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# Resistance to QoI Fungicide and Cytochrome *b* Diversity in the Hungarian *Botrytis cinerea* Population

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## ABSTRACT

**Quinol oxidation inhibitors (QoIs) are one of the most important classes of fungicides used in agriculture. They block electron transfer between cytochrome *b* and cytochrome *c*1, thereby impeding the production of ATP via oxidative phosphorylation. QoI fungicides are generally at high risk of provoking resistance in fungal phytopathogens. Resistance has been reported in more than thirty species, amongst others, in *Botrytis cinerea*. In various QoI-resistant monosporic *B. cinerea* isolates from Hungary, a G-to-C point mutation was identified in the mitochondrial gene that encodes the QoI target, cytochrome *b*, resulting in a glycine to alanine substitution at position 143 (G143A). Analysis of Hungarian group I and group II strains further indicated the frequent occurrence of an additional group I-type intron in the *cytb* gene directly downstream of the glycine-143 codon. Mutual presence of distinct mitochondrial DNAs specifying different *cytb* alleles (heteroplasmy) has also been detected in monosporic strains. Remarkably, a number of group II field isolates were found to be highly resistant to azoxystrobin although they did not appear to carry the G-to-C mutation (G143A) generally associated with fungal QoI-resistance.**

**Keywords:** Azoxystrobin resistance, *Botrytis pseudocinerea*, Group I intron, Heteroplasmy, Quinol Oxidation Inhibitor.

## INTRODUCTION

The necrotrophic fungus *Botrytis cinerea* Pers. Fr. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel) is the causal agent of grey mould, a disease affecting many economically important crops. At the present, *B. cinerea* is considered a species complex consisting of two phylogenetic or cryptic "species", group I (also called *B. pseudocinerea*) and group II (*B. cinerea sensu stricto*). Albeit extremely difficult to be distinguished by classical taxonomic criteria, they can be identified easily using certain molecular markers (Fournier *et al.,* 2003; Walker *et al.,* 2011; Fekete *et al.,* 2012).

Chemical control is the classical and, hence, the most frequently used disease containment approach in agriculture. However, inappropriate use of singular-target fungicides often provokes the progressive development of resistance. Resistance of *B. cinerea* to benzimidazoles, dicarboximides, anilinopyrimidines and fenhexamid already occurs worldwide (Banno *et al.,* 2008; Baroffio *et al.,* 2003; Esterio *et al.,* 2007; Ma and Michailides,

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2005). Moreover, there is an ever increasing negative public perception regarding the impact of pesticide use on human health and the environment. In order to establish strategies for sustainable fungicide management on the field, information on the population dynamics of fungicide resistance of pathogens is essential (Yoon *et al.,* 2008; McDermott and McDonald, 1993; Leroux *et al.,* 2002; Gullino and Kuijpers, 1994).

Quinol oxidation inhibitors (QoIs), including strobilurins such as azoxystrobin and kresoxim-methyl, are amongst the most effective anti-fungal agents used in agriculture. QoIs inhibit mitochondrial respiration by binding to the outer quinol-oxidation (Qo) site of the fungal cytochrome *bc*1 complex, blocking electron transfer and reducing ATP synthesis considerably (Fernández-Ortuño *et al.,* 2008). In reverse, QoIs are at high risk of provoking resistance in target pathogens. Resistance to QoIs have been found in many plant pathogens, like *Alternaria alternata* (Ma *et al*., 2003), *Colletotrichum graminicola* (Avila-Adame *et al*., 2003), *Pyrenophora teres* (Sierotzki *et al*., 2007), *Corynespora cassiicola* and *Mycovellosiella nattrassii* (Ishii *et al.,* 2007), *Pseudoperonospora cubensis* (Zhang *et al.,* 2008) and *Venturia inaequalis* (Lesniak *et al.,* 2011) as well as in *Botrytis cinerea* (Wedge *et al.,* 2007; Jiang *et al.,* 2009; Ishii *et al.,* 2009; Leroux *et al.,* 2010; Samuel *et al.,* 2011; Weber and Hahn, 2011).

