Detection of the Genus *Phytophthora* and the Species *Phytophthora nicotianae* by LAMP with a QProbe

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Abstract

Phytophthora is an oomycete genus with worldwide distribution, and many of its species cause destructive diseases. In Japan, *Phytophthora* species are listed as quarantine organisms with the exception of *Phytophthora nicotianae*. For effective quarantine control, we designed a *Phytophthora* genus-specific loop-mediated isothermal amplification (LAMP) primer set and a *P. nicotianae* species-specific quenching probe (QProbe) to establish a simultaneous LAMP-based detection method. We confirmed the specificity of the genus-specific primers, and all 161 taxa were detected. No other species in the closely related genera *Pythium* and *Phytopythium* gave positive results with the exception of two species, *Phytopythium delawarensense* and *Phytopythium*

fagopyri. These two species gave inconsistent results. We used annealing curve analysis with the QProbe to demonstrate that *P. nicotianae* could be distinguished from other species. DNA from inoculated and naturally infected plants was extracted using a time-saving extraction kit and subjected to the simultaneous detection method. We confirmed that all *Phytophthora* DNAs in the plant samples were detected, and *P. nicotianae* was specifically identified. This simultaneous detection method will make quarantine inspections faster and easier.

Keywords: loop-mediated isothermal amplification, *Phytophthora*, *Phytophthora nicotianae*, oomycetes, pathogen detection, quenching probe

Most species in the oomycete genus *Phytophthora* are known as plant pathogens that cause serious commercial losses. An invasion of *Phytophthora* species in a new region will create unexpected threats to that region's ecosystems. *Phytophthora infestans* (Mont.) de Bary (de Bary 1876), which causes potato late blight, was responsible for the Irish potato famine in continental Europe in 1845–1849. More recently, the economic impact of the disease in the EU has been estimated at more than €1 billion per year (Haverkort et al. 2008). In the U.S.A., the costs of the disease are estimated at \$77.1 million in fungicides and \$210.7 million in lost revenue due to decreased yield, quality, and storability (Guenther et al. 2001). Population genetic analyses based on microsatellite markers and nuclear gene sequences suggested that *P. infestans* was introduced to Europe from central Mexico in the 1840s (Goss et al. 2014).

Another destructive plant pathogen is *Phytophthora ramorum* Werres, de Cock & Man in't Veld (Werres et al. 2001), which causes sudden oak death and has infected a range of forest and nursery trees in North America and Europe (Hansen 2015). The recent appearance of four lineages of *P. ramorum* (NA1, NA2, EU1, and EU2) is due to the independent introduction of NA1 and NA2 into North America and EU1 and EU2 into Europe (Goss et al. 2009; Van Poucke et al. 2012). A 2011 analysis of the EU1 lineage obtained from nurseries in Europe and North America suggested that the lineage had migrated from Europe to North America more commonly than in the other direction (Goss et al. 2011). The economic

impacts of sudden oak death were estimated in the tens of millions of dollars in the U.S.A. (Grünwald et al. 2008), and in England and Wales, £1.446 billion of public revenue is at risk per year from the uncontrolled spread of *P. ramorum* and *Phytophthora kernoviae* Brasier, Beales & Kirk (Brasier et al. 2005; Drake and Jones 2017). *P. kernoviae* is another introduced, invasive species that has been found in the U.K. and New Zealand. The origin of *P. ramorum* is currently unknown, but one hypothesis is that it came from the Himalayan region of Asia (Hansen 2015).

In Australia, forest dieback caused by the introduced species *Phytophthora cinnamomi* Rands (1922) has had negative impacts on fauna habitats (Garkaklis et al. 2004). The origin of *P. cinnamomi* is also unknown, although speculation centers on Taiwan and the islands of Southeast Asia and the Pacific (Hansen 2015).

In Japan, *Phytophthora* species are listed as quarantine organisms, with the exception of *Phytophthora nicotianae*, because it is already a dominant pathogen in Japan, infecting at least 77 plant species in 41 families (Database of Plant Disease in Japan in the Genebank Project, National Agriculture and Food Research Organization). In February 2015, discolored leaves were found on a *Rhododendron* sp. cultivar Loch Lomond, which was being imported from Scotland to Japan, and the isolate was identified as *P. ramorum* (Sakoda et al. 2017). This detection at entry suggests that careful import inspections are required, even if a phytosanitary certificate is attached to imported plants (Sakoda et al. 2017).

For the effective quarantine control of plant pathogens, rapid and accurate detection methods are essential. Previously, *Phytophthora* genus-specific PCR primers have been designed using the following sequences: the ribosomal DNA internal transcribed spacer (rDNA-ITS) region (Scibetta et al. 2012); the *Ypt1* gene that encodes the ras-like GTP-binding protein (Schena et al. 2006, 2008); the mitochondrial cytochrome c oxidase subunit 1 and subunit 2 genes (Martin et al. 2004); and two mitochondrial regions, *trnM-trnM* and *atp9-nad9* (Bilodeau et al. 2014). Another detection technique, called recombinase polymerase amplification (RPA), has been established for *Phytophthora* spp. and provides species-specific assays for *P. ramorum* and *P. kernoviae* (Miles et al. 2015). However, as far as we know these detection methods have been tested only with a limited number of species: at most, 136 of 188 available *Phytophthora* taxa including subspecies,

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*The e-Xtra logo stands for “electronic extra” and indicates that one supplementary table and three supplementary figures are published online.

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Table 1. Isolates used in this study with their QProbe region allele numbers (see Table 3) and results of assays with the *Phytophthora* genus-specific LAMP primers with or without the *Phytophthora nicotianae*-specific QProbe

