available at www.sciencedirect.com

SCIENCE DIRECT®

journal homepage: www.elsevier.com/locate/mycres

A molecular protocol using quenched FRET probes for the quarantine surveillance of *Tilletia indica*, the causal agent of Karnal bunt of wheat

Mui-Keng TAN[a,*], *Gordon M. MURRAY*[b]

[a]NSW Department of Primary Industries, Elizabeth Macarthur Agricultural Institute, PMB8, Camden, NSW 2570, Australia
[b]NSW Department of Primary Industries, EH Graham Centre for Agricultural Innovation, Wagga Wagga Agricultural Institute, PMB, Wagga Wagga, NSW 2650, Australia

ARTICLE INFO

Article history:
Received 15 April 2005
Received in revised form
9 August 2005
Accepted 25 August 2005
Corresponding Editor: Michael Weiß

Keywords:
Crop protection
Molecular identification
Tilletiales
Ustilaginomycete
Wheat exports

ABSTRACT

The current surveillance protocol for Karnal bunt of wheat in most countries, including the USA, European Union (EU), and Australia, involves the tentative identification of the spores based on morphology followed by a molecular analysis. Germination of spores is required for confirmation which incurs a delay of about two weeks, which is highly unsatisfactory in a quarantine situation. A two-step PCR protocol using FRET probes for the direct detection and identification of *Tilletia indica* from a very few number of spores ($\leq$10) is presented. The protocol involves amplification of the ITS1 DNA segment in the highly repeated rDNA unit from any *Tilletia* species, followed by FRET analysis to detect and unequivocably distinguish *T. indica* and the closely related *T. walkeri*. This rapid, highly sensitive, fluorescent molecular tool is species-specific, and could supersede the conventional microscopic diagnosis used in a quarantine surveillance protocol for Karnal bunt which is often confounded by overlapping morphological characters of closely related species.

Introduction

Tilletia indica, the cause of Karnal bunt of wheat (Mitra 1931, 1935), is a floret-infecting fungus that results in the production of bunted grains. Bunted grains emit a rotting fish smell due to the production of trimethylamine, and wheat products made from such severely infected grain are unpalatable (Sekhon *et al.* 1980; Singh & Bedi 1985).

This fungus is the target of strict quarantine regulations by many wheat growing countries and its presence raises trade barriers to wheat exports (Rush *et al.* 2005). Karnal bunt was first identified as a new disease in wheat near the town of Karnal in India in 1931 (Warham 1986). It has since been reported from Pakistan, Nepal (Singh *et al.* 1989), Afghanistan, Mexico (Joshi *et al.* 1983), Syria (Williams 1983), Brazil (Diekman & Putter 1995), the USA (Bonde *et al.* 1997; Ykema *et al.* 1996), Iran (Torarbi *et al.* 1996), and South Africa (Rong 2000; Crous *et al.* 2001). Australia is at a considerable distance from known infested areas and currently has a status of being free of Karnal bunt. Thus, quarantine is extremely important for Australia where a large proportion of wheat production is traded internationally. Any incursion would cause severe disruption to Australia's international wheat trade and consequent huge losses in export markets (Brennan & Warham 1990; Brennan *et al.* 1992; Murray & Brennan 1998).

A morphologically similar fungus, *T. walkeri*, was widely detected during the USA 1996 National Karnal Bunt Survey (Castlebury & Carris 1999). When first detected, the spores

* Corresponding author.
E-mail address: mui-keng.tan@dpi.nsw.gov.au
0953-7562/$ – see front matter © 2005 The British Mycological Society. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.mycres.2005.08.006

were described as *T. indica*-like teliospores, and were found on ryegrass growing alongside or in wheat fields (Bonde *et al.* 1997; Cunfer & Castlebury 1999). Thus, the spores were found in wheat seed washes in south-eastern USA (Castlebury & Carris 1999). *T. walkeri* only causes bunt of ryegrass and does not infect wheat, so unequivocal differentiation of this closely related species from *T. indica* is critical from a quarantine perspective.

Phylogenetic analysis has revealed that *T. indica* and *T. walkeri* have a closer genetic relationship with each other than with many other tuberculate-spored *Tilletia* species (Pimentel *et al.* 1998; Levy *et al.* 2001; Pascoe *et al.* 2005). Conventional taxonomic separation based on colour, spore sizes, and ornamentation, is labour-intensive and requires a considerable quantity of about 50 spores of each species for a statistically significant determination (Inman *et al.* 2003).

Real time PCR using dual-labelled probes to differentiate *T. indica* and *T. walkeri* has been reported (Frederick *et al.* 2000) and is currently one of the molecular methods routinely used in the USA (Frederick *et al.* 2000), European Union (Inman *et al.* 2003) and Australia (Wright *et al.* 2003). This assay is based on the differential amplifications of a target mitochondrial fragment using a pair of *T. indica*-specific primers and a pair of *T. walkeri*-specific primers in two separate reactions (Frederick *et al.* 2000).

The ITS region between the nuclear small and large subunit ribosomal DNA (rDNA) has proved useful for distinguishing closely related species of fungi (e.g. Chen *et al.* 1992; Lee & Taylor 1992; Tan & Niessen 2003). Molecular characterisation using RFLPs of the ITS region was reported to differentiate *T. indica* and *T. walkeri* (Levy *et al.* 2001). Sequence analysis of the ITS fragments (comprising ITS1, 5.8SrDNA, ITS2) revealed only 2 nucleotide differences out of 612 nucleotides between *T. walkeri* and *T. indica*, with both nucleotide differences located in the ITS1 fragment of 258 bp.

