SHORT COMMUNICATION

A PRELIMINARY REPORT ON GENETIC DIVERSITY IN POPULATIONS OF SUGARCANE RUST IN KWAZULU-NATAL

PILLAY L, McFARLANE S A and RUTHERFORD R S

South African Sugarcane Research Institute, P/Bag X02, Mount Edgecombe, 4300, South Africa
lugashni.pillay@sugar.org.za, sharon.mcfarlane@sugar.org.za, stuart.Rutherford@sugar.org.za

Abstract

In 2000, the newly released sugarcane variety N29, that had shown only mild rust symptoms during selection, became severely infected with brown rust, caused by *Puccinia melanocephala*. This change in rust susceptibility could have been due to a change in climatic conditions or to the development of a new race of *P. melanocephala*. Concerns were also raised about the possibility of orange rust, caused by *P. kuehnii*, being present in the South African sugar industry for the first time, particularly when orange pustules were observed in some areas. Microscopic observations of the orange, brown and black spores collected from four different areas in KwaZulu-Natal indicated the presence of *P. melanocephala* and not *P. kuehnii*, and this was confirmed by amplifying the D1/D2 region, which is the most variable region in the large ribosomal sub-unit of the genome, using NL1 and NL4 primers and sequencing the cloned products. Those sequences that could be successfully aligned indicated that genetic diversity existed in the different samples of *P. melanocephala* collected from three of the four sampled areas in KwaZulu-Natal. Further work will be carried out to confirm this result and to identify different populations in other sugarcane growing areas.

Keywords: Puccinia melanocephala, brown rust, urediniospores, phylogenetics

Introduction

Two types of rust are known to infect sugarcane; brown rust caused by *Puccinia melanocephala* and orange rust caused by *Puccinia kuehnii* (Egan, 1964). Rust was first recorded in South Africa in 1941 on variety Co301. The pathogen was incorrectly identified at that time as *P. kuehnii*, but it was later accepted that *P. melanocephala* had in fact caused the rust outbreak (Bailey, 1979). Orange rust occurs mainly in the Asian-Oceana region and has not been reported in South Africa (Magarey, 2000).

In 2000, the newly released variety N29, that had shown only mild rust symptoms during selection, became severely infected with brown rust (Cadet *et al*., 2003). This change in rust susceptibility could have been due to a change in climatic conditions or to the development of a new race of *P. melanocephala*. The purpose of this study was to compare populations of rust collected from four areas in KwaZulu-Natal (KZN) to determine whether differences in their morphology and genetic makeup were evident. Concerns were also raised about the possibility of orange rust being present in the South African sugar industry, particularly when orange pustules were observed in some areas. Although the urediniospores of *P. melanocephala* and *P. kuehnii* can be distinguished microscopically, part of the study also aimed at developing a molecular tool for reliably identifying the two rust pathogens.

Materials and methods

Rust-infected leaves were collected from four climatically distinct areas in KZN. Plant sections containing urediniospores were prepared and viewed using the scanning electron microscope (SEM) at the Electron Microscope Unit at the University of KwaZulu-Natal, Durban.

Leaves were examined under a Leica dissecting microscope at 100 X magnification and individual spores were collected for DNA isolation. *P. melanocephala* and *P. kuehnii* DNA was also obtained from Dr K Braithwaite, Bureau of Sugar Experiment Stations in Australia, for comparative purposes.

The Carlson lysis method for DNA extraction was carried out according to Carlson *et al*, (1991). The variable D1/D2 region of the large subunit ribosomal DNA was amplified with the NL1/NL4 primer combination using polymerase chain reaction (PCR) (Virtudazo *et al*, 2001a). The PCR cycling conditions were 1 cycle at 95ºC for 3 minutes; 35 cycles each at 94ºC for 30 seconds, 45ºC for 1 minute and 72ºC for 1 min 30 seconds; 40 cycles at 72ºC for 10 minutes. Amplified DNA fragments were separated on a 1% agarose gel. Gel slices containing the bands were excised and the DNA fragments were purified using the QIAquick Gel Extraction Kit (Qiagen). Cloning was performed using the pGEM®-T Easy Vector Kit (Promega) according to the manufacturer's protocol. Plasmid DNA was purified using Qiagen Purification Kit (Qiagen). Sequencing of the purified DNA was carried out using the BigDye Terminator Kit® (ABI) according to the manufacturer's protocol. Automated sequencing was conducted by the Department of Biotechnology at SASRI using an ABI 310 Genetic Analyser. Raw DNA sequences were edited using the Staden software package. Sequence identity was confirmed using Blast and sequences were aligned using the CLUSTAL X program for phylogenetic analysis.

