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Detection of orchid Phytophthora disease by nested PCR

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ABSTRACT. Orchid disease caused by *Phytophthora* has long been a major threat to cultivation of orchids in Taiwan. *Phytophthora* spp. known to infect orchids include mainly *P. palmivora* and *P. parasitica*. Identification of *Phytophthora* species by the conventional method includes the use of selective media to obtain *Phytophthora* isolates and examination by microscopy. The procedures are rather labor-intensive and time-consuming. In order to accelerate and simplify the process of diagnosis, we have developed a nested PCR assay for rapid and accurate detection of *Phytophthora* pathogens infecting orchids. After isolation of DNA from the plant tissue, PCR was performed using a primer set specific for *Phytophthora*. Amplification of DNA fragments of approximately 1 kb in length indicated the presence of *Phytophthora* pathogens. To identify the species, nested PCR was then performed using amplified product from the first PCR as the template and species-specific oligonucleotides as the primers. Amplification of specific DNA fragments would tell whether the orchids were infected by *P. palmivora*, *P. parasitica*, or both. Furthermore, the sensitivity of detection was greatly enhanced. This assay provides a rapid and sensitive method for detection of *Phytophthora* pathogens in infected orchids as well as infested media used for cultivation of orchids, and thus can assist growers in early diagnosis of the devastating orchid Phytophthora disease.

Keywords: Internal transcribed spacer (ITS); Nested PCR; Orchid Phytophthora disease; *Phytophthora palmivora*; *Phytophthora parasitica*; Rapid detection.

INTRODUCTION

Due to their beauty both as cutting flowers and potted flowering plants, orchids (*Orchidaceae* L.) have become the most important floriculture crops of Taiwan in recent years. Fungal diseases known to attack orchids include anthracnose, Botrytis petal blight, and Southern blight (Leu, 1994; Huang and Lee, 1994). Besides, some species of *Phytophthora*, which belong to the Oomycete group of Stramenopiles, were found to cause severe black rot in orchids, including *P. cactorum* (Leb. and Cohn) Schröeter (Burnett, 1974), *P. erythroseptica* Pethybridge var. *erythroseptica* (Hall, 1989), *P. parasitica* Dastur *(=P. nitcotianae* Breda de Haan) (Ann, 1995), *P. palmivora* (Butler) Butler (Ann, 1995; Yehm et al., 1998), and *P. multivesiculata* Ilieva, Man in 't Veld, Veenbaas-Rijks et Pieters. sp. nov. (Ilieva et al., 1998). In Taiwan, *P. palmivora* and *P. parasitica* are known to attack a wide variety of orchids, including *Cattleya*, *Cymbidium*, *Dendrobium*, *Oncidium*, and *Phalaenopsis*, to mention only the most important ones (Ann, 1995; Yehm et al., 1998), while *P. multivesiculata* was reported only in one case, infecting *C. tracyanum* (Chern and Ann, 1996; Ilieva et al., 1998). Diagnosis of orchid Phytophthora disease is complicated by the observation that symptoms caused by *Phytophthora* are hard to distinguish with those caused by the bacterial pathogen *Erwinia carotovora* subsp. *carotovora* (Su and Leu, 1992), and even worse, these pathogens might infect orchids simultaneously. Traditionally, diagnosis of the orchid Phytophthora disease was performed by isolation of *Phytophthora* pure culture from diseased plants, followed by identification based on morphological characteristics, which might take more than one week to identify a pathogen. In the present study, a nested polymerase chain reaction (PCR) method was developed in order to simplify and speed up the procedure for disease diagnosis.

PCR is now used extensively for detection of plant pathogens due to advantages of sensitivity, speed, and high sample throughput (Martin et al., 2000). The key step for development of a PCR method is to design oligonucleotide primers with good specificity. For *P. parasitica*, primers have been designed based on a variety of sequences, including the sequence of a *P. parasitica*-specific DNA

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segment obtained from the genomic library (Érsek et al., 1994), the *parA1* gene which encodes the elicitin (Kamoun et al., 1993; Lacourt and Duncan, 1997; Kong et al., 2003), and ribosomal internal transcribed spacer (ITS) sequence (Ippolito et al., 2002); the latter is a good candidate for designing PCR primers due to its high copy number, which was estimated to be 820 copies per diploid nucleus in *P. infestans* (Judelson and Randall, 1998). Indeed, sequences of high copy number have been the choice of many studies in order to enhance the sensitivity of detection by PCR (Judelson and Tooley, 2000; Jyan et al., 2002; Martin et al., 2004). Besides, sensitivity may be improved by the use of nested PCR. In this method, two primer pairs were designed based on the sequence of a DNA segment, with one pair nested within the other. PCR was first run with the outer primers. Afterwards, a second PCR was performed using the inner pair as the primer and the amplification product from the first PCR as the template. Thus, in addition to sensitivity, specificity of detection may be improved by nested PCR (Martin et al., 2000). In this paper, a nested PCR method for rapid detection of *Phytophthora* pathogens was developed in order to assist early diagnosis of the orchid *Phytophthora* disease.

