Master Thesis

Diversity and comparative studies of Oomycetes in cropping sites between the Philippines and Japan

Graduate School of Bioresources
Mie University

Muhammad Nur Akhyar Azelan
July 2023

This research is submitted to the Graduate School of Bioresources, Mie University for the purpose of obtaining a degree.

Acknowledgement

بِسْمِ اللهِ الرَّحْمٰنِ الرَّحِيْمِ

First and foremost, I would like to praise Allah the Almighty, the Most Gracious, and the Most Merciful for His blessing given to me during my study and in completing this thesis. May Allah's blessing goes to His final Prophet Muhammad (peace be up on him), his family and his companions.

Secondly, I would like to thank my supervisor, Prof. Dr. Chiharu Nakashima for helping me and guiding me all the way through from the beginning of journey until the end. His insights and opinions have been significantly helped me navigating through this journey.

I would like to also thank my friends from the Laboratory of Phytopathology who have provided supports in all the times I have known them. Knowing them and spending time together is what makes life tolerable and something to look forward to.

Finally, I would like to thank myself for putting up with all the pressure, deadlines, and requirements needed along voyage to finally earn this master's degree. A lot has happened, and it helped me to grow better as a person. Here's to hope that everything I have done will be enough.

CONTENTS

Chapter 1

General Introduction

Background of the study

Oomycetes, commonly known as water moulds, are a group of devastating plant pathogens that are filamentous, have a motile stage, and reproduce sexually by producing oospores. Oomycetes are found in a wide variety of habitats, including freshwater, saltwater, soil, and plant tissues and pathogenic which can cause serious diseases in plants, fish, and humans.

However, oomycetes are not true fungi, but are instead classified in the kingdom *Chromista*. Some observed distinctions that differentiate oomycetes and true fungi apart are cell wall compositions and structure of reproduction (Fawke et al, 2015). Oomycetes cell wall contain cellulose in contrast with true fungi cell wall, which is made of chitin. Also, sporangia of oomycetes produced motile zoospores equipped with two types of flagella, namely whiplash and tinsel assisting in movement for dispersal (Thines & Kamoun, 2010) whereas true fungi disperse through various mechanisms such as wind assisted.

As provided by (Maneveldt & Keats, 2003) and WoRMS (2023), The Kingdom *Chromista*, a diverse group of organisms such as diatoms, brown algae, and water moulds (Cavalier-Smith, 2018) is house to the subkingdom *Harosa* in which Infrakingdom *Heterokonta*, a taxonomic group of organisms with ciliary hairs on their flagella (Cavalier-Smith, 2018) are placed under. Within the *Heterokonta*, the Phylum *Oomycota* resides, and Class *Peronosporea* belongs under the Phylum. The Subclass of *Peronosporidae* and *Saprolegniidae* are placed under the class and consist of Order *Peronosporales* and *Pythiales* respectively. In *Peronosporales*, the Family

Peronosporacea (de Bary, 1863) housed the Genus *Phytophthora*, which is known to consists multiple important plant pathogens species. Whereas for the Family *Pythiaceae* (J. Schrot, 1893), it is placed under *Pyhthiales* and where the Genus *Globisporangium* (Uzuhasi et al, 2010), *Phytopythium* (Abad et al, 2010), and *Pythium* (Pringsheim, 1858) located.

Oomycetes are a group of important plant pathogens that causes wide variety of diseases including downy mildew of grapevine, sudden oak death, root, and stem rot of soybean (Fry & Grünwald, 2010). The Irish Potato Famine in 19th century (Stevens, 1933) is caused by a member of the oomycetes, the infamous *Phytophthora infestans*. Due to infection, the blight caused potato to rot underground making it inedible leading to acute shortage of food supply for the Irish. Scarcity of potato lead to death of a million and migration of the Irish (Ristaino, 2002) to the America continent.

The oomycete group is house to many important causal agents of devastating diseases with wide host ranges and proves to be difficult to be contained and controlled. Infection from members of the group negatively impact the livelihood of crops economy and its value (Bebber & Gurr, 2015). Soil-borne oomycetes, such as *Phytopthora palmivora* in cropping sites of cacao plants are responsible in inflicting great profit losses upon infection (Rodriguez-Polanco, 2020; Ramirez et al, 2021). Such species thrive in tropical climate which are favoured for growth and dispersion.

However, the species diversity of oomycetes in cropping sites are poorly recorded which leads to the difficulty in early detection of diseases present on-site and trouble for proper planning of steps to control and overcome the problems. Hence, isolates collected from the cropping sites between the Philippines and Japan were identified to observe oomycetes species diversity based on modern taxonomical criteria.

Objectives and Outline of the Study

Throughout this research, isolates collected from cropping sites in the Philippines and Japan were analysed to establish an inventory of *Oomycota* from various ecological niches, saprophytes, and plant pathogens. Also, the phylogeny and morphologies of the hitherto pathogens will be revealed and described based on modern taxonomical criteria.

In Chapter 2, diversity of oomycetes associated with cropping sites of *Theobromae cacao* L. between the Philippines and Japan will be outlined through baiting method from soil samples collected with NARM selective medium. As for phylogenetic analyses, the rDNA Internal Transcribed Spacer (ITS) and Cytochrome c oxidase 1 (COX1) loci were analysed to draw the phylogenetic tree, which are known to be the species barcodes of *Oomycota*. As much as 24 isolates collected are identified as *Phytopythium cucurbitacearum*, *Pythium acanthophoron*, *P. nunn*, and *P. plurisporium*.

In Chapter 3, diversity of oomycetes across *Cannabis sativa* L. cropping sites in Japan will be summarized by baiting method from soil samples using leaves of *Quercus glauca* and *Morus Alba* and NARM selective medium. Isolates collected were identified according to the current taxonomical criteria such as multi-locus phylogenetic analysis of ITS and COX1 regions.

This analysis will reveal *Oomycota* species diversity in the soil of cropping farms between the Philippines and Japan based on current taxonomical criteria. An inventory of *Oomycota* will be established and phylogeny and morphology of the pathogens will be revealed and described.

References

Bebber, D. P., & Gurr, S. J. (2015). Crop-destroying fungal and oomycete pathogens challenge food security. *Fungal Genetics and Biology*, 74, 62-64.

Cavalier-Smith, T. (2018). Kingdom Chromista and its eight phyla: a new synthesis emphasising periplastid protein targeting, cytoskeletal and periplastid evolution, and ancient divergences. *Protoplasma*, 255, 297-357.

Fawke, S., Doumane, M., & Schornack, S. (2015). Oomycete interactions with plants: infection strategies and resistance principles. *Microbiology and Molecular Biology Reviews*, 79(3), 263-280.

Fry, W. E., & Grünwald, N. J. (2010). Introduction to oomycetes. *The Plant Health Instructor*.

Maneveldt, G., & Keats, D. (2003). The chromista. *Engineering in Life Sciences*.

Ramírez Martínez, J., Cárdenas Toquica, M., Guevara-Suarez, M., Duarte, D., Victorino Jimenez, L. D., Argüello Bernal, B. K., & Restrepo Restrepo, S. (2021). Oomycete species associated with *Theobroma cacao* crops in Colombia. *Plant Pathology*, 70(7), 1695-1707.

Ristaino, J. B. (2002). Tracking historic migrations of the Irish potato famine pathogen, *Phytophthora infestans*. *Microbes and infection*, 4(13), 1369-1377.

Rodríguez-Polanco, E., Morales, J. G., Muñoz-Agudelo, M., Segura, J. D., & Carrero, M. L. (2020). Morphological, molecular, and pathogenic characterization of *Phytophthora palmivora* isolates causing black pod rot of cacao in Colombia. *Spanish Journal of Agricultural Research*, 18(2), e1003-e1003.

Stevens, N. E. (1933). The dark ages in Plant Pathology in America: 1830—1870. *Journal of the Washington Academy of Sciences*, 23(9), 435-446.

Thines, M., & Kamoun, S. (2010). Oomycete–plant coevolution: recent advances and prospects. *Current opinion in plant biology*, 13(4), 427-433.

Uzuhashi, S., Kakishima, M., & Tojo, M. (2010). Phylogeny of the genus *Pythium* and description of new genera. *Mycoscience*, 51(5), 337-365.

WoRMS Editorial Board (2023). World Register of Marine Species. Available from https://www.marinespecies.org at VLIZ. Accessed 2023-07-20.

Chapter 2: Oomycetes associated with cacao cropping sites in the Philippines and Japan

Introduction

Theobromae cacao L., commonly known as cacao, is an important plant species that captured the hearts of chocolate lovers around the world. It belongs to the *Malvaceae* family and is native to the tropical regions of Central and South America (Diaz-Valderrama, 2020). In higher classification of cacao, it is further placed under the order *Malvales* which belongs to the class of *Magnoliopsida* (Kongor et al, 2016). Based on (de Souza et al, 2018), cacao tree originates from tropical American rainforest regions. There are three cultivars of cacao, namely Criollo, Forastero and Trinitario (Avila-Lovera et al, 2016). Criollo is the name used to differentiate cacao trees that originates from Venezuela (Lachenaud & Montamayor, 2017) where Forastero is used for outside cacao trees that are brought into Venezuela. Whereas for Trinitario, the cultivar is a hybrid between the Criollo and Forastero cultivars (Bekele et al, 2020; Motilal et al, 2010). Each cultivars have distinct qualities and tastes that according to (Elwers et al, 2009), phenolic compounds in each cultivars affect the brown colour of raw cocoa and chocolate hence influencing cacao bitterness and astringency, both as important factors regarding cacao flavours.

Throughout the years, cacao cultivation and trade have significantly impacted the economies of various nations, contributing to the global chocolate industry that delights millions of people today. Moreover, health benefits associated with consuming cacao have been discovered (Vega & Kwik-Uribe, 2012). As cocoa is a rich source of antioxidants, it can help to protect the body against damage caused by free radicals and improve blood flow and lower blood pressure (Latif, 2013). As cacao-based products are popularized since its discovery, (Toma & Saseanu, 2020) observed that all sizes and types of business organizations have entered the chocolate industry. According to data collected by International Cocoa Organization (ICCO) in the year 2023,

production of cocoa beans is projected to be 4.98 million kilograms comprised of Africa, America, Oceania, and Asia continents. Chocolates and other cocoa-based confectionaries are made from the raw material; cocoa beans as primary source to the multi-billion-dollar industries (Wickramasuriya & Dunwell, 2018) further stressed the impact of cacao-based industries.

Cacao, along with other perennial plants are susceptible to infections and diseases by pathogens such as oomycetes which negatively impact the livelihood of economies that revolve around it. Black pod rot caused by the oomycete, P*hytophthora* spp. (Adeniyi, 2019; Delgado-Ospina et al, 2021) is an important disease of cacao as it contributes to huge yield loss of cacao-related economies. (Acebo-Guerrero, 2012; Erwin & Ribeiro, 1996; Guest, 2007; Perrine-Walker, 2020) stated that in global scale, black pod rot of cacao inflicted 30% losses of pod and 10% losses of trees each year. Pathogenic agents infecting cacao plants will inhibit and/or stunt growth thus negatively impact the livelihood of harvest values of cacao. (Delgado-Ospina, 2021; Hebbar, 2007; Ploetz, 2016) listed several diseases with high impact to cacao plants, such as witches' broom and frosty pod rot by *Moniliophthora perniciosa* and *Moniliophthora* roreri respectively, Ceratocystis wilt of cocoa or "Mal de machete" caused by *Ceratosystis cacaofunesta*. Previously, it was proposed that *Phytophthora palmivora* was the sole causal agent of black pod rot. However, (Adeniyi, 2019) highlighted that more than one *Phytophthora* species, namely *Phytophthora megakarya* and *Phytophthora capsisci* are identified to be part of the complex causing black pod rot disease. *Pyhtophthora citrophthora* is also listed as one of the causal agents of black pod rot disease by (Rodríguez-Polanco, 2020).

To mitigate the impact of infection and diseases on cacao productions, necessary steps should be taken immediately. One of the first approaches as recommended by (Adeniyi, 2019), cultural control practice should prioritize in promoting crop growth to inhibit and obstruct the growth of pathogen. Integrated Pest Management (IPM) as reported by (Hebbar, 2007) in countries

that directly related to the productions of cocoa or indirectly related have took place for years by authorities to curb the problems posed by pathogens causes diseases. (Nieves-Orduña et al, 2023) listed several findings that suppress several diseases incidence such as black pod rot, witches' broom, and frosty pod with success. (Guest, 2007) had lay out extensive research carried out to control disease from surfacing using biological control. (Acebo Guerrero et al, 2012) had listed several organisms as biocontrol agents from research in combating black pod rot disease.

Addressing the source of infection in cacao plants is crucial to control disease severity in cacao cropping sites. However, oomycetes species diversity associated with cacao cropping sites are poorly recorded. Hence, in this chapter, an inventory of oomycetes associated with cacao cropping sites will be listed and the phylogeny and morphologies of the hitherto pathogens will be addressed based on modern taxonomical criteria.

Materials and Methods

Collection of oomycetes isolates

In June 2022, soil samples subjected to baiting method were collected from a cacao farm in Pantabangan, Nueva Ecija, the Philippines. Baiting method was carried out using perilla seeds and NARM selective medium. In 18 ml of sterilized water, two g of soil is added and shaken gently. Five pieces of sterilized perilla seeds are then added into the mixture and incubated for 24 hours at room temperature. After incubation, seeds are then collected and transferred onto Corn Meal Agar (CMA) amended with Nystatin, Ampicillin, Rifampicin, and Miconazole (NARM) selective medium and further incubated in dark at 30˚C for 24 hours. Mycelia observed after incubation period is then transferred onto Potato Dextrose Agar (PDA) and incubated at 20˚C-25˚C for culture purification.

In February 2023, soil samples were obtained from a cacao cropping site in Mie prefecture, Japan. Soil samples are subjected to baiting method following (Pérez-Sierra et al, 2022) recommendations with modifications. Young, healthy leaves of *Quercus glauca* and *Morus alba* are used for baiting method. 200-500 g of soils are submerged in 700-1000 ml of water collected from a well. Then, collected leaves are blanched in boiling water for 45-60 seconds and then dried using paper towel to remove excess water. The leaves are then floated in the soil water mixture and left incubated for seven days at 20˚C-25˚C. After incubation, leaves are carefully removed and washed briefly with distilled water. Leaves are then cut into two to five mm smaller pieces and placed onto NARM selective medium. Next, plated pieces are incubated in dark for 48 hours at 20˚C-25˚C. After two days, observed mycelia is transferred onto PDA and incubated at 20˚C-25˚C for culture purification.

From Mie University Culture Collection (MUCC), two isolates MUCC3486 and MUCC3487, namely *Phytophtora palmivora* are also included in the study.

Molecular and Phylogenetic analyses

Using DNeasy Ultra Clean Microbial Kit (Qiagen, Germany) following manufacturer's protocol, genomic DNA of isolates grown on PDA were extracted from the aerial mycelia. Targeted sequences of Internal Transcribed Spacer (ITS) and Cytochrome c oxidase subunit I (COX1) loci were amplified with in a thermal cycler (Bio-rad T100, Tokyo, Japan). The PCR conditions and primer sets for each locus are presented in Table 1.

The PCR mixture for ITS with a total volume of 12.5 µL was made up of 1 µL DNA, 8.2 µL double distilled H_2O, 0.5 mM $MgCl_2$, 1.25 µL 10x NH4 Reaction Buffer (Bioline, London, UK), 0.25 µM dNTPs (Bioline), 0.5 µM of each primer, 0.2 µL Bovine Serum Albumin (BSA) solution (Wako, Japan) and 0.1 U of Taq DNA polymerase (Bioline).

Also, The PCR mixture for COX1 with a total volume of 12.5 µL was made up of 1 µL DNA, 6.35 µL double distilled H_2O, 0.5 mM $MgCl_2$, 1.25 µL 10x NH4 Reaction Buffer (Bioline, London, UK), 2.5 µM dNTPs (Bioline), 0.3 µM of each primer, 0.2 µL Bovine Serum Albumin (BSA) solution (Wako, Japan) and 0.1 U of Taq DNA polymerase (Bioline).

Amplicons are then sequenced in both directions using the respective PCR primers and Big Dye Terminator v3.1 Cycle Sequencing (Applied Biosystems, USA) on an Applied Biosystems 3730xl DNA analyser installed in Mie University Advanced Science Research Promotion Centre (Mie, Japan). Sequences are then assembled and aligned using MEGA11: Molecular Evolutionary Genetics Analysis version 11 (Tamura, Stecher, and Kumar 2021).

Sequenced DNAs are then analysed with verified reference sequences in GenBank database using Nucleotide Basic Local Alignment Search Tool (Nucleotide BLAST) queries of National Centre for Biotechnology Institute (NCBI) (http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi). Sequences identified as oomycetes through searches are then aligned with matrices of ITS and COX1 loci for oomycetes provided by (Robideau et al, 2011) in TreeBASE study S11552.

To determine phylogenetic relationships of the sequences, Maximum likelihood (ML) analysis is performed using RAxML-NG software (Kozlov et al, 2019). The best-fit substitution model for ITS marker is GTR+I+G4 whereas for COX1 marker is TIM3+I+G4 determined based on Akaike Information Criterion (AIC) computed by ModelTest-NG (Darriba et al, 2019). The internal branches' strength of resultant trees is tested by bootstrap analysis (BS) (Felsenstein, 1985) using 100 replications.

Locus	Primer F	Primer R	PCR conditions
ITS	ITS5	ITS4	95˚C 4 mins
	GGA AGT AAA AGT CGT AACA	TCC TCC GCT TAT TGA TATG	95˚C 40 secs
	(White et al, 1990)	(White et al, 1990)	55˚C 40 secs (37 cycle)
			72˚C 1 min
			72˚C 5 mins
COX1	OomCox1-levup	OomCox1-levlo	95˚C 4 mins
	TCA WCW MGA TGG CTT TTT TCA AC	CYT CHG GRT GWC CRA AAA ACC AAA	95˚C 40 secs
			56˚C 40 secs (40 cycle)
	(Robideau et al, 2011)	(Robideau et al, 2011)	72˚C 1 min
			72˚C 5 mins

Table 1: Primer sets and PCR conditions.

Results

Diversity of oomycetes isolates from the Philippines and Japan

In ITS data matrix, there are 259 sequences with a total character of 1805 whereas for COX1 data matrix, there are 254 sequences with 677 characters including alignment gaps. Maximum Likelihood (ML) trees of ITS (Fig. 1) and COX1 (Fig. 2) data matrices are generated using the software, RAxML-NG. As much as 24 oomycetes isolates from the Philippines and five isolates from Japan are amplified for ITS and COX1 regions with around 1050 base pairs (bp) and 700 bp respectively. Outgroup for the generated ML trees is (FI373) *Eurychasma dicksonii*, based on (Robideau et al, 2011). Among the isolates from the Philippines, 21 isolates are in the same clade with (CBS74896) *Phytopythium cucurbitacaerum*, one isolate with (CBS33729) *Pythium acanthophoron*, one isolate with (CBS80896) *Pythium nunn*, and one isolate with (CBS100530) *Pyhtium plurisporium*.

As for isolates collected in Japan, all five isolates are in the same clade with (CBS33729) *Pythium acanthophoron*.

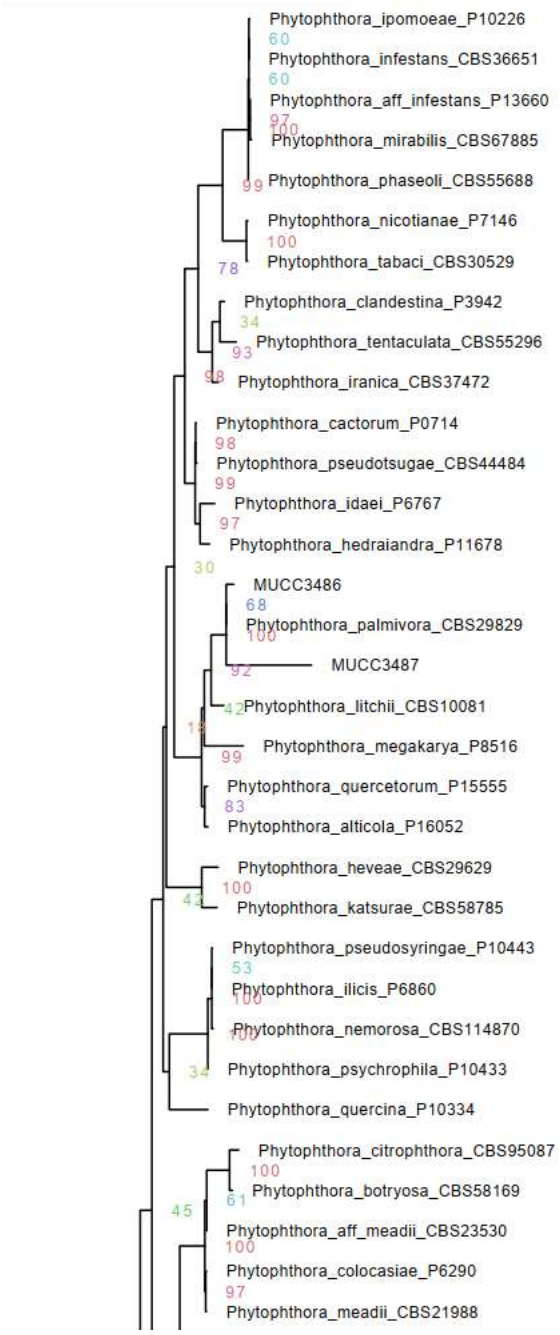


Figure 1: Phylogenetic tree of Maximum Likelihood (ML) analysis of ITS region for the isolates collected from the Philippines and Japan using data matrix provided by (Robideau et al, 2011). Bootstrap values are calculated at 100 replicates. “PH” encodes for isolates from the Philippines whereas “JP” encodes isolates from Japan.

