QoI Resistance and Mitochondrial Genetic Structure of *Zymoseptoria tritici* in Morocco

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Abstract

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In total, 230 single-conidial isolates of the fungal wheat pathogen *Zymoseptoria tritici* (formerly *Septoria tritici*, teleomorph: *Mycosphaerella graminicola*) were sampled in Morocco in 2008 and 2010 to assess resistance against quinone outside inhibitors (QoIs), a widely used group of fungicides in wheat pest management. All 134 isolates sampled in 2008 were QoI sensitive. In contrast, 9 of the 96 isolates from the 2010 collection were resistant, suggesting a recent emergence of the resistance. Mitochondrial (mt)DNA-sequence analyses identified four haplotypes among the resistant isolates. Wright's F statistics (F_{ST}) analyses from mtDNA sequences revealed a shallow population struc-

ture of *Z. tritici* within Morocco and a substantial asymmetric gene flow from Europe into Morocco. A phylogenetic reconstruction including Moroccan and European isolates clustered the haplotypes regardless of their geographic origin. The four Moroccan QoI-resistant mitochondrial haplotypes clustered in two distinct clades in the tree topology, suggesting at least two independent origins of the resistance. This study reported, for the first time, the occurrence of QoI-resistant genotypes of *Z. tritici* in Morocco. Our findings are consistent with the hypothesis that QoI resistance emerged very recently through parallel genetic adaptation in Morocco, although gene flow from Europe cannot be excluded.

Septoria tritici blotch, caused by *Zymoseptoria tritici* (formerly *Septoria tritici*, teleomorph: *Mycosphaerella graminicola*), is currently among the most frequent foliar diseases on both bread wheat (*Triticum aestivum*) and durum wheat (*T. durum*) in Morocco and worldwide. Severe disease epidemics can reduce yields by 35 to 50% (19). *Z. tritici* undergoes both asexual and sexual reproduction in the field. During asexual reproduction, it produces pycnidiospores that are locally dispersed by rain splash (19). Through sexual reproduction, the pathogen produces ascospores that are dispersed over long distances by wind (15). Although ascospores are responsible for most of the initial infections of wheat in the autumn, they also contribute to disease progression during the growing season because sexual reproduction occurs year-round (15). As a result, the pathogen population is highly diverse, even at the field scale (22,30).

Because host resistance against *Z. tritici* is not completely effective in most wheat cultivars, fungicides are used as a primary method of disease control (11). Methyl benzimidazole carbamates (MBCs) and sterol biosynthesis inhibitor (SBI) fungicides have been the most widely used groups of fungicides to control *Z. tritici* since early and late 1970s, respectively (5,16). Since their introduction in 1996, strobilurin fungicides, or quinone outside inhibitor (QoI) fungicides, were intensively applied for the management of most agricultural fungal pathogens, including *Z. tritici* (2). QoI fungicides inhibit mitochondrial respiration in fungi by binding to the Qo binding site of the cytochrome bc1 enzyme complex, thereby blocking electron transfer and halting ATP synthesis (14).

The success of QoI fungicides is due to their higher efficacy and their additional favorable effects on plant physiology (12).

The intensive use of QoI fungicides led to a rapid development of resistant genotypes of *Z. tritici* in Europe (12). As for most other fungi, fungicide resistance in *Z. tritici* is conferred by a point mutation in the mitochondrial cytochrome *b* (*cytb*) gene, giving rise to an amino-acid substitution from glycine to alanine at position 143 (G143A) (11,12). This mutation prevents binding of QoI fungicides to the Qo site, hence restoring the ability of the fungus to carry out mitochondrial respiration.

The first resistant isolates of *Z. tritici* were detected in the United Kingdom in 2001 (12), and the frequency rapidly increased across Europe (7,13,21,26). Frequencies of resistant genotypes were higher in the north-European countries such as Belgium, Denmark, Ireland, the United Kingdom, northern France, and northern Germany (1,13,16,18,21,26) than south-European areas such as southern France and southern Germany (13,16,21). This gradient of resistance was thought to be due to differences in the intensity of QoI fungicide use, because lower disease pressure occurs in southern regions (21,26).