Species	Clade ^a	Isolate ^b	Allele number of QProbe region ^c	Detection of genus by LAMP ^d	Detection of genus by LAMP with QProbe ^d	Detection of <i>P. nicotianae</i> by LAMP with QProbe ^d
<i>Phytophthora</i>						
<i>P. nicotianae</i>	1	CBS 305.29	Original	+	+	+
<i>P. nicotianae</i>	1	CBS 535.92	Original	+	+	+
<i>P. nicotianae</i>	1	MAFF 235436	Original	+	+	+
<i>P. nicotianae</i>	1	MAFF 237653	Original	+	+	+
<i>P. nicotianae</i>	1	MAFF 238148	Original	+	+	+
<i>P. nicotianae</i>	1	MAFF 238150	Original	+	+	+
<i>P. nicotianae</i>	1	MAFF 238152	Original	+	+	+
<i>P. nicotianae</i>	1	MAFF 238154	Original	+	+	+
<i>P. nicotianae</i>	1	MAFF 239554	Original	+	+	+
<i>P. nicotianae</i>	1	MAFF 242197	Original	+	+	+
<i>P. nicotianae</i>	1	MAFF 305591	Original	+	+	+
<i>P. nicotianae</i>	1	MAFF 305795	Original	+	+	+
<i>P. nicotianae</i>	1	MAFF 305796	Original	+	+	+
<i>P. nicotianae</i>	1	MAFF 305797	Original	+	+	+
<i>P. nicotianae</i>	1	MAFF 305799	Original	+	+	+
<i>P. nicotianae</i>	1	MAFF 305926	Original	+	+	+
<i>P. nicotianae</i>	1	MAFF 305939	Original	+	+	+
<i>P. nicotianae</i>	1	MAFF 305940	Original	+	+	+
<i>P. nicotianae</i>	1	MAFF 712194	Original	+	+	+
<i>P. nicotianae</i>	1	MAFF 712287	Original	+	+	+
<i>P. nicotianae</i>	1	MAFF 712342	Original	+	+	+
<i>P. aleatoria</i>	1	NZFS 4037	Allele 1	NT	NT	NT
<i>P. cactorum</i>	1a	MAFF 731066	Allele 1	+	+	—
<i>P. hedraiaandra</i>	1a	CBS 111725	Allele 1	+	+	—
<i>P. idaei</i>	1a	WPC P6767	Allele 1	+	+	—
<i>P. pseudotsugae</i>	1a	WPC P10339	Allele 1	+	+	—
<i>P. clandestina</i>	1b	WPC P3943	Allele 12	+	+	—
<i>P. iranica</i>	1b	WPC P3882	Allele 12	+	+	—
<i>P. tentaculata</i>	1b	CBS 552.96	Allele 18	+	+	—
<i>P. andina</i>	1c	CBS 115547	Allele 5	+	+	—
<i>P. infestans</i>	1c	MAFF 236324	Allele 5	+	+	—
<i>P. ipomoeae</i>	1c	WPC P10225	Allele 21	+	+	—
<i>P. mirabilis</i>	1c	WPC P3005	Allele 5	+	+	—
<i>P. phaseoli</i>	1c	WPC P10145	Allele 5	+	+	—
<i>P. urerae</i>	1c	PSR27	Allele 5	NT	NT	NT
<i>P. acaciae</i>	2	AN02	Allele 10	NT	NT	NT
<i>P. menzei</i>	2	ATCC MYA-4554	Allele 1	+	NT	NT
<i>P. multivesiculata</i>	2	CBS 545.96	Allele 1	+	NT	NT
<i>P. siskiyouensis</i>	2	ATCC MYA-4187	Allele 1	+	NT	NT
<i>P. tropicalis</i>	2	CBS 434.91	Allele 1	+	NT	NT
<i>P. oleae</i>	2	Po1a	Allele 1	NT	NT	NT
<i>P. botryosa</i>	2a	CBS 581.69	Allele 1	+	NT	NT
<i>P. citrophthora</i>	2a	WPC P0479	Allele 1	+	+	—
<i>P. colocasiae</i>	2a	WPC P6317	Allele 1	+	NT	NT
<i>P. himalsilva</i>	2a	CBS 128767	Allele 1	+	NT	NT
<i>P. meadii</i>	2a	CBS 219.88	Allele 1	+	NT	NT
<i>P. mekongensis</i>	2a	CBS 135136	Allele 1	+	NT	NT
<i>P. occultans</i>	2a	CBS 101557	Allele 1	+	NT	NT
<i>P. terminalis</i>	2a	WPC P19956	Allele 1	+	NT	NT
<i>P. amaranthi</i>	2b	WPC P20892	Allele 1	+	NT	NT
<i>P. capsici</i>	2b	MAFF 305920	Allele 1	+	+	—
<i>P. glovera</i>	2b	CBS 121969	Allele 1	+	NT	NT
<i>P. acerina</i>	2c	CBS 133931	Allele 1	+	NT	NT
<i>P. capensis</i>	2c	WPC P1819	Allele 1	+	NT	NT
<i>P. caryae</i>	2c	ATCC TSD-54	Allele 1	+	NT	NT
<i>P. citricola</i>	2c	CH95PHE28	Allele 1	+	+	—

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^a Clade designations are from Abad et al. (2019).

^b ATCC, American Type Culture Collection; CBS, Westerdijk Fungal Biodiversity Institute; CCIBt, Institute of Botany Culture Collection; ICMP, International Collection of Microorganisms from Plants; IMI, International Mycological Institute Herbarium; MAFF, Japanese Ministry of Agriculture Forestry and Fisheries; NBRC, National Institute of Technology and Evaluation Biological Resource Center; NZFS, New Zealand Forest Service; WPC, World Phytophthora Genetic Resource Collection.

^c The sequences of the QProbe regions are classified into alleles (original plus numbers 1 to 26; see Table 3) based on the rDNA-ITS sequences with NCBI accession numbers listed in Supplementary Table S1.

^d At least two repeats were performed. +, detected within 60 min; —, not detected within 60 min; NT, not tested. Asterisks indicate samples that were tested and confirmed using the ABI StepOnePlus Real-Time PCR System.

Table 1. (Continued from previous page)

