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Characterization of *Phytopythium* and *Pythium* species from freshwater area based on Morphological traits and ITS sequence

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Abstract Twenty isolates were identified as *Globisporangium nunn*, *Pythium acanthicum*, *Phytopythium chameahyphon*, *Phytopythium cucurbitacearum*, *Phytopythium helicoides*, *Phytopythium iriomotense*, *Pythium torulosum*, *Phytopythium vexans* and unknown specific epithet *Phytopythium* species. The reconstruction of phylogenetic trees using Neighbour-joining, maximum-likelihood, and maximum-parsimony algorithms revealed that the majority of isolates belonged to clade K. Furthermore, this is the first report of *Phytopythium iriomotense* in Thailand. The current information possible that the discovered oomycetes will be useful in future research and applications.

Keywords: Oomycetes, *Phytopythium* sp., *Pythium* sp., ITS sequence, Diversity

Introduction

The phylum Oomycota contained 800 species that were saprobic and parasitic on terrestrial or aquatic plants and animals. (Rossman and Palm, 2006), particularly *Pythium* sp., a member of the class Oomycetes (Peronosporales, Pythiaceae) is a well-known oomycete genus with over 355 species discovered (Ho, 2018). There have been numerous reports that some plant pathogenic species, such as *Pythium ultimum* cause a severe damage to corn and soybean (Broders *et al*., 2007) or *Pythium dissotocum* that cause diseases in leafy greens on hydroponic systems (Luecke *et al*., 2022). Whereas *Pythium insidiosum* is an important mammalian pythiosis infectious agent (Mendoza, 1998). However, some members of this group, such as *Pythium oligandrum*, have demonstrated exceptional potential as a biological control agent due to their ability to stimulate plant growth, induce plant defence, and exhibit antimicrobial effects on a wide range of plant pathogens (Benhamou *et al*., 2012).

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In the past, classification of *Pythium* spp. was based on morphological traits which was the most tools available to identify and describe species description of the genus *Pythium* such as mycelium and colony formation as chrysanthemum, rosette, and cottony pattern. *Pythium* is reproduced by developing zoosporangia and zoospores (asexual reproduction) or oogonia and antheridia (sexual reproduction) (Van der Plaats Niterink's, 1981).

However, the morphological traits to classify *Pythium* and related species may be insufficient data because those characteristics are easily acquired or lost during the evolution process. As a result, molecular approaches and systematic analysis played a key role in classifying oomycetes. The analysis of *Pythium* phylogeny by using the ITS (internal transcribed spacer) region of the nuclear ribosomal DNA revealed the formation of 11 major clades (Clades A–K) (Levesque and de Cock, 2004). Some species in clade K, such as *Pythium helicoides*, *Pythium cucurbitacearum* and *Pythium vexans* were later designated to *Phytopythium*, a new species in the family Pythiaceae, demonstrating that *Pythium* in clade K which were distinct from others and closely related to *Phytophthora* species (De Cock *et al*., 2015).

The objective of this study was to investigate the diversity of *Phytopythium* and *Pythium* species found in natural environment in Kanchanaburi and Nakhon Nayok, Thailand and to study for their morphological structure. ITS sequences were submitted to GenBank as a guideline for future research.

Materials and methods

Isolation and morphological study

Soil samples and decaying plant debris were collected from river (14.2073666, 99.0598677), waterfall and forest (14.3519008, 98.9226392), (14.3590037, 99.1410994), (14.3110038, 101.2546416), (14.3350209, 101.3225350), (14.3364226, 101.3068337). The numbers represent the sampling locations latitudes and longitudes. Oomycetes were isolated from soil and freshwater samples using the soil plate techniques (Masago *et al*., 1977) and soil baiting techniques (Dhingra and Sinclair 1994), respectively. Rose bengal, and BNPRA (benomyl 10 ppm, nystatin 25 ppm, pentachloronitrobenzene 25 ppm, rifampicin 10 ppm, and amplicillin 500 ppm) was amended to avoid contamination of bacteria in potato dextrose agar (PDA) (Masago *et al*., 1977). The fully grown colony was transferred the hyphal tips to new PDA plates to get pure culture. Morphology was observed in 6 days after inoculated on PDA, V8 juice agar (V8A), and corn meal agar (CMA). Agar plugs of obtained isolates were floated in mineral water in Petri dish for

24 hours under fluorescens to induce sporangium development. Oogonium, oospore and antheridium were observed after culturing the isolate in V8A for 7 days. All structures were observed under microscope using eyepiece wifi camera (MC-500W) and measured using the Pixit pro program.

DNA extraction and sequencing analysis

The mycelia were cultured in V8 broth medium for 14 days before filtering and rinsed with distilled water before drying. A GF-1 Fungus DNA Extraction Kit was used to extract DNA (Vivantis, Malaysia). A spectrophotometer (Eppendorf, USA) was used to measure DNA purity at 280 nm. Amplifications of DNA fragments were performed using the Polymerase Chain Reaction technique (PCR). Master Mix PCR (Vivantis, Malaysia), forward universal primer ITS6 (5' GAAGGTGAAGTCGTAACAAGG 3') and reverse universal primer ITS4 (5' TCCTCCGCTTATTGATATGC 3') (Hung *et al*., 2015) were used to amplify partial sequence of internal transcribed spacer (ITS) rDNA region. PCRs condition consisted of 1 cycle of 95℃ for 2 min; 30 cycles of 95℃ for 20 s, 55℃ for 25 s, 72℃ for 50 s; and a final cycle of 72℃ for 10 min (Cooke *et al*., 2000). The quality of PCR yields obtained by gel electrophoresis were then examined. PCR products sequences were then analyzed by Bionics Co., Ltd (Seoul, Korea) and compared with the sequences from Genbank database (The National Center for Biotechnology; NCBI; Bethesda, USA) using Basic Local Alignment Search Tool (BLAST). All nucleotide data were then submitted into the Genbank database (NCBI database).

Phylogenetic analyses

All sequences were examined and edited using the Finch TV 1.4 sequence analysis software (https://finchtv.software.informer.com/1.4/). DNA Sequence FASTA files were used to assemble Contiguous DNA Sequence using the BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Phylogenetic tree was analyzed by using MEGA 7 software (https://www.megasoftware.net/), Each *Pythium* sp. and *Phytopythium* sp. sequence were aligned by using ClustalW function. Neighbor-joining algorithm (Saitou and Nei, 1987) was used to reconstruct the phylogenetic tree. Maximum-likelihood (Felsenstein, 1985) and maximum-parsimony algorithm have also been used to compare the undifferentiated branches (Kluge and Farris, 1969). The model of Jukes and Cantor was used for calculating evolutionary distances (Jukes and Cantor, 1969). The phylogenetic tree reliability was evaluated using a 1000 repeated bootstrap support values

analysis (Felsenstein, 1985). *Aphanomyces salsuginosus* (Oomycetes, Saproleginales) was used as an outgroup.

