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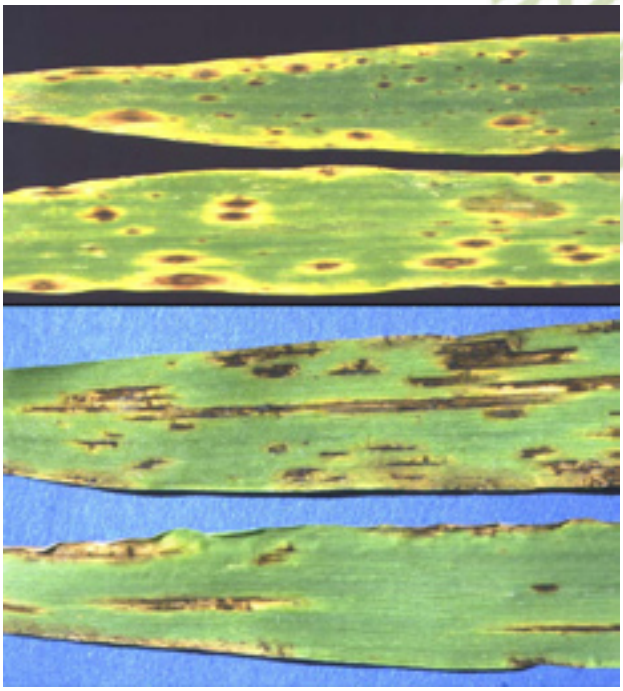
Doctoral Dissertation

Marjo Serenius



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Academic Dissertation
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MTT Agrifood Research Finland

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Abstract

Barley, *Hordeum vulgare* L., is the most important crop in Finland based on cultivated land area. Net blotch, a disease caused by *Pyrenophora teres* Drech., is the most damaging disease of barley in Finland but is also important in other barley cultivating areas. The pressure to improve the economics and efficiency of agriculture has increased the need for more efficient plant protection methods. Development of durable host-plant resistance to net blotch is a promising possibility. However, deployment of disease resistant crops could initiate selection pressure on the pathogen (*P. teres*) population. The aim of this study was to understand the population biology of *P. teres* and to estimate the evolutionary potential of *P. teres* under selective pressure following deployment of resistance genes and application of fungicides.

The study included mainly Finnish *P. teres* isolates, but population samples from Russia and Australia were also included. Using AFLP markers substantial genotypic variation in *P. teres* populations was identified among only a few clones. Differences among isolates were least within Finnish fields and significantly higher in Krasnodar, Russia. Genetic differentiation was identified among several populations from northern Europe and from Australia, and between the two forms of *P. teres*, spot and net type, in Australia. Differentiation among populations was also identified based on virulence (capacity to infect a particular host genotype) between Finnish and Russian populations, and based on prochloraz (fungicide) tolerance in barley fields in the Häme region in Finland. In contrast to previous surveys, the Finnish *P. teres* populations comprised only *P. teres* f. *teres* (net form of net blotch), which was also recovered from Russia, whereas both forms (net and spot: *P. teres* f. *teres* and *P. teres* f. *maculata*) were found within several fields in Australia. Surprisingly, both mating types were equally common in all but the Krasnodar population. This finding supported the assumptions of sexual reproduction of *P. teres* in those areas.

In conclusion, *P. teres* populations are differentiated at several levels. Human assistance in dispersal of *P. teres* on infected barley seed might decrease the differentiation, which would increase plant protection problems caused by this pathogen. *P. teres* is capable of sexual reproduction in several areas. Based on these findings it is apparent that *P. teres* has the potential to pose more serious problems in barley cultivation if plant protection is neglected. Therefore, good

agricultural practices, including crop rotation and use of healthy seed, are recommended. Problems can be lessened if the spread of this pathogen is reduced. Special attention should be paid to use of fungicides with different modes of action and alternation of fungicides. When resistance genes against net form of net blotch are deployed in barley cultivation in Finland, spot form of net blotch might become more prevalent. This possibility needs to be taken into consideration because the shift in occurrence of forms of *P. teres* seems to have occurred previously in Finland.

Key words: crop production, fungal diseases of plants, plant pathology, population genetics

Ohranverkkolaikun aiheuttajan, *Pyrenophora teres*, populaatiorakenne

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Tiivistelmä

Pinta-alansa perusteella ohra on tärkein viljelykasvi Suomessa, mutta ohran satoa heikentää *Pyrenophora teres* Drech. -sienen aiheuttama ohranverkkolaikkutauti. Ohranverkkolaikku on ohran merkittävin tauti Suomessa. Tauti vähentää sadon määrää ja mallastuslaatua. Taudille aroilla lajikkeilla sairastunut lehtipinta-ala on vuosittain keskimäärin 40 % Maa- ja elintarviketalouden tutkimuskeskuksen (MTT) virallisten lajikekokeiden perusteella. Viljelyn tehostuessa on pyritty taloudellisempaan ja kilpailukykyisempään tuotantoon, joka tarkoittaa mm. tilojen erikoistumista tiettyyn tuotantosuuntaan (yksipuolistumista) ja aikaa vievien muokkausmenetelmien korvaamista nopeammilla suorakylvömenetelmillä. Viljelyn yksipuolistuminen ja kasvijätteen määrän lisääntyminen pellon pinnassa ovat omiaan edistämään kasvitautien, kuten ohranverkkolaikun, säilymistä vuodesta toiseen. Lisääntyvä tautipaine saattaa vaatia enemmän kemiallista kasvinsuojelua, jolle ympäristöystävällisempi vaihtoehto olisi ohranverkkolaikun kestävä ohralajike.

Tässä tutkimuksessa selvitettiin ohranverkkolaikkutaudin aiheuttajan populaation rakennetta. Saadun tiedon avulla voidaan arvioida taudinaiheuttajan populaation muunteluun vaikuttavia tekijöitä ja valikoitumisen nopeutta viljelyolosuhteissamme. Kasvinsuojeluaineiden käyttö sekä taudinkestävien lajikkeiden viljely voivat muokata taudinaiheuttajan populaatiota torjuntamenetelmiä paremmin kestäväksi. Taudinaiheuttajan populaation muunteluun vaikuttavien tekijöiden ymmärtäminen auttaa arvioimaan, kuinka nopeasti populaatio voi valikoitua torjuntamenetelmiä kestäväksi. Tässä työssä keskityttiin pääasiassa tutkimaan suomalaisia *P. teres* populaatioita, joita edustavat tautikannat oli kerätty vuosien 1989-2003 välillä eri puolilta Suomea. Varsinaiset peltopopulaatiot kerättiin Lounais-Hämeestä sekä Ylistarosta. Lisäksi mukana oli kaksi venäläistä peltopopulaatiota sekä kokoelma australialaisia *P. teres* -kantoja.

Tutkimus osoitti, että *P. teres* -kannat olivat erittäin muuntelevia molekyylimerkkien perusteella (AFLP), mutta suomalaiset populaatiot olivat vähiten muuntelevia. Populaatiot olivat erilaistuneet niin peltojen kuin paikkakuntienkin välillä. Ohranverkkolaikkutaudin aiheuttajan erilaistuminen peltojen välillä oli ilmeistä myös virulenssitestauksen ja prokloratsi-kasvinsuojeluaineen siedon perusteella. Prokloratsin sietotesti antoi viitteitä, että kasvinsuojeluaine voi valikoida tautikantoja. Tästä syystä kemiallisessa torjunnassa tulisi välttää

saman tehoaineen käyttöä sekä peittaus- että kasvukauden aikaisena kasvinsuojeluaineena. Suomesta ja Venäjältä kerätyt taudinaiheuttajakannat olivat kaikki ohranverkkolaikun verkkotyyppiä, vaikka 70-luvulla taudin molemmat oiretyypit: verkko- ja laikkutyyppi olivat yhtä yleisiä Suomessa. Taudinaiheuttajan molempia pariutumistyyppejä löydettiin yhtä yleisesti, vaikka suvullisen lisääntymisen merkitystä ja esiintymistä Suomessa on epäilty. Populaationäyte Krasnodarista, Mustanmeren rannalta, erosi kaikista muista populaatioista ollen erittäin muunteleva, mutta vain toista pariutumistyyppiä. Australiassa molemmat pariutumistyypit sekä oiretyypit olivat yleisiä. Tutkimuksessa ei varsinaisesti selvitetty syitä, jotka ovat johtaneet populaatioiden erilaistumiseen paikkakuntien välillä.

Tämän työn havainnot (suvullisen lisääntyminen, toisen oiretyypin yleistyminen Suomessa ja populaatioiden erilaistuminen usealla tasolla) korostavat monipuolisen kasvinsuojelun tarvetta, vaikka viljelymenetelmät muuttuvatkin. Siemenen terveydestä huolehtimalla voidaan taudinaiheuttajan leviämistä estää niin peltojen kuin eri maidenkin välillä. Suvullisen lisääntymisen mahdollistavia olosuhteita ei tutkittu tässä työssä, mutta sairastuneen kasvijätteen jääminen pellon pintaan ja ohran viljely yksipuolisesti todennäköisesti lisäävät suvullisen lisääntymisen mahdollisuutta. Taudinaiheuttajan suvullinen lisääntyminen lisää taudinaiheuttajan muuntelumahdollisuuksia ja torjuntatarvetta. Viljelykierrolla voidaan vähentää taudinaiheuttajan säilymistä, kun valitaan ei-isäntäkasvi seuraavaksi vuodeksi, vaikka tautista kasvimateriaalia jäisikin pellolle. Verkkotyypille kestävien ohralajikkeiden tullessa viljelyyn tulisi kiinnittää huomiota laikkutyypin mahdolliseen uudelleen yleistymiseen. Tässä tutkimuksessa kerätyt näytteet olivat kaikki verkkotyyppiä, vaikka molemmat oiretyypit ovat olleet yhtä yleisiä 70-luvulla. Syytä muutokseen ei tiedetä. Tämä tutkimus vahvisti oletusta, että oiretyyppien aiheuttajat olisivat luonnossa erillisiä populaatioita, jotka eivät luonnossa lisäänny keskenään. Taudinkestävyyden kestävä käyttö ohran viljelyssä vaatii taudinaiheuttajan muuntelun seuraamista jatkossakin sekä kasvinsuojelun muuttuviin tarpeisiin vastaamista. Taudinaiheuttajan populaatio valikoituu helpoimmin, jos yleisesti käytetään yhteen torjuntamekanismiin perustuvaa menetelmää kuten yhden tehoaineen kasvinsuojeluainetta tai yhteen suurivaikutteiseen geeniin perustuvaa taudinkestävyyttä. Torjuntatehon säilymistä tulisi seurata ja tarvittaessa käyttää vaihdellen tehoaineita ja tehoaineyhdistelmiä tai vastaavasti vaihdella ohralajikkeita, joissa on eri suurivaikutteisiin geeneihin perustuva taudinkestävyys tai käyttää kvantitatiivista (pienivaikutteisiin, useaan geeniin perustuvaa) taudinkestävyyttä.

Avainsanat: kasvintuotanto, kasvitaudit, populaatio genetiikka

List of original articles

The thesis is a summary and discussion of the following articles, which are referred to by their Roman numerals:

I Marjo Serenius, Nina Mironenko and Outi Manninen (2005) Genetic variation, occurrence of mating types and different forms of *Pyrenophora teres* causing net blotch of barley in Finland. *Mycological Research* **109(7)**: 809-817. Copyright Elsevier

II Marjo Serenius and Outi Manninen. Prochloraz tolerance in net blotch of barley in Finland. *Agricultural and Food Science* vol 15: (in press) Copyright MTT

III Marjo Serenius, Outi Manninen, Hugh Wallwork and Kevin Williams. Genetic differentiation in *Pyrenophora teres* populations measured with AFLP markers. Manuscript, submitted.

IV Marjo Serenius, Nina Mironenko, Olga Filatova and Outi Manninen. Comparison of virulence and AFLP markers as population diversity measurements in *Pyrenophora teres*. Manuscript, submitted.

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Contents

1 Introduction

1.1 Importance of phytopathogens in cereals in Finland

Cereal crops are cultivated on 54% of the 2.2 million hectares of agricultural land in Finland (Finfood News and Information Agency 2005). Barley, *Hordeum vulgare* L., is the most important cereal crop in Finland and was sown on 594,200 ha in 2005. Barley grain is mainly used as feed (63%) or in the malting and starch industries (29%) (Information Centre of the Ministry of Agriculture and Forestry 2006). Phytopathogens of barley are important as they diminish the photosynthetic leaf area, and therefore decrease grain yield and quality. Grain quality is especially important in malting barley production. The two most important malting barley cultivars, Saana (38% of production) and Scarlett (32%) (Plant Production Inspection Centre 2005), are among the most disease resistant barleys cultivated in Finland (Kangas et al. 2005a).

Phytopathogens of barley are usually controlled either with a seed dressing (chemical fungicide or biological control agent) or by foliar application of fungicide during the growing season. Fungicides are used annually on 30% of the barley growing area in Finland (Salopelto 2004). The total value of fungicide sales was 12.6 million euros in Finland in 2004 and 65% of all marketed fungicides were used on cereals (Savela and Hynninen 2004). Prochloraz and imazalil, which belong to the same imidazole class of sterol 14α-demethylation inhibitors (DMIs) are the active ingredients for half of the chemical products approved for seed coating of barley in Finland (Plant Protection Inspection Centre 2005). Prochloraz alone is the leading fungicide used for cereals in Finland according to the sales of fungicides (Savela and Hynninen 2004). Other, more pro-environmental plant protection, methods are available and effective against barley pathogens, including crop rotations and deep ploughing. The use of major resistance genes in barley cultivars against net blotch, caused by *Pyrenophora teres* Drechslera, remains largely unexploited in Finland. However, resistance genes represent a pro-environmental and economically sound method to protect plants against phytopathogens. The resistance gene (*mlo*), active against *Erysiphe graminis* DC. f. sp. *hordei* Marchal, is a good example of durable disease resistance in barley (Wolfe and McDermott 1994).

