Development of a PCR-Based Method for Identification of *Tilletia indica*, Causal Agent of Karnal Bunt of Wheat

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ABSTRACT

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The polymerase chain reaction (PCR) was used to identify *Tilletia indica*, the causal agent of Karnal bunt of wheat. The method used two sets of oligonucleotide primers developed by sequence analysis of cloned *Dra*I fragments of mitochondrial DNA of *T. indica*. The primer pair TI17M1 (5′-TCCCCTTGGATCAGAACGTA-3′) and TI17M2 (5′-AGAAGTCTAACTCCCCCCTCT-3′), derived from clone pTI-MD17, amplified a single 825-bp product from all isolates of *T. indica* and no products for other *Tilletia* spp. In addition, the primer pair TI57M1 (5′-TTTTCCCTCTCTCCTTTTTTCA-3′) and TI57M2 (5′-AGCAAAGACAAAGTAGGCTTCC-3′), derived from clone pTI-MD57, produced a product of 118 bp which was unique to *T. indica*. Specificity of the primers was evaluated using 78 isolates of *T. indica* and 79 isolates of five other *Tilletia* spp., including 69 isolates of *T. barclayana*, from geographically diverse locations. The specificity of amplification products for *T. indica* was confirmed by Southern-blot hybridization using pTI-MD17 or pTI-MD57 as ^{32}P-labeled probes. The method also employed a control PCR assay that used primers to conserved binding sites that amplified an internal transcribed spacer (ITS) region of ribosomal DNA reported in the literature for several groups of fungi. All *Tilletia* spp. produced a 420-bp product using the primers ITS3 and ITS4 in the control assay. These results demonstrated that the negative PCR results obtained with *T. barclayana* and other *Tilletia* spp. using *T. indica*-specific primers were not associated with mycelial DNA degradation or the presence of PCR inhibitors. Using teliospores germinated from a seed wash extraction method of infested grain, we demonstrated that *T. indica* can be reliably detected at an infestation level of five teliospores per 50-g grain sample.

Tilletia indica Mitra is a fungal plant pathogen that causes Karnal bunt of wheat (*Triticum aestivum* L.) (13). This nonsystemic smut pathogen is spread by airborne sporidia at the time of flowering. The resulting infection leads to partial bunting of wheat kernels and a characteristic fishlike odor which emanates from infected grain (19). The major impact of the disease is on wheat quality and not yield reduction (3).

Karnal bunt is found in several tropical and subtropical areas of the world, including India (13) and Mexico (10). The disease has not been reported in the United States (4,15); however, because of several interceptions of teliospores in wheat shipments entering the United States from Mexico (10), it has become a significant concern to the U.S. wheat industry and U.S. Department of Agriculture (USDA).

The movement of wheat into the United States and other Karnal bunt-free countries is the subject of strict quarantine regulations (15). A major problem encountered with the identification of *T. indica*-contaminated wheat shipments is that at least one other smut pathogen produces teliospores that are morphologically similar. This nonquarantined pathogen, *T. barclayana* (Bref.) Sacc. & Syd., is the causal agent of kernel smut of rice (*Oryza sativa* L.). Teliospores of *T. barclayana* often contaminate wheat shipments because wheat transport systems, harvesting equipment, and storage facilities also process rice. In a recent survey of U.S. wheat export samples in southern ports, 8% of the composite samples taken from 308 ships were contaminated with *T. barclayana* teliospores (G. L. Peterson and M. R. Bonde, *unpublished data*). Presently, isozyme analysis is used to distinguish isolates of *T. indica* from *T. barclayana* using proteins extracted from germinated teliospores; however, this method requires considerable experience with interpretation of complex isozyme polymorphisms associated with these species (1,2). For these reasons, isozyme analysis is not considered a practical approach for the routine identification of *T. indica*.

This paper reports results of evaluating the polymerase chain reaction (PCR) (14) for distinguishing *T. indica* from *T. barclayana*. Using *T. indica*-specific primers derived from mitochondrial DNA sequence analysis, our results showed that this technology was a reliable method for differentiating *T. indica* from *T. barclayana*. Employing a seed wash extraction method for infested grain samples followed by PCR testing of germinated teliospores,

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we demonstrated that *T. indica* can be reliably detected at a level of five teliospores per 50-g grain sample. Preliminary results of this research have appeared in abstract form (17).

MATERIALS AND METHODS

Fungal isolates and DNA extraction. Seventy-eight isolates derived from 32 separate field collections of *T. indica* were tested. Of these, 10 isolates were each composed of a mixture of germinated teliospores from a single collection, 51 from single-teliospore isolations, and 17 from single-basidiospore isolations. Seventeen field collections of *T. barclayana* were the source for 69 isolates, including four isolates each composed of a mixture of germinated teliospores from a single collection, and 65 single-teliospore isolates. In addition, two isolates of *T. tritici* (Bjerk.) G. Wint. in Rabenh., one of *T. laevis* Kühn in Rabenh., four of *T. controversa* Kühn in Rabenh., and three of *T. fusca* Ellis & Everhart were also included in the studies. The identities of all teliospore collections of *T. indica* used in this study were confirmed by pathogenicity on the highly susceptible wheat cultivar WL-711. Plant inoculations were performed as described by Warham et al. (20). The identity of many of the *T. barclayana* collections was confirmed on the rice cultivar Blue Bonnet by the same inoculation method. Although pathogenicity results are not available for some isolates of *T. barclayana*, their identities were determined by morphological characteristics, place of origin, host from which first isolated, and the presence of a pink-red pigment that is often, but not always, produced by this organism in culture

TABLE 1. Fungal isolates and results of polymerase chain reaction (PCR) testing using two pairs of *Tilletia indica*-specific primers and a control primer pair that amplify a ribosomal DNA sequence present in all species

