Indonesian Journal of Biotechnology

VOLUME 31(2), 2026, 106-118 | RESEARCH ARTICLE

Metabarcoding analysis identifies the phyllosphere fungal communities associated with a newly emerged leaf fall disease in rubber plants in Indonesia

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SUBMITTED 20 January 2026 **REVISED** 15 March 2026 **ACCEPTED** 11 May 2026

ABSTRACT A leaf disease causing leaf fall in *Hevea brasiliensis* recently appeared in Southeast Asia and threatens rubber production. While tentatively associated with the *Pestalotiopsis*, *Colletotrichum*, and *Neopestalotiopsis* species, its etiology remains poorly characterized. This study aims to identify the fungal community associated with symptomatic rubber leaves using culture-independent ITS amplicon sequencing. Leaf samples representing three levels of symptom severity were collected from Central Java and South Sumatra. Fungal community analysis revealed that geographic origin, rather than symptom severity, was the driver of community clustering. *Phyllosticta* and *Colletotrichum* were consistently dominant across all samples, with *Phyllosticta* displaying higher abundance. Minor proportions of *Pestalotiopsis*, *Neopestalotiopsis*, *Corynespora*, and *Pestalotia* were also detected. Fungal isolates collected from diseased leaves in the same regions were identified as *Neopestalotiopsis*, *Pestalotiopsis*, and *Colletotrichum* and placed in a two-loci (ITS and β-tubulin) phylogenetic tree to clarify their identity at species level. Pathogenicity assays confirmed that representative isolates reproduced field symptoms. However, no *Phyllosticta* isolate was obtained, probably due to slow growth. This metabarcoding assessment of the phyllosphere mycobiota in diseased rubber leaves identified *Phyllosticta* as a critical yet overlooked component of the leaf fall disease complex, providing insights into fungal ecology and pathogenic interactions underlying this emerging production threat.

KEYWORDS Fungal community; ITS amplicon sequencing; Leaf fall disease; *Phyllosticta*; Rubber

1. Introduction

A new infectious plant disease, presumably caused by a consortium of pathogens, causes leaf fall in rubber tree (*Hevea brasiliensis*) in Indonesia and other Asian countries. The first severe outbreak of the disease was reported in North Sumatra-Indonesia in 2017 (Junaidi et al. 2018), although the disease may have emerged as soon as 2016 (Febbiyanti et al. 2018). In early 2018 and 2019, the disease had spread to other regions in Indonesia (Febbiyanti and Fairuzah 2020). In 2019, the disease was also reported in Johor-Malaysia Palai-India, and Sri Lanka, as reviewed in Aliya et al. (2022). In September 2020, an outbreak was reported in Thailand (Pornsuriya et al. 2020). The Rubber Research Institute of Vietnam stated that similar symptoms were detected in Dong Nai and Binh Phuoc Province, Vietnam, in September 2021 (Hiệu et al. 2022).

Fusicoccum sp. and *Colletotrichum* sp. were initially suspected to cause the disease in North Sumatra-Indonesia in September-October 2017 bases on morphological observations (Junaidi et al. 2018). With molecular identification and pathogenicity tests, further studies conducted in various countries identified *Pestalotiopsis microspora*, *Pestalotiopsis jesteri*, *Neopestalotiopsis cubana*, *N. formicarum*, *Colletotrichum siamense*, and *Colletotrichum fructicola* as causal pathogens, acting alone or in association (Aliya et al. 2022; Kusdiana 2021; Pornsuriya et al. 2020; Vineeth et al. 2024). The diversity of fungal pathogenic groups identified with the same symptom indicates that the disease is influenced by complex microorganism communities.

To date, few studies have been conducted on the microbiome composition of the rubber tree phyllosphere, especially on fungal communities associated with leaf dis-

ease. Several metabarcoding studies on rubber include soil bacteria composition, richness and biodiversity of rubber rhizosphere in West Java-Indonesia (Effendi et al. 2019, 2020); fungal ITS amplicon for analyzing soil microbial composition and diversity between mixed and monoculture rubber plantations in Hainan-China (Jatoi et al. 2019); and virome analysis using small RNA deep sequencing that identified the presence of a putative new virus named *Hevea brasiliensis* virus (HBrV) in reference to its host (Fonseca et al. 2018, 2022; Lan et al. 2024; Wei et al. 2022). Metabarcoding analysis may also help clarifying the fungal community structure and diversity in diseased rubber trees, thereby facilitating more accurate and timely disease diagnosis.

This study was conducted to identify the differences in fungal pathogen community associated with newly emerged leaf fall disease from two locations, South Sumatra, and Central Java. Three levels of necrosis symptoms were compared at each location. The disease has affected all rubber clones currently planted in Indonesia. No clones have been found to be resistant to this disease. The clones severely affected in Indonesia include BPM 1, BPM 24, GT 1, IRR 112, IRR 220, PB 217, PB 260, PB 330, PB 340, RRIC 100, RRIM 712, RRIM 911, and RRIM 937 (Junaidi et al. 2018; Febbiyanti and Fairuzah 2020; Kusnedy et al. 2021). GT 1 was chosen for this study because of its well-known susceptibility to rubber leaf diseases (Mei et al. 2016). The symptoms observed in the nursery, budwood nursery, immature, and mature plantation varied greatly in severity (Febbiyanti and Fairuzah 2020).

Higher disease severity has been reported during the rainy season, especially in locations with wet environmental conditions. Environmental factors, including humidity, rainfall, and the number of rainy days, contribute to the increased disease severity. Kusdiana (2021) reported that higher disease severity occurred in the humidity range of 87–89%, rainfall exceeding 100 mm/month, and more than 15 rainy days/month. Sampling of diseased leaves was carried out in April, which is the rainy season with rainfall exceeding 100 mm/month in both locations.

