Genetic Variation Among Natural Populations of *Tilletia controversa* and *T. bromi*

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ABSTRACT

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Isolates of *Tilletia controversa* and *T. bromi* were sampled from wheat and two *Bromus* species hosts, respectively, in the Pacific Northwest, and genetic variation within and among populations was determined. Fifty-one random amplified polymorphic DNA markers from eleven primers were treated as phenotypic 1 and 0 character state data to estimate similarities and analyze molecular variance (AMOVA) among populations and as putative genetic loci to carry out analyses of gene diversity. Phenotypic analysis of *T. controversa* and *T. bromi* isolates revealed two distinct clusters that were 37% similar. The *T. bromi* cluster was subdivided further into two groups, corresponding to host, with 40% similarity. Cluster analysis based on allele frequencies produced similar results and also

supported two *T. bromi* groups based on host. No evidence of natural hybridization and introgression was detected between the *T. controversa* and *T. bromi* populations. Both AMOVA and gene diversity analyses detected moderate levels of differentiation among *T. controversa* populations, whereas *T. bromi* populations were highly differentiated. The level of genetic differentiation observed between the *T. bromi* populations on different *Bromus* species hosts supports the hypothesis that a high degree of host specificity exists in the wild grass-infecting smuts. We speculate that the higher level of genetic differentiation among the *T. bromi* populations compared with the *T. controversa* populations on wheat may be due to selection by a more genetically diverse host population.

Additional keywords: dwarf bunt, PCR, population genetics, sympatric populations.

Tilletia controversa Kühn, the causal agent of dwarf bunt of wheat, and *T. bromi* (Brockm.) Brockm., a complex of taxa infecting *Bromus* species (3), are obligate parasites that coexist in wheat fields in the Pacific Northwest. The only common host for *T. controversa* and *T. bromi* is *Bromus carinatus* Hook. & Arnott, a perennial brome grass that occurs in North America from the Rocky Mountains west to the Pacific Coast (17). In the United States, *T. controversa* was the focus of a quarantine by the People's Republic of China established in 1973 and lifted in April 1999 (42). Members of the *T. bromi* complex also have been the target of quarantines because they are similar to *T. controversa* in biology and teliospore morphology, and both species are reproductively compatible under artificial conditions (4,22). *T. controversa* and *T. bromi* reproduce sexually via a bipolar mating system with multiple alleles (9; L. M. Carris unpublished data). Their life cycles depend on the ability to infect and sporulate in a susceptible host. Meiosis occurs during germination of diploid teliospores, and compatible haploid primary basidiospores fuse rapidly to form the infective dikaryon (H-bodies) while still attached to the basidium (12). H-bodies germinate to produce infective hyphae that penetrate the coleoptile of a susceptible host, grow inter- and intracellularly into the floral primordia prior to internodal elongation, and sporulate in the ovarian tissue (11).

The origin of new pathotypes in *Tilletia* species populations is not clearly understood. Some factors that may be involved include selection by host, mutation, and natural hybridization within and among *Tilletia* species (9). Annual surveys of *T. controversa* and the common bunt species, *T. tritici* and *T. laevis*, were conducted in the Pacific Northwest from 1900 to 1960 to determine the effect of wheat cultivars on race composition (19,25,26). After the de-

ployment of new wheat cultivars, new smut pathotypes appeared within the first year (25), suggesting heterozygosity in pathogenic populations and strong selection exerted by host resistance genes. Based on these observations, it was inferred that the amount of genetic variability present in wheat bunt populations was high, and the variation has contributed to the difficulty in controlling dwarf and common bunt through host resistance (26). Although *T. controversa* still causes sporadic outbreaks in some areas of the Pacific Northwest, the wheat bunt pathogens have been controlled successfully in the United States by a combination of fungicides and resistant cultivars (11). There may be relatively greater potential for outcrossing in *T. controversa* than in *T. tritici* and *T. laevis* due to the multiallelic system for mating in *T. controversa* versus the biallelic system in the common bunts. However, high levels of inbreeding within *Tilletia* species populations also may be expected due to the rapid fusion of compatible primary basidiospores formed from the same teliospore, reducing the overall population heterozygosity.

