Electrophoretic Karyotypes of *Tilletia caries*, *T. controversa*, and Their F_1 Progeny: Further Evidence for Conspecific Status

Brian W. Russell[2] and Dallice Mills[1,2]

[1]Department of Botany and Plant Pathology and the [2]Genetics Program, Oregon State University, Corvallis 97331-2902 U.S.A.

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Electrophoretic karyotypes were obtained from intact sporidia and mycelia of *Tilletia controversa* and *T. caries*, and hybrid progeny were obtained by crossing these pathogens. The chromosomes typically ranged from approximately 850 to 4,490 kilobases (kb) for all strains, and they were variable in number with 19 or 20 for strains of *T. controversa*, 14–20 for *T. caries*, and from 19 to 22 for the hybrid progeny. The estimated genome size varied from 28 to 42 megabases (Mb) for these strains. Radiolabeled probes made of single copy DNA fragments and a heterologous actin gene identified four linkage groups among all strains that exhibited maximum chromosome length polymorphisms of 14% or less. The chromosomes carrying the rDNA genes, representing a fifth linkage group, exhibited length polymorphisms of approximately 40%. The actin gene and a rDNA probe hybridized with one or more bands in these strains, suggesting that some of the variability in chromosome number may result from aneuploidy. The karyotypes of the hybrid progeny revealed chromosome numbers and genome sizes essentially identical to each parental strain, clearly indicating that the reduction division stage of meiosis had occurred. These data and other corroborative genetic data provide substantial evidence that *T. controversa* and *T. caries* are not different species, but variants of a single species.

Additional keywords: CHEF, fungal taxonomy, smut fungi.

Tilletia controversa Kuhn and *T. caries* (DC) Tul are closely related filamentous basidiomycetes that cause dwarf and common bunt of wheat, respectively. Young (1935) recognized dwarf bunt disease as being distinct from common bunt, but he attributed the disease to a variant strain of *T. caries*. Subsequently, Conners (1954) identified the causal agent of dwarf bunt as *T. controversa*, a species distinct from other *Tilletia* spp. In recent years, dwarf bunt disease in the United States has achieved international importance because the People's Republic of China prohibits importation of wheat containing teliospores of *T. controversa* (Hoffmann 1982). Criteria presently used to distinguish between these pathogens include the optimum temperatures for teliospore germination, teliospore wall morphology, and disease symptoms, all of which are problematic because genetic variability within natural populations of these pathogens precludes using any single criterion for their identification (Fischer 1953; Holton and Kendrick 1956; Holton *et al.* 1968).

Molecular and genetic analyses generally have not proven more reliable in distinguishing *T. controversa* from *T. caries*. Monoclonal antibodies made to teliospore wall antigens of *T. controversa* react with teliospores of either pathogen (Banowetz *et al.* 1984), and phenol-soluble polypeptides extracted from the teliospore walls were shown to be indistinguishable (Kawchuk *et al.* 1988). The walls of mature teliospores of *T. controversa* autofluoresce, whereas the teliospores of *T. caries* generally do not (Stockwell and Trione 1986), but exceptions have been noted (Russell and Mills 1992). *T. controversa* and *T. caries* strains are sexually compatible (Holton and Kendrick 1956; Silbernagel 1964), and the hybrid progeny do not contain a complement of chromosomes from each parent, as expected if the parental strains were different species (Russell and Mills 1991). Furthermore, the mating-type genes and a single gene for cycloheximide resistance were shown in a hybrid cross to assort independently among F_1 basidiospores as predicted from inheritance of unlinked genes (Trail and Mills 1990). Numerous physiologic races of both pathogens have been described that follow gene-for-gene interactions (Flor 1953), and the interaction of a single plant resistance gene can confer incompatibility with races of either pathogen (Hoffmann and Metzger 1976).

The ability to generate electrophoretic karyotypes is seen as a useful addendum to existing criteria for the proper taxonomic classification of some fungi. For example, the greatly dissimilar karyotypes of the closely related yeasts *Kluyveromyces marxianus* var. *marxianus* and *K. m.* var. *lactis* (Steensma *et al.* 1988; Sor and Fukuhara 1989) were used as supporting evidence that these organisms are different species. Furthermore, the highly virulent and weakly virulent isolates of *Leptosphaeria maculans*, which also fail to mate, have very dissimilar molecular karyotypes, and it has been argued that they are different species (Taylor *et al.* 1991).

Because genetic, physiological, and morphological criteria have failed to distinguish *T. caries* from *T. controversa* at the species level, the goal of this research was to obtain

Corresponding author: D. Mills.

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molecular karyotypes of these pathogens and their hybrid progeny that would provide useful information for their taxonomic classification. The karyotypes, genome sizes, and assignment of cloned restriction fragments and genes to linkage groups support the hypothesis that these pathogens are variants of a single species.

RESULTS

Molecular karyotypes and genome sizes of *T. controversa* and *T. caries*.

