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Inbreeding of the avirulent physiologic race 8 strains of *Ustilago hordei* was purported to have increased its pathogenicity in a stepwise manner that led to a highly pathogenic race, designated race 14. The analysis of electrophoretic karyotypes and restriction fragment length polymorphism profiles detected with a telomere-specific probe (TelomereRFLP) in races 8 and 14, and progeny obtained by selfing race 8 strains, revealed substantially changed patterns in the purported progeny of the second generation of selfing, and the race 14 strain. The telomereRFLP patterns in strains purported to be the progeny of the second generation of inbreeding of race 8, were unlike both race 8 and race 14 strains, and identical to those of *U. hordei* physiologic races 10 and 13. These data suggest that the progeny believed to have been derived from the second selfing of race 8 strains were clonal lineages from either race 10 or race 13 strains, rather than the

products of meiosis of race 8 teliospores. The electrophoretic karyotypes resolved from race 8 and 14, revealed chromosome-length polymorphisms (CLPs) that were similar in magnitude to those reported among strains of the fourteen physiologic races of *U. hordei*, rejecting the postulate that race 14 is a lineage derivative from race 8.

Electrophoretic karyotyping and ribosomal DNA (rDNA) sequence analysis of eight *Ustilago* species revealed that the four sporidium-forming species, *U. avenae, U. hordei, U. kolleri,* and *U. nigra,* form a coherent group. Ten probes detected a maximum of 15% CLPs among the putative homologous chromosomes of theses species, and the internal transcribed spacer (ITS) amplified from these species shared 97-99% sequence identity. The taxonomic distinctness of *U. maydis* from the rest of the smut fungi was evidenced by its divergence at 7 nucleotides in the 5.8S rDNA coding region.

Molecular Fingerprinting, rDNA Internal Transcribed Spacer Sequence, and Karyotype Analysis of *Ustilago hordei* and Related Smut Fungi

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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CHAPTER I

INTRODUCTION

General introduction

Fungi have provided geneticists with novel genetic systems and the opportunity to study practical problems with greater ease and precision than is possible with other organisms (Burnett, 1975). Plant pathologists, plant breeders, and industrialists have utilized knowledge of fungal genetics to either prevent or utilize the activities of fungi. Phytopathogenic fungi engage in a wide variety of interactions with plants, ranging from those that are highly mutualistic, through benign forms of parasitisms, to economically devastating crop diseases. To better exploit or guard against these intriguing interactions it is necessary to understand the biochemical mechanisms controlling both their intimacy and specificity.

The smut fungi are a large group of plant-pathogenic fungi that belong to the order Ustilaginales of the Basidiomycetes (Fischer and Holton, 1957; Agrios, 1988). They attack cereal crops and wild grasses, and have been known to humanity since ancient times. Woolman and Humphrey (1924) believed that stinking smut of wheat was known to Theophrastus, Vergil, Pliny, and other ancient Romans. At the beginning of the twentieth century cereal crops represented 73 percent of total food consumed by mankind

(Stevens and Stevens 1952), making any phytopathogen that attacks these crops an immediate target for scrutiny and efforts to limit its disease incidence. A wealth of information has been collected about smut epidemiology and the yield losses they engender (Fischer and Holton 1957). The myriad of investigations were confined primarily to the economically important smuts, i.e., *Ustilago maydis* (corn smut disease), *U. nuda* (loose smut of barley), *U. avenae* (loose smut of oats), *U. tritici* (loose smut of wheat), and *U. hordei* (covered smut of barley). The pathogenic reactions of these fungi were regarded as a particular form of interaction between two organisms, the fungus and its host.

General aspects of the life cycle of *Ustilago* species

The genus *Ustilago* comprises approximately 200 species that share basic features of their life cycle. Several of these fungi are dimorphic during their life cycle, exhibiting a yeast-like form, marking the haplophase, and a filamentous form, marking the heterokaryotic phase. The filamentous form arises after mating of two yeast-like, sexually compatible strains. Until recently, all economically important smut fungi, except *U. maydis*, were thought to have a bipolar mating system, consisting of one locus with two alleles that control sexual compatibility (Nielsen 1988). *U. maydis* has a tetrapolar mating system consisting of two unlinked loci, *a* and *b*, that control sexual development (Holliday 1974; Banuett and Herskowitz 1988). The *a* locus consisits of a gene complex

encoding pheromones and pheromone receptors (Froeliger and Leong 1991, Bolker et al. 1992), and controls fusion of mating partners. The *b* locus has at least 25 naturally occuring alleles (Puhalla 1968) and controls events after fusion including establishment of the infectious dikaryon and sexual development within the plant (Day et al. 1971). The cloning of two alleles of the multiallelic *b* locus of *U. maydis* (Kronstad and Leong 1989) revealed that it encodes at least two polypeptides, containing a region with similarity to the homeodomain of regulatory proteins (Kronstad and Leong 1990). The *a* and *b* mating-type loci of *U. maydis* hybridized with DNA sequences from other smut fungi (Bakkeren et al. 1992). This conservation was not only structural but also functional (Bakkeren and Kronstad 1993), as the mating-type locus of *U. hordei* was found to replace some functions of the *b* locus of *U. maydis*.

The general life cycle of *Ustilago* fungi generally resembles that of other basidiomycete yeasts of the Ustilaginales (Alexopoulos and Mims 1979), except that formation of cells capable of undergoing meiosis requires infection of plant hosts. During the haplophase the cells grow vegetatively by budding, and they are termed primary sporidia. The dikaryotic phase starts when the cytoplasm of two compatible sporidia merge to form a hyphal-shaped cell. Hyphal growth seems to occur by apical extension of the hyphal tip (Thomas 1988). Dikaryotic hyphae are capable of infecting the host and grow in association with the differentiating host tissues. Eventually, smut sori are produced in the developing plant organs. At maturity the sori spread teliospores, each of which contain a diploid nucleus produced by fusion of the two haploid nuclei of

the dikaryon. In the laboratory, teliospores germinate on a variety of growth media and form four haploid primary basidiospores that can be separated by micro-manipulation. *U. hordei* and other basidium-forming species produce ordered tetrads which makes them attractive for genetic studies. *U. tritici* and *U. nuda* are distinct from other smut fungi by virtue of their inability to produce haploid basidiospores from germinating teliospores. Indeed, compatible adjoining cells of the basidium conjugate immediately after germination, forming knee-like junctions from which a dikaryotic hypha immediately develops (Ingold, 1983).

Taxonomic relationships among *Ustilago* species

Variation in both morphology and physiology abounds within the genus *Ustilago.* Since morphology is used as a basic taxonomic criterion in the smut fungi, any variation in primary morphological features becomes significant in relation to species delimitation. Size, shape, color, and external ornamentation of the teliospores are the principal features employed as criteria in species delineation in the smut fungi. Unfortunately, all of these criteria are highly variable, and overlapping features present problems in species description. For example, Fischer and Holton (1957) describe the teliospores of the bunt fungus, *Tilletia caries* (DC) Tul., as polygonal reticulate, but occasionally somewhat cerebriform. However, the range in spore diameter may vary by at least 75 percent, the shape of the spores can vary from globose to ovoid, and spore color may display shades

of yellow, gray, and brown. Duran and Fischer (1961) advanced a concept advocating that the genus *Tilletia* represents a composite of variants embracing several former 'species' now relegated to synonymy on the premise of morphological similarity.

All morphological characteristics of smut spores are genetically controlled, although in most cases the mode of inheritance is yet to be defined (Fischer and Holton 1957; Halisky 1965). Since most of the smut species investigated are interfertile, much of the variation in their heritable characters probably is related to natural hybridization. Nielsen (1988) and Thomas (1988) established that the ornamentation on teliospores of *U. hordei*, *U. nigra*, *U. kolleri*, and *U. avenae* is controlled by two genes, and at least one additional gene modifies the height of the rounded echunilations. Moreover, both investigations showed that hybrids made between a species with smooth spores and a species with echunilate spores will yield segregants of each parental type and numerous variants.

Two grasses, *Agropyron tsukushiense* var. *transiens* and *Hordeum compressum*, were found to be hosts for all *Ustilago* species pathogenic on wheat, oats, and barley (Nielsen 1978). These grasses allowed the study of the taxonomic relationships of the smuts through hybridization between the taxa from different hosts (Huang and Nielsen 1983). The fact that *U. hordei*, *U. nigra*, *U. avenae*, and *U. kolleri*, which are pathogenic to oats and barley, can hybridize in all possible combinations, showed that they are related and share a common ancestry. Regarding the barley pathogens, *U. hordei* and *U. nigra*, a single gene controlling the morphology of the spore wall appears to separate the

two taxa. A similar example of relatedness of *U. avenae* and *U. kolleri,* which parasitize oats, peaked the interest of early investigators. Hanna and Popp (1930) and Holton (1930) found that the two species could be readily hybridized, and the progeny germinated normally, but most of the primary sporidia were capable of only a few cell divisions followed by cell death. This phenomenon, termed lysis, was thought to be caused by multiple suppressive genes of *U. kolleri* acting on sensitive genes of *U. avenae* in the nuclei of hybrid sporidia (Nielsen 1968). Huang and Nielsen (1984) showed that this incompatibility, as well as complete compatibility, can occur in both intra- and inter-specific hybrids of *U. avenae* and *U. kolleri* depending on the genetic background of the partners of the cross.

Spores of *U. tritici* and *U. nuda* which cause loose smut of wheat and barley, respectively, germinate without producing primary sporidia, and the infection type, spore morphology, and morphological characteristics of the germination products are identical. These similarities have led to various proposals that these two species are synonymous, or are only specialized varieties or forms of the same species. Cunningham (1924), Rodenhiser (1926), and Fischer (1943) suggested that the principal difference between the two species lay in their host specificity. The only morphological difference known between the loose smuts of barley and wheat was reported by Nielsen (1972), who showed that the promycelium of *U. tritici* is slightly curved and about twice as long as that of *U. nuda*

Genetics of *Ustilago hordei* host specificity

Covered smut of barley has been used as a model system for investigating the nature of the effects of pathogenicity genes (Thomas and Person 1965; Person et al., 1987, Christ and Person 1987). Fungal phytopathogenic model systems used to investigate the interactions with host plants typically have four characteristics (Catten et al., 1984): i) the fungus is amenable to crossing and genetic analysis, ii) the pathogenicity of field isolates can be readily assessed in a quantitative way, iii) the pathogen exhibits variation in virulence, and iv) the effects of factors not under investigation, such as variation in host resistance, inoculum size, and plant growth conditions, can be controlled so as to minimize environmental influences. *U. hordei* satisfies all of these requirements.

Pathogenic organisms are quite variable in their interaction with their hosts. Accordingly whether they are virulent or avirulent on each of the host differential varieties has been used to classify strains into different physiologic races (Stakman 1917 and 1918). During the period from 1920 to 1940, studies on the genetics of pathogen virulence showed that virulence and avirulence are Mendelian traits (Johnson and Newton 1940). A fundamental feature of these interactions was first described by Flor in a series of studies in the 1940s in which he demonstrated a gene-for-gene relationship between each resistance/susceptibility gene locus in flax and each avirulence/virulence gene locus in *Milampsora lini* (Flor 1942; 1946; and 1947). Since then, the gene-for-gene model has been shown to hold, not only for plant-fungus interactions (Barrett 1985; Islam and Sheperd 1991; Thomas 1991), but also for other interactions including viruses,

bacteria, nematodes, and insects (Day 1974; Sidhu 1986). Physiologic specialization in *U. hordei* was alluded to as early as 1924 (Faris 1924).

Variation in virulence is revealed by different patterns of compatibility (> 5% infected plants) and incompatibility (< 5% infected plants) with specific barley cultivars (Tapke 1937). Thirteen physiological races of *U. hordei* were reported based upon the differential response of eight barley cultivars (Tapke 1945). The differences in virulence were determined by allele pairs at single loci which interact with host genes in a typical gene-for-gene relationship (Thomas and Person 1965; Sidhu and Person 1971). Sidhu and Person (1972) studied the genetics of resistance of three barley varieties to two known virulence genes present in two test cultures of *U. hordei.* Segregants among F_3 progeny derived from crossing barley differential cultivars revealed that resistance and susceptibility to the two test cultures were inherited independently from one another, and that resistance was expressed as a dominant characteristic at both loci. The interrelationships between two loci for resistance in the host, and the two corresponding loci for virulence in the pathogen, led the investigators to conclude that a gene-for-gene relationship exists in the *Hordeum vulgare*: *U. hordei* system. Ebba and Person (1975) elaborated on these pathogenicity investigations by testing the percentage of infection under changed environmental conditions and different genetic backgrounds. They found that the V_2v_2:R_2R_2 interaction, for which in previous tests the parasite had been consistently avirulent, is subject to influence by modifier genes and by the effect of the environment on these genes. In the same study, virulence on two barley differentials,

Keystone and Himalaya, was attributed to control by duplicate recessive genes present at either one of two genetic loci.

Several genetic investigations elucidated the existence of six virulence/avirulence genes involved in the interaction between *U. hordei* and its host, *H. vulgare* (Thomas 1988). Allele V_1 controls virulence on Hannchen (Sidhu and Person 1971), V_2 controls virulence on Excelsior and is under modification by other genes and by the environment, V_3 is linked to V_2 and controls virulence on Nepal and Pannier (Sidhu and Person 1972), V_4 and V_5 are duplicate at two different genetic loci and control virulence on Keystone and Himalaya (Ebba and Person 1975), and V_6 controls virulence on Lion and Plush (Thomas 1976).

Race 8 of *U. hordei* is pathogenic only on the differential barley cultivar Odessa, which is susceptible to all known races of the fungus. Selfing of sexually compatible strains from ordered tetrads of race 8, a previously stable race, surprisingly increased its pathogenicity on the other barley differential cultivars in a stepwise progression (Jensen 1971; Pedersen and Kiesling 1979). In these investigations the teliospores of race 8 were germinated and the primary sporidia were selfed. Certain crosses produced sori on the previously resistant cultivar Hannchen. Teliospores collected from Hannchen were germinated and primary sporidia were selfed. Some crosses produced dikaryons that were pathogenic on four additional cultivars: Nepal, Lion, Pannier, and Trebi. Subsequent selfing of primary sporidia from germinated teliospores from these four cultivars produced dikaryons that were pathogenic on all eight differential cultivars. This

shift toward pathogenicity was not readily understood and several explanations were advanced without firm data. It was suggested that the results could be attributed to parental heterozygocity at several virulence/avirulence loci and/or mutation followed by somatic recombination of gene(s) regulating the virulence/avirulence genes. However, these interpretations were admittedly inconsistent with previous observations regarding the high level of stability of race 8 strains.

The term aggressiveness has been used to to describe variability in severity of disease reactions among virulent biotypes of a pathogen in a host (Burnett 1975). Variation in aggressiveness may be revealed by differences in the proportion of smutted plants or in the number of smutted spikes per plant, in response to infection by different dikaryons that are compatible with the host. Emara (1972) tested the level of infection of different matings on the cultivar Odessa, and reported it to vary between 1.7% and 41.9%. One-half of this variability was attributed to the environment and the remainder to chromosomal genes. In a later study, Emara and Sidhu (1974) showed that variation in aggressiveness was controlled by polygenes that are mainly additive in their effect. The genetic determination of aggressiveness was examined in progeny populations derived from dikaryons that differed in both origin and cultural history (Catten et al. 1984). The findings of this investigation suggested that approximately 60% of the genetic effect was additive and 40% was dominant. The authors noted a difference between opposite mating-type segregants, suggesting the presence of a factor which affected aggressiveness and that was linked to one mating-type.

Further studies of virulence and aggressiveness genes involved in the interaction between *U. hordei* and the differential cultivars Odessa and Tribi (Pope and Wehrhahn 1990) allowed the distinction between additive and non-additive (dominant and epistatic) gene effects that control total disease incidence variation on both cultivars. The additive genetic variation was 2.5 times higher on Trebi than on Odessa, and nonadditive variance effects were higher by a factor of 1.5 on Odessa than on Trebi. Analysis of F_2 populations suggested that five effective factors, a virulence gene and four aggressiveness factors, control the disease incidence on both cultivars. It is not clear whether any, or all, of the five factors identified on Trebi were the same as those identified on Odessa.

In an exhaustive study Gaudet and Kiesling (1991) investigated the components of aggressiveness and disease severity among 13 physiologic races of *U. hordei*. Variation was observed for the aggressiveness components of peduncle compaction and extent of sorus formation in heads, leaves, and nodes. Race 7 and 12 strains were the most variable for these components, and race 1 and 8 strains were the least variable.

Pulsed-field gel electrophoresis applications to fungal genetics

Recent advances in the separation of relatively high molecular weight DNA have led to a physical basis for analysis and comparison of genomes of plant-pathogenic fungi. Since its introduction, pulsed-field gel electrophoresis (PFGE) (Carle and Olson 1984; Schwartz and Cantor 1984) has been hailed by mycologists as a method by which

chromosomes that otherwise were too small to be distinguished by light microscopy can be visualized. PFGE, thus, offers a new tool to dissect genomic structure and to clarify evolutionary relationships between isolates.

One of the earliest applications of PFGE involved the study of the chromosomal makeup of the unicellular ascomycete *Saccharomyces cerevisiae* (Schwartz and Cantor 1984). The investigators used a PFGE device consisting of two perpendicularly oriented alternately pulsing electrical fields, to separate DNA molecules up to 2000 kilobasepairs (kb) in size. This device suffers from variable migration of DNA molecules in different lanes, making lane-to-lane comparison and accurate size determination extremely difficult. This problem was addressed in the orthogonal field-alternation gel electrophoresis (OFAGE) system (Carle and Olson 1984), which applies two alternately pulsing inhomogeneous fields to the DNA. In this system the angle between the electrical fields varies from the top to the bottom of the gel, causing DNA molecules to migrate at different rates in different positions in the gel, which typically results in the curved migration of DNA in the outer lanes. OFAGE was used to resolve the electrophoretic karyotypes of *S. cerevisiae* (Carle and Olson 1984; Carle et al. 1986), *Candida spp.* (Magee and Magee 1987), and *U. maydis* (Kinscherf and Leong 1988). OFAGE did not represent a significant advance over PFGE as judged from the resolution of the karyotype of *S. cerevisiae,* as both techniques resolved 11 chromosomal bands.

The resolution of electrophoretic karyotypes of *S. cerevisiae* (Carle et al. 1986) and *Candida albicans* (Lott et al. 1987) was improved with the development of field-

inversion gel electrophoresis (FIGE) (Carle et al. 1986). FIGE allowed the resolution of DNA species up to 10 megabasepairs (Mb) in size. However, like PFGE and OFAGE, FIGE suffered from the lack of uniformity of electrical fields across lanes. This problem was overcome with the development of contour-clamped homogeneous electric field (CHEF) gel electrophoresis (Chu et al. 1986) and transverse alternating-field electrophoresis (TAFE) (Gardinier et al. 1986). The CHEF system employs a hexagonal array of 24 electrodes that surround the gel and produce a contour-clamped homogeneous electrical field that alternates between two orientations at an angle of 120°. CHEF PFGE allowed the separation of DNA molecules up to 12 Mb in size (Orbach et al. 1988).

Recent developments in CHEF PFGE have made it possible to separate the chromosomes of fungi according to their size in agarose gels. Southern hybridization of various cloned genes to CHEF PFGE-fractionated chromosomes allowed the development of detailed karyotypes in several filamentous fungi (Skinner et al. 1991). The technique was first used to resolve the karyotype of *S. cerevisiae* (Chu et al. 1986). In typical strains, the sixteen chromosomal DNA molecules were separated into thirteen bands (ten singlets and three doublets). Hybridization of cloned single-copy genes to Southern transfers of the gels, showed unique bands of hybridization, indicating that each band was composed largely of a single chromosome. The success in visualizing the chromosomes of *S. cerevisiae* by CHEF PFGE led to the separation of the chromosomes of a wide range of filamentous fungi, including *Neurospora crassa* (Orbach et al. 1988), *Phytophthora megasperma* (Howlett 1989), *U. hordei* (McCluskey and Mills 1990),

Tolypocladium inflatum (Stimberg et al. 1992), *Tilletia caries* and *T. controversa* (Russell and Mills, 1993), *Fusarium oxysporum* (Boehm et al. 1994), *Gibberella fujikuroi* (Xu et al. 1995).

Several investigations focused on the resolution of the karyotype of the human pathogen *C. albicans* (Magee and Magee 1987; Magee et al. 1988, Trash-Bingham and Gorman 1992; Rustchenko-Bulgac and Howard 1993). Iwaguchi et al. (1990) examined the electrophoretic karyotypes of twenty seven strains of *C. albicans*. The chromosomes of these strains were resolved into seven to twelve bands ranging in size from 0.42 to 3.0 Mb. Most of the separated chromosomal bands hybridized with eight cloned *C. albicans* DNA probes, suggesting that the haploid number of chromosomes in this yeast species is eight. Moreover, with the exception of one probe, each of the other probes hybridized specifically to one or two bands of similar size in most strains. A similar study reported by Thrash-Bingham and Gorman (1992) corroborated the initial finding that the haploid number of chromosomes in *C. albicans* strains is eight. In this study the investigators used CHEF PFGE to compare the electrophoretic karyotypes of six *C. albicans* isolates. The hybridization pattern of 22 cloned sequences demonstrated that linkage groups were generally conserved, suggesting that, in spite of gross karyotype differences, there is an underlying similarity in the genome organization of different isolates. The hybridization data also provided direct evidence that DNA transposition and reciprocal translocations contribute to chromosome-length polymorphisms in *C. albicans*.

