Research Article

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QoI resistance emerged independently at least 4 times in European populations of *Mycosphaerella graminicola*

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Abstract

BACKGROUND: QoI fungicides or quinone outside inhibitors (also called strobilurins) have been widely used to control agriculturally important fungal pathogens since their introduction in 1996. Strobilurins block the respiration pathway by inhibiting the cytochrome bc1 complex in mitochondria. Several plant pathogenic fungi have developed field resistance. The first QoI resistance in *Mycosphaerella graminicola* (Fuckel) Schroter was detected retrospectively in UK in 2001 at a low frequency in QoI-treated plots. During the following seasons, resistance reached high frequencies across northern Europe. The aim of this study was to identify the main evolutionary forces driving the rapid emergence and spread of QoI resistance in *M. graminicola* populations.

RESULTS: The G143A mutation causing QoI resistance was first detected during 2002 in all tested populations and in eight distinct mtDNA sequence haplotypes. By 2004, 24 different mtDNA haplotypes contained the G143A mutation. Phylogenetic analysis showed that strobilurin resistance was acquired independently through at least four recurrent mutations at the same site of cytochrome b. Estimates of directional migration rates showed that the majority of gene flow in Europe had occurred in a west-to-east direction.

CONCLUSION: This study demonstrated that recurring mutations independently introduced the QoI resistance allele into different genetic and geographic backgrounds. The resistant haplotypes then increased in frequency owing to the strong fungicide selection and spread eastward through wind dispersal of ascospores.

Keywords: mitochondrial genome (mtDNA); *Septoria tritici*; QoI fungicides; strobilurins; resistance

1 INTRODUCTION

Mycosphaerella graminicola (Fuckel) Schroter (anamorph: *Septoria tritici* Roberge) is the causal agent of Septoria tritici leaf blotch, one of the most important foliar diseases of wheat in many parts of the world.[1,2] *Mycosphaerella graminicola* is a haploid heterothallic ascomycete characterized by both sexual and asexual reproduction. Sexual spores play an important role as the primary source of inoculum initiating an epidemic,[3–5] but they also contribute to secondary infection and can be dispersed over tens of kilometres.[6] The asexual pycnidiospores are disseminated over limited distances from plant to plant via rain splash, and they contribute only to secondary infection.[7]

Mycosphaerella graminicola was controlled mainly by demethylation-inhibiting (DMI) fungicides until the introduction of strobilurin-based fungicides in 1996. This new class of fungicides provided a superior disease control and additional favourable effects on the physiology of the plant.[8] QoI fungicides act by inhibiting the Qo site of the cytochrome bc1 complex in the mitochondria, thereby blocking the electron transfer process in the respiration chain and causing an energy deficiency due to a lack of adenosine triphosphate (ATP).[9] In most pathogens, including *M. graminicola*, resistance to strobilurins is conferred by a single nucleotide change in the mitochondrial cytochrome b gene (*cyt b*), leading to an amino acid change at codon 143 from glycine to alanine (G143A).[10] In some fungi, such as *Alternaria solani* Sorauer[11] or *Pyrenophora teres* Drechsler,[12] another mutation in *cyt b* (F129L) has been identified, but this mutation results in a less resistant phenotype compared with the G143A mutation.[10,13] Recently it was postulated that, for fungi such as *A. solani* and *P. teres* that possess an intron placed directly after G143, the mutation leading to the G143A change cannot occur, because this mutation would strongly affect the splicing process, causing a deficient cytochrome b.[14] Other unknown mechanisms leading to resistance to QoI have been reported in *Venturia inaequalis* (Cooke) Winter[15] and *Podosphaera fusca* (Fr.) Braun & Shishkoff.[16]

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The first QoI-resistant isolates of *M. graminicola* were detected retrospectively in the UK in 2001 at low frequency in QoI-treated plots,[17] and subsequently in 2002 in five European countries.[18] During 2003 and 2004 the frequency of resistant isolates increased rapidly in northern Europe. The resistance is now widespread across the entire UK,[17] whereas in France and Germany resistance levels are higher in the north than in the south.[18] The north–south gradient in resistance distribution is thought to be due to differences in the intensity of QoI use because of lower disease pressure in southern regions.

The aim of this study was to clarify the evolutionary mechanisms behind the emergence and spread of QoI-resistant isolates in *M. graminicola*. The main hypotheses tested were:

1. The G143A mutation occurred only once or very few times, potentially in only one mtDNA haplotype and/or one region, and was subsequently distributed to other regions by migration.
2. The G143A mutation occurred independently in several different mtDNA haplotypes and/or geographic regions.

