Population genetics analysis of *Phytophthora nicotianae* associated with heart rot in pineapple revealed geneflow between population

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Abstract. *Afandi A, Subandiyah S, Wibowo A, Hieno A, Afandi, Loekito S, Suga H, Kageyama K. 2021. Population genetics analysis of* Phytophthora nicotianae *associated with heart rot in pineapple revealed gene flow between populations. Biodiversitas 22: 3342-3348.* *Phytophthora nicotianae* has caused heart rot in pineapple. This study identified the population genetic diversity of *P. nicotianae*, collecting 90 isolates of *P. nicotianae* from the pineapple plantation sites in three provinces of Indonesia and, as comparisons, seven isolates from non-pineapple sites. Six polymorphic microsatellite markers amplified the non-coding region was used to characterize the population diversity. The phylogenetic tree constructed by MEGA revealed three major clades; the first and second clades were dominated by the isolates from pineapple plantation sites, and the third clade contained isolates from the non-pineapple sites. The allelic pattern analysis using Genalex software revealed the local alleles specific to Lampung and Blitar populations. The AMOVA of microsatellite genotypes data showed that the isolates had a low diversity among the population (6%) but high diversity within individual. Conclusively, *P. nicotianae* population associated with heart rot in pineapple showed geneflow between populations.

Keywords: Analysis of molecular variation, genetic variation, microsatellite, *Phytophthora nicotianae*, population structure

INTRODUCTION

Some pathogens cause heart rot in pineapple but the most common in Indonesia is *Phytophthora nicotianae*. Held responsible for drastically diminishing annual yield of fruit harvest (Ratti et al. 2018), this disease shows symptoms of soft rotting of the basal white tissues of the youngest leaves at the heart of the apical meristem. The infected leaves are easily pulled from the plant, and as the disease progresses sufficiently, the plants die. In fruit-bearing plants of susceptible varieties, the infection can move up through the peduncle and rot the fruit (Green and Nelson 2015).

To suppress the pathogen, pineapple farmers maintain their soil pH below 3.8. Although seemingly effective in suppressing the growth of Phytophthora, applying this method for years in pineapple plantations can alter the soil microbiome condition and render severe soil damage in the future. Another safer method to control the pathogen is by dipping the seed materials in a fungicide suspension before planting (Radmer et al. 2017). However, recent studies reported the emergence of fungicide-resistant *P. nicotianae* among the natural population (Panabiries et al. 2016); an alarming sign for farmers who rely heavily on fungicide to treat the pathogen.

Phytophthora nicotianae has arachnoid branching mycelium and non-cadoucus sporangia (Bush et al. 2006). Most *P. nicotianae* isolates are heterothallic, and some can be homothallic. The antheridia are amphigynous and spherical or oval and $9\text{-}10 \times 10\text{-}12$ µm in size (Waterhouse and Waterston, 1964a; 1964b). The Oogonia are smooth and spherical with a diameter of 15-64 µm (av. 26.8 µm), a 1-2 µm thick wall, and aplerotic oospores, 13-35 µm in diameter. This pathogen was first isolated in Indonesia in 1896 by de Haan. To date, *P. nicotianae* remains the most destructive plant pathogen with a broad range of hosts and habitats (Panabieres et al. 2016), responsible for 100% yield loss in tobacco and severe damage in citrus and tomato industries in Australia. As a soil-borne pathogen, *P. nicotianae* can be found indigenously in its natural environments, such as mountainous areas (Vettraino et al. 2009), forest soil (Jung et al. 2016), and irrigation water (Hong and Moorman 2005).

The characteristics of *P. nicotianae* population are specific according to geography and host. In South India, the host-specific lineages are reported on Brinjal, Ridge Gourd, and Tomato (Chowdappa et al. 2016). On the other hand, *P. nicotianae* population in Georgia is associated with geographical location (Li et al. 2017). Interestingly, recent studies using various hosts and geographic locations

across Japan reported the absence of geographical structure and suggested human facilitated migration (Afandi et al. 2019).

