UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
FACULDADE DE CIÊNCIAS AGRONÔMICAS
CAMPUS DE BOTUCATU

ESTRUTURA DA POPULAÇÃO E EPIDEMIOLOGIA DE *Moniliophthora roreri* NO MAGDALENA MEDIO COLOMBIANO

YEIRME YANETH JAIMES SUÁREZ

Tese apresentada à Faculdade de Ciências Agronômicas da UNESP – Campus de Botucatu, para obtenção do título de Doutor em Agronomia (Proteção de Plantas).

BOTUCATU – SP
Fevereiro – 2016

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1. RESUMO

A Moniliase do cacaueiro, causada pelo fungo *Moniliophthora roreri*, é uma das doenças mais devastadoras do cacaueiro na região oeste da América do Sul e Central, por exemplo, a região do Vale do Magdalena na Colômbia, considerada o possível centro de origem para á espécie. Para analisar a diversidade genética foram utilizados isolados dos estados de Santander, Antioquia, Tolima e Huila da Colômbia utilizando vinte-três marcadores microssatélites (SSR). No total, 117 genotipos multilocus diferentes se encontraram entre os 120 isolados, cada um representado como um haplotipo único. O índice de associação observado e estandardizado (I_A e $\check{r}d$) indicaram que as populações de *M. roreri* são clonais. Além disso, dada a alta diversidade de haplotipos com desequilíbrio de ligação se sugere que *M. roreri* poderia ser uma espécie assexual possivelmente com recombinação rara ou parcial devida à parasexualidade. Enquanto a estrutura populacional, três grupos geográficos foram reconhecidos entre os isolados utilizando métodos de agrupamento bayesianos. Resultados similares se obtiveram depois do analises discriminante de componentes principais (DAPC), analise de coordenadas principais (PCA) e a arvore de semelhança com os loci dos microssatélites baseados na distância de Nei. A identificação destes agrupamentos explicasse pela diferenciação geográfica e clones de cacaueiro e variáveis ambientais não contribuem significativamente à diferenciação genéticas entre os grupos. Em relação a epidemiologia da doença foi monitorada a incidência da moniliase no tempo e no espaço em clones com diferente grau de resistência em quatro localidades do Estado de Santander entre Julho de 2013 até Maio de 2015. O modelo linear generalizado se utilizou para analisar o progresso temporal e espacial da Moniliase. A distribuição de Poisson com função de ligação logarítmica se selecionou pela superdispersão dos dados. Uma interação significativa se

encontrou entre os clones e localidades sobre os parâmetros epidemiológicos. O clone suscetível SCC 61 apresentou o maior intensidade de doença na maioria das localidades, a exceção de San Vicente onde tive o mais baixo nível. A localidade de San Vicente apresentou a mais baixa intensidade de doença em todos os clones de cacaueiro, quando comparado com as outras localidades. Depois de aplicar o teste de Moran I aos dados de frutos doentes cortados no centro de pesquisa La Suiza, foi evidente a autocorrelação espacial. O estudo da dinâmica demostrou que as epidemias da moniliase variam de acorde com a localidade e o material vegetal e sua localização no campo. As informações obtidas neste trabalho podem ser empregadas para melhorar o manejo da moniliase ao nível regional e as práticas deverão ser ajustadas considerando a variação do microambiente.

Palavras chave: Moniliase, diversidade genética, *Theobroma cacao*, epidemias

***Moniliophthora roreri* POPULATION STRUCTURE AND EPIDEMIOLOGY IN THE COLOMBIAN MIDDLE MAGDALENA REGION**

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2. SUMMARY

Frosty pod rot disease (FPR) on cocoa, caused by *Moniliophthora roreri*, is one of the most devastating cocoa diseases in the Western Hemisphere, including the Magdalena Valley areas in Colombia, which is considered the possible center of origin for the species. We analyzed the genetic diversity of isolates from the states Santander, Antioquia, Tolima and Huila of Colombia using twenty-three simple sequence repeats (SSR) markers. In total, 117 different multilocus genotypes were found among 120 isolates, each one represented as a unique haplotype. The observed and standardized index of association (I_A and *řd*) indicates that the populations of *M. roreri* are clonal populations. Furthermore, given the high haplotype diversity with linkage disequilibrium are suggest that *M. roreri* could be an asexual species possibly undergoing rare recombination or partial recombination due to parasexuality. Three geographical groups were recognized among the isolates using

Bayesian clustering methods. Similar results were obtained after discriminant analysis of principal components (DAPC), principal coordinate analysis (PCA) and a neighbor-joining tree from microsatellite loci based on Nei distance. The identified clusters where explained by geographical differentiation and cacao clones and environmental variables did not contribute significantly to the genetic differentiation between groups. Regarding to the disease epidemiology, incidence of Frosty Pod Rot (FPR) disease, caused by *Moniliophthora roreri*, in time and space on clones with different level of resistance were investigated in four localities of Santander State between July 2013 and May 2015. Generalized linear model was used to analyze the temporal and spatial progress of FPR. Poisson distribution with logarithm link function was chosen because of data overdispersion. A significant interaction was found between clones and localities on epidemics parameters. The susceptible clone SCC61 had the higher levels of disease in the most localities, excepted in San Vicente where it obtained the lower level. The locality in San Vicente had the lower levels of disease in all cacao clones, when compared with the other localities. After applied Moran's I test to data of cut disease pod in La Suiza Research Center, were evident in spatial autocorrelation. The dynamic study provide that FPR epidemics vary according to location and plant material and its location on the plot. The information obtained in this word could be used to improve the management of FPR at the regional level and the practices will be adjusted to local level considering microenvironmental variation.

Keywords: Frosty pod rot, genetic diversity, *Theobroma cacao*, epidemics

3. INTRODUÇÃO GERAL

O cacau, produzido por *Theobroma cacao* L., é a matéria-prima para o chocolate e constitui-se na base de uma agroindústria com conexões econômicas e sociais em todo o mundo. Cerca de três milhões de toneladas de grão de cacau seco são consumidos anualmente, portanto este produto é considerado uma das "commodities" mais importante nas regiões tropicais. A agricultura familiar de pequena escala é responsável pelo 80% a 90% da produção mundial deste produto, sendo a principal fonte de renda para aproximadamente seis milhões de pequenos produtores (WFC, 2014).

No mundo, estima-se que aproximadamente entre o 30% e 40% da produção de cacau se dana pela ação de pragas e patógenos. Em condições ótimas para o desenvolvimento destes agentes causais, as perdas podem exceder o 80%. Em termos econômicos, as perdas totais são de $2 bilhões de dólares anuais, onde o impacto direto, nesta cadeia de produção, é sobre a renda familiar dos produtores. (DUFFEY, 2009).

Na América Latina, as doenças causadas por *Moniliophthora roreri*, *Moniliophthora perniciosa* e *Phytophthora palmivora* são os mais limitantes da produção de cacau. Sendo que a moniliase é a doença fúngica mais grave (LEACH; MUMFORD; KRAUSS, 2002). A presença desta doença nas árvores de cacaueiro tem efeitos tão devastadores na produção que a viabilidade econômica em longo prazo pode ser comprometida. Nos diferentes países da América Latina, onde ela se encontra estabelecida, as perdas atribuídas tem sido tão serias que as culturas inteiras têm sido abandonadas (PHILLIPS-MORA; AIME; WILKINSON, 2007).

M. roreri é um fungo altamente especializado que destrói os frutos de espécies pertencentes aos gêneros *Theobroma* e *Herrania*. O fungo é capaz de crescer sob uma vasta gama de condições ambientais. Este alto nível de adaptação do fungo e o grande

número de esporos de longa vida que produz fazem de *M. roreri* um fitopatógeno altamente eficaz e um formidável invasor de novas regiões geográficas (PHILLIPS-MORA; AIME; WILKINSON, 2007).

A moniliase manifesta-se como lesões marrons espalhadas na superfície do fruto, e no final produz-se nela uma massa de esporos cor creme. Geralmente, os frutos infetados em fases muito precoces morrem. Em alguns casos, na superfície dos frutos podem aparecem deformações, antes da formação dos conídios (GRIFFITH *et al*., 2003). Os frutos doentes tornam-se na principal fonte de inoculo, já que o fungo produz um grande número de esporos na superfície da lesão (cerca de 7 bilhões de esporos por fruto (PHILLIPS-MORA; WILKINSON, 2007).

O controle químico da moniliase não é economicamente viável, adota-se o manejo integrado desta doença, que envolve melhores práticas agronômicas combinadas com materiais de plantio melhorados para resistência e o controle biológico. Na atualidade, estas são as únicas estratégias factíveis para o manejo desta doença (ALI *et al*., 2015).

Para o manejo desta doença também deve-se considerar a epidemiologia deste patógeno, ou seja, estabelecer as bases teóricas para compreender as epidemias no tempo e no espaço (MILGROOM; PEEVER, 2003). Da moniliase conhece-se que a dispersão dos esporos do patógeno depende da temperatura e a umidade, da mesma forma que o aumento da incidência e da quantidade de tecido doente tem uma relação positiva com a quantidade da chuva em 2 a 3 meses antes da infecção. A dispersão dos propágulos se dá por meio da água, material vegetal infectado, animais, ferramentas ou o homem (LEANDRO, 2011).

No que se refere a resistência, não há materiais de cacaueiro conhecidos com resistência do tipo imune a *M. roreri*, só há matérias resistentes ao fungo desenvolvidos pelo CATIE, na Costa Rica. A classificação dos materiais de cacaueiro se baseou no índice de severidade em resistente (0 – 1.25), moderadamente resistente (1,260 – 2,50), moderadamente susceptíveis (2,51 – 3,75) e susceptível (3,76 – 5) (PHILLIPS-MORA *et al*., 2005). Porém, a perda de resistência na maioria dos clones selecionados tem aumentado ao longo dos últimos 15 anos (ALI *et al*., 2015). A infeção de clones com alta resistência por *M. roreri* tem sido observada em ambientes desfavoráveis ao hospedeiro, incluindo alterações no metabolismo neste patógeno e indução de respostas a estresses para enganar os mecanismos de defesa da planta (BAILEY *et al*., 2014).

Uma boa parte desta adaptação é devida a genética na população do fungo, o que permite que indivíduos específicos na população se adaptem às mudanças das condições ambientais, em particular ao tipo de resistência apresentada pelo hospedeiro, a partir da pressão seletiva natural ou a modificação do hospedeiro feita pelos fitomelhoradores. Portanto, o conhecimento apropriado da variação genética na população de *M. roreri* é fundamental para o estabelecimento de um programa de melhoramento genético de resistência eficaz e sustentável. Com a recente designação do Nordeste da Colômbia como o centro de diversidade e origem deste fitopatógeno, estudo como este pode ser relevante no entendimento deste patossistema, se a pesquisa for efetuada nesta região (ALI *et al*., 2015).

Neste sentido, para elucidar a variabilidade genética dos isolado de *M. roreri* no Magdalena Medio Colombiano e a dinâmica temporal e espacial da doença. Assim, o presente trabalho teve como objetivos:

a) estudar a variação e estrutura genética entre as populações de *M. roreri* no centro de origem.

b) estudar a relação da dinâmica temporal e espacial em diferentes ambientes, para a moniliase.

A tese foi dividida em dois capítulos na forma de artigos científicos, sendo o primeiro capítulo intitulado: “Geographic Differentiation and Genetic Structure of *Moniliophthora roreri* in the Principal Cocoa Production Areas in Colombia”, e o segundo capitulo intitulado: “Spatio-temporal dynamic of frosty pod rot in the main cocoa producing areas of Santander State, Colombia” ambos regidos conforme as normas da revista Plant Disease**.**

4. REVISÃO BIBLIOGRÁFICA

4.1. A cultura do cacaueiro

Theobroma cacao L. é uma espécie de arvore frutífera diploide (2n = 2x = 20) endêmica da floresta tropical do Amazonas na América do Sul. Esta espécie foi domesticada cerca de 3.000 anos atrás, na América Central (ARGOUT *et al*., 2011). Seu produto, os grãos de cacau ou sementes, é o principal ingrediente dos chocolates e os produtos derivados desta indústria. Citações indicam que os grãos de cacau foram consumidos pelos Maias e Aztecas, e provavelmente pelos Olmecas (1.500 – 400 AC), e hoje são um importante componente da economia de muitos países produtores e processadores (LOPES *et al*., 2011).

Para o período entre 2013 e 2014, a produção mundial anual de grãos de cacau foi estimada em 4,36 bilhões de toneladas e o consumo mundial anual de grãos de cacau em 4,3 bilhões de toneladas (ICCO, 2015). No mundo, entre 5 a 6 milhões de pequenos produtores são responsáveis por mais do 80% da produção (WCF, 2014). A Colômbia é o decimo produtor mundial de cacau e o quinto na América Latina com uma produção atual de 47 toneladas aproximadamente (FAO, 2015), sendo o estado de Santander o primeiro produtor com mais de 27,07% da produção. Na exploração desta cultura estão envolvidas mais de 30 mil famílias, onde o 95% são pequenos produtores (MADR, 2013).

As regiões cacaueiras estão em grande parte centradas em importantes "hotspots" de biodiversidade, em 13 das regiões do mundo mais diversas biologicamente. Por esta razão o cacaueiro, uma arvore perene, é cultivado na sombra com um ciclo produtivo de mais de 50 anos. A cultura de cacaueiro prove benefícios ambientais tais como corredores migratórios de aves realçando a diversidade, conservação do solo e

bacias hidrográficas e como zonas tampão próximas de habitats de florestas em extinção (GUILTINAN *et al*., 2008).

Contudo, como a maioria das culturas, o cacaueiro é atacado por um grande número de patógenos responsáveis por 30% dos danos na produção mundial. Entre estas doenças, a moniliase é considerada a doença do cacaueiro mais destrutiva. Além de constituir uma ameaça para diversas regiões produtoras do mundo; como o Brasil e África. A introdução deste patógeno no continente africano encontra-se entre as maiores ameaças para economia mundial do cacau (PLOETZ, 2007; JAIMES *et al*., 2011).

4.1.1. Composição genética do cacaueiro cultivado

A maior diversidade genética do cacaueiro silvestre se localiza na região do Amazonas. Más, durante sua domesticação só uma pequena parte desta diversidade se exportou a diferentes destinações para ser cultivados (LOOR *et al*., 2009). Assim, os tipos de cacaueiro cultivado na atualidade se subdividem em três grandes grupos: forasteiro, crioulo (domesticado pelos indígenas na América Central) e trinitários (híbridos entre forasteiro e crioulo originários de Trinidad e Tobago) (AIKPOKPODION, 2012). O grupo forasteiro se compõe de populações muito diversas com diferentes origens geográficos: Alto-Amazonas, Baixo-Amazonas, Orinoco e as Guianas (MOTAMAYOR *et al*., 2002). As arvores deste grupo se caracterizam por ser vigorosas e com maior resistência as doenças (AIKPOKPODION, 2012). Contudo, os forasteiros são pouco valorados por ter grãos fermentados de sabor de menor qualidade, embora existam exceções como é a variedade de forasteiro "Nacional" do Equador (YANG *et al*., 2012).

O grupo crioulo, cultivado originalmente pelos Maias da América Central), representa o primeiro cacaueiro domesticado (LOOR *et al*., 2009). Este grupo se caracteriza por ter grãos de cacau de um sabor de alta qualidade, más tem menor vigor e produtividade (MOTAMAYOR *et al*., 2002). Os crioulos são conhecidos por ser o grupo mais susceptível a varias doenças do cacaueiro (YANG *et al*., 2012). Desde 1825, estes materiais têm sido substituídos por clones trinitários mais resistentes e produtivos em países como a Venezuela (MOTAMAYOR *et al*., 2012).

O grupo trinitário é uma população hibrida desenvolvida de hibridações naturais entre cacau crioulo e forasteiro do Baixo-Amazonas importados à ilha de Trinidad quando as plantações de crioulo foram destruídas por um desastre natural no final do século XVIII (LOOR *et al*., 2009). Estes híbridos amostraram variação na sua

combinação de caracteres parentais e a seleção do programa se baseou no inicio na produtividade, conduzindo à identificação de 100 arvores trinitários na atualidade conhecidos como acessões Imperial Collection Selection (ICS). Nos anos 40s do século passado, o Ministério de Agricultura na Trinidade estabeleceu um programa de melhoramento genético baseado na hibridação de trinitários selecionados com genótipos da América do Sul e se gero as acessões Trinidad Selected Hybrids (TSH), considerados a base dos programas de melhoramento moderno da cultura do cacaueiro (YANG *et al*., 2012).

Contudo, os programas de melhoramento modernos do cacaueiro se focalizam no estabelecimento de variedades de cacaueiro melhoradas através do cruzamento seletivo de arvores com características agronómicas e resistência desejada (LIVINGSTONE *et al*., 2011). Más, no futuro a economia mundial do cacaueiro vai depender significativamente do uso de germoplasma com uma ampla base genética para produzir novas variedades com resistência a doenças e pragas, características de qualidade desejáveis, e a capacidade de se adaptar a mudanças climáticas (ZHANG; MOTILAL, 2016).

4.1.2. Principais doenças do cacaueiro

Varias doenças causadas por fungos, vírus e nematoides têm sido reportadas na cultura do cacaueiro, más só poucas destas têm sido reconhecidas por ter importância ao nível global e muito destas são só importantes ao nível regional ou local. As doenças com importância a o nível global são: a podridão-parda causada por *Phytophthora* spp. (*P. palmivora, P. capsici, P. citrophthora, P. heveae,* e *P. megakaria*); a vassoura-de-bruxa causada por *Moniliophthora perniciosa*; o vírus do rebroto inchado (CSSV); e a moniliase causada por *M. roreri*. Este patógenos contribuem à redução da produção mundial até num 40%, más esta redução pode alcançar o 90-100% em casos de doenças como a Vassoura-de-bruxa (BHATTACHARJEE; KUMAR, 2007).