MATERIALS AND METHODS

Fungal cultures and growth conditions

Isolates of *Phytophthora* spp. and other fungi used in the study were listed in Table 1. Isolates of *Phytophthora* spp. and *Peronophthora litchii* were provided by the third author (Dr. P. J. Ann), *Pythium myriotylum* and *Py. ultimum* by Dr. P. H. Wang (Dept. Life Science, Tunghai University, Taichung, Taiwan), *Fusarium* spp. and *Rhizoctonia solani* by Dr. S. S. Tzean (Dept. Plant Pathology and Microbiology, National Taiwan University). To prepare mycelia for isolation of DNA, isolates were grown on liquid media, harvested by filtration, and frozen at -80°C until use. *Phytophthora infestans* was grown on Rye B medium at 20°C for 20 days (Caten and Jinks, 1968), other *Phytophthora* spp. on 5% V8 agar (5% Campbell's V8 juice, 0.02% $CaCO_3$, and 2% Bacto agar) at 25°C for 10 days, *Pythium* spp. on 10% V8 agar (10% Campbell's V8 juice, 0.02% $CaCO_3$, and 2% Bacto agar) at 30°C for 5 days, and other fungi on potato dextrose broth (Difco Laboratories, Detroit, Michigan) at 25°C for 8 days.

Isolation of DNA

DNA was isolated by using the DNeasy Plant Mini Kit (Qiagen®, Basel, Switzerland). The concentration of DNA was determined by spectrophotometry, using GeneQuant II (Amersham Biosciences, Uppsala, Sweden).

Design of oligonucleotide primers for PCR

To design oligonucleotide primers for PCR, sequences of 28S rRNA and ITS1-5.8S rRNA-ITS2 from a variety of *Phytophthora* spp. were collected from the NCBI website (http://www.ncbi.nlm.nih.gov/) and analyzed by multiple sequence alignment using Clustal X (Thompson et al., 1994). In addition, to confirm the accuracy of sequences, ITS1-5.8S rRNA-ITS2 sequences of representative *Phytophthora* spp. analyzed in this study were cloned by PCR using primers ITS1 and ITS4 (White et al., 1990).

Table 1. *Phytophthora* spp. and other isolates analyzed in this study.

Species	Isolate	Location	Host	Ribosomal ITS accession number
P. botryosa	ATCC26479			AY251664
	ATCC52221			AY251665
P. cactorum				AY251663
P. capsici	21170	Yunlin	*Capsicum annuum* (sweet pepper)	AY251662
P. cinnamomi	PC97	Chiayi		AY251661
	94006	Wufeng, Taichung	*Persea Americana* (Avocado)	AY251660
P. citricola	9023	Linluo, Pingtung	*Syzygium samarangese* (wax apple)	
	9024	Linluo, Pingtung	*Syzygium samarangese* (wax apple)	
	9025	Sinyuan, Pingtung	*Syzygium samarangese* (wax apple)	
	9026	Sinyuan, Pingtung	*Syzygium samarangese* (wax apple)	
P. citrophthora	95004	Ilan	*Fortunella* sp. (kumquant)	AY251659
			Annona squamosa (custardapple)	
	98165	Ilan	*Fortunella* sp. (kumquant)	AY251658
	98167	Ilan	*Citrus tankan* (tankan)	
	95004	Ilan	Calamondin	
	97083	Taiping, Taichung	*Averrhoa carambola* (star fruit)	

Table 1. (Continued)

