Restriction Endonuclease Map of the Mitochondrial DNA of *Mycosphaerella graminicola*

Kelly Hogan
University Undergraduate Research Fellow, 1994-95
Texas A&M University
Department of Plant Pathology

APPROVED
Undergraduate Advisor Bruce A. McDonald

Exec. Dir, Honors Program

Fungal diseases cause significant economic losses in agriculturally important crops. On average a 5-10% loss of yield per year due to pathogenic fungi is typical in industrialized countries (James 1974). A knowledge of the genetic structure of populations of pathogenic fungi is necessary before effective methods of control for fungal disease can be developed. Genetic structure of a population refers to allele frequencies and genetic diversity in a population. The more genetic diversity in a population the more likely that the fungi will be able to adapt to overcome controls. In the past genetic variation of pathogen populations has been determined by virulence markers and isozymes. Since virulence genes and isozymes are under such strict selection, they are inadequate to determine the true genetic diversity of a population (McDonald and McDermott 1993). More recently, restriction fragment length polymorphisms (RFLP) have been used to determine genetic diversity. "RFLPs are based on the hybridization of DNA probes to fragments of DNA that have been digested with specific restriction endonucleases and size fractionated on an agarose gel" (McDonald and McDermott 1993). RFLP markers, which are selectively neutral, have the advantage of being highly variable and codominant. These qualities make RFLPs much better markers than virulence genes. The focus of many studies of pathogenic fungi have been RFLPs in mitochondrial DNA (McDonald and McDermott 1993) Study of the mitochondrial genome has been an important aspect in the study of the genetic structure of fungal populations in a variety of ways. Mitochondrial DNA has been used in determining taxonomic relationships, in some species of fungi mitchondrial DNA is host specific, and in still other species of fungi mitochondrial DNA has been used as individual fingerprint (Borromea et al 1993 and Milgroom and Lipari 1993).

Over the past several years the genetic structure of populations of the fungus *Mycosphaerella graminicola* have been studied using RFLP analysis

of the nuclear genome. In addition to a large amount of genetic diversity found in each population studied, similar allele were found at similar frequencies in all populations studied including international populations. This data supports the hypothesis that gene flow is occurring between populations (McDonald, personal communication). The exception to the large amount of genetic diversity found in *M. graminicola* populations is found in the mitochondrial genome. Figures 1 and 2 illustrate the difference between the diversity in the nuclear and mitochondrial DNA. Only eight different mitochondrial haplotypes have been found world-wide, Fig 3 is a representation of six of the eight PstI haplotypes. This is unusual since studies of other fungi have shown that the amount of mitochondrial variation is similar to the amount of variation in the nuclear genome (Milgroom and Lipari 1993). The most common haplotype, haplotype 1, has been found in all but one of the populations internationally (McDonald, Personal communication).

My objectives in the experiment described here were to determine if the most common mitochondrial haplotypes, type 1 and type 2 were actually the same in international populations. Also a restriction site map of the mitochondrial genome is necessary before any hypothesis can be made as to why there is so little variation in mitochondrial genome. In addition, I wished to determine if there was evidence for selection of the mitochondrial haplotype based on Host genotypes.

DNA isolation:

DNA was extracted using a modified CTAB extraction procedure. Tissue, cultured in YSB (1% sucrose, 1% yeast extract), was harvested after 7-10 days, then frozen and lyophilized for 48 hrs. The lyophilized tissue was ground into a powder, dispersed in 5 ml of CTAB extraction buffer [700

