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Population genetic structure of *Mycosphaerella graminicola* and Quinone Outside Inhibitor (QoI) resistance in the Czech Republic

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Abstract Damage caused by the wheat pathogen *Mycosphaerella graminicola* increased rapidly during the last two decades in the Czech Republic. We collected isolates from naturally infected fields in seven wheat-growing locations and analysed these using eight microsatellite markers. All markers were highly polymorphic. We found a high degree of genetic diversity and low clonality within all sampled Czech populations. We identified 158 unique multilocus haplotypes among 184 isolates. Field populations showed weak genetic structure but we detected more differentiation between climatic regions within the Czech Republic. We compared the Czech field populations to populations from the United Kingdom, Germany and Switzerland and found a marked differentiation between Czech populations and Western European populations. We hypothesize that decades of different agricultural practices, including the use of different wheat cultivars, may explain this genetic differentiation. We detected a rapid increase in QoI fungicide resistance during the sampling period from 2005 to 2011, coinciding with the widespread application of this class of fungicides in the Czech Republic. *M. graminicola* populations in the Czech Republic underwent a rapid adaptive evolution from sensitivity to resistance similar to what was described earlier in Western Europe.

Keywords *Septoria tritici* · Migration rates · Eastern Europe · Fungicide resistance

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Introduction

Mycosphaerella graminicola (Fuckel) Schröt. in Cohn is a haploid, heterothallic ascomycete and the causal agent of Septoria tritici blotch (STB) on wheat. The anamorph, *Septoria tritici* Roberge in Desmaz., was first described by Desmazières in France in 1842 (Desmazières 1842), but the sexual stage *M. graminicola* was not connected with the anamorph until more than a century later (Sanderson 1972). The complex para- and polyphyletic nature of the *Mycosphaerella* genus was recently reorganized and a new genus was created to accommodate *Septoria*-like species found on graminicolous hosts under the new genus name *Zymoseptoria* (Quaedvlieg et al. 2011). Hence, *Mycosphaerella graminicola* was proposed to be renamed *Z. tritici*.

M. graminicola has a worldwide distribution and causes significant yield loss in many wheat-growing areas of the world (Eyal 1999), including the Czech Republic. *M. graminicola* propagates both by asexual pycnidiospores and by sexually produced ascospores

that play a major role in shaping the genetic structure of populations (Chen and McDonald 1996; Zhan et al. 2003). Equal distribution of both mating type idiomorphs (MAT1-1 and MAT1-2) was found in most populations worldwide, consistent with ubiquitous sexual reproduction (Waalwijk et al. 2002; Zhan et al. 2002b; Siah et al. 2010). Knowledge of the genetic structure of pathogen populations provides important insight into the evolutionary processes that shaped populations in the past and may allow prediction of the evolutionary potential of the pathogen. Knowledge of evolutionary principles can inform disease management in agricultural ecosystems (e.g., McDonald and Linde 2002a). Fungal populations with high levels of genetic variation are more likely to adapt rapidly to fungicides or overcome host resistance than populations with less genetic variation. For instance, the cultivar 'Gene' was highly resistant to *M. graminicola* when it was released but became susceptible only 5 years after exposure to field populations of the pathogen (Cowger et al. 2000). Knowledge of the evolutionary potential of a pathogen may allow us to predict a pathogen's response to different control strategies and develop new approaches for sustainable disease management (McDonald and Linde 2002b).

Populations of *M. graminicola* have been characterized using a variety of molecular markers: RFLP markers were used for global studies (Linde et al. 2002; Zhan et al. 2002b; Jürgens et al. 2006); Razavi and Hughes (2004) assessed genetic variability of Canadian populations using RAPD markers; and populations in Germany, the USA and Iran were characterized using AFLP markers (Schnieder et al. 2001; Kabbage et al. 2009; Abrinbana et al. 2010). Owen et al. (1998) described the first nine microsatellite markers in *M. graminicola*. Almost 100 additional microsatellite markers were identified later using an EST sequence database (Goodwin et al. 2007). In recent years, several *M. graminicola* population genetic studies were performed using microsatellite markers (Banke and McDonald 2005; El Chartouni et al. 2011; Gurung et al. 2011; Boukef et al. 2012).

Population genetic studies from different geographical regions showed that the genotypic diversities of *M. graminicola* field populations are very high and that there is little genetic differentiation among populations within a region, suggesting significant homogenizing gene flow (Schnieder et al. 2001; Linde et al. 2002; Zhan et al. 2003; Banke and McDonald 2005). As exceptions, some genetic differentiation was found between populations sampled in California, Indiana, Kansas and North Dakota (Kabbage et al. 2009; Gurung et al. 2011) and strong differentiation was found among populations sampled from different wheat-growing provinces in Iran (Abrinbana et al. 2010). In the latter case, a high genetic diversity could be consistent with the hypothesis that *M. graminicola* adapted to wheat during the domestication process in the Fertile Crescent approximately 10,000 years ago (Stukenbrock et al. 2007). The pathogen is thought to have dispersed throughout the wheat-growing areas of the world during the spread of agriculture. An analysis of populations of sister species of *M. graminicola* found on wild grasses in Iran suggested that the speciation process was not only associated with host adaptation but also accompanied by significant genetic differentiation (Stukenbrock et al. 2007).