During recent years economical and environmental issues have become more important in Finnish agriculture (Kola 1998, Rikkonen 2005). Direct drilling and no-till methods have become more popular. Farm sizes are increasing alongside production increases (Rikkonen 2005). Therefore, more time-efficient cultivation methods are required. Moreover, the regulations to limit land area without vegetation, for erosion control, favour conservation tillage methods. However,

infected plant debris, including barley stubble, which is no longer ploughed deep into the soil, can act as an inoculum reservoir for infection of the next crop (Jordan 1981, van den Berg and Rossnagel 1991). In addition, the larger farms are specialising in one production sector (either cereal production or animal production), which reduces the number of crop species in rotation, and increases the succession of cereals. All the above-mentioned changes in agriculture may favour the survival of phytopathogens over growing seasons and increase the inoculum source of phytopathogens. In order to avoid increasing demands for use of fungicides under stronger disease pressure, deployment of host plant resistance genes could represent one solution in pathogen management.

Before deploying major gene resistance in barley cultivation in Finland, more knowledge is needed to evaluate the risk of pathogens evolving under selection pressure. It has been shown that resistance genes and use of fungicides expose pathogen populations to selective pressures that render them more tolerant to host-plant resistance and fungicides (McDonald and Linde 2002a, b). The speed with which a pathogen population evolves under the selection pressure depends on several factors. McDonald and Linde (2002a, b) developed a model to predict the evolutionary potential of pathogen populations in which they included the mating system (sexual, asexual or mixed reproduction), dispersal range and effective population size of a pathogen. According to this model, pathogens that pose the greatest risk of breaking down resistance genes have a mixed reproduction system, a high potential for genotype flow, large effective population sizes, and high mutation rates. Based on their model, several ascomycete pathogens were placed in one and the same risk category. The population biology of *P. teres* is not yet well enough described to enable evaluation of the evolutionary potential of this pathogen.

1.2 Net blotch is caused by two forms of *P. teres*

Net blotch of barley is caused by *P. teres* (anamorph *Drechslera teres* [Sacc.] Shoemaker syn. *Helminthosporium teres*). *P. teres* belongs to the phylum: Ascomycotina, subphylum: Pezizomycotina, class: Dothideomycetes, family: Pleosporacea (Eriksson 2005). Class Dothideomycetes encompasses genera including the most important pathogens of wheat, legumes, barley, apples, bananas, and rapeseed. Net blotch is one of the most important diseases of barley in all major barley growing regions of the world (Mathre 1997). The infection rate of net blotch is on average 40% on susceptible barley varieties in Finland (Kangas et al. 2005a), and a significant improvement in yield can be gained through the use of fungicides to control it (Kangas et al. 2005b). The disease symptoms are most commonly seen on barley leaves and leaf sheaths, but they can also occur on flowers and grain (Jordan 1981). *P. teres* is known to produce two different symptoms (McDonald 1967), and based on these Smedegård-Petersen (1971) differentiated the forms of *P. teres* as *P. teres* f. *teres,* which produces brown

netted lesions and *P. teres* f. *maculata,* which produces brown spot lesions (Smedegård-Petersen 1971). The morphological characters of spores overlap between the forms of *P. teres*, and therefore it is difficult to identify the forms based solely on morphology (Crous et al. 1995, Stevens et al. 1998). Originally the spot form of net blotch was named as a new *Pyrenophora* species: *P. japonica* Ito & Kuribayashi However, *P. japonica* has been recognised as being *P. teres* (Crous et al. 1995). The spot form has also been referred to as *P. hordei* Wallwork, Lichon & Sivanesan (Wallwork et al. 1992, Stevens et al. 1998). Both forms of *P. teres* can cause variable symptoms according to environment and host genotype (Scott 1992, Williams et al. 2001). Difficulties in identification of the two forms of *P. teres*, have sometimes led to misidentification (Rau et al. 2003). Currently there are specific molecular markers available, which are based on the polymerase chain reaction (PCR), which facilitate identification. These markers rely on differences in deoxyribonucleic acid (DNA) sequences (Williams et al. 2001, Leisova et al. 2005a). The resistance of barley cultivars to the two forms of *P. teres* differs (Tekauz 1990, Scott 1992), which makes the identification of the prevalent cause of net blotch (the form of *P. teres*) important in the field. Identification of the form of net blotch makes implementation of appropriate major gene resistance programs possible.

1.3 Life cycle of *P. teres*

The life cycle of *P. teres* is illustrated in Figure 1. Net and spot forms of *P. teres* can overwinter on infested plant residues, on barley seed (Jordan 1981, van den Berg and Rossnagel 1991) or on alternate host plants (Shipton et al. 1973, Sampson and Watson 1985, Brown et al. 1993). During the beginning of the growing season, *P. teres* produces asexual conidia and sexual ascospores on barley residues and infects sprouting barley (primary infection) (Jordan 1981, van den Berg and Rossnagel 1991). Barley residues act as an inoculum reservoir (van den Berg and Rossnagel 1991). *P. teres* can continue to produce spores at least after two winters in Canada (Duczek et al. 1999). During the growing season *P. teres* reproduces mainly asexually on barley leaves. Passively liberated haploid conidia are dispersed by wind and rain splashes to surrounding plants and upper leaves mainly within the same field (secondary infection) (van den Berg and Rossnagel 1991). Conidia production requires leaf surface wetness, high relative humidity and conducive temperature regimes (>6°C and <25°C), and occurs only on senescent or dead host tissue (van den Berg and Rossnagel 1991). To complete the life cycle, sexual reproduction occurs on straw debris and leaf fragments (Jordan 1981). During sexual reproduction *P. teres* is diploid and forms bitunicate asci within perithecia (Eriksson 2005). The ascus is associated with paraphyses-like structures and therefore some authors, including Crous et al. (1995), have used the term pseudothecia rather than perithecia. Two isolates of different mating type are needed for sexual reproduction since *P. teres* is heterothallic and self-sterile (McDonald 1963). The maturation of perithecia

takes six to 15 months under laboratory conditions (Smedegård-Petersen 1978). A perithecium contains several asci and each ascus normally contains eight ascospores (McDonald 1963, Jordan 1981).

Even though the life cycle of *P. teres* is well documented, the importance of different stages of the life cycle to occurrence of net blotch symptoms is still not entirely clear. The contribution of different stages (sexual and asexual reproduction) of the life cycle to diversity of *P. teres* population varies. In addition, human assistance in long-distance dispersal via seed increases the possibilities for this pathogen to evolve rapidly. As Peever and Milgroom (1994) expressed: 'The wide host range, capacity for long-distance dispersal, and mixed reproductive system in this pathogen raises interesting questions about the genetic structure of *P. teres* populations.' This statement still holds true. Our current knowledge of the population structure of *P. teres* remains limited.

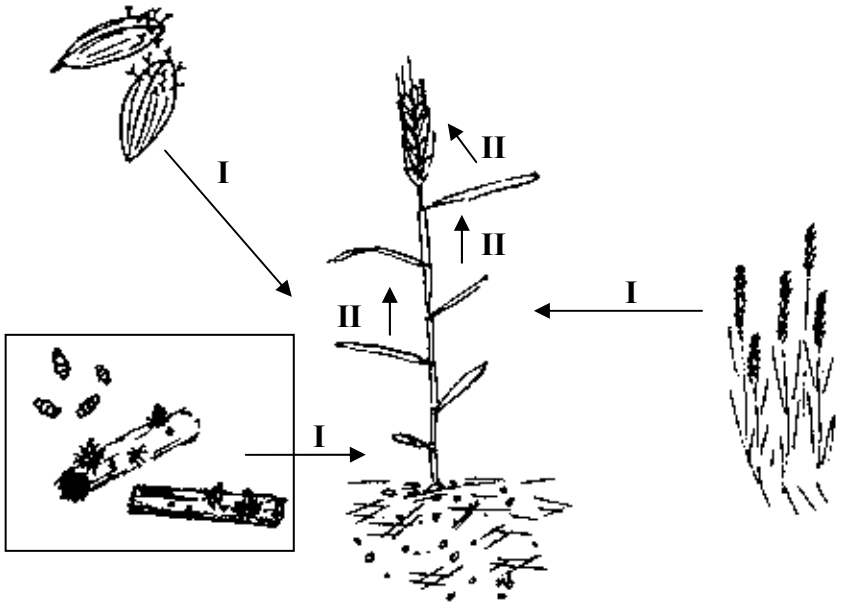

Figure 1. Illustration of life cycle of *Pyrenophora teres*, cause of net blotch of barley. Primary infection originates from infected seed, alternate host plants or stubble, which is the main source of sexual ascospores. Asexual conidia are dispersed by wind or rain splash on upper parts of the plant, head or on to neighbouring plants (secondary infection).

1.4 Occurrence of *P. teres* f. *teres* and *P. teres* f. *maculata*

Net blotch occurs worldwide, but spot and net forms are not uniformly distributed. Both forms were reported to occur in the Czech Republic (Leisova et al. 2005b), Italy (Rau et al. 2003), Australia (Williams 2001), South Africa (Scott 1992), Canada (Tekauz and Mills 1974, Tekauz 1990), Finland (Mäkelä 1972), Norway (Hansen and Magnus 1968) and in the United States of America (Bockelman et al. 1983). In general, the net form of net blotch (*P. teres* f. *teres*) has been known as a barley disease for longer than the spot form (McDonald 1967, Hansen and Magnus 1968, Smedegård-Petersen 1971, Khan and Tekauz 1982, Scott 1992). The first known barley leaves with net form of net blotch infection in Norway dates back to 1880, but the spot form only became common in the 1960s (Hansen and Magnus 1968). The net form has declined in several regions as the spot form has become more prevalent, for instance in Denmark (Jørgensen et al. 2000), in Australia (Khan and Tekauz 1982), in Canada (Tekauz 1990, van den Berg and Rossnagel 1991) and in South Africa (Campbell et al. 1999). However, the net form of *P. teres* is currently more prevalent in Norway, the spot form being restricted to the northernmost cereal growing region of Trøndelag, which represents less than 10% of the barley acreage in Norway (Oleif Elen, Planteforsk Plantevernet, personal communication). Trøndelag was one of the first locations in Norway where the spot form was recorded (Hansen and Magnus 1968). The relative occurrence of the two forms of net blotch might have changed similarly in Finland as both forms were equally common in the 1970s (Mäkelä 1972), but recent publications consider *P. teres* f. *teres* only (Peltonen et al. 1996, Jalli and Robinson 2000, Robinson and Mattila 2000). It is uncertain, why the occurrence of spot and net forms has changed in some areas.

The two forms of net blotch are known to differ in several respects: there are differences in host differentiation (Sampson and Watson 1985, Brown et al. 1993), at the genome level according to molecular analysis (Bulat and Mironenko 1990, Williams et al. 2001, Campbell et al. 2002, Rau et al. 2003, Leisova et al. 2005b), and in host-pathogen interaction (Khan and Tekauz 1982, Tekauz 1990, Scott 1992, Williams et al. 1999), which could affect adaptation to environmental changes. Cultivated barley varieties might cause a shift from one form to the other if they are resistant to a single form of the pathogen (Khan and Tekauz 1982). According to the biological species concept, *P. teres* is one species with two forms (lesion types) that resemble each other in spore morphology (Crous et al. 1995, Stevens et al. 1998) and which are able to reproduce sexually with each other in the laboratory (Smedegård-Petersen 1978). It is still uncertain whether recombination and hybrids occur in nature (Scott 1991, Campbell and Crous 2003, Rau et al. 2003).

1.5 Population biology and diversity

Mutation, gene flow (migration) and recombination are considered to increase diversity in pathogen populations (Burdon and Silk 1997, McDonald and Linde 2002a, b). The evolutionary potential of a pathogen population is reflected in its population genetic structure which refers to the amount and distribution of genetic diversity (McDonald and Linde 2002a, b). In a haploid asexually reproducing pathogen, a non-lethal mutation will survive for generations due to clonal reproduction. In a heterozygotic randomly mating population the chance of a recessive mutation appearing in the next generation is only 25% (Falconer and Mackay 1996). Point mutations, small deletions, insertions into or transpositions of the existing coding DNA sequence have been an important source of variation in old pathogen populations (Burdon and Silk 1997). Gene flow brings novel genotypes into a population and increases variation (Falconer and Mackay 1996). In addition, there are several examples of plant pathogens where variation in the population has increased due to migration of the other mating type (Burdon and Silk 1997). Recombination combines the parental genotypes to produce new combinations. In a widely spread population, mating is random only within local groups (Falconer and Mackay 1996). This is assumed also in agricultural plant pathogen populations where there is no dispersal between countries due to quarantine regulations. In addition, when random drift occurs independently in different sub-populations it leads to genetic differentiation between the sub-populations (Falconer and Mackay 1996).

