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Comparison of *Botrytis cinerea* populations isolated from two open-field cultivated host plants

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ABSTRACT

The necrotrophic fungus *Botrytis cinerea* is reported to infect more than 220 host plants worldwide. In phylogenetical–taxonomical terms, the pathogen is considered a complex of two cryptic species, group I and group II. We sampled populations of *B. cinerea* on sympatric strawberry and raspberry cultivars in the North-East of Hungary for three years during flowering and the harvest period. Four hundred and ninety group II *B. cinerea* isolates were analyzed for the current study. Three different data sets were generated: (i) PCR-RFLP patterns of the ADP-ATP translocase and nitrate reductase genes, (ii) MSB1 minisatellite sequence data, and (iii) the fragment sizes of five microsatellite loci. The structures of the different populations were similar as indicated by Nei's gene diversity and haplotype diversity. The *F* statistics (F_{st}, G_{st}), and the gene flow indicated ongoing differentiation within sympatric populations. The population genetic parameters were influenced by polymorphisms within the three data sets as assessed using Bayesian algorithms. Data Mining analysis pointed towards the five microsatellite loci as the most defining markers to study differentiation in the 490 isolates. The results suggest the occurrence of host-specific, sympatric divergence of generalist phytoparasites in perennial hosts.

1. Introduction

Fungi can be used as models to study speciation processes, facilitated by their short life cycle and metabolic versatility, enabling rapid adaptation, specialization and/or diversification. Many fungi feature vegetative as well as sexual life cycles and often occur in large swaths of the world's geography. Numerous species complexes are described in the fungal kingdom, encompassing recently diverged sister species, which are suitable to investigate the early stages of speciation (Giraud et al. 2008; Kohn 2005; Pringle et al. 2005).

Sympatric speciation is one of the modes of natural speciation by which (a) new species evolve(s) from a single ancestor in the absence of geographical barriers. Sympatric speciation has been studied in several phytopathogenic fungi like *Aspergillus flavus* (Grubisha and Cotty 2010), *Venturia inaequalis* (Gladieux et al. 2011), *Mycosphaerella graminicola* (Stukenbrock et al. 2007), *Rhizoctonia solani* (González-Vera et al. 2010), *Podosphaera leucotricha* (Kiss et al. 2011) and also in some yeast species (Gonçalves et al. 2011). Nevertheless, it remains one of the most contentious concepts in evolutionary biology (Barluenga et al. 2006; Fitzpatrick et al. 2008; Ridley 2004).

The necrotrophic fungus *Botrytis cinerea* Pers. Fr. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel) is the causal agent of grey mould in many economically important crops. Several studies have suggested that *B. cinerea* is not one clearly defined species but rather a species complex, with a restricted gene flow between different cryptic genetic groups (Giraud et al. 1997; Albertini et al. 2002; Fournier et al. 2003; Fournier et al. 2005; Fekete et al. 2012). Giraud et al. (1997) studied genetic diversity of *B. cinerea* in France using a range of molecular markers including transposable elements Boty and Flipper, and restriction fragment length polymorphisms (RFLPs). This pioneer study revealed that the *B. cinerea* population investigated was genetically very diverse, without clonal lineages of importance, suggesting a significant role for recombinational processes. The sampled population appeared to be composed of

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two subgroups, *transposa* (featuring both transposons) and *vacuma* (without either transposon), that were genetically distinct but concurred in sympatry on the same host plants and in the same region. In a subsequent study, Giraud et al. (1999) described that there was a significant genetic differentiation among isolates collected from different host plants in France while distinct levels of fungicide resistance were observed between *transposa* and *vacuma*. Genetic diversity of *B. cinerea* was also studied in Spain by Alfonso et al. (2000). This study showed that the population as a whole was highly heterogeneous, albeit with little differentiation within subpopulations isolated in different greenhouses. Muñoz et al. (2002) assessed *B. cinerea* populations infecting grapevine or tomato in Chile and found that the sampled population was genetically very diverse without the emergence of important clonal lineages, not even in relation with fungicide selection pressure. Ma and Michailides (2005) studied the genetic structure of *B. cinerea* populations in California looking at transposable elements and microsatellite primed (MP)-PCR and found no differentiation between populations isolated from different hosts.

B. cinerea group I (*Botrytis pseudocinerea*) and group II (*B. cinerea sensu stricto*) variants can be identified easily using certain molecular markers (Fournier et al. 2003; Walker et al. 2011; Fekete et al. 2012). Fournier and Giraud (2008) reported a significant genetic structure variation within *B. cinerea* group II comparing samples isolated from two natural host plants, grapevine and bramble, using microsatellite markers. That indicated a restricted gene flow, even in sympatry. Similarly, Rajaguru and Shaw (2010) and Muñoz et al. (2010) found genetic differentiation and diversity correlations between hosts and locations in populations of *B. cinerea* in southern England and Argentina, respectively. On the other hand, Karchani-Balma et al. (2008) studied the genetic structure of *B. cinerea* populations in Tunisia on different hosts in various regions using microsatellite markers but could not detect a clear correlation between the plant host and fungal differentiation.

Here we investigate the genetic structure of *B. cinerea* group II sympatric populations, infecting two cultivated perennial hosts (strawberry and raspberry) in the open field in Hungary. We employed three different molecular methods (RFLP analysis microsatellites and a minisatellite) to study the population genetics during three years.

2. Materials and methods

2.1. Botrytis cinerea isolates and DNA extraction

Monosporic isolates of *B. cinerea* were collected from infected strawberries and raspberries (total 490 isolates) on the 17 ha of plantations of the Benedek Gyümölcsfarm in Nagyréde district in the North-East of Hungary in 2007, 2008 and 2009 (Table 1). All sampling was done in an area of 2 km × 2 km. "Autumn Bliss" raspberries occupied 2 ha in the centre of the area and were planted in 2003. The "Honeoye" strawberry fields were so close to each other that they could be considered one continuous field comprising some 12 ha. The remaining hectares of the farm were planted with wheat, and involved in the rotation of strawberry; strawberries are typically maintained for three years. Hence, one third of the strawberry plants were the same throughout our study. Each year, the entire area was screened for grey mould at multiple occasions during the flowering and fruiting season (April–October), and all encountered were collected. The large majority of grey mould samples (94%) were collected from fruits. In 2007, the strawberry plants were treated twice with the anti-botrytis fungicide thiophanate-methyl (Topsin-M) on the 13th of May and the 20th May, and once with fenhexamid (Teldor) on the 1st of June. Raspberry needed no fungicide treatment in 2007. Next year, strawberry was treated with Topsin-M on the 9th of May, and with azoxystrobin (Quadris) on the 18th of May. In 2008, raspberry was treated with mankoceb (INDOFIL M-45) on the 23rd of May. In the third and final year of this study (2009), strawberry was treated twice with Topsin-M (19th and 26th of April), while raspberry was treated twice with Topsin-M (19th of May, 5th of June) and once with Teldor (28th of June).

