

Taxonomic identity of the Sterile Red Fungus inferred using nuclear rDNA ITS 1 sequences

Vera ANDJIC*, Anthony L. J. COLE and John D. KLENA‡

Department of Plant and Microbial Sciences, Private Bag 4800, University of Canterbury, Christchurch, New Zealand.
E-mail: v.andjic@murdoch.edu.au

Received 29 May 2003; accepted 13 September 2004.

The taxonomic position and properties of an unidentified fungus isolated from wheat roots was investigated. The Sterile Red Fungus (SRF) is characterised by its fast growing habit, red pigmentation, non-sporing nature and mycelial form resembling some *Laetisaria* and *Limonomyces* species. rDNA variation was used to study the relationship of the SRF to *Laetisaria* spp. and *Limonomyces* spp. Nucleotide sequence obtained from eight *Laetisaria* and three *Limonomyces* species representing the ITS 1 region, were analysed by PCR and direct sequencing. Plant growth promoting properties of the five taxa were also determined. The SRF had closest identity (98%) to British material of *Limonomyces roseipellis*. UPGMA analysis of ITS 1 DNA sequence support a close relationship between SRF and *L. roseipellis*. The relationship inferred by nucleotide sequence data was supported by phenotypic analysis; both *L. roseipellis* and the SRF were shown to promote the growth of wheat plants. Unexpectedly, *Laetisaria arvalis* and material named as *Limonomyces roseipellis* from New Zealand appeared to be closely related with 98% rDNA sequence identity, suggesting the misidentification of the New Zealand collection.

INTRODUCTION

The Sterile Red Fungus (SRF) is an endophyte associated with *Gramineae*. It was originally isolated from surface-sterilised roots of wheat (*Triticum aestivum*) and ryegrass (*Lolium rigidum*) grown in Western Australia (Dewan & Sivasithamparam 1988). The major characteristics of the fungus are its high rhizosphere competence on roots of wheat and ryegrass, and its ability to promote growth of wheat plants (Gillespie-Sasse *et al.* 1991). The SRF may have the potential to be an important biocontrol agent because it is capable of growth over a range of temperature and pH, is a rapid coloniser of roots, and stimulates growth of plant roots and shoots (Dewan & Sivasithamparam 1990), has a wide host range and has been shown to protect wheat against the 'take all' pathogen *Gaeumannomyces graminis* var. *tritici* in laboratory, glasshouse, and limited field trials in Western Australia (Dewan & Sivasithamparam 1988, 1989a, b, c). In view of the potential importance of this fungus, it was important to ascertain its taxonomic affiliation.

SRF is sterile but has been observed to produce clamp connections and is, therefore, basidiomyceteous in nature. However, its formal taxonomic status is unknown. Certain characteristics, especially its rapid growth rate, red pigmentation and association with cereals are similar to those reported for some *Laetisaria* spp. and *Limonomyces* spp. also associated with *Gramineae*. In an attempt to resolve the taxonomic position of SRF, DNA sequencing of the ITS 1 region of rDNA was performed and the resultant sequence was used in a phylogenetic analysis of ITS 1 regions obtained from morphologically similar *Laetisaria* and *Limonomyces* isolates.

Nucleotide sequences coding for rDNA change very slowly over time and are useful for the evolutionary comparison of relatively distant organisms. rDNA consists of highly conserved regions interspersed with variable regions such as internal transcribed spacer regions. ITS regions separate the 18S cistron from 5.8S cistron and the 5.8S cistron from 28S cistron. They contain a high level of nucleotide sequence divergence and may be used to analyse the phylogeny of very closely related species or even isolates of the same species (Fedoroff 1979). Analysis of ITS regions have been useful in clarifying a number of problems associated with fungal classification such as determining the phylogenetic relationship amongst isolates of *Pyrenophora*

* Corresponding author. Present address: School of Biological Sciences and Biotechnology, South Street, Murdoch University, Perth, Western Australia 6150, Australia.