Results and discussion

Scanning electron microscopy

Urediniospores collected from rust-infected leaf material from Eston were orange in colour and appeared smaller when compared with the usual brown spores under the dissecting microscope. It is likely that these spores were immature urediniospores, or the colour difference may have been due to the cooler temperatures in the Midlands area at the time of pustule development.

SEM of a ruptured rust pustule showed an abundance of urediniospores that varied in shape and size (Figure 1a). Urediniospores that varied in shape and size in the ruptured pustule were likely to have been a mixture of immature, developing and mature urediniospores (Figure 1b). The number, size and arrangement of the echinulations matched previous reports (Virtudazo *et al*, 2001b) that compared urediniospores of *P. melanocephala* with those of *P. kuehnii* in Japan. Urediniospores from KZN that were observed using SEM clearly exhibited a dense arrangement of spines, especially surrounding the germ pore (Figure 1c), whereas *P. kuehnii* has been shown to have a very sparse arrangement of spines and has no spines near the germ pores. These morphological differences, the size of the urediniospore and the presence or absence of apical thickening can be used to differentiate the two species of rust in sugarcane, and clearly indicated that *P. melanocephala* was present in KZN, and not *P. kuehnii*. Figure 1d shows three germ tubes extending from three of the four germ pores on a

urediniospore. One of the germ tubes formed an appressorium over the stomatal aperture, which is necessary for infection of the host tissue (Sotomayor *et al*, 1983).