Several PCR-based mutation-detection strategies using fluorescence resonance energy transfer (FRET) chemistry (Livak *et al.* 1995; Tyagi *et al.* 1998) can be used to discriminate allelic homologous genomic fragments with only one nucleotide difference. Conventional FRET analysis requires the use of two sequence-specific oligonucleotides labelled with two different fluorescent dyes which hybridize in a head to tail arrangement to an amplified target sequence (Wittwer *et al.* 1997). The donor probe which is fluorescently labelled at the 3′ end absorbs light emitted from a diode and emits light. This emitted light is then absorbed by a compatible fluorophore at the 5′ end of the acceptor probe when both donor and acceptor probes have annealed to the amplified target sequence. The acceptor probe then emits fluorescence at a different wavelength. The melting temperature (Tm) of the short, amplified target DNA duplex (usually between 150-200 bp) is sequence dependent and so the annealing temperature of the FRET probes to the amplicon during melt analysis is directly correlated with the sequence specificity of the amplicon. Thus the monitoring of fluorescence in a melt curve analysis allows the genotyping of mutations or single nucleotide polymorphisms (SNPs) in allelic amplicons. FRET-based PCR assays have been utilised to detect SNPs or mutations in different strains of microorganisms (Koo & Jaykus 2002; Orrů *et al.* 2004) and in different gene alleles (Dufresne *et al.* 2004).

This paper reports the use of a modification of the FRET approach where a quencher molecule is used in place of the second fluorophore to detect one A/G nucleotide difference in the ITS1 region to distinguish *T. indica* and *T. walkeri* (Fig 1). In the quenched FRET analysis, the amplification products are heated gradually after amplification and the change in fluorescent energy is monitored continuously to generate a melt curve profile, which only measures the increase in energy of the donor probe during the melt as denaturation occurs. This has the advantage of using less spectral bandwidth per probe set. Background fluorescence before the start of melt curve analysis is minimised by a denaturation step, followed by an annealing step, before commencing the melt curve analysis. The differential of the melt curve gives the melting temperature (Tm) for each allelic amplicon.

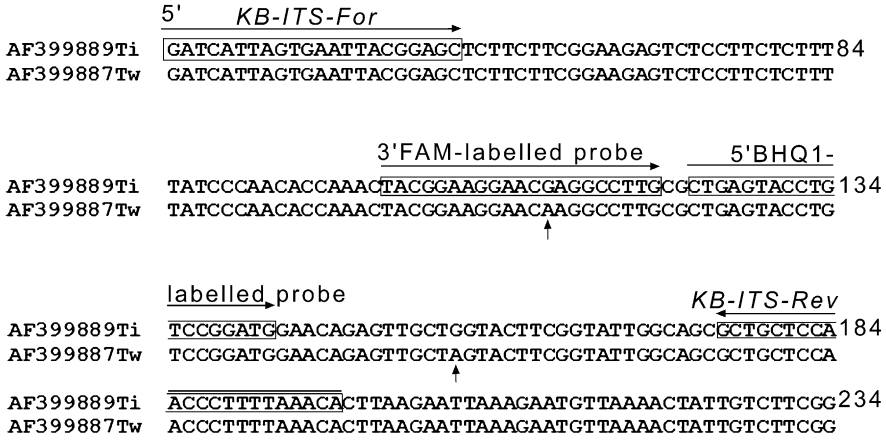

Fig 1 – Sequences and positions of primers and probes used in the diagnosis of *Tilletia indica* (Ti) and *T. walkeri* (Tw). The 3′FAM labelled probe hybridizes to a region with one nucleotide mismatch (arrow). The other mismatch (arrow) is outside the probe region. The 5′BHQ1-labelled probe was labelled with phosphate at the 3′ end to prevent any extension during PCR. The direction of the arrows indicates the orientation of the primers or probes. Numbers on the right refer to nucleotide positions in GenBank accession no. AF399889.

Materials and methods

Genomic DNA samples

The DNA samples of the *Tilletia* species used in this study (Table 1) with the exception of the DAR isolate were supplied from overseas laboratories on an AQIS permit 200322422.

Ten spores of a dried specimen of *T. walkeri* (DAR16720) were obtained for whole genome amplification using the GenomiPhi DNA amplification kit according to manufacturer's instructions (Amersham, Uppsala, Sweden). The walls of spores were broken to release the DNA by at least three freeze-thaw cycles. The spores were suspended in 20 μl of TE (pH 8), rapidly frozen at −70 °C in liquid nitrogen for 5 min and then rapidly heated to 95 °C by immersing the tube in a heated water bath for 5 min. The freeze-heat cycles were repeated at least three times. The DNA extract obtained was centrifuged at 11 500 *g* for 5 min. One microliter of the released nucleic acid was amplified according to the manufacturer's protocol and purified by alcohol precipitation.

Oligonucleotide primers and probes for FRET analysis

The GenBank ITS sequences of various *Tilletia* species, including *T. indica* (AF310174-AF310179, AF399888-9, AF135434, AF398434), *T. walkeri* (AF310180-1, AF310183-5, AF399887), *T. barclayana* (AF399894, AF310168-90), *T. horrida* (AF310171-3, AF399891-3, AF398435), *T. nigrifaciens* (AY309481), *T. ehrhartae* (AY770433), *T. laevis* (AF310166, AF398445), *T. fusca* (AF398455), *T. bromi* (AF398461), *T. caries* (AF310167), *T. goloskokovii* (AF310190), *T. cerebrina* (AF310188), *T. setariae* (AF399883), and *T. controversa* (AF398451), were aligned for the design of a reverse primer, Tilletia-R (5′-CAAGAGATCCGTTGTCAAAAGTTG, nt 215-277 of AF310176), which in conjunction with primer MK56 (Tan *et al.* 1996) allowed the amplification of the ITS1 region of the various *Tilletia* species.