The chromosome-sized DNAs obtained from intact cells, which henceforth will be referred to as chromosomes of *T. caries* and *T. controversa*, were resolved using several electrophoretic parameters (Fig. 1). Each strain used in this study had a karyotype that was reproducible from different preparations and that was unique relative to all other strains (Table 1). The number of chromosome bands varied from 13 to 17, and from 14 to 18, respectively, for *T. caries* and *T. controversa*. The chromosomes of all strains ranged in size from approximately 850 kilobase pairs (kb) to 4,490 kb, except for strain tc3060 of *T. caries* which had two small chromosomes approximately 300 kb in size (Fig. 1 and Table 1). Bands that appeared brighter than others of similar size (Fig. 1) were determined by

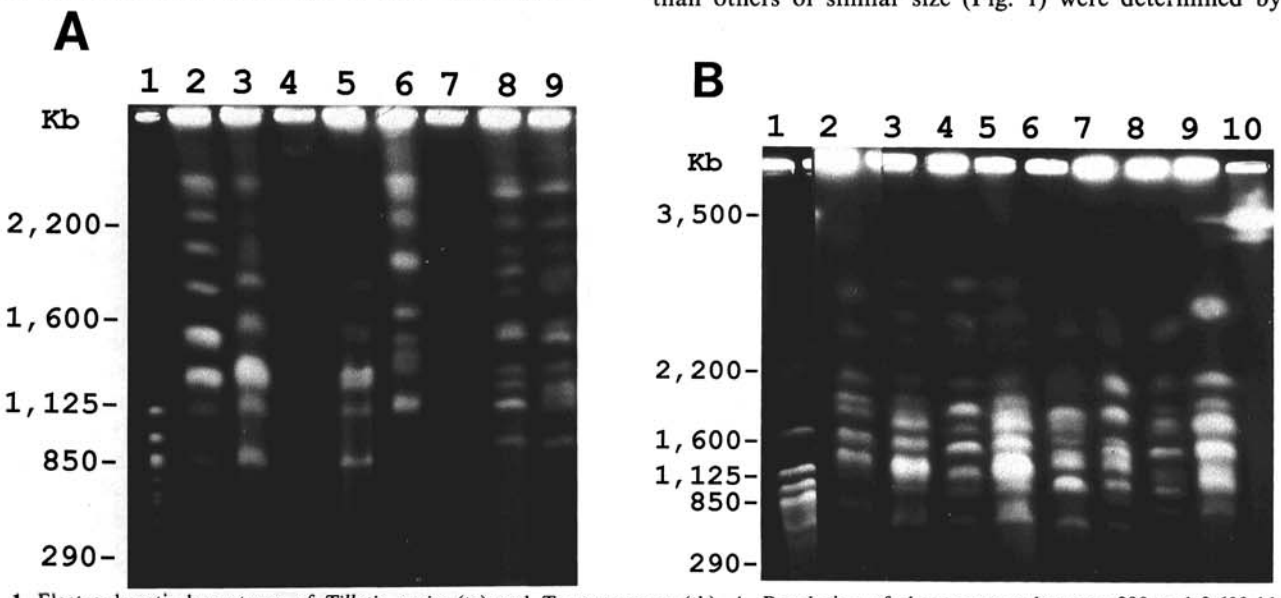

Fig. 1. Electrophoretic karyotypes of *Tilletia caries* (tc) and *T. controversa* (tk). **A,** Resolution of chromosomes between 290 and 2,600 kb. **B,** Resolution of chromosomes between 2,200 and 5,000 kb. Lanes 2–5, strains tc3060, tc120, tc960, and tc920, respectively; lanes 6–9, strains tk660, tk880, tk310, and tk220, respectively. Lanes 1 and 10, molecular size markers of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* chromosomes, respectively.

Table 1. Estimated sizes, number of chromosomes, and genome sizes of four stains each of *Tilletia caries* and *T. controversa*

Band number	T. caries				T. controversa			
	tc3060	tc120	tc960	tc920	tk660	tk810	tk310	tk220
18					3,600			
17	3,960[a]	3,880		4,490	3,340			
16	3,370	3,690		3,670	2,890	3,800		
15	3,060	3,090		3,090	2,650[b]	3,480		4,490
14	2,720	2,780		2,780	2,360	2,890	4,000	2,990[b]
13	2,190	2,530	4,050	2,530	2,230	2,650[b]	3,290	2,260[b]
12	2,020	2,150	3,140	2,150	2,160	2,290	2,700[b]	2,030
11	1,800	2,040	2,810	2,040	1,960[b]	2,170	2,170[b]	1,880
10	1,560	1,980	2,550	1,980	1,930	2,070[b]	2,030	1,810
9	1,500[b]	1,860[b]	2,140	1,860[b]	1,860	1,920	1,910	1,640
8	1,340	1,650	2,000[b]	1,650	1,700	1,720	1,790	1,580
7	1,280[b]	1,610	1,820	1,610	1,550	1,600[b]	1,620	1,560[b]
6	1,180	1,390[b]	1,610	1,390[b]	1,480	1,500[b]	1,580[b]	1,400
5	1,160	1,330[b]	1,560	1,330[b]	1,410	1,380	1,390	1,320
4	900	1,340	1,340	1,190	1,240	1,280	1,300	1,270
3	880	1,280	1,280	1,170	1,180	1,240	1,200[b]	1,210
2	300	1,140	1,140	870	870	1,110	1,020	1,040
1	290	870	870	850	850	860	1,000	1,020
Chromosome no.[c]	19	20	14	20	20	20	20	19
Totals (Mb)[d]	32.25	38.70	28.30	39.23	39.87	39.88	36.15	34.31

[a] Band sizes are expressed in kilobase pairs.
[b] Bands are presumed to be doublets and to contain twice the amount of DNA.
[c] Values represent the estimated number of chromosomes in each strain.
[d] Estimated genome size in megabase pairs (Mb) of each strain.

densitometric tracing to contain at least twice the amount of DNA, and they are listed as doublets in Table 1. Because the doublet bands contained an additional chromosome, the estimated minimum number of chromosomes ranged from 14 to 20 among the four strains of *T. caries*, and was either 19 or 20 for *T. controversa*. The genome sizes for these strains, which varied from about 28 to 40 megabase pairs (Mb) (Table 1), were estimated by summing the individual bands, and assuming twofold values for doublet bands.

