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Comparison of AFLP fingerprints and ITS sequences as phylogenetic markers in Ustilaginomycetes

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Abstract: We have compared the use of DNA sequences from the genomic internal transcribed spacer (ITS) ribosomal RNA region, with a newer method, the amplified fragment length polymorphism (AFLP) technique. ITS sequences encompass only a small part of the genome but normally reveal sufficient variability to distinguish isolates at the genus and often the species level. Although the AFLP technology reveals genome-wide restriction fragment length polymorphisms, it has not been employed extensively in establishing phylogenetic relationships. We have adapted the AFLP technology for fungal genomes and compared AFLP fingerprints generated from several fungal species and isolates from the order Ustilaginales: *Ustilago hordei, U. nigra, U. aegilopsidis, U. avenae, U. kolleri, U. bullata, U. nuda, U. tritici, U. maydis, U. scitaminea, Sporisorium reilianum,* and Tilletiales: *Tilletia indica* and *T. walkeri*. Geographical isolates of *U. hordei* and related species, particularly those infecting small-grain cereals, were difficult to distinguish when comparing ITS sequences, but were clearly separated when comparing AFLP fingerprints. The abundance of polymorphisms makes the AFLP technique more suitable to distinguish organisms in clusters of closely related species and at the isolate level. Phylogenetic analyses of the data sets generated with the two methods revealed that the AFLP-derived phylogenetic relationships were not in disagreement with the ITS-derived tree. The fungal phylogenetic tree correlated additionally with one from the graminaceous hosts generated from literature data, suggesting coevolution of some specialized host-pathogen systems. The clustering of small grain-infecting smuts due to limited genetic variability, in combination with other molecular, mating and literature data, suggests reclassification of this group possibly to include *varietas* designations to define host range.

Key Words: basidiomycete, bunt fungus, host plant, pathogen, phylogeny, smut, taxonomy, Ustilaginales, *Ustilago*

INTRODUCTION

One often-used taxonomic method compares DNA sequences from one particular area in the genome, the nuclear ribosomal RNA gene complex. There are normally many tandem arrays of this complex per genome and their DNA sequence identity is preserved by a mechanism of continuous recombination and selection for function. The comparison of DNA sequences of several of the components of this unit, such as the 5.8S, 18S and 28S RNA encoding genes in eukaryotes, allows separation of organisms at the genus level (Blanz and Gottschalk 1984, White et al 1990, Berbee and Taylor 1992, Swann et al 1999). The more variable regions between the units, the internally transcribed spacer (ITS) regions, frequently allows the distinction of organisms at the species level (Ferris et al 1993, Zambino and Szabo 1993, Rehner and Uecker 1994, Kropp et al 1997). However, sometimes species and certainly geographic isolates of one species, have similar or identical ITS sequences. When other (e.g., morphological) characters are identical too, different or larger areas of the genome must be analyzed to distinguish isolates. The amplification and sequencing of other specific areas of the genome such as known genes using the polymerase chain reaction (PCR) and specific primers is feasible but costly, time consuming, and limited in scope.

In recent years, several techniques for genome analysis have been developed that reveal genome-wide DNA polymorphisms in DNA fingerprints (Caetano-Anolles and Gresshoff 1997). Comparisons of polymorphisms from different individuals have been used to assess the level of genetic variation or relatedness between them. Some of the techniques are based on PCR amplification needing only small

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amounts of genomic DNA and do not require prior knowledge of actual DNA sequences, such as randomly amplified polymorphic DNA (RAPD) analysis (Williams et al 1993, Kolmer et al 1995) and the recent amplified fragment length polymorphism (AFLP) technology (Vos et al 1995, Vos and Kuiper 1997). Compared with other fingerprinting methods, the AFLP technique is robust, reproducible and generates numerous restriction fragment length polymorphisms (RFLPs) per single reaction that originate from random locations in the genome. Previously, the applicability of the AFLP fingerprinting technology as a new taxonomic tool has been investigated in bacterial populations (for example, Janssen et al 1996, Huys et al 1996), for the detection of genetic variation between isolates of bacterial and fungal species (Aarts et al 1998, Majer et al 1996) and in the construction of a genetic map (van der Lee et al 1997).

Many basidiomycete genera have been classified using rRNA gene sequences (Swann and Taylor 1993, Swann et al 1999). From this phylum, several genera of Ustilaginomycetes have been classified using 5S (Blanz and Gottschalk 1984) or 28S rRNA gene sequences (Begerow et al 1997). For a recent comprehensive study on the classification of the smuts based on ultrastructural characters, see Bauer et al (1997). Smuts occur world-wide and are persistent pathogens mainly of the Gramineae, such as cereal crops and forage grasses. We focussed on selected smut fungi of the Ustilaginales, *Ustilago hordei* (Pers.) Lagerh. (causative agent of covered smut of barley and oat), *U. nigra* Tapke (black loose smut of barley), *U. aegilopsidis* Picbauer (head smut of aegilops), *U. avenae* (Pers.) Rostr. (loose smut of oat), *U. kolleri* Willie (covered smut of oat), *U. bullata* Berk. (head smut of temperate grasses), *U. nuda* (Jens.) Rostr. (loose smut of barley), *U. tritici* (Pers.) Rostr. (loose smut of wheat), *U. maydis* (DC.) Corda (smut of corn), *U. scitaminea* Syd. & P. Syd. (sugarcane culmicolous smut), *Sporisorium reilianum* (Kuehn) Langd. (head smut of sorghum and maize), and employed two species from the Tilletiales, *Tilletia indica* Mitra (wheat karnal bunt) and *T. walkeri* Castlebury & Carris (partial bunt of ryegrass); for reference see TABLE I. Some *Ustilago* species that infect small grain cereals and related wild (grass) hosts, *U. avenae, U. kolleri,* and *U. nigra, U. hordei* and *U. aegilopsidis* are closely related and have been the subject of taxonomic controversy before. Lindeberg and Nannfeldt (cited in Huang and Nielsen 1984) proposed to unite the first four smuts in the species *U. segetum* (Pers.) Roussel, and relegate the two morphologically different spore forms to varieties: *U. segetum* var. *avenae* (Pers.) Brun. with echinulate spores (*U. avenae* and *U. nigra*), and *U. segetum* var. *hordei* (Pers.) Rbh. with smooth spores (*U. kolleri* and *U. hordei*). However, characters generally used to distinguish species, such as sorus type, spore morphology and host range are not consistent in this sub-group (Huang and Nielsen 1984, Thomas 1989). For example, sorus type is known to be affected by environmental factors, and Huang and Nielsen (1984) have shown that spore wall echinulation is determined by only two genes and that this character can be easily transmitted between *U. nigra* and *U. hordei,* and between *U. avenae* and *U. kolleri.* These species are also sexually compatible and are mainly divided according to host range although some hosts are shared (Fischer and Holton 1957, Nielsen 1978). These previous studies combined with sequence comparisons of mating-type genes from our laboratory (Bakkeren et al 1992, Bakkeren and Kronstad 1993) and the work presented here, support the placement of some of the small grain-infecting smuts in a common species.

