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RESEARCH ARTICLE

# Morphological characterization, molecular identification, and phylogenetic analysis of *Lasiodiplodia theobromae* associated with CCN-51 cacao plants in Ecuador

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**Abstract**

Necrotrophic fungi are pathogens that cause tissue death in plants, which negatively impacts their growth and productivity. This study focused on identifying the presence of *Lasiodiplodia theobromae* in CCN-51 cacao plants in the Simón Bolívar canton in Ecuador. We sampled cacao pods exhibiting necrotic lesions and obtained fungal isolates for morphological and molecular characterization. Techniques, such as culturing on selective media, microscopy, and DNA sequencing were used to confirm the fungal identity. We compared our results with international databases and assessed the genetic variability of the isolates. Morphological characterization placed the fungal isolates within the family *Botryosphaeriaceae*, and molecular analysis using ITS and EF1-α regions confirmed the species as *Lasiodiplodia theobromae*, with 100% DNA quality for amplicon analysis and 100% sequence similarity in GenBank. We constructed phylogenetic trees using maximum likelihood methods, which revealed high genetic similarity and recent divergence among sequences despite their varied geographic origins. The fungal isolates specifically confirmed the presence of *L. theobromae* as the causal agent of necrotic lesions in CCN-51 cacao pods from Simón Bolívar. These findings underscore the importance of studying necrotrophic fungi in cacao plants to inform control strategies, improve crop resistance, and support sustainable production, essential to the global cacao trade.

**Keywords**: Disease; species; ITS region; molecular identification; necrosis; rot.

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## 1. Introduction

Cacao (*Theobroma cacao*) is one of the main economic resources of tropical countries, particularly in Latin America, where it represents a significant agricultural and commercial activity (Lander et al., 2025). In Ecuador, cacao cultivation has historically been a cornerstone of the rural economy, with the CCN-51 variety standing out due to its disease resistance and high yield (Vera Rodríguez et al., 2021). However, in recent years, various diseases have negatively affected cacao productivity and quality, with *Lasiodiplodia theobromae* emerging as one of the most concerning pathogens for local farmers (Alvarez-Romero et al., 2025).

*L. theobromae* is a necrotrophic fungus from the genus *Lasiodiplodia*, within the family *Botryosphaeriaceae* (Ko et al., 2025). This pathogen is known for causing tissue rot in plants and is characterized by its ability to infect various parts of the plant, including branches, trunk, and fruit (Cambero-Ayón et al., 2024). In cacao cultivation, this fungus represents a major concern for the industry, as it can drastically reduce both yield and harvest quality (Flores Hernández et al., 2021).

Infection by *L. theobromae* primarily manifests as necrotic lesions on branches and fruit, often leading to their premature drop (Nurhikmah Mutmainna et al., 2025). The decomposition of plant tissues may affect the structural integrity of the plant, compromising its ability to photosynthesize and thereby reducing yield (Sultana et al., 2025). Studying the identification of this fungus in cacao is crucial for understanding the factors that facilitate its spread and for implementing effective control strategies (Yang et al., 2025).

Agroclimatic conditions and agricultural management practices can significantly influence the prevalence of *L. theobromae* (Clavijo, 2022). Other factors such as high humidity, elevated temperatures, and the presence of plant debris promote the growth and spread of this pathogen (Plasencia-Vázquez et al., 2022). In addition, the frequent use of susceptible varieties and the lack of preventive measures contribute to the rapid propagation of the disease in cacao plantations (Sudha et al., 2025). The disease caused by *L. theobromae* in cacao plants has become an emerging threat in several cacao-growing areas (Canchignia-Martínez et al., 2025). This is the case in Simón Bolívar canton, located in Guayas province, Ecuador, where CCN-51 cacao is grown in diverse soil types and a tropical climate that favors pathogen development. Although CCN-51 has shown tolerance to certain diseases, its susceptibility to *L. theobromae* poses a challenge to its long-term viability (Jiménez et al., 2022).

