Sexual Recombination in the *Botrytis cinerea* Populations in Hungarian Vineyards

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ABSTRACT

Váczy, K. Z., Sándor, E., Karaffa, L., Fekete, E., Fekete, É., Árnayasi, M., Czeglédi, L., Kövics, G. J., Druzhinina, I. S., and Kubicek, C. P. 2008. Sexual recombination in the *Botrytis cinerea* populations in Hungarian vineyards. *Phytopathology* 98:1312-1319.

Botrytis cinerea (anamorph of *Botryotinia fuckeliana*) causes gray mold on a high number of crop plants including grapes. In this study, we investigated the genetic properties of a grape pathogenic population of *B. cinerea* in the area of Eger, Hungary. A total of 109 isolates from 12 areas were sampled. Based on the sequence of the β -tubulin (*tub1*) locus, they all belong to group II, a phylogenetic species within *B. cinerea*. Seventy-four isolates were classified as *transposa*, with both the Flipper and Boty transposons, and 10 were classified as *vacuma*, lacking both transposons. The remaining isolates contained either only Flipper (13) or Boty (12). Multilocus analysis of sequences from *tub1* and two other loci (elonga-

tion factor 1-alpha, *tef1*, and a minisatellite from the intron of an ATPase, *MSB1*) led to poor phylogenetic resolution of strains in individual clades. Analysis of five microsatellites (Bc2, Bc3, Bc5, Bc6, and Bc10) resulted in 55 microsatellite haplotypes within the 109 strains. No correlation was detected among individual haplotypes and the presence/absence of Flipper and/or Boty, the geographic origin, or the year of isolation. Application of the index of association, the chi-square test, and the phi test consistently indicated that the population of Hungarian isolates of *B. cinerea* undergoes sexual reproduction. However, the index of association test suggested the presence of some clonality, and the fixation index showed a low or occasionally moderate level of fixation in the Flipper populations. We conclude that the *B. cinerea* populations in Hungary consist of a strongly recombining group II phylogenetic species.

Additional keywords: transposable elements.

Botrytis cinerea Pers.:Fr., the anamorph of *Botryotinia fuckeliana* (de Bary ex de Bary) Whetzel, causes gray mold on a number of crop plants in the temperate zones worldwide including grapes (17). In grape vineyards, the frequent occurrence of *B. cinerea* prior to harvest results in serious losses of fruits and deterioration of wine quality. *B. cinerea* was for a long time believed to have no host specificity (17) and to consist of a single anamorphic population whose somatic and phenotypic diversity was due to aneuploidy (6) and heterokaryosis (2,21). In contrast, the application of multiple gene genealogies (8) showed that *B. cinerea* in fact consists of two phylogenetic species (named group I and group II). In group I, no recombination was detected (though clonality was not demonstrated either), it showed a reduced host range, and lacked the two transposable elements that were characterized from *B. cinerea* Boty (5) and Flipper (20). Group II, in contrast, exhibited strong recombination, showed a wider host range, and most strains contained both transposons. The presence (*transposa* genotype) or absence (*vacuma* genotype) of these two transposons in isolates of group II has been associated with differences in the growth rate and conidiospore size (8,23) but does not seem to influence the virulence or host specificity.

B. cinerea is already resistant against most fungicides, thus making alternative strategies for its control essential. A clue to these strategies lies in the understanding of the genetic structure and dynamics of its populations. Interestingly, only a few such studies have been published (8,11,24,27).

The area around the town of Eger, Hungary, is a major and traditional wine growing region in Central Europe where *B. cinerea* is considered to be the third most important grapevine pathogen after downy mildew (*Plasmopara viticola*/Berk. & Curt ex de Bary/Berl. & de Toni) and powdery mildew (*Erysiphe necator* Schwein. var. *necator* [syn. *Uncinula necator*/Schwein./Burrill var. *necator*]), with an estimated annual loss of up to 15 to 20%.

The objectives of the present study were to determine the structure of *B. cinerea* populations, which are occurring as vineyard pathogens in this area, and to compare these findings with those in French and Chilean vineyards.

MATERIALS AND METHODS

Isolation. Strains of *B. cinerea* were collected from vineyards in various locations of the Eger wine districts (Fig. 1). They were isolated from infected berries in 2003 and 2004 during the vintage period (September to October). Single-spore isolates were prepared from each strain and maintained on potato dextrose agar (PDA). Conidial suspensions were stored in 50% glycerol at -80°C . Numbering of strains was done according to the

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chronology of collection, irrespective to the local provenance (Table 1).