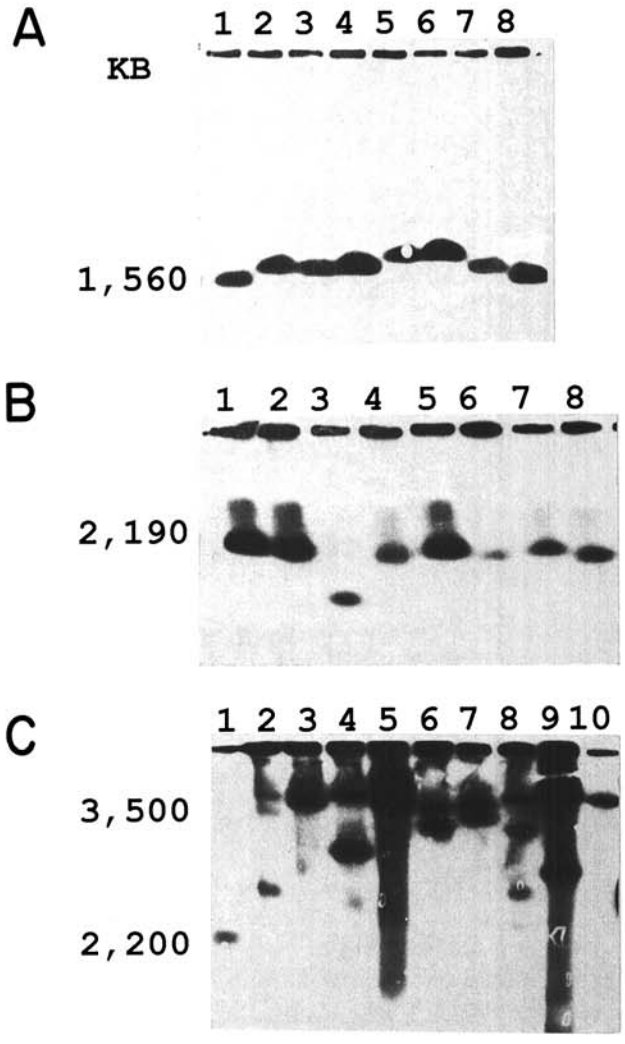

Fig. 2. Assignment of DNA fragments to linkage groups of *Tilletia caries* (tc) and *T. controversa* (tk). Chromosomes fractionated by CHEF PFGE were blotted and hybridized with probes made of the, **A,** 2.5-kb *Eco*RI fragment from pOSU1003, and, **B,** the 0.5-kb *Eco*RI fragment from pOSU1006. Lanes 1–4, strains tc3060, tc120, tc960, and tc920, respectively; lanes 5–8, strains tk660, tk880, tk310, and tk220, respectively. **C,** Hybridization of probe made of the 4.5-kb *Eco*RI fragment from *T. caries* (pOSU1101) that has homology with *Neurospora crassa* 17S rDNA (pRW612). Lanes 2–5, strains tc3060, tc120, tc960, and tc920, respectively; lanes 6–9, strains tk660, tk880, tk310, and tk220, respectively. Lanes 1 and 10, molecular size markers of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* chromosomes, respectively.

Karyotypic relatedness and analysis of linkage groups by Southern hybridization.

Southern-blots of CHEF-separated chromosomes were probed with restriction fragments from either pathogen and with conserved heterologous genes to examine the amount of chromosome heterogeneity among the strains. DNA probes made from the inserts in pOSU1003, pOSU1006 (Fig. 2A,B and Table 2), and pOSU1105 (Table 2) hybridized to a single chromosome band in all eight strains of *T. controversa* and *T. caries*. The maximum length polymorphisms for these chromosomes was less than 10% (Table 2).

The inserts in pOSU1001 and pOSU1004 contain highly repeated sequences, and only one to five chromosome bands among these strains failed to hybridize to these probes (Table 2). Moreover, the intensity of hybridization was different for single bands in several strains, suggesting that the copy number of these repeated sequences may vary for some chromosomes (data not shown). An internal 1-kb fragment from the 17S *Neurospora crassa* rDNA repeat cloned in pRW612 (P. J. Russell, personal communication) has homology with the 1.4-kb *Eco*RI fragment cloned in pOSU1101 and produces the same hybridization pattern. The insert from pOSU1101 hybridized with the

Table 2. Number of bands, average size, and maximum variability of chromosomes of *Tilletia caries*, *T. controversa*, and F_1 progeny identified by Southern hybridization with homologous and heterologous DNA probes