Species	Clade ^a	Isolate ^b	Allele number of QProbe region ^c	Detection of genus by LAMP ^d	Detection of genus by LAMP with QProbe ^d	Detection of <i>P. nicotianae</i> by LAMP with QProbe ^d
<i>P. multivora</i>	2c	NBRC 31016	Allele 1	+	NT	NT
<i>P. pachypleura</i>	2c	IMI 502404	Allele 1	+	NT	NT
<i>P. pini</i>	2c	CBS 181.25	Allele 1	+	NT	NT
<i>P. plurivora</i>	2c	CBS 124093	Allele 1	+	NT	NT
<i>P. bishii</i>	2d	CBS 122081	Allele 10	+	NT	NT
<i>P. elongata</i>	2d	CBS 125799	Allele 1	+	NT	NT
<i>P. frigida</i>	2d	CBS 121941	Allele 20	+	+	–
<i>P. ilicis</i>	3a	WPC P3939	Allele 1	+	NT	NT
<i>P. nemorosa</i>	3a	C71	Allele 1	+	+	–
<i>P. pluvialis</i>	3a	ATCC MYA-4930	Allele 1	+	NT	NT
<i>P. pseudosyringae</i>	3a	CBS 111772	Allele 1	+	NT	NT
<i>P. psychrophila</i>	3a	CBS 803.95	Allele 1	+	NT	NT
<i>P. castanetorum</i>	3b	CBS 142299	Allele 1	+	NT	NT
<i>P. quercina</i>	3b	WPC P10439	Allele 1	+	NT	NT
<i>P. tubulina</i>	3b	CBS 141212	Allele 23	+	+	–
<i>P. alticola</i>	4	CBS 121939	Allele 1	+	NT	NT
<i>P. arenaria</i>	4	CBS 127950	Allele 1	+	NT	NT
<i>P. boodjera</i>	4	CBS 138637	Allele 1	+	NT	NT
<i>P. megakarya</i>	4	WPC P1672	Allele 10	+	NT	NT
<i>P. palmivora</i>	4	CH88-1	Allele 10	+	+	–
<i>P. quercetorum</i>	4	WPC P15555	Allele 1	+	NT	NT
<i>P. agathidicida</i>	5	ICMP 17027	Allele 5	+	NT	NT
<i>P. castaneae</i>	5	ICMP 19434	Allele 5	+	NT	NT
<i>P. cocois</i>	5	ICMP 16948	Allele 5	+	NT	NT
<i>P. heveae</i>	5	WPC P1102	Allele 5	+	+	–
<i>P. sp. novaeguineae</i>	5	ICMP 19637	Allele 5	+	NT	NT
<i>P. asparagi</i>	6	CBS 132095	Allele 19	+	+	–
<i>P. lacustris</i>	6	WPC P10337	Allele 2	+	NT	NT
<i>P. balyanboodja</i>	6a	CBS 143058	Allele 2	NT	NT	NT
<i>P. chesapeakeensis</i>	6a	CBS 140655	Allele 2	NT	NT	NT
<i>P. condilina</i>	6a	CBS 143059	Allele 2	NT	NT	NT
<i>P. cooljarloo</i>	6a	CBS 143062	Allele 2	NT	NT	NT
<i>P. gemini</i>	6a	CBS 123381	Allele 2	+	NT	NT
<i>P. humicola</i>	6a	CBS 200.81	Allele 2	+	+	–
<i>P. inundata</i>	6a	WPC P8478	Allele 2	+	NT	NT
<i>P. kwongonina</i>	6a	CBS 143060	Allele 2	NT	NT	NT
<i>P. oreophila</i>	6a	U11	Allele 2	NT	NT	NT
<i>P. pseudorosacearum</i>	6a	CBS 143061	Allele 2	NT	NT	NT
<i>P. rosacearum</i>	6a	ATCC MYA-4456	Allele 2	+	NT	NT
<i>P. xstagnum</i>	6b	ATCC MYA-4926	Allele 2	+	NT	NT
<i>P. amnicola</i>	6b	CBS 131652	Allele 2	+	NT	NT
<i>P. bilorbang</i>	6b	CBS 131653	Allele 2	+	NT	NT
<i>P. borealis</i>	6b	ATCC MYA-4881	Allele 2	+	NT	NT
<i>P. chlamydospora</i>	6b	WPC P6133	Allele 2	+	NT	NT
<i>P. crassamura</i>	6b	CBS 140357	Allele 2	+	NT	NT
<i>P. fluvialis</i>	6b	CBS 129424	Allele 2	+	NT	NT
<i>P. gibbosa</i>	6b	CBS 127951	Allele 2	+	NT	NT
<i>P. gonapodyides</i>	6b	WPC P7050	Allele 2	+	NT	NT
<i>P. gregata</i>	6b	CH97TUL2	Allele 2	+	NT	NT
<i>P. litoralis</i>	6b	CBS 127953	Allele 2	+	NT	NT
<i>P. megasperma</i>	6b	CBS 402.72	Allele 2	+	NT	NT
<i>P. mississippiae</i>	6b	ATCC MYA-4946	Allele 2	+	NT	NT
<i>P. moyootj</i>	6b	CBS 138759	Allele 2	+	NT	NT
<i>P. ornamentata</i>	6b	CBS 140647	Allele 2	+	NT	NT
<i>P. pinifolia</i>	6b	CBS 122924	Allele 2	+	NT	NT
<i>P. riparia</i>	6b	ATCC MYA-4882	Allele 2	+	NT	NT
<i>P. thermophila</i>	6b	CBS 127954	Allele 2	+	NT	NT
<i>P. xcambivora</i>	7a	CBS 141218	Allele 3	+	NT	NT
<i>P. xheterohybrida</i>	7a	CBS 141207	Allele 3	+	NT	NT
<i>P. xincrassata</i>	7a	CBS 141209	Allele 3	+	NT	NT
<i>P. attenuata</i>	7a	CBS 141199	Allele 3	+	NT	NT
<i>P. cinnamomi</i>	7a	NBRC 33180	Allele 3	+	NT	NT
<i>P. europaea</i>	7a	CBS 109049	Allele 3	+	NT	NT
<i>P. flexuosa</i>	7a	CBS 141201	Allele 3	+	NT	NT
<i>P. formosa</i>	7a	CBS 141203	Allele 3	+	NT	NT
<i>P. fragariae</i>	7a	CBS 209.46	Allele 3	+	NT	NT

(Continued on next page)

Table 1. (Continued from previous page)

Species	Clade ^a	Isolate ^b	Allele number of QProbe region ^c	Detection of genus by LAMP ^d	Detection of genus by LAMP with QProbe ^d	Detection of <i>P. nicotianae</i> by LAMP with QProbe ^d
<i>P. intricata</i>	7a	CBS 141211	Allele 3	+	NT	NT
<i>P. parvispora</i>	7a	CBS 411.96	Allele 3	+	NT	NT
<i>P. rubi</i>	7a	CBS 967.95	Allele 3	+	NT	NT
<i>P. tyrhenica</i>	7a	CBS 142301	Allele 3	+	NT	NT
<i>P. uliginosa</i>	7a	CBS 109054	Allele 3	+	NT	NT
<i>P. vulcanica</i>	7a	CBS 141216	Allele 3	+	NT	NT
<i>P. asiatica</i>	7b	CBS 133347	Allele 3	+	NT	NT
<i>P. cajani</i>	7b	WPC P3105	Allele 3	+	+	–
<i>P. melonis</i>	7b	CBS 582.69	Allele 3	+	NT	NT
<i>P. niederhauserii</i>	7b	CH96HE1	Allele 3	+	NT	NT
<i>P. pisi</i>	7b	CBS 130350	Allele 3	+	NT	NT
<i>P. pistaciae</i>	7b	CBS 137185	Allele 3	+	NT	NT
<i>P. sojae</i>	7b	Pm-1	Allele 3	+	NT	NT
<i>P. vignae</i>	7b	NBRC 30613	Allele 3	+	NT	NT
<i>P. cinnamomi</i> var. <i>robiniae</i>	7c	ATCC 90456	Allele 3	+	NT	NT
<i>P. fragariaefolia</i>	7c	CBS 135747	Allele 3	+	NT	NT
<i>P. nagaii</i>	7c	CBS 133248	Allele 3	+	NT	NT
<i>P. himalayensis</i>	8	CBS 357.59	Allele 7	NT	NT	NT
<i>P. cryptogea</i>	8a	CBS 113.19	Allele 7	+	+	–
<i>P. drechsleri</i>	8a	CBS 292.35	Allele 25	+	+	–
<i>P. erythroseptica</i>	8a	WPC P0340	Allele 7	+	+	–
<i>P. medicaginis</i>	8a	WPC P7029	Allele 6	+	NT	NT
<i>P. pseudocryptogea</i>	8a	CBS 139749	Allele 7	+	NT	NT
<i>P. richardiae</i>	8a	CBS 240.30	Allele 14	+	+	–
<i>P. sansomeana</i>	8a	WPC P3163	Allele 7	+	+	–
<i>P. sp. kelmaniana</i>	8a	GF649	Allele 7	+	NT	NT
<i>P. trifolii</i>	8a	WPC P6980	Allele 6	+	NT	NT
<i>P. brassicae</i>	8b	CBS 179.87	Allele 6	+	+	–
<i>P. cichorii</i>	8b	CBS 115029	Allele 22	+	+	–
<i>P. dauci</i>	8b	CBS 127102	Allele 6	+	+	–
<i>P. lactucae</i>	8b	BPIC 1985	Allele 6	+	NT	NT
<i>P. porri</i>	8b	CBS 140.87	Allele 6	+	NT	NT
<i>P. primulae</i>	8b	WPC P10333	Allele 6	+	NT	NT
<i>P. pseudolactucae</i>	8b	CBS 137103	Allele 6	+	NT	NT
<i>P. foliorum</i>	8c	WPC P10974	Allele 8	+	+	–
<i>P. hibernalis</i>	8c	WPC P3822	Allele 9	+	+	–
<i>P. lateralis</i>	8c	CBS 168.42	Allele 9	+	+	–
<i>P. ramorum</i>	8c	CBS 101553	Allele 9	+	+	–
<i>P. ramorum</i>	8c	Pr-1	Allele 9	+	NT	NT
<i>P. austrocedri</i>	8d	ATCC MYA-4074	Allele 8	+	NT	NT
<i>P. obscura</i>	8d	CBS 129273	Allele 8	+	NT	NT
<i>P. syringae</i>	8d	FIUm1	Allele 8	+	+	–
<i>P. cacuminis</i>	9	U40	Allele 11	NT	NT	NT
<i>P. estuarina</i>	9	CCIBt 4157	Allele 16	NT	NT	NT
<i>P. pseudopolonica</i>	9	CBS 142610	Allele 17	NT	NT	NT
<i>P. rhizophorae</i>	9	CCIBt 4152	Allele 4	NT	NT	NT
<i>P. aquimorbida</i>	9a	ATCC MYA-4578	Allele 16	+	+	–
<i>P. chrysanthemi</i>	9a	CBS 123163	Allele 4	+	NT	NT
<i>P. hydrogenae</i>	9a	ATCC MYA-4919	Allele 4	+	NT	NT
<i>P. hydropathica</i>	9a	ATCC MYA-4460	Allele 15	+	NT	NT
<i>P. irrigata</i>	9a	ATCC MYA-4457	Allele 26	+	+	–
<i>P. macilentosa</i>	9a	ATCC MYA-4945	Allele 24	+	NT	NT
<i>P. parsiana</i>	9a	C25	Allele 15	+	+	–
<i>P. sp. cuyabensis</i>	9a	WPC P8213	Allele 4	+	NT	NT
<i>P. sp. lagoariana</i>	9a	WPC P8217	Allele 4	+	NT	NT
<i>P. virginiana</i>	9a	ATCC MYA-4927	Allele 4	+	NT	NT
<i>P. insolita</i>	9b	WPC P6195	Allele 13	+	+	–
<i>P. polonica</i>	9b	CBS 119650	Allele 17	+	+	–
<i>P. prodigiosa</i>	9b	CBS 135138	Allele 13	+	+	–
<i>P. captiosa</i>	10a	NZFS 310C	Allele 11	NT	NT	NT
<i>P. constricta</i>	10a	CBS 125801	Allele 11	+	NT	NT
<i>P. fallax</i>	10a	NZFS310L	Allele 11	+	NT	NT
<i>P. macrochlamydospora</i>	10a	WPC P10263	Allele 14	+	NT	NT
<i>P. quininea</i>	10a	CBS 407.48	Allele 8	+	NT	NT
<i>P. richardiae</i>	10a	WPC P7789	Allele 14	+	+	–