DNA isolation. DNA was extracted from aerial mycelium of *B. cinerea* strains grown on PDA medium for 10 days at 20°C. Mag-nalyser (Roche, Osterode, Germany) was used for the disruption of fungal cells, and DNA was isolated with the Plant DNA Purification Kit (Qiagen, Hilden, Germany).

Detection of transposable elements Boty and Flipper. 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 (1,250 bp) probe. Primers used were LTR98 (5'-AGC-CTGTAGAATCACCAACG-3') and LTR728 (5'-CGGTATTCT-GGTTGGCA-3') for Boty and F300 (5'-GCACAAAACCTA-CAGAAGA-3') and F1500 (5'-ATTCGTTTCTTGGACTGTA-3') for Flipper (20,27). 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 manu-facturer's protocol.

PCR amplification and sequencing of single copy genes. A portion of *tef1* was amplified and sequenced with primers EF1 (5'-ATGGGTAAGGA(A/G)GACAAGAC-3') and EF2 (5'-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3') (16); a portion of *tub1* was amplified and sequenced with primers 155 (5'-CAA-CCTTCAAAATGCGTGAG-3') and 1174 (5'-AGATGGGTT-GCTGAGCTTCA-3') (8); and a fragment of the intron of the ATPase gene *atp1* containing the minisatellite MSB1 with primers MSB1fw (5'-AAGTTGCTGGTTCCTTGA-3') and MSB1rev (5'-GTTGCAACCGGCGTAGAT-3') (10). Purified PCR products were subjected to automatic sequencing at MWG-Biotech AG, Ebers-berg, Germany. Sequences were edited manually and deposited in NCBI GenBank (accession nos. EU194917 to EU194951).

Sequence analysis. DNA sequences were aligned first with Clustal X 1.81 (33) and then visually adjusted with Genedoc 2.6 (29). Molecular phylogenies were analyzed with MEGA 3.1 (19), PhyML (13; provided online by PHYML), and Mr. Bayes version 3.0B4 (14). For the latter, MODELTEST3-06 (MODELTEST is available online) was used to compare the likelihood of different nested models of DNA substitution and select the best-fit

Fig. 1. Geographic map of the Eger sampling regions. Capital letters specify the subregions, as given in Table 1. Circles with italic numbers (I to V) indicate the larger, more homogenous regions (valleys) used for the fixation index (F_{ST}) determinations (Table 3).

model for the investigated data set using Akaike information criterion (AIC) output strategies. For construction of haplotype networks, sequences were collapsed to haplotypes with DNAsp (32). Sequences, one per each haplotype, were aligned and converted to a NEXUS file, and the networks computed by statistical parsimony analysis as implemented in TCS version 2.11 (4).

Microsatellite amplification and analysis. Five microsatellites (Bc2, Bc3, Bc6, Bc7, and Bc10) were analyzed. Amplification protocols and the primers used were identical to those described by Fournier et al. (9). The 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.

Detection of recombination and population differentiation. Recombination within *B. cinerea* isolates was tested by (i) the

index of association (I_A) test, which measures whether the alleles from the different loci in a population are randomly or non-randomly associated in the analyzed genomes (18), and which was computed with the linkage disequilibrium analysis available on the MLST website (published online by Multi Locus Sequence Typing), and significance was gauged from 1,000 random permutations of the data; (ii) the maximum chi-square test (25), with the modification that all expected >2 are considered (30); and (iii) the phi test as implemented in SplitsTree version 3.8 (15). This approach is particularly useful in distinguishing recurrent mutation from recombination when assumptions such as a single, randomly mating, and constant-size population are not met.

Computation of the fixation index (F_{ST}) as a measure of population differentiation was done with POPGENE32 version 1.31 (provided online by the University of Alberta, CA) and by calculating G_{ST} , which is equal to the weighted average of F_{ST} for all multiple alleles (28).