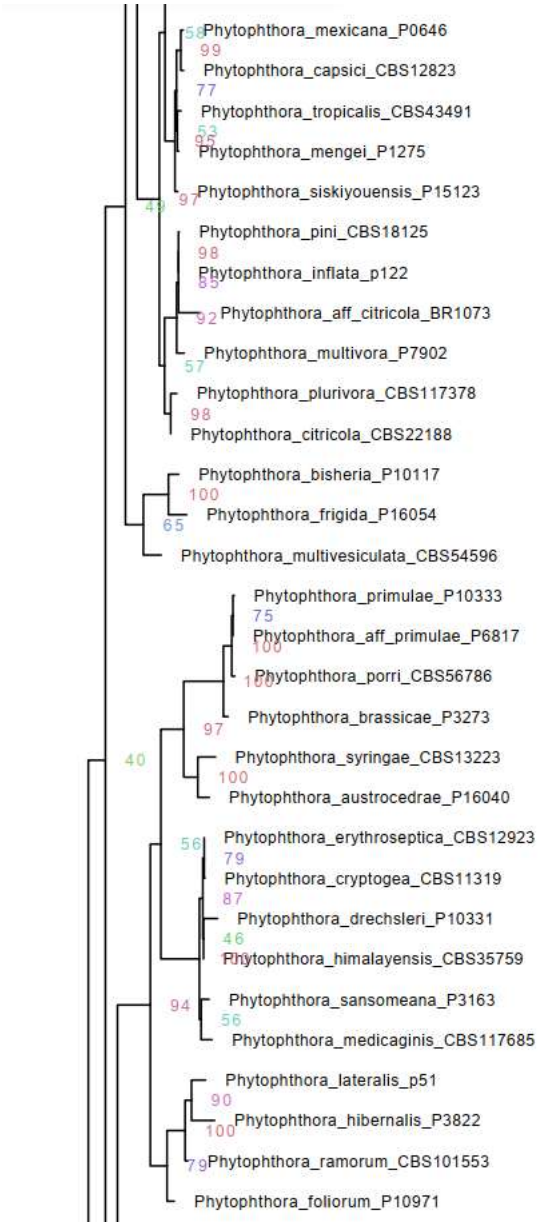


Figure 1 (continued)

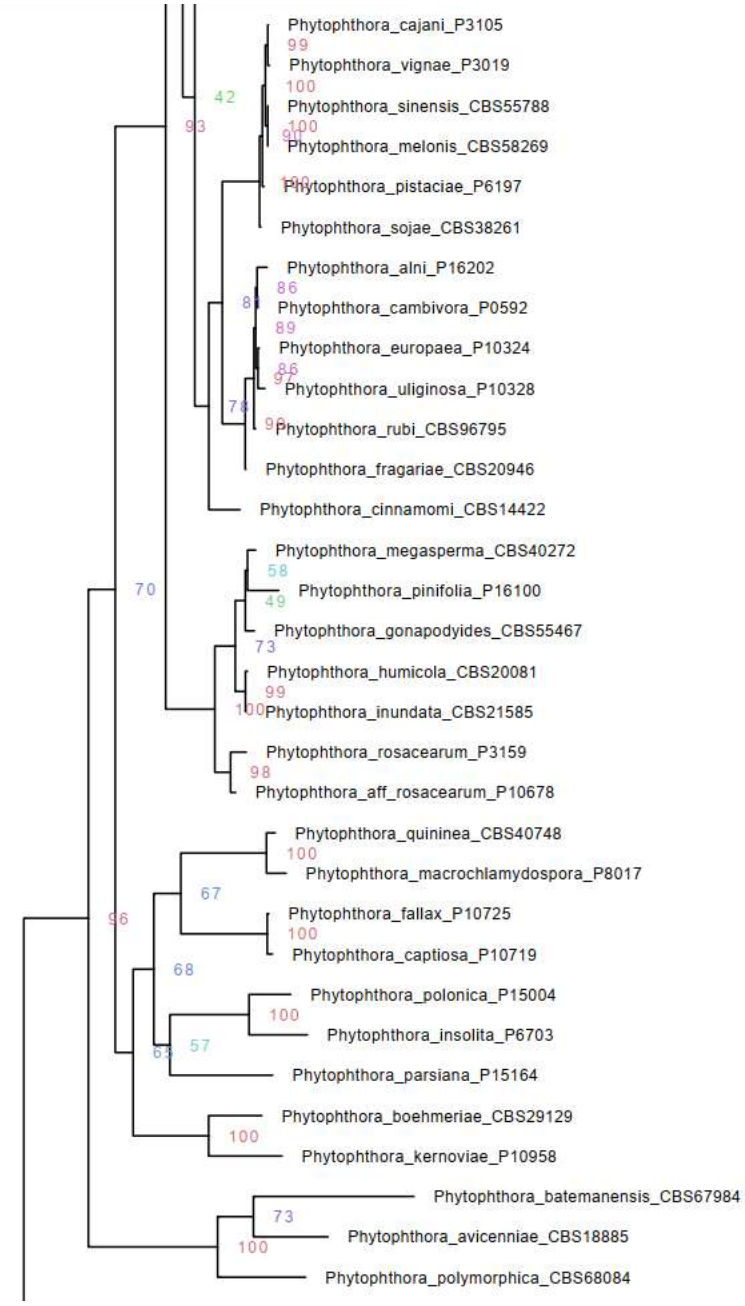


Figure 1 (continued)

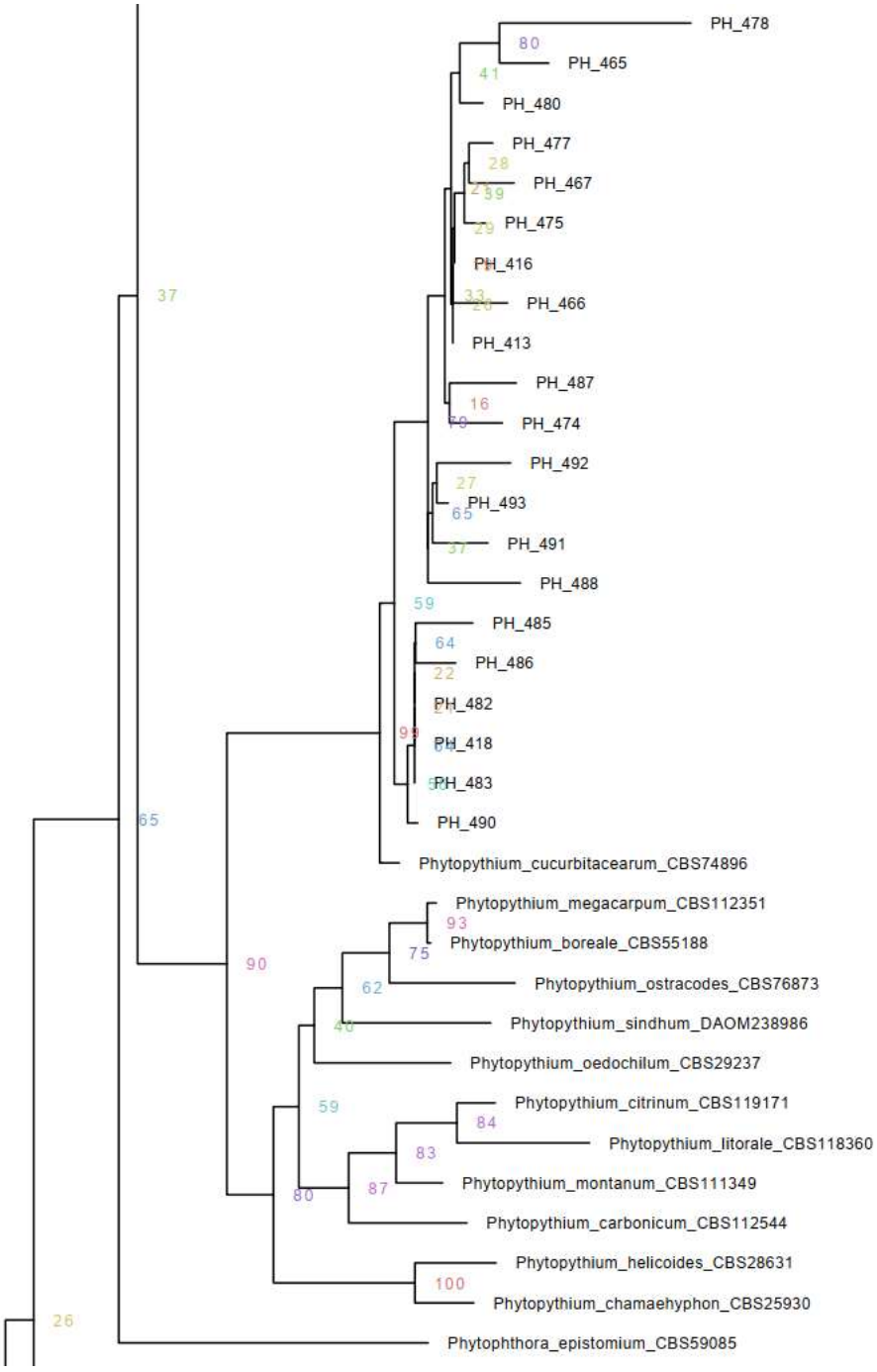


Figure 1 (continued)

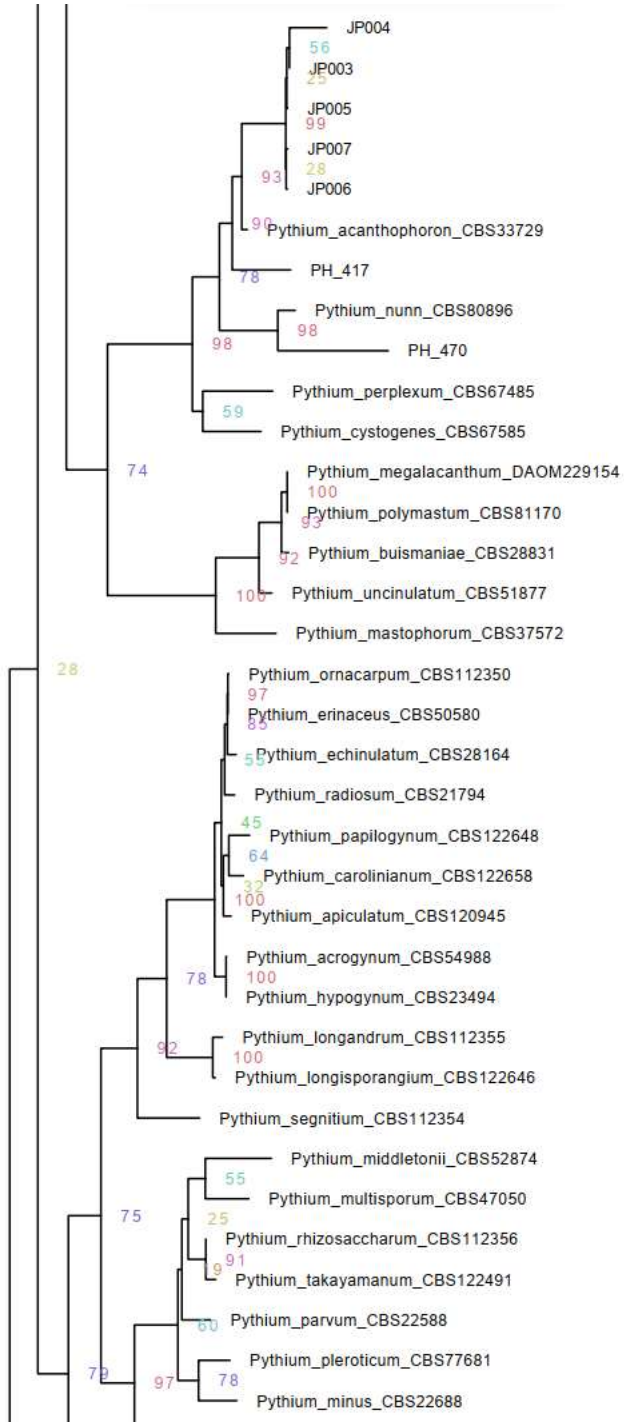


Figure 1 (continued)

Figure 1 (continued)

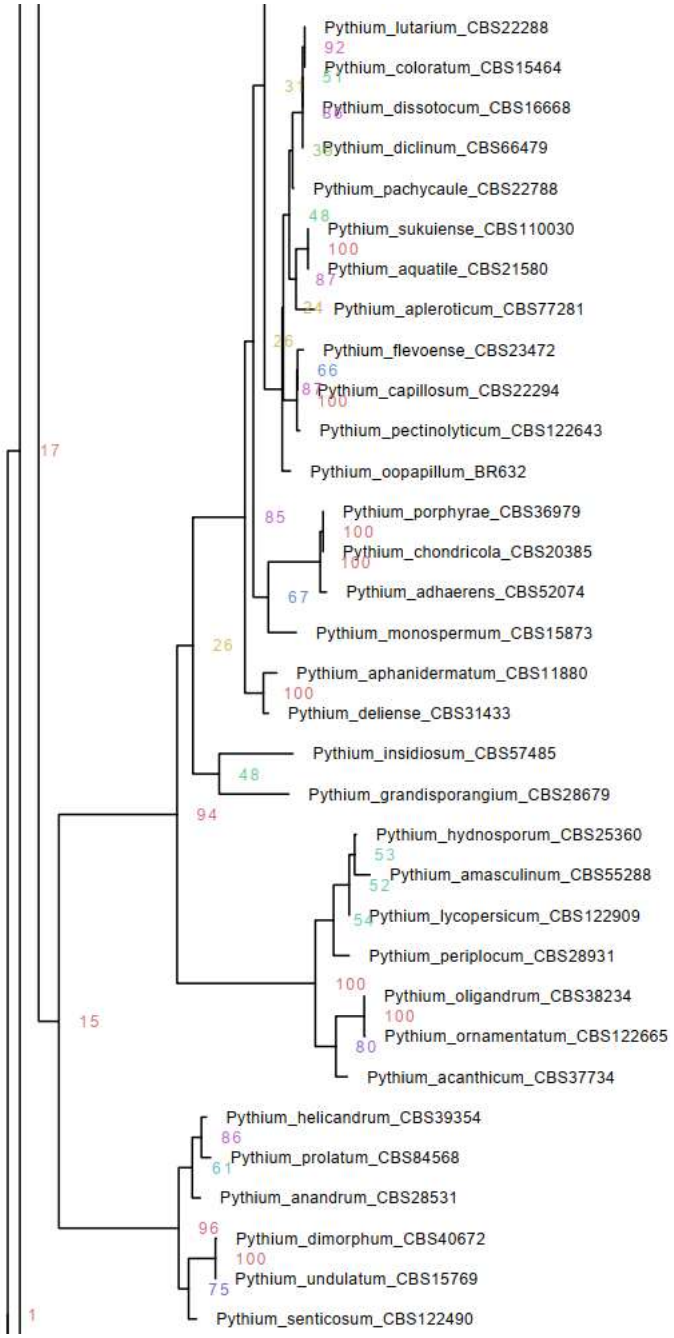


Figure 1 (continued)

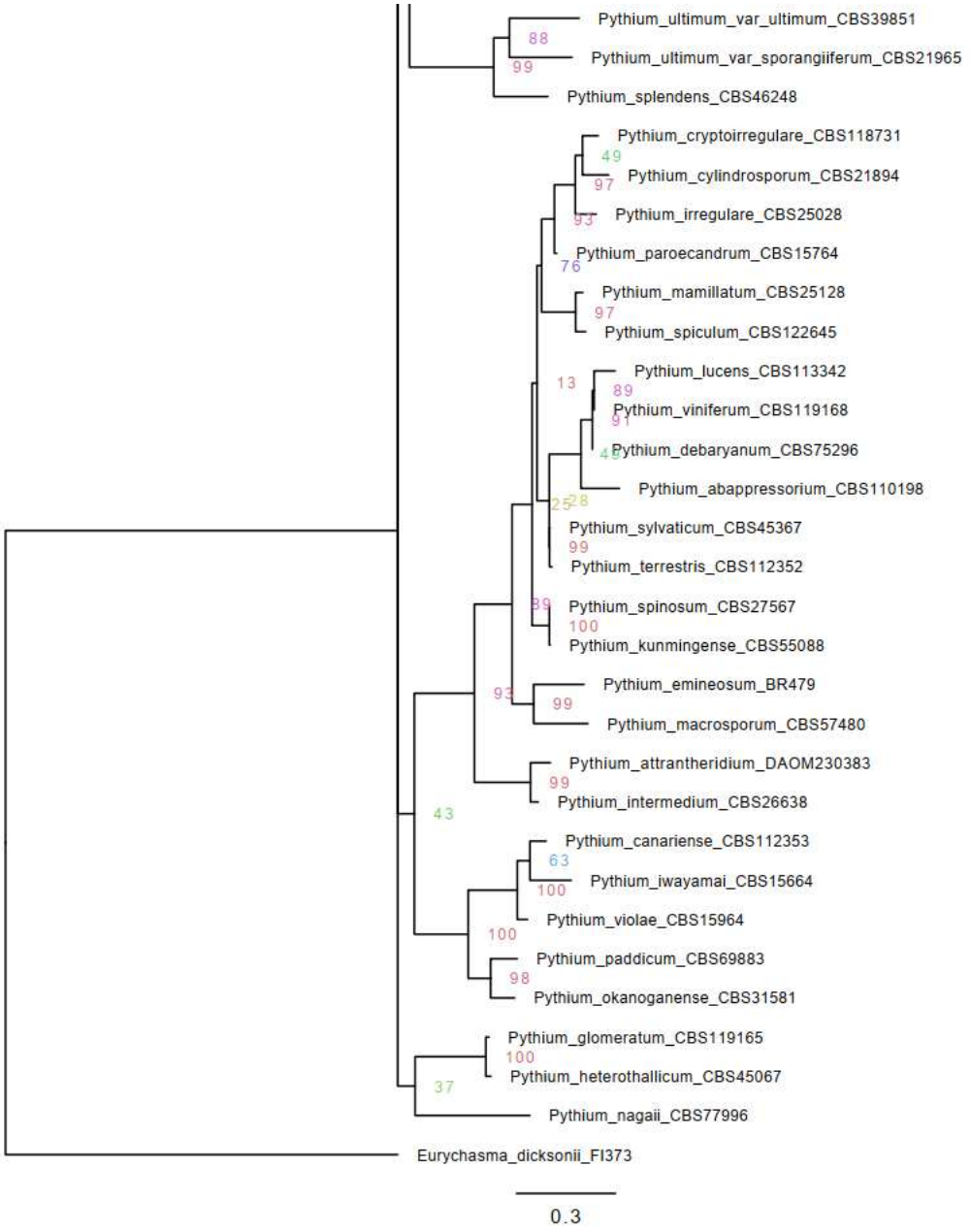


Figure 1 (continued)

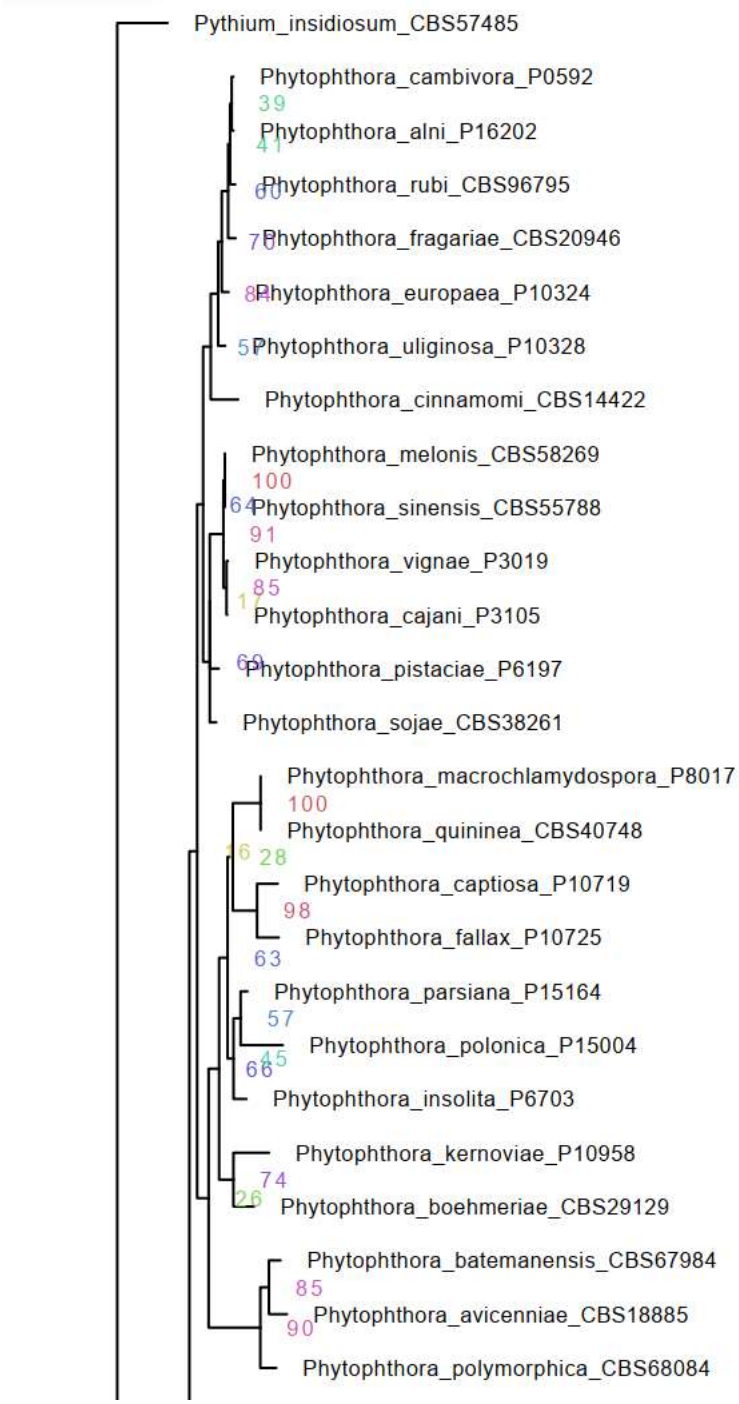


Figure 2: Phylogenetic tree of Maximum Likelihood (ML) analysis of COX1 region for the isolates collected from the Philippines and Japan using data matrix provided by (Robideau et al, 2011). Bootstrap values are calculated at 100 replicates. “PH” encodes for isolates from the Philippines.

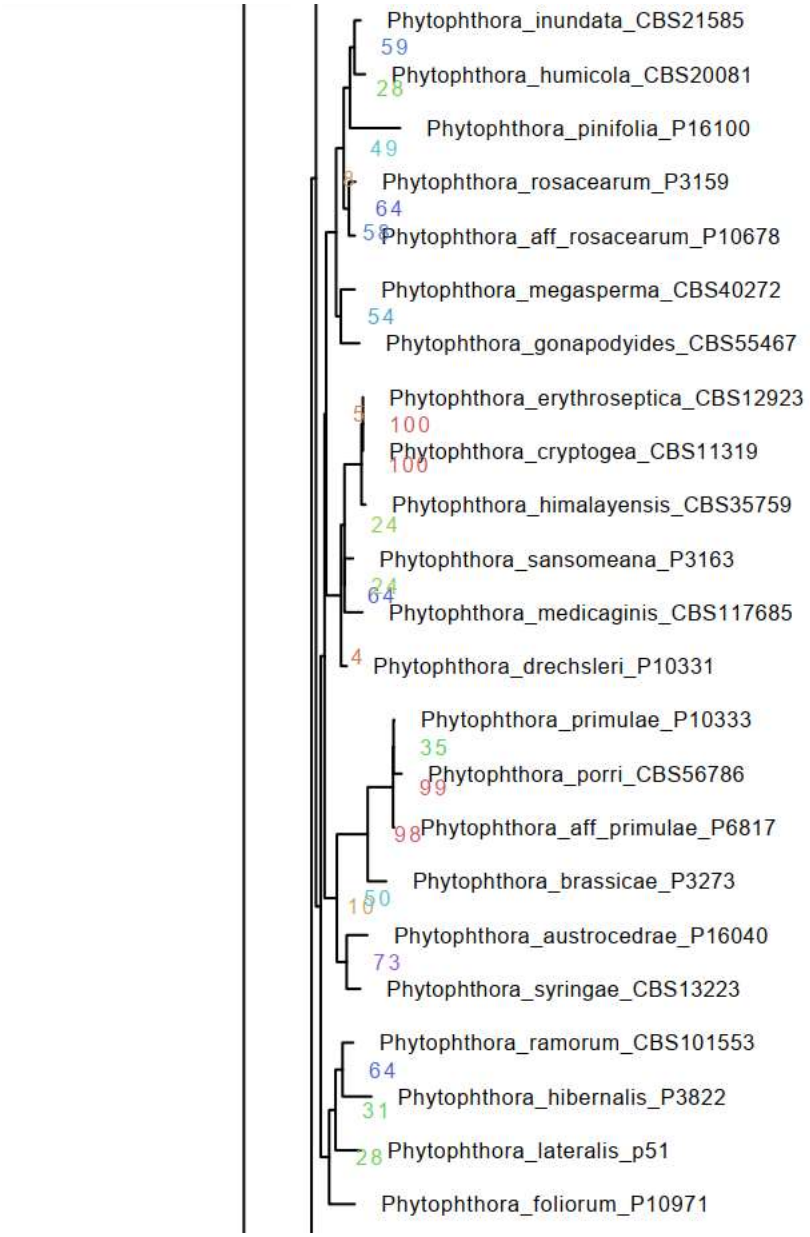


Figure 2 (continued)

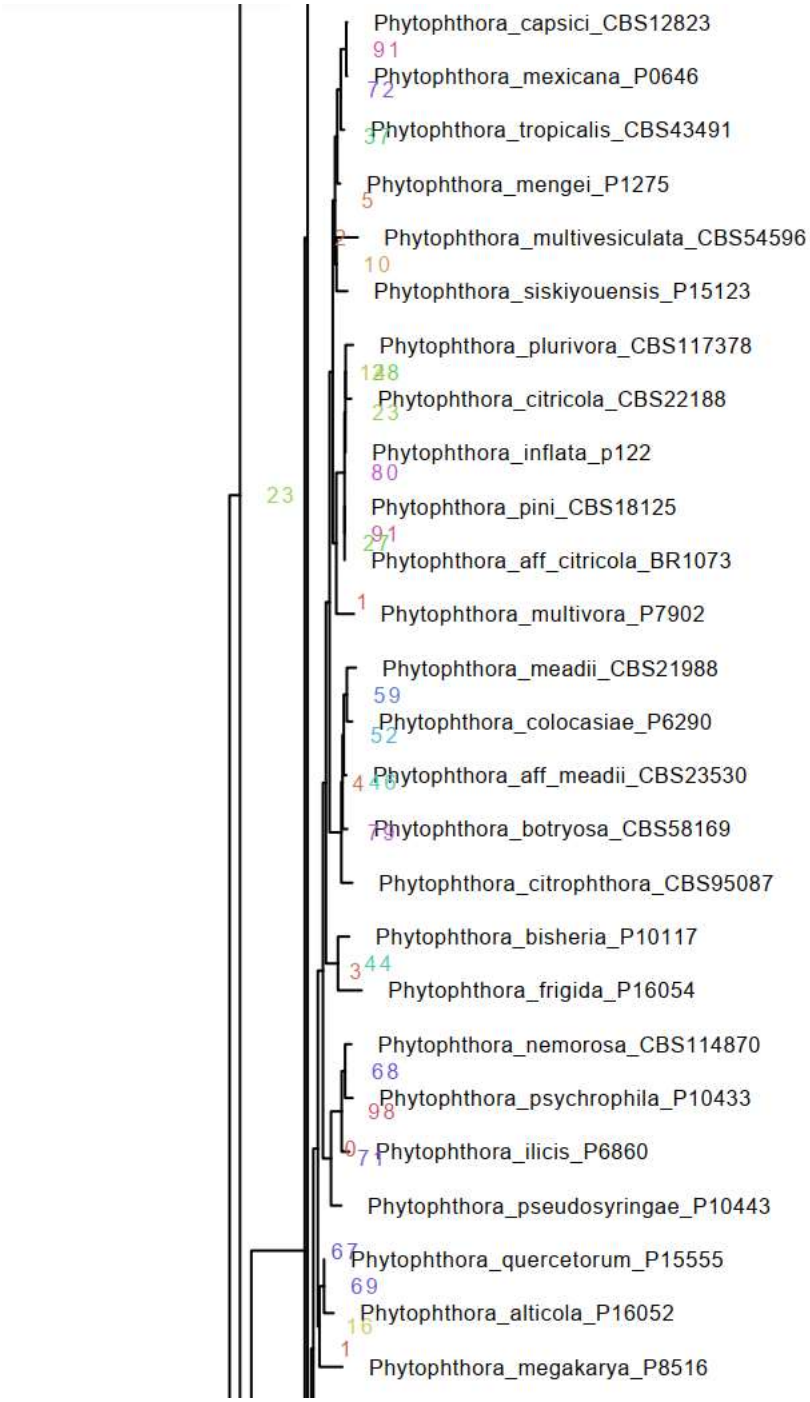


Figure 2 (continued)

