Online journal ISSN - 1676-5680
GMR Genetics and Molecular Research
EVOLUTION AND TECHNOLOGY

# Identification of *Glomerella cingulata* f. sp *phaseoli* recombinants by RAPD markers

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Genet. Mol. Res. 6 (3): 607-615 (2007)
Received June 4, 2007
Accepted August 10, 2007
Published September 30, 2007

**ABSTRACT.** We examined the capacity of strains of *Glomerella cingulata* f. sp *phaseoli* fungus (*Colletotrichum lindemuthianum* sexual stage) to form recombinants, using random amplified polymorphic DNA (RAPD). Crosses of all possible combinations between strains 40, 42, 20, 21, 22, 23, 24, 25, and 26 were made on Petri dishes using $M_3$ culture medium. The 42 x 21 cross produced the largest number of perithecia and five asci; the respective ascospores were isolated. RAPD analysis was performed on the parents and descendants. The 62 polymorphic RAPD bands obtained were used to assess the genetic similarity using the method of Sorence and Dice and clustering analysis in the form of a dendrogram by the UPGMA method. The RAPD markers allowed identification of recombinants from the cross between strains 42 and 21 of *G. cingulata* f. sp *phaseoli* and 40 ascospores presented 63 and 49% genetic similarity with parents 2 (strain 42) and 1 (strain 21), respectively.

**Key words:** *Colletotrichum lindemuthianum*, Sexual phase, DNA marker, Recombinants, Clustering analysis

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# INTRODUCTION

Anthracnose, caused by *Colletotrichum lindemuthianum* (Sacc. & Magn.) Scribner is one of the most important diseases in the common bean (*Phaseolus vulgaris* L.). The use of resistant cultivars is the main control measure for this disease, but the high genetic variability presented by the fungus (González et al., 1998; Sartorato, 2002; Talamini et al., 2006) hinders disease management with genetic resistance, as it can be overcome by new races in the pathogen population (Sartorato, 2002).

The mechanisms responsible for this wide-genetic variability are very little understood. Shear and Wood (1913) described the sexual stage, which was named *Glomerella lindemuthiana*, but their study raised doubts, especially because of the lack of proof regarding pathogenicity. There was no further reference to the teleomorphic state of *C. lindemuthianum* in the literature until 1970, when Kimati and Galli reported the formation of fertile perithecia in certain pairings of typical self-sterile conidial strains. The latter authors proposed the name *Glomerella cingulata* f. sp *phaseoli*.

There are genetic studies in the literature about reproductive behavior of this pathogen, including those by Batista and Chaves (1982), Kimati and Galli (1970), Bryson (1990), Mendes-Costa (1996), and Rodriguez-Guerra et al. (2005). In most of the studies that used morphological markers, evidence was observed of *G. cingulata* f. sp *phaseoli* recombinants. Rodriguez-Guerra et al. (2005) analyzed 19 Mexican isolates and found three types of response: negative (only mycelial growth), potential (spherical perithecia-like structures) and positive (perithecia containing asci and ascospores). All strains were self-sterile and only one combination produced fertile perithecia. Forty-four monoascospore cultures were characterized with amplified fragment length polymorphisms, confirming that these individuals were progeny from a sexual cross between the original two *G. cingulata* f. sp *phaseoli* and that sexual reproduction is heterothallic in nature. Attempts to isolate and analyze the eight ascospores from a single ascus were unsuccessful preventing an accurate genetic analysis. Consequently, doubts persist on the origin of the descendents.

Although sexual reproduction occurs, it has been little investigated. We examined recombinants obtained from crosses between *Glomerella cingulata* f. sp *phaseoli* strains using random amplified polymorphic DNA (RAPD) markers, which could be useful for future genetic and molecular studies.

# MATERIAL AND METHODS

## Fungal isolates

Nine *Colletotrichum lindemuthianum* strains, collected in Minas Gerais State, Brazil, from stems, leaves and pods of common bean plants showing anthracnose symptoms were used in this study. These strains belong to the culture collection of the Biology Department of the Federal University of Lavras.

