Phylogenetic relationship of coffee leaf rust in the central jungle of Peru

Relación filogenética de roya del café en la selva central de Perú

Delia Palmira Gamarra Gamarra [iD][1,3], Gilberto Torres Suarez [iD][1,4], Charo Milagros Villar Quiñonez [iD][1,5], Alistair R. McTaggart [iD][2,6], Emerson Clovis Carrasco Lozano [iD][1,7].

Abstract

Coffee leaf rust is the main disease that causes significant losses in *Coffea arabica*. In Peru, this disease caused epidemics between 2008 and 2013 with production losses of 35 %. The objective was to identify *H. vastatrix* using a morphological and molecular approach based on a phylogenetic species concept. Coffee leaf samples with symptoms of chlorotic lesions with the presence of yellow uredospores at different severity stages of different cultivars were collected from 11 locations in the departments of Pasco and Junin during 2017-2018. DNA was purified as proposed by Cristancho and coworkers. The major subunit of ribosomal DNA was amplified with universal primers LR0R and LR5, and sequenced by Macrogen and deposited in GenBank. Sequences from the genera *Achrotelium*, *Blastospora*, *Cystopsora*, *Hemileia*, and *Mikronegeria* were included for phylogenetic analysis. The results showed that the rust was distributed in coffee growing regions of Pasco: Villa Rica (Catimor, Caturra, and Gran Colombia); Oxapampa (Yellow Caturra), and Junín: San Luis de Shuaro (Catimor), Chanchamayo (Catimor), San Ramón (Catimor), Vitoc (Caturra), Pichanaki (Caturra), Río Negro (Caturra), Pangoa (Yellow Caturra, Gran Colombia, Limani). It was also grouped into a single clade with isolated *H. vastatrix* from Mexico and Australia, suggesting that they come from a common ancestor. This is the first confirmed report using molecular barcoding of *H. vastatrix* in the central jungle of Peru.

Keywords: *Coffea arabica*, DNA barcode, *Hemileia vastatrix*, Peru, phylogeny.

Resumen

La roya del café es la principal enfermedad que causa pérdidas significativas en *Coffea arabica*. En Perú esta enfermedad causó epidemias entre 2008 y 2013 con pérdidas de 35 % de la producción. El objetivo fue identificar *H. vastatrix* mediante un enfoque morfológico y molecular basado en un concepto de especie filogenética. Se recogieron muestras de hojas de café con síntomas de lesiones cloróticas con presencia de uredosporas amarillas en diferentes estados de severidad de diferentes cultivares en 11 localidades de los departamentos de Pasco y Junín durante el período 2017-2018. El ADN se purificó según lo propuesto por Cristancho y sus colaboradores. Se amplificó la subunidad mayor del ADN ribosomal con primers universales, LR0R y LR5, y fue secuenciada por Macrogen y depositadas en GenBank. Para el análisis filogenético se incluyeron secuencias de los géneros *Achrotelium*, *Blastospora*, *Cystopsora*, *Hemileia* y *Mikronegeria*. Los resultados mostraron que la roya se distribuyó en regiones cafetaleras de Pasco: Villa Rica (Catimor, Caturra y Gran Colombia), Oxapampa (Caturra Amarilla) y Junín: San Luis de Shuaro (Catimor), Chanchamayo (Catimor), San Ramón (Catimor), Vitoc (Caturra), Pichanaki (Caturra), Río Negro (Caturra), Pangoa (Caturra Amarilla, Gran Colombia, Limani). Asimismo, se agrupó en un solo clado con *H. vastatrix* aislados de México y Australia, lo cual sugiere que provienen de un ancestro común. Este es el primer informe confirmado que utiliza un código de barras molecular de *H. vastatrix* en la selva central del Perú.

Palabras claves: *Coffea arabica*, DNA barcode, *Hemileia vastatrix*, Perú, filogenia.

Introduction

Over 2.25 billion cups of coffee are consumed daily (Ponte, 2002), and the production of coffee is an economically important industry for countries of South America such as Brazil, Colombia, and Peru (Hoffmann, 2014). Coffee leaf rust is the main disease that causes significant losses in susceptible varieties (McCook, 2006). This disease caused epidemics between 2008-2013 with average losses of 35 % in Colombia, Ecuador, El Salvador, Honduras, Mexico, Nicaragua, Panama, and Peru (Avelino *et al.,* 2015) and 50 % losses in Brazil (Zambolim, 2016).

