana	9a	ATCC MYA-4927	Water	Virginia, USA	+	
Ph. polonica	9b	1	Unknown	Unknown	+	*
		2	Unknown	Unknown	+	*
		P19445	Unknown	Unknown	+	
		P131445	*Alnus glutinosa*	Wielkopolska Province, Poland	+	*
Ph. prodigiosa	9b	CBS 135138	*Citrus grandis*	Vinh Long Province, Vietnam	+	
Ph. captiosa	10a	P10719	*Eucalyptus saligna*	New Zealand	+	*
Ph. constricta	10a	CBS 125801	Soil	Great Southern, Western Australia, Australia	+	*
Ph. fallax	10a	P10722	*Eucalyptus delegatensis*	Southland, New Zealand	+	
Ph. macrochlamydospora	10a	P10263	Soybean	South East Queensland, Queensland, Australia	+	*
Ph. quininea	10a	CBS 407.48	*Cinchona officinalis*	Region of Tingo María, Peru	+	
Ph. richardiae	10a	P7789	*Zantedeschia aethiopica*	USA	+	
Ph. boehmeriae	10b	P6950	*Boehmeria nivea*	Taiwan	+	
Ph. gallica	10b	P16826	*Quercus robur*	Grand Est, France	+	

Table 1 (continued)

Species		Clade	Isolate	Host/source	Location	PCR	
	Ph. intercalaris	10b	CBS 140632	Water	Virginia, USA	+	
	Ph. kernoviae	10b	P19875	*Lactuca sativa*	Greece	+	*
			P1571	*Fagus sylvatica*	England, UK	+	
	Ph. morindae	10b	CBS 121982	*Morinda citrifolia* cv. Noni	Island of Hawaii, Hawaii, USA	+	
	Ph. stricta	New clade	ATCC MYA-4944	Water	Mississippi, USA	+	*
	Ph. lilii	New clade	CBS 135746	*Lilium longiflorum*	Kagoshima, Japan	+	
Phytopythium							
	Pp. aichiense	1	CBS 137195	Sludge	Aichi, Japan	–	*
	Pp. boreale	1	CBS 551.88	Soil	Beijing, China	–	*
	Pp. iriomotense	1	GUGC 0025	Water	Okinawa, Japan	–	*
		1	GUGC 0028	Water	Okinawa, Japan	–	*
		1	GUGC 0036	Water	Okinawa, Japan	–	*
	Pp. chamaehyphon	1	CBS 259.30	*Carica papaya*	Unknown	–	*
		1	NBRC 107394	Water	Okinawa, Japan	–	*
		1	NBRC 107441	Water	Okinawa, Japan	–	*
	Pp. carbonicum	1	CBS 112544	Soil	France	–	*
	Pp. citrinum	1	CBS 119171	Soil	Bourgogne-Franche-Comté, France	–	*
	Pp. cucurbitacearum	1	CBS 748.96	Unknown	Northern Territory, Australia	+	
	Pp. delawarense	1	382B	Soybean	Ohio, USA	–	*
	Pp. fagopyri	1	MAFF 242908	*Fagopyrum esculentum*	Akita, Japan	–	*
		1	NBRC 113135	*Fagopyrum esculentum*	Fukui, Japan	–	*
	Pp. helicoides	1	NBRC 100107	*Rosa hybrida*	Gifu, Japan	–	
	Pp. litrale	1	GUGC 1072	Water	Okinawa, Japan	–	*
		1	GUGC 1132	Water	Shizuoka, Japan	–	*
	Pp. megacrpum	1	CBS 112351	Soil	Hauts-de-France, France	–	*
	Pp. mercuriale	1	CBS 122443	Soil	Limpopo, South Africa	–	*
		1	SuTo5SST3	Soil	Shizuoka, Japan	–	*
		1	SZ14S6	Strawberry field	Shizuoka, Japan	+	*
	Pp. montanum	1	CBS 111349	Soil	Bavaria Alps, Germany	–	*
	Pp. oedochilum	1	CBS 292.37	Unknown	USA	–	*
		1	GUGC 5078	Chrysanthemum	Toyama, Japan	–	*
		1	MAFF 242907	*Smallanthus sonchifolius*	Hokkaido, Japan	–	
	Pp. ostracodes	1	CBS 768.73	Soil	Ibiza, Spain	–	
	Pp. vexans	1	02A2 16-3	Soil	Gifu, Japan	+	*
		1	2D111	Soil	Gifu, Japan	+	
		1	2D4S072	Soil	Gifu, Japan	–	*
		1	NBRC 107381	Water	Okinawa, Japan	–	*
		1	NBRC 107393	Water	Okinawa, Japan	–	*
		1	NBRC 107397	Soil	Okinawa, Japan	–	*
		1	N01A5 6-3	Soil	Gifu, Japan	–	*
		1	N02A3 18-3	Soil	Gifu, Japan	–	*
Pythium							
	P. adhaerens	3	CBS 520.74	Soil	Flevoland, Netherlands	+	
	P. aphanidermatum	3	TA114	Soil	Gifu, Japan	–	*
		3	TJu132	Soil	Gifu, Japan	–	*
	P. vanterpoolii	3	DK1-6-3D	Zoysia grass	Gifu, Japan	–	*
	P. arrhenomanes	3	NBRC 100102	*Zoysia tenuifolia*	Hyogo, Japan	–	*

Table 1 (continued)

