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Research paper

Single nucleotide polymorphism-based multiplex PCR for identification and genotyping of the oomycete *Pythium insidiosum* from humans, animals and the environment

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ABSTRACT

Pythium insidiosum causes a life-threatening infectious disease, called pythiosis, in humans and animals worldwide. Diagnosis of pythiosis is difficult and often delayed. Surgical removal of infected tissue is the main treatment option. Disabilities and death are common outcomes for pythiosis patients. Reports of *Py. insidiosum* infections are rising. While it would be useful for clinical, epidemiological, and microbiological studies, information on genetic variation in *Py. insidiosum* strains is limited. This limitation is, at least in part, due to the cost and time-requirements of DNA sequencing procedures. rDNA-sequence-based phylogenetic analyses categorize *Py. insidiosum* into three groups, in relation to geographic distribution: Clade-I (American strains), Clade-II (American, Asian, and Australian strains), and Clade-III (Thai and American strains). In rDNA sequence analyses, we observed single nucleotide polymorphisms (SNP) that were associated with the phylogenetic clades of *Py. insidiosum*. In this study, we aim to develop a multiplex PCR assay, targeting the identified SNPs, for rapid genotyping of *Py. insidiosum*. We also aim to assess diagnostic efficiency of the assay for identification of *Py. insidiosum*. Fifty-three isolates of *Py. insidiosum* from humans (n = 35), animals (n = 14), and the environment (n = 4), and 22 negative-control fungi were recruited for assay evaluation. Based on the pattern of amplicons, the multiplex PCR correctly assigned phylogenetic clades in 98% of the *Py. insidiosum* isolates tested. The assay exhibited 100% sensitivity and specificity for identification of *Py. insidiosum*. The assay successfully identified and genotyped the first proven isolate of *Py. insidiosum* from an animal with pythiosis in Thailand. In conclusion, the multiplex PCR provided accurate, sensitive and specific results for identifying and genotyping *Py. insidiosum*. Thus, this multiplex-PCR assay could be a simple, rapid, and cost-effective alternative to DNA sequencing for the identification and genotyping of *Py. insidiosum*.

1. Introduction

Pythium insidiosum is a member of the oomycetes, which is a unique group of morphologically fungus-like microorganisms that belong to the Kingdom Stramenopila (De Cock et al., 1987; Gaastra et al., 2010; Kamoun, 2003; Mendoza et al., 1996). While most pathogenic oomycetes are capable of infecting plants, *Py. insidiosum* can cause a life-threatening infectious condition, called pythiosis, in humans and animals (horses, dogs, cats, cattle) (Gaastra et al., 2010; Kamoun, 2003; Mendoza et al., 1996). Zoospores are the infective agents of *Py. insidiosum*, as they can adhere and germinate into host tissue (Mendoza et al., 1993). Target organs for infection include skin, intestines, blood vessels and eyes (Gaastra et al., 2010; Krajaejun et al., 2004, 2006; Mendoza et al., 1996). With the lack of other diagnostic tools (Chareonsirisuthigul et al., 2013; Inkomlue et al., 2016; Intaramat et al., 2016; Keeratijarut et al., 2009, 2015), diagnosis of pythiosis relies on culture identification, which is laborious and time-consuming. Conventional antimicrobial drugs are not generally effective against *Py. insidiosum*. Extensive surgical removal of the infected organ is the inescapable treatment option, and most often leads to disabilities in the

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affected patients. Post-surgery recurrence of the *Py. insidiosum* infection and death are the final outcomes in many patients with pythiosis (Krajaejun et al., 2004, 2006).

Pythiosis in animals has been increasingly diagnosed in tropical and subtropical regions, whereas the disease in humans has been mostly found in Thailand, where no animal case has been reported thus far (Gaastra et al., 2010; Krajaejun et al., 2006; Mendoza et al., 1996). The successful isolations of *Py. insidiosum* from swampy areas in Australia, Thailand, and the United States of America have been described (Miller, 1983; Presser and Goss, 2015; Supabandhu et al., 2008), suggesting that the pathogen is distributed worldwide. Several genes [i.e., ribosomal DNA (rDNA), *exo1*, and *cox II*] have been used to explore phylogenetic relationship of *Py. insidiosum* isolated from different hosts and geographic areas (Azevedo et al., 2012; Chaiprasert et al., 2009; Kammarnjesadakul et al., 2011; Krajaejun et al., 2010; Ribeiro et al., 2016; Schurko et al., 2003; Supabandhu et al., 2008). Among them, rDNA [which consists of 18S rRNA, internal transcribed spacer 1 (ITS1), 5.8S rRNA, ITS2, and 28S rRNA] has been a frequent target for phylogenetic studies of *Py. insidiosum* (Chaiprasert et al., 2009; Lerksuthirat et al., 2015; Schurko et al., 2003; Supabandhu et al., 2008; Vanittanakom et al., 2014). The rDNA-based phylogenetic analyses categorize *Py. insidiosum* into three groups, in association with geographic origins: Clade-I (containing American strains), Clade-II (American, Asian, and Australian strains), and Clade-III (mostly Thai strains) (Chaiprasert et al., 2009; Schurko et al., 2003).

