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Short Note

First Report of *Phytopythium vexans* (de Barry) Abad, de Cock, Bala, Robideau, Lodhi & Lévesque Causing Potato Tuber Rot in Indonesia

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ABSTRACT

Phytopythium vexans (de Barry) Abad, de Cock, Bala, Robideau, Lodhi & Lévesque was successfully isolated from soil of potato fields in Ngablak, Magelang. This research aimed to obtain knowledge of *P. vexans* potency as a pathogen on potatoes, and also morphologically and molecularly identify *P. vexans* compared to Oomycetes, *Phytophthora* and *Pythium*. Morphological identification was conducted by observing macroscopic colony pattern that grew on PDA medium for five days and microscopic observation on its hyphae, sporangia, papillate, and chlamydospore. Molecular identification was conducted using multigene analysis, ITS and LSU. The in vitro pathogenicity test was done by inoculating *P. vexans* inoculum to healthy potato leaves and tubers. Results of morphological observation showed that *P. vexans* had a stellate pattern, aseptate hyphae, ovoid shaped sporangium and completed with semipapillate. Chlamydospores were also found and often used for survival. This research revealed that the morphology characters of *P. vexans* were combination between the characteristics of *Pythium* and *Phytophthora*. Whereas, based on molecular analysis using ITS and LSU, *Phytopythium* spp. was more closely related to *Phytophthora* spp. rather than *Pythium* spp. The pathogenicity test of *P. vexans* showed that it could infect the flesh of potato tubers by showing a brown lesions symptom.

Keywords: ITS; LSU; oomycetes; potato; *Phytopythium*

INTRODUCTION

Potato (*Solanum tuberosum* L.) is an essential non-cereal staple food and is widely cultivated in more than 100 countries. The tubers contain high nutrients, including carbohydrates, protein, minerals, fiber, and vitamins (Zhang *et al.*, 2017). In Indonesia, potato production centres area spread in the highlands across Sumatra and Java. Based on Central Statistics Agencies of Magelang Regency in 2018, Ngablak is one of the potato production areas located in Central Java that produced 44,540 quintals from 141 ha cultivated area (*Badan Pusat Statistik Kabupaten Magelang*, 2018). Nevertheless, pathogens and pests are still a severe potato production problem.

The most destructive soil-borne pathogen is *Phytophthora infestans* and is globally known as the causal agent of potato late blight. Millions of people died due of the Ireland-Irish famine (Hammond-Kosack, 2014). Another pathogen of potato production in Indonesia, *Pythium vexans*, was mentioned by Centre of Agriculture and Biosciences International. (1987). No further information was found about this pathogen, especially from potato production areas in Indonesia. Lévesque and de Cook (2008) have proposed a new genus called *Phytopythium* spp. based on the molecular analysis to separate the clade K from the *Pythium* genus, one of the clade K members is *Phytopythium vexans*.

In the last five years, *P. vexans* was first reported by many countries and also its destructive potential on various host plants, including dieback disease in Morocco (Jabiri *et al.,* 2020), decline syndrome of kiwi in Italy (Prencipe *et al.*, 2020), rot and collar rot of Kiwi in Turkey (Polat *et al.*, 2017), and root rot on Mandarin (*Citrus reticulate* L. cv. Sainampueng) in Thailand (Noireung *et al.*, 2020). Rapid and proper identification are required before controlling pathogens. The Internal Transcribed Spacer (ITS) region is the most common region of DNA used to identify Oomycetes. Nevertheless, due to the accurate comparison issues, Robideau *et al.* (2011) zused a multigene analysis method using ITS, Cytochrome c Oxidase subunit I (COX1), and nuclear, large subunit (LSU) rDNA with 28S rDNA to define genus including *Pythium, Phytophthora*, and *Phytopythium* from Oomycetes. In Indonesia, especially in potato productions, studies about *Phytopythium* have not been conducted to our knowledge. Therefore it is essential to study *P. vexans* isolates based on molecular, morphology, and pathogenicity of this Oomycetia soil-borne pathogen found in potato production.

