Analysis of the Genetic Relationships Among the Wheat Bunt Fungi Using RAPD and Ribosomal DNA Markers

Y. L. Shi, P. Loomis, D. Christian, L. M. Carris, and H. Leung

Department of Plant Pathology, Washington State University, Pullman 99164-6430.
This work was supported in part by USDA-CSRS grant 90-37151-5433.
We thank B. Goates, J. Waldher, and J. Sitton for providing many of the fungal samples. We thank P. Gray and S. Thompson for their help in this study, and I. Yap and R. Nelson for providing the Winboot program.
Accepted for publication 30 November 1995.

ABSTRACT

Shi, Y. L., Loomis, P., Christian, D., Carris, L. M., and Leung, H. 1996. Analysis of the genetic relationships among the wheat bunt fungi using RAPD and ribosomal DNA markers. Phytopathology 86:311-318.

Ninety-five isolates of *Tilletia controversa*, *T. tritici*, *T. laevis*, and *T. fusca* var. *bromi-tectorum* were assayed for random amplified polymorphic DNA (RAPD). Based on 23 RAPD markers, two distinct RAPD groups (RG I and RG II) with 12% similarity were obtained using the distance matrix method. RG I included all 66 isolates of the wheat bunt fungi and RG II contained all 29 isolates of *T. fusca* var. *bromi-tectorum*, which was considered as an outgroup. RG I was further divided into two subgroups RG IA and RG IB with 75% similarity: RG IA containing 19 isolates of *T. controversa* and RG IB containing 38 isolates of *T. tritici*, *T. laevis*, and six isolates of *T. controversa*. Bootstrap analysis supported the separation between isolates from wheat and isolates from cheatgrass, but not the clustering of isolates within the wheat bunt group. However, the g1 statistic, a measure of the skewness of the tree-length distribution, indicated a significant difference between the dwarf bunt and common bunt clusters ($P < 0.05$). Restriction digestion analysis of the 5.8s and internal transcribed spacer region of ribosomal DNA in a subset of wheat bunt fungi (28 isolates of *T. controversa*, 19 isolates of *T. tritici*, and 12 isolates of *T. laevis*) showed two distinct patterns. Haplotype A was associated with 24 of 28 isolates of *T. controversa* and haplotype B was associated with all isolates of common bunt fungi and four isolates of *T. controversa*. The data suggested that the wheat bunt fungi descended from a common ancestral population that subsequently differentiated into two sublineages. The fact that a considerable number of isolates have reciprocal characteristics of both dwarf and common bunt fungi raises the question of whether natural hybridization is responsible for the apparent recombination of characters.

Additional keyword: phylogenetic lineage.

Wheat bunt fungi, *Tilletia tritici* (Bjerk.) Wint., *T. laevis* Kühn, and *T. controversa* Kühn, are closely related filamentous basidiomycetes that are highly destructive pathogens of wheat worldwide. *T. tritici* and *T. laevis*, the causal agents of common bunt, have been controlled in the United States by a combination of chemical seed treatment and the use of resistant cultivars (13). On the other hand, *T. controversa*, the cause of dwarf bunt, has not been controlled by seed treatment and still causes sporadic outbreaks in some wheat growing areas. The most important economic impact of *T. controversa* results from the contamination of wheat shipments by teliospores. As a result of the quarantine against *T. controversa* imposed by China, the wheat trade between the United States and China has been seriously disrupted (38).

Teliospore morphology and germination requirements are two major criteria for the identification of the wheat bunt fungi. *T. laevis* was easily differentiated from *T. tritici* and *T. controversa* by its smooth teliospore wall. The exospores of *T. tritici* have small and shallow polygonal reticulation, whereas *T. controversa* has large, regular, and deep polygonal areolae on its exospore. At 16°C, teliospores of *T. tritici* and *T. laevis* take only 3 to 5 days to germinate. In contrast, teliospores of *T. controversa* only germinate after 3 to 6 weeks of incubation at 4°C.

Early genetic studies of the wheat bunt fungi focused on control of sexual compatibility. Flor (7) first demonstrated the heterothallic nature of *T. tritici* and *T. laevis* by showing that smutted heads were produced on wheat following inoculation with pairs of monosporidial lines, but not with single monosporidial lines. A number of sexually compatible groups were found in his study. Hanna (11) provided further evidence for heterothallism in *Tilletia*, but detected only two sexual groups. Becker (1) designated the two sex factors as plus and minus in *T. tritici*. By fusion between secondary sporidia and inoculation of paired monosporidial lines, Silbernagel (34) found that *T. controversa* could be sexually compatible with both the plus and minus lines of *T. tritici*, indicating a bisexual nature of *T. controversa*. The discovery of heterothallism of the wheat bunt fungi led to a series of studies aimed at understanding the role of sexual hybridization in generating variability. Holton (15) reported new pathogenic races derived from a cross between *T. tritici* and *T. laevis*. By crossing different races of *T. tritici*, hybrids that had pathogenic properties different from those of their parents and of other known races were obtained. In addition, hybrid-like spores were found in field collections (19). These results suggested that intraspecific and interspecific hybridization might play an important role in the production of new and more virulent races.

In most genetic studies of the wheat bunt fungi, phenotypic characteristics such as teliospore morphology, sorus color and morphology, germination requirements of teliospores, and pathogenicity were used as genetic markers. Auxotrophic mutants with a requirement for adenine, uracil, and glycine were used to force the formation of heterokaryons in planta (3,4). Antibiotic resistance was used to analyze a cross between *T. tritici* and *T. controversa*, and independent segregation between mating type and cycloheximide resistance was demonstrated (37). However, a shortage of genetic markers remains a barrier for more detailed analyses of the genetic relationships among the wheat bunt fungi.

Corresponding author: H. Leung; E-mail address: hleung@mail.wsu.edu

Publication no. P-1996-0131-02R

Genetic markers generated by random amplified polymorphic DNA (RAPD) (40) have been proven to be useful for studying genetics and phylogenetic relationships in a wide range of organisms (5,8,9). Tibayrenc et al. (36) demonstrated that multilocus isozymes and RAPD markers gave almost identical phylogenetic trees in five parasitic protozoa, further confirming RAPD analysis as a valid tool for investigating phylogenetic relationships among strains within a species. RAPD markers are particularly suitable for the genetic study of slow-growing organisms such as *Tilletia* species, from which large amounts of DNA are difficult to obtain. The objectives of this study were to use RAPD and ribosomal DNA markers to determine the genetic relationships among the wheat bunt fungi, and to infer the potential for genetic exchange among *Tilletia* species. In addition to the wheat bunt fungi, *T. fusca* Ellis & Everh. var. *bromi-tectorum* J. de Urries, a parasite on cheatgrass (*Bromus tectorum* L.), was also investigated. *T. fusca* var. *bromi-tectorum* is widely distributed in the Pacific Northwest and found abundantly in wheat fields. It was included to provide a relative measure of the genetic relatedness among *Tilletia* species within and outside the wheat bunt group.