(Continued on next page)

Table 1. (Continued from previous page)

Species	Clade ^a	Isolate ^b	Allele number of QProbe region ^c	Detection of genus by LAMP ^d	Detection of genus by LAMP with QProbe ^d	Detection of <i>P. nicotianae</i> by LAMP with QProbe ^d
<i>P. boehmeriae</i>	10b	WPC P6950	Allele 4	+	NT	NT
<i>P. gallica</i>	10b	WPC P16826	Allele 4	+	NT	NT
<i>P. gondwanensis</i>	10b	CBS 139336	Allele 4	NT	NT	NT
<i>P. intercalaris</i>	10b	CBS 140632	Allele 4	+	NT	NT
<i>P. kernoviae</i>	10b	P1571	Allele 4	+	+	–
<i>P. morindae</i>	10b	CBS 121982	Allele 4	+	NT	NT
<i>P. lilii</i>	New clade	CBS 135746	Allele 1	+	+	–
<i>P. stricta</i>	New clade	ATCC MYA-4944	Allele 1	+	NT	NT
<i>Pythium</i>						
<i>Py. adhaerens</i>	A	CBS 520.74		–*	–	–
<i>Py. myriotylum</i>	B1	NBRC 100113		–*	–	–
<i>Py. pyrilobum</i>	B1/B2	NBRC 107365		–*	–	–
<i>Py. dissotocum</i>	B2	MAFF 305576		–*	–	–
<i>Py. acanthicum</i>	D	MAFF 241099		–*	–	–
<i>Py. hypogynum</i>	E1	CBS 234.94		–*	–	–
<i>Py. middletonii</i>	E2	CBS 528.74		–*	–	–
<i>Py. irregulare</i>	F	CBS 263.30		–*	–	–
<i>Py. paddicum</i>	G	MAFF 241108		–*	–	–
<i>Py. anandrum</i>	H	CBS 285.31		–*	–	–
<i>Py. ultimum</i> var. <i>ultimum</i>	I	NBRC 100122		–*	–	–
<i>Py. nunn</i>	J	CBS 808.96		–*	–	–
<i>Phytophthora</i>						
<i>Pp. aichiense</i>	–	CBS 137195		–	NT	NT
<i>Pp. boreale</i>	–	CBS 551.88		–	NT	NT
<i>Pp. carbonicum</i>	–	CBS 112544		–	NT	NT
<i>Pp. chamaeophyphom</i>	–	CBS 259.30		–	NT	NT
<i>Pp. citrinum</i>	–	CBS 119171		–	NT	NT
<i>Pp. cucurbitacearum</i>	–	CBS 748.96		–	NT	NT
<i>Pp. delawarens</i>	–	382B		+	–	–
<i>Pp. fagopyri</i>	–	CBS 137105		+	+	–
<i>Pp. helicoides</i>	–	NBRC 100107		–*	–	–
<i>Pp. iriomotense</i>	–	CBS 137104		–	NT	NT
<i>Pp. litorale</i>	–	T1a2-17		–	NT	NT
<i>Pp. megacrumpum</i>	–	CBS 112351		–	NT	NT
<i>Pp. mercuriale</i>	–	CBS 122443		–	NT	NT
<i>Pp. montanum</i>	–	CBS 111349		–	NT	NT
<i>Pp. oedocheilum</i>	–	CBS 292.37		–	NT	NT
<i>Pp. ostracodes</i>	–	CBS 768.73		–	NT	NT
<i>Pp. vexans</i>	–	2D111		–	–	–
Other soil-borne pathogens						
<i>Aphanomyces cochlioides</i>	–	GFHT6		–	–	–
<i>Fusarium oxysporum</i> f. sp. <i>fragariae</i>	–	MAFF 727510		–	–	–
<i>Plasmodiophora brassicae</i>	–	HY		–	–	–
<i>Rhizoctonia solani</i>	–	S02		–	–	–
<i>Saprolegnia parasitica</i>	–	NBRC 32708		–	–	–
<i>Sclerotinia sclerotiorum</i>	–	AiTog		–	–	–
<i>Verticillium albo-atrum</i>	–	Vaal 130308		–	–	–

varieties, and hybrids. The potential number of *Phytophthora* species is estimated to be between 200 and 600 (Brasier 2009). Accordingly, we have updated the genus-specific detection method and confirmed its specificity using a wide range of taxa including some newly discovered *Phytophthora* species.

