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GENETIC VARIATION IN A WORLDWIDE COLLECTION OF THE SUGARCANE SMUT FUNGUS *USTILAGO SCITAMINEA*

By

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Abstract

THE sugarcane smut fungus, *Ustilago scitaminea,* first appeared in Australia in the Ord River Irrigation Area (ORIA) in July 1998. The most likely source of this infection was thought to be wind blown spores from Indonesia. Currently, the sugar industries of eastern Australia, Fiji and Papua New Guinea are still free of the disease. However, the risk of a smut incursion into eastern Australia is very high. Australian sugarcane cultivars are currently being screened in Indonesia and the ORIA to obtain smut resistance ratings, and results show that 70% of Australian cultivars are susceptible. As the use of resistant cultivars is the best option for long-term control of smut, a high priority of Australian sugarcane breeding programs is to increase the level of smut resistance in commercial cultivars. However, successful disease control requires an understanding of the level of diversity in the pathogen population. Information on the smut pathogen present in Australia and neighbouring sugar industries will enable plant breeders to select appropriate breeding strategies, including germplasm selection, for increased resistance in Australian sugarcane. The DNA fingerprinting technique of amplified fragment length polymorphisms (AFLPs) was used to assess genetic variation between isolates of the sugarcane smut fungus. The fungal collection comprised 38 isolates from 13 countries with some isolates collected from the same sugar industry 15 years apart. The technique revealed a low level of variation at the genomic DNA level, but a divergent group of isolates from Southeast Asia was identified. Sugarcane smut spores from this region could show different virulence patterns on Australian cultivars and could constitute another incursion threat to the Australian sugar industry.

Introduction

Smut disease of sugarcane, caused by the fungus *Ustilago scitaminea,* can cause considerable yield losses and reduction in cane quality (Ferreira and Comstock, 1989). Sugarcane smut was first reported in South Africa in 1877, and many observations were made in Africa and Asia in the following decades (reviewed by Antoine, 1961; Presley, 1978). Smut remained confined to the Eastern hemisphere until it was found in Argentina in 1940. It has since been recorded in most sugarcane producing countries of the world (Presley, 1978).

In July 1998, sugarcane smut was observed in Australia for the first time in the Ord River Irrigation Area (ORIA) of Western Australia. The most likely source of this infection was thought

to be wind blown spores from Indonesia (Riley *et al*., 1999). Currently, the sugar industries of eastern Australia, Fiji and Papua New Guinea are still free of the disease. However, the risk of a smut incursion into eastern Australia, where over 99% of Australian sugar is produced, is very high.

Australian sugarcane cultivars are currently being screened in Indonesia to obtain smut resistance ratings, and results show that 70% of Australian cultivars are susceptible (Croft *et al*., 2000). As the use of resistant cultivars is the best option for long-term control of smut, a high priority of Australian sugarcane breeding programs is to increase the level of smut resistance in commercial cultivars.

While various countries have reported the presence of races of *U. scitaminea*, the actual number of races and their prevalence are unknown (Ferreira and Comstock, 1989). For the Australian sugar industry, it is critical to know if neighbouring sugar industries harbour different races of *U. scitaminea* so that the sugarcane breeding program can target resistance to those races that are the greatest threat to this industry.

Successful disease management strategies and deployment of resistance requires an understanding of variation in the pathogen population. While neutral genetic markers such as AFLPs may not detect changes in pathogenicity genes, such markers can track changes in pathogen populations across geographic areas and over time, determine whether resistance screening trials are being done in the appropriate location, and reveal evidence of pathogen genotype by host genotype interactions (Peever *et al.*, 2000).

Our study was initiated to assess the extent of genetic variation among isolates of sugarcane smut collected from various sugar industries, with emphasis on the Asian region using the technique of amplified fragment length polymorphisms (AFLPs, Vos *et al*., 1995).

Materials and methods

A world collection of *U. scitaminea* isolates was assembled at the Pacific Agri-Food Research Centre (Agriculture & Agri-Food Canada) in late 2000–early 2001. Another world collection made in 1984–1985 and held by Dr Ken Damann (Louisiana State University) was also available for this study. Isolates were selected so that all sugar industries in the two collections were represented, with the major emphasis on Asia, because of its proximity to Australia.