Species		Clade	Isolate	Host/source	Location	PCR	
	P. catenulatum	3	CBS 842.68	Turf grass	South Carolina, USA	–	*
		3	CBS 843.68	Turf grass	South Carolina, USA	–	*
		3	ATCC 10950	Unknown	Unknown	–	*
		3	How1-1	Water	Hokkaido, Japan	–	*
		3	1207 Cu1	Sludge	Aichi, Japan	–	*
	P. graminicola	3	MAFF 425415	Soil	Kumamoto, Japan	–	*
	P. myriotylum	3	NBRC 100113	*Phaseolus vulgaris*	Hokkaido, Japan	–	
	P. periillum	3	CBS 169.68	Soil	Florida, USA	–	*
		3	YGS1T3	Soil	Yamagata, Japan	–	*
		3	S2-8-1S	Zoysia grass	Gifu, Japan	+	*
	P. plurisporium	3	CBS 100530	*Agrostis palustris*	North Carolina, USA	+	
	P. sulcatum	3	NBRC 100117	*Daucus carota* var. *sativus*	Gifu, Japan	–	*
	P. torulosum	3	TJu143	Carrot	Gifu, Japan	–	*
	P. aquatile	3	NBRC 107450	Water	Hokkaido, Japan	–	*
	P. dissotocum	3	MAFF 305576	Soil	Chiba, Jpan	–	
		3	N02E2 3-4	Soil	Gifu, Japan	–	*
	P. pyrilobum	3	NBRC 107365	Water	Hokkaido, Japan	–	
	P. acanthicum	3	MAFF 241099	Soil	Hokkaido, Japan	–	
	P. periplocum	3	NBRC 100114	*Zoysia tenuifolia*	Gifu, Japan	–	*
	P. oligandrum	3	GFSt2-1	Soil	Gifu, Japan	–	*
Globisporangium							
	G. hypogynum	4	CBS 234.94	Soil	Hauts-de-France, France	–	
	G. rostratum	4	NBRC 100115	*Zoysia tenuifolia*	Gifu, Japan	–	*
	G. middletonii	4	CBS 528.74	Soil	Flevoland, Netherlands	–	
	G. parvum	4	N01B4 16-2	Water	Gifu, Japan	–	*
	G. takayamanum	4	NBRC 104223	Soil	Gifu, Japan	–	*
	G. intermedium	4	CBS 266.38	*Agrostis stolonifera*	South Holland, Netherlands	–	*
	G. irregulare (DNA type I)	4	NBRC 100108	Carrot	Gifu, Japan	–	*
	G. irregulare (DNA type II)	4	CBS 263.30	*Nicotiana tabacum*	Kentucky, USA	–	
	G. spinosum	4	NBRC 100116	Soil	Gifu, Japan	–	*
	G. sylvaticum	4	NBRC 100119	Soil	Gifu, Japan	–	*
	G. nagaii	4	CO132	Soil	Fukuoka, Japan	–	*
	G. paddicum	4	MAFF 241108	*Triticum aestivum*	Hokkaido, Japan	–	
	G. heterothallicum	4	CBS 450.67	Soil	Alberta, Canada	–	*
		4	CBS 143.69	Soil	Utrecht, Netherlands	+	*
		4	CBS 207.68	Soil	Netherlands	+	
		4	N02C1 3-3	Soil	Gifu, Japan	+	*
		4	1D2S021	Soil	Gifu, Japan	+	
		4	KA5 20-1	Soil	Gifu, Japan	+	*
		4	CA224	Soil	Kumamoto, Japan	+	*
	G. splendens	4	C101	Kangaroopaw	Chiba, Japan	–	*
		4	ATCC 142852	*Luculia gratissima*	New Zealand	–	*
		4	MAFF 425469	*Cucumis melo*	Chiba, Japan	–	*
		4	CBS 266.69	Ericaceae	East Flanders, Belgium	–	*
		4	CBS 462.48	Unknown	USA	–	*
		4	CBS 267.69	Ericaceae	Gent, Belgium	–	*

Table 1 (continued)

Species		Clade	Isolate	Host/source	Location	PCR	
		4	CBS 268.69	Unknown	Zaire	–	*
		4	CH90LPY1	Unknown	Unknown	–	*
		4	CH82-52	Melon	Chiba, Japan	–	*
		4	On11S 12-1	Soil	Okinawa, Japan	–	*
	G. ultimum	4	NBRC 100122	*Beta vulgaris*	Hokkaido, Japan	–	
	G. nunn	4	CBS 808.96	Soil	Colorado, USA	–	
	G. polymastum	4	CBS 811.70	*Lactuca sativa*	Gelderland, Netherlands	–	*
	G. nodosum	4	MAFF 305905	Soil	Kochi, Japan	–	*
Elongisporangium							
	E. anandrum	5	CBS 285.31	*Rheum rhaponticum*	Unknown	–	
	E. senticosum	5	NBRC 104222	Soil	Gifu, Japan	–	*
	E. undulatum	5	NBRC 107363		Gifu, Japan	–	*
		5	P7505	*Larix* sp.	Scotland, UK	–	*
		5	P9315	Unknown	Unknown	–	*
Others							
	Aphanomyces sp.	–	GFHT6	Spinach	Gifu, Japan	–	*
	Fusaium oxysporum	–	MAFF 727510	Strawberry	Nara, Japan	–	*
	Plasmodiophora brassicae	–	HY	Chinese cabbage	Hyogo, Japan	–	
	Rhizoctonia solani	–	S02	Sutera	Shizuoka, Japan	–	*
	Saprolegnia sp.	–	NBRC 32708	Brown trout	UK	–	*
	Sclerotinia sclerotiorum	–	AiTog	Winter melon	Aichi, Japan	–	*
	Verticillium albo-atrum	–	Vaal 130308		Japan	–	*