The advantages of loop-mediated isothermal amplification (LAMP)-based detection methods include rapidity (detection in about 30 min), high tolerance to inhibitors from biological sources (Kaneko et al. 2007), and broader applicability from the laboratory to the field, as demonstrated by numerous studies (Mori and Notomi 2009; Mori et al. 2001; Nagamine et al. 2002; Notomi et al. 2000). The use of a quenching probe (QProbe)

(Kurata et al. 2001) allows for the simultaneous, quantitative detection of a specific mutation among sequences amplified in the LAMP assay. A QProbe is a single-stranded oligonucleotide that has a cytosine labeled with a fluorophore. When the QProbe hybridizes with its target sequence, the fluorescence is quenched by electron transfer between the fluorophore and a guanine residue in the target. Even a single nucleotide mutation in the target sequence of a LAMP product can be detected using a QProbe (Ayukawa et al. 2017).

In this study, we developed a LAMP-based simultaneous detection method for the genus *Phytophthora* and the species *P. nicotianae*, by using a genus-specific LAMP primer and a species-specific QProbe.

Materials and Methods

Isolates and mycelial DNA extraction. The isolates used in this study are listed in Table 1. They were grown on V8 agar plates at 25°C. Mycelial samples were collected by scraping with an inoculating needle, and the DNA was extracted in the PrepMan Ultra Sample Preparation Reagent (Thermo Fisher Scientific, Waltham, MA) using the manufacturer's protocol. Total DNA concentration was measured using the QuantiFluor dsDNA System (Promega, Tokyo, Japan) with a Qubit2.0 Fluorometer (Invitrogen, Tokyo, Japan). The concentration was adjusted to 100 pg/μl with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and the samples were stored at 4°C until used.

LAMP primer and QProbe design. The conserved regions within the rDNA-ITS regions of *Phytophthora* species were identified using a multiple alignment analysis (Supplementary Figure S1), and LAMP primers were designed using Primer Explorer V5 software (<https://primerexplorer.jp>). The LAMP primer set included the F3, B3, FIP (F1c to F2), and BIP (B1c to B2) primers without F- and B-loop primers. For simultaneous detection, a *P. nicotianae*-specific QProbe was designed using a QProbe design tool in Microsoft Excel (provided by Nippon Steel Eco-Tech Corp., Tsukuba, Japan). The sequences of the LAMP primers and QProbe are based on the rDNA-

ITS sequence (NCBI: HQ643363; <https://www.ncbi.nlm.nih.gov/nucleotide/>) of *P. nicotianae* isolate CBS 305.29 (from the CBS-KNAW collection, Westerdijk Fungal Biodiversity Institute). The NCBI accession numbers of other rDNA-ITS sequences used in the multiple alignments are shown in Supplementary Table S1.

DNA sample preparation from inoculated and naturally infected plants. To inoculate plants, *P. nicotianae* and *P. capsici* isolates were grown on V8 agar for 4 days, and 7 mm diameter mycelial disks were taken from actively growing colonies. Tomato and eggplant fruits were placed on wet paper towels in plastic trays and inoculated with the mycelial disks. The trays were covered with polyethylene bags to maintain high humidity and incubated in a growth chamber (12-h light/dark cycle) at 25°C for 3 days. After incubation, 5-mm squares of infected tissue were cut into small pieces with razor blades and collected in 1.5-ml Eppendorf tubes, then subjected to DNA extraction. Lesions of naturally infected periwinkle and tobacco stems were shaved off the stems with a knife, and two samples (5 mm lengths) from each plant were cut into small pieces and collected in 1.5-ml Eppendorf tubes, then subjected to DNA extraction.

DNA extraction was performed using the Kaneka Easy DNA Extraction Kit version 2, following the manufacture's protocol. All

Table 2. Sequences of the *Phytophthora* genus-specific LAMP primer set and the *Phytophthora nicotianae*-specific QProbe

	Name	Sequence (5'-3')	Length (bp)
<i>Phytophthora</i> genus-specific LAMP primer set	Physp F3	TCTGCTTTTAACTAGATAGC	20
	B3	CACCACTTTTCGAGCAAAGA	20
	FIP	GCATACTTCCAGGACTAACCCTAATGCGAATTGCAGGA	38
	BIP	CCTGTATCAGTGTCCTGATCTCTCCACCGACTACACG	40
<i>P. nicotianae</i> -specific QProbe	PnicQP QProbe-3G	CCGTACATTAACTTGACTTTCTTCC	26

Table 3. Allele numbers of the QProbe regions with their sequences and experimentally identified negative peak temperatures

Allele number ^a	Number of species (Total: 175)	Sequence of QProbe region ^b	Representative species	Isolate ^c	Negative peak temperature (°C) (Average ± SE, n = 3)	Delta temperature (°C) (Allele – Original)
Original	1	CCGTACATTAACTTGACTTTCTTCC	<i>Phytophthora nicotianae</i>	CBS 305.29	53.06 ± 0.03	–
Allele 1	43	CCGTACATCAAACCTTGgCTTTCCTTCC	<i>P. cactorum</i>	MAFF 731066	42.07 ± 0.03	–11.00
Allele 2	30	CCGTACATCAAACCTTGgtTTTCTTCC	<i>P. humicola</i>	CBS 200.81	<40	<–13
Allele 3	27	CCGTACATCAAACCTTGgCTcCTTCC	<i>P. cajani</i>	WPC P3105	<40	<–13
Allele 4	12	CCGTACATCAAACCTTGgCTTcCTTCC	<i>P. kernoviae</i>	P1571	<40	<–13
Allele 5	10	CCGTACAaCAAACCTTGgCTTTCCTTCC	<i>P. infestans</i>	MAFF 236324	42.03 ± 0.02	–11.03
Allele 6	8	CCGTACAaTAAACCTTGgCTccCTTCC	<i>P. brassicae</i>	CBS 179.87	<40	<–13
Allele 7	6	CCGTACAaTAAACCTTGgCTccCTTCC	<i>P. cryptogea</i>	CBS 113.19	<40	<–13
Allele 8	5	CCGTACATCAAACCTTGgCTccCTTCC	<i>P. syringae</i>	FIUm1	<40	<–13
Allele 9	4	CCGTACATCAAACCTTGcCTccCTTCC	<i>P. ramorum</i>	CBS 101553	<40	<–13
Allele 10	4	CCGTACATCAAACCTTGgtTTTCTTCC	<i>P. palmivora</i>	CH88-1	<40	<–13
Allele 11	4	CCGTATaTCAAACCTTGcCTTcCTTCC	<i>P. fallax</i>	NZFS310L	<40	<–13
Allele 12	2	CCGTACATCAAACCTTGACTTTCTTCC	<i>P. clandestina</i>	WPC P3943	48.84 ± 0.18	–4.22
Allele 13	2	CCGTACATgAAACCTTGgCTTcCTTCC	<i>P. prodigiosa</i>	CBS 135138	<40	<–13
Allele 14	2	CCGTACATgAAACCTTGgCTccCTTCC	<i>P. richardiae</i>	WPC P7789	<40	<–13
Allele 15	2	CCGTACATcAAcCTTGgCTccCTTCC	<i>P. parsiana</i>	C25	<40	<–13
Allele 16	2	CCGTACATcAAcCTTGgCTTcCTTCC	<i>P. aquimorbida</i>	ATCC MYA-4578	<40	<–13
Allele 17	2	CCGTACAaCAAACCTTGgCTTcCTTCC	<i>P. polonica</i>	CBS 119650	<40	<–13
Allele 18	1	CCGTACATCAAACCTTGACTTTCTTtC	<i>P. tentaculata</i>	CBS 552.96	40.94 ± 0.0003	–12.13
Allele 19	1	CCGTACATCAAACCTTGgCTTTCCTTCC	<i>P. asparagi</i>	CBS 132095	<40	<–13
Allele 20	1	CCGTACATcAAAaTTGgCTTTCCTTCC	<i>P. frigida</i>	CBS 121941	<40	<–13
Allele 21	1	CCGTACAaTAAACCTTGgCTTTCCTTCC	<i>P. ipomoeae</i>	WPC P10225	42.15 ± 0.08	–10.91
Allele 22	1	CCGTACAaTAAACCTTGcCTccCTTCC	<i>P. cichorii</i>	CBS 115029	<40	<–13
Allele 23	1	CCGTACATcAAcCTTGgCTTTCCTTCC	<i>P. tubulina</i>	CBS 141212	<40	<–13
Allele 24	1	CCGTACATgAAcCTTGtCTTcCTTCC	<i>P. macilentosa</i>	ATCC MYA-4945	<40	<–13
Allele 25	1	CCGTACgTAAACCTTGgCTccCTTCC	<i>P. drechsleri</i>	CBS 292.35	<40	<–13
Allele 26	1	CCGTACATgAAcCTTGtCTTcCTTCC	<i>P. irrigata</i>	ATCC MYA-4457	<40	<–13

