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GENOTYPIC POPULATION STRUCTURE OF *ASCOCHYTA PINODES* AND *PHOMA PINODELLA* IN POLAND

ABSTRACT

The genotypic populations structure of *Ascochyta pinodes* and *Phoma pinodella* was determined across a hierarchy of spatial and temporal scale by using vegetative incompatibility system as phenotypic genetic marker. Components of genetic variation within and between populations included the following values: indices of diversity H', richness (g), indices of evenness E_1, E_2 and E_5, spatio-temporal analysis of genotypic variation (H_T and H_S values), pairwise comparisons of genotype diversity, distribution of VCGs across spatio-temporal scale (G_{ST} value) and analysis of variance calculated on VCGs genotype frequencies within populations. It has been found that genotypic populations structure of *Ascochyta pinodes* and *Phoma pinodella* belong to mixed population structure clonal and recombining. Three regional populations of *Ascochyta pinodes* and *Phoma pinodella* differed significantly in both of VCGs genotype frequencies and their distribution on a spatio-temporal scale indicating on the low or moderate degree of genotype migration between regions of Poland.

Keywords: *Mycosphaerella* (*Didymella*) *pinodes anamorph Ascochyta pinodes*, *Phoma pinodella (*teleomorph *Didymella)*, genotypic population structure, components the genetic variation of population, VCG frequency, spatial and temporal VCG diversity

INTRODUCTION

Ascochyta blight of pea is caused primarily by *Ascochyta pinodes* (Berk et Blox) Jones teleomorph *Mycosphaerella pinodes* (Berk et Blox) Vesterg = *Didymella pinodes*, Pertrak and *Phoma medicaginis var pinodella* (Jones) Boerema = *A.pinodella* Jones (teleomorph *Didymella*). This disease provides a good example of the difficulties in seed infections controlling and epidemics of *Ascochyta blight* causes by pathogens that are highly host-specific and are capable of causing serious damage in a conductive environment. Since the area of pea has systematically increased in some countries of the world, Ascochyta blight of pea has become one of the most important diseases in pea production. Except of direct yield losses, Ascochyta blight of pea reduces of seed quality and seeds are often contaminated by mycotoxins.

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In Poland there have not been obtained pea cultivars of high resistance to *A.pinodes* and *P.pinodella* despite considerable efforts in breeding for disease resistance. Little knowledge of genetic population structure of *A.pinodes* and *P.pinodella* as well of the host resistance mainly due to the quantitative nature of both of them make problems in plant breeding for disease resistance.

There is also not enough information about the source of resistance and the population biology of *A.pinodes* and *P.pinodella* at molecular levels. Another limitation is due to lack or little knowledge about gene flow and genotypes migration within and between Polish, European or transcontinental populations of *A.pinodes* and *P.pinodella* and on the phylogenetic relationship between geographically distant populations. Nevertheless over the last decade pea breeders have concerned their works regarding the resistance of lequminose species to some pathogens and viruses.

Although the number of studies on fungal species has increased last years, the most comprehensive work was focused on quantitative resistance pea to *F.oxysporum f.sp. pisi, F.solani f.sp. pisi, Aphanomyces euteiches* and *A.pinodes*. Similar researches have conducted regarding the resistance chickpea to *F.oxysporum f.sp. ciceris* and *Ascochyta rabiei,* faba-bean to *A.fabae* and lentis to *A.lentis*. Substantial progress in the field of understanding the quantitative resistance of pea, chickpea, faba-been and lensis to pathogens has been made (McPhee *et al.* 2006, Muehlbauer and Chen 2006, Cubero *et al.* 2005, Ford *et al.* 1999, Prioul *et al.* 2004, Taylor and Ford 2006, Baranger *et al.* 2006, Tivoli *et al.* 2006, Fondevilla *et al.* 2006).

Genetic analysis of *A.pinodes* population was hindered for a long time due to the inability to perform genetic crosses regarding the homothallic mating system of this fungus. Similarly still is impossible to make the conventional genetic sexual crossing and obtain pseudothecia regarding the heterothallic species of *Ascochyta* (*Didymella*) *pisi* and *Phoma* (*Didymella*) *pinodella* due to lack of mating type testers and sensitive molecular markers. More detailed analyses on population genetics of *A.pisi, A.pinodes* and *P.pinodella* have begun since 1997 when molecular markers for the first time were used (Bouznad *et al.* 1995, 1996, Barve *et al.* 2003, Faris-Mokaiesh *et al.* 1996, Fatehi and Bridge 1998, Fatehi 2000, Fatehi *et al.* 2003, Onfroy *et al.* 1999, Tohamy and Mohamed 1998, Lubeck *et al.* 1998, Armstrong *et al.* 2001, Peever *et al.* 2002, 2004, Fondevilla *et al.* 2006, Zhang *et al.* 2000, 2006).

At present using of molecular markers is giving different approaches of genetic diversity examining within populations of *A.pinodes* and *P.pinodella* by possibility of insight into the reproductive strategy and population genetics. In theory, the comparison the diversity of RAPDs or AFLP-PCR-fingerprints haplotype patterns within and between populations is a way to analyze genetic population structure of heterothallic *P.pinodella* (*Didymella*), *A.pisi* (*Didymella*) and in less extence of

homothallic *Mycosphaerella* (*Didymella*) *pinodes* . Looking for the number of single and rare genotypes in populations is a second one to estimate genetic population structure. The finding of a few dominant genotypes within a local population is the compelling evidence for clonal population structure and asexual reproduction. On the contrary, the finding that most isolates of *P.pinodella* or *A.pinodes* belong to different genotypes can be concerned as the evidence for mixed population structure, (clonal and recombining) and for sexual reproduction under field conditions (Milgroom 1995, 1996, Milgroom and Fry 1997, McDonald 1997, McDonald and Linde 2002, Zhan *et al.* 2000).

Given the seemingly asexual nature of *A.pinodes* or sexual homothallic nature of *Mycosphaerella (Didymella) pinodes* and heterothallic nature of *P.pinodella* (*Didymella*) and their wide distribution worldwide it would be interesting to examine the extent of gene and genotypic diversity within and between regional populations and the test the null hypothesis that *A.pinodes* and *P.pinodella* are clonally distributed across Poland.

Understanding the populations structure of *A.pinodes* and *P.pinodella* and their evolution is necessary for design of management practices aimed at limiting of the appearance and spreadingof the new genotypes and more virulent pathotypes of the pathogens.

Knowledge on the genetic population structure and asexual or sexual strategies of reproduction of *A.pinodes* and *P.pinodella* on a local scale gives insight into the evolutionary processes that shaped the local populations in the past and offers insight into the future of evolutionary potential populations of these fungi. Knowledge on the evolutionary potential of *A.pinodes* and *P.pinodella* may prove useful to optimize the management of resistance genes, applied of fungicides, maximize their useful and minimize the losses in efficacy of these control methods. Current research interest cover a broad range of subjects related to *A.pinodes* and *P.pinodella* biology including: genetics and taxonomy at molecular level, evolution of these pathogens and host-pathogen interactions, gene flow and genotype migration, occurrence of sexual reproduction in Poland and other countries and phylogenetic relationships between strains and different worldwide subpopulations of *A.pinodes* and *P.pinodella* (Barve *et al.* 2003, Baranger *et al.* 2006, Coyne *et al.* 2000, Cubero *et al.* 2005, Fatehi 2000, Fondevilla *et al.* 2006, McPhee *et al.* 2006, Peever *et al.* 2002, 2004, Prioul *et al.* 2004, Timmerman – Vaughan *et al.* 2000, 2002, Tivoli *et al.* 2006, Wroth 1999, Xue and Warkentin 2001, Zhang *et al.* 2006).

The purpose of this study was to evaluate the pattern of spatio-temporal VCGs genotype diversities and their distribution within the three regional Polish populations of *A.pinodes* and *P.pinodella*.

