

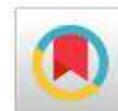


## Diversity of aquatic *Pythium* and *Phytopythium* spp. from rivers and a pond of Gifu city, Japan

Hani M. A. Abdelzaher<sup>1,2\*</sup>; Koji Kageyama<sup>3</sup>

<sup>1</sup>Biology Department, College of Science, Jouf University, P.O. Box: 2014, Sakaka, Saudi Arabia; <sup>2</sup>Department of Botany and Microbiology, Faculty of Science, Minia University, El-Minia, 61511, Egypt; <sup>3</sup>Laboratory of Molecular Biology, River Basin Research Center, Gifu University, Japan

\*Corresponding author E-mail: [hmdaher@ju.edu.sa](mailto:hmdaher@ju.edu.sa)



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### Abstract

Pythiaceae fungi have variety of occurrences in different environments. If these fungi are present in water intended to irrigate crops, they pose high risk as pathogens. *Pythium adhaerens*, *P. aquatile*, *P. diclinum*, *P. dissotocum*, *P. pachycaule* and *P. torulosum*, in addition to asexual isolates of *P. dissotocum* (*Pythium* “group F”) and *Phytopythium helicoides* (the former, *Pythium* “group P”) were isolated from 5 rivers and a pond in Gifu, Japan, and their diversity were studied. All of the isolated species have been previously recorded from aquatic habitats except for *P. pachycaule*. Sequencing of the internal transcribed spacer regions of ribosomal DNA (rDNA-ITS) including the 5.8SrDNA of these fungi confirmed primary identification based on morphological characteristics. This study proves the dense presence of different species of these Pythiaceae fungi, based on the latest modern identification methods, through which a new genus of *Pythium* was recorded and divided into two species. The aim of this study was to study the biological diversity of *Pythium*, which is pathologically important for many crop plants that are irrigated with water from these five rivers and the pond in Gifu, Japan.

**Keywords:** Aquatic habitats, Gifu-Japan, DNA (rDNA-ITS), *Pythium* spp., *Phytopythium*

### 1. Introduction

In 1858, Pringsheim was the first scientist to establish the Genus of *Pythium* ([Pringsheim, 1858](#)). As a result of the fact that sexual reproduction of this genus occur by the formation of oospores, the status of these fungi was placed in Oomycetes, which was established according to their oospores. Recently, [Abdelzaher et al., \(2020\)](#) revealed that about 150

species of *Pythium* have been identified. *Pythium* spp. are unique in their interactions with the other organisms, they can be pathogenic, saprophytic, varying in their virulence, and may be intrusive on other fungi (including species of the same genus), or cause inhibition of growth of other bacteria or invertebrates ([Abdelzaher et al., 2020](#)). Moreover, a

previous study conducted by [Abdelzaher et al., \(1995\)](#) reported that these fungi are diverse in their livelihoods, sometimes found in aquatic habitats and sometimes in terrestrial, and some species live in both water and under dry conditions, but prefer to live in humid environments, as their asexual zoospores have flagella for motility. Since the beginning of localization of the genus *Pythium* ([Pringsheim, 1858](#)), classification of its species has undergone many changes. According to [Abdelzaher et al., \(1995\)](#); [Abdelzaher, \(1999\)](#); [Senda et al., \(2009\)](#); [Uzuhashi et al., \(2015\)](#), the genus *Pythium* has been divided into 4 different genera including; *Ovatisporangium*, *Elongisporangium*, *Globisporangium* and *Pilasporangium*. A previous study conducted by [Uzuhashi et al., \(2010\)](#) listed the genus *Pythium* on those species that possess filamentous zoosporangia and created four new genera, as previously mentioned. In addition, [Bala et al., \(2010\)](#) introduced a new genus *Phytopythium* to those species containing globose to ovate zoosporangia, often papillate and are internally proliferating zoosporangia. Recently, [De Cock et al., \(2015\)](#) issued molecular evidence that members of clade K *Pythium* have their place to the genus *Phytopythium*.

A recent study conducted by [Abdelzaher et al., \(2020\)](#) revealed that most species of the genus *Pythium* replicate asexually; under appropriate growth conditions, by the formation of swimming zoospores; however, some species have lost their ability to produce zoospores and reproduce asexually by the formation of hyphal swellings and/or segments of hyphae. As a result of their asexual reproduction by forming swimming zoospores, these fungi flourish in the aquatic habitats of rivers, lakes, swamps and any fresh or brackish water pools. A study of [Al-Sheikh and Abdelzaher, \(2012\)](#) recorded the presence of some species of *Pythium* in salty water. According to [Abdelzaher et al., \(2020\)](#), sexual reproduction usually occurs under inappropriate growth conditions. There are many forms of antheridia (male structure) and oogonia (female structure) that depending on their shape and adhesion; are used to help for species

identification. The classification and identification of genus *Pythium* are mainly based on the morphological characteristics of zoosporangia; antheridia, oogonia and oospores.

[Abdelzaher et al., \(1995\)](#) postulated that the ecology of fresh-water *Pythium* spp. did not undertake the same studies like terrestrial *Pythium* spp. Until 1995, reports on the occurrence of aquatic *Pythium* spp. in Japan, however, were scarce. [Abdelzaher, \(1994\)](#) studied the occurrence of *Pythium* spp. in 3 ponds in Osaka, Japan. At that time, identification of *Pythium* species was based primarily on morphological characteristics, as molecular identification was not commonly used yet.

The objective of this study was to describe the isolation, identification (morphological and molecular) of *Pythium* spp. and an asexual isolate of *Phytopythium helicoides*, recovered from 5 rivers and a pond waters in Gifu, Japan, during the spring and summer periods of 2008.

## 2. Material and methods

### 2.1. Isolation and identification of the aquatic pythiaceous fungi

Five replicate water samples were collected from 5 rivers including; Toba, Ijira, Nagara, Shinhoru and the connection zone between Ijira and Nagara, as well as a pond (Ijira) located in Gifu, Japan, during the two seasons of spring (April) and summer (June), 2008. Surface water was sampled through areas of 10 meters apart and two meters from the shore, from a depth of 30 cm, in sterilized bottles.