Population genetics research has used different genetic markers extensively, including mitochondrial DNA (mtDNA), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), single nucleotide polymorphism (SNP), and microsatellite markers.

To date, understanding the population structure of *P. nicotianae* collected from pineapple plantation that suffers from heart rot disease remains an issue. Although genetic variability within a population directly impacts the virulence and ecology of certain pathogens, a highly variable gene pool allows them to adapt more quickly to environmental change; hence, increasing their potential to produce new virulent variants. This study analyzed the population genetics of *P. nicotianae* causing heart rot disease in pineapple to better understand the disease management strategies.

MATERIALS AND METHODS

Research sites

Phytophthora nicotianae used in this study was part of the Gifu University Culture Collection. The isolates were collected from 39 locations at Central Lampung, Lampung Province; Blitar, East Java; Subang, West Java; and Pemalang, Central Java. In comparison, *P. nicotianae* isolated from the non-pineapple host were collected from Getasan, Godean, and Sleman (Table 1). The isolates were previously identified molecularly using ITS primer. The DNA extraction was performed at Biotechnology research center, Universitas Gadjah Mada, the microsatellite amplification was done at Oomycete Research Laboratory, River Basin Research Center, Gifu University, Japan and the fragment analysis was performed at genomic research laboratory, Life Science Research Center, Gifu University, Japan.

DNA extraction

The total DNA was extracted using the PrepMan Ultra Reagent (Applied Biosystem). First, the isolates were cultured at v8 medium to increase the hyphal growth. A small loopful of mycelia was transferred into 100 μl of the PrepMan Ultra Sample Preparation Reagent in a microcentrifuge tube for a vigorous vortex for 30 s. The tube was heated at 100 °C for 10 minutes and then spun in the microcentrifuge for 2 minutes at the highest speed. At last, 50 μl of the supernatant was transferred to a new microcentrifuge tube and stored at -4 °C.

Microsatellite genotyping

Six polymorphic loci were used to analyze the *P. nicotianae* population (Afandi et al. 2019). The primers were labeled at the 5′ end separately with two fluorescent dyes, FAM (6-carboxy-fluorescein), or HEX (4,7,2′,4′,5′,7′-hexachloro-6-carboxyfluorescein) (Lees et al.2006). All selected primers were administered to amplify DNA consecutively as follows: one cycle at 94ºC for 5 min, 35 cycles at 94ºC for 30 s, 58ºC for 30 s, 72ºC for 30 s, and finally extended to 72ºC for seven minutes. The total reaction volume was 25 μl containing 2 μL 1 ng DNA, 2.5 μL 10 x PCR buffer (Takara, Japan), 2.5 μl 4 mg/mL BSA, 2.5 μL 10 mM primer forward and reverse, 2 μL 2.5 mM dNTP mix (Takara, Japan), 0.1 μL rTaq polymerase (Takara, Japan), and 10.9 μL ddH2O. The PCR amplification products were evaluated by GelRed staining (Biotium) using 2% agarose gel in 0.5×Tris-Acetate-EDTA buffer and observed under UV light. The LIZ 250 DNA ladder was utilized as a marker to analyze these fragments on the ABI3100 and ABI3130 genetic analyzers (Applied Biosystem), then the electropherogram was manually scored.

Data analysis

The genetic diversity analysis for this study included statistical analysis and phylogenetic tree construction. The statistical analysis was performed on molecular variance, allelic pattern across population, and the calculation of fixation index GenAlex 5.6.3 (Peakall and Smouse 2006 2012). The phylogenetic tree was constructed using the neighbor-joining algorithm by MEGA 6 (Tamura et al. 2013).