Major European wheat cultivars vary widely in Septoria tritici blotch resistance (STB). The durability of major *Stb* resistance genes is doubtful in the absence of appropriate breeding strategies and disease management. Quantitative resistance is likely to be more durable than single-gene resistance but it involves more complex breeding strategies. A successful application of quantitative resistance breeding for *M. graminicola* resistance was recently demonstrated by Risser et al. (2011). In the absence of useful resistance, fungicide treatment is the main control measure to manage STB. Two major categories of fungicides have been used against STB: quinone outside inhibitors (QoI), also known as strobilurins, and sterol demethylation inhibitors (DMIs), also known as azoles. In addition, benzimidazole (or MBC) fungicides were used. The most recent fungicides include multisite fungicides, such as chlorothalonil, and SDHI fungicides inhibiting the complex II. QoI fungicides act by inhibiting the mitochondrial respiration by binding at the Qo site of the cytochrome *bc*1 complex, blocking electron transfer and disrupting the energy cycle within the fungus (Barlett et al. 2002). QoI fungicides were first used in 1996 but resistant *M. graminicola* strains emerged rapidly and spread throughout many European countries (Fraaije et al. 2005). In most fungal pathogens, QoI resistance is conferred by a single point mutation in the mitochondrial cytochrome *b* gene leading to an amino acid change (Gisi et al. 2002). The amino acid glycine at

codon 143 is replaced by alanine in resistant strains of *M. graminicola*. Interestingly, the G143A mutation emerged independently in different genetic and geographic backgrounds (Torriani et al. 2009).

Wheat is the most important cereal in the Czech Republic. The damage caused by *M. graminicola* increased rapidly during the last two decades, leading to severe yield losses (Šíp 2003). The main control strategy against *M. graminicola* in the Czech Republic is the application of fungicides. The application of strobilurin fungicides on cereals increased rapidly between 2000 and 2008 (according to data from the State Phytosanitary Administration). Based on observations from other European *M. graminicola* populations (Fraaije et al. 2005; Torriani et al. 2009), a rapid emergence of resistant strains is expected in the Czech Republic due to extensive use of QoI fungicides during the past decade. However, no population genetic data are currently available to support this hypothesis.

The first objective of this study was to analyze the genetic structure of *M. graminicola* populations across different agriculturally important regions of the Czech Republic. The second objective was to assess the frequency of QoI-resistant genotypes in Czech populations and to determine the extent and origin of the QoI resistance. So far, many *M. graminicola* studies focused on populations collected from Western European countries. Here, we provide the first population genetic study from the Czech Republic, an Eastern European country that has experienced very different agricultural practices due to its political history.

Material and methods

Sampling and DNA extraction

A total of 192 *M. graminicola* isolates was collected from seven geographical locations in the Czech Republic (Fig. 1a, Table 1). Two locations (I and IV) consisted of fields with small-scale experimental plots, while the others were conventional agricultural fields. Locations I and IV were not treated with fungicides prior to sampling but the other locations were treated with fungicides. The different locations varied in elevation and amount of precipitation. Elevation ranged from 220 m (IV) to 530 m (V) above sea level. Locations I, II, and IV are in drier and warmer regions, while locations III, V, VI, and VII are in regions with higher humidity and lower temperature (Table 1). Samples were collected using a transect sampling scheme in each field. Distances between samples were 2 m in small-scale plots and 10 m in normal fields. From each field, we collected ~30 leaves. Different fields within one location were ~5 km apart. All fields were naturally infected by *M. graminicola* and infected leaf tissue was taken from different wheat cultivars. In three cases more than one strain (3–5) was isolated from one lesion, otherwise each strain was obtained from a different plant. The number of strains isolated per population varied between 23 and 40. Ninety six isolates were collected during the years 2005–2009. In 2011 an additional 96 samples were isolated from two populations (IV and V) already sampled in the 2005–2009 collection, and two new populations (VI and VII) were added.

Air-dried wheat leaves were surface-sterilized using a routine disinfection technique (70 % ethanol for 10 s, 0.5 % sodium hypochlorite solution for 60 s and rinsed in distilled water for 10 s) and placed into Petri dishes containing wet filter paper. Petri dishes were incubated at 18 °C for 48 h to induce oozing of spore-containing cirri. A sterile needle was used to transfer the contents of pycnidia onto new Petri dishes containing yeast malt dextrose agar broth (YMDA; 4 g of yeast extract, 4 g of maltose extract, 4 g of dextrose, 15 g of agar, 1 l of distilled H_2O). After incubation at 18 °C for 1–2 weeks, one colony per dish was randomly selected and plated on a fresh YMDA plate. After an additional 2 weeks of incubation at 18 °C, the cultures were harvested by gently scraping with a sterile spatula. The harvested spores were used immediately for DNA extraction.

Mycelia of *M. graminicola* isolates (~100 mg) were frozen in liquid nitrogen and ground to a fine powder with mortar and pestle. DNA was extracted using the established CTAB extraction protocol (Schnieder et al. 1998). The DNA concentration of each sample was adjusted to 25 ng/ml and samples were stored at −20 °C.

Microsatellite analyses

Template DNA was amplified with eight microsatellite markers (TCC_09, AC_01, CCA_03, GGC_01, AG_11, GT_03, AG_06 and TCC_08; Goodwin et al. 2007). PCR reaction volumes of

