Molecular Variation of *Sporisorium scitamineum* in Mainland China Revealed by RAPD and SRAP Markers

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Abstract

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Sugarcane smut caused by *Sporisorium scitamineum* occurs worldwide, causing serious losses in sugar yield and quality. To study the molecular variation of *S. scitamineum*, 23 *S. scitamineum* isolates collected from the six primary sugarcane production areas in mainland China (Guangxi, Yunnan, Guangdong, Hainan, Fujian, and Jiangxi provinces) were assessed by random amplified polymorphic DNA (RAPD) and sequence-related amplified polymorphism (SRAP) markers. The results of RAPD, SRAP, and RAPD-SRAP combined analysis

showed that, whereas the molecular variation of *S. scitamineum* was associated with geographic origin, there was no evidence of co-evolution between sugarcane and the pathogen. The results of RAPD, SRAP, or RAPD-SRAP combined analysis also did not provide any information about race differentiation of *S. scitamineum*. This suggests that the mixture of spores from sori collected from different areas should be used in artificial inoculations for resistance breeding and selection.

Sugarcane smut, caused by *Sporisorium scitamineum* (Basionym: *Ustilago scitaminea*) (25,33), occurs worldwide and is one of the most prevalent fungal diseases of sugarcane in China. The disease can cause serious losses in stalk yield and sucrose content in susceptible cultivars (16). However, sugarcane smut does not always pose a serious problem because incidence and severity vary depending on cultivars grown and environmental conditions. In recent years, the incidence of the disease has increased, resulting in greater damage to mainland China sugarcane, especially in the most important sugarcane production area of Guangxi province, where the infection rate was as high as 20% in ratoon cane (L. Xu and Y. Que, unpublished). Although several methods have been recommended for the control of smut, only the use of resistant cultivars is practical and economical (8,36).

To increase the understanding of the interaction between sugarcane and *S. scitamineum* and to improve breeding for smut-resistance cultivars, an investigation of the molecular variation of *S. scitamineum* was studied. An international group of researchers led by the International Society of Sugar Cane Technologists was established in 2004 to investigate the genetic diversity of *S. scitamineum*. Raboin et al. (28) analyzed a collection of *S. scitamineum* populations collected from 15 sugarcane-producing countries for polymorphisms at 17 microsatellite loci. They found that genetic diversity was extremely low among the American and African populations. Higher genetic diversity, however, was observed among the Asian populations, particularly those from the Philippines. The American and African *S. scitamineum* populations all belonged to a single lineage, which was also found in some populations in Asia.

The researchers concluded from the results that the fungus migrated from Asia to other continents, probably through movement of infected plant material.

Several molecular methods have proven useful for analysis of genetic variation in pathogenic fungi (21,35). For example, rapid amplification of polymorphic DNA (RAPD; 26,30), amplified fragment length polymorphism (AFLP; 4), and internal transcribed spacer (ITS) sequence analysis (32,33) have been used to assess the intraspecific diversity within *S. scitamineum* populations. Unlike the traditional polymerase chain reaction (PCR) analysis, RAPD does not require any previous DNA sequence information of the target organism, because the primers will bind anywhere in the genome, depending on sequences that are complementary to the primers. This makes it more feasible in the phylogenetic study of various plant-pathogenic fungi (5,12). Sequence-related amplified polymorphism (SRAP), which combines simplicity, reliability, moderate through-put ratio, and easy sequencing of selected bands, is a relatively new but powerful molecular marker system (20). It uses two types of primers (forward and reverse) with the sequence CCGG in the forward primer and AATT in the reverse primer. This method targets the open read frames (ORFs) of genes and can detect polymorphisms due to length variance of introns, promoters, and spacers. Compared with AFLP and inter-simple sequence repeat, the higher annealing temperature of 50°C or above and the longer length of primers allows for a large number of robust polymorphic bands in SRAP amplification, leading to desirable discrimination ability (6). The objective of this study was to describe the genetic variation of populations of *S. scitamineum* isolated from the six primary sugarcane production areas in mainland China (Guangxi, Yunnan, Guangdong, Hainan, Fujian, and Jiangxi provinces) with two techniques, RAPD and SRAP, in order to gain a better understanding of the molecular variation within this species.

Materials and Methods

Test materials. Twenty-three sugarcane *S. scitamineum* isolates were collected from the six primary sugarcane-producing provinces in mainland China (Table 1).

DNA extraction and PCR identification. Mycelia derived from a single spore were used in DNA extraction by a procedure described by Que et al. (26,27). Quality and quantity of DNA samples were determined by 1.0% agarose gel electrophoresis and

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ultraviolet spectrophotometer analysis. Before RAPD and SRAP analysis, the identification of all DNA was verified using PCR to amplify the *bE* mating-type gene of *S. scitamineum* (2,29).

RAPD analysis. PCR amplification of genomic DNA was performed using arbitrary primers listed in Table 2. The S series primer set was originally obtained from the Shanghai Sangon Biological Engineering Technology & Services Co. Ltd.; Operon primers were purchased from Operon Technologies. RAPD amplification of *S. scitamineum* DNA was performed using the 13 random primers in Table 2. PCR amplification was performed in a final volume of 20 µl containing 50 ng of *S. scitamineum* DNA and 0.5 µM 10-mer random primer. The reaction mixture also contained a dNTP concentration of 0.15 mM and 2.5 µl of 10× PCR reaction buffer (containing 15 mM MgCl₂) and 1 unit of Taq DNA polymerase (Takara Bio Inc.). The volume was adjusted to 20 µl with the addition of sterile distilled water. Amplification was performed in an Eppendorf thermal cycler, with an initial denaturation at 96°C for 5 min, followed by 40 cycles each of denaturation at 94°C for 1 min, annealing at 37°C for 1 min and extension at 72°C for 1.5 min. Immediately after thermal cycling, a final extension at 72°C for 8 min was done and samples were cooled and held at 4°C until use. PCR products were loaded into a 1.0% agarose gel containing 0.1% EB, subjected to electrophoresis in 1× Tris-acetate-EDTA (40 mM Tris; 20 mM glacial acetic acid; and 1 mM EDTA, pH 8.0) for about 2 h. The gel image was analyzed using Quantity One software (version 4.6.2; Bio-Rad Laboratories, Inc.).