It is presumed that if major resistance genes are deployed, the best adapted (virulent) pathogen isolates can survive. This is termed selection, which basically decreases the variation in a population (Burdon and Silk 1997, McDonald and Linde 2002a, b). Prolonged exposure to the same resistance genes or fungicides encourages the pathogen population overcome them to the extent that they are no longer effective against the pathogen (McDonald and Linde 2002a, b). According to McDonald and Linde (2002a, b) population size, dispersal range and reproduction system of a pathogen mainly determine how quickly the pathogen population evolves and overcomes the resistance genes or fungicides. The pathogen's ability to evolve under selection pressure is termed evolutionary potential (McDonald and Linde 2002a, b). The lowest risk is posed by pathogens with small population size, dispersal range within 5 metres and asexual reproduction. The evolutionary potential increases as the exchange of genetic material between locations or individuals increases (McDonald and Linde 2002a, b). Fungicides act in the same way as resistance genes in shaping pathogen populations.

Several cereal pathogens that have a mixed reproduction system like that of *P. teres*, and disperse mainly within a field, are placed in the same category (risk 6-8) in the evolutionary risk model (maximum rating 9) of McDonald and Linde

(2002a, b). They are characterised by having most genetic diversity within a small geographical area and little differentiation among locations. Such pathogens include *Phaeosphaeria nodorum* (Müller) Hedjaroude, cause of glume blotch (Keller et al. 1997), *Tapesia yallundae* Wallwork & Spooner, cause of cereal eyespot (Douhan et al. 2002), *Rhynchosporium secalis* (Oudem.) Davis, cause of scald (Salamati et al. 2000), and *Mycosphaerella graminicola* (Fuckel) Schröt. in Cohn, cause of *Septoria tritici* leaf blotch (Schnieder et al. 2001, Zhan et al. 2003). Zhan and McDonald (2004) concluded that major factors decreasing genetic variation in the wheat pathogen *M. graminicola* are random drift and natural selection, whereas factors increasing genetic variation are mutation, gene flow and sexual recombination.

1.6 Expectations on population structure of *P. teres*

It is presumed that the global population of *P. teres* would have a similar population structure as other Ascomycete pathogens with a mixed reproduction system and a within-field dispersal range. The occurrence of net and spot forms of *P. teres* increases the overall diversity in *P. teres* populations. Mutation is a powerful source of novel variation in pathogens with a simple genome like *P. teres*, which is haploid for most of its life cycle. *P. teres* is capable of long distance dispersal with human assistance, for example on infected seed. Since *P. teres* is able to reproduce both sexually and asexually, it is presumed to be evolutionarily efficient. Based on these arguments the population of *P. teres* is presumed to be highly diverse. No genetic differentiation was found between samples from various locations in Finland (Peltonen et al. 1996), the Czech Republic (Leisova et al. 2005a, b), Sweden (Jonsson et al. 2000) or North America (Peever and Milgroom 1994). In addition, random amplified polymorphic DNA (RAPD) markers revealed substantial genetic diversity within Finnish *P. teres* populations (Peltonen et al. 1996). Peltonen et al. (1996) suggested that the *P. teres* population does not change markedly between growing seasons because no differentiation was established over several years of sampling. The phenotypic diversity for virulence within and between *P. teres* f. *teres* populations was relatively similar over Scandinavia and northern Europe (Jalli and Robinson 2000, Robinson and Mattila 2000). Until now, no major gene resistance has been deployed against *P. teres* in Finland thus no selective pressure, which would shape the population and decrease the variation, has been exerted on the Finnish *P. teres* population by the major resistance genes (Jalli and Robinson 2000).

In contrast to the predictions, a clear difference in virulence was observed between *P. teres* isolates from Finland, eastern Europe and the Russian Federation (Afanasenko 2001), and a significant genetic differentiation was recorded among isolates from states in North America (G_{st} 0.33), locations in Italy (F_{st} 0.43) (Rau

et al. 2003), and between European and North American *P. teres* populations (G_{st} 0.46) (Peever and Milgroom 1994, 1995). Peever and Milgroom (1994) suggested that the genetic differentiation in *P. teres* was due to local adaptation to alternate hosts, restricted migration and genetic drift. Genetic drift might be important in areas where the pathogen population size changes substantially between years. Winter, deep ploughing and crop rotation are among the factors that could reduce the population size of *P. teres* in Finland.

In conclusion, the Finnish *P. teres* population is most likely i) to be similar over years and among regions based on the results of Peltonen et al. (1996), Jalli and Robinson (2000), and Robinson and Mattila (2000), and ii) the *P. teres* isolates do not differ markedly from other *P. teres* isolates from northern Europe (Jalli and Robinson 2000, Robinson and Mattila 2000), but iii) differ from Russian *P. teres* populations (Afanasenko 2001). The current occurrence of *P. teres* f. *maculata* (spot form), both mating types i.e. random mating and the alternate host species in Finland is not recorded. The analysis of the genetic structure of *P. teres* populations in Finland will reveal the distribution and degree of diversity and help estimate the sources and factors that limit diversity in *P. teres* populations.

1.7 Objectives

In order to understand better the forces shaping the *P. teres* populations and to estimate the durability of deployed major genes in Finland, several questions were posed. The original papers addressing these issues are indicated with Roman numerals.

1. Is the (Finnish) *P. teres* population clonal or does it vary at the genotype level, in virulence, or in fungicide tolerance? (I-IV)

2. How much genotypic variation is distributed on a small scale (e.g. field) compared to on a broader scale? (I-IV)

3. How diverse is the Finnish *P. teres* population compared with those of other regions? (III, IV)

4. Are *P. teres* populations differentiated among locations based on AFLP (Amplified Fragment Length Polymorphism) markers, virulence, or fungicide tolerance? (I-IV)

5. How prevalent are the two forms of *P. teres*? Do hybrids occur? (I, III)

6. Is sexual reproduction prevalent? (I, III)

7. What is the evolutionary potential of *P. teres*? (III)

2 Materials and methods

2.1 *Pyrenophora teres* isolates

Barley leaves with symptoms of net blotch were collected from three farmers' and experimental barley fields in Finnish Häme region (six fields altogether) and from one experimental field at the MTT research station in Ylistaro, Finland (I-IV). Net blotch symptoms were mostly typical of *P. teres* f. *teres*, except on Farm 2 from Häme (II), where leaves were collected from the fairly resistant barley cultivar 'Annabell' (Kangas et al. 2005a), and were more of the *P. teres* f. *maculata* type. Barley leaves were collected in Finland during the growing seasons of 2001-2003 (I-IV). Infected barley leaves collected from a field in Krasnodar, Russia were received from O. Afanasenko, All-Russian Research Institute for Plant Protection (VIZR) (Figure 2) (III). Single-conidial isolates were established from infected leaf material according to McDonald (1967), as explained later. Another Russian collection of *P. teres* isolates from Rozhdestveno was received from VIZR as single conidial isolates and as DNA samples (IV, Figure 2). Both Russian populations were collected during the growing season of 2002. Several random *P. teres* isolates (both forms of *P. teres*) were received from researchers or collected during the years 1958–2002. These isolates covered not only the entire barley growing region in Finland (Peltonen et al. 1996), but also other European and North American countries and Australia (I, III). These isolates are currently part of the fungal collection at MTT in Jokioinen, Finland. Data from *P. teres* isolates from South Australian Research and Development Institute (SARDI), but not the isolates themselves, were received from K. Williams and H. Wallwork, who collected, maintained and analysed the *P. teres* isolates in SARDI (Figure 2). This collection included both forms of *P. teres* isolates. Only single-conidial isolates of *P. teres* were analysed with molecular markers or in virulence and fungicide tests. Isolates of same origin (e.g. a field) are termed a population later in the text. More detailed information on the isolates is given in the original papers I-IV.

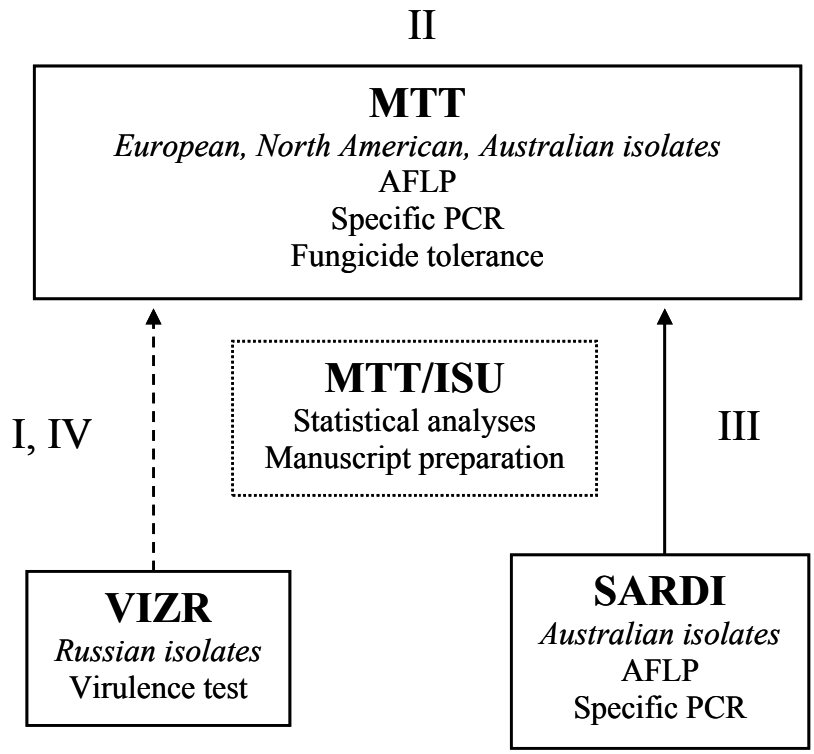

Figure 2. This study was a collaborative effort between three research institutes: MTT, SARDI and VIZR. Origin of *Pyrenophora teres* isolates used in this study, and belonging to the collections of the institutes involved, is shown in italics. Material received at MTT is indicated accordingly: solid line=data, dashed line=isolates, leaves and DNA. Mating types and forms of *P. teres* were analysed with specific PCR markers. Manuscripts prepared in collaboration are indicated with Roman numerals on the edges of the illustration. The final three papers were prepared at Iowa State University, USA (ISU).

2.2 Methods

2.2.1 DNA extraction

All *P. teres* isolates were first grown from infected leaf tissue on a V8 medium under near ultra violet light at +18°C according to McDonald (1967). Single-conidial isolates were prepared by transferring one conidial spore from the original mycelium culture with a sterile needle on to a new growth medium (I-IV). Before any analyses were done, single conidial isolates were grown on V8 medium and plugs of mycelium were stored in a freezer in plastic 1.5 ml tubes at –70°C for later use. Isolates were grown in yeast extract liquid medium (I, III, IV) prior to DNA extraction. DNA was extracted from freeze-dried mycelium (lyophilised) (I, III, IV). Before analysing the population samples, several

DNA extraction methods were tested: CTAB protocol (Tinker et al. 1993) and commercial kits: GenElute Plant Genomic DNAkit (SIGMA) and Plant Easy Mini kit (Qiagen) both together with FastPrep machine (Q-biogene). Plant Easy Mini kit was chosen because of the good quality of DNA and the efficiency of the method. The same DNA extraction method was used at MTT for all of the *P. teres* DNA extractions (I, III, IV). Different DNA extraction methods were used in SARDI and in VIZR, according to Raeder and Broda (1985) and Bulat et al. (1998), respectively.

2.2.2 Genotypic variation

Molecular variation at the genome level of *P. teres* was studied using AFLP fingerprinting (Vos et al. 1995, Williams et al. 2001) (I, III, IV). AFLP (Vos et al. 1995, reviewed in Bensch and Åkesson 2005) is a fingerprinting method where DNA is first cut with two different restriction enzymes. In this study the methylation sensitive *Pst*I restriction enzyme was used (I, III, IV). Methylation of cytosine (C) is a common epigenetic modification in most eukaryotic organisms (Bensch and Åkesson 2005). *Pst*I will not cut the DNA (based on sequence 5'CTGCA*G3') if the restriction site (*) is symmetrically methylated at either the internal or external cytosine (C) residue (McClelland et al. 1994). Secondly, specific adaptors corresponding to the restriction cut-site were attached with a ligation enzyme to both ends of the DNA fragment (I, III, IV). Thirdly, these fragments were amplified in a thermal cycler using specific PCR incorporating two primers specific to the adaptors. The *Pst*I-primer had a selective nucleotide (adenine, A) at the 3' end of a primer sequence. Lastly, fragments were amplified by using two selective nucleotides at the 3' end of both primer sequences (corresponding to *Pst*I and *Mse*I). The amplified fragments were separated on an automated capillary electrophoresis MegaBASE^{TM} 500 (Amersham Pharmacia Biotech). *Pst*I -primers were labelled with a fluorescent tag, which was used to identify the amplified fragments after the electrophoresis.

Before analysing the populations, two methylation sensitive AFLP protocols (Campbell et al. 1999, Williams et al. 2001) were compared. The method of Williams et al. (2001) was easier to adapt to the MTT laboratory, and therefore all AFLP analyses were conducted according to that protocol. Williams et al. (2001) protocol was used also in SARDI for analysing the Australian *P. teres* isolates included in this study (Figure 1) (III).