For the isolation of aquatic *Pythium* spp., baits of 4 autoclaved grass leaf pieces (0.5×1.0 cm) were placed in 30 ml of water samples in sterilized Petri-plates. Hemp-seeds and internal rinds of *Citrus reticulata* plant were also used as baits. After 7 d of incubation at room temperature (25 °C ±2), baits were detached using sterile forceps from the water and then dried between sterile tissue papers under aseptic conditions. After that, the baits were cultivated on the surface of

NARM [(Nystatin (10 mg\ 1), ampicillin (250 mg\ 1), rifampicin (10 mg\ 1) and miconazole (1 mg\ 1)], in cornmeal agar (CMA) medium at 20°C for selective isolation of *Pythium* spp. until appearance of fungal colonies, according to [Senda et al., \(2009\)](#). The fungal colonies growing on CMA were picked, purified using a single spore colony ([Abdelzaher et al., 2020](#)), and then morphologically identified using the keys of [Plaats-Niterink, \(1981\)](#); [Dick, \(1990\)](#).

## 2.2. Relationship between fungal growth with incubation temperature

Effects of cardinal temperatures on growth of each fungus were determined on CMA. Fungi were grown on CMA plates by inoculating the middle of each plate, and then incubating these plates at different temperatures such as; 3, 5, 10, 15, 20, 25, 28, 30, 35 and 40 °C. Minimum, optimum and maximum cardinal temperatures giving maximum mycelial growth of each fungus were evaluated at the tested incubation temperatures, by measuring the diameter of mycelial growth every 24 h using a calibrated ruler, according to [Abdelzaher et al., \(2020\)](#).

## 2.3. Molecular identification of the fungal isolates

### 2.3.1. Extraction of fungal DNA

According to [Senda et al., \(2009\)](#), fungal mycelia were collected from V8 agar medium after incubation for 7 d at 25 °C. Total genomic DNA were extracted from mycelia grown on culture plates suspended in 200 µl of PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, CN, USA) in a 2.0 ml micro-centrifuge tube. Samples were vortexed for 30 sec. followed by boiling for 10 min. at 100 °C in a water bath (Takara Bio Inc., Shiga, Japan), and then were centrifuged for 30 min. at 15000 g. Supernatants were placed into new micro-centrifuge tubes and prepared for Polymerase chain reaction (PCR).

### 2.3.2. Amplification of the DNA

In the rDNA, the ITS region was amplified with common primers pair ITS1 and ITS4 ([White et](#)

[al., 1990](#)). Reaction mixture of 25 ml consisted of 1µm of each primer, 0.625 units Taq DNA polymerase (Takara Bio Inc., Shiga, Japan), 0.2 mM dNTPs mixture, PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl and 1.5 mM MgCl<sub>2</sub>) and 1 ml template 5 ng DNA, in reference to [White et al., \(1990\)](#); [Matsumoto et al., \(1999\)](#). The amplicons were 700-900 bp long. On the other hand, 563 bp of the cox II gene in certain *Pythia* was amplified using the primer pair FM66 (5' TAGGATTTCAAGATCCTGC 3') and FM58 (5' CCACAAATTTCACTACATTGA 3'), according to [Martin, \(2000\)](#).

Amplification of the sequencing template was carried out using DNA Thermal Cycler 2700 (Applied Biosystems) with a cycling profile of pre-PCR at 94 °C for 5 min., followed by denaturation at 94 °C for 1 min., primer annealing (1 min.) at 55°C for ITS, 52°C for cox II and elongation at 72 °C, 2 min. for 40 cycles, with a 7 min. extension at 72 °C after the final cycle. To check the presence of PCR products, 5 µl of the PCR reaction mixture was loaded in 2% L03 (Takara Bio) agarose gel, electrophoresed at 100 V for 20-30 min., and then stained with ethidium bromide. The sequencing templates were purified with GenElute PCR Clean-up kit (Sigma Chemical Co., St Louis, Missouri, USA) as recommended by the manufacturer's instructions. Sequencing was performed using BigDye Terminator v3.1 Cycle Sequencing Reaction kit (Applied Biosystems) by the initial primers in the PCR trial. The sequencer (ABI3100 DNA) was used to run the purifying sequencer reaction mixture, through ethanol-precipitation, as reported in the method conducted by [Senda et al., \(2009\)](#).

### 2.3.3. Cloning and sequencing of the PCR products

Some PCR products were cloned in the pT7Blue T-vector (Takara Bio) with the Ligation kit (Takara Bio) using the manufacturer's instructions. The cloned ITS region was amplified using M13M4 (5' GTTTTCCAGTCACGAC 3') and M13Rv (5' CAGGAAACAGCTATGAC 3') primers. Resulting PCR products were purified using the Gene Elute PCR

cleaning kit (Sigma, Ronkonkoma, NY, USA). The BigDye™ Terminator Cycle Sequencing Ready Reaction kit \*Applied Biosystems) was used for cycle sequencing with primers M13M4 and M13Rv, according to the manufacturer's instructions. Purification of the sequencing reaction mixture was performed through ethanol precipitation and then run on an ABI 3100 DNA sequencer (Applied Biosystems). The consensus sequences were generated based on the forward and reverse primer sequences, in reference to [Kageyama et al., \(2007\)](#).

### 3. Results

#### 3.1. Occurrence and distribution of *Pythium* spp.

About 133 isolates of *Pythium* spp. and 11 isolates of *Saprolegnia* sp. are obtained from the studied area in Gifu, Japan, as shown in Table (1). Of these, a total of 6 species could be identified on the basis of their morphological and molecular standards. However, the remaining isolates, which lacked sexual structures, have filamentous sporangia and one of them is identified molecularly as *P. dissotocum*, while the others which lacked sexuality and have proliferated sporangia are identified as *Phytopythium helicoides* (the former *Pythium* "group P"). An isolate of *Saprolegnia* is sequenced using ITS region and gave a boundary species near to *Saprolegnia*, but it needs further studies.

