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Research article

Morphological and phylogenetic diversity of *Pythium* and related genera (*Pythiaceae*, *Pythiales*) from some areas in eastern Thailand

Rattasit Saelee[a,†], Kanungnid Busarakam[b,†], Prommart Koohakan[a,*]

[a] *Department of Plant Production Technology, School of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok 10520, Thailand*

[b] *Biodiversity Research Centre, Thailand Institute of Scientific and Technological Research, Pathum Thani 12120, Thailand*

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Abstract

Importance of the work: *Pythium* and related genera in the class *Oomycetes* are an important microorganism group that affects environmental stability due to their diverse lifestyle patterns. However, full information on their distribution is limited in Thailand.

Objectives: To describe their oomycetes distribution in Chanthaburi, Rayong, and Trat provinces, eastern Thailand and provide their diversity and phylogenetic data.

Materials & Methods: Composite soils samples were collected July–September 2019. Three techniques (soil baiting, soil dilution and soil plating) were used for isolation. Isolated oomycetes species were obtained from different locations (mangrove forest, natural forest, cultivated field and a river). The isolates were characterized using combined data of morphological traits and sequences analysis of the internal transcribed spacer region (ITS).

Results: In total, 52 oomycetes were isolated and assigned to 11 species (*Globisporangium carolinianum, Globisporangium splendens*, *Pythium acanthicum*, *Pythium deliense*, *Pythium diclinum*, *Pythium longipapillum*, *Pythium myriotylum*, *Pythium torulosum*, *Phytopythium cucurbitacearum*, *Phytopythium helicoides* and *Phytopythium vexans*). Phylogenetic trees of the ITS region analysis indicated that the species obtained from the various locations in this investigation shared a similar biological trait of *Pythium* and related genera within clades A, B, D, I and K.

Main finding: This was the first report of *P. longipapillum* recovery in Thailand and perhaps the first publication of this species in Thailand and Southeast Asia. Identification and descriptions of *Pythium* and related genera were provided, as well as useful information to understand their distribution in Thailand's unique ecosystem.

† Equal contribution.
* Corresponding author.
E-mail address: prommart.ko@kmitl.ac.th (P. Koohakan)

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Introduction

Fungal-like organisms in the class *Oomycetes* exist in almost every type of ecosystem (Thines, 2014). Some oomycetes cause diseases in plants and animals (Derevnina et al., 2016). Most oomycetes are plant pathogens (particularly *Phytophthora* and *Pythium*, which are economically important) that cause significant diseases in many economic plants, including vegetables, field crops and fruit crops (Agrios, 2005; Hung et al., 2015a; Boevink et al., 2020). Oomycetes can live in a wide range of environments resulting in their high diversity, intraspecific variation and overlapping morphological characteristics (McLeod et al., 2009).

While classification to the genus level of oomycetes can be achieved using a conventional procedure, such as a morphological study (sporangium, oogonium and antheridium, Van der Plaats-Niterink, 1981) by an experienced observer, the identification can be more difficult due to the intraspecific variation and overlapping of morphological features. Molecular and phylogenetic analyses are essential tools to distinguish between specimens using data from the internal transcribed space (ITS) region, the 5.8S gene of nuclear ribosomal DNA, *cytochrome oxidase I* (*cox I*) and *II* gene (*cox II*) and the NADH dehydrogenase subunit and *β-tubulin* gene (Martin, 2000; Lévesque and De Cock, 2004; Villa et al., 2006; Uzuhashi et al., 2010). Lévesque and De Cock (2004) applied a set of primers to amplify the ITS regions and the 5.8S gene of nuclear ribosomal DNA together with the morphological characteristics, such as the sporangium, oogonium and antheridium. The results allowed them to describe and classify most *Pythium* species into 11 clades (A–K). Some reports, based on former *Pythium* and related genera studies, reorganized the former taxonomical system into supported clades (1–5) or split it into several genera, *Elongisporangium, Globisporangium, Ovatisporangium* (*Phytopythium*) and *Pilasporangium* (Uzuhashi et al., 2010).

Pythium and related genera are distributed in a variety of regions from tropical to temperate (Van der Plaats-Niterink, 1981) and several species are plant pathogens in both monocotyledons and dicotyledons, such as damping off, tuber rot and root rot diseases. There are only a few domestic reports on *Pythium* distribution in Thailand because most studies only focused on phytopathogenic species (Rujirawat et al., 2017; Suksiri et al., 2018; Charoenrak et al., 2019; Hattapanichaporn et al., 2020). Although the preliminary work of Saelee et al. (2021) investigated in Rayong province, their report focused on the morphology of the genera. Therefore, the current study aimed to provide new data on *Pythium* and related genera distribution from three provinces in eastern Thailand (Chanthaburi, Rayong and Trat) and to describe some new strains of known species using morphological characteristics, ITS regions and 5.8S ribosomal RNA gene sequence data.

Materials and Methods

Sampling location and isolation

Vertical soil samples (300 mm) were collected in a two-way, diagonal pattern (Estefan et al., 2013) from both degraded forest and a cultivated field and in a straight pattern from a mangrove forest and a river, which represented an undisturbed inland soil, a cultivated soil, a marine environment and a freshwater area, respectively. Sixteen soil samples were collected from each area of each province (Table 1), including leaf debris from the mangrove forest and the river. The samples were placed in an icebox before use. Some of the collected soils from each environment were pooled together for fungal isolation. Three techniques—soil plate (Warcup, 1950), soil dilution (Stanghellini and Kronland, 1985) and soil baiting (Dhingra and Sinclair, 1994)—were used for isolation based on selective agar medium, corn meal agar (CMA) + BNPRA (Benomyl 10 ppm, Nystatin 25 ppm, Pentachloronitrobenzene 25 ppm, Rifampicin 10 ppm, Ampicillin 500 ppm and rose Bengal) (Masago et al., 1977; Chamswarng and Cook, 1985). For the soil plate technique, soil particle was sprinkled on the selective medium, while for the soil dilution technique, 1 g of a soil sample was prepared to give 1×10^{-4} dilutions that were spread over plates of the selective agar medium and incubated at room temperature (30±2 °C). The baiting technique was prepared by placing 1 g of a soil sample from each area on separate Petri dishes, prior to adding 10 mL of sterile distilled water and cucumber seeds (10 seeds/plate) that were incubated at room temperature for 24 h. Then, the cucumber seeds were rinsed and placed on selective medium. To obtain a pure culture, *Pythium* colonies were randomly selected from selective isolation plates of each method and sub-cultured individually onto CMA+BNPRA and potato dextrose agar (PDA) media.

