Current Applied Science and Technology Vol. 21 No. 1 (January-March 2021)

Morphological Characterization and Phylogeny of *Pythium* and Related Genera in Rayong Province, Thailand

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Received: 12 March 2020, Revised: 5 August 2020, Accepted: 21 August 2020

Abstract

Most well-known microorganisms in the class Oomycetes (notably genera *Phytophthora* and *Pythium*) are pathogenic to both animals and plants due to their diverse lifestyle patterns. This study was designed to recover *Pythium* from composite soils (cultivated and forest soils) and water sources (fresh and brackish water) from Rayong Province. Twenty isolates of hyaline and non-septate fungal-like organisms were isolated from those sources. The primer pair ITS4 and ITS6 were used to amplify approximately 900 bp products from Internal transcribed spacer (ITS) region and morphological characteristics including sporangium, oogonium, antheridium and oospore, were noted. Morphological characteristics data of recovered *Pythium* strain can be classified into 12 source groups. ITS sequencing results revealed that eight closely related species had been recovered: *Globisporangium splendens*, *Pythium cucurbitacearum*, *Pythium acanthichum*, *Pythium deliense*, *Pythium diclinum*, *Pythium torulosum*, *Phytopythium vexans* and *Phytopythium helicoides*, which had similarities in the range 94.67-100% values at between 656 and 922 locations. Most of these species were reported as plant pathogens. Therefore, this report can be used as a guide for disease control planning.

Keywords: Oomycetes; identification; phylogeny; Rayong Province; *Pythium*
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1. Introduction

The microbes in class Oomycetes are classified in kingdom Chromista and subphylum Oomycota. Some species: like *Pythium* sp., live in many types of ecosystems, including a wide range of soil and water sources [1]. Many species in this class affect the environment and economy due to their capability to be plant and animal pathogens [2, 3]. *Pythium* and related genera in family Pythiaceae are one of the most important Oomycete distributed worldwide. They can survive under different location and environments such as tropical forests, natural and agricultural ecosystems, arid zones, temperate zones or even polar regions [4], because they have an ability to produce thick-walled resting spore or sexual reproductive structure called oospore, and asexual reproductive structure called sporangium which form zoospore inside which can be released through vesicle discharge tube

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[1]. *Pythium* and related genera can be isolated from both terrestrial and aquatic habitats, and many of them are plant pathogens. Oomycetes have a high distribution rate, which results in a infecting a wide range of host plants, notably succulent plants causing pre- and post- emergence damping off disease [1, 5, 6]. Moreover, *Pythium* can infect mammals, mainly in tropical and subtropical area, and causes pythiosis disease [7-9]. However, there were many reports about the capability of *Pythium* spp. as biological control agent (BCA), for example: *Pythium oligandrum* [10], *Pythium periplocum* and *Pythium acanthicum* [11]. Moreover, there have been a few reports that indicated that *Pythium* could also produce cellulolytic enzymes [12, 13]. Then, it can be seen that *Pythium* exists in every type of ecosystems, and can cause both positive and negative effects on a wide range of hosts. Therefore, good cultivation plan and pathogenicity data are necessary for disease control measures. Simultaneously, it can be applied as additional data for *Pythium* and related genera distribution in Thailand. Generally, a conventional procedure such as morphological study has been widely used to identify Oomycetes genera and the internal transcribed spacer (or ITS) sequences have also been used to classify to species level [1]. Thus, the purpose of this investigation was to study the diversity group of *Pythium* spp. that could be isolated from cultured-dependent methods. The samples were collected from cultivated areas, natural forests, mangrove forests and rivers in Rayong province <12.686277, 101.271261>. All *Pythium* isolates in this study were classified using morphological characteristics and ITS sequence data.

2. Materials and Methods

2.1 Sampling and isolation

Vertical soil samples (300 mm soil depth) were obtained from a cultivated field<12.85099178, 101.55733498>, a natural forest <12.849345, 101.555479>, a mangrove soil <12.698767, 101.707131> and a river <12.776806, 101.714779>. Moreover, plant debris from river and mangrove were also collected. Three techniques were used for isolation:

1) Modified soil plate technique [14]: Approximately 1g of soil sample was put on the surface of selectiveagar media (CMA (corn meal agar) + BNPRA (benomyl 10 ppm, Nystatin 25 ppm, Pentachloronitrobenzene 25 ppm, Rifampicin 10 ppm and Amplicillin 500 ppm) media + Rose Bengal(0.05 g/liters) [15]. Then an agar plug was transferred onto new agar media (CMA, potato dextrose ager (PDA) and V8 juice agar) to obtain a pure culture.

2) Soil baiting technique [16]: Approximately 1g soil or 1ml water sample was mixed with 9 ml sterile distilled water in a Petri dish, then 10 cucumber seeds were added and spread carefully. The sample was incubated (room temperature, 24 h), and a seed was transferred onto selective agar media. Then again an agar plug was transferred onto CMA, PDA and V8 agar media to obtain a pure culture.

3) Soil dilution technique [17]: Approximately 1g soil sample was mixed with 9 ml of sterile distilled water in a test tube and serially diluted to obtain a 10^{-4} dilution. One milliliter of the soil suspension was then pipetted onto Petri dishes containing CMA + BNPRA + Rose Bengal media and then an agar plug was transferred to CMA, PDA and V8 agar media to obtain a pure culture.

2.2 Morphology identification

Water culture, grass blade culture and low nutrient media were used to study asexual reproduction (Sporangium development) [18]. All techniques used were as follows:

1) The water culture technique: an agar plug of pure culture was placed on a Petri dish filled with sterile distilled water.

2) Grass blade culture technique: a boiled grass leaf was placed in a Petri dish and sterile distilled water was then added.

3) Low nutrient media culture: each isolate was cultured in CMA and then checked for asexual structures under a light microscope.

4) Checking for sporangium and zoospore formation within 24-48 h to study sexual reproduction: each isolate was cultured in V8 juice agar and the sexual organs (antheridia, oogonia, and oospores) were observed under a light microscope. All experiments were performed with 3 replicates and observed within 7 days. The taxonomic key used for identification was referred to Van der Plaats-Niterink [19]

2.3 DNA extraction, PCR amplification and sequencing

Oomycetes strains were cultured in PDA at room temperature and genomic DNA was extracted according to Ivors protocol [20]. The internal transcribed spacer (ITS) regions were amplified using the primer ITS6 (5'-GAAGGTGAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGC TTATTGATATGC-3) [21]. The PCR conditions were the same as those used by Cooked *et al.* [22]. The PCR products were analyzed by gel electrophoresis. Gels were extracted and purified using GeneJET Gel Extraction Kit (Thermo scientific). The purified products were stored at -20C° until required. The sequencing of ITS region was determined by Bionics Co. Ltd.

2.4 Phylogenetic analyses

Sequences were determined by the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology (NCBI; Bathesda, USA). The aligned sequences were used to construct phylogenetic trees. The neighbour-joining algorithm [23] was from the MEGA X program. The resultant tree was evaluated in bootstrap analyses [24] based on 1000 resamplings of the neighbour-joining dataset from the PHYLIP package. All DNA sequences were submitted to GenBank (NCBI database).

