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Pythium ultimum and *Phytopythium vexans*, the Potential Pathogen Isolated from Potato Rhizosphere in Central Java, Indonesia

Pythium ultimum dan *Phytoppythium vexans*, Patogen Potensial yang Diisolasi dari Risosfer Kentang di Jawa Tengah Indonesia

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ABSTRAK

Phytophthora dan *Pythium* merupakan kelompok *Oomycetes* yang banyak berasosiasi dengan penyakit pada tanaman kentang. Penelitian ini bertujuan untuk mengidentifikasi *Oomycetes* yang berasosiasi dengan rizosfer kentang yang menunjukkan gejala penyakit hawar daun. Empat isolat berhasil dikoleksi dari empat wilayah sentra pertanaman kentang di Jawa Tengah (isolat UGM_St_TM, UGM_St_BK , UGM_St_BNJ , dan UGM_St_KJ berturut-turut dari Temanggung, Bakal, Banjarnegara dan Kejajar) dan satu isolat UGM_St_NG koleksi dari Laboratorium Ilmu Penyakit Tumbuhan. Identifikasi molekuler dari semua isolat dilakukan menggunakan penanda gen internal transscribed spacer (ITS1/ITS4), Nuclear large-ribosomal subunit (LSU), dan cytochrome C oxidase subunit 1 (COX1). Berdasarkan hasil penelitian, isolat UGM_St_TM, UGM_St_BK, dan UGM_St_BNJ teridentifikasi sebagai *Phythium ultimum* sedangkan isolat UGM_St_KJ dan UGM_St_NG teridentifikasi sebagai *Phytopythium vexans*.

Kata kunci: identifikasi, karakter morfologi, *oomycetes*, penanda gen

ABSTRACT

Phytophthora and *Pythium* are a group of *Oomycetes* that are widely associated with diseases in potato plants. Therefore, this study was conducted to identify the *Oomycetes* associated with the rhizosphere of infected potato plants showing leaf blight. Four isolates were collected from four regions in Central Java (UGM_St_TM, UGM_St_BK, UGM_St_BNJ, and UGM_St_KJ, the isolate from Temanggung, Bakal, Banjarnegara, Kejajar respectively), and UGM_St_NG isolate as culture collection from Laboratory of Plant Pathology. Molecular identification of all isolates was carried out using the internal transcribed spacer (ITS1/ITS4), nuclear large-ribosomal subunit (LSU), and cytochrome C oxidase subunit 1 (COX1) gene markers. Based on the results, the isolates UGM_St_TM, UGM_St_BK, and UGM_St_BNJ were identified as *Phythium ultimum* while UGM_St_KJ and UGM_St_NG isolate were identified as *Phytopythium vexans*.

Keywords: gene marker, identification, morphology characters, *oomycetes*

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INTRODUCTION

Potato (*Solanum tuberosum*) is an important horticultural commodity with high economic value (Asgar 2013). Infection of fungal pathogens has been reported worldwide as one of the limiting factors for potato production. *Phytophthora infestans* from the class *Oomycetes* is known as the primary pathogen in potatoes that causes leaf blight which may lead to yield losses of up to 100% (Nathasia *et al.* 2014). *Phytopythium* is a new genus of *Oomycetes* that has been reported to cause disease in many crops (Baten *et al.* 2015). The genus *Phytopythium* belongs to the family *Peronosporaceae* and to the order *Peronosporales* (Beakes *et al.* 2014). It has similar morphological and physiological characteristics to the genera *Pythium* and *Phytophthora*, such as having a mechanism of zoospore release similar to *Pythium*, producing papillary sporangium with a globose to ovoid shape, and the presence of internal proliferation as in *Phytophthora* (Bala *et al.* 2010). de Cock *et al.* (2015) also reported that the genus *Phytopythium* was grouped between *Pythium* and *Phytophthora* based on phylogenetic analysis using ITS, LSU, SSU, and COI markers.

Phytopythium vexans was first reported as the cause of root rot in kiwifruit in Turkey (Polat *et al.* 2017), while Santoso *et al.* (2015) also reported an association of *P. vexans* and a disease in durian plants in Indonesia. Later on, Santika *et al.* (2021) identified *P. vexans* from the rhizosphere of potato plants in Ngablak, Magelang, Central Java. Therefore, this study aims to identify the *Oomycetes* from the potato rhizosphere associated with rot disease and determine its ability to infect potato plants.