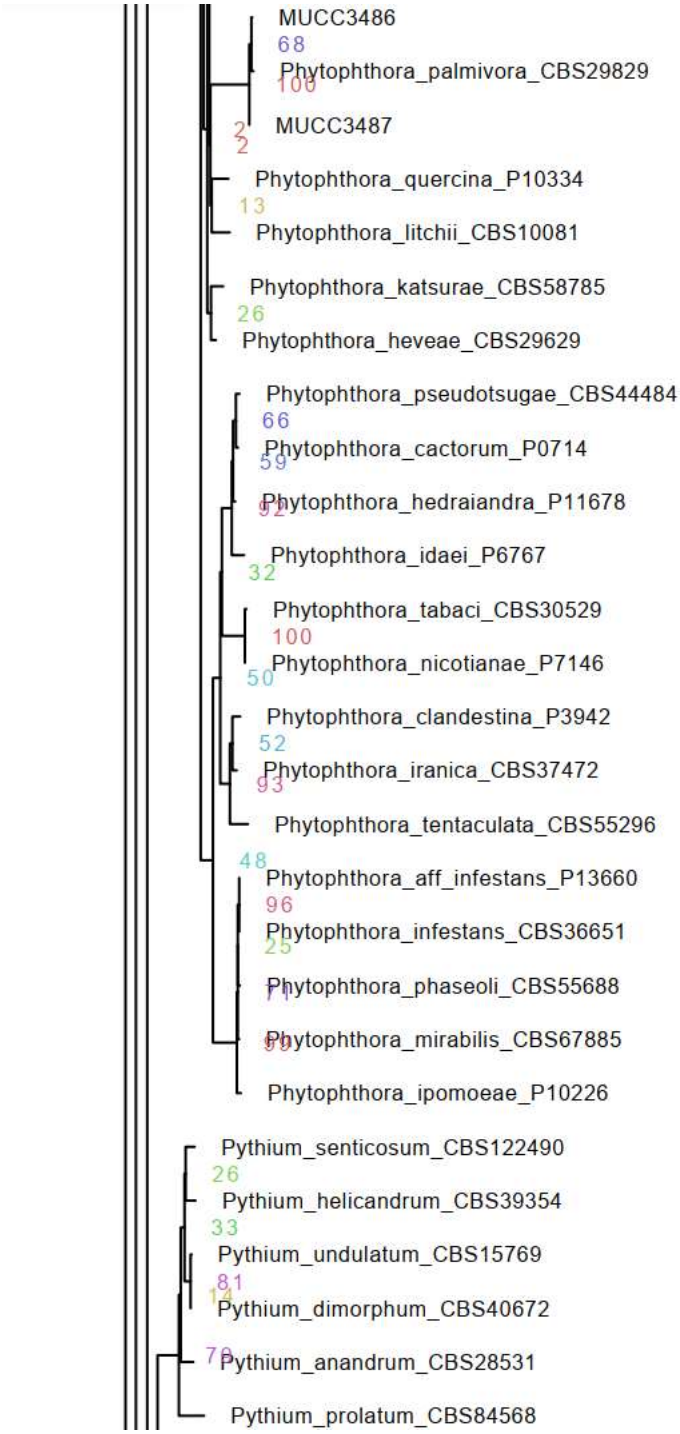


Figure 2 (continued)

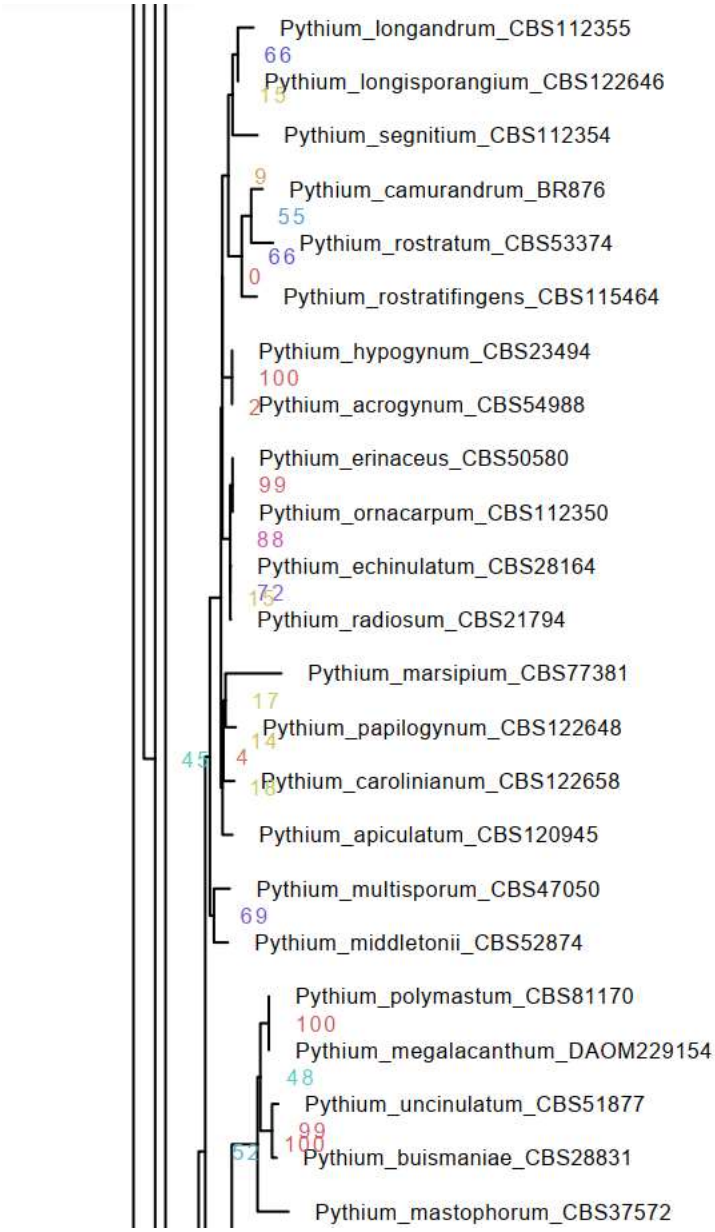


Figure 2 (continued)

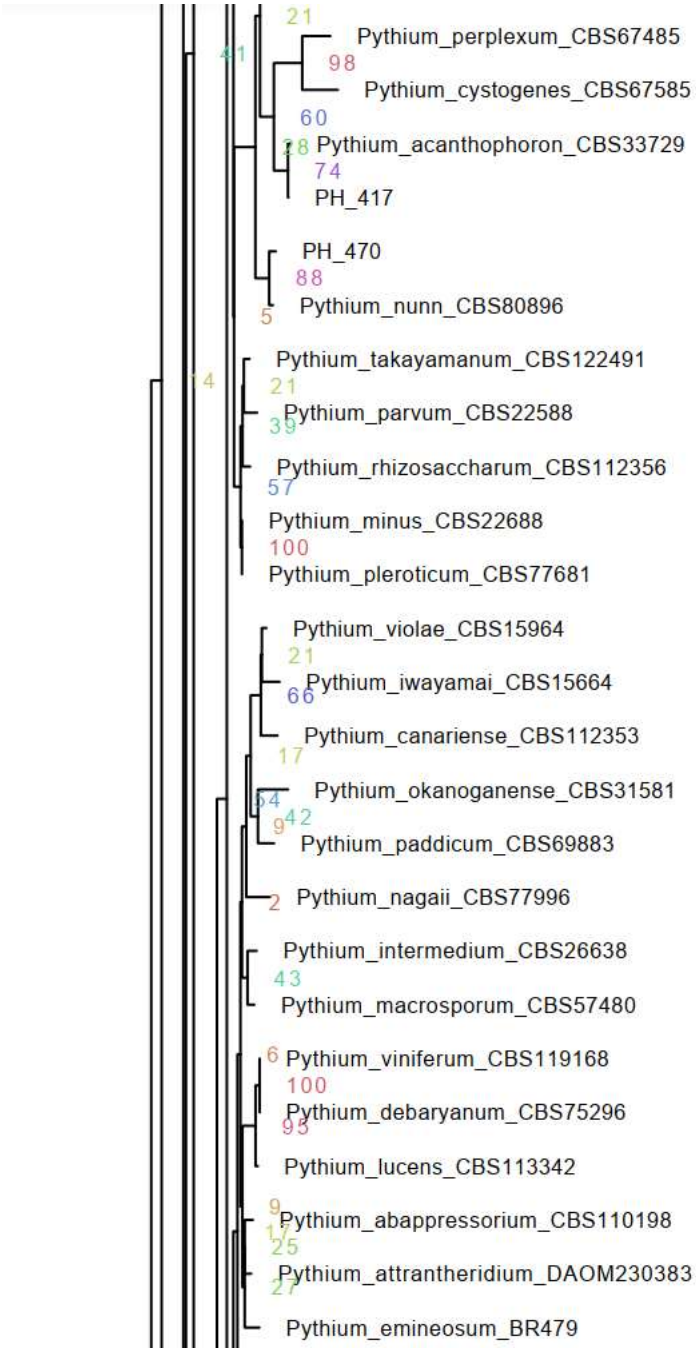


Figure 2 (continued)

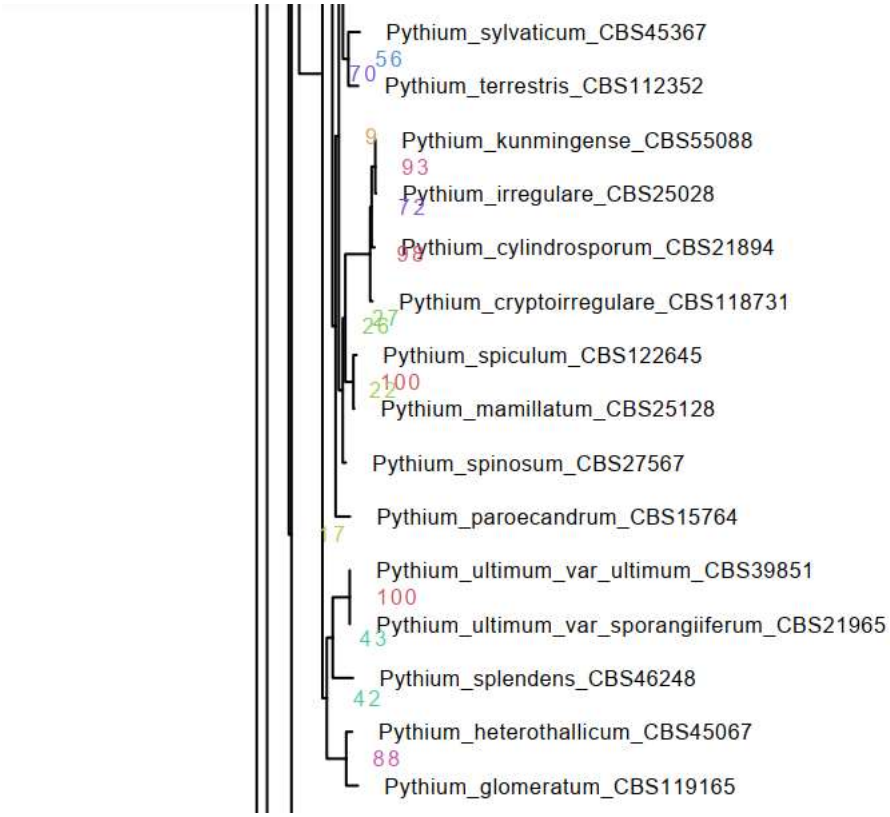


Figure 2 (continued)

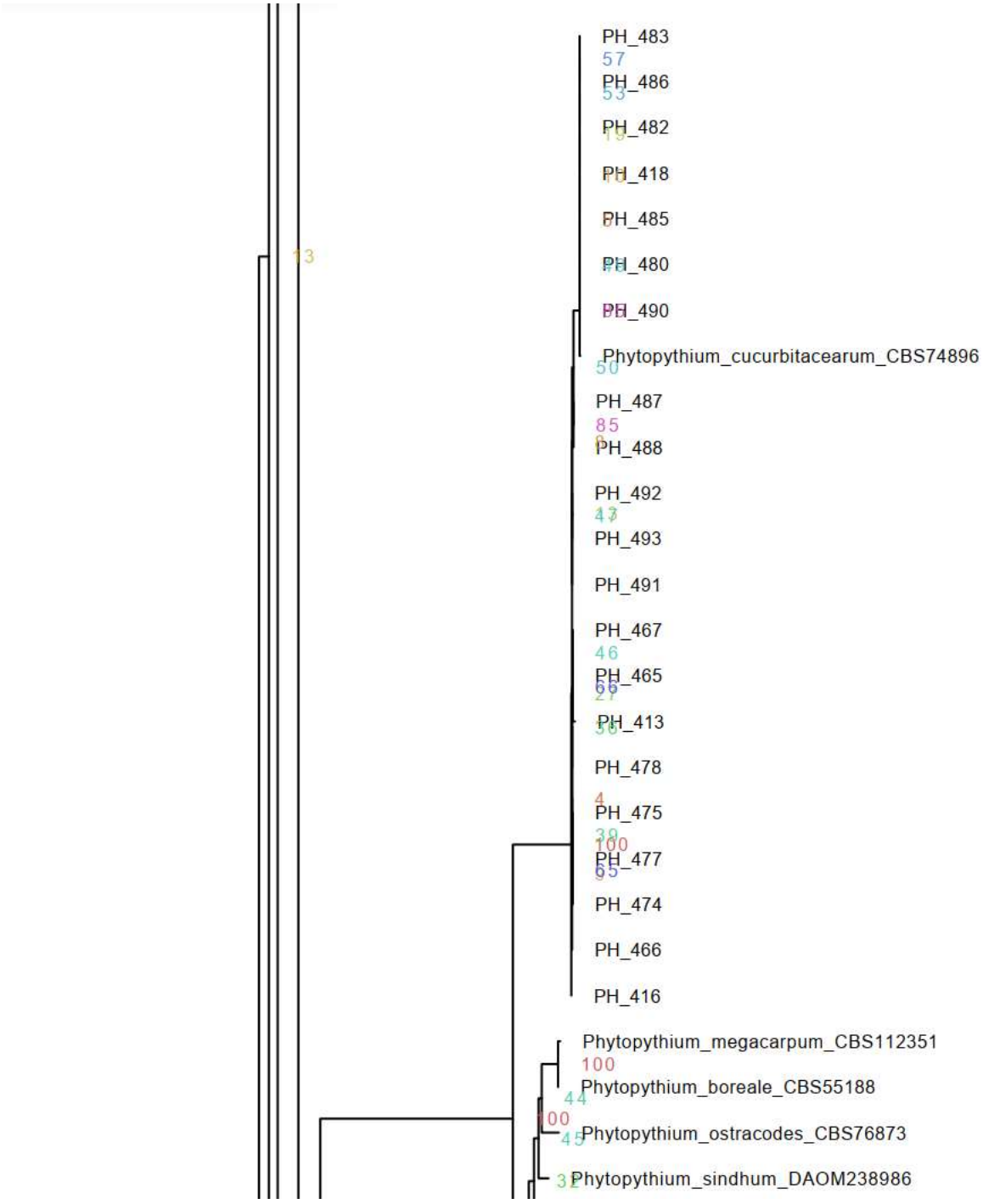


Figure 2 (continued)

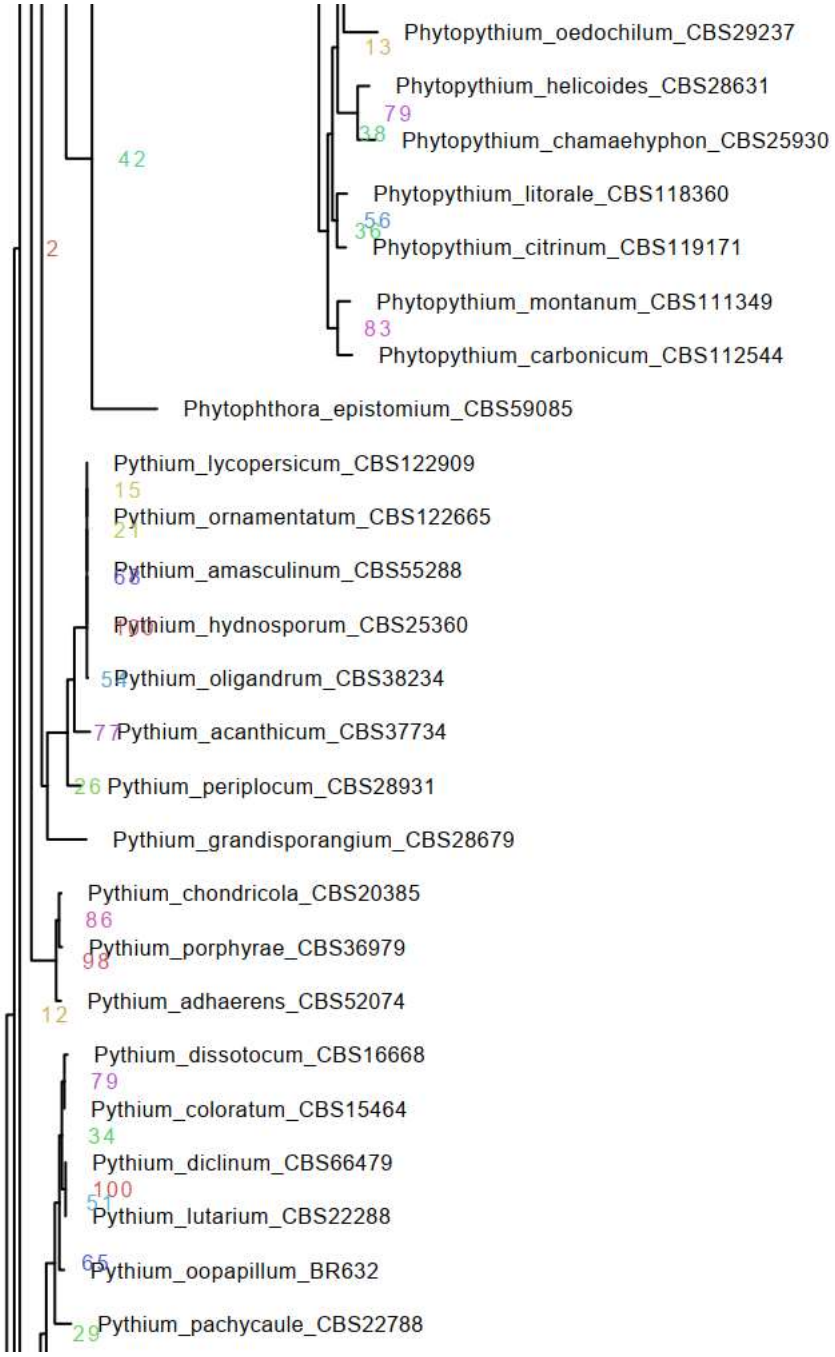


Figure 2 (continued)

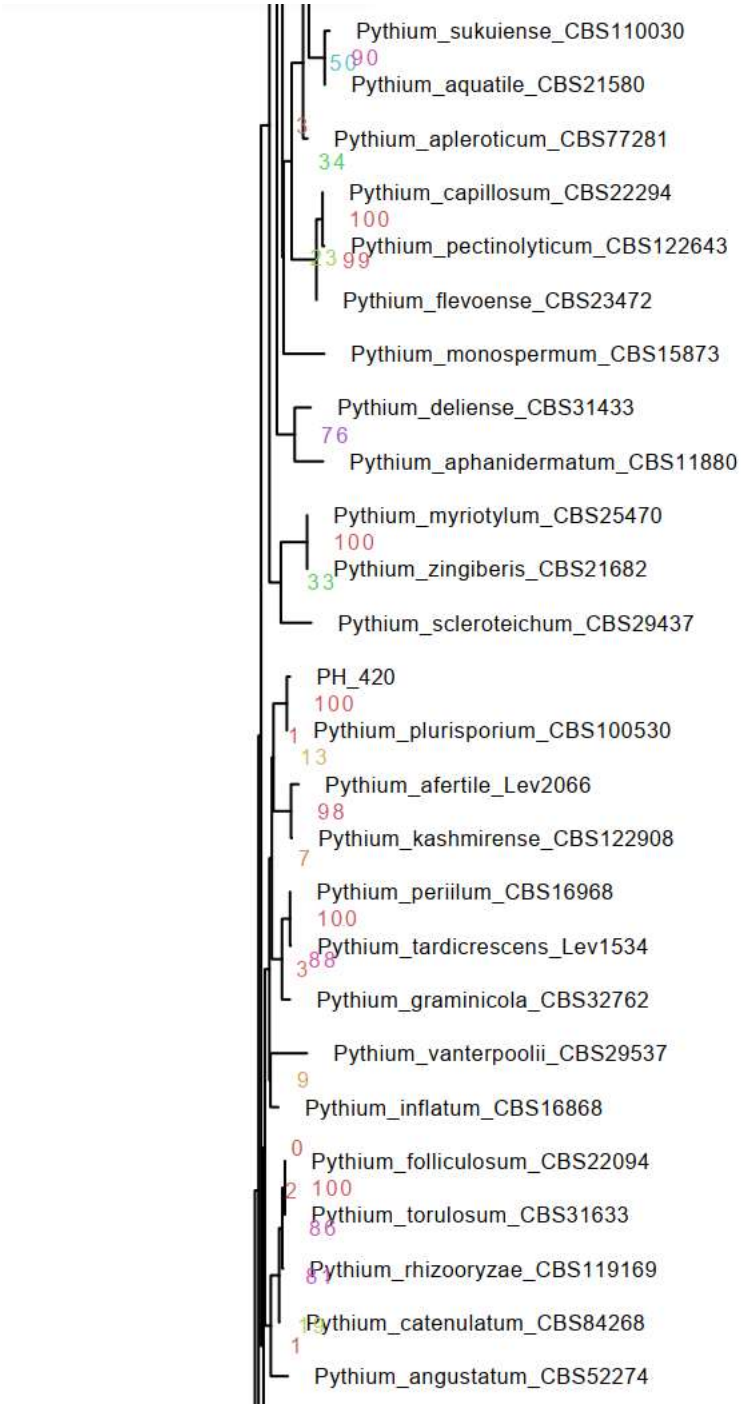


Figure 2 (continued)

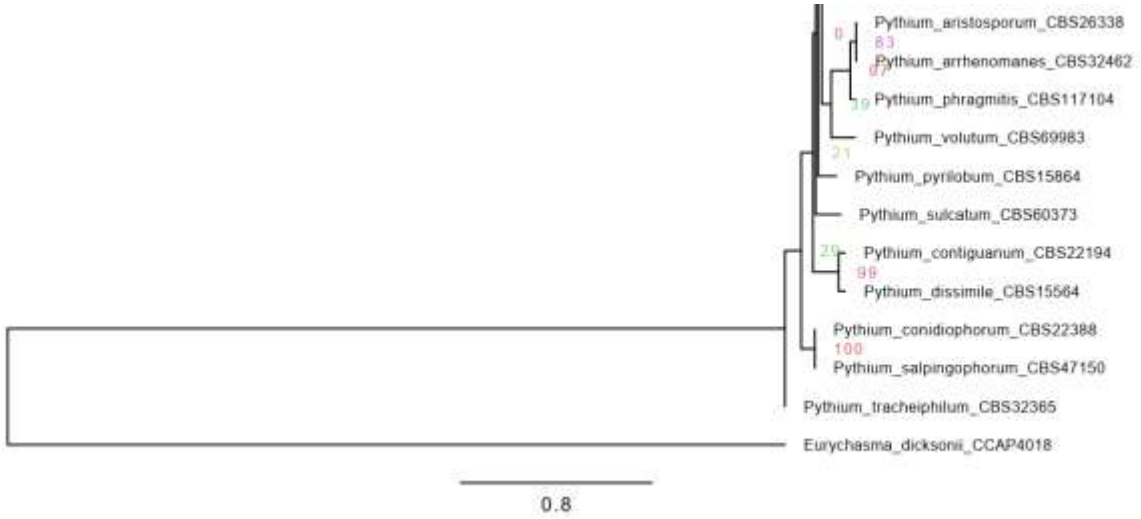


Figure 2 (continued)

Isolates collected from the Philippines

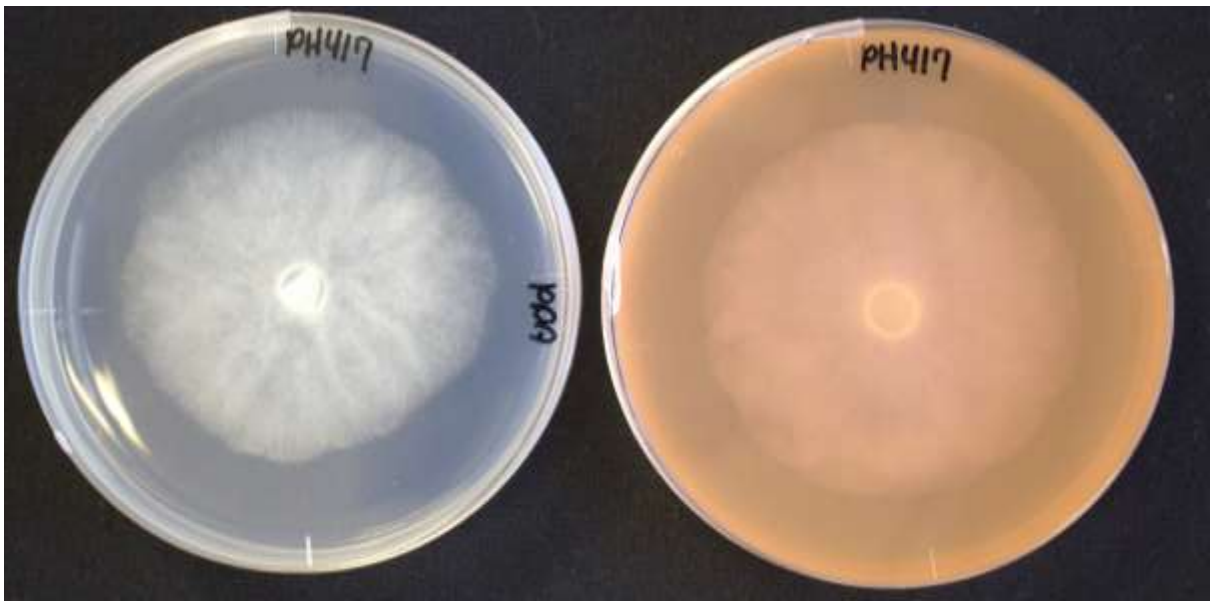


Figure 3: Left to right, isolate PH417 grown on Potato Dextrose Agar (PDA) and Carrot Agar (CA). Photos are taken two days after incubation.

