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Population structure and temporal maintenance of the multihost fungal pathogen *Botrytis cinerea*: causes and implications for disease management

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Abstract

Understanding the causes of population subdivision is of fundamental importance, as studying barriers to gene flow between populations may reveal key aspects of the process of adaptive divergence and, for pathogens, may help forecasting disease emergence and implementing sound management strategies. Here, we investigated population subdivision in the multi-host fungus *Botrytis cinerea*, based on comprehensive multiyear sampling on different hosts in three French regions. Analyses revealed a weak association between population structure and geography, but a clear differentiation according to the host plant of origin. This was consistent with adaptation to hosts, but the distribution of inferred genetic clusters and the frequency of admixed individuals indicated a lack of strict host-specificity. Differentiation between individuals collected in the greenhouse (on *Solanum*) and outdoor (on *Vitis* and *Rubus*) was stronger than that observed between individuals from the two outdoor hosts, probably reflecting an additional isolating effect associated with the cropping system. Three genetic clusters coexisted on *Vitis*, but did not persist over time. Linkage disequilibrium analysis indicated that outdoor populations were regularly recombining, whereas clonality was predominant in the greenhouse. Our findings open up new perspectives for disease control by managing plant debris in outdoor conditions, and reinforcing prophylactic measures indoor.

Keywords: population structure, reproductive isolation, divergent selection, crop management, reproductive system, migration, host plant, gray mold, *Botrytis cinerea*

INTRODUCTION

Most eukaryotic microbial pathogens are subdivided into distinct populations (Taylor et al., 2006). Understanding the causes of population subdivision is of fundamental importance to population biologists, as studying barriers to gene flow in populations that are not yet completely reproductively isolated may reveal key aspects of the process of adaptive divergence before they become confounded by other factors. Knowledge on the processes that shaped population structure should ultimately allow efficient forecasting and preventing the emergence of genotypes, populations or species with negative effects on ecosystem health and human welfare (McDonald and Linde, 2002; Giraud et al., 2010; Williams, 2010; Gladieux et al., 2011b). An accurate description of the population structure of pathogens is also needed to answer questions about the existence of pathogen reservoirs and the transmissibility or longevity of populations (Milgroom and Peever, 2003; Taylor and Fisher, 2003; Gladieux et al., 2011b; Simwami et al., 2011).

Population differentiation may be adaptive or non adaptive, and may be caused by limited dispersal, limited mating preferences and/or limited adaptation, the consequence being the divergence of gene frequencies between demes. Host-specific or geographic differentiation has been extensively investigated in fungal plant pathogens, and subdivision into multiple populations associated with different hosts or regions has been demonstrated for many species (Peever et al., 2000; Giraud et al., 2006; Gladieux et al., 2008; Gladieux et al., 2011a; Dutech et al., 2012; Robert et al., 2012). The role of other structuring factors, such as i) ecosystem features, *e.g.* wild *vs*. agricultural ecosystems (Munkacsi et al., 2008; Stukenbrock and McDonald, 2008; Gladieux et al., 2010); ii) abiotic factors, *e.g.* temperature (Frenkel et al., 2010; Zhan and McDonald, 2011; Mboup et al., 2012); iii) agrosystem subunits or cropping systems, *e.g.* nursery *vs* commercial fields (Peever et al., 2000), has been much less thoroughly investigated. Temporal changes in population structure have also seldom been investigated (Ali et al., 2013). Multiyear sampling can provide access to key features of pathogen population dynamics, such as variation in migration intensity over time and space, the existence of barriers to gene flow, or the prevalence of disease spillover (*i.e.* cross-species disease transmission) and hybridization (Gladieux et al., 2011b).

Botrytis cinerea (teleomorph *Botryotinia fuckeliana*) is a filamentous, heterothallic ascomycete fungus causing gray mold on more than 220 host plants, including high-value crops, such as grapevine and tomato, and wild species, such as bramble (Elad et al., 2004). This fungal pathogen can also develop saprophytically, and it is widespread in the environment (Martinez et al., 2005; Gordon and Leveau, 2010). The pathogen spreads through asexual cycles in spring and summer, mostly dispersed by wind and human activities (Elmer and Michailides, 2004). Although signatures of recombination indicate the existence of sexual reproduction in overwintering field populations (Giraud et al., 1997; Fournier and Giraud, 2008), and although sex can be elicited in the lab, ascocarps are rarely observed in field conditions (Beever and Weeds, 2004). *Botrytis cinerea* was long thought to be a single, though morphologically variable and generalist species. However, several recent studies have shown *B. cinerea* to be a species complex, the cryptic species *Botrytis pseudocinerea* (teleomorph *Botryotinia pseudofuckeliana*)

being found in sympatry with *B. cinerea,* but at low frequency (Albertini et al., 2003; Fournier et al., 2003; Fournier et al., 2005; Martinez et al., 2008; Walker et al., 2011; Johnston et al., 2013). Population genetic surveys using microsatellite and transposable element markers have reported contrasting patterns of host-specific differentiation within *B. cinerea*. No significant genetic differentiation was found between isolates collected from grape, kiwifruit, pea, and squash in the Californian Central Valley (Ma and Michailides, 2005), but other studies revealed significant differentiation between isolates collected from grape, tomato, kiwifruit and bramble in Chile (Munoz et al., 2002), from grape and bramble in France (Fournier and Giraud, 2008), from grape, tomato, faba bean and strawberry in Tunisia (Karchani-Balma et al., 2008) and also between wild hosts in UK (Rajaguru and Shaw, 2010). The existence of host-specific differentiation raises the question of the role of adaptation to the host in the establishment of barriers to gene flow between sympatric *B. cinerea* populations. The components of the life cycle of *B. cinerea* are not fully understood. However, as in many ascomycete pathogens that reproduce on the plant on which their spores initially landed, dispersal between selection on the host and mating may be limited in *B. cinerea* thereby facilitating host-specific differentiation (Giraud et al., 2006; Giraud et al., 2010). In addition to the divergent selection pressures exerted by hosts, other factors may shape the population structure of *B. cinerea*. The role of the saprotrophic phase of *B. cinerea* life cycle is of particular interest as it might serve as a source of inoculum for new epidemics, or lead to the appearance of recombinant genotypes through mating between populations adapted to different habitats. Another major factor that should be considered is geographic distance. Previous studies have shown differentiation between populations of *B. cinerea* from different continents (South Asia and Australia; Isenegger et al., 2008), but patterns of geographical subdivision seemed to be weaker at smaller scales (Fournier and Giraud, 2008; Karchani-Balma et al., 2008).

Here, considering this context, we hypothesize that *B. cinerea* populations may evolve according to time, space, host and anthropic activities but that evolution patterns still need to be understood in crop production conditions specific to France. Therefore, we investigated the population structure of *B. cinerea* using a comprehensive hierarchical sampling over a two-year period, with four sampling dates, and several host plants, regions and cropping systems in France. We addressed the following questions: (i) Are sampling date, host plant and geographic location structuring genetic variation? (ii) Is the same population structure observed in different regions? (iii) Is population structure stable over time? (iv) What is the role of sexual reproduction in the temporal maintenance of populations?