For the Florida, Louisiana, Mauritius and South African sugar industries, one isolate each collected 16 years earlier was included with the 2000–2001 isolates, to give an indication of any genetic change that may have occurred over time. The final selection of 38 isolates used in the AFLP study is shown in Table 1. One isolate each of *Ustilago hordei* (covered smut of barley; isolate Uh364) and *Ustilago maydis* (corn smut; isolate Um521) were included in the analysis as outgroups.

Teliospores were germinated on potato dextrose agar and single sporidia were isolated randomly and their mating type (either *MAT-1* or *MAT-2* in this bipolar fungus) was determined (Bakkeren and Kronstad, 1994). For consistency, the genetic diversity study used only sporidial cultures of the same mating type (*MAT-1)*. Cultures were grown in potato dextrose broth and genomic DNA was extracted as described in Bakkeren *et al*. (2000), modified by using a Qiagen DNeasy Plant kit.

AFLP templates were prepared by digesting the DNA with *Bam*HI and *Mse*I, followed by ligation of *Bam*HI and *Mse*I adaptors. Polymerase chain reaction (PCR) amplification used one of 16 Bam.Pxx primers and one of four fluorescently labelled (FAM, NED or JOE) Mse.Px primers (where x stands for any of the four specifying nucleotides). Primer and adaptor details and PCR conditions are given in Table 2 and in Bakkeren *et al*. (2000).

For AFLP analysis, three differently labelled PCR products (containing either FAM, JOE or NED label) and a ROX-labelled internal size standard were combined, denatured and subjected to electrophoresis on a Prism ABI310 Genetic Analyser (Applied Biosystems). Sizing of the AFLP fragments was performed using GeneScan 3.1.2 software (Applied Biosystems). Polymorphic peaks were detected both by eye and by using Genotyper 2.5 software (Applied Biosystems). The presence or absence of a peak was recorded as 1 or 0, respectively.

The binomial data set was converted to a nexus file and phylogenetic analysis was performed using PAUP (Phylogenetic Analysis Using Parsimony software, Swofford, 1999). The data were analysed using Parsimony with Neighbour Joining followed by bootstrapping with 1000 replicates.

Results

We screened 38 isolates with 12 primer combinations (Table 2) with all PCR reactions and AFLP runs done in duplicate. Each primer combination could detect approximately 40 fragments between 40 to 600 base pairs in size, but only polymorphic fragments were considered informative and were used in the phylogenetic analysis.

Fragments which were not present in both duplicates were not included in the analysis. Only 67 scorable polymorphic peaks were detected (Table 2). Although *U. hordei* and *U. maydis* were included as outgroups in the phylogenetic analysis, there were too few fragments in common between *U. hordei*, *U. maydis* and *U. scitaminea* to allow for a meaningful comparative analysis.

Preliminary phylogenetic analysis revealed four clusters loosely based on geographic origin (data not shown). Three clusters consisted of predominantly Indonesian and Western Australian isolates, North and South American isolates, and African isolates, respectively. Isolates within these three clusters diverged by one to seven polymorphic fragments.

The fourth cluster consisted of seven Asian isolates and displayed from 12 (Tw1) to 40 (Tw2) polymorphisms. However, once bootstrap analysis had been performed on the data, only the cluster containing the seven Asian isolates remained as statistically significant, whereas the 31 isolates in the minor clusters were considered as homogeneous (Figure 1).

Eleven of the 12 primer combinations were able to generate at least one polymorphism within the seven variable Asian isolates. After bootstrapping, no differences were observed among isolates collected 16 years apart from the same sugar industry (Fl1 and Fl511 from Florida; Lo1 and Lo538 from Louisiana; Ma2, Ma4 and Ma500 from Mauritius; SA1, SA5, SA6, SA7 and SA527 from South Africa).

Table 1. The 38 *U. scitaminea* isolates used in this study.

