Tropical Plant Pathology (2018) 43:473–476
https://doi.org/10.1007/s40858-018-0246-9

SHORT COMMUNICATION

Population genetic structure of the coffee pathogen *Hemileia vastatrix* in Minas Gerais, Brazil

Mateus F. Santana[1] · Eunize M. Zambolim[2] · Eveline T. Caixeta[3] · Laércio Zambolim[2]

Received: 6 October 2017 / Accepted: 31 July 2018 / Published online: 4 September 2018

Abstract

The biotrophic fungus *Hemileia vastatrix* Berk & Broome is the most destructive coffee pathogen in Brazil. Better understanding of the population genetics of *H. vastatrix* would provide important insights into its biology, epidemiology, and evolutionary potential. The aim of the present study was to assess the genetic diversity and population structure of *H. vastatrix* in Minas Gerais (Brazil) using ribosomal DNA (rDNA) sequences. The analyzes were performed by sequencing the internal transcribed spacers ITS1 and ITS2, and the 5.8S gene from 15 *H. vastatrix* populations. Of the 82 sequences obtained, 68 ribotypes were found, as defined by 108 nucleotide substitutions and five indels. Of the 68 ribotypes, 64 were exclusively found in one population. Analysis of molecular variance (AMOVA) and F_{ST} fixation index indicated moderate genetic differentiation among field populations, which were divided according to geographic origin. In conclusion, analysis of the nuclear ITS1–5.8S-ITS2 rDNA sequence diversity in the *H. vastatrix* population of Minas Gerais revealed that most ribotypes are restricted to a single population and that there exists greater genetic diversity within than among field populations.

Keywords *Coffea arabica* · Coffee rust · ITS polymorphism · Population structure

Brazil is the largest coffee producer in the world, and the state of Minas Gerais accounts for approximately 50% of the Brazilian coffee production (Conab 2017). However, several diseases affect coffee trees; among them, coffee leaf rust (caused by the biotrophic fungus *Hemileia vastatrix*) is the most important. Currently, this disease can be found in all coffee producing regions of Brazil and other countries (Avelino et al. 2015; Zambolim 2016; Talhinhas et al. 2017). One major consequence of leaf rust is the defoliation of coffee trees, which reduces flowering and fruiting, and may lead to a 30–90% decrease in coffee production (Avelino et al. 2015; Zambolim 2016). Chemical control is effective, but its impact on the microbial community may exacerbate other coffee tree diseases and pests, as well as facilitate the emergence of resistance in the pathogen population through natural selection (Várzea and Marques 2005). Another form of coffee rust control is the use of resistant cultivars, which has less environmental impact. However, several resistant cultivars have become susceptible to the disease, owing to the high genetic diversity of *H. vastatrix* (Várzea and Marques 2005).

The Coffee Rust Research Centre (CIFC, Oeiras, Portugal) has identified more than 50 races of *H. vastatrix* through infection patterns on a series of differential coffee clones (Várzea and Marques 2005). Fifteen races have been identified in Brazil (Cabral et al. 2009; Capucho et al. 2012; Zambolim 2016). However, the mechanisms underlying the appearance of new pathotypes/races remain unknown. Genetic diversity can result from mutation (Várzea and Marques 2005), criptossexuality (Carvalho et al. 2011), and the activity of transposable elements (Cristancho et al. 2014). However, information about the genetic diversity and population structure of *H. vastatrix* is scarce and the few reported studies are based on random amplified polymorphic DNA (RAPD) (Gouveia et al. 2005; Nunes et al. 2009) and amplified fragment-length polymorphism (AFLP)

Section Editor: Diana Fernandez

✉ Mateus F. Santana
mateus.santana@ufv.br

✉ Laércio Zambolim
zambolim@ufv.br

[1] Department of Microbiology, BIOAGRO, 36570-000, Universidade Federal de Viçosa, Viçosa, MG, Brazil

[2] Department of Phytopathology, BIOAGRO, BIOCAFÉ, Universidade Federal de Viçosa, Viçosa, MG 3650–000, Brazil

[3] Embrapa Café, BIOAGRO, BIOCAFÉ, Universidade Federal de Viçosa, Viçosa, MG 3650–000, Brazil

(Maia et al. 2013; Cabral et al. 2015). In the present study, we investigated the use of ribosomal DNA (rDNA) sequences to assess the genetic diversity and genetic structure of *H. vastatrix* populations. This is the first time these sequences are used to study the genetic diversity of the *H. vastatrix* populations from Brazil.