Asterisk indicates no replication. No asterisk indicates there are at least two replications

Clade designations of Abad et al. (2022) for *Phytophthora* spp. and of Uzuhashi et al. (2010) for *Phytopythium* spp. (syn. *Ovatisporangium*), *Pythium* spp., *Globisporangium* spp., *Elongisporangium* spp. were used

+, amplified; –, not amplified

surface was covered with hyphae (~ 4 days). The broth was then removed with a filter paper, and the mycelia were homogenized with 100 mL of sterilized distilled water at 3,000 rpm for 5 min. Young ivy leaves were then detached and placed in the mycelial suspension for 7–10 days at 20 °C until symptoms were obvious.

Detached leaves of rhododendron, Japanese andromeda and camellia were inoculated with one or two mycelial disks (7 mm) *P. ramorum* (CBS 101553), *P. kernoviae* (P19875) and *P. lateralis* (P3361) in a plastic box as described by Hieno et al. (2021) with 12 h light at 20 °C/12 h dark at 15 °C for 3–5 days until symptoms were seen.

Symptomatic stems of periwinkle from Gifu, Japan and tobacco plants from Java island, Indonesia were used as naturally infected samples.

For DNA extraction from symptomatic leaves or stems, a 5 × 5 mm leaf piece or 0.2 g of epidermis shaved from the stem was shredded with a blade, then all material for the test plant was placed in a 1.5-ml Eppendorf tube. DNA was extracted using the Kaneka Easy DNA Extraction Kit version 2 (Kaneka, Tokyo, Japan) and the manufacturer's protocol. All extracts were diluted 20 times with TE buffer and stored at 4 °C until further use.

Primers specific for *Phytophthora*

In our previous study (Bi et al. 2019), we designed a primer pair specific for *Phytophthora* (Yph1F_mod2: CGA CCATKGTGGACTTTG, Yph2R_mod2: ACGTTCTCR CAGGCGTATCTG) based on the *Phytophthora*-specific primers (Yph1F and Yph2R) of Schena et al. (2008). In the present study, we further tested the specificity and applicability of our genus-specific primer pair (Bi et al. 2019) using 222 *Phytophthora* isolates that represented 155 taxa and 104 isolates that belonged to other genera (Table 1). We then used this primer pair to develop the multiplex PCR assay (described later) to detect *Phytophthora* species from infected plants.

Simplex PCR using genus-specific primers for *Phytophthora*

The reaction mixture contained 0.5 µM of each primer (Yph1F_mod2, Yph2R_mod2), 0.625 U *Taq* HS DNA polymerase (Takara Bio, Kusatsu, Shiga, Japan), 0.2 mM dNTP mixture, 1 × PCR buffer (10 mM Tris–HCl pH 8.9, 50 mM KCl and 1.5 mM $MgCl_2$), 10 ng of bovine serum albumin (Merck KGaA, Darmstadt, Germany), and 0.1 ng of DNA template, in a total volume of 25 µl. The PCR was run in a BioRad T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) at 95 °C for 2 min; 40 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 45 s, an extension at 72 °C for 30 s; and a final extension at 72 °C for 10 min.

Applicability of the primers was evaluated in simplex PCR for a wide range of strains with different molecular phylogenetic groups (André Lévesque and De Cock 2004; Abad et al. 2022) as shown in Table 1. The sensitivity of the simplex PCR was tested with serial dilutions (10 pg–1 fg per reaction) of mycelial DNA of *P. ramorum* (Pr-1), *P. kernoviae* (P19875) and *P. nicotianae* (CBS 305.29). The simplex PCR was also tested for detectability of DNA from inoculated and naturally infested plants (Supplementary Table S1).

Duplex and triplex PCR

Plant primer pair (FMPl-2b and FMPl-3b) reported by Martin et al. (2004), which amplifies *cox1*, was used as an internal control to determine the success or failure of DNA extraction and PCR of the test samples. The plant species used to test primer detectability are shown in Table 2. We extracted DNA from plant samples and used it in PCR tests as described for the simplex PCR. The same DNA thermal cycler was used, but thermocyling conditions were 95 °C for 8.5 min; 40 cycles at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min; and 72 °C for 10 min.

Duplex PCR for plant–*Phytophthora* spp. was also done as described for the simplex PCR above except for the concentrations of $MgCl_2$ and plant primers. $MgCl_2$ and the plant primers were tested in combinations of 1.5, 2.0, 2.5 mM and 0.05, 0.125, 0.25, 0.5 µM, respectively, to determine the optimal concentration of each.

Triplex PCR for plant–*Phytophthora* spp.–*P. nicotianae* was performed as described above for the duplex PCR, but using the primer pair specific for *P. nicotianae*, Nic-F1/Nic-R1 reported by Li et al. (2011). The primers were tested at 0.05, 0.1, 0.25, 0.5, 1.0 µM to determine the optimal concentration.

