Differentiation of *Tilletia* Species by rep-PCR Genomic Fingerprinting

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ABSTRACT

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The potential of repetitive-sequence-based polymerase chain reaction (rep-PCR) fingerprinting of fungal genomic DNA as a rapid and simple alternative to random amplified polymorphic DNA (RAPD) analysis in the study of phylogenetic relationships, and also as a diagnostic method, was investigated with species of *Tilletia*. DNA primers (BOX, ERIC, and REP) corresponding to conserved repetitive element motifs, originally described in prokaryotes, were used to generate genomic fingerprints of *T. indica*, *T. walkeri*, *T. controversa*, *T. laevis*, *T. tritici*, *T. goloskokovii*, *T. barclayana*, and members of the *T. fusca* complex. Computer-assisted analysis of the database of combined fingerprints clearly distinguished each taxon and indicated phylogenetic relationships consistent with previously reported RAPD analyses. There were three main clusters with isolates showing 35 to 40% similarity. Group 1 included *T. indica* and *T. walkeri*; group 2 included members of the *T. fusca* complex, as well as *T. controversa*, *T. laevis*, *T. tritici*, and *T. goloskokovii*; and group 3 included only *T. barclayana*. If, as is likely, the conserved repetitive element motifs on which this technique is based are widespread or universal in fungal species, rep-PCR shows strong potential, not only as a simple generic taxonomic tool, but also as a diagnostic method.

There is increasing interest in the application of polymerase chain reaction (PCR) technology for the identification of plant pathogenic fungi. Such methods offer the advantage of reducing or eliminating the need for lengthy culturing and difficult morphological identification procedures (11,27). The potential benefits of this technology have been especially recognized in the regulatory field, where timeliness and accuracy of identifications are crucial (10,22).

An important area of application has been in tests to confirm the identity of teliospores of *Tilletia* spp. found in seed washes. With the discovery in 1996 of *Tilletia indica* in Arizona (26), there was a need to conduct national surveys of wheat-producing regions of the United States for Karnal bunt infection. In order to distinguish teliospores of *T. indica* from the morphologically similar *T. barclayana*, a PCR test was used that distinguished the two (22). This test used a single set of mitochondrial DNA primers that had also been validated against a number of other *Tilletia* spp. that were known to be common contaminants in grain shipments.

However, soon after the survey was initiated, *T. indica*-like spores were unexpectedly found in seed lots of annual ryegrass (*Lolium multiflorum*), and they tested positive in the PCR confirmation test (16). After considerable further investigation, it was determined that the ryegrass smut was a distinct species, named *T. walkeri* (4,5,18), and several new sets of mitochondrial DNA primers were developed that would make this distinction (8). This chronology was thus instructive on the risks associated with basing a PCR test on a single set of primers.

An alternative to identification using specifically targeted PCR primers is the use of PCR-based genomic fingerprinting. Whole genome techniques, such as random amplified polymorphic DNA (RAPD) analysis and amplified fragment length polymorphisms (AFLP), have been shown to be relatively robust and discriminatory (14). They are widely used in population and taxonomic studies but, due to their complexity, are not frequently used for identification. In taxonomic studies of *Tilletia* spp., including such species as *T. indica*, *T. barclayana*, *T. walkeri*, *T. controversa*, *T. goloskokovii*, *T. laevis*, *T. tritici*, and the *T. fusca* species complex, RAPD as well as the less sensitive PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of the ITS region of rDNA have been used (2,3,18,21). In general, these methods have identified groupings that were consistent with taxa that have been established principally on the basis of teliospore morphology, although they have also suggested significant divi-

sions within those taxa based upon host specificity, indicating the need for further taxonomic refinements. One such division was in *T. fusca* var. *fusca*, where RAPD profiles of isolates on *Vulpia octoflora* shared only 20 to 35% similarity with isolates from *Vulpia microstachys* (2,3).

