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FINAL REPORT - SRDC PROJECT BSS258
ASSESSING THE IMPACT THAT PATHOGEN VARIATION HAS ON THE SUGARCANE BREEDING PROGRAM

by

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SUMMARY

In recent years, two fungal diseases of sugarcane have had a huge impact on the Australian sugar industry and the BSES-CSIRO breeding and pathology programs. Sugarcane smut, caused by *Ustilago scitaminea*, first appeared in Australia in 1998, whilst orange rust, caused by *Puccinia kuehnii*, was a minor pathogen until an epidemic devastated the industry in 2000. In the case of both pathogens, the industry had to respond to the outbreaks without a full understanding of the pathogen's population structure. Successful disease-management strategies and deployment of resistance require an understanding of variation in the pathogen population. This project has clarified the genetic makeup of the two pathogens and has identified a number of quarantine issues that could place the Australian sugar industry at risk.

Orange rust devastated the Australian sugar industry in 2000, causing estimated losses to growers of A$100-140 million and A$150-210 million to the industry as a whole. Because orange rust was, until 2000, a minor pathogen, there had been little basic research into the pathogen and the disease. At the start of the project, the conditions for spore storage, retrieval, germination and infection were not optimal. Conditions for short- and long-term storage for germination and DNA extractions have now been developed. Reliable spore germination conditions have been determined and incorporated into a contained detached-leaf inoculation system.

Circumstantial evidence suggested that the 2000 epidemic was due to the appearance of a new race, which may have developed in Australia or have been introduced from overseas. To determine if the source of the new race could be identified, a large collection of Australian field isolates was compared together, then a sub-sample of Australian isolates was compared to isolates from Papua New Guinea, Indonesia, China and historical herbarium collections using DNA-sequence data from three ribosomal DNA regions: the intergenic spacer (IGS), the internal transcribed spacer (ITS) and a portion of the large subunit (LSU).

Genetic diversity among 28 Australian field isolates, determined by sequencing the IGS, was very limited. This suggests that there is only a single dominant genotype present in the current population of orange rust in Australian commercial sugarcane fields. However, there was considerable genetic variation among the overseas rusts infecting wild *Saccharum* canes. All isolates thought to be orange rust clustered into three groups, and this clustering was supported by data from all three ribosomal DNA regions. All isolates from commercial canes grouped into cluster 1. Several Indonesian and PNG isolates separated into a second cluster, and rust isolated from *Erianthus* formed a third cluster. Isolates in clusters 2 and 3 appeared similar to 'typical' orange rust, based on symptoms and spore shape.

This study has shown that Australian orange rust is not unique and probably did not arise from an accidental introduction from overseas. It is possible that the new race appeared as a mutation in only a single gene. Host resistance should be effective across a wide range of isolates if the pathogen population is genetically uniform. This project has also shown that close to Australia are a number of diverse rusts infecting wild *Saccharum* canes. Any potential disease risks posed by these new pathogens to the Australian sugar industry are

unknown at this stage, but must be considered as a quarantine threat to the Australian sugar industry.

Sugarcane smut was reported for the first time in Australia in the Ord River Irrigation Area (ORIA) of Western Australia in July 1998. The most likely source of the smut incursion in the ORIA was wind-blown spores from Indonesia. Screening trials in Indonesia and the ORIA have shown that 75% of Australian varieties are susceptible to the local isolates. An earlier genetic diversity study based on amplified fragment length polymorphisms (AFLPs) showed that Indonesian and Western Australian isolates were identical, but genetically divergent isolates were present in Taiwan, Philippines and Thailand. These South East Asian countries, due to their proximity to Australia, represent a potential risk to the Australian sugar industry. Results from the initial study suggested that there was a need to assess the genetic diversity in a greater range of Asian isolates and a need to screen Q canes in areas where divergent isolates occur.

Two world collections of smut isolates were already available at Pacific Agri-Food Research Centre (PARC), Canada, and a third collection was assembled with isolates from Thailand, Malaysia, Vietnam, India, China and Hawaii. In this project, genetic diversity was detected by Diversity Arrays Technology (DArTs). DNA was assembled from 95 smut samples, including all 38 isolates from the first study and 46 isolates from the new collection. Isolates were derived from either germinated sporidia (haploid) or teliospores (dikaryon).

Both a Principal Component Analysis and a dendrogram clearly showed that only six isolates from Vietnam, China, Taiwan and the Philippines were distinctly different. As was found with AFLPs, the overall variation within the world population of sugarcane smut is surprisingly low. When results from the DArT and AFLP studies are considered together, China, Taiwan, the Philippines, Vietnam and Thailand were found to harbour diverse sugarcane smut isolates, compared to the rest of the world. The DArT study confirmed the earlier finding that Indonesian and Western Australian isolates are closely related.

These genetic diversity studies have indicated that South East Asia contains isolates that are genetically variable compared to the rest of the world. The resistance of Australian sugarcane varieties towards isolates of smut from this region was unknown. Originally, it was planned to send approximately 10 Australian varieties to PHILSURIN in the Philippines and the Taiwan Sugar Research Institute to determine their smut reaction to local isolates in those countries. However, Taiwan was replaced by the Yunnan Sugar Research Institute, China, as the second location for screening. The varieties chosen, based on their reaction in trials performed in the ORIA and Indonesia, were Q117, Q138, Q170$^{\Phi}$ (susceptible); Q183$^{\Phi}$, Q120, Q124, Q135, Q190$^{\Phi}$ (intermediate); and Q96, Q182$^{\Phi}$ and Q171$^{\Phi}$ (resistant). Q120 replaced Q96 in China. For both countries, the canes have passed through the appropriate quarantine procedures and will be planted in smut-resistance trials in 2006. When the results are available, the smut resistance of the varieties will be compared to their reaction in trials performed in the ORIA and Indonesia.

1.0 BACKGROUND

In recent years, two fungal diseases of sugarcane have had a huge impact on the BSES-CSIRO breeding program and have consumed considerable resources from the pathology and breeding teams. Sugarcane smut, caused by *Ustilago scitaminea*, first appeared in Australia in 1998, whilst orange rust, caused by *Puccinia kuehnii*, was a minor pathogen until an epidemic swept through the industry in 2000. In both situations, rapid responses had to be made, often without a full understanding of the pathogen's biology and population structure. This report describes work carried out to gain a better understanding of the pathogens in order to guide future decisions about disease control.

Orange rust, once considered a minor pathogen in the Australian sugar industry, was observed in the year 2000 on Q124, a cultivar previously considered resistant. At that time, Q124 was the major cultivar in Queensland, accounting for 45% of the crop. In the Central and Herbert regions, it accounted for 87% and 67%, respectively. The heavy reliance on Q124 meant that the 2000 orange rust epidemic had devastating consequences in those areas. 2000 was also a year for poor weather, and the two factors led to an estimated loss to growers of A$100-140 million and A$150-210 million to the industry as a whole. This makes the 2000 orange rust epidemic the most economically important in the history of the Australian sugar industry. The disease was managed in the short term with fungicides, until sufficient resistant replacement cultivars became available.

Because orange rust was, until 2000, a minor pathogen, there had been little basic research into the pathogen and the disease. Circumstantial evidence suggested that a new race had appeared. It had been observed that orange rust, common in Papua New Guinea (PNG), did not infect Q124, whereas in Indonesia, Q124 was affected by this pathogen. In addition, prior to the epidemic, orange rust was occasionally seen on Q78 in north Queensland but Q78 remained unaffected during the epidemic. The new race may have developed in Australia or it may have been introduced from overseas. To determine if the source of the new race could be identified, orange rust isolates from Queensland, Papua New Guinea, Indonesia and historical isolates maintained at the DPI&F Plant Pathology Herbarium were compared using DNA-sequencing techniques. A basic understanding of why this pathogen has changed from a minor to a devastating pest is important for preparing for future changes in the pathogen and in directing breeding for resistance to this new strain and any future strains of the pathogen.

Sugarcane smut was reported for the first time in Australia in the Ord River Irrigation Area (ORIA) of Western Australia in July 1998. The disease quickly spread through the susceptible cultivars NCo310 and Q117, which comprised 30% of the crop. Quarantine regulations were enacted in Queensland and New South Wales to reduce the risk of spread by plant material or appliances. Extensive surveys in Queensland and New South Wales failed to detect sugarcane smut. The most likely source of the smut incursion in the ORIA was wind-blown spores from Indonesia or possibly South Africa. In preparation for the possible appearance of smut in eastern Australia, BSES, in cooperation with the Indonesian Sugar Research Institute and the Sugar Research and Development Corporation, began a screening program on Madura Island. Results show that 75% of Australian varieties are susceptible to the smut isolates present in Indonesia. Breeding for smut resistance is underway and smut-resistant cultivars have been propagated in preparation for possible incursions.

A scoping study funded by the CRC for Tropical Plant Protection (CRC-TPP) and BSES carried out at the Pacific Agri-Food Research Centre (PARC), Summerland, Canada, measured the genetic diversity within a worldwide collection of isolates using amplified fragment length polymorphisms (AFLPs). A major aim of that study was to compare isolates from the ORIA with those from Indonesia and South Africa, and the choice of isolates reflected this. Results showed that the Indonesian and Western Australian isolates were identical. The results also revealed that genetically divergent isolates were present in Taiwan, Philippines and Thailand. These Southeast Asian countries, due to their proximity to Australia, represent a potential risk to the Australian sugar industry. The resistance of Australian cane cultivars towards these Asian isolates is completely unknown. These results suggested two areas of further work were needed:

1. Assess the genetic diversity in a greater range of Asian isolates;
2. Screen Q canes in areas where divergent isolates occur.

An understanding of the diversity in the fungus and the susceptibility of Q canes to these diverse strains will ensure that the Australian sugar industry is prepared for any further incursions of this serious pathogen.

2.0 OBJECTIVES

This project aimed to understand how genetic variation within two major pathogens of sugarcane, smut and orange rust, would affect the breeding program. The specific objectives were:

1. Use molecular markers to assess the genetic diversity within orange rust from Australia, Papua New Guinea, Indonesia and historical herbarium collections from Australia.
2. Extend a preliminary study on genetic diversity in sugarcane smut to focus on strains from Southeast Asia.
3. Use the information gained from the diversity studies to estimate the risk of new strains appearing and make recommendations to assist plant breeders.

Each objective was achieved as summarised below:

Objective 1. Use molecular markers to assess the genetic diversity within orange rust from Australia, Papua New Guinea, Indonesia and historical herbarium collections from Australia.

DNA-sequence data from three ribosomal DNA regions was used to estimate genetic diversity in orange rust collections. The final collection comprised 28 Australian isolates from sugarcane fields collected since 2002, one from an old commercial cultivar known to be susceptible to orange rust before 2000, one from wild cane growing in the Northern Territory, three historical isolates collected before the epidemic (from 1898, 1955 and 1961), four from Papua New Guinea, eight from Indonesia, and one from China. Initially, genetic diversity among all Australian isolates was determined by sequencing the intergenic spacer (IGS) region. The extent of variation among isolates was minor, with

only three isolates showed DNA base pair changes, compared to the consensus sequence. A sub-sample of field isolates was then compared to the historical, PNG, Indonesian, and Chinese isolates. Comparisons were based on sequence data from three ribosomal gene regions: the IGS, the internal transcribed spacer (ITS) and a portion of the large subunit (LSU).

Sequencing revealed considerable genetic variation among the overseas rusts infecting wild *Saccharum* canes. All isolates thought to be orange rust clustered into three groups and the clustering was supported by data from all three ribosomal DNA regions. All isolates from commercial canes grouped into cluster 1. Several Indonesian and PNG isolates separated into a second cluster. Rust isolated from *Erianthus* formed a third cluster. Isolates in clusters 2 and 3 appeared similar to 'typical' orange rust, based on symptoms and spore shape. Comparison to published rust sequences has revealed that there is greater diversity in sugarcane rusts than previously thought.

Objective 2. Extend a preliminary study on genetic diversity in sugarcane smut to focus on strains from Southeast Asia.

Diversity Arrays Technology (DArT) was used to assess diversity between 95 sugarcane smut samples. 112 polymorphic markers were used to screen the DNA displayed in an array format. The Asian countries represented in the study included China, Philippines, Taiwan, Vietnam, Thailand, Malaysia, Indonesia and India. The array of isolates included all 38 samples derived from haploid spores from the first study and 46 isolates from a new collection, all derived from germinated teliospores.

DArT fingerprinting revealed that overall there was very little genetic variation in the world smut population. However, six genetically diverse isolates from China, Philippines, Taiwan and Vietnam were detected. Isolates from Western Australia and Indonesia were closely related. These findings are in agreement with the earlier study using AFLPs as the molecular marker. Combining results from the two studies, diverse isolates have now been detected in China, Philippines, Taiwan, Vietnam and Thailand. Thus, the Indo-China region of eastern Asia appears to be an area of genetic diversity for sugarcane smut.

Objective 3. Use the information gained from the diversity studies to estimate the risk of new strains appearing and make recommendations to assist plant breeders.

The DNA-sequence data revealed limited genetic variation within the current population of orange rust isolates in commercial sugarcane fields. This suggests that there is only a single dominant genotype present in the field, and, if this is so, then host resistance should be effective across a wide range of isolates. The phylogenetic clustering grouped isolates from commercial and wild cane in Australia, China, PNG, Indonesia and from 1898. Thus, the orange rust in Australia is not unique and probably did not arise from an accidental introduction from overseas. It is possible that the new race appeared as a mutation in only a single gene, and such a change would not be detected by sequencing ribosomal genes. During epidemics, the presence of high amounts of inoculum in the environment can provide selection pressure for more races to appear. The level of resistance in the BSES-CSIRO breeding program is high and, since 2000, new resistant

cultivars have been deployed to replace Q124. Fortunately, there have not been reports of new outbreaks, indicating that resistance is holding well.

Considerable diversity was found within the PNG and Indonesian collection of rusts, indicating that close to Australia are a number of different rusts infecting wild *Saccharum* canes. The significance of three rust clusters is unknown at present. Based on symptoms and spore morphology, the rusts from wild *Saccharum* canes appear similar to 'typical' orange rust. Any potential disease risks posed by these new pathogens to the Australian sugar industry are unknown at this stage, but all must be considered as a quarantine threat.

The sugarcane-smut diversity work has shown that Indonesian and Australian isolates are closely related genetically. The resistance-screening results carried out in Indonesia and the ORIA have shown that Australian cultivars react comparably to local isolates in the two locations. Thus, both the genetic data and pathogenicity data confirm the close relationship between isolates from the two countries. As the most likely source of a smut incursion to eastern Australia is either Indonesia or the ORIA, the resistance screening results should be applicable to the smut disease pressure presently existing in Australia.

Both the AFLP and DArT studies have shown that Southeast Asia is an area of high risk for smut introductions to the Australian sugar industry. The reaction of Australian sugarcane varieties to isolates from these regions is unknown at present. Results from smut testing in China and the Philippines will be available in another 1-2 years. As only 10 Australian clones are being tested, this should be considered a preliminary study. Any large discrepancies between the ratings obtained in China and the Philippines, compared to results obtained in the ORIA and Indonesia, will indicate that a more comprehensive testing program is required.

3.0 ORANGE RUST

3.1 Introduction

The two most common rusts of sugarcane are brown rust, caused by *Puccinia melanocephala*, and orange rust, caused by *Puccinia kuehnii*. The two can be distinguished by spore colour, shape and ornamentation, and pustule colour. Orange rust is prevalent in humid summer conditions, and brown rust occurs more in the spring. Brown rust, formerly known as common rust, has been considered the more serious pathogen since its worldwide spread during the 1970s, whilst orange rust was considered a minor pathogen and confined to the Asian-Australian-Pacific region. Orange rust has been present in Australia since the 1880s. Because orange rust was a minor pathogen in the past, there has been little basic research into the pathogen and the disease in Australia and the amount of published literature is extremely limited. This lack of very basic information influenced the approaches that were taken during the molecular analyses.

3.2 Establish a collection of orange-rust isolates

3.2.1 Australian field isolates

Over 50 isolates of orange rust were collected from Queensland and NSW sugarcane fields by BSES and other industry staff from December 2001 until January 2004. Twenty-eight isolates were used in the genetic diversity study (Table 1). Approximately half of these isolates were from Q124 growing from Meringa to Ballina. The remaining 13 isolates were from 11 other cultivars.

3.2.2 Other Australian isolates

An orange-rust isolate from cultivar Q78 growing in the Tully Experiment Station museum block was included in the study. This cultivar is important because, prior to the 2000 epidemic, it was one of the few Australian canes susceptible to orange rust and yet at the height of the epidemic Q78 had very light infection, whereas Q124 close by was heavily infected (Magarey *et al.* 2001b). This suggested that the epidemic was caused by a new race of orange rust. Unfortunately, no rust from Q78 prior to 2000 has been preserved; the particular isolate included in this study was collected in 2004.