Dentro as doenças mais devastadoras se encontram: a vassoura-de-bruxa que em 10 anos reduziu a produção do Brasil num 75% (desde 400000 t a 100000 t); a moniliase que tem devastado a produção de pequenos cacauicultores na América Central e América do Sul; e a podridão parda causada por *P. megakarya*, patógeno altamente virulento que ameaça a produção da África Ocidental. A introdução acidental destes três patógenos é considerada como uma ameaça potencial (HEBBAR, 2007). Porém, a moniliase é a doença de fúngica do cacaueiro mais severa em 12 países da América Latina (MEINHARDT *et al*., 2014). Esta doença é considerada uma ameaça latente para a produção

mundial de cacau, e sua presença tem efeitos devastadores sobre produção que ao longo do tempo compromete a viabilidade econômica desta cultura (PHILLIPS-MORA; AIME; WILKINSON, 2007).

4.2. *Moniliophthora roreri*

M. roreri, agente causal da moniliase, é um fungo parasítico de plantas pertencente à família Marasmiaceae. Por muitos anos, este fungo foi considerado a forma anamórfica de um ascomiceto. Mas, técnicas taxonômicas e moleculares indicaram a posição de *M. roreri* como um basidiomiceto e demostraram também sua estreita relação genética como *M. perniciosa* (BAILEY *et al*., 2013). Embora tenha um genoma de maior tamanho, 52,3 Mbp, o genoma de *M. roreri* é estrutural e organizacionalmente semelhante a *M. perniciosa*. A maior parte da diferença no tamanho se associa com DNA repetitivo, mas o número de genes localizados em cada genoma é semelhante, assim como o número de elementos de transposição com longas repetições terminais entre os genomas (MEINHARDT *et al*., 2014).

4.2.1. Origem do patógeno

No início, o Equador foi considerado o centro de origem da moniliase, pelo fato que em 1917 o fitopatologista Rorer J.B fez a primeira ocorrência oficial do agente causal da moniliase naquele país, coletando amostras que se enviaram a Smith R.E, da Universidade de Califórnia que identificou o agente causal da moniliase como *Monilia* sp. (JAIMES; ARANZAZU, 2010). Segundo a pesquisa desenvolvida por Phillips-Mora (2003), a moniliase do cacau apareceu pela primeira vez no Norte de Santander, na Colômbia em 1817, de acordo com os relatos da época e com o padrão de variabilidade do patógeno existente nesta região. Assim, a região nordeste da Colômbia foi designada como o centro de diversidade e origem de *M. roreri* (PHILLIPS-MORA; AIME; WILKINSON, 2007).

4.2.2. Taxonomia

Ciferri e Parodi (1933) classificaram inicialmente o fungo como um ascomiceto anamórfico, pela aparente ausência do estado meiótico e de estruturas sexuais, e pela similaridade morfológica com outros fitopatógenos do gênero *Monilinia*. Contudo, Evans *et al*. (1978) encontraram a presença de septo doliporo no micélio vegetativo, septo

característico dos fungos Homobasidiomicetes. Também, observou-se que o processo de formação do conídio primário holoblástico, e a diferenciação do conídio primário, um segmento da hifa fértil abaixo do seu septo basal que incha e se diferencia num novo propágulo e assim por diante (EVANS *et al.,* 1978). Portanto, devido a estas inconsistências com sua classificação no gênero *Monilinia*, o fungo causador da moniliase se reclassificou como *Moniliophthora roreri* (EVANS *et al*., 1978; GRIFFITH *et al*., 2003). Assim, na atualidade *M. roreri* é membro do reino Fungi, filo Basidiomycota, Subfilo Agaricomycotina, Classe Agaricomicetes, Ordem Agaricales, família Marasmiaceae, gênero *Moniliophthora* e espécie *M. roreri*.

4.2.3. Características culturais

O crescimento da colônia em ágar malte é lento. Depois de duas semanas, a colônia atinge o diâmetro entre 8 a 15 mm. As margens são lisas ou ligeiramente elevadas. As colônias têm textura lanuda a feltrosa, a cor inicial é salmão pálido ocreado ou rosa amarelado claro, e a cor final é canela amarelada clara a cor de argila ou localmente cor marrom da madeira a cor de abricó amarelada clara. Às vezes, as colônias têm textura pubescente ou farinácea, cor branca a creme com matizes ocre. O odor é indistinto (EVANS *et al*., 1978).

4.2.4. Morfologia

As hifas são hialinas, com parede delgada, septadas e ás vezes levemente irregulares com inchaço com 1,5 até 5 µm de comprimento. *M. roreri* tem dois tipos de hifas aéreas, as hifas presentes na zona de progresso com paredes levemente engrossadas, e as hifas hialinas a marrom pálido com parede espessa não septadas, com 1 até 3 µm de largo, esqueléticas, raras vezes ramificadas. Os conidióforos são ramificados, dão origem a uma cadeia madura basipetal de conídios. Os conídios se separam facilmente, estes têm parede espessa, cor amarelo pálido, em massas são cor marrom, e tipicamente são globosas a subglobosas com diâmetros entre 6,5 até 25 µm, ás vezes são elipsoides entre 8 – 20 µm ou 5 – 14 µm e parede de até 2 µm de espessura. Igualmente, apresentam-se conídios com parede fina, provavelmente provenientes das cadeias imaturas (EVANS *et al*., 1978).

4.2.5. Hospedeiros

M. roreri ataca frutos das espécies dos gêneros *Theobroma* e *Herrania*. Em 1918 se reportou a ocorrência desta doença nos frutos de *T. bicolor* e *H. balaensis* no Equador (RORER, 1918). Baker *et al*. (1954) reportaram infecções em *T. gireli*. Evans *et al*. (1981) reportaram o ataque de *M. roreri* em frutos de *T. mammosum*, *T. simiarum*, *T. sylvestre*, *T. angustifolium*, *H. nítida* e *H. pulcherrima*. Mais tarde, Enriquez (1981) identificaram o patógeno em *T. grandiflora* e *H. purpúrea* (MOREIRA, 2006).

4.2.6. Variabilidade genética

No estudo de Moreira (2006) utilizando marcadores de Seqüências simples repetidas ou Microssatélites (também conhecidos como SSR - Simple Sequence Repeats) desenvolvidos para *M. perniciosa* e Polimorfismo de DNA amplificado ao acaso (RAPD - Random Amplified Polymorphic DNA), observou-se um nível moderado de multialelismo nos *loci* avaliados. Os *loci* de microssatélites avaliados serviram para detectar uma média de 7,5 diferentes genótipos multilocus entre isolados oriundos do Equador e Peru. Mas, na população do Peru se encontrou a mais alta diversidade genética total, indicando uma adaptação do patógeno às condições ambientais e do hospedeiro neste país. Contudo, nas populações de *M. roreri* do Equador se encontrou a maior diversidade alélica em relação às populações do Peru. Isto sugere que nestas populações poderiam estar atuando processos evolutivos tais como mutação, seleção ou barreiras genéticas (“genetic drifit”).

Análises com marcadores de Polimorfismo de comprimento de fragmentos amplificados (AFLP - Amlified Fragment Length Polymorphism) e sequências simples repetitivas internas (ISSR – Inter simple sequence repeat) mostraram que *Moniliophthora roreri* tem cinco grupos genéticos. Os grupos Co-East e Co-central aparentemente estão centrados no nordeste da Colômbia, o grupo Bolívar é o mais amplamente distribuído, encontra-se no nordeste da Colômbia, Venezuela e Peru, assim como o grupo Co-west distribuído no noroeste da Colômbia, Centro América e o Equador. No caso do grupo Gileri, encontra-se restrito ao norte do Equador. A distribuição e variabilidade dos grupos genéticos tem alguma relevância sobre a designação do centro de diversidade e origem das espécies. Neste estudo, quatro dos grupos genéticos se encontraram na Colômbia, sendo os mais variáveis os grupos Co-East e Co-Central, aparentemente endêmicos deste país. Portanto, estas observações apontaram coletivamente em direção à

Colômbia como centro de origem de *M. roreri* (PHILLIPS-MORA; AIME; WILKINSON, 2007).

Ao contrario com os cinco grupos genéticos previamente reportados, a análise com marcadores de polimorfismo de nucleotídeo simples (SNP - Single nucleotide polymorphism) encontrou que os isolados de *M. roreri* se agrupam em dois grandes grupos, o grupo denominado de "Grupo *b*" disperso em termos de variação genética e distância geográfica. Enquanto ao "Grupo *a*" está confinado à Colômbia, e tem uma diversidade genética significativa resultando num único grupo de isolados, mas com quatro subgrupos. O subgrupo I está centrado em torno do vale alto do rio Magdalena e atravessa os Andes Central através do lado norte e aí se move ao sul pelo vale do Cauca. De maneira semelhante, o subgrupo II e IV se movem do este do vale alto do rio Magdalena e atravessam os Andes do Oeste e se movem ao sul ao longo do litoral do Pacífico até atingir o Equador. No caso do subgrupo III atinge os Andes do Leste. Por outro lado, os isolados do grupo filogenético *b* se movem de norte ao oeste do vale alto do rio Magdalena e poucos isolados são capazes de atingir e de se mover ao sul ao longo do litoral (ALI *et al*, 2015).

Na publicação de Phillips-Mora, Aime e Wilkinson (2007), doze isolados de *M. roreri* se agruparam em dois grupos genéticos restritos à região central da Colômbia e a Santander. No entanto, Ali *et al* (2015) amostraram uma maior diversidade do patógeno em termos de distribuição no interior e dispersão desde as regiões de Santander. Neste estudo também se verificou que o *M. roreri* mantém um estado clonal, e os autores sugeriram uma dispersão de clones do patógeno desde o Vale Alto do rio Magdalena ao longo do tempo e subsequente deriva genética localizada e associada com estes isolados em eventos únicos. Caso que a reprodução do patógeno seja restrito à assexual ou clonal, o risco de dispersão de novos patotipos às populações vizinhas seria baixa neste cenário, e o incremento na diversidade genética se limitariam às mutações acumuladas com um número limitado de origens genéticas (linhagens clonais), com fluxo de genes em distâncias mais longas mediadas pelo homem (TIVOLI; BANNIZA, 2007).

Contudo, pode-se dizer que é necessário conhecer mais a estrutura das populações de *M. roreri*. As populações além de sua diversidade específica, também se caracterizam por sua estrutura genética, na sua vez influenciados pela deriva genética, a pressão de seleção ou pelos processos de migração. A estrutura genética das populações se refere à quantidade e distribuição da variabilidade genética dentro e entre populações. A definição da estrutura genética é a primeira etapa lógica nos estudos de genética de

populações de um fungo, já que esta é o reflexo se sua história e potencial evolutivo (MACDONALD, 1997).

4.2.7. Ciclo de vida

O clima e a quantidade de esporos livres no ambiente são fatores determinantes no ciclo de vida de *M. roreri*. O ciclo se inicia com a estação seca, nesta época se encontram o maior número de esporos dispersos no ambiente. Contudo, para que se inicie a infecção o fungo precisa de condições de alta umidade. Na Colômbia, as condições favoráveis ao processo de infeção e desenvolvimento da moniliase se apresentam o ano todo devido à distribuição das precipitações. Porém, precisa-se considerar o intervalo entre os períodos de produção (JAIMES; ARANZAZU, 2010). No Equador, o fator crítico no ciclo da doença está marcado pela época seca, daí sua importância para determinar a sobrevivência de *M. roreri* entre períodos produtivos e a disponibilidade de fonte de inoculo ao início da estação úmida (EVANS, 1981).

Os esporos germinam somente na presença de um filme de água, obtendo a maior porcentagem de germinação aos 24 °C. Em condições de laboratório, os esporos têm uma viabilidade e capacidades infecciosa de até 22 meses (MERCHÁN, 1981). A germinação dos esporos ocorre em aproximadamente 6 até 8 h depois da deposição. A hifa infectiva do fungo penetra a epidermes do fruto e coloniza inter e intracelularmente o tecido vegetal da subepidermes e exocarpo. A colonização do tecido atinge até as sementes, nesse momento se inicia a necrose do tecido doente. Dos 25 até 35 dias da infecção, externamente aparecem os primeiros sintomas como pontos oleosos circulares de tamanho muito pequeno ou deformações do tecido. Estes se tornam lesões necróticas irregulares de cor amarelo até marrom. Isto acontece entre 50 até 70 dias depois do início da infecção. Os sinais da doença aparecem aos 3 ou 4 dias com o desenvolvimento de micélio branco sobre as lesões. Posteriormente, aparecem os esporos conferindo uma cor creme a marrom sobre o tecido doente (JAIMES; ARANZAZU, 2010).

4.2.8. Epidemiologia da moniliase

A esporulação de *M. roreri* sobre o tecido do fruto é densa e inúmeros esporos são liberados e transportados pelo vento, chuva e em menor proporção pelos insetos (EVANS, 1986). Estima-se que a densidade de esporos produzidos pelo fungo sobre o fruto aproximasse a 44 milhões de esporos·cm^{-2} de tecido. Um fruto esporulado

localizado numa altura de 2 m aproximadamente tem um gradiente de dispersão de até 20 m, com um 40% de capacidade de infecção (MERCHAN, 1981). Existe uma correlação positiva entre o número de esporos dispersos no ar e a temperatura, e uma correlação negativa com respeito à umidade relativa (PORRAS, 1983).

O nível máximo de esporulação se observa durante poucas semanas depois de seu início, reduzindo-se a quantidade de esporos produzidos depois da décima semana quando se torna quase insignificante. Os esporos podem ser disseminados de frutos mumificados, mesmo despois de 1 ano da infecção, o que garante a disponibilidade de inóculo durante esse período (EVANS, 1981). Até o 90% dos esporos podem germinar sobre meios de cultura artificiais, más só o 10% conseguem germinar em agua (RAM; VALLE; ARÉVALO, 2004).

Os frutos mumificados e esporulados no dossel da árvore são considerados a principal fonte de inóculo para iniciar a epidemia, disseminando-se os esporos de maneira descendente. A presença de água livre além de permitir a germinação dos esporos, transporta o inóculo em direção os frutos localizados na parte baixa da árvore (JAIMES; ARANZAZU, 2010). A eliminação e deposição de frutos com sintoma de mancha no solo além de permitir e acelerar a decomposição deste tecido vegetal permite diminuir a disseminação do patógeno (CUBILLOS, 1981; ARANZAZU, 1987).

Existe uma estreita correlação entre a quantidade de chuva, o período de floração e a formação dos frutos com a ocorrência da moniliase (LOPES; MARTINS, 2005). Na Colômbia, os frutos são infectados nos períodos entre novembro e janeiro e entre março e julho. A esporulação sobre os frutos se apresenta nos períodos entre março e abril e entre setembro e outubro coincidindo com as safras. Mas, durante o ano todo se encontram frutos doentes, acreditando-se que ocorrem várias infecções secundárias durante os períodos chuvosos (JAIMES; ARANZAZU, 2010). Ainda não se tem esclarecido a distância que alcançam os esporos desde uma fonte de inóculo, tem-se sugerido distancias de até 1 km (EVANS, 1981; LOPES; MARTINS, 2005), enquanto que autores mais conservadores sugerem distâncias entre 30 e 375 m (LOPES; MARTINS, 2005).

Na Colômbia, o ciclo da doença pode se iniciar em qualquer momento, devido a fonte de inoculo primário estar presente constantemente pela distribuição de chuvas ao longo do ano, e a correlação positiva entre a incidência da moniliase e as chuvas que ocorreram há dois meses (MERCHAN, 1981). Contudo, os períodos críticos se

apresentam entre novembro e janeiro e entre março e julho, quando se inicia a floração e os frutos se encontram na idade de maior suscetibilidade (JAIMES; ARANZAZU, 2010).

Em Tabasco (México), se avaliou o progresso temporal da moniliase entre o ano 2008 e 2009. Neste período se registraram três epidemias coincidindo com igual número de formação de frutos. As epidemias coincidiram como os períodos de produção na região conhecidos como: "Venturero Loco" entre o 15 de junho e 15 de setembro, "Alegrón-invernada" entre o 16 de setembro e 15 de fevereiro; e "Cosecha" entre fevereiro e junho. A marcada separação das epidemias sugere a possibilidade de manejar independentemente cada epidemia com ações integradas que reduzam a incidência da moniliase no estado reprodutivo do cacaueiro de alta importância econômica. A moniliase se apresentou durante os 11 meses consecutivos, indicando a presença de condições favoráveis para as continuas infeções nesse ano de produção em Tabasco (TORRES DE LA CRUZ *et al*., 2011).

Caso contraditório se encontrou no estudo de Leandro (2011), neste o crescimento aparente da curva de progresso da moniliase e sua respectiva linearização se ajustou a monociclos independentes, apresentando como fundamentos o fato que os frutos de cacau são mais susceptíveis nos primeiros meses de idade e o período é relativamente prolongado em comparação com outros patógenos tal como *Phytophthora* sp. Também, se associou uma correlação positiva entre o número de frutos formados e a incidência da moniliase, assim como a relação da incidência da doença com a fenologia do cacaueiro (LEANDRO, 2011).

As epidemias de moniliase em Tabasco se ajustaram adequadamente ao modelo descrito por Weibull. A incidência da doença se correlacionou positivamente com temperatura baixas entre 20 até 26,9 °C e umidade relativa acima de 90%, condições apresentadas 49 dias antes da aparição dos sintomas. Desrosiers e Suárez (1974) encontraram que aparição de sintomas se encontra relacionada com a idade dos frutos, com aparição de sintomas aos 40 dias quando os frutos são infectados aos 20, 30, 40 e 60 dias de idade. Em frutos maiores aos 80 dias de idade os sintomas aparecem em 60 dias (TORRES DE LA CRUZ *et al*., 2011).

Na Costa Rica, monitorou-se a intensidade da moniliase em 36 sistemas agroflorestais com cacaueiro, a fim de determinar o efeito da composição vegetal e estrutura espacial na intensidade da doença. Neste estudo se encontrou que: 1) a composição do hospedeiro é uma fonte de dissolução da intensidade da doença; 2) a densidade das

árvores de cacaueiro afeta a intensidade pelo autosombreamento que se gera; e 3) a distribuição homogênea e não agregada das árvores florestais de sombreamento ou com distribuição ao azar reduzem a intensidade da doença quando comparados com parcelas com baixa densidade de arvores florestais (GIDOIN *et al*., 2014).