Variation in electrophoretic karyotypes and chromosome-length polymorphisms (CLPs) were reported in several plant-pathogenic fungi (Kistler and Miao 1992). Karyotypic polymorphisms in the form of variation in chromosome size and number were first reported among isolates of *U. maydis* (Kinscherf and Leong 1988). Similar electrophoretic karyotype variability was later reported in the related smut fungus *U. hordei* (McCluskey and Mills 1990). High quality karyotypes of smut fungi were later resolved without first generating protoplasts (McCluskey et al. 1990; Mills et al. 1995), and this technique may be applicable to a wide variety of fungi (Russell and Mills 1993; Plummer and Howlett 1993).

Differences in chromosome sizes and numbers were reported among the fourteen physiologic races of *U. hordei* (McCluskey and Mills 1990). Fifteen to nineteen chromosomal bands varying in size between 170 kb and 3,150 kb were resolved from representative strains of the fourteen races. Chromosome-specific probes, anonymous genomic probes, and heterologous conserved genes have been utilized for detection of CLPs among strains representing the fourteen races (McCluskey et al. 1994), and chromosomes of similar size did not necessarily hybridize to the same probe. The magnitude of CLP detected by the different probes ranged between 10 and 15%, relative to the average size for each homologous chromosome.

Significant karyotypic variability and CLPs have been observed in related strains of other phytopathogenic fungi, including *Colletotricum gloeosporioides* (Masel et al. 1993), *Tilletia spp.* (Russell and Mills 1992 and 1993), *Fusarium oxysporum* (Boehm et

al. 1994), and *Leptosphaeria maculans* (Morales et al. 1993; Plummer and Howlett 1993). It has been hypothesized (Kistler and Miao 1992; Stimberg et al. 1992) that CLPs in plant-pathogenic fungi are related to the lack of meiosis under field conditions, and they most likely arise from genome rearrangements, such as translocations (Orbach et al. 1988), deletions (McCluskey et al. 1994, Agnan and Mills 1994), and insertions (Ono and Ishino-Arao 1988). Molecular karyotyping offers a tool to detect these types of rearrangements. A filamentous morphology mutant of *U. hordei*, recovered following heat shock treatment, has a karyotypic polymorphism relative to the wild type sporidial strain (McCluskey et al. 1994; Agnan and Mills 1994). A 50 kb deletion in a 940-kb chromosome was found to cosegregate with the filamentous phenotype.

Masel et al. (1993) used CHEF PFGE to compare the karyotypes of two pathotypes, Type A and Type B, of *Colletotrichum gloeosporioides*. A 1.2 Mb minichromosome was present only in isolates of Type B. Southern analyses with probes specific to the 1.2 Mb minichromosome demonstrated that the variation in the occurrence of this chromosome did not arise by rearrangement of the genome of a progenitor strain, but involved either larger scale deletion or addition of DNA.

The chromosome complements from several isolates of *Cochliobolus heterostrophus* were compared (Tzeng et al. 1992), and a total of fourteen bands were detected in the moderately virulent strains, designated C-strains, whereas twelve bands were resolved from the toxin-producing, T-strains. One hundred and twenty eight restriction fragment length polymorphism (RFLP) probes were assigned to the different

chromosomes in both strains, allowing the detection of a supernumerary chromosome in the C-strains, and a branched linkage associated with the virulence locus, *Tox-1*, in the T-strains. The branched linkage is brought about by a reciprocal translocation at or near the *Tox-1* locus.

CHEF PFGE has been used as an addendum for phylogenetic studies within genera of phytopathogenic fungi. Highly and weakly virulent strains of *L. maculans* have been speculated to fit into distinct species based on the major differences found in the chromosome complements of the two strains (Taylor et al. 1991). A significant karyotypic variability was detected among eight species of the *Fusarium* sections Arthrosporiella and Sporotrichiella, corroborating their classification as distinct species despite their morphological and physiological similarities (Fekete et al. 1993). However, the karyotypes of field isolates of *T. caries* and *T. controversa* collected from Oregon, Pakistan, and Turkey were essentially identical (Russell and Mills 1993), advocating their conspecific classification.

Molecular typing of plant-pathogenic fungi

Knowledge of genetic variability in populations of plant pathogens is important in understanding host coevolution in plant pathosystems (McDonald et al. 1989). Several molecular techniques have been developed to study taxonomic and phylogenetic relationships among taxa, and to differentiate pathovars and subgroups intraspecifically.

Recent studies of the phylogenetic relationships among taxa of plant-pathogenic fungi have relied heavily upon the analysis of DNA sequence data from regions of the ribosomal gene repeat amplified by the polymerase chain reaction (PCR) (White et al. 1990, Berbee and Taylor 1992). Nuclear ribosomal sequences are well characterized in many organisms because they are repeated, and because some regions are highly conserved (Metzenberg 1991). Primer sites common to many fungi have been identified that flank regions of the 18S, 5.8S, and 28S genes, and the internal transcribed spacer (ITS) (White et al. 1990). Although the noncoding region (ITS) is more highly variable in sequence, it cannot be assumed that it has evolved synchronously, or individually according to a predictive molecular clock model. The correlation rate of sequence divergence with time has been controversial, but it is the foundation for theories of molecular evolution and some approaches to phylogenetic reconstruction (Moritz and Hillis 1990).

Phylogenetic relatedness among strains and *formae speciales* of *Puccinia coronata*, *P. graminis*, *P. recondita*, and other cereal and grass rusts and related species were recently investigated (Zambino and Szabo 1993) using ribosomal DNA sequencing of two regions, the ITS and the 5' end of the region coding for the large-subunit rRNA. The ITS sequence data provided sufficient variabilty for phylogenetic analysis, whereas the 5' end of the 28S gene had little variation. At the species level, corelated species were confirmed as species that clustered with each other and had little sequence divergence in the ITS region compared to other species. Within species, identical ITS

sequences have indicated *formae speciales* that are interfertile or otherwise closely related.

Whereas intraspecific variability in the nuclear genome will reflect patterns of sexual recombination, the mitochondrial genome of filamentous fungi appears to rarely undergo recombination in nature (Kohn 1992). Therefore, even in sexually reproducing organisms, mitochondrial lineages may be a reliable indicator of intraspecific phylogeny (Avise et al. 1987). However, large-sized length mutations occur at high frequency in fungal mitochondrial DNA (Taylor et al. 1986, Bruns et al. 1988) making alignments of restriction site maps and recognition of site similarities difficult (Bruns and Palmer 1989).

Where little is known of the genetic constitution of a particular fungus, direct analysis of polymorphisms in DNA can provide useful information on variation both within and between species. Several methods have been used with varying degrees of success to differentiate pathovars or subgroups within taxa of plant-pathogenic fungi. For example, RFLP analyses of nuclear DNA have proved to be a powerful method for studying variation within a range of species. (Hulbert and Michelmore 1988, McDonald and Martinez 1990). However, detection of polymorphysms by techniques involving Southern hybridization analysis is time consuming and laborious. An alternative approach has been to utilize the PCR (Saiki et al. 1985, 1988) to examine for example, length variation in mini- and micro-satellite arrays (Love et al. 1990). Such an approach requires sequence information which is available only for well characterized species. The amplification of genomic DNA with single primers of arbitrary nucleotide sequence

rapidly generates polymorphic markers (William et al. 1990). The primers were shown to reproducibly detect polymorphisms in the absence of specific nucleotide sequence, and the term RAPD (random amplified polymorphic DNA) has been applied to these polymorphisms.

Hamer et al. (1989) identified a family of dispersed repetitive DNA sequences, called MGR, that was conserved in the genome of *Magnaporthe grisea,* a rice pathogen. The investigators detected 40 to 50 copies of MGR in the genome of isolates that infect rice, while isolates that do not infect rice contain relatively few copies of the MGR sequence. Thus, MGR-DNA sequences diagnostically mark populations of *M. grrisea* that have a rice-specific pathogen genotype (Hamer 1991). MGR probes have also been used to investigate pathotype variation and stability within a collection of U.S. blast fungus isolates (Levy et al. 1991). The MGR probes were used to construct genotype-specific profiles based on RFLPs (MGR-DNA fingerprints) of 42 isolates representing eight major blast fungus pathotypes found in the region. The study showed that isolates of the same pathotype, collected from different areas at different times, had diagnostically similar MGR-DNA fingerprints.

Objectives of the research

The present research sought to explore the techniques of pulsed-field gel electrophoresis, Southern-blot analysis with a telomere-specific probe, and sequence

analysis of the internal transcribed spacer (ITS) regions of the rDNA gene, as means to characterize strain lineages within the covered smut fungus, *U. hordei*, and delineate species among the genus *Ustilgo*.

Inbreeding of the avirulent physiologic race 8 of *U. hordei* was purported to have increased its pathogenicity and given rise to a new virulent physiologic race, designated race 14. The comparison of the electrotrophoretic karyotypes of representative strains of race 8 and 14 was investigated for providing insights related to the mechanisms that might have led to the increased pathogenicity in race 14. Chromosome-specific probes were used to detect possible chromosome rearrangements that could be correlated with the change in the pathogenicity profile of race 8. To further characterize the lineage relationship between race 8 and 14 strains, the level of polymorphism detected by a telomere-specific probe was used as a measure for genomic variability among race 8 and 14 strains, and progeny purported to be obtained from the inbreeding of race 8 strains for one or two generations.

The usefulness of electrophoretic karyotypes as an addendum for species delineation was investigated among eight *Ustilago* species. The electrophoretic karyotypes and magnitudes of chromosome length polymorphisms depicted by Southern-CHEF blot hybridizations was used to elucidate the relatedness among these smut fungi. The relatedness inferred from the analysis of the electrophoretic karyotypes was compared to taxonomic relationships drawn from the sequence analysis of the ITS regions.

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CHAPTER II

DELINEATION OF *Ustilago hordei* STRAINS BY ELECTROPHORETIC KARYOTYPING AND TELOMERE-SPECIFIC RFLP PATTERNS

Abstract

Electrophoretic karyotype profiles, chromosome-length polymorphism (CLP) and fingerprints of restriction fragment length polymorphisms of chromosomal termini (telomereRFLP) were used to assess genome stability among a collection of *Ustilago hordei* strains that purportedly comprise a lineage which exhibited a change to increased virulence in successive stages of inbreeding. The comparative analyses were made for race 8 strains which are avirulent on 8 barley differential cultivars, teliospore collection 447, progeny of the first inbred generation of race 8, and pathogenic to the universal susceptible cultivar and one additional cultivar, teliospore collection 1279, progeny of the second inbred generation of race 8, and pathogenic to 6 barley differential cultivars, and race 14 strains, which are pathogenic to all 8 barley differential cultivars. The frequency of occurrence of CLPs and telomereRFLPs were first examined in recently collected progeny from 2 ordered tetrads representing 2 generations of inbreeding race 8 strains, and 3 randomly collected basidiospores. No evidence was obtained for the generation of either CLPs or telomereRFLPs for *Bam*HI and *Bgl*II restriction sites in any of these

strains. In a similar analysis, strains representative of teliospore collection 447 were determined to be indistinguishable from race 8 strains, whereas strains representative of teliospore collection 1279 had numerous CLPs and telomereRFLPs for both *Bam*HI and *Bgl*II restriction sites. In a comparison with other races, the electrophoretic karyotype and telomereRFLP obtained from teliospore collection 1279 and strains representing races 10 and 13 were indistinguishable. Race 10 and 13 strains appear to have a single, but different avirulence gene, and are pathogenic to 7 of the 8 barley differentials. A telomereRFLP was detected for at least half of the *Bam*HI and *Bgl*II restriction sites of race 14 strains when compared with either strain 1279, or race 8 strains. Visually detectable CLPs ranging of 8 percent or less of the average size of each chromomosome were observed for 24 putative homologous chromosomes in race 8 and 14 strains, a magnitude characteristic of variability detected among all physiologic races except races 10 and 13. These results support the premise that teliospore collection 447 was derived from race 8 strains, but do not support the premise that teliospore collection 1279 and race 14 strains were derived from race 8 through inbreeding. Telomere RFLP profiles offer a powerful tool for following strain lineages in *Ustilago spp.* and in natural populations of other inbred fungal species.

Key Words: Electrophoretic karyotype, chromosome-length polymorphism, telomere-RFLP, *Ustilago hordei.*

Introduction

The heterothallic basidiomycete fungus, *Ustilago hordei* (Pers.) Lagerth causes covered smut disease of barley (*Hordeum vulgare* L.). The life cycle of the fungus favors a high level of inbreeding and only rarely is there opportunity for out crossing (Fischer and Holton 1957). Diploid teliospores which are produced in the spikes of susceptible barley plants become scattered and frequently lodge on healthy seed where they undergo meiosis and germinate to form a probasidium on which the four products of meiosis known as primary sporidia or basidiospores emerge. A single locus with two alleles, *MAT*1 and *MAT*2, controls mating which usually occurs between basidiospores on the probasidium, thereby reducing opportunities to outcross. The resulting infectious dikaryon penetrates the barley coleoptile and maintains a quiescent state in the meristematic tissue until the onset of flowering, at which time it differentiates into masses of teliospores that are contained within a membranous sac. In the laboratory, the basidiospores which form an ordered tetrad, can be micromanipulated from the probasidium and grown on a variety of synthetic media where they form yeast-like colonies. Sexually compatible strains may also be mated to form the infectious dikaryon.

The interaction between *U. hordei* and its host, barley, obeys the gene-for-gene model (Flor 1942; Ellingboe 1981) indicating that dominant avirulence genes of the fungus interact with dominant resistance genes in the host to affect an incompatible interaction (Sidhu and Person 1971). Thirteen physiologic races were identified on a

series of 8 barley cultivars by Tapke (1945) and among these, only race 8 strains were observed to produce an incompatible interaction with all cultivars except Odessa (C.I. 934), the universally susceptible cultivar which is not known to have any resistance gene. In separate laboratory studies (Jansen 1971; Pedersen and Kiesling 1979) crosses of race 8 strains were purported to produce progeny with increased virulence on an additional cultivar, Hannchen. Progeny obtained from teliospores collected from smutted heads of Hannchen were crossed producing in excess of 40 dikaryons that were bioassayed for increased virulence in both studies. One dikaryon produced increased virulence on cultivars Nepal, Lion, Pannier and Trebi (Jensen 1971). In the study by Pedersen and Kiesling (1979), a single dikyaryon was obtained with increased virulence on Lion and Trebi, but subsequent rounds of inbreeding led to strains with increased virulence on all 8 cultivars. As none of the 13 races described by Tapke were virulent on all 8 cultivars, these latter strains represented a new physiologic race, designated race 14. It appeared therefore that inbreeding of race 8 strains could lead to a rare, but sequential change to increased virulence.

Four possibe hypotheses were advanced to explain the association between inbreeding and the shift toward pathogenicity (Pedersen and Kiesling 1979): i) the presence of recessive pathogenicity genes in the parental teliospore that segregated independently from the avirulence genes during inbreeding, ii) inbreeding affected regulator genes that control expression of avirulence, iii) pathogenicity genes are subject to a high rate of mutation during meiosis, and iv) contamination of the progenitor teliospores with other

pathogenic races. The race 8 teliospores used in the two studies (Jensen 1971; Pedersen and Kiesling 1979) had no identifiable genetic markers and no molecular techniques were known to monitor progeny resulting from the crosses. However, recent advances in molecular technology as, for example, isoelectric focusing polyacrylamide-gel electorphoresis (Thomas et al. 1987), isozyme analysis (Hellmann and Christ, 1991) and electrophoretic karyotyping (McCluskey and Mills 1990; McCluskey et al. 1994) have offered new promise to differentiate among strains representative of the 14 physiologic races of *U. hordei* . Restriction fragment length polymorphisms (RFLP) offer yet another method of analysis especially among strains that reproduce essentially by inbreeding.

This study reveals the feasibility of using electrophoretic karyotype profiles, chromosome-length polymorphisms (CLPs), and fingerprints of restriction fragment length polymorphisms of chromosomal termini (telomereRFLP) in combination to follow strain lineages. Strains with increased virulence purported to have arisen from inbreeding *U. hordei* race 8 strains and maintained from the collection of Jensen (1971) were used in this analysis.

Materials and Methods

Fungal strains: *U. hordei* strains used in this study are listed in Table II.1. All strains were grown in potato dextrose broth (PDB, Difco Laboratories, Detroit, MI) and maintained on potato dextrose agar (PDA) slants. For preparation of intact chromosomes the fungal strains were cultivated in 200 ml of PDB on a rotary shaker (250 rpm) at 28°C for 48 hr.

Table II.1. *Ustilago hordei* strains used in this study

Strains	Relevant characteristics	Origin	Source
8.2a	Monosporidial basidiospore isolated from a race 8 teliospore designated 8.2, *MAT1*.	Race 8 collection of teliospores	W.L.Pedersen[a]
8.2c	Monosporidial basidiospore isolated from a race 8 teliospore designated 8.2, *MAT2*.	Race 8 collection of teliospores	W.L.Pedersen
8.A	Monosporidial basidiospore micromanipulated randomly from race 8 teliospores.	Race 8 collection of teliospores	W.L.Pedersen
8.B	Monosporidial basidiospore micromanipulated randomly from race 8 teliospores.	Race 8 collection of teliospores	W.L.Pedersen
8.C	Monosporidial basidiospore micromanipulated randomly from race 8 teliospores.	Race 8 collection of teliospores	W.L.Pedersen
14.1d	Monosporidial basidiospore isolated from a race 14 teliospore designated 14.1, *MAT2*.	Race 14 collection of teliospore	W.L.Pedersen

Table II.1. (continued)

Strains	Relevant characteristics	Origin	Source
10.1a	Monosporidial basidiospore isolated from race 10 teliospore collection.	Race 10 collection of teliospore	W.L.Pedersen
13.1a	Monosporidial basidiospore isolated from race 13 teliospore collection.	Race 13 collection of teliospore	W.L.Pedersen
88.1a-d	Progeny of a tetrad obtained from crossing 8.2a X 8.2c. Teliospores collected from barley differential Odessa.	This study	
88.2a-d	Ordered tetrad obtained by crossing 88.1a (*MAT1*)X 88.1b (*MAT2*) and inoculating the barley differential culticar Odessa.	This study	
447.1a-d	Progeny of a germinated teliospore from collection 447 isolated from the barley cultivar Hannchen. Represents first inbred generation.	D.A.Gaudet[b]	Jensen 1971

Table II.1. (continued)

Strains	Relevant characteristics	Origin	Source
447.2a-d	Progeny of a germinated teliospore from collection 447 isolated from the barley cultivar Hannchen. Represents first inbred generation.	D.A.Gaudet[b]	Jensen 1971
1279.1a-d	Progeny from a germinated teliospore produced by crossing sexually compatible 447 strains. Represents second inbred generation.	D.A. Gaudet	Jensen 1971
1279.4a-d	Progeny from a germinated teliospore produced by crossing sexually compatible 447 strains. Represents second inbred generation.	D.A. Gaudet	Jensen 1971

[a]: University of Illinois, Urbana

[b]: Agriculture Canada, research Station, Lethbridge, Alberta

Two monosporidial strains of opposite mating type obtained from a collection of race 8 teliospores were crossed on PDA medium, and the resulting dikaryon was used to inoculate the coleoptiles of the barley cultivars Odessa, Hannchen, and Plush following the method described by Pedersen and Kiesling (1979). The teliospores produced on Odessa plants were germinated on PDA medium, and the four basidiospores of an ordered tetrad, designated 88.1, were recovered using a Sensaur Pneumatic Micro-manipulator (ALOE Scientific, St. Louis, MO). The mating type of each basidiospore was determined using the Bauch test (Lade and Jensen 1967). The products of tetrad 88.1 were crossed in all possible combinations to produce the progeny of the second generation of selfing, which were tested for their pathogenicity on the same three barley cultivars. A tetrad from the third inbred generation, designated 88.2, was micromanipulated from a smutted head collected from the universal susceptible cultivar.

Contour clamped homogeneous electric field pulsed-field gel electrophoresis (CHEF PFGE): CHEF electrophoresis (Chu et al. 1986) was used to separate intact chromosomes with a Bio-Rad CHEF DR-II apparatus (Bio-Rad Laboratories, Richmond, CA). Chromosome samples were prepared without the need of protoplasting (McCluskey et al. 1990). For the separation of chromosomes smaller than 1.6 megabase pairs (Mb), electrophoresis was performed in 1% agarose (Agarose Type I-A, Sigma, St. Louis, MO) gels, in 0.5X TBE running buffer (1X TBE is 0.9MTris-borate, 2mM EDTA) at 12°C, using the parameters reported previously (McCluskey and Mills 1990). Chromosomes larger than 1.6 Mb were separated in 0.8% "Fast Lane" agarose (FMC Bioproducts, Rockland, ME) in 1X TAE (4 mM Tris-acetate, 1mM EDTA) at 10°C. Running

parameters were as follow: 75V, ramped switch time of 400-1200 sec. for 96 hr. To visualize the chromosomes, CHEF gels were stained in the running buffer with 0.5 μg/ml ethidium bromide.

