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SID 5 Research Project Final Report

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Project identification

1. Defra Project code: PH0302

2. Project title:
   Plant Health Fellowship 1: Development of molecular techniques to distinguish vegetative compatibility groups in fungi: Rhizoctonia spp. a case study

3. Contractor organisation(s):
   Central Science Laboratory
   Sand Hutton
   York
   YO41 1LZ

4. Total Defra project costs: £ 15000

5. Project: start date ..... 01 March 2004

   end date ..... 30 April 2005

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Executive Summary

7. The executive summary must not exceed 2 sides in total of A4 and should be understandable to the intelligent non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

In recent years the development of diagnostic conventional and real-time (TaqMan®) PCR assays has increasingly focussed on the use of nuclear ribosomal DNA as a target sequence. Due to the availability of reliable universal primers many studies have utilised the internal transcribed spacer (ITS) region. The ITS region is non-coding, and like many other non-coding regions (for example introns), evolves rapidly compared to coding regions, where even single base pair mutations could alter the protein structure considerably. This rapid evolution increases the chance of finding the unique sequences required for diagnostic assay design. More recently protein coding regions such as cytochrome oxidase (COX) and β-tubulin have proven valuable for discrimination between closely related groups. The use of the COX region for designing sequence specific primers is being investigated in project PH0305. This coupled with the lack of suitable primers to amplify the COX region of *Rhizoctonia* spp. meant this study concentrated on the β-tubulin region.

Rhizoctonia is a large, diverse and complex genus of fungi. Due to morphological similarities, the species is divided into anastomosis groups (AGs) and sub-groups on the basis of somatic or vegetative incompatibility between isolates in culture. The genus *Rhizoctonia* will be used as a model system for determining the suitability of the β tubulin region for the design of diagnostic assays.

A collection of 120 isolates of *Rhizoctonia* anamorphs were selected from the CSL culture collection. ITS and β tubulin sequences were generated and a comparison made between clustering trees generated using each area.

Some difficulty was experienced using primer pairs designed to amplify the β-tubulin region. Non-specific primer annealing affected the production of readable fragments for some isolates. The ITS and β tubulin region were sequenced for 58 *Rhizoctonia* isolates. The β-tubulin sequences of Rhizoctonia *spp.* were structured differently to other related fungi. Such splicing polymorphism offers the opportunity for unique sequences to arise, but also diminishes the possibility of universal primers working on all fungi. The difficulties experienced, whilst surmountable, highlight the need for the design of truly specific primer to amplify the tubulin genes.

Overall the ITS and β-tubulin exhibits similar sequence divergence between intraspecies and interspecies comparisons. Binucleate clades were successfully resolved from multinucleate clades with maximum bootstrap confidence for both ITS and β-tubulin data sets.

Several test assays were designed to β-tubulin sequences for AGs where previous ITS-based assays had failed to discriminate specific AGs. The β-tubulin sequences were easier to align and potentially successful sequence polymorphisms were identified for four problem groups.

A better understanding of the protein coding regions such as β-tubulin increases the chances of identifying truly diagnostic sequences. In the future this will enable us to more rapidly target appropriate regions of sequence for the design of diagnostic assays to both non-quarantine and quarantine fungal pathogens. This work provides a good starting point for the fungal aspects of objective 1 of the project PH0305 'Novel diagnostic techniques for the detection of pests and diseases of statutory concern' which aims to investigate which gene or genes can be used to discriminate species for bacterial pathogens (Multi Locus Sequence Typing), fungal pathogens and invertebrate pests. In this project the aim is to investigate sequences in addition to the currently used ITS regions for *Phytophthora ramorum*. The aim is to further the study of COI sequences for this and related species, but the development of tubulin primer sets in this project will allow further analysis of not just ITS and COI but also β-tubulin sequence, which have been shown here to be useful from the point of view of designing discriminatory PCR based assays.

Further analysis of the data is required to elucidate evolutionary relatedness between different subgroups within the genus *Rhizoctonia*. This analysis will be concluded in the remaining two years of a PhD, initiated through this project, in association with Reading University.

