THE USE OF VIRULENCE AND AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP) TO STUDY THE GENETIC DIVERSITY OF FIELD ISOLATES OF *Ustilago tritici*

BY

ZLATKO POPOVIC

A Thesis

Submitted to the Faculty of Graduate Studies

In Partial Fulfillment of the Requirements

for the Degree of

MASTER OF SCIENCE

Department of Botany

University of Manitoba

Winnipeg, Manitoba

August, 2002

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

*****

COPYRIGHT PERMISSION PAGE

THE USE OF VIRULENCE AND AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP) TO STUDY THE GENETIC DIVERSITY OF FIELD ISOLATES OF *Ustilago tritici*

BY

ZLATKO POPOVIC

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

Master of Science

Abstract

Ustilago tritici (Pers.) Rostr. is the casual agent of loose smut of wheat. The pathogen is seed-borne and rarely devastating, rather causing low to moderate annual losses. In Canada the disease is normally controlled by using resistant cultivars. Annual surveys of wheat fields allow us to monitor the incidence and severity of *U. tritici*. Virulence analysis of collected isolates provides information to assess the effectiveness of host resistance genes and study genetic diversity. Traditionally, isolates of *U. tritici* are collected during extensive surveys of large areas by sampling one smutted head from each infested field encountered. This study was conducted to determine if an intensive sampling of a few fields (20 smutted heads per field from four fields) could yield as much information on genetic diversity as the traditional extensive sampling method (81 smutted heads collected from 81 fields), using AFLP and virulence data as a measure of diversity.

Six wheat differentials were used in the virulence study. Of the161 isolates of *U. tritici* assessed, 17 different virulence combinations were observed. AMOVA F-test showed no significant differences in variability between the extensive and intensive collections using virulence data. In the AFLP analysis, 23 polymorphic loci were common for the extensive and intensive collections, and 40 different AFLP banding patterns were observed for isolates from the intensive collection and 59 for isolates from the extensive collections. Unlike virulence data, AMOVA F-test for AFLP data showed highly significant differences in variability between the extensive and intensive collections..

The intensive sampling of 20 smutted heads per field from four fields resulted in a more genetically diverse collection than the extensive sampling of 81 heads from

individual fields as determined using AFLP analysis. Virulence analysis results showed that these two collections were equally diverse. So, in terms of genetic diversity we can conclude that the intensive sampling of a small number of fields yielded a similar or more genetically diverse collection of *U. tritici* than the extensive sampling method.

Acknowledgments

Thank you to James Menzies for his guidance and patience. Gratitude is also extended to my committe members: Dr. Lakhdar Lamari and Dr. Chris Rampitsch. I greatly appreciate the technical advice and friendship shared by Cheryl, Graham, Frank, Cheri, Marcy, Chris, Sung, and Colin. Assistance with figures and slides was provided by Reg, Kathy and Mike. Assistance with statistical analysis was provided by Sheila Woods. I especially want to thank my wife Mira for her continued love and support.

Table of Contents

List of Tables

List of Figures

I. INTRODUCTION

Ustilago tritici (Pers.) Rost., the casual agent of loose smut of wheat, occurs wherever wheat is cultivated (Nielsen and Thomas 1996, Saari et al. 1996). The pathogen is seed-borne and usually spread from place to place through infected seed; rarely is it disseminated over long distances by wind. Loose smut is rarely devastating, but causes low to moderate annual losses. It is more common in regions with a cool moist climate during flowering of the host. Yet, even in dry warm climates, economic losses occur. Since percentage infection equals loss in yield (Green at al. 1968), and since most of the monetary return from one field goes into cost of production, even 1-2% infection can reduce profit to the farmer by 5-20%. Loose smut of wheat was first reported to cause serious losses in western Canada in the 1890's (Johnson 1961). Although the majority of these reports dealt with losses caused by bunt, in 1888, Fletcher (Johnson 1961) was releasing reports to farmers on how to control Loose smut of wheat using hot water treatments. Because of the importance of bunt of wheat and the effectiveness of 'bluestone' (copper sulphate) and other seed treatments in controlling bunt, actual figures for losses caused by loose smut in wheat at that time are difficult to obtain. Wheat yield losses in the mid to late 1900's were at low levels (Thomas 1992,1995, Menzies et al. IN PRESS). Data from annual smut surveys indicate that, on average, 10 to 20% of fields of common bread wheat are infested with Loose smut at trace (<0.1%) levels. Although the levels of infection are low, farmers are still losing income to this disease, and the use of seed treatment to control this disease is adding cost to farming operations, as well as creating environmental concerns and health risks. These economic, environmental and

health costs are avoidable if resistant varieties are grown.

Ustilago tritici infects most *Triticum* species (Nielsen and Thomas 1996). The pathogen also infects *Aegilops* species and is likely to have parasitized *Aegilops* before cultivated wheat evolved. The study of races of loose smut started in the 1920's and 1930's (Tiemann 1925, Piekenbroch 1927, Garvel 1930), and quickly became based on the principles of 1) resistance to each race was monogenic, 2) the virulence pattern of a race does not depend on its geographic origin, but on the host resistance genes the pathogen is exposed to, 3) selection pressure by the prevalent cultivars selects cultivar-specific virulent races and 4) the same race can occur in different geographical areas (Nielsen 1987).

Races are currently identified by inoculation of a differential host series with teliospores from a single spike of a field collection (Nielsen 1987). A new race is identified when an isolate of *U. tritici* displays virulence on a cultivar that was previously known to be resistant to all known races or on a series of cultivars in which the combination of virulence gene had not been previously reported. A new race could arise by mutation and/or recombination of preexisting virulence genes.

In western Canada, Loose smut of wheat is normally controlled by growing resistant cultivars or by using fungicide seed treatment. Annual surveys of wheat fields allow a monitoring of the incidence and severity of loose smut and opportunities to collect isolates of *U. tritici*. Virulence or race analysis of collected isolates provides information to assess the effectiveness of host resistance. Therefore, it is important when surveying the pathogen population to obtain a genetically diverse collection of the pathogen to

ensure that the virulence information obtained from the sampled isolates gives an accurate assessment of the virulence characteristics of the entire pathogen population in the area being surveyed. The conventional way to ensure a genetically diverse collection of *U. tritici* has been to sample one smutted head per field from distant fields over a large area, i.e. western Canada. It was thought that this method was more efficient at sampling the genetic variation in the pathogen population than making more numerous collection from smaller areas.

This study was conducted to determine if an intensive sampling of a few fields (20 smutted heads per field from four fields) could yield as much information on genetic diversity as the traditional sampling method (81 smutted heads collected from 81 fields), using AFLP and virulence data as a measure of diversity.

2. LITERATURE REVIEW

2.1 *Ustilago tritici*

Loose smut has been associated with wheat cultivation since the beginning of recorded history (Saari at al. 1996). It was first recorded by Theophrastus (384-332 BC) and was also known to the Romans, who named it Ustilago (which means burnt in Latin). Smut was illustrated in 1556 in Hieronymus Dock's Herbal. In 1890, three distinct fungal species were shown to cause the loose smuts of wheat, barley and oats (Nielsen 1987). The disease undoubtedly originated in the same center of origin (the near east) as wheat and its relatives (Saari et al. 1996). The pathogen is seed-borne and usually spread over long distances by man. European settlers introduced *U. tritici* to North and South America, Australia and South Africa when they brought infected wheat seed with them. In North America, loose smut was reported as early as 1832 (Hendry and Hanson 1934).

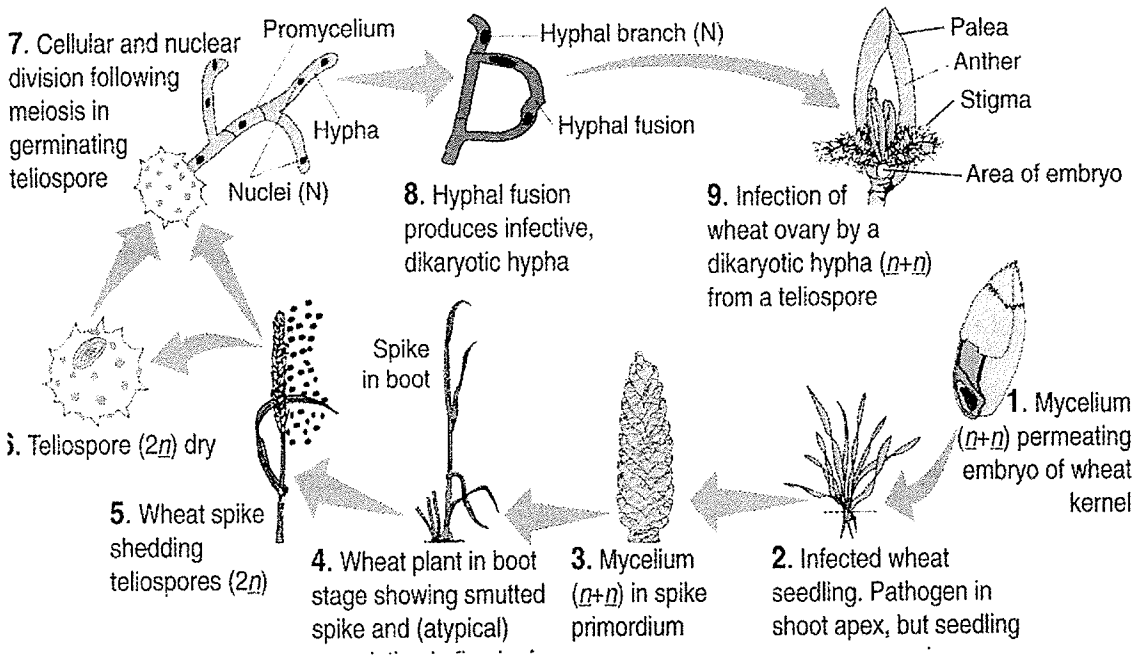
Loose smut of wheat is caused by the heterobasidiomycetous fungus *Ustilago tritici* (Pers.) Rost. It is the only species of *Ustilago* that occurs on wheat. The normal teliospore mass of *U. tritici* is dark olivaceous brown (Nielsen and Thomas 1996). Teliospores do not exhibit dormancy and the fungus is heterothallic and bipolar. At spore germination, the single diploid nucleus of the spore divides meiotically and a slender, slightly curved, promycelium emerges on the lighter colored side of the spore. The promycelium consists of four cells, each with a single haploid nucleus. These cells may divide before producing a short hypha within 18 hours at 20^0C, the optimum temperature for germination, in either light or darkness. The haploid monokaryotic hypha, or a

mycelium developed from it, can be either MAT-1 or MAT-2 mating type (Bakkeren and Kronstad 1993). Mating type is determined in a fusion test with two standards of known mating type, on a medium low in nutrients such as potato-dextrose agar at 1/10 normal nutrient concentration (Nielsen & Thomas 1996). Hyphae of opposite compatible mating type readily fuse to form a dikaryotic hypha. In axenic culture, a dikaryotic cell soon divides into one dikaryotic and two monokaryotic cells, each of the latter of opposite mating type.

Maddox determined that *U. tritici* infects the wheat plant through the ovary in 1896 (Nielsen and Thomas 1996). Infection can occur only during flowering of the wheat plant. Teliospores of the loose smut fungus that land in flowers of healthy plants germinate, and the developing mycelium penetrates the ovary and establishes itself in the embryo (Nielsen and Thomas 1996). As the seed matures the mycelium becomes dormant and the appearance of the infected seed does not differ from healthy seed. When an infected seed germinates, the mycelium also starts growing again and penetrates the growing point of the plant. As the head begins to form it is so thoroughly invaded by the fungus that a mass of teliospores develops instead of the normal spikelets. The spores mature by heading time and are dispersed mainly by wind and less so by rain to the flowers of healthy plants, completing the disease cycle (Fig. 1).

The disease expresses itself only on wheat heads, the other parts of an infected plant appear normal (Nielsen and Thomas 1996). Infected heads emerge at the same time as healthy ones and usually all their parts except the central stalk or rachis are replaced by the loose mass of dark brown spores. The spores are blown away by wind or washed off

Figure 1: Life cycle of *Ustilago tritici* (Wilcoxoson and Saari 1996)


7. Cellular and nuclear division following meiosis in germinating teliospore
Promycelium
Hypha
Nuclei (N)
Hyphal branch (N)
Hyphal fusion
8. Hyphal fusion produces infective, dikaryotic hypha
Palea
Anther
Stigma
Area of embryo
9. Infection of wheat ovary by a dikaryotic hypha (n+n) from a teliospore
1. Mycelium (n+n) permeating embryo of wheat kernel
2. Infected wheat seedling. Pathogen in shoot apex, but seedling
3. Mycelium (n+n) in spike primordium
Spike in boot
4. Wheat plant in boot stage showing smutted spike and (atypical)
5. Wheat spike shedding teliospores (2n)
6. Teliospore (2n) dry

by rain, so that at maturity, only the bare stalk remains. Traces of the dark teliospores still remain visible on the stalk, as well as some grayish remnants of glumes or awns if the tissues of the host plant are not completely destroyed.

The first studies of races of *U. tritici* started in the 1920s and 30s (Tiemann 1925, Piekenbrock 1927, Gravel, 1930). Some principles that were developed at the time are still in use today, such as; 1) resistance to every race is monofactorial, 2) the virulence pattern of a race is determined by the cultivar on which it occurred, not by geographical origin, 3) cultivars select the races that are virulent on them, and 4) the same race could occur in different wheat growing areas of the world.

Races of *U. tritici* are determined by inoculating a series of differential cultivars. This method is labor-intensive and time-consuming. Several sets of different cultivars were used in the past and in different countries mostly based on reactions on local cultivars (Tiemmann 1925, Piekenbrock 1927, Gravel 1930, Oort 1947, Bever 1953). The present set of spring wheat differentials contains 19 cultivars, (Nielsen 1987, Nielsen and Dyck 1988). Every one of these differentials possesses different resistance genes, or different combinations of resistance genes. Inoculation of collections onto the standard set of the differentials shows the race or viurlence phenotype of the pathogen. Races of loose smut seem to arise and spread slowly. A new race can arise by mutations at loci responsible for virulence or recombination of pre-existing virulence genes. A new race is identified if teliospores from a single spike of a field collection yields a pattern of virulence that is different from any known race. A new race can also be identified if teliospores from field collection are virulent on a line that has been resistant to all known

races (Nielsen 1983). The virulence of over 300 isolates of *U. tritici* collected from hexaploid wheats during the period of 1964 to 1998 from across Canada, but mostly from Manitoba and Saskatchewan, was assessed by inoculation onto the differential host series (Menzies and Knox, unpublished data). Races T-5 (30%) and T-10 (36%)were the most commonly identified races of *U. tritici*. Thirteen new races of *U. tritici* isolated from hexaploid wheats were identified in western Canada but there does not appear to be a greater diversity of races in the 1990s than in previous years. The identification of so many new races is likely the result of an increased sampling during this period. A number of the new races are very similar to previously identified races except for the presence or absence of one or two virulence genes. Given that the pathogen undergoes sexual reproduction every time it infects a host (Nielsen and Thomas 1996), it is not surprising that new races are found that represent a reassortment of virulence genes found in previously identified and common races of the pathogen (Menzies at al. unpublished data).

Ideally, phytopathologists hope to develop a differential host series which is composed of host lines each differing in one unique gene for resistance. In the absence of single gene differentials, however, one can use the data collected from inoculation of the differential host series with different races of the pathogen to reveal the complexity of resistance within the host.

2.2 Genetic markers

In the last 10-15 years, plant pathologists have started to employ genetic markers to gain more knowledge about the genetic structure of plant pathogens. Even though a large number of genetic studies have been carried out, most of the work has been done on a relatively small number of fungal pathogens. Molecular genetic studies have not been conducted on *U. tritici.*

Genetics of plant pathogens must be studied continuously because plant pathogens evolve. Pathogens constantly adapt to changes in their environment in order to survive. For pathogens of agricultural crops, those changes include environment, host resistance, fungicides and crop management practices. Mechanisms of evolution of the pathogen can be attributed to mutation, sexual and asexual recombination and gene flow.