Fragments amplified by AFLP were detected with gel electrophoresis on 6% sequencing gels in an Applied Biosystems 373 DNA Sequencer in SARDI (Williams et al. 2001). The computerized capillary electrophoresis was established at the MTT core facility in summer 2001 at the beginning of this project. The system was tested several times with both forms of *P. teres* isolates and with different dilutions before analysing the population samples. The population sample

analyses began at the start of 2002. The automated capillary electrophoresis at MTT is operated by one person (Ms. Anneli Virta) to minimise the random experimental variation. Using the capillary electrophoresis, 48 samples are analysed simultaneously. This capacity affected the sampling sizes used in this study. A inner size standard (ET Rox-900, Amersham Pharmacia Biotech) was used in every capillary to standardise the sizes of the amplified fragments. Data was processed with Genetic Profiler Software (Amersham Pharmacia Biotech). The intensity of the fluorescent labels in the size standard (Rox) and in the samples (Fitch, Hex or Tet) was detected with a laser beam. Four fluorescent labels were analysed simultaneously in one capillary. In this study, however, only Fitch and Hex labelled primers, which produced the best quality identifiable fragments, were further analysed (I, III, IV). Fam and Tet fluorescent labels were used in SARDI (Williams et al. 2001).

In addition to AFLP fingerprinting, specific PCR markers were used to identify the mating types (I, III), and forms of *P. teres* (I, III, IV), namely *MAT1* and *MAT2* for mating types (Rau et al. 2005), and *PTT* for *P. teres* f. *teres* and *PTM* for *P. teres* f. *maculata* (Williams et al. 2001). The primer sequences are given in the original papers and in the references. Both specific PCR assays were tested with control isolates before testing the population samples, as explained in I. The mating type identity was verified by crossings of control isolates (I).

2.2.3 Phenotypic diversity

Virulence (IV) and fungicide (prochloraz) tolerance (II) were tested to describe the phenotypic variation in *P. teres* within and between locations, and to compare the phenotypic, namely virulence, and genotypic variation (AFLP) (IV). Isolates were grown on PDA before the fungicide tolerance test (II) and on V8 medium before the virulence test (IV).

2.2.3.1 Virulence

The diversity in pathogen populations is commonly analysed by testing the variation in virulence. Virulence of *P. teres* isolates within and between two Finnish fields and a Russian field were analysed. In total, 124 *P. teres* single-conidial isolates, collected from barley cultivar ‘Inari’, were tested on the following barley accessions: 1) Canadian Lake Shore (CI 2750), 2) Harbin (CI 4929), 3) c-8755, 4) c-20019, 5) CI 4207, 6) Manchurian (CI 739), 7) Tifang (CI 4407-1), 8) Diamant (c-29192), 9) CI 9825, 10) CI 5791, 11) CI 9819, 12) c-23874, 13) c-19979, 14) c-15811, 15) c-15812. Barley accessions with an initial ‘c’ belong to the species collections of The N.I. Vavilov All-Russian Scientific Research Institute of Plant Industry (VIR). The first 11 barley accessions are among the 12 barley accessions recommended for an international differential set by Afanasenko et al. (1995). The Finnish barley cultivar Pirkka was used as a

susceptible control. Virulence of an isolate was tested on segments of a first leaf of barley in a benzimidazole assay (Afanasenko et al. 1995) (IV). The virulence test was conducted at VIZR and was repeated once in 2003.

2.2.3.2 Fungicide tolerance

Fungicide tolerance is a phenotypic trait used to describe the variation within and among plant pathogen populations. Tolerance of 364 single conidial isolates of *P. teres* against prochloraz (1-{*N*-propyl-*N*-[2-(2,4,6-trichlorophenoxy)ethyl]carbamoyl}-imidazole, the most common fungicide used in barley production in Finland (commercial names in Finland: Sportak and Prelude) was tested in a radial growth assay on Petri dishes (II). Isolates originated from three farmers' fields and from one experimental field of MTT, all in Häme region. Isolates were tested on two dilutions; 0.1 μg ml^{-1} and 1.0 μg ml^{-1} of prochloraz on potato dextrose agar (PDA) medium based on a preliminary experiment. Radial growth of *P. teres* mycelium on dishes was measured after 7 days post inoculation in two successive experiments, both of two replicates per isolate for each fungicide dilution and for control medium. Medium without fungicide was used as a control since all the dilutions were made in sterile water. Details of the experimental design and the preliminary experiment are given in the original paper (II).

2.2.4 Statistical methods

Statistical analyses were performed on all raw data in the same manner. Statistical methods were mainly used to compare the differences and similarities in frequency distributions of molecular (AFLP), virulence and fungicide tolerance data among samples. The following software packages were used to conduct the statistical analyses: Arlequin version 2.00 (Schneider et al. 1997) and version 3.0 (Excoffier et al. 2005) (I, III, IV), MultiLocus version 1.2.2. (Agapow and Burt 2000) (III), Popgene version 1.32 (Yeh et al. 1999) (III, IV), and SAS® Proprietary Software Release 8.2 (SAS) (SAS Institute Inc., Cary, NC, USA) (II). The basic descriptive indices were calculated from AFLP data using Arlequin. Frequency distributions were compared with Arleguin for AFLP data (I, III, IV) and for virulence data (IV) and with SAS for fungicide tolerance data (II). The sources of variation in fungicide tolerance were analysed with ANOVA in SAS PROC GLM (II). Multilocus associations and hypothesis of random mating was tested with MultiLocus (III). More details are provided in the original papers.

2.2.4.1 Genetic variation and migration based on AFLP data

The analyses of molecular variance (AMOVA) (Weir and Cockerham 1984, Excoffier et al. 1992) and the partitioning of total variation based on AFLP were calculated with Arlequin software (I, III, IV). A distance matrix for all pairwise comparisons was calculated using squared Euclidean distances. Groups of isolates were defined and their grouping was subsequently tested. The total variance was partitioned into covariance components in a hierarchical analysis of variance due to within-individual differences, between individual differences and within-population differences. Then the covariance components were used to compute the fixation indices in terms of coalescent times. The equations are given and the methodology is described in more details in Excoffier et al. (2005). It is assumed that the effects of group, population and isolates within a population within a group was additive, random, independent, and had the associated covariance components. The covariance components were summarised to count the total molecular variance. *P. teres* isolates are haploid, which simplifies the analyses. The significance of the indices was tested using a non-parametric permutation (reviewed in Excoffier et al. 2005).

Migration rate was calculated with Arlequin (I) assuming that two populations of size *N* were drawn from a large pool of populations, a fraction *m* of migrants was exchanged in every generation and that the mutation rate *u* was negligible as compared with migration rate *m* (reviewed in Schneider et al. 1997). Equilibrium between migration and drift was assumed and the absolute number of migrants *M* exchanged between the populations was calculated as $M=1-F_{ST}/2F_{ST}$.

2.2.4.2 Mating and phenotypic variation

The hypothesis of random mating between *P. teres* isolates within a population was tested with indices of multilocus association I_A and r_d (Brown et al. 1980, Maynard Smith et al. 1993). Multilocus association indices were calculated among isolates of the same origin and within forms of *P. teres* with MultiLocus v1.2.2 (Agapow & Burt 2000) (III). The number of loci at which two isolates differ is first calculated between all pairs of individuals, and the variance of these differences is compared to that expected if there is no linkage disequilibrium. The program calculates I_A as,

where

$$I_A = \frac{V_D}{\sum \mathrm{var}_j} - 1$$

$$V_D = \frac{\sum D^2 - \frac{(\sum D)^2}{n_p}}{n_p}$$

and

$$\mathrm{var}_j = \frac{\sum d^2 - \frac{(\sum d)^2}{n_p}}{n_p}$$

In both above mentioned equations the summations of n_p=n(n-1)2 possible pairs of isolates; D is the total number of differences between two isolates over all loci; d is the difference (0 or 1) in loci j.

The goodness of fit of distribution of mating types, which was identified with specific PCR as described earlier, was tested with χ^2-statistics (a 1:1 expected ratio) (Ranta et al. 1997, Moore and Novak Frazer 2002) within sampling sites and within forms of net blotch (I, III).

$$\chi^2 = \sum_{i=1}^{k} \frac{(o_i - e_i)^2}{e_i}$$

where k is the number of observational classes, o_i is the observed frequency and e_i the expected frequency of observation i (Ranta et al. 1997).

Genetic differentiation among isolates belonging to different mating types within a field was tested with AMOVA as described above (I).

Frequency distributions of avirulent and virulent isolates (II) and frequency of mating types among field populations were compared with G^2–test (III) with Popgene v1.31 (Yeh et al. 1999). The χ^2 and G^2–tests are basically similar (Ranta et al. 1997). Shannon's information index (*H'*) was calculated with Popgene to measure the variation in virulence and in AFLP markers within populations (III, IV).

$$H' = -\sum_{i=1}^{k} p_i \ln p_i$$

Shannon's information index counts for abundance of classes, where k is the number of all classes, p_i is the proportional frequency of observations in class i. Shannon's information index is the only suitable measurement of variation dispersion on nominal scale data (Ranta et al. 1997).

3 Results

3.1 Clonal or variable populations

3.1.1 Population structures as determined by AFLP

P. teres genotypes were identified using AFLP fingerprinting (I, III, IV). Not all the same indices were originally calculated for all the *P. teres* populations. The mismatch distribution and the sum of identified fixed and polymorphic AFLP markers (frequency<1) within a population were therefore calculated from the original data. These indices and the observed mating type ratio are given in Figure 3. Part of the information was drawn from the original papers (I, III, IV).

The mismatch distribution of AFLP markers was normally distributed when data from each field location were studied separately (I and Figure 3). The only exception was the distribution of differences among isolates from Krasnodar, Russia (Figure 3), where markedly more differences were observed than elsewhere. The mismatch distribution differed from a normal distribution when the 'population' represented several collection years or several field locations (Figure 3: Finland 1989-1995). The fit of the normal distribution was not tested statistically. The frequency of putative clones (0 differences between isolates) was comparatively low within all samples, but highest among Finnish isolates collected in 1989-1995 from several locations (Figure 3 and I, III, IV). The overall genotype diversity was high since almost every isolate had a unique AFLP fingerprint (Figure 3 and I, III, IV). Few putative clones were found between collection years and locations in Finland and Australia (III), but overall frequency of putative clones was low even within a sample from a single field (I, III, IV). In Finland one putative clone was found during 1989-1995 in four of the survey years and from several locations (III). These isolates were collected from official variety trials at MTT research stations. Usually same seed lot is used for each of the cultivars in the variety trials throughout the country (Kangas et al. 2005a, b). Another putative clone was found three times in a field in Ylistaro in 2001 (Figure 3 and I), and it was re-sampled up to 100 m distance (I).

In contrast to Finnish *P. teres* isolates, those from Krasnodar were very different from each other (III, Figure 3). The number of pairwise differences in AFLP markers between isolates in Krasnodar ranged from 8 to 106, the average being 53 (III, Figure 3). The maximum number of mismatches between isolates within a population ranged from 22 to 37 AFLP markers in all of the other populations (Figure 3). Results show that *P. teres* field populations of different origin can differ from each other, but putative clones represent only a small fraction of the total population.

Australian isolates of mainly the spot form of *P. teres* were analysed in SARDI with fewer AFLP markers than were used for the isolates analysed in Finland (III). A few putative clones were identified from several field locations within states in Australia, but none of the putative clones were found in more than one state (III). The higher numbers of putative clones found among the Australian states (III), compared with the MTT collection (I, III, IV), may have been due to a smaller number of AFLP markers used in analyses at SARDI. Since different AFLP primer combinations were used in SARDI than in MTT, the results are not directly comparable and thus the isolates from MTT and SARDI were not compared with each other.

The number of putative clones was small among isolates in this study. When 16 isolates from Ylistaro, additional to the original 36 isolates (I), with half of the distance between isolates (III), were analysed, the average pairwise difference between isolates (12 AFLP markers) did not change. This showed that the distribution of differences between isolates did not change markedly, when 1.5 times more isolates were sampled from a field at closer distances (10 m instead of 20 m) (compare Figure 3 and I). Isolates representing the same leaf or the same host plant were not studied here. Based on these results, the field population structure of *P. teres* is more similar to that of a sexually reproducing than of a truly clonal population.