8. As a guide this report should be no longer than 20 sides of A4. This report is to provide Defra with details of the outputs of the research project for internal purposes; to meet the terms of the contract; and to allow Defra to publish details of the outputs to meet Environmental Information Regulation or Freedom of Information obligations. This short report to Defra does not preclude contractors from also seeking to publish a full, formal scientific report/paper in an appropriate scientific or other journal/publication. Indeed, Defra actively encourages such publications as part of the contract terms. The report to Defra should include:
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   - the extent to which the objectives set out in the contract have been met;
   - details of methods used and the results obtained, including statistical analysis (if appropriate);
   - a discussion of the results and their reliability;
   - the main implications of the findings;
   - possible future work; and
   - any action resulting from the research (e.g. IP, Knowledge Transfer).

Introduction

In recent years the development of diagnostic conventional and real-time (TaqMan®) PCR assays has increasingly focussed on the use of nuclear ribosomal DNA as a target sequence. Due to the availability of reliable universal primers many studies have utilised the internal transcribed spacer (ITS) region. The ITS region is non-coding, and like many other non-coding regions (for example introns), evolves rapidly compared to coding regions, where even single base pair mutations could alter the protein structure considerably. This rapid evolution increases the chance of finding the unique sequences required for diagnostic assay design.

The ideal diagnostic sequence would evolve rapidly enough to generate useful sequence differences at the species or sub-species level, but conversely, evolve slow enough to produce minimal sequence variability between individuals within that level. Recent research has indicated that ITS regions of the nuclear rRNA may evolve so quickly that sequence polymorphisms exist within populations or even within a single fungal isolate (Justesen *et al.*, 2001). Practically, the large variation in ITS regions often presents difficulties when analysing sequence alignments for small differences. Also, for some species, particularly if investigating below the species level, (e.g. vegetative incompatibility or mating types) sequence differences often prove to be too small to exploit (e.g. *Phytophthora ramorum* A1 and A2 mating types).

More recently protein coding regions such as cytochrome oxidase (COX) and β-tubulin have proven valuable for discrimination between closely related groups including *Diabrotica* spp. (Roehrdanz *et al.*, 2003), *Phytophthora* spp. (Martin & Tooley, 2003), *Amanita* spp. (Oda *et al.*, 2004) and Colletotrichum spp. (Lubbe *et al.,* 2004). The tubulin gene family is composed of three major groups, the α, β and γ tubulins. α and β tubulins produce heterodimers which are the basic building blocks for microtubules. Microtubules are minute filaments that form an extensive network essential for the construction of the cytoskeleton (Nogales *et al.*, 1998). Benzimidazole fungicides, such as benomyl or carbendazim, target microtubule formation adding an interesting fungicide resistance angle to generating tubulin sequence data. The use of the COX region for designing sequence specific primers is being investigated in project PH0305. This coupled with the lack of suitable primers to amplify the COX region of *Rhizoctonia* spp. meant this study concentrated on the β-tubulin region.

Rhizoctonia is a large, diverse and complex genus of fungi. *Rhizoctonia solani* is a member of the Basidiomycotina whose teleomorph is *Thanatephorus cucumeris*. The fungus is characterised by brown septate hyphae with right angle branching and a slight kink in the hyphae prior to branching. Due to morphological similarities, the species is divided into anastomosis groups (AGs) and sub-groups on the basis of somatic or vegetative incompatibility between isolates in culture. In this study, the genus *Rhizoctonia* will be used as a model system for determining the suitability of the β tubulin region for the design of diagnostic assays. A comparison of the phylogenetic trees generated using sequence data from both ITS and β-tubulin regions for *Rhizoctonia* spp. and AGs will provide insight into the relative merits of these two target regions for discriminating fungal pathogens at and below the species barrier.

Methods

Storage and recovery of isolates

A collection of 120 isolates of *Rhizoctonia* anamorphs were selected from the CSL culture collection. In order to store isolates, barley kernels were placed in universals containing an equal volume of water and autoclaved for 30 min at 121 C on 2 successive days. For each isolate 5 cores (No. 4) were taken from the edge of an actively growing colony on PDA (amended with 0.6% Penicillin G and 2.0% streptomycin sulphate) and transferred to a 30 ml universal contained sterile barley seed. Universals were agitated and left to incubate for 14-21 days at 18°C until the fungus had fully colonised the seed. The barley was then dried for 14 days prior to being placed in storage at –20°C until required. Each isolate was recovered by placing 3 seeds onto PDA plate and incubating at 18°C.

