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Genetic diversity of a *Botrytis cinerea* cryptic species complex in Hungary

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ABSTRACT

Botrytis cinerea has been described as a species complex containing two cryptic species, referred to as groups I and II. The first *B. cinerea* group I strains outside of Western Europe were collected in Hungary in 2008 from strawberry and rape plants. Sympatric *B. cinerea* cryptic species were analyzed using a population genetic approach and phenotypic markers. Statistically significant, but moderate population differentiation was found between the two groups in Hungary. Group I was originally typified by the lack of the transposable elements Boty and Flipper. However, all the Hungarian group I isolates carried the Boty element and one isolate additionally contained Flipper, indicating a much wider genetic variation than previously believed. Vegetative compatibility analyses showed that twelve of the thirteen *B. cinerea* group I isolates studied belonged to a unique vegetative compatibility group (VCG), but VCGs overlapped between groups. Phenotypic markers such as fenhexamid resistance or asexual spore size were found unsuitable to differentiate between the cryptic species. The results did not confirm the complete separation of the two cryptic species, previously determined with genealogical concordance of the phylogenetic species recognition using multiple gene sequences, and suggest instead the possibility of information exchange between them.

Introduction

A microbial species can be defined as a group of taxa with a common evolutionary history and, consequently, shared exclusive characters (Simpson 1951). The three most commonly discussed species concepts are those based on morphological, biological, and phylogenetic characteristics. Nowadays, population genetic analysis for species recognition is getting widespread mainly to identify asexual organisms (de Queiroz 2007; Grünig et al. 2007; Birky et al. 2010). Most fungal species have been determined by Morphological Species Recognition (MSR) based on the morphology of asexual and sexual reproductive structures, but the number of morphological characters is often limited and variable. Biological species concept (Mayr 1942) is restricted only for fungi that feature a sexual life cycle. Taylor et al. (2000) proposed the Genealogical Concordance Phylogenetic Species Recognition (GCPSR) for species definition, which could be an attractive alternative or complement to the morphological species concept. GCPSR requires parallel analysis of several unlinked genes and implies that the phylogenetic position of a true species is concordant in at least some of them and may not be contradicted in the others. GCPSR has been applied to the *Botrytis* genus (Staats et al. 2005) and *Botrytis cinerea* (Fournier et al. 2005). Both GCPSR and population genetic species recognition are the practical applications of the Evolution Species Concept to recognize the reproductive isolation of groups of strains to define species. Whereas GSPSR is based on the analysis of single strains, often originated from different locations, the population genetic analysis operates with groups of strains (isolates) representing populations. Population genetic species recognition was successfully used for various eukaryotic organisms (Birky et al. 2010; Robeson et al. 2011), and fungi (Grünig et al. 2007; Queloz et al. 2010; Chala et al. 2011).

The necrotrophic phytopathogenic ascomycetous fungus *Botryotinia fuckeliana* (de Bary) Whetzel – anamorphic stage, *B. cinerea* Pers. Fr. – causes grey mould disease worldwide. In viticulture, it is commonly known as “botrytis bunch rot”; in horticulture, it is usually called “grey mould” or “grey mould”. Within the *Botrytis* genus, *B. cinerea* has the widest host range, including more than 230 plant species, mainly dicotyledonous plants (Prins 2000). The great adaptability of this fungus is apparent from its polyphagia

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Table 1
Botrytis cinerea group I and group II strains.

Strain	Number of isolates	Isolation date	Host	*β-tubulin* accession numbers	*β-tubulin* sequence[a]	*Bc-hch* + *Hha* I[b]
Group I						
8001–8008	8	24 April 2008	Rape	HQ890436–HQ890443	1	1
8029–8032	4	18 May 2008	Strawberry	HQ890444–HQ890447	1	1
8047	1	07 June 2008	Strawberry	HQ890448	1	1
Group II						
8009–8022	14	18 May 2008.	Raspberry	HQ890449–HQ890451	2	2
8023–8028 and 8033–8046	20	18 May 2008	Strawberry	HQ890452–HQ890458	2	2
8048–8092	45	07 June 2008	Strawberry	HQ890459–HQ890461	2	2

[a] 1: group I sequencing result; 2: group II sequencing result.
[b] 1: group I RFLP pattern; 2: group II RFLP pattern.

and the diversity of the target organs (leaf, berry, flower petal, and stem). The fungus infects flowers, setting fruits, mature fruits, and leaves as well.

Several authors have shown that *B. cinerea* is a species complex with restricted gene flow between different cryptic genetic groups (Giraud et al. 1997, 1999; Albertini et al. 2002; Munoz et al. 2002; Fournier et al. 2003). Initially, two sympatric sibling species or transposon types were described: (1) *transposa*, that specifies the presence of Boty and Flipper transposons and (2) *vacuma*, which is (apparently) transposon-free (Diolez et al. 1995; Levis et al. 1997; Giraud et al. 1997).

