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HISTORICAL AND CONTEMPORARY GENOTYPIC POPULATION STRUCTURE OF PEA ASCOCHYTA BLIGHT PATHOGENS *ASCOCHYTA PINODES* AND *PHOMA PINODELLA*

ABSTRACT

The historical and contemporary population genotypic structure of pea blight pathogens, *A.pinodes* and *P.pinodella* was determined on temporal and spatial scale by using vegetative incompatibility system as phenotypic genetic marker. It has been indicated that since new 1976 VCG genotypes had been introduced into contemporary population in Poland. Significantly genotypic difference was found among old historical subpopulations sampled from different pea cultivars that were grown in three regions of Poland and historical subpopulations sampled from resistance nurseries artificially infested and long – term monocultures of pea. The difference between historical and contemporary populations also was significant indicating the Occurrence of two distinct populations of *A.pinodes* and *P.pinodella* during 1975 – 2004 period. It has been shown that VCG genotype diversity was significantly different between the historical and contemporary populations. This genotypic difference was particularly significant between subpopulation sampled from pea cultivars and subpopulation sampled from breeding resistant lines. Relatively high of VCGs diversity within contemporary population indicated importance of the sexual stage of *A.pinodes* and *P.pinodella* as the source of inoculum for pea *Ascochyta* blight epidemic and potentially adaptative of genotypic diversity.

Key words: *Ascochyta pinodes, Phoma pinodella,* genotypic population structure, VCGs genotype frequencies, spatio-temporal scale, distribution of genotypes

INTRODUCTION

Ascochyta blight of pea is caused by three related fungal species: *Ascochyta pisi* (Lib), *Mycosphaerella pinodes* (Berk et Blox) Vestergr = *Didymella pinodes* (Berk et Blox) Pertak; stade asexue: *Ascochyta pinodes* (Berk et Blox) Jones and *Phoma medicaginis* var *pinodella* (Jones) Boerema = *A.pinodella* Jones. It is already well known that *Phoma pinodella* (teleomorph *Didymella*) and *Ascochyta pisi* (teleomorph *Didymella*) belong to heterothallic ascomycete fungal species. In contrast *Ascochyta pinodes* belongs to homothallic species. It has been suggested that the life cycle of these fungi consists of a single sexual generation during vegetation, which develop on senescence bottom leaves and on ripening pea plants or on infected plant de-

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bris left on the soil. Occurrence of several asexual generations during the growing season of the pea depended on weather especially on weather moisture. Three species cause disease on all above-ground parts of pea, including pods which leads to seeds infection. Infected seeds are the primary mode of transmission of the pathogens into regions previously free of the disease and the Ascochyta blight is now present in most areas of the world where peas are grown (Zhang *et al.* 2003, Shoeny *et al.* 2006, Bouznad *et al.* 1995, 1996, McPhee *et al.* 2006, Bretag *et al.* 1995, Furgał-Węgrzycka 1984, 1990, 1991, 1992 Marcinkowska 1996, Onfroy *et al.* 1999, Kraft *et al.* 1996, Wroth and Khan 1999, Xue *et al.* 1996).

Based on heterothallic mating system of *P.pinodella* or *A.pisi*, it is assumed that sexual recombination could occurs infrequently or regularly in the life cycle of these two fungal species primarily in regions where both mating types occurs (Peever *et al.* 2002). It is worth noticed that the mating type system of *P.pinodella* and *A.pisi* has not been determined in detail worldwide and in Poland. Nevertheless it is proposed that similar to the heterothallic species *A.rabiei* teleomorph *Didymella rabiei* (Kovacheski) v. Arx that causes Ascochyta blight of chickpea ascospores from the sexual stage can represent recombinant progeny that could contribute to increase genotypic diversity within regional populations. This variation is potentially adaptative, allowing the pathogens to evolve by increasing virulence on resistant cultivars and/or to develop resistance to fungicides (Peever *et al.* 2002, 2004, Kaiser 1997, Milgroom and Peever 2003). It is worth noticed that up to date only the *Mat* locus controlling mating system in *A.rabiei* was cloned and was characterized in detail, what allowed the development and using of *Mat-1-2* as genetic marker in genetic studies regarding the genetic population structure and phylogenetic relationship between *Ascochyta / Phoma* complex species associated with leguminouse (Peever *et al.* 2002, 2004, Peever 2004, Barve *et al.* 2003).

The genetic population structure of fungal plant pathogens is influenced by several evolutionary forces including mutation, selection, gene flow and genotype migration, genetic drift and recombination (McDonald 1997; Milgroom 1996), Molecular markers have been revolutionized studies regarding the population genetics of many fungal species including *Ascochyta* and *Phoma* species complex sampled from leguminose hosts. Using the sensitive molecular markers such as AFLP or microsatellite it is now a relatively straightforward task to collect allele frequencies data from multiple, putatively neutral genetic loci and use this variation to test hypothesis about the biology and evolution of plant pathogenic fungi (Peever *et al.* 2004; Douhan *et al.* 2002, 2003, Dyer *et al.* 2001, Milgroom 2001, McDonald 1997, McDonald and Linde 2002, Fatehi and Bridge 1998, Fatechi *et al.* 2003). The understanding of the genetic structure of *P.pinodella* and *A.pinodes* populations and their variation on a spatial and temporal scale can provide insight into their biology and evolution and may also have important implications for plant disease management and breeding programmes (Milgroom 1996, 2001; Milgroom and Fry 1997; Milgroom and Peever 2003, Peever 2003, 2004, Peever *et al.* 2002, 2004). In addition to studies regarding the local population

structure also research on mating type system should be estimated because the knowledge about the strategies of reproduction have an important implications for understanding the biology and epidemiology of Ascochyta blight pathogens (Milgroom and Peever 2003).

Little is known regarding the worldwide population genetic structure of *A.pinodes, A.pisi* and *P.pinodella* even though several different molecular markers have been recently used to study genetic variation within and among populations of related pathogens such as *A.rabiei* or *A.lentis* (Banniza and Vandenberg 2006, Fatehi 2000, Ford *et al.* 1996, Peever *et al.* 2002, Tivoli *et al.* 2000, Zhang *et al.* 2006). Peever *et al.* (2004) have been used AFLP and STMS markers and found the significant genetic variation in the populations of *A.rabiei* sampled from susceptible cultivars of chickpea and a low gene and genotypic diversity within populations sampled from resistant breeding lines and resistant cultivars. It is worth noticed that Peever *et al.* (2004) for the first time have compared historical and contemporary genetic population structure of *A.rabiei*. In this case the local populations of *A.rabiei* that consisted of two subpopulations historical and contemporary were sampled from resistant and susceptible cultivars or breeding resistant lines of chickpea.

It should be noticed that almost identical breeding resistance programme of pea lines regarding *Ascochyta* blight disease as have been conducted by Kaiser, Muehlbauer, Kraft and their colleagues I have begun since 1975. The breeding programme was based primarily on random bulk population selection and next on single descent breeding selection methods and were conducted at first in resistance screening nursery artificially infested every year of mixture of inoculum of *A.pisi*, *A.pinodes* and *P.pinodella*. After selection from commercial cultivars Flavanda, Allround, Porta and Auralia resistant breeding lines, the further resistance breeding works were conducted in long term monoculture of pea naturally infested since 1975 due to infected debris of pea left on soil surface.

In addition to resistance breeding programme I have conducted typical studies on genotypic population structure regarding historical and contemporary populations of *A.pinodes* and *P.pinodella* sampled from cultivars of pea that were grown at this time in three regions of Poland. Historical population included also the subpopulations of these pathogens sampled from resistance nursery and from natural long-term monoculture where were grown worldwide resistant lines or resistant cultivars.