Coffee leaf rust is caused by *Hemileia vastatrix* (Berkeley & Broome, 1869) (Pucciniales, Pucciniomycotina), a biotrophic fungus that parasitizes leaves of *Coffea*, particularly *C. arabica* (Zuluaga & Céspedes, 2009). Currently, according to the Coffee Rust Research Center (CIFC) 50 physiological races of the fungus have been reported (Talhinhas *et al.,* 2017). The greatest diversity of races is found on the Asian continent, mainly in India (Várzea & Marques, 2005), while in Latin America race II predominates (Cristancho *et al.*, 2007; Cabral *et al.,* 2016; Zambolim, 2016).

According to Schieber and Zentmyer (1984) race II was first reported from Peru in the province of Satipo, department of Junín, in 1979. Coffee production in Peru was most impacted by coffee leaf rust in 2013, with losses that reached 35 % of the production (Avelino *et al.,* 2015). The National Coffee Board showed the cultivated area and production decreased between 2013 (429.000 ha and 252.800 tn) and 2014 (390.000 ha and 181.700 tn). The main departments affected by the disease were Junín (107.903.85 ha, 24 % of national production), San Martin (93.687 ha, 22 %), Cajamarca (73.098 ha, 17.2 %), Cusco (52.222 ha, 12.3 %), and Amazonas (42.744 ha, 10 %) (JNC, 2016).

Research on the identity of fungi was traditionally based on approaches based on cultivation and observation of morphological characteristics; however, as these characteristics are variable, this induces inaccurate identification. Currently, identity studies based on nucleic acids are accurate and may even reveal new species (Hibbett *et al.,* 2016; Tedersoo *et al.,* 2018). Multiple regions of the ribosomal RNA genes have been used for studies of fungal taxonomy and genetic diversity; these include the small subunit (SSU), large subunit (LSU), and internal transcribed spacer (ITS) region that separate the two ribosomal genes (Vilgalys & Hester, 1990; White *et al.,* 1990; Asemaninejad *et al.,* 2016). The ITS region is more variable and has multiple copies, so it is difficult to align due to differences in the length of the amplicons obtained (Eberhardt, 2012; Talhinhas *et al.,* 2017), whereas the LSU region contains two variable regions in length and structure called D1 and D2, which are bordered by highly preserved sequences in most fungi. This arrangement facilitates the alignment of the LSU region for phylogenetic analysis (Hibbett *et al.,* 2016). There are several reports for the phylogenetic classification of rust using the LSU molecular marker (Aime, 2006; Zuluaga *et al.,* 2011; McTaggart *et al.,* 2016).

We used a morphological and molecular approach based on a phylogenetic species concept to identify this rust fungus in the localities of Oxapampa (Pasco), Satipo, and Chanchamayo (Junín), and report its spread through coffee-growing areas. Knowledge of disease etiology and distribution of the pathogen is important for integrated management strategies, both of which rely on accurate identification of the causal agent.

Materials and methods

Sample collection. An average of 50 leaves from 10 coffee plants with typical symptoms of chlorotic lesions with presence of yellow uredospores at different stages of severity were collected from different cultivars in 11 locations in the departments of Pasco and Junin, during the period 2017 and 2018 (Table 1). The samples were transported in Ziploc bags to the Center for Plant Molecular Biology Research from Universidad Nacional del Centro del Perú. The collected leaves were observed with a stereomicroscope to eliminate contaminants and then the spores were collected in an eppendorf tube and stored at 4 °C for processing and later analysis. For morphological description, spores collected from all localities were observed with a Leica ICC50 W microscope. In addition, all specimens were registered in the Herbarium of Phytopathology of the Faculty of Agronomy of the Universidad Nacional del Centro del Perú.

