Two Sibling Species of the *Botrytis cinerea* Complex, *transposa* and *vacuma*, Are Found in Sympatry on Numerous Host Plants

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ABSTRACT

Giraud, T., Fortini, D., Levis, C., Lamarque, C., Leroux, P., LoBuglio, K., and Brygoo, Y. 1999. Two sibling species of the *Botrytis cinerea* complex, *transposa* and *vacuma*, are found in sympatry on numerous host plants. *Phytopathology* 89:967-973.

Strains of *Botrytis cinerea* (the anamorph of *Botryotinia fuckeliana*) were collected from 21 different plant species around vineyards in the Champagne region (France). Strains were analyzed using three new polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) markers that were found by SWAPP (sequencing with arbitrary primer pairs), in addition to 15 other markers (PCR-RFLP, transposable elements, and resistance to fungicides). The markers revealed a high degree of genetic diversity and were used to investigate population structure. The two sym-

patric species *transposa* and *vacuma*, previously identified on grapes in these vineyards, were also detected on many of the plant species sampled. A new type of strain was also detected, having only the transposable element *Boty*. We did not detect any differentiation between strains from different organs or locations, but the prevalences of *transposa* and *vacuma* were significantly different on the different host plants. Fungicide resistance frequencies were significantly different in *transposa* and *vacuma* species. This study confirms that *B. cinerea* is a complex of sibling species and shows that the sibling species occur sympatrically on many host plants. However, the two species seemed to have different pathogenic behaviors. These findings contradict the traditional view of *B. cinerea* as a clonal population without specialization.

Botrytis cinerea, the anamorph of *Botryotinia fuckeliana*, attacks at least 235 plant species in temperate regions (19) and causes grey mold on many economically important crops including vegetables (tomato and cucumber), ornamentals (gerbera and rose), bulbs (onions), and fruits (strawberry and grape). It is also a saprophyte on senescent or already invaded plant materials. Because of the wide range of hosts and tissues that it attacks, and because any strain can infect all plants and organs under laboratory conditions, it had long been thought that *B. cinerea* was not specialized, unlike the other species of this genus. For example, *B. tulipae* is found only on tulips, *B. allii* on *Allium* spp., and *B. fabae* on Leguminosae. However, laboratory experiments may not reflect the natural conditions of infection. In a previous study on grapes in the Champagne region, we showed that *B. cinerea* consists of at least two sympatric sibling species, *vacuma* and *transposa* (10). Although both species were present on grapes, there was a dramatic decrease in the percentage of *vacuma* from June to October. We previously suggested that *transposa* may be a species more specific to grapes, whereas *vacuma* may be a migrant species from other hosts. The species *transposa* was characterized by the presence of two transposable elements, *Boty* and *Flipper*, whereas strains of *vacuma* had neither (10). *B. cinerea* had been thought to be entirely clonal, but we also showed that it regularly reproduces sexually on grapes in Champagne (10,12).

An understanding of the mode of reproduction and the specialization of phytopathogenic fungi in field conditions is essential for effective disease control. Population dynamics data could be helpful in deciding the best time to apply chemicals, and awareness of

the existence of differentiated populations may make it possible to find more efficient methods of control.

The existence of specialization would also be very interesting from a theoretical point of view. It may have played an important role in the speciation of the two sibling sympatric species in *B. cinerea*. There is still no consensus about whether species can arise sympatrically, but it is often argued that specialization on different resources could indeed lead to speciation (8,9,20,24). Sibling species (species indistinguishable on the basis of morphological characters) are often sympatric and specialized on different resources. They may, therefore, have appeared by sympatric speciation due to specialization (8,9,20,24). In fungi, there are many examples of sibling species (2), and most of them are phytopathogens, in which each sibling species uses different resources (hosts, organs, etc.). For example, *Puccinia graminis* (13,25,27), *Erysiphe graminis* (14,15), and *Fusarium oxysporum* (1) all display numerous formae speciales.

To determine whether the two sympatric sibling species *transposa* and *vacuma* are specialized, we collected strains from 21 different plants from around grapevines in Champagne. The samples were taken from the same locations as in a previous study on grapes (10). The strains were analyzed using the markers described by Giraud et al. (10): (i) presence of the transposable elements *Boty* and *Flipper*; (ii) sensitivity to fungicides (carbendazim, diethofencarb, and vinclozolin, as in the previous study, plus an experimental botryticide, fenhexamid); (iii) polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) markers; and (iv) length of the minisatellite MSB1 (first minisatellite of *Botrytis*) (11). In addition, we used the SWAPP technique (sequencing with arbitrary primer pairs) (4) to find new PCR-RFLP markers. The objectives of this study were to determine (i) whether the genetic diversity of the complex *B. cinerea* was as great as that on grapes; (ii) whether both *transposa* and *vacuma* species were present on other plant species; and (iii) whether the population was structured by the host, location, or organ from which it was collected.

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MATERIALS AND METHODS

Strains and sampling. A total of 107 field strains of *B. cinerea* was collected in June 1997 from various plants growing near vineyards in the Champagne region (France). The same three locations (Boursault, Plumecoq, and Trepail) were sampled as in the study on grape strains (10). The strains are listed in Table 1, showing the location, the organ, and the host plant from which each was collected. The strains were not collected inside vine-

yards but from a 10-m ring around the vineyard, except in the case of strains from green peas, which were sampled in fields of peas located 100 m from the vineyards. Some strains were obtained from disease lesions on living plants and others by isolation from asymptomatic tissues. The strains are typed as S and P, respectively, in Table 1.

