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Brief Report

Fungal Diversity Detected by ITS-5.8S from *Coffea arabica* Leaves Infected by Rust (*Hemileia vastatrix*) in Southern Ecuador

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Abstract: Coffee production worldwide is affected by the pathogen *Hemileia vastatrix*, which causes the "coffee rust" disease and may be associated with other fungi. Ecuador lacks studies on fungal diversity associated with coffee rust, which could potentially control or escalate pathogen activity. Using the ITS-5.8S nrDNA region, we randomly detected a small preliminary fungi diversity related to coffee rust in Ecuador, which we report here for the first time. Ten coffee farms (four in Loja, three in Calvas, and three in Quilanga) from the Loja Province were sampled to analyze the genetic diversity of the pathogen *Hemileia vastatrix* in rust lesions on coffee leaves. A high number of selected sequences (Sanger sequencing) showed the presence of 48 OTUs (Operational Taxonomic Units) or "hypothetical species" of Ascomycetes and Basidiomycetes distributed across all the sampled farms. The genera *Akanthomyces*, *Ceramothyrium*, *Cladosporium*, *Didymella*, *Fusarium*, *Mycosphaerella*, *Neoceratosperma*, and *Trichothecium* of Ascomycetes, as well as *Bulleribasidium*, *Hannaella*, and *Meira* of Basidiomycetes, were the most abundant. To avoid taxonomic conflict, some sequences were placed into Capnodiales (Ascomycetes) and Tremelalles (Basidiomycetes) without a genus definition. A new phylogenetic group of sequences is considered *Incertae Sedis* from Basidiomycetes. Additionally, morphospecies of *Akanthomyces* (synonymous with some *Lecanicillium* species) and *Colletotrichum* were observed macroscopically and microscopically growing closely with rust. Most of the OTUs probably correspond to rust mycoparasites, as previously reported in the literature. However, this study is limited by the number of sequences analyzed phylogenetically, which may hinder the discovery of significant insights. Future studies are needed to determine whether this preliminary fungal diversity is associated with the rust fungus or corresponds to ubiquitous airborne fungi. Furthermore, research into the function of these species may reveal whether they promote rust pathogenicity or enhance plant responses by activating resistance mechanisms.

Keywords: Ascomycete; Basidiomycete; coffee rust; fungal diversity; mycoparasites; OTUs

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1. Introduction

Coffee (*Coffea* spp.), with its most cultivated species *Coffea arabica* L. (common name Arabica coffee) and *Coffea canephora* Pierre ex A. Froehner (common name Robusta coffee), is one of the main crops, representing export produce with a high economic value for Ecuador [1,2]. Moreover, coffee cultivation supports the preservation of flora and fauna within agroforestry systems [3], while coffee consumption has been linked to the prevention of certain health issues, such as type 2 diabetes [4] and neurodegenerative diseases [5]. However, coffee production is highly susceptible to attacks by the Basidiomycete fungus

Hemileia vastatrix Berk. and Broome, which causes the "coffee rust" disease [6–8]. This pathogenic fungus mainly attacks the coffee leaf stomata, causing premature defoliation, a reduction in photosynthetic capacity, and low fruit yield [9]. This disease causes economic losses of up to a billion dollars annually and affects all types of crops, regardless of their production regime (organic or inorganic) [10].

The rust disease requires adequate control through resistant plant varieties [11] or intensified fungicide applications [12]. The application of bio-controllers, for example, fungi belonging to *Calcarisporium*, *Clonostachys*, *Lecanicillium*, *Sporothrix*, and *Simplicillium*, has proven efficient against coffee rust [12,13]. These fungi may also enhance or beneficially regulate plant resistance [11]. On the other hand, interactions between microorganisms through metabolite secretion could favor pathogenic activity [14]. In this context, it is crucial to know the microorganism diversity that could be either promoting rust pathogenicity or enhancing plant functionality by activation of their resistance genes [11,12]. Furthermore, different fungi occupy different ecological niches, such as coffee fruits [15] or coffee roots [16], and have different functional roles [17,18]. The diversity of these fungi—whether pathogenic, antagonistic, or decomposers—may decrease or increase when cultivated areas are abandoned and regenerated [19]. Currently, the ITS-5.8S nrDNA marker, known as a Universal barcode for fungi, along with next-generation sequencing and metagenomic techniques, allows for the detection and identification of fungal diversity based on Operational Taxonomic Units (OTUs) [20,21]. Molecular identification is recommended as a high-throughput quantification tool to address population dynamics, community ecology, as well as host–microorganism associations [22].