MATERIAL AND METHODS

Exploration and Sample Collection

A disease survey was conducted in August 2020 in several potato-growing areas in Central Java, including Kledung (Temanggung), Bakal (Banjarnegara), Pejawaran (Banjarnegara), and Kejajar (Wonosobo). Soil samples were taken at a 5–10 cm depth around the rhizosphere of potato plants showing leaf or stem blight symptoms. In addition, the isolate collection of the Laboratory of Plant Pathology, Universitas Gadjah Mada from Ngablak, Magelang (isolates UGM_St_NG) was included in this study. Nucleotide sequences of this isolate have been submitted to GenBank, i.e., MW898226 (ITS) and MW911663 (LSU).

Fungal Isolation and Purification

Fungal isolation was carried out following the modified soil baiting method (Santoso *et al.* 2015). Apples were the first surface disinfected using 70% alcohol and perforated using a cork borer (0.5 cm in diameter) at four opposite points. The hole was then filled with soil samples and covered with tape. Thereafter, incubation was carried out in containers at room temperature. Observations were made every day until the initial symptoms of soft brown spots appeared (more or less 2–3 days after inoculation). Symptomatic tissues were cut out at the border between healthy and symptomatic parts, around 2 × 3 mm in size, and then isolated on potato dextrose agar (PDA) medium and incubated for 2–3 days. Furthermore, the fungi that grew from the slices were transferred to a water agar (WA) medium and cultured as a pure culture. The morphology of each isolate, including sporangium, hyphae, chlamydospore, and colony shape, was observed according to Abad *et al.* (2019) 7 days after the incubation period.

DNA Extraction, PCR Amplification, and Sequencing

Five days old fungal cultures were cut using a scalpel and weighed up to 0.5 g extraction and amplification of DNA were carried out using the CTAB procedure (Doyle and Doyle 1990) and modified multigenic analysis method, respectively, based on polymerase chain reaction (PCR) using ITS1/ITS4 (Ochoa *et al.* 2012), LSU (Schurko *et al.* 2003), and COX1 (Martin and Tooley 2003) as shown in Table 1. Each amplification reaction was carried out in a total volume of 13 µL, which was composed of 1 µL DNA template for each isolate, 6.5 µL of PCR mix (My Taq HS Red Mix: Geneaid), 4.5 µL of dDH2O, and 0.5 µL each of primers pair. The PCR products were loaded into 1%

Table 1 Primers used for PCR amplification and DNA sequencing

Gene	Primer	Primer Sequence (5'-3')	Reference
ITS	ITS 1	TCCGTAGGTGAACCTGCGG	Ochoa *et al*. (2012)
	ITS 4	TCCTCCGCTTATTGATATGC	
LSU	UN-up28S40	5-GCATATCAATAAGCGGAGGAAAAG-3	Schurko *et al*. (2003)
	UN-LO28S576B	5-CTCCTTGGTCCGTGTTTCAAGACG-3	Bakkeren (2000)
COX1	OomCoxILevup	5-TCAWCWMGATGGCTTTTTTCAAC-3	Martin and Tooley (2003)
	Fm85mod	5-RRHWACKTGACTDATRATACCAAA-3	

agarose gel (w/v), electrophorized with a voltage of 55 V for 50 minutes, and visualized with UV light after ethidium bromide staining. DNA sequencing analysis was carried out at the Integrated Laboratory for Researching and Testing of Gadjah Mada University, Yogyakarta.

Phylogenetic Analysis

Sequence data from the *Oomycetes* isolates were aligned and compiled using the ClustalW method. The results were used as input data to construct phylogenetic trees using the maximum likelihood method with 1000 bootstrap values. Furthermore, the phylogenetic tree was generated using Mega-X software (Kumar *et al.* 2016). The DNA sequence data were used to analyze the isolates' similarities compared to data from GeneBank using the NCBI BLAST-N program (Basic Local Alignment Search Tool) on http://www.ncbi.nih.gov/BLAST (Table 2).

