RANDOM AMPLIFIED POLYMORPHIC DNA TYPING AND PHYLOGENY OF *PYTHIUM INSIDIOSUM* CLINICAL ISOLATES IN THAILAND

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Abstract. Forty-three *Pythium insidiosum* clinical isolates recovered from human pythiosis cases in Thailand were characterized by random amplified polymorphic DNA (RAPD) analysis. Three random oligonucleotide primers, OPW11, OPW12 and OPX13 generated 39, 34 and 35 DNA patterns with high value of typeability (100%), reproducibility (98.5, 88.8 and 93.3%) and discriminatory power (0.83, 0.82 and 0.77), respectively. Using GelCompar software based on band similarity, the 43 clinical isolates of *P. insidiosum* could be arranged into 9, 13 and 11 clades using OPW11, OPW12 and OPX13, respectively and the combination of all three primers revealed 36 RAPD patterns. Members in each RAPD pattern varied in both clinical forms and/or geographical locations. RAPD pattern 15 was found in 6 isolates, half of which were found in central region of Thailand. Isolates MCC15 and MCC16 isolated from different patients exhibited identical pattern with all three primers. Our results revealed high genetic heterogeneity among *Pythium insidiosum* isolates in Thailand. RAPD method should be appropriate for future epidemiological studies of *P. insidiosum* strains from patients and from natural habitats.

INTRODUCTION

Members of the genus *Pythium* have been described as aquatic organism-like fungi and classified in the Kingdom Chromista (Kwon-Chung, 1994; Martin, 2000). In addition, they are recognized as ubiquitous pathogen of plant and fish throughout the world (Paul *et al*, 1998; Bernard and Isaac, 2000). *Pythium insidiosum* is the only species reported as a mammalian pathogen and causes pythiosis insidiosi, a rare disease but with high morbidity and mortality. Human pythiosis was first reported in 1985 (Thianprasit, 1986), and was described in the patients from Thailand in 1996 (Thianprasit *et*

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al, 1996). Recently, 102 cases have been reported in the literature (Prasertwitayakij *et al*, 2003; Krajaejun *et al*, 2006); however, some cases were not documented informatively. In Thailand, clinical manifestations of human pythiosis are usually represented as subcutaneous/cutaneous, systemic and ophthalmic infections (Wanachiwanawin *et al*, 1993; Imwidthaya, 1994; Thianprasit *et al*, 1996; Krajaejun *et al,* 2006). Systemic pythiosis or vasculitis forms occur mostly in patient who have underlying disease such as thalassemia and hemoglobinopathy syndrome. The reason why human pythiosis in Thailand has a higher prevalence than animal pythiosis compared to other countries (Prasertwitayakij *et al*, 2003; Krajaejun *et al,* 2006) needs to be explored.

The severity of pythiosis may be due to virulence levels of etiologic agents (Vincent *et al,* 1986; Spitzer *et al*, 1989; Keath *et al*, 1992) or to host immunity. In order to study the virulent strains, typing is a meaningful implement for preliminary investigation. In addition, recent evidences show a high degree of genomic heterogeneity of *P. insidiosum* from different geographical regions, including isolates from Thailand (Schurko *et al*, 2003a,b), which are present in two clades isolated from Australia, Asia and Japan. This has led us to investigate the genomic variation of *P. insidiosum* isolated from human pythiosis cases in Thailand by using a common molecular technique, random amplified polymorphic DNA (RAPD) method.

MATERIALS AND METHODS

P. insidiosum isolates

A total of 43 clinical isolates of *P. insidiosum* obtained from three different clinical manifestation, cutaneous/subcutaneous (n = 4), systemic (n = 17) and ophthalmic pythiosis (n = 22), were used in this study (Table 1). Of these, 9 isolates (No. 35 to 43) were kindly provided from Dr Theerapong Krajaejun, Clinical Immunology Laboratory, Department of Pathology, Ramathibodi Hospital. All isolates were routinely identified and confirmed as *P. insidiosum* by immunodiffusion test (Imwidthaya and Srimuang, 1989) and zoospore induction (Chaiprasert *et al*, 1990). In addition, other two *Pythium* spp (*P. middletonii* and *P. aphanidermatum* CBS 118.80) causing disease in plant were also used as out-group in this study for comparison of band polymorphisms.