MATERIAL AND METHODS

Quadrate based methods were used to quantified VCGs genotype frequencies and their distribution within and between regional Polish populations of *A.pinodes* and *P.pinodella* on the spatio-temporal scale. Diseased plants were hierarchically sampled from the farmer's fields of the size of minimum from about 0.5 to one hectare in size. Three different area of the farmer fields were chosen in three regions of Poland: northern, central and southern. The three regions were separated and the distance from one to other was at least 600 km. Within each region two locations (two farmer fields) were selected, both separated about 1 km or less and usually belonging to farmers being the neighbours who used the same seed material and machine equipment. The third location (farmer field) was separated from those two locations approximately 200 km and planted with different cultivars usually not closely related with the environmental habitat.

In northern region two closely situated fields were located near Olsztyn and these fields were about 1-6 km apart. Third field was located near Ełk in close proximity to the Russian border.

In southern region two fields were located near Wrocław at the distance of about 2-3 km. The third field in southern region was located about 300 km from Wrocław near the borderof the Czech Republic and Slovakia.

Fields in central region were located near Warsaw and Poznań.

Three regional populations were also sampled according to temporal scale: First temporal population was sampled in 1990-1992, second in 1995-1997 and third in the period of 2002-2004. In this researches Polish populations of *A.pinodes* and *P.pinodella* covered three geographically distant regions and a broad temporal scale. In this study the quadrate base method was defined as the 0.5-1 m^2 areas called also as infection foci. The infected pea plants and sampled isolates within them were evaluated for quantification and distribution of genotypic variation both within and between infection foci as on the smallest spatial scale. Within single infection focus of about ten infected leaves were sampled if possible. Because within a single pea field the number of infection foci and their distribution 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. Infected leaves were dried at room temperature in laboratory. Dried samples were stored at 5°C for further analyzed.

Isolation of *A.pinodes* and *P.pinodella* from infected leaves, species designation and preparation of monoconidial populations were identical as described previously by Furgał–Węgrzycka (1990). When isolation of *A.pinodes* and *P.pinodella* from infected leaves was difficult due to the occurrence the low level of symptoms, collection of isolates was prepared from infected seeds after harvest.

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

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 (Leslie 1993, 1996, Katan 1999). Test for vegetative compatibility was performed as described by Milgroom and Cortesi (1999). Strains were paired about 1-3 cm apart on PDA medium amended with active charcoal and on freshly prepared oat medium (OA) amended with bromocresol green pH indicator dye. It has been shown that freshly prepared oat mealt 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 two colonies of *A.pinodes* or *P.pinodella* indicates 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* indicates the vegetative incompatibility. Petri dishes with paired strains were incubated in the dark at 22°C for 30 days. All strains that were vegetative compatible belong 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 null hypothesis testing that VCGs genotype 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 included: Shannon's diversity index (H'), Stoddart and Taylor index (), evenness (E_1, E_2 and E_5), richness (g), genotypic diversity within each subpopulation (H_s) average diversity (H_T) and genotypic population subdivision G_{ST} values.

Genotypic variation in each spatio-temporal population was quantified by partitioned the total genotype diversity H_T and H' into components among regions, years, fields and among infection foci within single field used the hierarchical genotype diversity analysis. Hierarchical analysis was also used to partition genotypic diversity into two levels regarding the years: variation within single temporal population and between 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 using chi-square test and *t* test values. These statistical tests can result in significance values when one or few of rare genotypes were present at all spatio-temporal subpopulations. In

such a case when there were obtained significant departures from null hypothesis that genotypes were equally distributed across all spatio-temporal subpopulations, pair wise comparisons subpopulation by subpopulation were used to determine the subpopulation pairs responsible for significant departures for null hypothesis and for overall evenness. Pair wise measures and genetic population differentiation G_{ST} was calculated as:

$$G_{ST} \quad \frac{H_T \quad H_S}{H_T}$$

where: H_T was the average of genotypic diversity within populations and was calculated by treating all genotypes as if they belong to a single population, 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 the spatio-temporal scale (e.g. regions, locations and consecutive years). All statistical measures regarding the obtained results and genotypic diversity within Polish population of *A.pinodes* and *P.pinodella* were calculated by computer program described by Węgrzycki Michał.

RESULTS

The genotypic populations structure of *A.pinodes* and *P.pinodella* was determined across the hierarchy of spatial and temporal scales by using vegetative compatibility test as phenotypic genetic marker.

Summary regarding the genetic population structure was based on statistical information that included: indices of diversity H', N_1 and , richness (g) and indices of evenness E_1, E_2 and E_5. Obtained results were given inTables 1 and 8. Spatio-temporal analysis of genotypic variation H_T and H_S values, pair wise comparisons of genotypes diversities and their distribution across spatio-temporal scale (G_{ST}, chi-square and *t* values) and analysis of variance based on VCG genotype frequencies were given in Tables 2, 3, 4, 5, 6 and 7 for *A.pinodes* and in Tables 9,10,11,12,13 and 14 regarding to *P.pinodella*.

The number of VCG genotypes of *A.pinodes* and *P.pinodella* found in three regional Polish populations ranged from 34 to 55 respectively with the average of 29 VCG genotypes per region (Tables 1 and 8). Genotype diversity within regional populations of *A.pinodes* and *P.pinodella* was H_T=(0.217-0.413) for *A.pinodes* and H_T = (0.270 - 0.412) for *P.pinodella* Tables 6 and 13). Stoddart-Taylor measure of genotype diversity () ranged from 21.2% to 50.7% of its theoretical maximum across three spatio-tempo-

Table 1

Polish population structure of *Ascochyta pinodes*

Statistic	Southern region	Northern region	Central region
Sample size n	120	300	80
Indices of richness gobs	31	22	34
$E_{(gn)}$ - no of expected genotypes	30.8	21.1	33.7
$E_{(gn)}$ - no of expected genotypes to the smallest sample size	30.64	19.9	30.2
$g_{max} = 2^6$ based on number of vic loci	64	64	64
Indices of diversity: H'	2.73 (2.67-2.96)	1.26 (1.16-1.58)	2.98 (2.59-3.08)
N_1	15.7 (14.4-18.9)	13.21 (12.94-13.24)	16.94 (14.4-18.9)
N_2	10.49 (9.53-17.7)	15.70 (9.68-16.6)	12.57 (9.53-13.7)
Indices of evenness E_1	0.774	0.684	0.799
E_2	0.371	0.564	0.369
E_5	0.620 (0.61-0.75)	0.427 (0.38-0.54)	0.672 (0.61-0.75)

Description of population structure are based on Grunwald *et al*. (2003) data.

Table 2

Variance component estimates for the VCG genotypes frequencies for Polish population of *Ascochyta pinodes*

Source of variation	Variance component	Percent of total variance	P
Difference in genotypes: among regions	0.640**	16.5	0.01
Among fields	0.302*	8.9	0.01
Between foci within single field	3.211**	74.6	0.01

*. ** = indicate significance at P=0.05 and P=0.01respectively

Table 3

Hierarchical distribution of genetic variance within Polish population of *Ascochyta pinodes*

Source of variation Distribution of genotypic diversity:	Variance component	Percent of total variance	P
Temporal scale 1990-1992			
Within and among regions	2.658	33.17	0.01
Within single field	7.322	66.83	
Temporal scale 1995-1997			
Within and among regions	1.762	28.11	0.01
Within single field	7.340	71.89	
Temporal scale 2002-2004			
Within and among regions	0.720	10.42	0.01
Within single field	7.453	80.58	
Spatial scale - Northern population vs Central-Southern populations			
Within and among regions	2.112	29.34	0.01
Within single field	7.311	70.66	
All locations (all fields)			
Among subpopulations	4.629	25.4	0.01
Within subpopulations	7.299	74.6	