Hybridization probes and Southern-blot analysis of karyotypes: The chromosomal bands in race 8 and 14 strains were resolved using electrophoretic parameters that separated small- to medium-sized bands (i.e. bands smaller than 1.6 Mb). Chromosome-specific libraries were made in the vector pUC19 (Yanisch-Perron et al. 1983) following the protocol described in Mills et al. (1995). Plasmids were maintained in *Escherichia coli* DH5α (Bethesda Research Laboratories, Gaithersberg, MD) and grown on Lauria-Bertain medium supplemented with 100 μg/ml ampicillin. Only inserts from the chromosome-specific libraries that hybridized with a single fragment in a Southern blot (Southern 1975) of total digested genomic DNAs, from race 8 and 14 strains, were used to probe CHEF blots to identify putative homologous chromosomes. At least three different single copy probes, from each chromosome-specific library, were hybridized to CHEF blots in order to determine the putative homologous chromosome in race 8 and 14 strains. A total of 70 anonymous probes from the chromosome-specific libraries were used for hybridization to CHEF blots.

The chromosomes of race 8 and 14 strains were resolved, denatured in 0.4N NaOH, and transferred in the same solution to Zeta-Probe^{R} nylon membranes (Bio-Rad Laboratories), and hybridization reactions were carried out at 65 °C according to the manufacturer's recommendations. Radiolabelled probes were prepared using the random priming technique of Feinberg and Volstein (1983).

Genomic typing with a telomere-specific probe:

To study the genomic relatedness of race 8 strains and the progeny purported to have arisen through its inbreeding, total genomic DNA was isolated from monosporidial strains obtained from each selfing following the method of McDonald and Martinez (1990). The DNA was digested with *Bam*HI and *Bgl*II (Gibco-BRL, Grand Island, NY) in separate reactions, Southern-blotted, and hybridized at 65°C with the radiolabelled telomere-specific tandem repeat $[TTAGGG]_{18}$ (Powell and Kistler 1990).

To estimate the number of chromosomes in each strain, chromosome bands were resolved in 1% low melting temperature agarose (FMC Bioproducts, Rockland, ME), excised under UV illumination, and the DNA in each band was extracted by the freeze-thaw method (Mills et al. 1995). The DNA from each band was digested with *Bam*HI and *Bgl*II restriction enzymes in separate reactions, and the fragments were fractionated in 0.8% agarose gels, and Southern-blotted onto Zeta-Probe^R nylon membranes. The telomere-specific probe was radiolabelled (Sambrook et al. 1989) and hybridized to these Southern blots. The number of chromosomes in each band was estimated to be equal to half the number of restriction fragments detected by the telomere-specific probe (McCluskey et al. 1994, Mills et al. 1995).

Results

Electrophoretic karyotypes and chromosome length polymorphisms (CLPs): To establish whether electrophoretic karyotypic profiles could be used as a criterion for predicting strain lineage within the race 8 collection of teliospores, it was first important to determine the extent to which karyotyic variability naturally occurs. Although strains representing the 14 races of *U. hordei* have unique karyotypes (McCluskey and Mills, 1990), the extent to which CLP occur in strains in successive generations of selfing had not been established. Karyotypic profiles were therefore established in randomly isolated basidiospores of race 8, tetrads obtained through the inbreeding of race 8 teliospores for one or two generations, the number of generations that produced numerous changes to increased virulence in previous studies (Fig. II.1). The electrophoretic karyotypes of random basidiospores micromanipulated from race 8 teliospores, and the selfing progeny obtained in this study, were invariant (Fig. II.2), and it could be strongly argued that inbreeding would have only a very limited effect, if any, on the occurrence of CLPs for strains derived from race 8 teliospores.

A comparison of the electrophoretic karyotypes was then obtained for strain 8.2a, representative of race 8, strains representative of the second and third inbred generations obtained in Jensen (1971), 447-1a and 1279-1a, respectively, and strain 14.1d representative of race 14 (Fig. II.3). No CLPs were detected between the karyotypes of strains 8.2a and 447-1a, representative of the second inbred generation that had increased virulence only on Hannchen

	First Inbred Generation			Second Inbred Generation		
	Teliospore designation	Pathogenicity profile	Basidiospores crossed	Teliospore designation	Pathogenicity profile	Basidiospores crossed
A	R8A	Odessa	2 x 1 →	447	Odessa Hannchen	1 x 2 →
B	K	Odessa	1 x 2 1 x 4 3 x 2 3 x 4 →	K-2	Odessa Hannchen	1 x 3 1 x 4 2 x 3 2 x 4 →
C	8.2	Odessa	a x c a x d b x c b x d →	88.1	Odessa	a x b a x c d x b d x c →

Fig. II.1. Pathogenicity profile obtained by inbreeding progeny of race 8 strains of *Ustilago hordei.* A, initial study of Jensen (1971); B, study by Pedersen and Kiesling (1979); C, results of this study.

	Third Inbred Generation			Subsequent Selfing
	Teliospore designation	Pathogenicity profile	Basidiospores crossed	
A	1279	Odessa Hannchen Lion Nepal Pannier Trebi	1x3 1x4 2x3 2x4	
B	K-2-8	Odessa Hannchen Lion Trebi	1 x 2 1 x 3 4 x 2 4 x 3	----------→ Race 14
C	88.2	Odessa	a x b a x c d x b d x c	

Fig. II.1. (Continued)

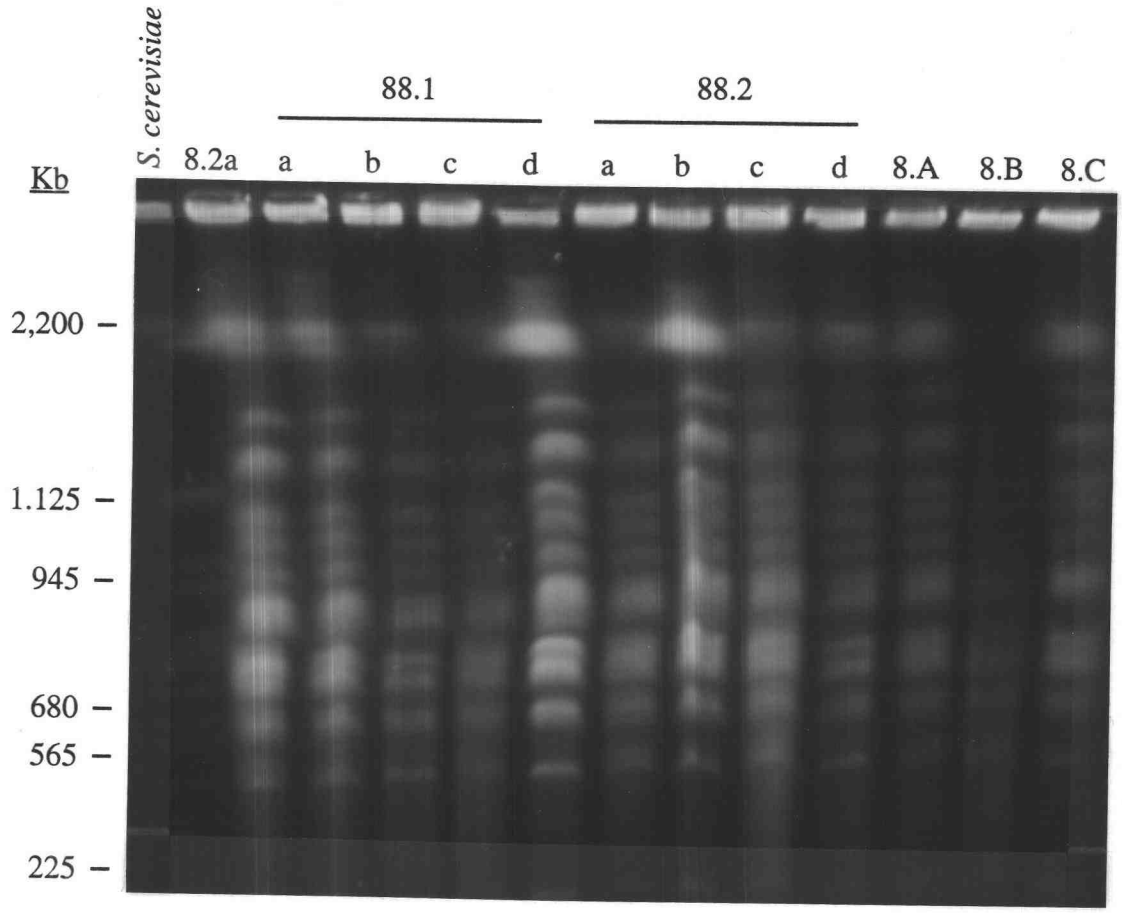

Fig. II.2. Electrophoretic karyotypes of race 8 strains of *Ustilago hordei*. Lanes designated a, b, c, and d represent the progeny of ordered tetrads 88.1 and 88.2.

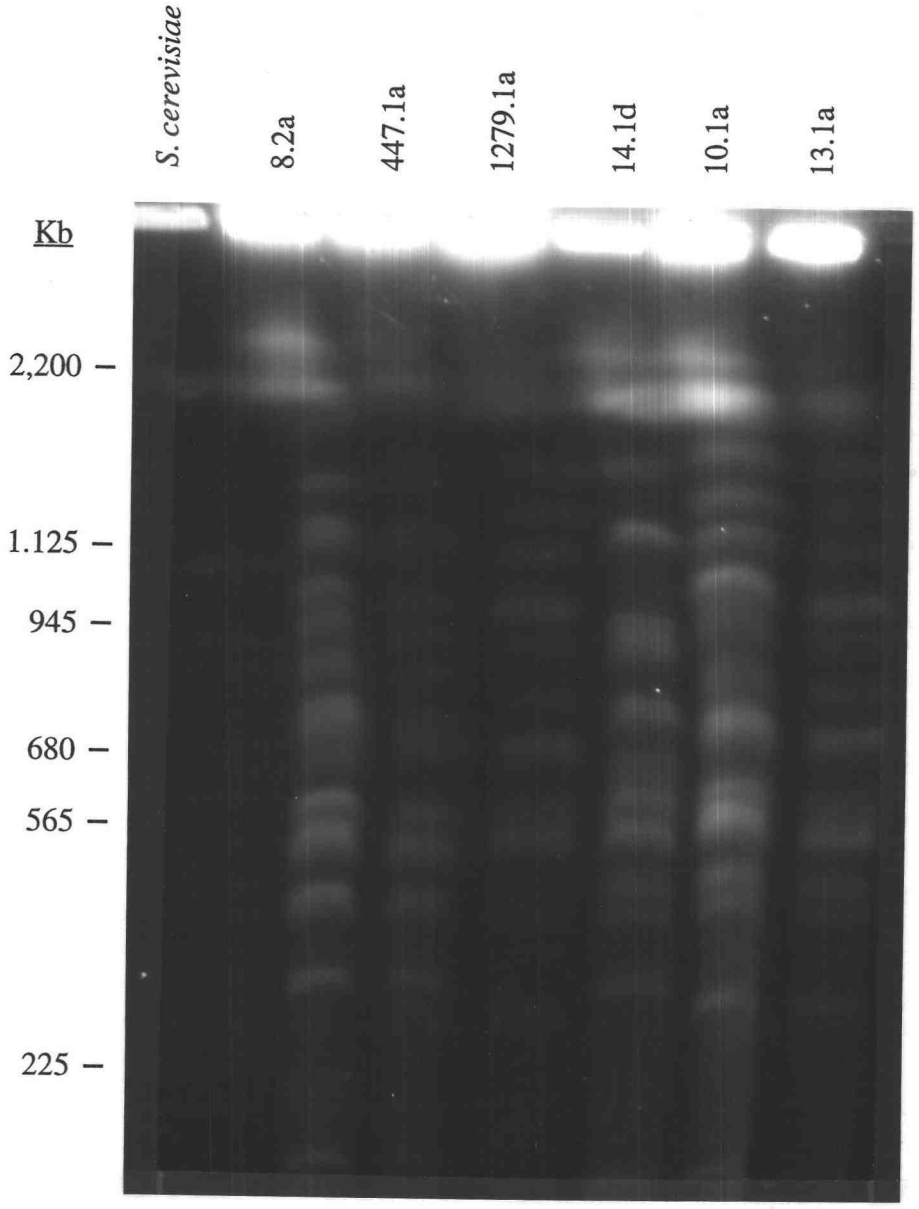

Fig. II.3. Comparative electrophoretic karyotypes of race 8, its purported inbreeding progeny, and strains of physiologic races 10 and 13.

(Fig. II.1; Jensen 1971). However, numerous CLPS were observed in comparisons between the race 8 strain and strains from the 1279 and race 14 collection of teliospores. Teliospore collection 1279 represents the third inbred generation in the inbreeding scheme of race 8 (Fig II.1, Jensen 1971), and was thought to be a direct lineage descendent from the inbreeding of the 447 teliospore collection. Race 14 which has increased virulence on all barley differentials, is most distant from race 8 strains in the purported lineage, having been obtained by additional rounds of inbreeding beyond the third inbred generation (Fig. II.1; Pedersen and Kiesling 1979).

Southern CHEF-blot anaysis with chromosome-specific probes and telomere analysis (Mills et al. 1995) were used to determine putative homologous chromosomes, and the number of chromosomes in strains 8.2a and 14.1d. To obtain an estimate of the magnitude of CLPs for putative homologous chromosomes in strains 8.2a and 14.1d, sublibraries were made for each chromosomal band separated by CHEF PFGE, and typically at least 3 single copy restriction fragments from each sublibrary were radiolabelled and used to probe CHEF blots (Fig. II.4). Measurable CLPs which ranged up to a maximum of approximately 8 percent of the average size were detected for putative homologous chromosomes (Table II.2). CLPs of this magnitude were characteristic of the variability observed among strains representative of the 14 physiologic races of *U. hordei* (McCluskey et al. 1994). An estimate of the number of chromosomes in each band was also obtained by probing Southern blotted restriction fragments from each chromosomal band with a telomere-specific DNA probe. The telomere allowed to estimate 24 and 23 chromosomes in strains 8.2a and 14.1d, respectively.

Table II.2. Identification of putative homologous chromosomes and CLPs in *Ustilago hordei* strains 8.2a and 14.1d by Southern CHEF-blot analysis.

8.2a			14.1d			
Chromosomal band[a]	**Size (kb)**	**Number of chromosomes[b]**	**Chromosomal band**	**Size (kb)**	**Number of chromosomes**	**CLP (%)[c]**
1[d]	200	1	1	190	1	4
2	450	1	2	460	1	2
3	590	2	3	590	2	0
4	650	2	4	630	2	2
5	720	3	5	720	1	0
6[e]	850	2	6[e]	760	2	8
			7[e]	830	2	2

Table II.2. (Continued)

8.2a			14.1d			
Chromosomal bands	**Size (kb)**	**Number of chromosomes**	**Chromosomal bands**	**Size (kb)**	**Number of chromosomes**	**CLP (%)[c]**
7[f]	920	3	8[f]	920	3	0
8[f]	980	1				4
9	1,040	1	9	1,020	1	4
10	1,090	1	10	1,060	1	1
11	1,150	1	11	1,170	1	2
12	1,260	2	12	1,260	2	1
R.B.[g]	2,200	4	R.B.	2,200	4	nd[h]

[a]: Band resolved by CHEF PFGE

[b]: Estimate of the number of chromosomes per band based on hybridization with the telomere-specific probe.

[c]: CLP is: (standard deviation from the average size / statistical mean of sizes of homologous chromosomes detected by the chromosome-specific probes) X 100

[d]: Chromosomal bands on the same row are identified to be homologous, based on Southern hybridization with at least three different chromosome-specific probes.

Table II.2. (Continued)

[e]: Chromosome-specific probes found homology on two different chromosomal bands in strain 14.1d.
[f]: Chromosome-specific probes found homology on two different chromosomal bands in strain 8.2a.
[g]: R.B.: Reptation band comprises chromosomes that were not resolved with the short run parameters.
[h]: Not determined

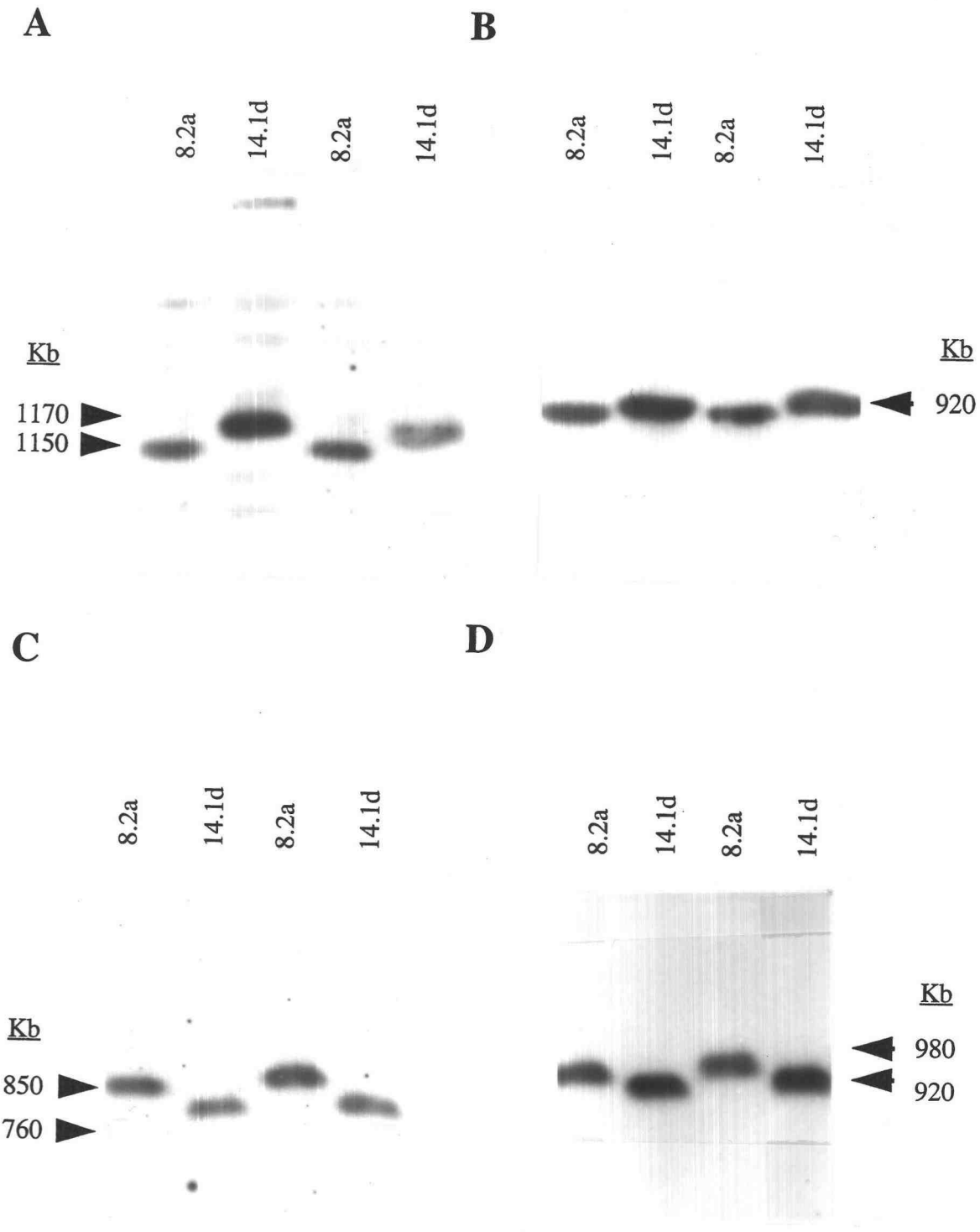

Fig. II 4. Chromosome-length polymorphisms between *Ustilago hordei* strains 8.2a and 14.1d.

Fig. II.4. (legend continued)

CHEF blots with alternate lanes of intact chromosomal DNAs of *U. hordei* strains 8.2a and 14.1d were hybridized with pOSU1170-14 (panel A), pOSU920-8 (panel B), pOSU760-14 (panel C), and pOSU980-8 (panel D). pOSU1170-14 (1.2-kb insert) is a chromosome-specific probe from chromosomal band 11 of strain 14.1d. pOSU920-8 (2.5-kb insert) is a chromosome-specific probe from chromosomal band 7 of strain 8.2a. pOSU760-14 (1.6-kb insert) is a chromosome-specific probe from chromosomal band 6 of strain 14.1d. pOSU980-8 (1.8-kb insert) is a chromosome-specific probe from chromosomal band 8 of strain 8.2a.

TelomereRFLP (TelRFLP) as a sensitive method of following strain lineage: The telomere-specific probe $[TAAGGG]_{18}$ was used to obtain RFLP profiles for terminal *Bam*HI and *Bgl*II restriction fragments from chromosomes of strains that included 3 inbred generations of race 8 obtained in the course of this study, strains from ordered tetrads obtained from the 447 and 1279 teliospore collections, and a strain representing race 14. The *Bam*HI and *Bgl*II TelRFLP profiles for the race 8 strain and the progeny of inbreeding obtained in this study, tetrads 88.1 and 88.2, were identical (Figure II.5). Not all of the putative terminal *Bam*HI and *Bgl*II fragments from the 24 chromosomes were resolved in Southern blots of total genomic DNA with this probe. However, the invariant profiles suggest that these strains are homozygous at approximately 96 restriction sites at the chromosomal termini. Strains obtained from the 447 teliospore collection had *Bam*HI and *Bgl*II TelRFLP profiles identical to race 8 strains, whereas strains from the 1279 and race 14 collection of teliospores had TelRFLP profiles that differed from the race 8 strains and from each other (Fig. II.6). The TelRFLP profiles of strain 8.2a and the monosporidial strains from collection 1279 differed at more than 60% of the terminal *Bgl*II fragements, while the same comparison between strain 14.1d and the 1279 strains revealed identity at approximately 50% of the terminal *Bgl*II sites (Fig. II.6).

