VARIABILITY OF *PYRENOPHORA TERES* f. *TERES* IN HUNGARY AS REVEALED BY MATING TYPE AND RAPD ANALYSES

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SUMMARY

This study was conducted to investigate for the first time the genetic variability among Hungarian isolates of *P. teres* f. *teres*, the causal agent of the net form of net blotch of barley. A total of 68 monoconidial isolates were tested for mating type (MAT) and RAPD profiles using a MAT-specific PCR assay and 30 random decamer primers, respectively. Isolates were derived from symptomatic leaves of barley sampled in commercial fields and experimental plots of four geographical regions from 2006-2010. In total, 29 MAT1 and 39 MAT2 cultures were identified. Both mating types were found on different spatial scales, i.e. the same leaf, the same field or geographical region, and across several years, indicating the potential for sexual reproduction. Of the 188 distinct RAPD bands 171 were polymorphic. All isolates exhibited unique RAPD patterns. UPGMA clustering revealed four main isolate groups, but no general correlation between clusters and mating type or geographical origin of the isolates was detected. Nei's gene diversity analysis showed that genetic diversity within sampling units accounted for most of the total genetic diversity, while that between sampling units represented a small proportion of the total diversity. Genetic differentiation was not observed between MAT1 and MAT2 isolates. Although a low level of allelic differentiation was obtained according to field type (commercial vs. experimental) and geographical origin of the isolates, both UPGMA clustering of multilocus RAPD haplotypes and Nei's gene diversity analysis supported the view that seasonal changes had the greatest effect on the genetic divergence of isolates.

Key words: barley, genetic diversity, net blotch, mating types.

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INTRODUCTION

Pyrenophora teres Drechsler [anamorph: *Drechslera teres* (Sacc.) Shoem.], the causal agent of net blotch of barley (*Hordeum vulgare* L.), is a widepread important pathogen and causes considerable reductions in yield and quality of feed and malting barley in many countries (Deadman and Cooke, 1987; Khan, 1987; Steffenson *et al*., 1991; Murray and Brennan, 2010). The fungus causes net- or spot-like necrotic leaf lesions elicited by two morphologically indistinguishable forms of the pathogen, *P. teres* f. *teres* (PTT) and *P. teres* f. *maculata* (PTM), respectively (Smedegård-Petersen, 1971, 1977).

P. teres is hemi-biotrophic and capable of reproducing both asexually and sexually. In the asexual cycle, the fungus spreads and causes multi-cyclic disease by air-borne conidia in the host growing season, whereas in the sexual cycle ascospores are produced in pseudothecia, which serve as over-seasoning structures on infested plant debris (Mathre, 1982). The pathogen can also survive on and spread with infected seed (Shipton *et al*., 1973). Since *P. teres* is heterothallic with two mating types designated as MAT1 and MAT2 (McDonald, 1963; Rau *et al*., 2005), the sexual cycle is initiated only when two fungal strains of different mating types interact (Kronstad and Staben, 1997). In contrast to clonal propagation, sexual outcrossing may generate new pathogen genotypes with increased genetic diversity and rapid adaptability to environmental conditions and agronomical control practices such as the use of resistant cultivars or fungicides (Elliot, 1994). The concomitance of MAT1 and MAT2 net blotch isolates may vary in both small and large geographical scales. The two mating types of *P. teres* forms have been reported to be equally common, or nearly so, in several locations of Australia, Finland, Lithuania and Poland, but only MAT2 was identified in Krasnodar, Russia (Serenius *et al*., 2005, 2007; Bogacki *et al*., 2010; McLean *et al*., 2010; Statkeviciute *et al*., 2010, 2012; Baturo-Ciesniewska *et al*., 2012). Jalli (2011) also identified both mating types of PTT in Finland, but MAT2 was predominant.

Pathogen populations with more variable gene pools have a higher evolutionary potential to develop new genotypes and aggressive/virulent strains (McDonald and Linde, 2002). Therefore, investigating the genetic variability of local populations of plant pathogens has important implications for research into the organisms and for disease management (Tibayrenc *et al*., 1991).

Several studies have examined the genetic variability of *P. teres* populations in various regions of the world. Techniques such as random amplification of polymorphic DNA (RAPD) (Peever and Milgroom, 1994; Peltonen *et al*., 1996; Weiland *et al*., 1999; Jonsson *et al*., 2000; Campbell *et al*., 2002; Frazzon *et al*., 2002; Bakonyi and Justesen, 2007; Baturo-Ciesniewska *et al*., 2012), restriction fragment length polymorphism (RFLP) (Wu *et al*., 2003), amplified fragment length polymorphism (AFLP) (Rau *et al*., 2003; Leišova *et al*., 2005a, 2005b; Serenius *et al*., 2007; Lehmensiek *et al*., 2010; Bentata *et al*., 2011; Statkeviciute *et al*., 2012) as well as microsatellite and inter simple sequence repeat (ISSR) analyses (Bogacki *et al*., 2010; Statkeviciute *et al*., 2010) have largely been used.