Species	Isolate	Location	Host	Ribosomal ITS accession number
P. colocasiae	9177	Minsyong, Chiayi	*Colocasie esculenta* (taro)	AY251657
	97066	Shueili, Nantow	*Colocasie esculenta* (taro)	AY251656
	20216	Kinmen	*Colocasie esculenta* (taro)	AY251655
	98115	Houli, Taichung	*Colocasie esculenta* (taro)	
P. cryptogea	90130		*Euphobia pulcherrima* (poinsettia)	AY251653
	98067	Dounan, Yunlin	*Solanum tuberosum* (potato)	
	98176		*Lycopersicum esculentum* (tomato)	
	94011	Yongjing, Changhua	*Gerbera jamesonii* (transvaal daisy)	AY251654
P. drechsleri (*P. melonis*)	98141	Pusin, Changhua	*Cucumis sativus* (cucumber)	AY251650
	96032		*Benincasa hispida* (wax gourd)	AY251651
	98107	Tainan	*Momordica charantia* (bitter gourd)	
	CH-1		*Momordica charantia* (bitter gourd)	
P. infestans	98029	Dounan, Yunlin	*Solanum tuberosum* (potato)	
	20040	Rueiyen, Hawlian	*Lycopersicum esculentum* (tomato)	
	99017	Houli, Taichung	*Solanum tuberosum* (potato)	
	20060	Houli, Taichung	*Solanum tuberosum* (potato)	
P. meadii	NTU-01	Taipei	*Zantedeschia aethiopica* (white arum lily)	AY251649
P. palmivora	93105	Dacheng, Changhua	*Cattleya* sp. (orchid)	AY251647
	9253	Taitung	*Phalaenopsis aphrodite*	AY251648
	9257	Taitung	*Cattleya* sp. (orchid)	
	PpaA1-5	Madou, Tainan	*Persea Americana* (Avocado)	
	9150		*Hedera japonica* (English ivy)	
	8829	Nansi, Tainan	*Citrus sinensis* Osb. (sweet orange)	
	9097	Yuli, Hualien	*Carica papaya* (papaya)	
P. parasitica	92171	Chiayi	*Peperomia* sp.	
	92033	Taitung	*Saintpulia ionantha* (African violet)	
	991-3	United States	*Citrus* sp.	
	92145	Jhongpu, Chiayi	*Sinningia speciosa* (glozimia)	
	92143	Jhongpu, Chiayi	*Sinningia speciosa* (glozimia)	
	98161	Wufeng, Taichung	*Adenium obesum* (Desert rose)	
	98151		*Pandanus odorus*	
Peronophythora litchii	90113	Minsyong, Chiayi	*Litchi chinensis* (litchi)	AY251666
Pythium aphanidermatum				
Py. myriotylum				
Py. splendens	Pys10	Puli, Nanto		
Py. sylvaticum	CCRC33460			
Py. ultimum				
Fusarium moniliforme	CCRC 31492			
F. oxysporum f. sp. *melonis*	CCRC 32121	France	*Cucumis melo* (muskmelon)	
F. oxysporum f. sp. *momordicae*	CCRC 35046	Dahu, Miaoli	*Momordica charantia* (bitter gourd)	
F. roseum Link	CCRC 35115	Wufeng, Taichung	*Musa sapientum* (banana)	
F. solani (Martius) Saccardo	CCRC 32448	New Zealand		
F. verticillioides Saccardo	CCRC 35113	Wufeng, Taichung	*Sorghum bicolor* (sorghum)	
Rhizoctonia solani	AG-3 021122			

Nucleotide sequences of the recombinant clones were determined on both strands of DNA, using the BigDye terminator cycle sequencing ready reaction kit and an ABI Prism 310 Genetic Analyzer apparatus (Applied Biosystems, Foster City, CA, USA), followed by analyses using programs in the GCG software package (Genetics Computer Group, Wisconsin Package Version 10.0). Genus-specific primers were designed based on the highly conserved regions of ITS1 and 28S rRNA, while species-specific primers based on the ITS1 and ITS2 sequences which are highly diverged among species (Table 2). Besides, a primer set was designed based on the conserved sequences of plant 18S ribosomal DNA to serve as a positive control for PCR while using DNA prepared from plants as the template (Table 2).

Test of primer specificity by PCR

PCR was performed in a 20-µl reaction, which contained 10 ng of template DNA, 1.25 µM of oligonucleotide primers, 0.2 mM dNTP, 1X PCR buffer, and 1 U of DyNazyme™ II DNA polymerase (Finnzymes, Espoo, Finland). Amplification was initiated by denaturation at 94°C for 5 min, followed by 25 cycles of [95°C/30 sec-58°C/30 sec-72°C/1 min] and a 10-min extension at 72°C in a thermocycler (GeneAmp PCR System 2400, Perkin elmer, Foster City, CA, USA). PCR amplified products were analyzed by 1.5% agarose gel electrophoresis in 1× TAE.

Nested PCR

To carry out the nested PCR, the first PCR was performed using Phy1s/Phy2a as the primer pair. After completion of the amplification reaction, the PCR mixture was diluted 100× with sterilized ddH_2O, followed by the initiation of a second PCR using 3 µl of the diluted mixture as the template and species-specific oligonucleotides as the primers. The second PCR was performed according to procedures described in the previous section except that, instead of 58°C, the annealing temperature was set at 67°C. PCR amplified products were analyzed by 1.5% agarose gel electrophoresis in 1× TAE.

Detection of orchid *Phytophthora* disease by nested PCR

Diseased *Oncidium* sp. (Ramsey) was collected from Ping Tong, Taiwan. Crude extract was prepared from *Oncidium* tissues accroding to the method developed by Wang et al. (1993) with some modifications. A small piece of the *Oncidium* tissue (approximately 0.2 g) was immersed in 500 µl of 0.5 N NaOH and macerated with a homogenizer. Following centrifuation at 17,000 *g* for 5 min, the supernatant was collected and mixed thoroughly with 9 volumes of 0.1 M Tris (pH 8.0). Aliquots of the mixture were then used as the template for PCR as described in the previous section.

Isolation of *Phytophthora* spp. from the culture media of orchids

To find out whether the culture media were contaminated, *Phytophthora* spp. were trapped according to procedures described by Grimm and Alexander (1973) with some modifications. Culture media collected from a orchid garden located on the Ping Tong area of Taiwan were soaked in water for 3-4 days at room temperature, with the addition of six leaf pieces (1×1 cm^2) excised from the orchid. Afterwards, the baits were collected and used for extraction of DNA and nested PCR as described in the previous section.

