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MOLECULAR CHARACTERIZATION OF *BOTRYTIS CINEREA* MOROCCAN ISOLATES INFECTING GRAPEVINE AND THE CRYPTIC SPECIES STATUS IN NORTH AFRICA/ EUROPE

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ABSTRACT

Grey mould is a serious disease that limits grapevine cultivation in some regions around the world. It causes severe losses in the quality and the volume of grapes harvested for wine production or fruit market. The genetic diversity of the *Botrytis cinerea* populations within Moroccan vineyards related to transposable elements (TEs) has been unexplored. Such information can help manage more effective strategies for controlling the disease. In the current study, 122 grapevine isolates were genotyped in order to check for transposable elements. It demonstrates that *B. cinerea* Moroccan populations is harbored all four transposable element states: both *Boty* and *Flipper*, *Boty* only, *Flipper* only and no transposable elements, relating to the four TEs genotypes *Transposa*, *Boty*, *Flipper* and *Vacuma* respectively. After frequency investigation, results showed that *Transposa* individuals containing *Boty* and *Flipper* TEs was the predominant type (78.69%) in grapevine populations, whereas only (2.46%) belonged to the *Vacuma* individuals lacking the two TEs types. Furthermore, *Botrytis cinerea* was mentioned to contain two cryptic species as species cluster, Groups I and II. Assessment of genetic polymorphism of the Bc-hch haplotyping locus using PCR and restriction enzyme digestion identified only the *B. cinerea* population isolates corresponding to one allelic type, so all the analysed isolates were classified as Group II *B. cinerea stricto sensu* (according to the most recent proposed classification). Study of genetic distribution of populations based on group I/group II cryptic species between North Africa and Europe studies reports revealed possible populations migration.

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INTRODUCTION

The phytopathogenic ascomycetous fungus *Botrytis cinerea* Pers. Fr. (also referred to as the teleomorphic stage *Botryotinia fuckeliana* [de Bary] Whetzel) is the causative agent of Gray mold, a biotic factor that hinders the improvement of grape berry yield and quality. As an endophyte, it can colonize plants without causing disease or stress symptoms (Van Kan *et al*., 2014). *B. cinerea* causes blight of leaf, shoot and inflorescence but the fruit rots engender the most important damages impacting harvest (Diolez *et al*., 1995; Kerssies *et al*., 1997; Samuel *et al*., 2012; Zhou *et al*., 2014). Additionally, this pathogen results in both quality and quantity losses across various plant species (De Miccolis *et al*., 2016).

Because of its scientific and economic impacts, *B. cinerea* has established itself as a prominent fungal pathogen in

the field of molecular plant pathology research. It serves as a model organism for investigating the infection process in necrotrophic pathogens (Dean *et al*., 2012).

Numerous studies on the population genetics of *B. cinerea* have revealed that this fungus involves complex species with limited gene flow occurring between different cryptic genetic groups (Abdel Wahab, 2015; Albertini *et al*., 2002; Alfonso *et al*., 2000; Diolez *et al*., 1995; Fournier & Giraud, 2008; Fournier *et al*., 2005, 2003, Giraud *et al*., 1999, 1997; Kerssies *et al*., 1997; Ma & Michailides, 2005; Muñoz *et al*., 2002; Rajaguru & Shaw, 2010; Vercesi *et al*., 2014; Yourman *et al*., 2001).

Two sympatric sibling species or transposable elements (TEs) types were initially identified in populations isolated from different or similar hosts. They were characterized based on the presence or the absence of two mobile (TEs) *Boty* and *Flipper*, such as *Transposa* genotype that contains *Boty* and *Flipper* transposons while *Vacuma* genotype is transposon free (Diolez *et al*., 1995; Giraud *et al*., 1999, 1997; Levis *et al*., 1997). It has been suggested that *Transposa* isolates are better adapted to infect grapes berries, while *Vacuma* isolates preferably infect the grape leaves (Giraud *et al*., 1999).