#### 3.2. Morphological identification of the recovered fungal isolates

Several fungi including; *P. adhaerens*, *P. aquatile*, *P. diclinum*, *P. dissotocum* and *P. torulosum* as well as an isolate of *Saprolegnia* sp. were isolated using hemp-seed as baits. Whereas, *P. aquatile*, *P. diclinum*, *P. pachycaule*, an asexual isolate of *Phytopythium helicoides* (the former *Pythium* "group P") as well as *Saprolegnia* sp. were recovered on using internal rinds of mandarin as baits. On the other hand, *P. diclinum* and *Phytopythium helicoides* (the former, *Pythium* "group P") as well as *Saprolegnia* sp. were obtained on using grass leaf segments as baits. *Pythium* "group F" were obtained from all samples on using all types

of baits. All these isolates are described below using observations of only one representative isolate generated from a single hyphal tip.

#### *Pythium adhaerens* (Sparrow)

Colonies were submerged on CMA, with random shaped pattern. Main hyphae is up to 7 µm wide. Zoosporangia are strictly filamentous (Fig. 1.1-9). Zoospores liberated at 20 °C. Encysted zoospores are about 5-10 µm in diameter. Oogonia are globose, smooth, terminal or intercalary, 11-24 µm (av. 18 µm) diameter (Fig. 1.8-10). Antheridia are 1-4 rarely up to 6 per oogonium, borne on a single stalk or rarely on distinct stalks, diclinous, often surrounding the oogonium; antheridial cells are hook-necked, 14×7 µm (Fig. 1.8-10). Oospores are not filling the oogonium (aplerotic); 7-23 µm (av. 15) diameter, wall 2 µm thick (Fig. 1.8-10). Principal cardinal temperatures are: minimum at 5 °C, optimum at 25 °C and maximum at 35 °C. This fungus has been deposited in River Basin Research Center, Gifu University, Japan RBRC, with isolate number of KH56.

#### *P. aquatile* (Höhnk)

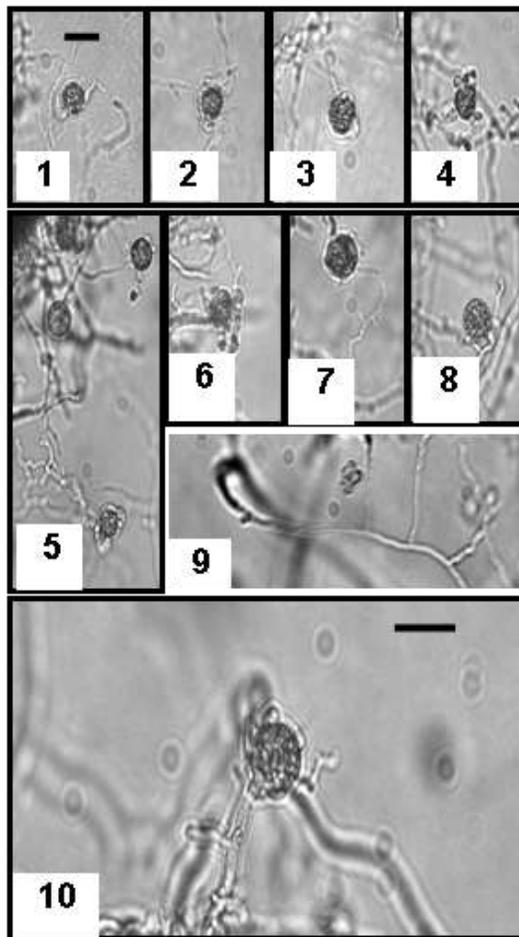
Colonies on CMA are submersible not fluffy, with rose pattern. Main hyphae is up to 7 µm wide. Zoosporangia are filamentous or slightly swollen forming some dendroid structures (Fig. 2.1). Zoospores liberated at 20°C from vesicles of about 30 µm diameter, with discharge tubes up to 250 µm long or more; and 4 µm wide. Encysted zoospores are about 10 µm diameter. Oogonia are spherical, soft without appendages or thorns, terminal and sometimes are intercalary, 18-24 µm (av. 21 µm) diameter (Fig. 2.2-10). Antheridia are monoclinal, originating from the oogonial stalk at 2-10 µm or more below the oogonium, or from the parent hypha; about 1 or rarely 2 per oogonium (Fig. 2.2-10). Oospores are applerotic; 15-20 µm (av. 18) diameter, wall 2-3 µm thick (Fig. 2.2-10). Principal cardinal temperatures are: minimum at 5 °C, optimum at 25 °C and maximum at 35 °C. This fungus has been deposited in RBRC, Gifu University, with isolate number of KH4.

**Table 1.** Isolation of different *Pythium* spp. from Ijira pond and river water (Ijira, Shinhori, Toba, the connection zone between Ijira and Nagara, and Nagara), during spring and summer of 2008