Genomic DNA extraction

Cultures were maintained and grown on Sabouraud dextrose agar plate for 5 days at 25ºC. The mycelial-attached agars were transferred to Sabouraud dextrose broth and subsequently incubated with shaking for 5 days at room temperature. Chromosomal DNA was extracted by grinding of mycelial mass with mortar and pestle in liquid nitrogen and lysis buffer, and thereafter purified by phenol-chloroform method as described previously (Jackson *et al*, 1999). DNA concentration and purity were estimated by measurement of an optical density (OD) at wavelength 260 nm and 280 nm in spectrophotometer (Shimadzu U160A, Japan). DNA integrity was also checked by 1% agarose gel-electrophoresis.

RAPD analysis

Five different primers, namely OPU6 (5′-ACCTTTGCGG-3′), OPU14 (5′-TGGGTC CCTC-3′), OPW11 (5′-CTGATGCGTG-3′), OPW12 (5′-TGGGCAGAAG-3′) and OPX13 (5′-ACGGGAGCAA-3′) (Invitrogen, Tokyo, Japan), were selected from the previous study (Siriwattanametanon, 2000) and used to generate bands in RAPD. Components in 50 μl of RAPD reaction composed of 25 ng *P. insidiosum* DNA, 1xPCR buffer [10 mM Tris-HCl pH 8.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.001% (w/v gelatin)], 0.4 mM of each of the four deoxynucleoside triphosphates (dNTPs) (Amersham Pharmacia, UK), 20 pmol each of oligonucleotide primers, and 2 units of *Taq* DNA polymerase (Amersham Pharmacia, UK).

To reduce variations of PCR mixture preparation, a number of master mix was prepared equal to the number of tested isolates, and aliquots were stored at -20ºC until used. Amplifications were performed in a thermal cycler (Perkin-Elmer 480, Cetus, USA), which consisted of initial denaturation at 94ºC for 5 minutes, followed by 40 cycles of denaturation at 94ºC for 1 minute, annealing at 36ºC for 1 minute, extension at 72ºC for 2 minutes and final extension at 72ºC for 10 minutes. Amplified products were then fractionated by 1.4% ultra pure agarose gel-electrophroresis in 1xTBE buffer (90 mM Tris-borate, 2 mM EDTA, pH 8.0) at 5.0 V/cm using EC 360 maxicell gel system (20x20 cm) (Gibco BRL, USA). Gels were stained with ethidium bromide (0.5 μg/ml), and photographed on UV transilluminator (Spectroline TC-321, USA) using a Polaroid camera (Polaroid MP4+, USA) to record the DNA patterns. The experiments were performed on at least two different occasions in duplicate. To test reproducibility, repeat amplification at two different times and duplicative run of each amplification were performed. In addition, *P. insidiosum* CBS 673.85 was used as a positive control and included with each amplification in order to observe the stability of DNA pattern. If there were discordances, a third experiment was performed.

Dendrogram construction

The DNA patterns were stored in a Tag Image File Format (TIFF). The GelComparII software version 1.5 (Applied Maths, Belgium) was used for estimation of the DNA fragment molecular weights and normalization prior to analysis. For dendrogram construction, band sizes ranging from 200 bp to 2,000 bp were selected for analysis by autosearch facility of the software and later assigned by eyes. Cluster analysis was performed using the Jaccard coefficient and the unweighted pair group method with arithmetic averages (UPGMA). Typeability percentage was the ratio of the number of strains producing banding pattern and the number of strains tested (Bidet *et al*, 2000). Reproducibility percentage was calculated from the number of isolates that gave the same result on repeated testing (Bidet *et al*, 2000). Discriminatory power was the ability to distinguish between unrelated strains and was reported as a discrimination index (*D*) using the following equation (Hunter and Gaston, 1988):

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} n_j (n_j - 1)$$

where N is the total number of isolates in the sample population, s is the total number of types obtained, and n_j is the number of isolates belonging to the j^{th} type.

RESULTS

Typeability and reproducibility

Two primers, OPU6 and OPU14, were excluded in RAPD analysis, due to their poor priming ability. The other primers, OPW11, OPW12 and OPX13, exhibited high variability among the isolates. All 43 isolates of *P. insidiosum* and 2 pathogenic plant species were typeable with these three primers. Different patterns occasionally occurred in some isolates (two, five and three isolates using OPW11, OPW12 and OPX13, respectively). The actual patterns of those isolates were determined in a third amplification. The reproducibility of primer OPW11, OPW12 and OPX13 was 95.5, 88.8 and 93.3%, respectively.

