New Aggressive *Colletotrichum gloeosporioides* Strains on *Stylosanthes scabra* Detected by Virulence and DNA Analysis

S. Chakraborty and R. Perrott, Commonwealth Scientific and Industrial Research Organization, Tropical Agriculture, Co-operative Research Centre for Tropical Plant Pathology, University of Queensland, Australia 4072; and N. Ellis and M. R. Thomas, CSIRO Mathematical and Information Sciences, 306 Carmody Road, St. Lucia, Queensland, Australia 4067

ABSTRACT

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Widespread use of *Stylosanthes scabra* cv. Seca to improve native pastures in northern Australia makes it necessary to monitor changes in the anthracnose pathogen, *Colletotrichum gloeosporioides*, because new damaging races have arisen to devastate many agronomically promising cultivars in the past. A total of 103 isolates collected during the past 20 years were analyzed by using virulence and molecular markers to determine whether aggressive strains have evolved in the field. Data on severity for eight host differentials were obtained from a greenhouse assay and analyzed by linear discriminant functions developed from existing data on 182 isolates of known races. A molecular analysis of a subset of 21 isolates by random amplified polymorphic DNA (RAPD) and electrophoretic karyotyping gave comparable results for both markers, although electrophoretic karyotyping detected a higher level of polymorphism. The majority of the 103 isolates were placed into three groups on the basis of RAPD analysis. Virulence analysis detected isolates that were highly aggressive toward Seca. During the past 20 years, the frequency of aggressive isolates has increased. Many of the aggressive isolates were collected during the 1990s, and one had a distinct RAPD genotype. The lack of a strong association between RAPD genotypes and pathogen races suggests that races have arisen independently within each genetic group. This work highlights the need for regular monitoring of the pathogen population.

Additional keywords: genetic groups, multivariate analysis, pathogenic groups, race surveys

Pasture legumes of the genus *Stylosanthes*, introduced from Central and South America, have been among the most successful in improving livestock nutrition and production and soil fertility in the tropical and subtropical regions of Australia. More than 1 million ha of native pastures have been oversown with cultivars of mainly *S. scabra* Vog. and *S. hamata* (L.) Taub. This area is currently increasing by 100,000 ha per year (6). The recent release of two new cultivars of *S. seabrana* will further extend the range of soil types and climatic zones in which *Stylosanthes* spp. can be successfully utilized in Australia.

Anthracnose, caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz., remains a major threat to commercial utilization of *Stylosanthes* spp. (13). During the 1970s, this disease devastated an estimated 500,000 ha of highly susceptible cultivars of *S. humilis* Kunth (6). Al-

though this species has since been replaced with more resistant cultivars of *S. scabra* and *S. hamata*, anthracnose continues to cause losses in seed and herbage yield (9), since none of the commercial cultivars is completely resistant.

C. gloeosporioides shows considerable variation in virulence in Australia, and two biotypes of the pathogen, distinguished by host range, morphology, symptomatology and genetic characteristics (13,21), have been recorded. Biotype A is pathogenic on all species of *Stylosanthes* while biotype B is pathogenic mainly on *S. guianensis* (Aubl.) Sw. Because *S. guianensis* cultivars are suited only to a limited geographical area in Australia, biotype A is economically more significant, and discussions in this paper are restricted to this biotype.

C. gloeosporioides has readily adapted to resistant cultivars following their release. *S. scabra* cv. Fitzroy was productive over a large area of central Queensland but was discarded within 5 years of its release after highly aggressive strains severely damaged stands. Similarly, a new race infecting *S. scabra* cv. Seca was discovered within 6 years of release of this highly resistant cultivar (8). Although only minor damage to commercial Seca was noticed in an earlier work (14), industry reliance on Seca

as the single most important cultivar dictates that regular surveys be carried out to determine whether more aggressive strains are developing on Seca. Given that virulence also measures relative aggressiveness (25), tools such as a recently described multivariate technique for the analysis of pathogenic variation in *C. gloeosporioides* (5) can be used to analyze aggressiveness.

DNA markers can replace time-consuming and often variable (4) pathogenicity assays if there is strong correlation between pathogenicity and molecular markers. Selection-neutral random amplified polymorphic DNA (RAPD) markers have been used to differentiate races of *Fusarium solani* f. sp. *cucurbitae* (7). Although differences in restriction fragment length polymorphism (RFLP) between the two biotypes of *C. gloeosporioides* that infect *Stylosanthes* are well established (1), only limited information is available on the level of genetic variation within each biotype, and correlation between molecular groups and races has not been studied. Using four biotype A and seven biotype B isolates, Masel et al. (22) found that none of the isolates had the same minichromosome complement, indicating that electrophoretic karyotyping (EK) can be a powerful tool in the analysis of the *C. gloeosporioides* population. A detailed molecular analysis of the Australian biotype A pathogen population is currently lacking.