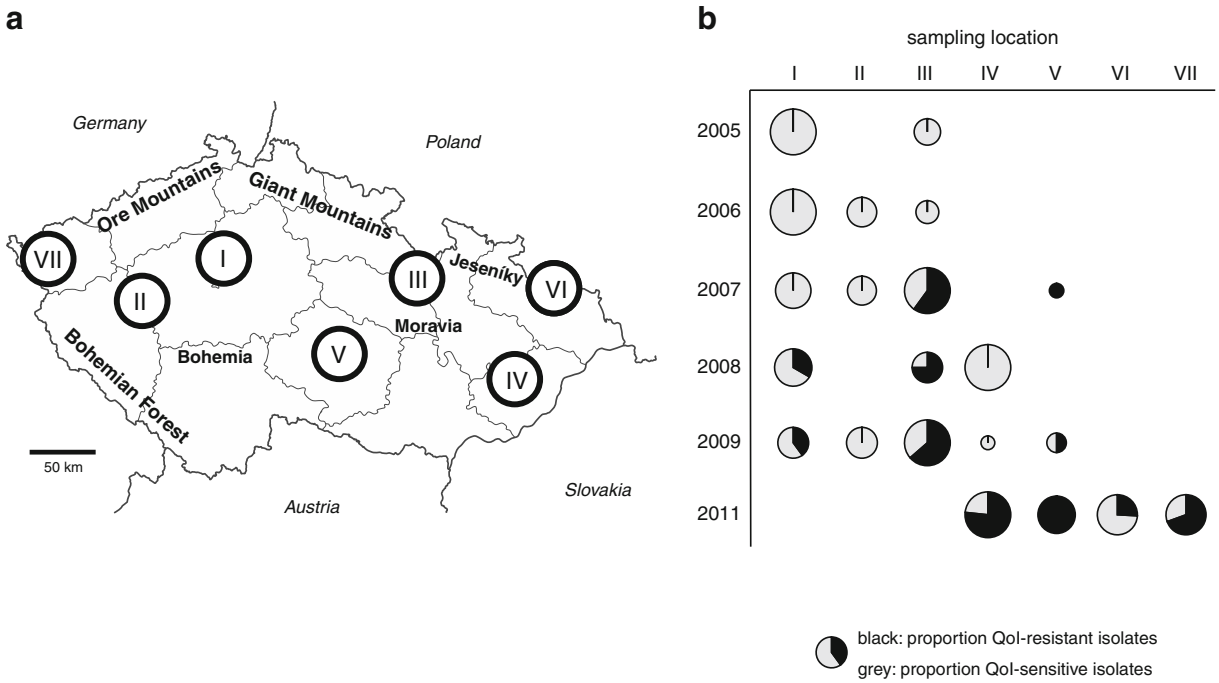

Fig. 1 **a** Geographical locations of sampled *Mycosphaerella graminicola* field populations in the Czech Republic. Within *circles*, roman numerals indicate the population number. *Bold names* indicate mountain ranges and higher elevation areas in the Czech Republic. **b** Frequency of QoI resistance alleles in *M. graminicola* populations in the Czech Republic sampled during the years 2005–2011. Pie areas within one sampling year are proportional to the sample size

20 µl contained 0.5 µM of each primer (one was labelled with a fluorescent dye), 0.25 mM of dNTPs, reaction buffer and 0.04 U of Taq polymerase (Fermentas, Mont-sur-Lausanne, Switzerland). PCR amplification was carried out using a thermal cycler (Biometra, Göttingen, Germany) with the following conditions: initial denaturation at 96 °C for 5 min, followed by 35 cycles of 96 °C for 30 s, 53 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min. Amplified fragments were separated on an ABI capillary sequencer (Life technologies, Carlsbad CA, USA) using

Table 1 Average annual temperatures and precipitation in each sampling location of the Czech Republic. Data obtained from the Czech Hydrometeorological Institute. The extent of the agricultural area in each region is indicated including the percentage of winter and spring wheat

Location	Annual mean temperature (°C)	January mean temperature (°C)	July mean temperature (°C)	Precipitation range (mm)	Vegetation days[a]	Agricultural area total (hectares)	% winter wheat of total area	% spring wheat of total area
I	8–9	−3	18	500–600	160–177	488,800	35	3
II	7–8	−3	17	500–600	142–159	195,000	31	2
III	6–8	−4	16	700–800	142–159	175,600	28	2
IV	8–9	−3	18	500–600	160–177	96,300	35	2
V	6–7	−4	16	600–700	124–141	279,000	25	2
VI	7–8	−3	17	600–700	160–177	124,200	32	2
VII	6–7	−4	16	600–800	142–159	32,800	38	2

[a] Number of days per year with a temperature >10 °C (Moravec and Votýpka 1998)

GeneScan-500 LIZ as internal size standard (Life Technologies, Carlsbad CA, USA).

Mating type determination

The mating type idiomorphs were determined by a multiplex PCR amplification using MAT1-1 and MAT1-2 primer pairs corresponding to the two idiomorphs of the MAT locus (Waalwijk et al. 2002). Each 25 µl PCR reaction contained 0.5 µM of each of the four primers, 0.25 mM of dNTPs, reaction buffer and 0.04 unit of Taq polymerase (Fermentas, Mont-sur-Lausanne, Switzerland). PCR amplification was carried out using a thermal cycler (MJResearch PTC-200; Bio-Rad, Hercules CA, USA) with the following conditions: initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 68 °C for 30 s, 72 °C for 60 s and final extension at 72 °C for 10 min. PCR products were separated on a 1 % agarose gel in 1X TBE buffer at 100 V for 1 h. Isolates carrying the MAT 1-1 allele could be scored by a specific 340 bp PCR product and isolates carrying the MAT1-2 allele by a specific 600 bp PCR product.

Detection of QoI fungicide resistance genotypes

Detection of the QoI resistance A143 allele was performed using a PCR-RFLP technique described by Torriani et al. (2009). Amplification of the mitochondrial *cytb* gene was followed by a restriction digestion with the enzyme *SatI* (Fermentas, Mont-sur-Lausanne, Switzerland). Digestion products were separated on agarose gels as described above. Resistant isolates carrying an A143 allele generated three restriction fragments (298 bp, 210 bp and 144 bp) and sensitive isolates with a G143 allele generated two restriction fragments (442 bp and 210 bp).

Fungicide resistance assays based on mycelial growth

We tested the effect of carrying different QoI resistance alleles on mycelial growth. We randomly selected three isolates carrying the QoI-resistant allele each from a different population (III, V, VI). In addition, we randomly selected 12 isolates carrying the QoI-sensitive allele. These isolates were selected from all seven populations. We used 5 µl of a calibrated spore suspension (100 spores/µl) to inoculate YMA plates containing 0.5 mM salicylhydroxamic acid (SHAM) in order to control for the effect of a potential alternative respiration pathway. In the fungicide assay plates, we added 1 ppm of technical grade azoxystrobin (Amistar, Syngenta, Prague, Czech Republic). Both the SHAM and azoxystrobin were dissolved in methanol. The final concentration of methanol in the media was 0.3 %v/v for all treatments. Each isolate was replicated three times per plate and all plates were replicated three times. The inoculated YMA plates were incubated in the dark at 20 °C for 14 days.