MATERIALS AND METHODS

Fungal isolates. Twenty-eight isolates of *T. controversa*, 24 isolates of *T. tritici*, 14 isolates of *T. laevis*, and 29 isolates of *T. fusca* var. *bromi-tectorum* were used for phylogenetic analysis. Most isolates collected in 1990 to 1992 were field teliospore collections from smutted heads from a single plant, whereas older collections were multiplied on wheat hosts by artificial inoculation. The origin, dates of collection and inoculum increase, spore

TABLE 1. Geographic origin, date of collection and inoculum increase, spore morphology, and germination requirement of isolates of *Tilletia controversa*, *T. tritici*, *T. laevis*, and *T. fusca* var. *bromi-tectorum*

Isolate	Place and year of collection[a]	Year increased	Days to germinate[b]	Spore morphology	Source[c]	Reference
T. controversa						
TCK 3	Troy, ID 1990		7	Reticulate	This study	
TCK 5	Petersons, ID 1990		45	Reticulate	This study	
TCK 7	Petersons, ID 1990		21	Reticulate	This study	
TCK 29	Pullman, WA 1954	1980	14	Reticulate	This study	
TCK 9110	Troy, ID 1991		34	Reticulate	This study	
TCK 9118	Troy, ID 1991		22	Reticulate	This study	
TCK 90-002	Deary, ID 1991		34	Reticulate	Sitton	
TCK 90-006	Haines, OR 1990		33	Reticulate	Sitton	
TCK 90-0008	Bennington, ID 1990		41	Reticulate	Sitton	
TCK 90-0009	Montpelier, ID 1990		28	Reticulate	Sitton	
TCK 90-0020	Copland, ID 1990		19	Reticulate	Sitton	
TCK 90-0022	Worley, ID 1990		26	Reticulate	Sitton	
DB 32	Nephi, UT 1954	1980	38	Reticulate	Goates	
DB 36	Mt. Hope, WA 1957	1980	32	Reticulate	Goates	
DB 44	Preston, ID 1958	1980	33	Reticulate	Goates	
DB 108	Box Elder Co., UT 1962	1980	29	Reticulate	Goates	
DB 129	Tremonton, UT 1962	1980	47	Reticulate	Goates	
DB 224	Newton, UT 1967	1978	28	Reticulate	Goates	
DB 295	Box Elder Co., UT	1980	29	Reticulate	Goates	
DB 460	Davenport, WA	1978	33	Reticulate	Goates	
DB 611	Waterville, WA	1984	25	Reticulate	Goates	
DB 90-30	Elgin, OR	1990	28	Reticulate	Goates	
DB-OR	Baker Co., OR	1990	46	Reticulate	Goates	
TCKTR 78	Turkey	1981	22	Reticulate	Goates	
TCK 283	Logan, UT 1975	1984	28	Reticulate	Goates	
TCK 492	Cavendish, ID	1978	25	Reticulate	Goates	
TCK 572	Afton, WY	1982	37	Reticulate	Goates	
TCKTR 115	Tatvan, Turkey	1982	10	Reticulate	Goates	
T. tritici						
T-1		1984	7	Shallow reticulate	Goates	28
T-1 (20Y)			16	Shallow reticulate	Goates	28
T-1 (20Y)A			16	Shallow reticulate	Goates	28
T-3		1979	10	Shallow reticulate	Goates	28
T-4		1990	5	Shallow reticulate	Goates	28
T-5		1988	19	Shallow reticulate	Goates	28
T-7		1990	10	Shallow reticulate	Goates	28
T-9		1990	6	Shallow reticulate	Goates	28
T-9 (20Y)			11	Shallow reticulate	Goates	28
T-10		1990	6	Shallow reticulate	Goates	28
T-11		1990	7	Shallow reticulate	Goates	28
T-14		1990	20	Shallow reticulate	Goates	20
T-15		1978	21	Shallow reticulate	Goates	15
T-18		1990	5	Shallow reticulate	Goates	21
T-20		1990	6	Shallow reticulate	Goates	21
T-22		1990	5	Shallow reticulate	Goates	23

(*continued on next page*)

[a] Isolates DB 90-30, DB-OR, DB 513, TCKTR 78, TCK 492, TCK 572, and TCKTR 115 do not have a record of original collection date. Isolates of *T. tritici* (T-1 to T-30) and *T. laevis* (L-1 to L-16) do not have a collection record. The race designations of these collections are described in the references cited. Each culture collection was maintained at the disease nursery at Aberdeen, ID, by B. Goates.

[b] Number of days for teliospores to begin to germinate at 8°C; ND = no data.

[c] Affiliations of sample suppliers: B. Goates, USDA-ARS, Aberdeen, ID; J. Waldher, USDA-ARS, Washington State University, Pullman (retired); J. Sitton, Washington State University, Pullman; and R. Metzger, USDA-ARS, Oregon State University, Corvallis (retired).

morphology, and requirements for spore germination are summarized in Table 1. Spore morphology and germination requirements were two criteria for the initial species designation. Teliospores from at least two sori were examined from each isolate and the time for teliospore germination was recorded. All isolates were maintained as haploid, monosporidial cultures.

Culturing conditions. To obtain monosporidial lines, teliospores from a single sorus of each isolate were germinated on 3% water agar at 8°C. Primary sporidia were randomly isolated from the germinating teliospores with a Chambers micromanipulator (17), placed on 5 × 5 × 2-mm block of T19 agar (24), and incubated at 8°C. After a colony was formed, the culture was transferred onto a potato-sucrose agar (PSA) slant and maintained at 8°C. The culture was transferred to 50 ml of potato-sucrose broth (PSB) and grown at 15°C on an orbital shaker for 20 to 30 days. For long-term maintenance, the monosporidial cultures were stored in 15% glycerol at –70°C (22).

