Mitochondrial DNA of *Puccinia graminis* f. sp. *avenae:* Molecular Cloning, Restriction Map, and Copy Number

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ABSTRACT

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Mitochondrial DNA (mtDNA) from the oat stem rust fungus *Puccinia graminis* f. sp. *avenae* is circular and 80.15 kb in length. Cloned restriction fragments from *P. g. avenae* race 3 and *P. g. tritici* race 126-Anz-6,7, covering 99.7% of the rust mtDNA, were used for mapping the mitochondrial genome of *P. g. avenae* race 3. A physical map containing 148 restriction sites revealed no repeat structures. Cloned genes from *Schizophyllum commune* mtDNA were used in Southern hybridizations to determine the approximate location of four mitochondrial genes on the restriction map. No restriction fragment length polymorphism was detected between the mtDNAs of *P. g. avenae* races 3 and 8A, which differ for maternally inherited virulence on host lines with resistance gene *Pg-3.* The copy number of *P. g. avenae* mtDNA was estimated to be 19–29 per germinated urediniospore.

Additional keyword: avirulence gene.

Host-parasite interactions in cereal rust diseases are governed by a complementary genetic relationship that has been referred to as a gene-for-gene relationship (5,19). The essential element of a gene-for-gene system is that specific genes in the host and in the parasite interact to condition a compatible or incompatible relationship. No avirulence gene or product in a rust fungus, or corresponding resistance gene or product in the host, has been isolated and cloned. Avirulence gene *avr-9* from *Cladosporium fulvum* is the only fungal avirulence gene that has been isolated and characterized on a molecular level (3).

Genetic studies (9) with reciprocal crosses of *Puccinia graminis* Pers.:Pers. f. sp. *avenae* Eriks. & E. Henn., the causal agent of stem rust of oats, determined that most avirulence genes segregated in the F_2 populations in normal Mendelian segregation ratios. However, avirulence to resistance gene *Pg-3* (*avr-3*) showed maternal inheritance in crosses between races 3 and 8A. Maternal inheritance implied that the *avr-3* gene is not located on the nuclear genome of *P. g. avenae* but is located on a cytoplasmic or mitochondrial plasmid or on the mitochondrial genome. In extensive preliminary experiments, we were unable to detect a plasmid in either of the parental isolates used in the reciprocal *P. g. avenae* crosses made by Green and McKenzie (9). We concluded that the *avr-3* gene in *P. g. avenae* is located on the mitochondrial genome. The much smaller size of this genome, compared with the nuclear DNA, increases the chances for isolating this avirulence gene on a molecular level.

This paper describes the cloning and restriction mapping of the *P. g. avenae* race 3 mitochondrial genome that presumably contains the maternally inherited avirulence gene *avr-3*. Restriction analysis of the mitochondrial DNA (mtDNA) from both parental *P. g. avenae* cultures of the reciprocal crosses was done with 18 restriction endonucleases in order to determine the mtDNA size and fragment order and to detect restriction fragment length polymorphisms (RFLPs). The approximate location of four mitochondrial genes on the restriction map was determined by heterologous hybridization with cloned mitochondrial genes from *Schizophyllum commune* (23). We also estimated the copy number of mtDNA per binucleate, dihaploid germinated urediniosporeling by dot blot hybridization of cloned mtDNA and total DNA from *P. g. avenae* race 3.

MATERIALS AND METHODS

***P. g. avenae* isolates.** Both parental cultures of races 3 and 8A and seven F_2-progeny cultures from both reciprocal crosses of *P. g. avenae* (9) were examined in this study (Table 1). Urediniospores of all cultures were increased on adult plants of the susceptible oat cultivar Makuru. Plants were grown in compost soil in growth cabinets at 18 C with a 16-h photoperiod of incandescent and fluorescent lighting. Virulence phenotypes were evaluated on the *Pga* differential sets (Table 1) (16). Urediniospores were used for DNA extraction only after careful screening to remove foreign material and to ensure racial purity.

TABLE 1. Virulence phenotypes of *Puccinia graminis* f. sp. *avenae* parent and progeny isolates used in this study

Parent and progeny	Host resistance genes[a]	
	Effective	Ineffective
Parental isolates		
60 (4) = race 3[b]	2,3,4,8,13,16	1,9,15
60 (45) = race 8A[c]	1,8,13,16	2,3,4,9,15
F_2 progeny from cross 64 (1); 60 (4) × 60 (45)		
65A20	3,4,8,13,16	1,2,9,15
65B4	3,4,8,16	1,2,9,13,15
65A24	3,4,8,16	1,2,9,13,15
65A41	3,4,8,13,16	1,2,9,15
65A3	3,4,8,13,16	1,2,9,15
65A16	3,4,8,13,16	1,2,9,15
65A37	3,4,8,13,16	1,2,9,15
F_2 progeny from cross 64 (16); 60 (45) × 60 (4)		
65E10	4,8,13,16	1,2,3,9,15
65E9	4,8,13,16	1,2,3,9,15
65F9	8,13,16	1,2,3,4,9,15
65F16	8,13,16	1,2,3,4,9,15
65F22	4,8,16	1,2,3,9,13,15
65B3	8,13,16	1,2,3,4,9,15
65D3	4,8,16	1,2,3,9,13,15

[a] Identified on *Pga* differential sets (16).
[b] Race 3 is homozygous for avirulence gene *avr3* (9).
[c] Race 8A is homozygous virulent on oat cultivar Jostrain (*Pg-3*) (9).