MATERIALS AND METHODS

Soil-Borne Pathogen Isolation

The pathogen was isolated from soil of potato fields located at Ngablak, Magelang (7º23′57.386″ S 110º23′52.242″ E) in 2019. The isolation was conducted using the *soil baiting method* according to Purwantisari and Hastuti (2009) with modification. Apple cv. Manalagi were bored using 0.5 cm boring tool and filled with collected soil. Holes were closed with tips and preserved in a plastic box completed with wet cotton to keep the high moisture levels. It was incubated at room temperature for 3–4 days until brown lesions appeared. Test apples were cut at regions with both healthy and lesion portions, then cultured on *Carrot Sucrose Agar,* amended with Rifampicin 1000 ppm (20 ml/L), and incubated at 18ºC in dark condition. After 5–6 days, the isolates that appeared were subcultured to PDA. In this research, we have found a unique culture with a papillate pattern named NG isolate and used for further studies.

Morphological Observation

Morphological observations were conducted based on the macroscopic and microscopic characteristics of the five-day-old NG isolate. Macroscopic observation described the pattern and color of the colony. Microscopic observation described the shape and size of sporangia, chlamydospore, hyphae from the isolate. Observations were conducted under a binocular microscope (Olympus X32). The results were compared to literatures for genus identification based on the morphological characteristics (Paul *et al.*, 2006; Bridge *et al.*, 2008; Santoso, 2016; Bennett *et al.*, 2017; Ho, 2018; Nam & Choi, 2019; Gómez-González *et al.*, 2020; Shimelash & Dessie, 2020).

In Vitro-Pathogenicity Test

The pathogenicity test of *P. vexans* was conducted using the NG isolate and inoculated on healthy potato leaves and tubers. For the potato tuber, the pathogenicity test was conducted according to Gherbawy *et al.* (2019) with modification. Healthy potato tubers weighing 100–150 g were selected for this test. Potatoes were cut into 1 cm thick pieces. While for potato leaves, healthy leaves were sterilized in sodium hypochlorite solution for 3 minutes, rinsed using sterile distilled water three times, and dried under a laminar flow. The tubers and leaves were placed in a petri dish amended with a small ball of wet cotton to maintain the moisture inside. Then, an agar cut (± 1 cm^2) with active mycelia from NG isolate was placed in the center of potato tubers and leaves for inoculation. Other treatments used different inoculum. The positive control was used a piece of potato leaves consisting of *P. infestans'* spores and the negative control used water agar. All treatments were incubated in the dark at 18°C for seven days.

Molecular Identification

The DNA extraction from five days-old NG isolates was carried out using Genomic DNA Mini Kit (Plant) Protocol (GeneAid, Australia) under the kit protocol. Internal transcribed spacer (ITS) and Large Subunit Ribosomal (LSU) were amplified using ITS1/ITS4 and UN-up28S40/UN-lo28S576B primers (Robideau *et al.*, 2011). PCR reactions were performed in 25 μl mixture of 12.5 μl of 2x DNA Taq polymerase (MyTaq HS Red Mix; Bioline, London, United Kingdom), 9.5 μl miliQ sterile water, 1 μl of 0.08 lM each marker forward and reverse, and 1 μl of DNA template in T100 Thermal Cycler machine (Biorad, California, United States). The temperature adjustment on PCR cycling was based on Standard MyTaq HS Red Mix Protocol and annealing temperatures were set to

55°C for ITS and 51°C for LSU. The PCR products were evaluated on 1% agarose gel and run in 100 V for 30 min. Then, the gel was colored with ethidium bromide for 15 min and visualized under UV Transilluminator. Sequence analysis was carried out at 1st BASE, Malaysian service providers.

Phylogenetic Analysis

Phylogenetic analysis was constructed from NG isolate and consensus sequences from the previous research by Robideau *et al.* (2011). The consensus sequences were selected and explored as representatives from Oomycetes (*Phytopythium, Pythium,* and *Phytophthora*) using BLAST in Table 1. All sequences were aligned by ClustalW using MEGA X. The phylogenetic tree was constructed from ITS and LSU sequences by adjusting the Maximum Likelihood (ML) method with the Kimura 2-parameter model and tested by 1000 bootstrap replications.