RAPD analysis

Amplification with OPW11, OPW12 and OPX13 primers displayed 39, 34 and 35 different RAPD patterns among 45 isolates, respectively (data not shown). By using 38% band similarity as the cut-off value to separate *P. aphanidermatum* and *P. middletonii* from *P. insidiosum*, results of the dendrogram construction revealed 9, 13 and 11 different

Table 1
Sources of reference strains and clinical isolates.

No	Reference number	Age/ Sex	Year	Sources of specimen/clinical form	Residential province (region of Thailand)
1	CBS 673.85 [Kr]	23/Male	1985	Cutaneous infection	NA
2	SIMI 6666 [C-R]	42/Male	1986	Pus from corneal ulcer	Kamphaeng Phet (North)
3	MCC 4 [BS]	25/Male	1986	Ramathibodi/Arteritis	Suphan Buri (Central)
4	MCC 9 [LA]	46/Female	1986	Ramathibodi/Arteritis	Phichit (North)
5	SIMI 7873 [P-S]	31/Female	1988	Thrombus from Lt illiac artery	Chanthaburi (East)
6	SIMI 7874 [P-S]	31/Female	1988	Tissue from Lt illiac artery	Chanthaburi (East)
7	SIMI 8659 [M-K]	52/Male	1988	Tissue from skin, thrombus	Suphan Buri (Central)
8	SIMI 8727 [P-M]	19/Male	1988	Pus and bone tissue from leg	Yasothon (Northeast)
9	MCC 7 [PU]	62/Male	1988	Ramathibodi/Ophthalmic	Ayutthaya (Central)
10	MCC 1 [S-T]	20/Male	1989	Tissue from corneal ulcer	Samut Prakan (Central)
11	SIMI 9642 [O-C]	53/Female	1989	Pus from corneal ulcer	Yasothon (Northeast)
12	SIMI 9743 [O-C]	53/Female	1989	Tissue from cornea	Yasothon (Northeast)
13	SIMI 10201 [N-S]	23/Female	1989	Corneal scraping	Phatthalung (South)
14	MCC 3 [BC]	40/Male	1991	Ramathibodi/Cutaneous	Phichit (North)
15	MCC 2(477-35) [S-Ko]	33/Female	1992	Corneal ulcer	Chai Nat (Central)
16	MCC 8 [TO]	62/Male	1992	Occular	Narathiwat (South)
17	M29 [MU]	48/Male	1993	Known as systemic (arteritis)	Ratchaburi (Central)
18	MCC 5 [P-L]	49/Female	1993	Siriraj/ Arteritis	Bangkok (Central)
19	SIMI 16068 [N-K]	26/Female	1994	Corneal ulcer (left eye)	NA
20	SIMI 240-37 [P-D]	78/Male	1994	Known as ophthalmic	Samut Sakhon (Central)
21	SIMI 322-37 [W-Ch]	26/Male	1994	Fibrin from corneal ulcer	NA
22	SIMI 348-37 [D-C]	36/Male	1994	Ophthalmic	Bangkok (Central)
23	SIMI 18093 [J-J]	50/Female	1995	Ophthalmic	Samut Sakhon (Central)
24	SIMI 283-40 [P-H]	58/Female	1997	Discharge and corneal scrape from eye	Bangkok (Central)
25	SIMI 149-41[K-S]	14/Male	1998	Thrombus in femoral (Rt) artery	Lop Buri (Central)
26	SIMI 2989-42 [L-P]	72/Male	1999	Tissue and clot blood	Suphan Buri (Central)
27	MCC 10 [PC]	12/Male	2000	Ramathibodi/Gastrointestinal tract	Saraburi (Central)
28	MCC 11 [RP]	48/Female	2000	Ramathibodi/Cornea	Chon Buri (East)
29	SIMI 3306-44 [J-U]	40/Female	2001	Corneal pus	Nakhon Si Thammarat (South)
30	SIMI 4763 [B-J]	40/Male	2001	Tissue	Ratchaburi (Central)
31	MCC 12 [No]	46/Male	2001	Ramathibodi/Cornea	Nakhon Ratchasima (Northeast)
32	MCC16 [Pr-Kr]	56/Male	2001	Chiang Mai 2/ subcutaneous tissue	Lampang (North)
33	SIMI 2921-45 [H-R]	75/Female	2002	Corneal tissue	Ratchaburi (Central)
34	SIMI 4523-45 [J-M]	37/Male	2002	Corneal recipient	Pathum Thani (Central)
35	MCC13 [P-K]	15/Male	2002	CU1 /Acute cellutitis	Saraburi (Central)
36	MCC14 [SA]	63/Female	2002	CU2 / Arteritis	Ayutthaya (Central)
37	MCC15 [S-S]	44/Male	2002	Chiang Mai 1/Arteritis	Nan (North)
38	MCC17 [J-R]	26.5Female	2002	Chiang Mai 3 /Brain tissue	Chiang Mai (North)
39	MCC18 [S-P]	47/Male	2002	Chiang Mai 4 / Cornea	Chiang Mai (North)
40	SIMI 916-46 [S-Y]	31/Female	2003	Corneal recipient	Nakhon Ratchasima (Northeast)
41	SIMI 1126-46 [M-M]	55/Male	2003	Vessel	Suphan Buri (Central)
42	SIMI 1839-46 [K-C]	19/Female	2003	Tissue from leg	Nakhon Pathom (Central)
43	MCC 6 [PYII]	-	NA	Siriraj/ Occular	NA
44	MCC19 [CBS 118.80]	Unknown	NA	*Pythium aphanidermatum*	France
45	MCC20	-	NA	*Pythium middletonii*	KU