Fungicide resistance monitoring is particularly important to ensure effective disease control and is crucial for understanding the distribution, evolution, and the impact of fungicide resistance in the field. Although QoI resistance in *Z. tritici* is currently widespread throughout Europe, recent investigations revealed an absence of fungicide resistance in Africa (4,24). To date, there is no information available regarding QoI resistance in Morocco, despite the use of QoI fungicides to control wheat pathogens, including *Z. tritici* (10). The main objectives of this work were (i) to investigate whether QoI resistance occurs in the Moroccan population of *Z. tritici* and, if present, (ii) whether resistance emerged locally or was introduced from Europe.

Materials and Methods

Fungal sampling and isolation. A set of 230 single-conidial isolates of *Z. tritici* were sampled from six distinct wheat produc-

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ing locations in Morocco (Table 1). In all, 134 isolates were first collected in 2008 from Chaouia, Gharb-Chrarda, Meknes-Tafilalet, and Tadla-Azilal. In 2010, 96 additional isolates were collected from these regions and two new locations, Doukkala-Abda and Fes-Boulmane (Table 1). Each set of isolates sampled from each location at the same time point will hereafter be referred to as a subpopulation. The isolates were collected according to the protocol described by Siah et al. (23) from nonfungicide-treated and naturally infected wheat plants. All isolates were grown on potato dextrose agar (PDA) medium for 2 weeks and stored at -80°C for further analyses.

DNA extraction. DNA was extracted from 2-week-old PDA-grown cultures according to Siah et al. (21), with modifications. Briefly, approximately 50 mg of cirrus were harvested from a PDA-grown culture of each isolate and transferred into a 1.5-ml microcentrifuge tube. After the addition of 200 μl of sterile distilled water, each tube was vortexed and sonicated (Deltasonic) for 20 min. Phenolchloroform (400 μl) was added to each tube, vortexed, and centrifuged for 5 min at 13,000 rpm and 4°C (Hettich, Zentrifugen). The aqueous phase was collected and transferred into a clean microcentrifuge tube. After addition of 300 μl of chloroform, the tubes were vortexed and centrifuged at 13,000 rpm and 4°C for 3 min. This operation was repeated twice. The aqueous phase was transferred to new clean tubes. Total DNA was precipitated overnight at -20°C after the addition of 10 μl of NaCl (5 M) and 500 μl of absolute ethanol. After centrifugation for 30 min at 14,000 rpm and 4°C , the pellets were washed with 70% ethanol. The tubes were then centrifuged for 15 min at 14,000 rpm and 4°C before they were dried for 20 min in desiccators. DNAs were solubilized in 40 μl of sterile distilled water for 20 min at 37°C and the concentrations were determined at 260 nm using a UV light spectrophotometer (UV Light). The tubes containing DNAs were then stored at -20°C for further investigations.

Polymerase chain reaction mismatch to determine G143A substitution. Sensitivity to QoI fungicides among the isolates was determined using a mismatch amplification mutation assay on part of the *cytb* gene (21,28). Primers were designed with a mismatch on the penultimate nucleotide of the 3' end of the forward primer, in which the ultimate nucleotide was at the point mutation position of codon 143. The primer set used to amplify a 639-bp DNA fragment in sensitive isolates was sense primer StrobSNP2fwd (5'-CTTATGGTCAAATGCTTTATGATG-3', nucleotides 404 to 428 of the *cytb* gene sequence), with a mismatch of T instead of G at nucleotide position 427, and antisense primer StrobSNP1rvs (5'-GGTGACTCAACGTGATAGC-3', nucleotides 1,024 to 1,043 of *cytb*). The primer set used to amplify a 302-bp DNA fragment in resistant isolates was antisense primer StrobSNPrcF7 (5'-CAA TAAGTTAGTTATAACTGTTGCGG-3', nucleotides 428 to 453 of the *cytb* gene sequence), with a mismatch of T instead of G at nucleotide 429, and sense primer StrobSNPrcR1 (5'-CTATGC ATTATAACCCTAGCGT-3', nucleotides 152 to 173). This resulted in a single-nucleotide mismatch on the sequence of the sensitive isolates and a double-nucleotide mismatch on the sequence of the resistant isolates, and vice versa for each primer set. Polymerase chain reaction (PCR) reactions and PCR-thermal cycling conditions were performed according to Siah et al. (21). PCR products were separated by electrophoresis on 1.5% agarose gels run at 100 V for 45 min. After ethidium bromide staining and visualizing under UV light, images were captured with a digital camera (Clara Vision) and scored manually.