Studies on the molecular mechanism of QoI resistance showed that it arises from a target-site-based selection and involves mutations in the mitochondrial gene for cytochrome *b* (*cyt b*) (Gisi *et al.*, 2002; reviewed by Kuck and Gisi, 2007). Three amino acid substitutions – glycine to alanine at position 143 (G143A), glycine to arginine at position 137 (G137R) and phenylalanine to leucine at position 129 (F129L) – have been identified in the cytochrome *b* of phytopathogenic fungi that had acquired resistance to QoIs. Isolates carrying F129L or G137R featured moderate resistance, which is usually overcome by the recommended dosages of the fungicide. In contrast, isolates with G143A exhibited high resistance, to the extent that QoIs failed to contain their detrimental impact (on the field) (Gisi *et al.,* 2002; Kim *et al*., 2003).

The coding region of the *B. cinerea* gene is usually interrupted by three group I-type self-splicing introns, typical to eukaryote mDNA (Haugen *et al*., 2005). These introns appear to be ancestral as the last two also occur in the closely related species *Sclerotinia sclerotiorum* (Accession AAGT01000680: coordinates 65282-57982) while all three of them are position-conserved in the *cyt b* gene of the unrelated orbiliomycete *Arthrobotrys oligospora* (ADOT01000075: 12369-3811). However, Grasso *et al*. (2006) reported that some *B. cinerea* variants, including the reference strain B05.10 (AAID01004537: 15913-22520), feature a fourth, alternatively present group-I intron, situated directly behind codon 143.

A fungal cell usually has multiple mitochondria and distinct mitochondrial genomes (mtDNA) may co-exist in single cells of one individual, a phenomenon known as heteroplasmy, which has been described in *Podosphaera leucotricha* (Lesemann *et al.,* 2006), *Corynespora cassiicola* and *Mycovellosiella nattrassii* (Ishii *et al.,* 2007), as well as in *B. cinerea* (Ishii *et al.,* 2009). Newly acquired resistance to QoIs in one cell may be transmitted - by means of proliferation of mitochondria -, and cell-to-cell migration of mitochondria carrying the mutated *cyt b* gene (Gisi *et al.,* 2002).

The aims of the current study were (i) to characterize extant QoI fungicide resistance among Hungarian *B. cinerea* field isolates, (ii) to examine the genetic basis of this resistance, and (iii) to examine heteroplasmy with respect to wild-type and (possible) mutant *cyt b* alleles.





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## MATERIALS AND METHODS

### Botrytis cinerea Isolates

A total of 157 monosporic isolates of *B. cinerea* were collected from infected oilseed rape, strawberries, and raspberries from Nagyréde and Hatvan districts in the North-East of Hungary in 2008 and 2009 (Figure 1 and Table 1). Isolates were maintained on potato dextrose agar (PDA, Scharlau, Spain). Conidial suspensions were stored in 50% glycerol at –80°C. All isolates had been assigned previously to either group I or II (Fekete *et al.,* 2012).

### Fungicide Sensitivity Assay

Sensitivity of *B. cinerea* to azoxystrobin was determined by measuring radial growth on plates. Quadris (Syngenta, UK), a product that contains 250 g $L^{-1}$ azoxystrobin, was added to PDA medium after sterilization to give final concentrations of 0, 1 and 100 mg azoxystrobin $L^{-1}$. To inhibit the alternative respiratory pathway (Wood and Hollomon, 2003), 100 mg $L^{-1}$ salicylhydroxamic acid (SHAM) (Sigma–Aldrich Kft, Hungary) was also added. A 10 mm mycelial plug was taken from the edge of a 3-day-old colony and placed on the center of PDA plates containing SHAM and azoxystrobin. Three parallel replicates of each concentration were prepared for each isolate. Plates were incubated at 25ºC for 3 days in the dark and, subsequently, the diameter of the colonies was measured. Isolates that exhibited considerable colonial growth (colony diameter> 50% of that on the fungicide-free control medium) on PDA plus 100 mg $L^{-1}$ azoxystrobin were designated as highly resistant (HR) isolates. Isolates that grew out on PDA plus 1 mg $L^{-1}$ azoxystrobin (colony diameter> 50% of that on control medium) but were unable to grow on PDA plus 100 mg/L azoxystrobin, were designated as lowly resistant (LR) isolates, while those that suffered severe growth inhibition in the presence of the fungicide (colony diameter on 1 mg $L^{-1}$< 50% of that on control medium) were designated as azoxystrobin-sensitive (S) isolates.

