Genotypic variation in *Rhynchosporium secalis* pathotypes collected in the Western Cape Province of South Africa

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An anonymous multilocus DNA probe was used to characterize the genotypic structure of *Rhynchosporium secalis* isolates previously characterized according to their virulence spectra on a set of differential barley cultivars. The maximum percentage of genotypic diversity of 47 *R. secalis* isolates from the Western Cape Province of South Africa was 46.5%. In comparison with diversity observed at DNA level, less variation was observed in pathogenicity for *R. secalis*. DNA polymorphisms in *R. secalis* seemed to be independent of variation in virulence. No correlation between any particular fingerprint pattern, race, district, field or lesion was observed. The two most frequently observed races, 4 and 7, did not share the same genotypes, even when isolated from the same field or lesion. The high level of genotypic variation observed in the South African *R. secalis* population resembles the genotypic diversity observed in other cereal pathotypes with known sexual states. Although no teleomorph has yet been observed, these data suggest that sexual recombination may operate within the South African population of *R. secalis*.

The barley scald fungus *Rhynchosporium secalis* (Oud.) Davis is an important pathogen of barley and occurs in all barley-producing countries, including South Africa. Barley scald has been responsible for yield reductions of up to 37% in South Africa.[1] Considerable variation in pathogenicity has been reported in populations of *R. secalis*.[2–8] Although new resistant barley cultivars are routinely introduced, this pathogen has been found easily to overcome race-specific resistance.[9] High levels of virulence polymorphism have also been observed within fields, and in lesions on the same leaf.[5] The mechanism of this variation for pathogenicity is, however, still uncertain,[3,10–12] since no teleomorph has yet been reported for *R. secalis*. Furthermore, *R. secalis* pathotypes appear to be unstable and results could often not be repeated between laboratories.[2–4,13,14] Hansen and Magnus[3] found large intra-isolate variation, and were able to recover multiple pathotypes from plant lesions infected with a culture derived from a single conidium. However, those pathotypes were never confirmed by DNA fingerprinting techniques, and thus contamination cannot be ruled out. Zhang *et al.*[15] pointed out that many studies[16–19] had highly repeatable results and reported no intra-isolate variability when the inoculation and incubation method of Jackson and Webster[9] was used.

It is well known that different environmental conditions during pathogenicity studies will affect pathotype analysis[20–23]. In previous studies dealing with *R. secalis*, different rating scales, differential cultivars, as well as different environmental conditions were used to determine pathotypes, making a comparison of results impossible.[5,14] However, neutral molecular markers such as RFLPs (which are highly repeatable) and isozymes, have proved to be useful means to compare the variability of *R. secalis* populations from different studies.[11,24,25]

Recently, McDonald *et al.*[26] developed anonymous RFLP markers for use in *R. secalis*. Probes were selected from a random sample of total genomic DNA fragments of *R. secalis*. These probes were used to assay the genetic structure of *R. secalis* in Australia, where genetic variation on a fine (within 3.24 m^2) and large scale (within 200 m^2) was investigated.[26] Most of the genetic diversity was observed on a fine spatial scale. Since the virulence spectra of the isolates tested were not known, McDonald *et al.*[26] could only speculate about the possible relationships between virulence and genetic diversity. The purpose of the present study was thus to investigate the genotypic variation of South African *R. secalis* isolates using RFLP fingerprinting and to determine if any relationship exists between genotypes and pathotypes.

Materials and methods

Isolates. The 47 *R. secalis* isolates investigated were selected in a previous study, where they had been characterized according to their virulence spectra on a set of 17 differential barley cultivars.[27] The resistance genes reported for this set of differential barley cultivars were summarized by Goodwin *et al.*[28] The 47 isolates represented 20 races, originating from 27 different fields in the Western Cape (Table 1). Ten fields were represented by one isolate each, 14 fields were represented by two isolates (where isolates from seven fields originated from the same lesion) and the remaining three fields were represented by three isolates from three different lesions in each field (Table 1).

