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ABSTRACT

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Karnal bunt of wheat, caused by *Tilletia indica*, was found in regions of the southwestern United States in 1996. Yield losses due to Karnal bunt are slight, and the greatest threat of Karnal bunt to the U.S. wheat industry is the loss of its export market. Many countries either prohibit or restrict wheat imports from countries with Karnal bunt. In 1997, teliospores morphologically resembling *T. indica* were isolated from bunted ryegrass seeds and wheat seed washes. Previously developed PCR assays failed to differentiate *T. indica* from the recently discovered ryegrass pathogen, *T. walkeri*. The nucleotide sequence of a 2.3 kb region of mitochondrial DNA, previously amplified by PCR only from *T. indica*, was determined for three isolates of *T. indica* and three isolates of *T. walkeri*. There was greater than 99% identity within either the *T. indica* group or the *T. walkeri* group of isolates, whereas there was ≈3% divergence between isolates of these two *Tilletia* species. Five sets of PCR primers were made specific to *T. indica*, and three sets were designed specifically for *T. walkeri* based upon nucleotide differences within the mitochondrial DNA region. In addition, a 212 bp amplicon was developed as a target sequence in a fluorogenic 5′ nuclease PCR assay using the TaqMan system for the detection and discrimination of *T. indica* and *T. walkeri*.

Tilletia indica Mitra infects wheat, durum wheat, and triticale and causes a disease commonly referred to as Karnal bunt. The disease was first described in the village of Karnal, India in 1930 (16). Since then Karnal bunt has been found in the neighboring countries of Pakistan, Nepal, Iraq, and Afghanistan (4). Karnal bunt was reported first on the North American continent in 1972 when it was found in Mexico (10), and by 1982 the disease had become well established in wheat fields in northwestern Mexico (5). Subsequently, in March of 1996, Karnal bunt was discovered in wheat in the United States in Arizona and later that year in California (5,26). In 1997, the disease was discovered in a small area of central Texas (5).

Yield reductions in wheat from Karnal bunt are slight; however, the disease is of extreme economic importance. Many countries, including the U.S., have a zero tolerance for Karnal bunt and refuse wheat shipments from quarantined countries and/or areas (18). Since the U.S. is the world's leading exporter of wheat with an estimated annual value of $5 billion, Karnal bunt poses a serious threat to international trade for the U.S. wheat industry (7,18).

Prior to the discovery of Karnal bunt in the U.S., polymerase chain reaction (PCR) primers were developed at our research facility to distinguish *T. indica* from other smut fungi (11,23). *T. barclayana*, which causes rice kernel smut, sometimes is present as a contaminant in harvested or stored wheat and can be misidentified as *T. indica*. These primers were used to confirm the identity of the pathogen as *T. indica* following discovery of teliospores in the southwestern U.S.

During the 1996 National Karnal Bunt Survey conducted by the Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA), teliospores morphologically similar to *T. indica* were discovered as free spores in seed washes of wheat from the southeastern United States and in forage-mix seed lots from Oregon, but no infected wheat seeds were found (5,8,18). Further investigation revealed that the teliospores originated from bunted annual ryegrass (*Lolium multflorum*) seeds present as contaminants in wheat from fields in these areas. The existing PCR primers did not distinguish this newly discovered ryegrass smut pathogen from *T. indica*. The ryegrass smut was described recently as *T. walkeri* Castlebury and Carris on the basis of teliospore morphology and ornamentation (7). Distinguishing *T. indica* from *T. walkeri* requires examination of many teliospores by trained mycologists. This process is inefficient, tedious, and subject to misinterpretation.

In this study, nucleotide sequences of a 2.30-kilobase region of mitochondrial DNA (mtDNA), previously amplified by PCR only from *T. indica* (11), were determined for three isolates each of *T. indica* and *T. walkeri*. Based upon nucleotide differences, we report

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here the development of improved classical PCR assays and a new TaqMan test for the detection and discrimination of *T. indica* from *T. walkeri* and other related smut species. Preliminary reports of this research have been made (12,13).

MATERIALS AND METHODS

Fungal isolates and growth conditions. A list of the *Tilletia* isolates used in this study is shown in Table 1. The isolates were from bunted seed samples or seed washes. Mycelial cultures were grown on potato dextrose agar (PDA) plates at 21°C for 7 to 10 days. Secondary sporidia were produced by placing mycelial plugs onto 2% water-agar plates and incubating them at 21°C for 3 to 4 days. Mycelial tissue used for extracting genomic DNA was grown and prepared as follows: Mycelial plugs (each 3 cm^2) cut from water-agar plates were placed inverted on the inside surface cover of a 100 × 15 mm petri plate containing sterile potato dextrose broth. After approximately 10 days of incubation at 21°C, mycelia and spores floating on the broth were harvested by filtration through a Buchner funnel onto Whatman filter paper, and the tissue was blotted dry and stored at –80°C.

DNA extraction and recombinant DNA techniques. Genomic DNA from *T. indica* and *T. walkeri* was extracted from 0.5 to 1.0 g frozen mycelial tissue. The tissue was placed into 1.5-ml microcentrifuge tubes with 75 µl lysis buffer and ground with a sterile pestle attached to a power drill. An additional 75 µl of lysis buffer was added, and DNA was extracted using a Puregene Genomic DNA Isolation kit (Gentra Systems, Minneapolis, MN) according to the manufacturer's directions. DNA concentrations were determined by UV spectrophotometry at 260 nm.

A 2.3-kb fragment was amplified from three *T. indica* isolates (Yv3b, WL1562, and Bpop) and three *T. walkeri* isolates (210G, YRG-001, and TNRG-02) by PCR using the oligonucleotide primers Ti-1 and Ti-4 (synthesized by Life Technologies/Gibco BRL, Gaithersburg, MD) as described previously (11). The fragments from these isolates were cloned into the TA cloning vector pCR2.1 (Invitrogen Corp., Carlsbad, CA) and transformed into *E. coli* INV∝F´ cells according to the manufacturer's directions.

DNA sequencing and analysis. Plasmid DNA containing the 2.3-kb mtDNA fragment from *T. indica* or *T. walkeri* isolates was extracted from *E. coli* using a Qiagen Plasmid DNA kit following the manufacturer's procedure (Qiagen, Inc., Chatsworth, CA). DNA concentrations were determined by UV spectrophotometry at 260 nm, and nucleotide sequence was determined (Sequetech Corp., Mountain View, CA). Nucleotide sequences were compared and aligned in our laboratory using the Bestfit and Pileup programs of the Genetics Computer Group computer package (version 9.0) (9) at the Advanced Biomedical Computing Center of the National Cancer Institute, Frederick, MD.