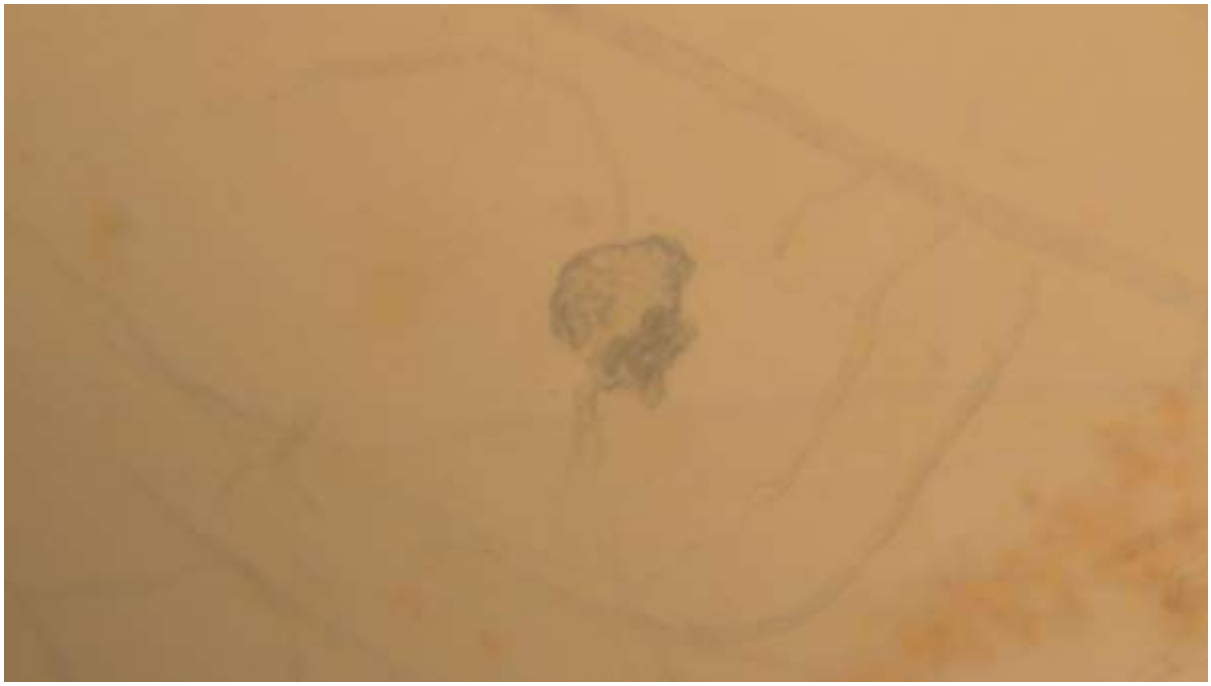


Figure 3a: Structure observed in PH417 grown on CA.

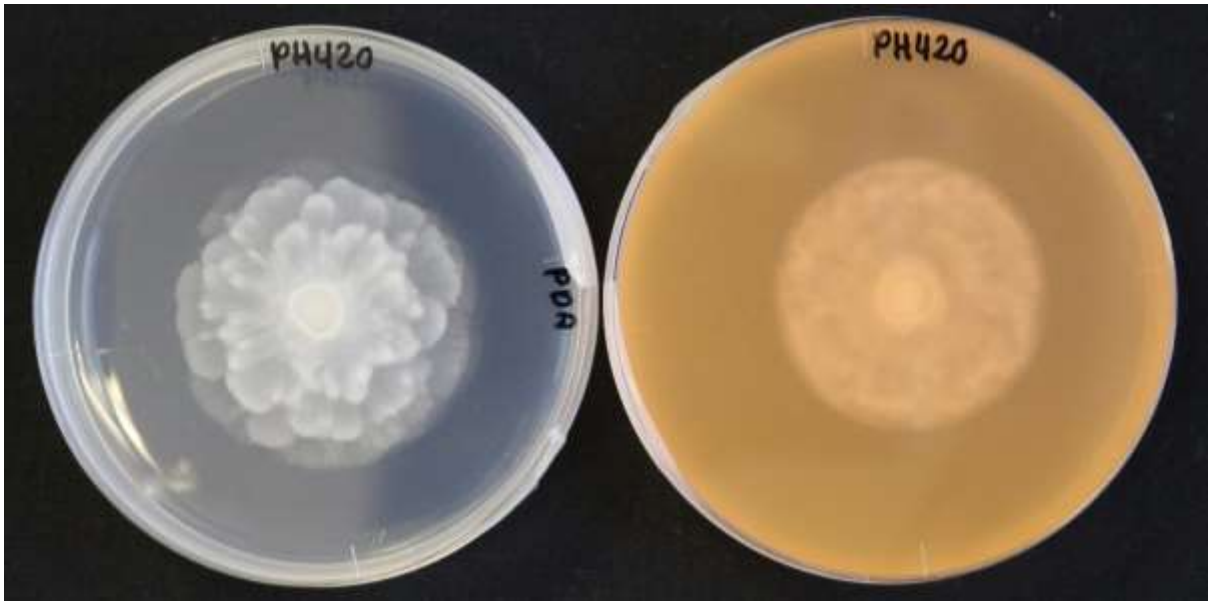


Figure 4: Left to right, isolate PH420 grown on Potato Dextrose Agar (PDA) and Carrot Agar (CA). Photos are taken two days after incubation.

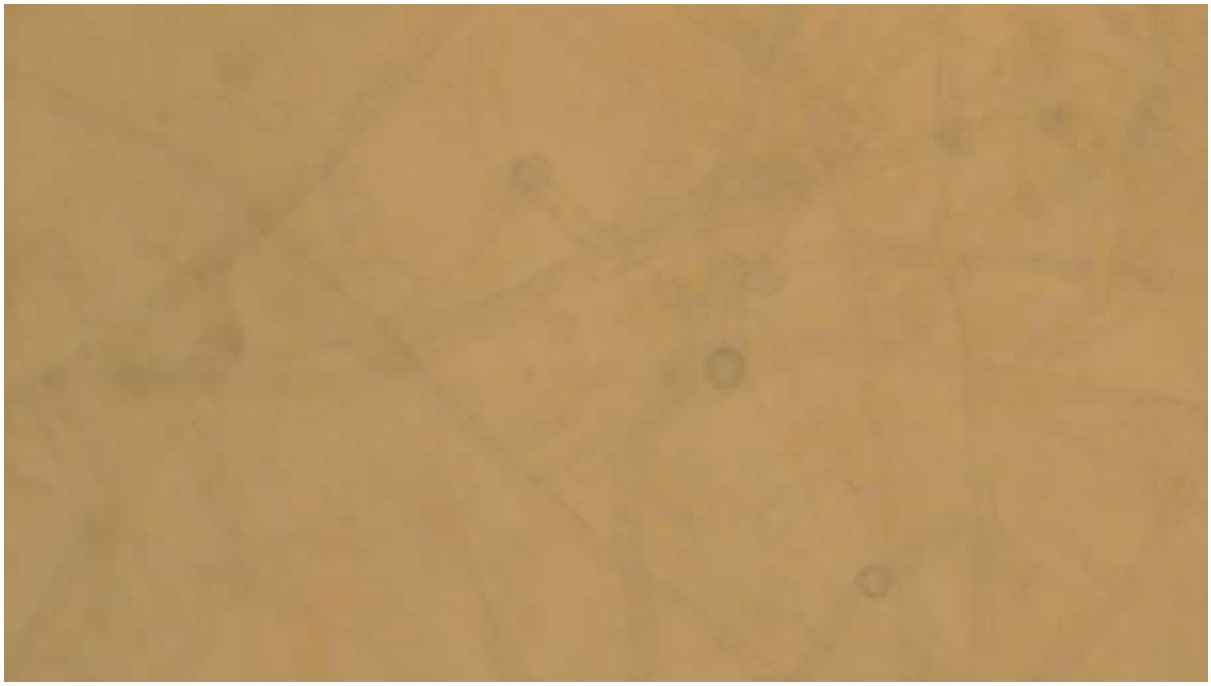


Figure 4a: Structure observed in PH420 grown on CA.

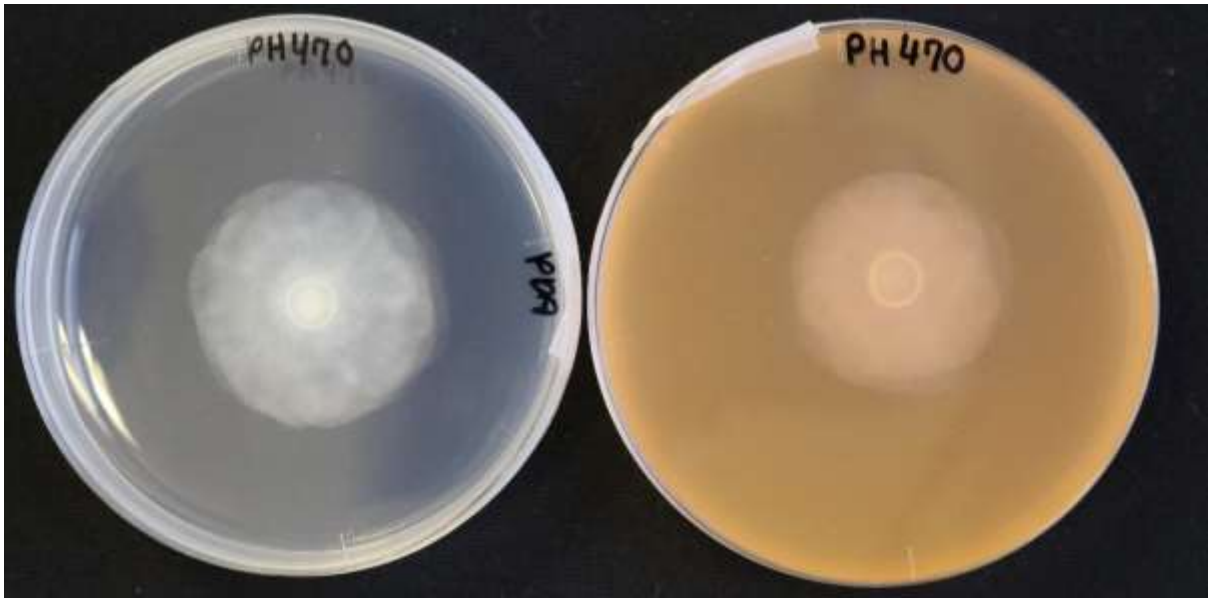


Figure 5: Left to right, isolate PH470 grown on Potato Dextrose Agar (PDA) and Carrot Agar (CA). Photos are taken two days after incubation.

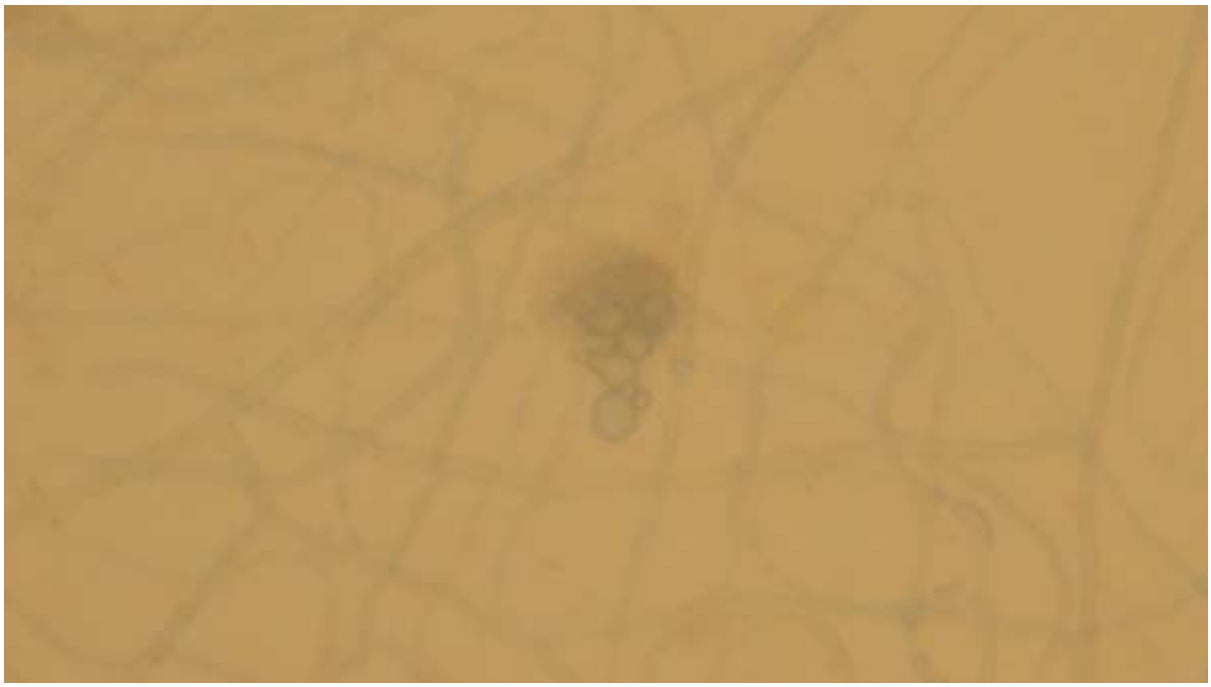


Figure 5a: Structure observed in PH470 grown on CA.

Figure 6: Left to right, isolate PH413, PH483 and PH493 grown on Potato Dextrose Agar (PDA) and Carrot Agar (CA). Photos are taken two days after incubation.

These three isolates are chosen as representative for isolates that clade with (CBS74896) *Phytopythium cucurbitacaerum*.

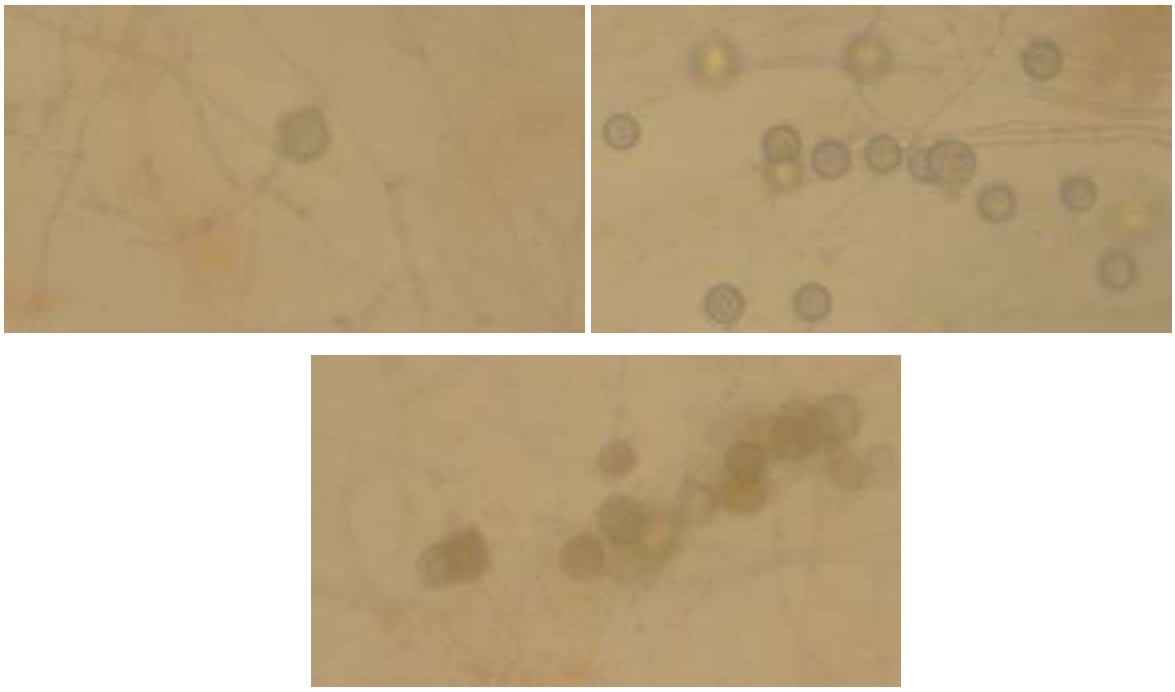


Figure 6a: Structures observed in PH413 (left-top), PH483 (right-top), and PH493 (bottom) grown on CA.

Isolates collected from Japan

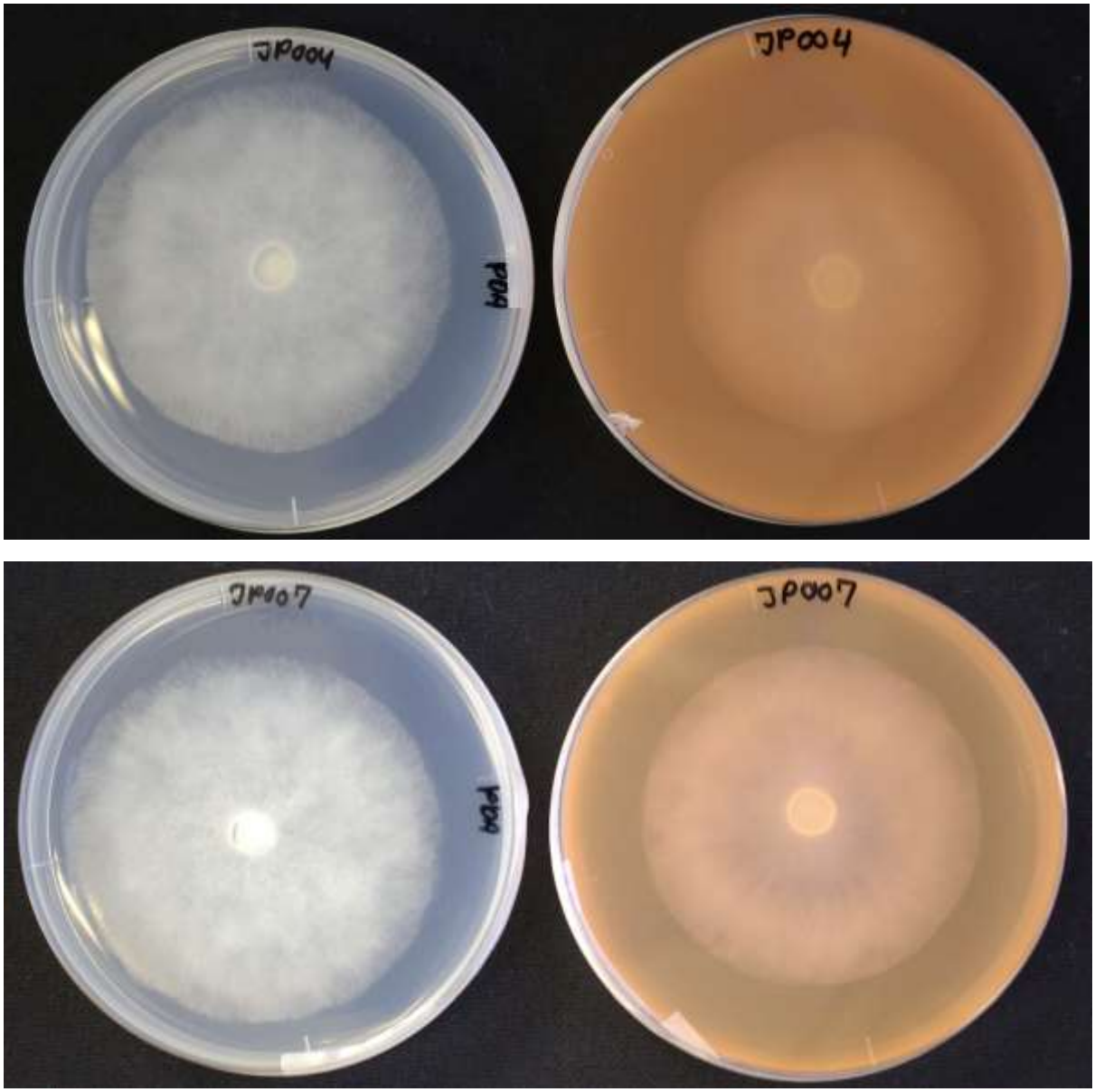


Figure 7: Left to right, isolate JP004 (Top) and isolate JP007 (Bottom), grown on Potato Dextrose Agar (PDA) and Carrot Agar (CA). Photos are taken two days after incubation.

Two isolates are chosen as representative for isolates that clade with (CBS33729) *Pythium acanthophoron*.

Figure 7a: Structure observed in JP007 grown on CA.

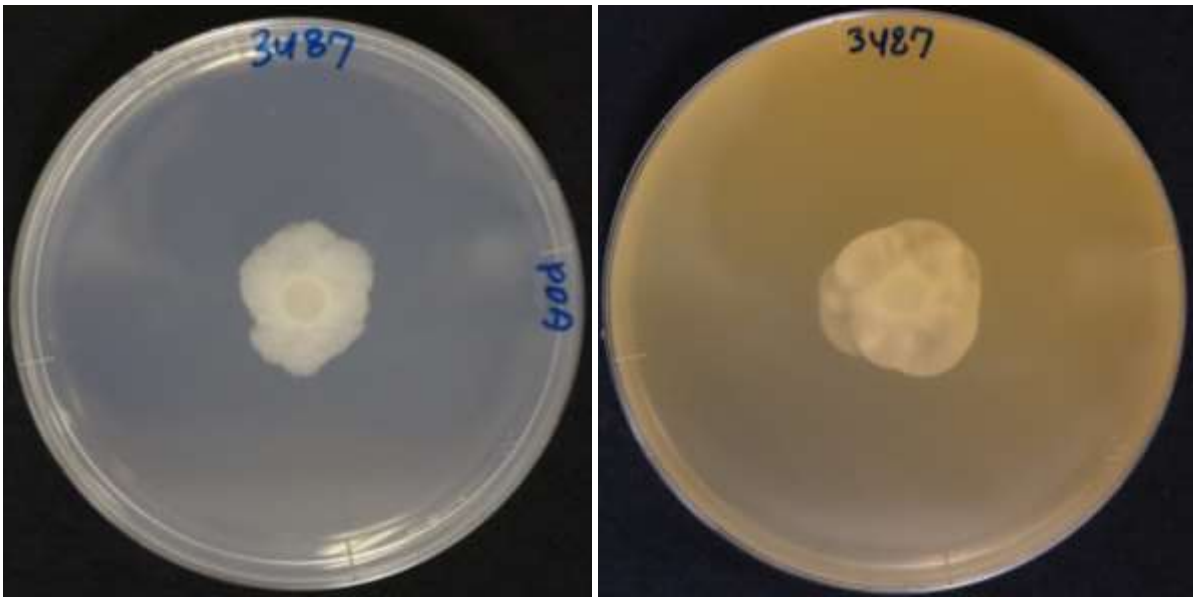


Figure 8: Left to right, isolate MUCC3487, *Phytophthora palmivora* grown on Potato Dextrose Agar (PDA) and Carrot Agar (CA). Photos are taken two days after incubation.

