Endophytic bacteria associated with healthy and rust-infected *Coffea arabica* L. leaves

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Coffea arabica L., the most commercialized species of coffee, has been continuously devastated by the Coffee Leaf Rust (CLR) disease worldwide. A recent outbreak of the disease in Central America caused the loss of more than 616 millon USD in coffee production. However, the control and prevention of CLR remains to be challenging especially now that climate change is significantly affecting environments globally and there is limited information known about the biotic interactions that surrounds this infection. Hence, in this study, the endophytic bacteria associated with healthy and CLR infected *C. arabica* var. *yellow bourbon* (a susceptible variety) leaves as well as leaves from *C. canephora* var. *robusta* (a resistant variety) were examined individually via culture-independent and culture independent methods to determine whether there are differences in the biotic composition of each. *Achromobacter, Alcaligenes, Citrobacter, Delftia, Erwinia, Exiguobacterium, Hafnia, Hydrogenophaga, Janthinobacterium, Luteibacter, Novosphingobium, Staphylococcus, Thiobacillus,* and *Wolbachia* were identified as new records of bacterial endophytes in coffee leaves. CLR infected leaf samples had the most diverse endophytic bacterial community with a total of 13 genera while healthy Yellow Bourbon and resistant Robusta leaves had 8 and 9 genera, respectively. This study is the first to report the difference in the microbiome of healthy and CLR infected coffee leaves. Unique species per leaf sample were also identified. Using culture-dependent isolation, *Bacillus, Curtobacterium, Enterobacter, Erwinia, Hafnia, Novosphingobium,* and *Stenotrophomonas* were found only in the CLR infected Yellow Bourbon leaves. On the other hand, *Staphylococcus* was only isolated from healthy Yellow Bourbon leaves, while *Achromobacter, Alcaligenes*, *Citrobacter*, and *Luteibacter* were only isolated from the resistant Robusta leaves. In the case of the culture-independent method, additional genera like *Hydrogenophaga* and *Wolbachia* were identified as unique in healthy leaves, while *Achromobacter, Alcaligenes,* and *Citrobacter* were found to be unique in resistant leaves. *Hydrogenophaga* and *Achromobacter* are known plant-growth promoting bacteria, which can be studied further for their possible biocontrol properties against *H. vastatrix* while unique phytopathogens in infected leaves, such as, *Erwinia* can be studied for their association in CLR development.

KEYWORDS

coffee leaf rust, *Hemileia vastatrix*, endophytic bacteria, plant-growth promoting bacteria, phytopathogen

INTRODUCTION

Coffee (*Coffea* sp.) reached the status of being a global commodity in as early as the eighteenth century and it significantly contributes to the economy of more than sixty countries as the main source of income of more than one hundred million people (Talhinhas et al. 2017). In 2019 alone, 169.3 M bags (in 60 kg) or 10.2 M metric tons of coffee beans were consumed globally and in the Philippines, 3.35 M bags (in 60 kg) or 201,000 metric tons were consumed (International Coffee Board, 2020). However, coffee production in the country is still not fully self-sufficient since the total coffee produced in 2019 amounted to only 60,040 metric tons (Philippine Statistic Authority, 2019).

Out of the several *Coffea* species, only two are highly commercialized worldwide and these are *Coffea arabica* L. and *Coffea canephora*. Pierre ex A. Froehner. Between these two, *C. arabica* is the most valued in the market due to its superior taste, rich aroma and low caffeine content (Mishra and Slater, 2012). However, *C. arabica* production has been greatly affected by many diseases due to its low genetic diversity (Bertrand et al. 2003). Diseases that infect this species include fungal infections like Coffee Leaf Rust (CLR) caused by *Hemileia vastatrix,* Coffee Berry Disease (CBD) caused by *Colletotrichum kahawae,* and Coffee Wilt Disease (CWD) caused by *Gibberella xylarioides* (Hindorf and Omondi, 2011). It can also be infested

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by pests such as the: (1) coffee berry borer, *Hypothenemus hampei,* (2) coffee leaf miner, *Leucoptera coffeella,* and (3) root mealybugs under the family Pseudococcidae (Barrera, 2008).

The most devastating disease among these because of its adverse impact on coffee production worldwide is considered to be CLR. In fact, simultaneous outbreaks that lasted for five years recently affected tropical countries (Avelino et al. 2015). The International Coffee Organization reported that in Central America alone, it had caused the loss of more than 616 million USD (Mccook and Vandermeer, 2015; Talhinhas et al. 2014). CLR has also caused the major wipeout of *Coffea arabica* during the 1890s in the Philippines. This impeded the country's role as a major leading exporter of coffee in the nineteenth century and, until now, the country has not been able to recover its former status (Bamber et al. 2017; Philippine Coffee Board, 2012). Low levels of CLR infection are still evident in many of the coffee farms in the country and the lack of sufficient knowledge about *H. vastatrix* heightens the risk of an outbreak.

Evidently, the control and prevention of CLR epidemics is a very complex task especially now that the global environment is rapidly changing due to climate change. Relying solely on a single management strategy may not be as effective (Toniutti et al. 2017). Enough information about how the plant is affected by these changes in the environment is needed so that alternative solutions that will help the plant to withstand these changes can be proposed. Several abiotic factors have already been identified to affect the life cycle of *H. vastatrix* (Avelino et al. 2004; Talhinhas et al. 2014). However, the association of biotic factors to this disease has not yet been examined in detail.

Studies on the impact of the present microbial community on disease development in plants are often lacking because of the traditional concept that plant infections are only confined in the three-party relationship between the plant host, the pathogen and the environment (Rastogi et al. 2013); when in fact, in the invasion of the plant tissue by a pathogen, it also encounters a diverse microbial community that exists outside and inside the plant.

Profiles of plant microbial communities have been observed in several studies to vary as a result of infection (Araújo et al. 2002; Bogas et al. 2015; Koskella et al. 2017; Lebreton et al. 2019; Purahong et al. 2018; Suda et al. 2009; Trivedi et al. 2010). An example of this is the infection caused by *Pseudomonas syringae* pv. *actinidiae* (Psa) in kiwifruits. In the study of Purahong and co-workers (2018), it was observed that Psa infection is associated with the disappearance of dominant bacterial species in the kiwifruit plant while it also caused the proliferation of *P. syringae* pv. *syringae* (Pss).

Bacteria present during the establishment of infection can also in turn, regulate plant pathogens. Unique bacterial species from healthy plant leaves were seen to portray antagonistic relationships with pathogens through the production of antifungal compounds (Balint-Kurti et al. 2010; Ritpitakphong et al. 2016; Santhanam et al. 2015). These compounds are able to reduce spore germination and disease severity under greenhouse and field conditions just like in the response of *Pseudomonas putida* P286 and *Bacillus thuringiensis* B157 against *Hemileia vastatrix* (Haddad et al. 2013). Some bacteria can also help plants achieve a healthy physiological state and thus, enable the plant to adapt to stresses (Rosenblueth and Martínez-Romero, 2006; Wu et al. 2009).

Other bacteria may even help pathogens to colonize plant hosts. In the study of citrus plants, it was found that *Methylobacterium* species were observed to have a direct association with the development of symptoms of citrus variegated chlorosis (CVC) caused by *Xylella fastidiosa* (Araujo et al. 2002). It was suggested that CVC may be triggered by the synergistic interaction of *Methylobacterium* spp. with *X. fastidiosa* since it has previously been noted to positively affect the pathogen's growth (Araujo et al. 2002).

This study specifically aimed to: 1) determine the endophytic bacterial composition of healthy and CLR infected *C. arabica* leaves via culture-independent and culture independent methods, 2) compare the differences in bacteria found in healthy and CLR infected *C. arabica* leaves as well as in the resistant *C. canephora* leaves, and 3) identify unique endophytic bacteria present in healthy and CLR infected *C. arabica* leaves. The analysis of the differences between these communities could help elucidate how the presence of certain bacterial species regulates the suppression or establishment of CLR infection.

MATERIALS AND METHODS

Collection and Surface Sterilization

Leaves of *C. arabica* var. *yellow bourbon* (susceptible variety), and *C. canephora* var. *robusta* (resistant variety) were collected from the Bureau of Plant Industry in Baguio City, Benguet. Samples were collected from five different trees per species and from each tree, five leaves were collected. In the case of *C. arabica* var. *yellow bourbon*,a set of five infected leaves and another set of five apparently healthy or asymptomatic leaves were separately collected. CLR infected leaves were those observed with clusters of orange-colored powder on the abaxial surface. These clusters were microscopically identified to be spores of *H. vastatrix*. Leaves were considered healthy if there were no visible signs of any infection on both the adaxial and abaxial surfaces. Leaves were then kept in separately labeled plastic bags and stored at -20°C until further processing (Donegan et al. 1991).

The leaves were surface sterilized in order to ensure that only the bacteria inside the plant tissues will be recovered. Each leaf was washed in running water, disinfected in 70% ethanol for 1 min, sodium hypochlorite (2% Cl-) for 4 min., 70% ethanol for 30s, and rinsed three times with sterile distilled water (De Oliveira Costa *et al*., 2012). To test the effectiveness of the sterilization process, 100 µL of the last distilled water wash was plated in nutrient agar (NA) and incubated at around 25°Cfor 24-48 hours.

