Doctoral Thesis

Determination of the relative role of evolutionary forces in the development of epidemics of the plant pathogenic fungus Phaeosphaeria nodorum

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Determination of the relative role of evolutionary forces in the development of epidemics of the plant pathogenic fungus *Phaeosphaeria nodorum*

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presented by

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The world’s population is constantly growing and so is the demand for food and renewable energy. This mounting demand has forced agriculture to increase its productivity without increasing agricultural surfaces. Because pest-derived (e.g. insects, viruses, bacteria and fungal pathogens) agricultural losses may reach one third of the potential production, a sound way of achieving this goal is reducing crop losses through efficient crop protection. Because populations of plant pathogens are continuously evolving, control strategies can sometimes be difficult to implement. To effectively control pathogen damage, and manage the control strategies in a sustainable way, it is therefore important to understand how populations will evolve allowing them to escape the control strategies. Different factors contribute to the pathogen’s evolution. These factors include selection, mutation, gene flow, mating system and random genetic drift. Determining the relative contribution of these forces to a pathogen populations’ structure and epidemiology can contribute to our understanding of its evolutionary potential and improve disease control.

Among the world’s most important agricultural crops (and source of food and energy) is wheat (*Triticum aestivum*). Wheat is the leading cereal traded in the world today, and provides food for nearly 35% of its population. Because of its multiple uses and because of the rapid population growth, demand for wheat will probably grow faster than any other crop in the near future. In the past new and better performing wheat varieties were introduced to satisfy the growing demand. However the narrowed genetic diversity of these high yielding varieties rendered them extremely susceptible to pathogens such as the fungal pathogen *Phaeosphaeria nodorum*. *Phaeosphaeria nodorum* (one of the major wheat pathogens) is a fungal pathogen with a mixed mating system, which is able to infect all above-ground plant parts during the parasitic phase. While the importance of the asexual stage is undisputed, it is not clear what role sexual reproduction plays in the population genetic structure and in the epidemiology of the disease. To shed light on the evolutionary potential and the relative contribution of sexual and asexual reproduction to the population structure and epidemics of *P. nodorum*, we conducted a two year mark-release-recapture field experiment in which we released ten
SUMMARY

genetically marked isolates on four cultivars (with different levels of resistance) and a 1:1 mixture. The experimental part was complemented by an empirical part in which global populations of the pathogen were analyzed.

To investigate the relevance of the sexual and asexual cycle to the epidemics of *P. nodorum* we complemented our two year mark-release-recapture experiment by investigating the frequency and distribution of its mating types. The mating type study revealed that both mating types were found at equal frequencies and at the smallest spatial scales tested; while our two year experiment revealed a within season increase in diversity and the absence of linkage disequilibrium in the populations. Additional isolate assignment tests indicated that most novel isolates recovered in the experimental plots were derived from recombination. The mating type analysis and the mark-release-recapture experiment corroborated the idea that recombination plays an important role in the epidemic development even during the same year as the initial infection. These results also substantiate that the sexually generated and windblown ascospores can serve as primary inoculum and initiate new primary infection in pathogen-free fields at considerable distance.

The same two year mark-release-recapture experiment was used to investigate the evolution of the pathogen in the agricultural environment and to study the effect of host diversity on populations of *P. nodorum*. The findings indicated that genetic diversity and different levels of resistance in the host populations may affect evolutionary rates in pathogen populations. In fact the genetic structure of *P. nodorum* populations sampled from a cultivar mixture did not change over two years and exhibited the lowest variation in selection coefficients among the inoculated isolates, while partially resistant hosts had pathogen populations that evolved more slowly than susceptible hosts. We also found evidence for differential selection on *P. nodorum* during the parasitic and saprophytic phases of its life cycle. These findings support the hypothesis that increasing genetic heterogeneity in host populations will retard the rate of evolution in pathogen populations and that tillage is an effective disease control mechanism.

The findings were complemented by a comparative mitochondrial-nuclear genome analysis aimed at investigating the effects of selection, gene flow, and sexual reproduction on the population genetic structure and evolutionary history of the wheat
pathogen *P. nodorum*. In this study we compared patterns of mitochondrial diversity with patterns of nuclear diversity of fungal isolates from Texas, Oregon, and Switzerland. The results revealed unexpectedly high levels of mitochondrial diversity and the presence of two dominant mtDNA haplotypes present in every region. The comparative analysis revealed high levels of gene flow, low levels of population subdivision, and the absence of host specificity and cyto-nuclear disequilibrium. The concordance in estimates of population genetic parameters from both genomes suggested that both genomes underwent similar evolutionary processes indicating that either of them could be used to
RIASSUNTO

Il recente aumento della popolazione a livello mondiale ha generato un aumento della domanda di cibo e fonti di energie alternative. Questo incremento della domanda impone all’agricoltura di aumentare la produzione, possibilmente senza incrementare la superficie coltivabile. Dato che fino ad un terzo della produzione agricola totale é distrutto dai parassiti, quali i funghi patogeni, un modo efficiente per raggiungere questo obbiettivo é ridurre la perdite dovute a quest’ultimi. I metodi di controllo possono però essere difficili da implementare poiché i parassiti fungini possono evolvere rapidamente annullandone l’efficacia. Per migliorare l’efficacia dei rimedi fitosanitari é quindi necessario conoscere il potenziale evolutivo di questi patogeni. Potenziale evolutivo che é influenzato da cinque fattori quali la pressione di selezione, il grado di mutazione, l’immigrazione, la deriva genetica e il modo di riproduzione. Determinare il contributo di queste forze al potenziale evolutivo e all’epidemiologia della malattia contribuirà a migliorare i metodi di controllo.

Il fungo Phaeosphaeria nodorum é uno dei maggiori patogeni del frumento a livello mondiale. Questo patogeno con sistema riproduttivo misto può infettare tutte le parti aeree della pianta. Mentre l’importanza e il contributo della fase asessuata alla struttura della popolazione e all’epidemiologia di questo patogeno é ben conosciuta, non lo é alquanto quella della fase sessuata, conoscenza che é di fondamentale per determinarne il potenziale evolutivo e quindi migliorare i metodi di prevenzione. Per chiarire il ruolo della fase sessuata, al potenziale evolutivo e all’epidemiologia di P. nodorum, abbiamo condotto un esperimento di due anni (biannuale) nel quale abbiamo rilasciato dieci isolati marcati geneticamente su quattro varietà di frumento con diversi livelli di resistenza. I risultati dell’esperimento sono stati completati da analisi di popolazione a livello globale. Per studiare l’importanza relativa del ciclo sessuato e di quello asessuato alle epidemie di P. nodorum abbiamo analizzato la frequenza e la distribuzione dei loro “mating types” (ideomorfi) a livello globale oltre al esperimento biannuale “in situ” sopra citato. Il nostro studio sulla frequenza e distribuzione dei mating type hanno rivelato che ambo due gli ideomorfi erano presenti in frequenze simili per fino sulla stessa foglia; mentre l’esperimento biannuale ha rivelato che la diversità genetica aumenta durante la stagione.
RIASSUNTO

I test hanno pure rivelato un equilibrio gametico ed assegnato la maggior parte degli isolati con un background genetico diverso da quelli rilasciati alla categoria dei ricombinanti. I risultati dei due studi sostengono l’idea che il ciclo sessuato ha avuto un ruolo importante anche durante la stagione di crescita del frumento. I risultati evidenziano pure l’importanza della ascospore nell’iniziare nuove epidemie.

Lo stesso esperimento biannuale è stato usato per studiare l’evoluzione del patogeno in un ambiente agricolo e di come questo sia influenzato dalla diversità genetica dell’oste. I risultati indicano che la diversità genetica e i livelli di resistenza presenti nell’oste influenzano la popolazione del patogeno. In effetti, la struttura genetica delle popolazioni di P. nodorum campionata delle varietà più resistenti e dall’assemblaggio di varietà indica che la velocità di evoluzione si rallenta con l’aumentare della complessità genetica dell’oste. I risultati hanno pure rivelato che ci sono delle diverse pressioni di selezione durante la fase parassitica e quella saprofita.

Questi risultati sono stati completati da uno studio comparativo condotto tra la diversità genetica presente nel DNA mitocondriale e in quello nucleare incentrato sul scoprire gli effetti che hanno avuto (e che avranno) sulle popolazioni di P. nodorum, la migrazione, le riproduzione sessuata, e la selezione. I risultati dello studio, nel quale sono stati paragonati i livelli di variazione dei due genomi, di popolazioni provenienti dal Texas, dal Oregon a dalla Svizzera, hanno rivelato dei livelli di diversità mitocondriale inaspettatamente alti. I risultati indicano che ci sono state delle importanti migrazioni in passato, che le popolazioni sono molto simili globalmente e che vi è un’assenza d’adattamento ad una varietà specifica. I risultati di equilibrio cyto-nucleare indicano, ancora una volta, che la riproduzione sessuata gioca un ruolo importante nel creare nuova diversità e quindi nel epidemiologia della malattia, dato che le ascospore prodotte sessualmente sono capaci di volare a lunghe distanze e di iniziare un nuovo ciclo infettivo.
CHAPTER 1

General introduction
CHAPTER 1

GENERAL INTRODUCTION:
AGRICULTURE, PATHOGENS AND EVOLUTION

By supplying humanity with food, feed, fiber, fuels and other goods agriculture has played a key role in the development of human civilization. The domestication of plants and animals allowed humans to settle down and give up their previous hunter-gatherer lifestyle during the neolithic revolution. Shifts in agricultural practices occurring after the neolithic revolution allowed to increase agricultural productivity and sustain strong population growth, eventually freeing up a significant percentage of the workforce which helped drive the industrial revolution. In the twentieth century the greatest changes in agriculture occurred during the green revolution. During this period new techniques and improved cultivars increased productivity enough to support further population expansion. In the more recent years the rapid growth of developing countries such as China and India, and the decline in oil reserves have forced agriculture to increase its productivity, again, to satisfy the increasing demand for resources, such as food and alternative energy.

As growing demand must be met primarily by increasing production on land already under cultivation one obvious strategy is to reduce crop losses. Because pest derived (e.g. insects, viruses, bacteria and fungal pathogens) agricultural losses may reach one third of the potential production (Oerke, 2006), efficient crop protection is a sound way to achieve this goal. Pests and diseases can be controlled and managed in a number of ways, which include chemical control (e.g. fungicides), sound agricultural management practices (e.g. cultivar mixtures), biological control (e.g. antagonistic organisms), plant resistance and disease exclusion/escape (e.g. quarantine). Because of the growing environmental concerns the goal is to integrate all of these control methods, putting less emphasis on chemical control and more on biological control such as plant resistance and genetically modified crops. However as populations of plant pathogens in continually adapt the control strategies can some times be difficult to implement. Pathogen may escape chemical control or plant defense mechanisms by developing chemical resistances or new virulences, or may be able to enter new areas because of
their movement potential. To effectively control pathogen damage and manage the different control strategies in a sustainable way, it is therefore important to understand how populations will evolve and try to elude different control strategies.

Many factors contribute to the pathogens’ evolution allowing them to escape the control strategies. These factors include, selection, mutation, gene flow, mating system and random genetic drift (population size) (McDonald, 1997). In fungal populations mutation allows organisms to create new diversity, possibly conferring fitness advantages to certain individuals (e.g. virulence or fungicide resistance) (McDonald and Linde, 2002). Individuals carrying these selective advantages will increase in frequency through natural selection. Gene flow (movement of individuals) will move this new diversity and the selected characters into new areas and populations. In agricultural systems gene flow may occur by movement of infected material such as seed and straw or airborne propagules, and may be affected by the mode of reproduction as the different reproductive strategies can produce propagules with different means of dispersal, which can influence the source of the primary inoculum. Mode of reproduction does not only affect gene flow but also the levels of diversity. While asexual reproduction can rapidly increase the frequency of selected individuals (asexual reproduction) sexual reproduction increases genetic variation of pathogen population by generating new alleles (O’Donnell et al. 2000) or allele combinations, potentially increasing pathogenic fitness (Cowger et al. 2002). The combination of high gene flow potential and increased genetic variation may allow pathogens with sexual reproduction to evolve and adapt more rapidly to changing environments, reducing the life span of fungicides and resistant cultivars (McDonald and Linde 2002). Contrary to the previous evolutionary mechanism, which usually increased genetic diversity and potentially conferred advantageous traits, genetic drift tends to decrease genetic diversity and population fitness. Genetic drift is favored by small population sizes. In agricultural environments it is not uncommon to find small populations or severe reductions in population size (called "bottlenecks"). after crop harvest and founder events (when a new population starts from a small number of individuals). Because of the negative effects of genetic drift any management program that keeps pathogen population sizes small assists disease control (McDonald and Linde, 2002). In plant pathology thus it is important to determine the relative contribution of the
five evolutionary forces to the genetic structure of pathogen populations as it affects disease control and reflects the pathogens evolutionary potential (McDonald, 1997). Knowledge of a pathogens evolutionary potential can improve disease management in agricultural ecosystems improving productivity while reducing crop losses without affecting the environment.
CHAPTER 1

THE HOST

*Triticum aestivum.* Wheat (*Triticum aestivum*) is the leading cereal traded in the world today and provides food for nearly 35% of its population. This cereal, believed to be domesticated 10-12'000 years ago in the area of the Fertile Crescent (Smith, 1994), is widely cultivated as a cash crop because of its good yields, and extreme versatile usage. The grains, in fact, can be used to make flour for bread and pasta; and for fermentation to make beer, vodka, and biofuel. Because of its multiple usage and because of the rapid population growth, demand for wheat will probably grow faster than any other crop (Rajaram, 1999) in the near future.

To satisfy the growing demand wheat yields doubled following the introduction of genetically uniform, high yielding, and fertilizer-responsive wheat varieties (mainly during the green revolution). However the narrowed genetic diversity of these high yielding wheat varieties rendered them extremely susceptible to the effects of epidemics, as, due to its genetic uniformity one virulent pathogen strain has the potential to infect the entire field. Because of their vulnerability, efficient and sustainable crop protection is fundamental to maintain high yields. To achieve effective and sustainable crop protection, though, plant breeders and agronomists will have to improve the knowledge of the evolutionary potential of the pathogen they are dealing with.
THE PATHOGEN *PHAESPHAERIA NODORUM*

**Importance.** *Phaeosphaeria nodorum* is a major wheat pathogen in most wheat-growing areas of the world (Wiese, 1987). The pathogen can cause severe crop and economical damage. In most severe cases the pathogen can cause yield losses of up to 31% (Bhathal et al., 2003). In the past decades, however the relative importance of *P. nodorum* has declined in Europe, but not so in the United States and Australia. In Europe *Septoria tritici*, a pathogen with a similar life cycle to *P. nodorum*, replaced *P. nodorum* as the most important foliar disease of wheat (Eyal, 1999; Hardwick et al., 2001). The shift is to be attributed to differential responses by the pathogens to factors such as cultivars, fungicides employed, and to altered cultural practices or climatic conditions (Eyal, 1999).

**Taxonomy.** The taxonomy and nomenclature of *Stagonospora nodorum* has been importantly revised during the past 100 years. After Berkeley’s description of the asexual stage (*Septoria nodorum*) in 1845 it took more than a century before the sexual fruiting bodies were discovered. The teleomorph of *Stagonospora nodorum* was discovered in 1952 and classified as a species of the *Leptosphaeria* grouping (Müller, 1952). In 1968 Hedjaroude transferred *Leptosphaeria nodorum* to *Phaeosphaeria*. Several subsequent taxonomic studies recognize *Phaeosphaeria nodorum* (E. Müll.) Hedjar. as the teleomorphs of *Stagonospora nodorum* (Shearer, 1977a,b). Today the official name of the pathogen is *Stagonospora nodorum* (Berk.) Castellani and Germano (syn. *Septoria nodorum* Berk.), the anamorph form of *Phaeosphaeria nodorum* (E. Müller) Hedjaroude (syn. *Leptosphaeria nodorum* E. Muller).

**Symptoms and biology.** *Stagonospora nodorum*, which causes leaf and glume blotch on wheat (*Triticum aestivum* L.), is able to infect all above-ground plant parts during the parasitic phase. The major sources of primary inoculum are thought to be asexually infected seeds and crop residue (Bennet et al., 2007), and sexually produced windborne ascospores (Eyal et al., 1999; Sommerhalder et al., 2006). The pathogen
overwinters during the saprophytic stage on infected stubble (Shaner, 1981), where the sexual stage is thought to occur (Cowger and Silva-Rojas, 2006; Shaner, 1981). In the spring the fungus grows on tissues that have been infected in fall or early spring. Secondary spread of the pathogen usually occurs via asexually produced and splash-dispersed pycnidiospores (Shah et al., 2001). These spores contribute to epidemic development for the remainder of the growing season, in particular under wet and warm weather conditions (Arseniuk et al., 1998). It is likely that 2–4 cycles of asexual infection are necessary for the fungus to produce significant disease and, in particular, to infect the wheat heads (Shah and Bergstrom, 2002). Symptoms usually appear 7-14 day after infection as dark brown flecks or spots, often surrounded by a yellow halo. These small irregular lesions expand into oval light brown lesions with dark brown centers. On the wheat heads the lesions begin as either grayish or brownish spots on the chaff, usually on the upper third of the glume. As the lesions enlarge, they become dark brown and the centers turn grayish-white in color as tiny brown pycnidia develop within them. Pycnidia, which are translucent at very early stages, expand to form pale brown growths (Douaiher et al., 2004). The release of pycnidiospores, usually in a mass of pink-pigmented cirrus, is preceded by the swelling of a single point on the pycnidial surface forming a protuberance which ruptures the cuticle. Once the entire leaf collapses and chlorosis is total, the fungus quickly ramifies through the tissue and asexual sporulation begins en masse.

Mode of reproduction. Sexual reproduction in this heterothallic loculoascomycetes requires mating among two genetically distinct fungal isolates carrying alternate forms of mating types (something like male and female in higher organisms), called MAT1-1 and MAT1-2. Once the mating occurred asci carrying eight ascospores are formed. These spores are usually actively dispersed to a distance of 0.5 to 2.0 cm and then dispersed by air currents (Eyal et al. 1987; Halama and Lacoste, 1992). Though it is known that the fungus can undergo sexual reproduction, it is not clear what role sexual reproduction plays in the population genetic structure and the epidemiology of the pathogen. Results from Bathgate and Loughman (2001), Keller et al. (1997a, b) and McDonald et al. (1994) indicated that sexual reproduction plays an important role in the
pathogen’s population genetic structure, and dispersal potential, while others (Bennet et al., 2003) focused on the importance of the asexual stage.

**Population structure.** Over the past decade, the population genetic structure of this fungus has been well characterized by RFLP, microsatellite, and mating-type analyses of its nuclear genome (Bennet et al., 2003; Halama, 2002; Keller et al., 1997a, b; McDonald et al., 1994; Solomon et al., 2004; Sommerhalder et al., 2006; Stukenbrock et al., 2006). The molecular analyses revealed that the fungal populations were characterized by high nuclear diversity distributed on very small spatial scales, high degrees of genetic similarity and low levels of population subdivision (Keller et al., 1997a, b; Stukenbrock et al., 2006) over larger scales. Most populations exhibited high levels of gametic equilibrium and equal mating type frequencies, suggesting the importance of the sexual stage in shaping the population structure (Sommerhalder et al., 2006).

**Control.** A number of control practices, such as crop rotation, tillage, fertilization, disease-free seed, resistant cultivars, cultivar blends and fungicides can be used to control the disease. Because crop residue is one of the most important means of disease carryover crop rotation, burning and tillage are very effective in reducing the disease. Because of the seed-borne nature of *P. nodorum* the use of clean seed will be efficient in helping to prevent the epidemics. Nevertheless effective disease control should integrate the above-mentioned control methods with other cultural practices as windblown ascospores, are a further source of inoculum capable of initiating an epidemic.

Together with the cultural practices fungicide treatment is a critical part of crop management as correct application usually controls the outbreak of *P. nodorum* epidemics. However fungicides are becoming inefficient, are expensive and thus may not always be economically advantageous (Stover et al., 1996). Increase in productivity or at least the enhancement of yield stability can also be achieved through genetic control, which is a more sustainable and economically affordable disease control method. Often, not only is it a valid alternative to the fungicide use, but constitutes the only available
option in many parts of the world. The degree of resistance varies in different cultivars from highly susceptible to moderate (Solomon et al., 2006). It is believed that resistance is inherited as a quantitative trait (Czembor et al., 2003) and that resistance to leaf and glume infection is controlled by different genes (Wicki et al., 1999). Cultivar mixtures and multilines are other valid genetic control methods as they reduce sporulation and apply disruptive selection on the population lowering population fitness.
CHAPTER 1

OBJECTIVES OF THE STUDY

Objectives. In this study we wanted to determine the relative contribution of the evolutionary forces to the development of epidemics and population structure of the fungal wheat pathogen *P. nodorum*. The knowledge drawn from these experiments should illuminate some general aspects of evolutionary biology and epidemiology, which should be applicable to other agricultural parasites with life cycles similar to *P. nodorum*. The specific objectives were: i) to determine the main source of primary inoculum; ii) to measure the relative contribution of sexual, asexual reproduction and immigration to the genetic structure of *P. nodorum*; iii) to determine the geographical distribution of mating type idiomorphs; and iv) to investigate the effects of host genotypes on the genetic structure of *P. nodorum* populations.

Chapter 2. In the first study (chapter 2) we analyzed the frequency and distribution of *P. nodorum* mating types to investigate the importance of sexual reproduction in the population genetics and epidemiology of this pathogen. The outcome of the mating type analysis was accompanied by other population genetic data such as measures of genotypic diversity, measures of gametic equilibrium/disequilibrium and direct observation of the teleomorph. The consequences of equal mating type frequencies, high genetic diversity and genetic connectivity were discussed.