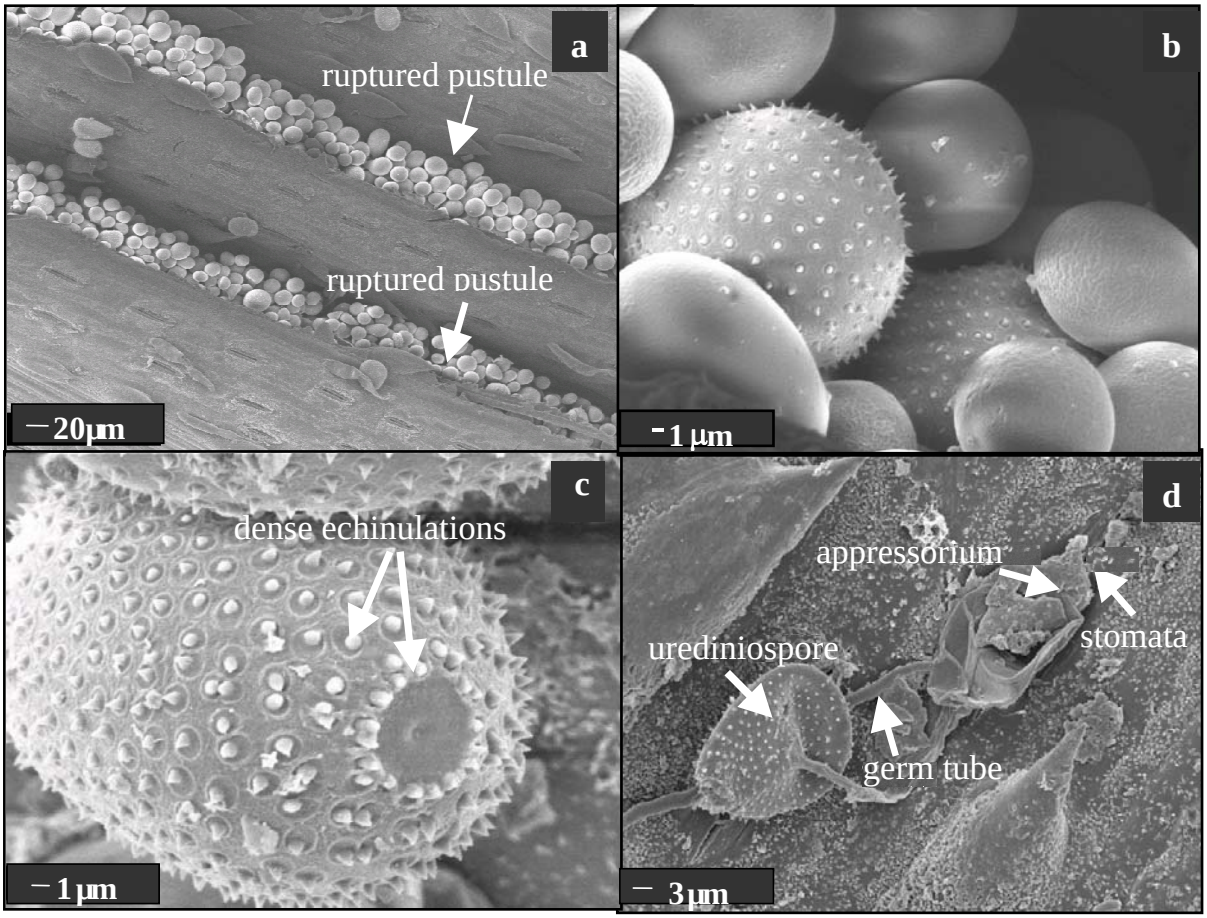

Figure 1. Scanning electron micrographs of (a) ruptured *P. melanocephala* pustules, (b) mature and immature urediniospores of *P. melanocephala,* (c) arrangement of echinulations on the surface of a mature urediniospore and (d) development of germ tubes from a urediniospore.

Molecular methods

By viewing the rust-infected leaf material under a dissecting microscope, individual urediniospores could be selected using a dissecting needle. This effectively eliminated most plant debris and was expected to reduce the possibility of extracting DNA from the spores and mycelium of contaminating fungi.

DNA yields were low when the original Carlson lysis method was used, possibly because of the limited sample size and the presence of a thick spore coat. The method was therefore modified to include an additional freeze step (30 minutes at –20ºC) and heat step (30 minutes at 74ºC), which aided in breaking down the thick spore coat and increased the amount of DNA released. NL1 and NL4 primers (Virtudazo *et al*, 2001a) proved to be the best combination for the reliable amplification of the region of interest. Products were visible using 4 ng/µl DNA per PCR reaction, and decreased when concentrations as high as 16 ng/µl were used. The KZN PCR amplified fragments produced were approximately 620 base pairs in length, which is the expected size for the targeted region (Virtudazo *et al*, 2001a).

Blast searches revealed that a significant number of sequences were from fungi other than *Puccinia* indicating that fungal contaminants were present. *Ustilago scitaminea* (sugarcane smut) was detected as a contaminant of rust pustules from the Umfolozi area, where smut remains a significant problem due to the continued cultivation of susceptible varieties such as NCo376.

Other contaminants were tentatively identified as fungi known to be hyperparasitic on rusts such as *Cladosporium uredinicola* and *C. tenuissimum* (Ryan and Wilson, 1981; Moricca *et al*, 2001). Others showed phylogenetic similarity to *Phoma glomerata*, a species known to be antagonistic or hyperparasitic to fungal plant pathogens (Ouimet *et al*, 1997; Sullivan and White, 2000). These results suggest that other fungi often inhabit rust pustules, and several may be important components of a hyperparasitic guild of fungi that naturally restrict the progress of rust epiphytotics. Further study is warranted to evaluate the effectiveness of hyperparasitic control of rust pathogens.