RESULTS

We collected 3546 *B. cinerea* strains over the period of two years, in three regions of France (North-East, South-West, South-East), on litter and on three hosts (*Solanum lycopersicum, Vitis vinifera* and *Rubus fruticosus*) under two different management regimes (indoor and outdoor) (Fig. 1; Table 1). All strains were genotyped using eight microsatellite markers (Fournier et al., 2002).

Population subdivision

We first examined the partitioning of genetic variation among the different potential factors shaping population subdivision (sampling date, host plant and geographic location; Table 1) using hierarchical analyses of molecular variance (AMOVA) (Table 2). In a first AMOVA in which populations of the full dataset were organized by sampling date, variation among dates was highly significant ($P < 0.0001$; $F_{ST} = 0.32$) even if variation within dates accounted for most of the molecular variance (67.43 %; $P < 0.0001$). In a second set of AMOVAs performed separately for each sampling date, we explored the distribution of genetic variation among regions and among hosts of origin (*Vitis, Rubus* or *Solanum*) nested within the three regions (Northeast, Southeast and Southwest). Variation within populations accounted for most of the molecular variance (70-77%; $P < 0.001$; $0.19<F_{ST}<0.29$). Variation among hosts within regions accounted for 23 to 34% of molecular variance ($P<0.001$), whereas variation among regions was never significant. These results suggest that the two main factors significantly affecting the genetic variance in our dataset were sampling date and host of origin, whereas geographic origin played a minor role.

We investigated patterns of population subdivision using the clustering method implemented in STRUCTURE, assuming a model with admixture and correlated allele frequencies. Analyses were performed independently for each sampling date, without using prior information regarding the host or region of origin of genotypes. The rate of change in the log probability of data between successive K values (ΔK) exhibited a mode at K=5 for the June 2006, September 2006 and June 2007 datasets, and at K=2 for the September 2005 dataset (Supporting Information Table 1). We then compared the results obtained with STRUCTURE, which is a model-based clustering method assuming linkage and Hardy-Weinberg equilibrium within subpopulations, with those obtained with DAPC, a non-parametric multivariate clustering method. This latter analysis revealed essentially the same pattern as STRUCTURE analyses, with five distinct groups inferred whatever the sampling date considered (Supporting Information Fig. 1). In subsequent analyses, we used the clustering patterns inferred with STRUCTURE for K=5 at all sampling dates (Fig. 2 lines A, B, D, F).

Whatever the sampling date considered, one cluster consisted mostly of isolates collected in greenhouses on *Solanum* (on average per season, 83% of genotypes from this habitat had membership coefficients of 0.73-0.98 in the same cluster, with a mean of 0.96). Another cluster was mostly composed of isolates collected on *Rubus* (on average per season, 47% of genotypes collected on this host had membership coefficient of 0.72-0.98 in the same cluster, with a mean of 0.94). The proportion of isolates collected on *Rubus* grouping in this cluster was higher in spring (95% and 89% in June 2006 and 2007, respectively), than in fall (57% and 63% in September 2005 and 2006, respectively). The last three clusters grouped together isolates collected mostly from *Vitis*, with an average of 69% of the isolates collected on this host having membership coefficients above 0.70 in one of the three clusters (mean 0.90). No cluster specifically grouped together isolates collected on litter, which were distributed mostly across the three *Vitis* clusters (4.7%-25.3% of the genotypes assigned to these clusters were collected on litter). Litter was thus considered to be essentially the same as *Vitis* in subsequent analyses.

Across sampling dates, pairwise F_{ST} between clusters ranged between 0.12 and 0.65 (mean of 0.40, all F_{ST} values being significant), and population differentiation was always greater between clusters grouping genotypes from different hosts (range: 0.20–0.65, mean 0.43) than between the clusters coexisting on *Vitis* (range 0.12–0.30, mean 0.23; Supporting Information Table 2). Hence, consistent with the results of AMOVA, clustering analyses indicated that whatever the sampling date, genetic variation in *B. cinerea* populations was mainly structured according to the host plant, with geographic location having a much weaker effect.

Temporal maintenance of population subdivision

To investigate the maintenance over time of the pattern of population subdivision in five genetic clusters we first performed a PCA to visualize the relationships among the 20 groups formed by the five genetic clusters inferred at each season (Fig. 3). Only genotypes with membership greater than 0.7 in a given cluster were considered. The four clusters inferred on *Solanum* (*SOLANUM* clusters) at the different sampling dates were clearly grouped together. The four clusters inferred on *Rubus* (*RUBUS* clusters) were also separated from the others, but formed two groups, one group for spring sampling dates, the other for fall sampling dates. The 12 clusters inferred on *Vitis* (*VITIS* clusters) formed a third undifferentiated group.

We also investigated the temporal maintenance of subdivision using the option in STRUCTURE allowing assignment of focal individuals to populations defined *a priori* as reference populations. Here, we considered all individuals from a given sampling date (*t*), and inferred their proportion of ancestry in the 5 genetic clusters inferred at the previous sampling date (*t*-1) (the 5 'reference' populations). To increase the stringency of the analysis, the five genetic clusters of date *t-1* were defined as reference populations on the basis of genotypes that had membership coefficients greater than 0.9 in any of these clusters. Genotypes from date *t* were considered 'assigned' to a given cluster from data *t-1* if their membership coefficient in this cluster was above 0.70. Across seasons, 66.8% to 85.9% of the genotypes sampled on *Solanum* at date *t* were assigned to the *SOLANUM* cluster inferred at date *t*-1 (Fig. 2; Table 3). Only 0.5% to 11.7% of genotypes from *Solanum* were assigned to the *VITIS* or *RUBUS* clusters, and the remaining genotypes were admixed and could not be assigned to any cluster. Individuals sampled on *Rubus* in June 2006 and September 2006 were mostly assigned to the *RUBUS* clusters from date *t-1* (40.6% to 75.6% of genotypes), while individuals collected in June 2007 were mostly assigned to the *VITIS* cluster (56.8% of genotypes), and 15.1% to 36.7% of genotypes were admixed and could not be assigned to any cluster (Fig. 2; Table 3). Genotypes sampled on *Vitis* at a given date *t* tended to be assigned to the *VITIS* clusters of date *t-1* (25.3% to 48.7% of genotypes) but a relatively greater proportion of genotypes, compared to other host plants, was not assigned to any of the three clusters identified at date *t-1* (50.0 to 59.2% of genotypes) showing that the three *VITIS* clusters were not stable over time, probably due to regular recombination among individuals infecting this plant (Fig. 2; Table 3). Individuals collected in litter were assigned to the *RUBUS* clusters (0% to 21.6% of genotypes), to the *VITIS* clusters (21.6% to 63.0% of genotypes) or not assigned to any cluster (37.0% to 56.8% of genotypes), but none was assigned to the *SOLANUM* clusters. Altogether, these results indicate that the population subdivision of *B. cinerea* is globally maintained throughout time.