DNA extraction. Because of the slow growth habit of *Tilletia* species, a miniextraction procedure modified from the cetyltrimethylammonium bromide (CTAB) procedure (25) was used. Mixtures of sporidial and mycelial growth in PSB from single sporidial cultures were harvested by filtration through one layer of Miracloth (Calbiochem-Behring, La Jolla, CA) or by centrifugation, and rinsed twice with sterile water. The collected materials were frozen at –70°C overnight and then lyophilized. The lyophilized samples were ground with a small amount of sterile sand into a fine powder in a porcelain plate using the end of a 15-ml Corex tube (Corex Inc., Piqua, OH). The fine powder was transferred to a 1.5-ml microcentrifuge tube, suspended in 1 ml of extraction buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, and

TABLE 1. (*continued from preceding page*)

Isolate	Place and year of collection[a]	Year increased	Days to germinate[b]	Spore morphology	Source[c]	Reference
T-23		1983	6	Shallow reticulate	Goates	23
T-25-A			15	Shallow reticulate	Metzger	23
T-25		1990	19	Shallow reticulate	Goates	23
T-29-A			7	Shallow reticulate	Metzger	23
T-29		1984	7	Shallow reticulate	Goates	23
T-30			7	Shallow reticulate	Metzger	23
T-31		1988	9	Shallow reticulate	Goates	
T-OK	Oklahoma	1978	7	Shallow reticulate	Goates	
T. laevis						
L-1990	Heyden, CO 1990		6	Smooth	Goates	
L-1		1990	3	Smooth	Goates	28
L-1 (20Y)			13	Smooth	Goates	28
L-3		1990	5	Smooth	Goates	28
L-4		1990	6	Smooth	Goates	28
L-4 (20Y)			11	Smooth	Goates	28
L-5		1979	7	Smooth	Goates	28
L-5 (20Y)			9	Smooth	Goates	28
L-7		1979	4	Smooth	Goates	28
L-10		1990	6	Smooth	Goates	28
L-16		1984	5	Smooth	Goates	14
L-OK	Oklahoma 1990		8	Smooth	Goates	
L-CO	Berthoud, CO 1988		6	Smooth	Goates	
L-90-61	Heyden, CO 1990		4	Smooth	Goates	
T. fusca var. *bromi-tectorum*						
Tbt 9101	Craigmont, ID 1991		ND	Shallow reticulate	This study	
Tbt 9103	Grace, ID 1991		ND	Shallow reticulate	This study	
Tbt 9104	Thache, ID 1991		ND	Shallow reticulate	This study	
Tbt 9107	Hansel Valley, UT 1991		ND	Shallow reticulate	This study	
Tbt 9108	Clifton, ID 1991		ND	Shallow reticulate	This study	
Tbt 9110	Weston, ID 1991		ND	Shallow reticulate	This study	
Tbt 9115	Georgeton, ID 1991		ND	Shallow reticulate	This study	
Tbt 9116	Cascade, ID 1991		ND	Shallow reticulate	This study	
Tbt 9119	Garfield Co., WA 1991		ND	Shallow reticulate	This study	
Tbt 1	Kahlotus, WA 1991		ND	Shallow reticulate	This study	
Tbt 3	Ritzville, WA 1991		ND	Shallow reticulate	This study	
Tbt 4	Pampa, WA 1991		ND	Shallow reticulate	This study	
Tbt 25	Corfu, WA 1991		ND	Shallow reticulate	This study	
Tbt 28	Ewartsville, WA 1991		ND	Shallow reticulate	This study	
Tbt 29	Hooper, WA 1991		ND	Shallow reticulate	This study	
Tbt 51	Farmington, WA 1991		ND	Shallow reticulate	This study	
Tbt 52	Kamiak Butte, WA 1991		ND	Shallow reticulate	This study	
Tbt 61	Cavendish, ID 1991		ND	Shallow reticulate	This study	
Tbt 68	Moscow, ID 1991		ND	Shallow reticulate	This study	
Tbt 79	Joseph Canyon, OR 1991		ND	Shallow reticulate	This study	
Tbt 96	Spring Valley, ID 1991		ND	Shallow reticulate	This study	
Tbt 99	Princeton, ID 1991		ND	Shallow reticulate	This study	
Tbt 116	Tetonia, ID 1991		ND	Shallow reticulate	This study	
Tbt 118	Cascade, ID 1991		ND	Shallow reticulate	This study	
Tbt 131	Smiths Ferry, ID 1991		ND	Shallow reticulate	This study	
Tbt 133	Smiths Ferry, ID 1991		ND	Shallow reticulate	This study	
Tbt 134	Donnelly, ID 1991		ND	Shallow reticulate	This study	
Tbt 135	Meadows, ID 1991		ND	Shallow reticulate	This study	
Tbt 138	Avery, ID 1991		ND	Shallow reticulate	This study	

100 mM EDTA [pH 8.0]) by vortexing, and then divided into two tubes. Twenty-five microliters of 20% sodium dodecyl sulfate (SDS) was added, mixed, and the tubes were incubated at room temperature with gentle shaking for 1 h. Seventy-five microliters of 5 M NaCl and 65 µl of 10% CTAB/NaCl (10% CTAB in 0.7 M NaCl) were added serially, mixed, and incubated at 65°C for 15 min. An equal volume of chloroform/isoamyl alcohol (24:1) was added to denature proteins, and then centrifuged at 13,000 × *g* at room temperature for 12 min. The aqueous phase was removed to a fresh tube and the DNA was precipitated by adding 0.6 volumes of cold isopropanol, incubated at –20°C for 30 min, and then centrifuged at room temperature for 5 min. The pellet was rinsed twice with cold 70% ethanol and dried at room temperature. The pellet was dissolved in 50 µl of Tris-EDTA (TE; 10 mM Tris-HCl and 1 mM EDTA [pH 8.0]) plus 1 µl of ribonuclease (10 mg/ml) and incubated at 37°C for 30 min. The DNA was reprecipitated with 0.1 volumes of 3 M sodium acetate and two volumes of 95% ethanol at –20°C for 30 min, centrifuged, and dried. The genomic DNA was dissolved in 40 µl of TE, and quantified using the minigel method (32).