Using field surveys, we collected isolates of *C. gloeosporioides* from commercial seed-production plots and other sites in Queensland, Australia, where *Stylosanthes* is used in commercial pastures. We examined the level of diversity in 103 isolates collected during more than 20 years using virulence and molecular analysis. The objectives of this study were to determine (i) whether more aggressive strains capable of damaging the cultivar Seca were present in the pathogen population collected during a period of time and (ii) whether there was a relationship between DNA polymorphism and variation in virulence and aggressiveness in the pathogen population that would allow the use of molecular markers to identify pathogen races.

MATERIALS AND METHODS

Isolates. Samples of infected *Stylosanthes* leaves and stems were collected from different areas in Queensland, Aus-

Corresponding author: S. Chakraborty
E-mail: sukumar.chakraborty@tag.csiro.au

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tralia, and single-spore isolates of *C. gloeosporioides* were obtained from these samples. Selected single-spore isolates were also obtained from the existing collections of J. Irwin (University of Queensland) and R. Davis (Queensland Department of Primary Industries). All 103 isolates, including reference isolates cs89, sr24, 21365, wrs20, and wrs32, which represent the current biotype A races in Australia, were grown on oatmeal agar and stored as lyophilized cultures (Table 1). All reference isolates are virulent on Fitzroy and avirulent on accession 93116 (5). Race 1, represented by the reference isolate cs89, is virulent only on Fitzroy. Race 2 (isolate 21365) is virulent on *S. viscosa* Sw. accession 33941 (13). Race 3 (isolate sr24) is virulent on

Seca. Races 4 (wrs32) and 4a (wrs20) are most severe on 36260. Differences in susceptibility to races 4 and 4a in Q10042 are quantitative, and in some tests similar disease severity levels on Q10042 have been recorded with both races (4). These two isolates could not be distinguished from each other by multivariate analysis and have since been grouped as race 4 (5).

Assessment and analysis of pathogenic variation. Pathogenic variation in *C. gloeosporioides* isolates infecting *S. scabra* was assessed by using six differentials comprising cultivars Fitzroy and Seca and accessions 36260, 55860, Q10042, and 93116 (5). Of these, Fitzroy, Seca, 36260, Q10042, and 93116 form the main group of differentials necessary to identify races.

Accession 55860 is partially resistant to all isolates tested so far and does not contribute to the separation of races (5). In this work, 55860 was replaced with 110354 to detect potential virulence toward this highly resistant accession. In addition, two *S. viscosa* accessions, 33941 and 34904, were used to detect race 2 (13). Six-week-old seedlings of the differentials were raised in a greenhouse in sandy loam soil in 50-mm² plastic pots (Kwik pots, Arthur Yates & Co., Australia). Thirty pots were randomized in a tray to contain three to four replicate seedlings of each host differential. Seedlings in each tray were inoculated with a suspension (10⁶ conidia/ml) of a single isolate of *C. gloeosporioides* by spraying to incipient run off and then

Table 1. Source and characteristics of *Colletotrichum gloeosporioides* isolates used in this study^a