Patterns of genetic variability

Having evidenced patterns of population subdivision, we next investigated how genetic variability varied within each subpopulation in order to get insight into population dynamics. We estimated genetic variability within each clusters at each sampling date, by estimating the mean number of alleles per locus, A_r, and the genic diversity, H_e (Fig. 4). The *SOLANUM* clusters were the least variable, with A_r values ranging between 2.63 and 3.88 over time, and H_e not exceeding 0.32. Genetic variability was higher in all other clusters, but with different ranking among them, depending on the sampling date. In the *RUBUS* cluster, the inter-date standard deviations of A_r and H_e were 5.19 and 0.21, respectively (A_r range: 3.75-16.00; H_e range: 0.34-0.81). In the three *VITIS* clusters, A_r ranged from 3.63 to 15.38 and H_e ranged from 0.31 to 0.81. Inter-date standard deviations could not be computed for these latter clusters, as they did not appear to be maintained through time.

Mode of reproduction

To investigate the reproductive mode within each *B. cinerea* subpopulation we used the proportion of genotypes repeated at multiple times (clonal fraction) and an estimate of multilocus linkage disequilibrium: the r_D index (Fig. 5). For each season, we also considered all possible pairs of loci and determined whether these pairs were significantly linked or not, using Fisher's exact tests, and estimated the proportion of pairs for which linkage status changed over time. These data were represented using "transition graphs" from on sampling date to the other (Supporting Information Fig. 2).

The highest clonal fraction was found in the *SOLANUM* clusters (from 0.75 to 0.91). In these clusters, multilocus linkage disequilibrium, estimated with the r_D index, varied from 0.18 to 0.34 over time and was lower in June samples than for those collected at the previous sampling date (September). Transition graphs showed that in the *SOLANUM* cluster, most pairs of loci (57 to 76%) remained linked over time (Supporting Information Fig. 2). Together, these results suggest that asexual reproduction is probably the main mode of reproduction in the *SOLANUM* cluster.

In the *RUBUS* clusters, r_D values were low and varied little between seasons (range 0.07-0.14), contrasting with the *SOLANUM* cluster (Fig. 5). The clonal fraction was below 0.50 and peaked at the end of summer (0.14 and 0.16 in September 2005 and September 2006, respectively). Transition graphs (Supporting Information Fig. 2) confirmed that 36% to 39% of the statistical associations between pairs of loci were not observed anymore between fall and the following spring. This suggests that regular recombination events occurred within the *RUBUS* cluster, and that reproduction probably took place during the winter.

In the *VITIS* clusters, r_D values were always lower than in the *RUBUS* cluster, varying between 0.02 and 0.14, depending on the collecting date. The clonal fraction ranged between 0.10 and 0.58, and was always lower in spring (0.11-0.37 for June 2006, 0.10-0.31 for June 2007) than in fall (0.32-0.55 for September 2005, 0.34-0.58 for September 2006). Thus, as for the *RUBUS* cluster, recombination probably occurred regularly within and among the *VITIS* clusters, although the time period in which it occurred cannot be inferred due to the lack of any shift in statistical associations between pairs of loci. Since the *VITIS* clusters do not persist over

time (Fig. 2, Table 3), it was not relevant to analyze how linkage disequilibrium between pairs of loci was generated or broken between consecutive dates.

DISCUSSION

We analyzed associations over seasons between the population structure and the region and host plant of origin of multilocus microsatellite genotypes of the multihost plant pathogen *B. cinerea*. For all sites and all sampling dates, populations collected from different hosts were significantly differentiated. Genetic variation was also significantly structured according to sampling date. At each date, individuals collected from *Rubus* and *Solanum*, respectively, had high membership in distinct clusters (*RUBUS* and *SOLANUM* clusters), which persisted over time. Individuals collected on *Vitis* had a high membership in three different clusters (*VITIS* clusters) that did not seem to be fully maintained over time, possibly indicating within-host disruptive selective pressures that do not persist through time. Asexual reproduction seemed to be the main reproductive mode in the *SOLANUM* cluster, which also displayed little admixture with other clusters, whereas footprints of regular recombination and higher levels of admixture were found in the other clusters.

Geography and host plant as factors of isolation

AMOVA and clustering analyses showed that population structure was only weakly associated with geographic location at the (regional and national) scale used in our study, as already observed at the scale of Britain, French, New-Zealand and Tunisia (Fournier and Giraud, 2008; Karchani-Balma et al., 2008; Rajaguru and Shaw, 2010; Johnston et al., 2013). Previous studies also found that geographic differentiation in chickpea-associated *Botrytis* populations was only observed at the inter-continental level (Isenegger et al., 2008). The lack of geographic structure suggests extensive migration and/or high population sizes impeding the differentiation of allele frequencies at the scale of our study.

Unlike the geographic origin of samples, the host plant of origin significantly accounted for population subdivision in *B. cinerea*. Host-specific population differentiation has already been described in *B. cinerea* (Munoz et al., 2002; Fournier and Giraud, 2008; Karchani-Balma et al., 2008; Rajaguru and Shaw, 2010; Samuel et al., 2012), even between hosts as phylogenetically close as strawberry and blackberry (Rajaguru and Shaw, 2010). The divergent selection pressures exerted by hosts may explain the stable pattern of differentiation observed in *B. cinerea* populations over time. Defense mechanisms specific to different hosts may require distinct sets of pathogenicity alleles/genes involved in necrotrophy (Choquer et al., 2007; Williamson et al., 2007). Genomic sequencing of *B. cinerea* revealed a large number of secondary metabolites such as fungal toxins (Amselem et al., 2011), that might play a role in ecological specialization to different hosts (Giraud et al., 2010). Barriers to gene flow might also be associated with differences in host phenology or periods of receptivity (Desprez-Loustau et al., 2010).

Different genetic clusters were also observed on the same host, *Vitis*. This suggests that *B. cinerea* isolates on *Vitis* might be partitioned into different ecological niches at very fine scale, which would be interesting to identify in a future study. These clusters might be associated to various host tissues and/or to the degree of physiological maturity of these tissues, as suggested for *Zymoseptoria tritici* (Brunner et al., 2013). However, we found clear signatures of admixture among these clusters and a lack of persistence through time, suggesting that the putative fine-scale niche partitioning does not prevent gene flow.

Unlike the plant of origin, the nature of the substrate of origin (living plant organs or litter) had no significant effect on the genetic structure of outdoor populations. Litter isolates predominantly clustered with the *VITIS* populations, consistent with the large surface occupied by vineyards in the three regions under study. The overwintering of grapevine debris was also demonstrated as an important source of *B. cinerea* inoculum (Elmer and Michailides, 2004; Jaspers et al., 2013). Large amounts of inoculum released from grapevine may furthermore readily colonize dead material present in the immediate neighborhood, which is not systematically sprayed with fungicides and may therefore serve as a reservoir of fungicide-susceptible inoculum. This opens up new possibilities for gray mold management. More drastic prophylactic measures (such as the removal of potential substrates for the pathogen; (Elmer and Michailides, 2004; Boyd-Wilson et al., 2013; Jaspers et al., 2013) could contribute to decrease the amount of resistant inoculum on litter in *Vitis* crop and to preserve a limited proportion of susceptible inocula kept on outside hosts, able to hinder the evolution of drug resistance.