Polymerase chain reaction (PCR) and electrophoresis. Amplification of DNA was performed in a 12.5-µl PCR (that included 0.25 mM each of dATP, dCTP, dGTP, and dTTP [Sigma Chemical Co., St. Louis]; 2.5 µl of 25 mM $MgCl_2$; 1.25 µl of 10× *Taq* polymerase buffer; and 0.03 units of *Taq* DNA polymerase [Promega Corp., Madison, WI]), 1 µl of random primer (about 4 pmole/µl) (Operon Technologies Inc., Alameda, CA); 1 µl of genomic DNA (0.5 ng/µl); 5.7 µl of sterile water; and 25 µl of sterile mineral oil overlay. As a negative control, 1 µl of sterile water was used instead of the genomic DNA. Amplification was performed in a Perkin-Elmer model 480 thermal cycler (Perkin-Elmer Corp., Norwalk, CT) programmed for an initial denaturation of 7 min at 94°C; 45 cycles of denaturation at 94°C for 1.5 min, annealing at 37°C for 2.5 min, and extension at 72°C for 3 min; followed by a final extension at 72°C for 10 min. The shortest ramp time was used. After amplification, 5 µl of the 12.5-µl reaction was electrophoresed in a 1.5% agarose gel in 0.5× Tris-borate-EDTA (TBE; 0.045 M Tris-borate, 0.045 M boric acid, and 0.001 M EDTA [pH 8.0]). Three microliters of 1-kb size marker ladder (0.05 µg/µl) (Gibco BRL, Bethesda, MD) was used to estimate the molecular weight of the PCR products. The gel was stained with ethidium bromide (0.5 µg/ml) for 30 min, and photographed using a UV transilluminator.

A subset of the wheat bunt isolates (total 59; 28 isolates of *T. controversa*, 19 isolates of *T. tritici*, and 12 isolates of *T. laevis*) was analyzed for polymorphism in the internal transcribed spacer (ITS) region of the small subunit of nuclear ribosomal DNA (rDNA). Amplification of the ITS region defined by primers ITS3 (5′-GCATCGATGAAGAACGCAGC-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (39; primers purchased from Operon Technologies Inc.) was performed in a 100-µl volume. The same PCR parameters described above were used except that the annealing temperature was set at 55°C.

Restriction digestion of PCR products. To obtain DNA of the ITS region of rDNA for restriction analysis, the PCR products amplified with primers ITS3 and ITS4 were extracted with 100 µl of chloroform. The aqueous phase was transferred to a fresh tube and precipitated with sodium acetate and ethanol as described above. The DNA was resuspended in 50 µl of TE and was subjected to restriction digestion with the four-base recognition endonucleases *Alu*I, *Msp*I, and *Taq*I (Life Technologies, Inc., Grand Island, NY), according to the supplier's instruction. Digestion products were electrophoresed in a 4% agarose gel.

Data analysis. DNA banding patterns generated by RAPD were scored as "1" for the presence of an amplified band and "0" for its absence. Three isolates of *T. controversa* and one isolate each of *T. tritici* and *T. laevis* were used initially to screen a collection of random primers. The primers that produced consistent banding patterns were chosen to analyze the entire collection of isolates. All RAPD assays were performed twice and only the reproducible bands were scored. The same binary method was used to score the restriction fragment length polymorphisms (RFLPs) of the rDNA region.

Dendrograms were constructed based on RAPD data and RFLPs of the rDNA using Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc) version 1.80 (29), the mixed method parsimony program of PHYLIP version 3.5 (6), and PAUP version 3.1 (35). With NTSYS-pc analysis, matrixes of Dice and simple matching coefficients were computed in the SIMQUAL program and the phenogram was generated by the unweighted pair-group, arithmetic average method (UPGMA) in the SAHN program. To assess the strength of the clusters generated by the tree-making programs, the data were subjected to bootstrap analysis with 500 replications using the Winboot program (26). With PHYLIP analysis, 100 trees were generated with the mixed method parsimony program and were used to produce a consensus tree using the CONSENSE program. The significance of phylogenetic signal shown by the consensus tree was tested using the method described by Hillis and Huelsenbeck (12). This method involved plotting the tree lengths of the random trees generated by the data set. The g1 statistic generated in the PAUP program was used to measure the skewness of the tree-length distribution from symmetry (random distribution). A g1 value that deviated from that generated from a random data set would indicate significant phylogenetic signal over random noise.

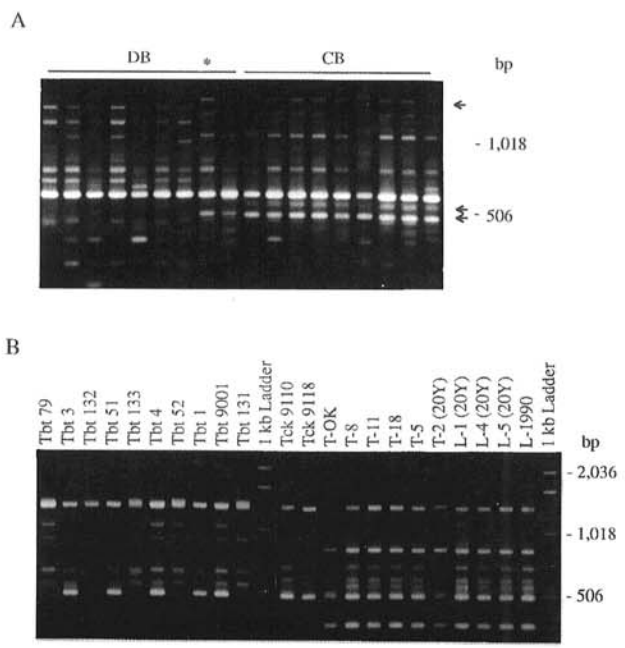

Fig. 1. Banding patterns of random amplified polymorphic DNA (RAPD) of *Tilletia controversa* (dwarf bunt [DB]), *T. tritici* and *T. laevis* (common bunt [CB]), and *T. fusca* var. *bromi-tectorum* (bunt on cheatgrass). The polymerase chain reaction products were electrophoresed on 1.5% agarose gel. **A,** Nine isolates of *T. controversa* (DB) and nine isolates of *T. tritici* and *T. laevis* (CB) using primer OPE-07. Molecular sizes are indicated on the right margin. The banding patterns of the two groups of isolates were similar except at a few band positions (arrows). The starred lane (TCK 9110) in the dwarf bunt group had a pattern similar to the common bunt group. **B,** Twenty-two isolates of *T. controversa*, *T. tritici*, *T. laevis*, and *T. fusca* var. *bromi-tectorum* using primer OPA-03.

