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Morphological characterization and genetic analysis of *Drechslera teres* isolates

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Abstract

Net blotch, caused by the phytopathogen *Drechslera teres*, is a common disease of barley (*Hordeum vulgare* L) and is responsible for large economic losses in some barley growing areas. In this study the morphology and genetic variability of eight *D. teres* isolates from different regions of the Brazilian state of Rio Grande do Sul were investigated. Colony morphology was studied on potato-dextrose-agar (PDA) and genetic variability investigated using the random amplified polymorphic-DNA (RAPD) technique. 27 commercially available primers were tested of which 16 were selected for use in polymorphic analysis due to their good resolution and reproducibility. Similarity coefficients were used to construct dendrograms based on colony morphology and RAPD data showing the relationship between the eight isolates studied. Colony morphology showed variability between the isolates while RAPD assays showed high similarity coefficients, but grouping of the isolates according to the geographic origins of the seeds from which they were isolated was not possible.

Key words: Drechslera teres, barley, net blotch, DNA polymorphism, RAPD.

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Introduction

Barley (*Hordeum vulgare* L) is an intensively cultivated cereal grown worldwide, being cultivated in Brazil mainly in the southern state of Rio Grande do Sul (Leal, 1972). The most important disease of barley is net blot, caused by the phytopathogenic fungus *Drechslera teres* (Sacc) Schoemaker; teleomorphic stage: *Pyrenophora teres* Drechs., (syn. *Helminthosporium teres* Sacc.) (Alexopoulus and Mims, 1985; Mather, 1987). Both the seeds and leaves of barley can be infected by *D. teres*, which can also persist in straw (Mather, 1987). Heterokaryosis, sexual recombination and parasexuality all contribut to the enormous variability and diversity of this fungus (Azevedo, 1976).

Random amplified polymorphic DNA (RAPD, Williams *et al.* 1990), has often been used in characterization studies of phytopathogenic fungi, for example in the study of *Fusarium oxysporum* races and *formae speciales* (Jesus *et al.*, 1995) and to investigate variations between isolates of *Rhizoctonia solani* (Duncan, *et al.*, 1993), *Puccinia striiforms* (Chen, *et al.*, 1993) and other fungi.

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The aim of the work reported in this paper was to investigate morphological aspects of different *D. teres* isolates using traditional microbiological methods and chromosomal DNA polymorphisms using RAPD analysis.

Material and Methods

Isolation of fungi

Seeds of Barley (*Hordeum vulgare* L) cultivar MN599 were obtained from a malting-house (*Maltaria Navegantes*) in Porto Alegre, Rio Grande do Sul, Brazil. The geographic origin of the seeds from which the *D. teres* were isolated are given in Table I. The fungi were isolated from the seeds by sanitizing the seeds by immersion in 2% aqueous sodium hypochlorite for 120 s, washing them 3 times in sterile water and incubating them on wet filter paper in a tray for 10 days at 24 °C with a 12 h photoperiod. Conidia of *D. teres* were collected from each seed and inoculated onto plates of potato-dextrose-agar (PDA) which were also incubated for 7 days at 24 °C with a 12 h photoperiod. After confirmation of the vegetative structure of the fungi, conidia were transferred to PDA slants and

Table I - Isolation of *Drechslera teres* from barley seeds.

Geographic origin of the host seeds	*D. teres* code
Sananduva[a] (high plateau region)[b]	Sanad599
Ipiranga do Sul (middle plateau region)	Ipira599
Palmeira das Missões (middle plateau region)	Palme599
São Gabriel (prairie region)	Saog599
Encruzilhada do Sul (southeastern mountains region)	Encru599
Vacaria (high plateau region)	Vacar599
Piratini (southeastern mountains region)	Pirat599
Carazinho (middle plateau region)	Caraz599

[a]Nearest town to where the seeds were collected.
[b]Geographic region of Rio Grande do Sul state, Brazil.

incubated as before. All strains were stored as conidia and hyphae at 4 °C.