To evaluate the sensitivity of the duplex and triplex PCRs, we used mixtures of plant DNA extracted from aseptically cultivated plants (tomato and cucumber) and mycelial DNA of *P. nicotianae* (CBS 305.29), *P. capsici* (P1319) or *P. melonis* (P6870), which was serially diluted (10 pg–1 fg per reaction). Tomato and cucumber seeds were soaked in 10% v/v H_2O_2 solution for 20 min and then washed three times with sterile distilled water. The sterilized seeds were sown in 75 × 75 × 10 mm plant boxes on Murashige-Skoog agar (Fujifilm Wako, Osaka, Japan; 0.8% agar). The plants were grown at 25 °C for 2 weeks in a growth chamber. For aseptically grown plants, DNA was extracted from 5-mm lengths of the hypocotyl for tomato and 5 × 5 mm piece of the main leaf for cucumber as described above.

The DNA detectability of the duplex or triplex PCR was tested using DNA extracted as described above from inoculated and naturally infected plants (Supplementary Table S1).

PCR products were separated by electrophoresis in 2.5% Agarose S (Fujifilm Wako, Osaka, Japan). Gels were stained with Gel Red (10,000 ×, Biotium, Fremont, CA, USA) and photographed under ultraviolet light. All experiments were done at least twice.

Results

Specificity and sensitivity of *Phytophthora* genus-specific primers

The modified primers were tested with more genera, species, and strains to examine the reliability of the detection of the genus *Phytophthora*. The simplex PCR assay enabled the amplification of mycelial DNA of all 222 *Phytophthora* isolates among 155 taxa (Table 1). The amplification efficiency varied among species, and *P. aquimorbida* was the most difficult to detect (detectable in three of five replicate reactions using at least two DNA extractions from the same isolate).

None of the DNA was amplified from the 97 non-target isolates, representing 55 species of *Phytopythium*, *Pythium*, *Globisporangium*, *Elongisporangium*, and one isolate each of seven species of soil-borne pathogens (Table 1). However, DNA from some closely related species was nonspecifically amplified including *Pythium adhaerens* and *Py. plurisporium*, one of three isolates of *Py. periilum*, and six of seven isolates of *Globisporangium heterothallicum*, *Phytopythium cucurbitacearum*, two of nine isolates of *Pp. vexans* and one of three isolates of *Pp. mercuriale* (Table 1).

The detection limit of the modified primer pair using mycelial DNA of *P. ramorum*, *P. kernoviae*, and *P. nicotianae* was determined to be 100 fg (Supplementary Fig. S1).

Application of simplex PCR using diseased plants

The genus-specific simplex PCR assay using the modified primer pair was tested in symptomatic plants (ivy,

Table 2 List of various plants used for the versatility test of the plant PCR primers and amplification results

Order	Family	Species	PCR result
Apiales	Apiaceae	*Daucus carota* subsp. *sativus*	+
	Araliaceae	*Hedera helix*	+
	Pittosporaceae	*Pittosporum tobira*	+
Aquifoliales	Aquifoliaceae	*Ilex integra*	+
Asterales	Asteraceae	*Zinnia elegans*	+
Austrobaileyales	Schisandraceae	*Illicium anisatum*	+
Brassicales	Brassicaceae	*Brassica oleracea* var. *capitata*	+
		Brassica rapa var. *pekinensis*	+
Cornales	Cornaceae	*Cornus florida*	+
	Hydrangeaceae	*Hydrangea macrophylla*	+
Cucurbitales	Cucurbitaceae	*Cucumis sativus*	+
Ericales	Ericaceae	*Rhododendron* × *pulchrum*	+
		Rhododendron indicum	+
		Rhododendron sp.	+
		Vaccinium sect. *Cyanococcus*	+
	Pentaphylacaceae	*Cleyera japonica*	+
	Primulaceae	*Ardisia crenata*	+
	Theaceae	*Camellia japonica*	+
Fabales	Fabaceae	*Robinia pseudoacacia*	+
		Quercus serrata	+
Garryales	Garryaceae	*Aucuba japonica*	+
Gentianales	Apocynaceae	*Catharanthus roseus*	+
		Nerium oleander	+
	Gentianaceae	*Eustoma* sp.	+
Ginkgoales	Ginkgoaceae	*Ginkgo biloba*	+
Lamiales	Lamiaceae	*Salvia rosmarinus*	+
	Linderniaceae	*Torenia* sp.	+
	Oleaceae	*Osmanthus fragrans* var. *aurantiacus*	+
	Plantaginaceae	*Antirrhinum majus*	+
Magnoliales	Magnoliaceae	*Magnolia obovata*	+
Pinales	Cupressaceae	*Juniperus chinensis* cv. Kaizuka	+
	Podocarpaceae	*Podocarpus macrophyllus*	+
Plunbaginales	Plumbaginaceae	*Limonium sinuatum*	+
Proteales	Platanaceae	*Platanus* sp.	+
Ranunculales	Berberidaceae	*Nandina domestica*	+
	Lardizabalaceae	*Akebia quinata*	+
Rosales	Rosaceae	*Photinia* × *fraseri*	+
		Rosa sp.	+
Sapindales	Sapindaceae	*Acer* sp.	+
Solanales	Solanaceae	*Nicotiana* sp.	+
		Solanum lycopersicum	+

+, amplified

tomato, hydrangea, rhododendron, Japanese andromeda, and camellia) that had been inoculated with or naturally infected (periwinkle and tobacco) with various *Phytophthora* species. *Phytophthora* was detected in all inoculated and naturally infected plants (Supplementary Table S1), and no amplicons were obtained using healthy plant tissues.