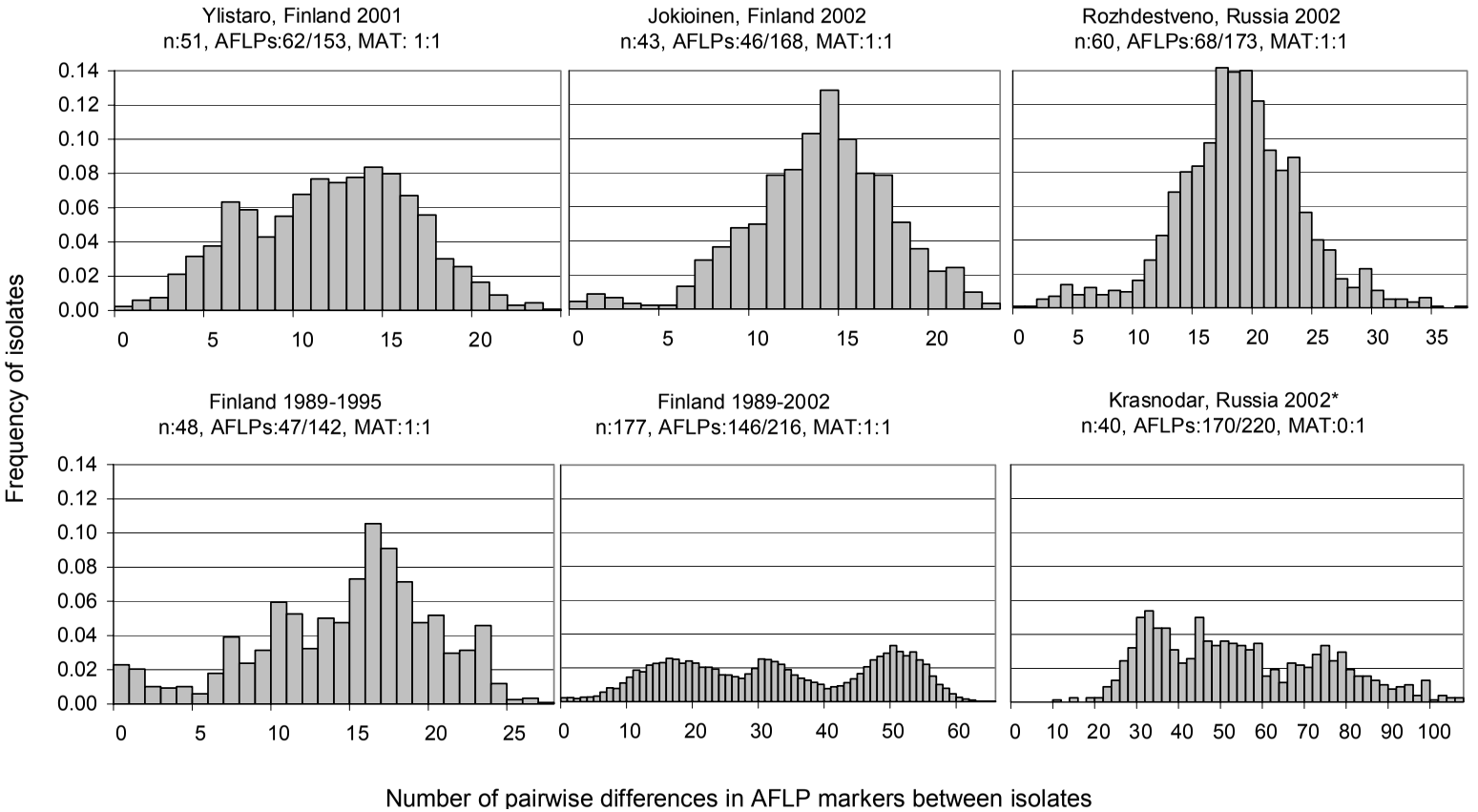

Figure 3. Mismatch distribution among *Pyrenophora teres* isolates within four fields (Ylistaro, Jokioinen, Rozhdestveno and Krasnodar) and among Finnish isolates 1989-1995 and 1989-2002. Abbreviations: n=number of isolates, AFLPs=number of polymorphic AFLP markers per all AFLP markers within population, MAT-ratio=mating type ratio identified with specific PCR markers. *Two mismatch classes combined to reduce the size of the picture.

3.1.2 Population structures as described by virulence and fungicide tolerance tests

Among 124 isolates, 56 different virulent races were identified (IV). The same virulent phenotypes were recovered more often than isolates with same AFLP fingerprint (IV). The least number of different virulent phenotypes were identified in Jokioinen in 2001, and the largest number of virulent phenotypes were identified in Rozhdestveno in 2002 (IV). The highest percentage of avirulent isolates (40%) was recovered from Jokioinen in 2001 (IV). Those isolates also had the lowest overall mean virulence, but the avirulence level varied among isolates. Isolates from Rozhdestveno were the most diverse based on virulence tests (IV).

Prochloraz tolerance varied among and within the Finnish *P. teres* isolates and field populations in 2003 (II). Tolerance to prochloraz was continuously distributed among isolates. *P. teres* isolates originating from fields where prochloraz was used during the growing season on average grew better on the fungicide medium than other isolates (II). The growth of those isolates was more variable especially on the stronger prochloraz PDA medium (II). Radial growth of *P. teres* isolates on prochloraz PDA media varied less in fields where fungicides other than prochloraz had been used during the growing season.

3.2 Variation on a field compared to a wider geographical region

Nearly similar levels of variation were found within each field studied in 2001-2002 in Finland as among several isolates collected during 1989-1995 and covering the whole barley growing area in Finland based on Shannon's information index and the number of pairwise differences in AFLP markers between isolates (Figure 3 and I, III). The 2001-2002 populations differed markedly form the 1989-1995 isolates, and thus the differences between all Finnish isolates were substantial (Figure 3). The same pattern was recorded in Australia in the SARDI collection among both forms of *P. teres*; the total variation was higher than within the field samples (III). The overall diversity in a *P. teres* f. *teres* collection consisting of 162 isolates representing Europe, North-America and Australia, was higher than that within the countries (III). The only exception was for Krasnodar, Russia, where the variation was much higher than the total variation observed among all other *P. teres* f. *teres* isolates (III and Figure 3).

3.3 Variation in *P. teres* in Finland compared with that in other countries

3.3.1 Variation detected with AFLP

167 *P. teres* isolates representing a large geographical area were analysed from the MTT collection using the same AFLP primer combination (III). Among the Finnish *P. teres* f. *teres* isolates there were on average 11-15 different AFLP markers (Figure 3 and I, III, IV). The overall number of differences in AFLP fingerprint among isolates within a sample was less than 30 over all Finnish samples. Both Russian field samples were more variable than the Finnish samples (Figure 3 and III, IV). The average number of differences among isolates within Russian samples was more than 18 and the total number of differences among isolates was more than 37 AFLP markers (Figure 3 and III, IV). The average number of differences among *P. teres* f. *teres* isolates in Australia, Canada and in Sweden was also higher than in Finland, namely 27, 33 and 26, respectively (III). The Finnish samples were the least variable compared with all other *P. teres* f. *teres* samples that were analysed using the same AFLP primer combination (III, IV). The SARDI collection (III) was analysed with different AFLP markers and therefore direct comparison between the Finnish isolates and the SARDI collection was not possible.

3.3.2 Variation detected with virulence test

Variation in virulence was lower in Jokioinen, Finland than in Rozhdestveno, Russia in 2002 (IV). There were more avirulent isolates and more variation in level of avirulence (ratings varied between 0-2 in virulence test) in Jokioinen than in Rozhdestveno (IV), whereas in Rozhdestveno there were more virulent isolates and pathotypes (virulent against several barley accessions) (IV). The interpretation of virulence results varies depending on which data, original or transformed (binary data), were used for the calculations (IV). Three barley accessions (CI 739, c-20019 and CI 9819) had similar reactions to all Finnish and Russian *P. teres* f. *teres* isolates, the two first being the least resistant and the last being the most resistant of all tested barley accessions. The remaining eight tested barley accessions exhibited variable reactions to the Finnish and the Russian *P. teres* isolates (IV).

3.4 Differentiation among populations based on AFLP, virulence and fungicide tolerance

A significant ($P<0.05$) genetic differentiation among *P. teres* populations was recorded with AFLP markers at several levels: between fields in Finland (F_{ST}

0.31-0.39) (I, II), among states in Australia within *P. teres* f. *teres* (F_{ST} 0.57) and *P. teres* f. *maculata* (F_{ST} 0.22) (III), between Finnish and Russian *P. teres* f. *teres* populations (F_{ST} 0.51) (III, IV), and between forms of *P. teres* in Australia (F_{ST} 0.79), and in the MTT collection (F_{ST} 0.40) (I, III). The genetic differentiation among bio-geographical provinces (F_{ST} 0.02), years (F_{ST} 0.11) and locations (F_{ST} 0.00-0.16) in Finland was not clear, probably due to insufficient numbers of isolates (<10) representing particular locations (III). Moreover, no significant differentiation (F_{ST} 0.00-0.14) was established for fields within states in Australia (III), probably due to insufficient numbers of isolates or AFLP markers. Genetic differentiation among populations was significant when several (>10) isolates from the same origin were analysed (I, II, III, IV). No genetic differentiation (F_{ST} 0.02) was recorded among *P. teres* isolates originating from close fields in Jokioinen, separated by a narrow road and sown with the same barley seed (IV).

Finnish field populations of *P. teres* were differentiated from each other, not only in AFLP fingerprint (I, III, IV), but also by some degree in virulence (IV). Mean virulence to barley accessions differed in Jokioinen in 2001 and 2002(IV). There were more common pathotypes among the Finnish (2001 and 2002) and Russian population than among the two Finnish populations(IV). Barley accessions CI 739 and c-20019 had the highest mean infection response (IR) values (least resistant), and the frequency of virulent isolatesto those accessions was similaramong fields, being close to 0.40 (IV). Virulence to the most resistant accession, CI 9819, was found in low frequency (<0.15) within all tested fields. The distribution of virulence was homogeneous within fields to CI 739, c-20019, and CI 9819 based on a G^2 test (P=0.79, P=0.48, P=0.82, respectively), whereas the distribution of virulence was heterogeneous (P<0.004) within fields to barley accessions c-25285, c-8755, CI 4407-1, c-29192, c-223874, c-19979, c-15811 and c-15812 (IV).

When testing the prochloraz tolerance the effect of field origin of *P. teres* isolates on radial growth was significant F=212, P<0.0001 and F=267, P<0.0001 at 0.1 μg ml^{-1} and at 1.0 μg ml^{-1} prochloraz on PDA medium, respectively (II). Most of the total variation was between fields, namely 38.0% and 43.5% on 0.1 μg ml^{-1} and on 1.0 μg ml^{-1} prochloraz PDA medium, respectively. Differentiation between fields was obvious, but the experimental design did not able intrepretation of the factors that affected the differentiation (II). This study was a preliminary survey and further studies are needed to determine if tolerant isolates evolved due to prochloraz use (II). Tolerance to prochloraz was similar between fields, where prochloraz was the main fungicide used during the growing season. Lower tolerance to prochloraz was observed in fields where other fungicides were used (II).

3.5 No hybrids between *P. teres* f. *teres* and *P. teres* f. *maculata*

There are only six *P. teres* f. *maculata* isolates in the MTT collection and five of those were included in this study. The isolates originated from Australia, Canada, Czech Republic, Sweden and two from Finland. The Finnish isolates were collected in 2000. All new Finnish and Russian isolates collected during this study were *P. teres* f. *teres* according to specific PCR markers (I, III). The amplified PCR product is not annotated. It is uncertain if the PCR product has function or biological meaning or if it is just associated with the form of net blotch. Symptoms on leaves of the moderately resistant variety Annabell in one field appeared to be atypical lesions of the net form of net blotch (II). Those isolates were not analysed using molecular methods and thus their identity remains uncertain. The two forms of *P. teres* were clearly distinguished in AFLP analyses (III). The differences between the spot and net forms of *P. teres* were always larger than differences among isolates within a single form (I, III). As a result, isolates were clearly clustered based on AFLP according to net and spot forms (I, III). AFLP fingerprinting did not reveal any subcluster between spot and net forms (I, III). However, a low but significant genetic differentiation was estimated between Krasnodar, Russia and between groups of both forms of *P. teres*, although Krasnodar isolates were all *P. teres* f. *teres* (III). This might have been due to the overall very high diversity recorded among isolates from Krasnodar (Figure 3 and III). Krasnodar possibly represents an old population of *P. teres*, which will be further discussed later.

The proportion of *P. teres* f. *maculata* isolates was comparatively higher in Australia (III). In addition, both forms were found in the same field in New South Wales, in Queensland and in Western Australia (III). No intermediate isolates were found and all the isolates were clearly clustered based on AFLP into two distinct groups, net and spot forms (III). The genetic differentiation between the forms of net blotch was high and significant (III).

3.6 Sexual reproduction

Both mating types were common within all populations, except in Krasnodar, where only *MAT2* mating type was present (Figure 3 and III). The reason for the occurrence (or identification) of only one mating type in Krasnodar is uncertain and will be discussed later. The fraction of clonal isolates among all samples was comparatively low (Figure 3) and because the mating type ratio did not significantly differ from a 1:1 ratio based on χ^2 (I, III), random mating was expected among all samples other than those from Krasnodar.

The index of multilocus associations was analysed. Surprisingly, based on the

indices, the hypothesis of random mating was rejected among samples belonging to the MTT collection (III). The indices were also calculated from samples for which putative clones were represented only once, but the random mating hypothesis was still rejected among Finnish samples (III). In the SARDI collection from Australia the multilocus associations were closer to the hypothesis of random mating (III).

In conclusion, these results indicate that sexual reproduction may be common in most *P. teres* populations since both mating types were equally common and the clonal faction of isolates within fields was low (Figure 3 and I, III). In addition, no genetic differentiation based on AFLPs was observed between isolates of different mating type (I), which is an indication of genetic exchange among isolates belonging to different mating types. The reason why the test for multilocus associations resulted in rejecting the random mating hypothesis will be discussed later.

4 Discussion

Diversity of *P. teres* populations was studied at the molecular level and in terms of virulence and prochloraz tolerance. The objective was to address several questions concerning the population biology of *P. teres* and then estimate the potential of *P. teres* to evolve under selection pressure exerted through deployment of resistance genes and use of fungicides in barley cultivation. *P. teres* isolates from Finnish fields were studied in the main, but populations from Russia and Australia were also included in this study. AFLP markers revealed a high degree of genotypic variation in *P. teres* populations, and extensive genetic differentiation among populations and between forms of *P. teres*. Surprisingly, the Finnish *P. teres* populations comprised only *P. teres* f. *teres* and both mating types were equally common. These results support the earlier suggestion of possible sexual reproduction in *P. teres* populations in Finland (Robinson and Mattila 2000), and the report of substantial genetic differentiation in *P. teres* populations (Peever and Milgroom 1994). Results obtained in this study and the limitations in their interpretation are discussed.