Another genomic fingerprinting technique that is beginning to be used for identification, as well as taxonomic purposes, is repetitive-sequence-based polymerase chain reaction (rep-PCR) (24). The technique is based on PCR-mediated amplification of DNA sequences located between specific interspersed repeated sequences in prokaryotic genomes. These repeated elements are termed BOX, REP, and ERIC elements. Amplification of the DNA sequences between primers based on these repeated elements generates an array of different-sized DNA fragments from the genomes of individual strains. The separation of these fragments on agarose gels yields highly specific DNA fingerprints that can be either visually compared or subjected to computer assisted pattern analysis (19). Because of the simplicity of this approach, and also because rep-PCR can tolerate a wider range of DNA concentrations than RAPD analysis in generating reproducible results (14), it has proven useful not only in characterizing the variability within bacterial species but also in the identification of bacterial species, including plant pathogens (14).

Although rep-PCR primers were designed for repeated elements in prokaryotic genomes, BOX, ERIC, and REP primers have been shown to also amplify inter-repeat sequences of some fungal genomes and have been used to characterize variability at inter- and/or intra-specific levels of several fungal genera, including *Verticillium* (1), *Fusarium* (7), *Stagonospora* (6), *Septoria* (6), and *Leptosphaeria* (13). Additional endogenous repetitive DNA elements have also been identified in fungi and used to generate genomic fingerprints (9,13). However, there have been no studies to determine the applicability of rep-PCR genomic fingerprinting for the identification of fungal species, whether for taxonomic or other purposes. Therefore, the current study was initiated to examine this potential with respect to species of *Tilletia*.

MATERIALS AND METHODS

Fungal isolates and DNA extraction. Sixty-seven isolates representing 11 *Tilletia* taxa were used (Table 1). Teliospores

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of *T. indica* and *T. barclayana* were surface-sterilized with a 10% commercial bleach (sodium hypochlorite) solution, while the remainder were treated with a 5% solution. They were then streaked on 2% water agar to germinate. Incubation temperatures were: room temperature for *T. indica*, *T. walkeri*, and *T. barclayana*; 16°C for *T. tritici*, *T. laevis*, *T. goloskokovii*, and *T. fusca*; and 5°C for *T. controversa*. After germination, the procedures of Smith et al. (22) were used to obtain a mycelial mat. DNA was then extracted from the mycelial mat using the procedure of Möller et al. (15).

Rep-PCR amplification and analysis. DNA from the fungal isolates listed in Table 1 was subjected to rep-PCR genomic fingerprinting using primer sets corresponding to the BOX, ERIC, and REP elements (24). The primer sets included BOX element 1A primer 1R (5'-CTACGGCAAGGCGACGCTGACG-3'); ERIC primers 1R (5'-ATGTAAGCTCCTGGGGATTAC-3') and 2I (5'-AAGTAA GTGACTGGGGTGAGCG-3'); and REP primers 1R (5'-IIICGICGICATCIGGC-3') and 2I (5'-ICGICTTATCIGGCCTAC-3'). The rep-PCR protocols were carried out as described by Rademaker et al. (20). Briefly, 50 ng of purified DNA was used as template in a 25-µl reaction mixture containing 50 µM of each primer, 900 µM deoxynucleotide triphosphates, and 2 units of *Taq* DNA polymerase (Gibco BRL, Burlington, ON) in a reaction buffer containing 67 mM Tris-HCl (pH 8.8), 16.6 mM (NH₄)₂SO₄, 6.7 mM MgCl₂, 6.7 mM EDTA, 30 mM 2-mercaptoethanol, 4 µg of bovine serum albumin, and 10% dimethylsulfoxide (vol/vol). PCR amplifications were performed in a model PE 9700 Perkin-Elmer thermocycler (Perkin-Elmer, Norwalk, CT) using the following conditions: an initial denaturation at 95°C for 7 min; 35 cycles consisting of 94°C for 3 s, 92°C for 30 s, then either 40°C (REP primers) or 50°C (ERIC/BOX primers) for 1 min; extension at 65°C for 8 min; and a single final extension at 65°C for 8 min, followed by cooling at 4°C. To determine reproducibility, all samples were subjected at least twice to independent PCR and electrophoresis, and reaction products were duplicated on the same gel.