Hierarchical genotypic diversity based on ANOVA analysis

Table 4

Pairwise comparisons (G_{ST}) of contemporary population of *Ascocha pinodes*

Fields	Northern region			Central region			Southern region		
	A	B	C	D	F	G	H	K	L
A	-	4.91*	0.97NS	3.51***	2.01**	2.01*	1.95*	5.49***	6.20***
B	0.068a	-	6.08***	2.56*	1.95*	5.27***	3.08**	2.15*	1.95*
C	0.074	0.092	-	5.49***	2.01**	1.95*	5.27**	6.38***	10.72***
D	0.111	0.094	0.093	-	2.01*	3.08***	2.15*	1.95*	1.79**
F	0.115	0.083	0.113	0.087	-	2.01**	0.80NS	2.15*	2.01*
G	0.142	0.170	0.097	0.162	0.073	-	2.56*	3.64**	4.26**
H	0.102	0.196	0.104	0.111	0.172	0.193	-	1.95*	2.15*
K	0.116	0.189	0.170	0.173	0.163	0.191	0.093	-	0.42*
L	0.159	0.111	0.162	0.069	0.083	0.122	0.072	0.052	-

[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

Table 5

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

Temporal distribution	Hierarchical distribution of number of genotypes (^{2} values)	Hierarchical distribution of genotypic diversity (t-values)
First survey (1990-1992)	16.1*	1.79*
Second survey (1995-1997)	29.7*	2.56*
Third survey (2002-2004)	10.8**	3.08**
1st versus 2nd survey	19.5*	0.42*
1st versus 3rd survey	28.6**	2.01**
2nd versus 3rd survey	14.1*	1.63*
Spatial distribution - 1st survey		
Differences between regions	15.23**	2.01**
Between fields within single region	5.4*	1.95*
Between foci within single fields	4.31*	5.56*
Spatial distribution - 2nd survey		
Differences between regions	28.6**	3.08**
Between fields within single region	19.1*	2.01**
Between foci within single fields	1.6*	0.80*
Spatial distribution - 3rd survey		
Differences between regions	22.9**	6.38**
Between fields within single region	29.7**	4.26**
Between foci within single fields	1.7*	0.42*

*. ** = 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

Table 6

Polish genotypic population structure of *Ascochyta pinodes* determined across a temporal scale.

Years	HT	HS	G_{ST} values	Evenness index values E_5	H'	$\hat{G}$
1990	0.341	0.153	0.199	0.514	0.846	21.2
1991	0.217	0.192	0.142	0.431	0.798	25.3
1992	0.399	0.287	0.111	0.612	1.100	39.9
1995	0.223	0.153	0.092	0.493	0.731	30.7
1996	0.394	0.192	0.089	0.679	0.993	28.3
1997	0.372	0.283	0.094	0.711	1.320	39.2
2002	0.341	0.264	0.071	0.683	1.900	49.1
2003	0.384	0.306	0.082	0.614	1.720	41.9
2004	0.413	0.292	0.064	0.632	2.130	48.9

G_{ST} – values for genetic population differentiation between years

Genotype diversity expressed as: H' of Shannon index. $\hat{G}$ 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 temporal populations

Table 7

Polish genotypic population structure of *Ascochyta pinodes* determined across a hierarchy spatial scale

Years	Northern region			Central region			Southern region		
	A	B	C	D	F	G	H	K	L
1990	0.259	0.269	0.264	0.289	0.317	0.391	0.373	0.278	0.229
1991	0.229	0.248	0.383	0.399	0.379	0.387	0.298	0.312	0.289
1992	0.173	0.197	0.299	0.377	0.349	0.364	0.274	0.306	0.399
1995	0.317	0.228	0.314	0.318	0.316	0.367	0.301	0.318	0.319
1996	0.229	0.276	0.347	0.312	0.319	0.312	0.315	0.323	0.416
1997	0.312	0.392	0.382	0.306	0.312	0.392	0.377	0.398	0.362
2002	0.398	0.388	0.368	0.320	0.344	0.397	0.321	0.314	0.415
2003	0.310	0.399	0.417	0.393	0.399	0.375	0.397	0.411	0.413
2004	0.432	0.415	0.419	0.411	0.422	0.396	0.413	0.401	0.411

The total genotype diversity (H_T) was partitioned into different spatial components with a hierarchical genotype diversity analysis. Spatial scales are based on comparison among fields. among locations and among regions

Table 8

Structure of Polish population of *Phoma pinodella*

Statistics	Northern region	Southern region	Central region
Sample size n	300	100	100
Indices of richness gobs	46	52	55
$E_{(gn)}$ - no of expected genotypes	45.83	51.80	54.97
$E_{(gn)}$ - no of expected genotypes to the smallest sample size	32.11	32.17	34.20
g_{max} = 26 based on number of vic loci	64	64	64
Indices of diversity: H'	2.91 (2.94-3.24)	3.19 (2.94-3.24)	3.21 (2.94-3.24)
N_1	19.0 (19.1-24.8)	20.6 (19.0-24.9)	22.9 (19.0-24-9)
N_2	14.3 (13.0-17.8)	15.09 (12.8-17.5)	15.2 (12.8-17.5)
Indices of evenness E_1	0.684	0.774	0.789
E_2	0.289	0.299	0.331
E_5	0.642 (0.63-0.74)	0.672 (0.62-0.73)	0.678 (0.62-0.73)

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 9

Variance component estimates for the VCG genotypes frequencies for Polish population of *Phoma pinodella*

Source of variation	Variance component	Percent of total variance	P
Difference in genotypes: among regions	0.520**	10.2	0.01
Among fields	0.275*	5.4	0.01
Between foci within single field	4.26**	84.4	0.01

*. **= indicate significance at P=0.05 and P=0.01 respectively

Table 10

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

Source of variation Distribution of genotypic diversity:	Variance component	Percent of total variance	P
Temporal scale 1990-1992			
Within and among regions	0.994	26.1	0.01
Within single field	7.001	73.9	
Temporal scale 1995-1997			
Within and among regions	0.593	17.83	0.01
Within single field	7.083	82.17	
Temporal scale 2002-2004			
Within and among regions	0.216	7.80	0.01
Within single field	6.080	92.20	
Spatial scale - Northern population vs Central-Southern populations			
Within and among regions	2.546	25.34	0.01
Within single field	7.011	74.66	
All locations (all fields)			
Among subpopulations	1.997	15.73	0.01
Within subpopulations	6.998	84.27	

Hierarchical genotypic diversity based on ANOVA analysis

Table 11

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

Fields	Northern region			Central region			Southern region		
	A	B	C	D	F	G	H	K	L
A	-	2.15*	3.51***	0.97*	0.54*	0.42*	1.62*	1.79*	5.49***
B	0.053a	-	5.27**	1.95*	1.79*	2.01*	2.56*	1.62**	1.63**
C	0.094	0.074	-	6.20***	10.72***	5.56***	6.38**	2.15**	0.94*
D	0.074	0.099	0.153	-	0.043*	0.97*	1.63**	1.59**	5.56***
F	0.093	0.083	0.142	0.043	-	0.032*	1.77*	1.95*	10.72***
G	0.091	0.093	0.172	0.064	0.072	-	0.052*	1.78**	5.56***
H	0.089	0.111	0.138	0.052	0.049	0.059	-	0.86*	5.27**
K	0.124	0.090	0.083	0.084	0.062	0.083	0.743	-	0.20*
L	0.183	0.120	0.192	0.145	0.132	0.158	0.093	0.743	-

[a] = G_{ST} values for genetic population differentiation
above diagonal – t values, below diagonal – G_{ST} values

Table 12

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

Temporal distribution	Hierarchical distribution of number of genotypes (^{2} values)	Hierarchical distribution of genotypic diversity (t-values)
First survey (1990-1992)	28.4**	1.62**
Second survey (1995-1997)	29.7**	2.56**
Third survey (2002-2004)	5.4*	3.03**
1st versus 2nd survey	16.0*	2.01**
1st versus 3rd survey	10.8**	5.56**
2nd versus 3rd survey	14.6*	2.15**
Spatial distribution - 1st survey		
Differences between regions	4.5**	2.01**
Between fields within single region	5.4*	1.79*
Between foci within single fields	1.59*	0.94*
Spatial distribution - 2nd survey		
Differences between regions	19.5**	3.08**
Between fields within single region	5.4*	2.01*
Between foci within single fields	1.6*	0.80*
Spatial distribution - 3rd survey		
Differences between regions	22.9*	3.03*
Between fields within single region	29.7*	2.15*
Between foci within single fields	2.56**	0.94**

*. ** = 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

Table 13

Polish genotypic population structure of *Phoma pinodella* determined across a temporal scale.