2. Materials and Methods

2.1. Samples collection for metabarcoding analysis

Samples were collected from one of the areas heavily affected by the disease, which is South Sumatra (S), and from the area recently affected by the disease, namely Central Java (J), and all samples consisted of mature rubber leaves at the same developmental stage. Three samples with different levels of infection (Figure 1; 0 = no symptom, 1 = mild, and 2 = severe symptoms) were collected from each area and stored at room temperature in conical tubes filled with absolute ethanol. One sample was a composite from 3–5 leaves per tree, cut leaf fragments of the same size around the symptom, 3–5 trees of each location. Samples were collected during rainy season (April) in both areas. A total of six samples were taken in this study, consisting of three levels of infection from South Sumatra (S0B, S1B, S2B) and three levels of infection from Central Java (J0B, J1B, J2B). Samples were collected from the plantation without any surface disinfection and transported to the laboratory in absolute ethanol.

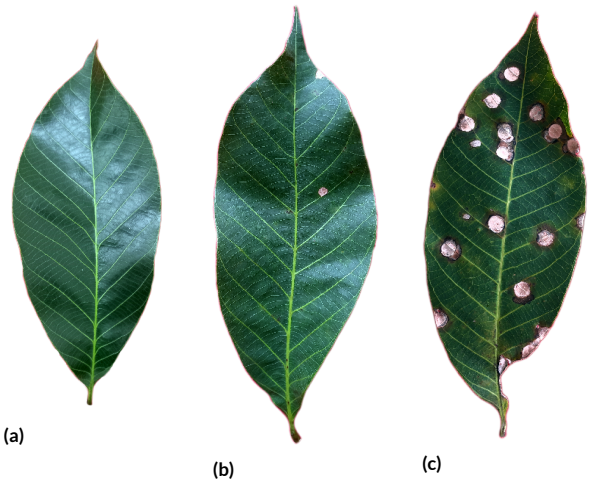

FIGURE 1 Symptom levels of the new leaf fall disease of rubber tree (*Hevea brasiliensis*), on clone GT 1 used in this study: a. No symptom; b. Mild symptom; c. Severe symptom.

2.2. Total DNA extraction

Genomic DNA was extracted from 100 mg of ground leaf tissue using the Genomic DNA Mini Kit Plant (Geneaid), following the manufacturer's protocol, and DNA quantity was measured using a spectrophotometer (MN-913A MaestroNano Pro; MaestroGen, Taiwan).

2.3. Sequencing library preparation and data analysis

The analysis was performed using Illumina NovaSeq 6000 with the fragment length of 380 bp (Novogen, Korea) based on the ITS2 regions of rDNA. The amplicons were amplified using ITS3-2024F (5′-GCATCGATGAAGAACGCAGC-3′) and ITS4-2409R (5′- TCCTCCGCTTATTGATATGC-3′) primer sets for fungal ITS (Zheng et al. 2024). All PCR reactions were conducted with Phusion High-Fidelity PCR master mix (New England Biolabs). The PCR products with proper size were selected by 2% agarose gel electrophoresis. Same amount of PCR products from each sample was pooled, end-repaired, A-tailed, and subsequently ligated with Illumina adapters. Libraries were examined with a Qubit® 2.0 Fluorometer (Thermo Scientific) and an Agilent Bioanalyzer 2100 system for size distribution detection. Libraries were sequenced on a paired-end Illumina platform to generate 250 bp paired-end raw reads. Paired-end reads were merged using FLASH software (v1.2.7) (Magoč and Salzberg 2011) resulting raw tags. The high-quality clean tags (Bokulich et al. 2013) were obtained using filtering tool of Qiime 2 (Bolyen et al. 2019) software. These tags were compared with the Unite (v8.2) reference database using UCHIME algorithm to detect chimera sequences. Furthermore, the chimera sequences

were removed (Haas et al. 2011) to obtain the effective tags. Sequences of all the effective tags were then be analyzed using Uparse (v7.0.1090) software (Edgar 2013) and clustered into operational taxonomical units (OTU). Sequences with ≥97% similarity were assigned to the same OTUs. Representative sequence for each OTU was annotated at each taxonomic rank using BLAST according to Blastall (v2.2.25) and Unite (v8.2) database. The MUSCLE version 5.1 software (Edgar 2022) was used in comparison of the multiple sequences to obtain phylogenetic relationship of all OTUs representative sequences. OTUs abundance information was normalized using a standard of sequence number corresponding to the sample with the least sequences. Beta diversity analysis on unweighted unifrac was calculated by Qiime 2 software to evaluate differences of samples in species complexity. Principal Coordinate Analysis (PCoA) was performed to get principal coordinates and visualize from complex, multidimensional data, which were then displayed by WGCNA package (Langfelder and Horvath 2008), stat packages and ggplot2 package (Villanueva and Chen 2019) in R (v2.15.3) software.

2.4. Pathogen isolation

Fungal isolates were obtained from diseased rubber tree leaves, i.e. leaves exhibiting typical pathogen induced disease symptoms (such as circular lesions and necrotic spots), approximately 1–24 months before the metabarcoding sampling at the same locations. Mature leaves showing distinct symptoms were collected from infected trees, placed in paper bags and transported to Laboratory of Plant Protection, Research Unit of Bogor-Getas, Salatiga, where they were processed for fungal isolation following Nyaka Ngobisa et al. (2017) with slight modification. Pieces of diseased leaf tissues were cut 4–5 mm^2 from the edges of the lesions, surface-disinfected by immersion in ethanol 70% for 1 min, immersed in 0.1% sodium hypochlorite (NaOCl) for 1 min, rinsed three times with sterile distilled water and inoculated onto potato dextrose agar (PDA) plates. The plates were incubated at room temperature. The putative pathogens were isolated as a particular colony emerging from the disinfected leaf tissue. As soon as a fungal colony could be observed with a characteristic of targeted pathogens, namely *Colletotrichum* and/or *Pestalotiopsis*, a small piece of agar containing the colony was transferred to a new PDA in a petri dish and preserved at room temperature.

2.5. Single spore isolation

Single spore culture technique was performed following Hsu et al. (2024) with slight modification. Conidiomata were immersed in 10 mL of sterile distilled water in a test tube and stirred for 30 s on a vortex mixer. A 200 µL spore suspension was transferred with a sterile pipette onto the surface of a petri dish with a thin layer of water agar (WA) as the media. The petri dish was left overnight to germinate and then observed using microscope within 12 h. Single germinated spores were transferred onto a potato dextrose agar (PDA) plate and incubated at room temperature.