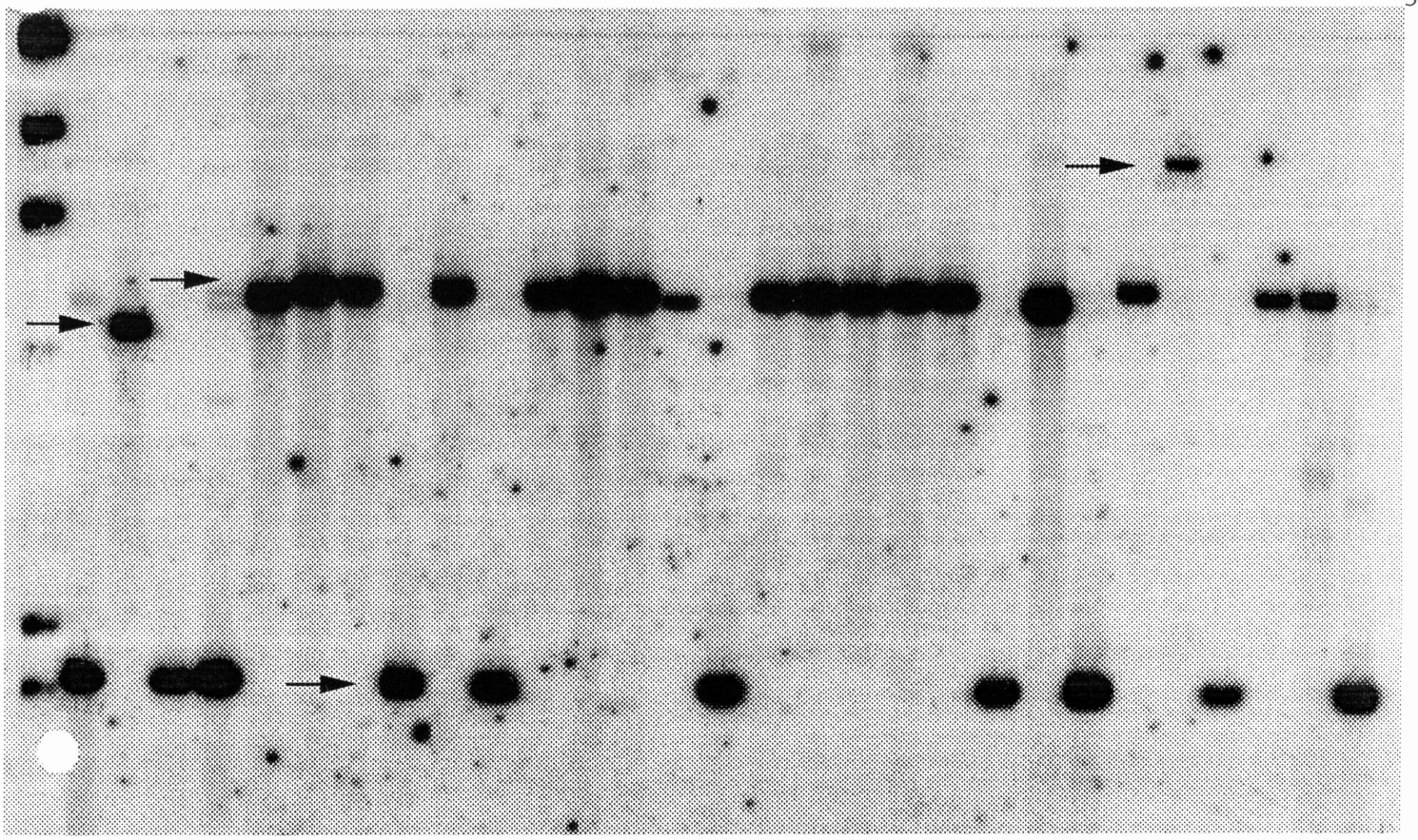


Figure 1: This is a picture of a population of M. graminicola taken from a single field in Indiana. The DNA was digested with Pst I, size fractionated on an 0.8% agarose gel and Southern blotted to a nylon membrane. An anonymous nuclear probe was used. The arrows indicate the four different alleles found in the population.

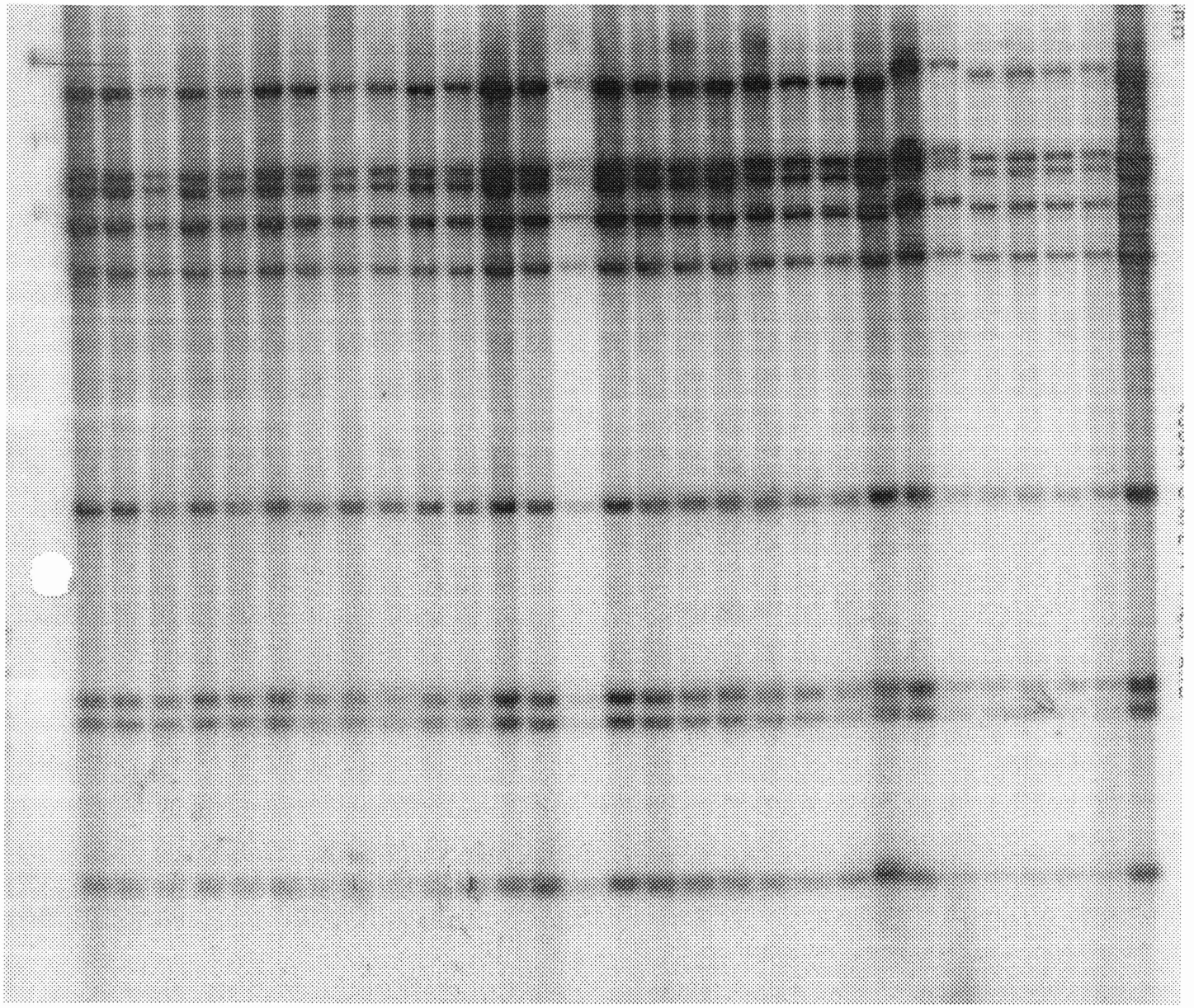


Figure 2: This is the same Indiana population shown in the previous figure, with mtDNA used as a probe. Notice that all the mt haplotypes are identical.