NA=Not available, KU=Kasetsart University, MCC=Mycology culture collection, CBS=Centraal bureau voor Schimmel cultures

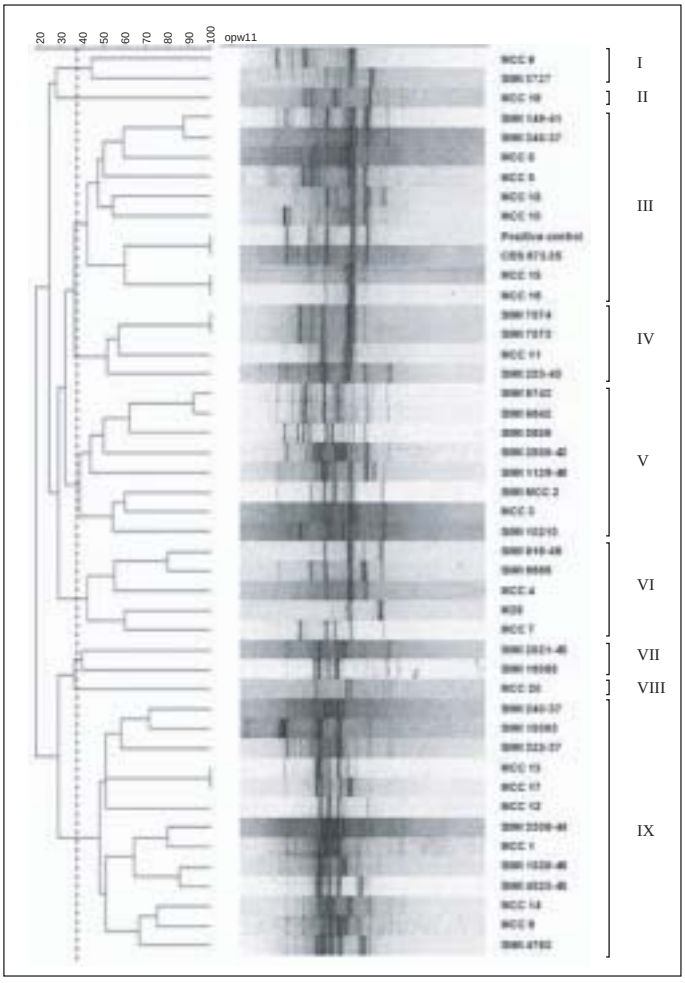

Fig 1–Dendrogram construction obtained from OPW11 primer showing 9 distinctive groups after cluster analysis. The reference strain, *Pythium insidiosum* CBS 673.85, was used as control and indicated as positive control and CBS 673.85. Similarity percentages determined by Dice correlation coefficient (S_{xy}) are shown on the horizontal axis. The names of reference strains and clinical isolates are indicated on the vertical axis.

groups from OPW11, OPW12 and OPX13 primer, respectively (Fig1). Discriminatory power calculated from each primer was 0.83, 0.82 and 0.77, respectively.