Morphology identification

Three different media (PDA, CMA and V8 juice agar) were

used in these experiments. Asexual reproduction was studied using water culture, grass blade culture and poor media. For the water culture technique, the sample was placed on an agar plug of pure culture on a Petri dish filled with sterile distilled water, then checked for sporangium and zoospore production in the Petri dish after incubation at 28 °C under fluorescent light for 24–48 h (Hung et al., 2015a), which would typically be observed after 7 d. For the grass blade culture technique, pieces of boiled grass leaf were placed in a Petri dish that contained sterile water, then agar plugs of pure culture were placed in the water. The plates were incubated at 28 °C, with checking for sporangium and zoospore formation within 24–48 h. In low nutrient media culture, each isolate was cultured in CMA, then checked for asexual structures under a light microscope. To study sexual reproduction, each isolate was cultured in V8 juice agar and observed after incubation at room temperature for 6 d.

DNA extraction, polymerase chain reaction amplification and sequencing

Total genomic DNA was extracted using a modification of the method of Ivors (2015). A sample of 1–2 µL of each crude DNA was used as a DNA template. The DNA was amplified using the forward primer ITS6 (5'- GAAGGTGAAGTCGTAACAAGG-3') and the reverse primer ITS4 (5'- TCCTCCGCTTATTGATATGC-3') to amplify ITS1, ITS2 and fragments of 5.8S subunit ribosomal RNA in a polymerase chain reaction (PCR; Hung et al., 2015a). The PCR reactions were conducted as follows: initial denaturation at 95 °C for 2 min, 30 cycles of 95 °C for 20 s, 55 °C for 25 s 72 °C for 50 s and finally 72 °C for 10 min (Cooke et al., 2000). The PCR products were checked for quality and purified using a GeneJET Gel Extraction Kit (Thermo Scientific, USA). Sequences of the ITS region were determined by Bionics Co. Ltd (Korea).

Phylogenetic analyses

The chromatogram files in ABI were read using the FinchTV software (Patterson et al., 2004) and converted to a FASTA file. The similarities were determined using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology (NCBI; USA). All sequences were submitted to GenBank (NCBI database). The sequences of a species with the highest similarity to the isolates used in this study, including the ex-type sequences described by Lévesque and De Cock (2004), were downloaded from GenBank. The MEGA X software was used to perform sequence alignment using the ClustalW algorithm (Tamura et al., 2011; Kumar et al., 2018). For evolutionary analyses, phylogenetic trees were reconstructed from aligned sequences and generated using the neighbor-joining (Saitou and Nei, 1987) maximum-likelihood (Felsenstein, 1985) and maximum-parsimony (Kluge and Farris, 1969) algorithms.

Neighbor-joining analyses were conducted using the MEGA X software. The pairwise deletion option was used to remove all ambiguous positions of each sequence pair and the evolutionary distance model of Jukes and Cantor (1969) was applied to generate evolutionary distance matrices. The raxmlGUI v2.0.7 software (Silvestro and Michalak, 2012; Edler et al., 2020) was used to construct a maximum-likelihood (ML) tree. The ML search and rapid bootstrap function was selected with GTRGAMMA as the substitution model. The TreeViewX program (Page, 1996) was used to visualize the ML tree. Maximum parsimony analysis was conducted using PAUP v. 4.0a169 (Swofford, 2002) and a heuristic search. Gaps were treated as missing data. A phylogram was constructed using FigTree v1.4.4 (Rambaut, 2018). The topology of all trees was evaluated in bootstrap analyses (Felsenstein, 1985) based on 1,000 samplings of a dataset and combined into one set of tree data. *Halophytophthora avicenniae* and *Halophytophthora polymorphica* were used as the outgroup.

Results and Discussion

Isolation and morphological observation

In total, 52 isolates were recovered (2 isolates from the marine environment, 11 isolates from undisturbed inland soil, 12 isolates from cultivated soil and 27 isolates from the fresh water area). Most isolates produced both asexual and sexual structures. Obtained isolates were assigned to *Globisporangium carolinianum*, *Globisporangium splendens, Pythium acanthicum, Phytopythium cucurbitacearum, Pythium deliense, Pythium diclinum, Pythium longipapillum, Pythium myriotylum, Pythium torulosum, Phytopythium helicoides* and *Phytopythium vexans*. Based on morphological characteristics (Figs.1–3, Table 4) and sequence similarity (Tables 1–3), 9 species produced sporangia (Fig. 2) and 8 species produced sexual structures (Fig. 3). The original reference descriptions and key to *Pythium* species were based on Van der Plaats-Niterink (1981), except for *P. longipapillum* (Salmaninezhad and Mostowfizadeh-Ghalamfarsa, 2019).