TABLE 1. *Botrytis cinerea* strains, origin of location, haplotypes, and accession numbers

Year	Grape	Strain no.	Haplotype		Bc2 msat ^a	Bc3 msat	Bc6 msat	Bc7 msat	Bc10 msat	Presence of Flipper ^b	Presence of Boty	MSB1		tef11		tub1	
			Multi-locus	Transposon								Haplo-type	Accession number	Haplo-type	Accession number	Haplo-type	Accession number
2003	Zenit	3	1	1	166	217	116	118	179	1	1	1	EU194924	4	EU194925	2	EU194936
2003	Olaszrizling	9	2	2	166	223	116	118	165	1	1	2	EU194920	4	EU194925	4	EU194938
2003	Leányka	13	2	3	166	219	116	110	179	1	0	2	EU194921	4	EU194925	4	EU194938
2003	Olaszrizling	17	2	4	160	221	116	114	181	1	1	2	EU194921	4	EU194925	4	EU194941
2003	Hárslevelű	18	26	5	168	211	160	110	171	0	1	3	EU194923	4	EU194926	9	EU194948
2003	Medina	22	2	6	160	219	116	118	181	1	1	2	EU194921	4	EU194928	4	EU194941
2003	Zenit	29	10	7	168	219	120	116	187	1	1	4	EU194917	2	EU194927	4	EU194938
2003	Zenit	30	15	8	166	213	116	110	179	1	1	2	EU194921	3	EU194929	4	EU194938
2003	Olaszrizling	31	7	9	166	221	116	118	165	1	0	4	EU194917	4	EU194925	5	EU194943
2003	Olaszrizling	32	15	8	166	213	116	110	179	1	1	2	EU194921	3	EU194929	4	EU194938
2003	Leányka	33	3	10	166	221	116	118	181	1	1	2	EU194921	4	EU194925	10	EU194949
2003	Leányka	34	8	9	166	221	116	118	165	1	1	4	EU194917	4	EU194925	4	EU194941
2003	Kékfrankos	36	9	11	166	221	116	116	165	1	1	4	EU194917	4	EU194925	12	EU194951
2003	Olaszrizling	38	4	12	166	219	116	110	181	1	1	2	EU194921	4	EU194925	1	EU194932
2003	Hárslevelű	41	6	13	168	219	116	116	181	1	0	2	EU194921	4	EU194925	3	EU194936
2003	Eger csillaga	43	10	14	166	219	116	118	181	1	1	4	EU194917	2	EU194927	4	EU194938
2003	Leányka	45	1	15	166	213	116	118	179	1	1	1	EU194922	4	EU194925	2	EU194937
2003	Leányka	46	2	9	166	221	116	118	165	1	1	2	EU194921	4	EU194925	4	EU194942
2003	Leányka	47	24	16	160	219	116	116	177	0	1	1	EU194922	2	EU194927	4	EU194938
2003	Leányka	49	15	17	166	217	116	110	179	1	1	2	EU194921	3	EU194929	4	EU194933
2003	Kékoportó	53	8	18	160	221	116	118	181	1	0	4	EU194917	4	EU194925	4	EU194938
2003	Kékoportó	54	2	19	168	217	116	116	183	1	1	2	EU194921	4	EU194925	4	EU194938
2003	Kékoportó	55	17	20	180	219	116	110	177	1	1	2	EU194921	2	EU194927	4	EU194938
2003	Leányka	56	16	21	166	217	116	118	169	1	1	2	EU194921	3	EU194929	2	EU194936
2003	Leányka	57	26	22	168	211	128	110	173	0	1	3	EU194923	4	EU194926	9	EU194948
2003	Leányka	58	8	23	166	219	116	118	165	1	1	4	EU194917	4	EU194925	4	EU194938
2003	Leányka	59	27	24	168	211	116	118	173	0	0	3	EU194923	4	EU194926	4	EU194938
2003	Leányka	60	8	25	168	219	116	116	177	1	1	4	EU194917	4	EU194925	4	EU194938
2003	Leányka	61	12	25	168	219	116	116	177	1	1	4	EU194918	4	EU194925	6	EU194945
2003	Kékfrankos	63	11	26	166	219	116	114	179	1	1	4	EU194917	3	EU194929	2	EU194936
2003	Leányka	64	15	27	180	217	116	118	181	1	1	2	EU194921	3	EU194929	4	EU194938
2003	Leányka	65	2	14	166	219	116	118	181	1	0	2	EU194921	4	EU194925	4	EU194938
2003	Leányka	67	2	11	166	221	116	116	165	1	1	2	EU194921	4	EU194925	4	EU194938
2003	Zengő	68	3	11	166	221	116	116	165	0	1	2	EU194921	4	EU194925	10	EU194949
2003	Zengő	70	10	28	164	219	116	110	165	1	1	4	EU194918	2	EU194927	4	EU194938
2003	Hárslevelű	75	25	29	180	219	116	118	189	1	1	1	EU194922	3	EU194928	4	EU194938
2003	Hárslevelű	76	8	11	166	221	116	116	165	1	1	4	EU194918	4	EU194925	4	EU194938
2003	Kékfrankos	86	8	30	180	219	116	116	181	1	1	4	EU194917	4	EU194925	4	EU194938
2004	Leányka	401	19	31	166	219	116	116	183	1	0	1	EU194922	4	EU194925	4	EU194940
2004	Leányka	402	19	31	166	219	116	116	183	0	0	1	EU194922	4	EU194925	4	EU194938
2004	Chardonnay	405	1	1	166	217	116	118	179	0	1	1	EU194924	4	EU194925	2	EU194936
2004	Blauburger	407	18	32	180	219	122	110	177	0	0	1	EU194922	5	EU194930	4	EU194939
2004	Blauburger	408	18	32	180	219	122	110	177	0	1	1	EU194922	5	EU194930	4	EU194938
2004	Néró	409	8	33	166	221	116	116	181	1	1	4	EU194918	4	EU194925	4	EU194938
2004	Néró	410	8	33	166	221	116	116	181	1	0	4	EU194918	4	EU194925	4	EU194938
2004	Néró	411	8	33	166	221	116	116	181	0	1	4	EU194918	4	EU194925	4	EU194938
2004	Pinot noir	412	14	34	170	217	150	110	171	0	0	3	EU194919	1	EU194928	9	EU194948
2004	Pinot noir	413	14	35	170	219	150	110	171	0	0	3	EU194919	1	EU194928	9	EU194948
2004	Chardonnay	414	24	36	180	219	116	118	177	0	0	1	EU194922	2	EU194927	4	EU194938
2004	Chardonnay	415	10	37	180	219	116	114	177	0	0	4	EU194918	2	EU194927	4	EU194938
2004	Sauvignon blanc	417	10	38	160	219	120	120	181	0	0	4	EU194918	2	EU194927	4	EU194938
2004	Cabernet sauvignon	419	8	54	166	219	116	116	181	1	1	4	EU194918	4	EU194925	4	EU194938