DNA extraction from fungal cultures

DNA preparations were prepared using Wizard® Magnetic DNA Purification System for Food (Promega, FF3750) in conjunction with a Kingfisher ML magnetic particle processor (Thermo Electron Corporation). Mycelium was scraped from the surface of an actively growing culture and placed into a 2 ml screw cap tube containing 0.5 ml of 1 mm zirconia beads and 1ml Lysis Buffer A containing 10% antifoam B emulsion (Sigma). Samples were shaken at full speed for 30 s on a mini-beadbeater (BioSpec Products, Inc.) before following the standard Promega extraction protocol. The extraction was completed using the gDNA program including the optional heating stage on the Kingfisher ML. Samples were eluted into molecular grade water and stored at -30°C until required.

PCR amplification

The ITS regions were amplified using the primer pair ITS1 and ITS4 which amplifies ITS1, 5.8S rDNA, ITS2 with flanking regions of 18S and 28S rDNA (White *et al.*, 1990). The reaction mixture contained 5 pmol of each primer, 37.5 mmol $MgCl_2$, 5 mmol dNTP, 0.75 U of BIO-X-ACT Long DNA Polymerase (Bioline, BIO-21049) and 1 µl of template. Samples were cycled at 95°C for 4 min followed by 35 cycles of 95°C for 1 min, 52°C for 1 min and 72°C for 1 min with a final extension phase at 72°C for 2 mins using a T3 triple-block thermocycler (Anachem, UK).

Amplification of the partial sequence of the β-tubulin gene was achieved using degenerate primers B-TUB-IF and B-TUB-IR (Oda *et al.*, 2004). This primer set was designed to amplify a 500 bp region of the of β-tubulin gene of *Amanita* spp. including three exons and two introns. The reaction mixture and cycling conditions were identical to those described above except 20 pmol of each primer was used and the annealing temperature was reduced to 50°C.

Purification of PCR products and sequencing

PCR products were purified using a QIAquick PCR purification Kit (Qiagen) following manufacturers instructions. Purified products were quantified by visualising alongside a quantifiable marker (Hyperladder I, Bioline) on 1% agarose gels in 1 x TBE (90mM Tris-HCl pH 8.3, 90mM Boric Acid, 2 mM EDTA). Quantified products were diluted to 1.3 ng/µl and sent to the University of Dundee for sequencing.

Sequence alignment and analysis

Sequences alignments and percentage identity estimates were generated using clustal W within the MEGALIGN package (DNAstar, Lasergene6). Sequence distances were estimated using the algorithm of Jukes and Canter (1969), taking into account all positions and all omissions and deletions. Tree topology was inferred using the neighbour-joining algorithm of TREECON version 1.3b with 1000 bootstrap resamples (Van de Peer and de Wachter, 1993). Rooted clustering trees were drawn using TREECON version 1.3b and the sequence of isolate 1986 (*Rhizoctonia zeae*) was used as an outgroup. Select β-tubulin sequences were analysed for the location of open reading frames (ORFs) using EditSeq and GeneQuest (DNAstar, Lasergene6). Putative gene products were translated and NCBI protein database interrogated.

Real-time(TaqMan®) primer and probe design

Specific real-time primers and probes were designed to 4 subgroups (AG3, AG4HGii, AG5 and AG9) of *Rhizoctonia* using Primer Express software (Applied Biosystems, USA). Sequences of β-tubulin were aligned and unique sequence patterns identified. Assays were tested by interrogating 80 β-tubulin sequences from related *Rhizoctonia* species.

Results

PCR amplification and sequencing

PCR products of the expected size were obtained for all 120 isolates selected from the collection using the universal ITS primers. Utilisation of degenerate primer pair B-TUB-IF and B-TUB-IR for the amplification of the partial sequence of β-tubulin gene only yielded PCR products from around a third of the isolates tested. To obtain a primer pair with greater specificity for *Rhizoctonia* spp., β-tubulin fragments from a range of AGs were cloned into a PGMT-easy vector. The inserted fragments were sequenced and primer pair B-TUB-3F (GAYAAYTTYGTDTTYGGHCAGA) and B-TUB-2R (TANARBGCYTCGTTRTCKATRCARA) designed. The combination of both sets of β-tubulin primers amplified 96/120 isolates. The quantity of β-tubulin PCR products fluctuated between isolates (Figure 1).