### PCR Amplification and Analysis of *Cyt b* Gene Sequences

DNA was extracted from aerial mycelium of *B. cinerea* strains grown on PDA plates for 10 days at 20°C. Magnalyser (Roche,

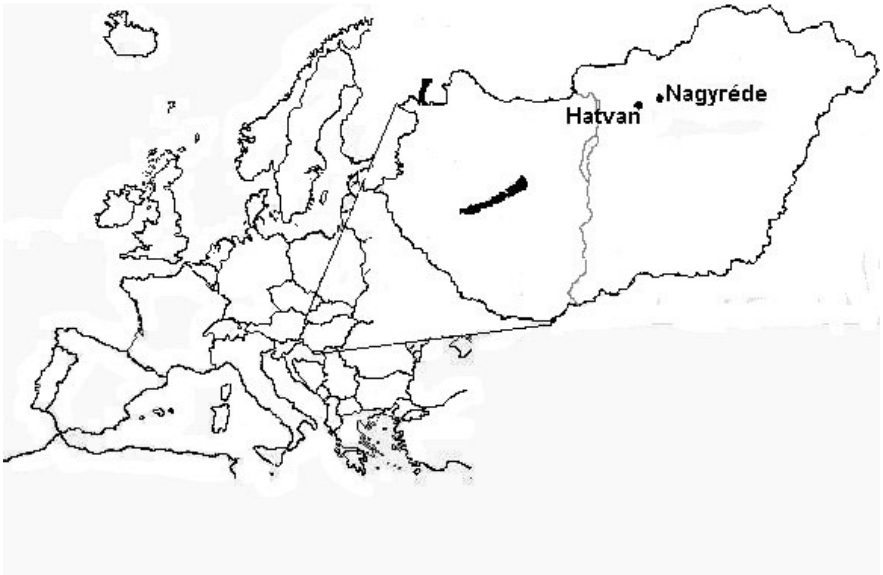


**Figure 1.** Geographic origins of the 157 Hungarian *B. cinerea* field isolates used in this study.





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**Table 1**. Origin, azoxystrobin resistance and molecular characteristics of *Botrytis cinerea* isolates.

| N[a] | Origin | Host | Sensitivity to azoxystrobin[b] | *Cyt b* fragment size[c] | Allele-specific PCR [d] | PCR-RFLP[d] | Accession number of *Cyt b* fragment |
|---|---|---|---|---|---|---|---|
| Group I | | | | | | | |
| 6 | Hatvan | Rape | S | 1768 bp | - | - | |
| 1 | Hatvan | Rape | LR | 1768 bp | - | - | |
| 5 | Nagyréde | Strawberry | S | 1768 bp | - | - | |
| Group II | | | | | | | |
| 1 | Nagyréde | Raspberry | LR | 1768 bp and 564 bp | - | - | |
| 29 | Nagyréde | Raspberry | LR | 564 bp | - | - | |
| 4 | Nagyréde | Raspberry | LR | 1768 bp | - | - | |
| 2 | Nagyréde | Raspberry | HR | 1768 bp | - | - | |
| 1 | Nagyréde | Raspberry | HR | 564 bp | - | - | |
| 1 | Nagyréde | Raspberry | HR | 1768 bp and 564 bp | - | - | |
| 1 | Nagyréde | Raspberry | S | 1768 bp and 564 bp | - | - | |
| 2 | Nagyréde | Raspberry | S | 1768 bp | - | - | |
| 16 | Nagyréde | Raspberry | S | 564 bp | - | - | |
| 15 | Nagyréde | Strawberry | S | 1768 bp | - | - | |
| 29 | Nagyréde | Strawberry | S | 564 bp | - | - | JQ362461-64, JQ362466-67 |
| 1 | Nagyréde | Strawberry | S | 1768 bp and 564 bp | - | - | |
| 5 | Nagyréde | Strawberry | S | 564 bp | + | - | |
| 1 | Nagyréde | Strawberry | S | 1768 bp | + | - | |
| 8 | Nagyréde | Strawberry | LR | 1768 bp | - | - | |
| 5 | Nagyréde | Strawberry | LR | 564 bp | - | - | |
| 18 | Nagyréde | Strawberry | HR | 564 bp | + | + | JQ362466, JQ362468-70 |
| 3 | Nagyréde | Strawberry | HR | 564 bp | - | - | |
| 2 | Nagyréde | Strawberry | HR | 1768 bp | - | - | |
| 1 | Nagyréde | Strawberry | HR | 1768 bp and 564 bp | + | - | |