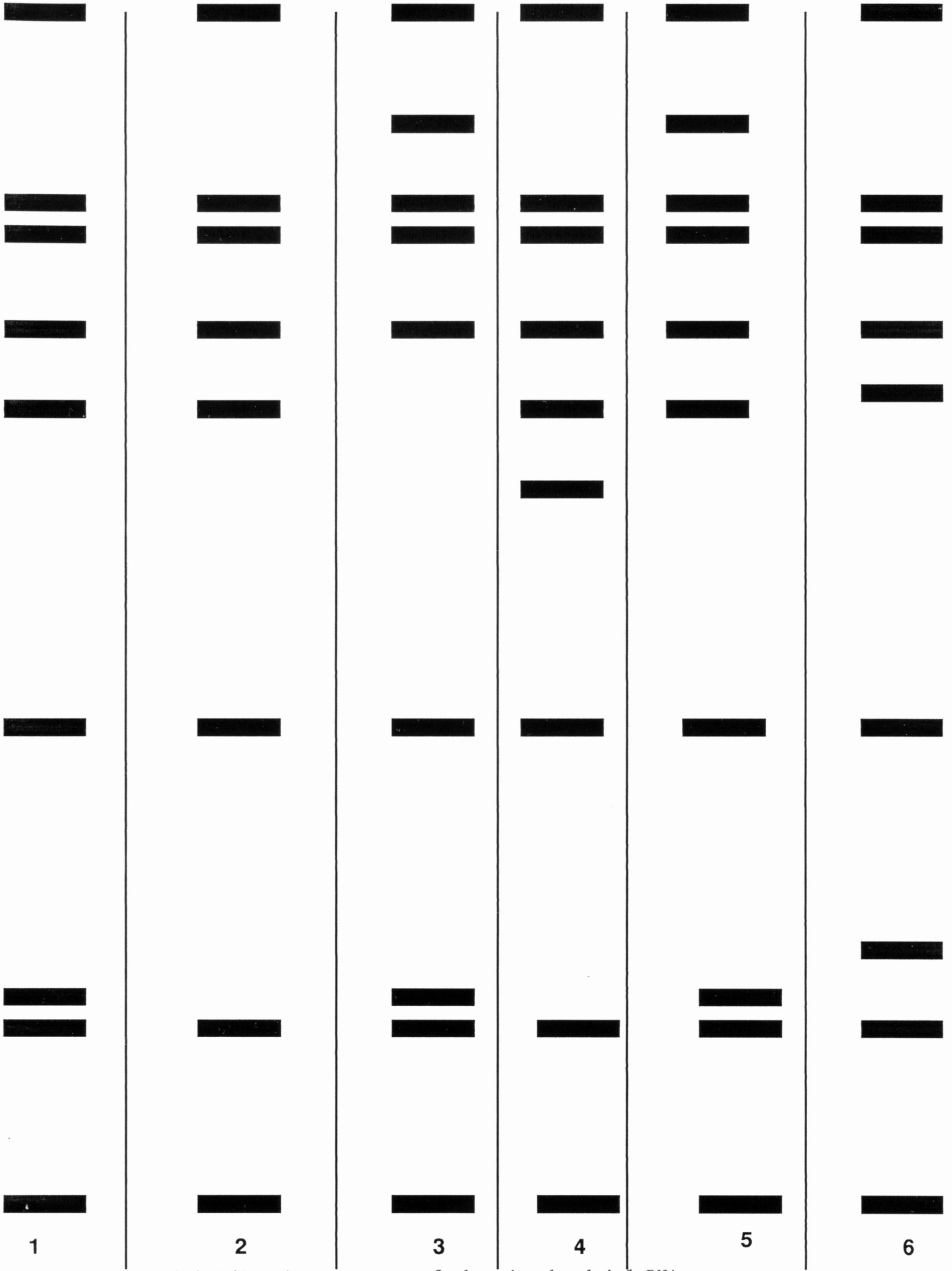


Figure 3: PstI hybridization patterns of the mitochondrial DNA.

mM NaCl, 50 mM Tris-HCl(pH 8.0), 10 mM EDTA, 1% 2-mercaptoethanol (v:v), and 1% CTAB (w:v)] in a 15-ml centrifuge tube, and placed in a 60 C water bath for 30-45 min. An equal volume of chloroform:iosamyl alcohol (24:1, v:v) was added and the tube mixed by inversion to form an emulsion. The emulsion was spun 10 min in a centrifuge at 12,900 X g, at 4 C and the upper aqueous phase was transferred to a fresh 15-ml tube. A second equal volume of chloroform:isoamyl alcohol was added and the tube mixed by inversion to form an emulsion. The emulsion was spun 10 min in a centrifuge at 12,900 X g at 4 C and the upper aqueous phase was transferred to a fresh 15-ml tube. Nucleic acids were precipitated by adding 0.8 volumes of isopropanol, inverting tube several times, and allowing the tube to sit at least 10 min before centrifuging 6 min at 10,400 X g at 25 C. The nucleic acid pellet was dissolved in 2 ml of TE (10mM Tris, 1 mM EDTA, pH 8.0); it was then precipitated by adding 1 ml of 7.5 M NH_4OAc and 4 ml of 95% ethanol, inverting tube several times, and allowing the tube to sit for at least 10 min before centrifuging 6 min at 10,400 X g at 25 C. The pellet was dissolved in 0.4 ml of TE and transferred to a 1.5-ml microfuge tube, and nucleic acids were precipitated by adding 0.2 ml of 7.5 M NH_4OAc and 0.8 ml of 95% ethanol, inverting tubes several times, and allowing to sit for at least 10 min before centrifuging for 10 min at 16,000 X g. Nucleic acid pellets were allowed to air dry for 30 min then resuspended in minimal amounts of TE.(McDonald and Martinez, 1990)

Probes:

The probes used had been previously cloned into pGem4 which is used as a vector. 3 ml of LB-Amp (75 µg/ml) with a single *E. coli* colony bearing the plasmid containing the probe and incubated at 37 C with shaking (200 rpm) until the culture reaches the stationary growth phase (18-20 hrs). 1.5

ml of cells were pelletted in a 1.5 ml microfuge tube by spinning at full speed for 30 sec and resuspended in 350 µl of STET (50 mM Tris, 50 mM EDTA, 0.5% Triton X-100, 8.0% sucrose) by vortexing vigorously for 20-30 sec. To the resuspended cells 15µl of fresh lysozyme (10mg/ml of TE?) was added, vortexed briefly to mix and let sit at room temperature for 3-5 minutes. The microfuge tube was placed in a boiling water bath for 45 sec then removed and placed on ice until the tube had cooled room temperature (5-10 min) The tube was centrifuged at top speed for 15-20 minutes (The chromosomal DNA occupied no more than 20% of the volume.) The chromosomal pellet was removed with a sterile toothpick. The plasmid was precipitated with 150µl of 7.5 M NH_4OAc and 900 µl of 95% ethanol, inverting tube several times and allowing mixture to sit for at least 10 min before centrifuging at full speed for 10 min. Pellet was allowed to air dry and then resuspended in 50 µl of TE. The plasmid containing the probe was digested with HindIII and incubated overnight. The digest was size-fractionated on a 1..0% low-melt agarose gel run at 20V. The DNA was visualized using ethidium bromide and UV radiation. The procedure for extraction of DNA from low-melt agarose was modified from Maniatis. While wearing protective clothing the gel was placed on a 312nm UV transilluminator and the probe DNA was cut from the gel using a new razor blade. The transilluminator was turned off and the agarose plug was placed in a 15-ml tube. 5 volumes of TE was added to the agarose and placed at 65 C for 5 min to melt the agarose. An equal volume of phenol was added to the mixture, briefly vortexed, centrifuged at 4,000 X g for 5 min, and the upper aqueous layer was transferred to a clean tube. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v:v:v) was added to the mixture, briefly vortexed to form an emulsion, centrifuged at 6,600 X g for 10 min,

