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Molecular Markers Dispute the Existence of the Afro-Andean Group of the Bean Angular Leaf Spot Pathogen, *Phaeoisariopsis griseola*

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ABSTRACT

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Coevolution of the angular leaf spot pathogen, *Phaeoisariopsis griseola*, with its common bean host has been demonstrated, and *P. griseola* isolates have been divided into Andean and Mesoamerican groups that correspond to defined bean gene pools. Recent characterization of *P. griseola* isolates from Africa has identified a group of isolates classified as Andean using random amplified polymorphic DNA (RAPD), but which are able to infect some Mesoamerican differential varieties. These isolates, designated Afro-Andean, have been identified only in Africa. Random amplified microsatellites, RAPD, and restriction digestion of amplified ribosomal intergenic spacer region were used to elucidate the relationships among the Afro-Andean, Andean, and Mesoamerican groups of *P. griseola*. Cluster and multiple correspondence analysis of molecular data separated isolates into Andean and Mesoamerican groups, and the Afro-Andean isolates clustered with Andean isolates. Analysis of molecular variance ascribed 2.8% of the total genetic variation to differences between Afro-Andean and Andean isolates from Africa. Gene diversity analysis revealed no genetic differentiation (G_{ST} = 0.004) between Afro-Andean and Andean isolates from Africa. However, significant levels of genetic differentiation (G_{ST} = 0.39) were observed between Afro-Andean or Andean isolates from Africa and Andean isolates from Latin America, revealing significant geographical differentiation within the Andean lineage. Results from this study showed that Afro-Andean isolates do not constitute a new *P. griseola* group and do not represent long-term evolution of the pathogen genome, but rather are likely the consequents of point mutations in genes for virulence. This finding has significant implications in the deployment of resistant bean genotypes.

The knowledge that single gene changes in pathogen isolates can affect the durability of resistance in host plants has led to management strategies that require monitoring of the pathogen (27,32,40). Identification of the range of genetic variation that exists in a pathogen population improves the ability to screen and select for resistant germ plasm, facilitates the breeding of resistant cultivars, and refines the development of cultivars for specific locations. The choice of isolates and selection of field test locations for breeding purposes also depend on pathogen subdivisions, whereas strategies for deploying resistant cultivars differ for clonal and sexually recombining organisms (31,32). Understanding the geographical range over which genetic differentiation among isolates occurs is important for breeding programs because it allows development of strategies that target variation of different populations in different regions.

Angular leaf spot (ALS) of common bean, caused by *Phaeoisariopsis griseola* (Sacc.) Ferraris (43), is a major constraint to common bean production in tropical and subtropical countries. On susceptible cultivars, ALS causes premature defoliation, shriveled pods, shrunken seeds, and losses in yield as high as 80% (3,13,20,42,43,45). In Africa, where beans constitute the most important source of dietary protein, with some countries such as Burundi recording the highest consumption per capital, ALS is considered the number one constraint to bean production (38) with annual losses estimated at 374,800 t (50).

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With the intensification of bean production, differences in the pathogenic and ecological adaptation of the ALS fungus have become apparent. An understanding of the population genetics of this pathogen is important to better understand disease outbreaks, predict future disease development, and develop effective strategies for breeding for resistance to this disease (27,32). In addition, this should reveal the evolutionary history of the pathogen, its potential to evolve (30), and the role of specific evolutionary processes in maintaining or influencing the pathogen population structure.

Previous studies have reported pathotypic and genetic variation for *P. griseola* (7,9,12,15,19,29,44), but they have been based on small sample sizes and often address specific geographical regions. On the basis of pathogenicity, *P. griseola* isolates have been divided into two groups, Andean and Mesoamerican, which correspond to the two gene pools defined for its common bean host (12,37,46) and originating from the two main centers of diversity. This grouping has also been revealed by random amplified polymorphic DNA (RAPD) (9,19,29) and isozyme markers (5,12). Andean isolates exhibit a narrow host range, attacking only beans belonging to the Andean gene pool. Although isolates collected from Mesoamerican genotypes are more virulent and aggressive on Mesoamerican beans, they have a wide virulence spectrum, infecting both Mesoamerican and Andean beans (10,19). These differences in *P. griseola* isolates are thought to reflect the diversity found in the bean gene pools (4).

The two main pathogen groups also occur in Africa. However, in 1997, a group of *P. griseola* isolates that were identified as typical Andean based on RAPD analysis were found to colonize some Mesoamerican host differential genotypes (11). This un-

expected reaction appears unique to Africa, and so far has been observed only with isolates from Africa, and were tentatively placed in a new group called "Afro-Andean" (11). The origin and ecological significance of Afro-Andean isolates is not known, but it is possible that these isolates represent a new group within *P. griseola* that arose through mutation or recombination, followed by ecological adaptation under agroecological conditions found in Africa. This variation is shaped by the forces of selection and genetic drift. Major selective forces may be imposed by the degree of specialization in host–pathogen interactions, control measures, or more general environmental constraints. These factors act to generate differences in the distribution of phenotypic and genotypic variation among plant pathogen populations that can lead to the emergence of new pathogen forms. It is possible that any one of these factors alone or in combination may have resulted in the emergence of the Afro-Andean group.

In Africa, unlike in Latin America, Andean and Mesoamerican bean varieties are grown together (41,50). Farmers grow mixtures to ensure elevated and stable yield and consciously manage them for different conditions such as soil, crop associations, or seasons (47). Smaller seeded cultivars are selected for less fertile soils and home consumption, larger for fertile soils and sale. The diversity in mixtures is often great (16,48), and the number of seed types and colors per farmer mixture can be as high as 20. However, in Latin America, Andean beans and Mesoamerica beans are cultivated in separate regions, in the Andean and Middle America regions, respectively. This difference in production systems between Africa and Latin America might influence the composition and evolution of *P. griseola* populations.

