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Geography, Plants, and Growing Systems Shape the Genetic Structure of Tunisian *Botrytis cinerea* Populations

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ABSTRACT

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Botrytis cinerea, considered for a long time as a generalist fungal pathogen of a multitude of plants, was recently shown to exhibit significant population structure in France according to the host, suggesting sympatric specialization. Recent models also showed that adaptation to new hosts may facilitate the process of sympatric speciation in fungal plant pathogens. The present work aimed at investigating if host plants, combined with geographic origin and growing systems, shape the diversity and structure of Tunisian populations of *B. cinerea*. We genotyped 153 isolates with 9 microsatellites. In all the investigated populations, the

fungus reproduced mainly sexually. Gene flow was significantly reduced between greenhouses and open fields from strawberry but not from grapevine. Populations from tomatoes, sampled under greenhouses only, exhibited a low genotypic diversity. The effects of plant and geography from open fields were investigated on a sample of 74 isolates. Six populations were inferred, mainly structured according to a geographic barrier corresponding to the Grande Dorsale Mountain. However, this effect could not be separated from the host plant origin of isolates. The analysis of 63 isolates recovered from strawberries and faba beans in the Cap Bon and Centre regions did not reveal any significant effect of plant on pathogen population differentiation.

Additional keyword: parasite.

The adaptive potential of a species conditions their response to selective pressures, hence their evolutionary trajectories. Fungal plant pathogens show numerous cases of adaptive evolution (e.g., plant resistance breakdowns, host jumps, or evolution of resistance to antifungal compounds) (34). Deciphering the adaptive potential of economically damaging phytopathogenic fungi is then crucial to set up durable resistance strategies. The adaptive potential depends on several processes, among which are contemporary gene flow and reproductive mode. Both processes may be shaped by different factors, such as landscape structure and geographic distance, host plant species, and host plant growing system. In the present study, we were interested in describing these two processes and examining the main factors explaining them in the plant pathogenic fungus *Botrytis cinerea*.

B. cinerea Pers.: Fr. (teleomorph *Botryotinia fuckeliana* [de Bary] Whetzel) is a haploid, heterothallic Ascomycete. It causes gray mold on more than 200 Eudicotyledon species (43), among which many cultivated and ornamental crops, being responsible for serious economic losses in pre- and postharvest. This fungus exhibits a great morphological, physiological, and genetic variability (5,8,14,16,19,30,48). Its life cycle is divided in a saprophytic and a parasitic phase, to which various pathogenicity factors are associated (9,45). Several studies reported a gradual decrease of the efficiency of some fungicides in the field (12,23,27,40).

Phenotypic variability was attributed to parasexuality (6,46). At the genetic level *B. cinerea* has been considered a clonal species

for a long time, due to the great production of conidia and the scarcity of sexual structures observed in natural conditions. This view, however, was totally revised during the last decade. For instance, several molecular studies using random amplified polymorphism DNA (RAPD) (35,38,44), or restriction fragment length polymorphism (RFLP), and transposable elements (19) showed a great genetic variability and a lack of linkage disequilibrium compatible with the existence of genetic recombination probably due to regular sexual reproduction, at least for France, Spain, Italy, and Chile (3,5,19,36).

Recent studies based on multiple gene genealogies (2,14,16) showed that *B. cinerea* was in fact a complex of at least two sibling species, called *B. cinerea* groups I and II, that diverged without any remnant gene flow since at least 10^7 generations. The species *B. cinerea* group I exhibits restricted host and geographic ranges, while the species *B. cinerea* group II remains the most widespread and damaging species on grapevine and other hosts at harvest (14,31). Within *B. cinerea* group II, isolates have been distinguished according to the presence or absence of transposable elements (TE) in their genome, *transposa* isolates carrying the two TE Boty and Flipper, and *vacuma* isolates carrying none (19). However, no reduction of gene flow was observed between *vacuma* and *transposa* isolates (13). The species *B. cinerea* group I includes only *vacuma* isolates (14).

Is the species *B. cinerea* group II panmictic or are there important barriers to gene flow, structuring its genetic variability and its reproductive mode, hence its adaptive potential? Besides the geographic origin of isolates, the host plant on which isolates are developing is an important factor that may shape fungal pathogen genetic structure. Indeed, parasites and their hosts are continually evolving in response to each other (24), and different host species exert strong disruptive selection. Giraud et al. (20) used a theoretical approach to show that, because of their life cycle, speciali-

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zation of a fungal pathogen on different plants would be sufficient alone to allow both adaptation to the host plant and reproductive isolation, thus facilitating sympatric speciation. Several studies of *B. cinerea* (*sensu lato*) on various host plants showed very weak geographic differentiation, if any, agreeing with large scale dispersal of asexual spores (5,19,36). Muñoz et al. (36) reported a significant genetic differentiation between grapevine and tomato isolates in Chile, and similar results were obtained in the Champagne region of France for *B. cinerea* on numerous plants (18). However, these studies did not distinguish between *B. cinerea* groups I and II, which may have biased the results. More recently, a significant genetic structure was found within *B. cinerea* group II according to two natural host plants, grapevine and bramble, using microsatellite markers (13). This raises the question of the generality of such a host plant effect, according to other plants and other parts of the world.

Another factor that may have an important effect on pathogen population structure is the cropping system for the considered host plant, especially open field versus greenhouses growing systems. Indeed, greenhouses may exhibit very particular population resulting from bottleneck effects due to restricted migrations from outer areas. Several works have studied the epidemiology and disease installation and progression within and between different greenhouses (3,10,22,39), but to our knowledge no study

has ever compared closed systems to open field conditions. Moreover, due to the preference of *B. cinerea* for wet conditions, the questions of the respective roles of geography, host plant, and cultural modes in the differentiation of *B. cinerea* group II populations have not been addressed to date in dry Mediterranean areas, where *B. cinerea* is yet also an important problem on many cultivated plants.

The aim of the present work was, therefore, to study the genetic variability, mode of reproduction, and differentiation according to host, geography, and growing system, in several populations of *B. cinerea* group II from Tunisia, using nine polymorphic microsatellite markers (15). We specifically addressed the following questions. (i) Does the growing system influence the genetic structure of *B. cinerea* group II populations? (ii) In open field systems, are the *B. cinerea* group II populations genetically differentiated as a function of geography and host plants?