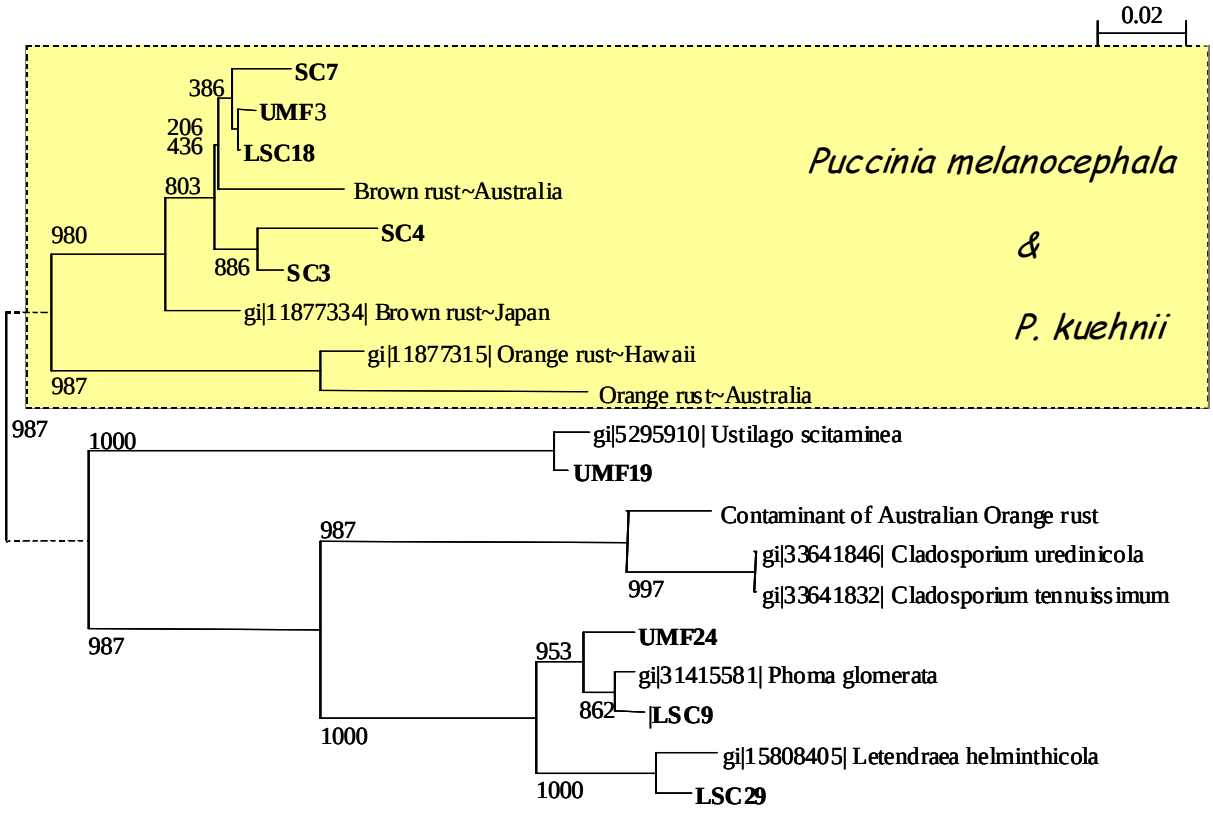

Figure 2. Neighbour-Joining phylogenetic tree of LSU rDNA sequences from rust pustules in KZN and samples from Australia. Entries with gi|numbers| are from GenBank. (SC–South Coast; LSC–Lower South Coast; UMF–Umfolozi)

Twenty-two out of the 44 DNA fragments that were successfully cloned and sequenced were tentatively identified as *P. melanocephala*. Five of these sequences, four from the South Coast and one from the Umfolozi area, were of sufficient quality for phylogenetic analysis. These sequences along with GenBank sequences from Japan and Hawaii were compared in a Neighbor-Joining phylogenetic tree (Figure 2). This analysis suggests the presence of two or possibly three different *P. melanocephala* races. DNA isolated in Australia from orange urediniospores was identified as *P. kuehnii*, while that isolated from brown urediniospores was identified as *P. melanocephala*. This shows that the cloning and sequencing products

amplified using NL1 and NL4 primers could be used to differentiate the two species of rust infecting sugarcane.

Further studies of *P. melanocephala* populations may indicate differences in pathogenicity, which could have resulted in changes in the susceptibility of certain varieties to brown rust. A database has now been established to provide gene sequences of *P. melanocephala* for comparative purposes in the event of future outbreaks.

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