In Hungary, barley is an important crop occupying the third largest cultivation area amongst cereals (*ca*. 250.000 ha per year). Losses caused by *P. teres* increased significantly in the 1980s (Sipos *et al*., 1989), then the pathogen became one of the most important biotic agents threatening the production of barley (Palágyi and Tomcsányi, 2006; Tomcsányi *et al*., 2006). The fungus has also been isolated from wheat (Tóth *et al*., 2008). Information from breeders and producers has suggested that the net form is widely distributed and has long been associated with net blotch disease of barley in Hungary, whereas the spot form has been identified only recently (Ficsor *et al*., 2010).

In spite of the economical importance of PTT in Hungary, data are not available on the pathogen's variability. Therefore, the aim of our study was to determine the mating type and genetic diversity of PTT isolates derived from leaf samples collected in the course of routine disease surveys from commercial and experimental fields in different years and Hungarian regions. The specific objectives were: (i) to investigate if both mating types of the pathogen occur in Hungary, i.e. is there a chance for sexual reproduction to take place, and (ii) to measure the level of genetic differentiation in a collection of isolates.

MATERIALS AND METHODS

Sample collection and fungal isolation. Diseased barley leaves were collected at random in the course of routine disease surveys from various spring and winter barley cultivars/breeding lines in experimental plots of distantly located breeding stations and commercial fields from 2006 to 2010 (Table 1). Leaf samples were stored in paper bags in the laboratory until processing. To induce conidiogenesis, leaf segments with necrotic lesions were placed into a moist chamber in Petri dishes that were kept on a laboratory bench at ambient temperature or incubated under white light (OSRAM model L36W/640) in a 16 h light/8 h dark cycle for 1-3 days at 20-22°C. Monoconidial isolates were then cultured by transferring single conidia from the conidiophores to potato dextrose agar (PDA) plates with a sterile needle, using a Leica MZ6 stereomicroscope at 300-400× magnification in a laminar air flow cabinet. Single-conidial isolates were incubated for 10-14 days in the dark at 20-22°C, and used as inocula for stock and potato dextrose broth cultures. Stock cultures were grown on PDA slants for 7-10 days in the dark at 20-22°C, then kept under mineral oil at 15°C.

Extraction and analysis of fungal DNA. Monoconidial isolates were cultivated in potato dextrose broth in steady cultures at 22-25°C for 7-10 days. Mycelia were harvested by vacuum filtration, washed with sterile deionised water, freeze-dried and ground in liquid nitrogen. Pulverized mycelia were kept at –70°C and used for DNA extraction. Total genomic DNA was isolated using the MasterPure™ Yeast DNA Purification Kit (Epicentre Biotechnologies, USA) according to the manufacturer's instructions. To confirm species identity and differentiate between forms of *P. teres*, DNA of each culture was subjected to specific PCR assays as described by Taylor *et al*. (2001) and Williams *et al*. (2001). PTT strains were then further subjected to PCR-based mating type and RAPD assays. MAT1 and MAT2 isolates were identified using the mating type-specific primer pairs (MAT1-F/MAT1-R and MAT2-F/MAT2-R) and the PCR procedure developed by Rau *et al*. (2005). Primers for net/spot form- and mating type-specific PCRs were synthesized by Bio Basic Canada Inc. (Markham, Ontario, Canada). To measure genetic variability, RAPD analyses were carried out with arbitrary decamer primers. Two random primers, each amplifying reproducible DNA fragments of different size in preliminary tests with a subset of PTT isolates, were combined in a reaction mixture to reduce costs, and altogether 30 RAPD primers were applied in 15 combinations: OPB-06/OPB-12; OPH-09/OPE-07; OPA-14/OPE-03; OPB-07/OPB-10; OPW-03/OPA-07; OPW-07/OPW-17; OPH-20/OPH-12; OPE-20/OPE-17; OPB-11/OPB-09; OPB-16/OPC-13; OPG-04/OPB-01; OPE-15/OPC-16; OPH-03/OPE-16; OPE-01/OPA-02; OPI-12/OPE-19 (Eurofins MWG Operon, Germany). The 20 µl RAPD reaction mixture contained 1× DreamTaq™ PCR Master Mix (Fermentas, Lithuania), 20 pmol of each primer and 20 ng of DNA. RAPD thermal profile consisted of an initial denaturing step of 94°C for 2 min, followed by 45 cycles of 92°C for 1 min, 35°C for 1 min and 72°C for 2 min, and a final extension step at 72°C for 5 min. RAPD amplifications were repeated three times. All PCRs were performed in an Eppendorf Mastercycler nexus thermal controller. Amplification products were separated by electrophoresis in 1.7% agarose gels, stained with ethidium bromide, and visualized under UV light.

