Genetic Diversity in *Colletotrichum gloeosporioides* from *Stylosanthes* spp. at Centers of Origin and Utilization

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ABSTRACT

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Using molecular markers, this work compares the genetic diversity in *Colletotrichum gloeosporioides* infecting species of the tropical forage legume *Stylosanthes* at the center of origin in Brazil and Colombia with that of Australia, China, and India, where *Stylosanthes* spp. have been introduced for commercial use. There was extensive diversity in the pathogen population from Brazil, Colombia, China, and India. The Australian pathogen population was least diverse probably due to its geographical isolation and effective quarantine. The extensive diversity in China and India means that threats from exotic pathogen races to *Stylosanthes* pastures can potentially come from countries outside the South American center of origin. In Brazil and India, both with native

Stylosanthes populations, a high level of genetic differentiation in the pathogen population was associated with sites where native or naturalized host population was widely distributed. There was limited genetic diversity at germplasm evaluation sites, with a large proportion of isolates having identical haplotypes. This contrasts recent pathogenicity results for 78 of the Brazilian isolates that show hot spots of complex races are more common around research stations where host germplasm are tested, but few are found at sites containing wild host populations. For a pathogen in which the same races arise convergently from different genetic backgrounds, this study highlights the importance of using both virulence and selectively neutral markers to understand pathogen population structure.

Additional keywords: anthracnose, pathogen surveys, random amplified polymorphic DNA.

Species of the tropical pasture and forage legume *Stylosanthes* are widely distributed in South America, with Brazil and Colombia among the major centers of origin. Only 4 out of over 40 species are native to Africa and Asia (27). Extensive research efforts have been made in the collection of native germplasm and selection and breeding to improve performance of *Stylosanthes* spp. since the late 1960s in Brazil and Colombia (25). Of the nine *Stylosanthes* cultivars released in South America, all except multiline cv. Estilosantes Campo Grande released in 2001 (9) were developed directly from natural germplasm accessions. It is too early to assess the success of the multiline cultivar, but most of the previously released cultivars have failed to maintain productivity in the field to support animal production beyond the first 2 years (20). Anthracnose disease, caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., has been the most significant biotic factor limiting the productivity, persistence, and utilization of *Stylosanthes* spp. in South America (17).

Because of their adaptation to acid and infertile soils in semi-arid environments, *Stylosanthes* spp. have been introduced to many countries including Australia, China, India, Thailand, and the Philippines to improve animal production and restore soil nitrogen (10). In general, commercial utilization of *Stylosanthes* spp. has been more successful at these centers of utilization, away

from the center of origin. *S. humilis* was accidentally introduced into Australia from Brazil in the early 1900s. Introductions of other species have occurred at intervals since then. Plant introduction and an active breeding program have produced several new cultivars that are used in improved pastures mainly in the dry tropics of northern Queensland and the Northern Territory (2,8). In Australia, over 1 million ha of native pasture has been over-sown with *Stylosanthes* spp.

Introductions of *Stylosanthes* spp. to China from Australia, Africa, and South America began in the late 1960s and have continued to the present. *Stylosanthes* spp. are principally grown in Hainan and Guangdong provinces as an annual crop for cut-and-carry forage, leaf meal, and hay (10). *S. fruticosa* is endemic to the southern peninsular regions of India (11). In addition, South American species were first introduced in 1945 and introductions from Australia, Africa, South America, and the United States continue to the present day (24). In India, *Stylosanthes* spp. are regarded as the most important tropical legume for the semiarid and arid regions and are mainly used in wasteland reclamation, as cut-and-carry forage in horticultural systems and as a component of dryland mixed cropping (24).

Anthracnose is a serious impediment to *Stylosanthes* utilization in all countries where it has been introduced to improve pasture production. Research in Australia has identified two biotypes of *C. gloeosporioides* infecting *Stylosanthes* spp. (12). Studies in Colombia and Brazil have confirmed the presence of the two biotypes, although there are strains that do not clearly belong to either biotypes (3,13). Similarly, biotypes A and B and a putative biotype C have been described from Africa (22), but no information on pathogen populations is available from Asia.

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The two biotypes are genetically distinct (4,5,16), which indicates separate clonal descent from two separate introductions into Australia (18). Similar introductions through germplasm exchange between countries are the most likely source of spread of this pathogen to countries such as India and China. However, the discovery of new genotypes of highly aggressive strains in Australia (5) demonstrates that new variants can arise within a region. A transposable element and the transfer of specific chromosome between biotypes are mechanisms that generate variation in this pathogen (18). This genetic plasticity in *C. gloeosporioides* has contributed to the development of new races to devastate once-productive cultivars in Australia (6) and most likely in South America (20).