RESULTS

Genetic relationship based on RAPD markers. Of 80 primers screened, 48 primers (60%) gave amplification (Fig. 1A and B). Six random primers (OPA-03, OPA-09, OPB-18, OPD-18,

OPE-07, and OPX-17; primer sequences available from Operon Technologies) gave 32 consistent RAPD markers in which 9 were monomorphic and 23 were polymorphic. Based on the RAPD patterns, none of the 23 polymorphic markers gave a clear separation at the species level because of an overlapping of the markers among the species. Only one marker (OPB-18-D) appeared to be unique to *T. fusca* var. *bromi-tectorum*. By UPGMA cluster analysis, 95 isolates of *T. controversa*, *T. tritici*, *T. laevis*, and *T. fusca* var. *bromi-tectorum* were clustered as two distinct RAPD groups (RG I and RG II) (Fig. 2). RG I contained all isolates of

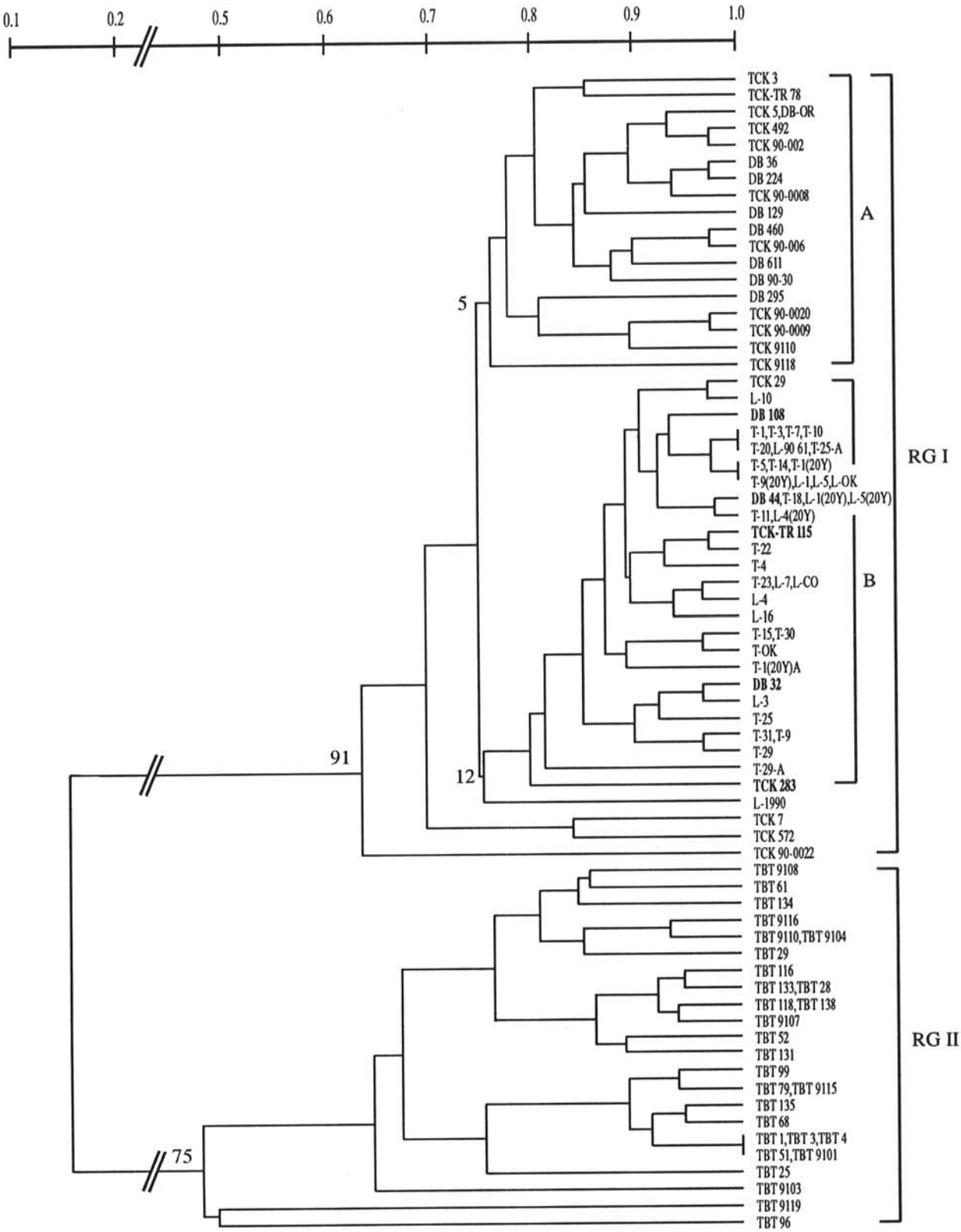

Fig. 2. Dendrogram depicting the genetic relationships among 95 isolates of *Tilletia controversa*, *T. laevis*, *T. tritici*, and *T. fusca* var. *bromi-tectorum* based on 23 random amplified polymorphic DNA markers. All analyses were done using Numerical Taxonomy and Multivariate Analysis System (NTSYS) version 1.80 (29). A similarity matrix was calculated using SIMQUAL based on Dice's coefficient. The tree was generated from the similarity matrix by unweighted pair-group, arithmetic mean (UPGMA) of the SAHN procedure. Boldfaced isolates show a disagreement between phylogenetic grouping and conventional taxonomy (Table 2). Numbers at the nodes of major clusters represent bootstrap values generated by 500 replicates using the Winboot program (26).

the wheat bunt fungi and RG II included all isolates of *T. fusca* var. *bromi-tectorum*. RG I was further divided below the 75% similarity level into two subgroups RG IA and RG IB. RG IA contained 19 isolates of *T. controversa*; RG IB contained 38 isolates of *T. tritici*, *T. laevis*, and six isolates of *T. controversa*. Three *T. controversa* isolates (TCK 7, TCK 572, and TCK 90-0022) within RG I were not clustered in either RG IA or RG IB. Bootstrap analysis of the data generated by Dice's similarity coefficient supported the separation between the wheat isolates (bootstrap value 91%) and the cheatgrass isolates (bootstrap value 75%). However, the bootstrap values for clusters within the wheat isolates were low (5 and 12%), suggesting that the clustering of RG IA and RG IB was not supported by this analysis.