Morphological analysis

For each *D. teres* isolate a 0.5 cm diameter paper disk containing isolated conidia was placed at the center of a PDA plate, five replicates being made for each strain. Using the key produced by Nobles (1958), the macroscopic characters (color, sector, border and texture) of each colony were recorded at 24-hour interval until one of the colonies had reached the border of the plate.

DNA extraction

Genomic DNA was extracted from mycelium using a modified Ashktorab and Cohen (1992) method. Isolates of *D. teres* were grown in 100 mL of potato-dextrose broth (20% potato, 2% dextrose (w/v)) for 10 days at 24 ± 2 °C with 12 h photoperiod. After which 20 g of wet mycelia were separated by filtration, washed three times with sterile water, transferred to a mortar and ground to a fine powder in liquid nitrogen. To this powder was added 20 mL of extraction buffer (200 mM Tris-HCl pH 8.0; 250 mM NaCl; 25 mM EDTA pH 8.0; 50 μg/mL proteinase K; 1% (v/v) of sodium dodecyl sulfate and 1%(v/v) β-mercaptanol). The suspension was incubated for 1 h at 65 °C. The suspension was then centrifuged at 4.000 rpm for 20 min and the supernatant extracted once with phenol, twice with phenol-chloroform and once with chloroform. The DNA was precipitated with 100% ethanol and 3 M NaCl, washed with 70% ethanol, dried, re-suspended in TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) and stored at -20 °C.

Primers and amplification conditions

The primers (Table II) were obtained from Byodinamics SRL (Buenos Aires, Argentina) and OPERON Technology (Alameda, CA). The RAPD reactions were performed in 25 μL volumes containing 10 mM Tris HCl

Table II - Sequences of the 16 primers used in this study and size in kilobases (kb) of the amplified *Drechslera teres* DNA fragment.

Primer	Sequence 5'-3'	Fragment size (kb)	*D. teres* isolate
A01	CCCAAGGTCC	1.69	Vacar599
		0.97	Vacar599
		0.74	Sanad599, Saog599, Encru599
		0.67	All isolates
		0.61	All isolates
A02	GGTGCGGGAA	1.68	All isolates
		1.39	Ipira599
		1.02	All isolates
A06	GAGTCTCAGG	1.77	All isolates
		1.23	Ipira599
		0.96	All isolates
		0.81	Saog599, Vacar599, Ipira599, Palme599
		0.73	Sanad599, Pirat599, Saog599
A08	ACGCACAACC	1.78	All isolates
		1.20	Pirat599
		0.88	Ipira599
		0.80	All isolates
B01	TCGAAGTCCT	1.93	Caraz599, Pirat599, Saog599, Vacar599, Palme599, Encru599, Ipira599
		1.49	Caraz599, Sanad599, Saog599, Vacar599, Palme599, Encru599, Ipira599
		1.21	Sanad599, Saog599, Vacar599, Palm599, Encru599, Ipira599, Pirat599
		0.81	Pirat599
B02	GCATGTCAGA	1.21	All isolates
		0.96	All isolates
		0.48	Sanad599, Ipira599
B06	GTGACATGCC	1.05	All isolates
		0.81	All isolates
		0.47	Caraz599, Sanad599, Saog599, Vacar599, Palme599, Encru599, Ipira599

Table I (cont.)

Primer	Sequence 5'-3'	Fragment size (kb)	*D. teres* isolate
B07	AGATGCAGCC	1.59	All isolates
		1.34	All isolates
		0.78	All isolates
		0.51	All isolates
B08	TCACCACGGT	1.81	Caraz599, Sanad599, Saog599, Vacar599, Palme599, Ipira599
		1.38	All isolates
		0.93	All isolates
B09	ATGGCTCAG	1.40	All isolates
B10	CAGGCACTA	1.88	All isolates
		1.51	Saog599
		0.77	Saog599, Vacar599
		0.73	Caraz599
		0.69	Sanad599, Pirat599, Palme599 Caraz599, Encru599, Ipira599
		0.64	Sanad599, Pirat599, Palme599, Encru599, Ipira599
OPC02	GTGAGGCGT	1.98	All isolates
		1.38	All isolates
OPC03	GTCGCCGTC	1.98	All isolates
		1.64	All isolates
		1.52	All isolates
		1.32	All isolates
OPC05	GATGACCGC	1.87	All isolates
		1.36	Saog599, Vacar599, Caraz599, Encru599, Ipira599
		0.91	All isolates
OPC13	AAGCCTCGT	1.67	All isolates
		1.38	Caraz599, Sanad599, Saog599, Vacar599, Palme599, Encru599, Ipira599
		1.00	All isolates
		0.64	Palme599
		0.51	Pirat599
		0.42	Sanad599
OPB17	AGGGAACGA	1.87	Sanad599
		1.52	Sanad599
		1.07	Sanad599, Caraz599, Saog599,Vacar599, Palme599
		0.56	Encru599, Ipira599
		0.47	Sanad599
			All isolates

pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 0.001% (w/v) gelatin; 2.5 mM of each dNTP; 30 ng of primers; 30 ng genomic DNA and 1 U *Taq* polymerase. Amplification was carried out in a thermalcycler (Mini Cycler™ MJ Research) as follows: one cycle of 1 min at 94 °C, 5 min at 35 °C and 2 min at 72 °C, followed by 45 cycles of 1 min at 94 °C, 1 min at 35 °C and 2 min at 72 °C. Amplification products were separated by electrophoresis in 2% (w/v) agarose gel, stained with ethidium bromide and photographed under UV light. All amplifications were repeated at least twice for each isolate in separate experiments.

Data analysis

Dendrograms were constructed based on morphological analysis and RAPD data using the statistical package of the social sciences (SPSS) software, 2nd. Similarity between isolates was assessed by calculating the simple association coefficient and cluster analysis using the unweighted pair group method with arithmetical averages (UPGMA). The presence or absence (coded as 1 or 0, respectively) of RAPD bands was used as an additional character for matrix construction.

Results and Discussion

The *Drechslera teres* isolates used in this work presented variations in mycelium color, with isolates Pirat599, Encru599, Sanad599 and Saog599 having a Nobles key color of U-5-1° (dark ash) while isolates Palme599, Vacar599 and Caraz599 had a value of U-15-1° (ash) and isolate Ipira599 being white.

Santos (1996), studying *Drechslera triti-repentis* isolates grown on PDA, and McDonald (1967), working with *Pyrenophora teres* isolated from different locations, both observed some variability in mycelium color while Valim-Labres *et al.* (1997), Oliveira *et al.* (1998) and Matsumura (1991) all found variability in mycelium color and colony

morphology in *Bipolaris sorokiniana* isolates grown on PDA.

The isolates Sanad599 and Pirat599 exhibited white tufts while isolate Palme599 showed fan shaped sectors, although the surface of all isolates was plain. Santos (1996) also observed white tufts in some *D. tritici-repentis* isolates and Valim-Labres *et al.* (1997) and Oliveira *et al.* (1998) found sectors in the form of white and pink tufts in some *B. sorokiniana* isolates.

Of the 27 primers tested, 16 produced reproducible well resolved DNA products, the remaining 11 primers showing either faint or no fragments at all on electrophoresis. Genomic DNA amplification of all isolates produced 62 bands which were submitted to cluster analysis, 56.4% of the fragments being polymorphic. The sequences of the 16 primers used in this work and their DNA products are shown in Table II. Figures 1 and 2 show the results of RAPD analysis with primers B02, B07 and B10. Four primers B07, B08, OPC03 and OPC02 produced monomorphic fragments with all *D. teres* isolates. Schnell *et al.* (1995), studying mango isolates (*Mangifera indica* L.), observed monomorphic amplification with 19 primers. Monomorphic fragments have also been found by Peever and Milgroon (1994) who investigated 16 *Pyrenophora teres* isolates and by Hamelin *et al.* (1993) who worked with two *Gremmeniella abientina* races

The dendrogram constructed with both the RAPD data and morphological characters (Figure 3) presents a major group subdivided into two subgroups, variation in similarity coefficients being between 0.8714 and 0.7286. Isolates Vacar599 and Saog599, and Encru599 and