RESULTS AND DISCUSSION

In total, 97 isolates of *P. nicotianae* were collected for genetic diversity using six microsatellite markers: 90 from diseased pineapple plant or rhizosphere and seven from the non-pineapple, for comparison (Table 1). The isolates associated with heart rot disease in pineapple were collected in four provinces in Indonesia, namely 41 isolates in Lampung (Terbanggi Besar and Punggur area), ten isolates in West Java (Subang area), eight isolates in Central Java (Pemalang area), and 31 isolates in East Java (Blitar area). The non-pineapple isolates were collected from chrysanthemum in Sleman, chili and tobacco in Getasan, and cabbage in Dieng.

A total of 27 multilocus genotypes (MLGs) were observed from six groups of *P. nicotianae* populations represented by 97 isolates (Table 2). A multilocus genotype is a unique combination of alleles that span two or more loci. For *P. nicotianae* that most likely reproduce asexually, multilocus genotypes are extremely valuable for identifying the pattern and spread of the pathogen (Kamvar and Grunwald 2021). The highest number of MLG was found in *P. nicotianae* population collected in Central Lampung (12), followed by Blitar (10), the non-pineapple group (6), Pemalang (5), Subang (2), and the least was Punggur (1). The high number of MLG in one population illustrates the high variability of *P. nicotianae*. In this case, the Central Lampung population had the highest variability.