DNA extraction from *H. vastatrix*. For the extraction of genomic DNA from *H. vastatrix*, the methodology proposed by Cristancho *et al.* (2007) was used. For this purpose, uredospores were deposited in new 1.5 mL eppendorf tubes. For cell lysis, 350 µL of lysis buffer (50 mM EDTA pH 8.0; 3 % SDS; 50 mM tris HCl pH 7.5; 1- Mercaptoethanol), 25 µL of Zymolase (10 mg/mL) were added and incubated at 65 °C for one hour. 10 µL Proteinase K (0.5 mg/mL) was added, incubated at 37 °C for 30 min and centrifuged at 14.000 rpm for 10 min; the supernatant was transferred to a new 1.5 mL eppendorf tube. For purification, a volume of Phenol:Chloroform:Isoamyl alcohol (25:24:1), and 1 volume of Chloroform:Isoamyl alcohol (24:1), was used. After centrifugation at 12.000 rpm for 10 min, the supernatant was transferred to an eppendorf tube and 1 volume of Chloroform was added and centrifuged at 12.000 rpm for 15 min, the supernatant was transferred to a new eppendorf tube. For DNA precipitation, a volume of cold Isopropanol was added and incubated for 12 hours. Subsequently, it was centrifuged at maximum speed for 10 min, the

Table 1. Geographical origin of *Hemileia vastatrix* samples used for molecular analysis.

Herbarium code	GenBank Code	Cultivar	Collection site	Altitude (m.a.s.l.)
RAC001	MN386212	Catimor	Finca Pampa encantada-Villa Rica-Oxapampa-Pasco	1.416
RAC002	MN386213	Caturra roja	Rancho Zevallos-Villa Rica-Oxapampa-Pasco	1.505
RAC003	MN386214	Gran Colombia	Rancho Sho'llet-Villa Rica-Oxapampa-Pasco	1.653
RAC004	MN386215	Caturra amarilla	Oxapampa-Oxapampa-Pasco	1.813
RAC005	MN386216	Catimor	Estación experimental UNCP-vivero forestal la esperanza San Ramón-Chanchamayo-Junín	869
RAC006	MN386217	Caturra roja	Centro poblado Santa Ana-Vitoc-Chanchamayo-Junín	965
RAC007	MN386218	Catimor	San Luis de Shuaro-Chanchamayo-Junín	757
RAC008	MN386219	Caturra roja	Anexo de Pucusani-Pichanaki-Chanchamayo-Junín	922
RAC009	MN386220	Caturra roja	Centro Poblado Kanariaki-Rio Negro-Satipo-Junín	951
RAC010	MN386221	Gran Colombia	Finca 2-Centro Poblado de Canaan-Pangoa-Satipo-Junín	990
RAC011	MN386222	Limani	Finca 3-Centro Poblado de Canaan-Pangoa-Satipo-Junín	1.150

DNA pellet was washed with 500 µL of 75 % ethanol and resuspended in 50 µL of TE buffer. Also, the DNA was extracted using a Geneiad® kit as recommended by the manufacturer. The DNA extractions were subjected to electrophoresis in 1 % agarose gel with 0.5 µL of ethidium bromide (10 mg/mL) and displayed using photo documentation equipment (Image software Lab™ DocXR+Systems Biorad®). Finally, the purified DNA was quantified with a spectrophotometer NanoDrop (Thermo Scientific™).

PCR and sequencing. Universal primers LR0R (5' ACCCGCTGAACTTAAGC 3') and LR5 (5' TCCTGAGGAAACTTCG 3') were used to perform PCR, amplifying the larger rDNA subunit (28 S) containing the D1 and D2 domains (Vilgalys & Hester, 1990; Eberhardt, 2012). A reaction mixture was prepared with the following components: 0.4 µM of each primer; 0.2 µM of dNTPs; 2 µM of MgCl$_2$; 1X PCR Buffer; 0.05 U/µL of Taq DNA polymerase (ThermoScientific™, USA) and 30 ng/µL of genomic DNA from each rust sample. A C1000 Thermocycler (Biorad®) was used with the following program: the initial stage at 94 °C for 5 min; followed by 35 denaturation cycles at 94 °C for 60 s, alignment at 55 °C for 60 s and extension at 72 °C for 60 s; and the final stage of extension at 72 °C for 10 min. The amplified fragments were purified from 1.5 % agarose gels using the Wizard PCR Preps DNA Purification System (Promega®) as recommended by the manufacturer and sequenced by Macrogen by the Sanger method using the primers from PCR.

