Use of Inter-Simple Sequence Repeats and Amplified Fragment Length Polymorphisms to Analyze Genetic Relationships Among Small Grain-Infecting Species of *Ustilago*

J. G. Menzies, G. Bakkeren, F. Matheson, J. D. Procunier, and S. Woods

First, third, fourth, and fifth authors: Cereal Research Centre, Agriculture and Agri-Food Canada, 195 Dafoe Road, Winnipeg, MB, Canada, R3T 2M9; and second author: Pacific Agri-Food Research Centre, Agriculture and Agri-Food Canada, Highway 97, Summerland, BC, Canada, V0H 1Z0.
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ABSTRACT

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In the smut fungi, few features are available for use as taxonomic criteria (spore size, shape, morphology, germination type, and host range). DNA-based molecular techniques are useful in expanding the traits considered in determining relationships among these fungi. We examined the phylogenetic relationships among seven species of *Ustilago* (*U. avenae*, *U. bullata*, *U. hordei*, *U. kolleri*, *U. nigra*, *U. nuda*, and *U. tritici*) using inter-simple sequence repeats (ISSRs) and amplified fragment length polymorphisms (AFLPs) to compare their DNA profiles. Fifty-four isolates of different *Ustilago* spp. were analyzed using ISSR primers, and 16 isolates of *Ustilago* were studied using AFLP primers. The variability among isolates within species was low for all species except *U. bullata*. The isolates of *U. bullata*, *U. nuda*, and *U. tritici* were well separated and our data supports their speciation. *U. avenae* and *U. kolleri* isolates did not separate from each other and there was little variability between these species. *U. hordei* and *U. nigra* isolates also showed little variability between species, but the isolates from each species grouped together. Our data suggest that *U. avenae* and *U. kolleri* are monophyletic and should be considered one species, as should *U. hordei* and *U. nigra*.

The parasitic species of the genus *Ustilago* which cause the smut diseases of barley, oat, and wheat traditionally have been classified using characteristics such as spore size, shape, morphology, and germination, and, to a lesser extent, host range. Although these characteristics are useful and relatively easy to work with, a system of classification which attracts general consensus among the people who work with these fungi has been illusive. Stevenson and Johnson (41) proposed a system in which the loose smuts with embryo infection and mycelial germination are classified as *U. nuda* (Jensen) Kellerm. & Swingle on barley and *U. tritici* (Pers.) Rostr., on wheat; the seedling-infecting species with sporidial germination and echinulate spores are classified as *U. avenae* (Pers.) Rostr. on oat and *U. nigra* Tapke (syn. *U. avenae* (Pers.) Rostr.) on barley; and those with smooth spores are classified as *U. kolleri* Wille (syn. *U. hordei* (Pers.) Lagerh.) on oat and *U. hordei* (Pers.) Lagerh. on barley. This has been popular among plant pathologists likely because it reflects the ease of determining the different species (simple spore morphology and germination tests) and the convenience of the pathogen species aligning with the host plant. However, the very simplicity of this system does not ensure its accuracy. Fischer and Shaw (15) and Vánky (45) have warned that, because there are comparatively few morphological features available as taxonomic criteria, it is difficult to draw conclusions about relationships within smut fungi and between smut fungi and other groups. In addition, Fischer (14) warns about putting too much emphasis on the host plant in the classification of these fungi, while disregarding the actual characteristics of the fungi. It is recognized that knowledge of many characteristics is needed to establish relationships among these fungi (45), including the biological relationships between the species of *Ustilago* (32).

In attempting to gain more knowledge about the relationships among the different species of *Ustilago*, researchers have expanded the traits that are considered in classification. In other studies on the morphology of the smuts, Bauer et al. (6) used electron microscopy and studied the differences in hyphal septation and zones of host–parasite interactions to identify monophyletic groups within the smut fungi and allied taxa. Their results were useful in sorting out some relationships at the order and genus levels, but were not as helpful at the species level. Other researchers have studied the ability of the different *Ustilago* spp. to successfully hybridize. Distinction has to be made between intercompatibility and fecundity. Intercompatibility is revealed in petri dish mating assays where quite different species readily fuse because of compatible mating type factors such as promiscuous pheromone and pheromone receptors. However, often no teliospores will be formed on (common) host plants. In certain combinations, some F_1 teliospores will be formed that are incapable of germinating. Hybridization ranges from the formation of such teliospores which, when germinating, will produce haploid basidiospores that subsequently lyse to a degree related to the degree of relatedness of the parents, to fecundity defined as true hybridization producing fertile F_1 haplonts (33). Hybridization studies were pioneered by Hanna and Popp (18) and Holton (20) using *U. avenae* and *U. kolleri* and summarized by Nielsen (36). It appears that, in the species *U. nuda*, *U. tritici*, *U. hordei*, *U. nigra*, *U. avenae*, *U. kolleri*, *U. bullata* Berk. in Hook., *U. aegilopsidis* Picbauer, *U. phrygica* Magnus, and *U. turcomanica* Tranzschel ex K. Vánky, the mating of haplonts is controlled by a bipolar mating

Corresponding author: J. G. Menzies; E-mail address: jmenzies@agr.gc.ca

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system. The lines having mating type 1 (*MAT-1*, formerly called *a*) (3,4) of any of these species are compatible and will form infective dikaryons with lines having mating type 2 (*MAT-2*, formerly called *A*) (3,4) of any other of the species. Two common hosts have been described which allow the production of teliospores (32). It is important to realize, however, that there can be a low compatibility between certain collections or races within a species of *Ustilago* such as *U. bullata* (24) and *U. tritici* (33). Nielsen (33) suggested that the low compatibility between some isolates or races is probably caused by evolution during long-term association with host populations. This also may explain some of the karyotype differences observed among isolates within a species. These studies of interspecific hybridization, combined with studies of the inheritance of certain traits between species such as virulence genes (16) and spore shape (29,43), were useful in demonstrating how closely related some of these "species" appear to be.

TABLE 1. Fifty-four isolates of *Ustilago* spp. used for inter-simple sequence repeats (ISSR) analysis