3. Results and Discussion

3.1 Isolation and morphological identification

Twenty isolates containing 2 genera, *Pythium* and *Phytopythium*, were obtained from soil and water samples and could be classified into 8 species. They were well delineated into 12 groups based on the origin (cultivated field, degraded forest, fresh water or marine). Most isolates produced both asexual and sexual structures, but some isolates did not. RYS-13, RYS-6, RYS- 7, RYS- 13 and RYS- 16 showed no asexual organs, while RYS-9, RYS-10, RYS-12, RYS-13, RYS-14, RYS-15, RYS-16 and RYS-17 presented no sexual organs (Table 1, Figures 1-12). However, in mangrove soil, there were no isolates of Oomycetes found.

Group 1: No zoospoarangia, produce only sexual reproductive structure obtained from cultivated soil (Figure 1).
Group 2: Subglobose or pyriform proliferating zoosporangia and smooth wall oospores obtained from natural forest soil (Figure 2).
Group 3: No zoosporangia formation, produced only sexual reproductive organs obtained from natural forest soil (Figure 3).
Group 4: Oomycete with non-internal and internal proliferating subglobose or pyriform zoosporangia, acute spines oospore obtained from natural forest soil (Figure 4).
Group 5: Subglobose or pyriform zoosporangia with papillae and smooth wall oospores obtained from natural forest soil (Figure 5).
Group 6: Subglobose or pyriform of non-papillate zoosporangia obtained from natural forest soil (Figure 6).
Group 7: Non- inflated zoosporangia obtained from river water (Figure 7).
Group 8: Inflated zoosporangia with smooth wall oospore obtained from river leaf debris (Figure 8).
Group 9: Papillate subglobose or pyriform zoosporangia obtained from river leaf debris (Figure 9).
Group 10: Inflated filamentous sporangia (Figure 10)
Group 11: Non-internal and internal proliferating subglobose or pyriform zoosporangia, acutely spine oospores obtained from river soil (Figure 11).
Group 12: Subglobose or pyriform zoosporangia with papillae and smooth wall oospores obtained from river soil (Figure 12). The distribution of all isolates is shown in Figure 15.

Table 1. Morphology of Oomycetes isolates

Isolate	**Full growth (days)**			**Sporangia**	**Oogonia**	**Antheridia**	**Oospores (µm)**	**Source group**
	PDA	CMA	V8					
RYS-1	2	2	2	-	Smooth wall, intercalary	3-4 monoclinous antheridia per oogonia	Aplerotic (68.76)	1
RYS-2	2	2	2	Subglobose or pyriform proliferating	Smooth wall, terminal or intercalary	1 monoclinous or hypogynous antheridia per oogonia	Nearly Plerotic (47.31)	2
RYS-3	2	2	2	-	Smooth wall, intercalary	1 monoclinous or hyphogynous antheridia per oogonia	Aplerotic (56.38)	3
RYS-4	5	5	4	Subglobose with discharge tube	Ornamented, terminal or intercalary	1-2 monoclinous antheridia per oogonia	Plerotic (44.42)	4

Table 1. (cont.)

RYS-5	5	5	5	Subglobose with discharge tube	Ornamented, terminal or intercalary	1-2 monoclinous antheridia per oogonia	Plerotic (42.53)	4
RYS-6	4	4	5	-	Ornamented, terminal or intercalary	1 monoclinous of hyphogynous antheridia per oogonia	Plerotic (39.65)	4
RYS-7	7	7	6	-	Ornamented, terminal or intercalary	1-2 monoclinous antheridia per oogonia	Plerotic (44.64)	4
RYS-8	6	6	6	Subglobose or pyriform, papillate	Smooth wall, terminal or intercalary	-	Plerotic (32.48)	5
RYS-9	2	2	2	Subglobose or pyriform	Smooth wall, terminal or intercalary	1 monoclinous antheridia per oogonia	-	6
RYS-10	5	5	5	Non-inflated filamentous	-	-	-	7
RYS-11	2	2	2	Inflated filamentous with vesicle	Smooth wall, terminal and intercalary	1 monoclinous antheridia per oogonia	Aplerotic (50.81)	8
RYS-12	2	2	2	Subglobose or pyriform	Smooth wall, terminal and intercalary	1 monoclinous antheridia per oogonia	-	9
RYS-13	2	2	2	-	-	-	-	9
RYS-14	5	5	4	Inflated filamentous	-	-	-	10
RYS-15	2	2	2	Subglobose or pyriform	Smooth wall, terminal and intercalary	-	-	9
RYS-16	4	4	3	-	-	-	-	10
RYS-17	2	2	2	Subglobose or pyriform	-	-	-	9
RYS-18	5	5	4	Subglobose	Ornamented, terminal or intercalary	1 monoclinous antheridia per oogonia	Plerotic (28.11)	11

Table 1. (cont.)

RYS-19	3	3	3	Subglobose	Ornamented, terminal or intercalary	1 monoclinous antheridia per oogonia	Plerotic (42.75)	11
RYS-20	5	5	5	Pyriform	Smooth wall, terminal or intercalary	1-2 monoclinous antheridia per oogonia	Plerotic (34.11)	12

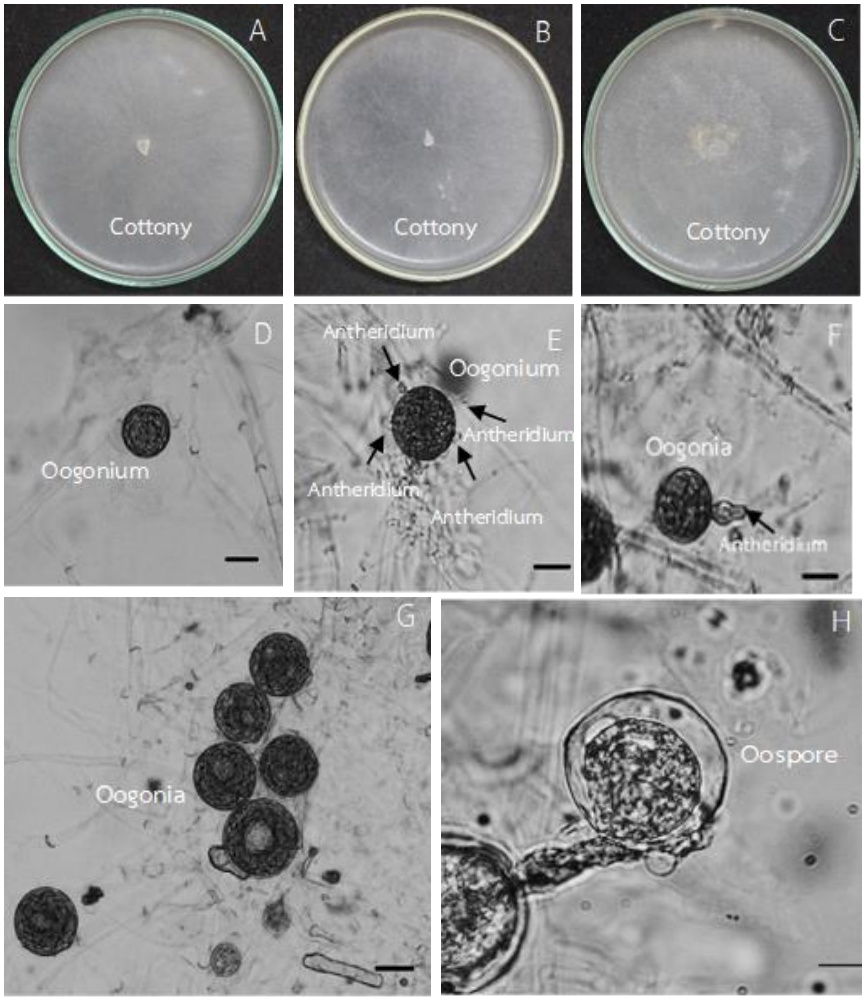

Figure 1. Morphology of an isolate obtained from cultivated soil (group 1: RYS-1). A-C: Colony patterns on CMA (A), PDA (B) and V8 agar (C); D-G: 10×; H: 40× (scale bars D-H: 20 µm); black arrows indicate antheridia.