According to the growing number of publications on pythiosis, awareness of the disease and its causative agent, *Py. insidiosum*, has increased among healthcare professionals and microbiologists. However, in order to support clinical, epidemiological and microbiological studies, a faster and simpler method is needed for identification and genotyping than that based on DNA sequence analysis. After analysis of the rDNA sequences previously-reported by our group (Lerksuthirat et al., 2015), we observed single nucleotide polymorphisms (SNP) within the rDNA region that show associations with the phylogenetic clades of *Py. insidiosum* (i.e., Clade-I, -II, and -III). SNPs are genetic markers widely used in biological and clinical studies, such as, genome-wide association, population genetics, pharmacogenomics, and disease susceptibility (Kim and Misra, 2007; Seeb et al., 2011; Vignal et al., 2002). To our knowledge, no SNP markers have been identified and utilized for molecular studies of *Py. insidiosum*. In the current study, we report on the development and evaluation of a multiplex PCR assay, targeting the SNPs identified in the rDNA region, for rapid and cost-effective genotyping of *Py. insidiosum*. We also test the diagnostic efficiency of the assay for identification of *Py. insidiosum* isolates. Finally, we demonstrate the efficacy of the multiplex PCR to identify and genotype the first proven isolate of *Py. insidiosum* from an infected animal in Thailand.

2. Materials and methods

2.1. Microorganisms and genomic DNA extraction

Fifty-three isolates of *Py. insidiosum* from human patients with pythiosis (n = 35), equine patients with pythiosis (n = 13), mosquito larva (n = 1), and environmental samples (n = 4) were used for this study (Table 1). Identities of all *Py. insidiosum* isolates were molecularly confirmed by rDNA sequence analysis with 99–100% homology to the rDNA sequences of *Py. insidiosum* obtained from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) (Badenoch et al., 2001). Each isolate was incubated in a Petri dish with 10 ml of Sabouraud dextrose broth (pH 7.2), at room temperature for 10 days. The hyphae were removed from the broth, briefly washed with distilled water, and filtered through Whatman No.1 filter paper for genomic DNA (gDNA) extraction, using the salt-extract protocol described by Lohnoo and co-workers (Lohnoo et al., 2014).

To serve as negative controls, 22 clinically relevant fungi (4 *Candida* species, 2 *Mucor* species, 2 *Fusarium* species, 2 *Trichophyton* species, 2 *Trichosporon* species, and one isolate each of *Cryptococcus neoformans*, *Penicillium marneffei*, *Torulopsis glabrata*, *Scedeosporium apiospermum*, *Rhizopus* species, *Conidiobolus* species, *Aspergillus* species, *Acremorium* species, *Microsporum* species, and *Curvularia* species) were obtained from the Clinical Microbiology Laboratory, Department of Pathology, Ramathibodi Hospital, Bangkok, Thailand. The identity of each filamentous fungus was confirmed by colony and conidia morphology, while that of yeast was confirmed by carbohydrate and nitrogen assimilation, carbohydrate fermentation, and phenol oxidase/urease enzyme production. Fungal gDNAs were prepared using the gDNA extraction protocol described by Keeratijarut and co-workers (Keeratijarut et al., 2014). Quantity and purity of each gDNA sample was determined by a NanoDrop 2000 spectrophotometer (Thermo Scientific). All extracted gDNA were stored in Tris-EDTA buffer (pH 8.0) at − 20 °C.

2.2. Sequence alignment and primer design

The rDNA sequences of 18 *Py. insidiosum* isolates, classified in three different phylogenetic clades (Lerksuthirat et al., 2015), and six other oomycete microorganisms [i.e., *Pythium deliense* (accession number, AY151181.1), *Pythium aphanidermatum* (AY598622.2), *Phytophthora capsici* (AY726623.1), *Phytophthora infestans* (HQ643247.1), *Phytophthora parasitica* (KC768775.1), and *Hyaloperonospora arabidopsidis* (AY531434.1)] were retrieved from the NCBI database and aligned by using the BioEdit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html). The Primer-BLAST program (Ye et al., 2012) was used to design the multiplex-PCR reverse primers R1 (5′-CCTCACATTCTG-CCATCTCG-3′), R2 (5′-ATACCGCCAATAGAGGTCAT-3′), and R3 (5′-T-TACCCGAAGGCGTCAAAGA-3′), based upon the clade-specific SNPs presented in the rDNA sequences of *Py. insidiosum* (Fig. 1). The fungal universal primer ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) was used as the shared multiplex-PCR forward primer, and also with the fungal universal ITS4 (5′-TCCTCCGCTAATTGATATGC-3′) for quality evaluation of the extracted gDNA samples (see below). The diagram, demonstrating all primer annealing locations, was prepared using the IBS program (Liu et al., 2015). All primer sequences were BLAST searched against the draft genome sequence of *Py. insidiosum* (Rujirawat et al., 2015) and the NCBI database for potential off-target priming.