SRAP analysis. From 45 possible primer pairs of five forward and nine reverse primers (Table 3), 18 combinations that produced a few strong, reproducible, and polymorphic bands were selected. These primer combinations were used to amplify the 23 *S. scitamineum* genotypes. PCR amplification was performed in a 20-µl reaction volume containing 2 µl of 10× PCR reaction buffer (containing 15 mM/L MgCl₂), a dNTP concentration of 0.75 mM, forward and reverse primers each at 0.3 mM, 40 ng of *S. scitamineum* DNA, and 2.5 units of Taq DNA polymerase (Takara Bio Inc.). Samples were subjected to the following PCR program: 5 min of denaturing at 94°C and five cycles of three steps consisting of 1 min of denaturing at 94°C, 1 min of annealing at 35°C, and 2 min of elongation at 72°C. In the following 30 cycles, the annealing temperature was increased to 50°C, with a final elongation step of 5 min at 72°C. PCR products were resolved by electrophoresis on 1.6% agarose gel stained with 0.1% ethidium bromide in 0.5× Tris-borate-EDTA buffer. The gel image was analyzed using Quantity One software (version 4.6.2; Bio-Rad Laboratories).

Table 1. Origins and sugarcane cultivar hosts of 23 isolates of *Sporisorium scitamineum* used in this study

Isolates	Origin	Host
1	Fujian	Badila
2	Fujian	Co1001
3	Fujian	F134
4	Fujian	NCo310
5	Fujian	NCo376
6	Fujian	Mintang76-2
7	Fujian	Guitang94-119
8	Jiangxi	Guitang94-119
9	Jiangxi	Mintang95-354
10	Guangdong	Yuetang93-159
11	Guangdong	ROC16
12	Guangxi	ROC16
13	Guangxi	Guitang21
14	Hainan	Guitang16
15	Hainan	Yuetang92-126
16	Yunnan	CP34-85
17	Yunnan	Chuanzhe2
18	Yunnan	Yacheng55-1
19	Yunnan	Yacheng73-498
20	Yunnan	Yacheng84-37
21	Yunnan	Yuetang75-897
22	Yunnan	F172
23	Yunnan	ROC22

Data analysis. For both RAPD and SRAP analyses, the presence and absence of each band was coded by 1 and 0, respectively, and was scored using a binary data matrix (0 to 1). Genetic distances ($1 - S_{ij}$) among isolates were calculated according to the Nei and Li (24) similarity coefficient $S_{ij} = 2a/(2a + b + c)$, where S_{ij} is the similarity between two individuals i and j , a is the number of shared bands, b is the number of bands exclusively amplified by i , and c is the number of bands exclusively amplified by j . Then, DPS2000 software (Zhejiang University, China) was used in the corresponding data analyses, and resulted in the construction of a phylogenetic tree and dissimilarity coefficient matrix. During the data processing, the distance matrix was subjected to cluster analysis by the unweighted pair-group method with arithmetic means (UPGMA). In RAPD-SRAP combined analysis, the data from RAPD and SRAP were combined and analyzed with the same procedure as above.

Results

RAPD analysis. Thirteen RAPD primers were used for the evaluation of genetic relationships among 23 *S. scitamineum* isolates. In total, 189 bands was scored. Among them, 102 bands (54%) were polymorphic between at least two individuals. The highest and lowest rates of polymorphism (number of polymorphic amplified bands/total monomorphic and polymorphic bands within a paired comparison) among the amplified bands were 88 and 31%, respectively. The length of amplification bands ranged from 200 to 3,000 bp, with an average band number of 15 per primer. Genetic dissimilarities among all isolates ranged from 0.071 to 0.825 (Table 4). The Nei-Li similarity coefficient and UPGMA clustering algorithm grouped the genotypes into four clusters at the dissimilarity coefficient of 0.50 using the RAPD dendrogram (Fig. 1). Grouping of genotypes by RAPD analysis suggests that the molecular variation of *S. scitamineum* is geographically specific because isolates from four of the six locations clustered according

Table 2. Sequences of primers used in random amplified polymorphic DNA analysis of *Sporisorium scitamineum*

Primers	Sequence
S65	GATGACCGCC
S104	GGAAGTCGCC
S105	AGTCGTCCCC
S302	TTCCGCCACC
S307	GAGCGAGGCT
S350	AAGCCCGAGG
S469	GTGGTCCGCA
S471	AACGAGTCGG
OPC08 (S68)	TGGACCGGTG
OPH19 (S119)	CTGACCAGCC
OPM13 (S413)	GGTGGTCAAG
OPM14 (S414)	AGGGTCGTTT
OPR12 (S512)	ACAGGTGCGT

Table 3. Sequences of primers used in sequence-related amplified polymorphism analysis of *Sporisorium scitamineum* (20)