Given the unique characteristic exhibited by the Afro-Andean group, this study was carried out to determine whether the group was a separate subgroup within *P. griseola* that has not been described previously. Molecular markers, i.e., random amplified microsatellites (RAMS) (21,22,34), RAPD, and restriction fragment length polymorphism of the amplified ribosomal intergenic spacer region (IGS-RFLP) (2,14,28,36,51), were used to analyze the *P. griseola* genome and test this hypothesis. In addition, the genetic diversity within *P. griseola* isolates collected from Latin America was compared with that occurring in Africa. The specific objectives were to (i) elucidate the relationship between isolates belonging to the Afro-Andean, Andean, and Mesoamerican groups of *P. griseola*, and (ii) determine the level of genetic diversity within Andean and Mesoamerican *P. griseola* groups within and between Latin America and Africa.

MATERIALS AND METHODS

Selection and characterization of *P. griseola* isolates. A total of 131 monosporic *P. griseola* isolates were used in this study. The isolates were obtained from naturally infected common bean tissues collected from farmers' fields in Africa and Latin America (Table 1). Isolation, purification, single spore production, and preservation of the isolates were done as described by Pastor-Corrales et al. (38). Virulence of monosporic cultures was determined on a set of 12 common bean differential genotypes recommended during the first ALS workshop held by CIAT in 1995. Six of the differentials belong to the Andean gene pool, and the other six belong to the Mesoamerican gene pool (Table 1). Fifty-two isolates belonged to the Andean pathogen group, 52 were Mesoamerican, and 27 were Afro-Andean (11). Isolates that were classified as Afro-Andean had previously been found to have RAPD profiles typical of Andean isolates, but infected some Mesoamerican differential genotypes. Lyophilized monosporic cultures were revived by adding a sucrose-peptone solution to make a spore suspension that was plated on V8-juice agar medium and incubated at approximately 24°C. The virulence phenotypes of these isolates were reconfirmed as described by Pastor-Corrales et al. (38).

DNA extraction. To produce *P. griseola* mycelium for DNA extraction, 10 fungal disks (1 cm in diameter), collected from the tips of actively growing monosporic cultures, were introduced into Erlenmeyer flasks (200 ml) containing 60 ml of liquid V8-juice medium. The cultures were placed on a rotary shaker (134.5 × *g*) and incubated at room temperature for 12 days. Mycelia were harvested by filtration through cheesecloth and freeze-dried. For DNA extraction, freeze-dried mycelia (0.25 g) were frozen in liquid nitrogen and ground to a fine powder in a mortar. Ground mycelium was transferred to a 2-ml microcentrifuge tube, and DNA was extracted by the sodium dodecyl sulfate method as described by Möller et al. (33). The pellet was washed with 70% ethanol, dried, and resuspended in 1× Tris-EDTA (TE) buffer containing 10 mg/ml of RNase A. Tubes were incubated at 37°C for 1 h, and the DNA was precipitated with 1/10 volume of 3 M NaAc, pH 5.2, and 2 volumes of 95% ethanol. The pellet was dried and finally suspended in 0.1× TE buffer. To determine the quality, extracted DNA was electrophoresed on 0.7% agarose gels. The DNA concentration was measured with a fluorometer (Hoefer DyNA Quant 2000; Pharmacia Biotech, Piscataway, NJ) and adjusted to 5 ng/μl in 0.1× TE buffer.

RAMS-polymerase chain reaction. Seven RAMS primers [(CCA)n, (CGA)n, (GT)n, (AG)n, (CA)n, (TG)n, and (CT)n] (21) anchored at the 5′ end were used to amplify microsatellite regions within the *P. griseola* genome. Polymerase chain reactions (PCR) were performed in 25 μl of final volume containing 0.2 mM dNTPs, 1× PCR buffer, 1.5 mM $MgCl_2$, 2 μM primer, 5 ng of genomic DNA, and 0.5 units of *Taq* DNA polymerase. Amplifications were done in a thermal cycler (MJ Research, Waltham, MA) programmed for an initial step of DNA denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 58°C (GT), 61°C (CGA), 50°C (AG), 55°C (TG), 41°C (CA and CT), and 55°C (CCA) for 45 s, 72°C for 2 min, and a final extension of 7 min at 72°C.

RAPD analysis. Seven RAPD primers (OPA2, OPA11, OPA18, OPB15, OPC5, OPF1, and OPH8 [Operon Technologies, Inc., Alameda, CA]) that exhibited polymorphisms and yielded consistent banding patterns were used to amplify *P. griseola* DNA. DNA amplification was performed in a thermal cycler (MJ Research) with initial step at 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min, and a final cycle at 72°C for 10 min. Reactions were carried out in 25-μl final volumes containing 1× DNA polymerase buffer (50 mM Tris-HCl [pH 8.5], 2.0 mM $MgCl_2$, 50 mM KCl, and 0.1% Triton X-100), 0.2 mM each dNTP, 0.4 μM primer, 1 unit of Ampli*Taq* DNA polymerase, and 20 ng of genomic DNA.

Electrophoresis. DNA amplification products from RAPD and RAMS-PCR were separated in 1.2% agarose gels at a constant 60 volts in 1× Tris-borate-EDTA buffer for 5 to 6 h at room temperature. Fragments were visualized under 300 nm UV light after staining with ethidium bromide. Gel images were captured with a documentation system (Eagle Eye Gel; Stratagene, La Jolla, CA), and band position was determined using the Quantity One Scientific Software, version 4 (Bio-Rad Laboratories, Hercules, CA). For each primer, nonreproducible fragments between repetitions were disregarded. Profiles for each primer were compared on the basis of the presence or absence of a fragment presumed to be the same length, and fragments of the same size were scored as identical.