RESULTS AND DISCUSSION

Morphological Observation

The morphological description of the *P. vexans* colony was described from five-day-old cultures growing on PDA. *P. vexans* colony, which was incubated at dark conditions and 18ºC, was white, had thin mycelia, and stellate patterns. This Oomycetes colony grew fast, covering 9 cm of petri dish in 5 days, as shown in Figure 1. Further microscopic observation was conducted from this isolate, as shown in Figure 2. In Table 2, *P. vexans'* characteristics were compared to *Phytophthora*, *Pythium*, and *Phytopythium*.

Based on macroscopic observation results, the colony pattern of *P. vexans* was similar to *Phytopythium*, especially *P. vexans* from durian, which colony had *patellate* or *stellate* patterns (Santoso, 2016). Nam and Choi (2019) also isolated *P. vexans* on several media for 72 hours and resulting in different mycelial diameters, such as 35–40 mm on PDA, >70 mm on V8 medium and 55–60 mm on CMA (Corn Meal Agar). However, it did not change the pattern of the mycelial colonies. *P. infestans* had a radiate growth pattern on various mediums such as on lime bean agar (LBA), on Rye A and Carrot agar (AZ) (Gómez-González *et al.*, 2020), as well as *Pythium* (Paul *et al.,* 2006).

Based on microscopic observations, the seven-day-old culture of NG isolates showed hyphae sizes of 3.36–4.60 μm, was less than 5 μm and similar to *Phytopythium*. Comparison between *Phytopythium* to *Phytophthora* and *Pythium*, both would have a larger diameter of hyphae, 5–7μm and 4–6μm respectively (Ho, 2018). Further observations were conducted until the culture of *P. vexans* reached 30 days old and at 25ºC, chlamydospore and sporangium were found. Chlamydospore is known as the survival structure

Table 1. List of isolates and accession numbers of *Phytopythium, Pythium*, and *Phytophthora* used as a reference in the phylogenetic analysis

No.	Species	Isolate	Genebank Accesion Number	
			ITS	LSU
1	*Pythium insidiosum*	CBS 574.85	HQ643570	HQ665273
2	*Pythium aphanidermatum*	CBS 118.80	HQ643438	HQ665084
3	*Pythium deliense*	CBS 314.33	HQ643522	HQ665204
4	*Phytopythium vexans*	P3980	HQ261730	EU080487
5	*Phytopythium vexans*	CBS 119.8	HQ643400	HQ665090
6	*Phytopyhium litorale*	CBS 122662	HQ643385	HQ665114
7	*Phytopythium boreale*	CBS 551.88	HQ643372	HQ665261
8	*Phytopythium ostracodes*	CBS 768.73	HQ643395	HQ665295
9	*Phytophthora vignae*	P3019	HQ261724	EU079787
10	*Phytophthora palmivora*	CBS298.29	HQ643307	HQ665195
11	*Phytophthora sojae*	P3114	HQ261677	EU079794
12	*Phytophthora infestans*	P10650	HQ261589	EU079630
13	*Phytophthora tentaculata*	*CBS 55296*	HQ643365	HQ665264
14	*Eurychasma dicksoni**	FI373	HQ643131	HQ665307

*outgroup

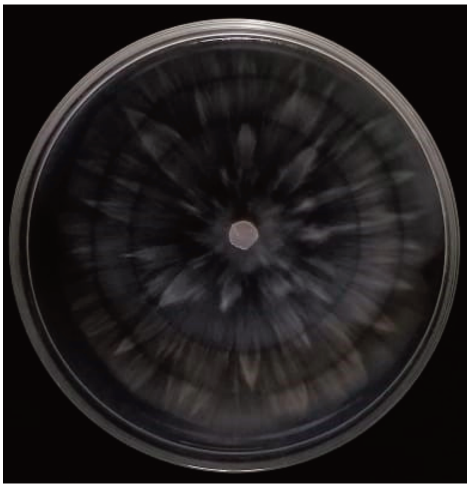

Figure 1. The stellate pattern of *Phytopythium vexans* incubated at 18°C five days old on Potato Dextrose Agar (PDA) media