Between the two *Phytophthora palmivora* isolates, MUCC3487 is chosen as representative.

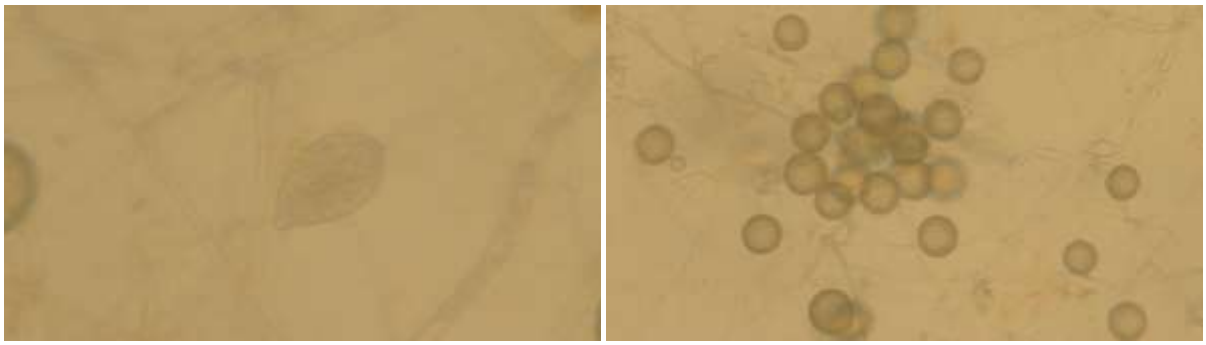


Figure 8a: Structures observed in MUCC3487 Pytophthora palmivora grown on CA.

Isolate PH420 is grouped with (CBS100530) *Pythium plurisporium* in COX1 ML phylogenetic tree, is strongly supported with 100% bootstrap as compared in ITS ML phylogenetic tree with 96% bootstrap support. For isolate PH470, it is phylogenetically identified with (CBS80896) *Pythium nunn* with high bootstrap support of 98% in ITS ML followed by 88% in COX1 ML.

Next, isolate PH417 is grouped with (CBS33729) *Pythium acanthophoron* in COX1 ML supported by 74% bootstrap value whereas in ITS ML, the isolate is positioned separately from the reference isolate with 90% bootstrap value. JP003, JP004, JP005, JP006, and JP007 are in monophyletic sub-clade with (CBS33729) *Pythium acanthophoron* with boostrap value of 93%.

All the other PH isolates that clade with (CBS74896) *Phytopythium cucurbitacearum* are strongly supported with 99% bootstrap value in ITS ML and 100% in COX1 ML. However, the position of the isolates in ITS ML are not clear as compared to in COX1 ML.

Discussion

Pythium acanthophoron was isolated originally in Hawaii, from diseased leaves of pineapple, *Ananas sativus* (Van der Plaats-Niterink, 1981). This species is placed in Clade J of generated phylogenetic tree as reported by (Levesque & De Cock, 2004). *Pythium acanthophoron*, along with *Pythium oligandrum* and *Pythium nunn* are known for its mycoparasitic properties as demonstrated by (Jones & Deacon, 1995), where it is aggressive combating *Fusarium oxysporum* in plate cultures. Currently, the accepted name of this species is *Globisporangium acanthophoron* as proposed by (Uzuhashi et al, 2010) for its globose sporangia characteristics and now placed in Clade 4, as the relationship between isolates in this clade is unclear and low supported to be consider as monophyletic group.

As described earlier, *Pythium nunn* shares similar antagonistic properties as *Pythium acanthophoron* and clade together in Clade J (Levesque & De Cock, 2004). Then, it was revised by (Uzuhasih et al, 2010) as *Globisporangium nunn*, in Clade 4. In both studies, they isolated *Pythium nunn* from soils in Japan and United States of America respectively. It is reported in Japan, *Pythium nunn* is effective in suppressing damping off in cucumber seedlings caused by *Pythium ultimum* var *ultimum* (Kobayashi et al, 2010).

On the other hand, *Pythium plurisporium* was identified and isolated from bentgrass *Agrostis palustris* suffering from Pythium crown and root rot in North Carolina, USA by (Abad et al, 1995). They described the species to be distinct as it produced multiple oospores and non-pathogenic towards bentgrass although it is a type of secondary colonizer of bentgrass's roots. In the study conducted by (Levesque & De Cock, 2004). It was placed in subclade B1, Clade B with filamentous-inflated sporangia characteristic. Then, (Uzuhashi et al, 2010) further refined the Clade system proposed by (Levesque & De Cock, 2004) into 5 moderately supported Clade 1 until Clade 5. In this new system, *Pythium plurisporium* is placed in Clade 3, which represent a monophyletic group.

As (Levesque & De Cock, 2004) came up with 11 clades for the genus Pythium, previously named *Pythium cucurbitacearum* was placed in Clade K with characteristic of sporangia with ovoid shape. Also, isolates placed in this clade requires high and maximum temperature for growth, 30˚C to 40˚C. Later in 2010, Uzuhashi et al ethad renamed the isolate into *Ovatisporangium cucurbitacearum* and then placed in Clade 1 with the characteristic of ovoid sporangia. They also stated that through this study, Clade 1 is established as an independent genus, putting end to concern raised by (Belbahri et al, 2008) on whether the species belong to the genus *Pythium*.

However, based on findings of (De Cock et al, 2015), the genus *Ovatisporangium* is synonym to genus *Phytopythium* then based on Maximum Likelihood (ML) analyses of LSU, SSU, and COX1, *Phytopythium* is an independent monophyletic group. Thus, genus *Phytopythium* is an intermediate between the genus Phytophthora and Pythium. Even though the conflict had resolved, *Pythium cucurbitacearum* was an invalid taxon due to missing Latin diagnosis (De Cock et al, 2015). Nevertheless, (Ramírez Martínez et al, 2021) argued that based on their results, *Phytopythium cucurbitacearum* is an independent clade, not part of the *Phytopythium vexans* species complex. They also suggested that a complete Latin diagnosis should be carried out to validate the taxon.

In this study, variation among sequences was not evaluated. As performed by (Belbahri, 2008; Rahman et al, 2013), sequence data of an isolate is compared with established reference sequences by comparing sequence variation to identify potential new species and clarifying the relationship between isolates. Both authors pointed out that evaluating sequence variation is important in identifying and detecting isolates or species from various niches, that share not all but some similarities with each other. They also highlighted that by identifying intra-specific variations among isolates, isolates of same species could be distinguished as novel. (Robideau et al, 2011) suggested that intraspecific and within-isolate sequence variation in ITS should be

acknowledged as large number of insertions and deletion occurred in large scale, posing difficulties in comparing and distinguishing species as this is the locus of oomycetes DNA barcoding. Insertion and deletion will further cause difficulty in direct sequencing of PCR products, due to differences and multiple copies among isolates as was observed in this current study involving the ITS region. Alignment of PCR product on ITS region of the isolates collected was difficult to handle and comprehend, unlike COX region. This is also supported by (Choi et al, 2015) where they underlined inaccuracies of ITS region as DNA barcoding due to large insertion making it difficult to amplify and sequence the loci leading to incomplete or inaccurate data. As they observed that ITS DNA barcoding exhibits insufficient variability for closely related oomycetes species, they deduced that COX2 locus is far more suited to be DNA barcoding of oomycetes due to ease of amplification with higher discriminatory power at species level and diverse amount of COX2 datasets on oomycetes are available. (Sapkota & Nicolaisen, 2015) managed to retrieve oomycetes sequences with increased proportion by evaluating the amplification protocol, which increasing annealing temperature during PCR by 95%. This observation was carried on ITS region, due to its high variation and easy amplification using universal primers. Such technique could be applied in the current research for a better results' pool. (Burgess et al, 2022) recommended that to identify species and assigning to Operational Taxonomical Unit (OTU), precautions such as technical replicates, usage of primer combinations and phylogenetic approach should be considered. Also, sampling substrate should be treated with care as hybrid species might be present. Some of the recommendations were carried out during the experiment period of the current research. They also mentioned that intraspecific variation in ITS can be exploited to delineate subpopulations of closely related species. (Schardl & Craven, 2003) suggested that usage of multiple loci in phylogenetic and taxonomic analyses is recommended for better reflection for the oomycete's diversity, as it was performed in this study using ITS and COX1 loci. (Martin & Tooley, 2003)

encouraged the use of COX 1 and COX2 genes to infer phylogenetic relationship in oomycetes due to absence of mutations at genus level, simplifying the sequence alignment. They also invalidate combining data set of sequences between ITS and COX as alignment of ITS sequences are assumed to be incorrectly assumed and presence of evolutionary divergence with different rate between the two, thus inaccurately reflecting true phylogenetic relationship. In this study, the ITS and COX1 phylogenetic analyses were carried out independent of each other. In addition, intraspecific and internal sequence variation of ITS region is significant in identifying closely related species and understanding genetic diversity within a population (Soanes et al, 2007). (Schoch et al, 2012) presented that ITS region is proposed as the barcode region in fungal barcoding. However, limitation such as incapability to differentiate recently diverged species should be reckoned with thus preventing it as the only tool to species delimitation. Nevertheless, (Winkworth et al, 2022) carried out complete mitochondrial genome sequences analyses in *Peronosporaceae* and noted that with such usage and analysis, origin, diversification, and evolutionary relationships are more accurately inferred and potentially informative in distinguishing closely related taxa. By their standard, current taxonomy of *Peronosporaceae* does not capture the evolutionary relationship within.

With all the presented information, several limitations listed out in other studies are also observed in the current research. To produce better and accurate results, recommendations and suggestions from other authors should be weighed in. Based on the listed findings, proposing new species require a whole lot of experience and technique familiarisations. Nonetheless, inexperience could be overcome with more efforts taken in the future to further resolve the inferring of isolates collected in the Philippines and Japan as new species or closely related taxa with well-established species. In this chapter, analysis on some of isolates' COX1 region is still under investigation. Problems were encountered during the experimentation period. Reparation actions have been taken to overcome the issues encountered.

References

Abad, G., Shew, H. D., Grand, L. F., & Lucas, L. T. (1995). A new species of *Pythium* producing multiple oospores isolated from bentgrass in North Carolina. *Mycologia*, 87(6), 896-901.

Acebo-Guerrero, Y., Hernández-Rodríguez, A., Heydrich-Pérez, M., El Jaziri, M., & Hernández-Lauzardo, A. N. (2012). Management of black pod rot in cacao (*Theobroma cacao* L.): a review. *Fruits*, 67(1), 41-48.

Adeniyi, D. (2019). Diversity of cacao pathogens and impact on yield and global production. Theobroma Cacao-Deploying Science for Sustainability of Global Cocoa Economy.

Avila-Lovera, E., Coronel, I., Jaimez, R., Urich, R., Pereyra, G., Araque, O., & Tezara, W. (2016). Ecophysiological traits of adult trees of Criollo cocoa cultivars (*Theobroma cacao* L.) from a germplasm bank in Venezuela. *Experimental Agriculture*, 52(1), 137-153.

Belbahri, L., McLeod, A., Paul, B., Calmin, G., Moralejo, E., Spies, C. F., & Lefort, F. (2008). Intraspecific and within-isolate sequence variation in the ITS rRNA gene region of *Pythium mercuriale* sp. nov. (Pythiaceae). *FEMS microbiology letters*, 284(1), 17-27.

Bekele, Frances L., Gillian G. Bidaisee, Harjit Singh, and Duraisamy Saravanakumar. "Morphological characterisation and evaluation of cacao (*Theobroma cacao* L.) in Trinidad to facilitate utilisation of Trinitario cacao globally." *Genetic Resources and Crop Evolution* 67, no. 3 (2020): 621-643.

Burgess, T. I., White, D., & Sapsford, S. J. (2022). Comparison of primers for the detection of *Phytophthora* (and other oomycetes) from environmental samples. *Journal of Fungi*, 8(9), 980.

Choi, Y. J., Beakes, G., Glockling, S., Kruse, J., Nam, B., Nigrelli, L., & Thines, M. (2015). Towards a universal barcode of oomycetes–a comparison of the cox1 and cox2 loci. *Molecular ecology resources*, 15(6), 1275-1288.

Darriba, D., Posada, D., Kozlov, A. M., Stamatakis, A., Morel, B., & Flouri, T. (2020). ModelTest-NG: a new and scalable tool for the selection of DNA and protein evolutionary models. *Molecular biology and evolution*, 37(1), 291-294.

De Cock, A. W. A. M., Lodhi, A. M., Rintoul, T. L., Bala, K., Robideau, G. P., Abad, Z. G., & Lévesque, C. A. (2015). *Phytopythium*: molecular phylogeny and systematics. *Persoonia-Molecular phylogeny and evolution of fungi*, 34(1), 25-39.

Delgado-Ospina, J., Molina-Hernández, J. B., Chaves-López, C., Romanazzi, G., & Paparella, A. (2021). The role of fungi in the cocoa production chain and the challenge of climate change. *Journal of Fungi*, 7(3), 202.

de Souza, P. A., Moreira, L. F., Sarmento, D. H., & da Costa, F. B. (2018). Cacao—Theobroma cacao. In *Exotic fruits* (pp. 69-76). Academic Press.

Díaz-Valderrama, J. R., Leiva-Espinoza, S. T., & Aime, M. C. (2020). The history of cacao and its diseases in the Americas. *Phytopathology®*, 110(10), 1604-1619.

Elwers, S., Zambrano, A., Rohsius, C., & Lieberei, R. (2009). Differences between the content of phenolic compounds in Criollo, Forastero and Trinitario cocoa seed (*Theobroma cacao* L.). *European Food Research and Technology*, 229, 937-948.

Erwin, D. C., & Ribeiro, O. K. (1996). *Phytophthora diseases worldwide*. American Phytopathological Society (APS Press).

Guest, D. (2007). Black pod: diverse pathogens with a global impact on cocoa yield. *Phytopathology*, 97(12), 1650-1653.

Hebbar, P. K. (2007). Cacao diseases: a global perspective from an industry point of view. *Phytopathology*, 97(12), 1658-1663.

Jones E. E. & Deacon J. W. (1995): Comparative Physiology and Behaviour of the Mycoparasites *Pythium acanthophoron, P. oligandrum* and *P. mycoparasiticum*, *Biocontrol Science and Technology*, 5:1, 27-40

Kobayashi, S., Uzuhashi, S., Tojo, M., & Kakishima, M. (2010). Characterization of *Pythium nunn* newly recorded in Japan and its antagonistic activity *against P. ultimum var. ultimum. Journal of general plant pathology*, 76, 278-283.

Kongor, J. E., Hinneh, M., Van de Walle, D., Afoakwa, E. O., Boeckx, P., & Dewettinck, K. (2016). Factors influencing quality variation in cocoa (*Theobroma cacao*) bean flavour profile—A review. *Food Research International*, 82, 44-52.

Kozlov, A. M., Darriba, D., Flouri, T., Morel, B., & Stamatakis, A. (2019). RAxML-NG: a fast, scalable and user-friendly tool for maximum likelihood phylogenetic inference. *Bioinformatics*, 35(21), 4453-4455.

Lachenaud, P., & Motamayor, J. C. (2017). The Criollo cacao tree (*Theobroma cacao* L.): a review. *Genetic Resources and Crop Evolution*, 64(8), 1807-1820.

Latif, R. (2013). Health benefits of cocoa. *Current opinion in clinical nutrition & metabolic care*, 16(6), 669-674.

Levesque, C. A., & De Cock, A. W. (2004). Molecular phylogeny and taxonomy of the genus *Pythium. Mycological research*, 108(12), 1363-1383.

Martin, F. N., & Tooley, P. W. (2003). Phylogenetic relationships among *Phytophthora* species inferred from sequence analysis of mitochondrially encoded cytochrome oxidase I and II genes. Mycologia, 95(2), 269-284.

Motilal, L. A., Zhang, D., Umaharan, P., Mischke, S., Mooleedhar, V., & Meinhardt, L. W. (2010). The relic Criollo cacao in Belize–genetic diversity and relationship with Trinitario and

other cacao clones held in the International Cocoa Genebank, Trinidad. *Plant Genetic Resources*, 8(2), 106-115.

Nieves-Orduña, H. E., Krutovsky, K. V., & Gailing, O. (2023). Geographic distribution, conservation, and genomic resources of cacao *Theobroma cacao* L. *Crop Science*.

Pérez-Sierra, A., Jung, M. H., & Jung, T. (2022). Survey and monitoring of *Phytophthora* species in natural ecosystems: methods for sampling, isolation, purification, storage, and pathogenicity tests. In *Plant Pathology: Method and Protocols* (pp. 13-49). New York, NY: Springer US.

Perrine-Walker, F. (2020). *Phytophthora palmivora*–cocoa interaction. *Journal of Fungi*, 6(3), 167.

Ploetz, R. C. (2007). Cacao diseases: important threats to chocolate production worldwide. *Phytopathology*, 97(12), 1634-1639.

Rahman, M. Z., Uematsu, S., Coffey, M. D., Uzuhashi, S., Suga, H., & Kageyama, K. (2013). Re-evaluation of Japanese *Phytophthora* isolates based on molecular phylogenetic analyses. Mycoscience, 55(4), 314-327.

Ramírez Martínez, J., Cárdenas Toquica, M., Guevara-Suarez, M., Duarte, D., Victorino Jimenez, L. D., Argüello Bernal, B. K., & Restrepo Restrepo, S. (2021). Oomycete species associated with *Theobroma cacao* crops in Colombia. *Plant Pathology*, 70(7), 1695-1707.

Robideau, G. P., De Cock, A. W., Coffey, M. D., Voglmayr, H., Brouwer, H., Bala, K., & André Lévesque, C. (2011). DNA barcoding of oomycetes with cytochrome c oxidase subunit I and internal transcribed spacer. *Molecular ecology resources*, 11(6), 1002-1011.

Sapkota, R., & Nicolaisen, M. (2015). An improved high throughput sequencing method for studying oomycete communities. *Journal of microbiological methods*, 110, 33-39.

Schardl, C. L., & Craven, K. D. (2003). Interspecific hybridization in plant-associated fungi and oomycetes: a review. *Molecular Ecology*, 12(11), 2861-2873.

Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., & White, M. M. (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the national academy of sciences*, 109(16), 6241-6246.

Soanes, D. M., Richards, T. A., & Talbot, N. J. (2007). Insights from sequencing fungal and oomycete genomes: what can we learn about plant disease and the evolution of pathogenicity? *The Plant Cell*, 19(11), 3318-3326.

Toma, S., & Săseanu, A. (2020, June). Chocolate, a global business. In *BASIQ 2020 International Conference. Messina, Italy* (p. 1233).

Uzuhashi, S., Kakishima, M., & Tojo, M. (2010). Phylogeny of the genus *Pythium* and description of new genera. *Mycoscience*, 51(5), 337-365.

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

Vega, C., & Kwik-Uribe, C. (2012). Theobroma cacao—An introduction to the plant, its composition, uses, and health benefits. In *Cocoa butter and related compounds* (pp. 35-62). AOCS Press.

Winkworth, R. C., Neal, G., Ogas, R. A., Nelson, B. C., McLenachan, P. A., Bellgard, S. E., & Lockhart, P. J. (2022). Comparative analyses of complete peronosporaceae (oomycota) mitogenome sequences—insights into structural evolution and phylogeny. *Genome Biology and Evolution, 14*(4), evac049.

White, T. J., Bruns, T., Lee, S. J. W. T., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications, 18*(1), 315-322.

Chapter 3 Oomycetes associated with hemp cropping sites in Japan

Introduction

Cannabis sativa L. is a controversial plant with long history of domestication yet has multiple important uses and benefits towards mankind in several industries such as medical and textile. Hemp, a variety of Cannabis sativa L. is cultivated as a source of fibre in textile industries and oil extracted from the seeds in healthcare for its psychoactive compounds making it a multipurpose crop (Andre et al, 2016; Russo et al, 2008; Skoglund et al, 2013). Issue surrounding the one of the psychoactive compounds of *Cannabis sativa* L. namely tetrahydrocannabinol (THC) is upon usage, users will be intoxicated like drugs abuse (Aizpurua-Olaizola et al, 2016). However, (Andre et al, 2016) have listed multiple benefits from ingesting hemp chemical compounds such as cannabidiol (CBD) that act as therapeutic agents and anti-inflammatory, also confirmed through the findings of (Burstein, 2015; van Bakel, 2011).

Due to its vigour and robust characteristics, hemp is intensively cultivated across multiple countries. Versatility of the crop starting from seed sowing to growing the plants, with recorded resistance towards hot weather and pest problems (Andre et al, 2016) has made the plant to be extensively studied to molecular level (van Bakel et al, 2011). In North America, Canada, and the United States of America (USA), growing hemp had gained popularity in which both countries have developed guidelines to propagate the industries revolving around hemp (Ellison, 2021; Punja, 2021).

While hemp is rigorously cultivated due to the belief that this plant is immune to infection and diseases, time has proven that hemp in fact, does suffer from diseases through infections from various pathogens. (Thiessen et al, 2020) reported that several diseases are observed in hemp

grown commercially in the USA such as gray mold caused by *Botrytis cinerea*, Pythium root and crown rot caused by *Pythium myriotylum*, and *Pythium ultimum*. *Fusarium* spp. are also recorded to cause diseases like Fusarium wilt, Fusarium canker and Fusarium foliar and flower blight (Thiessen et al, 2020; Punja et al,2019; Punja, 2021). Disease-causing pathogen towards hemps cultivated under field conditions have not been rigorously investigated due to restriction by authorities (Punja & Rodriguez, 2018). Other than fungal and oomycetes infections, hemps also suffer infections from bacteria, virus and nematodes which are recorded by (McPartland, 1996) such as bacterial blight and stunted growth leading to yield loss.

Numerous actions have been taken to manage diseases from spreading in hemp cultivation areas. (Scott & Punja, 2023) have reported that using *Trichoderma harzianum* and *Trichoderma virens*, infection from *Fusarium oxysporum* and *Pythium* spp. could be controlled through pre-colonization with biocontrol agents. (Abbey et al, 2019; Mahmoud et al, 2019) also reported that bud rot caused by *Botrytis cinerea* could be reduced using *Trichoderma* spp. as biocontrol agents. (Punja, 2021) has come up with management strategies to reduce risk of infection and disease incidence through sanitation, controlling environment in closed cultivation areas and ensuring plant stock to be “clean” from infections and diseases.

With the reports of diseases recorded on *Cannabis sativa* L., addressing the source of infection in hemp is crucial to contain the severity caused in hemp growing sites hampering the status of economy. Thus, in this chapter, an inventory of oomycetes associated with hemp cropping sites will be listed and the phylogeny and morphologies of the hitherto pathogens will be addressed based on modern taxonomical criteria.

Materials and Methods

Collection of oomycetes isolates

In June and July 2023, hemp seeds, hemp seedlings and soil samples are obtained from several hemp cropping sites in Mie prefecture, Japan. Hemp seeds and soil samples are subjected to baiting method following (Pérez-Sierra et al, 2022) recommendations with modifications. Young, healthy leaves of *Quercus glauca* and *Morus alba* are used for baiting method. The seeds are submerged in 50 ml of well water whereas 200-500 g of soils are submerged in 700-1000 ml of water collected from a well. Then, collected leaves are blanched in boiling water for 45-60 seconds and then dried using paper towel to remove excess water. The leaves are then floated on water surface and left incubated for seven days at 20˚C-25˚C. After incubation, leaves are carefully removed and washed briefly with distilled water. Leaves are then cut into two to five mm smaller pieces and placed onto NARM selective medium. Next, plated pieces are incubated in dark for 48 hours at 20˚C-25˚C. After two days, observed mycelia is transferred onto PDA and incubated at 20˚C-25˚C for culture purification.

