Internal Transcribed Spacer Sequence-Based Phylogeny and Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Differentiation of *Tilletia walkeri* and *T. indica*

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ABSTRACT

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A polymerase chain reaction-restriction fragment length polymorphism assay to distinguish *Tilletia walkeri*, a rye grass bunt fungus that occurs in the southeastern United States and Oregon, from *T. indica*, the Karnal

bunt fungus, is described. The internal transcribed spacer (ITS) region of the ribosomal DNA repeat unit was amplified and sequenced for isolates of *T. indica*, *T. walkeri*, *T. horrida*, and a number of other taxa in the genus *Tilletia*. A unique restriction digest site in the ITS1 region of *T. walkeri* was identified that distinguishes it from the other taxa in the genus. Phylogenetic analysis of the taxa based on ITS sequence data revealed a close relationship between *T. indica* and *T. walkeri*, but more distant relationships between these two species and other morphologically similar taxa.

Tilletia indica Mitra, the causal agent of Karnal bunt of wheat, was first described on wheat near Karnal, India in 1931 (16,17). *T. indica*, a floret-infecting fungus, causes a partial bunt of wheat seeds and infected seeds have a trimethylamine odor (12,17). Since its discovery, it has been reported from Pakistan, Nepal, Afghanistan, Mexico, and the United States. (2,26). This fungus is the target of international quarantines by most wheat-growing nations and its presence poses serious risks to the ability of a country to export its wheat. Following the discovery of *T. indica* in the southwestern United States in 1996 (26), the Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) initiated the National Karnal Bunt Survey to determine the extent of this outbreak in the United States. Survey samples were sent to the USDA-APHIS National Plant Germplasm Quarantine Laboratory at Beltsville, MD, for polymerase chain reaction (PCR)-based identification using the method of Smith et al. (21), and to the APHIS National Mycologist and the USDA-Agricultural Research Service (ARS) Systematic Botany and Mycology Laboratory for morphological identification.

At that time, the most morphologically similar species of *Tilletia* known to occur in the United States and the most likely to be found in grain handling systems was *T. horrida* Takah. (*T. barclayana* (Bref.) Sacc. & Syd. in Sacc.). *T. horrida*, the rice (*Oryza sativa* L.) kernel smut fungus, produces dark-brown tuberculate teliospores similar to those of *T. indica*, although teliospores of *T. horrida* are, on average, smaller (6). Wheat samples from the southern United States may be contaminated with *T. horrida* due to the common practice of grain processors handling both wheat and rice. To distinguish *T. indica* from *T. horrida*, a

PCR test was developed (21). This test was based on PCR primers that amplified a *T. indica*-specific mitochondrial DNA fragment and did not amplify products from *T. horrida* DNA. Primers specific for the internal transcribed spacer (ITS) 2 region of the nuclear ribosomal DNA (rDNA) repeat unit were used as positive controls (24).

Until November 1996, results from the National Karnal Bunt Survey based on the PCR assay indicated that the *T. indica* outbreak was confined to the southwestern United States. However, in late 1996 and early 1997, teliospores from survey samples of pasture seed mixtures originating from Oregon tested positive for *T. indica*. Eventually, these teliospores were traced to bunted annual rye grass seeds (*Lolium multiflorum* L.) (2). Morphologically similar teliospores also were found in wheat seed washes from the southeastern United States, but bunted wheat seeds were never found in survey samples from Oregon or the southeastern United States (2,6,8). Mycologists with APHIS and ARS observed morphological differences between *T. indica* and this previously unknown *Tilletia* sp. on rye grass, which was eventually described as *T. walkeri* Castlebury & Carris (6).

During the course of the National Karnal Bunt Survey, fragment length variation was observed in the ITS positive controls of the various taxa tested with the PCR assay of Smith et al. (21). Fungal rDNA has been previously found to contain regions of variability within genera (20,24). Recent studies using restriction fragment length polymorphism (RFLP) of the ITS region have identified taxon-specific markers among the common and dwarf wheat bunts and related taxa (3-5,18). In this study, we amplified and sequenced the ITS1 and ITS2 regions of the rDNA, including the 5.8S rDNA, from isolates of *T. walkeri*, *T. indica*, *T. horrida*, and other taxa in the genus *Tilletia*. Our specific objective was to exploit any sequence variation to differentiate *T. walkeri* from *T. indica* and possibly other taxa in the genus *Tilletia*, and to determine which taxa were most closely related to *T. indica* in order to better select the most appropriate taxa for testing identification methods both now and in the future.