Although anthracnose disease caused by *C. gloeosporioides* is considered the most significant limiting factor for *Stylosanthes* production in both India (24) and China (10), no information on genetic diversity or pathogenicity is available for these countries. A comparative analysis of genetic variation at centers of origin and utilization will help explain the overall structure of the pathogen population, and the diversity within each country will better define quarantine risks from exotic strains to better align plant-breeding programs against potentially damaging strains.

A number of selectively neutral markers are available to study pathogen populations (19). Codominant markers such as restriction fragment length polymorphism (RFLP) and isozymes can be used to reveal allelic forms of genetic loci. The dominant polymerase chain reaction (PCR)-based random amplified polymorphic DNA (RAPD) markers, on the other hand, do not detect heterozygotes and have limited use in sexual genetic studies (19). Both biotypes of *C. gloeosporioides* from *Stylosanthes* spp. are anamorphic (23), and RFLP (1) analysis and genome size suggest haploidy. With the more direct correspondence between genotype and phenotype in a haploid organism, the choice of markers is not as crucial for this pathogen, making the use of RAPD markers a suitable, inexpensive, and high throughput option, if reproducibility is adequately addressed.

This paper aims to provide a comparative analysis of genetic diversity in *C. gloeosporioides* populations originating mainly from species other than *S. guianensis* at centers of origin and utilization. For Brazil and India where native *Stylosanthes* populations exist, an additional aim was to examine whether genetic diversity is higher on native populations than on cultivated germplasm.

MATERIALS AND METHODS

Media and growth conditions. Potato dextrose agar (PDA, Sigma Chemical, St. Louis) and oatmeal agar (OMA, 20 g of rolled oats per liter and 20 g of agar per liter) were used for routine culturing. Liquid V8 (L-V8) medium was prepared by clarifying V8 juice (Campbell's, 250 ml liter⁻¹) with 14 g of CaCO₃ per liter and centrifuging for 20 min at 4,000 rpm. Isolations were made by surface sterilizing excised anthracnose lesions in commercial bleach with 1% available chlorine for 5 min, washing twice in sterile water, blotting on sterile blotting paper to remove excess water, and transferring to OMA or PDA. Monoconidial isolates were established by transferring single conidia onto PDA. Cultures were grown at room temperature (25 to 28°C) under a 12-h cycle of fluorescent white light and long-wavelength UV light and stored as mycelium on PDA in sterile water at room temperature.

Isolates. A total of 334 monoconidial *C. gloeosporioides* isolates were available for this study. All foreign isolates were imported under a quarantine permit, and DNA extraction, RAPD, and other genetic analyses were carried out at the Cooperative Research Center for Tropical Plant Protection laboratories in Brisbane, Australia.

Field surveys were generally used to collect samples of infected plant materials from Australia, Brazil, China, Colombia, and

India. Each collection site was delineated into one to two transects depending on its size, and samples were collected from a number of points along each transect. The transect length was varied to suit each site and several samples were usually collected from each point along a transect. This stratified sampling allowed a hierarchical comparison of isolate diversity between and within lesions, leaves, transects, and sites.

In Brazil, samples of infected *Stylosanthes* leaves and stems were collected either from field sites where *Stylosanthes* germplasm has been under evaluation for the past 3 to 4 years or from native *Stylosanthes* populations. Isolates from native *Stylosanthes* populations came from field surveys in Minas Gerais and Goias states where *S. capitata*, *S. scabra*, *S. hamata*, and *S. macrocephala* are naturally distributed, but it was not always possible to confirm the identity of the host species during field surveys. Data on location, latitude, and longitude held in germplasm banks in Brazil and Australia were utilized to locate areas of natural distribution of these species using a global positioning system (GPS II Plus; Garmin Corporation, Olathe, KS). The latitude and longitude of sample collection sites ranged between 5°02'01"S, 42°48'05"W and 20°27'02"S, 54°43'16"W.

In India, isolates were obtained from regional germplasm evaluation sites spread throughout the country, but focused on peninsular India (8°25'33.2"N, 76°59'21.0"E to 15°29'0.26"N, 74°58'49.3"E) where *Stylosanthes* spp. show most promise. Isolates at some sites came from *S. fruticosa*, which is native to India (11), and other *Stylosanthes* spp. naturalized over the last 50 years.