Comparative TelRFLP profiles of 1279 strains and other races. The dissimilar electrophoretic karyotypes and the *Bam*HI and *Bgl*II TelRFLP profiles of race 8 strains and strains from the 1279 teliospore collection suggested that the lineage had been interruped at the putative third inbred generation (Fig. II.1). To test one hypothesis advanced by Pedersen and

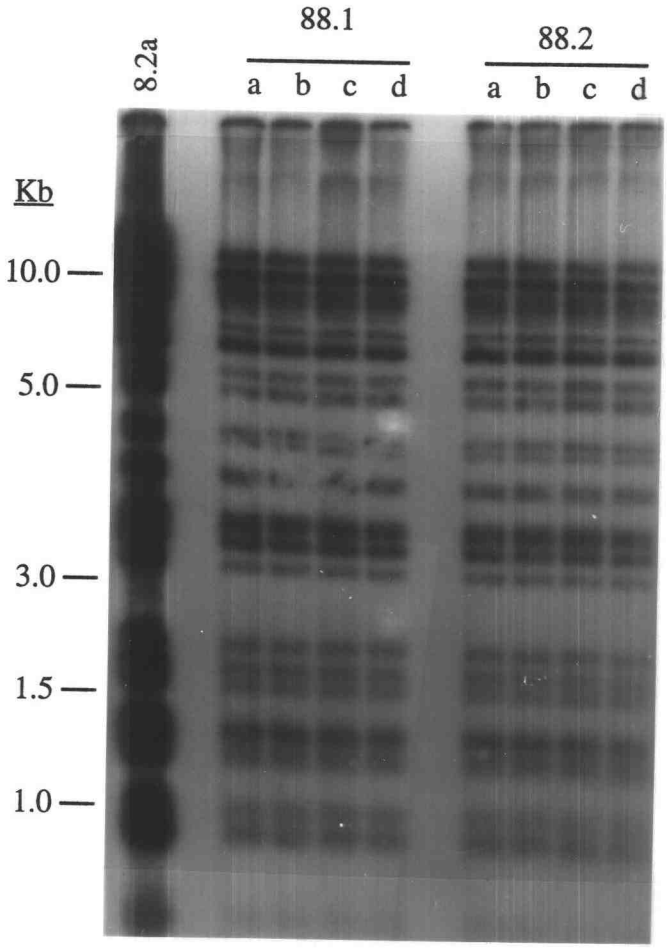

Fig. II.5. Hybridization of the telomere-specific probe onto *Bam*HI-digested total genomic DNA from *Ustilago hordei* strain 8.2a and the monosporidial products of tetrads 88.1 and 88.2.

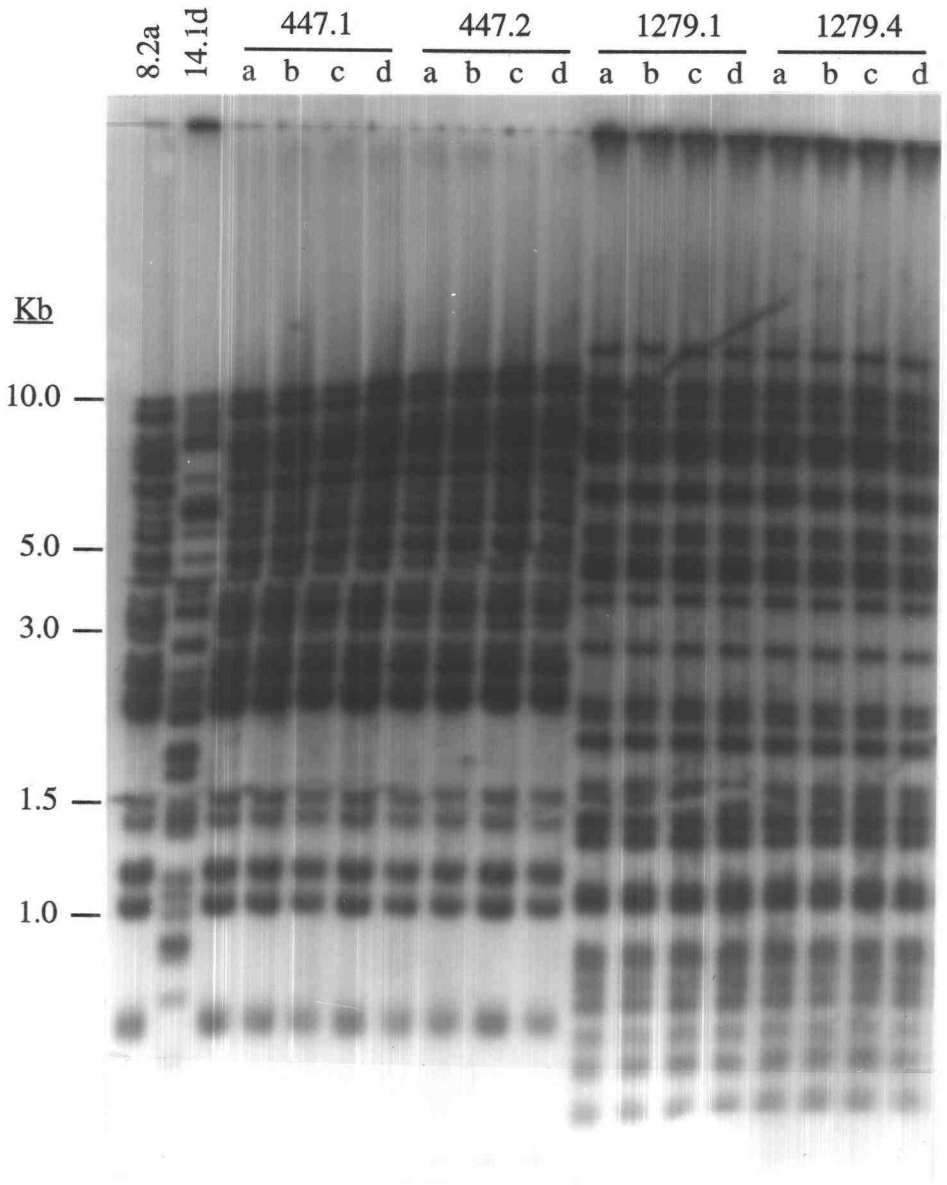

Fig. II.6. TelomereRFLP profiles of *Ustilago hordei* strains 8.2a, 14.1d, and the monosporidial products of ordered tetrads 447.1, 447.2, 1279.1, 1279.4. Total genomic DNA from the different strains were digested with *Bgl*II and probed with the telomere-specific repeat $[TTAGGG]_{18}$.

Kiesling (1979), that the change to increased virulence could have resulted from a contaminant, a comparative analysis was made between the TelRFLP of strains from the 1279 collection and the TelRFLP profiles obtained by Agnan and Mills (unpublished data) for strains representing the 14 physiologic races of *U. hordei*. The *Bam*HI and *Bgl*II TelRFLP were identical for 1279 strains and strains representing races 10 and 13 (Fig. II.7). A comparison of the electrophoretic karyotypes of strains representing the race 10, race 13 and 1279 collections of teliospores also revealed no evidence for CLPs (Fig. II.3)

Discussion

There is often the need to know whether field isolates share a common ancestry or linage, but without genetic markers, there is considerable uncentainty. In the laboratory, naturally occurring drug resistance and, more recently, introduced foreign DNA, have been used to follow strains in crosses through successive generations. In this study, electrophoretic karyotypes and the TelRFLP profiles of the naturally inbred smut fungus, *U. hordei*, were determined to be highly accurate methods of assessing strain lineages and provided insight into the probable reason for the purported change to increased virulence associated with a strain lineage.

U. hordei race 8 strains were initially shown to be nonpathogenic on a series of 8 differential barley cultivars (Tapke 1945). In subsequent independent studies of Jensen

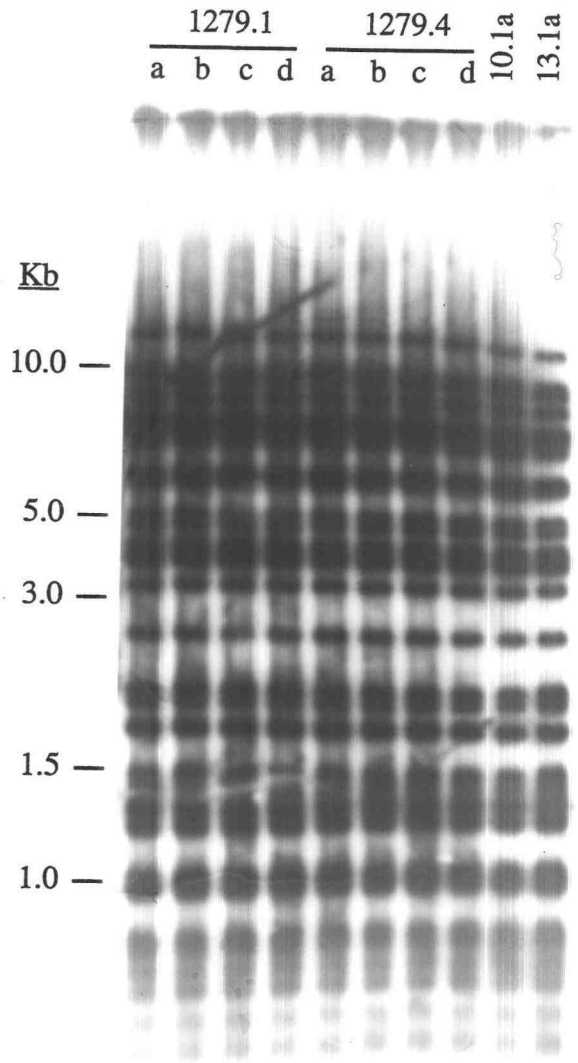

Fig. II.7. TelomereRFLP profiles of *Bgl*II digests of *Ustilago hordei* strains 10.1a, 13.1a, and the monosporidial products of ordered tetrads 1279.1 and 1279.4.

(1971) and Pedersen and Kiesling (1979) race 8 strains were observed to be unstable and acquire increased virulence in a stepwise manner in sequential stages of inbreeding. In the absence of genetic markers, the possibility that the field collection of race 8 teliospores was inhomogeneous could not be excluded. This fungus rarely outcrosses and strains representing races that are spatially isolated have unique electrophoretic karyotypes (McCluskey and Mills 1990) because of CLPs that apparently are fixed within the isolated populations, aneuploidy and possibly the occurrence of supernumerary chromosomes (Mills and McCluskey 1990).

Although strains representing the various races have unique karyotypes, the degree to which gross changes such as inversions, deletions and duplications or other loci are made homozygous through inbreeding is unknown. CLPs are expected during meiosis upon recombination between homologous chromosomes of unequal sizes (Russell and Mills 1993, Plummer and Howlet 1993), and crosses between non polymorphic strains of *Coprinus cinereus* revealed karyotypes similar to that of the parental strains (Zolan et al., 1994). The difference in sizes between homologous chromosomes is usually traced to the presence of dispersed repeats (Valent and Chumley, 1991; Zolan et al., 1994). Repeated DNA sequences which had homology to all chromosomes of races 8 and 14 strains, 8.2a and 14.1d, respectively, were found in several of the chromosome-specific libraries. However it is not known whether these elements are repeated in tandem or distributed as a single copy on each chromosome. Even though almost 25% of the library clones screened in the course of this study contained repeated DNA, it is unlikely that the CLPs between

races 8 and 14 strains originated from meiotic recombinations. Indeed, since karyotypes resolved from race 8 strains were nonpolymorphic (Fig. II.2), little, if any, CLPs would be expected in the descending inbreeding progeny. The inbreeding progeny obtained in this study, tetrads 88.1 and 88.2, second and third inbred generations of race 8 teliospores, respectively, have a karyotype indistinguishable from that of race 8 (Fig. II.2). Likewise, the second inbred generation obtained by Jensen (1971) has a karyotype identical to that of strain 8.2a. It was, thus, surprising to detect karyotypic polymorphisms between teliospore collection 1279, the third inbred generation obtained by Jensen (1971), and strain 8.2a. The fact that the karyotype of teliospore collection 1279 was identical to karyotypes of race 10 and 13 strains led to the speculation that the 1279 collection contained a population of race 10 or 13, or both types.

To investigate the genetic variability among race 8 and 14 strains, and the putative progeny of inbreeding race 8 strains, the telomere-specific RFLP profiles were analyzed. Strains from similar lineages were predicted to have identical telomere-RFLP profiles, while distant lineages should yield polymorphic telomere profiles. Telomeric DNA has been advanced as a universal probe for detecting inter- and intra-species variation in fungi (Coleman et al. 1993). Similar studies in plant species showed that inbred lines of tomato and melon transmit their telomere-region patterns (Broun et al, 1992). Hybridization of the telomere-specific probe to *Bam*HI- and *Bgl*II-digested total genomic DNAs from the different strains, showed that the second inbred generation, teliospore collection 447obtained by Jensen (1971), has TelRFLP profiles identical to that of the race 8 strain;

however, its purported selfing progeny, teliospore collection 1279, revealed TelRFLP profiles that differed at more than 50% of the terminal *Bam*HI and *Bgl*II sites, relative to the TelRFLP profiles recovered from the race 8 strain. The telRFLP profiles depicted from teliospore collection 1279 were identical to those of race 10 and 13 strains. The data of Southern-analysis with the telomere probe corroborate the finding by karyotypic analysis, which indicates that the 447 teliospore collection was derived from the race 8 teliospores, whereas the 1279 collection of teliospores was not derived from the 447 teliospore, and is probably the product of unadvertent contamination with race 10 and/or race 13 teliospores.

In this study, inbreeding of race 8 did not yield matings more pathogenic than the progenitor teliospores. However, selfing of race 8 of *U. hordei* was reported, in two independent studies, to increase its pathogenicity (Jensen 1971, Pedersen and Kiesling 1979). In both studies the progeny of the first inbreeding were pathogenic on the universally sueceptible cultivar, Odessa, and the barley cultivar Hannchen. The recurrence of this result points to the possibility that the avirulence gene, controling the interaction of the fungus on Hannchen, is heterozygous in the progenitor teliospores, or is a site of a high rate of mutation. Both studies reported further increase of pathogenicity upon inbreeding of tetrads collected from Hannchen. However, Jensen (1971) reported a shift toward higher pathogenicity on four more barley cultivars, while Pedersen and Kiesling (1979) detected pathogenicity on only two more barley cultivars. The telomereRFLP profiles, as well as the molecular karyotype analyses, provide evidence advocating that the progeny obtained by Jensen (1971) after two generations of inbreeding has a genome organization identical to

that of race 10 and 13 strains, and is not a direct lineage derivative from teliospore collection 447. Races 10 and 13 are avirulent on a single barley differential cultivar each, Excelssior and Pannier, respectively (Tapke 1945). It is, thus, more likely that teliospore collection 1279 was derived from physiologic race 10 of *U. hordei*. A thorough test of pathogenicity is needed to ascertain whether teliospore collection 1279 is synonymous to race 10 or race 13.

The telomere profile of race 14 is distinct from the profiles depicted from all 13 physiologic races. It is unlikely that race 14 is a direct lineage derivative from any of the 13 physiological races. The examination of the TelRFLP profiles, obtained from strain 14.1d and teliospore collection 1279, revealed that approximately 50% of the terminal *Bgl*II sites are not polymorphic (Fig. II.6), suggesting that race 14 could have arisen from the outcrossing of a race 10 or 13 basidiospore with a basidiospore of another *U. hordei* physiologic race. It is unlikely that the second basidiospore is derived from a race 8 teliospore, since the TelRFLP profiles of races 8 and 14 are polymorphic for more than 60% of the *Bam*HI and *Bgl*II terminal sites (Fig. II.6). Alternatively, it is possible that race 14 is a highly virulent field isolate that inadvertedly contaminated the teliospores used in the investigation of Pedersen and Kiesling (1979) during the later steps of inbreeding race 8.

Previous reports suggested that aneuploidy might account for much of the variability in fungi (Tolmsoff, 1983), including the ability of phytopathogenic fungi to produce new host-specific physiologic races (Day, 1972). The telomere-specific probe was used to estimate the number of chromosomes in each chromosomal band fractionated by PFGE

(Mills et al. 1995, McCluskey et al. 1994). Because none of the enzymes used cut within the telomere repeat, the number of restriction fragments detected by the probe in each digest represents the minimal number of copies of telomeres in the genome. This procedure is limited by the possibility that some terminal fragments are fortuitously of similar size. The number of chromosomes in each genome is equal to half the number of telomeres. Using this methodology, *U. hordei* strains 8.2a and 14.1d were estimated to have 24 and 23 chromosomes, respectively. The haploid number of chromosomes has not been determined in *U. hordei*, but is estimated to vary between 19 and 23 (Agnan and Mills, unpublished data). The discrepancy in the number of chromosomes estimated by telomere analysis could stem to the variability in resolving the number of telomere-specific ends, or to the contamination of chromosomal bands with DNA from neighboring bands. In this investigation, strain 8.2a was estimated to have an additional chromosome relative to the race 14 strain 14.1d. It is unknown whether the extra chromosome in race 8 is a supernumerary chromosome or a duplicated copy of an indispensable chromosome.

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CHAPTER III

A MOLECULAR ANALYSIS OF ELECTROPHORETIC KARYOTYPES AND TAXONOMIC RELATIONSHIPS WITHIN THE GENUS *Ustilago*

Abstract

The electrophoretic karyotypes of seven species of the order *Ustilaginales* were determined by pulsed field-gel electrophoresis. Ten to seventeen chromosomal bands ranging in size from 0.2 to 6.3 megabase pairs were observed in these species. Radiolabelled probes made from anonymous DNA fragments from the genome of *Ustilago hordei* and 3 conserved fungal genes were used in Southern analyses to identify putative homologous chromosomes in the karyotypes of the various species, and to obtain a measure of chromosome-length polymorphisms (CLPs). Patterns of similarity emerged among the karyotype of sporidium-forming species in pairwise analysis of the CLPs, and substantiated the synonymy proposed for some species. The minimum CLP of less than 15% was detected in the comparison of *U. avenae* and *U. kolleri* karyotypes. A high degree of similarity was observed between *U. hordei* and *U. nigra* karyotypes where only one probe detected CLP exceeding 15%. Homology was not detected when *U. hordei* probes were hybridized to the chromosomes of the nonsporidium-forming species, *U, nuda* and *U. tritici,* at high stringency, suggesting that these species are more distantly related to the sporidium-forming species.

Key words: electrophoretic karyotype, chromosome-length polymorphisms, pulsed field-gel electrophoresis, *Ustilago spp.*

Introduction

The *Ustilaginales* comprise at least 33 genera (Fischer and Holton 1957) commonly known as smut fungi. The genus *Ustilago* occurs on the *Gramineae* and its economic importance stems from the fact that it has a world-wide distribution and it parasitizes some of man's most important crops, as well as numerous wild species. Macroscopically, the disease symptoms caused by many *Ustilago* species have the same general appearance. However the spores are microscopically variable with respect to the size and ornamentation. Fischer and Shaw (1953) have proposed a species concept based chiefly on spore morphology, but which recognizes host specialization at the host family level. More recently, molecular techniques have been used to supplement morphological taxonomy in fungi. Restriction fragment length polymorphisms (Michelmore and Hulbert 1987), ribosomal RNA sequences (Logrieco et al. 1991), and randomly amplified polymorphic DNA (Crowhurst et al. 1991) have been used as addenda to morphological classification in the fungal kingdom.

In recent years major strides in fungal taxonomy have also been realized through the development of pulsed field gel electrophoresis (PFGE) (Schwartz and Cantor 1984), a technique first used to study aspects of genome organization in the yeast *Saccharomyces cerevisiae* (Carle and Olson 1985; Schwartz and Cantor 1984). The development of contour clamped homogeneous electric field (CHEF) (Birren et al. 1988) technology permitted the separation of DNA molecules by PFGE of up to 10 megabase pairs (Mb) in size (Orbach et al. 1988). The CHEF PFGE technique has been used to establish

electrophoretic karyotypes in different fungi (for review see Mills and McCluskey 1990; Skinner et al. 1991). Using CHEF PFGE, genomic heterogeneities were detected in inter- and intra-species investigations. The electrophoretic karyotypes of haploid strains representative of the fourteen physiological races of *U. hordei* have been resolved (McCluskey and Mills 1990) and variations in chromosome sizes and numbers were detected. However, Southern hybridizations with anonymous probes typically revealed length polymorphisms of less than 15% per chromosome (McCluskey et al. 1994). The electrophoretic karyotypes of three bunt fungi, *Tilletia caries, T. controversa*, and *T. foetida* were essentially indistinguishable (Russell and Mills 1993, 1994) which corroborates genetic and biochemical attributes that advocate their synonymy (Kawchuk et al. 1988; Trail and Mills 1990). Conversely, the electrophoretic karyotypes of highly and weakly virulent strains of *Leptosphaeria maculans* were very different, suggesting that these strains are distinct species (Taylor et al. 1991).

In this study, CHEF PFGE and a rapid protocol for chromosome sample preparation described in McCluskey et al. (1990) were used to obtain electrophoretic karyotypes of seven *Ustilago* species. DNA probes made from anonymous fragments from the genome of *U. hordei* and heterologous conserved fungal genes were used to identify putative homologous chromosomes in the *Ustilago* species, to identify linkage among the different probes, and to ascertain the extent of chromosome plasticity among the different species.