Results

Morphological characteristics

Twenty isolates of *Pythium* spp. and *Phytopythium* spp. were identified, twelve isolates were obtained from Kanchanaburi soil sample. Five and three isolates of *Pythium* spp. and *Phytopythium* spp. were collected from Nakhon Nayok soil and decaying plant debris samples, respectively. The average growth of isolates in 2-4 days was observed in V8A, PDA, and CMA media. Ingeneral, colony were colorless or slightly yellowish with three types of pattern formation were considered as chrysanthemum, cottony, and rosette. Hyphae were coenocytic and hyaline. Both sexual and asexual structures were discovered as seen in Table 1. All isolates are categorized into 9 species as follows:

Isolate KS-18 is similar to *Globisporangium nunn*, colony pattern is chrysanthemum, hypha (av. 3.9 µm). The sporangium structure is absent. Oogonium smooth wall (av. 21.9x21.65 µm) and thin-walled (av. 1.8 µm), oogonium with diclinous antheridium (av. 7.0x8.6 µm) (Figure 1).

Table 1. Morphological study of the isolates

Isolate	Full growth (Days)			Morphological structures					Host/ Location
	V8A	PDA	CMA	Hypha e (um)	Sporangia (asexual) (µm, length × wide)	Oogonia (µm, length × wide)	Oospore (µm, length × wide)	Antheridia (Sexual)	
KS-18	4	4	4	(av. 3.9)	-	Smooth wall, oogonium (av. 21.9x21.65) and thin-walled (av. 1.8)		diclinous antheridium (av. 7.0x8.6)	Soil/ Kanchanaburi
KS-17	3	3	4	(av. 2.9)	-	terminal or intercalary, globose, (av. 19.2x19.8), thin-walled (av. 1.7), projections conical with a blunt tip (av. 2.5x2.6)	-	1 or 2 antheridium per oogonium (av. 5.6x6.6)	Soil/ Kanchanaburi

av. = average

Table 1. (Continue)

Isolate	Full growth (Days)			Morphological structures					Host/ Location
	V8A	PDA	CMA	Hyphae (um)	Sporangia (asexual) (µm, length × wide)	Oogonia (µm, length × wide)	Oospore (µm, length × wide)	Antheridia (Sexual)	
NS-1	3	3	3	(av. 3.2)	subglobose or Obovoid, non-papillate sporangia (av. 19.8x27.7) with more than 1 discharge tubes (av. 3.8x12.2)	Smooth wall (av. 24.9x25.3)	Aplerotic oospores (av. 23.0x23.7) thick wall (av. 2.5)	1 antheridium per oogonium, diclinous antheridium	Soil/ Nakhon Nayok
KS-9	4	4	4	(av. 3.6)	Globose sporangium (av. 19.0x18.2) with zoospore (av. 7.8x9.9)	Smooth wall (av. 15.2x16.4)	Aplerotic oospores (av. 18.7x19.3) and thick wall (av. 1.9)	monoclinous antheridium	Soil/ Kanchanaburi
KS-11	4	4	4						
NS-20	3	3	3						
KS-13	4	3	4	(av. 5.2)	Subglobose sporangium, proliferous sporangium (av. 25.5x27.9) with papilla (av. 4.8x4.9) and discharge tubes arising apically (av. 6.5x20.4)	Aplerotic, smooth wall (av. 15.8x15.6)	-	1 antheridium per oogonium winding around the oogonial stalk (av. 3.5x8.0)	Soil/ Kanchanaburi
NL-18	2	2	2						Decaying plant debris/ Nakhon Nayok
NL-6	3	3	3	(av. 6.0)	Globose and non-proliferating sporangia (av. 25.5x24.9)	-	-	-	decaying plant debris/ Nakhon Nayok
KS-15	4	4	3	(av. 5.2)	Filamentous and inflated filamentous sporangium	Smooth wall (av. 21.3x21.8)	Plerotic oospores (av. 19.7x19.5) thick wall (av. 2.6)	-	Soil/ Kanchanaburi
KS-16	4	4	3						

av. = average

Table 1. (Continue)

Isolate	Full growth (Days)			Morphological structures					Host/ Location
	V8A	PD	CMA	Hyphae (um)	Sporangia (asexual) (µm, length × wide)	Oogonia (µm, length × wide)	Oospore (µm, length × wide)	Antheridia (Sexual)	
KS-7	4	4	4	(av. 5.2)	Globose sporangium (av. 18.6x18.4) and zoospore (av.5.9x8.8)	Smooth wall (av. 21.2x21.5)	Aplerotic oospores (av. 17.2x18.9) and thick wall (av. 2.4)	1-3 antheridium per oogonium	Soil/ Kanchanaburi
KS-12	4	3	4						
NS-3	3	3	3						
NS-4	3	3	3						
NS-9	3	4	4						
NL-19	3	3	3						
KS-1	4	3	4	(av. 3.6)	Globose sporangium (av. 21.3x21.7) with more than 1 discharge tubes (av. 4.2x6.5) and zoospore (av. 6.8x10.0)	-	Smooth wall and plerotic oospores (av. 23.9x24.1) with thin-walled (av. 1.6)	-	Soil/ Kanchanaburi
KS-3	3	3	4						
KS-5	4	4	4						

av. = average

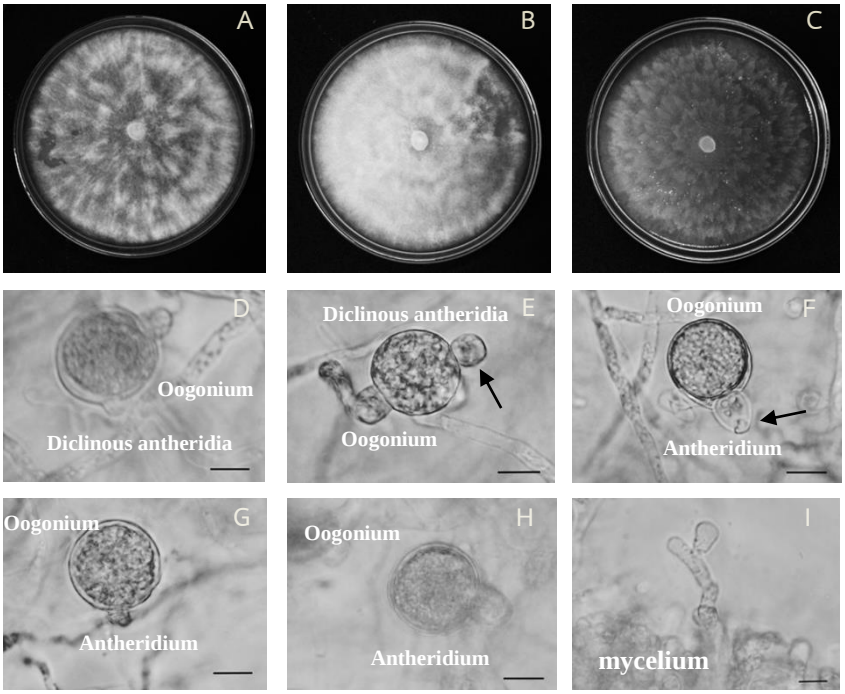

Figure 1. Morphology of *Globisporangium nunn* isolate KS-18. A-C: Colony pattern on V8A (A), PDA (B) and CMA (C) medium at 6 Days; D-H: oogonium and antheridium (40×); I: mycelium (40×); (scale bars at 40×: 10 µm); The arrow represents antheridium

Isolate KS-17 is similar to *Pythium acanthicum*, colony pattern is rosette, hypha (av.2.9 µm). The sporangium structure is absent. Oogonium terminal or intercalary (av. 19.2x19.8 µm) thin-walled (av. 1.7 µm). Ornamented oogonia are projections conical with a blunt tip (av. 2.5x2.6 µm), antheridia 1-2 per oogonium (av. 5.6x6.6 µm) (Figure 2).