Recently, molecular studies of different nuclear genes have suggested that *B. cinerea* populations are grouped into two different clades in the various gene phylogenies, group I and group II, i.e., phylogenetic species (Albertini et al. 2002; Fournier et al. 2003, 2005). Described group I isolates are exclusively from the *vacuma* transposon type, while group II can feature *transposa*, *flipper* (containing only Flipper), *boty* (containing only Boty) or *vacuma* genotype (Fournier et al. 2005; Ma and Michailides 2005; Munoz et al. 2002; Albertini et al. 2002; Ben Ahmed and Hamada 2005; Milicevic et al. 2006; Isenegger et al. 2008; Váczy et al. 2008; Rajaguru and Shaw 2010; Esterio et al. 2011). DNA polymorphism and vegetative incompatibility studies revealed that the genetic diversity is lower in group I than group II (Fournier et al. 2005). In addition, the two groups present differences in morphology, phenotypic characteristics and host range, e.g., asexual spores from group I isolates are reported to be significantly larger than those from group II strains (Fournier et al. 2005). While group I isolates have been mainly found in spring on grapevine, group II isolates have been collected both in spring and in fall (Martinez et al. 2005). Group I appears to have a narrower host range than group II. It is likely that the various differences observed between *vacuma* and *transposa* isolates correlate with the divergent evolution of group I and group II and/or to the variable proportion of *vacuma* and *transposa* isolates of the two cryptic species (Fournier et al. 2005).

The aim of this study was to (i) recognize group I and group II *B. cinerea* isolates in Hungary and (ii) to analyze the sympatric populations of *B. cinerea* cryptic species. To this end, we employed several genetic and phenotypic markers and compared their suitability for assessing the population structure and diversity of this widespread plant pathogenic fungus.

Materials and methods

B. cinerea strains

Strains of *B. cinerea* were collected from different hosts from the Nagyréde district, in North-East Hungary from strawberries and raspberries from the same farm, and from rape grown within 10 km of the farm. They were isolated from infected oilseed rape stalk and from infected fruits of strawberries and raspberries in 2008. Single-spore isolates were prepared from each strain and maintained on potato dextrose agar (PDA, Scharlau, Spain). Conidial suspensions were stored in 50% glycerol at −80 °C. Numbering of strains was done according to the chronology of collection, irrespective of the local provenance (Table 1).

DNA extraction

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 cells, and DNA was isolated with the Plant II DNA Purification Kit (Macherey-Nagel GmbH and Co. KG, Germany).

Bc-hch amplification and digestion

PCR-RFLP of the *Bc-hch* gene (the *B. cinerea* homolog of the *Neurospora crassa het-c* vegetative incompatibility locus) was used to identify group I and group II isolates as it was described by Fournier et al. (2003). Two primers: 262 (5′-AAGCCCTTCGATGTCTTGGA-3′) and 520L (5′-ACGGATTCCGAACTAAGTAA-3′), were used to amplify the *Bc-hch* gene. These primers amplified a 1171 pb fragment between position 701 and 1871 of the *Bc-hch* gene. The amplification was conducted in a final volume of 50 μl containing 4 μM of each primer, 100 ng of fungal DNA, 25 μl of Green Master Mix (Promega). Reactions were performed in a Primus (MWG-Biotech, Germany) thermal cycler programmed as follows: 1 cycle of 2 min at 95 °C, followed by 30 cycles of 1 min at 95 °C, 1 min at 51 °C, 1 min and 15 s at 72 °C. A final extension of 10 min was carried out at 72 °C. Amplified products were purified with Wizard® SV Gel and PCR Clean-Up System (Promega) and finally eluted with 40 μl nuclease free water. Digestion was carried out on 10 μl of the purified PCR product using 2 μl of FastDigest® *Hha* I restriction enzyme (Fermentas). Incubation was performed for 2 h at 37 °C. Fragments were separated on a native 1% agarose gel by electrophoresis and visualized by ethidium bromide under UV illumination. Fournier et al. (2003, 2005) proved that the *Bc-hch* fragment contains five *Hha* I restriction sites in group II isolates (positions 119, 274, 283, 367, 884), whereas only four sites in group I. The two restriction profiles thus differ in the size of the upper band (601 bp in group I, and 517 bp in group II).

β-tubulin amplification and sequencing

A portion of *tub1* was amplified and sequenced with primers and sequenced with primers 155 (5′-CAACCTTCAAAATGCGTGAG-3′) and 1174 (5′-AGATGGGTTGCTGAGCTTCA-3′) (Fournier et al. 2005). Amplification conditions were the same as described for *Bc-hch* gene except for 55 °C annealing temperature. Purified PCR products were sequenced at MWG-Biotech AG, Ebersberg, Germany.

Sequences were edited manually and deposited at GenBank (accession nos. HQ890436–HQ890461).

Microsatellite amplification and analysis

Microsatellite analysis was performed as previously described in Váczy et al. (2008). Five of the nine microsatellites described by Fournier et al. (2002) were analyzed. Amplification protocols and the primers used for amplification of Bc2, Bc3, Bc6, Bc7, and Bc10 microsatellites were identical to those described by Fournier et al. (2002). Fragment analysis was performed in an automated single-capillary genetic analyzer (ABI PRISM 310 Genetic Analyzer). The 5′ ends of the forward primers were labeled fluorescently (Bc2: FAM, Bc3: NED, Bc6: NED, Bc7: FAM, Bc10: VIC) as suggested by the analyzer's manufacturer.

Transposon detection

The presence of transposons was detected with dot-blot method (Váczy et al. 2008). Genomic DNA (200 ng) was spotted onto positive charged nylon membrane (Roche), denaturated in 0.5 M NaOH and 1.5 M NaCl for 20 min, and neutralized in 0.5 M Tris–HCl (pH 7.5) and 1.5 M NaCl for 20 min. The membrane was equilibrated in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) 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 (648 bp) or Flipper (1250 bp) probe. Primers used were LTR98 (5′-AGCCTGTAGAATCACCAACG-3′) and LTR728 (5′-CGGTATTTCTGGTTGGCA-3′) for Boty and F300 (5′-GCACAAAACCTACAGAAGA-3′) and F1500 (5′-ATTCGTTTCTTGGACTGTA-3′) for Flipper (Levis et al. 1997; Munoz et al. 2002). The amplification protocol consisted of an initial denaturation step (3 min, 95 °C) followed by five cycles with a denaturation (1 min, 95 °C), an annealing (1 min, 60 °C), and an elongation step (1 min 72 °C), and 30 slightly different subsequent cycles with a denaturation (1 min, 90 °C), an annealing (1 min, 60 °C), and an elongation step (1 min, 72 °C). Reaction was completed with a final 15 min elongation step at 72 °C. Probes were DIG-labeled with the polymerase chain reaction (PCR) DIG Probe Synthesis Kit (Roche), following the manufacturer's protocol.