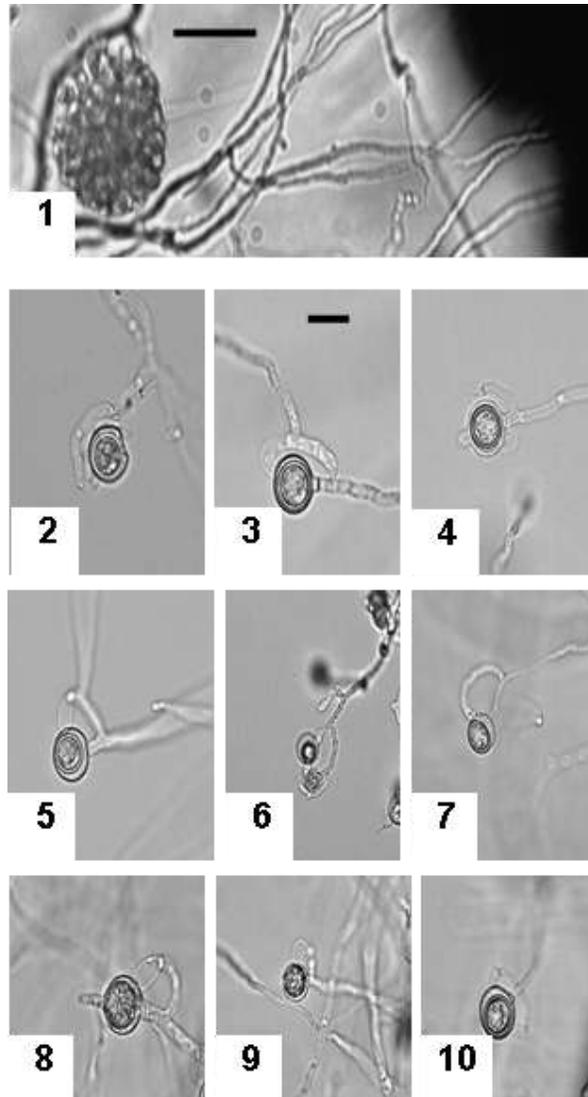
Sampling sites	Spring (April)*/ Summer (June)**		
	Grass	Hemp-seed	Mandarin
Ijira pond	*“group F” (100%)	* <i>Saprolegnia</i> sp. (25%)	*“group F” (100%)
	**“group F” (100%)	*“group F” (75%)	**“group F” (100%)
		** <i>P. aquatile</i> (25%)	
		**“group F” (75%)	
Ijira river	*“group F” (100%)	*“group F” (100%)	* <i>P. aquatile</i> (25%)
	** <i>Saprolegnia</i> sp. (25%)	** <i>Saprolegnia</i> sp. (50%)	* <i>P. pachycaule</i> (25%)
	**“group F” (75%)	**“group F” (50%)	*“group F” (50%)
			** <i>Saprolegnia</i> sp. (25%)
			**“group F” (75%)
Shinhori river	*“group F” (100%)	* <i>P. aquatile</i> (25%)	*“group F” (100%)
	** “group P” (25%)	* <i>P. torulosum</i> (50%)	** <i>Saprolegnia</i> sp. (25%)
	**“group F” (75%)	* <i>Saprolegnia</i> sp. (25%)	**“group F” (75%)
		** <i>P. diclinum</i> (25%)	
		** <i>Saprolegnia</i> sp. (25%)	
		**“group F” (50%)	
Toba river	*“group F” (100%)	*“group F” (100%)	*“group F” (100%)
	** <i>Saprolegnia</i> sp. (25%)	** <i>Saprolegnia</i> sp. (25%)	** <i>Saprolegnia</i> sp. (25%)
	**“group F” (75%)	**“group F” (75%)	**“group F” (75%)

	*N.D.	*N.D.	N.D.
<b>Connection</b>	**“group F” (75%)	** <i>P. torulosum</i> (25%)	**“group F” (75%)
<b>(Ijira &amp; Nagara)</b>	**“group P” (25%)	**“group F” (50%)	**“group P” (25%)
		** <i>Saprolegnia</i> sp. (25%)	
	**“group F” (100%)	**“group F” (100%)	**“group F” (100%)
<b>Nagara river</b>	**“group F” (100%)	** <i>P. adhaerens</i> (25%)	**“group F” (100%)
		**“group F” (75%)	

Where; each value represents the percentage (%) of colonies obtained on NARM selective medium, using 4 types of baits for each sample. N.D = not detected.



**Fig. 1.** Morphology of *P. adhaerens*. Fig. (1.1-10) show dichinous antheridia and oogonia. Fig. (1.7) demonstrates three antheridia on a single stalk. Fig. (1.9) show zoosporangium and a vesicle. Bar (20  $\mu$ m) on Fig. (1.10) is only for this figure, while bar (20  $\mu$ m) on Fig. (1.1) is applicable to the rest of figures.

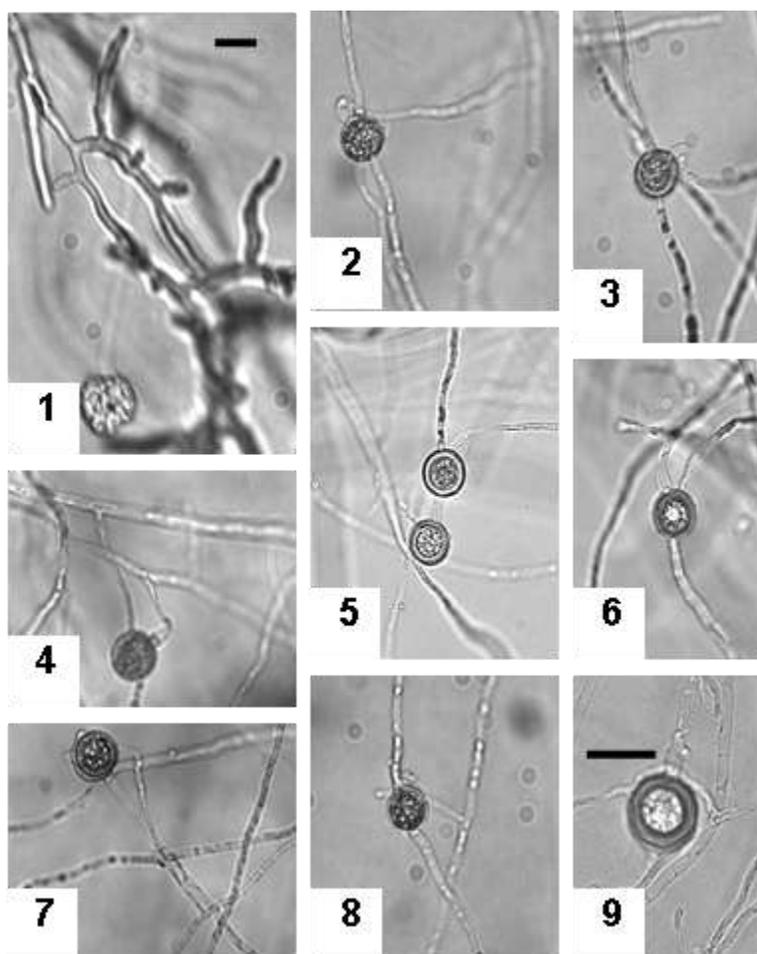


**Fig. 2.** Morphology of *P. aquatile*. Fig. (2.1) shows long evacuation tube and a big vesicle. Fig. (2-9) show monoclinous, one antheridium is attached to the oogonium. Fig. (2.10) demonstrates monoclinous containing two antheridia. Bar (20 $\mu$ m) on Fig. (2.1) is only for this photo, while bar (20  $\mu$ m) on Fig. (2.5) is applicable to the rest figures.