Ten ITS sequences of *T. indica* (AF310174-AF310179, AF399888-AF399889, AF398434, AF135434), and six of *T. walkeri* (AF310180-AF310185, AF399887), were aligned for the design of primers and probes in FRET analysis (Fig 1). The probes were designed against *T. indica* with the aid of the program, Primer3 (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Both probes were synthesized by Biosearch Technologies (Novato, CA).

Table 1 – *Tilletia* species, their host, and geographical origin and their suppliers used in this study

	Host	Origin/Year	Supplier[a]
T. indica			
Ti 1	wheat	Sonnora, Mexico, 1996	1
Ti 2	wheat	Sonnora, Mexico, 1991	1
Ti 3	wheat	Sonnora, Mexico, 1994	1
Ti 6	wheat	Pakistan, 1997	1
Ti 7	wheat	Dakka, India, 1995	1
Ti 8	wheat	Ropar, India, 1997	1
Ti 9	wheat	Guerdersmir, India, 1997	1
Ti 10	wheat	California, USA, 1997	1
WL1562	wheat	India, 1983	1
T. walkeri			
210G	SW[b]	Oregon, USA, 1997	1
Tw4	ryegrass	Georgia, USA, 1997	1
[c] DAR16720	ryegrass	Kangaroo Valley, NSW, Australia	2
T. horrida			
Th2	rice	California, USA, 1998	1
T. tritici			
S4	wheat	Sejet, Denmark, 1999	3
S6	wheat	Sejet, Denmark, 1999	3

a 1, K. Huges, Central Science Laboratory, Sand Hutton, York, Y0411LZ, UK; 2, M. Priest, Orange Agricultural Institute, NSW Department of Primary Industries, Orange, NSW 2800, Australia; 3, S. K. Christiansen, Plant Research Department, Risø National Laboratory, P.O. Box 49, DK-4000 Roskilde, Denmark.
b SW, isolate derived from seed wash.
c DAR, NSW Department of Primary Industries Herbarium, Orange, NSW 2800, Australia.

Dual-labelled probe

Real-time PCR assays using the dual-labelled probe and oligonucleotide primers, as published in Frederick *et al.* (2000) were performed on different DNA concentrations of *Tilletia indica* using the instrument Rotor-Gene 2000 (Corbett Research, Australia). The dual-labelled probe was labelled at the 5′ end with the fluorescent dye, 6-carboxy-fluorescin (FAM) and at the 3′ end with the quencher molecule, BHQ1. The optimal ratio of the primer combination, Tin3:Tin10 (Frederick *et al.* 2000) was determined to be 0.9:0.1 μM with the probe concentration at 0.25 μM.

Each reaction was performed in 20 μl of 5 mM $MgCl_2$, 200 μM of each of the four deoxynucleotides, dATP, dTTP, dCTP and dTTP; 0.25 μM of the dual-labelled probe, primer combination, Tin3:Tin10 (Frederick *et al.* 2000) of 0.9:0.1 μM, 0.5 U TaqPCRxDNA Polymerase (Invitrogen, Carlsbad, CA) in 1× buffer (20 mM Tris-HCl, pH8.4, 50 mM KCl). The thermal cycling parameters included an initial cycle of 94 °C for 5 min, followed by 45 cycles of 94 °C for 15 s, 68 °C for 30 s [data acquiring to cycling A (FAM)], and 72 °C for 30 s.

PCR amplification with and without SYBR Green I

The ITS1 region of various *Tilletia* species (Table 1) was amplified using primers MK56 and Tilletia-R. Each non-fluorescent PCR reaction was performed in 20 μl of 5 mM $MgCl_2$, 200 μM of each of the four deoxynucleotides dATP, dTTP, dCTP and dTTP; 0.25 μM of each of the primer pair, MK56 and Tilletia-R, 0.5 U TaqPCRxDNA Polymerase (Invitrogen) in 1× buffer (20 mM Tris-HCl, pH8.4; 50 mM KCl). Amplified fragments were electrophoresed in 2 % agarose gels and visualized by ethidium bromide staining under UV light.

For amplicon detection using SYBR Green I fluorescence dye, the dye (S-7563, Invitrogen, Carlsbad, CA) was added to a final concentration of 1X in each PCR reaction. Concentration of primers in the reaction was optimized to reduce the presence of primer dimers.

Analysis using quenched FRET probes

The forward primer, *KB-ITS-For*, and the probes are in the same orientation (Fig 1) and hence optimization of the real-time PCR was performed using decreasing concentration of the forward primer from a starting concentration of 0.5 μM to 0.0039 μM. The optimum concentration of the ratio of forward primer:reverse primer used in this study was determined to be *KB-ITS-For*:*KB-ITS-Rev* = 0.0625:0.2.

The instrument used was Rotor-Gene 2000 (Corbett Research, Mortlake, NSW, Australia) with software Rotor-Gene 6.0. Each reaction was performed in 20 μl of 5 mM $MgCl_2$, 200 μM of each of the four deoxynucleotides dATP, dTTP, dCTP and dTTP; 4 pmoles of each of the two probes, 2 pmoles *KB-ITS-Rev*, 0.3125 pmoles *KB-ITS-For*, 0.5 U TaqPCRxDNA polymerase (Invitrogen) in 1× buffer (20 mM Tris-HCl, pH8.4; 50 mM KCl). The template DNA was 10-20 ng of the ITS1 DNA fragment amplified with the primers, MK56 and Tilletia-R.