(Continued on next page)

^a Bc2, Bc3, Bc6, Bc7, and Bc10 msat indicate the five microsatellites analyzed in this study.

^b Presence or absence of a particular transposon is indicated by 1 or 0, respectively.

RESULTS

The Hungarian *B. cinerea* populations consist entirely of group II. Fournier et al. (8) recently applied the genealogical concordance phylogenetic species recognition concept (GCPSR) to divide *B. cinerea* into two phylogenetic species, groups I and II. Since the two groups were clearly distinguishable in all four genes used, we used one of the genes from their investigation (*tub1*) to assign the strains from our study to these two groups. Up to 109 strains from 12 locations (Fig. 1) were thus sampled and sequenced. TBLASTN analysis against the NCBI database indicated that they all were identical to group II strains sequenced by Fournier et al. (8).

Transposon genotypes in the Hungarian *B. cinerea* group II species. *B. cinerea* group II has been described to mostly exhibit the *transposa* genotype (i.e., contain both Boty and Flipper) and

occasionally the *vacuma* genotype (i.e., contain neither). To determine whether this also applies to the Hungarian population, we tested for the presence of both transposons in 109 strains by the dot blot hybridization method. The results documented that indeed the vast majority of isolates (74, corresponding to 67.8%) contained both Flipper and Boty and was thus *transposa*. However, some of isolates also contained only Flipper (11.9%) (13) or only Boty (11%) (12), respectively. Ten isolates (9.1%) were *vacuma*, i.e., they contained neither transposon.

Multilocus structure of the Hungarian *B. cinerea* group II population. In order to determine the population structure of the Hungarian *B. cinerea* isolates, we sequenced two more loci: a fragment of the elongation factor 1- α encoding gene *tef1*, containing the fourth and fifth intron; and the minisatellite-containing intron of the *atp1* gene encoding an ATPase. A BLAST search against the *B. cinerea* genome database confirmed that

TABLE 1. (Continued from previous page)