To mitigate the impact of this causal agent in cacao plantations, it is essential to understand its taxonomy, biology, and ecology (Huda-Shakirah et al., 2022). Early symptom detection, continuous monitoring, and targeted control are commonly used strategies to reduce the damage caused by this pathogen (Huaman-Pilco et al., 2024). Nevertheless, the implementation of sustainable control measures (Moreira et al., 2021), along with the use of resistant varieties, is a key step toward improving cacao productivity in regions affected by this fungus (Ferreira de Souza et al., 2025).

The primary goal of this study was to identify the presence of *L. theobromae* in CCN-51 cacao plantations in Simón Bolívar canton. Due to the limited research available in this region, this study contributes to a deeper understanding of the pathogen and its effects on cacao crops. Furthermore, the results obtained will serve as a foundation for designing more effective management strategies that promote plant health and support farmers in addressing the challenges posed by this disease, thereby contributing to economic stability and food security in the region.

## 2. Methodology

The collection of samples (infected cacao pods) from CCN-51 variety cacao plants was carried out on three properties located in the Hermanos Quito area, within the rural administrative division of Lorenzo Garaicoa, Simón Bolívar canton, Guayas province, Ecuador. The isolation and identification of the fungal agent were conducted at the microbiology laboratory of the Faculty of Science and Engineering at the State University of Milagro, Ecuador.

### Field Sampling

The field sampling was carried out on three cacao-producing farms in the area previously described, identified as H770, H771, and H629, all located within a 1.5 km radius. The producers reported fruiting losses exceeding 40%, along with signs of anthracnose and plant death. For this purpose, blackened cacao pods were collected as samples, labeled, and stored at 8 °C for transport to the laboratory.

### Fungal Isolation

The samples were washed with neutral soap and distilled water, then submerged in 1% sodium hypochlorite for 2 minutes and subsequently sterilized in 96% ethanol for 20 seconds. To inoculate the infected tissue onto the culture medium, initial cuts were made on the surface of the pod's exocarp, and 10 $mm^2$ fragments of the mesocarp were extracted and placed onto potato dextrose agar (PDA, 39 g/L) in 90 mm glass Petri dishes. The plates were sealed with parafilm and incubated at 28 °C and 75% relative humidity for 7 days. During the purification phase, 5 $mm^2$ fragments of fungal mycelium were transferred to PDA supplemented with V8 juice in a 10:2 ratio and incubated under the same temperature and humidity conditions for 14 days (Figure 1).

### Morphological Characterization

To confirm that the fungal isolates belonged to the *Botryosphaeriaceae* family, we conducted both macroscopic (color, shape, and mycelial growth) and microscopic observations. Microscopic features, such as hyphae, conidiophores, and conidia, were examined using a Motic™ Panthera S trinocular microscope (manufactured in China) under a 40x objective. These observations were then compared against taxonomic keys.

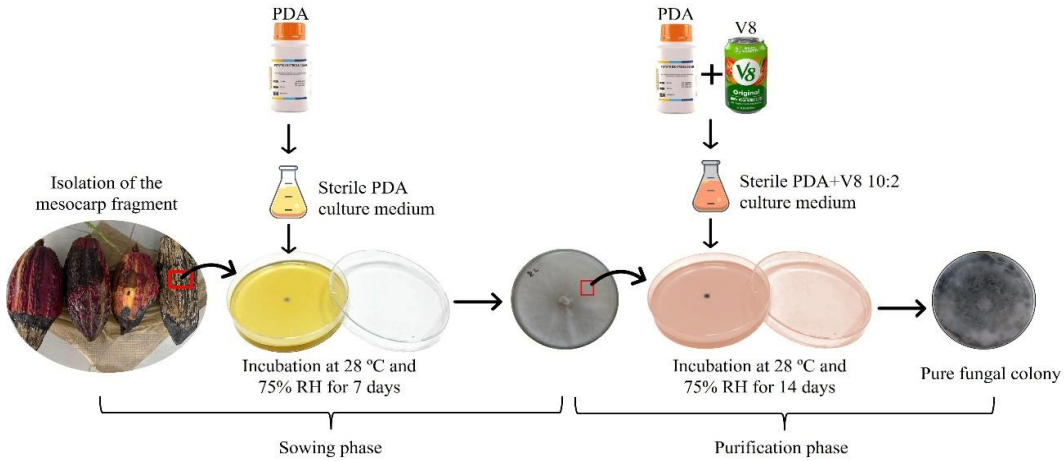


Figure 1. Schematic of the fungal isolation process.