Analysis studies of different nuclear genes and the screening for polymorphism using PCR-RFLP of the Bc-hch gene (the *B. cinerea* homolog of the *Neurospora crassa* het-c vegetative incompatibility locus) revealed the existence of two genetically distinct groups, group I and group II, phylogenetic species in populations of *B. cinerea* (Fournier *et al*., 2005, 2003).

The group I isolates of *B. cinerea* belong to the *Vacuma* TEs genotype and displayed natural resistance to the fungicide fenhexamid (Fekete *et al*., 2012; Leroux *et al*., 2002); Whereas group II isolates has been described to feature all four possible genotypes: *Transposa*, *Flipper* (containing only *Flipper*), *Boty* (containing only *Boty*) and *Vacuma* genotype (Esterio *et al*., 2011; Fekete *et al*., 2012; Fournier *et al*., 2002; Leroux *et al*., 2002; Milicevic *et al*., 2006; Rajaguru *et al*., 2010; Váczy *et al*., 2008).

According to evidence from the above-mentioned molecular studies and the sexual cross experiment (ascospore production), the group I isolates of *B. cinerea* was defined as species like *Botrytis pseudocinerea* (Walker *et al*., 2011) and in as last review a newly *B. cinerea* group I species was designated as *Botrytis medusa* (Harper *et al*., 2019).

Therefore, this study was conducted to fulfil these objectives: (i) exploring the group I and group II cryptic species through Bc-hch RFLP (Reverse Forward Length Polymorphism), (ii) assessing molecular characterization of *B. cinerea* isolates from Moroccan vineyards relating to transposable elements types, and (iii) comparing the status of *B. cinerea* cryptic species migration in North Africa with Europe.

MATERIALS AND METHODS

Fungal Isolates

Strains of *B. cinerea* used in this study were derived from Moroccan vineyards belonging to ten locations in different regions (Rabat, Meknes, Fes, Tetouan, El Jadida and Marrakesh). Three isolates were added to the collection (two isolates from tomato and one from Strawberry plants), they were obtained from naturally infected tomato and strawberry leaves. A total of one hundred and twenty five isolates were established after single spore isolation. All isolates were grown on potato dextrose agar (PDA) as described by Amselem *et al*. (2011), cultures were harvested and lyophilised.

DNA Extraction

Genomic DNA was extracted according to the method described by Möller *et al*. (1992). 20 mg of lyophilised mycelium was ground in a mixer mill then disrupted in a 1.5ml tube in an extraction buffer (100 mM Tris-HCl, pH 8, 10 mM EDTA and 2% SDS). After adding 70µg K Proteinase, the tube was incubated at 60°C for 1h and gently mixed. The mixture was amended with 5 M NaCl and 10% CTAB and then incubated at 65°C for 10 min. SEVAG was added to the mixture and kept for 30 min on ice before centrifugation at 13,000 rpm for 10 min. The supernatant was transferred to 1.5ml tube, 5 M acetate ammonium was added and the mixture was kept for 30 min on ice and centrifuged at 13,000 rpm for 5 min. Isopropanol was added to the fresh tube transferred supernatant and centrifuged at 13,000 rpm for 10min to collect DNA.

The pellet was washed twice with 70% ethanol, dried and resuspended in TE (10 mM Tris-HCl at pH 8 and 1 mM EDTA). The DNA extractions quality and quantity were checked by using the NanoDrop 2000, Thermo Scientific spectrophotometer.

Bc-hch Haplotyping: PCR-RFLP Amplification and Digestion

PCR-RFLP haplotyping of the Bc-hch locus was assessed for *B. cinerea* isolates collection in order to identify