Culture and harvesting of fungal strains. A spore suspension of each *R. secalis* isolate was inoculated into flasks with liquid lima bean broth (62 g l^{-1} distilled water) (pH 5.9) and grown at 20°C in a shake incubator (120 rev/min). After two weeks the mycelium was harvested through a sieve and squeezed between filter paper to remove excess liquid. The dried mycelium was immersed in liquid nitrogen, ground with a mortar and pestle to a fine powder, and transferred aseptically into sterile Eppendorf tubes.

Solutions for DNA preparation. Extraction buffer: 200 mmol Tris HCl pH 8.5, 250 mmol NaCl, 25 mmol EDTA, 0.5% SDS; phenol; RNAse A: e.g. Boehringer Mannheim No. 109169, 10 mg ml^{-1} in ddH_20, boiled for 30 min, stored at –20°C.

DNA preparation. Ground mycelium in an Eppendorf tube was resuspended in three volumes SDS extraction buffer and mixed homogeneously. Samples were placed in a boiling bath for 2 min and afterwards snap-frozen by floating tubes in liquid nitrogen for 2 min. Tubes were later placed in a water-bath at 60°C for 10 min to thaw the samples. One volume of phenol was added to one volume of the sample, inverted for 10 min and centrifuged for 20 min in an Eppendorf centrifuge (14 000 × *g*). The upper aqueous phase was immediately removed and transferred to a sterile Eppendorf tube. Phenol-chloroform-isoamylalcohol (2:1:1) was added to one volume of the sample, inverted for 10 min and centrifuged for 10 min at 14 000 × *g*. The upper phase was transferred into a sterile Eppendorf tube and mixed with about one volume isopropanol and 20 µl sodium acetate. DNA precipitated visibly into a lump and was incubated overnight at –20°C for total precipitation. The lump of DNA was spooled out and transferred to a sterile 2 ml tube containing 1 ml TE (pH 8.0). Twenty-five microlitres RNAse A solution was added and the sample incubated overnight at 37°C. Proteinase K (15 µl, 2%) and SDS (5 µl, 20%) were added to the solution and incubated at 60°C

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Table 1. Collection data and race typification of *Rhynchosporium secalis* isolates studied.