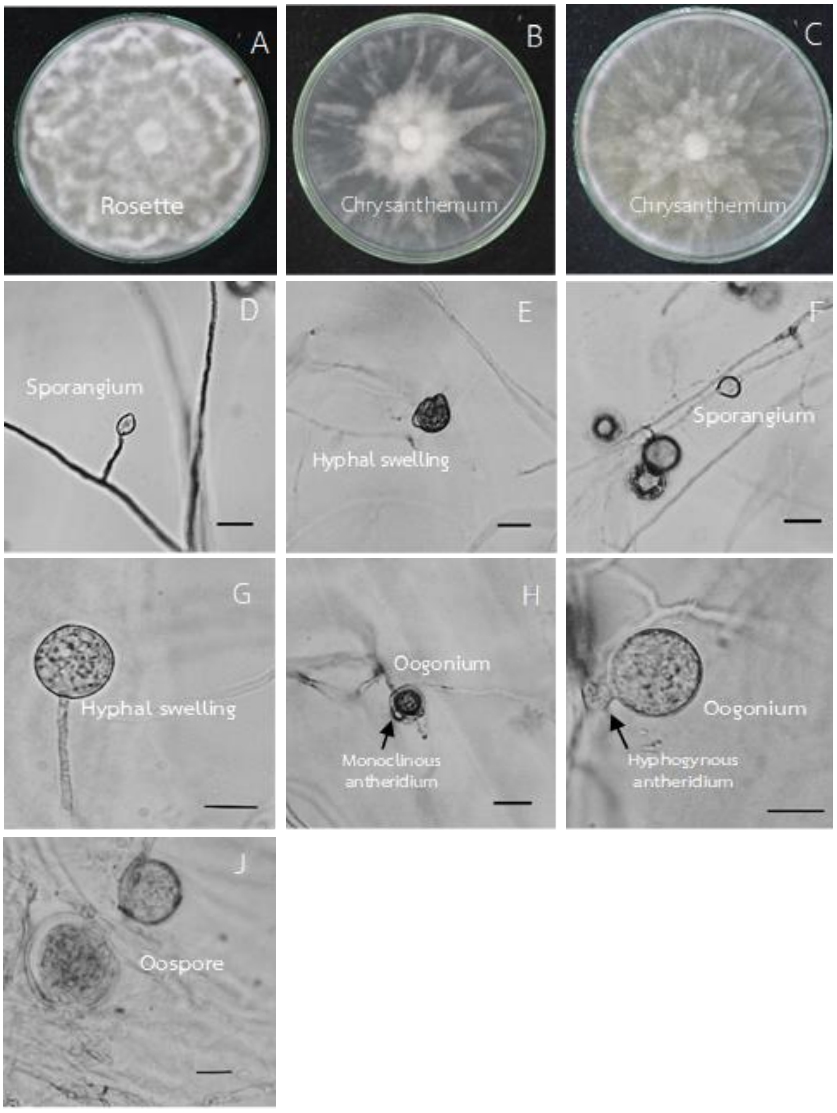

Figure 2. Morphology of an isolate obtained from natural forest soil (group 2: RYS-2). A-C: Colony patterns on CMA (A), PDA (B) and V8 a gar (C); D-F and H: 10×; G, I and J: 40× (scale bars D-J: 20 μm); black arrows indicate antheridia.

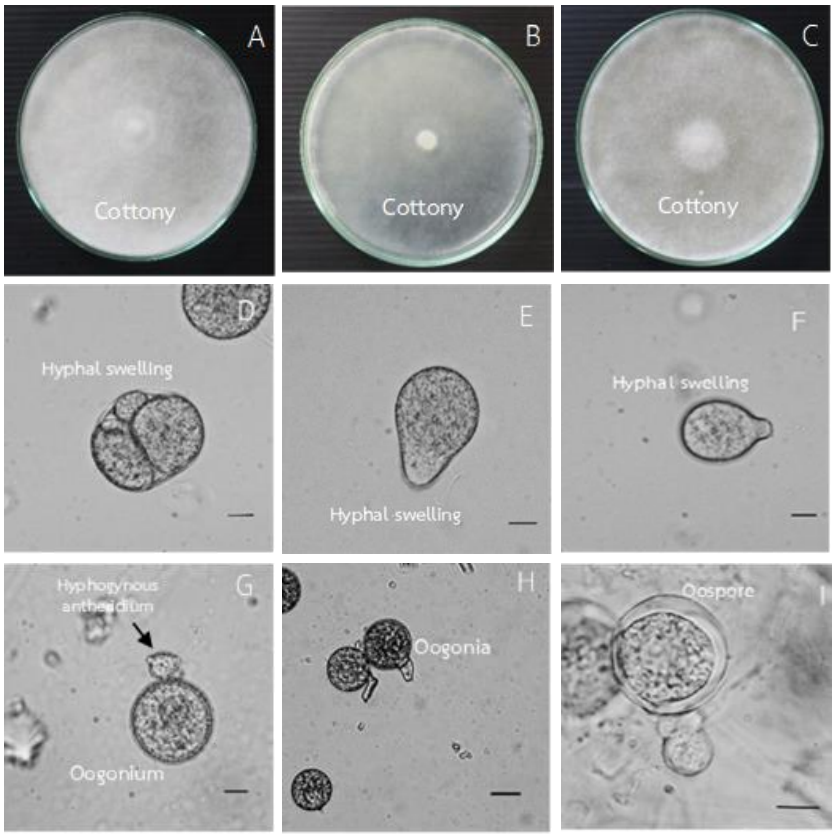

Figure 3. Morphology of an isolate obtained from natural forest soil (group 3: RYS-3). A-C: Colony patterns on CMA (A), PDA (B) and V8 agar (C); D-H: 10×; I: 40× (scale bars D-I: 20 µm); black arrows indicate antheridia.