Microsatellite data analysis

The software Popgene Version 1.31 (Yeh and Boyle 1999, provided online by the University of Alberta, CA) was used for data analysis. Allele frequencies, effective number of alleles and gene diversity were determined in the total sample and in both cryptic species. Nei's coefficient of gene variation (G_{ST}) was calculated which is equal to the weighted average of F_{ST} for all multiple alleles (Nei 1973). Gene flow (N_m) was estimated using the equation $N_m = 0.05\,(1 - G_{ST})/G_{ST}$ (McDermott and McDonald 1993). Nei's (1972) genetic identity and genetic distance also were calculated with this programme.

A second programme, called Multilocus 1.3b (Agapow and Burt 2003, published online by Multi Locus Sequence Typing), was used to calculate the genotypic diversity in relation to the number of loci employed. This analysis provides information on whether the inclusion of additional markers is needed to capture the breadth of the genotypic diversity present in the two cryptic species. 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) were also calculated using Multilocus 1.3b. The r_D is independent of the number of loci considered, and varies between 0 (complete panmixia) and 1 (no recombination). The null hypothesis of complete panmixia ($r_D = 0$) was tested with the procedure implemented in the software, by comparing the observed data set to 100 randomized data sets in which infinite recombination has been imposed by randomly shuffling the alleles among individuals, independently for each locus.

Population differentiation was tested by comparing allele frequencies among the cryptic species using Weir and Cockerham's θ (F_{ST}) value (Weir and Cockerham 1984) beyond G_{ST}. 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 data sets in which isolates have been randomized across populations 10,000 times using the programme Multilocus 1.3b.

The null hypothesis of lack of genetic differentiation between the two cryptic species was tested using the χ^2 test (Nei 1987) implemented in Popgene software.

Mycelial incompatibility

Mycelial incompatibility was tested by observing the interaction zone between paired colonies of wild-type *B. cinerea* strains on malt-extract agar amended with NaCl (Beever and Parkes 1993; Beever and Weeds 2004). Mycelial plug inoculums (10 mm diameter) were taken from the actively growing margin of 4–5-day-old PDA colonies. Four plugs from two different isolates were placed in each dish. Cultures were incubated in the dark in 20–22 °C. The pairings were examined 2 weeks after inoculation. Strains that formed dark pigmentation or exhibited sparse mycelium, with or without dark pigmentation, along the line of confrontation were considered incompatible. Incompatibility between two strains was registered as strong if it was evident and stable in replications; it was registered as weak if the line of confrontation was vague and/or it was inconsistent in the replications.

Fungicide resistant tests

Fungicides used in the study were the commercial formulations of fenhexamid (Teldor 500 SC, Bayer Crop Science, Hungary). The fungicides were dissolved in sterilized distilled water, and stock solutions were prepared. Autoclaved PDA was cooled to 50 °C and amended with aqueous fungicide solutions at discriminatory doses of 0, 2, and 5 mg l^{-1} fenhexamid by adding appropriate volumes of the fungicide stock solutions into the medium while it was still liquid. For determining pathogen sensitivity to the fungicide, a minimal medium was used containing 10 g glucose, 1.5 g K_2HPO, 2 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 5 g $MgSO_4 \cdot 7H_2O$, 2 g yeast extract (Scharlau), and 12.5 g agar (Scharlau) per liter (Leroux et al. 1999). Control medium was not amended with fungicides. Tests for each isolate were replicated three times per concentration. Mycelial plugs were removed, with the aid of a 10 mm in diameter cork borer, from the colony margins of actively growing 3-day-old colonies on PDA and placed upside down on the centers of 9 cm diameter plastic Petri dishes containing the fungicide-amended or -unamended media. Cultures were incubated at 20 °C in the dark for 3 days. Then, the mean colony diameter was measured and expressed as percentage of the mean diameter of the untreated control. Three phenotypes were distinguished: sensitive strains were resistant to estimated effective fenhexamid concentrations lower than 2 mg l^{-1}; moderately resistant isolates were resistant to estimated effective fenhexamid concentrations between 2 and 5 mg l^{-1}; and resistant isolates survived estimated effective fenhexamid concentrations higher than 5 mg l^{-1}.