Culture-dependent Analysis

Surface sterilized leaves were cut into disks (0.5 cm^2) using a sterile cork borer (Das et al. 2017). Two leaf sections obtained from each leaf sample were plated equidistantly on a NA plate with the anti-fungal agent nystatin (20 U/mL) to ensure that only bacterial endophytes will grow. Then the plates were incubated at around 25°C for 72 hours (modified from Gagne-Bourgue et al. 2013). For the infected leaves, 0.5 cm^2 disks were excised from areas with obvious CLR spots. Colonies that grew around the leaf sections underwent at least two rounds of colony isolation by re-streaking on new NA plates. Colonies isolated were generally chosen based on their distinct morphological characteristics.

In preparation for DNA extraction, 1-2 colonies per isolate were sub-cultured into tubes of Luria-Bertani (LB) broth and were incubated at around 25°C for about 36-48 hours (modified from Gagne-Bourgue et al. 2013). DNA extraction was done via the conventional boiling method (modified from Junior et al. 2016). Bacterial cells were collected from the LB culture by discarding the supernatant after centrifugation at 14,000 RPM for 3 minutes then the bacterial pellet was washed by mixing 500 µL of UltraPure™ distilled water. The previous step was repeated for

further washing. The tubes were then incubated at 100°C for 15 minutes and immediately cooled in ice for 10 minutes. Lastly, the tubes were centrifuged at 14,000 RPM for 8 minutes then the supernatant that contains the DNA were transferred into a new tube.

DNA extraction was followed by DNA amplification through the use of 16S rDNA bacterial primers: 27f: 5'-AGAGTTTGATCMTGGCTCAG and 1492r: 5'-ACGGYTACCTTGTTACGACTT (Reysenbach and Pace, 1995). Amplification was done using T100TM Thermal Cycler (Bio-rad, Singapore). The PCR cycle condition was as follows:initial denaturation for 3 minutes at 95°C; 35 cycles of a denaturation step for 30 seconds at 95°C, an annealing step for 30 seconds at 50°C and an extension step for 1 minute at 72°C; final extension for 4 minutes at 72°C.

Subsequently, amplicons were run through a 1% agarose gel and then crisp bright bands of about 1500 bp in length were excised then purified using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific Baltics, UAB) and sent to the DNA Sequencing Core Facility (DSCF) of the Philippine Genome Center (PGC), University of the Philippines, Diliman, Quezon City for Sanger sequencing. Sequences were assembled using STADEN package v2.0.0 (Staden *et al*., 1998) and then consensus sequences were used in performing a nucleotide BLAST (BLASTn) search in the NCBI site for the putative identification of each isolate.

Sequences of all the isolates and two of their closest relatives were compiled and aligned using MAFFT v7 (Katoh et al. 2017). A phylogenetic tree was constructed using the optimal model determined via the Bayesian Information Criterion (BIC) test performed in the software IQ-Tree multicore version 1.6.9 (Nguyen et al. 2018). Using the same software, the maximum-likelihood (ML) method was applied for the tree construction and support for the nodes of the trees were obtained through bootstrap resampling method with 1000 replicates. The tree was finally visualized using the online software Interactive Tree of Life (iTOL) version 4.4.2 (Letunic and Bork, 2019).

Culture-independent Analysis via PCR-DGGE

Total endophytic bacterial community DNA was extracted from pooled surface sterilized leaf sections from each tree sample using the DNeasy® PowerSoil® kit (Qiagen Inc., Germany). Three sets of amplification strategies (Table 1) that made use of various primers (Table 2) were sequentially tested in order to optimize the analysis of the diversity of endophytic bacteria and also to reduce contamination of eukaryotic DNA.

Table 1: Amplification strategies conducted in order to optimize DGGE profiles.

Strategy No.	PCR Type	Primer combinations	Reference
1	Nested	Round 1: 27f and 1492r Round 2: GC338f and 518r	Mahmood *et al*., 2005
2	Conventional	GC968f and 1401r	Nübel *et al*.1996
3	Nested	Round 1: 799-m7 and 1492r Round 2: 968f and 1401r	Chelius and Triplett, 2001

The amplicons were then subjected to DGGE on 8% and 6% polyacrylamide gels for the target hypervariable regions V3 and V6-V8, respectively. The linear gradient used for all gels ranged from 35% to 65% urea-formamide denaturant (modified from Muyzer *et al*., 1993). A 0%-denaturing solution and a 100%-denaturing solution were prepared with the latter containing 7M urea and 40% formamide. Migration was carried out at 100 V for 10 min and then maintained at 60 V for 16 h in 0.5X TAE

Table 2: PCR primers used in the amplification of target 16S hypervariable regions that were used for DGGE analysis.

Primer name	Sequence (5'-3')	16S region	Reference
27f	AGAGTTTGATCMTGGCTCAG	V1 - V9	Reysenbach and Pace, 1995
1492r	ACGGYTACCTTGTTACGACTT		Reysenbach and Pace, 1995
GC338f*	CCTACGGGAGGCAGCAG	V3	Muyzer, 1993
518r	ATTACCGCGGCTGCTGG		Muyzer, 1993
GC968f*	AACGCGAAGAACCTTAC	V6 - V8	Nübel *et al*., 1996
1401r	CGGTGTGTACAAGACCC		Nübel *et al*.1996
799f-m7	GATTAGATACCCKGGT	V5 - V9	Hanshew *et al*. 2013
1492r	ACGGYTACCTTGTTACGACTT		Reysenbach and Pace, 1995

*GC-clamp (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG) is attached on the 5' end.

buffer, using the DCode™ Universal Mutation Detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Gels were stained with ethidium bromide nucleic acid stain for 30 minutes to 1 hour and then viewed under UV light using GenoSens 1860 (Clinx Science Instruments Co., Ltd.). Distinct bands were excised and the DNA fragments were extracted using the QIAquick® Gel Extraction Kit (Qiagen Inc., Germany). All the extracted bands were then re-amplified using the corresponding primers per target region without the GC clamp. PCR products were sent to Macrogen Inc. (Seoul, Korea) for Sanger sequencing. Consensus sequences were assembled using STADEN package v2.0.0 (Staden et al. 1998) and subsequently used to conduct a BLASTn search for putative identification.

RESULTS AND DISCUSSION

Culture-based Isolation

A total of twenty colonies were isolated from CLR infected Yellow Bourbon leaves while only nine and ten colonies were isolated from healthy Yellow Bourbon and Resistant Robusta leaves, respectively. Putative identification of each isolate was initially conducted using the analysis of 16S rDNA sequences; wherein based on the NCBI reference database, the percent similarity of all isolates compared to their closest relatives range from 97-100% (Table 3).

This analysis also revealed that Proteobacteria was the most abundant phylum across all leaf samples (Figure 1A). Furthermore, the profile of each leaf sample showed that infected leaves had the highest diversity representing three phyla, namely, Actinobacteria (5%), Firmicutes (5%), and Proteobacteria with classes Gammaproteobacteria (75%) and Alphaproteobacteria (15%). On the other hand, healthy leaves were composed of Firmicutes (22%), Gammaproteobacteria (67%), and Alphaproteobacteria (11%). Meanwhile, resistant leaves were composed of only Gammaproteobacteria (60%) and Betaproteobacteria (40%).

All coffee leaves were also dominated by *Pseudomonas* (Figure 1B). In fact, *Pseudomonas* sp. accounts for ≥40% of the total bacterial composition across all samples. It was the only genus found to overlap on all leaf samples. Furthermore, comparison of all isolate sequences even showed that *Pseudomonas* isolates I3B2, H1B1, and R2D1, which comes from each leaf sample, were actually identical. Hence, this provides proof that *C. arabica* and *C. canephora* leaves possess similarities in their endophytic microbiota.

Table 3: Nucleotide BLAST search results for the closest relatives of endophytic isolates from coffee leaf samples.