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CAPÍTULO I

Geographic Differentiation and Genetic Structure of *Moniliophthora roreri* in the Principal Cocoa Production Areas in Colombia [1]

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Geographic Differentiation and Genetic Structure of *Moniliophthora roreri* in the Principal Cocoa Production Areas in Colombia

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Abstract

Frosty pod rot disease (FPR) on cocoa, caused by *Moniliophthora roreri* is one of the most devastating cocoa disease in the Western Hemisphere. In Colombia, the disease is particularly severe in the Magdalena Valley, which is considered the possible center of origin for the pathogen species. We analyzed the genetic diversity of isolates from the states of Santander, Antioquia, Tolima and Huila in Colombia using twenty-three simple sequence repeats (SSR) markers. In total, 117 different multilocus genotypes were found among 120 isolates, each one representing a unique haplotype. The observed and standardized indexes of association (I_A and *řd*) indicate that the populations of *M. roreri* are clonal. Furthermore, given the high haplotype diversity with linkage disequilibrium we hypothesize that *M. roreri* could be a preferentially asexual species undergoing sporadic recombination or partial recombination through parasexuality. Three geographical groups were recognized among the isolates using Bayesian clustering methods. Similar results were obtained after discriminant analysis of principal components (DAPC), principal coordinate analysis (PCA) and a neighbor-joining tree from microsatellite loci based on Nei distance. The identified clusters were explained by geographical differentiation. Cacao clones and environmental variables did not contribute significantly to the genetic differentiation of the groups. We discuss how this information could be used to improve the management of FPR at the regional level.

Key words: Frosty pod rot, genetic diversity, microsatellite markers, population structure, *Moniliophthora roreri*

Introduction

Moniliophthora roreri H.C. Evans, Stalpers, Samson & Benny, causal agent of frosty pod rot (FPR), is a parasitic fungus of plants belonging to the Marasmiaceae family. For many years, *M. roreri* was considered the anamorph of an ascomycete. Taxonomic and molecular techniques clarified its position as a basidiomycete and demonstrated its close genetic relationship to the witches' broom pathogen *Moniliophthora perniciosa* (Bailey et al. 2013). The FPR disease occurs in the major cacao-producing areas in the Western hemisphere. *M. roreri*, a highly specialized fungus, attacks only fruits of the species of the close genera *Herrania* and *Theobroma*, causing internal and external pod damage that results in total pod loss. The presence of the disease in the cacao tree (*Theobroma cacao*) has had such devastating effects on yield that long-term economic viability of the crop has been compromised (Phillips-Mora et al. 2007).

At this moment, the feasible strategies for managing FPR are better agronomic practices combined with improved planting materials and biological control methods, under an integrated management approach (Krauss and Soberanis 2001; Ali et al. 2015). To date, there are not cacao clones immune to *M. roreri*, and the higher tolerant clones developed by CATIE (*Centro Agronómico Tropical de Investigación y Enseñanza*) in Costa Rica have slowly decreased in tolerance after 15 years. These observations raise questions about the possibility that the pathogen may be in the process of overcoming or breaking tolerance (Ali et al. 2015). A previous study has characterized the differential expression of genes during the successful infections when controlled infections on tolerant and susceptible clones infections are compared (Bailey et al. 2014). Some of these differentially expressed genes are involved in changes in

metabolism and the induction of fungal stress responses putatively designed to mitigate plant defense mechanisms (Bailey et al. 2014). In general, *M. roreri* is a highly adaptable fungus able to thrive under a wide range of environmental conditions. It can be found from sea level to over 1000 m above sea level, and microclimates that range from very dry to very humid (Evans 1981; Phillips-Mora and Wilkinson 2007). This level of adaptation to different environments, and the huge number of long-lived spores that are generated by each infection has made *M. roreri* a very effective pathogen and a formidable invader of new geographical regions (Phillips-Mora and Wilkinson 2007).

It is a general understanding that increased genetic variation in fungi populations would allow these pathogens to readily adapt to changing environmental conditions and breeder or natural selective pressures, such as hosts with different degrees of tolerance or resistance to the infections (McDonald 1997; Thrall et al. 2002). Therefore, a proper understanding of *M. roreri* genetic variation is fundamental to establish a breeding program based in an effective and sustainable resistance (Ali et al. 2015). One of the main limitations in the study of non-model organisms like *M. roreri* is the lack of species-specific markers such as simple sequence repeats (SSR) or SNPs, which are considered robust for detailed genotypic assessment of fungi and have become commonly used to assess genetic diversity (Dutech et al. 2007; Frenkel et al. 2012). These markers are highly reproducible and they allow genotyping directly with DNA extracted from diseased plant tissue. Of the two, the SSR markers may be a more economical and rapid method for genetic diversity studies (Jai et al. 2015), especially in countries with limited access to resources for the widespread development of high throughput SNP detection technologies.

The development of genetic resources for the study of *M. roreri* although limited, has provided the possibility to start examining some of these questions. Previous studies performed using amplified fragment length polymorphisms (AFLP) and inter simple sequence repeats (ISSR) analyses have shown that *M. roreri* has five genetic groups (Phillips-Mora et al. 2007). The groups Co-East, Co-Central and Bolivar apparently are centered in the Northeast of Colombia. Of these, the Bolivar group presents the widest distribution, which includes Venezuela and Peru. Another group, the Co-West also presents a wide geographic distribution, and can be found in the Northwest of Colombia, Central America and Ecuador. Finally, the group Gireli is restricted to Northern Ecuador (Phillips-Mora et al. 2007).

In contrast with the five groups reported by Phillips-Mora et al. (2007), single nucleotide polymorphisms (SNP) marker analysis showed that *M. roreri* isolates could be grouped into two major clusters (Ali et al. 2015). The 'Group b' has wider geographic range and higher genetic differentiation between locations and lower local genetic diversity. The 'Group a', which is confined to Colombia, has a significant diversity resulting in one group with four subgroups. The geographic distribution of subgroup 'I' is centered on the Upper Magdalena Valley, and crosses the Central Cordillera of the Andes through the north side and then it moves to the south by the Cauca Valley. Similarly, the geographic distribution of subgroups 'II' and 'IV' runs from the east of Upper Magdalena Valley to the west along to the Pacific Coast down to Ecuador. The subgroup 'III' is located towards the Eastern Cordillera of the Andes. It has been observed that isolates from 'Group b' have dispersed from the north of Upper Magdalena Valley to the west and few isolates were able to reach and move to the south along the coast (Ali et al. 2015).

In the Phillips-Mora et al. (2007) study, twelve *M. roreri* isolates restricted to Santander and the central region of Colombia were grouped in two genetic groups. On the other hand, Ali et al. (2015) showed a major diversity of the pathogen in terms of distribution within and dispersion from Santander regions to other areas. Based on these results, this region is considered as the possible center of origin for *M. roreri*. In addition, the clonal state of *M. roreri* was reported, suggesting a dispersion of the pathogen clones from the Upper Magdalena Valley through time and subsequent localized genetic drift associated with these unique isolates and events. This result was obtained in the absence of heterozygosity for SNP markers within Colombia suggesting that *M. roreri* isolates does not reproduce by sexual recombination very often (Ali et al. 2015).

The number of isolates employed in the studies aforementioned has been rather limited, making it difficult to unravel the geographic structure and pattern of dispersion of fungus in Colombia and the rest of the region. In order to obtain a clearer picture of the genetic and geographic pattern of differentiation within Colombia, more isolates, especially from Upper Magdalena Valley should be analyzed. Using the recently published sequenced genome of *M. roreri* (Meinhardt et al. 2014), we developed 23 specific SSR markers for the analysis of isolates of the Co-Central, Co-West and Co-West genetic groups reported by Phillips-Mora et al. (2007). The rationale for focusing our analysis on isolates belonging to these groups is based on the observations that the Co-East and Co-Central groups are the most variable and endemic in South America, suggesting that the center of genetic diversity and potentially geographic origin of the species is located in Colombia (Phillips-Mora et al. 2007). Our analysis of 120 isolates of *M. roreri* obtained from the four main cocoa producing states of Colombia allowed us to identify three main genetic groups using a Bayesian clustering algorithm. We discuss the implications

of these results in terms of the origin of the species and the potential for adaptation and management of the disease caused by this pathogen of enormous economic importance for the region.

Materials and Methods

Fungal material and DNA extraction. From 2013 to 2014, pods with FPR symptoms were collected from four main cocoa producing States of Colombian from susceptible trees (ICS 39, ICS 60 or ICS 1) and from tolerant trees (ICS 95) in farmer fields. Five farmer fields were selected in each locality using as criteria the origin of the available vegetal material and the distance from each other. In total, thirteen localities were sampled: Apartado, Turbo, and Maceo in Antioquia State, Campo Alegre and Rivera in Huila State, Ataco and Planadas in Tolima State and Cimitarra, El Carmen, San Vicente, Lebrija, El Playon and Rionegro in Santander State (Fig. 1). The samples were isolated from fruits showing initial or intermediary stages of external necrosis and grown in Sabouraud 4% dextrose Agar (Merck) supplemented with V8 Juice (20% w/v V8 Juice) at 27 °C ± 1 °C. The 120 strains of *M. roreri* obtained were purified in petri dishes on Sabouraud 4% dextrose Agar (Merck) at 27 °C (Table 1).

Morphological characterization of fungal isolates. One mycelium plug (5 mm diameter) taken out from colonies of twelve days-old was transferred to the center of 15 cm petri dish on Sabouraud 4% dextrose Agar (Merck) supplemented with V8 Juice (20% w/v V8 Juice). The petri dish containing the mycelium plug was sealed and incubated in a 12 h light-dark

photoperiod at 27 °C for 16 days. Each isolate was replicated four times. Mean diameter of colony was recorded each 24 h and these data were used to calculate the relative growth rate of colony. At the sixteenth day, certain characteristics of colony were registered (color using the Munsell soil color chart (1990), shape, margin, presence/absence of sectors, texture, rings position and spores/cm^2). Finally, length and width of 30 spores per isolate were registered using a Motic digital camera adapted to optical microscope CX41 (Olympus) at 40X. The data were obtained from processed images with the Motic Images Plus software version 2.0. All variables were classified by experimental unit and were submitted to principal component analysis (PCS) in the software InfoStat 2014 version.

Microsatellite loci detection and Primer design. We identified potential regions with SSR in the *M. roreri* genome available on GenBank (Meinhart et al. 2014) under accession number MCA2977, using GMATO (Wang et al. 2013) (default settings). To reduce the large number of potential microsatellite loci detected, sequences containing microsatellite with two nucleotide motifs were discarded as well as those located in regions encoding genes with conserved function. Thereby, 23 SSRs with three motifs were selected and the primers were designed with the primer-Blast tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). The major parameters of primer selection were as follow: length of PCR products to four different size classes (50-120, 120-200. 200-300, 300-400), length of each primer of 18-22 bp, melting temperature at 56-60 °C, and G plus C content in any primer of 45% to 55%. The primers were labeled with 6-FAM, VID, NED and ROX fluorophore. To establish high throughput SSR analysis in this study, we placed 23 SSR markers

arranged into three PCR multiplexed panels according to its fragment size and fluorescent dye label (Table 2).

Genetic diversity and population structure of *Moniliophthora roreri* strains using microsatellite data (SSR). Microsatellite amplification and genotyping. Genomic DNA was extracted from isolates of *M. roreri* using the ZR Fungal/Bacterial MiniPrep Kit (Zymo Research), according to manufacturer's instructions. The genomic DNA of *M. roreri* used in this study was extracted and purified from fungal mycelium incubated on Sabouraud 4% dextrose Agar (Merck) for 15 days. For PCR amplification, the reactions were performed in a total volume of 25 µl with ≅ 50 ng of genomic DNA, 200 µM of each dNTP, 1 µM of forward primer labeled either with 6-FAM, VID, NED or ROX fluorophore, 1 µM of non-labeled reverse primer, 1.5 mM $MgCl_2$, 1X Buffer Gold and AmpliTaq Gold polymerase (Applied Biosystems).

The PCR was carried out using an icycler thermal cycler (Bio-Rad) with the following conditions: an initial denaturation at 94 °C for 10 min; 37 cycles of denaturation at 92 °C for 90 s, annealing at 56 °C for 60 s with increase of 1 °C every cycle until reaching 60 °C, and elongation at 72 °C for 90 s; and ending with an extension at 72 °C for 52 min. Labeled amplification products were resolved onto an automated 310 ABI PRISM DNA genetic analyzer (Applied Biosystems), using Performance Optimized Polymer 4 (POP–4) as an internal size standard. The PCR fragments were detected with the GeneScan™ analysis software version 3.1 (Applied Biosystems). Multilocus genotypes (MLG) were determined using the set of twenty-three loci nuclear microsatellite developed for this study. Analysis was conducted for 120 isolates of *M. roreri* (Table 1).

Population genetic analysis and structure. For the population genetic analysis, a multilocus genotype (MLG) was assigned to each isolate with the *Poppr* package (Kawvar et al. 2014) implemented in R software v1.0.3. This R package introduces core functions for analysis, including options that consider mixed modes of reproduction (asexual and sexual), typically observed in microbial populations. Isolates with the same MLG were treated as clones using clones correction implemented in *Poppr* package in order to eliminate the redundancy in the collection that results from asexual reproduction of the isolates (Chen and McDonald 1996; Kumar et al. 1999). The population genetic structure was estimated for all isolates of *M. roreri* using four levels of stratification: I) Clone, II) Locality, III) State and IV) Altitude. For each stratum, frequencies within subpopulations at each locus were tested for departures from Hardy-Weinberg equilibrium (HWE) in *Poppr* package (Kamvar et al. 2014). This test was performed to explore random mating (Michalakis and Excoffier 1996). Genotypic diversity (Nei 1987), evenness and the Shannon-Wiener index of diversity and populations summary statistic were also determined using *Poppr* package in R software v1.0.3. Each statistic was calculated for each data subset with and without corrections for samples size (Chao and Shen 2003). Additionally, we compared the ratio of observed multilocus genotypic diversity to that expected under conditions of sexual reproductions, as described by Stoddart and Taylor (1988).

In order to determine the degree of clonality and the potential for recombination in the population we estimated linkage disequilibrium (LD) across al SSR markers using the standardized index of association (I_A), and also with a measure corrected for the number of loci ($\check{r}d$) (Agapow and Burt 2001). A lack of association between any pair of loci may be interpreted

as being due to recombination, whereas linkage or homoplasy might cause deviations from expected genotypic frequencies under random mating in some pairs of loci (Agapow and Burt 2001). For this analysis, we used 1000 random permutations and missing data were not considered.

We tested for admixture under a model without supervision using Bayesian model based clustering software *Structure* v2.3 (Pritchard et al. 2000). We explored clustering under two different models, one allowing for admixture (individuals may have mixed ancestry, which would be consistent with a scenario in which some degree of recombination has occurred historically) and a no-admixture model, allowing correlated allele frequencies (Falush et al. 2003). Searches with ancestral number of cluster ranging from K=1 to 15 were performed in independent runs with 100,000 generations and a burn in of 5,000. K was established taking in account, the mean estimated likelihood probability (Pritchard et al. 2000) and the second order rate of change in the log probability (ΔK) (Evanno et al. 2005), implemented in Structure Harvester v.0.6.93 (Earl vonHoldt 2012).

In addition to the structure analysis, we performed a discriminant analysis of principal components (DAPC). This technique extracts information from genetic datasets (multivariate in nature) by first performing a principal component analysis (PCA) on pre-defined groups or populations, and then using the PCA factors as variables for a discriminant analysis (DA), which seeks to maximize the intergroup component of variation (Jombart et al. 2010). DAPC was implemented in the *adegenet* package for R (http://adegenet.r-forge.r-project.org/) (Jombart 2008). Level of clustering was assessed with the function *find.clusters* ran over 150 components. We used Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) to choose

the best model, and therefore the number and nature of clusters. Additionally, we also computed a principal coordinate analysis (PCA) using the *adegenet* R (http://adegenet.r-forge.r-project.org/) (Jombart 2008), plotting individuals in the most significant axes space according to their genetic similarities. Genetic divergence among isolates was quantified by calculating the neighbor-joining trees from microsatellite loci with 1000 bootstrap iterations based on Nei distance. We explored the population structure with a diverse set of relatively orthogonal methods in order to ensure robustness of our results.

Results

Morphological characteristics. Growth on agar Sabouraud 4% dextrose Agar (Merk) supplemented with V8 juice (20% w/v V8 Juice) was slow: colonies attained a diameter from 11 to 90 mm after 16 days. A feathery margin was a predominant characteristic in all isolates. Most isolates presented rings in the colony, but their distribution varied among isolates. Colony colors alternated between whites (7.5 YR 8/1 and 10YR 8/1), light to dark browns (7.5YR 6/4, 10YR 7/3, 10 YR 5/3, 10 YR 4/3, 7.5YR 5/2, 10 YR 4/3 and 10YR 3/3), light grays (10YR 7/2 and 10YR 7/1) and light to dark brownish grays (10YR 6/2 and 10YR 4/2). Few colonies showed weak or pale red colors (10R 4/3 and 10 R 6/2), presence of sector and absence of rings in the colony. Spores of all isolates were typically globoses to subgloboses in shape with 6.64-9 X 8.64-13.28 μm of diameters.

The first and second components of the PCA analysis performed with morphological data account for 19.6% (PCI) and 13.4% (PCII) of the total variation, respectively. Higher morphological variability was observed within than between isolates analyzed. Morphological

characterization, based on the variables measured in this study did not allow for a clear discrimination of geographic origin in *M. roreri* (Fig. 2), suggesting that underlying causes for phenotypic variation did not link to the genetic differentiation between localities (see below).