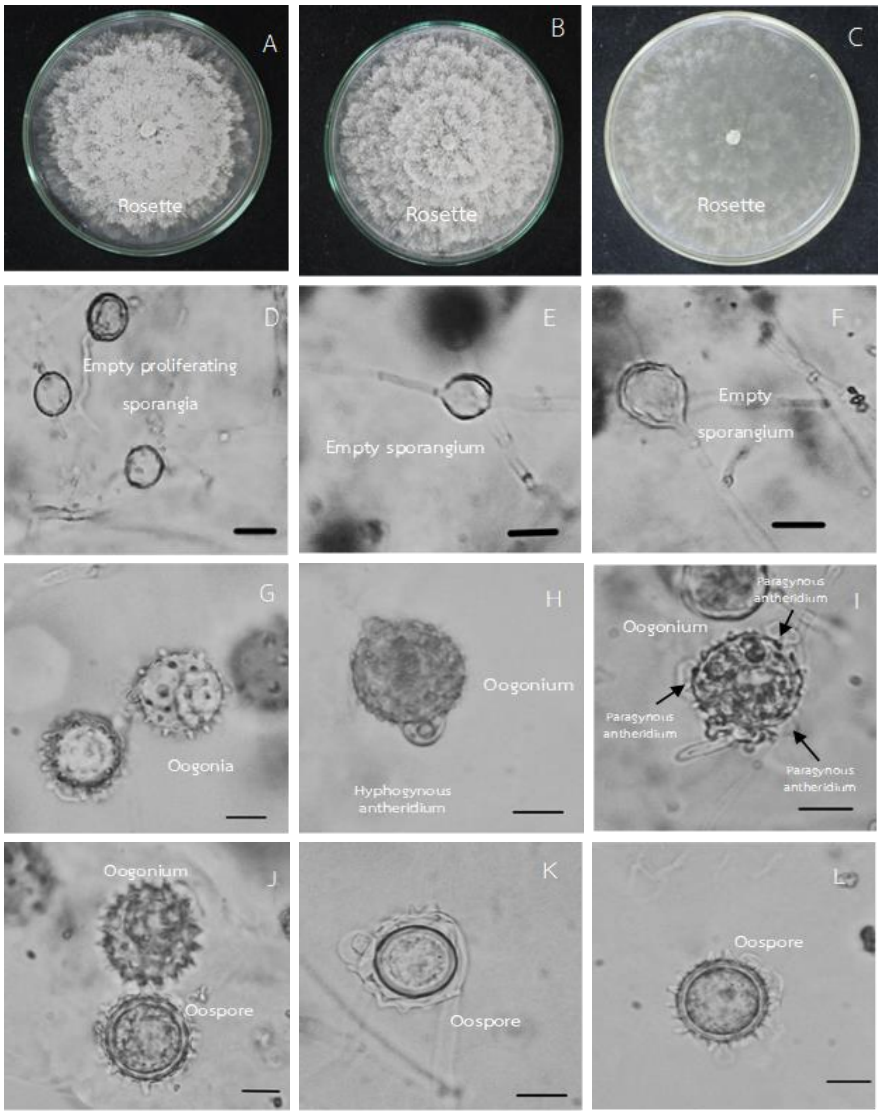

Figure 4. Morphology of an isolate obtained from natural forest soil (group 4: RYS-4, RYS-5, RYS-6 and RYS-7).A-C: Colony patterns on CMA (A), PDA (B) and V8 agar (C); D-F: 10×; G-L: 40× (scale bars D-L: 20 µm); black arrows indicate antheridia.

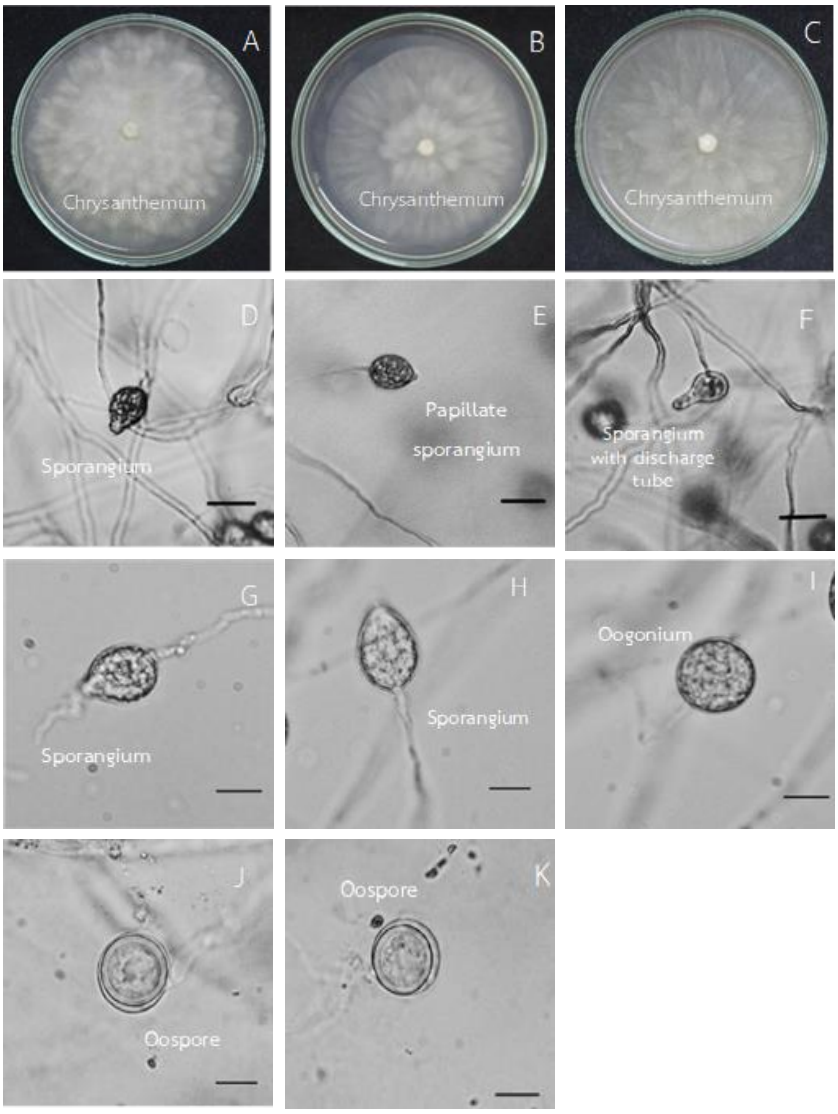

Figure 5. Morphology of an isolate obtained from natural forest soil (group 5: RYS-8). A-C: Colony patterns on CMA (A), PDA (B) and V8 agar (C); D-F: 10×; G-K: 40× (scale bars D-K: 20 µm)