Population genetic analyses

Raw electropherograms from the capillary sequencer were scored with GeneMapper 4.0 software (Life technologies, Carlsbad CA, USA). Isolates showing identical alleles for all eight loci within populations were considered clones and only a single representative haplotype was used for subsequent analyses. The GenoDive 2.0 software (Meirmans and Van Tienderen 2004) was used to calculate allele frequencies, Nei's genotypic diversity (G), gene diversity (H) and the fixation index (G_{ST}) of Nei (1973) for each population. We used the $\chi 2$ statistic to test for even distribution of MAT1-1 and MAT1-2 alleles in the populations. Randomness of associations among microsatellite loci was tested by measuring the index of association *IA* using LIAN 3.5 (Haubold and Hudson 2000).

Genetic differentiation and gene flow among European populations

To obtain a broader picture of the population genetic relationships among European populations, we included microsatellite data from a recent study by Boukef et al. (2012). This study included samples from three major wheat-growing regions in Western Europe: Great Britain (n=32), Germany (n=32) and Switzerland (n=32), typed using the same eight microsatellite loci as the Czech populations. We used the hierarchical analysis of molecular variance (AMOVA) implemented in GenoDive 2.0 to determine the degree of genetic differentiation among populations and among countries of origin.

We used the Bayesian algorithms implemented in the software STRUCTURE (Pritchard et al. 2000) to identify population structure without predefined entities. Initial runs with 20 replicates for groups of K=1

to 15 consisted of a burn-in period of 5000 and a Markov chain Monte Carlo (MCMC) sampling of 50,000 generations. We identified the most likely number of groups by plotting the ΔK values according to Evanno et al. (2005), which determines the second-order rate of change in the distribution of the L(K) values. We repeated the analyses for the most likely K values with a burn-in of 500,000 and a MCMC sampling of 1,000,000.

Estimates of recent migration rates were performed with the software BayesAss (version 3; Rannala and Yang 2003). The Bayesian approach estimates the inferred posterior mean of m, the proportion of genotypes in a population composed of migrants over the last few generations. We used the following priors to perform the MCMC. The mixing parameter for allele frequencies was initially set to 0.3 and the mixing parameter for inbreeding coefficients was set to 0.1. We allowed for a burn-in of 1,000,000 iterations and a MCMC sampling of 10,000,000 iterations. We verified that the large log-probability fluctuations were restricted to the burn-in phase and that during the MCMC sampling no major oscillations occurred biasing parameter estimates.

In addition to the estimates of recent migration rates, we used the maximum-likelihood approach implemented in the software MIGRATE-N (version 3.2.16; Beerli 2011) to estimate historic migration rates. We encoded the haploid data as diploids with one phase as missing values, according to the software's instructions. The starting values for the migration rates were estimates based on pairwise F_{ST}. We employed 10 short chains and three long chains with a burn-in phase of 10,000 trees for each chain and a four-temperature, adaptive heating scheme. To account for the mutation mode at microsatellite loci, we set the parameters to account for a Brownian mutation model for microsatellite locus mutations. The Brownian mutation model closely follows the classic stepwise mutation model but is less computationally costly.

Results

Genetic diversity and structure of Czech populations

All microsatellite loci were polymorphic. The total number of alleles was 74 and the number per locus ranged from 4 (TCC-08) to 14 (GGC-01). Eight isolates that did not amplify for more than two loci were removed from the data set. In total, we included 184 isolates in the analyses and found 158 unique multilocus haplotypes. 17 clones were found within lesions or field populations but nine haplotypes were shared among different populations. In two cases identical haplotypes had different mating types and were therefore not considered clones. Six haplotypes occurred multiple times in different populations during the same sampling year. Furthermore, we found four haplotypes at the same site multiple times but in different sampling years. The most frequent haplotype was shared among five populations (I, III-VI). The genotype diversity was highest in population VII (n=23 unique haplotypes) and lowest in population III (n=24 unique haplotypes). Nei's measure of gene diversity per population ranged from 0.44 to 0.58 (Table 2).

We found low genetic differentiation among Czech populations. Pair-wise comparisons among populations are given in Table 3. The highest genetic differentiation was found between populations I and VIII (G_{ST}=0.07, p=0.001). We found no significant effect of geographic distance on the genetic differentiation among pairs of populations (Mantel test; Z-statistic=54.597, p=0.601). AMOVA analyses showed that 97.6 % of genetic variation could be explained by differences among individuals within field populations and 2.4 % by differences among populations. We found higher differentiation after forming two groups of populations representing either more humid or drier regions of the Czech Republic (hierarchical G_{ST}= 0.03, p=0.004).

Mating type distribution and sexual reproduction

The PCR amplification of the mating type idiomorphs produced single amplicons in all isolates corresponding to either MAT1-1 or MAT1-2. In the clone-corrected set of isolates, 47 % were MAT1-1 and 53 % were MAT1-2. The ratio of mating type idiomorphs calculated individually for each population did not differ significantly from the expected 1:1 ratio (Table 2). We detected no significant deviation from random marker association based on the standardized index of association.