RESULTS

Primer design and specificity

To design oligonucleotide primers for PCR, sequences of 28S rRNA and ITS1-5.8S rRNA-ITS2 from a variety of *Phytophthora* spp. were collected from the NCBI website and analyzed by multiple sequence alignment using Clustal X. Besides, to ensure that *Phytophthora* isolates analyzed in this study contained the same ribosomal ITS sequences as those obtained from the NCBI website, sequences encompassing the ITS1-5.8S rRNA-ITS2 regions of representative isolates of *Phytophthora* spp. and *Pe. litchii* were cloned by PCR and analyzed. The resultant data were deposited in the GenBank

Table 2. PCR primers used in this study.

Target	Name	Primer sequence (5′ to 3′)	Location in rDNA	Expected size of PCR product (bp)
Phytophthora spp.	Phy1s	ACT TTC CAC GTG AAC CGT ATC A	ITS1	~1000
	Phy2a	GCA CGA GCC ACT CAG GGA TG	28S	
P. palmivora	Pal1s	CAC GTG AAC CGT ATC AAA ACT	ITS1	648
	Pal2a	CAA TCA TAC CAC CAC AGC TGA	ITS2	
P. parasitica	Par1s	ACG TTT GGG CTT CGG CCT GAT T	ITS1	680
	Par2a	GAT GCA TAC CGA AGT ACA CAT TA	ITS2	
Plant	Pl1s	GGT CGT ACG CAC GAG CCA CT	18S	678
	Pl2a	ATT ACT CCG ATC CCG AAG G	18S	

with accession numbers AY251647- AY251651 and AY251653-AY251666 (Table 1). Analysis by Blastn of NCBI indicated that, with the exception of AY251649, the best hits of all sequences were ribosomal ITS sequences obtained from the same species by other laboratories, and thus confirmed the identity of the aforementioned *Phytophthora* and *Pe. litchii* isolates. Analysis by multiple sequence alignment also indicated that sequences of ITS1-5.8S rRNA-ITS2 were very homogeneous within species of *Phytophthora* (data not shown). The sequence of AY251649 was cloned from *P. meadii*, which was known to infect white arum lily (*Zantedeschia aethiopica*) (Liou et al., 1999). Thus far, there is no other ribosomal sequence from this species available in the GenBank.

