Student Paper

Genotype diversity and transposable elements in Mycosphaerella graminicola
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Part 1: *Mycosphaerella graminicola*: genotypic diversity and spread of asexual spores within a field

Part 2: Transposable elements in the genome of *M. graminicola*

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**Introduction**

*Current knowledge about the genetics M. graminicola*

«Genetic variability in plant pathogens has often been studied on a macro-geographical scale but rarely at the level of individual populations. Most surveys to date have evaluated genetic variation from a limited number of isolates collected from a wide geographical area. Such surveys provide insight into the range of variability existing over large geographical areas, but the samples may have been collected from a large number of discrete pathogen populations, and thus they are probably inadequate to provide definitive data on the population genetics of plant pathogens. An alternative sampling strategy is to collect large numbers of isolates from individual populations on a micro-geographical scale » (B. A. McDonald, 1989).

*Septoria tritici* (anamorph) Roberge in Desmaz. (teleomorph *Mycosphaerella graminicola* (Fuckel) J. Schröt. In Cohn) is an ascomycete fungus in the family Dothideaceae and causes Septoria tritici leaf blotch, a significant disease of wheat worldwide (King et al., 1983). *M. graminicola* is principally a foliar pathogen, but it can infect wheat heads. Yield reductions resulting from infection by *M. graminicola* can approach 10-20% where fungicides are not used for disease control. Reduced grain yield weights are the result of reduced tillering of plants, reduced seed set, poor grain fill and loss of shriveled grain with chaff during harvest (Hershman, 1992).

![Fig. 1: Seasonal life cycle of M. graminicola](image)

The incidence and economic significance of Septoria tritici leaf blotch increased steadily since the 1960s as a result of the widespread and rapid replacement of local cultivars with semi-
dwarf, early-maturing, higher-yielding cultivars that are susceptible to this pathogen. Changes in cultural practices (higher densities, phosphorus fertilizers) also contributed significantly to the increased disease incidence.

*M. graminicola* produces haploid vegetative hyphae, asexual spores (conidia) in a pycnidium, and sexual spores (ascospores) in a pseudothecium (Sanderson, 1977). Lesions of *M. graminicola* start as small yellow flecks, usually on lower leaves that are in contact with the soil. Flecks expand into red-brown rectangular lesions, which follow the leaf veins. Lesions will have numerous black pycnidia dispersed throughout.

Infection by *M. graminicola* is highly temperature-dependent and requires rather cool, wet conditions in order to occur. If conditions are favourable, infection will occur at any stage of plant development. However, because of the temperature and moisture requirements, infections by *M. graminicola* are most common in early to mid-season when temperatures are coolest (Hershman, 1992). The fungus survives from year to year in diseased wheat straw of previous crops and volunteer wheat.

The conidia are dispersed by rain splash, hence having limited potential to move long distances. In the absence of rain to provide splash, spores may also move by rubbing between adjacent plants and plant parts, but the horizontal distance of this process must be less than splash and the efficiency seems likely to be poor (Shaw and Royle, 1993). Ascospores are wind dispersed, hence having the potential to move longer distances. The relative importance of sexual and asexual reproduction in the population genetics of this organism was not clear until recently. Several epidemiological studies provided evidence that the primary inoculum leading to disease in wheat fields was airborne, which suggests that ascospores play a major role in initiating disease (Shaw and Royle, 1989). Several analyses show that wind-blown ascospores are produced throughout the growing season (DiLone et al., 1997).

**Transposable elements**

A significant percentage of the genome of many organisms lies outside of gene regions and an even greater portion is not translated into proteins. A portion of this non-genic sequence is comprised of repeat elements and some of these repeats can be classified as transposable or transposable-like elements. Transposable genetic elements are commonplace and often exhibit easily recognized features that can aid in their identification and annotation (http://nucleus.cshl.org/protarab/TnAnnotation.htm).

A DNA sequence that possesses the ability to place replicates of itself into a DNA helix is called a transposon or a "jumping gene". The transposition event is called a translocation or "jump". In fact in most transposition events the transposon is not excised and replaced elsewhere in the cells DNA but a copy (replicate) of the transposon is inserted elsewhere than the original sequence.