‡ Present address: School of Molecular Biosciences, Washington State University, Pullman, WA 99164-4234, USA.

spp. that infect barley and oats (Stevens *et al.* 1998). Sequence analysis of the ITS region has also been used to identify species and isolates of pore fungi *Antrodia* spp and *Oligoporus placenta* (Schmidt & Moreth 2003).

MATERIALS AND METHODS

Origin and source of isolates

The fungal isolates used, with information on the host, environment, and country of origin, source, and ITS-1 sequence GenBank accession no. were as follows:

- (1) Sterile Red Fungus 1, wheat root, Western Australia (University of Western Australia), ITS 1 AY266047.
- (2) Sterile Red Fungus 2, wheat root, Western Australia (University of Western Australia), AY266048.
- (3) *Laetisaria arvalis*, agricultural soil, Delaware Co., Ohio, USA, CBS 339.81), ITS 1 AY266049.
- (4) *Laetisaria fuciformis*, turf grass, UK (CBS 170.36), ITS 1 AY613908.
- (5) *L. fuciformis*, turf grass, Vogelenzang, The Netherlands (CBS 182.49), ITS 1 AY613910.
- (6) *L. fuciformis*, *Lolium perenne*, Wolfhere, Papandal, Sportvelden NS, The Netherlands (CBS 443.80), ITS 1 AY613913.
- (7) *L. fuciformis*, *Lolium perenne*, Wolfhere, Papandal, Sportvelden NS, The Netherlands (CBS 444.80), ITS 1 AY613911.
- (8) *L. fuciformis*, *Lolium perenne*, Wolfhere, Papandal, Sportvelden NS, The Netherlands (CBS 445.80), ITS 1 AY613909.
- (9) *L. fuciformis*, *Festuca* sp., RIVRO experimental fields, Sevenum, The Netherlands (CBS 447.80), ITS 1 AY613912.
- (10) *L. fuciformis*, *Lolium perenne*, Wageningen garden, Plantenziektenkundige, The Netherlands (CBS 617.80), ITS 1 AY613914.
- (11) *Limonomyces culmigenus*, authentic strain from *Auriscalpium vulgare* in North Woolton, UK (CBS 136.69), ITS 1 AY266050.
- (12) *L. roseipellis*, UK (University of Uppsala, UPS 2373), ITS 1 AY266051.
- (13) *L. roseipellis* (Auckland), leaves of cotula (*Leptirella* sp.), (AgResearch, Auckland), ITS 1 AY613915.

Culture conditions

All isolates were grown in 250 ml Erlenmeyer flasks containing 100 ml of potato dextrose broth at 20 °C and aerated, using an orbital shaker, for 3 wk. Mycelium was harvested by filtration through muslin then freeze dried.

DNA extraction

DNA was extracted from freeze dried mycelia by grinding in liquid nitrogen using a pestle and mortar. One ml of genomic DNA isolation reagent (DNAzol; Gibco BRL, Life Technologies, LA) was added to 60 mg

of the dry mycelium mycelia in a tube, mixed by inversion and incubated at ambient temperature for 1 h. After incubation, mixtures were centrifuged for 15 min at 14 700 *g* and viscous supernatants were transferred to fresh tubes. 0.5 ml of ice cold isopropanol was added to each sample, mixed by inversion and incubated at ambient temperature for 1 h. After incubation, each mixture was centrifuged for 10 min at 14 700 *g*, supernatant discarded and DNA washed with 1 ml 70 % ice cold ethanol by centrifuging at 14 700 *g* for 5 min. After centrifuging, ethanol was decanted and DNA allowed to air dry for 15 min. DNA was resuspended in 50 µl of sterile double distilled water (DDW).

PCR amplification

PCR was performed using a Corbett Research FTS-320 Thermal Sequencer. Primers ITS 1 and ITS 2 (Gibco BRL; final concentration 100 pmol in both cases). Each 100 µl reaction mixture contained: sterile DDW, 10 × *Taq* reaction buffer (Qiagen, Chatsworth, LA), dNTP's master mix (final concentration 200 µM), ITS 1 (final concentration 100 pmol), ITS 2 (final concentration 100 pmol), template DNA (approx. concentration 20 µM), *Taq* polymerase (two units, Qiagen) and overlaid with of sterile mineral oil. PCR conditions consisted of 26 cycles of 94 °, 1 min, 42 °, 2 min, 74 °, 3 min and 4 ° 5–10 min (Ward & Gray 1992). To detect possible contamination in the amplification reaction, a negative control which contained all reaction components except the fungal template DNA was used with every reaction. After PCR was completed 15 µl of PCR product was applied to a 2 % agarose gel and subjected to electrophoresis. The size of the amplicons was close to 300 bp.