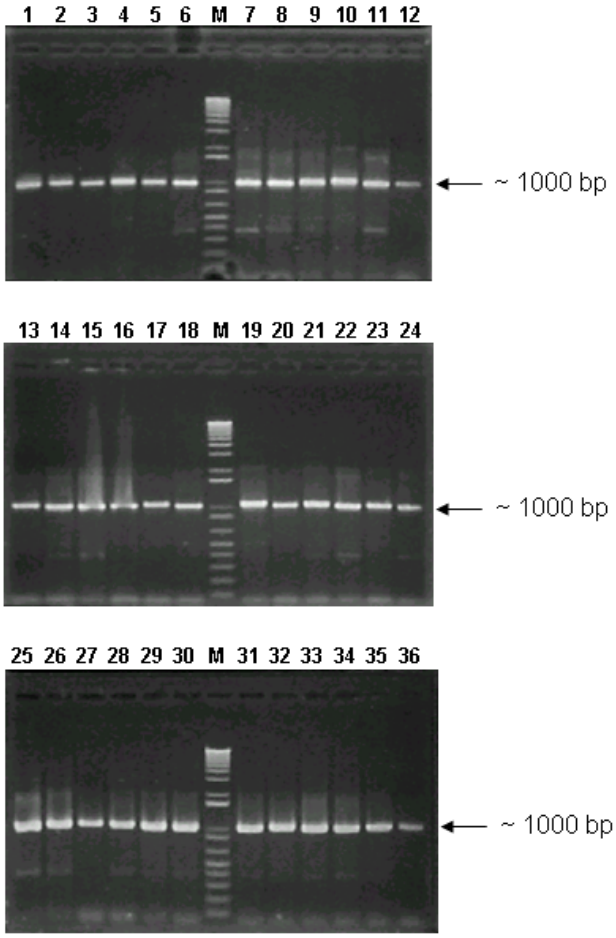

Figure 1. Specificity test of the primer pair Phy1s/Phy2a. PCR was performed using Phy1s/Phy2a as primers, and DNA from representative *Phytophthora* spp. as the template. The amplified products were analyzed by 1.5% agarose gel electrophoresis. Lane 1: *P. citricola* 9025; 2: *P. drechsleri* (*P. melonis*); 3: *P. infestans* 98029; 4: *P. parasitica* 92143; 5: *P. cryptogea*; 6: *P. colocasiae* 98115; 7: *P. palmivora* 93105; 8: *P. botryosa*; 9: *P. citricola*; 10: *P. cinnamomi* PC97; 11: *P. citrophthora* 95004; 12: *P. parasitica* 991-3; 13: *P. parasitica* 92145; 14: *P. citrophthora* 97083; 15: *P. colocasiae* 9177; 16: *P. infestans* 20040; 17: *P. drechsleri* 96032; 18: *P. cryptogea* 98176; 19: *P. cinnamomi* 94006; 20: *P. cactorum*; 21: *P. citricola* 9023; 22: *P. colocasiae* 20216; 23: *P. parasitica* 98151; 24: *P. cryptogea* 94011; 25: *P. capsici* 21170; 26: *P. citrophthora* 98165; 27: *P. parasitica* 98161; 28: *P. cryptogea* 90130; 29: *P. citrophthora* 98167; 30: *P. meadii*; 31: *P. botryosa* 52221; 32: *P. citricola* 9026; 33: *P. citrophthora*; 34: *P. colocasiae* 97066; 35: *P. citricola* 9024; 36: *P. drechsleri* 98141; M: 1 kb plus DNA ladder (Invitrogen).

Analysis by multiple sequence alignment indicated that some regions of ITS1 and 28S rRNA sequences were conserved among different species of *Phytophthora*. Three primers were designed accordingly, including Phy1s, Phy2a, and Phy2a-1, which would make up two primer pairs for PCR: Phy1s/Phy2a and Phy1s/Phy2a-1, respectively (Table 2). To evaluate the specificity of the primers, PCR was performed using Phy1s/Phy2a or Phy1s/Phy2a-1 as the primer set, and DNA from 13 *Phytophthora* spp. (a total of 47 isolates) and other species as the template (Table 1). When PCR was performed using Phy1s/Phy2a as the primers, analysis of the amplified products indicated that DNA fragments of approximately 1,000 bp in length were obtained from all the *Phytophthora* spp. analyzed, including *P. botryosa*, *P. cactorum*, *P. capsici*, *P. cinnamomi*, *P. citricola*, *P. citrophthora*, *P. colocasiae*, *P. cryptogea*, *P. drechsleri*, *P. infestans*, *P. meadii*, *P. palmivora*, and *P. parasitica* (Figure 1). No amplification signal was detected, however, when PCR was performed using DNA from *Pythium* spp. or other fungal isolates as the templates, with the exception of *Pe. litchii* (data not shown). PCR with Phy1s/Phy2a-1, in contrast, gave rise to amplified products not only from *Phytophthora* spp., but also from *Pythium*. As a result, this primer set was not used in the following experiments.

Specificity of the species-specific primer sets, Pal1s/Pal2a and Par1s/Par2a for *P. palmivora* and *P. parasitica*, respectively, was evaluated in a similar way. When PCR was primed with Pal1s/Pal2a, DNA fragment of the expected length (648 bp) was obtained only when DNA from *P. palmivora* was used as the template (Figure 2A, lane 15). When the same experiments were performed using Par1s/Par2a as the primers, DNA fragment of the expected length (680 bp) was obtained only when DNA from *P. parasitica* was used as the template (Figure 2B, lanes 16 and 22). These results confirmed the specificity of these two primer sets toward their respective targets and thus supported their applications for detection of *P. palmivora* and *P. parasitica*, respectively.

Detection of *P. palmivora* and *P. parasitica* by nested PCR and sensitivity test

To detect *P. palmivora* and *P. parasitica* by nested PCR, the first PCR reaction was primed with Phy1s/Phy2a, while the second PCR with Pal1s/Pal2a or Par1s/Par2a. To determine the minimal amount of the template DNA that is enough for generation of the amplified product, the first PCR reaction was performed using different amounts of *P. palmivora* or *P. parasitica* DNA as the templates. As shown in Figure 3, the PCR-amplified product from