It should be noticed those thirty years ago for the first time I have used the vegetative incompatibility system as phenotypic genetic marker in studies regarding historical and contemporary genotypic population structure of *A.pinodes* and *P.pinodella*. Studies regarding to VCGs genotype diversity within historical and contemporary populations of *A.pinodes* and *P.pinodella I* have conducted during 1975-2004. Research regarding the genotypic diversity within historical and contemporary populations I have begun with three elementary questions from the population genetics: How much genetic diversity was present within populations? How was genetic diversity distributed within and among populations? How were stable populations of *A.pinodes* and *P.pinodella* over time?

The two specific hypotheses were tested. The first and most general hypothesis was that historical Polish populations of *A.pinodes* and *P.pinodella* have changed substantially and had acquired the feature of new contemporary populations. I have expected that these new contemporary populations should be characterized by broad spectrum of virulence and a higher genotypic diversity as a genetic consequence of recombination, presence or absence of genotype migration and introduction of new genotypes with imported seeds. The second hypothesis was that genotypic diversity within population of *P.pinodella* would be relatively high within contemporary populations as a consequence of the occurrence of *Mat-1-1* and *Mat-1-2* sexual genotypes and equal distribution of mating type genotypes. In addition the genotypes composition within contemporary population should be different comparison to historical old population due to the host selection on base of selection of group of genotypes with higher level of virulence, higher fitness and better adapted to changed environment. Based on population genetic theory that temporal and spatial genotypic population structure of plant pathogenic fungal species result primarily from interactions among natural selection, genetic drift, and gene or genotype flow, I have expected to find significant genotype difference between old historical population and contemporary population of *A.pinodes* and *P.pinodella*.

Genetic drift can cause random fixation of different alleles in regional Polish or European populations, leading to non-adaptative genetic differentiation. In the absence of gene flow or genotype migration among regional populations, the accumulation of both adaptative and non-adaptative genetic differences will lead to spatial genetic structure. In agricultural ecosystems, natural selection and genetic drift are expected to play more important role in the spatial genetic structure of *Ascochyta / Phoma* complex populations due to selection for corresponding virulence alleles and repeating cycles of extinction and re-colonization of local populations resulting from pea as host dynamics. On the other hand, under natural conditions mutation and genetic drift rather than selection play the dominant role.

The genetic structures of *A.pinodes* and *P.pinodella* populations have not been studied extensively. This is a contrast to *A.rabiei* which genetic population structure has been studied for over a decade. The field experiments conducted in the past by Kaiser and Muehlbauer research group (Muehlbauer 1996, Muehlbauer *et al.* 1997, Kaiser and Muehlbauer 1984, 1988) and recently by Peever and colleagues (Peever *et al.* 2004) have indicated that host selection plays an important role in the population genetic of this pathogen. Though the *A.rabiei* is a haploid pathogen and the local or worldwide populations are strongly affected by natural selection, it has been revealed recently that genetic variations of *A.rabiei* was low in historical population and was high in contemporary population and the majority of genetic variation was distributed over an area of single field (Peever *et al.* 2004, Milgroom and Peever 2003).

Many evolutionary and demographic factors can affect temporal variation regarding genetic structure of a population. The major forces governing this change may vary among pathogenic fungal species causes the disease of

leguminose crops. This difference is related to ecology, biology and life history of particularly fungal species. In agricultural ecosystems, the selection due to widespread growing of resistant cultivars plays a significant role in temporal changes in population of leguminose pathogenic fungal species. The selection of particular pathogen genotypes also could lead to the loss of female fertility within heterothallic populations of *A.pisi,* and *P.pinodella*. Such selection or genetic drift has led to arising of clonal lineages that could evolve into asexual population of *A.pisi* and *P.pinodella*. On the other hand, to my knowledge the roles of genetic drift and migration have not been extensively studied regarding the Polish and worldwide populations of *A.pinodes* and *P.pinodella*. It should be noticed that genetic drift and migration probably play major roles regarding the temporal genetic variation primarily on local populations. During annual epidemic cycles of Ascochyta blight of pea the local *A.pinodes* and *P.pinodella* populations usually undergo annual cycles of expansion and contraction. In this case during the end of each epidemic cycle only a small fractions of the population of these pathogens successfully survive over seasons and serve as the founding populations for the next epidemic cycle. If this numerical bottleneck is significant, the resulting genetic drift usually leads to significant changes in the genetic structure of local population from year to year. It should be noticed that the knowledge of the possible role of temporal variation in population genetic structure is still unknown. It has been proposed that studies on the population genetic structures of *A.pinodes* and *P.pinodella* should be conducted in order to determine the spatial distribution of genetic variations, the importance of gene and genotype flow, genetic drifts, mating systems and selections in the evolutionary biology of these species.

It is important to notice that the ability to compare the historical population with contemporary population of *A.pinodes* and *P.pinodella* has led to better understanding the changes in the population structure not only on a spatial scale, but also on temporal scale.

The objectives of this research were (1) to determine the historical and contemporary populations structure of *A.pinodes* and *P.pinodella* in three regions of Poland and to follow the changes in population structure through the time (2) to estimate the difference in genotypic diversity among populations of *A.pinodes* and *P.pinodella* sampled from pea cultivars actually grown across three regions of Poland and among populations sampled from resistant breeding lines obtained as results of long-term of resistance breeding programme.

MATERIALS AND METHODS

Sampling

Three different historical subpopulations of *A.pinodes* and *P.pinodella* were obtained from genetically diverse sources during 1976-1990. Two historical subpopulations were obtained form resistant breeding lines that were grown in two resistance screening nurseries. First was grown during the 1975 - 1986 in the

experimental plot. This resistance nursery was every year artificially infested with mixture inoculum of equal proportion of highly virulent isolates of *A.pisi, A.pinodes* or *P.pinodella.* The second was a long-term monoculture located since 1975 in the Breeding Station Zielonki near Warsaw. It is worth noticed that in long-term monoculture there were tested the level of resistance of worldwide pea breeding lines, new bred cultivars and my resistant breeding lines obtained from cultivars Allround, Flavanda, Porta and Auralia (Furgał-Węgrzycka 1990). The third historical subpopulation of *A.pinodes* and *P.pinodella* was obtained from several pea cultivars that were grown across three regions of Poland: Northern, Southern and Central. These historical subpopulations were obtained from the cultivars of pea grown in Poland during 1975-1990 and had no known resistance to *Ascochyta* blight of pea. The artificially infested nursery had approximately 0.5 ha and consisted of 3 m rows of replicated resistant pea lines arranged in 3 x 3-m plots. A highly susceptible breeding lines or susceptible pea cultivars were planted between plots as a control and the nursery was irrigated during the growing seasons to promote disease development. The resistance nursery was located approximately 3 km from Olsztyn and approximately 200 km from Warsaw. The long - term monoculture had about 1 ha where international breeding lines and worldwide collection of pea cultivars were grown in order to test their resistance to complex pathogens of pea. Number of isolates of *A.pinodes* and *P.pinodella* sampled from resistance nurseries and from pea fields according to historical populations is given in Tables 1 and 6.

The contemporary populations of *A.pinodes* and *P.pinodella* were sampled during 1991 – 2004. Sampling of contemporary populations has focused primarily on years when epidemic of Ascochyta blight has occurred due to optimal environmental conditions promoting disease development. The contemporary population consisted with 500 isolates of *A.pinodes* or *P.pinodella*.

Four populations of *A.pinodes* and *P.pinodella* were collected primarily from infection foci located in each pea fields or resistance nursery. Within each pea fields or nurseries, if possible five to ten infection foci were randomly chosen depended on the area of pea fields or plots. Ten to fifteen leaves from each focus were sampled based on the occurrence on leaves large lesions with pycnidia. It should be noticed that in two resistance nurseries. It was no problem to find the many of infection foci and sampled leaves covered with large lesions with pycnidia. In addition it was also no problem sampling pods with lesions and pycnidia.