IGS-RFLP. The IGS of rDNA was amplified with the primers CLN12 (5′-CTGAACCGCCTCTAAGTCAG-3′) and CLNTS1 (5′-AATGAGCCATTCGCAGTTTC-3′), with priming sites at the 3′ end of the 28S gene and the 5′ end of the 18S gene, respectively (28). PCR reactions were performed in 50 μl containing 0.2 mM dNTPs, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.25 μM each primer, 50 ng of genomic DNA, and 2 units of Ampli*Taq* DNA polymerase. Amplification conditions consisted of an initial step of DNA denaturation at 94°C for 5 min, followed by 35 cycles of

TABLE 1. Pathotype designation, virulence phenotype and group, and the origin of Andean, Afro-Andean, and Mesoamerican *Phaeoisariopsis griseola* isolates used in this study

Pathotype[a]	Virulence phenotype[b]	Virulence group[c]	No. of isolates	Origin
14-0	bcde	Andean	10	Kenya, Malawi, Zambia, Rwanda, Uganda, Colombia, Ecuador
31-0	abcde	Andean	10	Burundi, Congo, Malawi, Rep. South Africa, Tanzania, Malawi, Zambia, Ecuador, Colombia
30-0	bcde	Andean	9	Kenya, Burundi, Uganda, Zambia, Malawi, Rwanda, Colombia, Ecuador
63-0	abcdef	Andean	5	Uganda, Colombia
15-0	abcd	Andean	4	Kenya, Malawi, Madagascar, Colombia
62-0	bcdef	Andean	3	Kenya, Ethiopia, Colombia
12-0	cd	Andean	2	Colombia
29-0	acde	Andean	2	Colombia, Ecuador
47-0	abcdf	Andean	2	Colombia, Uganda
13-0	acd	Andean	1	Ecuador
28-0	cde	Andean	1	Colombia
46-0	bcdf	Andean	1	Colombia
61-0	acdef	Andean	1	Colombia
58-0	bdef	Andean	1	Congo
63-32	abcdefl	Afro-Andean	5	Ethiopia, Kenya
59-32	abdefl	Afro-Andean	2	Rwanda, Ethiopia
31-1	abcdeg	Afro-Andean	2	Malawi
63-33	abcdefgl	Afro-Andean	2	Ethiopia, Rwanda
63-36	abcdefil	Afro-Andean	2	Kenya, Ethiopia
63-17	abcdefgk	Afro-Andean	1	Uganda
31-36	abcdeil	Afro-Andean	1	Uganda
47-1	abcdfg	Afro-Andean	1	Uganda
63-1	abcdefg	Afro-Andean	1	Uganda
62-4	bcdefi	Afro-Andean	1	Kenya
14-5	bcdgi	Afro-Andean	1	Malawi
30-5	bcdegi	Afro-Andean	1	Malawi
46-6	bcdfhi	Afro-Andean	1	Kenya
63-6	abcdefhi	Afro-Andean	1	Kenya
62-32	bcdefl	Afro-Andean	1	Kenya
62-33	bcdefgl	Afro-Andean	1	Rwanda
56-32	defl	Afro-Andean	1	Ethiopia
39-36	abcfil	Afro-Andean	1	Malawi
59-36	abdefil	Afro-Andean	1	Uganda
31-39	abcdeghil	Mesoamerican	6	Uganda, Kenya, Malawi, Honduras, Puerto Rico
31-47	abcdeghijl	Mesoamerican	4	Uganda, México, Honduras, Panama
63-39	abcdefghil	Mesoamerican	4	Brazil, Uganda, Rwanda, Malawi
63-7	abcdefghi	Mesoamerican	2	Uganda
63-63	abcdefghijkl	Mesoamerican	3	Honduras, Nicaragua
63-37	abcdefgil	Mesoamerican	2	Ethiopia, Malawi
63-47	abcdefghijl	Mesoamerican	2	Malawi, El Salvador
63-31	abcdefcghijk	Mesoamerican	2	El Salvador, Brazil
7-55	abcghikl	Mesoamerican	2	Congo, Guatemala
63-21	abcdefgik	Mesoamerican	1	Uganda
3-7	abghi	Mesoamerican	1	Uganda
7-7	abcghi	Mesoamerican	1	Uganda
21-7	abeghi	Mesoamerican	1	Uganda
23-7	abceghi	Mesoamerican	1	Rwanda
31-7	abcdeghi	Mesoamerican	1	Uganda
7-23	abcghik	Mesoamerican	1	Malawi
31-23	abcdeghik	Mesoamerican	1	Malawi
3-39	abghil	Mesoamerican	1	Kenya
21-39	abeghil	Mesoamerican	1	Malawi
23-39	abceghil	Mesoamerican	1	Congo
61-39	acdefghil	Mesoamerican	1	Uganda
31-55	abcdeghikl	Mesoamerican	1	Kenya
15-7	abcdghi	Mesoamerican	1	Puerto Rico
63-11	abcdefghj	Mesoamerican	1	Honduras
31-15	abcdeghij	Mesoamerican	1	El Salvador
7-31	acghijk	Mesoamerican	1	México
31-31	acdeghijk	Mesoamerican	1	Nicaragua
7-43	acghjl	Mesoamerican	1	Nicaragua
47-47	acdfghijl	Mesoamerican	1	Brazil
31-51	acdeghkl	Mesoamerican	1	Nicaragua
15-55	acdghikl	Mesoamerican	1	Brazil
15-63	acdghijkl	Mesoamerican	1	Honduras
23-63	aceghijkl	Mesoamerican	1	Guatemala
31-63	acdeghijkl	Mesoamerican	1	México

[a] Pathotype designation is based on the sum (binary values) of susceptible cultivars based on a CIAT 1-to-9 scale, in which ratings of 1 to 3 are resistant and 3 and greater are susceptible. The first number is based on isolate reaction to Andean differentials, and the last number on reaction to Mesoamerican differential genotypes.

[b] Virulence phenotype is based on susceptible (compatible) response to each isolate on a set of 12 differential varieties: Andean differential genotypes are A = Don Timoteo, B = G 11796, C = Bolon Bayo, D = Montcalm, E = Amendoim, and F = G5686; Mesoamerican differential genotypes are G = PAN 72, H = G2858, I = Flor de Mayo, J = Mexico 54, K = BAT 332, and L = Cornell 49242. Lowercase letters (a to i) indicate compatibility of the respective common bean differential genotype to the specific isolate or pathotype of *P. griseola.*

[c] Virulence grouping is based on the bean variety from which the isolate was collected as confirmed by differential interaction of the isolate with Andean or Mesoamerican differential genotypes under greenhouse conditions.