The isolates collected on plants were purified by monospore isolation and, therefore, can be referred to as strains. They were preserved in paraffin oil at 16°C. The fungi were cultured on

TABLE 1. Haplotypes of *Botrytis cinerea* strains and the location, organ, and host plant from which strains were collected^o

Isolate	Op	F ^d	B ^r	76 ^s											Nitrate reductase ^s		Fungicides			Host ^y	Organ ^z	Location	
				1 ^s			ADP ^s		IGS ^s						RsaI	V ^t	Ben ^u						
				HaeIII	HincII	MSB1 ^s	EcoRI	AvaI	RsaI	BamHI	HindIII	HaeIII	Hin6I	200 ^v			198 ^w	F ^x					
1148	S	1	1	0	0	1	0	1	1	2	1	1	1	2	1	0	1	1	0	<i>C. arvensis</i>	Bindwind	L	Boursault
1177	S	1	1	0	0		0	1	1	2	1	1	1	2	1	0	1	1	0	<i>C. arvensis</i>	Bindwind	L	Boursault
1178	S	1	1				0	1	1	2	1	1	1	2	1	0	1	1	0	<i>C. arvensis</i>	Bindwind	L	Boursault
1204	P	1	1	0	0	1	0	1	1	1	0	1	1	2	1	0	1	1	0	<i>C. arvensis</i>	Bindwind	F	Boursault
1149	S	1	1	0	0	1	0	1	1	3	0	1	5	2	1	0	1	1	0	<i>Rumex acetosella</i>	Sheep sorrel	L	Boursault
1179	S	0	0	0	0	1	0	1	1	1	1	1	5	2	0	0	1	0	0	<i>Rumex acetosella</i>	Sheep sorrel	L	Boursault
1185	S	1	1	0	0	1	0	1	1	3	0	1	1	2	0	0	1	1	0	<i>Rumex acetosella</i>	Sheep sorrel	L	Boursault
1152	S	1	1	0			0	1	1	1	1	1	1	2	1	0	1	1	0	<i>Anagallis arvensis</i>	Scarlet pimpernel	F	Boursault
1153	S	1	1	0			0	1	1	1	1	1	1	2	1	0	1	1	0	<i>Anagallis arvensis</i>	Scarlet pimpernel	F	Boursault
1156	S	1	1	0			2	1	1	1	1	1	1	2	1	0	1	1	0	<i>Fraxinus</i> sp.	Ash	L	Boursault
1157	S	1	1	0			2	1	1	1	1	1	1	2	1	0	1	1	0	<i>Fraxinus</i> sp.	Ash	L	Boursault
1225	S	1	1				1		3		0	0	9	6		0	1			<i>Fraxinus</i> sp.	Ash	L	Boursault
1158	P	1	1	1			0	1	1	1	0	1	1	2	1	0	0	0	0	<i>G. robertianum</i>	Herb Robert	F	Boursault
1159	P	1	1	0	0	1	0	1	1	1	0	1	1	2	1	0	0	0	0	<i>G. robertianum</i>	Herb Robert	F	Boursault
1160	P	1	1	0	0	1	0	1	1	1	1	1	1	2	1	1	1	1	0	<i>G. robertianum</i>	Herb Robert	F	Boursault
1161	P	1	1		0	1	2	1	1	1	1	1	1	2	1	0	1	1	0	<i>G. robertianum</i>	Herb Robert	F	Boursault
1162	P	1	1	0	0	1	0	1	1	1	1	1	1	2	1	1	1	1	0	<i>G. robertianum</i>	Herb Robert	F	Boursault
1163	P	1	1	0			0	1	1	1	0	1	1	2	0	0	0	0	0	<i>G. robertianum</i>	Herb Robert	F	Boursault
1182	P	1	1	0	0		0	1	1	1	1	1	1	2	1	0	1	1	0	<i>G. robertianum</i>	Herb Robert	F	Boursault
1183	P	1	1	0	0	1	1	1	1	1	1	1	0	2	1	1	1	1	0	<i>G. robertianum</i>	Herb Robert	F	Boursault
1164	P	0	0	1	0		1	1	0	1	1	1	3	2	0	0	1			<i>S. officinale</i>	Common comphrey	F	Boursault
1165	P	0	0	1			1	1	0	1	1	1	3	2	0	0	1			<i>S. officinale</i>	Common comphrey	F	Boursault
1184	P	0	1	0	0	1	4	1	1	1	1	1	1	2	0	0	1	0	0	<i>S. officinale</i>	Common comphrey	F	Boursault
1166	S	0	0				0	1	0	1	1	1	3	2	0	0	1		0	<i>P. acerifolia</i>	Plane tree	L	Boursault
1167	S	0	0	1	0	0	0	1	0	1	1	1	3	2	0	0	1			<i>P. acerifolia</i>	Plane tree	L	Boursault
1226	S	1	1	0			2	1	1	1	1	1	0	2		0	1			<i>P. acerifolia</i>	Plane tree	L	Boursault
1150	P	0	0				0	0	0	0	1	1	5	0	0	0	1	1	0	<i>Rubus</i> sp.	Bramble	P	Boursault
1151	P	0	0	1			0	0	0	0	1	1	5	0	0	0	1	1	0	<i>Rubus</i> sp.	Bramble	F	Boursault
1168	P	0	0	0	1	1	5	0	1	0	1	1	2	2	0	0	1		0	<i>Rubus</i> sp.	Bramble	F	Boursault
1169	P	0	0		2	2	1	1	0	1	1	1	1	2	0	0	1	1	1	<i>Rubus</i> sp.	Bramble	F	Boursault
1170	P	1	1	0	0	1	0	1	1	1	1	1	1	2	1	0	1	1	0	<i>Rubus</i> sp.	Bramble	F	Boursault
1171	P	0	0	1			2	1	0	0	1	1	6	0	1	0	1	0	0	<i>Rubus</i> sp.	Bramble	F	Boursault
1172	P	0	1	0			0	1	1	1	0	1	1	2	0	0	1	1	0	<i>Rubus</i> sp.	Bramble	F	Boursault
1173	P	0	1	0			0	1	1	1	0	1	1	2	0	0	1	1	0	<i>Rubus</i> sp.	Bramble	F	Boursault
1174	P	0	0	0	0	1	1	1	1	1	0	0	0	0	0	0	1	0	0	<i>Rubus</i> sp.	Bramble	F	Boursault
1186	P	0	0	0	0	1	0	0	0	0	1	1	1	0	0	0	1	1	0	<i>Rubus</i> sp.	Bramble	F	Boursault
1207	S	1	1	0	0	1	0	1	1	1	1	1	1	2	1	0	1	1	0	<i>Rubus</i> sp.	Bramble	L	Boursault
1208	S	1	1	0	0	1	0	1	1	1	1	1	1	2	1	0	1	1	0	<i>Rubus</i> sp.	Bramble	L	Boursault
1209	S	1	1	0	0	1	0	1	1	1	1	1	1	2	1	0	1	1	0	<i>Rubus</i> sp.	Bramble	L	Boursault
1211	P	1	1	0	0	1	0	1	1	1	1	1	1	2	1	0	1	1	0	<i>Rubus</i> sp.	Bramble	P	Boursault
1212	S	1	1	0	0	1	0	1	0	1	1	1	1	2	1	1	1	1	0	<i>Rubus</i> sp.	Bramble	L	Boursault
1213	P	1	1	0	0	1	0	1	1	1	1	1	1	2	1	0	1	1	0	<i>Rubus</i> sp.	Bramble	ST	Boursault
1214	P	1	1	0	0	1	0	1	1	1	0	1	1	2	1	0	1	1	0	<i>Rubus</i> sp.	Bramble	ST	Boursault
1175	S	1	1	0	0	1	0	1	1	1	1	1	5	2	0	1	1	1	0	<i>Tilia</i> sp.	Lime tree	L	Boursault
1176	S	1	1	0	0		4	1	1	1	1	0	1	2	1	0	1	1	0	<i>Tilia</i> sp.	Lime tree	L	Boursault
1188	S	1	1	0	0	1	0	1	1	1	1	1	1	2	1	0	1	1	0	<i>Tilia</i> sp.	Lime tree	L	Boursault