Parallel genetic adaptation to drugs and pesticides has been widely studied in animals for insecticides[19,20] and nematicides,[21] in plants for herbicides[22] and in bacteria for antibiotics.[23,24] However, very little is presently known about the processes driving the development of fungicide resistance alleles in plant pathogens. The unique features of mitochondrial genomes, including their uniparental inheritance, the near absence of genetic recombination, their small size and the fixed complement of genes, make it possible to clearly differentiate between multiple or single occurrences and origins of fungicide resistance in *M. graminicola*. To accomplish this, the authors sequenced three loci of the mtDNA genome and assessed the QoI resistance/sensitivity in each isolate by identifying the presence/absence of the G143A mutation.

2 MATERIALS AND METHODS

2.1 Fungal isolates

Isolates from geographically distinct European populations of *M. graminicola* were obtained from infected leaves collected from locations in Germany (GER), England (UK), Denmark (DN), Ireland (IR) and France (FR). To address the temporal dimension of evolution of QoI resistance, isolates from the same regions, whenever possible, included samples collected before the use of QoI (pre-QoI) and samples collected after the first occurrence of QoI-resistant isolates (post-QoI). In total, 181 *M. graminicola* isolates obtained from infected leaf samples by the methods previously described[25,26] were used in this study (Table 1). The pre-QoI isolates from 1992 and 1994 were part of a previously described worldwide collection of *M. graminicola*.[25,27–29] The post-QoI isolates from 2002 were supplied by Syngenta Switzerland,[18] and the German isolates from 2004 were collected close to Dedelow in the north-eastern German state of Brandenburg (Ger-D) and close to Quarnbeck in the north-western German state of Schleswig-Holstein (GER-Q).[30]

2.2 PCR-RFLP to detect the QoI-resistant allele

All 181 isolates were assayed using a PCR-RFLP approach to detect the QoI-resistant A143 allele. Using the recently published mitochondrial genome of *M. graminicola* (EU090 238),[31] two primers were designed to amplify *cyt b*. The total PCR reaction volume was 20 µL per well, containing 10 pmol of each primer (MgcytbF 5′-TCG TTA CTG GTG TTA CAC TTG C-3′ and MgcytbR 5′-GCC ATA ACA TAA TTC TCG CTG TCA CC-3′), 100 µM of each nucleotide, 2 µL of 10× PCR buffer {1× PCR buffer: 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, 2 mM $MgSO_4$, 0.1% Triton X-100, [pH 8.8]}, 1 U of *Taq* DNA polymerase (New England Biolabs, England) and 4 µL of *M. graminicola* genomic DNA (5–10 ng final DNA concentration). Thermal cycling conditions were as follows: initial hold at 96 °C for 2 min, 35 cycles of 96 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min. The final products were incubated for 5 min at 72 °C. A portion of each PCR product (10 µL) was digested using 1 U *Fnu4*HI (New England BioLabs, England) for 4 h at 37 °C. Digests were visualized on a 1% agarose gel with 1× Tris-borate-EDTA (TBE) buffer (Fig. 1).

2.3 Sequencing and sequence analysis

The mtDNA loci *Mg1*, *Mg2* and *Mg3*[31] were amplified and sequenced for all 181 isolates. Sequences were aligned and edited manually using Sequencher 4.5 (Gene Code Corporation, Ann Arbor, MI). The concatenated nucleotide sequences were then collapsed into haplotypes recording insertions and deletions (indels) with the program SNAP Workbench (Table 1; Fig. 2).[32] DnaSP[33] was used to test for recombination within and among the tested loci.

2.4 Data analysis

The Kishino–Hasegawa (KH) test,[34] as implemented in PAUP* 4.0b.10,[35] was used to test different hypotheses of resistance appearance by comparing tree topologies using the full mtDNA dataset (all codon positions). The authors compared the unconstrained trees obtained by maximum likelihood (ML) and maximum parsimony (MP) analyses, respectively, with two alternative hypotheses of haplotype genealogy: (i) resistant (R) haplotypes were assumed to be monophyletic; (ii) sensitive (S) haplotypes were assumed to be monophyletic. Results and probability values (two-tailed test) were obtained through 1000 non-parametric bootstrap replicates and the 'full optimization' criterion (Table 2).