Years	HT	HS	G_{ST} values	Evenness index values E_5	H'	$\hat{G}$
1990	0.270	0.160	0.119	0.663	1.17	26.6
1991	0.320	0.192	0.141	0.511	1.22	31.2
1992	0.380	0.243	0.091	0.642	1.99	42.2
1995	0.299	0.147	0.115	0.538	2.14	41.8
1996	0.315	0.274	0.093	0.698	2.98	43.6
1997	0.360	0.250	0.066	0.779	2.43	42.2
2002	0.399	0.210	0.082	0.783	2.99	45.2
2003	0.370	0.300	0.072	0.742	2.49	49.3
2004	0.432	0.320	0.078	0.777	3.17	50.7

G_{ST} – values for genetic population differentiation between years

Genotype diversity expressed as: H' of Shannon index. $\hat{G}$ 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 temporal populations

ral populations (Tables 6 and 13). Three spatio-temporal populations differed significantly both in VCGs genotype frequencies and their distribution and regarding the components of genetic variation such as: genotypes diversities, richness and evenness (Tables 1 and 8). The majority of genotype diversity was distributed on a small spatial scale within single field primarily within and between infections foci occurred in fields.

Table 14

Polish genotypic population structure of *Phoma pinodella* determined across a hierarchy spatial scale

Years	Northern region			Central region			Southern region		
	A	B	C	D	F	G	H	K	L
1990	0.315	0.329	0.229	0.368	0.329	0.369	0.349	0.283	0.392
1991	0.319	0.314	0.217	0.384	0.369	0.333	0.337	0.384	0.311
1992	0.329	0.369	0.283	0.421	0.328	0.349	0.399	0.376	0.296
1995	0.334	0.301	0.399	0.382	0.307	0.348	0.366	0.302	0.330
1996	0.399	0.333	0.317	0.361	0.358	0.372	0.399	0.311	0.357
1997	0.342	0.378	0.393	0.369	0.311	0.397	0.378	0.345	0.320
2002	0.402	0.341	0.372	0.320	0.424	0.392	0.316	0.311	0.398
2003	0.401	0.396	0.353	0.474	0.303	0.398	0.302	0.406	0.401
2004	0.403	0.442	0.464	0.488	0.419	0.488	0.438	0.409	0.359

The total genotype diversity (H_T) was partitioned into different spatial components with a hierarchical genotype diversity analysis. Spatial scales are based on comparison among fields. among locations and among regions

Hierarchical genotype diversity analysis indicated that about 20%-30% of the total genotype diversity (H_T) was distributed between the regional populations and 30% among three temporal populations of *A.pinodes* and *P.pinodella*. The majority of genotypic diversity (70%-80%) was distributed within and between foci located in single field of pea (Tables 2, 3, 9 and 10). The identical VCG genotypes were found primarily within single focus and in less frequency between different foci. The average 2 to 5 identical VCG genotypes were found in different foci within single field. Genotypic population structure at the smallest spatial scale regarding the infection foci seem to belong to clonal population structure. These clonal foci subpopulations of *A.pinodes* had the lower H_T values than mixed population and H_T ranged from 0.217 to 0.223 and genotypic diversity of Stoddart-Taylor's () values varied from 21.2 to 27.4% of its maximal value. Shannon indices H' ranged from 0.731 to 0.798 (table 6). The clonal foci subpopulations of *P.pinodella* H_T values ranged from 0.270 to 0.299, Stoddart-Taylor's values ranged from 26.6 to 29.9% and Shannon indices H' ranged from 0.99 to 1.17 (Table 13).

Regarding foci subpopulations, genotype diversity values, and evenness values particularly E_5 value were greatly influenced by the presence few dominated VCG genotypes despite the occurrence also rare genotypes

within single field subpopulations. Regarding the single field *A.pinodes* subpopulations, evenness values E_5 were low and ranged from 0.431 to 0.493 (Table 6) despite their richness values were relatively moderate. The foci subpopulations of *P.pinodella* E_5 values ranged from 0.511 to 0.538 (Table 13). The three regional populations of *A.pinodes* and *P.pinodella* differed significantly (P=0.01) in both the VCGs genotype frequencies and their distribution on the spatial as well as on the temporal scales (Tables 5 and 12). The degree of genetic differentiation G_{ST} ranged from 0.064 to 0.199 for *A.pinodes* (Table 6) and G_{ST} values ranged from 0.058 to 0.141 for *P.pinodella* (Table 13). G_{ST} values usually correlated with geographical distance between locations or regions and temporal surveys. In contrast, the level of population differentiation between closely related fields in region was relatively low and G_{ST} values ranged from 0,043 to 0,068 (Tables 4 and 11). At the smallest spatial scale, genotypic population differentiation between foci within single field was low (G_{ST} ranged from 0.03 to 0.05) and genotypic identity (evenness) between foci was high indicating the low within single field genotypic sub structuring of population. Relatively moderate genotypic differentiation regarding the regional populations of *A.pinodes* or *P.pinodella* indicating the low degree of genotype migration among these three spatio-temporal populations.

Pair wise comparison of VCGs genotype diversities and their distribution between pea fields located in three distant regions of Poland were shown in Tables 4, 5, 11 and 12. Significantly differences (P=0.01) concerned genotype frequencies and their distribution were found in comparisons between Northern population and Central population and between first temporal population and third temporal population. Chi-square test and *t* test statistic values showed the significance (P=0.01) regarding the differences in genotypic diversities and their distribution across not only on the space scales but also on the temporal scales (Tables 5 and 12).

Analysis of variance demonstrated that differences in genotypes frequencies and their distribution between regions were significant (P=0.01). Based on analysis of variance it has been confirmed that about 60-75% of the total genotypic variations were distributed within single field particularly within single focus and rest between locations and regions (Tables 3 and 10).

In summary: the findings from the results on Polish genotypic structures of *A.pinodes* and *P.pinodella* taken from three regions and three temporal surveys in which there were 34 VCG genotypes of *A.pinodes* and 55 VCG genotypes of *P.pinodella* clearly indicated that these two fungal species possesses a mixed population structure, clonal and recombining.

On the average Central subpopulation possessed more rare or unique VCG genotypes found only within single field than Northern subpopulation. VCGs genotype frequencies and their distribution were different both within and between three spatio-temporal populations. Gener-

ally, third temporal subpopulation of *A.pinodes* and *P.pinodella*, which belonged to actually contemporal population showed relatively high degree of genotypic variability. It should be noticed that Stoddart-Taylors genotype diversity measure varied between 21.2% and 50.7% of its theoretical maximum. Pair wise genotype diversity values and genetic population subdivision G_{ST} values differed significantly among pairs of single field subpopulations, which were compared each other and when tested the null hypothesis that VCG genotypes were equally distributed (P=0.01). G_{ST} differentiation values were up to 0.196 between three regions and G_{ST} values were up to 0.199 between three temporal populations. The statistical values regarding the tested the null hypothesis such as chi-square value and *t* test were significant for the three comparing spatio-temporal populations (P=0.01). Hierarchical genotype diversity analysis showed that the total genotype diversity (H_T) across all spatio-temporal populations was up to H_T=0,413. Genotypic diversity between three regions contributed up to 30% of the total diversity. In contrast, the majority of the total genotypic diversity (70-80%) was distributed within single field. Of this value about 70% of VCG diversity was distributed within single focus of approximately 0.3-0.5 m^2 area in size.

Though the VCGs frequencies and their distribution were significantly different between regions and years, but G_{ST} values were low within single field or between closely fields within single region. The estimation of G_{ST} values suggested that the genotypes migration between the three Polish populations of *A.pinodes* and *P.pinodella* were restricted not only on the spatial scales but also on the temporal scales. Population differentiation on the temporal scales were relatively moderate. There were only significant differences regarding the genotype diversities and genotype distributions between the first temporal populations and third temporal populations. The similarity among populations on the regional scales, within single field or between closely related fields in single region suggest that genotypes migration occurred over the spatial scales of at least to 20-30 kilometers and over single time period e.g. over three consecutive years. This hypothesis was supported by the findings that genotypic population differentiation within single field and over single time survey was small. On the other hand, population differentiation between fields located in distant regions and among three temporal populations primarily between first subpopulations and third subpopulation were much higher.

