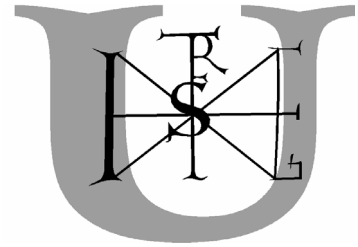

SZENT ISTVÁN UNIVERSITY

INTRA- AND INTERSPECIFIC VARIABILITY OF BARLEY-PATHOGENIC *PYRENOPHORA* SPECIES

PhD Theses

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1. BACKGROUND AND OBJECTIVES

Barley (*Hordeum vulgare* L.) is a very valuable and important world-wide grown cereal. Based on the growing area, barley is in the fourth place among cereals in the world, while third in Hungary. World barley production was 141.16 million tons, and it was harvested from 49.2 million hectares in 2014/15 (USDA data). Hungary produced 322 thousand tons of spring barley harvested from 82 thousand hectares and 957 thousand tons of winter barley from 205 thousand hectares in 2014. Barley is valuable forage, important raw material for malt and beer-making, and also suitable for human consumption.

Barley production is influenced by lots of diseases (MATHRE 1997). In Hungary the most considerable ones are – depending on the growing season and the varieties produced –yellow dwarf caused by viruses, powdery mildew, leaf rust, leaf blotches caused by *Pyrenophora* spp. and *Fusarium* ear blight. However, the frequencies of scald and *Ramularia* leaf spot have also been increasing (MANNINGER and MURÁNYI 2009, TOMCSÁNYI et al. 2006).
The pyrenophora leaf spot diseases of barely are caused by ascomycete fungi belonging to the genus *Pyrenophora* Fr. (imperfect stage: *Drechslera* Ito) and placed in the genus *Helminthosporium* formerly. Although the occurrence of more than a dozen *Pyrenophora*/*Drechslera* spp. was reported on *Hordeum* species worldwide (SIVANESAN 1987), the leaf stripe pathogen *P. graminea* Ito et Kurib. (anamorph: *D. graminea* (Rabenh. ex Schlecht.) Shoem.) and the net blotch pathogen *P. teres* Drechs. (anamorph: *D. teres* (Sacc.) Shoem.) are the most frequent ones on barley. These two pathogens are abundant in the world and in Hungary, too. Yield loss caused by these pathogens is generally 20-30 %, but might be even higher (KHAN 1987, STEFFENSON et al. 1991, PALÁGYI and TOMCSÁNYI 2006, TOMCSÁNYI et al. 2006). SMEDEGÅRD-PETERSEN (1971) sorted *P. teres* isolates into two sub-specific groups based on their symptoms. The isolates causing net-like longitudinal and

transverse necrotic lines are named *P. teres* Drechs. f. *teres* Smed.-Pet. (PTT), while the isolates causing spot-type necrotic blotches are named *P. teres* Drechs. f. *maculata* Smed.-Pet. (PTM).

P. graminea and the two forms of *P. teres* are very similar both morphologically and genetically, their accurate phylogenic relationship is not clarified. In traditional way, they can only be separated from each other accurately based on the typical disease symptoms. Recent research results suggest that their current, morphology-based classification may not be in agreement with their true phylogenetic relationship. The first doubts about the classification of *P. graminea* and *P. teres* arose when stable and fertile interspecific hybrids were created between them in laboratory crossing, indicating a tight genetic relationship (SMEDEGÅRD-PETERSEN 1983). In addition, it was not possible to separate the barley pathogenic *Pyrenophora* species from each other unambiguously based on nuclear ribosomal DNA ITS sequence analysis (STEVENS et al. 1998), which is often used as a marker region for differentiation at species level.

The pathogens try to adapt to changes in environmental conditions and host plant population continuously to survive. The bigger the genetic variability of a pathogen is, the higher potential it has to adapt, e.g. the new pathotypes and more aggressive and/or fungicide-resistant strains may develop in the population. Therefore it is important to study the genetic variability and population structure of pathogens present in given geographical regions. Both *P. graminea* and *P. teres* are capable of reproducing both sexually and asexually, therefore the frequency and ratio of the sexual and asexual reproduction have a great effect on the population genetic structure of these fungi (LIU et al. 2011). Being heterothallic fungi, the sexual cycle, which is the major source of the genetic variability in general, is initiated only when both fungal strains (MAT1 and MAT2) of the different mating types interact. To estimate these pathogens' adaptive ability to change, we must study their population genetic structure and

the occurrence and frequency of the two mating types. This may help to understand how the population evolve and protect against plant pathogens (SOMMERHALDER et al. 2006). In Hungary, there have no been such data available about these pathogens.