2.6. DNA extraction and PCR amplification

DNA extraction of the selected isolates (Supplementary Table S4) was carried out by Genomic DNA Mini Kit Plant (Geneaid). Two-day-old fungal colonies (100 mg) were cut and ground to a fine powder then transferred to a 1.5 mL microcentrifuge tube. The next procedures were in accordance with the manufacturers protocol previously used in this study. Amplification of multiple loci was performed by a 2720 Thermal Cycler (Thermo Fisher Scientific) using standard primers for the gene regions ITS and β-tubulin. Primer sequence information and references as well as PCR cycles conditions are presented in Supplementary Table S1. Amplification of each gene region was carried out with a 25 µL PCR mixture consisting of 12.5 µL MyTaq™ HS Red Mix (Bioline Meridian Bioscience), 2 µL DNA template, 2 µL of each primer set, and 6.5 µL nuclease-free water. PCR products were observed by gel electrophoresis with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific).

2.7. DNA sequencing and analysis

The PCR products obtained with the ITS and β-tubulin primers (Supplementary Table S1) were purified and sequenced (by Genetika Science Indonesia) using the same primers as for PCR amplification. Sequences of isolates from this study were compared to the known DNA sequences in the NCBI (National Center for Biotechnology Information) database using BLAST searches to assign each isolate to the appropriate genus. On this basis, strains representing multiple described species within the same genus as our isolates (not restricted to the closest BLAST hits) were selected, and their ITS and β-tubulin (TUB) sequences were retrieved from NCBI (Supplementary Table S2) to serve as reference taxa in the multilocus analysis, in order to resolve the identity of the isolates at species level. All sequences were adjusted as required and aligned using Clustal W (Sievers and Higgins 2017). Phylogenetic analysis of the combined ITS and β-tubulin (TUB) sequences was then performed in MEGA version 12 using Maximum Likelihood and Bayesian clustering approaches under the Jukes–Cantor or Tamura–Nei nucleotide substitution models, with 1,000 bootstrap replicates to assess branch support (Kumar et al. 2024).

2.8. Pathogenicity tests

Pathogenicity tests were conducted to assess Koch's postulates using mycelial plugs to confirm whether the isolates were rubber pathogens. The isolates used in this test correspond to those listed in Supplementary Table S4 and were selected based on their collection sites, which were located within the same area as the sampling locations used for metabarcoding. The rubber clone GT1, from which each fungal strain was isolated, was used as the test host plant. Seven-day-old fungal isolates grown on PDA were inoculated onto detached rubber tree leaflets follow-

ing the method of Pornsuriya et al. (2020) with modifications. Each leaflet was wounded with a fine needle on both sides of the midrib, three spots per side, and each spot was treated with a mycelial plug of the fungal pathogen. Incubation was carried out in a covered moist box at room temperature (25 to 30 °C) for five days. Control leaves were inoculated with PDA only. Symptoms were recorded after the incubation period, and pathogens were subsequently re-isolated from the inoculated leaves. The re-isolated cultures were compared with the original isolates to confirm Koch's postulates. The fungal isolate was confirmed as causal agent of the disease if it fulfilled the four criteria of Robert Koch's postulates (Bhunjun et al. 2021).

3. Results and Discussion

In this study, the six samples were obtained from two locations, consisting of three levels of infection from each location (Table 1).

3.1. Beta diversity analysis

The data show that samples from the same location have tendency to be in the same group, based on the principal component of axis 1, with contributions from the weighted and unweighted UniFrac values of 86.37% and 30.51%, respectively (Supplementary Figure S4). Principal coordinate analysis (PCoA) plot based on the species composition and the relative abundance of species indicates that the fungal community of the samples from Central Java has a distinct cluster, separated from the samples from South Sumatra (S1B and S2B), except for sample with no symptom (S0B), which is in the same cluster as Central Java cluster.

Comparison of weighted UniFrac dissimilarities between pairwise samples showed that each sample in this study was relatively different from the other samples based on fungal community composition and abundance, with dissimilarity coefficient ranging from 0.434 to 0.633. Regardless of the abundance of fungal species, the three samples from Java showed significant differences compared to the samples from South Sumatera with mild symptom (unweighted UniFrac dissimilarity coefficients of 0.777 to 0.907), but they showed similarities with the sample from South Sumatera with no symptoms (0.066 to 0.176) (Figure 2).

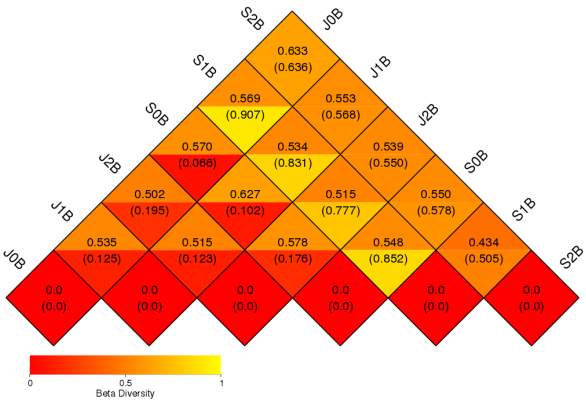

FIGURE 2 Heatmap based on dissimilarity coefficients between pairwise samples, in which weighted Unifrac distances are shown above and unweighted Unifrac distances are displayed below, where codes are defined in Table 1.

3.2. Alpha diversity analysis

The analysis showed that the number of species, or species richness, increased with the severity of symptoms, especially in the samples from South Sumatra. The average species richness of the samples from South Sumatra was higher than that of those samples from Central Java. The samples with severe symptom from South Sumatra (S2B) had the highest observed species richness (424 species) and phylogenetic diversity (PD whole tree = 82.728), while S1B exhibited the highest Shannon diversity index (3.406), indicating a more even distribution of species. In contrast, the samples with no symptoms (J0B and S0B) showed that they had the lowest diversity levels across most parameters. Goods coverage values were consistently high (close to 1.000) for all samples, suggesting that the sampling was highly adequate (Supplementary Table S3). Overall, S2B and S1B were the most diverse and phylogenetically enriched communities among the groups analyzed, but also had species that dominate the community, with Simpson index of 0.722 and 0.807, respectively.