Strong isolation of populations infecting *Solanum*

Several lines of evidence indicated that the *SOLANUM* cluster was clearly differentiated from other clusters. This relative isolation of populations infecting *Solanum* may result from a combination of habitat isolation (the greenhouse acts as physical barrier), reduced frequency of sexual reproduction in the greenhouse (decreasing the probability of mating with genotypes adapted to other hosts) and strong natural selection against migrants or hybrids from divergent habitats. These latter ecologically-based barriers to gene flow may be associated with factors such as effective prophylaxis indoors, divergent abiotic conditions between outdoor and indoor environments, or divergent selective pressures exerted by hosts (*Solanum* belongs to the Asterids subclass, whereas *Vitis* and *Rubus* belong to the Rosids subclass (The Angiosperm Phylogeny, 2009)). Additional sampling, comparing locally populations from greenhouse or open-field tomatoes would certainly refine our results.

We also observed differences in diversity between *Solanum* populations from different regions (Supplementary Information Fig. 3), possibly reflecting differences in the prophylactic measures implemented, the greenhouse structure, climatic conditions or historical factors (number of founding propagules). Nevertheless, clonal fraction in low-tech greenhouses, likely to be exchanging more migrants with outdoor populations, was greater than the one in other outdoor clusters, which confirms that the reproduction mode is mostly asexual in all greenhouses even if some heterogeneity exists among them. The higher relative contribution of asexual reproduction of *B. cinerea* populations in indoor conditions is consistent with previous studies (Karchani-Balma et al., 2008; Decognet et al., 2009).

Altogether, our results suggest that cropping system is an important structuring factor in *B. cinerea* populations. Prophylactic measures should be implemented to strengthen isolation, such as disinfection between crops and seedlings, quarantine, filtering of the incoming air, weed management in the areas surrounding greenhouses and the use of techniques limiting the introduction of diversity. The confinement of greenhouse populations would ensure that fungicide resistance selected on outdoor crops does not introgress indoor populations. Indeed, analyses of the distribution of resistant genotypes in greenhouse populations have revealed isolates resistant to the limited number of fungicide types used indoors, whereas greater phenotypic diversity is encountered in the surrounding populations collected on grapevine, for which different modes of action are authorized (AS Walker, unpublished data).

Mode of reproduction

The low clonal fraction and low linkage disequilibrium values are signatures of regular recombination within the *RUBUS* and *VITIS* clusters. These findings are consistent with previous studies (Giraud et al., 1997; Fournier and Giraud, 2008), that suggested regular cryptic sexual reproduction in *B. cinerea* populations, despite the fact that sexual structures are hardly observed in natural conditions (Beever and Weeds, 2004).

When possible (*i.e.*, for clusters that persisted over time), the examination of how linkage between pairs of loci changed across time suggested that recombination events in outdoor populations take place during the cold season. In the *RUBUS* clusters in particular, the proportion of linked pairs was higher at the end of summer than in spring, indicating that linkage disequilibrium is "broken" during winter, probably due to recombination. The period occurrence of recombination could not be inferred with confidence in the three *Vitis* clusters but previous findings concerning the epidemiology of gray mold suggested that cold conditions are favorable to sexual reproduction of *B. cinerea* (Elmer and Michailides, 2004). Parasexuality due to anastomosis has been hypothesized in *B. fuckeliana* (Beever and Parkes, 2003; Roca et al., 2012). However, given the observation of the sexual cycle in the lab we favor the hypothesis of a sexual cycle occurring regularly but inconspicuously in nature.

Concluding remarks

Our comprehensive analysis of *B. cinerea* population structure and dynamics has direct applications for disease management. Understanding the causes of population subdivision, its temporal maintenance, and the flows of genotypes among demes will help to implement management strategies aiming at restricting genetic exchanges between populations. For example, limiting the density of potential host plants in the vicinity of greenhouses or improving litter and wild hosts management in vineyards might significantly decrease the intensity of epidemics but also delay fungicide resistance evolution. The efficiency of these prophylactic measures (detailed in Elmer and Michailides, 2004) are well-known by epidemiologists and agronomists, but our findings underline the genetic mechanisms underlying their success. Such knowledge may be used to better optimize the joint use of the various control methods and hence contribute to increase the sustainability of agricultural production for crops susceptible to *B. cinerea*.

EXPERIMENTAL PROCEDURES

Sample collection

Samples were collected on four dates: September 2005, June 2006, September 2006 and June 2007 (Table 1). June corresponds to the start of the cropping season and the samples collected in this month were presumed to have undergone sexual reproduction during the winter. By contrast, September corresponds to the end of the cropping season, after the occurrence of asexual multiplication on the host. Samples were collected in the Northeast (Champagne), Southwest (Aquitaine) and Southeast (Provence and Côte d'Azur) of France, at two to six separate sites per region (Fig. 1). Sampling sites were separated from 3-180 km within a region and from 340-684 km between regions. In each region, samples were collected from four different hosts/substrates: (i) tomato (*Solanum lycopersicum)* in greenhouses, (ii) grapevine (*Vitis vinifera*), (iii) bramble (*Rubus fruticosus*), from bushes surrounding vineyards or greenhouses, and (iv) litter in open-field conditions, on various dead wild plants and/or grapevine debris, on the soil within and/or outside the grapevine plots (Table 1). Each local population corresponds to a minimum of 30 samples randomly collected within the plot/greenhouse. Strains were collected on cotton swabs, from sporulating lesions for greenhouse crops, grapevine and bramble in September, and from asymptomatic grapevine flower caps and dried blackberry flowers in June. For June and for litter samples, the fungus was collected following the incubation of the plant material in a moist chamber at room temperature until conspicuous sporulation was observed.

Strains were grown on malt-yeast-agar (MYA) medium (20 $g.l^{-1}$malt extract, 5 $g.l^{-1}$ yeast extract and 12.5 $g.l^{-1}$ agar) or potato dextrose agar (PDA; 39 $g.l^{-1}$ ready-to-mix DIFCO potato dextrose agar), at 19°C to 21°C, under continuous illumination, to induce sporulation. Single-spore cultures, referred to hereafter as "isolates", were obtained for all strains. Stocks of spore suspensions for each isolate were stored in 20% glycerol, at -80°C, until required.

Microsatellite genotyping

For each isolate, DNA was extracted after seven days of culture on MYA or PDA medium at 21°C. DNA was extracted in an automated system, with the DNeasy adapted kit (Qiagen), or manually (Martinez et al., 2008; Decognet et al., 2009). All samples were genotyped for eight microsatellite markers — Bc1, Bc2, Bc3, Bc4, Bc5, Bc6, Bc7 and Bc10 (Fournier et al., 2002) — either in multiplex PCR, as previously described (Leroux et al., 2010), or in simplex PCR. Automatic allele recognition and annotation (binning analysis, Beckmann Coulter CEQ 8000 software) was carried out for the microsatellites for the multiplex analyses, after manual parametrization following the observation of a large number of samples covering as much diversity as possible for each locus. In addition, as genotyping was carried out in several laboratories, a panel of 21 reference isolates was distributed to all the laboratories and used to cross-validate allele assignment. We excluded isolates of the cryptic species *B. pseudocinerea*, which is morphologically undistinguishable from *B. cinerea*, on the basis of a previously described diagnostic allele at locus Bc6 (Walker et al., 2011). Only isolates genotyped at all microsatellite markers were included in the analyses.