Our aims were as follows:

- Identify *Pyrenophora graminea* and the two forms of *P. teres* and their mating types, as well as to survey their distribution and frequency on barley in different growing areas in Hungary.
- Study the genetic variability and population structure of *P. teres* f. *teres*, the causal agent of net type of net blotch in Hungary.
- Study the phylogenetic relationship between *P. graminea* and *P. teres* by multigene sequence analysis.

2. MATERIAL AND METHODS

2.1.Isolate collection

Altogether, 157 leaf samples of winter barley, spring barley and winter wheat with typical, atypical or unknown symptoms were collected countrywide (western, central, south-eastern and north-eastern region) from 2006 to 2010, mainly with the help of the county Government Office. Additionally, we received 51 isolates collected from winter wheat by Dr. Mária Csősz. Leaf samples were stored in paper bags in the laboratory at room temperature until they were processed. Single-conidial specimens were made on potato-dextrose agar plates from the conidia formed on the leaves in moist chamber, using a dissecting microscope. Stock cultures were kept on PDA slants under sterile mineral oil at 15°C.

2.2.Morphological and pathogenicity study of isolates from atypical leaf symptom

We selected four isolates collected in Kiszombor, Röjtökmuzsaj, Szeged-kecskéstelep and Táplánszentkereszt from leaves showing unconventional necrotic symptoms of presumably pyrenophora infection. Two strains of *P. teres* f. *teres*, identified by us previously using molecular methods, were included as control in these laboratory tests.

We tested the culture morphology and growth rate of our isolates on potato dextrose agar (both home-made and commercial (Nebotrade, Budapest)), corn meal agar (Difco), malt extract agar (Sigma-Aldrich, USA) and clarified V8-juice agar (ERWIN and RIBEIRO 1996) in 9 cm Petri dishes (20 ml medium/dish) at 22°C.

To verify Koch-postulates, we sprayed leaves of the six-row barley cv. Botond at 2–4 leaf stage with mycelial suspension (5 plants/pot, 2 pots/isolate). Control plants were sprayed with water. After the infection plants were kept in

100% relative humidity at 20°C for 24 hours, than in 70% relative humidity at 24°C/20°C light/dark cycles for three weeks. After that the strains were re-isolated and identified by using morphological and molecular genetics method. The shape and size parameters of 25 conidia per isolates were recorded under microscope at ×200–400 magnification. Statistical analyses of our data were carried out with T-test and one-way variance analysis.

2.3. Molecular genetic methods

DNA extraction

Total genomic DNA were extracted from lyophilized mycelium powder with three different methods. We applied the CTAB-based method (AUSUBEL et al. 1994) with slight modifications, the faster and modern MasterPure^{TM} Yeast DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA) and the NucleoSpin Plant II Genomic DNA Purification Kit (Macherey-Nagel GBMH & Co. KG, Düren, Germany) according to the manufacturers' instructions.

Identification of pathogens with specific PCRs

All isolates were tested with three primer-pairs developed for specific identification of *P. teres* f. *teres*, *P. teres* f. *maculata* and *P. graminea*. Total volume of the PCR-mix was 25 µl (50 ng DNA, 1.5 mM or 2.2 nM $MgCl_2$, 0.2 mM or 0.25 mM dNTP, 0.4 µM primer, 1 U Taq DNA polymerase, 1× Taq puffer). DNA amplification with each primer-pair was carried out in different reaction tubes according to WILLIAMS et al. (2001) and TAYLOR et al. (2002) with the use of Bio-Rad C1000 thermocycler (Table 1). Control reactions contained sterile Milli-Q water instead of DNA. Amplified PCR products were stained with ethidium bromide and visualized under UV light.

Identification of mating types with specific PCR

The frequency of MAT1 and MAT2 isolates was examined by using the mating type-specific primer pair and PCR procedure (Table 1) developed by RAU et al. (2005). Out of the 20 µl PCR products (1× puffer /20nM Tris HCl (pH 8.4), 50 nM KCl/, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 4 pmol per primer, 1 U Taq polymerase enzyme, 20 ng DNA), 5 µl per sample were subjected to electrophoresis in 1.2 % agarose gels (Gibco), and photographed under UV light, based on existing or lack of the product.