Materials and Methods

Strains and media: The strains and plasmids used in this study are listed in Table III.1. Teliospores of the sporidium-forming species, *U. avenae, U. bullata, U. hordei, U. kolleri, U. maydis, and U. nigra,* were germinated on Potato Dextrose Agar (PDA, Difco Laboratories, Detroit, MI) at room temperature. Teliospores of the nonsporidium-forming species, *U. nuda* and *U. tritici*, were germinated at room temperature on a modified culture medium described by Newcombe and Thomas (1990) consisting of 1.3% (w/v) glucose; 0.4% (w/v) yeast extract; 0.09% (w/v) asparagine; 0.05% proline (w/v); 20 ml of Vogel's complete salt solution per liter of medium (Vogel 1956) and 1.5% agar. Single spores of the sporidium-forming species and promycelial cells from nonsporidium-forming species were isolated from the germinating teliospores using a Sensaur pneumatic micro-manipulator (ALOE Scientific, St. Louis, MO). Colonies grown from single spores were transferred to PDA slants where they were maintained at room temperature and transferred bimonthly. Mycelia from basidiospores of nonsporidium-forming species were grown in liquid culture medium in shaking flasks at 200 rpm at room temperature, and transferred every two weeks. Chromosomes of *Hansenula wingei, S. cerevisiae,* and *Schizosaccharomyces pombe* (Bio-Rad Laboratories, Hercules, CA) were used as molecular size standards. The plasmids used in this study were maintained in *Escherichia coli* DH5α (Bethesda Research Laboratories, Gaithersberg, MD) which was grown on Lauria-Bertain medium (Sambrook *et al.* 1989) containing ampicillin at 100 μg/ml.

Table III.1. Fungal strains and plasmids used in this study.

Strains and plasmids	Relevant characteristics	Designation	Source or reference
Ustilago avenae			
A1031	Product of a single spore	A1031	L. J. Szabo[a]
A1036	Product of a single spore	A1036	B.J. Goates[b]
U. bullata			
B1032	Product of a single spore	B1032	B.J. Goates
B1033	Product of a single spore	B1033	B.J. Goates
B1034	Product of a single spore	B1034	B.J. Goates
U. hordei			
H8.2a	Product of a single spore of race 8	H8.2a	W. L. Pedersen[c]
U. kolleri			
K1013	Product of a single spore	K1013	P. Koepsell[d]
K1014	Product of a single spore	K1014	P. Thomas[e]
U. maydis			
M1004	Product of a single spore	M1004	K. B. Johnson[d]
M1005	Product of a single spore	M1005	J. Sherwood[f]

Table III.1. (Continued)

Strains and plasmids	Relevant Characteristics	Designation	Source or reference
U. nigra			
Ng1015	Product of a single spore	Ng1015	P. Thomas
Ng1016	Product of a single spore	Ng1016	P. Koepsell
U. nuda			
Nd1042	Product of a promycelium	Nd1042	B.J. Goates
Nd1043	Product of a promycelium	Nd1043	B.J. Goates
U. tritici			
T1007	Product of a promycelium	T1037	L.J. Szabo
T1038	Product of a promycelium	T1038	B.J. Goates
Escherichia coli			
DH5α			Focus 8(2):9, 1986[g]
Plasmids			
pUC19	Sequencing and cloning vectors, Ap^r		Yanich-Perron et al. 1985

Table III.1. (Continued)

Strains and plasmids	Relevant characteristics	Designation	Source or Reference
pOSU1090	2.0-kb *Bam*HI fragment from *U. hordei* cloned in pUC19		This study
pOSU1040	1.2-kb *Bam*HI fragment from *U. hordei* cloned in pUC19		This study
pOSU980	1.5-kb *Bam*HI fragment from *U. hordei* cloned in pUC19		This study
pOSU920	500-bp *Sau*3AI fragment from *U. hordei* cloned in pUC19		This study
pOSU850	750-bp *Sau*3AI fragment from *U. hordei* cloned in pUC19		This study
pOSU650	200-bp *Sau*3AI fragment from *U. hordei* cloned in pUC19		This study
pOSU560	800-bp *Sau*3AI fragment from *U. hordei* cloned in pUC19		This study

Table III.1. (Continued)

Strains and plasmids	Relevant characteristics	Designation	Source or reference
pOSU1101	4.5-kb *Eco*RI fragment from genomic DNA of *Tilletia controversa*, has homology with the 17S rDNA of *Neurospora crassa*		Russell and Mills 1993
pSF8	The actin gene from *Aspergillus nidulans* cloned on a 5.0-kb *Hind*III fragment		Fidel et al. 1988
pBH100	1.5-kb *Bam*HI fragment from mating type gene *b* East in *U. hordei*		Bakkeren and Kronstad 1993
pNLA17	$[TTAGGG]_{18}$ telomere repeat from *Fusarium oxysporum* cloned into pUC19		Powell and Kistler 1990

[a] USDA-ARS, Univ. of Minnesota, St Paul

[b] USDA-ARS, Aberdeen, ID

[c] Univ. of Illinois, Urbana

[d] Oregon State Univ., Corvallis

[e] Agriculture Canada, Winnipeg

[f] Montana State Univ., Bozeman

[g] Published by Bethesda Research Labs., Gaithersburg, MD

Ap^r: ampicillin resistance

Preparation of samples for CHEF PFGE: *U. avenae, U. bullata, U. hordei, U. kolleri, U. maydis,* and *U. nigra* chromosome samples were prepared from overnight cultures grown in Potato Dextrose Broth (PDB, Difco Laboratories) as described in McCluskey et al. (1990), whereas, the chromosome samples for *U. nuda* and *U. tritici* were prepared according to the procedure of Russell and Mills (1993). The plugs were stored at 4°C for up to 6 months in 0.5M ethylene diamine tetraacetic acid (EDTA).

CHEF electrophoresis conditions: CHEF PFGE was performed using the CHEF DR-II electrophoresis system (Bio-Rad Laboratories). For resolution of bands in the 100 kilobase pair (Kb) to 1.6 Mb range, plugs were loaded in the wells of 1% agarose (Sigma type II, medium EEO) gels maintained at 14 °C in 0.5X TBE buffer (Sambrook *et al.* 1989) and subjected to switch times ramped from 70 to 90 s for 21 h at 200 V, followed by a switch interval of 120 s for 15 h at 200 V. For resolution of bands larger than 1.6 Mb, electrophoresis was performed in 0.8% Fast Lane agarose (FMC Bioproducts, Rockland, ME) gels in 1X TAE buffer (Sambrook *et al.* 1989) maintained at 10 °C. Two sets of conditions have been used, each targeting a different size range. The first conditions resolved DNA molecules in the 2.0 to 5.7 Mb range and consisted of a 3600 s pulse time for 96 h followed by 72 h of switch times ramped from 480 to 900 s. The second conditions resolved DNA molecules larger than 5.0 Mb and consisted of a 3600 s pulse time for 96 h followed by 2700 s pulse time for 72 h. Gels were stained with 0.5 mg/ml ethidium bromide in the running buffer, and photographed under UV illumination after destaining in distilled water overnight. Chromosome sizes were estimated after three independent

fractionations. Cricket Graph (Cricket Graphics, Inc., Philadelphia, PA) was used to configure a standard curve derived from migration distances of molecular size markers. Genome sizes of the different strains were calculated by summation of the individual band sizes and counting density staining bands as doublets.

Probes and hybridization conditions: The separated chromosomes were transferred in 0.4N NaOH solution to Zeta-Probe^R GT nylon membranes (Bio-Rad Laboratories) using capillary transfer overnight (Southern 1975). Hybridization reactions were carried out at high stringency conditions according to manufacturer's recommendations. Hybridization probes were made by the random priming method (Feinberg and Vogelstein 1983) and consisted of seven anonymous fragments cloned from *U. hordei*, a genomic DNA fragment of *T. controversa* with homology to the 17S ribosomal DNA (rDNA) from *Neurospora crassa* (Russell and Mills 1993), the actin gene from *Aspergillus nidulans* (Fidel et al. 1988), and the mating type gene from *U. hordei* (Bakkeren and Kronstad 1993).

To obtain a better estimate of the chromosome number of *U. nuda*, total genomic DNA was extracted according to McDonald and Martinez (1990), digested with *Eco*RI, and electrophoresed in 0.8% agarose gel before Southern transfer onto a Zeta-Probe^R GT nylon membrane. The minimum number of chromosomes in each strain was estimated to equal one-half the number of fragments that hybridized to a telomere-specific probe from *Fusarium oxysporum* (Mills et al. 1995).

Results

Preparation of chromosome samples without generating protoplasts: All *Ustilago spp.* used in this study were amenable to production of chromosome samples without first preparing protoplasts. Protoplasting improved neither the sharpness of the chromosomal bands nor their intensities as compared with the non-protoplasting method (data not shown). However, plugs made from both strains of *U. nuda* resolved only 3 or 4 faint bands which suggested that only a partial karyotype was resolved for this species. The electrophoretic karyotypes resolved were reproducible from one preparation to another.

Electrophoretic karyotypes of Ustilago spp.: Typical electrophoretic karyotypes of representative strains of eight *Ustilago spp.* are presented in Figures III.1 and III.2. Electrophoretic parameters that resolved chromosomes smaller than 1.6 Mb produced partial karyotypes that differentiated some species from others (Fig. III.1). Electrophoretic parameters that separated the chromosomes of *Hensenula wingei,* resolved the large-sized chromosomes in all sporidium making *Ustilago spp.* (Fig. III.2A), and parameters known to resolve chromosomes larger than 5.7 Mb did not resolve additional DNA molecules in these species. Longuer runs with wider switching times (see materials and methods) were needed to separated the chromosomes of the nonsporiium-making species, *U. nuda* and *U. tritici* (Fig. III.2B). The approximate numbers and sizes of the chromosome bands observed in the karyotypes shown in Figs. III.1 and III.2 are schematically presented in Fig. III.3. Some

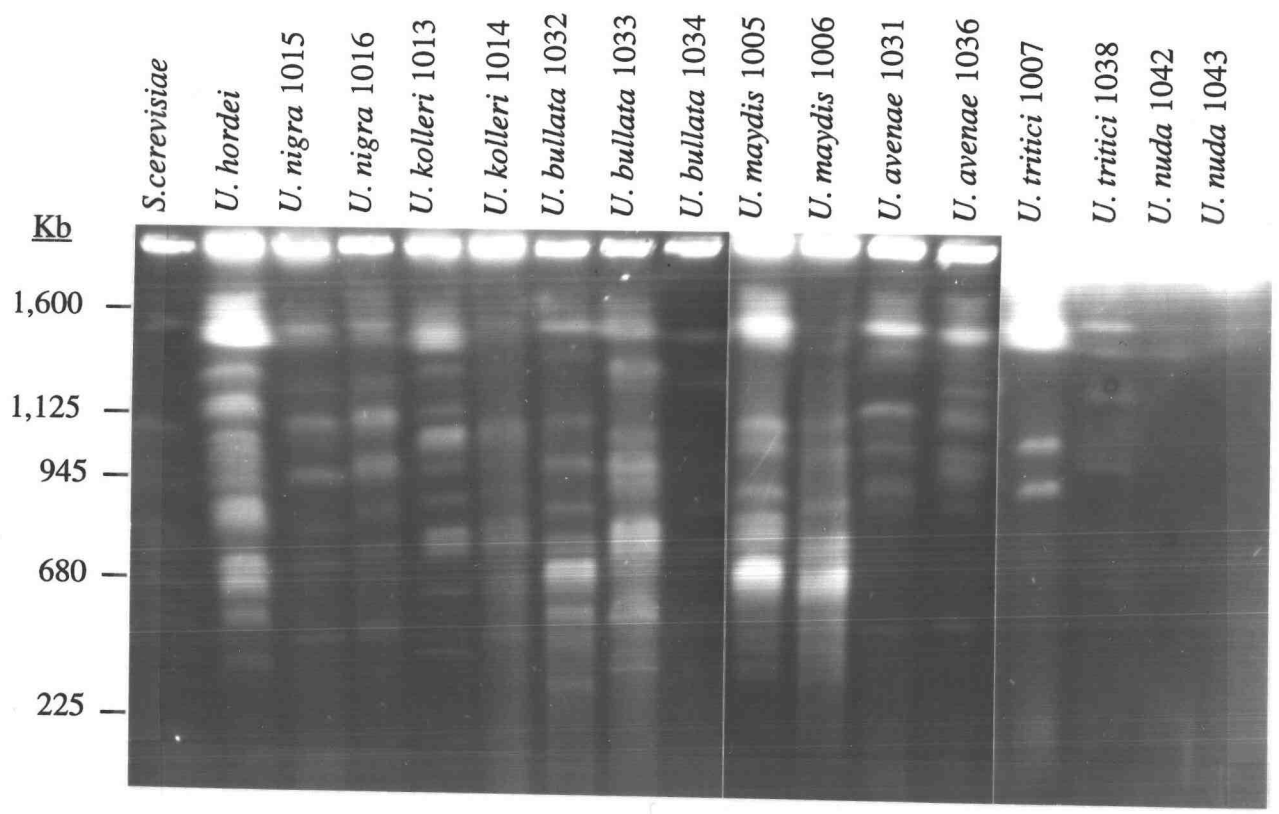

Fig. III.1. Electrophoretic karyotypes of eight *Ustilago spp.* Separation of small- and medium-sized chromosomes.

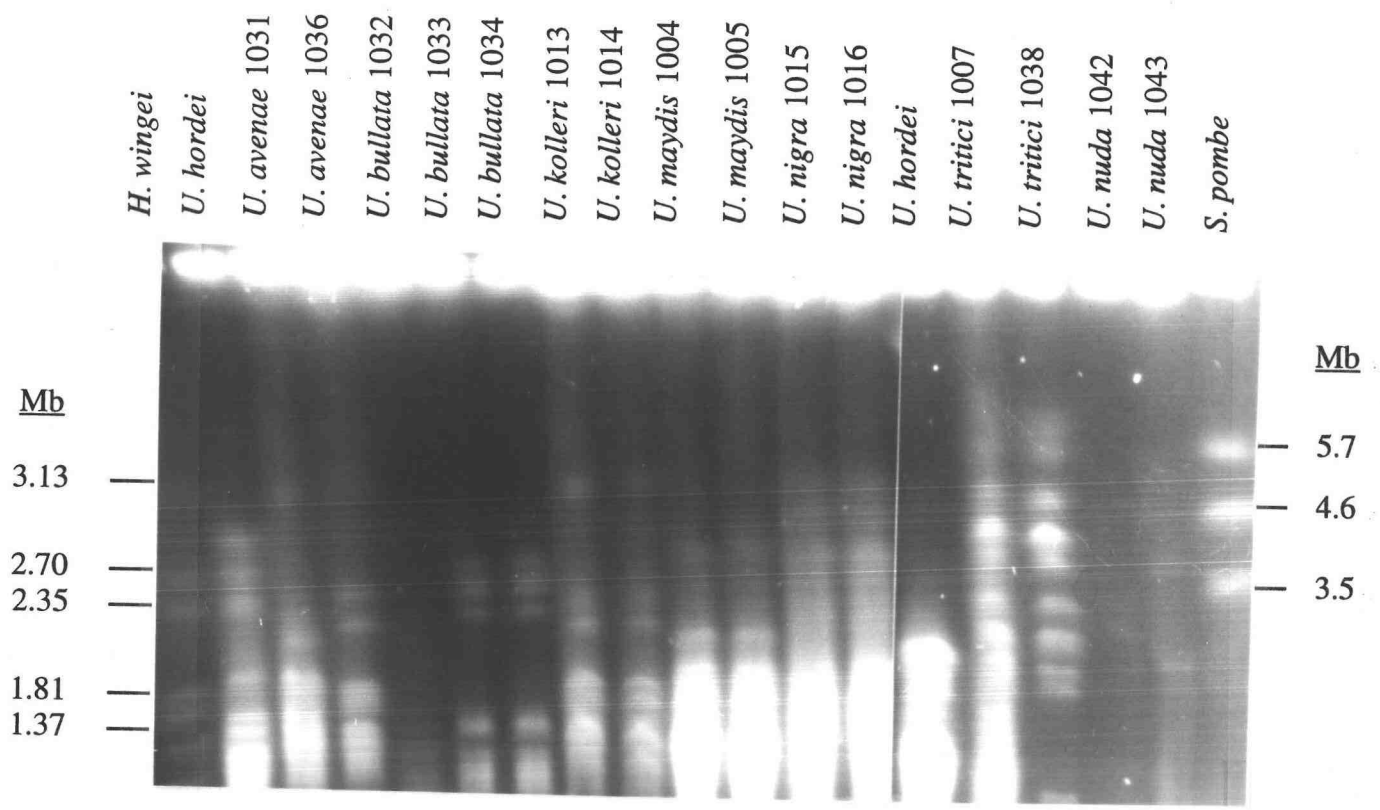

Fig. III.2. Electrophoretic karyotypes of eight *Ustilago spp*. Separation of large-sized chromosomes.

Table III.2. Estimates of chromosome sizes from *Ustilago* species as determined by CHEF analysis

	U. avenae		*U. bullata*			*U. hordei*
	A1031[a]	**A1036**	**B1032**	**B1033**	**B1034**	**H8.2a**
	3580[b]	3600	2720	2770	2770	3000
	3380[c]	3500	2670	2700	2700	2760
	2770	3380	2300	2320	2320	2380
	2690	2820[c]	1850	1750	1750	1900
	2580	2700	1330	1615	1615	1260[c]
	2260[c]	1900	1150	1280	1300	1170
	1900	1830	1090[c]	1160	1220	1090
	1760	1790	980	1010	1030	1040
	1570	1700	840	900	935	980
	1440	1440	750	825[c]	875	920[c]
	1270	1270	720[c]	720	795[c]	850
	1085[c]	1180	660	660	720	720[c]
	1035	1070	570	520	625	650
	965	965	420	430	515	560
	865	875	285	270	435	450
	790	730			300	200
	745	610				
	650	425				
	390					
N° bands[e]	19	18	15	15	16	16
Σ[f]	36.2	33.7	20.1	19.7	20.7	22.8

Table III.2. (Continued)

U. kolleri		*U. maydis*		*U. nigra*	
K1013	**K1014**	**M1004**	**M1005**	**Ng1015**	**Ng1016**
3600	3600	2800	2800	3400	3400
3480	3480	2100	2100	3330	3350
2650	2650	1900	1900	3130	3130
2220	2250	1700	1700	2350	2350
2150	2190	1395[c]	1450[c]	1900	1900
1800	1850	1130	1090	1820	1820
1780	1690	1020	1010	1700	1700
1690	1490	875	865	1530	1530
1490	1430	760[c]	780[c]	1335	1335
1360	1200	710	745	1120[c]	1200[c]
1210	1110	630[d]	630[d]	1075	1035
1150	1045[c]	530	505	1010	955
1070[c]	920	450	420	885	885
965	760[c]	335	335	780[c]	780[c]
810	480			720	670
715	255			620	620
610				460	500
470				320	320
270					
N° bands[e] 19	16	14	14	18	18
Σ[f] 30.6	28.2	21.6	21.7	29.4	29.4

Table III.2. (Continued)

	U. nuda		*U. tritici*	
	Nd1042	**Nd1043**	**T1007**	**T1038**
	3450	5560	6300	6200
	2740	4100	5800	5800
	2410	2600	4600	4600
	2350		3720[c]	3700[c]
			3600	3620
			3250	3280
			2500[c]	2500[c]
			2370	2400
			1185	1320
			940	1010
N° bands[e]	4	3	10	10
Σ^f	10.1	12.3	40.50	40.60

[a] For abbreviation see Table 1.

[b] Values in kilobase pairs (Kb) are the means of three independent estimations.

[c] Bands interpreted as clusters of chromosomes based on intensity of ethidium bromide staining.

[d] Bands interpreted as a cluster of six chromosomes (Kinccherf and Leong 1988).

[e] Estimated number of DNA bands resolved in each strain.

[f] Estimated genome size in megabase pairs (Mb) of each strain.