Lesions collected in the field yielded the sexual phase directly in medium (potato dextrose agar, $M_3$; Junqueira et al., 1984), without the use of inducers.

The isolates were cultivated and maintained on $M_3$ medium. Monoascosporic strains were obtained from perithecia by micromanipulation.

## Pathogenicity test

The set of 12 differential international cultivars was used to identify the *C. lindemuthianum* pathotypes using the methodology established during the "Primer Taller Latinoamericano de Antracnosis de Frijol", carried out at Centro Internacional de Agricultura Tropical - CIAT (1988). The symptoms were assessed seven to nine days after inoculation using the scale described by Rava et al. (1993).

## Crossing tests

Crosses were made of all possible combinations among the strains 40, 42, 20, 21, 22, 23, 24, 25, and 26. After 22 to 27 days of incubation at 20°C in the dark (Figure 1), ridges of well-developed perithecia formed quickly on lines of contact among the compatible strains. Single ascospore cultures, from each ascus, were examined. The eight ascospores of each ascus were then transferred separately to Petri dishes with $M_3$ culture medium. The dishes were then taken to a BOD type incubator where they remained for approximately 25 days.

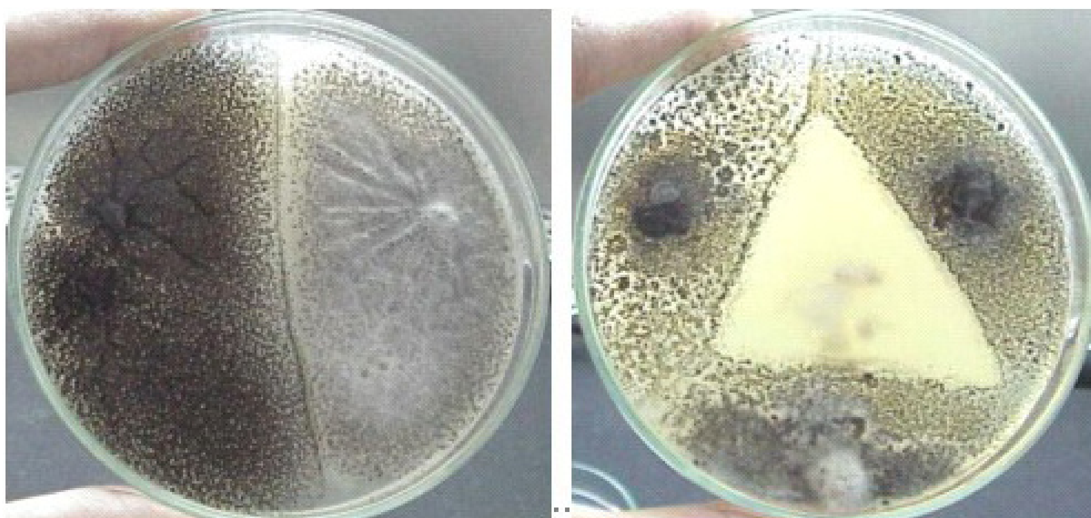

**Figure 1.** Cross between two strains (42 x 21) of *Glomerella cingulata* f. sp *phaseoli* fungus.

## DNA manipulation

The ascospores of each micromanipulated ascus were cultured and transferred to Erlenmeyer flasks with 150 mL of liquid $M_3$ culture medium plus Chemectein®. The cultures were placed in a rotary shaker (110 rpm) and incubated at 22°C for five days. Mycelia were harvested by filtration through Whatmann No. 1 filter paper placed on a funnel inserted in a filtering flask attached to a vacuum pump. After filtration, the harvested mycelia were lyophilized.

DNA extraction was performed according to Raeder and Broda (1985) adapted for the methodology by Rogers and Bendich (1988).