Phylogenetic analysis. Sequences were assembled with CLC Genomics Workbench 7.0 (Qiagen®), deposited in GenBank, and aligned with sequences of rust fungi from the Mikronegeriaceae, including genera such as *Achrotelium*, *Blastospora*, *Cystopsora*, *Hemileia*, and *Mikronegeria*, and taxon sampling to include families recovered by Aime (2006) and McTaggart *et al.* (2016). We used available sequences of *H. vastatrix* in GenBank to provide a phylogenetic species hypothesis for the samples collected in Peru (Table 2). We aligned sequences using Guidance2 (Sela *et al.,* 2015) and searched for the most likely tree with IQTree v.1.7 beta (Nguyen *et al.,* 2015) with a model test (command-m TEST), 10.000 ultrafast bootstraps (Hoang *et al.,* 2018) and a probability ratio test with approximately 10.000 replicates (Guindon *et al.,* 2010).

Results

The urediniospores were prepared in slides with immersion oil and observed under the microscope. Rust occurred on the adaxial side of leaves, and symptoms of chlorotic spots were accompanied by suprastomatal uredinal pustules on the abaxial leaf surface. As infection progressed, small lesions coalesced and produced orange uredinia (Figure 1B). The adaxial leaf surface became necrotic followed by defoliation (Figure 1A). Urediniospores are structures of survival, dispersion, infection, and sexual reproduction (Carvalho *et al.,* 2011). The urediniospores of all the samples observed, presented similar characteristics as irregularly shaped, reniform, echinulate on upper halves and ventrally smooth, 22-36 × 18-28 µm in diameter with the presence of lipidic bodies containing carotenoids (Figure 1C). Spores had similar dimensions to those reported by de Castro *et al.,* (2009). The primers used, amplified fragments of approximately 950 bp, the DNA sequences were deposited in GenBank (Table 1). Peruvian rust specimens were identified as *H. vastatrix*. Our analyses supported that *H. vastatrix* has spread to the central rainforest into the coffee regions. The rust isolates of the following localities of the department of Pasco: Villa Rica (Catimor, Caturra, and Gran Colombia), Oxapampa (Caturra Amarilla), and the following localities of the department of Junín: San Luis de

Table 2. Species of rust, host and GenBank numbers of taxa included in the analyses.