RESULTS

Morphological Characteristics of Isolate

There were 4 isolates successfully collected from the potato rhizosphere, namely UGM_St_TM (Kledung-Temanggung), UGM_St_BK (Bakal-Banjarnegara), UGM_St_BNJ (Pejawaran-Banjarnegara), and UGM_St_KJ (Kejajar-Wonosobo). All of the isolates, including UGM_St_NG (laboratory collection), showed rapid growth on the PDA medium or with an average of 3–4 days after incubation. General characteristics observed from all isolates were white colonies, aseptic hyphae, and thick-walled chlamydospores (Figure 1). Moreover, the isolates of UGM_St_TM, UGM_St_BK, and UGM_St_BNJ had cottony-shaped colonies, while UGM_St_KJ and UGM_St_NG had chrysanthemum and rosaceous stellate-shaped colonies, respectively. Sporangium grew on tenth day after incubation at 25 °C. While UGM_St_TM, UGM_St_BK, and UGM_St_BNJ isolates produced papillary and non-papillary sporangium with globose, ovoid, and obovoid shapes, UGM_St_NG isolate produced no sporangium. The morphological characteristics of isolates are presented in Figure 1 and Table 3.

Analysis of Phylogenetic Tree

Maximum likelihood analysis based on ITS, LSU, and COX1 with a bootstrap value of 1000 was presented in a phylogenetic tree (Figure 2). Redundant sequences of GenBank accessions were identified, and those with 100% identity to other included taxa were removed from the analyses. These duplicates are cataloged in Table 2. The cladogram showed that the isolates UGM_St_TM, UGM_St_BK, and UGM_St_BNJ were in the same group as other *P. ultimum* isolates. In contrast, the isolates UGM_St_KJ and UGM_St_NG (MW898226) were found in the group of *P. vexans*. Based on the LSU analysis, isolates UGM_St_TM, UGM_St_BK, and UGM_St_BNJ have the closest relationship with *P. ultimum* from Japan (AB513047); while UGM_St_KJ and UGM_St_NG (MW911663) isolates have the closest relationship with *P. vexans* from Canada (HQ665090) and Iran (MT729990), respectively. Furthermore, phylogenetic analysis based on COX1 showed that isolates UGM_St_TM, UGM_St_BK, and UGM_St_BNJ were closely related to *P. ultimum* from the Netherlands (HG708919) and America (KF761145), while the isolates of UGM_St_

Table 2 GenBank accessions of *Pythium* and *Phytopythium* involved in phylogenetic analysis

Species	ITS		LSU		COX1	
	Country	Genbank Accession Number	Country	Genbank Accession Number	Country	Genbank Accession Number
Phytopythium vexans	Thailand	MT758165	Canada	HQ665090	Brazil	KX429661
Phytopythium vexans	China	MF196966	China	KX092469	America	MT076052
Phytopythium vexans	Pakistan	MK035704	Japan	AB468722	Italy	MN510424
Phytopythium vexans	Turkey	MW425424	Iran	MT729990		
Phytopythium vexans	Japan	AB468784	Turkey	KY024344		
Phytopythium vexans	Indonesia	KP183960	Italy	MN510428		
Phytopythium vexans	India	MN227195				
Phytopythium vexans	UK	MF115286				
Phytopythium vexans	Pakistan	MG799213				
Phytopythium vexans	Vietnam	MN872764				
Phythium ultimum	USA	MK326555	Canada	HQ665227	America	KF761145
Phythium ultimum	Canada	MH023356	Canada	HQ665103	South Africa	GU071815
Phythium ultimum	Uruguay	KY433893	Japan	AB468719	Canada	KJ639172
Phythium ultimum	UK	MF115493	Japan	AB513047	Canada	HQ708963
Phythium ultimum	China	KU746661			Netherlands	HQ708919
Phythium ultimum					UK	KJ639184
Phythium ultimum					Lebanon	KJ639178
**Phytophthora infestan*	Ethiopia	KM078914	Netherlands	HQ708309	Ethiopia	KM078914
**Phytophthora infestan*	China	MN458152	UK	MH760241	China	MN458152
**Phytophthora infestan*	Ireland	AF348606	Russia	HQ261335	America	AF348599
**Phytophthora infestan*	USA	AF348598			Netherlands	AY564150
**Phytophthora infestan*	USA	AF348599				
**Phytophthora infestan*	Netherlands	AY564150				
**Phytophthora infestan*	Ecuador	MG869099				

