Chapter 12
Plant Pathogenic Oomycetes Inhabiting River Water Are a Potential Source of Infestation in Agricultural Areas

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Abstract In recent years, plant pathogenic oomycetes have become a cause of destruction of nature due to their contribution to new forest diseases and damages to agricultural production, and have attracted attention as important pathogens. It is essential to understand the ecology of pathogenic fungi to elucidate the causes of disease outbreaks. However, there has been little focus on how pathogenic fungi behave in the environment. In this study, we investigated several parameters of two representative genera of plant pathogenic oomycetes, *Pythium* and *Phytopythium* species, in terms of (a) their distribution in Japan, (b) seasonal variation from upstream to downstream of rivers with different anthropogenic impacts, (c) morphological and molecular phylogenetic characteristics of river water isolates and (d) pathogenicity. The results suggest that phytopathogenic oomycetes are naturally found in rivers and can act as pathogens when they invade agricultural production sites, indicating the need to pay attention to river water and irrigation water as one of the pathogen transmission routes.

Keywords *Pythium* · *Phytopythium* · Irrigation · Water supply · Crop production · Horticulture

12.1 Introduction

Oomycetes grow on filamentous mycelia like true fungi, but they belong to the kingdom Stramenopile (Chromista) and are genetically distantly related to true fungi (Cavalier-Smith and Chao 2006; Schroeder et al. 2013). In recent years, plant pathogenic oomycetes have attracted attention as important pathogens by

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F. Li et al. (eds.), *River Basin Environment: Evaluation, Management and Conservation*, https://doi.org/10.1007/978-981-19-4070-5_12

contributing to the destruction of nature through spreading new forest diseases and causing damage to agricultural production through new diseases (Kageyama 2015). The first step in disease control is to identify and classify the species of pathogens that cause the disease. In recent years, the introduction of molecular biological methods has opened major avenues for species classification (Kageyama 2014). In addition, it is essential to understand the ecology of pathogenic fungi to elucidate the causes of disease outbreaks. However, there has been little research on how pathogenic fungi behave in the environment.

12.1.1 Classification of Phytopathogenic Oomycetes

A molecular phylogenetic analysis of the taxonomic system within the oomycetes was conducted based on the nucleotide sequences of the small subunit (SSU), large subunit (LSU), internal transcribed spacer region of nuclear ribosomal DNA, and the mitochondrially encoded cytochrome oxidase gene 1 and 2 (Beakes and Sekimoto 2009; Martin 2000; Matsumoto et al. 1999; Robideau et al. 2011). Based on this analysis, oomycetes are divided into "basal oomycetes" and "crown oomycetes". The "basal oomycetes" include five orders, Haptoglossales, Eurychasmales, Haliphthorales, Olpidiopsidales, and Atkinsiellales, that do not form sexual organs, but form sporangium and zoospore, the asexual organs, instead. The "crown oomycetes" include six orders, Rhipidiales, Leptomitales, Pythiales, Albuginales, Saprolegniales, and Peronosporales, all of which have oogonium, antheridium and oospore as sexual organs, in addition to the asexual organ. This system was developed not only from the molecular phylogenetic analysis, but also considering their morphological, histological and ecological characteristics. The orders reported as plant pathogens are the Leptomitales, Pythiales, Albuginales, Saprolegniales, and Peronosporales, which consist of a highly evolved group of oomycetes. The phytopathogenic fungi used in this study are species that belong to the genera *Pythium* and *Phytopythium* of the order Pythiales.

It is estimated that more than 200 species of *Pythium* and *Phytopythium* have been described, of which over 50 new species have been described since 2000, and more than 300 species have not been described due to insufficient comparison with other species or lack of type strains (Schroeder et al. 2013).

12.1.2 Morphological Characteristics of Pythium *and* Phytopythium *Species*

Both these species grow into a mycelium when active. The nuclear phase of the mycelium is diploid, whereas fungi are usually haploid. The cell wall is made of cellulose and does not contain chitin, unlike fungi. The mycelium is usually

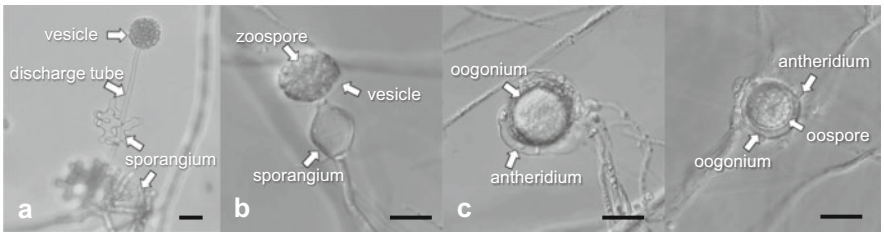

Fig. 12.1 Morphological characteristics of *Pythium* and *Phytopythium* species. (**a**, **b**) asexual propagative structures, filamentous sporangium (**a**), spherical sporangium (**b**). (**c**, **d**) sexual propagative structures, oogonia, antheridia, oospore. Bars = 20 μm

coenocytic without septa, and septa are found at the borders of various reproductive organs (van der Plaats-Niterink 1981; Waterhouse 1968).

During asexual reproduction, a spherical or filamentous sporangium is formed (Fig. 12.1a, b), and zoospores are produced in the spherical vesicle formed by the transfer of protoplasm from the sporangium through the discharge tube. The zoospores have two flagella of different lengths, a feather-shaped one, extending forward from the side of the cell, and a sheath shaped one extending backward. The zoospores play a role in expanding the habitat by swimming in water. The sexual reproductive organs are the oogonia in the female, the antheridia in the male, and the oospores are fertilized by these organs (Fig. 12.1c, d). In sexual reproduction, the diversity of the species is maintained by crossbreeding, and the thick-walled oospore formed by fertilization is a survival organ that can withstand the environment when nutrients are depleted until a new source of nutrients is found.

12.1.3 Habitat and Life Cycle of Pythium *and* Phytopythium *Species*

The habitats of *Pythium* and *Phytopythium* spp. vary from aquatic to terrestrial. For example, *Py. porphyrae* is a marine organism and is an important pathogen causing red rot in seaweed cultivation (Kawamura et al. 2005). *Phytopythium helicoides* is both aquatic and terrestrial and causes root rot in many plants such as rose, kalanchoe and poinsettia (Kageyama et al. 2002; Takahashi et al. 2014; Watanabe et al. 2007) and is soil-transmitted by its oospore. *Pythium intermedium* is a less virulent pathogen of crops and does not form zoospores. Instead, it forms hyphal swellings as asexual organs and adapts to the soil (Li et al. 2010, 2021).

In terms of their life cycle, they adapt to their living environment by strategically forming asexual and sexual reproductive organs. When infecting organic matter or roots, the mycelium actively absorbs nutrients while simultaneously the sporangium is formed and the zoospores with flagella released from the sporangium are used to swim and spread through the water. They are attracted to new organic matter and to attractants leached from the roots of the host plant. After attaching to organic matter

or roots, the flagella are discarded and the zoospore becomes an encysted spore. The encysted spores germinate on the surface to which they are attached and infect organic matter and roots by invading through their mycelium. After absorbing all the nutrients, they form sexual structures. The oospores germinate in response to new organic matter or host-approaching attractants and become infectious (Kageyama 2014).