Chapter 3. In the second study (chapter 3) we conducted a two-year mark-release-recapture experiment to investigate the relative contribution of sexual and asexual reproduction to the genetic structure of *P. nodorum*. To conduct this experiment and initiate the epidemic we released ten *P. nodorum* isolates marked with 11 microsatellite markers onto five replicated host populations. The relative importance of sexual and asexual reproduction to the population genetic structure of *P. nodorum* was discussed.
Chapter 4. In the third study (chapter 4) the same two-year mark-release-recapture experiment was used to investigate the effect of host resistance and cultivar mixtures on the rate of pathogen evolution, and the different selection pressures occurring during the parasitic and saprophytic phases. The effect of host resistance and differential selection during the saprophytic and parasitic phase were discussed.

Chapter 5. In fourth experiment (chapter 5) we conducted a comparative analysis using the mitochondrial genome and the nuclear genome to investigate the effects of selection, gene flow, and sexual reproduction on the population genetic structure and evolutionary history *P. nodorum*. The effects of the five evolutionary forces on both genomes were discussed.
CHAPTER 1

LITERATURE


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The frequencies and spatial distribution of mating types in *Stagonospora nodorum* are consistent with recurring sexual reproduction.
ABSTRACT

To test the hypothesis that *Stagonospora nodorum* undergoes regular cycles of sexual recombination, a total of 1207 isolates sampled from 18 fields in 12 geographical regions in six countries on four continents were analyzed for mating type frequency and distribution using PCR amplification of the mating type locus. RFLP and RAPD fingerprints were used to clone-correct the data sets. Both mating types were often found on the smallest spatial scales tested, including within the same lesion, the same leaf, and the same 1 m$^2$ plot. In only one case out of the 18 fields tested was there a significant departure from the expected 1:1 ratio. Combining this result with previous data on the population structure of *S. nodorum*, we conclude that this pathogen undergoes regular cycles of sexual recombination in all regions we examined.

*Sommerhalder, R. J., McDonald, B. A, and Zhan, J. 2006.*
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INTRODUCTION

The heterothallic loculoascomycete *Stagonospora nodorum* (Berk.) Castellani and Germano (syn. *Septoria nodorum* Berk.), the anamorph form of *Phaeosphaeria nodorum* (E. Müller) Hedjaroude (syn. *Leptosphaeria nodorum* E. Müller) causes *Stagonospora nodorum* glume blotch on wheat (*Triticum aestivum* L.), a major disease of wheat globally (Eyal, 1999; Eyal et al., 1987; King et al 1983). It is able to infect all above-ground plant parts and overwinters in the field on infected stubble (Shaner, 1981) where the sexual stage occurs (Halama, 2002; Halama and Lacoste, 1991). Teleomorph fruiting bodies have been detected in Australia (Bathgate and Laughman, 1994), Europe (Arseniuk et al., 1998a; Mittelstädt and Fehrman, 1987), North Carolina (Cowger and Silva-Rojas, 2004), Oregon (C. Cowger, personal communication), Texas (B. McDonald, unpublished data), and western Canada (Duczek et al., 1999), but have not been detected in Georgia (Cunfer, 1998) or New York (Bennet et al., 2003) although both mating types were found in these locations. These observations suggest that the ability of the fungus to undergo sexual reproduction might vary among locations (Shaw, 1999).

Though it is known that the fungus can undergo sexual reproduction, it is not clear what role sexual reproduction plays in the population genetic structure of the pathogen and the epidemiology of the disease. Results from Bathgate and Loughman (1994), Keller et al. (Keller et al., 1997a, b) and McDonald et al. (1994) indicated that sexual reproduction plays an important role in the pathogen’s population genetic structure, and the authors suggested that windborne ascospores are the main source of primary inoculum. Other studies argued against the importance of the sexual stage (Bennet et al., 2003) and hypothesized that the seed-borne pycnidiospores were the main source of primary inoculum (Arseniuk et al., 1998b; Shah et al., 1995). In either case, it is clear that following primary infection, asexually produced, rain-splashed pycnidiospores contribute to epidemic development during the remainder of the growing season, in particular under wet and warm weather conditions (Arseniuk et al., 1998a; Mittelstädt and Fehrman1987).
Knowledge of the extent of sexual reproduction and its contribution to primary inoculum is needed to understand the evolutionary potential and epidemiology of pathogens. Sexual populations may have evolutionary advantages over asexual populations because more fit genotypes can arise rapidly through recombination (Brassier et al., 1999; Brown, 1999; Milgroom 1996). If the sexual spores (ascospores in this case) are wind-dispersed, they have a greater potential for long distance gene flow, and therefore can move selected characters such as virulence or fungicide resistance efficiently over large areas. Increased genetic variation combined with high gene flow potential increases the ability of a pathogen to evolve rapidly in response to changing environments, and therefore can reduce the useful life span of fungicides or cultivars carrying single major resistance genes. On the other hand, if the pathogen is limited to asexual reproduction and the main source of primary inoculum is seed-borne or splash-dispersed pycnidiospores, the risk of the spread of new pathotypes among neighboring populations would be low. Under this scenario, the increase of genetic diversity would be limited to mutations within a limited number of genetic backgrounds (clonal lineages), and gene flow over longer distances would have to be man-mediated. In this case the spread of the pathogen could be more easily limited with appropriate control measures, such as using clean seeds and crop rotations.

Sexual reproduction in heterothallic loculoascomycetes such as *S. nodorum* requires the presence of two genetically distinct fungal isolates carrying alternate forms of mating types, called *MAT1*-1 and *MAT1*-2, at the same geographical locations. These alternate forms are termed idiomorphs instead of alleles because they lack significant sequence similarity (Coppin et al., 1997; Metzenberg and Glass, 1990; Pöggler, 2001; Turgeon and Yoder, 2000). Each idiomorph carries a single regulatory gene that encodes proteins with confirmed or putative DNA binding motifs (Pöggler, 2001). The *MAT1*-1 idiomorph of loculoascomycetes encodes a DNA binding protein containing an alpha (α) domain, while *MAT1*-2 encodes a DNA binding protein containing a high mobility group (HMG) domain (Coppin et al., 1997; Pöggler, 2001; Turgeon and Yoder, 2000).

The hypothesis that populations of *S. nodorum* undergo regular cycles of sexual reproduction could be tested by studying the frequencies and distributions of the two mating types in field populations. In populations where sexual reproduction is common,
ascospores could be a significant source of primary inoculum. If the pathogen population within a field undergoes regular cycles of sexual reproduction and ascospores are a major source of primary inoculum, then both mating types should occur at equal frequencies within the field as a consequence of frequency-dependent selection operating on MAT genes (Milgroom, 1996). Skewed ratios, on the other hand, would be consistent with rarity of sexual reproduction, and in the case of S. nodorum, with a major role played by seedborne inoculum. This approach has been successfully applied to infer the importance of sexual recombination for several other fungi (Barve et al., 2003; Dayakar et al. 2000; Dyer et al. 2001; Linde et al., 2003; Zhan et al., 2002). The outcome of the test can be strengthened by combining the results with other population genetic data such as measures of genotypic diversity, measures of gametic equilibrium/disequilibrium and direct observation of the teleomorph.

Previous reports on mating type frequencies of S. nodorum were inconsistent. Halama (Halama, 2002) found skewed mating type frequencies using in vitro crosses against known tester strains to assign the mating type. In Halama’s test, 56 of 101 isolates were assigned to MAT1-1, 5 to MAT1-2, and the remaining 40 isolates could not be assigned to a specific mating type. Solomon et al. (2004) found that frequencies of the two mating types in 23 isolates sampled from Western Australia did not deviate significantly from the 1:1 ratio while Bennett et al. (2003) found both skewed and equal mating type ratios in New York. Earlier studies on mating type frequencies of S. nodorum were either based on relatively small sample sizes or lacked genotype information. Small sample sizes lead to a low statistical confidence and lack of genotype information could lead to an overrepresentation of one or more common clonal genotypes, thus biasing the results (Zhan et al., 2002).

The objectives in this experiment were to determine the frequencies and hierarchical distribution of mating types over spatial scales ranging from lesions to continents to test the hypothesis that S. nodorum populations undergo regular cycles of recombination and to determine if the same pattern would exist across a large number of fields and environments representing major wheat-production areas on four continents.
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MATERIALS AND METHODS

Fungal isolates. A total of 1207 *S. nodorum* isolates collected from 18 fields in 12 geographical regions in six countries on four continents were included in this study. Most collections were made following a hierarchical sampling strategy (McDonald et al., 1999) from naturally infected flag leaves or heads at a single point in time (Table 1). With the exception of the Swiss populations, single or multiple pycnidial isolations were made from each of ten infected leaves collected at each sampled site (plot) within the field. The Australia collection was made from a single field located north of Norragin following an eight-site hierarchical sampling strategy. The total area covered by this sampling strategy was ~300 m$^2$. Swiss isolates were collected from nine fields in three locations, each of them planted with one of three wheat cultivars (Arina, Galaxy, and Tamaro; Keller et al., 1997a). Only five infected flag leaves were collected at each plot within these fields. The total sampling area covered for each Swiss field was approximately 400 m$^2$ and the geographic distances among these fields ranged from 0.1 km to 9.0 km. The North Dakota collection was made in Fargo following a six-site hierarchical sampling scheme. The total area covered in this field was approximately 200 m$^2$. Hierarchical sampling strategies were also used for Oregon and Texas. The isolates from Oregon were collected from flag leaves and heads from three sites in a field in Hyslop. In Overton, Texas, collections were made at three different plots in two separate fields as previously described (McDonald et al., 1994). The New York collection was made from naturally infected flag leaves sampled along parallel transects in a single field located at the Ithaca experiment station, covering an area of 400 m$^2$. The Chinese collection was made from a farmer's field located at Louyuan in the east part of China. The infected leaves were collected at 1 m intervals along eight 15-meter long transects; representing a total area of approximately 105 m$^2$. In Arkansas isolations were made from seedlings grown from infected seed lots grown in a greenhouse or directly from infected seed. Mexican isolates were collected from a CIMMYT disease nursery in Patzcuaro. The South Africa population was collected at random from an area of 10,000 m$^2$ located in the Gouda area (South-Western Cape).
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DNA extraction and PCR amplification of mating type idiomorphs. DNA extraction of the American populations was as described previously by McDonald et al. (McDonald et al., 1994). DNA of Australian, Chinese, Swiss, and South African populations was extracted using DNeasy Plant Mini DNA extraction kit (Qiagen, GmbH, Hilden, Germany) following the manufacturer’s instructions. Mating types of the fungal strains were determined by PCR amplification using four mating-type-specific primers according to Bennett et al. (6) (Figure 1). The combination of primers 5’- CTT CAC GAC CGG CCA GAT AGT - 3’ (F) and 5’- CAG AGG CTT GTC GGG TTC AT - 3’ (R) amplified a ~360 bp fragment in MAT1-1 isolates. A ~510 bp fragment was amplified in MAT1-2 isolates by using the combination of primers 5’- ACC CCG CCC CAT GAA CAA GTG - 3’(F) and 5’- CTA GAC CGG CCC GAT CAA GAC CAA AGA AG - 3’(R). PCR amplifications were performed using a mixture of all four primers (multiplex PCR) and were carried out in 96 well plates (Greiner) using a Biometra thermocycler. The total PCR reaction volume was 20 µl per well, consisting of 1 µl (10 µM) of each primer, 1 µl (2 mM) of dNTPs (dATP, dCTP, dTTP, and dGTP), 2 µl of 10x reaction buffer, 1 U of Taq polymerase (Amersham Pharmacia Biotech), and 5 µl of re-suspended S. nodorum genomic DNA (5-10 ng final DNA concentration). Thermal cycling conditions were as follows: initial hold at 96 °C for 2 min, 39 cycles of 96 °C for 1 min, 59 °C for 1 min, 72 °C for 1 min. Finally, the products were incubated for 5 min at 72 °C. Amplicons were separated by agarose gel electrophoresis (1% gel, 0.5 x TBE) at 80 V for 3 hours and visualized by staining the gels with ethidium bromide.

Genotype determination All populations, apart from the Australian and the Chinese, were clone-corrected using RFLP markers. Seven single locus probes and one fingerprinting probe (pSNS4) were used for the clone-correction (McDonald et al., 1994; Keller et al., 1997b). Combined results from two RAPD primers (9442 and 9498) (Czembor and Arseniuk, 1999) were used to clone-correct populations from China and Australia because the RFLP fingerprinting probe pSNS4 was no longer available. The RAPD PCR conditions were as described by Czembor and Arseniuk (1999). Isolates with the same multi-locus haplotypes (MLHT), RFLP fingerprints and/or RAPD fingerprints
were considered clones. The clonal fraction for each sampling unit was calculated (Zhan et al., 2002) by dividing the number of clones by the total number of isolates.

**Data analysis.** Hierarchical pooling of the isolates enabled us to determine the frequency distribution of mating types across different spatial scales, including within lesions, leaves, field plots, fields, locations, and countries. Frequencies of mating types were calculated using both clone-corrected and uncorrected data. For clone-corrected data, only one representative haplotype per clone was included in the analysis. A $\chi^2$-test (Ott, 1999) was applied to determine whether the frequencies of the two mating types departed from the null hypothesis of a 1:1 ratio within sampling units. Heterogeneity in mating type frequencies among sampling units was evaluated using a contingency $\chi^2$-test (Everitt, 1977). Only isolates with complete mating type and fingerprint data were included in the analysis.
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RESULTS

**Multiplex PCR.** A total of 1207 fungal isolates were assayed for mating type frequencies, following PCR amplifications of part of the α-domain and HMG-box with specific primers. Among these isolates, 1080 produced a single amplicon of ~550 or ~360 bp corresponding either to the *MAT1-1* or the *MAT1-2* idiomorph (Table 2). Four isolates produced a 100 bp amplicon in addition to the *MAT1-2* amplicon. Eight isolates produced both *MAT1-1* and *MAT1-2* amplicons, possibly due to mixed cultures or contamination during preparation of microtiter plates. These latter 12 isolates were excluded from the analysis of mating type frequencies. 115 isolates did not produce any amplicons, probably due to low DNA concentrations or DNA degradation. Of the 1080 isolates that produced single amplicon of the expected sizes, DNA fingerprint (RFLP or RAPD) and multilocus haplotype (MLHT) data were available for 914 (75.7%) of these isolates. A total of 814 distinct genotypes were identified among these 914 isolates. The clonal fractions for most populations were low (Table 3).

**Spatial distribution of mating types within and among leaves.** It was common to find both mating types among isolates sampled from the smallest spatial scales, including different pycnidia within the same lesion, different lesions on a leaf or different kernels on the same head. Both mating types were found in four of 24 cases where isolations were made from different pycnidia within the same lesion. In 55 cases across 5 geographical regions (China, Mexico, North Dakota, Oregon, and Texas) where isolations were made from different lesions on the same leaf, 23% (12 out of 55) were occupied by both mating types and about 98% (54 out of 55 leaves) were occupied by different genotypes. In the population sampled from Oregon, 20% (3 of 15) of heads were infected by both mating types.

**Spatial distribution of mating types among plots within fields.** In the 104 1 m² plots sampled from four countries or states across 14 fields, both mating types were found in
all but 13 of the 1 m² plots (Table 4). In the Australian population, both mating types were found in all plots. Both mating types were found in 87% (78 of 90) of the plots in Swiss populations. In Oregon both mating types were found in all six plots. In Texas, eight of nine sites, located in three fields at two locations, had both mating types (Table 4).

Spatial distribution of mating types among fields and regions within countries. Different fields from the same geographical regions in Switzerland and Texas were analyzed for mating type frequencies and tested for deviation from the null hypothesis of a 1:1 ratio. In Switzerland χ² analysis revealed no significant differences in the frequencies of two mating types within and among fields (Table 5) and locations (Table 6). In Texas both mating types were identified in the fields at both locations and χ² tests revealed no difference in mating type frequencies within and among locations.

Spatial distribution of mating types among countries and continents. With the exception of Oregon, the null hypothesis of a 1:1 ratio between the two mating types was not rejected at regional and continental scales when clone-corrected data were used (Table 3). In Oregon, the frequencies of the two mating types deviated significantly from the expected 1:1 ratio (χ² =3.95, P < 0.05). The null hypothesis was rejected in 5 out of 11 populations when data were not clone-corrected (Table 3).
DISCUSSION

The primary objective of this study was to analyze the frequency and distribution of *S. nodorum* mating types in order to gain insight into the role of sexual reproduction in the population genetics and epidemiology of this pathogen. We achieved this objective by using a large collection of genotyped strains sampled hierarchically from 18 wheat fields on four continents. We found that both mating types were present at all spatial scales and were at similar frequencies in the clone-corrected datasets. It was common to find both mating types in the same lesion and the same leaf when multiple isolations were made, suggesting that close physical proximity of the two mating types is common in agricultural fields. The close proximity of opposite mating types increases the likelihood that the teleomorph will form. Most of the sampled plots and fields did not depart significantly from the null hypothesis of 1:1 ratio. The finding of skewed ratios between the two mating types in five populations when data were not clone-corrected does not contradict our null hypothesis. Rather, this finding illustrates the importance of genotyping the strains to identify clones that would otherwise distort the analysis. Most of the samples with skewed ratios in the uncorrected datasets had the highest clonal fractions.

In only one case (Oregon) was there a significant departure from a 1:1 ratio of the two mating types in a clone-corrected dataset. Several processes can explain this finding. Selection (e.g. due to a fungicide application or a resistant cultivar) that favors one or the other idiomorph, may affect the frequency and distribution of mating types. It has been reported that mating type genes may be associated with other functions, such as cellular processes (Kunz, and Haynes, 1981), maintenance of cell wall integrity (Verma and Ballaster, 1999), and virulence (Kronstad and Staben, 1997; Kwong-Chung et al., 1992). It is possible that the products of the mating type genes may affect pathways influencing ecological functions, such as fungicide resistance or that these genes are linked with other genes that confer fungicide resistance. Another possibility is that the significant differences found in Oregon were due to a false rejection of the null hypothesis (Type I error, Ott, 1999). As the probability of the Type I error for tests conducted at the $P = 0.05$
level is 0.05, the likely number of falsely rejected null hypotheses in our 25 tests (Tables 3, 5, 6) is 1.3, which is similar to what we obtained.

Our results are consistent with findings made by Solomon et al. (Solomon et al., 2004) who found 9 MATI-1 and 14 MATI-2 strains in a sample of 23 isolates, and partially in agreement with findings made in New York (2003) where clone-corrected field samples from Niagara County (22 single haplotypes) and Cayuga County (32 single haplotypes) were analyzed. A $\chi^2$ test showed that frequencies did not deviate significantly from 1:1 mating type ratio in Cayuga County ($\chi^2 = 0.32, P > 0.05$) but were different in Niagara County ($\chi^2 = 8.91, P < 0.05$). Deviation from the expected 1:1 ratio in this case could be due to selection (e.g. due to a fungicide application) or to non-representative sampling.

Compared to earlier studies (Bennet et al., 2003; Halama, 2002; Shaner et al., 1981), we used larger sample sizes and many more field collections to increase the statistical confidence of the results. We also clone-corrected the dataset to eliminate the possible overrepresentation of any clones present in pycnidal collections. The use of the PCR-technique allowed us to avoid the use of in vitro crosses to determine the mating types. The results from in vitro crosses may be affected by unsuccessful mating due to isolate-specific infertility and/or media-selection. Finally, the hierarchical sampling method we employed enabled us to analyze the geographical distribution of mating types over spatial scales ranging from 1 m$^2$ plots to continents, allowing us to show that the 1:1 ratio existed at all spatial scales and across many different environments and regions on four continents.

The finding of both mating types at all spatial scales coupled with an even distribution among all field populations suggest this fungus undergoes regular cycles of sexual reproduction. If the sexual cycle is occurring regularly, it is likely that the ascospores are making a significant contribution as a source of primary inoculum in epidemics of Stagonospora nodorum glume blotch. It has been hypothesized that equal mating type frequencies are mediated by frequency-dependent selection, which is a type of balancing selection that favors rare genotypes (Santos et al., 1986). Under frequency-dependent selection, the fitness of a phenotype is dependent on the relative frequency of other phenotypes in the population. In the case of skewed mating type ratios, frequency-
dependent selection will decrease the frequency of the dominant idiomorph as it will be less likely to find a mate, and will increase the frequency of the less frequent idiomorph until the probability of finding a compatible mate is maximized. The 1:1 ratio of mating types observed throughout the sampled locations clearly demonstrates the likelihood of recurrent sexual reproduction. Based on our data, we hypothesize that sexual reproduction occurs at least once a year in most locations. But we recognize that the absence of functional mating types or favorable environmental conditions may preclude recurrent sexual reproduction in some areas.