Isolates tested	Sample composition[a]	Origin/year	Supplier[b]	Alternate identification method[c]	Results of PCR[d]: *T. indica*-specific primers TI17M1/2	*T. indica*-specific primers TI57M1/2	Control primers ITS3/4
Tilletia indica							
MX-81A, MX-81B	T	Mexico, 1981	1	P	+	+	+
D3	T	Mexico, 1981	1	P	+	+	+
D3-S1, D3-S2, D3-S3, D3-S4	B	Mexico, 1981	1	P	+	+	+
CIANO 1982	T	Mexico, 1982	2	P	+	+	+
MX-82 pop	C	Mexico, 1982	1	P	+	+	+
CIANO 1985	C	Mexico, 1985	1	P	+	+	+
CIANO 1986	C	Mexico, 1986	1	P	+	+	+
Mali 1A, Mali 1B, Mali 1C	T	Mexico, 1986	1	P	+	+	+
MX-87	C	Mexico, 1987	1	P	+	+	+
MX-87A, MX-87B	T	Mexico, 1987	1	P	+	+	+
MX-88A, MX-88B, MX-88C	T	Mexico, 1988	1	P	+	+	+
Fuente-1A, Fuente-1C	T	Mexico, 1989	1	P	+	+	+
Mali-1D, Mali-1E	T	Mexico, 1989	1	P	+	+	+
Navajoa 1A	T	Mexico, 1989	1	P	+	+	+
Navajoa-pop	C	Mexico, 1989	1	P	+	+	+
Yaqui-3B, Yaqui-3C, Yaqui-3D	T	Mexico, 1989	1	P	+	+	+
MX-91A, MX-91B, MX-91C	T	Mexico, 1991	3	P	+	+	+
Toluca pop	C	Mexico, 1991	4	P	+	+	+
Toluca-1A, Toluca-1B	T	Mexico, 1991	4	P	+	+	+
S-9A, S-9B, S-9C, S-6197A, S-6197B, S-6197C, S-6197D, S-6210A, S-6210B, S-6210C, S-6210D, S-6210E	T	Mexico, 1993	1	P	+	+	+
Amritsar 1562	C	India, 1983	5	P	+	+	+
Sangar-83 pop	C	India, 1983	5	P	+	+	+
Sangar-1A	T	India, 1983	5	P	+	+	+
HD-2008A, HD-2288A, Punjab WL-711, Sample IIA, Sample IIB, TL-419A, WL-2265A	T	India, 1989	5	P	+	+	+
Pantanagar-91, A-pop	C	India, 1991	6	P	+	+	+
A3	T	India, 1991	6	P	+	+	+
A1-S1, A1-S2, A1-S3, A1-S4, A1-S5, A4-S2, A4-S3, A4-S4	B	India, 1991	6	P	+	+	+
B4	T	Pakistan, 1985	7	P	+	+	+
B3-S1, B4-S2, B4-S3, B4-S4, B4-S5	B	Pakistan, 1985	7	P	+	+	+
B-pop	C	Pakistan, 1985	7	P	+	+	+
BZ-1, BZ-5	T	Brazil, intercept[e]	4	P	+	+	+
Calexico CF1, Calex. CF2, Calex. CF3, Calex. CF4	T	1983 intercept from Mexico[f]	8	P	+	+	+

(*continued on next page*)

[a] C = isolate derived from mycelia grown from a mixture of germinating teliospores; T = isolate from the isolation of a single teliospore; B = isolate from a single basidiospore; U = unknown composition.

[b] 1, R. Kahn, USDA, APHIS, Hyattsville, MD; 2, G. Fuentes-Davila, CIMMYT, Mexico; 3, G. Peterson, USDA, ARS, Frederick, MD; 4, L. Butler, CIMMYT, El Batan, Mexico; 5, K. Gill, Punjab Agric. Univ., Ludhiana, India; 6, M. Bonde, USDA, ARS, Frederick, MD; 7, M. Royer, USDA, APHIS, BATTS, Hyattsville, MD; 8, T. Boratynski, APHIS, PPQ, El Centro, CA; 9, T. Matsumoto, Calif. Dept. of Agric., Sacramento, CA; 10, L. Lee, Univ. of Arkansas, Fayettville, AR; 11, G. Urskin, USDA, Fed. Grain Inspection Serv., Galveston, TX; 12, C. Castro, EMBRAPA, Brasilia, Brazil; 13, J. M. Bonman, Int. Rice Res. Inst., Manila, Philippines; 14, Z. Zhang, China Animal & Plant Quarantine Serv. (CAPQ), Beijing, PRC; 15, J. Peng, CAPQ, Dalian, PRC; 16, B. Goates, USDA, ARS, Aberdeen, ID; 17, G. White, Plant Protection and Quarantine, Agric. Canada, Ottawa, Canada; 18, R. Vyskumny, Piestany, Czechoslovakia; 19, I. Williams, Oklahoma State Univ., Stillwater, OK; 20, J. Hoffman, USDA, retired, Hanalei, HI.

[c] P = identity confirmed by host inoculation; M = identity based on morphology and origin; S = identity based on collection from infected host and morphology.

[d] + = 825-bp, 118-bp, and 420-bp products amplified using primer pairs TI17M1/M2, TI57M1/M2, and ITS3/4, respectively; – = no amplified product.

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

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

[g] DNA isolated from a mixture of fungi (no *T. indica* or *T. barclayana* observed) extracted by a wheat seed wash of 50 g of export wheat sample and grown in yeast malt (YM) shake culture for 7 days.