Table 1
Origin of *B. cinerea* samples used in this study.

No. of isolates	Host	Phenological stage of plant	Year	Date
81	Strawberry	Fruiting	2007	6-Jun
4	Raspberry	Fruiting	2007	6-Jun
24	Raspberry	Fruiting	2007	06.aug
14	Raspberry	Vegetative	2008	18-May
15	Strawberry	Fruiting	2008	18-May
50	Strawberry	Fruiting	2008	7-Jun
12	Strawberry	Fruiting	2008	14-Jun
36	Strawberry	Fruiting	2008	5-Jul
18	Raspberry	Fruiting	2008	5-Jul
12	Raspberry	Fruiting	2008	13-Jul
39	Raspberry	Fruiting	2008	06.aug
23	Raspberry	Fruiting	2008	6-Sep
20	Raspberry	Fruiting	2008	11-Oct
4	Strawberry	Vegetative	2008	11-Oct
8	Strawberry	Flowering	2009	18-Apr
10	Strawberry	Fruiting	2009	16-May
20	Strawberry	Fruiting	2009	28-May
36	Strawberry	Fruiting	2009	21-Jun
10	Strawberry	Fruiting	2009	27-Jun
14	Raspberry	Fruiting	2009	27-Jun
27	Raspberry	Fruiting	2009	4-Jul
13	Raspberry	Fruiting	2009	15-Jul

Isolates were maintained on potato dextrose agar (PDA, Scharlau, Spain). Conidial suspensions were stored in 50% glycerol at −80 °C. DNA was extracted from aerial mycelium of *B. cinerea* strains grown on PDA medium for 10 days at 20 °C. Magnalyser (Roche, Osterode, Germany) was used for the disruption of fungal biomass, and DNA was isolated with the Plant II DNA Purification Kit (Macherey-Nagel GmbH and Co. KG, Germany). Yield and integrity of the cleaned-up DNA were checked upon native agarose gel electrophoresis. Freshly isolated genomic DNA was used as template material for the subsequent PCR reactions (see below).

2.2. RFLPs and amplification length polymorphism

The primers used in PCR of the nitrate reductase- and ADP-ATP translocase genes were as described by Giraud et al. (1997). Amplification reactions were performed in a total volume of 50 μl containing 50 ng fungal DNA, 0.4 μM of each primer, and 25 μl GoTaq® Green Master Mix. Cycling conditions were 30 cycles of 1 min at 95 °C, 1 min at 52 °C, and 2 min at 72 °C for amplification of the nitrate reductase gene. For the ADP-ATP translocase gene, the PCR conditions were the same as described above, with the except that the anneal temperature was 51 °C, and the elongation time, 1 min 30 s. The ADP/ATP translocase amplifications were digested with *Eco*RI, while those of the nitrate reductase gene were cut either with *Rsa*I or with *Hinc*II. The DNA fragments were then separated by electrophoresis in 1.5% native agarose gels and 1× Tris/Acetate/EDTA buffer.

2.3. Microsatellite amplification

Five of the nine known *B. cinerea* microsatellites (Fournier et al. 2002) were analyzed. Amplification protocols and the primers used for amplification of Bc2, Bc3, Bc6, Bc7, and Bc10 microsatellites were as described previously (Váczy et al. 2008). Fragment length analysis was performed with an automated single-capillary genetic

analyzer (ABI PRISM 310 Genetic Analyzer). The 5′ ends of the forward primers were labelled with fluorescent dye (i.e., Bc2: FAM, Bc3: NED, Bc6: NED, Bc7: FAM, Bc10: VIC, respectively). Purified PCR products were subjected to fragment length analysis at MWG-Biotech AG, Ebersberg, Germany. GeneMapper 3.7 software (ABI, Perkin Elmer, Foster City, CA, USA) was used for genotype scoring.

2.4. Minisatellite amplification and analysis

A fragment of the intron of the ATPase gene *atp1* containing the minisatellite MSB1 was amplified with primers MSB1fw and MSB1rev (Giraud et al. 1998). Amplification was done in a total volume of 50 μl containing 50 ng fungal DNA, 0.4 μM of each primer, and 25 μl GoTaq® Green Master Mix. 30 cycles of 1 min at 95 °C, 1 min at 60 °C, and 2 min at 72 °C were applied. Purified PCR products were subjected to automated sequencing at MWG-Biotech AG, Ebersberg, Germany. DNA sequences were aligned first with Clustal X 1.81 (Thompson et al. 1997) and then visually adjusted with Genedoc 2.6 (Nicholas and Nicholas 1997). The results were analyzed with DnaSP software (Librado and Rozas 2009).

2.5. Transposon detection

The presence of transposons was detected with a dot-blot method (Váczy et al. 2008). Genomic DNA (200 ng) was spotted onto positive charged nylon membrane (Roche), alkali-denatured in 0.5 M NaOH and 1.5 M NaCl for 20 min, and subsequently re-neutralized in 0.5 M Tris–HCl (pH 7.5) and 1.5 M NaCl for 20 min. The membrane was then equilibrated in 20× SSC (1× SSC: 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) for 5 min, baked at 120 °C for 30 min, and prehybridized in DIG Easy Hyb (Roche) for 30 min.

Hybridizations were performed overnight at 42 °C in the same solution after addition of *Boty*- or *Flipper*-specific PCR-generated and DIG-labeled probes. Primers used were LTR98 and LTR728 for *Boty* and F300 and F1500 for *Flipper* (Levis et al. 1997; Muñoz et al. 2002). The amplification protocol consisted of an initial denaturation (3 min, 95 °C) followed by five cycles with a denaturation of 1 min at 95 °C, an annealing of 1 min at 60 °C, and an elongation (1 min 72 °C), and 30 additional cycles with the denaturation step at 90 °C. Reaction was completed with a 15-min post-cycling elongation at 72 °C. Probes were digoxigenin-labeled with the polymerase chain reaction (PCR) DIG Probe Synthesis Kit (Roche), following the manufacturer's protocol.