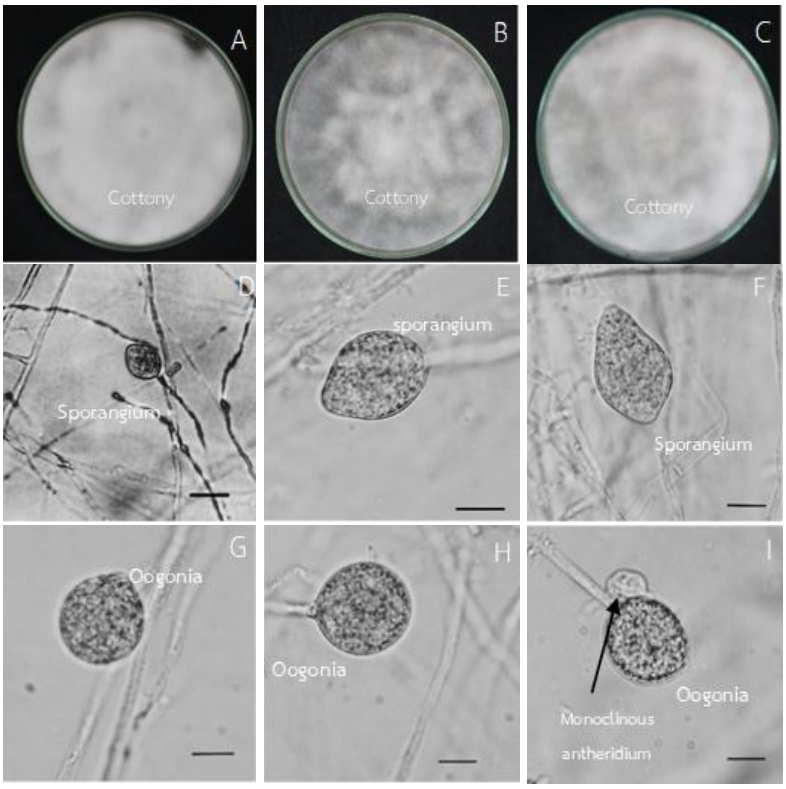

Figure 6. Morphology of an isolate obtained from natural forest soil (group 6: RYS-9). A-C: Colony patterns on CMA (A), PDA (B) and V8 agar (C); D and G: 10×; E-I: 40× (scale bars D-I: 20 µm); black arrows indicate antheridia.

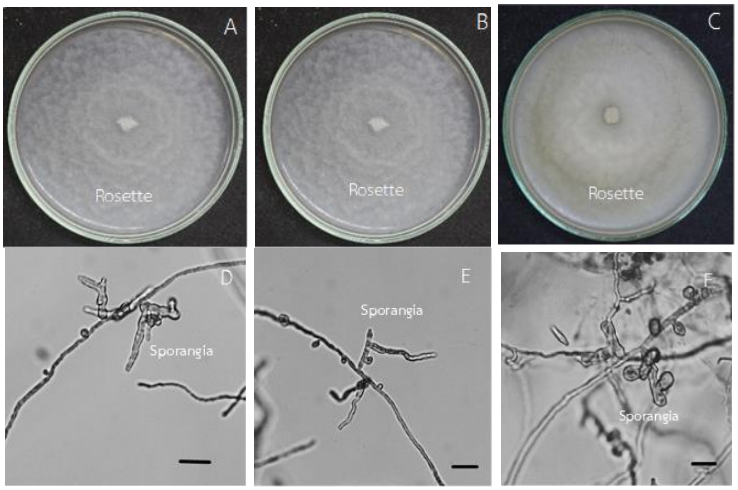

Figure 7. Morphology of an isolate obtained from river (fresh water; group 7: RYS-10).A-C: Colony patterns on CMA (A), PDA (B) and V8 agar (C); D-F: 10×; (scale bars D-F: 20 µm)

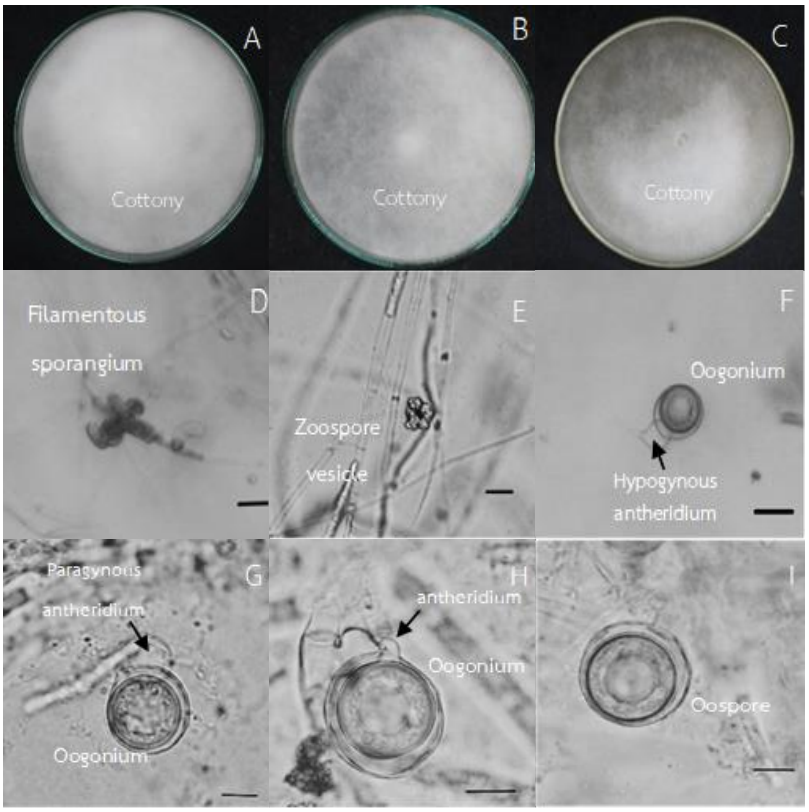

Figure 8. Morphology of an isolate obtained from river debris (group 8: RYS-11). A-C: Colony patterns on CMA (A), PDA (B) and V8 agar (C); D-F: 10×; G-I: 40× (scale bars D-I: 20 µm); black arrows indicate antheridia.

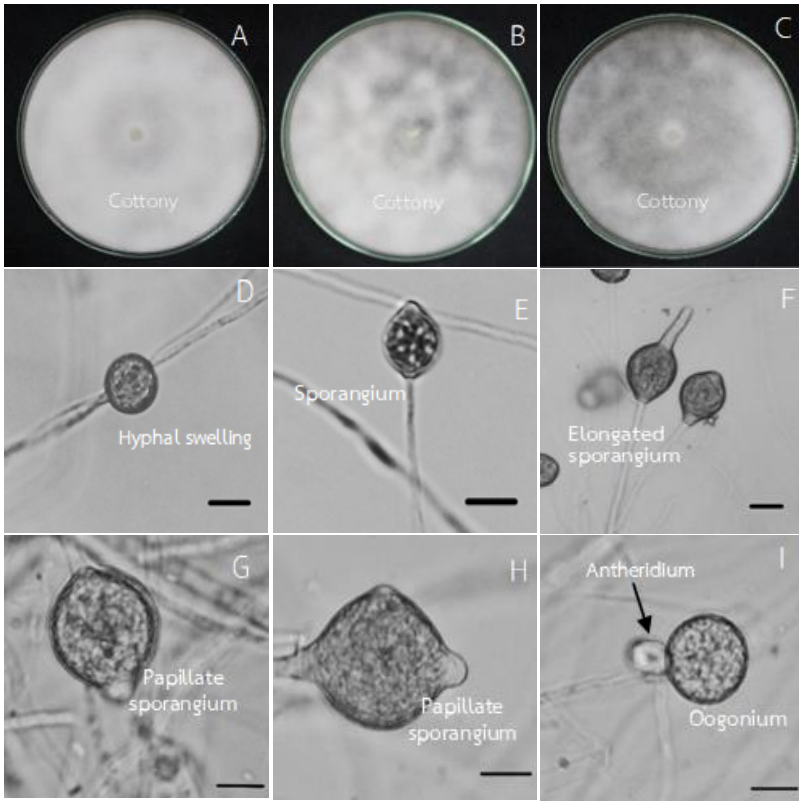

Figure 9. Morphology of an isolate obtained from river debris (group 9: RYS-12, RYS-13, RYS-15 and RYS-17). A-C: Colony patterns on CMA (A), PDA (B) and V8 agar (C); D-F: 10×; G-I: 40× (scale bars D-I: 20 µm); black arrows indicate antheridia.