The characteristics traditionally used to identify *Tilletia* species are teliospore and soral morphology, teliospore germination, and host specificity (6). Teliospore morphology can be variable and tends to overlap among species, making identification based solely on teliospores difficult. Teliospore variability has been cited as an indication that high levels of genetic variation exist in populations of *Tilletia* species (9,21), although the genetic basis of teliospore morphology has not been studied. Several studies have shown that *Tilletia* species are reproductively compatible under artificial conditions, and if hybridization occurs in nature, it may be a factor that contributes to the morphological and genetic variability of these fungi (4,10,20). For example, Holton and Kendrick (21) inferred that hybridization between *T. controversa* and the common bunt species, *T. tritici* and *T. laevis*, occurred under natural conditions based on the similarities in teliospore morphology and germination between field isolates and greenhouse hybrids. Shi et al. (40), using cluster analysis based on random amplified polymorphic DNA

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(RAPD) phenotypes, showed that several isolates morphologically identified as *T. controversa* clustered with isolates of the common bunts. They speculated that these isolates might represent natural interspecific hybrids. Holton and Siang (22) showed that primary basidiospores of *T. controversa* fused with primary basidiospores of *T. bromi* from *Bromus* spp. and *T. fusca* from *Vulpia* spp. under artificial conditions. Later, Carris and Gray (4) demonstrated that primary basidiospores of all three wheat bunt species fused with basidiospores of several wild grass-infecting *Tilletia* species under axenic conditions, but viable teliospores were formed only between the wheat bunts and *T. fusca*. Interspecific hybridization among wheat- and wild grass-infecting species of *Tilletia* in nature may contribute to genetic variability in wheat bunt populations and may allow the fungus to overcome disease control measures (15). However, it has not been demonstrated conclusively that hybridization occurs among *Tilletia* species in the field.

Previous studies documenting genetic variability and relationships among *Tilletia* species were performed with a limited number of isolates from the same geographic regions (2,36,40). However, studies reporting levels of genetic variation among and within populations of *Tilletia* species have not been done. The objectives of this research were to (i) quantify the genetic differentiation among *T. controversa* and *T. bromi* populations in the Pacific Northwest; (ii) determine whether hybridization or introgression has occurred among natural populations of *T. controversa* and *T. bromi*; and (iii) determine the importance of host specificity and geographic isolation in controlling the genetic structure of *T. controversa* and *T. bromi* populations.

MATERIALS AND METHODS

Sampling. Fifteen samples representing *T. controversa* on winter wheat (*Triticum aestivum* L.) and *T. bromi* on *B. tectorum* L. and *B. japonicus* Thunb. ex J.A. Murray were collected from wheat fields in the Pacific Northwest (Table 1). Samples of *T. controversa* were collected from wheat stubble remaining in fields after harvest. *T. bromi* was collected from within or around wheat fields after harvest. Each sample contained at least 26 isolates. Isolates from different plants of the same host species in a wheat field were considered a single population. To determine whether populations of *T. controversa* and *T. bromi* were differentiated among geographically separated locations in the Pacific Northwest, five populations were sampled for each species: three from Oregon, one from Idaho, and one from Washington (Table 1). Sympatric populations of *T. controversa* and *T. bromi* were collected from four of the same wheat fields: three in Oregon and one in Idaho (Table 1) to determine whether interspecific hybridization had occurred in nature.

DNA isolation and polymerase chain reaction (PCR) amplification with 10-mer primers. Genomic DNA was extracted from 35 to 45 mg of teliospores from multiple sori produced in a single inflorescence with an Omni Prep kit (GenoTechnology Inc., St. Louis, MO) following the manufacturer's instructions. DNA yields were 0.1 to 1.25 µg µl⁻¹, as quantified by UV spectrophotometry. RAPD amplifications were carried out according to the method of Pimentel et al. (36) with the following modifications. Amplifications were performed with 0.3 U of AmpliTaq Gold (Perkin Elmer, Branchburg, NJ) in either a GeneAmp PCR 9700 system (PE Applied Biosystems, Roche Molecular Systems Inc., Branchburg, NJ) or a Coy TempCycler II (Coy Laboratory Products Inc., Grass Lake, MI). The following cycling parameters were used: samples were incubated at 95°C for 9 min 30 s for initial activation of the polymerase and denaturation of DNA, followed by 45 cycles of denaturation at 94°C for 35 s, annealing at 48°C for 1 min 10 s, extension at 72°C for 1 min 40 s, and a final extension at 72°C for 7 min. The ramp time between all steps in the cycles was controlled at 80% of the regular speed. The more stringent conditions for annealing the primer and template were used to increase the reliability of the amplified product (28). Amplified PCR products (5 µl) were examined by electrophoresis (3.5 V cm⁻¹) in 1.5% agarose gel (UltraPure Agarose, GIBCO Bethesda Research Laboratories, Gaithersburg, MD) in 0.5× TBE buffer (45 mM Tris-borate, 45 mM boric acid, and 1 mM EDTA, pH 8.0). Gels were stained with ethidium bromide (0.5 µg ml⁻¹), and visualized with a UV transilluminator. Eleven random primers (Operon Technologies, Alameda, CA) were used in the study to generate reproducible PCR products (bands) (Table 2). All amplifications were repeated at least three times, and both faint and intense bands were scored among all isolates if reproducible in independent runs.