Analyses of population subdivision

We used hierarchical analyses of molecular variance (AMOVA) implemented in ARLEQUIN V3.5 (Excoffier and Lischer, 2010) to investigate the relative contributions of sampling date, region of origin and host plant species within regions to the partitioning of genetic variance.

Population subdivision was investigated by the Bayesian clustering method implemented in STRUCTURE (Pritchard et al., 2000). This model-based algorithm assumes linkage equilibrium within inferred genetic clusters, therefore is in principle appropriate for species experiencing regular recombination. However, STRUCTURE has been shown quite robust to departure from panmixia (Morgan et al., 2007; Dutech et al., 2010; Ali et al., 2014); moreover, as outlined earlier, linkage equilibrium within *B. cinerea* populations was observed in several previous studies. Therefore we assume that this method is appropriate to infer *B. cinera* population subdivision. We ran STRUCTURE with the admixture model and correlated allele frequencies. Burn-in length was set at 100,000 Markov Chain Monte Carlo iterations. The burn-in period was followed by a run phase of 500,000 iterations, with the number of clusters K ranging from 1 to 10 and 10 independent replicates for each value of K. STRUCTURE outputs were processed with CLUMPP (Jakobsson and Rosenberg, 2007); a G' statistic greater than 80% was used to assign groups of runs to a common mode (*i.e.* clustering solution). The amount of additional information explained by increasing K was determined by calculating the ΔK statistic (Evanno et al., 2005).

To confirm the pattern of population subdivision inferred using STRUCTURE, we used an alternative, non model-based, clustering method: the discriminant analysis of principal components (DAPC, (Jombart et al., 2010)). This multivariate method involves a discriminant analysis on genetic data transformed after principal component analysis. The DAPC was carried out with the *adegenet* 1.3-1 package in the R 2.13.1 environment. We used the K-means procedure implemented in the function *find.clusters* to determine K, the optimal number of clusters, by letting K vary between 1 and 30. We used the Bayesian information criterion (BIC) to determine the 'optimal' value of K, defined as the value for which BIC was minimal, or at which the rate of change of BIC changed abruptly.

The temporal maintenance of population subdivision was assessed using assignment tests implemented in STRUCTURE (USEPOPINFO option). Genotypes sampled at season t were assigned into the genetic clusters inferred with STRUCTURE at season $t-1$. Only genotypes having a membership coefficient over or equal to 0.9 in a single cluster at season $t-1$ were used as learning samples. Genotypes of season t were not included in updates of allele frequency estimates (option UPDATEPFROMPOPFLAGONLY). The population model and run length were the same as in clustering analyses. Five independent runs were performed to check for convergence of the algorithm. We also investigated the temporal maintenance of genetic clusters inferred at several season using a PCA on multilocus genotypes categorized by season and genetic clusters within seasons, as implemented in the ADEGENET package of R (Jombart, 2008).

Genetic variability, differentiation among clusters, and mode of reproduction

Calculations were performed on the clusters inferred using STRUCTURE, considering genotypes having a membership coefficient over 0.7 in a single cluster. GENETIX (Belkhir et al., 1996-2004) was used to estimate within-cluster genetic variability on the basis of genic diversity calculated

as multilocus observed heterozygosity (*He*), and allele richness (*Ar*). GENEPOP V4.1 (Raymond and Rousset, 1995) was used to calculate pairwise Weir & Cockerham's F_{ST} between pairs of clusters (Weir & Cockerham 1984).

The number of unique multilocus genotypes (G) and the clonal fraction (1-G/N) were calculated with MULTILOCUS V1.3b (Agapow and Burt, 2001). We also used this software to calculate the r_D index, an estimate of multilocus linkage disequilibrium. Unlike the I_A index, r_D is corrected for the number of loci considered, and ranges from 0 (complete panmixia) to 1 (strict clonality). Significance was established by comparing the observed values with the distributions obtained by 1000 randomizations (Agapow and Burt, 2001). We evaluated the significance of pairwise linkage disequilibrium by contingency tests (with default parameters) implemented in GENEPOP V4.1 (Raymond and Rousset, 1995).

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Table 1: Populations of *Botrytis cinerea* collected in three French regions, on various host plants and in different cropping systems, between 2005 and 2007

Region	Location	Host plant[a]	Cultivar	Sampling date				
				September 2005	June 2006	September 2006	June 2007	**Total**
Southwest	Couhins	*Vitis vinifera*	Merlot noir	54	24	63	59	200
	Fauguerolles	*Solanum lycopersicum*	Palmiro	26	16	32	28	102
	Fauillet	*Solanum lycopersicum*	Hipop	32	18	24	-	74
	Grande-Ferrade	*Rubus fruticosus*	Wild	26	33	25	27	111
		Vitis vinifera	Merlot noir	50	41	65	55	211
		Litter	-	30	42	73	46	191
	Saint Julien	*Rubus fruticosus*	Wild	28	31	28	27	114
		Vitis vinifera	Merlot noir	55	44	56	57	212
	Marmande	*Solanum lycopersicum*	Palmiro	-	19	11	30	60
Northeast	Courceroy	*Solanum lycopersicum*	Moneymaker	-	6	55	48	109
	Courteron	*Rubus fruticosus*	Wild	18	4	46	25	93
		Vitis vinifera	Pinot meunier	53	50	99	30	232
		Litter	-	19	1	-	-	20
	Foissy-sur-Vanne	*Solanum lycopersicum*	Moneymaker	24	50	-	-	74
		Rubus fruticosus	Wild	-	-	22	10	32
	Hautvillers	*Vitis vinifera*	Pinot noir	93	59	108	47	307
	Vandières	*Rubus fruticosus*	Wild	23	18	17	28	86
		Vitis vinifera	Pinot noir	63	84	85	29	261
Southeast	Carnoules	*Vitis vinifera*	Syrah	8	-	-	-	8
	La Farlède	*Vitis vinifera*	Rolle	20	7	14	7	48

	Litter	-	22	32	-	1	55
Lançon-Provence	*Rubus fruticosus*	Wild	16	-	31	18	65
	Vitis vinifera	Grenache	30	29	30	30	119
	Litter	-	30	13	24	30	97
	Solanum lycopersicum	Alison	30	30	30	30	120
Pierrelatte	*Solanum lycopersicum*	Hipop	30	30	30	30	120
Sarrians	*Rubus fruticosus*	Wild	31	-	30	31	92
	Vitis vinifera	Grenache	30	25	30	29	114
	Litter	-	39	-	30	30	99
	Solanum lycopersicum	Emotion/Alison	30	30	30	30	120
Total			910	736	1088	812	3546

[a] On grapevine (*Vitis vinifera)* and bramble (*Rubus fruticosus*), samples were collected from diseased berries in the fall, and from flower caps or decaying flower parts in spring. *Vitis* samples were collected from open-field cultivated plots. *Rubus* samples were collected from plants surrounding the grapevine plots. On litter, samples were collected from asymptomatic wild- or crop-plant debris on the ground within or very close to grapevine plots in all seasons. On tomato (*Solanum lycopsersicum*), samples were collected from diseased fruits in all seasons, either in high-tech (Lançon-Provence, Courceroy, Pierrelatte, Sarrians) or low-tech (Fauguerolles, Fauillet, Foissy sur Vanne, Marmande) greenhouses.