Four strains isolated from cutaneous/subcutaneous pythiosis revealed 4 RAPD patterns, namely, pattern 4, 10, 21 and 32. They were isolated from central and northern region of Thailand in 1985, 1991, 2001 and 2002 (Table 2). Twenty-two strains of *P. insidiosum* isolated from ophthalmic pythiosis from all parts of the country, from Chieng Mai to Narathiwat, since 1986 to 2003 (Table 2), showed 17 RAPD patterns. RAPD pattern 15 consisted of 6 strains; 4 of which were isolated from central part of the country, one from an unknown province and the other, one from Nakhorn Si Thammarat since 1991 to 2003 (Table 2). For arteritis form of pythiosis, 15 strains of *P. insidiosum* resulted in 14 patterns. Two isolates from the same patient obtained in 1988 revealed the same RAPD pattern 12. The remaining 13 strains were isolated from central, lower northern, eastern and northeastern region during 1986-2003. There were 2 strains obtained from other systemic forms of pythiosis which showed 2 RAPD patterns. The first strain was isolated from a case of brain from Chiang Mai in 2001 and revealed RAPD pattern 19. The other strain was obtained in 2000 from a patient in Saraburi, a province in central region of Thailand with gastrointestinal pythiosis and showed RAPD pattern 8.

From RAPD analysis using primers OPW11, OPW12 and OPX13, specific correlation between genotypes and clinical signs or the places where the agents isolated was not found in most of the tested isolates (Table 2). Combining results from all three primers generated a high value of discriminatory power (0.98) obtained from 36 different RAPD patterns. RAPD pattern 15 was predominated (6/

Table 2
RAPD patterns obtained from OPW11, OPW12 and OPX13 primers.

Reference number	Year	Clinical manifestation	Geography	Cluster			RAPD pattern no.
				OPW11	OPW12	OPX13	
SIMI 8727 [P-M]	1988	Arteritis	Yasothon (N-E)	I	I	I	1
MCC 8 [TO]	1992	Ophthalmitis	Narathiwat (S)	I	XI	III	2
MCC 9 [L-A]	1986	Arteritis	Phichit (N)	II	VI	III	3
MCC 15 [S-S]	2002	Arteritis	Nan (N)	III	III	I	4
MCC 16 [Pr-Kr]	2001	Subcutaneous	Lampang (N)	III	III	I	4
SIMI 348-37 [D-C]	1994	Ophthalmitis	Bangkok (C)	III	IX	I	5
MCC 18 [S-P]	2002	Ophthalmitis	Chiang Mai (N)	III	V	I	6
SIMI 149-41 [K-S]	1998	Arteritis	Lop Buri (C)	III	V	I	6
MCC 7 [PU]	1988	Ophthalmitis	Ayutthaya (C)	III	VI	III	7
MCC 10 [PC]	2000	Gastrointestinal infection	Saraburi (C)	III	VIII	I	8
MCC 4 [BS]	1986	Arteritis	Suphan Buri (C)	III	XI	I	9
CBS 673.85 [Kr]	1985	Cutaneous	NA	III	XII	I	10
SIMI 283-40 [P-H]	1997	Ophthalmitis	Bangkok (C)	IV	V	I	11
SIMI 7873 [P-S]	1988	Arteritis	Chanthaburi (E)	IV	VI	I	12
SIMI 7874 [P-S]	1988	Arteritis	Chanthaburi (E)	IV	VI	I	12
MCC 11 [RP]	2000	Ophthalmitis	Chon Buri (E)	IV	VIII	I	13
MCC 12 [No]	2001	Ophthalmitis	Nakhon Ratchasima (N-E)	IX	V	V	14
MCC 1 [S-T]	1991	Ophthalmitis	Samut Prakan (C)	IX	V	VI	15
SIMI 1839-46 [K-C]	2003	Arteritis	Nakhon Pathom (C)	IX	V	VI	15
SIMI 240-37 [P-D]	1994	Ophthalmitis	Samut Sakhon (C)	IX	V	VI	15
SIMI 322-37 [W-Ch]	1994	Ophthalmitis	NA	IX	V	VI	15
SIMI 3306-44 [J-U]	2001	Ophthalmitis	Nakhon Si Thammarat (S)	IX	V	VI	15
SIMI 4523-45 [J-M]	2002	Ophthalmitis	Pathum Thani (C)	IX	V	VI	15
SIMI 18093 [J-J]	1995	Ophthalmitis	Samut Sakhon (C)	IX	V	VIII	16
SIMI 4763 [B-J]	2001	Arteritis	Ratchaburi (C)	IX	V	XI	17
MCC 5 [P-L]	1993	Arteritis	Bangkok (C)	IX	VI	I	18
MCC 17 [B-V]	2001	Brain abscess	Chiang Mai (N)	IX	VII	VIII	19
MCC 14 [SA]	2002	Arteritis	Ayutthaya (C)	IX	VIII	IV	20
MCC 13 [P-K]	2002	Acute cellutitis	Saraburi (C)	IX	VIII	VI	21
MCC 19 (*P. aphanidermatum*)	-	-	France	V	II	X	22
SIMI 1126-46 [M-M]	2003	Arteritis	Suphan Buri (C)	V	V	I	23
SIMI 2989-42 [L-P]	1999	Arteritis	Suphan Buri (C)	V	V	IX	24
SIMI 10210 [N-N]	1989	Ophthalmitis	Phatthalung (S)	V	VI	I	25
SIMI 8659 [M-K]	1988	Arteritis	Suphan Buri (C)	V	VI	III	26
SIMI 9642 [O-C]	1989	Ophthalmitis	Yasothon (N-E)	V	VIII	III	27
SIMI 9743 [O-C]	1989	Ophthalmitis	Yasothon (N-E)	V	VIII	III	27
MCC 20 (*P. middletonii*)	-	-	KU	V	XIII	II	28
SIMI 916-46 [S-Y]	2003	Ophthalmitis	Nakhon Ratchasima (N-E)	VI	III	I	29
SIMI 6666 [C-R]	1986	Ophthalmitis	Kamphaeng Phet (N)	VI	V	III	30
MCC 6 [PYII]	-	Ophthalmitis	NA	VI	V	VI	31
MCC 3 [BC]	1991	Subcutaneous	Phichit (N)	VI	VI	I	32
M29 [MU]	1993	Arteritis	Ratchaburi (C)	VI	VI	III	33
SIMI 16068 [N-K]	1994	Ophthalmitis	NA	VII	IX	VI	34
SIMI 2921-45 [H-R]	2002	Ophthalmitis	Ratchaburi (C)	VII	X	VII	35
MCC 2 (477-35) [S-Ko]	1992	Ophthalmitis	Chai Nat (C)	VIII	III	III	36