4.1 Population diversity

4.1.1 Genotypic variation

In this study the isolates represented distinct locations and several years and almost every isolate was unique based on AFLP (Figure 3). However, isolates from Krasnodar differed each other comparatively more than isolates within other populations (Figure 3). The high degree of genotypic diversity in *P. teres*

populations is in agreement with earlier RAPD (Peltonen et al. 1996, Jonsson et al. 2000), restriction fragment length polymorphism (RFLP) (Wu et al. 2003), and AFLP (Rau et al. 2003) studies. It is presumed that AFLP can distinguish closely related individuals better than other DNA-based techniques (Purwantara et al. 2000) because several markers are amplified from whole genome. The number of putative clones in *P. teres* was low (Figure 3) based on AFLP compared with in *Tapesia yallundae* populations, where the most common multilocus genotype accounted for 11% of the collection (Douhan et al. 2002). In this study many more AFLP markers were used than by Douhan et al. (2002), which may influence the results on diversity. Similar high genotypic variation has been revealed with AFLP in other cereal pathogens: *Phaeosphaeria nodorum* (Bennett et al. 2005), *Gibberella zeae* (Schwein.) Petch (Zeller et al. 2004), and in *Rhynchosporium secalis* (Williams et al. 2003, Kiros-Meles et al. 2005). The methylation insensitive AFLP fingerprinting method revealed a smaller portion of unique genotypes in Italy (Rau et al. 2003) than in this study. This is in accordance with the information that methylation sensitive *Pst*I AFLP tends to produce more markers than methylation insensitive *Eco*RI AFLP (Powell et al. 1997, Haen et al. 2004, Yuan et al. 2004). Methylation insensitive and sensitive markers are differently distributed on the chromosomes (Powell et al. 1997), *Pst*I markers covering the whole genome (Nybom 2004).

Gene diversity (Nei 1973) is commonly used instead of genotypic diversity to measure population variation. Gene diversity is not as informative based on AFLP as when based on co-dominant markers since the true alleles are difficult to identify with AFLP. Therefore, it is usually assumed without any further support that AFLP loci have two alleles: the presence or absence of a locus. Here the gene diversity was low (0.07-0.21) (IV and Serenius, unpublished data), and increased slightly after the monomorphic, fixed markers were excluded from the original AFLP data (0.10-0.23) (IV). Similar gene diversities were observed based on AFLP data from *P. nodorum* (0.11-0.12) (Bennett et al. 2005) and significantly higher from *T. yallundae* (0.39-0.43) (Douhan et al. 2002).

When AFLP fingerprints are compared, the actual DNA sequence is unknown. Polymorphisms detected with AFLP can arise from loss or gain of restriction sites or insertions/deletions between restriction sites (reviewed in Bensch and Åkesson 2005). When mutation occurs between the restriction sites, it results in a DNA fragment of a different length, whereas a substitution that creates a new cut site between the restriction sites may result in an absent allele for one AFLP locus that can be scored as present at another AFLP locus (reviewed in Bensch and Åkesson 2005). Due to such problems, the number of differences between isolates is an underestimate of variation between isolates, but an overestimate of true AFLP loci. This is a common problem with AFLP markers. Therefore, isolates with identical AFLP fingerprints have been termed ‘putative clones’ earlier in the text.

It should be borne in mind that AFLP markers are not as informative for measuring the gene diversity as co-dominant markers. However since *P. teres* is haploid most of the problems of biallelic marker can be avoided in analysing the population structure. In this study over 150 AFLP markers were used for comparisons (I, III, IV), except for the SARDI isolates, which were compared with each other based on 37 polymorphic AFLP markers (III). The genome heterogeneity was high (I, III, IV). Douhan et al. (2002) noted that seven to eight AFLP markers, which segregated in 1:1 ratio, but were unlinked, were enough to account for 95-99% of the observed variation in *Tapesia yallundae*. Douhan et al. (2002) used only 10 , segregating AFLP markers to analyse *Tapesia yallundae* population structure. Isolates of *P. teres* were crossed in this study (I), but since no progeny were produced from *P. teres* f. *teres* crossings, there were no segregation data available. Therefore, all AFLP markers were analysed. Crossing *P. teres* f. *teres* and *P. teres* f. *maculata* at MTT (I) produced 180 segregating AFLP markers (Manninen et al. unpublished data). However, this information was not useful for this study as there were so few *P. teres* f. *maculata* isolates in the MTT collection (III) and the forms of *P. teres* differed significantly. In this study, AFLP was the only method used to identify variation at the molecular level. Due to *P. teres* being haploid and use of single-conidial isolates, the observed presence or absence of an AFLP marker represents the actual genotype of an isolate, which is an advantage. Most often about 50 polymorphic AFLP markers were used in this study to compare isolates of the same origin (population) (I, III, IV).

4.1.2 Phenotypic variation

In this study 56 *P. teres* pathotypes were identified among 124 isolates of net forms of the pathogen using 11 barley differentials (IV). Similarly, Tekauz (1990) identified 65 pathotypes among 219 isolates of *P. teres* f. *teres* and *P. teres* f. *maculata* in Canada using 12 barley accessions. Thus more pathotypes per isolate were identified in this study than in Canada. Fewer pathotypes were identified within field in Finland than in Rozhdestveno. However the total number of pathotypes among all Finnish isolates (Jokioinen 2001 and 2002) was higher than in Rozhdesteveno in this study (IV). Virulence among Nordic-Baltic isolates was shown to be comparatively similar (Robinson and Mattila 2000), but clear differences were established between Russian and Finnish isolates (Afanasenko 2001). Here more common pathotypes were found among Rozhdestveno population and the two Finnish populations than among the two Finnish populations(IV). The comparison of virulence test results across studies is difficult because of the differences in experimental conditions (field vs. greenhouse vs. laboratory; whole plants vs. detached leaves; first leaf vs. second or third leaf).

The seedling leaf used for inoculation (Tekauz 1990), but not the seed size (Jalli and Robinson 2000) affected the infection response, which can vary among

leaves on the same plant and even according to position on the same leaf (Tekauz 1990). The variation in infection reactions is likely due to physiological age of each leaf at the time of inoculation (Tekauz 1990). The cotyledon (first leaf) is more susceptible than subsequent leaves (Tekauz 1990). Differences in composition of the wax layer, which is important for the plant's self-defence, were identified in maize between juvenile and adult leaves (Sturaro et al. 2005). This might also be the case in barley. In this study the first leaf was used and it was cut into sections (detached leaf test) to represent replicates (Afanasenko et al. 1995). In contrast, Tekauz (1990) preferred to use central sections of the second and third seedling leaves, and host genotypes were more clearly differentiated into resistant and susceptible categories by scoring the infection response on the second seedling leaf rather than on the first (Robinson and Jalli 1996, Jalli and Robinson 2000). Scott (1992) identified cultivars that were susceptible to *P. teres* at the seedling stage, but which showed resistant reactions at the adult stage, whereas some cultivars were more susceptible to *P. japonica* (probably *P. teres* f. *maculata*, Crous et al. 1995) at the adult stage. In this case the detached leaf test did not reflect adult plant resistance. Tekauz (1990) suggested that symptoms can vary as the result of temporal differences in host penetration and/or subsequent lesion development by individual or grouped conidia. This was supported by the finding that inoculum concentration affects infection response (Jalli and Robinson 2000). In this study the inoculum was filtered through two layers of cheesecloth to diminish the amount of mycelium in the inoculum and to ensure that conidia were the source of infection and not pieces of mycelium. Toxins produced by *P. teres* might also affect the susceptibility of the host plant. *P. teres* produces three different types of aspergillomarasmine that are toxic to barley (Weiergang et al. 2002a). The form of *P. teres* is not correlated with toxin production and no clear correlation has been established between toxin B production and virulence (Weiergang et al. 2002b). In contrast, sensitivity of barley to toxins A and C and susceptibility of barley to *P. teres* was demonstrated (Weiergang et al. 2002a). Weiergang et al. (2002a) suggested that pure toxins A and C could be used to test the susceptibility of barley to *P. teres* in lieu of conidial isolates.

In accordance with Robinson and Jalli (1996) and Jalli and Robinson (2000), barley accession CI 9819 was resistant in our study and represented a promising source of resistance to net blotch for the Nordic-Baltic region. In this study CI 5791 and especially CI 739 were not as resistant as reported earlier (Khan and Tekauz 1982, Tekauz 1990, Robinson and Jalli 1996, Jalli and Robinson 2000, Robinson and Mattila 2000), but the virulence to CI 739 was similar among all samples (IV). Correspondingly, Arabi et al. (2003) reported lower resistance to CI 5791 than to other accessions. In this study more virulent isolates were identified among all populations to CI 739 (35-40%) and c-20019 (25-35%) than to any other barley accessions among the differential set (IV). Earlier 10.8% and 19.1% of *P. teres* isolates from Russia, Germany, Czech Republic and Slovakia

were virulent to CI 739 and c-20019, respectively (Afanasenko et al. 1995). Therefore, it is suggested that resistance represented by CI 739 or c-20019 might be more easily lost than resistance of other tested barley accessions. However, as mentioned earlier, several factors affect interpretation of virulence tests and susceptibility gauged from infection response on the first leaf may not represent that of an adult plant.

Understanding the diversity and monitoring the changes in virulence and pathotype composition are required for successful resistance breeding programs (Tekauz 1990). A virulence test measures a gene-for-gene interaction (Flor 1947), where the host resistance genes (receptors) recognise avirulence gene products of a pathogen. Also a partial resistance (quantitative resistance), which does not follow the gene-for-gene pattern, can be detected using a virulence test (Robinson and Jalli 1996, McDonald and Linde 2002a, b). A virulence or resistance test can also be used for finding new and more resistant barley accessions for plant breeding purposes. Several criteria are used in assessments to determine the resistance level. These include lesion type, percentage of infected leaf area, lesion size and number of lesions per unit leaf area, and an index combining lesion size and type (reviewed in Scott 1992). Nevertheless, the isolates are commonly classified into avirulent and virulent classes based on the extent of leaf chlorosis.

Afanasenko et al. (1995) suggested a standard differential set of barley accessions for international use to test the variation in *P. teres* populations. The genetic background of resistance in the differentials is largely unknown as pointed out (IV, Tekauz 1990, Afanasenko et al. 1995). In western Canada the virulence of the spot form of net blotch was shown to change rapidly, although the most commonly grown barley cultivars were susceptible, i.e. no selection pressure was exerted (Tekauz 1990). In order to improve the virulence test and to get more information, pathotypes should be named based on similarity of IR on barley differentials, i.e. the true occurrence of resistance genes as suggested originally by Limpert and Müller (1994). Their nomenclature of pathotypes is a good system and the names of pathotypes are provided according to triplets of barley accessions. The international, uniform nomenclature as well as use of uniform differential series, would help to monitor evolution of pathotypes. More research is needed to identify the genetic backgrounds of the barley accessions to avoid using unconsciously the same resistance genes or alleles in breeding programs.

Fungicide tolerance is as important a phenotypic trait as virulence. The prochloraz tolerance test indicated substantial variation between fields and among isolates in Finland (II). High levels of variation among isolates in radial growth and conidial production were recorded also earlier in Finland (Peltonen et al. 1996). Peever and Milgroom (1992, 1993, 1995) found that fungicide tolerant pheno-

types were continuously distributed in *P. teres* populations that had not previously been exposed to DMI fungicides. The distribution of prochloraz tolerance was continuous within fields in this study also, but the average tolerance differed between fields (II). In South Africa spot form of net blotch was more tolerant to DMIs than net form of net blotch (Campbell and Crous 2002). Prochloraz and imazalil, which both belong to the same imidazole class of DMIs, are the active ingredients in half of the chemical products approved for seed dressing barley in Finland (Plant Production Inspection Centre 2006a) and accounted for 56% of active ingredients in fungicide products sold in Finland in 2004 (Plant Production Inspection Centre 2004). DMIs inhibit the cytochrome P450 dependent oxidative demethylation of eburicol in filamentous fungi in the ergosterol biosynthesis pathway (Steffens et al. 1996). Reduced sensitivity of *P. teres* to DMIs in barley has been reported in New Zealand (New Zealand Plant Protection Society 2006). In addition, Peever and Milgroom (1992, 1993, 1995) found cross reactions between fungicides in the DMI class for *P. teres*. A concentration 1 $\mu g\ ml^{-1}$ of prochloraz resulted in 90% inhibition in growth of *P. teres* isolates in Sweden before prochloraz-based fungicides were in commercial use (Olvång 1988). In this study the overall mean inhibition of growth of *P. teres* isolates was 86% at the same prochloraz concentration (II). Since earlier results from Finland are lacking, it cannot be concluded that the effectiveness of prochloraz has changed over time. Prochloraz tolerance of *P. teres* might have been under selection because distribution of tolerance was different between the fields (II) and prochloraz has been common fungicide used in barley production in Finland (Plant Production Inspection Centre 2004).