From Mie University Culture Collection (MUCC), two isolates MUCC3486 and MUCC3487, namely *Phytophtora palmivora* are also included in the study.

Molecular and Phylogenetic analyses

Using DNeasy Ultra Clean Microbial Kit (Qiagen, Germany) following manufacturer's protocol, genomic DNA of isolates grown on PDA were extracted from the aerial mycelia. Targeted sequences of Internal Transcribed Spacer (ITS) and Cytochrome c oxidase subunit I (COX1) loci were amplified with in a thermal cycler (Bio-rad T100, Tokyo, Japan). The PCR conditions and primer sets for each locus are presented in Table 1.

The PCR mixture for ITS with a total volume of 12.5 µL was made up of 1 µL DNA, 8.2 µL double distilled H_2O, 0.5 mM $MgCl_2$, 1.25 µL 10x NH4 Reaction Buffer (Bioline, London,

UK), 0.25 µM dNTPs (Bioline), 0.5 µM of each primer, 0.2 µL Bovine Serum Albumin (BSA) solution (Wako, Japan) and 0.1 U of Taq DNA polymerase (Bioline).

Also, The PCR mixture for COX1 with a total volume of 12.5 µL was made up of 1 µL DNA, 6.35 µL double distilled H_2O, 0.5 mM $MgCl_2$, 1.25 µL 10x NH4 Reaction Buffer (Bioline, London, UK), 2.5 µM dNTPs (Bioline), 0.3 µM of each primer, 0.2 µL Bovine Serum Albumin (BSA) solution (Wako, Japan) and 0.1 U of Taq DNA polymerase (Bioline).

Amplicons are then sequenced in both directions using the respective PCR primers and Big Dye Terminator v3.1 Cycle Sequencing (Applied Biosystems, USA) on an Applied Biosystems 3730xl DNA analyser installed in Mie University Advanced Science Research Promotion Centre (Mie, Japan). Sequences are then assembled and aligned using MEGA11: Molecular Evolutionary Genetics Analysis version 11 (Tamura, Stecher, and Kumar 2021).

Sequenced DNAs are then analysed with verified reference sequences in GenBank database using Nucleotide Basic Local Alignment Search Tool (Nucleotide BLAST) queries of National Centre for Biotechnology Institute (NCBI) (http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi). Sequences identified as oomycetes through searches are then aligned with matrix of ITS locus for oomycetes provided by (Robideau et al, 2011) in TreeBASE study S11552.

To determine phylogenetic relationships of the sequences, Maximum likelihood (ML) analysis is performed using RAxML-NG software (Kozlov et al, 2019). The best-fit substitution model for ITS marker is GTR+I+G4 whereas determined based on Akaike Information Criterion (AIC) computed by ModelTest-NG (Darriba et al, 2019). The internal branches' strength of resultant trees is tested by bootstrap analysis (BS) (Felsenstein, 1985) using 100 replications.

Locus	Primer F	Primer R	PCR conditions
ITS	ITS5	ITS4	95˚C 4 mins
	GGA AGT AAA AGT CGT AACA	TCC TCC GCT TAT TGA TATG	95˚C 40 secs
	(White et al, 1990)	(White et al, 1990)	55˚C 40 secs (37 cycle)
			72˚C 1 min
			72˚C 5 mins
COX1	OomCox1-levup	OomCox1-levlo	95˚C 4 mins
	TCA WCW MGA TGG CTT TTT TCA AC	CYT CHG GRT GWC CRA AAA ACC AAA	95˚C 40 secs
			56˚C 40 secs (40 cycle)
	(Robideau et al, 2011)	(Robideau et al, 2011)	72˚C 1 min
			72˚C 5 mins

Table 1: Primer sets and PCR conditions.

Results

Oomycetes isolates obtained from Japan

In ITS data matrix, there are 249 sequences with a total character of 1851 including alignment gaps. Maximum Likelihood (ML) trees of ITS (Fig. 9) data matrix is generated using the software, RAxML-NG. As much as 14 oomycetes isolates from Japan are amplified for ITS region with around 1050 bp. Outgroup for the generated ML trees is (FI373) *Eurychasma dicksonii*, based on (Robideau et al, 2011). The isolates from Japan are grouped as follow, five isolates with (CBS11880) *Pyhtium aphanidermatum,* three isolates with (CBS119165) *Pythium glomeratum,* five isolates are in the same clade with (CBS25028) *Pythium irregulare*, and one isolate with (CBS115464) *Pythium rostratifingens*.

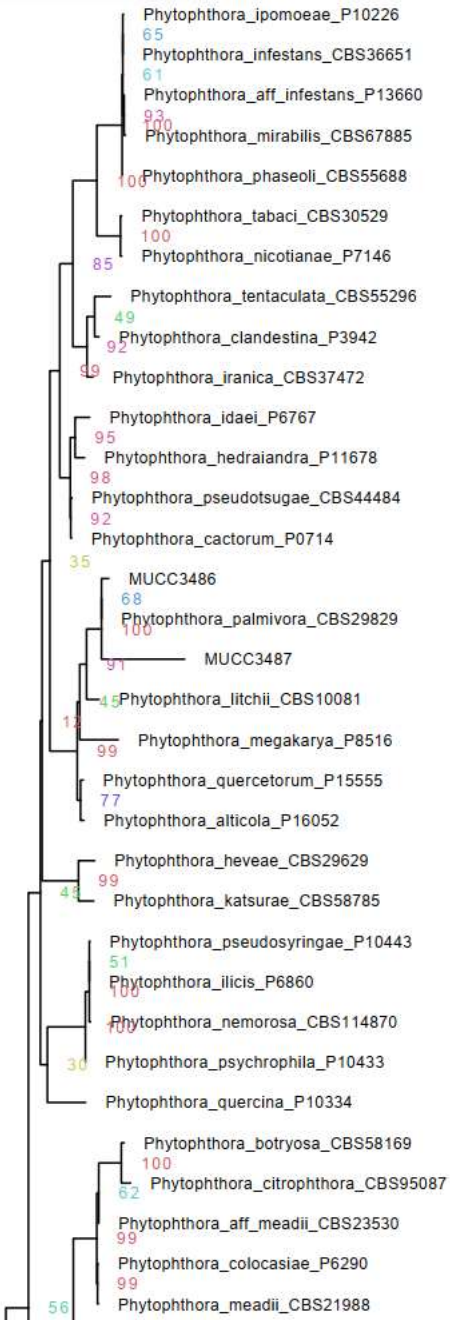


Figure 9: Phylogenetic tree of Maximum Likelihood (ML) analysis of ITS region for the isolates collected from Japan using data matrix provided by (Robideau et al, 2011). Bootstrap values are calculated at 100 replicates. “JP” encodes isolates from Japan.

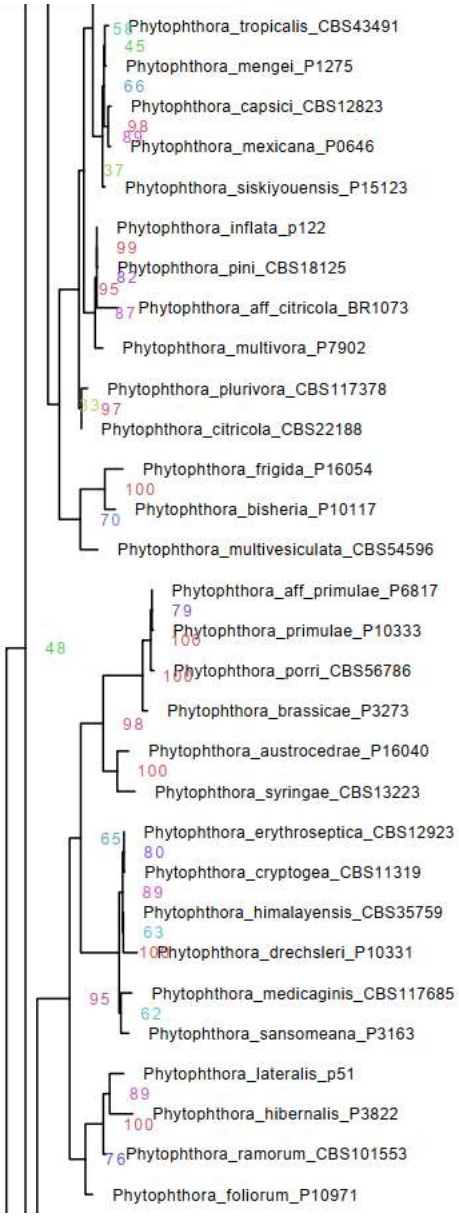


Figure 9 (continued)

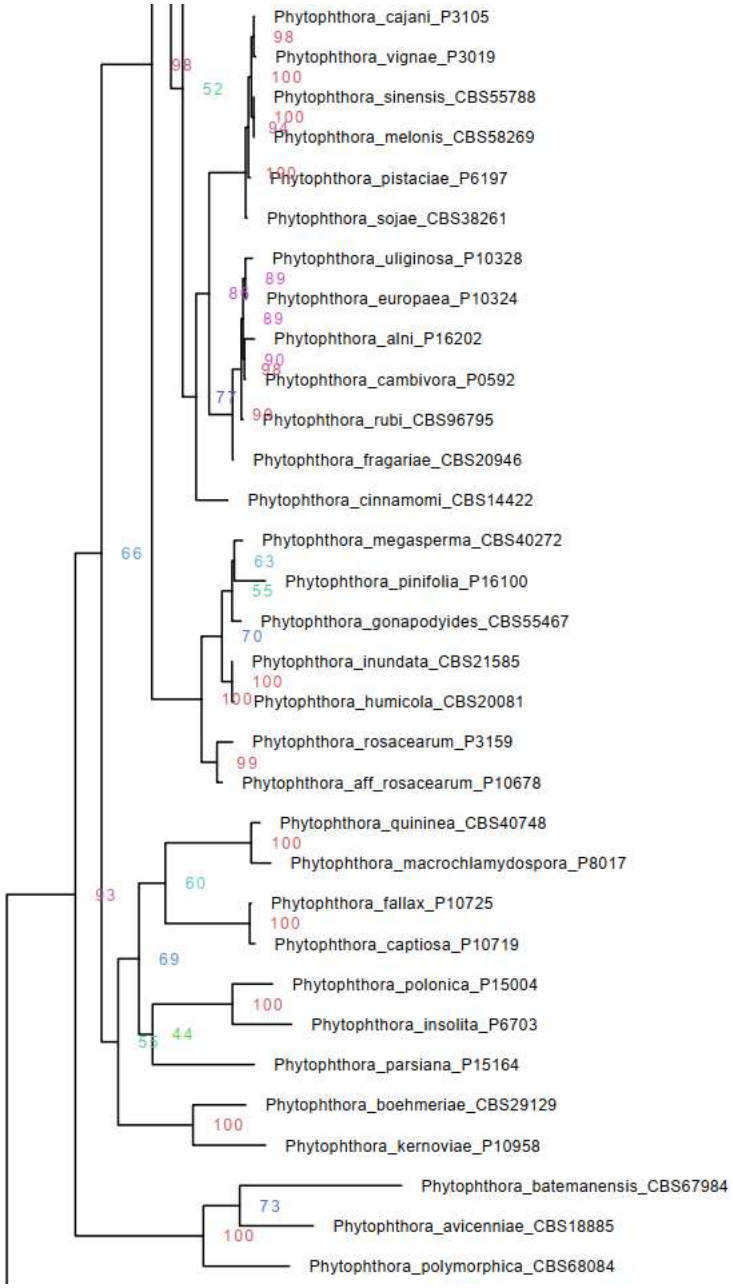


Figure 9 (continued)

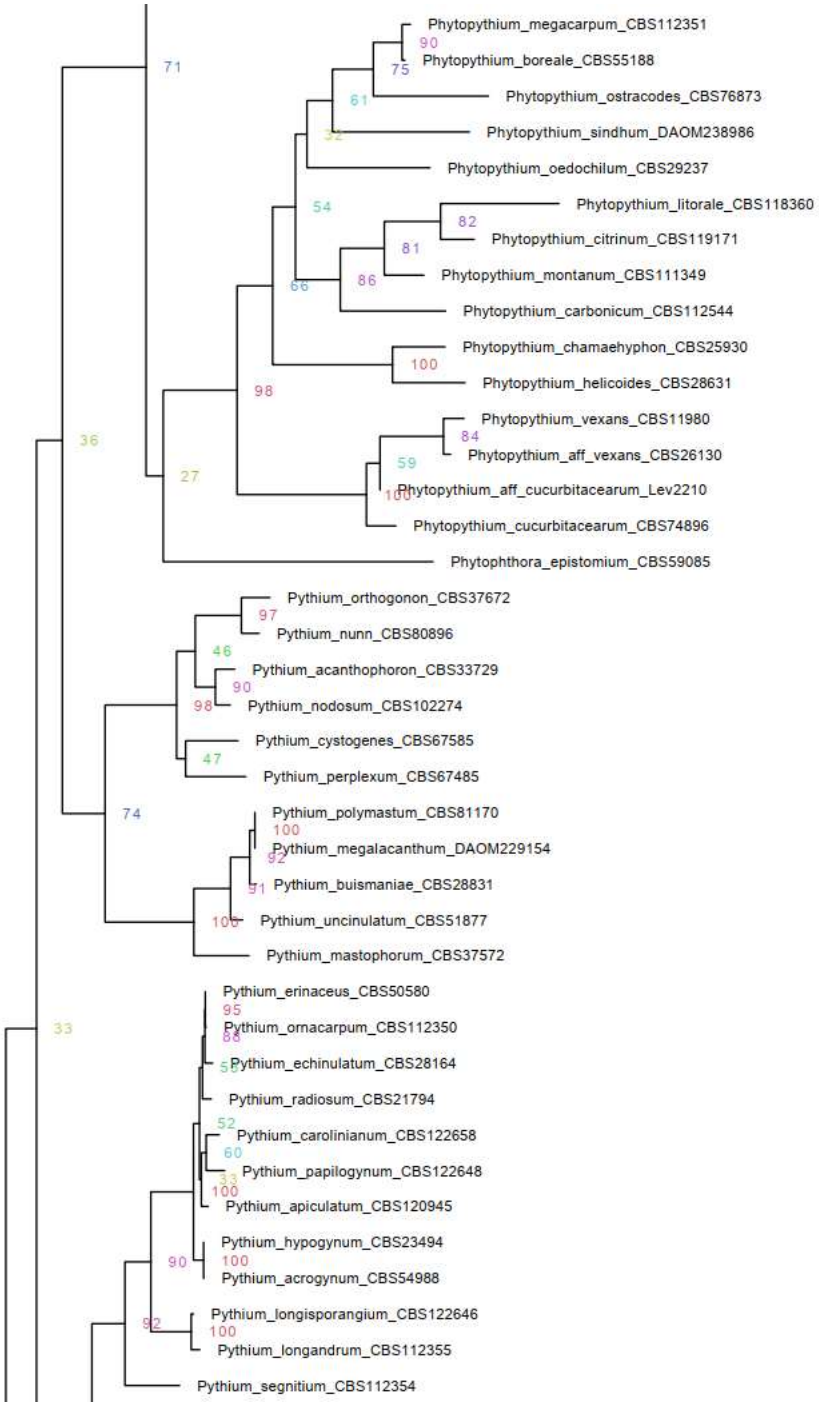


Figure 9 (continued)

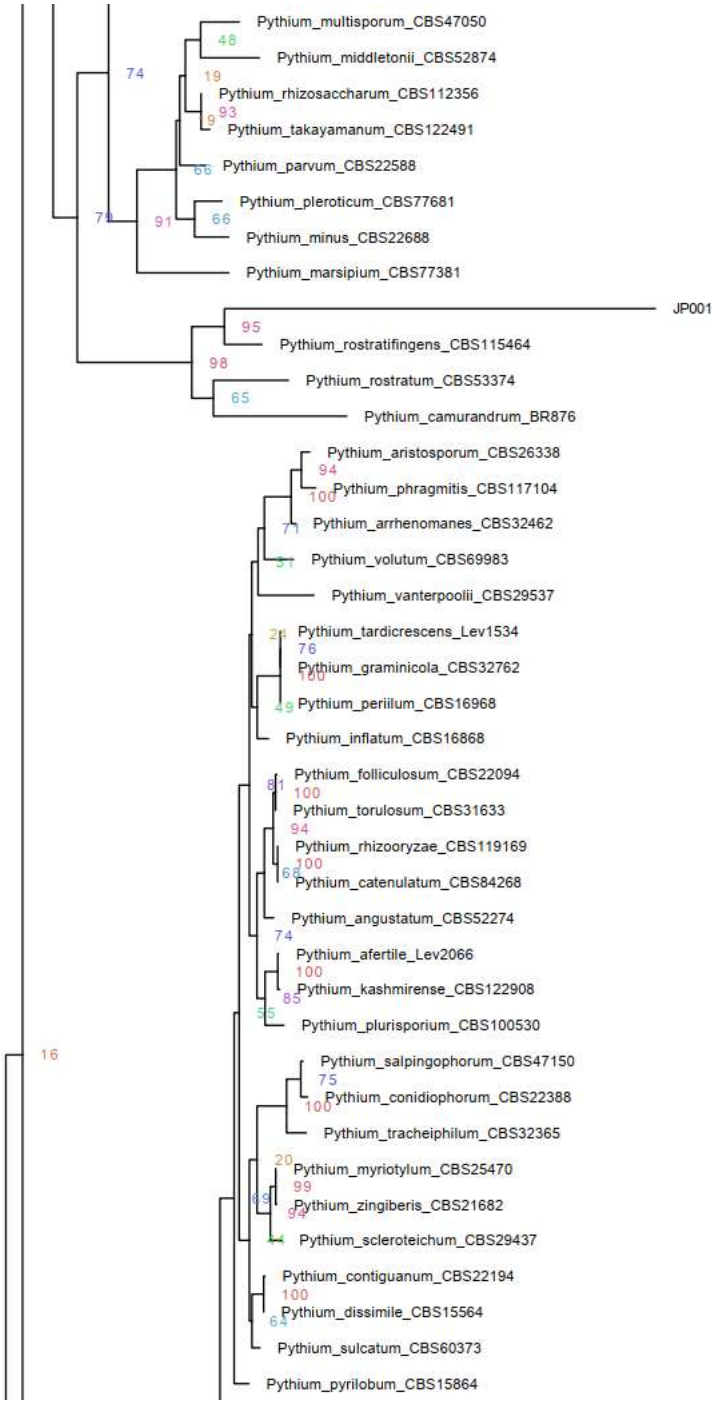


Figure 9 (continued)

Figure 9 (continued)

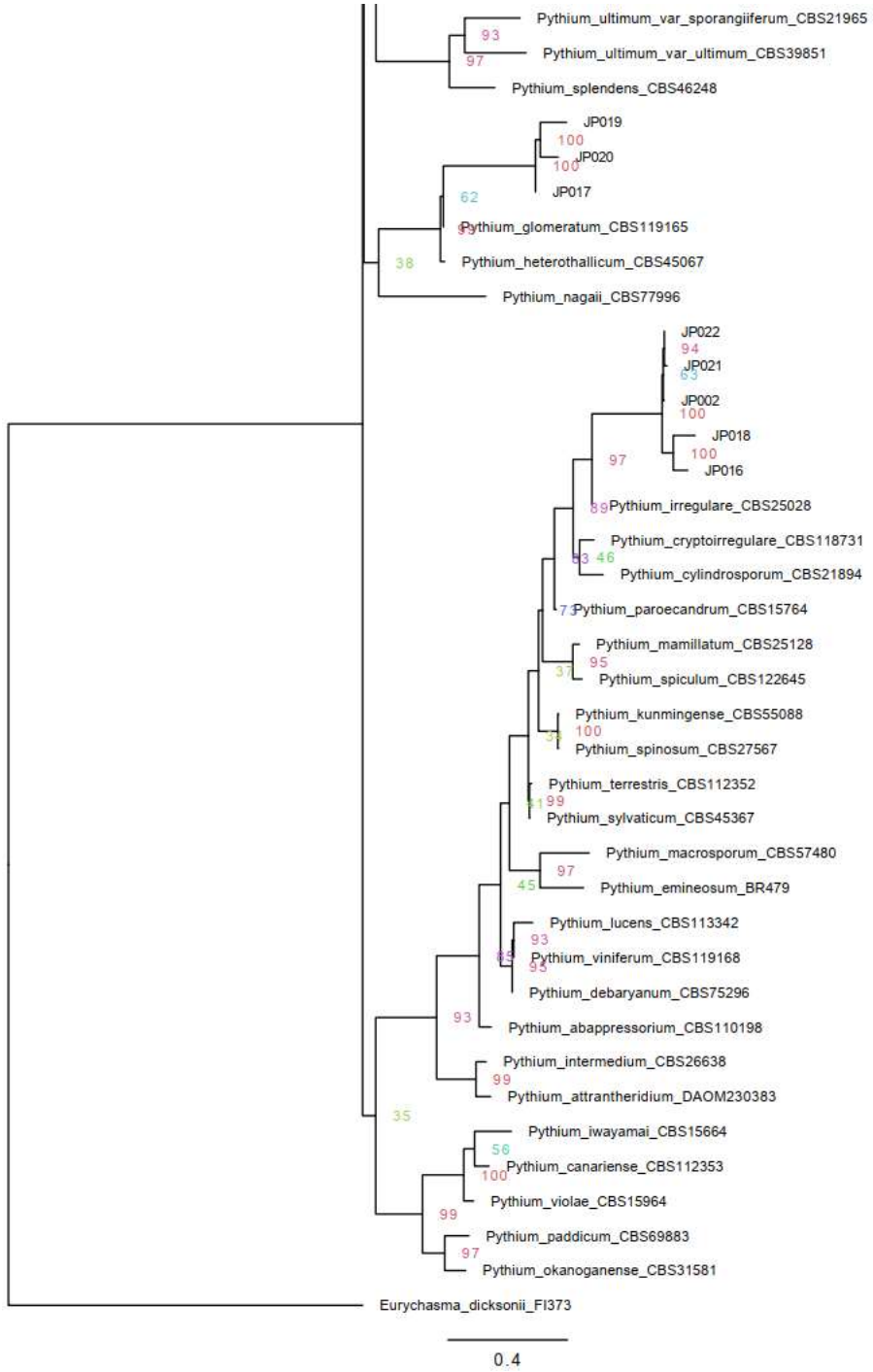


Figure 9 (continued)

Isolates collected from hemp seedlings

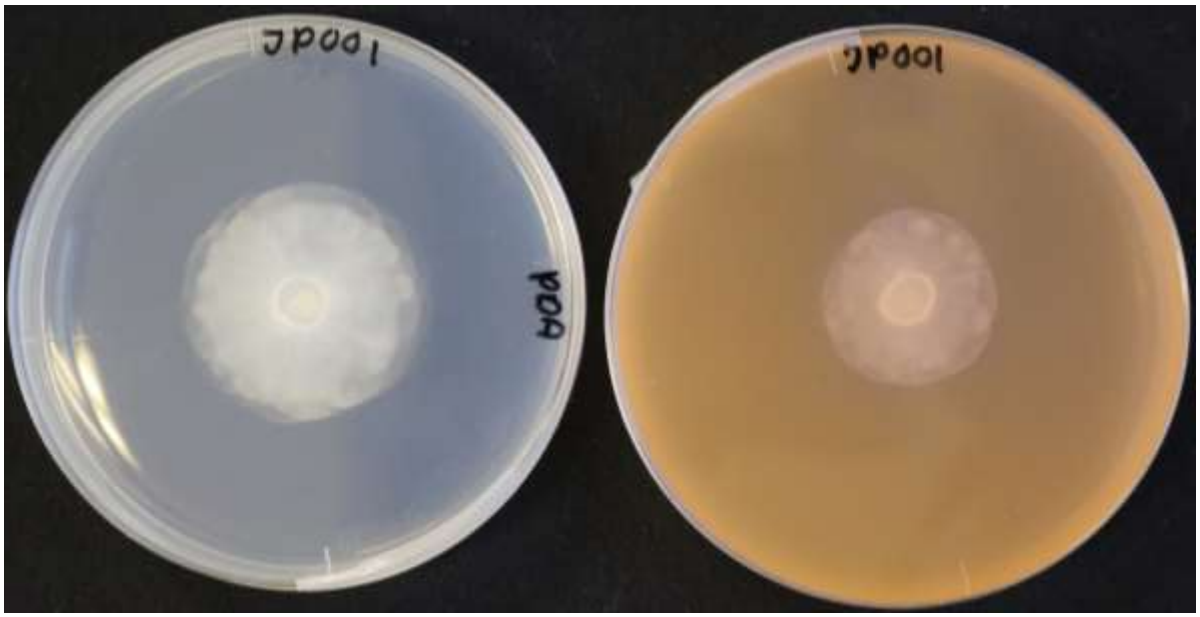


Figure 10: Left to right, isolate JP001 grown on Potato Dextrose Agar (PDA) and Carrot Agar (CA). Photos are taken two days after incubation.