	Average chromosome length and maximum variability								
	T. caries			*T. controversa*			F_1 Progeny		
Probe	No. bands identified	Chromosome length[a]	Maximum variability[b]	No. bands identified	Chromosome length[a]	Maximum variability[b]	No. bands identified	Chromosome length[a]	Maximum variability[b]
pOSU1003	1	1,618	90 (5)	1	1,650	140 (8)	1	1,730	70 (4)
pOSU1006	1	2,122	190 (9)	1	2,158	40 (2)	1	2,170	120 (5)
pOSU1105	1	1,365	50 (4)	1	1,395	30 (2)	1	1,412	120 (8)
pOSU1101	2–3	3,663	1,770 (39)	2–3	3,521	1,790 (40)	1–3	3,372	1,290 (35)
pSF8	1–2	1,160	50 (4)	1–2	1,188	130 (10)	1	1,144	180 (14)
pOSU1001	12–15	NA[c]	NA	11–15	NA	NA	NA	NA	NA
pOSU1004	13–16	NA	NA	12–16	NA	NA	14–17	NA	NA
pOSU1008	0[d]	NA	NA	0[d]	NA	NA	NA	NA	NA

[a] The average size of chromosomes in kilobase pairs identified by the probe.
[b] Variability is expressed in kilobase pairs (kb); numbers in parentheses represent maximum percent variability (calculated by dividing the difference between the largest and the smallest chromosomes identified and the average of those chromosomes identified by the probe).
[c] Not applicable or experiment not performed.
[d] pOSU1008 is a mitochondrial DNA probe that hybridized to DNA in the wells and to a diffuse region approximately 100–200 kb in size.

three largest chromosomes of tc3060 and tk310, and with the two largest chromosomes in all other strains (Fig. 2C and Table 2). Band 17 of strain tc920 and band 14 of strain tk310, which have homology with these probes, have length polymorphisms of approximately 1,770 kb (Table 2).

The single-copy actin gene of *Aspergillus nidulans* (pSF8) hybridized to two chromosomes of similar size (about 1,160 kb) in some strains of both pathogens and to a single chromosome in other strains. The maximum length variability of these chromosomes did not exceed 10% (Table 2).

To determine whether any of the bands were of mitochondrial origin, an *Eco*RI fragment (pOSU1008) from the mitochondrial genome of *T. caries* was probed to a CHEF blot. This fragment hybridized intensely with DNA in all of the sample wells (Table 2), and with a diffuse band below the smallest chromosome band in each strain.

Molecular karyotypes of F_1 hybrid progeny.

The karyotypes of five F_1 progeny from a cross of *T. controversa* × *T. caries* were unique with respect to each other and the parental strains (Fig. 3). The number of bands varied from 15 to 17 among the progeny, and with consideration of unresolved doublet bands, the number of chromosomes in these strains ranged from 19 to 22, whereas each parental strain had 20 (Fig. 3 and Table 3). The chromosomes of the progeny varied in length from approximately 830 to 3,830 kb, and the genome sizes ranged from approximately 37 to 42 Mb, which is approximately the size (39 Mb) of the parental genomes (Table 3).

Analysis of linkage groups in hybrid progeny.

The single-copy DNA probes previously used to identify unique linkage groups among strains of both pathogens were also used in similar analyses of the hybrid progeny. Each of the inserts of pOSU1003 (Fig. 4A and Table 2), pOSU1006 and pOSU1105 (Table 2), could be assigned to a single chromosome that was similar in size to the parental chromosome, although length polymorphisms of 4–8% were observed (Fig. 4A and Table 2). The single copy *A. nidulans* actin gene hybridized with two chromosomes ranging from 1,180 to 1,240 kb in each of the parental strains (data not shown), but with only a single chromosome of similar size in the progeny (Table 2). Although the probe hybridized with doublet bands in strains f120 and f1260, the signal was not greater than that observed for single bands, suggesting that these doublet bands were comprised of nonhomologous chromosomes.

A probe made of the ribosomal rDNA repeat hybridized with two chromosomes in both parental strains, but with one to three chromosomes in the progeny (Fig. 4B and Table 2). The karyotypes obtained from the same or different chromosome preparations have been consistently reproducible by CHEF PFGE. Hence, if any chromosome is running anomolously, its migration though the gel is repeatable. If it is assumed the rDNA probe hybridized with homologous chromosomes rather than with genes translocated to nonhomologous chromosomes, maximum length polymorphisms of 1,290, 1,770, and 1,790 kb exist among the progeny and the parental chromosomes, respectively (Table 2).

DISCUSSION

A procedure that utilizes intact fungal mycelia and sporidia rather than protoplasts (McCluskey *et al.* 1991) provided excellent material for producing electrophoretic karyotypes of strains of *T. caries*, *T. controversa*, and their hybrid progeny. Although the karyotypes of both pathogens differed somewhat, the karyotypic profiles were similar among all strains, including the hybrid progeny, suggesting that the chromosomes of both pathogens are