(continued on the next page)

^o Blanks within the table indicate that the data was not determined.

^p O = Obtained. S indicates that the strain was obtained from disease lesions on living plants, and P indicates that the strain was obtained from asymptomatic tissues.

^r F = *Flipper*. The absence of *Flipper* is coded by 0 and its presence by 1.

^t B = *Boty*. The absence of *Boty* is coded by 0 and its presence by 1.

^s Each allele of polymerase chain reaction-restriction fragment length polymorphism markers is coded by a different number.

^t V = Vinclozolin. The sensitivity to vinclozolin (ImiS) is coded by 0 and the resistance (ImiR) by 1.

^u Between the columns 200 and 198, the phenotype BenR2 (resistant to carbendazim and diethofencarb) is coded by 0-0, BenR1 (resistant to carbendazim and sensitive to diethofencarb) by 1-1, and BenS (sensitive to carbendazim and resistant to diethofencarb) by 1-0.

^w 200 = Codon 200. At the codon 200 of the β -tubulin, 1 is for the presence of a phenylalanine and 0 for the presence of tyrosine.

^w 198 = Codon 198. At the codon 198 of the β -tubulin, 1 is for the presence of an alanine and 0 for the presence of glutamic acid.

^x F = Fenhexamid. The sensitivity to fenhexamid (HydS) is coded by 0 and the resistance (HydR) by 1.

^y Hosts genuses include *Convolvulus* (C.), *Rumex*, *Anagallis*, *Fraxinus*, *Geranium* (G.), *Symphytum* (S.), *Platanus* (P.), *Rubus*, *Tilia*, *Fragaria*, *Conus*, *Coronilla*, *Sonchus*, *Corylus*, *Sambucus*, *Trifolium*, *Pisum*, *Brassica*, *Rosa*, *Epilobium*, and *Reseda*.

^z L = Leaf, F = flower, P = petal, ST = stamen, FR = fruit, PO = pod, and S = sepal.

plates containing 20 ml of nutrient yeast medium (20 g of malt per liter, 20 g of agar per liter, and 2 g of yeast extract per liter) at 25°C.

Fungicide sensitivity assays. Conidia were collected in sterile water from 2-week-old agar slant cultures. The resulting spore suspension (2×10^5 conidia per ml) was used to inoculate a glucose agar medium containing fungicides (17). Fungicides from four chemical groups were tested: benzimidazoles (carbendazim), phenylcarbamates (diethofencarb), dicarboximides (vinclozolin), and hydroxyanilides (fenhexamid). The discriminatory concentrations were 1 and

10 mg/liter for carbendazim, 10 mg/liter for diethofencarb, 5 mg/liter for vinclozolin, and 0.4 mg/liter for fenhexamid (Table 2). The response of the various strains was recorded after incubation for 1 or 2 days.