Isolate	Closest Relatives	Percent Identity	Accession Number
Infected Coffea arabica var. Yellow Bourbon leaves			
I1C1	*Curtobacterium* sp. strain IAE256	99.57	MK414963
	Curtobacterium sp. strain a113164	99.5	MK280704
I5E3	*Bacillus* sp. strain LY2	99.86	MK182267
	Bacillus velezensis strain FB11	99.86	MK828185
I1B2	*Pseudomonas* sp. strain SAP49_1	99.86	JN872540
	Pseudomonas extremorientalis strain FJAT-hcl-1	99.78	KY653093
I1C2	*Pseudomonas cichorii* strain MAFF 302699	99.57	AB724287
	Pseudomonas cichorii strain MAFF 302698	99.57	AB724286
I1E1	*Pseudomonas taiwanensis* strain PsTW DMC 234	99.43	MK598329
	Pseudomonas putida strain MG-Y2	99.43	EU179737
I2B1	*Pseudomonas* sp. strain PS22	99.86	MH884000
	Pseudomonas extremorientalis culture DSM:15824	99.86	KX186943
I2B2	*Pseudomonas* sp. strain S18	100	KM117221
	Pseudomonas fluorescens strain P201	100	JN228208
I2E1	*Uncultured Stenotrophomonas* sp. clone F10	99.58	KX456229
	Stenotrophomonas maltophilia strain SBR01	99.44	KX018308
I3B1	*Hafnia alvei* strain IC3211	98.87	AB244474
	Hafnia alvei strain ICMP 7619	98.87	MF682389
I3B2	*Pseudomonas* sp. strain 2-31	100	KX378937
	Pseudomonas fluorescens strain S16	100	DQ095904
I3C1	*Atlantibacter hermannii* strain S17_PA1R	99.72	MK883098
	Enterobacter cloacae strain WTB56	99.72	MK241852
I4A1	*Pseudomonas* sp. strain WL1(2016)	99.43	KU324481
	Pseudomonas putida strain M9	99.43	KF358272
I4B2	*Pseudomonas cichorii* strain SY-21	99.72	MF979525
	Pseudomonas cichorii strain Pc-Gd-5	99.71	KU923374
I5A2	*Stenotrophomonas* sp. strain CV81Nov	99.72	KJ482859
	Stenotrophomonas rhizophila strain ICE234	99.72	KX588618
I5C1	*Erwinia pyrifoliae* strain EpK1/15	97.15	KX966188
	Erwinia sp. strain fn_84	97.38	LC333541
I5D1	*Enterobacteriaceae bacterium* strain SAP817.4	98.37	JX067700
	Erwinia tasmaniensis strain Et1/99	98.3	NR_074869
I5A1	*Pseudomonas palleroniana* strain IHB B 7234	99.79	KJ767367
	Pseudomonas palleroniana strain Y1	99.86	JQ770187
I1B1	*Agrobacterium* sp. strain N11	99.63	GU086419
	Agrobacterium larrymoorei strain AF28	99.63	LC015600
I2A2	*Novosphingobium* sp. strain N8	99.63	GU086416
	Novosphingobium lindaniclasticum strain IIL-Asp23	99.56	KX380918
I2C1	*Rhizobium* sp. strain R-31762	99.63	AM403584
	Agrobacterium tumefaciens strain CH1-36	99.7	JX971560
Healthy Coffea arabica var. Yellow Bourbon leaves			
H1A1	*Staphylococcus* sp. strain InS-282-1	99.86	MF070515

	Staphylococcus epidermidis strain VITAPRRKCU-3	99.86	MH118521
H1C4	*Staphylococcus pasteuri* strain BMC3N7_1	100	MG996864
	Psychrobacter pulmonis strain BMC2N12_2	100	MG996850
H1A2	*Rhizobium* sp. strain DR 7-06	98.81	KM253034
	Agrobacterium rubi strain PgBe187	98.74	MH211278
H1B2	*Pseudomonas abietaniphila* culture DSM:6506	99.07	KX186935
	Pseudomonas graminis strain KF701	99.07	AB109886
H1B1	*Pseudomonas* sp. strain 2-31	100	KX378937
	Pseudomonas fluorescens strain S16	100	DQ095904
H1B4	*Pseudomonas extremorientalis* culture DSM:15824	99.86	KX186943
	Pseudomonas sp. strain PB21	99.86	KY228974
H2B1	*Pseudomonas extremorientalis* strain 9F	99.72	KC329818
	Pseudomonas poae strain Z6	99.86	HQ406827
H2B2	*Pseudomonas azotoformans* strain R3ScM3P1C23	100	KF147036
	Pseudomonas sp. strain SGb343	100	HQ224634
H3B2	*Pseudomonas* sp. strain A21	99.93	MK391954
	Pseudomonas azotoformans strain R3ScM3P1C23	99.93	KF147036
Resistant Coffea canephora leaves			
R1A2	*Luteibacter rhizovicinus* strain LL-C	99.86	EU022023
	Luteibacter rhizovicinus strain E4 - 6	99.72	KY938100
R1B1	*Pseudomonas putida* strain CFBP 5898	99.93	HF545843
	Pseudomonas sp. strain CBCEN8	99.93	EF427849
R1B2	*Citrobacter freundii* strain FC18565	99.51	MK561018
	Citrobacter sp. strain ChDC B346	99.51	KF733674
R1D	*Pseudomonas* sp. strain PS22	99.86	MH884000
	Pseudomonas extremorientalis strain CNU082017	99.86	KF979139
R2D1	*Pseudomonas* sp. strain 2-31	100	KX378937
	Pseudomonas fluorescens strain S16	100	DQ095904
R3B2	*Pseudomonas* sp. strain S1Bt5	99.93	MH463696
	Pseudomonas lurida strain CV9.2	99.93	MH379724
R1C	*Alcaligenes faecalis* subsp. *faecalis* strain SK12	99.72	KC790302
	Alcaligenes sp. strain JLT1515	99.79	KX989249
R2A1	*Achromobacter xylosoxidans* strain YJY2	99.22	KP973962
	Achromobacter marplatensis strain SY6	99.22	KC790321
R3A1	*Achromobacter denitrificans* strain RT10-2	98.43	MK014241
	Achromobacter denitrificans strain 1104	98.43	KT832691
R3A2	Uncultured *Alcaligenes* sp. clone VOTO2-F	99.64	EU169605
	Achromobacter insuavis strain LMG 26845	99.64	NR_117706