RAPD analysis of *Pyrenophora teres* f. *teres*

We used 30 primers (Operon Technologies Inc., Alameda, USA) for the RAPD analysis (OPB06-OPB12; OPH09-OPE07; OPA14-OPE03; OPB07-OPB10; OPW03-OPA07; OPW07-OPW17; OPH20-OPH12; OPE20-OPE17; OPB11-OPB09; OPB16-OPC13; OPG04-OPB01; OPE15-OPC16; OPH03-OPE16; OPE01-OPA02; OPI12-OPE19). The PCR-mix was 20 µl (10 µl Dream Taq™ PCR Master Mix, 2-2 µl primer, 4 µl sterile distillated water and 2 µl DNA). The DNA replication was performed in Eppendorf Mastercycler® 96-well PCR machine (pre-denaturation: 94°C – 2 min, 45 cycle (denaturation: 94°C – 1 min; annealing: 35°C – 1 min; elongation: 72 °C – 2 min) post polimerization: 72°C – 5 min), then the product was visualized under UV light after electrophoresis. All amplification was repeated three times.

Presence and absence of RAPD bands were scored as 1 and 0, respectively. Data were collected into a binary matrix and were used to calculate pair wise genetic distances between isolates based on Nei and Li's coefficient (NEI and LI 1979). Hierarchical clustering of individual isolates was carried out by employing the UPGMA method (Unweighted Pair-Group Method with Arithmetic mean) (SNEATH and SOKAL 1973), and we made a dendrogram showing the genetic distance between isolates. TREECON version 1.3b (VAN DE PEER and DE WACHTER 1994) was used for these computations.

To assess the genetic polymorphism and measure the differentiation within and between subgroups of isolates we counted the frequency of polymorphic loci and Nei's gene diversity (NEI 1973, 1987). The statistical reliability of Nei' gene diversity was studied by value of G^2 which was compared to tabulated values of X^2 (chi-square). To measure the genotypic diversity we used the Shannon's index and Simpson's index.

Phylogenetic relationship among barely-pathogenic *Pyrenophora* species

The isolates included in our phylogenetic study originated from various continents and countries. In total 13 *P. graminea*, 13 *P. teres* f. *maculata* and 15 *P. teres* f. *teres* strains were studied. Isolate WAC11137 of the closely related species *Pyrenophora tritici-repentis* was chosen as outgroup.

Four nuclear DNA regions, suitable for testing phylogenetic relationship of other fungi, were chosen for our phylogenetic studies. DNA amplifications of rDNA ITS, and segments of ß-tubulin, actin and glicerinaldehyde-3-phosphate-dehydrogenase genes were carried out in 50 µl PCR mix according to WHITE et al. (1990), GLASS and DONALDSON (1995), VOIGT et al. (2005) and BERBEE et al. (1999), respectively, using an Eppendorf Mastercycler®. For the electrophoresis 8 µl PCR product was used in 1 % agarose gel (1×TBE buffer, 200 V). DNA fragments were stained with GelStar and visualized in UV light. Amplicons were purified and sequenced in both directions with Sanger-sequencing by Macrogen Europe (Amsterdam, the Netherlands). Electropherograms were quality checked, then forward and reverse reads were compiled and repaired manually using the Staden Program Package (STADEN et al. 2000).

Sequences of each locus were aligned using the online MAFFT version 7 (http://mafft.cbrc.jp/alignment/server/) (KATOH and STANDLEY 2013) using the default settings, then we made a slight manual correction in the ITS alignment. From the aligned sequences of the four genome regions, a 2375

nucleotide long multigene dataset was made. Maximum Likelihood (ML) and Bayesian phylogenetic analysis were carried out based on previously selected best-fit-models for each gene partition. For ML phylogeny we used IQ-TREE 1.3.4 (NGUYEN et al. 2015). Bootstrap analysis (FELSENSTEIN 1985) with 1000 replicates was used to test the support of the branches. The Bayesian analyses were computed using MrBayes v3.2.3 (RONQUIST et al. 2011) running at „The CIPRES Science Gateway V. 3.1" on-line portal (MILLER et al. 2000).

Table 1 PCR cycling conditionsused in this study.