12.1.4 Pythium *and* Phytopythium *Species as Plant Pathogens*

Many species of *Pythium* and *Phytopythium* live as saprophytic fungi that decompose organic matter such as fallen leaves (Hendrix and Campbell 1973; Jacobs 1982). However, some species are parasitic on living plants such as crops and destroy them. Phytopathogenic species cause a wide range of plant diseases, including seed rot, seedling blight, and root rot of mature plants. In addition, a single species can be pathogenic to multiple plants. For example, *Py. aphanidermatum* has been reported on 43 species of crops in Japan, including soybean blight, cucumber root rot, watermelon rot, geranium stem rot, chrysanthemum blight, and Alstroemeria rhizome rot. *Phytopythium helicoides* has been reported on 14 species of crops in Japan, including strawberry root rot, chrysanthemum vertical blight, verruca root rot, gerbera root rot, and lotus rot. Occasionally, multiple *Pythium* and *Phytopythium* pathogens cause different diseases even on the same plant. Seedling blight of *Pp. vexans,* root rot of *Py. myriotylum*, and cotton rot of *Py. aphanidermatum* have been reported in tomato plants (Gene Bank of Agricultural Production Resources, https://www.gene.affrc.go.jp/databases-micro_pl_diseases.php).

In recent years, protected horticulture and hydroponic cultivation that are less affected by external environmental conditions have been developed to ensure stable agricultural production. In both cases, the plants are cultivated in a closed space and at a constant temperature, which is preferable for the plants, but since the temperature is kept at an optimum level with little temperature variation and the conditions are humid, they are conducive for the growth of *Pythium* and *Phytopythium* spp. In addition, in facility cultivation, anniversary cultivation is carried out for effective use of facilities. These conditions facilitate the growth of *Pythium* and *Phytopythium* spp. that are tolerant to high temperatures (optimum growth temperature around 35 °C) in the summer and induce damage.

12.1.5 *Distribution of Plant Pathogenic* Pythium *and* Phytopythium *Species*

The distribution of *Pythium* and *Phytopythium* spp. has been investigated in agricultural soils, irrigation water and reservoirs (Abdelzaher et al. 1994a, b, 1995;

Jacobs 1982; Hong and Moorman 2005; Nechwatal et al. 2008; Sánchez and Gallego 2000; Watanabe 1981, 1984), but few studies have been conducted in non-agricultural areas such as forests and rivers (Abdelzaher and Kageyama 2020). *Pythium, Phytophthora*, and *Phytopythium* spp. have been reported to be isolated from forest soil and river water in non-crop areas, and *Pp. helicoides* and *Py. myriotylum*, which are also plant pathogens, from forest soil and river water in the subtropical and cool temperate regions of Japan (Kageyama 2010). Although irrigation water has been investigated in other countries (Zappia and Hüberli 2014), there have only been few studies in non-agricultural areas, conducted in Korea (Nam and Choi 2019) and USA (Shrestha et al. 2013).

If plant pathogens are found in rivers, the use of river water through agricultural canals to irrigate crops and as culture media for nutrient cultivation would mean the artificial introduction of a primary source of infection. In addition, in recent years, guerrilla rains caused by abnormal weather conditions have resulted in river flooding, increasing the likelihood of river water intruding into farmland. Since river flooding is widespread, it may lead to the expansion of pathogen infestations regionally. Therefore, investigating *Pythium* and *Phytopythium* spp. that pose a threat to agricultural production in the natural environment can contribute to providing some guidelines for disease control measures.

12.1.6 Objectives of this Study

In this study, we investigated the hypothesis that pathogenic species such as *Pythium* and *Phytopythium* spp. present in river water invade agricultural production sites and cause damage to agricultural crops in Japan. Toward this end, we tested several parameters, including (a) the distribution of *Pythium* and *Phytopythium* spp. (b) seasonal variation of their distribution from upstream to downstream of rivers with different anthropogenic impacts, (c) morphological and molecular phylogenetic characteristics of river water isolates and (d) their pathogenicity.

12.2 Materials and Methods

12.2.1 Isolation of Pythium *and* Phytopythium *Species from Water*

Thirty to fifty grass blades cut into 5 mm pieces were placed in a 250 ml plastic bottle, which was sterilized by autoclaving and used as a sampling bottle. The water from the river was collected from a location where the river flow was slow and where organic matter such as fallen leaves had accumulated. Two samples were taken by filling the bottle with about 80% volume of river water (Fig. 12.2). After one week of

Fig. 12.2 Sampling and baiting methods for isolation of *Pythium* and *Phytopythium* species from water

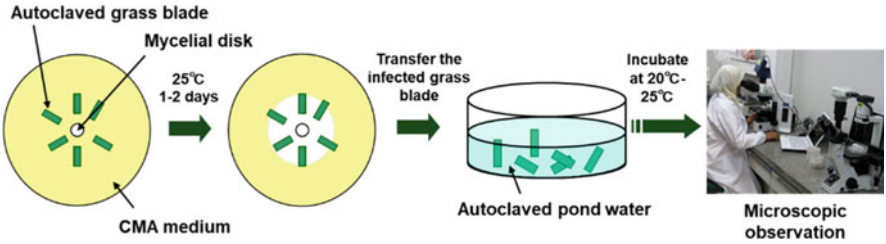

Fig. 12.3 Method for morphological observation

incubation at 25 °C, the grass pieces were collected on paper towels, lightly drained, and placed on NARM medium (Morita and Tojo 2007), a selective medium for *Pythium* spp.

The NARM medium was sterilized by adding 17 g/L of corn meal agar (Becton, Dickinson and Company) and 10 g/L of bacto agar (Becton, Dickinson and Company), and then cooled to about 50 °C. The mixture was prepared by adding 10 mg/L of nystatin, 250 mg/L of ampicillin sodium, 250 mg/L of rifampicin, and 1 mg/L of miconazole nitrate (all from Wako Pure Chemical Industries, Ltd.). The mycelial tips were transferred to CMA slope medium and used as isolates.

12.2.2 Species Identification of the Isolates

Morphological characteristics and DNA sequences were used to identify the species of isolates.

Morphological observations were made using the grass-blade method (Waterhouse 1967, 1968), which involves infecting grass blades with the isolate and floating it on water (Fig. 12.3). Sections (1.0–1.5 cm) of turfgrass were sterilized by high pressure steam and incubated in the dark at 25 °C for 1–3 days. Sections of grass blades infected with the fungus were floated in a 1: 2 mixture of filtered pond water and distilled water sterilized by high-pressure steam and incubated in the dark at 20 °C for 1–7 days. The morphology of asexual organs such as sporangium, spermatogonia, and oocysts was observed under a microscope.

In oomycetes, the internal transcribed spacer region of rDNA (the rDNA-ITS region) and mitochondrial cytochrome c oxidase subunit 1 gene coding region (*cox*1) are recognized as useful DNA barcode regions capable of accurate species identification (Robideau et al. 2011). Therefore, we carried out the BLAST search based on the sequences of ITS and *cox*1. DNA was extracted from mycelia using PrepMan Ultra Reagent (Thermo Fisher Scientific, Tokyo, Japan) according to the manufacturer's instructions. PCR was conducted using the ITS4 (5-′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5-′-GGAAGTAAAAGTCGTAACAAGG-3′) primers for the rDNA-ITS region, and OomCoxI-Levup (5′-TCAWCWMGATGGCTTTTTTCAAC-3′) and Fm85mod

(5′-RRHWACKTGACTDATRATACCAAA-3′) primers for *cox*1, according to the method described by White et al. (1990) and Robideau et al. (2011), respectively. The 25 μl reaction mixtures contained 1 μl DNA, 2 μM of each primer, 0.4 mg/ml BSA, 0.4 mM dNTPs, 0.125 U of TaKaRa Taq DNA polymerase (Takara Bio, Kusatsu, Japan), and PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, and 1.5 mM $MgCl_2$). The PCR reactions were carried out in a T100 DNA Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). The rDNA-ITS region and *cox*1 were amplified under the following conditions: 94 °C for 2 min, followed by 35 cycles at 94 °C for 1 min, 55 °C for 30 s (annealing step), 72 °C for 1 min, with a final extension at 72 °C for 10 min. PCR products were checked for successful amplification by electrophoresis in 2% (w/v) agarose gels (Agarose S, FUJIFILM WAKO, Tokyo, Japan). The PCR product was purified using ExoSAP-IT Express (Thermo Fisher Scientific). Sequencing of the purified PCR product was performed using BigDye Terminator v3.1 (Thermo Fisher Scientific).