MATERIALS AND METHODS

Fungal sampling and isolation. A total of 169 *B. cinerea* isolates were collected from April to September 2004, in four different Tunisian areas (Cap Bon, Nord, Grand Tunis, and Centre; Fig. 1) from four different host plants: faba bean (*Vicia fabae*), grapevine (*Vitis vinifera*), tomato (*Lycopersicon esculentum*), and strawberry (*Fragaria × ananassa* varieties). Isolates from faba bean were sampled exclusively in open field systems, isolates from tomato were sampled exclusively under greenhouses, whereas both growing systems were sampled for isolates from grapevine and strawberry (Table 1). Within a particular region, sample places were not more distant than 40 km, except the town of Bou Salem that was 130 km away from Mateur in the Nord region. Isolations were performed from decaying fruits on strawberry, from chocolate spots on faba bean leaves, and from sporulated lesions on stems and peduncles on tomato and grapevine. Small tissue pieces taken from lesions were incubated at 20°C during 5 to 10 days in petri dishes on potato dextrose agar (PDA) amended after autoclaving with 0.5 g/liter of streptomycin (Sigma-Aldrich) resuspended in 0.5% ethanol. Growing mycelium was transferred and isolates were genetically purified by monospore isolation and conserved on NY medium plates (20 g of malt, 2 g of yeast extract, and 20 g per liter of agar) at 4°C as well as spore suspensions in 20% glycerol at -80°C for long-term preservation.

DNA extraction and microsatellite genotyping. DNA extractions were performed using the QIAGEN Biorobot 3000 extraction kit from 5-day-old mycelial cultures grown on NY medium at 22°C. Biological material was first grinded in 25 µl of QIAGEN lysis buffer with tungsten microballs. DNA quality was checked by electrophoresis on 0.8% agarose gel. For each isolate, we amplified separately nine microsatellite markers previously developed by Fournier et al. (15), with forward fluorescence-labeled primers, and following author's polymerase chain reaction (PCR)

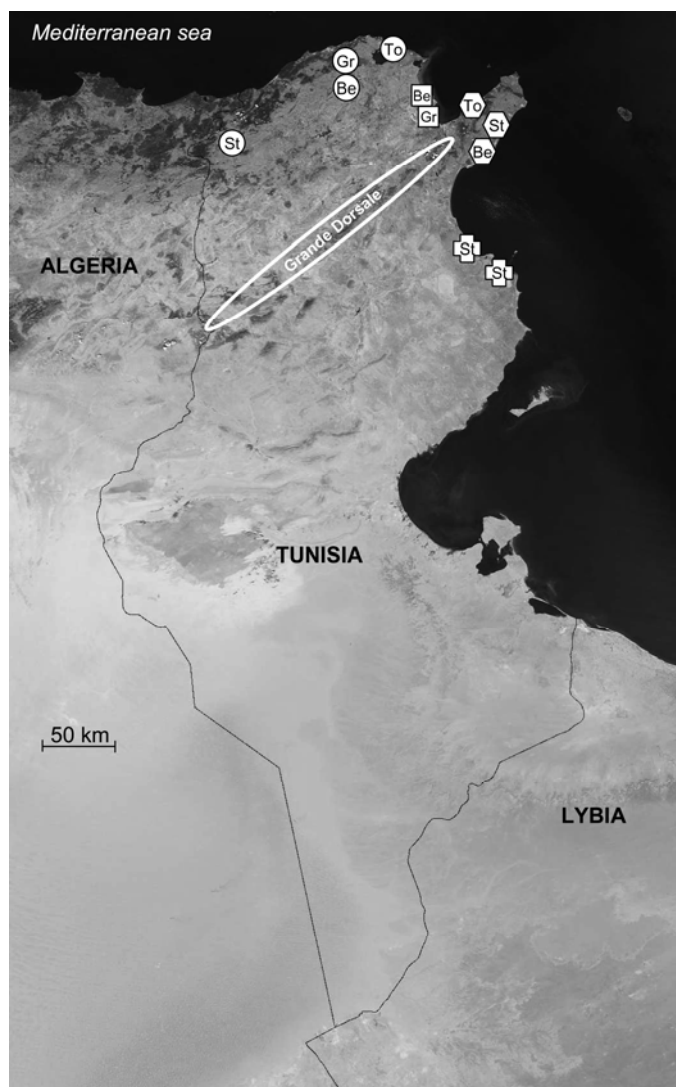


Fig. 1. Location of *Botrytis cinerea* sampling sites in Tunisia. St: strawberry; Gr: grapevine; To: tomato; and Be: bean. Populations from Nord are designated by circles, Grand Tunis by squares, Cap Bon by hexagons, and Centre by crosses.

TABLE 1. Number of *Botrytis cinerea* isolates entirely genotyped from the different regions of Tunisia, the different host plants, and the different growing systems

Host	Nord		Grand Tunis		Cap Bon		Centre	
	GR ^a	OP	GR	OP	GR	OP	GR	OP
Tomato	3				35 ^b			
Grapevine		9 ^{b,c}	22 ^{b,c}					
Strawberry		2			33 ^b	24 ^{b,c}		6 ^{b,c}
Bean		1		2		13 ^{b,c}		
Others				1				2

^a GR: greenhouse, and OP: open field.

^b Populations further used for the analysis of the effect of growing system.

^c Populations further used for the analysis of the effects of plant and geography.

conditions. PCR products were analyzed using a CEQ8000 DNA sequencer (Beckman Coulter, Fullerton, CA), with allele sizing and grouping manually performed by visual inspection of chromatograms. After isolation, purification, removal of group I isolates, DNA extraction, and genotyping process, a total of 153 out of the 169 isolates were finally available (complete genotypes) for subsequent analyses (Table 1).

Distinction between *B. cinerea* groups I and II isolates.

According to Fournier et al. (15), isolates from *B. cinerea* group I exhibit at least one private allele at four of the nine microsatellite markers. Further population studies (13, A.-S. Walker, *personal communication*) showed that the allele sizing 86-bp at locus Bc6 was diagnostic for *B. cinerea* group I. In the present study, none of the isolates genotyped carried this private allele, demonstrating that the whole sample belonged to the species *B. cinerea* group II. This result was further confirmed by a test of resistance to the fenhexamid fungicide (Teldor WG50, Bayer, Monheim, Germany), following the protocol of Albertini et al. (2). None of the isolates of the present study was phenotyped as HydR1, which corresponds to the high natural level of resistance exhibited only by *B. cinerea* group I representatives (2).