2.2.1 Isozymes

An isozyme is an enzyme that is catalytically and structurally similar to another enzyme within the same organism (Voet and Voet, 1990). Isozymes are usually differently charged enzyme molecules that can be separated using electrophoretic procedures (Market and Moller, 1959). Since enzymes catalyze specific biochemical reactions, it is possible to visualize the location of a particular enzyme on a gel by supplying the appropriate substrate and cofactors (and involving the product of the enzymatic reaction in a color-producing change). The color product forms in the gel, producing a visible band where a particular enzyme has been electrophoretically localized. Bands visualized from specific enzymes representing proteins, have a genetic

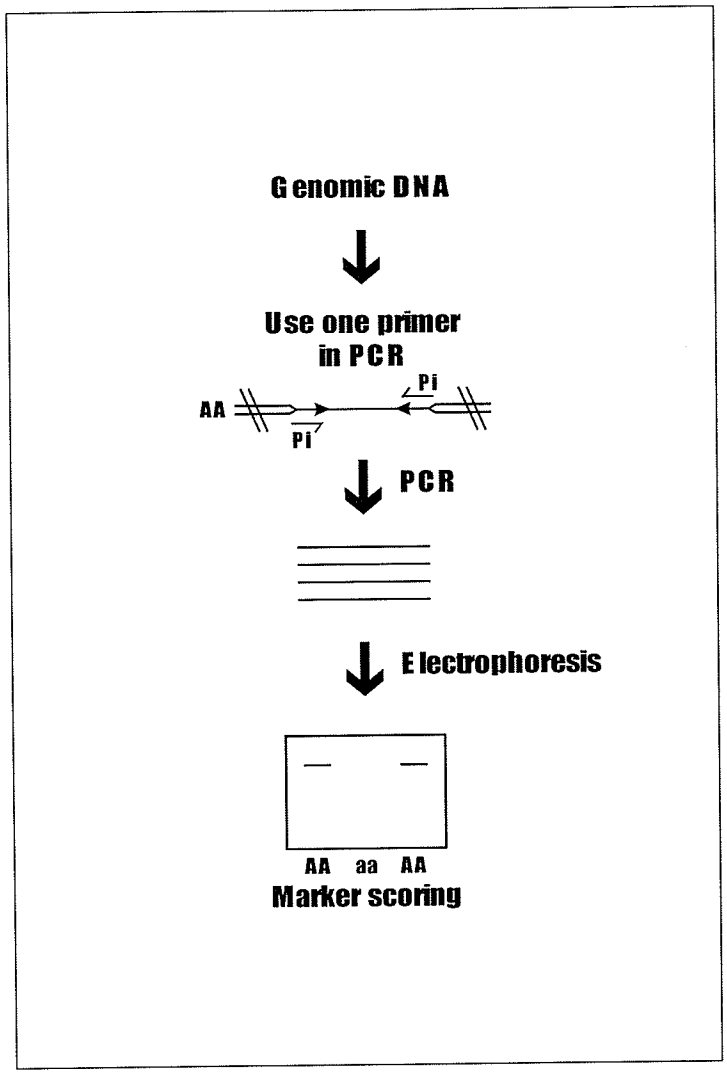
basis, and can provide genetic information as codominant markers.

Burden and Roelfs, 1986 examined isozyme polymorphism among isolates of *Puccinia graminis* (the wheat stem rust fungus) that differed in virulence as defined by their virulence phenotypes. They found that isolates with the same or similar virulence variation phenotype almost always had identical isozyme phenotypes whereas isolates in different virulence clusters had different isozyme phenotypes.

2.2.2 Random Amplified Polymorphic DNA (RAPD)

Random amplified polymorphic DNA markers are generated by PCR amplification of random genomic DNA segments with single primers (usually 10 nucleotides long) of arbitrary sequence (Williams *et al*, 1990). The primers are used as substrates for DNA polymerase to copy the genomic sequences 3' to the primers. Iteration of this process yields a discrete set of amplified DNA products that represent target sequences flanked by opposite-orientated primer annealing sites (Fig. 2). The primers amplify fragments of the genome and are capable of identifying polymorphic loci even if there is a single nucleotide sequence difference between two genomes at the site of primer binding Williams *et al*, 1990, Welsh and McClelland, 1990). This PCR technology is relatively easy to implement using very small amounts of DNA. RAPD data are easy to interpret because they are based on amplification or nonamplification of specific DNA sequences, generating data that are easy to analyze (the band is either present or absent). Although this is ideal for genetic mapping, it is not ideal for studies of genetic diversity as it is affected by the number of alleles at a locus. Also, results obtained with RAPDs can be

Figure 2: Random Amplified Polymorphic DNA (Staub at al 1996). Single 10 base primer is used in RAPDs in PCR and amplification of bands are present or absent (dominant marker system).


Genomic DNA
Use one primer
in PCR
AA
Pi
Pi
PCR
Electrophoresis
AA aa AA
Marker scoring

difficult to reproduce among laboratories and sometimes within a single laboratory because of several technical limitations such as use of different PCR equipment and Taq polymerase in the optimization of PCR reactions conditions for RAPD.

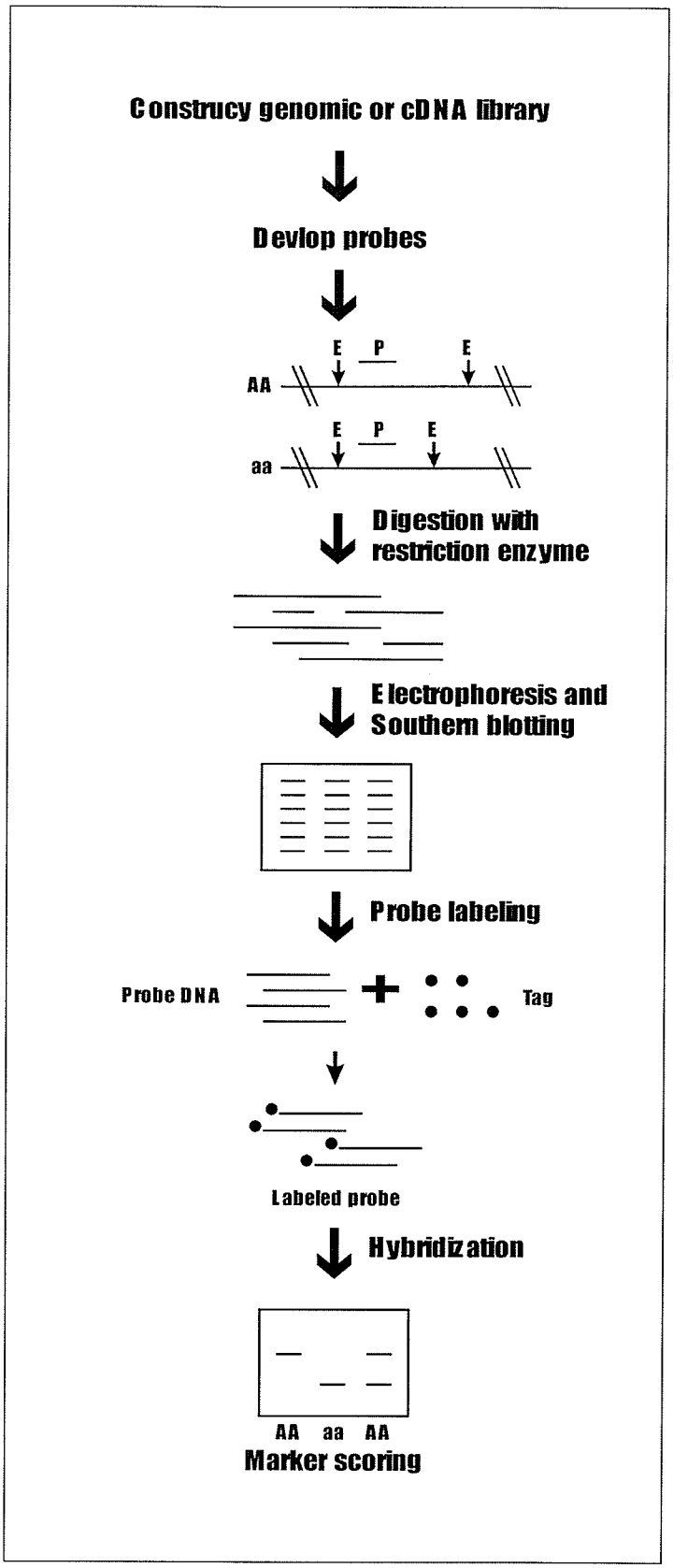
RAPD fragments have been used in crop species as linked markers for traits of agronomic importance and genome maps (Penner, 1995). Chen et al (1993) compared RAPD analysis of *Puccinia striiformis* races to the virulence spectrum of the isolates and found that there was little correlation between the two. Nevertheless, cluster analysis of RAPD fragments did show evidence of the evolutionary relationships of isolates. RAPD analysis has also been used to differentiate between species and individuals within a species such as *Magnaporthe poae* (Huff *et al*, 1994). A second round of amplification conducted on single amplicons excised from agarose gels resulted in a number of bands. When probed with the original RAPD primer, Southern blot analysis showed that all of the reamplified fragments contained binding sites, indicating that the original band was made up of repeats. Kolmer et al. (1995) analyzed isolates of leaf rust (*Puccinia recondita f. sp. tritici*) for virulence polymorphisms on near-isogenic lines of wheat and for randomly amplified polymorphic DNA. They found that molecular variation was greatest among isolates of different virulence phenotypes. The virulence and RAPD data were used in combination and separately in cluster analysis. The RAPD data were more effective in distinguishing among the major clusters of *P. recondita f. sp. tritici* isolates compared to the virulence data. On the other hand, virulence polymorphisms were better in distinguishing among isolates within major clusters compared to molecular polymorphisms. Gang *at al* (1996) used RAPDs to analyze the genetic relationship and

variability among three species of wheat bunt fungi (*Tilletia controversa, T. foetida* and *T. caries*). They identified a high level of genetic variability among species, races and even individuals in the same race of the *Tilletia species*. Cluster analysis of the RAPD banding patterns distinguished races of *T. caries, T. foetida and T. controversa.*

2.2.3 Restriction Fragment Length Polymorphisms (RFLP)

Restriction fragment length polymorphism is the result of a sequence change at a restriction enzyme target site that results in a difference in the length of the fragment produced upon cleavage with the corresponding enzyme (Fig. 3) (Lewin, 1990). RFLPs are based on DNA-DNA hybridization. They are technically more difficult than RAPDs but offer the advantage of being more reproducible. RFLPs are a coodominant marker system and can exhibit a potentially unlimited number of alleles per locus. One disadvantage of RFLPs is that they require large amounts of DNA from each individual, which can be a major problem in working with fungi. It also requires more technical expertise including cloning, Southern blotting and radioactive labeling. RFLPs were identified in *Verticillium* species which could differentiate *Verticillium albo-atrum* from *V. dahlie* (Carder and Barbera, 1991). RFLPs identified U.S. strains of the bacteria *Xanthomonas campestris oryzae* as genetically unique from strains from other geographic origins (Xu and Gonzales, 1991). RFLPs were also used as genetic markers in mapping a rust resistance gene in *Triticum tauschii* (Gill et al, 1991). Restriction fragment length polymorphism markers were used to determine genetic variation of Australian field populations of the barley scald pathogen *Rhynchosporium secalis* (McDonald at al, 1999).

Figure 3: Restriction Fragment Length Polymorphism (Staub at al 1996). To start RFLP, probes must be developed from cDNA library. DNS is then digested with restriction enzymes. Next step is electrophoresis and Southern blotting. That product is than labeled with probe DNA and after hybridization bends appear as shifts if any differences are present (codominant marker system).

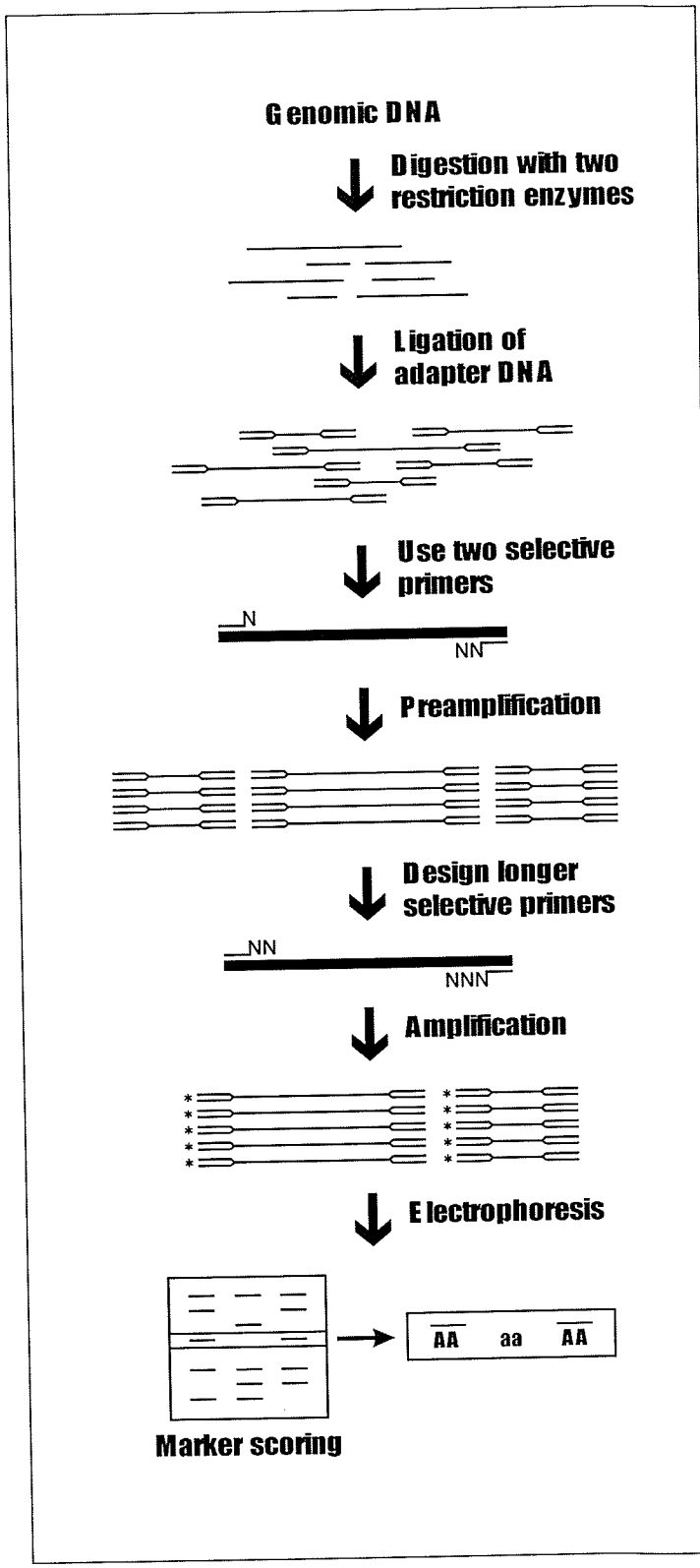

Construcy genomic or cDNA library
Devlop probes
E
P
E
AA
E
P
E
aa
Digestion with
restriction enzyme
Electrophoresis and
Southern blotting
Probe labeling
Probe DNA
Tag
Labeled probe
Hybridization
AA
aa
AA
Marker scoring

They collected isolates from five infected fields throughout Australia and observed genetic variation among fields as well as within fields. Their results showed a large amount of genetic diversity among and within fields, in contrast to their hypothesis that *R. secalis* would exhibit low genetic variability, because the fungus is thought to reproduce exclusively asexually.

2.2.4 Amplified Fragment Length Polymorphisms (AFLP)

Production of AFLPs is based on selective amplification of restriction enzyme-digested DNA fragments. Multiple bands are generated in each amplification reaction that contains DNA markers of random origin (Fig. 4). Amplified fragment length polymorphisms offer a potentially powerful tool to detect polymorphic DNA sequences in fungi. AFLPs share many characteristics with RAPDs. They are dominant and usually only have two alleles per locus. AFLPs have an advantage over RAPDs in that more loci are screened in each reaction and their use of primers makes it more likely that an AFLP will be reproducible. AFLPs are likely to be used for DNA fingerprinting applications because a large number of loci can be screened in one reaction. The disadvantages of AFLPs are that they require more technical expertise than RAPDs including ligation, restriction enzyme digestion and polyacrylamide gels. The AFLP technique represents a conceptual and practical advance in DNA fingerprinting. It does not require prior knowledge of DNA sequences and produces an unlimited source of template DNA. From this source, DNA fingerprints are generated with greater resolution and information content than is possible by conventional RAPD and RFLP techniques (Zabeau and Vos, 1993).

Figure 4: Amplified Fragment Length Polymorphism (Staub at al 1996). In AFLPs genomic DNA is digested with two restricted enzymes. Next step is to ligate adaptors DNA and than preamplification using two pre-amp primers. Preamplification is then followed by selective amplification using two selective primers. Bands are viewed as present or absent (dominant marker system).


Genomic DNA
Digestion with two restriction enzymes
Ligation of adapter DNA
Use two selective primers
N
NN
Preamplification
Design longer selective primers
NN
NNN
Amplification
Electrophoresis
AA aa AA
Marker scoring

The AFLP technique has been used in genetic diversity studies in *Bacillus anthrachis* (Keim et al, 1996), the most molecularly monomorphic bacterium known. AFLP markers were used to analyze *B. anthracis* isolates and six related *Bacillus* species for molecular variation by generating 357 AFLP polymorphic fragments. Cluster analysis identified two very distinct lineages among the *Bacillus* isolates. AFLP was also used to assess the levels of genomic variations among species and isolates of the genus *Colletotrichum* (O'Neill et al, 1997). The authors attempted to distinguish between two alfalfa pathogen isolates Avl-NW and 57RR at the molecular level. Using the AFLP technique they succeeded in placing these two isolates within the species *C. triffoli* and *C. glaeoaporioides,* respectively.