Usually a single infected leaf was obtained from a single pea plant at each sampling foci. Number of chosen diseased plants from foci was different during the years and was correlated with kind of weather. Isolation of pathogens from infected leaves, species designation and preparation of monoconidial populations were identical as described previously by Furgał-Węgrzycka (1990).

Regarding the sampling contemporary populations from three regions of Poland the quadrate based methods were used to quantified VCG genotype frequencies and their distribution within and between regional Polish populations

of *A.pinodes* and *P.pinodella* on a spatio-temporal scale. Diseased plants were hierarchically sampled from the fields pea that had minimum about 0,5 to one hectare in size. The three regions were separated by at least 600 km apart. Within each region two locations (two pea fields) were separated about 1 km or less and usually belonged to neighbour farmers who has used the same seed materials and machine equipment. The third location (farmer field) within single region was separated from these two locations by approximately 200 km and was planted with different cultivars and usually was not closely related by environmental habitat. In Northern region two closely related fields were located near Olsztyn and these fields were about 1-6 km apart. Third field was located near Ełk in close aproximity to Russia border. In Southern region two fields were located near Wrocław and these fields were located about 2-3 km apart. Third field in Southern region was located about 300 km from Wrocław and usually was located near Czech and Slovakia border. In Central region fields were located near Warsaw or Poznań.

Three regional populations were also sampled according to temporal scale. First temporal population was sampled in 1991-1992, second in 1995-1997 and third during 2002-2004. In this case Polish populations of *A.pinodes* and *P.pinodella* covered three geographically distantly regions and a broad temporal scale. In this study a quadrate base method was defined as the 0,5-1 m^2 areas called also as infection foci within which the infected pea plants and sampled isolates were evaluated for quantification of genotypic variation and distribution of this variation within and between infection foci as on a smallest spatial scale. Because within single pea field the number of infection foci and their distribution were random and were different during particular years, minimum five or when it was possible even more infection foci were chosen. Samples of leaves from single infection focus were placed individually in brown paper envelope to prevent cross contamination and infected leaves were dried at room temperature in laboratory. Dried samples were stored at 5 ^{o}C until they could be analyzed further.

It should be noticed that in the situation when the isolation of *A.pinodes* and *P.pinodella* from infected leaves was difficult due to the occurrence of low level of symptoms, preparation the collection of isolates was made from infected seeds after harvest.

Studies on VCGs frequencies within populations of *A.pinodes* and *P.pinodella*

It should be noticed that unlike other plant pathogenic fungi *A.pinodes* and *P.pinodella* have few phenotypic genetic markers that can be used to study the population structure. In *A.pinodes* and *P.pinodella* nitrogen non-utilizing mutants (*nit* mutant) and vegetative compatibility groupings via *nit* mutant complementation test is not available as for *Fusarium* species. Test for vegetative compatibility was performed as described by Milgroom and Lipari (1955) and Milgroom and Cortesi (1999). Strains were paired about 1-3 cm apart on PDA medium amended with active charcoal and on freshly prepared oat me-

dium (OA) amended with bromocresol green pH indicator dye. It has been shown that freshly prepared oat meal agar was the best medium for detecting the black barrage reaction lines or discoloration along the zone of mycelial contact between expanding colonies (vegetative incompatibility reaction). Vegetative compatibility between paired isolates was assessed according to the merging/barrage reaction response or discoloration in the medium between colonies. Merging of the two colonies of *A.pinodes* or *P.pinodella* indicates the vegetative compatibility, whereas the formation of the barrage zone of dead cells (barrage) with or without conidia between two colonies of *A.pinodes* or *P.pinodella* indicated the vegetative incompatibility. Petri dishes with paired strains were incubated in the dark at 22^{o}C for 30 days. All strains that were vegetative compatible belonged to single VCG genotype.

Population genotypic diversity analysis

Sample set collected from single field during the single year was considered as subpopulation. The first step was the testing the null hypothesis that genotypes diversities and their distributions across spatio-temporal scale were equal. Statistic analysis according to genotypic population structure of *A.pinodes* and *P.pinodella* was based on Grunwald *et al.* (2003). Components of genotypic variation were included: Shannon's diversity index (H'), Stoddart and Taylor index (G), evenness (E), richness (g), genotypic diversity within each subpopulation (H_s), average diversity (H_T) and genotypic population subdivision G_{ST} values.

Richness values g or E(g) was calculated also according to Grunwald *et al.* (2003). Evenness values E_1, E_2 and E_5 were calculated as described Grunwald *et al.* (2003). Genotypic variation in each spatio-temporal population was quantified by genotype diversity. Total genotype diversity H_T and H' was partitioned into components among regions, years, among fields (locations) and among infection foci within single field or resistant nursery by hierarchical genotype diversity analysis. Hierarchical analysis was also used to partition genotypic diversity into two levels according to years: variation within single temporal population and among three temporal populations. To test the null hypothesis of equal frequencies of genotypes and their distribution within and between spatio-temporal subpopulations significance were tested by using chi-square test and *t* test values. These statistical tests can results in significance values when one or few of rare genotypes were present at all spatio-temporal subpopulations. In case in which significant departures from null hypothesis that genotypes were equally distributed across all spatio-temporal subpopulations have been obtained, pairwise comparisons subpopulation by subpopulation were used to determine the subpopulation pairs responsible for significant departures for null hypothesis and for overall evenness. Pairwise measures and genetic population differentiation G_{ST} was calculated as:

$$G_{ST} = \frac{H_T - H_S}{H_T}$$

H_T was the average of genotypic diversity within populations and was calculated by treating all genotypes as if they belong to a single metapopulation, H_S was the average of genotypic diversity for each spatio-temporal subpopulations and was calculated separately.

Genotypic population structure was also examined by analysis of variance. The analysis of variance was used to partition the variance of genotype frequencies within and among subpopulations and on a spatio-temporal scale (e.g. regions, locations and consecutive years). All statistical measures regarding obtained results and genotypic diversity within Polish population of *A.pinodes* and *P.pinodella* were calculated by computer program described by Węgrzycki.

It should be noticed that in theory estimation of genotypic diversity is an important component of the analysis of the local population structure of pathogen. Estimates of genotypic diversity are the function of both the number of VCG genotypes observed in the local population (genotypic richness) and the evenness of distribution of genotypes within and between populations. The number of genotypes finds in local population of *A.pinodes* or *P.pinodella* depends on the genetic marker used in the study on population structure and on sample size of compared subpopulations. If populations size were very similar, in this case the number of genotypes identified within and between local population consisted the values of richness. When sample size was different in each local subpopulation the rarefaction method to calculate richness should be used (Grunwald *et al.* 2003).

Rarefaction curves yield the number of genotypes expected in the smallest sample size and was as following:

$$E_{(g_n)} = \sum_{i=1}^{g} \left[1 - \left(\frac{\frac{N - n_i}{N}}{\frac{N}{n}} \right) \right]$$

The method to assess genotypic richness by using the rarefaction curve was implement in C using the function bico. This function returns a binomal coefficient using logarithms of factorials. The algorithm implemented in C computer language that calculate $E_{(g_n)}$ for each sample size from n=1 to N is available from Grunwald or Milgroom (Grunwald *et al.* 2003). $E_{(g_n)}$ computes the expected number of genotypes in a random sample size n as the sum of the probabilities that each genotype will be included in the sample. In this study a new bootstrap method for constructing confidence intervals on expected in local

population of the number of genotypes (richness) were used that offers some improvements (Grunwald *et al.* 2003). Confidence intervals contain the population mean with fixed probability determined by the confidence coefficient chosen to be 95%. Bootstrapping could conducted using SAS macro <jack-boot sas> available online at no cost from the website of SAS of the Institute Carry NC modified to calculate indices of diversity and evenness. Bootstrapping was calculated using 2000 resample at confidence intervals of 95% using the accelerated bootstrap procedure.