Southern hybridization among RAPD products. Southern hybridization of selected RAPD products from *T. controversa* and *T. bromi* showing similar electrophoretic mobility was performed to determine whether the products were homologous within and among *T. controversa* and *T. bromi* populations. RAPD products present in all taxa were recognized as shared (e.g., homologous alleles), whereas markers present in only one *Tilletia* species were considered exclusive. Amplified PCR products were diluted (1:15) in sterile distilled water and separated (6 µl) by electrophoresis in 1.65% agarose gel to generate DNA blots. DNA fragments were transferred from gels to Zeta-Probe GT Genomic blotting membranes (Bio-Rad Laboratories, Hercules, CA) by capillary transfer under alkaline conditions (39). Probes were obtained by reamplifying PCR products at a 55°C annealing temperature, followed by electrophoresis (6 µl) in 1.6% agarose gels. DNA fragments were excised from the agarose gel and purified with GenElute Agarose spin columns (Supelco, Bellefonte, PA) following the manufac-

TABLE 1. Collections of *Tilletia controversa* and *T. bromi* used in the study

<i>Tilletia</i> sp.	Collection no. ^a	n ^b	Location	Month collected	Host
<i>T. controversa</i>	282	33	Flora, OR	August 1994	<i>Triticum aestivum</i>
	285	31	Paradise (A), OR	August 1994	<i>T. aestivum</i>
	290	30	Paradise (B), OR	August 1994	<i>T. aestivum</i>
	295	30	Troy-Deary, ID	September 1994	<i>T. aestivum</i>
	355	32	Davenport-Miles, WA	August 1996	<i>T. aestivum</i>
<i>T. bromi</i>	284	30	Flora, OR	August 1994	<i>Bromus tectorum</i>
	287	26	Paradise (A), OR	August 1994	<i>B. tectorum</i>
	291	31	Paradise (B), OR	August 1994	<i>B. tectorum</i>
	297	31	Troy-Deary, ID	September 1994	<i>B. tectorum</i>
	350	31	Rice-Orin, WA	August 1996	<i>B. tectorum</i>
	283	31	Flora, OR	August 1994	<i>B. japonicus</i>
	286	30	Paradise (A), OR	August 1994	<i>B. japonicus</i>
	292	31	Paradise (B), OR	August 1994	<i>B. japonicus</i>
	296	27	Troy-Deary, ID	September 1994	<i>B. japonicus</i>
	351	32	Rice-Orin, WA	August 1996	<i>B. japonicus</i>

^a Accession numbers refer to the L. M. Carris' collection at Washington State University, Pullman.

^b Number of isolates per location.

turer's instructions. Probes were labeled with the nonradioactive AlkPhos direct labeling and detection system (Amersham Life Science, Arlington Heights, IL) and hybridized to blots containing RAPD bands from 10 isolates of each taxon collected at the same location. In addition, PCR products shown to be exclusive to *T. bromi* populations were hybridized to DNA blots containing RAPD products of *T. controversa* from different populations to determine whether the sequence was present. Membranes were prehybridized,

TABLE 2. Primer sequences and presence and absence of random amplified polymorphic DNA (RAPD) markers in *Tilletia controversa* (Tc) and *T. bromi* (Tb) populations

Primer	Sequence (5' → 3')	Locus	Tc ^a	Tb ^a	Southern hybridization ^b
OPA-03	AGTCAGCCAC	A3a	—	+	t
		A3b	—	+	t
		A3c	—	+	t
		A3d	+	+	nt
		A3e	+	—	nt
		A3f	+	—	nt
OPA-09	GGGTAACGCC	A9a	+	+	nt
		A9b	+	+	t
		A9c	—	+	t
		A9d	+	+	t
		A9e	+	—	nt
		A9f	+	—	nt
OPA-13	CAGCACCCAC	A13a	+	+	t
		A13c	+	—	t
OPA-18	AGGTGACCGT	A18a	—	+	nt
		A18b	—	+	t
		A18c	+	+	t
		A18d	+	—	t
		A18e	+	—	t
OPD-18	GAGAGCCAAC	D18b	—	+	t
		D18c	—	+	t
		D18d	—	+	nt
		D18e	+	—	t
OPE-02	GGTGCGGGAA	E2a	+	+	t
		E2b	—	+	nt
		E2c	—	+	t
		E2d	+	—	nt
		E2e	+	—	t
		E2f	—	+	t
		E2g	—	+	t
		E2h	—	+	nt
OPE-07	AGATGCAGCC	E7a	+	+	t
		E7b	—	+	t
		E7c	—	+	nt
		E7d	—	+	nt
		E7e	—	+	t
		E7f	—	+	t
OPX-01	CTGGGCACGA	X1a	+	+	t
		X1b	+	+	t
		X1c	+	+	nt
		X1d	—	+	t
		X1e	+	—	nt
OPX-08	CAGGGGTGGA	X8a	+	—	t
		X8b	+	+	t
		X8c	—	+	nt
		X8d	—	+	t
		X8e	+	+	t
		X8f	—	+	t
OPX-15	CAGACAAGCC	X15a	+	+	nt
		X15b	+	+	nt
OPY-03	ACAGCCTGCT	Y3a	+	+	nt

^a — indicates positive allele not present in any isolate in the population; + indicates presence of positive allele in at least one isolate in the population.