2.6. PCR-RFLP and microsatellite fragment length analysis

PCR-RFLP and microsatellite analysis was performed as previously described by Fekete et al. (2012). Popgene Version 1.31 software (Yeh and Boyle 1999) was used for data analysis. Allele frequencies, effective number of alleles and gene diversity were determined for the six individual populations (defined by the host and the year of isolation) as well as for the entire collection. Nei's (1972) genetic identity is the normalized identity of genes between two populations and varies between 0 (the compared populations are different), and 1 (the compared populations are identical).

Population differentiation was tested by comparing allele frequencies among the six populations using Weir and Cockerham's θ (F_{st}) value (Weir and Cockerham 1984). The θ-value was estimated under the null hypothesis of non-differentiation among subpopulations, when $\theta = 0$. Statistical analysis was done by comparing the calculated θ-values to those of data sets in which isolates had been randomized across populations 10,000 times using the programme Multilocus 1.3b. Gene flow (N_m – equivalent to the number of migrants per generation) was determined using the equation $N_m = 0.5(1-\theta)/\theta$ (McDermott and McDonald 1993).

Multilocus 1.3b (Multi Locus Sequence Typing) was used to calculate the number of different multilocus genotypes (MLG) and the standardized version of the index of association r_D (Agapow and Burt 2001; Giraud et al. 2006). The null hypothesis of complete panmixia ($r_D = 0$) was tested by comparing the observed data set to 100 randomized data sets in which infinite recombination was simulated upon randomly shuffling the alleles among individuals, independently for each locus.

2.7. Bayesian inference

The existence of (a) defined population structure(s) in the total sample was investigated using the Bayesian approach implemented in Structure Version 2.3 software. This method infers the number of clusters of individuals (K) while maximizing Hardy–Weinberg equilibrium and minimizing the linkage disequilibrium within clusters. Individual isolates in the sample are assigned probabilistically to one of these K populations, or jointly to two or more populations if their genotypes indicate that they are mixed irrespective of the host plant of origin (Pritchard et al. 2000; Falush et al. 2003).

The scores of individuals in the clusters corresponded to the probability of ancestry in each and any one of them. We varied K from 1 to 20, each with three independent simulations to check the consistency of the results. Each simulation consisted in 500,000 Monte-Carlo Markov Chain (MCMC) iterations preceded by a burn-in period of 500,000 iterations. (NB: The burn-in period is the first set of iterations of the MCMC that is presumed to be dependent on the configuration at the start of the simulation – these iterations are not incorporated in the final calculation of the posterior probability.) Using a distribution of Maximum Likelihood, we calculated the posterior probability for each K to determine the most probable population structure. According to Evanno et al. (2005), the best estimation of K was that associated with highest ΔK, an ad hoc quantity related to the second order rate of change of the log probability (likelihood) of the data (Eq. (12) in Pritchard et al. 2000) with respect K.

2.8. Data Mining

Knowledge Discovery in Data (Data Mining) is a relatively new field in science used to extract valuable knowledge from huge data sets (Han et al. 2011). Data Mining has been scarcely applied to biological systems; it has previously been used in the evaluation of anti-viral therapies (Kim et al. 2008; Nanni et al. 2011; You et al. 2005). Here, we have used Data Mining to address Attributes Importance, i.e., the suitability of the various molecular markers employed in this work to differentiate between sympatric populations using Rapidminer (http://rapid-i.com) and Weka (http://www.cs.waikato.ac.nz/ml/weka) software. Discriminating attributes for the fungal populations on the two hosts (strawberry and raspberry) were defined and combined in three different attributes datasets (Supplementary Tables S1 and S2). "Decision Trees" and "Rules Induction" algorithms are applied to obtain attributes importance information (Esmaeili and Fazekas 2009; Maimon and Rokach 2005; Wu et al. 2008). Selection measures "Gain Ratio" and "Chi-Square" provide an attributes ranking according to their suitability to correctly classify *Botrytis* isolates.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.micres.2012.12.008.

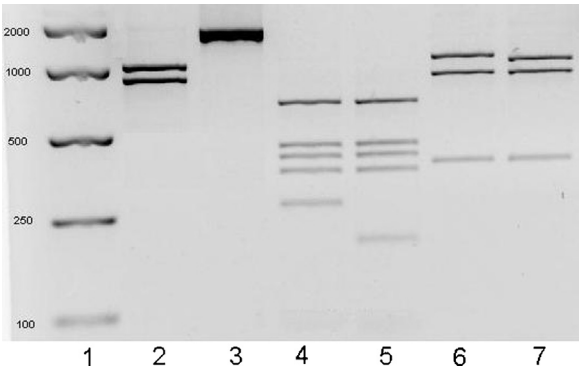

Fig. 1. ADP/ATP translocase and nitrate reductase gene alleles detected by PCR-RFLP. At the left, molecular weight markers (Easy Ladder I, BioRad); The numbers give marker length in bp. Fragments from ADP/ATP translocase alleles 1 and 2 upon *Eco*RI digestion, lanes 2 and 3, respectively; *Rsa*I-fragments from nitrate reductase alleles 1 and 2, lanes 4 and 5; *Hinc*II-fragments from nitrate reductase alleles 1 and 2, lanes 6 and 7.

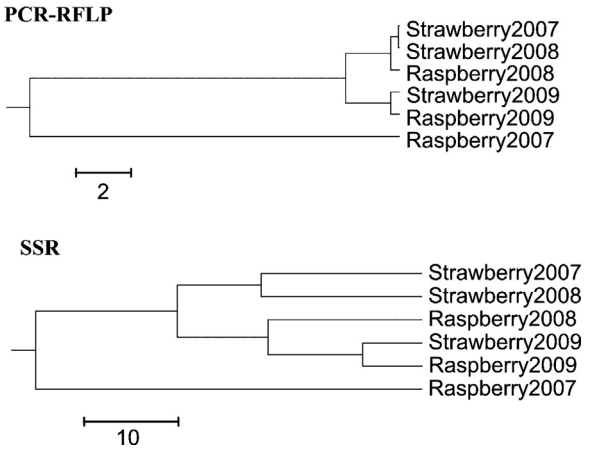

Fig. 2. Dendograms of *B. cinerea* populations (host/year) based on Nei's similarity coefficients (Nei 1978) using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) as the clustering method calculated from PCR-RFLP and microsatellite fragment length (SSR) data with Popgene.