Estimates of diversity are often presented without any measure of confidence in the estimate. Also comparisons of genotype diversity between populations that depend on the rarefaction method a new bootstrap method for constructing confidence intervals on expected genotypes within and between local subpopulation (richness) should be used. Two indices of genotypic diversity have been used most frequently in the estimation of genotypic diversity within and between the local populations of a given pathogen: first indices is Stoddart and Taylor's G and second is Shannon-Weiner's indices H'.

Stoddart and Taylor's G has a minimum value of 1 and a maximum value, which is equal to the sample size. The normalized $\hat{G}$ value, which is obtained by dividing G by sample size, has a minimum 1% and maximum of 100%.

Evenness values measure how genotypes were distributed within and between local populations and how genotypes were distributed on a temporal scale. If within a population one or few VCG genotypes dominated, in this case the evenness and diversity values also were low. If local population was considered with great number of genotypes and their distribution within and between populations were equal then evenness and diversity values were very high.

Stoddart and Taylor index was calculated as follows:

$$G = \frac{1}{\sum p_i^2}$$

or

$$G = \frac{1}{\sum (fx) \times \left(\frac{x_i}{n} \right)^2}$$

where n was the sample size, fx was the number of genotypes observed x times;

Shannon-Weiner's H' was calculated as follows:

$$H' = -\sum_i p_i \times \ln(p_i)$$

Where pi was the frequency of ith genotype in the population. The maximal value of the Shannon-Weiner index H' occurs when each p=1/g or when every isolate has unique genotype. Because indices of diversity combine both richness and evenness thus Shannon-Weiner's H' increase as richness or evenness increase.

The maximal value for Shannon-Wiener index H'_{max} occurs when each $p_i=1/g$ (e.g. when every isolate belong to an unique genotype). Thus H'_{max} was calculated as follows:

$$H'_{\max} = -g\left(\frac{1}{g}\ln\left(\frac{1}{g}\right)\right) = -\ln\left(\frac{1}{g}\right)$$

It is observed that, in the case where $p_i=1/g$ it also gets get $p_i=1/n$, because n=g. However, when diversity is low, then n may be much greater than g and ln(n) > ln(g).

According to Grunwald *et al.* (2003) H' should be scaled as $\ln(g_{max})$ where g_{max} is the maximum number of genotypes expected in population and strongly depend on the genetic marker used in the study. In this case

$$H' = E_1 = H' \Big/ \ln(g)$$

and the index reflects how uniformly genotypes are distributed within population.

Grunwald *et al.* (2003) developed a conceptual framework for analysis of diversity and have given the statistic Na, N_1 and N_2 which were calculated as follows:

$$N_a = \left(\sum_{i=1}^{g} p_1^a\right)^{1/(1-a)}$$

In which pi was the frequency of ith genotype

$N_i = e \times H'$

Where *H'* was the Shannon-Weiner H' indices.

N_i represents the number of equally common genotypes which would produce identical diversity value as *H'*.

$N_2 = 1/\lambda$

Where λ was Simpson index:

$$\lambda = \sum_{i=1}^{g} p_i^2$$

N_2 diversity values correlate with Stoddart-Taylor's genotypic diversity G and usually they are similar and weight the number of all genotypes in population including unique, rare and dominant genotypes in population. In contrast N_1 weights the occurrence of rare genotype and N_1 generally falls between N_0 and G values.

N_1 and G values increase linearly with increasing richness and have a maximum value equal to richness (g) when evenness is maximal. N_1 and G increase linearly as the number of genotypes (g) increase. In contrast H' increases nonlinearly as the number of genotypes increase because H' and N_1 is most sensitive to changes of number of rare genotypes within population.

Grunwald *et al.* (2003) have proposed three values of evenness: E_1, E_2, and E_5.

The most common index of evenness E_1 scales the Shannon-Weiner index by the maximally expected number of genotypes in population (g_{max}).

$$E_1 = \frac{H'}{\ln(g_{max})} = \frac{\ln(N_1)}{\ln(N_0)}$$

The statistic of E_2 was as follows:

$$E_2 = \frac{e^{H'}}{g} = \frac{N_1}{N_0}$$

E_5 was the ratio of the number of all genotypes that has been found in population to the number of rare genotypes. Value of E_5 ranges from 0 when one or a few VCG genotype dominated in population and was not affected by sample size and richness values. The statistic of E_5 was following:

$$E_5 = \frac{\left(1/\lambda\right) - 1}{e^{H'} - 1} = \frac{G - 1}{N_1 - 1}$$

RESULTS

The genotypic structure of field population of *A.pinodes* and *P.pinodella* was determined across the hierarchy of spatial and temporal scales by using vegetative compatibility test as phenotypic genetic markers. Spatial scale includes three different regions of Poland. In addition, historical population was composed with subpopulations sampled from resistant breeding lines that grown on long-term monoculture and on artificially infested of experimental plot.

Components of genotypic variation were included: indices of diversity H', N_1 and N_2, richness (g) and indices of evenness E_1, E_2 and E_5. They included also: spatio-temporal analysis of genotypic variation (H_T and H_S values), pairwise

comparisons of genotype diversity and their distribution across spatio-temporal scale (G_{ST} values) and analysis of variance based on VCG genotype frequencies (Tables from 1 to 5 for *A.pinodes* and from 6 to 10 for *P.pinodella)*.

Table 1

Historical population structure of *Ascochyta pinodes*

Statistic	Northern region	Central - Southern regions
Sample size n	120	80
Indices of richness gobs	15	26
$E_{(gn)}$ - number of expected genotypes	14.96	25.1
$E_{(gn)}$ - number of expected genotypes to the smallest sample size	14.90	24.4
g_{max} = 26 based on number of vic loci	64	64
Indices of diversity: H'	0.962 (0.56-1.02)	1.21 (1.16-1.58)
N_1	2.11 (1.45-2.25)	3.19 (2.94-3.24)
N_2	12.54 (9.21-16.2)	16.11 (9.68-16.6)
Indices of evenness E_1	0. 690	0. 732
E_2	0.552	0.568
E_5	0.452 (0.38-0.54)	0.673 (0.61-0.75)

Description of population structure are based on Grunwald *et al.* (2003) data.
Numbers in parentheses indicate confidence intervals calculated by the bootstrapping approach for the common sample size of the smallest population

Table 2

Hierarchical distribution of genetic variance within historical population of *A.pinodes*

Source of variation	Variance component	Percent of total variance	P
Genotypic diversity distribution			
Temporal scale 1975-1980			
Within and among regions	2.658	39.17	< 0.01
Within single field	6.322	60.83	
Temporal scale 1981-1985			
Within and among regions	1.762	34.11	< 0.01
within single field	6.340	65.89	
Temporal scale 1986-1990			
Within and among regions	0.720	29.42	< 0.01
Within single field	5.453	70.58	
Spatial scale - Northern population vs Central-Southern populations			
Within and among regions	2.112	36.34	< 0.01
Within single field	6.311	63.66	
All locations (all fields)			
Among subpopulations	4.629	38.67	< 0.01
Within subpopulations	7.299	61.33	

Table 3

Pairwise comparisons of historical population (G_{ST}) of *Ascochyta pinodes*

Fields	Northern region			Central-Southern regions		
	A	B	C	D	E	F
A	-	0.97*	1.95*	2.01*	3.63**	6.20**
B	0.074a	-	2.15*	5.27*	4.91**	5.56**
C	0.096	0.093	-	5.49**	3.64**	10.72**
D	0.132	0.141	0.168	-	0.54*	1.79**
E	0.156	0.138	0.177	0.096	-	3.51**
F	0.190	0.169	0.115	0.089	0.913	-
Indices of diversity						
Stoddart-Taylor	39.6%	37.8	21.2	34.2	36.6	30.8
H'	1.172	1.24	0.983	0.974	1.873	1.731
HT	0.327	0.294	0.274	0.210	0.345	0.293
Hs	0.210	0.183	0.174	0.111	0.234	0.193