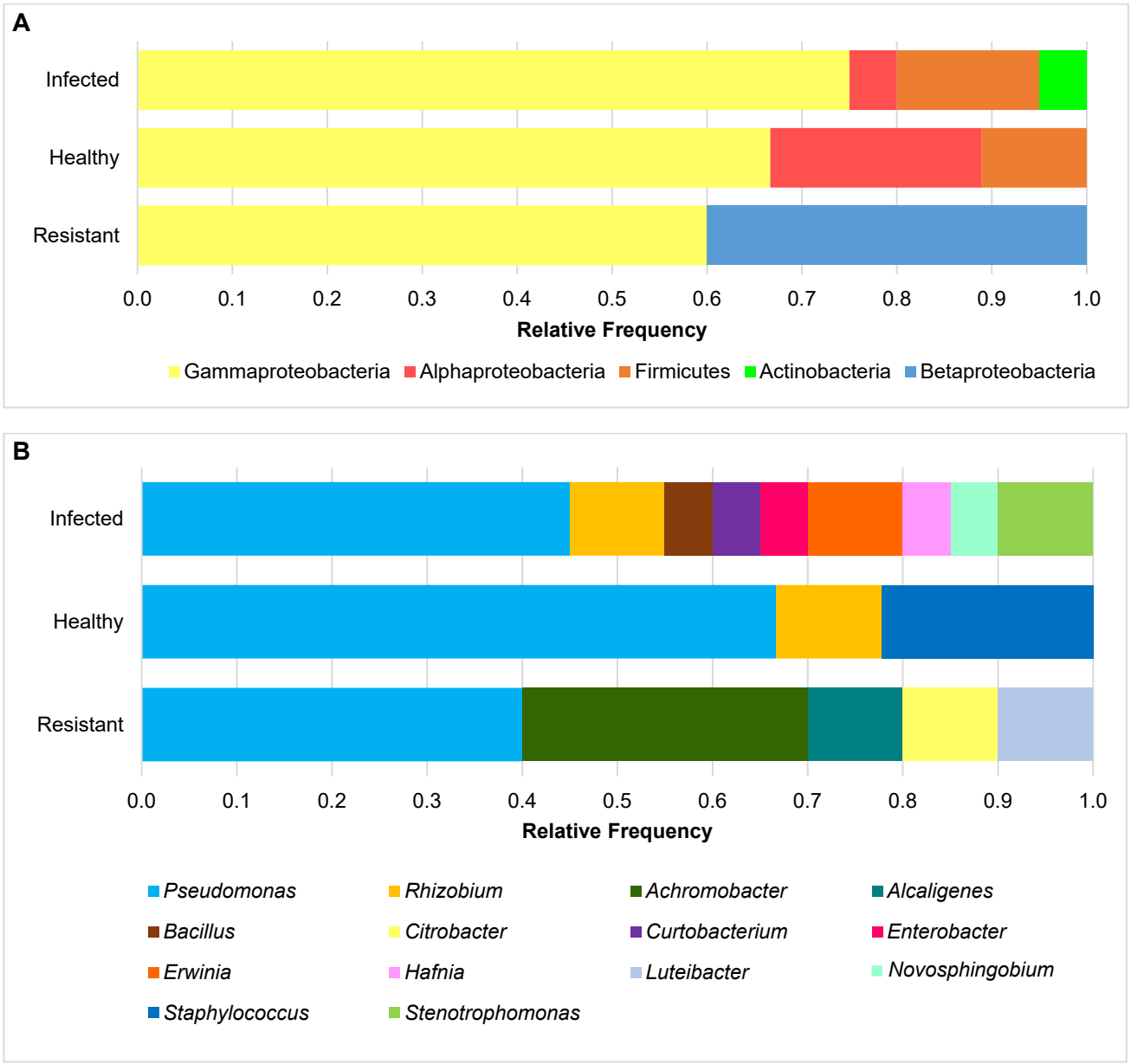


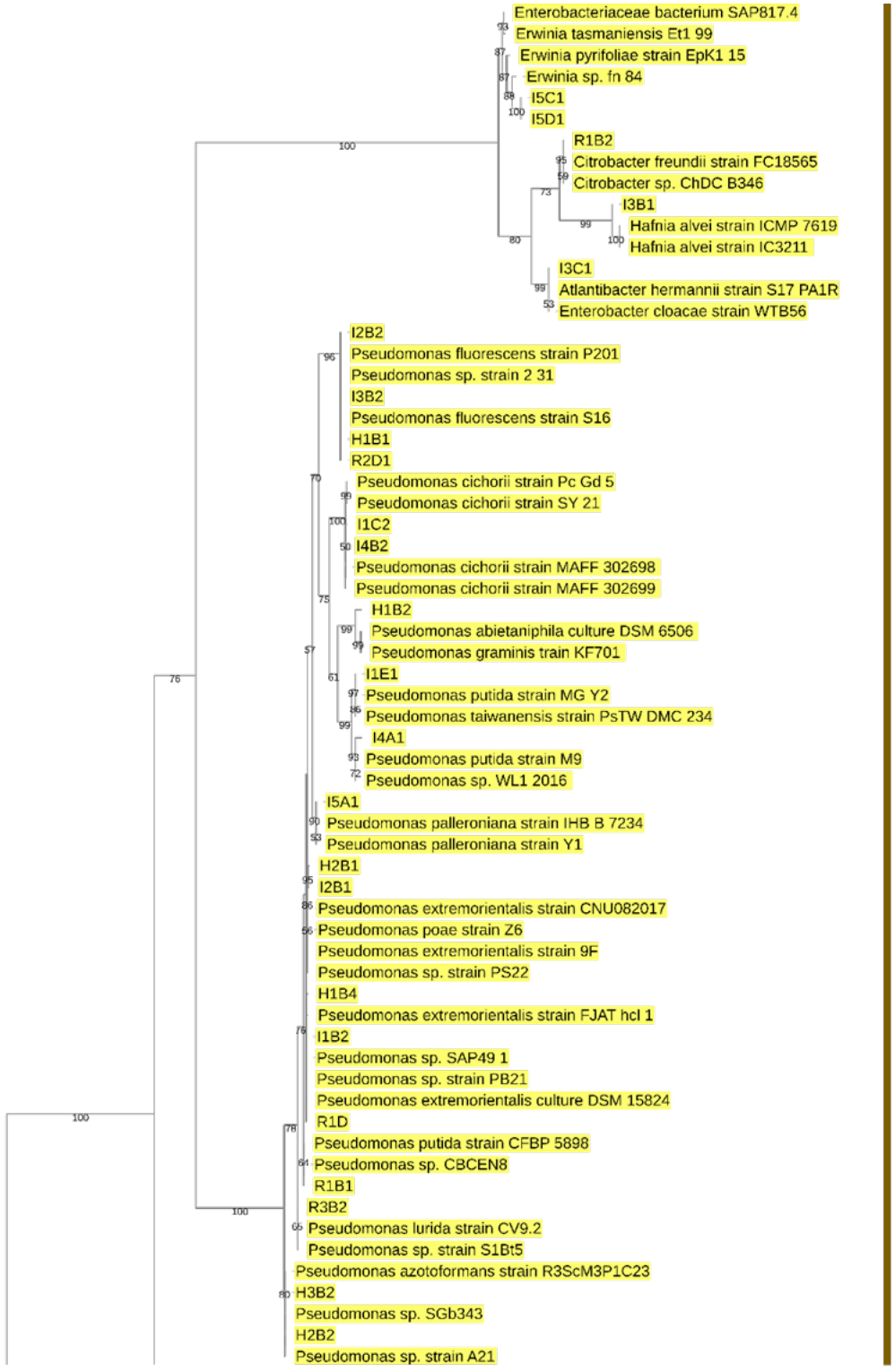
Figure 1: Endophytic bacterial profiles of coffee leaf samples based on culture-dependent isolation. The bar charts denote the relative frequency of the identified isolates at the (A) phylum and (B) genus level. For A, Phylum Proteobacteria was further divided into the class levels: gamma, alpha, and beta.

Apart from *Pseudomonas, Rhizobium* or *Agrobacterium* was also isolated in both CLR infected (I1B1) and healthy (H1A2) Yellow Bourbon leaves. The rest of the isolates identified, however, were unique to their leaf source. *Bacillus* (I5E3), *Curtobacterium* (I1C1), *Enterobacter* (I3C1), *Erwinia* (I5C1 and I5D1), *Hafnia* (I3B1), *Novosphingobium* (I2A2), and *Stenotrophomonas* (I2E1 and I5A2) were found only in the CLR infected Yellow Bourbon leaves. *Staphylococcus* (H1A1 and H1C4) was only isolated from healthy Yellow Bourbon leaves, while *Achromobacter* (R2A1, R3A1, and R3A2), *Alcaligenes* (R1C), *Citrobacter* (R1B2), and *Luteibacter* (R1A1) were only isolated from the resistant Robusta leaves.

Even though the percent similarities of all isolates were greater than 97%, a phylogenetic analysis was conducted in order to determine whether each isolate will consistently cluster with their closest relatives based on the reference database. This was also done in order to efficiently resolve the taxonomic position of each isolate. All 39 16S rDNA sequences of coffee leaf sample isolates as well as 71 16S rDNA sequences of the determined closest relatives were used for the phylogenetic tree construction.

The phylogenetic tree showed that isolates initially branched into two major clades (Figure 2). One clade is comprised of isolates from both Actinobacteria and Firmicutes (100% bootstraps) and the other isolates were classified under Proteobacteria (100% bootstraps). Additionally, a clear separation between Actinobacteria and Firmicutes (100% bootstraps) was also seen. Subclasses of Proteobacteia also formed distinct clades in which, Alphaproteobacteria branched out first (100% bootstraps) while the other two classes split later (100% bootstraps). However, some putatively identified isolates, namely, *Luteibacter* sp. R1A2, *Stenotrophomonas* sp. I2E1, and *Stenotrophomonas* sp. I5A4 clustered with Betaproteobacteria isolates (100% bootstraps) instead of other Gammaproteobacteria.

Looking at the genus level, phylogeny-based clustering was observed to be generally consistent with that of the homology-based search results. However, species identification of most isolates still cannot be made since they were found to cluster with other isolates as well as with various reference species (Figure 2). Hence, although the percent similarities of all isolates are greater than the conventional 97% cut-off value and most are even greater than the more stringent 98.65% (Table 3), species



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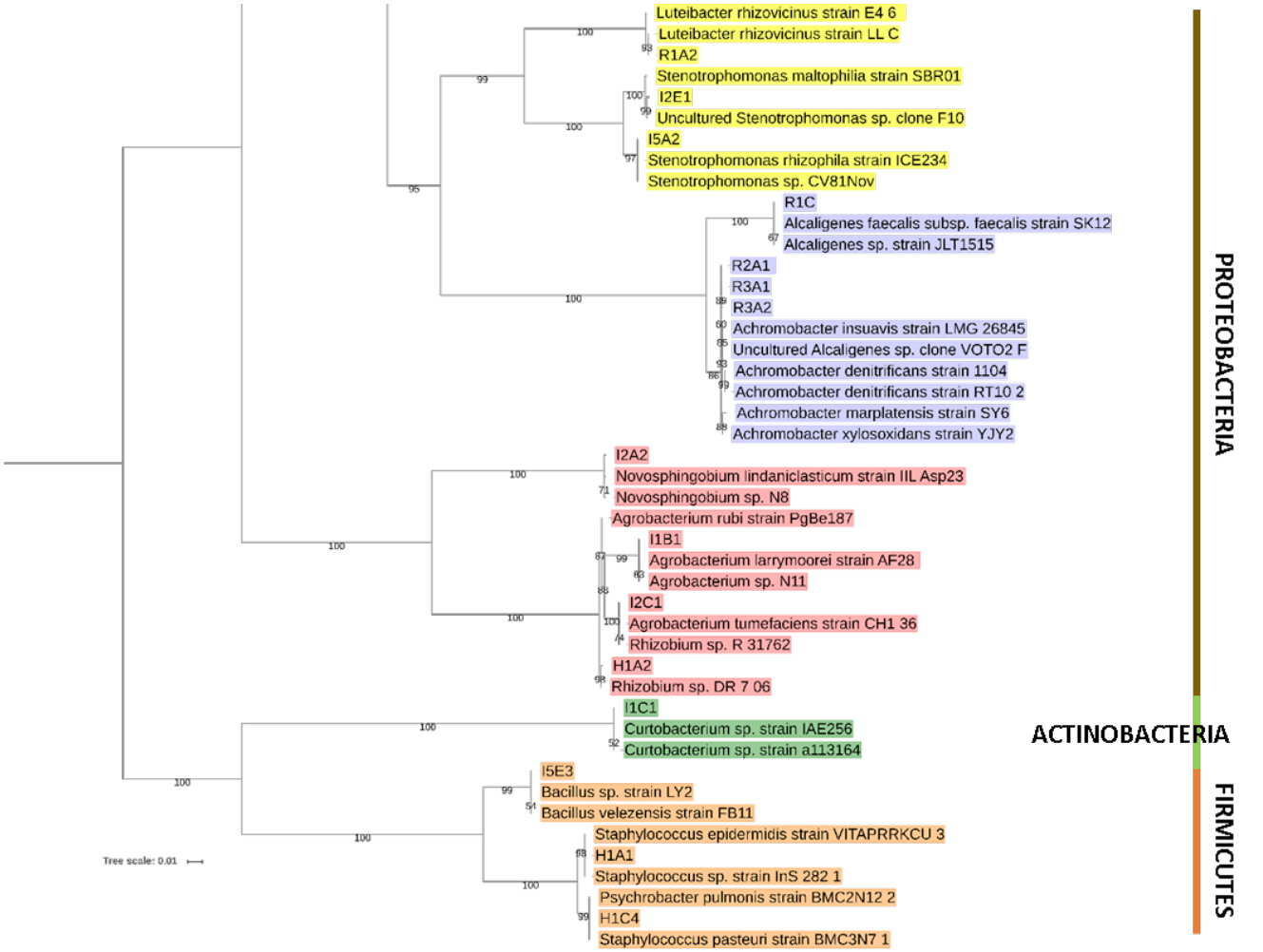