Year	Grape	Strain no.	Haplotype		Bc2 msat ^a	Bc3 msat	Bc6 msat	Bc7 msat	Bc10 msat	Presence of Flipper ^b	Presence of Boty	<i>MSB1</i>		<i>tef1</i>		<i>tub1</i>	
			Multi-locus	Transposon								Haplo-type	Accession number	Haplo-type	Accession number	Haplo-type	Accession number
2004	Sauvignon blanc	421	19	39	168	219	116	110	179	1	1	1	EU194922	4	EU194926	4	EU194938
2004	Turán	423	19	40	166	219	116	116	179	1	1	1	EU194922	4	EU194925	4	EU194938
2004	Turán	424	19	40	166	219	116	116	179	1	1	1	EU194922	4	EU194925	4	EU194938
2004	Rizlingszilváni	425	1	1	166	217	116	118	179	1	1	1	EU194922	4	EU194925	2	EU194936
2004	Cabernet franc	427	8	31	166	219	116	116	183	1	1	4	EU194918	4	EU194925	4	EU194938
2004	Kékfrankos	431	13	1	166	217	116	118	179	1	1	4	EU194918	4	EU194926	2	EU194936
2004	Kékfrankos	432	20	41	180	219	116	116	179	1	1	1	EU194922	4	EU194926	7	EU194946
2004	Kékfrankos	433	19	41	180	219	116	116	179	1	1	1	EU194922	4	EU194926	4	EU194938
2004	Kékfrankos	435	10	42	166	221	122	118	181	0	0	4	EU194918	2	EU194927	4	EU194938
2004	Kékfrankos	437	8	43	166	219	116	120	181	1	1	4	EU194918	4	EU194926	4	EU194938
2004	Kékfrankos	438	13	44	166	217	116	120	179	1	1	4	EU194918	4	EU194926	2	EU194936
2004	Kékfrankos	439	8	12	166	219	116	110	181	1	1	4	EU194918	4	EU194926	4	EU194938
2004	Merlot	440	25	45	160	221	116	118	175	0	1	1	EU194922	3	EU194928	4	EU194938
2004	Merlot	441	25	45	160	221	116	118	175	0	1	1	EU194922	3	EU194928	4	EU194938
2004	Kékfrankos	442	1	17	166	217	116	110	179	1	1	1	EU194922	4	EU194926	2	EU194936
2004	Kékfrankos	443	8	54	166	219	116	116	181	1	1	4	EU194918	4	EU194926	4	EU194938
2004	Chaslas	445	8	30	180	219	116	116	181	0	0	4	EU194918	4	EU194925	4	EU194938
2004	Olaszrizling	448	19	46	166	217	116	116	181	1	1	1	EU194922	4	EU194926	4	EU194944
2004	Rizlingszilváni	449	1	1	166	217	116	118	179	1	1	1	EU194922	4	EU194926	2	EU194936
2004	Rizlingszilváni	450	1	1	166	217	116	118	179	1	1	1	EU194922	4	EU194926	2	EU194936
2004	Rizlingszilváni	451	1	1	166	217	116	118	179	1	1	1	EU194922	4	EU194926	2	EU194936
2004	Hárslevelű	454	1	47	166	217	116	110	169	1	1	1	EU194922	4	EU194926	2	EU194936
2004	Hárslevelű	455	19	48	166	219	116	110	183	1	1	1	EU194922	4	EU194926	4	EU194938
2004	Hárslevelű	456	19	49	166	219	116	118	183	1	1	1	EU194922	4	EU194926	4	EU194938
2004	Hárslevelű	457	23	50	166	219	116	120	183	0	1	1	EU194922	4	EU194926	11	EU194950
2004	Hárslevelű	458	22	58	166	219	116	110	183	0	1	1	EU194922	4	EU194926	8	EU194947
2004	Bianka	460	1	15	166	213	116	118	179	1	1	1	EU194922	4	EU194925	2	EU194936