Wild sugarcane (*Saccharum spontaneum*) has been growing on the Daly River in the Northern Territory since at least 1946. Northern Australian Quarantine Strategy (NAQS) officers found orange rust on the cane in 2004 and forwarded a sample to BSES. This finding has particular interest to the ORIA, which is still free of orange rust.

3.2.3 Asia-Pacific isolates

A collection of rust isolates from Papua New Guinea, Indonesia and China was made by Rob Magarey and Barry Croft during disease surveys conducted as parts of projects for the Australian Centre for International Agricultural Research (ACIAR). A small number of samples were stored as dried leaves in $CaCl_2$ and brought to BSES under permit from AQIS. All remaining samples were stored as dry leaves, pressed between newspapers, or in paper bags at the DPI&F Herbarium, Indooroopilly. Samples were either heat-treated or irradiated to make the pathogens non-viable. Samples were identified as orange rust (*Puccinia kuehnii*), brown rust (*Puccinia melanocephala*) or simply 'rust', based on the symptoms observed when collected. Later microscopic examination of selected samples by Roger Shivas at the Herbarium confirmed some of them as orange rust and herbarium accession numbers were assigned to these. As the samples were subject to quarantine, no attempt was made to propagate the spores. The total number of PNG and Indonesian isolates at the herbarium is large, but only a subset of the total number could be included in the diversity study, due to problems experienced with DNA quality (described below).

3.2.4 Historical herbarium orange-rust isolates

Three historical samples of orange rust (collected in 1898, 1955 and 1961) were available at the DPI&F Plant Pathology Herbarium. These represent the only known samples in Brisbane from before the 2000 epidemic. Unfortunately, the DNA extracted from these samples was also of poor quality.

3.2.5 Other rusts

A small collection of brown rust was assembled during the project. This included one isolate from the Ord River Irrigation Area, representing the first report of brown rust in the ORIA. Sorghum rust (*Puccinia purpurea*) and corn rust (*Puccinia sorghi*) were also included for comparison.

3.3 Development of handling conditions

At the start of the project, the conditions for spore storage, retrieval, germination and infection for *P. kuehnii* were not known. Work was underway in BSES Tully to determine optimal conditions for disease development. Although the optimal germination temperature had been determined (Magarey *et al.* 2001a), reliable and consistent germination was still difficult. A contained system had to be developed for work in the biotechnology laboratory at BSES Indooroopilly, for quarantine reasons. A modified detached-leaf system based on that used for brown rust (Taylor 1992) was found to be the optimal method. After much trial and error, the handling conditions were optimized as:

Storage of viable spores for later inoculations

- Optimal:
  - Long-term storage: harvested spores stored at -70°C
  - Short-term storage (1-2 weeks): fresh leaves stored at room temperature (RT) in paper bags
- Conditions that give poor germination:
  - Dried spores remain viable for a few days and must be rehydrated first
  - Leaves standing in water or in plastic bags will produce viable spores, but contamination levels will be high
  - Leaves can be stored in the refrigerator for 1 day, but spores must be heat shocked first before they will germinate
- Conditions that give no germination:
  - Long-term dried leaves
  - Leaves or spores stored in the refrigerator for longer than one day

Storage of leaf material for DNA extractions (Spores do not need to be viable)

- Optimal: Infected leaves dried over $CaCl_2$
- Leaves dried over silica gel, leaves frozen at -70°C or harvested spores frozen at -70°C were also suitable
- Storage of leaves between sheets of newspaper (as is done in the herbarium) is extremely detrimental to good DNA quality

Spore germination test

Plastic P1000 pipette tip boxes were used as the humidity chambers. Distilled water (not tap water) was used in the bottom chamber to generate the humidity.

- Remove frozen spores from -70°C and heat shock (42°C for 2.5min)
- Remove the required aliquot of spores and return unused spores to the freezer
- Leave the spores in an open microfuge tube on the rack in the humidity box overnight at RT to rehydrate
- Suspend the spores in 100 µL water and spread on 1% water-agar slide
- Germination of stored spores may be improved if they are suspended in the germination stimulator 1-nonanol at 0.002%
- Slide is placed in the humidity box in the dark at 19-21°C for 5 hours or overnight
- Germinated spores on the slide (Figure 1) are observed under a microscope (without a coverslip)

Notes:

- The use of tap water in the base of the humidity box inhibits germination
- 1-nonanol is not necessary for fresh spores and has no effect on non-viable spores

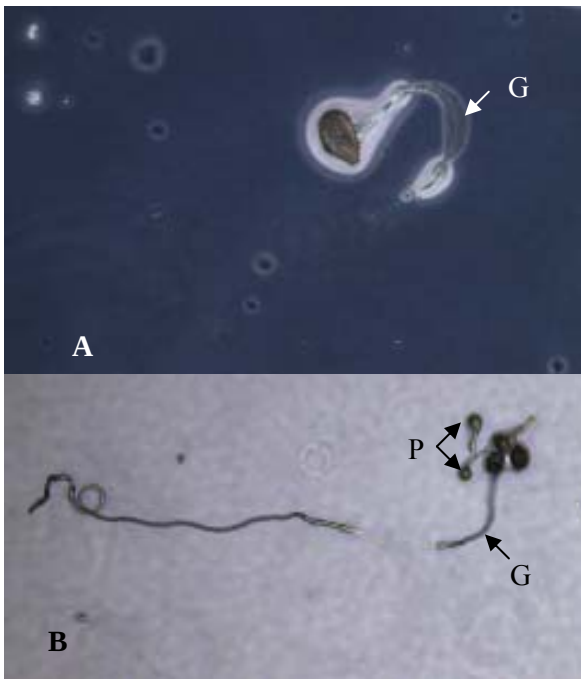

Figure 1 **Germinating rust spores showing the germ tube (G). A. Orange rust. B. Brown rust, can be distinguished from orange rust by the presence of parapheses (P).**

Detached-leaf inoculation set-up

- Select young, healthy, undamaged sugarcane leaves and cut into 11 cm pieces. Discard the base and top third of the leaf. Wash pieces under running water
- The leaf pieces are arranged abaxial side up on the rack in the pipette tip box and held in place with rubber bands
- Viable spores are heat shocked, hydrated and suspended in 100 µL water or 1-nonanol as described above
- Small (approx. 5 µL) drops of the spore suspension are placed on the leaf (Figure 2A)
- The closed box, with distilled water in the base, is kept in the dark at 19-21°C overnight
- The leaves are placed upright in a sealed 400 mL tube containing sterile water and left on a laboratory bench (Figure 2B)
- Pustules should appear where the droplets were placed after 10 days to 2 weeks (Figure 2C) and spores can be harvested with a toothpick from leaves observed under the stereo microscope

Notes:

- Leaf pieces with any physical or insect damage will succumb to fungal infection quickly
- The leaf tip wilts and senesces quickly so should not be used
- After 2 weeks in the tube, contaminating fungi will grow on the leaf

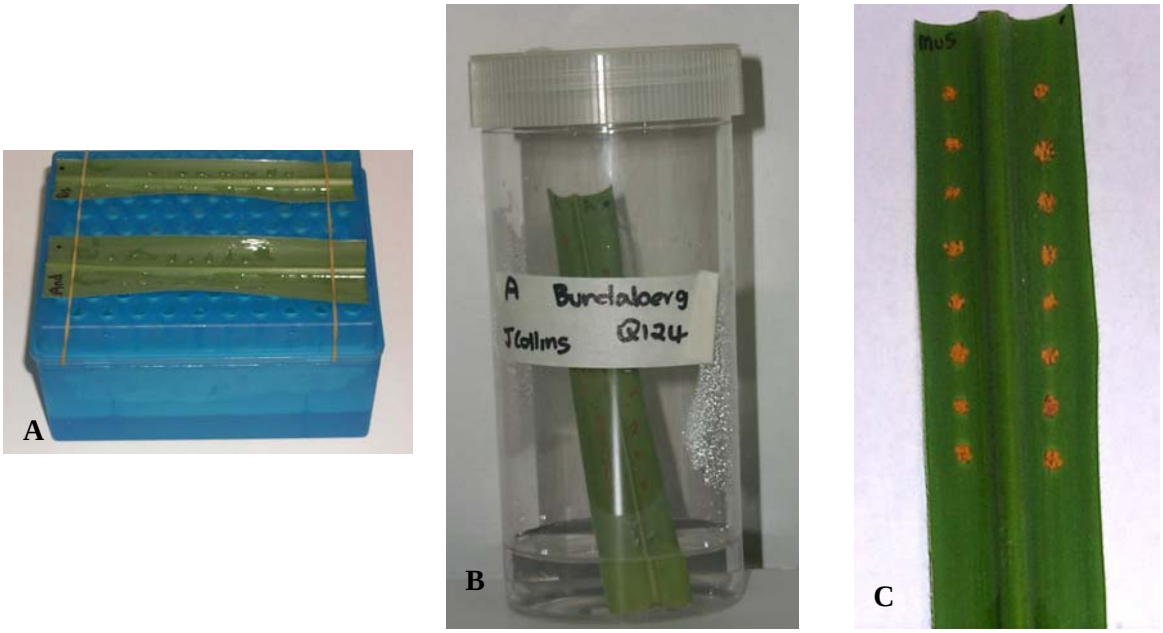

Figure 2 **Detached-leaf inoculation set up. A. Leaf pieces placed abaxial side up and inoculated with drops of a spore suspension. B. Inoculated detached leaves placed in water in a sterile tube. C. Orange rust pustules are visible.**

3.4 Choice of molecular-marker system

3.4.1 Australian field isolates

Rust fungi present certain challenges that affect the choice of molecular marker to be used. Rusts are biotrophic organisms, thus they are unable to be grown easily in axenic culture. Fungal DNA-fingerprinting studies usually rely on initially establishing a pure culture from a single spore. Establishing an infection from a single pustule can be considered an acceptable substitute to single-sporing.

Orange rust-infected sugarcane leaves collected from the field were infected with a wide range of organisms, including saprophytic fungi, rust hyperparasites such as *Cladosporium uredinicola, Darluca filum,* and *Trichothecium roseum,* and insect larvae from the family Cecidomyiidae that feed within rust pustules (Ryan and Egan 1989). The larvae could be removed by sieving harvested spores through a 58-µm sieve, but the contaminating fungi were usually transferred during inoculation experiments. The growth of contaminating organisms was reduced, but not eliminated, by: (i) ensuring that the leaves to be inoculated were healthy and undamaged; (ii) using a toothpick to select spores from a 'clean' pustule under the stereomicroscope; and (iii) minimising the time that the inoculated leaves were kept in the tubes.

The initial work plan proposed the use of random fingerprinting techniques, such as rep-PCR (repetitive sequence-based polymerase chain reaction; Versalovic *et al.* 1994) for assessing variation among Australian field isolates. Unfortunately, it was found that every field sample generated a different fingerprint, due to contaminating organisms found in association with the rust pustules. DNA-fingerprinting techniques have been used successfully to assess variation in populations of other rust pathogens (eg Keiper *et al.* 2003), but require adequate quantities of pure, single pustule-derived material for DNA extractions. The reliable germination conditions described in section 3.3 were not determined until the summer of year 2, and, by the winter of 2004, only eight single pustule-derived spore collections had been generated. In order to progress the project, it was apparent that a fingerprinting method specific to rust fungi, and not contaminants, was required quickly. It was decided that cloning and sequencing of ribosomal coding regions would have to be used.

3.4.2 Overseas and herbarium isolates

Overseas material could not be propagated in Australia for quarantine reasons and the herbarium samples were in poor condition and no longer viable. For these reasons, the initial work plan did not propose the use of random fingerprinting techniques for these isolates.

3.4.3 The ribosomal genes

The nuclear-encoded ribosomal-RNA genes (rDNA) of fungi are used widely in phylogenetic and population genetic studies. The genes exist in multiple copies, arranged head to toe. Each repeat unit contains coding regions (small subunit (SSU, 18S), large subunit (LSU, 25-28S) and 5.8S), internal transcribed spacers (ITS), and intergenic spacers (IGS) between the units (Figure 3). Most Basidiomycetes and some Ascomycete

yeasts also contain the 5S gene within the repeat unit. Other fungi have the 5S gene on another chromosome. The spacer regions are more variable than the conserved coding regions and, hence, are the best target for assessing intraspecific variation.

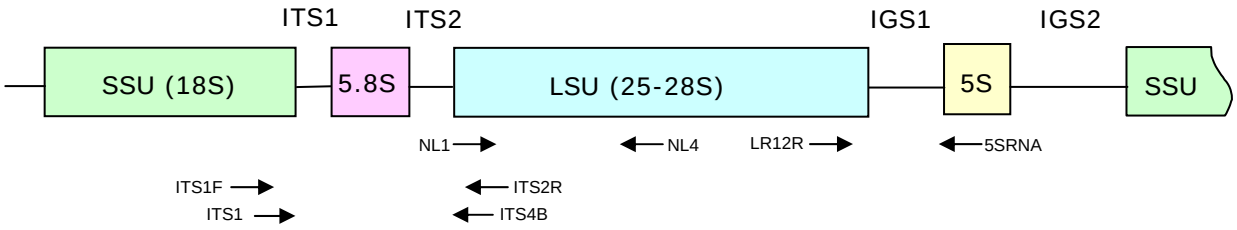

Figure 3 **Schematic representation of fungal ribosomal DNA genes (rDNA). The small subunit (SSU), 5.8S and large subunit (LSU) genes are separated by internal transcribed spacers (ITS), whilst individual repeat units are separated by intergenic spacers (IGS). The 5S gene is usually present in Basidiomycetes. The approximate locations of the primer combinations used in this study are shown.**

A pilot study was carried out to determine if the ribosomal spacer regions would be useful targets for sugarcane orange rust. Three sets of universal primers were tested. The strategy was to amplify PCR products from mixed DNA templates (rust-infected sugarcane leaves), clone them into the Promega pGEM-T Easy vector using the manufacturer's instructions, and transform into *E. coli*. Two replicate plasmids were sequenced using Big Dye 3.1 (ABI Prism) at the Australian Genome Research Facility (AGRF). The putative identity of the sequences was determined from BLASTN searches of databases using programs provided by the Australian National Genomic Information Service (ANGIS; http://www.angis.org.au).

The IGS primer pair (LR12R / 5SRNA; James *et al*. 2000) showed good specificity, clear bands, and the PCR products were identified as being from Basidiomycetes. Two ITS primer sets (ITS1 / ITS4; White *et al*. 1990 and ITS1F / ITS4B; Gardes and Bruns 1993) consistently gave non-specific amplification from rust-infected leaves. Thus, the initial cloning and sequencing of the Australian field isolates was based on the IGS region, which appeared to be a more robust technique for amplifying orange-rust DNA.

3.4.4 DNA extraction methods

DNA was extracted from the majority of rust isolates, using the BIO101 FastPrep kit. This method was able to extract sufficient amounts of pure DNA from spores, and fresh, frozen or $CaCl_2$ dried infected leaves.

Small quantities of crude DNA were extracted from single pustules of several field samples using the method of Virtudazo *et al*. (2001). This DNA was suitable for PCR amplification of ribosomal genes, but was not of sufficient quality for fingerprinting techniques such as REP-PCR.

Initially the herbarium samples were extracted using the BIO101 FastPrep kit. It proved to be extremely difficult to amplify from DNA extracted from some herbarium samples, particularly when using the ITS primers, and this delayed progress on the project. A number of fungal DNA-extraction techniques were trialled. Many of the methods, especially those involving CTAB, were not successful. A method based on a Tris/EDTA/SDS extraction buffer developed by Bruns *et al.* (1990) was more successful. However, some herbarium samples were still difficult to amplify (described later).

3.5 Genetic variation within Australian field isolates based on IGS sequencing

The IGS primer pair LR12R/5SRNA (James *et al*. 2001) are designed to recognise the large subunit and 5S genes and amplify across the spacer region known as IGS1 (Figure 3). Basidiomycetes have the 5S gene within the RNA operon, but many other fungi have the 5S gene on another chromosome. Thus, if the 5SRNA primer is able to successfully bind and generate a PCR product with LR12R, it is a good indication that the template is a Basidiomycete. The IGS has been reported to show more inter- and intra-specific variability that the ITS (James *et al.* 2001; Roose-Amsaleg *et al.* 2002). For these reasons, the IGS was initially chosen as the target region for the 28 Australian field isolates (Table 1). The final PCR reagent mix after optimisation was $MgCl_2$ 3mM, dNTPs 200µM each, primers 0.2µM each, *Taq* DNA polymerase (Fisher Biotech) 1.65 units and 10x buffer supplied with the enzyme, in 25 µL. PCR cycling conditions were: 95°C for 5 min; 35 or 45 cycles of 95°C for 30 sec, 50°C for 30 sec, 72°C for 1 min; followed by 72°C for 10 min. Cloning and sequencing was as described in section 3.4.