Isolate	Year	Host	Location	Race	RAPD cluster
*uq 14	1970s	<i>Stylosanthes scabra</i>	North Qld.	3	G
*21365	1978	<i>S. viscosa</i> 33941	Townsville	2	J
sr13	1980s	<i>S. fruticosa</i> 41219A2	Southedge	1	J
sr23	1982	<i>S. guianensis</i> 18750	Southedge	1	C
wrs8	1982	<i>S. montevidensis</i> 11494	Walkamin	1	C
wrs10	1982	<i>S. hamata</i> 75169	Walkamin	1	J
wrs15	1982	<i>S. hamata</i> 57248	Walkamin	1	J
wrs18	1982	<i>S. fruticosa</i> 60354	Walkamin	1	J
*sr4	1982	<i>S. scabra</i> cv. Fitzroy	Southedge	1	J
sr4-1	1982	<i>S. scabra</i> cv. Fitzroy	Southedge	3	D
wrs14	1982	<i>S. hamata</i> 75164	Walkamin	3	J
*wrs20	1982	<i>S. scabra</i> 36260	Walkamin	4a	G
wrs3	1982	<i>S. capitata</i> 40240B	Walkamin	Unclassified	J
wrs11	1982	<i>S. capitata</i> 55840	Walkamin	Unclassified	A
wrs34	1982	<i>S. guianensis</i> cv. Endeavour	Walkamin	Unclassified	J
*sr30	1984	<i>S. hamata</i> cv. Verano	Southedge	1	J
wrs35	1984	<i>S. guianensis</i> 79639	Walkamin	1	L
*wrs33	1984	<i>S. scabra</i> 36260	Walkamin	3	D
*sr24	1984	<i>S. scabra</i> cv. Seca	Southedge	3	D
*wrs51	1984	<i>S. fruticosa</i> 41219	Walkamin	4	G
*wrs32	1984	<i>S. scabra</i> Q10042	Walkamin	4	G
*cs18	1986	<i>S. hamata</i> 75166	Woodleigh	1	J
cs20	1986	<i>S. hamata</i> cv. Verano	Springs Rd.	1	J
cs133	1986	<i>Stylosanthes</i> mixture	Springs Rd.	3	H
cs137	1986	<i>Stylosanthes</i> mixture	Springs Rd.	3	G
T7G22	1986	<i>Stylosanthes</i> mixture	Springs Rd.	4	I
T8G7	1986	<i>Stylosanthes</i> mixture	Springs Rd.	4	J
cs136	1986	<i>Stylosanthes</i> mixture	Springs Rd.	4	J
*cs116	1987	<i>S. scabra</i> Q10042	Samford	1	J
cs68	1987	<i>S. scabra</i> Q10042	Boowoogum	1	J
*cs89	1987	<i>S. scabra</i> cv. Fitzroy	Samford	1	J
*cs105	1987	<i>S. scabra</i> cv. Fitzroy	Samford	3	D
*cs87	1987	<i>S. scabra</i> cv. Fitzroy	Samford	3	D
cs69	1987	<i>S. scabra</i> cv. Fitzroy	Boowoogum	3	K
*cs231	1988	<i>S. scabra</i> cv. Seca	Samford	1	D
cs157	1988	<i>S. scabra</i> 55860	Byrne valley	1	G
*cs232	1988	<i>S. scabra</i> cv. Fitzroy	Samford	3	J
*cs218	1988	<i>S. scabra</i> Q10042	Samford	3	D
cs154	1988	<i>S. scabra</i> cv. Fitzroy	Byrne valley	3	G
cs158	1988	<i>S. scabra</i> Q10042	Byrne valley	3	G
cs141	1988	<i>S. scabra</i> cv. Seca	Springmount	3	G
cs144	1988	<i>S. scabra</i> mixture	Springmount	3	F
*cs239	1988	<i>S. scabra</i> cv. Seca	Samford	3	D
*cs204	1988	<i>S. scabra</i> cv. Seca	Samford	3	D
cs199	1988	<i>S. scabra</i> Q10042	Samford	4	G
*cs233	1988	<i>S. scabra</i> 36260	Samford	4	G
wrs52	1989	<i>S. guianensis</i>	Walkamin	1	B
cs309	1989	<i>S. scabra</i> Q10042	Boowoogum	1	G
cs310	1989	<i>S. scabra</i> cv. Fitzroy	Wycheproof	1	F
*cs273	1989	<i>S. scabra</i> 55857	Cedarvale	1	G

(continued on next page)

^a The 21 isolates used to compare polymorphism by electrophoretic karyotype and random amplified polymorphic DNA (RAPD) markers are indicated by asterisks.

maintained in a dew chamber for 48 h. Isolates were grown on oatmeal agar for 7 days, and cultures were flooded with sterile distilled water to obtain conidial suspensions. Seedlings were visually examined for percentage of leaf area diseased 10 days after inoculation and rated on a scale of 0 to 9 (0, no visible symptoms; 1, 1 to 3%; 2, 4 to 6%; 3, 7 to 12%; 4, 13 to 25%; 5, 26 to 50%; 6, 51 to 75%; 7, 76 to 87%; 8, 88 to 94%; and 9, 95 to 100% leaf area diseased) (4). Because of limited greenhouse space, only a small number of isolates were screened at any one time. At each inoculation, one or more of the reference isolates were included for comparison.

The bioassay was repeated once, and data from the two runs were averaged for

an analysis of pathogenic variation. Data on disease severity of each isolate on the host differentials were $\log_e(\text{severity} + 1)$ transformed and analyzed by a multivariate technique. In this analysis, a data set containing disease severity on the host differentials for isolates of known races was used to develop linear discriminant functions (LDF), which were applied to the data on disease severity of new isolates to ascertain their racial grouping. Previous work on 182 Australian isolates found three major races within the population infecting *S. scabra*. Data on these 182 isolates were used as the training set to develop LDF for the three races. Details of this procedure have been reported (5).