In Mexico, multiple OTUs, referred to as "hypothetical species" within the genera *Cladosporium* spp., *Lecanicillium* spp., or *Trichothecium* spp. along with "species" from the order Tremellales, were detected in coffee rust lesions using metagenomics; they are considered putative mycoparasites of coffee rust [12]. Gómez-De La Cruz et al. [23], from Veracruz, Mexico, reported the positive in vitro activity of isolates from similar genera, such as *Lecanicillium*, *Calcarisporium*, *Sporothrix*, and *Simplicillium*, which act as mycoparasites against rust. Likewise, Guatimosim et al. [24] reported the biocontrol capacity of "*Mycosphaerella yunnanensis*" (currently *Neoceratosperma yunnanensis*) against rust. In the Neotropical region (e.g., Peru, Brazil, Colombia, and Ecuador), several studies have been conducted, mainly targeting rust genetic diversity [18,25,26] or directly assessing the mycoparasitic activity of fungi against the rust [23,27,28], though studies identifying the fungal diversity associated with rust are rare [15].

In Ecuador, no research on the fungal diversity, molecularly associated or related to rust, has been found in the reviewed literature. In this context, the present research aims to (a) detect, for the first time, preliminary fungal diversity related to rust lesions in coffee leaves from 10 farms in the Province of Loja using the ITS-5.8S nrDNA region; (b) investigate how the diversity of fungi detected is distributed across different coffee varieties and farms. These results are the first step towards describing and understanding the fungal diversity closely related to coffee rust and its potential functional role in disease prevention and management.

2. Materials and Methods

2.1. Study Area

This study was carried out in Loja Province across 10 coffee farms (obtaining farmer consent) located at altitudes between 1642 and 2090 m.a.s.l (Table 1; Figure 1). Four farms were in the Loja canton, three in the Calvas canton, and three in the Quilanga canton. *Coffee arabica*, recognized by its long, oblong fruits, is generally found growing at higher altitudes (approx. 800 to 2000 m.a.s.l). In the studied area, coffee is usually grown in open ecosystems, often intercropped with crops such as banana (*Musa* × *paradisiaca* L., Sp. Pl.) and orange (*Citrus* × *aurantium* var. *sinensis* L., Sp. Pl. (Linnaeus)), under the shade of eucalyptus (*Eucalyptus globulus* Labill., Voy. Rech. Pérouse) or local plants such as porotillo (*Erythrina smithiana* Krukoff, Brittonia) (Figure 2).

Table 1. Information for the analyzed samples in this study.

Canton	Coffee Farms by Sector	Number of Samples	Plant Varieties	Latitude and Longitude	Altitude m.a.s.l
Loja	El Cristal	6	Typica/criolla	−4.1206; −79.1993	1973.7
	San Pedro de Vilcabamba. Farm 1	14	Typica/criolla	−4.2343; −79.2206	1678.6
	San Pedro de Vilcabamba. Farm 2	4	Typica/criolla	−4.2341; −79.2215	1706.7
	San Pedro de Vilcabamba. Farm 3	4	Typica/criolla	−4.2342; −79.2225	1730.5
Calvas	Jiropamba	14	Paca, yellow Bourbon, red Bourbon,	−4.3556; −79.5789	2090.7
	Surununa	9	Catucai, yellow Bourbon.	−4.2996; −79.7188	2001.6
	Cango Bajo	11	Typica/criolla, yellow Bourbon, red Bourbon	−4.3448; −79.5789	1908.4
Quilanga	San José. Farm 1	8	Catucai, San Salvador	−4.3743; −79.3948	1582.8
	San José. Farm 2	11	criollo, Paca, San Salvador	−4.3719; −79.4031	1706.8
	San José. Farm 3	3	caturra	−4.3746; −79.3997	1642.4
Total		84			

Plant varieties were classified according to the Asociación Nacional del Café ANACAFE and Velásquez [29].