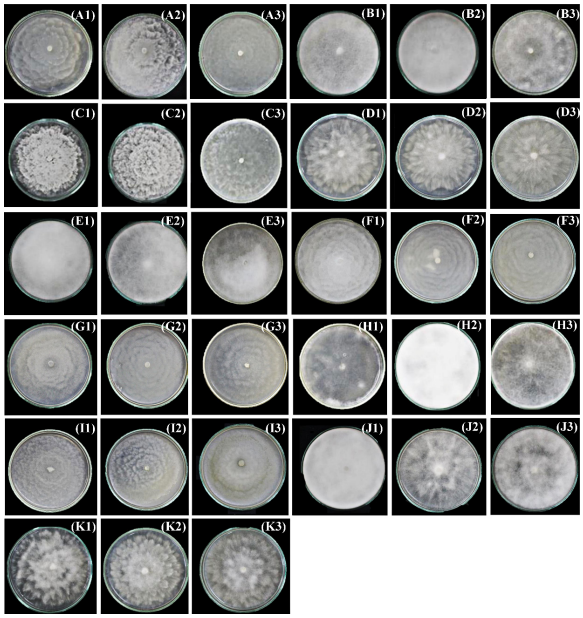

Fig. 1 Colony morphology of *Pythium* and related genera on potato dextrose agar (1), corn meal agar (2) and V8-agar (3) media after incubation at room temperature: (A1–A3) *G. carolinianum*; (B1–B3) *G. splendens*; (C1–C3) *P. acanthicum*; (D1–D3) *Ph. cucurbitacearum*; (E1–E3) *P. deliense*; (F1–F3) *P. diclinum*; (G1–G3) *P. longipapillum*; (H1–H3) *P. myriotylum*; (I1–I3) *P. torulosum*; (J1–J3) *Ph. helicoides*; (K1–K3) *Ph. vexans*

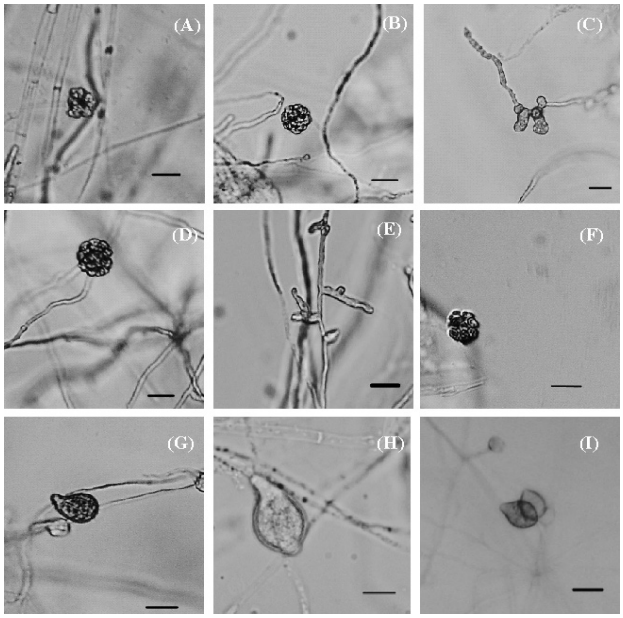

Fig. 2 Asexual structures of all isolates: (A) vesicle of *P. deliense*; (B) vesicle of *G. Carolinianum*; (C) inflated sporangium of *P. myriotylum*; (D) vesicle of *P. longipapillum*; (E) strictly filamentous sporangium of *P. torulosum*; (F) vesicle of *P. diclinum*; (G) papillate sporangium of *Ph. vexans*; (H) ovoid sporangium of *Ph. helicoides* and (I) non-papillated ovoid sporangium of *Ph. cucurbitacearum*, where scale bars are 20 µm (A–G and I) and 10 µm (H)

Table 1 Internal transcribed spacer (ITS) sequence similarity of *Pythium* and related genera in some areas of eastern Thailand: clade A and B

Clade	Isolate	GenBank Accession no. (ITS sequence)	Isolation area	Host or source	Closest species	Sequence length (bp)	Similarity (%)
A	RYS-11	MT758174	Rayong province <12.776806, 101.714779>	Leaf debris (river)	*P. deliense* MN365090.1	823	99
B	CHS-9	MW412827	Chanthaburi province <12.711763, 102.181276>	River soil	*P. catenulatum* JX569196.1	837	99
	CHS-10	MW412828	Chanthaburi province <12.711763, 102.181276>	River soil	*P. catenulatum* JX569196.1	769	99
	CHS-11	MW412829	Chanthaburi province <12.711763, 102.181276>	Leaf debris (river)	*P. myriotylum* KY019264.1	864	99
	CHS-14	MW412832	Chanthaburi province <12.711763, 102.181276>	Leaf debris (river)	*P. longipapillum* KX228104.1	797	99
	CHS-15	MW412833	Chanthaburi province <12.711763, 102.181276>	Leaf debris (river)	*P. longipapillum* KX228104.1	744	99
	CHS-16	MW412834	Chanthaburi province <12.711763, 102.181276>	Leaf debris (river)	*P. longipapillum* KX228104.1	856	99
	CHS-17	MW412835	Chanthaburi province <12.711763, 102.181276>	Leaf debris (river)	*P. longipapillum* KX228104.1	820	99
	RYS-10	MT758173	Rayong province <12.776806, 101.714779>	Fresh water	*P. torulosum* MK015674.1	877	99
	RYS-14	MT758177	Rayong province <12.776806, 101.714779>	Leaf debris (river)	*P. diclinum* MK015676.1	782	99
	RYS-16	MT758179	Rayong province <12.776806, 101.714779>	Leaf debris (river)	*P. diclinum* MK015676.1	774	99
	TS-13	MW426387	Trat province <12.35619623,102.440991>	Leaf debris (river)	*P. diclinum* MK015676.1	725	97
	TS-15	MW426389	Trat province <12.35619623,102.440991>	Leaf debris (river)	*P. diclinum* MK015676.1	726	99