and the upper aqueous layer was transferred to a clean tube. An equal volume of chloroform:isoamyl (24:1, v:v) was added to the aqueous layer, vortexed briefly to emulsify, centrifuged at 9,000 X g for 7 min and the upper aqueous layer was transferred to a clean tube. To precipitate the nucleic acids 0.5 volumes of NH_4OAc and 2 volumes of 95% ethanol were added and the mixture was allowed to sit overnight at 4 C. The nucleic acids were pelletted by centrifuging at top speed and resuspended in a minimal amount of TE.

Southern blotting and hybridization:

2.5 μg of total cellular DNA from each isolate was incubated overnight with 25 U of enzyme. HindIII, PstI, XhoI, HindIII and PstI, and HindIII and XhoI were used for the isolates used to make the restriction maps. PstI, HindIII, BamHI, DraI, EcoRI, EcoRV, HaeIII, KpnI, NspV, PvuII, TaqI were used to digest the type 1 and type 2 isolates. The DNA fragments were separated on 0.8% agarose gels in TBE (Tris-borate-EDTA) buffer at 0.5 V/cm. Following electrophoresis, the fragments were transferred to Zetaprobe membranes (Biorad, Hercules, CA) by the alkaline transfer method according to the manufacturer's directions.

Membranes were prehybridized in a buffer containing 250 mM disodium phosphate and 7% SDS at 60 C for at least 1 hour. Probes were labeled with ^{32}P by nick translation according to manufacturer's directions. Probes were hybridized to membranes overnight 250 mM disodium phosphate and 7% SDS at 60 C in hybridization incubator. Following hybridization, membranes were washed twice for 1.5 hrs at 60 C in 20 mM disodium phosphate and 5% and then twice for 1.5 hrs at 60 C in 20mM disodium phosphate and 1% SDS.

The hybridization patterns were visualized by exposing X-ray film to the blots for 4-16 hrs. at -80 C.

Results:

Analysis of type 1 and type 2 mitochondrial haplotypes:

Ten type 1 isolates and 5 type 2 isolates were digested with ten different restriction endonuleases, and probed with total mitochondrial DNA. From this experiment one type 1 isolate from the United Kingdom was found that had a deletion that the other nine isolates did not contain. Other than this one variation, the other isolates had identical haplotypes with all 11 endonucleases used. The type 2 isolates proved more interesting. Two isolates from the UK and Germany had the loss of a DraI restriction site that was not found in the other isolates. Also different isolates from the UK and the US had the loss of a PvuII restriction site that was not found in the other isolates.

Restriction Maps:

The following four figures show the restriction maps made of type 1-type 4. In making the restriction maps of the mt DNA of *M. graminicola* two of the haplotypes previously thought to be independent haplotypes were determined to be artifacts. The PstI haplotype shown in fig. 3 was found to be haplotype 3. It is possible that the original isolate found to be type 5 was a hybrid containing both type 1 and type 3 mitochondrial haplotypes. Also PstI haplotype 6 was found to be identical to type 1. This is understandable since the difference between type 1 and type 6 was only a couple of hundred base pairs found in a 1.6 Kb piece as shown in fig. 3. The further study that making the restriction map allowed showed no measurable difference between these types. Previously, eight different