Table 1. *Phytophthora nicotianae* isolates from pineapple and other plant hosts

Collection code	Origin	Host plant	Isolation year
AA 129D 2	Central Lampung	Pineapple	2016
AA 71A S1	Central Lampung	Pineapple	2016
AA 114K HS 2	Central Lampung	Pineapple	2016
AA 71A 2	Central Lampung	Pineapple	2016
AA 36G	Central Lampung	Pineapple	2016
TBC GTS	Getasan	Tobacco	2016
AA 71A 3	Central Lampung	Pineapple	2016
TBC GTS 4	Getasan	Tobacco	2016
AA 10A 1	Central Lampung	Pineapple	2016
AA 114K HS 1	Central Lampung	Pineapple	2016
AA 114K HS 3	Central Lampung	Pineapple	2016
AA 114K S3	Central Lampung	Pineapple	2016
AA 114K S2	Central Lampung	Pineapple	2016
AA 129D 1	Central Lampung	Pineapple	2016
AA 129D4	Central Lampung	Pineapple	2016
AA 35A1	Central Lampung	Pineapple	2016
AA NIA S	Central Lampung	Pineapple	2016
AA 36G 1A	Central Lampung	Pineapple	2016
AA 71A S1	Central Lampung	Pineapple	2016
AA71A 3	Central Lampung	Pineapple	2016
ORC GOD	Godean	Orchid	2016
CHL KDT A	Getasan	Chili	2016
CHL S A2	Sleman	Chili	2016
AA 51402R01	Central Lampung	Pineapple	2016
CS GH2 2	Sleman	Chrysanthemum	2016
CS GH3 1	Sleman	Chrysanthemum	2016
CBG DIENG 1-1	Dieng	Cabbage	2016
51402R2b	Central Lampung	Pineapple	2016
aa 71 a 2 b	Central Lampung	Pineapple	2016
aa 25a06 pt1	Central Lampung	Pineapple	2016
AA 36G 2A	Central Lampung	Pineapple	2016
NBRC 31423	Taiwan	Pineapple	1984
155A 3	Blitar	Pineapple	2018
B156 C3	Blitar	Pineapple	2018
P31C	Pemalang	Pineapple	2018
155 A 2	Blitar	Pineapple	2018
PUNG 36 2	Punggur	Pineapple	2018
B 151 B3	Blitar	Pineapple	2018
B 156 B2	Blitar	Pineapple	2018
B 159 C	Blitar	Pineapple	2018
S 69	Subang	Pineapple	2018
B 156 C	Blitar	Pineapple	2018
S 63	Subang	Pineapple	2018
PUNG 152 2	Punggur	Pineapple	2018
B 150 A	Blitar	Pineapple	2018
B 154 B1	Blitar	Pineapple	2018
B 156 A2	Blitar	Pineapple	2018
B 157 A 2	Blitar	Pineapple	2018
PUNG 182 N	Punggur	Pineapple	2018
B 156 C2	Blitar	Pineapple	2018
1725A3	Central Lampung	Pineapple	2017
17124C3	Central Lampung	Pineapple	2017
1725A06	Central Lampung	Pineapple	2017
17114K2	Central Lampung	Pineapple	2017
1736G2	Central Lampung	Pineapple	2017
129D1	Central Lampung	Pineapple	2017
17124D2	Central Lampung	Pineapple	2017
17514042	Central Lampung	Pineapple	2017
P32B	Pemalang	Pineapple	2017
B156B3	Blitar	Pineapple	2018
159C2	Blitar	Pineapple	2018
B156C2	Blitar	Pineapple	2018
151A1	Blitar	Pineapple	2018
155A1	Blitar	Pineapple	2018
B157B3	Blitar	Pineapple	2018
153B1	Blitar	Pineapple	2018
159C2	Blitar	Pineapple	2018
153A2	Blitar	Pineapple	2018
159A1	Blitar	Pineapple	2018
151C	Blitar	Pineapple	2018
157A1	Blitar	Pineapple	2018
156C3	Blitar	Pineapple	2018
155A1	Blitar	Pineapple	2018
B154C2	Blitar	Pineapple	2018
B156C1	Blitar	Pineapple	2018
157A2	Blitar	Pineapple	2018
155A3	Blitar	Pineapple	2018
B157B2	Blitar	Pineapple	2018
1771A1	Central Lampung	Pineapple	2017
P3B2	Pemalang	Pineapple	2018
1736G3	Central Lampung	Pineapple	2017
S51	Subang	Pineapple	2018
1736G1B	Central Lampung	Pineapple	2017
1725A062	Central Lampung	Pineapple	2017
P31B	Pemalang	Pineapple	2018
S33A	Subang	Pineapple	2018
S1R2	Subang	Pineapple	2018
P31E	Pemalang	Pineapple	2018
1710A7	Central Lampung	Pineapple	2017
S1R1	Subang	Pineapple	2018
P34E	Pemalang	Pineapple	2018
P33F	Pemalang	Pineapple	2018
S71	Subang	Pineapple	2018
S63	Subang	Pineapple	2018
P33C	Pemalang	Pineapple	2018
S62	Subang	Pineapple	2018
1736B	Central Lampung	Pineapple	2017
S1R3	Subang	Pineapple	2018
1710A1	Central Lampung	Pineapple	2017

A deeper probe into the multilocus genotype analysis (Table 2) revealed that several isolates of *P. nicotianae* from Central Lampung and Blitar shared common MLGs, indicative of the same source of infection, which probably derived from the same pineapple seedling because the pineapple plantations in both Central Lampung and Blitar belong to one company.

The isolates from the non-pineapple host showed distinctive MLG compared to the pineapple counterparts and the rest of the populations. It is indicative of zero interaction between the non-pineapple and pineapple population.

Analysis of molecular variance (AMOVA) estimated the population differentiation directly from molecular data. The AMOVA of microsatellite genotypes data showed that the isolates had a low diversity among the population (6%) but high diversity within the individual (Table 3), evidenced from the low number of Fst (0.056) that represents the variance of allele frequencies among populations. The result of allelic pattern analysis using Genalex software demonstrated that the private alleles were specific to a population. Central Lampung population had the highest frequency of private alleles which evolve only in specific places (Table 4). Additionally, Central Lampung had highest number of locally common alleles which take

up 50% of the populations with >5% frequency in a specific population. The highest Shannon's Information Index was in the non-pineapple group followed by Subang, Pemalang, Punggur, Central Lampung, and Blitar. A previous study also reported a high variability within the population in *P. infestans* collected in Nordic European countries (Brurberg et al. 2011).