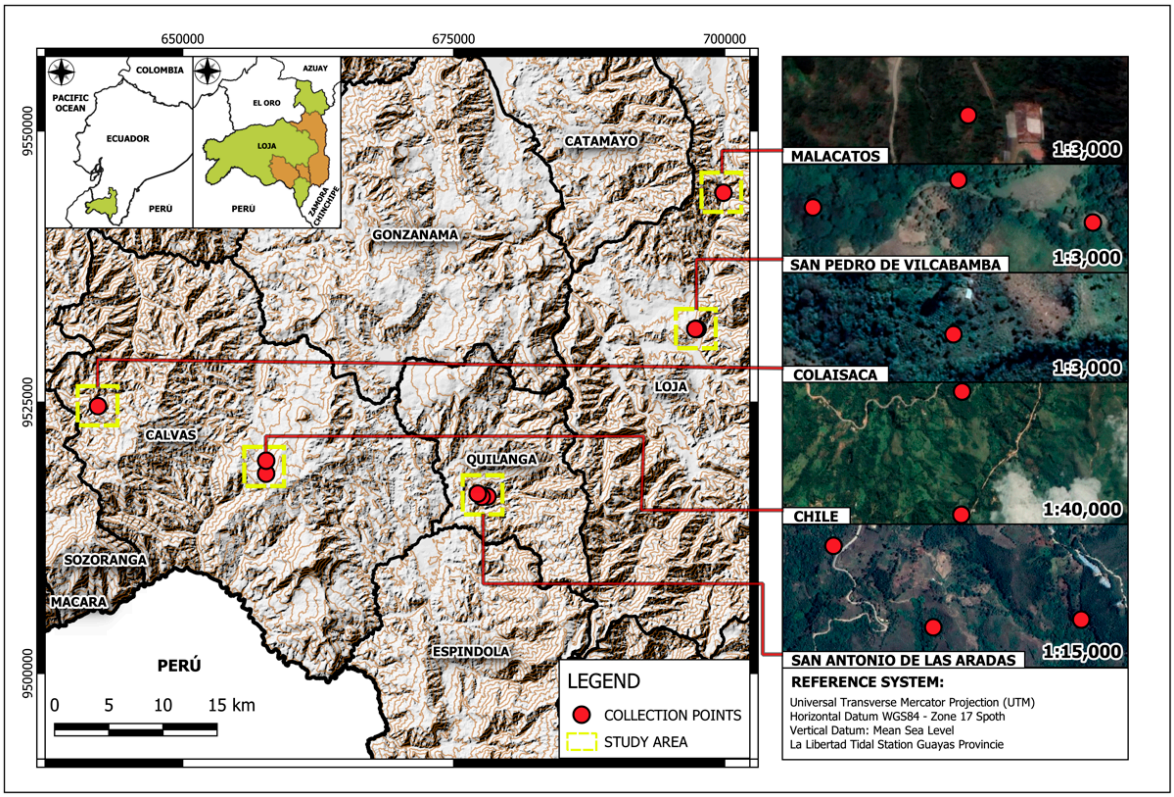

Figure 1. Topographic map showing the elevation and geographical location of the coffee farms sampled in the cantons of Calvas, Loja, and Quilanga in Loja Province.

Figure 2. General coffee-growing ecosystems from the sampled farms in the different cantons: (**A**) cultivation with bananas and other plants (Calvas: Jiropamba and Cango Bajo; and Loja: El Cristal); (**B**) growing in the shade of eucalyptus (black arrowheads) (Quilanga); (**C**) growing in more open areas (Loja: San Pedro de Vilcabamba); and (**D**) growing along with "porotillo" plants (black arrowhead) (Quilanga).

These study areas primarily encompass arid and humid–dry ecosystems, featuring a rainy season in April and May, followed by a dry season characterized by low rainfall from June to November/December [30]. Annual temperatures range from 15.6 to 21 °C, while average rainfall varies between 668 and 1149 mm per year [31]. The land cover is predominantly composed of dry deciduous forests, with maize and coffee production being the main agricultural activities, along with sparse sugar cane cultivation in the secondary valleys (e.g., Malacatos and Vilcabamba), as recorded in Ochoa et al. [31].

2.2. Data Collection

In each of the 10 coffee farms (Figure 1), 10 coffee plants were selected; from each plant, 10 leaves with yellowish to orange "rust-like" lesion symptoms (considered to be generally caused by *H. vastatrix*) were collected. A total of 100 samples were generated (10 leaves per sample). The leaves were stored in plastic bags at room temperature and analyzed in the laboratory on the same day.

2.3. Microscopic Examination of the Samples

All samples were examined using a stereomicroscope (Stemi Carl Zeiss AG; Oberkochen; Germany) and a microscope (Axiostar Plus, Carl Zeiss AG; Oberkochen; Germany) by scraping the rust sporulation area (about 5 mm^2). The slide preparations included a drop of 10% KOH or H_2O for observation under light microscopy to 40× and 100× magnification. This method allowed us to rule out samples corresponding to other infections. Different structures, such as uredospores and teliospores, were recorded.

2.4. DNA Extraction and PCR

The selected samples mostly consisted of rust uredospores (approximately 4 mg) scraped from the rust lesion area of each sample. Total DNA was extracted and amplified using PCR (Polymerase Chain Reaction) following the protocol established in the Phire Plant Direct PCR kit (Thermo Scientific, Waltham, MA, USA), as previously described by Apolo et al. [32].

The ITS-5.8S region of nrDNA was amplified with the primer combination ITS1F 5′-CTTGGTCATTTAGAGGAAGTAA-3′/ITS4 3′-TCCTCCGCTTATTGATATATGC-5′ [33]. The PCR parameters for 20 µL (final reaction volume) were as follows: initial denaturation at 98 °C for 5 min, followed by 40 cycles. Each cycle consisted of a denaturation step at 98 °C (30 s), annealing at 55 °C (30 s), and a final extension at 72 °C (30 s). PCR results were analyzed via electrophoresis, combining 2 µL of the PCR product with 1 µL of bromophenol blue (Sigma-Aldrich, St. Louis, MO, USA), and run on a 1% agarose gel in 1× GelRed™ Safe Nucleic Acid Gel Stain solution (Biotium, Hayward, CA, USA). Positive and negative controls were included in the PCR process.

Positive PCR products were purified using the PureLink PCR Purification Kit following the manufacturer's protocol (Invitrogen, Thermo Scientific, MA, USA). The amplicons were sequenced (Sanger sequencing) using the same set of PCR primers at Macrogen (Teheran-ro, Gangnam-gu, Seoul, Republic of Korea).