The Australian isolates came from five commercial properties and research stations in Queensland. Isolates from China were collected at 12 research or commercial properties in southern China (18°27'00"N, 108°53'00"E to 24°20'00"N, 115°45'00"E). *C. gloeosporioides* isolates from *Stylosanthes* spp. in other countries were included for comparison and these included one each from Peru, Nigeria, Ivory Coast, Burundi, Thailand, and the Philippines. Also included were selected *C. gloeosporioides* isolated from hosts other than *Stylosanthes* from Israel and New Zealand. In addition to the 334 *C. gloeosporioides* isolates, two *C. acutatum* isolates obtained from Israel were used.

DNA extraction. Isolates were grown on PDA until sporulation, flooded with 15 ml of L-V8, and the resulting spore suspension was divided between two 250-ml flasks, each containing 150 ml of L-V8 to obtain two DNA replicates per isolate. The flasks were incubated on a rotary shaker at 160 rpm at room temperature for 3 days, and mycelium was vacuum-filtered through Miracloth (Calbiochem, La Jolla, CA), washed in deionized water, freeze-dried, and stored at -80°C. DNA was extracted from 0.6 to 1.0 g of freeze-dried mycelium according to the method of Edwards et al. (7), modified by inclusion of a second phenol/chloroform step, a second ethanol wash, and the addition of NaCl (final concentration of 2 M) in the final ethanol wash to remove polysaccharides. All DNA was quantified with a Hoefer DNA 200 fluorometer (Hoefer Scientific Instruments, San Francisco) and adjusted to a standard concentration of 10 ng/μl.

RAPD analysis. Eight arbitrary 10-base oligonucleotide primers (Table 1, Pacific Oligos), giving simple clear polymorphic banding patterns, were selected from a previous study of 143 primers (4). RAPD-PCR was carried out in a 25-μl reaction mix containing Tris-HCl (67 mM, pH 8.8 at 25°C), [NH₄]₂SO₄ (16 mM), Triton X-100 (0.45%), Gelatin (0.2 mg ml⁻¹), dNTPs (0.2 mM) distributed as 5× buffer (Agenix, North Ryde, NSW, Australia), MgCl₂ (4 mM), primer (10 pM), DNA (30 ng), and *Taq* DNA polymerase (1.65 units, Agenix). A water control (DNA replaced with sterile water) was included with each set of 15 isolates. Amplifications were performed in a programmable thermocycler (MJ Research, Waterhouse, MA) under a program of 40 cycles of 1-min denaturation at 94°C, 1-min annealing at 37°C, and 2-min primer extension at 72°C and a 10-min extension at 72°C. The amplification product (15 μl) was electrophoresed in modified

Tris-borate-EDTA buffer (45 mM Tris-borate and 0.127 mM EDTA at pH 8.0) containing ethidium bromide (0.17 µg ml⁻¹) for 16 h at 51 V. DNA fragments were separated on a 1.6% (wt/vol) agarose gel (DNA grade; Progen Industries, Darra, Brisbane) containing ethidium bromide (0.24 µg ml⁻¹) and photographed under UV light (Polaroid 667; Polaroid Corp., Cambridge, MA). Replicate reactions (different PCR runs) were run on separate gels for each primer-isolate combination.

Isolate tests. A preliminary cluster analysis of the RAPD data for all 334 isolates showed that over 90% of the isolates could be placed in one of five clusters, whereas the remaining isolates could not be easily placed in any cluster. PCR amplification of targeted sequences from the variable internal transcribed spacer region 1 (ITS1) of *C. gloeosporioides* with primer ITS4 and a 20-nucleotide (nt) CgInt sequence (GGC CTC CCG CCT CCG GGC GG) (21) gave a distinctive 450-bp band that was used as a putative diagnostic marker for *C. gloeosporioides* (21,22). Preliminary tests with these primers gave variable results and the method was modified by including a 22-nt sequence variation in the CgInt region found in some *C. gloeosporioides* isolates from *Stylosanthes* spp., GGC CTC CCG CCC CCC CGG GCG G (CgInt2). A combination of all three primers (ITS4, CgInt, and CgInt2) gave a clear 450-bp band for almost all *C. gloeosporioides* isolated from *Stylosanthes* spp., but not for *C. acutatum*, *C. miyabeana*, *C. destructivum*, *C. orbiculare*, *C. coccoides*, *C. lindemuthianum*, *C. trifolii*, and *C. dematium*.

These three primers were used to analyze a subset of 214 isolates, including ones outside of the five clusters and other isolates representative of the overall diversity within the five groups. The PCR reaction mix for this test was the same as used for RAPD, except 20 pM of each primer and 60 ng of template DNA was used. Amplifications were performed using a 7-min denaturation at 94°C followed by 30 cycles of 1.5-min denaturation at 94°C, 2-min annealing at 68°C, and 3-min primer extension at 72°C and a 7-min extension at 72°C. The PCR product (15 µl) was electrophoresed as before and the presence or absence of the target fragment (450 bp) was recorded.