Samples group from South Sumatra (S) consistently showed a higher OTU richness than samples group from Central Java (J) at all sequencing depths, indicating greater microbial diversity. Both groups had curves plateau, suggesting sufficient sequencing depth to capture most of the diversity, with S reaching over 320 OTUs and J leveling off below 250 (Supplementary Figure S1).

The distribution of fungal operational taxonomic units

TABLE 1 Sample codes.

Sample Code	Location	Coordinates	Symptom Levels
J0B	Cilacap, Central Java	7o19'50" S 108o35'34" E	No Symptom
J1B	Cilacap, Central Java	7o19'50" S 108o35'34" E	Mild Symptom
J2B	Cilacap, Central Java	7o19'50" S 108o35'34" E	Severe Symptom
S0B	Banyuasin, South Sumatera	2°55'51.2"S 104°32'24.1"E	No Symptom
S1B	Banyuasin, South Sumatera	2o55'51" S 104o32'24" E	Mild Symptom
S2B	Banyuasin, South Sumatera	2o55'51" S 104o32'24" E	Severe Symptom

(OTUs) across all samples are illustrated in a flower diagram (Supplementary Figure S2). A total of 71 OTUs are shared among all samples, representing the core fungal community in rubber leaves in the areas affected by this disease. Each sample also has a number of unique OTUs, with S2B exhibiting the highest number of unique OTUs (94), indicating a distinct and highly diverse fungal composition compared to the other samples. Other samples also contain unique OTUs to varying extents, such as J1B (64), J0B (23), J2B (24), S1B (25), and S0B (31). These findings suggest both stable core microbiome and sample-specific variations, with samples from Sumatra, particularly S2B, showing greater fungal uniqueness compared to samples from Java.

Taxonomic composition analysis based on the unweighted UniFrac dendrogram and relative abundance at the phylum level further shows clear distinctions between the two groups. All samples are dominated by the phylum Ascomycota, followed by Basidiomycota (Supplementary Figure S3).

The distinct fungal profiles were displayed by the J group and the S group, with certain taxa highly abundant in J samples but nearly absent in S samples, and vice versa. This variation indicates that the fungal community composition differs substantially between the two groups. These genera-specific variations contribute to the overall divergence in fungal community structure between the two sample sets. Several genera as potential pathogens of rubber showed distinct group-specific patterns. For example, some genera (e.g., *Phyllosticta* and *Neopestalotiopsis*) exhibited higher relative abundance in the S group, while others (e.g., *Colletotrichum* and *Corynespora*) were more prominent in the J group (Figure 3). These genera were always found in high abundance in the samples with severe symptoms (J2B and S2B).

Several fungal genera were detected among six leaf samples (S0B, S1B, S2B, J0B, J1B, and J2B). *Phyllosticta* were the most abundant and consistently detected fungal genera, especially dominant in sample S2B (20.32%) and S1B (5.25%), with much lower levels in J-group samples. *Colletotrichum* also showed significant presence, particularly in J2B (6.63%) and S1B (3.07%). Other genera, such as *Diaporthe*, *Pestalotiopsis*, *Neopestalotiopsis*, *Corynespora*, *Pestalotia* and others, were detected at trace levels or not detected (0) in most samples (Table 2). The results indicated a site-specific distribution, with S2B exhibiting the highest fungal load, dominated by Phyllosticta, suggesting a possible association between this taxon and disease symptoms in the location.

3.3. Multigene phylogenetic analysis

The isolates were selected based on their geographic origin, to match the locations used for metabarcoding sampling, and on BLAST results (on ITS sequences) indicating that they belonged to the genera *Pestalotiopsis*, *Neopestalotiopsis* or *Colletotrichum*, previously reported as potential causal agents of this new leaf disease. A phylogenetic analysis based on two concatenated genes (ITS and β-tubulin) was conducted to tentatively resolve the identity of the selected isolates at species levels. Strains representative of distinct species within each genus were selected from public databases and incorporated into the phylogenetic analysis. The selected species were namely *Neopestalotiopsis formicarum*, *N. cubana*, *N. protearum*, *N. aotearoa*, *N. thailandica*, *N. foedans*, *N. saprophytica*, *Pestalotiopsis microspora*, *P. diploclisiae*, *P. endophytica*, *Colletotrichum siamense*, and *C. gloeosporioides*. Figure 4 shows the placement of the seven isolates from our study in the phylogeny. Among the Neopestalotiopsis cluster, isolate P01 is more closely related to the *N. protearum* species; isolates P54 is closely related to *N. aotearoa*; P226 appears to be *N. thailandica*; P187 is more distantly related to the Neopestalotiopsis species of this study but cannot be assigned to one specific species. Only one isolate (P71) is a confirmed *Pestalotiopsis*, of the *P. diploclisiae* species or closely related. The two *Colletotrichum* isolates (C52 and C228) belong to the *C. gloeosporiodes* species complex but are distinct from the two *C. siamense* isolates.

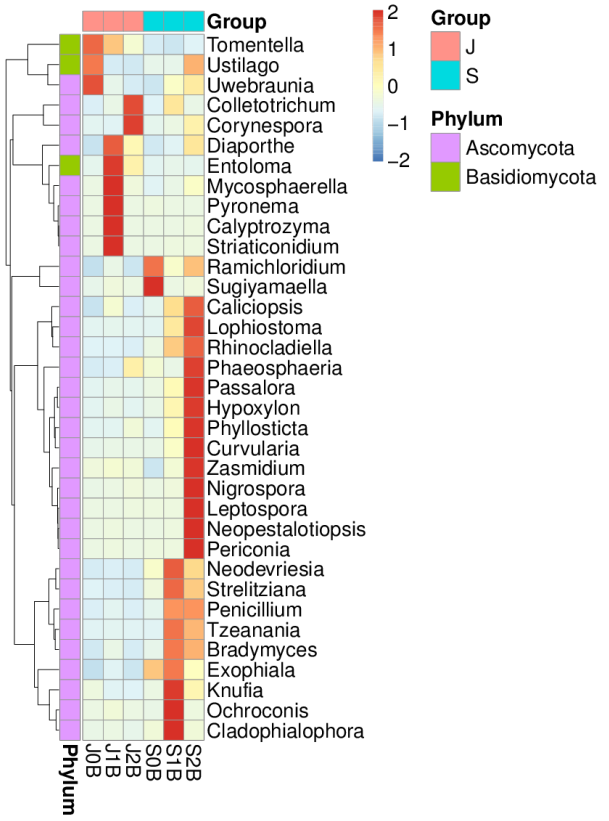

FIGURE 3 Heatmap of fungal taxa abundance across different samples from Central Java (J) and South Sumatra (S), with color intensity indicating relative abundance, where codes are defined in Table 1.