Race	Isolates	Virulence spectrum[a]	Octal name	District	Year isolated	Collector
1	11-1-1	000000000010000001	00001001	Caledon	1993	C.L. Lennox
1	35-4-2	000000000010000001	00001001	Philadelphia	1995	B. Robbertse
2	3-1-3	000100000010000001	02001001	Riversdal	1993	C.L. Lennox
2	30-5-1	000100000010000001	02001001	Mooreesburg	1995	B. Robbertse
3	37-4-2	010100100000000001	12200001	Caledon	1995	B. Robbertse
4	5-2-1	000100100010000001	02201001	Swellendam	1993	C.L. Lennox
4	6-5-2	000100100010000001	02201001	Bredasdorp	1993	C.L. Lennox
4	9-3-2	000100100010000001	02201001	Bredasdorp	1993	C.L. Lennox
4	9-4-1	000100100010000001	02201001	Bredasdorp	1993	C.L. Lennox
4	10-3-3	000100100010000001	02201001	Bredasdorp	1993	C.L. Lennox
4	12-1-3	000100100010000001	02201001	Heidelberg	1993	C.L. Lennox
4	13-1-1	000100100010000001	02201001	Heidelberg	1993	C.L. Lennox
4	15-1-1	000100100010000001	02201001	Riversdal	1993	C.L. Lennox
4	31-6-3	000100100010000001	02201001	Caledon	1995	B. Robbertse
4	33-5-1	000100100010000001	02201001	Caledon	1995	B. Robbertse
4	37-1-1	000100100010000001	02201001	Caledon	1995	B. Robbertse
5	36-5-3	000100110010000001	02301001	Caledon	1995	B. Robbertse
6	8-3-1	000100100010010001	02201021	Bredasdorp	1993	C.L. Lennox
7	3-2-1	010100100010000001	12201001	Riversdal	1993	C.L. Lennox
7	4-5-1	010100100010000001	12201001	Caledon	1993	C.L. Lennox
7	5-1-1	010100100010000001	12201001	Swellendam	1993	C.L. Lennox
7	7-1-3	010100100010000001	12201001	Caledon	1993	C.L. Lennox
7	7-4-1	010100100010000001	12201001	Caledon	1993	C.L. Lennox
7	8-5-1	010100100010000001	12201001	Bredasdorp	1993	C.L. Lennox
7	13-1-3	010100100010000001	12201001	Heidelberg	1993	C.L. Lennox
7	14-2-2	010100100010000001	12201001	Swellendam	1993	C.L. Lennox
7	38-4-1	010100100010000001	12201001	Caledon	1995	B. Robbertse
7	42-2-3	010100100010000001	12201001	Caledon	1995	B. Robbertse
7	45-8-6	010100100010000001	12201001	Bredasdorp	1995	B. Robbertse
8	33-5-2	000100100010001001	02201011	Caledon	1995	B. Robbertse
9	30-3-1	010100101010000001	12211001	Mooreesburg	1995	B. Robbertse
9	5-3-2	010100101010000001	12211001	Swellendam	1993	C.L. Lennox
10	6-1-3	010100110010000001	12301001	Bredasdorp	1993	C.L. Lennox
10	7-5-1	010100110010000001	12301001	Caledon	1993	C.L. Lennox
10	12-1-2	010100110010000001	12301001	Heidelberg	1993	C.L. Lennox
10	27-4-1	010100110010000001	12301001	Caledon	1995	B. Robbertse
10	28-3-3	010100110010000001	12301001	Caledon	1995	B. Robbertse
11	27-2-2	110110010010000001	33101001	Caledon	1995	B. Robbertse
12	39-1-1	010100110010100001	12301101	Heidelberg	1995	B. Robbertse
13	4-5-3	110110100010000001	33201001	Caledon	1993	C.L. Lennox
14	35-2-3	010100111110000001	12312001	Philadelphia	1995	B. Robbertse
15	40-5-1	110110110010000001	33301001	Swellendam	1995	B. Robbertse
16	23-4-5	110100111010100001	32311101	Bredasdorp	1994	C.L. Lennox
18	39-1-3	110100111010011001	32311031	Heidelberg	1995	B. Robbertse
19	11-1-3	110110111010101001	33311111	Caledon	1993	C.L. Lennox
20	27-3-1	110110111010101011	33311113	Caledon	1995	B. Robbertse
21	28-3-1	111110111010101011	37311113	Caledon	1995	B. Robbertse

[a]According to the octal nomenclature proposed by Goodwin *et al.*[28]. Pathogenicity to the differential cultivars C.I.2226, Jet, Psaknon, West China, Nigrinudum, C.I. 3515, C.I. 8618, C.I. 4364, Abyssinian, Osiris, Steudelli, Turk, Modoc, Brier, Wisconsin Winter × Glabron, Atlas 46, La Mesita and the susceptible cultivar Clipper are listed from left to right, where 1 = susceptible and 0 = not susceptible. (Abyssinian was used as the source of the Rh9 resistance instead of Kithchin; cultivars proposed for digit 8 were not evaluated in this study, instead Jet and C.I.2226 were incorporated).

for two hours. Phenol and chloroform-isoamylalchohol were added to the sample (1:1:2) and inverted a few times before being centrifuged for 10 min at 14 000 × *g*. The upper phase was transferred into a sterile Eppendorf tube and mixed with about one volume isopropanol and 20 μl sodium acetate (3 M, pH 6.0), and incubated at –20°C for two hours. The tube was centrifuged for 1 min to concentrate the precipitate into a DNA pellet. As much liquid as possible was decanted and the pellet rinsed with 70% ethanol, dried in an oven at 50°C for 30 min and resuspended in 50–100 μl TE (pH 8.0) according to the amount of DNA.