*Outgroup

KJ and UGM_St_NG were closely related to *P. vexans* from Italy (MN510424). The phylogenetic tree analysis based on the three primers showed that UGM_St_TM, UGM_St_BK, and UGM_St_BNJ were identified as *P. ultimum* with a similarity level of 99%–100%. Meanwhile, UGM_St_KJ and UGM_St_NG were identified as *P. vexans* based on ITS and LSU with a similarity level of 99%–100%, and based on COX1 with a similarity of 98%–99% (data not shown) (Table 4).

DISCUSSION

Five *Oomycetes* isolates from Central Java potato fields had similar morphological characteristics. The hyphae were generally hyaline and aseptate, while the chlamydospore had thick walls and was located intercalary and terminally. In addition, the isolates mostly had a globose to ovoid shape of the sporangium, while colony morphology varied from cottony to chrysanthemum and rossaceous-stellate. Therefore, morphological characteristics observed in this study were unable to distinguish the presence of different genus between the isolates. These morphology characters were in line with the characteristics of *Pythium* and *Phytopythium* described by Bala *et al.* (2010) and Uzuhashi *et al.* (2010). Other references showed that *P. vexans* produced stellate colonies when incubated at 18 °C on a PDA medium (Santika *et al.* 2021). *P. vexans* and several types of *Pythium* had a cottony aerial colony with a spreading pattern when grown on V8, PDA, and corn meal agar (CMA) medium at 25 °C (Nam and Choi 2019).

Further molecular identification of these *Oomycetes* isolates was carried out to observe their genetic relationship. The use of ITS, LSU, and COX1 as molecular markers for