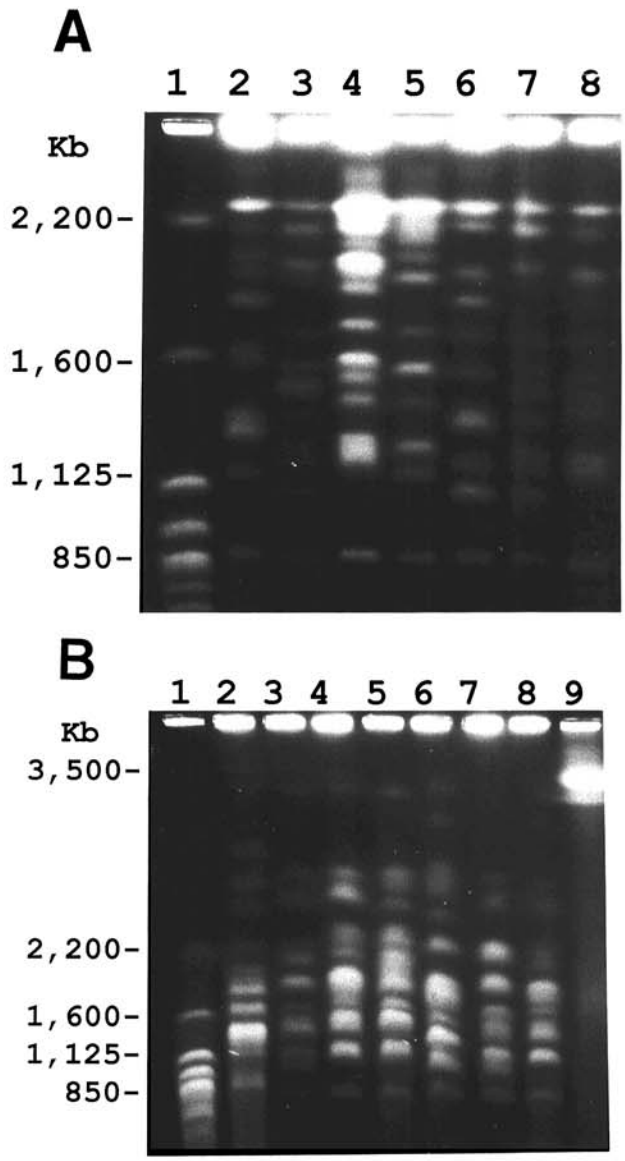

Fig. 3. Electrophoretic karyotypes of *Tilletia caries* (tc) and *T. controversa* (tk), parental strains and F_1 hybrid progeny. **A,** Resolution of chromosomes between 830 and 2,600 kb. **B,** Resolution of chromosomes between 2,200 and 5,000 kb. Lanes 3–7, progeny strains f30, f40, f80, f120, and f1260, respectively; lanes 2 and 8 parental strains tc920 and tk660, respectively. Lanes 1 and 9, molecular size markers of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* chromosomes, respectively.

Table 3. Estimated sizes, number of chromosomes, and the genome sizes of parental strains of *Tilletia caries*, *T. controversa*, and five F_1 progeny

Band number	*T. caries* tc920	F_1 progeny f30	f40	f80	f120	f1260	*T. controversa* tk660
18							3,600
17	4,490[a]		3,740				3,340
16	3,670	3,830	3,610			3,640	2,890
15	3,090	3,640	2,870	3,610	3,640	3,430	2,650[b]
14	2,780	2,810	2,660[b]	2,870	3,300	2,840	2,360
13	2,530	2,660[b]	2,310	2,750	2,930	2,590[b]	2,230
12	2,150	2,200	2,260	2,560	2,780[b]	2,140[b]	2,160
11	2,040	2,140[b]	2,160[b]	2,310	2,450	2,010[b]	1,960[b]
10	1,980	2,010[b]	2,030[b]	2,260	2,150	1,990	1,930
9	1,860[b]	1,720	1,920	2,170[b]	1,970	1,700	1,860
8	1,650	1,690	1,770	2,040	1,850[b]	1,580	1,700
7	1,610	1,600	1,640[b]	1,960	1,710	1,490	1,550
6	1,390[b]	1,510[b]	1,560	1,750	1,560	1,380	1,480
5	1,330[b]	1,380	1,480	1,600[b]	1,400	1,250	1,410
4	1,190	1,270	1,320	1,460	1,360	1,190	1,240
3	1,170	1,210	1,270	1,280[b]	1,200	1,080[b]	1,180
2	870	1,100	1,240	1,180	1,090[b]	1,070	870
1	850	830	850[b]	840[b]	830[b]	830[b]	850
Chromosome no.[c]	20	20	21	19	19	22	20
Totals (Mb)[d]	39.23	39.92	41.87	36.53	36.77	38.86	39.87

[a] Band sizes are expressed in kilobase pairs.
[b] Bands are presumed to be doublets and to contain twice the amount of DNA.
[c] Values represent the estimated number of chromosomes in each strain.
[d] Estimated genome size in megabase pairs (Mb) of each strain.

