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Estimation of genetic structure of a *Mycosphaerella musicola* population using inter-simple sequence repeat markers

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ABSTRACT. Among the diseases affecting banana (*Musa* sp), yellow Sigatoka, caused by the fungal pathogen *Mycosphaerella musicola* Leach, is considered one of the most important in Brazil, causing losses throughout the year. Understanding the genetic structure of pathogen populations will provide insight into the life history of pathogens, including the evolutionary processes occurring in agrosystems. Tools for estimating the possible emergence of pathogen variants with altered pathogenicity, virulence, or aggressiveness, as well as resistance to systemic fungicides, can also be developed from such data. The objective of this study was to analyze the genetic diversity and population genetics of *M. musicola* in the main banana-producing regions in Brazil. A total of 83 isolates collected from different banana cultivars in the Brazilian states of Bahia, Rio Grande do Norte, and Minas Gerais were evaluated using inter-simple sequence repeat markers. High variability was detected between the isolates, and 85.5% of the haplotypes were singletons in the populations. The highest source of genetic diversity

(97.22%) was attributed to variations within populations. Bayesian cluster analysis revealed the presence of 2 probable ancestral groups, however, showed no relationship to population structure in terms of collection site, state of origin, or cultivar. Similarly, we detected noevidence of genetic recombination between individuals within different states, indicating that asexual cycles play a major role in *M. musicola* reproduction and that long-distance dispersal of the pathogen is the main factor contributing to the lack of population structure in the fungus.

Key words: Inter-simple sequence repeat markers; *Musa* spp; *Mycosphaerella musicola*; Population structure

INTRODUCTION

Fruticulture is one of the fastest growing agricultural sectors in tropical and subtropical countries worldwide. Bananas (*Musa* sp) are among the most economically important fruits; they are cultivated in more than 130 countries and have a global production of approximately 100 million tons (FAO, 2014). In Brazil, the Northeast is the largest banana-producing region, accounting for 35.4% of national production, while Bahia as the principal banana-producing state, producing 44.5% of the crop in the Northeast (IBGE, 2014).

Although banana is one of the world's most important edible crops, the crop is consistently affected by abiotic factors such as drought and root lodging, as well as biotic factors such as pests and diseases, including nematodes, viruses, bacteria, and fungi. Among the fungal diseases, 3 ascomycete species of the genus *Mycosphaerella* are important foliar pathogens. As members of the Sigatoka disease complex, *Mycosphaerella fijiensis* causes black leaf streak disease, or black Sigatoka, *Mycosphaerella musicola* causes Sigatoka leaf spot disease, or yellow Sigatoka, and *Mycosphaerella musae* causes Eumusae leaf spot. Yellow Sigatoka is particularly relevant, as it is found in banana plantations across the country. This disease causes foliar lesions, decreasing the photosynthetic area andreducing fruit production and fruit quality (Ramsey et al., 1990; Chilllet et al., 2009).

The spores involved in the disease cycle of *M. musicola* consist of wind-dispersed sexual ascospores and water-dispersed asexual conidiospores (Rocha, 2012). As spore formation is continuous in humid climates, dispersaloccurs through water accumulation on leaf surfaces following rainfall or dew formation; thus, this disease is very important in such climates, and severe infections are often observed in tillers located under the most mature and infected plants (Cordeiro et al., 2011).

Most commercial banana cultivars are susceptible to yellow Sigatoka and black Sigatoka. The control of these fungi in most regions is largely reliant upon application of systematic fungicides. This long-term dependence on agrochemical control has a negative impact of 15-20% on the final cost of the marketed fruit (Ploetz, 2001).

Information regarding genetic variability in a pathogen population is important for determining appropriate disease management strategies, particularly those related to the establishment of host resistance. Plant breeding programs aimed at disease resistance may benefit from population biology studies of plant pathogens (McDonald and Linde, 2002), as the genetic structure of a population reflects both its history and its evolutionary potential (Leung

et al., 1993).

Genetic markers can be applied to assess genetic diversity and intraspecific and interspecific phylogenetic relationships. By measuring the degree of population subdivision among populations at different spatial scales, the potential for gene flow among populations can be estimated. This information can be used for decision-making in disease control strategies and thus be used to support breeding programs (Rivas et al., 2004; Hayden et al., 2003, 2005; Halkett et al., 2010; Rieux et al., 2011; Robert et al., 2012).