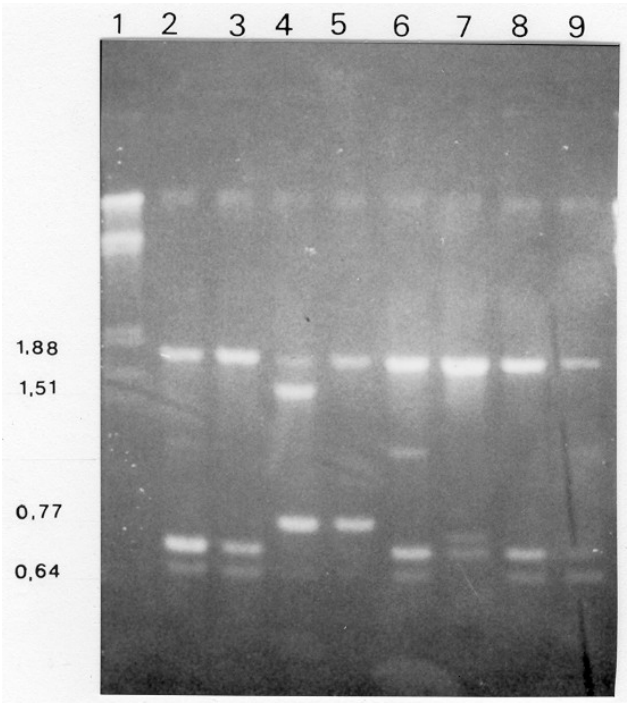

Figure 2 - Amplification products generated from *Drechslera teres* isolates with primer B10. Lane (1)Lambda DNA digested with *Eco*RI and *Hind*III used as molecular weight marker. Lane: (2) isolates Sanad599; (3) Pirat599; (4) Saog599; (5)Vacar599; (6) Palme599; (7) Caraz599; (8) Encru599 (9); Ipira599. Fragment sizes in base pairs are indicated on the right.