[a] = G_{ST} values for genetic population differentiation, above diagonal – t values, below diagonal – G_{ST} values
*, ** = indicate significance at P=0.05 and P=0.01 respectively,
H' = genotype diversity expressed as Shannon index, H_T = total genotypic diversity

The number of VCG genotypes of *A.pinodes* and *P.pinodella* found in all historical subpopulations and in three regions of Poland ranged from fifteen to 27 (Tables 1 and 6). Average genotype diversities within Polish historical populations of *A.pinodes* and *P.pinodella* ranged from H_T=0.227 for *A.pinodes* to H_T=0.317 for *P.pinodella* (Tables 5 and 10). Stoddart-Taylor measure of genotype diversity ranged from 22.4 to 30.2% of its theoretical maximum across all the historical populations (Tables 5 and 10). It has been shown that the three historical subpopulations differed significantly both in VCGs genotype frequencies and their distribution in following statistical values: genotype diversity, richness and evenness (Tables 1, 3, 5, 6, 8 and 10). The majority of genotype diversity was distributed on a small spatial scale within single focus ranging about 0.3 - 0.5 m^2 in size. Hierarchical genotype diversity analysis indicated that about 20% - 30% of the total genotype diversity (H_T) was distributed among the three regional historical populations. In all case the majority of genotypic diversity (60%-70%) was distributed within single field of pea (Tables 2 and 7). The same VCG genotypes could be found primarily within old historical population sampled during 1975 - 1980. The genetic population structure of old historical population of *A.pinodes* or *P.pinodella* probably belongs to clonal population and an average from 2 to 5 identical VCG genotypes could be found within foci localized in the single pea field.

In this case genotype diversity values and evenness values, particularly E_5 value, were greatly influenced by the presence of few dominant of VCG genotypes, particularly within historical subpopulation sampled from resis-

Table 4

Differences of VCG frequencies and differences in genotypic diversity among historical subpopulations of *Ascochyta pinodes* compared on spatio-temporal scale

Temporal distribution	Hierarchical distribution of number of genotypes (χ^2 values)	Hierarchical distribution of genotypic diversity (t values)
First survey (1975-1980)	5.56*	1.66*
Second survey (1981-1985)	6.37*	2.06*
Third survey (1986-1990)	9.0**	6.28**
1st versus 2nd survey	6.26*	1.79*
1st versus 3rd survey	10.80**	4.73**
2nd versus 3rd survey	5.95*	2.01*
Spatial distribution - 1st survey		
Differences between regions	12.57*	2.41*
Between fields within single region	6.26*	1.95*
Between foci within single fields	12.60	6.38
Spatial distribution - 2nd survey		
Differences between regions	7.51*	2.33*
Between fields within single region	5.95*	2.01*
Between foci within single fields	4.31	1.77
Spatial distribution - 3rd survey		
Differences between regions	4.92**	2.56**
Between fields within single region	4.20*	1.63*
Between foci within single fields	6.63*	1.79*

*, ** = indicate significance at P=0.05 and P=0.01 respectively
Total number of genotypes and total genotypic diversity was partitioned into different temporal and spatial scale components based on a hierarchical analysis

tant lines (Tables 11 and 12). In addition for the old historical population sampled from commercial cultivars of pea, evenness value of E_5 was also low and ranged from 0,388 for *A.pinodes* to 0,398 for *P.pinodella* despite the richness value was relatively moderate (Tables 1, 5, 6 and 10). The historical populations of *A.pinodes* and *P.pinodella* differed significantly in both of VCGs genotype frequencies and their distribution on a spatial scale. The degree of genetic differentiation G_{ST} ranged from 0.074 to 0.190 for historical populations of *A.pinodes* and from 0.083 to 0.199 for *P.pinodella* (Tables 3 and 8). The level of population differentiation G_{ST} values between closely related fields located few kilometers apart within historical populations was relatively low and ranged from G_{ST}=0,074 to G_{ST}=0,096. At the smallest spatial scale the genotypic population differentiation among infection foci within single field was low and genotypic identity (evenness) between infection foci was high. Relatively moderate genotypic differentiation of populations of *A.pinodes* and *P.pinodella* suggested a low degree of genotype migration among spatio-temporal populations.

Table 5
Historical genotypic population structure of *Ascochyta pinodes* determined across spatio-temporal scale

Subpopulations	H_T	H_S	G_{ST}	evenness E_5	H'	$\hat{G}$
Temporal scale						
First survey (1975-1980)	0.191	0.113	0.089*	0.388	0.839	22.4
Second survey (1981-1985)	0.194	0.170	0.212**	0.412	1.12	27.6
Third survey (1986-1990)	0.277	0.121	0.112**	0.577	1.32	32.2
Spatial scale - 1st survey						
Between regions	0.283	0.112	0.143*	0.397	0.937	38.2
Between locations	0.210	0.119	0.111*	0.444	0.788	34.7
Between foci within single field	0.212	0.132	0.094	0.394	1.22	29.3
Spatial scale - 2nd survey						
Between regions	0.220	0.130	0.174**	0.394	1.42	39.9
Between locations	0.190	0.121	0.121*	0.411	1.12	29.9
Between foci within single field	0.210	0.199	0.084	0.607	1.57	30.1
Spatial scale - 3rd survey						
Between regions	0.226	0.110	0.199**	0.402	1.69	41.7
Between locations	0.210	0.121	0.110*	0.513	1.12	31.1
Between foci within single field	0.246	0.190	0.093*	0.619	2.11	38.4

G_{ST} – values for genetic population differentiation between years and across spatial scale

Genotype diversity as: H' of Shannon index, $\hat{G}$ as percentage of possible maximum of Stoddart and Taylor values.

H_T – total genotypic diversity
E_5 – the eveness index that reflecting whether or not one or few of VCG genotypes dominate within spatio-temporal populations
*, ** - indicate significance at P = 0,05 and P = 0,01 respectively

Significant differences in genotype frequencies and their distribution were found between historical population and contemporary population sampled from cultivars of pea and between two historical subpopulations sampled from resistant breeding lines of pea. Chi-square test and *t* test statistic values showed significant differences between historical and contemporary populations. Obtained results suggested that historical population of *A.pinodes* and *P. pinodella* had different genotypic structure comparing to contemporary population of these pathogens (Tables 4, 9, 13 and 14).

It has been found that genotypic diversity was significantly lower in old historical population including also resistant lines subpopulation comparing to the last young contemporary populations collected from actually growing in Poland pea cultivars. Genotypic diversity of population sampled from resistant breeding lines ranged from H_T = 0,199 to H_T = 0,214 for *P.pinodella* and ranged from H_T=0,115 to H_T= 0,210 for *A.pinodes*. Pairwise comparisons of all historical subpopulations revealed that subpopulations of *A.pinodes* and *P.pinodella* sampled from resistant breeding

Table 6

Historical population structure of *Phoma pinodella*

Statistics	Northern region	Central - Southern regions
Sample size n	120	80
Indices of richness gobs	21	27
$E_{(gn)}$ - number of expected genotypes	19.93	26.40
$E_{(gn)}$ - number of expected genotypes to the smallest sample size	19.11	26.16
g_{max} = 26 based on number of vic loci	64	64
Indices of diversity: H'	1.79 (2.57-3.08)	2.01 (2.59-3.07)
N_1	12.8 (13.6-20.2)	20.04 (13.8-20.1)
N_2	10.46 (9.21-16.2)	15.96 (9.68-16.6)
Indices of evenness E_1	0.620	0.610
E_2	0.593	0.557
E_5	0.607 (0.62-0.85)	0.596 (0.64-0.88)

Description of population structure are based on Grunwald *et al*. (2003) data.
Numbers in parentheses indicate confidence intervals calculated by the bootstrapping approach for the common sample size of the smallest population

Table 7

Hierarchical distribution of genetic variance within historical population of *Phoma pinodella*