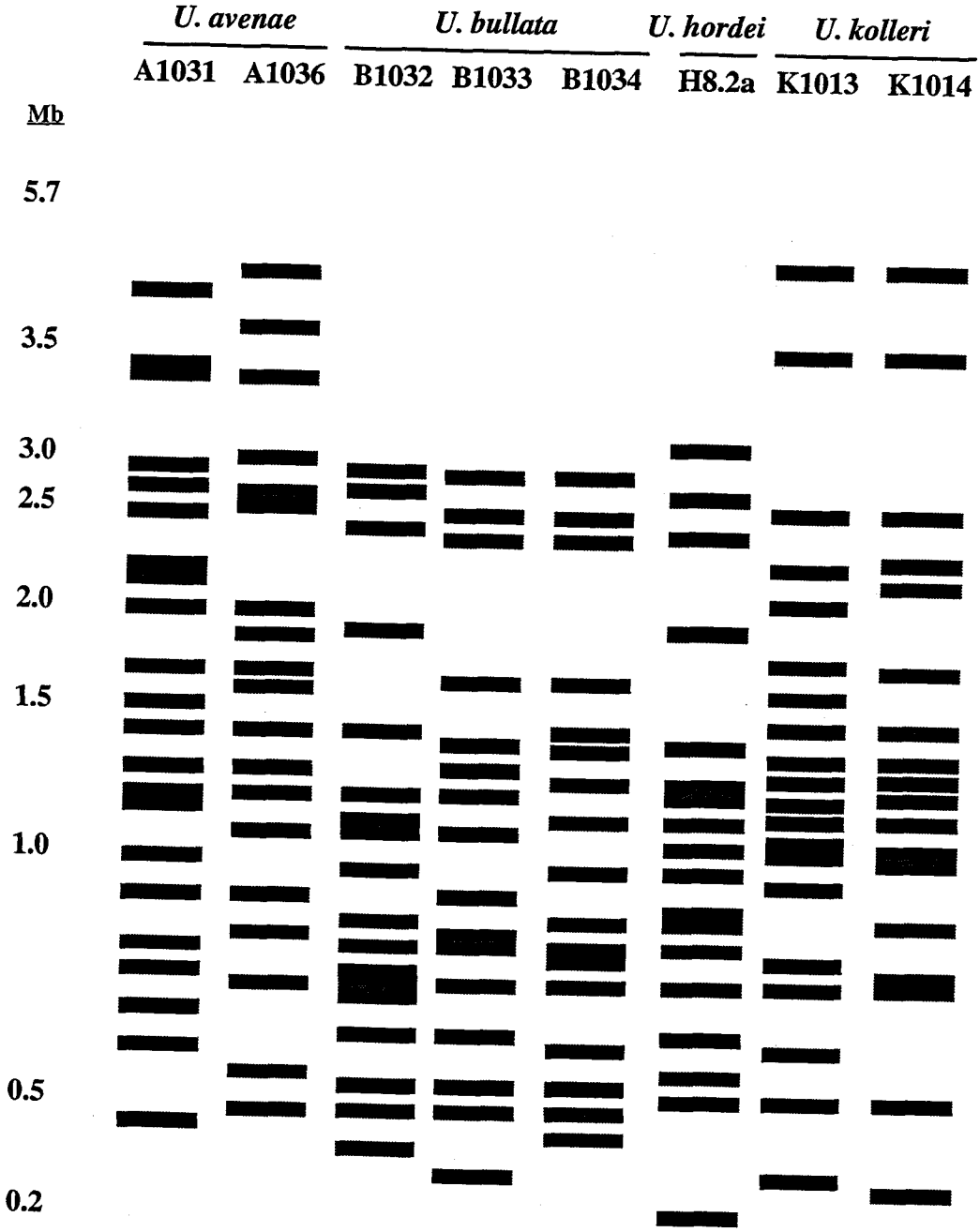

Fig. III.3. Schematic representations of chromosomes of the different strains of *Ustilago spp.* Thicker bands identify chromosome clusters.

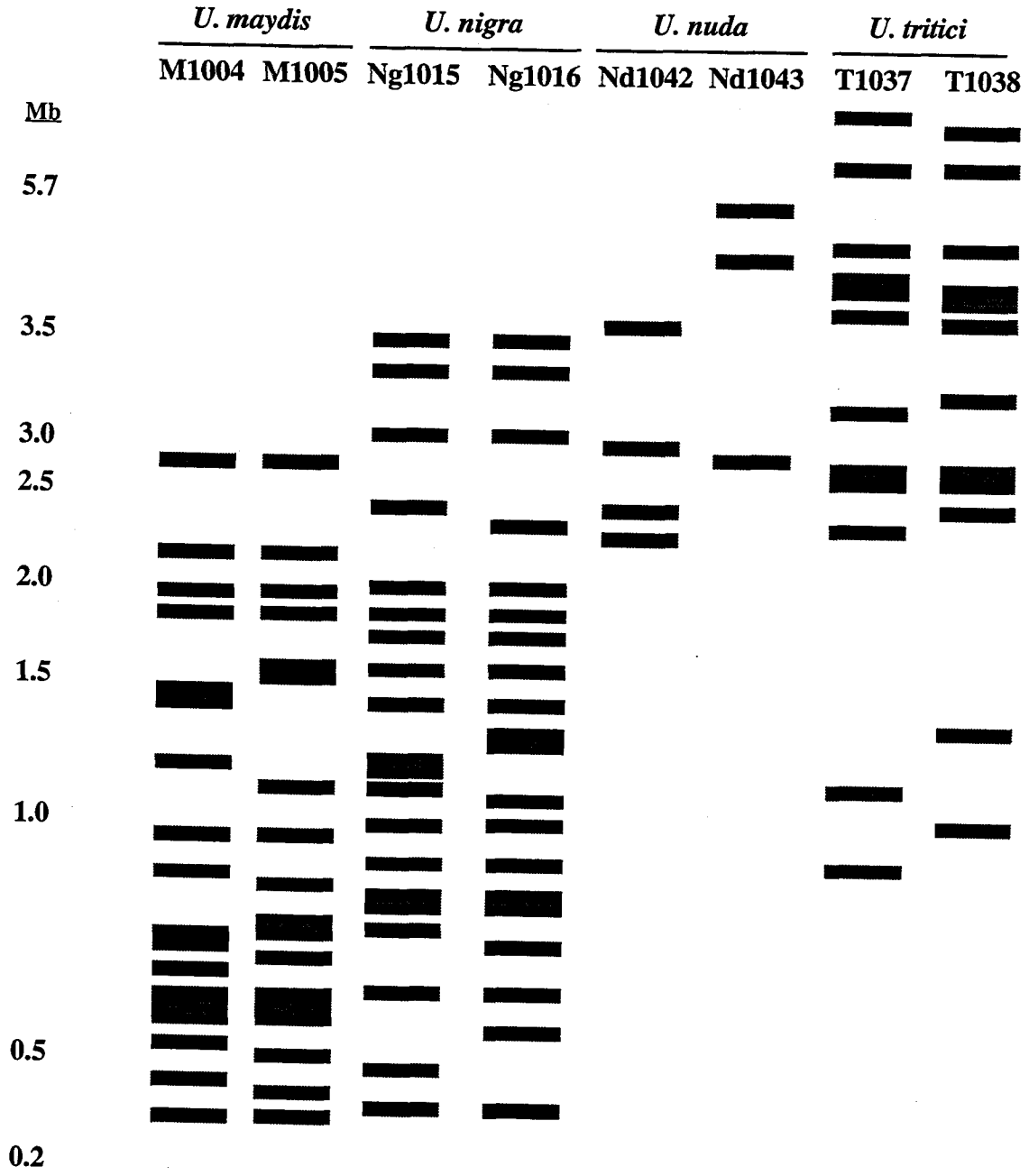

Fig. III.3. (Continued)

bands stained more intensely than others with ethidium bromide and were interpreted to contain multiple chromosomes of similar size.

The karyotypes differed among the eight species, and also among strains of the same species. The electrophoretic karyotype of *U.tritici*, and possibly *U. nuda*, was dramatically different from those of the nonsporidium-forming species. The smallest chromosomes of *U. tritici* were approximately 4 fold larger than the smallest chromosomes of the sporidium-forming species (Table III.2). The largest chromosomes of all species, excluding *U. tritici* and *U. nuda*, range from ca. 2.1 to 3.6 Mb. The electrophoretic karyotype of *U. nuda* strains revealed only 3 or 4 chromosomes that ranged from ca. 2.3 to 5.6 Mb, suggesting that the karyotype was not fully resolved.

To ascertain whether *U. nuda* has additional undetected chromosomes, a telomere-specific probe was used to estimate the minimum number of chromosomes in this species (Mills et al. 1995). In genomic digests of both strains, 14 or 16 bands hybridized with the probe (Fig. 4) suggesting that the karyotypes of these strains are comprised of at least 7 chromosomes which are not fully resolved by CHEF PFGE. *U. tritici* strains have karyotypes with 10 bands which range in size from 940 Kb to 6.3 Mb. The largest chromosomal band in both strains of *U. tritici* was larger than chromosome III (5.7 Mb) of *S. pombe,* and its size was estimated by extrapolating the standard curve based on *S. pombe* chromosomes. Strains of *U. tritici* had the largest genome sizes among all species.

The sporidium-forming species have a wider distribution of chromosome sizes and the number of chromosome bands resolved varied between 13 and 17. The chromosomes

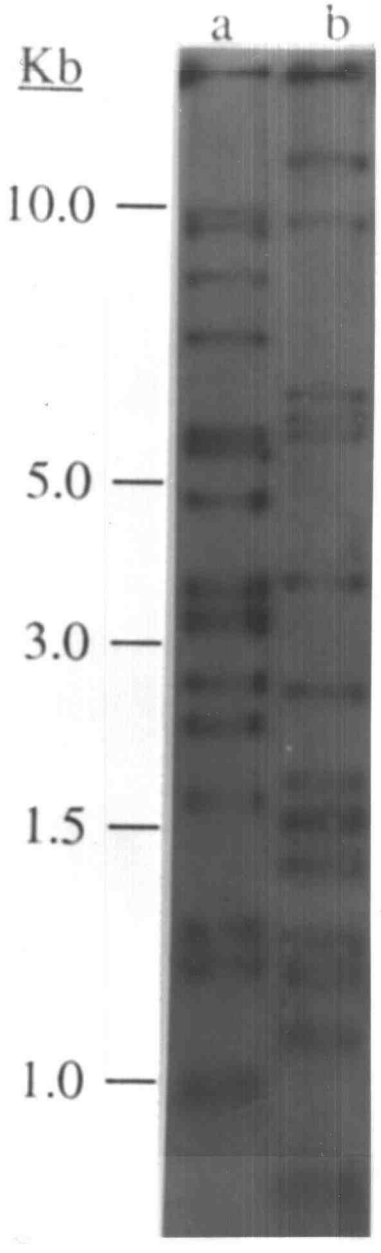

Fig. III.4. Estimation of the minimum number of chromosomes in *Ustilago nuda* strains. Lanes: a, *U. nuda* 1042; b, *U. nuda* 1043. Southern-blot hybridization of total genomic DNA from *U. nuda* digested with *Eco*RI and hybridized with the telomere repeat, $[TTAGGG]_{18}$, from *Fusarium oxysporum.*

of *U. bullata*, *U. hordei*, and *U. maydis* have a similar size distribution, with as many as half of the bands smaller than 1.0 Mb. The strains of *U. avenae, U. kolleri*, and *U. nigra* have similar overall karyotypes in that most of the bands are larger than 1.0 Mb and chromosomal bands larger than 3 Mb were resolved in the karyotypes of all three species(Fig. III.2). The genome sizes of the sporidium-forming species ranged from 17 to 27 Mb.

Assignment of DNA probes to chromosomes: A variety of DNA fragments was used to identify homologs in the eight species and to look for possible linkage among the probes. Some of the Southern hybridizations are presented in Figure III.5, and the results of all hybridizations are summarized in Table III.3. Probes made of seven unlinked anonymous fragments from the genome of *U. hordei* did not hybridize to the chromosomes of *U. nuda* and *U. tritici*, and no signal was detected when 2 of these probes were hybridized at low stringency to a blot containing *Eco*RI-digested total genomic DNA from these two species. Among these probes, only three hybridized to *U. maydis* chromosomes, and one probe was not homologous with *U. bullata* chromosomes.

Fragments that were unlinked in one strain may be linked in a second strain of the same species. For example, probes pOSU650 and pOSU560 were linked on the 530 Kb chromosome of *U. maydis* strain M1004, while they hybridized to two distinct chromosomes in *U. maydis* strain M1005. Patterns of similarity emerged, however, as the linkage groups of strains from different species appeared to have similar organization. The similarities were most striking between strains of *U. avenae* and *U. kolleri* where 5 of 10

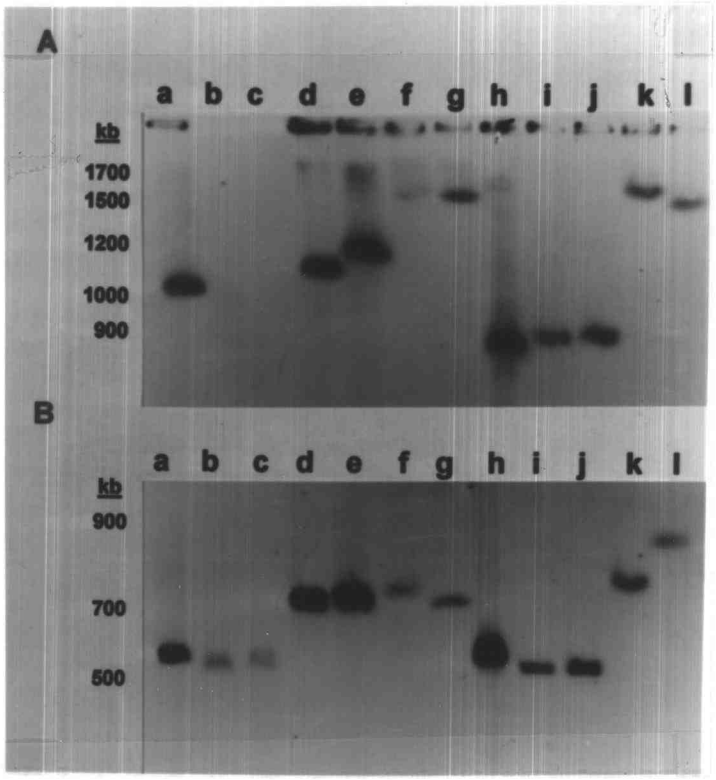

Fig. III.5. Hybridization of two anonymous probes, pOSU1090 (A) and pOSU650 (B), from the genome of *U. hordei* to the chromosomes of *Ustilago spp.* Lanes: a, *U. hordei* 8.2a; b, *U. maydis* 1004; c, *U. maydis* 1005; d, *U. nigra* 1015; e, *U. nigra* 1016; f, *U. kolleri* 1013; g, *U. kolleri* 1014; h, *U. bullata* 1032; i, *U. bullata* 1033; j, *U. bullata* 1034; k, *U. avenae* 1031; l, *U. avenae* 1036.

Table III.3. Localization of anonymous DNA fragments and heterologous genes to chromosomes of *Ustilago* species.

	Strains					
	U. avenae		***U. bullata***			***U. hordei***
Probes	A1031[a]	A1036	B1032	B1033	B1034	H8.2a
pOSU1090	1760[b]	1700	840	900	935	1090
pOSU1040	1085	1070	840	900	875	1040
pOSU980	1085	1070	-[c]	-	-	980
pOSU920	1085	1180	720	1010	1030	920
	1035	1070			720	720
		965				
pOSU850	965	965	750	720	875	850
pOSU650	865	965	420	825	625	650
pOSU560	865	965	570	520	515	560
pSF8	865	965	420	430	435	560
pOSU1101	2770	2820	1330	1615	1615	1900
	2690					
pBH100	3580	3600	2720	2750	2750	3000

Table III.3. (Continued)

Probes	Strains					
	U. kolleri		*U. maydis*		*U. nigra*	
	K1013	**K1014**	**M1004**	**M1005**	**Ng1015**	**Ng1016**
pOSU1090	1690	1690	-	-	1120	1200
pOSU1040	1070	1045	-	-	1075	1035
pOSU980	1070	1045	530	505	1075	1035
pOSU920	1150	1110	-	-	1120	1200
	1070	1045			1075	1035
					885	885
pOSU850	1070	1045	-	-	1010	955
pOSU650	810	760	530	505	780	780
pOSU560	810	760	530	505	780	780
pSF8	810	760	450	420	620	620
pOSU1101	2150	2190	1700	1700	2350	2300
pBH100	3600	3600	2800	2800	3400	3400

Table III.3. (Continued)

Probes	Strains			
	U. nuda		*U. tritici*	
	Nd1042	**Nd1043**	**T1007**	**T1038**
pOSU1090	-	-	-	-
pOSU1040	-	-	-	-
pOSU980	-	-	-	-
pOSU920	-	-	-	-
pOSU850	-	-	-	-
pOSU650	-	-	-	-
pOSU560	-	-	-	-
pSF8	2350	2600	1185	1320
pOSU1101	2740	4100	4600	4600
pBH100	-	-	3600	3620

[a] For strain designations see Table 1

[b] Chromosome band size, in Kb, identified by the probe

[c] -: no homology detected

probes were linked to two chromosomes in strains of both species. Two of the 10 probes, pOSU1040 and pOSU980, hybridized with a chromosome that was of ca. 1070 Kb in all strains of these species, whereas, pOSU650, pOSU560, and pSF8 hybridized with a chromosome of ca. 820 ± 50 Kb. Of the remaining probes, 2 anonymous probes and the probe made of the *b* East region of the mating type gene of *U. hordei,* each hybridized with a different chromosome, and probe pOSU920 hybridized to either 2 or 3 chromosomes in strains of *U. avenae* and *U. kolleri.* Probe pOSU920 also hybridized with 2 or more chromosomes in *U. hordei, U. nigra,* and *U. bulatta,* with the exception that in strain B1032 of *U. bulatta,* it hybridized to a cluster band. The actin gene probe, pSF8, hybridized to the fifth smallest chromosome in *U. avenae, U. kolleri,* and *U. nigra* strains; the third smallest chromosome in *U. bullata, U. hordei* and *U. tritici* strains, and the second smallest chromosome in *U. maydis* strains. The rDNA probe, pOSU1101, hybridized to one chromosome in all strains, except in strain A1036 of *U. avenae,* in which two chromosomal bands hybridized to the probe. The mating type gene probe, pBH100, hybridized to a single band either the largest or second largest chromosome, in all strains except in *U. tritici* strains, which ranged from 2.1 to 3.6 Mb. The probe did not hybridize with any chromosomes in the partial karyotype of *U. nuda.*

Chromosome-length polymorphisms: Chromosome-length polymorphisms among strains of the sporidium-forming species, *U. avenae, U. bullata, U. hordei, U. kolleri, U. maydis,* and *U. nigra,* were further analyzed by determining a statistical coefficient of variability for chromosomes identified by each probe (Table III.4). The smallest values were observed in

Table III.4. Pairwise analysis of chromosome length polymorphisms among sporidium-forming *Ustilago* species

	Pairs examined for chromosome length polymorphisms						
	U. av	***U. av***	***U. av***	***U. av***	***U. av***	***U. b***	***U. b***
	X	***X***	***X***	***X***	***X***	***X***	***X***
Probe	***U. b***	***U. h***	***U. k***	***U. m***	***U. ng***	***U. h***	***U. k***
pOSU1090	38[a]	24	2	-	25	11	36
pOSU1040	12	2	2	-	2	10	11
pOSU980	-[b]	5	2	35	2	-	-
pOSU920	16	17	2	-	3	9	17
pOSU850	11	7	5	-	3	7	15
pOSU650	34	19	10	33	11	19	26
pOSU560	31	26	10	33	11	5	22
pSF8	43	26	10	42	23	14	34
pOSU1101	32	17	13	25	9	14	21
pBH100	15	10	0.2	14	3	5	15

Table III.4. (Continued)

	Pairs examined for chromosome length polymorphisms							
	U. b X	*U. b* X	*U. h* X	*U. h* X	*U. h* X	*U. k* X	*U. k* X	*U. m* X
Probe	*U. m*	*U. ng*	*U. k*	*U. m*	*U. ng*	*U. m*	*U. ng*	*U. ng*
pOSU1090	-	13	23	-	2	-	23	-
pOSU1040	-	11	2	-	2	-	2	-
pOSU980	-	-	5	33	5	34	2	34
pOSU920	-	14	18	-	14	-	4	-
pOSU850	-	12	12	-	9	-	5	-
pOSU650	14	25	11	14	10	24	3	23
pOSU560	5	21	19	5	18	24	3	23
pSF8	3	21	19	15	6	33	14	20
pOSU1101	10	25	8	7	11	14	4	18
pBH100	1	12	10	4	7	14	3	11

[a]: Percentage of variability depicted by the probe = (standard deviation from the average size / statistical mean of sizes of chromosomes detected by the probe) X 100

[b]-: Probe did not detect homology with either of the two species

pairwise comparison of *U. avenae* and *U. kolleri* chromosomesad, where CLP values ranged between 2 and 11%. The highest CLP values were detected between *U. avenae* and *U. maydis* with all probes depicting more than 15% variability. *U. hordei* had the least CLPs when compared to *U. nigra* with all values in the range of 2 to 18%, and only 3 of the 10 probes detected more than 10% variability. *U. bullata* had the smallest values of polymorphism when compared to *U. maydis*. However, four of the probes that did not hybridize to *U. maydis* did hybridize with *U. bullata*, and one probe that did not hybridize with *U. bullata* chromosomes did hybridize with *U. maydis* chromosomes.

Discussion

Electrophoretic karyotypes were obtained from intact spores or mycelia from seven *Ustilago* species without the need for protoplasting (McCluskey et al. 1991; McCluskey 1991). However, only an incomplete karyotype was obtained from two strains of *U. nuda*, and neither protoplasting nor nonprotoplasting procedures were effective in resolving clear bands, thereby precluding an estimate of the number of chromosomes per haploid cell and the size of its genome. The karyotypes of these smut species differed both in respect to the numbers of bands detected in the representative strains of any species, and the overall distribution of the sizes of the bands among the species. The number of chromosome bands varied between 10 and 17, and the minimum genome sizes of these species were estimated to be between 17 and 39 Mb. The estimated sizes of the four largest chromosomes of *U.*

hordei strain 8.2a were approximately 300 to 500 Kb smaller than previous estimates (McCluskey and Mills 1990). The previous estimates were obtained by extrapolation from a standard curve of *S. cerevisiae* chromosomes, the largest of which is 2.2 Kb, whereas, in this study, the size standards extended to 5.7 Mb providing more accurate estimates.