Classic estimates of migration rates assume that populations have reached equilibrium between drift and migration and are of equal size. However, the historical timeframe under investigation is probably too short for haplotypes drawn from populations to have sorted into distinct lineages,[36] and hence these populations are probably not in equilibrium. Thus, the likelihood-based coalescent method implemented in MIGRATE version 2.1.3,[37] which does not rely on the assumption of populations in equilibrium or equal population size and therefore provides a biologically more realistic approach, was applied. Furthermore, MIGRATE estimates asymmetric/directional gene flow among populations, making this approach particularly useful in epidemiological settings. Following the authors' recommendations, a first MIGRATE run was made with the default values, using FST to find the starting parameters for the four tested populations (UK-1994, DN-1994, GER-Q-2004 and GER-D). Parameters obtained from this initial run were used to rerun the program with the following Markov chain settings: short chains 10, long chains 3, averaging over three replicates and a four-chain heating scheme.

3 RESULTS

A total of 181 strains were analysed, including 111 sensitive and 70 resistant isolates (Table 1). The first QoI-resistant isolates were found in 2002 in four sampled locations. All 77 pre-QoI samples

Table 1. Characterization of mtDNA haplotypes found in *Mycosphaerella graminicola* isolates (the number of isolates is shown in parentheses)

Haplotype	GER(20) 1992	UK (44) 1994	DN (13) 1994	UK (6) 2002	IR (5) 2002	DN (1) 2002	FR (9) 2002	GER-Q (49) 2004	GER-D (34) 2004	R (70)[a]	S (111)
H1 (5)	-	2	-	-	-	-	-	**1**	2	**1**	4
H2 (11)	4	4	-	-	-	-	2	**1**	-	**1**	10
H3 (28)	6	8	2	-	-	-	**1**	**7**	4	**8**	20
H4 (19)	3	2	1	-	-	-	-	**6**	1 6	**6**	13
H5 (2)	1	-	1	-	-	-	-	-	-	-	2
H6 (8)	-	3	-	-	-	-	**1**	**2**	2	**3**	5
H7 (2)	-	2	-	-	-	-	-	-	-	-	2
H8 (4)	-	-	-	-	-	-	-	**3**	1	**3**	1
H9 (1)	-	-	-	-	-	-	-	-	1	-	1
H10 (2)	-	-	-	-	-	**1**	-	-	1	**1**	1
H11 (1)	-	-	-	-	-	-	-	-	1	-	1
H12 (1)	-	-	-	-	-	-	-	**1**	-	**1**	-
H13 (4)	-	-	-	-	-	-	-	**4**	-	**4**	-
H14 (1)	-	1	-	-	-	-	-	-	-	-	1
H15 (1)	-	-	-	-	-	-	-	-	**1**	**1**	-
H16 (4)	-	2	1	-	-	-	-	-	1	-	4
H17 (1)	-	-	-	-	-	-	-	**1**	-	**1**	-
H18 (2)	-	-	-	-	-	-	**2**	-	-	**2**	-
H19 (1)	-	-	-	-	-	-	-	-	1	-	1
H20 (2)	-	-	-	-	-	-	-	**1**	**1**	**2**	-
H21 (1)	-	-	-	-	-	-	-	-	**1**	**1**	-
H22 (3)	-	2	1	-	-	-	-	-	-	-	3
H23 (5)	-	2	-	-	**3**	-	-	-	-	**3**	2
H24 (8)	-	2	3	**2**	-	-	-	**1**	-	**3**	5
H25 (2)	1	1	-	-	-	-	-	-	-	-	2
H26 (1)	-	1	-	-	-	-	-	-	-	-	1
H27 (1)	-	1	-	-	-	-	-	-	-	-	1
H28 (6)	2	-	3	-	-	-	1	-	-	-	6
H29 (1)	1	-	-	-	-	-	-	-	-	-	1
H30 (1)	-	1	-	-	-	-	-	-	-	-	1
H31 (1)	-	1	-	-	-	-	-	-	-	-	1
H32 (1)	-	-	-	-	-	-	-	**1**	-	**1**	-
H33 (21)	1	3	-	2	2	-	-	**11**	**2**	**13**	8
H34 (1)	-	-	-	-	-	-	-	-	1	-	1
H35 (1)	-	-	-	-	-	-	-	**1**	-	**1**	-
H36 (1)	-	1	-	-	-	-	-	-	-	-	1
H37 (1)	-	-	-	-	-	-	-	-	1	-	1
H38 (1)	-	-	-	-	-	-	1	-	-	-	1
H39 (1)	-	1	-	-	-	-	-	-	-	-	1
H40 (1)	-	1	-	-	-	-	-	-	-	-	1
H41 (5)	-	1	1	1	-	-	-	**1**	**1**	**2**	3
H42 (1)	-	-	-	-	-	-	-	-	**1**	**1**	-
H43 (2)	1	-	-	-	-	-	-	-	1	-	2
H44 (1)	-	-	-	-	-	-	-	-	1	-	1
H45 (1)	-	-	-	-	-	-	-	**1**	-	**1**	-
H46 (1)	-	-	-	-	-	-	-	**1**	-	**1**	-
H47 (1)	-	-	-	-	-	-	-	**1**	-	**1**	-
H48 (3)	-	-	-	-	-	-	-	**1**	**2**	**3**	-
H49 (4)	-	1	-	-	-	-	**1**	**1**	**1**	**3**	1
H50 (1)	-	-	-	-	-	-	-	**1**	-	**1**	-
H51 (1)	-	1	-	-	-	-	-	-	-	-	1
H52 (1)	-	-	-	**1**	-	-	-	-	-	**1**	-