Isolate no.	*Ustilago* spp.	Origin
4	*U. nuda*	Italy, 1997
6	*U. nuda*	Lacombe, Alberta, Canada, 1996
7	*U. nuda*	Tyndall, Manitoba, Canada, 1997
8	*U. nuda*	Ontario, Canada, 1995
9	*U. nuda*	Ontario, Canada, 1995
11	*U. nigra*	Morden, Manitoba, Canada, 1997
12[a]	*U. nigra*	The former USSR
13[a]	*U. nigra*	The former USSR
14	*U. nigra*	Gimili, Manitoba, Canada, 1997
15	*U. nigra*	Binscarth, Manitoba, Canada, 1997
16	*U. nigra*	Gimili, Manitoba, Canada, 1985
17	*U. nigra*	Canada
18[a]	*U. nigra*	Winnipeg, Manitoba, Canada, 1996
21[a]	*U. hordei*	Morden, Manitoba, Canada, 1997
22[a]	*U. hordei*	Edran, Manitoba, Canada, 1997
23[a]	*U. hordei*	Kenya
24[a]	*U. hordei*	Turkey
25	*U. hordei*	Kenya
26	*U. hordei*	Ethiopia
27	*U. hordei*	Portugal
28[a]	*U. hordei*	Canada
29	*U. hordei*	South Australia
30	*U. hordei*	Iran
31	*U. tritici*	Canada (Race T2)[b]
32	*U. tritici*	Argentina (Race T12)[b]
33	*U. tritici*	Czechoslovakia (Race T9)[b]
34[a]	*U. tritici*	Turkey (Race T26)[b]
35	*U. tritici*	Canada (Race T33)[b]
36[a]	*U. tritici*	China (Race 37)[b]
38[a]	*U. tritici*	Israel (Race T38)[b]
39	*U. tritici*	Tunisia (Race T14)[b]
40[a]	*U. tritici*	Melita, Manitoba, Canada, 1997
41[a]	*U. avenae*	Creelman, Saskatchewan, Canada 1973
42	*U. avenae*	United States of America[c]
43	*U. avenae*	United States of America[c]
44[a]	*U. avenae*	Canada
45	*U. avenae*	Canada
46	*U. avenae*	Europe, 1992
47[a]	*U. avenae*	Europe, 1992
48	*U. avenae*	United States of America[c]
51	*U. kolleri*	Morden, Manitoba, Canada, 1997
52	*U. kolleri*	Bolivia, 1991
54	*U. kolleri*	Vinton, Quebec, Canada 1945
55	*U. kolleri*	Olhao, Portugal, 1980
56	*U. kolleri*	South-West Turkey
57[a]	*U. kolleri*	Kapunda, Australia, 1978
58[a]	*U. kolleri*	1977
59[a]	*U. kolleri*	1974
60[a]	*U. kolleri*	Melfort, Saskatchewan, Canada, 1997
69[d]	*U. bullata*	Golden Prairie, Saskatchewan, Canada
70[d]	*U. bullata*	Piapot, Saskatchewan, Canada
71[d]	*U. bullata*	Meadow Lake, Saskatchewan, Canada
72[d]	*U. bullata*	Nipawin, Saskatchewan, Canada
73[d]	*U. bullata*	Tisdale, Saskatchewan, Canada

[a] These 19 isolates were studied using 7 ISSR primers, but the other 35 isolates were studied using all 11 primers.
[b] Described by Nielsen (35).
[c] Represents three different races provided by C. S. Holton to J. Nielsen, CRC, AAFC in 1968.
[d] Ub69 and Ub70 were isolated from Downy Brome (*Bromus tectorum* L.), Ub71 and Ub73 were isolated from Foxtail Barley (*Hordeum jubatum* L.), and Ub72 was isolated from Meadow Brome (*B. riparius* Rehmann).

The electrophoretic analysis of proteins also has been useful in studying the relationships of the different *Ustilago* spp. Bradford et al. (10) examined 14 species of *Ustilago* with graminaceous and nongraminaceous hosts using gel electrophoresis. They found that they could not characterize species using α-esterase, phosphatase, and leucine aminopeptidase zymograms, but the leucine aminopeptidase zymograms were similar for graminaceous smuts. Kim et al. (23) studied detergent soluble polypeptides separated by two-dimensional isoelectric-focusing polyacrylamide gel electrophoresis for six different smut species. They found that *U. nuda* and *U. tritici* differed greatly in their polypeptide composition and *U. nigra*, *U. hordei*, *U. avenae*, and *U. kolleri* differed very little.

The use of DNA-based molecular techniques to examine relationships within and among different fungi at the species, subspecies, forma specialis, and race levels also has been applied to the smut fungi in recent years. Blanz and Gottschalk (9) studied the 5S ribosomal RNA nucleotide sequences and Begerow et al. (8) studied the ribosomal DNA sequences of some smut fungi and related taxa, including some different species of *Ustilago*. They were mostly concerned with determining the appropriateness of the different genera of smuts in taxonomic orders. They demonstrated that these techniques can be useful in helping to understand genetic relationships among the different smut species. Bakkeren et al. (5) compared the use of DNA sequences from the genomic internal transcribed spacer (ITS) ribosomal RNA region with the amplified fragment length polymorphism (AFLP) technique while examining phylogenetic relationships among the cereal- and grass-infecting *Ustilago* spp. They found that, when comparing five geographic isolates of *U. hordei* and individual isolates of related species, it was difficult to distinguish isolates and some related species using the ITS sequences, but the *U. hordei* isolates were well separated from the different species isolates when using AFLP fingerprints. The AFLP technique, which is based on the selective polymerase chain reaction (PCR) amplification of restriction fragments from a total digest of genomic DNA, is highly sensitive to differences in DNA sequences and can provide more information on variation than any other molecular technique (26). The genetic relationships among different species of bunt fungi (*Tilletia* spp.) also have been examined using molecular techniques such as random amplified polymorphic DNA (RAPD) (17,40), the random fragment length polymorphism (RFLP) analysis of the 5.8S rRNA and the ITS region (40), and AFLP (A. Laroche and G. Bakkeren, *unpublished data*).

Inter-simple sequence repeats (ISSRs) have been used as another method to characterize genetic variation within fungi but, to date, not with the smut fungi. This technique was first demonstrated as a technique for measuring genetic diversity in plants and animals by Zietkiewicz et al. (47) and then shown by Hantula et al. (19) to be able to generate DNA markers in a variety of fungi as well. This technique using primers containing microsatellite sequences and degenerate anchors at the 5′ end, is highly reproducible, allows detection of interspecific and intraspecific DNA-polymorphisms, and is applicable to a diverse range of fungal species (19). The work of Hanulta et al. (19) supported the hypothesis that there would be a greater likelihood of finding polymorphisms with ISSRs (referred to as RAMS in their work) than with most other techniques, including RAPDs, because the evolutionary rate within ISSRs is considerably higher than in most other types of DNA (11). Hanulta et al. (19) also found that ISSRs are common in *Ustilago* genomes by conducting a computer search on all available *Ustilago* sequences.

The objective of this work was to use ISSRs to generate DNA profiles of different isolates of seven species of *Ustilago* to examine the phylogenetic relationships among this group of small grain-infecting fungi. Previous work by Bakkeren et al. (5) indicated that *U. nigra* and *U. hordei* are very closely related; therefore, to more closely examine this relationship, apart from the ISSR experiments, the AFLP technique was employed to study the phylogenetic relationship between *U. nigra* and *U. hordei*.

MATERIALS AND METHODS

Fungal isolates. ISSRs from 54 isolates of different *Ustilago* spp. were amplified using anchored ISSR primers (Table 1). The species studied included *U. nuda*, *U. nigra*, *U. hordei*, *U. tritici*, *U. avenae*, *U. kolleri*, and *U. bullata* (the *U. bullata* isolates were provided by B. Gossen, AAFC, Saskatoon, SK, Canada). A pure culture of each isolate was obtained by streaking teliospores onto half-strength potato dextrose with 2% agar (0.5 PDA) and incubating them for 24 h to allow them to germinate. Single germinated teliospores were isolated and transferred to fresh 0.5 PDA. Pure cultures were maintained at 4°C.

To prepare fungal cells for DNA extraction, a loop of cells from the 0.5 PDA was inoculated into 15 ml of sterile *Ustilago* medium (2) and incubated in shake culture (200 rpm) at 22°C for 5 to 7 days. After incubation, the culture was transferred to 15-ml sterile centrifuge tubes, centrifuged at 2,300 × *g* for 20 min, and the liquid decanted. The pellet was washed twice with 9 ml of sterile distilled water, mixing with a vortex and recentrifugation. The cells were frozen at –20°C until lyophilized. Lyophilized cells were stored at –70°C.