(22). A list of collections used in this study is presented in Table 1. All pathogen manipulations were conducted in the containment laboratory at Frederick, MD (12). Mycelial cultures were maintained on potato-dextrose agar (PDA) plates at 18°C. Mycelial plugs were transferred to 2% water agar to induce the production of abundant secondary sporidia. These sporulating cultures were transferred to shaker culture (200 rpm), grown at room temperature (21 to 23°C) for 5 to 7 days in 200 ml of yeast malt extract broth (Difco Laboratories, Detroit), collected by gravity filtration on Miracloth (Calbiochem-Behring, La Jolla, CA), and stored at –70°C prior to freeze-drying. Total DNA was extracted from 1 g of freeze-dried mycelium as described by Crownhurst et al. (5). Mitochondrial DNA (mtDNA) was isolated from total DNA of one isolate of *T. indica* (Pantanagar-91) and one isolate of *T. barclayana* (CA-1) by cesium chloride-bis-benzimide gradient ultracentrifugation as described by Karlovsky and de Cock (9). DNA concentrations were determined by UV spectroscopy at 260 nm (16).

Southern-blot analysis of mtDNA. Mitochondrial DNA (0.5 µg) from *T. indica* (Pantanagar-91) and *T. barclayana* (CA-1) was incubated for 4 h at 37°C with 10 units of *Dra*I in a 20-µl reaction volume. Fragments were resolved in a 1.2% agarose gel using 0.5× Tris-borate-EDTA (TBE) buffer, stained with ethidium bromide, and photographed (1× TBE is 89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA, pH 8.3). The agarose gel was alkaline-blotted to a Zeta-Probe GT membrane (Bio-Rad Laboratories, Richmond, CA), prehybridized, hybridized, and washed as described by the manufacturer. One microgram of *T. bar-*

TABLE 1. (*continued from preceding page*)

Isolates tested	Sample composition[a]	Origin/year	Supplier[b]	Alternate identification method[c]	Results of PCR[d]		
					T. indica-specific primers		Control primers
					TI17M1/2	TI57M1/2	ITS3/4
Calexico RR1	T	1983 rail car from Mexico[f]	8	P	+	+	+
T. barclayana							
CA-1, CA-2, CA-3, CA-4, CA-5, CA-6, CA-7	T	California, 1985	9	P	–	–	+
Wash-5, Wash-7, WAT-T01, WAT-T07, WAT-T12	T	Washington, 1985	1	P	–	–	+
AK-pop	C	Arkansas, 1986	7	P	–	–	+
AK-T01, AK-T02, AK-T03, AK-T04, AK-T07, AK-T08, AK-T09	T	Arkansas, 1986	7	P	–	–	+
Cross T1, Cross T2, Miss. T1, Miss. T3, Loudon T1, Loudon T2, Ark T1, Ark T2, Ark T3	T	Arkansas, 1993	10	P	–	–	+
R-1 TX comp	C	Galveston, TX, 1991	9	M	–	–	+
SL-7C, SL-7D, SL-7E, SL-10B, SL-11A	T	Galveston, TX, 1991	11	M	–	–	+
Tb TX-11, Tb TX-12	T	Galveston, TX, 1991	3	P	–	–	+
63212F, 63335a, 63335b	T	Galveston, TX, 1991	3	M	–	–	+
TM-6C	T	Galveston, TX, 1991	9	M	–	–	+
55457Fa, 55486L	T	Beaumont, TX, 1991	3	M	–	–	+
TS-137a, TS-139aII, TS-140a	U	Brazil, 1991	12	S	–	–	+
Philip-1, Philip-2, Philip-3, Philip-4, Philip-5, Philip-6, Philip-7, TbP-T01, TbP-T02, TbP-T04, TbP-T05, TbP-T07, TbP-T08	T	Philippines, 1989	13	P	–	–	+
TbP-pop	C	Philippines, 1989	13	P	–	–	+
PRC 1990	C	China, 1990	14	S	–	–	+
PRC 1991	C	China, 1991	15	S	–	–	+
PJ-T03, PJ-T06, PJ-T07, PJ-T12, PJ-T13, PJ-T14	T	China, 1991	15	S	–	–	+
IND-90A, IND-90E	T	India, 1990	5	S	–	–	+
IND-91A, IND-91B, IND-91C	T	India, 1991	6	S	–	–	+
T. controversa							
D-107	C	Craigmont, ID, 1989	16	P	–	–	+
D-131	C	Creston, MT, 1989	16	P	–	–	+
D-035	C	Ontario, Canada, 1989	17	S	–	–	+
D-046	C	Czechoslovakia, 1989	18	S	–	–	+
T. tritici							
C-100	C	Pullman, WA, 1990	16	P	–	–	+
C-125	C	Cavendish, ID, 1990	16	P	–	–	+
T. laevis							
F-008	C	Stillwater, OK, 1989	19	S	–	–	+
T. fusca							
G-105	C	Boise, ID, 1990	20	S	–	–	+
G-110	C	Flora, OR, 1990	20	S	–	–	+
G-112	C	Logan, UT, 1990	20	S	–	–	+
Seed wash[g]							
SW-1	C	Belle Chase, LA	3	NA	–	–	+
SW-2	C	Beaumont, TX	3	NA	–	–	+

clayana mtDNA (CA-1) was radiolabeled by random priming (Ready-To-Go Kit; Pharmacia LKB Biotechnology Inc., Piscataway, NJ) using [α-^{32}P]dCTP (3,000 Ci/mmol) (Amersham Corp., Arlington Heights, IL) and added to the hybridization solution to a final concentration of 1×10^6 cpm/ml. Autoradiography was performed at –70°C for 3 days with intensifying screens.