3. Results

3.1. PCR-RFLP markers

We sampled populations of *B. cinerea* on sympatric strawberry and raspberry cultivars for three years. Altogether, 490 of group II *B. cinerea* isolates were analyzed. Isolates were first analyzed with RFLPs, amplifying two coding sequences (the genes encoding a nitrate reductase and an ADP/ATP translocase) subsequently digested with appropriate restriction enzymes (see below). The sizes of the RFLP-fragments of both genes among our Hungarian isolates were essentially identical to those detected in French and Chilean *B. cinerea* isolates (Giraud et al. 1997; Muñoz et al. 2002). Two alleles were identified for both genes with PCR-RFLP analysis (Fig. 1). For the first marker (ADP/ATP translocase), 368 strains (75% of the total) could be digested with *Eco*RI, while the remaining 25% of the isolates could not. For nitrate reductase, literature suggested to use two restriction enzymes, *Rsa*I and *Hinc*II (Giraud et al. 1997; Muñoz et al. 2002). The amplicons from 464 (95%) of the strains were digested with *Rsa*I in five fragments (774 bp, 503 bp, 457 bp, 404 bp, 303 bp) while 26 strains (5%) also gave five fragments but with slightly different sizes except for the smallest fragment (785 bp, 515 bp, 465 bp, 409 bp, 222 bp) (Fig. 1). Upon digestion with *Hinc*II, the nitrate reductase amplification of 79 (16%) of the strains yielded three fragments (1431 bp, 1090 bp, 442 bp) while for 411 strains (84%) three fragments of different size were observed (1379 bp, 1117 bp, 451 bp).

POPGENE32 Version 1.31 was used to compute basic population parameters like "observed number of alleles" (n_a), and "effective number of alleles" (n_e), as well as gene diversity indexes Nei's gene diversity (h) in the total sample and in each population (host and year) (Table 2). The calculations suggested that the population structures of sympatric *B. cinerea* samples were similar, and that both loci were polymorph. Nei's gene diversity indicated similar gene diversity for all populations, except for the isolates from raspberry *anno* 2009. The clone-corrected standardized index of association (r_D) was high in all six populations (0.222–0.5), indicating high clonality in each.

Similarity between the fungal samples from two hosts was computed as Nei's genetic identity, while the opposite feature, genetic distance, is reflected by Weir and Cockerham's θ (F_{st}) and the gene flow (N_m) in the total sample and in each population (host and year) (Table 3). The θ (F_{st}) and N_m values indicate considerable differentiation and restricted gene flow between the two sympatric populations exclusively in 2007, i.e., when fungi could only be collected from the strawberries in June while the large majority of raspberry samples were from August (see Table 1 and Fig. 4). Except for the strawberry populations from 2008 and 2009, the *B. cinerea* populations isolated from the same host in two subsequent years were divers (Table 3 and Fig. 2).

Table 2
Genetic diversity indexes for *Botrytis cinerea* populations isolated from different hosts in 2007–2009 calculated from PCR-RFLP data.

Host	n[a]	n_a[b]	n_e[c]	h[d]	No. of distinct MLG[e]	Max. no. of repeated MLG[f]	Genotypic diversity	r_D[g]
Strawberry 2007	81	2.00 (0.00)	1.45 (0.44)	0.26 (0.23)	5	20	0.484*	0.389
Raspberry 2007	28	2.00 (0.00)	1.30 (0.10)	0.22 (0.06)	5	20	0.484*	0.222
Strawberry 2008	117	1.67 (0.58)	1.41 (0.39)	0.25 (0.22)	4	59	0.631	0.5
Raspberry 2008	126	1.67 (0.00)	1.30 (0.28)	0.20 (0.18)	4	80	0.537	0.5
Strawberry 2009	84	1.17 (0.58)	1.29 (0.25)	0.20 (0.17)	4	57	0.505	0.5
Raspberry 2009	54	1.67 (0.58)	1.15 (0.17)	0.12 (0.13)	4	45	0.297*	0.5
Total	490	2.00 (0.00)	1.36 (0.24)	0.25 (0.14)	7	284	0.601	0.057

Standard deviation between brackets.
[a] n: sample size.
[b] n_a: observed number of alleles.
[c] n_e: effective number of alleles.
[d] h: Nei's gene diversity.
[e] No. of distinct MLG: number of distinct multilocus genotypes.
[f] Max. no. of repeated MLG: maximum number of repeated multilocus genotypes.
[g] r_D: standardized index of association (clone corrected).
* $P < 0.1$.

Table 3
F statistics calculated from RFLP data for the six populations. Weir and Cockerham's θ (F_{st}) (rows), and N_m (columns) were calculated with Multilocus 1.3b; $N_m = 0.5(1-\theta)/\theta$. Light grey background indicates moderate (θ: 0.05–0.15), dark grey big (θ: 0.15–0.25), black very big (θ: >0.25) differences between populations.

F_{st} / N_m	Strawberry 2007	Raspberry 2007	Strawberry 2008	Raspberry 2008	Strawberry 2009	Raspberry 2009
Strawberry 2007		0.390*	0.001	0.284	0.105	0.199
Raspberry 2007	0.782051		0.369*	0.421*	0.516*	0.585*
Strawberry 2008	166.1667	0.855014		0.007	0.096	0.164
Raspberry 2008	1.260563	0.687648	6.642857		0.069	0.113
Strawberry 2009	4.261905	0.468992	4.708333	6.746377		0.028
Raspberry 2009	2.012563	0.354701	2.54878	3.924779	17.34464	

*$P < 0.001$.

3.2. Microsatellite analysis

Fragment size analysis of the five microsatellite loci produced 167 haplotypes among 469 isolates (Table 4). More than one allele at one or more of the microsatellite loci were detected in 21 (4%) isolates and these individuals were not taken in consideration in the statistical analysis (below). The number of alleles (n_a), effective number of alleles (n_e) and Nei's gene diversity (h) were computed by POPGENE32 (Table 5).

Similar to the results with the ADP/ATP translocase and nitrate reductase genes, all five microsatellite loci were polymorph, and population structure of sympatric *B. cinerea* from the two different hosts showed similarity except for the fungi collected from raspberries anno 2007. (NB: By far the smallest batch of samples collected.) Nei's gene diversity indicated similar gene diversity for all populations, except again for the 2007 raspberry isolates. Hence, populations showed similar structures with the microsatellite analysis, with the exception of the population with the smallest sample of isolates (viz. Table 5, column 2).

The clone-corrected standardized index of association (r_D) (computed on the total sample of 469 isolates) was significantly different from zero ($r_D = 0.154$, $P < 0.01$, Table 5), indicating genetic differentiation between populations. The estimate of linkage disequilibrium (Table 5) varied between 0.056 (raspberry 2007) and 0.516 (raspberry 2009) at $P < 0.01$ in all cases. This suggests a sexual reproductive mode with a significant level of clonality for all populations. Nevertheless, we have never encountered apothecia, typical for the teleomorph form of *B. cinerea*, on host plants in the field.