### Molecular identification

The fungal isolates were subjected to molecular identification through ITS and EF1-α barcoding. Genomic DNA was extracted from 100 mg of sample using conventional methods. The quality and quantity of the extracted DNA were assessed using a NanoDrop microvolume spectrophotometer (Thermo Scientific ND 2000C, Waltham, USA) and visualized by 1% agarose gel electrophoresis (Thermo Scientific B1A, Waltham, USA). Specific genomic regions were amplified by PCR (Macrogen and BlasTaq™) using the primers ITS1/ITS4 (White et al., 1990) and EF1-983F/EF1-2218R (Rehner & Buckley, 2005). The amplification products were purified and sequenced using the Sanger method. The resulting sequences were bioinformatically processed using Geneious version 11.1.2 and compared with reference databases such as GenBank to determine species identity.

### Phylogenetic analysis

A phylogenetic analysis was conducted on the DNA sequences obtained from the samples. The sequences were assembled and aligned using the MEGA-X software and compared using the BLAST tool. Maximum likelihood analysis was applied separately to the ITS and EF1-α regions, using reference sequences from previous studies involving the same species, particularly those reported near the study area. These sequences were retrieved from the NCBI GenBank database to evaluate the genetic variation in each region among the different isolates from the province.

## 3. Results and discussion

### Isolation of *L. theobromae*

All isolates from the different farms exhibited circular mycelial growth that was initially whitish in color, gradually turning dark black over time when cultured on PDA medium, regardless of their location, as shown in Figure 2a and Figure 2b. The morphological analysis of the isolates confirmed their classification within the *Botryosphaeriaceae* family, based on microscopic observation at 40x magnification, as illustrated in Figure 2c.

The mycelial growth observed in the Petri dishes from all farms exhibited the same uniform and continuous development, covering the full 90 mm diameter of the plate within 7 days. The colonies formed abundant aerial mycelium with a white to grayish coloration. The dark pigmentation observed at 14 days is associated with compounds released by the fungus into the culture medium. The morphological results described are consistent with those reported by Tiznado et al. (2018), who indicated that *L. theobromae* exhibited uniform in vitro growth, reaching an average of 4.6 cm in five days, with white-grayish cottony mycelium that turned olive-gray and then black. By day 20, they observed *pycnidial conidiomata*, with young conidia being oval and hyaline, while mature conidia were dark brown, thick-walled, and displayed longitudinal striations. Similarly, the study by Phillips et al. (2013) on the *Botryosphaeriaceae* family described hyphae as septate and either hyaline or pigmented; conidia as unicellular or multicellular, with varied shapes and wall thicknesses; and conidiophores as filamentous structures that may be branched or unbranched, also exhibiting differences in pigmentation.

### Molecular identification

The sizes of the amplicons obtained from all *Botryosphaeriaceae* isolates were recorded as shown in Figure 3: H770 (313 base pairs [bp] for the ITS marker and 948 bp for the EF1-α marker), H771 (516 bp for the ITS marker and 964 bp for the EF1-α marker), and H629 (424 bp for the ITS marker and 963 bp for the EF1-α marker).

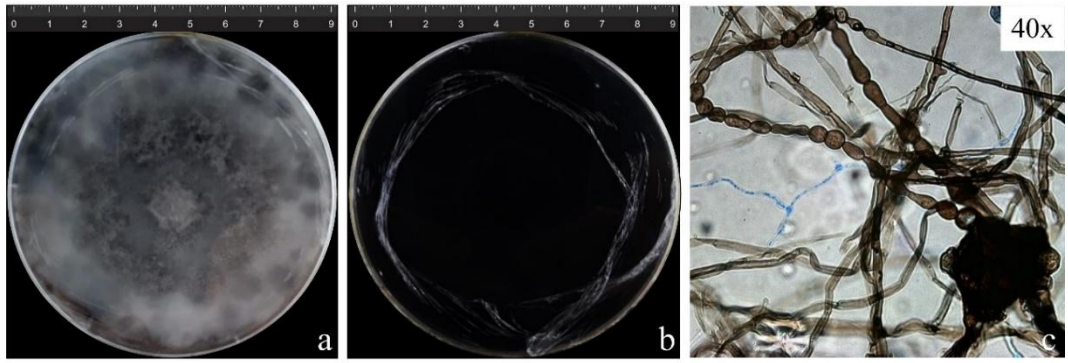


Figure 2. Macroscopic and microscopic morphological characterization. (a) Top view of the Petri dish showing fungal growth after 14 days on PDA medium. (b) Bottom view of the Petri dish observed at 14 days. (c) Hyphae, conidiophores, and conidia.