PCR assay and Southern blot analysis. Oligonucleotide primers specific to either *T. indica* or *T. walkeri* were synthesized to unique sequences within the 2.3-kb mtDNA regions (Life Technologies/Gibco BRL). Classical PCR reactions were performed in a Gene AMP PCR System 9700 thermocycler (Perkin-Elmer/Applied Biosystems, Inc., Foster City, CA) using 25 ng of genomic DNA. PCR was performed in a total volume of 25 µl containing: 10 mM Tris-HCl; 50 mM KCl (pH 8.3); 1.5 mM $MgCl_2$; 0.001% (wt/vol) gelatin; dATP, dGTP, dCTP, and dTTP, each at a concentration of 100 mM; each primer at a concentration of 100 nM; and 0.5 U of *AmpliTaq* DNA polymerase (Perkin-Elmer/Applied Biosystems). The *T. indica*-specific PCR primers Tin3 (5′-CAATGTTGGCGTGGCGGCGC-3′)/Tin10 (5′-AGCTCCGCCTCAAGTTCCTC-3′), F3 (5′-GGCACCAGAGTACAGCTGTCGTT3′)/R1 (5′-GTCGGATTTGCGGACACTTTC-3′), Tin3/Tin6 (5′-GGCGGACTACCACTCGAGCT-3′), Tin5 (5′-GACGTCGAGGCCGACCGTAT-3′)/Tin6, and Tin3/Tin4 (5′-CAACTCCAGTGATGGCTCCG-3′) were used with the following cycling conditions: 94°C denaturation for 1 min, 25 cycles of 94°C for 15 s, 65°C for 15 s, and 72°C for 15 s, followed by an extension step of 72°C for 6 min.

The *T. walkeri*-specific PCR primers Tin11 (5′-TAATGTTGGCGTGGCGGCAT-3′)/Tin4 were used at the same conditions as described for the *T. indica*-specific PCR primers, however for the *T. walkeri* primers Tin7 (5′GTTTGAGCCACGCTATGACC-3′)/Tin8 (5′-GGCTCATCTACGCATACGTT-3′) the number of cycles was increased to 30. For the *T. walkeri* primers Tin11/Tin10 (5′-AGCTCCGCCTCAAGTTCCTC-3′), an annealing temperature of 55°C was employed for 45 cycles.

Negative controls were tested by using the same reaction mixture under the amplification conditions described above without template DNA. To verify that DNA extracted from *T. indica* and *T. walkeri* isolates could be amplified by PCR, primers ITS2 and ITS5 (25) were used to amplify an internal transcribed spacer region of the rDNA. DNA fragments produced by PCR were analyzed by electrophoresis on 1.4% agarose gels in 0.5 X TBE buffer stained with ethidium bromide (2).

Gels for Southern blots were denatured and blotted onto positively charged nylon membranes (Boehringer-Mannheim, Indianapolis) (2), and the blots were hybridized for 14 to 16 h at 55°C with the 2.3-kb mtDNA region from *T. indica* isolate WL1562 that was labeled with digoxigenin according to the manufacturer's directions (Boehringer-Mannheim). Chemiluminescent detection was performed as directed by the manufacturer (Boehringer-Mannheim), and the membranes were exposed to X-ray film (Blue sensitive; Molecular Technologies, St. Louis) at room temperature for up to 20 min.

TaqMan 5´ nuclease PCR assay. The PCR conditions were a modification of the classical PCR assays described above for the *T. indica*-specific primers Tin3/Tin10 and the *T. walkeri*-specific primers Tin11/Tin10. Cycling conditions consisted of the following: 50°C for 2 min, 95°C for 10 min, and 34 cycles of 95°C for 15 s and 60°C for 1 min. The 5′ nuclease assays were performed using an ABI Prism 7700 Sequence Detection System (Perkin-Elmer/Applied Biosystems) in a total volume of 25 µl containing 1X TaqMan Universal Master Mix (Perkin-Elmer/Applied Biosystems), 400 nM of either Tin3/Tin10 or Tin11/Tin10, and 12.5 ng of genomic DNA. The TaqMan probe, 5′-ATTCCCGGCTTCGGCGTCACT-3′, was labeled at the 5′-end with the fluorescent reporter dye, 6-carboxy-fluorescin (FAM) and at the 3′ end with the quencher dye, 6-carboxy-tetramethyl-rhodamine (TAMRA) (Perkin-Elmer/Applied Biosystems). The probe was used at 400 nM in both the *T. indica*-specific and the *T. walkeri*-specific assays. Dilution series of genomic DNA extracted from *T. indica* isolate Bpop and *T. walkeri* isolate YRG-001 were prepared in sterile distilled water.

Nucleotide sequence accession numbers. The 2,300 bp mtDNA sequence of *T. indica* isolates Yv3b, WL1562, and Bpop and *T. walkeri* isolates 210G, TNRG-02, and YRG-001 have been deposited at GenBank and have been assigned the accession numbers AF218058, AF218059, AF218060, AF218061, AF218062, and 218063, respectively.

RESULTS

Sequence comparison of a 2.3-kb region of mtDNA from *T. indica* and *T. walkeri*. A 2.3-kb region of mtDNA was amplified by PCR using the primers Ti-1 and Ti-4 from three isolates each of *T. indica* and *T. walkeri*, and the PCR products were cloned. The nucleotide sequence was determined for each isolate, and comparisons were made among the isolates from the same species and between the two *Tilletia* species. Among the three *T. indica* isolates, only two differences were found among the 2,297 nucleotides (>99.9% similarity). Likewise, when the three *T. walkeri* sequences were analyzed, 13 differences were observed among the 2,300 nucleotides (99.4% similarity). The comparison of *T. indica* and *T. walkeri* sequences revealed 69 nucleotide differences (Fig. 1),

most of which were randomly scattered throughout the 2.3-kb region. In each case, however, three nucleotides (CAG) were absent from the *T. indica* sequences corresponding to nucleotide positions 214 through 216 in *T. walkeri*. BLAST searches of nucleic acid and protein databases with the *T. indica* and *T. walkeri* 2.3-kb mtDNA sequences did not reveal any significant matches in either database (1).