The thermal cycling parameters for the FRET analysis included an initial cycle of 94 °C for 5 min, followed by 45 cycles of 94 °C for 15 s, 56 °C for 40 s, 72 °C for 20 s. The products were then denatured at 94 °C for 15 s, re-annealed at 50 °C for 1 min and followed by a melting curve analysis from 50 °C to 84 °C.

Results

Real time PCR using dual-labelled probe

Real time PCR using different concentrations of *Tilletia indica* with the primer pair, Tin3/Tin10 (Frederick *et al.* 2000), demonstrated that the threshold cycle of detection, C_T, increased with the increasing dilution of the DNA concentration (Fig 2a). The results could be represented in a standard curve (Fig 2b) to determine an unknown DNA concentration of *T. indica*. The closely related species, *T. walkeri* has a much higher C_T value (Fig 2a).

Amplification of ITS1 from various Tilletia *species*

The primer pair, MK56 and Tilletia-R, amplified the ITS1 fragment of 258 bp from *Tilletia indica*, *T. walkeri*, *T. horrida* and *T. tritici* as expected from alignment analysis. Detection by SYBR Green I using melt curve analysis of the amplicon was also performed (Fig 3). The presence of a peak corresponding to a primer dimer (Fig 3a) in the melt curve analysis can be reduced or eliminated by the optimisation of the concentration of the primer pair or by increasing the number of cycles of PCR. This work showed that the primer-dimer peak was considerably reduced by decreasing the concentration of the primer pairs from 0.25 μM to between 0.15 μM and 0.20 μM (Fig 3b) and/or increasing the number of cycles of PCR (data not shown).

FRET analysis

ITS sequence alignment of ten sequences of *Tilletia indica* (AF310174-AF310179, AF399888-AF399889, AF398434, AF135434) and six sequences of *T. walkeri* (AF310180-AF310185, AF399887) revealed only two nucleotide differences

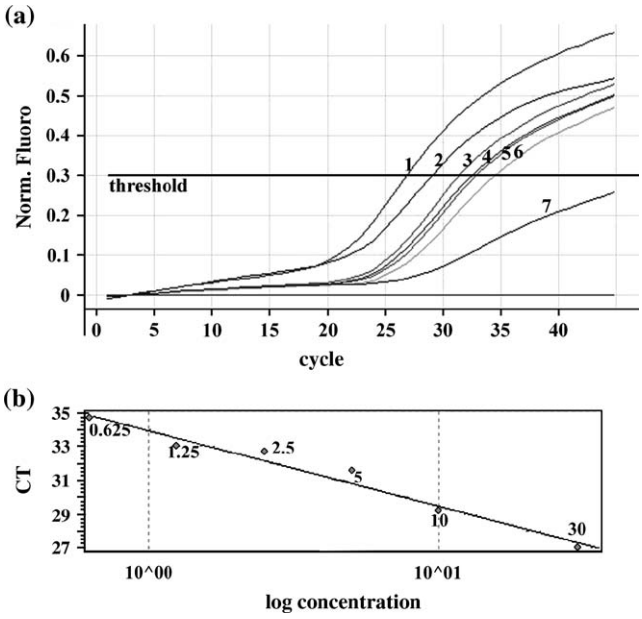

Fig 2 – (a) Normalized fluorescence versus cycle number for real-time assays using dual-labelled probe and primer pair Tin3/Tin10 (Frederick *et al.* 2000) on a range of concentrations of DNA of *Tilletia indica*; 1, 30 ng μl^{-1}; 2, 10 ng μl^{-1}; 3, 5 ng μl^{-1}; 4, 2.5 ng μl^{-1}; 5, 1.25 ng μl^{-1}; 6, 0.625 ng μl^{-1}; and 7, *T. walkeri*, 30 ng μl^{-1}. Reactions were performed on Rotor-Gene 2000 (Corbett Research) and using software Rotor-gene 6.0 for analysis. (b) A standard curve correlating the threshold cycle of amplification, C_T with the log DNA concentrations of *T. indica*, with the concentrations in ng μl^{-1} denoted at the appropriate points.

(nt. 78 and nt. 121 of GenBank AF310176) out of 258 nucleotide bases between the ITS1 sequences of the 2 species. Both sites are A/G transitions with the 'A' nucleotide belonging to *T. walkeri* and the 'G' nucleotide to *T. indica*.

The ITS1 amplicon generated in the presence of SYBR green 1 (see above) was diluted to about 50 ng μl^{-1} and used directly as templates for FRET analysis. PCR using quenched FRET probes followed by melt curve analysis gave two clearly distinguishable peaks with Tm maximums of 63.5 °C and 69 °C (Fig 4) corresponding to *T. walkeri* and *T. indica* respectively.