3. MATERIALS AND METHODS

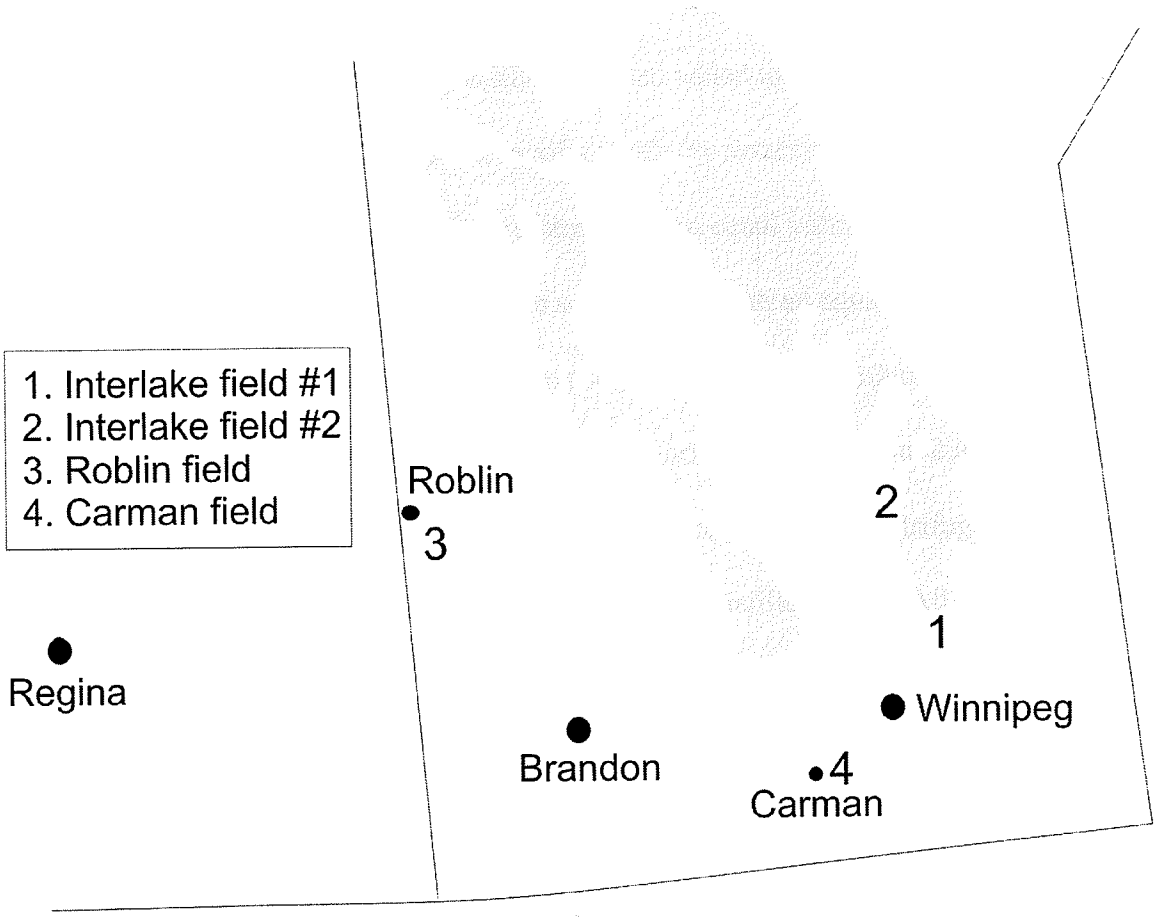
3.1 Field survey, Single spore isolation and Culture maintenance

One hundred sixty one isolates of *Ustilago tritici* were collected in 1995 and 1996 in Manitoba, Saskatchewan and Alberta (Figure 4), (Table 1 and 2). Teliospores collected from infected plants were stored as individual spikes in paper envelopes at 5^0C. In order to obtain single spore cultures, teliospores were spread on one half strength potato dextrose agar in a Petri dish under aseptic conditions using a laminar flow hood. When telispores germinated they were transferred individually (using a sterile transfer needle) to separate dishes containing one half strength PDA medium. An alternative way of obtaining pure cultures of the fungus is to pass the isolate through the host a number of times, collecting one smutted head each time (Nielsen 1987). In this way, the host (wheat) will purify the culture. This method is time consuming and laborious. The pure cultures were maintained on one half strength potato dextrose agar at 5^0C, and rejuvinated every six months.

3.2. DNA Extraction

DNA was extracted using a modified CTAB technique (Hexadecyltri-Methylammonium Bromide), (Kim et al. 1990). Pure cultures of *U. tritici* were transferred, using a sterile needle, from Petri dishes into 125 ml flasks containing 15 ml of Vogel liquid media (Atlas et al. 1993). The cultures were grown for one week in an incubator set at 22^0C (the optimum growth temperature for *U. tritici*) and aerated by

Figure 5: A map of Manitoba and Saskatchewan covering most of the surveying area and the locations were the intensive field isolates were collected.


1. Interlake field #1
2. Interlake field #2
3. Roblin field
4. Carman field
Roblin
3
2
1
Regina
Winnipeg
Brandon
4
Carman

constant orbital shaking. Mycelia were harvested by centrifuging at 2300 g using a bench top centrifuge for 10 minutes. Harvested mycelia were lyophilized and stored at -20^{0}C. To extract DNA, lyophilized mycelia were ground with sterile sand using a mortar and pestle. Ground material was placed into 2 ml Eppendorf tubes and 800 µl of extraction buffer were added (1M Tris/HCl pH 8.0, 0.5 mM EDTA, 5M NaCl, 10% CTAB). Prior to extraction 100 µl of freshly prepared proteinase K (1mg/ml) were added. The CTAB extraction buffer was added to the tubes containing the ground mycelia, and vortexed for 5-10 seconds. Eighty-eight µl of 20% SDS was also added to each tube and all material was incubated at 65^{0}C for three hours, mixing gently by inversion every 30 minutes. The supernatant was recovered twice; the first time with an equal volume of phenol and chloroform:isoamyl alcohol (24:1) and the second time with an equal voulme of chloroform:isoamyl alcohol (24:1). The next step was adding 0.6 volume of isopropanol and mixing it with the supernatant. After centrifugation for 5 minutes at 2300 g, a DNA pellet was obtained. The pellet was washed in 70% ethanol and re-centrifuged. The dried pellet was resuspended in 200 µl of autoclaved double distilled water. All samples were treated with 2µl of RNAse A (1 mg/ml) at 37^{0}C for one hour. Resuspended DNA was precipitated by the addition of 0.5 volume 7.5M NH_4 acetate and 2.5 volumes of 95% cold ethanol and incubated at -20^{0}C for a minimum of 30 minutes (generally one hour or longer) and centrifuged as before. Precipitated DNA was then washed twice in 70% cold ethanol and re-centrifuged. The resulting pellet was resuspended in 50-250 µl of 0.1 TE buffer (1mM Tris, 0.1mM EDTA). Concentrated stocks of resuspended DNA were stored at 5^{0}C. The concentration of extracted DNA was quantified by A_{260} readings on a Varian

Table 1: Eighty one isolates of *U. tritici* collected from 81 individual fields from Manitoba, Saskatchewan and Alberta and their percent of smut.

Isolate	Location	Cereal	Percent of Smut
1	Manitoba	Awned Wheat	Trace
2	Manitoba	Wheat	0.50%
3	Manitoba	Wheat	0.10%
4	Manitoba	Wheat	Trace
5	Manitoba	Wheat	0.10%
6	Manitoba	Wheat	Trace
7	Manitoba	Wheat	Trace
8	Manitoba	Wheat	Trace
9	Manitoba	Wheat	Trace
10	Manitoba	Wheat	Trace
11	Manitoba	Wheat	Trace
12	Manitoba	Wheat	0.10%
13	Manitoba	Wheat	0.10%
14	Manitoba	Wheat	Trace
15	Manitoba	Wheat	0.50%
16	Manitoba	Awned Wheat	0.20%
17	Manitoba	Wheat	0.20%
18	Manitoba	Wheat	0.10%
19	Manitoba	Wheat	0.30%
20	Manitoba	Awned Wheat	0.10%
21	Manitoba	Wheat	0.10%
22	Manitoba	Wheat	Trace
23	Manitoba	Wheat	0.20%
24	Manitoba	Wheat-A Wheat	Trace
25	Manitoba	Wheat-A Wheat	Trace
26	Manitoba	Wheat	Trace
27	Manitoba	Wheat	0.10%
28	Manitoba	Wheat-A Wheat	Trace
29	Manitoba	Wheat	Trace
30	Manitoba	Wheat	Trace
31	Manitoba	Wheat	Trace
32	Manitoba	Wheat	Trace
33	Saskatchewan	Wheat	Trace
34	Saskatchewan	Wheat	Trace

35	Saskatchewan	Awned Wheat	0.10%
36	Saskatchewan	Wheat	Trace
37	Manitoba	Awned Wheat	Trace
38	Manitoba	Awned Wheat	0.50%
39	Manitoba	Wheat	0.10%
40	Manitoba	Awned Wheat	0.10%
41	Manitoba	Awned Wheat	0.10%
42	Manitoba	Wheat	Trace
43	Manitoba	Wheat	Trace
44	Manitoba	Wheat	Trace
45	Manitoba	Wheat	0.10%
46	Manitoba	Wheat-A Wheat	0.10%
47	Manitoba	Wheat	Trace
48	Manitoba	Wheat	0.10%
49	Manitoba	Wheat-A Wheat	0.10%
50	Manitoba	Wheat	Trace
51	Manitoba	Wheat	0.10%
52	Manitoba	Wheat-A Wheat	Trace
53	Manitoba	Wheat	Trace
54	Manitoba	Wheat	Trace
55	Manitoba	Wheat	Trace
56	Manitoba	Wheat	Trace
57	Manitoba	Wheat	Trace
58	Manitoba	Wheat	Trace
59	Manitoba	Wheat	Trace
60	Manitoba	Wheat	0.50%
61	Manitoba	Wheat	0.10%
62	Manitoba	Wheat	0.10%
63	Manitoba	Awned Wheat	0.10%
64	Manitoba	Wheat	Trace
65	Saskatchewan	Wheat	0.10%
66	Saskatchewan	Wheat	0.10%
67	Saskatchewan	Wheat	Trace
68	Saskatchewan	Awned Wheat	0.30%
69	Saskatchewan	Awned Wheat	0.20%
70	Saskatchewan	Awned Wheat	0.30%
71	Saskatchewan	Awned Wheat	0.10%
72	Saskatchewan	Wheat	Trace
73	Saskatchewan	Awned Wheat	Trace
74	Saskatchewan	Wheat	Trace
75	Saskatchewan	Awned Wheat	Trace

76	Manitoba	Awned Wheat	0.10%
77	Alberta	Wheat	0.10%
78	Alberta	Wheat	0.10%
79	Alberta	Wheat	0.10%
80	Alberta	Wheat	0.10%
81	Manitoba	Wheat	0.10%

Table 2: Eighty isolates of *U. tritici* collected from four fields in Manitoba and their percent of smut.

Isolate	Location	Cereal	Percent of Smut
1	Interlake1 Manitoba	Wheat	0.50%
2	Interlake1 Manitoba	Wheat	0.50%
3	Interlake1 Manitoba	Wheat	0.50%
4	Interlake1 Manitoba	Wheat	0.50%
5	Interlake1 Manitoba	Wheat	0.50%
6	Interlake1 Manitoba	Wheat	0.50%
7	Interlake1 Manitoba	Wheat	0.50%
8	Interlake1 Manitoba	Wheat	0.50%
9	Interlake1 Manitoba	Wheat	0.50%
10	Interlake1 Manitoba	Wheat	0.50%
11	Interlake1 Manitoba	Wheat	0.50%
12	Interlake1 Manitoba	Wheat	0.50%
13	Interlake1 Manitoba	Wheat	0.50%
14	Interlake1 Manitoba	Wheat	0.50%
15	Interlake1 Manitoba	Wheat	0.50%
16	Interlake1 Manitoba	Wheat	0.50%
17	Interlake1 Manitoba	Wheat	0.50%
18	Interlake1 Manitoba	Wheat	0.50%
19	Interlake1 Manitoba	Wheat	0.50%
20	Interlake1 Manitoba	Wheat	0.50%
21	Interlake 2 Manitoba	Wheat	0.30%
22	Interlake 2 Manitoba	Wheat	0.30%
23	Interlake 2 Manitoba	Wheat	0.30%
24	Interlake 2 Manitoba	Wheat	0.30%
25	Interlake 2 Manitoba	Wheat	0.30%
26	Interlake 2 Manitoba	Wheat	0.30%
27	Interlake 2 Manitoba	Wheat	0.30%
28	Interlake 2 Manitoba	Wheat	0.30%
29	Interlake 2 Manitoba	Wheat	0.30%
30	Interlake 2 Manitoba	Wheat	0.30%
31	Interlake 2 Manitoba	Wheat	0.30%
32	Interlake 2 Manitoba	Wheat	0.30%
33	Interlake 2 Manitoba	Wheat	0.30%
34	Interlake 2 Manitoba	Wheat	0.30%

35	Interlake 2 Manitoba	Wheat	0.30%
36	Interlake 2 Manitoba	Wheat	0.30%
37	Interlake 2 Manitoba	Wheat	0.30%
38	Interlake 2 Manitoba	Wheat	0.30%
39	Interlake 2 Manitoba	Wheat	0.30%
40	Interlake 2 Manitoba	Wheat	0.30%
41	Roblin Manitoba	Awned Wheat	0.50%
42	Roblin Manitoba	Awned Wheat	0.50%
43	Roblin Manitoba	Awned Wheat	0.50%
44	Roblin Manitoba	Awned Wheat	0.50%
45	Roblin Manitoba	Awned Wheat	0.50%
46	Roblin Manitoba	Awned Wheat	0.50%
47	Roblin Manitoba	Awned Wheat	0.50%
48	Roblin Manitoba	Awned Wheat	0.50%
49	Roblin Manitoba	Awned Wheat	0.50%
50	Roblin Manitoba	Awned Wheat	0.50%
51	Roblin Manitoba	Awned Wheat	0.50%
52	Roblin Manitoba	Awned Wheat	0.50%
53	Roblin Manitoba	Awned Wheat	0.50%
54	Roblin Manitoba	Awned Wheat	0.50%
55	Roblin Manitoba	Awned Wheat	0.50%
56	Roblin Manitoba	Awned Wheat	0.50%
57	Roblin Manitoba	Awned Wheat	0.50%
58	Roblin Manitoba	Awned Wheat	0.50%
59	Roblin Manitoba	Awned Wheat	0.50%
60	Roblin Manitoba	Awned Wheat	0.50%
61	Carmen Manitoba	Wheat	Trace
62	Carmen Manitoba	Wheat	Trace
63	Carmen Manitoba	Wheat	Trace
64	Carmen Manitoba	Wheat	Trace
65	Carmen Manitoba	Wheat	Trace
66	Carmen Manitoba	Wheat	Trace
67	Carmen Manitoba	Wheat	Trace
68	Carmen Manitoba	Wheat	Trace
69	Carmen Manitoba	Wheat	Trace
70	Carmen Manitoba	Wheat	Trace
71	Carmen Manitoba	Wheat	Trace
72	Carmen Manitoba	Wheat	Trace
73	Carmen Manitoba	Wheat	Trace
74	Carmen Manitoba	Wheat	Trace
75	Carmen Manitoba	Wheat	Trace

76	Carmen Manitoba	Wheat	Trace
77	Carmen Manitoba	Wheat	Trace
78	Carmen Manitoba	Wheat	Trace
79	Carmen Manitoba	Wheat	Trace
80	Carmen Manitoba	Wheat	Trace

DMS 1000 spectrophotometer (A_{260} x 0.05 x dilution factor = μg/μl). Aliquots were diluted to a concentration of 250 ng/μl and the remaining DNA was precipitated and stored at -20^{0}C in 95% ethanol.

3.3 Amplified Fragment Length Polymorphism procedure

The AFLP procedure was performed following the protocol of Keygene N.V. (Zabeau and Vos 1993). The AFLP technique is based on the selective polymerase chain reaction (PCR) amplification of restriction fragments from a total digest of genomic DNA. The technique involves three steps: 1. restriction of the DNA and ligation of oligonucleotide adapters, 2. selective amplification of sets of restriction fragments and 3. gel analysis of the amplified fragments.

3.3.1 Restriction of DNA

Two hundred and fifty ng of genomic DNA were digested at 37^{0}C for two hours using 5 units of MseI enzyme (1 unit = amount of enzyme required to cleave 1 μg of DNA in 1 hour at 37^0 C) and REact 1 buffer (50 mM Tris-HCl pH 8.0 and 10 mM $MgCl_2$,Gibco BRL) in total volume of 10 μl. After two hours 5 units of EcoRI enzyme and React 3 buffer (60 mM Tris-HCl pH 8.0, 60 mM $MgCl_2$,1500mM NaCl and 10 mM of DDT, Gibco BRL) were added to the 10 μl MseI digest in a total volume of 50μl at 37^{0}C for an additional two hours.

3.3.2 Ligation of DNA

Ligation of DNA was performed using 25 µl of digested DNA, T4 DNA ligase buffer (250 mM Tris-HCl pH 8.0, 50 mM $MgCl_2$ 5 mM ATP, 5mM DDT and 25% (w/v) polyethylene glycol-8000), 50 pmol MseI adaptor, 5 pmol EcoRI adaptor and one unit of T4 DNA ligase in total of 50 µl and incubated at room temperature for two hours. The AFLP adapters consist of a core sequence and an enzyme specific sequence.

The structure of the EcoRI adapter is:

5'-CTCGTAGACTGCGTACC

CATCTGACGCATGGTTAA-5'

The structure of the MseI adapter is:

5'-GACGATGAGTCCTGAG

TACTCAGGACTCAT-5'

Ligated DNA was diluted one in four with sterile double distilled water.

3.3.3 Preamplification of template DNA

Preamplification (nonselective amplification) of secondary template was performed with primers complementary to the core of the adapter sequences. A solution was made of 15 ng/µl of MseI pre-amp primer (GATGAGTCCTGAGTAA+C = M+C), 15 ng/µl of EcoRI pre-amp primer (GACTGCGTACCAATTC+A = E+A), Taq activity buffer (50 mM Tris/HCl pH 9.0, 20 mM NaCl, 1% (v/v) Triton X-100) (Perkin Elmer), 0.8 mM dNTPs, 1.5 mM $MgCl_2$, one unit of Taq polymerase (1 unit incorporates 10 nmol of deoxyribonucleotide into acid-precipitable material in 1 hour at 72^0 C, Gibco BRL) and

2 µl of diluted ligated DNA up to 25µl. The PCR reaction was performed in a MJ PTC 200 thermocycler using the following temperature profile: 30 sec. at 94^{0}C; 60 sec. at 56^{0}C; 60 sec. at 72^{0}C for 20 cycles. Pre-amplified PCR products were diluted three folds with sterile distilled water.