Taxon	Host	GenBank number	Reference
Achrotelium ichnocarpi	Ichnocarpus frutescens	KT199393	McTaggart et al. (2016)
Allodus podophylli	Podophyllum peltatum	DQ354543	Aime (2006)
Austropuccinia psidii	NA	KF318445	Pegg et al. (2014)
Blastospora smilacis	Smilax sieboldii	DQ354568	Aime (2006)
Caeoma torreyae	Torreya californica	AF522183	Szaro and Bruns (unpublished)
Coleosporium senecionis	NA	AY512840	Begerow et al. (2003) (unpublished)
Cronartium flaccidum	NA	AF426239	Maier et al. (2003)
Cystopsora notelaeae	Notelaea microcarpa	KT199396	McTaggart et al. (2016)
Dasyspora gregaria	Xylopia cayennensis	JF263477	Beenken et al. (2012)
Desmella aneimiae	Nephrolepis hirsutula	KM249867	McTaggart et al. (2014)
Endoraecium acaciae	Acacia koa	DQ323916	Scholler and Aime (2006)
Eocronartium muscicola	NA	AF014825	Bruns and Szaro (unpublished)
Gerwasia rubi	Rubus sp.	KT199397	McTaggart et al. (2016)
Hemileia vastatrix	Coffea robusta	KT199399	McTaggart et al. (2016)
Hemileia wrightiae	Wrightia sp.	KT199400	McTaggart et al. (2016)
Hemileia vastatrix	Coffea arábica	DQ354566	Aime (2006)
Hemileia vastatrix	Coffea arabica x Coffea canephora	MF417754	Pelayo-Sanchez et al. (2017) (unpublished)
Hemileia vastatrix	Coffea arabica cv. Oro Azteca	MF417753	Pelayo-Sanchez et al. (2017) (unpublished)
Hemileia vastatrix	Coffea arabica var. Caturra Rojo	MF417752	Pelayo-Sanchez et al. (2017) (unpublished)
Hemileia colombiana	Mesechites sp.	EU851165	Zuluaga et al. (2011)
Hyalopsora polypodii	Deparia petersenii	KJ698627	Padamsee and McKenzie (2014)
Maravalia cryptostegiae	Cryptostegia grandiflora	KT199401	McTaggart et al. (2016)
Melampsoridium betulinum	Alnus sp.	DQ354561	Aime (2006)
Melampsora euphorbiae	Euphorbia heterophylla	DQ351722	Deadman et al. (2006)
Melampsora larici-populina	Populus canadensis	AY125395	Wingßeld et al. (2004)
Mikronegeria alba	Nothofagus nervosa	DQ354569	Aime (2006)
Mikronegeria fuchsiae	Fuchsia excorticata	KX985771	Padamsee and McKenzie (2017)
Naohidemyces vaccinii	Vaccinium ovatum	DQ354563	Aime (2006)
Phakopsora pachyrhizi	Glycine max	DQ354537	Aime (2006)
Phragmidium mucronatum	Rosa sp.	HQ412646	Deadman et al. (2011)
Pucciniastrum epilobii	NA	AF522178	Szaro and Bruns (2002) (unpublished)
Puccinia graminis	NA	AF522177	Szaro and Bruns (2002) (unpublished)
Ravenelia evansii	NA	KP661595	Ebinghaus et al. (2015) (unpublished)
Sphaerophragmium acaciae	Albizzia sp.	KJ862350	McTaggart et al. (2015)
Thekopsora areolata	NA	KJ546894	Kaitera et al. (2014)
Trachyspora intrusa	Alchemilla vulgaris	DQ354550	Aime (2006)
Tranzschelia discolor	Prunus domestica	DQ354542	Aime (2006)
Uredinopsis filicina	Phegopteris connectilis	AF426237	Maier et al. (2003)
Uromyces appendiculatis	Phaseolus vulgaris	AF522182	Szaro and Bruns (2002) (unpublished)
Uromycladium simplex	Acacia pycnantha	KJ632990	Doungsa-ard et al. (2015)

Shuaro (Catimor), Chanchamayo (Catimor), San Ramón (Catimor), Vitoc (Caturra), Pichanaki (Caturra), Río Negro (Caturra), Pangoa (Caturra Amarilla, Gran Colombia, Limani) these were grouped into a single clade that includes the isolates of *H. vastatrix* isolates from Mexico and Australia (Figure 3), suggesting that they come from a common ancestor (Aime, 2006; McTaggart *et al.,* 2016). Coffee production in the central jungle of Peru is affected by factors such as climate, altitude, and the shading system, which can generate microclimates that form small thermal microhabitats that favor pests and diseases (Liebig *et al.,* 2019).
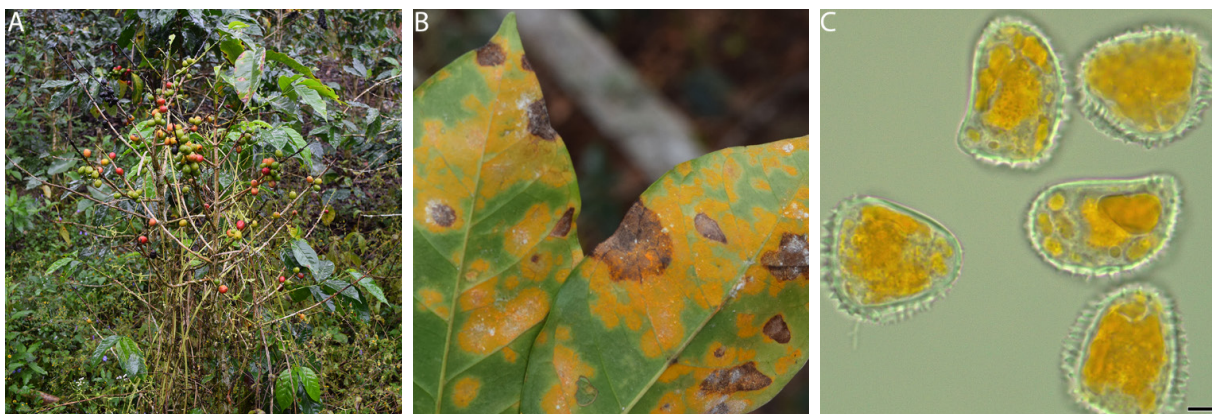
Figure 1. A. Defoliated symptoms for rust on a coffee plant (*Coffea arábica*); **B.** Symptoms of *H. vastatrix* on the abaxial surface of the leaf; **C.** Urediniospores of *H. vastatrix* observed 100× magnification with a Leica ICC50 W microscope, scale = 10 μm. Fuente: Gamarra-Gamarra, D.