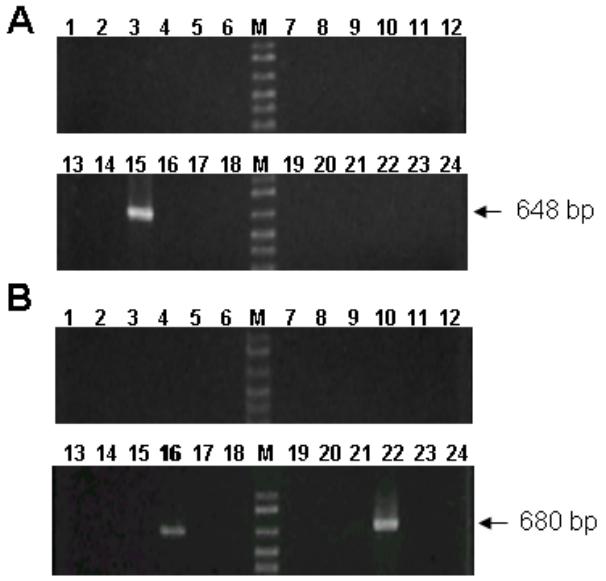

Figure 2. Specificity test of primer pairs Pal1s/Pal2a and Par1s/Par2a. PCR was performed using Pal1s/Pal2a (A) or Par1s/Par2a (B) as primers, and DNA from representative *Phytophthora* and *Pythium* species as the template. The amplified products were analyzed by 1.5% agarose gel electrophoresis. Lane 1: *P. infestans*; 2: *P. citricola* 9025; 3: *Py. myriotylum*; 4: *P. drechsleri* (*melonis*); 5: *Py. aphanidermatum*; 6: *P. cinnamomi*; 7: *P. capsici*; 8: *Py. splendens*; 9: *P. cryptogea* 98067; 10: *P. colocasiae* 9177; 11: *P. citrophthora* 95004; 12: *Peronophythora litchii*; 13: *Py. sylvaticum*; 14: *P. cryptogea* 98176; 15: *P. palmivora* 93105; 16: *P. parasitica* 98151; 17: *P. drechsleri* 96032; 18: *P. cactorum*; 19: *P. cryptogea* 94011; 20: *Py. ultimum*; 21: *P. meadii*; 22: *P. parasitica* 98151; 23: *P. botryosa* 52221; 24: ddH_2O; M: 1 kb plus DNA ladder (Invitrogen). Arrows indicated the location of the DNA fragments obtained from *P. palmivora* (A, lane 15) and *P. parasitica* (B, lanes 16 and 22), respectively.

1 pg of *P. palmivora* DNA was detectable by ethidium bromide staining of the agarose gel (Figure 3A, lane 5). No signal was observed, however, as the amount of DNA template decreased. When the second PCR was performed using Pal1s/Pal2a as the primers, and diluted (1:100) amplified product from the first PCR as the template, amplification signal was detectable even when only 10 fg of *P. palmivora* DNA was used as the template for the first PCR (Figure 3B, lane 7).

The same experiments were performed using Par1s/Par2a and *P. parasitica* DNA. As shown in Figure 4, the PCR-amplified product from 0.1 pg of *P. parasitica* DNA was detectable by ethidium bromide staining of the agarose gel (Figure 4A, lane 6). No signal was observed when the amount of DNA template decreased. When the second PCR was performed using Par1s/Par2a as the primers and diluted amplified product from the first PCR as the template, amplification signal was detectable while 10 fg of *P. parasitica* DNA was used as the template for the first PCR (Figure 4B, lane 7). It was thus obvious that, with nested PCR, the sensitivity of detection was enhanced by 10-100 folds.

Detection of orchid Phytophthora diseases by nested PCR

To develop a method for rapid detection of orchid Phytophthora diseases by nested PCR, sample tissues were collected from leaves, pseudostems, and roots of diseased *Oncidium*. Furthermore, *Phytophthora* pathogens which might exist in the culture media were trapped with leaf pieces of the orchid. Crude extract was then prepared from each of these samples by the NaOH method and analyzed by PCR. In addition to Phy1s/Phy2a, the primer set Pl1s/Pl2a (Table 2), which was designed based on the conserved sequences of plant 18S rRNA, was included

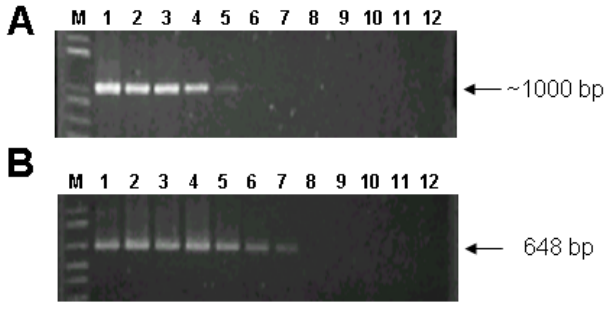

Figure 3. Sensitivity analysis of nested PCR for *Phytophthora palmivora*. The first PCR was performed using Phy1s/Phy2a as the primer pair, and indicated amounts of *P. palmivora* DNA as the template (A). The second PCR was performed using Pal1s/Pal2a as the primer pair and diluted amplified products from the first PCR as the template (B). The amplified products were analyzed by 1.5% agarose gel electrophoresis. Lane 1: 10 ng; 2: 1 ng; 3: 100 pg; 4: 10 pg; 5: 1 pg; 6: 100 fg; 7: 10 fg; 8: 1 fg; 9: 100 ag; 10: 10 ag; 11: 1 ag; 12: ddH_2O; M: 1 kb plus DNA ladder (Invitrogen).

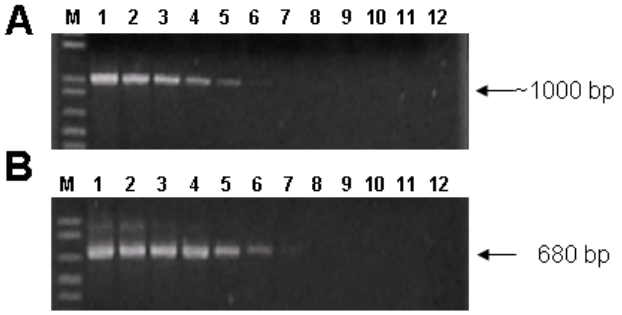

Figure 4. Sensitivity analysis of nested PCR for *Phytophthora parasitica*. The first PCR was performed using Phy1s/Phy2a as the primer pair, and indicated amounts of *P. parasitica* DNA as the template (A). The second PCR was performed using Par1s/Par2a as the primer pair and diluted amplified products from the first PCR as the template (B). The amplified products were analyzed by 1.5% agarose gel electrophoresis. Lane 1: 10 ng; 2: 1 ng; 3: 100 pg; 4: 10 pg; 5: 1 pg; 6: 100 fg; 7: 10 fg; 8: 1 fg; 9: 100 ag; 10: 10 ag; 11: 1 ag; 12: ddH_2O; M: 1 kb plus DNA ladder (Invitrogen).