2004	Chardonnay	461	5	15	166	213	116	118	179	1	1	2	EU194921	4	EU194925	2	EU194936
2004	Merlot	464	8	14	166	219	116	118	181	1	1	4	EU194918	4	EU194925	4	EU194938
2004	Chaslas	465	2	11	166	221	116	116	165	1	1	2	EU194921	4	EU194925	4	EU194938
2004	Chaslas	466	2	51	166	219	116	116	165	1	1	2	EU194921	4	EU194925	4	EU194938
2004	Kékfrankos	468	8	33	166	221	116	116	181	1	1	4	EU194918	4	EU194925	4	EU194938
2004	Cabernet sauvignon	471	2	52	164	219	116	118	181	1	0	2	EU194921	4	EU194926	4	EU194938
2004	Hárslevelű	473	19	14	166	219	116	118	181	1	0	1	EU194922	4	EU194925	4	EU194938
2004	Hárslevelű	474	19	14	166	219	116	118	181	1	0	1	EU194922	4	EU194925	4	EU194938
2004	Hárslevelű	475	2	23	166	219	116	118	165	1	1	2	EU194921	4	EU194925	4	EU194938
2004	Hárslevelű	476	2	31	166	219	116	116	183	1	1	2	EU194921	4	EU194926	4	EU194935
2004	Hárslevelű	478	8	11	166	221	116	116	165	1	1	4	EU194918	4	EU194925	4	EU194938
2004	Kékfrankos	488	21	53	164	213	116	118	181	0	1	1	EU194922	4	EU194926	5	EU194934
2004	Kékfrankos	489	10	54	166	219	116	116	181	1	1	4	EU194918	2	EU194927	4	EU194938
2004	Kékfrankos	490	10	14	166	219	116	118	181	1	1	4	EU194918	2	EU194927	4	EU194938
2004	Kékfrankos	491	19	11	166	221	116	116	165	1	1	1	EU194922	4	EU194925	4	EU194938
2004	Pinot blanc	4107	8	11	166	221	116	116	165	1	0	4	EU194918	4	EU194925	4	EU194938
2004	Olaszrizling	4147	2	54	166	219	116	116	181	1	1	2	EU194921	4	EU194925	4	EU194938
2004	Turán	4149	5	15	166	213	116	118	179	1	0	2	EU194921	4	EU194925	2	EU194936
2004	Turán	4150	2	33	166	221	116	116	181	1	0	2	EU194921	4	EU194925	4	EU194938
2004	Turán	4151	2	33	166	221	116	116	181	1	1	2	EU194921	4	EU194925	4	EU194938
2004	Turán	4152	2	33	166	221	116	116	181	1	1	2	EU194921	4	EU194925	4	EU194938
2004	Leányka	4163	2	14	166	219	116	118	181	1	1	2	EU194921	4	EU194925	4	EU194938
2004	Leányka	4165	2	9	166	221	116	118	165	1	1	2	EU194921	4	EU194925	4	EU194938
2004	Leányka	4167	2	55	166	223	116	114	165	1	1	2	EU194921	4	EU194925	4	EU194938
2004	Leányka	4168	2	51	166	219	116	116	165	1	1	2	EU194921	4	EU194925	4	EU194938
2004	Leányka	4171	2	31	166	219	116	116	183	1	1	2	EU194921	4	EU194931	4	EU194938
2004	Leányka	4183	2	9	166	221	116	118	165	1	1	2	EU194921	4	EU194931	4	EU194938
2004	Olaszrizling	4185	2	49	166	219	116	118	183	1	1	2	EU194921	4	EU194931	4	EU194938
2004	Olaszrizling	4186	2	49	166	219	116	118	183	1	1	2	EU194921	4	EU194931	4	EU194938
2004	Rizlingszilváni	4188	19	40	166	219	116	116	179	1	1	1	EU194922	4	EU194926	4	EU194938