BOX, ERIC, and REP primers were synthesized by Gibco BRL. DNA fragments in 8 µl of the amplified PCR product were separated by gel electrophoresis at 4°C on 1.5% agarose (Seakem LE, FMC BioProducts, Rockland, ME) gels in Tris-acetate-EDTA (TAE; 0.4 M Tris-acetate and 1 mM EDTA, pH 8.0) buffer for 18 to 19 h at 2 V/cm, stained with ethidium bromide, and photographed on a UV transilluminator using a Speedlight Platinum Jr. digital image capture gel documentation system (Lighttools Research, Encinitas, CA). Digital images of the gels were subjected to analysis using the commercially

available GelCompar software (version 4.0; Applied Maths, Kortrijk, Belgium). Gels were normalized using the same standards (1-kb DNA ladder; Gibco BRL), loaded on both sides and in the center of 20-lane gels. The "rolling disk" background subtraction option was applied. The BOX-, ERIC-, and REP-PCR genomic fingerprints were linearly combined using the "combined gel" option of GelCompar. The similarity between pairs of linearly combined fingerprints was calculated using the product-moment correlation coefficient (*r* value) (17) and applied to the whole densitometric curves of the gel tracks (12). Cluster analysis of the pairwise similarity values was performed using the unweighted pair group method using arithmetic averages (23) clustering technique. Fingerprint patterns were reconstructed as described (19).

RESULTS AND DISCUSSION

Amplification of total DNA from the different isolates of *Tilletia* with the ERIC-, BOX-, and REP-primers followed by agarose gel electrophoresis of the PCR prod-

ucts resulted in complex fingerprint patterns (Fig. 1). Rep-PCR fingerprint patterns for an isolate of each *Tilletia* species were examined. The size of the amplification products ranged from approximately 100 bp up to 4,000 bp. Each primer set generated ~25 to 30 bands visible on the densitometric curves of the gel tracks.

In analyzing the reproducibility of the rep-PCR, comparison of the resulting fingerprint patterns yielded a similarity of ~90 to 95% when the patterns were generated in the same PCR experiment and resolved on the same gel, while independent PCR and electrophoresis of repeated samples resulted in similarities of ~85 to 90% (data not shown). These values are consistent with those from rep-PCR studies on prokaryotes (25).

Analysis of the combined BOX-, ERIC-, and REP-PCR patterns revealed that the isolates clustered together into three major groups designated 1 to 3 (Fig. 2). Group 1 contained two separate clusters of *T. indica* and *T. walkeri* isolates that shared a similarity of ~30%. The isolates of *T. indica* and *T. walkeri* shared ~40% and ~75%

Table 1. Collections of *Tilletia* species isolates used in study designated by isolate number, origin, date collected, host and supplier