and used as a positive control to ensure quality of plant DNA extraction. As shown in Figure 5A, DNA fragments of approximately 1,000 bp long were observed with DNA from the pseudostem, root, and leaf of *Oncidium* sp., as well as that from the leaf baits (Figure 5A, lanes 1-4), indicating the presence of *Phytophthora* pathogens in these specimens. This fragment was also detected while PCR was performed using DNA from *P. palmivora* as the template (Figure 5A, lane 5), but not with DNA from the orchid (Figure 5A, lane 6). Besides, DNA fragments of 678 bp in length were amplified from genes encoding plant 18S rRNA (Figure 5A, lanes 1-4 and 6). To identify the *Phytophthora* species, a second PCR was performed using diluted amplified product from the first PCR as the template. Amplified products of the expected size were observed only when PCR was primed with Pal1s/Pal2a (Figure 5B), but not Par1s/Par2a (data not shown), indicating the pathogen which caused disease in *Oncidium* sp. was *P. palmivora*, rather than *P. parasitica*. DNA fragments about 1,000 bp in length, which appeared as bands of weaker intensity above the 648-bp fragments, were the DNA templates obtained from the first PCR (Figure 5B).

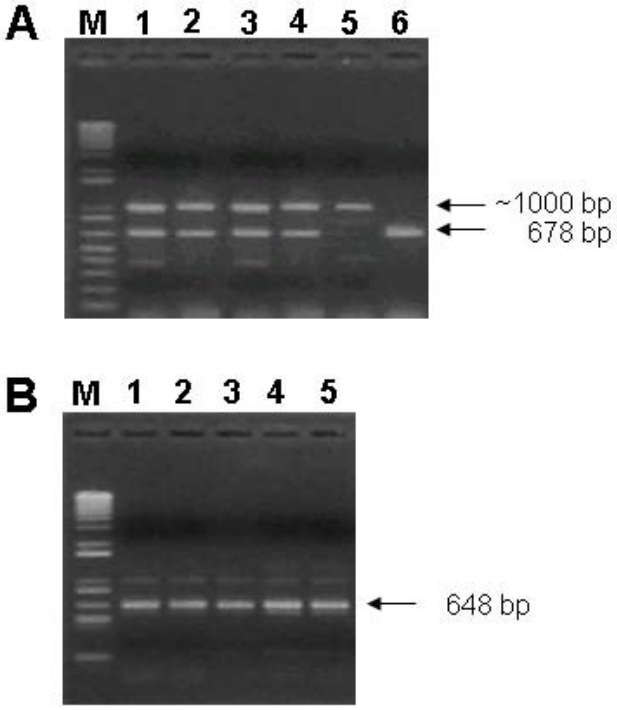

Figure 5. Detection of the orchid Phytophthora disease by nested PCR. Samples from infected tissues of *Oncidium* sp. were process by the NaOH method and analyzed by the nested PCR. The first PCR was performed using Phy1s/Phy2a and Pl1s/pl2a as the primer pairs (A), while for the second PCR, Pal1s/Pal2a was included in the PCR reaction (B). The amplified products were analyzed by 1.5% agarose gel electrophoresis. For both (A) and (B), lane 1: the leaf baits from infested media; 2: diseased orchid leaves; 3: diseased orchid roots; 4: diseased orchid pseudostems; 5: *Phytophthora palmivora*. Lane 6 of (A): leaves from a healthy orchid. M: 1 kb plus DNA ladder (Invitrogen). Arrows indicated the location of the amplified DNA fragments.