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MATERIALS AND METHODS

Spore germination, culture conditions, and DNA isolation.

Strains of *Tilletia* used in this study are listed in Table 1. All cultures used in this study were single teliospore or monobasidiospore lines from bunted seeds or seed washes. Teliospores removed from bunted wheat kernels were soaked in 5 ml of distilled water or Tween-water (1 to 2 drops of Tween 20 in 100 ml of distilled water) for ≈ 24 h at 22 to 25°C. Teliospores were pelleted by centrifugation at $\approx 1,000 \times g$ in an IEC CL centrifuge for 3 min. The supernatant was discarded and the pellet was resuspended in 10 ml of 0.42% NaClO (8% vol/vol commercial bleach) and immediately centrifuged as described above for 1 min. The supernatant was discarded and the pellet washed twice with 14 ml of sterile water followed by a 1-min centrifugation as above. Telio-

spores were resuspended in 1.8 ml of sterile water, which was divided in half and spread on water agar (WA) supplemented with streptomycin and ampicillin at 100 mg/liter each and incubated at 19°C. Small blocks of agar with individual germinating teliospores were aseptically removed with a scalpel and affixed to the lid of a petri dish containing potato dextrose broth so that secondary basidiospores were discharged into the broth. After 7 to 14 days, mycelia were removed from the dish, blotted on filter paper, vacuum-dried in 1.5-ml microcentrifuge tubes, and stored at -20°C.

To extract DNA, vacuum-dried mycelium was frozen in liquid nitrogen and pulverized with a microtube pestle. Genomic DNA was isolated using the PureGene DNA isolation kit (Gentra Systems, Inc., Research Triangle, NC) according to the manufacturer's instructions.

TABLE 1. Geographic origin and host of *Tilletia* isolates used in the study, and results from *ScaI* restriction digest of the internal transcribed spacer 1 (ITS1) region of the ribosomal DNA

Species, collection no. ^a	Origin ^b	Host/source	ITS1/ <i>ScaI</i> digest ^c	GenBank Accession
<i>Tilletia indica</i>				
WL-7562 ^d	Amristar, India, 1983	<i>Triticum</i> sp.	+/-	...
A-3 ^d	Pantanagar, India	<i>Triticum</i> sp.	+/-	...
Sangar ^d	India, 1983	<i>Triticum</i> sp.	+/-	...
HD 2288 ^d	India, 1989	<i>Triticum</i> sp.	+/-	...
BC-388 ^e	Jagraon, India, 1995	<i>Triticum</i> sp.	+/-	AF31079
BC-389 ^e	Jagraon, India, 1995	<i>Triticum</i> sp.	+/-	...
B-pop ^d	Pakistan, 1985	<i>Triticum</i> sp.	+/-	...
BC-392	Pakistan, 1996	<i>Triticum</i> sp.	+/-	AF310177
BC-393 ^e	Pakistan, 1996	<i>Triticum</i> sp.	+/-	...
Yaqui Valley ^d	Yaqui Valley, Mexico, 1989	<i>Triticum</i> sp.	+/-	...
FV-1a ^d	Fuente Valley, Mexico, 1989	<i>Triticum</i> sp.	+/-	...
Toluca Valley ^d	Mexico	<i>Triticum</i> sp.	+/-	...
MX-82 ^d	Ciudad Obregon, Mexico 1981	<i>Triticum</i> sp.	+/-	...
Navajoa ^d	Mexico, 1989	<i>Triticum</i> sp.	+/-	...
MX-81 ^d	Ciudad Obregon, Mexico, 1981	<i>Triticum</i> sp.	+/-	...
MV-1a ^d	Mayo Valley, Mexico, 1989	<i>Triticum</i> sp.	+/-	...
BC-386 ^e	Ciudad Obregon, Mexico 1991	<i>Triticum</i> sp.	+/-	AF310174
BC-398 ^e	Blythe, CA, 1996	<i>Triticum</i> sp.	+/-	AF310175
BC-399 ^e	Blythe, CA, 1996	<i>Triticum</i> sp.	+/-	...
BC-404 ^e	Poston, AZ, 5 Jun 1996	<i>Triticum durum</i>	+/-	...
BC-405 ^e	Poston, AZ, 5 Jun 1996	<i>T. durum</i>	+/-	AF310176
BC-406 ^e	Poston, AZ, 5 Jun 1996	<i>T. durum</i>	+/-	...
BC-341 ^e	Yuma, AZ, Mar 1996	Seed wash	+/-	...
KB96-1733 ^e	Pinal Co., AZ, May 1996	Seed wash.	+/-	...
KB96-3845 ^e	Arizona, 1996	Seed wash	+/-	...
KB96-515 ^e	Arizona, 1996	Seed wash	+/-	...
KB96-2114 ^e	Arizona, 1996	Seed wash	+/-	...
KB96-6109 ^e	Arizona, 1996	Seed wash	+/-	...
KB96-93 ^e	Arizona, 1996	Seed wash	+/-	...
BC 494 ^e	San Saba Co., TX, 1997	<i>Triticum</i> sp.	+/-	...
BC 495 ^e	San Saba Co., TX, 1997	<i>Triticum</i> sp.	+/-	...
BC 496 ^e	San Saba Co., TX, 1997	<i>Triticum</i> sp.	+/-	...
BC 497 ^e	San Saba Co., TX, 1997	<i>Triticum</i> sp.	+/-	...
BC 498 ^e	San Saba Co., TX, 1997	<i>Triticum</i> sp.	+/-	...
BC 499 ^e	San Saba Co., TX, 1997	<i>Triticum</i> sp.	+/-	...
LC 1012 ^d	San Saba Co., TX, 1997	<i>Triticum</i> sp.	+/-	AF310178
<i>Tilletia walkeri</i>				
BC 447 ^e	Oregon, 1996	<i>Lolium multiflorum</i>	+/+	AF310185
BC 449 ^e	Oregon, 1996	<i>L. multiflorum</i>	+/+	AF310182
BC 451 ^e	Oregon, 1996	Seed wash	+/+	...
BC 456 ^e	Oregon, 1996	Seed wash	+/+	AF310184