2.3. Polymerase chain reaction

Multiplex-PCR amplification was carried out in a 25-μl reaction, which contained 100 ng of gDNA template, 0.65 U of Taq DNA polymerase (Fermentas, USA), 1 × Taq buffer with KCl, 2 mM of $MgCl_2$, 0.2 mM of dNTPs, 0.03 μM of the forward primer ITS1, and 0.07 μM each of the reverse primers R1, R2, and R3. The PCR reaction was performed in a Mastercycler Nexus thermal cycle machine (Eppendorf, Germany), using the following conditions: initial denaturation at 95 °C for 5 min, 20 cycles of denaturation at 95 °C for 30 s, annealing at 59 °C for 30 s, and extension at 72 °C for 45 s, and final extension at 72 °C for 10 min. To check for the presence of amplifiable DNA in the samples, conventional PCR amplification was conducted, using the above-mentioned PCR conditions, with two modifications: (i) using the primers ITS1 and ITS4 (0.1 μM each), and (ii) changing the annealing step to 25 cycles at 55 °C. The obtained PCR products were separated in a 1% agarose gel, stained with ethidium bromide, and visualized by a Gel Doc XR + Molecular Imager (Bio-Rad, USA).

2.4. DNA sequencing and nucleotide sequence accession numbers

The rDNA ~920-bp amplicons generated with primers ITS1 and ITS4 using genomic DNA from the 53 isolates of *Py. insidiosum* were sequenced using the primers ITS1 and ITS4, an ABI PRISM BigDyeTM terminator cycle sequencing ready reaction kit (Applied Biosystems, USA), and an ABI 3100 Genetic Analyzer (Applied Biosystems, USA).

Table 1
Fifty-three isolates of *Py. insidiosum* used for evaluation of the multiplex PCR assay. Information on strain ID, reference strain [indicate the strains that have been phylogenetically categorized by other investigators (Chaiprasert et al., 2009; Schurko et al., 2003)], sources of isolation (affected hosts and countries), phylogenetic clades, accession number for the rDNA sequences, and PCR products of each set of the PCR primers, are summarized in the table ('bp', base pair; '+', PCR product is detected; 'ND', PCR product is not detected; NA, data not available).

Strain ID	Reference strain	Source	Country (province, city or state) of origin	Phylogenetic clade	Accession number of rDNA	PCR product (bp)			
						Primers ITS1/ ITS4 (~920)	Primers ITS1/ R1 (~490)	Primers ITS1/ R2 (~660)	Primers ITS1/ R3 (~800)
Pi01	ATCC58639	Equine	Costa Rica (NA)	Clade-I	LC199875	+	+	+	ND
Pi02	ATCC58638	Equine	Costa Rica (NA)	Clade-I	AB971176	+	+	+	ND
Pi03	ATCC58640	Equine	Costa Rica (NA)	Clade-I	AB971177	+	+	+	ND
Pi04	ATCC58641	Equine	Costa Rica (NA)	Clade-I	AB898106	+	+	+	ND
Pi05	ATCC58642	Equine	Costa Rica (NA)	Clade-I	AB971178	+	+	+	ND
Pi06	ATCC58643	Equine	Costa Rica (NA)	Clade-I	AB971179	+	+	+	ND
Pi07	ATCC58644	Equine	Costa Rica (NA)	Clade-I	AY151158	+	+	+	ND
Pi08	ATCC58637	Equine	Costa Rica (NA)	Clade-I	AB898107	+	+	+	ND
Pi09	394	Equine	Brazil (NA)	Clade-I	AY151163	+	+	+	ND
Pi10	M6	Human	USA (Tennessee)	Clade-I	AB898108	+	+	+	ND
Pi11	–	Human	Thailand (NA)	Clade-II	AB898109	+	ND	+	ND
Pi12	Hu20	Human	Thailand (Lopburi)	Clade-II	LC199876	+	ND	+	ND
Pi13	–	Human	Thailand (NA)	Clade-II	AB898110	+	ND	+	ND
Pi14	Hu24	Human	Thailand (NA)	Clade-II	LC199877	+	ND	+	ND
Pi15	–	Human	Thailand (NA)	Clade-II	AB898111	+	ND	+	ND
Pi16	CBS119452	Human	Thailand (Nan)	Clade-II	AB971182	+	ND	+	ND
Pi17	–	Human	Thailand (NA)	Clade-II	AB898112	+	ND	+	ND
Pi18	CBS119453	Human	Thailand (Lumpang)	Clade-II	EF016853	+	ND	+	ND
Pi19	–	Human	Thailand (NA)	Clade-II	AB898113	+	ND	+	ND
Pi20	CBS119455	Human	Thailand (Chiang Mai)	Clade-II	EF016855	+	ND	+	ND
Pi21	–	Human	Thailand (NA)	Clade-II	AB898114	+	ND	+	ND
Pi22	Hu22	Human	Thailand (Yasothon)	Clade-II	GU137338	+	ND	+	ND
Pi23	–	Human	Thailand (Saraburi)	Clade-II	AB898115	+	ND	+	ND
Pi24	Hu10	Human	Thailand (Chonburi)	Clade-II	GU137327	+	ND	+	ND
Pi25	–	Human	Thailand (NA)	Clade-II	AB898116	+	ND	+	ND
Pi26		Human	Thailand (Patomthani)	Clade-II	AB898117	+	ND	+	ND
Pi27	Hu12	Human	Thailand (Korat)	Clade-II	GU137329	+	ND	+	ND
Pi28	–	Human	Thailand (NA)	Clade-II	AB898118	+	ND	+	ND
Pi29	Hu13	Human	Thailand (Suphanburi)	Clade-II	GU137330	+	ND	+	ND
Pi30	–	Human	Thailand (NA)	Clade-II	AB898119	+	ND	+	ND
Pi31	–	Human	Thailand (NA)	Clade-II	AB898120	+	ND	+	ND
Pi32	–	Human	Thailand (NA)	Clade-II	AB898121	+	ND	+	ND
Pi33	–	Human	Thailand (NA)	Clade-II	AB898122	+	ND	+	ND
Pi34	–	Human	Thailand (NA)	Clade-II	AB898123	+	ND	+	ND