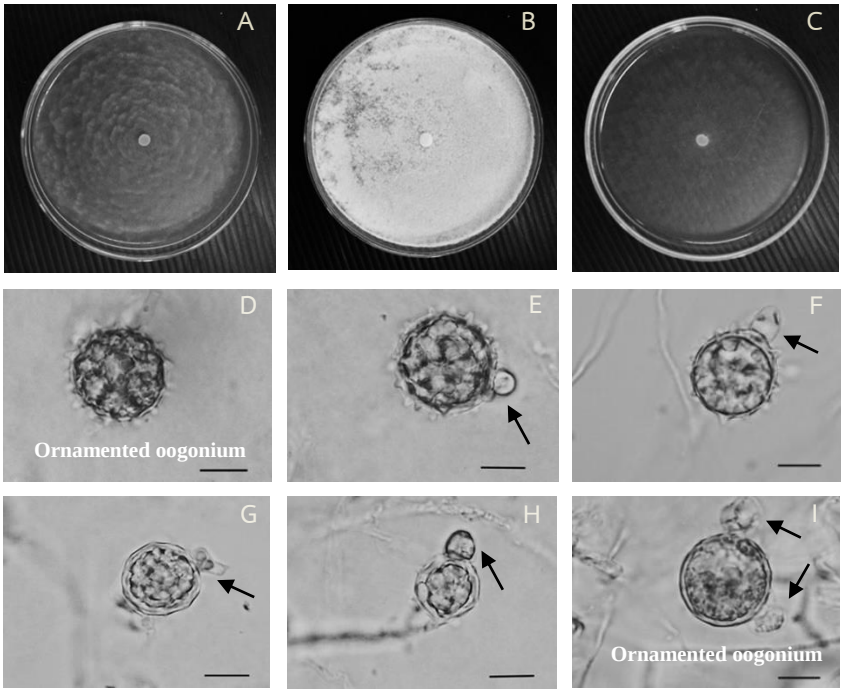

Figure 2. Morphology of *Pythium acanthicum* isolates KS-17. A-C: Colony pattern on V8A (A), PDA (B) and CMA (C) medium at 6 Days; D-I: oogonia and antheridium (40×); (scale bars at 40×: 10 µm); The arrow represents antheridium

Isolate NS-1 is similar to *Phytopythium chamaehyphon*, colony pattern is chrysanthemum, hypha (av. 3.2 µm). Subglobose or obovoid and non-papillate sporangia (av. 19.8x27.7 µm), Sporangium with 1-2 long discharge tube (av. 3.8x12.2 µm) and sporangium are large. Aplerotic oospores (av. 23.0x23.7 µm), thick-walled (av. 2.5 µm) and smooth wall, oogonium (av. 24.9x25.3 µm), 1 antheridium per oogonium, diclinous antheridium (Figure 3).

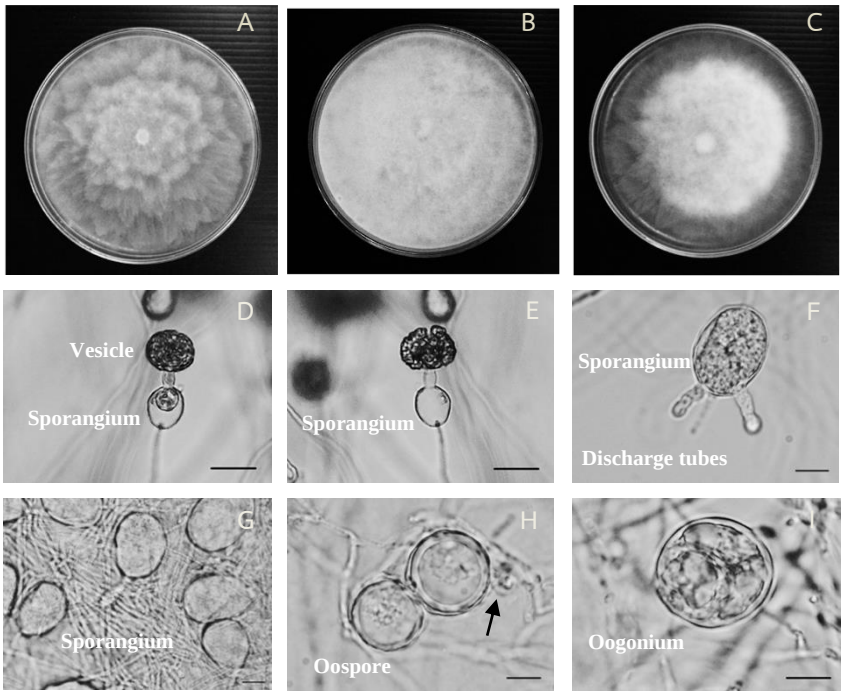

Figure 3. Morphology of *Phytopythium chamaehyphon* isolate NS-1. A-C: Colony pattern on V8A (A), PDA (B) and CMA (C) medium at 6 Days; D-E: sporangium forming vesicle (10×); F-G: sporangium with discharge tube (40×); H: oospore (40×); I: oogonium (40×); (scale bars at 10×: 20 µm; 40×: 10 µm); The arrow represents antheridium

Isolate KS-9, KS-11 and NS-20 are similar to *Phytopythium cucurbitacearum*, colony patterns are chrysanthemum, hypha (av. 3.6 µm). Globose and non-proliferating sporangia (av. 19.0x18.2 µm), encysted zoospore (av. 7.8x9.9 um). Aplerotic oospores (av. 18.7x19.3 um) and thick-walled (av. 1.9), smooth wall, oogonium (av. 15.2x16.4 µm) monoclinous antheridium near and sometime winding the oogonium stalk, antheridia 1 per oogonium (Figure 4).

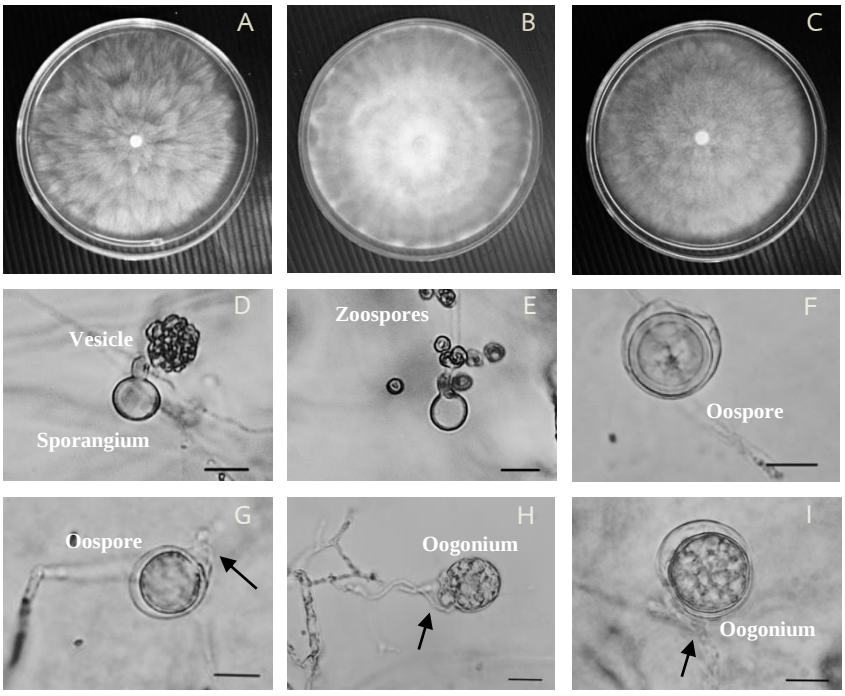

Figure 4. Morphology of *Phytopythium cucurbitacearum* isolates KS-9, KS-11, NS-20 () A-C: Colony pattern on V8A (A), PDA (B) and CMA (C) medium at 6 Days; D: sporangia forming vesicle (10×); E: zoospores liberating from vesicle (10×); F: oospore (40×); G: oospore and antheridium (40×); H: oogonium and antheridium (40×); (scale bars at 10×: 20 µm; 40×: 10 µm); The arrow represents antheridium