The LR12R/5SRNA fragment amplified from Australian orange rust was usually either 848 bp or 833 bp; the difference due to a variable number of 15 bp repeat sequences within the spacer (Figure 4). Seven copies of the repeat were most common, followed by six copies, with five copies observed only once. For 11 of the 28 fungal isolates, the two replicate clones sequenced differed in the number of repeats. This highlights the importance of sequencing replicates, otherwise, variation among isolates would have been overestimated. Length polymorphisms due to the presence of repeats have also been observed in the IGS of *Puccinia striiformis* f.sp. *tritici* (Roose-Amsaleg *et al.* 2002) and *P. graminis* f.sp. *tritici* (Kim *et al.* 1992) and many other basidiomycetes. The presence of two versions of the IGS within one isolate is suggested to be due to the dikaryotic nature of rust fungi, with the two nuclei heterozygotic for two different IGS types (Roose-Amsaleg *et al.* 2002).

Aside from the length polymorphism described above, the extent of variation among 28 Australian field isolates of orange rust collected since 2002 was minor, as only three isolates (shown as *** in Table 1) showed single base-pair changes in both replicates, compared to the consensus sequence. Minor base changes are most likely due to PCR errors. A low level of variation was also observed with *Puccinia striiformis* f.sp. *tritici*, believed to be due to strict asexual reproduction (Roose-Amsaleg *et al.* 2002). In contrast, Kim *et al.* (1992) found that races of *P. graminis* f.sp *tritici* could be distinguished by IGS variation and this high level of polymorphism was possibly due to sexual recombination before the eradication of barberry, the alternate host plant. Teliospores of orange rust are rarely present (Ryan and Egan 1989). This suggests that the single IGS consensus

sequence amplified from the 28 isolates represents the single dominant genotype of orange rust present in Australia since the 2000 epidemic. A single mutation may have occurred to produce a race virulent to Q124. This new genotype then spread rapidly throughout the industry during the epidemic by asexual spores (uredospores).

An alternative explanation for the low level of variation identified in this study is that the IGS represents only a small fragment of the entire genome and polymorphisms within this region may not correlate with differences in the virulence genes. Thus for this fungus, IGS sequencing was not able to distinguish closely related isolates. Random fingerprinting techniques, such as rep-PCR, have the advantage of widely sampling the organism's genome. As described above, this could not be done without single-spore-derived isolates.

Table 1 The 28 Australian field isolates used in the genetic variation study. The DNA sequence of the ribosomal intergenic spacer region (IGS) was determined. Only three isolates (marked *) differed by one base pair change.**