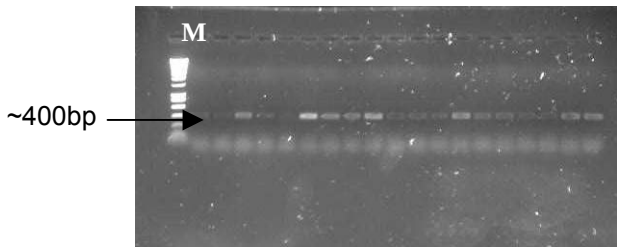


Figure 1. Gel image showing variation in quantity of PCR products between *Rhizoctonia* isolates compare to Hyperladder I marker (M).

Some β-tubulin sequence reads were unintelligible. Analysis of the chromatographs revealed multiple peaks for individual nucleotides, suggesting non-specific binding of the degenerate β-tubulin primers. In all, 80 β-tubulin sequences of approximately 360 bp were sequenced. Analysis of the β-tubulin sequence from isolate 1885 for ORFs suggests the sequence begins with a single intron running into a single exon encoding the β-tubulin gene.

Sequence alignment and analysis

After alignment and trimming, ITS and β-tubulin sequences from 58 isolates were compared (Table 2). Clustering trees were constructed using 450 bp of ITS and 225 bp of β-tubulin sequence. Isolates clustered in the same groups in both the ITS or β-tubulin trees although some positional rearrangement of clusters did occur (Figure 2).

Table 2. Anastomosis (AG/CAG) and subgroup designation, origin and source of *Rhizoctonia* species for which both ITS and β-tubulin sequence data was obtained.

Isolate	Origin	Subgroup	Source
1825 (USDA1)	-	AG1-IC	Dr. Chris Thornton, University of Exeter
1834 (CBS 101775, IPO 04R02)	Sugar beet, Japan	AG4HGii	Dr. Chris Thornton, University of Exeter
1836 (CBS 159.83)	Lilium sp., Netherlands	AG5	Dr. Chris Thornton, University of Exeter
1838 (CBS 101779, IPO 06R02)	Soil, Japan	AG6	Dr. Chris Thornton, University of Exeter
1842 (CBS 96996, ATCC90331)	Soil, USA	AG9	Dr. Chris Thornton, University of Exeter
1843 (CBS 97096, ATCC90334)	Potato, USA	AG9	Dr. Chris Thornton, University of Exeter
1851 (R31)	Orchard grass, Japan	AG1-IB	Prof. Hyakumachi, Gifu University
1866 (H5-354)	Soybean, Japan	AG2-3	Prof. Hyakumachi, Gifu University
1885 (21-01, Ps-4)	Pea, Japan	AG2-1	Dr. Hans Schneider, IRS
1888 (03-01, ST-11-6)	Potato, Japan	AG3	Dr. Hans Schneider, IRS
1889 (04-03, Rh165)	Sugar Beet, Japan	AG4HGii	Dr. Hans Schneider, IRS
1901 (R14, 1/97 T1)	Potato, N. Ireland	AG3	Dr. Alison Lees, SCRI
1918	-	AG2-1A	Dr. Tony Keinath, Clemson University
1924	-	AG7	Dr. Tony Keinath, Clemson University
1926	-	AG9	Dr. Tony Keinath, Clemson University
1929 (01R11, PD 77/679)	Gladiolus, Netherlands	AG1-IC	Dr. Paul van den Boogert, PRI
1931 (22R29)	-	AG2-2 IV	Dr. Paul van den Boogert, PRI
1933 (2tabacR01)	-	AG2tabac	Dr. Paul van den Boogert, PRI
1935 (ATCC 66154, 21R01)	-	AG2-1	Dr. Paul van den Boogert, PRI
1936 (ATCC 76167)	-	AG3	Dr. Paul van den Boogert, PRI
1940 (06R01, OMT-1-1)	Soil, Japan	AG6	Dr. Paul van den Boogert, PRI
1942 (08R31, 1512)	Barley, Australia	AG8	Dr. Paul van den Boogert, PRI
1943 (ATCC 62804)	-	AG9	Dr. Paul van den Boogert, PRI
1944 (ATCC 76107)	Barley, USA	AG10	Dr. Paul van den Boogert, PRI
1945 (ATCC 90857)	-	AG11	Dr. Paul van den Boogert, PRI
1947	Cauliflower, UK	AG2-1	CSL collection
1950	Cauliflower, UK	AG2-1	CSL collection
1953	Cauliflower, UK	AG2-1	CSL collection
1960	Lettuce, UK	AG2-1	Cathryn Lambourne, STC
1966	Cauliflower, UK	AG2-1	Mandy Shepherd, ADAS
1968	Spring Cabbage, UK	AG4HGii	CSL collection
1970	Spring Cabbage, UK	AG2-1	CSL collection
1971	Broccoli, UK	AG2-1	CSL collection
1973	Brussels Sprouts, UK	AG2-1	CSL collection
1975	Brussels Sprouts, UK	AG2-1	CSL collection
1976	Brussels Sprouts, UK	AG2-1	CSL collection
1977	Brussels Sprouts, UK	AG2-1	CSL collection
1986	Soil, UK	R. zeae	CSL collection
1990	Cabbage, UK	AG2-1	CSL collection
1991	Cabbage, UK	AG2-1	CSL collection
2001	Savoy cabbage, UK	AG2-1	CSL collection
2004	Cauliflower, UK	AG2-1	CSL collection
2022	Cauliflower, UK	AG2-1	CSL collection
2023	Cauliflower, UK	AG2-1	CSL collection
2307	Cauliflower, UK	AG4HGii	CSL collection
2308	Broccoli, UK	AG4HGii	CSL collection
2310	Broccoli, UK	AG4HGii	CSL collection
2315	Cauliflower, UK	AG4HGii	CSL collection
2316	Cauliflower, UK	AG4HGii	CSL collection
2318	Cauliflower, UK	AG4HGii	CSL collection
2319	Cauliflower, UK	AG4HGii	CSL collection
2325	Broccoli, UK	AG2-1	CSL collection
2335	Broccoli, UK	AG2-1	CSL collection
09 (BN31)	Peanut, GA	CAG-3	Dr. Marc Cubeta, NCSU, USA
10 (BN38)	Soybean, GA	CAG-4	Dr. Marc Cubeta, NCSU, USA
13 (C-610)	Unknown, Japan	AGD	Dr. Marc Cubeta, NCSU, USA
41	Soil, Japan	AGH	Dr. Marc Cubeta, NCSU, USA
167 (RS-44)	-	AG4HGiii	Dr. Marc Cubeta, NCSU, USA