4.2 Distribution of variation and differentiation

It was assumed that the Finnish *P. teres* population was likely to be i) fairly similar over years and regions based on the results of Peltonen et al. (1996), Jalli and Robinson (2000), and Robinson and Mattila (2000), and ii) the Finnish *P. teres* isolates do not differ markedly from other *P. teres* isolates from northern Europe (Jalli and Robinson 2000, Robinson and Mattila 2000), but iii) differ from Russian *P. teres* populations (Afanasenko 2001). In this study, higher diversity was observed within large groups of isolates than within field samples based on AFLP data analysed by Shannon's information index (Figure 3, III, IV). Significant genetic differentiation was identified based on AMOVA among populations when several isolates were analysed from the same field (Figure 3, I, III, IV). Similar results were obtained earlier by Peever and Milgroom (1994) and Rau et al. (2003). These results are in accordance with short-distance dispersal of *P. teres*. The restricted dispersal among fields could lead to differentiation between them. In contrast, population subdivision has not been reported for several other cereal fungal pathogens: *T. yallundae* (Douhan et al. 2002), *P. nodorum* (Keller et al. 1997, Bennett et al. 2005), *G. zeae* (Zeller et al. 2004), *R. secalis* (Salamati et al. 2000) or *M. graminicola* (Schnieder et al. 2001, Zhan et

al. 2003). All of the above-mentioned cereal pathogens are similar to *P. teres* in their dispersal characteristics and long-distance dispersal is not possible without human assistance. Accordingly, no genetic differentiation in the *P. teres* populations was established among sampling locations (few samples only) in Finland (Peltonen et al. 1996, III) or in the Czech Republic (Leisova et al. 2005a, b) or among populations separated by 20 km in Sweden (Jonsson et al. 2000) or in North America (Peever and Milgroom 1994). Genetic differentiation was low or insignificant between bio-geographical provinces (F_{ST} 0.02)and sampling years (F_{ST} 0.11) based on few isolates in Finland and within states in Australia (F_{ST} 0.00-0.16) (III). Recently, Banke and McDonald (2005) identified subdivision among populations of *M. graminicola*, in contrast to earlier results of Schnieder et al. (2001) and Zhan et al. (2003). This supports our findings.

In this study AMOVA was used to estimate differentiation among populations. It is nowadays even more widely used than G_{ST} (coefficient of gene differentiation, Nei 1973) for partitioning genetic variability (Nybom 2004). Values for Nei's G_{ST} and for the AMOVA-derived F_{ST} (Weir and Cockerham 1984) are usually similar when calculated for the same data set (reviewed in Nybom 2004). The basic assumptions behind the two indices that consider the evolutionary development lineages differ (Nei and Kumar 2000). Nei and Kumar (2000) criticized that assumptions of equality of relationships among populations, which characterise F_{ST}, are unnatural. This study concentrated on measuring the variation in *P. teres* populations and recording the differentiation among populations and thus the assumptions were accepted. Estimation of genetic diversity or distance from AFLP data is straightforward (Hill and Weir 2004), and the type of dominant marker (AFLP or RAPD) is probably of relatively little importance for AMOVA-derived among population variability (Nybom 2004). In conclusion, results obtained here with AFLP are considered valid.

In this study differentiation was recorded based on virulence tests among *P. teres* field populations . This might be due to differences in the barley cultivars grown among regions, which may have exerted different selective pressure on the pathogen (Tekauz 1990). In accordance, quantitative resistance was reported earlier among Nordic barley genotypes and the Finnish *P. teres* isolates differed in virulence according to the host barley (Robinson and Jalli 1996). *P. teres* is capable of rapid adaptation, as evidenced by the change in the proportion of the forms of *P. teres* in several geographical regions (Tekauz 1990). Our finding of differentiation on virulence is in line with the findings of Afanasenko (2001). In this study no genetic differentiation was observed between virulent and avirulent isolates within a field (F_{ST} 0.03-0.06) when isolates were grouped according to virulence reaction and the genetic variation in AFLPs was compared among groups (IV).

4.3 Sources of variation in *P. teres* populations

4.3.1 Asexual and sexual reproduction

Robinson and Mattila (2000) suggested that *P. teres* reproduces mainly asexually under natural conditions in Finland. Asexual reproduction must be important in the life cycle of *P. teres* since it is presumed to be the main form of spore dispersal during the growing season (Jordan 1981). Robinson and Mattila (2000) also suggested that the apparent diversity in *P. teres* populations might come through, as yet unobserved sexual or parasexual reproduction or high mutation rates. The results of this study support the assumption of sexual reproduction in Finland. Both mating types were found and they were equally common within sampling locations (I, III). Furthermore, no genetic differentiation was found between mating types, which supports random mating rather than strictly asexual reproduction of isolates (I). Based on this study, the overall population structure of *P. teres* seems to be more of a sexually reproducing than a truly clonal population in Finland, in Russia and in Australia (Figure 3 and I, III, IV). A significant proportion of clonal isolates is expected in the population if asexual reproduction is predominant in the life cycle of a pathogen (Kohli and Kohn 1998). The frequency of clonal isolates was close to 80% based on DNA fingerprinting in a *Sclerotinia sclerotiorum* (Lib.) de Bary population (Kohli and Kohn 1998).

The only exception to mating type ratios within sampling location in this study was for the Krasnodar population (III). Surprisingly, only one mating type was found in Krasnodar, where instead of putative clones, large differences were identified among isolates (III). The high diversity in Krasnodar might come through retrotransposons (Taylor et al. 2004) or high mutation if Krasnodar represents an old population. It is presumed that these results does not exclude the possibility of sexual reproduction or high mutation rates in Krasnodar. On the contrary, both mating types should occur in Krasnodar according to the hypothesis that the region is close to the centre of origin of barley (Blattner 2006). The mating primers were developed based on two isolates only (Rau et al. 2005) and if mutation has occurred on mating type region it is possible that these spesific primers does not identify the other mating type (MAT1) among Krasnodar isolates. Further sampling is necessary to confirm or disprove the occurrence of both mating types in Krasnodar.

The occurrence of sexual reproduction was also studied using multilocus association (III). The multilocus association analysis rejected the hypothesis of random mating within some populations (III). There are several reasons why the multilocus association indices reject random mating even if the population reproduces sexually (Maynard Smith et al. 1993). Even if both mating types occur in the same area, recombination may not be frequent enough for multilocus associations to approach zero or the population might actually be a mixture of

several populations or have an epidemic structure (Maynard Smith et al. 1993). In the Finnish fields recombination may not be frequent enough to break up the multilocus associations. The samples may also comprise isolates of different origin if the original inoculum was seed borne (possible mixture of seed lots) and derived also from plant debris. This might account for rejecting the hypothesis of random mating based on multilocus associations even though both mating types were equally common and not genetically differentiated (I, III).

Factors affecting the frequency of sexual reproduction in *P. teres* in Finland remain speculative. Jordan (1981) indicated that sexual reproduction is important as a source of infection; he noted symptoms on healthy tillers after 5-10 days of exposure to straw bearing the mature perithecia with ascospores. In this study, *P. teres* isolates from Jokioinen were crossed on autoclaved barley straw after identifying the mating types with specific PCR markers. Straw was inoculated with *P. teres* isolate mixtures of both mating types and placed outdoors on the ground in a nylon-mesh bag in October 2002. Control straw was inoculated with one mating type only. Straw was assessed in 2003, but perithecia were not observed (Serenius, unpublished data). More research is needed to understand the conditions that determine if *P. teres* reproduces sexually in Finland. The temperature regimes should be suitable for perithecia development since the process requires cool temperatures (10-15°C) for one to six months (McDonald 1963), but development may take up to 11-15 months (Smedegård-Petersen 1978). Based on these results sexual reproduction occurs both in Finland and in Australia (I, III), but possibly infrequently.

4.3.2 *P. teres* f. *teres* and *P. teres* f. *maculata*

4.3.2.1 Occurrence of forms of net blotch

The status of *P. teres* f. *maculata* (spot form) in Finland is currently uncertain, but both forms have been equally common based on symptom types (Mäkelä 1972). It appears that *P. teres* now comprises only *P. teres* f. *teres* in Finland (I, III). This might be a result of unintentional selection, which is supported by the findings of Jørgenssen et al. (2000), who indicated that several Nordic barley cultivars have some levels of resistance to *P. teres* f. *maculata*. According to this study, the prevalence of *P. teres* f. *teres* has increased significantly since the 1970s (Mäkelä 1972) presuming that the forms of *P. teres* were correctly identified earlier. However, at that time the spot form was also reported from other Scandinavian countries (Hansen and Magnus 1968, Smedegård-Petersen 1971). The reason for the change in occurrence of forms of *P. teres* was not elucidated during this study. As Hyvönen (2004) pointed out, during the last decades agriculture has become more efficient and simultaneously the amount of pasture land has declined in Finland, whereas the use of fertilisers and pesticides has increased. This change has affected e.g. weed composition in fields (Hyvönen

2004). Some of the weed species and pasture plants might bealternate hosts to plant pathogens such as *P. teres*, and therefore the change in weed composition or in the amount of pasture affects pathogen populations. The forms of *P. teres* differ somewhat in their host speciation (compare Sampson and Watson 1985, Brown et al. 1993). However, it is uncertain if changes in weed composition and area of pasture are the reasons for the change in form composition of *P. teres* in Finland. *P. japonica* (synonym for *P. teres* f. *maculata*) was found on barley cultivars and on wild barley in South Africa (Scott 1991). In contrast, *P. teres* was found only on barley in the 1970s in Finland, although other closely related phytopathogens species (*Helminthosporium* spp.) were found on other cereals and grasses (Mäkelä 1975). *P. teres* f. *teres* was recently found on *Agropyron repens* L. in Finland (M. Jalli, MTT, personal communication).

The hypothesis of changes in agriculture affecting selection of pathogen populations is in accordance with the suggestion that the epidemic caused by *Pyrenophora tritici-repentis* (Died.) Drechs. on wheat in Canada in the 1970s was due to changes in agronomic practices (Strelkov and Lamari 2003). This hypothesis is further supported by a recent report showing that the spot form of *P. teres* increased together with adoption of no-tillage stubble management systems in Western Australia (Jayasena et al. 2002). Regional increases in occurrence of disease have also been attributed to increased use of susceptible cultivars over several years (Jayasena et al. 2002). In conclusion, there are several factors in the agricultural environment that could affect selection of pathogen populations. This requires further research.

4.3.2.2 Hybridization

In this study the *P. teres* isolates were clearly clustered according to their symptom form determined by PCR and AFLP analysis (III, Williams et al. 2001). This was in accordance with Rau et al. (2003) and Leisova et al. (2005a). Since the two forms of *P. teres* are able to cross (Smedegård-Petersen 1978, Campbell and Crous 2003, I), the hybrid form of the two can exist. Some possible hybrids have been identified through molecular methods (Stevens et al. 1998, Campbell et al. 2002) and the genetic stability of the hybrid progeny under laboratory conditions has been reported (Campbell and Crous 2003). In contrast, based on the results of this study and other AFLP studies on *P. teres* (Williams et al. 2001, Rau et al. 2003, Leisova et al. 2005a), it seems unlikely that recombination occurs in nature frequently between the two forms of *P. teres*. This was supported also by results from crossing the two forms of *P. teres in vitro*, as incomplete numbers of asci and abnormal ascospores were produced in perithecia (I). Campbell and Crous (2003) were able to produce only 14 hybrid isolates. In addition, Rau et al. (2006) found little variation in mating type gene sequences within forms of *P. teres*, but high genetic differentiation (F_{ST} 0.837 for MAT1 and F_{ST} 0.879 for MAT2; $P<0.001$) was found between the forms of *P. teres*. Moreover, they

concluded that hybridization between forms of net blotch is rare or absent under field conditions.

In this study comparatively less differentiation was recorded between *P. teres* f. *teres* isolates from Krasnodar and *P. teres* f. *maculata* isolates than other *P. teres* f. *teres* isolates and *P. teres* f. *maculata* in the MTT collection (III). Blattner (2006) suggested that the genus *Hordeum* originated from Eurasia, close to the Krasnodar region. It is uncertain if *P. teres* has evolved together with barley or if it became pathogenic later in the host evolution. If parallel evolution is presumed, then Eurasia could be the origin and centre of diversity of *P. teres*. This theorem is supported by the finding that isolates from Krasnodar were the most diverse; 220 polymorphic loci and substantial differences between isolates (Figure 3 and III), but closest to *P. teres* f. *maculata* isolates (III). Krasnodar is relatively close to the Fertile Crescent, which was suggested to be the area of origin of *M. graminicola* (Banke and McDonald 2005). Krasnodar representing an old population is supported by suggestion that southern Russia is also the origin of *Fusarium graminearum* Schwabe (Gagkaeva and Yli-Mattila 2004). Based on the current results, the Krasnodar population might provide interesting information on evolution of the *Pyrenophora* species.

4.3.3 Gene flow

The observed gene flow between *P. teres* populations in different field locations was low since the genetic differentiation between locations was high in this study (I, III, IV). Thus there must be factors limiting the dispersal. Global marketing of barley seeds makes long distance dispersal of *P. teres* possible, which would not otherwise spread over such distances. Seed-borne inoculum is a well-established source of primary infection of *P. teres* and even one percent of infected seed could represent a significant base source for infections (Jordan 1981). Commercial barley seed is inspected in Finland and 24.7% of seed samples had more than five percent *Pyrenophora* species infection (*P. graminea* and *P. teres* not specified) in 2005 (Plant Production Inspection Centre 2006b). It is speculated that the *P. teres* f. *maculata* was introduced into Canada by plant breeders in infected seed from Scandinavia (Tekauz and Mills 1974). This hypothesis was supported by these results since among the five spot form isolates in the MTT collection, the Canadian isolate was most similar to the Finnish isolate (Serenius, unpublished data). The Australian spot form isolate was most similar to the Swedish isolate (Serenius, unpublished data). These observations are preliminary and based on a few isolates only. Current results show that the differentiation between net form isolates from Sweden and Canada was also low (III) and the isolate from USA was most similar to the Canadian and Finnish *P. teres* f. *teres* isolates (Serenius, unpublished data). The *P. teres* f. *maculata*, as well as several other phytopathogens, has been introduced into Australia from the Old World (Khan and Tekauz 1982). The migration pattern of *M. gramini-*

cola (Fertile Crescent->Europe->New World) supports this theory (Banke and McDonald 2005). An example of possible natural spread of *P. teres* includes increased prevalence of the spot form in Saskatchewan and Alberta between 1976 and 1985 (Tekauz 1990).