Source of variation	Variance component	Percent of total variance	P
Temporal scale 1975-1980			
Distribution of genotypic diversity: within and among regions	2.933	44.6	< 0.01
Within single field		55.4	
Temporal scale 1981-1985			
Within and among regions	1.463	30.2	< 0.01
Within single field		69.80	
Temporal scale 1986-1990			
Within and among regions	0.942	26.0	< 0.01
Within single field		74.0	
Spatial scale - Northern population vs Central-Southern populations			
Within and among regions	1.842	30.6	< 0.01
Within single field		69.4	
All locations (all fields)			
Among subpopulations	0.640	22.7	< 0.01
Within subpopulations	6.207	77.3	

Hierarchical genotypic diversity based on Anova analysis

Table 8

Pairwise comparisons of historical population (G_{ST}) of *Phoma pinodella*

Fields	Northern region			Central - Southern regions		
	A	B	C	D	E	F
A	-	0.34*	3.51*	0.97*	1.47*	17.4**
B	0.083[a]	-	2.56*	1.13*	1.59*	5.56*
C	0.096	0.085	-	16.0*	2.15*	19.5*
D	0.156	0.091	0.174	-	0.48*	4.91*
E	0.162	0.143	0.169	0.124	-	1.79*
F	0.179	0.080	0.199	0.146	0.152	-
Indices of diversity						
Stoddart-Taylor	36.7%	39.8	37.2	33.4	39.5	29.9
H'	1.19	1.30	1.22	0.99	1.60	2.12
H_T	0.348	0.332	0.342	0.377	0.386	0.411
H_s	0.249	0.201	0.232	0.211	0.262	0.347

[a] = G_{ST} values for genetic population differentiation; above diagonal – t -values, below diagonal – G_{ST} values
*, **, *** = indicate significance at P=0.05, P=0.01 and P=0.001 respectively
H' = genotype diversity expressed and Shannon index, H_T = total genotypic diversity

Table 9

Differences of VCG frequencies and differences in genotypic diversity among historical subpopulations of *Phoma pinodella* compared on spatio-temporal scale

Temporal distribution	Hierarchical distribution of number of genotypes (χ^2 values)	Hierarchical distribution of genotypic diversity (t-values)
First survey (1975-1980)	9.33**	4.26**
Second survey (1981-1985)	4.0*	3.03*
Third survey (1986-1990)	3.33*	2.01*
1st versus 2nd survey	4.31*	1.95*
1st versus 3rd survey	11.54**	6.20**
2nd versus 3rd survey	4.31*	1.79*
Spatial distribution - 1st survey		
Differences between regions	9.33**	5.56**
Between fields within single region	5.40*	2.56*
Between foci within single fields	4.27*	1.77*
Spatial distribution - 2nd survey		
Differences between regions	6.26**	4.26**
Between fields within single region	3.33*	1.62*
Between foci within single fields	4.00*	0.54 *
Spatial distribution - 3rd survey		
Differences between regions	4.19**	1.79**
Between fields within single region	4.31*	1.95*
Between foci within single fields	5.40*	2.01*

*, ** = indicate significance at P=0.05 and P=0.01 respectively
Total number of genotypes and total genotypic diversity was partitioned into different temporal and spatial scale components based on a hierarchical analysis

lines grown in resistance nursery or in long-term monoculture were significantly different comparing to the population sampled from commercial pea fields. In this case population sampled from resistant breeding lines was significantly different from old historical population and the last young contemporary population sampled from commercial pea cultivars (Tables 11, 12, 13 and 14).

Table 10

Historical genotypic population structure of *Phoma pinodella* determined across spatio-temporal scale

Subpopulations	H_T	H_S	G_{ST}	evennessE_5	H'	$\hat{G}$
Temporal scale						
First survey (1975-1980)	0.214	0.110	0.097*	0.398	1.030	28.3
Second survey (1981-1985)	0.259	0.120	0.110**	0.412	0.983	26.6
Third survey (1986-1990)	0.317	0.214	0.150**	0.603	1.120	30.2
Spatial scale - 1st survey						
Between regions	0.314	0.217	0.179**	0.403	0.930	29.9
Between locations	0.220	0.121	0.093*	0.422	0.710	38.8
Between foci within single field	0.352	0.298	0.083*	0.219	0.820	41.7
Spatial scale - 2nd survey						
Between regions	0.260	0.132	0.160**	0.422	0.996	30.2
Between locations	0.230	0.151	0.120*	0.598	0.832	22.8
Between foci within single field	0.390	0.212	0.072*	0.649	1.140	49.2
Spatial scale - 3rd survey						
Between regions	0.270	0.129	0.138**	0.399	0.910	22.7
Between locations	0.215	0.110	0.110**	0.460	0.810	49.7
Between foci within single field	0.333	0.211	0.089*	0.680	1.200	54.7

G_{ST} – values for genetic population differentiation between years and across spatial scale
Genotype diversity expressed as: H' of Shannon index as percentage of maximum possible of Stoddart and Taylor values.
H_T – total genotypic diversity
E_5 – the eveness index that reflecting whether or not one or few of VCG genotypes dominate within spatio-temporal populations
*, ** - indicate significance at P = 0,05 and P = 0,01 respectively

Genotypic diversity was averaged across all historical subpopulations and contemporary population and *t* or chi-square statistic values indicated highly significant (P = 0.01) differentiation between historical and contemporary populations. Also pairwise comparison G_{ST} between three historical subpopulations revealed that population sampled from resistant breeding lines was significantly different from the subpopulations sampled from cultivars grown during this period across Poland (Tables 13 and 14). It is worth noticed that also historical or contemporary subpopulations sampled from susceptible breeding lines or susceptible cultivars grown in monoculture was significantly different from historical or contemporary subpopulations sampled from resistant breeding lines tested in long term monoculture. In this case G_{ST} values ranged from 0.159 to 0.345 for *A.pinodes* and from 0.198 to 0.296 for *P.pinodella* (Table 14). In conclusion: Based on obtain results, populations of *A.pinodes* and *P.pinodella* could be divided into four groups. One

Table 11

Historical population structure of *Ascochyta pinodes* sampled form resistant breeding lines of pea

Statistics	Artificially infested plot	Monoculture
Sample size n	100	100
Indices of richness gobs	4	5
$E_{(gn)}$ - number of expected genotypes	3.66	4.32
$E_{(gn)}$ - number of expected genotypes to the smallest sample size	2.70	4.70
$g_{max} = 2^6$ based on number of vic loci	64	64
Sample size n	0.412 (0.39-0.84)	0.610 (0.54-0.93)
N_1	2.11 (1.45 -2.25)	2.05 (1.68-2.49)
N_2	1.84 (1.45-2.25)	1.53 (1.38-1.64)
Stoddart and Taylor $\hat{G}$ of its maximal value	16.4%	18.8%
H_T	0.115	0.210
H_S	0.111	0.117
Indices of evenness E_1	0.502	0.461
E_2	0.311	0.320
E_5	0.317 (0.39-0.84)	0.363 (0.47-0.71)

Description of population structure are based on Grunwald *et al.* (2003) data. Numbers in parentheses indicate confidence intervals calculated by the bootstrapping approach for the common sample size of the smallest population.