Primer codes	Sequences (5'-3')
me1	5'-TGAGTCCAAACCGGATA-3'
me2	5'-TGAGTCCAAACCGGAGC-3'
me3	5'-TGAGTCCAAACCGGAAT-3'
me4	5'-TGAGTCCAAACCGGACC-3'
me5	5'-TGAGTCCAAACCGGAAG-3'
em2	5'-GACTGCGTACGAATTGCG-3'
em3	5'-GACTGCGTACGAATTGAC-3'
em4	5'-GACTGCGTACGAATTGGA-3'
ern5	5'-GACTGCGTACGAATTAAAC-3'
em6	5'-GACTGCGTACGAATTGCA-3'
em7	5'-GACTGCGTACGAATTATG-3'
em12	5'-GACTGCGTACGAATTGTC-3'
em13	5'-GACTGCGTACGAATTGGT-3'
em15	5'-GACTGCGTACGAATTCTG-3'

to their respective locations (Hainan isolates 14 and 15, Yunnan isolates 16–23, Jiangxi isolates 8 and 9, and Guangdong isolates 10 and 11). The results do not support host-specific grouping because two sets of isolates from the same host varieties (7 and 8 from Guitang94-119 and 11 and 12 from ROC16) did not cluster according to their host origins. However, RAPD analysis did not group all the isolates based on their geographical origin. The two isolates from Guangxi (isolate 12 and 13) belonged to two separate clusters.

SRAP analysis. In total, 201 distinct amplified bands was produced by the 18 SRAP primer combinations. Among them, 126 (63%) were polymorphic between at least two individuals. Among the 18 primer combinations, the highest and lowest polymorphism rates of amplified bands for a single primer were 85 and 29%, respectively. The length of amplification bands ranged from 200 to 3,000 bp, with an average band number of 11 per primer. The re-

sults revealed a relatively high level of genetic distance among most isolates tested and the genetic dissimilarities among all isolates ranged from 0.045 to 0.947 (Table 5). The Nei-Li similarity coefficient and the UPGMA clustering algorithm grouped the genotypes into six clusters at the dissimilarity coefficient of 0.50 using the SRAP dendrogram, including two clusters with multiple isolates. Four isolates remained unclustered (Fig. 2). Three isolates from Fujian (isolates 1, 5, and 6) formed one cluster, while the other four isolates from Fujian (isolates 2, 3, 4, and 7) were part of a second cluster. In addition to the four Fujian isolates, the second cluster included the two isolates from Jiangxi (isolates 8 and 9), the two isolates from Guangdong (isolates 10 and 11), seven of eight isolates from Yunnan (isolates 16–21,23), and one of two isolates from Guangxi (isolate 13). With the exception of isolate 22, which formed a separate single-isolate cluster, the second-largest cluster identified with SRAP analysis was the same as the largest cluster

Table 4. Dissimilarity coefficients of *Sporisorium scitamineum* isolates 1 to 23 (Iso.) at DNA level revealed by random amplified polymorphic DNA analysis

Iso.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	1.000	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2	0.735	1.000	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
3	0.630	0.405	1.000	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
4	0.755	0.324	0.425	1.000	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
5	0.341	0.609	0.415	0.612	1.000	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
6	0.366	0.600	0.476	0.686	0.275	1.000	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
7	0.696	0.467	0.409	0.378	0.529	0.577	1.000	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
8	0.580	0.477	0.419	0.422	0.447	0.500	0.304	1.000	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
9	0.778	0.524	0.600	0.390	0.667	0.686	0.413	0.457	1.000	...	...	...	...	...	...	...	...	...	...	...	...	...	...
10	0.551	0.543	0.381	0.521	0.413	0.500	0.340	0.190	0.551	1.000	...	...	...	...	...	...	...	...	...	...	...	...	...
11	0.673	0.556	0.500	0.532	0.608	0.627	0.417	0.426	0.592	0.356	1.000	...	...	...	...	...	...	...	...	...	...	...	...
12	0.704	0.565	0.604	0.571	0.615	0.608	0.547	0.500	0.571	0.529	0.596	1.000	...	...	...	...	...	...	...	...	...	...	...
13	0.750	0.456	0.500	0.295	0.625	0.667	0.423	0.431	0.370	0.519	0.500	0.420	1.000	...	...	...	...	...	...	...	...	...	...
14	0.438	0.775	0.714	0.750	0.547	0.449	0.656	0.571	0.750	0.621	0.700	0.638	0.727	1.000	...	...	...	...	...	...	...	...	...
15	0.580	0.825	0.786	0.817	0.673	0.588	0.742	0.733	0.797	0.754	0.724	0.707	0.750	0.261	1.000	...	...	...	...	...	...	...	...
16	0.690	0.500	0.540	0.383	0.582	0.600	0.404	0.347	0.233	0.442	0.537	0.519	0.300	0.672	0.714	1.000	...	...	...	...	...	...	...
17	0.611	0.422	0.364	0.370	0.429	0.510	0.255	0.222	0.500	0.298	0.408	0.589	0.415	0.576	0.689	0.333	1.000	...	...	...	...	...	...
18	0.667	0.381	0.395	0.364	0.490	0.540	0.283	0.289	0.531	0.362	0.404	0.618	0.442	0.627	0.738	0.392	0.071	1.000	...	...	...	...	...
19	0.691	0.381	0.432	0.364	0.520	0.596	0.319	0.326	0.500	0.396	0.469	0.618	0.380	0.672	0.738	0.360	0.159	0.143	1.000	...	...	...	...
20	0.636	0.456	0.468	0.370	0.490	0.538	0.327	0.298	0.500	0.333	0.408	0.564	0.415	0.623	0.710	0.333	0.174	0.159	0.200	1.000	...	...	...
21	0.684	0.456	0.468	0.295	0.519	0.593	0.292	0.333	0.470	0.400	0.440	0.614	0.385	0.645	0.730	0.333	0.133	0.116	0.159	0.091	1.000	...	...
22	0.588	0.522	0.500	0.531	0.458	0.569	0.509	0.490	0.615	0.490	0.558	0.618	0.527	0.579	0.574	0.481	0.412	0.471	0.408	0.412	0.442	1.000	...
23	0.630	0.511	0.457	0.426	0.480	0.585	0.412	0.451	0.549	0.420	0.460	0.607	0.491	0.639	0.661	0.444	0.340	0.333	0.333	0.271	0.306	0.261	1.000