A.

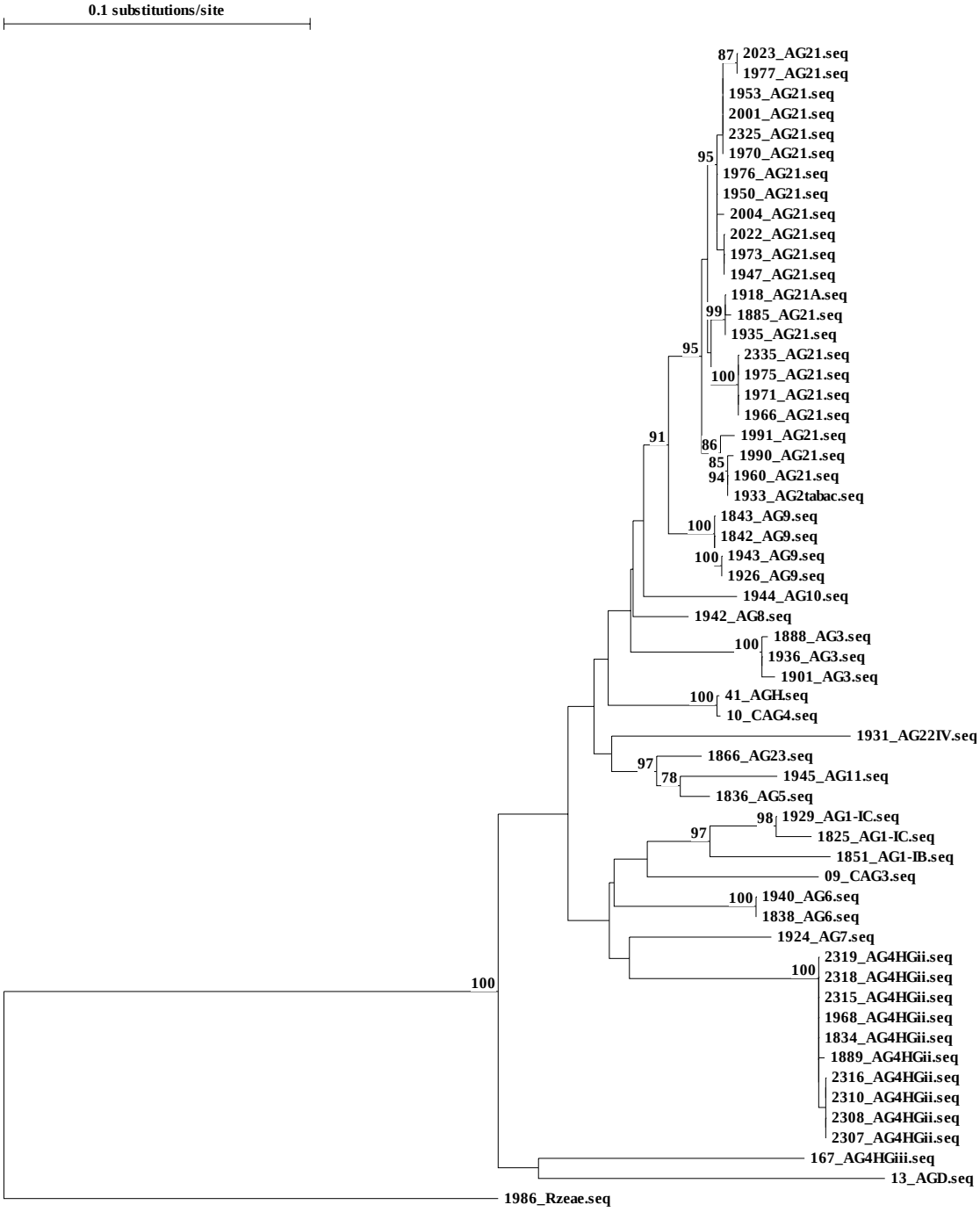