^a The sequences of the QProbe regions are classified into alleles (original plus numbers 1 to 26) based on the rDNA-ITS sequences with NCBI accession numbers listed in Supplementary Table S1.

^b Small characters and underlines indicate mismatches with the QProbe sequence.

^c ATCC, American Type Culture Collection; CBS, Westerdijk Fungal Biodiversity Institute; MAFF, Japanese Ministry of Agriculture Forestry and Fisheries; NZFS, New Zealand Forest Service; WPC, World Phytophthora Genetic Resource Collection.

extracts were diluted 20 times with TE buffer, then stored at 4°C until used as templates.

LAMP assay using the GenieII system, with or without the QProbe. The LAMP reaction was conducted in a total volume of 25 µl containing 1× Isothermal Master Mix (fluorescent dye, Optigene, Horsham, U.K.), 1.6 µM each of FIP and BIP primer, 0.2 µM each of F3 and B3 primer, and 1 µl of DNA template. In all assays except the assay used to determine sensitivity and the assays of plant extracts, the mycelial DNA concentration was adjusted to 100 pg/µl. A reaction mix containing 1 µl sterile distilled water instead of DNA was used as a negative control. The LAMP assay was performed using the portable real-time fluorometer GenieII (Optigene) with the following conditions: preheat 5 min at 68°C; amplification 60 min at 68°C; and annealing curve analysis at 98°C to 80°C, ramping at 0.05°C per sec. The fluorescence curve was created using Microsoft Excel from raw fluorescence data obtained using the Genie Explorer version 2.0.6.3 software (Optigene). The results were based on the amplification curve for detecting positive or negative amplification, and the annealing curve for checking specific amplification with a single peak.

For LAMP assays with the QProbe, the reaction mixture described above also contained 1 µM of the specific QProbe for *P. nicotianae*. The LAMP assay was performed as described above except that the annealing curve analysis was extended from 98°C to 40°C with ramping at 0.05°C per sec.

The specificity of the LAMP assay, with or without the QProbe, was assessed at least two times with each DNA sample. The sensitivity of the LAMP assay with or without the QProbe, and the average peak temperature of the QProbe, were determined in at least three experiments.

LAMP assay using the StepOnePlus system, with or without the QProbe. The LAMP reaction, with or without the QProbe, was also performed with the ABI StepOnePlus Real-Time PCR System. The reaction mixture was the same as that used with the GenieII system, except the concentration of the QProbe was 0.5 µM. First, short prerun (70°C, 1 min) was carried out with empty plate to reduce the heating time for the main run. The main reaction was conducted with the following conditions: 68°C for 60 min (1 min × 60 cycles), followed by an annealing step with 98°C for 10 s, then 80°C for 30 s (for QProbe: 45°C for 30 s), then a melting step with gradual

temperature increments of 0.3°C/s to 98°C with “step and hold” detection, and finally 98°C for 10 s. The fluorescence derivative data were collected during the melting phase. The assay was performed at least twice without the QProbe and at least twice with the QProbe.

Results

Design of genus-specific LAMP primers and *P. nicotianae*-specific QProbe. For the genus-specific detection of *Phytophthora*, we designed a LAMP primer set based on multiple alignments of the rDNA-ITS sequences from 27 species of *Phytophthora* (clades 1 to 10) (Abad et al. 2019), 11 species of *Pythium* (clades A to J) (Robideau et al. 2011), two species of *Phytophythium*, and one species of *Halophytophthora* (Supplementary Figure S1). The *Pythium*, *Phytophythium*, and *Halophytophthora* genera are closely related to *Phytophthora* (Robideau et al. 2011), and we included species from those genera in order to design primers that would be specific for *Phytophthora*. We also designed a *P. nicotianae*-specific quenching probe (QProbe) using the same multiple alignment (Supplementary Figure S1). The sequences of the primers and probe were based on the rDNA-ITS sequence (NCBI accession no. HQ643363) of *P. nicotianae* CBS 305.29 (Supplementary Figure S1 and Table 2).

Specificity of the LAMP assays, with and without the QProbe. Experiments to test the specificity and sensitivity of the LAMP primers and QProbe were performed using the GenieII system, which is a compact, lightweight instrument for performing real-time isothermal reactions with fluorescence measurement. This portable instrument has the advantage that it can be used in the field as well as the laboratory.

The specificity of the genus-specific LAMP primer set was assessed using mycelial DNA obtained from *Phytophthora* and closely related genera: 161 taxa (including subspecies, varieties, and hybrids) of *Phytophthora*, 12 species (12 isolates) of *Pythium*, 17 species (17 isolates) of *Phytophythium*, and one isolate each of the following soil-borne pathogens: *Aphanomyces cochlioides*, *Fusarium oxysporum* f. sp. *fragariae*, *Plasmodiophora brassicae*, *Rhizoctonia solani*, *Saprolegnia parasitica*, *Sclerotinia sclerotiorum*, and *Verticillium albo-atrum* (Table 1). Among the tested *Phytophthora* species, 159 taxa were detected within 60 min, and most of these were detected within 30 min, in duplicate assays. Two species, *P. macrochlamydospora* and *P. insolita*, were detected within

Fig. 1. Specificity of the *Phytophthora* genus-specific LAMP assay with the *Phytophthora nicotianae*-specific QProbe. Mycelial DNA samples were extracted from representative *Phytophthora* species in clade 1. After amplification at 68°C for 60 min, fluorescence derivative data during the annealing phase (98 to 40°C) were obtained.