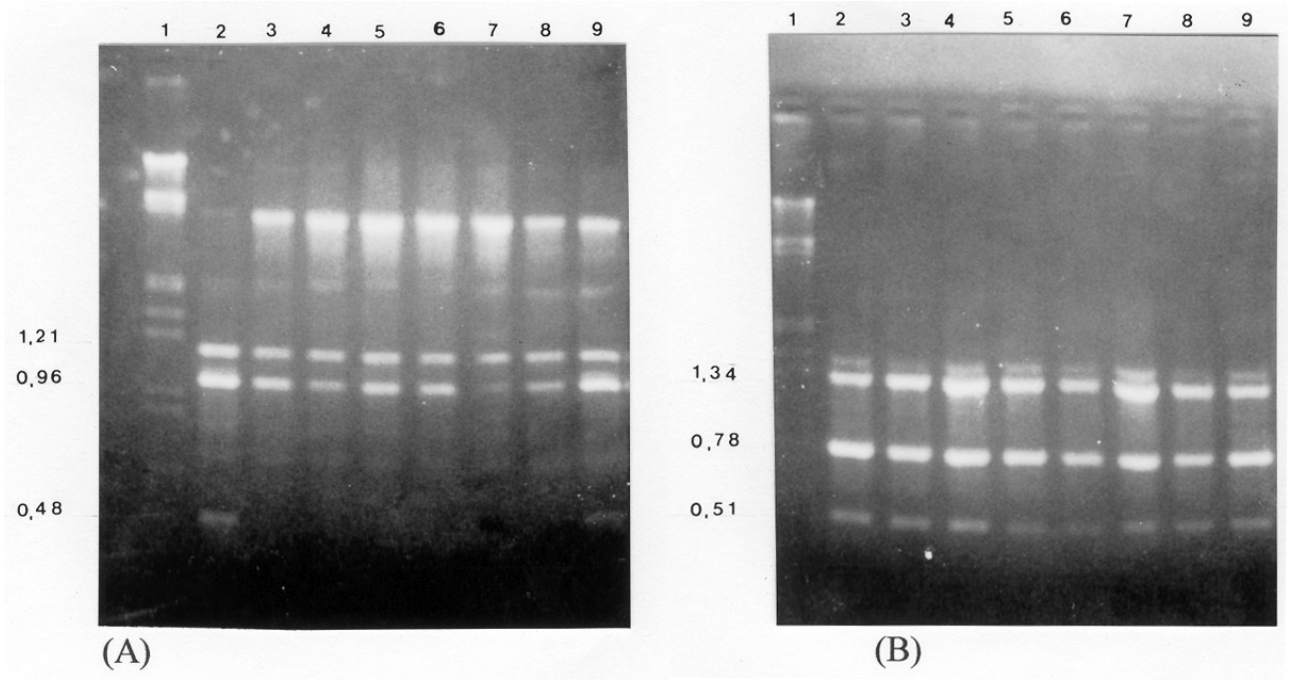

Figure 1 - Gel showing amplification products from *Drechslera teres* isolates with primers B02 (A) e B07 (B). Lane (1) Lambda DNA digested with *Eco*RI and *Hind*III used as molecular weight marker. Lane: (2) isolates Sanad599; (3) Pirat599; (4) Saog599; (5)Vacar599; (6) Palme599; (7) Caraz599; (8) Encru599; (9) Ipira599. Fragment sizes in base pairs are indicated on the right.

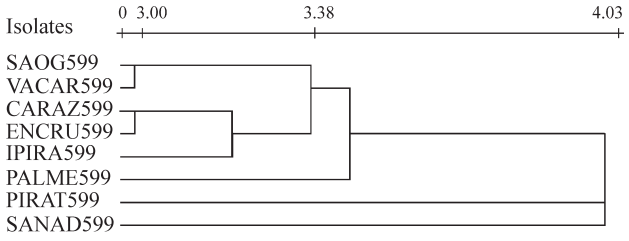

Figure 3 - Dendrogram of the relationships between *Drechslera teres* isolates based on both colony morphology (edge, texture, color and sector) and random amplified polymorphic DNA (RAPD) analysis.

Caraz599 showed the highest similarity coefficient (0.8714) while the lowest (0.7286) was obtained for isolates Sanad599 and Vacar599. All other isolates presented a similarity coefficient between 0.8571 and 0.7429 (Table III).

In this study a correlation between genetic and morphological variability and the geographic origin of the seeds from which the isolates were obtained could not be established. These findings being similar to those obtained by Valim-Labres (1995) and Oliveira (1995) who worked with *B. sorokiniana* and Santos (1996) who studied *D. tritici-repentis*. In our work, this lack of correlation was perhaps due to the lack of multiple samples from the same field or geographic region. Increasing the number of samples and using different primers might result in a correlation. Comparing samples from very different regions of Brazil, or even from different countries, might also allow us to confirm the degree of similarity observed in this work.

Considering only colony morphology the similarity coefficients between isolates were 1.00 to 0.50 (Table IV). The combined culture characteristics of all isolates were analyzed using simple matching coefficients and the dendrogram was constructed (Figure 4). This dendrogram also shows two distinct groups with different similarity levels, one group being formed by isolates Encru599 and Sanad599 (with the highest similarity coefficient of 1.00) and Pirat599 and Vacar599, and a second group which contains all the other isolates.

When only the RAPD data was used cluster analysis showed a main group with decreasing similarity levels, leading to two subgroups. The highest similarity coefficient (0.9032) was observed between isolates Caraz599 and Encru599, and Saog599 and Vacar599. The lowest coefficient in this analysis was 0.7581, which was seen for isolate Vacar599 in relation to isolates Pirat599 and Sanad599, as well as for isolate Saog599 in relation to isolate Pirat599 (Table V). The similarity coefficient for the 16 primers used in this study was between 75 and 90%. The RAPD results suggest low genetic diversity among the *D. teres* isolates from different geographic regions of Rio Grande do Sul (Figure 5).

Considering all the dendrograms together, it seems that separating morphologic characters and RAPD data did not help in finding a correlation between isolate variability and geographic origin. However Guthrie *et al*. (1992) and Assigbetse *et al*. (1994) did succeed in using RAPD analysis to separate isolates based on their geographic origins and DNA amplification products.