Selection of species-specific primers and the development of PCR assays. Although the overall sequence similarity is high

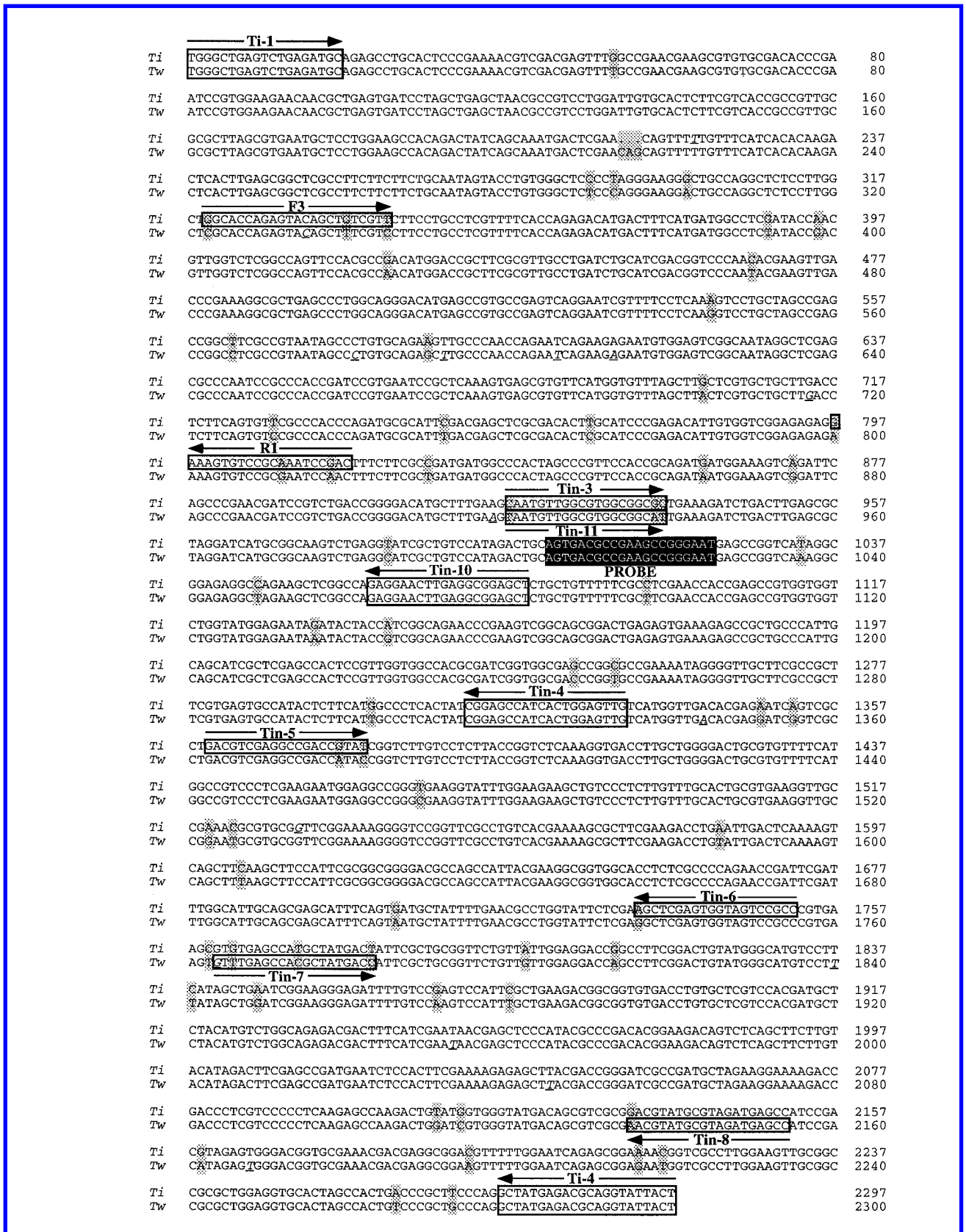

Fig. 1. Alignment of the 2.3-kilobase mitochondrial DNA nucleotide sequences from the *Tilletia indica* isolate Bpop (*Ti*) and the *T. walkeri* isolate 210G (*Tw*) Nucleotide differences that occur among either the *T. indica* isolates or the *T. walkeri* isolates are italicized and underlined, and nucleotide differences between *T. indica* and *T. walkeri* isolates are highlighted by shaded boxes. The location of PCR primers is depicted by open boxes, and 5′ to 3′ direction of each primer is shown by the arrow. The FAM-labeled TaqMan probe sequence is indicated by the black box.

between *T. indica* and *T. walkeri* within this mtDNA region (97.0% similarity), selective sites were identified for PCR primer development. Primer design incorporated differences at the final nucleotide position at the 3′-end of the oligonucleotide. Because *Taq* polymerase does not extend primers with 3′-terminal mismatches (17), PCR products are produced only in those reactions where complete annealing occurs at the 3′-end between the DNA template and the oligonucleotide primer. Using this strategy, five sets of PCR primers were designed from regions where there is a conserved nucleotide among the three *T. indica* isolates but not among the three *T. walkeri* isolates. The five primer sets designed specifically for *T. indica* are: Tin3/Tin10, F3/R1, Tin3/Tin6, Tin5/Tin6, and Tin3/Tin4, that amplify DNA fragments of: 212, 497, 885, 392, and 414 bp, respectively (Fig. 2A).

Three sets of PCR primers were selected as *T. walkeri*-specific based upon characteristic nucleotides at the final nucleotide position at the 3′-end of the oligonucleotide. The *T. walkeri* PCR primer sets, Tin11/Tin10, Tin7/Tin8, and Tin11/Tin4, amplified PCR products of 212, 391, and 414 bp, respectively (Fig. 2B). PCR reaction conditions were optimized for each set of PCR primers by adjusting the annealing temperature or the number of amplification cycles.

In order to verify the specificity of the *T. indica* and *T. walkeri* PCR primers, DNA was extracted from *T. indica* and *T. walkeri* isolates from different geographic areas (Table 1) and tested with each PCR primer set. The five *T. indica* PCR primer sets produced single bands using DNA from 34 *T. indica* isolates, while none of the 10 *T. walkeri* isolates produced a PCR product with these primers. Southern blot hybridizations confirmed the identity of the PCR product for each of the *T. indica* samples, and no detectable bands were observed with any of the *T. walkeri* samples (Southern blots not shown). Three *T. walkeri* PCR primer sets produced single bands only with DNA extracted from *T. walkeri* isolates, and no detectable DNA product was found from the *T. indica* isolates (Southern blots not shown).