Recombinant DNA techniques. *T. indica* mtDNA was cleaved with *Dra*I and cloned into the *Sma*I site of the plasmid vector pBLUESCRIPT II SK+ (Stratagene Inc., La Jolla, CA) using DH5α host cells and standard recombinant DNA techniques (16). Ampicillin-resistant transformants were subjected to blue/white screening, and the size of inserts from 80 white colonies was determined by PCR (7) using T7 and T3 primers (Gibco BRL, Gaithersburg, MD) which flank the multiple cloning site of the vector. Selected recombinant clones were grown by standard protocols (16) and plasmid DNA was isolated using a Qiagen Plasmid Midi Kit (Qiagen, Inc., Chatsworth, CA). Plasmid DNA was sequenced using the Sequenase Kit (United States Biochemical Corp., Cleveland, OH) and T3 or T7 primers. Alternatively, plasmid DNA was sequenced using a Prism Ready Reaction Dye-Deoxy Terminator Cycle Sequencing Kit and a Model 373A DNA Sequencing System (Applied Biosystems, Inc., Foster City, CA). The resulting data were analyzed using the sequence analysis software package (version 7.0) of the Genetics Computer Group (6). The program PRIMER (version 0.5) (Whitehead Institute/MIT, Cambridge, MA) was used to assist with the selection of PCR primer pairs from DNA sequence data. Primers were synthesized commercially (Genosys Biotechnologies, Inc., The Woodlands, TX).

PCR method. Amplification reactions contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 10 μg/ml of gelatin, 200 μM each dNTP, 1 μM each primer, 25 ng of DNA, and 1.25 units of *Taq* DNA polymerase (Perkin-Elmer Corp., Norwalk, CT) in a final volume of 50 μl. After overlaying the reaction mixture with 50 μl of mineral oil, amplifications were performed in a Perkin-Elmer Thermal Cycler 480 (Perkin-Elmer Corp.). Samples were first heated at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min. A final step at 72°C for 5 min was added to complete primer extension. In addition, using these same conditions, control PCR assays were conducted using the primers ITS3 and ITS4. These primers anneal to conserved binding sites and amplify an internal transcribed spacer (ITS) region of ribosomal DNA for several different fungi (21). Products greater than 400 bp were analyzed using 1.2% agarose gels in Tris-acetate-EDTA (TAE) buffer (40 mM Tris-acetate [pH 8.0],

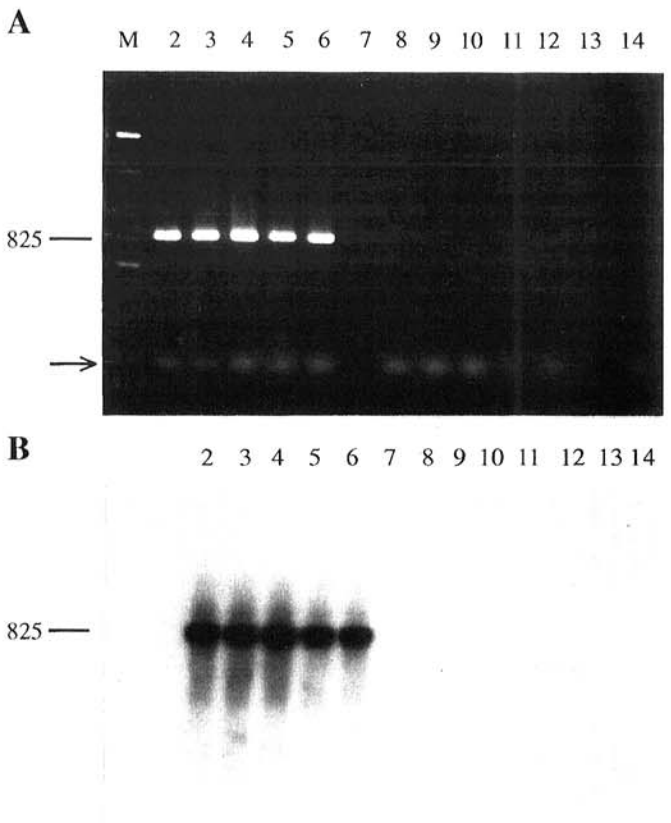

Fig. 1. A, Agarose gel electrophoresis of polymerase chain reaction (PCR) products from *Tilletia indica* and *T. barclayana* using mycelial DNA amplified with the primer pair TI17M1 and TI17M2. Lanes 2 to 6 contain PCR products from *T. indica* isolates MX-91A, Navajoa-pop, Amritsar 1562, Pantanagar-91, and MX-81A, respectively. Lanes 8 to 12 contain PCR products from *T. barclayana* isolates PRC 1990, IND-91A, PRC 1991, AK-pop, and CA-1, respectively. Lanes 7 and 13 are blank. Lane 14 contains a PCR water control. The DNA fragment has an estimated size of 825 bp and was amplified from isolates of *T. indica* only. The arrow marks the position of unreacted primers. **B,** Southern-blot hybridization analysis of **A** using ^{32}P-labeled pTI-MD17 as probe. Hybridization was observed for PCR products from isolates of *T. indica* only. Table 1 contains additional information on fungal isolates.