Variations in chromosome numbers and genomes sizes have been reported for species of the genus *Trichoderma* (Herrera-Estella et al. 1993), *Tolypocladium* (Stimberg et al. 1992), and strains of *Fusarium spp.* (Migheli et al. 1993; Fekete et al. 1993). Karyotypic variability was also observed among strains of each species in this study, which is not unique to the smut fungi, as polymorphic chromosomal patterns were previously reported for 14 physiologic races of *U. hordei* (McCluskey and Mills 1990), for isolates of bunt fungi (Russell and Mills 1993, 1994) and vegetative compatibility groups of *F. oxysporum* f. sp. *cubense* (Boehm et al. 1994). The intra-species electrophoretic karyotype variation could be attributed to a variety of genetic phenomena including changes resulting from deletions (Masel et al. 1993; Agnan and Mills 1994), translocations (Thrash-Bingham and Gorman 1992), tolerance of aneuploidy (Bakalinsky and Snow 1990), and the presence of supernumerary chromosomes (Miao et al. 1991; Kistler and Benny 1992).

One goal in undertaking this study was to determine whether electrophoretic karyoptypes and the assignment of markers to chromosomes by Southern hybridization could provide a useful tool for delineating species within the *Ustilaginales.* Some of the species used in this study have been the subject of taxonomic uncertainty due to their similarities in spore morphology and biology. Using spore morphology as a criterion,

Lindeberg and Nannfeldt (1959) proposed to unite the sporidium-forming species *U. avenae, U. kolleri, U. hordei,* and *U. nigra* into a single species *U. segetum.* Subsequently, Langdon at al. (1976) proposed that *U. nigra* and *U. avenae*, which cause false loose smut of barley and loose smut of oats, respectively, are synonymous, as were *U. hordei* and *U. kolleri,* which cause covered smut of barley and oats, respectively. Huang and Nielsen (1984) supported a con-specific classification scheme for all four species based on sexual compatibility and the production of viable offspring. However, a positive mating reaction alone may not constitute sufficient evidence to support a con-specific status. The *b* East region of the *b* mating type gene of *U. maydis* was demonstrated by Southern hybridization to have homology with DNA from 8 *Ustilago* species including *U. avenae, U. hordei, U. kolleri,* and *U. nigra* (Bakkeren et al. 1992). Bakkeren and Kronstad (1993) subsequently cloned the mating type gene of *U. hordei* by homology with the *b* locus of *U. maydis.* When the *b* gene complex from *U. hordei* was introduced into *U. maydis*, the resulting haploid transformants were weakly pathogenic on maize, demonstrating that structurally and functionally conserved *b* genes are present in these two distinct species.

In this study we used chromosome-length polymorphisms as a criterion to ascertain or reject synonymies proposed in previous reports. Chromosome-length differences of as much as 20 percent were detected for some homologous chromosomes of *Septoria tritici*, whereas maximum variability of 15 percent was typical for homologous chromosomes of *U. hordei* (McCluskey et al. 1994), and *T. caries* and *T. controversa* (Russell and Mills 1993, 1994). Values for the smut and bunt fungi were calculated as the range in the size of the

chromosomes to which a probe hybridized, divided by the mean of the sizes of those chromosomes. Using the statistical method described in Table III.4, which minimizes over-estimation of variability attributed to outliers, CLPs of 5% or less were observed for eight chromosomes among strains representing the 14 physiologic races of *U. hordei* (McCluskey et al. 1994), and for chromosomes of the field isolates of *T. caries* and *T. controversa* (Russell and Mills 1993, 1994, and Mills, unpublished data). Using this statistical method to evaluate similarities among putative homologous chromosomes of *Ustilago* species suggests some credence for a con-specific status for certain of these fungi. For example, the karyotypes of *U. avenae* and *U. kolleri* are similar, and their genomes appear to have a similar organization based on the hybridization pattern of the 10 probes (Table III.3). Chromosome-length polymorphisms varied from 2 to 11 % supporting the synonymy proposed between these two species. Pairwise comparisons with *U. nigra* revealed that this species is more similar to *U. hordei* than to *U. avenae* and *U. kolleri* (Table III.4). Only one probe, pOSU560, detected a polymorphism of more than 15% between *U. nigra* and *U. hordei*, while 2 probes revealed more than 15% polymorphism among strains of *U. nigra* and strains of *U. avenae* or *U. kolleri.* Linkage relationships also differed between chromosomes of *U. nigra* and those of *U. avenae* and *U. kolleri.* For example, the actin gene and two anonymous probes, pOSU650 and POSU560, are linked in *U. avenae* and *U. kolleri,* but are unlinked in *U. nigra.* Even larger CLPs were observed between chromosomes of *U. hordei* and putative homologs of *U. avenae* and *U. kolleri.* The lesser divergence between strains of *U. hordei* and *U. nigra* could be attributed to sexual

compatibility and growth on the same host. The magnitudes of CLPs detected by pairwise analysis among the seedling-infecting *Ustilago spp.* of barley and oats, *U, avenae, U. kolleri, U. hordei,* and *U. nigra*, corroborate the interspecific hybridization data reported by Huang and Nielsen (1984) which revealed that more viable offspring could be recovered from the interspecific cross *U. avenae* X *U. kolleri* than in the cross *U. hordei* X *U. kolleri.* The differences in genome organization we report between *U. hordei* and *U. kolleri* could explain the low viability of progeny from crosses of these two species, while the similarity in the genome organization between *U. avenae* and *U. kolleri* leads to a more efficient pairing of the homologs during meiosis and, hence, greater viability of the offspring.

Pairwise analysis of chromosome-length polymorphisms revealed that *U. bullata* diverged more from *U. avenae, U. kolleri,* and *U. nigra* than from *U. hordei.* Only one probe detected more than 15% polymorphisms between *U. bullata* and *U. hordei*, while greater CLPs were detected between *U. bullata* and each of the other bipolar smut fungi with at least 4 probes depicting more than 20% polymorphisms. Among the seven probes from the genome of *U. hordei* only three had homology with *U. maydis* chromosomes, supporting the taxonomic distinctness of this species from the rest of the smut fungi. These results are also consistent with the analysis of 5S rRNA sequences (Blanz and Gottschalk 1984) which showed that *U. maydis* is evolutionarily separated from the other smut fungi.

Based on infection type and spore morphology, *U. nuda* and *U. tritici* which cause loose smut on barley and wheat, respectively, have been classified as specialized varieties of the same species (Fischer and Holton 1957). A proline requirement that is linked to one of

the two mating types of *U. nuda* has been used as a criterion to distinguish *U. nuda* from *U. tritici* (Nielsen 1972). Southern analysis with the telomere-specific probe detected at least 7 chromosomes in two strains of *U. nuda*, while CHEF PFGE resolved 10 chromosomes in strains of *U. tritici* suggesting that the karyotypes of these two species have similar numbers of chromosomes. However, until a complete karyotype of *U. nuda* is resolved, this methodology can not be used to support or reject the synonymy of these species. The chromosomes of both species did not hybridize to 7 radiolabeled anonymous probes from the genome of *U. hordei.* A probe from the mating type gene of *U. hordei* revealed only weak homology with *U. tritici* chromosomes and none with *U. nuda.* These Southern hybridization analyses suggest an ancestry of these two species that is more divergent from that of the sporidium-forming species.

The results of this study demonstrate that although the electrophoretic karyotypes of the smut fungi are variable, they could be used as a useful and practical addendum for analyzing the taxonomy in the order *Ustilaginales.* The chromosome-length polymorphisms detected by Southern hybridizations could be explained by gross mutations (duplications, insertions, and deletions) that became fixed in the course of evolution through the high rate of inbreeding that characterizes the genus *Ustilago.* The cloning of pathogenicity genes will further our insights into the mechanisms of adaptation to the host that led to the divergence among these species.

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CHAPTER IV

SEQUENCE ANALYSIS OF THE INTERNAL TRANSCRIBED SPACER REGION IN EIGHT *Ustilago* SPECIES AND IMPLICATIONS FOR TAXONOMIC RELATIONSHIPS

Abstract

The regions coding for the 5.8S ribosomal RNA (rRNA) and the flanking internal transcribed spacers (ITS1 and ITS2) from eight *Ustilago* *U. avenae, U. bullata, U. hordei, U. kolleri, U. maydis, U. nigra, U. nuda*, and *U. tritici*, were amplified by the polymerase chain reaction (PCR) and sequenced. Direct double-stranded sequencing yielded approximately 700 bases for all species under investigation. The sequence of the 5.8S rDNA gene from *U. maydis* revealed seven nucleotide substitutions relative to the invariant consensus sequence determined from the other smut fungi. The ITS regions of *U. avenae, U. hordei, U. kolleri,* and *U. nigra*, were determined to have 97-99% sequence identity, providing additional support for their conspecific classification. The ITS regions of these four pathogens revealed 10-12% sequence divergence from *U. bullata, U. maydis, U. nuda*, and *U. tritici.* The taxonomic relatedness among these eight smut fungi based on the comparison of the sequences of their ITS regions, and the feasibility of using PCR primers specific to the ITS regions for the detection of these *Ustilago* species, are discussed.

Key words: ribosomal DNA, intergenic transcribed spacer, taxonomic relatedness, *Ustilago*

Introduction

Phylogenetic relationships between different fungal lineages have long been a matter of controversy, and different theories exist concerning their evolutionary origins (Burnett 1987). Fungi were traditionally classified according to various morphological, developmental, and biochemical characteristics (Heim 1948, Hughes 1953, Kendrick 1981, Kreger-VanRij 1984). Contradictory phylogenetic schemes usually reflect differences in traits used to infer relationships. The relative importance assigned to the same traits by different fungal systematists, and the phenotypic plasticity of the selected traits also have contributed to taxonomic uncertainties. Thus, the correct identity of a particular isolate and the inference of phylogenetic relationships remain controversial.

An alternative to morphotaxonomy is obtaining an estimate of the extent of genetic relatedness by comparing nucleic acids sequences. Phylogenetic relationships may be inferred objectively and quantitatively from similarities and differences in sequences of genes having a common ancestry (Sogin et al. 1989). Regions most commonly used for phylogenetic analysis are the regions coding for nuclear and mitochondrial large-subunit ribosomal RNAs (rRNAs), and the internal transcribed spacer (ITS) that resides between the regions coding for nuclear small- and large-subunit rRNA, and which also includes the coding region for the 5.8S rRNA. The sequences coding for the rRNAs (rDNAs) change very slowly and are useful for phylogenetic studies among relatively distant organisms (Berbee and Taylor 1992, Bruns et al. 1990).

On the other hand, the ITS regions change more rapidly, and are useful for the study of closely-related organisms (Hillis and Dixon 1991).

The ITS region is flanked by highly conserved rRNA coding regions, permitting its amplification by the polymerase chain reaction (PCR) technique (White et al. 1990). The sequence information generated from the resulting PCR products was useful in providing insights in phylogenetic studies of several fungal species groups. Morales et al. (1993) amplified the ITS regions from isolates of the blackleg pathogen *Leptosphaeria maculans,* and found that differences in ITS sequences correlated with pathogenicity grouping. Sequence variation of the ITS regions has been used to study relationships among *Phytophthora* species (Lee and Taylor 1992), cereal and grass rusts (Zambino and Szabo 1993), and ascomycetous yeasts (Geber et al. 1992). Species-specific primers from the ITS regions were used to amplify rDNAs probes that could detect verticillium wilt pathogens (Nazar et al. 1991), and two *Ophiosphaerella* species (Tisserat et al. 1994).

Classification problems are compounded when morphologically similar organisms produce similar disease symptoms and have overlapping host ranges, as is the case with different species in the genus *Ustilago*. In this genus, sorus morphology has been used to distinguish among different species (Fischer and Holton 1957). A *Ustilago spp.* with echunilate spores could be either *Ustilago tritici*, *U. nigra*, *U. nuda*, *U. avenae*, or *U. bullata*. The type of germination of the teliospores allows one to distinguish *U. tritici* and *U. nuda,* which form mycelia upon germination, from *U. nigra*, *U. avenae,* and *U. bullata,* which form sporidia upon germination. But, it is virtually impossible to

distinguish between *U. nigra*, *U. avenae*, and certain strains of *U. bullata* by any easily recognizable morphological trait, either macro- or microscopic. The same problem exists with *U. hordei* and *U. kolleri* which produce smooth-walled teliospores.

This study was undertaken to determine the extent of inter-species differences over the entire ITS region of eight *Ustilago* species. The ITS regions were amplified by PCR using universal primers (White et al. 1990). The sequence data and its correlation are discussed with respect to species delineation within the genus *Ustilago.*

Materials and Methods

Fungal isolates and DNA extraction: Isolates used in this study and their hosts are listed in Table IV.1. All cultures were maintained on potato dextrose agar (PDA, Difco Laboratories, Detroit, MI) slants. For extraction of total genomic DNA, sporidial and mycelial cultures were cultivated in 100 ml of potato dextrose broth (PDB) on a rotary shaker (250 rpm) at 28°C for 48 hr. DNA was extracted as described by Lee et al. (1988) and stored at -20°C until required for amplification.

PCR amplification and purification: PCR amplification was conducted in a total volume of 50 µl and contained 10-100 ng genomic DNA, 1.5 mM $MgCl_2$, 200 mM of each deoxyribonucleotide triphosphate, 100 µM of each of the oligonucleotide primers, and 2.5 units Amplitaq DNA polymerase (Perkin Elmer, Foster City, CA). Primers for PCR amplification of the ITS regions were ITS4 and ITS5 described by White et al.

Table IV.1. Fungal strains used in this study

Strains	Relevant characteristics	Host	Source
U. avenae 1031	Product of a single spore	Oats	L.J. Szabo[a]
U. avenae 1036	Product of a single spore	Oats	B.J.Goats[b]
U. bullata 1032	Product of a single spore	Wild grasses	B.J.Goats
U. bullata 1034	Product of a single spore	Wild grasses	B.J.Goats
U. hordei 1006	Product of a single spore	Barley	L.J. Szabo
U. hordei 8.2a	Product of a single spore	Barley	W.L. Pedersen[c]
U. kolleri 1013	Product of a single spore	Oats	P. Koepsell[d]
U. kolleri 1014	Product of a single spore	Oats	P. Thomas[e]
U. maydis 1004	Product of a single spore	Corn	K.B. Johnson[f]
U. maydis 1005	Product of a single spore	Corn	J. Sherwood[g]
U. nigra 1015	Product of a single spore	Barley	P. Thomas
U. nigra 1016	Product of a single spore	Barley	P. Koepsell
U. nuda 1042	Product of a promycelium	Barley	B.J.Goats
U. nuda 1043	Product of a promycelium	Barley	B.J.Goats
U. tritici 1007	Product of a promycelium	Wheat	L.J. Szabo
U. tritici 1038	Product of a promycelium	Wheat	B.J.Goats

[a]: USDA-ARS, Univ. of Minnesota, St Paul
[b]: ARS, Aberdeen, ID
[c]: Univ. of Illinois, Urbana
[d]: Oregon State Univ., Corvallis
[e]: Agriculture Canada, Winnipeg
[f]: Oregon State Univ., Corvallis
[g]: Montana State Univ., Bozeman

(1990), and synthesized by the central service laboratory at Oregon State University. The reactions were performed for 40 cycles in a DeltaCycler II™ DNA thermocycler (ERICOMP, San Diego, CA) following an initial 2 min at 94°C to denature the DNA, and 10 min at 72°C for extension at the conclusion of the final cycle. Each cycle consisted of 30s at 94°C followed by 60s at 55°C for annealing and 2 min at 72°C for extension.

Double-stranded PCR products were purified by preparative gel electrophoresis of the amplified products in 1% low melting point agarose (FMC Bioproducts, Rockland, ME). Bands were excised under UV illumination and the DNAs were eluted using a QIAaquick gel extraction kit (QIAGEN, Chatsworth, CA).

DNA sequencing and sequence alignment: Direct sequencing of PCR products was done using the two amplification primers, ITS4 and ITS5, and two internal primers, ITS 2 and ITS3 (White et al. 1990), in an automated sequencer(Applied Biosystem 373A, ABI, Foster City, CA) by the Taq DyeDeoxi terminator™ cycle system. DNA sequences were analyzed with the Genetic Computing Group (GCG) package (Devereux et al. 1984), using BestFit for pairwise alignments and determining the percentages of sequence similarity, and Pretty for the multisequence alignment.

Results

ITS4 and ITS5 PCR primers (White et al. 1990) amplify a region of DNA extending from the 3' end of the gene coding for the small ribosomal subunit to the 5' end of the gene coding for the large ribosomal subunit, including the 5.8S rDNA gene which separates ITS regions ITS1 and ITS2 (Fig IV.1). Approximately 750 base pairs (bp) were amplified by primer pair ITS4/ITS5 for two strains of the 8 smut species (Table IV.1). Aligned sequences for each pair of strains revealed only a maximum of 5 nucleotide changes. Therefore, the ITS sequence from only one strain of each species is presented in Figure IV.2. Typically, complete sequences were obtained using primers ITS3, ITS4, and ITS5 in all eight smut fungi, whereas sequence data could not be obtained using the internal primer ITS2. A pairwise comparison of rDNA sequence similarity among the eight *Ustilago* species is presented in Table IV.2.

To determine the position of the ITS regions and the 5.8S rDNA gene, the sequence data obtained from the *U. hordei* strain was used to search for sequence homology in other fungi. A search in the GenBank data base revealed that the sequence from nucleotides 124-330 has extensive homology with the 5.8S rDNA genes of different fungi. Hence, the nucleotide region 1-123 has been designated ITS1, and nucleotide region 331-700 was termed the ITS2 region.

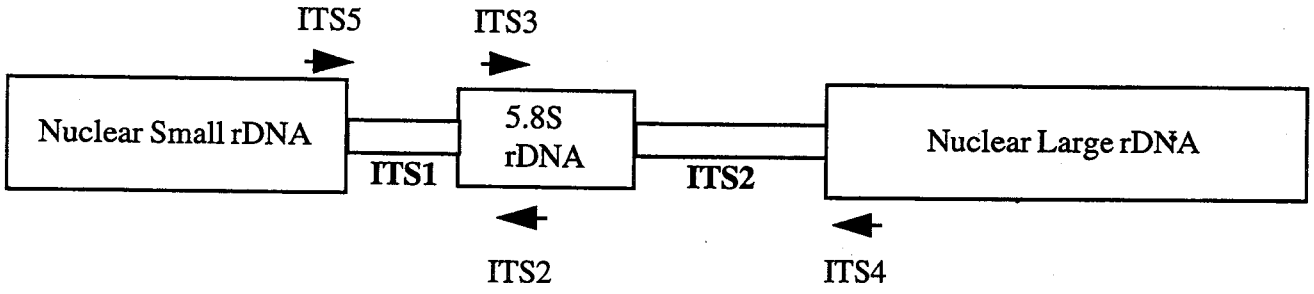

Fig. IV.1. Universal oligonucleotide primers used for the PCR amplification and direct sequencing of nuclear rDNA in *Ustilago spp.* Arrows represent the primers used in PCR amplifications and direct sequencing reactions (adapted from White et al. 1990).

```
                        1                                                  50
Ustilago hordei         TAGTAGGTGA ACCTGCAGAT GGATCATTTC GATGGAAAAA CCTTTTTTCA
Ustilago avenae         ---------- ---------- ---------- ------ggg- ----------
Ustilago bullata        ---------- ---------- attcg----- t---cgc--- ----------
Ustilago kolleri        ---------- ---------- ---------- ----a-a--c -t--------
Ustilago maydis         ---------- ---------- ---------- ----a-ctgc g----g----
Ustilago nigra          ---------- ---------- ---------- ----c-t--t ----------
Ustilago nuda           ---------- ---------a ---------a -gatc-tt-- tgaa-c--t-
Ustilago tritici        ---------- ---------g ---------- ----a-c--t ----------

                        51                                                 100
Ustilago hordei         GAGGTGTGGC TCGCACCTGT CCAACTAAAC TTGAGCTACC TTTTTCAACA
Ustilago avenae         ---------- ---------- ---------- ---------- ----------
Ustilago bullata        --------tt --cac----- t-gcta--ct -gagct-c-- c---------
Ustilago kolleri        ---------- ---------- ---------- ---------t ----------
Ustilago maydis         ---------- --t--g---- gg-t------ ---------- g---------
Ustilago nigra          --------c- --a------- ---------- ---------- ----------
Ustilago nuda           tga-gcc-ta --t-tt---- a--t-g--t- -aa---c-t- a-c-att-tc
Ustilago tritici        tga-gcc-ta ------g--- ----gc--g- --tgagct-- cc---t-ca-

                        101                                                150
Ustilago hordei         CGGTTGCATC GGTCGGCCTG TCAAACAGTG CGACGCAAGG AGAAAATCCT
Ustilago avenae         ---------- ------g--- ---------- ---------- ----------
Ustilago bullata        ---g------ --gtc-g-ct gtc------- ---------- ----------
Ustilago kolleri        ---------- ---------- ---------- ---------- ----------
Ustilago maydis         a--c------ ---------- -----ac--- t--------- ----------
```