TaqMan 5′ nuclease assay. Fluorogenic 5′ nuclease assays were developed for the TaqMan system, utilizing either the *T. indica*-specific primer set Tin3/Tin10 or the *T. walkeri*-specific primer set Tin11/Tin10 plus an internal 5′-FAM-labeled oligonucleotide probe. In both TaqMan assays, the amplicon was 212 bp. The ΔRQ values were measured at the conclusion of each amplification cycle, illustrated by the amplification plots *of T. indica* isolate Bpop and *T. walkeri* isolate YRG-001 (Fig. 3). In the *T. indica*-specific TaqMan assay,

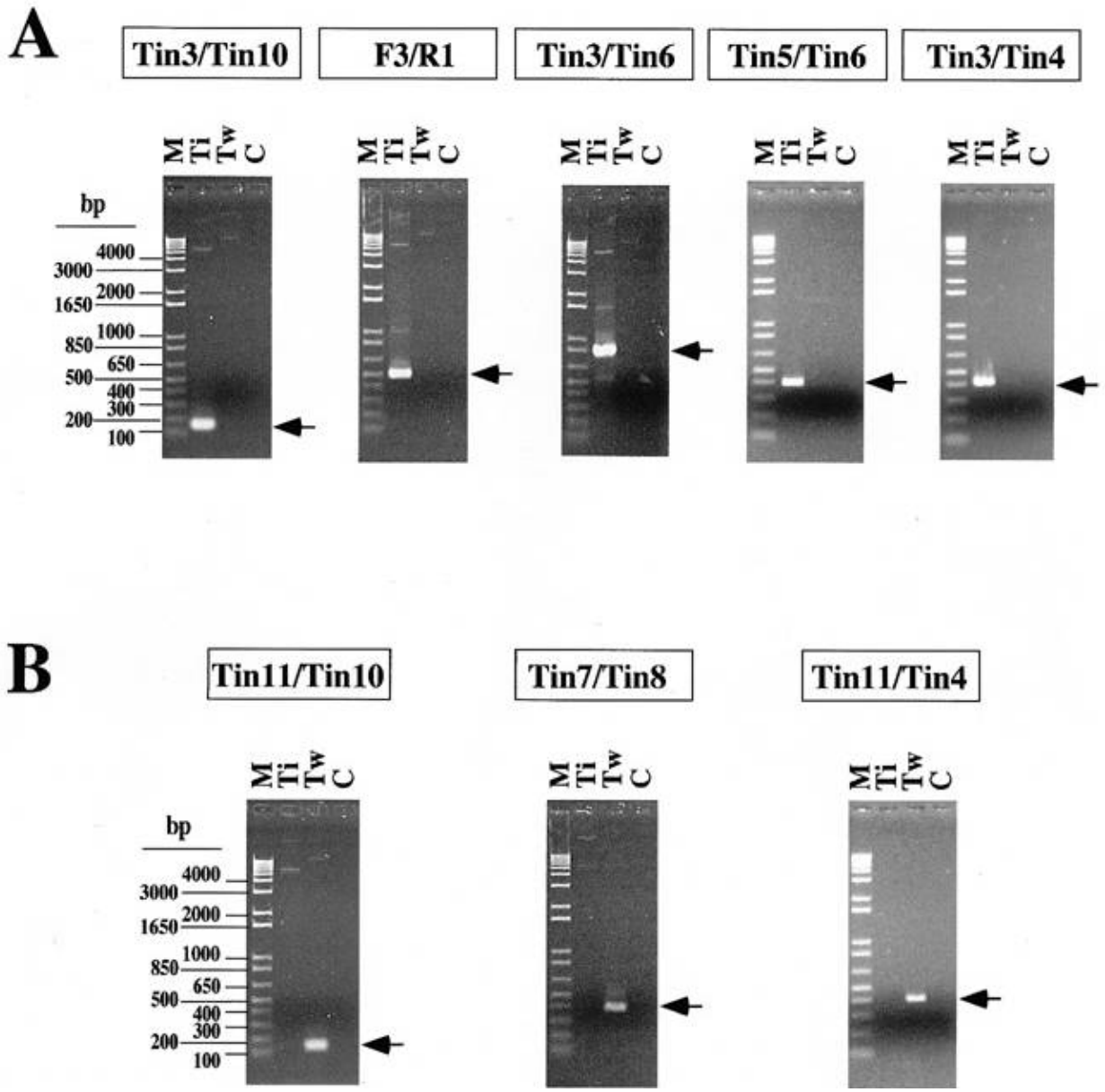

Fig. 2. Agarose gels of classical PCR assays using *Thletia indica*-specific (A) or *T. walkeri*-specific (B) primers. M = molecular weight markers, Ti = *T. indica* isolate Bpop; Tw = *T. walkeri* isolate YRG-001; and C = no DNA template control. PCR products are indicated by arrows.

TABLE 1. Isolates of *Tilletia* spp. used in this study

Isolate	Sample composition[a]	Host	Origin/Year	Supplier[b]
T. Walkeri				
210G	SW		Oregon, 1997	1
BPPC-0410	C	Ryegrass	Oregon, 1997	2
BPPC-0518	T	Ryegrass	Georgia, 1997	1
BPPC-0519	T	Ryegrass	Georgia, 1997	1
YRG-001	T	Ryegrass	Oregon, 1997	3
YRG-002	T	Ryegrass	Oregon, 1997	3
YRG-009	T	Ryegrass	Oregon, 1997	3
TNRG-02	T	Ryegrass	Tennessee, 1997	3
TNRG-03	T	Ryegrass	Tennessee, 1997	3
TNRG-10	T	Ryegrass	Tennessee, 1997	3
T. indica				
Bpop	C	Wheat	Pakistan, 1985	4
B4	T	Wheat	Pakistan, 1985	4
B4-S3	B	Wheat	Pakistan, 1985	4
B4-S5	B	Wheat	Pakistan, 1985	4
A3	T	Wheat	India, 1991	5
A1-S3	B	Wheat	India, 1991	5
A1-S4	B	Wheat	India, 1991	5
A1-S5	B	Wheat	India, 1991	5
A4-S4	B	Wheat	India, 1991	5
Pantanagar 1991	C	Wheat	India, 1991	5
Sangar	C	Wheat	India, 1991	6
WL1562	C	Wheat	India, 1983	6
Sample IIB	T	Wheat	India, 1989	6
HD2288 A	T	Wheat	India, 1989	6
WL711	T	Wheat	India, 1989	6
WL2265 A	T	Wheat	India, 1989	6
Mx-81A	T	Wheat	Mexico, 1981	7
D3 1981 Sonora	T	Wheat	Mexico, 1981	7
D3-S1	B	Wheat	Mexico, 1981	7
D3-S2	B	Wheat	Mexico, 1981	7
D3-S3	B	Wheat	Mexico, 1981	7
D3-S4	B	Wheat	Mexico, 1981	7
Mx-82	C	Wheat	Mexico, 1982	7
Mx-85	C	Wheat	Mexico, 1985	7
Ciano 1982	C	Wheat	Mexico, 1982	8
Calexico	T	Wheat	Mexico, 1983[c]	9
Mexicali CF	C	Wheat	Mexicali, CA, 1990[c]	9
Navajoa 1989 MX	C	Wheat	Mexico, 1989	7
Yv3b	T	Wheat	Mexico, 1989	7
Yv3c	T	Wheat	Mexico, 1989	7
Mv-1a	T	Wheat	Mexico, 1989	7
Mv-1c	T	Wheat	Mexico, 1989	7
Fv-1c	T	Wheat	Mexico, 1989	7
Brazil T5	T	Wheat	Brazil, intercept, 1991[d]	10
T. barclayana				
PJ-11	B	Rice	China, 1991	11
L201	B	Rice	California, 1985	12
AK-T2	C	Rice	Arkansas, 1986	4
P-5	B	Rice	Philippines, 1989	13
137aI	B	Rice	Brazil, 1991	14
Tsp14	B	Rice	California, 1982	2
Tsp5	B	*Paspalum distichum*	Washington, 1982	2
T. tritici				
C-100	C	Wheat	Washington, 1990	15
C-125	C	Wheat	Idaho, 1990	15
T. laevis				
F-008	C	Wheat	Oklahoma, 1989	16
T. controversa				
DB-107	C	Wheat	Idaho, 1989	15
DB-046	C	Wheat	Czechoslovakia, 1989	18
DB-131	C	Wheat	Montana, 1989	15
T. fusca var. *bromitectorum*				
G-105	C	*Bromus tectorum*	Utah, 1990	19
T. fusca var. *guyotiana*				
G-110	C	*Bromus brizaeformis*	Idaho, 1990	19
G-112	C	*Bromus japonicus*	Oregon, 1990	19