Isolate	Host cultivar	Location	Other details	Collector	Date
Q124-Meri	Q124	Meringa		A. Rizzo	8-May-02
Q124-Tul	Q124	Tully	Cyclone spore trap	W.Neilsen	7-Mar-02
Q124-Burd***	Q124	Burdekin	Cyclone spore trap	W.Neilsen	7-Mar-02
Q124-Ing	Q124	Ingham		S.Brumbley	27-May-02
Q124-Mack1	Q124	Mackay		T.Staier	6-Dec-01
Q124-Mack2***	Q124	Mackay	M10 few pustules	T.Staier	21-Jan-03
Q124-Mack3	Q124	Mackay	M11 one pustule	T.Staier	21-Jan-03
Q124-Mack4	Q124	Mackay		T.Staier	28-Jan-04
Q124-Isis***	Q124	Isis	Kingston	B.Croft	1-Mar-02
Q124-Fair	Q124	Fairymead	Charlie Francis	J.Collins	3-Feb-03
Q124-Eum	Q124	Moreton	Eumundi	T.Blatch	4-Feb-03
Q124-Bli	Q124	Moreton	Ron Clarkson, Lefos Rd, Bli Bli	T.Blatch	29-Jan-03
Q124-Kal	Q124	Kallangar	Wegner	B.Croft	5-Mar-02
Q124-Cond	Q124	Condong	Stainlay	P.McGuire	30-Jan-03
Q124-Ball	Q124	Ballina	Anderson 4345 Block 20 Newrybar	A.Cattle	14-Feb-03
Q137-Cond	Q137	Condong	Stainlay	P.McGuire	30-Jan-03
Q141-Fair	Q141	Fairymead	DK & HM Schulte SP 8-A	D.Bush	30-Jan-02
Q141-Bund	Q141	Bundaberg	KG Plants A. Read	D.Bush	11-Apr-03
Q151-Mack	Q151	Mackay		D.Appo	15-Apr-02
Q151-Fair	Q151	Fairymead	AP mother plot	D.Bush	30-Jan-02
Q155-Fair	Q155	Fairymead	AP mother plot	D.Bush	30-Jan-02
Q170-Fair	Q170$^{\phi}$	Fairymead	R.Tucker	B.Croft	1-Mar-02
Q174-Mack	Q174$^{\phi}$	Mackay		D.Appo	15-Apr-02
Q177-Mack	Q177$^{\phi}$	Mackay		D.Appo	15-Apr-02
Q182-Bing	Q182$^{\phi}$	Bingera	Bundaberg Sugar	J.Collins	3-Feb-03
Q189-Mack	Q189$^{\phi}$	Mackay		D.Appo	15-Apr-02
Q205-Bund	Q205$^{\phi}$	Bundaberg	KG Plants	D.Bush	11-Apr-03
RB72-Bal	RB72-454	Ballina	Risso 4322 Block 12 Newrybar	A.Cattle	14-Feb-03

```
           466                                                       525
124_Eum1   ATTCTTACTTTAGGG CTATTTACTTTAGGG CTATTTACTTCAGGG CTATTTACTTCAGGG
124_Eum2   ATTCTTACTTTAGGG CTATTTACTTTAGGG CTATTTACTTCAGGG CTATTTACTTCAGGG

124_Fair3  ATTCTTACTTTAGGG CTATTTACTTTAGGG CTATTTACTTCAGGG CTATTTACTTCAGGG
124_Fair4  ATTCTTACTTTAGGG CTATTTACTTTAGGG CTATTTACTTCAGGG CTATTTACTTCAGGG

124_Kall1  ATTCTTACTTTAGGG CTATTTACTTTAGGG CTATTTACTTCAGGG CTATTTACTTCAGGG
124_Kall2  ATTCTTACTTTAGGG CTATTTACTTTAGGG CTATTTACTTCAGGG CTATTTACTTCAGGG

141_Bund2  ATTCTTACTTTAGGG CTATTTACTTTAGGG CTATTTACTTCAGGG CTATTTACTTCAGGG
141_Bund3  ATTCTTACTTTAGGG CTATTTACTTTAGGG CTATTTACTTCAGG. ...............

           526                                       570
124_Eum1   CTATTTACTTCAGGG CTATTTACTTTAGGG TTAATTACTTTAGGC
124_Eum2   CTATTTACTTCAGGG CTATTTACTTTAGGA TTAATTACTTTAGGC

124_Fair3  CTATTTACTTTAGGG ............... TTAATTACTTTAGGC
124_Fair4  CTATTTACTTTAGGG ............... TTAATTACTTTAGGC

124_Kall1  CTATTTACTTCAGGG CTATTTACTTTAGGG TTAATTACTTTAGGC
124_Kall2  CTATTTACTTTAGGG ............... TTAATTACTTTAGGT

141_Bund2  CTATTTACTTTAGGG ............... TTAATTACTTTAGGC
141_Bund3  CTATTTACTTTAGGG ............... TTAATTACTTTAGGC
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Figure 4 **Examples of variation in the number of 15 bp sub-repeats within the IGS. Q124-Eumundi replicate plasmids 1 and 2 both have seven copies. Q124-Fairymead replicate plasmids 3 and 4 both have six copies. Q124-Kallangar replicate plasmids 1 and 2 have seven and six copies respectively, while Q141-Bundaberg plasmids 2 and 3 have six and five copies, respectively.**

3.6 Genetic variation within brown rust based on IGS sequencing

Although a genetic analysis of brown rust (*Puccinia melanocephala)* was not part of the original project proposal, several unusual outbreaks of rusts other than orange rust occurred during the life of the project. The first incident involved the appearance of brown rust on the cultivar Q157, previously thought to be resistant. The second, more important incident, was the appearance, for the first time, of brown rust in the ORIA. The third report was of rust on corn from farms in the Bundaberg area. Some farmers using corn in crop rotation observed the rust and were concerned that brown rust had transferred to a new host. Field staff who reported the problems collected samples and sent them to Indooroopilly. Other samples of brown rust and sorghum rust were included for comparison (Table 2). The IGS was PCR amplified, cloned and sequenced in the same way as the orange-rust samples.

Table 2 Collections of brown rust, corn and sorghum rusts used in this study. Isolates were compared by amplifying, cloning and sequencing the ribosomal intergenic spacer region.

Host	Location	Notes	Collector	Date
Brown rust				
Q157	Proserpine	First record on Q157	T.Staier	5-Aug-03
Q157	South Mackay		T.Staier	7-Oct-03
Q99	ORIA	First record in ORIA	B.Webb	28-Mar-04
Q190[Φ]	Proserpine		T.Staier	5-Aug-03
Q102	Woodford		B.Croft	8-Jan-04
Unknown	Univ of Qld		S.Hermann	Jan 2002
Other rusts				
Corn	Bundaberg	common corn rust, *Puccinia sorghi*	T.Linedale	24-Sep-03
Sorghum	Univ of Qld	sorghum rust, *Puccinia purpurea*	S. Hermann	Jun-03

In contrast to orange rust, the size of the LR12R/5SRNA fragment amplified from brown rust was only 781 bp. Variation among the six isolates was minor. Only the Q99 isolate showed a single base-pair change in both replicates, compared to the consensus sequence. Thus, the sequencing results confirm that the new outbreaks on Q157 and in the ORIA were caused by brown rust isolates similar to those already present in Australian sugarcane fields.

The size of the LR12R/5SRNA fragments amplified from corn and sorghum rusts were 786 bp and 724 bp, respectively. The sequence data clearly showed that the rust on corn was not brown rust (Figure 5). Microscopic examination of the spores confirmed this. The four rusts studied here, orange, brown, corn and sorghum, were all clearly distinguished by IGS sequence data.

3.7 Comparisons among Australian, overseas and historical rusts samples

A sub-sample of eight Australian orange-rust isolates was compared to isolates from overseas (Indonesia, PNG, China), historical isolates from before the epidemic and two other Australian isolates (from a museum collection and from wild cane) and a brown-rust isolate. This final collection comprised 27 isolates (Table 3). Variation among these isolates was assessed from sequence data from the IGS, ITS and LSU ribosomal DNA regions.

The findings presented in this report were compared to those published by Japanese researchers (Virtudazo *et al*. 2001) who sequenced the ITS and a fragment of the LSU from a collection of Japanese "*Puccinia* sp." (PSP) isolates and compared them to herbarium orange-rust (PKU) samples. Based on ITS sequences, the Japanese isolates fell into two clusters (ITS Group I and II), despite appearing morphologically similar and occurring in similar locations. In contrast, the LSU data clustered all Japanese isolates together. Virtudazo *et al*. (2001) concluded that the presence of two Japanese ITS clusters but only one Japanese LSU cluster was due to the LSU being more conserved than the spacer region. They suggested that the two clusters represent morphologically indistinguishable, but reproductively isolated, sympatric species within *P. kuehnii*. The availability of the Japanese sequences on GenBank provided an opportunity to compare

the isolates collected in this project to the published Japanese sequences. The published sequences used in this study are listed in Table 4.

Sequence data were aligned using ECLUSTALW (Thompson *et al.* 1994, supplied by ANGIS) and relationship trees prepared using Neighbour Joining with 1000 bootstraps (supplied by Mega 2.1, Kumar *et al.* 2001).

Table 3 Collection of isolates used to compare Australian orange rust to overseas and historical rusts by ribosomal-DNA sequencing.

Isolate	Host	Location	Collector	Date	Storage method prior to DNA ext
Field Australia					
Q124-Burd	Q124	Burdekin	W.Neilsen	7-Mar-02	Frozen spores
Q124-Cond	Q124	Condong	P.McGuire	30-Jan-03	Silica gel dried leaf
Q124-Mack4	Q124	Mackay	T.Staier	28-Jan-04	Frozen spores
Q124-Tul	Q124	Tully	W.Neilsen	7-Mar-02	Frozen spores
Q141-Bund	Q141	Bundaberg	D.Bush	11-Apr-03	Silica gel dried leaf
Q155-Fair	Q155	Fairymead	D.Bush	30-Jan-02	Frozen spores
Q177-Mack	Q177	Mackay	D.Appo	15-Apr-02	Fresh spores
RB72-Bal	RB72-454	Ballina	A.Cattle	14-Feb-03	Silica gel dried leaf
Other Australia					
Q78-Mus	Q78	Museum Tully Station	J.Bull	30-Mar-04	Frozen spores
DalyR	*S. spontaneum*	Alligator Bend, Daly River, NT	P.Stephens	21-Oct-04	$CaCl_2$ dried leaf
Historical					
Aust-1898		Brisbane	H Tryon	1898	Herbarium
Aust-1955		Bundaberg	?	1955	Herbarium
WS-1961		Savaii, Western Samoa	A Johnston	13-Jun-61	Herbarium
PNG					
PNG-37	*S. edule*	Jayapura	R. Magarey	May-01	Herbarium
PNG-75	*S. officinarum*	Wewak	R. Magarey	May-01	Herbarium
PNG-143	*S. robustum*	New Britain	R. Magarey	May-01	Herbarium
PNG-214	*S. edule*	Alotau	R. Magarey	May-01	Herbarium
Indonesia					
Ind-312	*S. officinarum*	Lewa-Paku (Sumba)	B.Croft	1-Jun-02	$CaCl_2$ dried leaf
Ind-322	*S. spontaneum*	Kelimutu (Flores)	B.Croft	Jun-02	Herbarium
Ind-337	Hybrid	Utan-Sabiduk (Sumbawa)	B.Croft	Jun-02	Herbarium
Ind-338	Hybrid	Utan-Jorok/Sugar Mill	B.Croft	6-Jun-02	$CaCl_2$ dried leaf
Ind-345	*S. officinarum*	Kopang Jelunjuk (Lombok)	B.Croft	7-Jun-02	$CaCl_2$ dried leaf
Ind-346	*Erianthus*	Anjani (Lombok)	B.Croft	Jun-02	$CaCl_2$ dried leaf
Ind-355	*S. spontaneum*	Sanur (Bali)	B.Croft	Jun-02	Herbarium
Ind-Lam		Lampung (Sumatra)	Irawan	29-Feb-04	$CaCl_2$ dried leaf
China					
China	YT94-128	Zhanjiang China	B.Croft	16-Sep-03	$CaCl_2$ dried leaf
Brown rust					
BR102W	Q102	Woodford	B.Croft	8-Jan-04	Frozen spores

Table 4 GenBank sequences from Virtudazo *et al*. (2001) used in this study.

Isolate	Location	Date	Identity according to Virtudazo *et al.*	LSU GenBank Accession and cluster group	ITS GenBank Accession and cluster group
BPI-79612	Australia	1935	*P. kuehnii*	PKU 296524 Group I	PKU 406048 Group I
BPI-79624	Hawaii	1916	*P. kuehnii*	PKU 296525 Group I	PKU 406049 Group I
TSH-R11108	Japan	1996	*Puccinia* sp.	PSP 296534 Group I	PSP 406058 Group II
TSH-R11129	Japan	1996	*Puccinia* sp.	PSP 296535 Group I	PSP 406059 Group II
TSH-R11403	Japan	1996	*P. melanocephala*	PME 296543	PME 406069
TSH-R11407	Japan	1996	*P. melanocephala*	PME 296544	
TSH-R11402	Japan	1997	*P. melanocephala*		PME 406064

3.7.1 Genetic variation within rust isolates based on IGS sequencing

The IGS region was amplified, cloned and sequenced as described section 3.5. Two replicate clones were sequenced where possible. As described in section 3.5, the entire PCR product amplified by LR12R/5SRNA from Australian field isolates is 833-848 bp. However, as the primer binding sites are within the large subunit and 5S genes respectively, the sequences were edited so that only the spacer sequence data (approximately 500 bp in Australian isolates) were used in the phylogenetic analysis.

The results show that all isolates thought to be orange rust clustered into three groups (Figure 5). Within each cluster, variation among different isolates was in the same range as variation between replicates. The largest cluster (IGS Cluster 1) contained all modern Australian field isolates. The historical Australian samples, the Q78 isolate, the wild *S. spontaneum* isolate from the NT, the Chinese isolate and several Indonesian and one PNG isolate also clustered in this group. The second cluster (IGS Cluster 2) contained several Indonesian and PNG isolates and the Western Samoan isolate from 1961. There is no apparent correlation between either host or location to explain this cluster, but no isolates from commercial hybrid canes were in this cluster. The isolate from *Erianthus* (Ind-346) fell into a third, unique cluster (IGS Cluster 3). Three replicate clones from two DNA extractions were sequenced to confirm this result. No Indonesian or PNG isolate in this study clustered with brown rust. Brown and orange rusts were clearly distinguished from each other. Based on IGS sequences, brown rust appears more closely related to sorghum rust than to orange rust.

3.7.2 Genetic variation within rust isolates based on LSU sequencing

The ribosomal large subunit is a large coding region comprising various domains. Coding regions are generally more conserved than spacer regions (such as the ITS and IGS). However, the D1/D2 domain at the 5' end of the LSU is considered a relatively variable region within the LSU. This region was amplified using the primers NL1 / NL4 (Figure 3), the PCR reagent mix described in section 3.5, and the PCR program described in Virtudazo *et al*. (2001). PCR products were easily generated from most DNA templates, including those extracted from the herbarium samples, but were smaller than expected. BLASTN database searches indicated that these products were not from rust fungi. Considerable screening of plasmid minipreps had to be done to select products that did match rust and were the correct size (699 bp for clusters 1 and 3, 660 bp for cluster 2). All samples in Table 3 were eventually cloned, except for PNG-75. Cloning, sequencing

and phylogenetic analysis was as described above for the IGS. Selected GenBank accessions from Virtudazo *et al*. (2001) were included in the analyses.

The clustering pattern generated for LSU sequences is very similar to that seen for the IGS sequences, in that three major 'orange rust' clusters are present (LSU Clusters 1, 2 and 3; Figure 6). However, there is an unexpected addition to LSU cluster 3. Both replicates of the Australian 1955 isolate now cluster with the *Erianthus* isolate (Ind-346). PNG-37 now falls into cluster 1, although it was in IGS cluster 2.

The Japanese PSP and PKU sequences have not unexpectedly grouped into cluster 1. Virtudazo *et al*. (2001) suggested that the PSP and PKU sequences clustered together because the LSU is a conserved coding region and the differences among those isolates must be minor. As this study could identify three groups of orange rust that could be clearly distinguished by LSU sequencing, the differences among the isolates in these clusters must be very robust.