The low number of Fst and percentage of variance (Table 3) indicated non-random mating or clonal reproduction in *P. nicotianae* associated with pineapple's decayed root. It is confirmed by allelic patterns that revealed private alleles in Lampung, Blitar, and Subang populations (Table 4). The private allele is most likely produced by clonal reproduction and geographic isolation. *P. nicotianae* can undergo sexual and asexual reproduction, thus taking advantage of the strictly asexual or sexual pathogens. Likewise, mitotic recombination is common among Phytophthora species. The pathogen is not necessarily performing sexual reproduction at any chance (Dobrowlski et al. 2003). The previous studies reported private alleles specific to certain populations due to clonal reproduction in *P. infestans* (Gavino et al. 2000; Montarry et al. 2010) and *P. sojae* (Wu et al. 2017) or due to geographic isolation as observed in *P. alni* (Aguayo et al. 2012).

The number of alleles was highest in Central Lampung population (3.167) followed by Blitar (2.833) Subang (2.667), and the non-pineapple group (2.500), while the least were Pemalang (2.167) and Subang (2.167) (Table 4). This allelic richness is indicative of a population's long-term potential for adaptability and persistence (Greenbaum et al. 2015). Therefore, the population of *P. nicotianae* in Central Lampung is more likely to be adaptive to the environmental change rather than the other populations.

Table 2. Multi Locus Genotype (MLG) frequencies per population

MLG	Blitar	Central Lampung	Pemalang	Punggur	Subang	Central Java (Non-Pineapple)
1	0.15	0.03				
2	0.15	0.45				
3	0.02		0.28	1		
4	0.02	0.09				
5	0.24	0.29				
6						0.37
7	0.05	0.03				
8		0.06				
9	0.09	0.06				
10					0.40	
11		0.06				
12			0.42			
13	0.02	0.06				
14					0.60	
15			0.14			
16		0.03				
17	0.02					
18	0.02					
19		0.03				
20		0.03				
21						0.12
22			0.14			
23						0.12
24						0.12
25						0.12
26			0.14			
27						0.12

Table 3. Summary of analysis molecular variance

Source	df	SS	MS	Est. Var.	%	Fst	P-value
Among Populations	5	15.525	3.105	0.065	6%	0.056	0.001
Among Individuals	92	118.383	1.287	0.192	17%		
Within Individuals	98	88.500	0.903	0.903	78%		
Total	195	222.408		1.159	100%		

Note: df: degree of freedom; SS: sum of squares; MS: mean square; Est. var. estimated variance; %: percentage of variance, FST: Fixation Index.

Table 4. Allelic pattern across population

Population	Central Lampung	Blitar	Pemalang	Punggur	Subang	Central Java (Non-pineapple)
Na	3.167	2.833	2.167	2.167	2.667	2.500
Ne	1.546	1.477	1.900	1.900	1.789	1.750
I	0.577	0.513	0.596	0.588	0.615	0.640
No. Private Alleles	0.167	0.167	0.000	0.000	0.167	0.000
No. LComm Alleles (<=50%)	0.667	0.500	0.167	0.333	0.500	0.500
He	0.318	0.285	0.371	0.361	0.364	0.383
uHe	0.323	0.290	0.397	0.433	0.383	0.412

Note: Na: No. of Different Alleles; Ne: No. of Effective Alleles; I: Shannon's Information Index; No. Private Alleles: No. of Alleles Unique to a Single Population; No. LComm Alleles (<=50%) = Number of Locally Common Alleles (Freq. >= 5%) Found in 50% or Fewer Populations; He: Expected Heterozygosity; uHe: Unbiased Expected Heterozygosity

Shannon's information index is a vital criterion to understand the population variation because it distinguishes genetic diversity in a population combining abundance and evenness (Ali et al. 2019). Table 4 shows that Shannon's information index (I) in this study was generally high (0.513 to 0.640), indicative of high genetic variance and even distribution across populations. The highest was the non-pineapple group (0.640), followed by the pineapple group from Subang (0.615), Pemalang (0.596), Punggur (0.588), Central Lampung (0.577), and Blitar (0.513). It is evident that the population in Subang had higher and more evenly distributed genetic variance compared to that in Central Lampung.