Phenotypic evaluation of fungicide resistance. Phenotypic assays of QoI sensitivity were performed for 18 Moroccan isolates (9 isolates with the G143 allele and 9 isolates with the A143 allele) in clear and sterile flat-bottomed polystyrene microplates (Iwaki, Asahi techno glass) with eight rows of 12 wells. The QoI fungicide azoxystrobin (Sigma-Aldrich) was added to the medium at 50°C after autoclaving and after suspension in 0.5 ml of dimethyl sulfoxide (DMSO) (Merck). Final concentration of DMSO in the medium, including controls without fungicide, did not exceed 0.5 ml/liter of medium. Plate wells were filled with 150 μl of liquid

glucose peptone medium (dextrose [VWR] at 14.3 g/liter, bacto-peptone [Difco Laboratories] at 7.1 g/liter, and yeast extract [Merck] at 1.4 g/liter) amended with azoxystrobin at concentrations 0.006, 0.025, 0.1, 0.4, 1.6, 6.3, 25, and 100 ppm (final concentrations in 200 μl of medium). Aliquots of $50 \mu\text{l}$ of 2×10^5 spores/ml, which were obtained by washing a 7 day-old PDA fungal culture grown at 20°C with sterile distilled water, were added to the plate wells. Eight wells were used as replicates for each condition. Controls without fungus and without fungicide as well as medium with fungus and without fungicide were used for each isolate. After incubation for 6 days at 20°C in darkness and under horizontal shaking at 140 rpm, fungal growth was measured in a plate reader (MRX; Dynex Technologies) at 405 nm. The fungicide sensitivity of each isolate was determined as half maximal inhibitory concentration (IC_{50}) calculated using a dose-response relationship and nonlinear regression model according to the GraphPad Prism 5 software (Hearne Scientific Software). The bioassay was replicated twice and similar IC_{50} values were obtained with both replications for each isolate.

Mitochondrial DNA-sequence amplification and sequencing. Three mitochondrial (mt)DNA sequences, named *Mg1*, *Mg2*, and *Mg3* (27), were examined to estimate the mtDNA diversity of *Z. tritici* in Morocco and to reconstruct the phylogenetic relationship among Moroccan and European mtDNA haplotypes. These loci are located in different regions of the mtDNA and display different degrees of polymorphism in the comparison between the STBB1 and IPO323 isolates (27). These three sequences were amplified for 152 isolates: 100 isolates from Morocco (50 from each year of collection) and 52 reference isolates from Europe (26). Primer sets used for the amplifications were *Mg1F* (5'-CCGGTCCCTCTA ATAGTTCTGG-3') and *Mg1R* (5'-TAATCCGCCATTACTTCT CAGG-3'), *Mg2F* (5'-GGTCCCAATGGGTTTAAATGCTA-3') and *Mg2R* (5'-TGGGTGTAGCTAGAAACCCTTC-3'), and *Mg3F* (5'-AAGCTACGCCTATGGCTAACAC-3') and *Mg3R* (5'-AGGTAA GACGCACGCATTTC-3'). PCR reactions were performed in a 50- μl volume containing 0.1 μl (20 μM) of each primer, 4 μl (2.5 mM) of dNTPs, 5 μl of 10 $\times$ reaction buffer, 8 μl (25 mM) of MgCl_2 , 0.25 U of Taq polymerase (Ampli Taq Gold, Applied Biosystem, Roche), and 5 μl of fungal DNA (1 ng/ μl final DNA concentration). The amplifications were carried out using a Peltier thermal cycler (MJ Research) under the following temperature program: initial denaturation at 94°C for 10 min; followed by 40 cycles of 94°C for 1 min, 56°C for 30 s, and 72°C for 1 min; with a final extension at 72°C for 10 min. The PCR products were sequenced using the primers *Mg1F*, *Mg2F*, and *Mg3R*, generating a total of 733 concatenated nucleotides. Sequencing reactions were performed as described previously by Torriani et al. (27) for STBB1. DNA sequence alignments were done using the BioEdit software (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>).

Genetic data analyses. Standard population genetic parameters were estimated using DnaSP 5.10 (17). Hierarchical analysis of molecular variance (AMOVA) implemented in ARLEQUIN 3.5 (9) was used to determine the degree of genetic differentiation at three levels: (i) within subpopulations, (ii) among subpopulations within 2008 and 2010 populations, and (iii) between 2008 and 2010 populations. ARLEQUIN 3.5 was also used to assess Wright's F statistics (F_{ST}) for population differentiation for all population pairs.