DNA extraction and molecular markers. The mycelium was cultured as described by Diolez et al. (6). Genomic DNA was isolated by the method of Möller et al. (21). The primers, the amplification, and the digestion conditions used for the PCR-RFLP markers were the same as those described by Giraud et al. (10). The length

TABLE 1. (continued from the preceding page)

Isolate	Op	F ^q	B ^r	76 ^s		1 ^s		ADP ^s		IGS ^s						Nitrate reductase ^s		Fungicides			Host ^y	Organ ^z	Location
				RsaI	HaeIII	HincII	MSB1 ^s	EcoRI	AvaI	RsaI	BamHI	HindIII	HaeIII	Hin6I	RsaI	V ^t	Ben ^h						
																	200 ^v	198 ^w	F ^x				
1189	P	0	0	0	2	2	1	0	0	1	1	1	3	2	0	0	1	1	1	<i>Tilia</i> sp.	Lime tree	F	Boursault
1190	P	0	0						0	4	2	0	1	4						<i>Tilia</i> sp.	Lime tree	F	Boursault
1180	S	0	0						2	2	0	0	7	3						<i>Fragaria</i> sp.	Strawberry	FR	Boursault
1192	P	1	1	0	0		1	1	1	1	1	1	1	2	0	0	1	0	0	<i>Conus mas</i>	Cornel tree	F	Boursault
1193	P	1	1	0			1	1	1	1	1	1	1	2	1	0	1	0	0	<i>Conus mas</i>	Cornel tree	F	Boursault
1194	P	1	1	0	2	2	0	1	1	1	1	1	1	2	0	1	1	1	0	<i>Conus mas</i>	Cornel tree	F	Boursault
1195	P	1	1				1	1	1	1	1	1	1	2	1	1	1	1	0	<i>Conus mas</i>	Cornel tree	F	Boursault
1197	P	1	1	0			1	1	1	1	0	1	1	2	1	0	1	1	0	<i>Coronilla coronata</i>	Coronilla	F	Boursault
1198		0	0				1	1	1	1	1	1		2		0	1	1	1	<i>Sonchus arvensis</i>	Milkweed	F	Boursault
1199		0	0	1			1	1	0	1	1	1	3	2	0	0	1	1	1	<i>Sonchus arvensis</i>	Milkweed	F	Boursault
1200		1	1	0	0	1	2	1	1	1	1	1	0	2	0	0	1	1	0	<i>Sonchus arvensis</i>	Milkweed	F	Boursault
1202		1	1	0	0	1	2	1	1	1	1	1	0	2	0	0	1	1	0	<i>Sonchus arvensis</i>	Milkweed	F	Boursault
1203		1	1	0	0	1	2	1	1	1	1	1	0	2	1	0	1	1	0	<i>Sonchus arvensis</i>	Milkweed	F	Boursault
1205	S	1	1	0	0	1	2	1	1	1	1	1	1	2	1	0	1	1	0	<i>Corylus avellana</i>	Bazel tree	F	Boursault
1206	S	1	1				2	1	1	1	1	1	1	2	1	0	1	1	0	<i>Corylus avellana</i>	Bazel tree	F	Boursault
1215	S	0	0	0	1		0	0	0	0	1	1	3	0	1	0	1	1	0	<i>Sambucus nigra</i>	Common elder	L	Boursault
1217	S	1	1	0	0		0	1	1	1	1	1	0	2	0	0	1	1	0	<i>Sambucus nigra</i>	Common elder	L	Boursault
1218	S	1	1	0	0		0	1	1	1	1	1	1	2	1	0	1	1	0	<i>Sambucus nigra</i>	Common elder	L	Boursault
1219	S	1	1				0	1	1	1	1	1	1	2	1	0	1	1	0	<i>Sambucus nigra</i>	Common elder	L	Boursault
1220	S	1	1	0	1		0	1	1	1	1	1	1	2	0	0	1	1	0	<i>Sambucus nigra</i>	Common elder	L	Boursault
1221	P	0	0	1	2	2	0	1	0	1	1	1	3	2	0	0	1	1	1	<i>Trifolium repens</i>	White clover	F	Boursault
1222	P	0	0	1	0		1	1	0	1	1	1	3	2	0	0	1	1	1	<i>Trifolium repens</i>	White clover	F	Boursault
1223	P	0	0	1	2	2	1	1	0	1	1	1	6	2	0	0	1			<i>Trifolium repens</i>	White clover	F	Boursault
1272	P	1	1	0			0		1	1	0	1	1	2		0	1	1	0	<i>Trifolium repens</i>	White clover	L	Plumecoq
1229	P	0	0	1			1	1			0				1	0	1	1	1	<i>Pisum sativum</i>	Green pea	F	Trépail
1230	P	0	0	1	2	2	1	1	0	1	0	1	3	2		0	1			<i>Pisum sativum</i>	Green pea	F	Trépail