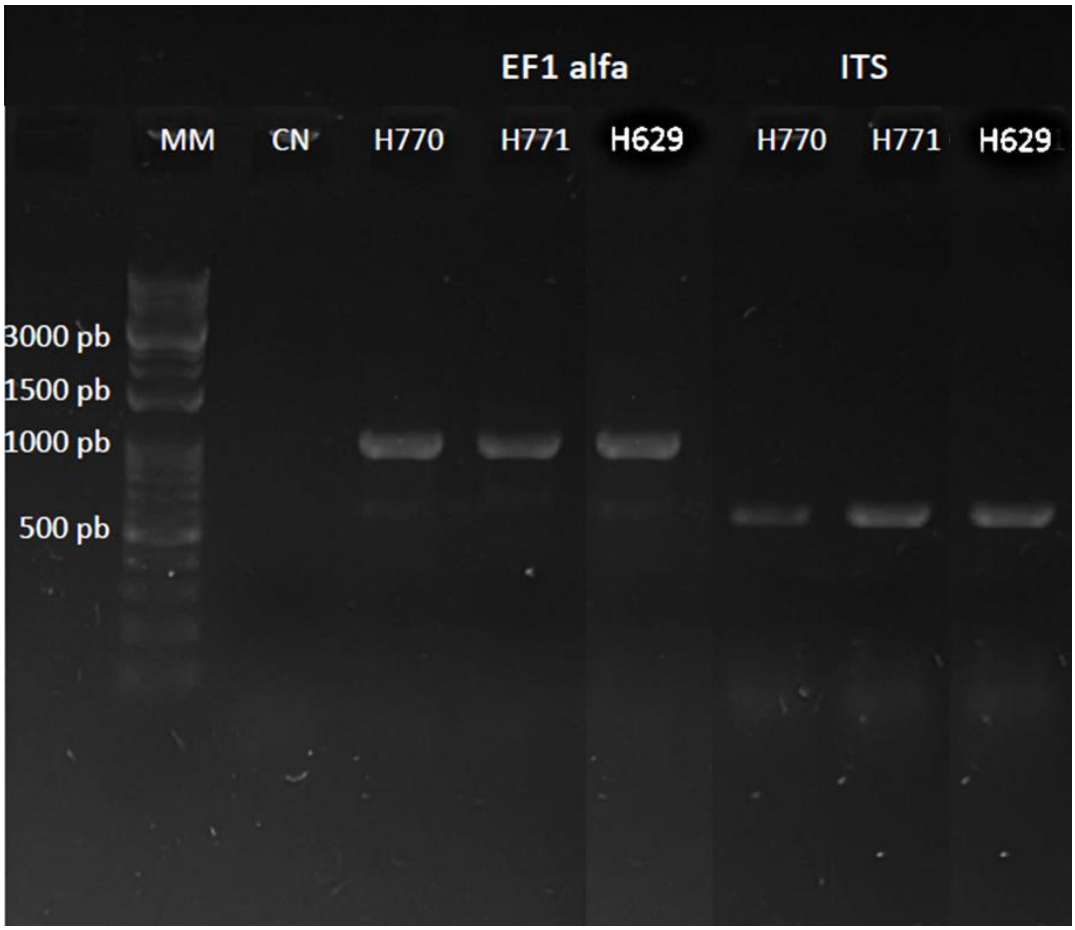


Figure 3. 1% Agarose Gel Electrophoresis. PCR products for the ITS and EF1-alpha fragments. MM = Molecular weight marker, CN = Negative control. Continuing with the molecular description, the number of base pairs obtained for the ITS and EF1-α genes is comparable to the values reported by Jiménez et al. (2022), who observed approximately 520 base pairs (bp) for the ITS region and 450 bp for EF1-α.