Development of duplex PCR assay

As an internal control to determine the success or failure of DNA extraction and PCR detection, we developed a duplex PCR to combine our genus-specific primers with the plant primers reported by Martin et al. (2004). The plant primers amplified DNA from all 41 tested plant species of 33

families belonging to 21 orders (Table 2). In the tests for optimal concentrations of magnesium and each primer, in the assay, the higher the magnesium concentration, the higher the activity of the polymerase and the higher the amplification efficiency, but excessive polymerase activity resulted in a loss of specificity and non-target amplification. When we tested several combinations of magnesium concentrations (1.5, 2.0, 2.5 mM) and plant primer concentrations (0.05, 0.0625, 0.125, 0.025, 0.5 µM) using the same concentrations of genus-specific primers as in the simplex PCR reaction solution, DNA extracted from several diseased plant species were amplified stably using 2.5 mM $MgCl_2$ and 0.05 µM of each plant primer (1/10 the concentration of the genus-specific primers) (data not shown). The detection limit of the duplex PCR with the mixture of DNA from aseptically grown plants and from mycelia was 10 to 100 fg for plants and 1 pg for *Phytophthora* spp. (Supplementary Fig. S2).

Development of the triplex PCR assay

Because *P. nicotianae* is already present in Japan and thus exempt from quarantine inspections, we needed a triplex PCR assay to simultaneously detect *Phytophthora* species and *P. nicotianae* in plant samples. In the tests of concentrations for the specific primers for *P. nicotianae* in the triplex PCR, the DNA for the three targets was amplified almost equally by using *P. nicotianae* specific primers at 1/10 the concentration of the genus-specific primers, as found for the plant primers. The detection limit of this triplex PCR using the mixture of DNA from aseptically grown plants and mycelia, was 1 pg for *Phytophthora* spp. and 100 fg for plants and *P. nicotianae* (Supplementary Fig. S3).

Detection of *Phytophthora* species in diseased plants using duplex and triplex PCR

The results of the PCRs using plants that were inoculated (eggplant, tomato, and pumpkin) or naturally infected (periwinkle and tobacco) are shown in Supplementary Table S1, with representative agarose gels of the amplicons in Figs. 1 and 2. The *Phytophthora* species were detected in all the diseased plant samples, and amplification of the plant DNA (*cox1*) was confirmed in all samples. These results indicate that DNA was extracted using the Kaneka Easy DNA Extraction Kit version 2 from diseased samples, and the presence or absence of *Phytophthora* was accurately determined. Triplex PCR results showed that the *P. nicotianae*-specific primers detected *P. nicotianae* from eggplant and tomato plants that were inoculated with the pathogen and from naturally infected periwinkle and tobacco; no amplicons were obtained from pumpkins inoculated with *P. capsici*. Thus, the duplex and triplex PCR tests confirmed the success of DNA extraction and PCR detection and determined whether the cause was, in fact, *Phytophthora*, and if the pathogen was determined to be in the triplex PCR, whether or not it was *P. nicotianae*, using only one tube.

Discussion

Here we tested our previously designed primer pair to detect *Phytophthora* genus-specific, which were based on the primers of Schena et al. (2008) and Bi et al. (2019), using mycelial DNA from 155 taxa (including subspecies, varieties and hybrids), representing members from all *Phytophthora* clades (1–10; Abad et al. 2022). All the tested taxa were detected (Table 1). When non-targets of the primer pair were tested, among 97 isolates representing 55 species of closely related genera, DNA was amplified from only three species and from a small portion of the isolates tested for four species. Thus, our improved primers detected all species of *Phytophthora* tested, although a few closely related species of *Pythium*, *Globisporangium* and *Phytopythium* yielded false positives (Table 1). Many more taxa were tested than in previous reports of genus-specific detection: 101 species (Bilodeau et al. 2014), 45 species (Scibetta et al. 2012), 35 species (Schena et al. 2008), and 136 taxa (Miles et al. 2015). Therefore, our primers will benefit quarantine efforts, where strict border control is required and detection without omission is of utmost importance.

The detection limit of the simplex PCR using mycelial DNA of *P. ramorum*, *P. kernoviae*, and *P. nicotianae* was 100 fg for all three species (Supplementary Fig. S1), the same detection limit as that of the nested PCR of Schena et al. (2008). We were able to achieve the same level of sensitivity as that of Schen et al. (2008) in one round of amplification. In a comparison of the electrophoretic results for the 155 taxa in the specificity test, the intensity of the amplicon band varied among species, even though 100 pg of DNA was used in all cases (data not shown). Amplification failed only for a few PCR replicate tests for *P. aquimorbida* (2 of 5 tests) and *P. macilentosa* (1 of 4 tests), both minor pathogens. Thus, the detection results for plant samples that are potentially infected with those two species should be evaluated carefully. However, for other species, positive results were obtained in all repeated tests.