Amplification of genomic DNA from herbarium spores

Genomic DNA was successfully amplified from ten spores of the reference material of one *T. walkeri* specimen (DAR16720; Table 1) using the GenomiPhi DNA amplification kit (Amersham). The walls of fungal spores (suspended in 20 μl of TE) were broken to release the DNA by at least three rapid freeze-heat cycles where the spore suspension was rapidly frozen in liquid nitrogen, followed by rapid thaw by immersing in a water bath at 95 °C. One microliter of the released nucleic acid was used for the amplification of genomic DNA using the GenomiPhi kit. The amplified genomic DNA was suspended in a total volume of 15 μl with a concentration of approx. 10 ng μl^{-1}. The identity of the reference sample was

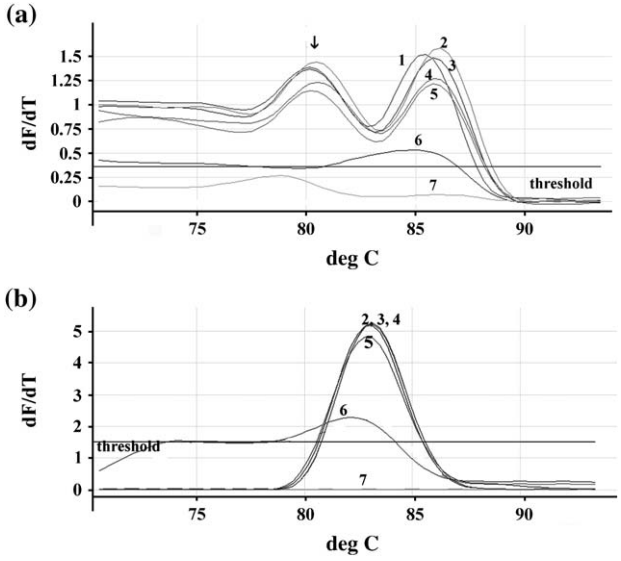

Fig 3 – Melting curve analysis giving a plot of the rate of change of fluorescence against temperature generated on the DNA fragments amplified by primer pairs, MK56 and Tilletia-R using the Sybr Green I fluorescent dye (software Rotor-gene 6.0). The presence of primer dimers (arrowed in Fig 3a) or their absence (Fig 3b) in the analysis depends on the concentration of primers and the number of PCR cycles in temperature profiles (see Results and Discussion) used. The samples in both analyses are: 1, 210G (*Tilletia walkeri*); 2, WL1562 (*T. indica*); 3, Ti9 (*T. indica*); 4, Ti6 (*T. indica*); 5, Ti1 (*T. indica*); 6, S4 (*T. tritici*); and 7, no template control.

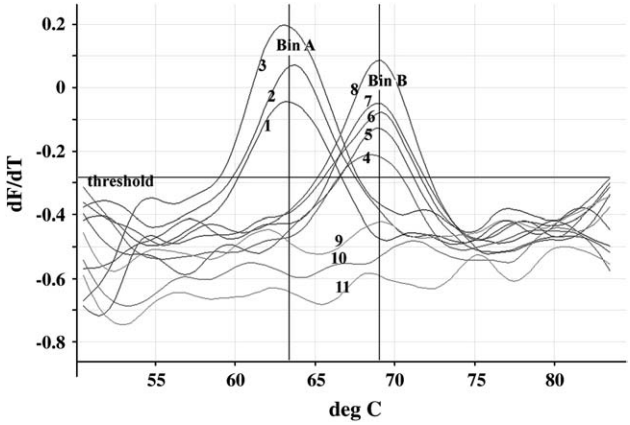

Fig 4 – A melting curve analysis giving a plot of the rate of change of fluorescence against temperature generated by quenched FRET probes (see Materials and methods). The samples are: 1, 210G (*Tilletia walkeri*); 2, Tw (*T. walkeri*); 3, DAR16720 (*T. walkeri*); 4, WL1562 (*T. indica*); 5, Ti2 (*T. indica*); 6, Ti8 (*T. indica*); 7, Ti5 (*T. indica*); 8, Ti3 (*T. indica*); 9, Th2 (*T. horrida*); 10, S6 (*T. tritici*); 11, no template control. Samples 1-3 (*T. walkeri*) are in Bin A (63.4 °C). Samples 4-8 (*T. indica*) are in Bin B (69 °C). *T. indica* has a 'G' nucleotide homologous to the 3′FAM-labelled probe, whereas *T. walkeri* has an 'A' nucleotide at the corresponding position which explains for the higher denaturing temperature, Tm, for *T. indica*. The two species *T. horrida* and *T. tritici* were not detected in this FRET analysis as predicted (see 'Results' and 'Discussion').

confirmed by sequencing the ITS region (GenBank DQ143991) and FRET analysis (Fig 4).

Discussion

Molecular diagnosis of *Tilletia indica* using the dual-labelled probe in real-time PCR (Frederick *et al.* 2000) found that the threshold cycle of detection, C_T, increased with increasing dilution of the DNA concentration (Fig 2a). The results could be represented in a standard curve (Fig 2b) to determine an unknown DNA concentration of *T. indica*.

Dual-labelled probes in real-time PCR are commonly used to analyse quantitative data, for example to determine the unknown concentration of a molecular marker in two different states, diseased versus healthy state. The fluorescence in this assay is emitted when the 5′ fluorophore of the probe is cleaved by the 5′ exonuclease activity of Taq Polymerase when the probe anneals to the target amplified sequence. This assay is thus very sensitive for the detection and quantification of pathogens but not reliable for distinguishing two closely related species.

Currently, this assay is used in the USA, EU, and Australia, to distinguish the two closely related species based on the differential amplifications of a target mitochondrial fragment using a pair of *T. indica*-specific primers and a pair of *T. walkeri*-specific primers in two separate reactions (Frederick *et al.* 2000). Thus a very low quantity of *T. indica* DNA may be confounded by an almost identical result from *T. walkeri*. A quarantine situation often involves very few numbers of spores. Hence confirmation requiring germination of the teliospores and further analysis using both microscopy and molecular tests is necessary.