3.7.3 Genetic variation within rust isolates based on ITS sequencing

Development of PCR conditions for the ITS

As described in section 3.4, the ITS primers, ITS1, ITS1F, ITS4 and ITS4B (White *et al.* 1990; Gardes and Bruns 1993) consistently gave non-specific amplification from rust-infected leaves. The original intention was to replicate the primer combination (ITS1F/ITS4) and PCR conditions used by Virtudazo *et al.* (2001). However, they used DNA extracted from spores, not from infected leaves. BLASTN searches showed that Basidiomycete sequences were being amplified, but they did not match rust fungi.

Fortunately, the reverse primer is located within the LSU D1/D2 region. D1/D2 sequences generated from LSU Cluster 1 and 2 isolates (in section 3.7.2) were aligned and compared to GenBank sequences from a range of other rusts and Basidiomycestes. A series of new primers (ITS 1R, 2R, 3R and 4R) specific to rusts were designed. Primer ITS2R (5'TTC AAT GAT GCT GCT TTT CAA C3') in combination with ITS1F (Figure 3) was found to give more consistent results. Using this primer combination, the PCR reagent mix described in section 3.5 and the PCR program described in Gardes and Bruns (1993) (except that all annealing temperatures were 45°C), clear single bands were obtained for DNA extracted from spores and leaves dried with $CaCl_2$ or silica gel. Cloning, sequencing and phylogenetic analysis was as described previously. Selected GenBank accessions from Virtudazo *et al.* (2001) were included in the analyses.

PCR amplification from the herbarium samples was difficult and surprisingly this was not related to the length of time in the herbarium (ie the 2002 samples were as difficult to work with as the historical samples). It appears that the slow drying of leaves between newspaper leads to degradation of the DNA. The rapid drying that results from the use of $CaCl_2$ gives much better quality DNA. To obtain ITS sequence results from the herbarium samples, a combination of approaches was needed:

1. A DNA extraction method based on a Tris/EDTA/SDS extraction buffer (Bruns *et al.* 1990) gave good results for some samples. Diluting the DNA 1/5 was also beneficial.
2. The Corbett Gradient PCR machine, which allows a gradient of annealing temperatures to be used in a single PCR run, was used to determine that the optimal

annealing temperature was 49°C. The program described in Gardes and Bruns (1993) was modified accordingly, and the total number of cycles was increased to 60.

3. A nested-PCR approach was developed to increase the sensitivity of the PCR. The first round PCR used ITS1F/ITS2R as described above. Then 1 µL of the first-round product was amplified with ITS1/ITS4B (Figure 3) at 58°C for 60 cycles.
4. PCR enhancers were trialled, and it was found that 1M Betaine (Sigma) was beneficial.

Using a combination of these four approaches, most of the herbarium samples could be amplified, cloned and sequenced. However, despite many attempts, no orange-rust products could be amplified from the 1898 sample.

Results from ITS sequencing

The clustering pattern generated for ITS sequences differs slightly from that seen for the IGS and LSU sequences. While clusters 1 and 2 are still distinct, cluster 3, containing the *Erianthus* isolate, is now a subgroup of cluster 1 (Figure 7). PNG-75 and PNG-37 fall into ITS cluster 1, although they were in IGS cluster 2.

The results of Virtudazo *et al.* (2001) were not replicated here. The PSP isolates that grouped into Group II in Virtudazo *et al.* (2001) fall into Cluster I in this study. The PKU isolates used by Virtudazo *et al.* (2001) were herbarium *P. kuehnii* isolates from Australia (collected in 1935) and Hawaii (collected in 1916) and formed Group I. No sequence from this study clustered with those sequences. Figure 7 shows that the PKU sequences are very unrelated to all the other sequences in this study including brown rust. Based on the difficulties encountered in amplifying rust sequences with universal ITS primers, it is possible that the Group I sequences amplified by Virtudazo *et al.* (2001) were from other organisms. In fact, the highest BLASTN matches for the PKU sequences was to the rust *Cronartium* and not *Puccinia* as Virtudazo *et al.* (2001) expected.

3.7.4 Discussion

The clustering patterns observed for all isolates are summarised in Table 5. The main finding is that there is extensive genetic variation within the rusts infecting sugarcane and its relatives. Three very distinct groups of isolates comprise 'orange rust'. This clustering is supported by sequence data from three ribosomal DNA regions: two spacers (ITS and IGS) and a coding region (LSU). As the LSU coding region is more conserved than the spacers, this suggests that the genetic differences are real.

Three isolates (Aust-1955, PNG-37 and PNG-75) did not give the same clustering pattern across the three ribosomal regions (Table 6). These isolates could contain more than one form of ribosomal gene in each of the dikaryotic nuclei, as described in section 3.5. Unlike the 15 bp repeat difference observed within the IGS of Australian isolates (and all other isolates in IGS cluster 1), the differences among the three clusters are extensive. The greatest difference is between IGS cluster 1 and 2, which differ in length by 152-167 bp. In this case, the dikaryotic nuclei must contain very distinctly different ribosomal types, or it is possible that the clustering patterns observed for Aust-1955, PNG-37 and PNG-75 are due to the presence of more that one rust on the leaves.

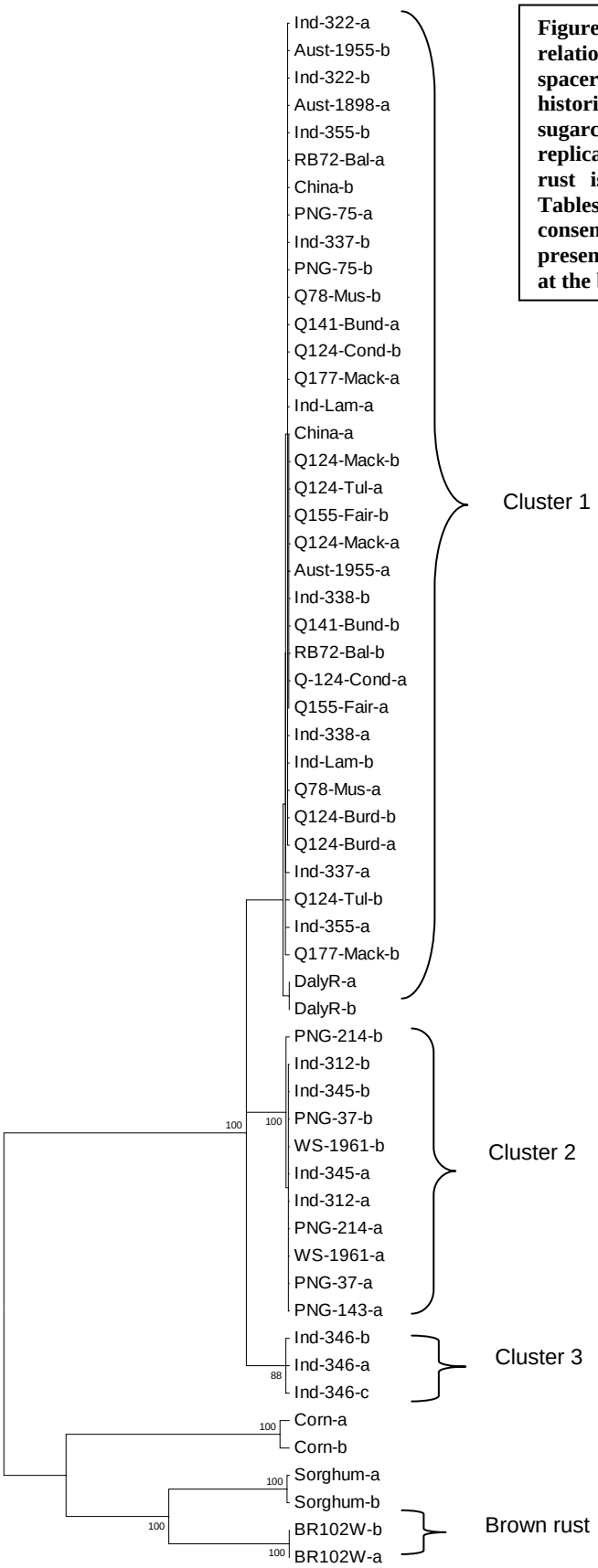

Figure 5 Dendrogram depicting the relationships between ribosomal intergenic spacer (IGS) sequences from Australian, historical, Indonesian, PNG and Chinese sugarcane rust isolates. One to three (a, b, c) replicate plasmids were sequenced for each rust isolate. Isolate codes are explained in Tables 2 and 3. The Neighbour Joining consensus tree from 1000 bootstrap data sets is presented. Bootstrap values >80% are shown at the branch points.

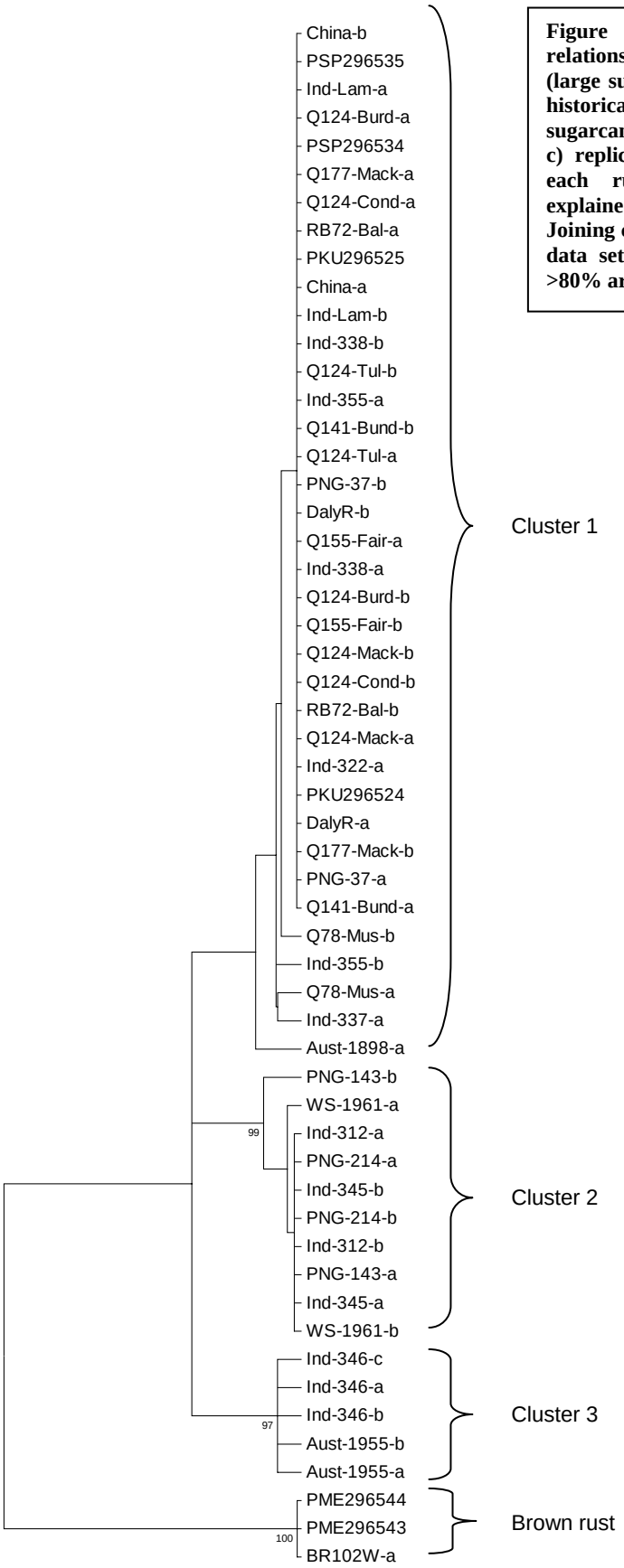

Figure 6 Dendrogram depicting the relationships between ribosomal D1/D2 (large subunit) sequences from Australian, historical, Indonesian, PNG and Chinese sugarcane rust isolates. One to three (a, b, c) replicate plasmids were sequenced for each rust isolate. Isolate codes are explained in Tables 3 and 4. The Neighbour Joining consensus tree from 1000 bootstrap data sets is presented. Bootstrap values >80% are shown at the branch points.

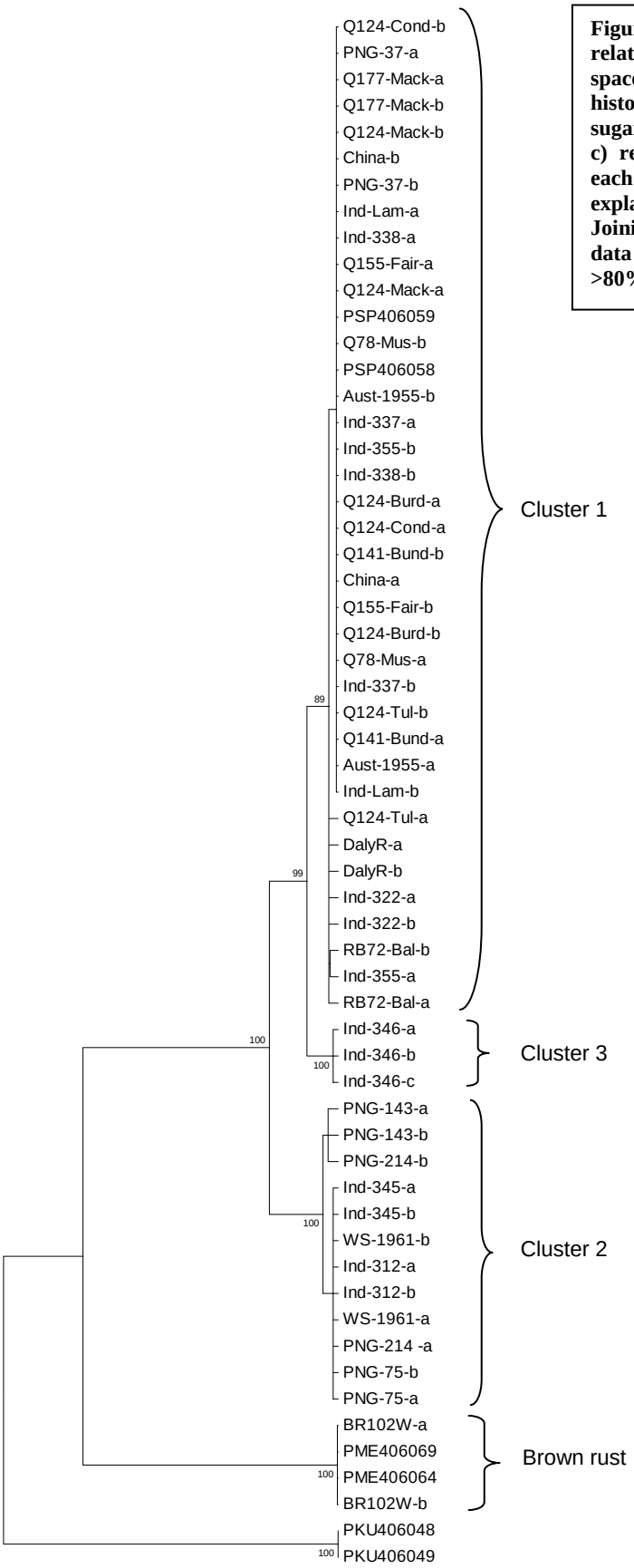

Figure 7 Dendrogram depicting the relationships between ribosomal intergenic spacer (ITS) sequences from Australian, historical, Indonesian, PNG and Chinese sugarcane rust isolates. One to three (a, b, c) replicate plasmids were sequenced for each rust isolate. Isolate codes are explained in Tables 3 and 4. The Neighbour Joining consensus tree from 1000 bootstrap data sets is presented. Bootstrap values >80% are shown at the branch points.

Table 5 Phylogenetic clustering patterns based on sequence data from three ribosomal regions, the intergenic spacer (IGS), a portion of the large subunit (LSU) and the internal transcribed spacer (ITS).

Isolate	Host	Location	IGS	LSU	ITS
Field Australia					
Q124-Burd	Q124	Burdekin	1	1	1
Q124-Cond	Q124	Condong	1	1	1
Q124-Mack4	Q124	Mackay	1	1	1
Q124-Tul	Q124	Tully	1	1	1
Q141-Bund	Q141	Bundaberg	1	1	1
Q155-Fair	Q155	Fairymead	1	1	1
Q177-Mack	Q177[Φ]	Mackay	1	1	1
RB72-Bal	RB72-454	Ballina	1	1	1
Other Australia					
Q78-Mus	Q78	Museum Tully Station	1	1	1
DalyR	*S. spontaneum*	Daly River, NT	1	1	1
Historical					
Aust-1898		Brisbane	1	1	NR
Aust-1955		Bundaberg	1	3	1
WS-1961		Savaii, Western Samoa	2	2	2
PNG					
PNG-37	*S. edule*	Jayapura	2	1	1
PNG-75	*S. officinarum*	Wewak	1	NR	2
PNG-143	*S. robustum*	New Britain	2	2	2
PNG-214	*S. edule*	Alotau	2	2	2
Indonesia					
Ind-312	*S. officinarum*	Lewa-Paku (Sumba)	2	2	2
Ind-322	*S. spontaneum*	Kelimutu (Flores)	1	1	1
Ind-337	Hybrid	Utan-Sabiduk (Sumbawa)	1	1	1
Ind-338	Hybrid	Utan-Jorok/Sugar Mill	1	1	1
Ind-345	*S. officinarum*	Kopang Jelunjuk (Lombok)	2	2	2
Ind-346	*Erianthus*	Anjani (Lombok)	3	3	3
Ind-355	*S. spontaneum*	Sanur (Bali)	1	1	1
Ind-Lam		Lampung (Sumatra)	1	1	1
China					
China	YT94-128	Zhanjiang China	1	1	1
Virtudazo *et al.* 2001					
BPI-79612 (PKU)		Australia	NT	1	unique
BPI-79624 (PKU)		Hawaii	NT	1	unique
TSH-R11108 (PSP)		Japan	NT	1	1
TSH-R11129 (PSP)		Japan	NT	1	1

NR: no result
NT: not tested

The significance of the three orange-rust clusters is unknown. There is no apparent correlation among host, location or symptoms to explain clusters 2 and 3, although isolates from commercial hybrid canes are only in cluster 1. Isolates from Indonesia are distributed throughout the three clusters. The Australian field isolates clustered with rusts from diverse locations; thus, it appears that the new race change in 2000 was not due to an accidental introduction from overseas.

During the ACIAR surveys in Indonesia and PNG, diseased samples were photographed and the symptoms recorded. The rust samples in this study were described as either "rust" or "orange rust"; no unusual symptoms were recorded for these isolates (see Figure 6 for

examples). No Indonesian or PNG isolate in this study clustered with brown rust. Brown rust was reported for the first time in PNG in 1991 in commercial sugarcane. The PNG isolates used in this study were collected from garden plots and not from the commercial plantation.

Spores and pustules from selected isolates were examined by Roger Shivas at the DPI&F Plant Pathology Herbarium. Ind-345 and PNG-214 (cluster 2) and Ind-346 (cluster 3) were examined by light microscopy and looked no different from typical orange rust (data not shown). Ind-337 (cluster 1), Ind-312 (cluster 2), Ind-346 (cluster 3) and brown rust from the ORIA were examined by scanning electron microscopy (Figure 7). Brown rust was distinguished by the smaller, more numerous spines. Ind-337, Ind-312 and Ind-346 looked no different from typical orange rust. Thus, the rusts comprising the three clusters appear morphologically similar. While the biological relationships between these rusts are not understood, they could pose a quarantine threat to Australian sugarcane. Any potential risks posed by these new pathogens to the Australian sugar industry are unknown at this stage.

Pathogenicity testing of isolates from the different clusters should be undertaken to determine the potential quarantine significance of isolates from the three clusters. This could be conducted in an institute isolated from commercial sugarcane crops, such as CIRAD in France.

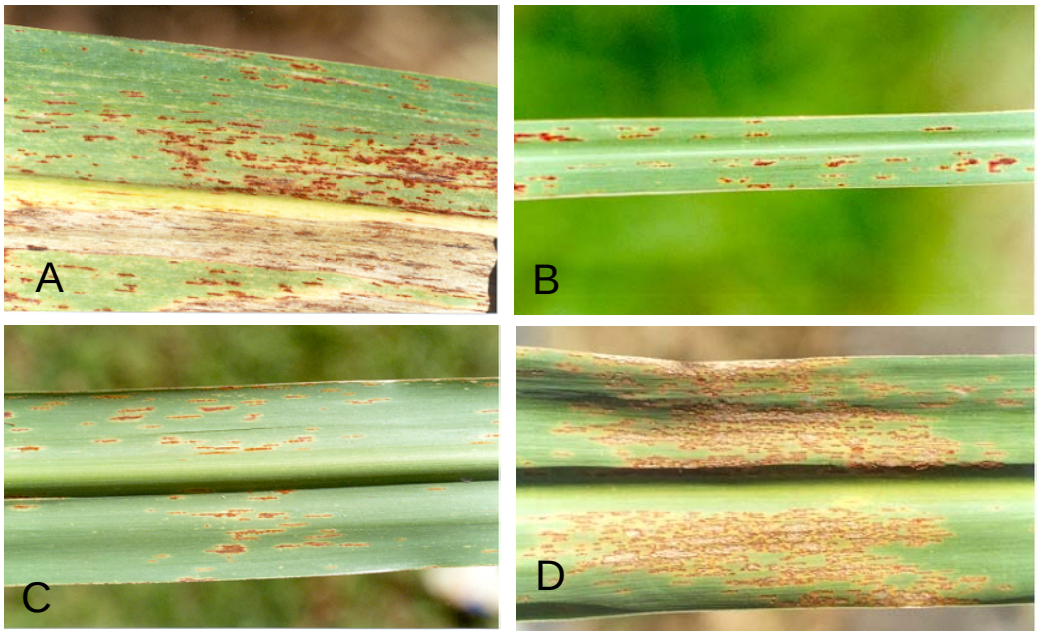

Figure 8 Photographs of symptoms taken during the ACIAR survey in PNG. A.Ind-338: orange rust on a hybrid cane (cluster 1); B. Ind-355: orange rust on *S. spontaneum* (cluster 1); C. Ind-345: orange rust on *S. officinarum* (cluster 2); D. Ind-346: rust on *Erianthus* (cluster 3).

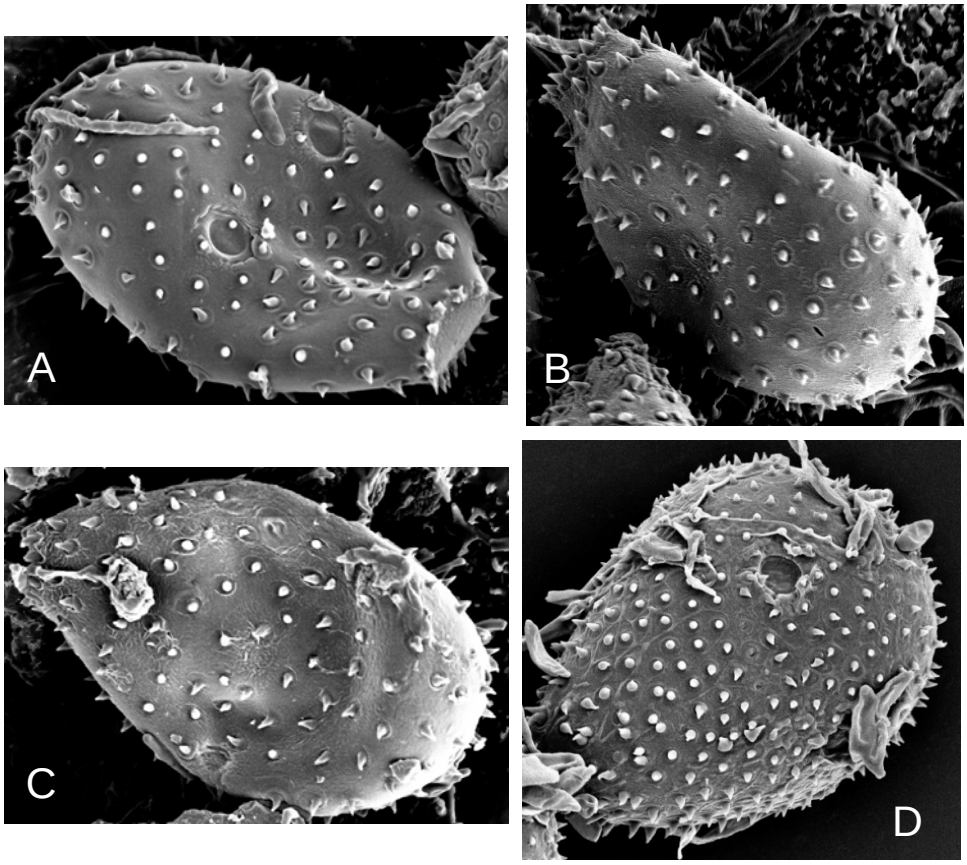

Figure 9 Scanning electron microscope images of sugarcane rust spores. A. Ind-337 (cluster 1); B. Ind-312 (cluster 2); C. Ind-346 (cluster 3); D. brown rust from Q99 in the ORIA.

4.0 SMUT

4.1 Introduction

The first smut genetic-diversity study (Braithwaite *et al.* 2004), funded by the CRC-TPP and BSES, was carried out in Dr Guus Bakkeren's laboratory at the Pacific Agri-Food Research Centre (PARC), Agriculture & Agri-Food Canada, during April-June 2001. Two world collections of *Ustilago scitaminea* isolates were assembled at PARC for the first study: one coordinated by Kathy Braithwaite and assembled in late 2000/early 2001; and an earlier collection made in 1984/1985 and held by Dr Ken Damann (Louisiana State University). Due to time constraints, the genetic diversity study comprised only 38 isolates from the two collections. Isolates were selected so that all sugar industries in the two collections were represented, with the major emphasis on Asia, due to its proximity to Australia. Several isolates were collected from the same sugar industry 15 years apart. The molecular marker technique of AFLPs (amplified fragment length polymorphisms) was chosen because it had been used successfully in Dr Bakkeren's laboratory to screen other *Ustilago* species (Bakkeren *et al*. 2000). Results showed that genetically divergent sugarcane smut isolates were present in Taiwan, Philippines and Thailand. The resistance of Australian cane varieties towards smut isolates from this region is completely unknown. These results suggested two areas for further work:

1. Assess the genetic diversity in a greater range of Asian isolates;

2. Screen Q canes in areas where divergent isolates occur.

4.2 Assemble a third collection of smut isolates

4.2.1 Coordinate a collection of smut isolates with emphasis on Asia

A third collection of sugarcane smut isolates with a greater focus on South East Asia was assembled at the Pacific Agri-Food Research Centre (PARC), Canada in 2003 to extend the initial study on genetic diversity. Once again, pathologists from various sugar industry organisations were contacted via email or in person at the 2003 ISSCT Plant Pathology Workshop and asked to collect smut samples. Canadian import permits were obtained and the material sent to Dr Guus Bakkeren at PARC, Canada.