DNA extraction. DNA extraction was conducted using a modified procedure from Kim et al. (22). The lyophilized pellet was broken apart using a sterile glass rod under sterile conditions. To each tube, 2 ml of 3-mm glass beads and 1 ml of sterile sand (both prewashed with HCl) were added and shaken on a paint shaker for 3 min. After shaking, 6 ml of extraction buffer (1.0 M Tris-HCl, pH 8.7, 0.5 M EDTA, pH 8.0, 5.0 M NaCl, and 10% cetyltrimethylammonium bromide) and Proteinase K at 50 µg/ml (10 mg/ml in TE [10 mm Tris-HCl, 1 mm EDTA, pH 7.6]) (Boehringer Mannheim) (lyophilizate at ≈20 units/mg at 37°C with hemoglobin as substrate; 1 unit is the enzyme activity which liberates in 1 min Folin-positive amino acids and peptides corresponding to 1 µmol tyrosine) were added. The tubes were inverted and gently shaken to mix the extraction buffer and pellet, followed by 10 s on a vortex machine. After mixing, sodium dodecyl sulfate was added to a final concentration of 1% and then the tubes were placed in a water bath at 65°C for 2 h, with a gentle shaking of the tube every 0.5 h. After cooling to 30°C, 6 ml of chloroform/isoamyl alcohol (24:1) was added and the tubes were incubated on an orbital shaker (60 rpm) at room temperature for 20 min. The tubes were then centrifuged at 2,300 × *g* for 20 min, after which the supernatant was transferred to a clean 15-ml centrifuge tube. Isopropanol (0.6 vol) was added and the solution slowly mixed by gentle inversion to precipitate the DNA. The DNA pellet was then obtained by centrifugation for 1 min at 2,300 × *g* and the supernatant decanted off. Five milliliters of 70% ETOH was added to the tube, at which point the DNA pellet was stored at 4°C.

The DNA pellet then was dissolved in 1.3 ml of sterile water and 0.5 ml of 5× TE was added along with RNAse (Sigma, 4.3 K units/ml of solution) to a final concentration of 50 µg/ml of solution. The suspension was gently mixed by inversion, centrifuged for 20 s at 2,300 × *g* and then incubated for 1.5 h in a water bath at 37°C. The suspension was transferred to a microcentrifuge tube (2.0 ml) containing 900 µl of DNA solution and 350 µl of buffered phenol. The solution was gently mixed by inversion for 1 min, centrifuged at 2,300 × *g* for 20 s, and 350 µl of chloroform/isoamyl alcohol (24:1) was added to the supernatant. The tubes were mixed again by inversion for 1 min and recentrifuged at 2,300 × *g* for 10 min. The supernatant was transferred to clean tubes containing 700 µl of chloroform/isoamyl alcohol (24:1) mixed by inversion for 1 min and then centrifuged at 2,300 × *g* for 10 min. The supernatant was collected in a clean 15-ml tube.

The DNA was precipitated by adding 1/10 vol of a 3 M NaCl solution and 2.5 vol of 75% ethanol. After centrifugation, the DNA pellet was washed three times with 70% ethanol, dried, and resuspended in sterile OPTIMA water (Fisher Scientific, Fair Lawn, NJ). Estimates of DNA concentration were obtained by A_{260} measurements using an Ultrospec 3000 (Amersham Pharmacia Biotech, Baie de Urfé, QC, Canada). Working solutions of PCR-ready DNA were diluted to a concentration of 20 ng/µl using sterile OPTIMA water.

ISSR amplification and electrophoresis. The DNA samples underwent Hot Start PCR following the methods of Procunier et al. (38). The PCR was carried out for 35 cycles in an MJ1 thermal cycler (MJ Research, Watertown, MA). In the cycle program, the annealing temperature was modified for specific primers (Table 2).

Gel electrophoresis was carried out using standard protocols. When scoring the gels, the presence of a particular band was given the value of 1 whereas the absence of this band was scored as 0.

AFLP analysis. A second set of experiments was conducted to examine more closely the relationship between *U. nigra* and *U. hordei* using AFLP primers. In these experiments, 16 single-basidiospore smut isolates were studied, of which 5 were *U. hordei* and 7 were *U. nigra*; and 1 each of *U. aegilopsidis*, *U. avenae*, *U. kolleri*, and *U. nuda* were added as control isolates (Table 3). DNA template preparation and AFLP analysis were conducted as described in Bakkeren et al. (5). No preamplification was performed. PCR reactions were performed on BamHI/Mse1 templates using primer combinations BamPat + MsePa.FAM, BamPat + MsePt.JOE, BamPaa + MsePt.JOE, and BamPaa + MsePc.FAM, whereby the lowercase letters denote the specifying nucleotides for Bam+2 and Mse+1, respectively, and 6-FAM and JOE are fluorophores. Primers were custom made by Applied Biosystems (Foster City, CA). PCR reactions were run on a 96-well TwinBlock System (Ericomp) using the following touchdown profile: cycle 1 = 3 min at 94°C, 30 s at 65°C, and 2 min at 72°C; cycles 2 to 9 = 1 min at 94°C; 30 s at 64°C (lowering the annealing temperature each cycle by 1°); 2 min at 72°C; 23 cycles of 1 min at 94°C, 30 s at 56°C, and 2 min at 72°C; and a final extension cycle of 30 min at 60°C.

For analysis, the techniques of Bakkeren et al. (5) were followed except that only two differently labeled fluorescent AFLP products (6-FAM and JOE) were used. AFLP fingerprints were compared and polymorphisms were scored as 1 (presence of fragment peak) or 0 (absence of fragment peak).

TABLE 2. Inter-simple sequence repeat (ISSR) primers used in developing DNA profiles of different isolates of *Ustilago* spp.

ISSR primer no.	Primer sequence (5′ to 3′)[a]	Working annealing temperature (°C)	Working DNA concentration (ng/µl)
2[b]	DVD[AAG]5	45	20
7	HBH[GCC]5	60	20
12[b]	DVD[ATC]5	45	20
16[b]	BDB[ACA]5	45	20
17[b]	VDV[GG]7	60	20
24	BHB[GTT]5	55	60
26	HVH[TGT]5	55	60
27	VHV[GTG]5	60	50
29	BDB[CCA]5	60	50
31	HBH[AGC]5	55	50
32	DBD[AAC]5	60	60

[a] Single-letter abbreviations for mixed base positions: B = C, G, or T, not A; D = A, G, or T, not C; H = A, C, or T, not G; V = A, C, or G, not T; 5 or 7 indicates the number of repeats.

[b] Excluded from the analysis of all 54 isolates (Table 1).

Data analysis. A matrix of squared distances between pairs of isolates was constructed from the ISSR data. This was used to determine the average distance within and between species of *Ustilago*, and to generate a dendrogram based on average linkage using the SAS procedures Cluster and Tree (SAS 2000; SAS Institute, Cary, NC). The squared distance was computed as the number of different bands between the two isolates divided by the total number of polymorphic bands (simple matching).

Parsimony analysis of the various data sets in phylogenetic analysis using parsimony (PAUP) software (42) was used as a phylogenetic inference method. Heuristic searches with random stepwise addition and 10 replicates were performed (MAXTREES set at 100). Trees were rooted through the outgroup, *U. nuda*. The bootstrap analysis was done using the full heuristic search option (50% majority rule consensus tree after 100 or 1,000 bootstrap replicates).