A consensus tree from 100 trees generated by parsimony analysis (mixed method) of PHYLIP (6) showed similar phylogenetic relationships as those generated by the distance matrix method in NTSYS (data not shown). In the parsimony analysis, all the isolates of RG I defined by the cluster analysis were tightly grouped together, whereas most of the isolates of *T. controversa* constituted a subgroup equivalent to RG IA. Isolate TCK 283, which was in the common bunt cluster (RG IB), was grouped with other *T. controversa* isolates by parsimony analysis. All *T. fusca* var. *bromi-tectorum* isolates were widely separated from the wheat bunt fungi. To test whether the generated trees revealed phylogenetic signal, g1 statistics were calculated by generating 10,000 random trees with data from all isolates and from the wheat bunt fungi only. The g1 values derived from the entire data set and from the wheat bunt group were –0.33 and –0.23, respectively (critical values of g1 are –0.18 at $P = 0.05$ and –0.24 at $P = 0.01$) (12), indicating significant skewness from symmetry.

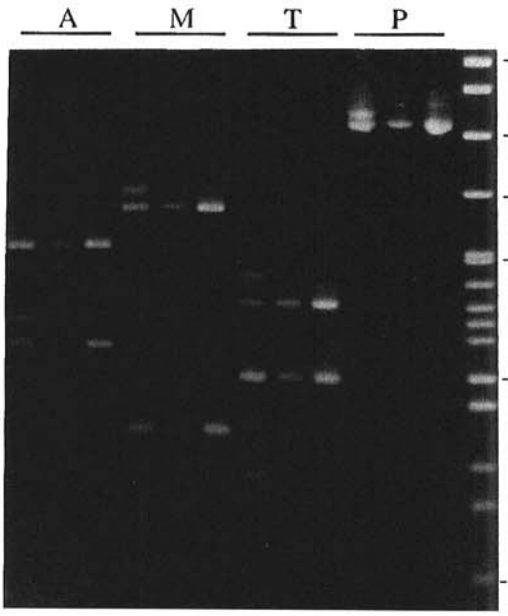

Fig. 3. Patterns of restriction digestion of the 5.8s rDNA and the intergenic transcribed spacer (ITS) region of *Tilletia controversa*, *T. laevis*, and *T. tritici*. P indicates the original polymerase chain reaction (PCR) products amplified using primers ITS3 and ITS4; A, M, and T indicate digestion of the PCR products with restriction endonucleases *Alu*I, *Msp*I, and *Taq*I, respectively. Each group of enzyme digestion, from left to right, contains *Tilletia controversa* (DB 492), *T. laevis* (L-5), and *T. tritici* (T-22). The far right lane is *Msp*I-digested pBR322 DNA (New England Biolabs, Beverly, MA). Molecular sizes are indicated on the right margin. The PCR products and digestion products were electrophoresed on 4% agarose gel.

TABLE 2. Summary of *Tilletia* isolates showing random association of conventional taxonomic characteristics and molecular markers[a]

Isolates	Geographic origin	No. of days to germinate	Spore morphology	RAPD group	rDNA haplotype
DB 32	Neiphi, UT	38	Reticulate	RG IB	B
DB-OR	Baker Co., OR	46	Reticulate	RG IA	B
TCKTR 78	Turkey	22	Reticulate	RG IA	B
TCKTR 115	Tatvan, Turkey	10	Reticulate	RG IB	B
TCK 283[b]	Logan, UT	28	Reticulate	RG IB	A
DB 44	Preston, ID	33	Reticulate	RG IB	A
DB 108	Box Elder Co., UT	29	Reticulate	RG IB	A

[a] Late germination (>28 days) and reticulated teliospores are considered typical of *T. controversa*. Phylogenetic analysis showed that most *T. controversa* isolates were in RG IA and had rDNA haplotype A, whereas all *T. tritici* and *T. laevis* isolates were in RG IB and had rDNA haplotype B.

[b] TCK 283 was grouped in the common bunt cluster based on parsimony analysis.

Genetic relationship based on the RFLPs of the 5.8s rRNA and the ITS region. A DNA fragment of approximately 430 bp was amplified uniformly from all isolates of the wheat bunt fungi using primers ITS3 and ITS4. Under high resolution gel electrophoresis (4% agarose gel), an additional 450-bp fragment was observed in 24 of 28 isolates that were identified as *T. controversa* based on teliospore morphology. Digestion of the PCR products amplified by primers ITS3 and ITS4 with three restriction endonucleases (*Alu*I, *Msp*I, and *Taq*I) produced two distinct RFLP patterns designated haplotype A and B (Fig. 3). Haplotype A included 24 of the 28 isolates of *T. controversa* and haplotype B comprised all isolates of the common bunt fungi and four isolates of *T. controversa* (TCKTR 78, TCKTR 115, DB-OR, and DB 32).

Although the dendrogram separated the wheat bunt fungi into two sublineages, the grouping of isolates in the lineages was not perfectly correlated with the taxonomic placement of *T. controversa*, *T. tritici*, and *T. laevis* (Table 2). Five isolates (TCK 283, TCKTR 115, DB 44, DB 32, and DB 108) identified as *T. controversa* based on teliospore morphology were grouped in the common bunt cluster (RG IB) based on RAPD markers. Based upon rDNA restriction patterns, TCK 283, DB 44, and DB 108 were in the dwarf bunt group, whereas TCKTR 115 and DB 32 were in the common bunt group. On the other hand, two *T. controversa* isolates (TCKTR 78 and DB-OR) in the dwarf bunt group (RG IA) had the typical rDNA haplotype B of the common bunt fungi.

DISCUSSION

Early genetic studies of the wheat bunt fungi have shown that sexual hybridization between the common and dwarf bunt fungi is possible in artificial mating, and that intraspecific and interspecific hybridization may play a role in the production of new races in nature. However, because of the lack of genetic markers, interpretation of the genetic relationships among wheat bunt fungi has not been conclusive. Our results showed that RAPD and RFLPs of the ITS region of rDNA were useful genetic markers for the phylogenetic study of *Tilletia* and that the data generated could be used to address questions concerning the potential significance of interspecific hybridizations between the common and dwarf bunt fungi.