Isolate ^a	Collection ID	Origin/year	Host	Supplier ^b
<i>Tilletia indica</i>				
10	960507	Mexico, 1996	<i>Triticum</i> sp.	GPW
11	970402	India, 1997	<i>Triticum</i> sp.	SSC
32	P91	India, 1991	<i>Triticum</i> sp.	MRB
34	D97	India, 1997	<i>Triticum</i> sp.	MRB
35	G97	India, 1997	<i>Triticum</i> sp.	MRB
36	H97	India, 1997	<i>Triticum</i> sp.	MRB
37	J97	India, 1997	<i>Triticum</i> sp.	MRB
38	CO81	Mexico, 1981	<i>Triticum</i> sp.	MRB
39	S85	Mexico, 1985	<i>Triticum</i> sp.	MRB
42	CO96	Mexico, 1996	<i>Triticum</i> sp.	MRB
44	P85	Pakistan, 1985	<i>Triticum</i> sp.	MRB
45	P86	Pakistan, 1986	<i>Triticum</i> sp.	MRB
84	17377	Pakistan, 1997	<i>Triticum</i> sp.	GPW
85	17202	Pakistan, 1997	<i>Triticum</i> sp.	GPW
90	17375	Pakistan, 1997	<i>Triticum</i> sp.	GPW
97	15993	Pakistan, 1997	<i>Triticum</i> sp.	GPW
98	16005	Pakistan, 1997	<i>Triticum</i> sp.	GPW
101	16014	Pakistan, 1997	<i>Triticum</i> sp.	GPW
112	15514	Pakistan, 1997	<i>Triticum</i> sp.	GPW
115	15044	Pakistan, 1997	<i>Triticum</i> sp.	GPW
<i>T. walkeri</i>				
1	M96447	Oregon, USA, 1996	<i>Lolium multiflorum</i>	GPW
2	M96504	Oregon, USA, 1996	<i>L. multiflorum</i>	GPW
3	P195GR3	Oregon, USA, 1996	<i>L. multiflorum</i>	GMI
4	LA146AT5	Oregon, USA, 1996	<i>L. multiflorum</i>	GMI
23	770	Oregon, USA, 1997	<i>L. multiflorum</i>	BJG
71	P195GR3	Oregon, USA, 1998	<i>L. multiflorum</i>	GMI
147 ^c	196	Oregon, USA, 1996	<i>L. multiflorum</i>	LMC
148 ^c	770	Tennessee, USA, 1997	<i>L. multiflorum</i>	LMC
149 ^c	TR1T11	Tennessee, USA, 1996	<i>L. multiflorum</i>	LMC
171	M96424	Oregon, USA, 1996	<i>L. multiflorum</i>	GPW
173	M964596	Oregon, USA, 1996	<i>L. multiflorum</i>	GPW

(continued on next page)

^a From single teliospores, except where indicated.

^b GPW = G. P. White, CFIA, Nepean, ON, Canada; SSC = S. S. Chahal, Department of Plant Pathology, PAW, Ludhiana, India; MRB = M. R. Bonde, USDA-ARS, Frederick, MD; GMI = G. M. Milbrath, Plant Division, Oregon Department of Agriculture, Salem, OR; BJG = B. J. Goates, USDA, Aberdeen, ID; LMC = L. M. Carris, Department of Plant Pathology, Washington State University, Pullman; GMU = G. M. Murray, NSW Agriculture, Wagga Wagga, New South Wales, Australia.

^c Provided as a single sporangial culture.

similarity, respectively. Group 2 contained separate clusters of *T. fusca* var. *fusca* on *V. microstachys*, *T. goloskokovii*, *T. fusca* var. *fusca*, on *V. octoflora*, *T. fusca* var. *bromi-tectorum*, *T. fusca* var. *guyotiana*, *T. controversa*, *T. tritici*, and *T. laevis* isolates that shared a similarity of ~40%. Within the group, isolates of the two *T. fusca* var. *fusca* taxa and *T. goloskokovii* each clustered separately and showed only ~45% similarity to each other. The remaining taxa formed a cluster with ~55% similarity, and *T. fusca* var. *bromi-tectorum* and *T. fusca* var. *guyotiana* formed one subcluster with ~60% similarity, while *T. controversa*, *T. laevis*, and *T. tritici* formed another subcluster with ~70% similarity. Group 3 included just *T. barclayana* isolates, and these shared a similarity of ~30%.

Previous genomic fingerprinting studies of *Tilletia* species, using RAPD analysis, have used various random primers to analyze different subsets of taxa (2,3,18,21). Consequently, a combined analysis of all these taxa, with a common set of PCR primers, has not previously been at-

tempted. However, the relationships revealed in this study are generally consistent with the RAPD analyses that have been made (2,3,18,21). It should be noted, however, that the present study differs from previous analyses by principally using polysporidial rather than monosporidial lines.