Mutation plays an important role in the evolution of organisms. Translocation is considered to be a genome mutation. Most mutation events in the genome have lethal or weakening consequences for the offspring but a small fraction of mutations can provide a benefit for the organism, especially when the mutation provides a better adaptation to the environmental conditions.
Goal of this work

Two different subprojects (Part 1/2) were conducted during the course of the experiments. Disease monitoring requires knowledge of spatial variability of the disease within the crop during the course of season. In Part 1 we tried to estimate the amount and distribution of genotypic variability in a *M. graminicola* field population and the following questions were investigated: (1) What is the genotypic diversity of a natural population of *M. graminicola* on a micro-geographical scale i.e. within a few square meters in a field, (2) how persistent are individual clones and are particular clones detectable at different times during the season at the same location, (3) how far can a particular clone move over time?

In Part 2 our goal was to detect transposons or transposon activity respectively in the genome of a *M. graminicola* population from an inoculated field.

**Materials and Methods**

**Methods (Part 1: Genotypic Diversity and Movement of Conidia)**

**Collection and maintenance of isolates**

Leaf tissue infected with *M. graminicola* was collected in February (early season), March (mid season) and July (late season) 1994 from a wheat field in Hillsboro (Oregon, USA). Infected leaves were collected from the same plot in February and March and from a third plot in July. The variety of winter wheat was Stephens. The plots measured 1.5 × 4 m. About 120 infected leaves were collected from the middle three rows of each plot. Leaf tissue from each plot was placed in a paper envelope and allowed to air dry for one week.

Dried leaf tissue was rewetted by immersion in 70% Ethanol and the surface was sterilized by immersion in a 0.5% sodium hypochlorite solution. Sterilized tissue was placed on a moistened filter paper in a petri dish overnight at room temperature to encourage extrusion of cirrhi from pycnidia. Pycnidiospores were streaked onto fresh PDA (39 g of potato dextrose agar in 1000 ml of water) petri plates to separate individual spores. Single colonies were isolated for further analysis. Isolates were grown in a yeast-sucrose broth (YSB, see Appendix).

Leaf tissue infected with *S. tritici* was collected in July 1994 from the same field as mentioned previously. Ten plots (A-J), each measuring 12 × 13 inches, were blocked out in the middle 4 rows of a long 6-row plot. From each plot, 12-15 infected leaves were collected that were either flag or F-1 leaves. When available, leaves possessing more than one discrete lesion were collected. Leaf tissue from each plot was placed in a paper envelope and allowed to air dry for one week. Isolations from leaves were made as described previously.

**DNA extraction**

DNA was extracted as follows: tissue frozen in liquid nitrogen was ground into a powder, dispersed in 5 ml of CTAB extraction buffer in a 15-ml centrifuge tube and placed in a 60°C water bath for 30-45 min. An equal volume of chloroform:isoamyl 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 × g, and the upper aqueous phase was transferred to a fresh 15-ml tube. Nucleic acids were precipitated by adding an equal volume of isopropanol and centrifuging for
5 min at 10,400 x g. The nucleic acid pellet was dissolved in 2 ml TE (10 mM Tris, 1 mM EDTA, pH 8.0); it was then precipitated by adding 1 ml of 7.5 M NH₄OAc and 5 ml 95% ethanol and centrifuging for 5 min at 10’400 x g. The pellet was dissolved in 0.4 ml TE and transferred to a 1.5-ml microfuge tube and nucleic acids were precipitated by adding 0.8 ml of 95% ethanol and centrifuging 2 min at 16’000 x g. Nucleic acid pellets were dried in a desiccator and then dissolved in 40-80 µl of TE (MCDONALD AND MARTINEZ, 1990).