### Random amplified polymorphic DNA analysis

The RAPD reactions were carried out with 200 10-base oligonucleotide primers (Operon Technology, Alameda, CA, USA). The RAPD reaction mixture consisted of 200 μM dNTP's, 0.6 units Taq DNA polymerase, 0.4 μM primer, reaction buffer, pH 8.0 (50 mM Tris, 2.0 mM $MgCl_2$, 20 mM KCl, 250 μg/mL cattle serum albumin, 1% Ficoll 400, 1 mM tartrazine), 20 ng genomic DNA and bi-distilled water to a volume of 15 μL. Amplification was carried out in a thermocycler (Idaho Technology) and consisted of one initial DNA denaturation step for one cycle of 2 min at 91°C, followed by 38 cycles of 1 s at 91°C, 7 s at 42°C and 70 s at 72°C, and a final extension step of 4 min at 72°C. Amplification products were separated by electrophoresis on 1.5% agarose gels and observed under UV light after staining with ethidium bromide.

The DNA bands amplified in the RAPD reactions were then scored according to their presence (1) or absence (0) for each individual. The similarity index and their associated errors between individuals were estimated by using the Sorence and Dice (Rohlf, 1992) and Skroch et al. (1992) methods, respectively. The matrix that was generated was used to produce a dendrogram by the unweighted pair group method with arithmetic averages by using the NTSYS-PC program (Rohlf, 1992).

## RESULTS

### Identification of compatible crosses

Seventy-two combinations were carried out and 13 crosses were compatible (Table 1). All *G. cingulata* f. sp *phaseoli* strains used in our study were self-fertile. Perithecia were produced in clusters forming large dark clots all over the Petri dish medium surface. The data presented in this study are restricted to the 42 x 21 cross due to the large amount of perithecia produced.

**Table 1.** Crosses among the 42, 40, 20, 21, 22, 23, 24, 25, and 26 strains of *Glomerella cingulata* f. sp *phaseoli* fungus.

| Strains | Strains | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 42 | 40 | 20 | 21 | 22 | 23 | 24 | 25 |
| 40 | - | | | | | | | |
| 20 | - | - | | | | | | |
| 21 | + | + | + | | | | | |
| 22 | + | + | - | - | | | | |
| 23 | + | + | + | - | - | | | |
| 24 | - | + | - | - | - | - | | |
| 25 | + | + | - | - | - | - | - | |
| 26 | + | + | - | - | - | - | - | - |

+, Fertile perithecia at the contact line between colonies; -, no perithecia production.

Strains 42 and 21 of *C. lindemuthianum* were classified as pathotypes 73 and 65, respectively. The pathogenicity test was carried out using the sexual stage (*G. cingulata* f. sp *phaseoli*) and the symptoms that resulted from inoculation were very mild compared to those from the asexual stage.

We found that 33 primers gave polymorphism. A total of 62 polymorphic bands were obtained, with an average of 1.87 bands/primer.

The similarity analysis and the clustering of all the asci and respective ascospores (Figure 2) showed that the genetic similarity among the asci varied from 66 to 77%. In general, the 40 descendent ascospores that were assessed were genetically closest to parent 2 (strain 42) because they presented 63% genetic similarity with this parent. The genetic similarity was 49% for parent 1 (strain 21). These values were close to 50%, which proved that the descendents assessed were products of meiosis from the cross among the parent strains. These results showed that the RAPD markers used allowed identification of recombinants derived from crossing among the *G. cingulata* f. sp *phaseoli* strains.

The descendent ascospores were clustered within their respective asci (Figure 2).