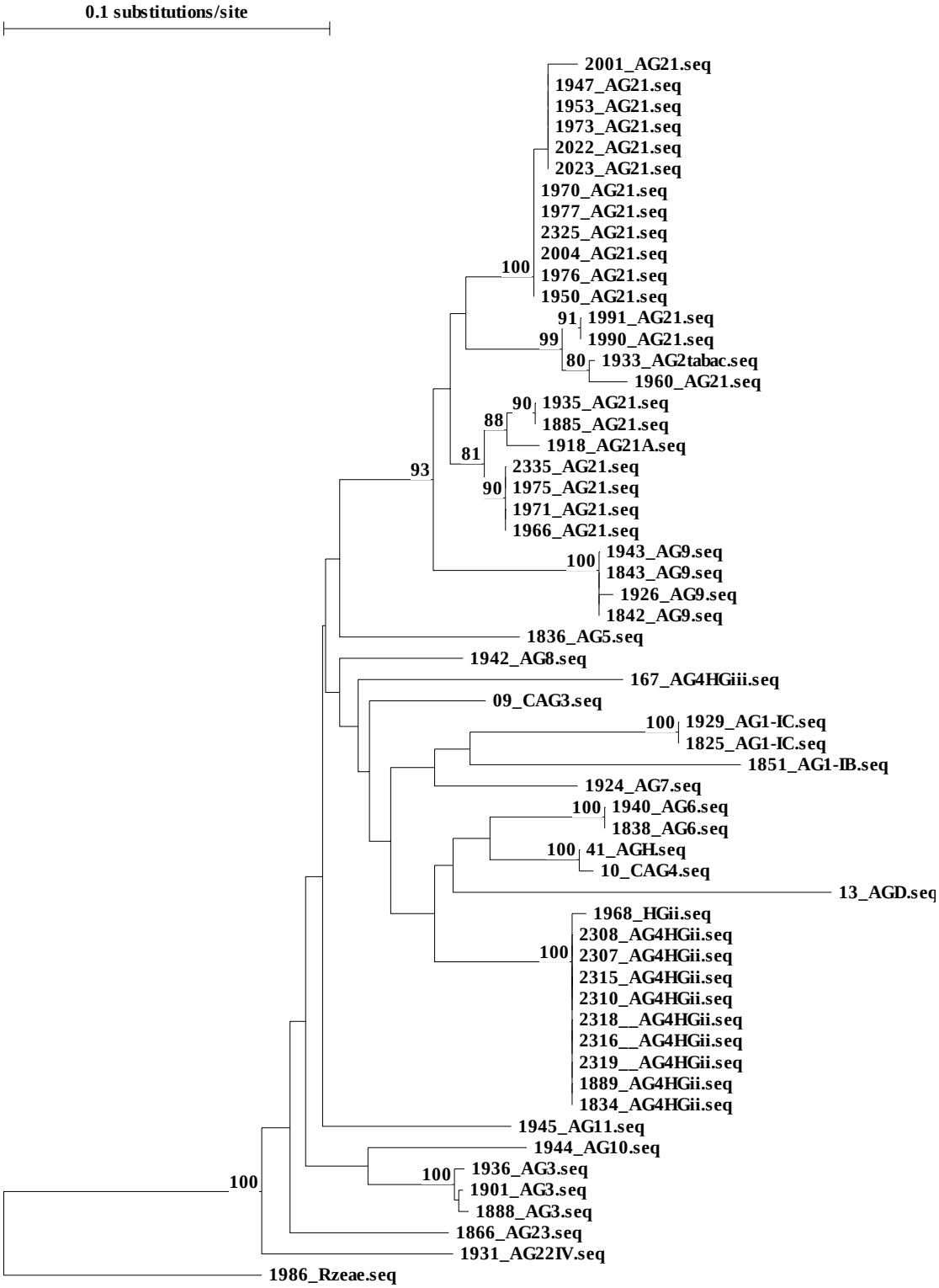