[a] SW = isolate derived from seed wash; C = isolate derived from mycelia grown from a mixture of teliospores; T = isolate from the isolation of a single teliospore; B = isolate from a single basidiospore.

[b] 1, R. Meyer, USDA APHIS PPQ, Beltsville, MD; 2, L. Carris, Washington State University; Pullman, WA; 3, G. Peterson, USDA ARS, Ft. Detrick, MD; 4, M. Royer, USDA APHIS, Beltsville, MD; 5, M. Bonde, USDA ARS, Ft. Detrick, MD; 6, K. Gill, Punjab Agricultural University, Ludhiana, India; 7, R. Kahn, USDA APHIS, Beltsville, MD; 8, G. Fuentes-Davila, CIMMYT, Mexico; 9, T. Boratynski, APHIS PPQ, El Centro, CA; 10, L. Butler, CIMMYT, El Batan, Mexico; 11, P. Jinhov, Dalian, China; 12, T. Matsumoto, California Department of Agriculture, Sacramento, CA; 13, J. M. Bonman, International Rice Research Institute, Manila, Philippines; 14, C. Castro, EMBRAPA, Brasilia, Brazil; 15, B. Goates, USDA ARS, Aberdeen, ID; 16, I. Williams, Oklahoma State University; Stillwater, OK; 17, G. White, Plant Protection and Quarantine, Agriculture Canada, Ottawa, Canada; 18, R. Vyskumny, Piestany, Czechoslovakia; 19, J. Huffman, USDA, retired, Hanalei, HI.

[c] Quarantine interception from Mexico by U.S. Animal and Plant Health Inspection Service (APHIS).

[d] Quarantine interception by Empresa Brazileira de Pesquisa Agropecaris (EMBRAPA).

the Bpop samples began to cross the threshold at the 20th cyling step, whereas YRG-001 did not begin to cross the threshold until the 33rd cycling step. In the *T. walkeri*-specific TaqMan assay, YRG-001 crossed the threshold at the 20th cycling step, while Bpop did not cross the threshold until the 33rd cycling step.

In order to evaluate the accuracy of the two TaqMan assays, DNA extracted from the 34 *T. indica* and 10 *T. walkeri* isolates was tested and compared to the classical PCR assays. For the *T. indica* TaqMan assays, all 34 *T. indica* isolates displayed ΔRQ values of at least 1.22, whereas ΔRQ values for the 10 *T. walkeri* isolates were no greater than 0.12 after 34 cycles (Fig. 4A). Conversely, all *T. walkeri* isolates had ΔRQ values of at least 2.21, and none of the *T. indica* isolates had ΔRQ values of more than 0.57 using the *T. walkeri*-specific primers Tin11/Tin10 (Fig. 4B). In each instance, the TaqMan assays produced the same results as classical PCR assays using either the *T. indica*-specific primers Tin3/Tin10 or the *T. walkeri*-specific primers Tin11/Tin10.

To determine the sensitivity limits of both the *T. indica*- and *T. walkeri*-specific 5′ fluorogenic assays, dilutions of purified total mycelial DNA from *T. indica* isolate Bpop and *T. walkeri* isolate YRG-001 were examined (Fig. 5). In both assays, 5 pg of total DNA produced detectable levels of fluorescence (0.06 and 0.04 ΔRQ values).

In order to verify the range of species specificity of the *T. indica*- and *T. walkeri*-specific 5′ fluorogenic assays, purified total mycelial DNA of several different *Tilletia* species were evaluated (Fig. 6). DNA extracted from *T. barclayana*, *T. tritici*, *T. laevis*, *T. controversa*, or *T. fusca* isolates did not amplify in either the *T. indica*- or *T. walkeri*-specific assay.

DISCUSSION

Methods for the detection and unambiguous identification of *T. indica* are crucial in order to alleviate unnecessary restrictions that prevent the movement of wheat free of Karnal bunt and hinder international trade of U.S. wheat. Such procedures also serve to clarify issues of pathogen (fungal) taxonomy that may cause confusion in the regulatory processes. Contamination of wheat with teliospores of the ryegrass smut pathogen, *T. walkeri*, is a case in point, raising the need for new diagnostic tests that can discriminate between these two closely related pathogens. Concerns exist that U.S. wheat shipments could be rejected at international ports due to the misidentification of *T. walkeri* teliospores as *T. indica*. Even though *T. walkeri* has been classified as a new *Tilletia* species based upon teliospore morphology and ornamentation (7), identification based solely upon a visual assessment of teliospore