For the strains that showed two bands in electrophoresis, the DNA was collected by cutting the band of the target size from the electrophoresed gel. For DNA extraction from the gel, Thermostable β-Agarase (NIPPON GENE CO., LTD., Tokyo, Japan) was used according to the manufacturer's instructions, and BigDye Terminator v3.1 was used for sequencing as before.

The rDNA-ITS region could not be sequenced in some cases due to intra-strain polymorphism; in which case, PCR products were cloned using the TOPO TA Cloning Kit for Sequencing (Thermo Fisher Scientific) according to the instructions. Direct PCR was performed from the colonies formed on the culture medium by cloning. The primers used were M13M4 (5'-GTTTTCCCAGTCACGAC-3′) and M13rv (5'-CAGGAAACAGCTATGAC-3′). The PCR was performed using the same reaction solution composition and reaction conditions as for the ITS region. For sequencing, BigDye Terminator v3.1 was used as before.

The nucleotide sequences were subjected to BLAST searches using DNA Data Bank of Japan (DDBJ; http: //www.ddbj.nig.ac.jp/index-j.html), and species with high homology were selected as candidate species.

Phylogenetic analysis of representative isolates in all species recovered from river water was conducted based on the sequences of the rDNA ITS region (Table 12.1). The phylogenetic tree was constructed from the sequences aligned using Clustal X and MEGA X software (Kumar et al. 2018) with the maximum-likelihood method and Tamura-Nei model (Tamura and Nei 1993). Bootstrap values were calculated with 1000 replicates.

12.2.3 Pathogenicity Test

To determine whether the widely distributed riverine strains of *Pp. helicoides, Py. myriotylum* and *Py. dissotocum* are actually pathogenic to plants, we conducted pathogenicity tests on crops that have been reported as hosts. Inoculation tests were

Table 12.1 List of *Pythium* and *Phytopythium* isolates used in phylogenetic analysis

Isolate	Isolation year	Location	Species
FIW5–11	2010	Fukui	*Py. aphanidermatum*
FIW5–13	2010	Fukui	*Py. arpisium*
GF19JW1–1-6	2019	Gifu	*Py. arrhenomanes*
GF19JW4–2-3	2019	Gifu	*Py. apleroticum*
GF20AW2–1-1	2020	Gifu	*Py. oopapillum*
GU20SRW1–2-1	2020	Gifu	*Pp. vexans*
HOW1–6	2010	Hokkaido	*Pp. litorale*
HSW1–11	2013	Hiroshima	*Py. myriotylum*
HSW2–1	2013	Hiroshima	*Py. catenulatum*
HSW3–11	2013	Hiroshima	*Py. oopapillum*
IKW1–6	2014	Ishikawa	*Py. inflatum*
ITW1–3	2014	Iwate	*Py. apleroticum*
ITW2–4	2014	Iwate	*Pp. helicoides*
MGW1–9	2014	Miyagi	*Py. dissotocum*
NRW2–3	2010	Nara	*Py. irregulare*
On11W3–2	2011	Okinawa	*Py. myriotylum*
On11W3–3	2011	Okinawa	*Pp. helicoides*
On11W6–2	2011	Okinawa	*Py. inflatum*
On11W9–8	2011	Okinawa	*Pp. vexans*
SGW1–1	2010	Saga	*Py. aphanidermatum*
SZW4–2	2012	Sizuoka	*Pp. litorale*
SZW10–14	2012	Shizuoka	*Py. catenulatum*
SZW12–2	2012	Shizuoka	*Py. marsipium*
TTW2–7	2012	Tottori	*Py. dissotocum*
TTW3–1	2012	Tottori	*Py. arrhenomanes*

conducted on sedum and gerbera plants grown in soil, and on lettuce and tomato plants grown hydroponically.

12.2.3.1 Pathogenicity Test by Soil Cultivation

Three isolates of *Pp. helicoides*, GF19AgW 2–2-3, GF19AgW 3–2-2, and GF19OW 4–2-3, were isolated from the Ijira river in Gifu Prefecture, Japan.

Pathogenicity tests by soil inoculation were conducted on sedum and gerbera plants, which have been reported as hosts of *Pp. helicoides* (Hayashi et al. 2018; Suzuki et al. 2009). In the case of sedum, cell trays (40 mm × 40 mm × 40 mm/cell) were filled with horticultural culture soil (Hanogokoro Co., LTD., Nagoya, Japan) that had been sterilized by high-pressure steam (120 °C, 60 min). The plants were cultivated for three weeks (25 °C, 12 h light: 12 h dark). For gerbera, the seeds (variety: Festival Scarlet Eye) were treated with 10% hydrogen peroxide water for 20 minutes and washed three times with sterile water to sterilize the surface. Two

seeds per square were sown in cell trays prepared in the same way as for sedum, and the trays were thinned as necessary so that one seedling remained and cultivated for four weeks.

The inoculum source was prepared as follows: 2 g of bentgrass seeds and 10 ml of distilled water were placed in a 200 ml flask and sterilized by high-pressure steam. Five sheets of fungus-containing agar were placed in the flask, and the tips of the mycelium of the test strain, which had been cultured on CMA medium for 2 days (25 °C, dark), were punched out with a 4-mm diameter cork borer. After 3 weeks, 50 ml of sterile water and the culture were mixed in a homogenizer and this fungal suspension was used as the inoculum source. The control was cultured in the same way with plain agar instead of fungus-containing agar.

For inoculation, five sedum seedlings were transplanted into a polycarbonate box (13.2 cm × 8.9 cm × 5.8 cm) filled with sterilized culture soil, and 5 ml of the fungal suspension was inoculated per plant. After inoculation, the plants were cultivated in an artificial climate for 4 weeks (25 °C, 12 h light: 12 h dark). For gerbera, the roots of the grown seedlings were washed under running water and then immersed in the fungal suspension for 1 minute. After that, gerberas were planted in cell trays (50 mm × 50 mm × 50 mm/cell) with high-pressure steam sterilized culture soil and cultivated for two weeks in an artificial climate machine under the same conditions.

For investigating disease development, the soil on the roots was washed with running water and the condition of the roots was evaluated. Disease development was evaluated by the disease index (0 = no disease to 3 = significant delay in growth and severe root browning) based on the condition of the above-ground parts and roots. The disease severity was calculated from the disease index by the following formula:

$$\text{Disease severity} = \{\Sigma(\text{disease index} \times \text{number of plants})/(\text{number of seeds} \times 3)\} \times 100$$

For re-isolation, diseased parts of five individual plants were placed on a 9 cm Petri dish of NARM medium (see above) after the disease survey. This was incubated in an incubator (25 °C, in the dark) for 1–2 days to check for mycelial growth.

12.2.3.2 Pathogenicity Test by Hydroponics

For hydroponic cultures, the following isolates were used: for *Pp. helicoides*, GF19AgW2–2-3, GF19AgW3–2-2, and GF19OW4–2-3; for *P. dissotocum*, GF19AW4–1-3, GF19AgW1–2-1, and GF19OW1–2-2, and for *P. myriotylum*, GF20JW2–2-3, GF20AgW2–2-2, and GF19OW4–2-3.

Lettuce, which has been reported as a host for *Pp. helicoides* and *Py. dissotocum* (Stanghellini and Kronland 1986) and tomato, which has been reported as a host for *Py. myriotylum* (Feng et al. 2018), were used as test plants. Surface sterilization was

performed for both by treating them with 10% hydrogen peroxide water for 20 min followed by rinsing with sterile water three times.