Table 1. Number and origin of Moroccan *Zymoseptoria tritici* isolates used in this study

Location	Wheat species	Number of isolates		
		2008	2010	Total
Meknes-Tafilalet	<i>Triticum aestivum</i>	45	22	67
Gharb-Chrarda	<i>T. aestivum</i>	55	6	61
Tadla-Azilal	<i>T. aestivum</i>	11	27	37
Chaouia	<i>T. aestivum</i>	23	8	37
Doukkala-Abda	<i>T. durum</i>	...	22	17
Fes-Boulmane	<i>T. aestivum</i>	...	12	11
Total	...	134	96	230

Significance of differentiation ($P < 0.05$) was estimated with the implemented Markov chain-based exact test of population differentiation.

Maximum likelihood (ML) phylogenetic trees were constructed using MEGA version 5 (25). The ML analyses were performed using the Tamura 3-parameter model with a discrete γ with five rate categories. The model search algorithm implemented in MEGA selected this model as having the lowest Bayesian Information Criterion score, which is considered to describe the nucleotide substitution pattern the best. All positions with less than 70% site coverage were eliminated. Statistical node support was estimated using 500 bootstrap replications.

Long-term migration rates between Europe and Morocco were estimated using the ML approach implemented in MIGRATE (3).

Based on the F_{ST} analyses that suggested low genetic differentiation between populations sampled at different time points (see results below), all populations from Morocco were pooled. The starting values for the migration rates were estimates based on pairwise F_{ST} . Markov chain settings were 15 short chains, 3 long chains with a burn-in of 10,000 trees, and averaging over long chains. The analysis was repeated three times to ensure convergence of parameter estimates.

Results

Frequency and distribution of sensitive and resistant alleles.

Sensitive and resistant isolates generated the expected single fragments of 639 and 302 bp, respectively (Fig. 1). All isolates sampled in 2008 had the 639-bp allele associated with sensitivity (Fig. 2).

Fig. 1. Mismatch amplification mutation assay on a part of the *cytochrome b* gene to determine G143 or A143 allele in 18 *Zymoseptoria tritici* isolates from Morocco. Sensitive isolates carrying G143 allele generated an amplicon of 639 bp. Resistant isolates carrying A143 allele generated an amplicon of 302 bp. Lane M = molecular size standard XIV (100-bp ladder).

Fig. 2. Map of northern Morocco showing the frequencies of quinone outside inhibitor (QoI)-sensitive and QoI-resistant *Zymoseptoria tritici* isolates within the locations sampled in A, 2008 or B, 2010. Within the circles, sensitive isolates are represented by clear gray and resistant isolates are represented by dark gray. A = Gharb-Chrarda, B = Doukkala-Abda, C = Chaouia, D = Tadra-Azilal, E = Meknes-Tafilalet, F = Fes-Boulmane.

However, nine isolates (9.4%) sampled in 2010 (four from Gharb-Chrarda and five from Fes-Boulmane) had the A143 allele that is associated with resistance (Figs. 1 and 2). The resistance of these isolates was confirmed phenotypically with the microplate bioassay. The nine sensitive isolates used as controls displayed very low IC₅₀ values compared with the nine resistant isolates, resulting in a sharp bimodal distribution pattern for IC₅₀ values (Table 2). IC₅₀ values were 0.003 to 0.15 ppm for sensitive isolates and 7.15 to >100 ppm for resistant isolates.

Mitochondrial genetic structure. The concatenated mtDNA sequences (733 bp) allowed us to identify 28 distinct haplotypes among the 100 Moroccan isolates analyzed (Table 3). The four

resistant isolates from Gharb-Chrarda shared one haplotype, while the five resistant isolates from Fes-Boulmane had four haplotypes. The resistant haplotype M 4 was found both in Gharb-Chrarda and Fes-Boulmane (Table 4). Although very similar to each other, no Moroccan haplotype was identical to any of the European haplotypes previously described by Torriani et al. (26). There was no significant difference in haplotype diversity between the 2008 and the 2010 collections (Table 3).