Enzyme digestion and DNA fingerprint. Total DNA (3 μg) was digested overnight with the restriction enzyme *Eco*R1. Digested DNA (2.5 μg per lane) was separated in an 0.8% agarose TAE (0.5 X) gel for 21 hours at 1.75 V/cm using gel electrophoresis. Upward capillary transfer of DNA to a nylon$^+$ membrane (Magnagraph-N+, MSI) was conducted overnight. DNA was fixed to the membrane by UV cross-linking on a 312 nm transilluminator for 3 min. A PCR DIG Probe synthesis kit (Roche) was used to label the pRs26-DNA probe.[26] Pre-hybridization and hybridization reactions were performed according to the manufacturer's instructions in a hybridization oven (Personalhyb, Stratagene) at 40°C. Hybridization reactions were allowed to proceed for 16–18 hours. After hybridization the membrane was washed twice at room temperature in 2XSSC;

0.1% w/v SDS for 5 min each time, followed by two 15-min washes in 0.5XSSC; 0.1% w/v SDS at 65°C. Detection of the hybridized probe and bound antibody conjugate was performed using a chemiluminescent alkaline phosphatase substrate (CDP-Star, Roche) according to the manufacturers instructions. Film (X-Omat AR, Kodak) was placed on the membrane and exposed for 10–20 min depending on the strength of the light signal. A multicopy DNA probe (pRs26) was provided by B. McDonald (Federal Institute of Technology, ETH-Zentrum, Switzerland), who identified it as being useful for fingerprinting isolates of *R. secalis*.[26]

Data analysis. Different multilocus haplotypes were assumed to represent different genotypes. The maximum percentage of genotype diversity ($\hat{G}$) and its variance were quantified according to Stoddart and Taylor[32].

Pathogenic diversity within and among clonal genotypes was quantified with the Shannon information statistic according to the description in Goodwin *et al.*[29] This statistic can be partitioned into within- and among-genotype components,[30] analogous to Nei's gene diversity analysis,[31] but is more suitable for phenotypic data.

Results

The maximum percentage of genotypic diversity of 47 *Rhynchosporium secalis* isolates from the Western Cape was 46.5% of its theoretical maximum diversity according to the measure of Stoddart and Taylor[32] (Table 2).

Rhynchosporium secalis isolates were genotypically more diverse than pathogenically and the spatial distribution of geno- and pathotypes differed (Fig. 1; Table 3). In four of seven cases where isolates originated from the same lesion, they represented different fingerprints, whereas isolates within each lesion always differed in pathotype (Table 3). Genotypes 1 and 4 were scattered over two districts and genotype 5 was scattered over three districts (Table 3). Genotypes 1 and 5 were present in the 1993 as well as in the 1995 *R. secalis* population. No association between genotype characteristic and pathotype was found, as isolates with the same genotype frequently represented different pathotypes (Table 3). The highest pathogenic variation was found in the most frequently observed genotype, namely genotype 1 (Table 4).

Discussion

In this study it was shown that 47 South African isolates of *R. secalis* exhibited a high degree of genotypic diversity (determined with DNA fingerprinting), as well as high degrees of variation in pathogenicity (determined on a set of differential barley cultivars). High degrees of genotypic variation has also been reported for *R. secalis* in other studies,[11,26,33] which is quite unexpected for a fungus with no known teleomorph.

No correlation between any particular multilocus haplotype and race at any district, field or lesion was observed. This supports the findings of isozyme studies by Newman[34] and Burdon *et al.*,[11] who did not find any correlation between any particular isozyme band or pattern with either host cultivar or