94°C for 40 s, 63°C for 1 min, and 72°C for 3 min. This was followed by a final extension at 72°C for 10 min. The size of the IGS fragment was estimated on agarose gels using a 100-bp step ladder (Promega, Madison, WI).

Following amplification, the IGS-PCR products were digested with *Rsa*I, *Hae*III, *Cfo*I, *Alu*I, or *Taq* αI according to the manufacturer's recommendations. The IGS-RFLP fragments were separated in 1.2% agarose gels and visualized under UV light. The images were captured with an Eagle Eye Gel documentation system (Stratagene). Band positions were determined using the Quantity One Scientific Software, version 4 (Bio-Rad), as described previously.

Analysis of genetic similarity. To estimate the genetic relationships among isolates, a phylogenetic tree was constructed from virulence, RAPD, RAMS, and IGS-RFLP data. The genetic similarity between two isolates was calculated based on Dice's coefficient with the SimQual program of NTSYSpc version 1.8 (Exeter Software, Setauket, NY). For each coefficient, the similarity matrix was used to construct dendrograms with the help of the unweighted pair grouping by mathematical averaging (UPGMA) methods using the SAHN and TREE programs in NTSYS. In addition, multiple correspondence analysis was used to assign isolates to RAMS, RAPD, and virulence groups (clusters) to avoid defining every clonally related pair of haplotypes as a separate group. Each such UPGMA-defined group of isolates was considered a separate genetic lineage. Statistical support for phenogram branching in qualitative analyses was obtained using 1,000 bootstrapped analysis in WINBOOT (52). Because the RAPD and RAMS markers were highly correlated ($r = 0.90$), using MXCOMP analysis in the NTSYSpc version 1.8 program, data from the two markers were combined and subsequently analyzed as a single data set.

Analysis of molecular variance. Analysis of molecular variance (AMOVA) was used to partition the amount of variation into that attributable to differences within and among *P. griseola* groups. In the first test, isolates were separated into Andean, Afro-Andean, and Mesoamerican based on virulence analysis. For the second analysis, isolates within each group (Andean and Mesoamerican) were separated according to geographic origin. A nonparametric permutational procedure computed the significance of the variance components. We employed the AMOVA-prep program (M. P. Miller, Department of Biological Sciences, Northern Arizona University, Flagstaff) to generate distance matrices based on Dice statistics and to create the group, distance, and population files. In addition to AMOVA, haplotypic diversity was calculated for each population (isolates belonging to the same group were considered a population following cluster analysis) using equation 1 (35):

$$H = 1 - J, \text{ and } J = \sum_k 1 - x_k^2 \quad (1)$$

where x_k is the frequency of the *i*th haplotype in each subpopulation or entire population.

The extent to which subpopulations were differentiated was assessed by calculating the coefficient of genetic differentiation (27) using equation 2:

$$G_{ST} = (H_{Total} - H_{Pop})/H_{Total} \quad (2)$$

H_{Tot} is the average genotypic diversity within the species and was calculated by treating all genotypes as if they belonged to a single population. H_{Pop} is the average genotypic diversity for each population and was calculated separately for each population using the formula above.

RESULTS

Virulence analysis of *P. griseola*. Virulence analysis of 131 *P. griseola* isolates defined 67 pathotypes, of which 19 were described as Afro-Andean based on their ability to infect Mesoamerican differential genotypes Pan 72, Flor de Mayo, or Cornell 49242 (Table 1). Thirty-four of the pathotypes were Mesoamerican, while 14 were typical Andean (Table 1). Twenty-three of the sixty-seven pathotypes (34.3%) had more than one isolate in the group. The Andean pathotype 31-0 was widely distributed and was recovered from both Africa (Burundi, Congo, Malawi, South Africa, Tanzania, and Zambia) and Latin America (Colombia and Ecuador) (Table 1). Similarly, pathotype 14-0 was widely distributed and was also recovered from Africa (Kenya, Malawi, Zambia, Rwanda, and Uganda) and South America (Ecuador and Colombia). Cluster analysis of virulence data separated pathotypes into three major groups, Mesoamerican, Andean, and the Afro-Andean (Table 1). In addition, virulence diversity (calculated using Nei's genetic differentiation [35]) was significantly higher for Mesoamerican isolates (0.93) compared with Andean isolates (0.79) (Table 2).

IGS-RFLP analysis. The size of IGS-PCR products obtained with primers CLN12 and CLNTS1 varied from 2.6 to 3.0 kb (Fig. 1), and there was no apparent association of the IGS fragment size to a particular *P. griseola* group. A total of 90 polymorphic fragments were obtained with the five restriction enzymes used in this study. Cluster and multiple correspondence analysis showed that all *P. griseola* isolates could be separated into two major groups, Andean and Mesoamerican. Isolates that had been designated Afro-Andean by host differential genotypes fell within the Andean group, as did Andean isolates from Africa (data not shown). Haplotype diversity calculated from IGS-RFLP data was 0.85 for the Andean group and 0.90 for the Mesoamerican group (Table 2).