Pairwise F_{ST} comparisons among populations revealed very low or nonsignificant genetic differentiation between most Moroccan subpopulations (Table 5), with the exception of the 2010 Tadla-Azilal subpopulation that was significantly different at $P = 0.05$ (Table 5). AMOVA analyses supported the low mtDNA-based population structure found in Morocco and showed that 96.3% of genetic variation could be explained by differences among indi-

Table 2. Half maximal inhibitory concentrations (IC₅₀) obtained with azoxystrobin for 18 Moroccan *Zymoseptoria tritici* isolates using the microplate assay

Isolate	Location	Allele ^a	IC ₅₀ (mg/liter)	S or R ^b
T01743	Fes-Boulmane	G143	0.003	S
T01718	Meknes-Tafilalet	G143	0.01	S
T01796	Tadla-Azilal	G143	0.02	S
T01778	Chaouia	G143	0.02	S
T01756	Fes-Boulmane	G143	0.04	S
T01789	Tadla-Azilal	G143	0.09	S
T01701	Tadla-Azilal	G143	0.15	S
T01750	Fes-Boulmane	G143	0.15	S
T01736	Gharb-Chrarda	G143	0.15	S
T01744	Fes-Boulmane	A143	7.15	R
T01748	Fes-Boulmane	A143	12.99	R
T01746	Fes-Boulmane	A143	20.74	R
T01741	Fes-Boulmane	A143	>100	R
T01752	Fes-Boulmane	A143	>100	R
T01737	Gharb-Chrarda	A143	>100	R
T01738	Gharb-Chrarda	A143	>100	R
T01739	Gharb-Chrarda	A143	>100	R
T01740	Gharb-Chrarda	A143	>100	R

^a Presence of G143 or A143 allele.

^b Sensitive (S) or resistant (R).

Table 3. Population genetic parameters of 10 Moroccan *Zymoseptoria tritici* subpopulations sampled at two time points^a

Year, location	<i>n</i>	<i>S</i>	<i>H</i>	<i>Hd</i>
2008				
Gharb-Chrarda	22	7	8	0.84
Meknes-Tafilalet	19	19	12	0.94
Chaouia	7	11	6	0.95
Tadla-Azilal	2	1	2	1.00
All 2008	50	21	20	0.90
2010				
Gharb-Chrarda	5	4	2	0.40
Meknes-Tafilalet	9	5	3	0.67
Chaouia	8	12	5	0.86
Tadla-Azilal	7	7	3	0.76
Doukkala-Abda	7	3	3	0.76
Fes-Boulmane	14	16	9	0.91
All 2010	50	17	14	0.86
Total	100	21	28	0.88

^a Estimates were calculated from three concatenated mtDNA loci (total 733 bp) using the program DnaSP v.5; *n* = number of samples, *S* = polymorphic sites, *H* = number of distinct haplotypes, and *Hd* = haplotype diversity.

Table 4. Geographic and temporal distribution of mtDNA haplotypes among 10 Moroccan *Zymoseptoria tritici* subpopulations sampled at two time points^a

Haplotype	2008				2010					
	Gharb-Chrarda	Meknes-Tafilalet	Chaouia	Tadla-Azilal	Gharb-Chrarda	Meknes-Tafilalet	Chaouia	Tadla-Azilal	Doukkala-Abda	Fes-Boulmane
M 1	2	—	—	—	—	—	—	—	—	—
M 2	3	—	—	—	—	—	—	—	—	1R
M 3	2	—	1	1	—	—	—	—	3	1
M 4	8	4	1	1	4R	5	—	2	—	3 + 1R
M 5	3	1	1	—	—	2	3	—	2	2
M 6	2	—	—	—	—	—	—	—	—	—
M 7	1	—	—	—	—	—	—	—	—	—
M 8	1	—	—	—	—	—	2	—	2	—
M 9	—	1	—	—	—	—	—	—	—	—
M 10	—	1	—	—	—	—	—	—	—	—
M 11	—	1	1	—	—	—	1	—	—	1
M 12	—	2	—	—	—	—	—	—	—	—
M 13	—	2	—	—	—	—	—	—	—	—
M 14	—	2	—	—	—	—	—	—	—	—
M 15	—	1	—	—	—	—	—	—	—	—
M 16	—	1	—	—	—	—	—	—	—	—
M 17	—	1	—	—	—	—	—	—	—	—
M 18	—	2	—	—	—	—	—	—	—	—
M 19	—	—	2	—	—	—	—	—	—	—
M 20	—	—	1	—	—	—	—	—	—	—
M 21	—	—	—	—	—	—	—	2	—	—
M 22	—	—	—	—	1	2	—	—	—	—
M 23	—	—	—	—	—	—	—	—	—	1R
M 24	—	—	—	—	—	—	—	—	—	2R
M 25	—	—	—	—	—	—	—	—	—	1
M 26	—	—	—	—	—	—	—	—	—	1
M 27	—	—	—	—	—	—	1	—	—	—
M 28	—	—	—	—	—	—	1	3	—	—