Table 12

Historical population structure of *Phoma pinodella* sampled form resistant breeding lines of pea

Statistics	Artificially infested plot	Monoculture
Sample size n	100	100
Indices of richness gobs	9	4
$E_{(gn)}$ - number of expected genotypes	8.84	3.78
$E_{(gn)}$ - number of expected genotypes to the smallest sample size	7.12	2.83
gmax = 2^6 based on number of vic loci	64	64
Indices of diversity: H'	0.693 (0.39-0.84)	0.710 (0.39-0.83)
N_1	2.55 (1.72-2.69)	1.59 (1.261-1.87)
N_2	2.03 (1.45-2.25)	1.86 (1.26-1.87)
Stoddart and Taylor $\hat{G}$ of its maximal value	23.3%	19.5%
H_T	0.199	0.214
H_S	0.120	0.110
Indices of evenness E_1	0.381	0.550
E_2	0.470	0.333
E_5	0.353 (0.38-0.54)	0.394 (0.44-0.61)

Description of population structure are based on Grunwald *et al.* (2003) data. Numbers in parentheses indicate confidence intervals calculated by the bootstrapping approach for the common sample size of the smallest population

main group consisted of historical population and another group consisted of contemporary population. The historical group could be divided into two additional groups consisting the resistance nursery subpopulations sampled from highly resistant lines and historical subpopulations sampled from susceptible cultivars of pea.

Table 13

Pairwise comparisons of genotype frequencies between populations of *Ascochyta pinodes* and *Phoma pinodella* sampled from resitant breeding lines of pea (χ^2 values)

Pea lines	Resistant	Partial resistant	Susceptible
Resistant	—	0.97^{NS}	26.24***
Partial resistant	6.08*	—	10.80***
Susceptible	10.72***	2.41***	—

*, **, *** - indicate significance at P=0.05, P=0,01 and P=0.001 respectively, ^{NS} - non-significant

Table 14

Pairwise comparisons of historical populations of *Ascochyta pinodes* and *Phoma pinodella* sampled from resistant breeding lines of pea

Comparisons	Ascochyta pinodes		Phoma pinodella	
	GST[a]	P[b]	G_{ST}	P
Nursery population vs historical population of cultivars of pea	0.244	< 0.001	0.278	< 0.001
Historical population of pea cultivars vs contemporary population of cultivars	0.159	< 0.001	0.198	< 0.001
Susceptible lines vs resistant lines	0.345	< 0.001	0.296	< 0.001

a - G_{ST} value for genetic population differentiation
b – indicate significance at P=0.001

DISCUSSION

Knowledge of the possible range of temporal variation in population genetic structure is limited for most pathogenic fungal species. Nevertheless it has been indicated that majority of fungal populations sampled from different geographical regions were stable over the several years according to their genetic population structure. On the other hand, it is well known that populations of pathogenic fungi exhibit the potential to undergo important shifts over the short periods of time. McDonald *et al.* (2002) in studying the population structure of *Mycosphaerella graminicola* and *Phaeosphaeria nodorum* clearly have indicated that the selection operates on specific pathogen genotypes under field conditions and that selection has not led to widespread clones that were adapted to specific host cultivars or breeding lines differing in their level of resistance. In addition no genotypes usually were shared among fields planted with the same

cultivar of wheat. In this case the isolates with the same DNA fingerprint haplotypes always came from the same infection foci within a field. It has been also indicated that a lesion of *M.graminicola* or *P.nodorum* had few dominant genotypes (usually up to two), which occupied the majority of the lesion area, and other rare genotypes were interspersed among the dominant genotypes. The selection operates on the population instead of the individual genotypes (McDonald and Linde 2002, Zhan *et al.* 2000, 2001, McDonald 1997, McDonald *et al.* 1996).

Genotypes are ephemeral but alleles persist in populations through the times. Though selection is able to increase the frequency of particular genotypes over the time, in my opinion particular genotypes of *A.pinodes* or *P.pinodella* are unlikely to reach high frequencies within field populations due to the limited dispersal potential of conidia. On the other hand, the alleles in the fit individuals usually persist and are able to recombine that leads to create new genotypes in the next growing season. During the years the selection is able to change the frequency of alleles and genotypes that affect adaptation to pea as host but in this case the new genotypes should appear each year. Zhan *et al.* (2000, 2001) have indicated that the genetic structure of *M.graminicola* populations were stable over the 6 years period and the common alleles at each RFLP locus were present at similar frequencies each year. More than 99% of gene diversity was distributed within populations sampled from the same year and less than 1% was attributed to differences among years. Isolates belonging to identical genotypes usually were sampled form the same fields and no identical genotypes were found between populations sampled from different years.

In regard to comparisons of variability between historical and contemporary populations it has been shown that few VCG genotypes of *A. pinodes* and *P.pinodella* were found in the historical population sampled during 1975-1990 from commercial cultivars of pea which were probably sown with infected seeds or belonged to local Polish endemic population. By 2004, many more VCG genotypes were detected within populations of *A.pinodes* or *P.pinodella* sampled form the same three regions of Poland and commercial cultivars of pea. These results suggest that a large number of genotypes could be introduced between 1975 and 2004 years. The occurrence of several rare or unique VCG genotypes within last "young" contemporary populations of *A.pinodes* and *P.pinodella* sampled during 2003-2004 also indicated that these two fungi have had the potential to form the sexual stage since 1975 and that the sexual component of the life cycle has probably played a significant role in the epidemiology of pea Ascochyta blight in Poland. On the other hand, in order to confirm whether contemporary populations of *A.pinodes* or *P.pinodella* are recombining regularly, the molecular sensitive markers should be used in further study. The import of seed of pea cultivars that was very popular just after 2000 can cause long distance dispersal mechanism for Ascochyta blight pathogens and the introduction of new foreign genotypes also can cause actually to serious problems of epidemic. It is worth noticed that the large-scale of import of pea seed has been recorded in Poland for the past

ten years especially since 1990, which has not presumably prevented the introduction of new genotypes. The sources of the introduction of genotypes of *A.pinodes* and *P.pinodella* into Poland with seeds are probably from other countries of Europe where pea is grown on a large area. In addition Poland also imported seeds of pea from Canada and Australia. The additional sources of introduction could be the resistant wild lines of pea sampled from centre of origin, used of lines in resistance breeding programme. This hypothesis is currently being tested by the determination of worldwide population structure and phylogeography of *A.pisi, A. pinodes* and *P.pinodella* conducted by research groups from USA, Spain, France, Canada and Australia. These researches included isolates sampled from the putative centres of origin of pathogens and isolates sampled from all worldwide countries where pea is grown (Onfroy *et al.* 1999, Tivoli *et al.* 2006, Cubero *et al.* 2005, Kraft *et al.* 1998, Xue and Warkentin 2001, Xue *et al.* 1996, 1998, Wroth and Khan 1999, Wroth 1998, 1999, Zhang *et al.* 2003, 2006). This kind of studies is focused on genetic population structure and on origin and diversity of pathotypes within local populations of *A.pisi, A. pinodes* and *P.pinodella* sampled from growing local pea cultivars.

It is worth noticed that recently have been tested worldwide pea lines in order their resistance to Ascochyta blight of pea. Kraft *et al.* (1998) and Muehlbauer and Chen (2006) have screened over 2000 breeding lines or cultivars of pea from diverse origins and none showed a high level of resistance to Ascochyta blight of pea but some indicated partial resistance. In addition, within breeding lines from the USDA *Pisum* collection some lines with partial resistance have been identified and these lines are at present incorporated into resistance breeding programme. Also Xue and Warkentin (2001), Xue *et al.* (1996) and Zhang *et al.* (2006) from Canada have tested over 2000 germplasm accessions obtained from the more than 30 countries and did not find genotypes with complete resistance. It is worth noticed that worldwide breeding lines tested in England were susceptible in Canada due to the occurrence of different and local virulent pathotypes (Zhang *et al.* 2006, Xue *et al.* 1998, 1996). Similar results have been obtained by Wroth (1998, 1999), Wroth and Khan (1999), Baranger and Tivoli and their research group (Baranger *et al.* 2006) and by Rubiales and his colleagues (Cubero *et al.* 2005). Baranger, Tivoli, Kraft and Rubiales and their colleagues have suggested that all tested cultivars and breeding lines are rather susceptible than resistant or partial resistant to local pathotypes of *P.pinodella* or *A.pinodes* (Onfroy *et al.* 1999, Cubero *et al.* 2005; Fondevilla *et al.* 2006, Kraft *et al.* 1998, Prioul *et al.* 2004, Tivoli and Onfroy 1997, Tivoli *et al.* 2006, Baranger *et al.* 2006).