For the duplex and triplex PCR for diseased plants, the composition of the reaction solution, including the concentrations of primers and magnesium, were optimized so that differences in amplification between primers due to competition between polymerase and substrates could be reduced. The detection limits for *Phytophthora* DNA in the multiplexed PCRs using mixtures containing plant DNA were 10 times higher than those of simplex PCR using only mycelial DNA (Supplementary Figs. S1–S3). Knowing that detection

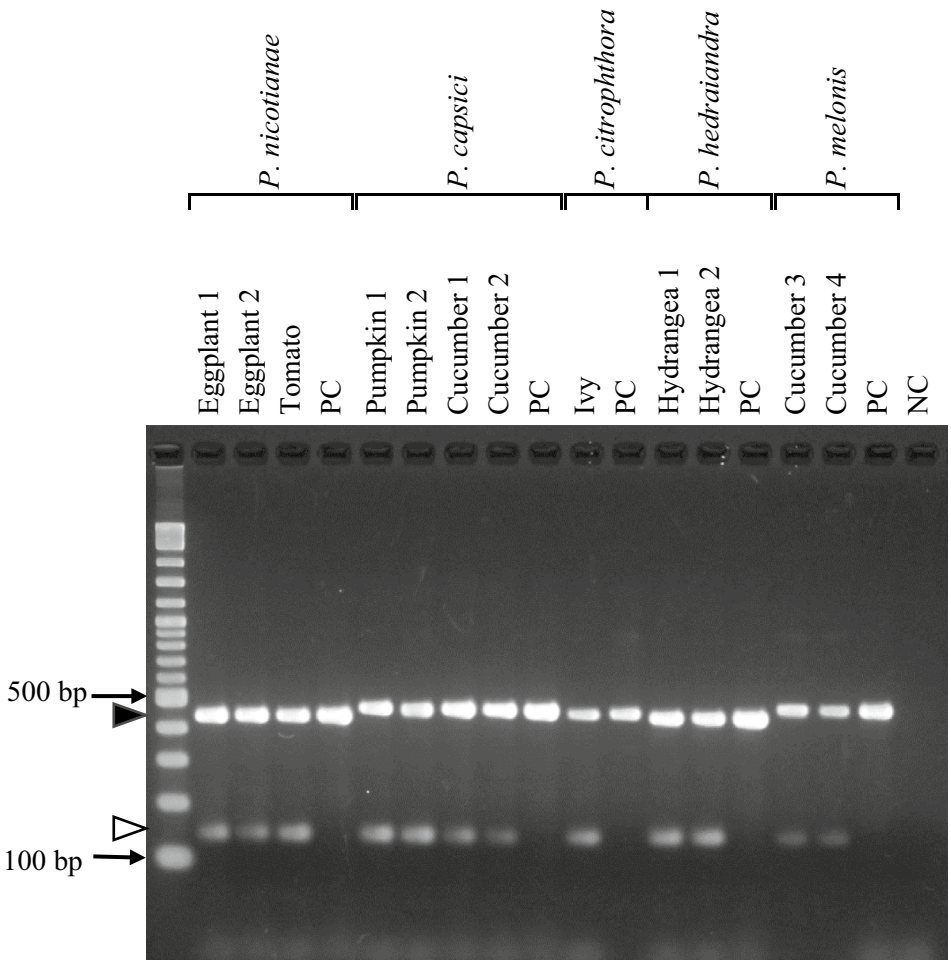

Fig. 1 Duplex PCR detection of *Phytophthora* spp. from various plant species inoculated with different pathogens. Plant–pathogen combinations: Eggplant 1 and tomato, *P. nicotianae* GK10Eg1; Eggplant 2, *P. nicotianae* 13Asp1-1; Pumpkin 1, cucumber 1 and 2, *P. capsici* CH01CUCU10; Pumpkin 2, *P. capsici* CH02UE0202; ivy, *P. citrophthora* CH94HE11; Hydrangea 1 and 2, *P. hedraiandra* TGTA1-1; Cucumber 3 and 4, *P. melonis* CH00ME21-21. PC: positive control, mycelial DNA of *P. nicotianae* CBS 305.29, *P. capsici* P0253. *P. citrophthora* P3693, *P. hedraiandra* P11725 and *P. melonis* P6870. NC: negative control, sterile distilled water. Black arrowhead: amplicons using *Phytophthora* genus-specific primers (around 470 bp). White arrowhead: amplicons using plant primers (approximately 140 bp)

sensitivity is better with higher quality DNA, we modified the extraction method of Kageyama et al. (2003) to obtain higher quality DNA and increased the sensitivity to 100 fg (data not shown). For the present study, considering the huge number of samples tested during quarantine inspections, we selected the Kaneka Easy DNA Extraction Kit version 2 method for its simple, rapid extraction of DNA (Hieno et al. 2019). Thus, users should choose DNA extraction and detection methods (simplex PCR or multiplex PCR) that are best for their situation and objectives.

Our newly designed triplex PCR was able to simultaneously determine the presence of a *Phytophthora* species and *P. nicotianae*, the only *Phytophthora* species not subject to quarantine in Japan, in a one-tube reaction. In addition, *Ypt1* regions have accumulated sufficient mutations to be highly discriminative of species so that the genus-specific primer can be used sequencing the amplicons to identify the species. In addition, the simplex PCR amplicons from DNA extracted from diseased plants (naturally infected tobacco sample no. 1 with *P. nicotianae* and rhododendron inoculated with *P. kernoviae* or *P. lateralis*; Supplementary Table S1) were subjected to a sequencing analysis, and these species were identified (data not shown). Although we previously developed a LAMP assay using a QProbe to simultaneously detect *Phytophthora* spp. and *P. nicotianae* (Hieno et al. 2020) that is a rapid, accurate highly applicable method for import/export quarantine inspections, it cannot be used to identify the actual species of *Phytophthora*, except for *P. nicotianae*. By comparing the advantages of each detection method, it is possible to select the method that best meets the conditions required by the user.