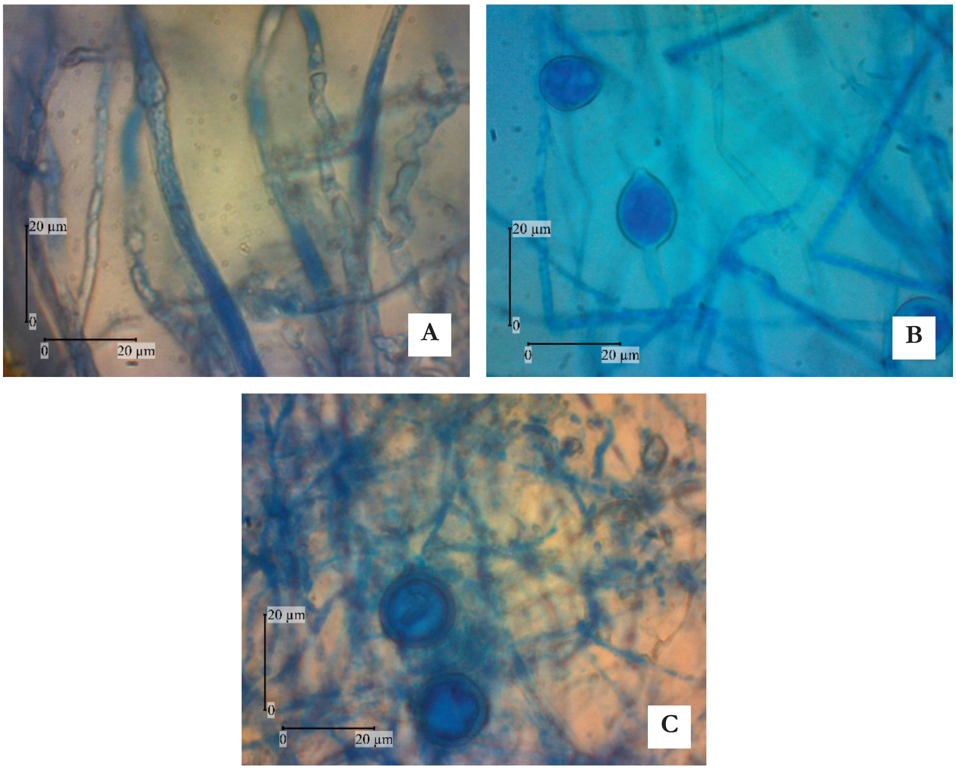

Figure 2. *Phytopythium vexans*; aseptate hyphae (A), sporangium (B), chlamydospore (C)

of pathogens in response to insufficient nutrients or unsuitable environment condition for optimum growth. The presence of chlamydospores from *P. infestans* Ic haplotype isolate collected from Ethiopia was found on rye and pea agar plates after 1 to 4 weeks of incubation of single cultures at 20°C in the dark (Shimelash & Dessie, 2020). Chlamydospore sizes of *Phytophthora* and *Pythium* were 20–25 μm and 40–70 μm, respectively (Bridge *et al.*, 2008).

Meanwhile, Bennett *et al.* (2017) reported that no chlamydospore were observed from *Phytopythium* spp. In this study, *P. vexans* chlamydospore diameters was 15.79 µm. *P. vexans* produced ovoid-shaped sporangia and completed with semipapillate. This papillate makes the *P. vexans* to be excluded from *Phytium* sp. Only *Phytophthora* and *Phytopythium* were completed with papillate (Ho, 2018). Both *Phytophthora* and *Phytopythium* have different mechanisms for

Table 2. List of characteristic differences of P*hytophthora infestans, Pythium, Phytopythium vexans*, and NG isolate

