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Development of a PCR-based diagnostic tool specific to wheat dwarf bunt, caused by *Tilletia controversa*

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Abstract Wheat dwarf bunt, one of the important international quarantine diseases, is caused by *Tilletia controversa*. *Tilletia caries* is a close relative species of *T. controversa* and the teliospore morphology and genomic structure of *T. caries* are very similar to those of *T. controversa*. In order to distinguish between them, a random amplified polymorphic DNA (RAPD) primer-mediated asymmetric-PCR (RM-PCR) was developed to screen differential sequences between the two pathogens. By RM-PCR, a 1,322 bp DNA fragment (PR32) was selected from 18 *T. controversa* and 29 *T. caries* strains. The PR32 genes were specific for *T. controversa* and almost had no homology to *T. caries* or other fungi in the present database. With primers designed from PR32, all 18 *T. controversa* strains were amplified, but no bands appeared in 29 *T. caries* strains by classical PCR. To identify *T. controversa* rapidly and accurately, SYBR Green I and TaqMan probe real-time PCR were established based on PR32. With TaqMan Real-Time PCR, different *T. controversa* strains and *T. caries* strains were detected. The results showed that all *T. controversa* strains were amplified with Ct from 19–29 and amplified curves were obtained. In contrast, the amplification of all *T. caries* strains did not show any signals, without Ct and amplified curves. Moreover, the developed TaqMan real-time PCR was used to detect *T. controversa* from asymptomatic wheat tissues successfully.

Keywords *Tilletia controversa* · *Tilletia caries* · Primer-mediated asymmetric PCR (RM-PCR) · Differential genes · Real-Time PCR

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Introduction

Wheat dwarf bunt caused by *Tilletia controversa* is one of the important international quarantine diseases. This disease is a pathogen historically limited in distribution by its very specific environmental requirements. The pathogen is an independent species that was separated from *Tilletia caries* in 1956, later based on morphology, physiology and genetics (Rusell and Mills 1994; Kim et al. 1990). However,

the teliospore morphology and genomic structure of both pathogens are so similar that until recently some researchers considered that they were the same species and that *T. controversa* was a mutant of *T. caries* (Gang and Weber 1996). This indicated that the differences between the two pathogens are so small that it is difficult to distinguish them based only on morphology, and that the development of an accurate identification method is a high priority for *T. controversa* quarantine purposes.

In recent years, studies on *Tilletia* species mainly focused on analysing the relationships between *T. controversa* and its close relatives by molecular biotechnology. Shi et al. (1996) researched *T. controversa* and *T. caries* by random amplified polymorphic DNA (RAPD). The results showed that wheat bunt fungi originated from a common ancestral population that subsequently differentiated into two sublineages, and a considerable number of isolates had reciprocal characteristics of both pathogens. McDonald et al. (2000) applied repetitive-sequence-based polymerase chain reaction (rep-PCR) to study phylogenetic relationships and to investigate species of *Tilletia*. Recently, Eibel et al. (2005) developed ELISA and PCR to detect *T. caries*; however no method could distinguish between *T. controversa* and *T. caries* completely.

RAPD (random amplified polymorphic DNA) primer-mediated asymmetric PCR (RM-PCR) is a new type of molecular marker based on the telomere repeat sequence (Shen and Zhu 1998a, b). This marker identifies differential genes among close relative species by analysing the polymorphism of amplified fragments and overcomes the disadvantages of differential display-reverse transcription PCR (DD-RT-PCR), restriction fragment length polymorphism (RFLP) and RAPD (Diatchenko et al. 1996; Money et al. 1996). With the advantages of small volume samples, high sensitivity and a high degree of reproducibility by RM-PCR, the amplified fragments can be analysed rapidly and the differential genes will be selected easily. In this study, we applied RM-PCR to screen differential sequences between *T. controversa* and *T. caries*, and established a real-time PCR detection system to identify *T. controversa* based on selected differential genes. The system could accurately detect *T. controversa* teliospores from seeds, soil, flowers and feeds, and infected mycelium from wheat tissue, and will help to develop scientific strategies in disease risk assessment and management.

Materials and methods

Sample collection and DNA extraction

A list of the strains of *T. controversa* and *T. caries* used in this study is given in Table 1; the host for all of these strains was wheat. Eighteen strains of *T. controversa* conserved by USDA-ARS (National Small Grains Germplasm Research Facility), were a gift given by Dr. Blair J. Goates. These *T. controversa* strains are different physiological races identified by inoculating the host (wheat) in wheat-planting areas in the north of the USA (Goates, 1996). Twenty-nine strains of *T. caries* also came from Dr. Blair J. Goates and from wheat fields in different provinces of China, identified by inoculating the host (wheat).