TABLE 1. Code and sequence for eight selected primers used to analyze genetic diversity of *Colletotrichum gloeosporioides* and the number and size range of polymorphic bands scored for each primer

Code	Sequence	Bands scored	Size range (kb)
Ac17	5'-CCTGGAGCTT-3'	7	0.4–2.8
An10	5'-CTGTGTGCTC-3'	10	0.4–2.6
An11	5'-GTCCATGCAG-3'	15	0.4–2.4
D18	5'-GAGAGCCAAC-3'	9	0.5–2.4
Q6	5'-GAGCGCCTTG-3'	12	0.4–2.0
Q20	5'-TCGCCAGTC-3'	15	0.3–2.3
Z10	5'-CCGACAAACC-3'	10	0.4–2.2
Z14	5'-TCGGAGGTTC-3'	11	0.4–2.8

Sixteen isolates did not produce a 450-bp band. These included six from Brazil, one from Australia, four from China, and five from India and were excluded from further analysis. All analysis of diversity at centers of origin and utilization were based on 318 *C. gloeosporioides* isolates (Table 2).

Data analysis. Photographs of agarose gels (Polaroid) were scanned as tagged-image format files, and RAPD profiles were compared using the Bio-Rad Molecular Analyst Fingerprinting Plus software package (Bio-Rad Laboratories, Hercules, CA) as follows. The two gel images from replicate DNA were normalized to a standard size through the alignment of DNA molecular weight markers and each set of 15 isolates included three DNA marker lanes for this purpose. Reference isolate CS89 was also included in each set, and for each primer, CS89 profiles were compared in each gel. Only gels in which the reference isolates gave the expected pattern were analyzed. Duplicate gel images were combined and consistent scorable bands were identified based on base pair size. The presence or absence of bands was coded into binary matrices for cluster analysis. Scorable bands were selected from an initial analysis of 29 diverse isolates selected from a previous study (4). Binary data were analyzed for band similarity using the unweighted pair group method with arithmetic averaging, and analyses were based on a paired association matrix derived using Jaccard's coefficient. SAS (SAS Institute, Cary, NC) and the multivariate statistical package MVSP (Kovach Computing Services, Pentraeth, Wales, UK) were used for statistical analysis and to generate dendrograms for each country group plus reference strains.

Each RAPD fragment of distinct size was treated as a marker for an individual locus with two alleles representing the presence or absence of a band. The combined allelic state of an isolate at all RAPD loci was designated as its multilocus haplotype. Genotypic diversity ($\hat{G}$) was calculated using the following formula (26):

$$\hat{G} = 1/\sum [f_x(x/N)^2]$$

where N is the sample size and f_x is the number of multilocus haplotypes observed x times in the sample. Within Brazil and India, genotypic diversity at selected sites containing native and cultivated *Stylosanthes* was also examined. The maximum possible value of $\hat{G}$ is the number of isolates in a sample. To compare $\hat{G}$ for populations with different sample sizes, $\hat{G}$ was expressed as the percentage of maximum diversity by dividing $\hat{G}$ by the sample size.

RESULTS

The eight primers generated a total of 89 reproducible bands ranging in size from 0.4 to 2.8 kb (Table 1). Polymorphisms

TABLE 2. Summary of *Colletotrichum gloeosporioides* isolates used in this study with their country and host species of origin

Country	Total	Host species ^a										Other
		<i>S. capitata</i>	<i>S. fruticosa</i>	<i>S. guianensis</i>	<i>S. gracilis</i>	<i>S. hamata</i>	<i>S. humilis</i>	<i>S. macrocephala</i>	<i>S. scabra</i>	<i>S. seabrana</i>	<i>Stylosanthes</i> sp. (unknown)	
Africa	3	1	...	2	...	...	...	...	...	...	...	...
Australia	23	...	...	3	...	1	...	...	10	5	3	1
Brazil	131	63	...	11	...	...	...	5	11	1	40	...
China	43	...	...	25	...	5	...	...	13	...	...	...
Colombia	13	9	...	...	2	...	...	...	1	...	1	...
India	95	...	10	15	...	10	5	...	31	24	...	...
Israel	1	...	...	...	...	...	...	...	...	...	...	1
Peru	1	1	...	...	...	...	...	...	...	...	...	...
Philippines	1	...	...	1	...	...	...	...	...	...	...	...
Thailand	1	...	...	...	...	1	...	...	...	...	...	...
New Zealand	6	...	...	...	...	...	...	...	...	...	...	6

^a Other indicates host other than *Stylosanthes* spp.

detected consistently in replicate gels (Fig. 1A) were scored after normalizing the gel images. The size markers allowed replicate gels to be combined and ensured consistent scoring of multiple gels (Fig. 1B).