Before inoculation, lettuce seeds (variety: Green Wave) were placed in a glass Petri dish (pore size: 8.5 cm) lined with a Kim-towel moistened with sterile water and grown in an artificial climate machine for 2 days. Tomatoes were grown in a hydroponic kit (20 cm × 6 cm × 5 cm, Living Farm Co., Ltd. Japan) by placing surface sterilized tomato seeds (cultivar: House Momotaro) in the slits on a sponge with slits. Liquid fertilizer was applied during the first week of culture.

For inoculation of lettuce, 4 mm diameter mycelium tips of the test strain cultivated on CMA medium for 2 days (25 °C, in the dark) were placed along with the seeds in the slits of the sponge of the hydroponics kit described above, and cultivated for 3 weeks at 20 °C with 12 h of light and 12 h of darkness. For tomatoes, fungus-containing agar made in the same way as for lettuce was placed in the sponge slit from the side of the tomato seedlings and incubated for three weeks at 25 °C with 12 h light and 12 h of darkness.

The disease survey and re-isolation were done in the same way as in the soil cultivation.

12.3 Results and Discussion

12.3.1 Distribution of Phytopathogenic Oomycetes in Water Systems in Japan

In order to investigate the flora of *Pythium* and *Phytopythium* species in water systems in Japan, we collected isolates from rivers and irrigation water in 10 regions and 20 prefectures throughout Japan from 2007 to 2020. Either or both species of both genera were detected in all survey sites. Many of the isolates did not form sexual organs even though the identified species originally sexually reproduced, and the asexual organs formed filamentous or swollen filamentous, earlobe-shaped, or club-shaped sporangia, which released a large number of zoospores in water. A BLAST search of these strains based on the nucleotide sequence of the rDNA ITS region or the *cox*1 gene, which is the DNA barcoding region of oomycetes (Robideau et al. 2011), revealed many strains that appeared to be new species.

Some of the isolates were reported as phytopathogenic species and their distribution pattern revealed that they are widely distributed in rivers, even though the survey sites, except for irrigation water, were mainly in forested areas upstream of rivers. We detected a large number of species, including 10 *Pythium* spp. and 3 *Phytopythium* spp. *Py. dissotocum*, *Py. catenulatum*, and *Pp. helicoides* detected in more than 10 prefectures. The next most frequent species were *Py. arrhenomanes*, *Py. marsupium*, *Py. myriotylum*, *Py. oopapillum*, and *Pp. litorale*, which were detected in 6 to 9 prefectures. The others were *Py. apleroticum*, *Py. aphanidermatum*, *Py. inflatum*, *Py. irregulare*, and *Pp. vexans* isolated in one to

three prefectures (Table 12.2, Fig. 12.4). *Pythium inflatum*, *Py. oopapillum*, *Pp. litorale* and *Pp. vexans* were also isolated from forest water in Korea (Nam and Choi 2019). *Pythium apleroticum*, *Pp. helicoides*, *Pp. litorale* and *Pp. vexans* were isolated in Tennessee, USA (Shrestha et al. 2013).

Comparing the species detected in irrigation water with those in river water, we found no unique species, with the same species detected in irrigation water also seen in river water. However, *Py. irregulare* was detected only at one site in irrigation water, and *Py. aphanidermatum* was detected at two sites, one of which was irrigation water.

We did not observe any regional bias in the species detected. For example, *Py. myriotylum* and *Pp. helicoides*, which are known to be tolerant to high temperatures, were not found more frequently in southern regions, but were found even in northern regions such as Yamagata and Iwate prefectures. In addition, there was no region where a particularly large number of species was detected.

12.3.2 *Positional and Seasonal Variations of Phytopathogenic* Pythium *and* Phytopythium *Fauna in Ijira River, Gifu, Japan*

Rivers are influenced by human activities such as agriculture and drainage as they move downstream. It is interesting to investigate whether the impact of human activities on rivers can be assessed by studying the changes in the diversity of *Pythium* and *Phytopythium* fauna depending on the location of the river. In addition, examining the seasonal variation of both the genera and the species at the same site will provide important information for setting the timing of the mycological survey and for discussing the results of the survey. We chose the Ijira river, which is a tributary of the Nagara river and flows from Yamagata City to Gifu City in Gifu Prefecture, as the study river, and four sites (A-D; A: upstream, B: upper midstream; C: lower midstream; D: downstream) (Table 12.3) were set up from upstream to downstream of this river. River water was collected every two months from February 2019 to December 2020, and the detected species were examined.

We detected 8 *Pythium* species and 2 *Phytopythium* species in the Ijira river, and the number of species detected was almost the same as the number of species detected in the Japan survey (10 and 3, respectively). The species that were not detected in the Ijira river were *Py. aphanidermatum, Py. irregulare* and *Pp. vexans*, all of which have rarely been detected in Japan (Table 12.3). On the other hand, *Py. dissotocum, Py. catenulatum, Py. marsipium, Py. oopapillum* and *Pp. helicoides* were detected in many sites as in other areas of Japan (Fig. 12.4). In comparison with the 18 other rivers and irrigation water sources in Gifu Prefecture, the number of species detected in the Ijira River was 10, whereas the number of species detected in Gifu Prefecture was 8, including *Phytopythium* species (Tables 12.2 and 12.3).

Table 12.2 Phytopathogenic *Phytopythium* and *Pythium* species isolated from rivers, lakes, and irrigation water from 2007 to 2018

Sampling site and year			Sites[a] and numbers		*Pythium*[b]										*Phytopythium*[c]		
					Aph	*Apl*	*Arr*	*Cat*	*Dis*	*Inf*	*Irr*	*Mar*	*Myr*	*Oop*	*Hel*	*Lit*	*Vex*
Hokkaido		2010, 2011	Ri	3					○							○	
Tohoku region																	
	Akita	2014	Ri	1					○								
	Iwate	2014	Ri	2		○			○					○	○		
	Yamagata	2014	Ri	3					○			○	○		○		
			Ic	3					○				○		○		
	Miyagi	2014	Ri	2					○					○	○		
Koshinetsu region																	
	Yamanashi	2012	Ri	5			○	○	○								
			Ic	1				○	○								
Hokuriku region																	
	Ishikawa	2014	Ri	4			○		○	○				○		○	○
	Fukui	2010	Ri	5	○			○					○		○		
Tokai region																	
	Shizuoka	2012, 2013	Ri	5			○	○	○			○			○		
			Ic	2				○	○			○			○	○	
	Gifu	2010, 2012	Ri	15				○	○			○	○	○	○		
		2014, 2019	Ic	3											○		
	Aichi	2008, 2009	Ic	7			○		○						○	○	
Kinki region																	
	Nara	2010	Ic	3							○				○		
Chugoku region																	
	Hiroshima	2013	Ri	3			○	○	○				○	○	○		
	Tottori	2012	Ri	4			○	○	○						○		

(continued)

Table 12.2 (continued)

Sampling site and year			Sites[a] and numbers		*Pythium*[b]										*Phytopythium*[c]		
					Aph	*Apl*	*Arr*	*Cat*	*Dis*	*Inf*	*Irr*	*Mar*	*Myr*	*Oop*	*Hel*	*Lit*	*Vex*
	Okayama	2013	Ri	2			○					○		○	○		
	Yamaguchi	2018	Ri	2									○		○		
Shikoku region																	
	Ehime	2012	Ri	6									○		○		
Kyushu region																	
	Saga	2010	Ic	4	○			○				○			○		
	Kagoshima (Amami Island)	2018	Ri	7					○						○	○	
Okinawa region																	
	Main Island	2011	Ri	4								○				○	○
			Ic	6						○			○		○		