Urediniospores were collected from several infected leaves and grouped into 15 *H. vastatrix* populations based on the collection site (Table 1). Ten out of the 15 *H. vastatrix* populations were collected in the main coffee production areas of Minas Gerais (MG; nine populations) and Espírito Santo (ES; one population) in Brazil (Table 1). Two (B1 and B3) of the remaining five populations originated from the CIFC in Oeiras, Portugal, and were previously characterized as Race II by inoculating a series of differential coffee trees. The other three populations (B2, B4 and B5) were collected in 1986 (Cardoso 1986) and were previously characterized as Races I, II, and III, respectively (Table 1).

The genomic DNA of each population was extracted from 50 mg of urediniospores according to a method described by Raeder and Broda (1985). The internal transcribed spacer 1 (ITS1), 5.8S, and ITS2 regions were amplified using universal primers: ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (White et al. 1990). Amplification was performed in a 25 μL total volume containing 25 ng of DNA, 10× PCR Buffer Plus (200 mM Tris-HCl pH 8.4, 15 mM $MgCl_2$, 500 mM KCl), 1 U GoTaq DNA polymerase (Promega), 10 mM of deoxynucleotide triphosphates (dNTPs), and 0.5 μM of primer (ITS1 and ITS4). The PCR was performed beginning with initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 2 min, and 70 °C for 1 min, and a final extension at 72 °C for 5 min (White et al. 1990).

The ITS1 and ITS4 primers amplified 950 bp and 600 bp fragments of rDNA regions of *H. vastatrix* and coffee (*Coffee arabica* L.), respectively. The PCR products were separated by electrophoresis in a 1% agarose gel and the band corresponding to *H. vastatrix* was excised and purified with a Wizard SV Gel and PCR Clean-Up System kit (Promega). The ITS amplicons of the 15 populations were cloned into pGEM-T Easy vector (Promega) and transformed into *Escherichia coli* DH5α. Four to seven transformed colonies were selected from each population, totalizing 82 sequenced clones (Table 2). Plasmid DNA from the selected colonies was purified with a Wizard SV Minipreps DNA Purification System kit (Promega), and the inserts were sequenced using a MegaBase DNA Analysis System 500 (Amersham Biosciences Corp.). The sequences corresponding to ITS1–5.8S-ITS2 region were edited, and aligned using CodonCode Aligner software (CodonCode Corporation) and deposited in GenBank (accession numbers MF627747-MF627828).

The number of ribotypes in the *H. vastatrix* population was estimated with the TCS 1.13 software (Clement et al. 2000). The gene and nucleotide diversity index (Nei 1987) and the F_{ST} fixation index of the field population were estimated using Arlequin software version 3.1 (Excoffier et al. 1992), which was also used for the analysis of molecular variance (AMOVA).

The alignment of the 82 sequences resulted in the identification of 68 ribotypes, which were defined by 108 nucleotide substitutions and five indels of one base each (Table 2). The length of the ITS1–5.8S-ITS2 regions ranged from 952 to 953 bp. The data indicated that the *H. vastatrix* ITS region exhibited both inter- and intra-population diversity. This result indicates that the genetic diversity of *H. vastatrix* within the populations is high and consists of a complex mixture of

Table 1 Geographic origins and races of *H. vastatrix* populations

Abbreviation	Population	Coffee Host	Geographic origin	Genotypic Diversity	Nucleotide Diversity
B1	Race II	*C. arabica*	CIFC, Oeiras - Portugal	0.90	0.0054
B2	Race II	*C. arabica* 'Mundo Novo'	Viçosa – Minas Gerais	1.00	0.0054
B3	Race II	*C. arabica*	CIFC, Oeiras - Portugal	1.00	0.0054
B4	Race I	Selection KP 423	Caratinga- MG	0.95	0.0059
B5	Race III	*C. arabica* 'Catuaí'	Campinas – SP	1.00	0.0047
I1	Field Population I	*C. arabica* 'Oeiras'	Piranga- MG	0.95	0.0040
I2	Field Population II	*C. arabica* 'Catuaí'	Araponga - MG	1.00	0.0054
I3	Field Population III	*C. arabica* 'Ubatam'	Mariana- MG	1.00	0.0020
I4	Field Population IV	*C. arabica* 'Catuaí'	Ervália- MG	0.70	0.0037
I5	Field Population V	UFV H514–7–10-1	Patrocínio- MG	1.00	0.0047
I6	Field Population VI	Timor Hibrid X 'Catuaí'	Senhora de Oliveira- MG	1.00	0.0062
I7	Field Population VII	*C. arabica* 'Catimor'	São Sebastião do Paraíso-MG	0.93	0.0078
I8	Field Population VIII	*C. arabica* 'Catuaí vermelho'	Capinópolis- MG	1.00	0.0066
I9	Field Population IX	*C. arabica* 'Catuaí'	Venda Nova dos Imigrantes-ES	0.95	0.0059
I10	Field Population X	*C. arabica* 'Catuaí vermelho'	Coimbra- MG	0.95	0.0034