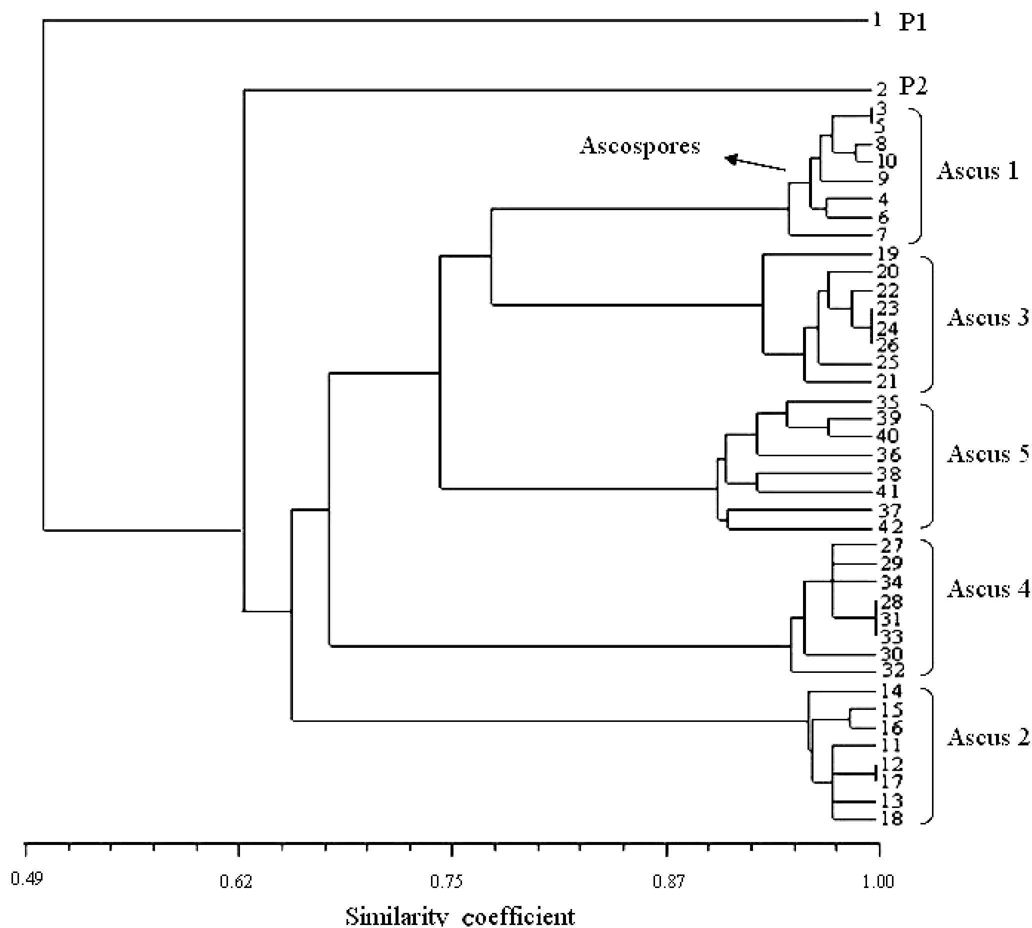


**Figure 2.** Cluster analysis among parents (21 and 42 strains) and their progeny (five asci and their respective ascospores).

To better visualize the results we made an analysis of individual similarity for each ascus within a 5% confidence interval. We observed (Table 2) that ascus 5 was the closest ascus to parent 1 (strain 21). This is due to the segregation of its respective ascospores that maintained the patterns of the parent strain (but none clustered with one of the parents, thus confirming that all the asci derive from sexual hybridization between strains 21 and 42).

**Table 2**. Confidence interval and mean error ($\bar{S}_{sg}$) of the genetic similarity estimates of each ascus with the parental strains.

| Ascus | P1 (strain 21) | P2 (strain 42) | Mean error ($\bar{S}_{sg}$) |
|---|---|---|---|
| 1 | (0.36-0.50)* | (0.71-0.85)* | 0.03648 |
| 2 | (0.32-0.48)* | (0.50-.066)* | 0.0448 |
| 3 | (0.32-0.46)* | (0.54-0.69)* | 0.0405 |
| 4 | (0.25-0.41)* | (0.55-0.69)* | 0.0437 |
| 5 | (0.45-0.65)* | (0.38-0.58)* | 0.0525 |

*Confidence interval 5%.