Population genetic diversity of *Moniliophthora roreri* isolates using microsatellite data (SSR). Genotyping and population statistics using microsatellite data. From the *M. roreri* genome published in GenBank, we identified 714 tri-nucleotides, 16 SSR penta-nucleotides and 5 SSR hepta-nucleotides. After a screening selection, the SSR located in conserved regions and those with less than five motifs repeats were discarded. Finally, twenty-three SSR tri-nucleotides with five or more motifs repeats were selected for marker development (Table 2). These specie-specific microsatellite loci were used to genotype 120 *M. roreri* strains. All of the loci were polymorphic in all populations except for Moni 11 (monomorphic for all isolates). An average of 4.87 alleles/locus were detected over 23 loci ranging from 1 (Moni 11) to 13 (Moni 19) alleles per locus. Both Moni 19 and Moni 21 were the most informative loci with 13 and 7 alleles, respectively (Table 2). The locus Moni 19 had the highest Simpson diversity (0.873). Moni 15 had the highest expected heterozygosity (0.9) and Moni 23 had the most evenly distributed alleles (0.956). We excluded those samples in the population with a proportion of missing data > 0.05. After Bonferroni correction for multiple comparison, almost all loci (21/23) showed an excess of homozygotes and significant deviation from HWE ($p > 0.05$), suggesting the existence in inbreeding and/or population subdivision (i.e., Wahlund effect).

In total, 117 MLG were detected for all populations (Table 3). The isolates showed high genetic diversity, with most MLGs being represented by a single isolate. The total of expected heterozygosity (Hexp) across all populations was 0.488 (Table 3). Considering a state stratum, the isolates from Santander had the highest expected heterozygosity (0.457; Table 3), whilst the Huila State had the lowest (0.277; Table 3).

For the uncorrected and corrected populations, the hypothesis of no linkage among markers was rejected ($P < 0.001$) for almost all populations (Fig. 3), indicating a clonal population. The *řd* of uncorrected or corrected populations had a little variation from 0.067 to 0.0672, respectively. Multilocus LD (I_A and *řd*) was significantly greater than zero within each population ($P < 0.001$). The isolates from Antioquia State had the highest I_A (2.550; Table 3) and *řd* (0.136; Table 3), whilst the Huila State had the lowest I_A (0.923; Table 3) and *řd* (0.057; Table 3), at level of State stratum. Given the strong support for linkage in our samples, we interpret the differential heterozygosity between populations as indicative of significant differences in effective population sizes.

Population Structure Analysis. The Bayesian statistic-based algorithm implemented in STRUCTURE assigned the 120 strains into two (under non-admixture) or three (under admixture) distinct genetic groups according to Evanno et al. (2005) (Fig. 4). For both analyses, the distribution of genetic groups exhibited high correlation with geographic location where isolates were collected. When a model without admixture was fitted to the data, we found that Santander isolates present a majority of ancestry from Cluster 2 (64.29% total ancestry from Cluster 2 to all individuals in Santander) and a lower contribution of Cluster 1 to their ancestry

(35.72%); Antioquia, Tolima and Huila isolates present a major component of ancestry from Cluster 1 (69.97%, 93.76% and 99.9%, respectively) and a progressively smaller contribution of ancestry from Cluster 2 (30.03%, 6.24% and 0.1%, respectively) (Fig. 4B).

Assuming the admixture model for *M. roreri* populations (Fig. 4A), Santander isolates have seem to conform an heterogeneous collection with most individual having a majority of ancestry from Cluster 3 (61.91% total ancestry of Cluster 3 for all individual in Santander). The second most frequent ancestry in individuals from Santander originates from Cluster 1 (31.84% total ancestry across individuals from Santander) and a minor contribution of ancestry from Cluster 2 (6.24%). Antioquia isolates on the other hand have a large ancestry contribution from Cluster 1 (50.77%), and the second largest contribution originating from Cluster 3 (34.61%) and a lesser contribution from Cluster 2 (14.61%). Lastly, Tolima and Huila isolates have present a major component of ancestry from Cluster 2 (85.16% and 90.03%, respectively), and much lesser contribution od ancestry from Cluster 3 (12.51% and 5.05%, respectively) and Cluster 1 (2.31% and 4.93%, respectively) (Fig. 4A).

The first and second components of the PCA analysis performed with SSR data accounted for 15.46% (PCI) and 9.28% (PCII) of the total variation, respectively. Principal coordinate analysis separated the isolates into major groups according to geographic location where isolates were collected, which was consistent with the genetic groups assignments generated by STRUCTURE (Fig. 5). Similar results were obtained with DAPC (Fig. 6). The strong support for linkage among analyzed, combined with the consistent assignment generated under the STRUCTURE and PCA analysis, allows us to propose that the population structure of *M. roreri* in Colombia can be explained by three main groups.

Interestingly, neighbor-joining tree, based on Nei genetic distance measured from microsatellite data, separated all isolates according to previous defined groupings correspond to geographic location (Fig. 7).

Discussion

This is the first study to thoroughly study the *M. roreri* populations in Colombia with a special emphasis in the regions where the pathogen originated. We showed that individual isolates of *M.* roreri could not be grouped according to their geographic origin based on the analysis of morphological characteristics, given that there is higher variation within than between populations. Ali et al (2015) obtained similar results in a study in which no differences in spore shapes and sizes were found for isolates from two groups, one from Colombia and another one from Ecuador. Since spores are the most important part of the *M. roreri* life cycle, they have been widely studied and it has been suggested that spore composition and compatible solutes and other protective proteins inside the spores are influenced by the environmental conditions during their formation (Ali et al. 2015). These results would suggest that most of the variation in morphological characteristics measured on the spores might not be heritable.

The 120 isolates of *M. roreri* from four cocoa producing states of Colombia showed a high level of genetic diversity, since almost each isolate was associated to a single haplotype (117/120). Phillips-Mora et al. (2007) reported similar results with a high level of polymorphism estimated with AFLP and ISSR profiles of isolates, under a model that assumes an asexual mode of reproduction of *M. roreri*, as it had been suggested for the species (Evans et al. 1981). Consistently with these results, Ali et al. (2015) reported limited genetic variation among *M.*

roreri isolates from five of the genetic groups previously reported, indicating that the heterozygosity observed was due to sequencing errors. The majority of the heterozygous SNP markers used in Ali et al. (2015) were associated with misalignments between paralogous sequences instead of unique genes (Ali et al. 2015).

We found a high genetic diversity at the population and isolate level in *M. roreri* indicating a relatively high level of genetic exchange within and among the different geographic locations. That said isolates collected from the same locality and state tend to be genetically more similar to each other and often clustered together. The high levels of heterozigosity found in this study are consistent with the high evolutionary rate of change of microsatellite markers (Hamblin et al. 2007). Additionally, the estimated I_A and *řd* values suggest that *M. roreri* populations are clonal populations. If a strict clonal reproduction in *M. roreri* is considered, high levels of gene flow together with a large effective population size would be required to account for such genotype diversity. Ali et al. (2015) suggested dispersal of *M. roreri* clones from the upper Magdalena Valley over time and the subsequent localized genetic drift associated with these unique isolates and events. However, given the high haplotype diversity with linkage disequilibrium, this study suggest that *M. roreri* could be an asexual species possibly undergoing sporadic or partial recombination due to parasexuality. Although parasexual genetic exchange may be limited in nature due to the prevalence of somatic incompatibility mechanisms in fungi (Anderson and Kohn 1995). Considering that fungal populations lacking the sexual cycle are still capable of rapid evolution, it could show through changes in virulence (Michelmore and Hulbert 1987). This could explain the highly virulent state of *M. roreri*, and the limited nature of resistance or tolerance against this fungus in the cacao germplasm and the observed increased in disease losses in some tolerant clones in Costa Rica (Ali et al. 2015).

In our study, the 120 isolates of *M. roreri* were collected primarily from different *T. cacao* clones with different genetic backgrounds but located in the same fields. In this study, the isolates from different clone showed genetic similarity. Nevertheless, the geographic location played a more important role in the shaping population structure. . Collecting a single isolate versus many isolates from individuals cacao clone represents an advantage for our analysis, because multiple isolates may increase the similarity of isolates in individual geographical population and overshadow environmental effects. Same effect was observed on the population structure of *Villosiclava virens* isolates, causal agent of rice false smut, in China. Higher genetic similarity from individual fields but different rice cultivar suggested that the geographic factor played a more important role in the selection of *V. virens* isolates than rice cultivar (Wang et al. 2014). The plots generated based on SSR were generally in agreement (Figs. 4-7). They showed that most isolates from the same cacao clone but from different state did not cluster together, while isolates from different clones of the same locality and state tended to cluster together.

A genetic analysis of the population structure of *M. roreri*, considering that one consequence of gene flow is population admixture, showed three major groups (Fig. 4A). Santander isolates presented large diversity followed by Antioquia isolates with a large ancestry contribution of ancestry from Cluster 3 and Cluster 1. Tolima and Huila isolates presented minor diversity and large ancestry contribution Cluster 2 with lesser genetic flow from Santander and Antioquia populations. Considering the geographical barriers, in our study the sampling sites were separated by mountain and Magdalena river. These may have caused patchy distribution of populations and limited gene flow among some of them. The major genetic flow was evidenced between Santander and Antioquia populations. DAPC showed genetic flow between Santander and Antioquia populations, and between Tolima, Huila and Antioquia populations

(Figs. 4 and 6D). The major Nei genetic distance was between Santander and Tolima and Huila populations (Fig. 7), with low levels of gene flow (Fig. 6D). These results are in agreement with Phillips-Mora et al. (2007), who assigned the group names according to origin region as Co-Central, Co-East and Co-West. However, the mediation by human activity on the early dispersal processes of *M. roreri* had influenced its dispersion over long distances involved and the huge physical barriers separating some of the relevant areas of cacao in Colombia (Phillips-Mora et al. 2007). It could explain the presence of Cluster 3 in Apartado and Turbo (Antioquia) (Fig. 4A), despite the large distances and physical barriers between these areas and Santander. On the other hand, Santander isolates had the larger genetic variability. This variability has been reported by Phillips-Mora et al. (2007) and Ali et al. (2015), so this region could be considered the probable center of origin of the species. Therefore, the Upper Magdalena Valley is considered the most important area in terms of FPR research (Ali et al. 2015).

Considering that, the results in this study showed that geographical environments influence genetic variation of *M. roreri* more than cacao clones. The information collected and analyzed here could be used to improve the management strategies for FPR by adjusting measures such as fertilizer, water, and planting density in the cacao fields to change the environments. Control measures such as fungicide applications or development of major resistance genes should be implemented with caution and measure to minimize gene flow, such as reducing the spread of *M. roreri* genotypes to new areas. Furthermore, disease control strategies should be designed at the regional scale in order to prevent the spread of this fungus from one locality to another.

The role of human activities in disseminating this disease across Latin America and its effect on pathogen population structure support the importance of quarantine politics in order to confine FPR disease to the Latin America area, as it recommended for the South American Leaf Blight (SALB) disease caused on rubber trees (*Hevea brasiliensis*) by *Microcyclus ulei* (Barrès et al. 2012). In areas that are still free of the disease, as Brazil, the avoidance is the best strategy to be followed (Phillips-Mora and Wilkinson 2007). Notwithstanding, it is important to consider that the slow natural spread of FPR to the Brazilian Amazon could be affected by increased human activity in the area (Evans 1986; Phillips-Mora and Wilkinson 2007). On the other hand, as reported by Phillips-Mora et al. (2007) and Ali et al. (2015), the Middle Magdalena Valley of Colombia are considered the most important area in terms of frosty pod rot research. The presence of individuals from different cluster in this area would assure breeding and screening programs of new cacao germplasm based in *M. roreri* resistance (Ali et al. 2015), as well as it could contain the more effective antagonist agents for biocontrol (Phillips-Mora et al. 2007).

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Table 1. Description and geographic origin of *M. roreri* isolates used in this study

Name	Collection date	State	Locality	Clone of *T. cacao*	Longitude	Latitude
Mon73	18/12/2014	Antioquia	Apartado	ICS1	76° 40.500' W	07° 55.417' N
Mon126	18/12/2014	Antioquia	Apartado	ICS1	76° 36.083' W	07° 55.250' N
Mon133	18/12/2014	Antioquia	Apartado	ICS1	76° 36.083' W	07° 55.250' N
Mon135	18/12/2014	Antioquia	Apartado	ICS1	76° 36.083' W	07° 55.250' N
Mon40	08/05/2014	Antioquia	Turbo	ICS60	76° 35.927' W	07° 56.014' N
Mon13	28/03/2014	Antioquia	Maceo	ICS39	74° 37.000' W	06° 33.000' N
Mon15	28/03/2014	Antioquia	Maceo	ICS39	74° 37.000' W	06° 33.000' N
Mon16	28/03/2014	Antioquia	Maceo	ICS95	74° 37.000' W	06° 33.000' N
Mon17	28/03/2014	Antioquia	Maceo	ICS95	74° 37.000' W	06° 33.000' N
Mon19	06/05/2014	Antioquia	Maceo	ICS39	74° 50.725' W	06° 29.001' N
Mon28	28/03/2014	Antioquia	Maceo	ICS39	74° 37.000' W	06° 33.000' N
Mon29	25/03/2014	Antioquia	Maceo	ICS39	74° 37.000' W	06° 33.000' N
Mon30	25/03/2014	Antioquia	Maceo	ICS95	74° 37.000' W	06° 33.000' N
Mon33	06/05/2014	Antioquia	Maceo	ICS39	74° 50.725' W	06° 29.001' N
Mon36	06/05/2014	Antioquia	Maceo	ICS39	74° 50.725' W	06° 29.001' N
Mon38	06/05/2014	Antioquia	Maceo	ICS95	74° 50.725' W	06° 29.001' N
Mon48	25/03/2014	Antioquia	Maceo	ICS95	74° 37.000' W	06° 33.000' N
Mon49	25/03/2014	Antioquia	Maceo	ICS39	74° 37.000' W	06° 33.000' N
Mon54	28/03/2014	Antioquia	Maceo	ICS39	74° 37.000' W	06° 33.000' N

Mon56	06/05/2014	Antioquia	Maceo	ICS95	74° 50.725’ W	06° 29.001’ N
Mon59	06/05/2014	Antioquia	Maceo	ICS95	74° 50.725’ W	06° 29.001’ N
Mon66	06/05/2014	Antioquia	Maceo	ICS95	74° 50.329’ W	06° 34.659’ N
Mon69	25/03/2014	Antioquia	Maceo	ICS95	74° 37.000’ W	06° 33.000’ N
Mon70	06/05/2014	Antioquia	Maceo	ICS39	74° 50.725’ W	06° 29.001’ N
Mon93	28/03/2014	Antioquia	Maceo	ICS39	74° 37.000’ W	06° 33.000’ N
Mon94	25/03/2014	Antioquia	Maceo	ICS95	74° 37.000’ W	06° 33.000’ N
Mon108	28/03/2014	Antioquia	Maceo	ICS39	74° 37.000’ W	06° 33.000’ N
Mon115	06/05/2014	Antioquia	Maceo	ICS39	74° 50.725’ W	06° 29.001’ N
Mon121	28/03/2014	Antioquia	Maceo	ICS39	74° 37.000’ W	06° 33.000’ N
Mon129	06/05/2014	Antioquia	Maceo	ICS95	74° 50.329’ W	06° 34.659’ N
Mon5	16/09/2013	Huila	Campo Alegre	ICS95	75° 17.548’ W	02° 41.292’ N
Mon8	16/09/2013	Huila	Campo Alegre	ICS95	75° 17.548’ W	02° 41.292’ N
Mon18	16/09/2013	Huila	Campo Alegre	ICS1	75° 17.840’ W	02° 41.395’ N
Mon50	16/09/2013	Huila	Campo Alegre	ICS39	75° 17.548’ W	02° 41.292’ N
Mon51	16/09/2013	Huila	Campo Alegre	ICS1	75° 17.840’ W	02° 41.395’ N
Mon75	18/09/2014	Huila	Campo Alegre	ICS95	75° 17.840’ W	02° 41.395’ N
Mon78	18/09/2014	Huila	Campo Alegre	ICS95	75° 17.840’ W	02° 41.395’ N
Mon97	16/09/2013	Huila	Campo Alegre	ICS1	75° 17.840’ W	02° 41.395’ N
Mon100	18/09/2014	Huila	Campo Alegre	ICS95	75° 17.840’ W	02° 41.395’ N
Mon106	18/09/2014	Huila	Campo Alegre	ICS95	75° 17.840’ W	02° 41.395’ N
Mon128	16/09/2013	Huila	Campo Alegre	ICS1	75° 17.840’ W	02° 41.395’ N
Mon130	18/09/2014	Huila	Campo Alegre	ICS95	75° 17.840’ W	02° 41.395’ N

Mon103	15/09/2014	Huila	Rivera	ICS95	75° 14 14.3’ W	02° 45 37.2’ N
Mon107	15/09/2014	Huila	Rivera	ICS95	75° 14 14.3’ W	02° 45 37.2’ N
Mon110	15/09/2014	Huila	Rivera	ICS39	75° 14.450’ W	02° 45.620’ N
Mon120	15/09/2014	Huila	Rivera	ICS95	75° 14 14.3’ W	02° 45 37.2’ N
Mon123	15/09/2014	Huila	Rivera	ICS95	75° 14 14.3’ W	02° 45 37.2’ N
Mon74	15/07/2014	Santander	Cimitarra	ICS60	63° 59.542’ W	06° 11.973’ N
Mon95	14/07/2014	Santander	Cimitarra	ICS60	73° 54.531’ W	06° 14.848’ N
Mon109	15/07/2014	Santander	Cimitarra	ICS39	73° 51.362’ W	06° 12.958’ N
Mon12	11/06/2014	Santander	El Carmen	ICS95	73° 30.495’ W	06° 42.183’ N
Mon14	10/06/2014	Santander	El Carmen	ICS95	73° 30.212’ W	06° 43.508’ N
Mon21	09/06/2014	Santander	El Carmen	ICS39	73° 27.627’ W	06° 29.001’ N
Mon25	11/06/2014	Santander	El Carmen	ICS39	73° 30.495’ W	06° 42.183’ N
Mon26	09/06/2014	Santander	El Carmen	ICS39	73° 34.068’ W	06° 44.447’ N
Mon31	09/06/2014	Santander	El Carmen	ICS39	73° 30.495’ W	06° 42.183’ N
Mon32	09/06/2104	Santander	El Carmen	ICS39	73° 27.627’ W	06° 29.001’ N
Mon47	10/06/2014	Santander	El Carmen	ICS95	73° 30.212’ W	06° 43.508’ N
Mon61	10/06/2104	Santander	El Carmen	ICS39	73° 27.627’ W	06° 29.001’ N
Mon62	09/06/2014	Santander	El Carmen	ICS95	73° 31.840’ W	06° 42.677’ N
Mon68	10/06/2104	Santander	El Carmen	ICS39	73° 27.627’ W	06° 29.001’ N
Mon72	09/06/2014	Santander	El Carmen	ICS39	73° 30 29.7’ W	06° 42 11.0’ N
Mon77	09/06/2014	Santander	El Carmen	ICS95	73° 31.840’ W	06° 42.677’ N
Mon112	09/06/2014	Santander	El Carmen	ICS39	73° 31.840’ W	06° 42.677’ N
Mon132	09/06/2014	Santander	El Carmen	ICS95	73° 31.840’ W	06° 42.677’ N