^b Southern hybridization performed using the RAPD-polymerase chain reaction product as the hybridization probe. t indicates product was tested and the presence or absence of the RAPD marker was confirmed; nt indicates the marker was not tested.

hybridized, and washed at 62°C and detected with autoradiography film (Hyperfilm ECL, Amersham) following the manufacturer's protocols.

Data analysis. Both phenotypic and genotypic approaches were used to determine similarities and genetic differentiation among populations of *T. controversa* and *T. bromi*. PCR products were scored as present or absent among all isolates in the study; however, they were treated differently in each approach. In the first approach, present or absent products were treated as phenotypic 1 and 0 character state data, respectively. In the genotypic approach, PCR products were treated as putative loci with two alleles: one positive (1) and one null (0).

Phenotypic approach. Cluster analyses were performed to establish similarities among *T. controversa* and *T. bromi* isolates from all populations and within each wheat field and to determine whether interspecific hybridization had occurred among sympatric populations. Phenograms were generated in NTSYS-pc (version 2.02j, Exeter Software, Setauket, NY). A similarity matrix was produced with the SIMQUAL program using Jaccard's distance coefficient (41), which measures the proportion of band mismatches between pairs of isolates. Jaccard's distance coefficient is defined as $a/(n - d)$, where a = number of positive matches (both having a common band), n = total sample size, and d = number of negative matches (both lacking the same band). Clustering was performed with the SAHN program using the unweighted pair-group method with arithmetic averages (UPGMA). Bootstrap support for the branches of the phenogram was generated with 1,000 bootstrapped samples in the WINBOOT program (46).

An analysis of molecular variance (AMOVA; version 1.55) was performed on *T. controversa* and *T. bromi* isolates to partition the total variance into among and within population components (7, 23). AMOVA uses a classical analysis of variance to compute molecular variance components at different hierarchical levels, using a matrix of squared molecular distances between pairs of observations (7). A Euclidean distance matrix between all pairs of multilocus RAPD phenotypes was calculated and used in the AMOVA (7,23). The variance components among populations and the F statistic analog, Φ_{st} , were tested statistically by nonparametric randomization analysis (7) with 1,000 repetitions.

Genotypic approach. Gene diversity analyses were done to determine the levels of genetic differentiation among populations of *T. controversa* and *T. bromi*. Allele frequencies at each locus were

Fig. 1. Southern blot showing sequence homology among random amplified polymorphic DNA (RAPD) products of *Tilletia controversa* and *T. bromi* isolates from the Troy-Deary, ID, subpopulations. Bar 1 = *T. controversa* isolates; bar 2 = *T. bromi* isolates from *Bromus tectorum*; bar 3 = *T. bromi* isolates from *B. japonicus*. **A**, DNA hybridization showing a RAPD sequence shared among isolates from all three hosts. DNA fragments were generated by primer OPX-01 and probed with marker X1b from *T. controversa*. **B**, DNA hybridization showing a RAPD sequence found only in *T. bromi* from *B. tectorum*. The DNA fragments were generated by primer OPE-07 and probed with marker E7b from *T. bromi* on *B. tectorum*.

estimated within each population and used to calculate standard population genetic statistics using POPGENE population genetics software (version 1.31, Molecular Biology and Biotechnology Centre, University of Alberta, Canada). Because teliospores are diploid, analyses of gene diversity were performed assuming conditions of Hardy-Weinberg (H/W) equilibrium ($F_{is} = 0$) and maximum disequilibrium ($F_{is} = 1$) due to the dominant nature of RAPD products (45). Estimates of gene diversity were calculated using Nei's equations (31). Genetic differentiation among populations was estimated using Nei's coefficient of genetic differentiation, G_{st} (31), as described in Hartl and Clark (16) and Goodwin et al. (14). G_{st} was calculated among all populations of *T. controversa* and *T. bromi*, among the *T. bromi* populations by host, and among the three Oregon populations for each taxon. The null hypothesis of no differentiation among populations ($G_{st} = 0$), assuming H/W disequilibrium, was tested using a Monte Carlo randomization approach that tested the observed G_{st} against a null distribution of G_{st} . The null distribution of G_{st} was generated by randomly assigning isolates to samples without replacement 1,000 times and calculating the G_{st} value after each iteration. The null hypothesis was rejected if fewer than 50 of the simulated G_{st} values exceeded the observed G_{st} ($P = 0.05$).