In agreement with the calculations for the ADP/ATP translocase and nitrate reductase RLPFs, the microsatellite data indicated that genetic diversity of *B. cinerea* from raspberry was atypically low in 2007, while the Weir and Cockerham's θ (F_{st}) and N_m values suggested very high level of differentiation between the two sympatric populations in 2007, and moderate differentiation in 2008 (Table 6). Fungal populations collected in subsequent years from the same host exhibited either moderate ($\theta = 0.066$–0.141) or big ($\theta = 0.206$) differences for both hosts (Table 6 and Fig. 2). Dendograms of *B. cinerea* populations based on Nei's similarity coefficients calculated from PCR-RFLP and microsatellite fragment length data exhibited a similar structure (Fig. 2). These results strongly suggest that every year, a new *B. cinerea* population colonizes the strawberry/raspberry fields, and that the impact of overwintering *B. cinerea* individuals is restricted. However, the consequent fungicide treatments at the onset of the new season could have contributed by eliminating any individuals that survived on the hosts from the previous year.

Table 4
Characteristics of the five amplified microsatellite loci.

Locus	Number of alleles	Allele size range (bp)
Bc2	20	138–198
Bc3	12	209–231
Bc5	11	82–149
Bc7	13	108–130
Bc10	14	161–190

3.3. Bayesian inference of population structure

To confirm the genetic differentiation of the populations on the two host plants, the microsatellite dataset (Table 4; see above) was further analyzed. With Structure analysis, we performed Bayesian clustering using the set of 469 group II isolates (see above). The highest magnitude of ΔK was found with $K = 2$ and therefore, two genetic clusters were detected (Fig. 3). Essentially identical results

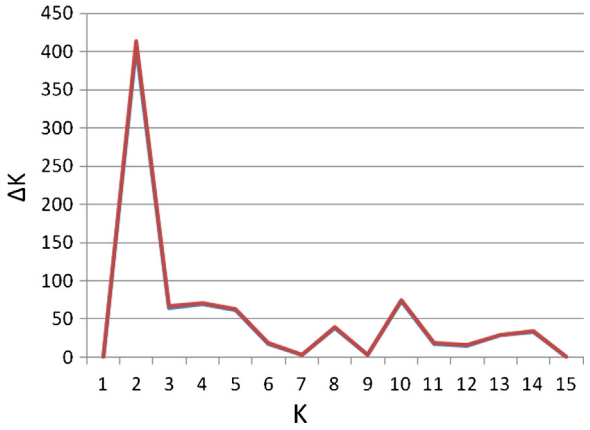

Fig. 3. The magnitude of ΔK at each level of K (1–15) used to determine the most probable number of genetic clusters ($K = 2$) of *B. cinerea* (*sensu stricto*) haplotypes from the Hungarian populations under study.

Table 5
Genetic diversity indexes for *Botrytis cinerea* populations calculated from microsatellite data.

Host	n[a]	n_a[b]	n_e[c]	h[d]	No. of distinct MLG[e]	Max. no. of repeated MLG[f]	Genotypic diversity	r_D[g]
Strawberry 2007	78	7.40 (0.89)	3.76 (0.21)	0.73 (0.01)	26	26	0.840	0.235**
Raspberry 2007	28	3.80 (1.48)	1.69 (0.36)	0.38 (0.15)	10	18	0.592	0.067*
Strawberry 2008	117	8.20 (0.83)	4.31 (1.12)	0.75 (0.07)	45	21	0.951	0.089**
Raspberry 2008	122	10.80 (3.11)	5.40 (1.23)	0.81 (0.05)	73	7	0.986	0.137**
Strawberry 2009	76	8.80 (2.49)	4.21 (1.65)	0.74 (0.07)	28	16	0.922	0.156**
Raspberry 2009	48	7.00 (1.87)	3.59 (1.12)	0.69 (0.13)	19	15	0.874	0.516**
Total	469	14.00 (3.53)	5.89 (1.22)	0.82 (0.03)	175	36	0.98	0.154**

Standard deviation between brackets.
[a] n: sample size.
[b] n_a: observed number of alleles.
[c] n_e: effective number of alleles.
[d] h: Nei's gene diversity.
[e] No. of distinct MLG: number of distinct multilocus genotypes.
[f] Max. no. of repeated MLG: maximum number of repeated multilocus genotypes.
[g] r_D: standardized index of association (clone corrected).
* $P<0.1$.
** $P<0.01$.

were found when samples from each year were analyzed separately (results not shown).

At $K=2$, the Structure Bar plot for isolates collected in the same year indicates clear separation of isolates from strawberries and raspberries in collection years 2007 and 2008 (Fig. 4). In 2007, all the samples from strawberries were collected at the same date (6th June), while all but four of the raspberry samples were collected two months later (6th August). In 2008, "better" meteorological conditions (i.e., longer humid periods with rain) resulted in a much higher infection ratio, and more isolates could be collected from both hosts between the middle of June and the first half of October. More samples could be collected at overlapping sampling times for the two hosts although most strawberry *B. cinerea* isolates were collected in June/early July (coinciding with the host's fruiting season). The majority of those strawberry samples belong to 2nd cluster (dark gray). On the other hand, fungi could be isolated from raspberries predominantly in August and September. The majority of those 2008 raspberry isolates were assigned by the STRUCTURE program to the other cluster (light grey). In the third year (2009), fungi could be collected from both hosts at the same time, in June, and the majority of them clustered to the 2nd (dark grey) population, irrespective of their hosts. However, the population dramatically changed in July 2009, following a fungicide treatment of the raspberries early in that month. Interestingly, a similar population shift was evident for strawberry isolates at the start of the flowering period (April–June) (before the plants started to bare fruit), again shortly after a double fungicide treatment.

3.4. Minisatellite analysis

The fragment of the intron of the ATPase gene *atp1* containing the MSB1 minisatellite was amplified and sequenced. 37 haplotypes among the 490 isolates could be detected (Table 7). One or two alleles were predominant (i.e., occurred in more than 50% of the isolates) in all six examined populations.

When assessed with minisatellite sequence variation, the genetic diversity of *B. cinerea* isolates from raspberry was lower in populations with fewer samples. G_{st} values indicated moderate (2009) to great (2007) differentiation of the sympatric populations.