3.4. Pathogenicity tests

Pathogenicity testing of 7 isolates predicted to cause the leaf fall disease (four *Neopestalotiopsis*, one *Pestalotiopsis* and two *Colletotrichum*) was performed using detached

TABLE 2 Relative abundance (%) of the fungal genera observed in the diseased leaves associated with rubber plants.

Observed Genera	S0B	S1B	S2B	J0B	J1B	J2B
Phyllosticta	0.24	5.25	20.32	0.08	0.19	2.49
Colletotrichum	0.06	3.16	0.61	0.05	0.69	6.63
Diaporthe	0.01	0.03	0.13	<0.01	0.25	0.10
Corynespora	<0.01	0.01	0.06	<0.01	<0.01	0.17
Mycosphaerella	<0.01	0.01	0.03	0.01	0.16	0.02
Curvularia	<0.01	0.03	0.19	<0.01	<0.01	<0.01
Periconia	<0.01	<0.01	0.19	0	<0.01	<0.01
Nigrospora	<0.01	<0.01	0.17	<0.01	<0.01	<0.01
Neopestalotiopsis	0	0	0	0	<0.01	0.14
Cercospora	<0.01	<0.01	0.02	<0.01	<0.01	0
Ganoderma	0	0	0	<0.01	0	<0.01
Pestalotiopsis	<0.01	0	<0.01	0	0	0
Pestalotia	<0.01	0	<0.01	0	0	0
Cytospora	0	0	<0.01	0	0	0

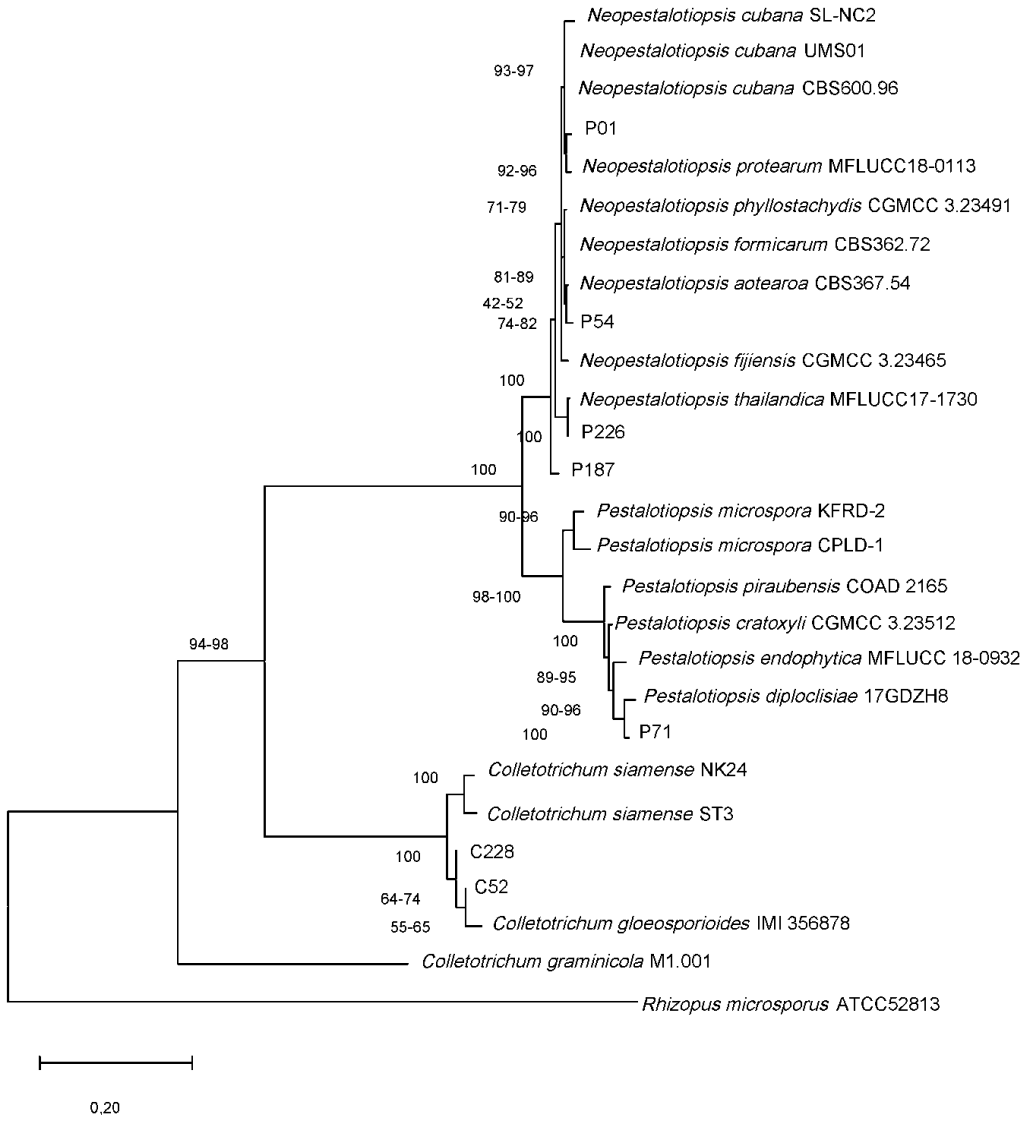

FIGURE 4 Two-loci phylogenetic tree showing the genetic relationship of the isolates from this study with various species within the same genera. The tree was constructed using a concatenated data set of ITS and β-tubulin gene sequences.

leaf assays on rubber tree leaflets. The selected isolates were inoculated onto healthy rubber leaves according to established *Pestalotiopsis* pathogenicity assessment protocols. The results indicated that the selected isolates could effectively induce symptoms that reproduced the disease symptoms observed in the field (Figure 5, panels a–b). Inoculated leaves exhibited circular brown to dark brown spots with/without yellow halos, evolving from small lesions to larger necrotic areas. The symptoms followed the typical progression pattern for Pestalotiopsis infection, beginning with small yellowish-brown spots that expanded into lesions with distinct boundaries. Lesion development demonstrated morphological traits consistent with those observed in the field across various leaf positions and developmental stages, displaying colors ranging from light/dark brown to a silvery center (Figure 5, panels c–i). Re-isolation from symptomatic tissues revealed identical fungal morphology compared to the original isolate.