Molecular detection methods can also be used for practical inspection of nonsymptomatic plants. Fichtner et al. (2012) pointed out that symptomless infections by *P. kernoviae* in North American native plants may thwart pathogen detection and underscore the importance of implementing a proactive and adaptive biosecurity plan. Harris and Webber (2016) also pointed out that symptomless infections of larch by *P. ramorum* can lead to an underestimation of infection plants. We are confident that our optimized detection method can contribute to more effective quarantine control.

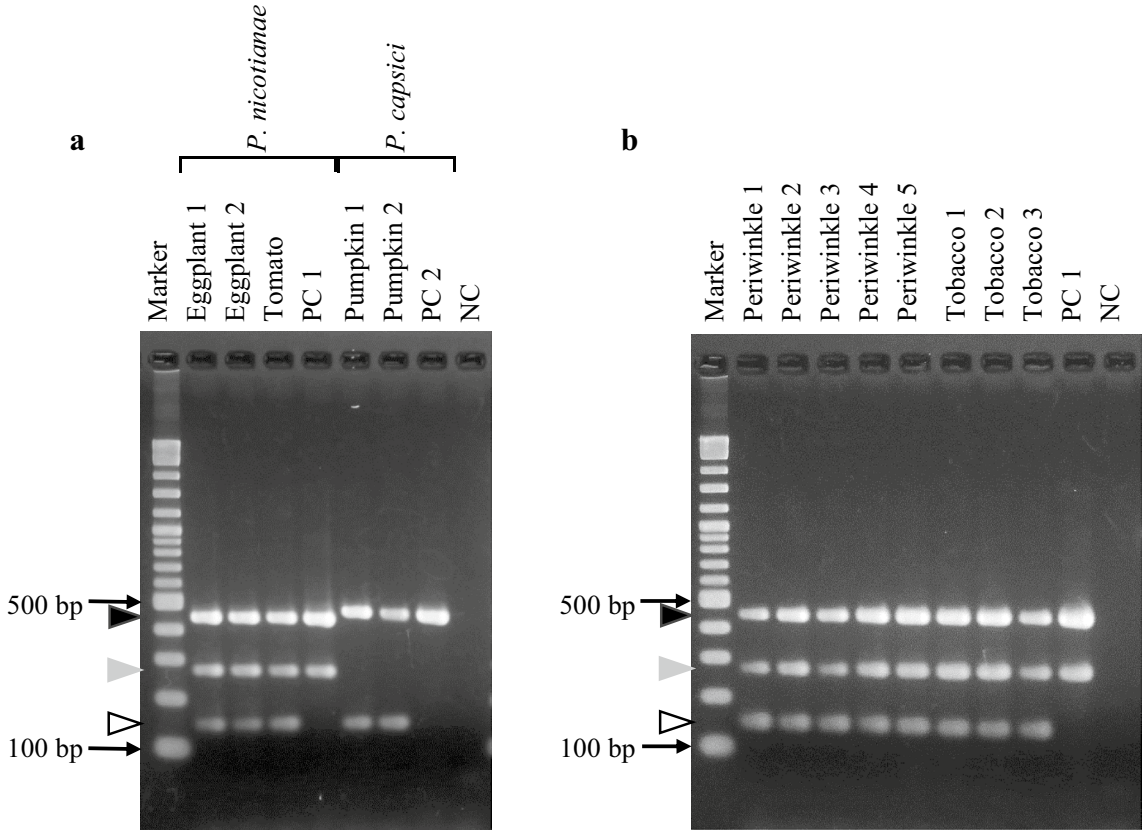

Fig. 2 Triplex PCR detection of *Phytophthora* spp. and *P. nicotianae* using DNA from symptomatic plants that were inoculated or naturally infected. a, Inoculated plants. Eggplant 1 and tomato were inoculatsed with *P. nicotianae* GK10Eg1. Eggplant 2 was inoculated with 13Asp1-1. Pumpkin 1 and 2 were inoculated with *P. capsici* CH01CUCU10 and CH02UE0202, respectively. b, Naturally infected plants. PC, positive controls using mycelial DNA of *P. nicotianae* CBS 305.29 (PC 1) or *P. capsici* P0253 (PC 2). NC, negative control using sterile distilled water. Black arrowhead: amplification with *Phytophthora* genus-specific primers (around 470 bp). Gray arrowhead amplicons using *P. nicotianae*-specific primers (267 bp). White arrowhead: amplicons using plant primers (approximately 140 bp)

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10327-024-01179-z.

Acknowledgements We thank Emeritus Prof. E. M. Hansen, Prof. M. D. Coffey, Dr. P. W. Tooley, Prof. C. Hong, Dr. B. S. Weir, Prof. T. I. Burgess, Dr. T. Jung, Mr. S. Uematsu, and Dr. H. Watanabe for providing many important isolates and Dr. S. Subandiyah and Dr. A. Wibowo for providing plant DNA samples. We also thank Mr. T. Sakoda and Mr. K. Ueda for technical assistance.

Author contributions KO, HS, KK and AH contributed to conceiving and designing the study. KO, ML and AA prepared materials and performed the experiments. KO and KK analyzed the data. KO, KK and AH wrote the manuscript.