Diversity at the center of origin. Pooled data from all eight primers were analyzed for the 144 isolates from Brazil and Colombia. In addition, 20 *C. gloeosporioides* isolates from *Stylosanthes* spp. in other countries, including one each from Peru, Nigeria, Ivory Coast, Burundi, Thailand, and the Philippines and from hosts other than *Stylosanthes* spp.; and two *C. acutatum* isolates were used for comparison. A cluster analysis was conducted in order to ascertain the number of natural groupings among these 166 isolates. The number of clusters was increased from 2 to 10 and the proportional reduction in residual sum of squares (R^2) was examined along with the frequency of isolates in each cluster and the cluster membership of isolates. A large majority of the isolates were grouped in the first three to four clusters for cluster solutions between 3 and 10 clusters and the

frequency of isolates in the first three clusters remained almost unchanged when the number of clusters increased from 7 to 10. R^2 increased from 0.77 to 0.91 when the number of clusters was increased from 2 to 10. Incremental improvements in R^2 for 3, 4, and 5 cluster solutions were 8, 4, and 2%, respectively. The 4-cluster solution was selected using an incremental R^2 improvement of >4% as the cut-off point.

Figure 2 illustrates the dendrogram generated for the 166 isolates with the four clusters marked. Cluster I contains 21 isolates from Brazil collected mainly from *S. capitata* in four different states. Cluster II contains 22 isolates from Brazil, Colombia, and Thailand and biotype A isolates from Australia. Cluster III contained 11 isolates from Brazil, Colombia, Peru, Africa, and all Australian biotype B isolates and were collected from *S. capitata*, *S. gracilis*, or *S. guianensis*. The 76 Brazilian isolates and 1 Colombian isolate in cluster IV came from a number of *Stylosanthes* spp. Included in this cluster is a subgroup containing 55 closely related isolates (Fig. 2, subgroup A), 44 of which had an

Fig. 1. A, Polymorphisms in replicate gels for 16 *Colletotrichum gloeosporioides* isolates determined by random amplified polymorphic DNA analysis with the arbitrary primer AN11. Lanes marked M contain combined molecular weight markers V111 (Roche Diagnostics, Castle Hill, Australia) and SPP1/*Eco*R1 (Geneworks, Rundle Mall, Adelaide, Australia). W = water control. **B,** Combined replicate AN11 gels for the 16 *C. gloeosporioides* isolates after normalization and band identification (white bars) using Molecular Analyst Fingerprinting Plus software (Bio-Rad Laboratories, Hercules, CA).

Fig. 2. Genetic diversity using random amplified polymorphic DNA (RAPD) fingerprinting of *Colletotrichum gloeosporioides* isolates from Brazil and Colombia and reference isolates from other locations (-----) showing the four major clusters (I, II, III, and IV) and the highly diverse group of isolates (shaded box, group V), which could not be classified as a cluster. Subgroup A contains 55 closely related isolates from Brazil, 44 of which have identical RAPD fingerprint (clone 1). T = isolates tested with *C. gloeosporioides* primers that gave a distinctive 450-bp band (*) or no band (**). Race = virulence cluster of isolates according to Chakraborty et al. (3).

identical fingerprint (Fig. 2, clone 1). These 44 isolates came from four cultivated host species grown in five locations in the adjacent states of Goiás and Matto Grosso do Sul (Table 3). Pathogenic diversity has been studied for 35 of the 44 isolates, representing six different virulence groups (3). The remaining 35 isolates outside the four major clusters were highly varied and included isolates of *C. gloeosporioides* and *C. acutatum* obtained from hosts other than *Stylosanthes* spp. Fifteen of these isolates from a variety of locations in Brazil, Colombia, the Philippines, and Africa are more similar to each other than the remaining 20 isolates. This group, tentatively designated as group V, is highlighted in gray in Figure 2. Included in this group was one isolate obtained from apple lesions in New Zealand. The remaining 20 isolates are highly diverse and remain ungrouped.

Hierarchical sampling allowed a comparison of diversity within and between leaves and there was a high degree of variation among isolates collected from the same leaf. Of the 16 pairs of isolates taken from different lesions on the same leaf, none were identical in their RAPD fingerprint and 5 pairs contained isolates from two different clusters.