3.5. Discussion

This metabarcoding analysis describes the phyllosphere fungal communities at different levels of symptoms of the rubber leaf disease. As a new emerging leaf fall disease of rubber tree, various aspects are still understudied, such as the causal agents, disease distribution, characterization, variability of the pathogen, and interaction with the host plant. In this study, fungal communities associated with the rubber tree phyllosphere affected or not by the new leaf fall disease, were identified. Samples were collected from two locations at the same season. The first location, Banyuasin Regency, South Sumatra, was selected as a representative location for rubber plantations that were severely affected by this disease. This disease was reported to cause large-scale leaf fall of more than 5000 hectares in South Sumatra in 2018, with 50–75% of the leaves falling (Febbiyanti and Fairuzah 2020). The second location, Cilacap Regency, Central Java, was selected as a representative for rubber plantation area more recently impacted by the disease, in 2021.

Principal coordinate analysis (PCoA) plot based on the species composition and the relative abundance of species indicated that there was no distinct clustering formed based on the differences of symptom severity. Another study on mycobiome of a symptomatic (leaf spot) oil palm leaf reported that there was no significant difference in the diversity and richness between symptomatic and asymptomatic trees in both sample types and location (Azeez et al. 2024). Their PCoA analysis of the beta diversity of all samples based on location and symptom type showed that there was no distinct clustering pattern among the individual samples. Earlier, the study on the fatal yellowing disease of oil palm by de Assis Costa et al. (2018) reported that there was no distinct clustering of FYD-symptomatic samples, but distinct clustering was observed from FYD-asymptomatic samples.

The comparison of dissimilarity between sample pairs

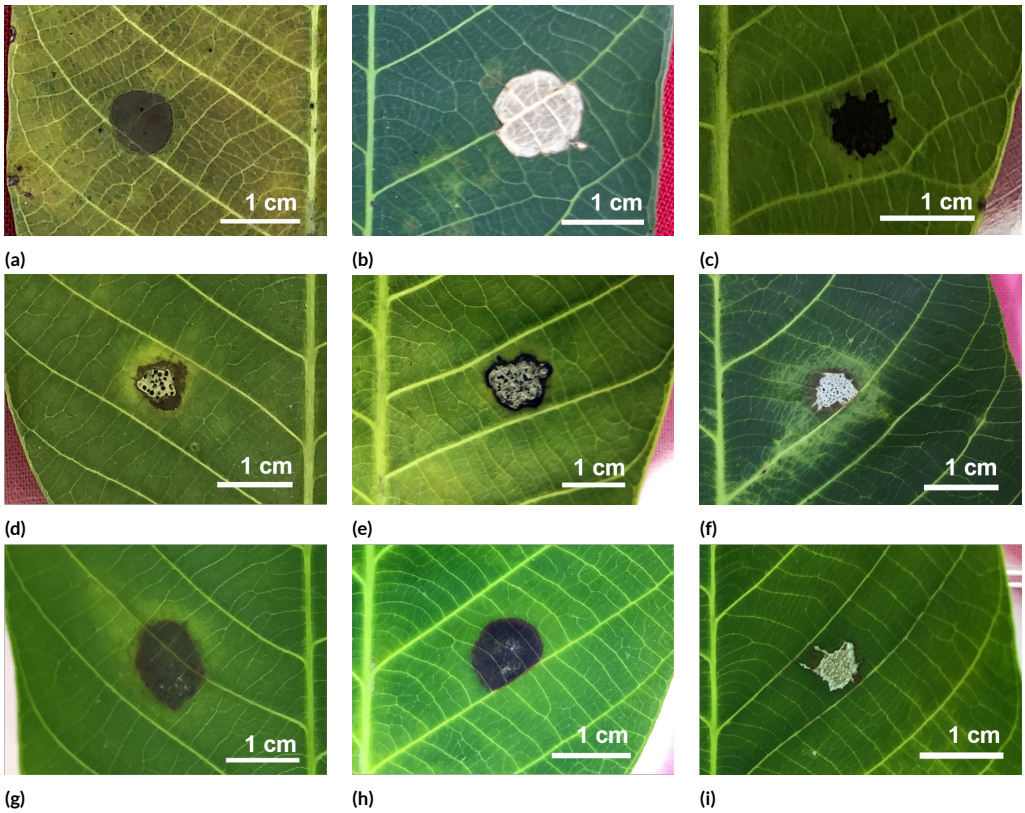

FIGURE 5 The symptoms formed by natural infections in the field with spots ranging from light/dark brown (a) to a silvery center coloration (b), and artificial infection of isolate P01 (c), P71 (d), P54 (e), P187 (f), C52 (g), C228 (h), P226 (i), on rubber detached leaflets, photo by B. Setyawan.

indicated that each sample in this study showed relatively distinct fungal community composition and abundance compared to the others. Pairwise beta diversity dissimilarities among samples within the same group (J–J or S–S) were generally lower than those between groups (J vs S), indicating that fungal communities were more similar within sites and more distinct between sites. Disregarding species abundance, the pairwise comparison of unique phylogenetic lineages across communities identified a wide range of beta diversity values (from 0.066 to 0.907), with no clear pattern associated with symptom levels or sampling locations. Similar result was reported by de Assis Costa et al. (2018) that fungal communities were not the same between samples at the same level of the symptomatic samples of fatal yellowing disease in oil palm. Similarly, a study on native rubber trees in the Brazilian Amazon identified specific phyllospheric core fungal communities across different plant parts and growth stages (Fonseca et al. 2022). The conversion of tropical rainforest to rubber plantations altered soil fungal community composition and reduced beta diversity, although alpha diversity remained comparable (Lan et al. 2020). In flowering dogwood, significant differences in microbial community composition were observed between healthy and diseased sites, with both genetic and environmental factors influencing foliar fungal communities (Pais et al. 2024). These studies highlight the complex relationships between plant hosts, fungal communities, and environmental factors in shaping fungal diversity in the rubber plant phyllosphere.