Table 1. Origin and mating type of *Pyrenophora teres* f. *teres* isolates used in this study.

Strain code[a]	Date/month/year of collection	Location[b]	Field type[c]	Barley cultivar/genotype	MAT[d]
Western Hungary					
H-230	13/06/2007	Chernelházadamonya (1)	C	Jubilant	1
H-196	12/06/2007	Egyházasrádóc (2)	C	Unknown	2
H-169	03/04/2007	Mosonmagyaróvár (3)	E	KH Korsó	2
H-198	13/06/2007	Püspökmolnári (4)	C	Pasadena	2
H-231	”	Sárvár (5)	C	Coralba	2
H-185[1]	2007	Táplánszentkereszt (6)	E	Unknown	1
H-306/1[1]	18/06/2008	”	E	Henley	2
H-294[1]	”	”	E	Unknown	1
H-296[1]	”	”	E	gks 419	2
H-303[1]	24/06/2008	”	E	Sebastian	1
H-477	26/05/2009	Hédervár (7)	C	Unknown	2
H-472	07/06/2009	Sárvár (5)	C	Scarlett	2
H-383/1[1]	22/06/2009	Táplánszentkereszt (6)	E	Scarlett	2
H-393/1[1]	”	”	E	GK Judy	1
H-396/1[1]	”	”	E	Unknown	2
H-497/3[1]	09/06/2010	”	E	Pasadena	2
H-500/1[1]	”	”	E	GK Mandolina	2
Central Hungary					
H-112	04/2006	Mór (8)	C	Unknown	2
H-327[1,2,3]	19/06/2008	Kocs (9)	C	Unknown	1
H-335[1,2,3]	”	”	C	Unknown	2
H-284[1]	16/05/2008	Martonvásár (10)	E	Kh Turul	2
H-287[1]	16/05/2008	”	E	F74-82/MANAS/ /SZD4002	2
H-288[1]	”	”	E	20899YH2/Petra	2
H-313/1[1]	”	”	E	Petra	2
H-317/1[1]	”	”	E	Nelly	1
H-321/1[1]	”	”	E	KH Center	2
H-322[1]	10/06/2008	”	E	Unknown	2
H-310/2[1]	12/06/2008	Tordas (11)	E	Unknown	1
H-314/1a	”	”	E	Unknown	2
H-323[1]	”	”	E	Unknown	1
H-324/1[1]	”	”	E	Unknown	2
H-325[1,2,3,4]	”	”	E	Unknown	1
H-336[1,2,3,4]	”	”	E	Unknown	1
South-Eastern Hungary					
H-159	2007	Kiszombor-Makó (12)	C	Unknown	2
H-201	11/07/2007	Miske (13)	C	Unknown	1
H-200	08/06/2007	Nemesnádudvar (14)	C	Ebson	2
H-204	2007	Öregcsertő (15)	C	Rex	1
H-463	28/05/2008	Derekegyház (16)	C	Unknown	1
H-337	26/05/2008	Kétsoprony (17)	C	KH Tas	1
H-339	27/05/2008	Murony (18)	C	Rex	2
H-357	27/05/2008	Orosháza (19)	C	Botond	1
H-469	06/06/2008	Ócsény (20)	C	Rex	1
H-308/2	22/05/2008	Székkutas (21)	E	Unknown	2
H-282	??/04/2008	Szolnok (22)	C	Nelly	1
H-447	02/06/2009	Jánoshalma (23)	C	Rex	1
H-478	2009	Kalocsa (24)	C	Unknown	1
H-410/2	02/06/2009	Kunszentmiklós (25)	C	Unknown	2
North-Eastern Hungary					
H-192	04/06/2007	Bátorliget (26)	C	Palinka	1
H-189	”	Fényeslitke (27)	C	Unknown	2
H-209	07/06/2007	Halmaj (28)	C	Scarlett	2
H-191	04/06/2007	Hodász (29)	C	KH Viktor	2
H-186	”	Kölcse (30)	C	Petra	1
H-190	”	Nagyvarsány (31)	C	Palinka	2
H-464	09/06/2008	Balassagyarmat (32)	C	Laverda	2
H-377	19/06/2008	Enőd (33)	C	Candesse	1
H-359	09/06/2008	Endrefalva (34)	C	Maress	2
H-358	”	Karancsság (35)	C	Unknown	1
H-340/1	19/06/2008	Igrici (36)	C	Candesse	2
H-360	18/06/2008	Sajópüspöki (37)	C	Pasadena	2
H-462	09/06/2008	Szügy (38)	C	Vanessa	1
H-361	17/06/2008	Tiszakarád (39)	C	Unknown	2
H-458	10/06/2008	Ujdombrád (40)	C	Unknown	2
H-428	07/06/2009	Apagy (41)	C	Unknown	1
H-438	05/06/2009	Cserháthaláp (42)	C	Ebson	2
H-429	04/06/2009	Karancsság (35)	C	Jubilant	1
H-473	16/06/2009	Mezőkeresztes (43)	C	Mauritia	2
H-433	17/06/2009	Tiszaszőlős (44)	C	Imperial	1
H-426	2009	Tomor (45)	C	Unknown	1