Population genetics analyses. The software Genetix 4.05 (K. Belkhir, P. Borsa, L. Chikhi, N. Raufaste, and F. Bonhomme, 1996) was used to compute unbiased genic diversity, H_{nb} (37), which is based on allele frequencies, and allelic richness over all loci in the total sample and in each population (i.e., for each region, host plant, and growing system combination) (Table 1). Genetix was also used to perform 2D-factorial correspondence analyses, allowing examining visually the distribution of the different multilocus genotypes in a two-dimensional space whose principal axes are linear combinations of different alleles at different loci. Lastly, we used Genetix to calculate pairwise Weir and Cockerham's (47) F_{ST} values between all pairs of populations, and to evaluate their significance by random permutations and progressive Bonferroni correction as described by Rice (41).

We used the program Multilocus 1.3b (1) to plot the genotypic diversity calculated from multilocus genotype frequencies (37) versus the number of loci, in order to see whether the nine microsatellites used in this study were sufficient to recover the maximal genotypic diversity, or whether it would be necessary to score more loci. The program randomly samples 100 times from 1 to $m-1$ loci (m being the total number of loci) from the data set and calculate the number of different genotypes and the genotypic diversity for each randomly shuffled data set. The m microsatellites are considered to be discriminating and powerful enough if the genotypic diversity recovered for 1 to $m-1$ loci reaches a plateau, that is, if increasing the number of scored loci does not increase the recovered genotypic diversity. The number of different multilocus genotypes (MLG) and the standardized version of the index of association r_D (1,21) were also calculated using Multilocus 1.3b. The index of association is a measure of the multilocus linkage disequilibrium (7,21,33), which gives information on whether two different individuals sharing the same allele at one locus are more likely to share the same allele at another locus. For any pair of individuals, the number of loci with respect to which they differ is calculated and the variance of this number is compared to that expected if there is no linkage disequilibrium. r_D , the standardized version of the index of association, is independent of the number of loci considered, and varies between 0 (complete panmixia) and 1 (no recombination). The null hypothesis of complete panmixia ($r_D = 0$) was tested with the procedure implemented in the software, by comparing the observed data set to 100 randomized data sets in which infinite recombination has been imposed by randomly shuffling the alleles amongst individuals, independently for each locus.

To analyze further the effect of host plant on the genetic structure of fungal populations, the relative contributions of the factors "plant species" and "sampling location" to the genetic variance

were estimated with a hierarchical analysis of molecular variance (AMOVA) using ARLEQUIN version 2.000 (S. Schneider, D. Roessli, and L. Excoffier, 2000), with plant used as a grouping factor. This analysis was performed on a subset of four populations sampled from grapevine in Nord and Grand Tunis, and from strawberry in Cap Bon and Centre, encompassing a total of 61 individuals. Finally, we used the software Structure version 2.1 (J. K. Pritchard, P. Stephens, and P. Donnelly, 2000) to analyze the respective roles of host plant of origin and geographic origin without using any a priori knowledge on population subdivision. The algorithm implemented in this software assumes that individuals in the sample are assigned probabilistically to K clusters, or jointly to two or more clusters if their genotypes indicate that they are admixed, without consideration of their region or host of origin. Each cluster is characterized by a set of allele frequencies at each locus, and loci are assumed at Hardy-Weinberg equilibrium and linkage equilibrium within the K clusters. However, this approach proved to be robust to deviations from these assumptions (11). Following Falush (11), we used the model with admixture, since we knew little about the existence of such a process in our data, and assumed uniform priors for the vector of proportion q_i of the individual i 's genome in each cluster. The scores of individuals in the clusters (i.e., the posterior estimates of the q_i) correspond to the probability of ancestry in any one of them. We varied K from 1 to 10, with three replicates for each K , and with each simulation consisting in 500,000 Monte-Carlo Markov Chain (MCMC) iterations preceded by a burn-in period of 100,000 iterations (the burn-in period is the first set of iterations of the MCMC that is dependent on the configuration at the start—the iterations are not incorporated in the final calculation of the posterior probability). The most probable structure was determined by computing the posterior probability for each K using the distribution of maximum likelihoods. When the probability of ancestry of an individual in a cluster was greater than the arbitrary threshold of 0.7, this individual was considered to be unambiguously assigned to this cluster.

RESULTS

Global genetic diversity and discrimination power of the microsatellites. Out of the 169 isolates collected, 153 (Table 1) were completely genotyped with the nine microsatellites. Consistent with previous studies (13,19), the overall level of genetic diversity was high with an unbiased genic diversity (H_{nb}) of 0.79 (± 0.12 standard deviation [SD]), and a mean number of alleles per locus reaching 12.80 (± 3.89 SD) (Table 2). The number of alleles per locus varied from four (locus Bc4) to 21 (locus Bc2), the latest contributing the most to the overall genic diversity ($H_{nb} = 0.90$) (Fig. 2).

Among these 153 genotyped isolates, 120 MLG were recovered. We used the program Multilocus to check for the discrimination power of the microsatellites. The genotypic diversity reached 0.77 with a single locus randomly chosen, and varied from 0.992 and 0.995 with five to nine loci; the proportion of different MLG detected in the total sample varied from 0.077 to 0.78, respectively, with one to nine randomly chosen loci (Fig. 3). These results indicate that the set of microsatellite markers used here was sufficient to correctly estimate the existing diversity.

As a preliminary inspection of the data, we performed two-dimensional analyses of factorial correspondences (AFC) in order to visually inspect the genetic structure of the total data set, according to growing system, host plant, and geographic origin (Fig. 4). No clear pattern appeared according to any of the three factors. However, these analyses showed that tomato and/or greenhouses populations were less genetically diverse.

Open field versus greenhouse populations for the different host plants. Since several studies previously showed that closed systems greatly influence the genetic structure and enhances the

level of clonality of *B. cinerea* populations (3,10), we first analyzed the influence of growing systems on the population diversity and structure in grapevine and strawberry populations. Population from these two host plants were analyzed separately since the host plant is also known to affect *B. cinerea* group II population structure (13).