Table 2 Population genetic summary of the sampled populations of *Mycosphaerella graminicola* in the Czech Republic based on eight microsatellite markers

Population	Location	Year(s)	Sample size	No of genotypes	G[a]	H[b]	MAT1-1[c]	MAT1-2[c]	$\chi 2$[d]
I	Praha	2005–2009	34	28	0,988	0,511	0,50	0,50	ns
II	Plzen	2005–2009	14	13	0,989	0,494	0,46	0,54	ns
III	Usti nad Orlici	2005–2009	31	24	0,981	0,439	0,43	0,57	ns
IV	Kromeriz	2008–2009, 2011	38	32	0,987	0,584	0,41	0,59	ns
V	Jihlava	2008–2009, 2011	21	18	0,986	0,512	0,45	0,55	ns
VI	Opava	2011	23	20	0,984	0,55	0,55	0,45	ns
VII	Cheb	2011	23	23	1	0,499	0,50	0,50	ns
	overall	–	184	158	0,998	0,523	0,47	0,53	ns

[a] Nei's genotypic diversity

[b] Nei's gene diversity

[c] MAT1-1 and MAT1-2: frequency of mating types

[d] $\chi 2$ test for deviation from a 50:50 ratio ($p<0.05$), ns: non-significant.

Genetic differentiation of Czech populations compared to Western Europe

The analysis of genetic differentiation including populations from Germany, Great Britain, Switzerland and the combined Czech populations showed that 3.2 % ($p=0.001$) of the total genetic variance was explained by country of origin. A further 1.7 % ($p=0.009$) of the total genetic variance was explained by variation among Czech populations. We found no shared haplotype between Western European and Czech populations.

We performed Bayesian analyses of population structure without prior assumptions using the software STRUCTURE. We determined that $K=2$ was the lowest number of groups supported by a high ΔK value. This assignment closely grouped the populations from Switzerland, United Kingdom and Germany into one group and all Czech Republic populations into the second group (Fig. 2b). All Czech populations showed some isolates with high posterior probability assignments to the Western European populations while one German isolate showed a high posterior probability of being assigned to the Czech population.

We estimated recent migration events using BayesAss. The MCMC sampling showed no major oscillations and auto-correlation over the sampling iterations, indicating good convergence for the migration rates. Significant migration was found from Germany and the United Kingdom to Switzerland, but this migration was asymmetrical as Switzerland contributed very few migrants to the United Kingdom and Germany (Table 4). Migration from Germany, Switzerland and the United Kingdom into the Czech Republic was relatively low, ranging between 0.71 % and 3.38 %, but all migration rates were significantly different from 0. Similarly, we detected low rates of migration from the Czech Republic into Western

Table 3 Population differentiation measured by G_{ST} (above the diagonal) among the seven Czech *M. graminicola* populations with p-values below the diagonal. *Asterisk* indicate significant *p*-values with a sequential Bonferroni correction ($\alpha=0.05$)

G_{ST}/*p*-value	I	II	III	IV	V	VI	VII
I	–	−0.01	−0.01	0,03	0	0.02	0.07
II	0,76	–	−0.03	0	−0.03	−0.01	0,05
III	0,63	0,95	–	0,03	0	0,03	0,04
IV	0,03	0,56	0,03	–	0.01	−0.02	0,04
V	0,44	0,91	0,60	0,25	–	0.01	0,04
VI	0,08	0,58	0,05	0,97	0,30	–	0.02
VII	0,00*	0,02	0,02	0,00*	0,02	0,07	–

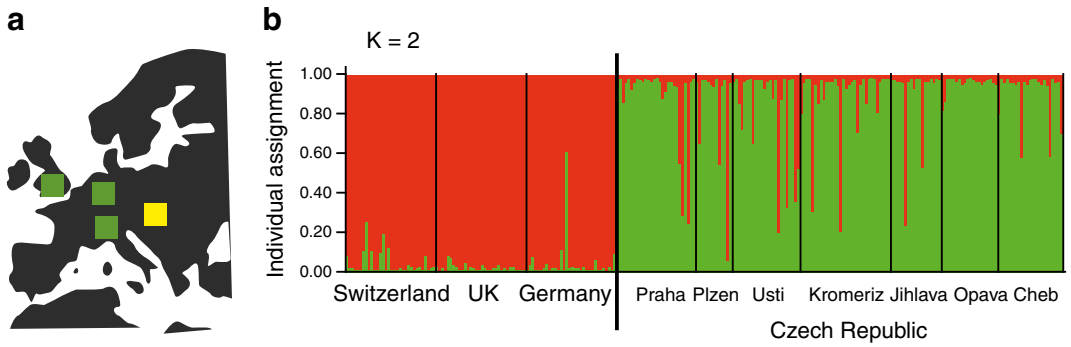

Fig. 2 Analysis of population structure using Bayesian individual assignment to clusters. **a** Schematic map of the sampling locations in the United Kingdom, Germany and Switzerland (*green squares*) and the Czech Republic (*yellow square*). **b** Posterior probability of the individual assignment to two clusters (K=2) showing the groupings Western Europe and the Czech Republic

European (0.78 – 3.69 %). Within the Czech Republic, we found a high contribution of immigrants to the Kromeriz population (IV) from all other Czech populations (20.78–24.76 %). This migration was very asymmetrical as opposing migration rate estimates were small, all ranging from 0.81 to 0.84 %.

Historical migration rates were estimated based on the maximum-likelihood estimates implemented in MIGRATE-N. We pooled all Czech populations and estimated the long-term migration rate M (scaled by the mutation rate) between Czech Republic and the three Western European populations. We found moderate levels of historic gene flow among the four major sampling locations within Europe (Fig. 3). Gene flow was generally similar to the estimated mutation rate (M=1). Significant asymmetric gene flow was detected both from Germany into Czech populations and from the United Kingdom into Czech populations.

QoI resistance in Czech populations

We found no resistant alleles at the *QoI* locus in the 32 isolates sampled prior to 2007. The first QoI resistance alleles were found in the sample collected in 2007 from the Usti nad Orlici population where six out of 10 isolates (60 %) carried the A143 resistance allele. Samples collected in neighbouring regions in 2008 showed a frequency of the A143 allele of 35 %. The most recent collection from 2011 showed the highest frequency of resistant isolates in all populations (average 42 %), with the Jihlava population being completely resistant (Fig. 1b, Table 5).