The hypothesis of recurrent sexual reproduction in *S. nodorum* also is consistent with high levels of genotypic diversity found in this fungus both in current and previous studies (Keller et al., 1997a; McDonald et al., 1994; Murphy et al., 2000). The finding of gametic equilibrium among neutral loci (Keller et al., 1997a) is a further indication that sexual reproduction is a major factor influencing the population genetic structure and epidemiology of *S. nodorum*. Two previous investigations (Keller et al., 1997b; Shah et al., 1995) showed that when clones were found, they were clustered in small areas within the field. These findings are also consistent with the hypothesis that the primary inoculum of *S. nodorum* is sexually produced ascospores. Under this scenario, the ascospores land on wheat and initiate the infection and then conidia are produced as secondary inoculum that is dispersed locally by rain splash, leading to clusters of the same genotypes in a small spatial area. If the fungus lacked sexual reproduction and the main source of primary inoculum were asexual pycnidiospores and/or infected seeds as proposed by other investigators (Bennet et al., 2003; Cunfer, 1978; Shah and Bergstrom, 1993; Shah and Bergstrom, 2000), we would be more likely to find the two mating types at unequal frequencies and a higher degree of clonality within field populations due to a combination of genetic drift and selection. It was proposed that mutations in other genes or environmental factors that limit sexual reproduction could explain inability to find the teleomorph in some locations (Bennet et al., 2003). Other possible explanations for not finding the teleomorph in these locations include inappropriate sampling methodology, examining too little plant material, or collecting plant material at the wrong point in the disease cycle.
Upon combining our findings of a 1:1 ratio of the two mating types at all spatial scales and in all locations surveyed, high genotypic diversity, random association among alleles among neutral loci, and the identification of the teleomorph (Bathgate and Laughman, 1994; Cowger and Silva-Rojas, 2004; Pedersen and Hughes, 1993), we conclude that *S. nodorum* populations undergo regular cycles of sexual reproduction. We acknowledge that seed-borne inoculum can contribute to the primary inoculum, and it is likely to be especially important as a mechanism for long-distance movement (gene flow) of the pathogen among regions and continents. But given our findings, we consider it more likely that the main primary inoculum of *S. nodorum* disease is airborne ascospores. If this is the case, clean seed and crop rotation may not be sufficient to prevent primary inoculum from entering a wheat field. We are presently conducting field experiments with mark-release-recapture techniques to quantify directly the amount of primary inoculum coming from the different possible sources.
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AKNOWLEDGEMENTS

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Table 1. Description of *Stagonospora nodorum* populations used in this study.

<table>
<thead>
<tr>
<th>Population</th>
<th>Year of collection</th>
<th>Number of fields</th>
<th>Area sampled (m²)</th>
<th>Collection method</th>
<th>Sample size</th>
<th>Clone-corrected sample size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arkansas</td>
<td>1995</td>
<td>--</td>
<td>--</td>
<td>Greenhouse and seed lots</td>
<td>142</td>
<td>100</td>
</tr>
<tr>
<td>Australia</td>
<td>2001</td>
<td>1</td>
<td>~300</td>
<td>Hierarchical</td>
<td>64</td>
<td>59</td>
</tr>
<tr>
<td>China</td>
<td>2001</td>
<td>1</td>
<td>~105</td>
<td>Parallel transects</td>
<td>100</td>
<td>84</td>
</tr>
<tr>
<td>Mexico</td>
<td>1993</td>
<td>1</td>
<td>~400</td>
<td>Random unordered</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td>New York</td>
<td>1991</td>
<td>1</td>
<td>~400</td>
<td>Parallel transects</td>
<td>21</td>
<td>15</td>
</tr>
<tr>
<td>North Dakota</td>
<td>1993</td>
<td>1</td>
<td>~200</td>
<td>Hierarchical</td>
<td>18</td>
<td>9</td>
</tr>
<tr>
<td>Oregon</td>
<td>1993</td>
<td>1</td>
<td>~60</td>
<td>Hierarchical</td>
<td>57</td>
<td>57</td>
</tr>
<tr>
<td>South Africa</td>
<td>1995</td>
<td>1</td>
<td>~10 000</td>
<td>Random unordered</td>
<td>45</td>
<td>25</td>
</tr>
<tr>
<td>Switzerland</td>
<td>1994</td>
<td>9</td>
<td>~3600</td>
<td>Hierarchical</td>
<td>410</td>
<td>410</td>
</tr>
<tr>
<td>Texas</td>
<td>1992</td>
<td>2</td>
<td>~100</td>
<td>Hierarchical</td>
<td>43</td>
<td>42</td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
<td>--</td>
<td>--</td>
<td></td>
<td>914</td>
<td>814</td>
</tr>
</tbody>
</table>
Table 2. Summary of results from PCR amplification of the mating type idiomorphs in a global collection of the wheat pathogen *Stagonospora nodorum*.

<table>
<thead>
<tr>
<th>PCR products</th>
<th>Number of isolates</th>
<th>Proportions</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>MAT1-1</em></td>
<td>446</td>
<td>0.37</td>
</tr>
<tr>
<td><em>MAT1-2</em></td>
<td>468</td>
<td>0.39</td>
</tr>
<tr>
<td>Both <em>MAT1-1</em> and <em>MAT1-2</em></td>
<td>8</td>
<td>0.01</td>
</tr>
<tr>
<td><em>MAT1-2</em> and 100 bp amplicon</td>
<td>4</td>
<td>0.00</td>
</tr>
<tr>
<td>No product</td>
<td>115</td>
<td>0.10</td>
</tr>
<tr>
<td>No fingerprint</td>
<td>166</td>
<td>0.14</td>
</tr>
<tr>
<td>Total</td>
<td>1207</td>
<td>1.00</td>
</tr>
</tbody>
</table>
Table 3. Mating type frequencies of *Stagonospora nodorum* in populations from Arkansas, Australia, China, Mexico, New York, North Dakota, Oregon, South Africa, Switzerland, and Texas.

<table>
<thead>
<tr>
<th>States/ Countries</th>
<th>Clone-corrected sample size</th>
<th>Clonal fraction</th>
<th>MAT1-1</th>
<th>MAT1-2</th>
<th>Within regions clone-corrected data</th>
<th>Within regions uncorrected data</th>
<th>Among regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arkansas</td>
<td>100</td>
<td>0.30</td>
<td>0.49</td>
<td>0.51</td>
<td>0.04</td>
<td>4.06*</td>
<td></td>
</tr>
<tr>
<td>Australia</td>
<td>59</td>
<td>0.08</td>
<td>0.37</td>
<td>0.63</td>
<td>3.82</td>
<td>4.00*</td>
<td></td>
</tr>
<tr>
<td>China</td>
<td>84</td>
<td>0.16</td>
<td>0.56</td>
<td>0.44</td>
<td>1.19</td>
<td>2.56</td>
<td></td>
</tr>
<tr>
<td>Mexico</td>
<td>13</td>
<td>0.07</td>
<td>0.31</td>
<td>0.69</td>
<td>1.92</td>
<td>2.57</td>
<td></td>
</tr>
<tr>
<td>New York</td>
<td>15</td>
<td>0.29</td>
<td>0.47</td>
<td>0.53</td>
<td>0.06</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>North Dakota</td>
<td>9</td>
<td>0.50</td>
<td>0.56</td>
<td>0.44</td>
<td>0.11</td>
<td>5.56*</td>
<td></td>
</tr>
<tr>
<td>Oregon</td>
<td>57</td>
<td>0.00</td>
<td>0.63</td>
<td>0.37</td>
<td>3.95*</td>
<td>3.95*</td>
<td></td>
</tr>
<tr>
<td>South Africa</td>
<td>25</td>
<td>0.44</td>
<td>0.44</td>
<td>0.56</td>
<td>0.36</td>
<td>6.42*</td>
<td></td>
</tr>
<tr>
<td>Switzerland</td>
<td>410</td>
<td>0.00</td>
<td>0.51</td>
<td>0.49</td>
<td>0.04</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Texas</td>
<td>42</td>
<td>0.02</td>
<td>0.43</td>
<td>0.57</td>
<td>0.86</td>
<td>1.14</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>814</td>
<td>0.11</td>
<td>0.49</td>
<td>0.51</td>
<td>0.01</td>
<td>0.53</td>
<td>12.34 (8)*</td>
</tr>
</tbody>
</table>

* indicates mating type frequencies which are significantly different at $P = 0.05$

* number in parenthesis indicates the degrees of freedom
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Table 4. Distribution of *Stagonospora nodorum* mating types among 1 m$^2$ plots.

<table>
<thead>
<tr>
<th>Population</th>
<th>Number of plots</th>
<th>Plots with a missing mating type</th>
<th>Average clonal fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australia</td>
<td>8</td>
<td>0</td>
<td>0.01</td>
</tr>
<tr>
<td>Switzerland 1</td>
<td>9</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>Switzerland 2</td>
<td>9</td>
<td>3</td>
<td>0.00</td>
</tr>
<tr>
<td>Switzerland 3</td>
<td>9</td>
<td>1</td>
<td>0.00</td>
</tr>
<tr>
<td>Switzerland 4</td>
<td>9</td>
<td>1</td>
<td>0.00</td>
</tr>
<tr>
<td>Switzerland 5</td>
<td>9</td>
<td>1</td>
<td>0.00</td>
</tr>
<tr>
<td>Switzerland 6</td>
<td>9</td>
<td>1</td>
<td>0.00</td>
</tr>
<tr>
<td>Switzerland 7</td>
<td>9</td>
<td>1</td>
<td>0.00</td>
</tr>
<tr>
<td>Switzerland 8</td>
<td>9</td>
<td>3</td>
<td>0.00</td>
</tr>
<tr>
<td>Switzerland 9</td>
<td>9</td>
<td>1</td>
<td>0.00</td>
</tr>
<tr>
<td>Oregon</td>
<td>6</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>Texas 1</td>
<td>2</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>Texas 2</td>
<td>2</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>Texas 3</td>
<td>5</td>
<td>1</td>
<td>0.03</td>
</tr>
<tr>
<td>Total</td>
<td>104</td>
<td>13</td>
<td>--</td>
</tr>
</tbody>
</table>
Table 5. *Stagonospora nodorum* mating type distribution in field collections from Switzerland and Texas, USA.

<table>
<thead>
<tr>
<th>Population</th>
<th>Clone-corrected sample size</th>
<th>Clonal fraction</th>
<th>MAT1-1</th>
<th>MAT1-2</th>
<th>χ² values&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Within fields</td>
<td>Among fields</td>
<td></td>
</tr>
<tr>
<td>Swiss 1</td>
<td>47</td>
<td>0.00</td>
<td>0.51</td>
<td>0.49</td>
<td>0.02 (9)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Swiss 2</td>
<td>45</td>
<td>0.00</td>
<td>0.56</td>
<td>0.43</td>
<td>0.82 (9)</td>
</tr>
<tr>
<td>Swiss 3</td>
<td>46</td>
<td>0.00</td>
<td>0.53</td>
<td>0.47</td>
<td>0.20 (9)</td>
</tr>
<tr>
<td>Swiss 4</td>
<td>45</td>
<td>0.00</td>
<td>0.42</td>
<td>0.58</td>
<td>1.09 (9)</td>
</tr>
<tr>
<td>Swiss 5</td>
<td>46</td>
<td>0.00</td>
<td>0.59</td>
<td>0.41</td>
<td>1.39 (9)</td>
</tr>
<tr>
<td>Swiss 6</td>
<td>45</td>
<td>0.00</td>
<td>0.42</td>
<td>0.58</td>
<td>1.09 (9)</td>
</tr>
<tr>
<td>Swiss 7</td>
<td>50</td>
<td>0.00</td>
<td>0.48</td>
<td>0.52</td>
<td>0.08 (9)</td>
</tr>
<tr>
<td>Swiss 8</td>
<td>44</td>
<td>0.00</td>
<td>0.63</td>
<td>0.37</td>
<td>3.13 (9)</td>
</tr>
<tr>
<td>Swiss 9</td>
<td>43</td>
<td>0.00</td>
<td>0.38</td>
<td>0.62</td>
<td>2.38 (9)</td>
</tr>
<tr>
<td>Texas 1</td>
<td>10</td>
<td>0.00</td>
<td>0.30</td>
<td>0.70</td>
<td>1.60 (4)</td>
</tr>
<tr>
<td>Texas 2</td>
<td>5</td>
<td>0.00</td>
<td>0.40</td>
<td>0.60</td>
<td>--&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Texas 3</td>
<td>25</td>
<td>0.04</td>
<td>0.52</td>
<td>0.48</td>
<td>0.04 (1)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Test on clone-corrected data

<sup>b</sup> Number in parenthesis indicates the degrees of freedom

<sup>c</sup> -- indicates sample size too small to perform χ² analysis
Table 6. *Stagonospora nodorum* mating type distribution among three Swiss locations separated by less than 10 km.

<table>
<thead>
<tr>
<th>Location</th>
<th>Clone-corrected sample size</th>
<th>Clonal Fraction</th>
<th>Frequencies</th>
<th>( \chi^2 ) values within fields(^a)</th>
<th>Among fields(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location CH1</td>
<td>136</td>
<td>0.00</td>
<td>0.54</td>
<td>0.46</td>
<td>0.74 (2)</td>
</tr>
<tr>
<td>Location CH2</td>
<td>136</td>
<td>0.00</td>
<td>0.48</td>
<td>0.52</td>
<td>0.26 (2)</td>
</tr>
<tr>
<td>Location CH3</td>
<td>138</td>
<td>0.00</td>
<td>0.50</td>
<td>0.50</td>
<td>0.00 (2)</td>
</tr>
</tbody>
</table>

\(^a\) test on clone-corrected data

\(^b\) number in parenthesis indicates the degrees of freedom
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Fig. 1. PCR amplification with mating type specific primers of 8 randomly chosen Swiss isolates of *S. nodorum*. The ~510 bp amplicon corresponds to the *MAT1-2* idiomorph while the ~360 bp amplicon corresponds to the *MAT1-1* idiomorph. First and last lanes are a 100 bp size latter.
CHAPTER 3

Sexual recombinants make a significant contribution to epidemics caused by the wheat pathogen *Phaeosphaeria nodorum*
CHAPTER 3

ABSTRACT

We conducted a two year mark-release-recapture experiment to quantify the relative contributions of sexual and asexual reproduction to epidemics of Stagonospora nodorum leaf blotch caused by *Phaeosphaeria nodorum*. The epidemic was initiated using equal proportions of ten *P. nodorum* isolates marked with unique microsatellite multilocus haplotypes (MLHTs). Inoculated and uninoculated plots were sampled four times across two growing seasons to assess the relative contributions of asexual and sexual reproduction to the epidemic. Only 51% of recovered isolates had MLHTs matching the marked inoculant strains while the remaining 49% of isolates originated from recombination among the inoculants or immigration. The proportion of the epidemic due to asexual reproduction decreased steadily over time. A significant proportion of the epidemic at the end of the first season was due to recombinant progeny originating from the 10 released strains or immigrant ascospores. A significant fraction of the epidemic in both years was due to sexual recombinants that emerged within the field during the growing season. The results indicate that recombinant offspring together with immigrant ascospores make a significant contribution to a Stagonospora nodorum leaf blotch epidemic during a growing season as well as between growing seasons.

Sommerhalder, R.J., McDonald, B.A., Mascher, F., Zhan, J. 2007
CHAPTER 3

INTRODUCTION

Reproduction mode has important epidemiological implications for many plant pathogens because the different reproductive strategies can produce propagules with different means of dispersal and can influence the source of the primary inoculum. For many plant pathogenic ascomycetes, the products of asexual reproduction are splash-dispersed spores that move only short distances (1-2 m), while the products of sexual reproduction are airborne ascospores that have the potential to travel long distances (Carter and Moller, 1961; Eyal et al. 1987 and 1999; Heald et al. 1915; Stensvand 1998), leading to rapid spread across large areas of important traits such as virulence or fungicide resistance. Sexual reproduction also increases genetic variation in pathogen population by generating new alleles (Brunner et al. 2008; O’Donnell et al. 2000) or allele combinations, potentially increasing mean fitness or resilience of the pathogen (Cowger et al. 2002). The combination of high gene flow potential and increased genetic variation through recombination may allow pathogens with sexual reproduction to evolve and adapt more rapidly to changing environments, reducing the useful life span of fungicides and resistant cultivars (McDonald and Linde 2002) and therefore, making disease control more difficult.

Combining neutral molecular markers with the analytical tools of population genetics has provided a powerful approach to infer the reproductive biology and epidemiological properties of many fungal pathogens for which the sexual structures are either known or unknown (Burt et al 1996; Koufopanou et al. 1997; Linde et al. 2003; LoBuglio and Taylor 2002; Zaffarano et al. 2006). Very few studies have combined these tools to directly quantify the relative contribution of sexual reproduction, asexual reproduction and immigration to the development of epidemics under controlled field experiments (Abang 2006; Bennet et al. 2007; Zhan et al. 1998). Many biological, ecological and experimental processes that affect hosts and pathogens can introduce "noise" that influences the interpretation of results. In this case, experimental studies under controllable and repeatable environments are important to obtain reliable results (Zhan et al., 2002; 2007).
The heterothallic loculoascomycete *Stagonospora nodorum* (Berk.) Castellani and Germano (syn. *Septoria nodorum* Berk.), the anamorph form of *Phaeosphaeria nodorum* (E. Müller) Hedjaroude (syn. *Leptosphaeria nodorum* E. Muller) which causes Stagonospora nodorum glume blotch on wheat (*Triticum aestivum* L.) is a major wheat pathogen globally (Eyal 1999; Eyal et al. 1987; King et al., 1983). The teleomorph has been observed (Bathgate and Loughman, 2001; Cowger and Silva-Rojas 2006) in many locations and the primary inoculum of the disease is thought to be composed mainly of sexually produced wind-dispersed ascospores (Brennan et al. 1985; Griffiths et al., 1976; Keller et al. 1997a, b), asexually infected seeds (Shah et al. 1995), and crop residue (Holmes and Colhuon, 1975). Though it is known that the fungus can undergo both asexual and sexual reproduction, it is not clear how often and when the latter occurs. The most common premise is that the sexual cycle occurs on infected straw left in the field at the end of the growing season, with the majority of ascospores release occurring through fall and spring, usually coinciding with the emergence of seedlings of winter wheat (Bathgate and Loughman, 2001; Bennett et al. 2007; Mittelstädt et al., 1997; O’Reilly et al. 1988; Sanderson and Hampton, 1978). While many studies (Adhikari et al. 2008; Keller et al, 1997a; Bathgate and Loughman; 2001; Sommerhalder et al. 2006 and 2007; Stukenbrock et al. 2006) indicate that sexual reproduction plays an important role in the population genetic structure of the pathogen, successful recover of parental inoculants from field experiments in some recent studies was interpreted as evidence that the sexual stage does not make a significant contribution to epidemic development (Bennett et al. 2003, 2007). We hypothesize that the sexual stage also plays an important epidemiological role to Stagonospora nodorum leaf blotch and ascospores make a significant contribution as both primary and secondary inoculum during the development of an epidemic.

The objective in this experiment was to elucidate the relative contributions of different sources of inoculum and their effects on the population genetics and epidemiology of *P. nodorum* on wheat. To achieve this objective, we conducted a two-year field experiment using a mark-release-recapture experimental design. The isolates recovered from infected leaves at different points in time isolates were assigned to different categories of source inoculum, namely asexual inoculants, immigrants, or
recombinants arising from within the experimental plots, using a combination of molecular genotyping, maximum likelihood estimate and posterior probabilities.
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MATERIAL AND METHODS

Experimental design. The mark-release-recapture experiment was conducted during 2003-2004 and 2004-2005 wheat growing seasons at the Agroscope Changins-Wädenswil research center in Changins, Switzerland. Four commercial Swiss wheat cultivars were used in this experiment. Runal and Tamaro were moderately resistant to *P. nodorum* on both leaves and glumes. Tirone was resistant to glume blotch but susceptible to leaf blotch. Levis was resistant on leaves but not on glumes. The four cultivars and a 1:1 mixture of Runal and Tamaro (5 host treatments in total) were planted in a randomized complete block design (RCBD) with four replications (Sommerhalder et al. *submitted*).

Ten Swiss *P. nodorum* isolates with distinct multi-locus microsatellite haplotypes were inoculated in equal proportions onto the field plots on 11 May 2004 as described earlier (Sommerhalder et al. *submitted*). PCR amplifications of the mating type idiomorphs showed that six of the isolates were Mat1-1 and the other four were Mat1-2 (Sommerhalder et al. *submitted*). The source of primary inoculum in the second year of the experiment (2004-2005) was infected straw and plant debris collected individually from each plot at the end of the first growing season. A total of four fungal collections were made during the experiment. The four collections, hereafter called 2004A, 2004B, 2005A and 2005B, were made on 4 June 2004, 2 July 2004, 11 April 2005 and 10 June 2005, respectively. For each collection, 30 to 40 leaves were collected from each plot at intervals of approximately 20 cm along transects within the inner rows of the field plots. One or two isolates were sampled from each infected leaf. When two isolates were taken from a leaf, each isolate originated from clearly separated lesions to minimize the possibility of sampling the same infection event.

DNA was extracted from each isolate using the DNeasy Plant Mini DNA extraction kit (Qiagen GmbH, Germany) according to the specifications of the manufacturer. The fungal isolates were assayed using ten microsatellites (SNOD1, SNOD3, SNOD5, SNOD11, SNOD15, SNOD16, SNOD17, SNOD21, SNOD22, and SNOD23) and one minisatellite marker (SNOD8) (Stukenbrock et al. 2005). Additional details on field layout, procedures used for inoculation and sampling and protocols for collecting SSR data from the fungal isolates are given in Sommerhalder et al. (*submitted*).
**Data analysis.** Alleles for each isolate were joined across the 11 loci to form a multilocus haplotype (MLHT). Isolates with the same MLHT as the inoculated genotypes were considered to be the asexual progeny of these inoculated genotypes (hereafter called parental genotypes). Unless specifically mentioned, only novel isolates, defined as isolates having MLHTs different from any of the ten inoculated isolates, were included in the estimates of genetic parameters throughout this manuscript. Because the contribution of mutation to the formation of new genotypes is expected to be trivial within the time scale of this experiment, these novel isolates were believed to originate either via immigration from outside of the experimental fields or by recombination among inoculated strains and/or immigrants within the experimental fields.

Analyses of allele frequencies and other population genetic parameters were performed on clone-corrected data using a single representative of isolates with the same MLHT. Allele number and Nei’s gene diversity (Nei 1973) were estimated using the POPGENE32 software (Yeh et al. 1999). Genotypic diversity was quantified as the clonal fraction of each population using the method of Zhan et al. (2003) and according to Stoddart and Taylor (1988) as described by McDonald et al. (1994). Comparisons of allele frequencies between control and inoculated plots were based on a contingency chi-squared test as described by Everitt (1977).