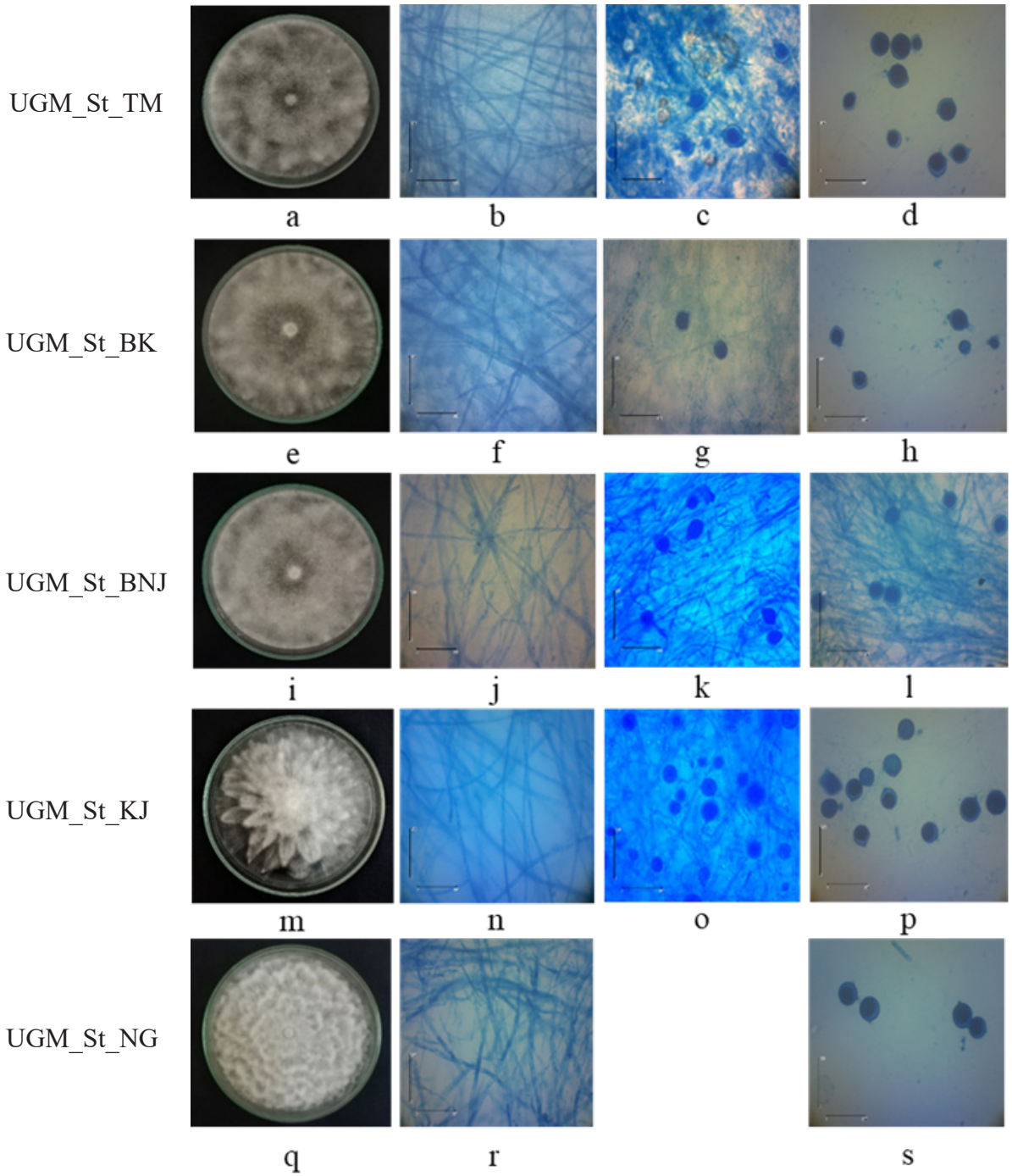

Figure 1 Morphology of fungal isolates. a, e, i, m, q are colony patterns; b, f, j, n, and r are aseptate hyphae; c, g, k, o are sporangium; d, h, l, p are terminal and intercalary chlamydospores with thick walls. Bar scale 50 µm.

Pythium and *Phytopythium* has been widely reported. The markers have been used to identify and characterize, among others *P. vexans* in Vietnam (Thao *et al.* 2020), *Pythium* spp. from soil samples in Illinois, America (Radmer *et al.* 2017), and a new species of *Phytopythium* and *Pythium* in Korean waters (Nam and Choi 2019).

Pythium ultimum and *P. vexans* species have been identified from the rhizosphere of potato plants in Central Java by using three different molecular markers. Both species had the same microscopic morphological characteristics, such as sporangium, hyphae, and chlamydospore, but had different colony forms. The temperature was one of the factors