Osterode, Germany) was used for the disruption of fungal cells, and DNA was isolated with the Plant II DNA Purification Kit (Macherey-Nagel GmbH and Co.KG, Germany) according to the manufacturer's instructions.

Based on the sequence of *cyt b* gene from *B. cinerea*, the pair of gene-specific PCR primers cytb-BcF and cytb-BcR was used as previously described (Jiang *et al.,* 2009) to amplify a *cyt b* fragment from all *B. cinerea* isolates. This *cyt b* fragment contained the codon corresponding to glycine residue 143, mutation of which is known to affect azoxystrobin sensitivity in many fungal species. PCR was programmed as follows: initial denaturation 5 minutes at 95ºC; 5 cycles of denaturation 1 minute at 95ºC; annealing 1 minute at 50ºC; and elongation 1 minute 30 seconds at 72ºC; 30 cycles of denaturation 1 minute at 90ºC; annealing 1 minute at 50ºC; and elongation 1 minute 30 seconds at 72ºC; and finally, a post elongation for 15 minutes at 72ºC.





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Amplifications were done in a final volume of 50 µL containing 0.4 µM of each primer, 100 ng of fungal DNA, 25 µL of Green Master Mix (Promega). PCR products were examined by electrophoresis in a 1.5% agarose gel in 1 X Tris–acetate-EDTA (TAE) buffer and stained with ethidium bromide. For a selected number of stains, this PCR fragment was purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega Corp., Madison, WI) and subsequently sequenced over both strands at MWG-Biotech AG (Ebersberg, Germany). Sequences were manually edited and deposited at GenBank (accession nos. JQ362461–JQ362470).

### Allele-specific PCR of the Mutated *cyt b* Gene

To detect the G-to-C point mutation in codon 143 of the *cyt b* gene, PCR primers BcAR-F and BcAR-R were used as described by Jiang *et al.* (2009). The primer BcAR-F was designed such that it could only amplify off genomic DNA carrying the G-to-C point mutation in the middle of codon 143. The primer BcAR-R was located within the last intron of the *cyt b* gene. The primer pair was expected to generate a ~260-bp fragment from mtDNA of HR isolates of *B. cinerea*. PCR was programmed as above, except using an annealing temperature of 55ºC and an elongation time of 30 seconds during cycling. PCR products were analyzed in a 1.5% agarose gel in TAE buffer and stained with ethidium bromide.

### Diagnosis of QoI Resistance by PCR-RFLP

PCR-RFLP was used to confirm fungicide-resistant isolates lacking the additional intron between codons 143 and 144. A PCR-RFLP protocol with the restriction enzyme *Ita*I was previously developed to identify resistance to strobilurin (Sierotzki *et al.,* 2000; Furuya *et al.,* 2009). *B. cinerea* DNA was amplified using primer set, cytb-BcF/cytb-BcR, purified and were digested with *Sat*I (*Fnu*4HI) (Fermentas, Biocenter Ltd., Szeged, Hungary), an isoschizomer of *Ita*I. The G-to-C point mutation resulting in the G143A substitution creates a *Sat*I restriction site (GC|NGC). Digests were run on a 1.5% agarose gel with TAE buffer and stained with ethidium bromide.