It is important to note that to date the gene flow between *P. teres* populations between closely linked regions like fields in Jokioinen (5 km distance and one year) (IV), Jokioinen and Ylistaro (in Finland, 400 km apart) (I) and Jokioinen and Rozhdestveno (500 km apart) (IV) seems to be low. On the other hand, these results could also indicate that *P. teres* adapts rapidly once it has emigrated, as was suggested by Tekauz (1990). The high diversity in *Rhynchosporium secalis* and capacity to evolve rapidly is suggested to arise through high mutation rates (Williams et al. 2003). *P. teres* might resemble *R. secalis* in this respect.

4.4 Sampling and assumptions

The experimental materials comprised *P. teres* single-conidial isolates collected either from a single field or from larger areas. The sample sizes varied according to sampling location and some locations were represented by only a single isolate (III). Sampling locations with only a single isolate were only included in comparisons between forms of *P. teres* (III). Among location comparisons were made when at least two isolates with different AFLP fingerprint per location were available (III), which represents a comparatively small sample size. Within location comparisons were based on larger sample sizes (36-67 isolates per location) among isolates from the MTT collection (I, III, IV). Smaller sample sizes per field were used from the SARDI collection (III), but more reliable results were obtained as the samples were pooled for among state comparisons (III). Samples sizes used in this study were similar to those of the earlier studies (Peever and Milgroom 1994, Jonsson et al. 2000, Rau et al. 2003).

In this study several *P. teres* isolates (less than 10) per sampling location were insufficient to detect genetic differentiation between bio-geographical provinces in Finland (F_{ST} 0.02) (III), years 1989-1995 in Finland (F_{ST} 0.11) (III) (III). This was in accordance with Peltonen et al. (1996), Jonsson et al. (2000), and Leisova et al. (2005b). Therefore, a minimum of 20 haplotypes of *P. teres* are recommended based on these results for population structure analyses (among locations) and for within sampling location analyses over years, whereas overall variation can be gauged by using several sampling locations and fewer or even a single representative isolate per location as in III, Peltonen et al. (1996) and Leisova et al. (2005b). A similar suggestion was made by Banke and McDonald (2005). In contrast, Nybom (2004) found no correlation between population genetics parameters derived from AFLP data and sampling strategies (number of populations, number of individuals sampled per population). Based on the current results (III, IV), the 'true' number for a sufficient sample size is even

larger than that suggested here because of the possibility of re-sampling the same putative clones, which weakens the test power to identify differentiation among populations.

4.5 Evolutionary potential

Based on these results, *P. teres* is able to reproduce both asexually and sexually in Finland and in Australia. However, the observed subdivision of variation in *P. teres* populations was somewhat surprising. Some other cereal pathogens for example *P. nodorum* (Bennett et al. 2005), *G. zeae* (Zeller et al. 2004) and *R. secalis* (Williams et al. 2003, Kiros-Meles et al. 2005), have a high genetic diversity within a small area. These pathogens resemble *P. teres* in their dispersal range and reproduction system. Therefore, it is concluded that there must be factors limiting spread and reproduction of *P. teres*. These may include the reduction of population size during winter or crop rotation. Consequently *P. teres* does not seem to pose as serious a risk as the other mentioned barley pathogens.

The model of McDonald and Linde (2002 a, b) predicts the evolutionary potential of a pathogen mainly based on dispersal range and reproduction system (asexual; sexual: inbreeding or outcrossing; mixed reproduction). The estimated capability to evolve increases as the dispersal range increases and as the reproduction system complicates. The results from this study show that *P. teres* differs from other cereal pathogens with similar population biology, at least in the geographical regions surveyed. The life cycle of plant pathogens may differ between geographical regions and depend, for example, on the cultivation methods (long crop rotations vs. succession, deep ploughing vs. no-tillage, spring and winter barley in cultivation vs. only one) or environmental factors (long-lasting snow cover vs. two seasons only). As a result, more information is needed on local plant pathogen populations. These conclusions are supported by Enjalbert et al. (2005), who reported a strong differentiation between northern and southern populations of *Puccinia striiformis* in France. The observed differentiation is in contrast to the long dispersal capacity of *P. striiformis.* They suggested that the differentiation was due to local adaptation to specific host, climatic conditions or host cultivars. *P. striiformis* is estimated to represent a comparatively lower evolutionary risk due to strictly asexual reproduction than pathogens with mixed reproduction system (McDonald and Linde 2002a, b). Moreover, Peever and Milgroom (1994) explained the observed differentiation between *P. teres* populations by local adaptation. Therefore, it is concluded that the evolutionary risk for *P. teres* might be lower in areas where the population is mainly asexually reproducing. A mixed reproduction system increases the evolutionary potential of a pathogen (McDonald and Linde 2002a, b). The above-mentioned examples and results from this study indicate the importance of analysing the local pathogen populations before deploying new resistance genes in cultivation. In addition, Kiros-Meles et al. (2005) suggested that local

pathogen populations should be studied before introducing host-plant resistance into cultivation because the efficacy of host-resistance management strategies is largely influenced by the local diversity of the pathogen.

4.6 Advantages and disadvantages of AFLP

In this study the results and conclusions on diversity in *P. teres* populations were mainly based on analyses of AFLP markers. AFLP markers are dominant, like RAPDs, which is a disadvantage in population studies. Medini et al. (2005) agreed with others that the polymorphic information content for simple sequence repeats (SSR) is greater (maximum 100%) than for AFLP (observed 31%). This is due to a larger number of alleles amplified with SSRs (Medini et al. 2005). However, Bensch and Åkesson (2005) concluded that AFLPs are currently the most suitable marker type for population structure studies since a large fraction of the genome can be easily covered. Since *P. teres* is haploid, there is no need to identify heterozygotes. Identification of heterozygotes is seldom possible with dominant markers and this is a disadvantage when analysing populations of diploid organisms.

4.6.1 Factors affecting results obtained with AFLP

4.6.1.1 Quality of DNA

The DNA extraction procedure is critical to AFLP reproducibility. The presence of inhibitors can result in incomplete digestion of the template DNA and cause amplification of DNA fragments that represent non-genetic polymorphisms (Bensch and Åkesson 2005). The distribution of the range of amplified fragments (normally 40–400 bp) may shift towards shorter fragments if the DNA template is partially digested and artefacts will be amplified instead of true fragments (Goulao et al. 2001). In this study most of the informative fragments were 90-300 bp. There were a certain number of fixed AFLP markers within populations and within forms of *P. teres* (I, III). Those fixed AFLP markers were used to check the quality of each isolate in this study.

In this study, a commercial DNeasy plant mini kit (Qiagen, Hilden) DNA was used to extract DNA from the *P. teres* isolates in the MTT collection. From the Rozhdestveno isolates DNA (IV) was extracted according to Bulat et al. (1998) and from the SARDI isolates (III) according to Raeder and Broda (1985). However, the SARDI isolates were not compared directly with any other isolates (III) hence the DNA extraction method is of minor consequence. The Rozhdestveno isolates were compared with Finnish isolates (IV) however. The highest level of diversity was observed among Krasnodar isolates (III, Figure 2), for which DNA was extracted using DNeasy plant mini kit (III). Therefore, the results from

both Russian populations are considered valid. Before analysing the population samples, the purity and quality of DNA of each isolate was first measured with GeneQuant II RNA/DNA Calculator (Amersham Pharmacia Biotech), and secondly the samples were separated by gel electrophoresis after pre-amplification to gauge the amplified fragment sizes.

According to Bensch and Åkesson (2005), variation in DNA concentration is less critical than variation in DNA quality. Nonetheless, DNA was diluted to equal concentrations, 25 ng μl^{-1}, before analyses in this study. Some isolates with an excessive number of bands were identified from one particular sampling location (Serenius, unpublished data). Those isolates were further grown *in vitro* and were found to be contaminated with *Cochliobolus sativus* (Ito & Kuribayashi) Drechs. and possibly with other *Pyrenophora* species. All isolates from that location were excluded. In this study, a few AFLP templates gave blank results, and those samples were reanalysed. In some cases, the fluorescent label was old (it ceased to fluoresce) and therefore no amplified fragments were observed in capillary electrophoresis.

4.6.1.2 Nuclear DNA content and ploidy level

The total genome size of *P. teres* is small, about 35 Mb, consisting of nine chromosomes (Aragona et al. 2000), and thus the 1C-value is 0.036 pg (Kullman et al. 2005). Fay et al. (2005) demonstrated that acceptable AFLP traces were obtained with the standard protocol when DNA content was low (1C-values 0.30–8.43 pg) and below this range the quality was improved by using 2-3 selective bases. Before analysing the populations, several primer combinations with 2-3 selective bases were tested with control isolates of both forms of *P. teres*. Two selective bases in both of the primers and in several combinations were selected for use because they gave the most reproducible and clear fingerprint. The same primer combinations were used throughout the analyses conducted at MTT (I, III, IV). A different set of selective bases were used in SARDI, but the AFLP fingerprints of Australian isolates were not compared directly with AFLP fingerprints analysed at MTT (III).

4.6.1.3 Detection of the amplified fragments

Fragments amplified with AFLPs can be detected either with gel electrophoresis on polyacrylamide gel or by automated capillary electrophoresis. In this study, AFLPs from the SARDI isolates were analysed on an automated gel sequencer (Williams et al. 2001) and others on capillary electrophoresis at MTT (I, III, IV). The SARDI isolates were not compared directly with any other isolates, but only with each other. A fluorescent label was used in both systems. The use of a fluorescent label instead of a radioactive label or silver staining makes the protocol much safer. Capillary electrophoresis can be scaled to high throughput

on automated analysis instruments (Lindstedt et al. 2000). Gel electrophoresis allows the recovery of fragments of interest (Dresler-Nurmi et al. 2000), which can be further used in sequencing or development of sequence characterized amplified region markers (SCAR) (e.g. Claverie et al. 2004, Williams et al. 2001). In this work no recovery of fragments was needed. All efforts were made to decrease the random variation and errors due to experimental conditions. Different AFLP protocols were tried before analysing the population samples. During the population analyses all suspicious results were re-analysed either by repeating the AFLP procedure or by first re-extracting the DNA and then repeating the AFLP analyses. The error rate, based on repeated tests on new DNA extracts from the same individuals, was estimated to be between 1.9% and 2.5% (reviewed in Bensch and Åkesson 2005). In this study the error rate was not estimated. Some isolates were excluded from the final analyses due to possible contamination as explained earlier. The standard AFLP procedure was used at MTT for analysing the *P. teres* isolates and the only exception was the different DNA extraction method used for Rozhdestveno isolates (IV). Possible misinterpretations of data are typical to AFLP, as described above, and must be kept in mind. However, AFLP was the most suitable molecular technique for the purposes of this study since RFLP was considered too laborious and SSRs were not available for *P. teres*.

5 Summary and conclusions

1. The *P. teres* population in Finland is less variable than the other *P. teres* populations included in this study. Based on AFLP markers, the proportion of clonal isolates is small, whereas the genotypic variation is high in all populations.

2. Sexual reproduction occurs in Finland and in Australia, which increases the pathogen's potential to evolve under selection pressure. Asexual reproduction is still important for *P. teres*.

3. The occurrence of *P. teres* f. *maculata* in Finland remains uncertain: all collected isolates were *P. teres* f. *teres*.

4. Both mating types were equally common within all populations except in Krasnodar, Russia.

5. The two forms of *P. teres* differ significantly from each other and hybrids are unlikely to occur in nature.

6. Probably both host genotype and fungicides exert selective pressure on and shape the *P. teres* populations.

5.1 Recommendations to plant protection

As revealed in this study, *P. teres* has all the required characteristics to pose a serious risk to barley cultivation if it is neglected. Based on current results, the dispersal of *P. teres* among fields in Finland and Russia is low. The evolutionary potential could increase if agricultural practices change to favour dispersal and spread of this pathogen. These factors include: increase of infected plant debris on the soil surface, barley in monoculture or use and spread of infected seed. All these issues should be addressed. The old recommendations for barley cultivation still hold: crop rotation, use of healthy seed and burying the stubble are still recommended.

The occurrence of *P. teres* f. *maculata* should be surveyed, especially if major gene resistance to *P. teres* f. *teres* is deployed in Finnish barleys in the near future. The *P. teres* population has changed from the 1970s and may change again since the introduction of net form resistance into barley cultivation may favour the spot form over the net form. To fully understand the population biology of *P. teres* more research should be concentrated on studying the different forms of this pathogen. To improve barley breeding for net blotch resistance, more international collaboration is needed among researchers to i) improve and standardise the virulence testing methods, and ii) name, characterize and map the resistance genes in barley accessions used in the differential set.

Seed hygiene is very important in order to prevent spread and introduction of highly virulent isolates into new areas. This is especially important if resistant varieties become very popular over large areas and if the resistance breaks in some areas. Maintaining the diverse genetic background in barley and providing resistance based on several different genes is important. For durable resistance to be developed it will be important to survey the virulence of *P. teres* populations in the future. This is also the case regarding fungicide use. If problems occur and the pathogen is able to evolve and overcome the deployed resistance, other plant protection methods should be implemented: crop rotations and tillage of plant debris. Understanding the local life cycle of plant pathogens assists greatly in crop management in general and plant protection in particular.

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