Figure 2: Maximum likelihood tree of all culture dependent isolates (in letter code) and their closest relatives based on 1212 nucleotides of the 16S rDNA gene. The tree is based on the TIM3+F+R3 substitution model. Values on the node represent bootstrap values based on 1000 replicates and bootstraps less than 50% are not shown. Scale bar represents five nucleotide substitutions for every one hundred nucleotides.

delineation based solely on this criterion can result to inaccurate species identification (Mysara et al. 2017).

Endophytic Bacterial Composition via PCR-DGGE

The molecular method, DGGE, was also employed in order to increase the coverage of diversity estimation of the bacterial endophytes in coffee leaves. It was assumed that through this method, other bacterial species that cannot be cultured or were not successfully cultured will be recovered. Detection of bacterial composition by DGGE is dependent on the nucleotide differences in the amplified sequences of each strain and is distinguished through their separation in a gel with a linear gradient of DNA denaturants (Kurtzman et al. 2011).

For the first analysis, a nested-PCR was done wherein, all samples were first amplified using 16S universal primers followed by the amplification using primers that target the V3 hypervariable region of the 16S rDNA. Fingerprints of the first gel showed bands in the lanes of all leaf samples (Figure 3). However, further examination of the excised bands through Sanger sequencing showed that only nine bands (A1–A9) correspond to bacteria, while bands with high GC content, found at the portion of high denaturation were all identified as plastid and mitochondrial DNA of the plant (Figure 3).

Bacterial strains identified were all classified as *Pseudomonas* sp. (Table 4). Moreover, these bands were observed in at least two out of five lanes of CLR infected Yellow Bourbon leaves but not in any of the lanes of healthy Yellow Bourbon and Robusta leaves (Figure 3). Although the presence of many *Pseudomonas* strains is consistent with the results of the culture-dependent analysis, their absence in other leaf samples is quite intriguing especially since they were observed to be dominant in all leaf samples.

Since the bacterial profile obtained from the V3 region was not able to give new and significant insights on the difference in the diversity of the leaf samples, another set of primers was used. For the second run, primers that target multiple regions (V6-V8) of the 16S rDNA were used for higher sensitivity. Direct amplification of the V6-V8 region was also done in order to reduce the possible amplification bias contributed by the use of the 16S universal primers in the first round of PCR in the previous run.

As expected, fewer bands were seen in the profile of the second DGGE (Figure 4). Lanes of infected Yellow Bourbon leaves still have the most number of bands but interestingly, lane R2 was observed to have a pretty similar banding pattern as lane I3 (Figure 4). However, just like in the first DGGE run, most of the bands were identified as plastid and mitochondrial DNA of coffee while only three bands were identified as bacterial species. Band B1 was putatively identified as *Luteibacter* sp. while bands B2 and B3 were identical *Pseudomonas* species (Table 4). Among these three, only B1 seems to have a corresponding band with the resistant Robusta leaves (lane R2). This confirms the culture-dependent result that *Luteibacter* can be isolated from Robusta leaves. This also further suggests that *Luteibacter* is also present in some CLR infected leaves of Yellow Bourbon

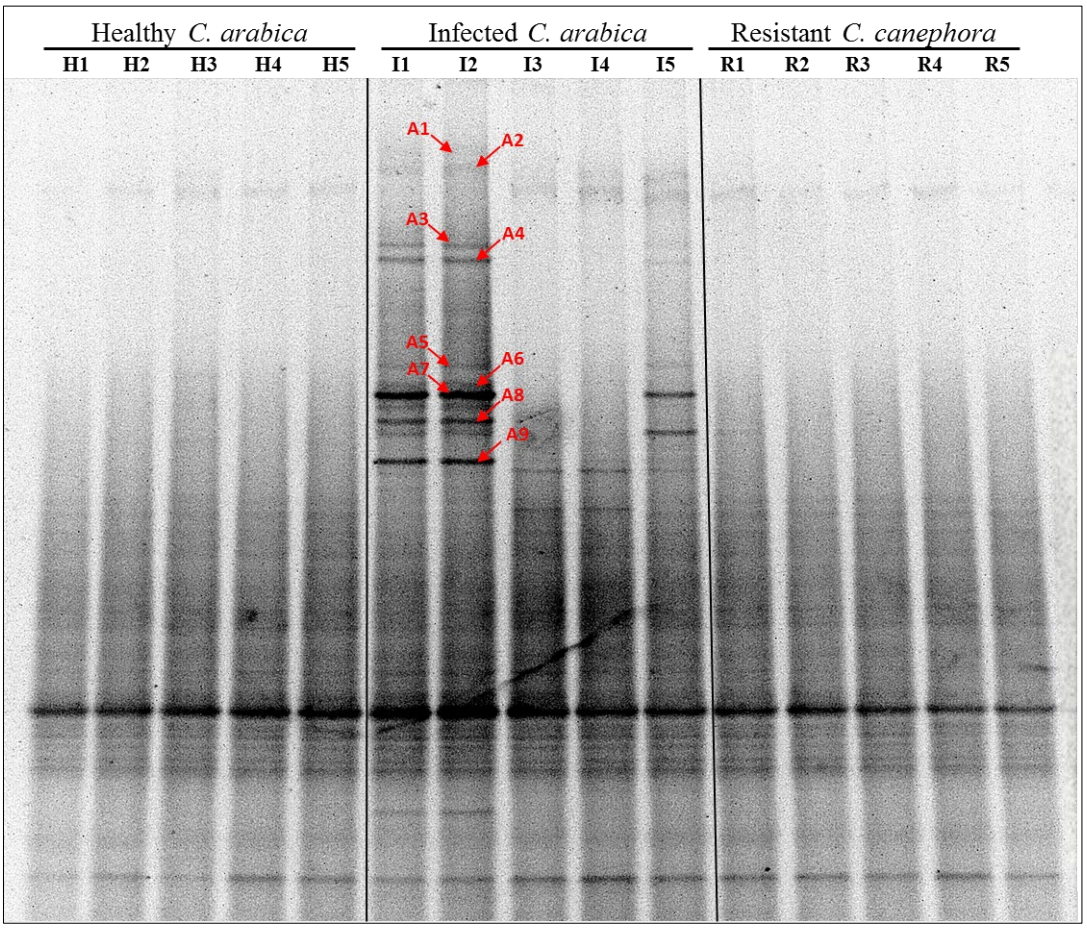

Figure 3: DGGE fingerprints of the nested-PCR amplified V3 hypervariable region of 16S from coffee leaves. Acrylamide gel linear gradient range from 35% to 65%. Numbered bands denote those that were sequenced and identified as bacterial species.