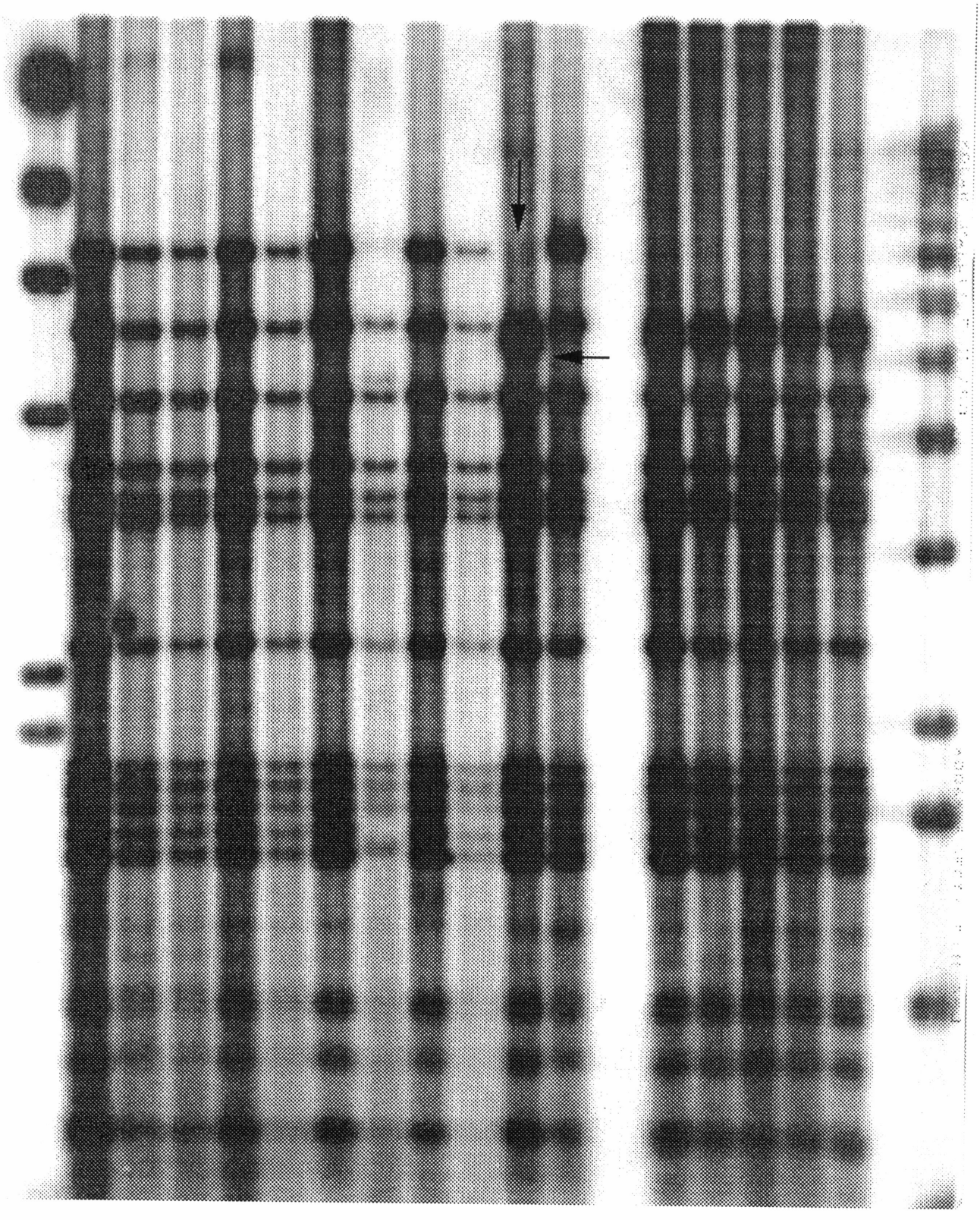


Figure 4: This is an audorad of the total mt DNA cut with HaeIII. The arrows indicate the deletion that occurs in the isolate from the UK.