Fig. 1. Dendrogram of 23 isolates of *Sporisorium scitamineum* based on random amplified polymorphic DNA data assembled by unweighted pair-group method analysis.

identified with RAPD analysis. Therefore, to some extent, isolates from the same geographical region grouped together, although there was a less clear pattern with SRAP than with RAPD analysis. This phenomenon might be due to the frequent cultivar or germplasm exchange in mainland China. Geography-specific molecular variation of *S. scitamineum* could explain the tendency for isolates collected from the same province to group together. Whereas the two isolates of Guitang94/119 (isolates 7 and 8) from Fujian and Jiangxi, respectively, fell into the same cluster, the two isolates of ROC16 (isolates 11 and 12) from Guangdong and Guangxi, respectively, were assigned to two different clusters, as that with RAPD analysis. The results suggest the lack of host-specific molecular variation in *S. scitamineum*. Compared with RAPD analysis above, the SRAP marker system seems to be a

more efficient marker system for better differentiating tested isolates.

RAPD-SRAP combined analysis. In RAPD-SRAP combined analysis, genetic dissimilarities among all isolates ranged from 0.026 to 0.838, with a mean similarity of 0.457 (Table 6). The Nei-Li similarity coefficient and the UPGMA clustering algorithm grouped the genotypes into five clusters with a dissimilarity coefficient of 0.32 using the RAPD-SRAP dendrogram (Fig. 3). As shown in RAPD-SRAP combined analysis, the distribution of various isolates in the dendrogram was somewhat geography specific and not host specific, similar to that in RAPD and SRAP analysis. Three isolates from Fujian (isolates 1, 5, and 6), six of eight isolates from Yunnan except isolates 16 and 22, and two isolates from Hainan (isolates 14 and 15) belong to three unique clusters, respectively.

Table 5. Dissimilarity coefficients of *Sporisorium scitamineum* isolates 1 to 23 (Iso.) at DNA level revealed by sequence-related amplified polymorphism analysis

Iso.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	1.000	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2	0.597	1.000	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
3	0.565	0.200	1.000	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
4	0.684	0.222	0.315	1.000	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
5	0.415	0.423	0.379	0.525	1.000	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
6	0.467	0.574	0.467	0.645	0.418	1.000	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
7	0.635	0.157	0.254	0.200	0.392	0.595	1.000	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
8	0.576	0.306	0.375	0.270	0.353	0.574	0.208	1.000	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
9	0.623	0.292	0.405	0.280	0.386	0.676	0.243	0.217	1.000	...	...	...	...	...	...	...	...	...	...	...	...	...	...
10	0.508	0.329	0.352	0.380	0.277	0.574	0.257	0.206	0.217	1.000	...	...	...	...	...	...	...	...	...	...	...	...	...
11	0.671	0.239	0.310	0.230	0.467	0.649	0.192	0.288	0.200	0.311	1.000	...	...	...	...	...	...	...	...	...	...	...	...
12	0.717	0.629	0.578	0.675	0.635	0.661	0.627	0.648	0.689	0.629	0.606	1.000	...	...	...	...	...	...	...	...	...	...	...
13	0.671	0.346	0.410	0.269	0.494	0.700	0.278	0.325	0.243	0.325	0.240	0.608	1.000	...	...	...	...	...	...	...	...	...	...
14	0.595	0.721	0.677	0.808	0.547	0.524	0.712	0.682	0.725	0.619	0.750	0.780	0.747	1.000	...	...	...	...	...	...	...	...	...
15	0.757	0.900	0.869	0.947	0.857	0.833	0.905	0.879	0.897	0.844	0.931	0.884	0.905	0.700	1.000	...	...	...	...	...	...	...	...
16	0.593	0.368	0.418	0.481	0.397	0.677	0.384	0.368	0.303	0.266	0.348	0.672	0.338	0.643	0.855	1.000	...	...	...	...	...	...	...
17	0.595	0.212	0.206	0.301	0.412	0.569	0.214	0.338	0.370	0.314	0.296	0.606	0.333	0.661	0.889	0.328	1.000	...	...	...	...	...	...
18	0.653	0.162	0.286	0.181	0.467	0.611	0.167	0.288	0.274	0.311	0.171	0.625	0.286	0.732	0.900	0.371	0.246	1.000	...	...	...	...	...
19	0.620	0.119	0.246	0.141	0.453	0.616	0.100	0.250	0.260	0.297	0.183	0.649	0.273	0.753	0.917	0.403	0.232	0.103	1.000	...	...	...	...
20	0.597	0.205	0.329	0.270	0.444	0.634	0.208	0.351	0.292	0.257	0.239	0.667	0.346	0.721	0.896	0.391	0.265	0.239	0.200	1.000	...	...	...
21	0.606	0.104	0.257	0.153	0.440	0.622	0.113	0.260	0.222	0.260	0.194	0.635	0.260	0.740	0.903	0.366	0.191	0.088	0.045	0.159	1.000	...	...
22	0.561	0.662	0.586	0.743	0.649	0.622	0.676	0.662	0.706	0.619	0.732	0.644	0.730	0.667	0.700	0.667	0.661	0.714	0.700	0.682	0.686	1.000	...
23	0.586	0.406	0.433	0.474	0.462	0.672	0.419	0.472	0.437	0.406	0.452	0.623	0.440	0.661	0.830	0.377	0.441	0.386	0.439	0.451	0.403	0.558	1.000

Fig. 2. Dendrogram of 23 isolates of *Sporisorium scitamineum* based on sequence-related amplified polymorphism data assembled by unweighted pair-group method analysis.