Molecular analysis. A subset of 21 isolates representing races 1, 2, 3, 4, and 4a

was analyzed by RAPD and EK to select an easy-to-use marker system for screening biotype A isolates. Arbitrary primers used for RAPD analysis were selected from a preliminary study in which seven isolates, including the five reference isolates, were screened with 143 arbitrary decanucleotide primers (Operon Technologies, Alameda, CA). Of these, six primers, AN 11 (GTC-CATGCAG), L02 (TGGGCGTCAA), L11 (ACGATGAGCC), P15 (GGAAGCCA-AC), P16 (CCAAGCTGCC) and Q20 (TCGCCCAGTC), gave scorable polymorphic bands. All 103 isolates were subsequently screened by RAPD analysis.

For RAPD analysis, single-spore cultures were multiplied on oatmeal agar, and DNA was extracted by the method of Edwards et

Table 1. (continued from preceding page)

Isolate	Year	Host	Location	Race	RAPD cluster
cs288	1989	<i>S. scabra</i> cv. Fitzroy	Lynford	1	G
cs282	1989	<i>S. scabra</i> cv. Seca	Byrne valley	3	G
cs295	1989	<i>S. scabra</i> mixture	Byrne valley	3	G
cs302	1989	<i>S. scabra</i> mixture	Byrne valley	3	G
cs325	1989	<i>S. scabra</i> mixture	Byrne valley	3	G
cs290	1989	<i>S. scabra</i> cv. Seca	Tedlands	3	G
cs298	1989	<i>S. scabra</i> 55860	Tedlands	3	G
cs312	1989	<i>S. scabra</i> cv. Fitzroy	Tedlands	3	G
cs276	1989	<i>S. scabra</i> cv. Fitzroy	Wycheproof	3	G
cs286	1989	<i>S. scabra</i> 55857	Wycheproof	3	G
cs287	1989	<i>S. scabra</i> Q10042	Wycheproof	3	G
cs299	1989	<i>S. scabra</i> 36260	Wycheproof	3	G
cs291	1989	<i>S. scabra</i> Q10042	Cedarvale	3	G
cs296	1989	<i>S. scabra</i> cv. Seca	Cedarvale	3	E
cs274	1989	<i>S. scabra</i> Q10042	Mt. Pluto	3	G
cs275	1989	<i>S. scabra</i> 55857	Mt. Pluto	3	G
cs280	1989	<i>S. scabra</i> mixture	Mt. Pluto	3	G
cs285	1989	<i>S. scabra</i> cv. Fitzroy	Mt. Pluto	3	G
cs305	1989	<i>S. scabra</i> mixture	Mt. Pluto	3	G
cs289	1989	<i>S. scabra</i> cv. Fitzroy	Boowoogum	4	G
cs292	1989	<i>S. scabra</i> Q10042	Boowoogum	4	G
cs297	1989	<i>S. scabra</i> 55860	Cedarvale	4	G
cs307	1989	<i>S. scabra</i> 36260	Cedarvale	4	G
cs300	1989	<i>S. scabra</i> 55857	Lynford	4	G
cs304	1989	<i>S. scabra</i> cv. Fitzroy	Byrne valley	Unclassified	G
cs294	1989	<i>S. scabra</i> mixture	Mt. Pluto	Unclassified	G
nq220	1992	<i>S. scabra</i> 93116	Springmount	Unclassified	D
nq244	1992	<i>S. scabra</i> 55860	Springmount	Unclassified	M
nq176	1992	<i>S. scabra</i> cv. Fitzroy	Springmount	Unclassified	D
nq301	1992	<i>S. scabra</i> cv. Fitzroy	Springmount	3	G
nq283	1992	<i>S. scabra</i> cv. Seca	Springmount	3	G
nq297	1992	<i>S. scabra</i> cv. Seca	Springmount	3	D
cs1106	1993	<i>S. scabra</i> mixture	Cedarvale	3	G
cs1096	1993	<i>S. scabra</i> mixture	Cedarvale	3	G
cs1095	1993	<i>S. scabra</i> mixture	Cedarvale	3	G
cs1074	1993	<i>S. scabra</i> cv. Seca	Cedarvale	Unclassified	G
cs1082	1993	<i>S. scabra</i> cv. Seca	Cedarvale	Unclassified	G
cs1076	1993	<i>S. scabra</i> cv. Seca	Cedarvale	3	G
nq124	1994	<i>S. scabra</i> cv. Seca	Springmount	Unclassified	G
nq151	1994	<i>S. scabra</i> 36260	Springmount	Unclassified	D
nq162	1994	<i>S. scabra</i> Q10042	Springmount	Unclassified	G
cs1248	1994	<i>S. scabra</i> mixture	Cedarvale	3	G
cs1199	1994	<i>S. scabra</i> mixture	Cedarvale	3	G
cs1374	1994	<i>S. scabra</i> cv. Seca	Cedarvale	Unclassified	G
cs1599	1996	<i>S. scabra</i> cv. Seca	T. Jepperson	Unclassified	G
cs1600	1996	<i>S. scabra</i> cv. Seca	T. Jepperson	3	G
cs1443	1996	<i>S. scabra</i> cv. Seca	T. Jepperson	Unclassified	G
cs1571	1996	<i>S. scabra</i> cv. Seca	Rains, Home	Unclassified	D
cs1588	1996	<i>S. scabra</i> cv. Seca	North Walsh	3	G
cs1499	1996	<i>S. scabra</i> cv. Seca	Wycheproof	1	G
cs1559	1996	<i>S. scabra</i> cv. Seca	Cuzzabo	Unclassified	D
cs1566	1996	<i>S. scabra</i> cv. Seca	Cuzzabo	Unclassified	G
cs1517	1996	<i>S. scabra</i> cv. Seca	Brian Pastures	1	G