(continued on next page)

^a Culture numbers are in parentheses. The presence of a city, town, or country name in the collection number does not necessarily indicate the presence of the fungus in that location.

^b Geographic origin and sample collection date.

^c ITS1 = polymerase chain reaction (PCR) amplification of ITS1 region DNA; + indicates amplification of a DNA band with primers ITS5 and ITS2. *ScaI* = restriction fragment length polymorphism analysis of the ITS1 PCR product with *ScaI*; + indicates digestion of the ITS1 PCR product into two bands of approximately 140 and 160 bp in size; - indicates the ITS1 PCR product was not digested with *ScaI*.

^d Samples provided as purified DNA from G. Peterson, USDA-ARS-FDWSRU.

^e Samples provided as freeze-dried mycelium from R. Meyer, USDA-APHIS-NPGQC.

^f Sample provided as bunted seed by M. Palm, USDA-APHIS.

^g Sample provided as bunted seed by B. Cunfer, University of Georgia.

^h Samples provided as freeze-dried mycelium from L. Carris, Washington State University.

ⁱ Sample provided as dry teliospores from P. Paulech, Slovak Academy of Science.

PCR and gel analysis. Purified DNA was diluted 1:10 with sterile water, and 1 µl was used in amplification reactions containing *T. indica*-specific primer pairs Ti17 M1/M2 and Ti57 M1/M2 (21). The ITS1 region was amplified using primer pair ITS5/2 as a positive control (24). These primers amplify a small portion of the small subunit rDNA, the ITS1 region, and the 5' end of the 5.8S rDNA. Fungal DNA was amplified in 50-µl reactions using a DNA amplification kit (Perkin-Elmer, Applied Biosystems, Foster City, CA) containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, gelatin at 10 µg/ml, 200 µM each dNTP, 20 pmol each primer, and 1.25 units of AmpliTaq. The thermal cycler program was as follows: 1 denaturation cycle of 94°C for 3 min, 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min, followed by a final extension step at 72°C for 10 min. PCR products were electrophoresed in 5% polyacrylamide in 1× TBE (89 mM Tris, 89 mM borate, and

2.5 mM EDTA) at 110 V for 2 h and visualized with silver nitrate. PCR products for sequencing were amplified under the same conditions as described above using the primer pairs ITS5/4 for the complete ITS region or ITS5/2 and ITS3/4 to amplify the region in two parts (24).