DNA quantification and restriction enzyme digestion

DNA concentration of all isolates (both experiments) was determined using the Tecan Robotic System based on picogreen fluorescence measuring. 5 µg of genomic DNA from each isolate was digested with 3.5 µl of the restriction enzyme PstI (15 units per µl, Amersham pharmacia). 5 µl of a buffer (500 mM Tris-HCl, pH 7.5; 100 mM MgCl₂; 10 mM Dithiothreitol; 1000 mM NaCl) was added to the DNA and enzyme and the emulsion was filled up with dd water to a total volume of 50 µl and incubated overnight at 37°C.

To make sure that the DNA was well digested, 5 µl of the restriction enzyme digestion reaction (for each isolate individually) were loaded together with 3 µl of a gel loading buffer (blue juice) into a 0.8% agarose gel (2.0 g of agarose in 250 ml of 0.5 x TBE, 4 µl ethidium bromide) and the gel was run at 100 volts for about 2-3 hours. A picture of the gel was taken (UV light) to determine whether any isolates were partial digestions.

DNA electrophoresis and Southern blotting

The rest of the restriction enzyme digestion reaction (for each isolate individually) was loaded together with 6 µl of a gel loading buffer (blue juice) into a 0.8% agarose gel (3.6 g of agarose in 450 ml of 1 x TBE). In the first lane, 3µl of the HindIII Lambda standard DNA (+ 3 µl of the gel loading buffer + 4 µl dd-H₂O ) were loaded on the gel. The gel was run for about 10 hours at 75 volts.

After the electrophoresis was completed, the gel was stained in ethidium bromide (0.5 µl ml⁻¹) for 20 min, distained in distilled water for another 20 min and photographed with UV light.

For the Southern blot, the gel was soaked first in 0.25 M HCL for 15-20 min in order to hydrolyse the DNA partially by acid depurination. A
platform was constructed and was covered with a wick made from three sheets of 3MM chromatography Whatman® paper, saturated with 0.4 M NaOH. The gel was placed on top of the platform. A nylon transfer membrane (Hybond N+, Amersham pharmacia) was wet with dd-H₂O and carefully laid out on the gel. Three pieces of 3MM Whatman® paper were wicked through 0.4 NaOH and placed on top of the membrane. In order to prevent an uneven transfer, air bubbles were rolled out on each layer using a glass pipette. A stack of absorbent paper towels was placed on top of the 3MM paper and a plexiglass weight of approximately 1 kg and placed on top of that.

The Southern transfer was conducted overnight. After dismantling, the membrane was washed in 2X SSC for 5 min with agitation. The membrane was placed between two pieces of filter paper (3MM) and baked in an oven at 80°C for 2 hours in order to fix the DNA to the membrane.

**Pre-hybridisation and Hybridisation**

Membranes were pre-hybridised overnight at 65°C in 50 ml pre-hybridization buffer (0.1 mg sonicated non-homologous DNA per ml (Boehringer Mannheim Cat. 1467-140)). Non-homologous DNA was denatured by heating at 100°C for 10 min, and then chilled on ice for 5 min before it was added to the pre-hybridisation buffer. The membranes were soaked in 2X SSC before pre-hybridisation buffer was added.

**Pre-hybridisation buffer**

- 0.5 M sodium phosphate*, pH 7.2
- 7% (w/v) SDS, pH 7.2
- 1% (w/v) BSA
- 1 mM EDTA, pH 8.0

(* 1 M sodium phosphate: 0.68 l Na₂HPO₄ (1 M) + 0.32 l NaH₂PO₄ (1 M), pH adjusted to 7.2 after combining the two solutions)

The only DNA probe used in the experiment, namely the fingerprint probe pSTL70, was labelled with the nick translation system following the manufacturer’s recommendations (Gibco BRL Nick Translation System Cat. 18160-010).