Isolates were received from Anusorn Kusalwong (Field Crop Research Institute, Thailand Department of Agriculture), Tan Tack Nee (Agricultural Research Division, Malaysia), Peter Nielsen (Nghe An Tate & Lyle, Vietnam), G.P. Rao (Society for Sugar Research & Promotion, India), Fan Yuanhong (Yunnan Sugarcane Research Institute, China), Chen Xiwen (Guangzhou Sugarcane Industry Research Institute, China). Isolates from Hawaii, provided by Susan Schenck (Hawaii Agriculture Research Centre), were also included in the study because of results presented at the 2003 ISSCT Plant Pathology Workshop that reported a new smut race on the Hawaiian island of Maui in 2001 on the previously resistant variety H78-7750.

4.2.2 Propagation of new smut isolates at PARC

At PARC, a technician Andrena Kamp was employed from May to September 2004 to process the new isolates and extract the DNA. The methods provided by Andrena Kamp and used to isolate single spore cultures, extract the DNA and put the material into long-term storage are included in Appendix 1. It is important to note that all isolates from the third collection were derived from single, germinated teliospores. Diploid teliospores germinate to produce haploid promycelia, sporidia or hyphae that fuse and grow to produce dikaryotic cultures (Alexander and Ramakrishnan 1977). The total number of new isolates processed at PARC was: Thailand 28; Malaysia 12; Vietnam 3; India 1; China 19; and Hawaii 4. Four isolates did not germinate, and so the final number of DNA extractions sent to BSES in October 2004 was 63.

4.2.3 Inclusion of isolates from the first study

The extent of genetic variation detected among the 38 isolates comprising the first study was unexpectedly low. This may be a true reflection of the sugarcane smut population or it may be due to the marker system used (AFLPs). Therefore, it was a clear intention of the second study to use an alternative marker system and to include isolates from the first study to make a comparison. The 38 isolates used in the initial study were derived from germinated single haploid sporidia as described in Croft and Braithwaite (2006), whereas the isolates prepared in 2004 represent single germinated teliospores. The DNA from the 38 isolates, extracted in 2001 and stored at -70°C, was sent to BSES in October 2004.

The 38 isolates used in the initial study were preserved as single, haploid sporidia of both mating types frozen at -70°C at PARC. Unfortunately, the -70°C freezer in Dr Bakkeren's laboratory failed in July 2004 and most of the preserved spore samples from the first study were lost. Fortunately, the original unused material from the first two world collections, comprising either harvested teliospores or pieces of whip, was still stored in Dr Bakkeren's laboratory at room temperature. Andrena Kamp established new single germinated teliospore cultures for many of the 38 isolates. The remaining samples could not be grown because the material was in very poor condition (eg damp, contaminated with yeast, tubes empty, etc). DNA from the teliospore cultures was sent to BSES in October 2004.

4.3 Choice of marker system

The original proposal was to use microsatellites or another marker other than AFLPs to fingerprint the smut collection. Through Guus Bakkeren, contacts were established with Andy Munkacsi, a graduate student from University of Minnesota working on genetic variation in corn smut. Approximately 110 corn-smut microsatellites were received from Andy Munkacsi and are stored at BSES. A collection of sugarcane-smut microsatellites was developed by CIRAD, but despite some negotiations early in the project, we were unable to gain access to this collection.

After some consideration the marker system known as Diversity Arrays Technology (DArT; DArT Pty Ltd, Canberra) was chosen. The technology is based on microarray

hybridizations that detect the presence versus absence of individual fragments in genomic representations. DArTs can detect and type DNA variation at several hundred genomic loci without relying on sequence information (Wenzl *et al.* 2004). The main advantages of DArT technology are:

- it is a reliable technology based on hybridising DNA to microarrays;
- it is not dependent on prior sequence information;
- it is high throughput due to a high level of multiplexing;
- it produces reproducible data at low cost; and
- it can be used in the evaluation of genetic diversity.

DArTs have been used successfully for numerous plant species, including rice, barley, Arabidopsis, cassava and sugarcane, and fungal pathogens of barley and the fungus *Mycosphaerella graminicola* (http://www.diversityarrays.com/default.htm).

4.4 Fingerprinting analysis of sugarcane smut DNA

4.4.1 Sugarcane-smut isolates used in the DArT fingerprinting project

The DArT platform is a 96-well array setup with one well kept spare for a control, leaving 95 isolates available for analysis. It was important to include both sporidial- and teliospore-derived versions of some isolates for comparison. In addition, as the focus was on the Asian region, only limited numbers of isolates were selected from non-Asian countries. However, several countries had large representations (ie 28 from Thailand and 19 from China) that were reduced. The final array comprised:

A. All 38 haploid isolates from the first study that were extracted in 2001 with 10 of those re-isolated as germinated teliospores and extracted in 2004, and a new isolate from Brazil (Table 6a);
B. 46 isolates from the new collection representing germinated teliospores extracted in 2004 (Table 6b).

4.4.2 Dart methodology used for sugarcane smut

Detailed methods and results provided by Alex Wittenberg and Andrzej Kilian are described in Appendix 2. Briefly, library preparation and screening was as follows:

Preparation of genomic representations: The DNA of 40 genotypes was pooled and used to construct a genomic representation. Three restriction endonucleases, HindIII and the frequent cutters MseI and RsaI, were used to reduce genome complexity. HindIII and MseI adapters were simultaneously ligated to the complementary overhangs with T4 DNA ligase.

Library construction and array preparation: A 16x384 plate HindIII-MseI-RsaI library was prepared and the library amplified by colony PCR. The amplification products were spotted in duplicate on polylysine-coated slides.

Target preparation and genotyping: Genomic representations of individual samples to be hybridised were generated by using the same complexity reduction method (HindII/MseI/RsaI) as was used to construct the array and labelled with Cy3-dUTP. Labelled representations, called targets, were hybridized to the processed microarrays.

Table 6a **Isolates from the first (1984/1985) and second (2000/2001) world collections used in the DArT fingerprinting study. Isolates with the code H were derived from germinated haploid sporidia and the DNA was extracted in 2001. The remaining isolates were derived from germinated teliospores and the DNA was extracted in 2004.**

ID[a]	Isolate	Location	Date	Collected by	Host	Tissue
1A	Ph1H	Batangas, Philippines	00/01	R. Cu	VMC84-524	haploid spore
1B	Ph3	Negros Island, Philippines	00/01	R. Cu	P85-83	teliospore
1C	Ph3H	Negros Island, Philippines	00/01	R. Cu	P85-83	haploid spore
1D	Ph6	Negros Island, Philippines	00/01	R. Cu	VMC98-126	teliospore
1E	Ph6H	Negros Island, Philippines	00/01	R. Cu	VMC98-126	haploid spore
1F	Th547	Sing Buri, Thailand	84/85	A. Kusalwong	Q83	teliospore
1G	Th547H	Sing Buri, Thailand	84/85	A. Kusalwong	Q83	haploid spore
1H	Th548	Supan Buri, Thailand	84/85	A. Kusalwong	Thai1	teliospore
2A	Th548H	Sing Buri, Thailand	84/85	A. Kusalwong	Thai1	haploid spore
2B	Th555	Kanchana, Buri	84/85	A. Kusalwong	F147	teliospore
2C	Th555H	Kanchana, Buri	84/85	A. Kusalwong	F147	haploid spore
2D	Tw1	Taiwan	00/01	Y. Liang	Race 1	teliospore
2E	Tw1H	Taiwan	00/01	Y. Liang	Race 1	haploid spore
2F	Tw2	Taiwan	00/01	Y. Liang	Race 2	teliospore
2G	Tw2H	Taiwan	00/01	Y. Liang	Race 2	haploid spore
2H	Tw3	Taiwan	00/01	Y. Liang	Race 3	teliospore
3A	Tw3H	Taiwan	00/01	Y. Liang	Race 3	haploid spore
3B	Wa1	Ord River, Western Australia	00/01	J. Engelke	nr[b]	teliospore
3C	Wa1H	Ord River, Western Australia	00/01	J. Engelke	nr	haploid spore
3D	Wa2H	Ord River, Western Australia	00/01	J. Engelke	nr	haploid spore
3E	Wa3H	Ord River, Western Australia	00/01	J. Engelke	nr	haploid spore
3F	Wa4H	Ord River, Western Australia	00/01	J. Engelke	nr	haploid spore
3G	Wa5H	Ord River, Western Australia	00/01	J. Engelke	nr	haploid spore
6G	In1	South Sumatra, Indonesia	00/01	U. Murdiyatmo	nr	teliospore
3H	In1H	South Sumatra, Indonesia	00/01	U. Murdiyatmo	nr	haploid spore
4A	In2H	West Java, Indonesia	00/01	U. Murdiyatmo	nr	haploid spore
4B	In3H	Central Java, Indonesia	00/01	U. Murdiyatmo	nr	haploid spore
4C	In4H	East Java, Indonesia	00/01	U. Murdiyatmo	nr	haploid spore
4D	In5H	South Sulawesi, Indonesia	00/01	U. Murdiyatmo	nr	haploid spore
4E	Sa1H	Pongola, South Africa	00/01	S. McFarlane	NCo310	haploid spore
4F	Sa5H	Umfolozi, South Africa	00/01	S. McFarlane	NCo376	haploid spore
4G	Sa6H	Eston, South Africa	00/01	S. McFarlane	nr	haploid spore
4H	Sa7H	Darnall, South Africa	00/01	S. McFarlane	nr	haploid spore
5A	Sa527H	Pongola, South Africa	84/85	R. Bailey	NCo376	haploid spore
5B	Zi522H	Experiment Station, Zimbabwe	84/85	P. Sinai	NCo376	haploid spore
5C	Ma2H	Petite Riviére, Mauritius	00/01	S. Saumtally	M1176/77	haploid spore
5D	Ma4H	Palmyre, Mauritius	00/01	S. Saumtally	M1030/71	haploid spore
5E	Ma500H	Mauritius	84/85	J. Autrey	nr	haploid spore
6F	Ar3H	Argentina	00/01	A. Rago	nr	haploid spore
G12	Br532	Sao Paulo, Brazil	84/85	J. Irvine	NA56-79	teliospore
6H	Br533H	Sao Paulo, Brazil	84/85	J. Irvine	NA56-79	haploid spore
6A	Co561H	Cucuta, Columbia	84/85	J. Victoria	CP57-603	haploid spore
6B	Ve530H	Maracay, Venezuala	84/85	G. Peterson	nr	haploid spore
6C	Fl1H	Canal Point, Florida	00/01	J. Comstock	nr	haploid spore
6D	Fl511H	Canal Point, Florida	84/85	J. Dean	CP73-1547	haploid spore
6E	Lo1H	Louisiana	00/01	J. Hoy	nr	haploid spore
6F	Lo538H	Houma, Louisiana	84/85	G. Benda	H74-14	haploid spore
6G	Ha524H	Hawaii	84/85	nr	Race A	haploid spore
6H	Tx529H	Sugar Mill, Texas	84/85	Ben Villalon	NCo310	haploid spore

Table 6b Isolates from the third (2003/2004) world collection used in the DArT fingerprinting study. All isolates were derived from germinated teliospores and the DNA was extracted in 2004.

ID[a]	Isolatee	Location	Date	Collected by	Host
7A	Id1	Uttar Pradesh, India	2003	G. P. Rao	CoSe98231
7B	Vt1	Vietnam	2003	P. Nielsen	F134
7C	Vt2	Vietnam	2003	P. Nielsen	ROC22
7D	Vt3	Vietnam	2003	P. Nielsen	QD 11
7E	Ha1	Kunia, Oahu Hawaii	2004	S. Schenck	78-7750
7F	Ha2	Maui, Field 902.1 Hawaii	2004	S. Schenck	78-7750
7G	Ha3	Maui, Field 919c Hawaii	2004	S. Schenck	83-7061
7H	Ha4	Maui, Field 903b Hawaii	2004	S. Schenck	65-7052
8A	Ml1	Malaysia	2003	T. Tack Nee	CP85-1432
8B	Ml2	Malaysia	2003	T. Tack Nee	F173
8C	Ml3	Malaysia	2003	T. Tack Nee	M387/85
8D	Ml4	Malaysia	2003	T. Tack Nee	NCo310
8E	Ml5	Malaysia	2003	T. Tack Nee	Q125
8F	Ml6	Malaysia	2003	T. Tack Nee	Q162
8G	Ml7	Malaysia	2003	T. Tack Nee	TC2
8H	Ml8	Malaysia	2003	T. Tack Nee	TC4
9A	Ml9	Malaysia	2003	T. Tack Nee	TC6
9B	Ml10	Malaysia	2003	T. Tack Nee	TC7
9C	Ml11	Malaysia	2003	T. Tack Nee	TC9
9D	Ml12	Malaysia	2003	T. Tack Nee	TC10
9E	Ch1	Kaiyuan, Yunnan, China	2003	A. James/Y. Fan	Gui 11
9F	Ch2	Kaiyuan, Yunnan, China	2003	A. James/Y. Fan	Arundinaceous hybrid
9G	Ch3	Kaiyuan, Yunnan, China	2003	A. James/Y. Fan	Spontaneum hybrid
9H	Ch5	Sanya, Hainan, China	1997	X-W. Chen	Spore mix
10A	Ch6	Yongyan, Guangdong, China	2002	X-W. Chen	Xintaitang 16
10B	Ch7	Zhanjiang, Guangdong, China	2002	X-W. Chen	YT89-113
10C	Ch8	Zhanjiang, Guangdong, China	2002	X-W. Chen	YT89-113
10D	Ch9	Zhanjiang, Guangdong, China	2002	X-W. Chen	YT89-113
10E	Ch10	Zhanjiang, Guangdong, China	2002	X-W. Chen	YT96-177
10F	Ch11	Guangzhou, Guangdong, China	2002	X-W. Chen	Q158
10G	Ch12	Guangzhou, Guangdong, China	2002	X-W. Chen	YT96-479
10H	Ch13	Guangzhou, Guangdong, China	2002	X-W. Chen	YT96-479
11A	Ch14	Guangzhou, Guangdong, China	2002	X-W. Chen	Xintaitang 10
11B	Ch15	Guangzhou, Guangdong, China	2002	X-W. Chen	YT96-177
11C	Th1	Kanchanaburi, Thailand	2003	A. Kusalwong	nr[b]
11D	Th2	Kanchanaburi, Thailand	2003	A. Kusalwong	nr
11E	Th28	Kamphaeng Phet, Thailand	2003	A. Kusalwong	nr
11F	Th3	Suphanburi, Thailand	2003	A. Kusalwong	Co1011
11G	Th4	Suphanburi, Thailand	2003	A. Kusalwong	0402 ROGME
11H	Th7	Suphanburi, Thailand	2003	A. Kusalwong	Fiji 121
12A	Th8	Suphanburi, Thailand	2003	A. Kusalwong	K84-69
12B	Th9	Suphanburi, Thailand	2003	A. Kusalwong	B41-211
12C	Th10	Suphanburi, Thailand	2003	A. Kusalwong	CP38-22
12D	Th11	Suphanburi, Thailand	2003	A. Kusalwong	N51-216
12E	Th12	Suphanburi Thailand	2003	A. Kusalwong	CP85-1382
12F	Th16	Suphanburi Thailand	2003	A. Kusalwong	Q101
12H	blank				

a ID: refers to the well position in a 96-well plate setup
b nr: data not recorded during sampling

Image analysis and polymorphism scoring: The barcoded slides were scanned using a laser scanner. The TIF images were subsequently analysed with DArTSoft, a software package developed in-house to both identify and score polymorphic markers. The scorings table was used as an input file to generate a Hamming distance matrix. The Hamming distance matrix was subsequently used as input for a Principal Component Analysis.

4.4.3 Results

There were 112 DArT markers identified among the samples tested. Most of the markers were scored identically in two independent experiments and the overall reproducibility of scoring was above 99%. The markers were scored as 1 (present) or 0 (absent). The percentage of score "1" (marker's presence in the representation) varied among the samples from 0.49 to 0.87, with the average at 0.77. This is the highest percentage of score "1" average recorded until now using DArT arrays, indicating that the array had an overrepresentation of clones from highly similar genotypes. The average Polymorphism Information Content (PIC) was lower than for other species (0.22).