Tests of gametic equilibrium were made for 2004B, 2005A, 2005B, the collection from control plots and on the putative immigrant and recombinant populations using the test for multilocus association (Brown et al., 1980) and the Phylogenetic Tree Length Permutation Test (PTLPT, Burt et al., 1996; Maynard Smith et al., 1993). The isolates recovered from the control plots in 2005A and 2005B were combined into one population because of their small sample sizes. Tests for gametic disequilibrium were not made for 2004A due to its small sample size (20 novel isolates). Multilocus association (Brown et al. 1980) was tested with POPGENE32 (Yeh et al., 1999) and using the PTLPT test implemented in PAUP 4.0b 10 (Swofford, 1998).

The relative contributions of sexual recombination and immigration to the generation of novel isolates in each collection were estimated with a maximum likelihood approach as described earlier (Zhan et al., 2000). The posterior probability of a novel
genotype resulting from immigration or sexual recombination between the inoculated isolates and/or between inoculated isolates and immigrants was calculated based on Bayesian theory by using the estimated immigration/migration rate as a priori using formula:

\[
\frac{mp_{G}\text{p}_{G}\text{i}}{mp_{G} + (1-m)p_{G}\text{r}}
\]

where \( p_{G}\text{r} \) and \( p_{G}\text{i} \) the prior probability of a novel isolate being recombinant and immigrant, respectively (Zhan et al., 1998) and \( m \) is the Maximum likelihood estimate of immigration rate. The formula differs slightly from previous formulas (e Paetkau et al., 1995; Rannala and Mountain 1997; Zhan et al., 1998). This difference reflects the prior informative knowledge of immigration rates. Formulae used previously were derived under the condition of a non-informative prior regarding immigration rates and assumes that a novel isolate is equally likely to be an immigrant or a recombinant.

Isolates with posterior probabilities \( \geq 90\% \) of being recombinants were assigned to the recombinant category. Isolates with posterior probabilities \( \geq 90\% \) of being immigrants were assigned to the immigrant category. Isolates with posterior probabilities between 10\% and 90\% of being immigrants or recombinants were assigned to the uncertain category (Zhan et al., 1998). The allele frequencies used to estimate the prior probability of a novel isolate being immigrant were estimated using 191 isolates from a Swiss collection collected in 1999 and previously described by Stukenbrock et al. (2006). Because high gene flow occurs among Swiss populations (Keller et al., 1997b) the large number of isolates in this Swiss collection provided more robust estimates of allele frequencies needed to estimate immigration rates compared to the relatively small number of immigrants identified in the uninoculated control plots. Allele frequencies used to estimate the prior probability of a novel isolate being recombinant were estimated from all isolates, including inoculated genotypes, present in inoculated plots in 2004A, 2004B, and 2005A. Due to the limited number of isolates recovered from the control plots across all 4 sampling periods, these isolates were combined to form a single population.
To determine if the hosts affected the recombination rate in their respective *P. nodorum* populations a Fisher’s least significant difference (LSD) and general linear model (GLM) analysis (SYSTAT 10.0 for Windows; SPSS Inc., Chicago) were conducted on the recombinant and immigrant population. Calculations were made only for the 2005B sample because of the large number of novel isolates present in these collections. The percentage of the recombinant and immigrant isolates in the different replications were subjected to arc sine transformation prior to conducting the analysis.
CHAPTER 3

RESULTS

Among the 1286 isolates assayed for the 11 microsatellite markers, 650 had MLHTs matching the 10 released strains, while 636 had MLHTs differing from the 10 released strains. Among the novel isolates, 550 (31 in 2004A, 138 in 2004B, 167 in 2005A and 214 in 2005B) were sampled from the inoculated plots and 86 (4 in 2004B, 69 in 2005 and 26 in 2005B) were sampled from the uninoculated control plots (Table 1). Among these, four (2004A) and five (2004B) were recovered more than once in the first year of the experiment, while thirteen (2005A) and seven (2005B) novel genotypes were recovered more than once in the second year of the experiment. The most frequent novel genotype was observed five times in 2005B. Thirteen isolates sampled from the control plots in 2005 (2005A and 2005B) had MLHTs identical to four (SNCH2.04; SNCH2.09, SNCH2.12; C1) of the 10 released isolates, suggesting movement of asexual inoculum into the control plots.

The average frequency of novel isolates in the inoculated plots increased steadily from 14% in the first collection to 75% by the fourth collection (Table 1). The increase was similar an all cultivars (Table 2). Novel alleles, defined as alleles not present in the inoculants, appeared in both control and inoculated plots. The average number of alleles increased over time in inoculated plots (Table 3) and the percentage of isolates carrying novel alleles increased from 35% to 57% (Table 1). The same pattern was found in the control plots. Comparisons between populations from the inoculated plots and the non-inoculated plots were made to determine if they had different sources of inoculum. The number of loci at which allele frequencies differed between the novel isolates from control and inoculated plots increased from 6 loci in 2004B to 8 loci in 2005B (Table 4).

Association tests failed to reject the hypothesis of random mating for the populations of novel isolates. The PTLPT analysis did not reject the hypothesis of random mating in the novel isolates treated as a single group (Table 1) and also when recombinant and immigrant populations were analyzed separately (data not shown). Brown’s analysis of multilocus association also showed an absence of significant association among loci in all populations composed only of novel isolates (Table 1) and when the recombinant populations and immigrant populations were analyzed separately (data not shown).
The MLE analysis conducted on the populations from different sampling dates indicated that 14% to 34% of the novel isolates in the inoculated plots originated from immigration (Table 5). With this estimated immigration rate, between 48% and 73% of the novel isolates could be assigned to the recombinant category (Table 5). All these results were consistent with the hypothesis that recombinants made a significant contribution to the epidemic.

The LSD (Table 6) and GLM analysis conducted on the 2005B population to investigate the possibility that recombination and immigration rates were affected by the different levels of resistance indicated that the host treatment did not affect the balance between asexual reproduction, recombination and immigration (P = 0.47; R = 0.72).
DISCUSSION

We conducted a two-year mark-release-recapture experiment to investigate the relative contributions of sexual and asexual reproduction to epidemics of Stagonospora nodorum leaf blotch caused by *P. nodorum*. Our results revealed that both modes of reproduction contributed significantly to the epidemic. 51% of isolates recovered from the inoculated plots had MLHTs matching the 10 inoculants and could be classified as asexual progeny, while the remaining 49% of isolates recovered had MLHTs differing from the released strains. Because we used disease-free seed and the plots were placed in fields that had not been planted with wheat or other hosts of *P. nodorum* for several years, we concluded that the novel isolates found in our experiments had originated through recombination or immigration. We have several lines of evidence supporting this hypothesis (i) there was a significant difference in the number of infections between the control plots and the inoculated plots, (2) high levels of genotypic diversity, (iii) gametic equilibrium found in the inoculated and control plots, (iv) a the steady increase in the frequency of novel genotypes over the course of the experiment.

The uninoculated control plots allowed us to detect possible carry-over of seedborne infection. If the main source of primary inoculum had been infected seeds, we would expect to find similar levels of disease in the inoculated and controlled plots. However, we found very low disease incidence in the control plots in the first year, compared to inoculated plots. No infected leaves were observed in the control plots at the 2004A sampling point, while many lesions were found distributed throughout the inoculated plots at the same point in time. Because our artificially infected plots were separated by 1.5m of the non-host triticale (Tridel), pycnidiospores or ascospores would need to disperse across at least 1.5 m to infect the control plots. The failure to find any released genotypes in the control plots in the first year indicates that few or no pycnidiospores traveled further than 1.5m during the growing season. We hypothesize that parental isolates in 2005 control plots were possibly due to infected straw that blew into the control plots.

Analyses of allelic associations and genotypic diversity are routinely used to infer the reproductive strategies of plant pathogens (Keller et al, 1997; Stukenbrock et al., 2006,
Zhan et al., 1998). The lack of allelic associations coupled with high levels of genotype diversity is generally considered a hallmark of sexual recombination. Two independent methods for detecting disequilibrium failed to detect disequilibrium in the novel isolate populations, consistent with our hypothesis that the novel isolates originated through recombination and implying an important role for ascospores in the epidemic.

In this experiment, ascospore infections could have originated either via airborne ascospores blowing in from outside the experimental plots (immigrants) or via recombination among inoculated and/or immigrant strains within the experimental plots (recombinants). The difference in allele frequency between the novel isolates from the inoculated and non-inoculated plots suggests that the sources of ascospore inoculum were different, with the majority of novel isolates in the control plots originating from immigration while the novel isolates in the inoculated plots originated from a combination of immigration and recombination.

To differentiate the source of novel isolates (immigrants from outside the field vs. recombinants from inside the field) we conducted a maximum likelihood analysis. The results revealed that recombinants originating from within the field were the largest source of novel genotypes. Specifically, 60% (2004B), 48% (2005A) and 72% (2005B) of the novel isolates were assigned to the recombinant category. The results from these assignment tests provide strong support for the hypothesis that sexual recombinants make a significant contribution to the development of leaf blotch epidemics.

The high levels of genotypic diversity and the gradual increase in the proportion of novel and recombinant isolates in inoculated plots further support the idea that recombination plays an important role in both the population genetics and epidemiology of this pathogen. In fact the average proportion of novel isolates increased from less than one-fifth in the 2004A sample to more than three quarters in the 2005B sample. These findings are in accordance with a similar study by Bennett et al. (2007) who also reported an increased recovery of novel genotypes between wheat tillering and flowering phases, consistent with constant arrival of immigrant ascospores or within-field recombination. Our results strengthen the Bennett et al findings and differentiate between the contributions of immigration and recombination.
The maximum likelihood analysis indicated that 5% to 18% of the novel isolates originated from immigration. The immigrant population was at gametic equilibrium and contained many novel alleles. These novel alleles were not present in the inoculants and could have originated either through mutation or immigration. The percentage of isolates carrying novel alleles increased from 35% in 2004B to 57% in 2005A and 45% in 2005B. We consider it very unlikely that mutation alone could have generated novel alleles in such a large fraction of the pathogen population. It is much more likely that strains carrying novel alleles were either immigrants or the offspring of crosses between immigrants and inoculants.

Other studies have presented evidence for the importance of sexual reproduction in the population biology of *P. nodorum*, mainly based on mating type frequency and disequilibrium analysis (Solomon et al. 2004; Sommerhalder et al. 2006 and 2007) or by capturing airborne ascospores. Bennett et al. (2007) found evidence for within season generation of novel genotypes. Arseniuk et al. (1998) reported recovering ascospores during the wheat growing season in Poland while Mittelstädt et al. (1987) reported the detection of ascospores throughout fall and spring in Germany. Cowger and Silva-Rojas et al. (2006) reported finding ascocarps in the summer months in North Carolina. Our findings provide strong supporting evidence that sexual recombination in *P. nodorum* occurs during the wheat growing season and that the resulting ascospores make a significant contribution to development of epidemics as a source of secondary inoculum.

The finding of 650 isolates with MLHTs matching the 10 released strains confirms that asexual reproduction also contributes significantly to the epidemic. By applying the crop residue collected at the end of the 2004 season to initiate the 2005 epidemic we were able corroborate in a quantitative way the earlier findings (Holmes and Colhoun, 1975) that crop residue can play an important role in initiating new epidemics. The recovery of isolates with MLHT matching the parental genotypes (47% of the population) in the inoculated plots in 2005 illustrates that *P. nodorum* isolates have the potential to persist on crop residue between growing seasons. However, despite providing optimal conditions for asexual carryover by storing the bags containing the infected straw in a protected place, the frequencies of the inoculants decreased sharply with time and they were rapidly replaced between spring and summer by novel genotypes that
originated through recombination or immigration (eg collection 2005B). The experimental design allowed us to exclude the possibility of a seed-borne contribution to the asexual population.

This mark-release-recapture study provided valuable insight into the relative contributions of asexual and sexual reproduction to epidemics of *P. nodorum*. These findings have epidemiological consequences because they show that windblown ascospores provide important sources of both primary and secondary inoculum. Although this experiment was not designed to measure long distance movement of ascospores, we consider it likely that ascospores can move over distances of at least a few kilometers. The ascospores provide wind-borne inoculum that can be transmitted to neighboring fields, both during and between growing seasons. Thus even wheat fields that have experienced the best sanitation practices, including multi year crop rotation (to eliminate infected straw from previous crops) and planting certified disease-free seeds, can be infected by ascospores that immigrate from neighboring fields. The potential for long-distance dissemination of air-borne ascospores also increases the risk for rapid dissemination of strains carrying novel virulence or fungicide resistance alleles. While we showed that the asexual inoculum can persist between seasons, there was a rapid decline in the frequency of the released genotypes over the course of the experiment. This suggests that clones will not persist over many years. On the contrary, we predict that populations will continuously change as new combinations of alleles come together through recombination events. Because of the important contribution made by air-borne propagules to the epidemiology of this pathogen, control strategies should be considered on a more regional basis (Mundt et al., 2002), as is the case with rust and powdery mildew diseases (Wolf et al., 1992).
ACKNOWLEDGEMENTS

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LITERATURE CITED


CHAPTER 3


Chapter 3


Yeh, F. C., Yang, R. C., Boyle, T. B. J., Ye, Z. H., and Mao, J. X. 1999. POPGENE, the user friendly shareware for population genetic analysis. Molecular Biology and Biotechnology Centre, University of Alberta, Canada.


Table 1. Characterization of the populations of *Phaeosphaeria nodorum* recovered from the control and inoculated plots over time, including measures of genotype diversity and disequilibrium.

<table>
<thead>
<tr>
<th>Collection</th>
<th>Isolates recovered (no.)</th>
<th>Parental isolates (%)</th>
<th>Novel isolates (no.)</th>
<th>CF&lt;sup&gt;a&lt;/sup&gt;</th>
<th>GD&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Brown Test</th>
<th>PTLPT&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>2004A</td>
<td>223</td>
<td>86%</td>
<td>14%</td>
<td>0.35</td>
<td>0.33</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>2004B</td>
<td>362</td>
<td>62%</td>
<td>38%</td>
<td>0.40</td>
<td>0.4</td>
<td>0.5</td>
<td>1.4</td>
</tr>
<tr>
<td>2005A</td>
<td>317</td>
<td>47%</td>
<td>53%</td>
<td>0.21</td>
<td>0.52</td>
<td>0.4</td>
<td>1.2</td>
</tr>
<tr>
<td>2005B</td>
<td>285</td>
<td>25%</td>
<td>75%</td>
<td>0.13</td>
<td>0.74</td>
<td>0.3</td>
<td>1.0</td>
</tr>
<tr>
<td>Control</td>
<td>99</td>
<td>13%</td>
<td>87%</td>
<td>0.13</td>
<td>0.58</td>
<td>0.76</td>
<td>1.46</td>
</tr>
<tr>
<td>Total</td>
<td>1286</td>
<td>51%</td>
<td>49%</td>
<td>0.20</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

<sup>a</sup> Clonal fraction

<sup>b</sup> Measures of genotypic diversity according to Stoddart and Taylor (1988)

<sup>c</sup> Observed variance of the number of heterozygous comparisons calculated using Brown’s multilocus association (Brown *et al.* 1980).

<sup>d</sup> L2 upper 95% confidence limit of S<sub>k</sub><sup>2</sup>

<sup>e</sup> PTLPT, Parsimony tree length permutation test (Maynard Smith *et al.* 1993). Null hypothesis of random mating was tested by comparing the length of the observed parsimony tree (L) to the lengths of 1000 randomized trees generated from artificially recombined data set (Burt *et al.*, 1996).
Table 2. Percentage of inoculant, recombinant, immigrant and uncertain genotypes recovered from each cultivar for each collection date. Uncertain novel genotypes could not be assigned to the immigrant or recombinant categories with greater than 90% confidence. Immigration estimates are based on allele frequencies from the Swiss reference population, while recombination estimates are based on allele frequencies from all isolates, present in inoculated plots in 2004A, 2004B, and 2005A.

<table>
<thead>
<tr>
<th></th>
<th>2004B</th>
<th>2005A</th>
<th>2005B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inoculant genotypes</td>
<td>Novel genotypes</td>
<td>Inoculant genotypes</td>
</tr>
<tr>
<td>MLE&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Levis</td>
<td>0.60</td>
<td>0.37</td>
<td>0.03</td>
</tr>
<tr>
<td>1:1 RuxTa</td>
<td>0.63</td>
<td>0.15</td>
<td>0.00</td>
</tr>
<tr>
<td>Runal</td>
<td>0.68</td>
<td>0.32</td>
<td>0.00</td>
</tr>
<tr>
<td>Tamaro</td>
<td>0.51</td>
<td>0.43</td>
<td>0.05</td>
</tr>
<tr>
<td>Tirone</td>
<td>0.62</td>
<td>0.23</td>
<td>0.15</td>
</tr>
</tbody>
</table>

<sup>a</sup> MLE of immigration calculated using the method of Zhan et al. (2000)
Table 3. Size range of SSR alleles and numbers of alleles found in the novel *Phaeosphaeria nodorum* isolates recovered from the inoculated and control plots.

<table>
<thead>
<tr>
<th>SSR locus</th>
<th>Size range</th>
<th>Inoculated plots</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2004A</td>
<td>2004B</td>
</tr>
<tr>
<td>SN15</td>
<td>158-168</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>SN3</td>
<td>303-312</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>SN11</td>
<td>164-170</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>SN5</td>
<td>426-450</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>SN1</td>
<td>284-298</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>SN17</td>
<td>98-138</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>SN8</td>
<td>364-403</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>SN22</td>
<td>234-255</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>SN21</td>
<td>200-212</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>SN23</td>
<td>312-364</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>SN16</td>
<td>191-201</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>2.55</td>
<td>4.09</td>
</tr>
</tbody>
</table>
Table 4. Chi-square test for homogeneity in allele frequencies between the novel isolates sampled from the inoculated and control plots.

<table>
<thead>
<tr>
<th>SSR locus</th>
<th>Control vs. 2004B</th>
<th>Control vs. 2005A</th>
<th>Control vs. 2005B</th>
</tr>
</thead>
<tbody>
<tr>
<td>SN15</td>
<td>7.33 (3)</td>
<td>5.22 (4)</td>
<td>13.22 (3)*</td>
</tr>
<tr>
<td>SN3</td>
<td>10.24 (2)**</td>
<td>7.01 (2)*</td>
<td>16.6 (2) **</td>
</tr>
<tr>
<td>SN11</td>
<td>2.27 (3)</td>
<td>2.70 (3)</td>
<td>5.79 (2)</td>
</tr>
<tr>
<td>SN5</td>
<td>50.41 (9)**</td>
<td>55.93 (9)**</td>
<td>54.29 (9)**</td>
</tr>
<tr>
<td>SN1</td>
<td>18.67 (8)*</td>
<td>34.30 (8)**</td>
<td>29.01 (7) **</td>
</tr>
<tr>
<td>SN17</td>
<td>30.98 (9)**</td>
<td>32.63 (8) **</td>
<td>47.31 (8) **</td>
</tr>
<tr>
<td>SN8</td>
<td>4.88(3)</td>
<td>5.06 (4)</td>
<td>5.94 (3)</td>
</tr>
<tr>
<td>SN22</td>
<td>55.26 (9)**</td>
<td>53.67 (8) **</td>
<td>51.84 (7) **</td>
</tr>
<tr>
<td>SN21</td>
<td>50.28 (6)**</td>
<td>34.75 (6) **</td>
<td>59.43 (6) **</td>
</tr>
<tr>
<td>SN23</td>
<td>1.76 (3)</td>
<td>0.842 (2)</td>
<td>0.01 (1)</td>
</tr>
<tr>
<td>SN16</td>
<td>10.81 (5)</td>
<td>13.99 (3) *</td>
<td>22.79 (3) **</td>
</tr>
</tbody>
</table>

*Significant at p = 0.05 level

**Significant at p = 0.01 level
CHAPTER 3

Table 5. The proportion of novel *Phaeosphaeria nodorum* isolates originating from immigration or sexual recombination using MLE methods. Immigration estimates are based on allele frequencies from the Swiss reference population.

<table>
<thead>
<tr>
<th>Collection</th>
<th>Immigration rate ( ^a )</th>
<th>Novel isolates</th>
<th>Immigrants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Recombinants</td>
<td>Unknown</td>
</tr>
<tr>
<td>2004B</td>
<td>0.23</td>
<td>0.60</td>
<td>0.32</td>
</tr>
<tr>
<td>2005A</td>
<td>0.34</td>
<td>0.50</td>
<td>0.33</td>
</tr>
<tr>
<td>2005B</td>
<td>0.14</td>
<td>0.74</td>
<td>0.21</td>
</tr>
</tbody>
</table>

\( ^a \) MLE of immigration calculated using the method of Zhan et al. (2000)
Table 6. P-values from LSD analysis on immigrant and recombinant 2005B populations of *Phaeosphaeria nodorum*. Immigrant population above the diagonal, recombinant populations below the diagonal. P ≤ 0.05 indicates significant differences among cultivars.