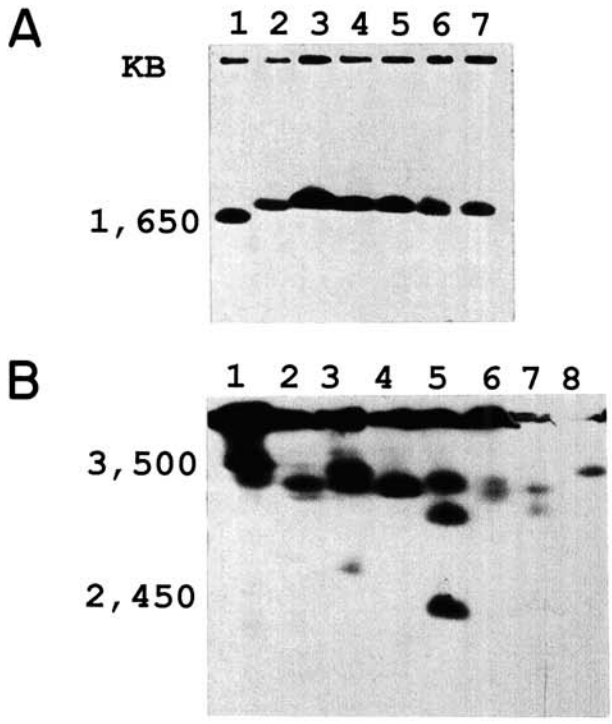

Fig. 4. Analysis of the number and size of chromosomes in the F_1 progeny that show homology with selected DNA probes. The progeny and parental chromosomes were blotted and hybridized with probe made of the **A**, 2.5-kb *Eco*RI fragment from pOSU1003, and, **B**, the 4.5-kb *Eco*RI fragment from pOSU1101, which has homology with *Neurospora crassa* rDNA. For both panels, lanes 2–6, progeny strains f30, f40, f80, f120, and f1260, respectively; lane 1 (tc920) and lane 7 (tk660) parental strains; Lane 8 (B only), molecular size marker of *Schizosaccharomyces pombe* chromosomes.

homologous. The karyotypic variability appears to be attributable to chromosome length polymorphisms and extra chromosome bands thought to result because of aneuploidy. Conversely, the karyotypes of different species of *Ustilago* (Skinner *et al.* 1991; K. McCluskey and D. Mills, unpublished data) and the weakly and highly virulent strains of *L. maculans,* which have recently been proposed to be different species (Taylor 1991), have chromosomes that differ greatly in relative size and number.

That the chromosomes of these pathogens are homologous is further supported by the observation that length polymorphisms among chromosomes representing four linkage groups varied by less than 10% among all field isolates. However, a fifth linkage group carrying the highly repeated rDNA genes varied by 40%. Excluding the chromosomes that carry the rDNA genes, chromosome length polymorphisms of approximately 10% have been observed among unrelated field isolates of *U. hordei* (McCluskey and Mills 1990) and *Septoria tritici* (McDonald and Martinez 1991). Hence, the karyotypic pattern determined by the number and sizes of the chromosomes, the length polymorphisms of four linkage groups analyzed and relative genome sizes could not be used as criteria for distinguishing strains of *T. caries* from *T. controversa.*

A striking feature of the karyotypes of these presumptive haploid strains was the variation in number of chromosome bands, which ultimately influences the genome size. Strain tc960 of *T. caries* with only 14 chromosomes and the smallest genome (28.3 Mb), has six fewer chromosomes and 11 Mb less DNA than observed in two other strains of *T. caries* (Table 1). Strain tc960 is prototrophic, and its growth rate is not reduced relative to other strains, suggesting that its chromosome numbers may be more

representative of the haplophase of *T. caries* and *T. controversa*. Variation in the number of chromosome bands has been reported for several fungi (for reviews, see Mills and McCluskey 1990; Skinner *et al.* 1991), and aneuploid strains may be common among field isolates (Tolmsoff 1983).

Using microfluorometry, the haploid genomes of *T. controversa* and *T. caries* were estimated to be approximately 115 and 100 Mb, respectively (Duran and Gray 1989). However, estimates of the genome sizes of *T. controversa* (34–40 Mb), of *T. caries* (28–39 Mb), and of the five hybrid progeny (37–42 Mb), from electrophoretic karyotypes, were much smaller, and the reason for the large discrepancy is unknown. The values reported here were in general agreement with the estimated genome size of the smut fungus *U. hordei* (20–26 Mb) (McCluskey and Mills 1990).

Genomic variability inherent in strains of *T. caries* (11 Mb), *T. controversa* (6 Mb), and the hybrid progeny (5 Mb) appears too large to be caused entirely by duplications and deletions. That some of the variability could result from aneuploidy is supported by probing CHEF blots with the rDNA probes and the *A. nidulans* actin gene. The rDNA probe hybridized with either two or three chromosomes in *T. caries* strains, one or two in *T. controversa* strains and one to three bands in the hybrid progeny. If the multiple bands that hybridized with the rDNA probe are homologous chromosomes rather than chromosomes with translocations, the additional copies of this large chromosome (3.1 Mb average size) could account for 3–6 Mb of additional DNA in strains with one or two extra copies.

The actin gene hybridized with either one or two chromosomes in the field isolates of both pathogens. Both parents of the hybrid cross had two bands that hybridized with the probe, whereas the progeny had only a single band. These bands varied by less than 10% among field isolates and less than 15% among the progeny, suggesting that the extra bands are homologous chromosomes. Translocation and transposition of single-copy genes could account for bands of different sizes hybridizing to a single-copy probe, although these events would not be expected to occur only between chromosomes of nearly identical size. Moreover, translocation and transposition events alone could not contribute to the variable number of chromosome bands observed in the karyotypes of these strains.

Some chromosomes have been observed to run anomolously in OFAGE gels (Carle and Olson 1985), which could affect the size of the genome. However, this problem appears to have been eliminated by CHEF gel electrophoresis because the karyotypes presented in Figures 1 and 3 were reproducible using either fresh material made from intact cells and protoplasts, or plugs stored for periods up to 12 mo. A complete linkage map derived by both molecular probing and genetic crosses will be essential to ascertain the ploidy level of these strains and the correct karyotype for the haplophase of these pathogens.