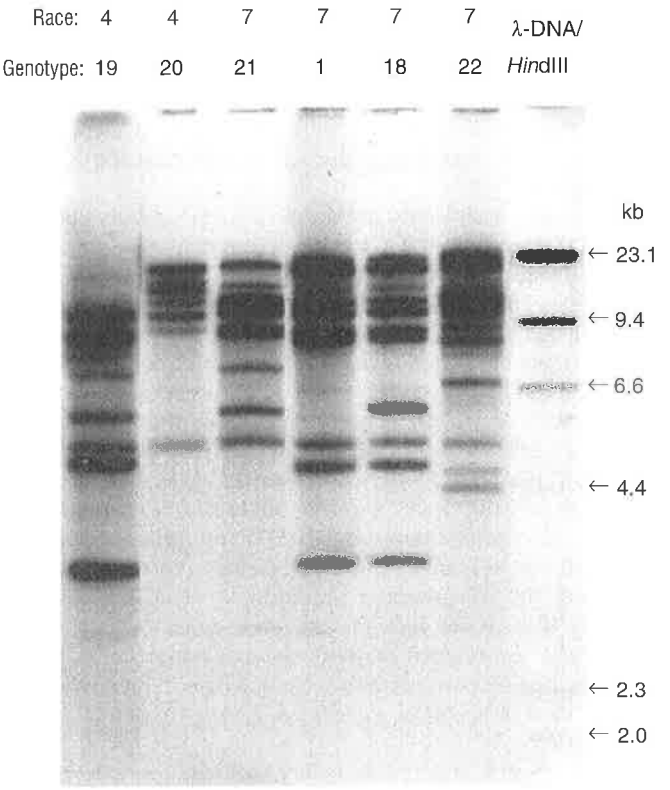

Fig. 1. DNA fingerprints of races 4 and 7 of *Rhynchosporium secalis*, produced by the multilocus probe pRS26 revealing six different genotypes. The last lane is a λ-*Hin*dIII standard and fragments are indicated in kilobases to the right.

site. In the present study, isolates that were identical according to DNA fingerprinting (and presumed to be clonal) represented different pathotypes. By contrast, Goodwin *et al.*[30] found association of particular electrophoretic genotypes (potentially clonal) with particular pathotypes within populations. However, in general no association between electrophoretic genotype and pathotype was evident.[30] It is possible, therefore, that the fingerprinting probe used in the present study may not be sensitive enough to distinguish between closely related clones. Little is known about the fingerprints produced by the pRs26 probe, as previous studies where it was employed did not include pathotype information. McDonald *et al.*,[26] using the method of Jeffreys *et al.*,[35] estimated that the average probability that two isolates chosen at random would have the same hybridization profile was $4 \times 10.^{-6}$ They suggested, therefore, that pRs26 could be useful for DNA fingerprinting in other populations.[26]

The maximum percentage of genotypic diversity was lower in the present study ($\hat{G}/n = 46.5$) than in that of McDonald *et al.*,[26] where the average genotypic diversity within a field population was 65% of the theoretical maximum. Results of the present study were consistent with previous observations, where the majority of genetic variation was distributed within fields on a

Table 2. Frequencies, diversity and clonal fraction of *Rhynchosporium secalis* genotypes.

Total sample	Total number of genotypes	Number of genotypes	Frequency	G	$\hat{G}/n$	Clonal fraction (%)
47	32	24	1	21.87	46.5%	32%
		5	2			
		2	4			
		1	5			

fine spatial scale and not between fields.[26] This distribution of genotypic diversity has also been found in other cereal pathogens such as *Septoria tritici* and *Stagonospora nodorum*.[36,37] Both these species have known teleomorphs and it was demonstrated that most genetic variation within populations of these pathogens was due to sexual reproduction, and to a lesser extent due to migration.[36,37]

The genotypic diversity of the two most frequently observed races in this study (4 and 7) indicated that they were genetically isolated from each other, sharing none of the same genotypes, even when isolated from the same field or lesion (Fig. 1). This is not unexpected, as the fingerprinting probe used was selected at random from the *R. secalis* genome, and therefore was probably not tightly linked to genes under selection such as virulence genes. Isolates within a pathotype were therefore not all expected to belong to a single multilocus haplotype. Eight different genotypes were identified among 11 isolates of each race. This indicates that races 4 and 7 originated from genetically diverse source populations, and that polymorphism at the DNA level in *R. secalis* is independent of variation in virulence. Furthermore, the independent variation in virulence observed could be due to the lack of resistance genes in local barley cultivars. The presence of particular resistance genes would have selected for corresponding races, which in turn would result in their associated genotypes being carried along by hitch-hiking.