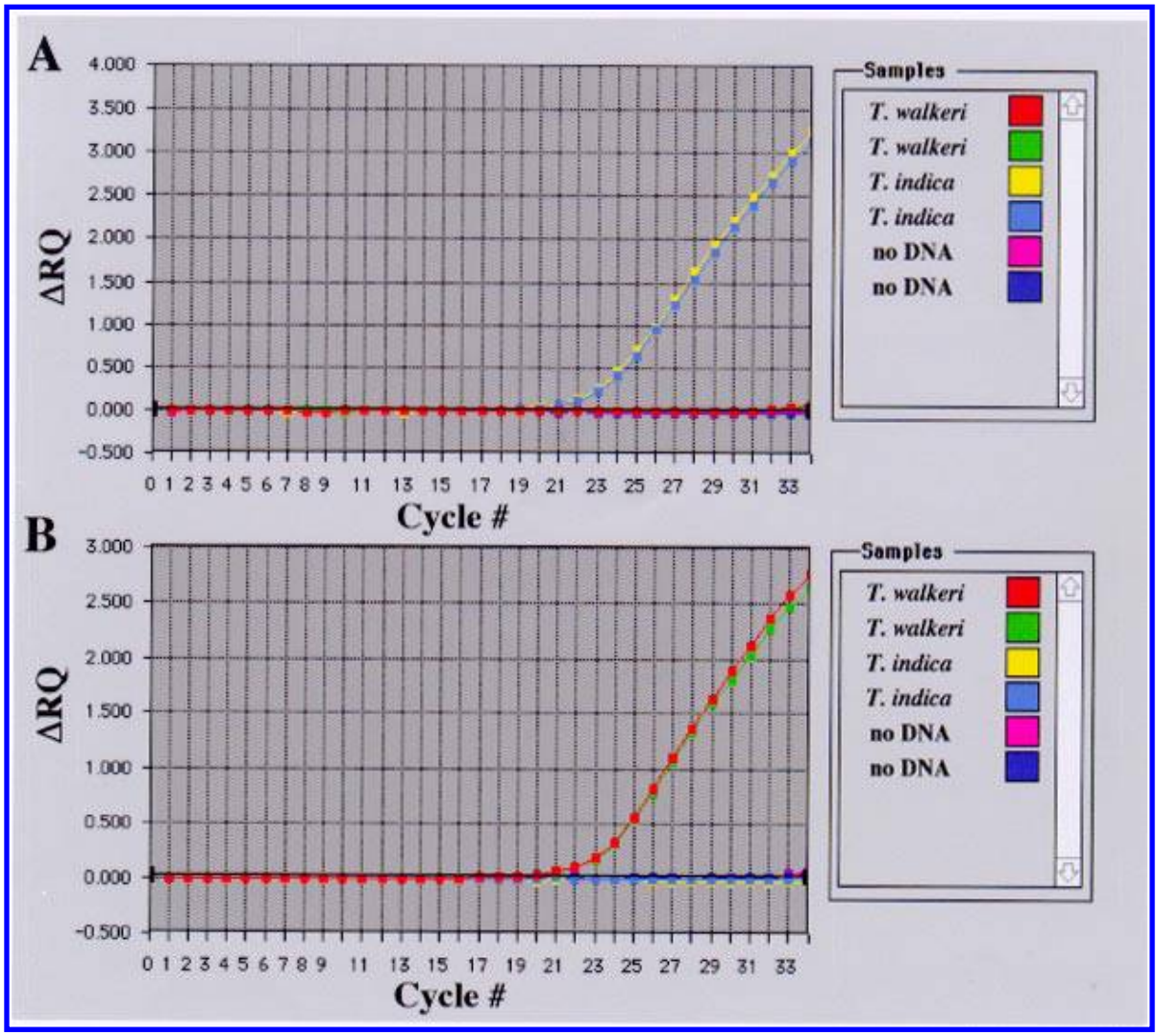

Fig. 3. Amplification of mitochondrial DNA of *Tilletia indica* isolate Bpop and *T. walkeri* isolate YRG-001 by TaqMan PCR using an ABI Prism 7700 Sequence Detection System (Perkin-Elmer/Applied biosystems, Inc., Foster City, CA). *T. indica*-specific flanking primers Tin3/Tin10 (A) or *T. walkeri*-specific flanking primers Tin11/Tin10 (B) were used with a 5′-FAM-labeled internal probe sequence. The left axis (ΔRQ) is the change in fluorescence that is a measure of probe cleavage efficiency, and the bottom axis is the PCR cycling stage. Two independent assays were analyzed using duplicate DNA samples for each isolate.

morphology is not acceptable. Therefore, we pursued the development of a molecular diagnostic test for the detection, identification and discrimination of the two *Tilletia* species: *T. indica* and *T. walkeri*.

Detection assays using classical PCR techniques have been developed for numerous plant pathogens, including bacteria, viruses, and fungi (14). These tests are attractive for several reasons. First, the assays are extremely sensitive and highly specific for the