Figure 10a: Structure observed in JP001 grown on CA.

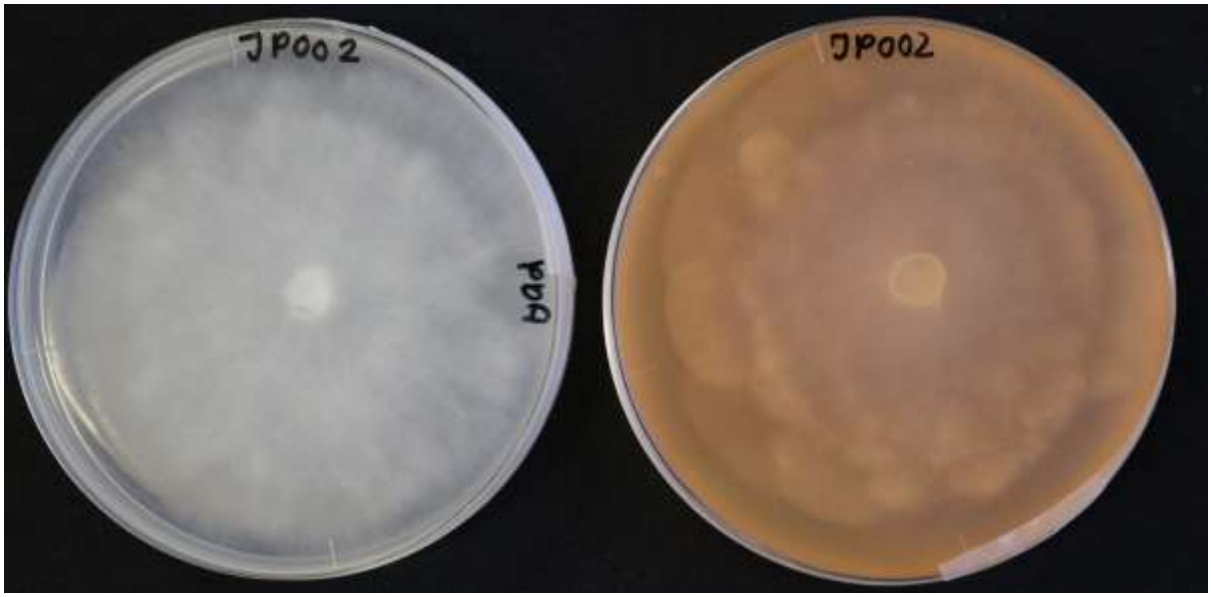


Figure 11: Left to right, isolate JP002 grown on Potato Dextrose Agar (PDA) and Carrot Agar (CA). Photos are taken two days after incubation.

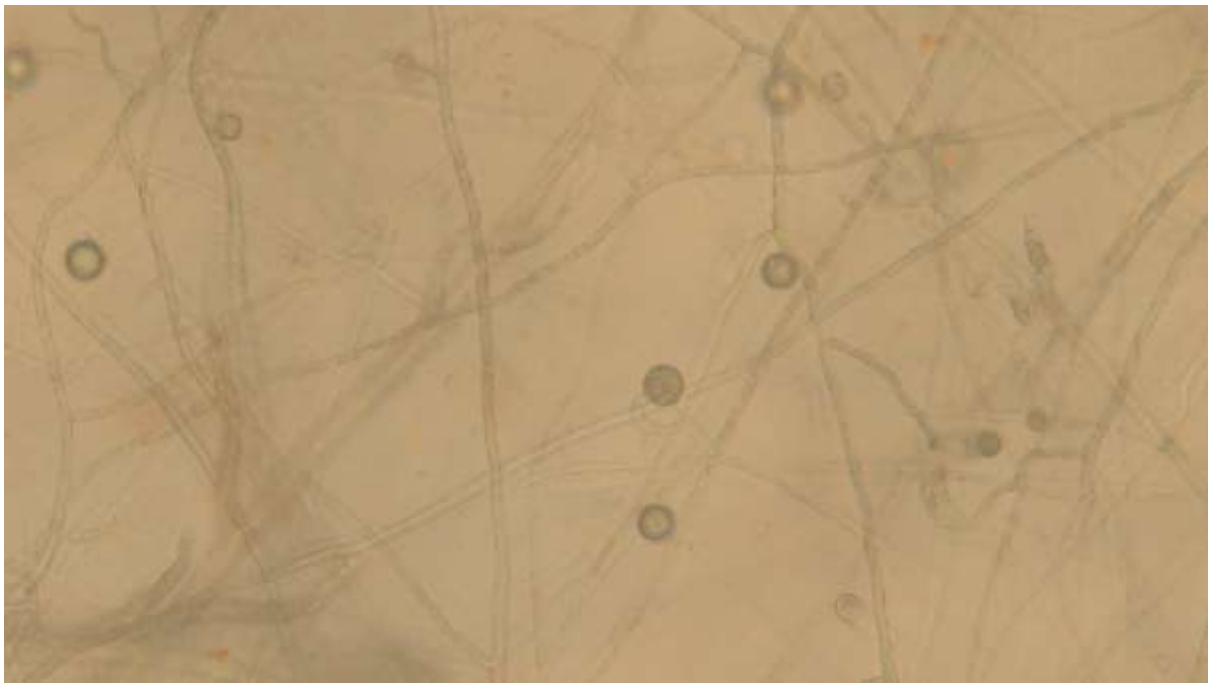


Figure 11a: Structures observed in JP002 grown on CA.

Isolates collected from soil samples

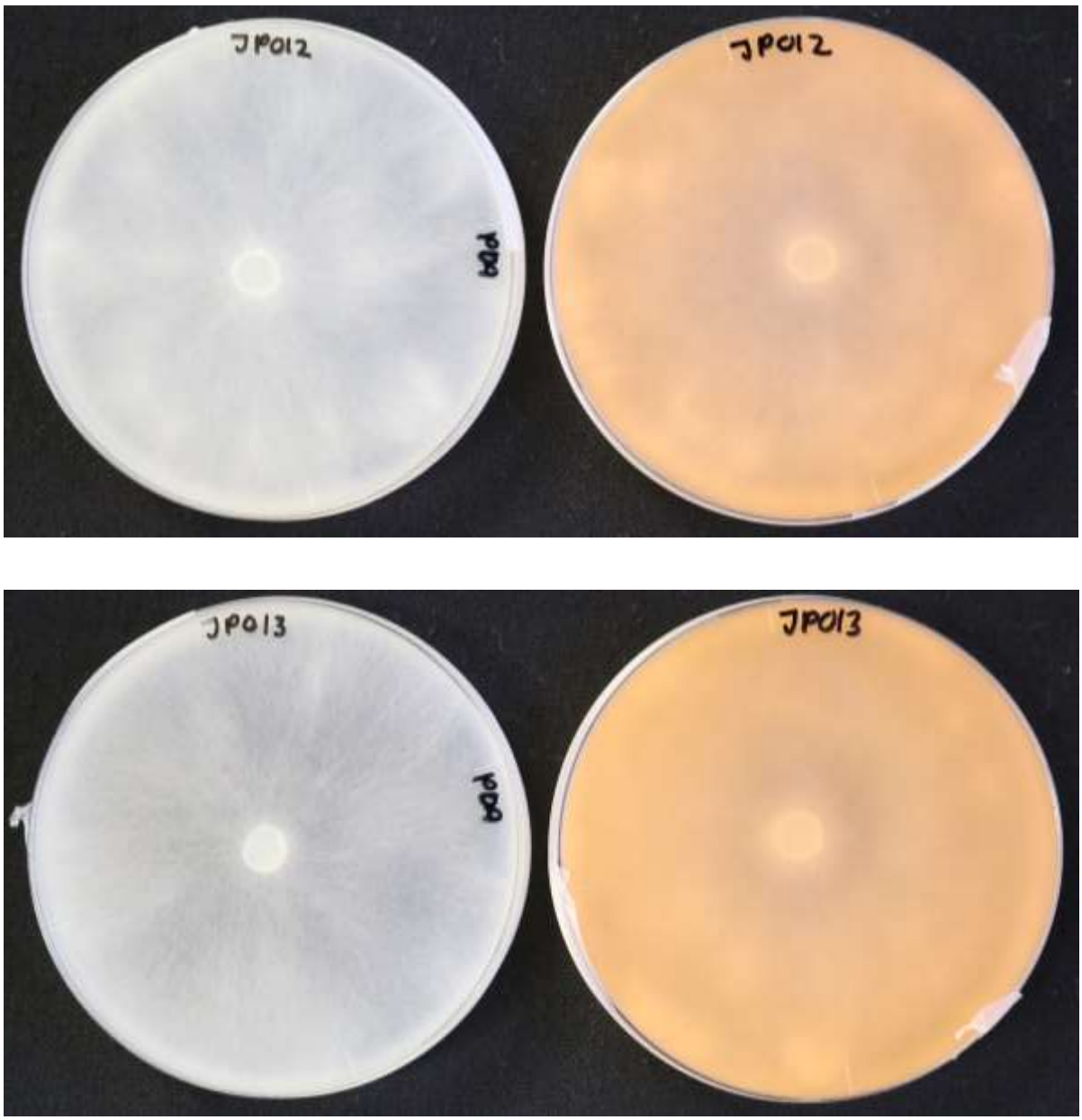


Figure 12: Left to right, isolate JP012 (Top) and isolate JP013 (Bottom) grown on Potato Dextrose Agar (PDA) and Carrot Agar (CA). Photos are taken two days after incubation.

These two isolates are representative for isolates that clade with (CBS11880) *Pythium aphanidermatum*.

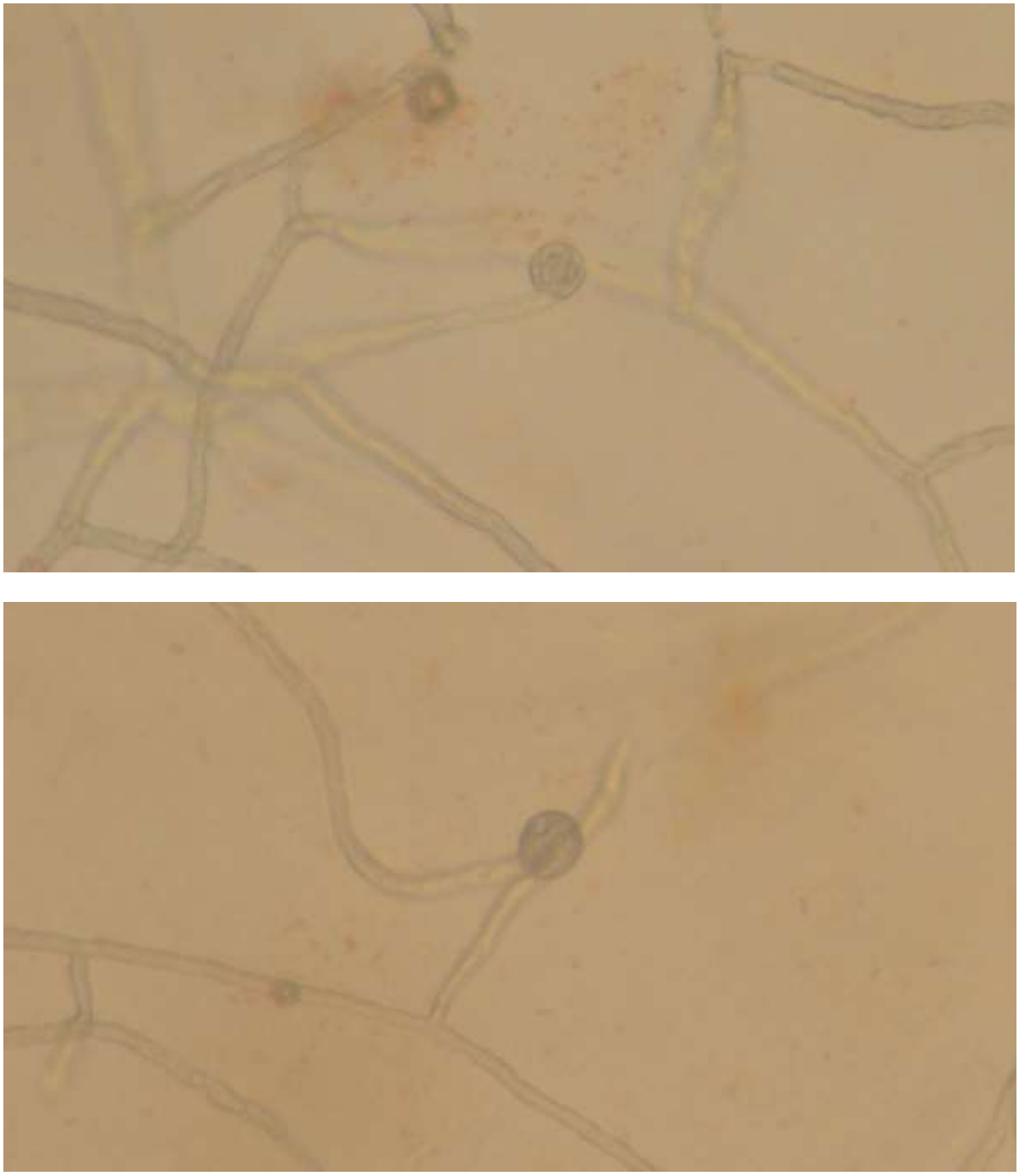


Figure 12a: Structures observed in JP012 (Top) and in JP013 (Bottom) grown on CA.

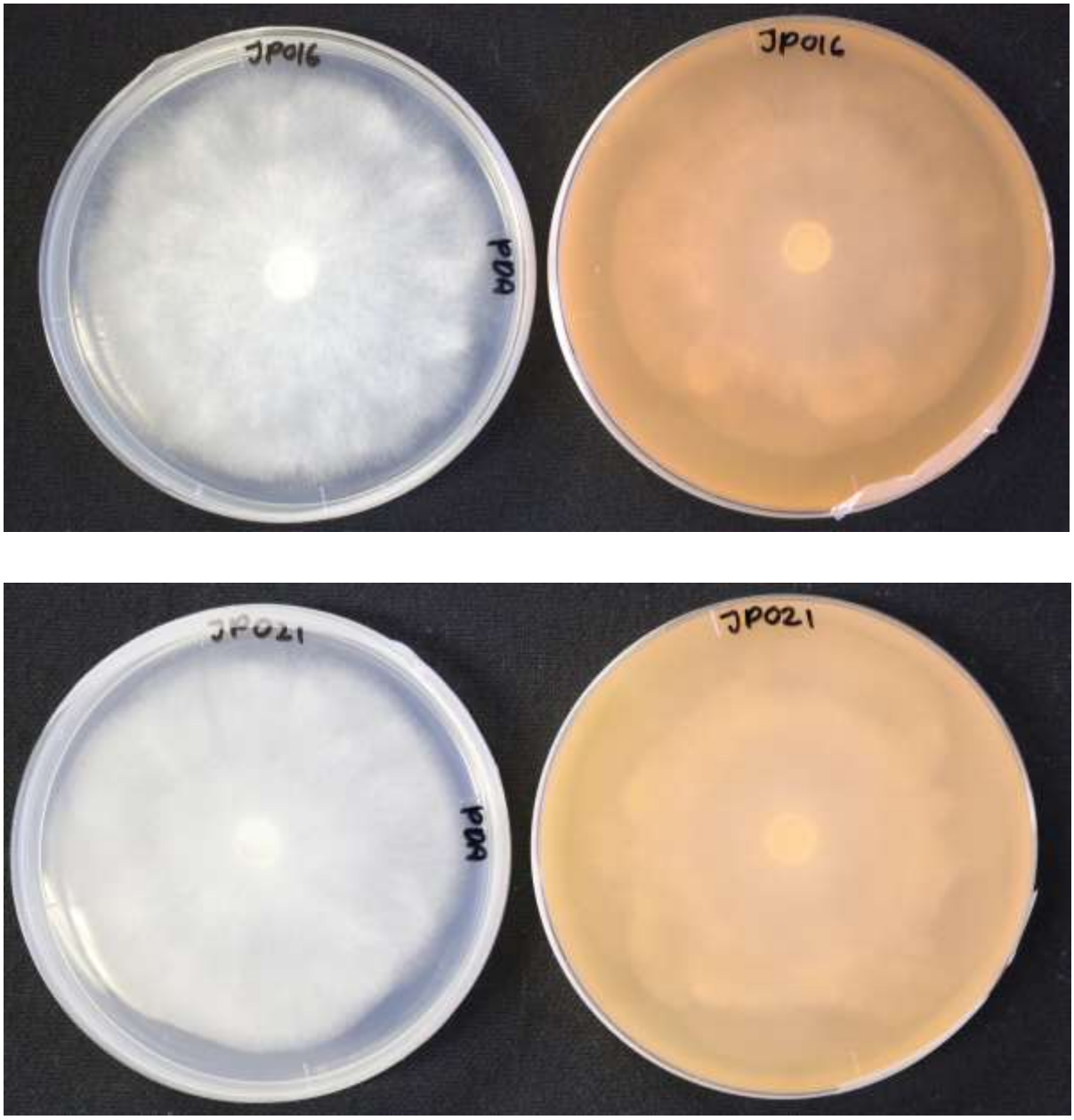


Figure 13: Left to right, isolate JP016 (Top) and isolate JP021 (Bottom) grown on Potato Dextrose Agar (PDA) and Carrot Agar (CA). Photos are taken two days after incubation.

These two isolates are representative for isolates that clade with (CBS25028) *Pythium irregulare*.

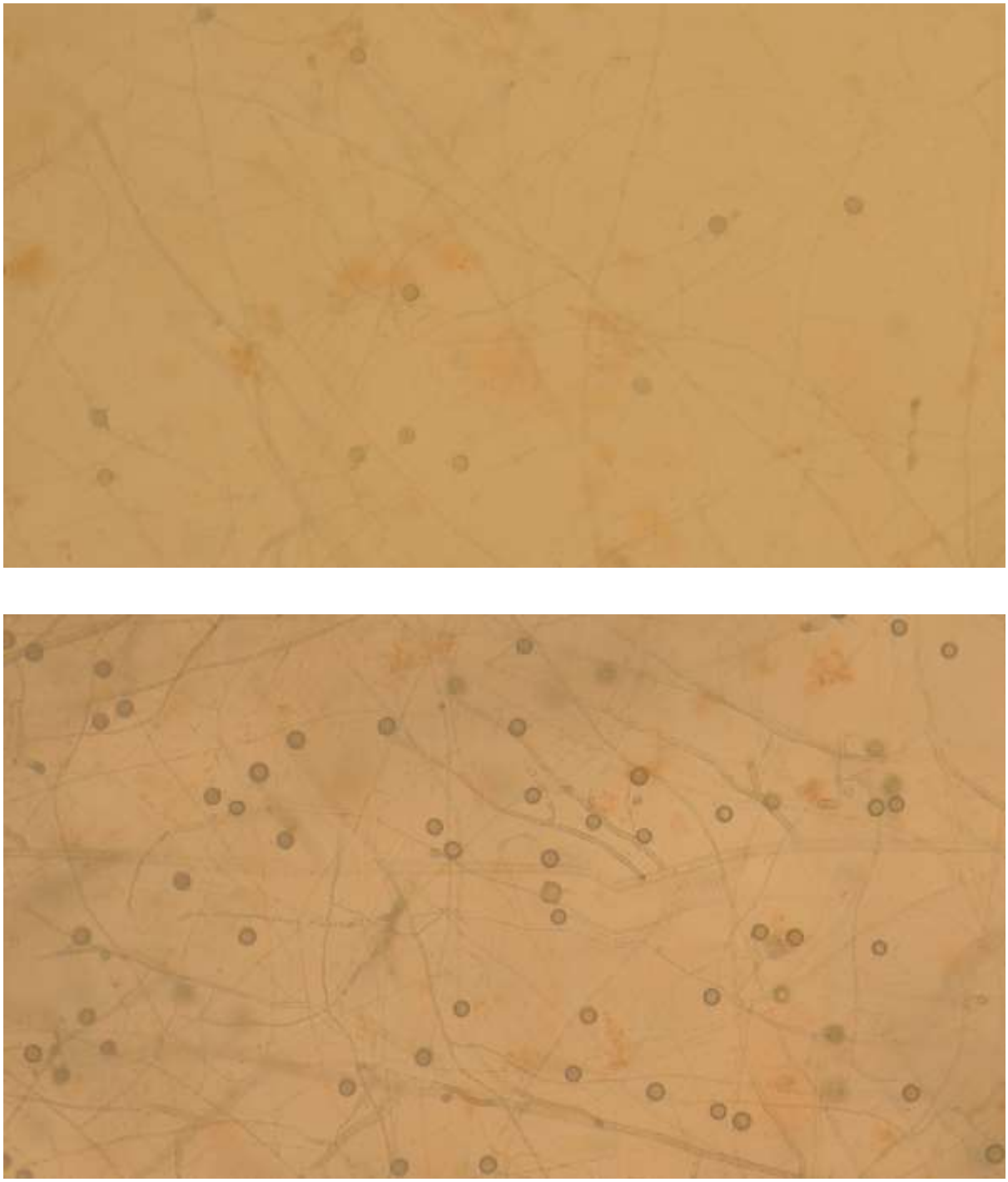


Figure 13a: Structures observed in JP016 (Top) and in JP021 (Bottom) grown on CA.

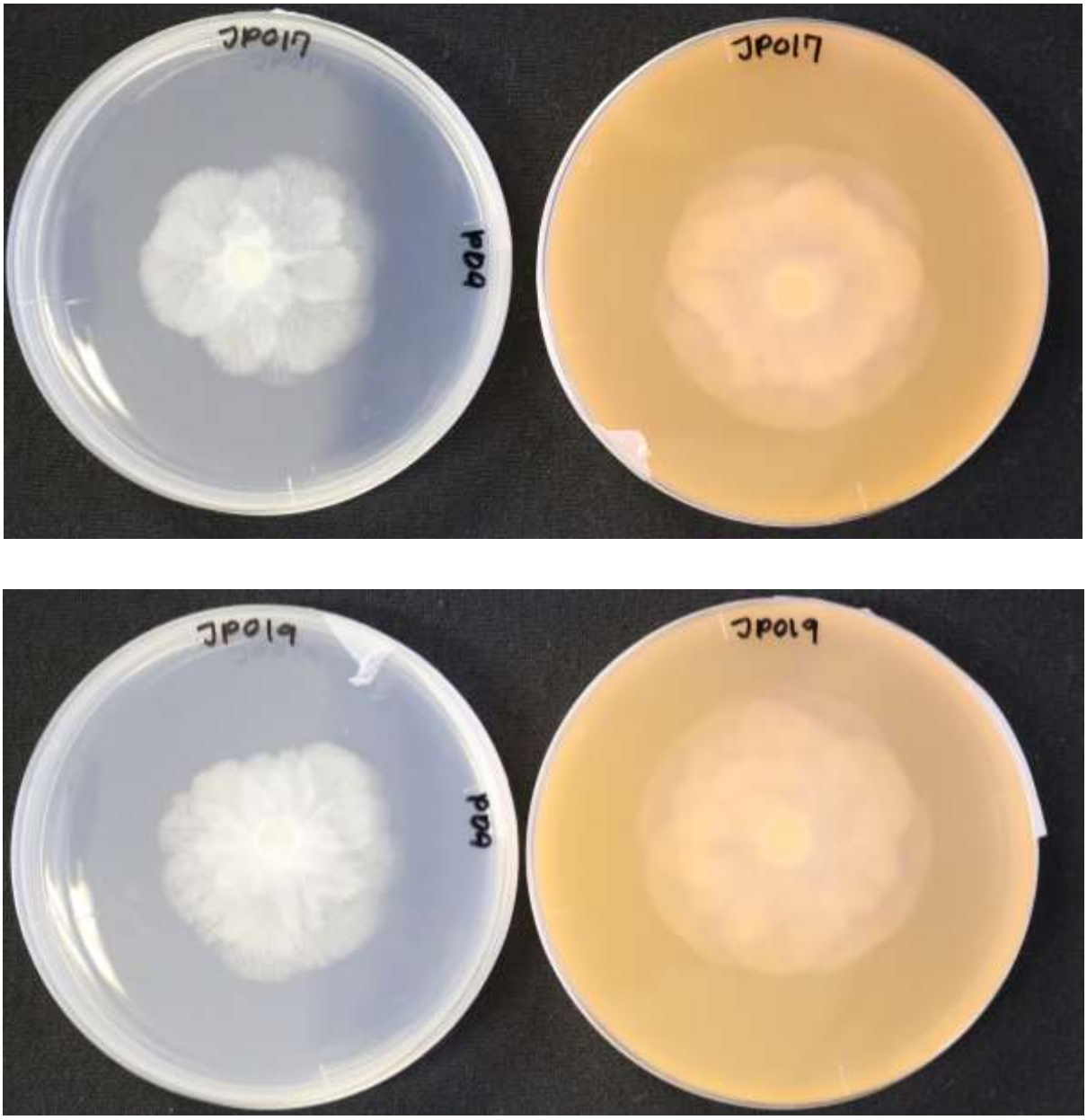


Figure 14: Left to right, isolate JP017 (Top) and isolate JP019 (Bottom) grown on Potato Dextrose Agar (PDA) and Carrot Agar (CA). Photos are taken two days after incubation.