group I and group II isolates. The Genbank accession numbers for the sequences of the Bc-hch locus are AY770143-AY770188. This allele contains five HhaI restriction sites in group II isolates (positions 119, 274, 283, 367, 884), while only four sites in group I, the site in Position 367 being mutated (Fournier *et al*., 2005, 2003). Thus, the two restriction profiles differ in size of the upper band (601 bp in group I and 517 bp in group II). Two specific primers were used as described by Fournier *et al*. (2003) : 262 (5'-AAGCCCTTCGATGTCTTGGA-3') and 520L (5'-ACGGATTCCGAACTAAGTAA-3'). These primers amplify a 1171bp fragment between position 701 and 1871 of the Bc-hch gene. The amplification was conducted as described by (Ben Ahmed & Hamada, 2005) in a final volume of 50µl containing 3µM of each primer, 100ng of fungal DNA and 25µl of MyTaqTM Mix (Bioline). PCR reactions were performed in a Multigene Optimax programmable thermal cycler: 1cycle at 94°C for 2 min; followed by 40 cycles of 94°C for 50s, 55°C for 50s, 72°C for 1 min and 30 s; followed by a final extension step at 72°C for 10 min. Digestion was carried out on 20µl of PCR product using 1U of HhaI restriction endonuclease. The amplicons in the PCR mixtures were incubated overnight at 37°C as described by (Zhou *et al*., 2014). Finally, digested products were separated by electrophoresis in 1.5% agarose gel in Tris-acetate (TAE) buffer; gels were stained by ethidium bromide and viewed under UV trans-illuminator.

Detection of Transposable Elements '*Flipper*' and '*Boty*'

To determine the presence of absence of transposable elements (TEs), separate PCR reactions were performed using primers previously described (Kretschmer & Hahn, 2008). To detect *Flipper* element, primers BcFlp1 (5'-GCACAAAACCTACAGAAGA-3') and BcFlp2 (5'-CCAAAGGTAAAAGTGCTCT-3') yielding a 484 bp fragment were used to amplify sequences. The detection of *Boty* element was tested using primers Bc-Boty1 (5'-CTTTACCGGAACACAAGCCAT- 3') and Boty2 (5'-GGTCTTCCATTCTTCGCCTTC-3'), yielding a 383 bp fragment. Negative samples for the presence of transposable elements were re-examined in separate reactions. The PCR-based detection of the transposable elements was performed using the same master mix except for the primers. The amplifications were conducted in a final volume of 20µl reaction containing 1x reaction buffer, 1.5mM Mgcl2, 100nM of each primer, 100µM dNTPs, 75ng DNA and 0.5U GoTaq (bioline). Reactions were performed in a Genius thermal cycler and consisted of an initial preheat step at 94°C for 5 min, followed by 40 cycles denaturing at 94°C for 30s, annealing at 60°C for *Boty* primers or 58°C for *Flipper* primers for 40s, and elongation step at 72°C for 1 min 30s. Reactions were completed with a final 7 min extension step at 72°C. Amplified products were separated by electrophoresis on 1.5% agarose gel in 1xTris-acetate (TAE) buffer, stained with ethidium bromide, viewed and photographed under UV light.

Strains carrying *Boty* and *Flipper* TEs were classified as *Transposa* strains, the absence of both TEs characterized the *Vacuma* strains and those with either *Boty* or *Flipper* elements were classified as *Boty* or *Flipper* strains.

Population Genetic Analysis

Study of genetic populations was based on group I/group II cryptic species Matrix between countries of North Africa (Tunisia and Morocco) and Europe (France, Germany, Italy, Greece and Hungary) published studies. We used the GENALEX Software (Peakall & Smouse, 2006, 2012) for Mantel test. We used data from molecular identified populations in: Tunisia (Ben Ahmed *et al*., 2005; Karchani-Balma *et al*., 2008), France (Fournier *et al*., 2003; Fabian Martinez *et al*., 2005), Germany (Fournier *et al*., 2003; Kretschmer *et al*., 2008), Hungary (Fekete *et al*., 2012), Italy (Vercesi *et al*., 2014) and Greece (Samuel *et al*., 2012). The genetic distance was performed based on Group I/ Group II frequencies encoded in binary and the geographic distance using the XY coordinates of the cities where isolates were collected (genetic distance and geographic distance data matrices are provided as supplementary materials).

The map was made with the Geographic Information System QGIS version 3.2.0 and data boundaries are downloaded from spatial data DIVA-GIS site: http://www.diva-gis.org.