these two and the *tub1* gene were unlinked and thus suitable for the analysis. Both Tajima's D-test as well as Fu's F-statistic did not reject the hypothesis of neutral evolution (Table 2), and we therefore considered the gene data set as appropriate for phylogenetic analysis.

The three loci included a total of 1,007 sites of which 26 were polymorphic (Table 2). However, only a low number of sites were parsimony informative. Maximum likelihood trees for individual loci are shown in Figure 2. Four, five, and twelve clades were obtained from the analysis of *MSB1*, *tef1*, and *tub1*, respectively. Likelihoods (loglk) were determined as -836.45626, -578.61042,

and -578.61042 for *tef1*, *tub1*, and *MSB1*, respectively. Most branches in these trees were only poorly supported, however, and also did not contain the same isolates, indicating incongruence between different trees. A consistent result was obtained when the individual gene sequences were collapsed to haplotypes and these analyzed by statistical parsimony (4). The corresponding networks are given in Figure 3, again indicating little hierarchical structure within them.

In order to learn whether any of these haplotypes would correlate with the presence or absence of Boty or Flipper, we mapped their absence/presence on the haplotype networks. The data,

TABLE 2. Nucleotide characteristics of *Botrytis cinerea* *tef1*, *tub1*, and *MSB1*

Characteristics	<i>tef1</i>	<i>MSB1</i>	<i>tub1</i>	Total
No of sites without gaps	249	254	504	1,007
No. of polymorphic sites	6	6	14	26
Parsimony informative sites	7	6	5	18
Pi	0.00384	0.00875	0.00120	
Theta	0.00530	0.00445	0.00513	
Haplotypes number	5	4	12	
Haplotype diversity	0.364 [±0.055]	0.697 [±0.013]	0.448 [±0.052]	
Fu and Li's D*	1.182	1.108	-3.783	
Tajima's D	-0.630	2.129	-2.035

Fig. 2. Maximum likelihood analysis of gene phylogenies for *tef1*, *tub1*, and *MSB1*. Only one sequence per haplotype was used for the analysis. The HKI model was used for all three genes. Numbers over branches indicate bootstrap supports (not shown below 50).

shown in Figure 3, indeed indicate that the sum of the minor haplotypes display a different ratio of the individual transposon genotypes than the population containing the major gene haplotype. However, since the two samples contain a significantly different number of strains, this finding is not statistically supported.

Recombination in the Hungarian *B. cinerea* population. The data above revealed incongruence between different gene trees, suggesting recombination within the Hungarian population of *B. cinerea*. Since the three genes used (*MSB1*, *tef1*, and *tub1*) did not contain sufficient genetic variation, we used microsatellites to test recombination. To this end, we used five microsatellites of *B. cinerea* (Bc2, Bc3, Bc6, Bc7, and Bc10). They yielded 6, 6, 6, 11, and 13 fragments of different length, respectively. Their occurrence per strain resulted in 55 different microsatellite genotypes. When these genotypes were compared with the gene haplotypes (Fig. 3), no correlation with any of them was obtained (data not shown). Also, no correlation was obtained with either the geographic origin of the isolates or the year of isolation (data not shown).

To test for recombination, we used the I_A test on a subset of clone-corrected data (i.e., individuals with identical genotypes at the five microsatellites were excluded so that each multilocus genotype was represented by only one individual [31]). The results from this test ($I_A = 0.166$; $P < 0.01$) revealed that panmixia alone cannot be inferred from the data and that some clonality must be present. Similar values were obtained when only the isolates from 2003 or 2004 were subjected to the I_A test ($I_A = 0.145$ and 0.187 ; $P < 0.01$ in both cases, respectively).

We also applied two tests on a concatenated set of our gene sequences, i.e., the maximum chi-square test and the phi test. The hypothesis of recombination was confirmed by both of them, i.e., the maximum chi-square test gave a value of 20.43 ($P = 0.009$); and the phi test resulted in $P = 9.564 \times 10^{-4}$.

Thus, the application of three different criteria and two different sets of genetic markers consistently indicates that the population of Hungarian isolates of *B. cinerea* in the Eger wine region undergoes sexual reproduction.

Genetic drift within the Hungarian *B. cinerea* population. Because of the absence of complete panmixia, we computed the F_{ST} values for all transposon populations and for all geographic populations. Qualitatively, an F_{ST} value within the range of 0.05 to 0.15 indicates moderate differentiation (=fixation of characters), within 0.15 to 0.25 indicates great differentiation, and greater than 0.25 indicates very great differentiation (34). As shown in Table 3, F_{ST} values between geographic populations were in the range of 0.018 to 0.077, indicating no or only very low fixation. A similar analysis of the four transposing populations showed low differentiation between most of them. Only the examination of the F_{ST} values between Flipper and *vacuma*—and less between *transposa* and *vacuma*—exhibited a moderate level of fixation (0.11 and 0.09, respectively).

DISCUSSION

The results presented in this paper demonstrate that the Hungarian wine-pathogenic population of *B. cinerea* exhibits significant recombination. Whether or not recombination occurs in *B. cinerea* has been a matter of debate for many years, mainly because of the detection of only asexual propagation (=conidiation) on the plants. Yet, the use of rigorous genetic tests such as linkage disequilibrium (12) clearly showed that a *B. cinerea* population from France indeed propagated by sexual recombination. On the other hand, Ma and Michailides (22), studying Californian populations of *B. cinerea*, obtained essentially contrary data suggesting clonality. The difference between these studies could be due to the different geography of the study or the use of different genetic markers (gene restriction fragments versus random amplified polymorphic DNA analysis, respectively). In the present study, a