Axenic culture of *P. g. tritici*. The mycelial culture of *Puccinia graminis* Pers.:Pers. f. sp. *tritici* Eriks. and E. Henn. race 126-Anz-6,7 was obtained from W. R. Bushnell, Cereal Rust Laboratory, USDA, University of Minnesota, St. Paul. Mycelium was cultured axenically (15) in the dark at 19 C and 100 rpm on a rotary shaker for 3 wk in 1-L flasks, each containing 200 ml of wheat stem rust medium: 1× Czapeks minerals, 0.3% (w/v) trisodium citrate, 3% (w/v) glucose, 0.4% (w/v) peptic peptone (U.S. Biochemical Corp., Cleveland, OH), and 0.4% (w/v) technical grade casamino acids (Difco, Detroit, MI), pH 6.0.

Gene probes. Plasmid pMF2 containing nuclear rDNA from *Neurospora crassa* (7) was obtained from R. Metzenberg, University of Wisconsin, Madison. Recombinant plasmids containing cloned mtDNA fragments *Eco*RI-4 with cytochrome oxidase subunit III (*CO III*), *Hin*dIII-2 with apocytochrome b (*Cyt b*), *Hin*dIII-4 with cytochrome oxidase subunit I (*CO I*), *Hin*dIII-10 with *S-rRNA* (*17S rRNA*), and *Xba*I-8 with *L-rRNA* (*25S rRNA*) from *S. commune* strain 4-40 (24) were supplied by R. C. Ullrich, University of Vermont, Burlington.

Urediniospore germination and DNA extraction. Urediniospores (1 g) were germinated for 16 h at 20 C (12), harvested, weighed, and frozen in liquid nitrogen. The frozen material was ground to a fine powder with dry ice in a precooled mortar. The material was suspended in lysis buffer (50 mM Tris-HCl, pH 7.4, 10 mM EDTA) containing 40 μg of proteinase K (Boehringer Mannheim, Indianapolis, IN) per milliliter. DNA was extracted and purified with the sodium dodecyl sulfate-cetyltrimethylammonium bromide (SDS-CTAB) method as modified by Kim et al (14). RNA was removed by digestion with RNase A (21). Total DNA from *P. g. avenae* was submitted to CsCl-bisbenzimide density gradient centrifugation (8) with a VTi 65 rotor (Beckman Instruments, Fullerton, CA) at 50,000 *g* for 42 h at 19 C.

Mitochondria were isolated from axenic mycelium of wheat stem rust following the procedure of Kim and Klassen (13) and Douce et al (4). DNA from the DNase I-treated, isolated mitochondria was extracted and purified as described above for total DNA from germinated urediniospores.

Restriction analysis. Restriction endonucleases were obtained from BRL (Bethesda Research Laboratories, Gaithersburg, MD), and digests were done under conditions specified by the manufacturer. Samples were electrophoresed in submarine agarose gels with Tris-borate-EDTA buffer (21). A 1-kb DNA ladder (BRL) was used as the size standard. Gels were stained with ethidium bromide (1 μg/ml) and photographed with Polaroid type 55 film. Fragment sizes were estimated with the Robust curve fitting program (20). Field inversion gel electrophoresis was performed in 1% agarose gels as described by McNabb and Klassen (17).

Cloning rust mtDNA. *Escherichia coli* strain DH5α was the host for plasmids pUC18 (BRL) (27) and pUCBM20 (Boehringer Mannheim). *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, and *Pst*I fragments of total DNA from *P. g. avenae* race 3 and *Hin*dIII fragments of mtDNA from *P. g. tritici* race 126-Anz-6,7 were cloned into pUC18. *Sty*I fragments of total DNA from *P. g. avenae* race 3 were cloned into pUCBM20. Transformants (Amp^r, Lac^-) containing mtDNA inserts were identified by colony hybridization with ^{32}P-labeled total mtDNA from *P. g. tritici* race 126-Anz-6,7. Cloning and screening procedures were performed as described by Sambrook et al (21). Plasmid DNA was purified from *E. coli* by the alkaline lysis method (21).