There was no clear relationship between RAPD clusters and the host species of origin (data not shown), and isolates from *S. capitata* and *S. guianensis* were present in almost all RAPD clusters. Likewise, no association was found between RAPD genotype clusters and pathogen race clusters for the 78 Brazilian isolates analyzed so far (3).

Diversity at centers of utilization. Bands were generated and analyzed similar to the Brazilian and Columbian isolates. Isolates from Australia, China, and India were analyzed separately. A subset of Brazilian and Columbian isolates representing the four clusters and group V and selected isolates of *C. gloeosporioides* and *C. acutatum* from other countries were included for reference. Clusters corresponding to the South American groups were identified with reference isolates and distance measure. Only a small number of clusters were represented and the pattern for each country was distinctive.

The Australian isolates showed very limited variation and all isolates could be allocated to either cluster II (19 isolates) or III (3 isolates) (Fig. 3). All known biotype B isolates (from *S. guianensis*) were allocated to group III. Isolate CS2435 was collected from a mango plantation adjacent to a *S. seabrana* seed production plot in north Queensland that had suffered serious anthracnose in 1998. This isolate was not pathogenic to *S. seabrana* (S. Chakraborty, unpublished data) and appears unrelated to the *Stylosanthes* pathogen.

In contrast to the pattern found in Australian isolates, the Chinese isolates (Fig. 4) showed a high level of diversity and isolates were allocated to clusters II (3 isolates), III (5 isolates), and the majority (35 isolates) were highly variable and placed in group V. As with the Australian isolates, all Chinese isolates in cluster III were from *S. guianensis*. Isolates from clusters II and III grouped closely with those from Australia, suggesting a possible Australian link.

The majority of the Indian isolates (Fig. 5) were grouped with clusters II and V but 17 highly variable isolates could not be placed in any of the South American clusters. Within cluster II there were two subgroups of five and eight isolates with identical RAPD fingerprint. Two isolates collected from the same lesion were never identical and did not always group together in the same cluster. Of the 10 isolate pairs obtained from the same lesion, half contained isolates from two different clusters. Ten isolates came from the endemic *S. fruticosa*, eight were in cluster II, one in the diverse group V, and one remained ungrouped. The identical RAPD fingerprint of isolates from *S. fruticosa* and the cultivated *S. scabra* and *S. seabrana* clearly indicates cross-infection of isolates between the naturalized and cultivated species.

Grouping of isolates at centers of utilization did not follow a discernable pattern with regard to host species (data not shown),

except that for the importing countries all cluster III isolates originated from *S. guianensis*.

Comparison of diversity between and within countries. Although there were 87 multilocus haplotypes among the 144 isolates from Brazil and Colombia, the genotypic diversity was low ($\hat{G} = 9$, only 7% of the theoretical maximum) due to the very high frequency (30% of all isolates) of clone 1 isolates. Clone 1 was obtained from cultivated *Stylosanthes* germplasm at evaluation sites and excluding these isolates from the data gave a higher genotypic diversity ($\hat{G} = 60$, 60% of maximum). Each of the 43 isolates from China had a distinct haplotype, giving the maximum possible genotypic diversity ($\hat{G} = 43$). This was followed by India with 82 haplotypes ($\hat{G} = 51$, 54% of maximum). With 14 haplotypes, the pathogen population in Australian was the least diverse ($\hat{G} = 6$, 27% of maximum) among countries where *Stylosanthes* spp. are commercially utilized.

In Brazil, 11 haplotypes were detected among 14 isolates collected from a population of native *Stylosanthes* spp. in Buritis ($\hat{G} = 9$, 63% of maximum). In contrast, there were 20 haplotypes among the 33 isolates from Campo Grande ($\hat{G} = 6$, 17% of maximum) and only 6 haplotypes among the 29 isolates from Chapadão do Sul ($\hat{G} = 2$, 5% of maximum). At both Campo

TABLE 3. Host plant, collection site, and race cluster of 44 Brazilian isolates of *Colletotrichum gloeosporioides* designated as clone 1 with identical random amplified polymorphic DNA fingerprints