The hybridisation reaction consisted of the following:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>d H₂O</td>
<td>30.0 µl</td>
</tr>
<tr>
<td>DNTPs (-cCTP)</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>DNA probe (100-1000 ng)</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>Lambda DNA (40 ng)</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>DNA pol/Dnase I</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>*³²P</td>
<td>4.0 µl</td>
</tr>
<tr>
<td></td>
<td>50.0 µl</td>
</tr>
</tbody>
</table>

For labelling of the probe, the hybridisation mixture was incubated at 15°C for 1 hour, and then denatured at 95°C for 10 min and placed for 2 min in ice. That probe mixture was added to the pre-hybridisation solution into the hybridisation bottle. Blots were hybridised overnight at 65°C, and then subjected to stringent washes (twice with 1X SSC, 0.1 % SDS, twice with 0.2X SSC, 0.1% SDS, and twice with 0.1X SSC, 0.1 % SDS). After washing blots were
drained and wrapped in Saran Wrap. Finally, blots were placed in autorad cassettes with Biomax MS intensifier screens, and exposed to Biomax MS film (Kodak) for 24 hours at –80°C in order to obtain an autoradiographic image. Autorads were visually scored for the presence of identical fingerprints.

In order to compare genotypic variation at the three sampling dates, a measure of genotypic diversity based on the number of genotypes in each sample was used (McDONALD and MARTINEZ, 1990). Genotypic diversity \( G \) in each population was calculated from following formula:

\[
G = \frac{1}{N} \sum_{x=0}^{N} \left[ (f_x) \cdot (x/N)^3 \right]
\]

where \( N \) is the sample size and \( f_x \) is the number of genotypes observed \( x \) times in the sample (STODDART and TAYLOR, 1988). The maximum possible value for \( G \), which occurs when each individual in the sample has a different genotype, is the number of individuals in the sample. To compare \( G \) in populations with different sample sizes, \( G \) from each sample was also divided by \( N \) to calculate the percentage of maximum diversity that was obtained.

Methods (Part 2: Detection of transposition events)

Collection and maintenance of isolates

The experiment was conducted at the Oregon State University Botany and Plant Pathology Field Laboratory in Corvallis (Oregon, USA) during the 1994-1995 winter wheat season. Field plots were arranged in a randomized complete block design with four replications. Wheat plots were planted in a checkerboard pattern with plots of barley (a non-host for this \( M. graminicola \) population) separating each plot of wheat. Each replication contained three plots planted either to the wheat cultivar Madsen or Stephens or a 50:50 mixture of Madsen and Stephens. Plots were 3.3 m (12 rows) x 5.3 m in size. The site used for this experiment was not planted to wheat in previous years, and the nearest wheat field was approximately 3 km distant.

Ten isolates of \( M. graminicola \) having different DNA fingerprints were inoculated onto the wheat seedlings in November 1994. Spores from the ten isolates were mixed in equal proportion and an aqueous suspension of one million spores per ml was sprayed onto three of the four replications using small, hand-carried sprayers. The fourth (control) replication was inoculated naturally by airborne ascospores. Samples consisting of 100 infected leaves were collected from the inoculated plots on 10 February, 3 April and 2 June 1995, hereafter referred to as early-, mid-, and late-season, respectively. For the early- and late-season collections, infected leaves were sampled from all nine inoculated plots. In the mid-season, only the three inoculated plots of Stephens were sampled. Sixty infected leaves were collected on 10 February 1995 from the control plots. In all cases, leaves were sampled from the inner 8 rows of each plot. Each leaf was sampled from a different plant. The leaves were air-dried at room temperature for two weeks before fungal isolations were made, as described previously (see Materials and Methods Part 1). Isolates were chosen from infected leaves and grown for DNA propagation as described previously (see Materials and Methods Part 1).
DNA isolation, enzyme digestion, electrophoresis, Southern blotting and hybridization

After the isolation the fungal DNA from each isolate was digested separately with the restriction enzyme PstI (for further information see Materials and Methods Part 1). After DNA electrophoresis, Southern blotting and pre-hybridisation the fingerprint probe pSTL70 was hybridised to the DNA. Autorad pictures of the blots were taken as described in Methods Part 1.