Characteristics	*Phytophthora infestans* [7), 8)]	*Pythium* [1), 2), 5)]	*Phytopythium vexans* [3), 4), 6)]	NG isolate
Colony Pattern	radiate	radiate	stellate or patellate	stellate
Hyphae diameter	5–7µm	4–6µm	Up to 5 µm	3,36–4,60 µm
Sporangia shape	lemon-shaped	globose	globose, ovoid, ellipsoid, lemoniform, and obpyriform	ovoid
Sporangia size (Length and width)	60.5 µm and 31.7 µm	15–55 µm in diameter	7–28 µm and 5–22 µm.	16.78 µm and 12.59 µm
Chlamydospore size	20–25 µm	40–70 µm	not observed	15.79 µm in diameter
Papillate	semipapillate	absence	papillate	semipapillate

[1)]Paul *et al.* (2006); [2)]Bridge *et al.* (2008); [3)]Santoso (2016); [4)]Bennett *et al.* (2017); [5)]Ho (2018); [6)]Nam & Choi (2019); [7)]Gómez-González *et al.* (2020); [8)]Shimelash & Dessie (2020).

zoospore discharge. In this case, *Phytophthora infestans* produces lemon-shaped sporangia and are completed with semipapillate. The zoospore was released from the papillate (Shimelash & Dessie, 2020). After being reclassified as a new genus, *Phytopythium* known for the morphologically and discharged of zoospore characteristic were intermediate between *Phytophthora* and *Pythium* (Ho, 2018). The sporangia size of *P. vexans* was 16.78 µm in length and 12.59 µm in width. This sporangia size was smaller than ones of *Phytophthora* with a length of 60.5 µm and a width of 31.7 µm (Gómez-González *et al.*, 2020). The sporangia of *Pythium*, with a globose shape, was 15–55 µm in diameter with an average of 32.6 µm (Paul *et al.*, 2006). Based on all of the morphological descriptions of *P. vexans*, this species had morphological characteristics between *Phytophthora* and *Pythium*.

In Vitro-Pathogenicity Test

The pathogenicity test was conducted for seven days, and the final result showed that no lesion symptoms appeared on potato leaves inoculated with *P. vexans*. The appearance was the same as the control inoculated with water agar (Figure 3). Nevertheless, it was different from healthy leaves inoculated with a small cut of leaves consisted of *P. infestans* sporangia. The color of the leaves turned black. The infected leaves appearance changes into water-soaked and die. On the lower surface of leaves, *P. infestans* could sporulate to whitish color (Schumann & D'Arcy, 2000 as cited in Ristaino *et al.*, 2018).

The observation was also done on potato tuber. *P. vexans* inoculated onto potato tubers showed the brown lesion surrounded the inoculum from a cut of mycelium. The brown lesion appeared rotten but dried. Furthermore, the control treatment where healthy tubers were inoculated with a small cuts of potato leaves with sporangium of *P. infestans* resulted in soft rot on tubers. Nevertheless, the control of potato tubers inoculated with water agar did not show any lesion symptoms, and the tubers were still in good condition.

The aggressiveness of *P. infestans* might be lethal for foliage and tubers of potato and tomato. This may cause this pathogen to be one of the most destructive pathogen in the potato field in the world. *Pythium* is also known as a saprophyte and opportunistic organism (Pegg *et al.*, 2015). Kilany *et al.* (2015) reported that 60% of genus *Pythium* caused damping-off in the nurseries and was challenging to control due to its wide host range and ability to survive for long terms in soil. In Indonesia, Santoso (2016) reported two pathogens that attacked durian orchard, *Phytophthora palmivora* and *P. vexans*. In Indonesia, *P. vexans* were reported to infect durian and cause bumps to appear on the trunk. In another case in Australia, Vawdrey *et al.* (2005) reported there were 13 durian orchards that were