The teliospores of *T. controversa* were incubated at 4–8°C for 3–5 weeks and those of *T. caries* were incubated at 18–20°C for 1 week for germination on water agar (WA) medium. The germinated teliospores of both pathogens were cultured for another 2–3 weeks at 15–25°C on potato dextrose agar (PDA) medium for mycelial growth. Then the mycelia were collected and the genomic DNA extracted according to Moller et al (1992).

Selection of differential genes between *T. controversa* and *T. caries*

To select differential genes, the mycelial DNA of 18 *T. controversa* strains and 29 *T. caries* strains was extracted and RM-PCR technique was established. One hundred random primers were synthesised at Shanghai Boya Biotechnology Company (China) and all primers were 10 bp long. The specific primer for the telomere of *T. controversa* ($CQUTCK_1$, 5′ CCCTAAACCCTAACCCTAACCCWAA 3′)was designed, in which the 5′ terminal sequence included two–three repeat oligonucleotides conserved among the telomere of the fungi and the 3′ terminal sequence had one–three selective bases. The RM-PCR reaction was performed in 25 μl reaction mixture containing 1× PCR buffer (Promega, USA), 0.25 μmol l^{-1} of random primer and specific primer $CQUTCK_1$,

Table 1 Species and strains used in this study

Tilletia species	Number	Physiological races
T. controversa	TCK 1	D1
	TCK 2	D2
	TCK 3	D3
	TCK 4	D4
	TCK 5	D5
	TCK 6	D6
	TCK 7	D7
	TCK 8	D8
	TCK 9	D9
	TCK 10	D10
	TCK 11	D11
	TCK 12	D12
	TCK 13	D13
	TCK 14	D14
	TCK 15	D15
	TCK 16	D16
	TCK 17	D17
	TCK 18	D18
T. caries	TCT 1	Sel 50077, Bt-7
	TCT 2	Heines VII, Bt-0
	TCT 3	Sel 1102, Bt-2
	TCT4	Sel 50077, Bt-7
	TCT 5	Bt-1
	TCT 6	Sel 50077, Bt-7
	TCT 7	Sel 1102, Bt-2
	TCT 8	Sel 1102, Bt-2
	TCT 9	Sel 50077, Bt-7
	TCT 10	Hohenheimer, Bt-5
	TCT 11	Ridit, Bt-3
	TCT 12	Sel 2092, Bt-1
	TCT 13	Thule III, Bt-13
	TCT 14	Sel 2092, Bt-1
	TCT 15	Bt-5
	TCT 16	Rio, Bt-6
	TCT 17	Bt-6
	TCT18	Bt-4
	TCT 19	Ridit, Bt-3
	TCT 20	Heines VII, Bt-0
	TCT 21	Bt-2
	TCT 22	CI15588, Bt-4
	TCT 23	M82-2098, Bt-9
	TCT 24	Bt-10
	TCT 25	Bt-10
	TCT 26	M82-2102, Bt-10
	TCT 27	Bt-9
	TCT 28	M90-387, Bt-9
	TCT 29	Bt-10

3.0 mmol l^{-1} $MgCl_2$ (Promega, USA), 0.4 mmol l^{-1} dNTPs (Promega, USA), 1 unit of Taq DNA polymerase (Promega, USA) and 1 ng of mycelial DNA of either pathogen. The PCR programme included 94°C for 4 min, five high stringency cycles each at 94°C for 30 s, 53°C for 45 s, 72°C for 1 min, ten compound cycles each including two high stringency cycles and one low stringency cycle (94°C for 30 s, 36°C for 45 s, 72°C for 1 min), and an extension step for 72°C for 10 min. PCR products were performed by electrophoresis in a 1.5% agarose gel prepared in 0.5× TAE buffer containing 0.5 g ml^{-1} of ethidium bromide at 80 V for 60 min, and was visualised on a UV transilluminator. Comparing amplified bands from both fungi, a differential DNA fragment specific for *T. controversa* was selected and then recovered from the gel. The recovered DNA was cloned into the *Escherichia coli* JM109 and positive clones were sent to Shanghai Boya Biotechnology Company to sequence. The sequenced result was aligned in GenBank using the BLASTn programme for analysis of the homology with *T. caries* and other *Tilletia* species.