Southern hybridizations. Total DNA (20 μg) from *P. g. avenae* race 3 or race 8A was restricted with two sets of nine endonucleases: set 1, *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I, *Stu*I, *Sty*I, and *Taq*I; set 2, *Ava*I, *Bcl*I, *Cla*I, *Dra*I, *Hae*III, *Msp*I, *Nco*I, *Sca*I, and *Xho*I. Total DNA from 14 F_2-progeny isolates was restricted with *Bgl*II, *Eco*RV, *Hin*dIII, and *Taq*I. Restriction digests were electrophoresed and transferred to MagnaGraph Nylon membranes (Micron Separations Inc., Westboro, MA) according to Southern (22). Total DNA from *P. g. avenae* race 3 or race 8A, mtDNA from *P. g. tritici* race 126-Anz-6,7, nuclear rDNA from *N. crassa* (pMF2), and cloned fragments of mtDNA from *P. g. avenae* race 3 or from *P. g. tritici* race 126-Anz-6,7

were labeled by nick translation or by random primer labeling with [α-^{32}P]dATP (21) and individually hybridized with the DNA on the Southern blots. Blocking was done for 3 h in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.2% (w/v) Carnation nonfat dry milk, and 0.1% (w/v) Nonidet P-40. Membranes were rinsed for 15 min with hybridization buffer (HSB, 6× SSC, 0.5% [w/v] SDS). Hybridizations were performed overnight in HSB containing 0.3 mg of denatured salmon sperm DNA per milliliter with a Hybaid Mini-Hybridization Oven (BIO-CAN, Mississauga, Ontario, Canada). Blots were washed twice for 15 min each in 5× SSC, 0.5% (w/v) SDS, twice for 15 min each in 1× SSC, 1% (w/v) SDS, and twice for 15 min each in 0.1× SSC, 1% (w/v) SDS.

For homologous hybridizations, all steps were done at 65 C. For hybridizations with mtDNA probes from wheat stem rust (WSR) and nuclear rDNA from *N. crassa* (pMF2), all steps were performed at 60 C under the conditions described above.

Labeled restriction fragments of mtDNA from *S. commune* were used as heterologous probes. Cloned rust mtDNA fragments were excised from recombinant plasmids, electrophoresed, and blotted. The blots were blocked and hybridized with the *S. commune* probes at 50 C under the conditions described above, and washes were done at 50 C in 3× SSC, 0.1% (w/v) SDS.

Autoradiography was performed at −70 C with Kodak X-OMAT AR film and Fisher Lightning Plus intensifying screens. Radiolabeled probes were removed from the membranes by agitation in 0.4 N NaOH at 45 C for 30 min followed by incubation in 0.1× SSC, 0.1% SDS, 0.2 M Tris-HCl, pH 7.5, at 45 C for 15 min.

Mapping. Single, double, or triple restriction digests of rust mtDNA recombinant plasmids were analyzed by standard methods to determine the order and size of fragments. The RZMAP computer program for mapping (6) was supplied by T. Graph, BioMolecular Engineering Research Center, Boston University.

Dot blot hybridization. Total DNA from *P. g. avenae* race 3 and plasmid DNA from clone pJS20S1 was dot blotted onto MagnaGraph Nylon membranes (Micron Separations Inc.) with a 96-well blotting manifold (Pierce Chemical Co., Rockford, IL). Each of the 10-fold serial dilutions of probe DNA (10 μg to 1 pg of DNA per dot) contained 1 μg of salmon sperm DNA per dot as carrier. Salmon sperm DNA (1 μg) was used for control dots, and all samples were done in triplicate. Plasmid pJS20S1 was labeled with [α-^{32}P]dATP by nick translation to a specific activity of 3.2×10^7 cpm/μg. Blocking and hybridization was performed at 68 C under the conditions described above. High stringency washes were done at 68 C in 0.1× SSC, 1% (w/v) SDS. After autoradiography, circles of the filter representing each dot were cut from the membrane, and the radioactivity of each dot was quantified by liquid scintillation counting.

RESULTS

Maternal inheritance of avirulence gene *avr3*. The parental isolates and 14 F_2 cultures of the reciprocal crosses of *P. g. avenae* race 3 and race 8A (9) were reevaluated for virulence phenotype (Table 1) with the *Pga* differential set (16). While virulence to resistance genes *Pg-4* and *Pg-13* segregated in the F_2 progenies, virulence to *Pg-3* did not. This confirmed that the recombinant isolates of Green and McKenzie (9) still showed maternal inheritance of virulence to *Pg-3*.