2.5. Phylogenetic Analysis and OTUs Placement

The resulting ITS-5.8S sequences (randomly detected) were visualized for quality control and edited using the software CodonCode Aligner 5.1.4 (CodonCode Corporation, Centerville, MA, USA). The edited sequences were compared in GenBank Blast [34], downloading the sequences with the highest similarity percentages, preferably with taxonomic identities (assigned species names). Subsequently, the resulting sequence sets for each taxonomic group (Ascomycetes and Basidiomycetes) were aligned using the MAFFT Version 7 software [35], using the G-INS-i strategy.

Each alignment (Ascomycetes and Basidiomycetes, respectively) was subjected to Operational Taxonomic Unit (OTU) analysis using OPTSIL [36], with a threshold of 3% [37,38] sequence difference and a 0.5 linkage fraction.

Four phylogenetic trees were constructed—two for Ascomycetes and two for Basidiomycetes—using the Neighbor-Joining and Maximum Likelihood algorithms [39], respectively. The Kimura-2 parameter model and G+I nucleotide substitution rate were applied, followed by 1000 Bootstrap replicates using MEGA X software [40]. OTUs and genotypes were positioned within the phylogeny.

The resulting Neighbor-Joining topologies for Ascomycetes and Basidiomycetes are shown in results, respectively.

3. Results

After microscopic examination, it was determined that 84 of the 100 samples corresponded with coffee rust characteristics (Table 1), so further analysis was carried out on these samples only.

3.1. Rust Lesions: Macroscopic and Microscopic Analysis

The 84 leaves that were examined macroscopically and microscopically showed symptoms and structures consistent with coffee rust infection (Figure 3A–D). The presence of hyphae that do not match rust infection was also detected (Figure 3E,F).