Analysis of RAMS and RAPD. A total of 112 polymorphic fragments were generated using seven RAMS primers. Figure 2 shows an example of the comparative banding patterns for some *P. griseola* isolates following amplification with the RAMS primer (CA)n. Cluster and multiple correspondence analysis of RAMS data defined 83 haplotypes among the 131 isolates analyzed. All haplotypes were distributed into two major groups, Andean and Mesoamerican. Within the Andean group, geographical differentiation was evident, with Andean isolates from Latin America forming a distinct cluster from Andean and Afro-Andean isolates from Africa. There was no apparent separation of Afro-Andean isolates, which clustered with the Andean isolates collected from Africa. Bootstrap analysis confirmed the separation of *P. griseola* isolates primarily by host gene pool, and within each group, by geographical origin. Average haplotype diversity (0.83) was high for the entire *P. griseola* population (Table 2). Similarly, cluster analysis of RAPD data defined 61 haplotypes that were separated into two major groups, Andean and Mesoamerican. Isolates classified as Afro-Andean separated with Andean isolates, and within this group, there was no apparent clustering of these isolates. The average haplotype diversity calculated from RAPD data was 0.835 (Table 2).

Molecular analysis of *P. griseola* data. Because RAPD and RAMS are neutral markers that are distributed throughout the

TABLE 2. Estimates of haplotype and pathogenic diversity within *Phaeoisariopsis griseola* groups

	P. griseola groups		
Type of marker[a]	Andean	Mesoamerican	Mean
RAPD	0.83	0.84	0.835
RAMS	0.75	0.90	0.825
IGS-RFLP	0.85	0.90	0.875
Virulence	0.79	0.93	0.860

[a] RAPD = random amplified polymorphic DNA, RAMS = random amplified microsatellites, and IGS-RFLP = restriction fragment length polymorphism digest of the amplified ribosomal intergenic spacer region. Virulence is based on differential interaction of each isolate on a set of 12 differential bean genotypes.

whole fungal genome, it is possible to correlate these two markers. We used Cophenetic correlation (COPH) and MXCOMP analysis in the NTSYSpc version 8.1 program to correlate RAPD and RAMS data and found that they were highly correlated (r = 0.90). Therefore, we combined the data and analyzed them as a single data set. Cluster analysis of combined data divided isolates into major groups corresponding to Andean and Mesoamerican (Fig. 3). The average similarity between the two groups was 44%. Multiple correspondence analysis separated isolates into six groups, and all Andean isolates were distributed into two groups (Fig. 3), with group 1 containing only Latin American isolates. Group 2 contained all isolates collected from Africa and identified as Andean, Afro-Andean using host differential interaction on a set of 12 common bean differential genotypes. Within this group, there was no apparent clustering of the Afro-Andean isolates. A three-dimensional plot of multiple correspondence analysis results from combined RAMS and RAPD data (Fig. 4) confirmed these results and showed significant levels of genetic and geographical separation of *P. griseola* isolates. Mesoamerican isolates from Latin America were more diverse and were distributed into groups 3, 4, 5, and 6, respectively, whereas all Mesoamerican isolates from Africa were distributed together with some isolates from Latin America in group 6 (Fig. 3). The average haplotype diversity within *P. griseola* population was 0.83, and the haplotype diversity in the species was 0.98.

AMOVA. To test if Afro-Andean isolates were significantly different genetically from *P. griseola* isolates from Africa, in order to warrant classification into a new group or subgroup, Afro-

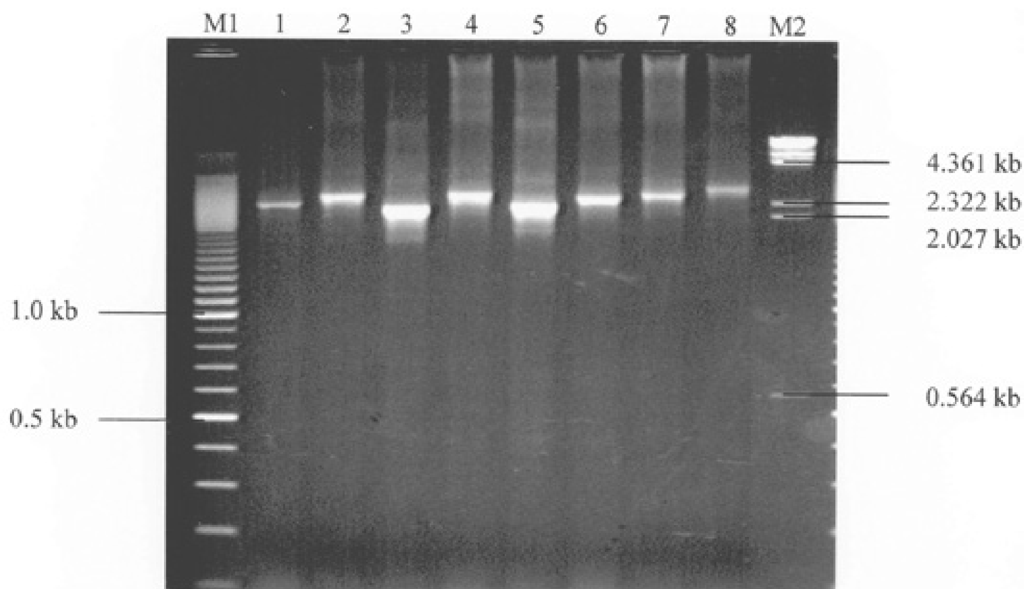


Fig. 1. Intergenic spacer amplification products of Andean, Afro-Andean, and Mesoamerican isolates of *Phaeoisariopsis griseola*. Lanes 1, 2, and 8 are Afro-Andean *P. griseola* isolates, lane 3, a Mesoamerican isolate, and isolates in lanes 4 to 7 are Andean. Lanes M1 and M2 correspond to a 100-bp step ladder and *Hin*dIII-digested lambda DNA, respectively.