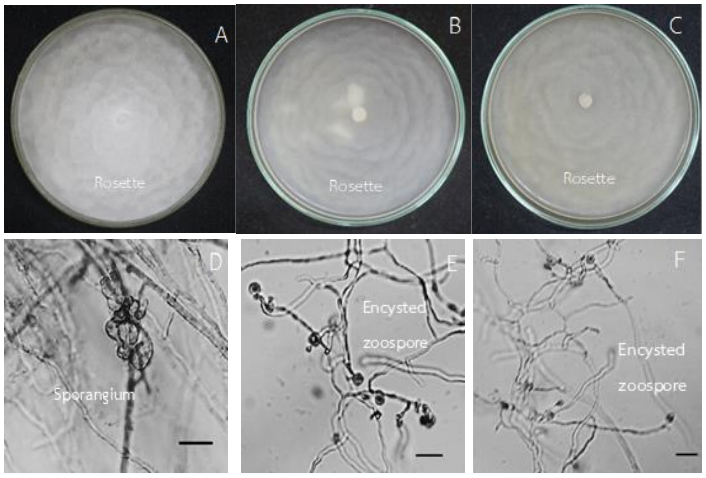

Figure 10. Morphology of isolate obtained from river debris (group 10: RYS-14 and RYS-16). A-C: Colony patterns on CMA (A), PDA (B) and V8 agar (C); D-F: 10×; (scale bars D-J: 20 µm)

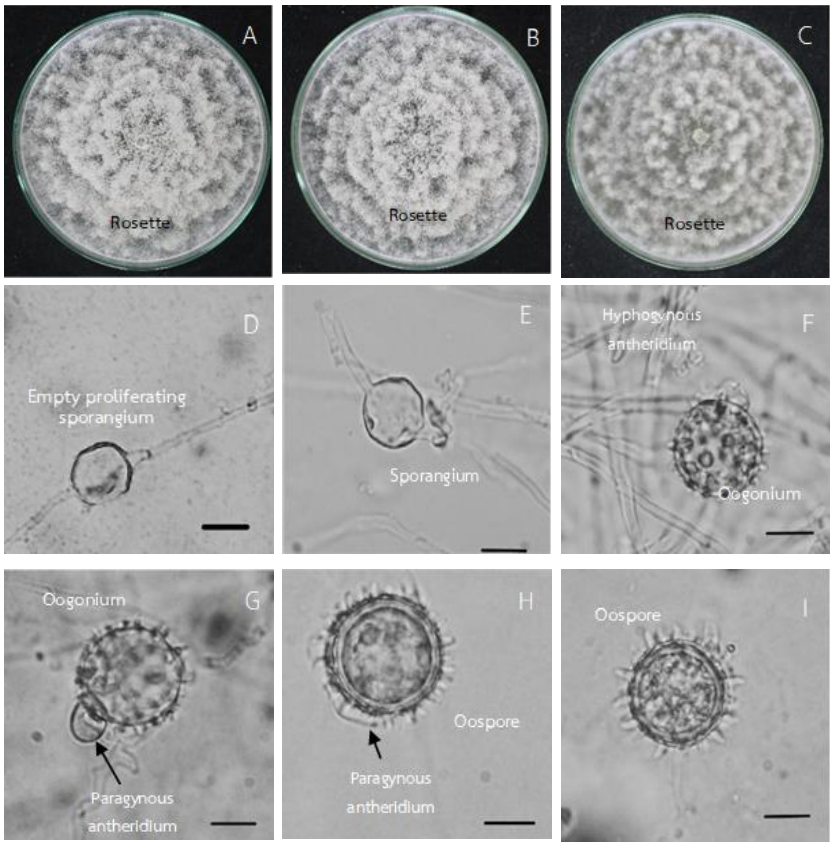

Figure 11. Morphology of an isolate obtained from river soil (group 11: RYS-18 and RYS-19). A-C: Colony patterns on CMA (A), PDA (B) and V8 agar (C); D: 10×; F-I: 40× (scale bars D-I: 20 µm); black arrows indicate antheridia.

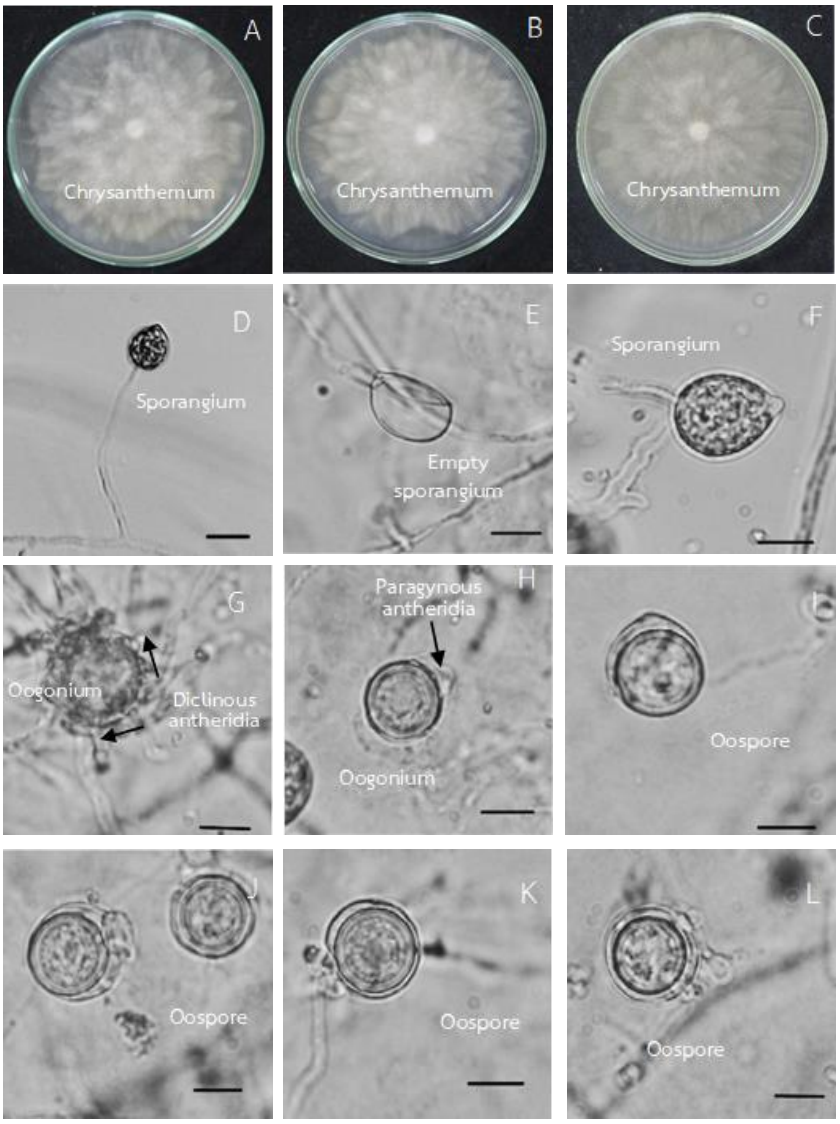

Figure 12. Morphology of an isolate obtained from river soil (group 12: RYS-20). A-C: Colony patterns on CMA (A), PDA (B) and V8 agar (C); D: 10×; E-L: 40× (scale bars D-I: 20 µm); black arrows indicate antheridia.