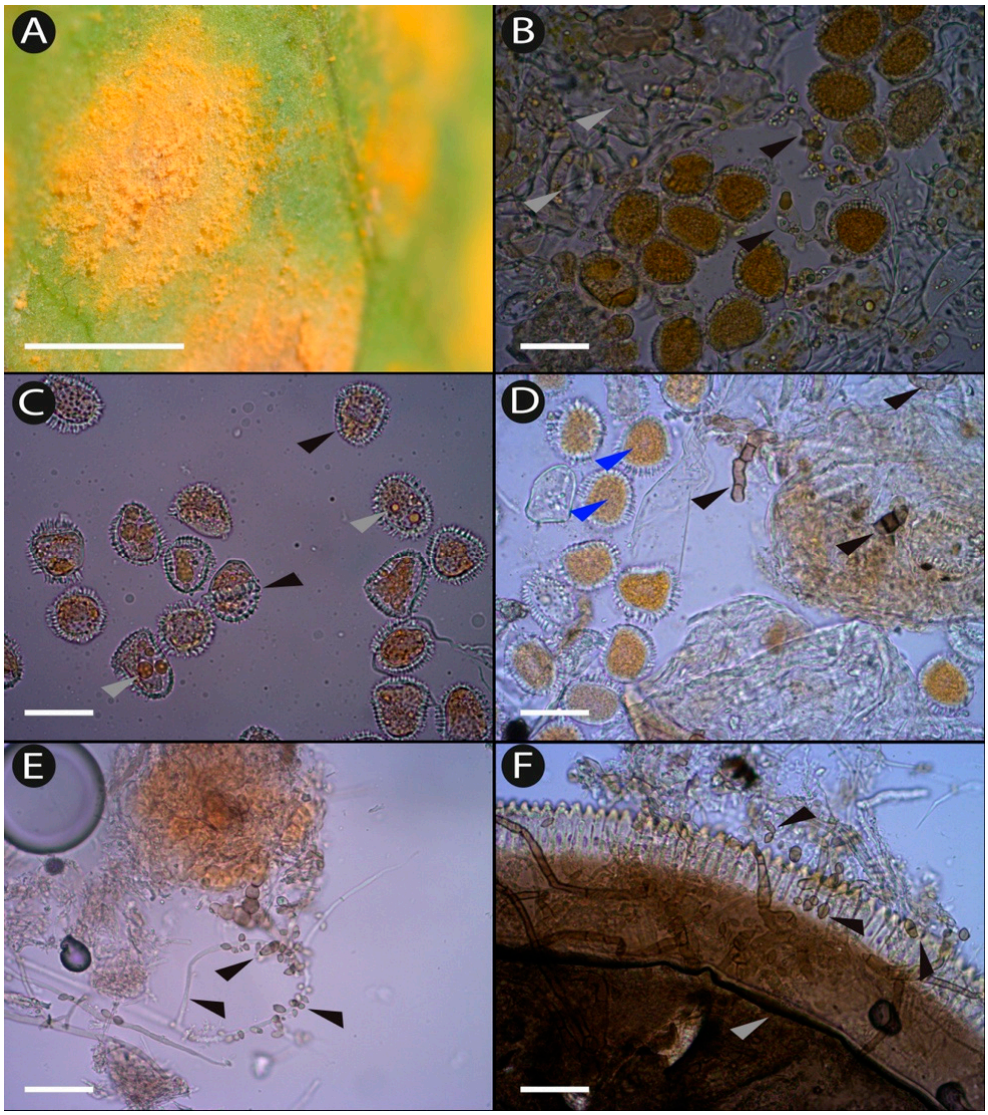

Figure 3. (**A**) Leaf of *Coffea arabica* L. 'red Bourbon' with foliar symptoms (yellow spots caused by rust). Bar = 1 cm; (**B**) uredospores generating germinating hyphae (black arrowhead) along leaf cells and stomata (grey arrowheads); (**C**) group of uredospores with thickened and warty upper walls (black arrowheads), with internal granules of orange–yellow carotenoid lipids (grey arrowheads); (**D**) mass of uredospores (blue arrowheads) close to hyphae and pigmented conidia belonging to other fungi (black arrowhead); (**E**) conidia and hyphae of *Cladosporium* spp. (black arrowheads) next to a rust lesion (orange zone); (**F**) conidia and hyphae of *Cladosporium* spp. (black arrowhead) colonizing the mealybug *Planococcus lilacinus* Risso (grey arrowhead) found next to a rust lesion. Bars in Figures (**B**–**F**) = 20 µm.

3.2. Phylogenetic Analysis and OTU Placement

From the 84 analyzed samples, 56 sequences (good quality chromatograms) that do not correspond to *Hemileia vastatrix* were randomly obtained: 38 sequences of Ascomycetes (Figure 4) and 18 sequences of Basidiomycetes (Figure 5). Only one sequence corresponding to coffee rust (*H. vastatrix*) was included in this analysis. Other twenty-eight sequences were eliminated because of their low quality (chromatograms with double peaks).