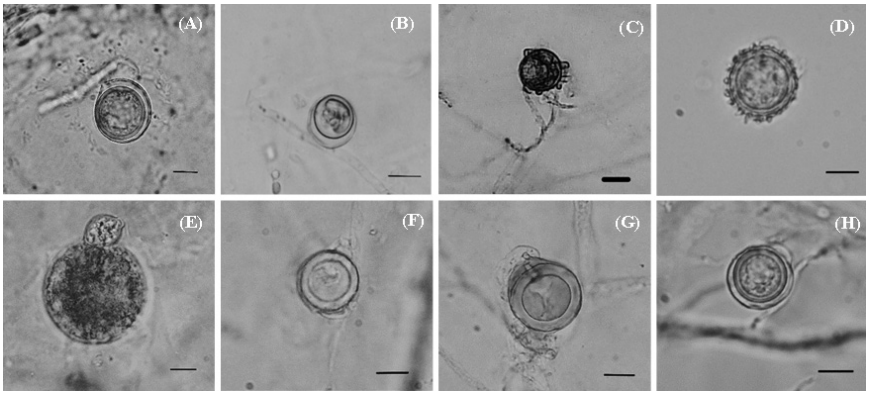

Fig. 3 Sexual structures of all isolates: (A) aplerotic oospore of *P. deliense*; (B) oogonium and diclinous antheridium of *G. carolinianum*; (C) oogonium of *P. myriotylum*; (D) oogonium and plerotic oospore of*P. acanthicum*; (E) large size oogonium of *G. splendens*; (F) aplerotic oospore of *Ph. vexans*; (G) aplerotic oospore of *Ph. helicoides*; (H) plerotic oospore of *Ph. cucurbitacearum*, where scale bars are 20 µm (C) and 10 µm (A, B and D–H)

Table 2 Internal transcribed spacer (ITS) sequence similarity of *Pythium* and related genera in some areas of eastern Thailand: clade D and I

Clade	Isolate	GenBank Accession no. (ITS sequence)	Isolation area (province)	Host or source	Closest species	Sequence length (bp)	Similarity (%)
D	RYS-4	MT758167	Rayong <12.849345, 101.555479>	Forest soil	*P. acanthicum* LC332027.1	772	98
	RYS-5	MT758168	Rayong <12.849345, 101.555479>	Forest soil	*P. acanthicum* KU210470.1	863	98
	RYS-6	MT758169	Rayong <12.849345, 101.555479>	Forest soil	*P. acanthicum* KU210470.1	871	98
	RYS-7	MT758170	Rayong <12.849345, 101.555479>	Forest soil	*P. acanthicum* KU210470.1	858	98
	RYS-18	MT758181	Rayong <12.849345, 101.555479>	River soil	*P. acanthicum* AY598617.2	822	99
	RYS-19	MT758182	Rayong <12.849345, 101.555479>	River soil	*P. acanthicum* HQ643411.1	770	98
	TS-4	MW426378	Trat <12.3554343,102.44117412>	Orchard soil	*P. acanthicum* KU210470.1	859	99
I	CHS-1	MW412819	Chanthaburi <12.7098950, 102.1804450>	Orchard soil	*P. splendens* AB796308.1	806	98
	CHS-2	MW412820	Chanthaburi <12.7098950, 102.1804450>	Orchard soil	*P. splendens* AB780629.1	803	99
	CHS-3	MW412821	Chanthaburi <12.7098950, 102.1804450>	Orchard soil	*P. splendens* AB780646.1	791	99
	CHS-4	MW412822	Chanthaburi <12.7098950, 102.1804450>	Orchard soil	*P. splendens* HQ237486.1	834	99
	CHS-5	MW412823	Chanthaburi <12.7098950, 102.1804450>	Orchard soil	*P. splendens* EU781675.1	822	99
	RYS-1	MT758164	Rayong <12.85099178, 101.55733498>	Orchard soil	*P. splendens* AY598655.2	853	99
	RYS-3	MT758166	Rayong <12.849345, 101.555479>	Forest soil	*P. splendens* KU724186.1	793	99
	TS-1	MW426375	Trat <12.3554343,102.44117412>	Orchard soil	*P. splendens* HQ237486.1	809	99
	TS-5	MW426379	Trat <12.3554343,102.44117412>	Orchard soil	*P. splendens* HQ237486.1	824	99
	TS-6	MW426380	Trat <12.3554343,102.44117412>	Orchard soil	*P. splendens* AB796287.1	800	95
	TS-9	MW426383	Trat <12.35619623,102.440991>	River soil	*P. splendens* EU781675.1	818	99

Table 3 Internal transcribed spacer (ITS) sequence similarity of *Pythium* and related genera in some areas of eastern Thailand: clade K

Clade	Isolate	GenBank Accession no. (ITS sequence)	Isolation area (province)	Host or source	Closest species	Sequence length (bp)	Similarity (%)
K	CHS-6	MW412824	Chanthaburi <12.711564, 102.177968>	Forest soil	*Ph. vexans* KP183941.1	824	99
	CHS-7	MW412825	Chanthaburi <12.573183, 101.903589>	Mangrove soil	*Ph. vexans* KP183944.1	831	99
	CHS-8	MW412826	Chanthaburi <12.573183, 101.903589>	Mangrove soil	*Ph. vexans* KP183944.1	827	99
	CHS-12	MW412830	Chanthaburi <12.711763, 102.181276>	Leaf debris (river)	*Ph. helicoides* KT750954.1	824	100
	CHS-13	MW412831	Chanthaburi <12.711763, 102.181276>	Leaf debris (river)	*Ph. helicoides* MG597190.1	679	99
	RYS-2	MT758165	Rayong <12.849345, 101.555479>	Forest soil	*Ph. vexans* MK011121.1	922	99
	RYS-8	MT758171	Rayong <12.849345, 101.555479>	Forest soil	*Ph. cucurbitacearum* KP183959.1	856	99
	RYS-9	MT758172	Rayong <12.849345, 101.555479>	Forest soil	*Ph. helicoides* KT750954.1	797	99
	RYS-12	MT758175	Rayong <12.776806, 101.714779>	Leaf debris (river)	*Ph. helicoides* KT595686.1	656	97
	RYS-13	MT758176	Rayong <12.776806, 101.714779>	Leaf debris (river)	*Ph. helicoides* KY084740.1	793	95
	RYS-15	MT758178	Rayong <12.776806, 101.714779>	Leaf debris (river)	*Ph. helicoides* KT750954.1	819	99
	RYS-17	MT758180	Rayong <12.776806, 101.714779>	Leaf debris (river)	*Ph. helicoides* KT750954.1	841	99
	RYS-20	MT758183	Rayong <12.776806, 101.714779>	River soil	*Ph. cucurbitacearum* MK416211.1	868	100
	TS-2	MW426376	Trat <12.3554343,102.44117412>	Orchard soil	*Ph. cucurbitacearum* KP183959.1	904	99
	TS-3	MW426377	Trat <12.3554343,102.44117412>	Orchard soil	*Ph. cucurbitacearum* KP183959.1	790	99
	TS-7	MW426381	Trat <12.15233197,102.53187095>	Forest soil	*Ph. vexans* MT647272.1	882	98
	TS-8	MW426382	Trat <12.15233197,102.53187095>	Forest soil	*Ph. vexans* KP183940.1	885	99
	TS-10	MW426384	Trat <12.35619623,102.440991>	River soil	*Ph. cucurbitacearum* KP183959.1	904	99
	TS-11	MW426385	Trat <12.35619623,102.440991>	River soil	*Ph. cucurbitacearum* KP183959.1	821	100
	TS-12	MW426386	Trat <12.35619623,102.440991>	Leaf debris (river)	*Ph. helicoides* KT750954.1	796	97
	TS-14	MW426388	Trat <12.35619623,102.440991>	Leaf debris (river)	*Ph. helicoides* KT750954.1	766	99