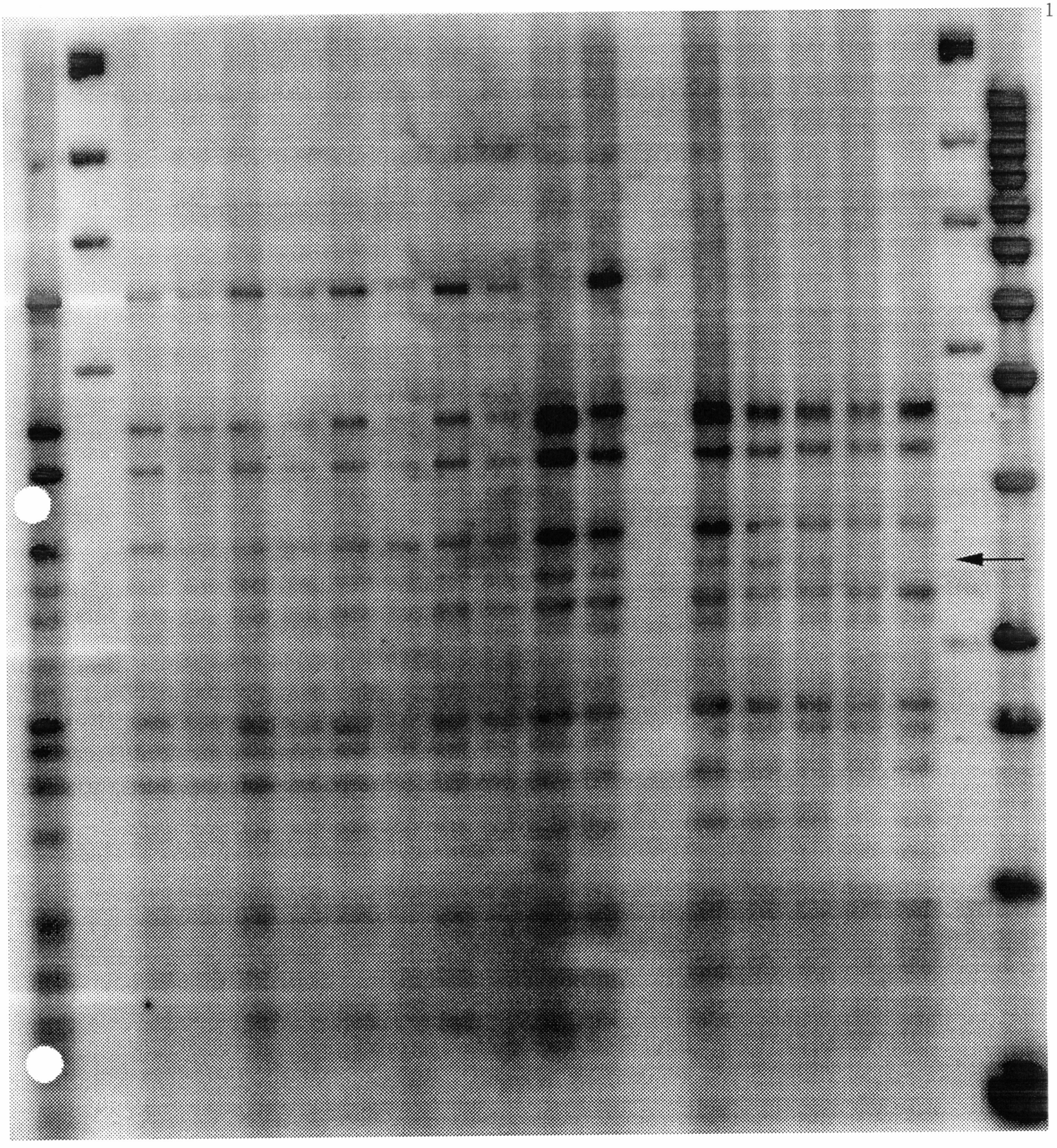
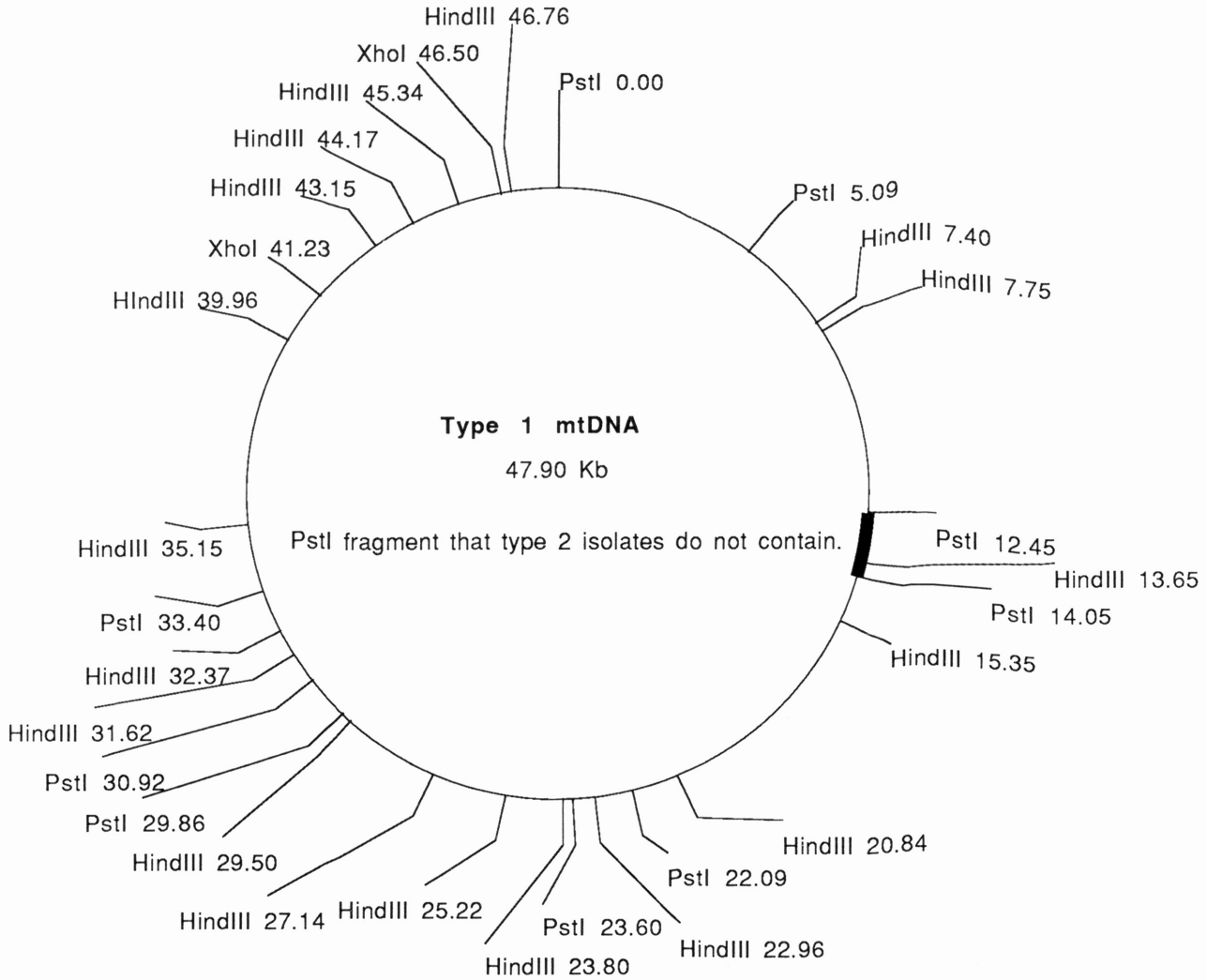
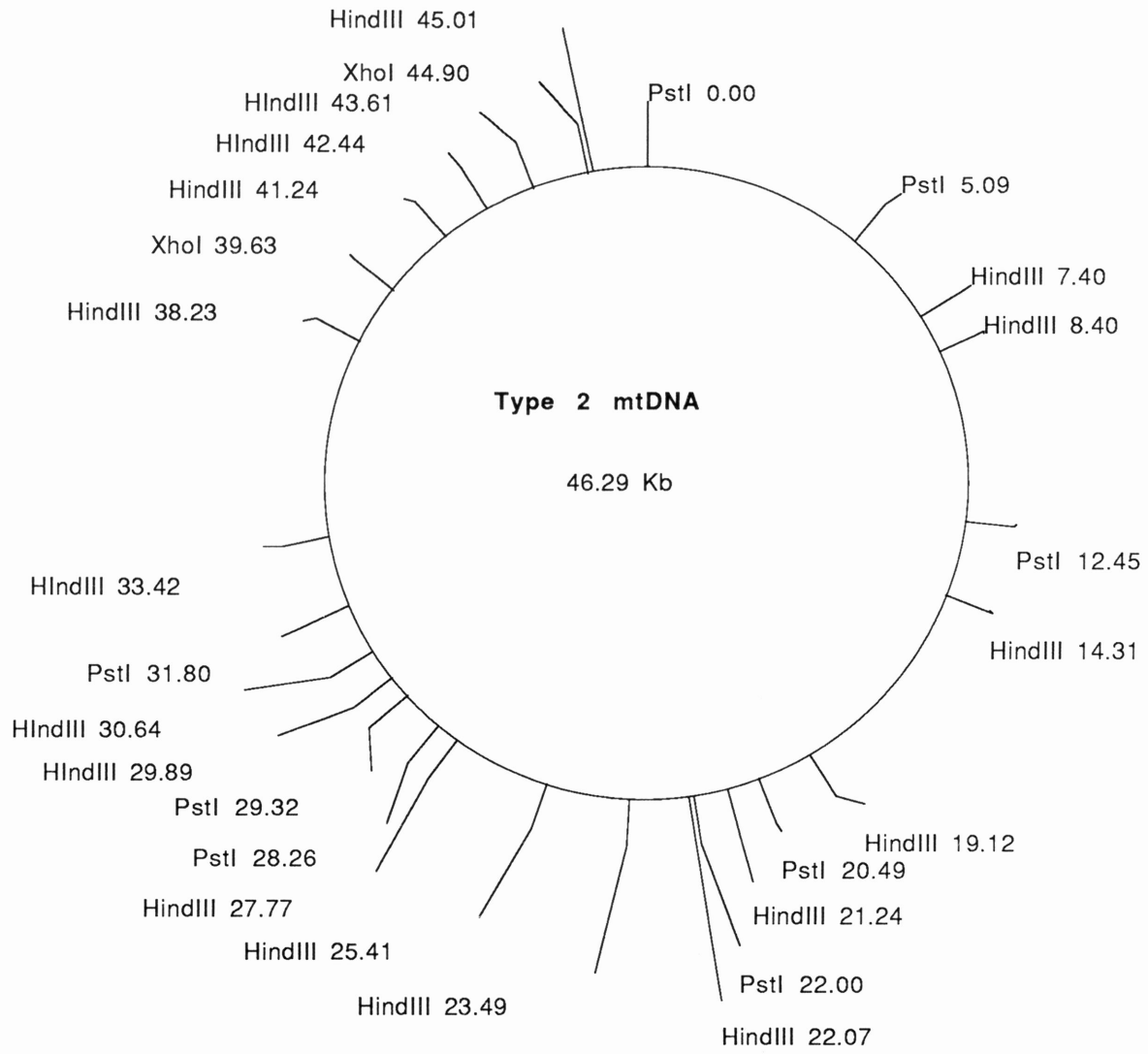