[a] Ri: River, Ic:irrigation canel

[b]Aph: *P. aphanidermatum*, *Apl: P. apleroticum, Arr: P. arrhenomanes, Cat: P. catenularum, Dis: P. dissotocum, Inf: P. inflatum,* Irr*: P. irregulare*, Mar: *P. marpsium*, Myr: *P. myriotylum*, Oop; *P. oopapillum*

[c]Hel: *Pp. helicoides*, Lit: *Pp. litorale*, Vex: *Pp. vexans*

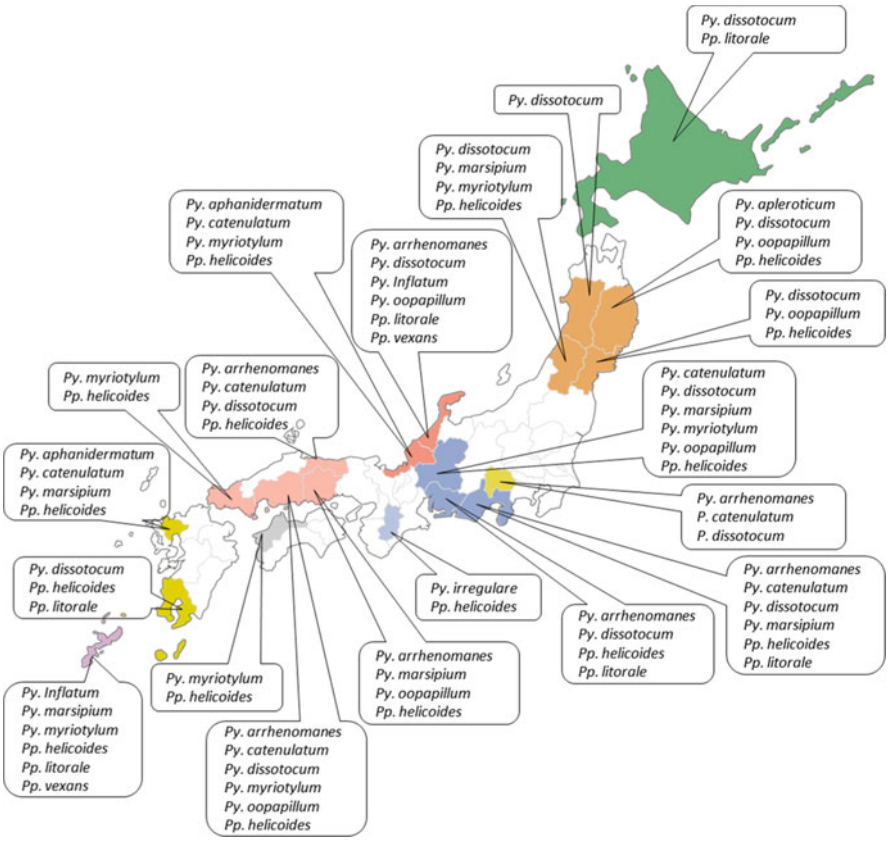

Fig. 12.4 Distribution of plant pathogenic *Pythium* and *Phytopythium* species in river waters in Japan

Pythium arrhenomanes, Py. apleroticum and *Pp. litorale* were detected in the upper to middle streams of the river, while *Py. catenulatum*, *Py. marsipium* and *Pp. helicoides* were not detected in the upper stream (Site A; Table 12.3). On the other hand, *Py. dissotocum* and *Py. oopapillum* were detected from upstream to downstream regardless of location (Table 12.3). The diversity tended to be low in the upstream of the river, with the highest diversity at site B in the upper-middle streams, where all eight species of *Pythium* spp. that were detected in other survey sites in the Ijira River were detected, and two of the three *Phytopythium* species was detected.

With regards to yearly variation (Table 12.3), *Pythium apleroticum* and *Pp. litorale* were detected only in 2019 in the upstream to midstream areas (sites A, B, and C, respectively). On the other hand, *P. inflatum* and *P. myriotylum* were detected only in 2020 in the upstream to midstream areas (sites B and C, respectively).

In terms of seasonal variation, high-temperature tolerant *Pp. helicoides* was not detected during the winter months of December to February (water temperature

Table 12.3 Positional and seasonal variation of plant pathogenic Phytopythium and Pythium fauna in water from upstream to downstream of Ijira river in Gifu, Japan

Sampling date		Isolated phytopathogenic species			
		Site A	Site B	Site C	Site D
2019	Feb.		*P. dissotocum*	*Phpy. litorale*	
		-		*P. catenulatum*	
				P. dissotocum	
	Apl.	*Phpy. litorale*	*P. oopapillum*	*P. dissotocum*	*P. dissotocum*
		P. dissotocum			
	Jun.	*P. oopapillum*	*Phpy. litorale*	*Phpy. litorale*	*Phpy. helicoides*
				P. catenulatum	
	Aug.	*P. arrhenomanes*	*Phpy. helicoides*	*Phpy. helicoides*	*Phpy. helicoides*
		P. dissotocum			
	Oct.	*P. dissotocum*	*P. apleroticum*	*Phpy. helicoides*	*P. marsipium*
		P. apleroticum	*P. marsipium*	*P. arrhenomanes*	*P. oopapillum*
	Dec.	*P. dissotocum*	*P. dissotocum*	*P. dissotocum*	
			P. oopapillum		
2020	Feb.		*P. dissotocum*	*P. dissotocum*	*P. dissotocum*
	Apl.	*P. dissotocum*	*P. dissotocum*	*P. catenulatum*	*Phpy. helicoides*
			P. oopapillum	*P. dissotocum*	*P. catenulatum*
					P. dissotocum
					P. oopapillum
	Jun.	*P. dissotocum*	*P. arrhenomanes*	*Phpy. helicoides*	*P. dissotocum*
			P. dissotocum	*P. dissotocum*	*P. inflatum*
			P. myriotylum	*P. marsipium*	*P. marsipium*
					P. oopapillum
	Aug.	*P. dissotocum*	*Phpy. helicoides*	*Phpy. helicoides*	
			P. marsipium	*P. marsipium*	
			P. myriotylum		
	Oct.		*Phpy. helicoides*	*P. dissotocum*	*Phpy. helicoides*
			P. dissotocum	*P. marsipium*	*P. catenulatum*
			P. marsipium		*P. dissotocum*
			P. oopapillum		*P. marsipium*
	Dec.		*P. catenulatum*	*P. dissotocum*	*P. catenulatum*
			P. dissotocum		*P. oopapillum*
			P. oopapillum		

8–18 °C, Table 12.3, Fig. 12.5). *P. myriotylum*, another species tolerant to similar levels of high temperatures, was detected only twice during the summer months of June and August (water temperature 24 °C and 31 °C, respectively). On the other hand, *Py. dissotocum* was detected throughout the year regardless of season, even though its optimum growth temperature was 25 °C, suggesting that it might have a wide range of temperature adaptation.

The number of species detected tended to be high in the relatively warm months between June and October (water temperature: 16–26 °C), although it was lower in

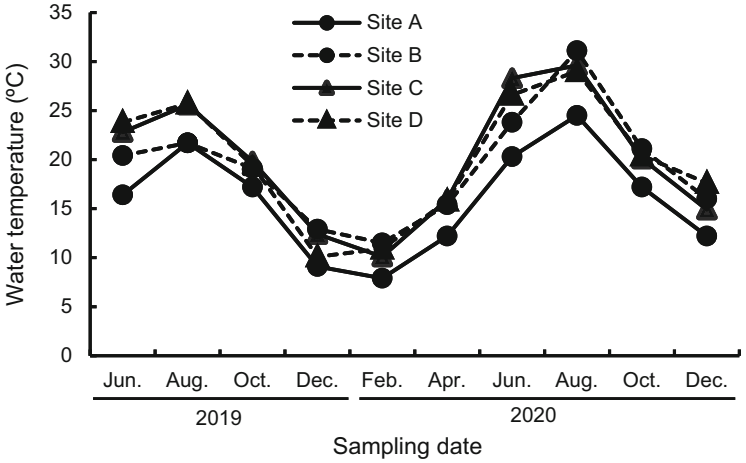

Fig. 12.5 Seasonal changes in water temperature in Ijira river located in Gifu prefecture, Japan

August (water temperature: 22–31 °C), the middle of summer, regardless of whether it was upstream or downstream. However, *Py. dissotocum*, *Py. catenulatum*, and *Py. oopapillum* were also detected in the winter months of December through February (water temperature 8–18 °C). It is not clear whether the survival form of *Py. dissotocum*, *Py. catenulatum*, and *Py. oopapillum* at this time was that of active zoospores or not.