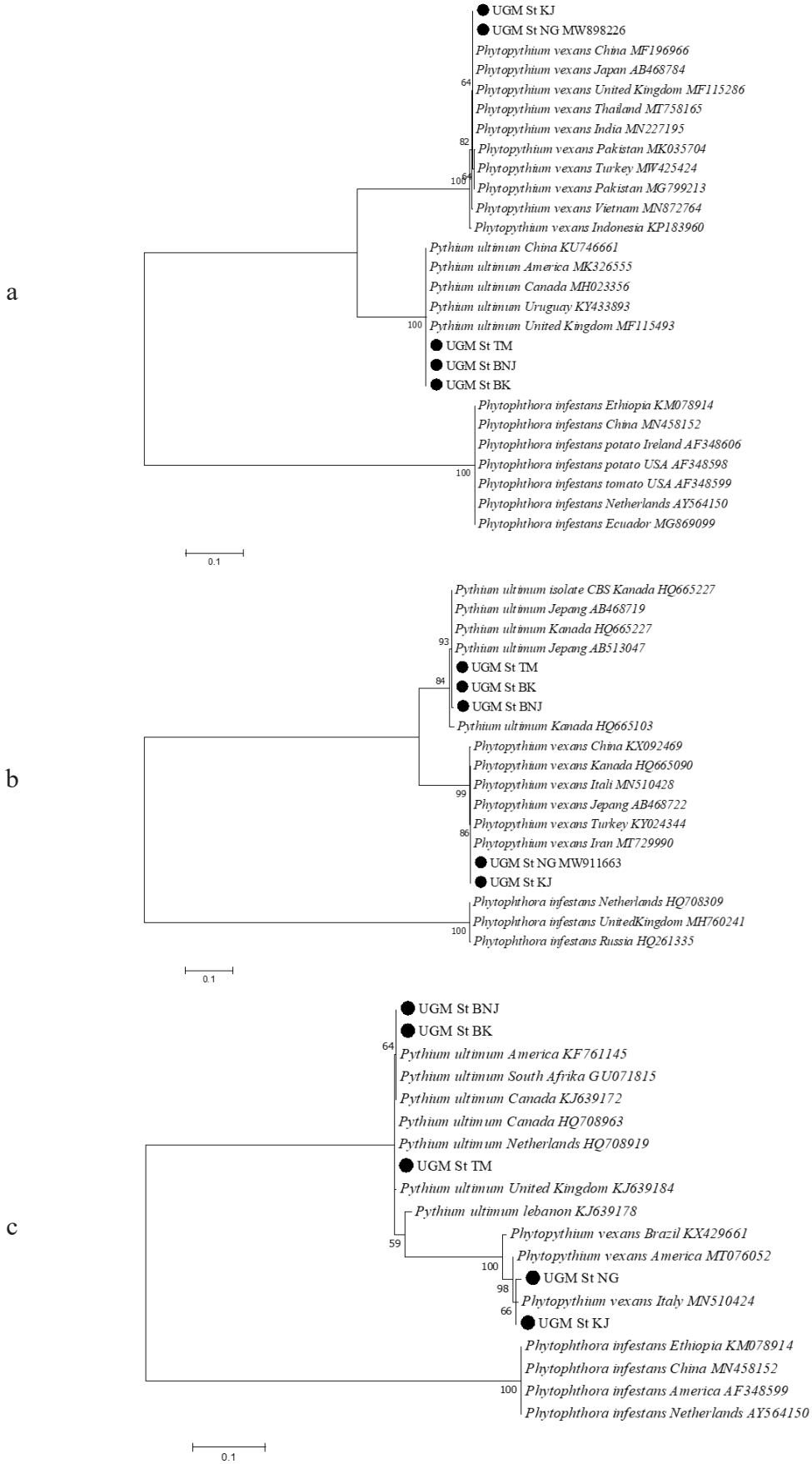

Figure 2 Maximum likelihood phylogenetic trees of: a, ITS; b, LSU ribosomal RNA region; and c, cytochrome c oxidase subunit 1 COX. Maximum likelihood 1000 bootstrap.

Table 3 Morphological characteristics of *Oomycetes* isolates from Central Java

Characters colony	UGM_St_TM	UGM_St_BK	UGM_St_BNJ	UGM_St_KJ	UGM_St_NG
Pattern	cottony	cottony	cottony	Rossaceous-stellate	Chrysanthemum
Color	white	white	white	white	white
Sporangium Size l × w (µm)	25.69 ± 8.22 × 21.10 ± 6.72	25.23 ± 2.94 × 23.00 ± 2.23	24.36 ± 4.49 × 19.29 ± 2.23	25.52 ± 3.21 × 23.00 ± 3.57	No present
Description	Globose to ovoid with papillae	Globose to ovoid semi-papillae	Globose and non-papillary obovoid	Globose non-papillary and ovoid with papillae	
Hyphae (µm)	4.19 ± 0.60	3.26 ± 0.79	3.60 ± 0.78	4.47 ± 1.37	4.07 ± 0.59
Chlamydospore Size l × w (µm)	21.38	21.71	18.24	15.71	17.21
Description				intercalar chlamydospores with thick walls	

*Isolate collection Laboratory of Plant Pathology Gadjah Mada University

Table 4 Identification of isolates from potato plants based on analysis of the phylogenetic relationship

Isolate code	Origin		Species
	Village	Districts	
UGM_St_TM	Kledung	Temanggung	*Pythium ultimum*
UGM_St_BK	Bakal	Banjarnegara	*Pythium ultimum*
UGM_St_BNJ	Pejawaran	Banjarnegara	*Pythium ultimum*
UGM_St_KJ	Kejajar	Wonosobo	*Phytopythium vexans*
*UGM_St_NG	Ngablak	Magelang	*Phytopythium vexans*

*Isolate collection Laboratory of Plant Pathology Gadjah Mada University

that influenced the growth of pathogens. The level of pathogenicity and resistance was influenced by the potato variety used. Potato varieties M07 and Grandia showed a lower level of resistance than the Granola variety.

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