The origin of the two atypically small chromosomes in tc3060 (Table 1) is uncertain, and their homologs have not been identified in other strains. Strain tc3060 was obtained from a collection of teliospores (designated race 30) resulting from a cross of two unrelated strains representing races 23 and 27 of *T. caries* (Metzger and Hoffman 1978). *T. caries* and *T. controversa* strains are highly inbred because matings typically occur between sexually compatible, primary sporidia within the whorl of a germinated teliospore (Buller and Vanterpool 1933). Therefore, crosses of highly inbred strains of different geographic origins could produce atypical, nonparental, recombinant chromosomes if the homologs have large length polymorphisms

Table 4. Strains and plasmids used in this study

Designation	Relevant characteristics	Source or reference
Tilletia caries		
Monokaryotic strains		
tc120	Race 1	Hoffman and Metzger 1976
tc920	Race 9	Hoffman and Metzger 1976
tc960	Race 9	Hoffman and Metzger 1976
tc3060	Race 30	Metzger and Hoffman 1978
T. controversa		
Monokaryotic strains		
tk220	Race unknown	Utah
tk310	Race unknown	Montana
tk660	Race unknown	Montana
tk810	Race unknown	Montana
F_1 progeny		
f30, f40, f80, f120, f1260	Monokaryotic strains obtained from crossing tk660 × tc920	Trail and Mills 1990
Plasmids		
pSF8	*Aspergillus nidulans* actin gene	Fidel *et al.* 1988
pRW612	*Neurospora crassa* rDNA genes	P. J. Russell[a]
pOSU1001	4.5-kb *Eco*RI *T. caries* fragment	This study
pOSU1003	2.5-kb *Eco*RI-*Pst*1 *T. caries* fragment	This study
pOSU1004	0.2-kb *Eco*RI *T. caries* fragment	This study
pOSU1006	0.5-kb *Eco*RI *T. caries* fragment	This study
pOSU1008	5.5-kb *Eco*RI fragment *T. caries* mitochondria	This study
pOSU1101	4.5-kb *Eco*RI *T. controversa* fragment	This study
pOSU1105	2.8-kb *Eco*RI *T. controversa* fragment	This study

[a] Reed College, Portland, OR.

resulting from gross rearrangements (e.g., translocations, deletions, duplications, and insertions). In *S. cerevisiae*, homologous chromosomes with a length polymorphism produced recombinant chromosomes that differed in size from either of the parental chromosomes, and the polymorphism which segregated 1:1 has been mapped (Ono and Ishino-Arao 1988).

The karyotypes of the five hybrid progeny provided additional evidence that parental strains tk660 of *T. controversa* and tc920 of *T. caries*, each of which have 20 chromosomes, are conspecific. The chromosome numbers of their progeny (19–22) indicated that the reduction division stage of meiosis had occurred. Furthermore, the single-copy probes hybridized with chromosomes that were of virtually identical size in the progeny and parental strains. In contrast, progeny of a cross of *K. m.* var. *marxianus* and *K. m.* var. *lactis*, which are proposed to be separate species (Steensma *et al.* 1988), exhibited no genetic exchange and produced progeny karyotypes that were the sum of the parental strains.

Presently, the classification of *T. caries* and *T. controversa* as separate species heavily relies upon the germination properties and wall morphology of teliospores (Fischer 1953). Unfortunately, there has been no clear way to correlate spore morphology with biological relationships (Duran and Fischer 1961), as morphological characteristics may be determined by only one, or a few, genes (Huang and Nielsen 1984). In discussing their classification of *Tilletia*, Duran and Fischer (1961) proposed a morphologic species concept, "in lieu of the more precise but as yet unavailable concept based on genetics." The earlier limitations of classical genetic analyses of members of the *Tilletia* genus are rapidly dissipating, and with recent advances in molecular genetics, more appropriate genetic criteria must be used in the taxonomic classification of these pathogens (for a Discussion, see Tolmsoff 1983).

The biological species concept has been defined as Mendelian populations in which gene exchange is limited or prevented by reproductive isolation, and organisms that produce recombinant offspring are included as one species (Dobzhansky 1976). Our results support the conclusion of Young (1935) who recognized dwarf bunt disease as being different from common bunt disease, but caused by a variant form of *T. caries*. Mutant alleles of key genes that control phenotypic traits such as teliospore morphology and germination could play a major role in criteria currently used for classification of these and other species of *Tilletia*. The molecular karyotypes of *T. controversa*, *T. caries*, and their hybrid progeny, together with preliminary linkage group data strongly argue that these pathogens are not different species. Previous genetic analyses (Trail and Mills 1990), and biochemical evidence (Kawchuk *et al.* 1988) also corroborate these conclusions.

MATERIALS AND METHODS

Strains, culture conditions, and plasmids.

Collections of teliospores of races 1, 9, and 30 of *T. caries*, and unknown races of *T. controversa* isolated from different geographic areas, were generously provided by W. Kronstad, Dept. Crop and Soil Science, Oregon State University, Corvallis, or previously described (Table 4). Hybrid progeny obtained by crossing strains of *T. controversa* and *T. caries* have been described (Trail and Mills 1990). Presumptive haploid strains used in this study (Table 4) were isolated from germinating teliospores as primary or secondary monokaryotic sporidia. Cultures were grown on agar-solidified or liquid T-19 minimal medium (T-19 MM) and stored as previously described (Mills and Churchill 1988).

The plasmids used in this study (Table 4) were maintained in *Escherichia coli* DH5α (Bethesda Research Laboratories, Gaithersberg, MD), which was grown on Luria-Bertani medium (Maniatis *et al.* 1982) containing 100 μg/ml of ampicillin.