Here we report on the amplification and sequence analysis of the ITS region of these smuts, and on the optimization of the AFLP technique to generate DNA fingerprints from fungi. We compared DNA polymorphisms generated with the two methods and investigated their use in establishing phylogenetic relationships among this limited set of different *Ustilago* species and isolates. The methods show no disagreement and complement each other.

MATERIALS AND METHODS

The hosts and geographic origins of the Ustilaginales and Tilletiales strains are given in TABLE I. Most strains were received as teliospore populations on smutted inflorescences; teliospores were germinated on potato dextrose agar (PDA, Difco Laboratories, Detroit, Michigan) and a single random basidiospore isolated. Strains were grown in PDB (broth) at 22 C and total genomic DNA was isolated using a glass beads/high-salt/phenol extraction procedure as described by Bakkeren et al (1992). Total genomic DNA preparations were received for *U. nuda, U. tritici* and the *Tilletia* strains.

ITS region analysis.—PCR-amplification and sequencing of the ITS region was done as follows. Ten to 20 ng of total genomic, RnaseA-treated DNA was amplified by PCR in 50 μL containing 0.8 μM primer UNUP18S42 (5′-CGTAACAAGGTTTCCGTAGGTGAAC, position 1707–1731 of the *Ustilago hordei* 18S rRNA gene; GenBank U00973), 0.8 μM primer UNLO28S576B (5′-CTCCTTGGTCCGTGTTTCAAGACG, position 595–618 of the *Boletus retipes* 28S rRNA gene; GenBank U11914), 200 μM dNTPs, 2 mM $MgCl_2$, 1 unit recombinant *Taq* polymerase (Bethesda Research Laboratories) in 1x buffer, overlaid with 20 μL mineral oil, and subjected to the following reaction profile in a Thermocycler PE480 (Perkin Elmer, Applied Biosystems, California):

TABLE 1. Ustilaginomycetes strains used

Fungal species	Host[a]	Alternative name/ genotype	Source
Sporisorium reilianum	*Sorghum vulgare*	unknown	Texas[b]
Tilletia indica	*Triticum aestivum*	#11	India[d]
Tilletia walkeri	*Lolium multiflorum*	#M96442	Oregon, USA[d]
Ustilago aegilopsidis	*Aegilops* sp.	#4327 (*MAT-2*)	Turkey[c]
Ustilago avenae	*Avena sativa*	#A-60 (*MAT-1*)	USA[c]
Ustilago bullata	*Bromus* sp.	#B-249 (*MAT-2*)	Manitoba, Canada[c]
Ustilago hordei, Uh316	*Hordeum vulgare*	#14.1 (*MAT-2*)	USA[c]
Ustilago hordei, Uh362	*Hordeum vulgare*	#4854(*MAT-2*)	Manitoba, Canada[c]
Ustilago hordei, Uh364	*Hordeum vulgare*	#4857 (*MAT-1*)	Manitoba, Canada[c]
Ustilago hordei, Uh521	*Hordeum vulgare*	#4640 (*MAT-2*)	Kenya[c]
Ustilago hordei, Uh523	*Hordeum vulgare*	#4632 (*MAT-2*)	Ethiopia[c]
Ustilago kolleri	*Avena sativa*	#K-45(*MAT-1*)	Quebec, Canada[c]
Ustilago maydis	*Zea mays*	#521(*a1b1*)	USA[f]
Ustilago nigra	*Hordeum vulgare*	#83-138 (*MAT-2*)	Manitoba, Canada[c]
Ustilago nuda	*Hordeum vulgare*	#96-253	Alberta, Canada[c]
Ustilago scitaminea	*Saccharum* sp.	#5-(*MAT-2*)	Louisiana, USA[g]
Ustilago tritici	*Triticum* sp.	#T-2	Manitoba, Canada[c]

[a] Only the major host is listed.
[b] R. Frederiksen, Dept. Plant Pathology and Microbiology, Texas A & M University.
[c] J. Menzies, P. Thomas, Agriculture and Agri-Food Canada, Winnipeg, MB, Canada.
[d] Via A. Laroche, Agriculture and Agri-Food Canada, Lethbridge, AB, Canada.
[e] D. Mills, Oregon State University, Corvallis, OR, USA. (McCluskey and Mills 1990).
[f] Described in Kronstad and Leong (1989).
[g] K. E. Damann via S. Schenk and H. Albert, Hawaiian Sugar Planters' Association, Aiea, Hawaii.