## BLAST Analysis (Basic Local Alignment Search Tool)

Using the previously aligned sequences, identification was carried out through the Basic Local Alignment Search Tool (BLAST). Table 1 presents the BLAST results for each sample and its corresponding code. The "Quality" column indicates the DNA quality percentage for the amplification process; the "Organism" column identifies the species with the highest match, along with the analyzed fragment and its similarity percentage. The final column provides the GenBank Accession Number corresponding to each species.

According to the GenBank database, the isolates obtained in this study correspond to the species

*Lasiodiplodia theobromae*, with a 100% sequence similarity. Pisco-Ortiz et al. (2024) reported the first occurrence of *L. theobromae* causing wilting in Theobroma cacao in Colombia. Through morphological and molecular analyses, they confirmed the identification of the pathogen and emphasized the need to implement management and control strategies to mitigate its impact on the cacao industry. This finding underscores the importance of monitoring and addressing emerging phytopathological threats in economically significant crops.

## Phylogenetic analysis

Two phylogenetic trees were constructed, one for the ITS gene (Figure 4) and another for EF1-α (Figure 5), using the maximum likelihood method for sequences closely related to the study location. The analysis allowed for the inference of evolutionary history; the trees display the most probable relationships based on the pairwise distance matrix, with branch lengths measured in the number of substitutions per site.

he observed phylogenetic tree shows the same topology for this region; all sequences belong to the same species, *L. theobromae*, involving six nucleotide sequences with the following accession numbers: PP532861.1 (Jaramillo-Aguilar et al., 2024); MT644474.1 (Jiménez et al., 2022); OQ879511.1 (Quiroz et al., 2024); OK056571.1 (Vélez-Zambrano et al., 2023); along with sequences OR116070.1 and OR116089.1. The extremely low numerical values on the branches indicate a very high genetic similarity among all sequences. This suggests that these isolates are closely related.

Table 1
Identification performed using BLAST. Closest matching species from GenBank for the isolates obtained

| Code | Quality | Organism | Gene | Identity | Nº Accesion |
|---|---|---|---|---|---|
| H629 | 99.7 | *Lasiodiplodia theobromae* | EF1-α | 100.00 | XM_035519539.1 |
| | 99.3 | *Lasiodiplodia theobromae* | ITS | 100.00 | OR116089.1 |
| H770 | 100 | *Lasiodiplodia theobromae* | EF1-α | 100.00 | XM_035519539.1 |
| | 100 | *Lasiodiplodia theobromae* | ITS | 100.00 | OR116070.1 |
| H771 | 100 | *Lasiodiplodia theobromae* | EF1-α | 100.00 | XM_035519539.1 |
| | 100 | *Lasiodiplodia theobromae* | ITS | 100.00 | OR116070.1 |

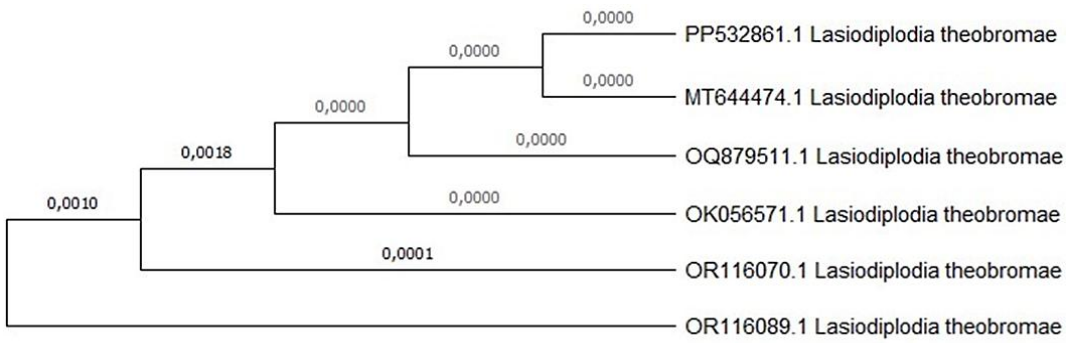


Figure 4. Phylogenetic tree. Maximum likelihood method based on ITS regions, constructed using six isolates closely related to the study location.