DNA sequencing

Prior to DNA sequencing, unincorporated nucleotides and residual oligonucleotide primers were removed from PCR amplicons using a Qiagen PCR clean-up kit. DNA sequencing was performed at the University of Waikato (New Zealand) sequence facility using an ABI Prism 377 DNA sequencer and fluorescent dye technology. Redundancy in the sequence was achieved using ITS 1 and ITS 2 as sequencing primers.

Analysis of DNA sequences

ITS 2-primed nucleotide sequences were reversed and complemented using the program DNA strider (Christian Marck, Commissariat à l'Énergie Atomique, Gif-sur-Yvette Cedex, France). ITS sequences were aligned using the program Clustal W using the default settings (Thompson *et al.* 1994). Edited DNA sequences were initially compared to similar sequences housed in GenBank using a BLAST search. Nucleotide sequences showing strong identity to the query sequence were retrieved from GenBank and included in a phylogenetic

analysis. The relationship between DNA sequences from different isolates was determined by construction of phylogenetics trees using the software package Mega 2.1 (Kumar *et al.* 2001). Phylogenetic trees were constructed using UPGMA analysis (Sneath & Sokal 1973) and evolutionary distances calculated using the method of Jukes and Cantor (Jukes & Cantor 1969). Bootstrap analysis (Felsenstein 1985) was performed using 1000 replicates.

Plant growth studies

The effect of SRF, *Laetisaria* spp. and *Limonomyces* spp. on height growth of wheat was compared in a wheat seedling assay as used by Dewan & Sivathamparam (1988) in non-sterile conditions. The isolates used in this study are listed above. *Gaeumannomyces graminis* var. *tritici* was used as positive control.

Preparation of inocula

Low-endophyte ryegrass seeds (Marathon, Wrightson, Christchurch, NZ) were washed and autoclaved at 120 ° for 50 min in 20 g lots in 250 ml flasks. Each flask of ryegrass seed was then inoculated with one 5 mm diameter disk from the growing margins of each fungal culture on PDA. The inoculated seeds were incubated for 14 d at ambient temperature (approx. 20 °).

Growth studies

The rye-grass inoculum (5 g) of each isolate was mixed with 1 kg of fine river sand (pH 7.1, N=0.04 %, C=0.062 %). For each fungal treatment, 200 g of sand with inoculum was placed into each of four replicates plastic cups. Wheat seeds (cv. 'Monad') were washed and surface sterilised with 1.25 % bleach. In order to break dormancy, seeds were placed on moistened Whatman No. 181 filter paper and incubated at 5 ° for 4 d after which time they were left to germinate for 24 h at approximately 20 °. Five seeds were planted in each of four cups and incubated in a growth chamber at 20 ° with a 12 h photoperiod. Sterilised wheat seeds were used as negative controls. Plants were watered every second day. After 3 wk plants were harvested, washed free of sand and plant height measured by placing the plant on graph paper.

Data analysis

To test the significance of differences in the shoot's average height between isolates analysis of variation (ANOVA) was applied using Microsoft Excel. The significance level was 95%. To obtain the multiple comparisons procedure between fungal isolates and control, Tukey's test was used (Statistical Analysis System SAS).