## RESULTS

A total of 157 Hungarian *B. cinerea* isolates were tested for QoI fungicide resistance on media containing azoxystrobin (Table 1). Twenty eight (18%) isolates were classified as highly resistant (HR) and forty eight (30%) exhibited a low resistance (LR), while the majority of the isolates, eighty one (52%), were sensitive (S) to azoxystrobin. High azoxystrobin resistance was exclusively encountered amongst group II strains, while group I isolates, with one exception, were all classed as sensitive to the QoI.

A major sequence variation in the *cyt b* gene was observed among the Hungarian *B. cinerea* isolates. Grasso *et al*. (2006) described a 1204-nt long, group I intron splitting exon 3 between codons 143 and 144, that featured alternatively in the *cyt b* gene of a number of *B. cinerea* variants (see Figure 2 and, e.g., Accession FJ390874). The primer pair cytb-BcF/cytb-BcR amplified two different PCR fragments (Figure 3). The larger, ~1750-bp, fragment indicated the presence of the alternative intron and was amplified in forty six (29%) isolates. The smaller ~ 560-bp fragment, without the alternative intron, was generated in 106 (68%) isolates. Interestingly, five (3%) isolates gave rise to both PCR fragments (Table 1 and Figure 3, slot 8 from the left), indicating the occurrence of *cyt b* heteroplasmy in the Hungarian *B. cinerea* population.

Isolates belonging to either of the two *B. cinerea* cryptic species i.e. group I and





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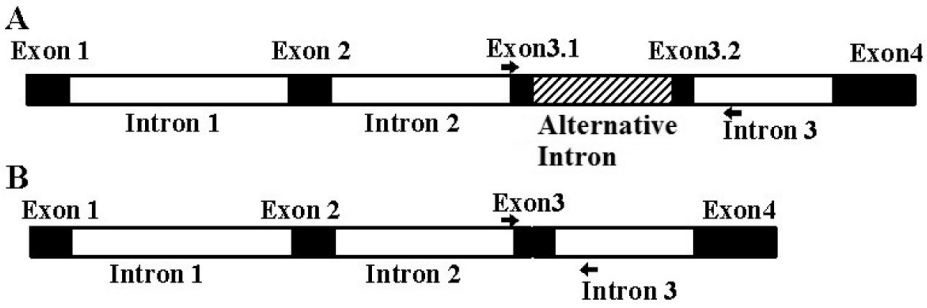


**Figure 2.** Schematic representation of the cytochrome *b* (*cyt b*) gene of *B. cinerea* with (A) and without (B) the alternative group-I intron. Black boxes indicate exons, white boxes indicate introns, while the hatched box signifies the alternative intron separating the codons for Gly143 and Ala144. Arrows indicate the approximate position of cytb-BcF and cytb-BcR primers used for amplification. Introns, exons and primers are not drawn to scale. Note that very recently, American isolates have been described that lack some or all *cyt b* introns (Yin *et al*., 2012); the new variants are not depicted here.

group II (Fekete *et al.,* 2012; Walker *et al.,* 2011) showed differences regarding the alternatively present intron in the *cyt b* gene between codons 143 and 144. It occurred in all twelve group I strains examined, while, among group II variants, two alleles of *cyt b* were present. Thirty four (23.5%) strains featured the alternative intron while 106 (73%) did not. All five isolates (3.5%) exhibiting heteroplasmy belonged to group II. Interestingly, four group II HR strains possessed the alternative intron in the *cyt b* gene while the remaining twenty four did not (Table 1).

Analysis of the sequences of the *cyt b* PCR products amplified with cytb-BcF/cytb-BcR from six S strains and four HR strains (Figure 4) showed that the wild-type GGT codon of the glycine at position 143 was present in S isolates where a GCT codon occurred in the HR isolates. The latter strains thus feature the glycine to alanine substitution at position 143 (G143A) of cytochrome *b*, associated with QoI resistance in *Alternaria* spp. (Ma *et al*., 2003), *Erysiphe graminis* (Sierotzki *et al*., 2000) and *Pyricularia grisea* (Gisi *et al.,* 2002; Kim *et al*., 2003). A pair of allele-