### *P. diclinum* (Tokunaga)

Mycelial growth on CMA is submerged, with radiate pattern and with little fluffy mycelia. The hyphae are up to 6  $\mu$ m in width. Zoosporangia are strictly filamentous, sometimes branched (Fig. 3.1). Zoospores are produced at 20 °C; about 2 or more in the vesicles. Encysted zoospores are about 7  $\mu$ m diameter. Oogonia ranged from globose, to ovoid, smooth, terminal or sub-terminal, sporadically

intercalary, 18-23  $\mu$ m (av. 20  $\mu$ m) diameter (Fig. 3.2-9). Antheridia are obviously diclinous, 1-2 per oogonium; about 12 $\times$ 15  $\mu$ m (Fig. 3, 3.2-9). Antheridial stalks are not branched (Fig. 3.2-9). Oospores are single, aplerotic; 17-19  $\mu$ m (av. 18) diameter, wall up to 3  $\mu$ m thick (Fig. 3.5,8,9). Cardinal temperatures are: minimum at 5 °C, optimum at 30 °C and maximum just below 40 °C. This fungus has been deposited in RBRC, Gifu University with isolate number of KH33.

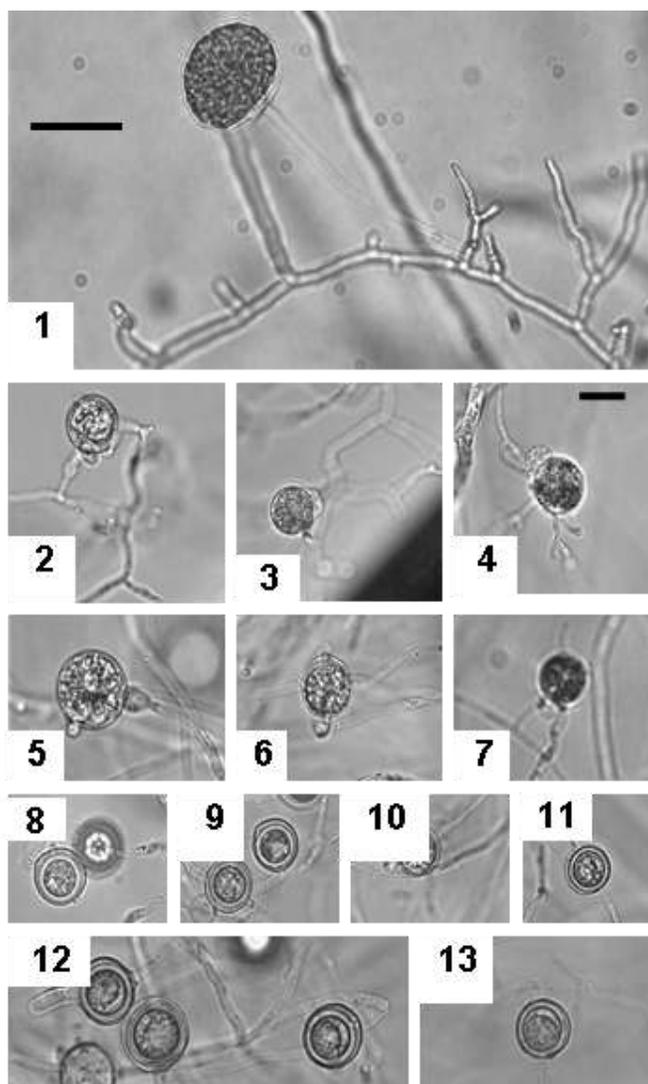


**Fig. 3.** Morphology of *P. diclinum*. Fig. (3.1) shows filamentous zoosporangium and a vesicle. Fig. (3.2, 4-8) demonstrate diclinous antheridia and intercalary oogonia. Fig. (3.3) shows terminal oogonium and diclinous antheridium. Bar (20  $\mu$ m) on Fig. (3.9) is only for this figure, while bar (20  $\mu$ m) on Fig. 3.1 is applicable to the rest figures.

### *P. dissotocum* (Drechsler)

Mycelial growth on CMA are devoid of aerial haphae, and without a special arrangement. The parent hyphae are up to 7  $\mu$ m wide. Zoosporangia are hyphal shaped, forming somewhat swollen dendroid structures (Fig. 4.1). Zoospores are produced at 20  $^{\circ}$ C, and after losing its flagella and resting, it becomes up to 9  $\mu$ m diameter. Oogonia are subspherical, lateral or intercalary, ranging from 20 to 24  $\mu$ m (av. 22  $\mu$ m) diameter (Fig. 4.2-13). Antheridia are commonly 1-3 occasionally 5 per oogonium; initiating from stalk of

the oogonium directly below it, sessile or originating on un-branched antheridial stalks at various distances from the oogonium. Oogonia range from monoclinal to diclinous (Fig. 4.2-9). Oospores are solitary possessing very narrow apertures; 19-23  $\mu$ m (av. 20) (Fig. 4.8-13) diameter. Oospores wall vary from 1-3  $\mu$ m thick (Fig. 4.8-13). Principal growth temperatures are: minimum at 5  $^{\circ}$ C, optimum at 30  $^{\circ}$ C and maximum at 35 $^{\circ}$ C. This fungus has been deposited in RBRC, Gifu University with isolate number of KH26.