Data analysis (scoring DNA fingerprints for possible transposition events)

Autoradiographs of DNA fingerprints from all isolates were examined as following: Fingerprints were scored for the ten genotypes occurring at the highest frequency in all collections (early-, mid- and late-season). Each individual of such a clone was compared with all other individuals of the same clone. Any additional or missing bands detected during comparison were noted and assessed. Additional bands (or dark spots on the autorad having the shape of a band) can have different origins:
- a certain amount of a former labelled probe can remain on insufficiently washed blotting membranes producing a weak band
- a partial digestion of an individual’s DNA can lead to an incorrect fingerprint pattern of the isolate with additional bands present
- background radiation centred in a small region possibly leads to a band-shaped spot on the autorad
- insertion of a transposable element (transposition event)

Results

Part 1

One hundred and twenty-four different clones were identified among the 167 isolates screened with no clone present at an absolute frequency greater than 8 (Table 1).

Genotypic diversity during the course of the season

Among the 16 isolates from the early-season collection (February), only one clone was sampled twice. This leads to a high value for the relative genotype diversity, namely 84% (100% would be the maximum value, meaning no genotype occurred more then once in the observed population).

Fifty-nine different genotypes were found among the 93 isolates sampled in March. The relative genotype diversity was 34%, in other words, a rather high degree of clonality was found as some genotypes could be isolated up to 6 or 8 times.

Unfortunately sampling in July couldn’t be repeated on the same plot within the field as in February and March. Hence, comparison and observation for persistent isolates could only be done in a restricted way. Seventy-one percent of the relative genotype diversity was obtained by 49 different clones among 58 isolates.
Table 1: Frequency distribution of clonal Mycosphaerella graminicola isolates and changes in genotype diversity in populations collected during February, March and July in Oregon.

<table>
<thead>
<tr>
<th></th>
<th>a4c&lt;sup&gt;a&lt;/sup&gt; (February)</th>
<th>b4c&lt;sup&gt;a&lt;/sup&gt; (March)</th>
<th>c4d&lt;sup&gt;a&lt;/sup&gt; (July)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of isolates N</td>
<td>16</td>
<td>93</td>
<td>58</td>
<td>167</td>
</tr>
<tr>
<td>Different clones</td>
<td>15</td>
<td>59</td>
<td>49</td>
<td>123</td>
</tr>
<tr>
<td>Clones occurring 1x</td>
<td>15</td>
<td>52</td>
<td>42</td>
<td>--</td>
</tr>
<tr>
<td>Clones occurring 2x</td>
<td>1</td>
<td>6</td>
<td>6</td>
<td>--</td>
</tr>
<tr>
<td>Clones occurring 3x</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>--</td>
</tr>
<tr>
<td>Clones occurring 4x</td>
<td>1</td>
<td>1</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Clones occurring 5x</td>
<td>1</td>
<td>1</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Clones occurring 6x</td>
<td>2</td>
<td>2</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Clones occurring 8x</td>
<td>1</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Genotype diversity</td>
<td>13.47</td>
<td>31.92</td>
<td>41.00</td>
<td>--</td>
</tr>
<tr>
<td>G/N (%)</td>
<td>84.2</td>
<td>34.3</td>
<td>70.7</td>
<td>--</td>
</tr>
</tbody>
</table>

Note: <sup>a</sup> Corresponds to the sampling date (a = early season, b = mid season, c = late season), treatment number (4 = wheat variety "Stephens"), and replication number.

Persistence of individual genotypes

To demonstrate that particular well-adapted and fit clones can persist at the same location within the field, we looked for identical genotypes from different sampling dates. No identical genotypes were detected during early-, mid- and late-season samplings in this analysis.

![DNA fingerprints](image)

Fig. 4: DNA fingerprints of Mycosphaerella graminicola isolates. Probe pSTL70 was hybridised to DNA from isolates digested with PstI. The first lane on the left side is a λHindIII size standard (values in kb).
Migration of clones within the field (plot) during the course of season

It was not possible to determine the precise coordinates of the isolates within the 10 subplots (A-J). However a certain range of dispersal distance can be calculated. The clone in Fig. 5 that was sampled from three different plots could have had a maximal dispersal of 97 cm corresponding to the diagonal formed by three neighbouring plots, and a minimal dispersal distance of one plot diameter (30.5 cm). The other clone in Fig. 5 that was sampled from two neighbouring plots would consequently have occurred in a range of 0 to 69.5 cm (69.5 cm is corresponding to the diagonal of two plots).

\[12 \text{ inches} = 30.5 \text{ cm}\]

\[13 \text{ inches} = 33 \text{ cm}\]

\[60 \text{ inches} = 1.52 \text{ m}\]

**Fig. 5**: Experimental design with two genotypes that occurred more than once and in different plots. Each plot measures 30.5 cm x 33 cm. One genotype was found in three different plots (H, I, J, red dots), the other was found in two neighbouring plots (I, J, blue dots). Leaf samples for this experiment were collected in July.