Size of asexual spores

Fournier et al. (2005) showed that asexual spores were significantly larger in *vacuma* group I (12.48 μm) isolates than in *vacuma*

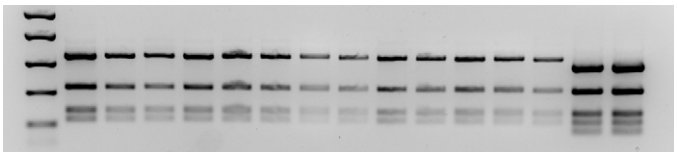


Fig. 1. Analysis of the fragment sizes produced by *Hha* I digestion of a *Bc-hch* gene amplification from thirteen *B. cinerea* group I strains. Samples from left: molecular marker, group I 8001–8008, 8029–8032, 8047, and 8049, 8061 strains. Molecular marker standards from top in base pairs: 2000, 1000, 500, 250, and 100.

group II (11.18 μm). The later has the same order of magnitude than the mean size of 11.62 μm for *transposa* group II isolates obtained by Giraud et al. (1997). In this study, we focused on the difference of spore size between transposons containing group I and group II. Nine group I strains (8001, 8002, 8004, 8005, 8006, 8007, 8029, 8030, 8031) and ten group II strains (8020, 8021, 8022, 8025, 8027, 8033, 8034, 8048, 8049, 8061) were grown on PDA medium for 14 days at 20 °C with a cycle of 12 h black light/12 h darkness. Spores were suspended in sterile water, observed under a Zeiss AxioImager equipped with AxioCam MRc5 camera. The longest diameter was calculated with AxioVision AC image analyser system for more than 50 spores of each isolate.

Results

Genotypic differentiation among group I and group II strains

More than a thousand *B. cinerea* samples, collected between 2007 and 2009, were analyzed to identify any group I isolates. Fungi were isolated from infected oilseed rapes, wine grapes, strawberries and raspberries (data not shown). The electrophoresis profile of a PCR-amplified *Bc-hch* fragment digested with the *Hha* I endonuclease resulted in two distinct restriction patterns. The size of the upper band was found to be either 601 bp for group I or 517 bp for group II strains (Fournier et al. 2003, 2005). The *Bc-hch* locus PCR-RFLP profile suggested that only 13 strains belong to the group I *B. cinerea* cryptic species isolated from oilseed rape and strawberries in April, May and early of June in 2008 (Fig. 1 and Table 1). These comprised only 14% of all the strains isolated from the same fields in 2008. Group II strains were isolated only from strawberry and raspberry hosts.

The parsimony analysis of the *β-tubulin* sequences clearly separates group I from group II. This clustering, supported by a bootstrap score of 100 (Fig. 2). The thirteen Hungarian and nine French isolates belonging to group I clustered into a monophyletic clade supported by a bootstrap value of 99. All the other Hungarian and French isolates clustered into a second clade, including the *B. fabae* isolate. The *β-tubulin* sequence analyses supported the results of *Bc-hch* PCR-RFLP results, identifying the same 13 isolates as belonging to the group I cryptic species (Table 1).

Transposon genotypes in group I and group II B. cinerea strains

B. cinerea group I has been described to exclusively feature the *vacuma* genotype, i.e., harboring neither Boty nor Flipper transposable elements. On the other hand, group II is characterized by a complex transposon spectrum representing four possible genotypes: (i) *vacuma*; (ii) *transposa*, harboring both Boty and Flipper elements; (iii) *flipper*, containing only Flipper; (iv) *boty*, containing only Boty. To determine whether these characteristics apply to our Hungarian isolates, we tested for the presence of the two transposons in 92 strains, including all 13 group I isolates identified above and a further 79 group II isolates, by means of dot blot hybridization. Intriguingly, transposable elements could be detected in the entire group I collection and in all but one of the

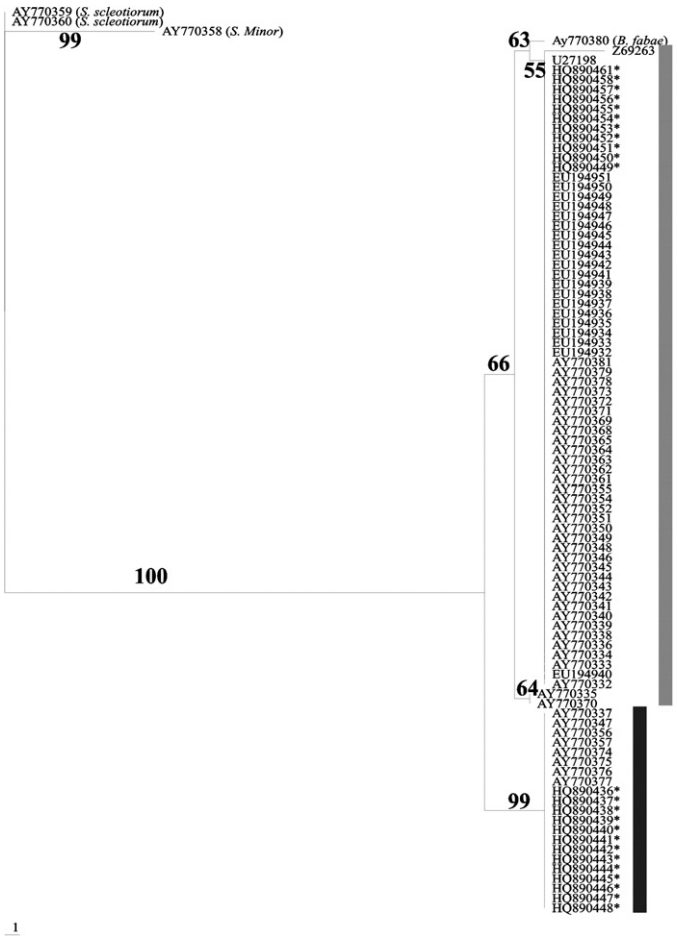


Fig. 2. One of the most parsimonious trees obtained for *β-tubulin*. Bootstrap values more than 50% ($N = 500$ replicates) are indicated on the corresponding nodes. Stars indicate Hungarian *Botrytis cinerea* isolates from 2008. Grey bar indicate group II; black bar indicate group I clade. The SPSS Inc. Sigma Plot for Windows program was used to create Fig. 3.

group II isolates (Table 2). The large majority (92%) of group I isolates harbored only the Boty transposon, while *transposa* (62%) and *boty* (34%) were the most frequent genotypes encountered among the group II isolates. Few group II isolates (2 strains) carried only the Flipper element (2.5%). We could not detect any transposon-specific hybridization in one of the group II isolates (1.26%).