Mon22	24/06/2014	Santander	El Playon	ICS60	73° 14.112’ W	07° 29.583’ N
Mon65	23/06/2014	Santander	El Playón	ICS95	73° 13.668’ W	07° 33.712’ N
Mon79	23/06/2014	Santander	El Playón	ICS95	73° 13.668’ W	07° 33.712’ N
Mon80	23/06/2014	Santander	El Playón	ICS95	73° 13.668’ W	07° 33.712’ N
Mon6	10/07/2014	Santander	Lebrija	ICS60	73° 16.212’ W	07° 06.793’ N
Mon7	10/07/2014	Santander	Lebrija	ICS95	73° 16.212’ W	07° 06.793’ N
Mon9	17/11/2013	Santander	Lebrija	ICS95	73° 16.212’ W	07° 06.793’ N
Mon10	17/11/2013	Santander	Lebrija	ICS60	73° 16.438’ W	07° 07.703’ N
Mon11	17/11/2013	Santander	Lebrija	ICS60	73° 10.872’ W	07° 22.400’ N
Mon23	09/07/2014	Santander	Lebrija	ICS95	73° 12.658’ W	07° 08.287’ N
Mon24	17/11/2013	Santander	Lebrija	ICS60	73° 14.000’ W	07° 08.000’ N
Mon27	09/07/2014	Santander	Lebrija	ICS95	73° 16.438’ W	07° 07.703’ N
Mon34	17/11/2013	Santander	Lebrija	ICS95	73° 16.438’ W	07° 07.703’ N
Mon37	17/11/2013	Santander	Lebrija	ICS60	73° 16.438’ W	07° 07.703’ N
Mon41	17/11/2013	Santander	Lebrija	ICS60	73° 12.658’ W	07° 08.287’ N
Mon42	17/11/2013	Santander	Lebrija	ICS60	73° 13.895’ W	07° 07.508’ N
Mon44	17/11/2013	Santander	Lebrija	ICS60	73° 16.438’ W	07° 07.703’ N
Mon46	17/11/2013	Santander	Lebrija	ICS95	73° 10.872’ W	07° 22.400’ N
Mon60	09/07/2014	Santander	Lebrija	ICS95	73° 16.438’ W	07° 07.703’ N
Mon64	10/07/2014	Santander	Lebrija	ICS60	73° 16.212’ W	07° 06.793’ N
Mon67	10/07/2014	Santander	Lebrija	ICS95	73° 13.895’ W	07° 07.508’ N
Mon76	17/11/2013	Santander	Lebrija	ICS60	73° 16.438’ W	07° 07.703’ N
Mon98	08/07/2014	Santander	Lebrija	ICS95	73° 16.438’ W	07° 07.703’ N

Mon102	10/07/2014	Santander	Lebrija	ICS95	73° 14.000’ W	07° 08.000’ N
Mon113	10/07/2014	Santander	Lebrija	ICS95	73° 13.895’ W	07° 07.508’ N
Mon117	10/07/2014	Santander	Lebrija	ICS95	73° 13.895’ W	07° 07.508’ N
Mon1	29/10/2013	Santander	Rionegro	ICS95	73° 10.000’ W	07° 22.000’ N
Mon39	17/02/2014	Santander	Rionegro	ICS95	73° 10.780’ W	07° 22.447’ N
Mon43	17/02/2014	Santander	Rionegro	ICS39	73° 10.780’ W	07° 22.447’ N
Mon45	17/02/2014	Santander	Rionegro	ICS95	73° 10.780’ W	07° 22.447’ N
Mon52	17/02/2014	Santander	Rionegro	ICS39	73° 10.780’ W	07° 22.447’ N
Mon53	17/02/2014	Santander	Rionegro	ICS39	73° 10.780’ W	07° 22.447’ N
Mon55	17/02/2014	Santander	Rionegro	ICS39	73° 10.780’ W	07° 22.447’ N
Mon104	17/02/2014	Santander	Rionegro	ICS39	73° 10.780’ W	07° 22.447’ N
Mon105	17/02/2014	Santander	Rionegro	ICS95	73° 10.780’ W	07° 22.447’ N
Mon114	17/02/2014	Santander	Rionegro	ICS39	73° 10.780’ W	07° 22.447’ N
Mon2	19/05/2014	Santander	San Vicente	ICS39	75° 22.950’ W	06° 55.783’ N
Mon20	19/05/2014	Santander	San Vicente	ICS95	73° 24.555’ W	06° 51.753’ N
Mon35	19/05/2014	Santander	San Vicente	ICS39	73° 24.555’ W	06° 51.753’ N
Mon63	21/05/2014	Santander	San Vicente	ICS95	73° 27.187’ W	06° 49.558’ N
Mon124	21/05/2014	Santander	San Vicente	ICS60	73° 27.187’ W	06° 49.558’ N
Mon3	14/10/2014	Tolima	Ataco	ICS95	75° 37.653’ W	03° 19.658’ N
Mon57	19/11/2013	Tolima	Ataco	ICS95	75° 37.653’ W	03° 19.658’ N
Mon96	19/11/2013	Tolima	Ataco	ICS95	75° 37.653’ W	03° 19.658’ N
Mon101	19/11/2013	Tolima	Ataco	ICS95	75° 37.653’ W	03° 19.658’ N
Mon116	19/11/2013	Tolima	Ataco	ICS95	75° 37.653’ W	03° 19.658’ N

Mon118	19/11/2013	Tolima	Ataco	ICS95	75° 37.653’ W	03° 19.658’ N
Mon125	19/11/2013	Tolima	Ataco	ICS95	75° 37.653’ W	03° 19.658’ N
Mon99	19/11/2014	Tolima	Planadas	ICS1	75° 38.000’ W	03° 16.710’ N
Mon111	19/11/2014	Tolima	Planadas	ICS1	75° 38.000’ W	03° 16.710’ N
Mon119	19/11/2014	Tolima	Planadas	ICS1	75° 38.000’ W	03° 16.710’ N
Mon122	19/11/2014	Tolima	Planadas	ICS1	75° 38.000’ W	03° 16.710’ N
Mon127	19/11/2014	Tolima	Planadas	ICS1	75° 38.000’ W	03° 16.710’ N
Mon131	19/11/2014	Tolima	Planadas	ICS1	75° 38.000’ W	03° 16.710’ N
Mon134	19/11/2014	Tolima	Planadas	ICS1	75° 38.000’ W	03° 16.710’ N

Table 2. Characterization of 23 polymorphic simple sequence repeat primer pairs and distribution of allele frequency used to assess genetic variation in *M. roreri*

Locus	**Multiplex panel**	**Target motif unit**	**Primer sequence (5’ – 3’)**	**Tm (°C)**	**Size (bp)**	**Allele**	**1-D[a]**	**Hexp[b]**	**Evenne**
Moni1	3	$(AAG)_6$	F: TCCATAGCAACAGAATCTTCCA R: CCTCTCCCCTTGTCCAGTTT	57.6 58.93	284 – 298	7.000	0.714	0.722	0.833
Moni2	1	$(GGT)_6$	F: TAGTGAGAAGAAGAGGGCGC R: CCATCTACCTGCTCCACCTT	59.18 58.79	103 – 109	3.000	0.220	0.221	0.492
Moni3	1	$(CTC)_7$	F: ACGCTTATCCTCCTCAACCTA R: TCGTGTGGGGAATGTATGGA	57.97 58.43	126 – 135	5.000	0.463	0.467	0.662

Moni4	2	$(CGG)_5$	F: CTCCTCCTCAACAAGACCCA R: GCTGATGGTTCGGGAAAGTC	58.65 58.91	200 – 210	4.000	0.433	0.437	0.763
Moni5	3	$(AAG)_6$	F: TCAAGCGGGCCATCTTCA R: GCTATCTCACCGTCACTTCC	58.92 57.79	190 – 200	2.000	0.017	0.017	0.341
Moni6	3	$(AGG)_{11}$	F: AGGAGGCGGTGGTGGTTC R: GCAGGAGAATGTCGAGGAGA	61.64 58.89	200 – 220	2.000	0.123	0.125	0.511
Moni7	1	$(AGG)_{10}$	F: CCTCACAAATCCAAACCCCG R: CAAGCGCATCCCATTCTTCT	59.11 58.61	180 – 190	2.000	0.127	0.128	0.515
Moni8	2	$(GAT)_{10}$	F: CCGTCACACCCAAAGTTCAT R: CTGTTCGTCCTCTGCCATCT	58.40 59.47	230 – 250	7.000	0.569	0.574	0.694
Moni9	1	$(GGA)_8$	F: GCTGACGAAGAGATGGACGA R: GCTTTGAGGTAAGGATGAGGG	59.55 58.08	126 – 146	3.000	0.574	0.579	0.836
Moni10	2	$(GGA)_8$	F: GCTGACGAAGAGATGGACGA R: GCTTTGAGGTAAGGATGAGGG	59.55 58.08	128 – 146	5.000	0.398	0.402	0.614
Moni11	1	$(TCA)_7$	F: CTTCCAATGCCCTCCAACAC R: TGAGACCGACGATGATACCG	59.11 59.06	160 – 170	1.000	.	.	.
Moni12	2	$(TGA)_{13}$	F: TGATTGGCATTCCTCCTCCA R: GCATTCCTGTTCCCGTTTGA	58.70 58.76	266 – 294	6.000	0.661	0.667	0.830
Moni13	2	$(TGG)_8$	F: ATGGCAGCTACCTCGGATAC R: AGAATCTCCTCACCCAACCG	59.03 59.09	138 – 146	4.000	0.624	0.629	0.762
Moni14	1	$(ACC)_9$	F: AGATACTGGGGTTCTTGGGC R: TCTTCAAGCGTTTCGGCAAT	59.08 58.77	187 – 205	5.000	0.688	0.694	0.802
Moni15	2	$(AAG)_{11}$	F: GCAGCAATGATAAAGAAGAGGC	58.04	340 – 350	4.000	0.720	0.900	0.922

			R: TCCGCTTAGATGCAGAAGTTG	58.38				
Moni16	2	$(GAC)_{10}$	F: GACGAGGAAGAGGAGGACG	58.90	143 – 161	7.000	0.571	0.576	0.734
			R: GGTCTGGTGGGTAGTCGAG	58.81				
Moni18	1	$(AGG)_{10}$	F: TCAACTTGTCCCACCCAACT	59.08	217 – 234	7.000	0.529	0.533	0.534
			R: CCAGATGTTCCACTAGCCGT	59.46				
Moni19	2	$(GGC)_{7}$	F: GCGATTCTGGAACCTGTTGA	58.19	247 – 276	13.000	0.873	0.899	0.767
			R: GATGTTGATGTAGGTGCGGA	57.69				
Moni20	3	$(GTG)_{10}$	F: GGCGGAGATGACATAGGCT	58.95	284 – 299	4.000	0.439	0.443	0.640
			R: AGAGTACACGAGCAGCAGAG	59.19				
Moni21	2	$(ACC)_{11}$	F: TCCAGCCAGACCAAATGCTA	59.01	294 – 318	8.000	0.395	0.399	0.483
			R: TCGGAGAGAATGAATGCCCT	58.50				
Moni22	1	$(AAC)_{8}$	F: CTACTGCTGGGTTGGATTGT	57.50	245 – 264	6.000	0.651	0.658	0.774
			R: AGAAGTGGAGGGTCTTGAGC	59.02				
Moni23	3	$(AGG)_{7}$	F: AGCCCTGGTCTTGTCTTGAG	59.31	212 – 224	5.000	0.784	0.791	0.956
			R: GGAGGAGGAGGAATACGACG	59.05				
Moni24	3	$(ACC)_{10}$	F: AAAACCCTTCTGCGACGAGT	59.89	290 – 308	2.000	0.364	0.367	0.780
			R: GGAACCGTGTTATCTGTGCG	59.28				
			Mean			4.870	0.475	0.488	0.693

[a] Simpson index

[b] Nei's 1978 genotypic diversity or Expected Heterozygosity

Table 3. Summary statistics of genetic variation within populations of *M. roreri* isolates according to four strata.

Stratum	Population	N	Number of MLGs	eMLG[a]	SE[b]	H[c]	G[d]	lambda	E_5[f]	Hexp[e]	I_A[g]	řd[h]
Clone	**ICS1**	18	17	14.3	4.64e-01	2.81	16.2	0.938	0.970	0.457	3.067	0.1783
	ICS39	34	34	15.0	1.61e-06	3.53	34.0	0.971	1.000	0.451	1.573	0.0842
	ICS95	53	52	14.9	2.65e-01	3.94	51.1	0.980	0.989	0.467	0.911	0.0513
	ICS60	15	15	15.0	0.00e+00	2.71	15.0	0.933	1.000	0.484	1.781	0.0899
	Total	120	117	14.9	2.41e-01	4.75	112.5	0.991	0.975	0.488	1.284	0.0670
Locality	**Rionegro (Rio)**	10	10	10.00	0.000	2.30	10.00	0.900	1.000	0.318	2.343	0.1594
	San Vicente (SV)	5	5	5.00	0.000	1.61	5.00	0.800	1.000	0.395	-0.404	-0.0315
	Ataco (At)	7	7	7.00	0.000	1.95	7.00	0.857	1.000	0.303	0.490	0.0379
	Campo Alegre (CA)	12	12	10.00	NaN	2.48	12.00	0.917	1.000	0.181	1.057	0.0768
	Lebrija (L)	21	21	10.00	NaN	3.04	21.00	0.952	1.000	0.449	2.067	0.1164
	El Carmen (EC)	15	13	8.98	0.711	2.49	10.71	0.907	0.880	0.311	1.822	0.1204
	Maceo (M)	25	25	10.00	NaN	3.22	25.00	0.960	1.000	0.355	2.441	0.1329
	El Playon (EP)	5	5	5.00	0.000	1.61	5.00	0.800	1.000	0.371	5.368	0.3838

	Apartado (A)	4	4	4.00	0.000	1.39	4.00	0.750	1.000	0.254	2.127	0.3061
	Turbo (T)	1	1	1.00	0.000	0.00	1.00	0.000	NaN	NaN	NA	NA
	Cimitarra (C)	3	3	3.00	0.000	1.10	3.00	0.667	1.000	0.333	1.625	0.2321
	Planadas (P)	7	6	6.00	0.000	1.75	5.44	0.816	0.937	0.251	2.438	0.2474
	Rivera (R)	5	5	5.00	0.000	1.61	5.00	0.800	1.000	0.359	0.153	0.0171
	Total	120	117	9.98	0.159	4.75	112.50	0.991	0.975	0.488	1.284	0.0670
	Santander (S)	59	57	12.9	3.59e-01	4.02	53.6	0.981	0.959	0.457	1.716	0.0894
	Tolima (T)	13	13	13.0	0.00e+00	2.56	13.0	0.923	1.000	0.316	0.938	0.0621
State	**Huila (H)**	18	18	13.0	2.79e-07	2.89	18.0	0.944	1.000	0.277	0.923	0.0577
	Antioquia (A)	30	30	13.0	NaN	3.40	30.0	0.967	1.000	0.413	2.550	0.1360
	Total	120	117	13.0	2.08e-01	4.75	112.5	0.991	0.975	0.488	1.284	0.0670
	A3 (400 – 600m)	18	17	9.71	4.56e-01	2.81	16.2	0.938	0.970	0.372	1.65	0.1013
	A5 (800 – 1000 m)	27	26	9.87	3.34e-01	3.24	25.1	0.960	0.979	0.465	1.27	0.0695
Altitude	**A6 (1000 – 1200m)**	28	28	10.00	3.41e-07	3.33	28.0	0.964	1.000	0.497	1.54	0.0873
	A4 (600 – 800m)	25	25	10.00	NaN	3.22	25.0	0.960	1.000	0.399	2.61	0.1495
	A2 (200 – 400m)	17	17	10.00	2.31e-07	2.83	17.0	0.941	1.000	0.284	2.25	0.1216

A1 (0 – 200m)	5	5	5.00	0.00e+00	1.61	5.0	0.800	1.000	0.208	1.30	0.186(
Total	120	117	9.98	1.59e-01	4.75	112.5	0.991	0.975	0.488	1.28	0.067(

[a] The number of expected MLG at the smallest sample size ≥10 based on rarefaction.

[b] Standard error based on eMLG

[c] Shannon-Wiener Index of MLG diversity

[d] Stoddart and Taylor's Index of MLG diversity

[e] Nei's 1978 genotypic diversity (corrected for sample size), or Expected Heterozygosity

[f] Evenness, E_5.

[g] The index of association, I_A

[h] The standardized index of association, řd

Figure 1. Geographical distribution of *M. roreri* isolates from four states in Colombia. Red points are located in the sampling areas. The map was obtained using the *RgoogleMaps* package for R Software v1.0.3.