Studies examining the genetic variability of *M. musicola* have been facilitated by the availability of molecular markers such as restriction fragment length polymorphism, random amplified polymorphic DNA, and simple sequence repeat (SSR) microsatellites, which aresuitable for detecting small genomic differences between isolates from different sources. Applications have included specific primer development for molecular detection of *M. musicola* (Molina et al., 2001; Zapater et al., 2008), molecular diversity assessment via random amplified polymorphic DNA, (Moreira et al., 2003; Oliveira et al., 2013), restriction fragment length polymorphism (Hayden et al., 2003), and SSRs (Rocha, 2008), as well as sensitivity to fungicides based on the cytochrome b gene (Gomes, 2012).

The objective of the present study was to evaluate the genetic diversity and population structure of *M. musicola* from banana-producing regions in the states of Bahia, Minas Gerais, and Rio Grande do Norte to support *Musa* breeding programs for the development of yellow Sigatoka-resistant cultivars.

MATERIAL AND METHODS

Genetic material

A total of 83 monosporic isolates of *M. musicola* were randomly collected from different banana genotypes in 16 municipalities of 6 major banana-producing regions in the states of Bahia, Rio Grande do Norte, and Minas Gerais (**Table S1** and Figure 1).

Figure 1. Sampling sites of *Mychosphaerella musicola* in the banana producing areas of Brazil (red dots). States: BA = Bahia; MG = Minas Gerais; RN = Rio Grande do Norte.

For isolation into pure culture, leaf segments with disease symptoms were maintained in a humid chamber at room temperature for 48 h. Conidia from the anamorph phase of *M. musicola* [*Pseudocercospora musae* (Zimm.) Deighton] were then transferred to V8 medium with the aid of a stereomicroscope. Monosporic isolates were preserved using different methods, including Castellani's method, potato dextrose agar+glycerol, glass beads, and paper strips with silica gel (Haddad et al., 2012).

DNA extraction and polymerase chain reaction amplification

Monosporic isolates were grown on V8 medium and incubated at 25°C for 11 days. Harvested mycelium was then dehydrated and macerated in liquid nitrogen. Total DNA was extracted using CTAB (Doyle and Doyle, 1990), with samples diluted to 10 ng/μL and stored at -20°C.

A total of 12 inter-simple sequence repeat (ISSR) primers (Table 1) were employed in the study (Table 1), with polymerase chain reaction amplifications performedon a PTC-100 thermocycler (MJ Research, Waltham, MA, USA) using the following program: initial denaturation at 94°C for 3 min; 30 cycles of denaturation at 94°C for 40 s, primer annealing at 48° or 50°C (depending on the primer) for 40 s, and extension at 72°C for 1 min; followed by a final extension step at 72°C for 4 min (Williams et al., 1990). Amplification products were separated by gel electrophoresis in 2.5% ultrapure agarose 1000 (Invitrogen, Carlsbad, CA, USA), treated with 0.5 μg/mL ethidium bromide, and visualized on an ultraviolet transilluminator. The band profiles obtained for each primer were transformed into a binary matrix (presence = '1' and absence = '0') and concatenated as a single multilocus profile for each isolate.

Table 1. ISSR primers used in *Mycospharella musicola* population study, motif, sequence and annealing temperature in amplification reaction.

Primer	Motif	Sequence 5'-3'	Ta
ISSR 12	$(GA)_8RC$	GAG AGA GAG AGA GAG ARC	48
ISSR 31	$CR(CAC)_5$	CRC ACC ACC ACC ACC AC	48
ISSR 33	$(CAG)_5$	CAG CAG CAG CAG CAG	50
ISSR 35	$(CAG)_5YC$	CAG CAG CAG CAG CAG YC	48
ISSR 36	$CR(CAG)_5$	CRC AGC AGC AGC AGC AG	50
ISSR 39	$(GTG)_5RC$	GTG GTG GTG GTG GTG RC	50
ISSR 58	$(ACG)_5RC$	ACG ACG ACG ACG ACG RC	50
ISSR 66	$(TAG)_5RC$	TAG TAG TAG TAG TAG RC	48
ISSR 86	$(CGA)_5RC$	CGA CGA CGA CGA CGA RC	50
ISSR 90	$(GAA)_5RC$	GAA GAA GAA GAA GAA RC	50
ISSR 94	$(GTA)_5RC$	GTA GTA GTA GTA GTA RC	48
ISSR 101	$(GGA)_5RC$	GGA GGA GGA GGA GGA RC	48

Ta = annealing temperature.