Isolate KS-13 and NL-18 are similar to *Phytopythium helicoides*, colony patterns are cottony, hypha (av. 5.2 µm). Subglobose or obovoid and proliferous sporangium (av. 25.5x27.9 µm) with papilla (av. 4.8x4.9 µm) and discharge tubes arising apically (av. 6.5x20.4 µm), encysted zoospore (av. 7.8x9.9 µm). Smooth wall oogonium (av. 15.8x15.6 µm). 1-2 antheridium per oogonium, sometimes winding around the oogonium stalk, elongates and attaches to the terminal oogonium (av. 3.5x8.0 µm) (Figure 5).

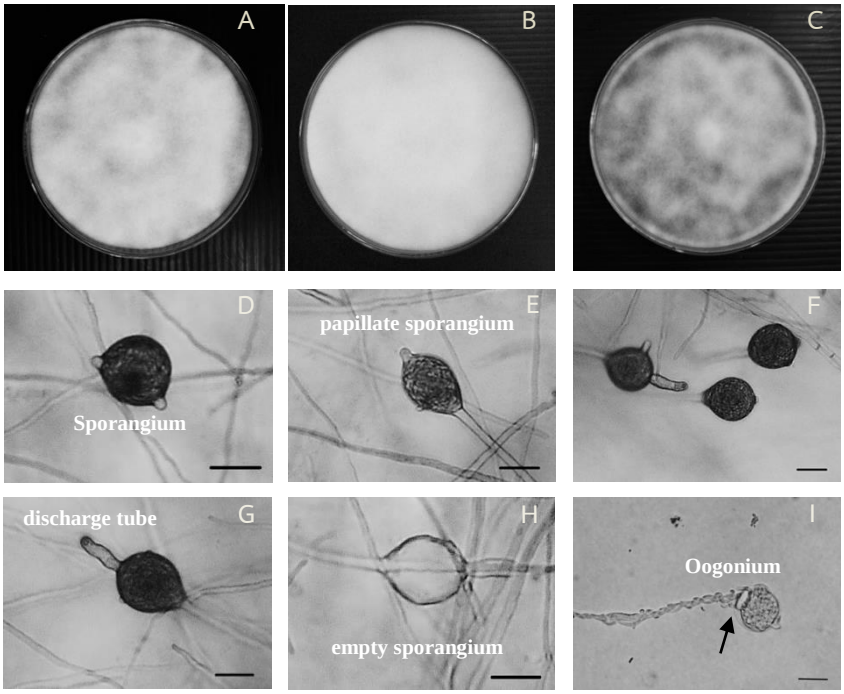

Figure 5. Morphology of *Phytopythium helicoides* isolates KS-13, NL-18. A-C: Colony pattern on V8A (A), PDA (B) and CMA (C) medium at 6 Days; D-E: papillate sporangium (10×); F-G: sporangium with discharge tube (10×); H: Empty sporangium (10×); I: Antheridium winding around the oogonial stalk; (scale bars at 10×: 20 µm; 40×: 10 µm); The arrow represents antheridium

Isolate NL-6 is similar to *Phytopythium iriomotense*, colony pattern is chrysanthemum, hypha (av. 6.0 μm) swelling and well branched, globose, papillated and non-proliferating sporangia (av. 25.5x24.9 um), oogonium and oospore structure is absent (Figure 6).

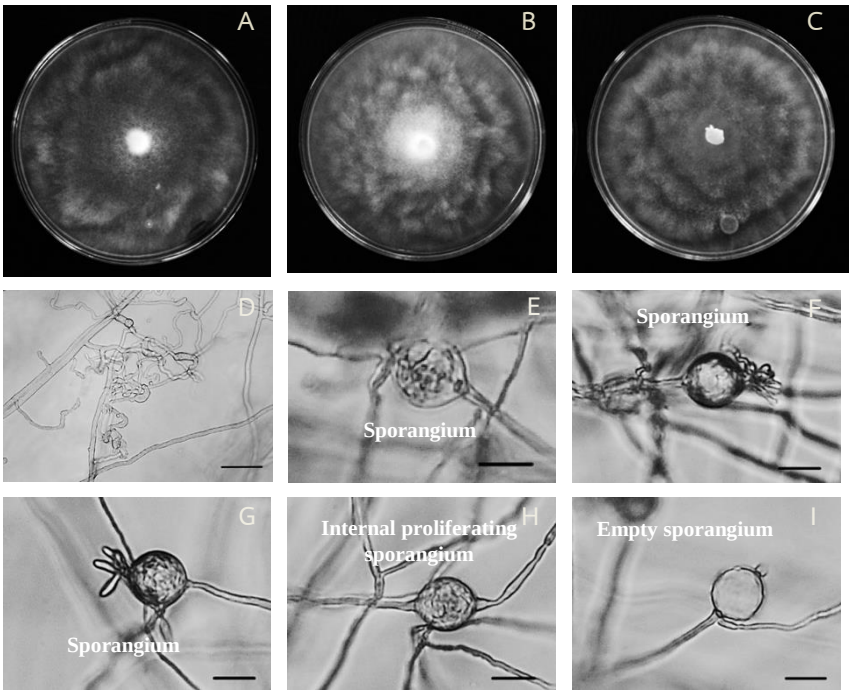

Figure 6. Morphology of *Phytopythium iriomotense* isolate NL-6. A-C: Colony pattern on V8A (A), PDA (B) and CMA (C) medium at 6 Days; D: hyphal (10×); E: sporangium (10×); F: contiguous sporangium (10×); G: sporangium (10×); H: internal proliferating sporangium (10×); I: empty sporangium (10×); (scale bars at 10×: 20 μm)

Isolate KS-15 and KS-16 are similar to *Pytopythium torulosum*, Colony patterns are rosette, hypha (av. 5.2 µm). Filamentous and inflated filamentous sporangium of various sizes. Oogonium smooth wall (av. 21.3x21.8 µm). Aplerotic oospores (av. 19.7x19.5 µm) and thick-walled (av. 2.6 µm). antheridium 1-3 per oogonium, monoclinous, or sometime diclinous antheridium (Figure 7).