The expected heterozygosity (He) was ranging between 0.383 to 0.318 (Table 4). The highest was group isolates collected from the non-pineapple plants (0.383) followed

by the pineapple associated isolates collected from Pemalang (0.371), Subang (0.364), Punggur (0.361), Central Lampung (0.318), and Blitar (0.285). The expected heterozygosity was used to assess genetic variation within populations. Estimation of this statistic could decrease in accuracy and precision when individuals are related or inbreed due to increasing dependence among allele copies in the sample (Harris and DeGiorgio 2016). Since *P. nicotianae* reproduction mostly by asexual rather than sexual, we calculated the unbiased expected heterozygosity and found that it ranged from 0.290 to 0.433 (Table 4). The highest was the population of *P. nicotianae* isolated from pineapple in Punggur (0.43) followed by the isolates collected from the non-pineapple (0.412), Pemalang population (0.397), Subang (0.383), Central Lampung (0.323), and Blitar (0.290).
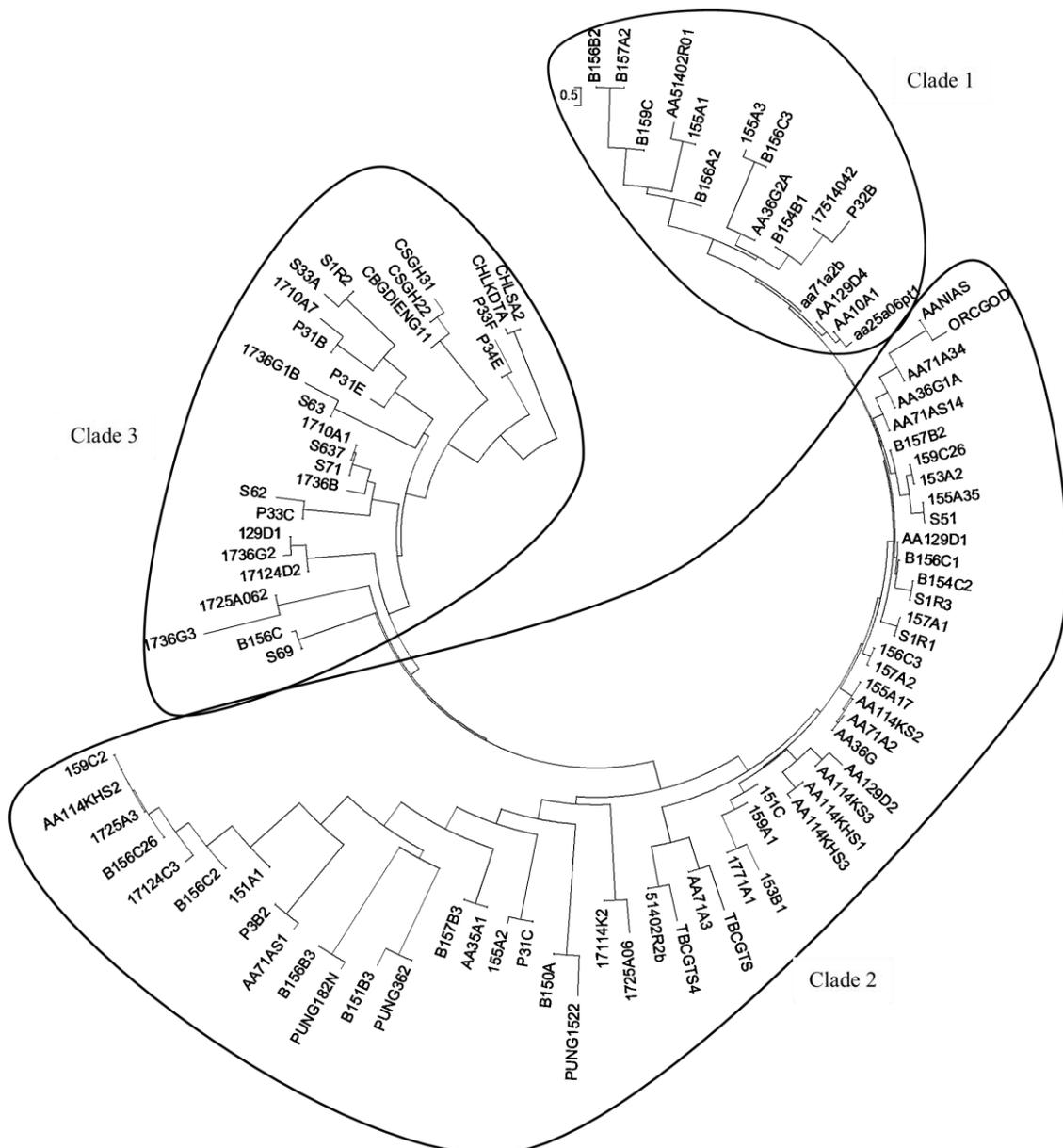