43) mostly recovered from ophthalmitis pythiosis. This combining of RAPD patterns revealed 4 groups (RAPD pattern 4, 6, 12 and 27) that consisted of 2 isolates in each group. Of these, the isolates obtained from the same patient revealed identical pattern. RAPD pattern 6 included isolates from ophthalmitis and arteritis pythiosis patients in northern and central region of Thailand, respectively. In pattern No. 4, isolate MCC15 and MCC16 exhibited identical patterns with all three primers. These 2 isolates were collected apart from 2 patients residing in North Thailand.

DISCUSSION

Molecular typing is a powerful test for preliminary investigation of strain characteristics. In recent years, a numbers of DNA-based typing methods have been developed for high resolution discrimination at the species level (Olive and Bean, 1999). RAPD technique is simple and easy to perform and is suitable for study of microorganisms that lack or have little known genetic information (Williams *et al*, 1990). The major disadvantage of RAPD method is the inconsistency of the patterns obtained from different laboratories (Tyler *et al*, 1997). There are several factors affecting the reproducibility of RAPD technique, such as PCR buffer (Hopkins and Hilton, 2001), dNTPs and Mg^{2+} concentration (Park and Kohel, 1994), model of thermalcycler (Saunders *et al*, 2001), source of *Taq* polymerase (Hughes *et al*, 1994), PCR condition (Benter *et al*, 1995), and concentration of DNA and primer (Davin-Regli *et al*, 1995). In this study, analysis of RAPD patterns by eyes revealed identical type or ≥ 90% similarity with GelCompar of clinical isolate strains SIMI 9642-SIMI 9743 and SIMI 7873-SIMI 7874. Each pair was obtained from the same patient at different times. With the high percentage of reproducibility (95.5, 88.8 and 93.3% for OPW11, OPW12 and OPX13, respectively), these results signified a good reproducibility of RAPD analysis in our study and demonstrated genomic stability of *P. insidiosum* cultures during laboratory maintenance as well.