Because sampling was done only once in July (late-season), it is highly probable that each clone found in this experiment came from a single dispersal event. If we consider that asexual reproduction takes place \(a\) times during the course of a season, the maximal dispersal within the field could be about \(a \times 0.5 \text{ m}\).

**Part 2**

**Transposable elements**

The fingerprints noted in Table 2 do not confirm a transposition event but in all the cases additional or missing bands seem to be caused by an event on the level of DNA sequence.
Table 2: Nineteen isolates within a collection from an inoculated field population (1994, Corvallis, Oregon, USA) assessed as individuals showing a putative transposition event in their DNA fingerprint. M1 to M5 are five different genotypes formerly collected from the wheat variety "Madsen" and propagated for inoculation. S1 to S5 are five different genotypes found on the wheat variety "Stephens".

<table>
<thead>
<tr>
<th>Clone</th>
<th>Isolate</th>
<th>Autorad</th>
<th>Band difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>AR4T5-67</td>
<td>JSZ-13</td>
<td>lowest band missing</td>
</tr>
<tr>
<td>M1</td>
<td>AR2T2-58</td>
<td>JSZ-20</td>
<td>additional band</td>
</tr>
<tr>
<td>M1</td>
<td>CR4T2-27</td>
<td>JSZ-24</td>
<td>band missing</td>
</tr>
<tr>
<td>M1</td>
<td>BR2T5-07</td>
<td>JSZ-30</td>
<td>band missing</td>
</tr>
<tr>
<td>M1</td>
<td>AR2T2-61</td>
<td>JSZ-33</td>
<td>band missing</td>
</tr>
<tr>
<td>M1</td>
<td>CR2T8-47</td>
<td>JSZ-41</td>
<td>band missing</td>
</tr>
<tr>
<td>M2</td>
<td>CR4T2-28</td>
<td>JSZ-24</td>
<td>partial digestion?</td>
</tr>
<tr>
<td>M3</td>
<td>BR4T5-67</td>
<td>JSZ-12</td>
<td>band missing</td>
</tr>
<tr>
<td>M3</td>
<td>AR2T5-64</td>
<td>JSZ-17</td>
<td>band missing</td>
</tr>
<tr>
<td>M3</td>
<td>CR2T5-43</td>
<td>JSZ-36</td>
<td>additional band</td>
</tr>
<tr>
<td>M5</td>
<td>CR4T2-66</td>
<td>JSZ-49</td>
<td>additional band</td>
</tr>
<tr>
<td>S1</td>
<td>AR1T8-61</td>
<td>JSZ-6</td>
<td>additional band</td>
</tr>
<tr>
<td>S1</td>
<td>CR2T8-63</td>
<td>JSZ-41</td>
<td>additional band</td>
</tr>
<tr>
<td>S1</td>
<td>CR3T8-39</td>
<td>JSZ-44</td>
<td>additional band</td>
</tr>
<tr>
<td>S2</td>
<td>CR2T8-33</td>
<td>JSZ-42</td>
<td>additional band</td>
</tr>
<tr>
<td>S3</td>
<td>AR1T8-15</td>
<td>JSZ-22</td>
<td>band missing</td>
</tr>
<tr>
<td>S3</td>
<td>AR4T2-40</td>
<td>JSZ-24</td>
<td>additional band</td>
</tr>
<tr>
<td>S3</td>
<td>CR2T8-36</td>
<td>JSZ-41</td>
<td>additional band</td>
</tr>
<tr>
<td>S3</td>
<td>CR1T8-54</td>
<td>JSZ-45</td>
<td>band missing</td>
</tr>
</tbody>
</table>