[a] Resistant isolates are in bold text.

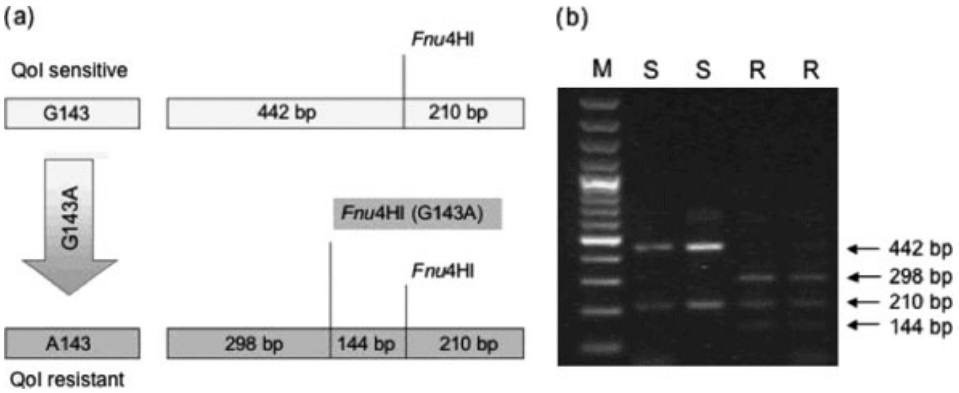

Figure 1. PCR-RFLP analysis of the *cyt b* locus for detection of the A143 strobilurin-resistant allele. (a) G143A mutation causes a second *Fnu*4HI restriction site in *cyt b*, conferring resistance to strobilurin. Molecular sizes are indicated in each fragment. (b) Example of the PCR-RFLP results for two sensitive (S) and two resistant (R) isolates. The first lane is a 100 bp size ladder (M). The size of the digested products is shown.