RESULTS

Bc-hch Polymorphism

Digestion of the amplified fragment corresponding to the region of the Bc-hch gene with the *Hha1* enzyme revealed one structure polymorphism. The restriction enzyme *HhaI* had 5 restriction sites in the Bc-hch2 allele and the restriction pattern demonstrates one upper band size 517bp in all samples (even strawberry and tomato samples) like example presented in Figure 1. Results indicated that all strains were identical to group II strains profile identified by (Fournier et al., 2003) who distinguish groups based on the polymorphism of Bc-hch.

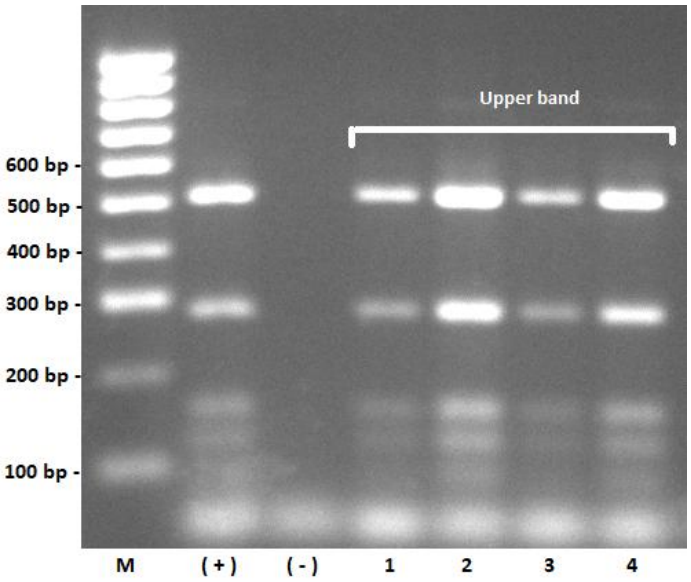

Figure 1. Example of agarose gel electrophoretic profile showing the result of the *HhaI* restriction enzyme digested Bc-hch gene amplicons. 1 to 4: some isolates of *B. cinerea* showing fragments from *HhaI* reductase alleles with 5 restriction sites in the Bc-hch2 allele and the 517 bp upper band size. (+): Positive Bc-hch2 control strain; (-): Negative *Flipper* control strain; M: Molecular weight marker 1 kb ladder.

Detection of Transposable Elements '*Flipper*' and '*Boty*'

The presence or absence of two transposable elements (*Flipper* and *Boty*) was tested in each strain using the PCR amplification reactions. Previously, *B. cinerea* group II has been described to mostly exhibit the *Transposa* genotype (contain both *Boty* and *Flipper*) and occasionally the *Vacuma* genotype (contain neither)(Giraud *et al*., 1997). Later, it was described like a complex transposon spectrum with four possible genotypes. Indeed, our results demonstrate that all four possible types of *B. cinerea* transposon types existed. Strains belonged to *Transposa* and *Vacuma* subpopulations were found in our samples, also was the third type of isolates having only the *Boty* transposable element and the fourth type having only the *Flipper* transposable element.

PCR amplification using *Flipper* forward and reverse primers has generated the expected 484 bp PCR fragment as shown in Figure 2. As well, the *Boty* primer pair has amplified a 383 bp PCR fragment showed in Figure 3.