<table>
<thead>
<tr>
<th></th>
<th>Levis</th>
<th>RuXTa</th>
<th>Runal</th>
<th>Tamaro</th>
<th>Tirone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Levis</td>
<td>-----</td>
<td>0.88</td>
<td>0.25</td>
<td>0.18</td>
<td>0.4</td>
</tr>
<tr>
<td>RuXTa</td>
<td>0.67</td>
<td>-----</td>
<td>0.29</td>
<td>0.22</td>
<td>0.48</td>
</tr>
<tr>
<td>Runal</td>
<td>0.5</td>
<td>0.33</td>
<td>-----</td>
<td>0.97</td>
<td>0.58</td>
</tr>
<tr>
<td>Tamaro</td>
<td>0.37</td>
<td>0.22</td>
<td>0.94</td>
<td>-----</td>
<td>0.54</td>
</tr>
<tr>
<td>Tirone</td>
<td>0.26</td>
<td>0.151</td>
<td>0.75</td>
<td>0.77</td>
<td>-----</td>
</tr>
</tbody>
</table>
CHAPTER 4

Slower evolution on cultivar mixtures and evidence of diversifying selection between pathogenic and saprophytic phases in experimental populations of *Phaeosphaeria nodorum*
ABSTRACT

The evolution of pathogen populations in an agricultural environment can be influenced by the diversity in host populations and through competition with other microbes during the saprophytic phase of the pathogen life cycle. Evolution in field populations of the wheat pathogen *Phaeosphaeria nodorum* was assessed using a mark-release-recapture experiment. Ten *P. nodorum* isolates marked with ten microsatellite and one minisatellite markers were released onto five replicated host populations to initiate epidemics of Stagonospora nodorum leaf blotch. A total of 1106 isolates were recovered from inoculated plots over the two-year experiment. Genetic diversity in the host populations affected the evolutionary rates of the corresponding *P. nodorum* populations. The relative frequencies of the ten inoculated isolates in the cultivar mixture did not change over the course of the experiment and exhibited the lowest variation in selection coefficients. These results support the hypothesis increasing genetic heterogeneity in host populations may retard the rate of evolution in associated pathogen populations. Our study also provides indirect evidence of fitness costs associated with host specialization in *P. nodorum* as indicated by differential selection on some strains during the parasitic and saprophytic phases.

Sommerhalder, R.J., McDonald, B.A., Mascher, F., and Zhan J. 2008
INTRODUCTION

The evolution of pathogens can be influenced by the type of resistance and amount of diversity found in host populations, with deployment options including partially resistant cultivars and cultivar mixtures (Lannou and Mundt, 1996; Mundt, 2002; Paillard et al., 2000). Host mixtures are considered to be an ecologically sound approach to control plant diseases, particularly for airborne pathogens of cereals (Mundt, 1989; 2002). It is thought that increasing genetic diversity in host populations by using cultivar mixtures could impose disruptive selection on pathogen populations (i.e., pathotypes that are favored on one host will have lower fitness on the other hosts) (Burdon, 1987; Dileone and Mundt, 1994; Higashi, 1995), impeding their ability to evolve towards higher pathogenicity (Ebert and Hamilton, 1996). On the other hand, because many fungal pathogens have large effective population sizes (Zhan et al., 2001) and exhibit a mixture of sexual and asexual reproduction (Anderson et al., 1992; Zhan et al., 2007b), they can rapidly develop new virulence through mutation and new combinations of virulence alleles through recombination and then maintain the novel allelic combinations through asexual reproduction. Thus extensive use of cultivar mixtures could lead to the development of complex races (Lannou, 2001; Parlevliet, 1981) that would be able to infect a large number of host genotypes.

Partial resistance is also thought to offer a more durable method to control plant diseases than major-gene resistance because it selects equally against all pathotypes (Simons, 1972; Vanderplank 1968). Partial resistance is usually not race-specific and is often inherited as a quantitative trait (Higashi, 1995; Maruyama et al., 1983; Robinson 1976), where each minor gene contributes a small portion to the overall resistance (Parlevliet 1993). However, even in pathogens lacking gene-for-gene interactions, selection may increase the frequencies of aggressiveness genes in pathogen populations and reduce the stability of quantitative resistance (Clifford and Clothier 1974; Gandon and Michalakis, 2002; Kolmer and Leonard 1986) though possibly at a slower pace (Zhan et al., 2002). However, partially resistant cultivars may also select for higher
aggressiveness (Pink et al. 1992, Schouten and Benires 1997) and enhance the rate of
sexual recombination in pathogens (Cowger and Mundt, 2002; Zhan et al., 2007a).

Evolution of pathogens may be further influenced by genotype x environment
interactions (Cory and Myers, 2004) and inter- or intra-specific competition during the
saprophytic phase. Differences in survival ability between parasitic and saprophytic
phases were observed previously (Abang et al., 2006) and can affect the emergence of
new virulence (Kiyosawa, 1982; Vanderplank, 1963).

Field experiments to directly measure the rate and direction of pathogen evolution
in agroecosystems are rare but have become feasible with the development of molecular
 genetic markers that can be used to differentiate among pathogen strains released into a
field setting. The mark-release-recapture strategy coupled with molecular genetic markers
has now been applied to cereal pathogens including Mycosphaerella graminicola (Zhan
et al., 1998; 2000; 2002), Rhynchosporium secalis (Abang et al., 2006) and
Phaeosphaeria nodorum (Bennett et al., 2007). In this study, we used this approach to
study the effects of partial resistance and cultivar mixtures on the evolution of field
populations of Phaeosphaeria nodorum.

The heterothallic loculoascomycete Phaeosphaeria nodorum (Berk.) Castellani
and Germano (syn. Septoria nodorum Berk.), the teleomorph form of Stagonospora
nodorum (E. Müller) Hedjaroude (syn. Leptosphaeria nodorum E. Muller), causes
Stagonospora nodorum leaf and glume blotch on wheat (Triticum aestivum L.). The
pathogen can undergo both asexual and sexual reproduction and is able to infect all
above-ground plant parts during the parasitic phase. The primary inoculum includes
infected seeds as well as conidia and ascospores produced on stubble. The pathogen
overwinters during the saprophytic stage on infected stubble (Shaner, 1981), where the
sexual stage occurs (Cowger and Silva-Rojas, 2006; Halama, 2002). Asexual
pycnidiospores are dispersed over short distances by rain-splash while sexual ascospores
are wind-dispersed, therefore having the potential for long distance movement (Arseniuk
et al., 1998; Brennan et al., 1983; Griffiths and Ao, 1976; Keller et al., 1997).
Observations of the teleomorph (Arseniuk et al., 1998; Bathgate and Loughman, 2001;
Bennett, et al., 2003; Cowger and Silva-Rojas, 2006) and analysis of mating type
frequency (Sommerhalder et al., 2006) suggest that the ability of the fungus to undergo
sexual reproduction may vary among locations (Shaw, 1999). The main methods used to control the disease include crop rotation, fungicides, and the use of partially resistant cultivars (Bostwick et al., 1993; Czembor et al., 2003; Wicki et al., 1999; Wilkinson et al., 1990).

The objectives of this study were to use the mark-release-recapture approach to: i) determine the effect of host resistance and cultivar mixtures on the rate of evolution in the pathogen population; and ii) compare selection during the parasitic and saprophytic phases of life cycle. Because the field experiments were based on artificial inoculation with equal proportions of ten $P. nodorum$ genotypes, our null hypothesis was that the fungal populations recovered from each host treatment would consist of the ten introduced genotypes present at equal frequencies over time during both parasitic and saprophytic phases of the disease cycle.
MATERIAL AND METHODS

Experimental design. Two field experiments were conducted at the Agroscope Changins-Wädenswil research center in Changins, Switzerland during 2003-2004 on Allotment 34 north and during 2004-2005 on Allotment 35 north. Both fields were grown with a permanent meadow for at least three years prior to the experiment. Four commercial Swiss wheat cultivars, Levis, Runal, Tamaro and Tirone, were used in this experiment. The varieties differed in quantitative resistance to leaf infection based on disease screening conducted at Changins between 2001 and 2002 (Mascher et al., unpublished). Levis, Tamaro and Runal were moderately resistant to *P. nodorum* on leaf blotch and their levels of resistance decreased in that order. Tirone was susceptible to leaf blotch. Levis was resistant on leaves but not on glumes. The four cultivars and a 1:1 mixture of Runal and Tamaro (5 host treatments in total) were planted in a randomized complete block design (RCBD) with four replications. The field plots were 1.5 m in width and 4.5 m in length. Each wheat plot was surrounded by four equal-sized plots planted with the highly resistant triticale variety Tridel. In the first year, the experiment was planted on 5 October 2003 with commercial seed treated with Coral (2.38% difenoconazole and 2.38% fludioxonil, 2 ml/kg seeds). The experiment in the second year was seeded on 17 October 2004 using clean but untreated seed harvested from separate, disease-free plots.

Ten *P. nodorum* isolates originating from naturally infected fields near Bern, Switzerland in 1999 were chosen as inoculants for the 2003-2004 experiment. Each of the isolates had distinct multi-locus haplotypes when assayed with seven single-locus RFLP markers (Keller et al., 1997), ten polymorphic EST-derived microsatellite markers and one minisatellite marker (Stukenbrock et al., 2005; Table 1). PCR amplifications of the mating type idiomorphs (Sommerhalder et al., 2006) showed that five of the ten isolates were Mat1-1 and the other five were Mat1-2 (Table 1). Only one of the ten isolates (SN99CH3.20a) carried the *ToxA* gene (Stukenbrock and McDonald, 2007). After completing the field experiments it was discovered that SN99CH3.19a had been replaced by a contaminant of unknown origin, hereafter called C1. Following removal from long-
term storage at -80°C, the strains were first grown on Yeast Maltose Agar (YMA, yeast 4 gl\(^{-1}\), maltose 4 gl\(^{-1}\), sucrose 4 gl\(^{-1}\), agar 10 gl\(^{-1}\)) at 21°C for ten days and then transferred to 1000 ml flasks containing 300 g of sterilized wheat kernels (cultivar Arina) in a dark incubator at 4°C. Three months later the infected wheat kernels were harvested and ground to a powder using a gristmill. The powdered kernels were mixed with distilled water and the spore suspension was filtered through cheese cloth and glass-wool. The spore suspension from each isolate was adjusted to 10\(^6\) spores per ml using a hemacytometer and the spores from each strain were mixed in equal proportions. A surfactant (Tween 20) was added to the spore suspension at the rate of one drop per 50 ml. The aqueous spore suspension was applied onto disease free wheat seedlings in three of the four replications at growth stage 31 on 11 May 2004. Each field plot was sprayed with 500 ml of the calibrated spore suspension. To optimize the humidity and increase the probability of infection, inoculations were carried out in the late afternoon on a cloudy day and the inoculated seedlings were covered with a plastic tarp for 24 hours. The fourth replication was not inoculated.

The source of primary inoculum in the 2004-2005 experiment was the infected straw and other plant debris saved from the first year’s experiment. After harvesting the grain at the end of July 2004, the straw and other plant debris in each plot was collected and stored separately in burlap potato sacks for 3 months. The sacks were stored in a dry, dark and cool room to allow the development of a saprophytic phase without the risk of excessive moulding. At the beginning of tillering (Zadoks stage 13 to 21), the straw was applied onto the corresponding host plots.

A total of four fungal collections were made during the two growing seasons. The first collection was made on 4 June 2004 from the third or fourth full leaf (Zadoks 1974) at three weeks after the artificial inoculation. The second collection was made on 2 July 2004 from flag leaves. The third collection (from the second true leaf) was made on 11 April 2005 and the last collection (from the third true leaf) was made on 10 June 2005. For each collection, 30 to 40 leaves were collected from each inoculated plot at intervals of approximately 20 cm along transects within the inner rows of the field plots. In most cases, only one isolate was made from each infected leaf. Because of low levels of infection, the total number of isolations made was much lower than the number of wheat
leaves collected. For some collections with very low infection, two isolations were made from the same leaf. In these cases, each isolation was made from clearly separated lesions to minimize the possibility of sampling the same infection event.

**DNA extraction and microsatellite data collection.** DNA was extracted from each isolate using the DNeasy Plant Mini DNA extraction kit (Qiagen GmbH, Germany) according to the specifications of the manufacturer. The genotype of each isolate was determined using the same ten EST-derived microsatellite markers (SN1, SN3, SN5, SN11, SN15, SN16, SN17, SN21, SN22, and SN23) and one minisatellite marker (SN8) used to tag the released strains. Multiplexed polymerase chain reactions (PCR) were carried out with fluorescently labeled primers using the same conditions described previously (41). The samples were cooled on ice for 2 min after denaturation and then separated on an ABI PRISM 3100 sequencer using the manufacturer’s instructions (Applied Biosystems). Alleles were assigned with the program GENESCAN version 3.7 (Applied Biosystems).

**Data analysis.** The multilocus haplotype (MLHT) for each strain was formed by joining the alleles at each of the 11 marker loci. Isolates with the same MLHT were considered to be clones, the products of asexual reproduction for a particular genotype. Because fungal collections were found to consist of both inoculated and novel isolates (novel isolates are defined as strains having MLHTs different from any of the ten inoculated isolates), only isolates derived from asexual reproduction of the ten released strains were included in calculating selection coefficients and determining the effects of host genotypes on the population genetic structure of *P. nodorum.* The novel isolates, which originated either via immigration from outside of the experimental fields or by recombination among inoculated strains and immigrants, will be considered fully in a separate paper.

Analysis of variance for genotype frequencies and selection coefficients were performed using ANOVA in SYSTAT 10.0 for Windows (SPSS Inc., Chicago). Selection coefficient of the inoculated strains within each host treatment were estimated simultaneously by setting the coefficient of the most fit strain (the strain with the greatest frequency increase over the considered time period) to zero as described by Zhan et al.
(2002). This method measures the over-all fitness of a genotype relative to the most-fit strain in a population during the entire disease cycle, including infection, colonization, reproduction and transmission (Zhan et al., 2002) and is useful to estimate relative fitness of genotypes in clonal organisms that exhibit asexual reproduction such as *P. nodorum*. To make more robust estimates, selection coefficients were estimated for each host treatment by pooling together data from different replications only for the six most common inoculated strains using 2004A and 2004B collections. The other three strains were recovered at frequencies too low to make meaningful estimates of selection coefficients. Selection coefficients were not estimated for the 2005 collections due to small sample sizes after the novel isolates were excluded. The means and standard deviations of the selection coefficients were generated based on 100 resamples of the original genotype frequencies using the Excel add-in PopTools 2.7 (CSIRO, Australia). Tukey’s significant differences implemented in SYSTAT were used to compare selection coefficients among the six inoculated genotypes. Genotype frequencies of the inoculated strains in populations from different hosts and sampling times were compared using contingency $\chi^2$ tests (Everitt, 1977).
RESULTS

Disease development and fungal isolation. Two field experiments were conducted during the 2003-2004 and 2004-2005 wheat growing seasons. The experiments consisted of five treatments, composed of replicated plots sown to wheat cultivars Levis, Runal, Tamaro, Tirone and a 1:1 mixture of Runal and Tamaro. Epidemics caused by *P. nodorum* were initiated by artificially inoculating the plots with ten strains having unique microsatellite multilocus haplotypes (MLHTs). In 2004 the weather was drier and hotter than normal, presenting sub-optimal conditions for infection. The amount of disease in 2004 was below average for the entire season. In 2005 weather conditions were conducive for epidemic development resulting in a greater number of earlier infections. The majority of infections occurred on leaves. Glume infections were rare in both years. As a result of these factors, the number of isolates recovered varied across time points and field plots.

The inoculated strains were recovered from the experimental plots on 4 June 2004 (hereafter called 2004A), 2 July 2004 (hereafter called 2004B), 11 April 2005 (hereafter called 2005A) and 10 June 2005 (hereafter called 2005B). The 1106 fungal isolates recovered from the inoculated plots across the experiments were assayed with 10 microsatellite and one minisatellite markers. Among these, 571 isolates were recovered from the 2003-2004 experiment and the remaining 535 isolates were recovered from the 2004-2005 experiment. 522 out of the 1106 isolates were novel genotypes with multilocus haplotypes that did not match the ten inoculated strains. All except one inoculated genotype were recovered from the experimental plots. The frequencies of inoculated genotypes in the fungal collections decreased gradually from an average of 87% in 2004A to 21% in 2005B (Table 2). Except for genotype C1, the majority of novel isolates were detected only once. C1 made up 5.9% of the total collection and was found in all of the inoculated plots but was not recovered from uninoculated control plots. For these reasons, we believe that C1 was a contaminant that replaced SN99CH3.19a during the production of inoculum.
CHAPTER 4

**Changes in genotype frequency among host populations over time.** A contingency $^2$-test was used to compare the frequency of the inoculated genotypes in the *P. nodorum* populations isolated from two (pairwise comparison) or more (multiple comparison) hosts. In pairwise comparisons, significant differences in genotype frequencies were detected between Tirone-Runal and Tirone-Tamaro in 2004B (Table 3) and between Levis-Tirone and Levis-Runal in 2005A (Table 4). No significant differences in genotype frequencies were detected between any other pairs of *P. nodorum* populations (Tables 3 and 4). In multiple population comparisons, significant differences in genotype frequencies were detected in collection 2005A but not in 2004A or 2004B (Table 5). Multiple population comparison was not conducted for the 2005B collection because of the small sample size.

There were highly significant differences in genotype (P < 0.001) frequencies among the ten inoculated genotypes in the fungal collections from the different host treatments. Isolate SN99CH2.12a established and performed well in 2004 and 2005 on all host treatments (Fig. 1). Isolate SN99CH2.09a established well at the beginning of the 2004 season on all host treatments. Its frequency increased from less than 20% in the 2004A sample to about 35% in the 2005B sample on the partially resistant cultivar Runal and increased from about 10% in 2004A to nearly 45% in 2005B on the partially resistant cultivar Tamaro. Isolate C1 established well on all host treatments and its frequency on Levis increased from less than 5% in 2004A to nearly 40% in 2005B. Isolate SN99CH3.23a was not recaptured during the course of the experiment.

Significant differences in selection coefficients were found on all treatments except on the mixture. The average selection coefficients of the six most common strains ranged from 0.03 to 0.83 across cultivars (Table 6). Isolates differed in their degree of adaptation to the different cultivars as indicated by the significant cultivar by isolate interaction in the analysis of variance (Table 7). For example, isolate SN99CH2.04a displayed the highest fitness on cultivar Runal and SN99CH2.09a displayed the highest fitness on cultivar Tamaro. SN99CH3.20a, which was the only inoculated strain carrying the *ToxA* gene, had selection coefficients ranging from 0.29 on Tirone to 0.80 on Runal. All isolates exhibited similar fitness on the mixture as indicated by no significant difference in their selection coefficients.
Changes in genotype frequency within host populations over time. Contingency $^2$-tests were used to compare the frequencies of the inoculated genotypes in the *P. nodorum* populations isolated from the same host but at different sampling dates. Between 2004A and 2004B, significant changes in genotype frequencies were found in *P. nodorum* populations collected from Runal, Tamaro, and Tirone but not from Levis (Table 8). Significant changes in genotype frequencies also occurred in the pathogen populations collected from Tamaro, Tirone and Levis between 2004B and 2005A (i.e. during the saprophytic phase) as well as between 2004A and 2005A. The changes in genotype frequency on the host mixture over the two years were not significant. When data from different replications of the same treatment at the same time point were pooled, all comparisons in genotype frequencies were significant and the differences in genotype frequencies among the pathogen populations increased (p-values decreased) over time (Table 9).
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DISCUSSION

Composition of *P. nodorum* populations. We initiated epidemics of Stagonospora nodorum leaf blotch using ten fungal strains marked with unique microsatellite multilocus haplotypes. Because the planted seeds were free of the pathogen and the fields used for the experiments had not been planted to wheat or other hosts of *P. nodorum* for at least three years, we expected that pathogen populations recovered from the experimental fields would consist only of the 10 released strains, under the assumption that no new genotypes would be introduced into the experimental plots after the inoculation. Instead, we found that a significant proportion of the isolates sampled from these fields consisted of strains with MLHTs differing from the ten released strains. The frequency of novel genotypes increased directionally over the course of the experiment. Except for strain C1, which we believe was a contaminant that replaced isolate SN99CH3.19a during the preparation of inoculum, these novel isolates originated either via immigration from outside of the experimental fields or by recombination among inoculated strains and/or immigrants. Further analysis with maximum likelihood estimates and posterior probability approaches (Zhan et al., 1998, 2000) indicated that more than half of the novel isolates were derived from sexual recombination involving the inoculants (data not shown), suggesting that ascospores contributed significantly to these epidemics of Stagonospora nodorum leaf blotch.

The effect of partial resistance and host diversity on the evolution of *P. nodorum*. Because the epidemics were initiated using ten *P. nodorum* strains mixed in equal proportions, our null hypotheses were that the frequencies of the ten released isolates would be nearly equal in different host populations and that the genetic structure of these populations would not change over time. Instead, we observed significant differences in the frequencies of the released isolates and the majority of *P. nodorum* populations sampled from the five host treatments changed significantly over time. The differences in genotype frequency among the inoculated strains within a population and among populations sampled from different points in time could be due to genetic drift or natural
selection, but we believe that the observed differences in this case should be attributed
mainly to selection. We have two lines of evidence supporting this hypothesis. 1) If
genetic drift was the main factor, we would expect a random change in genotype
frequency among the *P. nodorum* populations sampled from different hosts. Instead, we
found that changes in *P. nodorum* populations over time were dependent on host
populations (Table 8) and selection coefficients were strongly affected by host genotypes
(Table 6). 2) The differences in population genetic structure increased over time. Greater
differences in genotype frequencies were observed among *P. nodorum* populations
sampled from different hosts at late stages of the experiment compared to early stages of
the experiment (Tables 5 and 9).

It was hypothesized that increasing genetic diversity in host populations would
retard the rate at which pathogens evolve (Burdon, 1987; Dileone and Mundt, 1994;
Huang et al., 1994; Parlevliet, 1981) because heterogeneity in the host population would
lead to divergent selection pressure on the pathogen population (Ebert and Hamilton,
1996). Our results support this hypothesis. We found that the genetic structure of *P.
nodorum* populations sampled from the host mixture did not change significantly over
two years and displayed the lowest variation in selection coefficients. Slower pathogen
evolution on cultivar mixtures was also observed in a similar experiment conducted with
*M. graminicola* on wheat (Zhan et al., 2002) and was proposed for the *Blumeria hordei-
barley* plant-pathosystem (Chin and Wolfe, 1984).