- Indicates missing data, *i.e.* populations not collected because the disease was inexistent at this date or because samples never recovered living colonies.

Table 2: Hierarchical analyses of molecular variance (AMOVA) with (i) sampling dates as grouping factor (upper table), and (ii) geographic origin and host of origin nested within geographic origin as grouping factors (lower table).

	d.f.	Sum of squares	Variance components	Percentage of variation	*P*-value	Fixation indices
Among dates	1	90	0.03	1.09	**<0.001**	0.01
Within dates	3542	9915	2.80	98.91		
Total	3545	10005	2.83			

	d.f.	Sum of squares	Variance components	Percentage of variation	P-value	Fixation indices
September 2005						
Among regions	2	101	-0.16	-6.05	0.738	-0.06
Among hosts within regions	8	439	0.81	29.67	**<0.001**	0.28
Within hosts	869	1810	2.08	76.38	**<0.001**	0.24
Total	879	2350	2.73			
June 2006						
Among regions	2	130	-0.16	-5.36	0.728	-0.05
Among hosts within regions	8	476	1.05	34.85	**<0.001**	0.33
Within hosts	725	1534	2.12	70.51	**<0.001**	0.29
Total	735	2140	3.01			
September 2006						
Among regions	2	97	-0.17	-6.17	0.782	-0.06
Among hosts within regions	8	555	0.80	29.08	**<0.001**	0.27
Within hosts	1077	2288	2.12	77.09	**<0.001**	0.23
Total	1087	2940	2.75			
June 2007						
Among regions	2	57	-0.13	-4.40	0.845	-0.44
Among hosts within regions	8	405	0.70	23.44	**<0.001**	0.23
Within hosts	801	1950	2.43	80.95	**<0.001**	0.19
Total	811	2412	3.00			

P-values in bold are significant at the 5% confidence level.

Table 3. Assignment of genotypes collected at a given sampling date (*t*) to the 5 genetic clusters inferred at the previous sampling date (*t*-1).

Clusters from date *t-1*	Proportion of genotypes from date *t* assigned to the clusters identified at date *t-1*			
June 2006	*Solanum* (n=199)	*Rubus* (n=86)	*Vitis* (n=363)	Litter (n=88)
SOLANUM	0.859	0	0.011	0
RUBUS	0	0.756	0.143	0.216
VITIS	0.005	0.093	0.253	0.216
Not assigned	0.136	0.151	0.592	0.568
September 2006	*Solanum* (n=212)	*Rubus* (n=199)	*Vitis* (n=550)	Litter (n=127)
SOLANUM	0.821	0.020	0	0
RUBUS	0	0.090	0	0
VITIS	0.052	0.568	0.455	0.630
Not assigned	0.127	0.322	0.545	0.370
September 2006	*Solanum* (n=196)	*Rubus* (n=166)	*Vitis* (n=343)	Litter (n=107)
SOLANUM	0.668	0	0.006	0
RUBUS	0.087	0.404	0.087	0.121
VITIS	0.031	0.229	0.489	0.411
Not assigned	0.214	0.367	0.420	0.467

Analyses were carried out using USEPOPINFO option of the STRUCTURE program. To increase the stringency of the analysis, the 5 genetic clusters of date *t-1* were defined on the basis of genotypes that had membership coefficients greater than 0.9 in any of these clusters. Genotypes from date *t* were considered 'assigned' to a given cluster from data *t-1* if their membership coefficient in this cluster was above 0.70.

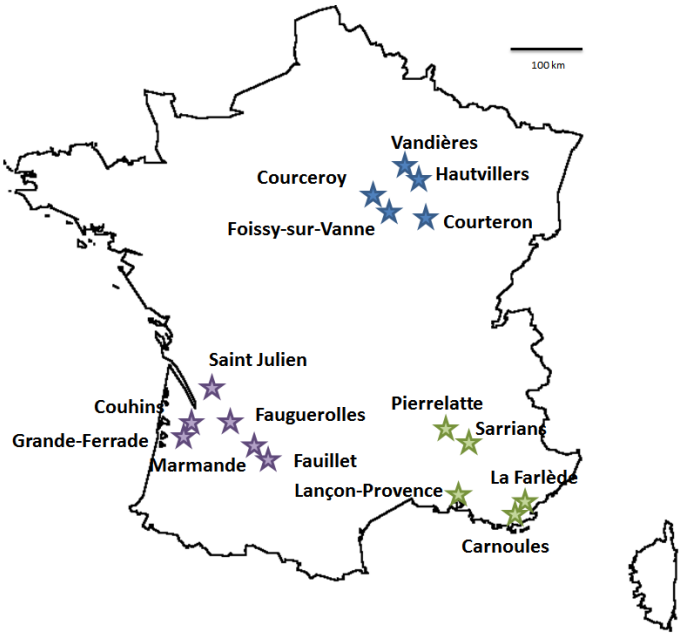

Figure 1: Map of *Botrytis cinerea* populations collected from three French regions on various host plants and in various cropping conditions, on four dates between 2005 and 2007.
Locations from the Northeast,Southwest and Southeast of France are shown in blue, purple, and green, respectively.