Of a greenhouse population of 22 isolates collected from grapevine (Table 2), at Grand Tunis, 20 distinct MLG were recovered,

displaying a genotypic diversity of 0.991. The Nord populations from open field exhibited very similar levels of diversity: 7 MLG were distinguished out of 9 individuals, for a genotypic diversity of 0.944. The pairwise F_{ST} (Table 3) between these two populations were not significant ($F_{ST} = 0.039$, $P = 0.084$ when all individuals were taken into account; $F_{ST} = -0.005$, $P = 0.5$ when repeated MLG were removed). Hence, neither the growing system nor the geographic distance affected the genetic differentiation

TABLE 2. Genetic diversity indexes within the pooled sample and in each population of *Botrytis cinerea* separated by host plant and by growing systems^a

Population	Geographic origin	N	H_{nb}	Mean no. of alleles/locus	No. of distinct MLG	Max. no. of repeated MLG	Genotypic diversity	r_D^b
Total sample	All	153	0.79 (0.12)	12.80 (3.89)	120	7	0.99	0.081S
Grapevine total	Nord - Grand Tunis	31	0.69 (0.16)	6.78 (2.19)	26	2	0.99	0.086S
Grapevine OP	Nord ^c	9	0.60 (0.21)	3.78 (1.13)	7	2	0.94	0.238S
Grapevine GR	Grand Tunis ^c	22	0.70 (0.14)	5.89 (1.79)	20	2	0.99	0.083S
Strawberry total	Cap Bon - Centre - Nord	65	0.79 (0.12)	11.11 (4.25)	58	3	0.99	0.107S
Strawberry OP	Cap Bon ^c	24	0.80 (0.07)	8.33 (2.49)	23	2	0.99	0.147S
	Centre ^c	6	0.70 (0.14)	3.78 (1.31)	6	1	1	0.310S
	Nord	2	–	–	–	–	–	–
Strawberry GR	Cap Bon ^c	33	0.70 (0.18)	7.11 (2.67)	29	3	0.99	0.104S
Tomato GR	Cap Bon + Nord	38	0.62 (0.11)	3.89 (1.10)	19	7	0.94	0.129S
	Cap Bon ^c	35	0.61 (0.12)	3.78 (1.13)	18	7	0.93	0.139S
	Nord	3	–	–	–	–	–	–
Faba bean OP	Cap Bon - Grand Tunis - Nord	16	0.75 (0.19)	6.33 (2.58)	16	1	1	0.111S
	Cap Bon ^c	13	0.72 (0.21)	5.44 (2.45)	13	1	1	0.13S
	Grand Tunis	2	–	–	–	–	–	–
	Nord	1	–	–	–	–	–	–
Others		3	–	–	–	–	–	–

^a OP: open field, GR: greenhouse. N: sample size. H_{nb} : nonbiased genic diversity (37), calculated with repeated genotypes (standard deviations between brackets). MLG: multilocus genotypes. All estimates were calculated only for populations counting more than six individuals.

^b r_D : standardized index of association (1,21). S indicates significant values, i.e., P values below 1% after 100 random resampling.

^c Same as in Table 1.

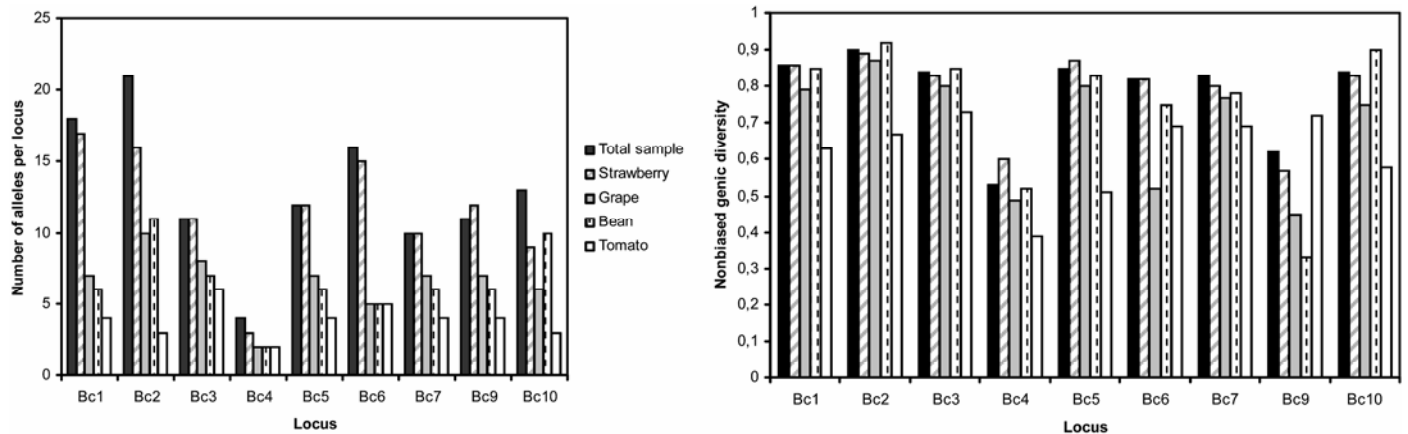


Fig. 2. Relative contribution of the different loci to the genetic diversity of *Botrytis cinerea* in the total sample and in the populations separated by host plants. Left panel: nonbiased genic diversity H_{nb} (37); right panel: number of alleles per locus.

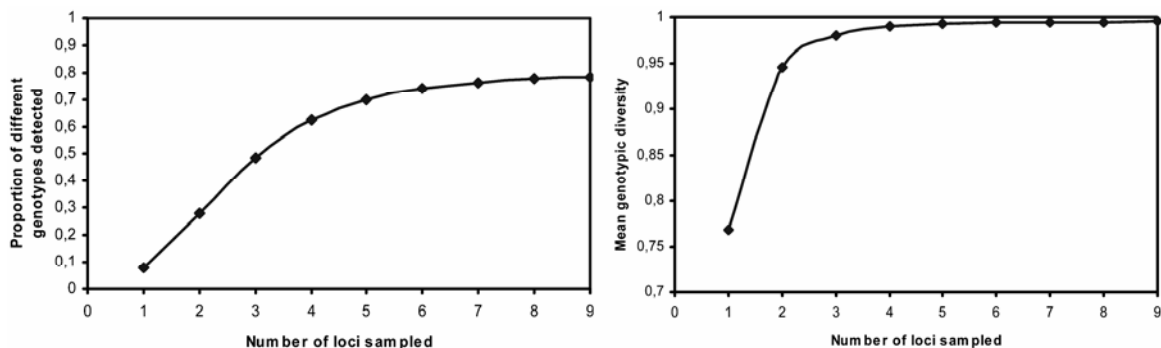


Fig. 3. Discrimination power of the nine microsatellite loci used in the present study. The proportion of different genotypes recovered from the total data set (GML/N) and the genotypic diversity were calculated from the data sets obtained after 100 random samples of one to eight loci.