3.3.4 Selective amplification of restriction fragments

Selective amplification was carried out using either 15 ng of EcoRI pre-amp primer or MseI pre-amp primer, 15 ng of either EcoRI selective primer (i.e. E+CCC) or MseI selective primer (i.e. M+AGC), Taq activity buffer, 0.8 mMdNTPs, 1.5 mM $MgCl_2$, one unit of Taq polymerase and 2 µl of diluted pre-amplified PCR product in total volume of 20 µl. The following PCR temperature profile (MJ PTC 200) was used: 60 sec at 94^{0}C (denature); 60 sec at 65^{0}C (anneal); 90 sec at 72^{0}C (extend) per cycle, the annealing temperature was decreased by 1^{0}C until it reached 56^{0}C in a total of 10 cycles. The initial cycles were followed by 23 cycles of 30 sec. at 94^{0}C; 30 sec. at 56^{0}C and 60 sec. at 72^{0}C.

3.3.5 Gel analysis

Following selective amplification reaction products were mixed with an equal volume (20 µl) of stop solution [98% formamide, 10mM EDTA pH8.0, bromophenol blue (0.25µg/ml), xylene cyanol (0.25µg/ml) and orange G (40 µg/ml)]. The resulting mixtures were denatured for 5 minutes at 95^{0}C and then quickly cooled on ice to keep the DNA denatured. Each sample (2.5 µl) was loaded on a 5% denaturing (sequencing)

polyacrylamide gel. The gel matrix was prepared using 667 mM of Urea, 10 % of 10X TBE (854 mM of Tris /887 mM of Boric Acid/ 4% of 0.5M pH 8 EDTA in 1 litre), 12.5 % of 40% acrylamide (19:1) and dd H_2O to make total of 100 ml gel solution. To enhance polymerization 100 µl of TEMED (Promega) and 100 µl of 10% (w/v) ammonim persulfate (Promega) were added. The gel was cast using a SequiGen 38x50 cm gel apparatus (Bio Rad Laboratories Inc. Hercules, CA USA).

Electrophoresis was performed in 10% TBE at constant power of 85 watts for ~ 2.5 hours. After electrophoresis, gels were fixed for 30 minutes in 10% acetic acid, stained with silver staining solution [0.1% (w/v) of $AgNO_3$ and 0.15 % of 37% formaldehyde in 2 liter of ultrapure water) for 30 minutes and developed (developing solution: 566 mM of $NaCo_3$, 0.3 % of 37% formaldehyde and 50.6 µM of sodium thiosulphate in 2 liter of ultrapure water). Gels were dried at room temperature and viewed using a light box. DNA fingerprints were evaluated independently by two individuals. Only the presence (+) or absence(-) of fragments were scored.

3.4 Virulence analysis

Six differential host lines were used to screen all isolates included in this study. The differentials that were used in this study were: TD4, TD9, TD12A, TD 15 and TD18 (TD = *U. tritici* differential). The isolates were also inoculated to TD 13 (universal suscept) to verify that the inoculation procedure was successful. TD-13 is the first known line to be susceptible to all races of loose smut collected on *Triticum* spp., but it is resistant to races collected on *Aegilops* spp (Nielsen, 1987). An isolate of *U. tritici* was

considered virulent on a differential if >10% of the plants arising from inoculated seed were smutted.

3.5 Biosys-1

Biosys-1 is a Fortran IV computer program for the analysis of electrophoretically detectable allelic variation. Despite the broad applicability of allozymic data, most programs currently available for the analysis of these data are of limited scope, forcing researchers to run several different programs for a complete analysis. Since this procedure requires that the output of one program be re-entered as input for another, it tends to be both inefficient and error-prone.

The program performs most types of electrophoretic data analysis commonly employed in biochemical population genetics and systematics. It can be used to compute allele frequencies and genetic variability measures, to test for deviation of genotype frequencies from Hardy-Weinberg equilibrium, to calculate F-statistics, to perform heterogeneity, chi-square analyses, to calculate a variety of similarity and distance coefficients, to construct phenograms using cluster analysis, and to estimate phylogenies by the distance Wagner procedure.

3.6 SAS/STAT and Hierclus

The cluster procedure hierarchically clusters the observations in a SAS data set using one of 11 methods. The data can be numeric coordinates or distances. Cluster creates an output data set from which the tree procedure can draw a tree diagram or

output clusters at a specified level of the tree. All methods are based on the usual agglomerative hierarchical clustering procedure. Each observation begins in a cluster by itself. The two closest clusters are merged to form a new cluster that replaces the two old clusters. Merging of the methods differ in how the distance between two clusters is computed. Hierclus is another statistical computer program and the same parameters were used as with SAS-STAT.

3.7 Analysis of molecular variance (AMOVA)

Analysis of molecular variance for F-value was used in this study. The Amova file computes mean squares and a pseudo-F statistics for comparing the average distance within and between groups (Excoffier et al. 1992).

3.8 Analysis of average squared distance

Analysis of average squared distance was done using SAS-STAT statistical program for the virulence and AFLP data. The squared distance between two isolates was the number of polymorphisms or virulence/avirulence differences. When these distances are pooled together the average squared distance is obtained.

4. RESULTS

The objective of this study was to compare two *U. tritici* survey protocols, namely: intensive sampling from a small number of fields *vs* extensive sampling from a large number of fields covering a wide geographical area. Consequently, the results section was structured to deal with 1) analysis of virulence data obtained on six host differentials lines and 2) analysis of the genetic structure of *U. tritici* populations using AFLP.

4.1 Virulence analysis

4.1.1 Virulence analysis between individual and four field isolates

The frequencies of the virulences to the six differential lines within the extensive and intensive isolates of *U. tritici* were very similar. The greatest difference between these two collections occurred on the differential TD-4 to which 83% of the individual field isolates and 71% of the four field isolates possessed virulence and on differential TD-12A, to which 80% of the extensive isolates and 89% of the four field isolates possessed virulence (Figure 6). For the other differentials, i.e. TD-9, TD-13, TD-15 and TD-18, the frequencies of virulences to these differentials differed by less than 3% between the extensive and intensive field isolates.

A total of 17 different (unique) virulence patterns were identified in the extensive and intensive collections of *U. tritici* on the six wheat differential lines (Table 4). There were 13 and 14 different virulence combinations among the 81 and 80 isolates from the

Figure 6: The percentage of virulence on six wheat differentials for the extensive and intensive field isolates of *U. tritici*. The percentage of virulence on the six differentials is also shown tor the 20 isolates from each field of the four intensively sampled fields.

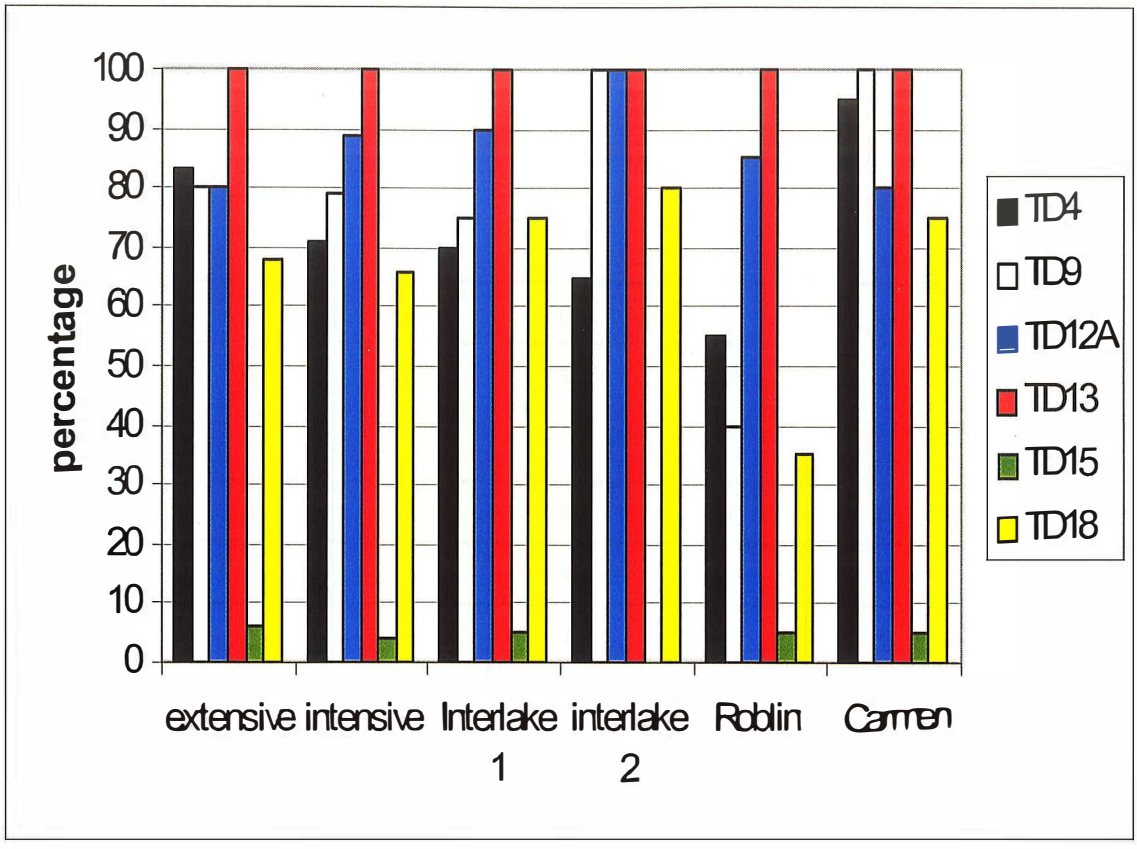

100
90
80
70
60
50
40
30
20
10
0
percentage
extensive
intensive
Interlake 1
interlake 2
Roblin
Carmen
TD4
TD9
TD12A
TD13
TD15
TD18

Table 3: The percent of isolates of *U. tritici* virulent on combinations of host differentials within 81extensive field isolates and 80 intensive field isolates, as well as within four fields of the intensive field isolates.

			Intensive field isolates			
Virulent on differential (TD-)	Extensive Field Isolates	Intensive Field Isolates	Interlake Field 1	Interlake Field 2	Roblin Field	Carmen Field
4, 9, 12A, 13, 18	60	51	50	60	35	65
12A, 13	7	11	5	0	40	0
9, 12A, 13	5	9	15	15	0	5
4, 9, 13	7	4	0	0	0	15
9, 12A, 13, 18	5	6	5	20	0	0
4, 13	6	3	0	0	10	0
4, 9, 13, 18	1	3	5	0	0	5
4, 12A, 13, 18	2	3	10	0	0	0
4, 9, 12A, 13	1	3	0	5	0	5
4, 9, 12A, 13, 15, 18	0	3	0	0	5	5
4, 12A, 13, 15, 18	2	0	0	0	0	0

4, 9, 13, 15	2	0	0	0	0	0
4, 13, 15	1	1	5	0	0	0
4, 12A, 13	1	1	0	0	5	0
12A, 13, 18	0	1	5	0	0	0
13	0	1	0	0	5	0
Total number of isolates	81	80	20	20	20	20

extensive and intensive collections, respectively (Table 3). The most common virulence combination was virulence to TD-4, TD-9, TD-12A, TD-13 and TD-18 in which 60% and 51% of the isolates from the individual fields and the four fields, respectively, contained this combination of virulence (Table 3). All other virulence combinations were found at frequencies of 11% or less. Some of the virulence combinations were observed from one collection of isolates, but not the other; for instance, isolates with the combined virulence to TD-12A, TD-13 and TD-18 were observed among the individual field isolates, but not the four field isolates (Table 3). However, isolates containing these virulence combinations were not common (<4 %) even in the group in which they were observed. No major differences were evident in the frequencies of isolates possessing the different virulence combinations between the individual field isolate and the four field isolate populations.

Virulence pattern 2 was the most common for both the extensive and intensive collections, accounting for more than 50 %. All of the other virulence patterns were present in 11 % or less in the rest of the isolates (Figure 7).

The average squared distance between isolates within and between extensive and intensive collections, as determined using virulence data, were very similar (Table 5) with the extensive collection isolates having an average squared distance of 1.49 and the intensive collection isolates having an average squared distance of 1.47 average squared distance.

The F-value for the virulence data (phenotypic data) computed between the intensive and extensive collection isolates using AMOVA was not significant (F=1).

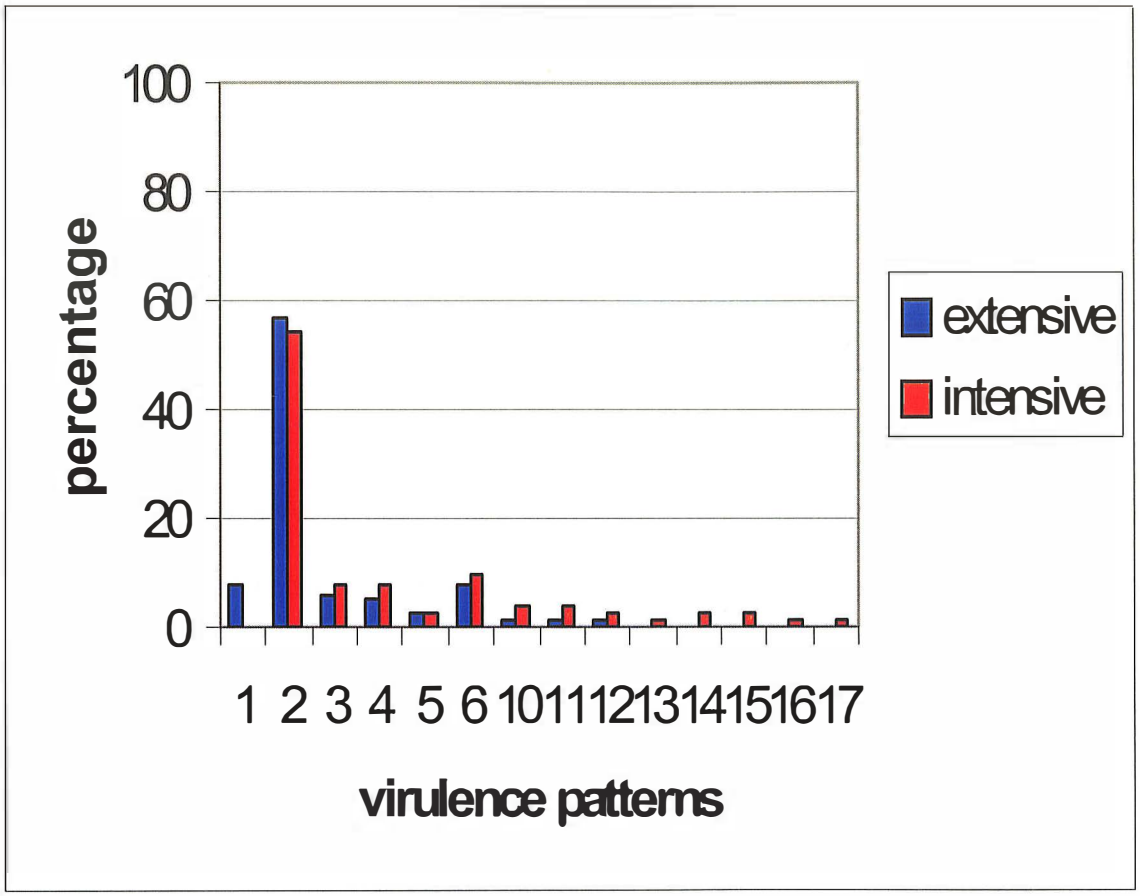
Table 4: Seventeen different virulence patterns found in extensive and intensive isolates of *U. tritici* on six wheat differential lines.

	Differentials					
Virulence patterns	TD 4	TD 9	TD 12A	TD 13	TD 15	TD 18
1	V	V	A	V	A	A
2	V	V	V	V	A	V
3	A	V	V	V	A	A
4	A	V	V	V	A	V
5	V	A	V	V	A	V
6	A	A	V	V	A	A
7	V	A	A	V	A	A
8	V	A	V	V	V	V
9	V	V	A	V	V	A
10	V	V	A	V	A	A
11	V	A	A	V	V	A
12	V	V	A	V	A	V
13	A	A	V	V	A	V
14	V	V	V	V	A	A
15	V	V	V	V	V	V
16	V	A	V	V	A	V
17	A	A	A	V	A	A

Table 5: The average squared distance within and between extensive and intensive isolates determined using virulence data. The squared distance between two isolates was the number of polymorphisms or virulence/avirulence differences. When these distances are pooled together the average squared distance is obtained.

Average Squared Distance				
	intensive isolates	extensive isolates	All Isolates	N
intensive isolates	1.47	1.49	1.48	80
extensive isolates		1.51	1.50	81
All Isolates			1.49	161

Figure 7: The percentage of virulence patterns found in intensive and extensive field isolates of *U.tritici* on six wheat differentials based on their viurlence or avirulence.