Note: The first letter in the labelling of the isolates corresponds to the sampling date (A = February, B = April, C = June).
Fig. 6: Six isolates of wild-type clone M1 showing a putative transposition event on their DNA fingerprint combined with 5 isolates of the wild-type M1. The band patterns of the six mutated isolates differ from the wild-type M1 only in having one band additional or missing. Arrows indicate the differences in fingerprint bands. Probe pSTL70 was hybridised to DNA from isolates digested with PstI. The first lane on the left side is a \( \lambda \)HindIII size standard. JSZ-X corresponds to the autorad.


**Discussion**

**Genotypic diversity**

The current work did not reveal any detailed information on the relative degree of sexual and asexual reproduction during the course of an epidemic. The samples collected in the late-season unfortunately had to be taken from a different plot than the early- and mid-season samples. In addition sample sizes were too unequal. However, genotypic diversity decreased during the course of the season, which is equivalent to an increasing importance of asexual reproduction.

In the majority of *M. graminicola* populations surveyed by MCDONALD and co-workers, each leaf usually was occupied by a unique *M. graminicola* genotype. In the cases where isolates with the same DNA fingerprint were repeatedly isolated, they often where sampled from the same leaf (=> low degree of clonality). This corresponds to our results. The genotypic diversity was quite high. A possible interpretation for the high degree of genotype diversity is that populations of *M. graminicola* undergo sexual cycles and that the primary inoculum is likely to be ascospores (CHEN and MCDONALD, 1996).

Estimates of the relative degrees of sexual and asexual reproduction may be affected by differences in the dispersal distances of sexual and asexual spores and by the spatial scale used for sampling. For example, if asexual spores are dispersed only over short distances (e.g., centimeters), the asexual fraction of a population may not be adequately represented if sampling is conducted at a larger scale (e.g., meters) (CHEN et al., 1996).

**Persistence of clones**

Do particular clones persist within the field during the course of the season? In this experiment no genotype was found at different sampling dates within the collections. This finding does not prove that there are no persistent clones present. It does indicate the low probability of finding persistent clones during the season, and therefore demonstrates low transmission abilities of asexual spores.

The experiment should be repeated for several reasons: on the one hand the sample size in the early-season was probably too low, on the other hand the individuals from the late-season had been collected from a different plot within the field. Several former studies (CHEN et al., 1994; MCDONALD et al., 1996) underlined the high genotypic diversity of *M. graminicola* in field populations and suggested that clones only move in the range of a few metres over the course of an epidemic. Hence the probability of finding the same clone at two different times within the growing season was rather low in our work. CHEN et al., 1994, found two cases among a total number of 617 genotypes where isolates with the same fingerprint occurred both early and late in the season.

**Migration of asexual spores**

In previous studies on the dissemination or spread of *M. graminicola* asexual spores (MCDONALD and MARTINEZ 1990; BOEGER et al., 1993), it was found that spores
disseminated only over short distances (a few meters). These results were always based on populations collected in a hierarchical structure, with a limited number of isolates from a small geographical area. This work provides a unique micro-geographical study where we determined the clonal spread of genotypes in a small area covering some square meters. In this study, clones were distributed across an area of approximately 1 m² and did not become widespread. This finding is consistent with limited spread of the splash-dispersed conidia for *M. graminicola* as found previously (CHEN et al., 1994) and also demonstrates the low probability of asexual spores spreading further than 1m. This is in contrast with findings of BRENNAN et al. (1985), studying a fungus with a similar life history, *Stagonospora nodorum*, and assessing that surrounding leaves may receive conidia up to distances of 3-4 m by splash during wind.

To confirm our findings statistically we would have to look at other populations and repeat sampling with larger sample sizes at different dates during the season (e.g. early-, mid- and late-season). A larger sample size will also increase the chance of finding the same genotype repeatedly.