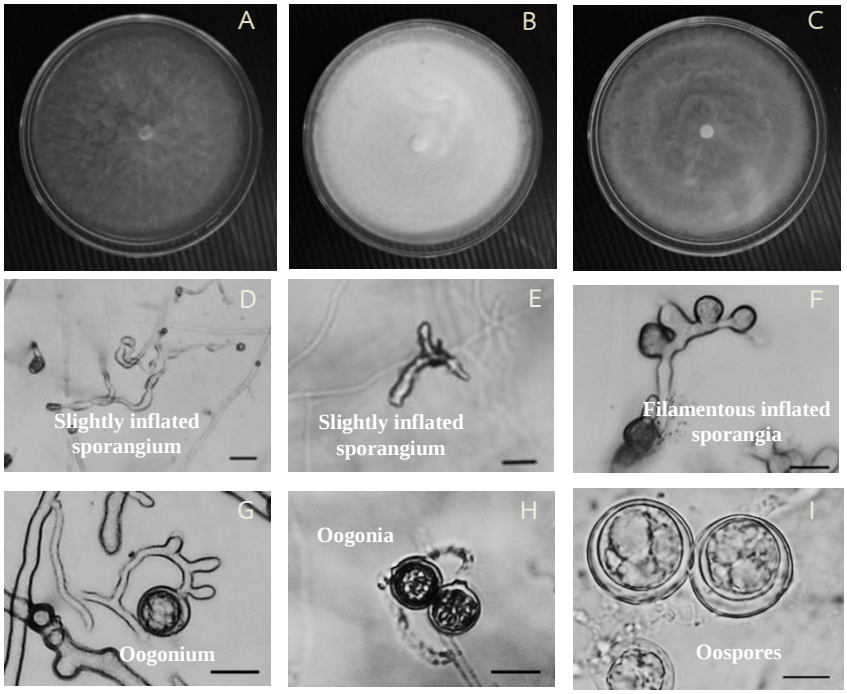

Figure 7. Morphology of *Pythium torulosum* isolates KS-15, KS-16. A-C: Colony pattern on V8A (A), PDA (B) and CMA (C) medium at 6 Days; D-F: inflated sporangium (10×); G-H: oogonia (10×); I: oospores (40×); (scale bars at 10×: 20 µm; 40×: 10 µm)

Isolate KS-7, KS-12, NS-3, NS-4, NS-9 and NL-19 are similar to *Phytopythium vexans*, colony patterns are chrysanthemum, hypha (av. 5.2 µm). globose or subglobose sporangium (av. 18.6x18.4 µm), Oogonium smooth wall (av. 21.2x21.5 µm), aplerotic oospores (av. 17.2x18.9 µm) and thick-walled (av. 2.4 µm), antheridium 1-3 per oogonium, monoclinous, or sometime diclinous antheridium (Figure 8-9).

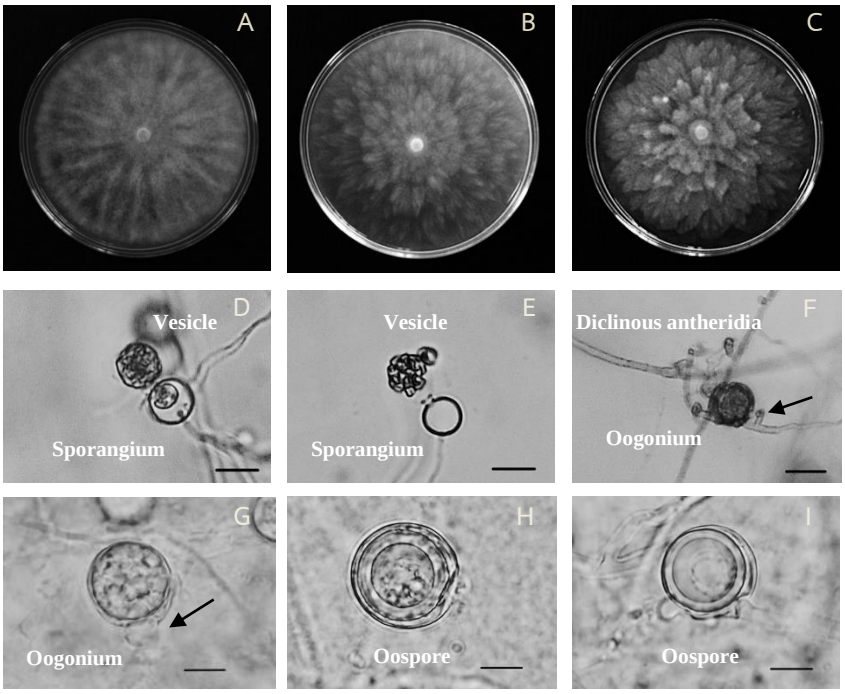

Figure 8. Morphology of *Phytopythium vexans* isolates KS-7, KS-12. A-C: Colony pattern on V8A (A), PDA (B) and CMA (C) medium at 6 Days; D: sporangium forming vesicle (10×); E: sporangium liberating zoospores (10×); F: oogonium and antheridium (10×); G: oogonium and antheridium (40×); H-I: oospore (40×); (scale bars 10×: 20 µm; 40×: 10 µm); The arrow represents antheridium

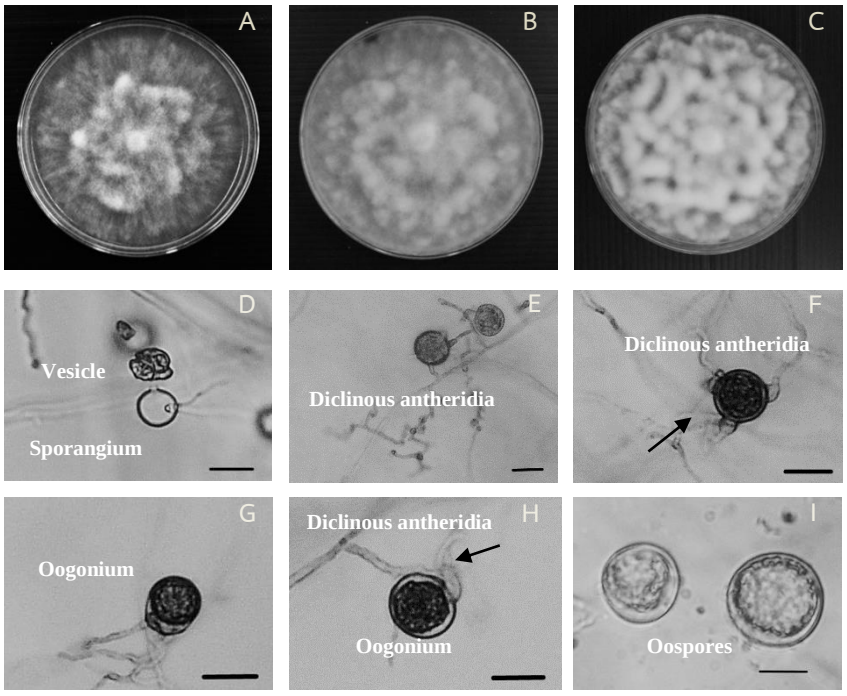

Figure 9. Morphology of *Phytopythium vexans* isolates NS-3, NS-4, NS-9, NL-19. A-C: Colony pattern on V8A (A), PDA (B) and CMA (C) medium at 6 Days; D: sporangium liberating zoospores (10×); E-H: oogonium and antheridium (10×); I: oospores (40×); (scale bars at 10×: 20 µm; 40×: 10 µm); The arrow represents antheridium

Isolate KS-1, KS-3 and KS-5 are similar to *Pythium* sp. (D37), colony patterns are chrysanthemum, Produce large amounts of globose sporangia (av. 21.3x21.7 µm) with many discharged tubes (av. 4.2x6.59 µm) and encysted zoospores (av. 6.8x10.0 µm). Plerotic oospores, smooth wall (av. 23.9x24.1 µm) with thin-walled (av. 1.6 µm) (Figure 10).