Discussion

To our knowledge, at least three different explanations for genetic diversity among fungi have been proposed. Some researchers conclude that genetic diversity of certain fungi can be related to geographical origin and the corresponding molecular variation is geography specific, as revealed by previous reports on *Pleurotus cytidiosus* and related taxa, *Sawadaca* spp., and *Beauveria* spp. (10,15,31). Other investigators argue that molecular differentiation of various fungi are related to the plant hosts (host specific) and, to some extent, to host–pathogen co-evolution mechanisms, as indicated in the studies of *Magnicellulatae* of *Podosphaera* section *Sphaerotheca* (Erysiphales) and *Podosphaera* (14,31). Third, there are claims that diversity may be due to differentiation determined by their intrinsic hereditary properties (7). Still other researchers proposed that genetic diver-

sity of various fungi is closely linked to both their geographical and host origins (9).

In this study, biogeographical patterns of variation within *S. scitamineum* were revealed by RAPD, SRAP, and RAPD-SRAP combined analysis. Opinions vary about the utility of combining data sets but, in some studies, the results obtained from individual versus combined molecular-markers data sets for the purpose of genetic diversity analysis were comparable (1,6,22). In this study, individual data sets of RAPD and SRAP analysis were combined to examine congruence and incongruence among the results. Our results demonstrated that combined data were concurrent with individual data when correlation among procedures was high, which is in accordance with Budak et al. (6).

Patterns of geographical subdivision in the genetic structure of the *S. scitamineum* isolates existed but occasional long-distance dispersal also possibly occurred. For example, while smaller

Table 6. Dissimilarity coefficients of *Sporisorium scitamineum* isolates 1 to 23 (Iso.) at DNA level revealed by random amplified polymorphic DNA–sequence-related amplified polymorphism combined analysis

Iso.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	1.000	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2	0.647	1.000	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
3	0.405	0.280	1.000	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
4	0.600	0.130	0.231	1.000	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
5	0.227	0.438	0.200	0.394	1.000	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
6	0.156	0.515	0.333	0.529	0.209	1.000	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
7	0.550	0.286	0.290	0.310	0.368	0.436	1.000	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
8	0.350	0.286	0.290	0.379	0.263	0.333	0.353	1.000	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
9	0.684	0.231	0.379	0.259	0.500	0.568	0.313	0.375	1.000	...	...	...	...	...	...	...	...	...	...	...	...	...	...
10	0.333	0.333	0.212	0.419	0.250	0.317	0.278	0.111	0.412	1.000	...	...	...	...	...	...	...	...	...	...	...	...	...
11	0.429	0.400	0.333	0.419	0.350	0.415	0.278	0.278	0.353	0.211	1.000	...	...	...	...	...	...	...	...	...	...	...	...
12	0.609	0.412	0.405	0.371	0.455	0.511	0.450	0.400	0.368	0.381	0.429	1.000	...	...	...	...	...	...	...	...	...	...	...
13	0.571	0.267	0.333	0.226	0.400	0.463	0.389	0.333	0.235	0.368	0.368	0.143	1.000	...	...	...	...	...	...	...	...	...	...
14	0.200	0.737	0.561	0.744	0.417	0.224	0.637	0.500	0.714	0.478	0.522	0.640	0.652	1.000	...	...	...	...	...	...	...	...	...
15	0.347	0.838	0.650	0.789	0.532	0.333	0.674	0.674	0.707	0.600	0.467	0.633	0.644	0.132	1.000	...	...	...	...	...	...	...	...
16	0.500	0.313	0.371	0.333	0.381	0.442	0.368	0.263	0.167	0.300	0.300	0.318	0.200	0.583	0.574	1.000	...	...	...	...	...	...	...
17	0.349	0.290	0.235	0.375	0.171	0.286	0.243	0.189	0.371	0.179	0.282	0.488	0.385	0.447	0.565	0.220	1.000	...	...	...	...	...	...
18	0.415	0.241	0.313	0.333	0.231	0.350	0.257	0.257	0.394	0.243	0.297	0.512	0.405	0.511	0.636	0.282	0.053	1.000	...	...	...	...	...
19	0.487	0.185	0.333	0.286	0.297	0.421	0.273	0.273	0.355	0.257	0.314	0.487	0.371	0.581	0.667	0.243	0.111	0.059	1.000	...	...	...	...
20	0.395	0.290	0.353	0.313	0.268	0.333	0.243	0.243	0.371	0.231	0.231	0.442	0.333	0.489	0.565	0.220	0.100	0.052	0.111	1.000	...	...	...
21	0.429	0.267	0.333	0.290	0.250	0.366	0.222	0.278	0.353	0.263	0.263	0.476	0.368	0.522	0.600	0.250	0.077	0.027	0.086	0.026	1.000	...	...
22	0.396	0.610	0.591	0.571	0.373	0.385	0.447	0.532	0.511	0.551	0.469	0.472	0.510	0.368	0.357	0.490	0.440	0.458	0.478	0.440	0.429	1.000	...
23	0.378	0.333	0.333	0.294	0.256	0.364	0.282	0.385	0.297	0.317	0.219	0.422	0.317	0.510	0.500	0.256	0.238	0.200	0.211	0.190	0.171	0.346	1.000