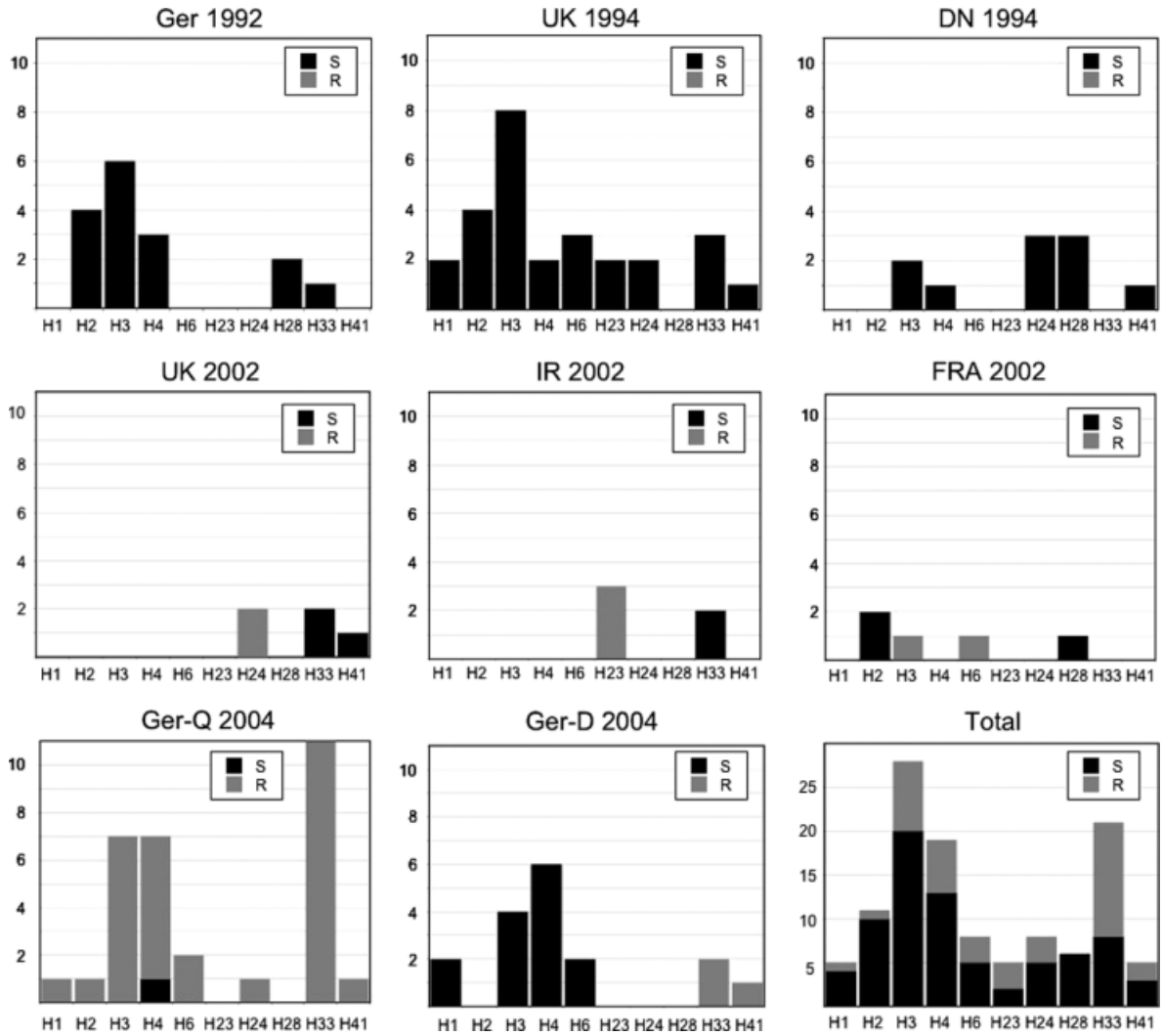

Figure 2. Comparison of sensitive (S, black) and resistant (R, grey) isolates belonging to the most frequent mitochondrial haplotypes (at least five isolates).

were sensitive, and 67% of post-QoI isolates were resistant. In 2004, GER-Q had only one sensitive isolate, while GER-D had 71% sensitive isolates (Table 1; Fig. 3a). The concatenated sequences of the 181 analysed isolates collapsed into 52 nucleotide haplotypes, designated as H1 to H52 (Table 1; Fig. 4), of which 29 included a single isolate and ten included at least five isolates (Table 1; Fig. 2). The ten most frequent haplotypes represented 64% of all isolates, including 69% of the pre-QoI and 54% of the post-QoI isolates. The remaining 42 'rare' haplotypes had on average 1.55 isolates per haplotype. The program DNAsp did not detect any recombination

Table 2. Kishino–Hasegawa tests for statistical assessment of different hypotheses of haplotype evolution by comparing tree topologies as implemented in PAUP. The unconstrained tree topologies obtained by maximum likelihood and maximum parsimony analyses, respectively, were compared with two alternative hypotheses of haplotype genealogy: (i) resistant (R) haplotypes were assumed to be monophyletic; (ii) sensitive (S) haplotypes were assumed to be monophyletic. The probabilities (*P*) of obtaining better trees were assessed using two-tailed tests, the full optimization criterion and 1000 bootstrap replicates

Tree	Tree score		Probability[a]
Maximum likelihood	$-\ln L$	$-\ln L$ difference	*P*
unconstrained	1042.71823	Best	
R-constrained	1072.48505	29.76681	0.016*
S-constrained	1072.48506	29.76683	0.016*
Maximum parsimony	Length	Length difference	
unconstrained	1158	best	
R-constrained	1207	49	<0.001*
S-constrained	1216	58	<0.001*

[a]* $P < 0.05$.

events within or between the tested mitochondrial loci *Mg1*, *Mg2* and *Mg3*.