According to three different data sets (i.e., PCR-RFLP pattern of ADP-ATP translocase and nitrate reductase genes, MSB1 minisatellite sequence data, and fragment size of five microsatellite loci) the structure of the different populations was similar as indicated by Nei's gene diversity and haplotype diversity. It should be noted, however, that a reduced sample size (most notably, raspberry in 2007) always appeared to co-incide with a reduced population diversity (see also below). The F statistics (the computed index of differentiation – G_{st}), and the gene flow indicated differentiation within the sympatric populations. However, the population genetic

Table 6
F statistics calculated from microsatellite data for the six populations. Weir and Cockerham's θ (F_{st}) (rows), and N_m (columns) were calculated with Multilocus 1.3b; $N_m = 0.5(1-\theta)/\theta$. Light grey background indicates moderate (θ: 0.05–0.15), dark grey big (θ: 0.15–0.25), black very big (θ: >0.25) differences between populations.

F_{st} / N_m	Strawberry 2007	Raspberry 2007	Strawberry 2008	Raspberry 2008	Strawberry 2009	Raspberry 2009
Strawberry 2007		0.272*	0.084	0.080*	0.122*	0.113
Raspberry 2007	1,338235		0.301*	0.206*	0.257*	0.312*
Strawberry 2008	5,452381	1,16113		0.087	0.141	0.137
Raspberry 2008	5,75	1,927184	5,247126		0.081	0.066
Strawberry 2009	3,598361	1,445525	3,046099	5,67284		0.036
Raspberry 2009	3,924779	1,102564	3,149635	7,075758	13,38889	

* $P<0.001$.

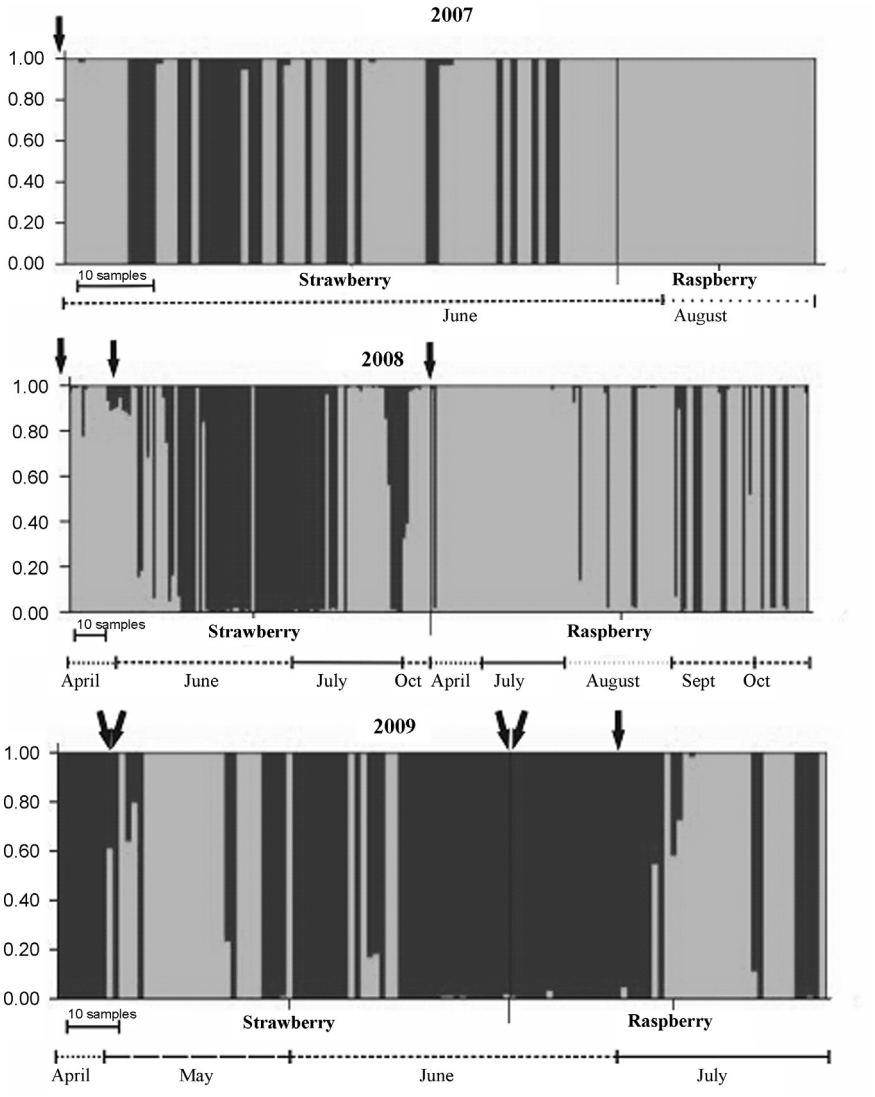

Fig. 4. Bayesian assignment of individuals to two population clusters. The Structure Bar-plot shows the assignment of haplotypes at $K = 2$ clusters for *B. cinerea* isolates from strawberry and raspberry. 2007, 2008, 2009 indicates the year of collection. Light grey bars represent population cluster 1; dark grey bars, cluster 2. Arrows indicate fungicide treatment of cultivars (see Section 2 for details). The timescale below the bar-plots indicates the time range of collection.

parameters were clearly influenced by the levels of polymorphism within the three data sets (Table 8).

3.5. Transposon elements

All 490 strains were screened for the presence or absence of the two transposable elements (Boty and Flipper) with dot blot hybridization. All four possible *B. cinerea* transposon genotypes could be distinguished (Table 9). The transposon content showed no correlation either to the host, the year of isolation or any fungicide treatment (Table 9).

3.6. Data Mining confirms microsatellites as useful markers to discriminate populations.

Three attributes datasets (see Section 2.8 and Supplementary Table S2.8) were tested with three algorithms (Rule Induction, J48, and Conjunctive Rule) and two criteria (Gain Ratio and Chi-Square).

Table 7
Analysis of *B. cinerea* MSB1 minisatellite sequencing results with DnaSP. Gaps were treated as a fifth character.

Year	Host	n	h	H_d	K	G_{st}
2007	Strawberry	79	11	0.806	29.089	
	Raspberry	28	5	0.384	19.928	0.2531
2008	Strawberry	131	15	0.842	63.268	
	Raspberry	112	11	0.768	32.346	0.1730
2009	Strawberry	83	13	0.801	40.998	
	Raspberry	54	8	0.638	16.919	0.1198
Total/mean		487	37	0.855	46.027	

n: sample size; h: number of haplotypes; H_d: haplotype diversity (Nei's 1987); K: average nucleotide diversity (Tajima 1983); G_{st}: index of differentiation.