Figure 2. Abandoned coffee production field in the province of Chanchamayo, department of Junin. Fuente: Gamarra-Gamarra, D.

Discussion

Peru exports coffee to 50 countries, the main ones being the United States, Germany, Belgium, etc. (JNC, 2019). However, since the first report of coffee leaf rust in Satipo-Peru, in 1979 (Schieber & Zentmyer, 1984), no importance has been given to the distribution of this pathogen. After the epidemic that was caused between 2012-2013 (Avelino *et al.,* 2015), efforts were made to manage the disease. However, according to the report from the National Coffee Board in the last campaign, more than 50 ha have been abandoned due to the presence of pests and diseases. In the department of Junín, only 90.000 ha were harvested due to the high production costs and devastating effects of the coffee leaf rust, so farmers abandoned their fields (Figure 2) replacing them with other crops, such as cocoa, coca, and bananas (JNC, 2019).

The Catimor cultivar is the most cultivated crop in the central jungle of Peru because it was reported as resistant to race II leaf rust (Avelino *et al.,* 2015). However, in the production fields, a disease incidence of 90 % was observed and a severity of 80 % in the leaves of Catimor (Silva-Acuña *et al.,* 1999) suggesting that this cultivar is no longer tolerant to *H. vastatrix*. Nevertheless, further knowledge is needed to test whether reproduction has occurred in Peru to overcome disease tolerance.