It was generally observed (Figures 3 to 6) that there were aberrant segregations in the descendants of individual asci when a single RAPD locus was assessed.

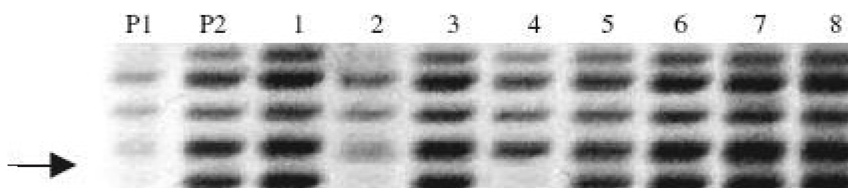


**Figure 3.** RAPD band pattern with the OPL-14 primer in the analysis of ascus 3 with the parents (21 and 42 strains) showing a 6:2 segregation.

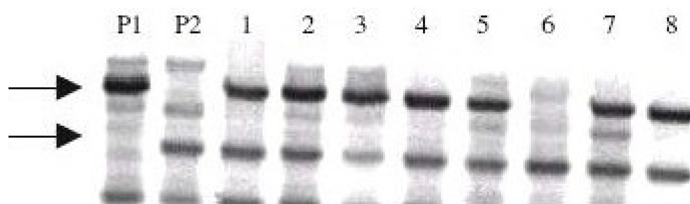


**Figure 4.** RAPD band pattern with OPAR-3 primer in analysis of ascus 1 with the parents (21 and 42 strains) showing 7:1 segregation (first arrow).

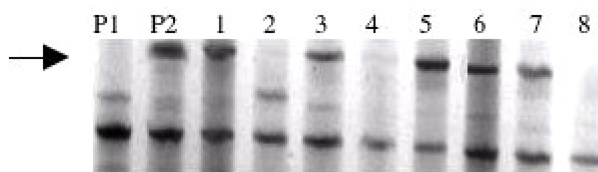


**Figure 5.** RAPD band pattern with OPO-4 primer in analysis of ascus 3 with the parents (21 and 42 strains) showing a 5:3 segregation.

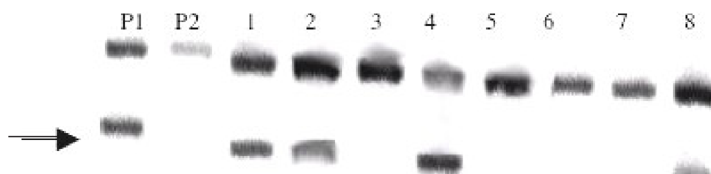


**Figure 6.** RAPD band pattern with OPL-4 primer in the analysis of ascus 5, with the parents (21 and 42 strains) showing a 4:4 segregation.

The occurrence of recombinants was ascertained for the two RAPD loci (Figure 4). All the descendants, except for the sixth descendant, presented bands of the two parents, showing the occurrence of recombinants.

## DISCUSSION

The sexual compatibility results (Table 1) proved the heterothallic nature of this fungus, confirming the finding of Rodriguez-Guerra et al. (2005). In all the compatible crosses, perithecia, asci and ascospore morphologies were similar to those described by Shear and Wood, 1913; Lucas, 1946; Kimati and Galli, 1970, and Bryson et al., 1992. The materials from these crosses were easy to manage and highly viable in the laboratory (Figure 1), contradicting earlier reports (Kimati and Galli, 1970; Batista and Chaves, 1982; Bryson et al., 1992; Rodriguez-Guerra et al., 2005).

No studies were found reporting the process of *G. cingulata* f. sp *phaseoli* infection in the host. However, there are various reports of the asexual stage (Mercer et al., 1975; Elliston et al., 1976; O'Connell and Bailey, 1991; Roca et al., 2003). We conclude that efforts should be made to understand the infection process of *G. cingulata* f. sp *phaseoli* in the common bean plant, which would contribute greatly to the understanding of the different symptoms of the disease in the plant depending on which stage (sexual or asexual) of the fungus was used in the inoculation. Likewise, the description of the life cycle could facilitate the sexual phase identification in common bean fields. The sexual stage is common, we and others have found (Roca et al., 2003) contradicting previous reports (Rodriguez-Guerra et al., 2005).