100
80
60
40
20
0
percentage
1 2 3 4 5 6 10 11 12 13 14 15 16 17
virulence patterns
extensive
intensive

4.1.2. Virulence analysis among isolates within the intensive collection

To assess field-to-field variation in virulence, separate analyses were conducted on isolates from individual fields (Interlake1, Interlake 2, Roblin and Carmen) (Figure 6). Differences in virulence were observed between fields. Virulence to TD-4 was observed in 95% of the isolates collected in the Carmen field, but only 55% of the isolates from the Roblin field (Figure 6). All of the isolates from the Interlake 2 and Carmen fields possess virulence to TD-9, as compared to 40% of the isolates from the Roblin field. Virulence to TD-12A was common in all four fields, and all of the isolates from the Interlake 2 field possessed this virulence. Virulence to TD-15 was not commonly possessed by the isolates from any field, and was absent in the Interlake 2 field isolates. Virulence to TD-18 was found in 75 to 80% of the isolates from all the fields except for the Roblin field isolates in which only 35% of the isolates possessed this virulence.

The frequencies of isolates with the different virulence combinations varied among the fields. The collections from the Interlake field 1, Interlake field 2 and Carmen field were dominated by isolates with the combination of virulence to TD-4, TD-9, TD-12A, TD-13 and TD-18. These isolates represented 50, 60 and 65% of all the isolates sampled from these fields, respectively (Table 3). This virulence combination was observed to have a frequency of 35% of the Roblin field isolates. The most common virulence combination among the Roblin field isolates (40%) was virulence to TD-12 and TD-13 only, which was only identified once in the other 60 isolates collected from the other three fields. Of the 14 virulence combinations observed among the 80 four field isolates, only seven virulence combinations were observed more than once in any one

field (Table 3).

Figure 8 shows the percentage of isolates of *U. tritici* from specific fields possessing specific virulence patterns collected from the four fields (20 isolates per field). Virulence pattern 2 was the most common accounting for 50-65 % of the pathogen isolates in the Interlake 1, Interlake 2 and Carmen fields. Only 35% of the Roblin field isolates had virulence pattern 2. On the other hand, the 40% of the Roblin field isolates were of virulence pattern 6.Virulence pattern 6 was unique to the Roblin field isolates. Each of the rest of the virulence patterns were represented by less than 20 % of the isolates from each field.

Cluster analysis using the Biosys statistical computer program showed that isolates from the four fields cluster well within field groups and have a high percent similarity for virulence (Fig. 9). The *U. tritici* isolates from the two fields from the interlake region of Manitoba (Interlake 1 and Interlake 2) were the most similar and the isolates collected from the Roblin field, which was geographically the farthest from the other three fields, were least similar to the isolates from the two interlake fields. Loose smut isolates collected from Carmen field were more similar for virulence to the Interlake 1 and Interlake 2 field isolates than to the Roblin field isolates. Overall isolate similarity for all four fields was over 90%. The average squared distance between isolates within the intensive collection, as determined using virulence data, was largest for the Roblin field and the smallest was for the Interlake field 2 (Table 6). The average squared distance between isolates among the four fields was largest when comparing the Roblin field to any of the other three fields, and the smallest between Interlake field 2 and the Carmen

fields.

The F-value for the virulence data (phenotypic) computed (AMOVA) among four fields was highly significant ($F>4.7$) indicating that there is significant differences among the four fields.

Figure8: The percentage of virulence patterns possessed by isolates of *U. tritici* collected by intensive sampling in four fields (Interlake1, Interlake 2, Roblin and Carmen fields). The virulence patterns are defined in Table 4.

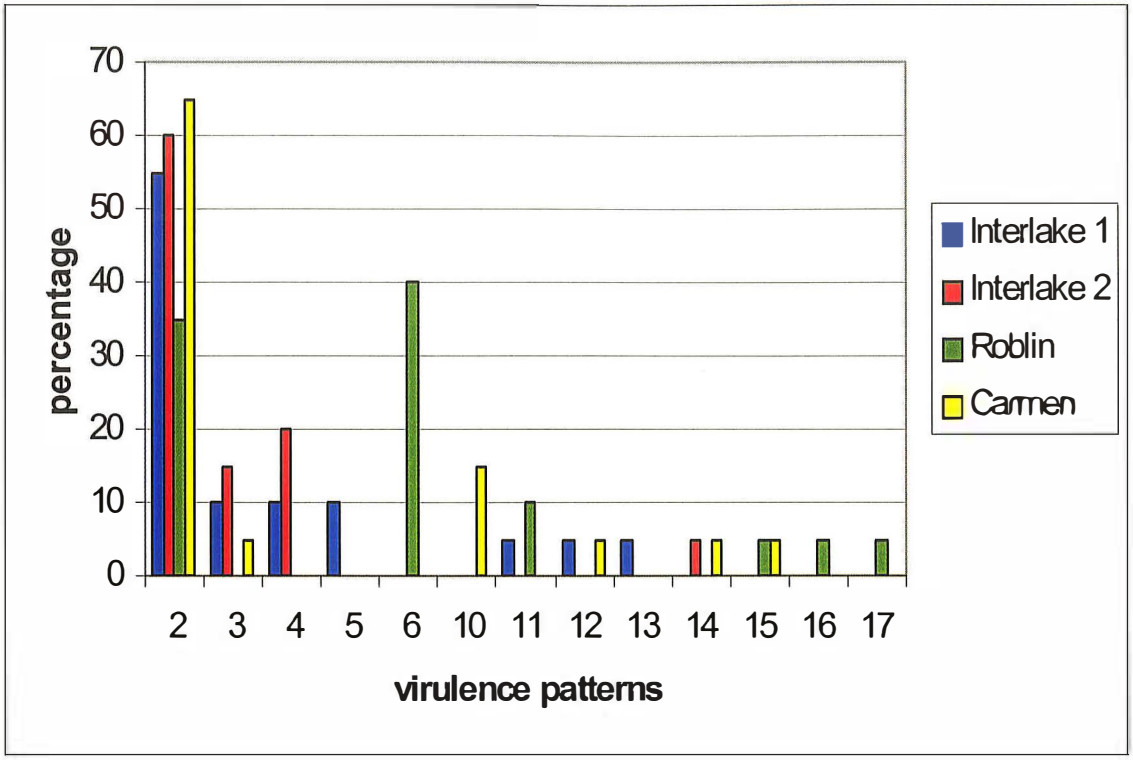

70
60
50
40
30
20
10
0
percentage
2
3
4
5
6
10
11
12
13
14
15
16
17
virulence patterns
Interlake 1
Interlake 2
Roblin
Carmen

Figure 9: Cluster analysis using the Biosys computer program based on 80 intensive isolates separated in four groups (Interlake 1, Interlake 2, Roblin and Carmen fields) based on virulence on six wheat differentials. The scale 0-1 represents percentage of similarity (their virulence/avirulence) among the four fields.

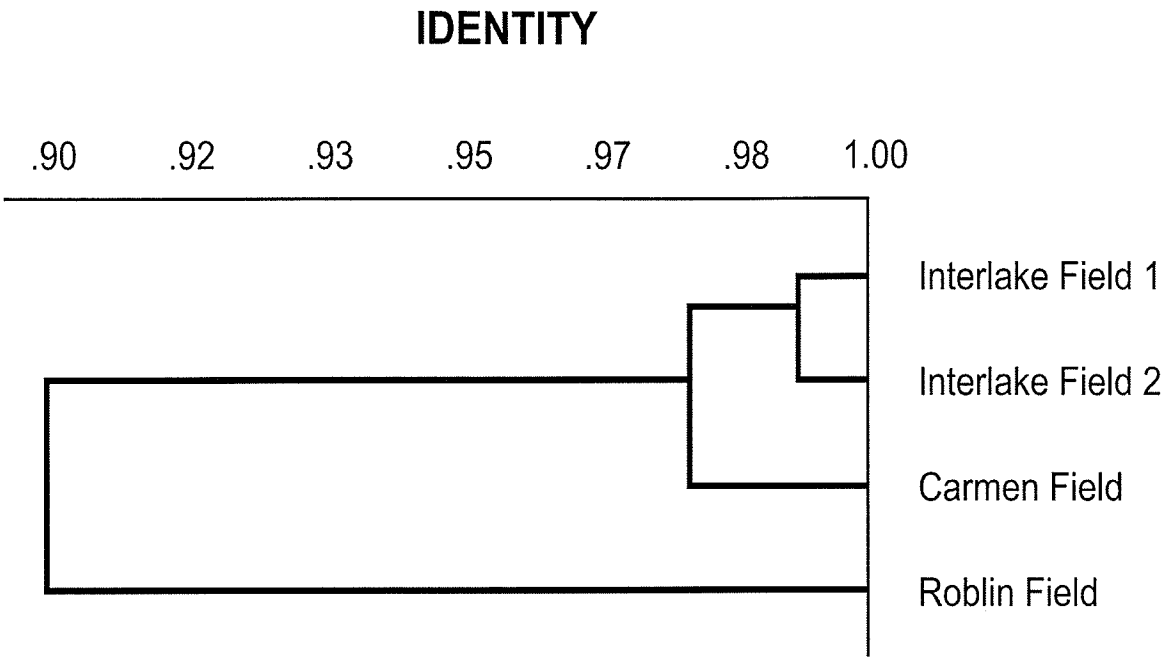

IDENTITY
.90
.92
.93
.95
.97
.98
1.00
Interlake Field 1
Interlake Field 2
Carmen Field
Roblin Field

Table 6: The average squared distance between isolates within the intensive collection, as determined using virulence data for 80 intensive isolates. The squared distance between two isolates was the number of polymorphisms or virulence/avirulence differences where they were different. When these distances are pooled together the average squared distance is obtained.

Average Squared distance						
	Interlake Field 1	Interlake Field 2	Roblin Field	Carmen field	All Isolates	N
Interlake Field 1	1.52	1.19	1.90	1.30	1.48	20
Interlake Field 2		0.82	1.85	0.97	1.21	20
Roblin Field			1.90	1.99	1.91	20
Carmen Field				0.93	1.30	20
All Isolates					1.47	80

4.2 Amplified Fragment Length Polymorphism analysis

4.2.1. AFLP analysis between individual and four field isolates

Of the 20 AFLP selective primers used for screening the *U. tritici* isolates in this study, only ten primer combinations yielded polymorphic bands (M+C/E+AGC, M+A/E+ACA, E+A/M+CAA, E+A/M+CCA, E+A/M+CCG, E+A/M+CCC, E+A/M+CCT. E+A/M+CTT, E+A/M+CGC AND E+A/M+CAC). Only polymorphic bands were used in the statistical analysis. Individual isolates that were collected from single wheat fields throughout Manitoba, Saskatchewan and Alberta yielded 35 polymorphic loci, while the four field isolates collected in Manitoba yielded 41 polymorphic loci. Twenty three polymorphic loci were common for both sets of isolates (individual and four field isolates). The common bands were determined visually and by using a molecular weight marker to [vector pGem ladder Promega)] measure the size of the bands that showed size difference (polymorphisms). The two dendrograms were constructed using 35 polymorphic loci generated by the AFLP analysis for the individual field isolates. The first was obtained using SAS-STAT (Fig. 10) and the second using Hierclus (Fig. 11) computer statistical program. The two dendrograms (Fig. 10 and 11) produced similar clustering. Isolates that clustered together were not necessarily collected from the same geographical area. Both dendrograms indicate that there is a low percentage of identity and high genetic variability among the *U. tritici* isolates, even though partial clustering is observed.

Figures 12 and 13 are dendrograms constructed by SAS-STAT and Hierclus

programs, using the 41 polymorphic loci generated by AFLP analysis of the four field isolates, (1-20 Interlake field 1, 21-40 Interlake field 2, 41-60 Roblin field 3 and 61-80 Carmen field 4). There was no definitive groupings of any kind in these dendrograms and the four field isolates appear to have high genetic diversity and a low percentage similarity.

Using the twenty three common polymorphic loci for both sets of U. tritici isolates (four field and individual isolates), statistical program cluster analysis dendrograms were constructed (SAS-STAT) for the four field and individual isolates. Individual (Figure 14) and four fields isolates (Figure 15) dendrograms made of 23 polymorphic loci were similar to the ones constructed using 35 and 41 polymorphic loci (Fig. 10 and 12). The dendrogram (Fig. 14), constructed using the 23 polymorphic loci for the individual field isolates had more isolates with the identical polymorphic banding pattern than the dendrogram constructed using the 35 polymorphisms (Fig. 12). There were two groups composed of more than 3 isolates that had identical banding patterns for the individual field isolates. One group was composed of 20 individual isolates (isolates: 3, 6, 7, 10, 11, 12, 14, 19, 34, 35, 47, 49, 50, 54, 56, 64, 68, 69, 76 and 80) and a second group was composed of 8 isolates (isolates: 27, 42, 53, 58, 65, 66, 70 and 78), (Fig. 14). Those isolates with identical banding patterns (by AFLP) did not appear to be localized to a particular area of Manitoba, Saskatchewan or Alberta but were scattered over the survey area. There was only one group composed of more than 3 isolates with identical banding patterns within the four field isolates. This group was composed of 5 isolates (isolates: 29, 35,40, 44 and 70) which were collected in 3 of the four fields sampled.

Figure 10: SAS-STAT cluster analysis based on 35 polymorphic AFLP loci generated using 81 *U. tritici* extensive collection isolates. The scale represents number of polymorphisams that these isolates differ.

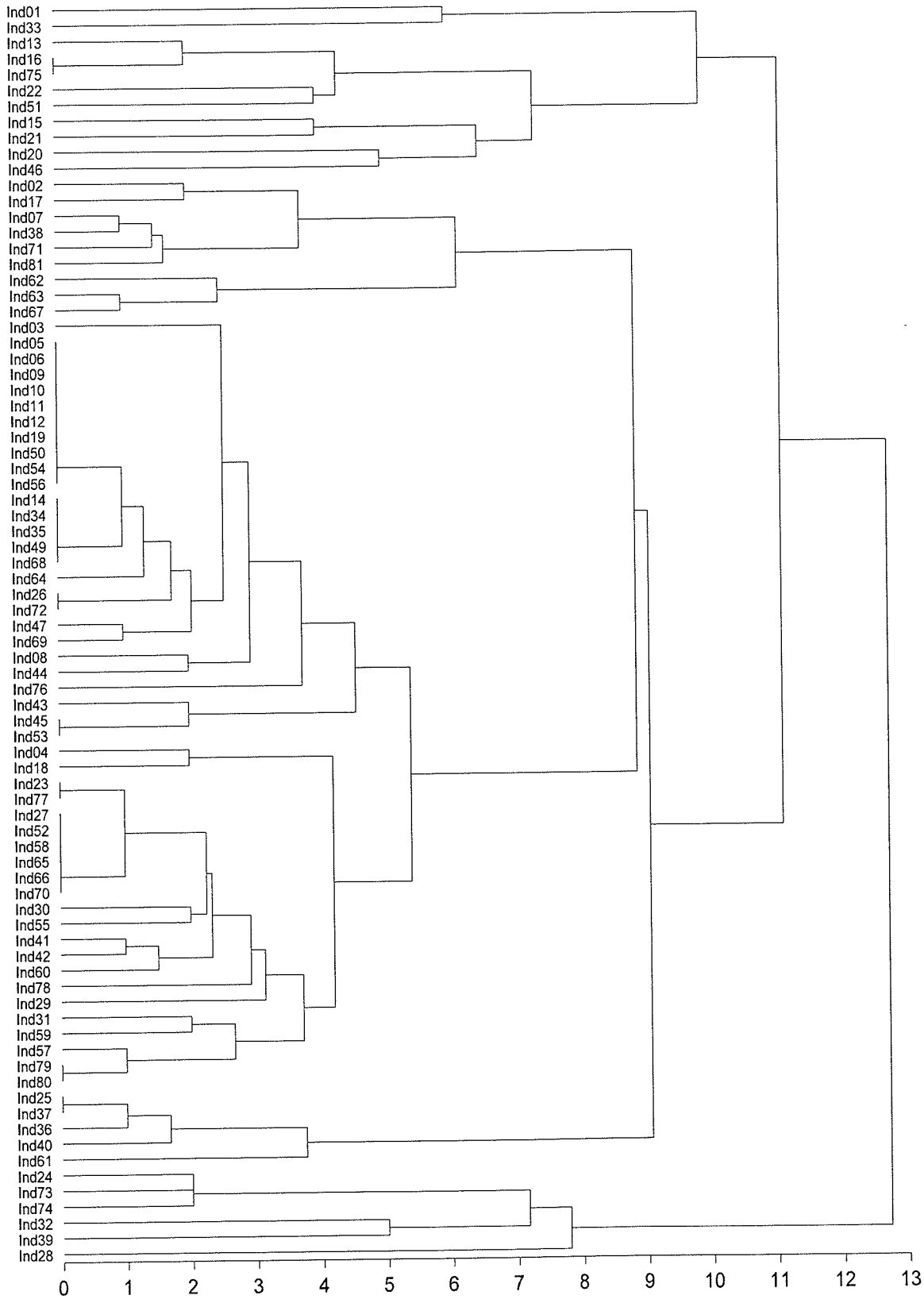

Ind01
Ind33
Ind13
Ind16
Ind75
Ind22
Ind51
Ind15
Ind21
Ind20
Ind46
Ind02
Ind17
Ind07
Ind38
Ind71
Ind81
Ind62
Ind63
Ind67
Ind03
Ind05
Ind06
Ind09
Ind10
Ind11
Ind12
Ind19
Ind50
Ind54
Ind56
Ind14
Ind34
Ind35
Ind49
Ind68
Ind64
Ind26
Ind72
Ind47
Ind69
Ind08
Ind44
Ind76
Ind43
Ind45
Ind53
Ind04
Ind18
Ind23
Ind77
Ind27
Ind52
Ind58
Ind65
Ind66
Ind70
Ind30
Ind55
Ind41
Ind42
Ind60
Ind78
Ind29
Ind31
Ind59
Ind57
Ind79
Ind80
Ind25
Ind37
Ind36
Ind40
Ind61
Ind24
Ind73
Ind74
Ind32
Ind39
Ind28
0
1
2
3
4
5
6
7
8
9
10
11
12
13

Figure 11: Hierclus cluster analysis based on 35 polymorphic AFLP loci generated using 81 *U. tritici* extensive collection isolates. The scale represents percentage of similarity among these isolates.