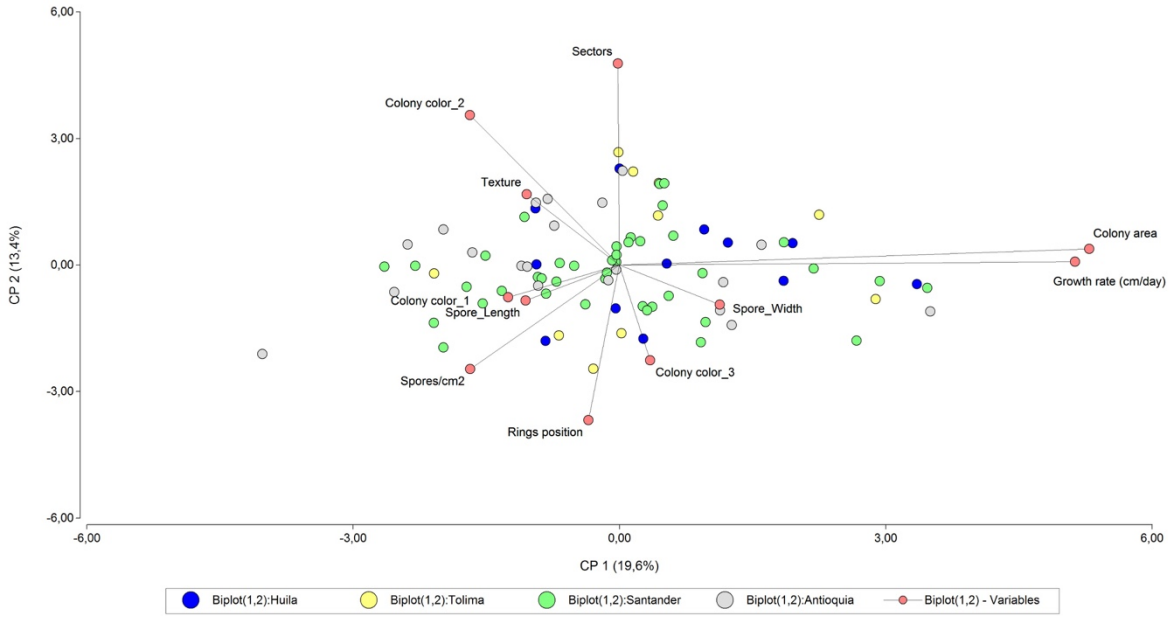

Figure 2. Bi-plot of morphological characteristics observed in *M. roreri* isolates based on principal coordinate analysis (PCA). First and second component of a principal components analysis (PCA) of 11 morphological variables.

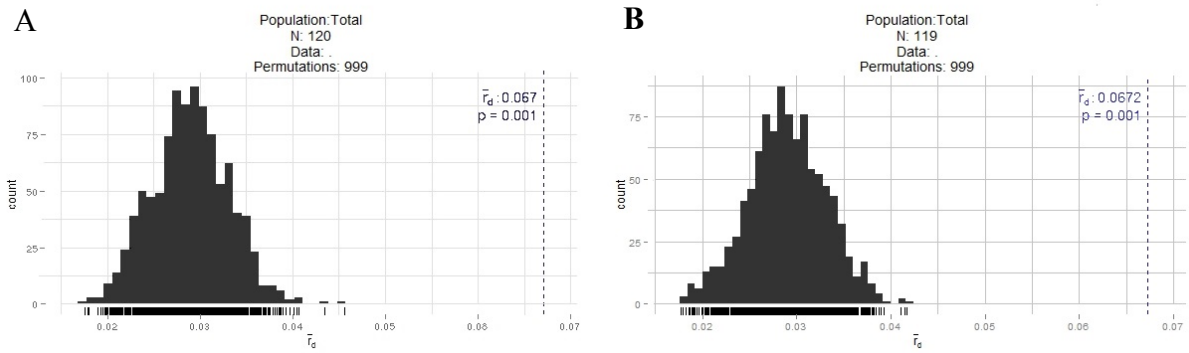

Figure 3. Index of association standardized to (A) uncorrected and (B) corrected data, using the *Poppr* package for R Software v1.0.3.

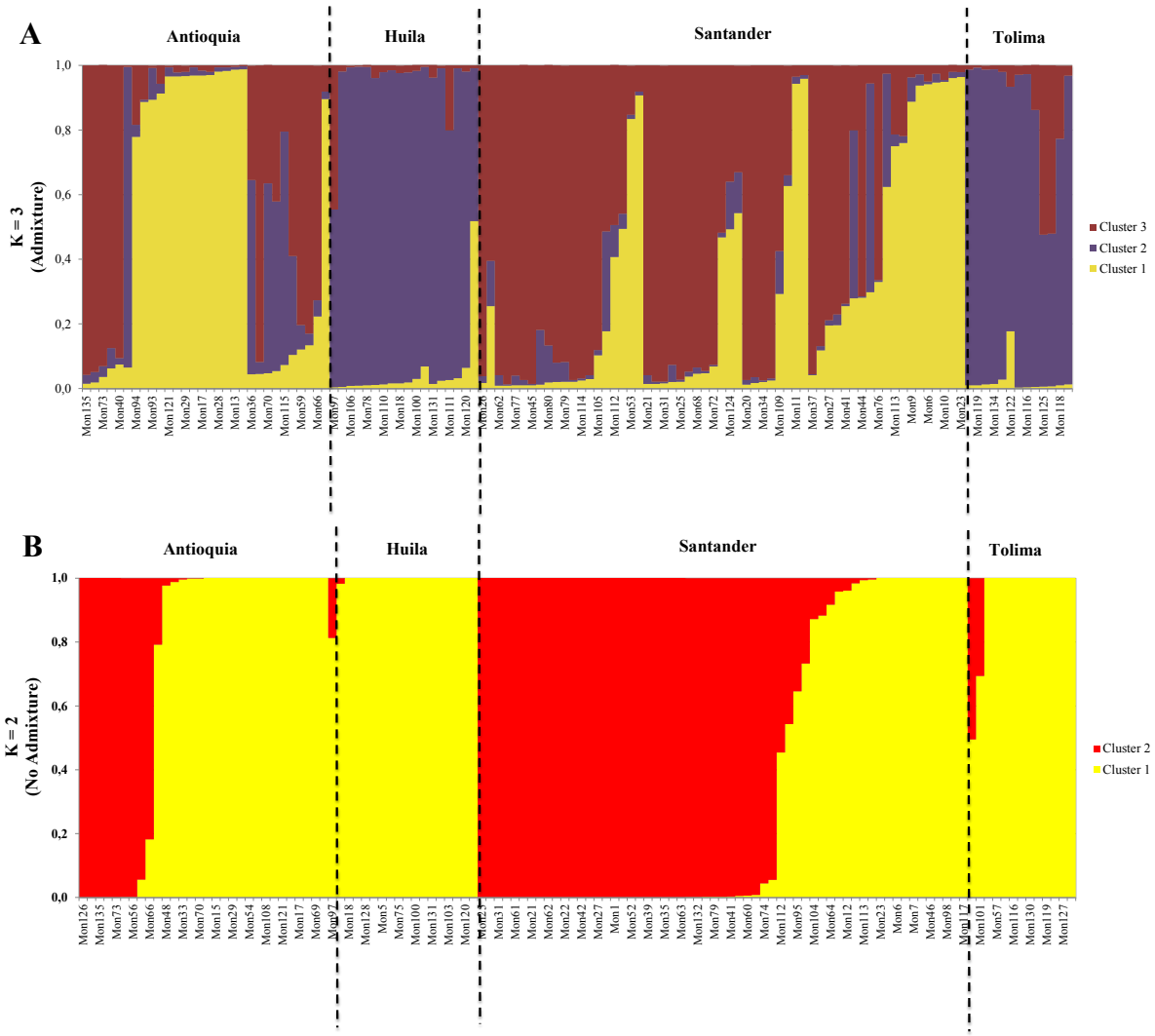

Figure 4. STRUCTURE analysis of 23 SSR markers for *Moniliophthora roreri* isolates. Plot of results for STRUCTURE output at different K organized by Q value. Each color represents one cluster defined by STRUCTURE. Vertical bars represent isolates, and the length of each colored segment in each vertical bar represents the proportion contributed by ancestral populations. A) Admixture model; B) No-admixture model.

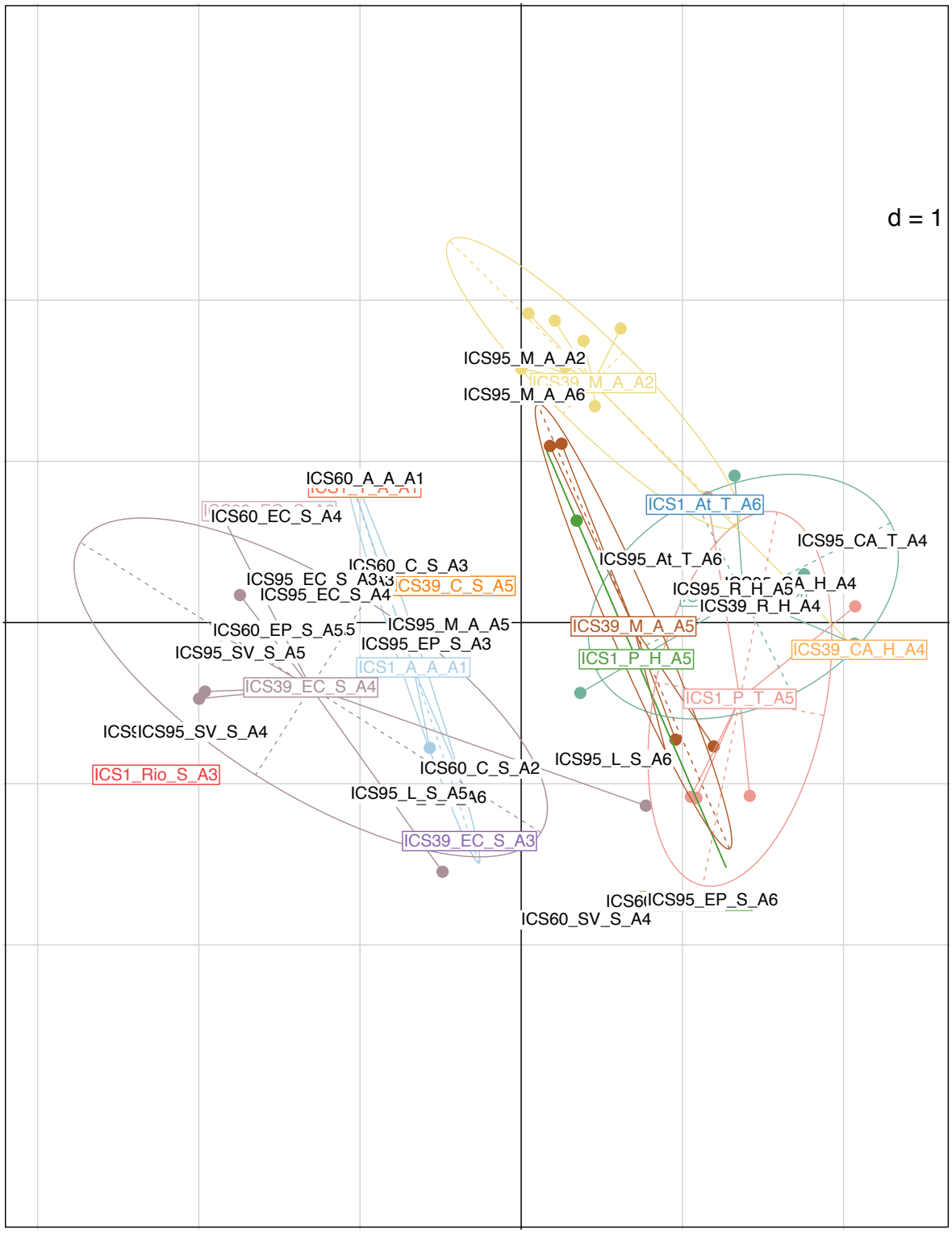

Figure 5. Scatterplot of the genetic differentiation observed in *M. roreri* isolates based on principal coordinate analysis (PCA). First and second component of a principal components analysis (PCA) for 23-locus microsatellite genotypes. Ellipses grouped isolates into geographic location.

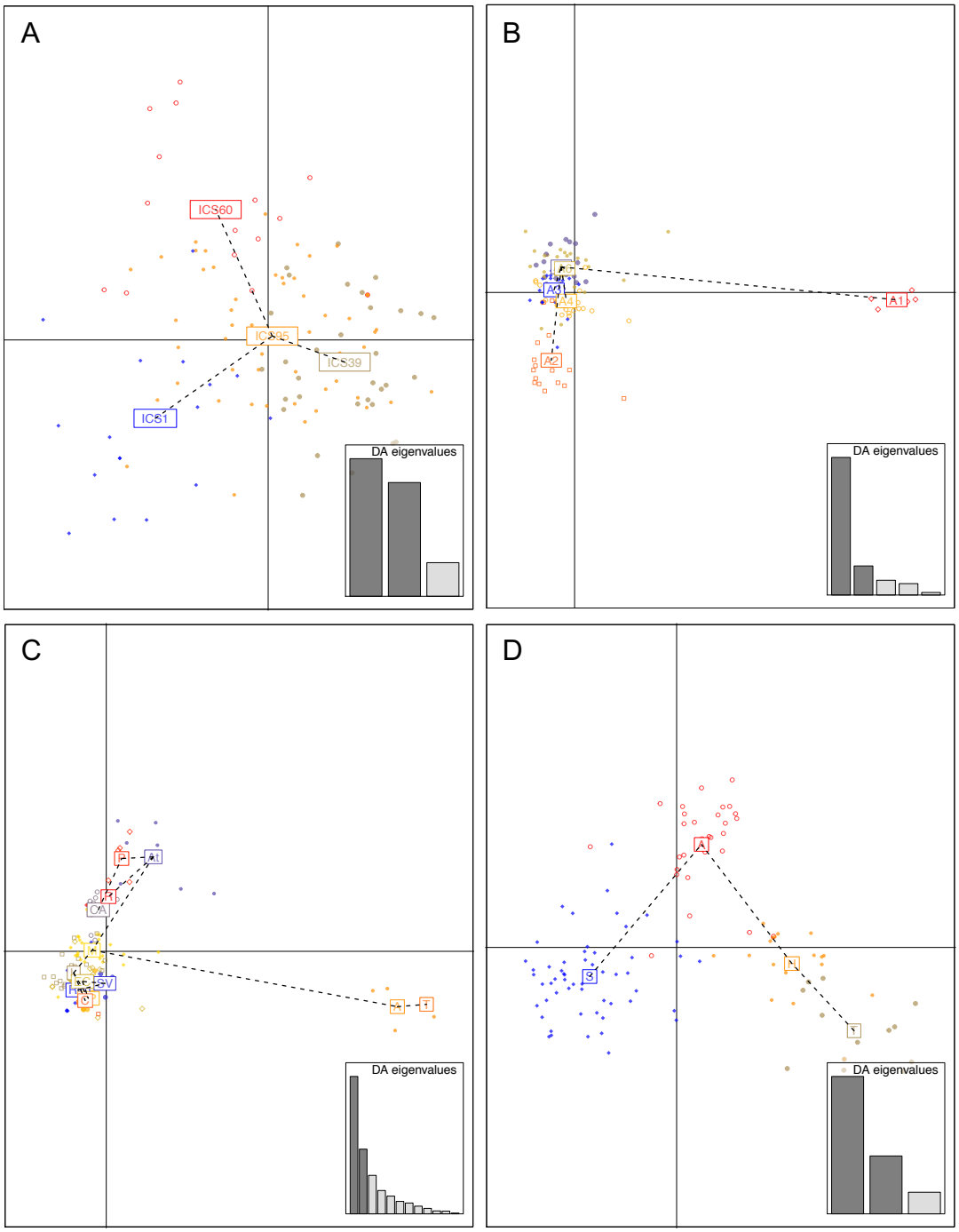

Figure 6. Summary of genetic relatedness among *M. roreri* obtained from Discriminant Analysis of Principal Components (DAPC). Scatter plot of the first two principal components from DAPC with a minimum-spanning tree based on the squared distance between populations, showing the connection between clusters for each stratum. A) Clone stratum; B) Altitude stratum; C) Locality stratum; and D) State stratum.