al. (10) with minor modifications. Mycelia and conidia were ground in an extraction buffer (250 mM Tris HCl, 200 mM NaCl, 25 mM EDTA, and 5% sodium dodecyl sulfate), centrifuged, cleaned with phenol chloroform, and washed with chloroform. The DNA was then precipitated in ethanol. DNA from duplicate samples of each isolate was amplified by polymerase chain reaction conducted in a programmable thermocycler (MJ Research, Watertown, MA). After an initial 5-min denaturation step at 94°C, 40 cycles consisting of 1 min of denaturation at 94°C, 1 min of annealing at 37°C, and 2 min of primer extension at 72°C were performed. The amplification product (30 µl) was electrophoresed in 1.5% agarose gel containing 40 µg of ethidium bromide. Gels were run in half-strength Tris-borate-EDTA buffer, and duplicate samples of each isolate were used for verification.

For EK analysis, conidia were obtained from single-spore cultures of isolates grown in V8 juice for 7 days with shaking. Spore blocks were prepared for pulse field electrophoresis according to Masel et al. (22). Conidia were centrifuged for 10 min at 4,000 rpm, and the pellet was washed with 50 mM EDTA and resuspended in 1% low-melting-point agarose in 125 mM EDTA and 50 mM Na citrate (pH 5.7) with a lysing enzyme (Sigma Chemical Co., St. Louis, MO). Agar blocks were set in a commercial sample mold (Bio-Rad Labo-

ratories, Richmond, CA). Conidia were lysed in 50 mM Na citrate with 0.4 M EDTA and 7.5% mercaptoethanol (pH 5.7) at 45°C for 24 h and in proteinase K (mg/ml) in NDS buffer (0.5 M EDTA, 10 mM Tris HCl, and 1% *N*-lauroylsarcosine adjusted to pH 8.0) at 50°C for 24 h.

A contour-clamped homogeneous electric field dynamically regulated system (CHEF-DR II, Bio-Rad) was used for gel electrophoresis in half-strength Tris-borate-EDTA buffer (pH 8.0) maintained at 14°C with constant buffer circulation. Spore blocks were placed in wells in a 0.8% agarose gel (14 × 12.7 × 1 cm) (Pharmacia, Uppsala, Sweden) and run with switch times of 20 h, 30 s followed by 9 h, 90 s. *Saccharomyces cerevisiae* chromosomes (Bio-Rad) were used as size markers. Gels were stained with ethidium bromide, destained in distilled water, and then examined with a UV-transilluminator.

Data obtained for the 21 isolates examined by RAPD and EK and the 103 isolates examined by RAPD were analyzed separately. Data on RAPD and EK were analyzed in a similar way. RAPD bands consistently present in duplicate samples of each isolate and bands representing putative minichromosomes were scored as present or absent. For each marker system, data were pooled to examine similarities from pairwise comparison of isolates. Similarity values were calculated with the formula $F = 2m_{xy}/(m_x + m_y)$, in which m_{xy} is

the number of shared bands and m_x and m_y are the number of bands in each isolate. A software package (SAS Institute, Cary, NC) was used for cluster analysis of the similarity values using the unweighted pair group method with arithmetic mean, and the procedure TREE (SAS) was used to generate a dendrogram. The SAS macro program DENDRO, developed by P. Nicholson of the University of Leeds, was used for this purpose. This program is available on-line from SAS.