Molecular population genetics approaches to assess the divergence between groups I and II

Microsatellite fragment length analysis

Microsatellites of *B. cinerea* are stable genetic markers for population studies (Ajouz et al. 2010). All 92 *B. cinerea* isolates were completely genotyped for five microsatellites (Table 3). The MULTILOCUS program was used to test for the discriminatory power of the five microsatellites. The genotypic diversity topped at 0.711 for a randomly chosen, single locus and increased to 0.913 and 0.927 for four or all five loci, respectively. These results indicate that the set of microsatellite markers used was sufficient to correctly estimate existing diversity.

All examined microsatellite loci were polymorphic. The five microsatellite markers exhibited between six and eight alleles, with an average of 7.0 alleles per marker in the examined *B. cinerea* samples (Table 3). The thirteen isolates of *B. cinerea* group I did not carried any typical allele for any of the five microsatellite loci (with respect to all the group II samples collected between 2007 and 2009,

Table 2
Transposon content and transposon types of group I and group II *B. cinerea* isolates collected in 2008.

	Transposon content (number of isolates)		Transposon type (number of isolates)			
	Boty	Flipper	*transposa*	*vacuma*	*flipper*	*boty*
Group I	13	1	1	0	0	12
Group II	76	51	49	1	2	27

Table 3
Characteristics of five examined microsatellite loci (Bc2, Bc3, Bc6, Bc7, and Bc10) isolated from *Botrytis cinerea* group I and group II samples.

	n[a]	Size range (bp)[b]					Number of alleles					Gene diversity[c]				
		Bc2	Bc3	Bc6	Bc7	Bc10	Bc2	Bc3	Bc6	Bc7	Bc10	Bc2	Bc3	Bc6	Bc7	Bc10
Group I	13	166	121–217	82–119	118–124	161–183	1	2	2	2	3	0.0000	0.4734	0.1528	0.3200	0.4615
Group II	79	138–172	209–221	82–149	110–130	161–188	6	6	6	6	8	0.6239	0.6303	0.5791	06630	0.6406
Total	92	138–172	209–221	82–149	110–130	161–188	6	6	6	6	8	0.6303	0.6628	0.6669	0.6545	0.6973

[a] Sample size.
[b] Base pairs. There was no amplification in some cases for Bc2, Bc6 and Bc7 both in group I and group II strains.
[c] Nei's (1973) gene diversity. Absence of a microsatellite was handled as missing value.

Table 4
Genetic diversity indexes within *Botrytis cinerea* cryptic species in Hungary calculated from microsatellite data.

	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]
Group I	13	2.0000 (0.7071)	1.4814 (0.3994)	0.2815 (0.2040)	5	6	0.756	0.238*
Group II	79	6.4000 (0.8944)	2.6977 (0.2153)	0.6274 (0.0308)	32	21	0.909	0.08*
Total	92	6.400 (0.8944)	2. 9742 (0.2170)	0.6624 (0.0241)	35	21	0.927	0.1005*

[a] Sample size.
[b] Observed number of alleles.
[c] Effective number of alleles.
[d] Nei's gene diversity.
[e] Number of distinct multilocus genotypes.
[f] Maximum number of repeated multilocus genotypes.
[g] Standardized index of association (clone corrected); standard deviation between brackets.
* $p < 0.01$.

data not shown) and no indistinguishable microsat haplotypes was found between group I and II isolates. The number of alleles and the genetic diversity appeared lower for all five microsatellites in group I, but it should be noted that we analyzed far more group II isolates (Table 3).

Consistent with previous studies (Fournier and Giraud 2008; Karchani-Balma et al. 2008; Rajaguru and Shaw 2010), the level of genetic diversity – indicated by the number of alleles, the effective number of alleles and Nei's gene diversity index – was higher for group II isolates than for group I strains (Table 4). Within the group II population of 79 isolates, 32 distinct multilocus genotypes (MLG) were recovered, displaying a genotypic diversity of 0.909. The group I population from the same area exhibited a lower level of diversity: 5 MLG could be distinguished among 13 strains, with a genotypic diversity of 0.756. The standardized version of the index of association (r_D) computed on a subset of clone-corrected data, on the total sample (containing both group I and II results) was significantly different from zero ($r_D = 0.100$, $P < 0.01$, Table 4), indicating genetic differentiation between populations. The estimate of linkage disequilibrium (Table 4) was lower in group II ($r_D = 0.08$, $P < 0.01$) than in group I ($r_D = 0.238$, $P < 0.01$). This suggests a sexual reproductive mode mainly with a low but significant level of clonality for both. We should like to note however, that the sample size for group I was rather small.

Statistically significant, but moderate population differentiation was found between the two sympatric *B. cinerea* cryptic species based on Weir and Cockerham's (1984) population differentiation statistics θ ($\theta = 0.1106$, $P = 0.007$) of clone corrected data. Similarly, both the calculated Nei's coefficient of gene variation ($G_{ST} = 0.1305$) and the estimated gene flow ($N_m = 3.3315$), indicated moderate differentiation and gene flow between the two sympatric populations. Nei's genetic identity (0.3837) and genetic distance (0.9579) indexes also suggested moderate identity between the two sampled populations.

Table 5
Tests of the null hypothesis of no genetic differentiation between groups I and II.