Morphological characterization

The 52 isolates were cultured on PDA, CMA and V8 agar and observed for morphological characteristics (Figs. 1–3, Table 4). The Mycobank, Index Fungorum and GBIF databases were used to clarify the current scientific name of the organism. The results indicated that 13, 18 and 21 isolates belonged to *Globisporangium*, *Pythium* and *Phytopythium*, respectively, whose generic diagnosis descriptions were amended as follows.

Globisporangium carolinianum (V.D. Matthews) Uzuhashi, Tojo and Kakish., Mycoscience 51(5): 361 (2010)

[Synonym: *Pythium catenulatum*, *Pythium carolinianum*]

Description: Colonies on PDA, CMA and V8 agar showed

Table 4 Morphological characteristics of *Pythium* and related genera found in this study

Species	Full growth (d)			Number of isolates	Sporangia (µm, length × wide)	Oogonia (µm)	Antheridia	Oospores (µm)
	PDA	CMA	V8					
G. carolinianum	3.5	3.5	3.5	2	Filamentous with vesicle (av. 18.37)	Terminal or intercalary (av. 18.85)	1–2 Monoclinous and diclinous	Plerotic or aplerotic (av. 16.14)
G. splendens	2.1	2.1	2.4	11	-	Terminal or intercalary, smooth-walled (av. 26.26)	1–3 Diclinous, monoclinous or hypogynous	Aplerotic (av. 35.10)
P. acanthicum	4.6	4.6	4	7	Subglobose with discharge tube (av. 21.71)	Ornamented (acute spines), terminal or intercalary (av. 35.48)	1 Monoclinous or hyphogynous antheridium per oogonia	Plerotic (av. 37.71)
P. deliense	2	2	2	1	Inflated filamentous with vesicle	Smooth wall, terminal and intercalary (av. 34.70)	1 Monoclinous antheridium per oogonia	Aplerotic (av. 50.81)
P. diclinum	4	4	3	4	Filamentous with vesicle	-	-	-
P. longipapillum	3.25	3.25	3	4	Filamentous with vesicle (av. 16.94)	-	-	-
P. myriotylum	2	2	2	1	Inflated filamentous	Terminal (33.20)	Diclinous antheridia	-
P. torulosum	5	5	5	1	Non-inflated sporangium	-	-	-
Ph. cucurbitacearum	5.2	5	5.2	6	Subglobose, ovoid or pyriform with papillae (18.58 × 9.89)	Smooth wall, terminal or intercalary (av. 24.94)	1–2 Monoclinous antheridia per oogonia	Plerotic (av. 24.62)
Ph. vexans	4.3	4.3	4.5	6	Subglobose, globose, ovoid, pyriform or limoniform (16.62 × 9.28)	Smooth wall, terminal or intercalary (av. 20.26)	1 Hyphogynous, 1–3 diclinous or 1–2 monoclinous	Aplerotic, sometimes nearly plerotic (av. 23.32)
Ph. helicoides	2.3	2.3	2.1	9	Subglobose, limoniform pyriform or obpyriform (21.78 × 12.69)	Smooth wall, terminal or intercalary (av. 27.53)	1–2 Monoclinous or diclinous	Aplerotic (av. 23.02)

PDA = potato dextrose agar; CMA = corn meal agar; av. = average

a rosette pattern. Catenulate hyphal swellings were present. Sporangium filamentous and slightly inflated. Oogonium spherical originated terminal or intercalary. Oospores were aplerotic. The morphological traits were similar to the description of *P. catenulatum* in the report of Van der Plaats-Niterink (1981). However, according to the GBIF and Index Fungorum databases, it seemed that *G. carolinianum* was a basionym of this species.

Globisporangium splendens (Hans Braun) Uzuhashi, Tojo and Kakish., Mycoscience 51(5): 363 (2010)

[Synonym: *Pythium splendens*]

Description: Colonies grew rapidly on agar medium (full growth in 48 h) and formed thick, cottony aerial mycelia. Hyphal swellings were abundant, but a sporangium was absent. Most isolates from Trat province were homothallic, while others were heterothallic. These isolates produced smooth-walled terminal or intercalary oogonia, monoclinous and diclinous antheridia and thick-walled aplerotic oospores.

Pythium acanthicum J. Wash. Acad. Sci. 20: 408 (1930)

Description: Colonies exhibited a stellate to rosette pattern on PDA, CMA and with a rosette pattern on V8 agar, submerged with some aerial mycelia. Sporangium sub-globose with a discharge tube. Ornamented oogonia terminal or intercalary with acute spines. There were 1–2 monoclinous antheridia per oogonium. Oospores were plerotic.