(continued on next page)

Table 1 *(continued)*

Strain ID	Reference strain	Source	Country (province, city or state) of origin	Phylogenetic clade	Accession number of rDNA	PCR product (bp)			
						Primers ITS1/ ITS4 (~920)	Primers ITS1/ R1 (~490)	Primers ITS1/ R2 (~660)	Primers ITS1/ R3 (~800)
Pi35	–	Human	Thailand (NA)	Clade-II	AB898124	+	ND	+	ND
Pi36	M23	Equine	Australia (NA)	Clade-II	AY151174	+	ND	+	ND
Pi37	M15	Equine	Papua New Guinea (NA)	Clade-II	AY151171	+	ND	+	ND
Pi38	393	Human	India (NA)	Clade-II	AB898125	+	ND	+	ND
Pi39	ATCC46947	Equine	Japan (NA)	Clade-II	AY151170	+	ND	+	ND
Pi40	296	Mosquito larva	India (NA)	Clade-II	AY151169	+	ND	+	ND
Pi41	E55	Environment	Thailand (Chiang Mai)	Clade-II	EF016862	+	ND	+	ND
Pi42	E14	Environment	Thailand (Chaing Rai)	Clade-II	EF016903	+	+ (weak)	+	ND
Pi43	–	Environment	Thailand (NA)	Clade-II	AB898126	+	ND	+	ND
Pi44	CBS119454	Human	Thailand (Chiang Mai)	Clade-III	AB971185	+	ND	ND	+
Pi45	Hu08	Human	Thailand (Saraburi)	Clade-III	AB971186	+	ND	ND	+
Pi46	Hu26	Human	Thailand (Nakorn Srithamarat)	Clade-III	AB971187	+	ND	ND	+
Pi47	Hu29	Human	Thailand (NA)	Clade-III	AB971188	+	ND	ND	+
Pi48	Hu21	Human	Thailand (Rachaburi)	Clade-III	AB971189	+	ND	ND	+
Pi49	–	Human	Thailand (NA)	Clade-III	AB898127	+	ND	ND	+
Pi50	M19	Human	USA (TX)	Clade-III	AB971190	+	ND	ND	+
Pi51	–	Environment	Thailand (NA)	Clade-III	AB898128	+	ND	ND	+
Pi52	–	Human	Thailand (NA)	Clade-II	LC199888	+	ND	+	ND
Pi53	–	Equine	Thailand (Bangkok)	Clade-II	LC199889	+	ND	+	ND

All new rDNA sequences have been submitted to the DNA data bank of Japan (DDBJ), under the accession numbers shown in Table 1.

2.5. Phylogenetic analyses

The rDNA sequences from 53 isolates of *Py. insidiosum* (Table 1) were subjected to online phylogenetic tree construction software at http://www.phylogeny.fr (Dereeper et al., 2008). In brief, the sequences were aligned by the MUSCLE program (Edgar, 2004) and phylogenetically analyzed using the Maximum-Likelihood method in the PhyML 3.0 program with 500 bootstrap replicates (Guindon et al., 2010). A tree was constructed using the locally-installed software Dendroscope 3 (Huson and Scornavacca, 2012). The outgroups included rDNA sequences from *Pythium deliense* (accession number, AY151181) and *Pythium grandisporangium* (AY151182).

3. Results

3.1. DNA sequence analysis

Conventional PCR amplifications, using the fungal universal rDNA primers ITS1 and ITS4 (Fig. 1), were performed to provide template for sequence and phylogenetic analyses and to check for the presence of amplifiable gDNA in the samples extracted from *Py. insidiosum* and from the control samples (Fig. 2A). Only the PCR-positive samples (*Py. insidiosum*, $n = 53$; controls, $n = 22$) were used for the downstream multiplex PCR analyses (see below).

3.2. Generation of a phylogenetic tree

The rDNA sequences (ITS1/ITS4 amplicon, ~920 bp) from 53 strains of *Py. insidiosum*, isolated from different hosts and geographic locations (Table 1) were aligned, and analyzed for phylogenetic relationships. A resulting Maximum Likelihood-based phylogenetic tree categorized the *Py. insidiosum* strains into Clade-I ($n = 10$), Clade-II ($n = 35$), and Clade-III ($n = 8$), which are in agreement with the previous studies (Chaiprasert et al., 2009; Schurko et al., 2003), as shown in Fig. 3 and Table 1.