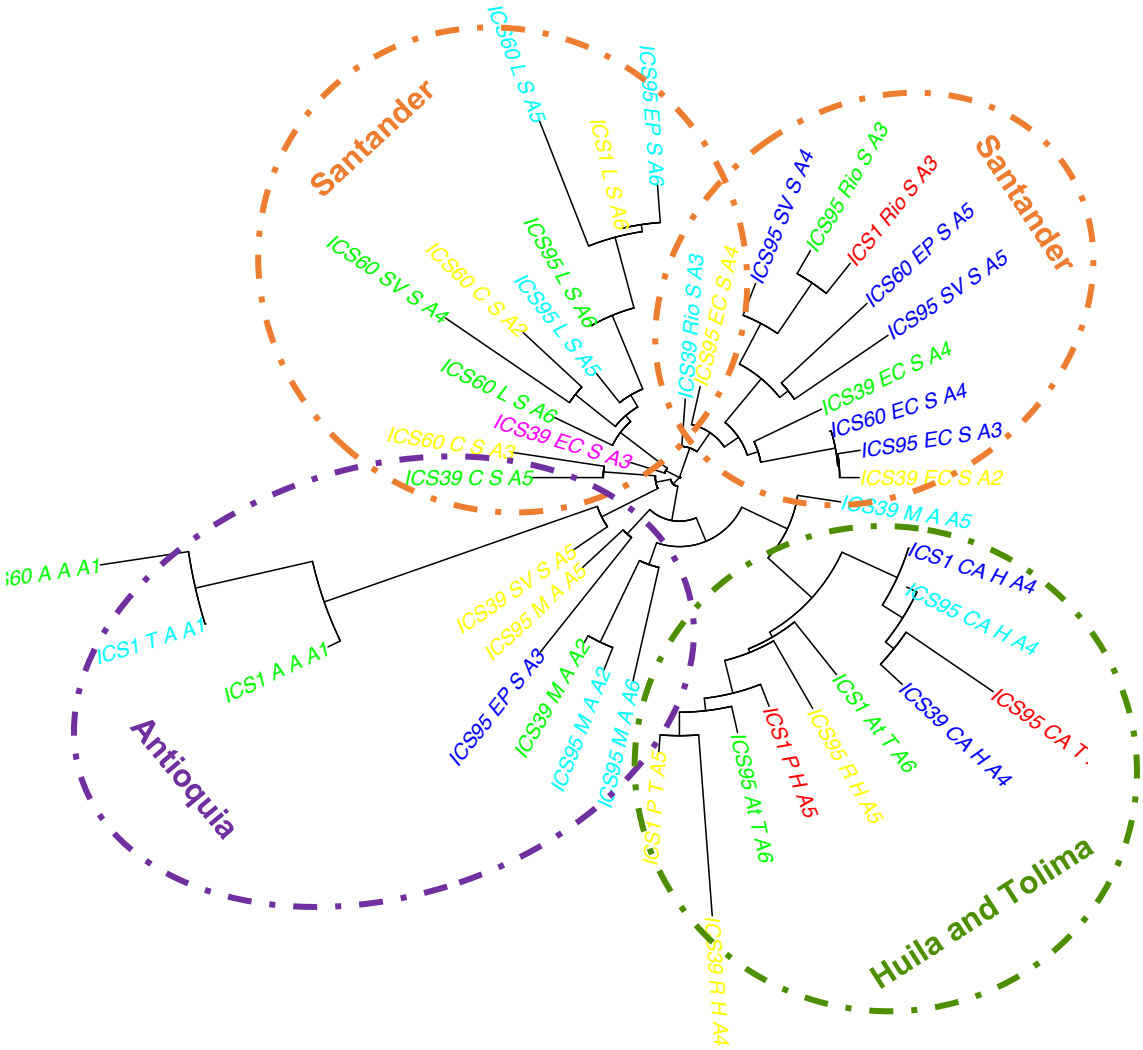

Figure 7. Dendrogram showing the relationships between *M. roreri* isolates using microsatellite markers. Distances among genotypes were calculated using Nei genetic distance and visualized with an unrooted neighbor-joining tree. Colors represent different geographic locations used in this study.

CAPÍTULO II

Spatio-temporal dynamic of frosty pod rot in the main cocoa producing areas of Santander State, Colombia [2]

[2] Redigido conforme as normas da revista Plant Pathology (Original Article)

Spatio-Temporal Dynamic of Frosty Pod Rot in the Main Cocoa Producing Areas of Santander State, Colombia

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Abstract

Incidence of Frosty pod rot disease (FPR), caused by *Moniliophthora roreri*, in time and space on clones with different levels of resistance was investigated in four localities of Santander between July 2013 and May 2015. Generalized linear model was used to analyze the temporal and spatial progress of FPR. Poisson distribution with a logarithm mic link function was chosen because of data overdispersion. A significant interaction was found

between clones and localities on epidemic parameters. The susceptible clones SCC 61 had the higher levels of disease in most localities, excepted in San Vicente where it showed the lower levels. The locality in San Vicente had the lower levels of disease in all cacao clones, when compared with the other localities. After Moran's I test was applied to data of cut diseased pods in La Suiza Research Center, spatial autocorrelation was evident. These results could suggest that FPR epidemics vary according to plot location and plant material location on the plot. With this, cacao clones must be evaluated for their behavior under different conditions and the FPR management practices must be adjusted to local situations considering the microenvironment variation.

Key words: Epidemics, generalized linear model, overdispersion, *Moniliophthora roreri*, *Theobroma cacao*

Introduction

Frosty pod rot (FPR) disease caused by *Moniliophthora roreri* can result in a considerable reduction in cocoa bean yields and is one of the most devastation disease of cocoa in tropical America, including Colombia (Evans et al., 2003; Phillips-Mora and Wilkinson, 2007; Jaimes and Aranzazu, 2010). Even though the FPR is a relatively minor disease in global terms due to its specific location, but the potential danger presented by the disease is enormous (Evans et al., 2003; Phillips-Mora and Wilkinson, 2007). The disease has been reported to be twice as destructive as black pod (*Phytophthora* spp.), and more dangerous and difficult to control than witches' broom (*Moniliophthora perniciosa*) (Phillips-Mora and Wilkinson, 2007). In Colombia, FPR is the most limiting disease for cacao production with losses above 40% depending on crop management (Jaimes and Aranzazu, 2010).

The fungal pathogen is a hemibiotroph and attacks only the fruit of species of genera *Herrania* and *Theobroma*, causing internal and external pod damage that result in total pod loss (Phillips-Mora et al., 2007). FPR development can be lengthy, progressing over the 5 or 6 months required for pod development and ripening (Bailey et al., 2013). Nevertheless, under favorable conditions the disease occurs 50 to 70 days after infection as brown spreading lesions on the pod surface, ending the life cycle 5 days later with the development of white mycelia on lesions with spore production and a color change from cream to brown (Jaimes and Aranzazu, 2010).

Management strategies for FPR at the moment are mainly agronomic practices combined with improved material and possibly biological control (Ali et al., 2015). However, some of these measures have been effective only on an experimental scale (Phillips-Mora et al., 2007), and the tolerant materials are limited with some of them having

gradual loss of resistance after 15 years of validation (Ali et al., 2015). Therefore, only those management strategies based on cultural practices are being adopted by smallholders (Phillips-Mora and Wilkinson, 2007). Though, to use these management practices efficiently as part of an integrated management program requires prior knowledge of the host-parasite-environment interaction that governs disease development (Mouen Bedimo et al., 2007).

In Costa Rica, the FPR progress was adjusted to independent monocycles and its incidence was correlated positively with the number of fruits and cacao phenology (Leandro, 2011). In Tabasco (Mexico), FPR epidemics were also associated with the number of fruits, suggesting that each epidemic must have independent integrated actions to management and reduce FPR incidence, especially in the cacao reproductive state for its economic importance. Also in this study, FPR epidemics were adjusted to Weibull models and associated positively with low temperature (20 – 26 °C) and relative humidity higher than 90% during 49 days to onset of symptoms (Torres de la Cruz et al., 2011). Contrary to these reports, in Colombia the FPR cycle can start at any time, because the source of primary inoculum is present throughout the year and favored by the rainfall distribution throughout the year. It is positively correlated with rainfall that occurs two months before the epidemics (Merchan, 1981).

A mixture of susceptible and resistant plants has the potential to slow, reduce and localize disease occurrence in natural and agriculture ecosystems (Severns et al., 2014). Though there are no cacao varieties with vertical resistance, some materials, such as ICS 95, have demonstrated horizontal resistance against FPR (Phillips-Mora et al., 2005; Ali et al., 2015), and this varied depending on pathogen aggressiveness (Phillips-Mora et al., 2005) and the environment (Jaimes et al., 2011). Moreover, trees selected for disease tolerance on one site, can be susceptible to the same disease on an unfavorable site. Hence, the study of

FPR epidemics which account for different environments and cacao varieties is required to understand their influence on the temporal and spatial dynamic of disease outbreak. The objectives of this research were therefore to monitor temporal and spatial dynamics of FPR in three clones at four localities of Santander State (Colombia), and to determine the relationship between FPR epidemics on the three cacao varieties with different grades of resistance at different environments.

Materials and Methods

Experimental sited and design. The study was conducted in El Trebol, El Venado, El Libano and the La Suiza Research Center of Corpoica (Table 1). An experimental plot with in the La Suiza Research Center was selected to recorded FPR incidence in the ICS 95, CCN51, SSC61 and ICS60 cacao varieties with different level of resistance according to Jaimes et al (2011) (Figure 1). To, FPR incidence was registered in a commercial field located in three different localities of Santander State (Colombia). These properties were selected for: (1) the cacao varieties available (ICS 95 – resistant, CCN 51 – moderately resistant and SCC 61 – susceptible), (2) the same crop agronomic management (pruning, fertilization after pruning according with onset of rainfall, weekly removal of disease fruits, weed control, and opportune harvest of fruit) as per the region's recommendation, and (3) different environment conditions.

To analyze disease progress over time in the La Suiza Research Center, FPR incidence was registered on the cacao varieties ICS 95 (Imperial College Selection), CCN 51 (Colección Castro Naranjal), SCC 61 (Selección Colombia Corpoica) and ICS 60. Five randomly were selected plants of each cacao variety per block in an experimental plot with four randomized complete blocks (Figure 1). Due the material available, in the other

localities the incidence was registered on the cacao varieties ICS 95, CCN 51 and SCC 61, selected five randomly plants of each cacao material per sampling site.

Disease assessment. To analyze disease progress over time for each plant, we recorded total pods, cherelle wilt, diseased pods with early symptoms of FPR, pods and cut diseased pods with advanced symptoms of FPR at intervals of 15 days between July 2013 and May 2015. The number of harvested pods was recorded only in La Suiza Research Center. To analyze disease progress over space we considered the spatial distribution of each plant in the experimental plot at the La Suiza Research Center.

Data analysis. Statistical analysis was performed using the *glm* function of the *Stats* package in R software v. 3.2.2. Generalized linear models (GLM) were used to analyze temporal and spatial variations in FPR progress. Because cut diseased pods with advanced symptoms of FPR are counts, Poisson distribution with a logarithmic link function was chosen. Cut diseased pods with advanced symptoms of FPR were used as response variables, and locations and clones were used as explanatory variables in the regression models with total pods as covariable. Relationships among clone, location and cut diseased pods with advanced symptoms of FPR were calculated using GLM as described above, but this time we used the sum of a given variable over the 34-month study period as response variables.

Spatial autocorrelation analysis was applied to data of cut diseased pods in La Suiza Research Center. Moran's *I* statistic of spatial autocorrelation was calculated using *moran.test* function of *spdep* package in R software v. 3.2.2. After an analysis of variance with block as factor, we did another test of spatial autocorrelation. Due to differential cacao material behavior depending on block, GLM was used to analyze spatial variations in FPR progress. Here we used the sum of a given variable over the 34-month study period as the response variable, and location and clone were used as explanatory variables in the

regression models with harvested pods as covariable. Also, the relationship between each variable with the number of harvested pods was calculated.

In all GLM analyses, overdispersion was taken in to count and the data were fitted with quasi-poisson regression (Zeileis et al., 2008). The presence of a significant interaction between location, clone and cut diseased pods indicates that disease progress differs between sites. Stepwise removal and pairwise comparison of sites was used to reveal the sites that differed significantly. For fitted models, an analysis of deviance was applied, providing a *p* value for the significance of each possible explanatory factor or covariate with a chi-squared test. In all analysis, effects were considered to be significant at a level of $p \leq 0.05$.

Results

Frosty pod disease was recorded in all locations studied at each sampling periods. A interaction was found between cut disease pods sum per tree on each clone and the locality (Figure 2). The cacao clone SCC 61 showed the greatest number of cut diseased pods in most localities studied, except in San Vicente de Chucuri and in the B1 block in La Suiza Research Center (Figure 2). Likewise, the cacao clone ICS 95 showed the lowest number of cut diseased pods in most localities, excepted in San Vicente de Chucuri. The first analysis suggested little disease occurrence in San Vicente and B1 block in Rionegro. In the other localities was observed different disease levels between clones and even between localities. But, data were often dispersed, that is that data often exhibited more variation than given by the mean. Therefore, to analyze cut diseased pods with advanced symptoms of FPR data a generalized linear model was used with Quasi-Poisson regression.

With the GLM analysis results a significant interaction was identified between cut diseased pods and location ($P < 2.2\ e^{-16}$), cut diseased pods and clone ($P < 2.2\ e^{-16}$) and between cut diseased pods and clone per locality ($P = 3.768\ e^{-05}$) (Table 2). Lower levels of disease were identified in San Vicente al B1 block compared to B3 block. Higher levels of disease were identified in B4 block compared to B2 block, El Carmen and Cimitarra (Table 3). Regarding cacao varieties, SCC 61 had more cut diseased pods than CCN 51, and ICS 95 was the cacao variety with lower cut diseased pods (Table 3).

Cut diseased pods were evident in spatial autocorrelation (Moran' *I* statistic standard deviate = 7.6644; $P = 8.986\ e^{-15}$) when considering all blocks. When the analysis was done considering the blocks, spatial autocorrelations was not found (Moran' *I* statistic standard deviate = 0.28195; *P* 0 0.389). It could be for the lower disease levels in the B1 block (Figure 3). As seen in different locations analyzed, different levels of FPR were observed in each clone between blocks of the same locality (Figure 4). Lower levels disease were observed in B1 block, where clones CCN51 and ICS 60 had the highest levels of FPR (Figure 4). In the B2 and B3 blocks intermediate levels of disease were observed, with variable behavior of clone CCN 51 (Figure 4). Highest levels of disease were observed in block 4, the susceptible clones SCC61 and ICS 60 had the highest levels of FPR disease (Figure 4). The behavior of cacao materials varied according to their location in the experimental plot (Figure 4), as it was observed in the analysis of different localities (Figure 2).

The GLM results for the blocks in La Suiza Research Center were similar to those obtained in the analysis with all localities. In the analysis, a significant interaction was identified between cut diseased pods and blocks ($P < 2.2.\ e^{-16}$), cut diseased pods and clone (P 0 $8.772\ e^{07}$) and between cut diseased pods and clones per block ($P = 0.001678$) (Table

4). Lower levels of FPR were identified in B1 and B2 blocks. Higher levels of disease were identified in B4 block compared to B3 (Table 5). Regarding cacao varieties, SCC 61 and ICS 60 had more cut diseased pods than CCN 51, and ICS 95 was the cacao variety with lower cut diseased pods (Table 5).

In all cacao varieties, few pods were harvested (Figure 5). In cacao clones ICS 95 and CCN 51, most pods was lost by wilting (Figure 5c – 5 d), with a relationship of 9.54 and 11.45 wilting pods per harvested pod, respectively. Regarding cut diseased pods in these clones, a relationship of 1.01 and 1.95 cut diseased pod per harvested pod was found. In the susceptible clones SCC 61 and ICS 60 less loss by wilting was observed (Figure 5a – 5b), compared to the levels of ICS 95 and CCN 51 (Figures 5c – 5d), with a relationship of 1.89 and 2.22 wilting pods per harvest pod, respectively. The relationship between cut diseased pods of SCC 61 and ICS 60 and harvested pods was 2.34 and 1.77, respectively. Even though we observed a considerable number of diseased fruits with early symptoms in all cacao varieties, few of these fruits completed the FPR cycle as cut diseased pods (Figure 5). This behavior was similar in all locations evaluated (data not shown).

Discussion

Preliminary studies suggest different models to describe FPR epidemics. Although, to described the temporal dynamic of FPR incidence in Costa Rica was considered that monomolecular model (Leandro, 2011) and in Mexico the Weibull model (Torres de la Cruz et al., 2011), the FPR incidence are correlated positively to cacao phenology as seen in our study (Figure 5). Notwithstanding, in the present study was observed that during the first month of fruit age the cherelle wilt was the principal cause of fruit loss. This was occurred during the exponential phase of fruit growth due to at this time the cacao tree eliminates pods

exceeding its load capacity (Valle et al., 1990), observing fruit loss greater than 80%. For this reason, the FPR incidence values were zero in the most case and the data were adjusted to Quasi-Poisson model considered the this adds to dispersion parameter to handle overdispersed count data..

In this study, the FPR epidemics varied both among clones, localities, and even blocks (spatial arrangement of the cacao varieties in the same plot). A significant interaction in temporal and spatial dynamic of disease throughout localities and cacao clones was found. Even though all localities have the same management, in Cimitarra, El Carmen and B4 block in Rionegro had the higher levels of FPR, compared to San Vicente and B1 block in Rionegro. Although, all localities have a tropical forest clime characterized by monthly rainfall above 60 mm throughout the year, annual rainfall exceeding evapotranspiration and no monthly average temperature below 18 °C, these varied in the altitude and in the case of the blocks in the plot localization and land topography. As reported by Merchan (1981), in Colombia the FPR cycle can begin at any time, however the critical periods are associated with the periods of greatest fruit formation (Figure 5) (Jaimes and Aranzazu, 2010; Torres de la Cruz et al., 2011), and the crop localization, including altitude and topography (Jaimes and Aranzazu, 2010).

Notwithstanding, the levels of resistance to FPR in the cacao clones ICS 95, CCN 51 and SCC 61 varies according to locality (Jaimes et al., 2011). According to Phillips-Mora et al. (2005), the cacao clones, as ICS 95, have partial resistance also called as horizontal resistance. This type of resistance is characterized to be not race-specific and the amount of disease could be high or low (Keane, 2012). For this, a cacao variety with horizontal resistance in one location may be unacceptably resistant or susceptible than another, as is the case with SCC 61. This means that resistant traits in cacao are environmentally sensitive

(Young, 1996). Beside the different resistance levels to FPR, the variation of spatial dynamic could be due to variation microenvironments given by forest tree spatial structure. In the study of Gidoin et al. (2013) it was found that disease intensity was lower in plots with a regular forest tree structure than in plots with low forest tree density.

Although currently recommend management practices were applied, epidemics still occurred. For this reason, an integrated disease management approach involving better agronomic practices, combined with improved planting materials and possibly biological control approaches will be the only feasible strategy for managing FPR at this time (Ali et al., 2015). Studies are required to evaluate zoning cacao material according to its behavior (both its productivity and resistance to FPR), as well as tree spatial structure. In addition, classical quantitative genetic research is required to understand the resistance mechanism in cacao clones and how these are affected under different environments, in order to define the additive or non-additive gene action and genotype-environment interaction (Young, 1996).

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Table 1. Location and data of properties monitored for FPR dynamic.