Phytopythium cucurbitacearum P.M. Kirk, Index Fungorum 280: 1 (2015)

Description: Colony showed chrysanthemum pattern,

submerged. All isolates produced papillate sporangium, sub-globose, ovoid or pyriform. Smooth-walled oogonium, terminal or intercalary. There were 1–2 monoclinous antheridia per oogonium. Thick-walled plerotic oospores.

Pythium deliense Meurs, Phytopath. Z. 7: 176 (1934)

Description: Fast-growing *Pythium* isolate, cottony pattern, forming inflated filamentous sporangium with vesicle. Smooth-walled globose oogonia terminal and intercalary with monoclinous antheridia. Oospores were aplerotic.

Pythium diclinum Tokun., in Ito & Tokunaga, Trans. Sapporo nat. Hist. Soc. 14(1): 12 (1935)

Description: Colonies with rosette pattern. Filamentous inflated sporangium. Sexual structures were not found in any isolate obtained from Rayong or Trat provinces, whereas in Chanthaburi province they produced smooth, spherical or ovoid oogonia, mostly terminal or subterminal. Antheridia were usually diclinous. Oospores were aplerotic.

Pythium longipapillum Mostowf. and Salmaninezhad, in Salmaninezhad and Mostowfizadeh-Ghalamfarsa, Mycologia 111(4): 700 (2019)

Description: Colonies with rosette pattern, submerged. Sporangia were filamentous that produced vesicles and zoospores. There were no sexual structures found in any isolate, but Salmaninezhad and Mostowfizadeh-Ghalamfarsa (2019) indicated that this species produced smooth-walled oogonia with crook-necked paragynous, monoclinous or diclinous antheridia. Oospores were aplerotic. As a result, it must be determined whether these isolates are heterothallic. Additional investigation is required.

Pythium myriotylum Drechsler, J. Wash. Acad. Sci. 20: 404 (1930)

Description: Obtained isolates produced cottony colonies with aerial mycelia on any agar medium. Inflated to lobulated filamentous sporangia, with no zoospores observed. Terminal globose oogonia with diclinous antheridia. No oospores were observed.

Pythium torulosum Coker and P. Patt., J. Elisha Mitchell scient. Soc. 42(3–4): 247 (1927)

Description: Colonies on PDA, CMA and V8 agar showed a rosette pattern. Sporangia inflated with various sizes. These isolates showed no sexual reproduction.

Phytopythium helicoides (Drechsler) Abad, de Cock, Bala, Robideau, A.M. Lodhi and Lévesque, Persoonia 34: 37 (2014)

[Synonym: *Pythium helicoides*]

Description: Most isolates grew very quickly. Colonies on any agar medium produced cottony aerial mycelia. Terminal or intercalary smooth-walled sporangia with sub-globose, limoniform, pyriform or obpyriform shapes (some isolates did not produce oogonia and antheridia). There were 1–2 monoclinous or diclinous antheridia per oogonium. Oospores were aplerotic.

Phytopythium vexans (de Bary) Abad, de Cock, Bala, Robideau, A.M. Lodhi and Lévesque, Persoonia 34: 37 (2014)

[Synonym: *Pythium vexans*]

Description: Colonies on PDA, CMA and V8 agar with aerial mycelia, with chrysanthemum and stellate patterns. Some isolates produced non-papillate sporangia, but some isolates occasionally produced a papillate type. Sporangia sub-globose, globose, ovoid, pyriform or limoniform. Oogonia smooth-walled, terminal or intercalary with 1 hyphogynous, 1–3 diclinous or 1–2 monoclinous. Oospores were aplerotic, sometimes nearly plerotic.

Phylogenetic analyses

All *Pythium* isolates were well delineated to clades A–K, according to Lévesque and De Cock (2004). From the current study, the isolates were assigned to 5 *Pythium* clades (Tables 1–3), containing clades A, B, D, I and K, with 1, 12, 7, 11 and 21 isolates, respectively (Fig. 4).

Clade A

Only one isolate, RYS-11, belonged to this clade and shared 99% similarity of the ITS sequence to *P. deliense* isolate S3-Iraq (MN365090.1) (Table 1). This isolate produced inflated filamentous sporangia and smooth-walled aplerotic oospores (Figs. 2 and 3, Table 4). *P. deliense* infected or colonized a wide range of plants and soil samples in Japan (Watanabe, 1981) and Thailand (Intaparn et al., 2019). Some species in this clade were waterborne or aquatic species (Nam and Choi, 2019). Lévesque and De Cock (2004) also mentioned that all the species in this clade originated from algae.