	χ^2[a]	P[b]	df[c]
Bc2	9.43	0.398	9
Bc3	7.18	0.517	8
Bc6	30.84	$<10^{-3}$	8
B7	30.04	$<10^{-4}$	7
Bc10	23.41	0.002	8

[a] Chi-square test for homogeneity (Nei 1987).
[b] Probability.
[c] Degree of freedom.

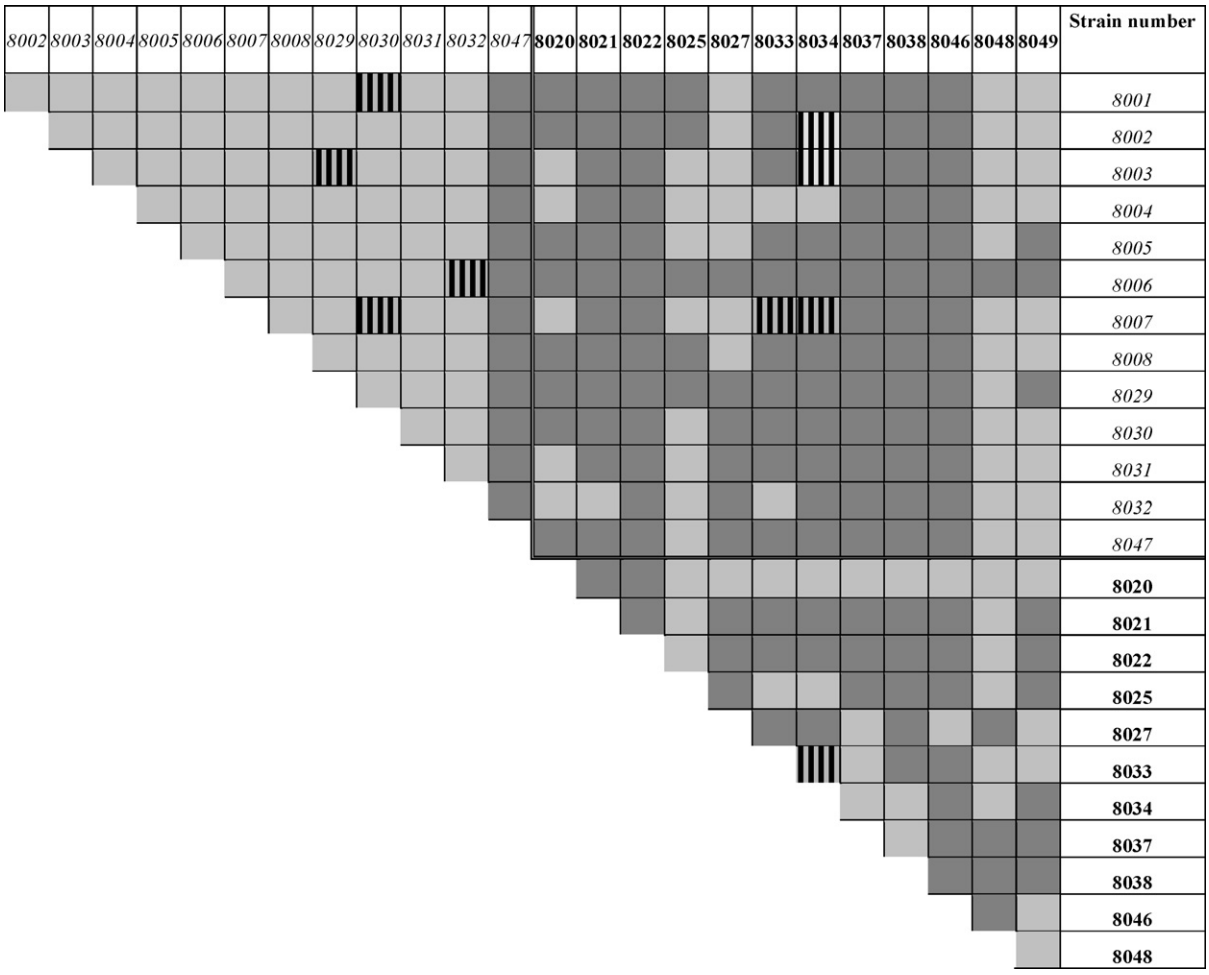
Results for Nei's χ^2 test were significant only for Bc6, Bc7 and Bc10 microsatellite loci (Table 5). However, for loci Bc2 and Bc3, the results of χ^2 test were not significant. Overall, these data indicated that the null hypothesis – no genetic differentiation between group I and group II – could not be completely rejected.

Classical population genetics approaches to assess the divergence between groups I and II

Vegetative compatibility tests

The thirteen group I *B. cinerea* isolates were further examined together with twelve group II *B. cinerea* strains. The latter were isolated from the same strawberry and neighboring raspberry fields at the same time day as (five of) the group I (8029–8032, and 8047)

Table 6
Compatibility tests of *Botrytis cinerea* isolates.[a]



[a] Italic numbers indicate *Botrytis cinerea* group I strains (8001–8008, 8029–8032, 8047); bold numbers indicate group II strains (8020, 8027, 8034, 8061); light grey: compatible; dark grey: non-compatible; barred: uncertain.

isolates (Table 1). Twelve group I isolates constituted a unique vegetative compatibility group (VCG). The 8047 group I isolate, however, was incompatible with all other group I strains but compatible with three of the group II strains (8025, 8048, 8049). Multiple VCGs were detected among group II isolates, and there were VCGs overlapped between the two groups (Table 6).