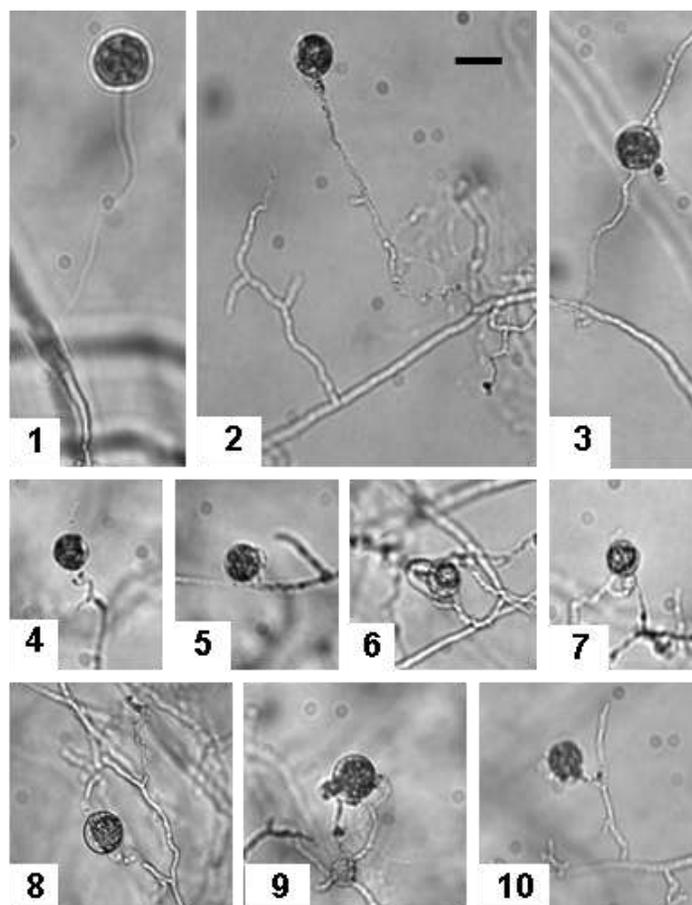


**Fig. 4.** Morphology of *P. dissotocum*. Fig. (4.1) shows filamentous zoosporangium with vesicle. Fig. (4.2,7,11) demonstrate diclinous antheridia. Fig. (4.4,8,9) show monoclinous antheridia. Fig. (4.8,9,11-13) demonstrate thick walled oospores. Bar (20  $\mu$ m) on Fig. (4.1) is only for this figure, while bar (20  $\mu$ m) on Fig. (4.4) is applicable to the rest of figures.

#### *P. pachycaule* (Shtayeh & Dick)

Mycelial growth on CMA have very little downy hyphae without a special pattern. The principle hyphae are up to 6  $\mu$ m wide. Zoosporangia are filamentous (Fig. 5.1). Formation of zoospores has been produced at 20  $^{\circ}$ C. Encysted zoospores are up to 8  $\mu$ m diameter. Oogonia are globose to subglobose, intercalary or lateral 19-23  $\mu$ m (av. 21  $\mu$ m) diameter

(Fig. 5.2-10). Antheridia are commonly 1-2 per oogonium, mostly diclinous, elongated and club-shaped, large 15 $\times$ 8  $\mu$ m (Fig. 5.6-9). Oospores are single, aplerotic; 18-22  $\mu$ m (av. 19) diameter, wall is thin (Fig. 5.6-9). Cardinal temperatures are: minimum at 5  $^{\circ}$ C, optimum at 25  $^{\circ}$ C and maximum just below 35  $^{\circ}$ C. This fungus was deposited in RBRC, Gifu University with isolate number of KH15. This isolate failed to reproduce sexually after sub-culturing.

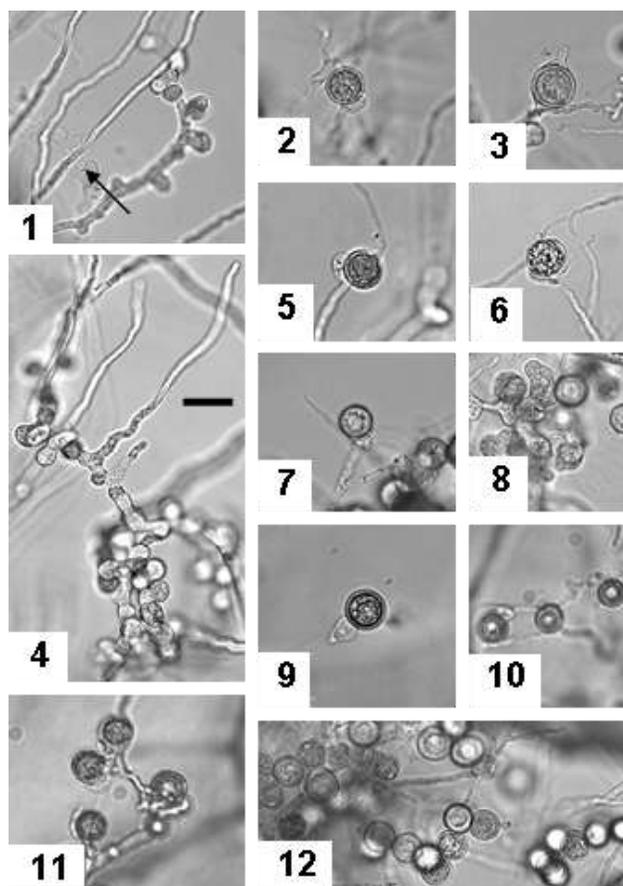


**Fig. 5.** Morphology of *P. pachycaule*. Fig. (5.1) demonstrates filamentous zoosporangia with vesicle. Fig. (5.2) shows diclinous antheridium. Fig. (5.3-10) demonstrate antheridia and oogonia. Fig. (5, 5.3,6,8,9) show typical elongated and club-shaped antheridia. Bar (20 µm) on Fig. (5.2) is applicable to the rest of figures.

### *P. torulosum* (Coker & Patterson)

Growth on CMA are submerged without a special morphological form, and with little downy aerial hyphae. The principal hyphae reached up to 6 µm wide. Zoosporangia resemble a group of connected peanuts (filamentous with flattened lateral growths). Discharge tubes are generally slender and long (Fig. 6.1,2,11). Zoospores generated at 20°C, from vesicles ranging from small to big dimensions. Encysted zoospores are up to 8 µm diameter. Oogonia are terminal or intercalary on short side branches, without cilia or thorns 13-22 µm (av. 15 µm) diameter (Fig.

6.3,8,7,9,10). Antheridia are 1-3 per oogonium, monoclinous (filled into the oogonium), sporadically diclinous (does not fill the oogonial space), generally initiating from the oogonial stalk. Oogonia are always vanished soon after fertilization. Antheridia are clavate, 5-10×3-6 µm, adhered apically to the oogonium (Fig. 6.3-7,10). Oospores showed plerotic form; 10-20 µm (av. 17) diameter, and their walls ranged from 1-2 µm thick (Fig. 6.4-9). Principal cardinal temperatures ranged from 5 °C (minimum), 30 °C (optimum), to 35 °C (maximum). This fungus has been deposited in RBRC, Gifu University with isolate number of KH29.