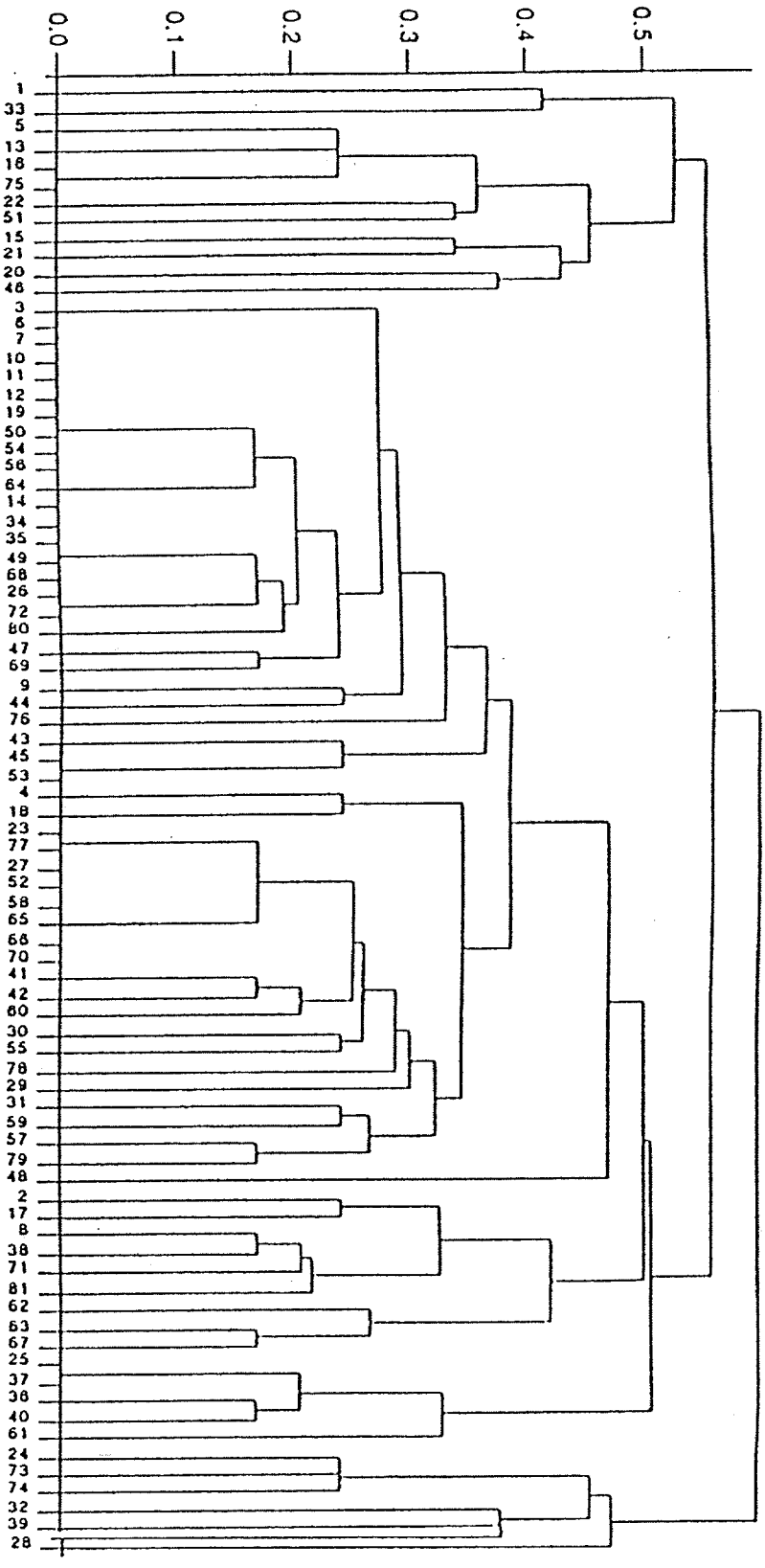

0.0
0.1
0.2
0.3
0.4
0.5
1
33
5
13
16
75
22
51
15
21
20
46
3
6
7
10
11
12
19
50
54
56
64
14
34
35
49
68
26
72
80
47
69
9
44
76
43
45
53
4
18
23
77
27
52
58
65
66
70
41
42
60
30
55
78
29
31
59
57
79
48
2
17
8
36
71
81
62
63
67
25
37
38
40
61
24
73
74
32
39
28

Figure 12: SAS-STAT cluster analysis based on 41 polymorphic AFLP loci generated using 80 *U. tritici* intensive collection isolates. The scale represents number of polymorphisams that these isolates differ.

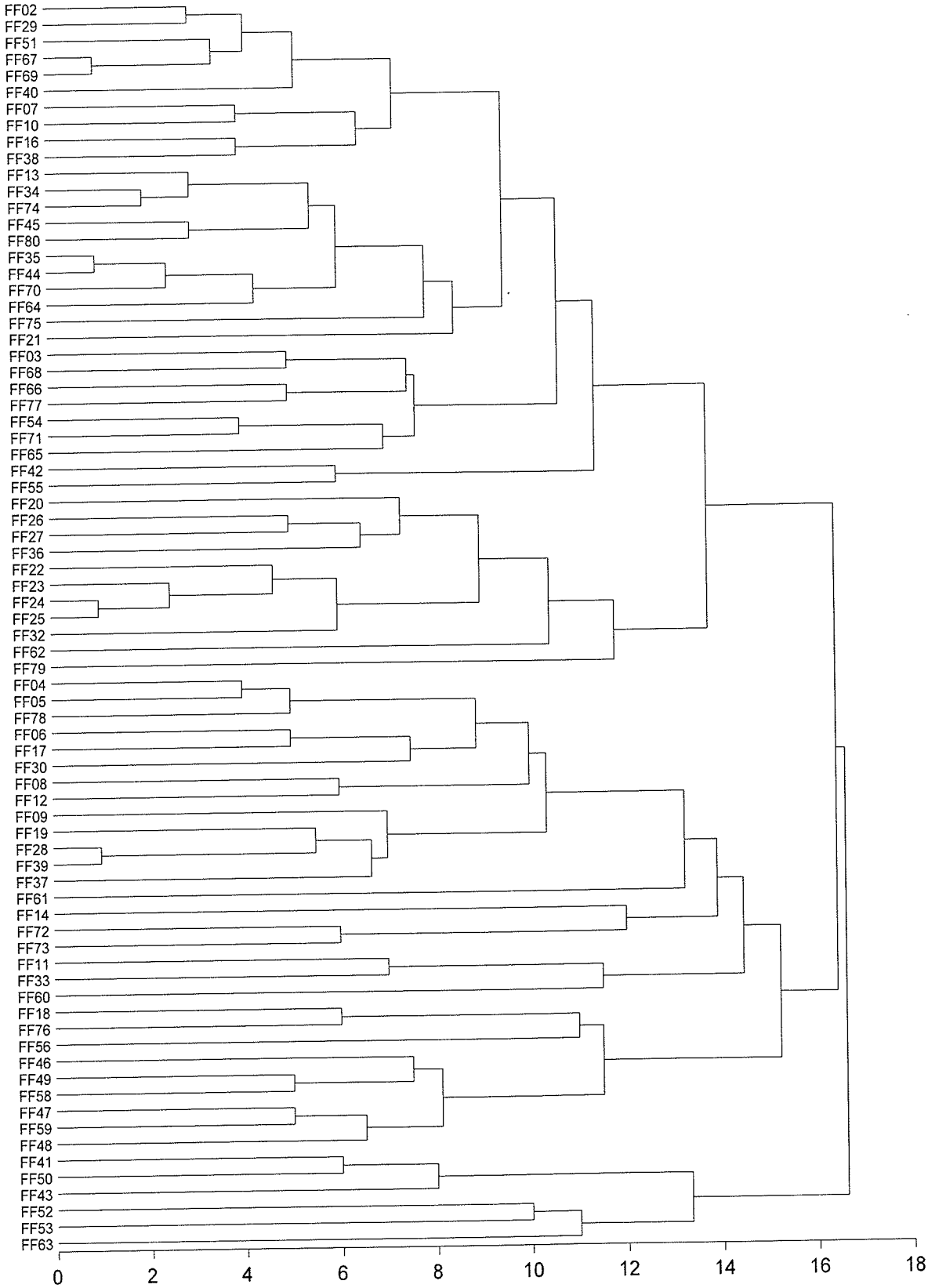
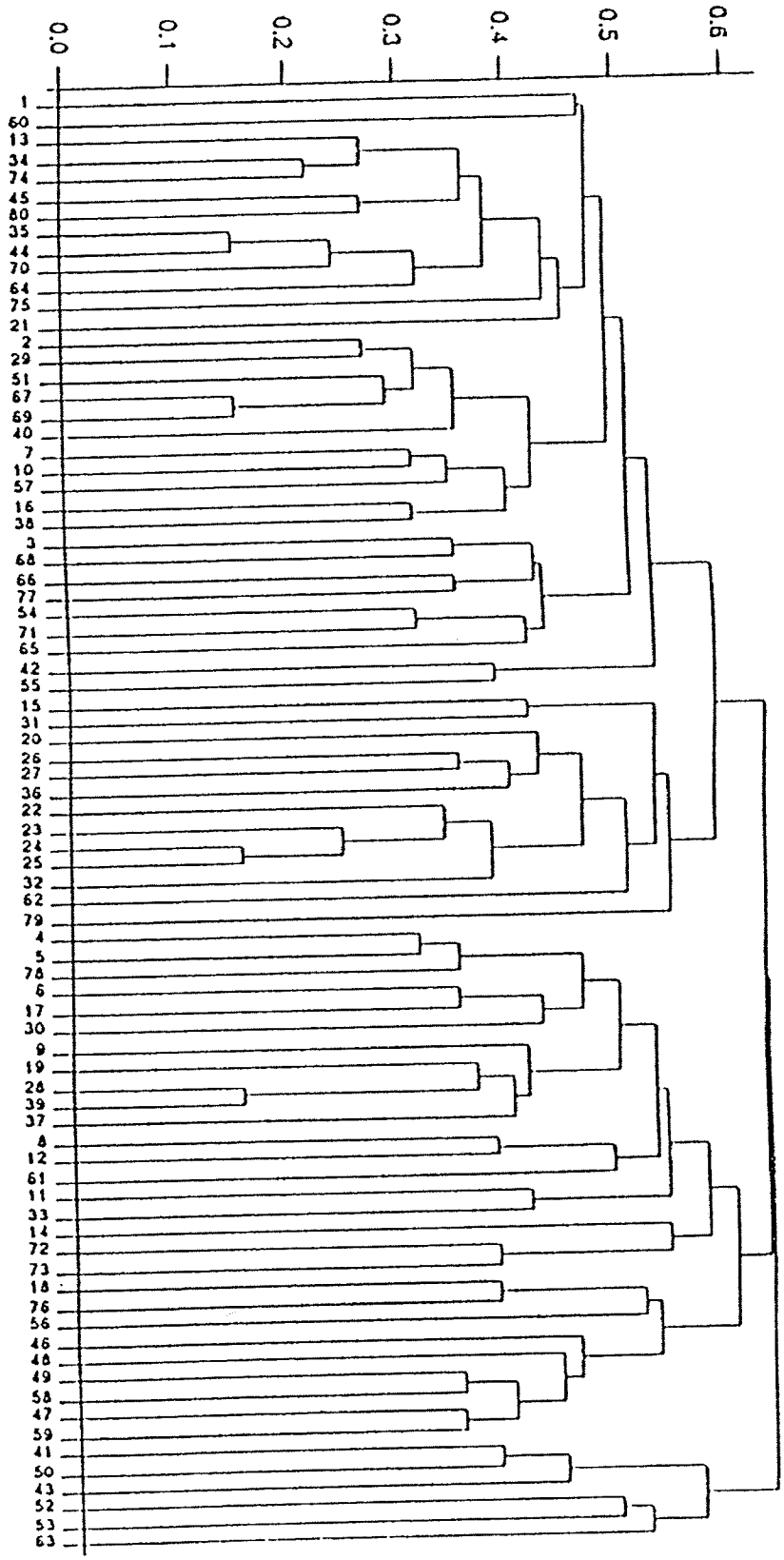

FF02
FF29
FF51
FF67
FF69
FF40
FF07
FF10
FF16
FF38
FF13
FF34
FF74
FF45
FF80
FF35
FF44
FF70
FF64
FF75
FF21
FF03
FF68
FF66
FF77
FF54
FF71
FF65
FF42
FF55
FF20
FF26
FF27
FF36
FF22
FF23
FF24
FF25
FF32
FF62
FF79
FF04
FF05
FF78
FF06
FF17
FF30
FF08
FF12
FF09
FF19
FF28
FF39
FF37
FF61
FF14
FF72
FF73
FF11
FF33
FF60
FF18
FF76
FF56
FF46
FF49
FF58
FF47
FF59
FF48
FF41
FF50
FF43
FF52
FF53
FF63
0
2
4
6
8
10
12
14
16
18

Figure 13: Hierclus cluster analysis based on 41 polymorphic AFLP loci generated using 80 *U. tritici* intensive collection isolates. The scale represents percentage of similarity among these isolates.


0.0
0.1
0.2
0.3
0.4
0.5
0.6
1
60
13
34
74
45
80
35
44
70
64
75
21
2
29
51
67
69
40
7
10
57
16
38
3
68
66
77
54
71
65
42
55
15
31
20
26
27
36
22
23
24
25
32
62
79
4
5
78
6
17
30
9
19
28
39
37
8
12
61
11
33
14
72
73
18
76
56
46
48
49
58
47
59
41
50
43
52
53
63

The frequencies of bands based on 23 common AFLP polymorphic loci for individual and four field isolates (Table 7) showed that polymorphic bands 2, 5, 9, 11, 14, 15, 16, 19, 20 and 22 differed by 10% or mote between the two collections. The greater difference in band frequency occurred with bands 15 and 16, where the difference between the band frequencies in the two collections was 21 and 36% respectively.

The average squared distance (determined using the 23 common AFLP polymorphic loci) between isolates within the extensive and intensive collections was greater for the intensive collection(Table 8). The average squared distance between isolates between the two collections was also less than the average squared distance between isolates within the intensive collection..

The F-values (generated by AMOVA) for AFLP data were done by comparing variability among the intensive collection as well as between the intensive and extensive collections. The F-value (generated by AMOVA for the AFLP data) for the difference between the extensive and intensive collections was highly significant ($F>6.5$). The extensive isolates were also divided into two groups; one group of isolates collected in Manitoba and another for isolates collected in Saskatchewan. The F-value computed between the Manitoba and Saskatchewan isolates was not significant ($F<1$).

Figure 14: SAS-STAT cluster analysis based on 23 (common for extensive and intensive collection isolates) polymorphic AFLP loci generated using 81 *U. tritici* extensive collection isolates. The scale represents number of polymorphisams that these isolates differ.