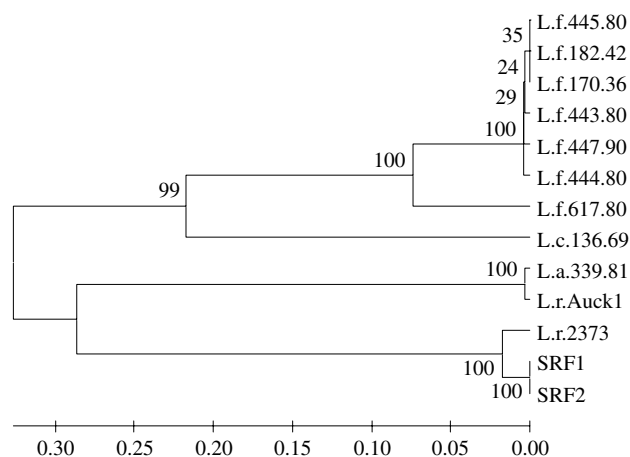


Fig. 1. Phylogenetic relationship of the Sterile Red Fungus to isolates of *Laetisaria* spp. and *Limonomyces* spp. The dendrogram was constructed using the ITS-1 nucleotide sequence (279 base pairs). DNA sequences were analysed using the UPGMA method (Sneath & Sokal 1973) and evolutionary distances calculated using the method of Jukes & Cantor (1969). Numbers at the branch nodes represent bootstrap values as described in the Materials and Methods. L.f., *Laetisaria fuciformis*; L.a., *Laetisaria arvalis*; L.r., *Limonomyces roseipellis*; SRF, Sterile Red Fungus.

RESULTS

Genetic relationships of the Sterile Red Fungus

Given its growth characteristics over a wide range of pH and temperature, as well as its ability to colonize and stimulate root growth, the SRF may have potential as a biocontrol agent against the 'take all' pathogen *Gaeumannomyces graminis* var. *tritici*. However, no genetic information was available regarding the relationship that SRF has with other morphologically similar fungi. Thus we determined the nucleotide sequence of the ITS 1 region from two independent isolates of the SRF. Additionally, we determined the sequence from isolates of *Laetisaria* spp. and *Limonomyces* spp. as they shared some morphological similarities to the SRF.

The ITS 1 region from the SRF was compared to nucleotide sequences in the National Center for BioInformatics database. Using the 279 bp of nucleotide sequence, the closest match to the SRF was obtained with *Russula liliacea* AY061731.1. However, a phylogenetic analysis of the SRF and *Russula liliacea* suggested that these fungi were less than 70% similar (data not shown). We therefore inferred the genetic relationship between the SRF isolates using the nucleotide sequence obtained from the ITS 1 region from several morphologically related fungi.

Analysis of the ITS 1 nucleotide sequence showed that the thirteen isolates separated into four distinct branches at the level of 80% identity (Fig. 1). One cluster consisted of the two isolates of the SRF and *Limonomyces roseipellis* (UK). The two isolates of the SRF were greater than 99.5% identical and these