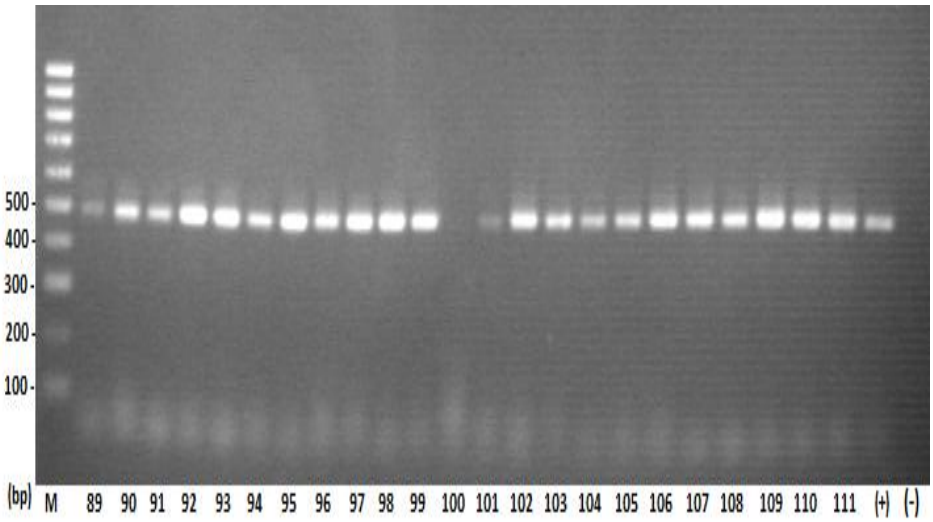

Figure 2. Example of agarose gel electrophoresis showing the result of the *Flipper* PCR amplification products (484bp) of some *Botrytis cinerea* isolates. (+): Positive *Flipper* control strain; (-): Negative *Flipper* control strain; M: Molecular weight marker 1 kb ladder.

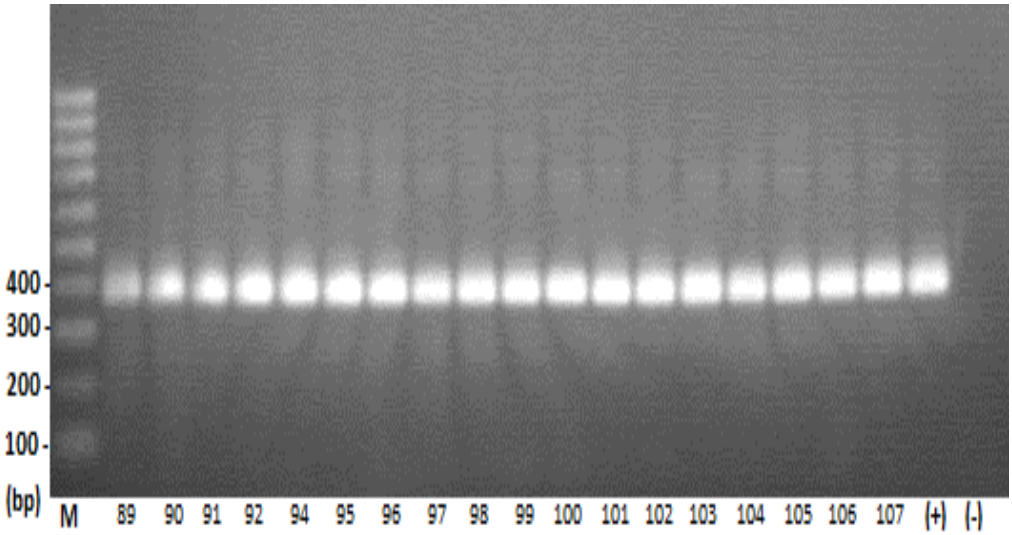

Figure 3. Example of agarose gel electrophoresis amplification of the *Boty* PCR products (383 bp) with DNA of *Botrytis cinerea* isolates. (+): Positive *Boty* control strain; (-): Negative *Boty* control strain; M: Molecular weight marker 1 kb ladder.

The results indicated that *B. cinerea* grapevine isolates containing both transposable elements were predominant (Table 1). Among 122 grapevine isolates tested, 96 isolates corresponding to 78.69% contained both *Flipper* and *Boty* TEs and belonged to *Transposa* type. Otherwise, only three isolates (2.46%) were *Vacuma*, and contained neither transposon. Sixteen isolates (13.11%) were found to harbor only *Boty* TEs, whereas seven isolates (5.74%) contained only the *Flipper* TEs. Furthermore, the three strains collected from tomato and strawberry plants harbored the two transposable elements and belonged then to *Transposa* type.