DISCUSSION

In this study we were interested in measure of genotype diversity, richness, genetic similarity (evenness) and genetic population subdivision G_{ST} of Polish population of *A.pinodes* and *P.pinodella.* The special attention placed on the large collection of isolates of *A.pinodes* and *P.pinodella* sam-

pled in the hierarchical scheme from three distant regions of Poland. Population genotypic structures were analyzed based on the number of VCG genotypes, genotypic diversity, G_{ST} values and the proportion of genotypic diversity attributed to differences between all subpopulations. Interestingly, although the number of *vic* loci is still underestimated in *A.pinodes* or *P.pinodella* it is thought that similar to other ascomycetes, vegetative incompatibility system is controlled by multiple *vic* loci with two or more alleles at single *vic* locus. Because *vic* loci are unlinked in fungal genomes, therefore VCG genotype diversity correlates with multilocus genetic structure of fungal pathogens where molecular markers were used (Glass *et al.* 2000, Leslie 1993, 1996, Katan 1999, Saupe 2000). It was indicated that VCGs genotype diversities within Polish populations of *A.pinodes* and *P.pinodella* were relatively moderate across the spatio-temporal scales. On the average 29 different VCG genotypes were present within three spatial and three temporal populations of *A.pinodes* or *P.pinodella*. Interestingly it has been found that distribution of VCG correlated with pea fields. Within each single field subpopulation (infection foci) of *A.pinodes* or *P.pinodella* between 2-4 VCG genotypes were present across three spatial and temporal populations.

In some fields three or more genotypes were commonly collected from a single focus. In other fields only single genotype primarily of *A.pinodes* was obtained from each lesion per leaf or from a single focus. Clones within single field were distributed across the area of approximately 0.2-0.5 square meters (across single foci) and did not become widespread. This finding is consistent with the limited spread the asexual conidia of *A.pinodes* and *P.pinodella.*

It has been found that isolates of *A.pinodes* or *P.pinodella* that belonged to the same VCG genotypes usually were sampled from the same infection foci within a field or from single necrosis on the leaf or eventually from the single pycnidium. Changes in genotype diversity and differences in genotypes distribution on a spatio-temporal scale could be resulted due to selection of genotypes with higher fitness. It has been proposed that selection operates on the population instead on individual VCG genotype when sexual recombination occurs within local population of pathogen and when pathogen ongoing regular sexual cycle. Similar results have obtained Czembor and Arseniuk (1999) regarding the genetic variability between isolates of *Stagonospora spp* and *Septoria tritici* sampled from single pycnidia, Linde *et al.* (2002) also have obtained similar results regarding number of molecular haplotypes collected from single lesion of *Mycosphaerella graminicola*. This pattern of genotypes diversities and their distribution on the spatio scale has been confirmed by Milgroom and Cortesi (1999), Cortesi and Milgroom (1998) for *Cryphonectria parasitica* and Cortesi and Milgroom (2001) regarding *Eutypa lata*. Based on obtained results in this study I have drawn conclusions regarding the evolutionary

forces that affect Polish populations structure of *A.pinodes* and *P.pinodella*.

For three regions, the population structure of *A.pinodes* and *P.pinodella* was the results of both sexual and asexual reproduction. Asexual reproduction might have an important impact on area of few square centimeters and within the single field. Sexual reproduction probably had much greater consequences concerning the Polish populations structure were biology of *A.pinodes* or *P.pinodella*. It is already well known that genotypes were ephemeral and usual did not persist in population over time that has led to sub structuring of populations (Milgroom 1995, 1996, McDonald 1997, McDonald *et al.* 1996, Peever *et al.* 2004, Milgroom and Peever 2003, Zhan *et al.* 2000)

In this study by using vegetative compatibility test as genetic marker it was also able to confirm that: Polish regional populations of *A.pinodes* or *P.pinodella* were moderately variable and the majority of genotypic variations were distributed within the single field on the fine spatial scales of 0.30 – 0.4 m^2. Strains of *A.pinodes* or *P.pinodella* sampled from different fields, locations and from three distant regions of Poland showed also the moderate degree of genetic variability. Similar results have obtained Tivoli *et al.* (1996), Roger and Tivoli (1996) and Roger *et al.* (1999) regarding the genetic variability between *A.pinodes* and *P.pinodella* subpopulations and Peever *et al.* (2004) according to populations structure *Aschochyta rabiei.* By using molecular markers, several dominant together with rare molecular haplotypes were recovered from each regional population. It is proposed that the level of clonality and the number of rare genotypes within populations of *A.pinodes*, *P.pinodella* or *A.rabiei* could have originated from genetically diverse source populations caused by founder effect. Used the hierarchical sampling method of isolates proposed by McDonald (1997) and Milgroom (1996, 2001) it was possible to partition the total genetic variation (H_T) between the spatio-temporal populations. The total genotypic diversity could be also partitioned into three components between regions, between fields and between foci within single field. The majority of molecular haplotypes or VCG genotypes always were distributed within foci across the single field covering the area maximum of 0,5 m^2. Only the small portion of genotypic diversity was distributed between geographically distant populations. These results indicated on mixed strategies of reproduction *A.pinodes*, *P.pinodella* and *A.rabiei* and that primarily conidia and a less extence also the ascopores were primary source of infection and were responsible for the major differences in the distribution of genotypic diversity on the spatial scales. It is well known that conidia of *A.pinodes, P.pinodella* and *A.rabiei* were dispersed primarily by rain splash up to 30 cm. In such case it is more likely that genotype migration or gene flow between distant populations occurs through infected seeds and seedborne inoculum plays the important role in epidemics of *Ascochyba*

blight. On the other hand, ascospores were dispersed by wind across the few kilometers to adjacent fields and this has led to gene flow and genotype migration only on the local scales (Roger *et al.* 1999, Roger and Tivoli 1996)

Tivoli and his colleagues have found that *A.pinodes* and *P.pinodella* survived winter as mycelia together with pseudothecia and then undergo sexual reproduction in the left debris. The fruiting bodies of *Mycosphaerella pinodes* (pseudothecia) containing the viable ascospores were developed on senescence part of pea plants, just after beginning the flowering stage and next in post-harvest residue left on the fields or on volunteer seedlings. Tivoli and his coworkers proposed that ascospores were able to play an important role as the primary inoculum source for pea Ascochyta blight epidemic, particularly in these regions, where pea cultivars including winter cultivars were extensively grown on the large areas (Roger *et al.* 1999, Roger and Tivoli 1996).

The fact that the majority of the total genotypic diversity was distributed within fields suggests that *A.pinodes, P.pinodella* and *A.rabiei* populations have the potential to evolve relatively quickly. High level of genetic diversity and field populations of *A.pinodes*, *P.pinodella* or *A.rabiei* which are composed of many genotypes distributed on the small spatial scales offers greater evolutionary potential than the strictly clonal population structure in which few genotypes dominate and are widely distributed (Amos and Hardwood 1998, Anderson and Kohn 1995, Chen and McDonald 1996, Brygoo *et al.* 1998).

Though the three regional populations exhibited moderate VCGs genotype diversities, Central subpopulation had significantly greater number of VCG genotypes per field comparing to Northern subpopulation of *A.pinodes* and *P.pinodella*. In addition Central subpopulation of these pathogens had a more even distribution of VCG genotypes on the spatio-temporal scales. In summary: Northern subpopulations exhibited lower VCG genotype diversity than other two regional populations. Northern subpopulations of *A.pinodes* and *P.pinodella* usually were composed of few dominant VCG genotypes comparing to Central subpopulations of *A.pinodes* and *P.pinodella.* At genetic point these findings were typical for clonal populations structure, restricted recombination in population and for pathogens that were introduced recently and likely reflects the founder effect. These finding suggests also that *A.pinodes* (*Mycospaherella pinodes*) or *P.pinodella* (teleomorph *Didymella*) have not undergone regular sexual reproduction under all fields in Poland and in the period of all years. Similar pattern the genotypic diversity was observed for three temporal populations of *A.pinodes* and *P.pinodella*. First temporal populations of *A.pinodes* and *P.pinodella* also indicated the lower VCG genotype diversity compare to third temporal population. When first temporal populations compared to third temporal populations, the statistic values ^{2} and t were significant at P=0.01. First temporal populations of *A.pinodes* and *P.pinodella* seem to be

clonal in structure. In contrast, third temporal populations primarily Central subpopulation of these fungi seems to be mixed population in structure clonal and recombining.