Nine of the ten most frequent haplotypes acquired the resistance mutation independently, with only haplotype H28 having all sensitive isolates (Fig. 2). Twelve mtDNA sequence haplotypes had both resistant and sensitive strains, 24 haplotypes were exclusively sensitive and 16 were exclusively resistant. Resistant isolates were first detected in 2002 in all tested geographical populations and belonged to eight distinct mtDNA sequence haplotypes out of 13 total mtDNA haplotypes detected in that year. In 2002, none of the resistant sequence haplotypes was found in more than one population. Two years later, 24 sequence haplotypes out of the 33 detected were resistant, and four of these haplotypes were found previously in 2002. KH tests were used for statistical testing of different hypotheses of haplotype evolution by comparing tree topologies. In both the ML and MP topologies, the unconstrained trees performed significantly better than the two alternative hypotheses, where trees with R-haplotypes or S-haplotypes were considered monophyletic respectively (Table 2). The unconstrained ML tree formed an unrooted cladogram and identified four major clades, all of which consisted of a mixture of R, S and mixed (RS) haplotypes (Fig. 4).

Estimates of directional migration rates indicated that the majority of gene flow in Europe had occurred in a west-to-east direction (Table 3; Fig. 3b). For example, estimates of gene flow were high ($Nm = 86$) from the UK to GER-Q, whereas there was no or little detectable gene flow in the opposite direction. Similarly, gene flow was 10 times higher from GER-Q into GER-D than in the opposite direction.

Closer inspection of the dynamics of individual haplotypes revealed some interesting patterns. H3 was the most common sequence haplotype (15.5%), found in six of the nine sampled populations and in four of the five countries sampled (Table 1; Fig. 2). H3 was the most frequent haplotype in the pre-QoI samples and the third most common in the post-QoI samples. H4 was the only sequence haplotype including resistant and sensitive individuals in the same population. The ten most frequent sequence haplotypes did not show a clear pattern of geographical association. Only H23 and H28 could be identified as 'local' haplotypes, because H23 was never found in continental

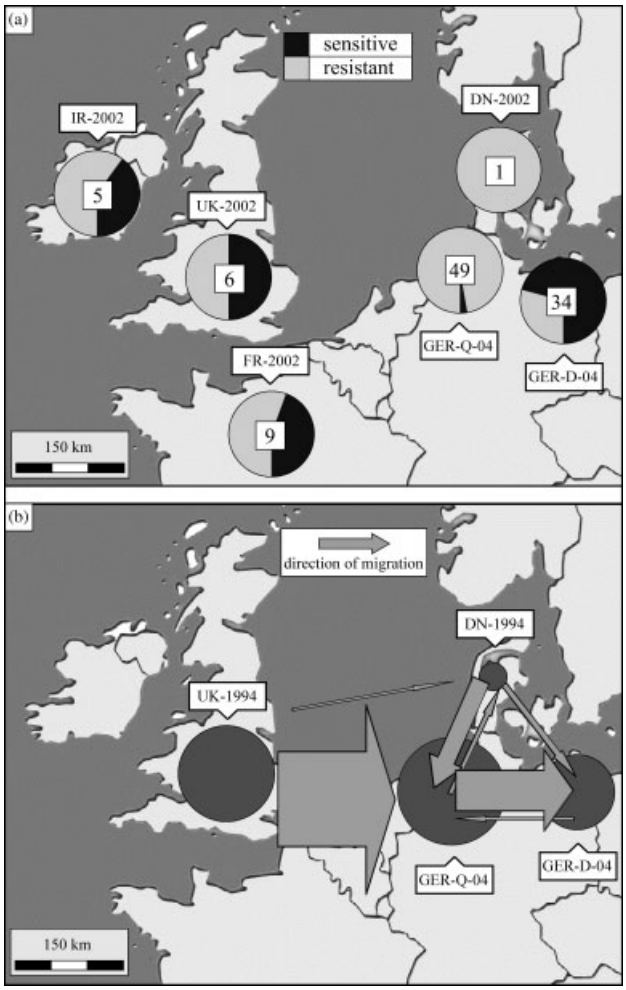

Figure 3. (a) Geographical locations of the six sampled post-QoI *Mycosphaerella graminicola* populations. The total number of isolates assayed for each population is indicated in the circles, which also show the frequencies of sensitive (black) and resistant (grey) strains. All pre-QoI populations were completely sensitive to strobilurins. (b) Migration patterns of *Mycosphaerella graminicola* among populations. Arrow sizes are proportional to the migration rate. The absence of an arrow indicates no or little detected migration ($Nm < 0.5$).

Europe, while H28 was found only on the continent. H28 had a frequency of 15.1% in GER-1992 and DN-1994 populations but was not present in the post-QoI samples of GER-Q and GER-D. This haplotype showed the strongest decrease in frequency after the introduction of strobilurins. H33 was the most frequent haplotype within post-QoI samples (16.3%), tripling in frequency compared with the pre-QoI period.