We performed a QoI sensitivity assay with azoxystrobin on three isolates carrying the QoI-resistant allele and 12 isolates carrying the QoI-sensitive allele. We found a consistent association with azoxystrobin sensitivity and the QoI-sensitive allele, as all tested isolates carrying the QoI-sensitive allele showed no mycelial growth at 1 ppm azoxystrobin (Fig. 4). Isolates carrying the QoI-resistant allele showed mycelial growth at the same azoxystrobin concentration.

Discussion

Our microsatellite analyses found a high degree of genetic diversity and low clonal fractions within all Czech *M. graminicola* populations. Both mating types of *M. graminicola* were found at equilibrium frequencies in all populations and no significant deviation from random association was found for the marker loci. Few haplotypes were shared among different populations. These findings are in agreement with previous studies, which revealed high genetic diversity in natural field populations across major wheat-producing areas worldwide (Zhan et al. 2003, 2002a). Our findings indicate that Czech populations of *M. graminicola* most likely experience frequent sexual reproduction. Regular sexual recombination is thought to allow a pathogen to adapt more rapidly to its environment, including the deployment of resistant host cultivars or new classes of fungicides (McDonald and Linde 2002b).

Czech *M. graminicola* populations showed very weak genetic differentiation. This is in agreement with

Table 4 Migration rate estimates (% of the population composed of recent immigrants) by BayesAss between all Czech Republic populations and the three Western European populations of *Mycosphaerella graminicola* with 95 % confidence intervals indicated in parentheses

Source of migrants		Destination of migrants				Czech Republic											
		Switzerland		UK		Germany		Praha		Plzen		Usti		Kromeriz		Jihlava		Opava		Cheb	
Switzerland		89.73	(3.48)	0.86	(0.83)	0.86	(0.84)	0.93	(0.90)	0.85	(0.83)	0.84	(0.83)	3.38	(2.98)	0.84	(0.81)	0.84	(0.82)	0.87	(0.84)
UK		26.22	(2.15)	67.49	(0.80)	0.71	(0.70)	0.71	(0.70)	0.71	(0.69)	0.72	(0.70)	1.29	(1.16)	0.71	(0.69)	0.72	(0.70)	0.72	(0.70)
Germany		26.18	(2.14)	0.72	(0.70)	67.50	(0.80)	0.72	(0.70)	0.71	(0.69)	0.71	(0.69)	1.32	(1.16)	0.71	(0.70)	0.72	(0.70)	0.72	(0.70)
Czech Republic	Praha	2.23	(2.00)	0.78	(0.75)	0.77	(0.75)	67.58	(0.87)	0.77	(0.75)	0.78	(0.76)	24.76	(2.76)	0.78	(0.75)	0.77	(0.75)	0.78	(0.75)
	Plzen	2.41	(2.24)	1.22	(1.17)	1.22	(1.16)	1.22	(1.18)	68.25	(1.46)	1.23	(1.18)	20.78	(3.57)	1.22	(1.17)	1.22	(1.16)	1.23	(1.17)
	Usti	1.99	(1.76)	0.87	(0.84)	0.86	(0.83)	0.87	(0.84)	0.87	(0.84)	67.71	(1.00)	24.21	(2.72)	0.87	(0.83)	0.87	(0.84)	0.87	(0.84)
	Kromeriz	3.69	(2.72)	0.82	(0.81)	0.83	(0.80)	0.84	(0.80)	0.82	(0.79)	0.81	(0.80)	89.75	(3.24)	0.81	(0.80)	0.83	(0.80)	0.82	(0.80)
	Jihlava	1.91	(1.90)	1.02	(0.98)	1.03	(0.99)	1.03	(0.98)	1.02	(0.98)	1.02	(0.98)	22.98	(3.12)	67.94	(1.20)	1.02	(0.98)	1.03	(0.98)
	Opava	1.30	(1.21)	0.96	(0.92)	0.95	(0.91)	0.96	(0.93)	0.97	(0.93)	0.96	(0.93)	24.13	(2.60)	0.96	(0.93)	67.85	(1.10)	0.96	(0.91)
	Cheb	1.70	(1.49)	0.88	(0.85)	0.88	(0.85)	0.87	(0.84)	0.87	(0.84)	0.86	(0.84)	24.47	(2.59)	0.88	(0.85)	0.87	(0.84)	67.72	(1.00)

relatively low levels of differentiation reported earlier in Western European populations (Schnieder et al. 2001; El Chartouni et al. 2011), as well as globally (Zhan et al. 2003). We detected an increased genetic differentiation between different climatic regions. However, geographic distance may also play a role in the observed differentiation, despite the lack of a significant isolation-by-distance effect among pairs of populations. *M. graminicola* generally causes more serious damage in wet and colder regions (Eyal et al. 1985). Thus, the genetic differentiation between the drier and more humid regions of the Czech Republic may reflect local adaptation to differences in fungicide applications, climate or wheat cultivars. Evidence for asymmetrical gene flow between populations was also found within the Czech Republic, with a much higher indicated influx of migrants into population IV (Kromeriz) compared to the other Czech populations. Kromeriz is located in the warmest and driest region of the Czech Republic and is also home to an important cereal breeding station. We hypothesize that the observed asymmetry of gene flow may be associated with the movement of infected seeds or plant material from around the country into this breeding station.