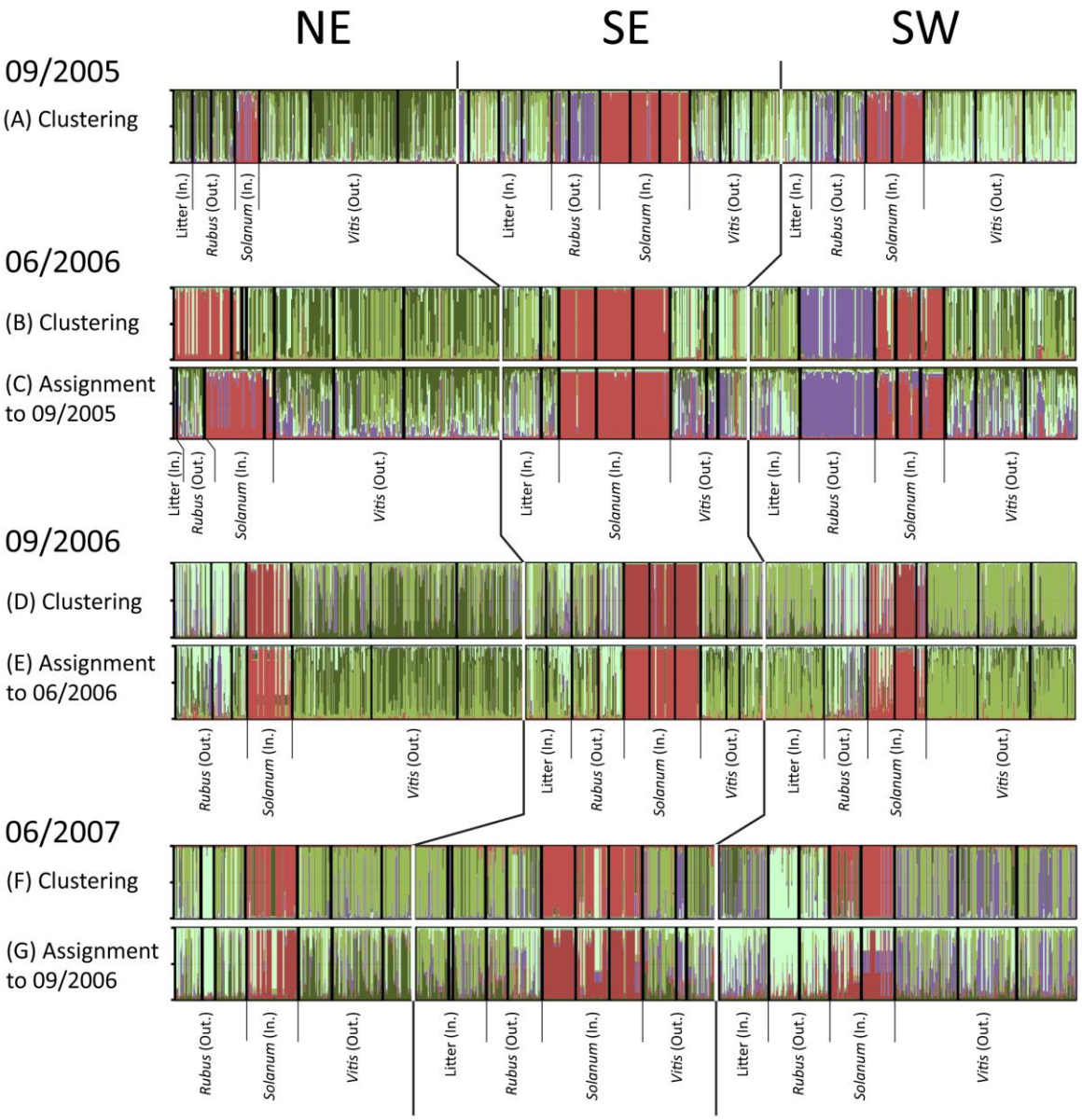

Figure 2: Barplots of the STRUCTURE analysis showing genetic subdivision of the 3546 *B. cinerea* isolates collected between 2005 and 2007 into five genetic clusters and the temporal maintenance of this subdivision.

The origin of isolates (host plant and geography) is indicated below each horizontal axis and on the top of the figure. The colours represent cluster membership of the individuals: red: *SOLANUM* clusters, purple: *RUBUS* clusters, green: *VITIS* clusters (*VITIS1*: dark green, *VITIS2*: medium green, *VITIS3*: light green).The cropping system is referred as "In" (indoor) or "Out" (outdoor). Plots A, B, D and F result from the assignment of the isolates collected at season *t* into the five genetic clusters. Plots C, E and G were built to assess whether the genetic subdivision assessed at one date was maintained at the following date. These plots represent how genotypes collected at season *t+1* are assigned into genetic clusters inferred at season *t* (these last being used as learning samples by STRUCTURE, and being defined using genotypes collected at season *t-1* and having a membership coefficient over or equal to 0.9 into a single cluster at this date).

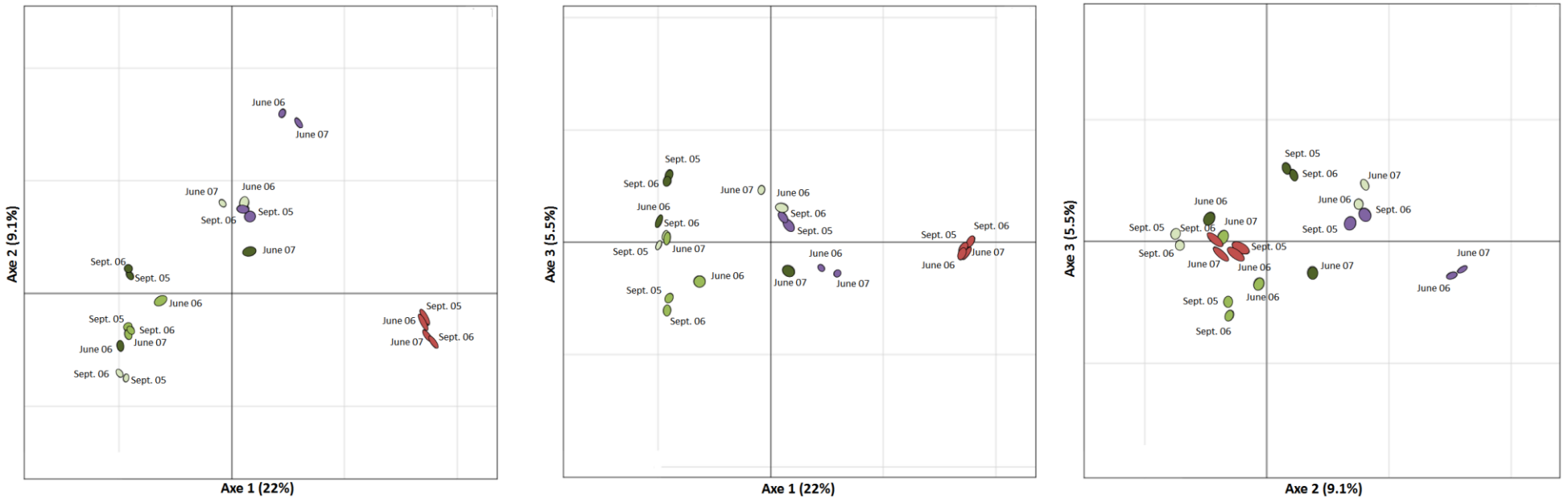

Figure 3: Principal components analysis (PCA) of the 3546 *B. cinerea* isolates collected between 2005 and 2007 on several hosts.
Individuals were organized into 20 groups (5 genetic clusters of origin x 4 sampling dates). The colours represent cluster membership of the individuals: red: *SOLANUM* clusters, purple: *RUBUS* clusters, green: *VITIS* clusters (*VITIS1*: dark green, *VITIS2*: medium green, *VITIS3*: light green). Only centers of inertia ellipses are represented