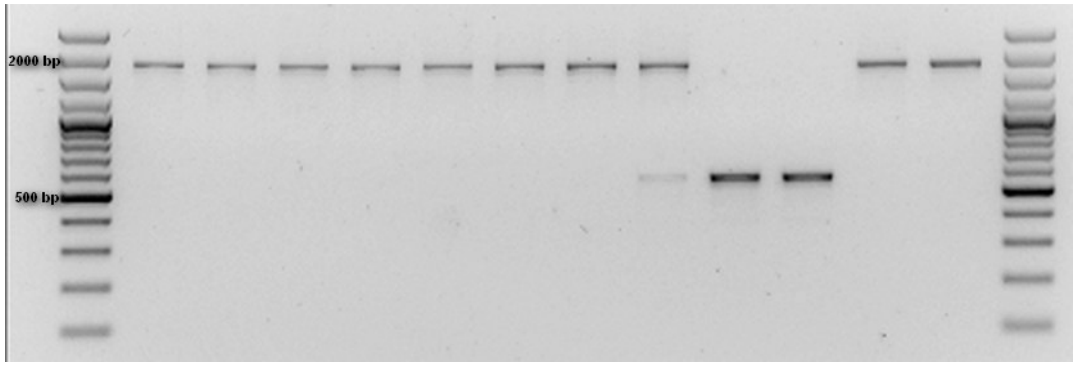


**Figure 3.** Cytochrome *b* (*cyt b*) gene size differentiation in the Hungarian *B.cinerea* field isolate collection. For each of the 157 monosporic strains, part of the *cyt b* gene was PCR-amplified with primers cytb-BcF and cytb-BcR. A representative selection of PCR amplifications is shown. First and last lane: molecular marker (O'GeneRulerTM 100bp plus DNA Ladder, Fermentas). The large fragment (~1750 bp) indicates the presence of the alternative intron between the codons 143 and 144.





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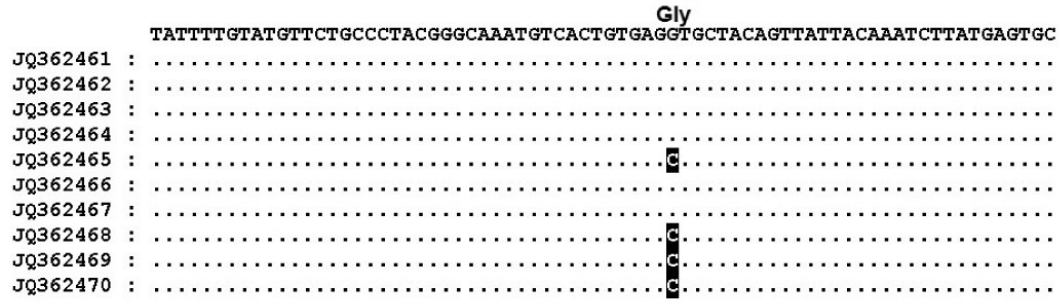


**Figure 4.** Partial *cyt b* sequences in Hungarian *B. cinerea* isolates. JQ362461-JQ362470 are the accession numbers of the sequences deposited at GenBank. JQ362465, JQ362468, JQ362469 and JQ362470 specify a C instead a G in the middle of codon 143, located in exon 3. The sequence at the top is the consensus sequence (with its conceptual translation given in three-letter code). Everywhere else, dots indicate nucleotides identical to consensus sequence.

specific primers BcAR-F and BcAR-R was used for the identification of *B. cinerea* isolates in the complete collection that carry the G-to-C point mutation leading to the G143A substitution. Amplification results (see Table 1, column 6) indicated the presence of the G-to-C mutation in twenty five isolates belonging to group II, of which, remarkably, nineteen were indeed highly resistant (HR), but the other six were sensitive (S) to azoxystrobin.