3 min at 94 C; 30 cycles of 45 s at 94 C, 45 s at 68 C, 45 s at 72 C; and a final extension cycle of 10 min at 72 C. The reaction product was purified on QIAquick Columns™ according to the specifications of the manufacturer (QIAGEN Inc, Ontario, Canada), whereafter approximately 90 ng of PCR product was subjected to sequencing using primers UNUP18S42 or ITS3 (White et al 1990) for the forward, or UNLO28S22 (5′-GTTTCTTTTCCTCCGCTTATTGATATG, which overlaps position 773–791 of the *U. hordei* 28S rRNA gene, GenBank AF105224, and position 1–12 of the mentioned *B. retipes* 28S rRNA gene) or ITS2 (White et al 1990) for the reverse reactions. Fluorescent dye-dideoxy-terminator cycle sequencing was performed in 20 µL according to the specifications of the manufacturer (Perkin Elmer), overlaid with 20 µL mineral oil, on a Thermocycler PE480 (Perkin Elmer). The reaction profile was 25 cycles of 30 s at 94 C, 4 min at 65 C (or 62 C for ITS2 and ITS3). Alternatively, cycle sequencing of approximately 30 ng of column-purified template DNA was done using BigDye Terminator-chemistry (Perkin Elmer) in a PE2400 Thermocycler (Perkin Elmer) without oil overlay using the following PCR profile: 30 s at 95 C followed by 25 cycles of 30 s at 95 C, 4 min 30 s at 58 C. The reaction products were analyzed on an automated Prism ABI310 Genetic Analyzer (Perkin Elmer). The DNA sequences of the ITS regions of 13 of the Ustilaginomycetes strains (compare TABLE I) have been deposited in GenBank; the accession numbers and total length of the sequences in bp are given in parentheses. *Ustilago bullata* (AF135423, 710 bp), *U. tritici* (AF135424, 707 bp), *U. avenae* (AF135425, 711 bp), *U. kolleri* (AF135426, 711 bp), *U. hordei* Uh362 (AF135427, 715 bp), *U. nigra* (AF135428, 715 bp), *U. aegilopsidis* (AF135429, 723 bp), *U. nuda* (AF135430, 709 bp), *U. maydis* (AF135431, 722 bp), *Sporisorium reilianum* (AF135432, 665 bp), *U. scitaminea* (AF135433, 677 bp), *Tilletia indica* (AF135434, 638 bp), *T. walkeri* (AF135435, 638 bp).

AFLP primers and adapters.—The fluorescently-labeled *Mse*I-primers (*Mse*I core = 5′-GATGAGTCCTGAGTAA + a.FAM, +c.FAM, +t.JOE, +g.NED, +aa.FAM, +ac.JOE, +ag.JOE or +at.NED; lower case letters denote the +1 or +2 specifying nucleotides) were kindly provided by Perkin-Elmer, Applied Biosystems. Other oligonucleotide primers were synthesized on a Beckmann Oligo 1000 M DNA synthesizer. Nucleotide sequences for *Eco*RI-, *Pst*I-, *Mse*I- and *Taq*I-adapters and primers were as described (Zabeau and Vos 1993, Vos et al 1995). *Bam*HI/*Bgl*II-adapter was prepared by mixing equimolar amounts of primers 5′-CTCGTGGACTGCGTAC and 5′-GATCGTACGCAGTCCAC and was ligated to ends of fragments generated by either *Bam*HI or *Bgl*II. Sixteen different *Bam*HI(+2)-primers, 5′-GGACTGCGTACGATCCxx (*Bam*Pxx), and 16 different *Bgl*II(+2)-primers, 5′-GGACTGCGTACGATCTxx (*Bgl*Pxx), were synthesized.

AFLP analysis.—Eight combinations of 6-cutter restriction enzymes, *Bam*HI, *Bgl*II, *Eco*RI or *Pst*I, and 4-cutter restriction enzymes, *Mse*I or *Taq*I, were employed and corresponding adapters were added. AFLP templates were pre-

pared essentially as described by Vos et al (1995). In short, 0.5 μg of total genomic DNA was digested for 1 h at 37 C in 40 μL containing 4 μL of a ×10 restriction ligation buffer (10xRL = 10 mM Tris-acetic acid, pH 7.5; 10 mM Mg acetate; 50 mM K acetate; 5 mM DTT, 50 ng/μL BSA), 10 units 6-cutter and 5 units 4-cutter restriction enzyme (enzymes purchased from BRL, *Mse*I from NewEngland Biolabs). Then, 10 μL containing 5 pMol 6-cutter-adapter, 50 pMol 4-cutter-adapter, 1 mM ATP, 1 unit T4 DNA Ligase (BRL) in 1xRL buffer, was added and incubation at 37 C was continued for an additional 3 h. Ten μL of this mix was run out on a 1.2 % agarose gel to verify complete digestion and conversion (adapters are designed in a way that does not allow recutting once ligated), and 360 μL 0.1xTE (10 mM Tris.HCL, pH 7.6; 0.1 mM EDTA) was added to the remaining 40 μL solution to give a template stock solution with a DNA concentration of ca 1 ng/μL.

PCR reactions were performed, for example on *Bam*HI/*Mse*I templates, as follows: 17 μL of a cocktail containing 2 μL ×10 *Taq* polymerase buffer (BRL), 2 μL dNTPs (from a stock solution of 2 mM each), 1 μL 50 mM $MgCl_2$, 1 μL [1 μM] *Bam*Pxx primer, 1 μL [1 μM] *Mse*Px-fluorescently labeled primer and 0.1 μL (5u/μL) Recombinant *Taq* polymerase (BRL), was added to 3 μL DNA template stock solution. The reaction was overlaid with 20 μL light mineral oil and run on a Gradient 96 model RoboCycler (Stratagene) using the following 'touch-down' profile: Cycle 1: 2 min at 94 C, 2 min at 65 C, 3 min at 72 C; Cycles 2–5: 30 s at 94 C, 2 min at 64 C lowering the annealing temperature each cycle 1 C, 3 min at 72 C; Cycles 6–9: 30 s at 94 C, 2 min at 60 C lowering the annealing temperature each cycle 1 C, 2 min 30 s at 72 C; 25 cycles of 30 s at 94 C, 2 min at 56 C, 2 min 30 s at 72 C; and a final extension cycle of 30 min at 60 C.

For analysis, 2 μL of each of three differently labeled fluorescent AFLP products (6-FAM, JOE, and NED) were combined with 0.5 μL GS2500 ROX-labeled internal size standard (Perkin Elmer) in 20 μL formamide, heated at 94 C for 3 min, cooled on ice, and subjected to electrophoresis on an automated Prism ABI310 Genetic Analyzer using 47 cm × 50 μm capillaries and POP4 polymer solution (Perkin Elmer). Typical analysis parameters were: injection for 5 to 10 s at 15 kV and a 30 min run at 13 kV and 60 C. Sizing and normalization of the bands (corresponding to single strand DNA molecules in nucleotides) on the output from an ABI310 Genetic Analyzer after capillary gel electrophoresis (electropherogram, see FIG. 1) was done with the aid of the internal size standard and the GeneScan 2.0.2 software package followed by alignment of the scans with the GenoTyper 2.0 software package (Perkin-Elmer).