Property	Locality of Santander State	Latitude	Longitude	Altitude (m)	Annual average temperature (°C)	Annual Rainfall (mm)	Annual average relative humidity (%)
El Trebol	Cimitarra	06° 15,10' N	073° 54,46' W	390	27,6	2766	85
El Venado	El Carmen	06° 43,713' N	073° 33,380' W	315	24,5	2156	85
El Libano	San Vicente	06° 49,723' N	073° 27,370' W	757	25,3	1820	85
La Suiza	Rionegro	7° 22' N	73° 10' W	500	28	1700	87

Table 2. Analysis of deviance of GLM results, degrees of freedom (d.f.), deviance, residuals degrees of freedom (Resid. d.f), residuals deviance (Resid. Dev) and probability value (*P)* of chi-squared test (>chi) for Cut disease pods with advanced symptoms of FPR as response variable and locality and clone as explanatory variables.

	d.f.	deviance	Resid. d.f	Resid. Dev	*P* (>Chi)
NULL			74	1047.85	
Locality	4	299.13	70	748.71	$< 2.2\ e^{-16}$ ***

Clone	2	332.52	68	416.19	$< 2.2\ e^{-16}$ ***
Log(Total_D)	1	312.46	67	103.73	$< 2.2\ e^{-16}$ ***
Locality:Clone	8	27.26	59	76.46	0.007059 **

Significance codes: 0 '***', 0.001 '**', 0.01 '*', 0.05 '.', 0.1 ' ', 1

Table 3. Summary of GLM results for Cut disease pods with advanced symptoms of FPR as response variable and locality and clone as explanatory variables, excluding San Vicente and block B1 of La Suiza Research Center localities by low levels of diseases.

	Estimate	**Standard Error**	**t value**	***P* (> [t])**
(Intercept)	- 0.41872	0.33101	- 1.265	0.21027
Locality-Cimitarra	- 0.22676	0.07768	- 2.919	0.00477 **
Locality-El Carmen	- 0.21030	0.06878	- 3.058	0.00320 **
Locality-B2	- 0.26703	0.09894	- 2.699	0.00880 **
Locality-B3	- 0.16629	0.10027	- 1.658	0.10191
Clone ICS95	- 0.15629	0.07620	- 2.051	0.04416 *
Clone SCC61	0.13873	0.06776	2.047	0.04454 *
log(Total_D)	0.88015	0.06803	12.938	$< 2\ e^{-16}$ ***

Significance codes: 0 '***', 0.001 '**', 0.01 '*', 0.05 '.', 0.1 ' ' 1

(Dispersion parameter for quasipoisson family taken to be 1.57)

Table 4. Analysis of deviance of GLM results, degrees of freedom (d.f.), deviance, residuals degrees of freedom (Resid. d.f), residuals deviance (Resid. Dev) and probability value (*P)* of chi-squared test (>chi) for Cut disease pods with advanced symptoms of FPR as response variable and block and clone as explanatory variables.

	d.f.	Deviance	Resid. d.f	Resid. Dev	*P* (>Chi)
NULL			79	1240.54	
Block	3	739.23	76	501.31	< 2.2 e^{-16} ***
Clone	3	92.84	73	408.47	8.772 e^{-7} ***
Log(Exposition)	1	119.29	72	289.17	2.889 e^{-10} ***
Log(Harvested)	1	25.71	71	263.47	0.003426 **
Block:Clone	9	79.59	62	183.87	0.001678 **

Significance codes: 0 '***', 0.001 '**', 0.01 '*', 0.05 '.', 0.1 ' ', 1

Table 5. Summary of GLM results for Cut disease pods with advanced symptoms of FPR as response variable and block and clone as explanatory variables.

	Estimate	Standard Error	t value	*P* (> [t])
(Intercept)	- 3.68517	1.31007	- 2.813	0.00821 **
Block 4	0.37781	0.12013	3.145	0.00350 **
Clone ICS60	0.62705	0.14088	4.451	9.20 e^{-05} ***
Clone ICS95	- 0.53079	0.15914	- 3.335	0.00212 **
Clone SCC61	0.69142	0.15464	4.471	8.68 e^{-05} ***
log(Exposition)	1.10602	0.21385	5.172	1.11e^{-05} ***
log(Harvested)	- 0.12600	0.08693	- 1.449	0.15664

Significance codes: 0 '***', 0.001 '**', 0.01 '*', 0.05 '.', 0.1 ' ' 1

(Dispersion parameter for quasipoisson family taken to be 3.654051)

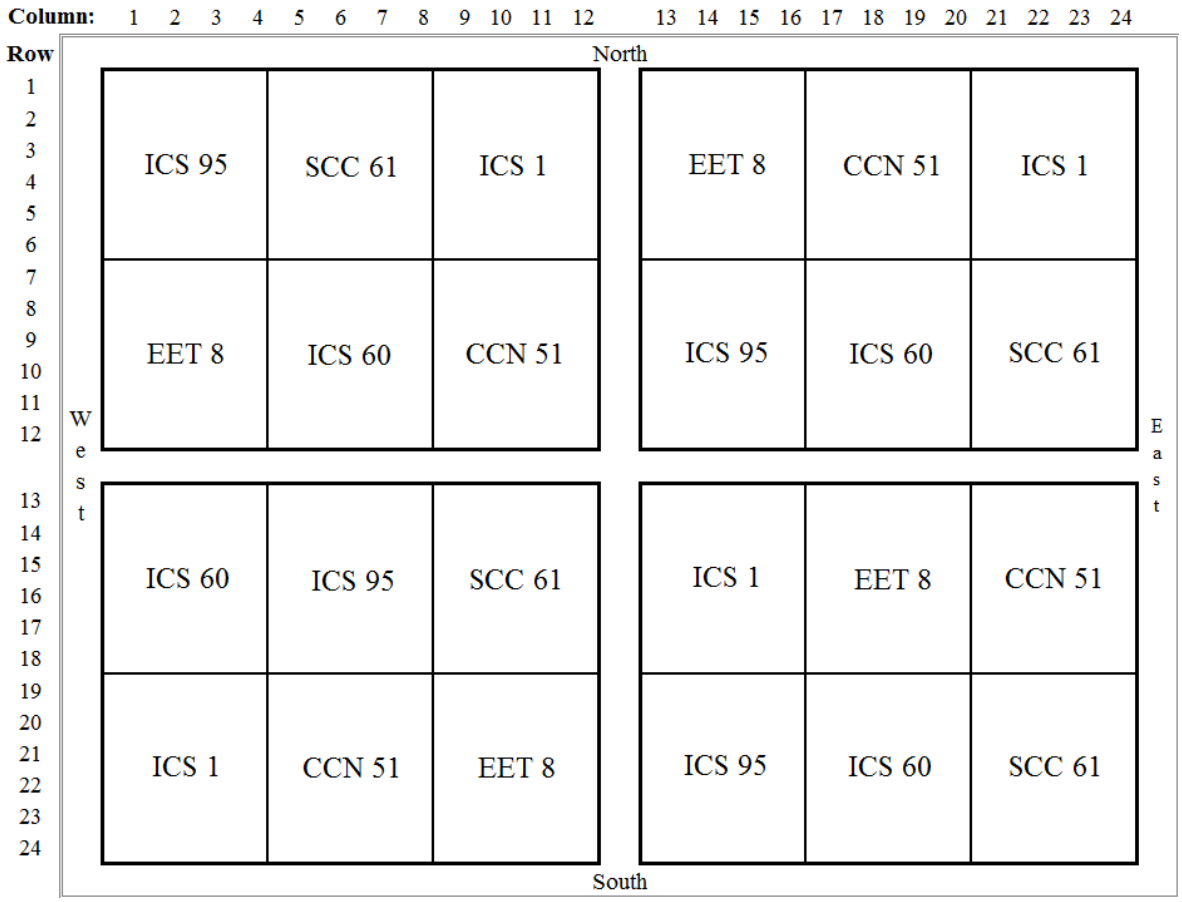

Figure 1. Design of experimental plot in La Suiza Research Center located in Rionegro (Santander – Colombia). 1 to 12 column and 1 to 12 row delimit the block 1 (B1); 13 to 24 column and 1 to 12 row delimit the block 2 (B2); 1 to 12 column and 13 to 24 row delimit the block 3 (B3); and 13 to 24 column and 13 to 24 row delimit the block 1 (B1).

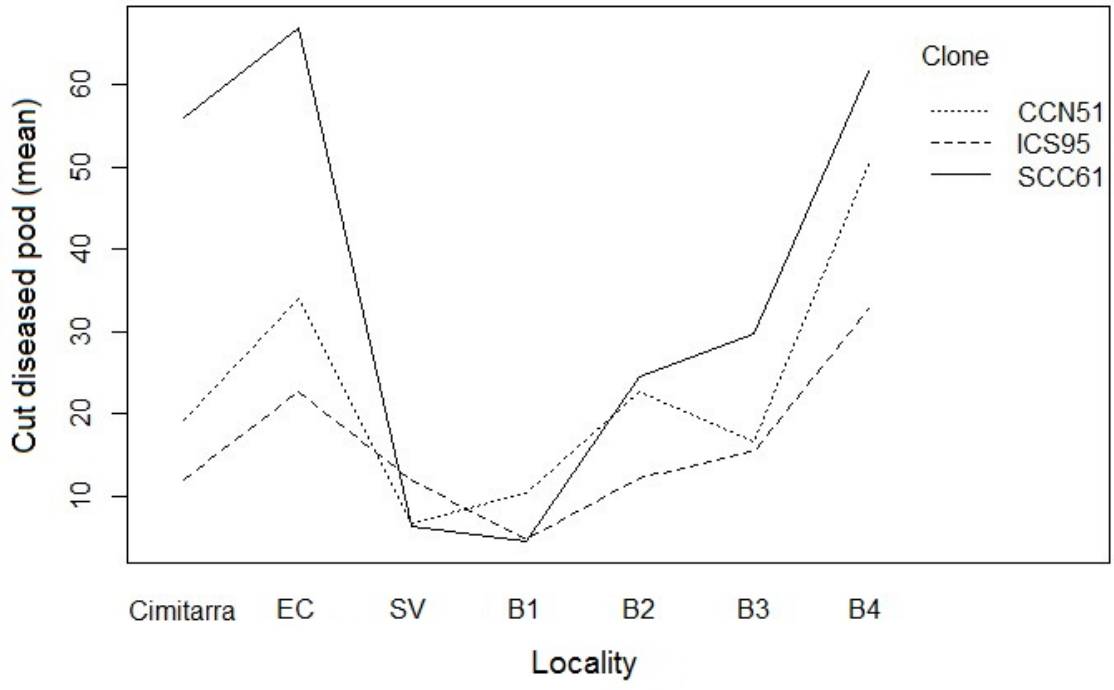

Figure 2. Interaction plot of Cut disease pods with advanced symptoms of FPR for ICS 95, CCN 51 and SCC 61 cacao clones in Cimitarra, El Carmen de Chucurí, San Vicente de Chucuri and B1, B2, B3 and B4 blocks in Rionegro (Santander – Colombia).

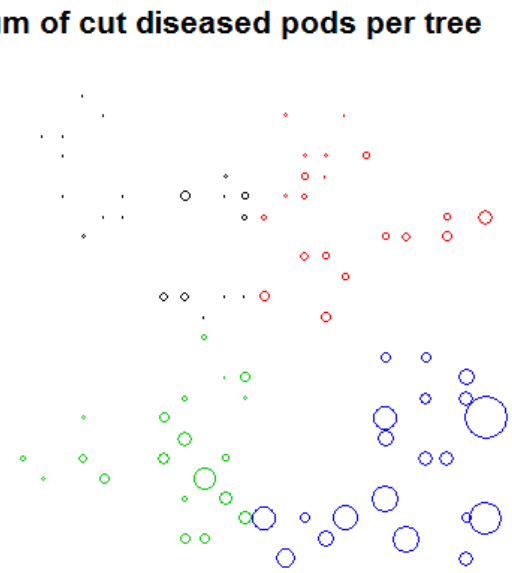

Figure 3. Spatial distribution of sum of Cut Disease pod with advanced symptoms of FPR at each block of experimental plot in La Suiza Research Center. Black circles represent block 1 (B1); red circles represent block 2 (B2); green circles represent block 3 (B3); and blue circles represent block 4 (B4).

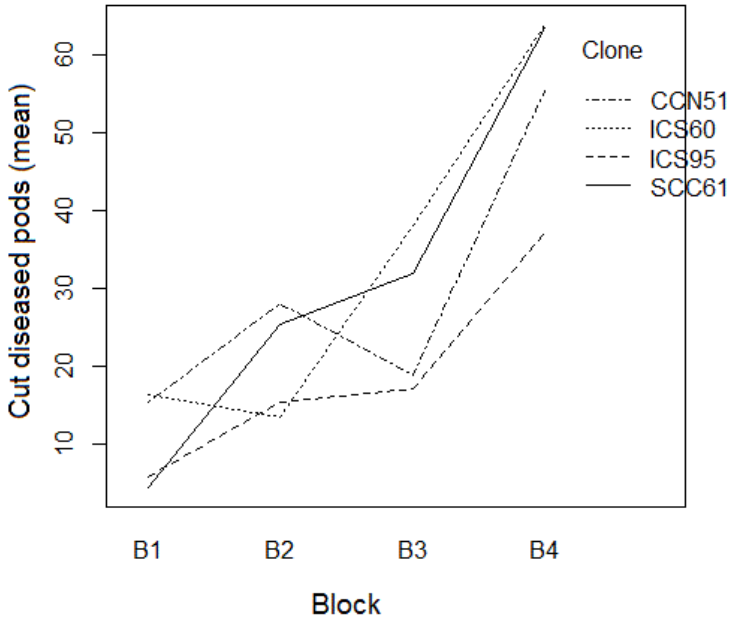

Figure 4. Interaction plot of Cut disease pods with advanced symptoms of FPR for ICS 95, CCN 51, ICS 60 and SCC 61 cacao clones in B1, B2, B3 and B4 blocks in Rionegro (Santander – Colombia).

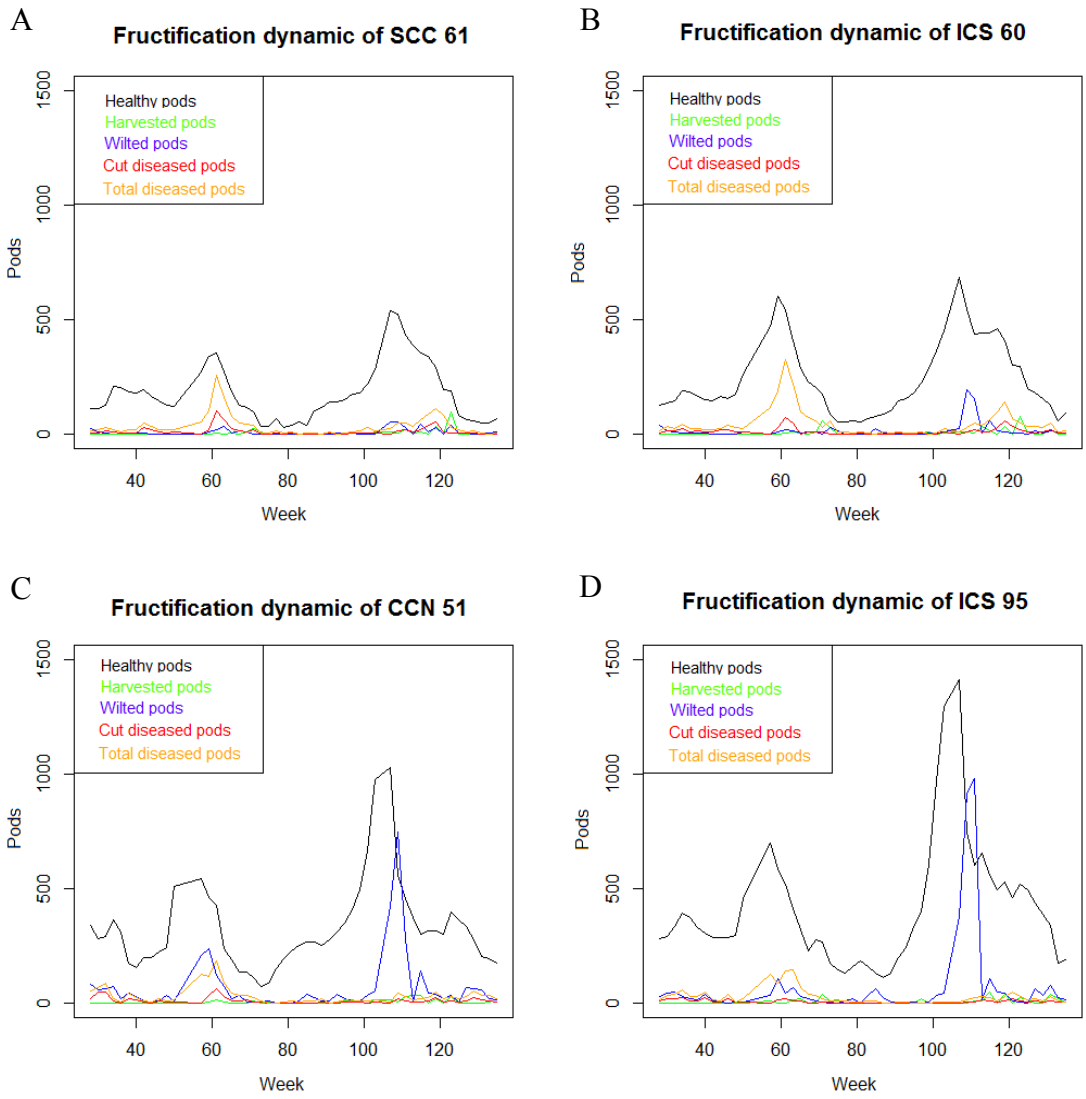

Figure 5. Dynamic of fructification of cacao varieties SCC61 (a), ICS 60 (b), CCN 51 (c) and ICS 95 (d) at experimental plot in La Suiza Research Center in Rionegro (Santander – Colombia).

CONCLUSÕES GERAIS

A alta variabilidade genética e a natureza clonal das populações de *M. roreri* nos principais estados produtores da Colômbia sugerem uma recombinação rara o parcial devida à parasexualidade.

As populações de *M. roreri* nos principais estados produtores da Colômbia agrupassem em três grupos genéticos associados à sua origem observando-se alto fluxo gênico entre ás populações.

Às epidemias da Moniliase estão influenciadas pelas localidades, e o material vegetal e sua localização no campo.