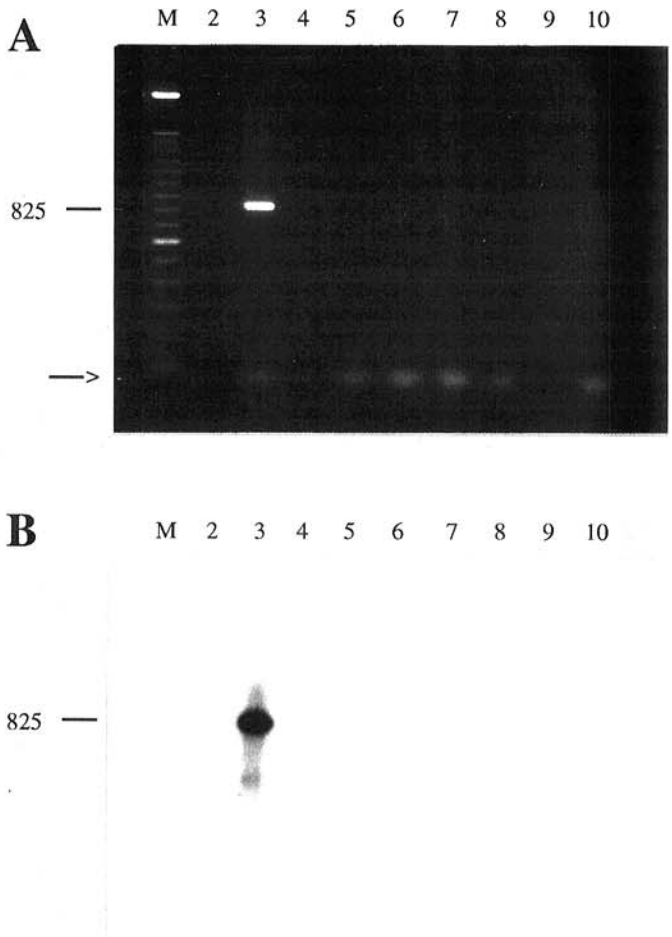

Fig. 2. A, Agarose gel electrophoresis of polymerase chain reaction (PCR) products from *Tilletia indica*, *T. tritici*, *T. controversa*, and *T. laevis* using mycelial DNA amplified with the primer pair TI17M1 and TI17M2. Lane M contains a 100-bp DNA ladder. Lane 3 contains amplified DNA from a *T. indica* isolate (Pantanagar-91). Lanes 5 to 8 contain PCR products from isolates of *T. tritici* (C-100), *T. tritici* (C-125), *T. controversa* (D-107), and *T. laevis* (F-008), respectively. Lane 10 contains a PCR water control. Lanes 2 and 4 are blank. The DNA fragment has an estimated size of 825 bp and was amplified from the *T. indica* isolate only. The arrow marks the position of unreacted primers. **B,** Southern-blot hybridization analysis of **A** using ^{32}P-labeled pTI-MD17 as probe. Hybridization was observed for the PCR product from the *T. indica* isolate only. Table 1 contains additional information on fungal isolates.

1 mM EDTA) followed by staining with ethidium bromide. Smaller products were analyzed using precast 20% acrylamide gels (NOVEX, Encinitas, CA) in 1× TBE buffer followed by staining with ethidium bromide.

Post-PCR Southern-blot analysis. Agarose gels were blotted to MagnaGraph nylon membranes (Micron Separations, Inc., Westboro, MA) using standard methodology (16). Polyacrylamide gels were electroblotted to NOVEX Nylon$^+$ membranes (NOVEX) for 2 h at 25 V as described by the manufacturer. Blots were prehybridized at 68°C for 2 h in 10 ml of 2× prehybridization/hybridization solution (Gibco BRL), and hybridization was conducted for 12 h at 68°C in 10 ml of prehybridization/hybridization solution containing 1×10^6 cpm/ml of ^{32}P-labeled plasmid DNA. Probes were prepared by random priming as described above. Blots were washed twice at room temperature for 15 min in 2× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate [pH 7.0]), 0.1% sodium dodecyl sulfate (SDS), and then twice at 68°C in 0.1× SSC, 0.1% SDS followed by autoradiography at –70°C for 6 to 72 h using an intensifying screen.