PCR-RFLP analysis (Ishii *et al.*, 2009; Saito *et al.* 2009) was carried out by digesting the cytb-BcF/cytb-BcR amplification products with *Sat*I restriction enzyme to confirm the results from (G143A) allele-specific PCR (see Material and Methods section for details). Figure 5 shows the results for a selection of strains that do not specify the alternative intron. None of the *cyt b* PCR fragments generated from group I strains (not shown) and azoxystrobin-sensitive (S) group II isolates were digested following enzyme treatment, whereas the ~560-bp *cyt b* fragments from eighteen HR group II strains were digested. The same strains were also identified with (G143A) allele-specific PCR.

On the other hand, the six azoxystrobin-sensitive (S) group II strains that responded positively with the (G143A) allele-specific primers featured a negative RFLP response

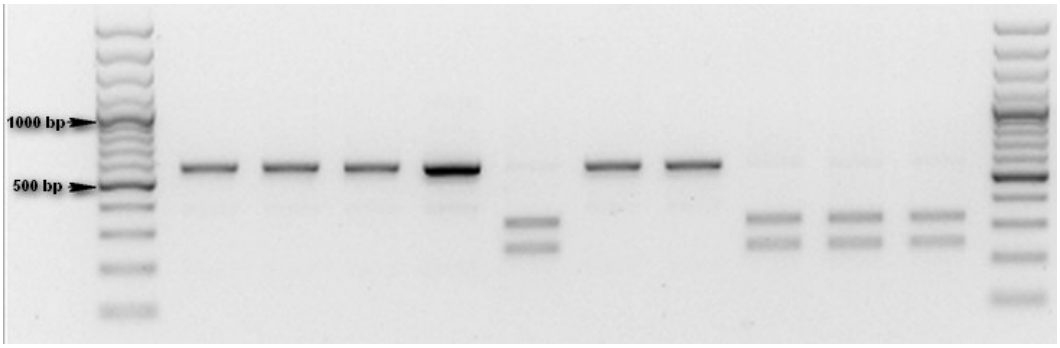


**Figure 5.** PCR-RFLP of the small *cyt b* amplification fragment (~560 bp) digested with *Sat*I endonuclease. First and last lane: molecular marker (O'GeneRulerTM 100bp plus DNA Ladder, Fermentas). Appearance of the two bands of lower molecular mass results from *Sat*I digestion of the fragment of higher molecular mass, evidencing the G-to-C mutation that causes the QoI-resistant phenotype. PCR-RFLP analysis of strains that carry the alternative intron was not done as in those strains, the G-to-C mutation does not result in a novel *Sat*I (GC|NGC) restriction site.





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(Figure 5, the six samples that were not cut). This would indicate imbalanced heteroplasmy with the large majority of mitochondria harbouring the wild-type (QoI-sensitive) *cyt b* gene and only a marginal presence of the resistance-conferring (normally semi-dominant) (G143A) *cyt b* allele in these strains.

## DISCUSSION

*Botrytis cinerea* poses a high-risk to develop resistance against single-target fungicides like QoIs, because of its high genetic variability, short life cycle, abundant inoculum production and ease to disseminate it. *B. cinerea* field isolates with azoxystrobin (a QoI fungicide) resistance have been found in several countries, such as France (Leroux *et al.,* 2010), Germany (Weber and Hahn, 2011), Greece (Samuel *et al.,* 2011), USA (Wedge *et al.,* 2007), China (Jiang *et al.,* 2009) and Japan (Ishii *et al.,* 2009). In the large majority of the cases, high resistance against QoIs is associated with one particular G-to-C point mutation in the cytochrome *b* (*cyt b*) gene. The resulting glycine-to-alanine mutation (G143A) changes the conformation at the azoxystrobin target binding site (Esser *et al.,* 2004). *Cyt b* is a mitochondrial gene and as a consequence; inheritance of fungal QoI resistance is uniparental (in analogy with mammals, via the maternal lineage) rather than Mendelian. Other mechanisms, such as bypassing the electron transfer chain blocked by QoI fungicides via the alternative oxidase pathway can also result in resistance to QoIs (Wood and Hollomon, 2003; Fernández-Ortuño *et al.,* 2008).