It was also hypothesized that partial resistance would retard the evolution of
pathogens and thus influence the durability of resistance (Parlevliet, 2002; Simons,
1972). However, experimental tests of this hypothesis under field conditions are very
rare. We tested the hypothesis in our experiments by comparing the changes in frequency
of marked pathogen strains competing on susceptible and partially resistant cultivars. We
believe that this approach enables a reasonable comparison of the relative evolutionary
rates of pathogens on hosts exhibiting race-specific and race non-specific resistance. In a
similar experiment in the wheat-*Mycosphaerella graminicola* pathosystem, we found that
the populations sampled from a partially resistant cultivar exhibited less change in
genetic structure over time and smaller selection coefficients than those from a
susceptible cultivar (Zhan et al., 2002), consistent with the theoretical hypothesis. But the
results from this experiment falsified this hypothesis. We detected significant changes in
*P. nodorum* populations sampled from the partially resistant cultivar Tamaro and Runal
as well as on the susceptible cultivar Tirone (Table 6 and 8), suggesting that rapid
directional selection also can occur in pathogen populations on partially resistant
cultivars, leading to the erosion of resistance as described in McDonald and Linde (2002).
The erosion of partial resistance was also observed in the barley scald pathogen
*Rhynchosporium secalis* (Abang et al., 2006).

**Differential evolution of *P. nodorum** during parasitic and saprophytic phases.**
Changes in population genetic structure of pathogens can occur not only during the
parasitic phase but also during the saprophytic phase (Kiyosawa, 1982; Vanderplank,
1968). Changes in the direction of natural selection between parasitic and saprophytic
phases could have a significant impact on the evolution of pathogens. Novel mutant
strains able to infect newly released cultivars may emerge more slowly than expected if
the increase in frequency of the mutants during the parasitic phase is offset by a decrease
in frequency during the saprophytic phase. Similar to earlier findings with *R. secalis* on
barley (Abang et al., 2006), we found some evidence of differential selection between
parasitic and saprophytic phases in *P. nodorum* in the pathogen populations sampled from
Runal. On this cultivar, a significant difference in population genetic structure was
observed between the *P. nodorum* populations from 2004A and 2004B but not between
2004A and 2005A, indicating that selection during the saprophytic phase may have offset
selection that occurred during the parasitic phase. Differential selection between parasitic
and saprophytic phases could also have occurred for isolate SN99CH2.04. This isolate
increased in frequency on four of the five treatments during the parasitic phase of the
disease cycle but decreased in frequency during the saprophytic phase except on the
cultivar mixture (Fig. 1), indicating it might have higher relative fitness during the
parasitic phase on the majority of living host tissue but lower competitive ability during
the saprophytic phase on the dead host tissue.

Lack of differential selection between parasitic and saprophytic phases in other
hosts and isolates may be partially attributed to our sampling strategy. 2005A was
sampled from infected plants several months after the application of the wheat stubble
inoculum. Thus at least one cycle of parasitic competition had likely occurred among the pathogen strains before the collection was made. If there was differential selection between the parasitic and saprophytic phases, selection for establishment and reproduction during the initiation of the 2005A epidemics may have partially offset the selection for saprophytic competition.

The only strain carrying ToxA, SN99CH3.20a, on average started in each host treatment at a relatively high frequency (17-27%) and always was present at a lower frequency (0-13%) by the final 2005B sample, with an average decrease in frequency of 17%. By comparison, the two other strains (SNCH3.08a and SNCH3.10a) that had an overall decrease in frequency on each host treatment between the first and the final collections showed a decrease averaging less than 2%. Though this experiment was not designed to determine whether there is a fitness cost associated with carrying ToxA, and we do not know if any of the Swiss wheat cultivars used in this experiment carry Tsn1, this observation suggests that ToxA may carry a fitness cost during both parasitic and saprophytic phases of selection. A fitness cost associated with ToxA was proposed earlier as an explanation for the difference in frequencies of ToxA positive strains among geographical P. nodorum populations (Stukenbrock and McDonald, 2007).

Understanding the evolutionary response of pathogen populations to host diversity and environmental changes (such as over-wintering or over-summering) is important for disease management. Many studies on host-pathogen interaction have focused on the development of mathematical models (Barrett, 1980; Lannou, 2001) to predict pathogen evolution in response to different strategies of resistance gene deployment (De Meaux and Mitchell-Olds, 2003; Long et al., 2006; Mundt, 2002; Paillard et al., 2000; Parlevliet, 2002). Very few studies have been conducted to test these theoretical models empirically in agroecosystems. Here, we present empirical evidence that strong selection occurs during both parasitic and saprophytic phases of the disease cycle. Selection during the parasitic phase occurred most slowly on the cultivar mixture. These results were largely similar to findings based on a similar experimental approach used with the wheat pathogen Mycosphaerella graminicola (Zhan et al., 2002) and the barley pathogen Rhynchosporium secalis (Abang et al., 2006), suggesting that the observed pattern of evolution may be applicable for other splash-dispersed pathogens on cereals.
AKNOWLEDGMENTS

This research was supported by the Swiss Federal Institute of Technology Grant TH-49a/02-1 and the Scottish Executive Environments and Rural Affairs Department. We thank S. Kellenberger for the field work, D. Gobbin, M. Lutz and P. Zaffarano for help with the data analysis, and V. Martinez, C. Phan, S. Seeholzer, and M. Zala for technical assistance.
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632 Zhan, J., Mundt, C.C. and McDonald, B.A. (2001) Using RFLPs to assess temporal variation and estimate the number of ascospores that initiate epidemics in field populations of *Mycosphaerella graminicola*. Phytopathology 91, 1011-1017.
difference in virulence between MAT1-1 and MAT1-2 isolates in the wheat pathogen
### TABLES AND FIGURES

Table 1. Microsatellite, mating type genotypes and presence or absence of the *ToxA* gene for the *Phaeosphaeria nodorum* isolates used in the field experiment.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>SN15</th>
<th>SN3</th>
<th>SN11</th>
<th>SN5</th>
<th>SN1</th>
<th>SN17</th>
<th>SN8</th>
<th>SN22</th>
<th>SN21</th>
<th>SN23</th>
<th>SN16</th>
<th>Mating type</th>
<th><em>ToxA</em> gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>SN99CH2.04a</td>
<td>162</td>
<td>303</td>
<td>168</td>
<td>426</td>
<td>287</td>
<td>126</td>
<td>403</td>
<td>244</td>
<td>212</td>
<td>312</td>
<td>191</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>SN99CH2.09a</td>
<td>164</td>
<td>303</td>
<td>168</td>
<td>446</td>
<td>289</td>
<td>98</td>
<td>364</td>
<td>250</td>
<td>203</td>
<td>364</td>
<td>201</td>
<td>2</td>
<td>-</td>
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<tr>
<td>SN99CH2.12a</td>
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<td>303</td>
<td>168</td>
<td>429</td>
<td>283</td>
<td>98</td>
<td>403</td>
<td>247</td>
<td>203</td>
<td>312</td>
<td>191</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>SN99CH2.35a</td>
<td>168</td>
<td>303</td>
<td>168</td>
<td>426</td>
<td>287</td>
<td>106</td>
<td>364</td>
<td>238</td>
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<td>312</td>
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<td>1</td>
<td>-</td>
</tr>
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<td>303</td>
<td>168</td>
<td>426</td>
<td>287</td>
<td>106</td>
<td>364</td>
<td>238</td>
<td>200</td>
<td>312</td>
<td>191</td>
<td>1</td>
<td>-</td>
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<tr>
<td>SN99CH3.09a</td>
<td>168</td>
<td>303</td>
<td>168</td>
<td>429</td>
<td>287</td>
<td>101</td>
<td>364</td>
<td>247</td>
<td>212</td>
<td>312</td>
<td>201</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>SN99CH3.10a</td>
<td>162</td>
<td>303</td>
<td>168</td>
<td>429</td>
<td>287</td>
<td>101</td>
<td>364</td>
<td>243</td>
<td>212</td>
<td>312</td>
<td>191</td>
<td>2</td>
<td>-</td>
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<td>SN99CH3.19a</td>
<td>168</td>
<td>303</td>
<td>168</td>
<td>426</td>
<td>388</td>
<td>101</td>
<td>364</td>
<td>243</td>
<td>209</td>
<td>312</td>
<td>191</td>
<td>2</td>
<td>-</td>
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<td>SN99CH3.20a</td>
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<td>303</td>
<td>168</td>
<td>429</td>
<td>298</td>
<td>98</td>
<td>364</td>
<td>238</td>
<td>203</td>
<td>312</td>
<td>201</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>C1</td>
<td>164</td>
<td>306</td>
<td>168</td>
<td>441</td>
<td>287</td>
<td>98</td>
<td>403</td>
<td>243</td>
<td>206</td>
<td>312</td>
<td>201</td>
<td>1</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 2. The percentage of inoculated genotypes (including C1) found in the *P. nodorum* populations recovered from host treatments over time. Sample size is shown in parentheses.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Levis</td>
<td>98.1% (52)</td>
<td>41.5% (82)</td>
<td>27.7% (47)</td>
<td>12.7% (55)</td>
</tr>
<tr>
<td>Runal</td>
<td>82.1% (39)</td>
<td>56.5% (59)</td>
<td>35.2% (45)</td>
<td>39.1% (23)</td>
</tr>
<tr>
<td>1:1 Mixture of Runal and Tamaro</td>
<td>86.1% (36)</td>
<td>60.5% (38)</td>
<td>40.0% (30)</td>
<td>8.5% (47)</td>
</tr>
<tr>
<td>Tamaro</td>
<td>88.6% (35)</td>
<td>54.5% (88)</td>
<td>48.0% (75)</td>
<td>22.5% (80)</td>
</tr>
<tr>
<td>Tirone</td>
<td>79.0% (52)</td>
<td>62.2% (90)</td>
<td>36.7% (79)</td>
<td>22.2% (54)</td>
</tr>
<tr>
<td>Average</td>
<td>86.8% (42.8)</td>
<td>55.0% (71.4)</td>
<td>37.5% (55.8)</td>
<td>21.0% (51.2)</td>
</tr>
</tbody>
</table>
Table 3. Chi square-test for differences in genotype frequencies between \textit{P. nodorum} collections made from different hosts in 2004. Above the diagonal are comparisons between populations sampled in early 2004 (2004A) and below the diagonal are comparisons between populations made in late 2004 (2004B). Degrees of freedom are shown in parentheses.

<table>
<thead>
<tr>
<th></th>
<th>Levis</th>
<th>Runal</th>
<th>Mixture</th>
<th>Tamaro</th>
<th>Tirone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Levis</td>
<td>---</td>
<td>10.24 (7)</td>
<td>7.43 (6)</td>
<td>14.06 (8)</td>
<td>9.07 (8)</td>
</tr>
<tr>
<td>Runal</td>
<td>12.66 (7)</td>
<td>---</td>
<td>5.53 (7)</td>
<td>7.38 (8)</td>
<td>8.41 (8)</td>
</tr>
<tr>
<td>1:1 Mixture of Runal and Tamaro</td>
<td>5.42 (7)</td>
<td>9.29 (7)</td>
<td>---</td>
<td>6.69 (8)</td>
<td>11.37 (8)</td>
</tr>
<tr>
<td>Tamaro</td>
<td>7.32 (7)</td>
<td>13.39 (7)</td>
<td>4.86 (7)</td>
<td>---</td>
<td>14.98 (8)</td>
</tr>
<tr>
<td>Tirone</td>
<td>7.68 (8)</td>
<td>20.51 (8)**</td>
<td>14.59 (8)</td>
<td>16.33 (8)*</td>
<td>---</td>
</tr>
</tbody>
</table>

*Significant at p = 0.05 level

**Significant at p = 0.01 level
Table 4. Chi square-test for differences in genotype frequencies between *P. nodorum* collections made from different hosts in 2005. Above the diagonal are comparisons between populations sampled in 2005A and below the diagonal are comparisons between populations made in 2005B. Degrees of freedom are in parentheses.

<table>
<thead>
<tr>
<th></th>
<th>Levis</th>
<th>Runal</th>
<th>Mixture</th>
<th>Tamaro</th>
<th>Tirone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Levis</td>
<td>---</td>
<td>42.65 (8)***</td>
<td>10.81 (6)</td>
<td>5.64 (4)</td>
<td>12.03 (5)*</td>
</tr>
<tr>
<td>Runal</td>
<td>3.28 (4)</td>
<td>---</td>
<td>1.92 (5)</td>
<td>8.32 (6)</td>
<td>3.98 (7)</td>
</tr>
<tr>
<td>1:1 Mixture of Runal and Tamaro</td>
<td>4.52 (4)</td>
<td>4.79 (3)</td>
<td>---</td>
<td>7.8 (5)</td>
<td>3.9 (6)</td>
</tr>
<tr>
<td>Tamaro</td>
<td>4.64 (5)</td>
<td>8.4 (6)</td>
<td>10.47 (6)</td>
<td>---</td>
<td>4.76 (6)</td>
</tr>
<tr>
<td>Tirone</td>
<td>6.30 (5)</td>
<td>6.5 (6)</td>
<td>5.69 (5)</td>
<td>9.04 (6)</td>
<td>---</td>
</tr>
</tbody>
</table>

* Significant at p = 0.05 level

*** Significant at p = 0.0001 level
Table 5. Multiple population comparison for genotype frequencies among *P. nodorum* populations sampled from different hosts. Degrees of freedom are in parentheses.

<table>
<thead>
<tr>
<th>Collection</th>
<th>Chi square</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2004A</td>
<td>37.99 (36)</td>
<td></td>
</tr>
<tr>
<td>2004B</td>
<td>46.12 (36)</td>
<td></td>
</tr>
<tr>
<td>2005A</td>
<td>55.48 (36) *</td>
<td></td>
</tr>
</tbody>
</table>

*Significant at p = 0.05 level*
Table 6. Average selection coefficients and their standard deviations for the six released fungal strains on each host treatment. Different letters following mean values in the same column indicate that selection coefficients differ significantly at $P = 0.05$.

<table>
<thead>
<tr>
<th></th>
<th>Levis</th>
<th>Runal</th>
<th>Mixture</th>
<th>Tamaro</th>
<th>Tirone</th>
</tr>
</thead>
<tbody>
<tr>
<td>SN99CH 2.04a</td>
<td>0.51 (0.16) a</td>
<td>0.03 (0.07) c</td>
<td>0.34 (0.29) a</td>
<td>0.15 (0.27) a</td>
<td>0.42 (0.18) c</td>
</tr>
<tr>
<td>SN99CH2.09a</td>
<td>0.48 (0.21) a</td>
<td>0.59 (0.19) a</td>
<td>0.44 (0.29) a</td>
<td>0.03 (0.06) a</td>
<td>0.82 (0.13) a</td>
</tr>
<tr>
<td>SN99CH2.12a</td>
<td>0.22 (0.20) b</td>
<td>0.33 (0.18) b</td>
<td>0.40 (0.29) a</td>
<td>0.47 (0.08) b</td>
<td>0.29 (0.19) d</td>
</tr>
<tr>
<td>SN99CH2.35a</td>
<td>0.48 (0.26) a</td>
<td>0.52 (0.23) a</td>
<td>0.39 (0.31) a</td>
<td>0.83 (0.12) c</td>
<td>0.61 (0.15) b</td>
</tr>
<tr>
<td>SN99CH3.20a</td>
<td>0.30 (0.22) b</td>
<td>0.80 (0.11) d</td>
<td>0.40 (0.32) a</td>
<td>0.71 (0.60) d</td>
<td>0.29 (0.19) d</td>
</tr>
<tr>
<td>C1</td>
<td>0.29 (0.25) b</td>
<td>0.83 (0.10) d</td>
<td>0.43 (0.29) a</td>
<td>0.67 (0.12) d</td>
<td>0.05 (0.10) e</td>
</tr>
<tr>
<td>Average</td>
<td>0.38</td>
<td>0.52</td>
<td>0.40</td>
<td>0.48</td>
<td>0.41</td>
</tr>
</tbody>
</table>
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Table 7. Analysis of variance for selection coefficients of the six most frequent inoculated strains of *Phaeosphaeria nodorum*.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>F-ratio</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultivar</td>
<td>4</td>
<td>15.56</td>
<td>0.0001</td>
</tr>
<tr>
<td>Isolate</td>
<td>5</td>
<td>135.14</td>
<td>0.0001</td>
</tr>
<tr>
<td>Cultivar * Isolate</td>
<td>12</td>
<td>145.87</td>
<td>0.0001</td>
</tr>
</tbody>
</table>
Table 8. Chi square-test for the difference of genotype frequencies between *P. nodorum* collections made from the same host treatment at different times. Degrees of freedom are in parentheses.

<table>
<thead>
<tr>
<th></th>
<th>2004A vs. 2004B</th>
<th>2004 B vs. 2005A</th>
<th>2004A vs. 2005A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Levis</td>
<td>10.16 (7)</td>
<td>25.02 (8)**</td>
<td>17.5 (7) *</td>
</tr>
<tr>
<td>Runal</td>
<td>16.28 (7)*</td>
<td>10.82 (6)</td>
<td>11.82 (7)</td>
</tr>
<tr>
<td>1:1 Mixture of Runal and Tamaro</td>
<td>10.87 (7)</td>
<td>10.97 (8)</td>
<td>10.38 (6)</td>
</tr>
<tr>
<td>Tamaro</td>
<td>17.8 (8)*</td>
<td>17.25 (7)*</td>
<td>15.87 (8) *</td>
</tr>
<tr>
<td>Tirone</td>
<td>15.8 (8) *</td>
<td>15.6 (8)*</td>
<td>19.54 (8) *</td>
</tr>
</tbody>
</table>

* Significant at P = 0.05
** Significant at P = 0.001
Table 9. Chi square-test for the difference of genotype frequencies between *P. nodorum* collections sampled at different times. P-values are given in parentheses.

<table>
<thead>
<tr>
<th></th>
<th>2004 B</th>
<th>2005A</th>
<th>2005B</th>
</tr>
</thead>
<tbody>
<tr>
<td>2004A</td>
<td>22.63 (0.0039)</td>
<td>24.00 (0.0023)</td>
<td>34.83 (0.0001)</td>
</tr>
<tr>
<td>2004B</td>
<td>22.98 (0.0034)</td>
<td>44.67 (0.0001)</td>
<td></td>
</tr>
<tr>
<td>2005A</td>
<td></td>
<td>40.57 (0.0001)</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 4

Figure 1. Frequencies of nine *Phaeosphaeria nodorum* isolates recovered from different host treatments during the 2004 and 2005 growing seasons. Only the last part of each isolate name (See Table 1 for full names) is shown in the figure. A) Levis; B) Runal; C) 1:1 Mixture of Runal and Tamaro; D) Tamaro and E) Tirone.
Concordant evolution of mitochondrial and nuclear genomes in the wheat pathogen *Phaeosphaeria nodorum*
ABSTRACT

We compared patterns of mitochondrial restriction fragment length polymorphism (RFLP) diversity with patterns of nuclear RFLP diversity to investigate the effects of selection, gene flow, and sexual reproduction on the population genetic structure and evolutionary history of the wheat pathogen *Phaeosphaeria nodorum*. A total of 315 fungal isolates from Texas, Oregon, and Switzerland were analyzed using seven nuclear RFLP probes that hybridized to discrete loci and purified mitochondrial DNA that hybridized to the entire mtDNA genome. Forty-two different mitochondrial haplotypes and 298 different nuclear haplotypes were detected. The two most frequent mtDNA haplotypes were present in every population and represented 32% of all isolates. High levels of gene flow, low levels of population subdivision, no evidence of host specificity and cyto-nuclear disequilibrium were inferred from the analysis of both genomes. The concordance in estimates of population genetic parameters from both genomes suggests that the two genomes underwent similar evolutionary processes.

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Patterns of genetic variation within a fungal species result from interactions among evolutionary forces of natural selection, genetic drift, gene flow, reproductive mode and mutation rate (Zhan and McDonald, 2004). Determining the amount and distribution of genetic variation can contribute to our understanding of the evolutionary history of fungi as well as their potential to adapt to changing environments (McDonald and Linde, 2002). Though each fungal cell contains two sets of genomes (i.e. mitochondrial and nuclear), the majority of our understanding of the evolutionary history of fungi is based on the information derived from genetic analysis of only one of the genomes (e.g. Douhan et al., 2002; Keller et al., 1997a, b). Because the nuclear (nu) and mitochondrial (mt) genomes may differ in their biological, evolutionary and demographic processes, the pattern of genetic variation may vary substantially across different genomes, as seen in other eukaryotes (e.g. Bensch et al., 2006; Sachidanandam, 2001). Under this scenario, information on the pattern of genetic variation in a species inferred from only one genome could be misleading.

Mitochondrial genomes are in general uniparentally inherited (Taylor, 1986; but see Saville et al., 1998). Due to the lack of recombination in mtDNA genomes, any evolutionary forces affecting one part of the genome would affect all other parts of the genome equally (Ballard and Whitlock, 2004). The fact that the entire mtDNA genome is genetically linked can be useful when addressing questions related to natural selection, migration, and relatedness of species. Because of these unique properties, mtDNA markers have been widely used in fungi to study genetic variation (Láday et al., 2004a; Miller et al., 1999; Xu et al., 1998), vegetative incompatibility (Gordon et al., 1992; Gray, 1989; Jacobson and Gordon, 1990; Smith et al., 1990), phylogenic relationships (Redberg et al., 2003) and selective sweeps (Zhan et al., 2004). Furthermore, due to its functional importance, changes in mtDNA sequence may have a substantial impact not only on the fitness of mitochondria themselves but also on their associations with nuclear genes, as the function of mitochondria depends on interactions between the two genomes (Olson and Stenlid, 2001). On the other hand, nuclear genomes are usually bi-parentally inherited and function as the primary genetic reservoir determining the biological and evolutionary processes of species and therefore are valuable for addressing
questions such as the effect of mating system on the evolution of species. Because of their contrasting modes of inheritance, combined analysis of nuclear and mitochondrial genomes can provide more comprehensive insights into the evolutionary forces acting on natural populations (Asmussen and Basten, 1994).