3.3. Setting up a multiplex PCR assay

To develop a simpler PCR-based assay for identification and genotyping (i.e., Clade identification) of *Py. insidiosum*, we used SNPs, identified in the sequence analyses, as the basis for primers for multiplex PCR. Three reverse primers (R1, R2, and R3) were designed to work in conjunction with the fungal universal forward primer ITS1 for the rDNA of *Py. insidiosum*, and to distinguish between the three different phylogenetic groups: Clade-I, -II, and -III (Fig. 1). The primer R1 should specifically prime the ITS-2 sequences of the Clade-I strains, the primer R2 should prime the ITS-2 sequences of both Clade-I and -II strains, and the primer R3 should only prime the ITS-2 sequences of the Clade-III strains (Fig. 1B). The three reverse primers did not match ITS-2 sequences of closely-related oomycete microorganisms, including *Py. deliense*, *Py. aphanidermatum*, *Ph. capsici*, *Ph. infestans*, *Ph. parasitica*, and *Hy. arabidopsidis* (Fig. 1B). To further check the annealing specificity, the primers were BLAST searched against the draft genome of *Py.*

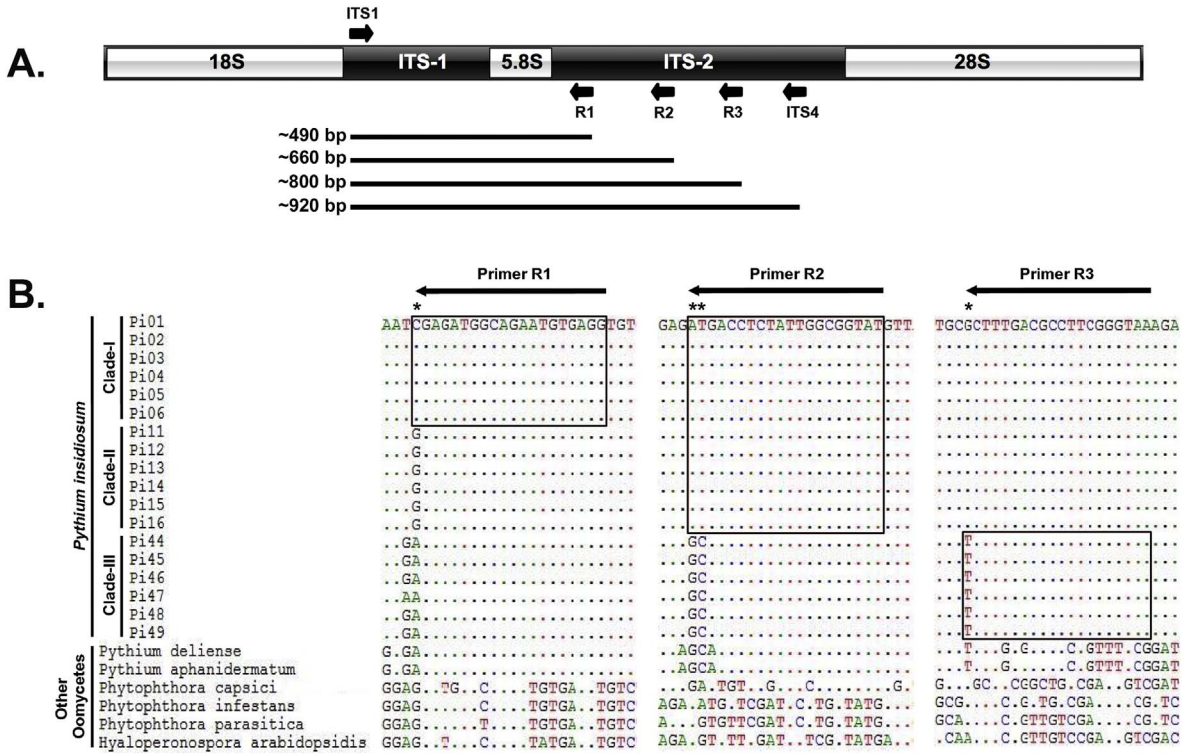

Fig. 1. The multiplex PCR primers used in this study: (A) The annealing locations of the primers ITS1, ITS2, R1, R2, and R3 in the rDNA region of *Py. insidiosum* (the horizontal bars indicate amplicon product sizes for each primer pair); (B) Alignment of the primer R1, R2, and R3-annealing sequences in the rDNA region of 18 *Py. insidiosum* isolates and 6 other oomycetes (the letters A, C, G, and T represent nucleotides; a dot represents the identical nucleotide; a box indicates the primer sequence).

insidiosum (Rujirawat et al., 2015), as well as, genes of all other organisms deposited in the NCBI database. No potential off-target annealing of these primers was identified. The reverse primers (R1, R2, and R3) and the shared forward primer ITS1 (Fig. 1A) were together used in the multiplex PCR assay. Among the strains tested, the amplicons obtained from each primer pair were similar in size with a few bases difference. The multiplex PCR assay resulted in the expected amplicon sizes of ~490 bp for the primer pair ITS-1/R1, ~660 bp for the primer pair ITS-1/R2, and ~800 bp for the primer pair ITS-1/R3 (Fig. 2B).