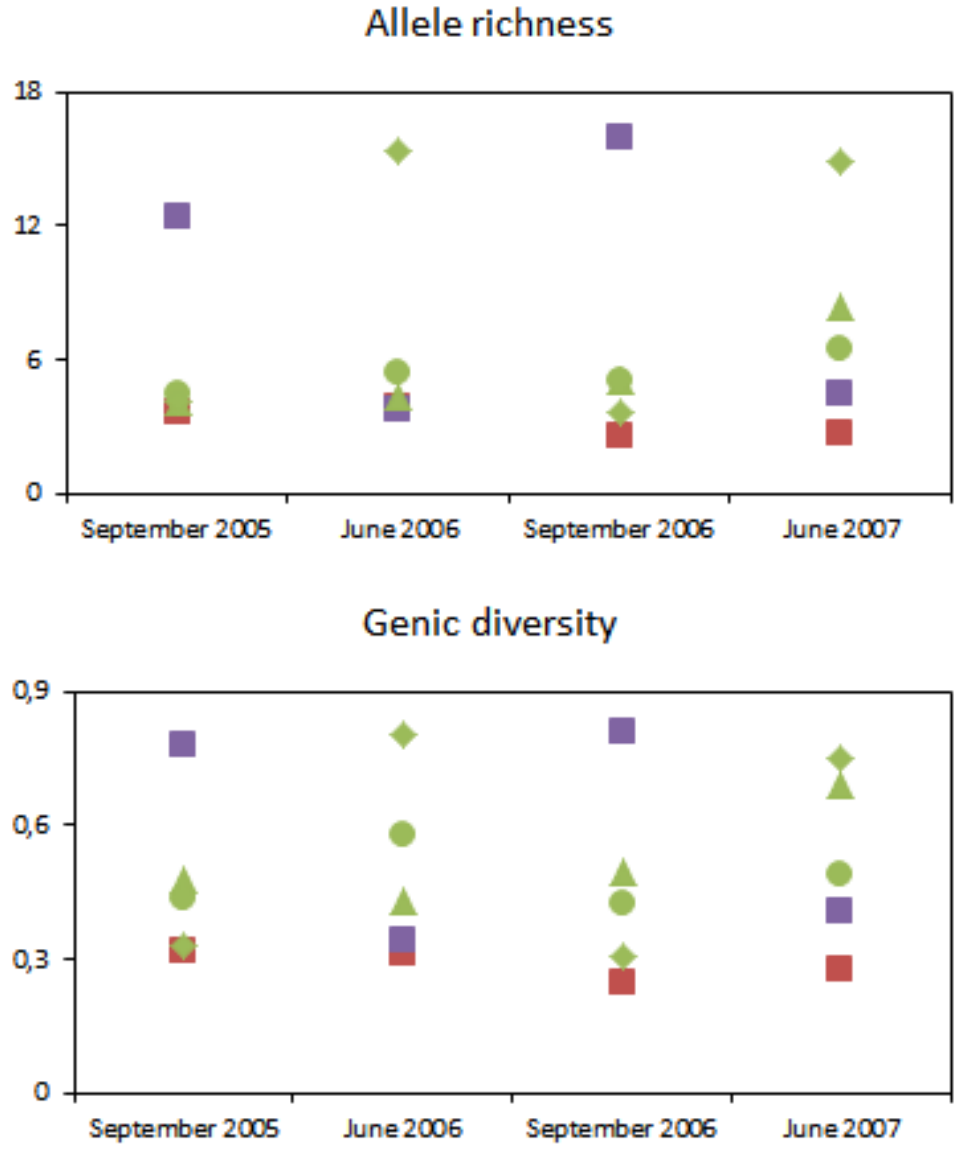

Figure 4: Diversity measured in the five *B. cinerea* clusters and for the four sampling dates. Colors and marks indicate clusters: *INDOOR* (red), *RUBUS* (purple), *VITIS1* (green, triangles), *VITIS2* (green, circles) and *VITIS3* (green, diamonds). Allele richness is calculated as the mean number of alleles per locus. Genic diversity is measured as the mean expected heterozygosity (H_e) over the eight loci. All the values were significant at the 5% confidence level.

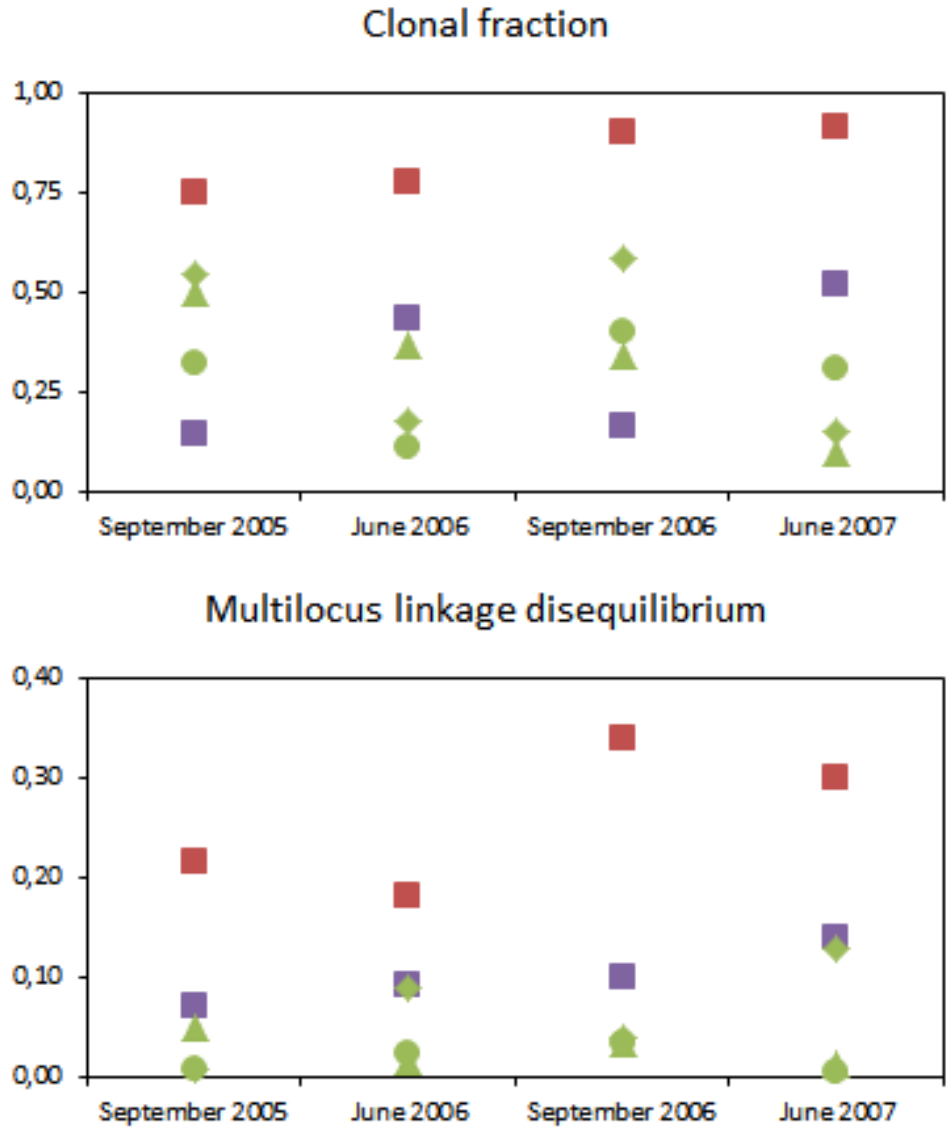

Figure 5: Clonal fraction, multilocus linkage disequilibrium, and numbers of significantly linked pairs of loci in the five clusters identified by clustering analyses. Clusters: *INDOOR* (red square), *RUBUS* (purple square), *VITIS1* (green triangles), *VITIS2* (green circles) and *VITIS3* (green diamonds). The clonal fraction represents the proportion of different multilocus genotypes. Multilocus linkage disequilibrium was estimated with the r_D index.