RESULTS

Thirty-one ISSR primers were tested singly using the DNA from a small number of isolates of different *Ustilago* spp. to determine which primers resulted in a clear banding pattern in agarose gels and yielded polymorphisms among the different species. From these, 11 primers were selected (Table 2). Not all primers gave results with the DNA of all the isolates. In some instances, no bands were produced with certain isolates using certain primers. Because of this, two sets of data were analyzed. In the first data set, the data from 11 primers scored for 35 isolates were used; in the second data set, the data from 7 primers for 54 isolates were used (Tables 1 and 2). This allowed us to determine if the results changed because of fewer isolates or fewer primers.

Amplification of the DNA from 35 isolates of *Ustilago* with the 11 ISSR primers resulted in the scoring of 205 polymorphic bands, and the amplification of the DNA from 54 *Ustilago* isolates with the 7 ISSR primers resulted in the scoring of 137 polymorphic bands.

The average squared distances between isolates within and between the same species of *Ustilago* for the 35 isolates and 11 ISSR primers data set and the 54 isolates and 7 ISSR primers data set are presented in Table 4. In general, the average squared distance between isolates within a species and between different species was similar for both data sets. The average squared distance between isolates within a species was 10 to 25% of the average squared distance between species. This was true for all species except for *U. bullata,* in which the average squared distance between isolates was higher and similar to the distances between species when compared with the other species of *Ustilago*. The average squared distance between *U. avenae* and *U. kolleri*, and between *U. hordei* and *U. nigra,* were much lower than the distances between other species of *Ustilago*. In both of these comparisons, the average squared distance between the different species was more similar to the distances obtained when comparing isolates within a species than when comparing between species, especially for *U. avenae* and *U. kolleri*.

The dendrograms produced using the distance matrix based on simple matching for the 35 isolates by 11 primer data sets and the 54 isolates by 7 primer data sets were similar (Figs. 1 and 2). In both figures, there are seven clusters in which the average distance between isolates within the clusters is less than 0.1 (10% of the bands are different), but the difference between each pair of clusters is more than 0.2 (>20% of the bands are different). Of the seven clusters, three are composed of isolates of *U. bullata*, one is *U. nuda*, one is *U. tritici*, one is *U. nigra* and *U. hordei,* and one is *U. avenae* and *U. kolleri*. The *U. nigra*/*U. hordei* and the *U. avenae*/*U. kolleri* clusters are the most similar. The three clusters of *U. bullata* suggest that these isolates are not as closely related as the isolates within the other species. It appears that Ub70, Ub72, Ub71, and Ub73 are more closely related to the isolates of the other *Ustilago* spp. than to Ub69. The difference between the two dendrograms is whether the *U. nuda* cluster or the four isolates of *U. bullata* (Ub70, Ub72, Ub71, and Ub73) join the clusters of the other species of *Ustilago* first. Although Figures 1 and 2 differ in this regard, the actual differences in average distance between clusters between these figures is small.

TABLE 3. Sixteen isolates of *Ustilago* spp. used for amplified fragment length polymorphism analysis

Isolate no.	*Ustilago* spp.	Origin
75	*U. hordei*	United States of America
76	*U. hordei*	Manitoba, Canada
77	*U. hordei*	Manitoba, Canada
78	*U. hordei*	Kenya
79	*U. hordei*	Ethiopia
80	*U. nigra*	Manitoba, Canada
81	*U. nigra*	Miles, Turkey[a]
82	*U. nigra*	Manitoba, Canada[b]
83	*U. nigra*	Manitoba, Canada
84	*U. nigra*	Manitoba, Canada[c]
85	*U. nigra*	Manitoba, Canada[c]
86	*U. nigra*	France
87	*U. aegilopsidis*	Turkey
6[d]	*U. nuda*	Lacombe, Alberta, Canada, 1996
42[d]	*U. avenae*	United States of America
54[d]	*U. kolleri*	Vinton, Quebec, Canada, 1945

[a] Isolated from the host, *Aegilopsides caudata*.
[b] Isolates from the host, *Hordeum agriocrithon*.
[c] Mentioned in Nielsen (32).
[d] Same as isolates 6, 42, and 54, respectively, in Table 1. Also used by Bakkeren et al. (5).

TABLE 4. Average squared distance within and between species of *Ustilago* based on simple matching using ungrouped bands (each band counted once)

	Average squared distance[a]						
Ustilago spp.	*U. avenae*	*U. bullata*	*U. hordei*	*U. kolleri*	*U. nigra*	*U. nuda*	*U. tritici*
Within species	0.023	0.277	0.055	0.024	0.025	0.028	0.025
U. avenae	…	0.342	0.239	0.026	0.245	0.359	0.301
U. bullata	0.377	…	0.372	0.339	0.378	0.364	0.366
U. hordei	0.232	0.369	…	0.231	0.085	0.306	0.302
U. kolleri	0.022	0.373	0.223	…	0.236	0.357	0.308
U. nigra	0.237	0.373	0.068	0.227	…	0.317	0.318
U. nuda	0.359	0.360	0.276	0.358	0.287	…	0.377
U. tritici	0.302	0.371	0.308	0.300	0.302	0.385	…
Within species	0.019	0.276	0.034	0.019	0.019	0.029	0.021
No. of isolates[b]	5/8	5/5	5/10	5/9	5/8	5/5	5/9

[a] Values in above diagonal are from data obtained from 35 isolates of *Ustilago* spp. using 11 inter-simple sequence repeat (ISSR) primers, and values in below diagonal are from data obtained from 54 isolates of *Ustilago* spp. and 7 ISSR primers.
[b] The first value in the number of isolates represents the number of isolates used for the 35 isolates of *Ustilago* and the second value represents the number of isolates used for the 54 isolates of *Ustilago*.

PAUP analysis of the data set generated with 35 *Ustilago* isolates and 11 ISSR primers resulted in the phylogenetic tree presented in Figure 3. PAUP analysis of the data set generated with 54 *Ustilago* isolates and 7 ISSR primers resulted in a phylogentic tree as presented in Figure 4. In both Figures 3 and 4, the *U. nuda* isolates are in a cluster and are separated from the rest of the *Ustilago* spp. According to the majority rule and bootstrap analysis, four of the isolates form a monophyletic cluster and one isolate (Unu4) varies from the other four, although not significantly so far as the bootstrap analysis is concerned in Figure 4. The next cluster is of the *U. bullata* isolates, which in this alternative analysis also isolates Ub69 but, once again, not significantly according to bootstrap analysis. The isolates of *U. tritici* cluster well together and the majority rule and bootstrap analyses suggest that they form a monophyletic group. Two more major clusters are formed in Figures 3 and 4, one of which contains the isolates of *U. nigra* and *U. hordei* and the other the isolates of *U. avenae* and *U. kolleri*. The majority rule and bootstrap analyses indicate that the cluster including the isolates of *U. avenae* and *U. kolleri* separates well from the isolates of the other *Ustilago* spp. but, within this cluster, the isolates of *U. avenae* and the isolates of *U. kolleri* are not separated into species-specific clusters. The majority rule and bootstrap analyses indicate that the *U. nigra* and *U. hordei* cluster separate well from the isolates of other *Ustilago* spp. In both Figures 3 and 4, the majority rule analysis separate the isolates of *U. nigra* into one cluster and the *U. hordei* isolates into another cluster and the bootstrap analysis values are moderately high (74 to 88%). Although, in both figures, the bootstrap analysis is significant for the *U. nigra* cluster, it is not significant for the *U. hordei* cluster, suggesting that these clusters are different, but the differences are not great.