Figure 2. Clustering trees generated from sequence data spanning partial sequence of ITS 1, ITS2, the 5.8S region of nuclear encoded ribosomal DNA (A) and intron and partial sequence of the β-tubulin gene (B). The results of bootstrap analysis are displayed as the percentage of trees from 1000 replicate samples displaying each node. Only bootstrap values greater or equal to 70 are displayed.

B.


0.1 substitutions/site
2001_AG21.seq
1947_AG21.seq
1953_AG21.seq
1973_AG21.seq
2022_AG21.seq
2023_AG21.seq
1970_AG21.seq
1977_AG21.seq
2325_AG21.seq
2004_AG21.seq
100
1976_AG21.seq
1950_AG21.seq
91
1991_AG21.seq
99
1990_AG21.seq
80
1933_AG2tabac.seq
1960_AG21.seq
90
1935_AG21.seq
88
1885_AG21.seq
1918_AG21A.seq
81
93
2335_AG21.seq
90
1975_AG21.seq
1971_AG21.seq
1966_AG21.seq
100
1943_AG9.seq
1843_AG9.seq
1926_AG9.seq
1842_AG9.seq
1836_AG5.seq
1942_AG8.seq
167_AG4HGiii.seq
09_CAG3.seq
100
1929_AG1-IC.seq
1825_AG1-IC.seq
1851_AG1-IB.seq
1924_AG7.seq
100
1940_AG6.seq
1838_AG6.seq
100
41_AGH.seq
10_CAG4.seq
13_AGD.seq
1968_HGii.seq
2308_AG4HGii.seq
100
2307_AG4HGii.seq
2315_AG4HGii.seq
2310_AG4HGii.seq
2318__AG4HGii.seq
2316__AG4HGii.seq
2319__AG4HGii.seq
1889_AG4HGii.seq
1834_AG4HGii.seq
1945_AG11.seq
1944_AG10.seq
100
1936_AG3.seq
100
1901_AG3.seq
1888_AG3.seq
1866_AG23.seq
1931_AG22IV.seq
1986_Rzeae.seq

Real-time(TaqMan®) primer and probe design
Real-time assays were developed to specifically detect 4 AG groups using aligned β-tubulin sequences. Sequences of isolates known to cross-react with the current AG3 assay (Lees *et al.*, 2002) were aligned with a sequence from an isolate of AG3. The real-time assay based on β-tubulin sequence contained more than twice the number of unique sequence differences to distinguish between the cross-reacting group and AG3 compared to original ITS based assay.

Discussion

Ease of access to ITS sequences has led to a large number of diagnostic assays utilising this sequence. ITS has many advantages; conserved flanking ribosomal subunits ensure primer specificity; a wealth of sequence data with which to compare to a target organism; the area is relatively free from functional constraints and evolves rapidly enough to present useful sequence divergence. More recently ITS regions have failed to distinguish between species where protein encoding regions have succeeded (Fahleson *et al.,* 2004). The aim of the current study was to assess the suitability of the protein encoding region of β-tubulin for the design of sub-species specific assays using genus *Rhizoctonia* as a model system.