Isolate	Host	Collection site	Race cluster ^a
CG008	<i>S. capitata</i>	Campo Grande-MS	6
CG011	<i>S. capitata</i>	Campo Grande-MS	4
CG073	<i>S. scabra</i>	Campo Grande-MS	8
CG075	<i>S. capitata</i>	Chapadão do Sul	ND
CG076	<i>S. macrocephala</i>	Chapadão do Sul	4
CG083	<i>S. capitata</i>	Chapadão do Sul	ND
CG093	<i>S. capitata</i>	Chapadão do Sul	ND
CG097	<i>S. capitata</i>	Chapadão do Sul	7
CG098	<i>S. capitata</i>	Chapadão do Sul	7
CG102	<i>S. capitata</i>	Chapadão do Sul	0
CG104	<i>S. capitata</i>	Chapadão do Sul	7
CG106	<i>S. capitata</i>	Chapadão do Sul	4
CG107	<i>S. scabra</i>	Chapadão do Sul	4
CG110	<i>S. capitata</i>	Chapadão do Sul	4
CG112	<i>S. guianensis</i>	Campo Grande-MS	ND
CG113	<i>S. capitata</i>	Campo Grande-MS	ND
CG114	<i>S. capitata</i>	Campo Grande-MS	8
CG117	<i>S. capitata</i>	Chapadão do Sul	4
CG119	<i>S. capitata</i>	Campo Grande-MS	6
CG120	<i>S. capitata</i>	Chapadão do Sul	4
CG122	<i>S. capitata</i>	Chapadão do Sul	8
CG124	<i>S. capitata</i>	Campo Grande-MS	4
CG125	<i>S. capitata</i>	Chapadão do Sul	7
CG126	<i>S. capitata</i>	Chapadão do Sul	4
CG128	<i>S. scabra</i>	Chapadão do Sul	7
CG129	<i>S. capitata</i>	Goiania-GO	7
CG130	<i>S. capitata</i>	Goiania-GO	8
CG133	<i>S. capitata</i>	Goiania-GO	ND
CG139	<i>S. guianensis</i>	Paranaíba-MS	ND
CG142	<i>S. capitata</i>	Goiania-GO	4
CG145	<i>S. capitata</i>	Goiania-GO	8
CG151	<i>S. macrocephala</i>	Chapadão do Sul	4
CG152	<i>S. capitata</i>	Chapadão do Sul	5
CG159	<i>S. capitata</i>	Chapadão do Sul	4
CG166	<i>S. capitata</i>	Campo Grande-MS	6
CG168	<i>S. capitata</i>	Campo Grande-MS	8
CG172	<i>S. macrocephala</i>	Chapadão do Sul	6
CG177	<i>S. capitata</i>	Campo Grande-MS	ND
CG179	<i>S. capitata</i>	Campo Grande-MS	6
CG187	<i>S. macrocephala</i>	Chapadão do Sul	7
CG189	<i>S. capitata</i>	Campo Grande-MS	7
CG204	<i>S. capitata</i>	Guapó-GO	6
CG208	<i>S. capitata</i>	Chapadão do Sul	ND
CG228	<i>S. scabra</i>	Goiania-GO	6

^a Race cluster according to Chakraborty et al. (3); 0 = complex isolate that could not be classified as one of eight existing races; ND = not determined.

Grande and Chapadão do Sul, isolates came from cultivated *Stylosanthes* germplasm under evaluation in small experimental plots. In India, isolates from Dhoni ($\hat{G} = 22$, 100% of maximum) and Rahuri ($\hat{G} = 13$, 100% of maximum) were more diverse than isolates from Dharwad ($\hat{G} = 19$, 91% of maximum). Areas in and around Dhoni and Rahuri have a history of growing naturalized *Stylosanthes* spp. since the early 1950s, whereas Dharwad has a relatively short history of seed production for cultivated germplasm.

DISCUSSION

To our knowledge, this is the first report of genetic diversity in *C. gloeosporioides* infecting *Stylosanthes* spp. in China and India and how this relates to pathogen diversity in Brazil, a center of host-pathogen diversity, and in Australia, a country where *Stylosanthes* spp. has been most successfully used on a commercial scale. This study has demonstrated extensive genetic diversity in *C. gloeosporioides* infecting *Stylosanthes* spp. in Brazil, which is similar to earlier findings on the pathogen infecting *S. guianensis* (14,16) and findings from a preliminary study with a limited number of isolates (4) from eight *Stylosanthes* spp. The high level of genetic differentiation in the pathogen population was associated with diverse host populations in both Brazil and India and at sites within these countries where native *Stylosanthes* populations were present. The use of a 450-bp region of the ITS sequence as a selection criteria confirms that this diversity is not due to *C. dematium*, which can also cause anthracnose of *Stylosanthes* (17).