These results suggest that *Py. dissotocum*, a pathogen of rice that is isolated more frequently upstream, may be a threat in rice paddies. *Pythium myriotylum* and *Pp. helicoides,* which have a wider host range, are found in the middle and down streams of the river, indicating that the use of river water for hydroponics is dangerous. It is also possible that these pathogens may enter the river with the effluent from the cultivation facilities.

From our results, it is apparent that the best time to conduct the survey of *Pythium* and *Phytopythium* fauna, is in June and October, when the water temperature is warm (15–25 °C). In addition, we consider that the middle streams of rivers are the best sites for the survey of plant pathogens.

12.3.3 Morphological Characteristics of Strains Isolated from Rivers

Next, we set out to understand the morphology of the sporangium, the organ that forms zoospores, and the characteristics of the sexual organs, which are durable survival organs in aquatic species compared to those species that live mainly in soil.

Pythium species we detected in water in this study were overwhelmingly the species that formed filamentous sporangia and had a high ability to form zoospores from the sporangia, even though they were not plant pathogens. Eight of the ten phytopathogenic *Pythium* species we detected (*Py. apleroticum*, *Py. aphanidermatum* (Fig. 12.6a-c), *Py. arrhenomanes* (Fig. 12.6d-f), *Py. catenulatum* (Fig. 12.6g-h), *Py. dissotocum* (Fig. 12.6i-k), *Py. inflatum*, *Py. myriotylum* (Fig. 12.7a-c) and *Py. oopapillum*) formed filamentous sporangia. For the genus *Phytopythium*, we detected three species in this study, *Pp. helicoides* (Fig. 12.7d-f), *Pp. littorale* (Fig. 12.7g-i), and *Pp. vexans* (Fig. 12.7j-l), with the first two forming sporangia with papillae and the latter forming sporangia without papillae.

Phytopythium litorale is not capable of forming sexual organs (Nechwatal and Mendgen 2006). The strains identified as *Py. inflatum, Py. marsipium*, and *Py. oopapillum* were reported to form sexual organs (van der Plaats-Niterink 1981), but in our observations, they only formed asexual organs. Among *Py. arrhenomanes*, *Py. dissotocum* and *Pp. helicoides*, although some strains formed sexual organs, most formed only asexual organs. Some strains of *Pp. helicoides* have been reported to have lost the ability to form sexual organs despite their pathogenicity (Kageyama et al. 2003) These asexual reproductive organ-dependent life cycles suggest that aquatic fungi have strong saprophytic capacity and lose the ability to form sexual organs as they are able to survive due to the constant supply of nutritive organic matter in water unlike in field soil, and don't need to resort to forming survival structures.

The characteristics of the sexual organs differ among species: the oogonia of *Py. arrhenomanes*, *Py. myriotylum*, and *Pp. helicoides* have a smooth surface and are large (around 30 μm), whereas those of *Py. dissotocum* and *Pp. vexans* are around 20 μm in size. *Pythium irregulare* has spines on the oogonia and is smaller than 20 μm in size. *Pythium catenulatum* does not form sexual organs in a single culture but forms them in mating culture with compatible strains. The sexual organs of the species isolated from water did not have any special features unlike that of asexual organs.

12.3.4 Molecular Phylogenetic Analysis of Strains Isolated from Rivers

In order to determine the molecular phylogenetic position of the isolates recovered from river and irrigation water, we constructed a molecular phylogenetic tree based on the nucleotide sequence of the rDNA-ITS region for both *Pythium* and *Phytopythium* (Table 12.1; Figs. 12.8 and 12.9).

In the genus *Pythium*, the morphological characteristic of the sporangium, (both filamentous and spherical sporangia), is to form a large cluster, which is the first divergence point in the molecular phylogeny (Lévesque and de Cock 2004; Matsumoto et al. 1999; Martin 2000; Villa et al. 2006). We identified that the