3.4. Multiplex PCR-based genotyping and detection

The multiplex PCR amplifications, using primers ITS1, R1, R2, and R3, provided the expected amplicons for gDNAs extracted from all *Py. insidiosum* isolates used in this study (Fig. 1). The amplicons had different sizes, so that the association with the phylogenetic groups of the organism, as previously defined by DNA sequence analyses, is dependent both on the presence of PCR products, as well as the sizes (Figs. 2B and 3). Two PCR products of ~490 and ~660 bp were amplified from all gDNAs of the Clade-I strains (n = 10). Only the PCR product of ~800 bp was observed in all Clade-III strains (n = 8). For the Clade-II strains (n = 35), 97% (n = 34) had one prominent (and expected) PCR product of ~660 bp. A single Cade-II strain (Pi42) showed two amplicons: a prominent band of ~660 bp and a weak band of ~490 bp. The multiplex PCR assay failed to amplify any product in all 22 negative-control gDNA samples extracted from various fungal species (The results of a few representative strains were shown in Fig. 2).

Because of the unexpected result of two amplicons with strain Pi42, we further analyzed the chromatogram of the rDNA sequence from strain Pi42 and found a unique SNP with a high intensity peak of guanosine triphosphate (G) and a relatively-low intensity peak of cytidine triphosphate (C), at the position corresponding to the annealing site of the 3′-end base of the primer R1 (Figs. 1B and 4D). No such finding was noted at the same SNP position in the rDNA sequences of the Clade-I strains (only C peak was observed; Fig. 4A), the Clade-III strains (only adenosine triphosphate (A) peak; Fig. 4C), and the other Clade-II strains (only G peak; Fig. 4B).

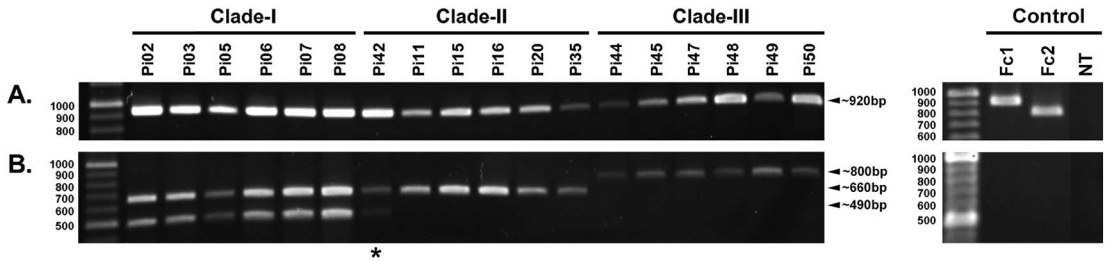

Fig. 2. PCR amplification of the rDNA sequences from representative strains of *Py. insidiosum* and control fungi: Agarose gel electrophoresis of PCR products amplified by (A) the primers ITS1/ITS4 (for checking the presence of gDNA; amplicon size, ~920 bp), and (B) the multiplex PCR primers ITS1, R1, R2, and R3 (the amplicon sizes for the primer pairs ITS1/R1, ITS1/R2, and ITS1/R3 are ~490 bp, ~660 bp, and ~800 bp, respectively). Fc1 and Fc2 represent the control fungi *Torulopsis glabrata* and *Conidiobolus* species, respectively. NT indicates no-template control. The 100-bp step-ladder markers are shown at the left side of the gel.