Preparation of samples for contour-clamped homogeneous field pulsed-field gel electrophoresis (CHEF PFGE).

Karyotypes were typically obtained from intact cells by culturing strains of *T. caries*, *T. controversa*, and F_1 progeny as described by McCluskey *et al.* (1991) with minor modifications. The homogenizing buffer was amended with 0.2 mM aurintricarboxylic acid (Sigma, St. Louis, MO) and, if necessary, the mycelia were briefly ground in a Pyrex tissue homogenizer to disaggregate mycelial mats. The digestion buffer also contained 1.5% sodium dodecyl sulfate (SDS) and the plugs were incubated at temperatures from 50° to 65° C, with the addition of fresh solution after 12 hr. Samples made from protoplasts for CHEF PFGE were prepared as previously described (McCluskey *et al.* 1990). The plugs were stored in 0.5 M EDTA (pH 8.0) at 4° C.

CHEF electrophoresis conditions.

The CHEF DR-II electrophoresis system (Bio-Rad Laboratories, Richmond, CA) was used for separation of chromosome-sized DNA molecules in ultrapure agarose (IBI, New Haven, CT). The DNAs were electrophoresed in 0.5× Tris-borate EDTA buffer (TBE) (Maniatis *et al.* 1982) with the buffer temperature maintained between 12° and 15° C. Chromosomes less than 2,000 kb in size were resolved in 1% agarose gels using an initial 16-hr period of 480-sec pulses at 100 V (3 V/cm), followed by 22 hr of ramped pulses from 480 to 240 sec at 100 V, and ending with 23 hr of ramped pulses from 240 to 120 sec at 150 V (4.5 V/cm). Chromosomes larger than 2,000 kb were resolved in 0.9% agarose gels using 96 hr of ramped pulses from 900 to 480 sec, beginning at 75 V (2.25 V/cm), with increases of 5 V at 24-hr intervals. Various other parameters were used to target DNA of a specific size range to resolve some bands assumed to be doublets. The bands were stained with ethidium bromide (3 μg/ml) in 0.5× TBE for 30 min and photographed after destaining overnight in 0.5× TBE. Photographic negatives of karyotypes with putative doublet bands were traced using an Ultrascan laser densitometer (LKB Bramma, Sweden) and analyzed with VCRB 2400 Gel Scan XL software. The lengths of the chromosome-sized DNAs were estimated using Cricket Graph (Cricket Graphics, Inc., Philadelphia, NJ) to configure a standard curve derived from migration distances of molecular size markers that included chromosomes of *Saccharomyces cerevisiae* and *Schizosaccharomyces*

pombe purchased from Bio-Rad Laboratories.

Sources of DNA probes.

Total genomic DNA was isolated from *T. caries* and *T. controversa* by the genomic mini-prep method of Lee *et al.* (1988), and recombinant plasmids were extracted using a modified method of Holmes and Quigley (1981), in which ammonium acetate was substituted for sodium acetate to precipitate DNA. Genomic *Eco*RI fragments were cloned into pUC18 or pUC19 (Yanisch-Perron *et al.* 1985) and used to transform *E. coli* DH5α (Maniatis *et al.* 1982). Cloned fragments and heterologous genes released from vectors by digestion with appropriate restriction enzymes were purified from agarose gels using Elutip-d (Schleicher & Schuell, Kleene, NH) following instructions provided by the manufacturer. The random priming technique of Feinberg and Voglestein (1983) was used to make radiolabeled DNA probes.

Isolation of mitochondrial DNA.

Mitochondrial DNA was isolated from *T. caries* strain tc920 using a modified procedure of Specht *et al.* (1983), in which total genomic DNA was subjected to CsCl density gradient centrifugation in the presence of bisbenzimide (Sigma). Each gradient contained 400–600 μg of DNA, 1.68 g/ml CsCl, and 120 μg/ml of bisbenzimide. The gradients were centrifuged at 50,000 rpm for 24 hr at 20° C in a Beckman 55 Ti rotor. The mitochondrial band was extracted and further purified by an additional centrifugation in CsCl without bisbenzimide.

DNA hybridizations.

CHEF-resolved chromosomes were nicked with ultraviolet light and blotted onto Genetran nylon membranes (Genetran, Plasco, Woburn, MA) using 0.4 N NaOH to denature and transfer the DNA. The blots were baked at 80° C for 2 hr, and hybridization reactions with homologous fragments were carried out in 50% formamide as described previously (Orbach *et al.* 1988). For heterologous probes the blots were prehybridized at 42° C for 12–22 hr in 20 ml of 3× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$) containing 30% formamide, 5× Denhardt's solution, 0.2% SDS, and 100 μg/ml of salmon sperm DNA. Hybridizations were then performed at 42° C for 12–22 hr in 10 ml of the prehybridization solution with 0.5% dextran sulfate and labeled probe DNA replacing the salmon sperm DNA. The membranes were initially washed at room temperature for 30 min with 3× SSPE containing 0.2% SDS, then at 42° C for 15–30 min with 1× SSPE (0.2% SDS), and finally with 0.5× SSPE (0.1% SDS). The membranes were blotted dry and exposed to Kodak X-Omat film. The blots were stripped in boiling 0.1× SSPE (0.1% SDS) and cooled to room temperature.

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