**Transposition events in the genome of *M. graminicola***

The candidates found in the examination have to be further analysed to confirm them as individuals showing a translocation on the fingerprint. As a next step the transposition candidates need to be re-run on the same gel together with the wild-type isolates where no transposition occurred, to get a direct comparison. The transposition isolates can then be sequenced like it was done by GOODWIN et al. (2001).

GOODWIN and co-workers (2001) did a study on the sequence level of *M. graminicola* examining two fungal clones in vitro. Their analysis of the *pSTL70* clone shows that it corresponds to part of a transposable element that was active during both sexual and asexual reproduction. During asexual transfers they identified isolates with gains and losses of bands that were probably due to movement of a transposon or possibly to rearrangements of DNA near transposon insertion sites. Some of the asexual progeny had lost two or three fingerprint bands during ten asexual transfers. To test the transposable element hypothesis, the DNA fingerprint probe *pSTL70* was sequenced, along with three other clones from a subgenomic library that hybridised with *pSTL70*. Analysis of these sequences revealed that *pSTL70* contains the 3’ end of a reverse transcriptase sequence plus 29- and 79-base pair direct repeats. These are characteristics of transposable elements identified in other organisms. To get an idea of the frequency of transposition events in field conditions, a future study could be conducted focussing on a limited number of inoculated fungal genotypes. Knowledge about the translocation frequency of certain transposons may provide more insight into the evolutionary potential of *M. graminicola*.

**Conclusions**

As found in numerous former studies (MCDONALD et al., 1995; CHEN and MCDONALD, 1996; BOEGER et al., 1993) we could confirm once more that *M. graminicola* exhibits a genetically diverse population structure within a farmer’s field. The degree of diversity decreased during the course of the season which is equivalent to an increasing importance of asexual reproduction. The individual genotypes seemed to be of little persistence. We discovered a minimal probability of finding persistent clones during the course of the season.
This study provides a unique observation on the migration behaviour of asexual spores of *Mycosphaerella graminicola*. Clones were distributed across an area of approximately 1m² and did not become widespread. Furthermore a large number of RFLP-fingerprints was analysed for transposition events. Probe pSTL70 had been hybridised to DNA of field isolates. Several isolates were assessed as individuals showing a putative transposition event in their DNA fingerprint.

**Literature Cited**


Parnell, L., (without year). In: [http://nucleus.cshl.org/protarab/TnAnnotation.htm](http://nucleus.cshl.org/protarab/TnAnnotation.htm) (June, 2001)


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Appendix

Materials

Culture media

- **Yeast-sucrose broth (YSB)**
  - 10 g Yeast extract
  - 10 g Sucrose
  - 1000 ml (1 l) Distilled water

Solutions and chemicals

All the solutions were made with dd-H₂O (unless there is written something else). The solutions were all autoclaved at 121°C for 20 min (unless another procedure is described).

- **10x Running Buffer tris-borate (10x TBE)** (Maniatis et al., 1989)
  - 0.9 M Tris
  - 0.9 M Boric acid
  - 0.02 M Na₂-EDTA

- **SDS (10% (w/v) Sodium dodecyl sulphate)**
  - 50 g SDS
  - 500 ml dH₂O

- **20 x SSC**
  - 346.6 g NaCl
  - 76.4 g Na₃ Citrate
  - pH 7.0

- **TE**
  - 10 mM Tris
  - 1 mM EDTA (0.5 M)
  - Equilibrated at pH 8.0 with HCl
• **Gel loading buffer (blue juice)** (Maniatis *et al.*, 1989, modified)
  0.25 % (w/v) Bromophenol blue
  30% (w/v) Glycerol

• **Gel of Agarose (0.8%)**
  3.6 g Agarose
  450 ml TBE (1x)

**Molecular biological work**

DNA nucleases

In this work the purified DNA was only digested once using *Pst I* (15 units/µl, Amersham pharcia) as restriction enzyme.

Size marker

*HindIII* digested DNA from the bacteriophage lambda was used as fragment size marker.

**Material for Southern blot**

• Nylon transfer membrane "Hybond H+" (Amersham pharcia)
• 3MM chromatography paper (Whatman)

**Storage of DNA and enzyme**

DNA and enzyme solutions were stored at –20°C.