Region	Isolate	Location	Year	Cultivar[a]	Supplier[a]
Australia	WA1	Ord River, WA	00/01	nr	J. Engelke
	WA2	Ord River, WA	00/01	nr	J. Engelke
	WA3	Ord River, WA	00/01	nr	J. Engelke
	WA4	Ord River, WA	00/01	nr	J. Engelke
	WA5	Ord River, WA	00/01	nr	J. Engelke
Asia	Ind1	South Sumatra, Indonesia	00/01	nr	U. Murdiyatmo
	Ind2	West Java, Indonesia	00/01	nr	U. Murdiyatmo
	Ind3	Central Java, Indonesia	00/01	nr	U. Murdiyatmo
	Ind4	East Java, Indonesia	00/01	nr	U. Murdiyatmo
	Ind5	South Sulawesi, Indonesia	00/01	nr	U. Murdiyatmo
	Th547	Sing Buri, Thailand	84/85	Q83	A. Kusalwong
	Th548	Supan Buri, Thailand	84/85	Thai1	A. Kusalwong
	TH555	Kanchana Buri, Thailand	84/85	F147	A. Kusalwong
	Ph1	Batangas, Philippines	00/01	VMC84-524	R. Cu
	Ph3	Negros Island, Philippines	00/01	P85-83	R. Cu
	Ph6	Negros Island, Philippines	00/01	VMC98-126	R. Cu
	Tw1	Taiwan, race 1	00/01	nr	Y. Liang
	Tw2	Taiwan, race 2	00/01	nr	Y. Liang
	Tw3	Taiwan, race 3	00/01	nr	Y. Liang
Africa	SA1	Pongola, South Africa	00/01	NCo310	S. McFarlane
	SA5	Umfolozi, South Africa	00/01	NCo376	S. McFarlane
	SA6	Eston, South Africa	00/01	nr	S. McFarlane
	SA7	Darnall, South Africa	00/01	nr	S. McFarlane
	SA527	Pongola, South Africa	84/85	NCo310	R. Bailey
	Zi522	Zimbabwe	84/85	NCo376	P. Sinai
	Ma2	Petite Riviere, Mauritius	00/01	M1176/77	S. Saumtally
	Ma4	Palmyre, Mauritius	00/01	M1030/71	S. Saumtally
	Ma500	Mauritius	84/85	nr	J. Autrey
USA	Ha524	Hawaii, race A	84/85	H73-7088	nr
	Fl1	Canal Point, Florida	00/01	nr	J. Comstock
	Fl511	Canal Point, Florida	84/85	CP73-1547	J. Dean
	Lo1	Louisiana	00/01	nr	J. Hoy
	Lo538	Houma, Louisiana	84/85	H74-14	G. Benda
	Tx529	Sugar Mill, Texas	84/85	NCo310	B. Villalon
S. America	Ar3	Argentina	00/01	nr	A. Rago
	Br533	Sao Paulo, Brazil	84/85	NA56-79	J. Irvine
	Co561	Cucuta, Columbia	84/85	CP57-603	J. Victoria
	Ve530	Maracay, Venezuala	84/85	nr	G. Peterson

[a]nr: information was not recorded when the isolate was collected.