Primer-pair	Development	pre-denaturation		number of cycles	denaturation		annealing		elongation		post-polimerization	
		s	°C	pieces	s	°C	s	°C	s	°C	s	°C
PTT-F/R	WILLIAMS et al. (2001)	150	94	35	30	94	35	65»56	35	72	120	72
PG-F/R	TAYLOR et al. (2001)	120	94	35	60	94	60	68	60	72	600	72
PTM-F/R	WILLIAMS et al. (2001)	150	94	35	30	94	35	65»56	35	72	120	72
MAT 1 F/R	RAU et al. (2005)	180	94	30	30	94	30	56	30	72	600	72
MAT 2 F/R	RAU et al. (2005)	180	94	30	30	94	30	57	30	72	600	72
ITS1 F/ ITS4R	WHITE et al. (1990)	180	94	35	45	94	30	57	60	72	600	72
T1-F Bt2b-R	GLASS and DONALDSON (1995)	300	94	40	60	94	60	57	120	72	420	72
actin17-F actin 18-R	VOIGT et al. (2005)	300	94	40	60	94	60	57	120	72	420	72
gpd 1-F gpd-2-R	BERBEE et al. (1999)	300	94	40	60	94	60	65	120	72	420	72

Comment: s=second; 65»56= temperature decreasing at a rate of 1°C/cycle from 65°C to 56°C.

3. RESULTS

3.1. Characteristics of isolates from atypical leaf symptom

Characteristics of four unknown *Pyrenophora* isolates from unusual leaf symptoms were compared to those of two *Pyrenophora teres* f. *teres* reference strains. The two groups had very similar parameters regarding conidiophores and the shape and size of the multi-cellular, cylindrical conidia. There were very small, albeit statistically significant, differences, between the two groups in the width of apical cells of conidiophores as well as the maximum breadth and number of septa of conidia, and the length and width of the basal cells and the width of the apical cells of conidia. Despite this, we found statistically not supported differences amongst individuals of the unknown isolates and the PTT strains in all traits examined, except for the number of septa in conidia.

The four unknown isolates could not be separated clearly from each other and from the PTT strains based on their culture morphology. The color of their colonies ranged from light grey to oil green, the margin was mostly entirely regular or rarely undulate. Dense and low aerial mycelium, occasionally with mycelial tufts, developed on all agar media except for corn meal agar. They did not develop sexual, asexual and resting structures in agar cultures. The mycelial growth rate of our unknown isolates differed considerably. The fastest growth was observed generally on the BDA, the slowest one on the malt extract agar, but the growth rate of our unknown isolates overlapped that of with PTT in case of all media except for malt extract agar.

In the pathogenicity test small necroses appeared on the leaves treated with our unknown isolates and the 2 PTT reference strains on the 4th day after inoculation. There were no differences in the symptoms at that time. The size of necroses increased gradually and finally two considerably different symptom-types developed on the 19th day. All of our unknown isolates caused small (a few mm long) and slightly elongated, oval-like necrotic spots, whereas the PTT

reference strains caused typical net-like necroses on the leaves. The control plants sprayed with water remained symptom-free.

3.2. Results of molecular studies

3.2.1. Identification of isolates according to specific PCRs

Altogether 208, infected barley and wheat leaf samples were collected in experimental plots of breeding stations and commercial fields from various cultivars or breeding lines in 2006-2010. Samples originated from eight locations in 2006, thirty-two in 2007, thirty-three in 2008, and fourteen in 2009 and two locations in 2010. The majority of cultures were collected in 2007 (29%) and 2008 (34%). Most isolates (32%) came from the Western region, whereas the least (13%) originated from the central region of Hungary. Most isolates were collected from winter barley (102 isolates, 49%), 26% from spring barley (55 isolates) and 25% from winter wheat (51 isolates).

We identified 142 (68%) *Pyrenophora teres* f. *teres* strains in our examination. The most of PTT (31%) originated from the North-East region, the least (15%) from the Central region. Interestingly, the PTT isolates often came from narrow, 1-2 cm long necrotic stripes (which did not resemble the leaf stripes caused by *P. graminea* in the entire length of the leaves) or small flecks, and not from typical net type of net blotches. PTT was the dominant pathogen both on barley and wheat in all years except for 2010, when only 5 isolates were tested.

Fifty-three (26%) *P. teres* f. *maculata* isolates from various parts of Hungary were identified, most of them (46%) were from the South-West region. PTM was isolated from the following types of symptoms: small necrotic flecks with irregular shape, typical PTM-type oval necrotic spots, with small flecks around, and short longitudinal necroses.

We identified 13 (6%) *P. graminea* strains, the majority of them (84.6%) came from the Western region. The isolates were derived from long necrotic

stripes spreading from the base to the top of leaf. We collected PG isolates in all years except for 2010.