Statistical analysis

The binary matrix of multilocus data was used to identify haplotypes using the SNAP Workbench software (Price and Carbone, 2005). Analysis of molecular variance was performed using the Arlequin 3.5.1.2 software (Excoffier and Lischer, 2010).

To calculate genetic differentiation between states, Wright's F statistic and its estimator, G_{ST}, were employed using the Popgene version 1.32 software (Yeh and Boyle, 1997). G_{ST} values were used to calculate the number of migrants per generation (N_m), using the formula:

$N_m = 0.5 (1 - G_{ST}) / G_{ST}$ (McDermott and McDonald, 1993). The relationship between genetic and geographic distance of the *M. musicola* isolates was analyzed using the Mantel test with 1000 permutations.

Richness values were estimated based on the number of haplotypes for the total population and its subpopulations (sites). To measure genotypic diversity (richness and equitability), Shannon-Wiener (H'), Hill (N_1) and Stoddart and Taylor (G') indices were calculated, in addition to the *E5* index = [(*G* - 1) / (*N1* - 1)] (Grünwald et al., 2003), which measures the evenness of the variability distribution. The richness, diversity indices, and the Mantel test were estimated using the 'vegan' package of the R software (Oksanen et al., 2013).

Linkage disequilibrium (I_A and r_d) was estimated in comparison with the expected distribution for loci when combined for the total population, states, and municipalities. The multilocus index for linkage disequilibrium r_d was estimated and the null hypothesis $r_d = 0$ (gametic equilibrium) was tested via 1000 permutations using the Multilocus software (Agapow and Burt, 2001).

To infer the population structure and the fraction of genetic clusters in each isolate, genetic cluster analysis was performed using the Structure 2.3.4 software (Pritchard et al., 2000). The dataset was analyzed using the mixed model 20 times with 100,000 Markov chain Monte Carlo chains after a "burn-in" of 25,000. The number of base groups (k) ranged from 1-30.

RESULTS AND DISCUSSION

Genetic population studies increase our understanding of evolutionary mechanisms present in pathogen populations and their epidemiological implications. This knowledge is important for developing more rational management strategies based on durable disease resistance and adequate fungicide management. However, few population studies have been conducted on fungi that cause Yellow and Black Sigatoka diseases, with particularly limited data for *M. musicola*. In previous studies, populations of *M. fijiensis* as well as *M. musicola* showed high genetic diversity, with sexual reproduction appearing to play a main role in the population genetic structure (Hayden et al., 2003, 2005).

In our study, high genetic variability was observed in the *M. musicola* population studied, with most (85.5%) isolates presenting unique haplotypes (Figure 2), and the greatest genetic variation occurring between haplotypes within municipalities (Table 2). However, no population structure was detected in regard to the collection sites and/or varieties from which they were isolated according to the Mantel test. Haplotype H 29 was the most frequent (N = 5), which was shared among isolates collected in different municipalities (Figure 2).

Despite the absolute differences in genetic diversity between different states, based on the H', G', and N_1 indices (Table 3), this may be because of the unequal sizes of samples obtained, as there was no significant difference between the richness and equitability data when the subpopulations were ranked using a rarefaction curve. According to the diversity indices, the populations showed high genotypic diversity with maximum values of richness and equitability, except for the population from Bahia (Table 3), in which some haplotypes shared among subpopulations were more frequent.

Based on population structure analysis, 2 probable ancestral groups (k = 2) were identified, corresponding to less than 15% of the genetic fraction shared among groups (Figure 3). Despite the different sample sizes between the states, 40% of the isolates from Bahia were grouped in 'cluster 1' (represented in green), whereas for those from Rio Grande do Norte, a total of 45% of the population, were grouped in 'cluster 2' (represented in red) (Figure 3). In

turn, the population from Minas Gerais (50%) comprised a group consisting of a mixture of the 2 ancestral groups. Furthermore, proportionate to the size of the population, Minas Gerais-presented the highest number of shared haplotypes in the population. Most of these shared haplotypes were grouped in 'cluster 1' (green), whereas most unique haplotypes were grouped in 'cluster 2' (red; Figure 3).