and diversity of *B. cinerea* populations from this plant. This also means that grapevine greenhouses cannot be considered as closed systems. Hereafter, all isolates from grapevine will therefore be analyzed as open field isolates. The estimates of multilocus linkage disequilibrium (Table 2) were low but significant in both populations (Grand Tunis greenhouse: $r_D = 0.083$, $P < 0.01$; Nord open field: $r_D = 0.238$, $P < 0.01$). This suggests a sexual reproductive mode mainly with a low but significant level of clonality.

Populations collected from strawberries in the Cap Bon and Centre regions presented a contrasting differentiation pattern (the only two isolates coming from other areas were not considered here) (Table 2). The open field population from the Centre region encompassed six isolates, all being distinct MLG, representing a genotypic diversity of one. This population was not significantly differentiated from the open field ($F_{ST} = 0.03$, $P = 0.20$) and greenhouse ($F_{ST} = 0.02$, $P = 0.17$) populations from the Cap Bon region, indicating an absence of geographic structure. However, gene flow was significantly reduced between the greenhouse and open field populations from the Cap Bon region ($F_{ST} = 0.095$, $P < 0.001$ with all individuals, $F_{ST} = 0.081$, $P < 0.001$ with non-repeated MLG only) (Table 3). Yet, both populations from the Cap Bon region exhibited similar levels of diversity (Table 2) (open field population showing 23 distinct MLG among 24 isolates with genotypic diversity = 0.996; and greenhouse population showing 29 MLG among 33 isolates with genotypic diversity = 0.99). Again, low but significant multilocus linkage disequilibria were recovered from both populations, indicating a significant departure from panmixia but not complete clonality (open field population from Cap Bon: $r_D = 0.147$, $P < 0.01$; greenhouse population from Cap Bon: $r_D = 0.104$, $P < 0.01$) (Table 2). Hence, the growing system apparently did not affect the population dynamics and reproductive mode, but clearly reduced the amount of gene flow between populations from strawberry in the same region. In the subsequent analyses regarding the respective effects of host plants and geography, only the open field populations from Cap Bon and Centre regions will hence be considered for isolates sampled on strawberries.

B. cinerea group II populations from faba beans were only sampled in open field conditions. The analysis of the Cap Bon population collected from this host showed that the diversity reached similar levels as open field populations from other plants (13 MLG among 13 isolates, leading to a genotypic diversity of 1). The multilocus linkage disequilibrium again showed the same trend of departure from panmixia ($r_D = 0.13$, $P < 0.001$).

We could not compare the effect of growing systems on *B. cinerea* group II populations sampled from tomato either because isolates from this host were sampled only under greenhouses. However, the AFC performed on the total sample already showed a particular genetic composition of this population (Fig. 4B). Indeed, the detailed analysis of the 35 isolates sampled on tomato in the Cap Bon region showed that only 18 distinct MLG were recovered (Table 2). In this population the lowest genotypic and genic diversities (0.936 and 0.62, respectively) were observed. However, the multilocus linkage disequilibrium was of the same range of magnitude as in other populations ($r_D = 0.139$, $P < 0.001$). This may indicate the existence of a bottleneck reducing the population diversity at the entrance of tomato greenhouses, without modifying the reproductive mode.

Respective effects of geography and host plant on open field populations from grapevine, strawberry, and bean. The above analyses led us to restrict the analysis of the effects of geography and host to populations from different plants grown in open field systems (including grapevine from the Grand Tunis region). Samples with less than six individuals were removed from this analysis, which thus included five populations: isolates sampled from grapevine in Nord (N = 9) and Grand Tunis (N = 22), strawberry in Cap Bon (N = 24) and Centre (N = 6), and faba bean in Cap Bon (N = 13), i.e., a total of 74 isolates (Table 1).

Pairwise F_{ST} , calculated between pairs of populations, showed that populations from different regions and from the same host were not significantly differentiated (Table 3), indicating that regardless of the host considered, the geographic distance did not ever reduce gene flow between populations. Only two F_{ST} were significant: grapevine populations from North versus strawberry population from Cap Bon on the one hand, grapevine population from Grand Tunis versus strawberry population from Cap Bon on the other hand. This pattern may be due either to the host plant or to the geographic origin or both. Indeed, the Grand Tunis and Nord regions are separated from the other sampling areas by the Tunisian Dorsale, a South-