Nei's (32) unbiased genetic distances between all pairs of *T. controversa* and *T. bromi* populations were measured using allele frequencies to determine whether the populations were genetically differentiated among regions. The genetic distance was calculated using the SIMGEND program of NTSYS-pc. Clustering was performed with the SAHN program using the UPGMA algorithm. Support for the phenogram branches was obtained with 1,000 bootstrap replicates using the SEQBOOT, GENDIST, NEIGHBOR, and CONSENSE programs in PHYLIP (Phylogeny Inference Package, version 3.573, Department of Genetics, University of Washington, Seattle).

RESULTS

Sequence homology among RAPD products of *T. controversa* and *T. bromi*. Fifty-one RAPD products were scored among the *Tilletia* taxa. Twelve and twenty-three RAPD products were found exclusively in the *T. controversa* and *T. bromi* populations, respectively (Table 2). Thirty-two RAPD products were selected at random and tested for sequence homology among isolates of *T. controversa* and *T. bromi* from different populations (Table 2). The tested RAPD products hybridized only to bands with similar electrophoretic mobility. RAPD markers found exclusively in *T. bromi* did not hybridize to any markers in *T. controversa* populations, suggesting that no homology existed in the other species and that no introgression had occurred between the sympatric populations (Fig. 1A and B).

Similarities and phenotypic variation among *Tilletia* isolates and natural populations. Cluster analysis of 456 isolates of *T. controversa* and *T. bromi* showed 2 major RAPD groups, cor-

responding to each host species, with 32% similarity (data not shown). The *T. bromi* cluster was divided into two groups, corresponding to the different *Bromus* species hosts, with 37% similarity. The percentage of similarity among all isolates of *T. controversa* was 68% and among *T. bromi* on *B. tectorum* and *B. japonicus* was 66 and 54%, respectively. No evidence was found for clustering of isolates by geographic region or collection site. Cluster analyses of *T. controversa* and *T. bromi* isolates from the

Fig. 2. Phenogram of *Tilletia controversa* and *T. bromi* populations collected from the same wheat field in Troy-Deary, ID. Multiple occurrences of isolates with the same random amplified polymorphic DNA phenotype are not shown. Bars to the right indicate three distinct clusters and correspond perfectly to the host. A = *T. controversa* from wheat; B = *T. bromi* from *Bromus tectorum*; and C = *T. bromi* from *B. japonicus*. Bootstrap values of major clusters (based on 1,000 replications) are indicated on internal branches.

TABLE 3. Analysis of molecular variance (AMOVA) for populations of <i>Tilletia controversa</i> and <i>T. bromi</i> , using random amplified polymorphic DNA phenotypes						
<i>Tilletia</i> sp.	Source	df	Variance	Total variance (%)	Φ statistic	P^a
<i>T. controversa</i>	Among populations	4	0.425	16.42	0.164	<0.001
	Within populations	151	2.165	83.58	...	...
<i>T. bromi</i>	Among populations	9	5.580	76.60	0.766	<0.001
	Within populations	290	1.705	23.40	...	...
<i>T. bromi</i> from <i>Bromus tectorum</i>	Among populations	4	0.710	36.55	0.365	<0.001
	Within populations	126	1.233	63.45	...	...
<i>T. bromi</i> from <i>B. japonicus</i>	Among populations	4	1.198	37.24	0.372	<0.001
	Within populations	146	2.019	62.76	...	...

^a Probability of having an equal or more extreme variance component and Φ statistic than the observed values by chance alone. Tested by nonparametric randomization analysis, using 1,000 repetitions.

same wheat field showed no evidence of interspecific hybridization (Fig. 2). The percentage of similarity among *T. controversa* isolates within each population ranged from 65 to 82%, whereas in *T. bromi* the percentage of similarity among isolates within populations ranged from 56 to 89%.

Based on AMOVA, the total variation in *T. controversa* populations was mostly attributable to diversity within populations (83.58%), whereas variation in *T. bromi* was attributable to diversity among populations (76.6%) (Table 3). The AMOVA was repeated separately for the *T. bromi* populations on *B. tectorum* and *B. japonicus*, and it showed high levels of variation within populations in each taxon (Table 3). In all cases, the observed phenotypic diversity among subpopulations of *T. controversa* and *T. bromi* was highly significant ($P < 0.001$).