[a]Superscript numbers after strain code indicate that a few particular isolates collected at the same location are from the same field ([1]), plot ([2]), leaf ([3]) or necrosis ([4]).

[b]Numbers in parentheses refers to numbers in Fig 1.

[c]C = commercial, E = experimental.

[d]Mating type: 1 = MAT1, 2 = MAT2.

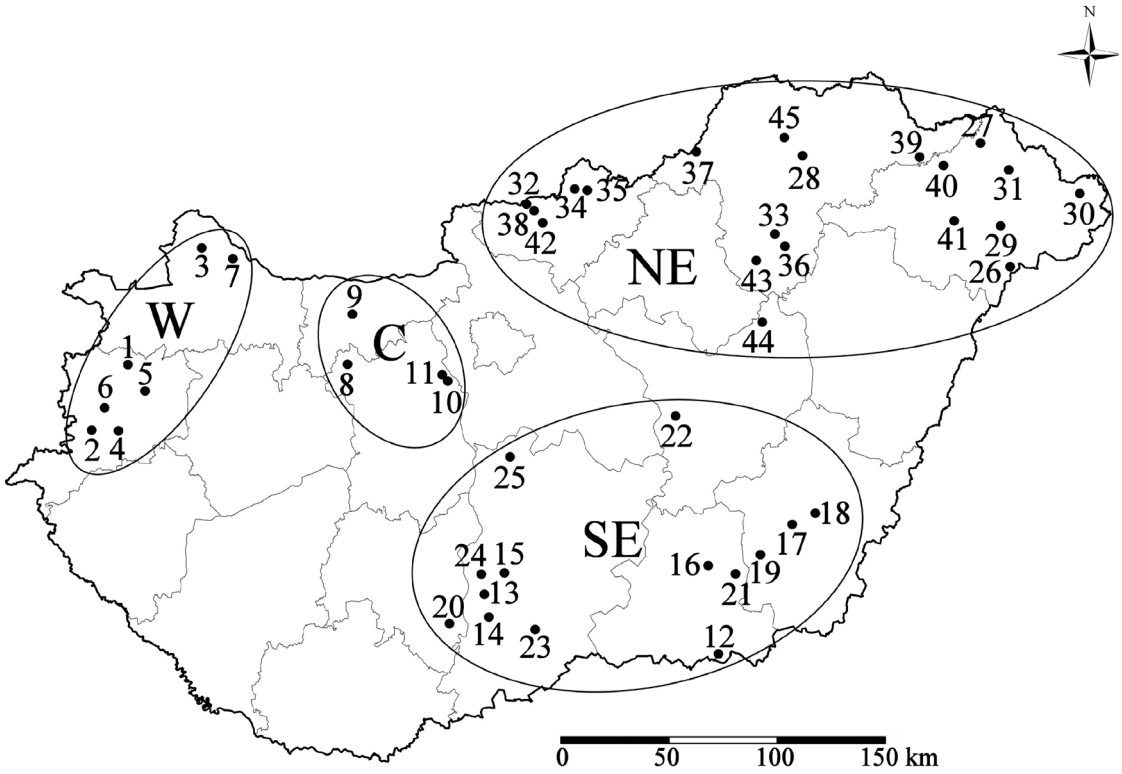

Fig. 1. Map of Hungary showing the locations (black dots) and geographical regions (W = west, C = central, SE = south-east, NE = north-east) where isolates were collected. Numbers next to dots refer to the name of locations as listed in Table 1.