^a “R” indicates quinone outside inhibitor-resistant isolates.

viduals within subpopulations and only 4.8% by differences among subpopulations within 2008 and 2010 populations (Table 6). There was no variation attributed to differences between the 2008 and 2010 populations (Table 6).

Geographical origin of resistant isolates. The concatenated nucleotide data from three mitochondrial loci were used to reconstruct the phylogenetic relationships among the Moroccan and European haplotypes of *Z. tritici*. The reconstructed ML tree clustered the haplotypes regardless of their geographic origin (e.g., European and Moroccan haplotypes did not cluster in distinct clades; Fig. 3). The four mitochondrial haplotypes included QoI-resistant isolates clustered in different clades. M 2 and M 23 are closely related and belong to the same clade, which was different from the one containing M 4 and M 24. These results indicate that the resistant allele originated in at least two unrelated genetic backgrounds. Interestingly, historical migration rates based on the ML estimates (MLEs) implemented in MIGRATE-N revealed a substantial asymmetric gene flow from Europe into Morocco (MLE = 84.29; 5th to 95th percentile range = 28.73 to 196.26). In contrast, no significant gene flow was inferred in the opposite direction (MLE = 0; 5th to 95th percentile range = 0.00 to 0.10).

Discussion

This is the first detection of QoI-resistant genotypes of *Z. tritici* in Africa. Boukef et al. (4) did not report any resistant A143 allele in a population of 357 *Z. tritici* isolates collected in Tunisia in 2007 and 2008. Likewise, Stammler et al. (24) analyzed phenotypically 52 Tunisian isolates sampled in 2010 and concluded that all isolates were sensitive to the QoI fungicide pyraclostrobin. The status of *Z. tritici* QoI resistance in Africa, including Morocco, differs strongly from the present situation in Western Europe, where high frequencies of resistance have been detected in most countries (http://www.frac.info/work/FRAC_QoI_Minutes2009Final.pdf). Such a difference is most likely explained by the lower fungicide usage in Morocco compared with Europe, where fungicide applications are more common (6,10). Recent research revealed that QoI resistance in *Z. tritici* is also widespread in Eastern Europe, especially in the Czech Republic, where a rapid increase in resistance was observed between 2005 and 2011, coinciding with the widespread application of QoI fungicides (7).

Two nonexclusive hypotheses were proposed to explain the rapid spread of QoI resistance over Europe: (i) the G143A mutation

occurred only once or at a very low local level and was subsequently distributed to other regions by migration of resistant individuals, and (ii) the G143A mutation occurred independently in several different mtDNA haplotypes or geographic regions. Torriani et al. (26) showed that the resistance in Europe emerged in at least four distinct genetic backgrounds and that the frequency of the resistant allele rapidly increased because of strong fungicide selection. Patterns of migrations were eastward, presumably through wind dispersal of ascospores (26). No QoI resistance has been reported in Asia, such as in Iran, where only the wild-type allele G143 was found in 89 *Z. tritici* isolates sampled from five provinces (20). More recently, Estep et al. (8) analyzed two fungal collections from two locations in western Oregon in the United States and found frequencies of 9.5% (16/169) and 83.4% (146/175) of resistant isolates in the two collections, respectively, hence reporting for the first time the occurrence of QoI-resistant genotypes of *Z. tritici* in North America.