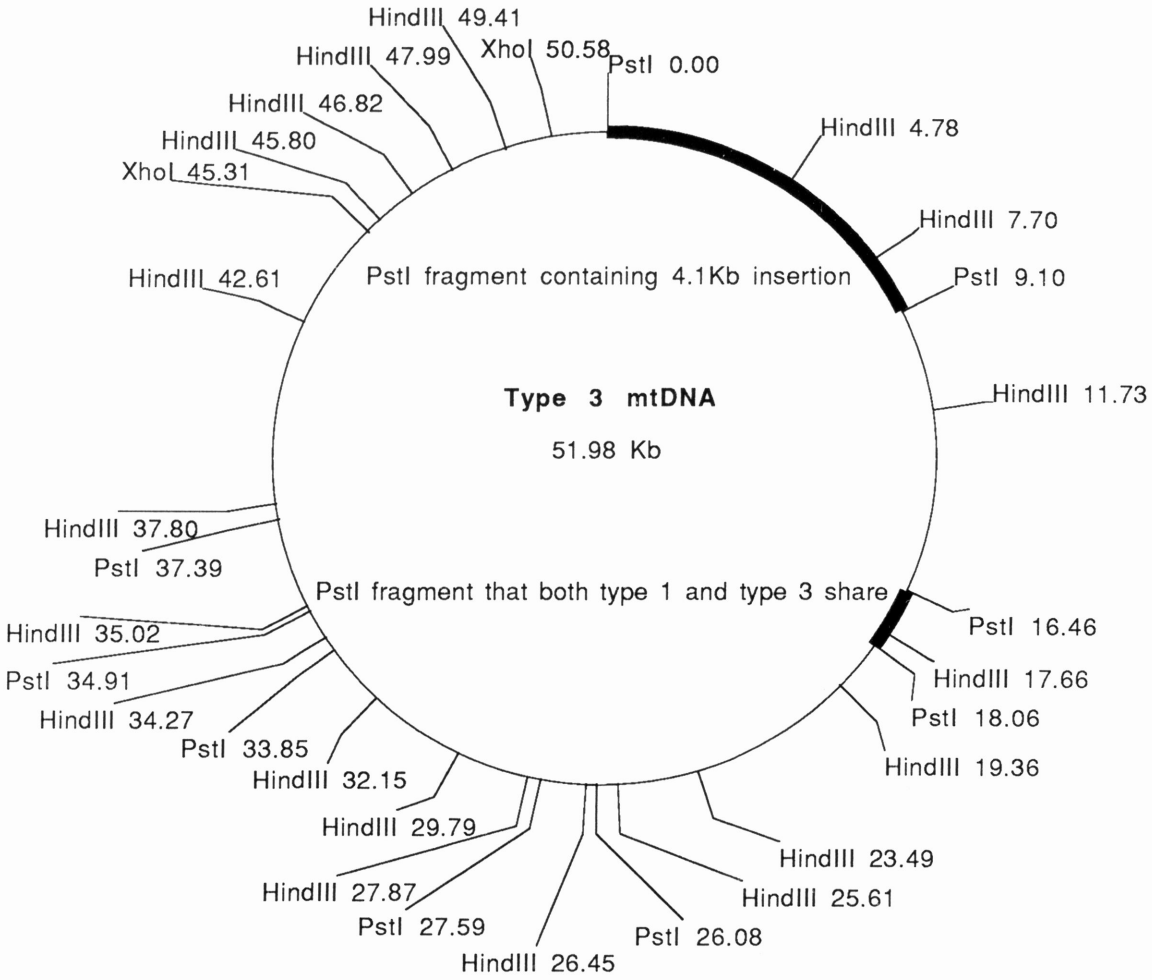
Figure 5: The arrow indicates the loss of a DraI restriction site in two of the isolates.



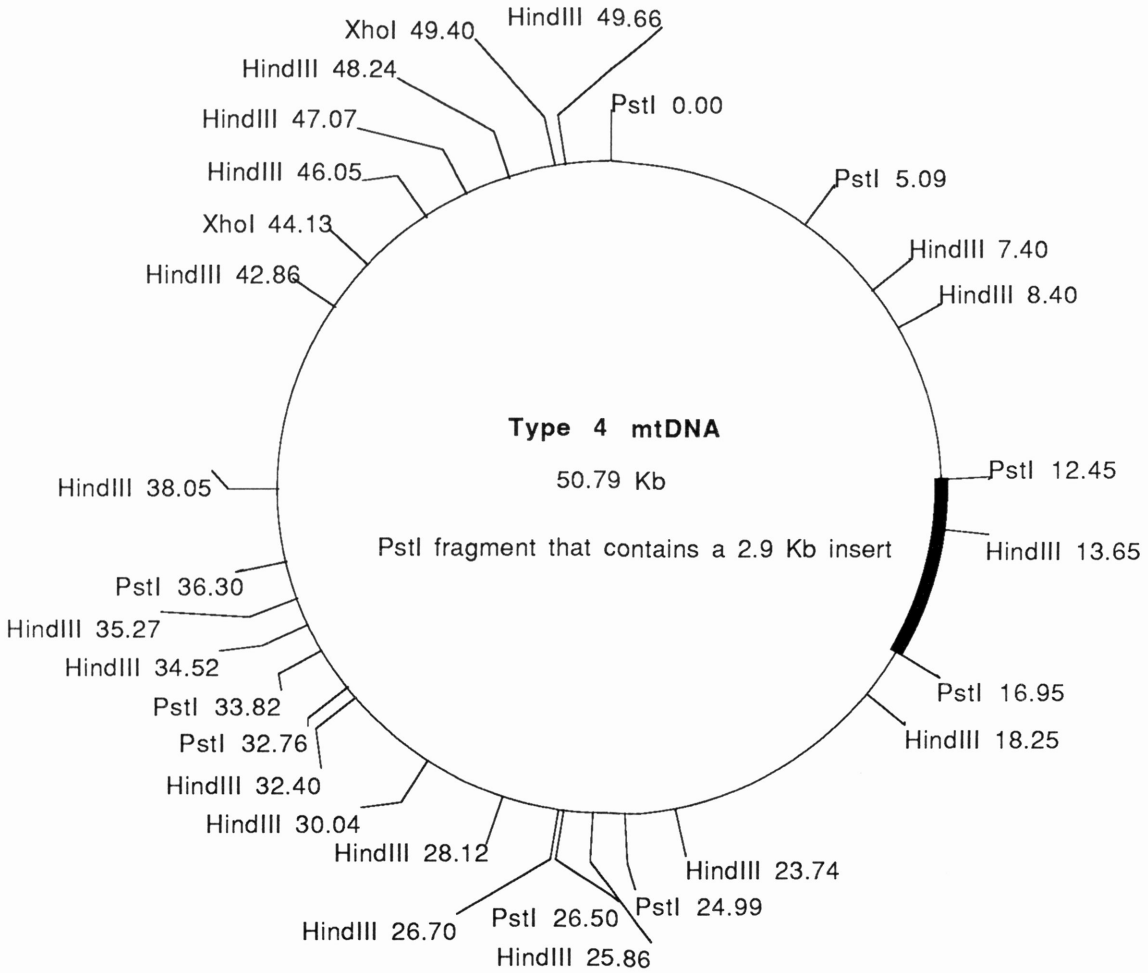
Plasmid Name: Type 1 mtDNA
Plasmid size: 47.90 kb
Constructed by: Kelly Hogan
Construction date: 3/29/95
Comments/References: Figure 6: Restriction Map of type 1 mt haplotype of M. graminicola. Higlighted region shows a 1.6 Kb insertion that is not present in the type 2 mt haplotype, but is present in type 3.