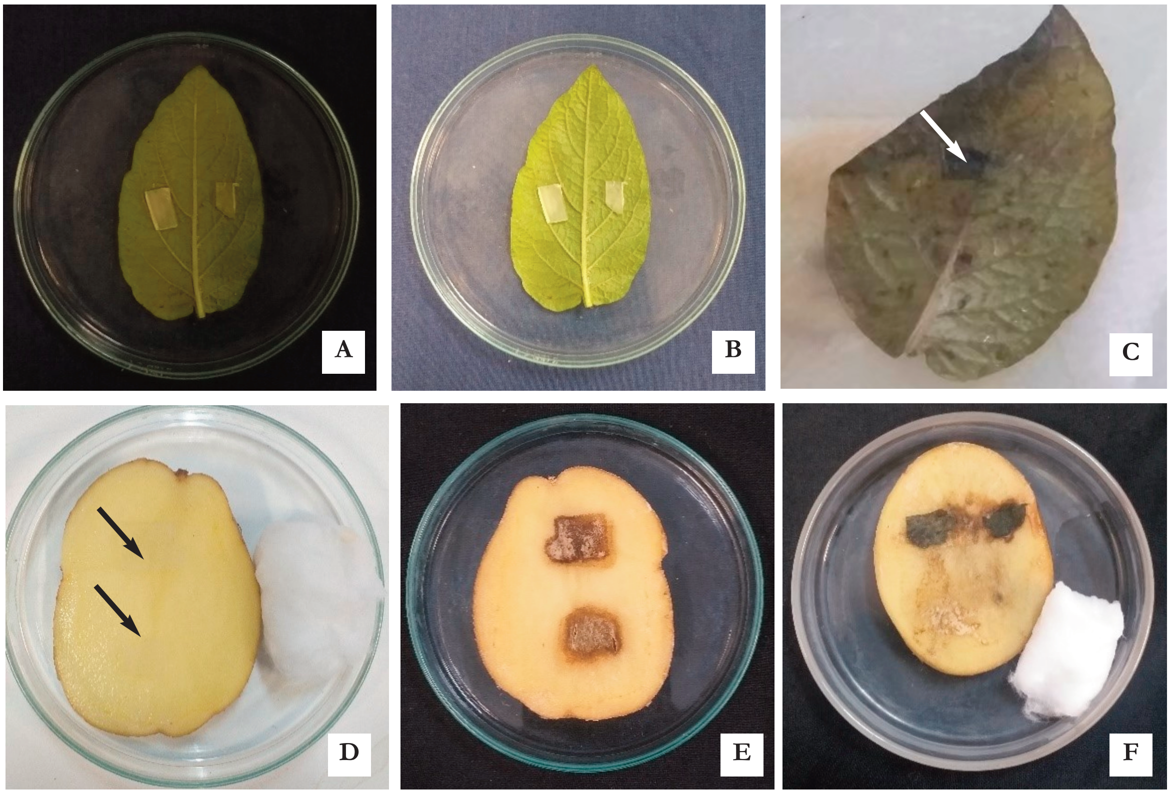

Figure 3. *In vitro* pathogenicity test on healthy potato leaves (A–C) and tubers (D–F); the materials were inoculated with water agar, negative control (A, D); *Phytopythium vexans* (B, E); and small cut of potato leaves infected by *Phytophthora infestans*, positive control (C, F) (→ : inoculum)

attacked by *P. palmivora* and *P. vexans*. Further experiments were conducted on the glasshouse trials and showed that healthy durian plants inoculated with *P. vexans* caused damage to the root and decreased the efficiency of the root to absorb the nutrient. *P. vexans* also can survive in wet or dry soil conditions. *P. vexans* is well known for causing root rot and damping-off on many ornamental plant pathogens (Yang & Hong, 2016).

This experiment revealed that *P. vexans* in Indonesia also infected potatoes with brownish blotches on tubers. It is the first reported pathogen from *Phytopythium* that could infect potatoes. After other Oomycetes like *P. infestans* known as a famous destructive pathogen on potatoes, *Phytophthora erythroseptica* as the causal agent of pink rot, and *Pythium ultimum* as the causal agent of the leak (Thompson *et al.,* 2007) were already reported. Potato tubers are plant parts with high economic and nutritious values for daily consumption. Unfortunately, it has a low tolerance for pathogen presence in tubers. The tubers infection by a soil-borne pathogen can lead to economic loss to farmers. Until nowadays, there is no effective management available to control the soil-borne pathogen on potatoes (Lazarovits *et al.,* 2008)