Highly significant genetic differentiation was detected between the historical and contemporary populations of *A.pinodes* and *P.pinodella* sampled from pea cultivars. When populations structure of *A.pinodes* and *P.pinodella* was inferred based on VCG diversity without regard to spatio-temporal scale, the global Polish population of these two pathogens could be subdivided onto three genetically different subpopulations. In this case to the first group belonged the isolates in the

historical samples collected from old cultivars of pea. The contemporary isolates were more likely to be assigned to second group. In addition the historical subpopulation sampled from resistant breeding lines belong to third group, quite different from the first and second groups. This suggests that the genotypes predominating in the first old historical subpopulation may have been replaced largely by different genotypes occurring in the last contemporary *A.pinodes* and *P.pinodella* populations sampled from new bred and modern cultivars of pea. The significant genotypic diversity differentiation that observed between the historical and contemporary populations were probably the result of pooling VCG genotype frequencies across old historical population and new contemporary population, which obscured the genetic variation within each population. The genotypic differentiation observed among the contemporary populations sampled from three distantly regions of Poland was probably due to genetic drift, restricted migration of genotypes and selection which causes by a new modern and more resistant cultivars of pea. Several different rare and unique VCG genotypes were found in three contemporary regional subpopulations suggested that migration among distantly regions of Poland was restricted.

It is worth noticed that the genotypic differentiation between resistant lines subpopulation and contemporary population also were highly significant. This data was consistent with the hypothesis that resistant breeding lines had selected new population of *A.pinodes* and *P.pinodella*, which might be better adapted to pea as a host. On the other hand, the potential role of random factors such as founder effects in determining which genotypes have become established in a given area must also be considered. It is worth to note that in further studies, the existence of two groups of virulence (pathotypes) in *A.pinodes* and *P.pinodella* populations have been found. One-pathotype groups that were characterized of low to intermediate level of virulence appear to predominate in historical and contemporary populations sampled from susceptible pea cultivars. The second group of pathotypes with high level of virulence predominates in population of *A.pinodes* and *P.pinodella* sampled from resistant breeding lines. It should be noticed that similar results have obtained Marcinkowska and Witkowska (1996) regarding the occurrence of pathotypes of *M.pinodes* within Polish population of this pathogen.

The existence of two virulence groups of pathotypes in *A.rabiei* populations that had been selected by resistant and susceptible cultivars of chickpea have been also found Chen *et al.* (2004), Cho *et al.* (2004) and Peever *et al.* (2004). Chen *et al.* (2004) clearly indicated the occurrence within populations of *A.rabiei* of two distinct groups of pathotypes. One group of pathotypes appears to predominate in historical population sampled from susceptible cultivars of chickpea and the second group of pathotypes appears to predominate in population sampled from resistant cultivars or from resistant breeding lines.

Similar results have been obtained by Xue *et al.* (1998) regarding the pathotypes variation within population of *A.pinodes* sampled from resistant breeding lines or resistant cultivars of pea. Within local population of *A.pinodes* sampled from re-

sistant breeding lines highly virulent pathotype 1 was dominated. In addition within population of *A.pinodes* sampled from cultivars of pea actually growing in Canada twenty two pathotypes have identified.

In the opposite to the results obtained by Peever *et al.* (2004) it should be noticed that in this study no significant genotypic differentiation was observed between *A.pinodes* and *P.pinodella* subpopulations sampled from the two resistance screening nurseries such as long - term monoculture and artificially infested nursery. In this case subpopulations of *A.pinodes* and *P.pinodella* sampled from resistant breeding lines usually consisted of up to three VCG genotypes and were characterized by the low genotypic diversities.

It is worth noticed that in the resistance nursery and in long-term monoculture *A.pinodes* regularly and every year developed pseudothecia. Regarding *P.pinodella* the situation was difficult to compare to *A.pinodes* because I was unable to find the sexual fruiting bodies during the end of growing season or in the left debris in autumn.

In this situation it was unable to reject the hypothesis of random mating in all historical populations of *P.pinodella* primarily according to two subpopulations sampled from infested plots or from long-term monoculture. In addition we cannot reject hypothesis of random mating according to contemporary population of *P.pinodella.* To resolve this problem, the sensitive molecular markers should be used in further study.

This information can be also obtained by using the conventional genetic analysis based on crossing of sampled isolates of *P.pinodella* with two testers *Mat-1-1* and *Mat-1-2* as male and female parents and looking for the occurrence of pseudothecia and for viable ascospores. This could be possible if only two testers would be available.

Random mating is not possible to detect at present as long as the sexual stage of *P.pinodella* will be reported in laboratory and under field condition and until two testers *Mat-1-1* and *Mat-1-2* will be available. On the other hand, it is also possible to conduct the special field experiments in the nearest future after cloning both mating type of *A.pisi* and *P.pinodella* or after cloning the genomes of these two heterothallic fungi. This special field experiment would be artificially infested by inoculum consisting with *Mat-1-1* and *Mat-1-2* mating types with the hope of obtaining the pseudothecia on the pea debris. Additional support for the importance of the sexual stage in the epidemiology of *Ascochyta* blight of pea comes from identical as Peever *et al.* (2004), observations according to *Ascochyta* blight development in the field on some leguminouse crops primarily on chickpea or lentis. I have observed that in artificially infested nursery or in long - term monoculture the occurrence of symptoms during vegetation is quite different comparing to with commercial pea fields. According to monoculture or artificially infested resistance nursery the symptoms of Ascochyta blight of pea are characterized by the uniform distribution of lesions on the upper leaves. The occurrence of lesions also on upper leaves particularly before or after flowering time is more consistent with the ascospores infection than with seedborne inoculum.

Milgroom and Peever (2003) and Peever *et al.* (2004) have proposed model based only on the occurrence of symptoms on seedlings or on upper leaves. This is particularly evident after flowering time and therefore, based on direct observations of the occurrence of Ascochyta blight symptoms, some conclusion according to the occurrence of sexual stages is possible. On the other hand, when sexual stage of *P.pinodella* or *A.pinodes* is absent the lesions should occur primarily on bottom leaves or on middle leaves and diseased pea plants should be concentrated within infection foci under field conditions due to the short distance of disperse of conidia comparing to ascospores.

The extent of genetic differentiation among subpopulations of *A.pinodes* or *P.pinodella* observed in this study was similar to that observed among *Cryphonectria parasitica* subpopulations in eastern North America (Milgroom and Lipari 1995), *A.rabiei* in Northern America (Milgroom and Peever 2003, Peever *et al.* 2004), but was lower than observed among *Pyrenophora teres* subpopulations which were sampled throughout North America (Peever and Milgroom 1994). But obtained results are not similar to other plant pathogens with similar biology to *P.pinodella. Mycosphaerella graminicola, Phaeosphaeria nodorum, Tapesia yallundae* and *T.accuformis* also have mixed population structures, clonal and recombining but they exhibit little genetic differentiation among geographically separated subpopulations, even on a worldwide sampling scale (Douhan *et al.* 2002, 2003, Zhan *et al.* 2000, 2001, McDonald *et al.* 1996, McDonald and Linde 2002). Regarding the suggestion of Peever research group it is not clear why *A.rabiei* exhibits significantly higher differentiation among subpopulations comparing to *M.graminicola* or *P.nodorum* (Peever *et al.* 2004). Peever *et al.* (2004) have suggested that this is due to greater restriction of migration and gene flow and/or a larger effect of genetic drift within subpopulations, which might be related to effective population sizes. Further study is required to determine what is the mechanism of these different patterns of genetic substructuring of different plant-pathogenic fungi with similar biology such as *A.pisi, P.pinodella, A.rabiei, M.graminicola* and *P.nodorum*.

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