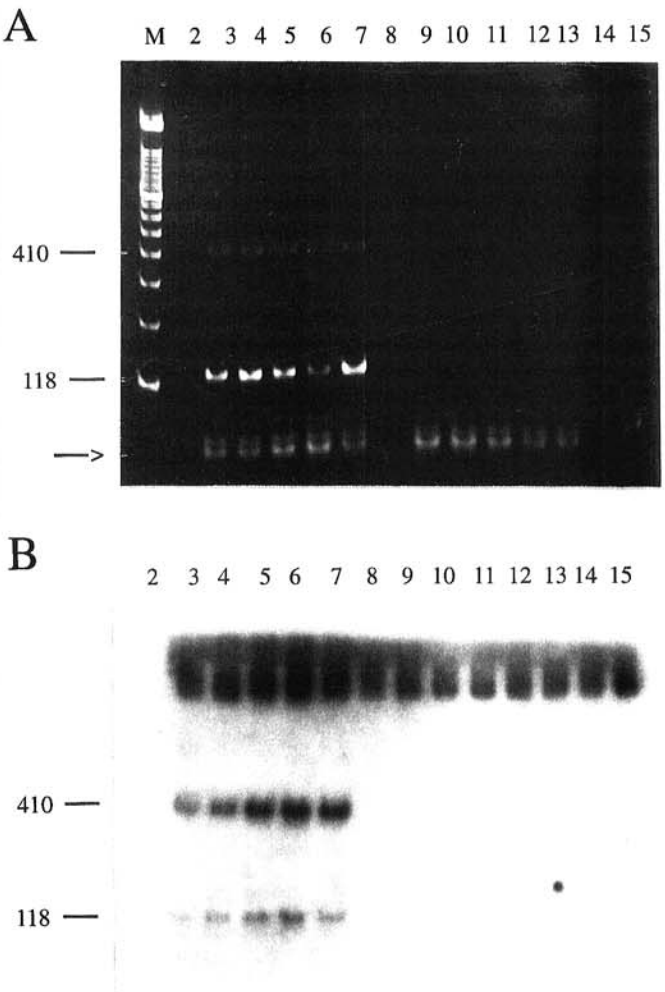

Fig. 3. A, Polyacrylamide gel electrophoresis of polymerase chain reaction (PCR) products from *Tilletia indica* and *T. barclayana* using mycelial DNA amplified with the primer pair TI57M1 and TI57M2. Lane M contains a 100-bp DNA ladder. Lanes 3 to 7 contain PCR products form *T. indica* isolates MX-91A, Navajoa-pop, Amritsar 1562, Pantanagar-91, and MX-81A, respectively. Lanes 9 to 13 contain PCR products from *T. barclayana* isolates PRC 1990, IND-91A, PRC 1991, AK-pop, and CA-1, respectively. Lane 15 contains a PCR water control. Lanes 2, 8, and 14 are blank. The major product has an estimated size of 118 bp and was amplified from isolates of *T. indica* only. A minor product with an estimated size of 410 bp also was detected. The arrow marks the position of unreacted primers. **B,** Southern-blot hybridization analysis of **A** using ^{32}P-labeled pTI-MD57 as probe. Hybridization was observed for both PCR products amplified from isolates *T. indica*. The 118-bp PCR product was not detected for isolates of *T. barclayana*; however, a faint autoradiographic signal corresponding to the 410-bp product was detected for DNA amplified from nine isolates (data not shown). Table 1 contains additional information on fungal isolates.

PCR assay of ungerminated teliospores from infected seed. Teliospore DNA was extracted using a Puregene DNA Isolation Kit (Gentra Systems, Inc., Research Triangle Park, NC) with modifications. Briefly, dry teliospores obtained from sori were hand-crushed between two siliconized glass slides and the debris suspended in 80 µl of cell lysis buffer (CLB). After transfer to a 1.5-ml microfuge tube containing 80 µl of CLB, 1 µl of proteinase K (20 mg/ml) was added followed by incubation at 55°C for 1 h. After addition of 1 µl of RNase A (4 mg/ml) and incubation at 37°C for 15 min, 50 µl of protein precipitation solution was added and DNA was recovered following the instructions of the manufacturer. The final teliospore DNA pellet was suspended in 20 µl of Tris-EDTA (TE) buffer (10 mM Tris [pH 8.0], 1 mM EDTA). The PCR assay was conducted as described above using 4-µl aliquots of each teliospore DNA solution. Teliospore amounts tested were 10,000, 5,000, 1,000, 500, 100, and 10 spores per slide as estimated by light microscope counting. Three collections of *T. indica* teliospores (MX-81, MX-91, and Pakistan) and one of *T. barclayana* (Arkansas) were tested.

Seed wash extraction of infested grain samples and PCR assay of germinated teliospores. Grain samples (50 g) were suspended in 100 ml of distilled water containing one to two drops of Tween 20 (Sigma Chemical Co., St. Louis). The suspension was poured through a 10-cm diameter, 52-µm Spectra/Mesh Nylon Filter (Spectrum, Inc., Houston, TX). After washing twice with 100 ml of distilled water, the filtrate was poured through a 10-cm diameter, 20-µm Spectra/Mesh Nylon Filter. The 20-µm filter was washed twice with 100 ml of distilled water and the debris was rinsed off the filter using 10 ml of distilled water and transferred to a 15-ml conical centrifuge tube. After incubation overnight at 20°C, samples were centrifuged for 3 min ($200 \times g$). The pellets were suspended in 10 ml of 0.525% sodium hypochlorite and immediately centrifuged for 1 min ($1{,}000 \times g$). The pellets were suspended in 10 ml of sterile distilled water and centrifuged 3 min ($200 \times g$) to wash the debris. This step was repeated and pellets were suspended in 500 µl of sterile distilled water.

Each 500-µl suspension was transferred to a 2% water agar (Difco Laboratories) plate containing 100 mg each of ampicillin and streptomycin sulfate followed by incubation at 20°C (12 h of light). After 6 to 10 days, one or more blocks of agar (about 1

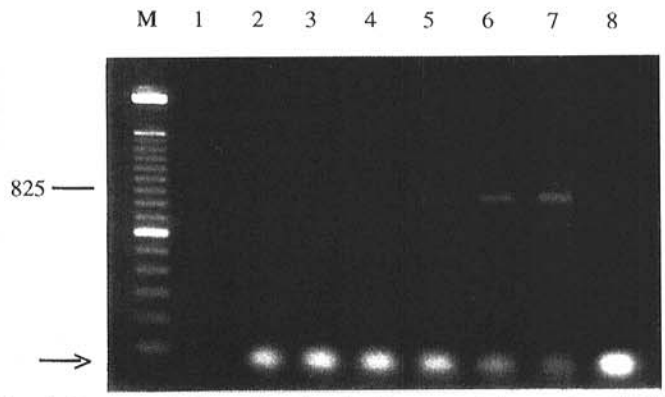

Fig. 4. Agarose gel electrophoresis of polymerase chain reaction (PCR) products from *Tilletia indica* using total DNA extracted from ungerminated teliospores and amplified with the primer pair TI17M1 and TI17M2. Lanes 2 to 7 contain PCR products from *T. indica* DNA extracted from 10, 100, 500, 1,000, 5,000, and 10,000 ungerminated teliospores, respectively. Lane 1 is blank. Lane 8 contains a PCR water control. The 825-bp product was detectable using DNA extracted from 1,000 (lane 5), 5,000 (lane 6), or 10,000 (lane 7) ungerminated teliospores. The arrow marks the position of unreacted primers.