Fig. 3. Haplotype networks for *tef1*, *tub1*, and *MSB1*, generated by statistical parsimony. *n* indicates number of isolates for each haplotypes, circles without *n* number consisted in single haplotypes only. Dots at the lines connecting haplotypes separate single mutations (= a line separated by no dot indicates a single mutation; one separated by a single dot indicates two mutations, etc.). Light gray sectors specify the proportion of isolates containing only Boty; and dark gray sectors specify isolates containing only Flipper. Black and white sectors indicate the proportion of isolates containing both and neither of them, respectively.

very high degree of linkage disequilibrium was shown both by analyses of gene sequences as well as of microsatellites, which minimizes a potential bias. Fournier and Giraud (7) also provided evidence for recombination in a French population of *B. cinerea*. Therefore, although the geographic argument still cannot be refuted, it appears that the European populations of *B. cinerea* indeed perform recombination by sexual reproduction. However, the I_A test also suggested an element of clonality. A corresponding analysis of the fixation index revealed that indeed the *vacuma* population exhibited some differentiation from both the Flipper and the *transposa* populations, which may explain these results. We could also detect some (albeit very moderate) differentiation between some geographic populations, particularly between II and IV and IV and V.

Previously, *B. cinerea* was believed to consist of two sympatric species, of which one (=transposa) contains both Flipper and Boty and the other (=vacuma) neither of them (12). *vacuma* and *transposa* were also found to significantly differ in phenotype such as conidial size, growth rates, and resistance against vinclozolin and diethofencarb (11). However, Fournier et al. (8), using GCPSR, provided the first evidence for two different sibling species of *B. cinerea* which they named group I and group II. In comparison to the *vacuma* and *transposa* concept, *B. cinerea* group I indeed contained only *vacuma* isolates. The results from the present study show that the *B. cinerea* populations in the Eger wine grape growing region in Hungary exhibit an unusual transposon composition; while the majority of isolates indeed were *transposa*, *vacuma* isolates were present in about the same percentage as isolates containing only Boty or only Flipper. The presence of only Flipper in *B. cinerea* has so far not been detected in isolates from California (22) and Chile (27), and seems to be strongly underrepresented in samples from England, France, and Tunisia (1,3). In contrast, a more recent study on *B. cinerea* isolates from Croatia (26) reported 26% of the isolates to contain only Flipper, whereas 41% were *transposa*. The higher number of isolates containing only Flipper raises the question of how these strains evolved, i.e., from *transposa* strains by loss of Boty, or from *vacuma* strains by gain of Flipper. Munoz et al. (27) attempted to explain the occurrence of Boty isolates in a Chilean population by crosses between *vacuma* and *transposa*, or by the existence of an invading population of the *vacuma* group. Because of the high linkage equilibrium in our genetic markers, we cannot distinguish between these two possibilities. In theory, the roughly equal number of Boty, Flipper, and *vacuma* isolates would be compatible with the speculation of a recombination of *transposa* with an invading population of *vacuma*. However, our data showed that the *vacuma* population exhibits the highest level of clonality of all transposon populations which makes this explanation rather unlikely. In any case, the evidence from our data, i.e., that *B. cinerea* group II also contains isolates which only contain Flipper or Boty, further underlines that the presence or absence of either transposon is not a valid character for speciation in *B. cinerea*.

In summary, our data underline a significant genetic variability within the Hungarian population of *B. cinerea*. From a phytopathological perspective, a more detailed insight into the mechanisms leading to the formation of the "Flipper-only" isolates will be worthwhile, as neither its physiological characters (fungicide

resistance and appearance during pathogenesis) nor the contribution of these isolates to the population structure of the pathogen have been examined so far.

ACKNOWLEDGMENTS

This work was supported by grants from the Ministry of Agriculture and Rural Development (33013/2003 and 46024/2004) and the National Office for Research and Technology (NKTH; A2-2006-0017). L. Karaffa was supported by the OTKA (Hungarian Scientific Research Fund; F042602 and K67667) and a Bolyai János Research Scholarship (BO/00107/06). E. Fekete and E. Sándor are recipients of an OTKA postdoctoral scholarship (D048617) and a Bolyai János Research Scholarship (BO/00446/04), respectively.

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TABLE 3. F_{ST} values for microsatellite markers between different geographic populations in the Eger wine region, Hungary

Location	II	III	IV	V
I	0.018	0.029	0.025	0.039
II		0.038	0.058	0.043
III			0.038	0.024
IV				0.077

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