Conventional PCR

Primers were designed based on selected differential genes using software primer 5.5 and were synthesised by Boya Biotechnology Company (Shanghai, China). The sequences of primers ($CQUTCK_2$/$CQUTCK_3$) were 5′ TCTAACTTACCTCGC GGATGG 3′ and 5′ ACG CAGTGACGGGTGGATA 3′. The PCR reaction was performed in 25 μl reaction mixture containing 1× PCR buffer (Promega, USA), 0.4 μmol l^{-1} of each primer, 1.5 mmol l^{-1} $MgCl_2$ (Promega, USA), 0.2 mmol l^{-1} dNTPs (Promega, USA), 1 unit of Taq DNA polymerase (Promega, USA) and 1 ng of mycelial DNA of either

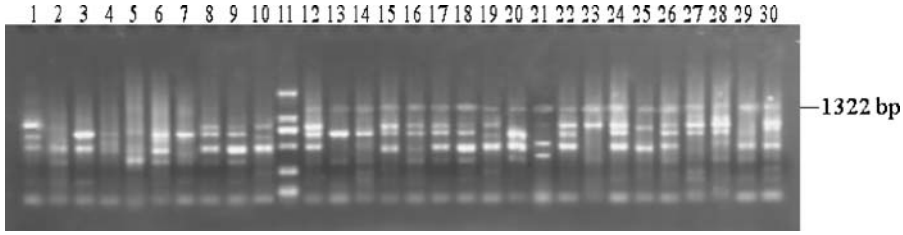

Fig. 1 Selection of differential genes by RM-PCR. *Lanes 1–10*: *T. caries*1–*T. caries*10; *lane 11*: Marker DL2000 (100, 250, 500, 750, 1,000, 2,000 bp); *lanes 12–29*: *T. controversa*1–*T. controversa*18; *lane 30*: *T. controversa* mixed template

pathogen. The PCR programme included 94°C for 5 min, 30 cycles each at 92°C for 20 s, 60°C for 20 s, 72°C for 45 s, and an extension step at 72°C for 7 min.

Real-Time PCR

For Real-Time PCR, the fluorescent-based TaqMan and SYBR Green systems were used. For TaqMan, The primer pair and probe were designed from the selected differential genes using the programme primer Express (ABI, Foster City, CA). The primer pair $CQUTCK_4$/$CQUTCK_5$(5′ AGTGCTGAGGCC GAAAAGGT 3′/5′ TTCTGGGCTCCACGACGTAT 3′) and Taq-man probe $CQUP_1$ (5′ ATGTGGCGAA ACTCTTTATCCACCCGTC 3′) were synthesised by Boya Biotechnology Company (Shanghai, China). The probe was labelled by 6-FAM as fluorescent dye at the 5′ end and TAMRA at the 3′ end as quench dye. The TaqMan PCR reaction was performed in 25 μl reaction mixture containing 1× PCR buffer (Bio-Rad, Master Mix, Richmond, CA), 0.4 μmol l^{-1} of each primer, 0.2 μmol l^{-1} TaqMan probe and 0.01–1 ng of extracted DNA sample. The ICycler^{iQ} Thermocycler (Bio-Rad) was used with the following programme for DNA amplification: 95°C for 5 min, 45 cycles each at 95°C for 15 s, 60°C for 45 s, and an extension step at 72°C for 7 min.

For SYBR Green, the primer pair $CQUTCK_4$/$CQUTCK_5$ was used and the PCR reaction was performed in 25 μl reaction mixture containing 1× PCR buffer (Bio-Rad, SYBR Green Master Mix), 0.4 μmol l^{-1} of primers ($CQUTCK_4$/$CQUTCK_5$) and 0.01–1 ng of extracted DNA sample. The ICycler^{iQ} was used with the following programme: 95°C for 5 min, 45 cycles each at 95°C for 15 s, 60°C for 45 s, and an extension step at 72°C for 7 min. Fluorescent signal was collected at the annealing step and the extending step (60°C for 45 s) of each cycle. To analyse the specificity of the PCR amplification, a melt curve programme was included as follows: 95°C for 1 min, 55°C for 1 min, and a single temperature rise of 0.5°C held for 10 s from 55°C to 95°C.

Standard curve for Real-Time PCR

The plasmid DNA containing the selected differential genes was transferred into pMD18-T vector and the plasmid extracted by UNIQ-10 spin column plasmid DNA extraction kit (Shanghai Sangon, Shanghai, China). The original plasmid standard solution was