In general, little differentiation has been found among *M. graminicola* populations worldwide (Zhan et al. 2003). But stronger population structure was observed in France (El Chartouni et al. 2011), between California, Kansas, Indiana and North Dakota in the USA (Kabbage et al. 2009; Gurung et al. 2011) and in Iran (Abrinbana et al. 2010). Local adaptation to climate and agricultural practices were proposed as explanations for the French and USA populations, while the elevated population structure and genetic diversity in the Iranian populations are consistent with the proposed centre of origin of *M. graminicola* in the Fertile Crescent (Stukenbrock et al. 2007). Even though genetic structure data are available for *M. graminicola* for many geographical regions, comparing the results of different studies can be problematic. Differences in the nature and number of molecular markers, the level of detected polymorphism and the statistical methods used for data analyses can make comparisons of different studies impractical. Different genetic markers are known to produce different levels of genetic structure for the same samples (see, e.g., Nybom 2004). A comprehensive analysis including the full geographic range of *M. graminicola* populations based on the same marker (e.g., microsatellites)

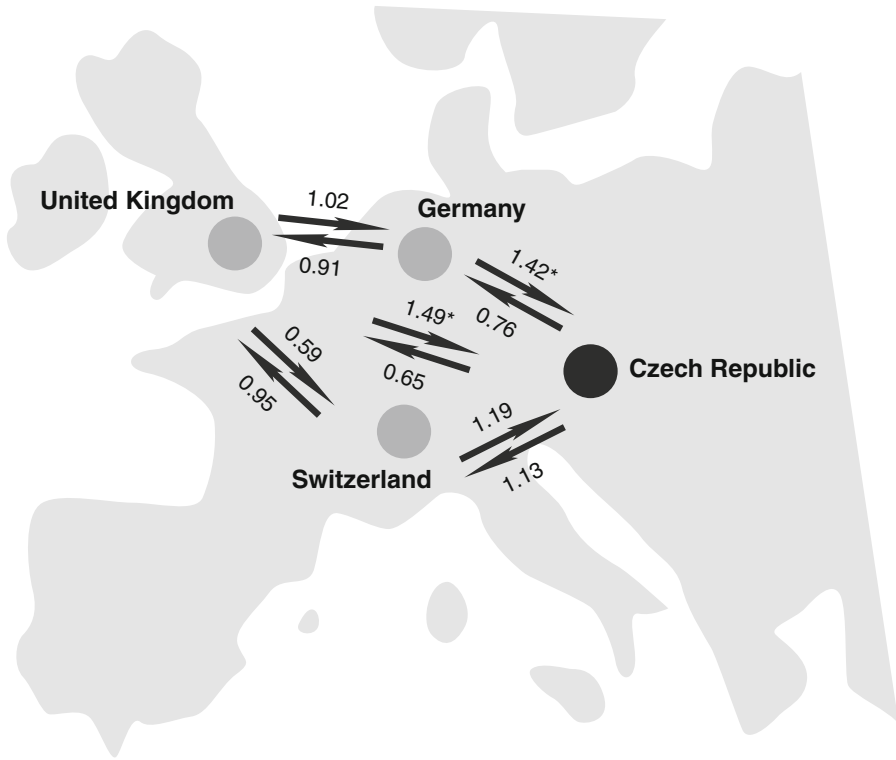

Fig. 3 Maximum-likelihood estimates of historic migration rates among the Czech Republic (treated as a single population), Switzerland, Germany and the United Kingdom. The numbers above the *arrows* indicate the directional migration rate M (scaled by the mutation rate). *Asterisks* indicate significant asymmetrical gene flow as per a separation of the 95 % confidence intervals of the maximum likelihood estimates of the migration rate M

would be a key step in better understanding the variable levels of population structure found in some geographic regions. A fundamental issue in accurately estimating dispersal rates in wind-borne organisms is the turbulent nature of wind dispersal. Dispersal in wind-borne organisms is, hence, significantly shaped by a power-law behaviour at the tail end of the distribution and more appropriate statistics for genetic differentiation should be applied (see, e.g., Wingen et al. 2007).

Based on the microsatellite analyses, Czech populations showed a marked differentiation compared to Western European populations (Switzerland, Great Britain and Germany). Within Western Europe, we observed very weak population differentiation. Genetic differentiation between populations may arise neutrally through reduced migration or through local adaptation. Very recent gene flow (over the past few generations) was detected mostly from Germany and the United Kingdom into the Swiss population. The

Table 5 Summary of all isolates of *Mycosphaerella graminicola* in each sampling location assayed for the G143A QoI fungicide-resistance mutation

Location	2005		2006		2007		2008		2009		2011	
	Total no	Resistant	Total no	Resistant	Total no	Resistant	Total no	Resistant	Total no	Resistant	Total no	Resistant
I	9	0	12	0	6	0	6	2	5	2	–	–
II	–	–	5	0	4	0	–	–	5	0	–	–
III	3	0	3	0	10	6	4	3	11	7	–	–
IV	–	–	–	–	–	–	9	0	1	0	30	23
V	–	–	–	–	1	1	–	–	2	1	20	20
VI	–	–	–	–	–	–	–	–	–	–	23	6
VII	–	–	–	–	–	–	–	–	–	–	23	16

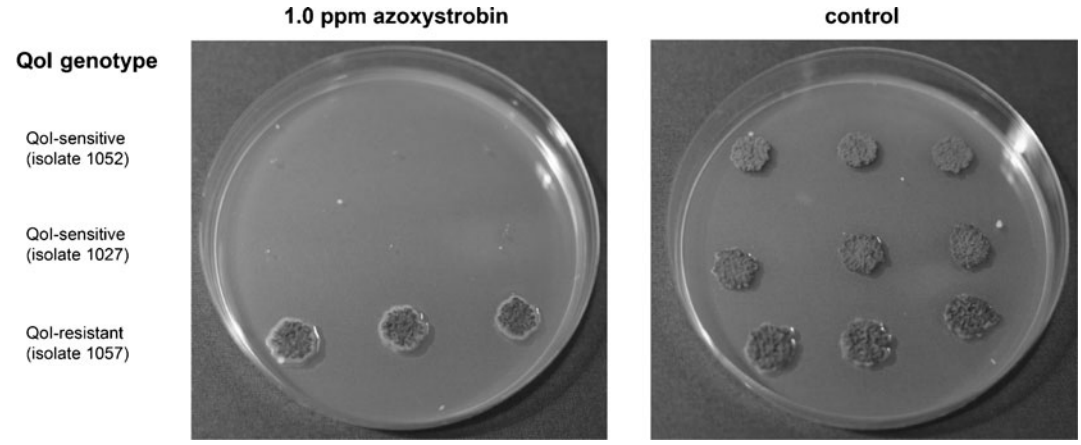

Fig. 4 In vitro QoI fungicide sensitivity assay. Two isolates carrying the QoI-sensitive allele and one isolate carrying the QoI-resistent allele are shown. Each isolate was replicated three times on each plate. The *left* image represents a YMA plate containing 1 ppm azoxystrobin and the *right* image shows a control YMA plate