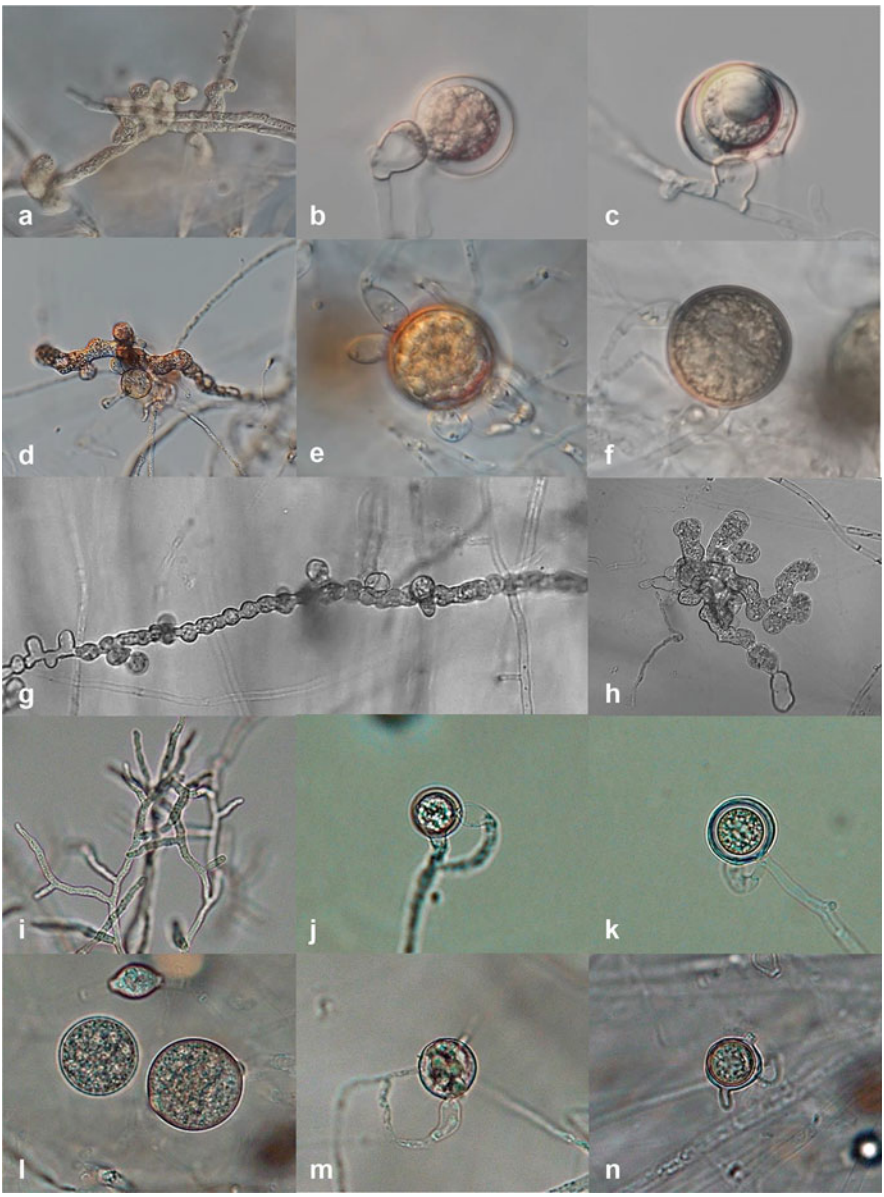

Fig. 12.6 Morphological characteristics of *Pythium aphanidermatum*, *Py. arrhenomanes*, *Py. catenulatum*, *Py. dissotocum* and *Py. irregulare*. (**a**-**c**) *Py. aphanidermatum*, (**a**) filamentous inflated sporangium, (**b**, **c**) smooth oogonium with an intercalary antheridium and aplerotic oospore, (**d**-**f**) *Py. arrhenomanes*, (**d**) lobulate sporangium, (**e**, **f**) smooth oogonia with multiple antheridia and nearly plerotic oospore, (**g**, **h**) *Py. catenulatum*, (**g**) catenulate hyphal swelling, (**h**) lobulate sporangia, (**i**-**k**) *Py. dissotocum*, (**i**) filamentous sporangia, (**j**, **k**) smooth oogonium with a monoclinous antheridium, aplerotic oospore, (**l**-**n**) *Py. irregulare*, (**l**) spherical sporangia, (**m**, **n**) irregularly spined oogonium with a monoclinous antheridium

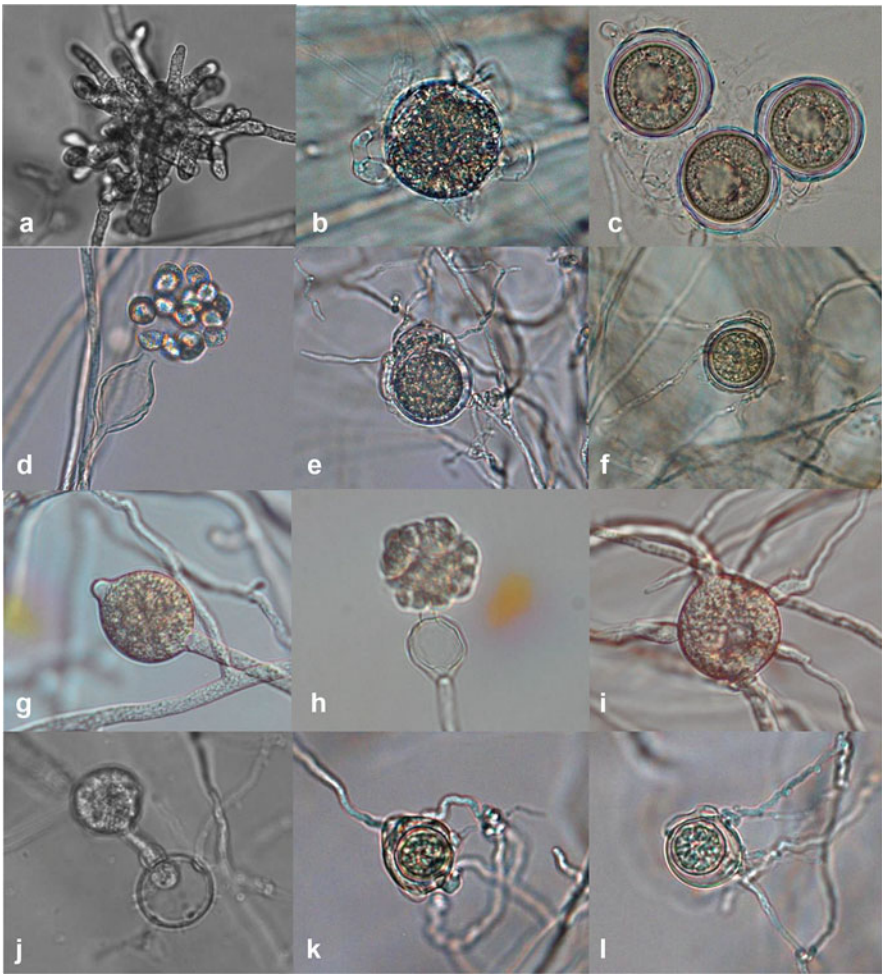

Fig. 12.7 Morphological characteristics of *Pythium myriotylum*, *Phytopythium helicoides*, *Pp. litorale* and *Pp. vexans*. (**a**-**c**) *Py. myriotylum*, (**a**) lobate sporangium, (**b**, **c**) smooth oogonium with multiple antheridia and aplerotic oospore, (**d**-**f**) *Pp. helicoides*, (**d**) zoospore formation in vesicle from sporangium, (**e**, **f**) smooth oogonium with broadly contacted antheridia and aplerotic oospore, (**g**-**i**) *Pp. litorale,* G; papillate sporangium, (**e**) zoospore formation in vesicle from sporangium, (**f**) direct germination of sporangium, (**j**-**l**) *Pp. vexans*, (**j**) spherical sporangia and vesicle formation from sporangia, (**k**, **l**) smooth oogonium with monoclinous antheridia and aplerotic oospore

phytopathogenic species isolated from rivers belonged to molecular phylogenies Clades A and B (to which filamentous sporangium-forming species belonged) as 8 out of 10 species were filamentous spore-forming species, and the species belonging to Clade B were dominant. *Py. aphanidermatum* belonged to Clade A. Other two phytopathogenic species, *Py. irregulare* and *Py. marsipium*, which form spherical

Fig. 12.8 Maximum likelihood tree of the river isolates with the ex-types of the genus *Pythium* inferred from the sequences of the rDNA ITS region. The isolate numbers used for the phylogenetic analysis are showed after the taxon name. The circled isolates were representative of the phytopathogenic species in the river isolates. *Phytopythium helicoides* was used as out group. The clade names referred to Lévesque and de Cock (2004). The numbers on the branches represent bootstrap values obtained from 1000 replications (only values greater than 60% are shown)

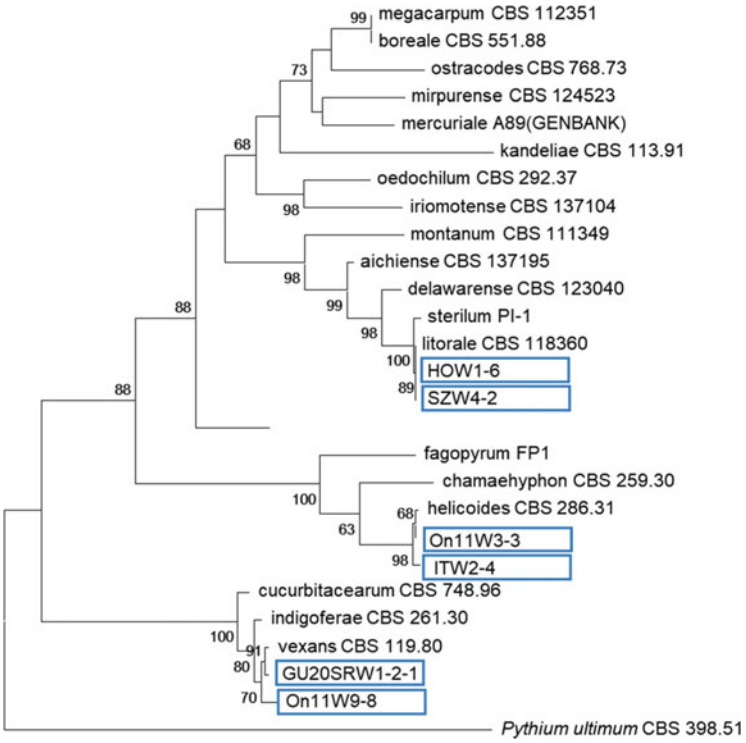

Fig. 12.9 Maximum likelihood tree of the river isolates with the ex-types of the genus *Phytopythium* inferred from the sequences of the rDNA ITS region. The isolate numbers used for the phylogenetic analysis are showed after the taxon name. The circled isolates were representative of the phytopathogenic species in the river isolates. *Pythium ultimum* was used as out group. The numbers on the branches represent bootstrap values obtained from 1000 replications (only values greater than 60% are shown)

sporangia, belong to Clade E and Clade F, respectively. These indicate that the species of *Pythium* present in the river are genetically related, although there are some exceptions.