In a study of *T. indica*, *T. walkeri*, and *T. barclayana* isolates, Pimentel et al. (18) also found that separate clusters of *T. indica* and *T. walkeri* formed a group that shared a similarity of ~25%. Also consistent with the present study was the fact that, within the clusters of *T. indica* and *T. walkeri*, isolates had similarities of 50 and 78%, respectively. Among the *T. barclayana* isolates, which are considered to be a species complex (18), they observed <10% similarity.

Isolates of *T. controversa*, *T. fusca* var. *bromi-tectorum*, *T. fusca* var. *guyotiana*, and *T. fusca* var. *fusca* on *V. microstachys* as well as *V. octoflora*, have been compared in two studies (2,3), with *T. goloskokovii* being included in one of the studies

Table 1. (continued)

Isolate ^a	Collection ID	Origin/year	Host	Supplier ^b
<i>T. controversa</i>				
19	756	Idaho, USA, 1997	<i>Triticum</i> sp.	BJG
20	638	Idaho, USA, 1997	<i>Triticum</i> sp.	BJG
54	M973111	Ontario, Canada, 1997	<i>Triticum</i> sp.	GPW
56	M973133	Ontario, Canada, 1997	<i>Triticum</i> sp.	GPW
57	M973144	Ontario, Canada, 1997	<i>Triticum</i> sp.	GPW
59	M973166	Ontario, Canada, 1997	<i>Triticum</i> sp.	GPW
60	M973177	Ontario, Canada, 1997	<i>Triticum</i> sp.	GPW
61	M973188	Ontario, Canada, 1997	<i>Triticum</i> sp.	GPW
62	M973199	Ontario, Canada, 1997	<i>Triticum</i> sp.	GPW
128	M98073	Ontario, Canada, 1997	<i>Triticum</i> sp.	GPW
151	177	Utah, USA, 1985	<i>Triticum</i> sp.	LMC
<i>T. barclayana</i>				
25	ST96	Arkansas, 1996	<i>Oryza sativa</i>	BJG
51	M97228	California, USA, 1997	<i>O. sativa</i>	GPW
67	M97016	China, 1997	<i>O. sativa</i>	GPW
69	M97619	Philippines, 1997	<i>O. sativa</i>	GPW
162	RDC9T5	Arkansas, 1992	<i>O. sativa</i>	LMC
163	FNL1	Arkansas, 1996	<i>O. sativa</i>	LMC
177	M98377	Arizona, 1998	<i>O. sativa</i>	GPW
<i>T. laevis</i>				
74	17399	Pakistan, 1997	<i>Triticum</i> sp.	GPW
124	72299	Australia, 1997	<i>Triticum</i> sp.	GMU
125	73300	Australia, 1997	<i>Triticum</i> sp.	GMU
127	73302	Australia, 1997	<i>Triticum</i> sp.	GMU
166	V-766	Iran, 1992	<i>Triticum</i> sp.	LMC
<i>T. tritici</i>				
21	T2	Idaho, USA, 1996	<i>Triticum</i> sp.	BJG
22	T16	Idaho, USA, 1996	<i>Triticum</i> sp.	BJG
63	M9732010	Ontario, Canada, 1997	<i>Triticum</i> sp.	GPW
<i>T. fusca</i> var. <i>bromi-tectorum</i>				
152	2	Washington, USA, 1991	<i>Bromus tectorum</i>	LMC
153	316	Washington, USA, 1995	<i>B. tectorum</i>	LMC
<i>T. fusca</i> var. <i>fusca</i>				
154	314A	Washington, USA, 1995	<i>Vulpia microstachys</i>	LMC
155	344	Washington, USA, 1996	<i>V. microstachys</i>	LMC
156	314A	Washington, USA, 1995	<i>V. octoflora</i>	LMC
157	318B	Washington, USA, 1995	<i>V. octoflora</i>	LMC
<i>T. fusca</i> var. <i>guyotiana</i>				
158	64	Idaho, USA, 1991	<i>B. japonicus</i>	LMC
159	74	Oregon, USA, 1991	<i>B. japonicus</i>	LMC
<i>T. goloskokovii</i>				
160	321	Washington, USA, 1995	<i>Aptera interrupta</i>	LMC
161	359	Washington, USA, 1998	<i>A. interrupta</i>	LMC