Table 1. Classification of *B. cinerea* isolates in TEs genetic groups

Hosts	Number of isolates	Transposable elements genetic groups			
		Transposa	*Vacuma*	*Boty*	*Flipper*
Grapevine	122	96	3	16	7
Tomato	2	2	-	-	-
Strawberry	1	1	-	-	-

Population Genetic Analysis

The study of genetic populations revealed a weak to moderate gene flow (1.22) between North Africa (Morocco/Tunisia) and Europe: in our study, the Mantel test indicated 0.261; P=0.01; 999 permutation.

The map (Figure 4) shows the distribution of *B. cinerea* cryptic species groups I and II in countries from North Africa and Europe. The group II was revealed in all studied countries; whereas, in Tunisia, Italy and Greece as Morocco the group I was not reported.

The map present also the histograms of approximate frequencies of transposable elements types and their distribution within countries. The approximate frequencies were deducted from data and/or diagrams studies that are presented in Table 2.

A great divergence in distribution of the TEs types was observed. The most prevalent transposon type was *Transposa*, it was reported in: Greece (48,67%), Hungary (54,35%), Germany (61,16%), France (71,75%) and Morocco (78.69%). However, it was underrepresented in Tunisia (18,18%) and Italy (31,03%). At the same time the group I was reported only in France, Germany and Hungary.

Table 2. Frequencies (by percent %) used for histogram distribution

Country	TEs types				Group	References
	Transposa	*Vacuma*	*Flipper*	*Boty*		
Morocco	78.69	2.46	5.74	13.11	II	Current study
Tunisia	18,18	27,27	27,27	27,27	II	Frequencies calculated from data table (Ben Ahmed *et al*., 2005)
Greece	48,67	28,17	16,17	7,00	II	Means calculated from approximate frequencies data diagram (Samuel *et al*., 2012)
Italy	31,03	51,79	1,79	15,38	II	Frequencies calculated from data table (Vercesi *et al*., 2014)
Hungary	54,35	1,09	2,17	42,39	I, II	Frequencies calculated from data table (Fekete *et al*., 2012)
Germany	61,16	17,69	-	21,15	I, II	Frequencies calculated from data (Kretschmer *et al*., 2008) and data table (Fournier *et al*., 2003)
France	71,75	28,25	-	-	I, II	Frequencies calculated from approximate data in diagrams (Fabian Martinez *et al*., 2005) and in data table (Fournier *et al*., 2003)