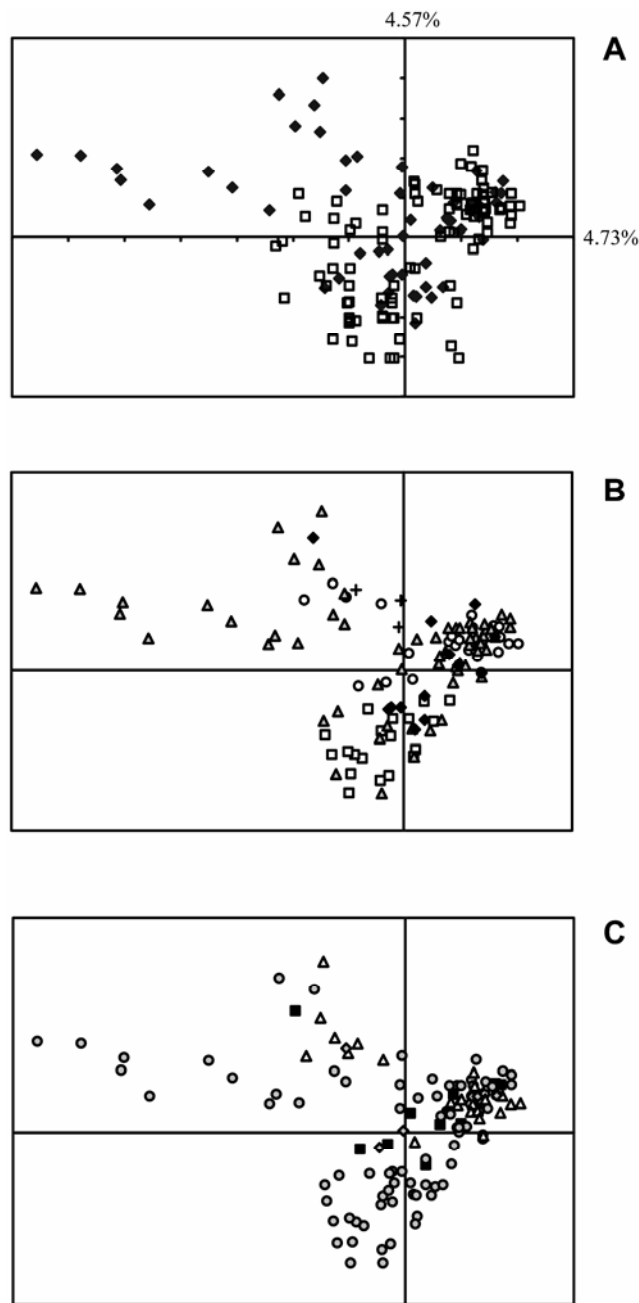


Fig. 4. Two-dimensional analyses of factorial correspondences performed on the total data set of *Botrytis cinerea* isolates. **A**, Populations distinguished by growing systems (□ isolates from greenhouses, ◆ isolates from open field cultures). **B**, Populations distinguished by host plants (□: tomato, ◆: bean, △: strawberry, ○: grapevine, +: others). **C**, Populations distinguished by geographic origin (■: Nord, ○: Cap Bon, △: Grand Tunis, ◆: Centre). Percentage values over each axis indicate the percentage of diversity explained by the corresponding axis.

East/North-East mountain range peaking at more than 1,500 m that could represent a geographic barrier to *B. cinerea* dispersion (Fig. 1).

To investigate the respective roles of host and geography, we performed a hierarchical AMOVA. We excluded isolates from faba bean since they were sampled in a single region; the AMOVA therefore concerned four of the five populations listed above (populations from grapevine sampled in Nord and Grand Tunis, and from strawberry in Cap Bon and Centre) (Table 4). The greatest part of the observed genetic variance came from the variation within each population. The host plant of origin had no significant effect on the partitioning of the total genetic diversity ($P = 0.17$), whereas the geographic origin has a marginally significant effect ($P = 0.05$), indicating that the observed pattern may be due mainly to a geographic barrier to gene flow.

Finally, we used a Bayesian approach to analyze the subset of five populations (74 individuals) with the Structure software, varying K from 1 to 10. The posterior probability of the data, $\ln\text{Pr}(X|K)$, reached a maximum for $K = 6$, and the posterior probability of K was also the highest ($P = 0.999$) for this value (Fig. 5A). The computation of the increase of likelihood ($\text{delta}[\ln\text{Pr}(X|K)]$; Fig. 5B), showed that the gain of explanatory power of the model when adding a new cluster to the analysis was the highest at K changing from 6 to 7, and then became null or negative. The frequencies of isolates in the six clusters (Fig. 5C) were significantly different according to their region and plant of origin ($\chi^2 = 46.5$, $P = 0.0002$, $\text{df} = 18$; Table 5). The major trend seemed to be a geographical structure opposing isolates from the Cap Bon and the North region. Indeed, clusters 2 and 3 encompassed exclusively isolates sampled in the Cap Bon area (7/7 from strawberries in cluster 2, 7 from strawberries, and 4 from faba beans in cluster 3), whereas clusters 5 and 6 comprised respectively 8/8 and 5/7 isolates sampled on grapevine from the Grand Tunis region (Table 5). The two other clusters represented a mixing of isolates from the different regions and plants. However, since all isolates from grapevine were sampled in the Nord and Grand Tunis regions, and all isolates from strawberry and faba beans in the Centre and Cap Bon regions, it was impossible again to truly separate geographic from host plant effects. We performed the same analysis to the restricted sample of 63 isolates from strawberries and faba beans from Cap Bon and Centre regions. The posterior probability of the data and associate indicators all peaked for $K = 4$ (Fig. 6A and B), but the probability of ancestry of isolates in each cluster (Fig. 6C) was not significantly associated with host plant or geographic origin

($\chi^2 = 13$, $P = 0.11$, $\text{df} = 8$; Table 5), showing that in this area, the host plant effect is probably very attenuated, if existing.

DISCUSSION

Although the sample of *B. cinerea* isolates analyzed in this study is not very big and could be optimized by adding new isolates in order to equilibrate the effectives per plant, geographic areas and growing systems, the results obtained here should be quite robust since we used unbiased estimates of genetic diversities and F_{ST} . Moreover, the analysis of genotypic diversity as a function of the number of microsatellite loci showed that the markers used here were powerful enough to detect and characterize the major part of the existing diversity. Several studies (19,31) showed that the frequencies of *vacuina* and *transposa* isolates (two categories characterized by the presence or absence of transposable elements in their genomes) in *B. cinerea* group II populations may vary during the growing season. However, no significant barrier to gene flow has been evidenced between *vacuina* and *transposa* French populations sampled on two different plant species at fall (13), and neither is there any convincing evidence of a correlation between seasonal variations in *vacuina* and *transposa* frequencies and changes in the genetic structure at neutral markers (A. S. Walker, *personal communication*). Hence, samples performed in one particular season may give a reasonable picture of how the genetic diversity is organized in this species.