These two isolates are representative for isolates that clade with (CBS119165) *Pythium glomeratum*.

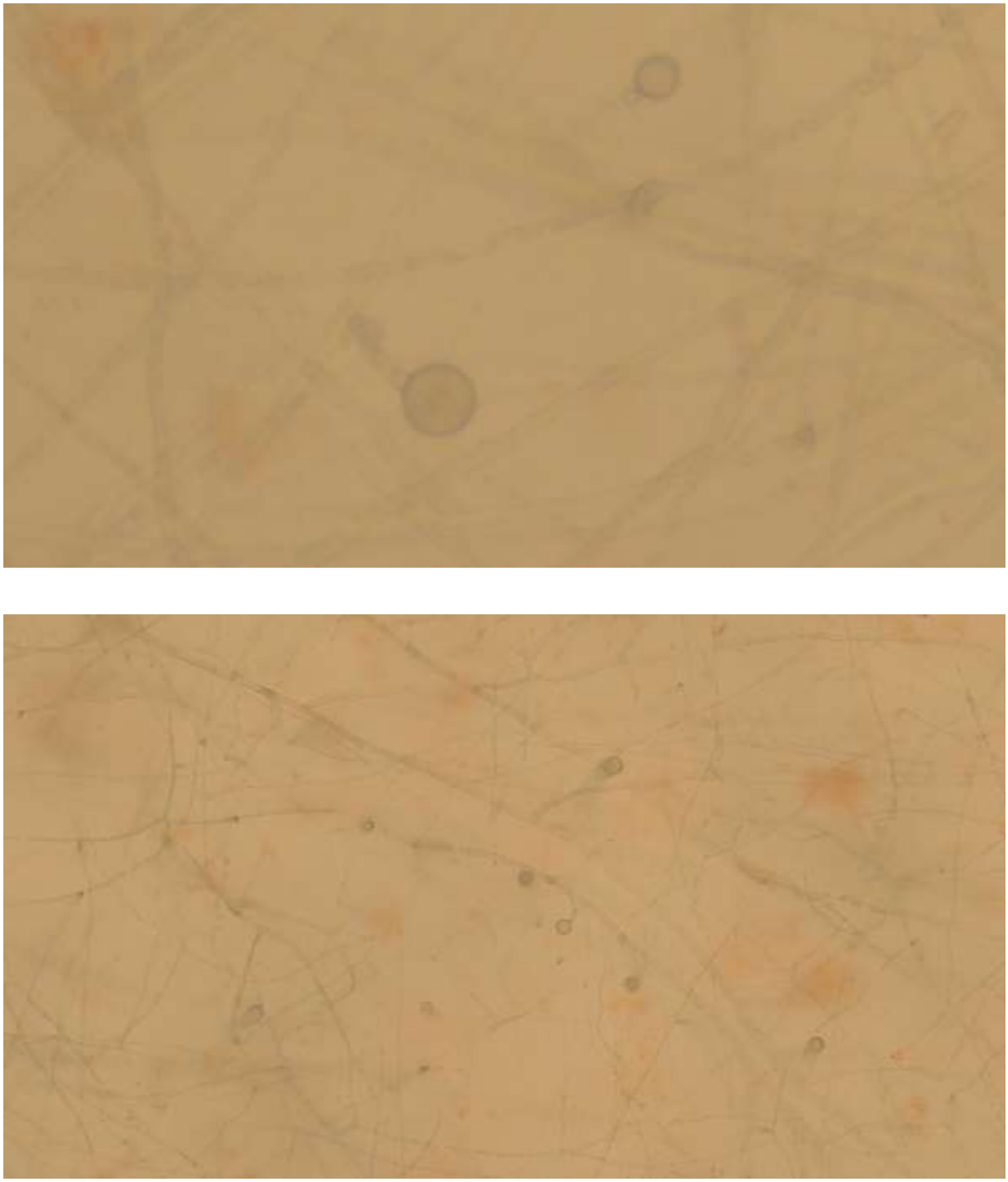


Figure 14a: Structures observed in JP017 (Top) and in JP019 (Bottom) grown on CA.

Isolate JP001, collected from hemp seedlings is grouped with (CBS115464) *Pythium rostratifingens* in ITS ML phylogenetic tree supported with 95% bootstrap. Another isolate collected from hemp seedlings, JP002, its position is unclear in the phylogenetic analysis as it placed in sub-clade with closely related species, (CBS25028) *Pythium irregulare* with high bootstrap support of 97% in the ITS ML.

Next, from soil samples of hemp field, isolate JP016, JP018, JP021, and JP022 are grouped with (CBS25028) *Pythium irregulare* like JP002, with unclear positions within the sub-clade in ITS ML supported by 97% bootstrap value.

JP017, JP019, and JP020 are in monophyletic sub-clade with (CBS119165) *Pythium glomeratum* with boostrap value of 62%, which also clade with (CBS45067) *Pythium heterothallicum* with 99% bootstrap value.

Other isolates, JP011, JP012, JP013, JP014, and JP015 are positioned in their sub-clade that clade with (CBS11880) *Phytopythium aphanidermatum* with low support of only 52% bootstrap value in ITS ML. The clade is also positioned separately in clade with (CBS31433) *Pythium delicense*, with only 82% bootstrap values in ITS ML.

Discussion

Pythium rostratifingens was first describe by (De Cock & Levesque, 2004) where it was isolated from several hosts such as leaf of Quercus sp., Canada, and soil under apple tree in the USA. (Mazzola et al, 2002) previously identified the species as *Pythium rostratum* and *Pythium aff. Rostratum*. This species is most like *Pythium rostratum*, morphologically and phylogenetically with difference in *Pythium rostratifingens* having smaller size of oogonia and sporangia (De Cock & Levesque, 2004). Next, (Uzuhashi et al, 2010) isolated the species from soil sample and wheat from Hokkaido, Japan. They then transferred the species under *Globisporangium rostratifingens* due to its globose sporangia characteristics. From (Robideau et al, 2011) findings, this species is placed under the Clade E based on (Levesque & De Cock, 2004) gene trees of *Pythium* species and in Clade 4 of (Uzuhashi et al, 2010). *Pythium rostratifingens* was reported to be isolated from Pyrethrum (*Tanacetum cinerariifolium*) crown tissues, in Australia (Liu et al, 2023).

Also, by (Liu et al, 2023), *Pythium irregulare* was isolated from crown and root of Pyrethrum (*Tanacetum cinerariifolium*) and soil sampled in Australia. This aggressive species caused severe seed rot and damping of in seedlings of Pyrethrum. In Japan, this species is associated with lettuce (*Lactuca sativa* L.) damping off in Kagawa Prefecture, isolated from diseased roots (Feng et al, 2019). (Mazzola et al, 2002) reported this species isolated from roots, to be virulent towards apple in Washington, USA by stunting growth of apple seedlings. (McGehee & Raudales, 2021) reported for the first time, that this species causing root rot on *Cannabis sativa* L. in Connecticut, USA. Isolates were collected from substrates used to grow the plant. In South Africa, (Spice et al, 2011) suggested that all isolates collected from infected vines in grapevine nurseries and vineyards comes from a single variable of *Pythium irregulare*.

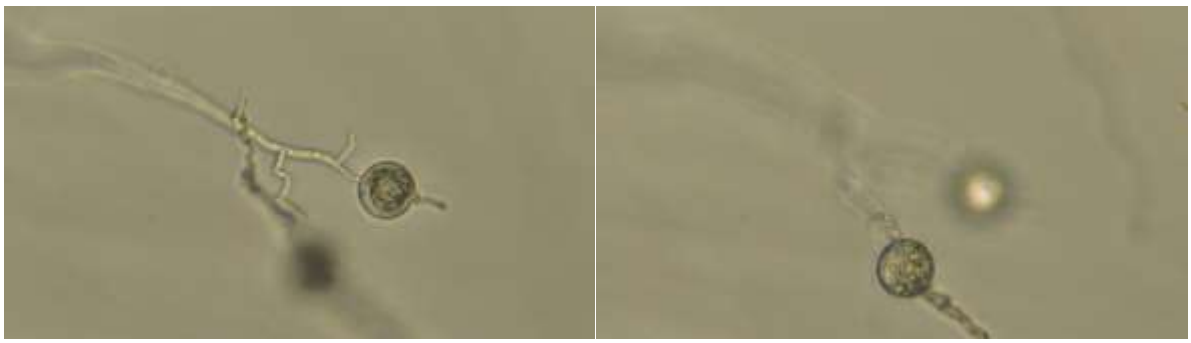
Moving on, *Pythium glomeratum* was first described by (Paul, 2003), which was isolated from soil samples in northern France. Previously, the species was recorded as *Pythium heterothallicum*. The study revealed that this species is closely related to *Pythium heterothallicum* with difference being, oogonia of *Pythium glomeratum* are larger in size and oogonia are wrapped around with antheridia. Based on the findings of (Levesque & De Cock, 2004), this species is placed under Clade I along with *Pythium heterothallicum*. (Uzuhashi et al, 2010) renamed this species as *Globisporangium glomeratum* and it is placed in Clade 4, with known characteristic of globose sporangia along with *Pythium heterothallicum* renamed to *Globisporangium heterothallicum*. *Pythium glomeratum* is associated with Aleppo pine seedlings (*Pinus halapensis*) in Algeria (Lazreg et al, 2016). The species was isolated from roots of the seedlings and proven pathogenic towards germination and root development, albeit lower. The study also revealed, *Pythium heterothallicum* isolated also from root, is more pathogenic, causing root rot and damping off in seedlings. (McLeod et al, 2009) revealed the diversity of *Pythium* species in South Africa, in which *Pythium heterothallicum* was reported for the first time to be isolated there. (Nam & Choi, 2019) reported for the first time also, *Pythium heterothallicum* was isolated from soil sediments collected in mountain stream in Korea.

(Van der Plaats-Niterink, 1981) described *Pythium aphanidermatum* as a pathogen with a wide hosts range. The species thrived in warm regions as higher temperature increased infection rate. This species also caused root rot, damping off, stalk and rhizome rot, soft rot, fruit rot, and cottony blight in countless species. This pathogen is placed under Clade A (Levesque & De Cock, 2004), followed with transition to Clade 3, by (Uzuhashi et al, 2010). They isolated the species from soil used for cultivation purposes in Nagano Prefecture, Japan. (McLeod et al, 2009) had isolated this species from South Africa, on host cherry tomato (*Lycopersicon esculentum*) and cucumber (*Cucumis sativus*) during the period of the study. (Punja &

Rodriguez, 2018; Punja et al, 2019) isolated this species from roots of *Cannabis sativa* L. in Canada causing damping off under greenhouse hydroponic conditions. (Sivan et al, 1984; Punja & Yip, 2003; Punja, 2021; Scott & Punja, 2023) suggested that biocontrol agents, *Gliocladium catenulatum*, *Trichoderma harzianum*, and *Trichoderma virens* are effective in reducing disease severity induced by *Pythium aphanidermatum*. (Mazzola et al, 2002) reported that this species was isolated from apple tree roots in Washington, USA and (Rosso et al, 2008) collected this species from soybean *Glycine max* L. cultivar Archer, showing resistance to damping off disease in Arkansas, USA. Root rot and crown rot in cucumber caused by *Pythium aphanidermatum* in Canada are suppressed with rhizobacteria that pre-colonized cucumber roots (Chen et al, 2002). Finally, (Thiessen et al, 2020) outlined this species is causing *Pythium* root rot, as observed from hemp samples used for commercial industry in North Carolina, USA.

As previously discussed in Chapter 2, variation among sequences in this chapter was also not evaluated. (Belbahri, 2008; Rahman et al, 2013) performed the usage of data sequences for identifying and clarifying relationships between isolates and species between isolates. Different studies (Burgess et al, 2022; Choi et al, 2015; Robideau et al, 2011, Sapkota & Nicolaisen, 2015; Schoch et al, 2012; Soanes et al, 2007) highlighted the importance of evaluating intraspecific and within isolates sequence variations to distinguish species as limitations of using ITS region for DNA barcoding such as large insertions are presence. COX2 locus is suggested as a more suitable DNA barcoding marker (Choi et al, 2015). Techniques, such as increasing annealing temperature (Sapkota & Nicolaisen, 2015) during PCR can improve results. Multiple loci, such as ITS and COX1 (Schardl & Craven, 2003) and COX1 and COX2 (Martin & Tooley, 2003), are recommended to come up with better phylogenetic analysis. The use of complete mitochondrial genome (Winkworth et al, 2022) sequences is also suggested to accurately infer evolutionary relationships in *Peronosporaceae*.

In this chapter, analysis on the isolates' COX1 region is still under investigation. Problems were encountered during the experimentation period. Reparation actions have been taken to overcome the issues encountered.



The images above are taken during the process of purifying the isolates. Observed structures are believed to be reproductive organs in oomycetes, the oogonia.

References

Abbey, J. A., Percival, D., Abbey, L., Asiedu, S. K., Prithiviraj, B., & Schilder, A. (2019). Biofungicides as alternative to synthetic fungicide control of grey mould (*Botrytis cinerea*)–prospects and challenges. *Biocontrol science and technology*, 29(3), 207-228.

Aizpurua-Olaizola, O., Soydaner, U., Öztürk, E., Schibano, D., Simsir, Y., Navarro, P., & Usobiaga, A. (2016). Evolution of the cannabinoid and terpene content during the growth of *Cannabis sativa* plants from different chemotypes. *Journal of natural products*, 79(2), 324-331.

Andre, C. M., Hausman, J. F., & Guerriero, G. (2016). *Cannabis sativa*: the plant of the thousand and one molecules. *Frontiers in plant science*, 19.

Burgess, T. I., White, D., & Sapsford, S. J. (2022). Comparison of primers for the detection of Phytophthora (and other oomycetes) from environmental samples. *Journal of Fungi*, 8(9), 980.

Burstein, S. (2015). CBD Receptor Binding Signaling Events Downstream Events. *Bioorg. Med. Chem.*

Chen, C., Belanger, R. R., Benhamou, N., & Paulitz, T. C. (2000). Defense enzymes induced in cucumber roots by treatment with plant growth-promoting rhizobacteria (PGPR) and *Pythium aphanidermatum*. *Physiological and molecular plant pathology*, 56(1), 13-23.

Choi, Y. J., Beakes, G., Glockling, S., Kruse, J., Nam, B., Nigrelli, L., & Thines, M. (2015). Towards a universal barcode of oomycetes–a comparison of the cox1 and cox2 loci. *Molecular ecology resources*, 15(6), 1275-1288.

Darriba, D., Posada, D., Kozlov, A. M., Stamatakis, A., Morel, B., & Flouri, T. (2020). ModelTest-NG: a new and scalable tool for the selection of DNA and protein evolutionary models. *Molecular biology and evolution*, 37(1), 291-294.

De Cock, A. W. A. M., & Lévesque, C. A. (2004). New species of *Pythium* and *Phytophthora*. *Studies in Mycology*, 50(2), 481-487.

Ellison, S. (2021). Hemp (*Cannabis sativa* L.) research priorities: Opinions from United States hemp stakeholders. *GCB Bioenergy*, 13(4), 562-569.

Feng, W., Hieno, A., Kusunoki, M., Suga, H., & Kageyama, K. (2019). LAMP detection of four plant-pathogenic oomycetes and its application in lettuce fields. *Plant Disease*, 103(2), 298-307.

Kozlov, A. M., Darriba, D., Flouri, T., Morel, B., & Stamatakis, A. (2019). RAxML-NG: a fast, scalable and user-friendly tool for maximum likelihood phylogenetic inference. *Bioinformatics*, 35(21), 4453-4455.

Lazreg, F., Belabid, L., Sánchez, J., & Gallego, E. (2016). Root rot and damping-off of Aleppo pine seedlings caused by *Pythium* spp. in Algerian forest nurseries. Journal of *Forest Science*, 62(7), 322-328.

Levesque, C. A., & De Cock, A. W. (2004). Molecular phylogeny and taxonomy of the genus *Pythium. Mycological research*, 108(12), 1363-1383.

Liu, Y., Vaghefi, N., Ades, P. K., Idnurm, A., Ahmed, A., & Taylor, P. W. (2023). *Globisporangium* and *Pythium* Species Associated with Yield Decline of Pyrethrum (*Tanacetum cinerariifolium*) in Australia. *Plants*, 12(6), 1361.

Mahmoud, M., BenRejeb, I., Punja, Z. K., Buirs, L., & Jabaji, S. (2023). Understanding bud rot development, caused by *Botrytis cinerea*, on cannabis (*Cannabis sativa* L.) plants grown under greenhouse conditions. *Botany.*

Martin, F. N., & Tooley, P. W. (2003). Phylogenetic relationships among *Phytophthora* species inferred from sequence analysis of mitochondrially encoded cytochrome oxidase I and II genes. *Mycologia*, 95(2), 269-284.

Mazzola, M., Andrews, P. K., Reganold, J. P., & Levesque, C. A. (2002). Frequency, virulence, and metalaxyl sensitivity of *Pythium* spp. isolated from apple roots under conventional and organic production systems. *Plant Disease*, 86(6), 669-675.

McGehee, C. S., & Raudales, R. E. (2021). Pathogenicity and mefenoxam sensitivity of *Pythium*, *Globisporangium*, and *Fusarium* isolates from coconut coir and rockwool in marijuana (*Cannabis sativa* L.) production. *Frontiers in Agronomy*, 3, 706138.

McLeod, A., Botha, W. J., Meitz, J. C., Spies, C. F., Tewoldemedhin, Y. T., & Mostert, L. (2009). Morphological and phylogenetic analyses of *Pythium* species in South Africa. *Mycological Research*, 113(9), 933-951.

McPartland, J. M. (1996). A review of Cannabis diseases. *Journal of the International Hemp Association*, 3(1), 19-23.

Nam, B., & Choi, Y. J. (2019). *Phytopythium* and *Pythium* species (Oomycota) isolated from freshwater environments of Korea. *Mycobiology*, 47(3), 261-272.

Paul, B. (2003). *Pythium glomeratum*, a new species isolated from agricultural soil taken in north-eastern France, its ITS region and its comparison with related species. *FEMS Microbiology Letters*, 225(1), 47-52.

Pérez-Sierra, A., Jung, M. H., & Jung, T. (2022). Survey and monitoring of *Phytophthora* species in natural ecosystems: methods for sampling, isolation, purification, storage, and pathogenicity tests. In *Plant Pathology: Method and Protocols* (pp. 13-49). New York, NY: Springer US.

Punja, Z. K. (2021). Emerging diseases of *Cannabis sativa* and sustainable management. *Pest management science*, 77(9), 3857-3870.

Punja, Z. K., Collyer, D., Scott, C., Lung, S., Holmes, J., & Sutton, D. (2019). Pathogens and molds affecting production and quality of *Cannabis sativa* L. *Frontiers in plant science*, 1120.

Punja, Z. K., & Rodriguez, G. (2018). *Fusarium* and *Pythium* species infecting roots of hydroponically grown marijuana (*Cannabis sativa* L.) plants. *Canadian Journal of Plant Pathology*, 40(4), 498-513.

Punja, Z. K., & Yip, R. (2003). Biological control of damping-off and root rot caused by *Pythium aphanidermatum* on greenhouse cucumbers. *Canadian Journal of Plant Pathology*, 25(4), 411-417.

Robideau, G. P., De Cock, A. W., Coffey, M. D., Voglmayr, H., Brouwer, H., Bala, K., & André Lévesque, C. (2011). DNA barcoding of oomycetes with cytochrome c oxidase subunit I and internal transcribed spacer. *Molecular ecology resources*, 11(6), 1002-1011.

Rosso, M. L., Rupe, J. C., Chen, P., & Mozzoni, L. A. (2008). Inheritance and genetic mapping of resistance to *Pythium* damping-off caused by *Pythium aphanidermatum* in 'Archer soybean. *Crop science*, 48(6), 2215-2222.

Russo, E. B., Jiang, H. E., Li, X., Sutton, A., Carboni, A., Del Bianco, F., & Li, C. S. (2008). Phytochemical and genetic analyses of ancient cannabis from Central Asia. *Journal of experimental botany*, 59(15), 4171-4182.

Sapkota, R., & Nicolaisen, M. (2015). An improved high throughput sequencing method for studying oomycete communities. *Journal of microbiological methods*, 110, 33-39.

Schardl, C. L., & Craven, K. D. (2003). Interspecific hybridization in plant-associated fungi and oomycetes: a review. *Molecular Ecology*, 12(11), 2861-2873.

Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., & White, M. M. (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the national academy of sciences*, 109(16), 6241-6246.

Scott, C., & Punja, Z. K. (2023). Biological control of *Fusarium oxysporum* causing damping-off and *Pythium myriotylum* causing root and crown rot on cannabis (*Cannabis sativa* L.) plants. *Canadian Journal of Plant Pathology*, 45(3), 238-252.

Sivan, A., Elad, Y., & Chet, I. (1984). Biological control effects of a new isolate of *Trichoderma harzianum* on *Pythium aphanidermatum*. *Phytopathology*, 74(4), 498-501.

Skoglund, G., Nockert, M., & Holst, B. (2013). Viking and early Middle Ages northern Scandinavian textiles proven to be made with hemp. *Scientific reports*, 3(1), 2686.

Soanes, D. M., Richards, T. A., & Talbot, N. J. (2007). Insights from sequencing fungal and oomycete genomes: what can we learn about plant disease and the evolution of pathogenicity? *The Plant Cell*, 19(11), 3318-3326.

Spies, C. F., Mazzola, M., Botha, W. J., Langenhoven, S. D., Mostert, L., & Mcleod, A. (2011). Molecular analyses of *Pythium irregulare* isolates from grapevines in South Africa suggest a single variable species. *Fungal biology*, 115(12), 1210-1224.

Thiessen, L. D., Schappe, T., Cochran, S., Hicks, K., & Post, A. R. (2020). Surveying for potential diseases and abiotic disorders of industrial hemp (Cannabis sativa) production. *Plant Health Progress*, 21(4), 321-332.

Uzuhashi, S., Kakishima, M., & Tojo, M. (2010). Phylogeny of the genus *Pythium* and description of new genera. *Mycoscience*, 51(5), 337-365.

van Bakel, H., Stout, J. M., Cote, A. G., Tallon, C. M., Sharpe, A. G., Hughes, T. R., & Page, J. E. (2011). The draft genome and transcriptome of *Cannabis sativa*. *Genome biology*, 12(10), 1-18.

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

Winkworth, R. C., Neal, G., Ogas, R. A., Nelson, B. C., McLenachan, P. A., Bellgard, S. E., & Lockhart, P. J. (2022). Comparative analyses of complete peronosporaceae (oomycota) mitogenome sequences—insights into structural evolution and phylogeny. *Genome Biology and Evolution*, 14(4), evac049.

White, T. J., Bruns, T., Lee, S. J. W. T., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications*, 18(1), 315-322.

Chapter 5 General Conclusion

In this research, isolates collected from the Philippines and Japan have been analysed to establish an inventory of *Oomycota* associated with cropping sites of *Theobroma cacao L*. and *Cannabis sativa* L. The phylogeny and morphologies of the hitherto pathogens have also been revealed and described based on modern taxonomical criteria. Based on the carried-out experiments, oomycetes isolates collected from cropping sites of cacao are currently identified as *Phytopythium cucurbitacearum*, *Pythium acanthophoron*, *Pythium nunn* and *Pythium plurisporium*, based on phylogenetic analysis using Maximum Likelihood analysis of ITS and COX1 regions. As for isolates collected from hemp cultivation areas in Japan, phylogenetic Maximum Likelihood analysis of ITS region revealed the isolates to be *Pythium aphanidermatum*, *Pythium glomeratum*, *Pythium irregulare*, and *Pythium rostratifingens*. This research contributes towards understanding species composition and species diversity in various ecological niches, saprophytes, and plant pathogens.

Throughout the process of oomycetes baiting, several non-oomycetes isolates were collected and analysed for identification. Species such as *Lasiodiplodia theobromae*, *Fusarium oxysporum* and *Colletotricum fructicola* were identified. These species are known to be important plant pathogens with wide host species. Such findings contribute more to emphasize and understand microbial communities composed of oomycetes and non-oomycetes members. Alas, these establishment of species inventory are valuable instruments for the cropping sites.

Every research carried out has plenty of room for improvements. As in this study, familiarization with the research field of the study is crucial to develop skills, vocabularies and knowledge required for successful isolation and identification of oomycetes. As oomycete is a diverse group, more research should be taken more critically to properly define this group of important plant pathogens.