The 43 clinical isolates generated 39, 34 and 35 RAPD types patterns from OPW11, OPW12 and OPX13 primer, respectively. These results indicated the genetic diversity of clinical isolates in Thailand which is different from other previous studies (Schurko *et al*, 2003a,b). Schurko *et al* (2003a) compared 28 isolates of *P. insidiosum* and *P. destruens* from Asia, Australia and the United States of America on the basis of restriction fragment length polymorphisms (RFLP) of amplified ribosomal intergenic spacer. They were grouped according to band profiles into three distinct clusters which revealed geographical correlation. These clusters were confirmed by sequence analysis of the ribosomal DNA internal transcribed spacer. Three isolates from Thai patients were classified in clades II and III with the strains from Asia (Schurko *et al* 2003b). As *P. insidiosum* has both sexual (oogamous) and asexual (zoosporogenesis) reproduction (De Cock *et al*, 1987) and zoospore itself is actively motile in aquatic environment, the diversity of RAPD patterns in our isolates may be associated with sexual recombination of the etiologic agent (Martin, 1995) and/or pasasexual recombination of zoospore in nature (De Cock *et al*, 1987). RAPD pattern 15 comprised of 6 isolates (14%), and was found in 5 ophthalmic cases and 1 arteritis case. Correlation of genetic variant of such isolates with clinical feature needs to be further investigated. Interestingly, RAPD profiles of MCC15 and MCC16 exhibited identical patterns using all three primers and the patients resided in north Thailand. Evidence of animal-to-animal and/or animal-to-human transmission of pythiosis has not been recorded (Imwidthaya, 1994; Thainprasit *et al*, 1996; Prasertwitayakij *et al*, 2003). Because RAPD analysis produces strain-specific fingerprint (Kersulyte *et al*, 1992), the identical RAPD pattern of MCC15 and MCC16 could be due to that the patients

were infected with the same strain of *P. insidiosum* or that *P. insidiosum* with this RAPD pattern is more virulent than others. Studies on pathogenesis of different *P. insidiosum* strains should be explored in the future.

In summary, RAPD method can be used for typing *P. insidiosum,* provides high reproducibility and reveals more discriminatory power than RFLP method. However, the genotypes were not specifically correlated with clinical forms of pythiosis or with the places where the agents were isolated. Nevertheless, the results presented here could serve as useful data for molecular epidemiology study of *P. insidiosum* in environmental niches in the future.

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REFERENCES

Benter T, Papadopoulos S, Pape M, Manns M, Poliwoda H, Optimization and reproducibility of random amplified polymorphic DNA in human. *Anal Biochem* 1995; 230: 92-100.

Bernard P, Isaac M. ITS I region of the nuclear ribosomal DNA of the mycoparasite *Pythium periplocum*, its taxonomy, and its comparison with related species. *FEMS Microbiol Lett* 2000; 189: 61-5.

Bidet P, Lalande V, Salauze B, *et al*, Comparison of PCR – ribotyping, arbitrarily primed PCR, and pulsed-field gel electrophoresis for typing *Clostridium difficile*. *J Clin Microbiol* 2000; 38: 2484-7.

Chaiprasert A, Samerpitak K, Wanachiwanawin W, Thasnakorn P. Induction of zoospore formation in Thai isolates of *Pythium insidiosum*. *Mycoses* 1990; 33: 317-23.

Davin-Regli A, Abed Y, Charrel RN, Bollet C, de Micco P. Variation in DNA concentrations significantly affect the reproducibility of RAPD fingerprint patterns. *Res Microbiol* 1995; 146: 561-8.

De Cock AWAM, Mendoza L, Padhye AA, Ajello L, Kaufman L. *Pythium insidiosum* sp. nov., the etiologic agent of pythiosis. *J Clin Microbiol* 1987; 25: 344-9.

Hopkins KL, Hilton AC. Optimization of random amplification of polymorphic DNA analysis for molecular subtyping of *Escherichia coli* 0157. *Lett Appl Microbiol* 2001; 32: 126-30.

Hughes MS, Beck LA, Skuce RA. Identification and elimination of DNA sequences in *Taq* DNA polymerase. *J Clin Microbiol* 1994; 32: 2007-8.

Hunter PR, Gaston MA. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *J Clin Microbiol* 1988; 26: 2465-6.

Imwidthaya P. Human pythiosis in Thailand. *Postgrad Med J* 1994; 70: 558-60.

Imwidthaya P, Srimuang S. Immunodiffusion test for diagnosing human pythiosis. *Mycopathologia* 1989; 106: 109-12.