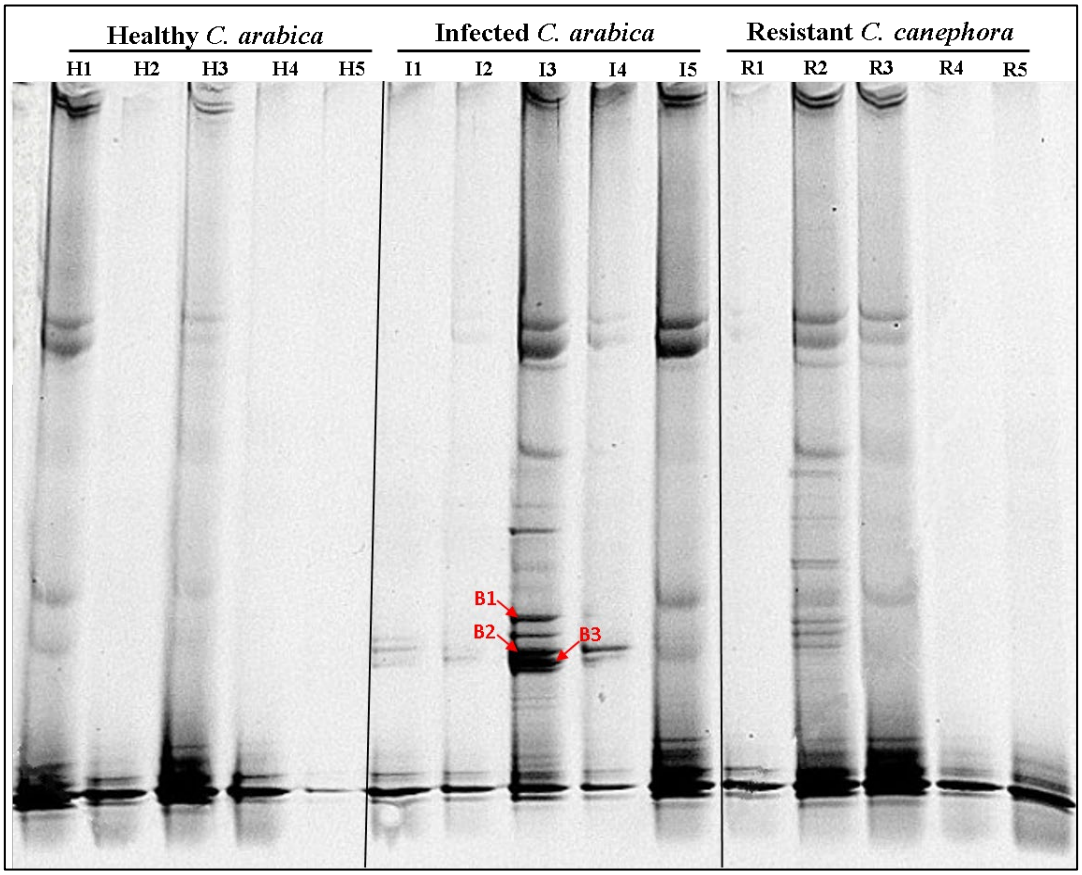

Figure 4: DGGE fingerprints of the amplified V6-V8 hypervariable regions of 16S from coffee leaves. Acrylamide gel linear gradient range from 35% to 65%. Numbered bands denote those that were sequenced and identified as bacterial species.

Table 4: Nucleotide BLAST search results for the closest relatives of bands excised from respective DGGE runs.

Classification	Band	Closest relatives	Percent Identity	Accession Number
DGGE 1: Nested PCR of V3 region				
γ-Proteobacteria	A1	*Pseudomonas* sp. strain L10.10	94.53	MH571536
		Pseudomonas sp. strain SHZ2.1	94.53	MF664158
γ-Proteobacteria	A2	*Pseudomonas baetica* strain D86 CV3R	99.19	MK883189
		Pseudomonas baetica strain D79 CV1R	99.19	MK883182
γ-Proteobacteria	A3	*Pseudomonas* sp. strain RS3	97.09	MH394447
		Pseudomonas viridiflava strain NK5	97.09	KU686696
γ-Proteobacteria	A4	Uncultured *Pseudomonas* sp. isolate DGGE gel band p6	97.25	MF034591
		Pseudomonas thivervalensis strain CR5	97.25	KX611491
γ-Proteobacteria	A5	*Pseudomonas putida* strain HM1	97.84	MK712480
		Pseudomonas fragi strain JLH 003	97.84	MK691449
γ-Proteobacteria	A6	*Pseudomonas putida* strain HM1	98.39	MK712480
		Pseudomonas fragi strain JLH 003	98.39	MK691449
γ-Proteobacteria	A7	Uncultured *Pseudomonas* sp. clone: DHUP34	99.42	AB451539
		Pseudomonas baetica strain S42	99.42	MK883123
γ-Proteobacteria	A8	*Pseudomonas putida* strain HM1	94.09	MK712480
		Pseudomonas fragi strain JLH 003	94.09	MK691449
γ-Proteobacteria	A9	*Pseudomonas putida* strain CP1V1-05	98.18	MK533941
		Pseudomonas sp. strain E1-8	98.18	KY938083
DGGE 2: Direct PCR of V6-V8 region				
γ-Proteobacteria	B1	Uncultured endophytic bacterium clone DKK2H06.M13-F	99.51	JN981884
		Luteibacter sp. strain L50	99.03	MK559964
γ-Proteobacteria	B2	*Pseudomonas* sp. strain NJ-NJ2-1021	99.46	MK863546
		Pseudomonas cichorii strain P-14	99.46	MH373661
γ-Proteobacteria	B3	*Pseudomonas* sp. strain NJ-NJ2-1021	100	MK863546
		Pseudomonas cichorii strain P-14	100	MH373661
DGGE 3: Nested PCR of V6-V8 region				
Bacilli	C1	*Exiguobacterium antarcticum* strain DW2	100	MK478815
		Exiguobacterium acetylicum strain SI17	100	MH719376
Bacilli	C2	*Exiguobacterium indicum* strain IHB_B_10090	99.75	KR233792
		Exiguobacterium indicum strain QW05	99.75	MK760069
Bacilli	C3	*Exiguobacterium indicum* strain QW05	99.46	MK760069
		Exiguobacterium sp. strain RS0S6	99.46	MH255949
Bacilli	C4	*Staphylococcus epidermidis* strain ATCC 14990	100	CP035288
		Staphylococcus epidermidis strain P32_BA1H	100	MK883070
Deinococci	C5	Uncultured bacterium clone CM44	94.07	EF580935
		Deinococcus aquaticus strain P76	90.79	MH504184
γ-Proteobacteria	C6	*Pseudomonas* sp. strain S3Bt38p	100	MH463751
		Pseudomonas mandelii strain 21-4(1)	99.64	KT369954
Bacilli	C7	*Exiguobacterium profundum* strain 80W	97.7	KY646127
		Exiguobacterium sp. HKG_126	97.67	HM01684
β-Proteobacteria	C8	*Hydrogenophaga* sp. strain BA0165	98.63	MK751587
		Hydrogenophaga sp. strain DI-97D	98.63	MH704930
α-Proteobacteria	C9	*Wolbachia* sp. strain China 1	99.47	CP016430

		Wolbachia sp. clone 17_3_1	99.47	LN829670
α-Proteobacteria	C10	*Wolbachia* sp. strain China 1	99.47	CP016430
		Wolbachia sp. clone 17_3_1	99.47	LN829670
β-Proteobacteria	C11	*Delftia lacustris* strain MB38	100	MK823230
		Delftia sp. strain IAE258	100	MH675503
α-Proteobacteria	C12	*Wolbachia* sp. strain China 1	97.87	CP016430
		Wolbachia sp. clone 17_3_1	97.87	LN829670
γ-Proteobacteria	C13	Uncultured bacterium clone 22-23	99.73	KT029984
		Pseudomonas sp. CC15M4	99.73	KM187195
γ-Proteobacteria	C14	Uncultured bacterium clone 22-23	99.2	KT029984
		Pseudomonas sp. CC15M4	99.2	KM187195
γ-Proteobacteria	C15	Uncultured bacterium clone 22-23	99.73	KT029984
		Pseudomonas sp. CC15M4	99.73	KM187195
γ-Proteobacteria	C16	Uncultured bacterium clone 22-23	99.73	KT029984
		Pseudomonas sp. CC15M4	99.73	KM187195
γ-Proteobacteria	C17	Pseudomonas sp. strain NJ-NJ2-1021	99.74	MK863546
		Pseudomonas cichorii strain P-14	99.74	MH373661
β-Proteobacteria	C18	Uncultured bacterium clone nbt05h08	99.69	EU535895
		Janthinobacterium sp. strain 197	100	KY682044
γ-Proteobacteria	C19	*Pseudomonas* sp. strain GZ22	100	MK999966
		Pseudomonas poae strain S46	100	MK883127
γ-Proteobacteria	C20	Uncultured bacterium clone EMIRGE_OTU_s2b2b_5119	98.8	JX222424
		Uncultured *Pseudomonas* sp. clone Bi1F02	97.19	JQ994185
Bacilli	C21	*Staphylococcus pasteuri* strain 2C	100	MH750037
		Sulfitobacter donghicola strain SB1155	100	CP026367
Bacilli	C22	*Staphylococcus* sp. A-19T0TMR-180-605	100	LC483353
		Caminibacter mediatlanticus TB-2	100	CP040463
β-Proteobacteria	C23	Uncultured bacterium clone TL-51	99.46	KP266499
		Uncultured *Thiobacillus* sp. clone F5OHPNU07H6X5H	99.46	HQ059114

even though it was not isolated via the culture-dependent method.