Here we conducted a comparative analysis of population genetic structure using the mitochondrial genome and the nuclear genome in fungus *Phaeosphaeria nodorum*. *P. nodorum* (E. Müller) Hedjaroude, anamorph *Stagonospora nodorum* (Berk.) Castellani & E.G. Germano is the causal agent of Septoria glume blotch on wheat and can be found in all wheat growing areas of the world (Eyal et al., 1987; Eyal, 1999). Over the past decade, the population genetic structure of this fungus has been well characterized by RFLP, microsatellite, and mating-type analyses of its nuclear genome (Bennet et al., 2003; Halama, 2002; Keller et al., 1997a, b; McDonald et al., 1994; Solomon et al., 2004; Sommerhalder et al., 2006; Stukenbrock et al., 2006). These molecular analyses revealed that the fungal populations were characterized by high nuclear diversity, low population subdivision (Keller et al., 1997a, b; Stukenbrock et al., 2006) and a mixed sexual and asexual reproductive system (Sommerhalder et al., 2006).

The main objectives of this project were: (1) to compare the patterns of population genetic structure in the mitochondrial and nuclear genomes and; (2) to determine the effect of natural selection on the population genetic structure of the mitochondrial genome. Earlier works on fungi (Fry et al., 1992; Griffith and Shaw, 1998; Ramsfield et al., 1999; Gosselin et al., 1999; Zhan et al., 2003, 2004) and other eukaryotes (Gerber et al., 2001; Nachman et al., 1994) have shown contrasting patterns of genetic variation between the two genomes and evidence for non-neutral evolution in the mitochondrial genome. In the most comprehensive analysis of mtDNA diversity in fungi to date, a global sample of nearly 1700 isolates of *Mycosphaerella graminicola*, a fungal pathogen sharing the same ecological niche as *P. nodorum* and having a nearly identical life cycle, were assayed for DNA fingerprints and RFLP markers in the nuclear and mitochondrial genomes. Genetic variation in the nuclear genome of *M. graminicola* was high for the majority of the populations surveyed, with more than 1300 distinct genotypes detected. In contrast, only seven mtDNA haplotypes were detected and the two most common mtDNA haplotypes represented approximately 93% of the world population (Zhan et al., 2003). Further analysis revealed a significant difference in the frequency of mtDNA haplotypes across different hosts (Zhan et al., 2004), suggesting the low genetic variation found in the nuclear
genome of *M. graminicola* was likely due to natural selection. Based on these earlier findings, we hypothesize: 1) the pattern of genetic variation in the nuclear genome of *P. nodorum* differs from the pattern of genetic variation in the mitochondrial genome for any population; 2) fungal populations sampled from different geographic regions and host genotypes differs in the frequency of mtDNA haplotypes; and 3) there is no random association between mtDNA haplotypes and nuclear alleles.
CHAPTER 5

MATERIALS AND METHODS

Fungal isolates. A total of 315 *S. nodorum* isolates were sampled from eight wheat fields in Texas, Oregon and Switzerland (Table 1) using a hierarchical sampling strategy described previously (McDonald et al., 1999a). The Texas population was sampled from two wheat nurseries located in Overton (McDonald et al., 1994). The Oregon population was collected from a commercial field sited in Hyslop (Keller et al., 1997b), and the Swiss population was sampled from five naturally infected commercial fields located in Eschikon, Nänikon, Oberembrach, and Wetzwil. Each of the Swiss fields was planted with one of three wheat cultivars (Arina, Galaxy, and Tamaro) as described earlier (Keller et al., 1997a).

RFLP and PCR analysis of fungal strains. DNA extraction was performed as described previously (McDonald et al., 1994). Total DNA from each isolate was digested with the restriction enzyme *Eco*RI. DNA fragments were separated by electrophoresis and transferred to nylon membranes by capillary Southern blotting (McDonald et al., 1994). Eight anonymous RFLP probes chosen from a *P. nodorum* genomic library (Keller et al., 1997a, b; McDonald et al., 1994) were hybridized in sequence to the Southern blots. Seven unlinked RFLP probes (pJSN3, pSNL13, pSNL15, pJSN27, pJSN35, pJSN73, and pJSN121) contains a total of 12,400 bp of cloned *P. nodorum* nuclear DNA and hybridize to individual nuclear loci. The remaining probe (pSNS4) hybridizes to a moderately repetitive DNA sequence dispersed across the nuclear genome. Mitochondrial haplotypes of the strains were determined by hybridizing the Southern blots to a purified sample of the entire mtDNA genome (approximately 55 kb in size, McDonald et al., 1999b) originating from a Texas isolate of *P. nodorum*. Pure mtDNA was isolated using the cesium chloride gradient ultracentrifugation method as described by Garber and Yoder (1983). The probes were radioactively labeled with dCT$^{32}$P by nick translation according to the manufacturer’s instructions (Bio-Rad) and then hybridized to the blots overnight in a hybridization incubator (McDonald et al., 1994). Mating types of the fungal strains were determined by PCR amplification using four mating-type-specific primers (Bennett et al., 2003) as described in Sommerhalder et al. (2006).
Data analysis. Fungal isolates were grouped into three regional populations (Oregon, Texas and Switzerland) according to their geographic origins. Swiss isolates were further grouped into three subpopulations according to locations (Wetzwil, Nänikon and Eschikon-Oberembrach) from which the isolates were collected. Subpopulation Wetzwil was sampled from two fields planted with wheat cultivars Arina and Tamaro, subpopulation Nänikon was sampled from a field planted with cultivar Galaxy, and subpopulation Eschikon-Oberembrach was sampled from two fields planted with cultivars Tamaro and Arina. The isolates from Switzerland were also grouped according to the cultivars from which they were collected (Arina, Galaxy, and Tamaro) to determine the effect of host selection on the population genetic structure of nuclear and mitochondrial genomes.

To analyze genotype diversity in the nuclear genome, multilocus haplotypes (MLHTs) were formed by joining the alleles present at each RFLP locus in the same order, creating a seven-digit number for each isolate. The series of hybridizing fragments generated by probe pSNS4 was treated as a DNA fingerprint (McDonald et al., 1994). To analyze mitochondrial diversity, isolates with different mtDNA RFLP profiles were considered as different mtDNA haplotypes. Isolates having the same MLHT, mating type idiomorph, mtDNA haplotype and DNA fingerprint were treated as a clone, the asexual progeny of the same genotype. Only one representative of each clone was used for the data analysis, i.e. analyses of allele frequencies and other population genetic parameters were performed on clone-corrected data.

Because of differences in sample size, populations from different regions were standardized to the size of the smallest regional population (Texas, n = 47) by nonparametric bootstrap analysis (Leberg, 2002; Zhan et al., 2003) and mitochondrial richness among populations was compared using the average estimate of 100 re-samples with replacement using the Resampling Stats software package (Version 5.02, 2000; Resampling Stats). Allele frequencies were calculated for each locus at the regional population level, local population level and cultivar level. Genetic variation in each population was quantified using measures of gene diversity (Nei, 1973). Contingency chi-square (Everitt, 1977) tests were used to detect differences in allele and mtDNA haplotype frequencies among *P. nodorum* collections originating from different cultivars in Switzerland. For this test, alleles and mtDNA haplotypes present at frequencies lower than 5% were pooled into a single category (Zhan and McDonald, 2005). The differences among regional and field populations were quantified by Nei’s measure
of population differentiation ($G_{ST}$) (Nei, 1973) and genetic distance (Nei, 1972, 1978) and average levels of gene flow ($N_m$) among the populations were estimated accordingly (Boeger et al., 1993). Differences in mtDNA haplotype richness were tested using a two-tailed $t$ test. Neutral evolution in mitochondrial genome was further tested with Tajima’s (Tajima, 1989) and Fu and Li’s index (Fu and Li, 1993) by using a computer program implemented in http://www.hgc.sph.uth.tmc.edu/fu/genealogy/test2/welcome.html.

Cyto-nuclear disequilibrium was evaluated using $\chi^2$ tests for the associations between nuclear RFLP loci and mitochondria (locus-by-locus comparisons) and between nuclear RFLP alleles and mitochondrial haplotypes (allele-by-allele comparisons) using methods described by Weir (1979) and implemented in the POPGENE 3.2 software package (Yeh and Boyle, 1997). The cyto-nuclear disequilibria were evaluated for each regional population and its subpopulations. For these tests, the entire mitochondrial genome was considered as a single locus and each mitochondrial haplotype was treated as an allele. For the locus-by-locus tests, alleles present at frequencies lower than 5% were pooled into a single category. In the allele-by-allele tests, the expected number of nonrandom allelic associations at the 5% level was calculated for each locus and compared to the observed number of nonrandom allelic associations to determine whether the observed number of nonrandom allelic associations arose from chance. A Bonferroni correction was applied to this analysis to avoid false rejections of the null hypothesis due to the large number of comparisons performed (Bonferroni, 1935).
CHAPTER 5

RESULTS

MtDNA haplotype variation. Of the 42 distinct mtDNA haplotypes found among the 315 isolates assayed, nine were shared by all populations and twenty were private to specific populations. Two dominant haplotypes (Types E and F, Fig. 1) were found in all locations and represented 32% of all isolates (Table 2). The region with the most mitochondrial haplotypes was Switzerland followed by Oregon and Texas (Table 1). Among the 20 private haplotypes, fourteen were detected in the Swiss population and three each in the Oregon and Texas populations (Table 1). After normalizing sample sizes and conducting bootstrap analysis, the difference in mitochondrial richness between Switzerland and the North American collections became smaller but remained significant. The number of mitochondrial haplotypes in Switzerland was reduced to seventeen while the number of mitochondrial haplotypes in Oregon and Texas was reduced to thirteen each.

NuDNA genetic variation. The number of alleles per locus ranged from two to six with an average of 4.7 alleles per locus (Table 1). Eleven private alleles were found among the seven RFLP loci analyzed, and all of them were detected in the Swiss population. Nei’s measure of gene diversity ranged from 0.42 to 0.52 for each population/subpopulation with an average of 0.49 across all populations (Table 1). All of the most common alleles in the nuclear genome were shared among the different populations, locations and cultivars. The combined analysis of nuclear RFLP loci, DNA fingerprint, mitochondrial haplotype and the MAT locus revealed no clones in the Swiss population. Thirteen and four clones were found in Texas and Oregon respectively. In all cases, the clones were found only among isolates sampled from the same fields, consistent with the splash-dispersal of asexual conidia.

Population subdivision and gene flow. In the nuclear genome, the average $G_{ST}$ was 0.01 (Table 3) and average genetic distance across the seven RFLP loci ranged from 0.01 to 0.02 (Table 3) when pairwise comparisons were made for populations from different locations within Switzerland. For pairwise comparisons of populations from different regions, the average $G_{ST}$
CHAPTER 5

across the seven RFLP loci ranged from 0.02 to 0.05 (Table 3) and the average genetic distance across the seven RFLP loci ranged from 0.04 to 0.15 (Table 3). In the mitochondrial genome, the pairwise $G_{ST}$ was 0.01 (Table 3) and average genetic distance ranged from 0.03 to 0.17 (Table 3) for pairwise comparisons within Switzerland. The pairwise $G_{ST}$ ranged from 0.01 to 0.02 (Table 2) and average genetic distance ranged from 0.09 to 0.22 (Table 3) when populations from different regions were compared.

Within Switzerland, the estimated average number of migrants exchanged among populations each generation assuming drift-migration equilibrium ($N_{e,m}$) ranged from 38 to 50 for the nuclear genome and from 45 to 83 for the mitochondrial genome (Table 3). $N_{e,m}$ ranged from 9 to 21 for the nuclear genome and from 33 to 66 for the mitochondrial genome when populations were compared across the regions.

**Effect of host cultivars on *P. nodorum***. Fungal isolates collected from different host cultivars shared all common nuclear RFLP alleles and mtDNA haplotypes. Chi square analysis revealed significant differences in allele frequency for two of seven nuclear loci when cultivar Arina was compared to cultivar Galaxy. No significant differences in nuclear allele frequency were found in other comparisons (Table 4). The chi-square test did not detect any statistical differences in mtDNA haplotype frequencies among *P. nodorum* isolates collected from different host cultivars (Table 4) and evidence of natural selection on the mitochondrial genome was not found (Table 5).

**Nuclear-mitochondrial association: Locus-by-locus analysis**: The probability of non-random association between seven nuclear RFLP loci and the mitochondrial haplotype ranged from 0.01-0.99 in the locus-by-locus analysis. Among the 42 comparisons, only two loci were in nuclear-mitochondrial disequilibrium. The significant disequilibria were found at the pJSN27 locus in the subpopulation originating from cultivar Tamaro and at the pJSN3 locus in the subpopulation from Wetzwil (Table 6).

**Nuclear-mitochondrial association: Allele-by-allele analysis**: Allele-by-allele tests showed that 47 of the 1224 allele-by-mtDNA haplotype combinations were significantly more or less frequent than expected in the Swiss population (Table 7). Among these, 26 were significant at P
≤ 0.05 and 21 were significant at P ≤ 0.01. Thirteen of 360 nuclear allele-mtDNA haplotype pairs in the Oregon population departed significantly from random association at P ≤ 0.05 in addition to another twelve pairs at P ≤ 0.01. In the population from Texas thirteen out of 374 nuclear allele-mtDNA haplotype pairs (6.7%) significantly deviated from the expectation of cytonuclear equilibrium at P ≤ 0.05 and another thirteen deviated from the expectation of cytonuclear equilibrium at P ≤ 0.01 (Table 7). Following Bonferroni correction of the data, only 49 of the 270 significant cases of disequilibrium found across all comparisons remained significant (Table 7). Further analysis revealed that all significant comparisons after the Bonferroni correction involved either rare (present at frequencies lower than 5%) combinations of mtDNA haplotypes and nuclear alleles or mtDNA haplotypes that were present only once in the population (data not shown). For example, in Oregon the only non-random association after Bonferroni correction involved a mtDNA haplotype that was present only once in the population. In Texas, two of four non-random associations involved mtDNA haplotypes present only once in the population while the remaining associations involved mtDNA haplotypes that were found only twice in the population. In Switzerland, one of eight cases involved a mtDNA haplotype found only once in the population and other seven cases involved mtDNA haplotypes present at frequencies lower than 5%.
Population genetic studies on *P. nodorum* in the past focused on the amount and distribution of genetic variation in its nuDNA (Keller et al., 1997a, b; Murphy et al., 2000), while little information was available regarding the pattern of genetic variation in the mtDNA. We conducted a comparative analysis of the genetic variation in both genomes on 315 fungal isolates sampled from three regions and three cultivars. This type of analysis is rare in fungi. Because of the reported low mtDNA mutation rate as compared to the nuDNA in fungi (Clark-Walker, 1991), we hypothesize that the mtDNA of *P. nodorum* would have lower genetic variation than the nuDNA, as found in *M. graminicola* (Zhan et al., 2003, 2004) and others (Carlisle et al., 2001; Ghimire et al., 2003; Láday et al., 2004a, b; Xu et al., 1998). We also hypothesize that there would be different patterns of spatial population genetic structure between the two genomes. Surprisingly, we found that a considerable amount of genetic variation existed in the mtDNA of *P. nodorum*. Forty two mtDNA haplotypes were detected among the 315 isolates, compared to an average of 4.7 alleles detected for the seven nuclear loci. It is possible that the higher mtDNA diversity detected in *P. nodorum* reflects the fact that the entire mtDNA genome was assayed, while only a small fraction of the nuclear genome was assayed. The probe used to distinguish mtDNA haplotypes represented the entire mtDNA genome (average size of approximately 55 kb), while probes used to detect variation in the nuclear genome covered only approximately 12 kb, representing a very small fraction (0.03%) of the approximately 37 Mb nuclear genome.

We also found that genetic variation in the two genomes was distributed in the same way among the different populations. Previous studies revealed a higher level of nuDNA diversity in the Swiss population of *P. nodorum* as compared to others (Keller et al., 1997b; Stukenbrock et al., 2006) and our results indicated the same pattern for the mtDNA. The difference in allele richness among fungal populations sampled from USA and Switzerland probably reflects migration routes of *P. nodorum* and its wheat host during colonization of the New World by Europeans. Wheat was introduced from Europe into North America about 500 years ago and a recent study on migration patterns of *P. nodorum* indicated that Europe is the most likely source of immigrants into North America (Stukenbrock et al., 2006). The finding of comparable
amounts of genetic variation coupled with concordant estimates of other population genetic parameters such as $G_{ST}$ and genetic distance (Table 3) suggests that the two genomes have undergone similar evolutionary processes. It also suggests that, in *P. nodorum*, population genetic parameters derived from either genome should be sufficient to understand the evolutionary history of the fungus.

The high amount of genetic variation found in the mtDNA of *P. nodorum* may reflect recurring gene flow among geographic locations as revealed by the nuDNA (Keller et al., 1997b; Stukenbrock et al., 2006). Nine mtDNA haplotypes were shared among populations sampled from different regions. The finding of a large number of shared mtDNA haplotypes across continents is congruent with substantial gene flow; which allows genetic connectivity (Lowe et al., 2004) among geographically separated areas. The estimates of 11-40 successful immigrants per generation among different regions are realistic considering that the wheat industry is global, with thousands of tons of grain moved around the world daily (Mercier, 1999). Movement among continents could occur on infected seed, which is known to occur commonly for this pathogen (Diekmann and Putter, 1995). At regional scales (eg over 100s of km), gene flow can occur by wind dispersal of airborne ascospores, either directly from one field to another or in a stepping-stone model.

The high genetic variation also suggests that natural selection may not have had a significant impact on the population genetic structure of the mtDNA of *P. nodorum*. We have three lines of evidence to support the neutral evolution of the mtDNA in this fungus. First, we did not find any difference in the frequencies of mtDNA haplotypes sampled from different cultivars within the same fields in Switzerland and among samples from different geographic regions. The major mtDNA haplotypes were shared among fungal populations sampled from different geographic regions and hosts. Second, we did not find evidence for cyto-nuclear association. An estimate of association between alleles in the nuDNA and haplotypes of cytoplasmically inherited organelles (Asmussen et al., 1987) such as mitochondria offers a powerful tool to study the nature of selection (Thomson and Klitz, 1987). Interactions between mitochondrial and nuclear genomes that confer differences in fitness among mtDNA haplotypes and nuclear alleles could lead to cyto-nuclear disequilibrium. Our calculation of mitochondrial-nuclear association revealed only two cases of statistically significant disequilibrium in the locus-by-locus analysis. Further analyses at the allele level revealed that all the allele-by-allele
comparisons which gave rise to disequilibrium involved nuclear alleles and/or mtDNA haplotypes that were present only once or at very low frequencies. Thus we believe that the disequilibrium found in these cases was not due to natural selection, but rather to the combined effects of chance and rare alleles, as extreme allele frequencies are known to provide misleading measures of disequilibrium (Asmussen and Basten, 1994). Third, tests for neutrality failed to detect any evidence of selection in the mtDNA (Table 5). Tajima’s D (Tajima, 1989) and Fu and Li’s D (Fu and Li 1993) are sensible to alleles with low frequency. There are many rare mitochondrial haplotypes (alleles) in *P. nodorum*. In this case, the two methods we used should be appropriate to detect selection in the mitochondrial of *P. nodorum*.

Drift could be common for pathogens in agricultural ecosystems due to the repeating epidemic cycles characterized by substantial expansion and contraction of population size (Zhan et al., 2004). Compared to the biparentally inherited nuDNA, the uniparentally inherited mtDNA is more prone to the effects of genetic drift (Korpelainen, 2004; Zhan and McDonald, 2005). The high genetic variation found in both *P. nodorum* genomes is consistent with a low degree of genetic drift, suggesting a large effective population size and a lack of population bottlenecks. The effective size of a species is related to its mating system, dispersal mechanisms and gene flow (Barton and Whitlock, 1997; Li, 1955; Wright, 1943). Large effective population size in *P. nodorum* is likely due to several factors, including recurring sexual outcrossing, long distance dispersal of ascospores and high levels of gene flow over large spatial scales mediated by movement of infected seeds. The findings of random association of RFLP and microsatellite markers in the nuclear genome (Keller et al., 1997b; Stukenbrock et al., 2006), equal frequencies of both mating types across the majority of field populations (Sommerhalder et al., 2006) and the lack of mitochondrial-nuclear disequilibrium (Table 6) support the hypothesis that random mating plays an important role in the population genetic structure of this fungus. Random mating among individuals tends to increase the effective population size (Wright, 1943) because the reproductive success of individuals in this case can be attributed to unrelated alleles at each locus (Nunney, 1999). The capacity for long distance dispersal of sexual ascospores may further enhance effective population size by decreasing the likelihood that related individuals will mate (Gilbert et al., 1991). Finally, high gene flow among *P. nodorum* populations (Table 3, Keller et al. 1997a, b; Stukenbrock et al., 2006) also increases the effective population size (Slatkin and Voelm, 1991) by linking fragmented populations across large regions.
AKNOWLEDGEMENTS

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CHAPTER 5

LITERATURE CITED


CHAPTER 5


CHAPTER 5


CHAPTER 5


### TABLE 1. Genetic diversity in nuclear and mitochondrial genomes in populations of *Phaeosphaeria nodorum*.

<table>
<thead>
<tr>
<th>Region</th>
<th>No. isolates&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. genotypes&lt;sup&gt;b&lt;/sup&gt;</th>
<th>No. mtDNA haplotypes (private)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Nuclear alleles/locus&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Nei’s nuclear gene diversity&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oregon</td>
<td>66</td>
<td>62</td>
<td>18 (3)</td>
<td>2.9</td>
<td>0.42</td>
</tr>
<tr>
<td>Texas</td>
<td>60</td>
<td>47</td>
<td>17 (3)</td>
<td>3.1</td>
<td>0.44</td>
</tr>
<tr>
<td>Switzerland</td>
<td>189</td>
<td>189</td>
<td>36 (14)</td>
<td>4.9</td>
<td>0.50</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wetzwil</td>
<td>62</td>
<td>62</td>
<td>26 (5)</td>
<td>4.0</td>
<td>0.50</td>
</tr>
<tr>
<td>Nänikon</td>
<td>41</td>
<td>41</td>
<td>19 (3)</td>
<td>3.3</td>
<td>0.48</td>
</tr>
<tr>
<td>Eschikon-Oberembrach</td>
<td>86</td>
<td>86</td>
<td>17 (4)</td>
<td>4.4</td>
<td>0.50</td>
</tr>
<tr>
<td>Cultivar</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arina</td>
<td>62</td>
<td>62</td>
<td>19 (3)</td>
<td>4.0</td>
<td>0.50</td>
</tr>
<tr>
<td>Galaxy</td>
<td>41</td>
<td>41</td>
<td>28 (6)</td>
<td>4.4</td>
<td>0.52</td>
</tr>
<tr>
<td>Tamaro</td>
<td>86</td>
<td>86</td>
<td>23 (4)</td>
<td>3.6</td>
<td>0.50</td>
</tr>
<tr>
<td>TOTAL</td>
<td>315</td>
<td>298</td>
<td>42 (20)</td>
<td>4.7</td>
<td>0.49</td>
</tr>
</tbody>
</table>

<sup>a</sup> Total number of isolates assayed in each population.