Table 8
Comparision of different genotypic markers used in this study.

Parameter	Number of MLG or haplotypes[a]			Gene diversity[b]			*F* statistics[c]		
Marker	PCR-RFLP MLG	SSR MLG	MSB *h*	PCR-RFLP *h*	SSR *h*	MSB H_d	PCR-RFLP F_{st}	SSR F_{st}	MSB G_{st}
Strawberry 2007	5	26	11	0.26 (0.23)	0.73 (0.01)	0.806			
Raspberry 2007	5	10	5	0.22 (0.06)	0.38 (0.15)	0.384	0.390*	0.272*	0.2531
Strawberry 2008	4	45	15	0.25 (0.22)	0.75 (0.07)	0.842			
Raspberry 2008	4	73	11	0.20 (0.18)	0.81 (0.05)	0.768	0.007	0.087	0.1730
Strawberry 2009	4	28	13	0.20 (0.17)	0.74 (0.07)	0.801			
Raspberry 2009	4	19	8	0.12 (0.13)	0.69 (0.13)	0.638	0.028	0.036	0.1198
Total	7	175	37	0.25 (0.14)	0.82 (0.03)	0.855			

[a] MLG: number of distinct multilocus genotypes; *h*: number of haplotypes.
[b] *h*: Nei's gene diversity (calculated with POPGENE); H_d: haplotype diversity (Nei 1987; calculated with DnaSP).
[c] θ: Weir and Cockerham's θ (F_{st}: Weir and Cockerham 1984); G_{st}: Nei's coefficient for gene variation (Nei 1973).
* $P < 0.001$.

Table 9
Transposon content of the studied *B. cinerea* populations.

Date	Host	*n*[a]	Transposa (%)[b]	Vacuma (%)[c]	Flipper only (%)	Boty only (%)	Flipper total (%)	Boty total (%)
06/06/2007	Strawberry	81	38.3	29.6	23.5	8.6	61.7	46.9
06/06/2007	Raspberry	4	75.0	25.0	0	0	75.0	75.0
06/08/2007	Raspberry	24	45.8	16.7	29.2	8.3	75.0	54.2
18/05/2008	Raspberry	14	85.7	0	0	14.3	85.7	10.0
18/05/2008	Strawberry	15	53.3	0	0	46.7	53.3	100.0
07/06/2008	Strawberry	50	58.0	2.0	4.0	36.0	62.0	94.0
14/06/2008	Strawberry	12	66.7	8.3	8.3	16.7	75.0	83.3
05/07/2008	Strawberry	36	58.3	0	0	41.7	58.3	100.0
05/07/2008	Raspberry	18	72.2	0	0	27.8	72.2	100.0
13/07/2008	Raspberry	12	66.7	0	0	33.3	66.7	100.0
06/08/2008	Raspberry	39	84.6	0	0	15.4	84.6	100.0
06/09/2008	Raspberry	23	39.1	8.7	0	52.2	39.1	91.3
11/10/2008	Raspberry	20	100	0	0	0	100.0	100.0
11/10/2008	Strawberry	4	75.0	0	0	25.0	75.0	100.0
18/04/2009	Strawberry	8	37.5	0	62.5	0	100.0	37.5
16/05/2009	Strawberry	10	70.0	0	30.0	0	100.0	70.0
28/05/2009	Strawberry	20	75.0	0	25.0	0	100.0	75.0
21/06/2009	Strawberry	35	77.1	0	22.9	0	100.0	77.1
27/06/2009	Strawberry	10	100.0	0	0	0	100.0	100.0
27/06/2009	Raspberry	14	50.0	0	50.0	0	100.0	50.0
04/07/2009	Raspberry	27	40.7	0.0	59.3	0.0	100.0	40.7
04/07/2009	Raspberry	13	69.2	0.0	30.8	0.0	100.0	69.2

[a] *n*: sample size (=100%).
[b] The transposa genotype features both Flipper and Boty transposons (Fekete et al. 2012).
[c] The vacuma genotype specifies neither the Flipper nor the Boty transposon.

Table 10
Data Mining: selected results for subsets of Dataset 1 and Dataset 3.[a]

Model classifier	Attributes importance high-to-low[b]		Accuracy[c]	
	Dataset 1[d]	Dataset 3	Dataset 1	Dataset 3
Rule Induction 1	Bc10, Bc7, Bc3, Bc2, Bc6, *Eco*RI, Boty, Flipper	Bc7, Bc10, Bc3, Bc6, Bc2, Minisatellite	91%	88%
Rule Induction 2	Bc10, Bc7, Bc3, Bc2, Bc6, *Eco*RI, *Hinc*II, Boty	Bc7, Bc10, Bc3, Bc6, Bc2	90%	81%
Decision Tree	Bc7	Bc7	68%	61%

[a] Complete Dataset 1: Host, year, Bc10, Bc7, Bc3, Bc2, Bc6, *Eco*RI, *Hinc*II, *RsaI*, Boty, Flipper; Complete Dataset 3: Bc7, Bc10, Bc3, Bc6, Bc2, Minisatellite.
[b] Set of ordered attributes by model.
[c] Model evaluation criterium.
[d] Bc2, Bc3, Bc6, Bc7, Bc10, are different microsatellites of *B. cinerea*; Flipper and Boty are transposons; *Eco*RI, *Hinc*II, *Rsa*I represents PRC-RFLPs of two *B. cinerea* genes, Minisatellite signifies MSB1 minisatellite.

Table 11
Data Mining: performance of single rule induction on datasets. The conjunctive rule induction model was applied using the Weka C4.5 algorithm.

Dataset	Rule[a]	Covered by the Rule[b]		Not covered by the Rule	
		Strawberry	Raspberry	Strawberry	Raspberry
Dataset 1	If (Flipper present) and (Boty present) then Host = Strawberry	0.545	0.455	0.623036	0.377
	If (Flipper not present) and (Boty present) then Host = Strawberry	0.617	0.383	0.567	0.433
	If (Flipper present) and (Boty not present) then Host = Strawberry	0.558	0.441	0.577	0.421
	If (Flipper not present) and (Boty not present) then Host = Strawberry	0.788	0.212	0.560	0.440
Dataset 2	If ($117 < Bc7 \leq 129$) and ($Bc10 \leq 176$) then Host = Strawberry	0.890	0.109	0.416	0.584
Dataset 3	If ($Bc7 > 117$) then Host = Strawberry	0.820	0.180	0.259	0.741

[a] Bc7, Bc10, are different microsatellites of *B. cinerea*; Flipper and Boty are different transposons of *B. cinerea*.
[b] Ratio of instances covered by rule (0-1).