Phylogenetic Analysis

In 2008, Lévesque and de Cock proposed the Clade K of *Pythium* dispersed from the *Pythium* family due to the molecular analysis using ITS, Large Subunit (28S) ribosomal *Cytochrome Oxidase* I (COI) known as *Phytopythium*. Therefore NG isolate was amplified using ITS and LSU, and the single band showed at 850 bp and 650 bp. Phylogenetic analyses were constructed using sequences from ITS and LSU amplification. Based on Figure 4, we concluded that the NG isolate from this experiment had the highest similarity with *P. vexans* CBS 119.8. Based on ITS, LSU, and COI markers, the phylogenetic tree showed that *Phytopythium* spp. were more closely related to *Phytophthora* spp. rather than with *Pythium* spp. This result was similar to Robideau *et al.* (2011). The sequences of NG isolate from ITS and LSU are registered in GeneBank

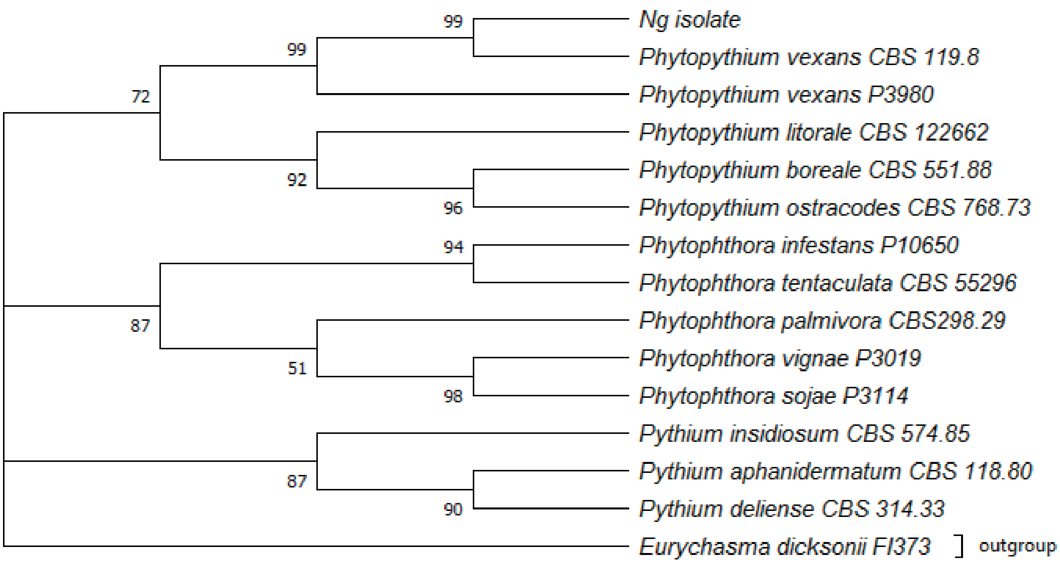

Figure 4. Phylogenetic analysis of NG Isolate Maximum likelihood phylogeny from multigene analysis ITS and LSU sequence alignment with 1000× bootstrap; *Eurychasma dicksonii* F1373 is used as an outgroup

with accession number as follow MW898226 and MW911663**.** This phylogenetic tree showed that *Pythium, Phytopythium,* and *Phytophthor*a came from the same ancestor. The evolution is responsible for the differences in phylogenetic identification due to those organisms' additional or missing genes (Rujirawat *et al.*, 2018).

CONCLUSION

Since the separation of *Pythium* clade K was proposed by Lévesque and de Cock (2008), numerous *Phytopythium* reports have been published, especially in identifying and reporting the disease in various plants. This research revealed that even the morphology of *P. vexans* is between *Pythium* and *Phytophthora*. However, the phylogenetic cluster showed that *P. vexans* are more closely related to the *Phytophthora* spp. rather than *Pythium* spp. This article is the first report of *P. vexans* found in the potato field in Indonesia. The pathogen infecting the tuber raised our concern about the soil-borne pathogen on potatoes. Further study is needed to understand *Phytopythium* better, especially in potato agricultural practices.

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