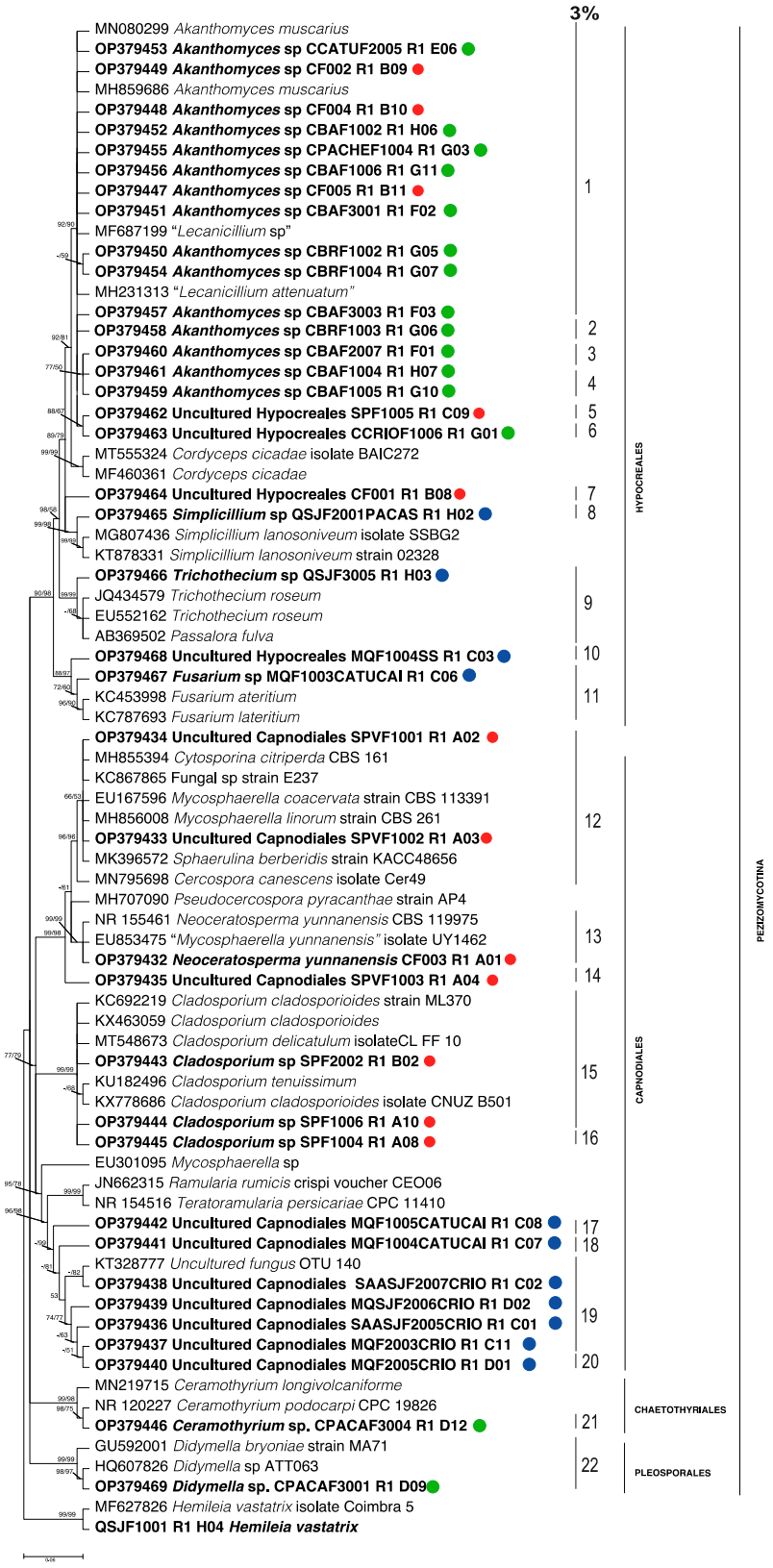

Figure 4. Neighbor-Joining phylogenetic tree for Ascomycetes, with bootstrap values ≥ 50 corresponding to Neighbor-Joining and Maximum Likelihood bootstrap, respectively. The tree is rooted with the outgroup *Hemileia vastatrix*. Here, 3% represents the applied threshold. OTU numbers are listed under the percentages. Colored circles show the origin of the samples: Calvas = green, Quilanga = blue, and Loja = red.

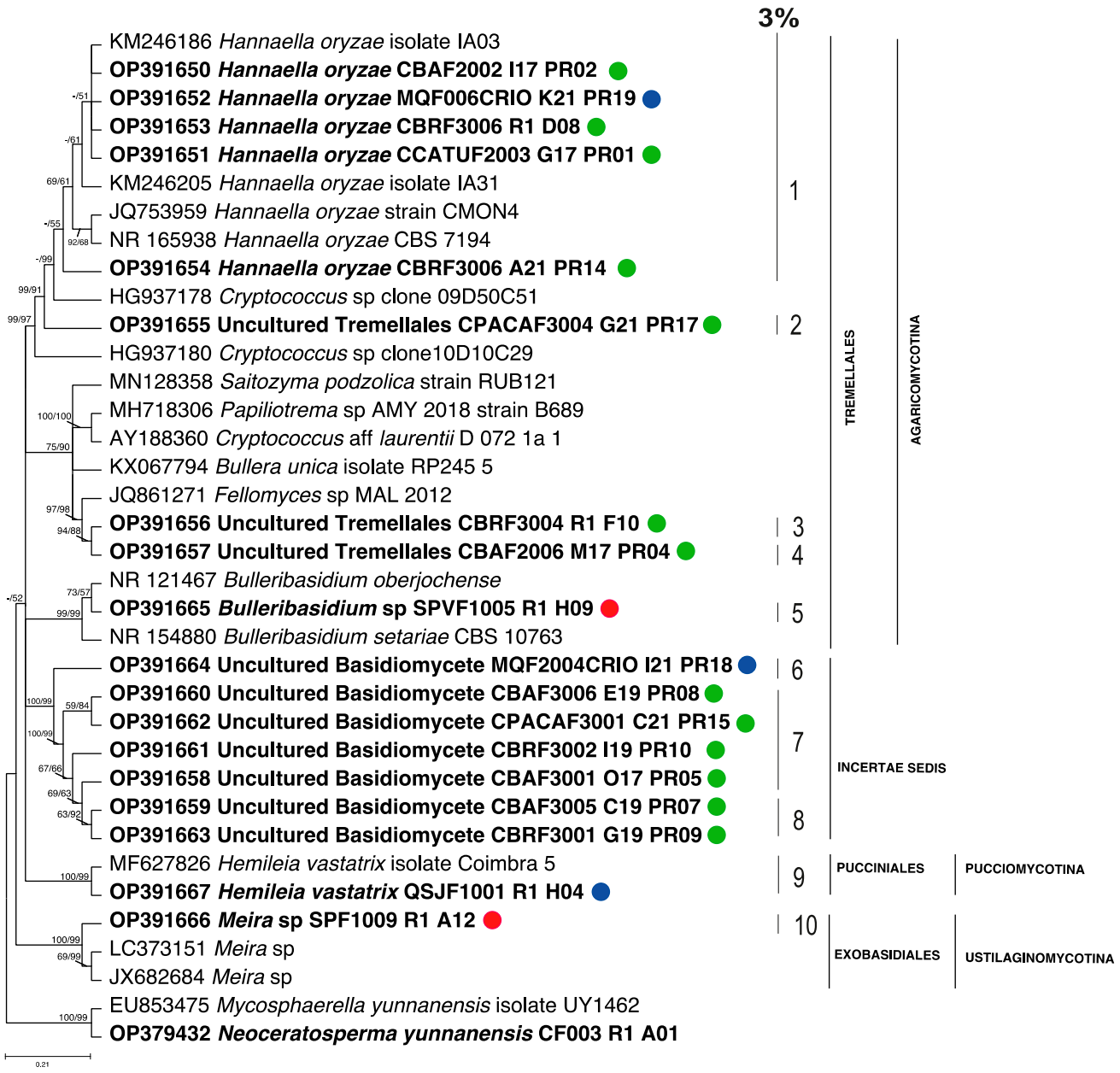

Figure 5. Neighbor-Joining phylogenetic tree for Basidiomycetes, with bootstrap values > 50 corresponding to Neighbor-Joining and Maximum Likelihood bootstrap, respectively. The tree is rooted with the outgroup *Mycosphaerella yunnanensis*. Here, 3% represents the applied threshold. OTU numbers are listed under the percentages. Colored circles show the origin of the samples: Calvas = green, Quilanga = blue, and Loja = red.