Genetic differentiation among *T. controversa* and *T. bromi* populations. Of 51 RAPD loci, positive alleles were observed at 28 and 39 loci among populations of *T. controversa* and *T. bromi*, respectively (Table 2). Nineteen loci were polymorphic in at least one *T. controversa* population, whereas *T. bromi* contained thirty-eight polymorphic loci. Gene frequencies in *T. controversa* and *T. bromi* populations based on conditions of H/W equilibrium and disequilibrium are not shown but are available on request. Gene diversity within populations, the total genetic diversity over all populations, and the coefficient of genetic differentiation among populations (G_{st}) are shown in Table 4. Slightly different G_{st} values were obtained for *T. controversa* and *T. bromi* depending on the H/W conditions used in the analysis (Table 4). G_{st} values are presented as ranges between H/W equilibrium and disequilibrium. In *T. controversa*, G_{st} ranged from 0.16 to 0.18 ($P < 0.001$), whereas in *T. bromi* G_{st} ranged from 0.62 to 0.75 ($P < 0.001$). These values indicate that 16 to 18% and 62 to 75% of the observed genetic variation in *T. controversa* and *T. bromi*, respectively, was due to genetic differentiation among populations. Be-

cause the *T. bromi* populations on each host appeared to be quite different, a separate G_{st} value was calculated for populations corresponding to the *Bromus* species (Table 5). Populations of *T. bromi* infecting *B. tectorum* had G_{st} values of 0.24 to 0.31 ($P < 0.001$), whereas the ones infecting *B. japonicus* had G_{st} values of 0.30 to 0.34 ($P < 0.001$). The G_{st} values among the three *T. controversa* populations from Oregon ranged from 0.09 to 0.13 ($P < 0.001$). In contrast, the *T. bromi* populations on *B. tectorum* and *B. japonicus* from Oregon had G_{st} values of 0.25 to 0.35 ($P < 0.001$) and 0.29 to 0.31 ($P < 0.001$), respectively. Pairwise G_{st} values for *T. controversa* and *T. bromi* populations suggested that the differentiation among populations could be attributed largely to differences between hosts (Tables 5 and 6).

The phenogram of genetic distances between pairs of populations showed three major clusters that corresponded to the different hosts and no evidence for any correlation between genetic distances and geographic isolation (Fig. 3). Overall genetic distance among the *T. controversa* populations was low (0.03%). Cluster analysis of the 10 populations of *T. bromi* showed two major clusters, corresponding to the different *Bromus* species hosts, with 50% genetic distance (Fig. 3). In both clusters, the overall genetic distance among the *T. bromi* populations on *B. tectorum* and *B. japonicus* was $<0.06\%$, and bootstrap analysis showed 100% support.

DISCUSSION

A high level of host specificity was observed among *T. controversa* and *T. bromi* isolates sampled from natural populations in the Pacific Northwest. Three distinct groups corresponding to *T. controversa* on wheat, *T. bromi* on *B. tectorum*, and *T. bromi* on *B. japonicus* were identified among the isolates by RAPD phenotypic clustering and genetic distance analyses. Previous studies on genetic variability among *Tilletia* species from diverse geographic

TABLE 4. Gene diversity estimates within *Tilletia controversa* and *T. bromi* subpopulations based on 51 random amplified polymorphic DNA loci assuming conditions of Hardy-Weinberg (H/W) equilibrium ($F_{is} = 0$) and disequilibrium ($F_{is} = 1$)

<i>Tilletia</i> sp.	No. of subpopulations ^a	H/W condition (F_{is})	H_s ^b	H_t ^c	G_{st} ^d
<i>T. controversa</i>	5	0	0.114	0.140	0.183
		1	0.082	0.097	0.159 ***
<i>T. bromi</i>	10	0	0.107	0.281	0.618
		1	0.065	0.263	0.753 ***
<i>T. bromi</i> from <i>Bromus tectorum</i>	5	0	0.088	0.116	0.242
		1	0.053	0.076	0.308 ***
<i>T. bromi</i> from <i>B. japonicus</i>	5	0	0.127	0.180	0.298
		1	0.077	0.117	0.338 ***

^a Number of subpopulations analyzed.

^b Genetic diversity within subpopulations (31).

^c Total genetic diversity over all subpopulations (31).

^d Genetic diversity among subpopulations as a proportion of total diversity, $G_{st} = (H_t - H_s)/H_t$ (31). Null hypothesis of no differentiation between locations assuming H/W disequilibrium. *** indicates $P < 0.001$, estimated using Monte Carlo simulations as described in text.