The molecular protocol suggested here consists of a simple two step process. The first step involves the use of a pair of primers, MK56 (Tan *et al.* 1996) and Tilletia-R, to amplify the ITS1 fragment of about 260 bp from various *Tilletia* species including *T. indica*, *T. walkeri*, *T. horrida* and *T. tritici*. The specificity of Tilletia-R was determined by BLAST with GenBank nucleotide database (http://www.ncbi.nlm.nih.gov/BLAST/). Absolute sequence alignments were observed with *Tilletia* species including *T. barclayana*, *T. nigrifaciens*, *T. laevis*, *T. ehrhartae*, *T. fusca*, *T. bromi*, *T. caries*, *T. goloskokovii*, *T. cerebrina*, *T. setariae* and, *T. controversa*. Complete homology of the primer, Tilletia-R was also observed with some species in two groups of fungi; *Basidiomycota* (including *Entyloma* sp., *Tilletiopsis* sp., and *Fistulina* sp.), and *Chytridiomycota* (including *Rhizophydium* sp., *Rhizophlyctis* sp.).

Detection of the ITS1 fragments can be made using conventional gel electrophoresis followed by visualization under UV light of ethidium bromide agarose gels or by melt curve analysis and detection using SYBR green I dye (Fig 3). The difference in Tm maximums of the melt curves of *T. indica* and *T. walkeri* was insufficient (less than 1 °C) for a clear separation of the two species (Fig 3a).

The second step is FRET analysis of the amplicon from step 1 using FRET probes to identify and differentiate the two closely

related species, *T. indica* and *T. walkeri* (Fig 4). Any *Tilletia* species (and some fungal species as discussed above) will give a positive fragment of about 260 bp in the first step. However, in the subsequent FRET analysis, a positive result will be observed only for *T. indica* and *T. walkeri*. This is due to the specificity of the FRET probes designed for the two closely related species whose DNA target fragments hybridising with the FRET probes differ by only one base (Fig 1). A negative result was observed in this work with *T. horrida* and *T. tritici* (Fig 4) as the probes are not homologous with the fragments from these species. From sequence alignment and their analysis, we would also expect that other *Tilletia* species would not be detected by these probes.

There are only two nucleotide differences out of 258 nucleotides between the ITS1 fragments of *T. walkeri* and *T. indica*, with *T. walkeri* possessing the two 'A' bases and *T. indica* the two 'G' bases. The pair of primers, *KB-ITS-For* and *KB-ITS-Rev* used in this study amplified a fragment of 163 bp, which harboured both the 'A/G' polymorphic sites present in the ITS1 region, even though only one of the polymorphic sites is located in the probe region (Fig 1). This explains for the relative large difference (5.5 °C) between the Tm maximums observed in the melt analysis, with the Tm of the DNA fragments of *T. walkeri* and *T. indica* being 63.5 °C and 69 °C respectively (Fig 4). This Tm difference enables distinct, unequivocal differentiation of these two closely related *Tilletia* species.

The number of samples, particularly the non *T. indica* samples screened in this study, was small (<10). However, the availability of a significant number of ITS sequences of *Tilletia* spp. in GenBank has been an invaluable resource for this study. The alignment of ITS sequences of ten isolates of *T. indica* (AF310174-AF310179, AF399888-AF399889, AF398434, AF135434), six isolates of *T. walkeri* (AF310180-AF310185, AF399887) and five isolates of *T. horrida* (AF310171-AF310173, AF399892-AF399893) revealed complete homology within each of these three species, but characteristic sequence differences between species. This thus confers on the FRET assay the diagnostic validity to accurately detect and distinguish these three tuberculate-spored *Tilletia* species without expending resources to screen a large number of isolates.

The genomic DNA in a few teliospores can first be amplified using the GenomiPhi amplification kit (Amersham) before molecular diagnosis thereby providing DNA reserves for further confirmation if necessary. Amplification of genomic DNA was performed from ten spores of one herbarium *T. walkeri* specimen, DAR16720 (Table 1), providing sufficient DNA for more than ten reactions per GenomiPhi amplification reaction. The target ITS1 segment is located in the rDNA unit which is highly repeated in long tandem arrays at one or a few chromosomal locations representing the nucleolar organizer regions in eukaryotes (Rogers & Bendich 1987). The high copy numbers of ITS1 templates in the genome facilitated the amplification of a high concentration of ITS1 fragments from very few spores (less than ten) in the genome amplification reaction thereby enabling reliable detection from ungerminated teliospores of the fungus or fungi under investigation.

The dual-labelled real-time assay (Frederick *et al.* 2000) was also conducted on the amplified genomic DNA template but without success (data not shown). According to the manufacturer's specifications, the GenomiPhi amplification kit amplifies linear genomic DNA and not circular genomes. Thus the circular mitochondrial genome was not amplified, which may possibly explain for the failure of the real-time assay. Amplification of linear genomic DNA and not circular genomes is a great advantage as the circular genomes of contaminating prokaryotes in a spore extraction from a surveillance operation will not be amplified and so the level of exogenous sources of DNA in a diagnosis is reduced.

A single *Sca* I site (nt. 121-126 of AF310180) is present in the ITSI of *T. walkeri* but not in *T. indica* or other *Tilletia* species. The ITS-RFLP method to distinguish *T. walkeri* and *T. indica* (Levy *et al.* 2001) thus used the restriction enzyme, *Sca* I, which is included as one of the options for molecular analysis in the European Union (Inman *et al.* 2003) and Australian (Wright *et al.* 2003) protocols. However, this approach is only suitable when spores from a single *Tilletia* species are present in a sample. A mixture of spores from two or more *Tilletia* species will give a hybrid RFLP profile of the ITS fragments with the resulting uncut fragment not allowing differentiation of which *Tilletia* species are present. This resulting uncertain interpretation of the findings becomes critical from a quarantine perspective if *T. indica* is one of the *Tilletia* species involved in the mixed sample. Hence accurate diagnosis requires microscopy and molecular analysis of DNA from mycelium produced from the germination of single pure teliospores, which takes approximately two weeks (Castlebury & Carris 1999; Holton 1949).