The similarity analysis and the clustering of all the asci and respective ascospores showed that the RAPD markers allowed identification of recombinants derived from the crosses among the *G. cingulata* f. sp *phaseoli* strains. Silva (1996), in a study on genetic characterization by RAPD of recombinants in *Trichoderma pseudokoningii*, also showed the efficiency of this technique, observing 70% genetic similarity in the recombinants with the parent they most resembled.

Considering that *G. cingulata* f. sp *phaseoli* presents four chromosomes (n = 4), eight different orientations are possible in metaphase 1 of meiosis. Therefore, these different orientations would probably not be the main cause of the separation of the five asci in the dendrogram (Figure 2), since only five ascus samples were used; that is, the probability of this event occurring is $(1/8)^5$. The probable cause of this clear distinction among the asci is intra-chromosome recombination (genetic exchange, transposable elements, etc.), which would have caused alteration in the annealing sites of the primers. Furthermore, interchromosome recombination would not alter the annealing sites of the primers. If each different orientation presented in metaphase I of meiosis ($S_{ij}$) indicates that intrachromosome mechanisms were random, generating a specific $S_{ij}$ for each ascus, that would also explain the small divergence observed within each ascus, among the 'sibling' ascospores (Figure 2).

Considering that the 62 polymorphic bands are distributed randomly in the four chromosomes, approximately 15 RAPD loci per chromosome were expected, on average, and intrachromosome recombination among these loci would account for most of the generation of genetic variability among the descendents, which allowed perfect distinction among the asci analyzed. This shows the potential of genetic variability that can be obtained by sexual reproduction of *G. cingulata* f. sp *phaseoli*, variability that is added to the independent distribution.

There have been other attempts to obtain *G. cingulata* f. sp *phaseoli* recombinants (Kimati and Galli, 1970; Batista and Chaves, 1982; Bryson, 1990; Mendes-Costa, 1996; Rodriguez-Guerra et al., 2005) that were apparently not very successful. These reports did not prove that the descendants obtained were really derived from crosses. Though Rodriguez-Guerra et al. (2005) confirmed sexual cross between two isolates by analysis of random ascospores by amplified fragment length polymorphism markers, the analysis of the eight ascospores from a single ascus was unsuccessful. We showed recombination within a single ascus, allowing detailed genetic analysis of the teleomorphic state of this fungus.

We observed aberrant segregations; this has also been reported in *Neurospora*, *Sordaria* and *Ascobolos immersus* (Mitchell, 1955; Olive, 1963) with morphological and nutritional markers. The results of these studies were explained from the theory of genetic conversion. There are many other studies in the literature on this subject (Roman, 1958; Strickland, 1958; Case and Giles, 1959, 1964; Marcou, 1969; Gutz, 1971; Detloff et al., 1991; Lamb and Zwolinski, 1992; Malone et al., 1992; Abla, 1994; Lamb, 1996; Chamnanpunt et al., 2001), thus overestimating segregation considered normal (4:4; 2:2:2:2). However, more on this subject needs to be researched and analyzed.

We confirm the hypothesis raised by Bryson et al. (1992) that asexuality in *C. lindemuthianum* has been exaggerated, especially because of the small sample of isolates assessed, the use of 'old' cultures, failure in testing combinations of strains and inadequate culture conditions. Therefore, efforts should continue to identify strains that show the sexual cycle and obtain recombinants by crosses among these strains, which would greatly facilitate genetic and molecular study on *G. cingulata* f. sp *phaseoli* and also would contribute to our understanding of the variability mechanisms of this pathogen, as in pathogenicity heredity studies.

## ACKNOWLEDGMENTS

Research supported by a fellowship to O.A. Camargo Junior from CAPES and financial support and scholarships from FAPEMIG.

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