DISCUSSION

Both *P. palmivora* and *P. parasitica* are important plant pathogens, able to cause severe diseases in a wide variety of crops (Erwin and Ribeiro, 1996). While infecting orchids, they caused severe orchid Phytophthora disease, which has been a major threat for cultivation of orchids in Taiwan (Ann, 1995). Traditional methods for identification of *Phytophthora* spp. are time-consuming and require considerable expertise to differentiate species of *Phytophthora* based on characteristics of morphology. In this study, a nested PCR assay was established for rapid detection of *Phytophthora* pathogens of orchids. Compared with conventional PCR, nested PCR has the advantages of higher sensitivity and better specificity, and thus has been used as the detection method in many studies (Grote et al., 2002; Ippolito et al., 2002; Martin et al., 2004). In our assay, oligonucleotides used to prime the first PCR, Phy1s and Phy2a, were designed according to the conserved sequences of ITS1 and 28S rRNA of *Phytophthora*. As shown by the specificity test, this primer set could amplify DNA from 13 *Phytophthora* spp. as well as *Pe. litchii*, an Oomycete pathogen known to cause blossom blight in litchi (*Litchi chinensis*) (Ann and Ko, 1984), but not pathogens belonging to the genus *Pythium*. Since *Pe. litchii* is known to infect only litchi, its interaction with Phy1s/Phy2a has not been a major concern. When PCR was primed with this primer set, appearance of an amplification signal with the expected size would indicate the presence of *Phytophthora* pathogen(s). Following the first PCR, nested PCR was then performed using species-specific oligonucleotides, Pal1s/Pal2a or Par1s/Par2a, as the primer set to identify the pathogen at the species level. Specificity of these two primer pairs was verified by PCR using DNA prepared from 13 *Phytophthora* spp., which represented species isolated from diseased plants collected from different areas of Taiwan in recent years with the exception of *P. botryose* and *P. cactorum*. *Phytophthora multivesticulata* was reported only once in 1996 (Chern and Ann, 1996), and thus was not included in the analysis. To exclude the possibility that Pal1s/Pal2a and Par1s/Par2a might interact with DNA of *P. multivesticulata*, we search the GenBank for the ribosomal ITS sequence of *P. multivesticulata*. There is only one sequence (DQ335639) available. Analysis of the sequence indicated that, because of sequence divergence, the aforementioned concern should not present a problem (data not shown).

The idea that nested PCR was carried out with the addition of both primer sets (Pal1s/Pal2a and Par1s/Par2a) simultaneously, namely by multiplex PCR, was very attractive. Results obtained from the experiments (data not shown), however, indicated that it is not applicable, due to the similarity in the size of amplified DNA fragments

obtained with Pal1s/Pal2a (648 bp) and Par1s/Par2a (680 bp), which were hard to distinguish by the regular agarose gel electrophoresis system used in this study. With nested PCR, as have been demonstrated in other studies (Ippolito et al., 2002; Martin et al., 2004), sensitivity of the test was enhanced 10-100 folds, and as less as 10 fg of DNA was enough for generating a significant amplification signal. However, there are risks of contamination when performing nested PCR in two rounds. Special precautions must be taken while setting up the reactions (Takahashi and Nakayama, 2006).

To detect the existence of *Phytophthora* pathogens in the orchids, DNA was extracted from the diseased plant tissues by the use of an alkali method (Wang et al., 1993). It took only a couple of minutes to obtain DNA to be used for PCR. Furthermore, to ensure that negative results from nested PCR were indeed indicative of the absence of *Phytophthora* pathogens, in addition to Phy1s/Phy2a, a second primer set (Pl1s/Pl2a) was included in the first PCR. This primer set was designed based on the conserved sequences of plant 18S rDNA and thus might serve as a positive control to check the quality of DNA, which was prepared from diseased plants and used as the template for PCR. Analysis by nested PCR indicated that the tested orchids were infected only by *P. palmivora*. The possibility that the absence of *P. parasitica* might result from failure of Par1s/Par2a to interact with DNA obtained from infected tissues was excluded, since it has been demonstrated previously that this primer set is useful for detection of *P. parasitica* in infected plants (data not shown). With the nested PCR assay, presence of *Phytophthora* spp. on diseased plants might be detected within a few hours, and thus provided a very useful tool for diagnosis of orchid Phytophthora disease. As mentioned in the previous section, *P. palmivora* and *P. parasitica* are important plant pathogens on numerous crops, including citrus, tomato, and a wide variety of ornamental crops (Erwin and Ribeiro, 1996). The method described here can also be adopted for detection of other plant diseases caused by these two pathogens.

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以 nested PCR 檢測蘭花疫病

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蘭花為蘭科 (Orchidaceae) 之草本花卉，除了可供切花外，也可做為盆花觀賞，是頗具特色與經濟價值之重要花卉。在台灣，可感染蘭花之疫病菌主要包括 *Phytophthora palmivora* 及 *P. parasitica*，但偶爾也可發現 *P. multivesticulata*。*P. palmivora* 及 *P. parasitica* 可危害之蘭花種類繁多，且因蔓延十分迅速，常在管理較差之蘭園造成嚴重經濟損失。為縮短及簡化蘭花疫病檢測流程，本研究根據疫病菌核醣體核酸序列設計 *Phytophthora* spp. 廣效性引子對 (Phy1s/Phy2a) 可用於檢測台灣常見疫病菌種類。此外，我們也針對 *P. palmivora* 及 *P. parasitica* 分別設計種專一性引子對，並配合 Phy1s/Phy2a 之應用，開發以 nested PCR 快速檢測上述兩種疫病菌之技術，可快速確定蘭花罹患疫病及栽培介質之帶菌情形，並確認所感染之疫病菌種類，以便儘早擬定防治策略，減少不必要之經濟損失。

關鍵詞：疫病菌；蘭花疫病；快速檢測；Internal transcribed spacer (ITS)；Nested PCR。