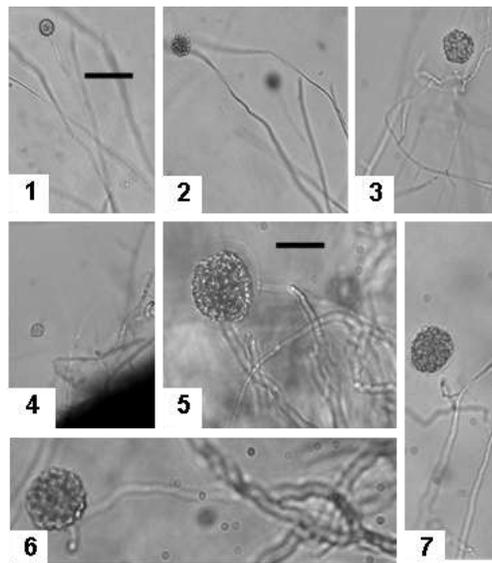
**Fig. 6.** Morphology of *P. torulosum*. Fig. (6.1,2) show young and empty zoosporangia with evacuation tube (arrow). Fig. (6.3-7,9,10) demonstrate antheridia and oogonia. (Fig. 6.8) presents many oospores. Bar (20  $\mu\text{m}$ ) on Fig. (6.2) is applicable to the rest of figures.

#### *Pythium* “group F” (Plasts Niterink)

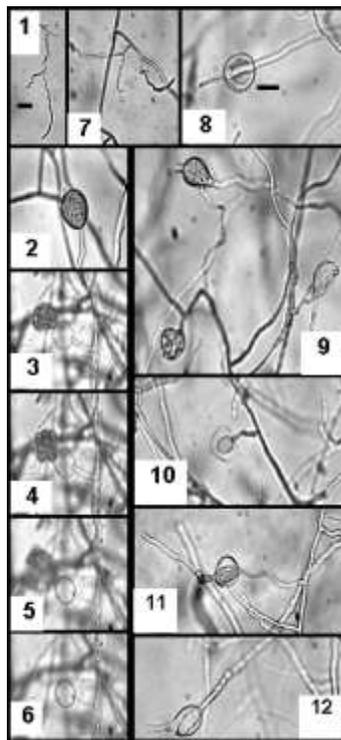
Colonies on CMA are submerged showing random pattern. Sporangia are obviously filamentous, as clear in Fig. (7). The size of the vesicles ranges from small containing 2 zoospores to large containing more than 100 zoospores. Zoospores liberated at 20°C. Encysted zoospores are 6-8 $\mu\text{m}$  diameter. There are no sexual structures and sexual reproduction in this group (Fig. 7).

#### *Phytophythium helicoides* (the former, *Pythium* “group P”)

Mycelial growth on CMA are submerged, with radiate shape and very little downy hyphae. The principal mycelia is up to 7  $\mu\text{m}$  thick (Fig. 8.1,7). Zoosporangia showed internal proliferation, often 20-30  $\mu\text{m}$  diameter; 27  $\mu\text{m}$  on average, with generally short evacuation tubes (Fig. 8.3-6, 8-12). Zoospores are produced from the exploding vesicles at 20°C. Encysted zoospores measured up to 13  $\mu\text{m}$  diameter. No sexual reproduction is observed. Cardinal temperatures ranged from 5 °C (minimum), 30 °C (optimum) and 35-40 °C (maximum) This fungus has been deposited in RBRC, Gifu University with isolate number of KH54.



**Fig. 7.** Morphology of *Pythium* “group F”. Fig. (7.1) shows a young (undifferentiated vesicle). Fig. (7.2) demonstrates early stage of vesicle differentiation Fig. (7.4) presents a small vesicle with fewer zoospores. Fig. (7.3,5,6,7) demonstrate differentiated vesicles containing many zoospores. Bar (20  $\mu\text{m}$ ) on Fig. (7.1) is applicable for Fig. (7.2,3,4), while bar (20  $\mu\text{m}$ ) on Fig. (7.5) is applicable to the rest of figures.

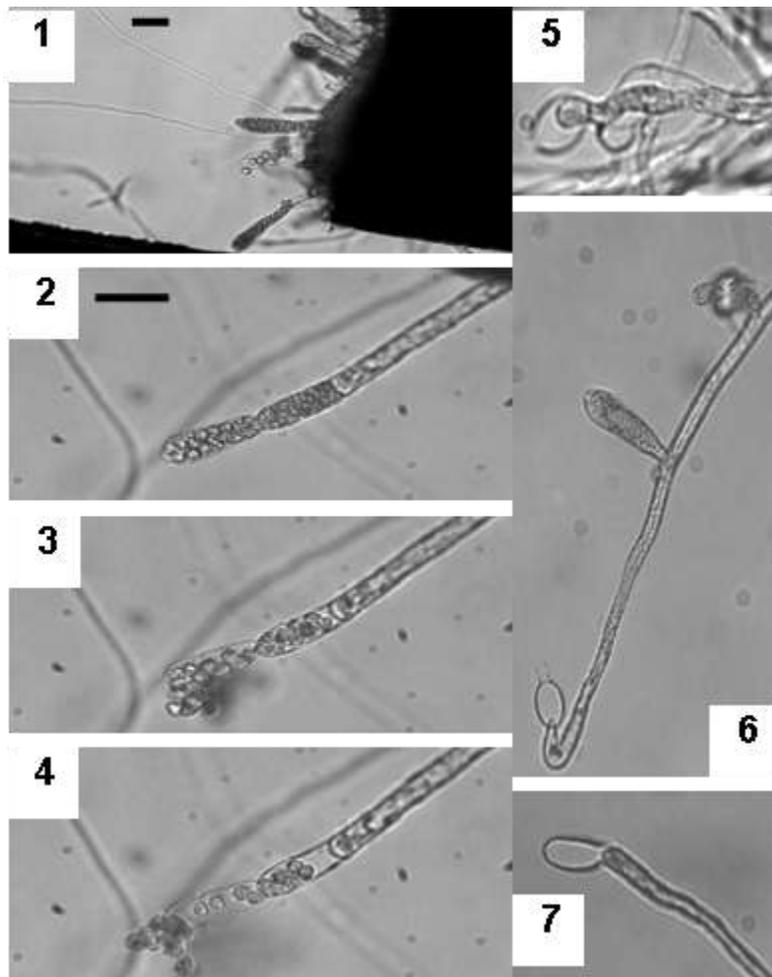


**Fig. 8.** Morphology of *Phytopythium helicoides* (the former, *Pythium* “group P”). Fig. (8.1,7) show the hyphae. Fig. (8.2) demonstrates a young papillated zoosporangia. Fig. (8.3-6) present zoospore formation. Fig. (8.8-12) demonstrate the proliferated zoosporangia. Bar (20  $\mu\text{m}$ ) is for Fig. (8.1,2), while bar (20  $\mu\text{m}$ ) in Fig. (8.8) is applicable to the rest of figures.