Four primer pair combinations yielded AFLP fingerprints from which a binomial data set is derived with a total of 136 characters (bands or fragments), of which 75 were parsimony-informative (polymorphic fragments) and 61 parsimony-uninformative. When *U. nuda* is treated as an "outgroup", three clusters can be distinguished (Fig. 5). One consists of the species *U. avenae* and *U. kolleri*, both pathogenic on *Avena* spp.; one encompasses *U. aegilopsidis*, pathogenic on *Aegilops;* and one is a smut isolated from *Aegilops caudata* (from Turkey) which previously had been identified as *U. nigra*. The third cluster consists of all the other *U. hordei* and *U. nigra* geographic isolates found on *Hordeum vulgare* (except for strain *U. nigra* 82 which was isolated from *H. agriocrithon*). In this third cluster, the *U. hordei* and *U. nigra* isolates are interspersed and the bootstrap analysis does not support the existing "species" boundaries.

DISCUSSION

The analysis of the two data sets (Table 4; Figs. 1 to 4) separated the isolates of *U. nuda*, *U. tritici*, *U. hordei*, *U. nigra*, *U. avenae,* and *U. kolleri* into four major clusters. The isolates of the two floral-infecting smuts, *U. nuda* and *U. tritici*, each formed their own cluster; the two seedborne smuts of barley, *U. hordei* and *U. nigra*, formed a third major cluster; and the two seedborne smuts of oat, *U. avenae*, and *U. kolleri,* formed a fourth major cluster. The isolates of the seedborne *U. bullata* were well separated from the other *Ustilago* spp., but did not group well together and seemed to form one to three separate clusters.

The development of two data sets in which the number of isolates of *Ustilago* and the number of primers used to analyze the DNA differed did not appear to affect the results of these experiments. The only difference of note between these two data sets

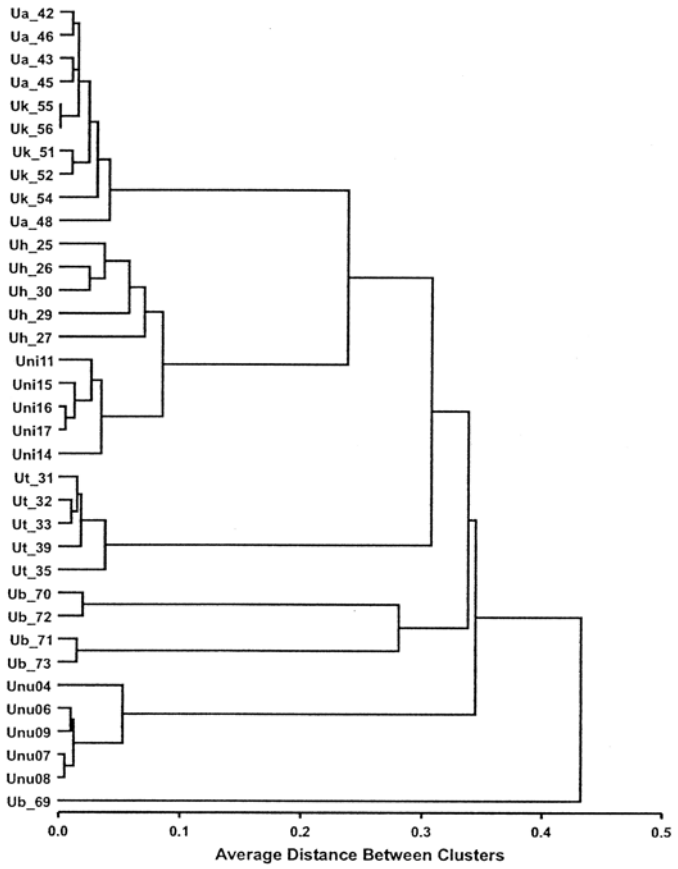

Fig. 1. Dendrogram of average distance (based on simple matching) between clusters for 35 isolates of small grain *Ustilago* spp. analyzed using 11 inter-simple sequence repeat primers.

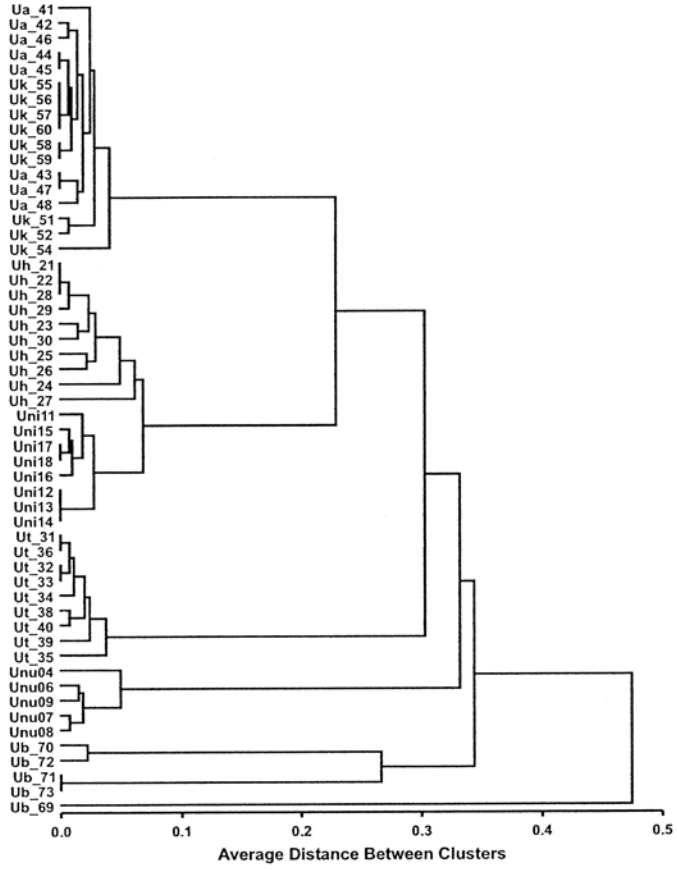

Fig. 2. Dendrogram of average distance (based on simple matching) between clusters for 54 isolates of small grain *Ustilago* spp. analyzed using seven inter-simple sequence repeat primers.

occurred in the dendrograms of average distance between clusters in Figures 1 and 2. In Figure 1 (35 isolates and 11 ISSR primers), the cluster of *U. bullata* isolates falls between the cluster of *U. tritici* isolates and the cluster of *U. nuda* isolates, whereas in Figure 2 (54 isolates and 7 ISSR primers), the cluster of *U. nuda* isolates is between the *U. tritici* cluster and the *U. bullata* cluster. This is reflected in the weak bootstrap support of the branch *U. bullata/U. tritici* which has been noted before (5). Otherwise, the differences in the average squared distance within and between species (Table 4) and the PAUP analysis (Figs. 3 and 4) between the two data sets are small and have little effect on interpretation of the results of the experiments. The lack of differences between these two data sets is likely a reflection of the number of polymorphic bands found among the isolates within the two data sets. Although only 35 isolates were used with the 11 ISSR primers, there were 205 polymorphic bands among the isolates; whereas, when the 54 isolates were used with only 7 ISSR primers, there were 137 polymorphic bands. Both data sets are adequate given the number of isolates for each species and polymorphic bands in each set.