Characterization of mtDNA. We routinely purified 2–3 mg of total DNA from 4–8 g (fresh weight) of germinated urediniospores. Attempts to isolate mtDNA from total DNA of *P. g. avenae* by CsCl-bisbenzimide density gradient centrifugation were unsuccessful. This result could be due to similar AT-GC ratios of nuclear DNA and mtDNA in *P. g. avenae* or to low amounts of mtDNA in germinated urediniosporelings. Since purification of mtDNA from mitochondria of *P. g. avenae* germinated urediniospores yielded insufficient amounts of mtDNA for cloning and hybridization experiments, we used mtDNA from axenically cultured mycelium of *P. g. tritici* race 126-Anz-6,7 as a probe for the identification of mtDNA from *P. g. avenae*. From 30 g (wet weight) of mycelium, about 3.6 μg of *P. g. tritici* mtDNA was recovered from DNase I-treated mitochondria.

Total DNA from *P. g. avenae* race 3 and race 8A was restricted with 18 endonucleases and examined by agarose gel electrophoresis and Southern hybridization. Results from set 1 of the digests are shown in Figure 1. Southern transfers were hybridized with radiolabeled total DNA from *P. g. avenae* race 8A (Fig. 1A), *P. g. avenae* race 3 (results were identical to *P. g. avenae* race 8A, data not shown), total mtDNA from *P. g. tritici* race 126-Anz-6,7 (Fig. 1B), rDNA from *N. crassa* (pMF2) (Fig. 1C), or cloned mtDNA fragments from *P. g. avenae* race 3 (Fig. 1D) and *P. g. tritici* race 126-Anz-6,7 (results not shown), respectively. Identical bands were detected when mtDNA from *P. g. tritici* and total DNA from *P. g. avenae* were used as probes, except that the rDNA fragments (for example, 9.0-kb *Bam*HI, 8.8-kb *Bgl*II, 3.2- and 2.6-kb *Eco*RI, and 5.8- and 3.2-kb *Pst*I) were absent when probed with mtDNA from *P. g. tritici* mycelium (Fig. 1A–C). The absence of the rDNA bands indicated that nuclear DNA was not present in the mtDNA preparation from DNase I-treated mitochondria. The rDNA fragments were the most prominent (nuclear) bands detected when total DNA from *P. g. avenae* was used as a probe (Fig. 1A and C).

Hybridization with mtDNA from *P. g. tritici* detected no RFLP in the mtDNA of parental *P. g. avenae* isolates with the 18 restriction enzymes (Fig. 1B; results for digests in set 2 not shown). The absence of RFLPs in the mtDNA after restriction with *Bgl*II, *Eco*RV, *Hin*dIII, and *Taq*I was confirmed by similarly analyzing blots of total DNA digests from parental and from all 14 F_2-progeny cultures (data not shown).

The prominent 1.28-kb *Taq*I fragment, detected in race 3 but not in race 8A digests after hybridization with labeled total DNA from *P. g. avenae* (Fig. 1A), was determined to be of nuclear origin, since it was present in all 14 F_2 cultures of the reciprocal crosses (data not shown).

The mitochondrial DNA fragment sizes obtained by digestion with eight endonucleases are listed in Table 2. The sizes of fragments larger than 12 kb were determined after pulsed field gel electrophoresis with high molecular weight DNA size markers (data not shown). Fragment sizes and identification of fragments occurring in doublet or triplet bands were confirmed by restriction analysis and hybridization data with cloned mtDNA fragments from *P. g. avenae* and *P. g. tritici*. The size of the mitochondrial genomes was estimated to be 80.15 kb (Table 2).

Cloning of the mitochondrial genome. All cloned mtDNA fragments from *P. g. avenae* hybridized with mtDNA from *P. g. tritici*. The cloned mtDNA fragments were identified first by double digestion of the plasmid DNA with a combination of the endonuclease used for cloning plus one of the following endonucleases: *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I, *Stu*I, and *Sty*I. Identity of cloned fragments was confirmed by hybridization of the labeled plasmid DNA to membrane-bound total DNA from *P. g. avenae* race 3 and race 8A that had been restricted with 18 endonucleases. Figure 1D demonstrates the results obtained by hybridizing radiolabeled plasmid pJS18B23 containing the mtDNA fragment *Bam*HI-5 (6.6 kb) from *P. g. avenae* race 3 with the same blot from Figure 1A–C. The probe hybridized with the homologous *Bam*HI-5 fragment and with fragments *Bgl*II-6, *Bgl*II-15, *Bgl*II-17, *Eco*RI-4, *Eco*RI-6, *Eco*RV-3, *Eco*RV-9, *Eco*RV-12, *Eco*RV-17, *Hin*dIII-3, *Hin*dIII-15, *Hin*dIII-18, *Hin*dIII-23, *Pst*I-1, *Stu*I-1, *Sty*I-2, *Sty*I-7, and *Sty*I-12 from *P. g. avenae* race 3 and race 8A (Fig. 1D, Table 2).

Restriction site map. There was no evidence of duplicated DNA. The mitochondrial DNA of race 3 and race 8A is a circular molecule 80.15 kb in length.