There were 87 haplotypes among the 144 isolates from Brazil and Colombia and the genotypic diversity was 60% of the theoretical maximum when all 44 clone 1 isolates were excluded from the analysis. Clusters I and IV, which contained 93 Brazilian

isolates, were not detected in Australia, India, or China. However, given the extensive geographical distribution of *Stylosanthes* spp. in South America, we have most likely only been able to sample a relatively small component of the overall diversity, whereas sampling has been more comprehensive in Australia, China, and on cultivated species in India due to the more limited distribution of the commercial pastures. Therefore, the diversity estimate for South America is likely to be conservative.

All 43 Chinese isolates had unique haplotypes and this extensive diversity is unexpected given that organized introduction and evaluation of *Stylosanthes* spp. only started in the 1960s (10). The Indian pathogen is also diverse with 82 haplotypes and some of the isolates represent a somewhat broader range of diversity than the most diverse South American isolates in group V. India is unique among the three countries utilizing *Stylosanthes* spp. to have a native population of *S. fruticosa*. A previous study of a small number of isolates from the Philippines and Thailand showed that new genotypes are present in Asia (18). Detailed studies of pathogen diversity in Asia are lacking and more extensive sampling and analysis are needed to better explain the source of this diversity.

The Australian pathogen population was least diverse. Although only 24 isolates were examined in this work, we have previously found similar low levels of genetic variation in over 100 isolates (3,4), and isolates used in this study represent the full range of diversity within Australia. All isolates collected in recent years (1996 to 1999) grouped close with those collected from 1982 to 1987. For instance, isolates SR24 and CS2321 were collected 15 years apart and had identical RAPD fingerprints. This suggests that Australia's geographical isolation and effective quarantine may have prevented the introduction of new exotic strains and the small amount of variation (5) has been generated within Australia. For China and India, extensive land borders with neighboring

Fig. 3. Allocation of Australian *Colletotrichum gloeosporioides* isolates into clusters (II and III) identified from an analysis of random amplified polymorphic DNA fingerprint of South American and other reference isolates (-----). T = isolates tested with *C. gloeosporioides* primers that gave a distinctive 450-bp band (*) or no band (**).

countries would make it difficult to patrol and hence quarantine may not be as effective. The extensive pathogen diversity in China and India means that for Australia and other centers utilizing *Stylosanthes* spp., potential threats from more damaging exotic races could come from countries such as India and China in addition to the center of origin.

We have shown that the level of genetic diversity differs between field sites within Brazil and India, the two countries with native *Stylosanthes* populations. The diversity among isolates from a population of native *Stylosanthes* spp. at Buritis is more extensive than those sampled from cultivated germplasm at Campo Grande or Chapadão do Sul and a large proportion of isolates from the germplasm evaluation sites had identical haplo-

types. Similarly in India, the pathogen population was more diverse at Rahuri and Dhoni than at Dharwad. Earlier studies have found site-specific genetic variation in *C. gloeosporioides* and its ability to infect different genotypes of *S. guianensis* in Colombia (14) and *S. capitata* in Brazil (15). Genetic diversity in the pathogen was more extensive at sites where the host had been grown for a long time (14) to allow the pathogen to diversify through migration and other means (18). This is consistent with our findings that diversity is extensive at sites where native or naturalized *Stylosanthes* populations are concentrated; whereas, only limited diversity occurs at sites such as Campo Grande, Chapadão do Sul, and Dharwad that have only been in use for a relatively short period.

Fig. 4. Allocation of Chinese *Colletotrichum gloeosporioides* isolates into clusters (II, III, and V) identified from an analysis of random amplified polymorphic DNA fingerprint of South American and other reference isolates (-----). T = isolates tested with *C. gloeosporioides* primers that gave a distinctive 450-bp band (*) or no band (**).

A recent study (3) of 296 Brazilian isolates, including 78 isolates used here, has shown that simple races dominate the pathogen population in the absence of widespread use of race-specific resistance. More complex races are distributed only at hot

spots around research stations that are centers of germplasm evaluation. Our current results show that this racial diversification at the germplasm evaluation sites has arisen from a limited genetic diversity. This is consistent with other findings that in *C.*

Fig. 5. Allocation of Indian *Colletotrichum gloeosporioides* isolates into clusters (II and V) identified from an analysis of random amplified polymorphic DNA fingerprint of South American and other reference isolates (-----). T = isolates tested with *C. gloeosporioides* primers that gave a distinctive 450-bp band (*) or no band (**).

gloeosporioides infecting *Stylosanthes* there is no strong link between genetic and pathogenic groupings (5,14) and pathogen races arise convergently from different genetic lineages. This highlights the importance of using both virulence and selectively neutral markers to understand pathogen population structure.

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