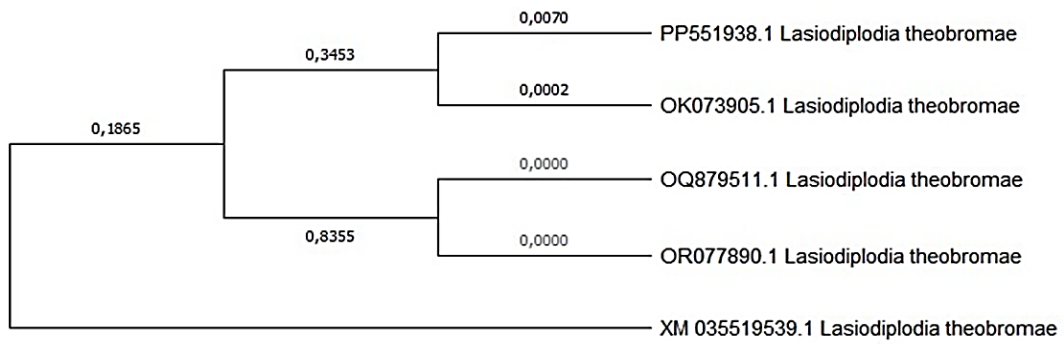


Figure 5. Phylogenetic tree. Maximum likelihood method based on EF1-α regions, constructed using five isolates closely related to the study location.

The tree structure indicates a very recent divergence among these species, as the proximity of all nodes to the root node suggests that these organisms separated evolutionarily quite recently. Mondragón-Flores et al. (2021) state that fungi belonging to the *Botryosphaeriaceae* family are responsible for diseases affecting various agricultural crops in temperate, tropical, and subtropical regions. Their ability to shift from endophyte to pathogen under stress poses a risk to crops under inadequate conditions.

This phylogenetic tree suggests that the analyzed *L. theobromae* isolates are closely related, involving five nucleotide sequences: PP551938.1 (Jaramillo-Aguilar et al., 2024); OQ879511.1 (Quiroz et al., 2024); OK073905.1 and OR077890.1 (Vélez-Zambrano et al., 2023); along with the sequence XM_035519539.1 and have undergone a recent divergence. Despite the overall high similarity, some genetic heterogeneity is observed among the species, particularly between the groups formed by PP551938.1 and OK073905.1 on one side, and the rest of the sequences on the other. This heterogeneity may indicate different geographic origins, local adaptations, or recombination events. Mendoza-Churape et al. (2024) note that the *Botryosphaeriales* family is the only group that includes pathogenic agents responsible for canker diseases in plantations. These characteristics were observed on the farms where the isolates were collected, which may explain the presence of *L. theobromae* in the current study, all associated with localized, regionalized, or widespread necrotic lesions on cacao plants.

## 4. Conclusions

This study confirmed the identity of the fungus in the collected samples through morphological and molecular analyses, identifying it as *Lasiodiplodia theobromae*, a pathogen that negatively affects cacao crops. Phylogenetic analysis revealed that the *L. theobromae* isolates studied are closely related to isolates from Ecuador reported by other authors, suggesting a recent divergence from a common ancestor. However, some genetic variability was also observed among the isolates, which may indicate local adaptations due to different geographic origins.

The results of this research have important implications for disease management in cacao cultivation. Accurate identification of *L. theobromae* as the causal agent of the observed infections is a crucial first step toward developing effective control strategies. Furthermore, understanding the genetic diversity of this pathogen can help predict its behavior and support the development of treatments or more resistant cacao varieties.

### Author contributions

**J. H. Vera-Rodríguez:** Investigation, conceptualization, methodology, writing, and project administration; **J. D. Sevilla-Carrasco** and **J. M. Duarte-Cuesta:** Laboratory analysis; **M. del R. Villamar-Aveiga** and **J. D. Ortiz-Mata**: Software; **G. Bucaram-Lara, C. S. Gavin-Moyano** and **L. R. Lucas-Vidal**: Formal analysis.

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### Conflicts of Interest

The authors declare no conflict of interest.

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