Analysis of genetic variability. Presence and absence of RAPD bands were scored as 1 and 0, respectively. Only bands present/absent in all replicate reactions were counted. Data were collated into a binary matrix used to calculate pairwise 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 unweighted pair-group method with arithmetic mean (UPGMA) (Sneath and Sokal, 1973), and statistical support for the topology of phenogram was tested by bootstrap analysis (1000 replications). TREECON version 1.3b (Van de Peer and De Wachter, 1994) was used for these computations.

Genotypic diversity of PTT isolates was measured by the normalised Shannon diversity index (H_s) (Sheldon, 1969)

$$H_s = -\Sigma\, g_i \ln g_i / \ln N$$

where g_i is the frequency of the ith multilocus genotype and N is the sample size. This index ranges from 0 (there is no diversity) to 1 (each isolate represents a unique genotype). In addition, Simpson's index ($D = \Sigma g_i^2$), indicating the probability that two isolates sampled at random and with replacement in the population belong to different multilocus genotype, was also calculated (Simpson, 1949). Simpson's index also ranges between 0 and 1, but in this case the low value indicates great sample diversity.

To test for differences in allele frequencies between subgroups of isolates, frequency of polymorphic loci and Nei's gene diversity parameters in subdivided populations (Nei, 1973, 1987) were computed with POPGENE (Yeh and Boyle, 1997). A locus was considered polymorphic if it had two alleles (0 or 1), regardless of the allele frequencies. To test whether or not allele frequencies differed significantly between sampling units (mating type, field type, region, year), likelihood ratio chi-square tests (G^2) implemented in POPGENE were performed with the null hypothesis of no differentiation (Brown, 1996). The observed values of G^2 were compared to tabulated values of chi-square.

RESULTS

Identity and origin of isolates. In total, 81 monoconidial isolates of *P. teres* were collected from necrotic barley leaves. Based on symptoms, isolates were expected to be PTT. Specific PCR assays confirmed this assumption for 68 strains derived from typical net blotches or small necrotic spots varying in size and shape from a few mm flecks to 1 to 2.5 cm long interveinal necroses scattered all over the leaf blades. Thirteen cultures, all from small flecks or short longitudinal necroses, proved to be PTM (data not shown). The 68 PTT isolates were collected in 43 commercial fields and four experimental stations representing altogether 45 locations in four geographical regions (West, Central, South-East and North-East) (Table 1, Fig. 1). For practical reasons, all plots within an experimental station

were considered to be the same field, considering the small distance amongst plots (up to *ca.* 1 km) and similar agronomical practices applied to them. One isolate was collected in 2006, sixteen were collected in 2007, thirty-five in 2008, fourteen in 2009, and two in 2010.

Distribution of mating types. Mating types of the 68 PTT isolates was determined: 29 were MAT1 and 39 were MAT2 (Table 1). Both MAT1 and MAT2 strains were found in 2007, 2008 and 2009 with a ratio of 6:10, 16:19 and 7:7, respectively. In addition, both mating types occurred in each geographical region as well as in both experimental and commercial fields. A leaf sample taken from a commercial field in Kocs in 2008 carried both MAT1 (H-327) and MAT2 (H-335) strains on different lesions. In the course of five sampling trips at three experimental stations, 2 to 7 isolates were collected within fields in 2008, 2009 or 2010, and in four cases both MAT1 and MAT2 isolates were found within the same field at each of these three experimental stations (Martonvásár, Táplánszentkereszt and Tordas).

Genetic variability of *P. teres* f. *teres*. RAPD analysis was used to investigate the genetic variability of the 68 PTT isolates. 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 seven (OPA14/OPE03) to 19 (OPG04/OPB01), with an average value of 12.5. Each isolate represented a unique multilocus RAPD haplotype. Consequently, normalized Shannon diversity index reached the maximum value of 1 and Simpson's index (0.0147) was close to zero, both supporting a high level of genotypic diversity.

Genetic relationship of isolates based on UPGMA cluster analysis is illustrated in Fig. 2. The percent genetic distance among the 68 PTT isolates ranged from 1.6 to 36% (average 9.5%). Fifty-seven isolates formed four main groups (Clusters I-IV). No general correlation between clustering and mating type or geographical origin of isolates could be detected as each cluster consisted of both MAT1 and MAT2 isolates from several regions. Clusters II and III contained isolates from three geographical regions, whereas Clusters I and IV contained isolates from all four geographical regions. Apparently, a slight degree of spatial substructuring was observed in only a few cases at subcluster level. For instance, four (H-294, H-296, H-303, H-306/1) of the six western Hungarian isolates, as well as three (H-322, H-323, H-327) from central Hungary in Cluster I, and four (H-426, H-428, H-429, H-433) from north-east Hungary in Cluster III grouped into their corresponding subclusters. 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