Finally, in order to reduce the contamination of plant DNA, the forward primer 799f-m7 was used for the first round of amplification of the third DGGE run (Hanshew et al. 2013). Through the use of this primer, plastid and mitochondrial DNA can be distinguished based on the size of the amplicon produced. Then for the second round of amplification, the same primers for the V6-V8 region were used.

A total of 23 bacterial strains were identified from the bands of the third run; these strains can be classified into 8 genera and 2 phyla (Figure 5 and Table 4). Ten bands were unique in healthy Yellow Bourbon leaves (C1, C2, C4, and C6-C12), five bands were unique in infected Yellow Bourbon leaves (C14-C19), and only band C20 was unique in resistant Robusta leaves (Figure 5). *Pseudomonas* species was again identified, but this time it was observed in at least a replicate of each leaf sample. *Staphylococcus* species were also identified in the healthy Yellow Bourbon and resistant Robusta leaves. Additionally, genera like *Delftia, Exiguobacterium, Hydrogenophaga, Janthinobacterium, Thiobacillus,* and *Wolbachia* were also identified even though they were not found in the culture-based isolation (Table 4).

Comparison of Bacterial Community Composition

Previous studies have evaluated the endophytic bacterial composition of coffee leaves (Silva et al. 2012; Vega et al. 2005) but this study is the first that tried to compare whether there are indeed differences in the bacterial endophyte composition between healthy and *H. vastatrix* infected coffee leaves. Figure 6 shows a Venn diagram representing the endophytic bacteria that were found in both culture-dependent and culture-independent analysis.

In all of the coffee leaf samples examined, *Pseudomonas* strains were observed with high relative frequency rates based on both culture-dependent and culture-independent results. This is consistent with previous findings wherein *Pseudomonas* was constantly among the bacteria identified in coffee leaves (de Sousa et al. 2018; Vega et al. 2005) as well asin the study of de Sousa et al (2018)in which it was observed to be the most abundant in *C. arabica* leaves. The prevalence exhibited by *Pseudomonas* reflects its metabolic and ecological versatility as

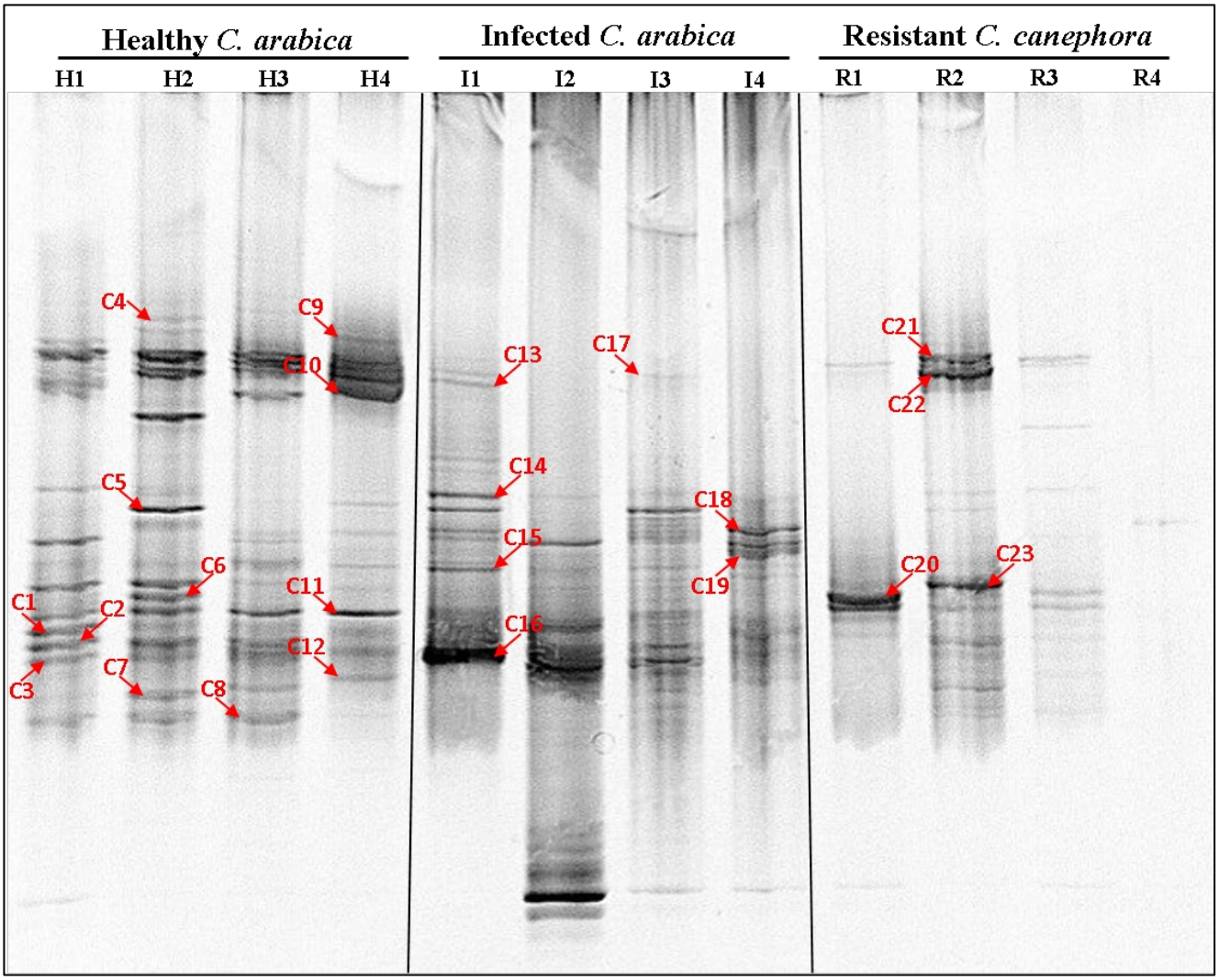

Figure 5: DGGE fingerprints of the nested-PCR amplified V6-V8 hypervariable regions of 16S from coffee leaves. Acrylamide gel linear gradient range from 35% to 65%. Numbered bands denote those that were sequenced and identified as bacterial species.

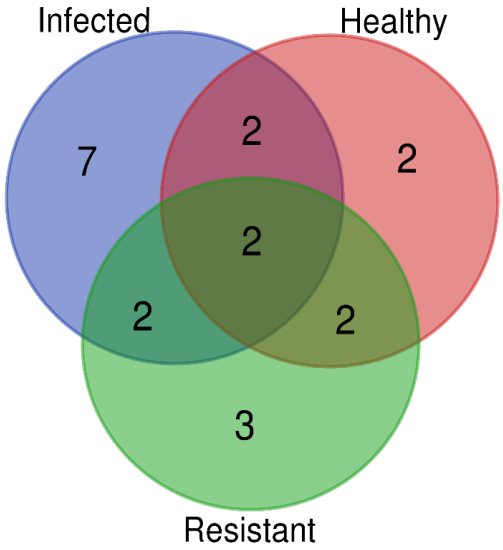

Figure 6: Venn diagram showing common and unique bacteria identified in the coffee leaf samples examined. Numbers refer to the number of bacterial genera found in: 1) all leaf samples: *Pseudomonas* and *Exiguobacterium*, 2) common in infected and healthy leaves: *Delftia* and Rhizobium, 3) common in healthy and resistant leaves: Thiobacillus and *Staphylococcus*, 4) common in infected and resistant: *Janthinobacterium* and *Luteibacter*, 5) unique in infected leaves: *Bacillus*, *Curtobacterium*, *Enterobacter*, *Stenotrophomonas*, *Erwinia*, *Hafnia*, and *Novosphingobium*, 6) unique in healthy leaves: *Hydrogenophaga* and *Wolbachia*, and 7) unique in resistant leaves: *Achromobacter*, *Alcaligenes*, and *Citrobacter*.

described by many studies in the past (Mercado-Blanco and Bakker, 2007).

Pseudomonas species in plants can generally be categorized as plant pathogens and plant mutualists. Some examples of phytopathogeic species are *P. syringae, P. cichorii* and *P. palleroniana* (Hofte and De Vos, 2006) while *P. fluorescens, P. putida* (Khan and Bano, 2016), *P. azotoformans* (Fang et al. 2016), and *P. extremorientalis* (Egamberdieva et al. 2013) are said to be beneficial for plants. Interestingly, *Pseudomonas* isolates (I1C2, I4B2, and I5A1) that were putatively identified as examples of pathogenic *Pseudomonas* species were found solely in the infected Yellow Bourbon leaves while the rest of the putatively identified plant beneficial isolates were distributed in all leaf samples. The presence of these phytopathogenic species of *Pseudomonas* in CLR infected leaves may be brought about by the compromised immune system of the plant as a result of CLR infection, thus giving the opportunity for other pathogenic bacteria to proliferate (Koeskella et al. 2017). Another possibility is that these species are positively associated with the pathogen such that simultaneous occurrence of both parties are needed for successful infection. This is exemplified by the study of Purahong et al (2018), in which they observed a direct correlation in the population growth of the pathogenic bacteria *P. syringae* pv. *syringae* (Pss) and *P. syringae* pv. *actinidiae* (Psa).