ITS PCR product purification, DNA sequencing, and analysis. PCR products were separated in 1.2% agarose electrophoresed in 1× TAE buffer (40 mM Tris-acetate, [pH 8.0], 1 mM EDTA) at 70 V for 1.2 h followed by staining with ethidium bromide. Bands were excised, crushed, and incubated overnight at 35°C with elution buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA) (14) at 400 µl per tube. Eluted DNA was filtered through 0.45-µm Spin-X columns (CoStar), precipitated, and concentrated by centrifugation. PCR products were further purified using the Wizard PCR Preps DNA purification kit according to the manufacturer's recommendations (Promega, Madison, WI).

TABLE 1. (continued from preceding page)

Species, collection no. ^a	Origin ^b	Host/source	ITS1/ <i>ScaI</i> digest ^c	GenBank Accession
BC 427 ^e	Oregon, 1996	Seed wash	+/+	...
BC 420 ^e	Oregon, 1996	Seed wash	+/+	...
BC 466 ^e	Tennessee, 1997	<i>L. multiflorum</i>	+/+	...
BC 464 ^e	Tennessee, 1997	<i>L. multiflorum</i>	+/+	...
BC 462 ^e	Tennessee, 1997	<i>L. multiflorum</i>	+/+	...
BC 199 ^e	Spain, 1996	Seed wash	+/+	...
BC 187 ^e	Australia, 1996	Seed wash	+/+	...
BC 188 ^e	Australia, 1996	Seed wash	+/+	AF310181
BC 249 ^e	Oregon, 1996	<i>L. multiflorum</i>	+/+	...
BC 027 ^e	Georgia, 1996	Seed wash	+/+	AF310183
98-8 (LC 1123) ^f	Georgia, 1998	<i>L. multiflorum</i>	+/+	AF310180
<i>Tilletia horrida</i>				
TS-137 A2 ^d	Brazil	<i>Oryza sativa</i>	+/-	...
Philip-1 ^d	Philippines	<i>O. sativa</i>	+/-	...
Frederick L-201 ^d	Glen Co., CA	<i>O. sativa</i>	+/-	...
CA-5 ^d	Yuba Co., CA	<i>O. sativa</i>	+/-	...
AK-T05 ^d	Arkansas Co., AR	<i>O. sativa</i>	+/-	...
Nb-1991A ^d	India	<i>O. sativa</i>	+/-	...
WASH-5 ^d	Unknown	<i>O. sativa</i>	+/-	...
PJ-T10 ^d	Peoples Republic of China	<i>O. sativa</i>	+/-	...
BA-1 (BC-491) ^g	Louisiana, 1997	<i>O. sativa</i>	+/-	...
BA-1h (BC-492) ^e	Louisiana, 1997	<i>O. sativa</i>	+/-	...
BA-1 (BC-493) ^g	Louisiana, 1997	<i>O. sativa</i>	+/-	...
337 ^h	Stuttgart, Arkansas, 1988	<i>O. sativa</i>	+/-	...
338 ^h	Cross Co., Arkansas	<i>O. sativa</i>	+/-	AF310172
340 ^h	Clay Co., Arkansas, 1995	<i>O. sativa</i>	+/-	...
358 ^h	Stuttgart, Arkansas	<i>O. sativa</i>	+/-	AF310173
69539 ^h	California, 1982	<i>O. sativa</i>	+/-	AF310171
<i>Tilletia barclayana</i>				...
637 ^h	Durango, Mexico, 6 Nov 1976	<i>Paspalum distichum</i>	+/-	AF310170
828 ^h	Durango, Mexico, 18 Oct 1978	<i>Panicum obtusum</i>	+/-	AF310169
832 ^h	Durango, Mexico, 18 Oct 1978	<i>Paspalum distichum</i>	+/-	AF310168
<i>Tilletia bromi</i>				
148 ^h	Dusty, WA, 24 Jun 1992	<i>Bromus tectorum</i>	+/-	AF310186
171 ^h	Flora, OR, 28 Jul 1992	<i>B. japonicus</i>	+/-	AF310187
<i>Tilletia fusca</i>				
141 ^h	Kahlotus, WA, 14 Jul 1971	<i>Vulpia microstachys</i>	+/-	AF310189
<i>Tilletia goloskokovii</i>				
321 ^h	Winchester-Ephrata, WA, 1995	<i>Apera interrupta</i>	+/-	AF310190
<i>Tilletia rugispora</i>				
357(a) ^h	Brazil, 30 Nov 1976	<i>Paspalum</i> sp.	+/-	...
357(b) ^h	Brazil, 30 Nov 1976	<i>Paspalum</i> sp.	+/-	...
<i>Tilletia cerebrina</i>				
168 ^h	Wind River Lake, WY, 1992	<i>Deschampsia caespitosa</i>	+/-	AF310188
<i>Tilletia controversa</i>				
V-412 ^h	Hungary	<i>Elymus hispidus</i>	+/-	AF310165
255 ^h	Malad-Weston, Idaho	<i>Secale cereale</i>	+/-	...
V-528 ^h	Bavaria, Germany	<i>Triticum aestivum</i>	+/-	...
<i>Tilletia laevis</i>				
V-766 ^h	Iran	<i>T. aestivum</i>	+/-	AF310166
<i>Tilletia tritici</i>				
T-1 ^h	Not available	<i>Triticum</i> sp.	+/-	AF310167
T-12 ^h	Not available	<i>Triticum</i> sp.	+/-	...
T-18 ^h	Not available	<i>Triticum</i> sp.	+/-	...
00138-1 ⁱ	Slovakia, Jul 1996	<i>T. aestivum</i>	+/-	...