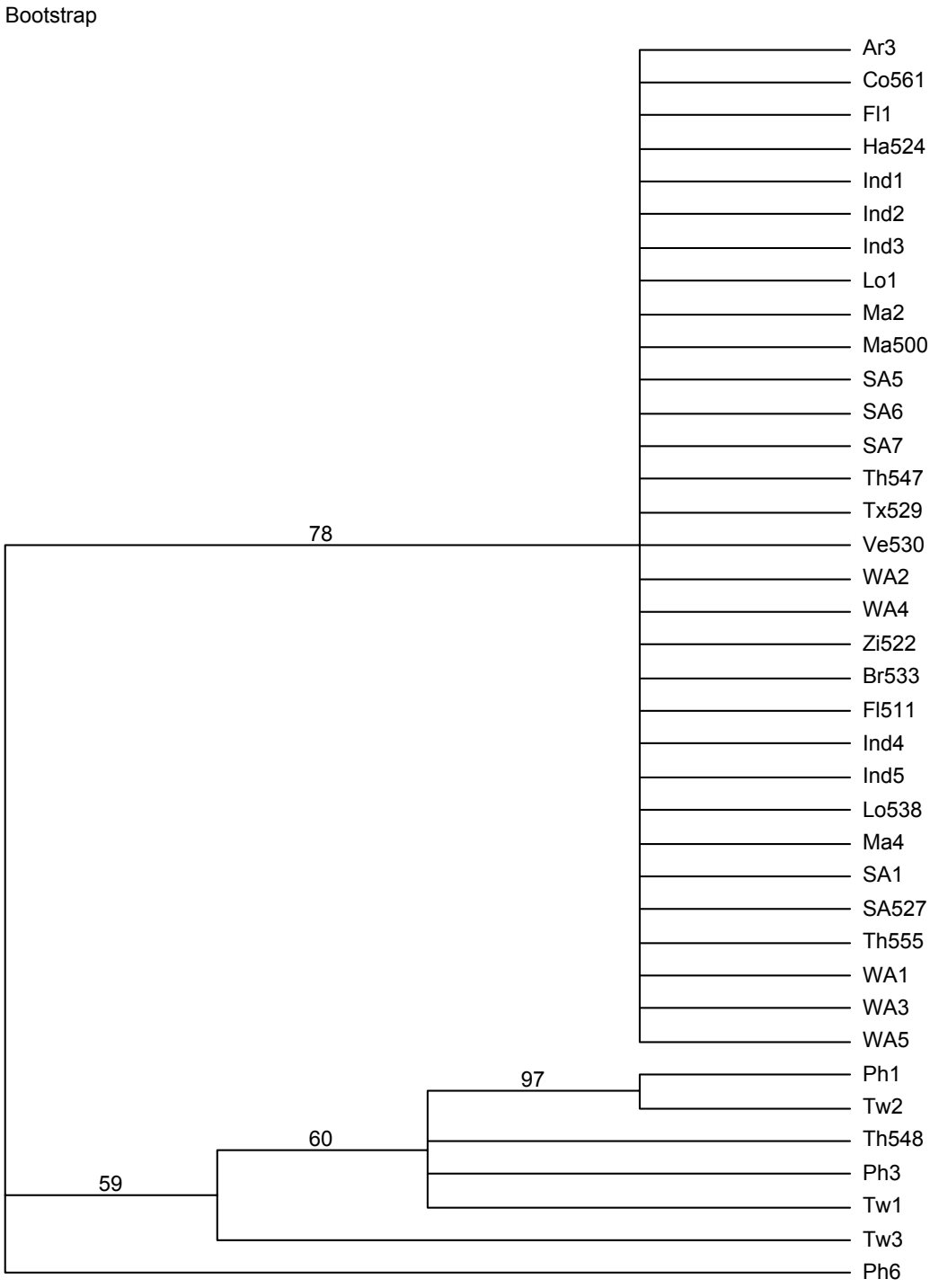

Fig 1—Phylogenetic relationships among 38 *U. scitaminea* isolates. Numbers above the branches refer to bootstrap values in percent (generated from 1000 replicates). Isolate codes are shown in Table 1.

Table 2—Primer combinations used in this study and the number of polymorphisms they generated within *U. scitaminea* isolates.

	BamHI primer	MseI primer			Number of polymorphisms
		FAM	JOE	NED	
1	Bam.Pca	Mse.Pa.FAM			0
2	Bam.Pca		Mse.Pt.JOE		5
3	Bam.Pca			Mse.Pg.NED	4
4	Bam.Pag	Mse.Pa.FAM			12
5	Bam.Pag		Mse.Pt.JOE		6
6	Bam.Pag			Mse.Pg.NED	3
7	Bam.Pga	Mse.Pc.FAM			9
8	Bam.Pag	Mse.Pc.FAM			3
9	Bam.Pca	Mse.Pc.FAM			4
10	Bam.Pgc		Mse.Pt.JOE		4
11	Bam.Ptt		Mse.Pt.JOE		10
12	Bam.Ptc		Mse.Pt.JOE		7
					Total = 67

Discussion

The AFLP fingerprinting technique was used to estimate the degree of genetic variation among 38 isolates of sugarcane smut collected from 16 sugar industries in 13 countries. The AFLPs revealed a low level of genetic variation among 31 isolates. However, seven isolates collected from Taiwan, the Philippines and Thailand, were genetically more divergent. There was no significant difference between isolates collected in 1984 and those collected 16 years later from the same sugar industry, suggesting that genetic change occurs slowly in this fungus and that no, or limited, new introductions of isolates have occurred in these existing populations. No genetic variation could be detected between the Indonesian and Australian isolates.

This finding is consistent with the resistance screening results obtained in Indonesia and the ORIA, which are comparable (Croft *et al*., 2000). As the most likely source of a smut incursion to eastern Australia is either Indonesia or the ORIA, these results should be applicable to the smut disease pressure presently existing in Australia. However, our results also show that a number of distinctive isolates are present in other parts of Asia and it is essential to obtain pathogenicity data to determine if these could constitute a disease threat to the Australian sugar industry. Arrangements are currently underway to test the reaction of some Australian cultivars to isolates in these regions.