TABLE 5. Pairwise comparisons of Nei's coefficient of genetic differentiation (G_{st}) values among *Tilletia bromi* populations from *Bromus tectorum* and *B. japonicus*

Host	Population	<i>B. tectorum</i> ^a				<i>B. japonicus</i> ^a			
		Paradise (A)	Paradise (B)	Troy	Rice-Orin	Flora	Paradise (A)	Paradise (B)	Troy
<i>B. tectorum</i>	Flora	0.110	0.269	0.169	0.120	0.728	0.531	0.564	0.574
	Paradise (A)		0.209	0.141	0.123	0.699	0.491	0.525	0.544
	Paradise (B)			0.117	0.243	0.725	0.485	0.537	0.555
	Troy				0.127	0.712	0.474	0.523	0.541
	Rice-Orin					0.695	0.489	0.514	0.540
<i>B. japonicus</i>	Flora						0.355	0.282	0.253
	Paradise (A)							0.084	0.138
	Paradise (B)								0.194
	Troy								0.047**

^a Genetic diversity between locations as a proportion of total diversity, $G_{st} = (H_t - H_s)/H_t$ (31), where H_t = total genetic diversity over all subpopulations and H_s = genetic diversity within subpopulations. Null hypothesis of no differentiation between locations was tested assuming Hardy-Weinberg (H/W) disequilibrium, using Monte Carlo simulations as described in text. All G_{st} values are significant at $P < 0.001$ unless indicated by **, in which case $P < 0.01$.

regions and different hosts have reported similar levels of differentiation among groups of different taxa (2,36,40). The low level of similarity and high genetic distance between *T. bromi* populations (50%) on different *Bromus* spp. suggests that the *T. bromi* isolates sampled from *B. tectorum* and *B. japonicus* are two distinct taxa. Based on previous studies on the genetic structure of fungal populations, G_{st} values ranging from 0 to 0.2 are considered low to moderate levels of genetic differentiation among populations (1,13,27). Both gene diversity analysis and AMOVA showed moderate levels of genetic differentiation among *T. controversa* populations (G_{st} = 0.16 to 0.18; Φ_{st} = 0.16), whereas high levels of differentiation were detected among *T. bromi* populations (G_{st} = 0.62 to 0.75; Φ_{st} = 0.77). High levels of genetic differentiation also were detected in *T. bromi* populations on *B. tectorum* (G_{st} = 0.24 to 0.31; Φ_{st} = 0.35) and *B. japonicus* (G_{st} = 0.3 to 0.34; Φ_{st} = 0.37). *T. bromi* populations infecting *Bromus* species were much more diverse than *T. controversa* populations regardless of whether we assumed that the fungus was inbreeding or outbreeding. Genetic structure analysis in *Pseudocercospora herpotrichoides*, similarly, has shown significantly lower gene diversity in populations on barley compared with wild grass hosts (M. Hocart, personal communication).

Levels of genetic differentiation among populations of fungal pathogens have been used to make inferences about the biology of the pathogen. For example, moderate levels of genetic differentiation detected among populations of *Phytophthora infestans* and *Rhynchosporium secalis* were attributed to either the recent origin of these populations from a common source or to gene flow between established populations (13,14). High levels of genetic differentiation (G_{st} = 0.51) among populations of *Peridermium harknessii* were attributed to selection of pathogen populations on different hosts (43). High levels of genetic differentiation (G_{st} = 0.46) among populations of *Pyrenophora teres* from North America and Germany were attributed to genetic drift combined with restricted migration among subpopulations (35).

Dissimilar levels of genetic differentiation among populations of *T. controversa* and *T. bromi* could reflect differences in evolutionary forces shaping their populations in nature. For example, selection due to host genotype could be an important factor in the genetic structure of *T. controversa* and *T. bromi* because both fungi need to infect and sporulate in a susceptible host to survive in nature. The high level of differentiation among the 10 populations of *T. bromi* suggests that host specificity and restricted gene flow are responsible for the differentiation seen among these populations. In contrast, the moderate level of differentiation among *T. controversa* populations and the lack of private alleles suggest that gene flow is occurring or has occurred among the Pacific Northwest populations. The introduction of *T. controversa* from a common source also could explain the moderate differentiation among populations. However, it is likely that *T. controversa* was introduced several times in the Pacific Northwest from the mid-1800s to the beginning of the 1900s in the wheat or grasses grown by European settlers (8,29), potentially introducing new genetic variants each time. Another evolutionary factor could be that

differences in the diversity of host genotypes present among host populations have a large impact on the genetic structure of *T. controversa* and *T. bromi*. *Bromus* species are likely to be much more genetically diverse than wheat cultivars. The high levels of differentiation detected among the five *T. bromi* populations on *B. tectorum* and *B. japonicus* from the Pacific Northwest and among the three Oregon populations separately support this hypothesis. Studies of genetic variation in *B. tectorum* populations from North America showed substantial genetic differentiation among populations (G_{st} = 0.48; [33]) likely due to the multiple introductions of *Bromus* species grasses as a grain contaminant in different regions of the western United States (29,34). *B. tectorum* and *B. japonicus* are common weeds in cultivated and dry wasteland areas in the Pacific Northwest. *T. bromi* is widely distributed on both hosts and can be readily found in wheat, rotating crops, and noncultivated areas. In contrast, *T. controversa* populations are limited to winter wheat, which is commonly grown in 2-year rotations with fallow in the Pacific Northwest (5). The absence of wheat during alternate years reduces the opportunity for *T. controversa* isolates to reproduce and may limit the opportunity for selection. Hence, the evolutionary time of *T. controversa* populations may be half that of *T. bromi* in the same field, and evolutionary forces such as selection by host may have a larger impact on the genetic structure of *T. bromi* than on *T. controversa*.