3.2.2. Distribution of mating types

Mating types of altogether 8 PG, 31 PTM and 109 PTT isolates were identified and surveyed in different regions, years and hosts in Hungary.

Among the 8 *P. graminea* isolates tested form mating type 5 belonged to MAT1 and 3 belonged to MAT2. Most of these (6 isolates) originated from the Western region. The isolates of *P. graminea* collected in Tálplánszentkereszt in 2006 represented both mating types. Regarding the distribution of matig types in different years the number of MAT1:MAT2 strains was 1:2 in 2006, 3:0 in 2007, and 1:1 in 2008.

In case of the 31 *P. teres* f. *maculata* isolates tested for mating type, 68 % belonged to MAT1 and 32 % to MAT2. Considering the whole sampling period, the distribution of mating types and the number of isolates from different regions were not equal in different regions: MAT1 was more abundant in the Central, South-East and Western regions, while MAT2 was more prevalent in the North-East region. In case of the isolates collected from wheat in Bóly in 2006 and the isolates collected from barley at Táplánszentkereszt in 2009, we identified both mating types from same location. In most years MAT1 was more frequent than MAT2, 5:2 in 2006; 8:4 in 2007; 2:2 in 2008; 4:2 in 2009 and 2:0 in 2010.

Most isolates with known mating type (109 isolates) belonged to *P. teres* f. *teres*, of which 56 (51%) and 53 (49%) were MA1 and MAT2, respectively. In 2006 and 2007, MAT1 isolates were in majority (MAT1:MAT2 ratio: 11:6 and 21:16, respectively), while in 2008 MAT2 isolates were more frequent (MAT1:MAT2 ratio: 17:22). The two mating types were collected in equal number (7:7) in 2009, and the two isolates from 2010 belonged to MAT2. Similarly to PTM, the ratio of mating types in the PTT population was not equal

within regions: MAT2 was prevalent in the Western, the North-East and the Central regions, while the opposite was true in the South-East region, where the MAT1 was in majority. We identified both mating types at the same locations (table): the isolates collected from wheat in Bóly and Székkutas in 2006, from winter barley in Kiszombor-Makó and from wheat in Táplánszentkereszt in 2007, from spring barley in Martonvásár, Tordas and Kocs in 2008, and from winter and spring barley in Táplánszentkereszt in 2009 and 2010 represented both MAT1 and MAT2.

As we mentioned previously, most PTT isolates (48%) originated from winter barley, while 28% from winter wheat and 24% from spring barley. MAT1 was slightly more frequent than MAT2 on all hosts with MAT1:MAT2 ratios of 24:17 (wheat), 38:32 (winter barley) and 18:17 (spring barley).

3.2.3. Genetically variability of PTT using RAPD analysis

The 68 PTT isolates tested with RAPD analysis were collected in 43 commercial fields and 4 experimental stations representing altogether 45 locations in 4 geographical regions (Western, Central, South-East and North-East). For practical reasons, all plots within an experimental station were considered to be the same field, considering the small distance among plots (up to ca. 1 km) and similar agronomical practice applied to them. One isolates was collected in 2006, sixteen were collected in 2007, thirty-five in 2008, fourteen in 2009 and two in 2010.

Altogether 171 (91%) polymorphic and 17 (9%) monomorphic DNA fragments. ranging in size from approximately 100 to 3000 bp, were scored. The average number of amplicons per locus was 1.9. The number of RAPD fragments amplified by different primer combinations varied from 7 (OPA14/OPE03) and 19 (OPG04/OPB01), with an average value of 12.5. Each isolates represented a unique multilocus RAPD haplotype. Consequently, normalized Shannon diversity index reached the maximum value of 1 and

Simpson's index (0.0147) was closed to zero, both supporting a high level of genotypic diversity.

The genetic relationship of isolates was studied with UPGMA cluster analysis. The genetic distance among the 68 PTT isolates ranged from 1.6 to 36% (average 9.5%). Fifty-seven isolates formed 4 main groups (Cluster I–IV). No general correlation between clustering and mating types or geographical origins of isolates could be detected as each cluster contained both MAT1 and MAT2 isolates from 3 to 4 regions. The isolates of Cluster II and III originated from three regions, while members of Cluster I and IV came from 4 regions. Apparently, a slight degree of spatial substructuring could be observed only in a few cases at subcluster level. Interestingly, the vast majority of strains in Clusters I, III and IV were collected within a year, whereas Cluster II alone represented three growing seasons (2008, 2009 and 2010). All but two cultures in Cluster I was obtained in 2008. Cluster III consisted of eight 2009 isolates plus a single 2008 strain, H-361, which was clearly separated from other members of Cluster III. Cluster IV was formed by isolates collected in 2007 plus a distantly related 2006 isolate (H-112). Those isolates which were collected from the same leaves did not represent the genetically most related RAPD haplotypes. Isolates H-327 and H-335, derived from two distinct lesions within a leaf and representing different mating types, were assigned to neighboring subclusters, whereas H-325 and H-336 originated from a single lesion and representing MAT1, were also separated from each other in Cluster I.