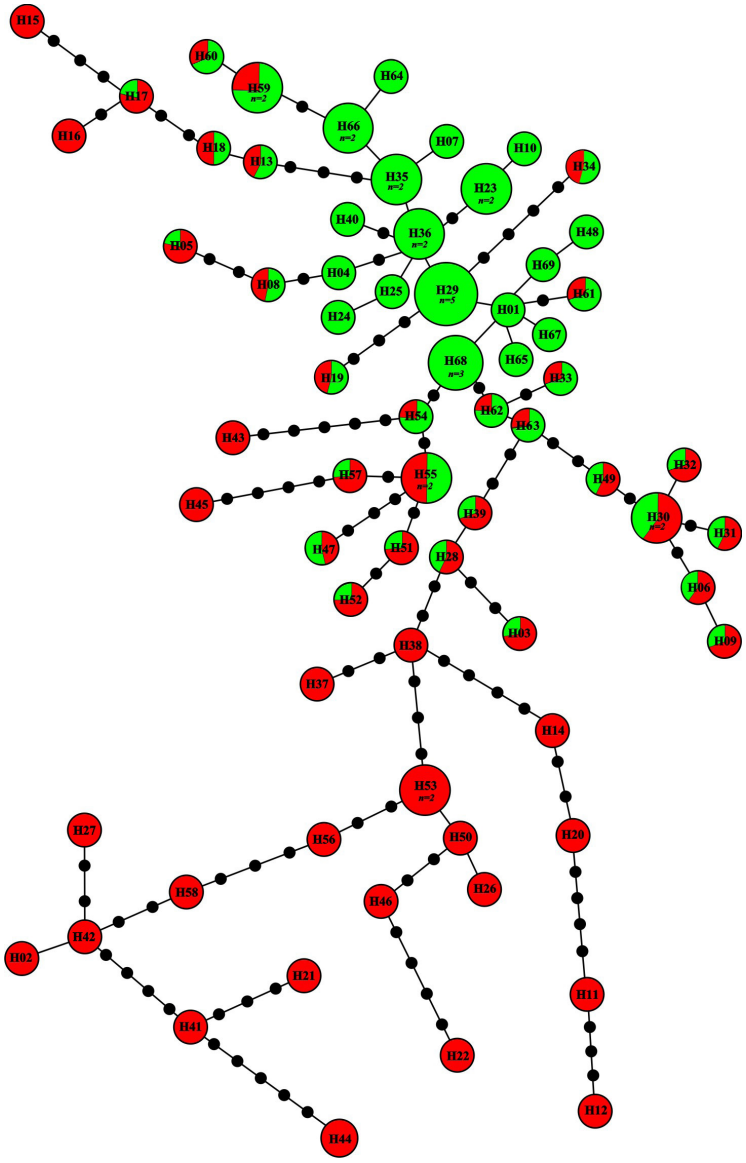

Figure 2. Minimum spanning tree of the population studied (83 strains). In the circles there are the names of haplotypes and number of strains. Branch length does not represent the genetic distances among the strains. Black dots along the branches represent a missing haplotype. Colors represent ancestral groups (k) according to Bayesian population structure analysis.

Table 2. Genetic variability of *Mycosphaerella musicola* isolates based on AMOVA.

Source of variation	d.f.	Variance components	Variation (%)	*F*	P
Among states	2	0.038	0.98	0.00977	0.37341
Among populations within states	13	0.070	1.80	0.01818	0.22190
Within populations	67	3.796	97.22	0.02778	0.15640
Total	82	3.905			

F = fixation index; P = significance, d.f. = degree of freedom.

Table 3. Genotypic diversity, richness and equitability of *Mycosphaerella musicola* populations in different locations.