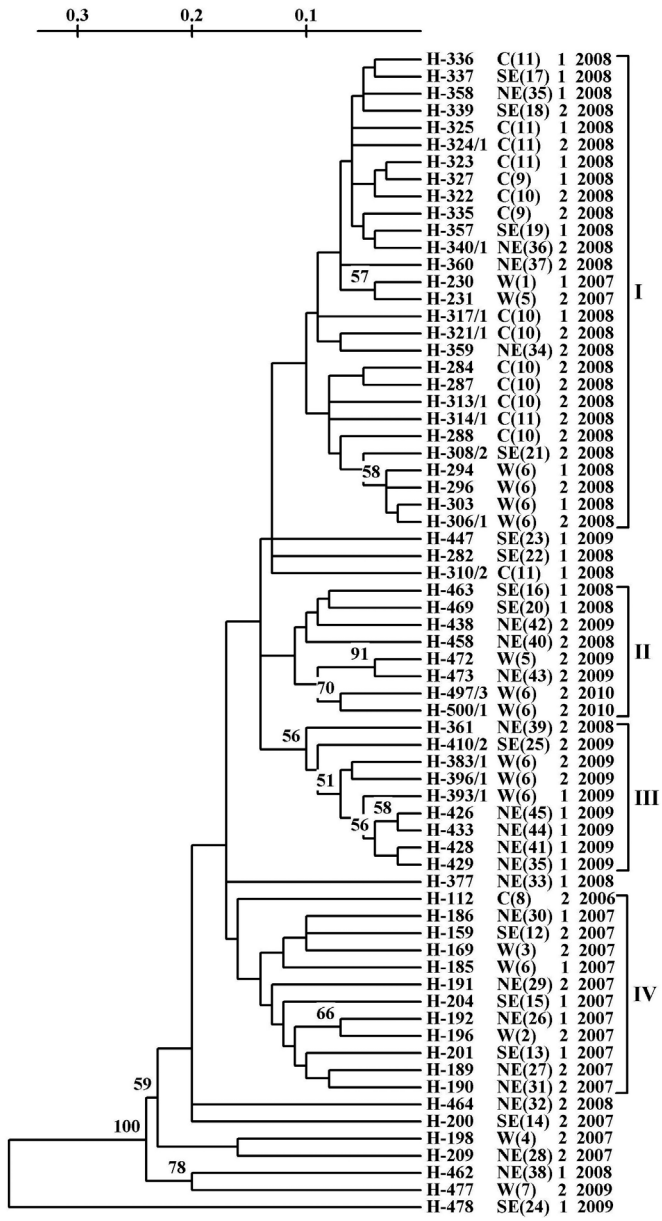

Fig. 2. UPGMA phenogram of *P. teres* f. *teres* isolates based on Nei and Li (1979) genetic distance coefficient. Numbers at nodes indicate bootstrap values of ≥50% (1,000 replications). Next to branches are isolate code, region (location), mating type and year of collection as shown in Table 1. Scale indicates level of genetic distance.

Cluster I were obtained in 2008. Cluster III consisted of eigth 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 most genetically related RAPD haplotypes. H-327 and H-335, derived from two distinct lesions within a leaf and representing different mating types, were assigned to neighbouring subclusters, whereas H-325 and H-336, originating from a single lesion and representing MAT1, were also separated from each other in Cluster I (Fig. 2).

Table 2. Genetic diversity as revealed by Nei's analysis of gene diversity in different sampling units of *P. teres* f. *teres.*

Sampling unit		H_S	H_T	D_{ST}	G_{ST}	G^2	P value
Mating type (MAT1 : MAT2)		0.1573	0.1595	0.0022	0.01379	188.7	0.1684
Field type (commercial : experimental)		0.1481	0.1537	0.0056	0.03643	319.5*	<0.0001
Regions							
	All regions	0.1478	0.1599	0.0121	0.07567	739.7*	<0.0001
	west : central	0.12849	0.13922	0.01073	0.07707	276.4*	<0.0001
	south-east : north-east	0.16729	0.17265	0.00536	0.03104	198.6*	0.0089
	west : south-east	0.16497	0.17188	0.00691	0.04020	205.7*	0.0047
	central : north-east	0.13073	0.1424	0.01167	0.08195	315.9*	<0.0001
	west : north-east	0.15313	0.15897	0.00584	0.036736	181.03*	0.0111
	central : south-east	0.14245	0.15047	0.00802	0.0533	251.5*	<0.0001
Years							
	2007 : 2008 : 2009	0.1411	0.1691	0.028	0.16558	1072.5*	<0.0001
	2007 : 2008	0.15354	0.1767	0.02316	0.13107	570.47*	<0.0001
	2007 : 2009	0.15621	0.18353	0.02732	0.14885	537.72*	<0.0001
	2008 : 2009	0.14143	0.15831	0.0273	0.1068	455.94*	<0.0001