Nei's gene diversity analysis revealed that genetic diversity within sampling units accounted for most of the total genetic diversity, while genetic diversity between sampling units represented a small proportion of the total diversity. Both average gene diversity within sampling units and total gene diversity were very similar in all kind of comparisons and ranged from 0.12849 to 0.16729 and from 0.13922 to 0.18353, respectively. A negligible and statistically not supported difference in allele frequencies was detected between

MAT1 and MAT2 isolates (G_{ST}=0.01379, G^2 was insignificant with a very high P value of 0.1684). Small, albeit extremely significant, differentiation was observed amongst isolates from commercial and experimental fields (G_{ST}=0.03643) or from the four geographical regions taken as a whole (G_{ST}=0.07567) with P value less than 0.0001 for G^2. This indicated that at least one regional subpopulation was differentiated from the others. In fact, heterogeneity in allele frequencies was found to be statistically supported in each pair wise regional comparison with G_{ST} ranging from 0.03104 to 0.08195 ($P \leq 0.0111$ for G^2). The highest level of genetic differentiation was obtained for sampling years 2007, 2008 and 2009 (G_{ST} =0.16558, $P<0.0001$ for G^2), meaning that approximately 16.5% of the total gene diversities was due to seasonal differences in the *P. teres* collection tested. Pair wise comparisons of the three years also resulted in statistically significant differentiation of 10.68 to 14.88% ($P<0.0001$ for G^2).

3.2.4. Result of phylogenetic relationship of PT and PG

The aligned multigene data set used for the phylogenetic analysis contained 2375 characters, of which actin, *ß*-tubulin, *gpd* and ITS1-5,8S-ITS2 partitions were represented by 762, 508, 596 and 509 characters, respectively. We identified 8, 7 and 6 multigene haplotypes in the 13 PG, the 13 PTM and the 15 PTT isolates, respectively, but the three pathogens did not have a common multigene haplotype and they showed small variability on the whole.

The actin sequences could be grouped into 3 haplotypes. The 13 Pg isolates had all three haplotypes, the 13 PTM isolates had two hyplotypes, and the 15 PTT isolates was fully homogeneous for this gene. Haplotype Akt-1 was more frequent in the PG strains, while Akt-2 was more frequent in the two forms of *P. teres*.

The ß-tubulin sequences could be divided into 4 haplotypes. PG was monomorphic. PG, PTM and PTT represented different haplotypes, except for isolates 26PTM and 21PTT, which contained the same haplotype.

PG had altogether 6, whereas PTM and PTT carried 3-3 different *gpd*-haplotypes. We did not find a common haplotype for the 3 pathogens, but isolate 38PG and several PTM isolates, and isolate 26PTM and several PTT isolates, and isolate 16PTT and several PG strain carried same sequences.

Each of PG and PTM represented 4, while PTT represented 5 ITS haplotypes. The same ITS sequence never appeared among any pairs of the three pathogens.

The multigene ML-tree, rooted to the outgroups species *P. tritici-repentis,* revealed 3 main phylogenetic clusters, corresponding in general to the 3 barley-pathogenic *Pyrenophora* species. Interestingly, isolate 26PTM separated from other 'f. *maculata'* isolates and was in basal position to the 'f. *teres*" cluster. According to the ML-tree, *P. teres* (and isolate 26PTM) separated first from the common ancestor of the 3 investigated *Pyrenophora* pathogens, whereas *P. graminea* and *P. teres* f. *maculata* followed a joint evolutionary pathway till they diverged. Except for the node connecting the outgroup to the rest of the ML-tree, bootstrap values did not reach the threshold of reliability (70%).