Cloned restriction fragments and eight restriction enzymes were used for mapping. Attempts to clone each *Hin*dIII fragment did not succeed. The 50 *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Pst*I, *Hin*dIII-*Pst*I, *Pst*I-*Hin*dIII, and *Sty*I fragments cloned from *P. g. avenae* race 3 (Table 2) represent 74.24 kb (92.6%) of the mitochondrial genome. Together with the fragment *Hin*dIII-5 (5.7 kb) cloned from *P. g. tritici* race 126-Anz-6,7, 79.94 kb (99.7%) of the mitochondrial genome were covered.

Each cloned fragment was examined for the presence of restriction sites for the other enzymes of the set. A first approximation for the order of fragments was based on hybridization data (Fig. 1D) by analyzing each of the 50 cloned fragments from *P. g. avenae* and 11 cloned fragments from *P. g. tritici*. For the precise order, double and triple restriction digests of all recombinant plasmids were analyzed with all appropriate combinations of endonucleases. Mapping was facilitated, since 54.42 kb representing 67.9% of the mitochondrial genome were cloned at least twice with different endonucleases. Restriction analysis and

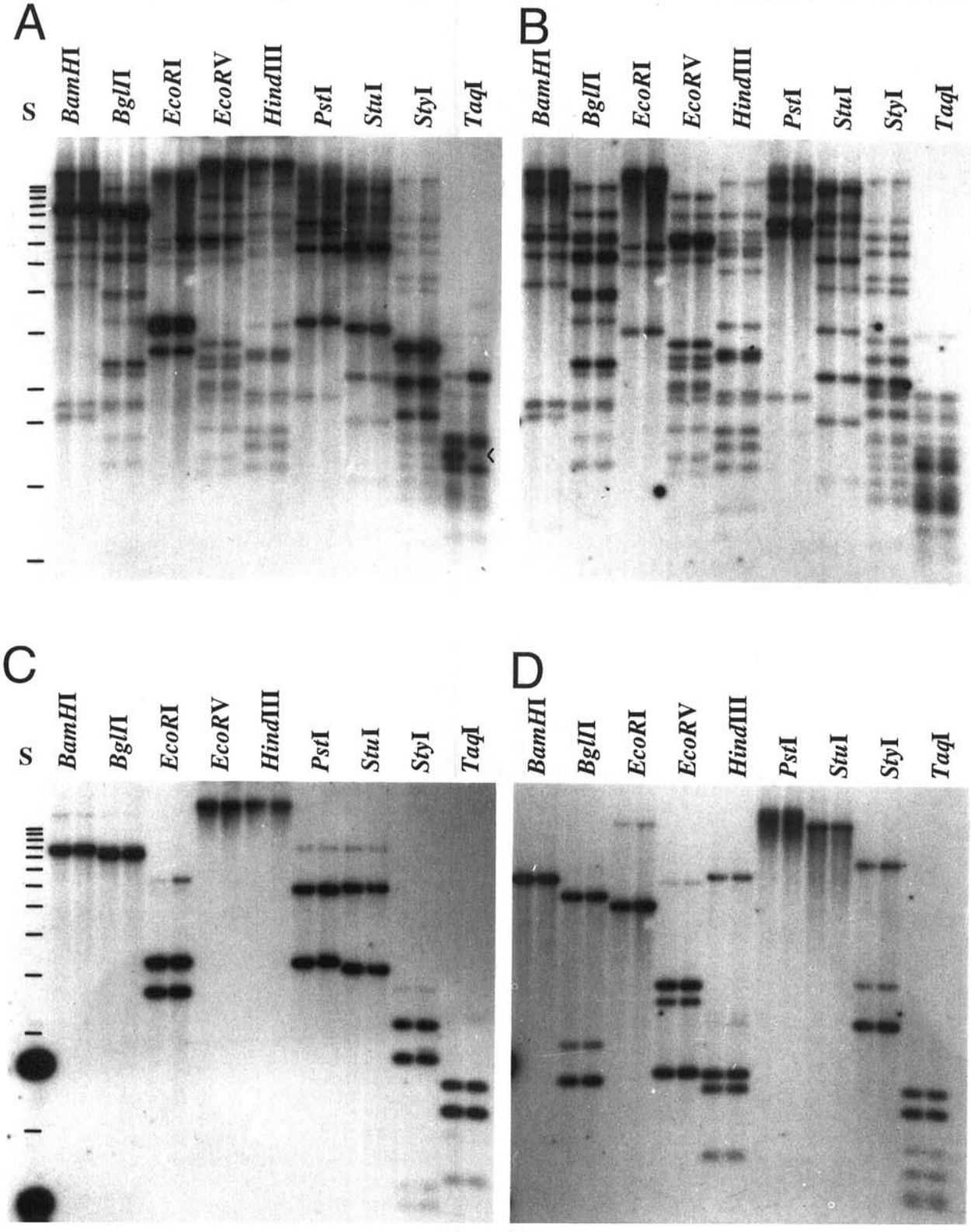

Fig. 1. Southern hybridizations of restricted total DNA from *Puccinia graminis* f. sp. *avenae* race 3 and race 8A. Total DNA from *P. g. avenae* race 3 (left lane of pair) and race 8A (right lane of pair) was digested with the endonuclease indicated, blotted, and hybridized sequentially with nick-translated total DNA from **A,** *P. g. avenae* race 8A, **B,** mtDNA from DNase I-treated mitochondria from *P. g. tritici* race 126-Anz-6,7, **C,** nuclear rDNA from *Neurospora crassa* (plasmid pMF2), and **D,** plasmid pJS18B23, containing the cloned 6.6-kb mtDNA fragment *Bam*HI-5 from *P. g. avenae* race 3. Sizes of the 1-kb DNA ladder (Bethesda Research Laboratories) fragments in lane S are 12.2, 11.2, 10.2, 9.2, 8.1, 7.1, 6.1, 5.1, 4.1, 3.1, 2.0, 1.6, 1.0, and 0.5 kb. The arrow in **A** indicates the polymorphic nuclear *Taq*I fragment present in race 3 but absent in race 8A.

hybridization data of overlapping, cloned restriction fragments provided sufficient information to confirm the order of fragments and the location of the restriction sites.

The orientation of the restriction fragments on the mitochondrial genome was determined by mapping the location of the unique *Eco*RV site in plasmid pJS4H2. This plasmid contains the 5.36-kb *Hin*dIII-*Pst*I fragment, originating from fragment *Hin*dIII-1 of *P. g. avenae* race 3, ligated into the appropriately double-restricted multiple cloning site of pUC18 (Table 2). Double digestions of pJS4H2 with *Hin*dIII and *Eco*RV as well as with *Pst*I and *Eco*RV revealed that the *Eco*RV site within the cloned fragment was present 4.51 kb from the *Hin*dIII restriction end (Fig. 2).