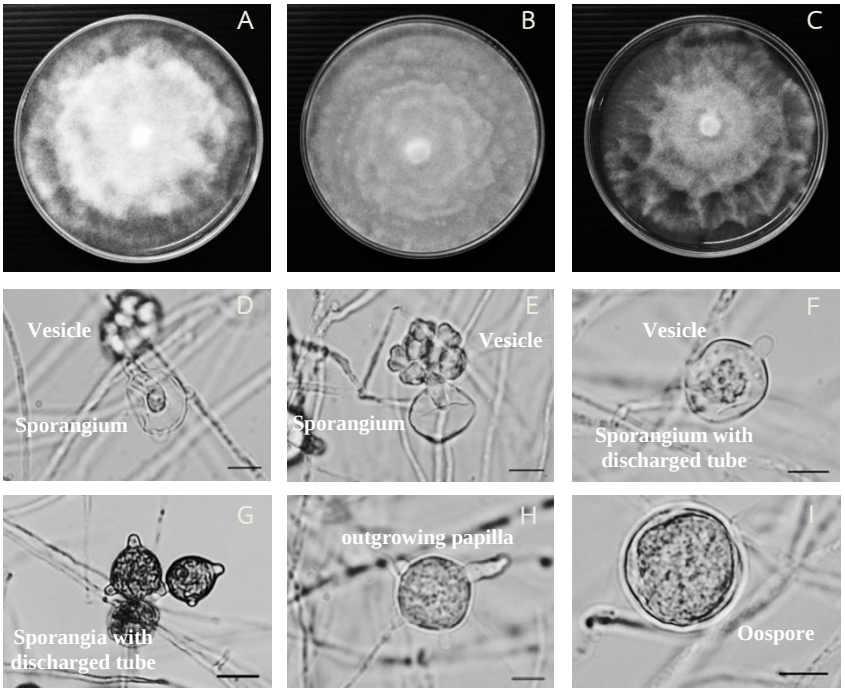

Figure 10. Morphology of *Phytopythium* sp. D37 isolates KS1, KS-3, KS-5. A-C: Colony pattern on V8A (A), PDA (B) and CMA (C) medium at 6 Days; D-E: sporangia forming vesicle (40×); F: sporangium with discharged tube (40×); G: sporangia with discharged tube (10×); H: sporangium with outgrowing papilla (40×); I: oospore; (scale bars at 10×: 20 µm; 40×: 10 µm)

Phylogenetic analysis

The phylogenetic tree was constructed using the obtained sequences and GenBank sequences revealed that the similarity ranged from 91 to 100 percent identity among twenty isolates with nucleotide lengths ranging from 750 to 950 bp. Mostly pathogenic species were in clade B, 2 isolates KS-15 and KS-16 are showed complete resemblance of 98% similarity to *Pythium torulosum* compared to sequence length at 869 and 874 base pairs, respectively. Clade D, 1 isolate KS-17 is 98% similar to *Pythium acanthicum* compared to sequence length 853 base pairs. Clade J, 1 isolate, KS-18 is 97% similar to *Globisporangiu nunn* as compared from sequence length 962 base pairs. Clade K, 16 isolates: 3 isolates KS-1, KS-3 and KS-5 are 98-99% similar to *Pythium* spp. (unknow) identified in the NCBI as isolate D37 as compared from sequence length 816-885 base pairs, 6 isolates KS-7, KS-12, NS-3, NS-4, NS-9 and NL-19 are 94-100% similar to *Phytopythium vexans* as compared from

sequence length 748-907 base pairs, 3 isolates KS-9, KS-11and NS-20 are 98-100% similar to *Phytopythium cucurbitacearum* as compared from sequence length 893-937 base pairs, 2 isolates KS-13 and NL-18 is 91-96% similar to *Phytopythium helicoides* as compared from sequence length 781-804 base pairs, 1 isolate NS-1 is 99% similar to *Phytopythium chamaehyphon* as compared from sequence length 833 base pairs, and 1 isolate NL-6 is 98% similar to *Phytopythium iriomotense* as compared from sequence length 798 base pairs. In which the phylogenetic tree shows clustering, evidently clade K is polyphyletic, clearly phylogenetically differentiated from other clade equal to 96% with a bootstrap support value of 1000 iterations (Table 2, Figure 11).

Table 2. Sequence identification data of the isolates

Clade	Isolates	GenBank Accession no.	Hits	Sequence length (bp)	Similar (%)	Variation ratio (Different/Total)
B	KS-15	ON394663	*P. torulosum* MT758173.1	869	99.65	3/869
	KS-16	ON394674	*P. torulosum* MT758173.1	874	99.66	3/874
D	KS-17	ON394672	*P. acanthicum* MW426378.1	853	98.71	11/854
J	KS-18	ON394671	*G. nunn* KU211484.1	962	97.72	22/964
K	KS-1	OM346740	*Pythium sp.* D37 KP183943.1	851	98.46	13/843
	KS-3	ON394659	*Pythium* sp. D37 KP183943.1	885	98.75	11/881
	KS-5	ON394661	*Pythium* sp. D37 KP183943.1	816	99.75	2/814
	KS-7	ON394660	*Ph. vexans* GU133594.1	748	94.10	38/644
	KS-9	ON394670	*Ph. Cucurbitacearum* KP183959.1	893	100	0/893

Table 2. (Continue)

Clade	Isolates	GenBank Accession no.	Hits	Sequence length (bp)	Similar (%)	Variation ratio (Different/ Total)
K	KS-11	ON394664	*Ph. cucurbitacearum* MW426384.1	899	100	0/899
	KS-12	ON394668	*Ph. vexans* MW426381.1	870	100	0/870
	KS-13	ON394665	*Ph. helicoides* KY084742.1	781	91.17	69/781
	NS-1	OM346740	*Ph. chamaehyphon* MN872766.1	833	99.73	2/735
	NS-3	ON533631	*Ph. vexans* MH671329.1	907	98.68	12/906
	NS-4	ON394667	*Ph. vexans* MT758165	880	99.54	4/875
	NL-6	ON394662	*Ph. iriomotense* AB690624.1	798	98.23	14/793
	NS-9	ON394669	*Ph. vexans* MT647272.1	871	99.54	4/871
	NL-18	ON394666	*Ph. helicoides* KY084740.1	804	96.64	27/804
	NL-19	ON394673	*Ph. vexans* MW426382.1	876	99.89	1/876
	NS-20	ON533632	*Ph. cucurbitacearum* KP183959.1	937	98.39	15/934