Overall, the fungal community in all samples is dominated by the phylum Ascomycota, followed by Basidiomycota, in both South Sumatra and Central Java plantations. Similar studies on rubber tree (*Hevea brasiliensis*) phyllosphere microbiomes also allowed significant insights into fungal community composition. The phylum Ascomycota dominated these communities, followed by Basidiomycota (Nizamani et al. 2023; Wei et al. 2022; Araújo et al. 2020; Kembel and Mueller 2014). At the genus level in this study, *Phyllosticta* and *Colletotrichum* were dominating in samples from both areas. The study by Araújo et al. (2020) on the diversity and distribution of endophytic fungi in different tissues of *Hevea brasiliensis*, native to the Brazilian Amazon forest, also revealed that the most abundant isolates belonged to the genus *Colletotrichum* (family Glomerellaceae). This result was similar to that of Musdalifah et al. (2025), reporting that various fungal genera, including potential pathogens like *Colletotrichum* and *Phyllosticta*, have been identified in rubber tree organs and soil. Several genera with pathogenic potential for the disease under study showed distinct group-specific patterns in their relative abundance between the S and J sample groups. For example, some genera (e.g., *Phyllosticta* and *Neopestalotiopsis*) exhibited relative higher abundance in the S group, while others (e.g., *Colletotrichum* and *Corynespora*) were more prominent in the J group (Figure 3). Notable distinctions in relative abundance between the two groups are evident throughout multiple taxonomic levels. These genera were always found in high abundance in the samples with severe symptoms. These taxonomic differences emphasize significant compositional variation in fungal community structure between the two affected areas. Kembel and Mueller (2014) reported that the host plant taxonomy and functional traits strongly influenced fungal community structure, explaining over half of the variation in composition. The observed genus-level differences may reflect the influence of environmental conditions, host-specific factors, or treatment effects that selectively enrich or suppress certain fungal groups. According to Wei et al. (2022), environmental factors, particularly mean annual temperature, play a crucial role in shaping fungal communities, leading to distinct biogeographical patterns.

Several potentially pathogenic fungal genera in rubber were detected among all samples, including *Phyllosticta*, *Colletotrichum*, *Diaporthe*, *Corynespora*, *Mycosphaerella*, *Curvularia*, *Periconia*, *Nigrospora*, *Neopestalotiopsis*, *Cercospora*, *Ganoderma*, *Pestalotiopsis*, *Pestalotia*, and *Cytospora*. All of these fungal genera have been previously reported to be associated with rubber plants (Senwanna 2021). Previous reports indicated that the main pathogens involved in the new leaf fall disease of rubber tree in Asia were genera belonging to the Sporocadaceae family (*Pestalotiopsis*, *Neopestalotiopsis*, *Pseudopestalotiopsis*) and/or *Colletotrichum* (Aliya et al. 2022; Febbiyanti and Fairuzah 2020; Pornsuriya et al. 2020). In Africa (Cameroon) *Pestalotiopsis* was identified as a pathogen causing leaf fall in rubber tree (Nyaka Ngobisa et al. 2017), although the symptoms described were not completely similar to those observed in Asia. Our metabarcoding analysis found that *Phyllosticta* dominated the population in all samples percentages ranging from 0.08 to 20.32% depending on the samples, compared to *Colletotrichum*, ranging from 0.05 to 6.63%. Meanwhile, other genera were found at a percentage less than 0.25%. The dominance of putatively assigned to *P. capitalensis* based on ITS and species belonging to the *Colletotrichum gloeosporioides* complex, in all rubber leaf samples in this study but more importantly in the samples with severe symptoms, suggested a potential role of these species in the development of the disease. *C. gloeosporioides* was commonly found as a pathogen in rubber (Brown and Soepena 1994; Wastie and Janardhanan 1970). *P. capitalensis* has been identified to be a pathogen of rubber foliar disease in India (Narayanan 2020), Sri Lanka (Herath et al. 2019) and was first reported in three plantations in the Eastern Plains region, Colombia, since 2020 (Guevara et al. 2022).

In this study, three isolates from South Sumatra (C52, P01, and P54) and four isolates from Central Java (C228, P71, P187, and P226) were selected based on the characteristics of *Colletotrichum* and *Pestalotiopsis*-like colonies, known as putative pathogens associated with this disease. The two-loci phylogenetic tree, constructed using a concatenated data set of ITS and β-tubulin gene sequences, clustered the isolates into three main clades. In

the first clade, corresponding to the *Neopestalotiopsis*, the isolates from South Sumatra (P01 and P54) and two isolates from Central Java (P187 and P226) were clustered into distinct subclades: P01 is closely related to *N. protearum*, in a group also containing *N. cubana*; P54 is closely related to *N. aotearoa*, in a group also containing *N. phyllostachydis* and *N. formicarum*. P226 may belong to the *N. thailandica* species, and P187 remains an undertermined *Neopestalotiapsis* sp. Previously, Pornsuriya et al. (2020) identified *N. cubana* and *N. formicarum* to be associated with the novel leaf fall disease of rubber trees in Thailand. Similarly, *N. aotearoa* was reported for the first time in China, as causing agent of the new leaf fall disease in rubber tree seedlings (Li et al. 2021). *N. protearum* was involved in oil tea seed rot (Tang et al. 2021). *N. thailandica* was found associated with leaf spots of *Rhizophora mucronata* Lam in Thailand (Norphanphoun 2019) and subsequently to leaf fall disease of *Hevea brasiliensis* in Indonesia (Kandila 2023; Febbiyanti and Matsui 2025). In the second clade, corresponding to the P*estalotiopsis* group, isolate P71 from Central Java is closely related to *P. diploclisiae* and, more distantly, to *P. endophytica*. *P cratoxyli* and *P. piraubensis*. Two *P. microspora* species form a distinct subclade among the *Pestalotiopsis*. Previous studies identified *P. microspora* (Kusdiana 2021) and *P. jesteri* (Aliya et al. 2022) as causing agent of the new leaf fall disease in Indonesia and Malaysia respectively. The last clade consists of *Colletotrichum* species of the *C. gloesporioides* species complex. The two *Colletotrichum* isolates from our study (C52 from South Sumatra and C228 from Central Java) are related to the type species *C. gloeosporioides* but distinct from the two *C. siamense* species. A previous study reported that *C. siamense* might be the primary causal pathogen of the new leaf spot disease of rubber in Malaysia (Aliya et al. 2022).