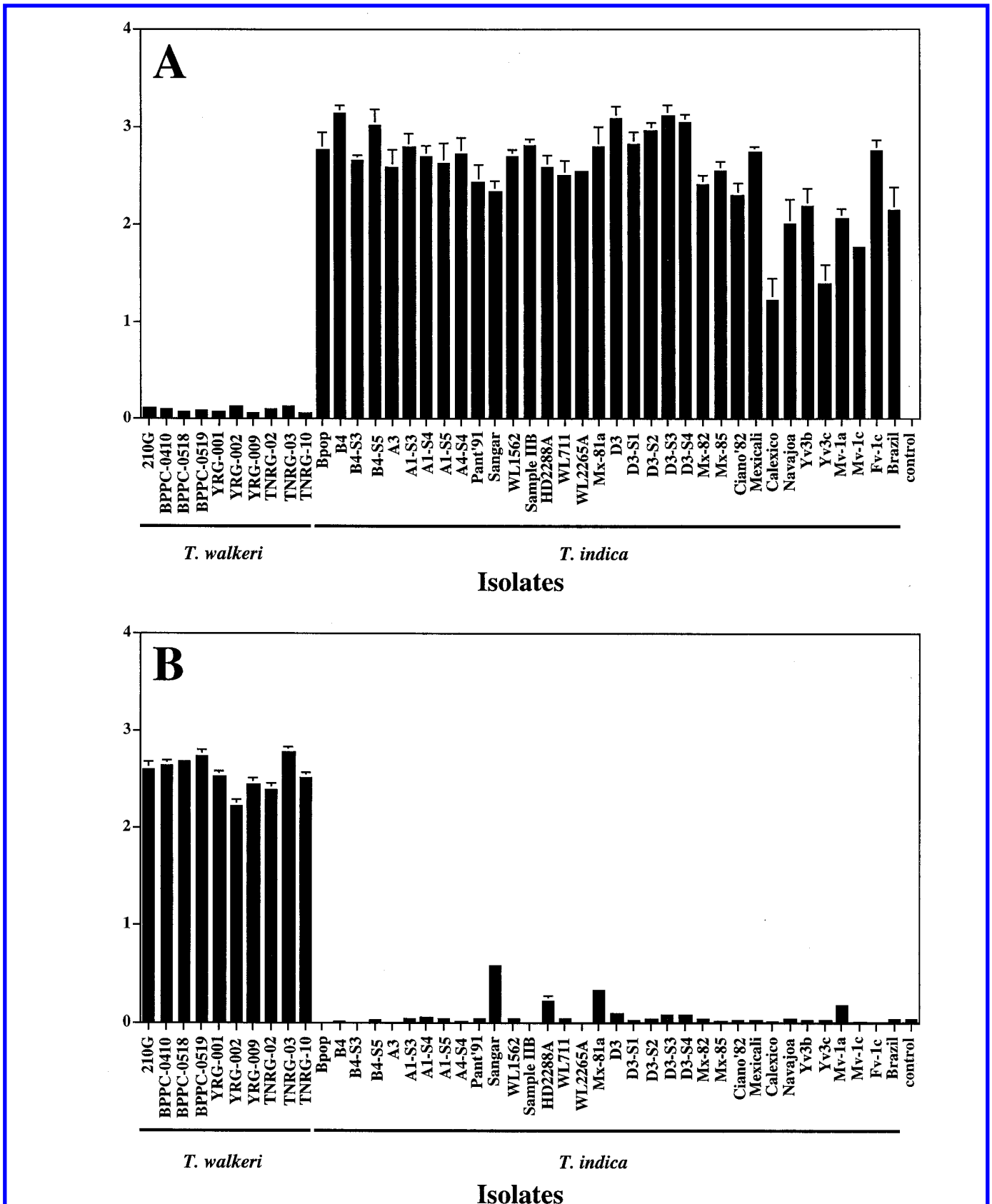

Fig. 4. TaqMan polymerase chain reaction amplification of mitochondrial DNA of *Tilletia indica* isolates and *T. walkeri* isolates using either the *T. indica*-specific flanking primers Tin3/Tin10 (A) or the *T. walkeri*-specific flanking primers Tin11/Tin10 (B) after 34 amplification cycles. The ΔRQ values are the means of two independent experiments with duplicate DNA samples. Error bars represent standard errors of the means.

pathogen in question. Second, PCR tests require minimal amounts of sample material, and commercial kits are available for extracting high quality genomic DNA from a wide variety of organisms. Finally, PCR reactions are relatively simple to set up and perform, and results can be obtained quickly, usually in a day.

Little is known about the genome or gene structure in *Tilletia* spp., including *T. indica*. Differences in chromosome number and size have been found among *T. indica* isolates (24), and genetic variation has been observed among isolates of *T. indica*, *T. barclayana*, and *T. walkeri*, either from PCR-RFLP analysis of the rDNA or from RAPD analysis (19). Any new PCR assay procedure to distinguish between *T. indica* and *T. walkeri* requires specific PCR primers to unique DNA sequences in each of the two species. Therefore, nucleotide sequence information from both *T. indica* and *T. walkeri* is a prerequisite for any PCR assay.

Previously, a 2.3-kb fragment was amplified by PCR using the primers Ti-1 and Ti-4 that was characteristic for *T. indica* isolates. DNA extracted from other smut fungi, including *T. barclayana*, *T. caries*, *T. controversa*, *T. laevis*, *T. fusca* var. *fusca*, and *T. fusca* var. *bromitectorum* , did not produce DNA bands using these two

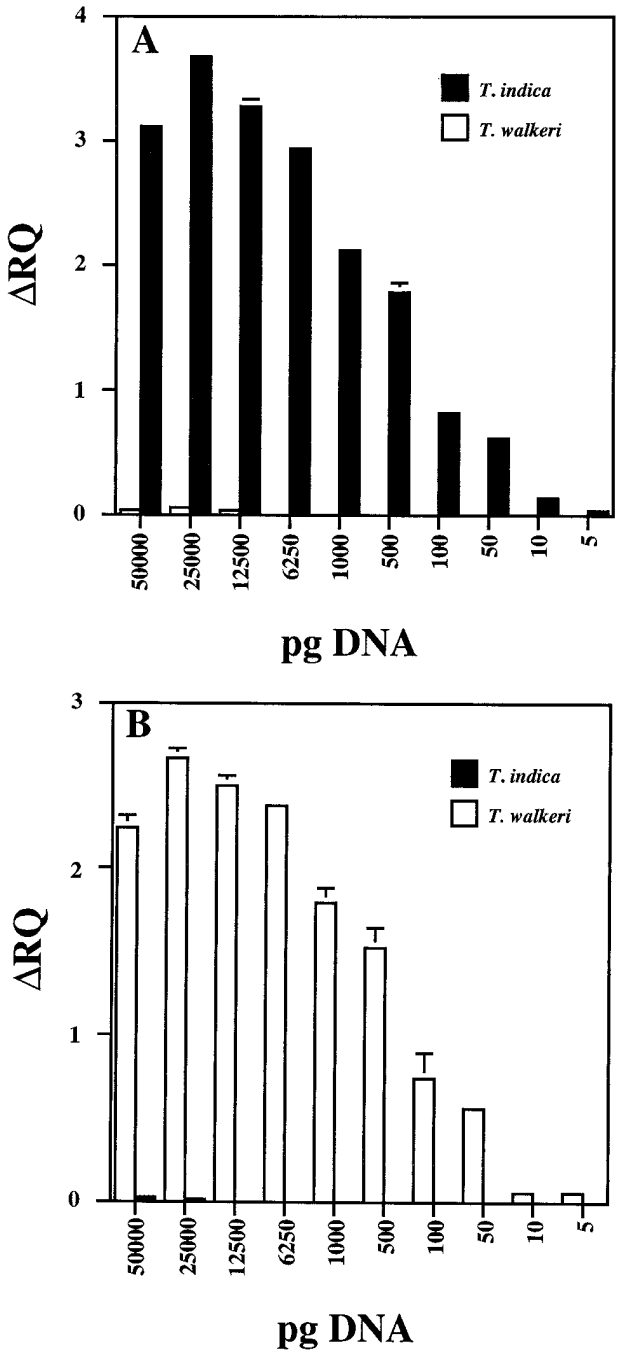

Fig. 5. Detection limits of *Tilletia indica* and *T. walkeri* DNA by the TaqMan polymerase chain reactions assays using either the *T. indica*-specific flanking primers Tin3/Tin10 (A) or the *T. walkeri*-specific flanking primers Tin11/Tin10 (B) after 34 amplification cycles. The ΔRQ values are the means of two independent experiments that had duplicate DNA samples. Error bars represent standard errors of the means. Dilution series of template DNA were prepared from *T. indica* isolate Bpop and *T. walkeri* isolate YRG-001.