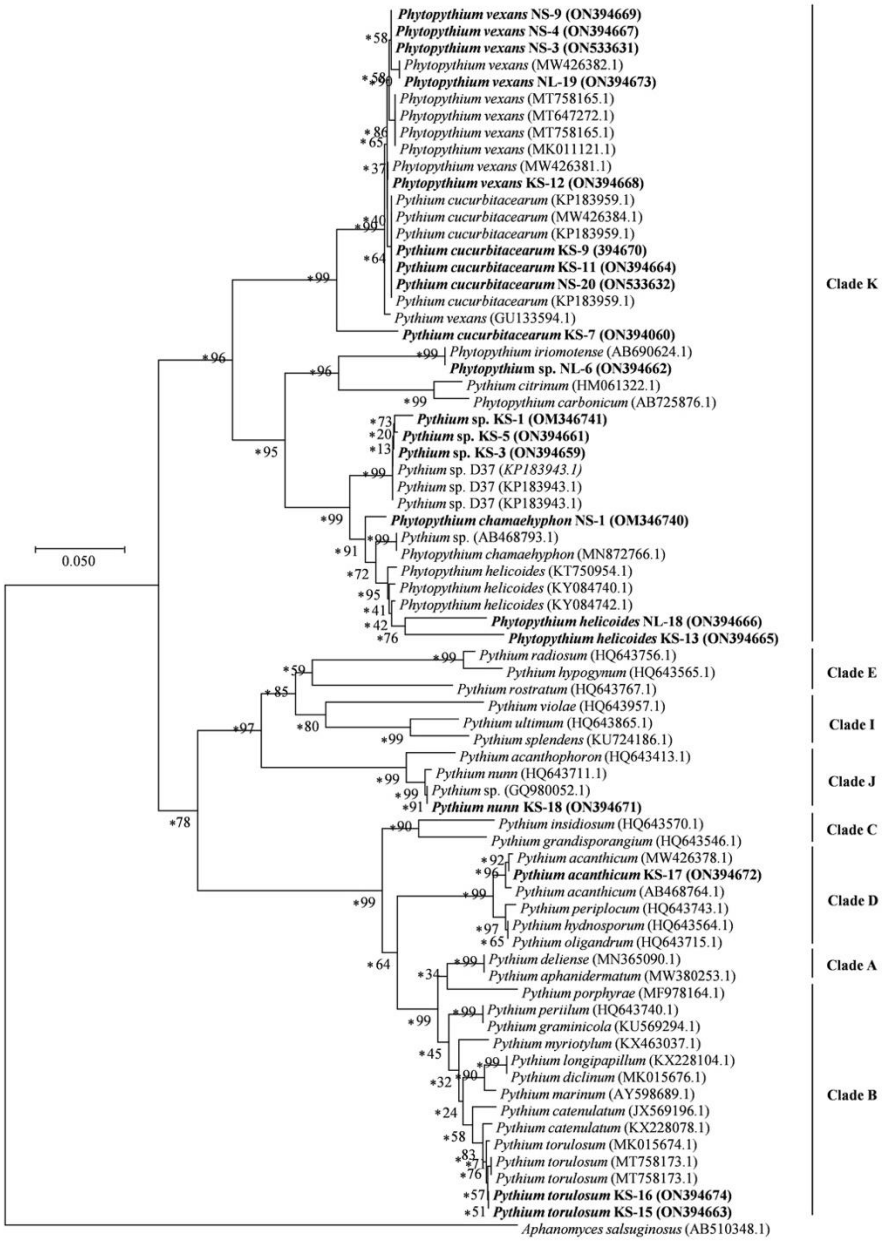

Figure 11. Phylogenetic tree of *Phytopythium* spp. and *Pythium* spp. using Neighbor-joining algorithm. The numbers shown on the node indicate the bootstrap support value of 1000 iterations, using the *Aphanomyces salsuginosus* nucleotide sequence as the outgroup. The scale bar represents a base displacement of 0.05 bases per nucleotide. An asterisk (*) denotes identical branches discovered by the maximum-likelihood and maximum-parsimony algorithms

Discussion

Based on the morphological characteristics, we identified twenty isolates of *Phytopythium* and *Pythium* obtained from various natural sources in Kanchanaburi and Nakhon Nayok provinces in Thailand's central area. After 48-96 hours of growing the mycelia on V8A, PDA, and CMA media, the colonies were classified into three pattern types (chrysanthemum, cottony, and rosette). The sporangia were mostly globose, while the oospores and oogonia had smooth walls.

Phylogenetics analysis based on the ITS region and fragments of the nuclear ribosomal DNA 5.8S gene suggested that the obtained isolates were similar to *Globisporangium nunn* (clade J), *Pythium acanthicum* (clade D), *Phytopythium chameahyphon* (clade K), *Phytopythium cucurbitacearum* (clade K), *Phytopythium helicoides* (clade K), *Phytopythium iriomotense* (clade K), *Pythium torulosum* (clade B), *Phytopythium vexans* (clade K) and unknown *Phytopythium* species (*Pythium* D37, clade K) which they were all found in the natural environment. Almost species we discovered were assigned to a polyphyletic group (clade K), with all the species in this clade classified as *Phytopythium*, which is consistent with the findings of Saelee *et al*. (2022), who studied the distribution of *Pythium* and related genera in Thailand's eastern region. These findings suggested that clade K is distributed broadly across various regions and has the potential to be a dominant species in Thailand's natural environment, which, like many other studies, have been reporting the discovery of the species for decades (Uzuhashi *et al*., 2010; De Cock *et al.,* 2015; Ho, 2018). There are studies reporting that the species in this clade can produce a large number of zoospores, living in a wide variety of areas that can tolerate and withstand the hot climate in tropical areas (Afandi *et al*., 2018), which may be why there are many reports on clade K discovery in Asia, and some have suggested that many species are also capable of causing disease in a variety of hosts. *Pythium* and *Phytopythium* is normally known to cause disease in seedlings, but recent findings suggest that can develop in other woody plants such as oak, citrus, apple, and durian trees etc (Jankowiak *et al*., 2015; Benfradj *et al*., 2017; Jabiri *et al*., 2020; Thao *et al*., 2020), including those that live in a wide range of environments even without a host (Nam and Choi, 2019, Tkaczyk, 2020).

We discovered a species with different colony growth characteristics which is similar to *Phytopythium iriomotense*. which is most likely the first occurrence in Thailand. The discovery of this species has previously been reported in Japan by Baten *et al.* (2015), suggests that this may be the first occurrence of *Phytopythium iriomotense* in Southeast Asia.

Interestingly, we also discovered *Pythium* D37, a previously unknown species. Although this species shares some similarities with *Pythium*, but phylogenetic analyses revealed that it does not include the most recent common ancestor of all reported *Pythium* species, suggesting that it is polyphyletic and clustered in *Phytopythium* taxa, indicating that they are diverged in lineage and have genetic divergence, related to the previous findings (Santoso *et al*., 2015., Intaparn *et al*., 2020). The mycelium on the agar media has chrysanthemum patterns, most of the sporangium is globose, had more discharged tubes than any other species, and had plerotic smooth walls oospores, implying that these unknown oomycetes strains could be a new *Phytopythium* species that has yet to be given a name.

The phylogeny of all species represents related to many reports (Martin, 2000; Levesque and de Cock, 2004; Villa *et al*, 2006; Uzuhashi *et al*., 2010) that there are the divergencies between the species from a basal clade that produced filamentous sporangium and the more distant clades that produced more diverse type of sporangium, whereas oogonia production appears unrelated to the phylogeny and evolutionary pattern. (Levesque and de Cock, 2004). The phylogenetic study and a previous work of Saelee *et al*. 2022 could be useful in determining the taxonomic position of the Thai strain which has been rarely documented and providing new information on the distribution of existing species in their natural habitats.