Fungicide resistance

To determine fungicide resistance profiles in group I and group II, sensitivity to fenhexamid was studied in all 13 isolates belonging to group I and 74 group II strains. In accordance with *in vitro* responses of these field strains of *B. cinerea*, three phenotypes could be distinguished: sensitive strains were resistant to estimated effective fenhexamid concentrations lower than 2 mg l^{-1}; moderately resistant isolates were resistant to estimated effective fenhexamid concentrations between 2 and 5 mg l^{-1}; and resistant isolates survived estimated effective fenhexamid concentrations higher than 5 mg l^{-1}. High resistance could be detected only among group I strains, while moderate resistant and sensitive isolates could be identified in both groups (Table 7). A majority of group II isolates classed as sensitive to fenhexamid.

Conidial size

Finally, conidial length was studied in some strains to investigate possible morphological differences between group I and group II samples. There were no significant differences between the vegetative spore size of the group I (11.65 ± 0.64 μm) and group II (10.73 ± 0.97 μm) isolates (Fig. 3). However, the tendencies we observed support the general view (Fournier et al. 2005) that *B. cinerea* group I strains usually produce somewhat longer conidia, while than group II strains exhibited more size variability.

Table 7
Fenhexamid resistance of group I and II *Botrytis cinerea* isolates.

	Number of isolates		
	Resistant[a]	Moderately resistant[b]	Sensitive[c]
Group I	9	0	4
Group II	0	4	70

[a] 5 mg l^{-1} < estimated EC_{50}.
[b] 2 mg l^{-1} < estimated EC_{50} < 5 mg l^{-1}.
[c] Estimated EC_{50} < 2 mg l^{-1}.

Discussion

Sympatric *B. cinerea* cryptic species were analyzed using a population genetic approach and phenotypic markers. The results have not confirmed a complete genetic differentiation of the two cryptic species (originally proposed by multiple gene sequence data analysis, GCPSR; Fournier et al. 2005), but point instead either towards a recent divergence or to the existence of genetic exchange between *B. cinerea* group I and group II cryptic species.

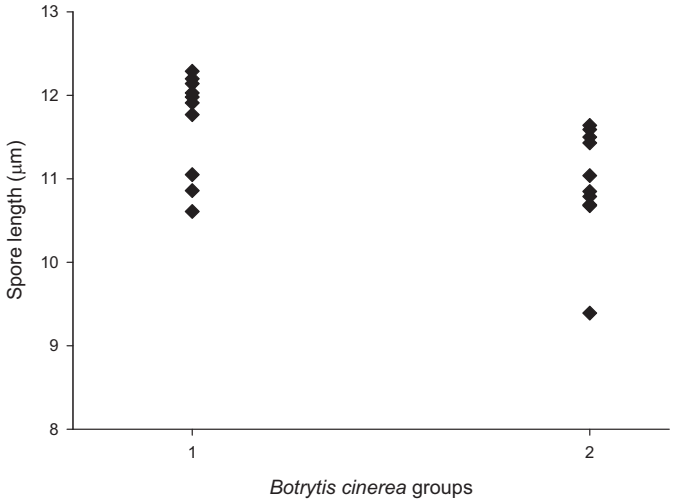


Fig. 3. Mean size of conidia for ten group I and nine group II isolates.

Before the discovery of appropriate genetic markers, *B. cinerea* was regarded as one, variable and polyphagous species exhibiting a great genetic diversity (Van der Vlugt-Bergmans et al. 1993; Diolez et al. 1995), and morphological variability (Grindle 1979; Di Lenna et al. 1981; Lorenz and Eichhorn 1983; Leone 1990; Movahedi and Heale 1990). Several genetic characterizations of *B. cinerea* populations revealed that this plant pathogen was genetically extremely diverse which suggested that there is no unique, large and panmictic population (Giraud et al. 1997, 1999; Albertini et al. 2002; Munoz et al. 2002; Fournier et al. 2003). Initially, two genotypes were described characterizing sympatric sibling species: (1) *transposa*, that harbors DNA transposons Boty and Flipper, and (2) transposonless *vacuma* (Diolez et al. 1995; Levis et al. 1997; Giraud et al. 1997). However, Fournier et al. (2005) showed that genetic differentiation determined from multiple gene sequences (genealogical concordance of the phylogenetic species recognition – GCPSR) was not in accordance with either of the previously described transposon genotypes (*transposa* or *vacuma*) and suggested partitioning of *B. cinerea* into group I and group II phylogenetic cryptic species. Group I is also known as '*Botrytis pseudocinerea*' while group II is referred to as '*B. cinerea sensu stricto*' (Fournier et al. 2005). The genetic diversity was lower within group I isolates, as revealed by DNA polymorphisms and vegetative incompatibility tests (Fournier et al. 2005). However, *B. cinerea* group II has been shown to be predominant on infected plants (Fournier et al. 2005; Isenegger et al. 2008; Karchani-Balma et al. 2008; Váczy et al. 2008).

Diagnostic molecular markers for the two cryptic species have been developed based on the difference of *Hha* I restriction pattern of PCR-amplified *Bc-hch* gene (Albertini et al. 2002; Fournier et al. 2003). To date, the *vacuma* transposon genotype has been detected within group I while in contrast, all four transposon genotypes (*vacuma*, *transposa*, *flipper*-only, and *boty*-only) have been detected in group II (Fournier et al. 2005; Ma and Michailides 2005; Isenegger et al. 2008; Karchani-Balma et al. 2008; Martinez et al. 2008; Váczy et al. 2008; Munoz et al. 2010; Rajaguru and Shaw 2010). High resistance to the fungicide fenhexamid appeared to characterize group I isolates (Fournier et al. 2005; Martinez et al. 2005).