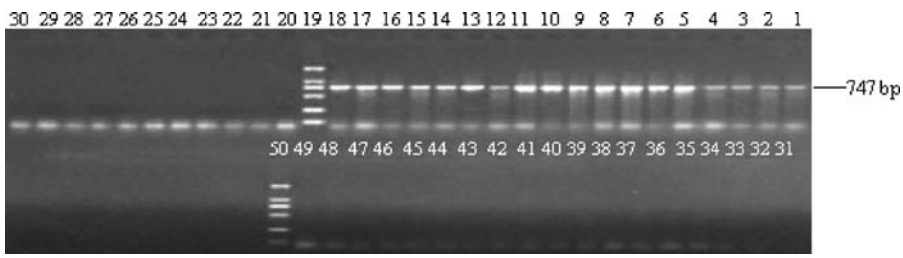

Fig. 2 Amplification of *T. controversa* and *T. caries* with specific primers $CQUTCK_2$/$CQUTCK_3$. *Lanes 1–18*: *T. controversa*1–*T. controversa*18; *lanes 19, 50*: Marker DL2000 (100, 250, 500, 750, 1,000, 2,000 bp); *lanes 20–48*: *T. caries*1–*T. caries*29; *lane 49*: negative control

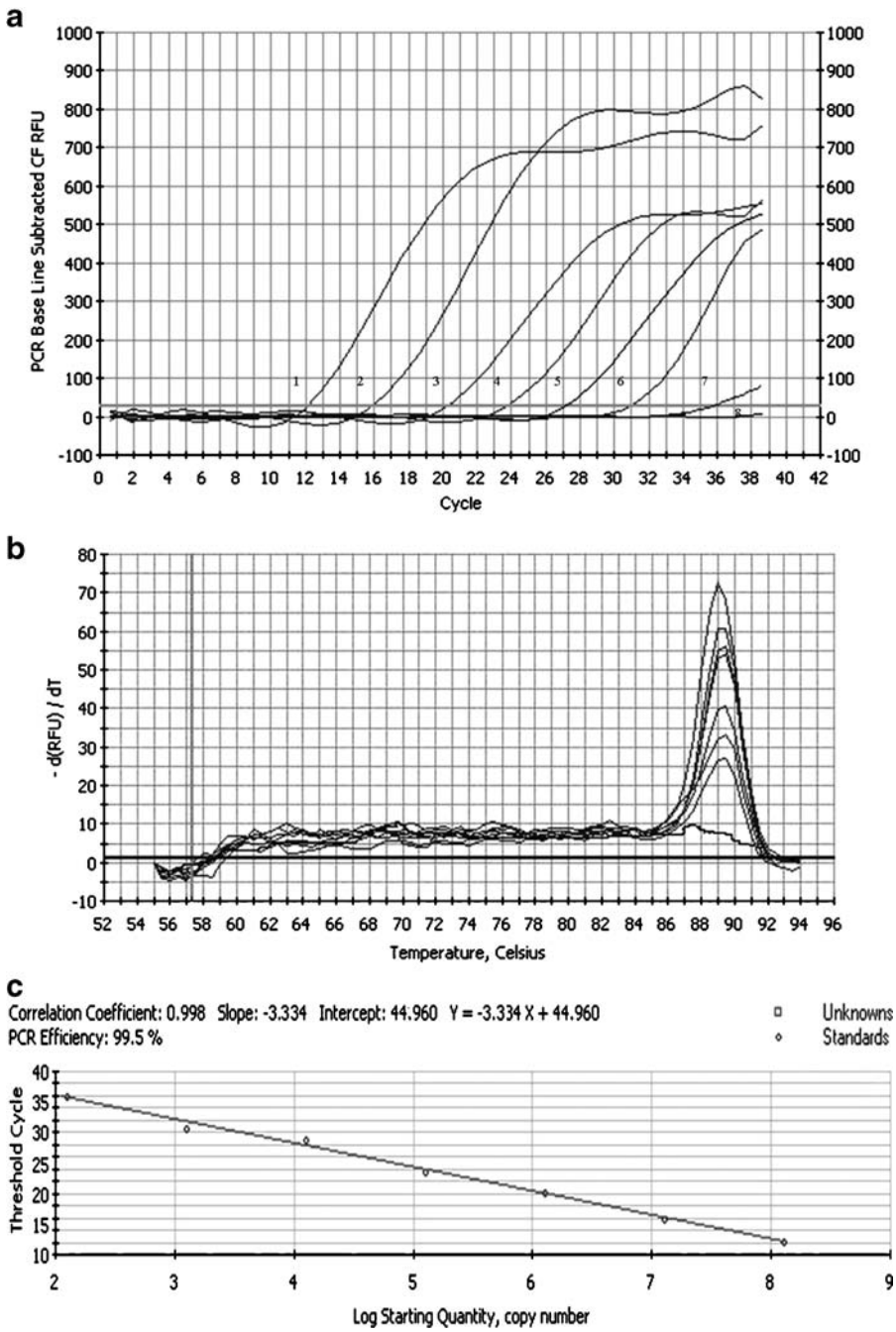

Fig. 3 Construction of standard curve by SYBR Real-Time PCR. **a** Real-Time amplified curves. *1–7*: ten-fold dilutions of recombined plasmid DNA (0.1 ng−0.1 fg, CN=2.31×10^{10}−2.31×10^{4}); *8*: negative control; **b** melt curve of SYBR Green I (peak temperature at 89°C). **c** Standard curve

quantified with a spectrophotometer (Beckman, USA) and then diluted in ten-fold serial dilutions which were used in Real-Time PCR to generate a standard-curve; each dilution was done in triplicate. With the standard-curve method, the iCycler iQ^{TM} could calculate the titer of pathogens in tested samples automatically. The unit of detection limitation was fg μl^{-1}, which had been changed into general form with the unit of copy number. The following formula was used to calculate the number (CN):

$$CN = \frac{M \times N}{L \times D}$$

where:

M = minimum concentration of nucleic acid detected (g ml^{-1})

N = Avogadro's number(6.022×10^{23}, molecules $mole^{-1}$)

L = length of nucleic acid in kilobase pairs (total length of plasmid + insert of DNA fragment)

D = conversion factor from 1 kb of nucleic acid to Daltons

dsDNA=6.6×10^{5}g $mole^{-1}$ kb^{-1}

Detection of samples by Taqman PCR

The mycelial DNA of 18 *T. controversa* strains and 29 *T. caries* strains was amplified by Taqman Real-Time PCR. The genomic DNA of healthy wheat was used as a negative control.

The artificially contaminated wheat seeds with *T. controversa* were sown in experimental fields and the asymptomatic wheat at different growth stages (trefoil stage, returning green stage, tillering stage, jointing stage, heading stage and filling stage) was analysed. The total DNA was extracted from the leaves of collected samples and detected with Real-Time TaqMan PCR in order to investigate the dynamic change of the titer of *T. controversa* in host plants throughout the year.

Results

Selection of differential genes between *T. controversa* and *T. caries*

The *T. controversa* and *T. caries* strains were amplified with the different random primers and a specific primer for the telomere by RM-PCR. The results showed that all the 18 *T. controversa* strains could be amplified well with specific 1,322 bp products, but the 1,322 bp bands did not appear in the 29 *T. caries* strains with random primer P23-BA0166 (5′ AAGGCGGCAG 3′) and $CQUTCK_1$ (Fig. 1). This indicated that the obtained 1,322 bp DNA fragment (PR32) was specific for *T. controversa*. The PR32 genes were cloned into *E. coli* JM109 and sequenced. The sequenced result was

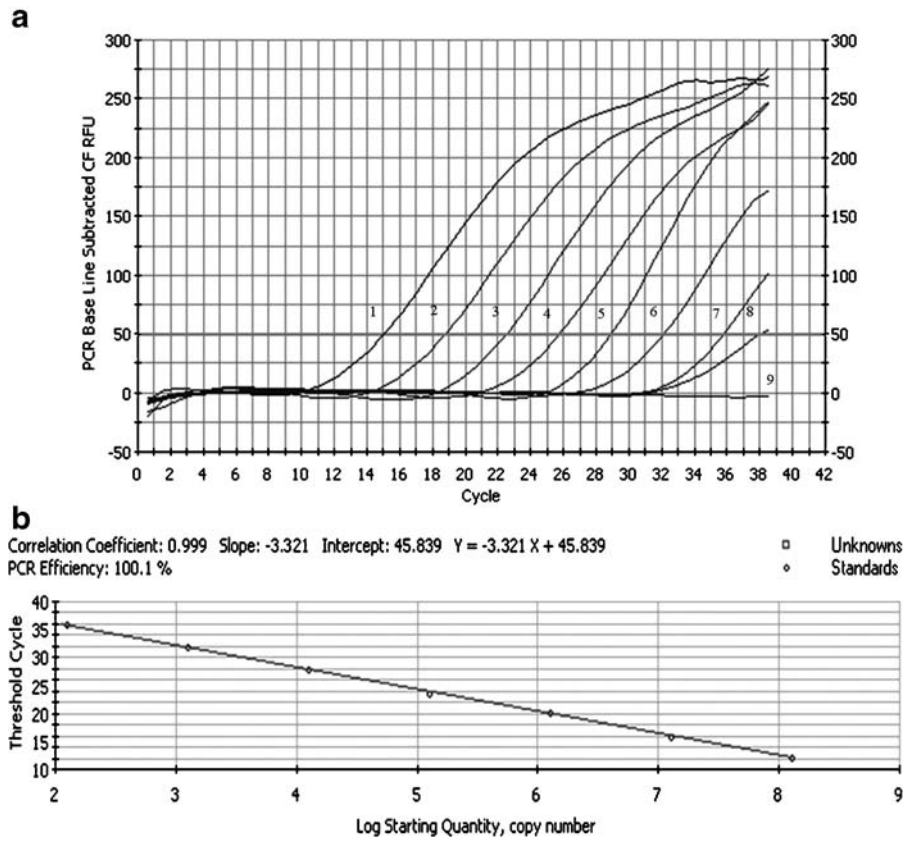

Fig. 4 Construction of standard curve by TaqMan probe Real-Time PCR. **a** Real-Time amplified curves. *1–8*: ten-fold dilutions of recombined plasmid DNA (1.0 ng−0.1 fg, CN=$2.31\times10^{11}-2.31\times10^{4}$); *9*: negative control; **b** standard curve