Smut disease on sugarcane was first recorded in South Africa in 1877 on the *Saccharum sinense* clone known as ‘China cane’. However, sugarcane smut was most likely present in Asia for much longer. There are circumstantial reports that smut has always been present in India (Antoine, 1961). Indian sugarcane (*Saccharum barberi*) is very susceptible to sugarcane smut and the wild grass *Saccharum spontaneum* is considered to be a collateral host of sugarcane smut and a major reservoir of inoculum in India (Chona, 1956). An Asian origin for *U. scitaminea* may explain the higher level of genetic variation among the Asian smut isolates that we studied. Further sampling from Asia, particularly India, and also from relatives of sugarcane (e.g. *S. spontaneum, S. barberi,*

S. sinense) may reveal more variation within the Asian region. Interestingly, the centre of origin of *Saccharum officinarum* is believed to be New Guinea (Daniels and Roach, 1987), and this region remains free of smut to this day.

Prior to the 20th century, sugar production was dependent on the Noble (*S. officinarum*), Indian (*S. barberi*), and Chinese (*S. sinsense*) canes. Early in the 20th century, Dutch plant breeders in Indonesia developed *Saccharum* interspecific hybrids from *S. officinarum* (having high sugar content) and *S. spontaneum* and others (having good agronomic traits) through a process known as nobilisation. Many modern commercial cultivars of sugarcane can be traced back to these early crosses. The 20th century saw the steady spread of sugarcane smut to almost all sugar industries of the world (reviewed by Presley, 1978). A widely adapted stable smut genotype may have been involved in this spread, explaining the lack of genetic variation in isolates collected from countries outside of Asia.

Pathogenic races of sugarcane smut have been observed in several countries including two races (A and B) from Hawaii (Comstock and Heinz, 1977) and three races (1, 2, 3) reported in Taiwan (Leu and Teng, 1972; Lee *et al.*, 1999). However, Ferreira and Comstock (1989) consider the true prevalence of races to be controversial. Many claims are based on the reaction of the same cultivar in different countries but the interpretation of these claims is confused by test-to-test variation and the use of different inoculation methods in different countries. Two international collaborations have attempted to standardise race typing. Gillaspie *et al*. (1983) performed race typing under glasshouse conditions to standardise the environment and six races were identified. Grisham (2001) co-ordinated a race typing study in nine countries using local isolates tested against a standardised set of 11 differential cultivars.

Although pathogenic variation throughout the world was observed, only Taiwan showed evidence of distinct races. Our AFLP results also indicated that Taiwan is an area of unusual diversity. Of the 38 isolates included in this study, three isolates from Taiwan (Tw1, Tw2, Tw3) and one from Hawaii (Ha524, race A) had previously been characterised as representing a distinct race. Our AFLPs easily distinguished the three Taiwanese races. Early studies found that the two Hawaiian races (A and B) could not be distinguished by isozymes (Anon., 1984), but could be distinguished by neutron activation analysis (Amire *et al*., 1982). Unfortunately, samples of spores representing race B are no longer available from Hawaii and could not be included in this study.

The technique of AFLPs was used by Bakkeren *et al*. (2000) to screen different *Ustilago* species. Five isolates of *U. hordei* were easily distinguished from each other using only four primer pairs. In contrast, within the *U. scitaminea* collection, we scored only 67 polymorphisms from 12 primer combinations. *U. scitaminea* is phenotypically variable with regard to morphology, cultural characteristics and pathogenicity (e.g. Muhammad and Kausar, 1962; Abo and Okusanya, 1996; Alexander, 1981; Pérez and Mauri, 1983). These phenotypic differences appear to be greater than the genetic differences detected by the neutral AFLP markers, suggesting that these phenotypes correlate with minor changes in the genome or possibly in single genes. They could also be indicative of environmental differences and/or gene expression differences. Alternative fingerprinting techniques, such as simple sequence repeats (SSRs or microsatellites), may provide higher sensitivity and generate more polymorphisms to allow us to verify the significance of the weak clustering we observed, or to reveal the existence of yet other clusters. We are in the process of collecting and analysing more isolates from Asia to investigate the extent of the genetic variation.

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REFERENCES

Abo, M.E. and Okusanya, B.A. (1996). Incidence and variability reaction of sugarcane smut (*Ustilago scitaminea* Syd.) isolates in greenhouse and laboratory tests in Nigeria. Discovery and Innovation, 8: 227–231.