The phylogenetic trees indicated that the Ascomycetes included four orders (Capnodiales, Chaetothyriales, Pleosporales, and Hypocreales) within the subphylum Pezizomycotina and two orders of Basidiomycetes (Tremellales and Exobasidiales) within the subphyla Agaricomycotina and Ustilaginomycotina.

According to the OTUs identified as "hypothetical species" obtained by applying the threshold (3% sequence difference), the Ascomycetes included 22 OTUs (Figure 4), while the Basidiomycetes included 10 OTUs (Figure 5). According to the analyzed sequences (Figures 4 and 5) and the 3% threshold, more genotypes were encountered in the Calvas canton (i.e., 13 OTUs corresponding to Ascomycetes and Basidiomycetes) than in the Loja and Quilanga cantons, with 10 to 11 OTUs, respectively (Table 2).

Table 2. Frequency of sequences randomly detected that are related to rust fungi in coffee leaves.

		Loja				Calvas			Quilanga		
Fungi	**OTU Number**	**El Cristal**	**San Pedro de Vilcabamba. Farm 1**	**San Pedro de Vilcabamba. Farm 2**	**San Pedro de Vilcabamba. Farm 3**	**Jiropamba**	**Surunuma**	**Cango Bajo**	**San José. Farm 1**	**San José. Farm 2**	**San José. Farm 3**
ASCOMYCETES											
Akanthomyces sp.	1	3					2	6			
Akanthomyces sp.	2							1			
Akanthomyces sp.	3							1			
Akanthomyces sp.	4							2			
Hypocreales	5		1								
Hypocreales	6					1					
Hypocreales	7			1							
Simplicillium sp.	8								1		
Trichothecium sp.	9								1		
Hypocreales	10									1	
Fusarium sp.	11									1	
Capnodiales	12		1	1							
Neoceratosperma yunnanensis	13	1									
Capnodiales	14		1								
Cladosporium sp.	15		1	1							
Cladosporium sp.	16			1							
Capnodiales	17								1		
Capnodiales	18								1		
Capnodiales	19								2		2
Capnodiales	20										1
Ceramothyrium sp.	21					1					
Didymella sp.	22					1					
BASIDIOMYCETES											
Hannaella oryzae	1						1	3	1		
Tremellales	2					1					
Tremellales	3							1			
Tremellales	4							1			
Bulleribasidium sp.	5				1						
Basidiomycete	6									1	
Basidiomycete	7						1	3			
Basidiomycete	8							2			
Hemileia vastatrix	9									1	
Meira sp.	10		1								

4. Discussion

This study is the first record of fungal diversity probably related to coffee rust in Ecuador, randomly detected through the molecular ITS-5.8S marker. The small portions of preliminary fungal diversity correspond to genotypes designated as OTUs, applying a 3% threshold. The number of OTUs may vary when applying a different threshold [e.g., 5% (Figures S1 and S2)], as previously discussed by Vrålstad [41]. In this analysis, a 3% sequence difference was used as a reference value because it is considered the universal barcoding threshold [42]. Ascomycetes, with 22 OTUs across four orders (Capnodiales, Chaetothyriales, Pleosporales, and Hypocreales), exhibited greater preliminary diversity compared to Basidiomycetes, which had 10 OTUs in two orders (Tremellales and Exobasiales) and one *Incertae Sedis* group. This diversity could be further explored using metagenomic techniques such as next-generation sequencing [20,21], a high-throughput tool recommended for quantifying population dynamics, community ecology, and host–microorganism interactions [22], though it was not applied here due to budget constraints.

The samples collected from farms in the Calvas canton were the most diverse, containing 13 OTUs of Ascomycetes and Basidiomycetes associated with rust. In contrast, the farms in Loja and Quilanga had 10 and 11 OTUs, respectively. This fungal diversity probably corresponds to ubiquitous airborne fungi [43,44] and may be higher depending on the type of coffee cultivation; for example, Calvas (Jiropamba) and Loja (El Cristal) use a combined cultivation system that includes fruit trees such as banana or orange. This observation supports the application of "Conservation Agriculture", which promotes the cultivation of coffee in forest ecosystems that host a higher diversity of microorganisms [45]. Similarly, Tomao et al. [46] indicate a positive correlation between fungal diversity and tree species diversity. This is probably also the case in our study since samples from open and shaded farms (e.g., Loja: San Pedro de Vilcabamba; all Quilanga farms) produced fewer sequences and OTUs (Table 2) for Ascomycetes or Basidiomycetes, respectively. The small portions of fungal molecular diversity reported in this study evidently do not encompass the whole diversity of fungi or other microorganisms that may be related to coffee rust. Some metagenomic studies report high levels of fungi diversity associated with coffee rust [20] and with fermentative processes in coffee fruits [47]. Some genera detected here within Capnodiales and Tremellaceae are similar to those reported by Kurtzman et al. [47] and James et al. [20] using metagenomic technique.