Here, we describe for the first time the existence of the *B. cinerea* group I ('*B. pseudocinerea*') cryptic species outside of Western Europe, possibly suggesting that it was able to spread beyond geographic barriers such as the Alps and to settle in the Carpathian Basin. In addition, oilseed rape was identified as a new host for *B. cinerea* (group I). GCPSR of *B. cinerea* cryptic species is based on the analysis of four nuclear loci, i.e., *β-tubulin*, *Bc-hch*, CYP51 (eburicol 14a-demethylase) and 63R (a noncoding region containing a microsatellite-like motif and flanking regions with numerous SNPs) (Fournier et al. 2005). The identification of Hungarian group I strains was based on (a) PCR-RFLP of *Bc-hch* gene (Albertini et al. 2002; Fournier et al. 2003) and (b) on sequence analyses of *β-tubulin* gene. Phylogenetic analysis of partial *β-tubulin* sequences put these Hungarian isolates into the same clade with Western-European group I isolates, collected earlier. However the population genetic analysis of the microsatellite fragment length data of the two sympatric *B. cinerea* cryptic species indicated only moderate population differentiation. Both population differentiation statistics (θ, G_{ST}, gene flow) and Nei's genetic identity index suggest moderate differences between the two sampled populations with a low level of gene flow. Finally, the standardized version of index of association (as computed on the total sample) was significantly different from zero, likewise indicating genetic differentiation between populations. However, the examined *B. cinerea* group I isolates did not carry any group-specific microsatellite alleles or haplotypes, while Nei's χ^2 test results supported the genetic differentiation between the two groups for only three out of the five examined microsatellites.

The group I (*B. pseudocinerea*) cryptic species represents between 2.5 and 15% of French isolates (Albertini et al. 2002; Fournier et al. 2003, 2005; Martinez et al. 2005) and was equally low (14%) among the Hungarian isolates collected in 2008 from different host plants from the same area. Group I strains were present at spring, in agreement with other studies (Fournier et al. 2005; Martinez et al. 2005).

In agreement with previous studies (Fournier and Giraud 2008; Karchani-Balma et al. 2008; Rajaguru and Shaw 2010), group II isolates (obtained predominantly from infected plants) showed higher genetic diversity than group I strains. This finding suggests a higher adaptive potential (e.g. towards new hosts), and in general may indicate that these strains are more successful pathogens.

Regarding transposon content, it was initially suggested that sympatric *transposa* and *vacuma* type *B. cinerea* strains were genetically isolated (Giraud et al. 1997, 1999). This definition was later revised by differentiating between group I *vacuma* isolates (Fournier et al. 2005) and group II isolates, that may feature *transposa*, *flipper*, *boty* or *vacuma* genotype (Fournier et al. 2005; Ma and Michailides 2005; Munoz et al. 2002; Albertini et al. 2002; Ben Ahmed and Hamada 2005; Milicevic et al. 2006; Isenegger et al. 2008; Váczy et al. 2008; Rajaguru and Shaw 2010). Phylogenetic studies (Albertini et al. 2002; Fournier et al. 2003, 2005; Isenegger et al. 2008) could not provide hard evidence for genetic differentiation between transposon genotypes. It must be mentioned, that transposon detection has many uncertainties in *B. cinerea* depending on the test used. Martinez et al. (2008) showed discrepancies between PCR and dot blot results of detecting Boty and Flipper transposon elements. Moreover, weak (or no) correlations were found between transposon frequency or transposon type and the geographic origin of the isolate (Munoz et al. 2002; Váczy et al. 2008), or the year of isolation (Váczy et al. 2008), while results were also contradictory regarding temporal distribution (Martinez et al. 2005, 2008) and host specificity (Ma and Michailides 2005). Our results indicated that molecular markers based on transposon content alone has limited value on defining population structures and are not useful in *B. cinerea* cryptic speciation either. Significant differences in transposon content could only be detected on a continental scale (Isenegger et al. 2008).

Vegetative compatibility analysis results of the Hungarian isolates suggest a more diverse genetic structure than previously described. The nine group I *B. cinerea* samples examined by Fournier et al. (2005) constituted one VCG, and no overlapping compatibilities could be observed between the two cryptic species. However one of the Hungarian group I isolates formed a different VCG, than the thirteen other group I. Moreover VCGs were uncovered overlapped between the two groups of sympatric isolates. Our results

support the occurrence of genetic gene transfers between group I and group II strains as indicated by the presence of transposable elements in both groups.

Our fenhexamid resistance studies confirm previous results of Albertini et al. (2002) and Fournier et al. (2005) that fenhexamid-resistant strains could only be found among group I isolates. However, three Hungarian group I strains were found sensitive to the fungicide. Moreover, fenhexamid-resistant group II isolates have been reported recently from Chile (Esterio et al. 2010). Therefore, fenhexamid resistance cannot be used for the identification of group I *B. cinerea* isolates. Unlike Fournier et al. (2005), we could not detect statistically relevant differences in conidial size among the Hungarian group I and II isolates, dismissing the spore morphology as a possible means to discriminate between the cryptic species.

Up until today, group I isolates could be detected in Europe only. As this and several other studies pointed out, they comprise a small minority of the *B. cinerea* isolates even there. While the reason for this unknown, we speculate that speciation may have occurred in this area, and the lower diversity indicates a bottleneck effect. Other explanations are that group I strains successfully adapted to non-crop hosts or have a mainly saprophytic lifestyle, thereby avoiding the attention of plant protection-oriented research programmes. Future studies will address this in more detail.

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