Fig. 3. Dendrogram of 23 isolates of *Sporisorium scitamineum* based on random amplified polymorphic DNA–sequence-related amplified polymorphism combined analysis assembled by unweighted pair-group method analysis.

dissimilarity coefficients were mostly found among isolates from the same province, large and small dissimilarity coefficients were also found among isolates from the same province and isolates from different provinces, respectively. In RAPD analysis, the smallest dissimilarity coefficient (0.071) existed between isolates 17 and 18 from Yunnan, while the largest occurred between isolate 2 from Fujian and isolate 15 from Hainan. In SRAP analysis, the smallest and largest dissimilarity coefficient existed between two isolates from Yunnan and two isolates from Fujian and Hainan, respectively. In RAPD-SRAP combined analysis, the smallest dissimilarity coefficient (0.026) occurred between two isolates from Hainan, while the largest existed between one isolate from Fujian and one isolate from Hainan. However, in RAPD analysis, the four isolates from Fujian exhibited relatively high dissimilarity coefficients. Another exception involved one isolate from Guangdong and one isolate from Yunnan. These exceptions were most probably due to intermingling of isolates involving germplasm exchange. The carriers responsible for long-distance dispersal in *S. scitamineum* are still poorly known but recent human-mediated dispersal through sugarcane germplasm exchange via stalk cuttings has been documented (26). James et al. (17) found a similar geographic pattern in the population structure of *Schizophyllum commune*, with examples of changes suggesting recent long-distant dispersal. The geography-specific characteristics of molecular variation in *Sporisorium scitamineum* isolates were also validated by phylogenetic analysis. Phylogenetic analysis of RAPD, SRAP, and RAPD-SRAP data divided *S. scitamineum* isolates into several lineages and, in each case, one of them corresponded largely with geographical groupings, especially for the three isolates from Fujian and six isolates from Yunnan. In RAPD and RAPD-SRAP combined analysis, two isolates from Hainan belonged to the same cluster. In the RAPD-SRAP combined analysis, the tendency toward geography-specific variation was even stronger. Isolates of *S. scitamineum* from China were not included in the earlier study by Raboin et al. (28), which demonstrated the greatest genetic diversity of this fungus among isolates from Asia. Therefore, further studies on genetic diversity of *S. scitamineum* should include isolates from China.

Analysis of RAPD, SRAP, and combined RAPD-SRAP data did not provide any proof of a co-evolution mechanism between sugarcane and *S. scitamineum*. Two isolates from Guitang94/119 (isolates 7 and 8 from Fujian and Jiangxi, respectively) and two isolates from ROC16 (isolates 11 and 12 from Guangdong and Guangxi, respectively) were included in this study to provide some information on whether the molecular variation of *S. scitamineum* was cultivar specific. In RAPD analysis, the two isolates from Guitang94/119, belonged to one clade in the phylogenetic tree, while the other two isolates from ROC16 (isolates 11 and 12) belonged to separate clades (Fig. 2). In SRAP analysis, the dissimilarity coefficient between isolates 7 and 8 was 0.208 and they belonged to the same cluster, while the dissimilarity coefficient between 11 and 12 was as high as 0.606 and they fell into two different clades. In RAPD-SRAP combined analysis, isolates 7 and 8 were distributed to two different clades, as were isolates 11 and 12. Therefore, RAPD, SRAP, and RAPD-SRAP combined analysis did not provide evidence for a co-evolution mechanism between sugarcane and *S. scitamineum*, which is different from the co-evolution phenomena proposed for *Magnicellulatae* and *Podosphaera* (14,34).

Research on the diversity or molecular variation of *S. scitamineum* and the association with geographical or host origin can be useful in breeding for sugarcane smut resistance. If the diversity is related to host origin, resistant cultivars should provide durable resistance. If variation is associated with the geographical origin, the inoculum for smut resistance testing should include isolates from various geographical origins, or potential parents or cultivars should be tested in different locations where other races are present. In this study, the molecular variation of *S. scitamineum* was more closely associated with geographical origin and not with host association. Therefore, resistance screens by artificial inoculation

or natural field infection should still be carried out in different sugarcane planting areas because of the risk of introducing new biotypes of *S. scitamineum* into different geographical regions. Genetic markers associated with resistance or susceptibility to geographically diverse *S. scitamineum* isolates would be useful for marker-assisted selection.