*Saprolegnia* sp. (Nees von Esenbeck)

Hyphae are slender 10-18 (av. 14  $\mu\text{m}$  diameter). Gemmae are sparse or absent. Zoosporangia are abundant, clavate or irregular, 50-80 (av. 65  $\mu\text{m}$ ) long

$\times 20-30$  (av. 25  $\mu\text{m}$ ) diameter, internally proliferated (Fig. 9.1,5,6). Zoospore is discharged saprolegnoid (Fig. 9.1-4). No sexual organs are detected.



**Fig. 9.** Morphology of *Saprolegnia* sp. Fig. (9.1-4) demonstrate zoosporangia and zoospore formation. Fig. (9.5,6) present the proliferation. Fig. (9.7) shows an empty Zoosporangium. Bar (30  $\mu\text{m}$ ) in Fig. (9.1) is only for this figure, while bar (30  $\mu\text{m}$ ) in Fig. (9.2) is applicable to the rest of figures.

### 3.3. Molecular identification

Sequencing of rDNA-ITS including the 5.8SrDNA was analyzed for the tested *Pythium* species, to confirm the species identification. The sequence of KH56 is closely related with that of *P. adhaerens* (AY598619) with a similarity of 98%. The sequence of KH4 is closely related to *P. aquatile* (AY598632) with 99% similarity. The sequence (with the aid of TA-cloning) of KH33 is identical to that of *P. diclinum* (EF153676) demonstrating 100% similarity. The sequence of KH26 showed a 99% similarity to that of *P. dissotocum* (AY598631). The sequence of KH15 is very close that of *P. pachycaule* (AY598687) showing 98% similarity. The sequence of KH29 presented 99% similarity with that of *P. torulosum* (AY598624). A single isolate of *Pythium* “group F” (KH16) was sequenced and gave a similarity of 99% with *P. dissotocum* (AY598634).

### 4. Discussion

This is the first study that dealt with aquatic pythia in several rivers and a pond in Gifu city, Japan. The species recovered during the current study were; *P. adhaerens*, *P. aquatile*, *P. diclinum*, *P. dissotocum*, *P. pachycaule*, *P. torulosum*, *Pythium* “group F” and an asexual isolate of *Phytopythium helicoides* (the former, *Pythium* “group P”). Morphological investigation of these fungal isolates showed that *P. adhaerens*, *P. aquatile*, *P. diclinum*, *P. dissotocum*, *P. pachycaule* and *Pythium* “group F” have filamentous sporangia, *P. torulosum* has inflated lobulate sporangia, whereas *Phytopythium helicoides* formed proliferated sporangia. An early study of [Plaats-Niterink, \(1981\)](#) documented that *P. adhaerens* was originally isolated from the green algae (*Rhizoclonium hieroglyphicum*) in the USA. Furthermore, [Plaats-Niterink, \(1981\)](#) revealed that *P. aquatile* was originally identified from a brackish water pool in Germany. *P. diclinum* was firstly isolated from a rice field in Japan, whereas *P. dissotocum* has also been recovered from water in Russia and Holland ([Plaats-Niterink, 1981](#)). According

to [Ali-Shtayeh and Dick, \(1985\)](#), *P. pachycaule* is the only *Pythium* sp. isolated from the soil. *P. torulosum* has also been isolated from aquatic habitats ([Coker and Patterson, 1927](#)). Recently, [Afandi et al., \(2018\)](#) reported that *Pythium* “group F” and the asexual isolate of *Phytopythium helicoides* were isolated several times from aquatic habitats. Therefore, current results of isolation of such species from water are in agreement with the previous studies.

Results of this study demonstrated that hemp-seed is very favorite bait for isolation of *Pythium*, *Phytopythium* and *Saprolegnia* spp. followed by internal rinds of mandarin, while fewer isolates are obtained using grass leaf segments. It is well known from previous study of [Abdelzaher et al., \(1995\)](#) that *Pythium* “group F” is abundant in the aquatic environment, which may not require the presence of thick walled sexual oospores. Meanwhile, *P. aquatile*, *P. diclinum* and *P. torulosum* were currently recovered in spring and summer, and this indicates that these fungi have a wide temperature range of existence under the aquatic environment. Only *P. pachycaule* was isolated in spring and cannot be obtained in summer, which reveals that this fungus is a mesophilic species, as recently reported by [Abdelzaher et al., \(2020\)](#). On the other hand, *P. adhaerens* and *Phytopythium helicoides* were isolated in summer, which reflects that these fungi prefer higher temperature than spring taxa, in reference to [Abdelzaher et al., \(2020\)](#).

It is worth to mention that, current results of the rDNA-ITS and cox II sequence analyses of the *Pythium* isolates approved the morphological identification of each isolate, and were very important factors for confirming the identification without any confusion or hesitation.

### Conclusion

Due to the alarming rate of the occurrence of *Pythium* spp. in irrigation water, this study was conducted to shed light on the diversity of these

important fungi recovered from five rivers and a pond in Gifu, Japan. It could be concluded that *P. adhaerens*, *P. aquatile*, *P. diclinum*, *P. dissotocum*, *P. pachycaule* and *P. torulosum*, asexual isolate of *P. dissotocum* (*Pythium* “group F”) and an asexual isolate of *Phytophythium helicoides* (the former, *Pythium* “group P”), were isolated and then identified using morphological and molecular criteria. Molecular identification was carried out using sequencing of the internal transcribed spacer regions of ribosomal DNA (rDNA-ITS) including the 5.8SrDNA.

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### Conflict of interest

The authors declare that there is no conflict of interests.

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### Ethical approval

Non-applicable

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