60 min at least once in triplicate assays. No species were not detected at all (Table 1, first data column). Among the other genera, only two *Phytophthora* species, *Pp. delawarens* and *Pp. fagopyri*, were detected within 60 min at least once in triplicate assays (Table 1, first data column).

Next, we tested the specificity of *P. nicotianae*-QProbe by using fluorescence derivative data at the annealing phase of the detection assay for *P. nicotianae*. We determined that the optimum concentration of the QProbe was 1 μ M, since this concentration showed a clear peak and was cost-effective (Supplementary Figure S2). The

Fig. 2. Sensitivity of the *Phytophthora* genus-specific LAMP assay. Mycelial DNA from the *Phytophthora* species *P. nicotianae* CBS 305.29 (**A** and **D**), *P. infestans* MAFF 236324 (**B** and **E**), and *P. ramorum* Pr-1 (**C** and **F**) were serially diluted and subjected to LAMP assays. **A–C**, Amplification curves. **D–F**, After amplification at 68°C for 60 min, fluorescence derivative data during the annealing phase (98 to 80°C) were obtained.

amplified target of the *Phytophthora* genus-specific primers was detected as a positive fluorescent peak at around 85°C, and the correct target of the *P. nicotianae*-specific QProbe was detected as a negative fluorescent peak at 53.06 ± 0.03°C (average ± SE, peak temperature of the original allele in Table 3). The stability of this negative peak temperature was confirmed using the 21 *P. nicotianae* isolates listed in Table 1 (data not shown). We found 27 variations of the QProbe region among 175 *Phytophthora* taxa, and categorized them as “original” (i.e., the sequence for *P. nicotianae* CBS 305.29) and alleles 1 to 26 (see Tables 1 and 3). A representative species with each allele was then subjected to a genus-specific LAMP assay with the *P. nicotianae*-specific QProbe. We calculated the differences between the negative peak temperature of the correct target and other alleles to obtain delta temperature values (Table 3). The species with allele 12 (*Phytophthora clandestina*) had the smallest delta temperature value (−4.22°C). All other alleles had negative peak temperatures that differed from that of the original allele by more than 10.9°C (Table 3). We then assayed 13 species from *Phytophthora* clade 1, including *P. nicotianae* and the two species carrying allele 12 (*P. clandestina* and *P. iranica*). As shown in Figure 1, the QProbe was able to distinguish *P. nicotianae* from all other species, including the two carrying allele 12. This result was confirmed in three experiments, with representative data shown in Figure 1.

To confirm the specificity of the QProbe, we assayed 29 species of *Phytophthora* (clades 1 to 10), 12 species of *Pythium* (clades A to J), one species of *Phytophythium*, and one isolate each of the seven other soil-borne pathogens. We used the genus-specific LAMP primers and the *P. nicotianae*-specific QProbe and tracked the fluorescence derivative data during the annealing phase. All the tested *Phytophthora* isolates were detected as positive peaks at around 85°C

Fig. 3. Sensitivity of the *Phytophthora* genus-specific LAMP assay with the *Phytophthora nicotianae*-specific QProbe. Mycelial DNA from *P. nicotianae* CBS 305.29 was serially diluted and used for the assay. After amplification at 68°C for 60 min, fluorescence derivative data during annealing phase (98 to 40°C) were obtained.

(Table 1, middle data column), while all 21 isolates of *P. nicotianae*, and no other isolates, were detected by the QProbe as negative peaks at around 53°C (Table 1, third data column).

Sensitivity of LAMP assay with and without the QProbe. To test the detection limit of the *Phytophthora* genus-specific LAMP assay, we used serially diluted DNA samples (ranging from 1 fg to 100 pg) from three representative species: *P. nicotianae* CBS 305.29 (clade 1), *P. infestans* MAFF 236324 (clade 1c), and *P. ramorum* Pr-1 (clade 8c). The detection limits for all three species were 100 fg, and these limits were the same in each of at least five experiments per species; representative data are shown in Figure 2. Next, we tested the detection limit for *P. nicotianae* in LAMP assays with the QProbe. Serially diluted DNA samples of *P. nicotianae* were assayed, and the detection limit was found to be 100 fg in 10 replicate experiments, with representative data shown in Figure 3.

Detection of *Phytophthora* spp. and *P. nicotianae* in inoculated and naturally infected plants. We tested the genus-specific LAMP assay with the *P. nicotianae*-specific QProbe in inoculated plants (tomato and eggplant) and naturally infected plants (periwinkle and tobacco). For these experiments, we extracted the DNA using the Kaneka Easy DNA Extraction Kit version 2, which we had previously determined was suitable for LAMP detection (Hieno et al. 2019). The LAMP assays, performed using the GenieII system with the *Phytophthora* genus-specific primers, amplified the target DNA in all samples within 30 min (Table 4, first data column). There was no amplification by LAMP assay with DNA extracted from healthy plant tissues (data not shown). When the LAMP assay was performed with the QProbe, all samples showed positive fluorescent peaks at around 85°C (Table 4, middle data column). Samples from plants infected with *P. nicotianae* also showed negative fluorescent peaks at around 53°C (Table 4, last data column). We also confirmed that there was no amplification from healthy plants in LAMP assays with the QProbe (data not shown). These results were confirmed in at least three experiments, and representative data are shown in Figure 4.

Application of the LAMP assay, with and without the QProbe, using an alternative real-time fluorometer. The GenieII system is versatile and portable, but it has the disadvantage that it can process no more than 16 samples at one time. Therefore, we applied the LAMP assay, with or without the QProbe to the ABI StepOnePlus Real-Time PCR System, which has the capacity for 96 samples. For this system we substituted a melting curve analysis for the annealing curve analysis (see Materials and Methods). First, we tested the optimal concentration of the QProbe and found it to be 0.5 µM, which is half of the optimal concentration of the GenieII system (Supplementary Figure S3). When a higher concentration, free QProbe was detected as non-negligible noise, although it did not interfere with the LAMP assay. Next, we checked the specificity of the assay using mycelial DNA from representative *Phytophthora*, *Pythium*, and *Phytophythium* species (* in Table 1) and DNA from infected plants (* in Table 4). There were no differences in the results between the GenieII and StepOnePlus systems. However, in the

Table 4. Results of assays of inoculated and naturally infected plants using the LAMP assay with or without the QProbe

Sample	Host	Tissue	<i>Phytophthora</i> species	Isolate	Location	Detection of genus by LAMP ^a	Detection of genus by LAMP with QProbe ^a	Detection of <i>P. nicotianae</i> by LAMP with QProbe ^a
Inoculated plants	Tomato	Fruit	<i>P. capsici</i>	CH01CUCU10	—	+	+	—
			<i>P. nicotianae</i>	GK10Eg1	—	+	+	+
	Eggplant	Fruit	<i>P. capsici</i>	CH01CUCU10	—	+	+	—
			<i>P. nicotianae</i>	GK10Eg1	—	+	+	+
Natural infected plants	Periwinkle	Stem	<i>P. nicotianae</i>	—	Gifu, Japan	+	+	+
	Tobacco	Stem	<i>P. nicotianae</i>	—	Java island, Indonesia	+	+	+

^a At least two repeats were performed. +, detected within 60 min; —, not detected within 60 min. Asterisks indicate samples that were tested and confirmed using the ABI StepOnePlus Real-Time PCR System.