In accordance with recent population studies conducted on *B. cinerea sensu lato* in several geographic areas (5,13,18,19,36), our results showed that the overall genetic diversity recovered from the 153 fully genotyped isolates from Tunisia was high in all populations. In all populations, the indirect evaluation of the reproductive mode, that is, the standardized index of multilocus linkage disequilibrium was always in accordance with a significant departure from total panmixia, but not reaching complete clonality. The best explanation for this pattern is that *B. cinerea* group II populations undergo regular recombination, separated by several cycles of clonal reproduction. This result agrees with former studies conducted in France (13,19). Hence, *B. cinerea* group II population dynamics and reproductive mode in Mediterranean areas do not seem to differ drastically from more septentrional and humid zones. Other studies conducted in California (29) did not evidence the existence of regular recombination in field populations, but the markers used (microsatellite-primed polymerase chain reaction) were likely to detect little amount of polymorphism, thus to overestimate the amount of clonality. On

TABLE 3. Pairwise F_{ST} among the five *Botrytis cinerea* populations (74 isolates) collected from strawberry, grapevine, and faba bean in different regions in open field systems

Population	St ^a Cap Bon	St Centre	Be ^a Cap Bon	Gr ^a Grand Tunis	Gr Nord
St Cap Bon	–	0.031 (0.026)	0.048 (0.047)	0.110S ^b (0.099 S ^b)	0.140 S ^b (0.102 S ^b)
St Centre		–	0 (0)	0.043 (0.033)	0.085 (0.047)
Be Cap Bon			–	0.024 (0.021)	0.083 (0.047)
Gr Grand Tunis				–	0.039 (0)
Gr Nord					–

^a St: strawberry, Gr: grapevine, and Be: bean.

^b Pairwise F_{ST} are calculated following Weir and Cockerham (47), with and without (values between brackets) repeated MLG. S indicates significant F_{ST} ($P < 0.01$) after 1,000 permutations and a Bonferroni correction.

TABLE 4. Hierarchical analysis of molecular variance performed using Arlequin 2.000 on the four following populations of *Botrytis cinerea*: isolates sampled from grapevine from Nord and Grand Tunis and strawberry from Cap Bon and Centre regions of Tunisia^a

Source of variation	df	Sum of squares	Variance component	Variation (%)	P
Plant species	1	14.570	0.289	7.64	0.17
Region within plant species	2	9.487	0.122	3.22	0.05
Within region	57	192.337	3.374	89.14	<0.001
Total	60	216.993	3.785	100	

^a The host plant of origin was used as a grouping factor.

the contrary, the nine microsatellite markers used here are sufficiently polymorphic and discriminatory to correctly estimate the existing diversity (Fig. 3).

Among the 169 isolates sampled during the present study, none appeared to belong to the species *B. cinerea* group I. This cryptic species, confounded in the past with *B. cinerea* group II, was characterized in several recent studies (2,14,16,31, and A. S. Walker, *personal communication*) in which isolates from *B. cinerea* group I were found in sympatry with *B. cinerea* group II populations at rather low frequencies varying between 2.5 and 15% according to the location and the season. Whereas *B. cinerea*

group I is totally absent from Tunisia or present only at very low frequencies and at particular periods remains to be further investigated.

The comparison between open field and greenhouse growing systems could be performed for two different host plants, leading to contrasting results. As regards isolates sampled from grapevine, the greenhouse population from Grand Tunis was not genetically differentiated from the open field population from Nord (Table 3).

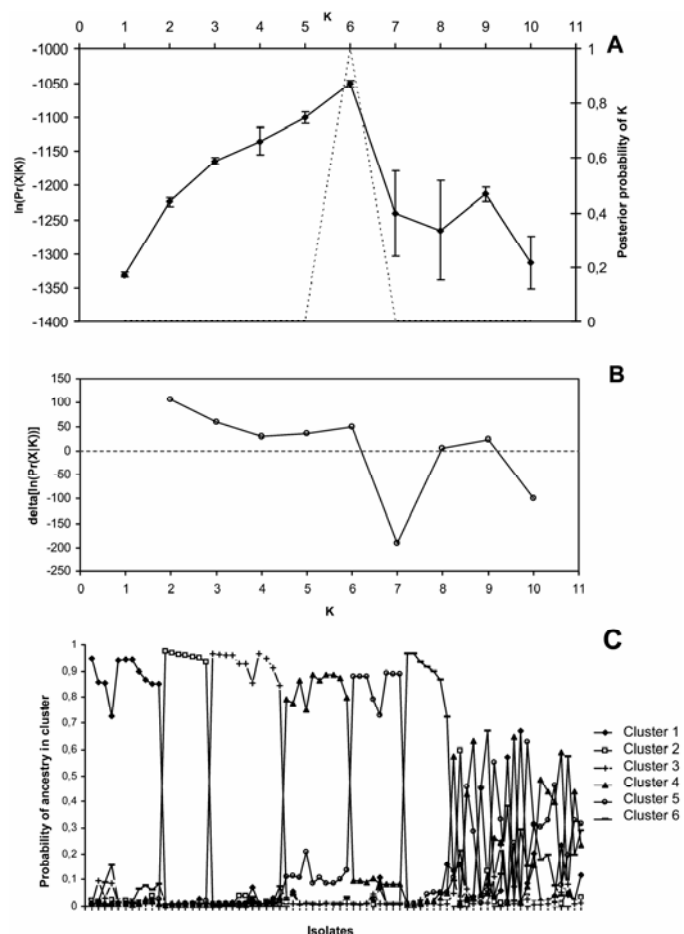


Fig. 5. Estimated number of populations from the 74 isolates of *Botrytis cinerea* from open field growing systems, analyzed with Structure. **A**, Mean (\pm standard deviation) probabilities of the data (left y axis, full line), and posterior probability of K (right y axis, dotted line) for each value of K . **B**, Mean variations of probabilities of the data ($\Delta[\ln(\Pr[X|K])]$) between successive K considered in the Structure analysis. For $K > 1$, $\Delta[\ln(\Pr[X|K])] = \ln(\Pr[X|K])_{k+1} - \ln(\Pr[X|K])_k$. **C**, Probability of ancestry of each individual in the different clusters for $K = 6$.