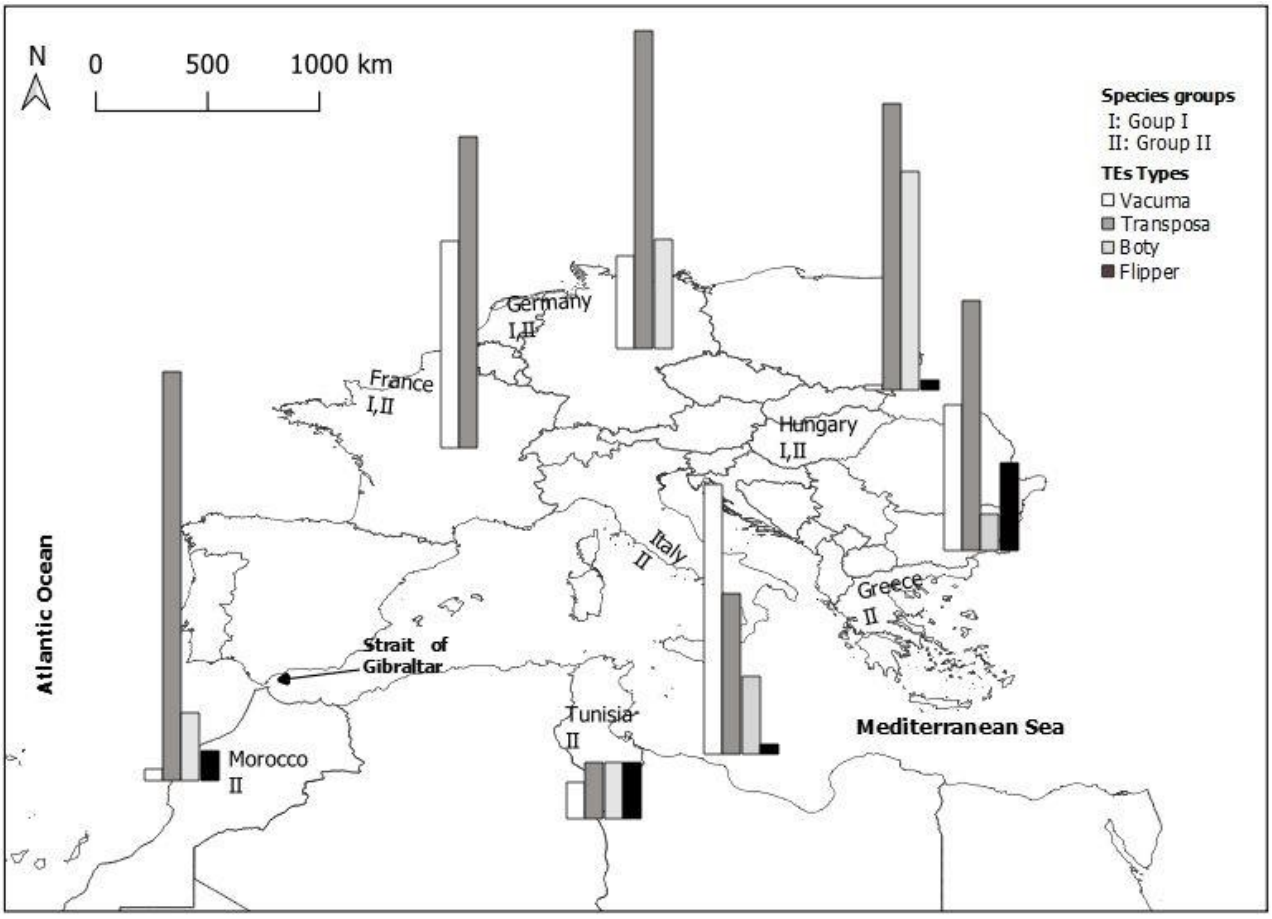

Figure 4. Overview of presence of *Botrytis cinerea* cryptic species groups in some countries from North Africa and Europe. I: Group I and II: group II. The strait of Gibraltar is arrowed and data boundaries of presented countries are downloaded from spatial data DIVA-GIS site. Histograms present approximate frequencies of transposable elements types in studies, which used TEs characterization.

DISCUSSION

In a first attempt to characterize genetically *B. cinerea* in Moroccan vineyards, the current study assessed the Bc-hch haplotyping to explore the group I and group II cryptic species. As a first finding, our study showed that only group II *B. cinerea* isolates were identified. Indeed, the vineyards populations of *B. cinerea* are related exclusively to group II known as *Botrytis cinerea stricto sensu*.

The Group I was mainly characterized by high resistance to the hydroxyanilide fungicide fenhexamid (Fournier *et al*., 2005; Fabian Martinez *et al*., 2005). A genetic isolation have been determined between group I and group II populations by genealogical concordance of the Phylogenetic Species Recognition (Fournier *et al*., 2005).

The group I cryptic species have been reported on grapevine in Europe: in France (Fournier *et al*., 2003; Fabian Martinez *et al*., 2005), in Germany (Kretschmer *et al*., 2008) and on raspberry and rape in Hungary (Fekete *et al*., 2012); also in America as resistant fenhexamid strains in California (Ma *et al*., 2005) and in new Zealand (Johnston *et al*., 2014). Even present, the group I seems to be considerably underrepresented with lower frequency levels (Fournier *et al*., 2005; F. Martinez *et al*., 2008; Fabian Martinez *et al*., 2005).

Among the isolates tested in our study, none belonged to group I as showed by PCR-RFLP of the Bc-hch locus profile. Similarly, group I strains were reported to be absent molecularly or as phenotypic strains in many studies in south Asia/Australia, Tunisia, Chile, Serbia and Egypt (Abdel Wahab, 2015; Esterio *et al*., 2011; Isenegger *et al*., 2008; Karchani-Balma *et al*., 2008; Tanovic *et al*., 2014).