Jackson CJ, Barton RC, Evans EGV. Species identification and strain differentiation of dermatophyte fungi by ribosomal-DNA intergenic spacer regions. *J Clin Microbiol* 1999; 37: 931-6.

Keath EJ, Kobayashi GS, Medoff G. Classification of *Histoplasma capsulatum* by restriction fragment length polymorphisms in a nuclear gene. *J Clin Microbiol* 1992; 30: 2104-7.

Kersulyte D, Woods JP, Keath EJ, Goldman WE, Berg DE. Diversity among clinical isolates of *Histoplasma capsulatum* detected by polymerase chain reaction with arbitrary primers. *J Bacteriol* 1992; 174: 7075-9.

Kwon-Chung KJ. Phylogenetic spectrum of fungi that are pathogenic to human. *Clin Infect Dis* 1994; 19(suppl): S1-S7.

Krajaejun T, Sathapatayavongs B, Pracharktam R, *et al.* Clinical and epidemiological analyses of human pythiosis in Thailand. *Clin Infect Dis* 2006; 43; 569-76.

Martin FN. Meiotic instability of *Pythium sylvaticum* as demonstrated by inheritance of nuclear markers and karyotype analysis. *Genetics*

1995; 139: 1233-46.

Martin FN. Phylogenetic relationships among some *Pythium* species inferred from sequence analysis of the mitochondrially encoded cytochrome oxidase II gene. *Mycologia* 2000; 92: 711-27.

Olive DM, Bean P. Principles and applications of methods for DNA-based typing of microbial organisms. *J Clin Microbiol* 1999; 37: 1661-9.

Park YH, Kohel RJ. Effect of concentration of $MgCl_2$ on random-amplified DNA polymorphism. *Biotechnique* 1994; 16: 652-6.

Paul B, Galland D, Bhatnagar T, Dulieu H. A new species of *Pythium* isolated from the Burgundy region in France. *FEMS Microbiol Lett* 1998; 158: 207-13.

Prasertwitayakij N, Louthrenoo W, Kasitanon N, Thamprasert K, Vanittanakom N. Human pythiosis, a rare cause of arteritis: case report and literature review. *Semin Arthritis Rheum* 2003; 33: 204-14.

Saunders GC, Dukes J, Parkes HC, Cornett JH. Interlaboratory study on thermal cycler performance in controlled PCR and random amplified polymorphic DNA analyses. *Clin Chem* 2001; 47: 47-55.

Schurko AM, Mendoza L, de Cock AWAM, Klassen GR. Evidence for geographic clusters: Molecular genetic differences among strains of *Pythium insidiosum* from Asia, Australia and the Americas are explored. *Mycologia* 2003a; 95: 200-8.

Schurko AM, Mendoza L, Lévesque CA, Désaulniers NL, De Cock AWMA, Klassen GR. A molecular phylogeny of *Pythium insidiosum*. *Mycol Res* 2003b; 107: 537-44.

Siriwattanametanon W. *Penicillium marneffei*: molecular typing, viability in acid medium, and drug susceptibility. Bangkok: Mahidol University, 2000. 99 pp. MSc Thesis.

Spitzer EE, Lasker BA, Travis SJ, Kobayashi GS, Medoff G. Use of mitochondrial and ribosomal DNA polymorphisms to classify clinical and soil isolates of *Histoplasma capsulatum*. *Infect Immun* 1989; 57: 1409-12.

Thianprasit M. Fungal infection in Thailand. *Jpn J Dermatol* 1986; 96: 1343-5.

Thianprasit M, Chaiprasert A, Imwidthaya P. Human pythiosis. *Curr Top Med Mycol* 1996; 7: 43-54.

Tyler KD, Wang G, Tyler SD, Johnson WM. Factors affecting reliability and reproducibility of amplification-based DNA fingerprinting of representative bacterial pathogens. *J Clin Microbiol* 1997; 35: 339-46.

Vincent RD, Goewert R, Goldman WE, Kobayashi GS, Lambowitz A M, Medoff G. Classification of *Histoplasma capsulatum* isolates by restriction fragment length polymorphisms. *J Bacteriol* 1986; 65: 813-8.

Wanachiwanawin W, Thianprasit M, Fucharoen S, *et al.* Fatal arteritis due to *Pythium insidiosum* infection in patients with thalassemia. *Trans R Soc Trop Med Hyg* 1993; 87: 296-8.

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. DNA polymophisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res* 1990; 18: 6531-5.