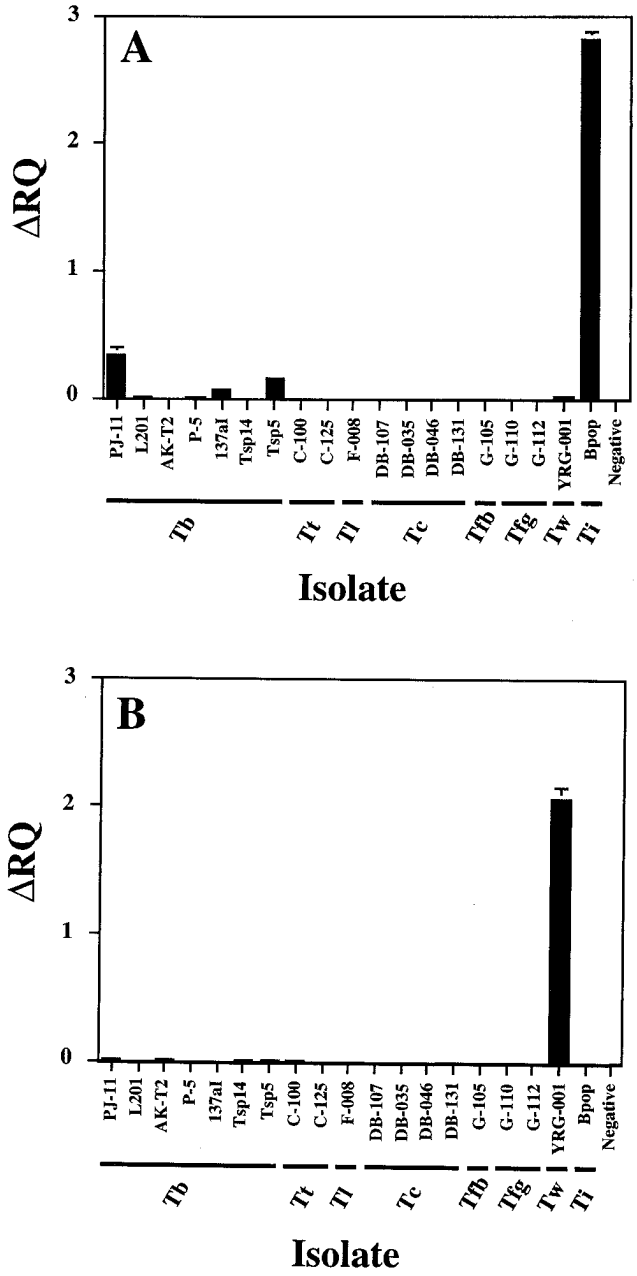

Fig. 6. TaqMan polymerase chain reaction amplification of mitochondrial DNA of *Tilletia* species using either the *T. indica*-specific flanking primers Tin3/Tin10 (A) or the *T. walkeri*-specific flanking primers Tin11/Tin10 (B) after 34 amplification cycles. Duplicate DNA samples were analyzed for each isolate. *T. barclayana* (Tb): PJ-11, L201, AK-T2, P-5, 137aI, Tsp14, and Tsp5; *T. tritici* (Tt): C-100, and C-125; *T. laevis* (Tl): F-008; *T. controversa* (Tc): DB-107, DB-035, DB046, and DB131; *T. fusca* var. *bromitectorumi* (Tfb): G-105; *T. fusca* var. *guyotiana* (Tfg): G-110, and G-112; *T. walkeri* (Tw): YRG-001; and *T. indica* (Ti): Bpop.

PCR primers (11). However, subsequent to the discovery of *T. walkeri*, a similar-sized 2.3-kb fragment was found to amplify from all available *T. walkeri* isolates using the PCR primers Ti-1 and Ti-4. We decided to clone the 2.3-kb fragment amplified by PCR using the primers Ti-1 and Ti-4 from several *T. walkeri* and *T. indica* isolates and compare the nucleotide sequences between these two *Tilletia* species.

Consistent nucleotide differences were found between *T. indica* and *T. walkeri* isolates that allowed for species-specific PCR primers to be designed. Five sets of PCR primers were made and shown to be highly specific only for *T. indica* isolates and not for any other *Tilletia* species, including *T. walkeri*. In addition, three PCR primer sets were developed that amplified single DNA bands only from *T. walkeri* isolates and not from any of the *T. indica* Isolates tested. In the future, smut teliospores isolated from wheat can be definitively identified either as *T. indica* or *T. walkeri* by using these species-specific PCR primers.

In addition to developing classical PCR assays, we adapted a set of *T. indica*- and *T. walkeri*-specific PCR primers for use with an internal 5′-FAM-labeled oligonucleotide probe sequence in a 5′-fluorogenic TaqMan PCR assay. In most 5′-fluorogenic TaqMan PCR assays, the flanking PCR primers are the same, and the internal fluorescent-labeled probe is designed to be characteristic for a specific sequence (3,15). Since the nucleotide differences within the 2.3-kb mtDNA region of *T. indica* and *T. walkeri* were randomly scattered, we chose instead to use the same probe sequence and to use different flanking primers specific either to *T. indica* or *T. walkeri*. Primer Tin3 provides specificity for *T. indica* isolates, while Tin11 is used for *T. walkeri* isolates. In both PCR assays, Tin10 is used with either Tin3 or Tin11, and the size of both amplicons is 212 bp.

The TaqMan detection assays offer several advantages over the classical PCR assays developed for *T. indica* or *T. walkeri*. The TaqMan assays combine the sensitivity of PCR along with hybridization of the internal oligonucleotide sequence that is present in a *T. indica* or *T. walkeri* mtDNA sequence. Following PCR, samples do not have to be separated on agarose gels, and the subsequent Southern blots and hybridization steps necessary to verify the identity of the PCR products are eliminated. Such additional post-PCR confirmation steps can add several days to the process of accurate pathogen identification. Using the TaqMan system, the *T. indica*- or *T. walkeri*-specific 5′-fluorogenic assays are completed within 2.5 h, and 96 samples can be tested during a single assay. As a result, large numbers of test samples can be accurately identified in a very short period of time with the TaqMan assay, and time can be critical when wheat shipments are being held at port due to the presence of smut teliospores.

Another advantage of the TaqMan system is the potential for multiplexing. 5′-fluorogenic assays have been used to detect other pathogenic microorganisms, including plant pathogenic bacteria (20) and viruses (21), and human fungal pathogens (6,22). Different fluorescent reporter dyes can be used to construct probes, and several different pathogen systems can be combined in the same PCR reaction. This reduces the labor costs that would be incurred if each of the tests were performed separately.

This is the first application of the TaqMan system for the detection and identification of plant pathogenic fungi. This assay will be extremely useful to resolve disputes regarding contamination of wheat with smut teliospores.

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