Czech populations showed higher genetic differentiation and reduced levels of recent gene flow compared to those in Western Europe. However, historic gene flow was asymmetrical from the United Kingdom and Germany into the Czech Republic. Gene flow originating from Germany and the United Kingdom into Switzerland and the Czech Republic may partially be explained by the large differences in wheat production among the different countries. Germany and the United Kingdom produced 24.11 million metric tons (MMT) and 14.88 MMT in 2010, respectively (FAOstat 2012). Switzerland and the Czech Republic produced 0.52 and 4.16 MMT, respectively. Large wheat producing areas may provide large source populations for *M. graminicola* spore dispersal.

Among the possible reasons for the distinct Czech populations may be the long period of collective farming (1950–1990) imposed by the socialist regime. Agricultural fields were collectivized after the Second World War and during this process German and French wheat cultivars were replaced by cultivars originating mostly from Eastern Europe. Effective replacement of susceptible cultivars may have been slow compared to Western European agricultural practice due to ineffective, centralized planning. Furthermore, following the velvet revolution in 1993, Czech agriculture experienced a decline due to ownership changes (Swinnen et al. 2009). To further elucidate the impact of the very different agricultural practices in the Czech Republic on *M. graminicola* populations, other Eastern European countries should be included in future comparative studies. An informative comparison would include collections from Slovakia and Poland as these countries had similar agricultural practices during the last century.

Two non-exclusive hypotheses may explain the emergence and spread of QoI resistance in the Czech Republic. Either the fungicide resistance emerged through gene flow from Western European populations that acquired QoI resistance earlier than populations in the Czech Republic, or the QoI resistance emerged independently in the Czech Republic. We found that *M. graminicola* populations underwent a rapid shift from almost complete sensitivity to almost complete resistance to the QoI class of fungicides within 6 years. Strobilurin fungicides were first sold in the Czech Republic in 1997. The application of strobilurins increased from 5,700 kg in 2000 to 18,700 kg in 2007 and to 31,700 kg in 2011 (State Phytosanitary Administration, 2012). Strobilurins are currently applied to approximately 90 % of the wheat fields (State Phytosanitary Administration, 2012). Hence, the rapid increase in QoI-resistant alleles, tracks the rise in strobilurin application across the Czech Republic. The first occurrence of resistant strains was most likely in the Českomoravská vrchovina region (populations III and V). This region is situated at relatively high elevation and receives higher amounts of precipitation than other regions. These are favorable conditions for most leaf pathogens and significant yield losses caused by *M. graminicola* were first reported from this region. Consequently,

more frequent fungicide treatments were most likely applied by affected farmers, thereby selecting for resistant strains that were already present but at a low frequency. Populations I and IV were sampled from small experimental field plots that had never been treated with fungicides in the years prior to our sampling. The high frequency of resistant isolates found in these fields suggests that resistant genotypes were immigrants from neighbouring treated fields, likely a result of wind-dispersed ascospores. A complicating factor in effective containment of QoI resistance is that the G143A mutation seems to incur no fitness cost to the fungus (Gisi et al. 2002). Therefore, a pathogen population gaining QoI resistance through a selective sweep imposed by applications of QoI fungicide is likely to maintain the resistance even after fungicide application is reduced or stopped entirely.

The emergence and rapid increase in frequency of QoI-resistant haplotypes was already reported in Western Europe (Fraaije et al. 2005). The resistance emerged independently in different genetic backgrounds and geographical regions and spread rapidly through wind dispersal of ascospores (Torriani et al. 2009). The historic asymmetric gene flow from Western Europe may have introduced QoI-resistant alleles into Czech populations. The significant levels of gene flow among populations in the Czech Republic would likely have contributed to the rapid subsequent spread of QoI resistance to the different regions once QoI resistance was established in the Czech Republic. However, two factors suggest that QoI resistance may have independently arisen in the Czech Republic. First, recent gene flow was more constrained between Western Europe and the Czech Republic, making it less likely that QoI-resistant alleles spread quickly from neighbouring countries. Second, the QoI-resistant alleles were first detected in the central region of the Czech Republic (locations III and V) and subsequently found in the neighbouring location I. This suggests that QoI resistance may have emerged and spread from the centre of the Czech Republic to the periphery. However, our collections do not contain isolates from all locations from the earlier sampling years and we may have missed some earlier emergence of QoI resistance. The current fungicide application strategy for Czech wheat fields consists of 1-2 applications of a combination of azoles and QoI fungicides (Jan Kazda, pers. comm.). However, the rapid emergence of QoI resistance casts doubt on the effectiveness of such fungicide treatments to contain STB. Broad resistance screening of European wheat cultivars (e.g., Schilly et al. 2011) may provide a more sustainable containment strategy for STB. Furthermore, a switch to modern combinations of multi-site and SDHI fungicides may help to contain yield losses.

Our analyses revealed a low degree of genetic differentiation among Czech populations and confirmed frequent sexual reproduction of the pathogen. Moderate differentiation was observed between Western European populations and the Czech Republic suggesting that differences in wheat cultivars and agricultural regime affected population structure. QoI resistance emerged later than in Western Europe and may have been an independent event. As elsewhere, the resistance rapidly increased in frequency spread across regions.

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