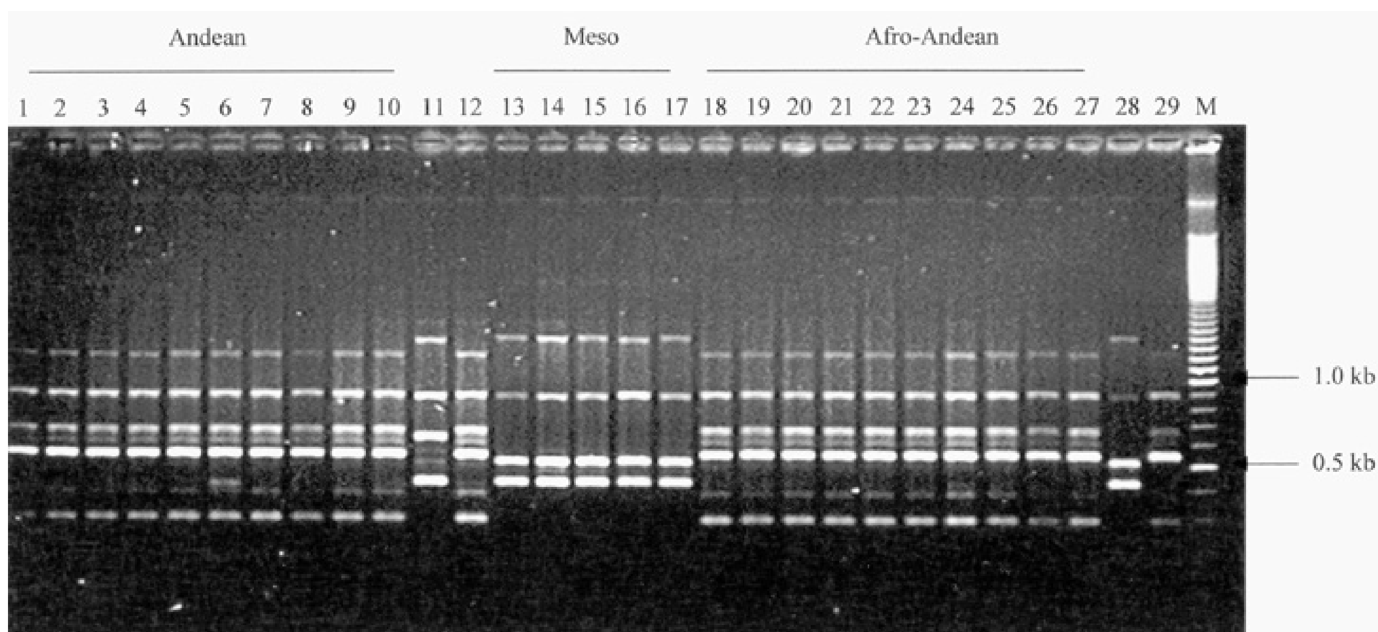
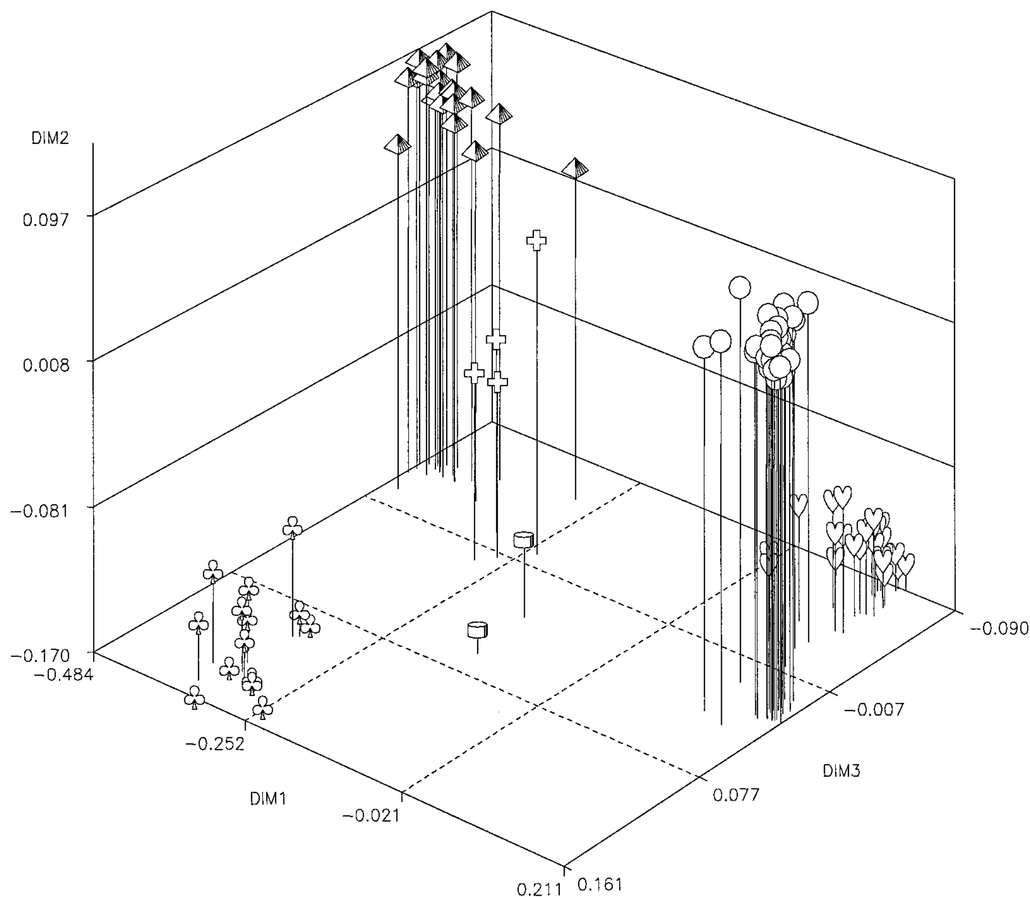


Fig. 2. Random amplified microsatellite sequence profiles of *Phaeoisariopsis griseola* isolates generated with primer (CA)n. Amplifications and electrophoresis were as described in text. Lanes 1 to 10 and 12 represent Andean *P. griseola* isolates; lanes 11, 13 to 17, and 28 represent Mesoamerican isolates; and lanes 18 to 27 and 29 are Afro-Andean isolates. Lane 30 is the 100-bp step ladder molecular size marker.