Mapping genes. Mitochondrial genes were located on the restriction map (Fig. 2) by heterologous hybridization of cloned mtDNA fragments from *P. g. avenae* or *P. g. tritici* with radiolabeled, cloned fragments of *S. commune* strain 4-40 mtDNA, each containing a known gene (24). Fragments *Eco*RI-7 (*CO I* gene originating from cloned fragment *Hin*dIII-4 [5.65 kb]), *Eco*RI-4 (*CO III* gene), *Hin*dIII-2 (*Cyt b* gene), and *Hin*dIII-10 (*S-rRNA*) or *Xba*I-8 (*L-RNA*) from *S. commune* were isolated after agarose gel electrophoresis, radiolabeled, and hybridized individually under conditions of reduced stringency to Southern transfers of restricted recombinant plasmids containing rust mtDNA in order to determine the approximate position of the known mitochondrial genes.

Of the probes tested, only the *S-rRNA* gene probe from *S. commune* did not hybridize to any of the 50 cloned mtDNA fragments from *P. g. avenae* or to 11 cloned mtDNA fragments from *P. g. tritici* (results not shown). Fragment *Hin*dIII-10 from *S. commune* contains only part of the *S-rRNA* gene (24), and the precise location of the coding region(s) in fragment *Hin*dIII-10 is unknown. It is possible that this probe from *S. commune* contains sequences of the *S-rRNA* gene that are not highly conserved among basidiomycetes. Insufficient homology of the gene probe from *S. commune* rather than the absence of the *S-rRNA* gene in *P. g. avenae* mtDNA may be the reason that this probe does not hybridize to *P. g. avenae* mtDNA.

Each of the other probes hybridized to a unique set of cloned mtDNA fragments from *P. g. avenae,* and the approximate gene locations given in Figure 2 represent the smallest portions of the mitochondrial genome tested to which the probes hybridized.

Copy number of mtDNA. The average copy number of mtDNA per nuclear genome of *P. g. avenae* germinated urediniospore was estimated; it was assumed that nuclear DNA and mtDNA are extracted with equal efficiency. The haploid genome size of *P. g. tritici,* determined with reassociation kinetics, is 6.7×10^7 bp (1). If one assumes that the DNA size of a binucleate, dihaploid germinated urediniospore from the closely related *P. g. avenae* is 1.34×10^8 bp, it is possible to estimate the copy number of mtDNA by dot blot hybridization.

Dot blot hybridization intensities of 10-fold serial dilutions of plasmid DNA from clone pJS20S1, containing the 7.4-kb fragment *Pst*I-6 from *P. g. avenae* race 3 mtDNA, and of total DNA from *P. g. avenae* race 3 were determined with radiolabeled plasmid pJS20S1 as the probe. The hybridization signal of 1 ng of plasmid DNA from pJS20S1 and of 1 μg of total DNA from *P. g. avenae* was 88 ± 21 cpm and 119 ± 28 cpm, respectively.