Table III - *Drechslera teres* similarity matrix coefficients based on both colony morphology and random amplified polymorphic DNA (RAPD) analysis.

Isolates	Caraz599	Encru599	Ipira599	Palme599	Pirat599	Sanad599	Saog599
Encruz599	0.8714						
Ipira599	0.8571	0.8429					
Palme599	0.8429	0.8000	0.8143				
Pirat599	0.7571	0.8000	0.7571	0.7714			
Sanad599	0.7571	0.8286	0.7571	0.7714	0.7714		
Saog599	0.8429	0.8571	0.8143	0.8286	0.7429	0.7714	
Vacr599	0.8571	0.8143	0.8286	0.8429	0.7571	0.7286	0.8714

Table IV - *Drechslera teres* similarity matrix coefficients based on colony morphology only.

Isolates	Caraz599	Encru599	Ipira599	Palme599	Sanad599	Pirat599	Saog599
Encruz599	0.6250						
Ipira599	0.7500	0.6250					
Palme599	0.7500	0.6250	0.7500				
Sanad599	0.6250	1.0000	0.6250	0.6250			
Pirat599	0.6250	0.7500	0.6250	0.6250	0.7500		
Saog599	0.7500	0.6250	0.7500	0.7500	0.6250	0.6250	
Vacr599	0.6250	0.5000	0.6250	0.6250	0.5000	0.7500	0.6250

Table V - *Drechslera teres* similarity matrix coefficients based on random amplified polymorphic DNA (RAPD) analysis.

Isolates	Caraz599	Encru599	Ipira599	Palme599	Pirat599	Sanad599	Saog599
Encruz599	0.9032						
Ipira599	0.8710	0.8710					
Palme599	0.8387	0.8387	0.8387				
Pirat599	0.7742	0.8065	0.7742	0.8065			
Sanad599	0.7742	0.8065	0.7742	0.8065	0.7742		
Saog599	0.8548	0.8871	0.8226	0.8226	0.7581	0.7903	
Vacr599	0.8871	0.8548	0.8548	0.8548	0.7581	0.7581	0.9032

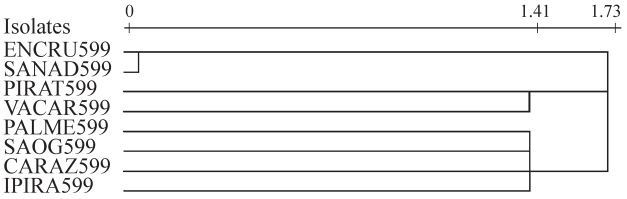

Figure 4 - Dendrogram of the relationships between *Drechslera teres* isolates based on colony morphology (edge, texture, color and sector).

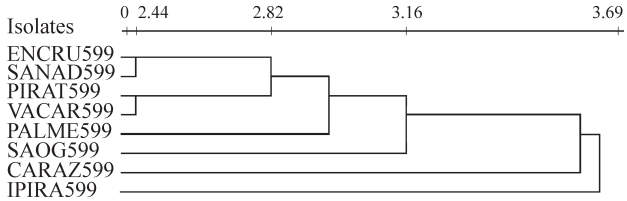

Figure 5 - Dendrogram of the relationships between *Drechslera teres* isolates based on random amplified polymorphic DNA (RAPD) analysis.

Many fungal populations have demonstrated low or moderate levels of genetic variability, and two different hypotheses may account for this. One hypothesis is that proposed by Goodwin *et al.* (1993), who explain the moderate level of variation seen in *Rhychosporium secalis* isolates from Europe, Australia and the USA by suggesting that these populations probably originated from the same ancestor. The other hypothesis, suggested by Boerger *et al.* (1993), proposes that the low genetic differences observed in *Mycosphaerella graminicola* populations from California and Oregon may be related to gene flow events involving these populations. Both these hypotheses may have a part to play in explaining the low level of genetic difference found among the *D. teres* isolates used in our study.

Acknowledgments

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