TABLE 2. Results of testing naturally infested grain samples for *Tilletia indica* using the seed wash extraction method and polymerase chain reaction (PCR) assay of germinated teliospores

Sample	Origin	Supplier[a]	Source[b]	Results of PCR[c]			Alternate identification method[d]
				T. indica-specific primers		Control primers	
				TI17M1/2	TI57M1/2	ITS3/4	
Tilletia indica							
MX88	Mexico, 1988	1	W	3/3	3/3	3/3	P
Calexico CF	California intercept[e]	2	W	5/5	5/5	5/5	P
Mexicali RR	California rail car[e]	2	T	1/1	1/1	1/1	P
S-9	Mexico, 1993	1	W	3/3	3/3	3/3	P
T. barclayana							
Loundon Co.	Loundon Co., AR, 1994	3	R	0/1	0/1	1/1	M
Cross Co.	Cross Co., AR, 1994	3	R	0/2	0/2	2/2	M
Glen Co.	Glen Co., CA, 1985	4	W	0/3	0/3	3/3	P
Philip	Philippines, 1989	5	R	0/5	0/5	5/5	P

[a] 1, G. Fuentes-Davila, CIMMYT, Mexico; 2, T. Bortynski, APHIS, PPQ, El Centro, CA; 3, F. Lee, Univ. of Arkansas, AR; 5. T. Matsumoto, Calif. Dept. of Agric., Sacramento, CA; 6, J. Bonman, Int. Rice Res. Inst., Manila, Philippines.
[b] W = wheat; R = rice; and T = railroad box car.
[c] Denominator is number of germinated teliospores tested. Numerator is number testing positive by PCR.
[d] P = identity confirmed by host inoculation; M = identity based on morphology/origin.
[e] Quarantine interception from Mexico by U.S. Animal and Plant Health Inspection Service (APHIS).

TABLE 3. Results of testing the sensitivity of the seed wash extraction method showing number of germinated teliospores recovered from artificially infested wheat samples and identified as *Tilletia indica* by polymerase chain reaction (PCR)

Replicate	Level of infestation[a]				
	0	1	2	5	10
1	0[b]	1	1	3	10
2	0	0	0	3	6
3	0	1	2	4	6
4	0	0	0	5	6
5	0	0	0	4	5
Frequency[c]	0.0	40	40	100	100

[a] Number of teliospores of *T. indica* used to artificially infest a 50-g grain sample.
[b] Number of germinated teliospores recovered and positively identified as *T. indica* by PCR using primer pairs TI17M1/M2 and TI57M1/M2.
[c] Percentage of samples testing positive by PCR for the presence of *T. indica* teliospores.

cm^2), each containing a germinated teliospore, were cut from the plate and transferred to the inside surface of the cover of a 100- × 15-mm petri plate (one agar block per plate). The bottom of the petri plate contained 10 ml of sterile potato-dextrose broth (Difco Laboratories). After 2 to 3 days, a floating mycelial matte (0.5- to 1.0-cm diameter) was produced from basidiospores and secondary sporidia that were deposited on the surface of the broth from the germinated teliospore.

The mycelial matte was removed by needle, touched to a piece of filter paper to remove excess media, and transferred to a 1.8-ml cryovial. The sample was frozen in liquid nitrogen and hand-pulverized using a precooled glass rod (8-mm diameter). DNA was extracted using a Puregene Isolation Kit and 150 µl of total CLB as described above. The final DNA pellet was suspended in 50 µl of TE and 1-µl aliquots were used for PCR as described above.

The sensitivity of the seed wash extraction method was determined by artificial infestation of 50-g wheat grain samples. The amounts of *T. indica* teliospores artificially added were 0, 1, 2, 5, and 10 teliospores per 50-g wheat sample. Five replicates were conducted for each level of artificial teliospore infestation.

RESULTS

Pathogenicity testing and morphological identification. All collections of *T. indica* infected the wheat cultivar WL-711. Collections of *T. barclayana* from the Philippines, Washington, Arkansas, California, and Texas produced disease in the rice cultivar Blue Bonnet. Collections of *T. barclayana* from China, Brazil, and India were obtained directly from infected rice, a nonhost for *T. indica* (15,19), and satisfied the basic morphological criteria for *T. barclayana* (22). The remaining collections of *T. barclayana* were isolated from U.S. grain elevators in Texas from wheat of U.S. origin and evaluated morphologically. Isolates SL-7C, SL-10B, SL-11A, R-1 TX Comp, and TM-6C did not produce pink-red pigment when cultured on PDA (22).

Selection of cloned *Dra*I fragments of *T. indica* mtDNA and primers. Clones containing inserts between 0.6- and 2.0-kbp were chosen for further analysis. Clones were selected as candidates for *T. indica*-specific sequences because Southern-blot analysis indicated that ^{32}P-labeled mtDNA from *T. barclayana* did not hybridize to the majority of *Dra*I restriction fragments of *T. indica* mtDNA in this size range (data not shown). One clone, designated pTI-MD17 and containing a 1.1-kbp insert, was partially sequenced and used for computer-assisted primer selection. Primer TI17M1 (5′-TCCCCTTGGATCAGAACGTA-3′) and primer TI17M2 (5′-AGAAGTCTAACTCCCCCCTCT-3′) amplified an 825-bp product using plasmid pTI-MD17 as a template (data not shown). Analysis of a second clone, designated pTI-MD57 and containing a 0.9-kbp insert, resulted in the selection of primer TI57M1 (5′-TTTTCCCTCTCTCCTTTTTTCA-3′) and primer TI57M2 (5′-AGCAAAGACAAAGTAGGCTTCC-3′). Using plasmid pTI-MD57, this primer pair yielded the predicted amplification product (118 bp) and an additional minor product with an estimated size of 410 bp (data not shown).

Amplification of mycelial DNA samples and specificity of mtDNA-derived primers. As summarized in Table 1, the primer pair TI17M1 and TI17M2 produced a 825-bp product for all isolates of *T. indica*. Attempts to amplify DNA from isolates of *T. barclayana* and four additional *Tilletia* spp. produced negative results (Table 1). Representative results of agarose gel electrophoresis are presented in Figures 1A and 2A. As summarized in Table 1 and presented in Figure 3A, similar analyses using the primer pair TI57M1 and TI57M2 demonstrated a 118-bp product that was specific for *T. indica*. In addition to the expected 118-bp product, a minor product with an estimated size of 410 bp was observed using these primers (Fig. 3A). This minor product was not unique to *T. indica* as determined by post-PCR hybridization analysis (see below). The results of testing the primers ITS3 and ITS4 demonstrated that all species and isolates of *Tilletia* produced a 420-bp product (Table 1).