Location[a]	N[b]	Statistics[c]					
		H'	N1	G'	E (gn)	E (g)	E5
Rio Grande do Norte	11	2.40 (2.06-2.73)[d]	11 (8.92-13.08)	11 (8.83-13.17)	11	5.0	1.0
Baraúna	5	1.6 (1.1-2.1)	5 (3.6-6.4)	5 (3.4-6.5)	5	5.0	1.0
Bahia	52	3.74 (3.54-3.93)	42.01 (37.28-46.74)	37.56 (31.96-43.15)	45	4.92	0.89
Barra do Choça	8	2.08 (1.69-2.47)	8 (6.19-9.81)	8 (6.13-9.87)	8	5.0	1.0
B. Jesus da Lapa	14	2.64 (2.34-2.94)	14 (11.68-16.32)	14 (11.47-16.53)	14	5.0	1.0
Itajuípe	9	2.20 (1.83-2.57)	9 (7.08-10.92)	9 (7.05-10.94)	9	5.0	1.0
P. Tancredo Neves	7	1.95 (1.52-2.37)	7 (5.26-8.74)	7 (5.23-8.77)	7	5.0	1.0
Minas Gerais	20	2.93 (2.66-3.20)	18.66 (15.72-21.57)	18.18 (15.06-21.30)	19	4.95	0.97
Lavras	9	2.20 (1.83-2.56)	9 (7.11-10.90)	9 (7.10-10.90)	9	5.0	1.0
Jaíba	11	2.27 (1.92-2.62)	9.70 (7.54-11.85)	9.30 (7.12-11.49)	11	4.82	0.95
Total	83	4.15 (3.99-4.30)	63.34 (57.20-69.48)	55.11 (47.87-62.36)	69	-	0.87

[a]Locations in bold represent the sampled states; locations in italic font represent the cities sampled; cities discarded due to sample size (less than five): Apodi; Ipanguaçu; Guandu; Governardor Mangabeira; Morro de São Paulo; Muritiba; Mutuípe; Teolândia; Wenceslau Guimarães. [b]N = total population. [c]H' = Shannon index; N1 = Hill index; G' = Stoddart & Taylor index; E(gn) = richness; E(g) = richness sorted based on the small sample number for regions (5); E_5 = evenness index estimated by (1 / λg) -1) / (N1 - 1). [d]Numbers in parentheses represent the lower and upper limits with 0.5% probability in the indexes estimated.

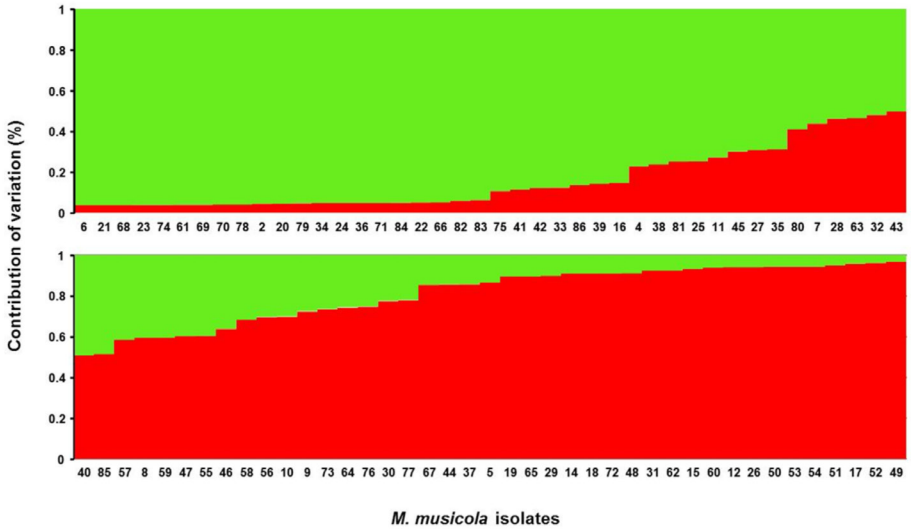

Figure 3. Cluster analysis of *Mycosphaerella musicola* from different locations using ISSR primers. Each bar in the figure divided into colors represents an individual whereas K is the number of clusters (assuming k = 2). Isolates are ordered according to the probability of inclusion in the group. Isolates are identified by their respective numbers as presented in Table 1.

Based on cluster analysis, the population studied was predominately clonal due to the number of groups identified. The low number of clusters may be associated with a limited number of sexual recombination events occurring in the fungus at the end of the epidemic cycle, as well as with the prevalence of asexual reproduction cycles. Because *M. musicola* has a mixed-type reproduction strategy, the genotypes generated by sexual recombination may have rapidly increasedtheir frequency through asexual reproduction (McDonald and Linde, 2002).