Phylogenetic analysis of ITS and AFLP data.—ITS DNA sequence data were aligned using the 'PILEUP' program of the Genetics Computer Group software package (Devereux et al 1984); parameter settings were: GapWeight = 1 and GapLength Weight = 0.1. AFLP fingerprints were compared and polymorphisms were scored as 1 (presence of fragment peak) or 0 (absence of fragment peak) whereafter a binomial data set was produced using the EXCEL software package (Microsoft, Washington state). Parsimony analysis of the data sets in PAUP (Phylogenetic Analysis Using Parsimony software, Swofford 1999) was used as a phylogenetic inference method. Heuristic searches with random step-wise addition and ten replicates were performed (MAXTREES set at 200 for ITS analysis and at 100 for the AFLP analysis). Gaps were treated as missing for the sequencing data. Trees were rooted through the outgroup, *Puccinia caricina* (Genbank U88234) plus the two *Tilletia* species for the ITS data, and *U. bullata* plus *U. tritici* for the AFLP data. The bootstrap analysis was done using the full heuristic search option (50% majority rule consensus tree after 250 (ITS data) and 1000 (AFLP data) bootstrap replicates).

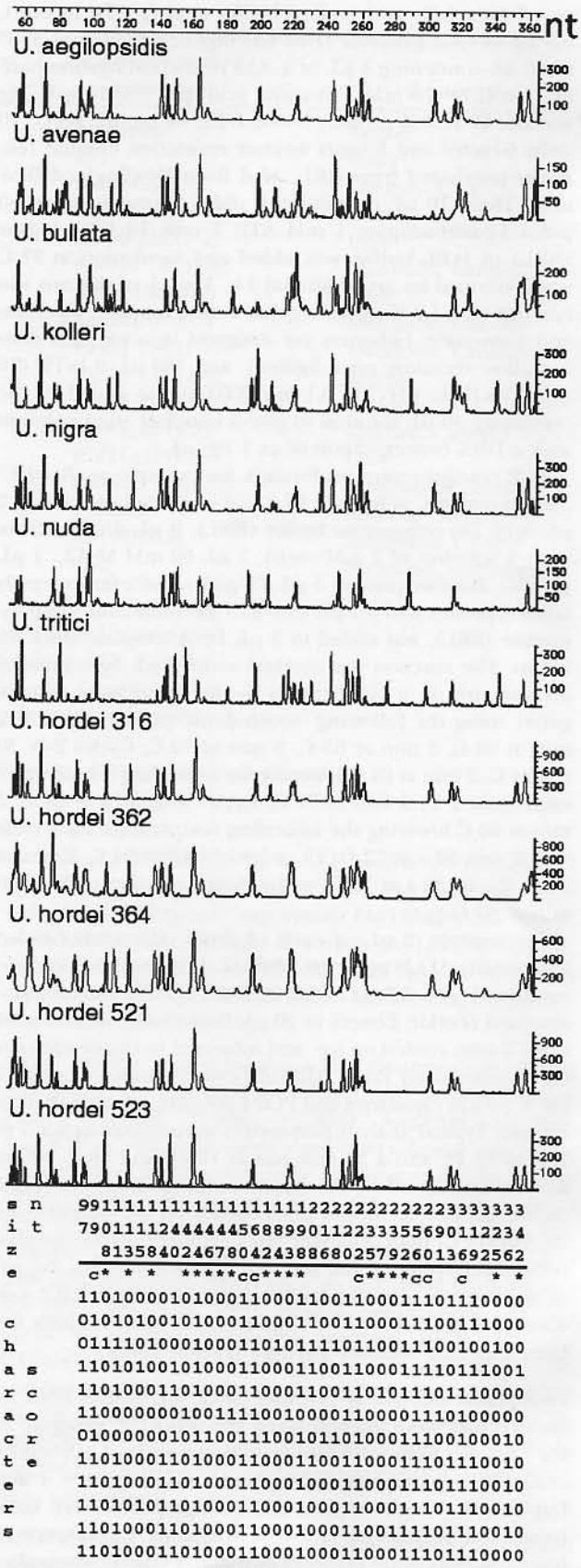
RESULTS

PCR amplification and sequencing of the ITS regions of fungi.—The ITS region has a tendency to form secondary structures, such as hairpins, that may form during PCR amplification at the recommended temperatures and consequently lead to failed sequence attempts. We designed additional primers for PCR and sequencing that can be used at elevated temperatures and still are able to anneal to DNA from a variety of fungal genera (materials and methods; C. A. Lévesque unpubl). A DNA sequence alignment of the ITS1/5.8S/ITS2 regions from 14 Ustilaginomycetes strains and a *Puccinia caricina* strain was obtained which could be retrieved from TreeBASE (M630). The alignment includes the sequences from only two *U. hordei* isolates, because the ITS region from this species is identical to the five sequenced isolates from a world-wide collection used in this study and to two recent *U. hordei* GenBank accessions, AF105224 (Willits and Sherwood 1999) and AF045866. In addition, the *U. hordei* sequences are identical to those of *U. nigra*. Indeed, very little sequence variability was found when comparing ITS sequences from the small grain-infecting species. For example, only eight base pair differences were found between *U. aegilopsidis* and *U. hordei,* and between *U. hordei* and *U. avenae* (in both cases all insertions/deletions), one between *U. avenae* and *U. kolleri,* six between *U. bullata* and *U. tritici,* and 13 between *U. tritici* and *U. nuda*. Overall, the majority of the base pair differences were found in an AT-rich insertion/deletion region approx 50 bp upstream of the 28S rRNA gene. The ITS sequences from the three smuts, *U. maydis, U. scitaminea* and *S. reilianum,* were rather different from the other clusters but also from each other and the base pair differences were scattered all along the ITS1 and ITS2 regions. The ITS sequences of two more of our species were compared with the sequences from different isolates found as recent GenBank accessions: *U. maydis* AF038826, which revealed 16 base pair differences with our isolate, and *S. reilianum* isolates AF045870 and AF038827, which

displayed one and six base pair differences with our isolate, respectively. The 5.8S rRNA gene was identical among the smuts, but differed slightly for the *Tilletia* species.