Based on G_{ST} values for genetic subdivision of populations it has been indicated that the distribution of genotypic diversity across three distant regions of Poland was probably consisted with the low level of gene flow and genotype migration. Common VCG genotypes between distant locations in region usually were not observed as well as between three regions. VCGs genotype distribution often were not quite similar within single region even between fields separated for example by one hundred of kilometers. This was the consequence that conidia were able to disperse only up to 30-40 m^2 and ascopores of *Mycosphaerella pinodes* were able to disperse only across the few kilometers (Onfroy *et al.* 1999, 2006, Roger and Tivoli 1996, Roger *et al.* 1999). This is in contrast to the results obtained by McDonald research group concerning genetic population structure of *Mycosphaerella graminicola, Sphaeospaeria nodorum* and *Rhynchosporium secalis* (McDonald 1997, Chen and McDonald 1996, Salamati *et al.* 2000). The primary sources of the infection of these cereal pathogens were ascospores, which were able to disperse across of hundred kilometers. This leads to absence the genetic subdivision even of transcontinental populations. In addition, the estimation of G_{ST} values were very low and did not significantly between different populations (Zhan *et al.* 2000, 2001). Polish populations of *A.pinodes* and *P.pinodella* exhibited moderate genetic population differentiation based on G_{ST} values. The estimation of G_{ST} values concerning genotypic subdivision of three spatio-temporal Polish populations reflects historic movement of *A.pinodes* and *P.pinodella* through Poland at least between Northern subpopulations and Central subpopulations. It is possible that gene flow and genotype migration is not significant evolutionary force regarding these pathogens at present or in the past.

The mechanism of genotype migration could be infected seeds, windblown straw or ascospores. Nevertheless the significant fraction of the total genotypic diversity was found within single plot called also as infection focus. McDonald *et al.* (1996) and Chen and McDonald (1996) in studies on genetic diversities of *M.graminicola* and *P.nodorum,* next Salamati *et al.* (2000) in similar studies regarding *Rhynchosporium secalis* and Peever *et al.* (2004) in studies on *A.rabiei* population structures have stated that pathogen populations exhibited the genetic substructuring where high percentage of the genetic variability was distributed on the small spatial scales might adapt more quickly to changes in the environment including resistant cultivars, fungicide application or other agricultural practices. Results obtained by these researchers are similar to the results obtained in this study.

Alternatively, subdivision of populations could be caused by differences in selection of *vic* genes in different populations but this mechanism of se-

lection is not known regarding *Ascochyta blight* pathogens of lequminouse. The role of selection on *vic* alleles and the consequence of this selection regarding population polymorphism has been studied in detailed primarily in *Neurospora crassa, Podospora anserina* and *Cyphonectria parasitica* (Glass *et al.* 2000, Saupe 2000, Milgroom and Cortesi 1999, Nauta and Hoekstra 1994, 1996, Cortesi and Milgroom 1998, 2001, Cortesi *et al.* 1996).

It has been proposed that significant subdivision of populations is also caused by founder effects and this subdivision has persisted due to restricted *vic* gene flow (Milgroom and Cortesi 1999). In theory, if *vic* genes are under selection, selection may cause gametic disequilibrium among *vic* genes if some VCG genotypes within Polish population of *A.pinodes* or *P.pinodella* have greater fitness than others. Genetic population structure of *A.pinodes* is also depended on homothallic nature of this fungus. Sexual structures of *Mycosphaerella pinodes* (pseudothecia) developed on senescence plants of pea or in the left deribs after harvest. Developed of pseudothecia in homothallic *Mycospaherella pinodes* results from self-fertilization and self-fertilization always produce progeny genetically identical compare to their parents. Nevertheless *Mycosphaerella pinodes* similar as homothallic species *Fusarium graminearum* (*Gibberella zeae*) under field conditions is able to outcrossing that leads to greater genotypic diversity (Onfroy *et al.* 1999, Bowden and Leslie 1999, Zeller *et al.* 2003, Roger and Tivoli 1996). Both asexual reproduction via conidia and selfing via ascospores could contribute to deviations from random mating and leads to clonal population structure. Regarding to *P.pinodella* (teleomorph *Didymella*) with still unobserved sexual structures on senescence pea plants or in the left debris and in laboratory sexual crosses, clonal population structure could be the results the dominance only one mating type within some local populations. Lack of second mating type usually female idiomorph *Mat-1-2* appears to prevent sexual reproduction in local populations (Leslie and Klein 1996).

The sources of genetic variation within local populations of fungi have been recently widely discussed. It has been found that populations of some pathogenic fungi revealed high and often unexpected picture of their spread and continuing evolution. At the microevolutionary level this had included the hidden spread of new pathogen genotypes with better adaptation to local environments and with higher levels of fitness.

Establishing links between fitness trait and population structure are critical for interpreting the changes in genetic population structure of *A.pinodes* and *P.pinodella* over the spatial or the temporal scales.

McDonald (1997), McDonald and Linde (2002), Milgroom (1996), Milgroom and Peever (2003) have proposed the following possible explanations for the spatio-temporal genotypic differences within populations of fungal species. The first possibility is the adaptation to the local environ-

ment climate, different regional agricultural practices and growing the different cultivars with different resistance level. This leads to genotypic differentiation and to substructuring populations of fungal species. Selection of virulence genes and selection of genotypes with higher fitness within local populations leads to adaptation to local environments and promote genotypic differences and subdivision of populations on the spatial and on the temporal scales. Generally selection within local populations primarily operate on genotypes best adapted to environment with high fitness value or pathotypes with greater level of virulence and causes populations of fungal species to differentiation on the spatio-temporal scales (Brown 2000, Amos and Harwood 1998, Anderson *et al.* 1992, Anderson and Kohn 1995, Nunney 1995, 1999, Brygoo *et al.* 1998, Bridge *et al.* 1999, Milgroom and Peever 2003, Kosman 1996).

The pattern of Polish genotypic population structure of *A.pinodes* and *P.pinodella* could be the results the interaction between several evolutionary forces such as mutation, gene flow, genotype migration, strategies of reproduction, heterothallic or homothallic mating system, genetic drift and at least selection. In order to understand the evolutionary forces that shaped structure of Polish *A.pinodes* and *P.pinodella* populations and infer the importance of the individual evolutionary factors detailed there is needed further study using both primarily AFLP-PCR and microsatellite molecular markers as it has been done recently in several laboratories around the world, regarding the populations structure of *A.pinodes, A.rabiei, A.lentis* and *A.fabae* (Peever *et al.* 2002, 2004, Rhaiem *et al.* 2006, Banniza and Vanderberg 2006, Zhang *et al.* 2003, 2006, Faris-Mokaiesh *et al.* 1996, Fatehi 2000, Wang *et al.* 2000, Onfroy *et al.* 1999, Wroth 1998, Xue *et al.* 1996, 1998, Tohamy and Mohamed 1998, Kaiser 1997).