Consistent genetic relationships were generated by the distance matrix method and parsimony analyses using 23 polymorphic RAPD markers. Bootstrap analysis supported the separation between the wheat bunt and grass bunt isolates, but not the subdivision within the wheat bunt group. On the other hand, the g1 statistic test suggested that the clustering of the dwarf bunt and common bunt groups represented significant phylogenetic signal. The analysis of RFLPs of the ITS region in rDNA also showed that *T. controversa* was associated with one haplotype, whereas *T. tritici* and *T. laevis* were associated with another. These results, taken together, suggested that dwarf bunt and common bunt fungi descended from a common ancestral population that subsequently differentiated into two sublineages. This was consistent with the view that the dwarf bunt and common bunt fungi are closely re-

lated species, but have evolved into distinct plant pathogens with different life cycles and adaptive characteristics.

The fact that a significant number of isolates have reciprocal characteristics of dwarf bunt and common bunt fungi raises the question of whether natural hybridization is responsible for the apparent mixing of characters that are presumably unique to each species. Riehm (27) hypothesized that the dwarf bunt fungus originated as a mutant of *T. tritici*. It was suggested that mutations conditioning cold requirement for spore germination may give rise to variants that adapt to new ecological niches, and subsequently may have led to the evolution of *T. controversa*. If Riehm's mutation hypothesis is true, the changes that confer typical *T. controversa* phenotypes must have occurred recently, since divergence between the genomes of the wheat bunt fungi is much less than that compared to the outgroup *T. fusca* var. *bromitectorum*.

An alternative explanation for the mixing of characters between isolates in the two sublineages is that some of the *T. controversa* isolates found in the common bunt sublineage might be interspecific hybrids between the dwarf bunt and common bunt fungi. The potential of hybridization between the dwarf and common bunt fungi has been demonstrated by artificial matings among *T. tritici*, *T. laevis*, and *T. controversa* (16,19,33). Natural hybridization between the dwarf and common bunt fungi has also been suggested by the presence of hybrid-like spores in field collections (18). Hybridization between the dwarf and common bunt fungi may result in "recombinants" that have the morphological and physiological characteristics of *T. controversa*. Based on analysis of teliospore characteristics, Russell and Mills (31) suggested that the combination of the genes governing the phenotypes of spore morphology and germination could result from mutation and recombination in a natural population. A delayed germination as a result of cold temperature requirement could be advantageous since it prolongs dormancy and allows the fungus to escape chemical seed treatment, which has been widely practiced since the 1950s. It is possible that the observed genetic relationships are the combined result of mutation and recombination. Further study of the segregation patterns of molecular markers in the interspecific hybrids may shed new light on the role of hybridization in the evolution of these pathogens.

We included *T. fusca* var. *bromi-tectorum* in our analysis to provide a measure of the genetic relationships among the wheat bunt fungi relative to an outgroup. The wheat bunt fungi are clearly more related to each other than to *T. fusca* var. *bromitectorum*, which parasitizes a different host. *T. fusca* var. *bromitectorum* is a variety of the species complex *T. fusca* that also includes *T. fusca* var. *fusca* and *T. fusca* var. *guyotiana*. These *Tilletia* species are parasitic on wild grasses and are frequently found around wheat fields in the Pacific Northwest. Carris and Gray (2) hybridized members of the *T. fusca* group with the wheat bunt fungi under axenic conditions. Although no genetic exchange between *T. fusca* var. *bromi-tectorum* and the wheat bunt fungi was detected in our study, more extensive sampling is needed to rule out the possibility of hybridization among the wheat and grass bunt fungi in nature.

A number of studies have raised questions about the conventional method for taxonomic classification of the wheat bunt fungi (20,30,31). Phenotypic characters such as germination requirement and teliospore morphology can be variable and an overlapping of the phenotypes among species has been reported (10). Kawchuk et al. (20) proposed classifying *T. controversa* and *T. laevis* as varieties of *T. tritici* based on the absence of species-specific polypeptides in the protein profiles of these species. This proposal was supported by the electrophoretic karyotype analysis and autofluorescence study of teliospore morphology of Russell and Mills (30,31). Russell and Mills (30) found almost random assortment of chromosome-size fragments in F_1 progeny from a cross between *T. tritici* and *T. controversa*. In their analysis of teliospore characteristics, two out of seven collections were ambiguous in species determination based on spore germination and autofluorescence (31). The close relationship between *T. tritici* and *T. laevis* shown in this study indicated little justification to consider *T. tritici* and *T. laevis* as two species. The separation of *T. controversa* from *T. tritici* and *T. laevis* is more relevant since the dwarf and common bunt are epidemiologically distinct diseases, and significant phylogenetic differences were shown by the g1 statistic used in this study. It is clear, however, that the differences among isolates within the wheat bunt group are substantially less than that between isolates of the wheat and grass bunt fungi. If the genetic divergence between *T. fusca* var. *bromitectorum* and wheat bunt fungi is used as a guide to designate species, classification of *T. controversa*, *T. tritici*, and *T. laevis* as separate species is questionable. Thus, a reexamination of the taxonomic classification of the wheat bunt fungi seems highly warranted. Given the potential for interbreeding among "species" of *Tilletia*, a meaningful revision of the taxonomy of the wheat bunt fungi must take into account the other *Tilletia* species that coexist in the same habitat.