The finding in this study indicated that clustering was not formed by location, since genetically related isolates from different locations grouped into one clade. Solarte et al. (2018) characterized 81 isolates from guava in different regions of Colombia and identified them as *Pestalotiopsis* and *Neopestalotiopsis* spp. based on the internal transcribed spacer, β-tubulin, and elongation factor genes. They reported that the diversity of the isolates did not correlate with geographical origin.

This study shows how combining bioinformatics predictions with experimental validation represents a contemporary approach to studying fungal pathogenesis. In this framework, computational genomics acts as a valuable tool for identifying virulence factors, which are then verified through biological assays. These predictions must be validated experimentally to ensure biological relevance (Josephin et al. 2024; Longsaward et al. 2024; Song et al. 2024). Our bioinformatics pipeline successfully identified strains predicted to contribute to the new leaf fall disease, with pathogenicity tests validating these predictions by reproducing characteristic circular brown leaf spots with distinct boundaries consistent with previously documented infection patterns across rubber-growing regions (Febbiyanti et al. 2021; Hasbunallah et al. 2023; Kusdiana 2021; Li et al. 2021; Solpot et al. 2023). This approach aligns with the revised Molecular Koch's postulates for experimental confirmation of in silico findings (Tan and Mohamad 2024). Our results contribute to growing evidence that bioinformatics-guided pathogen characterization, when coupled with appropriate biological validation, provides a reliable framework for identifying disease-causing agents or even understanding their mechanisms of action (Verly et al. 2025). This integrated approach is particularly valuable for emerging diseases such as the one hitting rubber trees in Asia where rapid identification and validation of pathogenic strains can inform management strategies across rubber plantations experiencing epidemic outbreaks in Malaysia, Indonesia, Thailand, and other major rubber-producing nations.

This study has a major limitation due to the temporal mismatch between the samplings for metabarcoding and for strain isolation, resulting in incomplete validation of computational predictions. The fungal isolation procedure that was used in this study was following previous publications and may have missed slow-growing fungi such as *Phyllosticta*, later identified by our metabarcoding analysis as a dominant contributor to the new leaf fall disease. Indeed, on synthetic media, *Phyllosticta* grows much more slowly than *Colletotrichum*, *Pestalotiopsis* and many other fungi associated with rubber (Pujade-Renaud, pers. com.). Future isolations will need to take this parameter into account.

Further studies on *Phyllosticta* are required, as it has also emerged as a potential pathogen causing defoliation in rubber plantations across other countries, such as India (Narayanan 2020) or Colombia (Guevara et al. 2022). Without biological validation of this predicted dominant pathogen, the pathogen complex driving the epidemic remains incompletely understood.

Future research should focus on isolating and testing *Phyllosticta* strains along with other bioinformatics-predicted candidates for thorough validation This case study illustrates the utility of the metabarcoding approach in revealing unculturable or hard-to-isolate fungi, overcoming limitations of culture-dependent methods, as shown previously (Pérez-Cobas et al. 2020). Integrating next generation sequencing approaches with traditional isolation methods can enhance the detection and characterization of fungal taxa, enable more thorough pathogen profiling, minimize biases toward easily culturable species, and emphasize those that had a greater role in disease progression, as demonstrated in other studies on tree diseases (Cho et al. 2025; Yang et al. 2022). These efforts would strengthen evidence for bioinformatics predictions and enhance insights into the multi-pathogen causes of new leaf fall epidemics in global rubber regions.

4. Conclusions

This metagenomic analysis revealed that rubber leaf fall disease results from a complex, location-dependent fungal community, rather than dominance by a single pathogen. While fungal diversity showed no consistent correlation with disease severity, *Phyllosticta* and *Colletotrichum* emerged as dominant taxa, with biogeographical variation suggesting that environmental and host factors shape pathogenic communities across regions. Phylogenetic analysis and pathogenicity testing confirmed *Neopestalotiopsis*, *Pestalotiopsis*, and *Colletotrichum* species as disease agents; however, without validation of the dominant *Phyllosticta capitalensis*, the complete pathogen complex remains incompletely understood. These findings demonstrate that multi-pathogen complexes drive epidemics in rubber plantations, and future research must integrate metagenomic discovery with comprehensive biological validation of all predicted dominant taxa to inform effective integrated disease management strategies across rubber-growing regions.

Acknowledgments

The authors thank the Kementerian Riset dan Teknologi/Badan Riset dan Inovasi Nasional of Indonesia [ID Proposal: a2ec43e0-7310-49ed-9b31-eb8b67f857f1] for their support for the project; Arsiyah for her assistance in laboratory; Alchemi Putri Juliantika Kusdiana and Tri Rapani Febbiyanti for kindly providing P01 isolate; and Ady Bayu Prakoso for helpful discussions at the beginning of the project; and Intan Berlian for kindly providing photograph used as a part of Figure 1.

Authors' contributions

BS, AW, VPR and SS conceived the study. BS performed all laboratory work and conducted the bioinformatics and statistical analyses, with assistance from AW and SS. BS contributed to sample collection from Central Java, while SS collected samples from South Sumatra. BS wrote the initial draft, which was revised by SS, followed by contributions from AW and VPR. The study was supervised by AW, VPR and SS. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare no competing interests or personal relationships that could have appeared to influence the work reported in this paper.

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