Figure IV.2. Aligned sequence data of the DNA fragments amplified by primers ITS4/ITS5 in eight *Ustilgo* species. A hyphen represents a base identical to that of the top sequence. A period represents a gap.

```
                        101                                                 150
Ustilago hordei         CGGTTGCATC GGTCGGCCTG TCAAACAGTG CGACGCAAGG AGAAAATCCT
Ustilago nigra          ---c------ ---------- ---------- ---------- ----------
Ustilago nuda           -aaaca--.. .....-gag- ggtgg----- ---------- ----------
Ustilago tritici        t--aaa--ca a-a-ta-gga --ggtg---- ---------- ----------

                        151                                                 200
Ustilago hordei         CGCGTCTGCT GGGCGACGGA CAGACAATTT TATTGAACAC TTTTTGATGA
Ustilago avenae         ---------- ---------- ---------- ---------- ----------
Ustilago bullata        ---------- ---------- ---------- ---------- ----------
Ustilago kolleri        ---------- ---------- ---------- ---------- ----------
Ustilago maydis         ttt------- ---------- ---------- g--------- ----g-----
Ustilago nigra          ---------- ---------- ---------- ---------- ----------
Ustilago nuda           ---------- ---------- ---------- ---------- ----------
Ustilago tritici        ---------- ---------- ---------- ---------- ----------

                        201                                                 250
Ustilago hordei         TCTAGGATTT GAAGGAGAAA AAGTCATTTT TACGAATGAA ATCGACTGGT
Ustilago avenae         ---------- ---------- ---------- ---------- ----------
Ustilago bullata        ---------- ---------- ---------- ---------- ----------
Ustilago kolleri        ---------- ---------- ---------- ---------- ----------
Ustilago maydis         ---------- ---------- --a------- ---a------ ----------
Ustilago nigra          ---------- ---------- ---------- ---------- ----------
Ustilago nuda           ---------- ---------- ---------- ---------- ----------
Ustilago tritici        ---------- ---------- ---------- ---------- ----------
```

Figure IV.2. (Continued)

```
                    251                                                 300
Ustilago hordei     AATGCGGTCG TCTAATTTTA AAAACAACTT TTGGCAACGG ATCTCTTGGT
Ustilago avenae     ---------- ---------- ---------- ---------- ----------
Ustilago bullata    ---------- ---------- ---------- ---------- ----------
Ustilago kolleri    ---------- ---------- ---------- ---------- ----------
Ustilago maydis     ---------- ---------- ---------- ---------- ----------
Ustilago nigra      ---------- ---------- ---------- ---------- ----------
Ustilago nuda       ---------- ---------- ---------- ---------- ----------
Ustilago tritici    ---------- ---------- ---------- ---------- ----------

                    301                                                 350
Ustilago hordei     TCTCCCATCG ATGAAGAACG CAGCGAATTG CGATAAGTAA TGTGAATTGC
Ustilago avenae     ---------- ---------- ---------- ---------- ----------
Ustilago bullata    ---------- ---------- ---------- ---a-g-a-t gtga-t-gca
Ustilago kolleri    ---------- ---------- ---------- ---------- ----------
Ustilago maydis     ---------- c--------- ---------- -------g-- --------c-
Ustilago nigra      ---------- ---------- ---------- ---------- ----------
Ustilago nuda       ---------- ---------- ---------- ---------- ----------
Ustilago tritici    ---------- ---------- ---------- ---------- --------a-

                    351                                                 400
Ustilago hordei     AGAAGTGAAT CATCGAATCT TTGAACGCAC CTTGCGCTCC CGGCAGATCT
Ustilago avenae     ---------- ---------- ---------- ---------- ----------
Ustilago bullata    ga-....... ..-----g-- --t------- ---------- t---------
Ustilago kolleri    ---------- ---------- ---------- ---------- ----------
Ustilago maydis     --------g- -------a-- --a------- ---------- ----------
Ustilago nigra      ---------- ---------- ---------- ---------- ----------
```

Figure IV.2. (Continued)

```
                        351                                                 400
Ustilago hordei         AGAAGTGAAT CATCGAATCT TTGAACGCAC CTTGCGCTCC CGGCAGATCT
Ustilago nuda           ---------- ---------- ---------- -----at--- -aagg-----
Ustilago tritici        --------t- -------g-- --t------- ---------- ----------

                        401                                                 450
Ustilago hordei         AATCTGGGGA GCATGCCTGT GTGAGGGCCG CGAATTGTTT CGAACGACAG
Ustilago avenae         ---------- ---------- t--------- ---------- ----------
Ustilago bullata        -c-------- --------cg t--------- ---------- ----------
Ustilago kolleri        ---------- ------g--- t-----c--- ---------- ----------
Ustilago maydis         -t-------- -------ccg t-tga-tg-c g--------- ----------
Ustilago nigra          ---------- ---------- t--------- ---------- ----------
Ustilago nuda           -c-------- -------at- ---------- -c----t--- ----------
Ustilago tritici        -c-------- --t---taac ---------- ---------- ----------

                        451                                                 500
Ustilago hordei         CTTTTTTCTT TTGGAAAAGG TTGACGGATC GGTATTGAGG GTTTGTGCCA
Ustilago avenae         ---------- ---------- ---------- ---------- -------g--
Ustilago bullata        -------g-- ---------- c--------- ---------- -----c--gc
Ustilago kolleri        ---------- ---------- ---------- ---------- ----t--g--
Ustilago maydis         -----c-t-- ---------- ---------- ---------- -----c--gc
Ustilago nigra          ---------- ---------- ---------- ---------- ----------
Ustilago nuda           g----c-t-g ---------- ---------- ------ac-- ------c-tg
Ustilago tritici        -------t-g ---------- ---------- ---------- ----c---tg
```

Figure IV.2. (Continued)

```
                    501                                                 550
Ustilago hordei     TTTACCGTGG CTCCCTtGAA ATAGATTAGC GCATCCATTT TATAGGCAAG
Ustilago avenae     ---------- ---------- ---------- ---------- ----------
Ustilago bullata    g--------- ---------- ---c------ tt-------- ----------
Ustilago kolleri    ---------- ---------- ---------- ---------- ----------
Ustilago maydis     ---------- ---------- ---at----- ag-------- ----------
Ustilago nigra      ---------- ---------- ---------- ---------- ----------
Ustilago nuda       ---------- ---------- ---at----- tt-------- ----------
Ustilago tritici    ------t--- a----a---- ----t---c- -----at--- a----ca-g-

                    551                                                 600
Ustilago hordei     ACGGACGAAA GCTCGATTTG TGCTCTCTCT TCCCTGCCGG GTTTTGATAC
Ustilago avenae     ---------- ---------- ---------- ---------- ----g-----
Ustilago bullata    ---------- ---g------ ---a--t--- --g-ct---- ----------
Ustilago kolleri    ---------- ---------- ---------- ---------- ----------
Ustilago maydis     ---------- ---gtga--- ----tct-tc -t---tg-c- -g---tgata
Ustilago nigra      ---------- ---------- ---------- ---------- ----------
Ustilago nuda       ---------t t--gat---- gc---gtct- ct-----g-c a----c----
Ustilago tritici    ---------- -g-g------ ------g--- --t------- ----------

                    601                                                 650
Ustilago hordei     TATCAGGACT TCGGAGAGGT TGAGATGGGT AGGAGCTCGA CGCAACGGCT
Ustilago avenae     ---------- c--------- --------t- ---------- ----------
Ustilago bullata    ---------- ---------- ---------- ------a--- a--t------
Ustilago kolleri    ---------- ---------- ---------- -------g-- ----------
Ustilago maydis     c--------- ---------- ---------- ------g--- ---c------
```

Figure IV.2. (Continued)

```
                        601                                                 650
Ustilago hordei         TATCAGGACT TCGGAGAGGT TGAGATGGGT AGGAGCTCGA CGCAACGGCT
Ustilago nigra          ---------- ---------- ---------- ---------- ----------
Ustilago nuda           ---------- ---------- ---------- ------c--- ---t------
Ustilago tritici        ---------- ---------- ---------- ------a--- -----t----

                        651                                                 700
Ustilago hordei         TGCTGTGTGG AGTGCTTCTG AAACCCGCCC AtGCCGAGTT TATTTTATTT
Ustilago avenae         ---------- ---------- ------cg-- c-tg------ ----------
Ustilago bullata        -ca------- ---------c --c--a---- ---------- ----------
Ustilago kolleri        ---------- ---------- ---------- --c------- ----------
Ustilago maydis         --------a- --------aa --g--g---- ---------- ----------
Ustilago nigra          -------at- ga-tgc-tct g--a--cg-- c-tg------ ----------
Ustilago nuda           --a-----t- ---------c --g--g---- ---------- ----------
Ustilago tritici        --a-----t- --------at --g---c--- ---------- ----------
```

Figure IV.2. (Continued)

Table IV.2. Pairwise comparisons of rDNA sequence identity among eight *Ustilago spp.*

	U. avenae	*U. bullata*	*U. hordei*	*U. kolleri*	*U. maydis*	*U. nigra*	*U. nuda*	*U. tritici*
U. avenae		92[a]	97	97	89	97	89	90
U. bullata			90	91	90	92	90	89
U. hordei				98	89	99	89	88
U. kolleri					90	98	89	89
U. maydis						89	89	89
U. nigra							89	89
U. nuda								90
U. tritici								

[a]: percentage of identity between the two sequences as determined by BestFit analysis

Analysis of the sequence data generated in this study revealed that all species, except *U. maydis*, had an identical sequence for the 5.8S rDNA gene (Fig. IV.2). The 5.8S rDNA in *U. maydis* had 7 nucleotide differences relative to the sequence obtained from the other smut fungi (Fig. IV.2). On the other hand, nucleotide differences were detected in the two ITS regions among the eight smut fungi.

Pairwise comparisons of the sequence data from *U. hordei, U. avenae, U. kolleri*, and *U. nigra* revealed 97%-99% sequence identity (Table IV.2). The most obvious sequence similarity was detected between the sequences amplified from *U. hordei* and *U. nigra*. These two species had sequence variation at six and five nucleotide positions in their ITS1 and ITS2 regions, respectively. Overall the DNA fragments amplified by ITS4/ITS5 in *U. hordei* and *U. nigra* shared 99% similarity (Table IV.2). The DNA fragments amplified by ITS4/ITS5 in *U. avenae* and *U. kolleri* had 97 and 98% sequence identity, respectively, with *U. hordei* (Table IV.2). Pairwise comparison between the ITS sequences of *U. avenae* and *U. kolleri* revealed 97% similarity (Table IV.2).

Alignment of the DNA fragments amplified in *U. bullata* and *U. maydis* to the DNA fragments amplified in *U. hordei, U. avenae, U. kolleri*, and *U. nigra* revealed substantial sequence divergence (Fig. IV.2), and most of the nucleotide differences were located in the ITS1 region. Pairwise analysis of the sequence data showed that the DNA fragments amplified in either *U. bullata* or *U. maydis* shared only 89-92% similarity with the sequences obtained from the four sporidium-forming species *U. hordei, U. avenae, U.*

kolleri, and *U. nigra* (Table IV.2). The ITS2 region in *U. bullata* had a 9 bases gap (nucleotide position 354-362) relative to the sequence in *U. hordei.*

The two nonsporidium-forming species, *U. nuda* and *U. tritici*, had a greater sequence divergence relative to the sporidium forming species (Fig. IV.2 and Table IV.2). The alignment of the sequence obtained from *U. nuda* to the sequences of the other smut fungi revealed a 7 nucleotide gap in the ITS1 region at position 109 to 115 (Fig. IV.2). Pairwise comparisons with the sequence data obtained from *U. nuda* and *U. tritici* revealed a maximum of 90% sequence similarity (Table IV.2).

Discussion

The nucleotide sequence extending from within the 3' end of the small nuclear rDNA gene, to within the 5' end of the large rDNA gene was used in a comparison intended to provide a means for estimating phylogenetic relationships among the eight *Ustilago* taxa: *U. avenae, U. bullata, U. hordei, U. kolleri, U. maydis, U. nigra, U. nuda,* and *U. tritici.* This technique has recently been used to study the taxonomic relatedness in several genera of phytopathogenic fungi, including *Phytophthora* (Lee and Taylor 1992), *Pythium* (Chen et al. 1992), *Verticillium* (Nazar et al. 1991), and isolates of *Leptosphaeria maculans* (Morales et al. 1993).

The sequence of the 5.8S rDNA gene was totally conserved among seven of the eight species, and nucleotide substitutions were detected at 7 positions in *U. maydis.* In a

previous investigation, sequence comparison of the 5S rRNA was used to discriminate among different species in the genus *Ustilago* (Blanz and Gottshalk 1984), differences at 12 nucleotides were reported between the species *U. hordei* and *U. maydis*. Based on this finding the two taxa were grouped into different clusters.

U. avenae and *U. kolleri* cause loose smut and covered smut of oats, respectively, while *U. hordei* and *U. nigra* cause covered smut and false loose smut of barley, respectively. All four species form sporidia upon germination of their teliospores, and are termed sporidium-forming species (Fischer and Holton, 1957). The only morphological criterion that differentiates *U. avenae* from *U. kolleri* is the presence of echinulations on the surface of the spore walls of *U. avenae*. This same criterion marks the unique morphological difference between *U. hordei* and *U. nigra*, with *U. hordei* being the smooth-spored species. Lindeberg and Nannfeldt (1959) postulated that these four species are merely specialized physiologic isolates of a single species, which they termed *U. segetum*. This proposal was later corroborated upon the finding that they are pathogenic to a common host, the grass *Agropyron tsukushiense* var. *transiens* (Nielsen 1978). The comparison of the electrophoretic karyotypes of these four species revealed chromosome-length polymorphisms reminiscent of specialized physiologic races of a single species (Abdennadher et al. submitted). Analysis of the ITS sequence data from these sporidium-forming species corroborates their conspecific classification. The four species shared 97%-99% sequence identity at their ITS regions, and differed at only 11 to 16 nucleotide positions over the entire length of the fragments amplified by ITS4/ITS5

(Fig. IV.2). These differences are assumed real since the error rate of *Taq* DNA polymerase for inserting the proper base has been estimated to be only 10^{-5} (Eckert and Kunkel 1990). The magnitude of sequence identity reported among the ITS regions of isolates of *Leptosphaeria maculans* was also in the range of 97-99% (Morales et al. 1993). *U. maydis* and *U. bullata* are taxonomically more separated from the other sporidium-forming smut fungi, based on the sequence divergence at their ITS regions. Indeed, the ITS regions amplified from *U. maydis* and *U. bullata* shared only 89 to 92 % sequence identity with the sporidium-forming species, *U. avenae, U. hordei, U. kolleri,* and *U. nigra.* A similar magnitude of sequence similarity (90%) was reported from the ITS1 regions of two distinct species of root-infecting fungi, *Ophiosphaerella korrae* and *O. herpotricha* (Tisserat et al. 1994).

U. tritici and *U. nuda* cause loose smuts on wheat and barley, respectively. These two species have been the central issue of many taxonomical controversies. Comparison of polypeptides produced in these two species provided differences significant enough to treat these taxa as separate species (Kim et al. 1984). However, teliospores with identical morphology, a similar germination type, identical mode of infection, and the ability to cross-hybridize in the laboratory, were criteria used to support the synonymy of these two species. Nielsen (1987) reported that the host-range of *U. tritici* and *U. nuda* overlap, with the latter having a narrower host-range, suggesting that *U. nuda* may have evolved from *U. tritici,* however these two species do not cross readily.in the laboratory. Results of rDNA sequence analysis of strains of diverse origins revealed only 3 base changes for

the two *U. tritici* strains, and 5 nucleotide substitutions for the two *U. nuda* strains, but contrasts the proposal of close relatedness between these two species. Indeed, the sequences of the ITS regions of these two species shared only 90% similarity, and were taxonomically as distant from each other, as they are from the other smut fungi (Table IV.2). In view of this finding, it would be of interest to compare the sequence of the ITS region from numerous strains of *U. tritici* and *U. nuda*, to those of other nonsporidium-forming smut fungi.

In the present study the usefulness of PCR-based sequencing of the rDNA region provided taxonomic insights in the genus *Ustilago*. Analysis of the ITS sequence data revealed high percentage of sequence similarity among the sporidium-forming species *U. avenae, U. hordei, U. kolleri*, and *U. nigra*, suggesting that these taxa form a coherent group within the genus *Ustilago*. The sequence analysis corroborated the taxonomic distinctness of *U. maydis* from the other smut species. Although the data are too preliminary to draw firm taxonomic conclusions, and the sample size was small, it is evident that the Ustilaginales are comprised of a heterogeneous group.

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CHAPTER V

GENERAL CONCLUSION

Electrophoretic karyotypes have been shown to be variable between strains in several fungal plant pathogens. Genomic rearrangements such as translocations, deletions, insertions, and duplications produce chromosome-length polymorphisms (CLPs). Also contributing to karyotypic variability is the presence and absence of supernumerary B chromosomes. Several studies have focused on the possible correlation between chromosomal rearrangements and the generation of new virulent forms of phytopathogens. If large rearrangements such as insertions, deletions, and some types of translocations, occur at loci that determine the plant-pathogen interaction, a change in the pathogenicity profile may ensue, and the chromosome alteration may be detectable by pulsed-field gel electrophoresis. However, if the change in pathogenicity resulted from a point mutation, a small deletion, an inversion, and/or insertion, it would be virtually impossible to correlate the shift in pathogenicity with a change in the electrophoretic karyotype.

Inbreeding of race 8 was purported to have increased its pathogenicity in succeeding generations in a stepwise manner, and ultimately led to the genesis of a new physiologic race pathogenic on all barley differential cultivars, designated race 14. Thus, strains of race 8 and 14, as well as the progeny obtained in the course of inbreeding race 8

teliospores, should be isogenic except for genes responsible for the interaction with the plant host. Genomic fingerprinting with a telomere-specific probe and analysis of CLPs were procedures used to examine the relatedness among strains of race 8 and 14, and the purported second and third inbred generations of race 8 strains, teliospore collections 447 and 1279, respectively. A comparative examination of the electrophoretic karyotypes of the second inbred generation (447 collection of teliospores) revealed no chromosome-size variation relative to the progenitor race 8 strain. On the other hand, the third inbred generation (1279 collection of teliospores) resolved karyotypes that were polymorphic relative to both race 8 and 14 strains.

In order to further investigate the lineage association among race 8 and the purported progeny of its inbreeding, a telomere-specific probe was used to investigate telomereRFLP profiles among these different strains. Hybridization of the telomere probe to *Bam*HI and *Bgl*II genomic fragments from a race 8 strain, and representative strains of the two generations of selfing race 8, revealed telomereRFLPs between the third inbred generation and the race 8 strain. The second inbred generation had terminal restriction sites unchanged relative to the race 8 strain. Surprisingly, the telomereRFLP profiles of the third inbred generation were identical to those of *U. hordei* physiologic races 10 and 13. These data together with the electrophoretic karyotype profiles, indicated that the population of teliospores thought to be the third inbred generation of race 8 strains, appear to be a clonal lineage from either race 10 or race 13. Inbreeding of race 8 strains in this study failed to reproduce the shift to increased pathogenicity.

Moreover, the progeny obtained after the first and second generation of inbreeding had karyotypes and telomere-RFLP patterns identical to that of the progenitor race 8 strain.

A comparison of the electrophoretic karyotypes of race 8 and 14 strains revealed CLPs, and the maximum size variation was ca. 90 Kb, representing 8% of the average size between the putative homologous chromosomes. Southern-blot analyses with 70 chromosome-specific probes failed to detect gross chromosome alterations. Southern-blot analysis with the telomere-specific probe onto digests of total genomic DNA from race 8 and race 14 strains revealed 12-16 telomereRFLPs in different restriction digests. The finding that the magnitude of CLPs detected between race 8 and 14 strains fell in the 0-15% range of variability, previously reported among the 14 physiologic races of *U. hordei*, and the difference in telomereRFLP profiles detected between the two races, challenge the postulate that these two races are isogenic except for the genes that determine race-specific interactions.

TelomereRFLP profiles for race 8, race 14, and strains from the 1279 collection of teliospores could be used to strongly argue that neither race 8 teliospores nor teliospore collection 1279 could be the progenitor for the highly pathogenic race 14. Although the mechanism(s) previously reported for the shift toward increased pathogenicity in the progeny of inbreeding were speculative, it is unlikely that race 14 is a direct lineage derivative from race 8 strains. The final population of teliospores, designated race 14, could have arisen from the fortuitous cross-hybridization between race 8 and a mixture of race 10 and 13.

Electrophoretic karyotyping also provided an additional tool for taxonomic studies of the smut fungi. In this respect, molecular linkage analysis and sequence analysis of the ribosomal DNA region were used for species delineation in the genus *Ustilago*. The electrophoretic karyotypes of *U. avenae, U. bullata, U. hordei, U. kolleri, U. maydis, U. nigra,* and *U. tritici* were resolved. An eighth species, *U. nuda,* was recalcitrant to electrophoretic karyotyping, and only a partial karyotype (3 or 4 chromosomes) was resolved. However, Southern-blot hybridization with the telomere-specific probe indicated that this species may have 7 or 8 chromosomes. The genome sizes, of the seven *Ustilago* species were found to be in the 17-41 megabase pairs (Mb) range. Southern hybridization analysis with anonymous DNA fragments from the genome of *U. hordei*, and conserved fungal genes, detected CLPs among the different species, and the magnitude of these CLPs were used as a measure of relatedness. The putative homologous chromosomes in the genomes of *U. avenae, U. hordei, U. kolleri*, and *U. nigra* were found to have less than 15% CLPs in pairwise comparisons. This synonymy was further corroborated by the 97-99% sequence similarity in the intergenic transcribed spacer region of these four species. *U. maydis*, on the other hand, appeared to be taxonomically distant from the rest of the *Ustilago* species. Indeed, more than half of the probes from the genome of *U. hordei* did not hybridize to the chromosomes of *U. maydis*, and the 5.8S rDNA gene in *U. maydis* had sequence divergence at seven nucleotides, relative to the unique sequence depicted in all the other seven species.

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