The absence of resistance in our 2008 collection and its detection in the 2010 collection suggests that QoI resistance arose recently in Moroccan populations of *Z. tritici*. On the other hand, the highlighted long-term gene flow from Europe into Morocco and the lack of geographic substructure in the phylogenetic reconstruction support the introduction of resistant haplotypes in Morocco via wind-dispersed ascospores from Europe. This conclusion might additionally be supported by the geographical origin of the resistant isolates that were found only in the northernmost locations, Gharb-Chrarda and Fes-Boulmane, close to Europe (Fig. 2). Thus, the emergence of resistance in Morocco could be the result of ascospore dispersal from southern France, where frequencies of QoI resistance are currently high (6). However, the MIGRATE analyses estimate long-term (i.e., past) gene flow. Hence, the gene flow detected from Europe into Morocco and the lack of geographic substructure in the phylogenetic tree are more likely the signatures of ancient exchanges, retained by the extremely conserved mtDNA of *Z. tritici* (27,29). Indeed, the observation that there were no shared haplotypes between Morocco and Europe suggests the lack of recent gene flow, negating the hypothesis of recent QoI resistance acquisition from Europe. Instead, the occurrence of the QoI-resistance mutation in different genetic backgrounds suggests an independent origin of the resistance in Morocco, which likely arose very recently at least twice. Additional analyses, for instance with highly variable microsatellite markers,

Table 5. Population differentiation measured by F_{ST} (above the diagonal) among 10 Moroccan *Zymoseptoria tritici* subpopulations^a

Subpopulations	Morocco 2008				Morocco 2010					
	Gharb-Chrarda	Meknes-Tafilalet	Chaouia	Tadla-Azilal	Meknes-Tafilalet	Gharb-Chrarda	Fes-Boulmane	Doukkala-Abda	Chaouia	Tadla-Azilal
2008										
Gharb-Chrarda	...	0.01	0.15	0.00	0.05	0.00	0.016	0.00	0.00	0.39
Meknes-Tafilalet	—	...	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.17
Chaouia	+	—	...	0.00	0.02	0.00	0.08	0.18	0.02	0.17
Tadla-Azilal	—	—	—	...	0.00	0.00	0.00	0.00	0.00	0.29
2010										
Meknes-Tafilalet	—	—	—	—	...	0.00	0.00	0.09	0.00	0.32
Gharb-Chrarda	—	—	—	—	—	...	0.00	0.07	0.00	0.31
Fes-Boulmane	—	—	—	—	—	—	...	0.08	0.00	0.28
Doukkala-Abda	—	—	—	—	—	—	—	...	0.03	0.52
Chaouia	—	—	—	—	—	—	—	—	...	0.24
Tadla-Azilal	+	+	+	—	+	+	+	+	+	...

^a Significance at $P < 0.05$ (below diagonal) was assessed using the exact test of population differentiation implemented in the program ARLEQUIN 3.5.

Table 6. Hierarchic analysis of molecular variance at three levels in the Moroccan *Zymoseptoria tritici* populations^a

Source of variation	df	Sum of squares	Variance components	Variation (%)
Between 2008 and 2010 populations	1	0.72	0.00 Va	0.0
Among subpopulations within populations	8	7.54	0.03 Vb	4.8
Within subpopulations	90	58.25	0.65 Vc	96.3
Total	99	66.50	0.67	...

^a Populations are pooled subpopulations sampled in 2008 or 2010, respectively. Subpopulations are set of isolates sampled in the corresponding locations.

Fig. 3. Molecular phylogenetic analysis by maximum likelihood method based on the Tamura 3-parameter model. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary relationship of 80 mitochondrial DNA *Zymoseptoria tritici* haplotypes. Haplotypes abbreviated as "E" are European haplotypes, described in a previous study (26). Haplotypes abbreviated as "M" and highlighted in gray are Moroccan haplotypes identified in this study. Suffixes "R" and "S" identify haplotypes found among quinone outside inhibitor (QoI)-resistant and QoI-sensitive isolates, respectively. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Arrows indicate the position of the four resistant Moroccan haplotypes.

would allow us to assess levels of recent gene flow (i.e., a few generations back) and to calculate probabilities as to whether the resistant Moroccan isolates arose locally.

In conclusion, this study highlighted the presence of QoI-resistant genotypes of *Z. tritici* in Morocco. However, the resistance mutation is at a low frequency and restricted locally, indicative of a recent emergence and early stage of resistance development. Therefore, appropriate QoI fungicide management is of utmost importance to preserve a low to moderate distribution of the resistance. Under these assumptions, we propose to use QoI fungicides in mixtures or in alternation with other fungicide classes to avoid further intraspecific parallel genetic adaptation of *Z. tritici* to QoI fungicides and, therefore, putatively reduce the spread of the resistance across Morocco and Northern Africa.

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