To date, several races of *S. scitamineum* are known to exist but the race type is still poorly understood or defined (4,8). Classification of *S. scitamineum* is based primarily on differences in spore morphology and the characteristics of germinating spores. Although attempts have been made to classify races of *S. scitamineum* according to the reaction of a standard set of sugarcane cultivars, a universally accepted set of standard control cultivars has not been established. Additionally, host-differential methods used to define races of pathogens are time and labor consuming. To insure reliable determination of a cultivar's smut resistance phenotype, inoculation and cultivation of two complete crops (i.e., two plant-cane crops or one plant-cane crop plus one ratoon crop) is normally required (19). The presence of races has been indicated or suggested when a sugarcane cultivar succumbs to smut after being grown successfully for several years (18,36). Experiments by Leu and Tang (19) demonstrated genetic control of pathogenicity in *S. scitamineum*. Several other reports have been published indicating the presence of races (3,8,11,23,30). At the 24th International Society of Sugar Cane Technologists Congress held in Brisbane, members of the Pathology Section agreed that there were only two races of sugarcane smut in the countries investigated, except in Chinese Taiwan, where a third race was identified. This conclusion was supported by an international project involving Argentina, Barbados, Colombia, India, South Africa, Sudan, Taiwan, the United States (including Hawaii), and Zimbabwe which did not reveal a race-cultivar interaction except in Taiwan (13). A set of 11 cultivars with different reactions to smut were distributed to participating countries, where they were inoculated in field trials with teliospores of indigenous populations of smut. However, a resistant cultivar in one location may be susceptible in another (30). In this study, two *S. scitamineum* isolates collected from two sugarcane varieties, NCo310 and F134, which were assumed to be susceptible to races 1 and 2, respectively, were also included to determine if RAPD or SRAP analysis would be useful in identifying smut races; however, the two isolates fell into the same clade. Among the 23 isolates in this study, there was no evidence that they could be divided into two races. Thus, sugarcane smut race classification through RAPD and SRAP analysis remains elusive. Moreover, the *S. scitamineum* isolate from ROC22 in Chinese Taiwan was reported to be race 3 but the dissimilarity coefficient between the isolate from ROC22 in Yunnan and all the other isolates, as revealed by RAPD and SRAP analysis, ranged from 0.261 to 0.661 and 0.377 to 0.830, respectively, indicating no evidence that it was race 3.

'Badila', a *Saccharum officinarum* cultivar used in early hybrid crosses, is widely recognized as a source of the smut resistance gene. NCo376 is the high resistant standard adopted in sugarcane smut resistance identification through artificial inoculation and field tests. During the several decades of research conducted at the Sugarcane Research Institute, Fujian Agricultural and Forestry University, only one *Sporisorium scitamineum* isolate was found on Badila and the infection rate of NCo376 was almost always 0. It was anticipated that the two isolates (isolates 1 and 5) collected from Badila and NCo376 would possess larger molecular discrepancy to all the other tested isolates. However, in the phylogenetic analysis, these two isolates plus isolate 6 from Fujian belonged to one clade, without significant molecular variation. Whether this is due to the cultivar degradation or molecular variation needs to be studied.

The present study showed that the variation in *S. scitamineum* at the molecular level was more closely associated with the origin of the isolate rather than with the cultivar from which it was associated. Additionally, the results indicated that, if an isolate is distinctly different from all others based on molecular analysis, its

pathogenicity should be tested using standard control cultivars to determine whether it represents a new race or only an isolate with greater molecular variation.