Fig. 3. Dendrogram of *Phaeoisariopsis griseola* isolates based on unweighted pair-group method with arithmetic average using the SAHN and TREE option in NTSYS program with similarity coefficients calculated from combined random amplified polymorphic DNA and random amplified microsatellites data. All isolates collected from Africa are shown in bold. Group A is based on multiple correspondence analysis in the SAS program, and group B is based on virulence characterization on a set of 12 differential common bean genotypes.

Andean isolates were compared with Andean and Mesoamerican isolates classified on the basis of virulence characterization. AMOVA revealed that 2.8% of the observed genetic variation was a result of differences between Andean and Afro-Andean populations (Table 3). When compared with Mesoamerican isolates, 68.2% of the total amount of genetic variation was ascribed to differences between Afro-Andean and Mesoamerican populations. The amount of genetic variation attributed to differences between Afro-Andean and Mesoamerican populations (68.2%) was similar to the amount of genetic variation observed between Mesoamerican and Andean isolates (67.3%), showing that Afro-Andean and Andean isolates from Africa consituted the same population. In addition, gene diversity analysis revealed no genetic differentiation (G_{ST} = 0.004) between Afro-Andean and Andean isolates from Africa. Andean and Afro-Andean isolates were therefore combined to constitute a single population in tests to see if *P. griseola* populations in Africa were similar to those in Latin America. AMOVA ascribed 67% of the total genetic variation to differences observed between Andean isolates from Africa and those from Latin America (Table 4). Gene diversity analysis showed that the Latin American Andean population was significantly different genetically (G_{ST} = 0.39), from the African Andean population, revealing significant levels of geographical differentiation. Although significant ($P < 0.05$), the total amount of genetic variation ascribed to differences between Mesoamerican isolates from Africa and Latin America was low (26.38%), as was the level of genetic differentiation (G_{ST} = 0.12). The total amount of genetic variation ascribed to differences between Andean and Mesoamerican isolates was higher (68.7%) for African isolates than for Latin American isolates (55.6%), revealing a great



♡ Group 1 - Andean isolates from Latin America

○ Group 2 - Andean and Afro-Andean isolates from Africa

Group 3 - Mesoamerican isolates from Latin America

Group 4 - Mesoamerican isolates from Latin America

♧ Group 5 - Mesoamerican isolates Latin America

Group 6 - Mesoamerican isolates from Africa and Latin America

Fig. 4. Three-dimensional graph based on multiple correspondence analysis of combined random amplified polymorphic DNA and random amplified microsatellites data and plotted using the spin platform of JMP program in SAS. Symbols indicate position of strains within each cluster. Group 1 consists of Andean isolates from Latin America, group 2 consists of Andean and Afro-Andean isolates from Africa, groups 3, 4, and 5 consist of Mesoamerican isolates from Latin America, and group 6 consists of Mesoamerican isolates from Africa and Latin America.

amount of genetic variation in the African *P. griseola* populations (Table 4). Furthermore, significant levels of genetic differentiation (G_{ST} = 0.57) were observed between Andean and Mesoamerican isolates, showing the strong influence of host genotype on the population structure of *P. griseola*. AMOVA showed that most of the variation resulted from genetic differences inherent between Andean and Mesoamerican groups (75%) rather than differences existing among individual isolates within a group (25%).

DISCUSSION

In this study, several molecular analyses were used to investigate genome diversity of *P. griseola* and to more precisely define the relationship among isolates belonging to the Afro-Andean, Andean, and Mesoamerican groups of *P. griseola*. This study revealed and confirmed that *P. griseola* displays high pathotypic and genomic diversity, concurring with previously published results (9,12,19,29,37). Although molecular and pathogenicity analyses divided *P. griseola* isolates principally into Andean and Mesoamerican groups, pathotypic and genomic diversity were not correlated. From studies with molecular markers, it was concluded that Afro-Andean isolates do not represent a new group within *P. griseola* but are Andean isolates that have evolved to colonize beans from the Mesoamerican gene pool.

Analysis of pathogen virulence revealed that the *P. griseola* populations in Africa and Latin America could be separated into two major groups, Andean and Mesoamerican, that corresponded to the gene pools that have been defined for its common bean host and confirming previous reports that *P. griseola* has coevolved with its host (9,12,18,19,29,38,47). However, a group of isolates designated Afro-Andean were described in Africa. These isolates are able to infect some Mesoamerican bean genotypes, but previous RAPD analysis classified these isolates with the Andean group (11). In this study, cluster analysis of molecular data generated using RAMS, IGS-RFLP, and RAPD distributed Afro-Andean and Andean isolates from Africa into the same cluster and within this group, no apparent clustering of Afro-Andean isolates was evident. Similarily, genetic diversity analysis revealed no genetic differentiation (G_{ST} = 0.004) between Afro-Andean and Andean isolates from Africa, whereas AMOVA revealed that Afro-Andean isolates were 97.2% similar to Andean isolates from Africa. Based on these results, Afro-Andeans are Andean isolates that have acquired the ability to infect some Mesoamerican cultivars but are not genetically distinct to be classified as a subgroup within the Andean group.

Due to the high level of similarity between the genomes of Afro-Andean and Andean isolates from Africa (G_{ST} = 0.004), major genome organization (i.e., loss or acquisition of a chromosome segment) is unlikely to be the event that lead to the emergence of these isolates. Therefore, Afro-Andean isolates do not represent long evolution of the whole pathogen genome, but probably reflects parasexual reproduction or point mutations in the genes conditioning virulence. Mesoamerican isolates infect both Andean and Mesoamerican bean genotypes (19,38,39), and multiple leaf infections by *P. griseola* isolates belonging to different groups might lead to parasexual recombination. Up to five pathotypes have been isolated from different lesions on the same leaf (*unpublished data*). Studies are underway to test the hypothesis of multiple infections by Andean and Mesoamerican *P. griseola* isolates.