Funding Open Access funding provided by Gifu University. The present study was supported by a Grant-in-Aid for "Development of detection and identification techniques of pests" under "Research and development of adaptation measures for global warming and abnormal weather" from the Ministry of Agriculture, Forestry, and Fisheries of Japan.

Declarations

Conflict of interest The authors declare no conflict of interest.

Human and animal rights This article does not contain any studies involving human participants or experimental animals.

References

Abad ZG, Burgess T, Bienapfl JC, Redford AJ, Coffey M, Knight L (2022) IDphy: molecular and morphological identification of *Phytophthora* based on the types. USDA APHIS PPQ S&T Beltsville Lab, USDA APHIS PPQ S&T ITP, Centre for Phytophthora Science and Management, and World Phytophthora Collection. https://idtools.org/id/phytophthora

André LéVesque C, De Cock AWAM (2004) Molecular phylogeny and taxonomy of the genus *Pythium*. Mycol Res 108:1363–1383

Bi X, Hieno A, Otsubo K, Kageyama K, Liu G, Li M (2019) A multiplex PCR assay for three pathogenic *Phytophthora* species related to kiwifruit diseases in China. J Gen Plant Pathol 85:12–22

Bilodeau GJ, Martin FN, Coffey MD, Blomquist CL (2014) Development of a multiplex assay for genus- and species-specific detection of *Phytophthora* based on differences in mitochondrial gene order. Phytopathology 104:733–748

Brasier CM (2008) The biosecurity threat to the UK and global environment from international trade in plants. Plant Pathol 57:792–808

Brasier C (2009) *Phytophthora* biodiversity: How many *Phytophthora* species are there? In: Proceedings of the 4th meeting of the international union of forest research organizations (JUFO) working party s07.02.09: Phythophthoras in forests and natural ecosystems. Goheen EM, Frankel SJ (tech. coords.) Gen Tech Rep PSW-GTR-221, U.S. Department Agriculture Forest Service, Pacific Southwest Research Station, Albany, CA, USA pp 101–105

Fichtner EJ, Rizzo DM, Kirk SA, Webber JF (2012) Infectivity and sporulation potential of *Phytophthora kernoviae* to select North American native plants. Plant Pathol 61:224–233

Harris AR, Webber JF (2016) Sporulation potential, symptom expression and detection of *Phytophthora ramorum* on larch needles and other foliar hosts. Plant Pathol 65:1441–1451

Hieno A, Li M, Afandi A, Otsubo K, Suga H, Kageyama K (2019) Rapid detection of *Phytophthora nicotianae* by simple DNA extraction and real-time loop-mediated isothermal amplification assay. J Phytopathol 167:174–184

Hieno A, Li M, Afandi A, Otsubo K, Suga H, Kageyama K (2020) Detection of the genus *Phytophthora* and the species *Phytophthora nicotianae* by LAMP with a QProbe. Plant Dis 104:2469–2480

Hieno A, Li M, Otsubo K, Suga H, Kageyama K (2021) Multiplex LAMP detection of the genus *Phytophthora* and four *Phytophthora* species *P. ramorum*, *P. lateralis*, *P. kernoviae*, and *P. nicotianae*, with a plant internal control. Microbes Environ 36:ME21019

Kageyama K, Komatsu T, Suga H (2003) Refined PCR protocol for detection of plant pathogens in soil. J Gen Plant Pathol 69:153–160

Li M, Asano T, Suga H, Kageyama K (2011) A multiplex PCR for the detection of *Phytophthora nicotianae* and *P. cactorum*, and a survey of their occurrence in strawberry production areas of Japan. Plant Dis 95:1270–1278

Martin FN, Tooley PW, Blomquist C (2004) Molecular detection of *Phytophthora ramorum*, the causal agent of sudden oak death in California, and two additional species commonly recovered from diseased plant material. Phytopathology 94:621–631

Miles TD, Martin FN, Coffey MD (2015) Development of rapid isothermal amplification assays for detection of *Phytophthora* spp. in plant tissue. Phytopathology 105:265–278

Miller PM (1955) V-8 juice agar as a general-purpose medium for fungal and bacteria. Phytopathology 45:461–462

Sakoda T, Goto H, Kanno T, Hiyama T, Hirakawa T, Nakanishi Y, Hirata T (2017) Ramorum blight of *Rhododendron* sp. caused by *Phytophthora ramorum* intercepted in plant quarantine inspection in Japan. Res Bull Plant Prot Serv Jpn 53:75–81

Schena L, Cooke DEL (2006) Assessing the potential of regions of the nuclear and mitochondrial genome to develop a "molecular tool box" for the detection and characterization of *Phytophthora* species. J Microbiol Methods 67:70–85

Schena L, Duncan JM, Cooke DEL (2008) Development and application of a PCR-based "molecular tool box" for the identification of *Phytophthora* species damaging forests and natural ecosystems. Plant Pathol 57:64–75

Scibetta S, Schena L, Chimento A, Cacciola SO, Cooke DEL (2012) A molecular method to assess *Phytophthora* diversity in environmental samples. J Microbiol Methods 88:356–368

Uzuhashi S, Tojo M, Kakishima M (2010) Phylogeny of the genus *Pythium* and description of new genera. Mycoscience 51:337–365

Yang X, Hong C (2018) Differential usefulness of nine commonly used genetic markers for identifying *Phytophthora* species. Front Microbiol 9:2334