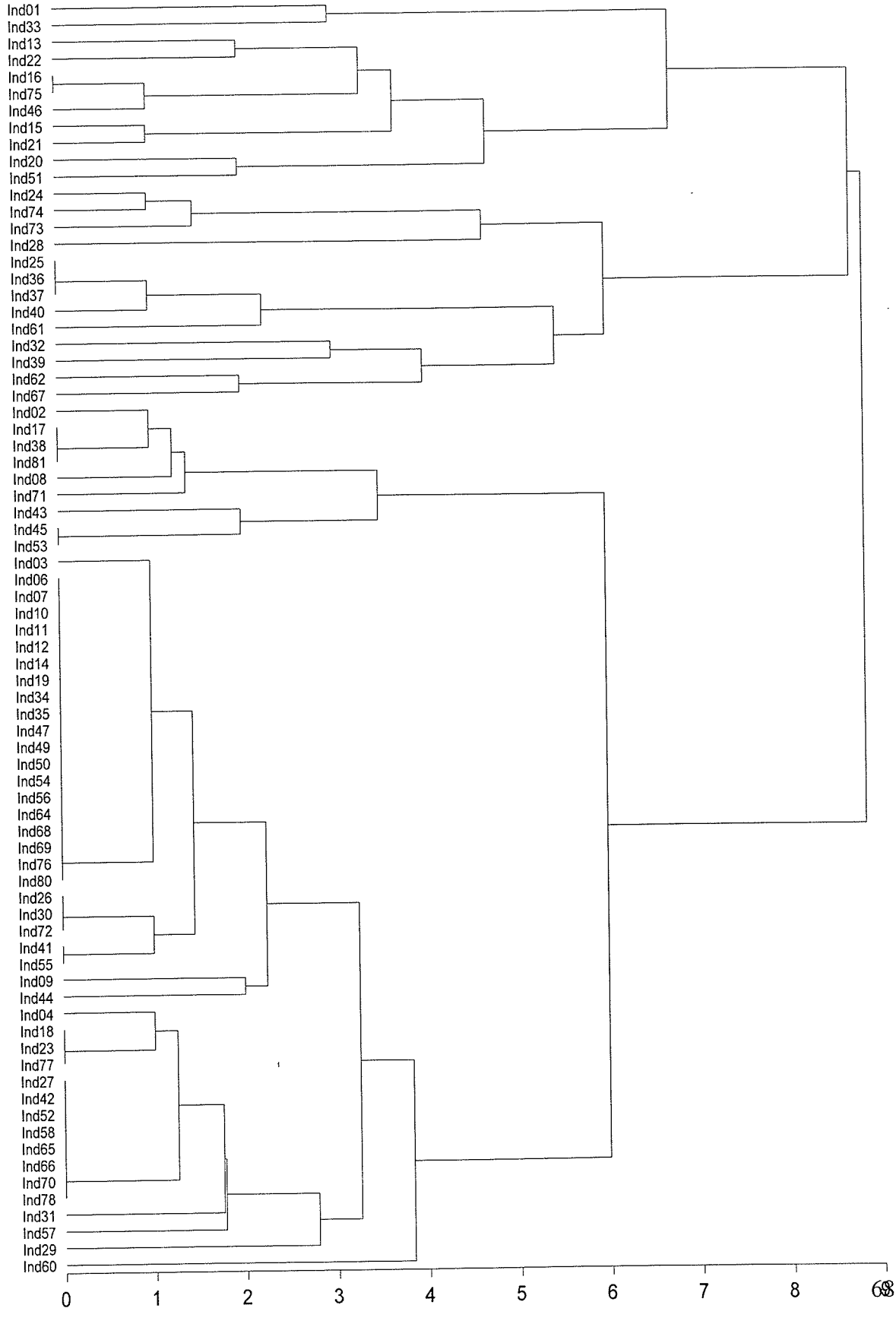

Ind01
Ind33
Ind13
Ind22
Ind16
Ind75
Ind46
Ind15
Ind21
Ind20
Ind51
Ind24
Ind74
Ind73
Ind28
Ind25
Ind36
Ind37
Ind40
Ind61
Ind32
Ind39
Ind62
Ind67
Ind02
Ind17
Ind38
Ind81
Ind08
Ind71
Ind43
Ind45
Ind53
Ind03
Ind06
Ind07
Ind10
Ind11
Ind12
Ind14
Ind19
Ind34
Ind35
Ind47
Ind49
Ind50
Ind54
Ind56
Ind64
Ind68
Ind69
Ind76
Ind80
Ind26
Ind30
Ind72
Ind41
Ind55
Ind09
Ind44
Ind04
Ind18
Ind23
Ind77
Ind27
Ind42
Ind52
Ind58
Ind65
Ind66
Ind70
Ind78
Ind31
Ind57
Ind29
Ind60
0
1
2
3
4
5
6
7
8

Figure 15: SAS-STAT cluster analysis based on 23 (common for extensive and intensive collection isolates) polymorphic AFLP loci generated using 80 *U. tritici* intensive collection isolates. The scale represents number of polymorphisams that these isolates differ.

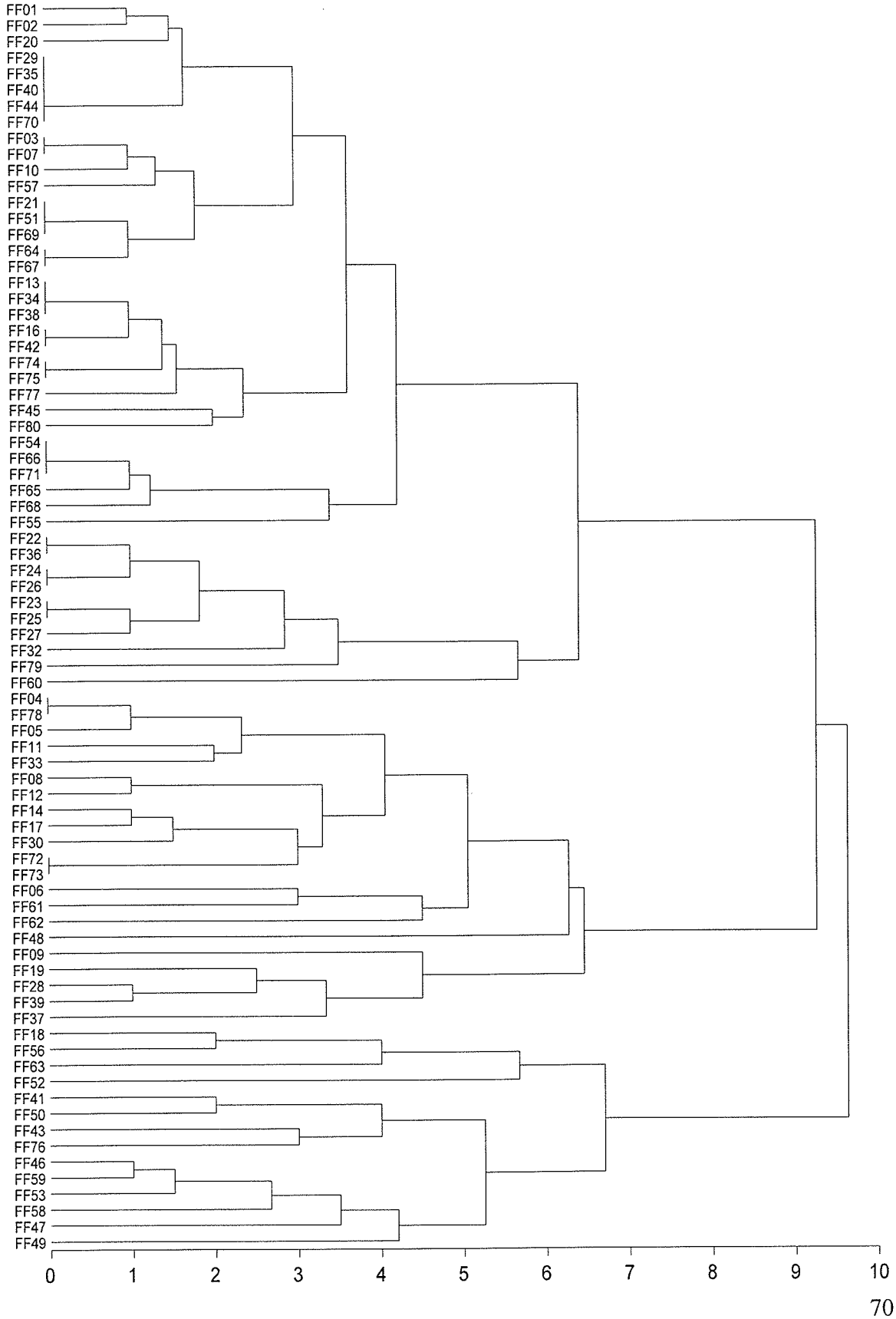

FF01
FF02
FF20
FF29
FF35
FF40
FF44
FF70
FF03
FF07
FF10
FF57
FF21
FF51
FF69
FF64
FF67
FF13
FF34
FF38
FF16
FF42
FF74
FF75
FF77
FF45
FF80
FF54
FF66
FF71
FF65
FF68
FF55
FF22
FF36
FF24
FF26
FF23
FF25
FF27
FF32
FF79
FF60
FF04
FF78
FF05
FF11
FF33
FF08
FF12
FF14
FF17
FF30
FF72
FF73
FF06
FF61
FF62
FF48
FF09
FF19
FF28
FF39
FF37
FF18
FF56
FF63
FF52
FF41
FF50
FF43
FF76
FF46
FF59
FF53
FF58
FF47
FF49
0
1
2
3
4
5
6
7
8
9
10

Table 7: Relative frequencies of present bands based on 23 common AFLP polymorphic loci for extensive and intensive collection isolates as well as four fields of the intensive collection isolates.

			Intensive field isolates			
poly-morphism	Extensive isolates (n=81)	Intensive isolates (n=80)	Interlake field 1 (n=20)	Interlake field 2 (n=20)	Roblin field (n=20)	Carmen field (n=20)
1	73	70	19	19	16	16
2	32	49	12	10	13	14
3	12	21	10	5	1	5
4	16	15	1	0	12	2
5	56	44	8	14	8	14
6	3	1	0	0	0	1
7	22	30	10	11	3	6
8	4	8	0	0	8	0
9	53	42	11	9	9	13
10	4	9	0	0	8	1
11	52	42	11	9	9	13
12	1	1	1	0	0	0
13	1	8	2	0	0	6
14	1	17	6	1	4	6
15	2	23	2	11	5	5
16	44	8	1	0	0	7
17	21	11	3	2	4	2
18	23	13	3	0	9	1
19	1	14	5	9	0	0
20	12	22	10	5	1	6
21	16	14	1	0	11	2
22	55	45	9	14	8	14
23	37	45	9	11	12	13

Table 8: The average squared distance within and between extensive and intensive isolates determined using 23 common polymorphic AFLP loci. The squared distance between two isolates was the number of polymorphisms or virulence/avirulence differences where they were different. When these distances are pooled together the average squared distance is obtained.

Average Squared Distance				
	Intensive Isolates	Extensive Isolates	All Isolates	N
Intensive Isolates	7.48	7.24	7.36	78
Extensive Isolates		6.03	6.64	76
All isolates			7.00	154

4.2.2. AFLP analysis among four field isolates

Figure 16 shows cluster analysis (using Biosys program) of the AFLP data for the four field isolates based on 41 polymorphic loci. The intensive isolates were closely clustered with a high percent similarity. The differences between Figures 12 and 13 *vs* Fig. 16 are because of the differences in the statistical programs in construction of the dendrograms and they should be seen as separate analyses. The Biosys program which is used in construction of the dendrogram in Figure 14 is used to observe differences among the four field isolates as groups, whereas the programs Hierclus and SAS-STAT look for the variability among the isolates as a whole.

The relative frequencies of a band being present based on the 41 AFLP polymorphic loci for the intensive isolates are presented in Table 9. Some bands were more commonly associated with isolates from particular fields as compared to others. For instance: polymorphic bands 7, 10, 19, 27 and 37 were more commonly found in isolates from the Roblin field than from the other three fields. In contrast, polymorphic bands 18 and 26 were not common at all in the Roblin field (band 18 was not detected) as compared to the other three fields. From the data presented in Table 9 and Figure 16 it appears that the Roblin field isolates are less similar to the isolates from the other three fields. It also appears that the isolates from the Interlake fields 1 and 2 are very similar and that Carmen field isolates were more similar to Interlake fields 1 and 2 than to Roblin field.

Analyzing the four field isolates it is noticeable that the Roblin field isolates differ in polymorphisms 7, 10, 18, 19, 27 and 37 from the other three fields.. The Carmen

Figure 16: Cluster analysis using the Byosis computer program based on 80 intensive collection isolates separated in four groups (Interlake 1, Interlake 2, Roblin and Carmen group) based on 41 AFLP polymorphic loci. The scale 0-1 represents percentage of similarity (their virulence/avirulence) among the four fields.

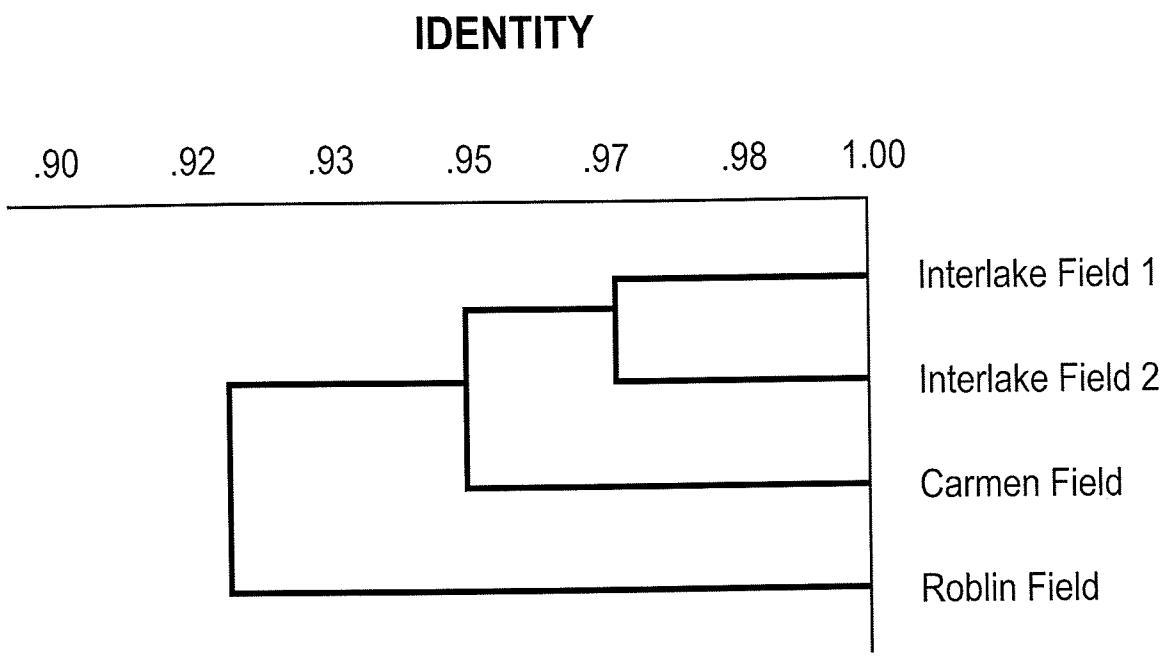

IDENTITY
.90
.92
.93
.95
.97
.98
1.00
Interlake Field 1
Interlake Field 2
Carmen Field
Roblin Field

Table 9: The relative frequencies of present bands based on 41 common AFLP polymorphic loci for the intensive collection isolates as well as four fields a of the intensive collection isolates.

poly-morphism	Intensive isolates (n+80)	Interlake 1 field (n=20)	Interlake 2 field (n=20)	Roblin field (n=20)	Carmen field (n=20)
1	31	9	11	6	5
2	61	16	13	16	16
3	46	11	11	14	10
4	45	15	12	8	10
5	68	17	19	17	15
6	19	10	5	1	3
7	15	1	0	12	2
8	44	8	14	8	14
9	45	9	11	12	13
10	9	0	0	8	1
11	42	11	9	9	13
12	17	2	5	6	4
13	28	4	9	5	10
14	42	6	11	12	13
15	31	3	7	11	10
16	70	19	19	16	16
17	49	12	10	13	14
18	22	10	5	0	7
19	14	1	0	11	2
20	45	9	14	8	14
21	6	2	0	4	0
22	35	5	10	11	9
23	45	9	12	12	12
24	25	4	2	5	14
25	1	0	0	0	1
26	29	10	10	3	6
27	8	0	0	8	0
28	42	11	9	9	13
29	8	2	0	4	2
30	5	1	0	3	1
31	1	1	0	0	0

32	8	2	0	0	6
33	17	6	1	4	6
34	22	2	11	5	4
35	9	1	0	1	7
36	11	3	2	4	2
37	13	3	0	9	1
38	14	5	9	0	0
39	16	3	1	5	7
40	18	5	7	3	3
41	43	11	12	8	12

field differed in polymorphisms 24, 32 and 35 from the other three fields whereas the Interlake fields 1 and 2 appear to be most common

The average squared distance between isolates within and between the four fields was determined with SAS-STAT using the 23 common polymorphic AFLP loci. In general, Interlake field 2 had the lowest average squared distances within the field of the four fields studied, with field 3 (Roblin field) having the largest average squared distance. Interlake field 1 and Interlake field 2 were very similar, having a lower average squared distance between isolates between the two fields than the average squared distance between isolates within Interlake field 1. The average squared distance between isolates from the Carmen field were greater than the Interlake fields, but these three fields appeared to be quite similar to each other. The greatest difference among the four fields were seen among the Roblin field and the other three fields. The Roblin field isolates also had the largest average squared distance between isolates within a field (Table 10).

The F-value generated by AMOVA for AFLP data among the intensive collection isolates showed that F-value for genetic variability among the four field isolates was highly significant ($F>3.6$).

Table 10: The average squared distance within and between intensive collection isolates determined using 23 common polymorphic AFLP loci. The squared distance between two isolates was the number of polymorphisms or virulence/avirulence differences where they were different. When these distances are pooled together the average squared distance is obtained.

Average Squared Distance						
	Interlake Field 1	Interlake Field 2	Roblin Field	Carmen Field	All Isolates	N
Interlake Field 1	6.95	6.75	8.56	7.22	7.39	19
Interlake Field 2		5.60	8.32	6.81	6.90	19
Roblin Field			7.57	8.41	8.22	20
Carmen Field				7.01	7.37	20
All Isolates					7.48	78

5. DISCUSSION

Traditionally, isolates of *U. tritici* are collected during surveys of large areas by sampling one smutted head from each infected field encountered. The reasoning behind this method was that the collection of isolates of *U. tritici* over a large area, such as the Canadian prairies (Manitoba, Saskatchewan and Alberta) would ensure the detection of genetic variability in the *U. tritici* collections and ensure that the collection was a reasonable representation of the western Canadian population. One smutted head was collected per field because the virulence analysis of *U. tritici* isolates is time and labour consuming such that more than one isolate per field could not be analyzed. In this study we made two collections: one was made by sampling 20 smutted heads per field from four wheat fields (intensive sampling) and the second consisted of 81 smutted heads from 81 different fields (extensive sampling). To our knowledge, a study to determine if more genetic variability is obtained by sampling one smutted head per field over a large area as compared to sampling numerous smutted heads per field from a few fields has not been conducted.