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Literature Cited

1. Ajmone-Marsan, P., Castiglino, P., Fusari, F., Kuiper, M., and Motto, M. 1998. Genetic diversity and its relationship to hybrid performance in maize as revealed by RFLP and AFLP markers. *Theor. Appl. Genet.* 96:219-227.
2. Albert, H. H., and Schenck, S. 1996. PCR amplification from a homolog of the *bE* mating-type gene as a sensitive assay for the presence of *Ustilago scitaminea* DNA. *Plant Dis.* 80:1189-1192.
3. Antoine, R. 1961. Smut. Pages 327-354 in: *Sugar-Cane Diseases of the World*, Vol. 1. J. P. Martin, E. V. Abbott, and C. G. Hughes, eds. Elsevier, Amsterdam.
4. Braithwaite, K. S., Bakkeren, G., Croft, B. J., and Brumbley, S. M. 2004. Genetic variation in a worldwide collection of the sugarcane smut fungus *Ustilago scitaminea*. *Proc. Aust. Soc. Sugar Cane Technol.* 26:48-56.
5. Borie, B., Jacquot, L., Jamaux-Despreaux, I., Larignon, P., and Peros, J. P. 2002. Genetic diversity in populations of the fungi *Phaeoemoniella chlamydospora* and *Phaeoacremonium aleophilum* on grapevine in France. *Plant Pathol.* 51:85-96.
6. Budak, H., Shearman, R. C., Parmaksiz, I., and Dweikat, I. 2004. Comparative analysis of seeded and vegetative biotype Buffalo-grasses based on phylogenetic relationship using ISSRs, SSRs, RAPDs, and SRAPs. *Theor. Appl. Genet.* 109:280-288.
7. Cantone, F. A., and Vandenberg, J. D. 1998. Intraspecific diversity in *Paecilomyces fumosoroseus*. *Mycol. Res.* 102:209-215.
8. Ferreira, S. A., and Comstock, J. C. 1989. Smut. Pages 211-229 in: *Diseases of Sugarcane—Major Diseases*. C. Ricaud, B. T. Egan, A. G. Gillaspie, Jr., and C. G. Hughes, eds. Elsevier, New York.
9. Fischer, M., and Binder, M. 2004. Species recognition, geographic distribution and host-pathogen relationships: a case study in a group of lignicolous basidiomycetes, *Phellinus* s.l. *Mycologia* 96:799-811.
10. Georgios, I. Z., Jean-Marc, M., and Rytas, V. 2004. Molecular phylogeny, biogeography and speciation of the mushroom species *Pleurotus cystidiosus* and allied taxa. *Microbiology* 150:715-726.
11. Gillaspie, A. G., Mock, R. G., and Dean, J. L. 1983. Differentiation of *Ustilago scitaminea* isolates in greenhouse tests. *Plant Dis.* 67:373-375.
12. Goodwin, P. H., and Annis, S. L. 1991. Rapid identification of genetic variation and pathotype of *Leptosphaeria maculans* by random amplified polymorphic DNA assay. *Appl. Environ. Microbiol.* 57:2482-2486.
13. Grisham, M. P. 2001. An international project on sugarcane variability within sugarcane smut. *Proc. Int. Soc. Sugar Cane Technol.* 24:459-461.
14. Hirata, T., Cunningham, J. H., Paksiri, U., Limkaisang, S., Shishkoff, N., Grigaliunaite, B., Sato, Y., and Takamatsu, S. 2000. Evolutionary analysis of subsection *Magnicellulatae* of *Podosphaera* section *Sphaerotheca* (*Erysiphales*) based on the rDNA internal transcribed spacer sequences with special reference to host plants. *Can. J. Bot.* 78:1521-1530.
15. Hirose, S., Tanda, S., Kiss, L., Grigaliunaite, B., Havrylenko, M., and Takamatsu, S. 2005. Molecular phylogeny and evolution of the maple powdery mildew (*Sawadaea*, *Erysiphaceae*) inferred from nuclear rDNA sequences. *Mycol. Res.* 109:912-922.
16. Hoy, J. W., Hollier, C. A., Fontenot, D. B., and Grelen, L. B. 1986. Incidence of sugarcane smut in Louisiana and its effects on yield. *Plant Dis.* 70:59-60.
17. James, T. Y., Moncalvo, J. M., Li, S., and Vilgalys, R. 2001. Polymorphism at the ribosomal DNA spacers and its relation to breeding structure of the widespread mushroom *Schizophyllum commune*. *Genetics* 157:149-161.
18. Lee-Lovick, G. 1978. Smut of sugarcane-*Ustilago scitaminea*. *Rev. Plant Pathol.* 147:181-188.
19. Leu, L. S., and Teng, W. S. 1972. Pathogenic strains of *Ustilago scitaminea* Sydow. *Sugarcane Pathol. Newsl.* 8:12-13.
20. Li, G., and Quiros, C. F. 2001. Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its application to mapping and gene tagging in *Brassica*. *Theor. Appl. Genet.* 103:455-461.
21. Lievens, B., and Thomma, B. P. H. J. 2005. Recent developments in pathogen detection arrays: implications for fungal plant pathogens and use in practice. *Phytopathology* 95:1374-1380.
22. Mohammadi, S. A., and Prasanna, B. M. 2003. Analysis of genetic diversity in crop plants—salient statistical tools and considerations. *Crop Sci.* 43:1235-1248.
23. Mundkur, B. B. 1939. Taxonomy of the sugar-cane smuts. *Kew Bull.* 10:525-533.
24. Nei, Y. M., and Li, W. H. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. USA* 76:5269-5273.
25. Piepenbring, M., Stoll, M., and Oberwinkler, F. 2002. The generic position of *Ustilago maydis*, *Ustilago scitaminea*, and *Ustilago esculenta* (*Ustilaginales*). *Mycol. Prog.* 1:71-80.
26. Que, Y. X., Xu, L. P., Zou, T. T., and Chen, R. K. 2004. Primary analysis of molecular diversity in populations of the fungus *Ustilago scitaminea* Syd. *Chin. J. Agric. Biotechnol.* 12:685-689.
27. Que, Y. X., Zhou, T. T., and Xu, L. P. 2004. The screening of medium for sugarcane *Ustilago scitaminea* and the technique for the isolation of its genomic DNA. *Acta Agric. Univ. Jiangxiensis* 6:353-355, 359.
28. Raboin, L.-M., Selvi, A., Oliveira, K. M., Paulet, F., Calatayud, C., Zapater, M.-F., Brottier, P., Luzaran, R., Garsmeur, O., Carlier, J., and D'Hont, A. 2007. Evidence for the dispersal of a unique lineage from Asia to America and Africa in the sugarcane fungal pathogen *Ustilago scitaminea*. *Fungal Genet. Biol.* 44:64-76.
29. Singh, N., Somai, B. M., and Pillay, D. 2004. Smut disease assessment by PCR and microscopy in inoculated tissue cultured sugarcane cultivars. *Plant Sci.* 167:987-994.
30. Singh, N., Somai, B. M., and Pillay, D. 2005. Molecular profiling demonstrates limited diversity amongst geographically separate strains of *Ustilago scitaminea*. *FEMS Microbiol. Lett.* 247:7-15.
31. St. Leger, R. J., Allee, L. L., May, B., Staples, R. C., and Robertset, D. W. 1992. World-wide distribution of genetic variation among isolates of *Beauveria* spp. *Mycol. Res.* 96:1007-1015.
32. Stoll, M., Begerow, D., and Piepenbring, M. 2005. Molecular phylogeny of *Ustilago*, *Sporisorium*, and related taxa based on combined analyses of rDNA sequences. *Mycol. Res.* 109:342-356.
33. Stoll, M., Piepenbring, M., Begerow, D., and Oberwinkler, F. 2003. Molecular phylogeny of *Ustilago* and *Sporisorium* species (*Basidiomycota*, *Ustilaginales*) based on internal transcribed spacer (ITS) sequences. *Can. J. Bot.* 81:976-984.
34. Takamatsu, S., Niinomi, S., Harada, M., and Havrylenko, M. 2010. Molecular phylogenetic analyses reveal a close evolutionary relationship between *Podosphaera* (*Erysiphales*: *Erysiphaceae*) and its rosaceous hosts. *Persoonia* 24:38-48.
35. Thomas, D. B., and Richard, P. S. 2004. Evolutionary studies of ectomycorrhizal fungi: recent advances and future directions. *Can. J. Bot.* 82:1122-1132.
36. Wu, K. K., Heinz, D. J., and Hogarth, D. M. 1988. Association and heritability of sugarcane smut resistance to races A and B in Hawaii. *Theor. Appl. Genet.* 75:754-760.