RESULTS

Virulence phenotypes. As noted before (5), Fitzroy, Seca, 36260, Q10042, and 93116 were the most important differentials in discriminating among the races. No virulence toward accession 110354 was detected in any isolate. Existing data on disease severity on the six host differentials for 182 Australian isolates from races 1, 3, and 4 (5) were used as the training set to develop LDF. Except for reference isolate 21365, none of the isolates matched the race 2 virulence pattern. No LDF were developed for race 2 because its host, *S. viscosa*, is not of commercial significance in Australia. The 103 isolates were each classified into one of the three race clusters by using the LDF. Isolates that were within the 95th percentile of the distribution of within-cluster Mahalanobis distance for a given race cluster were classified as members of that race (Fig. 1). Of the 84 isolates that could be classified by the LDF, 24 were classified as race 1, 47 as race 3, and 13 as race 4 (Table 1). Race 1 isolates, virulent only on the universal susceptible Fitzroy, originated from many different *Stylosanthes* species, including *S. hamata*, *S. scabra*, *S. guianensis*, *S. montevidensis*, and *S. fruticosa*. The more complex races 3 and 4 with virulence toward other differentials in addition to Fitzroy were isolated predominantly from *S. scabra* plants.

Fig. 1. Classification of 103 isolates of *Colletotrichum gloeosporioides* into race clusters (large numerals) by using linear discriminant functions developed from a training data set on disease severity of 182 isolates (small numerals) on differential host genotypes of *Stylosanthes scabra*. Unclassified isolates are represented by zeros (large numerals). The four original axes (arrows) have been projected onto the plot to relate the log-transformed scores on the differentials to the two canonical coordinates. The arrows originate from the point representing the overall mean severity of the training data set. The length of each arrow represents the change in canonical coordinates given a unit change from the mean score for each differential. For clarity, only the reference isolates have been labeled.

Fig. 2. Relationship between anthracnose severity on *Stylosanthes scabra* cultivars Fitzroy and Seca caused by *Colletotrichum gloeosporioides* isolates collected during 1970 to 1996.

Nineteen isolates that did not meet the 95th percentile criterion remained unclassified (Fig. 1). Seven isolates (cs304, cs1074, cs1599, nq162, wrs3, wrs11, and wrs34) were weakly pathogenic on all differentials (*data not shown*). The remaining 12 were highly aggressive on Seca with mean severity ratings >3. Five of these, cs1443, cs1571, nq124, nq151, and nq244, produced mean severity ratings between 4 and 6 (*data not shown*). This is much higher than the 1 to 2.7 range of severity (5) produced on Seca by the reference isolate and others used in developing the LDF.

Of the 12 highly aggressive isolates, 11 were collected between 1992 and 1996. To determine whether isolates had become progressively more aggressive toward Seca, the relationship between the year of isolation and the severity on selected differentials was examined by linear regression analysis. A significant ($P < 0.05$) linear relationship was detected for severity on Seca with severity increasing with time. Severity on all other differentials remained unchanged over time. Data are given for Fitzroy and Seca only (Fig. 2).

Comparison of RAPD and EK markers. A total of 75 reproducible RAPD bands ranging from 240 to 2,200 bp were obtained with the six selected primers on the 21 isolates (Fig. 3). Of the DNA fragments amplified with primer AN11, seven isolates (21365, cs18, sr30, cs116, cs232, cs89, and sr4) had a unique band of about 1,250 bp. Primers L11, P15, P16, and Q20 also amplified similar unique fragments in all seven isolates. Primer L2 amplified a unique 1,200-bp DNA fragment in these seven isolates and in isolates uq14, cs233, wrs20, wrs32, wrs51, and cs273. The 21 isolates were grouped into three clusters on the basis of an analysis of the pooled data for all six primers (Fig. 4A).

Six to 11 putative minichromosomes in the 300- to 615-kb range were resolved in the 21 isolates, and data are given for 15 of these isolates (Fig. 5). Of these, the 614-, 410-, and 367-kb fragments were present in all isolates; the 455-kb fragment was

present in all except sr30; and the 394-kb fragment was present in all except sr30 and cs18. Isolates could be classified into seven groups, with the majority (17) of isolates belonging to three groups. In group 1, eight isolates, cs87, cs105, cs204, cs218, cs231, cs239, sr24, and uq14, each had 10 minichromosomes of 614, 533, 511, 455, 410, 402, 394, 367, 342, and 312 kb. In group 2, five isolates, cs233, wrs20, wrs32, wrs33, and wrs51, each had eight minichromosomes of 614, 538, 485, 455, 410, 402, 394, and 367 kb. In group 3, four isolates, cs89, cs116, cs232, and sr4, each had six minichromosomes of 614, 538, 455, 410, 394, and 367 kb. The remaining four isolates, cs18, cs273, sr30, and 21365, each had a unique banding pattern with 7 to 11 minichromosomes. A cluster analysis was performed on EK data based on the presence or absence of the minichromosome bands, and a dendrogram, generated by the method outlined for RAPD, clearly shows the seven groups (Fig. 4B).