LITERATURE CITED

1. Becker, T. 1936. Untersuchungen uber sexualitat bei *Tilletia tritici* (Bjerk.) Wint. im Rahmen der immunitatszuchtung. Phytopathol. Z. 9:187-228.
2. Carris, L. M., and Gray, P. M. 1994. The ability of *Tilletia fusca* to hybridize with the wheat bunt species under axenic conditions. Mycologia 86:157-163.
3. Churchill, A. C. L., and Mills, D. 1984. Selection and culture of auxotrophic and drug-resistant mutants of *Tilletia caries*. Phytopathology 74:354-357.
4. Churchill, A. C. L., and Mills, D. 1985. Heterokaryon formation *in planta* by genetically marked strains of *Tilletia caries*. Can. J. Bot. 63:1924-1927.
5. Crowhurst, R. N., Hawthorne, B. T., Rikkerink, E. H. A., and Templeton, M. D. 1991. Differentiation of *Fusarium solani* f. sp. *cucurbitae* race 1 and 2 by random amplification of polymorphic DNA. Curr. Genet. 20:391-396.
6. Felsenstein, J. 1993. PHYLIP Manual, version 3.5. University of Washington, Seattle. Public domain software.
7. Flor, H. H. 1932. Heterothallism and hybridization in *Tilletia tritici* and *T. laevis*. J. Agric. Res. 44:49-58.
8. Goodwin, P. H., and Annis, S. L. 1991. Rapid identification of genetic variation and pathotype of *Leptosphaeria maculans* by random amplified polymorphic DNA assay. Appl. Environ. Microbiol. 57:2484-2486.
9. Guthrie, P. A. I., Magill, C. W., Frederiksen, R. A., and Odvody, G. N. 1992. Random amplified polymorphic DNA markers: A system for identifying and differentiating isolates of *Colletotrichum graminicola*. Phytopathology 82:832-835.
10. Halisky, P. M. 1965. Physiologic specification and genetics of the smut fungi III. Bot. Rev. 31:114-150.
11. Hanna, W. F. 1934. The physiologic specification and genetics of smut fungi. Pages 3195-3204 in: Proc. Pac. Sci. Congr., 5th.
12. Hillis, D. M., and Huelsenbeck, J. P. 1992. Signal, noise, and reliability in molecular phylogenetic analyses. J. Hered. 83:189-195.
13. Hoffmann, J. A. 1982. Bunt of wheat. Plant Dis. 66:979-986.
14. Hoffmann, J. A., and Metzger, R. J. 1976. Current status of virulence genes and pathogenic races of the wheat bunt fungi in the northwestern USA. Phytopathology 66:657-660.
15. Holton, C. S. 1938. A new pathogenically distinct race derived from a cross between *Tilletia tritici* and *T. laevis*. Phytopathology 28:371-372.
16. Holton, C. S. 1944. Inheritance of chlamydospore and sorus characters in species and race hybrids of *Tilletia caries* and *T. foetida*. Phytopathology 34:586-592.
17. Holton, C. S. 1951. Methods and results of studies on heterothallism and hybridization in *Tilletia caries* and *T. foetida*. Phytopathology 41:511-521.
18. Holton, C. S. 1954. Natural hybridization between common and dwarf bunt as related to the problem of delimitation of species of *Tilletia* occurring on wheat. (Abstr.) Phytopathology 44:493.
19. Holton, C. S., and Kendrick, E. L. 1956. Problems in the delimitation of species of *Tilletia* occurring on wheat. Res. Stud. Wash. State Univ. 24:318-325.
20. Kawchuk, L. M., Kim, W. K., and Nielsen, J. 1988. A comparison of

polypeptides from the wheat bunt fungi *Tilletia laevis*, *T. tritici*, and *T. controversa*. Can. J. Bot. 66:2367-2376.
21. Kendrick, E. L., and Holton, C. S. 1958. New physiologic races of *Tilletia caries* in the Pacific Northwest. Plant Dis. Rep. 42:15-17.
22. Loomis, P., and Leung, H. 1995. Low-temperature storage of monosporidial cultures of wheat bunt fungi. Can. J. Bot. 73:758-760.
23. Metzger, R. J., and Hoffmann, J. A. 1978. New races of common bunt fungi to determine resistance of wheat to dwarf bunt. Crop Sci. 18:49-51.
24. Mills, D., and Churchill, A. C. L. 1988. *Tilletia* spp., bunt fungi of the Gramineae. Adv. Plant Pathol. 6:401-414.
25. Murray, M. G., and Thompson, W. F. 1980. Rapid isolation of high-molecular-weight plant DNA. Nucleic Acids Res. 8:4321-4325.
26. Nelson, R. J., Baraoidan, M. R., Vera Cruz, C. M., Yap, I. V., Leach, J. E., Mew, T. W., and Leung, H. 1994. Relationship between phylogeny and pathotype for the bacterial blight pathogen of rice. Appl. Environ. Microbiol. 60:3275-3283.
27. Riehm, E. 1954. Der zwergbrand (*Tilletia brevifaciens* G. W. Fischer). Z. Pflanzenkr. Pflanzenpathol. Pflanzenschutz 61:129-139.
28. Rodenhiser, H. A., and Holton, C. S. 1945. Distribution of races of *Tilletia caries* and *Tilletia foetida* and their relative virulence on certain varieties and selections of wheat. Phytopathology 35:955-969.
29. Rohlf, F. J. 1993. NTSYS-pc: Numerical Taxonomy and Multivariate Analysis System, version 1.80. Exeter Software, Setauket, NY.
30. Russell, B. W., and Mills, D. 1993. Electrophoretic karyotypes of *Tilletia caries*, *T. controversa*, and their F_1 progeny: Further evidence for conspecific status. Mol. Plant-Microbe Interact. 6:66-74.
31. Russell, B. W., and Mills, D. 1994. Morphological, physiological, and genetic evidence in support of a conspecific status for *Tilletia caries*, *T. controversa*, and *T. foetida*. Phytopathology 84:576-582.
32. Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
33. Silbernagel, M. J. 1961. Hybridization between *Tilletia caries* and *T. controversa* on wheat. Ph.D. thesis. Washington State University, Pullman.
34. Silbernagel, M. J. 1964. Compatibility between *Tilletia caries* and *T. controversa*. Phytopathology 54:1117-1120.
35. Swofford, D. L., and Begle, D. P. 1993. PAUP – Phylogenetic Analysis Using Parsimony, version 3.1. Illinois Natural History Survey, Champaign, IL.
36. Tibayrenc, M., Neubauer, K., Barnabe, C., Guerrini, F., Skarecky, D., and Ayala, F. J. 1993. Genetic characterization of six parasitic protozoa: Parity between random-primer DNA typing and multilocus enzyme electrophoresis. Proc. Natl. Acad. Sci. U.S.A. 90:1335-1339.
37. Trail, F., and Mills, D. 1990. Growth of haploid *Tilletia* strains in planta and genetic analysis of a cross of *Tilletia caries* × *T. controversa*. Phytopathology 80:367-370.
38. Trione, E. J. 1982. Dwarf bunt of wheat and its importance in international wheat trade. Plant Dis. 66:1083-1088.
39. White, T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-332 in: PCR Protocols, a Guide to Methods and Amplifications. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, Inc., San Diego, CA.
40. Williams, J. G. K., Kubeilik, A. R., Livak, K. J., Rafalski, J. A., and Tingey, S. V. 1990. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18:6531-6535.