Because highly susceptible barley cultivars are grown commercially in the Western Cape,[38] a low level of selection pressure on the virulence genes in the *R. secalis* population would have been expected. Some measure of selection did function, however, since more frequently observed races (races 1, 2, 4, 7, 9, 10) were readily found in the 1993 and 1995 seasons. Only genotype 1 (represented by 4 isolates in 1993 and 1 in 1995) and genotype 5 (represented by 2 isolates in 1993 and 2 in 1995) persisted over a period of two years. The same geno- and pathotype combinations (genotype 1 and race 7; genotype 5 and race 4) were observed in two of the three instances where genotypes persisted over two years. Races 4 and 7 were the most prominent, and these genotypes were probably carried over as a result of hitch-hiking.

Multiple pathotypes recovered from single plant lesions in the present study and previously reported by Brown (1985) may in part be a result of different genotypes being present within the same lesion (Table 3). It is evident that host selection in concert with a source of variation plays an important role in the race-structure of the *R. secalis* population. Regular recombination not only generates diversity by continually reshuffling genes, but also prevents the dominance of a few clones. Infrequent sexual reproduction together with highly adapted and widespread clones would facilitate deterioration of host resistance or fungicide efficacy. Future research on the South African

Table 3. Pathogenicity and distribution of *Rhynchosporium secalis* genotypes.

Genotype	Race	Isolates	Year isolated	District
1	1	11-1-1*	1993	Caledon
1	6	8-3-1	1993	Bredasdorp
1	7	42-2-3	1993	Bredasdorp
1	7	8-5-1	1995	Caledon
1	19	11-1-3	1993	Caledon
2	7	4-5-1	1993	Caledon
2	7	7-1-3	1993	Caledon
2	7	7-4-1	1993	Caledon
2	10	7-5-1	1993	Caledon
3	13	4-5-3	1993	Caledon
4	3	37-4-2	1995	Caledon
4	7	45-8-6	1995	Bredasdorp
5	4	5-2-1	1993	Swellendam
5	4	15-1-1	1995	Caledon
5	4	31-6-3	1993	Riversdal
5	5	36-5-3	1995	Caledon
6	4	33-5-1	1995	Caledon
6	8	33-5-2	1995	Caledon
7	15	40-5-1	1995	Swellendam
8	16	23-4-5	1994	Bredasdorp
9	4	6-5-2	1993	Bredasdorp
9	4	9-4-1	1993	Bredasdorp
10	20	27-3-1	1995	Caledon
11	9	30-3-1	1995	Mooreesburg
12	10	6-1-3	1993	Bredasdorp
13	11	27-2-2	1995	Caledon
14	14	35-2-3	1995	Philadelphia
15	21	28-3-1	1995	Caledon
16	7	13-1-3	1993	Heidelberg
17	7	14-2-2	1993	Swellendam
18	2	3-1-3	1993	Riversdal
18	7	3-2-1	1993	Riversdal
19	4	12-1-3	1993	Heidelberg
19	10	12-1-2	1993	Heidelberg
20	4	37-1-1	1995	Caledon
21	7	5-1-1	1993	Swellendam
22	7	38-4-1	1995	Caledon
23	2	30-5-1	1995	Mooreesburg
24	12	39-1-1	1995	Heidelberg
25	1	35-4-2	1995	Philadelphia
26	4	9-3-2	1993	Bredasdorp
27	9	5-3-2	1993	Swellendam
28	10	28-3-3	1995	Caledon
29	18	39-1-3	1995	Heidelberg
30	4	13-1-1	1993	Heidelberg
31	4	10-3-3	1993	Bredasdorp
32	10	27-4-1	1995	Caledon

*Isolate number (location – lesion – spore).

Table 4. Mean pathogenic diversity within genotypes of the *Rhynchosporium secalis* population from the Western Cape Province of South Africa.

Genotype	Mean pathogenic diversity	Normalized pathogenic diversity	Number of isolates
SA1	0.304508	0.439405	5
SA2	0.033079	0.047732	4
SA4	0.040773	0.058836	2
SA5	0.066157	0.095465	4
SA6	0.040773	0.058836	2
SA9	0	0	2
SA18	0.081547	0.117672	2
SA19	0.081547	0.117672	2

R. secalis population should focus on the genetic structure of a population within a field, as this may reveal the role of the postulated sexual cycle.

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