Overall, a higher level of polymorphism was obtained by EK than with RAPD markers. Isolates within the three RAPD groups could be further discriminated by EK analysis. Sr30, cs18, and 21365, which formed part of one RAPD cluster, could be discriminated as individual isolates on the basis of their unique EK profiles. Similarly, the unique EK profile for isolate cs273 distinguished it from five other isolates that were in the same RAPD cluster. However, there was broad agreement between the three major genetic groups identified by the two marker systems. Of the eight isolates, sr24, cs239, cs204, wrs33, cs218, cs87, cs105, and cs231, which formed one RAPD cluster, all except wrs33 were grouped in the same EK cluster. Similarly, the majority of isolates from each of the other two RAPD clusters constituted a separate EK cluster. The logistics of the EK procedure, including the long sample-preparation time, make it unsuitable as a routine technique for screening large numbers of isolates. In addition, the diffused banding pattern obtained with EK can be

difficult to visualize and score. RAPD markers were therefore chosen as easy-to-use tools to characterize genetic differences between the 103 isolates.

Fig. 4. Dendrogram of 21 biotype A isolates of *Colletotrichum gloeosporioides* infecting *Stylosanthes* spp. developed by (A) random amplified polymorphic DNA analysis and (B) electrophoretic karyotype analysis.

Fig. 3. Polymorphism in duplicate samples of 21 *Colletotrichum gloeosporioides* isolates determined by random amplified polymorphic DNA analysis with the arbitrary primer AN11. The three unmarked lanes contain 100-bp ladder DNA (Pharmacia, Uppsala, Sweden) as size markers.

RAPD analysis of 103 isolates. A cluster analysis of 178 scorable bands was used to identify 13 clusters, designated A to M (Fig. 6). Three RAPD clusters, D, G, and J, contained more than 88% (91) of the isolates. Cluster G with 58 isolates represented the most dominant and widely distributed genetic group in all the major *Stylosanthes*-growing areas in Queensland (Table 1). Isolates in this cluster originated mainly from *S. scabra* plants. All 15 isolates in cluster D originated from *S. scabra*. In contrast, 14 of the 18 isolates in cluster J originated from host plants other than *S. scabra*. The remaining 12 isolates were contained in 10 other clusters, eight of which were represented by single isolates only (Fig. 6). Among these, five isolates, sr23, wrs8, wrs11, wrs35, and wrs52, were genetically distinct and originated from *Stylosanthes* spp. not grown widely in Australia. These isolates either belonged to race 1 or were weakly pathogenic.

There was no clear association between virulence groups and RAPD clusters among isolates studied in this work. Isolates of all three races came from more than one genetic background (Table 1). Seven different RAPD clusters were represented among isolates of each of races 1 and 3. Three RAPD clusters were represented among the smaller group of 13 race 4 isolates. However, a large number of isolates in each of the three races originated from a specific RAPD cluster. For instance, 12 of 24 race 1 isolates were from RAPD cluster J, and 32 of 47 race 3 isolates and 10 of 13 race 4 isolates were from RAPD cluster G.

Of the five most aggressive isolates, four, nq151, nq124, cs1443, and cs1571, were from RAPD clusters D and G, which contained other less aggressive isolates. Isolate nq244, on the other hand, is a new genotype with a distinct RAPD profile.

DISCUSSION

Highly aggressive isolates of *C. gloeosporioides* infecting the commercial cultivar Seca were detected from samples collected during recent years from a number

of field sites in Queensland. One aggressive isolate, nq244, has a new genotype with a distinct RAPD profile, while the others have arisen from genetic groups that also contain other less aggressive isolates. This is a significant finding, given the high level of genetic uniformity within the biotype A pathogen population in Australia demonstrated in this and other studies (1,20,21). Although a new race adapted to Seca was discovered within 6 years of its release (8), past surveys have shown that race 3 isolates cause only low to moderate damage to Seca (14). The 12 unclassified isolates did not represent a new virulence combination (race) but caused an increased amount of damage to Seca resulting from changes in their aggressiveness, as defined by Shaner et al. (25). When the search was narrowed from 103 to 19 isolates, the multivariate technique allowed the detection of the highly aggressive set of 12 isolates. Discovery of these isolates from different areas highlights a potential threat to the productivity and persistence of the most significant commercial *Stylosanthes* cultivar in Australia.