The extent of genetic differentiation among subpopulations observed in this study was similar to other plant pathogens such as *Cryphonectria parasitica* subpopulations (Cortesi *et al.* 1996, Milgroom and Lipari 1995), *A.rabiei* populations (Armstrong *et al.* 2001, Peever *et al.* 2004), *A.lentis* populations (Banniza and Vandenberg 2006) and *Aphanomyces euteiches* populations (Malvick and Percich 1998). Obtained results are not similar to some ascomycete plant pathogens with similar biology to heterothallic species *P.pinodella* (teleomorph *Didymella*). For example *Mycosphaerella graminicola, Phaeosphaeria nodorum, Rhynchosporium secalis* and *Tapesia yallundae* or *T.accuformis* also have mixed populations structure clonal and recombining but exhibit little differentiation of populations (G_{ST} < 0.05). It has been found the low G_{ST} values even between transcontinental populations (Douhan *et al.* 2002, 2003, Dyer *et al.* 2001, Salamati *et al.* 2000, Zhan *et al.* 2001). It is still debated why some fungal species exhibit significantly greater differentiation among subpopulations. It has been suggested that this is due to greater restriction of genotype migration and gene flow and/or the larger effect of genetic drift within subpopulations, which

might be related to effective population sizes (Zhan *et al.* 2000, McDonald *et al.* 1996, Milgroom and Peever 2003, Nunney 1995, 1999, Leslie and Klein 1996). Further study is required to determine the causes of these different patterns of population structure among plant-pathogenic fungi with similar biology such as *P.pinodella, A.rabiei* and *M.graminicola*.

The possibility the occurrence of restricted gene flow among geographically distant populations, which leads to populations subdivision need supported in future. This problem may be only resolved using molecular markers as did McDonald and Milgroom and their research groups (Linde *et al.* 2002, McDonald *et al.* 1996, Milgroom 1995, 1996, Salamati *et al.* 2000, Zhan *et al.* 2000). Restricted gene flow among geographically distant populations allows random fixation of different neutral alleles due to action of genetic drift. Under a model in which gene flow decrease with increasing distance, genetic isolation by distance and the significant negative correlation between of *Nm* and of the distance should be expected. This possibility can be tested with additional molecular markers. Also the degree of gametic equilibrium should be studied in future as an indirect measure of the significance of genetic exchange in recombination in the presumably outcrossing (mixed) population of *A.pinodes* and *P.pinodella.*

Studies on populations genetic of *Ascochyta* / *Phoma* complex species sampled from leguminose crops around the world are being conducted recently in order to reveal the primary sources of infection inoculum, the extent of genotype migration and gene flow among populations mode of reproduction during the epidemics and concern to breeding programme in order to find lines with partial resistance to ascochyta blight. These studies include also mapping the genome of host or the genome of pathogens in order to find QTLs loci conferring resistance or conferring aggressiveness or virulence and toxin synthesis (Barrange *et al.* 2006, Bretag *et al.* 1995, Cubero *et al.* 2005, Chen *et al.* 2004, Coyne *et al.* 2000, 1999, Cho *et al.* 2004, Fondevilla *et al.* 2006, Ford *et al.* 1999, Furgał-Węgrzycka 1984, 1990, 1991, 1992, Kraft *et al.* 1998, Kraft and Pfleger 2001, Marcinkowska 1996, 1998, Marcinkowska and Witkowska 1996, Pilet-Nayel *et al.* 2002, Muehlbauer and Chen 2006, Santra *et al.* 2000, 2001, Ratnaparkhe *et al.* 1998, Prioul *et al.* 2004, Taylor and Ford 2006, Prioul *et al.* 2004, Taylor and Ford 2006, Timmerman – Vaughan *et al.* 2000, 2002, Tivoli *et al.* 2006, Wroth 1999, Xue and Warkentin 2001).

Recently another aspect of research on biology of *A.pisi, A.pinodes* and *P.pinodella* was used based on phylogenetic analyses in order to determine the evolutionary relationship among isolates sampled from geographically distant regions. Understanding the mechanism of speciation of *A.pisi, A.pinodes* and *P.pinodella* strains towards to pea as the host would indicate the new approaches for developing improved disease management (Lubeck *et al.* 1998, Peever *et al.* 2004, Barve *et al.* 2003, Fatehi 2000, Fatehi and Bridge 1998).

Faris-Mokaiesh *et al.* (1996), Bouzand *et al.* (1995), Fatehi and Bridge (1998), Fatehi 2000, Onfroy *et al.* (1999), Lubeck *et al.* (1998), Tohamy and Mohamed (1998), Peveer *et al.* (2004) have used molecular markers in studies according the genetic variation within and between populations and on phylogenetic relationship between the three related fungal species commonly referred as the Ascochyta complex of pea. No sequence variation was found within the internal transcribed spacer (ITS1) region of rDNA of *A.pinodes* and *P.pinodella*. In further study the RAPD-PCR marker has been used to examine the existence the genetic variation within and between populations of *A.pinodes* and *P.pinodella*. Onfroy *et al.* (1999), Bouznad *et al.* (1995, 1996) and Faris-Mokaiesh *et al.* (1996) have indicated that although isolates of *A.pinodes* and *P.pinodella* showed genetic differences but there were not strongly clustering of haplotypes in UPGMA analysis of RAPDs and ITS/RFLPs. It has been proposed that the high genetic similarity at molecular level between isolates of *A.pinodes* and *P.pinodella* reflected the small founder effect and that both clonal propagation and recombination was important for the populations structure of these pathogenes. The results from molecular analysis imply the high degree a genetic conservation at the rDNA tandem repeat loci. The conserved status the ITS region of *A.pinodes* and *P.pinodella* is consistent with findings for several other plant pathogenic fungi sampled from leguminose. The absence of genetic polymorphism within the rDNA ITS fragments of *A.pinodes* and *P.pinodella* isolates sampled from different geographically distant regions suggested that these two fungi might have the relatively recent common ancestor and could provide a key to their host specificity (Peever *et al.* 2004, Lubeck *et al.* 1998, Fatehi 2000, Fatehi and Bridge 1998, Onfroy *et al.* 1999).

It is worth noticed that the traditional approach for determining the mating type and mating allele distribution within heterothallic *P.pinodella* (teleomorph *Didymella*) and *A.pisi* (teleomorph *Didymella*) populations or *A.fabae* (*Didymella*), *A.lentis* (*Didymella*) and *A.rabiei* (*Didymella*) populations is to attempt to cross of field isolates with each of two tester isolates which are already known to differ at the mating type locus (e.g. with *Mat-1-1* as male tester or with *Mat-1-2* as female tester). This is a time-consuming assay because sexual crosses in many heterothallic fungi take 4 to 8 weeks to complete. It also relies on finding the suitable and sexual fertile tester isolates, which could be unavailable for *P.pinodella* (*Didymella*) or *A.pisi* (*Didymella*) that have not been yet successfully crossed. For *P.pinodella* (*Didymella*) and *A.pisi* (*Didymella*) finding the compatible pairs of opposite mating types can be challenging. All tested isolates of *P.pinodella* or *A.pisi* in this case must be intercrossed, and the number of crosses that must be made in laboratory increases as the square of the number of isolates being tested. Furthermore, the likelihood of identifying sexually compatible pairs in many heterothallic species is reduced by the high

proportion of field isolates that are either female sterile or completely sterile and unable to mate with either tester isolate. This is a reason that actually searches for *Mat-1-1* and *Mat-1-2* testers of *P.pinodella* (*Didymella*) and *A.pisi* (*Didymella*) have relied upon PCR amplification of the *Mat-1-1* and *Mat-1-2* idiomorphs. Such kind of studies have undertaken recently in laboratory of Peever (Barve *et al.* 2003).

Similar to other species of ascomycetes the mating type is assessed with two primers set amplifying the diagnostic *alpha* and *HMG* box of the *Mat-1-1* and *Mat-1-2* genes respectively. By using PCR primers designated for *Mat* genes it has been shown for the first time that *Mat* type genes structure and their organization in *A. rabiei* (*Didymella*), *A.pinodes* (*Mycosphaerella pinodes*), *P.pinodella* (*Didymella*) or *A.pisi* (*Didymella*) belong to conserved and box sequence or *HMG-DNA* sequence cosegrate with mating type *Mat-1-1* or *Mat-1-2* in progeny obtained from traditional sexual crossing under laboratory conditions (Barve *et al.* 2003, Peever *et al.* 2004).