Adapting and optimizing the AFLP technology for fungi.—The complexity of AFLP patterns is dependent on the frequency with which a particular restriction enzyme (combination) cleaves DNA as a direct function of the (G+C)-content of the organism under study, on the number of generated template fragments which relates to the genome size, and on the number and combination of specifying nucleotides of the primers (Vos and Kuiper 1997). Fungal genomes are in general sufficiently small (approx 2×10^7 bp for *Ustilago*) to alleviate the need for pre-amplification. Eight different enzyme combinations were tested on two isolates of *U. hordei* (Uh362 and Uh364). Also, the number of specifying nucleotides for the 6-cutter/4-cutter restriction enzyme combinations was varied as follows: +1/+1, +1/+2, +1/+3, +2/+2 to +2/+1. Initially, primers were labeled with radioisotope ^{32}P according to Vos et al (1995) to study the complexity of the patterns without having to use numerous fluorescently-labeled primers. *Pst*1/*Mse*I templates generated AFLP-fragments that were too large to separate adequately. *EcoR*I, *Bam*HI and *Bgl*II in combination with either *Mse*I or *Taq*I gave profiles on polyacrylamide gels with fragments ranging from 50 to 1600 bp in length. The number of fragments in an average profile between 75 and 1000 bp was 150 or more for a +1/+1 combination, decreasing to approx 30 for a +1/+3 combination (data not shown).

AFLP fingerprints of Ustilaginales genomes.—FIGURE 1 shows a comparison of part of a profile from different *Ustilago* species and isolates and illustrates how similar but distinct fingerprints of related species can be. They demonstrate the utility of the method for rapidly comparing and distinguishing different fungal species and isolates. Indeed, using the same adapters and primer combinations, we have generated similar distinguishing fingerprints from isolates of the ascomycete, *Microsphaeropsis* (data not shown). We scored 207 fragments (bands or characters) of up to 500 nucleotides in size in fingerprints generated with four different primer combinations. Primer combination BamPaa + MsePc revealed 56 bands between 97 and 490 nucleotides, BamPaa + MsePt 41 between 66 and 500 nucleotides, BamPat + MsePa 50 between 77 and 405 nucleotides, and BamPat + MsePt 60 between 42 and 466 nucleotides. For example, 11 polymorphisms were found when comparing profiles of *U. hordei* isolates Uh523 and Uh316, and 124 between *U. hordei* isolate Uh362 and *U. bul-*



lata (FIG. 2). The profiles revealed that the eight different species share many of the same markers and that differences between these "species" are similar to the differences among isolates of the same species. The data matrix representing the 207 scored AFLP fragments has been deposited in TreeBASE as M631. AFLP profiles generated from the more distantly related Ustilaginomycetes, *U. maydis, U. scitaminea, S. reilianum,* and the two *Tilletia* strains, were too different to give meaningful phylogenetic data in comparison with the small grain-infecting cluster (data not shown).

Phylogeny using the ITS variability and the AFLP polymorphisms.—Phylogenetic analysis of the ITS DNA sequences by parsimony analysis revealed two major phylogenetic clusters supported by high bootstrap values among the studied species ("ITS data" in FIG. 2): cluster A containing smut species pathogenic on small grain cereals and related wild relatives, and cluster B consisting of the *Tilletia* species (bunts). The species pathogenic on members of the "Andropogoneae complex" fell between clusters A and B. Overall, the data set included an alignment of 805 nucleotides of which 217 were parsimony-informative. This resulted in a phylogenetic tree with a CI of 0.924, a RI of 0.890, and a tree length of 542. The robustness of the tree was further confirmed when a very similar tree and bootstrap values were obtained after PAUP analysis was performed with the exclusion of all gaps and corresponding bases from the sequence alignment. Moreover, neighbor joining (NJ), branch-and-bound (B&B) and UPGMA analyses of the data sets using PAUP with random addition options again confirmed the proposed tree. Within cluster A, the species revealed very little nucleotide variation implying a lack of differentiation and therefore a close genetic relationship. Indeed, the identical ITS sequences for the five *U. hordei* geographic isolates and *U. nigra* did not allow separation. The *Tilletia* species in cluster B are clearly separated from the *Ustilago* species due to rather different ITS sequences. This finding is corroborated by their mode of spore germination through distinctive probasidium and basidiospore formation and by their placement in a different subclass. Less homogeneity was revealed for the species between clusters A and B, but *U. scitaminea* and *S. reilianum* seemed related whereas *U. maydis* was consistently separated in several analyses and using different alignments. Moreover, the same results were obtained when the *Tilletia* and *Puccinia* species were deleted and *U. scitaminea* was used as an outgroup. Moreover, a compilation of our ITS sequences and other recent GenBank accessions, some of which are included in another phylogenetic study based on a larger group of different genera within the Ustilaginomycetes (Roux et al 1998), confirmed and expanded our phylogenetic tree (data not shown). For example, the *S. reilianum* and *U. maydis* isolates clustered with our isolates as shown in FIG. 2.

The analysis of the AFLP polymorphisms was targeted to cluster A to try to better distinguish those species and isolates. The data set of the four combined fingerprints yielded a total of 207 characters (bands or fragments) of which 106 were parsimony-informative (polymorphic bands), 24 were constant (bands shared among all strains) and 77 parsimony-uninformative characters (see FIG. 1 lower panel for an example). The complete data set has been deposited in TreeBASE (M631). This character set was more than sufficient to distinguish the eight smuts and even the five *U. hordei* geographic isolates. Using the parsimony analysis in PAUP, a heuristic search resulted in a phylogenetic tree with a CI of 0.732, a RI of 0.700 and a tree length of 250 ("AFLP data" in FIG. 2). Cluster A, including the *U. hordei* geographic isolates, was now clearly resolved and "species" boundaries were supported by high bootstrap values. NJ distance and B&B analyses of the data set revealed identical trees. Moreover, we found no disagreement between the phylogenetic trees generated with the ITS and AFLP data sets.