In this study, QoI resistance and related cytochrome *b* gene allelism in Hungarian group I and II *B. cinerea* populations were investigated. As could be expected, PCR-amplified cytochrome *b* gene fragments of many QoI-resistant field isolates showed the mutational change causing the glycine to alanine (Ala) (G143A) substitution and presence of the resistance-conferring G-to-C mutation was thus confirmed in Hungarian group II populations. All isolates, where the G-to-C mutation could be detected with both PCR-RFLP and allele-specific PCR, showed high resistance against azoxystrobin. In six cases, the mutation could only be detected with allele-specific PCR i.e., not with PCR-RFLP, and these strains were sensitive to azoxystrobin. This would indicate marginal presence of the resistance-conferring, mutated mtDNA and these strains may well develop resistance rapidly when faced with QoIs in the field.

PCR fragment length analysis of *cyt b* strongly suggested the presence of the alternative group I intron in a considerable part (> 32%) of the Hungarian *B. cinerea* field isolate collection. Some group II isolates gave rise to both fragments (~1750 bp and ~560 bp), which could imply *cyt b* heteroplasmy in monosporic isolates. Grasso *et al.* (2006) reported that the absence of the alternative intron immediately downstream the GGT codon for glycine 143 is positively correlated to the ability of plant pathogenic fungi to develop QoI resistance. This appears to be the case for the twelve Hungarian group-I isolates tested in this study. Neither of them were highly resistant to azoxystrobin and, in concordance, neither of them carried the G-to-C mutation in *cyt b* provoking QoI resistance while all possessed the alternative *cyt b* intron. However, Leroux *et al*. (2010) described at least one group-I isolate, I3, without the alternative intron (accession FJ217742).

On the contrary, four HR group-II strains were identified in this study that possessed the alternative intron (without detectable heteroplasmy) in the *cyt b* gene (Table 1). For one of those, amplification with the mutant-(G143A)-specific primer pair BcAR-F/BcAR-R was recorded (NB. *Sat*I-RFLP was not possible because of the presence of the alternative intron).

Remarkably, a considerable number of group-II strains (nine of twenty eight HR strains) exhibited high azoxystrobin resistance where the resistance-conferring G143A substitution could not be evidenced.





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We currently investigate these nine field isolates for potential alternative mutations that could have rendered them QoI resistant.

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# مقاومت به قارچ کش QoI و تنوع سیتوکروم *b* در جمعیت های کپک خاکستری مجارستان

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## چکیده

یکی از مهمترین قارچ کش‌های مورد استفاده در کشاورزی، قارچ کش‌های QoI هستند. آنها با مهار الکترون انتقالی بین سیتوکروم *b* و سیتوکروم *c*۱ در مرکز اکسیداسیون یوبی کینون باعث ایجاد اختلال در سیکل تولید ATP میشوند که در نهایت منجر به کم شدن انرژی در سلولهای قارچی میشود. این قارچ کش‌ها ریسک بالائی از مقاومت پاتوژنها را دارا می‌باشند. مقاومت به این قارچ کش‌ها در بیش از ۳۰ پاتوژن از جمله کپک خاکستری گزارش شده است. در میان نمونه‌های کپک خاکستری مقاوم به این قارچ کش‌ها در مجارستان، جهش در جایگاه ۱۴۳ در ژنوم سیتوکروم *b* میتوکندری شناسایی شد که باعث جایگزینی نوکلوتید سیتوزین به جای گوانین گردید که این تغییر منجر به تبدیل اسید آمینه گلیسین به آلانین شد. آنالیز نمونه‌های گروه I و II در مجارستان، حضور اینترون نوع I را در درصد بالایی از نمونه ها نشان داد که این اینترون مستقیماً بعد از کدون ۱۴۳ در ژنوم سیتوکروم *b* واقع شده است. حضور همزمان دو نوع DNA میتوکندری با آللهای متفاوت در سیتوکروم *b* نشان دهنده هتروپلاسمی در این نمونه هاست. شایان ذکر است که تعدادی از نمونه‌های گروه II که مقاوم به این قارچ کش‌ها بودند فاقد جهش در جایگاه ۱۴۳ بودند در صورتیکه معمولا این جهش با مقاومت به قارچ کش در ارتباط است.



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