StepOnePlus system, the negative peak temperature for the QProbe with *P. nicotianae* DNA was shifted slightly (+2°C) from ca. 53°C (Table 3) to ca. 55°C (Supplementary Figure S3). We also used the StepOnePlus system to perform the same experiments in Figures 2 and 3, to check the sensitivity of the LAMP assay with and without the QProbe and found no differences from the results obtained with the GenieII system (data not shown).

Discussion

We designed a *Phytophthora* genus-specific LAMP primer set based on 27 species in phylogenetic clades 1 to 10 (Abad et al. 2019), and this primer set was able to detect all 161 of the tested *Phytophthora* taxa (including subspecies, varieties, and hybrids) (Table 1). The number of species tested in this study is greater than the numbers tested in previous studies with PCR primers: 101 species (Bilodeau et al. 2014), 45 species (Scibetta et al. 2012), 35 species

(Schena et al. 2008), and RPA primers: 136 taxa (Miles et al. 2015). Based on these numbers, our LAMP primer set should be the most reliable for the specific detection of *Phytophthora* spp. However, among the closely related genera that we tested, *Pp. delawarensis* and *Pp. fagopyri* also gave positive results in our assays (Table 1), although the detection time for 100 pg of mycelial DNA from these species was 40 or more minutes (data not shown). These species are not common, and only one host has been reported for each of them (USDA Agricultural Research Service, <https://nt.ars-grin.gov/fungalatabases/fungushost/fungushost.cfm>). Therefore, we feel that our primer set will be useful for the specific detection of *Phytophthora* spp. in a quarantine setting.

There have only been a few studies on QProbes or QPrimer methods in LAMP assays. Most have been designed for the detection of clinical viruses, such as the real-time reverse transcription LAMP assay with a QProbe for the Middle East respiratory syndrome

Fig. 4. Detection from inoculated and naturally infected plants by the LAMP assay with the QProbe. Non-inoculated and inoculated fruits (**A**) and naturally infected plants (**B**) were subjected to the *Phytophthora* genus-specific LAMP assay with the *Phytophthora nicotianae*-specific QProbe. After amplification at 68°C for 60 min, fluorescence derivative data during the annealing phase (98 to 40°C) were obtained. A reaction mixture without DNA (Blank) was used as a negative control, and *P. nicotianae* mycelial DNA was used as a positive control. **A**, Tomato and eggplant fruits inoculated with *P. nicotianae* GK10Eg1 or *P. capsici* CH01CUCU10. Mycelial DNA from *P. capsici* was used as an additional negative control. **B**, Periwinkle and tobacco stems naturally infected with *P. nicotianae*.

coronavirus (Shirato et al. 2018), or the real-time reverse-transcription LAMP assay with a QPrimer for the influenza virus and the respiratory syncytial virus (Takayama et al. 2019). Among plant pathogens, a universal QProbe system was established for distinguishing races of *Fusarium oxysporum* f. sp. *lycopersici* (Ayukawa et al. 2017). This study is the second report on using a QProbe in a LAMP-based detection assay for a plant pathogenic microorganism.

P. nicotianae is a species with a broad host range and worldwide distribution, and it probably has a large amount of genetic variation (Afandi et al. 2019). Therefore, we confirmed the conservation of the *P. nicotianae*-specific QProbe sequence (Supplementary Figure S1, Table 2) using 100 rDNA-ITS sequences downloaded from the NCBI database (<https://www.ncbi.nlm.nih.gov/nucleotide/>). The LAMP assay with the *P. nicotianae*-specific QProbe was able to distinguish *P. nicotianae* from other tested *Phytophthora* taxa, which all had at least one sequence mismatch in the QProbe region (Fig. 1, Table 3). This high level of specificity was also found in the previous study on the specific detection of *F. oxysporum* f. sp. *lycopersici* races with single nucleotide variations (Ayukawa et al. 2017). It should be noted that the concentration of the QProbe affected the negative peak temperature: higher concentrations gave higher negative peak temperatures (Supplementary Figure S2).

Our method for the simultaneous detection of *Phytophthora* spp. and *P. nicotianae* is highly cost-effective when compared with separate assays using genus-specific primers (this study) and *P. nicotianae* species-specific primers (Hieno et al. 2019). The cost for reagents is about 20% lower for simultaneous detection than for separate assays, and there are significant savings in time and labor as well.

In this study, we developed the simultaneous detection method using the lightweight and portable GenieII system, and then confirmed that the method can also be used with a larger instrument, the ABI StepOnePlus Real-Time PCR System. The StepOnePlus system has the capacity for 96 samples; however, we found that nonspecific amplification occurred if we did not add a preheating step to the procedure (data not shown; see Materials and Methods).

As mentioned previously, several other molecular detection methods have been developed for the *Phytophthora* genus or for certain species within the genus. Schena et al. (2008) developed a PCR-based detection method for the genus and 15 species. Detection by PCR is a very popular approach, and it has the advantage that its products can be sequenced. On the other hand, PCR assays require long reaction times and the products are often detected using time-consuming gel electrophoresis. Miles et al. (2015) developed two multiplex RPA assays with fluorescent probes for detecting either the genus (with an internal plant control) or two species: *P. ramorum* and *P. kernoviae*. Like LAMP assays, RPA is an isothermal amplification method with a short reaction time, and the results are available immediately when using a real-time fluorometer. The use of the internal control provides the advantage of eliminating false negative results; however, the RPA-based detection method of Miles et al. (2015) requires two assays for detecting the genus and the individual *Phytophthora* species. Bilodeau et al. (2014) developed a multiplex real-time PCR method with TaqMan probes for detecting the genus, 13 species, and one species complex, and this method also includes an internal plant control. Both RPA- and TaqMan-based detection methods use fluorescent probes and are less likely to show nonspecific amplification than the LAMP method, which uses a double-stranded DNA-binding fluorescent dye. However, we have found that nonspecific amplification can be avoided if the reaction is started within 5 min after adding the template DNA. The detection method that we have developed, based on the LAMP assay with a QProbe, has the advantages of simultaneous detection in a single, rapid reaction, and immediate detection using a portable instrument. Portable real-time fluorometers for LAMP such as the GenieII are generally cheaper than real-time PCR systems.

In our previous study (Hieno et al. 2019), we designed a *P. nicotianae* species-specific LAMP primer and compared three methods for extracting DNA from diseased plants. We selected the Kaneka

Easy DNA Extraction Kit version 2 for future use because it was rapid (30 min needed for sampling and extraction) and the DNA was stable for use in LAMP assays and storable for around 2 years at 4°C. This study confirmed that the DNA extraction kit was applicable not only for LAMP assays, but also for LAMP assays with QProbes. The total detection time from sampling to results was around 120 min. Therefore, we have developed an effective, time-saving method for the simultaneous detection of all *Phytophthora* genus members and *P. nicotianae* specifically. Fichtner et al. (2012) identified symptomless *P. kernoviae* infections in North American native plants. Our detection method can be used in on-site quarantine inspections and will be effective for identifying *Phytophthora* even in asymptomatic imported plants.

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