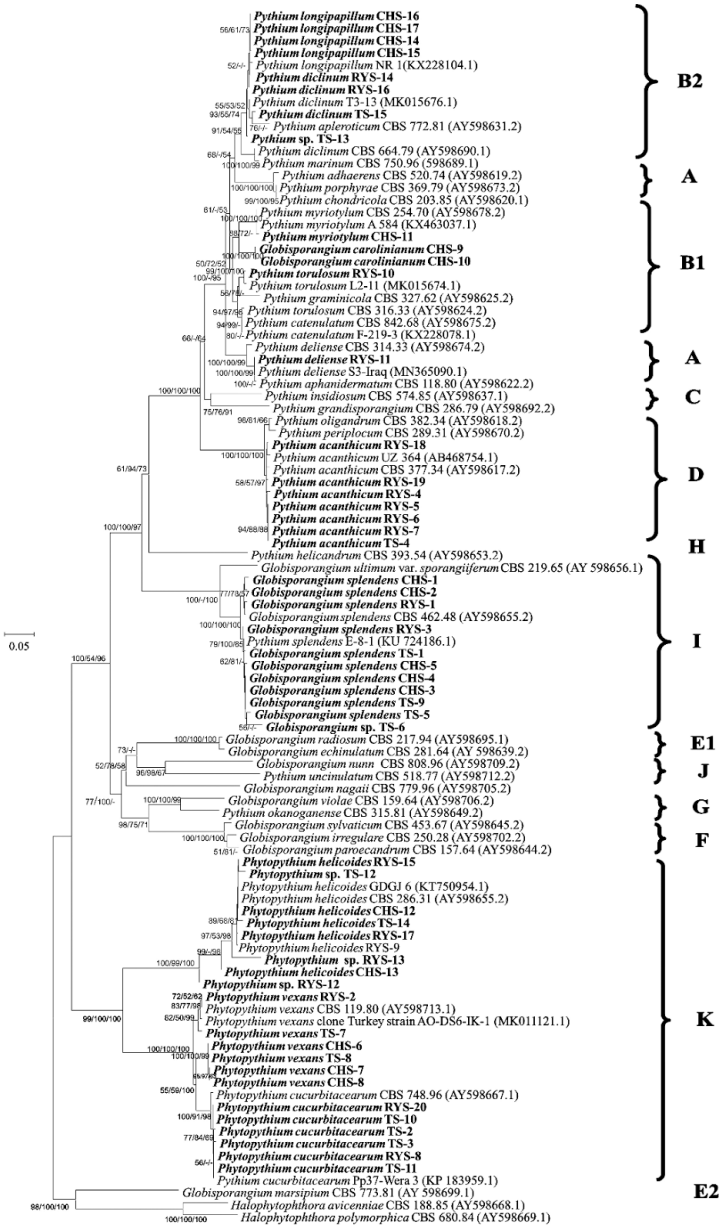

Fig. 4 Neighbor-joining phylogenetic tree reconstructed using MEGA X based on internal transcribed spacer region sequences of *Pythium* and related genera from all provinces, where numbers on nodes indicate bootstrap support from algorithms with 1,000 sampled datasets (values lower than 50 % not shown), braces indicate *Pythium* clades according to Lévesque and De Cock (2004), with *H. avicenniae* and *H. polymorphica* used as outgroup and scale bar = 0.05 substitutions per nucleotide position

Clade B

There were 12 isolates from Chanthaburi and Rayong provinces that were well delineated to clade B (CHS-9, CHS-10, CHS-11, CHS-14, CHS-15, CHS-16, CHS-17, RYS-10, RYS-14, RYS-16, TS-13 and TS-15), as shown in Table 1. Most of the isolates shared 99% partial ITS sequence similarity to their most closely related sequences, except for isolate TS-13 which shared 97.8% similarity. From this study, the isolates were closely related to *G. carolinianum, P. myriotylum, P. longipapillum*, *P. torulosum* and *P. diclinum* (Table 1). The species in this clade were waterborne, which was similar to clade A (Lévesque and De Cock, 2004; Nam and Choi, 2019). *Pythium* members in clade B produced filamentous, inflated sporangia and could be divided into two subclades, B1 and B2. From the current investigation, 4 isolates were recovered and assigned to subclade B1 as *G. carolinianum* (Synonym: *P. catenulatum*), *P. torulosum* and *P. myriotylum*. Isolates CHS-9 and CHS-10 were closely related to *P. catenulatum* JX569196. They produced both asexual and sexual reproductive structures (filamentous sporangia and plerotic oospores, respectively), as shown in Figs. 2 and 3 and Table 4. *Pythium* isolate RYS-10 was most closely related to *P. torulosum* MK015674.1. However, isolate RYS-10 produced only an asexual structure (Fig. 2). Furthermore, phylogenetic analysis showed that isolates CHS-9 and CHS-10 were clustered in a monophyletic clade with high bootstrap support (Fig. 4). Several other reports have claimed that *G. carolinianum* can be isolated from many countries in a wide range of environments, such as in irrigation water from the river (Sánchez and Gallego, 2001). *Pythium* isolate CHS-11 was assigned and most closely related to *P. myriotylum* KY019264.1. This isolate produced both asexual and sexual structures (Figs. 2 and 3). Reports have indicated the *P. myriotylum* caused virulent disease in many plants, such as lettuce and cucumber (Koohakan et al., 2008).

Seven isolates of *Pythium* subclade B2 were detected in this study that were assigned to *P. diclinum* and *P. longipapillum*. These were isolated from Rayong, Chanthaburi and Trat provinces. This subclade produced filamentous, non-inflated to slightly inflated sporangia (Lévesque and De Cock, 2004) with smooth-walled oogonia. *Pythium* isolates CHS-14, CHS-15, CHS-16 and CHS-17 were isolated from Chanthaburi province and assigned to *P. longipapillum*. They were most closely related to *P. longipapillum* KX228104.1. This species has been isolated from rice (*Oryza sativa*) in a paddy field, Iran (Salmaninezhad and Mostowfizadeh-Ghalamfarsa, 2019) and the current study was the first to report *P. longipapillum* in Thailand. All isolates obtained in the current study produced filamentous sporangia with vesicles (Fig. 2); however, the sexual reproductive structure was absent, despite a report that this species produced both oogonia and antheridia (Salmaninezhad and Mostowfizadeh-Ghalamfarsa, 2019). Thus, additional morphological research

is required for clarification. Phylogenetic analysis indicated that *P. longipapillum* isolate CHS-14, CHS-15, CHS-16 and CHS-17 were closely related to the referenced species from the Genbank database (Fig. 4) with 60–70% bootstrap support. However, it should be noted that all isolates of *P. longipapillum* were closely related with *P. diclinum*, suggesting that the obtained isolates might be conspecific.

Clade D

Seven isolates were isolated, with six (RYS-4, RYS-5, RYS-6, RYS-7, RYS-18 and RTS-19) from Rayong and one (TS-14) from Trat. All these isolates were well delineated to clade D and all were closely related to *P. acanthicum* (Table 2). They were obtained from forest, river and orchard soil sites. *P. acanthicum* produced oospores with spines (ornamented oospore with acute spines)(Van der Plaats-Niterink, 1981), as shown in Figs. 2 and 3 and Table 4. The *Pythium* species in this clade (*P. acanthicum, P. oligandrum, P. periplocum, P. amasculinum* and *P. hydnosporum*) have been reported as mycoparasites to some phytopathogenic fungi and oomycetes (Ali-Shtayeh and Saleh, 1999). Although some reports indicated that *P. acanthicum* is a non-pathogenic (Allain-Boulé et al., 2004) or low-virulent species (Sewell, 1981), some isolates could be an opportunistic pathogen in some plants.