The dissimilarity among the samples analysed was calculated as Hamming distance by DArTsoft, which measures the percentage of markers that discriminate between any pair of genotypes. There was a variation in the average distance value among the genotypes, but most samples were highly similar, with only seven samples having the average distance above 0.35.

The Hamming distance matrix data was used to prepare a dendrogram generated by Proc Tree (SAS, Figure 10). Dendrogram clusters have been numbered from 1 (most diverse) to 9. The Principal Components Analysis generated from the Hamming distance matrix is shown in Figure 11. Some of these dendrogram clusters can be resolved on the Principal Components Analysis. Both figures clearly show that six isolates, from Vietnam (Vt1), China (Ch7, Ch9 Ch10), Taiwan (Tw2) and the Philippines (Ph1), are distinctly different **(cluster 1).** Tw2 and Tw2H represent diploid and haploid versions of the same isolate, respectively. The previous AFLP study identified seven divergent isolates. Two of these (Tw2 and Ph1) were again found to be very divergent and grouped into cluster 1, while the remaining five (Ph3, Ph6, Tw1, Tw3, Th548) were also resolved by Principal Components Analysis into **cluster 5**. When results from the DArT and AFLP studies are considered together, Vietnam, China, Taiwan, the Philippines and Thailand, were found to harbour diverse sugarcane smut isolates, compared to the rest of the world.

The DArT study confirmed the earlier finding that Indonesian and Western Australian isolates are closely related. Four Western Australian (Wa2, Wa3, Wa4, Wa5) and four Indonesian (In1, In3, In4, In5) isolates grouped very closely together into **cluster 4**.

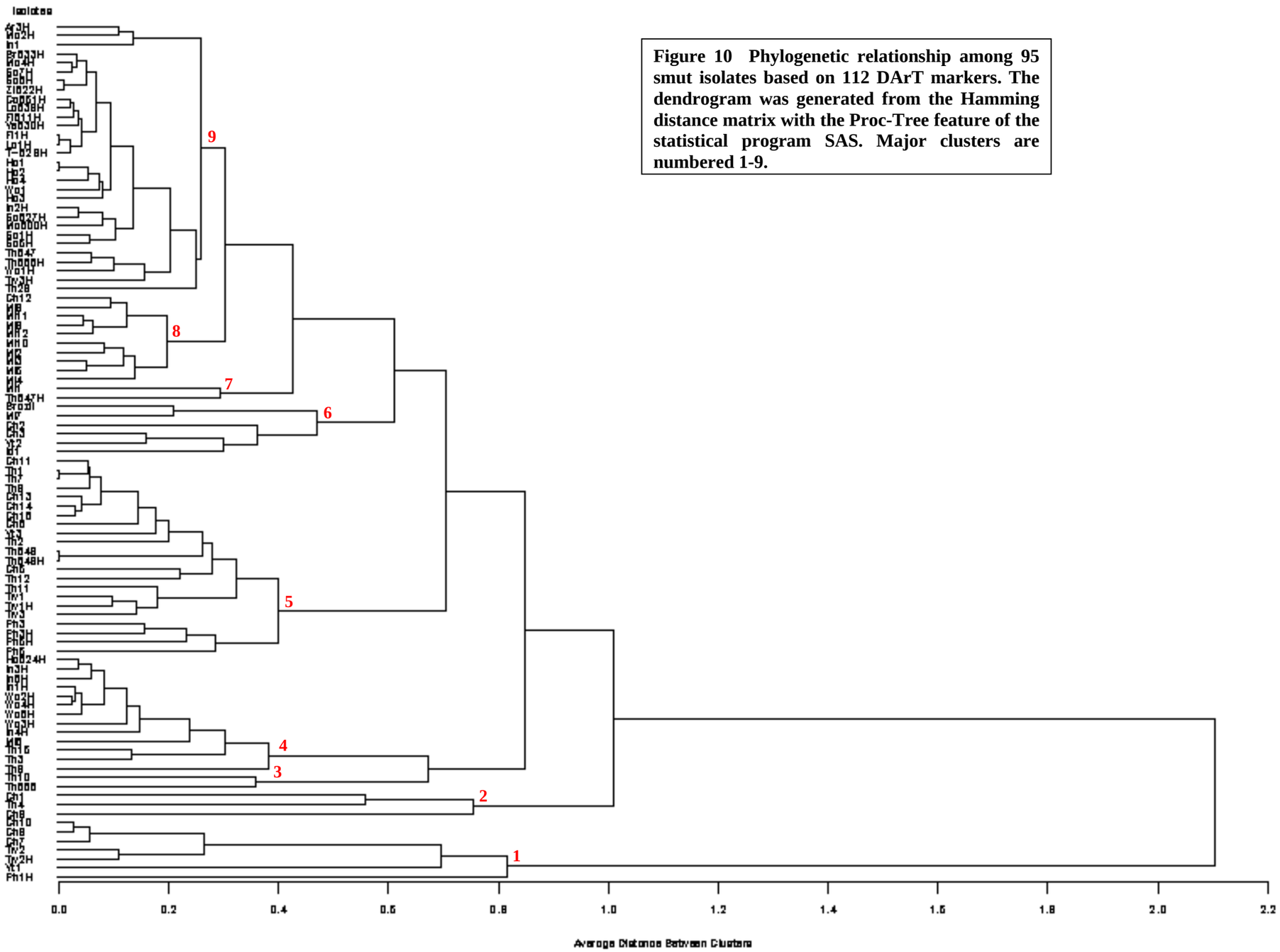

Figure 10 Phylogenetic relationship among 95 smut isolates based on 112 DArT markers. The dendrogram was generated from the Hamming distance matrix with the Proc-Tree feature of the statistical program SAS. Major clusters are numbered 1-9.

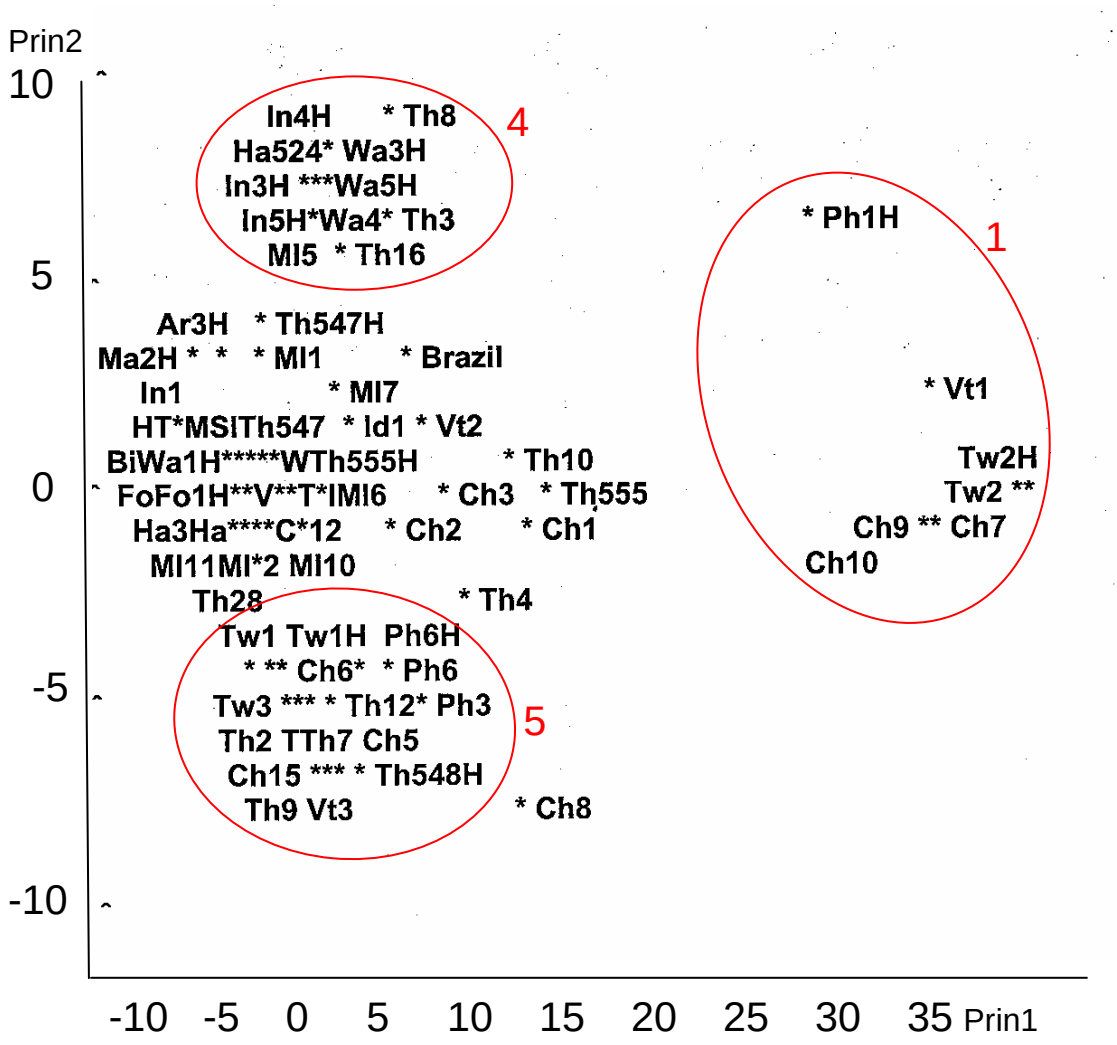

Figure 11 **Principal component analysis of 95 smut isolates based on 112 DArT markers. The analysis was calculated from the Hamming distance matrix. Clusters identified in the dendrogram that can be clearly resolved are show n. Note that 28 observations are hidden.**

As was found with AFLPs, the overall variation within the world population of sugarcane smut is surprisingly low. **Cluster 9** comprises isolates from diverse continents (North and South America, Africa, Asia, Australia). DArTs were unable to distinguish the four Hawaiian isolates representing different races. Isolates from several countries clustered closely together suggesting that they are clonally derived., eg many of the Malaysian isolates grouped together in **Cluster 8**. Some teliospore and haploid samples from the same collection (Tw3, Th547 and Th555) did not cluster together. It is surprising that such large differences would be due to ploidy. The differences may be due to the samples representing independent isolations from mixed material made several years apart.

4.4.4 Conclusions

The DArT array was developed and successfully applied to diversity analysis of *Ustilago scitaminea*. The level of polymorphism, both in the total number of markers and the dissimilarity among the samples was significantly lower than observed with other fungal pathogens analysed with DArT. The most comprehensive comparison can be made with *Mycosphaerella graminicola*, a fungal pathogen of wheat. Results suggest that the two species differ in at least one order of magnitude in the amount of genetic variation detected with DArT. This low level of variation is consistent with the previous AFLP analysis of 38 smut isolates.

Both the low PIC values for the markers and the relatively poor resolution of the samples suggest that the array had an overrepresentation of clones from highly similar genotypes. Using the more diverse isolates in an expanded array would be one approach to increase the resolution of the array. However, the current array enabled quite precise measurement of genetic diversity of the samples analysed and was able to identify groups of samples that are genetically nearly identical and several samples (a subset from China, Philippines and Vietnam) that were considerably more divergent.

4.5 Coordinate resistance screening of 10 Australian varieties

BSES has had a long history of cultivar exchange with many overseas sugarcane-breeding programs. As part of this exchange program, the cultivar reaction to diseases present in overseas countries is shared among the exchanging countries. However, cultivars can take up to 4 years to pass through pre-export and post-export quarantine and a further 2 years for propagation before planting in trials, then 2 more years before the results of the disease resistance trials are available (Croft and Braithwaite 2006). After the incursion of sugarcane smut in the ORIA, BSES (in partnership with SRDC) renegotiated a contract with the Indonesian Sugar Research Institute to expand and accelerate screening of Australian germplasm on the island of Madura. The quarantine period was reduced from the normal 2 years to 1 year and cultivars were propagated during the quarantine period. Later, clones were taken directly from quarantine glasshouses in Brisbane to be planted into the smut resistance trials. This allowed BSES to obtain information rapidly on new commercial cultivars and advanced clones in breeding programs (Croft and Berding 2004). Indonesia is a suitable location for conducting smut resistance screening because it is the most likely source for the spread of the pathogen to Australia (Croft and Berding 2004). Testing clones in Indonesia ensures that they will be tested against the biotypes of the pathogen that are most likely to enter Australia.

4.5.1 Selection of clones

Whilst the first genetic diversity study indicated that Australian and Indonesian isolates were identical, the Philippines and Taiwan isolates were genetically variable. The resistance of Australian cane clones towards isolates of smut from these regions is completely unknown. However, an indication of how Australian clones would respond to isolates from these regions is vital to ensure that Australia's sugar industry is prepared for further incursions of smut. Thus, the plan was to send approximately 10 Australian cultivars to PHILSURIN in the Philippines and the Taiwan Sugar Research Institute to

determine their smut reaction to local isolates in those countries. The cultivars were chosen based on their reaction in the early trials performed in the ORIA and Indonesia. Since then, five trials have been carried out in the ORIA, and seven carried out in Indonesia and data from poor trials has been excluded, giving more accurate information on the varieties. Consequently, some cultivars changed ratings during this time, in particular Q190[Φ], which was considered resistant but is now rated intermediate. Although some ratings have changed, the cultivars chosen still represent a good spread of ratings (Table 7).

In 2002 BSES breeders became aware that the Taiwan Sugar Research Institute would be suspending its sugarcane-breeding program. An alternative location for the smut testing had to be found. Fortunately, BSES and CSIRO have established close links with the Yunnan Sugar Research Institute (SRI), China, through:

- A MOU regarding exchange of germplasm;
- An ACIAR project involving CSIRO, BSES (Dr George Piperidis) and YSRI;
- The BSES quarantine officer (Mr Anthony James) spending 3 months working in YSRI in 2003.

The close proximity of Yunnan to Taiwan and a long exchange of germplasm between the areas in the past may mean that genetically similar isolates occur in the region. Thus, YSRI was approached and they agreed to carry out the testing.

Table 7 Australian cultivars chosen for smut testing in China and the Philippines. The rating is an average of all ORIA and Indonesian trials up to ORD5 and SM7, excluding those with poor data. The smut reaction is based on ratings of 1-3 considered resistant, 4-6 considered intermediate and 7-9 considered susceptible.

Cultivar	Smut reaction	Rating
Q117	Susceptible	8.1
Q138	Susceptible	8.0
Q170[Φ]	Susceptible	7.0
Q124	Intermediate	6.5
Q183[Φ]	Intermediate	6.3
Q120[a]	Intermediate	6.3
Q190[Φ]	Intermediate	5.8
Q135	Intermediate	5.5
Q96[Φ]	Intermediate	4.4
Q182[Φ]	Resistant	2.7
Q171[Φ]	Resistant	2.5

[a]Q120 replaced Q96 in China because there was insufficient planting material.

4.5.2 Progress on smut testing

As explained above, the screening program in Indonesia was accelerated to reduce the time in quarantine and for propagation. Unfortunately, no such arrangements are in place

at YSRI or PHILSURIN. Thus, the goal of this project was only to negotiate a screening program and assemble the canes, with the expectation that the screening would go beyond the life of the project. It was originally intended to visit PHILSURIN and the Taiwan Sugar Research Institute to observe the trials. However, the trials had not progressed far enough by the end of the project for the visit to take place.

China: Fortunately, many of the 10 cultivars were already present in Yunnan and did not need to be sent. Only Q96 had to be sent, and this was delayed until April 2004, because there was insufficient material in the quarantine glasshouse at Indooroopilly. It had to be replaced after DNA fingerprinting showed that the clone being propagated was not Q96. Q120, an intermediate variety**,** was also sent as backup. Yunnan staff have scheduled the planting of the trial for March-April 2006 after completion of a GxE trial for the ACIAR project, making the plant data available in 2007 and ratoon data available in 2008. In November 2005, Dr George Piperidis provided a progress report from China. Q96 will not be planted because of insufficient material. This cultivar will be replaced with Q120.

Philippines: The 10 canes were sent to PHILSURIN in November 2003 after negotiations with the director Dr Ramon Cu. Three cultivars (Q124, Q135 and Q171$^{(\phi)}$) did not germinate and were sent again in November 2004. In November 2005, Dr Ramon Cu provided a progress report. The Q cultivars were released from the quarantine screenhouse at IPB-UPLB last year. They are currently in the open field quarantine in the Island of Guimaras. They will be released sometime in January 2006 for smut testing in the field at Negros Island.

5.0 OUTPUTS

Orange rust

- A collection of rusts from around the centre of origin of *Saccharum officinarum* has been assembled.
- Techniques for storage and handling of a difficult host-pathogen interaction have been established.
- A detached-leaf inoculation assay has been established for Australian field isolates.
- Limited genetic variation has been detected in the Australian orange rust population using IGS sequencing.
- Phylogenetic analysis of ribosomal DNA sequences from overseas rust samples has revealed considerable genetic variation in sugarcane orange rust.
- Genetically diverse rusts appear morphologically indistinguishable from typical orange rust.

Smut

- A large world collection of sugarcane smut isolates has been assembled.
- Two independent molecular markers, AFLPs and DArTs, have been used to estimate genetic variation among isolates of sugarcane smut.
- Limited genetic variation has been detected in a world-wide collection of the sugarcane smut isolates.
- The southeast Asian region (China, Taiwan, Philippines, Vietnam and Thailand) contains genetically divergent isolates.
- Smut isolates from the ORIA were genetically closely related to isolates occurring in Indonesia
- Ten Australian sugarcane cultivars have been assembled in China and Philippines to assess their reaction to local smut isolates.

6.0 OUTCOMES

The main outcome from this project has been information about the genetic diversity in two important pathogens in the Australian sugar industry. Successful disease-management strategies and deployment of resistance require an understanding of variation in the pathogen population. In the absence of pathogenicity data, neutral genetic markers are widely used tools to estimate variation in pathogen populations. Whilst they 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.

This project has shown that there is only a single dominant genotype of orange rust in Australian sugarcane fields. The orange rust in Australia is not unique and probably did not arise from an accidental introduction from overseas. It is possible that the new race appeared as a mutation in only a single gene, which rapidly spread throughout the industry due to the high proportion of the susceptible cultivar Q124. Host resistance should be effective across a wide range of isolates if the pathogen population is genetically uniform. The level of resistance in the BSES-CSIRO breeding program is high and, since 2000, new resistant varieties have been deployed to replace Q124. Fortunately, there have been no reports of new outbreaks, indicating that resistance is holding well. This project has also shown that that close to Australia are a number of diverse rusts infecting wild *Saccharum* canes. Any potential disease risks posed by these new pathogens to the Australian sugar industry are unknown at this stage, but they must be considered as a quarantine threat to the Australian sugar industry.

For the Australian sugar industry, it is critical to know if neighbouring sugar industries harbour different races of sugarcane smut. One major outcome of the smut diversity work is that the Indonesian and Australian isolates tested in the AFLP and DArT studies are