Some difficulty was experienced using both primer pairs designed to amplify the β-tubulin region. Both sets of β-tubulin primers produced PCR products of differing concentration and in some cases no PCR product was detected. Poor sequencing reads for some isolates provides further evidence that non-specific primer annealing is occurring with these primers. Primer pair B-TUB-1F/R were designed for use on *Amanita* spp. where they amplified a 500 bp product consisting of three exons and two introns (Oda *et al.*, 2004). The positioning of introns in β-tubulin sequences of *Rhizoctonia* spp. was different, containing only one intron and one exon. Such splicing polymorphism offers opportunity for unique sequences to arise, but also diminishes the possibility of universal primers working on all fungi. The difficulties experienced, whilst surmountable, highlight the need for the design of truly specific primers to amplify the tubulin genes.

Percentage identity estimates for sequences from isolates within AG based clades followed similar patterns when comparing ITS and β-tubulin sequences. For example percentage identity estimates for the three isolates in the AG3 clade ranged from 99.1-99.6 for the β-tubulin sequence and 98.4-98.6 for the ITS. Previous research has found problems resolving differences between binucleate *Rhizoctonia* isolate BN38 CAG4 and AG6 using ITS sequences (Gonzalez *et al.*, 2001). Overall, sequence divergence was similar when comparing BN38 to AG6 isolates using β-tubulin (6.6) and ITS (10.0). Binucleate clades were successfully resolved from multinucleate clades with high bootstrap confidence (% figures) for both ITS and β-tubulin data sets.

Several studies have highlighted the large diversity in AG2-1 using isozyme polymorphism, DNA restriction analyses ITS sequences (Kuninaga *et al.*, 1997; Liu et al., 1992; Salazar *et al.*, 1999; Schneider *et al.*, 1997; Schneider *et al.*, 1997b). This study represents the first record of β-tubulin sequence from *Rhizoctonia* spp. Again data from β-tubulin and ITS sequences matches well, with the suggestion of between 3 and 5 clades within the subgroup of 2-1.

Several AGs have presented problems with cross-reaction when designing AG specific real-time assays using ITS regions. For example, the published assay for the specific detection of AG3 (Lees *et al.*, 2002) is known to cross-react with DNA preparations of AG2-1 isolates (data unpublished). In addition, an AG9 assay developed internally at CSL is thought to also detect AG2-1 isolates. Using alignments of β-tubulin sequence data, assays were designed to four problematic AGs. Although these assays are yet to be tested on 'real' DNA extracts, they prove that the β-tubulin region is potentially useful for designing diagnostic assays targeting below the species level. In addition, ITS sequences have been reported to be difficult to align (Gonzalez *et al.*, 2001). However, β-tubulin sequence aligned easily, making it far simpler to manually search for sequence differences, and predict (as far as possible) the outcome of the PCR.

Sequence alignment may become misleading due to gene rearrangements (e.g. inversion, transposition translocation) or unequal sequence length (Otu & Sayood, 2003). One alternate method for analysing the data would be to determine the relative complexity measure (RCM) for each sequence (Bastola *et al.*, 2004). This method obviates the need for sequence alignment and has been shown to be suitable for comparisons of whole genomes or sequences from single genes. Phylogenies constructed using RCM have been shown to be more robust than trees generated using methods based on alignment, retaining topology after the deletion of up to 50% of sequence data (Otu & Sayood, 2003). Further analysis of the data is required to elucidate evolutionary relatedness between different subgroups with the genus *Rhizoctonia* and to determine the significance of contrasting positions of several AG-based clades when comparing ITS and β-tubulin trees.

A better understanding of the protein coding regions such as β-tubulin increases the chances of identifying truly diagnostic sequences. In the future this will enable us to more rapidly target appropriate regions of sequence for the design of diagnostic assays to both non-quarantine and quarantine fungal pathogens.

This work provides a good starting point for the fungal aspects of objective 1 of the project PH0305 'Novel diagnostic techniques for the detection of pests and diseases of statutory concern' which aims to investigate which gene or genes can be used to discriminate species for bacterial pathogens (Multi Locus Sequence Typing), fungal pathogens and invertebrate pests. In this project the aim is to investigate sequences in addition to the currently used ITS regions for *Phytophthora ramorum*. The aim is to further the study of COI sequences for this and related species, but the development of tubulin primer sets in this project will allow further analysis of not just ITS and COI but also β-tubulin sequence, which have been shown here to be useful from the point of view of designing discriminatory PCR based assays.

References

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References to published material

9. This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project.