4 DISCUSSION

The main result of this study was to demonstrate unequivocally that QoI-resistant *M. graminicola* isolates arose independently in different genetic and geographic backgrounds, disproving the hypothesis that a single mutant haplotype emerged and subsequently spread across other regions in Europe. This finding indicates that recurring mutations introduced the resistance allele into several distinct mtDNA haplotypes, which then increased in frequency owing to the strong fungicide selection and spread eastward through wind dispersal of ascospores.

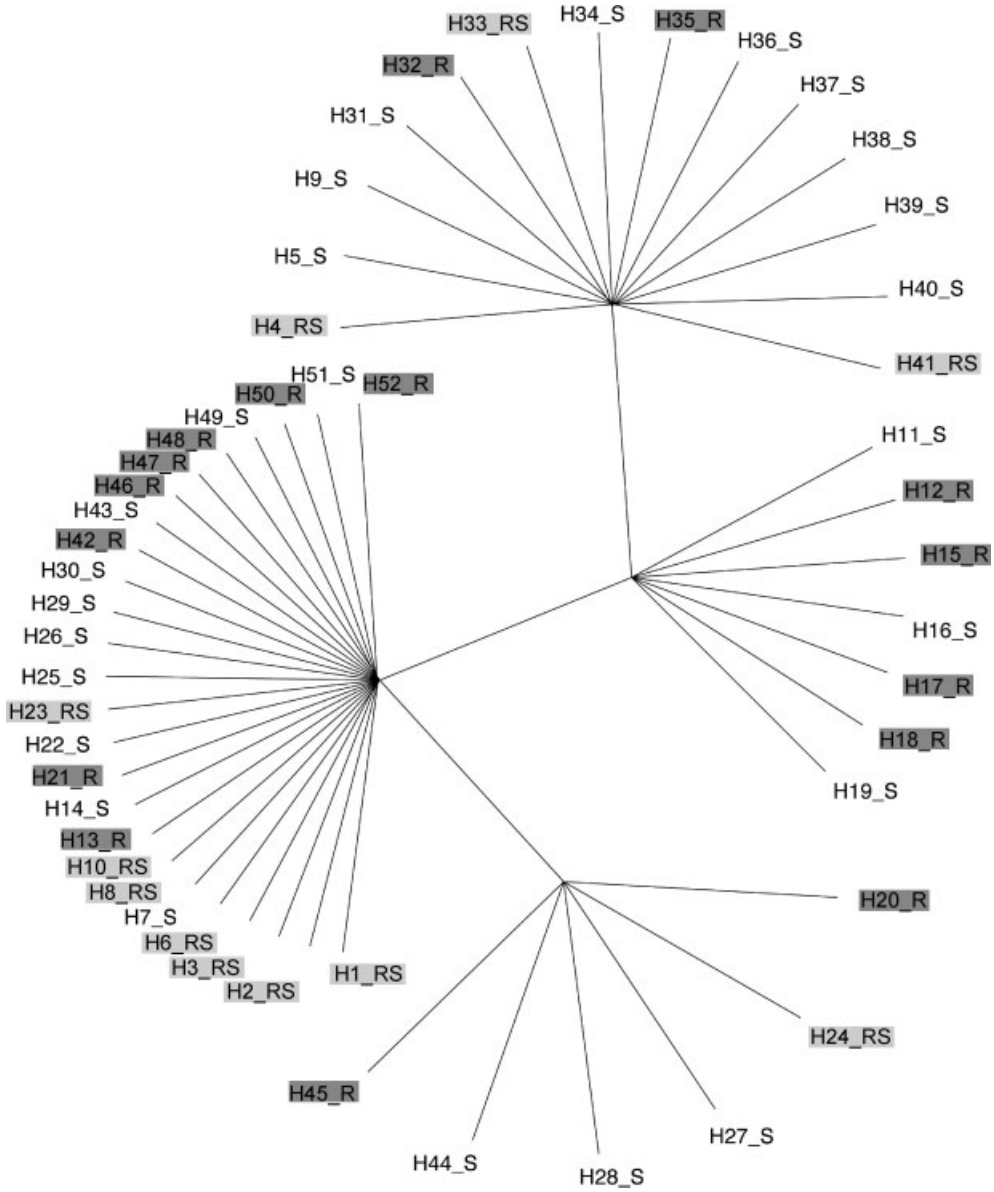

Figure 4. Unconstrained maximum likelihood tree obtained for all resistant (R, dark-grey hatched), susceptible (S, not hatched) and mixed (RS, soft-grey) mtDNA haplotypes.