Purified PCR products were sequenced in both directions using dye-terminator sequencing with an ABI 373A automated sequencing system at the DNA Sequencing Facility at the University of Maryland (College Park, MD) and an ABI 310 (Applied Biosystems) at the USDA Systematic Botany and Mycology Lab. The Lasergene software package (DNASTAR, Madison, WI) was used for processing and editing raw sequence data using the EditSeq function. The MapDraw feature of DNASTar was used to generate a list of restriction enzymes based on the sequences for the isolates sequenced.

Enzymatic digestion of ITS PCR products. ITS1 PCR products were digested directly with the restriction enzyme *ScaI* (Gibco BRL Corp., Gaithersburg, MD) using 3 µl of PCR product, 1.2 µl of buffer (10×), and 7.8 µl of sterile H₂O at 37°C for 2 to 4 h. Enzymatic digests were size fractionated in polyacrylamide and visualized with silver nitrate. PCR products (10 µl) amplified with ITS5/4 (ITS1, 5.8S rDNA, and ITS2 regions) were also digested with *ScaI* for 1 to 2 h, electrophoresed in 3% NuSieve 3:1 (FMC BioProducts, Rockland, ME) agarose at 100 V for 2 h, and visualized with ethidium bromide under UV light.

Phylogenetic analysis of ITS sequences. Sequences were manually aligned using GeneDoc (distributed by K. Nicholas). Phylogenetic analysis using parsimony (PAUP; version 4.08b, Sinauer, Sunderland, MA) was used to determine relationships of the taxa sequenced with the following settings: heuristic search option with random addition of sequences (10 replicates), tree-bisection-reconnection branch swapping, and steepest descent. Only

parsimony informative characters were included in the analysis and were treated as unordered with equal weights. Gaps were treated as missing data. One thousand bootstrap replicates were performed to estimate the relative support for the branches. Trees were midpoint rooted due to the unavailability of a true outgroup sequence.

RESULTS

PCR assays and restriction enzyme digestion. The PCR assay using the *T. indica*-specific primer pairs Ti17 M1/M2 and Ti57 M1/M2 amplified products of similar size from both *T. indica* and *T. walkeri*, but did not amplify products from *T. horrida* DNA (Fig. 1). Sequence analysis of the entire ITS region identified a *ScaI* restriction enzyme site in the ITS1 region of *T. walkeri* that was not present in *T. indica* or other *Tilletia* spp. included in the study. The ITS *ScaI* RFLP-PCR test (*ScaI* test) has been screened against 36 isolates of *T. indica*, 15 isolates of *T. walkeri*, and 34 isolates of taxa in the genus *Tilletia* from wheat and other grasses (Fig. 2; Table 1). Sequences deposited in GenBank are listed in Table 1.