Several analyses support the notion that *U. nuda* can be treated as an outgroup in these studies (Figs. 3 and 4; data not shown) in which PAUP analyses have been performed while forcing *U. tritici* or *U. bullata* as outgroups (5). Branches for the *U. nuda* cluster are well supported. In addition, the average squared distance analyses indicate that the five *U. nuda* isolates are closely related and form a monophyletic group (Table 4). The data analysis in this study seems to indicate that, on a relative basis, *U. nuda* is the least closely related to any of the other six *Ustilago* spp. Our data would support the separation of *U. nuda* from the other species in this study as a separate species.

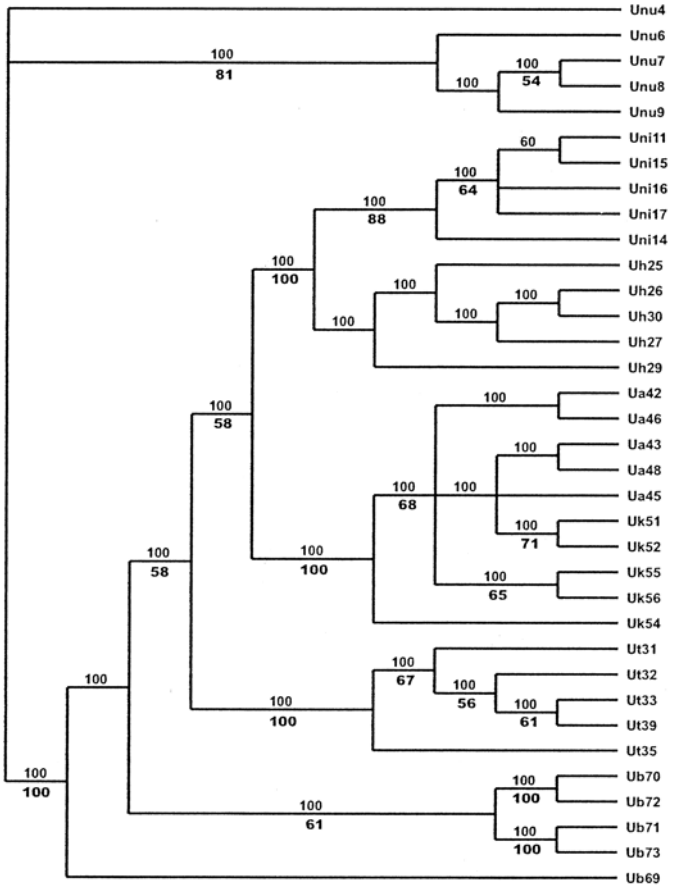

Fig. 3. Phylogenetic relationships among 35 isolates of small grain *Ustilago* spp. recalculated from analysis using 11 inter-simple sequence repeat primers. The data set harbored 205 characters, of which 168 were parsimony informative, 35 uninformative, and 2 constant. The consensus tree was generated with parsimony analysis (PAUP) using the heuristic search option (CI of 0.556, RI of 0.849, and a mean tree length of 365). Numbers above branches indicate consensus in percent (50% majority rule), bold numbers below branches refer to bootstrap values in percent after 100 replicates (full heuristic search option).

The other floral-infecting smut isolates of *Ustilago* (i.e., the *U. tritici* isolates) also were found to form a monophyletic group. These isolates had a small average squared distance within the *U. tritici* species (Table 4) and their branching was well supported (Figs. 3 and 4). Interestingly, the *U. tritici* isolates seem to be more closely related to the seed-infecting smuts of barley and oat than *U. nuda* and *U. bullata*, although this might not be significant. The data of Bakkeren et al. (5), obtained using AFLP analysis, indicated that *U. nuda* was more closely related to the seed-infecting smuts than *U. tritici* or *U. bullata*. As with *U. nuda*, our data would support the separation of the *U. tritici* isolates as a species.

The separation of the floral-infecting isolates into two separate species, *U. nuda* and *U. tritici*, has not been supported by all researchers. These fungi have been considered to be one species, with specialized forms infecting barley or wheat, by a number of authors of major works on the systematics of the smut fungi, such as Fischer (14), Ainsworth and Sampson (1), Fischer and Shaw (15), Lindeberg and Nannfeldt (25), and Punithalingam and Waterston (39). In general, they classified these fungi into the same species because they concluded that spore morphology and morphological characteristics after spore germination and infection type were identical. Others, in particular Nielsen, have disputed these findings and conclusions. Nielsen (31) agreed that both *U. tritici* and *U. nuda* germinate to form a four-celled promycelium, but argued that their respective promycelia differ in size and shape. The promycelium of *U. tritici* is slightly curved and about twice as long as the straight promycelium of *U. nuda*. In *U. tritici*, Nielsen found that each promycelial cell grows into a curved monokaryotic, haploid hypha, and compatible hyphae may fuse later to form dikaryons, each of which divides again into one dikaryon and two curved monokaryons. In *U. nuda*, however, Nielsen found that two dikaryons are formed by fusion of compatible promycelial cells. The dikaryons then divide again into one dikaryon and two monokaryons, each forming a mass of hyphae devoid of the regularity that is displayed by *U. tritici*. In addition, even though the two species have a compatible bipolar mating system, *U. nuda* has some peculiarities that separate its lines of different mating types from lines of *U. tritici* with the same mating types. Nielsen (29) found that all of the *U. nuda* lines with mating type 1 (*MAT-1*, formerly called *a*) (3,4) have a proline deficiency when grown on minimal medium and the gene controlling the proline synthesis in these lines is linked to the mating-type locus. It was also found that lines of *U. nuda* with mating type 2 (*MAT-2*, formerly called *A*) (3,4) are temperature sensitive, while those of *U. tritici* are not. Nevertheless, monokaryotic lines of certain races of the two species can be hybridized to form F_1 spores, but future generations from these spores are not viable (34).

The above-mentioned differences in spore morphology and characteristics of the fungi after spore germination are not the only characteristics that can be used to differentiate the fungi that cause loose smut of wheat from those that cause loose smut of barley. *U. nuda* and *U. tritici* share some of the same hosts, but *U. nuda* has a narrower host range than *U. tritici* (34). In addition, sori of *U. nuda* are always initially covered with a thin membrane, whereas those of *U. tritici* are always naked (34). The two fungi also can be differentiated by detergent-soluble polypeptides (23), soluble protein extracts (12), and DNA analysis (5). Kim et al. (23) found that *U. nuda* and *U. tritici* differed by 47 polypeptides, with *U. tritici* having 30 polypeptides which were absent in *U. nuda* and *U. nuda* having 17 which were absent in *U. tritici*. Dutrecq and Farro (12) reported that, when soluble protein

extracts were run using cellulose acetate electrophoresis, the two forms differed by at least one fast-moving protein band. Bakkeren et al. (5) compared the use of DNA sequences from the genomic ITS ribosomal RNA region with AFLP to distinguish among species and isolates from the Ustilaginomycetes. They found very little nucleotide variation among the ITS DNA sequences of the small grain-infecting smuts, implying a lack of differentiation and, therefore, a close relationship among the different species studied. Nevertheless, they were able to distinguish among the different species of Ustilaginomycetes, except for *U. nigra* and *U. hordei,* with *U. tritici* and *U. nuda* being distinctly different from each other. The AFLP analysis was able to separate all eight smut species they examined (*U. aegilopsidis* was included with the isolates of the seven *Ustilago* spp.) (5) and, once again, there was a clear separation between *U. tritici* and *U. nuda*. Given the data presented in this study and the findings of others listed above, we would conclude that *U. nuda* and *U. tritici* are two separate species.