Post-PCR hybridization analysis. The specificity of the amplification products produced by both pairs of mtDNA-derived primers from *T. indica* was evaluated by Southern-blot analysis for all isolates tested. As shown in Figure 1B, probe pTI-MD17 hybridized to the 825-bp product amplified from *T. indica* isolates, but not to products from *T. barclayana* (Fig. 1B) or three additional *Tilletia* spp. (Fig. 2B). Similar results were obtained with probe pTI-MD57 and its 118-bp product for *T. indica*, *T. barclayana* (Fig. 3B), and other *Tilletia* spp. (data not shown). In repeated Southern-blot hybridization experiments using probe pTI-MD57, a faint autoradiographic signal corresponding to the 410-bp product was detected for other *Tilletia* spp., including nine isolates of *T. barclayana*; however, hybridization to the 118-bp product was not observed (data not shown).

Application of PCR assay to identify ungerminated teliospores. The results of using DNA extracted from ungerminated teliospores of *T. indica* for PCR are shown in Figure 4. The primers TI17M1 and TI17M2 amplified the expected 825-bp product from DNA extracted from 10,000, 5,000, or 1,000 teliospores and the amount of product was proportional to the number of teliospores processed (Fig. 4). The PCR product was not detected using DNA extracted from 500 or fewer teliospores of *T. indica* (Fig. 4) and was not observed for DNA extracted from 10,000 teliospores of *T. barclayana* (data not shown).

Application of PCR assay to identify germinated teliospores extracted and germinated from grain samples. The results of testing teliospores extracted and germinated from naturally infested grain samples are summarized in Table 2. Both pairs of PCR primers readily identified *T. indica* from four grain samples. Similar assays conducted on grain samples infested with *T. barclayana* yielded negative results (Table 2).

To estimate the sensitivity of the method for recovering and identifying germinated teliospores, grain samples (50 g) artificially infested with ten, five, two, one, or zero teliospores were tested. As summarized in Table 3, germinated teliospores were recovered and positively identified by PCR for all samples infested with five or ten teliospores. Some samples infested with one or two teliospores were not identified by PCR because germinated teliospores were not recovered (Table 3).

DISCUSSION

The utility of PCR as a specific and sensitive assay for plant pathogen identification is well documented (8). Our results showed that *T. indica* can be readily distinguished from *T. barclayana* and other *Tilletia* spp. using PCR and the primer pair TI17M1 and TI17M2. This primer pair generated an 825-bp product that was specific to *T. indica*. The successful use of ITS primers to amplify a ribosomal DNA spacer element from all the fungal isolates indicated that the negative results obtained with *T. barclayana* and other *Tilletia* spp. were not associated with DNA degradation or the presence of PCR inhibitors. Additional tests conducted with the primer pair TI57M1 and TI57M2 demonstrated amplification of a 118-bp product which was unique to *T. indica*. The primers TI57M1 and TI57M2 produced a minor fragment (410 bp) which was not unique to *T. indica* based on Southern-blot hybridization analysis. These results indicated that the primer binding sites for the 410-bp product were present in other *Tilletia* spp., including *T. barclayana*. Amplification of the 410-bp product, using the plasmid pTI-MD57 as template, indicated these sites were present on the cloned mtDNA fragment. To determine the location of these primer binding sites, future work should include the complete sequence analysis of pTI-MD57.

Although teliospores of *T. tritici*, *T. laevis*, *T. controversa*, and *T. fusca* are morphologically distinct from *T. indica* and *T. barclayana*, they were included in our studies to confirm primer specificity because they are common contaminants found in grain shipments. For example, in 1991 we surveyed 308 wheat export shipments from U.S. southern ports and found that 38% of the ships were contaminated with *T. tritici* (G. L. Peterson and M. R. Bonde, *unpublished data*). In terms of the practical applications of our seed wash extraction and teliospore germination methodology, *T. tritici* and *T. laevis* would frequently be recovered with *T. indica* and *T. barclayana* because they share common germination requirements (18,19,22). In contrast, germinated teliospores of *T. controversa* and *T. fusca* would not be recovered because these species require colder temperatures and longer incubation periods for germination (11,18).

Our testing of ungerminated teliospores obtained from sori demonstrated that approximately 1,000 teliospores were needed for reliable identification by PCR (Fig. 4). This level of sensitivity was not adequate for practical applications which require the detection of *T. indica* in grain samples containing 10 or fewer teliospores. In order to satisfy this requirement, we developed an approach that used teliospores germinated from a seed wash extraction of infested grain followed by PCR testing. We successfully applied this methodology to the identification of *T. indica* in grain samples (Table 2) and demonstrated that the detection sensitivity level approximated five teliospores per 50-g grain sample (Table 3).

In comparison to the complex polymorphisms associated with isozymes, which can be difficult to interpret (1), the PCR assay described here is easier to interpret because it is based on the presence or absence of a single DNA fragment. We believe that the application of this method will be useful in international trade. The United States does not currently screen wheat exports for the presence of Karnal bunt teliospores because the disease has not been reported in the United States. However, the PCR-based method may indeed prove useful in disputes over the identity of teliospores found in U.S. grain exports or in confirming the presence of *T. indica* in foreign grain imports. The identification of *T. indica* in a grain shipment could have a serious economic impact on the supplier and, therefore, test results may require second party confirmation. An advantage of our assay method is the ability to establish a viable reference culture from the original germinated teliospore. Furthermore, if Karnal bunt were found in the United States, the PCR-based assay could be utilized for monitoring the distribution of the pathogen. This information would be useful in establishing quarantine areas and preventing the contamination of clean U.S. wheat shipments with teliospores from infested grain.

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