Sequence analysis. Among the 12 isolates of *T. indica* and *T. walkeri* sequenced, *T. walkeri* differed from *T. indica* at two positions, one of which was a part of the *ScaI* site. Both sites were A/G transitions located in the ITS1 region and did not vary between isolates of the two taxa. In addition, among *T. indica* isolates, LC 1012 differed from the other isolates at one position (C/A transversion), isolate BC 386 differed from the other isolates at one position (G/A transition), and isolate BC 398 also differed from the others by a G to A transition. Among *T. walkeri* isolates, BC 447 differed from all other *T. walkeri* and *T. indica* isolates by one substitution (G/A transition). All of these substitutions were located in the ITS2 region.

Fig. 1. *Tilletia indica*-specific polymerase chain reaction (PCR) assay of Smith et al. (21) showing PCR amplification from *T. indica* and *T. walkeri* but not *T. horrida* with the *T. indica*-specific primers and for all taxa with internal transcribed spacer region positive controls. Lanes 1 and 14, 100-bp marker; lanes 2, 3, 6, 7, 10, and 11, *T. indica*; lanes 4, 8, and 12, *T. walkeri*; and lanes 5, 9, and 13, *T. horrida*.

Fig. 2. Agarose gel electrophoresis of *ScaI* restriction enzyme digest of internal transcribed spacer 1 (ITS1), 5.8S, and ITS2 ribosomal DNA polymerase chain reaction products for morphologically similar taxa in the genus *Tilletia*. Lanes 1 and 20, 100-bp ladder; lanes 2 to 7, *T. indica*; lanes 8 to 13, *T. walkeri*; lanes 14 to 16, *T. horrida*; and lanes 17 to 19, *T. barclayana*.

Fig. 3. One of four most parsimonious trees showing internal transcribed spacer 1 (ITS1), 5.8S, and ITS2 ribosomal DNA phylogeny of taxa in the genus *Tilletia*. Consistency index = 0.902, retention index = 0.974, rescaled consistency index = 0.879. Bootstrap values greater than 50% are shown above the branches.

T. tritici (Bjerk.) G. Wint. in Rabenh. and *T. laevis* Kühn in Rabenh. differed by one insertion/deletion (indel) and *T. controversa* Kühn in Rabenh. differed from *T. tritici* and *T. laevis* at five positions (excluding the indel site difference between *T. tritici* and *T. laevis*). Sequences of *T. barclayana* and *T. horrida*, previously synonymized taxa (22), were highly diverged and difficult to align. Within *T. barclayana*, isolate 828 differed from isolates 832 and 637 by five substitutions and one indel, isolate 832 differed from 828 and 637 by two substitutions, and isolate 637 differed from 828 and 832 by two substitutions. Within *T. horrida*, isolate 338 differed from isolates 358 and 69539 by one substitution and isolate 358 differed from 338 and WSP 69539 by three substitutions.

The alignment consisted of 697 total characters, of which 219 were parsimony informative and was submitted to TreeBase (S618). Phylogenetic analysis resulted in four most-parsimonious trees 327 steps long (consistency index = 0.902, retention index = 0.974, rescaled consistency index = 0.879) differing only in the arrangement of taxa within terminal groups and showed *T. indica* and *T. walkeri* to be more closely related to each other than to other taxa included in the analysis (Fig. 3). *T. horrida*, previously assumed to be closely related to *T. indica* based on similar teliospore morphology and synonymous with *T. barclayana*, is quite distant from *T. indica* and *T. walkeri* as well as from *T. barclayana* from *Panicum* and *Paspalum*. The common and dwarf bunt fungi clustered most closely with isolates from the *T. fusca* Ellis & Everh. (*T. bromi* (Brockm.) Brockm.) complex with relatively short branch lengths indicating the small amounts of sequence variation among these fungi.

DISCUSSION

DNA sequencing and restriction digest results show that *T. walkeri* can be easily distinguished from *T. indica* and other morphologically similar bunt fungi with a *ScaI* restriction digest of either the ITS1 region alone or the entire ITS1, 5.8S, and ITS2 region of the nuclear rDNA repeat unit. Although *T. walkeri*, *T. indica*, and *T. horrida* can all be distinguished using morphological characters, identifications based on a single or a few teliospores can be problematic due to the intergradation of morphological characters (6). In many areas, rye grass may be growing alongside or within fields of wheat and accurate identifications are extremely important (2,6,8). Quarantine agencies require unequivocal decisions regarding the identities of quarantine pests and morphologically similar nonquarantine pests when millions of dollars of imports and exports are at stake.