Branching of the Bayes-tree were partly similar to that of the ML-tree in that the majority of isolates grouped according to the 3 pathogens, and isolate 26PTM grouped to PTT cluster again. However, in contrast to the ML-tree, Bayesian analysis revealed that *P. graminea* (and isolate 46PTM) separated first from the common ancestor of the 3 investigated *Pyrenophora* pathogens, whereas *P. graminea* and *P. teres* f. *maculata* followed a joint evolutionary pathway till they diverged. Except for the node connecting the PTM and PTT clusters, Bayesian posterior probability values were above the threshold of reliability (90%).

NEW SCIENTIFIC RESULTS

- We obtained novel data about the distribution of *Pyrenophora graminea*, *P. teres* f. *maculata* and *P. teres* f. *teres* in Hungary, and observed the wide-spread occurrence of the most abundant pathogen *P. teres* f. *teres*.
- We determined the occurrence of both mating types of *Pyrenophora graminea*, *P. teres* f. *maculata* and *P. teres* f. *teres* in Hungary.
- We were the first ones who demonstrated the occurrence of *Pyrenophora teres* f. *maculata* in Hungary according to the scientific requirements.
- We determined the genetically highly variable of the Hungarian PTT population which had a year change with the largest effect; and that its there is no statistically significant differentiation between MAT1 and MAT2 PTT isolates.
- In case of multigene phylogenetic analysis of *P. graminea, P. teres* f. *maculata* and *P. teres* f. *teres* we have revealed contradictory evolutionary relationships, namely Bayesian analysis supports the present taxonomical status of these pathogens, i.e. PTM and PTT are in a closer relationship, whereas ML analysis shows a closer relationship between PTM and PG.

4. CONCLUSIONS AND RECOMMENDATIONS

4.1. Identification of isolates from atypical leaf symptom

The conidia formed on atypical symptoms on barley leaves collected from 2006 to 2010 were very similar to the conidia of *P. teres*. Altogether four isolates from these unusual lesions were examined thoroughly with morphological, pathogenetic and molecular genetic methods to identify the unknown pathogen.

Based on conidia and conidiophores we could not separate the unknown isolates from the PTT reference strains unambiguously, despite the fact that there was little (few μm), but statistically significant differences in size of certain characters between the two groups. In our opinion, the real value of these little differences in the routine identification of these species is questionable, because of the variability of these parameters. The isolates from atypical leaf symptoms could not be separated from each other or from PTT reference strains on the basis of their culture morphology and colony growth rates.

All four isolates from unusual symptoms were pathogenic to young barley leaves and caused a few-millimeter, slightly elongated or oval spots, which were obviously different from the net blotch symptoms initiated by the PTT isolates. These suggested, but yet not prove our hypothesis that our unknown isolates are different from *P. teres* f. *teres*. However, with specific PCRs we proved unambiguously that they belonged to *P. teres* f. *maculata*.

We isolated *P. teres* f. *maculata* from leaf spots varying in shape and size. This shows the difficulties in symptom-based-identification, which has the biggest practical role at scoringdisease resistance. Since the resistance of barley varieties against the two forms of *P. teres* can be inherited independently and may differ (KHAN és TEKAUZ 1982, AFANASENKO et al. 1995, HO et al. 1996), the presence of *P. teres* f. *maculata* in Hungary is a new challenge for the breeding programs to develop complex resistance against leaf diseases.

4.2. Frequency and distribution of Pyrenophora graminea and P. teres forms and their mating types in Hungary

Regarding the distribution of the three pathogens in Hungary, most isolates (68%) belonged to *Pyrenophora teres* f. *teres*, while 53 (26%) were identified as *P. teres* f. *maculata*, both of which originated from different regions of the country, and only 13 isolates belonged to *P. graminea*, the majority of which came from the Western region of Hungary.

The low frequency of *P. graminea* may be explained by the use of fungicides for seed dressing, which effectively reduced the possibility of systemic infections. Additionally, although *P. graminea* produces conidia on infected leaves, it cannot infect new leaves, in contract to *P. teres,* which is able to initiate multi-cyclic disease in the growing season. Since host plant resistance against PG, PTM and PTT is not linked genetically in general, our data may be helpful to determine priorities in disease resistance breeding programs.