1231	P	0	0				1	1	0	1	1	1	3	2	0	0	1			<i>Pisum sativum</i>	Green pea	F	Trépail
1232	P	1	1	0	0	1	2	1	1	1	1	1	0	2		1	1	1	0	<i>Pisum sativum</i>	Green pea	F	Trépail
1233	P	0	1	0	0	1	1	1	1	1	0	1		2		0	1	1	0	<i>Pisum sativum</i>	Green pea	F	Trépail
1258	P	0	0	1			0	1	0	1	1	1			0	0	1	1	1	<i>Pisum sativum</i>	Green pea	F	Plumecoq
1259	P	0	0	0			0	0	1	0	1	1	5	1	1	0	1	1	0	<i>Pisum sativum</i>	Green pea	F	Plumecoq
1260	P	0	0	0	0	1	0	0	1	0	1	1	5	1	0	0	1	1	0	<i>Pisum sativum</i>	Green pea	F	Plumecoq
1261	P	0	0	0	0	1	0	0	1	0	1	1	5	2	0	0	1	1	0	<i>Pisum sativum</i>	Green pea	F	Plumecoq
1262	P	0	0	1	1	1	1	1	0	1	1	1		2	0	0	1	1	1	<i>Pisum sativum</i>	Green pea	PO	Plumecoq
1263	P	0	0	1			1	1	0	1	1	1		2	0	0	1	1	1	<i>Pisum sativum</i>	Green pea	PO	Plumecoq
1264	P	0	0	1			1	1				0			0	0	1	1	1	<i>Pisum sativum</i>	Green pea	PO	Plumecoq
1268	P	0	0	1			1	1			0			2	0	0	1	1	1	<i>Pisum sativum</i>	Green pea	PO	Plumecoq
1236	P	0	0	1	0	1	0	1	1	1	1	1	1	2	0	0	1	0	0	<i>Brassica napus</i>	Rapeseed	F	Trépail
1237	P	0	0	0	0	1	0	1	1	1	1	1	1	2	0	0	1	0	0	<i>Brassica napus</i>	Rapeseed	F	Trépail
1238	P	0	0	0	0	1	0	1	1	1	0	1	1	2	0	0	1	0	0	<i>Brassica napus</i>	Rapeseed	FR	Trépail
1239	P	0	0	0	0	1	0	1	1	1	0	1	1	2		0	1	0	0	<i>Brassica napus</i>	Rapeseed	FR	Trépail
1240	S	1	1	1	0		0	1	0	1	0	1	3	2	0	0	1	1	1	<i>Rosa canina</i>	Wild rose	P	Plumecoq
1241	S	1	1	0	0	1	2	1	0	0	1	1	3		0	0	1	1	0	<i>Rosa canina</i>	Wild rose	S	Plumecoq
1242	S	0	0	1	2	2	1	1	0	1	1	1	3	2	0	0	1	0	1	<i>Rosa canina</i>	Wild rose	S	Plumecoq
1243	S	0	0	1	2	2	1	1	0	1	0	1		2	0	0	1	0	1	<i>Rosa canina</i>	Wild rose	S	Plumecoq
1244	S	0	0	1			1	1	0	1	0	1		2	0	0	1	0	1	<i>Rosa canina</i>	Wild rose	S	Plumecoq
1245	S	0	0	1	2	2	1	1	0	1		1	3		1	0	1	0	1	<i>Rosa canina</i>	Wild rose	S	Plumecoq
1246	S	0	0	1	2	2	1	1	0		0	1		2	0	0	1	0	1	<i>Rosa canina</i>	Wild rose	S	Plumecoq
1247	S	0	0	0			1	1	0	1	1	1	3	2	0	0	1	0	1	<i>Rosa canina</i>	Wild rose	S	Plumecoq
1248	S	0	0	0			1	1	0		1	1		2	0	0	1	0	1	<i>Rosa canina</i>	Wild rose	S	Plumecoq
1249	S	1	1				4		2	4	1	0	8	5		0	1	0		<i>Rosa canina</i>	Wild rose	S	Plumecoq
1250	S	0	1				4		2	4	1	0	8	5		0	1	0		<i>Rosa canina</i>	Wild rose	L	Plumecoq
1251	S	0	1		1	1	4	1	2	4	1	0	8	5		0	1	0		<i>Rosa canina</i>	Wild rose	L	Plumecoq
1252	S	0	0	1			1	1	0	1	1	1		2	0	0	1	1	1	<i>Rosa canina</i>	Wild rose	L	Plumecoq
1253	P	0	0	0			2		0	1	1	1			0	0	1	0	0	<i>Rosa canina</i>	Wild rose	ST	Plumecoq
1254	P	1	1	0	0	1	4	1	1	1	1	1	1	2	1	1	1	1	0	<i>Rosa canina</i>	Wild rose	ST	Plumecoq
1256	S	0	0	1			1	1	0	1	1	1	3	2	0	0	1	1	1	<i>Epilobium</i> sp.	Willow herb	L	Plumecoq
1257	S	0	0	0	0	1	0	0	0							0	1	1	0	<i>Epilobium</i> sp.	Willow herb	L	Plumecoq
1269	P	0	1	0			1							2		0	1	0	0	<i>Reseda lutea</i>	Yellow weed	F	Plumecoq
1270	P	0	1	0	0	1	5	1	1	1	0	1	1	2		0	1	0	0	<i>Reseda lutea</i>	Yellow weed	F	Plumecoq
1271	P	1	1	0	0	1	0	1	1	1	0	0	3	2	1	0	1	0	0	<i>Reseda lutea</i>	Yellow weed	F	Plumecoq