TABLE 2. Fragment sizes (kilobase pairs) of mitochondrial DNA (mtDNA) from restriction endonuclease digestions of total DNA from *Puccinia graminis* f. sp. *avenae* race 3 and race 8A[a,b,c]

Fragment number	Restriction endonuclease							
	*Bam*HI	*Bgl*II	*Eco*RI	*Eco*RV	*Hin*dIII	*Pst*I	*Stu*I	*Sty*I
1	17.70	12.00	18.10	10.25	12.91[d]	24.44	13.85	13.50
2	16.60	8.00	15.80	8.10	8.04	13.60	13.60	7.75
3	11.30[d,e]	6.75	14.65	6.45	6.60[d,e]	10.10	12.05	6.45
4	7.55	6.55[d]	14.10	6.30	6.30[d,e]	7.40[d]	8.10	5.55
5	6.60[d]	6.30	5.80[d]	6.30	5.70[e]	7.40[d]	7.70	4.40
6	6.40[d]	5.60	5.00[d]	6.10	5.45[d]	7.40[d]	6.70	4.10
7	5.50[d]	5.40	3.00[d]	5.70	4.66	6.60[d]	5.20	2.80[d]
8	4.20[d]	5.20[d]	3.00[d]	5.30	3.10[d,e]	1.90[d]	4.25	2.80
9	1.90	4.00[d]	0.70	2.80	2.60[d]	0.55[d]	3.00	2.40
10	1.65[d]	3.85	...	2.70	2.50[d]	0.55	2.00	2.40
11	0.75[d]	3.20[d]	...	2.50	2.44[d]	0.21[d]	1.60	2.20
12	...	2.40[d]	...	2.45	2.40	...	1.10	2.15
13	...	2.40	...	2.35	1.90[d,e]	...	4.25	2.10
14	...	2.20[d]	...	2.10	1.80[d]	...	0.40	2.10
15	...	1.81	...	2.05	1.50	...	...	2.00
16	...	1.80[d]	...	1.90	1.45[d,e]	...	...	1.90
17	...	1.49	...	1.55	1.30[d,e]	...	...	1.70
18	...	1.20	...	1.25	1.30[d]	...	...	1.70
19	...	...	...	1.10	1.30[d]	...	...	1.50
20	...	...	...	0.80	1.20[d]	...	...	1.50
21	...	...	...	0.80	1.10[e]	...	...	1.40
22	...	...	...	0.60	0.86[d]	...	...	1.20
23	...	...	...	0.50	0.80[d,e]	...	...	1.15
24	...	...	...	0.20	0.65[d]	...	...	1.00[d]
25	...	...	...	...	0.64[d,e]	...	...	0.90
26	...	...	...	...	0.50	...	...	0.90
27	...	...	...	...	0.48[d]	...	...	0.70[d]
28	...	...	...	...	0.46[d,e]	...	...	0.60
29	...	...	...	...	0.21	...	...	0.40
30	...	...	...	...	...	...	...	0.35
31	...	...	...	...	...	...	...	0.35
32	...	...	...	...	...	...	...	0.20
Total	80.15	80.15	80.15	80.15	80.15	80.15	80.15	80.15

[a] Mitochondrial origin of restriction fragments was established by hybridization with mtDNA from DNase I-treated mitochondria, which had been purified from axenically cultured mycelium of *P. g. tritici* race 126-Anz-6,7.
[b] Fragment sizes were identical for mtDNA from race 3 and race 8A.
[c] Fragment sizes larger than 12 kb were determined by field inversion gel electrophoresis and by mapping with cloned restriction fragments.
[d] Fragment cloned from *P. g. avenae*.
[e] Fragment cloned from *P. g. tritici*.

The copy number of mtDNA can thus be estimated to be 24 ± 5 per binucleate, dihaploid cell of germinated urediniospore.

DISCUSSION

P. g. tritici has a relatively large haploid genome size of 6.7 × 10^7 bp containing 64% unique and 30% repetitive sequences (1). We used nick-translated total DNA as a probe in Southern hybridizations to locate the restriction fragments of repetitive DNA in restriction digests of total DNA from *P. g. avenae.* Almost all repetitive sequences detected by this method were identified to be mitochondrial and nuclear ribosomal DNA sequences. Unique sequence DNA remained undetected because of its low copy number. The same strategy has been used (23) to locate repetitive sequences in *S. commune.*

A restriction map of the mitochondrial genome was determined for *P. g. avenae* race 3, which presumably carries the maternally inherited avirulence gene 3, and race 8A, which lacks this gene. We conclude from our data that the mtDNA from both races is circular and 80.15 kb in length. This is similar to the length of 76 kb reported for mtDNA of *Ustilago cynodontis* (18). However, another member of the Ustilaginales, *U. violacea,* has a mitochondrial genome size of 45 kb (26). In fungi, including basidiomycetes, extensive variation in mitochondrial genome size has been found between related taxa (2,11,25).