Clade I

Eleven isolates were recovered and assigned to *G. splendens*: (CHS-1, CHS-2, CHS-3, CHS-4, CHS-5, RYS-1, RYS-3, TS-1, TS-5, TS-6 and TS-9), as shown in Table 2. They were isolated from Chanthaburi, Rayong and Trat provinces. The *G. splendens* sexual structure indicated that some isolates were homothallic. Recently, the genus *Pythium* in clade I was changed to *Globisporangium* because the production of an asexual structure was discovered (globose sporangia) (Uzuhashi et al., 2010; 2019). This resulted in three species of clade I being renamed from *P. ultimum*, *P. splendens* and *P. heterothallicum* to *Globisporangium ultimum, Globisporangium splendens* and *Globisporangium heterothallicum,* respectively (Uzuhashi et al., 2010). Some species in different clades were also amended and renamed to *Globisporangium* (Uzuhashi et al., 2019). In the current study, isolates of *G. splendens* were mostly isolated from orchard soil, except isolate TS-9 that was isolated from river soil (Table 2). Most of them produced large size oogonia (Fig. 3, Table 4) and hyphal swelling. All isolates were clustered in a monophyletic clade with high bootstrap support (Fig. 4). Interestingly, Suksiri et al. (2018) claimed the first report *P. splendens* (*G. splendens*) in infected durian, indicating the possibility of *G. splendens* spreading over cultivated soil in eastern Thailand. However, further study is needed to prove the possibility of *G. splendens* being a new dominant species.

Clade K

Phytopythium has been reported as *Pythium* clade K that produces *Phytophthora*-like sporangia, which also produced a vesicle. A biological trait (sporangium) that appeared to be a combination between *Pythium* and *Phytophthora* (Nam and Choi, 2019) indicated *Pythium* with this distinctive feature might be a new genus. The first successful attempt to officially classify the *Pythium* described above using morphological characteristics and phylogenetic analyses by Lévesque and De Cock (2004). The current study revealed that the *Pythium* in clade K is polyphyletic because of its differences in phylogeny to other clades. Although the species has both *Pythium* and *Phytophthora* characteristic traits, it was described by Bourret et al. (2018) that *Phytopythium* differed from *Pythium* and was likely to be an early-diverging species of all the genera in the *Peronosporaceae*. Due to this differentiation, some studies decided to rename *Pythium* species in clade K into a new genus, such as *Ovatisporagium* (Uzuhashi et al., 2010).

The 21 isolates in Clade K from the current study were assigned to *Ph. cucurbitacearum, Ph. vexans* and *Ph. helicoides*, which were found in every soil habitat (Table 3), especially *Ph. vexans* that was isolated from mangrove forest. This finding was related to the discovery by Bennett et al. (2018) of *Ph. helicoides* in mangrove leaf litter, while *Ph. cucurbitacearum* was also found in cultivated soil and plant hosts in Thailand (Suksiri et al., 2018; Hattapanichaporn et al., 2020). The similarity of these results suggested the ecological competence of this genus. The phylogenetic tree (Fig. 4) showed that all isolates in clade K were embedded in a polyphyletic clade, suggesting diverging phylogeny.

Information regarding *Pythium* and related genera in the class *Oomycetes* distribution in Thailand has been rarely documented. Many domestic studies have focused on well-known plant pathogenic species, such as *P. aphanidematum* (Charoenrak et al., 2019) or a species that is harmful to humans and animals, such as *P. insidiosum* (Rujirawat et al., 2017), while other studies only focused on a genus in a specific class (Hung et al, 2015b; Janruang and Unartngam, 2018; Hattapanichaporn et al., 2020). Therefore, this study, in conjunction with the work in Rayong province by Saelee

et al. (2021), has provided important information covering the extensive *Pythium* distribution in eastern Thailand (Fig. 5) and has established a new benchmark for future research on the distribution of Thai strains. The current studied identified most isolates as monophyletic, although some were polyphyletic and some species might be conspecific. However, the current study provided an introductory and preliminary experiment with overview data. Additional experimentation to study the diversity within a genome and environmental factors, such as a multi-gene study (*cox II*, *β-tubulin* and *EF-1α*) and a statistical method to estimate the relationships with an environmental variable, is required to gain a clearer understanding of relations within the genomes of each species and their relation with the environment, which may be either "determinant for" or "irrelevant to" *Pythium* distribution.

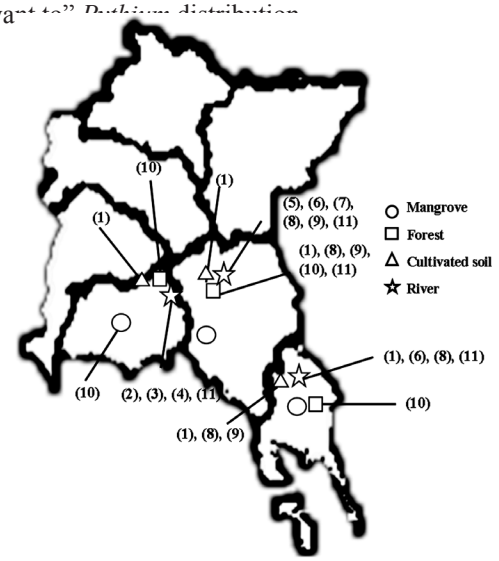

Fig. 5 Distribution of *Pythium* and related genera in Chanthaburi, Rayong and Trat provinces: (1) *G. splendens*; (2) *G. carolinianum*; (3) *P. longipapillum*; (4) *P. myriotylum*; (5) *P. deliense*; (6) *P. diclinum*; (7) *P. torulosum*; (8) *Ph. cucurbitacearum*; (9) *P. acanthicum*; (10) *Ph. vexans* and (11) *Ph. helicoides*

Conflict of Interest

The authors declare that there are no conflicts of interest.

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