Recently, species-specific PCR primers and a *TaqMan* test for both *T. indica* and *T. walkeri* have been developed (11) based on the mitochondrial DNA fragment that was the basis of the Smith et al. (21) PCR-based *T. indica* identification method. These species-specific PCR primers provide a presence/absence test for fragments specific to each of the two species. In such presence/absence diagnostic assays, it is necessary to include a positive control reaction so that negative results can be properly attributed to the absence of the primer annealing sites rather than a failure of the DNA to amplify due to degradation of the DNA, PCR inhibitors, or reagent problems. Previously mentioned PCR identification methods used the ITS3/ITS4 primer set, which amplifies the ITS2 region and part of the 5.8S rDNA (21), and the primer set ITS5/2, which amplifies the ITS1 region and part of the 5.8S rDNA (11), as positive controls. The *ScaI* test, using PCR primer set ITS5/2 or ITS5/4, can be used alone to provide molecular identification of *T. walkeri* or in conjunction with either of the published PCR-based identification systems to provide independent confirmation of results.

The methods of Smith et al. (21), Frederick et al. (11), and this method all essentially require that single spores be isolated either for germination or for direct PCR on individual spores. Although

spores can be collected from seed washes or swabs of grain-holding facilities, for maximum accuracy none of these PCR-based tests should be used on samples potentially containing a mixture of species. The ITS primers used in this method will amplify from nearly all fungal DNA, as well as from other eukaryotic DNA, so it is critical that spores first be examined morphologically to confirm that the fungus is a species of *Tilletia*. Currently, approximately 5 to 7 days are required for the germination of teliospores (6,13) and an additional 5 to 7 days for the production of enough mycelium for DNA extraction. McDonald et al. (15) reported direct amplification of DNA from species of *Tilletia* using the ITS3/4 primer set (24) as well as the Ti17 M1/M2 primers (21). Bonde et al. (1) reported the use of acidic electrolyzed water to enhance the germination of teliospores. Use of the above-mentioned methods in conjunction with the *ScaI*-ITS 1 restriction digest and other methods could reduce this time at least by half.

Sequence analysis showed relatively few differences in the ITS region among isolates of *T. indica* and *T. walkeri*. Similarly, there were few differences in the ITS region among isolates of closely related yet distinct taxa in the dwarf and common wheat bunt clade. At the same time, similar amounts of variation were shown among the *T. horrida* isolates and the *T. barclayana* isolates from Mexico, although sequences from these two taxa were highly diverged from each other and do not support their synonymy as reported by Tullis and Johnson (22). Whitney (25) had previously questioned the synonymy of *T. horrida* and *T. barclayana* based on host inoculation experiments and Royer and Rytter (19) were unable to artificially infect *Pennisetum clandestinum* Hochst. Ex Chiov. with *T. horrida* (as *T. barclayana*).

Although bootstrap values for the terminal clades are high, relationships of these clades to one another cannot truly be resolved due to the large amount of sequence variation between terminal clades, which resulted in some difficulty in aligning these sequences, the relatively small number of taxa included, and the lack of a true outgroup taxon. These data suggest that the rates of change in this region of the rDNA may vary widely across the genus and that ITS sequence analysis is not appropriate for determining broader relationships among these fungi. To date, there have been no published molecular phylogenetic studies of taxa within the genus *Tilletia* and the low level of variation in the ITS regions between closely related species in this study indicates that other gene sequences will be required to determine phylogenetic species and generic limits. Some authorities have considered the tuberculate-spored bunts to belong in a separate genus, *Neovossia* (23), while others have retained them in *Tilletia* (9,10). In addition to teliospore ornamentation, the tuberculate- and reticulate-spored bunt species differ in the time and location of dikaryon formation and host infection. The phylogenetic analysis based on ITS sequences shows that the reticulate-spored *Tilletia* spp. and the smooth-spored *T. laevis* form a single clade, whereas the tuberculate-spored taxa were in two strongly supported clades.

Although the results of this study indicate that the reticulate-spored *Tilletia* spp. included in this study are closely related, it fails to support the tuberculate-spored taxa as a monophyletic group. Additionally, phylogenetic analysis of the nuclear rDNA large subunit for these species has failed to support the monophyly of the tuberculate-spored species (7). The results of this study also point out the necessity of working with more than one isolate of different species in this genus for both molecular systematics and diagnostics. A better understanding of the taxonomic limits among these groups of bunt fungi is important for selecting the most appropriate taxa to test the specificity of diagnostic procedures.

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