The differences in the genetic proportions and the predominance of ancestral groups established in the 3 regions may be associated with their different environmental conditions. Altitude, humidity, and temperature are factors known to influence the prevalence and spatial distribution of Sigatoka species (Mouliom-Pefoura et al., 1996; Marín et al., 2003). In addition, environmental conditions can influence the predominance of the spore type.

Rocha et al. (2012) observed that disease severity spikes of Yellow Sigatoka during the dry season in a city in Minas Gerais were caused by high concentrations of ascospores. Therefore, the recombination rate and, consequently, the genetic variability of *M. musicola* populations may have been influenced by environmental conditions. The municipalities of Lavras and Jaíba have different altitudesand climatic conditions; the former has lower temperatures and higher altitude compared to the latter.

Movement of infected material between different sites and wind dispersal are principal factors that can contribute to the presence of isolates from different geographical regions in the same ancestral group. For example, haplotype H 68 was found in the municipalities of both Barra do Choça and Wenceslau Guimarães, which are 160 km apart from each other. These assumptions are consistent with the data obtained for differentiation between the subpopulations (between states) and the number of migrants per generation, given thatthe comparison of genetic differentiation between state populations showed low genetic differentiation and a high number of migrants. The states of Bahia and Minas Gerais presented the highest number of migrants between populations and thus lower genetic differentiation (Table 4). In addition, there was no correlation between genetic distance and geographic distance ($r = 0.03$; $P = 0.31$).

Table 4. Genetic differentiation estimated by the G_{ST} index (superior diagonal) and gene flow (inferior diagonal, Nm) for the three states: Bahia, Minas Gerais e Rio Grande do Norte.

Location	Bahia	Minas Gerais	Rio Grande do Norte
Bahia	-	0.018	0.046
Minas Gerais	27.29	-	0.040
Rio Grande do Norte	10.47	11.93	-

Gene flow was observed to be high between all states, particularly between Bahia and Minas Gerais (Table 4). This likely occurred because of the proximity of these states and the efficient long-distance dispersal of ascospores and/or movement of infected planting material. Gene flow, denoted by N_m, expresses a biological meaning for the mathematical value of population differentiation, indicated by G_{ST} (Linde et al., 2002). Hence, the N_m observed for the populations studied is considered to be high, given both that this number refers to the number of migrants per generation and that this fungus undergoes numerous reproductive cycles per year. Thus, under favorable conditions, a migrant individual can reach the same proportions as native individuals.

The differentiation between the sites may be related to topographical barriers, climatic differences, vegetation differences, genetic barriers followed by fungalcolonization events, or

a combination of these factors, which can all influence gene flow. A larger number of migrants leads to greater gene flow. This explains the introduction of new variants of a pathogen to a specific site, which may alter the pathogen relationships in the pathosystem (McDonald and Linde, 2002).

The high variability found in the *M. musicola* population, as explained based on the large number of unique haplotypes, may be related to sexual recombination events. Although not detected in the population studied (Figure 4), sexual reproduction is common for this pathogen (Cordeiro et al., 2005). Pathogens have a mixed reproduction system and thus a high rate of evolution. In this system, new genotypes generated during sexual recombination can be selected and adapted to the environmental conditions, subsequently increasing their frequency in the population through asexual reproduction (McDonald and Linde, 2002). The occurrence of new recombinant genotypes can also create new combinations of virulence alleles that are capable of supplanting disease resistance in improved plant genotypes (McDonald and Linde, 2002).