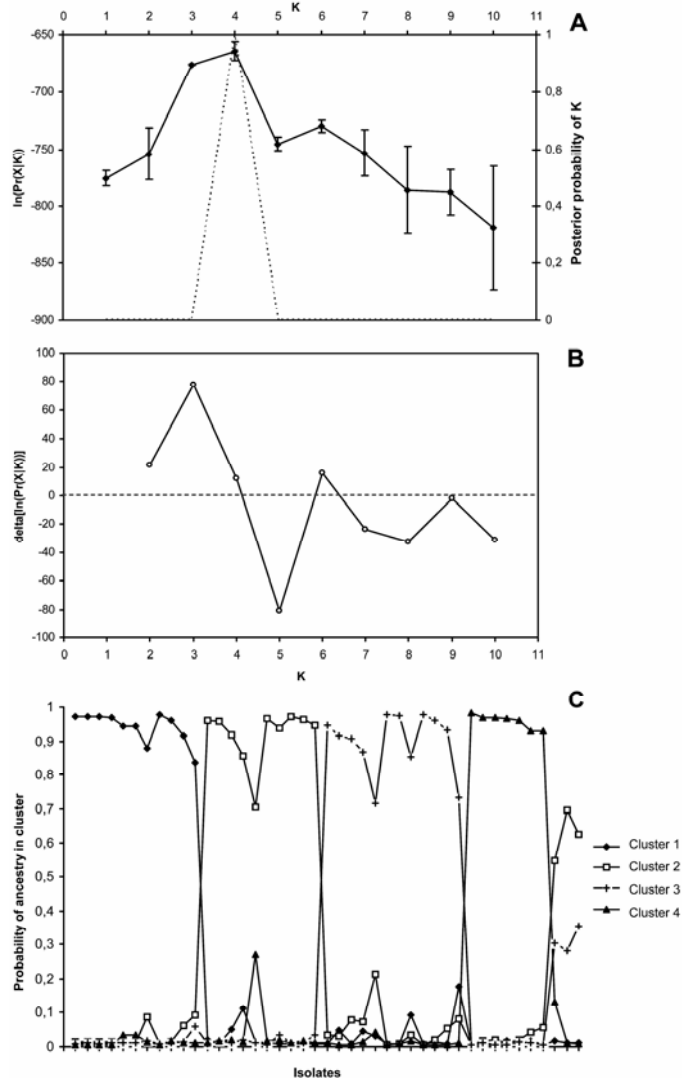


Fig. 6. Estimated number of populations from the 63 isolates of *Botrytis cinerea* from faba bean and strawberries sampled in the Cap Bon and Centre regions in Tunisia analyzed with Structure. The legends are the same as in Figure 5, except for Figure 6C where the probability of ancestry of each individual was calculated for $K = 4$.

TABLE 5. Frequencies of isolates of *Botrytis cinerea* from the different regions and host plants in the different clusters inferred with the Bayesian analyses using the Structure software

Sample analyzed	Plant and geographic region	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6	Not assigned
74 isolates (Fig. 5)	St ^a Cap Bon	4	7	7	2	0	0	4
	St Centre	1	0	0	2	0	0	3
	Be ^b Cap Bon	1	0	4	2	0	2	4
	Gr ^c Grand Tunis	5	0	0	4	8	5	9
63 isolates (Fig. 6)	St Cap Bon	7	5	5	7			0
	St Centre	0	2	3	0			1
	Be Cap Bon	3	3	4	0			2

^a St: strawberry.
^b Be: Faba bean.
^c Gr: grapevine.

Greenhouses used for grapevine cultivation probably serve above all as insect-proof nets without preventing plant contamination by external inoculum of airborne spores, thus cannot be assimilated to closed systems for *B. cinerea* populations. We assimilated them to open field systems in the rest of our analyses.

On the contrary, populations sampled on strawberries in open field conditions in the Cap Bon region were significantly differentiated from greenhouse populations from the same plant and region, whereas open field populations from this plant from Cap Bon and Centre were connected by an important amount of gene flow (Table 3). Hence, for *B. cinerea* coming from strawberries, the growing system has a major effect on population structure, stronger than geographic distance between populations. The genetic diversity, mean number of allele per locus, and number of different MLG, were not different in greenhouse populations from strawberry as compared to open field systems, and the r_D index showed equivalent levels of multilocus linkage disequilibria (Table 2).

The single population from tomato, encompassing 35 isolates sampled in a greenhouse from the Cap Bon region, exhibited the lowest genetic diversity (0.61) and the highest proportion of repeated MLG (51%). These reduced levels of diversity may be due to periodic bottlenecks at the entrance of external inocula, and/or to genetic drift acting within greenhouse populations if they are strongly disconnected from the outside (with no or very few external inoculum, entrance except at the time of greenhouse installation). These results also indicated that tomato greenhouses might be more pathogen-proof than strawberry greenhouses, imposing more severe bottlenecks to external inocula from the outside. However, the multilocus linkage disequilibrium was not more elevated in this population (Table 2), showing that the reproductive mode is not modified within tomato greenhouses.

As regards open field systems only, we analyzed 74 isolates coming from five populations of different plants and different regions (Table 3), in order to disentangle the respective roles of geography and host plants on *B. cinerea* group II populations. Indeed, pathogen populations and their host plants are continually co-evolving, conditioning the way parasites are adapting to their hosts, which remains a key problem in evolution. These long-term evolutionary processes may lead to sympatric ecological speciation of parasites by host shifts (4,17,20,28). In fact, many phytopathogenic fungi that had long been considered as broad generalists have recently been recognized as complexes of sibling species or isolated populations, specialized on different hosts (24–26, 32,42). In our study, classical population genetics analyses as well as Bayesian assignment approaches showed that *B. cinerea* group II open field populations were genetically structured. One important factor explaining the observed gene flow reduction between populations may be the Grande Dorsale Mountain, serving as a geographic barrier. Indeed, the highest pairwise F_{ST} values (0.83 to 0.14, Table 3) were obtained between populations from Cap Bon or Centre regions compared with populations from Nord or Grand Tunis, whereas pairwise F_{ST} between the different populations within the regions of Cap Bon and Centre, as well as between the different populations within the regions of Nord and Grand Tunis, did not exceed 0.4. Similar conclusions could be drawn from the Bayesian analysis with the Structure software, since four of the six clusters inferred from this approach were mainly explained by the geographic origin of isolates (Fig. 5). However, since all isolates from the Nord and Grand Tunis regions were sampled from grapevine, whereas isolates from Cap Bon and Centre regions came from strawberries and faba beans, it was impossible to disentangle geographic from host plant effect. The further analysis of the faba bean and strawberry isolates from the Cap Bon and Centre regions evidenced four clusters, without any clear pattern linking the probability of ancestry in one particular cluster to the geographic or host plant origin of the isolate (Fig. 6). This indicates that both factors (sampling location

and host plant) act together to shape the population genetic structure at this spatial scale. Hence, the host plant seems to remain an important feature driving population differentiation of *B. cinerea* group II, probably through adaptation, but this effect could not be separated clearly, in this study, from geographic effects. Further assignment analysis using individual spatial coordinates of isolates sampled in sympatry on different host plants, and in different regions, may be useful to depict more precisely these joint effects.

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