3.2 Phylogenetic analysis

After amplification of DNA sequence at ITS region using primers ITS4 and ITS6, approximately ~900 bp of PCR products were obtained (Figure 13). The comparison data between this study and the databases from NCBI found that the studied isolates were defined into eight Oomycetes species: *Globisporangium splendens* (formerly called *P. Splendens* [25], clade I), *Pythium cucurbitacearum* (clade K), *Pythium acanthichum* (clade D), *Pythium deliense* (clade A), *Pythium diclinum* (clade B), *Pythium torulosum* (clade B), *Phytopythium vexans* and *Phytopythium helicoides* (clade K) (Table

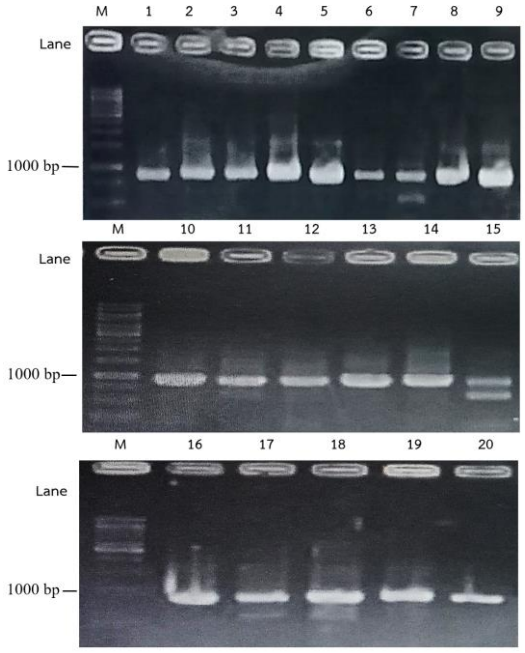

Figure 13. The primer pair ITS4 and ITS6 were used to amplified a 900 bp product compared with 1 kb marker (lane M); Lane 1-20: RYS-1 ~ RYS-20

2). All species showed the common morphological traits of each clade as reported by Lévesque and de Cock [26] and de Cock *et al.* [27]. It was found that *G. splendens* is a member of clade I in which most species in this clade do not produce zoospores. *Pythium cucurbitacearum* belongs to clade K with some common characteristics between *Pythium* and *Phytophthora* sp. like papillae sporangia, *Phytopythium* also belongs to this clade. *Pythium acanthichum* is in clade D, the members of which have oogonia with spines. Most of the species in this clade are mycoparasites, such as *P. oligandrum*. *Pythium deliense* belongs to clade A, which produce filimentous sporangia with intercalary antheridia. *Pythium diclinum* and *P. torulosum* are in clade B, which produce filimentous sporangia with smooth wall oogonia. Most species in this study are waterborne Oomycetes [28] (Figure 14). As stated, *Pythium* and related genera in the same class exist in many types of ecosystems. Therefore, the same genus can be found in a variety of habitats. A good example of this is *P. aphanidermatum*, now known to live in sea water [29] although this species was mostly found in cultivation area.

Table 2. Similarity and origin of each isolate

Species	GenBank Accession no. (ITS)	Origins	Hits	Seqence length (bp)	Similar (%)
G. splendens RYS-1	MT758164	Cultivated soil	*P. splendens* AY598655.2	853	98.71
P. vexans RYS-2	MT758165	Forest soil	*P. vexans* MK011121.1	922	99.21
G. splendens RYS-3	MT758166	Forest soil	*P. splendens* KU724186.1	793	99.62
P. acanthicum RYS-4	MT758167	Forest soil	*P. acanthicum* LC332027.1	772	98.71
P. acanthicum RYS-5	MT758168	Forest soil	*P. acanthicum* KU210470.1	863	98.61
P. acanthicum RYS-6	MT758169	Forest soil	*P. acanthicum* KU210470.1	871	98.74
P. acanthicum RYS-7	MT758170	Forest soil	*P. acanthicum* KU210470.1	858	98.49
P. cucurbitacearum RYS-8	MT758171	Forest soil	*P. cucurbitacearum* KP183959.1	856	99.42
P. helicoides RYS-9	MT758172	Forest soil	*P. helicoides* KT750954.1	797	99.75
P. torulosum RYS-10	MT758173	Fresh water	*P. torulosum* MK015674.1	877	99.42
P. deliense RYS-11	MT758174	Leaf debris	*P. deliense* MN365090.1	823	99.88
P. helicoides RYS-12	MT758175	Leaf debris	*P. helicoides* KT595686.1	656	96.68
P. helicoides RYS-13	MT758176	Leaf debris	*P. helicoides* KY084740.1	793	94.67
P. diclinum RYS-14	MT758177	Leaf debris	*P. diclinum* MK015676.1	782	99.22
P. helicoides RYS-15	MT758178	Leaf debris	*P. helicoides* KT750954.1	819	99.63
P. diclinum RYS-16	MT758179	Leaf debris	*P. diclinum* MK015676.1	774	99.21
P. helicoides RYS-17	MT758180	Leaf debris	*P. helicoides* KT750954.1	841	99.88
P. acanthicum RYS-18	MT758181	River soil	*P. acanthicum* AY598617.2	822	99.03
P. acanthicum RYS-19	MT758182	River soil	*P. acanthicum* HQ643411.1	770	98.83
P. cucurbitacearum RYS-20	MT758183	River soil	*P. cucurbitacearum* MK416211.1	868	100.00