<sup>b</sup> Total number of genotypes found in the population.

<sup>c</sup> Number of mtDNA haplotypes and number of private mtDNA haplotypes (in parenthesis) found in each population.

<sup>d</sup> Average number of nuclear alleles per locus in each population.

<sup>e</sup> Nei’s (1973) gene diversity.
CHAPTER 5

Table 2. Frequencies of the most common mitochondrial haplotypes in each population of *Phaeosphaeria nodorum*.

<table>
<thead>
<tr>
<th>MtDNA haplotype</th>
<th>E</th>
<th>F</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>L</th>
<th>a</th>
<th>G</th>
<th>Q</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oregon</td>
<td>0.24</td>
<td>0.18</td>
<td>0.00</td>
<td>0.02</td>
<td>0.16</td>
<td>0.08</td>
<td>0.03</td>
<td>0.00</td>
<td>0.00</td>
<td>0.03</td>
</tr>
<tr>
<td>Texas</td>
<td>0.19</td>
<td>0.17</td>
<td>0.09</td>
<td>0.09</td>
<td>0.06</td>
<td>0.02</td>
<td>0.02</td>
<td>0.00</td>
<td>0.09</td>
<td>0.00</td>
</tr>
<tr>
<td>Switzerland</td>
<td>0.15</td>
<td>0.13</td>
<td>0.13</td>
<td>0.09</td>
<td>0.04</td>
<td>0.05</td>
<td>0.04</td>
<td>0.05</td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>Total</td>
<td>0.17</td>
<td>0.15</td>
<td>0.10</td>
<td>0.07</td>
<td>0.07</td>
<td>0.05</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
</tr>
</tbody>
</table>
Table 3. Pairwise comparisons of population differentiation for mitochondrial and nuclear genomes in *Phaeosphaeria nodorum*.

<table>
<thead>
<tr>
<th>Regions</th>
<th>Swiss Locations</th>
<th>Cultivars</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Texas-Oregon</td>
<td>Swiss-Oregon</td>
</tr>
<tr>
<td>NuDNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$G_{ST}^a$</td>
<td>0.05</td>
<td>0.03</td>
</tr>
<tr>
<td>$D^b$</td>
<td>0.15</td>
<td>0.04</td>
</tr>
<tr>
<td>$N_e m^c$</td>
<td>9</td>
<td>17</td>
</tr>
<tr>
<td>MtDNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$G_{ST}$</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>$D$</td>
<td>0.15</td>
<td>0.22</td>
</tr>
<tr>
<td>$N_e m$</td>
<td>35</td>
<td>33</td>
</tr>
</tbody>
</table>

* $G_{ST}$: Nei’s coefficient of population differentiation.
* $D$: Nei’s unbiased measure of genetic distance.
* $N_e m$: Estimated number of migrants per generation.
Table 4. Chi-square test for homogeneity in mitochondrial haplotype frequencies and nuclear allele frequencies between *Phaeosphaeria nodorum* isolates collected from three different cultivars in Switzerland.

<table>
<thead>
<tr>
<th>Nuclear</th>
<th>Arina-Galaxy</th>
<th>Arina-Tamaro</th>
<th>Galaxy-Tamaro</th>
</tr>
</thead>
<tbody>
<tr>
<td>mtDNA</td>
<td>15.62 (15)</td>
<td>7.38 (9)</td>
<td>5.60 (13)</td>
</tr>
<tr>
<td>pJSN121-<em>Eco</em>RI</td>
<td>8.12* (2)</td>
<td>7.55 (3)</td>
<td>1.06 (3)</td>
</tr>
<tr>
<td>pJSN73-<em>Eco</em>RI</td>
<td>3.56 (3)</td>
<td>3.86 (4)</td>
<td>4.19 (4)</td>
</tr>
<tr>
<td>pJSNL15-<em>Eco</em>RI</td>
<td>0.60 (2)</td>
<td>0.95 (3)</td>
<td>0.53 (3)</td>
</tr>
<tr>
<td>pJSNL35-<em>Eco</em>RI</td>
<td>3.00 (2)</td>
<td>2.64 (2)</td>
<td>0.13 (2)</td>
</tr>
<tr>
<td>pJSN3-<em>Eco</em>RI</td>
<td>3.25 (3)</td>
<td>0.73 (2)</td>
<td>2.84 (3)</td>
</tr>
<tr>
<td>pJSN27-<em>Eco</em>RI</td>
<td>11.07* (3)</td>
<td>5.22 (3)</td>
<td>3.90 (3)</td>
</tr>
<tr>
<td>SNL13-<em>Eco</em>RI</td>
<td>0.33 (3)</td>
<td>0.83 (3)</td>
<td>1.53 (3)</td>
</tr>
</tbody>
</table>

* Degrees of freedom are in parenthesis.

* Significant at P ≤ 0.05.
Table 5 Neutrality tests for the mitochondrial genome of *P. nodorum* sampled from Oregon, Switzerland and Texas

<table>
<thead>
<tr>
<th>Populations</th>
<th>Tajima’s D</th>
<th>Fu and Li’s D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Texas</td>
<td>0.02 (-1.40)(^1)</td>
<td>1.30 (-1.90)</td>
</tr>
<tr>
<td>Oregon</td>
<td>-0.87 (-1.39)</td>
<td>2.47 (-1.93)</td>
</tr>
<tr>
<td>Switzerland</td>
<td>-1.05 (-1.38)</td>
<td>0.71 (-1.63)</td>
</tr>
<tr>
<td>Arina</td>
<td>0.32 (-1.36)</td>
<td>1.07 (-1.88)</td>
</tr>
<tr>
<td>Galaxy</td>
<td>-0.07 (-1.41)</td>
<td>0.15 (-1.85)</td>
</tr>
<tr>
<td>Tamaro</td>
<td>-1.25 (-1.38)</td>
<td>1.90 (-1.92)</td>
</tr>
</tbody>
</table>

\(^1\) Data within parenthesis is the significant value at p = 0.05. Estimated Ds larger than the significant values indicate the hypothesis for natural selection is not retained.
CHAPTER 5

Table 6. P-values for locus-by-locus tests for disequilibrium between nuclear and mitochondrial genomes.

<table>
<thead>
<tr>
<th>Locus-by-locus combination&lt;sup&gt;a&lt;/sup&gt;</th>
<th>pJSN121-mt haplotype</th>
<th>pJSN73-mt haplotype</th>
<th>pSNL15-mt haplotype</th>
<th>pJSN35-mt haplotype</th>
<th>pJSN3-mt haplotype</th>
<th>pJSN27-mt haplotype</th>
<th>SNL13-mt haplotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Region</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oregon</td>
<td>0.26</td>
<td>0.33</td>
<td>0.35</td>
<td>0.53</td>
<td>0.36</td>
<td>0.91</td>
<td>0.54</td>
</tr>
<tr>
<td>Texas</td>
<td>0.99</td>
<td>0.49</td>
<td>0.21</td>
<td>0.96</td>
<td>0.48</td>
<td>0.26</td>
<td>0.23</td>
</tr>
<tr>
<td>Switzerland</td>
<td>0.15</td>
<td>0.57</td>
<td>0.67</td>
<td>0.44</td>
<td>0.97</td>
<td>0.12</td>
<td>0.46</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wetzwil</td>
<td>0.59</td>
<td>0.94</td>
<td>0.82</td>
<td>0.26</td>
<td>0.01&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.23</td>
<td>0.36</td>
</tr>
<tr>
<td>Nänikon</td>
<td>0.20</td>
<td>0.99</td>
<td>0.59</td>
<td>0.23</td>
<td>0.62</td>
<td>0.20</td>
<td>0.35</td>
</tr>
<tr>
<td>Eschikon-Oberembrach</td>
<td>0.32</td>
<td>0.77</td>
<td>0.23</td>
<td>0.87</td>
<td>0.99</td>
<td>0.31</td>
<td>0.13</td>
</tr>
<tr>
<td>Cultivar</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arina</td>
<td>0.46</td>
<td>0.99</td>
<td>0.61</td>
<td>0.20</td>
<td>0.75</td>
<td>0.11</td>
<td>0.34</td>
</tr>
<tr>
<td>Galaxy</td>
<td>0.20</td>
<td>0.99</td>
<td>0.59</td>
<td>0.23</td>
<td>0.62</td>
<td>0.20</td>
<td>0.35</td>
</tr>
<tr>
<td>Tamaro</td>
<td>0.26</td>
<td>0.22</td>
<td>0.92</td>
<td>0.12</td>
<td>0.24</td>
<td>0.01&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.20</td>
</tr>
</tbody>
</table>

<sup>*</sup> Significant nuclear locus-by-mitochondrial haplotype association.
CHAPTER 5

Table 7. Allele-by-allele tests for disequilibrium between nuclear and mitochondrial genomes among the fungal isolates sampled from different cultivars, fields within Switzerland and different regions.

<table>
<thead>
<tr>
<th>Populations</th>
<th>Oregon</th>
<th>Texas</th>
<th>Switzerland</th>
<th>Wetzwil</th>
<th>Nänikon</th>
<th>Eschikon/Oberembrach</th>
<th>Arina</th>
<th>Galaxy</th>
<th>Tamaro</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total possible allele combinations</td>
<td>360</td>
<td>374</td>
<td>1224</td>
<td>728</td>
<td>418</td>
<td>527</td>
<td>713</td>
<td>418</td>
<td>868</td>
</tr>
<tr>
<td>P ≤ 0.05</td>
<td>13</td>
<td>13</td>
<td>21</td>
<td>17</td>
<td>7</td>
<td>14</td>
<td>19</td>
<td>7</td>
<td>17</td>
</tr>
<tr>
<td>P ≤ 0.01</td>
<td>12</td>
<td>13</td>
<td>26</td>
<td>22</td>
<td>17</td>
<td>10</td>
<td>7</td>
<td>17</td>
<td>18</td>
</tr>
<tr>
<td>Number in disequilibrium(^a)</td>
<td>25 (1)</td>
<td>26 (4)</td>
<td>47 (8)</td>
<td>39 (9)</td>
<td>24 (3)</td>
<td>24 (8)</td>
<td>26 (4)</td>
<td>24 (3)</td>
<td>35 (9)</td>
</tr>
<tr>
<td>Percentage in disequilibrium(^b)</td>
<td>6.9% (0.3%)</td>
<td>7.0% (1.1%)</td>
<td>3.8% (0.7%)</td>
<td>5.4% (1.0%)</td>
<td>5.7% (0.5%)</td>
<td>4.6% (1.5%)</td>
<td>3.6% (0.6%)</td>
<td>5.7% (0.7%)</td>
<td>4.0% (1.0%)</td>
</tr>
</tbody>
</table>

\(^a\) The number of observed pairs in disequilibrium before and after (in parenthesis) Bonferroni correction.

\(^b\) The percentage of observed pairs in disequilibrium before and after (in parenthesis) Bonferroni correction.
Figure 1. Composite autoradiogram showing the 10 most frequent mtDNA RFLP haplotypes of *Phaeosphaeria nodorum*. A λ *Hind*III size standard is shown in the right side.
CHAPTER 6

General discussion
GENERAL DISCUSSION

In this study we wanted to determine the relative contribution of the different evolutionary forces to the development of the epidemics, and to the population structure of the fungal wheat pathogen *P. nodorum*. This is important as the knowledge of a pathogen’s population structure can help explain how the pathogen will adapt to changes in agricultural environments, potentially improving disease control such as resistance breeding and fungicide management (Watson, 1981; McDonald et al., 1999; McDonald and Linde 2002).

Among the aims of this thesis was to determine the relative contribution of sexual recombination to the genetic structure of *P. nodorum*, and to determine the importance of the sexually derived ascospores as source of primary inoculum. To test the hypothesis that populations of *P. nodorum* undergo regular cycles of sexual recombination we conducted a study of the frequency and distribution of the different *P. nodorum* mating types on a global scale. The findings of equal mating type ratios (consequence of sexual dependant frequency dependent selection) even at the smallest scales accompanied by findings of high levels of genotypic diversity, and genetic connectivity among regions indicated that recombination played an important role in shaping the populations.

To support the empirical findings we conducted a two-year mark-release-recapture experiment in which we released ten known isolates on different wheat cultivars. At the end of the experiment we recovered an elevated number (48%) of novel isolate (isolates not matching the released ones). After analyzing the population structure of these novel isolates we concluded that they had to arise either through immigration or recombination. To investigate the hypothesis that immigration (ascospores) and recombination played an important role in increasing diversity in the experiments we conducted an assignment test, analyzed the levels of gametic equilibrium, and the increase in genotypic diversity. The MLE analysis (assignment test) revealed that the novel genotypes arose mostly through recombination and only in part through immigration. The findings of gametic equilibrium and the increase in genotypic diversity (found during both seasons) not only suggested that recombination takes place more often than expected, but also indicated that it occurs during the growing season. These findings on the population genetic structure of *P. nodorum* are
similar to other pathogens with known sexual stage such as *Venturia inaequalis* (Tenzer and Gessler, 1999), *Mycosphaerella graminicola* (Zhan et al., 2003) and *Tapesia yallundae* (Douhan et al., 2003).

A further aim of this study was to determine the effect of host resistance and cultivar mixtures on the rate of evolution, and analyze the changes that occur during the saprophytic phase of the pathogens life cycle. To analyze the effect of different levels of resistance and cultivar mixtures on the population structure of *P. nodorum* we released ten genetically marked isolates on to cultivars and mixtures with different levels of resistance. To analyze the differences in of selection during the saprophytic phase and the parasitic phase we studied the changes in population structure that occurred in summer and on stubble during the winter. The results revealed that selection occurs both during the parasitic and during the saprophytic phases of the disease cycle, and that selection during the parasitic phase occurred most rapidly on the most susceptible host, at intermediate rates on the partially resistant hosts, and most slowly on the cultivar mixture. These findings were analogous to findings based on a similar experimental approach conducted with the wheat pathogen *Mycosphaerella graminicola* (Zhan et al., 2002) and the barley pathogen *Rhynchosporium secalis* (Abang et al., 2006), suggesting that the observed pattern of evolution may be applicable for other splash-dispersed pathogens on cereals.

A third goal of this doctoral study was to determine the evolutionary history and the effect of natural selection on the populations of *P. nodorum*. To do this we compared the genetic variation present in the mitochondrial genome to the variation present in the nuclear genome of more than 300 isolates from Switzerland, Oregon and Texas. The results were surprising, as we found unexpectedly high levels of genetic variation in the mitochondrial genome. The results also reveled that the genetic variation was distributed in similar ways between the two genomes. The high genetic variation suggests that natural selection may not have had a significant impact on the population genetic structure of the mtDNA of *P. nodorum*. The similar distribution of genetic variation between genomes corroborates previous findings describing the importance gene flow among geographic locations (Keller et al., 1997; Stukenbrock et al., 2006). Our results also indicated low degrees of genetic drift, suggesting a lack of population bottlenecks and large effective population size. The findings of large effective population sizes in *P. nodorum* is likely due to several factors,
including recurring sexual out-crossing, long distance dispersal of ascospores and high levels of gene flow over large spatial scales mediated by movement of infected seeds (Barton and Whitlock, 1997; Li, 1955; Wright, 1943).

In conclusion, through this study we were able to contribute important knowledge to the understanding of the evolutionary response of populations of *P. nodorum* to host diversity and environmental changes (such as resistant cultivars, fungicides, over-wintering and over-summering). Contrary to previous studies on host-pathogen interaction, which focused on the development of mathematical models (Barrett, 1980; Lannou, 2001) to predict pathogen evolution in response environmental changes (De Meaux and Mitchell-Olds, 2003; Long et al., 2006; Paillard et al., 2000), we were able to test these theoretical models empirically in, both, natural and controlled agro-ecosystems. In fact we were able to validate the findings obtained in the controlled environment (two year experiment) with the results from natural populations of *P. nodorum*, underlining the validity of these types of experiments. Our findings of gametic equilibrium, equal mating type frequency across continents and the results from the assignment tests prove that random mating contributes in an important manner to the population genetic structure of this fungus and that ascospores are an important source of primary inoculum. Further we demonstrated that the level of cultivar resistance does not affect the population structure by increasing or decreasing the rate of evolution. The results of this study should be considered when trying to achieve effective disease control and when breeding for resistance. The evolutionary potential of this pathogen is in fact elevated, suggesting that it can easily adapt to environmental changes such as fungicide application and resistant cultivars. Any control method that will retard the evolutionary rate (e.g. cultivar mixtures and partial resistance) of this pathogen will be more durable, while efficient sanitary methods (tillage, crop removal) will have to accompany the use of clean seeds in order to prevent the outbreak of epidemic.
LITERATURE CITED


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• To my home town Carona, who gave me so much
• To my friends who helped my through the nice and difficult moments
• To my friend Alessia Della Torre, who not only helped me through the difficult moments but often treated me for lunches and coffees
• To my family, Nica, Ramon, my mom Niki, my dad Bruno; this thesis is also dedicated to them and to Zack (the dog).
• To my beloved NINA, whom I wish for always to my side. In addition to the first part of my family this thesis is dedicated also to her
Today’s job profile

PhD student at the Institute for Integrative Biology (ETH-Zürich)

During my dissertation I appreciated working in a goal oriented, high performing, serious and international environment. In this period I learned to analyze and interpret data in a methodological and analytical manner, thus data collection, elaboration, multitasking and creative problem solving are among my strengths.

Personal profile and education

Flexible and hard working Agronomical engineer (ETH-Zurich) with broad academic training, ranging from economics to mathematics, from chemistry to epidemiology. Additionally I complemented my background by attending law school and through International experience (U.S.A, Peru, Spain, India, and Middle East). I am curious and have good diplomatic and communicative skills.

Goals

Acquire deep business knowledge possibly in an international environment (consulting, commodity trading, market analyst, insurances). Internal schooling opportunities are a plus.
• **Particulars**
  - Name: Rubik Jaffarou
  - Surname: Sommerhalder
  - Date of birth: 21.05.1977
  - Place of birth: Sorengo, Ticino, Switzerland
  - Nationality: Swiss/USA
  - e-mail: rubiks@alumni.ethz.ch

• **Education**
  - 2004-2008 PhD at ETH-Zürich
  - 2005-2008 Law school at Uni Zürich I Liz Summer 08
  - 1997-2003 ETH-Zürich (Dipl. Ing. Agr.)
  - 2002 Universidad Politecnica, Valencia (Esp)
  - 1993-1997 High School-Lugano (CH), Federal Diploma

• **Working experience**

  **Dissertation at the ETH**
  - 2004-2008 PhD dissertation and research assistance at the Institute for integrative Biology (Prof. B.A. McDonald), ETH-Zürich
  - Achievements: Project Management Supervision and coaching of B.sc and M.sc students

  - 2005/2006 Member of the teaching staff for the traveling workshops in collaboration with ICARDA, (Aleppo, Syria) in Iran and Azerbaijan (2005), and in Morocco (2006)

  **Internships**
  - 2002 Internship at the International Potato Center (CIP), Lima, Perú
  - (evaluation and interpretation of potato genetic diversity data)
  - 1999-2000 Agronomical internship, H. Strassman, Münchenstein (BL)

  **Temporary jobs**
  - 1996 Summer job in USA warehouse
  - 1994-2003 Independent vendor
  - 1994-2008 Temporary as construction worker for B. Sommerhalder. Costruzioni S.A (Ticino)
**Languages**

- Italian  Native speaker  
- English  Native speaker  
- CH-German  Fluent  
- German  Fluent  
- French  Fluent  
- Spanish  Fluent  

**Qualification and strengths**

- Good team player, positive, responsible, reliable, honest, good negotiating skills  
- Open minded, flexible, and willing to learn, and work hard  

**Publications in referred journals and invited presentations**

**Publications**

- Sommerhalder, R.J., McDonald B.A., Zhan, J., 2007. Fungal Genetics and Biology 44- 764-772  
- ETH-EAWAG Library  

**Invited presentations**

- BSPP Presidential Meeting, Nottingham (UK) 2005  

**Hobbies and social activities**

- Traveling (3 months backpacking through S. America, 1 month through India)  
- Volunteer Fireman  
- Politics (Carona, member of the legislative since 1998, 2nd vice president in 2007)