of the minisatellite MSB1 was scored by PCR as described by Giraud et al. (11). The DNA fragments were then separated by electrophoresis in 1.5% agarose gels and in 1× Tris-borate-EDTA buffer.

Dot blots were performed as described by Giraud et al. (10) to detect the transposable elements *Boty* and *Flipper*. The presence or absence of *Flipper* was checked by PCR for some strains. The primers used were F300 (5'GCACAAAACCTACAGAAGA3') and F1500 (5'ATTCGTTTCTTGACTGTA3') (18). *Boty* is a 6-kilo-base gypsy-like retrotransposon (accession numbers X81790 and X81791) (6). *Flipper* is a 1,842-base pair (bp) class II element (accession number U74294) (18). Both were isolated from *B. cinerea*.

For the SWAPP technique, random amplification of polymorphic DNA (RAPD), and single-strand conformational polymorphism (SSCP) were done as described by Burt et al. (5). For sequencing, PCR products were purified using the Jetsorb gel extraction kit (Bioprobe Systems, Montreuil-sous-Bois, France) and sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase (Perkin-Elmer, Branchburg, NJ) and the ABI PRISM 310 Genetic Analyzer (Perkin-Elmer).

Data analysis. Allele frequencies and genetic differentiation were calculated using the GENEPOP program (23). The null hypothesis tested for genetic differentiation was H_0 (the allelic distribution is independent across populations). GENEPOP performs Fisher's exact tests using a Markov chain. The gene diversity (H_s) at each locus was calculated by the Nei's method (22): $H_s = 1 - \sum p_i^2$, in which p_i is the frequency of the i th allele. The mean gene diversity was the mean of H_s over loci.

RESULTS

SWAPP. The principle of the method is to amplify random segments of DNA using RAPDs with pairs of primers and then to select bands that are in all samples of interest but do not appear when either primer is used alone. These bands are then scored by SSCP, a technique that detects differences in conformation of single strands of DNA by migration in a denaturing gel. Polymorphic bands are then sequenced. We tried 31 primers in 42 arbitrary pairs

on 10 strains. Six primer pairs revealed eight polymorphic bands by SSCP (Table 3) that were then sequenced. None of the sequences showed a similarity with sequences present in GenBank. The sequences revealed polymorphic restriction sites (Table 3), and three enzymes were used in extensive population studies: *HaeII*, *HincII*, and *RsaI*. The enzymes *HaeII* and *HincII* had polymorphic restriction sites in the band amplified by the pair of primers no. 1 (primers ITS4 and ITS5) (Table 4), and *RsaI* had polymorphic restriction sites in the band amplified by the pair of primers no. 76 (primers L76-5' and L76-3'). We designed specific primers for these two bands (76U and 76L, 1BU and 1BL) (Table 4) and screened all the strains for polymorphism by digesting band 1 with *RsaI* and band 76 with *HaeII* and *HincII*. With marker 76, allele 0 (not cut by *RsaI*) was found in 71% of the strains and allele 1 (cut by *RsaI*) in 28.6%. With marker 1, a larger band (830 bp) was always coamplified with the 500-bp band. The 830-bp band exhibited a polymorphic amplification length: 15.4% of the strains displayed a higher and fainter band at 950 bp (Fig. 1, alleles 2). This 950-bp band is presumably homologous to the 830-bp band, since they never occurred together. After *HaeIII* digestion, allele 0 (not cut) was found in 76.9% of the strains and allele 1 (cut) in 7.7%. After *HincII* digestion, allele 0 (not cut) was found in 1.8% of the strains and allele 1 (cut) in 79.6%. The alleles are shown in Figure 1. These are good markers for population studies, since no allele is ever too prevalent.

Population analysis. The 107 strains analyzed are listed in Table 1. They were collected from 21 different plants around grapevines in Champagne, from the same locations as in the study on strains from grapes (10), and they were scored for 18 markers. Strains were scored for MSB1 length polymorphism (11) and for 11 PCR-RFLP markers by amplifying two coding sequences (nitrate reductase and ADP/ATP translocase), one noncoding repeated sequence (non-transcribed ribosomal intergenic spacer [IGS]), and the two anonymous SWAPP sequences (1 and 76). The IGS was digested by six enzymes (*AvaI*, *RsaI*, *BamHI*, *HindIII*, *HaeIII*, and *Hin6I*), the ADP/ATP translocase was digested by *EcoRI*, and the nitrate reductase was digested by *RsaI*. Restriction patterns were described by Giraud et al. (10). The band 1 obtained by SWAPP was digested by *HaeIII* and *HincII*, and the band 76 was digested by *RsaI*. Restriction patterns are shown in Figure 1. Different digestions of the IGS are considered to be different markers, because recombination can occur between these sites (10). The 107 strains were then scored for the presence of two transposable elements (*Boty* and *Flipper*) by dot blots.

The strains were also tested for their resistance to four fungicides (carbendazim, diethofencarb, vinclozolin, and fenhexamid). Several phenotypes were identified on the basis of in vitro effects of these fungicides on the various strains (Table 2). Three phenotypes were distinguished by their response to antimicrotubule fungicides: BenS (sensitive to carbendazim and resistant to diethofencarb), BenR1 (resistant to carbendazim and sensitive to diethofencarb), and BenR2 (resistant to both carbendazim and diethofencarb). Carbendazim-resistant strains differ from sensitive ones by two pairs of mutations in the beta-tubulin gene (28). Alanine replaces glu-

TABLE 2. *Botrytis cinerea* strains classified according to response of phenotypes to different fungicides

Phenotypes	Carbendazim		Diethofencarb	Vinclozolin	Fenhexamid
	1 ^x	10	10	5	
Wild types ^y	— ^z	—	+	—	—
BenR1	+	+	—	—	—
BenR2	+	—	+	—	—
ImiR	—	—	+	+	—
HydR	—	—	+	—	+

^x Fungicide concentrations are given in mg/liter.

^y Wild types include BenS, ImiS, and HydS.

^z — = Absence of germ tubes or presence of short germ tubes. + = Presence of long germ tubes.

TABLE 3. Polymorphism detected by SWAPP (sequencing with arbitrary primer pairs)^z

Primers	Band	Strains with polymorphic band by SSCP	No. of polymorphic base pairs detected by sequencing	No. of polymorphic restriction sites determined by sequencing	Enzymes
ITS4/ITS5		349, 111, 1081, 187	8	5	<i>MnII</i> , <i>MseI</i> , <i>HincII</i> , <i>HaeIII</i> , <i>NlaIII</i>
ITS4/NS24		111, 1081, 1069	2	1	<i>MboII</i>
TW14/Trpc1	A	169, 158	1	0	
TW14/Trpc1	B	169, 158	0	0	
MS2/NS4		158, 846	2	1	<i>FokI</i>
NS7/ML3.5	A	1069, 187	2	1	<i>ItaI</i>
NS7/ML3.5	B	1069, 187	0	0	
L76-5'/L76-3'		1069, 187	5	3	<i>RsaI</i> , <i>FokI</i> , <i>NsiI</i>

^z Primers that gave one or two polymorphic bands by single-strand conformational polymorphism (SSCP), polymorphism detected by sequencing these bands, and polymorphism of restriction.

tamic acid (codon 198) in BenR1, whereas tyrosine replaces phenylalanine (codon 200) in BenR2. The field strains were either sensitive (ImiS) or moderately resistant (ImiR) to vinclozolin. This resistance is encoded by a single gene (7), the function of which remains to be investigated. The in vitro tests conducted with the experimental hydroxylanilide botryticide, fenhexamid, distinguished sensitive (HydS) and resistant (HydR) strains. The genetic basis of this resistance is not known, but according to Suty et al. (26), it seems to be related to increased detoxification of this hydroxylanilide. All the fenhexamid-resistant strains, but none of the sensitive ones, exhibited increased sensitivity to fenpropimorph, as did the strains collected from grapes (16).