aligned in GenBank using BLASTn programme. It showed that the PR32 sequences were highly specific for *T. controversa* and almost had no homology with *T. caries* or other fungi present in the database. The genes were then submitted to GenBank and an accession number (DQ266258) was obtained.

In order to further confirm that the selected differential genes (PR32) were specific for *T. controversa*, a pair of primers ($CQUTCK_2$/$CQUTCK_3$) was designed from PR32 to amplify 18 *T. controversa* and 29 *T. caries* strains by conventional PCR. The results suggested that all the *T. controversa* strains were amplified and bands of about 747 bp were visualised, but no bands appeared in 29 *T. caries* strains (Fig. 2). This indicated that the differential genes (PR32) could be used to distinguish both pathogens.

Standard curve of Real-Time PCR

The SYBR Green I and Taqman Real-Time PCR for identifying *T. controversa* were established. For SYBR Green I, ten-fold serial dilutions of PR32 plasmid DNA ($CN=2.31\times10^{10}-2.31\times10^{4}$, 0.1 ng−0.1 fg) was used as a template. For each dilution, DRn (increase in fluorescence emission due to template amplification) was measured and plotted against cycle number. When the reaction was over, the melt curve was used for verifying the specificity of the PCR amplification. By plotting Ct values against the known copy number input, the standard curve was generated, with a linear range covering 7 log units. In Fig. 3, the correlation coefficient of the standard curve in SYBR Green I Real-Time PCR reached at 0.998 and the amplification efficiency was 99.5%. Furthermore, the amplification was specific by analysing the curve melt and its detection sensitivity was 0.1 fg ($CN=2.31\times10^{4}$), which showed that the SYBR Green I Real-Time PCR was successfully developed.

For Taqman probe Real-Time PCR, the standard curve was calculated on the serial dilutions of PR32 plasmid DNA ($CN=2.31\times10^{11}-2.31\times10^{4}$, 1.0 ng−0.1 fg). A linear relationship was observed between