Table 3. Pairwise likelihood estimates of directional migration rates between major geographical regions (95% confidence intervals in parentheses). Donor populations are shown on the left and receiving populations are given along the top. Migration rates are expressed as *Nm*, the number of immigrants per generation

	UK	DN	GER-Q	GER-D
UK	–	2.33 (0.54–5.74)	86.84 (57.67–121.55)	0.01 (0.00–0.01)
DN	0.46 (0.06–0.84)	–	16.11 (7.03–29.94)	5.52 (0.69–9.71)
GER-Q	0.25 (0.00–0.51)	4.21 (0.65–7.12)	–	37.82 (25.71–45.36)
GER-D	0.04 (0.00–0.10)	0.05 (0.00–0.08)	3.58 (0.45–5.43)	–

Since the introduction of QoI fungicides into the cereal market in 1996, QoI-fungicide-resistant isolates have been detected in field populations of a wide range of pathogen species, including *Blumeria graminis* Speer f. sp. *tritici* and *hordei*[38] and *Pyrenophora tritici-repentis* (Died.) Drechsler.[12] Resistance in *M. graminicola* emerged simultaneously in several locations during 2002, with a rapid increase in the frequency of resistant isolates during the following two seasons, reaching especially high levels in Northern Europe,[18] where the G143A resistance allele was found in more than half of the identified mtDNA sequence haplotypes (Table 1; Fig. 4). The hypothesis that the mutations to resistance emerged independently in different genetic backgrounds is clearly

supported by the unconstrained ML tree (Fig. 4), which shows that in *M. graminicola* the A143 allele was acquired through at least four distinct mutational events. Similar findings for the parallel emergence of fungicide resistance were identified for the grape pathogen *Plasmopara viticola* Berl. & de Toni.[39] This is the first time that intraspecific parallel evolution of fungicide resistance has been demonstrated for a cereal pathogen, and it confirms previous results from *P. viticola*, suggesting that *de novo* appearance of a resistance allele in distinct genetic backgrounds may be common for plant pathogenic fungi. The interspecific parallel evolution of resistance alleles was previously shown across a wide range of plant pathogens.[14]

No mutants were found when screening a worldwide collection of 1000 pre-QoI field isolates of *M. graminicola* for the presence of the A143 resistance allele. Assuming a binomial distribution for the mutation, the 95% confidence interval for the frequency of the G143A mutation in the global population ranged from 0 to 0.003 (McDonald and Sierotzki, unpublished). By 2002, eight mtDNA sequence haplotypes acquired the resistance allele. None of these resistance haplotypes was present in more than one population, indicating that 2002 was the starting point for the subsequent rapid emergence of resistant haplotypes. Published data show that the 143A allele was present at low frequency in QoI-treated plots in the UK in 2001.[17] The mutation was therefore present at detectable levels prior to 2002 but became much more common in 2002, a year of high Septoria disease pressure in NW Europe. While recurring mutations were important for driving the evolution of strobilurin resistance in *M. graminicola*, the subsequent role of natural or anthropogenic gene flow should not be underestimated. In fact, directional migration rates clearly showed the importance of gene flow in moving mtDNA haplotypes from Western to Eastern Europe (Table 3; Fig. 3b). The airborne ascospores have the potential to be dispersed over many kilometres,[40] following the prevailing wind directions across Europe. The same patterns of *M. graminicola* migration in Europe were found using the nuclear-encoded *Cyp51* locus.[30]

The unique properties of mtDNA made it possible to demonstrate unequivocally the intraspecific parallel evolution of QoI resistance. The lack of recombination found in the mtDNA of *M. graminicola* using the present and previous dataset[31] means that the G143A mutation had to emerge independently in different genetic backgrounds, instead of being shuffled into different backgrounds through sexual recombination. Most modern pesticides, such as DMI fungicides, have as their target nuclear-encoded proteins whose genes are subject to recombination.[30] For these resistances, recombination makes it difficult to determine how many times the mutation to resistance occurred, as the same mutation can be recombined into many different genetic backgrounds. The results arising from this study provide a better insight into the processes driving the development of fungicide resistance alleles in fungal populations. This study confirms the evidence for the independent multiple acquisition of the resistant allele to a fungicide at the intraspecific level in plant pathogenic fungi, and may provide new perspectives regarding the development of appropriate management strategies.

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