Population structure. The haplotypes of the 107 strains are shown in Table 1. We found, as in our previous study (10), that almost all strains either had both transposable elements or neither of them. That means that the two distinct species of *B. cinerea*, *transposa* and *vacuma*, are also found on hosts other than grapes. The percentage of *vacuma* is 45%, which is the same as that found in the strains collected from grapes in June 1994 and June 1995 (10). The sibling species *transposa* and *vacuma* were found to be sympatric on 11 out of the 16 plants from which more than two strains were collected. However, we also detected eight strains that had only the transposable element *Boty*. Such strains had not been detected before in France, but some were found in Chile (K. LoBuglio, unpublished data).

TABLE 4. Sequences of primers used for SWAPP (sequencing with arbitrary primer pairs)

Primer	Sequence (5'–3')
NS4	CTTCGTCGAATTCCTTTAAG
NS7	GAGGCAATAACAGGTCTGTGATGC
NS24	AAACCTTGTTACGACTTTTA
MS2	GCGGATTATCGAATTAAATAAC
L76-5'	CTGAGAAACCACTGCGTAG
L76-3'	CCATGAACAAATATGGC
ITS4	TCCTCCGCTTATTGATATGC
ITS5	GGAAGTAAAAGTCGGAACAAGG
TW14	GCTATCCTGAGGGAACTTC
ML3.5	GAGAATGCTGACATGAGTAAC
Trpc1	GTGCCAGTGTCTCTGTGCT
76U	AATTGAATTTGTGTGGTC
76L	ATTGCTATCCTGAGGGAAAC
1BU	ACAAAAGGGAGGGAGGTAT
1BL	TCGAGGGAAAGAAAGAAAGG

Fig. 1. Restriction patterns of the alleles of the three polymerase chain reaction-restriction fragment length polymorphism markers obtained for *Botrytis cinerea* by SWAPP (sequencing with arbitrary primer pairs).

To determine whether the population was structured, we subdivided it on the basis of organ, location, and host plant, and calculated the allele frequencies for each marker in each subgroup. We then performed a statistical test to see whether allele frequencies were significantly different in the subgroups. The plants from which less than four strains were collected were excluded from this analysis. The differences were considered significant if the probability (*P*) that they occurred by chance was less than 5%. There were no significant differences between the groups from different locations or between the groups from different organs. However, significant differences in allele frequencies were found for 11 out of the 18 markers among strains collected from different host plants (Table 5). Some plant species carried only *transposa* or only *vacuma*, and 11 plants carried both *transposa* and *vacuma*. But the frequencies of strains having *Boty* and *Flipper* were significantly different between the different plant hosts (Table 5). Thus, even though *transposa* and *vacuma* were sometimes found together on some plant species, their relative prevalences were significantly different. For example, *vacuma* is prevalent on green peas, whereas *transposa* is prevalent on bramble and herb Robert (Table 6). As *transposa* and *vacuma* have different

TABLE 5. Test of allelic differences among the strains of *Botrytis cinerea* collected from different plant hosts

Locus	<i>P</i> ^z
<i>Flipper</i>	0.00004*
<i>Boty</i>	0.00004*
76 <i>RsaI</i>	0.03354*
1 <i>HaeIII</i>	0.35576
1 <i>HincII</i>	0.01452*
ATP synthase	<0.000001*
ADP-ATP translocase/ <i>EcoRI</i>	0.60276
IGS/ <i>AvaI</i>	<0.000001*
IGS/ <i>RsaI</i>	0.002306*
IGS/ <i>BamHI</i>	0.31006
IGS/ <i>HindIII</i>	0.26588
IGS/ <i>HaeIII</i>	<0.000001*
IGS/ <i>Hin6I</i>	0.11486
Nitrate reductase/ <i>RsaI</i>	0.00040*
Vinclozolin	0.51474
β-Tubulin codon 200	0.10760
β-Tubulin codon 198	<0.000001*
Fenpropimorph	0.00072*

^z The *P* value indicates the probability that the difference in allelic frequency between the populations from the different plant hosts occurred only by chance. * = Probability is significant.

TABLE 6. Number of strains of *Botrytis cinerea* collected from each different plant species and percentage of strains that belong to the species *transposa*, considering only *transposa* and *vacuma* strains

Plant	No. of strains	Percentage <i>transposa</i>
Bindwind	4	100
Sheep sorrel	3	33
Scarlet pimpernel	2	100
Ash	3	100
Herb Robert	8	100
Common comphrey	3	33
Plane tree	3	33
Bramble	17	59
Lime tree	5	60
Strawberry	1	0
Cornel tree	4	100
Coronilla	1	100
Milkweed	5	60
Bazel tree	2	100
Common elder	5	80
White clover	4	25
Green peas	13	23
Rapeseed	4	0
Wild rose	15	40
Willow herb	2	0
Yellow weed	3	66

allele frequencies (10), the fact that the alleles of the markers other than *Boty* and *Flipper* have different frequencies on different plants could be attributable to different prevalences of *transposa* and *vacuma* on these plants.