Tilletia species reported as contaminants in Australian wheat grain included *T. tritici*, *T. laevis* and *T. ehrhartae*, and the apparently uncommon *T. walkeri* (Pascoe *et al.* 2005). These species will not interfere with the diagnosis of *T. indica* using the molecular protocol developed in this study. A positive fragment will be observed in step one of the protocol, but only *T. indica* and *T. walkeri* will be detected in the FRET analysis, which further allows clear differentiation of these two species based on different Tm values. As the molecular protocol is species-specific it allows accurate detection and identification of *T. indica* from samples which contain a mixture of spores from two or more *Tilletia* species. This is not possible with the currently accepted molecular protocol of Levy *et al.* (2001). The species specificity of this protocol could thus also supersede conventional microscopic diagnosis for *T. indica* which is time consuming and often confounded by overlapping morphological characters with other closely related species.

Modelling analysis by Stansbury *et al.* (2002) suggested that the time until first detection could range from 4-11 y depending on the rate of spread of the pathogen and the amount of resources allocated to detection. This time lag in detection could cause a potential economic impact which ranged from 8-24 % of the wheat production in WA alone. Sufficient resources must thus be directed for nation-wide baseline surveillance.

The weather conditions at anthesis in Australia are suitable for the infection of susceptible wheat crops by *T. indica*, and Karnal bunt could develop at many locations in particularly NSW and WA (Murray & Brennan 1998). Karnal Bunt is difficult to control once it is present in an area. Control options include the use of resistant cultivars, cultural practices, seed treatments, foliar treatments, and soil fumigation (Fuentes-Davila 1998). However these control options do not eradicate

the disease (Murray & Brennan 1998). Hence preventing an incursion is of a high priority in Australia and any wheat-growing country with no reported detection of *T. indica* to date.

If there is an incursion, successful containment and eradication of *T. indica* require early detection when the level of infection is not detected or extremely low. A highly sensitive detection tool which would allow direct diagnosis from a very few number of spores and which does not involve time-consuming and labour intensive germination of mycelium is thus not only extremely desirable but economically sustainable in a Karnal bunt surveillance program.

Acknowledgements

This study was undertaken in response to a survey of Australian wheat consigned for export conducted in March 2004 at the Department of Primary Industries, Victoria, Australia, using a national diagnostic protocol for detection and identification of *T. indica* (Wright *et al.* 2003). We thank Plant Health Australia for financial assistance. Thanks are also expressed to Lowan Turton for assistance with the diagrams and Steven Simpfendorfer for useful comments on the manuscript.

REFERENCES

Bonde MR, Peterson GL, Schaad NW, Smilanick JL, 1997. Karnal bunt of wheat. *Plant Disease* **81**: 1370–1377.

Brennan JP, Warham EJ, 1990. *Economic losses from Karnal bunt of wheat in Mexico*. [CIMMYT Economics Working Paper No. 90/02.] International Maize and Wheat Improvement Center, Mexico, D.F.

Brennan JP, Warham EJ, Byerlee D, Hernandez-Estrada J, 1992. Evaluating the economic impact of quality reducing, seedborne diseases: lessons from Karnal bunt of wheat. *Agricultural Economics* **6**: 345–352.

Castlebury LA, Carris LM, 1999. *Tilletia walkeri*, a new species on *Lolium multiflorum* and *L. perenne*. *Mycologia* **91**: 121–131.

Chen W, Hoy JW, Schneider RW, 1992. Species-specific polymorphisms in transcribed ribosomal DNA of five *Pythium* species. *Experimental Mycology* **16**: 22–34.

Crous PW, Van Jaarsveld AB, Castlebury LA, Carris LM, Frederick RD, Pretorius ZA, 2001. Karnal bunt of wheat newly reported from the African continent. *Plant Disease* **85**: 561.

Cunfer BM, Castlebury LA, 1999. *Tilletia walkeri* on annual ryegrass in wheat fields in the Southeastern United States. *Plant Disease* **83**: 685–689.

Diekman M, Putter CAJ, 1995. *Technical Guidelines for the Safe Movement of Germplasm. No. 14. Small Grain Temperate Cereals*. International Plant Genetic Resources Institute, Rome.

Dufresne PJ, Jenni S, Fortin MG, 2004. FRET hybridization probes for the rapid detection of disease resistance alleles in plants: detection of corky root resistance in lettuce. *Molecular Breeding* **13**: 323–332.

Frederick RD, Snyder KE, Tooley PW, Berthier-Schaad Y, Peterson GL, Bonde MR, Schaad NW, Knorr DA, 2000. Identification and differentiation of *Tilletia indica* and *T. walkeri* using the polymerase chain reaction. *Phytopathology* **90**: 951–960.

Fuentes-Davila G, 1998. Karnal bunt of wheat. In: Malik VS, Mathre DE (eds), *Bunts and Smuts of Wheat: an International Symposium*. North American Plant Protection Organisation, Ottawa, pp. 69–81.

Holton CS, 1949. Observations on *Neovossia indica*. *Indian Phytopathology* **2**: 1–5.

Inman AJ, Huges KJD, Bowyer RJ, 2003. EU recommended protocol for the diagnosis of a quarantine pathogen, *Tilletia indica*.http://www.csl.gov.uk/science/organ/ph/diagpro/tipro.pdf.