Some speculation has occurred on the relationships between *U. tritici* and *U. nuda* as to their mode of origin. Becerescu (7) suggested that *U. nuda* arose from *U. nigra* and *U. tritici* arose from either *U. nuda* or *U. nigra*. The germination type of *U. tritici* is intermediate between *U. nuda* and sporidial types like *U. nigra* (31). However, Nielsen (34) suggested that, because *U. nuda* has a narrower host range and is more specialized than *U. tritici*, *U. nuda* may have arisen from *U. tritici*. If, however, broadening a host range on related hosts is caused by the acquisition of virulence factors which might be equivalent to the loss of mutation of avirulence factors, one could argue that *U. tritici* evolved from *U. nuda*. Our results with the seven *Ustilago* spp. included in this study indicate that *U. tritici* and *U. nuda* are not closely related to *U. nigra*, although *U. tritici* does appear to be more closely related to *U. nigra* compared with *U. nuda* and *U. nigra*. This is in contrast to the work of Bakkeren et al. (5), who found that *U. nuda* was more closely related to *U. nigra*, *U. hordei*, *U. avenae*, and *U. kolleri* than was *U. tritici*, using ITS and AFLP data. The reasons for these differences between the results of Bakkeren et al. (5) and those presented here are not clear, except that more isolates of the different *Ustilago* spp. were used here. Also of interest in our results is the fact that *U. bullata* appeared to be an intermediate type between *U. tritici* and *U. nuda* (Figs. 1, 3, and 4). In the work of Bakkeren et al. (5), *U. bullata* and *U. tritici* were more closely related to each other than to *U. nuda*, although it was not a strong relationship. However, as Bakkeren et al. (4) suggest, it is tempting to speculate that even though *U. nuda* and *U. tritici* are both embryo-infecting smuts, this mode of infection may have arisen separately in these two species. Certainly, from our data, *U. bullata* seems to be an intermediate species between *U. nuda* and *U. tritici*.

The *U. bullata* isolates did not form a strong monophyletic group. The average squared distance among isolates of *U. bullata* was high and not comparable to the distances among isolates

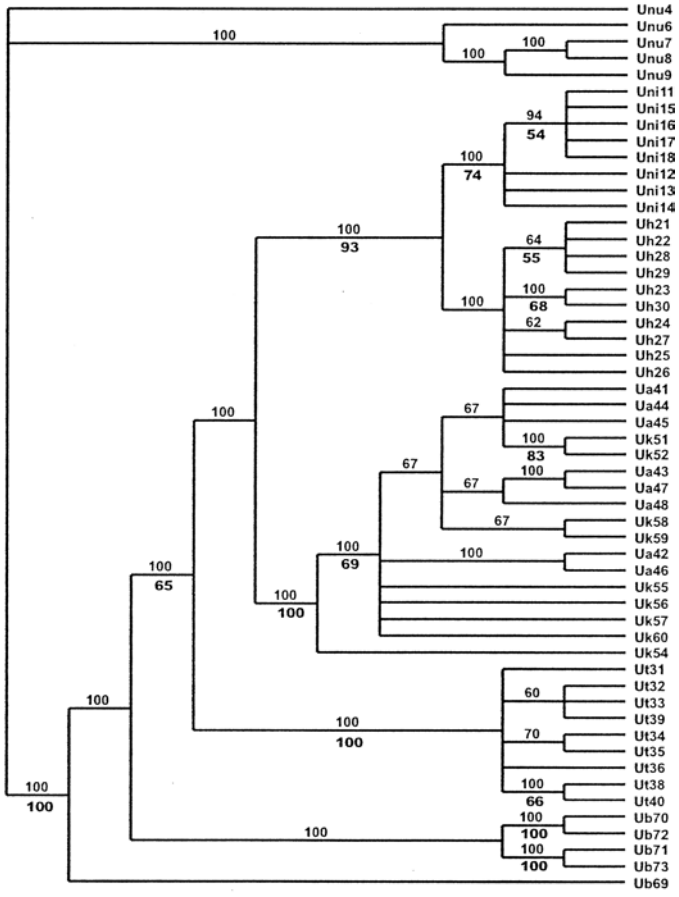

Fig. 4. Phylogenetic relationships among 54 isolates of small grain *Ustilago* spp. recalculated from analysis using seven inter-simple sequence repeat primers. The data set harbored 137 characters, of which 114 were parsimony-informative, and 23 uninformative. The consensus tree was generated with parsimony analysis (PAUP) using the heuristic search option (CI of 0.500, a RI of 0.871, and a mean tree length of 274). Numbers above branches indicate consensus in percent (50% majority rule), bold numbers below branches refer to bootstrap values in percent after 100 replicates (full heuristic search option).

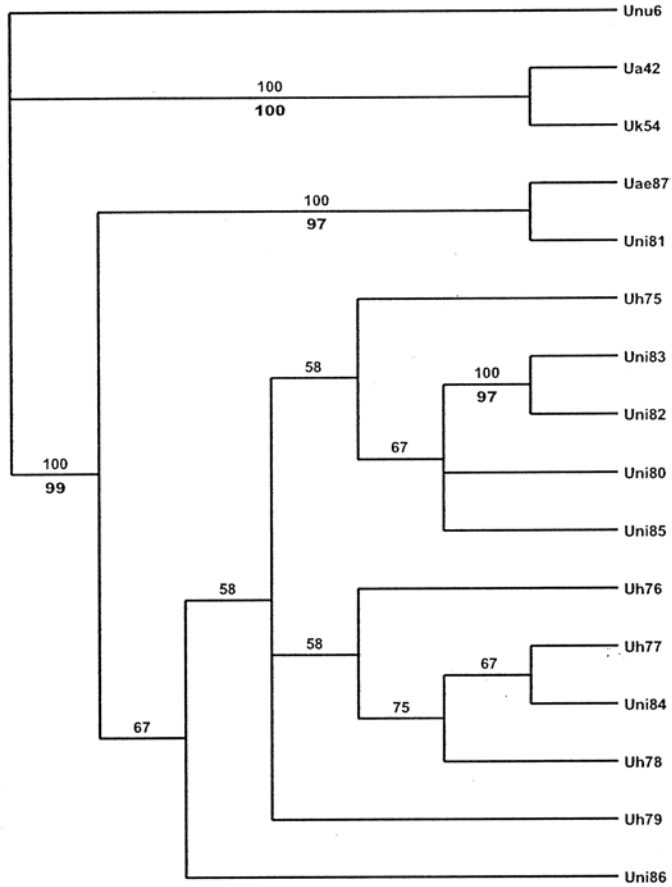

Fig. 5. Phylogenetic relationships among different small grain-infecting smut isolates calculated from amplified fragment length polymorphisms. The consensus tree was generated with parsimony analysis (PAUP) using the heuristic search option; *Ustilago nuda* 96-253 (U nu) was designated outgroup. Numbers above branches indicate consensus in percent (50% majority rule), bold numbers below branches refer to bootstrap values in percent after 1,000 replicates (full heuristic search option, giving a mean tree length of 214, a CI of 0.636, and a RI of 0.642).