Currently, the production of this crop is conditioned to the application of copper-based chemicals to counteract the disease. However, these products are easily leached out by heavy rainfall and are consequently contaminating the environment (Rivillas-Osorio *et al.,* 2011). Moreover, the application of non-lethal fungicides could increase the mutation rate and generate genetic variation in the population of fungal pathogens. For example, Amaradasa and Everhart (2016) evaluated the effect of commercial fungicides with different modes of action on susceptible isolates of *Sclerotinia sclerotiorum*, observing high allelic mutation rates. According to McDonald and Linde (2002) the evolutionary potential of a species is determined by factors such as mutation rate, population size, genotype flow, reproduction system, and selection pressure. The generation of genetic diversity in *H. vastatrix* is feasible due to its high mutation capacity (Avelino
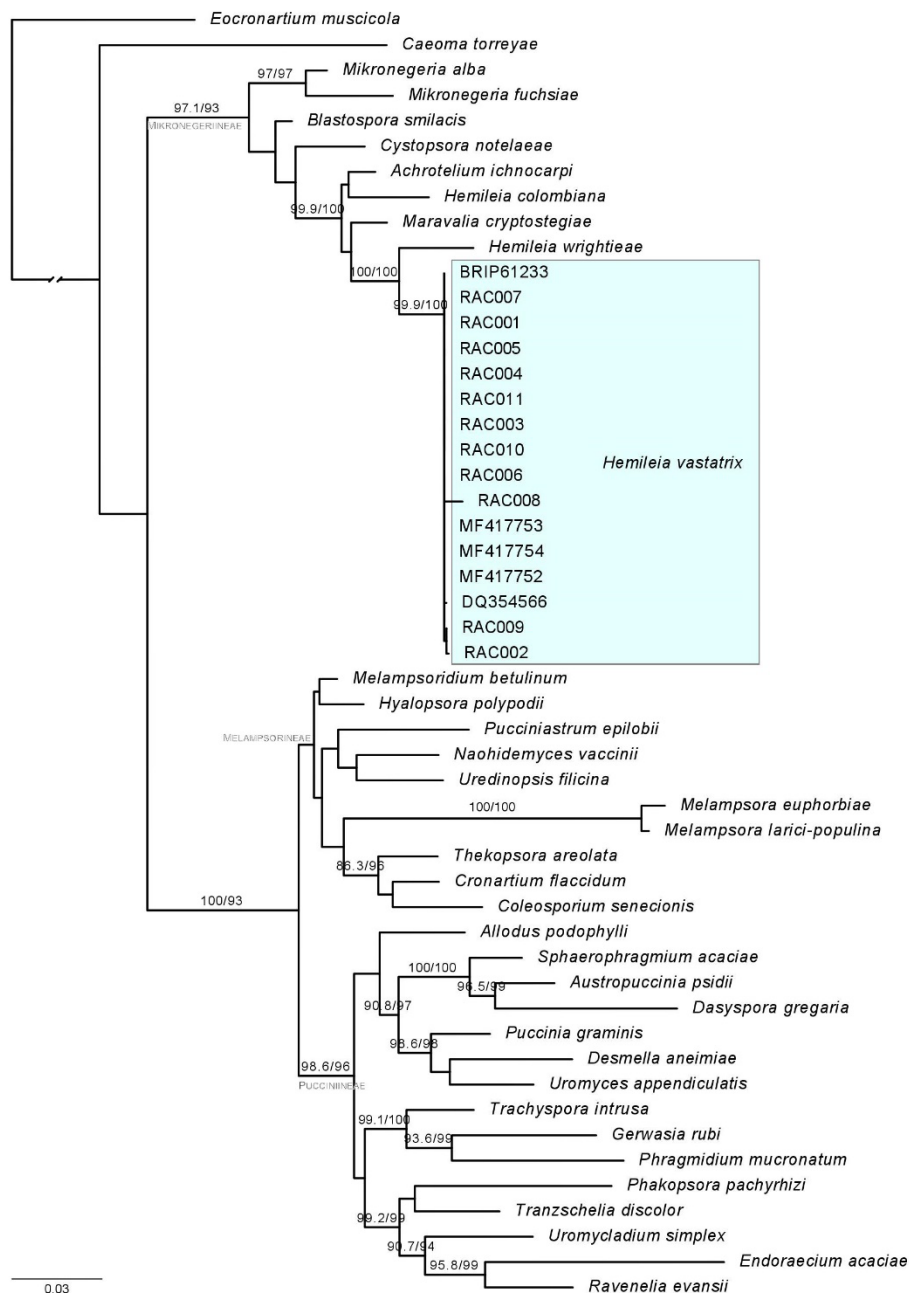
Figure 3. Phylogram obtained from a maximum likelihood search in IQTree v.1.7 beta, with a TVM+F+I+G4 model of evolution selected in a model test (command -m TEST). aRLT values (≥ 90 %) and ultrafast bootstrap values (≥ 95 %) from 10,000 replicates above nodes.

et al., 2015) and for presenting sexual reproduction and asexual multiplication capacity. It seems that this last feature is a mechanism used by the pathogen to generate new genetic variants. For example, Carvalho *et al.* (2011) report that sexual reproduction, through a phenomenon called 'cryptosexuality', could explain the emergence of new physiological races, thus overcoming host resistance, as reported in Brazil (Gouveia *et al.,* 2005) and Colombia (Cristancho *et al.,* 2007). In addition, Cabral *et al.* (2016) reported that at the population level in *H. vastatrix* there is a low degree of differentiation; however, at the subpopulation

level, there is a high degree of differentiation and this suggests a high rate of evolution of the pathogen which could partially explain the break of resistance to *H. vastatrix* of cultivars derived from the Hybrid of Timor and Icatu.

Our results on the phylogeny of *H. vastatrix* in the central jungle of Peru will allow predicting possible outbreaks from the dispersion of the pathogen to the different production areas. However, it is suggested that the genetic diversity of the pathogen should be determined in order to implement a genetic

improvement program that adopts or develops new cultivars with genotypic characteristics of horizontal resistance as a control strategy for this pathogen.

Conclusions

Hemileia vastatrix is reported from coffee production areas in Peru. Rust was collected on cultivars of coffee that were previously considered tolerant to *H. vastatrix*. The coffee leaf rust was grouped into a single clade that includes the *H. vastatrix* isolates from Mexico and Australia, suggesting that they come from a common ancestor. This is the first confirmed report using a molecular barcode of *H. vastatrix* in the central jungle of Peru.

Acknowledgments

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