The genera *Exiguobacterium* was also found in all leaf samples. Previous studies have shown that strains belonging to this genus are associated with plant-growth promoting and anti-pathogen effects. (Bharti et al, 2013; Dastager et al. 2010; Pandey and Bhatt, 2016; Rajendran et al. 2012). Isolates found variably in the three leaf samples, namely, *Janthinobacterium, Luteibacter, Hydrogenophaga, Delftia* and *Rhizobium*, were also considered beneficial to plants in the past due to their ability to provide nutrition and pathogen defense for plants (Agafonova et al. 2017; Egamberdieva et al. 2013; Guglielmetti et al. 2013; Haack et al. 2016; Kloepper et al. 1992; Mafia et al. 2009).

Staphylococcus was also found to be common in healthy Yellow Bourbon and resistant Robusta leaves. *Staphylococcus* is a commonly known human pathogen. However, the present study is the first to report it as an endophyte of coffee. Studies on other plant microbiomes have detected *Staphylococcus* like *S. epidermidis* that has been reported to also have a role in plant protection, growth and development (Chaudhry and Patil, 2016). In this study, since *Staphylococcus* was only identified in the leaves without CLR infection, it may be possible that *Staphylococcus* has a potential role in the protection of coffee against pathogens.

In line with this, exclusive genera found in resistant Robusta leaves were *Achromobacter, Alcaligenes* and *Citrobacter.* In a previous study, Robusta leaves were observed to be consistently dominated by the order Enterobacteriales in which *Citrobacter* is a member of (de Sousa *et al*., 2018). Members of this taxonomic group are often described as PGPB and biocontrol agents of plant pathogens (Walterson and Stavrinides, 2015). Their abundance in Robusta leaves may be one of the reasons why this species is resistant to CLR infection. The other isolates from Robusta leaves, *Achromobacter, Alcaligenes, Luteibacter,* and *Thiobacillus* have also been implicated in plant-growth-promoting and pathogen suppression activities (Awad et al. 2011; Felestrino et al. 2017; Guglielmetti et al. 2013; Moretti et al. 2008; Ray et al. 2016; Sayyed and Chincholkar, 2008).

Through PCR-DGGE, *Wolbachia* was identified in the healthy Yellow Bourbon leaves. *Wolbachia* is an endosymbiont of arthropod species and its isolation in coffee leaves is very intriguing. As a matter of fact, *Wolbachia* has been reported to infect *Hypothenemus hampei* also known as the coffee berry borer (CBB) (Vega et al. 2002). A reduction in the population of *Wolbachia* was correlated to the reduction of egg production and fertility by female CBBs and thus, suggesting that it significantly contributes to the reproductive success of CBBs (Mariño et al. 2017). Its presence in the coffee leaves may indicate that CBBs may have been able to transmit *Wolbachia* into coffee plants during their colonization of the seeds or through surface contact (Chrostek et al. 2017).

Unique bacteria found only in CLR-infected Yellow Bourbon were *Bacillus, Curtobacterium, Enterobacter, Stenotrophomonas, Erwinia, Hafnia,* and *Novosphingobium.* Based on previous reports, however, *Bacillus, Curtobacterium, Enterobacter,* and *Stenotrophomonas* were also isolated from healthy *C. arabica* leaves (de Sousa et al. 2018; Vega et al. 2005). Among these four, *Bacillus* has been greatly associated with the biological control of a broad range of plant pathogens (Fira et al. 2018; Shafi et al. 2017). *Bacillus* is often described as a reliable biocontrol agent of many studies because, apart from its ability to produce different antibiotics, it can also simultaneously act as a PGPB through the production of plant hormones and siderophores (Fira et al. 2018; Shafi et al. 2017).

Curtobacterium, Enterobacter and *Stenotrophomonas* on the other hand, are similar to *Pseudomonas* because of their flexible nature. *Curtobacterium flaccumfaciens,* for example, is a well-known phytopathogen that causes bacterial wilt in dry beans worldwide but in cucumber plants it has been reported to promote plant growth and disease protection (Osdaghi et al. 2015; Raupach and Kloepper, 2000). Recently, *Enterobacter* species is also being highlighted for its importance as a plant pathogen. It has been described to control fungal plant pathogens through the production of chitinolytic enzymes, antibiotics, and other antifungal metabolites and is also able to contribute to plant growth and nutrition by nitrogen fixation and soil phosphorus solubilization (Bhattacharyya and Jha, 2012; Chernin, 1995; Macedo-Raygoza et al. 2019). However, several reports have shown that it can also cause disease symptoms in crops like chili pepper (García-González et al. 2018), macadamia (Nishijima et al. 2007), mulberry (Wang et al. 2008), and onion (Shroeder et al. 2009). In contrast to *Curtobacterium* and *Enterobacter, Stenotrophomonas* is currently emerging as a human opportunistic pathogen and no known strain is considered to be phytopathogenic (Ryan et al. 2009). *Stenotrophomonas* is mainly isolated in the soil and the rhizosphere where it predominates other Gram-negative bacteria (Berg et al. 1996). It is also associated with the promotion of plant growth and suppression of pathogens in plants (Berg and Martinez, 2015; Messiha et al. 2007).

Meanwhile, *Erwinia, Hafnia* and *Novosphingobium* have not yet been reported in previous coffee microbiome studies. Members of the genus *Erwinia* are mainly plant-associated and many are phytopathogenic to plants like pome fruit trees and the Rosaceae family (Kado, 2006; Llop et al. 2011; Vrancken et al. 2013). Virulence factors associated with the pathogenicity of this group include the synthesis of exopolysaccharides (EPS), delivery of effector proteins via a type III secretion system, production of siderophores, as well as the presence of cell-wall degrading enzymes, metalloproteases, quorum-sensing systems, and plasmids (Holtappels et al. 2015; Llop et al. 2011; Piqué et al. 2015; Sjöblom, 2009; Vrancken et al. 2013).

Erwinia has also been found in the past to penetrate fungal hyphae and demonstrate a facultative endohyphal lifestyle (Baltrus et al. 2017). This is similar to the case of *Burkholderia rhizoxinica*, a bacterium found to be an endosymbiont of *Rhizopus*, which is known to cause rice seedling blight (Moebius et al. 2014). *B. rhizoxinica* releases the toxin rhizoxin that binds to β-tubulin and inhibits mitosis and arrests cell cycle in plants (Partida-Martinez & Hertweck, 2005). Thus, for future studies it would be interesting to examine whether *Erwinia* can establish symbiosis with *H. vastatrix.*

In contrast, there are only a few studies that relate *Hafnia* and *Novosphingobium* to plants and both are mostly related to human infections and illnesses (Albert et al. 1991; Kaplan, 2004; Podschun et al. 2001; Rutembemberwa et al. 2014; Stanic et al. 2015). Notably, *Novosphingobium* sp. was found to produce quorum sensing signals that can be linked to the regulation of pathways in pathogenic and symbiotic bacteria of plants (Gan et al. 2009).

CONCLUSIONS

A total of 21 unique genera were identified in the coffee leaves examined through the combination of culture-dependent and culture-independent methods. The culture-dependent method led to the discovery of 14 genera while 10 genera were identified using the culture-independent method. Only *Pseudomonas, Staphylococcus* and *Luteibacter* overlapped and the rest of the genera were unique to each technique used.

CLR infected leaf samples had the most diverse bacterial endophyte community in terms of species richness. A total of 13 genera were identified in this leaf sample. Additionally, healthy Yellow Bourbon leaves and resistant Robusta leaves had 8 and 9 genera, respectively. High diversity in the infected leaves may be associated with the compromised immune system of coffee, as was observed in previous studies.

Distinct endophytes from each of the leaf sample were also observed in this study. Using culture-dependent isolation, *Bacillus, Curtobacterium, Enterobacter, Erwinia, Hafnia, Novosphingobium,* and *Stenotrophomonas* were found only in the CLR infected Yellow Bourbon leaves. On the other hand, *Staphylococcus* was only isolated from healthy Yellow Bourbon leaves, while *Achromobacter, Alcaligenes*, *Citrobacter*, and *Luteibacter* were only isolated from the resistant Robusta leaves. In the case of the culture-independent method, additional genera like *Hydrogenophaga* and *Wolbachia* were identified as unique in healthy leaves, while *Achromobacter, Alcaligenes,* and *Citrobacter* were found to be unique in resistant leaves.

Overall, this study was able to provide new information on what kinds of bacteria reside inside the coffee leaf, such as, new records of *Achromobacter, Alcaligenes Citrobacter Delftia, Erwinia, Exiguobacterium, Hafnia, Hydrogenophaga, Janthinobacterium, Luteibacter, Novosphingobium, Staphylococcus, Thiobacillus,* and *Wolbachia.*

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CONFLICT OF INTEREST

None.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

A.G. Estopace – Project Leader, conceptualized the proposal, conducted the experiments, analyzed the data and wrote the manuscript. E.P. Cao – helped in analyzing and writing the manuscript.

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