Mean within sampling unit (H_S) and total (H_T) gene diversities were corrected for expected bias due to finite sampling (Nei and Chesser, 1983, equations 17 and 18). The absolute (D_{ST}) and relative (G_{ST}) measures of total gene diversity due to differentiation between sampling units were then estimated by $D_{ST} = H_T - H_S$ and $G_{ST} = D_{ST}/H_T$ (Nei, 1973). Likelihood ratio chi-square test over all loci were carried out under the null hypothesis of $G^2 = 0$ (Brown, 1996). Differences in allele frequencies were considered statistically significant and marked with an * if $P \leq 0.05$.

Nei's gene diversity analysis (Table 2) revealed that genetic diversity within sampling units (mating type, field type, region, year) 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 diversities within sampling units and total gene diversities were very similar across all comparisons made and ranged from 0.12849 to 0.16729 and from 0.13922 to 0.18353, respectively. A negligible and insignificant 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 ($G_{ST} = 0.03643$) and from the four geographical regions taken as a whole ($G_{ST} = 0.07567$) with P values 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 pairwise 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. Pairwise comparisons of the three years also resulted in statistically significant differentiation of 10.68 to 14.88% ($P < 0.0001$ for G^2).

DISCUSSION

Mating type and genetic variability of 68 monoconidial PTT isolates, the causal agent of the net form of net blotch disease of barley, were examined using a PCR-based mating type assay and RAPD analysis, respectively. The isolates were collected from barley leaves showing various types of lesions sampled in commercial fields and experimental plots in diverse geographical regions, mostly from 2007 to 2009. The large number of RAPD markers amplified and the high ratio of polymorphic loci in the collection support the suitability of using random oligonucleotides selected for the analysis. Although the majority of field isolates were identified accurately based on leaf lesions, classification of 13 isolates as PTM by specific PCR indicates the difficulties with symptom-based delineation of the two morphologically indistinguishable forms of *P. teres*. This is especially important for breeders and should be considered in their routine disease evaluation methodology.

The high genotypic diversity in our collection, as illustrated by the unique fingerprints for all isolates, compares well with results observed for several *P. teres* populations. For example, maximum genotypic diversity was obtained for 64 Swedish PTT isolates at two locations 20 km apart and tested with three arbitrary decanucleotide primers amplifying at 19 loci (Jonsson *et al*., 2000), and for 41 PTT strains sampled in four Polish provinces (Baturo-Ciesniewska *et al*., 2012). Additionally, each of the 30 Czech or Slovakian, 145 Lithuanian and almost all of the 72 Finnish plus 185 worldwide net form cultures showed individual AFLP genotypes (Leišova *et al*., 2005b; Serenius *et al*., 2005, 2007; Statkeviciute *et al*., 2012), whereas 84% of 76 South Australian PTT isolates from three field populations had unique multilocus haplotypes when subjected to microsatellite genotyping (Bogacki *et al*., 2010). A high genotypic diversity is frequently associated with sexual recombination (McDonald and Linde, 2002). Other mechanisms that may also contribute to populational variability are gene flow or migration, genetic drift, mutation, selection and parasexuality (Hartl and Clark, 1989). Long-distance travel of *P. teres* inoculum is possible by seed dispersal or

air-borne conidia, which can travel 10-200 km under favourable conditions (De Wolf *et al*., 1998). Such a dispersal of inoculum of different genetic background could also have contributed to the genetic variability observed in this study. Additionally, several pathotypes of both the spot and net form pathogens have been identified in Hungary earlier (J. Bakonyi, unpublished data). 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 pathotype diversity in the fungal population. Regarding parasexual (mitotic) recombination, as far as we know the phenomenon has not been reported for *P. teres*, and it is hard to estimate its role.

Leišova *et al*. (2005b) found that isolates of both forms of *P. teres* seemed to be grouped according to the year of sampling rather than to their geographical origin. Serenius *et al*. (2007) also reported a significant level of genetic differentiation for Finnish PTT isolates according to sampling years. Our data are in agreement with this in terms that both clustering and Nei's gene diversity analysis supported the view that temporal changes had a greater effect on the isolate's genetic divergence than the mating type, field type or geographical origin.