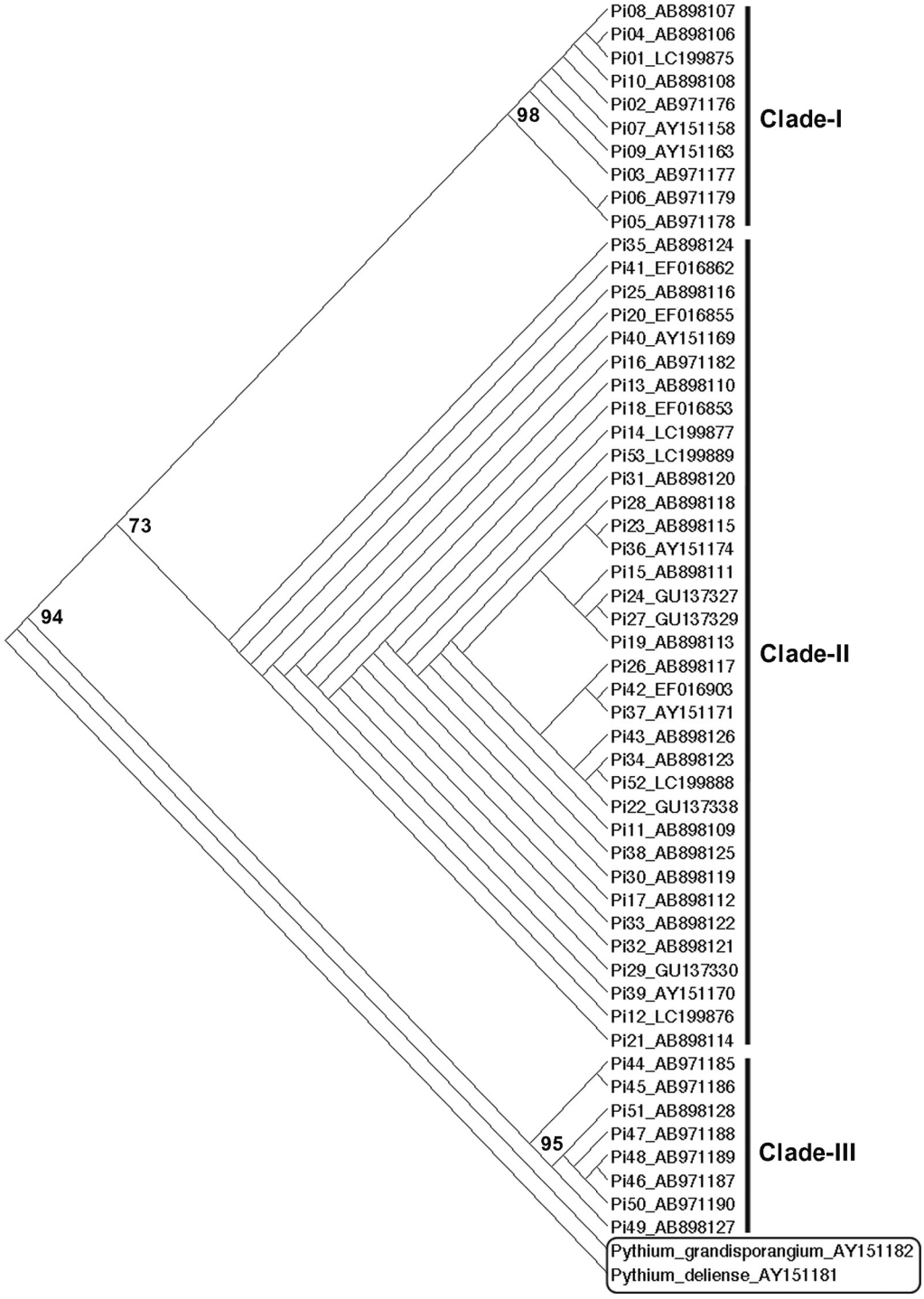

Fig. 3. Phylogenetic relationship of various *Py. insidiosum* isolates: rDNA sequences from 53 isolates of *Py. insidiosum* and 2 outgroup oomycetes (*Py. deliense* and *Py. grandisporangium*) are analyzed for phylogenetic relationship, using the Maximum Likelihood algorithm, with 500 bootstrap replicates. Only branch support values of 70% or more are shown.

4. Discussion

Currently, *Py. insidiosum* has been genotyped, using the costly, time-consuming, sequence-based phylogenetic analysis, which groups the organism into Clade-I (American strains), Clade-II (American, Asian, and Australian strains), and Clade-III (mostly Thai strains) (Chaiprasert et al., 2009; Schurko et al., 2003). Here, we have developed a multiplex PCR, targeting three SNPs identified in the rDNA region (Fig. 1), for simple and rapid genotyping of *Py. insidiosum*. Interpretation of the multiplex PCR-based genotyping relies on number and size of the resulting amplicons. The genotyping results from the multiplex PCR assay were generally consistent with that from the sequence-based phylogenetic analysis (Fig. 3 and Table 1). The Clade-I strains provided two amplicons (~490 and ~660 bp), whereas the Clades-II and -III strains showed only one amplicons (~660 and ~800 bp, respectively), with one exception for the strain Pi42 of Clade-II (Fig. 2). This finding indicates that the multiplex PCR assay provided ~98% overall accuracy for genotyping of *Py. insidiosum*.

The strain Pi42 was confirmed to be a Clade-II strain by the Maximum Likelihood-based phylogenetic analysis of the ITS rDNA region in this study (Fig. 3) and a previously reported study (Chaiprasert et al., 2009) (Table 1). Instead of having one PCR product (based on ITS1/R2 amplification), as usually observed in the other Clade-II strains, the strain Pi42 gave two PCR products that had compatible sizes