The genus *Phytopythium* was initially a molecular lineage Clade K of the genus *Pythium*. However, a molecular phylogenetic analysis by Villa et al. (2006), which included the genus *Phytophthora*, showed that it is a Clade genetically closer to *Phytophthora* than to *Pythium*, and a new genus was subsequently proposed by Bala et al. (2010). Three species detected in rivers did not belong to any special Clade (Baten et al. 2014; de Cock et al. 2015). With only about 20 species belonging to this genus, it was difficult to observe any special trends. However, many of the species in this genus were isolated from water in other countries (Shrestha et al. 2013; Nam and Choi 2019; Zappia and Hüberli 2014) and were considered to be a group of aquatic species.

Table 12.4 Pathogenicity of *Phytopythium helicoides* to sedum and gerbera plants in soil culture

	Disease incidence %	Disease severity	Re-isolation %
Sedum			
Control	0	0	0
GF19AgW2–2-3	100	75	100
GF19AgW3–2-2	100	73	100
GF19OW4–2-3	100	83	100
Gerbera			
Control	0	0	0
GF19AgW2–2-3	100	88	100
GF19AgW3–2-2	100	81	100
GF19OW4–2-3	100	79	100

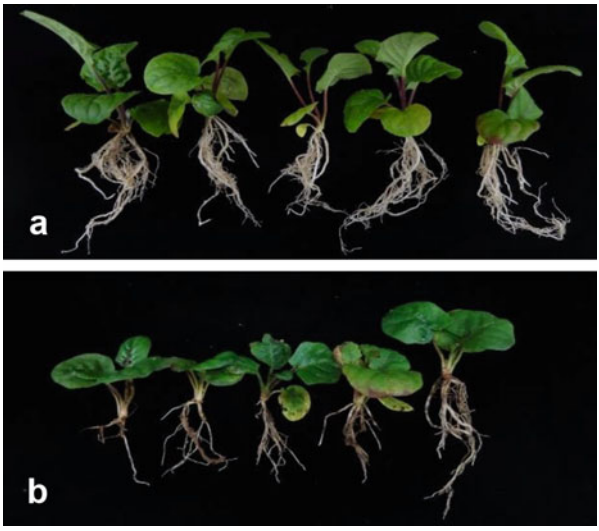

Fig. 12.10 Pathogenicity of *Phytopythium helicoides* from river water to gerbera plants grown in soil culture. (**a**) non-inoculated, (**b**) inoculated

12.3.5 Pathogenicity of Strains Isolated from River Water to Plants

In order to determine whether strains isolated from river water are actually pathogenic even if they are reported as plant pathogens, we examined the pathogenicity of the frequently encountered species, *Py. dissotocum*, *Py. myriotylum*, and *Pp. helicoides*, in soil and in hydroponic cultivation.

In soil cultivation, *Pp. helicoides* was tested for pathogenicity against sedum and gerbera plants, which have been reported as its natural targets. All three tested strains of *Pp. helicoides* caused significant root inhibition in sedum, and even when roots were produced, they were brown and decayed (Table 12.4). The growth of aboveground parts was also significantly inhibited. In gerbera, all three tested strains caused significant root brown rot and suppressed growth (Table 12.4, Fig. 12.10).

Table 12.5 Pathogenicity of *Phytopythium* and *Pythium* species in hydroponic culture

	Disease incidence %	Disease severity	Re-isolation %
Phytopythium helicoides to lettuce			
Control	0	0	0
GF19AgW2–2-3	100	85	80
GF19AgW3–2-2	100	80	90
GF19OW4–2-3	100	63	80
Pythium dissotocum to lettuce			
Control	0	0	0
GF19AW4–1-3	100	88	100
GF19AgW1–2-1	100	93	100
GF19OW1–2-2	100	88	100
Pythium myriotylum to tomato			
Control	0	0	0
GF20JW2–2-3	100	90	100
GF20AgW2–2-2	100	93	100

Fig. 12.11 Pathogenicity of *Pythium dissotocum* from river water to lettuce plants grown in hydroponic culture. (**a**) non-inoculated, (**b**) inoculated

Both *Pp. helicoides* and *Py. dissotocum* were tested for their pathogenicity against lettuce, which has been reported as their natural targets (McGehee et al. 2018). The pathogenicity of *Py. myriotylum* against tomato was also investigated, (Li et al. 2014; Feng et al. 2018) and both strains caused brown rot of the roots and consequent growth inhibition (Table 12.5, Fig. 12.11).

These results indicate that the strains isolated from river water are also pathogenic to plants as the strains isolated from diseased plants. In other words, we have clarified one of the important factors in testing the hypothesis that pathogenic species in river water invade agricultural production sites and cause damage to crops.

12.3.6 Conclusion

The presence of plant pathogens in river water has posed a problem for several plant species. In terms of the host range of plant pathogens detected in river and irrigation water, *Py. aphanidermatum* and *Py. myriotylum* have been reported on more than 40 plant species while *Py. irregulare* and *Pp. helicoides* on more than 20 plant species. These four species have been attracting attention not only in field crops but also in vegetables and flowering plants due to the widespread use of hydroponics and other greenhouse cultivation methods in recent years (Feng et al. 2015, 2018; Fukuta et al. 2013, 2014; Li et al. 2014; Suzuki et al. 2013; Takahashi et al. 2014). In particular, the three species other than *Py. irregulare* are important pathogens for tomatoes and flowering plants that are grown in greenhouses year-round even at high temperatures during the summer because of their heat tolerance and their ability to grow even at 40 °C, with an optimum growth at 30 °C or higher (Ishiguro et al. 2013). *Pythium dissotocum* is also a problematic pathogen in hydroponics, although its host range is not as wide as the four species mentioned above. Our results suggest that phytopathogenic oomycetes are naturally found in rivers and can act as pathogens when they invade agricultural production sites, emphasizing the need to pay attention to river water and irrigation water as one of the pathogen-transmission routes.

Although *Py. myriotylum* and *Pp. helicoids*, which were detected frequently, are high temperature tolerant species, their distribution shows that they are not necessarily found only in warm regions but are also present in the cold Hokkaido and Yamagata prefectures suggesting that they have a high ability to survive in winter. Considering the wide host range of these species, it is necessary to keep in mind that they can be harmful to plants even in cold areas.

Our morphological analyses showed that *Pythium irregulare* form spherical sporangia in water, but do not release zoospores. *Py. irregulare* has a wide host range and is commonly found in agricultural areas (Li et al. 2021). These findings suggest that the species was detected incidentally in river water due to the inflow of river water into agricultural fields caused by rising water levels, and their low detection frequency suggests that these species will be not active inhabitants of river water. If further studies prove this, this species could be used as a biological indicator of sediment inflow into rivers.

Although a broad host range is thought to be associated with a wide distribution, our results suggest that there need not be a strong correlation between the two factors. For example, *Py. myriotylum* and *Pp. helicoides* have a broad host range and are widely distributed, but *Py. aphanidermatum* and *Py. irregulare* do not have a

wide distribution in rivers but have a wide host range. Similarly, *Py. catenulatum*, *Py. marsupium*, and *Py. oopapillum*, which have a narrow host range (they have only been reported as pathogens of rice), have a wide distribution. Moreover, the life cycle of pathogens differs from species to species. While some aquatic species have acquired pathogenicity in crops, there may be others whose life cycle is mainly in soil but could still cause pathogenicity. It is necessary to consider the distribution of pathogens in soil as well as water for investigating the transmission modes of a plant pathogen.

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