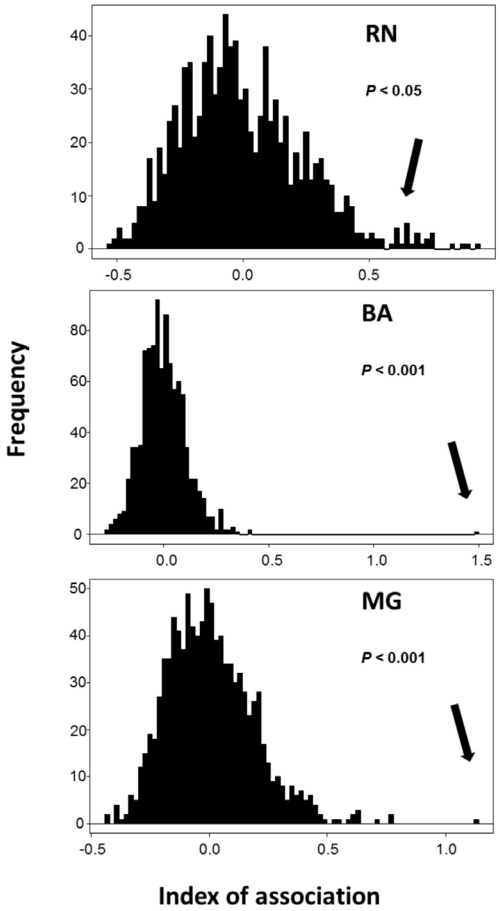

Figure 4. Histograms representing the distribution of randomly recombining populations of *Mycosphaerella musicola* from the states of Rio Grande do Norte, Bahia and Minas Gerais, using the Index of association (I_A). Observed values, indicated by arrows, within the randomized distribution represent the occurrence of recombination.

The genetic variability of *M. musicola* populations is often reported as moderate. Based upon restriction fragment length polymorphismdata, Hayden et al. (2005) reported moderate genetic and genotypic diversity in populations in Australia. In a global study, most of the examined *M. musicola* population (62%) consisted of unique haplotypes (Hayden et al., 2003).

In Brazil, relatively few studies have been conducted to assess the genetic diversity of *M. musicola*. These studies have either utilized less informative random amplified polymorphic DNA markers, as in the case of studies by Oliveira et al. (2013) and Montarroyos (2005), or were restricted to only one or few sample sites, as in the study conducted by Gomes (2012). Despite these limitations, all datasets revealed high genetic variability among *M. musicola* isolates, as indicated by the large number of unique haplotypes and high genotypic diversity indices.

Limited analysis of *M. musicola* populations in Brazil may also reflect the increased presence of black Sigatoka, which is typically the more threatening worldwide compared to Yellow Sigatoka. However, Yellow Sigatoka continues to be of significant economic importance in some areas in the states of Bahia, Minas Gerais, and Rio Grande do Norte, where black Sigatoka does not occur, or where *M. musicola* continues to be more prevalent, even in the presence *M. fijiensis*, because of the more favorable ecological conditions (Cordeiro et al., 2005).

Based on I_A and r_d, the hypothesis for sexual recombination was rejected for the 3 states (Figure 3), indicating that the subpopulations are not in linkage disequilibrium. This indicates that alleles are not randomly linked, thus showing a lack of sexual recombination. I_A is the traditional measure of multilocus linkage disequilibrium (Haubold et al., 1998); the "distance" (number of loci in which they differ) between all pairs of individuals is calculated, and the variation in these distances is compared against that expected in cases of no linkage disequilibrium. Despite the lack of evidence for sexual recombination in the population studied, its influence cannot be ruled out. The sexual cycle of *M. musicola* is known to occur predominantly at the end of the epidemic cycle, whereas reproduction is predominantly asexual during secondary disease cycles. The large number of asexual cycles, combined with a relatively narrow genetic base, as indicated by the low number of genetic groups based on analysis with the STRUCTURE software, likely influences the lack of recombination signals, given that there is a predominance of certain groups in relation to the different states.

Given the high diversity found in this study, together with a mixed reproduction system (both sexual and asexual reproduction) (Rocha et al., 2012), *M. musicola* shows a high evolutionary potential that is appropriate for overcoming plant host resistance and for the emergence of fungicide-resistant isolates.

The predominance of asexual cycles in *M. musicola* should be further examined. For example, if isolates resistant to the main systemic fungicides and/or with altered pathogenicity (virulence/severity) already exist in the pathogen population, selection for these traits may increase their prevalenceover a short time interval. Furthermore, the phytosanitary barrier systems to prevent the transport of infected plant material and reduce gene flow between Brazilian states should be examined.

Knowledge of the distribution of genetic variability within and among *M. musicola* populations is essential for adopting disease control strategies and maintaining durable resistance across different banana-producing regions.

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Supplementary material

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