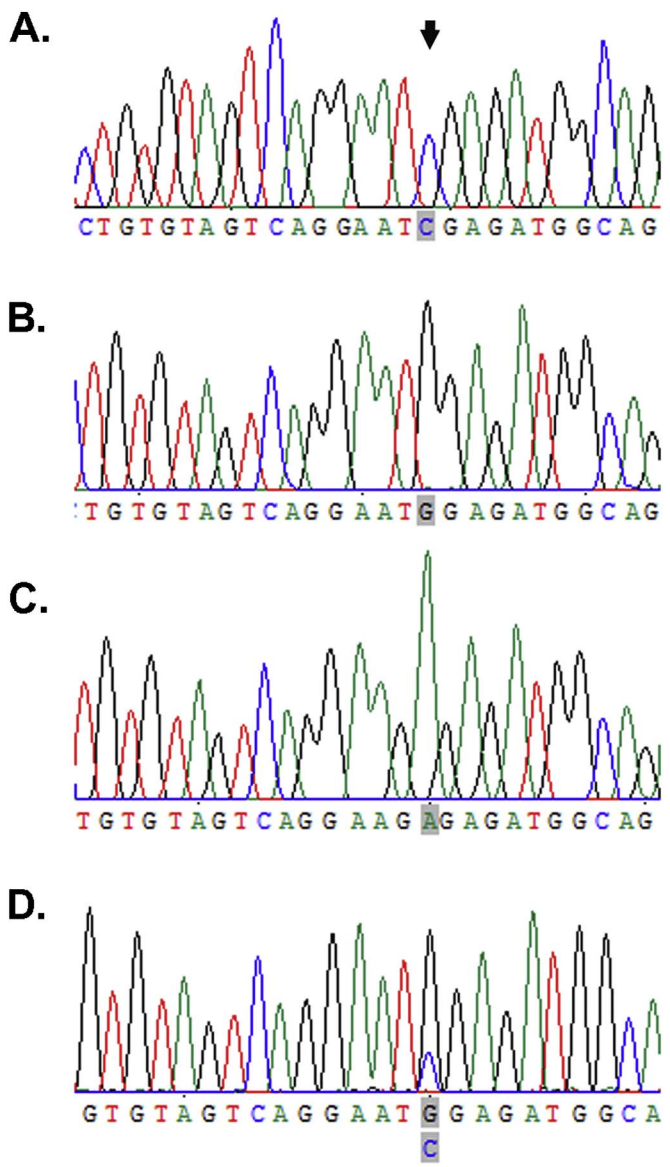

Fig. 4. Chromatograms of the rDNA sequences of *Py. insidiosum*: The arrow indicates the 3′-end nucleotide of the primer R1 annealing site in the rDNA region of the Clade-I (A), Clade-II (B), Clade-III (C), and Pi42 (D) strains [the letters A (green), C (blue), G (black), and T (red) represent nucleotides]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

with that of the Clade-I strains (with ITS1/R1 and ITS1/R2 products; Fig. 2). In addition, the chromatogram of the rDNA sequence of the strain Pi42, at the annealing site of the primer R1, shows two different peaks: a tall peak of nucleotide 'G' (the SNP found only in Clade-II strains) and an unexpected short peak of nucleotide 'C' (the SNP usually found in Clade-I strains) (Figs. 1B and 4D). Taken together with the fact that rDNA is a multi-copy locus (Grooters and Gee, 2002; Krajaejun et al., 2011), this information indicates that strain Pi42 has two different versions of the ITS region in its rDNA repeat sequence. In most of the genic units within the repeat, the sequence matches the expected Clade-II SNP, with a 'G', but in a minority of the genic units within the rDNA repeat, the 'G' is replaced with a 'C' (Clade-I SNP). This result with Pi42 suggests that in future use of this multiplex PCR for genotyping *Py. insidiosum*, if the gel analysis results are that Clade-I bands are not of the expected relative intensities, then further DNA analyses may be necessary to confirm the clade designation.

The multiplex PCR assay was also evaluated for its diagnostic efficiency for *Py. insidiosum*. The PCR results were read as positive when an expected PCR product is present, while read as negative if no PCR product is observed. All control samples from clinically relevant fungi (n = 22), including *Aspergillus* species, *Fusarium* species, and Zygomycetes (i.e., *Mucor*, *Rhizopus* and *Conidiobolus* species) that share microscopic features with *Py. insidiosum* were determined to be PCR negative, indicating that the assay has 100% detection specificity. On the other hand, all 53 gDNA samples extracted from *Py. insidiosum* were determined to be PCR positive, indicating that the assay has 100% detection sensitivity.

Among the *Py. insidiosum* samples, one gDNA, extracted from a strain of *Py. insidiosum* recently-isolated from an infected horse in Thailand (designated as the strain Pi53; Table 1), was also included in the multiplex PCR assay evaluation. There was only one PCR product at the size of ~660 bp, indicating that the organism was a Clade-II *Py. insidiosum* strain, as confirmed by the other methods, i.e., culture identification, sequence homology analysis, and rDNA-based phylogenetic study.

In conclusion, the SNP-based multiplex-PCR assay for identification and genotyping of *Py. insidiosum* was successfully developed. Based on the clade-specific patterns of PCR products, the assay provided 98% accuracy for genotyping, and 100% for both detection specificity and sensitivity. The newly-developed assay was also used to successfully identify, to our knowledge, the first proven isolate of *Py. insidiosum* from an animal in Thailand (clinical features of this animal pythiosis case will be described in details elsewhere). Taken together, the multiplex PCR is a simple and efficient assay for routine identification of *Py. insidiosum* isolated from clinical samples and the environment. The assay is also an alternative tool for convenient, rapid and cost-effective genotyping of the organism. Thus, correlation of the pathogen genotypes and clinical data is now more feasible, which can promote other areas of *Py. insidiosum* studies, i.e., molecular epidemiology, infection outbreaks, and host-pathogen associations.

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