Interspecific hybridization is another possible source of genetic variation in *Tilletia* species populations (6,20). In the wheat bunt species, morphological similarities between artificially produced hybrids and natural isolates and production of new pathotypes in the greenhouse were used as evidence to support the occurrence of hybridization in nature (9,20). Recently, we demonstrated that *T. controversa* and *T. bromi* from *B. tectorum* and *B. japonicus* hy-

TABLE 6. Pairwise comparisons of Nei's coefficient of genetic differentiation (G_{st}) values among *Tilletia controversa* populations^a

	Paradise (A)	Paradise (B)	Troy	Davenport-Miles
Flora	0.094	0.094	0.113	0.082
Paradise (A)		0.112	0.149	0.186
Paradise (B)			0.082	0.154
Troy				0.154

^a Genetic diversity between locations as a proportion of total diversity, $G_{st} = (H_t - H_s)/H_t$ (31), where H_t = total genetic diversity over all subpopulations and H_s = genetic diversity within subpopulations. Null hypothesis of no differentiation between locations was tested assuming Hardy-Weinberg (H/W) disequilibrium, using Monte Carlo simulations as described in text. All G_{st} values are significant at $P < 0.001$.

Fig. 3. Phenogram of *Tilletia controversa* and *T. bromi* populations from the Pacific Northwest based on Nei's unbiased genetic distances. A = collections of *T. controversa* from wheat; B and C = collections of *T. bromi* from *Bromus tectorum* and *B. japonicus*, respectively. Bootstrap values higher than 50% (based on 1,000 replications) are indicated on internal branches.

bridized in planta when mycelium of both species was inoculated in the coleoptile of wheat plants. However, the hybrid progeny had reduced fitness and were not able to reinfect wheat, indicating that these hybrids are probably not viable in nature (G. Pimentel, unpublished data). Our cluster and Southern analyses with RAPD products as probes showed no evidence of natural hybridization and introgression among sympatric populations. Perhaps the use of different molecular markers or screening of more populations could increase the probability of detecting hybridization between *T. controversa* and *T. bromi*. However, the rapid fusion of compatible basidiospores formed on the same basidium limits the outcrossing potential and likelihood of interspecific hybridization in these fungi. Hybridization could occur within host tissue if the established dikaryons dissociate after infection. However, host inoculation studies by Hoffmann and Meiners (18) and Meiners (30) showed that *T. bromi* does not infect wheat and that *T. controversa* does not infect any of the known hosts of *T. bromi*, except *B. carinatus*.

T. bromi isolates infecting *B. tectorum* and *B. japonicus* originally were described as *T. bromi-tectorum* Urries and *T. guyotiana* Har., respectively (6). Durán and Fischer (6) considered these species synonyms of *T. fusca* based on similarities in teliospore morphology, and Vánky (44) similarly recognized them as *T. bromi*. Recently, Boyd and Carris (2,3) combined host-specificity data, germination requirements, nuclear condition of primary basidiospores, and teliospore morphology to show that *T. fusca* and *T. bromi* are two distinct species. In addition, they suggested that *T. bromi* could be composed of more than one taxon based on the level of genetic similarity (55%) between isolates infecting different species of *Bromus* and differences in teliospore color (in mass) corresponding to host species. The high level of genetic differentiation observed in this study among *T. bromi* populations provides additional evidence to support the separation of *T. bromi* taxa based on their *Bromus* species hosts. Population structure analysis among three varieties of *Leptographium wagenieri* detected high levels of differentiation ($G_{st} = 0.86$) due to host specificity and supported their taxonomic designation as varieties (47). The taxonomic status of *T. bromi* on different grass hosts warrants further study of collections from outside the United States.

Although *T. controversa* and *T. bromi* coexist in nature and are reproductively compatible under artificial conditions, the results reported here support host specificity as a more important factor in shaping the genetic structure of these populations compared with natural hybridization and geographic isolation. Several greenhouse studies have demonstrated that *T. controversa* is reproductively compatible with *T. tritici* and *T. laevis* when coinoculated on wheat, and it has been assumed that hybridization can occur in nature (9). In addition, several studies have proposed classifying the three wheat bunt species as three varieties of a single species, in part because they are sexually compatible and their hybrid progeny result from normal meiotic events (24,37,38). Our study has shown that morphologically and biologically similar species of *Tilletia* can coexist in sympatric populations but are evolving independently on different grass hosts.

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