In concordance with Serenius *et al*. (2005), who used AFLP to investigate genetic variation within and between two remote Finnish PTT populations with no significant deviation from 1:1 mating type ratio, we did not see grouping according to mating type in cluster analysis, nor genetic differentiation between the two mating types. The statistically significant Gst values we obtained may reflect only very low (for field type and geographical regions) to moderate (for years) genetic differentiation. The very low level of differentiation between commercial and experimental fields indicates that certain level of gene flow is likely to have occurred between those fields during the sampling period. This is especially interesting from the point of view that screening for adult plant resistance against net blotch usually takes place at experimental stations with the predominant use of local fungal population/isolate(s).

Similarly to us, Baturo-Ciesniewska *et al*. (2012) and Peltonen *et al*. (1996) did not find a close link between RAPD clusters and geographical origin of *P. teres*. However, in contrast to our findings, they did not observe yearly differences either. Also, similar low levels or lack of significant regional differences in genetic composition of *P. teres* populations were found, in general, on a relatively small scale, e.g. between fields within a distance of up to 20 km in both Alberta (Canada) and Sweden (5% differentiation in both cases) (Peever and Milgroom, 1994; Jonsson *et al*., 2000), between several PTT subpopulations in Finland and within Australian states (Serenius *et al*., 2007; Bogacki *et al*., 2010). In contrast, considerable genetic differentiation was detected in PTT populations from Sardinia (Italy) (Rau *et al*., 2003), likely due to a low migration rate, and among geographically distantly related *P. teres* populations (e.g. between continents, different European or Australian states, and two Finnish locations 400 km apart) (Peever and Milgroom, 1994; Serenius *et al*., 2005, 2007).

Considering the genetic diversity of *P. teres* at the 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 (Fig. 2). These two isolates differed from each other at nine RAPD loci, five being present in H-325 and four in H-336. In the case of *Cochliobolus sativus* (anamorph: *Bipolaris sorokiniana*), Leišova-Svobodova *et al*. (2012) hypothesised that chromosomal rearrangements during conidiogenesis could be the most likely cause of genetic variation, particularly within lesions. Currently, we do not know whether the variation observed for isolates H-325 and H-336 could be attributed to a similar phenomenon or to simultaneous infections by different pathogen genotypes. A more sophisticated study which was out of the scope of our investigations is needed to answer this question.

There are two fundamental means by which fungi and other organisms transmit genes to the next generation: through asexual (clonal) reproduction or by sexual mating and recombination. In the case of clonal reproduction, each progeny has only one parent and its genome is an exact mitotic copy of its parent. Recombining populations develop through meiotic recombination following mating, or mitotic recombination through a parasexual cycle (Taylor *et al*., 1999). Recombination generates new genotypes and thus increases genetic diversity. Due to the heterothallic nature of *P. teres* and its ability for both asexual and sexual propagation, the genetic diversity derived from random mating in this fungal populations is largely dependent on the ratio and co-existence of two opposite mating types and the relative abundance of the two types of propagation in the life cycle of the fungus (Liu *et al*., 2011). Rau *et al*. (2003, 2005) found that sexual reproduction occurs at significant levels within both forms of *P. teres* in Sardinia and that the relative contribution of sexual and asexual reproduction varies among different environments. In contrast, Serenius *et al*. (2007) concluded that recombination was limited within both forms of *P. teres* within field, but was possible based on co-occurrence of mating types. Based on the high number of unique multilocus microsatellite haplotypes and a 1:1 distribution of mating types, Bogacki *et al*. (2010) suggested that sexual reproduction was predominant in two South Australian populations of both PTT and PTM, but an asexual reproductive component could not be excluded.

In our study, both MAT1 and MAT2 isolates were found in years with more than two isolates (2007, 2008 and 2009), all regions, within field and on the same leaf. This spatial and temporal distribution of mating types does not conclusively demonstrate sexual reproduction, but indicates the potential for it. The distribution of MAT1 and MAT2 strains within and among several clades on the

RAPD phenogram and the very low level of insignificant genetic differentiation between them may also be indicators for genetic exchange among isolates, possibly through sexual outcrossing.

In summary, this is the first study on mating types and genetic variability of *P. teres* f. *teres* in Hungary. The wide distribution of both MAT1 and MAT2 isolates indicates that a high potential for sexual reproduction exists in local populations of this pathogen. Although a low level of genetic differentiation was obtained between isolates from commercial and experimental fields as well as from different geographical regions, our data suggest that seasonal changes could be the main factors affecting the isolate's genetic divergence.

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