closely related. Thus, the genetic data is not inconsistent with the resistance screening results carried out in Indonesia and the ORIA that have shown that Australian cultivars react comparably to local isolates in the two locations. As the most likely source of a smut incursion to eastern Australia is either Indonesia or the ORIA, the resistance screening results should be applicable to the smut disease pressure presently existing in Australia. A second outcome of this study is that Southeast Asia has been identified as an area of high risk for smut introductions to the Australian sugar industry.

Both of these findings contribute significantly to the robustness of the BSES-CSIRO breeding program.

7.0 FUTURE WORK AND RECOMMENDATIONS

Whilst the Australian population of orange rust currently appears genetically uniform and stable, another race change can not be ruled out. During disease epidemics, the presence of high amounts of inoculum in the environment can provide selection pressure for more races to appear. Fortunately, there have not been reports of major outbreaks since the epidemic and the disease has been increasingly managed as new resistant cultivars have been deployed to replace Q124. It is important to be alert for any further race changes. The results presented here suggest that diverse rusts are present close to Australia's north.

While the most likely source of a smut incursion to eastern Australia is either Indonesia or the ORIA, there is also the risk of introduction of other strains from other regions of Asia. Results from the smut testing in China and the Philippines will not be available for another 1-2 years. Once obtained, the results will be compared to results obtained from Indonesian and ORIA trials. Large discrepancies between the ratings for any variety will need further investigation.

Recommendations from this project are:

1. The industry should not be complacent that further race changes of orange rust will not occur.
2. Continued quarantine vigilance against the introduction of both rust and smut into Australia is essential.
3. Continued quarantine vigilance against the spread of smut from the Ord to the east coast is essential.
4. Smut ratings from China and the Philippines must be compared to ratings from Indonesia and the ORIA, when available.

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APPENDIX 1 DNA extraction from single *U. scitaminia* teliospores (Andrena Kamp).

Culture Purification

Teliospores were transferred from teliospore collections (either as tubes of teliospores or as infected sugarcane whips) to 500 µL sterile ddH_2O using a sterile loop and vortexed to achieve a homogeneous teliospore suspension. The teliospores were then plated in dilution series on Potato Dextrose Agar (PDA) with 100 µg/mL ampicillin and 25 µg/mL chloramphenicol, and incubated at 22°C overnight to germinate the teliospores. **Single germinated teliospores** were identified and removed using a sterile loop under 100X magnification and streaked on PDA. Pure cultures were then incubated at 22°C for 2-3 days. Three copies of each germinated teliospore culture were prepared in order to increase material available for DNA extractions. The cultures were then left to grow for 5-7 days at 22°C.

DNA Extraction

Germinated teliospore cultures (from a single teliospore) were scraped off plates using a sterile spatula, frozen and stored at -80°C. Frozen fungal mats were then ground in liquid nitrogen using a mortar and pestle. DNA extractions from 2 x 100mg ground, frozen fungal tissue were carried out using the Qiagen DNeasy Plant Mini Kit (Cat. No. 69104) to yield roughly 50 µg purified DNA in 200 µL elution buffer. DNA was precipitated by adding 1/10 vol. 3M NaCl and 2.5 vol. 96% EtOH and incubation overnight at -20°C. Precipitated DNA was spun down, washed once with room temperature 70% EtOH and then air dried.

Strain Storage

Cultures from two separate, individual teliospores were grown shortly in potato dextrose broth (PDB) overnight. To 800 µL liquid culture 80 µL DMSO (10% v/v) was added, mixed and stored at -80°C. Each strain was stored in duplicate.

Remaining in our freezer(s):
1) Half of the extracted teliospore DNA.
2) Crushed fungal tissue of all teliospore cultures for later additional DNA extraction if required.
3) All stocked cultures in duplicate (in two, physically separated freezers!).

Problem Strains

Some teliospores were more difficult to germinate. The following steps were taken if germination did not occur overnight.

1) Plates were left for up to 7 days at 22°C. (Most germinated within 3-4 days)
2) Higher and lower concentrations of teliospores were plated out.
3) Germination of teliospores was attempted at 37°C.

4) Pieces of filter paper to which volatile nonanol (known to stimulate germination of rust urediniospores and some other spore types) was applied were affixed to the lid of the Petri dish.
5) Teliospores were plated on PDA that included 100 µg/mL ampicillin, 25 µg/mL chloramphenicol and nonanol in the medium.
6) Teliospores were plated on PDA that included 100 µg/mL ampicillin, 25 µg/mL chloramphenicol and nonanol in the medium. Pieces of filter paper to which volatile nonanol was applied were affixed to the lid of the Petri dish.
7) Teliospores were plated on water-agar medium that included nonanol.
8) Teliospores were plated on water agar that included nonanol and pieces of filter paper to which volatile nonanol was applied were affixed to the lid of the Petri dish.
9) A variety of the above conditions and repetitions were attempted for recalcitrant strains and as a last resort, alternate strains from the same geographic location were used.

Some strains were extremely contaminated with yeast and or other fungi, and where possible, single teliospores were isolated from dilute germination plates. Where this was not possible, teliospores were soaked for 1 hour in a 1.5% $CuSO_4$, washed three times with sterile ddH_2O and then plated in dilution series on PDA with ampicillin and chloramphenicol.

APPENDIX 2 DArT analysis of genetic diversity in *Ustilago scitaminea* (Alex Wittenberg and Andrzej Kilian)

Materials and Methods

Preparation of genomic representations

The DNA of 40 genotypes was pooled and used to construct a genomic representation. As a genome complexity reduction method three restriction endonucleases were used. Instead of using two six-base pair cutters to which adapters were ligated and a co-digesting enzyme, a combination of a six- and four-base pair cutter and a co-digesting enzyme were chosen. The representation was prepared by digesting 100 ng of DNA with 2 units of HindIII and the frequent cutters MseI and RsaI (NEB; New England Biolabs, USA). HindIII and MseI adapters (listed in Table 1) were simultaneously ligated to the complementary overhangs with T4 DNA ligase (NEB).

Library construction and array preparation

A 16x384 plate HindIII-MseI-RsaI library was prepared. Eight plates were prepared using the hybridization adapters and another 8 plates using the cloning adapters (Table 1). PCR products were dried and washed once with 70% ETOH and dried again, before re-suspending in 25 µL spotting buffer (DArTspotter II). The amplification products were spotted in duplicate on polylysine-coated slides (Erie Scientific, Portsmouth, NH, USA) with a MicroGrid II microarrayer (Biorobotics, Cambridge, UK). After printing, slides were processed by incubation in hot water (95°C) for 2 min, dipped in MQ and dried by centrifugation.

Target preparation and genotyping

Genomic representations of individual samples to be hybridized were generated by using the same complexity reduction method (HindII/MseI/RsaI) as was used to construct the array, with the exception that now only genotyping adapters were used (Table A1). The resulting 50 µL PCR-reactions were concentrated 10-fold by precipitation with 1 volume of isopropanol and denaturated at 95°C for 3 min. Each reaction was labelled with 0.1 µL of Cy3-dUTP (Amersham) using the exo Klenow fragment of *Escherichia coli* DNA polymerase I (NEB). Labelled representations, called targets, were added to 60 µL of a 50:5:1 mixture of ExpressHyb buffer (Clontech), 10 g L^{-1} herring sperm DNA, and the FAM-labelled polylinker fragment of the plasmid used for library construction as a reference. After denaturation, targets were hybridized to the processed microarrays overnight at 65°C. The next day slides were washed.

Image analysis and polymorphism scoring

The barcoded slides were scanned using a Tecan LS 300 (Grödig, Austria) laser scanner. The TIF images were stored directly in the database and subsequently analysed with DArTSoft (version 7.4.1), a software package developed in-house. DArTSoft was used to both identify and score polymorphic markers as described by Wenzl *et al*. (2004).

The scorings table was used as an input file to generate a Hamming distance matrix. The Hamming distance matrix was subsequently used as input for a software package for Principal Coordinate Analysis (PCO).

Results

There were 112 DArT markers identified among the samples tested. Most of the markers were scored identically in two independent experiments and the overall reproducibility of scoring was above 99%.

The average call rate (percentage of effective scores) of selected markers was high (97.4%), similar to what we obtain for other species. Most of the samples scored well (at least 100 scores for the 112 markers) with 109 markers scored on average. Only two samples were scoring poorly and inconsistently among the experiments: Sample Ch8 had 78 scores (78%) which were consistent among the experiments and sample Ma2H had 95 scores (85%).

The average quality of markers measured by function P of DArTsoft program was at 79, somewhat lower than usually observed. In addition, the average Polymorphism Information Content (PIC) was lower than for other species (0.22). The distribution of the PIC values for the clones identified (Figure A1) is somewhat discontinuous, suggesting strong genetic structure in the studied materials. The group of 20 markers with PIC value above 0.4 represents a subset of genomic clones with good discriminating power.

The percentage of score "1" (marker's presence in the representation) varied among the samples from 0.49 to 0.87 with the average at 0.77. This is the highest percentage of score "1" average recorded until now on our DArT arrays, indicating that the array developed had overrepresentation of clones from highly similar genotypes.

The dissimilarity among the samples analysed was calculated as Hamming distance by DArTsoft, which measures the percentage of markers that discriminate between any pair of genotypes. There was a variation in the average distance value among the genotypes, but most samples were highly similar, with only 7 samples having the average distance above 0.35 (Figure A2). The sample Ha3 from Hawaii was most similar to other samples (distance of 0.13), while the sample from Vietnam (Vt1) was the most distant from others (average distance of 0.58). Three samples from China (Ch7, Ch9 and Ch10) had average dissimilarity around 0.5, which means that on average 50% of the identified markers were polymorphic between these samples and other samples in the collection analysed. There was only one sample from the Philippines (Ph1), which showed similarly high level of divergence from the remaining samples (average Hamming distance of 0.48).

Conclusions

The DArT array was developed and successfully applied to diversity analysis of *Ustilago scitaminea*. The level of polymorphism, both in the total number of markers and dissimilarity among the samples was significantly lower than observed with a number of fungal pathogens analysed with DArT until now. Most comprehensive comparison can be

drawn for the work on wheat fungal pathogen, *Mycosphaerella graminicola*, as practically all the methods used (and the operator) were the same for the two genomes). Cursory review of the data obtained for the two species suggests at least one order of magnitude difference in the amount of genetic variation detected with DArT. Low level of variation seems to be consistent with previous AFLP analysis of a smaller subset of *Ustilago scitaminea* by the CRC team.

Both the low (on average) PIC values for the markers identified and relatively poor resolution of the samples suggests that the array developed had overrepresentation of clones from highly similar genotypes. Expansion of the array using samples representing better the diversity of the species would certainly increase the resolution of the array. However, the current array enabled quite precise measurement of genetic diversity of the samples analysed and therefore identification of groups of samples that are genetically nearly identical and several samples (a subset from China, Philippines and Vietnam) which were considerably more divergent.

Table A1 **Adapter and primer oligonucleotide sequences used for generation of the genomic representation (cloning) and hybridization to the micro-arrays (genotyping).**

Endonuclease and recognition site	Used for	Adapter sequences[a]	Primer sequences (5' to 3')
HindIII 5'-A↓AGCTT-3' 3'-TTCGA↑A-5'	Cloning	5'- CTCGTAGACTGCGTCAC -3' 3'- ATCTGACGCAGTGTCGA -5'	TAGACTGCGTCACAGCTT
	Genotyping	5'- GTGCTACAGTCGCTGAG -3' 3'- ATGTCAGCGACTCTCGA -5'	TACAGTCGCTGAGAGCTT
MseI 5'-T↓TAA-3' 3'-AAT↑T-5'	Cloning	5'- ACTCGATCCTCACACGTAAAGTATAGATCCCA -3' 3'- NH_2-TTCATATCTAGGGTAT -5'	ACTCGATCCTCACACGTA
	Genotyping	5'- AGTGCATGGTGAGAGCTAAACTATACATGGGA -3' 3'- NH_2-TTGATATGTACCCTAT -5'	AGTGCATGGTGAGAGCTA
RsaI 5'-GT↓AC-3' 3'-CA↑TG-5'	Co-digestion	-	-

[a] Adapter sequences were formed by annealing the strands whose sequences are listed. Complementary sequences are underlined.

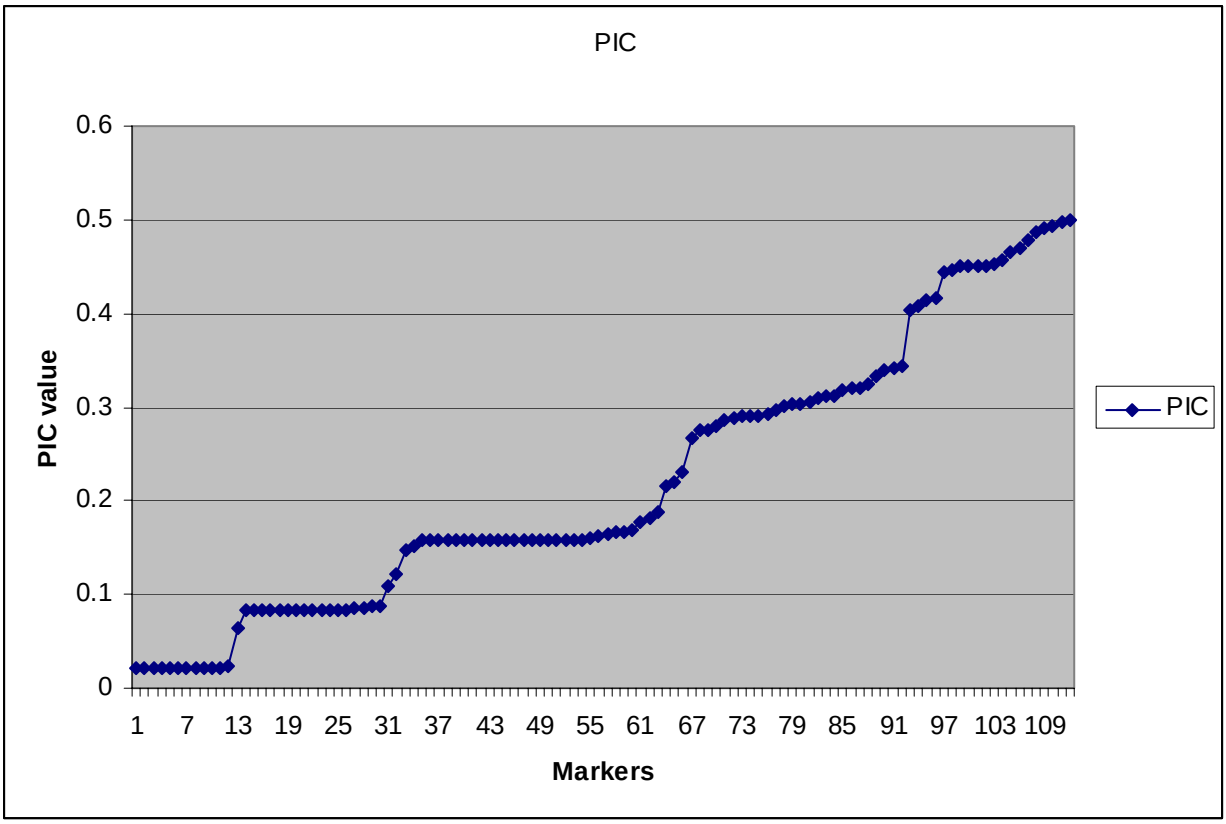

Figure A1 Distribution of PIC values for the DArT clones identified

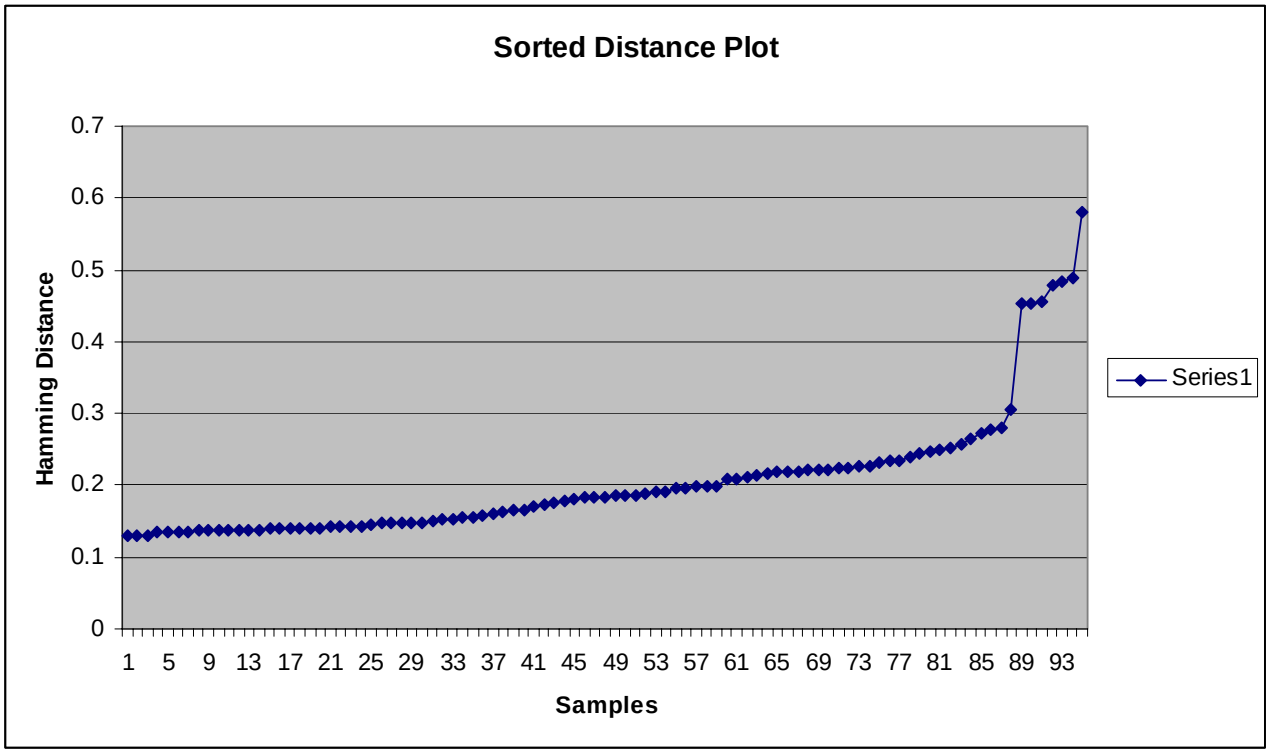

Figure A2 Sorted average Hamming Distance for the samples analysed.