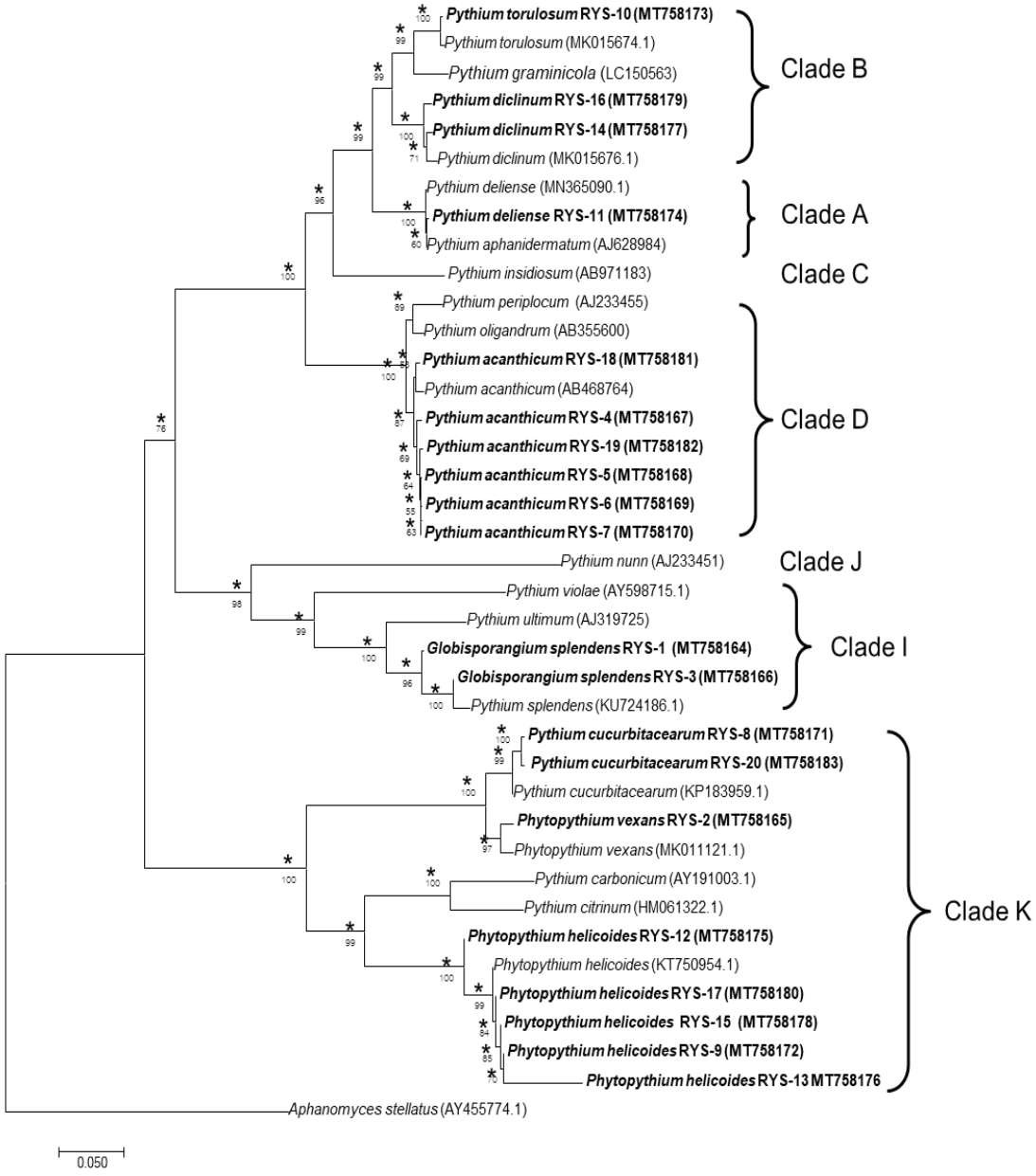

Figure 14. Neighbour-joining tree based on ITS region sequences (~900 bp) showing relationships between the studied-isolates and related *Pythium* species. Asterisks indicate branches of the tree that were also found using the maximum-likelihood and maximum-parsimony tree-making algorithms. Numbers of the nodes are percentage bootstrap values based on a neightbour-joining analysis of 1,000 sampled datasets. The root postion of the tree was determined using *Aphanomyces stellatus* AY455774.1. Bar, 0.05 substitutions per nucleotide position.

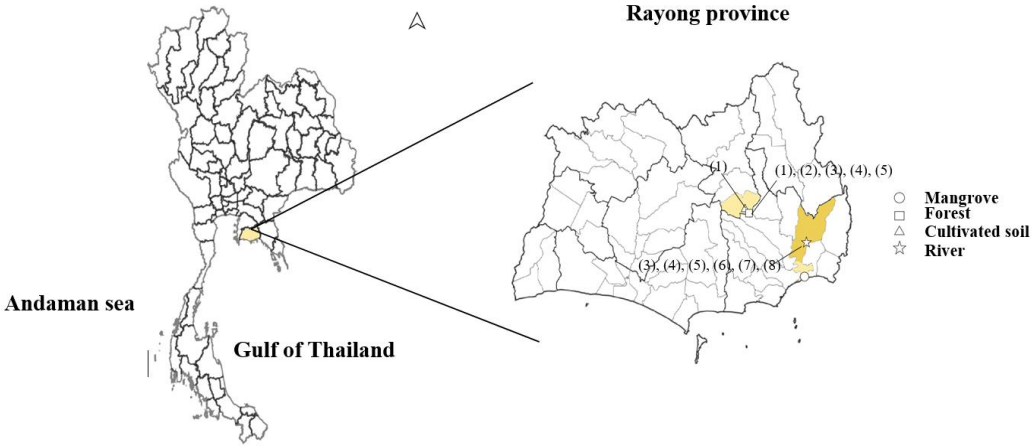

Figure 15. The distribution of *Pythium* and related genera in class Oomycetes; 1: *Globisporangium splendens*; 2: *Phytothora vexans*; 3: *Pythium acanthicum*; 4: *Pythium cucurbitacearum*; 5: *Phytothora helicoides*; 6: *Pythium torulosum*; 7: *Pythium deliense* and 8: *Pythium diclinum*

It can be seen that all strains of *Pythium* and related genera found in this study are more diverse in marginally disturbed or undisturbed habitats like natural forests or rivers, and less diverse in cultivated soil. Detection of these species was not that unexpected because there had been many reports of the discoveries of *Phytophthora* and *Pythium* species in similar locations. For example, *Phytophthora gonapodyides*, *Phy. lacustris*, *Pythium oopapillum*, etc., were discovered in rivers crossing the Polish-Ukrainian border area [30], *Pythium sukuiense*, from undisturbed natural forest in Taiwan [31], and *P. Aphanidermatum* was discovered a decades ago [32]. However, there have still been no discoveries of any Oomycetes species in mangrove soil. This might be because the condition of mangrove soil with obviously high salinity limits the diversity of soil and fresh-waterborne Oomycetes. There was a report about specific halotolerant *Pythium* species being able to inhabit in salt water and infect algae, but as mentioned, only a few particular species, such as *Pythium porphyrae*, can live in such conditions. However, there was also the discovery of *P. aphanidermatum* strain that inhabited leaf debris in sea water [29]. Based on this observation, the possibilities of finding well known plant or animal pathogenic Oomycetes in mangrove forests can not be ignored. A pathogenicity test can be carried out in a future study.

4. Conclusions

Fungal-like microorganisms in class Oomycetes are important in cultivation and environmental stability in many ways. Here, this paper provided new and detailed information about the distribution of *Pythium* and related genera in Rayong Province, Thailand. Eight *Pythium* species and related genera were identified, i.e. *Globisporangium splendens, Pythium cucurbitacearum, Pythium acanthichum, Pythium deliense, Pythium diclinum, Pythium torulosum, Phytopythium vexans* and *Phytopythium helicoides.* Moreover, it was found that Oomycetes in the undisturbed locations were more diverse than those found in disturbed locations. From the results, the distribution data can be used for advanced study or further field investigation. However, futher study of the obtained isolates is needed, and in particular further studies of pathogenicity and environmental factors that affect

Pythium and related genera will be required to formulate a universal overview of Oomycetes representives in Thailand.

5. Acknowledgements

This study was funded and supported by the Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang (KMITL) and Thailand Institute of Scientific and Technological Research (TISTR).

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