Alexander, K.C. (1981). Studies on variation in the smut disease organism (*Ustilago scitaminea* Syd.) of sugarcane. Sugarcane Pathologists' Newsletter, 27: 18–20.

Amire, O.A., Trione, E.J. and Schmitt, R.A. (1982). Characterisation of pathogenic races of the sugarcane smut fungus by neutron activation analysis. J. Radioanalytical Chemistry, 75: 195–203.

Anon. (1984). Smut race identification. Hawaiian Sugar Planters' Association Experiment Station Annual Report, Aiea, Hawaii, p 38.

Antoine, R. (1961). Smut. In: Martin, J.P., Abbott, E.V. and Hughes, C.G. ed. Sugar-cane Diseases of the World, 327–354. Elsevier Publishing Company, Amsterdam.

Bakkeren, G. and Kronstad, J.W. (1994). Linkage of mating-type loci distinguishes bipolar from tetrapolar mating in basidiomycetous smut fungi. Proc. Nat. Acad. Sci. USA, 91: 7085–7089.

Bakkeren, G., Kronstad, J.W. and Lévesque, C.A. (2000). Comparison of AFLP fingerprints and ITS sequences as phylogenetic markers in Ustilaginomycetes. Mycologia, 92: 510–521.

Chona, B.L. (1956). Chairman's opening address. Proc. Int. Soc. Sugar Cane Technol., 9(1): 975–986.

Comstock, J.C. and Heinz, D.J. (1977). A new race of culmicolous smut of sugarcane in Hawaii. Sugarcane Pathologists' Newsletter, 19: 24–25.

Croft, B.J., Irawan and Berding, N. (2000). Screening Australian sugarcane clones for smut reaction in Indonesia: initial results. Proc. Aust. Soc. Sugar Cane Technol., 22: 170–177.

Daniels, J. and Roach, B.T. (1987). Taxonomy and evolution. In: Heinz, D.J. ed. Sugarcane Improvement through Breeding, 7–84. Elsevier, Amsterdam.

Ferreira, S.A. and Comstock, J.C. (1989). Smut. In: Ricaud, C., Egan, B,T., Gillaspie Jr, A.G. and Hughes, C.G. ed. Diseases of Sugarcane, 211–229, Elsevier, Amsterdam.

Gillaspie Jr, A.G., Mock, R.G. and Dean, J.L. (1983). Differentiation of *Ustilago scitaminea* isolates in greenhouse tests. Plant Disease, 67: 373–375.

Grisham, M.P. (2001). An international project on genetic variability within sugarcane smut. Proc. Int. Soc. Sugar Cane Technol., 24(2): 459–461.

Lee, C.S., Yuan, C.H. and Liang, Y.G. (1999). Occurrence of a new pathogenic race of culmicolous smut of sugarcane in Taiwan. Proc. Int. Soc. Sugar Cane Technol., 23(2): 406–407.

Leu, L.S. and Teng, W.S. (1972). Pathogenic strains of *Ustilago scitaminea* Sydow. Sugarcane Pathologists' Newsletter, 8: 12–13.

Muhammad, S. and Kausar, A.G. (1962). Preliminary studies on the genetics of sugarcane smut, *Ustilago scitaminea* Sydow. Biologia, 8: 65–74.

Peever, T.L., Zeigler, R.S., Dorrance, A.E., Correa-Victoria, F.J. and St. Martin, S. (2000). Pathogen population genetics and breeding for disease resistance. www.apsnet.org/online/feature/PathPopGenetics/Top.html

Presley, J. (1978). The culmicolous smut of sugar cane. Sugar y Azucar, 73(10): 34–39.

Pérez, L. and Mauri, F. (1983). *Ustilago scitaminea*, Sydow in Cuba: Biology, physiology and varietal reaction. Proc. Int. Soc. Sugar Cane Technol., 18(2): 778–795.

Riley, I.T., Jubb, T.F., Egan, B.T. and Croft, B.J. (1999). First outbreak of sugarcane smut in Australia. Proc. Int. Soc. Sugar Cane Technol., 23(2): 333–336.

Swofford, D.L. (1999). PAUP: Phylogenetic analysis using parsimony. Version 4.0b2. Sinauer Associates, Sunderland, Massachusetts.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M. (1995). AFLP: a new technique for DNA fingerprinting. Nuc. Acids Res., 23: 4407–4414.