Because *P. teres* is a heterothallic fungus and capable of reproducing both asexually and sexually, the genetic structure and variability of *P. teres* populations greatly depend on the frequency and coincidence of the two opposite mating types as well as on the frequency of asexual and sexual reproduction in the growing season (LIU et al. 2011). This is also true for *P. graminea*, but as this pathogen can not cause new leaf infections by conidia, its asexual reproduction is restricted. Several studies have reported the significance of sexual reproduction in *P. teres* populations (PEEVER and MILGROOM 1994, RAU et al. 2003). By contrast, other researchers have found the widespread presence of asexual reproduction (CAMPBELL et al. 2002, LEHMENSIEK et al. 2010). We identified both mating types of all three pathogens during our research. Moreover, both MAT1 and MAT2 isolates of each fungal species/forms were distributed in almost all regions over several years (2007, 2008 és 2009). What is more, both mating types occurred on the same field/plot and leaf in case of *P. teres* f. *teres* (H-327 and H-335). Although

this spatial and temporal distribution of mating types does not verify that sexual reproduction took place in the Hungarian populations of these pathogens, it indicates the possibility of it.

4.3. Genetic variability of P. teres f. teres in Hungray

We have been the first to investigate the genetic variability of PTT populations in Hungary. Altogether 29 MAT1 and 39 MAT2 monospore isolates were investigated by RAPD analysis. Each isolate represented a unique multilocus RAPD haplotype. The high genotypic diversity in our collection coincides with results observed for several *P. teres* populations (Swedish, Polish, Czech, Slovakian, Lithuanian, Finnish, and Australian). The high genotypic diversity is frequently associated with sexual recombination (McDONALD and LINDE 2002). Other mechanisms that may also contribute to the populational variability are gene flow, migration, genetic drift, mutation, selection and parasexuality (HARTL and CLARK 1989). Since our isolates were collected from a large number of barley varieties/genotypes, selection pressure by different host genotypes could have theoretically affected the pathogen's genetic divergence through maintaining the pathotype diversity in the fungal population.

Both clustering and Nei's gene diversity analysis supported the view that temporal changes have a greater effect on the isolate's genetic divergence than the mating type, field type or geographical origin. In this sense, our results are in agreement with those obtained for Czech and Finnish populations. We did not see grouping according to mating type in cluster analysis, nor statistically supported genetic differentiation between the two mating types. The most probable explanation of this may be frequent sexual reproduction in the population. The statistically significant G_{ST} values we obtained may reflect only very low (for fileld type of geographical regions) to moderate (for years) genetic differentiation. The very low level of differentiation between commercial and

experimanetal fields indicates that certain level of gene flow could have occurred between those fields during the sampling period.

Similarly to us, BATURO-CIESNIEWSKA et al. (2012) and PELTONEN et al. (1996) did not find a close link between RAPD clusters and the geographical origin of *P. teres* isolates from four Polish regions or from various Finnish locations. However, in contrast to our data, they did not observe yearly differences. Considering the genetic diversity of *P. teres* at lesion level, it may be surprising that two MAT1 isolates (H-325 and H-336) from a single lesion represented different and clearly separated multilocus RAPD haplotypes in Cluster I. In the case of *Cochliobolus sativus* (anamorph: *Bipolaris sorokiniana*), chromosomal rearrangements during conidiogenesis were thought to be the most likely cause of genetic variation within lesions. Currently we do not know if the difference between out two isolates can be attributed to a similar phenomenon or simultaneous infections by different pathogens genotypes. A more sophisticated study, which was out of the scope of our investigations, is needed to answer this question.

4.4 Phylogenetic relationship of P. graminea and P. teres

Previous phylogenetic studies of barley pathogenic *Pyrenophora*-species only examined small number of isolates from a few geographical regions and used a few gene segments. To avoid these drawbacks, we used isolates from several continents and countries, and sequenced four gene regions. As an outgroup, we choose *P. tritici-repentis*, the causal agent of tan spot of wheat.

Our multigene phylogenetic analysis, which is the most detailed study of these *Pyrenophora* fungi so far, does not give an obvious answer to the exact phylogenetic relationship among PG, PTM and PTT. The primary reason for this is that ML and Bayesian analyses have revealed contradictory evolutionary relationships. Secondly, the statistical support of the branches on the ML tree is low. Bayesian analysis supports the present taxonomical status of these

pathogens, i.e. PTM and PTT are in a closer relationship, whereas ML analysis shows a closer relationship between PTM and PG. The result of ML analysis coincides with those of previous studies using AFLP and RAPD markers as well as MAT gene sequences (LEIŠOVÁ et al. 2005a, 2005b, BAKONYI and JUSTESEN 2007, RAU et al. 2007).

Considering the still existing uncertainties, additional molecular genetic data are required to clarify the phylogenetic relationship among barley pathogen *Pyrenophora* species.

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6. PUBLICATIONS RELATED TO DISSERTATION

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