Table 12
Data Mining: attributes importance based on Gain Ratio and Chi-Square criteria.

Dataset	Method[a]	Attributes importance (high-to-low)[b]
Dataset 1	Gain Ratio	Bc7, Bc6, Bc2, Bc10, Bc3, *Eco*RI, date, Flipper, *Hinc*II, Boty, *Rsa*I
	Chi-Square	Bc2, Bc7, Bc6, Bc10, Bc3, date, *Eco*RI, Flipper, *Hinc*II, Boty, *Rsa*I
Dataset 2	Gain Ratio	Bc7, Bc6, Bc2, Bc10, Bc3
	Chi-Square	Bc2, Bc7, Bc6, Bc10, Bc3
Dataset 3	Gain Ratio	Bc7, Minisatellite, Bc6, Bc2, Bc10, Bc3
	Chi-Square	Minisatellite, Bc7, Bc2, Bc10, Bc6, Bc3

[a] Attributes selection by two criteria: Gain Ratio, Chi-Square.
[b] Bc2, Bc3, Bc6, Bc7, Bc10, are different microsatellites; *Eco*RI, *Hinc*II, *Rsa*I represent PRC-RFLPs; Flipper and Boty are transposons; Minisatellite signifies the MSB1 minisatellite allele.

Table 10 shows the results for Attributes Dataset 1 and 3, where the right column specifies the prediction accuracy. Table 11 summarizes dataset analysis with one Rule and gives the computed coverage per class (i.e., Host = Strawberry or Raspberry). Gain Ratio and Chi-Square criteria were used to test attributes importance for ordering between the two classes (i.e., host plants) (Table 12).

The Conjunctive Rule model applied to Attributes Datasets 1, 2 and 3 (Table 11) suggested that if Bc7 is between 117 and 129 nucleotides (nt) in length, and Bc10 is smaller than 177 nt, the host plant is strawberry in almost 90% of the cases covered by the Rule. On the contrary, the same model applied to the *Flipper* and *Boty* transposons, widely used in *Botrytis* population genetics, strongly suggested that these markers were not particularly suitable for differentiating our strawberry and raspberry infecting *B. cinerea* populations. Applying three of the four possible Rules, roughly half of the isolates came from strawberry (Table 11). Only for vacuma isolates, the coverage of the Rule appeared to be better. However, only 33 of the 490 isolates were vacuma, 29 of which were collected in 2007. Data Mining analyses strongly suggested that the microsatellites are the most defining markers to differentiate between the populations under study (Table 12).

4. Discussion

B. cinerea was previously considered a generalist fungal plant pathogen. However, recently it was shown that significantly structured populations exist according to the host plant, suggesting sympatric specialization (Fournier and Giraud 2008). Differentiation among transposon genotypes within *B. cinerea* group II has been described and these genotypes were found in sympatry on various hosts irrespective of geography (Giraud et al. 1997; Giraud et al. 1999; Muñoz et al. 2002; Martinez et al. 2003; Samuel et al. 2012). On the other hand, Ma and Michailides (2005) studied the genetic structure of *B. cinerea* populations in California on different hosts using transposable elements as markers and found no differentiation between *B. cinerea* populations from different hosts. Phylogenetic studies by Albertini et al. (2002), Isenegger et al. (2008) and Fournier et al. (2003) and Fournier et al. (2005) provided only weak indications for genetic differentiation between transposon genotypes. Fournier and Giraud (2008) focused on the distribution of the *Flipper* element in isolates from different host plants and could not detect significant differences, while it varied significantly according to geographic location. The detection of all four possible transposon genotypes in Hungarian group II isolates in this work indicated that there is no significant barrier to gene flow of transposons or any relevant difference among isolates from the different host plants under study. Moreover, Data Mining analysis did not identify transposon content information as particularly important or even indicative for the differentiation of *B. cinerea* populations. Similar to our present results, weak correlations were found between transposon frequency or type and the *B. cinerea* cryptic species (Fekete et al. 2012), host specificity (Ma and Michailides 2005), or other observed variation within *B. cinerea* populations (Muñoz et al. 2002; Váczy et al. 2008).

Using PCR-RFLP markers, several studies (Giraud et al. 1999; Muñoz et al. 2002) indicated that *B. cinerea* group II populations are structured according to infected host plants, i.e., isolates from the same host exchange genes more frequently with each other than with isolates from another host. In the present study, we employed three different data sets (i.e., PCR-RFLP patterns of the *ADP-ATP translocase* and nitrate *reductase genes*, MSB1 minisatellite sequence data, and fragment size of five microsatellite loci) and conclude that the structure of the different populations under study was similar for all three datasets as indicated by Nei's gene diversity and haplotype diversity (in the case of the minisatellite markers). It should be noted that a reduced sample size (strawberry isolates in 2007) coincided with a reduced population diversity. Population genetic parameters were influenced by the extent of polymorphism within the data sets. Nevertheless, the F statistics and the gene flow strongly suggested differentiation within the sympatric populations on strawberry and raspberry. Data Mining analyses supported the thesis that there were significant differences between the *B. cinerea* sympatric populations infecting the two hosts in our study. The most informative markers turned out to be the microsatellites, while transposons were not informative for population differentiation in our study.

The Bayesian analysis of the microsatellite data set highlighted the basis of differentiation. Because of the different phenological characteristics of the perennial hosts, the influence of meteorology, and variable sensitivity during the annual life cycle (vegetative growth, flowering, fruit maturation) for *B. cinerea* infection, only a restricted number of isolates could be collected at the same time from the two hosts. The importance of spore migration and a subsequent, rapid change in *B. cinerea* populations in tomato greenhouses has been noticed previously (Alfonso et al., 2000; Decognet et al. 2009). The rapid shift in predominance between two population clusters with the progress of the annual phenology and after fungicide application(s) in 2008 and, particularly, in 2009 (Fig. 4) indicated that the same phenomena occurred with open-field hosts on adjacent fields. Sympatric specificity of *B. cinerea* growing on different hosts may be influenced by several parameters, like differences in the phenology of the hosts, as populations of migrating spores encounter host plants in a different phenophase. The sudden change of fungal population observed following fungicide treatment supports the hypothesis that a change of the *B. cinerea* population in the air, in the form of vegetative spores, could result in an abrupt change of *B. cinerea* populations on hosts. However, eventual host preferences of *B cinerea* variants may also play as role.

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