The absence of group I cryptic species of *B. cinerea* in Morocco and Tunisia may indicate the migration has been restricted between North Africa (Morocco/Tunisia) and Europe especially with the Mediterranean Sea (Figure 4). Nevertheless, other factors could also be considered including regional agricultural practices, pest management strategies especially the use of fenhexamid and climate factors as well as the characteristics of the host plant diversity between countries.

The Group I strains described in Hungary suggested the ability of the cryptic species to spread beyond geographic barriers like the Alps (Fekete *et al*., 2012). The presence of *B. cinerea* Group I cryptic species in Europe and the group fenhexamid resistant isolates in America may indicate that migration was occurred between these regions (Fournier *et al*., 2005; Isenegger *et al*., 2008; Ma *et al*., 2005).

A long distance migration was described as probably the result of human trade and colonisation (Beever & Weeds, 2007). In Australia, quarantine barriers performed for a long time could have contributed to restrict introduction of *B. cinerea* cryptic species group I, However the presence of group I in new Zealand (Johnston *et al*., 2014) required more investigation.

In the basis of the above findings, migration of *B. cinerea* species expectation is possible between Europe and North Africa and especially Morocco with the presence of the straits of Gibraltar (Figure 4). Hence, preventive activities like quarantine barriers could be an important method to manage and restrict *B. cinerea* external cryptic species group I. Otherwise, more sampling including other host plants, the use of pesticides like fenhexamid and seasonal factor remain to be further investigated.

Many previous studies reported that the group II isolates showed higher genetic diversity than group I strains (Fournier *et al*., 2008; Karchani-Balma *et al*., 2008; Rajaguru *et al*., 2010). In our study we investigate using transposable element markers in our group II isolates.

Transposable elements are a dynamic components of genomes, their presence in *B. cinerea* could explain its genetic variability as a source of genome evolution diversity (Beever *et al*., 2007).

Initially, two types of strains were described in France from grapes in the basis on the presence or absence of *Boty* and *Flipper* transposable elements, they were named *Transposa* (both TEs were present) and *vacuma* (both TEs were lacked) (Giraud *et al*., 1997), they were also reported by Fournier *et al*. (2002, 2003). After that, *B. cinerea* populations were reported to exhibit four TEs genotypes (F. Martinez *et al*., 2008) and were described at different proportions of *Transposa*, *Vacuma*, *Boty* (*Boty* TEs only) and *Flipper* (*Flipper* TEs only).

In our study, the Group II isolates exhibit the four TEs genotypes *Transposa*, *Vacuma*, *Flipper* only and *Boty* only. The *Transposa* genotype was the most prevalent (78.69%) similarl to the situation on grapes in France (F. Martinez *et al*., 2008), in Germany (Kretschmer *et al*., 2008), in California (Ma *et al*., 2005), in Chile (Esterio *et al*., 2011), in Egypt (Abdel Wahab, 2015) and in Greece (Samuel *et al*., 2012) also on strawberries and raspberries in Hungary (Fekete *et al*., 2012).

Nonetheless, in the republic of Macedonia the *Flipper*

genotype was the most prevalent (Kuzmanovska *et al*., 2012). The situation in the south Asia is such that *Flipper* and *Transposa* genotypes were the most prevalent in Bangladesh, most types from India/Nepal being *Boty* only and in Australia the *Transposa* and *Boty* genotypes were predominant with no *Flipper* strains detected (Isenegger *et al*., 2008), also no *Flipper* strains detected in Croatia (Topolovec-Pintaric *et al*., 2004).

This overview support on the one hand, the higher genetic diversity of group II isolates and its complexity. The frequency differences of transposable elements from different geographic regions suggest the higher adaptive potential of *B. cinerea* populations. On the other hand, for some countries the absence of group I *B. cinerea* naturally resistant to fungicide fenhexamid on grapevine may help in the typing of the population and then enhancing strategies of the integrated disease management in order to avoid epidemic situations, more host plants and fungicide sensitivity tests could be handled. Management of *B. cinerea* is as complex as also environment and fitness factors could be involved.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

All the authors contributed equally to this work.

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