Figure 1. Phylogenetic analyses of *P. nicotianae* constructed with Neighbor-Joining Algorithm using MEGA 6 was showed 3 major clades

The phylogenetic tree constructed with the neighbor-joining algorithm showed three major clades. The first and second clade were dominated by isolates associated with heart rot disease in pineapple, while the third clade consisted of isolates from the non-pineapple group (Figure 1). The first clade consisted of 16 *P. nicotianae* isolates from Blitar and Central Lampung, collected from pineapple plantations owned by the same private company which utilized vegetative propagation for pineapple cultivation and used the same source of planting materials. It explains the closer relationship between *P. nicotianae* in Blitar and Central Lampung.

The second clade had the most *P. nicotianae* isolates dominated by pineapple host in Central Lampung, Blitar, and Punggur, while the third clade had isolated from Subang, Pemalang, and the non-pineapple group. All non-pineapple isolates were collected from areas in Central Java which is in close proximity to Pemalang and Subang. Also, Central Lampung is relatively close to Punggur within Lampung Province.

It is evident from Figure 1 that most of the isolates from the same geographical origin are grouped into the same clade, while several other isolates from one population are scattered in the other clades. Most isolates collected from the non-pineapple plants show a distant relationship with pineapple isolates (Figure 1). The inconsistency of genotypic structure and geographical origin are common on demographic analysis of Phytophthora species, previous studies on *P. nicotianae* from various host and locations across Japan (Afandi, et al. 2019), *P. nicotianae* isolated on citrus (Biasi et al. 2016), *P. Plurivora* (Schoebel et al. 2014), and *P. colocasiae* (Nath et al. 2013) also showed moderate to high genetic diversity without consistency on the geographical origin. Pathogen transmission from one area to another may use human as their transporting agent. Since *P. nicotianae* is soil and waterborne and may long survive as chlamydospore for a long time, it is highly transmissible via agricultural products or water flow.

The isolates were obtained from different pineapple varieties. The private company in Central Lampung and Blitar planted the smooth cayenne variety, while the local farmers in Punggur, Subang, and Blitar planted the queen variety. To the best of our knowledge, there is no evidence of relationship between *P. nicotiana* genotypes and pineapple variety.

In conclusion, we reported a distinct genetic distance of *P. nicotianae* associated with root rot disease in the isolates of pineapple and the non-pineapple group. However, the low percentage of variance between *P. nicotianae* and other populations that shared common MLG indicated a gene flow between populations.

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