It should be noticed that the first step is to find female and male genotypes in heterothallic populations of *P.pinodella* (*Didymella*) or *A.pisi* (*Didymella*) and to confirm that *A.pinodes* (*Mycosphaerella pinodes*) is a homothallic species, primarily on molecular data by PCR amplification with mating type primers PCR. Peever (2002) and Barve *et al.* (2003) in an attempt to determine the mating type isolates of *A.pinodes, A.pisi* and *P.pinodella* primers *CHHMG-1* and *CHMG-2* have synthesized commercially and have used in PCR analyses. These primers amplified the high mobility domain (*HMG*) of mating type genes (*Mat-1-2*) in *A.pinodes, A.pisi* and *P.pinodella*. Barve *et al.* (2003) and Peever (2002) also have constructed primers, which could be used to prime PCR reactions that amplify *Mat-1-1* idiomorph. These results have opened the door in further study regarding to construct the phylograms generated from partial *Mat-1-1* and *Mat-1-2* DNA sequence data for *Didymella pinodes, Didymella pisi* and for *Didymella pinodella* populations. In further study Barve at al (2003) have confirmed that teleomorph of *A.pinodes* belong to genus of *Didymella* instead to the monophyletic genus of *Mycosphaerella sp.*

It has been expected that by direct sequencing of *Mat* locus in genome of *A.pinodes, A.pisi* and *P.pinodella* will be able to asses the mating potential of heterothallic *A.pisi* (*Didymella*), *P.pinodella* (*Didymella*) and homothallic *A.pinodes* (*Didymella pinodes*) and will be able to indicate for the potential to undergo of sexual reproduction under field conditions. It is worth noticed that *Mat* genes regarding the *Didymella* species complex could evolve at different rates and modes of reproduction under field condition could be quite different.

Peever and his colleagues have tested the function of *Mat* genes from *P.pinodella* (*Didymella*) by expressing them in *Mat* deletion strains of strictly sexual species of *A.rabiei* (*Didymella rabiei*). The null hypothesis is

that if *A.pinodella Mat* genes are able to function in *A.rabiei* this is evidence of ability to outcrossing under field condition (Barve *et al.* 2003).

Barve *et al.* (2003) in phylogenetic analyses based on ITS sequence data have revealed that *P.pinodella* and *A.pinodes* are very closely related. On the other hand *A,pinodes* could not belong to genus *Mycospherella* due to phylogenetic distance to other *Mycospherella* species like *M.graminicola*, *M.citri* and *M.fijiensis*. It is worth noticed that Bouznad *et al.* (1995, 1996), Onfroy *et al.* (1999), Fatehi and Bridge (1998), Fatehi 2000, Faris and Mokaiesh (1996) and Lubeck *et al.* (1998) also have indicated that *P.pinodella* and *A.pinodes* are very closely related and no intraspecific genetic variation of ITS sequence data have found. These two species could be clearly distinguished by RAPD-PCR molecular marker or by other molecular markers. On the other hand, *A.pisi* was not closely related to *A.pinodes* and *P.pinodella*. It is proposed that intraspecific variation of ITS sequence for *Ascochyta* species from leguminose in the *Didymella* genus averaged by minimum 1,0 nucleotides (nts) in the ITS region. Thus isolates with ITS sequences that differ by two or more nucleotides may belong to distinct species. In phylogenetic studies *A.pinodes* and *P.pinodella* belong to two distinct groups (subclade in the main *Didymella* clade) and could differed for example by 3-5 nts (Fatehi and Bridge 1998, Fatehi 2000).

It is worth noticed that studies according to the phylogeny of legume-associated *Ascochyta / Phoma* complex species in conjunction with detailed studies of the genetic control of sexual cycle would provide a better understanding the evolution, phylogenetic relationships between different species, speciation, and the genetics of host specificity towards different species of leguminose. In studies regarding the phylogenetically relationship between different species *Ascochyta* or *Phoma MAT* genes have been proposed as potentially useful regions of the genome for phylogenetic tree reconstruction. *MAT* genes evolve more quickly than other regions of the genome and are highly conserved within species thus belong to useful for phylogenetic analysis of closely related species.

In addition in studies on phylogenetic relationships between different species of *Ascochyta / Phoma* complex beside *Mat* locus sequence as molecular genetic marker also nuclear ribosomal ITS have been used. It has been indicated that in phylogenetic studies according to relationship between legume-associated *Ascochyta* and related Loculoascocmycete fungal species by using the sequence data from the nuclear ribosomal ITS clearly could be shown for monophyly of *Ascochyta / Didymella* complex species sampled from legume hosts but this molecular marker was insufficiently variable to differentiate of isolates obtained from different legume hosts. In contrast used the sequences of the HMG region of *MAT-1-2* as molecular marker significantly more variable results were obtained. In such case by using the HMG region of *MAT-1-2* as molecular marker fungal species sampled from leguminose crops belonged to seven clades.

In phylogenetic studies where of ITS and HMG have been used as genetic markers isolates *Ascochyta / Phoma* species sampled from particular legume hosts had identical ITS sequences. Estimation of the ITS phylogeny among *Didymella, Ascochyta, Phoma,* and *Mycosphaerella* spp. sampled from various legume hosts indicated two distinct clades. One clade contained *Mycosphaerella* spp. including *M. graminicola* and second clade contained *Ascochyta, Didymella* and *Phoma* from legumes. No significant differentiation was found among isolates sampled from legumes. In addition *A. pinodes* had identical ITS sequence to *P. pinodella.* In contrast and by comparison the HMG based phylogeny revealed significant more variation among the legume-associated *Ascochyta / Phoma* complex species than did the ITS based phylogeny. Based on HMG data, it has been found seven distinct clades that correlated to both host and to morphological and biological species. The exception was clade 4, which contained three isolates of *A. lentis* from lentil *(Lens culinaris)* and two isolates of an unknown *Ascochyta* sp. from hairy vetch *(Vicia villosa),* all of which had identical HMG sequence. In contrast to the ITS phylogeny, *A. pinodes* and *P. pinodella* in the HMG based phylogeny belonged to distinct clade which contained isolates of *A. pinodes* (clade 1) and *P. pinodella* (clade 2)*,* respectively sampled only from pea. Clade 3 contained isolates of an unknown *Ascochyta* species from alfalfa *(Medicago sa-tiva),* clade 4 contained *A. lentis* isolates from lentil and an unknown isolates of *Ascochyta* sp. obtained from hairy vetch and clade 5 contained *A. pisi* isolates sampled only from pea. clade 6 contained two *A. fabae* isolates from faba bean *(V. fabae)* and clade 7 contained two *A. rabiei* isolates from chickpea. Interestingly no sequence divergence was found among isolates sampled from the same host even though they were obtained from diverse geographic locations. The exception was only isolates of *A.pinodes, A.pinodella* and *A.pisi* where sequence divergence has been shown.

Interestingly it has been confirmed by the ITS phylogeny analysis which indicated that *Mycosphaerella* complex species were distantly related to *C. heterostrophus, A. alternata,* and *A. rabiei.* Barve *et al.* (2003) have shown that *A.pinodes* belong to *Didymella* species and in phylogenetic studies was distantly related to *M. graminicola* and to other *Mycosphaerella* complex species. Phylogeny studies based on ITS in this case clearly indicating for a monophyletic *Ascochyta / Didymella* clade that included all the legume-associated *Ascochyta / Phoma* species. The ITS data was not sufficiently variable to allow discrimination among any of the *Ascochyta / Phoma* species sampled from different leguminose hosts within this clade. In contrast, the HMG based phylogeny was considerably more variable and delimited clades that were highly correlated to the host and to morphological and biological species. It has been proposed that although ITS has preferred region of the genome for using according to fungal molecular systematics to date, other regions of the genome such as *MAT* loci

may provide much better resolution among closely related taxa. In this case Barve *et al.* (2003) have confirmed that the *Ascochyta / Phoma* complex sampled from pea are phylogenetically diverse and that *A. pinodes* and *P. medicaginis* are closely related to each other but distantly related to *A. pisi.* Based on ITS phylogeny analysis it has been proposed that *A. pinodes* from pea do not belong to *Mycosphaerella* but to *Didymella* genus. It is worth noted that similar results as obtained Peever and colleagueas also obtained Faris-Mokaiesh *et al.* (1996), Fatehi (2000), Bridge and Fatehi (1998) and Onfroy *et al.* (1999).

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