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FIG. 1. Comparison of AFLP fingerprints from 12 different *Ustilago* species and isolates. Example of a section of a representative electropherogram revealing fluorescent AFLP banding patterns generated with primer combination BamPaa + MsePc.FAM. Sizes in nucleotides (nt) of the single strand DNA fragments on the x-axis. Presence (1) or absence (0) of particular DNA polymorphisms (DNA fragments, or peaks in the Figure) represents the molecular characters and has been scored to create a binomial data set. A part of the data set corresponding to the AFLP fingerprints displayed is printed below; sizes of the fragments in nucleotides (nt) are given vertically above each column. Parsimony noninformative characters include the common bands ("c") and bands indicated with asterisks. Scale factor (arbitrary units on y-axis) varies according to the overall band intensities per sample. Note that due to this overall scale factor not all scored bands are revealed; the GenoTyper software can reveal polymorphisms of lower intensity. Refer to TreeBASE M631 for the complete data set.

DISCUSSION

We initiated this work to determine the usefulness of AFLP polymorphisms as molecular characters in resolving phylogenetic relationships among fungi. In this study we have employed and compared this

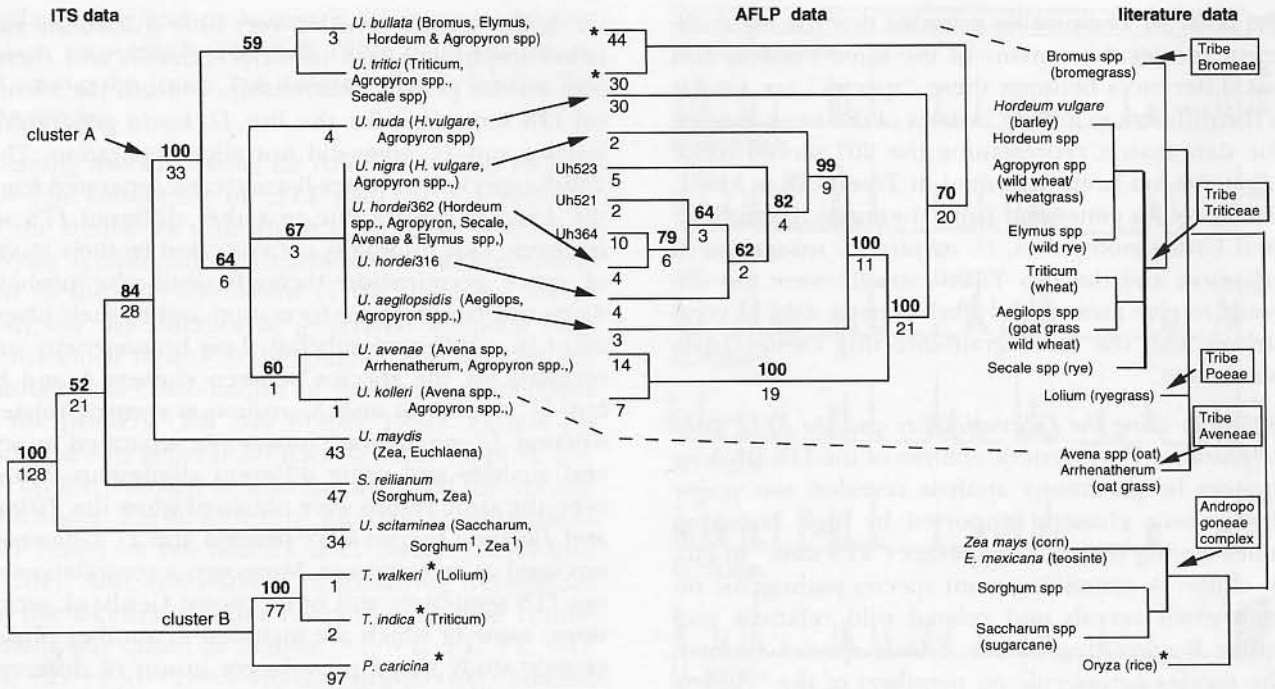

FIG. 2. Phylogenetic relationships among different isolates belonging to the Ustilaginomycetes and their plant hosts. The consensus trees are generated with parsimony analysis in PAUP. Refer to TreeBASE S430. Outgroups are indicated by asterisks and numbers in bold above nodes refer to bootstrap values in percent; numbers below branches indicate the number of character changes between branches (branch lengths not to scale). Following the fungal species name, the major known graminaceous host(s) is given in parentheses. The tree on the left is based on the ITS sequence alignment (CI = 0.924, RI = 0.890, tree length = 542). The tree in the middle is based on the AFLP data (CI = 0.732, RI = 0.700, tree length = 250). On the right, a tree showing a tentative phylogeny of some graminaceous hosts, improvised from literature data, is depicted. Note that sorghum and maize can both be hosts of *U. scitaminea* upon experimental inoculation procedures.

method with another method, ITS sequence comparison, to distinguish species and isolates from fungi belonging to the Ustilaginomycetes. ITS sequence data normally identify organisms at the genus level and can resolve most species. This was revealed in our study as well. However, in closely related groups where species do not exhibit clearly distinct morphological characters or are even interfertile such as the small grain-infecting smuts, species might not reveal a significant number of base pair changes in the ITS region to confidently distinguish them. Additional genes or DNA regions known to be hypervariable, such as the ribosomal intergenic spacer region, need to be analyzed. Whereas such labour and time consuming methods focus on specific, small regions of the genome, AFLP analysis scans the entire genome for polymorphisms. Our AFLP analysis showed that this method can distinguish species in closely related groups or geographic isolates of one species, and even among isolates such as Uh362 and Uh364. The latter two isolates are very related and Uh362 has been back-crossed with Uh364 two times. Recently, the AFLP technique has been used successfully to establish phylogenetic relationships in a large homogeneous clade of cichlid fish (Albertson et al 1999). In our study, the phylogenetic trees generated by the two methods identified similar evolutionary relationships. This suggests that for *Ustilago* species similar phylogenies can be obtained from the DNA in the localised ITS regions and from the genome at large.