Both *transposa* and *vacuma* strains were found on disease lesions on living plants and on asymptomatic tissues (Table 1). But *vacuma* strains were more often on asymptomatic tissues than on disease lesions, whereas *transposa* strains were more often on disease lesions than on asymptomatic tissues. A genetic differentiation test showed that these differences were significant ($P = 0.01$).

Genetic diversity. Another important finding is the high degree of genetic diversity. The 18 markers distinguished 74 different haplotypes in the 107 strains, the most common being found in only 8% of the strains. Several alleles were detected that had not been detected in strains from grapes, and several alleles from the grape populations were not present in these strains. Nei's mean gene diversity index, which measures the number of alleles and their evenness, was $H_s = 0.24$ for *transposa* and $H_s = 0.32$ for *vacuma*. These values are close to those calculated for the grape population for each species ($H_s = 0.32$ for *vacuma* and $H_s = 0.23$ for *transposa*) (10). As in the previous study (10), *vacuma* exhibited greater diversity than did *transposa*.

Fig. 2. Frequencies of resistance to various fungicides in strains of the two sibling species of *Botrytis cinerea*, *vacuma* and *transposa*, collected on 21 different plants near grapes in the Champagne region (France).

Since the number of strains collected from each plant was not sufficient to test for gametic disequilibria, we used the genotypic diversity $G_{\max}$ ($100 \times \text{number of haplotypes/number of strains}$) as an indicator of the occurrence of recombination (3). Genotypic diversity values were 55% for *transposa* and 57% for *vacuma*. These values are higher than those in asexual populations (3) and are close to those calculated for strains collected from grapes in Champagne (56% for *transposa* and 70% for *vacuma*).

Fungicide resistances. Figure 2 shows the percentages of fungicide resistance. As observed previously for the strains collected from grapes in June 1994 and June 1995 (10), (i) the BenR1 strains were predominant in both species; (ii) the BenR2 and ImiR phenotypes were present in *transposa*, but absent in *vacuma*; and (iii) only *vacuma* strains were resistant to fenhexamid.

DISCUSSION

The great genetic diversity detected in this study mirrors that found on grapes (10), in which the diversity was shown to result from both genetic recombination and population subdivision. We did not have enough individuals from each plant to test for gametic disequilibrium, but genetic diversity and genotypic diversity were as high as those on grapes. Gametic disequilibrium studies of the Champagne grape populations demonstrated that genetic recombination occurs, and our findings suggest that sexual reproduction also occurs frequently in this population.

This study shows that the two sibling species of *B. cinerea* identified on grapes also occur elsewhere. Therefore, *B. cinerea* is a complex of at least two species that are sympatric on many plants. However, we found few strains having only the transposable element *Boty*. Such strains had not been found before in France (10), but some have been identified in Chile (K. LoBuglio, unpublished data). More studies are needed to determine if this means that *Boty* is invading the species *vacuma* or if these strains belong to another population.

The genetic differentiation test shows that the frequencies of the markers *Flipper* and *Boty* are significantly different on different host plants. The species *transposa* and *vacuma* are differentiated by the presence of these transposable elements, such that, even if both occur on the same plants, their relative frequencies are significantly different. For example, *vacuma* is prevalent on green peas, as observed in 1994 (6), whereas *transposa* is prevalent on bramble and herb Robert (Table 6). This reinforces the idea that *transposa* and *vacuma* do not have the same ability to infect different hosts.

The frequencies of fungicide resistances were close to those found on grapes in June 1994 and June 1995 (10) and provided further evidence of this specialization (Fig. 2). The presence of BenR2 and ImiR phenotypes in *transposa* can be related to the intensive use of the corresponding fungicides in vineyards. Their absence in *vacuma* strains confirms that this species is not the major problem on grapes. BenR1 is present in *transposa* and *vacuma*, and this can be explained by the fact that carbendazim has long been used alone to spray on grapes and on several other crops. Resistance to fenhexamid, a fungicide that have never been used against grey mold or on any other crop, occurred only in *vacuma* strains. This resistance is related to increased detoxification of this hydroxylanilide, but this does not confer any direct advantage, because this fungicide is not used in vineyards. The specialization of *transposa* and *vacuma* is not yet understood, but there is undoubtedly a difference between the two species in their ability to infect certain plants.

The total percentage of *vacuma* was similar to those found on grapes in June 1994 and June 1995 (10). The study on grapes suggested a succession of different populations from June to October in Champagne; *vacuma* was more frequent in June and then decreased until October, when *transposa* was prevalent. The strains analyzed in this study were collected in June 1997 and they are more like the populations found on grapes in June 1994 and June 1995 than those isolated later in 1994 and 1995 from grapes. This

suggests that the same succession of populations may occur every year in Champagne.

vacuina Strains were more frequently found on asymptomatic tissues, whereas *transposa* strains were more frequent on disease lesions. This may reflect different behaviors of the two species, *vacuina* being more saprophytic and *transposa* being more like a parasite. It would be consistent with the observed succession of populations: *transposa* infecting first grapes as a parasite, followed by *vacuina* acting more saprophytically.

In conclusion, *B. cinerea* is not an unstructured clonal population devoid of specialization, as previously thought. On the contrary, *B. cinerea* undergoes regular sexual reproduction, and it comprises at least two sibling sympatric species, each of which probably has a different ecological niche. This study shows that the population genetics approach can give evidence for population structures and specializations that laboratory tests and field observations failed to detect. But pathogenicity tests are now necessary to confirm and to elucidate the nature of the specialization of the complex *B. cinerea*.

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