A high level of genetic differentiation was observed between Andean and Mesoamerican *P. griseola* groups (G_{ST} = 0.57), revealing the strong influence of host specialization on the population structure of the ALS fungus and confirming the subdivision of *P. griseola* isolates into groups structured along host gene pool lines (19,37,38). The genetic diversity within each group was very high (>81%), showing that sufficient genetic diversity is being maintained in this fungus. Given that sexual reproduction has not been reported for *P. griseola* (26), the sources of the high genetic diversity are not clear. Several factors, such as migration, mutations, and recombination, whether mitotic or parasexual (1,6,31,54), can interact to create and maintain high levels of genetic diversity as found in *P. griseola*. Zeigler et al. (54) showed evidence that high levels of haplotypic diversity can be maintained in asexually reproducing fungi through parasexual reproduction. Chromosomal diversions, deletions and loss of chromosome segments (25), and the presence of transposons (24) all have the capability to increase the diversity in fungi and contribute to high haplotypic diversity. Seed transmission of *P. griseola* has been adequately documented (12,44), and introductions of new haplotypes through contaminated seed cannot be ruled out as a source of the high genetic diversity (26). Some fungi, for example *Pythium ultimum*, have been observed to be mitotically unstable, and variation has been generated in this fungus in the absence of sex (17). A similar phenomenon may occur within *P. griseola*, but the importance of these factors in generating genetic variation in this fungus remains unclear. More studies are needed to quantify the effect of these factors on *P. griseola* genetic diversity. In addition, this study revealed significant levels of geographic differentiation within the Andean group, but only moderate geographic differentiation within the Mesoamerican group.

In addition to high levels of genetic differentiation between Mesoamerican and Andean groups, this study revealed significant levels of geographic differentiation within the Andean group. A high level of geographical differentiation (G_{ST} = 0.39) was observed for the Andean lineage, in which Andean isolates from Africa occupied a cluster distinct from that occupied by isolates from Latin America. Although the level of geographical differentiation within the Mesoamerican lineage was low (G_{ST} = 0.12), the results obtained reveal that Mesoamerican isolates from African are not a general sample of isolates found in Latin America but appear to be derived from a single subgroup. However, the high level of geographical differentiation observed indicates that different strategies should be employed when managing ALS in Africa

TABLE 3. Percentage of the total genetic variation ascribed to differences between *Phaeoisariopsis griseola* populations from Africa following analysis of molecular variance of combined random amplified polymorphic DNA and random amplified microsatellites data

P. griseola populations[a]	Andean	Afro-Andean	Mesoamerican
Andean	0.0	…	…
Afro-Andean	2.8	0.0	…
Mesoamerican	67.3	68.9	0.0

[a] *P. griseola* isolates were assigned to populations based on virulence analysis on a set of 12 differential bean genotypes (38), the bean genotype from which the isolate was collected, and geographic origin.

TABLE 4. Genetic distances between *Phaeoisariopsis griseola* populations following analysis of molecular variance of combined random amplified polymorphic DNA and random amplified microsatellites data

	P. griseola populations[a]			
	Andean-Africa	Andean-Latin America	Mesoamerican-Africa	Mesoamerican-Latin America
Andean-Africa	0.000	…	…	…
Andean-Latin America	0.6741	0.000	…	…
Mesoamerican (Africa)	0.6873	0.6470	0.000	…
Mesoamerican (Latin America)	0.6996	0.5560	0.2638	0.000

[a] *P. griseola* isolates were assigned to populations based on virulence analysis on a set of 12 differential bean genotypes (38), the bean genotype from which the isolate was collected, and geographic origin.

and Latin America, especially when using resistant varieties. Furthermore, these results show that there is little or no mixing of pathogen haplotypes between Latin America and Africa. Traditionally, Andean genotypes have been extensively cultivated in Africa, while Mesoamerican genotypes are recent introductions (26). The level of geographical differentiation reflects this trend of germ plasm introductions from Latin America to Africa (26) and might explain the low levels of geographical differentiation observed.

Several factors can contribute to the subdivision in pathogen populations (1,31). One of these is selection for different alleles and genotypes in different locations and in response to different environmental factors (6). Given the ecological niche of the two bean gene pools, the high levels of genetic differentiation within the ALS pathogen is not surprising. A high degree of asexual reproduction and clonal propagation of a limited number of the founder genotypes would also increase population subdivision (8). Because sexual reproduction has not been reported for *P. griseola* (26), this hypothesis may well explain the subdivision and geographical differentiation of the Andean group.

No relationship was observed between lineages defined by molecular markers and virulence analysis, although both types of analysis clearly divided isolates into Andean and Mesoamerican groups (38). Complete correspondence between genotype and phenotype may not always be assumed, even for asexually reproducing pathogens that are clonally propagated. Several studies have shown that different pathotypes can arise within the same clonal lineage, usually as a result of selection (23,49,53). Therefore, the lack of correlation between virulence and molecular lineages is not surprising. In addition, virulence analysis only targets a small region of the genome, whereas molecular markers sample the entire genome. Molecular markers are assumed to be neutral and therefore are not dependent on the interaction of the avirulence and resistance genes, which can be influenced by the type of inoculum and environmental conditions.

While confirming host–pathogen coevolution, this study has provided new and interesting findings regarding the population genetics of *P. griseola*. For the first time, geographical differentiation has been established within the two major groups of *P. griseola*. It is clear that potential sources of ALS resistance should be screened with more than one isolate, taking into consideration the host and geographical structuring of the fungus and the high genetic diversity displayed. The isolates classified as Afro-Andean by virulence analysis do not constitute a new *P. griseola* group but contain typical Andean isolates that evolved to colonize beans from the Mesoamerican gene pool. This group of isolates probably arose through point mutations in pathogenicity genes and have attained a sufficiently high frequency to be detected through selection under the production conditions found in Africa. Alternatively, these isolates may have acquired from the Mesoamerican isolates through parasexual recombination a chromosomal segment that contains pathogenicity genes, thus enabling them to infect Mesoamerican genotypes. Although this hypothesis needs to be tested further, results from this study show that the Afro-Andean group of isolates does not represent long-term evolution of the pathogen genome.

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