The use of an automated capillary gel electrophoresis system capable of detecting four different fluorescent dyes in one run greatly improved the speed, safety and ease of the technique. We selected *Bam*HI/*Mse*I and *Bgl*II/*Mse*I templates and fluorescently-labeled primers *Mse*I(+1) in combination with *Bam*HI(+2) or *Bgl*II(+2) primers because (i) these combinations generated reproducible profiles of 50 to 100 well-separated fragments between 75 and 1000 bp; (ii) the same adapter could be ligated to either *Bam*HI- or *Bgl*II-generated ends; (iii) only four different fluorescently-labeled primers were needed to allow for 64 different primer combinations on the same templates; and (iv) three AFLP reactions labeled with different fluorescent primers (6-FAM, JOE and NED) could be pooled with ROX-labeled internal size standard for separation and detection in the same electrophoresis run. The last three reasons en-

hance the cost- and time-effectiveness of the procedure. The technique proved very robust as identical profiles were generated on different Thermal Cyclers: a PE480 (Perkin Elmer), a 48-well OmniGene (Hybaid), a 96-well TwinBlock™ System (Ericomp) and a RoboCycler, Gradient 96 model (Stratagene). In general, fluorescent-primer chemistry is more sensitive than silver staining (allowing for low intensity fragments to be scored) and the automated data collection and software analysis alleviates the need for digitizing gel images. AFLP fingerprints can be useful identification tools, and polymorphisms detected in profiles from two parents and their progeny can be used to construct a genetic map (in progress for *U. hordei*).

Our AFLP analysis assumes that comigrating bands are homologous and originate from homologous locations in the genome. Only sequence data would be able to support this assumption, although a more extensive molecular analysis would be needed to distinguish homologous sequences originating from repetitive DNA. However, given the discriminatory power of this technique down to two-nucleotide resolution, the chance that comigrating fragments generated with two different restriction enzymes and +1 and +2 specifying nucleotides, respectively, originate from different genomic locations is very small in this genome of 2×10^7 base pairs. The molecular nature of the fragment polymorphisms is unknown. They could be due to a simple nucleotide mutation obscuring the restriction site, or they could involve an insertion or deletion of internal DNA. The molecular characterization of one AFLP marker in our laboratory revealed that this "polymorphic" fragment was deleted in the strain lacking the marker. Molecular characterization of AFLP bands would also determine whether there is phylogenetic incongruence among the "loci" they represent and estimate homoplasy among AFLP characters. Among the interfertile species of cluster A, incongruence could result from hybridization, lineage sorting, and/or horizontal transmission. Overall, the phylogenetic analysis takes into account AFLP data from four different fingerprints representing many polymorphic fragments which originate from many different regions of the genome. It is, therefore, unlikely that the phylogenetic relationships presented here will change because of incongruence among or comigration of some fragments.

The small number of isolates sampled per species obscures the inherent level of AFLP polymorphism in such a species to some degree. Although the number of AFLP polymorphisms between the species seems higher than among isolates, more isolates of each species would have to be analyzed to verify this observation statistically. Some relative height variation can be observed among some peaks in FIG. 1. This is seen sometimes when comparing different PCR runs. It seems to be an inherent phenomenon in such a complex template mixture and might be overcome by a pre-amplification using +1 nucleotide primers to reduce the complexity of the mixture. In these haploid organisms, it could potentially be due to amplification of repetitive sequences in some rare cases.

Cluster A encompasses the subgroup, *U. hordei, U. nigra, U. aegilopsidis, U. avenae* and *U. kolleri*. These species are all interfertile, which, according to Boidin (1986), would make them conspecific. Moreover, their homogeneity on the DNA level, as revealed by ITS sequences and AFLP fingerprints, might indicate recent separation from a common ancestor. Because spore wall echinulation is not a reliable character, being determined by only two genes which are easily transmitted between these species (Huang and Nielsen 1984), and considering the clustering suggested by our data regarding genetic variability, it seems that *U. segetum* var. *avena* should contain *U. avenae* and *U. kolleri,* as well as *U. segetum* var. *hordei, U. nigra* and *U. hordei* where the variety name reflects the major host. This would be a diversion of the proposal by Lindeberg and Nannfeldt (cited in Huang and Nielsen 1984). *Ustilago nigra* seems very closely related to *U. hordei* indeed given that the number of AFLP differences is comparable to those found between *U. hordei* isolates (FIG. 1). Moreover, experiments aimed at determining the number of physiological races of *U. hordei* and *U. nigra* on the same eight differential barley cultivars produced very similar disease ratings for several isolates from either smut (for example, *U. hordei* race 1 infects the exact same host cultivars as *U. nigra* race 2; compare Tapke 1945 and Tapke 1951, respectively). This suggests race overlap and could mean that *U. hordei* and *U. nigra* possibly share some identical avirulence genes and are conspecific. Close association between these two smuts was also concluded after observations of identical disease reactions on segregating populations of some crossed cultivars (Metcalf 1962, Fullerton and Nielsen 1973). The view of Durán (1987) who includes *U. nigra* in *U. segetum* var. *avenae* mainly on the basis of spore morphology, is not supported by our data. Earlier molecular data regarding polypeptide differences led Kim et al (1983) to the conclusion that these species "are indeed more closely related than their present taxonomic position indicates."

Ustilago aegilopsidis hybridizes readily with *U. nigra* and *U. hordei* but has a narrower host range. For example, while *U. aegilopsidis* does not infect barley

(*Hordeum vulgare*) by itself, crosses of *U. aegilopsidis* with *U. hordei* or *U. nigra* do cause infection (Thomas and Huang 1985). In that study, genetic analysis suggested that virulence on barley was under dominant, multigenic control in these smuts. The ease of hybridization, the finding that possibly only three loci are involved in host range differences, and the limited genetic variability shown in our data suggest that *U. aegilopsidis* is conspecific with *U. hordei* and therefore with *U. nigra*. It should probably be called *U. segetum* var. *aegilops*, thereby reflecting the specific host difference. However, our data do not support the view of Vánky (1994) that *U. aegilopsidis* is synonymous with *U. avenae*, along with *U. nigra*.

In *U. nuda* and *U. tritici*, adjacent cells of the basidium of germinated spores fuse immediately to establish the infective dikaryon thereby preventing outbreeding. These are embryo-infecting smuts whose dikaryotic mycelium mimics flower pollination and leads to a systemically contaminated seed. This is in contrast to the other small grain-infecting smuts that infect germinating seedlings. Although the bootstrap values are low, these two smuts seem to lack affinity, each infecting a different major host. It is tempting to speculate that embryo-infection has been derived separately in these species and that the two modes of infection do not constitute such a major difference. Bauer et al (1997) list some evolutionary trends in basidium generation in the smuts that support the idea of repeated changes and reversions. Of course, once this type of spore germination and infection was developed, the species became reproductively isolated. More embryo-infecting species would have to be analyzed to verify whether this character is mono- or polyphyletic.