Joshi LM, Singh DV, Srivastava KD, Wilcoxson RD, 1983. Karnal bunt, a minor disease that is now a threat to wheat. *Botanical Review* **49**: 309–330.

Koo K, Jaykus L-A, 2002. Detection of single nucleotide polymorphisms within the *Listeria* genus using an 'asymmetric' fluorogenic probe set and fluorescence resonance energy transfer based PCR. *Letters in Applied Microbiology* **35**: 513–517.

Lee SB, Taylor JW, 1992. Phylogeny of five fungus-like protoctistan *Phytophthora* species, inferred from the internal transcribed spacers of ribosomal DNA. *Molecular Biology and Evolution* **9**: 636–653.

Levy L, Castlebury LA, Carris LM, Meyer RJ, Pimentel G, 2001. Internal transcribed spacer sequence-based phylogeny and polymerase chain reaction-restriction fragment length polymorphism differentiation of *Tilletia walkeri* and *T. indica*. *Phytopathology* **91**: 935–940.

Livak KJ, Flood SJA, Marmaro J, Giusti W, Deetz K, 1995. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR Methods and Applications* **4**: 357–362.

Mitra M, 1931. A new bunt of wheat in India. *Annals of Applied Biology* **18**: 178–179.

Mitra M, 1935. Stinking smut (bunt) of wheat with special reference to *Tilletia indica*. *Indian Journal of Agricultural Science* **5**: 51–74.

Murray GM, Brennan JP, 1998. The risk to Australia from *Tilletia indica*, the cause of Karnal bunt of wheat. *Australasian Plant Pathology* **27**: 212–225.

Orrů G, De Santis P, Solinas F, Savini G, Piras V, Caporale V, 2004. Differentiation of Italian field and South African vaccine strains of bluetongue virus serotype 2 using real-time PCR. *Journal of Virological Methods* **122**: 37–43.

Pascoe IG, Priest MJ, Shivas RG, Cunnington JH, 2005. Ustilospores of *Tilletia ehrhartae*, a smut of *Ehrharta calycina*, are common contaminants of Australian wheat grain, and a potential source of confusion with *Tilletia indica*, the cause of Karnal bunt of wheat. *Plant Pathology* **54**: 161–168.

Pimentel G, Carris LM, Levy L, Meyer RJ, 1998. Genetic variability among isolates of *Tilletia barclayana*, *T. indica* and allied species based on RAPD and PCR-RFLP analysis. *Mycologia* **90**: 1017–1027.

Rogers SO, Bendich AJ, 1987. Ribosomal RNA genes in plants: variability in copy number and in the intergenic spacer. *Plant Molecular Biology* **9**: 509–520.

Rong I, 2000. Karnal bunt of wheat detected in South Africa. *Plant Protection News* **58**: 15–17.

Rush CM, Stein JM, Bowden RL, Riemenschneider R, Boratynski T, Royer MH, 2005. Status of Karnal bunt of wheat in the United States 1996 to 2004. *Plant Disease* **89**: 212–223.

Sekhon KS, Saxena AK, Randhawa SK, Gill KS, 1980. Effect of Karnal bunt disease on quality characteristics of wheat. *Bulletin of Grain Technology* **18**: 208–212.

Singh DV, Agarwal R, Shreshtha JK, Thapa BR, Dubin HJ, 1989. First report of *Neovossia indica* on wheat in Nepal. *Plant Disease* **73**: 277.

Singh PP, Bedi PS, 1985. Effect of Karnal bunt infection on gluten characteristics and protein fractionation of wheat grains. *Annals of Applied Biology* **1**: 223–225.

Stansbury CD, McKirdy SJ, Diggle AJ, Riley IT, 2002. Modeling the risk of entry, establishment, spread, containment, and

economic impact of *Tilletia indica*, the cause of Karnal bunt of wheat, using an Australian context. *Phytopathology* **92**: 321–331.

Tan MK, Niessen LM, 2003. Analysis of rDNA ITS sequences to determine genetic relationships among, and provide a basis for simplified diagnosis of, *Fusarium* species causing crown rot and head blight of cereals. *Mycological Research* **107**: 811–821.

Tan MK, Timmer LW, Broadbent P, Priest M, Cain P, 1996. Differentiation by molecular analysis of *Elsinoe* spp. causing scab diseases of citrus and its epidemiological implications. *Phytopathology* **86**: 1039–1044.

Torarbi M, Mardoukhi V, Jaliani N, 1996. First report on the occurrence of partial bunt on wheat in the Southern parts of Iran. *Seed and Plant* **12**: 8–9.

Tyagi S, Bratu DP, Kramer FR, 1998. Multicolor molecular beacons for allele discrimination. *Nature Biotechnology* **16**: 49–53.

Warham EJ, 1986. Karnal bunt disease of wheat: a literature review. *Tropical Pest Management* **32**: 229–242.

Williams PC, 1983. Incidence of stinking smut (*Tilletia* spp.) on commercial wheat samples in Northern Syria. *Rachis (ICARDA), Barley, Wheat and Triticale Newsletter* **2**: 21.

Wittwer CT, Herrmann MG, Moss MA, Rassmussen RP, 1997. Continuous fluorescence monitoring of rapid cycle DNA amplification. *Biotechniques* **22**: 130–138.

Wright D, Murray GM, Tan MK, 2003. *National Diagnostic Protocol for the identification of Tilletia indica, the cause of Karnal Bunt*. Deptartment of Agriculture, Perth, WA.

Ykema RE, Floyd JP, Palm ME, Peterson GL, 1996. First report of Karnal bunt of wheat in the United States. *Plant Disease* **80**: 1207.