Plasmid Name: Type 2 mtDNA
Plasmid size: 46.29 kb
Constructed by: Kelly Hogan
Construction date: 3/39/95
Comments/References: Figure 7: Restriction Map of type 2 mt haplotype in M. graminicola. Type 1 and type 3 contain a 1.6 Kb insert near the PstI site at 12.45 Kb



Plasmid Name: Type 3 mtDNA
Plasmid size: 51.98 kb
Constructed by: Kelly Hogan
Construction date: 3/29/95
Comments/References: Figure 8: Restricition Map of type 3 mt haplotype of M. graminicola. The highlighted region shows the PstI fragment that containsa 4.1 Kb insert.. The second highlighted area show the 1.6 Kb insert that type 1 also has



Plasmid Name: Type 4 mtDNA
Plasmid size: 50.79 kb
Constructed by: Kelly Hogan
Construction date: 3/30/95
Comments/References: Figure 9: Restriction Map of type 4 mt haplotype of M. graminicola. Highlighted region shows a 2.6 Kb insertion that is not present in any other mt haplotype

mitochondrial haplotypes were found and by making the restriction maps two of these isolates were determined to be artifacts.

The variation in the mitochondrial genome occurred in one of two places for all the isolates. Fig. 6 shows the restriction map of type 1. As shown in fig. 7 type 2 is missing a 1.6 Kb piece of DNA found in type 1 and type 3 isolates. Type 3 isolates have an insertion in another PstI fragment, close to where the first variation occurred , of 4.1 Kb as seen in fig. 8. In type 4 isolates the 1.6 Kb piece found in type 1 ad type 3 isolates contains an insertion that makes it 4.5 Kb, as shown in fig. 9. As can be seen from these maps the variation is not randomly distributed thought the mtDNA, but occur in specific locations.

Selection based on host specificity:

Isolates taken from Durham wheat and bread wheat had significantly different mitochondrial haplotypes. Isolates taken from Durham wheat had mainly type 4 mitochondrial haplotypes with a few type 2s. Isolates taken from bread wheat had type 1 and type 3 mitochondrial haplotypes.

Discussion:

Type 1 and type 2 mitochondrial haplotypes:

The loss of restriction sites shown in the type two isolates support the hypothesis of gene flow. In the past gene flow was indirectly detected by similarities in allele frequencies between populations. From the similarities in allele frequencies the number of migrant individuals necessary between populations to maintain the genetic similarity can be determined. In using this method past data has shown large amounts of gene flow between populations all over the world.

Since restriction endonucleases cut DNA at specific palindromic sequences a small amount of nucleotide sequence can be determined. From

the ten different restriction endonucleases I was able to determine the sequence approximately 900 base pairs. The loss of a restriction site is caused by one or more of the base pairs that the restriction endonulease recognizes to change. The chance that one of the restriction sites will be lost by two different isolates independently can be estimated by determining the probability that one of the base pairs detected would change in both isolates. The chance of independent events occurring is described by the multiplication rule of probabilities. That probability is 1 out of 6(900 x 900), 1 out of 4.8×10^6. This is a small probability, and the actual probability is much smaller since there are 45 kb in the mtDNA instead of the 900 I used as an estimate. Since there is such a small probability of the independent loss of a restriction site, it is likely that the loss of a restriction site that occurred in two different populations are not random events. There is a high probability that the mitochondria with this restriction site loss have a common ancestor. Therefore the loss of identical restriction sites gives a direct measure that supports gene flow between populations.

Also from the digestion of type 1 and type 2 mitochondrial haplotypes by several different restriction endonucleases a slightly higher level of variation was found, but these variation were small and still do not correspond to the expected mitochondrial diversity of a population with so much nuclear diversity.

Restriction Map:

The variation found in the mitochondrial genome all mapped to one of two places. The fact that the variations are not randomly distributed could be significant. Non-random mutation suggests a mutational or recombinational hotspot, also it suggests that the other areas of the mitochondria are necessary and mutations in other areas of the genome

prove lethal. Previous work has shown no selection among the RFLPs used in the nuclear genome. (B.A. McDonald, personal communication) But the non-random variation in the mitochondrial genome suggest the possibility of selection based on the mitochondria.

Host driven selection:

The partitioning of mt haplotypes between the different species of wheat suggest that there could be selection on the mtDNA is driven by the host genotype. This is interesting since the nuclear allele frequencies of Bread wheat and Durham wheat have no significant differences. (McDonald, Personal communication).

Conclusion:

The above data and discussion is just the beginning of study that must be completed before any understanding of the role the mtDNA plays in the genetic structure of *M, graminicola* can be gained. I hope the work I have done will lay a foundation for further study.

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