The portion of fungi diversity detected in our study includes species from several genera, such as *Ceramothyrium*, *Cladosporium*, *Didymella*, *Fusarium*, and *Mycosphaerella*, which are considered endophytes or parasites related to coffee lesions [48–51], or mycoparasites of rust [23]. Other less common fungi probably correspond to ubiquitous airborne fungi [43,44]; however, fungi such as "*Mycosphaerella yunnanensis*", currently known as *Neoceratosperma yunnanensis* [24], or *Trichothecium* sp., have also been reported from coffee leaves by James et al. [20], who speculated on their mycoparasitic activity against coffee rust. In the samples for Calvas, most sequences belonged to the genus *Akanthomyces*, which, according to the Index Fungorum, includes several species of *Lecanicillium*, known as natural rust controllers [28].

On the other hand, *Cladosporium* spp. and pigmented hyphae of Dotidiomycetes, which are very similar to those generated by *Colletotrichum* spp., were associated with rust; both these genera are considered endophytes of coffee plants and potentially mycoparasites of rust [51,52]. The DNA from these or other fungi was not detected, probably indicating that the use of specific primers, as suggested by Mosca et al. [53], is needed.

Another important group of detected fungal species was Basidiomycota, which, contrary to the study by Silva et al. [54], was less diverse in our study. However, among the encountered Basidiomycetes, there were several yeast species within the genera *Bulleribasidium*, *Meira*, and *Hannaella*, known to be plant pathogens of other crops such as rice, sugar cane, Japanese pear pepper, and others [55–57]. Employing metagenomics, James et al. [20] found some genera of fungi (e.g., *Bullera*) within the order Tremellales, which are closely related to *Bulleribasidium* and *Hannaella oryzae* found here. In another study, yeasts

from the family Tremellaceae were detected at a certain stage of coffee fermentation by De Oliveira et al. [15], which they attributed to possible external contamination. Species of yeast belonging to Tremellales appear to be related to coffee rust [20] and coffee fermentation processes [15], although further detailed taxonomic studies are needed to establish the functional implications of this relationship [45].

Molecular analyses need to be expanded by including metagenomics in order to further detect the diversity of fungi beyond the small portion presented here. This tool is highly applicable for the detection of plant pathogens [58] or for researching population dynamics, community ecology, and host–microorganism associations, as indicated by Tedersoo et al. [22]. The fungal species detected here could have various ecological roles, such as controlling coffee rust or other pathogens [48–51], activating plant defense genes [59], or regulating post-harvest fermentation processes called "coffee processing" [45]. An in-depth integrative study is needed to understand whether this fungal diversity is a mere coincidence or is really associated with rust fungus. In addition, studies on the antagonistic activity of fungal diversity (e.g., endophytes or fungi associated with rust) against *Hemileia vastatrix* (coffee rust) should be conducted, as described in Poma-Angamaraca et al. [60].

5. Conclusions

The conclusions of the present study are as follows: (a) various Ascomycetes and Basidiomycetes species were detected from *Coffea arabica* leaves infected by rust, but their functional roles remain to be defined; (b) among the identified species, the genus *Akanthomyces*, along with some synonymous species in *Lecanicillium*, is considered a natural controller of the rust pathogen and requires attention in future pest control studies; (c) the detected yeasts *Bulleribasidium* and *Hannaella* (from the Tremellaceae family) are closely related to those act in the fermentation process of the coffee fruit; future studies are required to identify this diversity and its effects.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d16100633/s1, Figure S1: Neighbor-Joining phylogenetic tree for Ascomycetes, with bootstrap values ≥ 50 corresponding to Neighbor-Joining and Maximum Likelihood bootstrap, respectively. The tree is rooted with the outgroup *Hemileia vastatrix*. Here, 3% and 5% represents the applied threshold. OTU numbers are listed under the percentages. Colored circles show the origin of the samples: Calvas = green, Quilanga = blue, and Loja = red.; Figure S2: Neighbor-Joining phylogenetic tree for Basidiomycetes, with bootstrap values > 50 corresponding to Neighbor-Joining and Maximum Likelihood bootstrap, respectively. The tree is rooted with the outgroup *Mycosphaerella yunnanensis*. Here, 3% and 5% represents the applied threshold. OTU numbers are listed under the percentages. Colored circles show the origin of the samples: Calvas = green, Quilanga = blue, and Loja = red.

Author Contributions: D.C. and M.C. contributed to the conceptualization and methodology of this study. D.C., M.C., and A.J.-R. carried out sampling. P.H. performed the OTU analyses and made a strong critical revision of the manuscript. R.A. contributed with general comments. All authors contributed with their experience to improve the whole manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study did not require ethical approval.

Data Availability Statement: Not applicable, as all molecular data are available in the GenBank database.

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Conflicts of Interest: The authors declare no conflicts of interest.

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