Southern hybridization experiments for ordering restriction fragments detected no regions of repeated DNA. Heterologous hybridization with cloned mitochondrial genes from *S. commune* revealed the approximate location on the restriction map of the *L-rRNA, CO I, CO III,* and *Cyt b* genes. The four mitochondrial genes located on the restriction map of *P. g. avenae* are arranged in the same order as those in *S. commune* (24), but the gene order in *P. g. avenae* differs from those reported for *Agaricus brunnescens* (10), two species of *Coprinus* (25), and five species of *Suillus* (2). To our knowledge, this is the first report on the mitochondrial genome size and organization of a member of the Uredinales. The average copy number of mtDNA in germinated urediniospores of *P. g. avenae* is similar to the number present in the filamentous mycelium of *S. commune* (23).

Restriction analysis of the mtDNA with 18 restriction endonucleases detected no RFLPs between the two *P. g. avenae* races we tested. This result indicates that the *avr3* gene probably does not involve large segments of mtDNA but may be the result of relatively small differences in base-pair sequences. The combined number of recognition sequence nucleotides used for restriction analysis comprised only about 1.8 kb (2.2%) of the *P. g. avenae* mitochondrial genome. The detection of melting behavior polymorphisms with denaturing gradient gel electrophoresis is a faster and more efficient method for revealing single base pair changes in mtDNA (28). Sequencing the entire mitochondrial genome presumably would localize the gene. However, identification may be more complicated, since it is likely that any two races may also differ in characters other than virulence. For the identification of the *avr3* gene in *P. g. avenae,* transformation of the rust mitochondria will be necessary.

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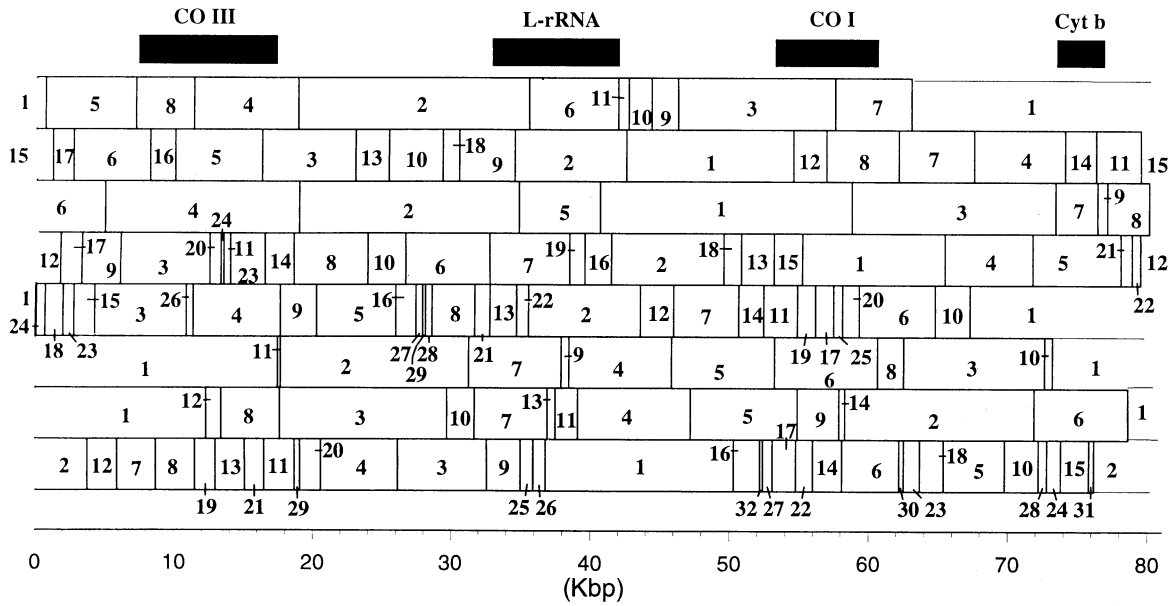

Fig. 2. Restriction site map of *Puccinia graminis* f. sp. *avenae* race 3 mtDNA. The circular genome is arbitrarily linearized at the *Eco*RI site between cloned fragments *Eco*RI-8 and *Eco*RI-6. Restriction sites (indicated by vertical bars) were mapped by hybridization with and digestion of cloned fragments. Restriction fragment numbers are the same as in Table 2. Gene locations are approximate and were determined by hybridization with *Schizophyllum commune* strain 4-40 cloned mtDNA fragments (24). *CO* = cytochrome oxidase subunits; *L-rRNA* = 25S ribosomal ribonucleic acid; *Cyt b* = apocytochrome b.

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