Studies into the genetic diversity of different populations of *U. tritici* have normally measured diversity by determining the virulence of the pathogen isolates to a set of wheat differential lines (Nielsen 1987). Although these studies focused on determining the races found in the *U. tritici* population, a number of studies assessed enough isolates (>50) to give a good indication of the genetic diversity in these populations (Rewal and Jhooty 1986, Nielsen and Tikhomirov 1993, Menzies et al, unpublished data). In these studies, the authors used 16 host differentials to determine the race structure of their

populations of *U. tritici*. In the present study only six host differentials, one being the universal suscept TD-13 (Nielsen 1987), were used to examine genetic diversity. Virulence to these six differentials is present at such levels that they can likely yield good information on genetic diversity within *U. tritici* isolates collected in western Canada. Virulence to TD-4, TD-9, TD-12A, TD-15 and TD-18 was found in 69 %, 49 %, 76 %, 71% and 47 % respectively, of all isolates collected and assessed for virulence from 1990 to 1995 (Menzies et al, unpublished data). To conduct a virulence assessment on all 161 isolates of *U. tritici* using the full 16 differential host lines would have required considerably more time and labor with the likelihood of the results being similar to those obtained with the six differentials used in this study.

To add further information to these studies, the assessment of genetic variability of the *U. tritici* isolates using the AFLP technique gives us genotypic data on the diversity of the different *U. tritici* populations, which combined with the phenotypic virulence data, gives us a better picture of how these populations differ.

The virulence data would indicate that there is similar genetic diversity within the *U. tritici* populations collected either as single smutted heads from individual fields over a large area or as twenty smutted heads per field from a small number of(four) fields. The virulence data showed little difference between the individual field and four field populations. Only the frequency of virulence to TD-4 and TD-12A differed between these two populations by more than 3% and the frequencies of virulence to these two differentials differed by only 12 and 9% respectively. As well, the frequency of occurrence of different combinations of virulence were also very similar. The most

common virulence combination (TD-4, TD-9, TD-12A, TD-13 and TD-18), was found in 60% of the individual field and 51% of the four field isolates. The other virulence combinations differed in frequency between the individual field and four field populations by 4% or less. The similarity in genetic diversity of the two collections of *U. tritici* for the virulence data was also indicated by the similar average squared distances between and within the two populations and the lack of significant differences between the two populations as determined by the AMOVA F-test.

The AFLP data indicated that there was dissimilarity between the individual and four field isolates with the four field isolates showing greater genetic diversity. Of the 23 common AFLP polymorphic loci, 19 were similar in their frequencies. Of the other four, three of them (loci 14, 15 and 19) were common at levels of 18 to 29% in the four field isolates but were found at levels of 1 to2% in the individual field isolates. The fourth locus (locus 16) was found in over 50% of the individual field isolates and was found in only 10% of the four field isolates. Within the individual field isolates, 45 of the isolates had the same banding pattern as at least one other isolate, such that nine groups of isolates of similar banding patterns were detected (Fig. 13). One of these groups represented 19 isolates (23 %) having the same banding pattern. Within the four field isolates there were 32 isolates which have the same banding pattern as at least one other isolate and these were divided into 13 groups (Fig. 14). The largest of these groups had only five isolates (6 %). The average square distance data also indicated that there was more variability within the four field than within individual field isolates. This is supported by the AMOVA analysis in which there were highly significant differences

between the individual and four field isolates.

The difference between the (phenotypic) virulence data and (genotypic) AFLP data in measuring the genetic diversity of the different populations of *U. tritici* may be the result of the nature of the data. As mentioned previously, there are only five differential host lines to determine virulence (TD-13 being universal suscept), whereas we had 23 polymorphic AFLP loci. The greater number of polymorphic loci gives a greater ability to assess the genetic diversity of the populations. The advantage of the virulence data is that we can relate it to phenotypic characteristics of the fungus in contrast to genotypic (AFLP) data.

It is important when conducting a study of this nature to realize that if one limits oneself to the sampling of a small number of fields that selection of the individual fields will play a significant role in the final outcome. Our analysis of the data obtained in the study show significant differences among the fields for both phenotypic and genotypic data. In terms of virulence and virulence combinations, the difference in some of the frequencies was greater among the four fields than in between the individual and four field isolates, i.e. TD-4, TD-9 and TD-18 (Table 3). The most common virulence combination (TD-4, TD-9, TD-12A, TD-13 and TD-18) in the individual and four field isolates was also the most common combination in the Interlake 1, Interlake 2 and Carmen fields at levels of 50-65%. In contrast in the Roblin field, this combination was found at 35% and the most common virulence combination was TD-12 and TD-13 at 40%. This virulence combination was found in just one other isolate among the other three fields and in 7% of the individual field isolates. As with the virulence data the

frequency of AFLP bands at different loci also varied greatly among the fields (Table 9).

Biosys analysis of virulence and AFLP data indicated that the four fields were similar, however, among the four fields the Roblin field was the least similar to the other three. Interestingly, the Biosys analysis using the virulence data showed more differentiation between the Roblin field and the other fields then the analysis using the AFLP data. This could be attributed to the fewer components in the virulence analysis (5 virulence differentials *vs* 23 polymorphic loci) such that each difference in the virulence data has a larger effect on the analysis. The greater variability within the Roblin field isolates and between the Roblin field isolates and the other three fields was also shown by the average square distance data. The AMOVA showed that there were significant differences among the four fields in their diversity as determined by virulence and AFLP data.

The differences among the four fields indicate that if few fields are going to be sampled, some thought must go into the number and types of field sampled. In our study the farther the fields were apart, geography wise, the less similar the fields were. The Byosis analysis for both the virulence and AFLP data were similar in showing that the Interlake field 1 and Interlake field 2, which were geographically the closest, were the most similar, followed by the Carmen field, which was geographically closer to the Interlake fields than the Roblin field, followed by the Roblin field. Another factor to be considered would be the class of wheat being sampled. The Roblin field was a field of awned wheat which was likely a Canada Prairie Spring cultivar (CPS) whereas the other three fields were unawned wheats and likely Canadien Western Red Spring (CWRS)

cultivars. According to the Manitoba Crop Insurance Corporation statistics and the Manitoba Seed Guide, over 90% of CWRS cultivars grown in Manitoba in 1995/96 had good to very good resistance to *U. tritici*, while over 90% of the CPS cultivars grown had poor to very poor resistance (Anon. 1999, 2001). This may explain the greater variability in the Roblin field because a CPS cultivar would likely have poor resistance to *U. tritici* and therefore less selection pressure would be applied to the pathogen population. More isolates of different genotypes could then be expected to successfully infect the wheat. This could be the reason why the Roblin field isolates differed significantly from the other three fields in this study. Conversely, the presence of CWRS cultivars, with their greater resistance in the other fields would be expected to have applied more selection pressure on the pathogen population, with a resulting decrease in genetic variability.

One of the interesting results of the AFLP analysis mentioned earlier was that more individual field isolates had identical banding patterns than the four field isolates. Common sense would suggest that the four field isolates should have had more isolates with identical banding patterns because they were sampled from smaller areas.

Knowledge of the amount and distribution of genetic variation within and among populations is an important part of understanding the population biology of pathogenic fungi. Genetic structure can be used to infer the relative impact of different forces that influence the evolution of pathogen populations. A better understanding of evolution could help us to predict the potential for plant pathogens to evolve in agricultural ecosystems. If for example, a large amount of genetic variability is present over a small geographical area, it suggests the possibility of rapid adaptation by a plant pathogen to a

change in it's environment (for example, new host resistance genes, fungicides etc.). On the other hand, a high degree of genetic similarity among populations collected from a large geographical areas suggests the occurrence of significant long-distance dispersal and gene flow. Gene flow over long distances may be a threat to the use of specific resistance genes in local pathogen populations, because introduced pathogen isolates can possess virulence genes that could overcome resistance in local host cultivars. New virulence genes could be incorporated into local pathogen populations through recombination (Braiser, 1988).

The results of the virulence and AFLP analysis indicate that the *U. tritici* population in Manitoba and Saskatchewan are genetically diverse. Out of 161 isolates of *U. tritici* we observed 16 different virulence combinations, even though one virulence combination accounted for 57% of the total number of isolates. As well, out of 81 individual field isolates there were 40 different AFLP banding patterns and within the 80 four field isolates there were 59 different banding patterns (determined using 23 common polymorphic loci). This might be expected given the nature of the pathogen and the wheat industry. *Ustilago tritici* undergoes sexual reproduction whenever it infects the host (Nielsen and Thomas 1996) which would result in genetic recombination. In studies conducted with *U. nuda* (Jens.) Rostr., the causal agent of loose smut of barley, Thomas (1978) found that the potential for hybridization was as much as 43 % of the amount expected from random mating between gametes produced by the teliospores in the florets of barley. The hybridization potential of *U. tritici* is not known but would likely be similar. Another source of variability would be the selection pressure applied to the

pathogen population by the different wheat cultivars grown in western Canada. Different wheat cultivars have different levels of resistance to the pathogen which would apply different levels of selection pressure to the pathogen population and although studies have not been conducted on the host cultivars, it is likely that the wheat cultivars differ in their resistance genes. The influx of new and different pathogens genotypes into western Canada is also a possible source of genetic variability. The importation of wheat seed from other countries of the world likely results in the introduction of new pathogen genotypes. Within western Canada, the commercial movement of wheat seed would be also important in distributing different genotypes of the pathogen throughout the area.

There have been very few studies conducted that are similar to the one presented here. Restriction fragment length polymorphism (RFLP) markers were used to determine genetic diversity of Australian field populations of the *Rynchosporium secalis* (Barley scald fungus), (McDonald at al, 1999). Fungal isolates were collected from five naturally infected fields in different geographic location on Australia. Similarly to our results, they observed genetic variation among fields as well as within fields. Their results showed a large amount of genetic diversity among and within fields, contrary to their hyphothesis that *R. secalis* would exhibit low genetic diversity because the pathogen is thought to reproduce exclusively asexually.

In the study presented here we were unable to associate AFLP banding pattern with virulence. Burdon and Roelfs (1980, 1985) examined isozyme polymorphism among isolates of the wheat stem rust fungus (*Puccinia graminis tritici*) that were in different clusters as defined by their virulence phenotypes. They found that isolates with the same

or similar virulence phenotypes almost always had identical isozyme genotypes. For *P. g. tritici* isozyme and virulence variation was highly correlated. Kolmer at al (1995) analyzed sixty four single-uredinial isolates of *Puccinia recondita* tritici collected from different regions of Canada for virulence polymorphism on 19 near-isogenic wheat differential lines and for randomly amplified polymorphic DNA. Thirty seven phenotypes of wheat leaf rust were distinguished by 19 host differential lines. Fifteen molecular phenotypes were distinguished using 10 RAPD primers. On the other hand, Chen at al (1993) compared RAPD analysis of *Puccinia striformis* races to the virulence spectrum of the isolates and found that there was little correlation between the two. It is important to realize, however, that in the present study, the wheat host differential series for *U. tritici* is not made of near isogenic lines and a number of differentials likely contain more than one and /or common resistance genes (Nielsen 1987, Nielsen and Thomas 1996, Menzies at al. manuscript in preparation).

It is important to mention that monomorphic bands weren't taken into account and that fifty percent of primers failed to produce any polymorphic DNA fragments. The AFLP marker selected system is known to generate a large number of fragments per primer combination (gel) (Vos and Zabeau, 1995). With a large number of fragments one would expect to find a lot of polymorphisms, which is one of the reasons for using AFLP in this study. Fifty percent of monomorphic primer combinations suggests high percentage of genetic uniformity of *U. tritici* as a species. Similar results were found by Matheson et al. (unpublished data) using Inter-Simple Sequence Repeats.

6. CONCLUSION

Considering the objective of this study it is evident that the sampling of the twenty smutted heads per field from four fields (intensive collection) resulted in a more genetically diverse collection than sampling 81 heads from individual fields (extensive collection) as determined using AFLP analysis. Virulence analysis would suggest that the two collections would be equally diverse. From this we can conclude that in terms of genetic diversity that the intensive sampling of the small number of fields is an appropriate survey method. However, the selection of the fields to sample is important to obtain a good representative sample of the *U. tritici* population in the area being studied. The traditional method of surveying (one smutted head per many different fields) which did not yield as much genetic diversity in this study, also has its advantages. The traditional method requires less care in field selection because large number of fields are sampled and allows for a larger area to be sampled, although less intensely. It also has the added advantage of allowing the surveyors to get an estimate of the severity of loose smut in the area of the survey.

7. REFERENCES

Anonymous. 2001. Management Plus Program. Manitoba Crop Insurance Corp. http://www.mmpp.com

Anonymous. 1999. Seed Manitoba 2000. ManitobaCo-operator. Winnipeg, Manitoba. 102 pages

Bakkeren, G. And J.W. Kronstad. 1994. Conservation of the *b* mating-type gene complex among bipolar and tetrapolar smut fungi. The Plant Cell 5: 123-136

Bever, W.M. 1953. Further studies on physiologic races of *Ustilago tritici.* Phytopathology 43: 681-683

Burdon, J.J. and Roelfs, A.P. 1985 Isozyme and virulence in asexually reproducing populations of wheat leaf and stem rust. Phytopathology 75: 907-913

Carder, J.H., Barbara, D.J. 1991. Molecular variation and restriction fragment length polymorphisms (RFLPs) within and between six species of *Verticilium.* Mycol. Res. 95: 935-942.

Chen, X., Line, R.F. Leung, H. 1993. Relationship between virulence variation and DNA polymorphism in *Puccinia striiformis*. Phytopathology 83: 1489-1497.

Gang, D. R. and Weber D. J. 1996. Using random amplified polymorphic DNA to analyze the genetic relationships and variability among three species of wheat smut (*Tilletia*). Bot. Bull. Acad. Sin.:173-180

Gill, K.S., Lubbers, E.L., Gill, B.S., Raupp, W.J., Cox, T.S. 1991. A genetic linkage map of *Triticum tauschii* (DD) and its relationship to the D genome of bread wheat (AABBDD). Genome 34: 362-374.

Green, G.J., Nielsen, J.I., Cherewick, W.J., and Samborski, D. J. 1968. The experimental approach in assessing disease losses in cereals: rusts and smuts. Can. Plant Dis. Surv. 48: 61-64.

Grevel, F.K. 1930. Untersuchungen über das Vorhandensein biologischer Rassen des Flugbrandes des Weizens (*Ustilago tritici*). Phytopathol. Z. 2: 209-234.

Hendry, G. W. and **H. N. Hansen**. 1934. The antiquity of *Puccinia graminis* and *Ustilago tritici* in California. Phytopathology 24: 1313-1314

Huff, D.R., Bunting, T.E., Plumley, K.A. 1994. Use of random amplified polymorphic DNA markers for the detection of genetic variation in *Magnaporthe poae.* Phytopathology 84: 1312-1316.

Kolmer, J. A., Liu, J.Q. and Sies, M. 1995. Virulence and molecular polymorphism in

Puccinia recondita f. sp. *tritici* in Canada. Phytopathology Vol. 85:276-285

Lewin, B. 1990. Genes IV. Published by Oxford University Press. P. 166 and 817.

McDonald, B. A., Zhan. J., and Burdon, J. J. 1999. Genetic structure of *Rhynchosporium secalis* in Australia. Phytopathology 89:639-645

Menzies, J. G., Thomas, P.L. and Woods, S. 1999. The effect of flowering stage in wheat on the infection efficiency of *Ustilago tritici*. Phytoprotection 80: 13-19

Nielsen J. 1987. Races of *Ustilago tritici* and techniques for their study. Can. J. Plant Pathol. 9: 91-105

Nielsen, J. and **P. L. Dyck**. 1988. Three improved differential hosts to identify races of *Ustilago tritici*. Can. J. Plant Pathol. 10: 327-331

Nielsen, J., and **Thomas, P.** 1996. Loose smut. IN Wilcoxson, R.D. and E.E. Saari, eds. Bunt and Smut Diseases of Wheat: Concepts and Methods of Disease Management. Mexico,

Oort, A. J. P. 1947. Specialization of lose smut of wheat, a problem for the breeder. Rijdskr.o.Plantez 53: 25-43

Penner, G.A. 1995. RAPD analysis of plant genomes Molecular Breeding 1: 349-354, 1995

Piekenbrock, P. 1927. Untersuchungen über das Verhalten der *Ustilago tritici* an Sorten und Kreuzungen. Kühn Archiv. 15: 411-456.

Rewal, H.S., and **Jhooty, J.S.** 1986. Physiologic specialization of loose smut of wheat in the Punjab state of India. Plant Dis. 70: 228-230.

Sarri, E. E., Mamluk, O.F. and Burnett, P. A. 1996. Bunts and Smuts of Wheat. pp I-II IN

Staub, J.E., Serquen, F. C. And Gupta, M. 1996. Genetic markers, map construction, and their application in plant breeding. Hort Science, Vol. 31 (5), Sept. 1996

Thomas, P. L. 1993. Cereal smut survey, 1993. Can. Plant Dis. Survey 74:70-70

Thomas, P. L. 1995. Cereal Smuts in the Prairie Provinces, 1895 to 1994. Can. J. Plant Pathol. 17: 92-92

Tiemann, A. 1925. Untersuchungen über die Empfänglichkeit des Sommerweizens für *Ustilago tritici*, und der Einfluss der äusseren Bedingungen dieser Krankheit. Kühn

Archiv. 9: 405-467

Voet, D., Voet, J.G. 1990. Biochemistry. Published by John Wiley and Sons Inc. P.184

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting Nucleic Acids Research, Vol. 23, No. 21 4407-4414

Welsh, J., McClelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res. 18: 7213-7218.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Antoni Rafalski, J., Tingey, S.V. 1990. DNA polymorphicms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18: 6531-6535.

Xu, G.W., Gonzales, C.F. 1991. Plasmid, genomic and bacteriocin diversity in U.S. strains of *Xanthomonas campestris* pv. *oryzae*. Phytopathology 81: 628-631.

Zabeau, M. and Vos, P. 1993. European Patent Application, publication no: EP 0534858