Several publications report intercompatibility in plate mating assays and hybridizations between all the eight species from cluster A (FIG. 2) on a common grass host, *Agropyron tsukushiense*, leading to teliospore production (Fischer and Holton 1957, Nielsen 1978). Fecundity might be compromised, however, because not all hybrid teliospores, depending on which isolates are used, give rise to viable basidiospores resulting from meiosis upon germination. Indeed, lysis of such basidiospores has been observed, although in a separate study hybrid F1 teliospores have been used as inoculum to produce a normal infection (Nielsen 1968, and citations therein). Thus, the hybrid dikaryon seems viable in the host but it might be difficult to obtain certain hybrid basidiospores for genetic analysis. In addition, previous molecular data from our laboratory concerning the analysis of the mating-type loci, revealed homologous *a* gene complexes and almost identical *b* gene complexes for several species of cluster A; *U. nuda* and *U. tritici* were not analyzed in this study (Bakkeren et al 1992, Bakkeren and Kronstad 1993, 1994). The weak bootstrap support for the phylogenetic analysis of the ITS sequences certainly indicates homogeneity in this first cluster of eight smuts (FIG. 2). Synonymy might only exist within the subgroup containing *U. hordei, U. nigra, U. aegilopsidis, U. avenae* and *U. kolleri* but in order to verify the revealed species boundaries, we would need to investigate multiple (geographic) isolates from each species and from different host genera.

Intercompatibility in plate mating assays has been observed between the species pathogenic on members of the Andropogoneae complex. The sorghum smut, *S. reilianum*, has been mated with the sugarcane smut, *U. scitaminea* (Damann pers comm), and mating has been observed between *U. scitaminea* and *U. maydis* (Bakkeren and Kronstad 1996) although in both cases no teliospores were produced. When mating capabilities are used to indicate relatedness (or even conspecificity) a distinction has to be made between intercompatibility (mating assays) and fecundity (true hybridization leading to fertile progeny). For example, we have also found weak natural intercompatibility between *U. scitaminea* and *U. hordei* (Bakkeren and Kronstad 1996). Moreover, intercompatibility, but not fecundity, could be forced by introducing a complete *a* gene complex, representing the pheromone and pheromone receptor for the alternate pheromone specificity, from *U. hordei* into *U. maydis* to allow this species to mate with a compatible *U. hordei* strain (Bakkeren and Kronstad 1996). Apparently, mating pathways are very conserved among related smuts and specificity resides with the pheromone and its cognate receptor. The ITS sequences among these three smuts revealed many base pair changes, and the AFLP fingerprints were too different to be compared meaningfully. Therefore, the genetic variability among these species is larger than among the smuts from cluster A and, consequently, are well separated from each other and from cluster A (FIG. 2). However, although *S. reilianum* and *U. scitaminea* belong to different genera, they consistently clustered together in several analyses using different parameters. Interestingly, it was found recently through comparative mapping that their respective hosts, sorghum and sugarcane, are highly related (Guimaraes et al 1997).

Over 900 species of Ustilaginomycetes belonging to 60 genera (300 species in *Ustilago*) have been described. Collectively they infect ca 4000 plant species belonging to 75 families, most of which belong to the Gramineae (Seymour 1929, Fischer 1953, Fischer and Holton 1957, Agrios 1988, Holliday 1992). However, the natural host range of most single species is rather

narrow. Some have only a single plant species as host while other species, such as *U. bullata,* seem to have a broader host range; it infects hosts originally from the Eurasian basin and might have been introduced to North-America in the mid 1800s where it expanded its host range to include grasses that are considered native. A similar scenario could apply to many species. Moreover, the natural host range of certain species can be expanded under controlled experimental conditions. Correlation of the phylogenetic relationships between the Ustilaginomycetes studied here with relationships between some of their major graminaceous hosts (FIG. 2, "literature data" compiled from Baum 1986, Davis and Soreng 1993, Liang and Hilu 1995, Hsiao et al 1995a, b, Guimaraes et al 1997) suggests a possible pathogen-host coevolution and niche partitioning. The smuts from cluster A, all pathogenic on small grain cereals and wild relatives contain a subgroup pathogenic mainly on the Triticeae Tribe which originated in the Eurasian basin (although *U. hordei* is also found on *Avenae* species; Nielsen 1993). The *Avenae* smuts have a narrower host range and seem to form the other subgroup correlating with the notion that their hosts belong to a separate Tribe, Aveneae (originating in the Mediterranean basin). Sorghum and sugarcane originated in South-East Asia and cluster, as do their respective smut pathogens. Corn and teosinte are considered indigenous Central-American plants and their smut pathogen, *U. maydis,* seems to be well-separated from the other smuts. However, *U. maydis* and *S. reilianum* are related and can even share the same host, *Zea mays.* Coincidently, they are the only smuts with a tetrapolar mating system in the fungi studied here; all the other smuts, and in fact the majority of smuts in general, have bipolar systems. It was found recently that the distinction between the two forms of mating type is only due to a different organization of the similar *a* and *b* mating type gene complexes on one versus two different chromosomes (Bakkeren and Kronstad 1994, Lee et al 1999). It would be interesting to analyze more tetrapolar smuts to see whether this particular mating type is a mono- or polyphyletic character and evolved occasionally simply through separation of the mating-type gene complexes, thereby increasing the outbreeding potential and contributing to successful niche formation.

Host-range studies naturally would be more informative from a taxonomic point of view if they involved analyses with indigenous or original wild hosts instead of with graminaceous crops, which represent a very recent, accelerated evolution through hybridization and selection by man. Indeed, small grain-infecting smuts seem to have additional hosts that are shared in some cases when wild relatives of cereal crops are considered. If, for example, host range differences on closely related (cereal) plants turn out to be due to a limited number of genes, this would argue for simplification of the species concept in these smuts. Logically, this would increase the host range of the unified species (Rehner and Uecker 1994). From a genetic point of view, man-bred and selected, smut-resistant crops and varieties, in combination with world-wide introductions of smut species and races, have exposed interesting host range differences and resistances that await molecular analysis.

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