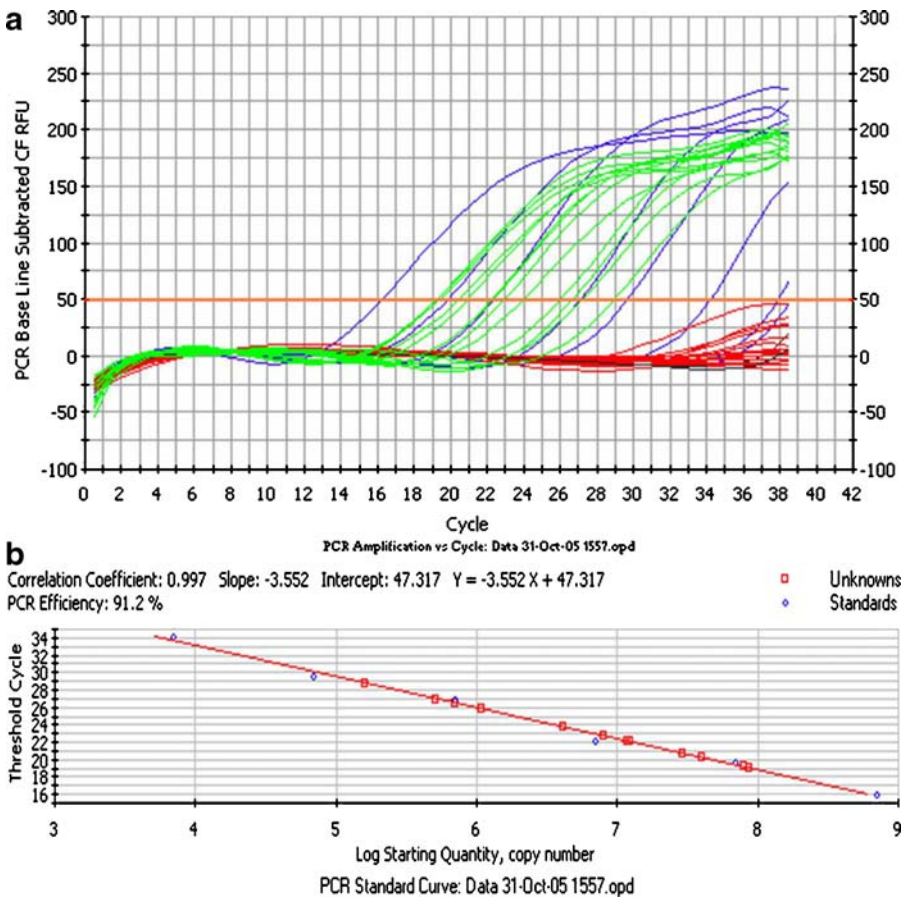

Fig. 5 Detection of *T. controversa* and *T. caries* strains by TaqMan probe Real-Time PCR. **a** Real-Time amplified curves; *blue lines* are the standard curves based on diluted PR32 plasmid (1.0 ng−0.1 fg, $CN=2.31\times10^{11}-2.31\times10^{4}$) and the *green lines* represent the amplified curves of *T. controversa*1–11. The *green lines* are the curve plots of *T. caries*1–11 and *black lines* are negative controls (DNA of healthy wheat). **b** Standard curve

Ct values and the log concentrations of recombinant plasmid DNA. In Fig. 4, the result of Taqman sensitivity detection showed that the sample containing DNA template 2.31×10^5 CN μl^{-1} had a Ct value range of 36, smaller than the acceptable threshold upper limit Ct 38, and sample dilutions lower than 2.31×10^5 CN μl^{-1} gave negative reactions (no Ct values obtained after 38 cycles, and no amplified curve of fluorescent signal). Therefore, the detection sensitivity of Taqman PCR for *T. controversa* was 1.0 fg, equal to 2.31×10^5 CN.

Identification of *T. controversa* using Taqman assay

The optimised Taqman probe Real-Time PCR was used to detect the different *T. controversa* strains and *T. caries* strains. In Fig. 5, the blue lines are the standard curve based on diluted PR32 plasmid (1.0 ng−0.1 fg, CN=$2.31\times10^{11}-31\times10^4$). All *T. controversa* strains were amplified with Ct from 19–29 and amplified curves obtained (green lines). In contrast, the amplification of all *T. caries* strains did not produce any signals, (no Ct and no amplified curves, red lines). The black line is the curve plot of the negative control (DNA of healthy wheat). The results confirmed that identification of *T. controversa* with Taqman probe Real-Time PCR was accurate and specific.

Detection of wheat samples using Taqman assay

The asymptomatic wheat samples were collected from experimental fields and detected by Taqman probe Real-Time PCR. The samples included all growth stages of wheat: trefoil stage, returning green stage, tillering stage, jointing stage, heading stage and filling stage. The DNA extracted from the wheat leaves was amplified by Taqman PCR and the DNA of healthy wheat was used as the negative control. *Tilletia controversa* was tested in the wheat samples at different growth stages: trefoil stage, returning green stage, jointing stage, heading stage and filling stage, but the pathogen could not be detected in the wheat samples at the tillering stage. This was because the amount of fungus in samples at the tillering stage was so low that it could not be detected. In Fig. 6, the blue lines are the standard curves in which plasmid DNA was $2.31\times10^{11}-2.31\times10^4$ CN and the red lines are the amplified curve plots of wheat DNA at different growth stages. The black lines are the negative controls.