Fig. 1. Agarose gel electrophoresis of polymerase chain reaction (PCR) fingerprint patterns obtained from isolates representing 11 taxa of *Tilletia* using primers corresponding to repetitive extrapalindromic sequences (rep-PCR). (A) BOX-PCR, (B) ERIC-PCR, and (C) REP-PCR. A 6-μl portion of each rep-PCR mixture was loaded onto a 1.5% agarose gel. Lane 1 = *T. indica* isolate 90; lane 2 = *T. walkeri* isolate 173; lane 3 = *T. fusca* var. *fusca* on *Vulpia microstachys* isolate 154; lane 4 = *T. goloskokovii* isolate 160; lane 5 = *T. fusca* var. *fusca* on *V. octoflora* isolate 157; lane 6 = *T. fusca* var. *bromi-tectorum* isolate 152; lane 7 = *T. fusca* var. *guyotiana* isolate 158; lane 8 = *T. controversa* isolate 19; lane 9 = *T. tritici* isolate 22; lane 10 = *T. laevis* isolate 74; lane 11 = *T. barclayana* isolate 47. Lane M = DNA molecular size markers (1 kbp ladder); sizes in base pairs.

(3). *T. fusca* var. *fusca* isolates on *V. microstachys* and *V. octoflora* showed the greatest differences from the other species, but these two taxa showed only 20 to 35% similarity with each other, which is con-

sistent with our results. Analyses of *T. controversa*, *T. fusca* var. *guyotiana*, *T. fusca* var. *bromi-tectorum*, and *T. goloskokovii* isolates revealed somewhat greater differences among these taxa than found here, in

that they were found to share only ~25% similarity (2,3). These latter two species were more similar to each other than they were to the others (3), which is also what our data show.

Fig. 2. Similarity of isolates of *Tilletia* spp. based on the presence and absence of bands generated using primers corresponding to repetitive extragenic palindromic sequences (REP), BOX1A sequences (BOX), enterobacterial repetitive intergenic consensus sequences (ERIC), and analyzed using the product-moment correlation coefficient (r); i = *T. indica* isolates, w = *T. walkeri*, ff-m = *T. fusca* var. *fusca* on *Vulpia microstachys*, g = *T. goloskokovii*, ff-o = *T. fusca* var. *fusca* on *V. octoflora*, fb = *T. fusca* var. *bromi-tectorum*, fg = *T. fusca* var. *guyotiana*, c = *T. controversa*, t = *T. tritici*, l = *T. laevis*, b = *T. barclayana*. Three major groupings are designated 1 to 3.

In a study of a large number of isolates of wheat bunt fungi (21), RAPD analysis identified considerable diversity among *T. controversa* isolates, with some isolates clustering together and others clustering with isolates of *T. laevis* and/or *T. tritici*. Rep-PCR analysis also identified considerable diversity among the *T. controversa* isolates and close similarity to isolates of *T. laevis* and *T. tritici*; however, each species clustered separately.

The results of this study demonstrate the usefulness of rep-PCR genomic fingerprinting as a complementary or alternate strategy to RAPD analysis for studying phylogenetic relationships of fungi. The major advantages of this approach are its simplicity, the universality of PCR primers, tolerance of a wider range of DNA concentrations in generating reproducible results, and amenability to computer database analysis. In addition, it has potential as a diagnostic method (14) and would be particularly applicable in situations where isolation and culture are a normal requirement and speed and accuracy are at a premium. For example, application of rep-PCR in support of the *T. indica* testing program would have readily determined the distinct identity of *T. indica*-like spores found in seed lots of annual ryegrass (4,16,18) and would have avoided the needless confusion and delay that resulted from this quarantine testing problem.

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