within species for the other *Ustilago* spp. (Table 4). This is corroborated in Figures 1 and 2, where the *U. bullata* isolates appear to be in three clusters, and in Figures 3 and 4, with high bootstrap support for branching within the cluster and the separation of isolate 69 of *U. bullata*. When grown in shake culture, isolate Ub69 produces round sporidia, while the other isolates produce oval sporidia (F. Matheson, *unpublished data*), also suggesting differences among the isolates. If the analysis in Table 4 is repeated while omitting Ub69, the average squared distances within the species (*U. bullata*) is reduced by 30 to 35% for both data sets (0.193 for the 35 isolates by 11 ISSR primers data set and 0.181 for the 54 isolates by 7 ISSR primers data set). This still is not comparable to the within-species values obtained for the other *Ustilago* spp. (0.019 to 0.055). The values obtained for the average squared distances among *U. bullata* and the other species also were reduced by ≈5 to 10% when Ub69 was omitted from the data sets, but this reduction did not affect the results of the analyses. Our data suggest that *U. bullata* is a more variable species than the other *Ustilago* spp. studied. Certainly, *U. bullata* has a wide host range, infecting species of Gramineae including *Agropyron*, *Brachypodium*, *Bromus* (including *Zerna*), *Elymus* (including *Clinelymus*), *Festuca*, *Hordeum*, *Lolium*, and *Sitanion* (46), and isolates of *U. bullata* often differ in host pathotypes (13,27). The isolates used in this study were isolated from Downy Brome (*Bromus tectorum* L, isolates Ub69 and Ub70), Foxtail Barley (*Hordeum jubatum* L, isolates Ub71 and Ub73), and Meadow Brome (*B. riparius* Rehmann, isolate Ub72). Turnbull and Gossen (44) found that the Saskatchewan and Alberta, Canada, populations of *U. bullata* were composed of three groups of pathotypes with specific hosts that did not necessarily overlap, suggesting a large degree of variability in these populations. In addition, Kirby and Mulley (24) found that, when they crossed two races of *U. bullata*, there was a high degree of inviability of primary sporidia from the hybrid teliospores. The variability among the *U. bullata* isolates in this manuscript also may be caused by not all the isolates being *U. bullata*, or the species being not well characterized. However, the *U. bullata* isolates as a group are well separated from the other *Ustilago* spp.

The last two clusters are composed of the seedling-infecting smut species *U. nigra*, *U. hordei*, *U. avenae*, and *U. kolleri*. These species separate into two significantly different clusters; one is composed of the oat-infecting *U. avenae* and *U. kolleri*, and the other is composed of the barley-infecting *U. nigra* and *U. hordei*. From our data, it appears that *U. avenae* and *U. kolleri* are very similar and likely monophyletic. The average squared distances between the two species are essentially the same as within the individual species (Table 4) and the isolates of the two species are well interspersed within the major cluster (Figs. 1 and 2). In addition, in the PAUP analysis, support for branching between *U. avenae* and *U. kolleri* isolates is weak.

The *U. nigra* and *U. hordei* isolates also appear to be closely related to each other but perhaps not as closely as the isolates of *U. avenae* and *U. kolleri*. The average squared distances between the *U. nigra* and the *U. hordei* isolates and the average distance between clusters were larger than those among the isolates within each species (except for *U. bullata*; Table 4; Figs. 1 to 3), but still small, suggesting a close relationship. This close relationship was also revealed in the dendrogram constructed from the AFLP data (Fig. 5). The *U. nigra* and *U. hordei* isolates cluster together using this analysis technique, and they appear to be monophyletic. However, in the ISSR analysis, *U. nigra* and *U. hordei* formed individual small clusters within the large cluster with supported branches (Figs. 3 and 4).

As previously mentioned, Stevenson and Johnson (41) proposed a nomenclature system in which the causal agents of loose and covered smut of oat and false loose and covered smuts of barley were separately classified as *U. avenae*, *U. kolleri*, *U. nigra*, and *U. hordei*, respectively. However, Fischer (14), Ainsworth and Sampson (1), and Vánky (46) reduced this group of fungi to two species based on spore morphology. In their systems, the echinulate spore types are united under the name of *U. avenae*, eliminating *U. nigra*, and the smooth spore types are united under *U. hordei*, eliminating *U. kolleri*. Lindeberg and Nannfeldt (25) went even further in reducing the number of species of *Ustilago* among this group by reducing these fungi to one species, *U. segetum* (Bull.:Pers.) Roussel, with two morphological distinct forms as varieties of this taxon, *U. segutum* var. *avenae* (Pers.) Brun which has echinulate spores, and *U. segutum* var. *hordei* (Pers.) Rbh., which has smooth spores. Support for the one-species concept of Lindeberg and Nannfeldt can be found in the ability of all four species to readily hybridize among themselves. It would appear from the work presented in this study and others that to delimit two morphologically distinct species on barley and two on oat does not appear to be tenable, and to classify these fungi based on spore morphology also does not appear to be tenable (21).

The taxonomic relationships among *U. avenae*, *U. kolleri*, *U. nigra*, and *U. hordei* are very close. All four species have been successfully hybridized with each other, indicating a close taxonomic relationship, but the only interspecific cross in which lysis has not been encountered is *U. nigra* × *U. hordei* (30), suggesting that these two species are closer to each other than to *U. avenae* or *U. kolleri*. The four species also share genes for spore echinulation. Studies have shown that echinulation of the spores in these four species are controlled by the same two dominant and complimentary genes (21,28,43). Perhaps this indicates that spore morphology should not be considered in determining species differences among these fungi because it represents only a small genetic difference among these four species of *Ustilago*. There also are similarities within particular pairs of these four species of *Ustilago*. *U. avenae* and *U. kolleri* have very similar host ranges (37) and share a recessive gene for buff teliospore color (28). *U. hordei* and *U. nigra* have been shown to possess identical genes for virulence to the barley cultivars Keystone and Conquest (16). Kim et al. (23) also were able to show differences and similarities among these four species through analysis of detergent-soluble polypeptides. They found that *U. avenae* and *U. kolleri* differed in only one peptide, indicating a very close relationship, and *U. nigra* and *U. hordei* differed in only six polypeptides. A comparison of *U. avenae* and *U. kolleri* with *U. nigra* and *U. hordei* indicated that these two groups differed by only seven polypeptides. They concluded that their work supported the proposal by Lindeberg and Nannfeldt (25) that the four species should be united under *U. segetum*. Bakkeren et al. (5) studied these four species using the DNA sequences from the ITS ribosomal RNA region and the AFLP technique. As was seen in our data, they found that *U. avenae* and *U. kolleri* clustered together (although only one isolate of each species was analyzed) and *U. nigra* and *U. hordei* clustered together. The differences between these two clusters were significant in both cases. Bakkeren et al. (5) suggested that these four species of fungi could be combined into *U. segetum* var. *avenae*, which would contain *U. avenae* and *U. kolleri,* and into *U. segetum* var. *hordei*, which would contain *U. hordei* and *U. nigra*, where the variety name would reflect the economically most important host species. Although our data are similar to those of Bakkeren et al. (5), they may not support the idea of one species (*U. segetum*) with two varieties (var. *avenae* and var. *hordei*). We suggest that *U. avenae* and *U. kolleri* be united under one species, *U. avenae; U. hordei* and *U. nigra* would be united under another species, *U. hordei*.

The use of molecular data in taxonomic studies can be very useful. For organisms such as the smut fungi, where few morphological features are available to use in taxonomy studies, the data can be invaluable. The ISSR and AFLP analysis of the seven smut species was useful in determining genetic variability among these species and the results were similar to those obtained by others

(5,23). The AFLP technique appeared to be more sensitive to differences in DNA sequences and provided more information on the variation between the different isolates than did the ISSR technique. However, the two techniques are complimentary. Establishing species boundaries from DNA polymorphisms should be done cautiously, however, and only with additional characteristics such as morphology, mating compatibilities, and life histories.

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