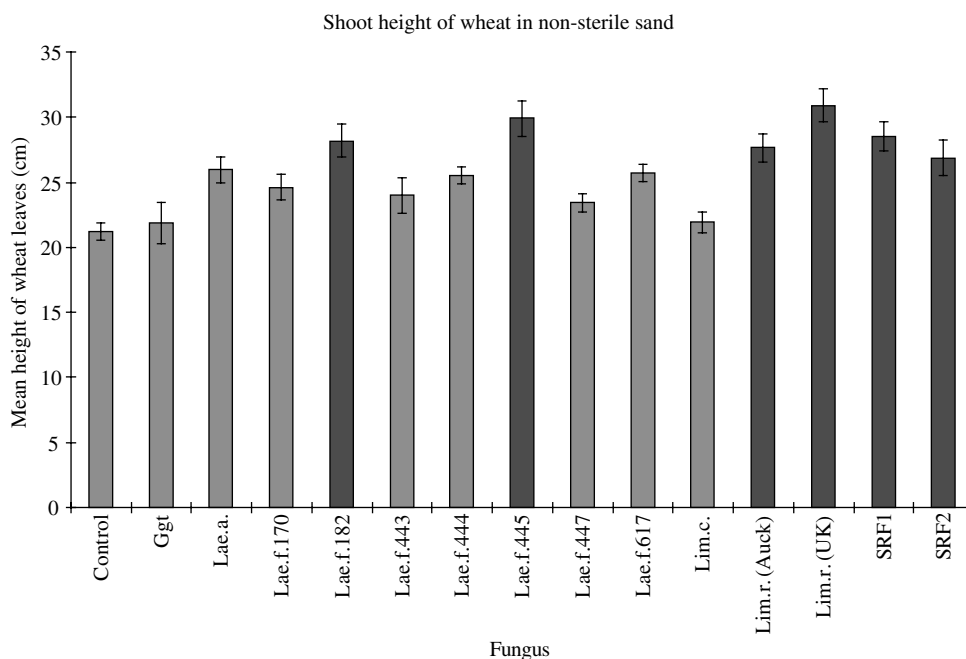


Fig. 2. Effect of the SRF, *Laetisaria* spp. and *Limonomyces* spp. on shoot height of wheat in non-sterile sand. Dark bars denote significant differences between each isolate and the control at 95% confidence level.

isolates were shown to be greater than 98% identical to *L. roseipellis* (UK). Bootstrap analysis suggested that the relationship between these isolates was highly significant. A second cluster, consisting of one isolate of *Laetisaria arvalis* and the *Limonomyces roseipellis* (Auckland) isolate (>99% identical) were less than 72.5% identical to the SRF cluster. *Limonomyces culmigenus* was the sole member of the third cluster. The fourth cluster consisted of the *Laetisaria fuciformis* isolates. Clusters 3 and 4 were less than 70% identical to the SRF isolates. Of interest is the apparent discrepancy in the genetic relationship amongst collections named as *Limonomyces roseipellis* as well as among isolates of *Laetisaria* spp.

Growth studies

At 95% confidence levels, significantly greater wheat shoot growth was seen with *Limonomyces roseipellis* (UK) (mean height 30.90 cm, 45.6%), *Laetisaria fuciformis* strain 445.80 (29.91 cm, 41%), SRF1 (28.51 cm, 34.4%), *Laetisaria fuciformis* strain 182.49 (28.16 cm, 32.7%), '*Limonomyces roseipellis*' (Auckland) (27.65 cm, 30.3%), and SRF2 (26.87 cm, 26.6%) when compared with the control (21.21 cm) (Fig. 2).

Limonomyces roseipellis (UK) produced the greatest increase in shoot height, but the average was only 0.99 cm greater than *Laetisaria fuciformis* 445.80. Both isolates of the SRF and of *Limonomyces roseipellis* and two isolates of *Laetisaria fuciformis* (182.49 and 445.80) gave a significant increase in wheat seedling growth.

DISCUSSION

In this study, the analysis of rDNA sequences of the ITS 1 region was useful in resolving the phylogenetic relationship between the SRF and the isolates of the genera *Laetisaria* and *Limonomyces*. Some members of *Laetisaria* spp. and *Limonomyces* spp. express phenotypes that resemble the SRF, notably rapid growth rate, red pigmentation and association with cereals. Based on our analysis, *Limonomyces roseipellis* (UK) is the closest species identified to date to the SRF. Given that the ITS-1 DNA sequence identity between these isolates was greater than 98%, we conclude that the SRF is an isolate of *Limonomyces roseipellis*.

Based on the previous identifications, we expected *L. roseipellis* (Auckland) to be highly related to *L. roseipellis* (UK) (Peter Buchanan, pers. comm.). We were therefore surprised to find that the ITS1 sequence data placed the two *L. roseipellis* isolates in different clusters. Of note is that *L. roseipellis* (Auckland) had growth characteristics indistinguishable from those of the phylogenetically similar *Laetisaria arvalis*. In addition, when inoculated on rye grass roots, indistinguishable symptoms were observed (data not shown). These symptoms were not present in rye grass roots inoculated with the SRF. Based on these observations, we believe that *Limonomyces roseipellis* (Auckland) should be reidentified as *Laetisaria arvalis*.

Further evidence of the close relationship of SRF to *Limonomyces roseipellis* is seen by their ability to promote the growth of wheat plants. Interestingly, two isolates of *Laetisaria fuciformis* also showed this ability, but this needs further studies.

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Corresponding Editor: D. S. Hibbett