A significant relationship between severity on Seca and the year of collection of an isolate suggests an increase in aggressiveness toward Seca in the pathogen population. Because of the relatively small number of isolates examined, it is not possible to clearly establish whether *C. gloeosporioides* infecting Seca has gradually adapted to become more aggressive in the last two decades or whether highly aggressive isolates, already present in low numbers, have increased in frequency during the sampling period. There is evidence of adaptation for increased aggressiveness in *Cochliobolus heterostrophus* toward partially resistant corn (16) within three sexual generations of the pathogen. Similar evidence exists for other plant pathogens for which controlled crossing has shown that aggressiveness is heritable and may be polygenically controlled (2). Despite the existence of teleomorphic strains of *C. gloeosporioides* with low virulence on *Stylosanthes* spp. (18), there is no evidence

of widespread genetic recombination between races and biotypes within Australia (20). However, heterokaryon formation through nitrate nonutilizing mutants in both biotypes and the introgression of a 2-Mbp biotype A chromosome into biotype B isolates (20,23) under laboratory conditions suggest the presence of parasexuality. Detailed genetic analysis of nq244 and other more aggressive isolates is needed to determine their origin.

In common with earlier work, we have observed variation in the number and size of DNA fragments, putatively referred to as minichromosomes (22). Masel et al. (24) classified isolates of *C. gloeosporioides* with fewer than six minichromosomes as Mtype 1 and isolates with six or more minichromosomes as Mtype 2. Six to 11 minichromosomes were resolved in the 21 isolates studied in this work. On the basis of earlier published work (22,24) and the results presented in this study, we conclude that all Australian biotype A isolates belong to the Mtype 2 group.

Previous studies showed highly variable karyotype among isolates within biotype B (22,24), despite the apparent monomorphism demonstrated by RFLP analysis (1,20). In this work on biotype A, we recorded similar differences in EK within the apparently clonal groups identified by RAPD analysis. He et al. (11) showed that although the minichromosomes themselves have a very high degree of sequence homology, karyotype polymorphism arises from DNA addition, deletion, and rearrangement (20).

The majority of isolates obtained from hosts other than the two most dominant species in Australia, *S. scabra* and *S. hamata*, are either weakly pathogenic or pathogenic only on Fitzroy. This includes the five genetically distinct isolates from *S. montevidensis*, *S. capitata*, and *S. guianensis*. Although only a small number of isolates from *S. hamata* were used in this work, we showed previously that all races are prevalent in *S. hamata* pastures (12). This suggests that the more simple races of the Australian biotype A tend to infect a broader range of species, whereas races that infect a large number of differentials (complex races) are commonly isolated from *S. scabra* and *S. hamata*.

The overall weak association between virulence and genetic groupings in our study is consistent with findings from studies on *C. gloeosporioides* infecting *S. guianensis* in which virulence and isozyme (17) and RAPD markers were used (15). These results indicate that pathogenic races have arisen independently within each genetic group. Leung et al. (19) concluded that the association between molecular markers and virulence patterns in plant pathogens can be perfect, partial, or absent. Close association between virulence and selection-neutral molecular markers, such as RAPD, has been recorded in some pathogens (7).

Fig. 5. Putative minichromosomes in isolates of *Colletotrichum gloeosporioides* determined with a contour-clamped homogeneous electric field dynamically regulated system used for electrophoretic karyotyping. Lanes marked "S.c" contain *Saccharomyces cerevisiae* chromosomes as size markers.

Fig. 6. Dendrogram of 103 single-spore isolates of *Colletotrichum gloeosporioides* infecting *Stylosanthes* spp. developed by random amplified polymorphic DNA analysis. Capital letter indicates the genetic group assigned to an isolate cluster.

The level of variation in the Australian population of *C. gloeosporioides* is limited compared with the extensive range of genetic and virulence diversity found at its center of origin in South America (3). The strict quarantine measures that exist today have helped to maintain this situation and must be continued for practical reasons. Given the potential for genetic recombination suggested by the EK analysis, any newly introduced overseas isolate may have the potential to recombine with well-adapted Australian races to pose a serious threat to the industry.

Within Australia, isolates of *C. gloeosporioides* have become progressively more aggressive toward *Seca*. The five highly aggressive isolates originated from different genetic backgrounds. This fact and the existence of the same pathogenic races within different genetic backgrounds suggest that host-mediated selection is a major determinant of the pathogen population structure. There is a similar link between genetic groups and host of origin in biotype B isolates collected from *S. guianensis* in South America (15). Regular surveys must be taken to ascertain whether new races are arising as a result of deployment of new cultivars.

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