Table 2 Number of ITS1–5.8S-ITS2 regions sequenced and the distribution of 68 ribotypes among *H. vastatrix* populations

	Ribotypes																																					
	N	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34			
B1	5	2																																				
B2	4											1			1																							
B3	5									1																	1										
B4	7		2																					1					1								
B5	4						1						1			1																					
I1	7		2																			1			1												
I2	6	1																1					1					1									
I3	4	1						1			1																										
I4	5		3		1																																
I5	4	1							1																								1	1			
I6	5																				1																
I7	6																1			2																	
I8	6					1								1																	1						
I9	7																									1				2	1						
I10	7	2		1															1																1		
Total	82	7	7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1			

	Ribotypes																																			
	N	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	HIo
B1	5																				1							1			1					4
B2	4		1											1																						4
B3	5	1																		1									1							5
B4	7								1							1	1																			6
B5	4				1																															4
I1	7																	1																1	1	6
I2	6						1																			1										6
I3	4									1																										4
I4	5																					1														3
I5	4																																			4
I6	5			1											1									1			1									5
I7	6										1	1	1																							5
I8	6							1																						1		1				6
I9	7																		1				1									1				6
I10	7					1																			1											6
Total	82	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1			

N: number of ITS1–5.8S-ITS2 regions sequenced in the population; Hlo: number of ribotypes found in the population

several ribotypes. The high number of singletons reinforced the idea that point mutations are important for generation of genetic diversity in the *H. vastatrix* genome (Table 2).

Arlequin software was used to estimate the genotypic and nucleotide diversity of the population. Genotypic diversity is a measure of the genetic diversity within the population that is dependent on allelic frequency. In contrast, nucleotide diversity is a measure of the degree of polymorphism within the population (Nei and Kumar 2000). The genotypic diversity ranged from 0.7 to 1.0 (maximum variability), while the nucleotide diversity ranged from 0.002 to 0.007 (Table 1). The high genotypic diversity and low nucleotide diversity in the *H. vastatrix* population suggests that although the mutations are few, they were exclusive to each ribotype. In addition, the high level of genotypic diversity observed is consistent with the high number of singletons.

Results of AMOVA suggest that most of the *H. vastatrix* genetic diversity is accounted for by within-population diversity (86.5%). This value could be explained by the large number of different ribotypes found within the same population. The F_{ST} value was 0.13, indicating a low to moderate genetic differentiation among populations. Nunes et al. (2009) and Cabral et al. (2015) obtained similar results using RAPD and AFLP markers, respectively, when studied the *H. vastatrix* populations from diverse coffee producing regions in Brazil. In contrast, Gouveia et al. (2005) used RAPD markers to reveal a high genetic differentiation among coffee rust isolates from Africa and Asia, but not among isolates from South America. The mixture of *H. vastatrix* genotypes observed in the present study is likely due to the wind dispersal of urediniospores, or else, to the transportation of infected leaves between crops, since coffee in Minas Gerais is planted in contiguous areas.

In conclusion, analysis of the genetic diversity of the nuclear ITS1–5.8S-ITS2 rDNA region in the *H. vastatrix* populations of Minas Gerais showed that most ribotypes are restricted to a single population, with greater genetic diversity within than among field populations. Therefore, analysis of the genetic diversity based on rDNA sequences allows a better understanding of the genetic population structure of *H. vastatrix*, which is very important for disease control and the success of coffee breeding programs.

Acknowledgements This work was sponsored by the Brazilian Coffee Research and Development Consortium (CBP&D/Café), Foundation for Research Support of the State of Minas Gerais (FAPEMIG), and National Council of Scientific and Technological Development (CNPq).

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