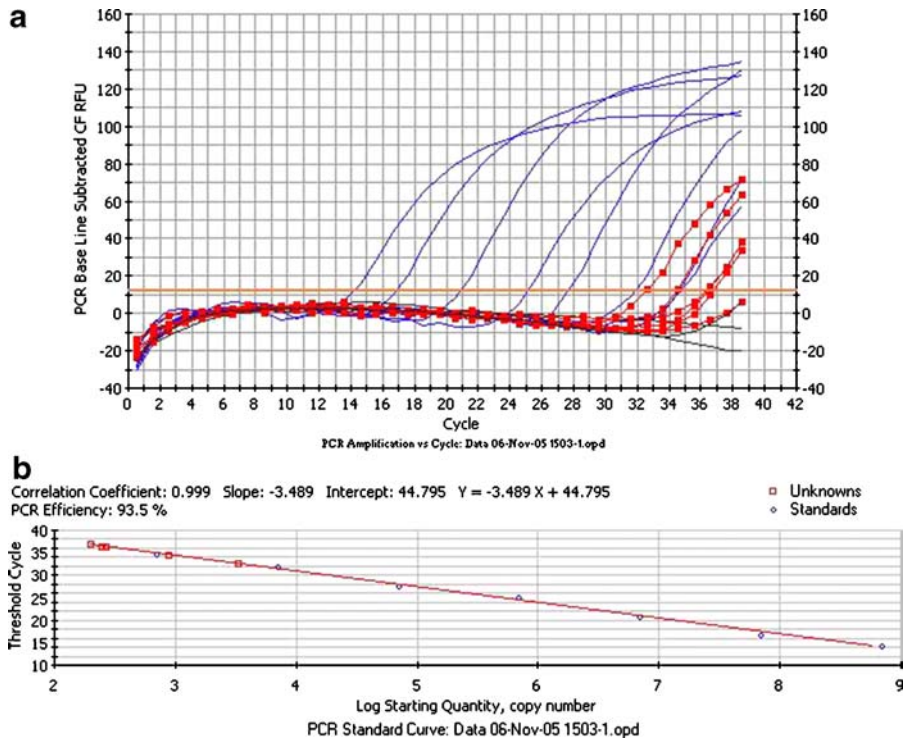

Fig. 6 Detection of *T. controversa* from wheat at different growth stages by TaqMan probe Real-Time PCR. **a** Real-Time amplified curves; the *blue lines* are the standard curves in which plasmid DNA was $2.31\times10^{11}-2.31\times10^4$ CN and the *red lines* are the amplified curves of wheat DNA at different growth stages. The *black lines* are negative controls. **b** Standard curve

Discussion

Tilletia caries is a close relative species of *T. controversa*, and the teliospore morphology, genomic structure and internal transcribed spacer region sequences (ITS) of the latter are highly similar to those of the former, so it is difficult to distinguish both fungi. Although many researchers have tried to identify and analyse species-specific sequences among *Tilletia* species by RAPD, repetitive-sequence-based polymerase chain reaction (rep-PCR)and genomic fingerprinting, none could differentiate *T. controversa* from *T. caries* completely (Shi et al. 1996; McDonald et al. 2000; Eibel et al. 2005). In our study, thermal asymmetric interlaced PCR (RM-PCR) was applied to amplify both pathogens with a specific primer and RAPD random primer. The specific primer was designed based on telomere associated sequence (TAS) and the sequences close to the telomere were amplified. During the RM-PCR process, high stringency PCR and low stringency PCR were used in turn. In high stringency PCR, only the primer specific for the telomere could amplify the target DNA, while the RAPD primer could do this only in low stringency PCR. The specific products amplified with the telomere primer and RAPD random primer were then more than the non-specific products amplified only using RAPD primers by adjusting the PCR reaction conditions (Levis et al. 1997; Shen and Zhu 1998a, b). In this study, the optimised RM-PCR was used to amplify 18 *T. controversa* and 29 *T. caries* strains and differential genes (PR32, 1,322 bp) specific for all *T. controversa* strains were successfully selected.

In order to identify *T. controversa* rapidly and accurately, SYBR Green I and Taqman Real-Time PCR were constructed. The detection sensitivity of SYBR Green I for *T. controversa* was 0.1 fg, equal to 2.31×10^4 CN and that of Taqman PCR was 1.0 fg, equal to 2.31×10^5 CN. However, the SYBR Green dye-based PCR assay may react with many other non-target DNA and requires a DNA melt curve to confirm the accuracy, especially in the case of a high Ct value. The TaqMan PCR approach has several advantages over conventional PCR including specificity, robustness, and fast speed (Schaad and Frederick 2002). The TaqMan assay is somewhat less sensitive than the SYBR Green I Real-Time PCR, but it has superior specificity and is considerably faster. Compared with molecular beacons and Scorpion probes, the Taqman probe provides a simple and straightforward design.

The developed Taqman Real-Time PCR was used to detect both fungi (Fig. 5). In normal scale view, it seemed that the threshold position was set to a high level (in fact it was done by IQ software automatically), while in logarithmic scale, the threshold position was still in logarithmic linear phase, and all reactions were reasonably close because all lines were parallel (data not shown). For Taqman Real-Time PCR, if the threshold position is set to linear phase, the Ct value is different, but the quantity of sample is the same. That is because the Δct between a certain sample and a standard sample does not vary. Therefore, the result of amplification by Taqman Real-Time PCR was correct.

In addition, the negative controls (black line) in Fig. 5 gave a small florescence signal. Different primer and probe concentrations were tried in the assay, but the same result occurred. For Taqman Real-Time PCR, accurate results normally come from ct values in the range of about 5 to 40, and this range depends on the quality of the TaqMan probe. After about 35 cycles, even a negative control will give increasing florescence signals due to the degradation of the probe during PCR. Sometimes the intensive light of the PCR instrument will result in a decline of florescence after long PCR cycles. Therefore the ct of around 40 can be regarded as suspicious in many cases.

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