However, we only used morphological and molecular data reference from Levesque and de Cock (2004), Van der Plaats Niterink (1981) which may insufficient to identify oomycetes in intraspecific level. Therefore, for reliable assertions, it may be necessary to use additional molecular markers for further study (Nam and Choi, 2019; Villa *et al*, 2006) such as *cytochrome c oxidase* subunit I (*Cox*I), *Cox*II mtDNA and *β-tubulin* gene combined with ITS rDNA sequences.

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References

Afandi, A., Murayama, E., Hieno, A., Suga, H. and Kageyama, K. (2018). Population structures of the water-borne plant pathogen *Phytopythium helicoides* reveal its possible origins and transmission modes in Japan. Plos one, 13(12).

Baten, M., Mingzhu, L., Motohashi, K., Ishiguro, Y., Rahman, M. Z., Suga, H. and Kageyama, K. (2015). Two new species, *Phytopythium iriomotense* sp. nov. and *P. aichiense* sp. nov., isolated from river water and water purification sludge in Japan. Mycological progress, 14:1-12.

Benfradj, N., Migliorini, D., Luchi, N., Santini, A. and Boughalleb-M'Hamdi, N. (2017). Occurrence of *Pythium* and *Phytopythium* species isolated from citrus trees infected with gummosis disease in Tunisia. Archives of Phytopathology and Plant Protection, 50:286-302.

Benhamou, N., Le Floch, G., Vallance, J., Gerbore, J., Grizard, D. and Rey, P. (2012). *Pythium oligandrum*: an example of opportunistic success. Microbiology, 158:2679-2694.

Broders, K. D., Lipps, P. E., Paul, P. A. and Dorrance, A. E. (2007). Characterization of *Pythium* spp. associated with corn and soybean seed and seedling disease in Ohio. Plant disease, 91:727-735.

Cooke, D. E. L., Drenth, A., Duncan, J. M., Wagels, G. and Brasier, C. M. (2000). A molecular phylogeny of *Phytophthora* and related oomycetes. Fungal genetics and biology, 30:17-32.

De Cock, A. W. A. M., Lodhi, A. M., Rintoul, T. L., Bala, K., Robideau, G. P., Abad, Z. G. and Lévesque, C. A. (2015). *Phytopythium*: molecular phylogeny and systematics. Persoonia-Molecular Phylogeny and Evolution of Fungi, 34:25-39.

Dhingra, O. D. and Sinclair, J. B. 1994. Basic plant pathology methods (2nd edition). Florida: CRC Press.

Felsenstein, J. (1985) Confidence Limits on Phylogenies: An Approach Using the Bootstrap. Evolution, 39:783-791.

Ho, H. H. (2018). The taxonomy and biology of *Phytophthora* and *Pythium*. Bacteriology & Mycology. Open Access, 6:40-45.

Hung, P. M., Wattanachai, P., Kasem, S. and Poeaim, S. (2015). Efficacy of Chaetomium species as biological control agents against *Phytophthora nicotianae* root rot in citrus. Mycobiology, 43:288-296.

Intaparn, P., Noireung, P., Maumoon, R., Poti, T., Wongwan, T., McGovern, R. J. and To-Anun, C. (2020). First record of Phytopythium sp. causing root and stem rot on Catharanthus roseus in Thailand. Plant Pathology & Quarantine, 10:10-20.

Jabiri, S., Lahlali, R., Bahra, C., Bendriss Amraoui, M., Tahiri, A. and Amiri, S. (2020). First report of Phytopythium vexans associated with dieback disease of apple trees in Morocco.Plant Pathology, 102:1319-1319.

Jankowiak, R., Stepniewska, H. and Bilanski, P. (2015). Notes on some *Phytopythium* and *Pythium* species occurring in oak forests in southern Poland. Acta Mycologica, 50(1).

Jukes, T. H. and Cantor, C. R. (1969) CHAPTER 24 - Evolution of Protein Molecules. In: H. N. Munro (Ed), Mammalian Protein Metabolism. Academic Press, pp. 21-132.

Kluge, A. G. and Farris, J. S. (1969). Quantitative phyletics and the evolution of anurans. Systematic Biology, 18:1-32.

Levesque, C. A. and De Cock, A. W. (2004). Molecular phylogeny and taxonomy of the genus Pythium. Mycological research, 108:1363-1383.

Luecke, N. C., Crawford, K. M., Koike, S. T., Stanghellini, H. and Burkhardt, A. (2022). First Report of Root Rot Caused by *Pythium dissotocum* on Hydroponically Grown Collard Greens (*Brassica oleracea* Var. *acephala*). Plant Disease, 106:1075.

Martin, F. N. (2000). Phylogenetic relationships among some *Pythium* species inferred from sequence analysis of the mitochondrially encoded cytochrome oxidase II gene. Mycologia, 92:711-727.

Masago, H., Yoshikawa, M., Fukada, M. and Nakanishi, N. 1977. Selective inhibition of *Pythium* spp. on a medium for direct isolation of *Phytophthora* spp. from soils and plants. Phytopathology 67:425-428.

Mendoza, L. (1998). *Pythium insidiosum*. History, 135:1-18.

Nam, B. and Choi, Y. J. (2019). *Phytopythium* and *Pythium* species (Oomycota) isolated from freshwater environments of Korea. Mycobiology, 47:261-272.

Rossman, A. Y. and Palm, M. E. (2006). Why are *Phytophthora* and other Oomycota not true fungi. Outlooks on Pest Management, 17:217.

Saelee, R., Busarakam, K. and Koohakan, P. (2022). Morphological and phylogenetic diversity of *Pythium* and related genera (Pythiaceae, Pythiales) from some areas in eastern Thailand. Agriculture and Natural Resources, 56:945-956.

Saitou, N. and Nei, M. (1987). The Neighbor-Joining Method: A New Method for Reconstructing Phylogenetic Trees. Molecular Biology and Evolution, 4:406-425.

Santoso, P. J., Aryantha, I. N. P., Pancoro, A. and Suhandono, S. (2015). Identification of *Pythium* and *Phytophthora* associated with durian (*Durio* sp.) in Indonesia: their molecular and morphological characteristics and distribution. Asian Journal of Plant Pathology, 9:59-71.

Thao, L., Hien, L., Liem, N., Thanh, H., Khanh, T., Binh, V. and Tu, T. (2020). First report of *Phytopythium vexans* causing root rot disease on durian in Vietnam. New Disease Reports, 41:2044-0588.

Tkaczyk, M. (2020). *Phytopythium*: origin, differences and meaning in modern plant pathology. Folia Forestalia Polonica, 62:227-232.

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

Van der Plaats-Niterink, A. J. (1981). Monograph of the genus *Pythium*. Studies in Mycology 21:1-242.

Villa, N. O., Kageyama, K., Asano, T. and Suga, H. (2006). Phylogenetic relationships of *Pythium* and *Phytophthora* species based on ITS rDNA, cytochrome oxidase II and β-tubulin gene sequences. Mycologia, 98:410-422.

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