New PCR primers applied to characterize distribution of *Botrytis cinerea* populations in French vineyards

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Summary

The phytopathogenic fungus *Botrytis cinerea* is a complex of two main genetic groups, Group-I and Group-II, the latter including different TE types distinguished by the presence or absence of two transposable elements (TE), *Boty* and *Flipper*. In populations from Bordeaux vineyards (n = 470), the frequency distribution into these genetic subdivisions showed that Group-I isolates were rare (2.3 %) whereas, within Group-II, four TE types were identified by dot blot in very different proportions: II-*transposa* (59.8 %), II-*boty* (21.3 %), II-*vacuma* (15.5 %) and II-*flipper* (1.1 %). To distinguish the TE types by PCR, a first primer pair was designed within the *Flipper* sequence which yielded a not-expected 2287 bp fragment. The 5' extremity of this fragment was sequenced revealing a potential genomic insertion site of the *Flipper* element allowing the design of a new overlapping PCR primer. Detection of the *Flipper* element by two newly developed PCR tests and a published one (F300-F1550) was consistent with dot blot results in Group-I and II-*transposa* (concordance rates from 94.6 % to 100 %). However, discrepancies between PCR and dot blot were noticeable especially in II-*boty*, but also in II-*vacuma* (concordance rates from 33.3 % to 38.0 % and from 62 % to 81.2 %, respectively). On the basis of TE-type identification strengthened by combining different PCR and dot blot results, the spatiotemporal distribution of the Group-II isolates was assessed according to the developmental stage and the host organ of grapevine. In Bordeaux as in Loire valley vineyards, similar distribution patterns were described showing significant differences between the most sampled TE types. The II-*transposa* isolates predominated on grape berries at the end of the season, whereas the II-*vacuma* ones were detected mostly at flowering. Lastly, the II-*boty* isolates were more often detected on grapevine leaves than on flowers or berries.

K e y   w o r d s : detection, dot blot, filamentous Ascomycete, grapevine, gray mold, identification, transposable element.

Introduction

Botrytis cinerea Pers., the anamorph of *Botryotinia fuckeliana,* is a phytopathogenic fungus which causes gray mold on a wide range of plant species in temperate regions (Coley-Smith *et al*. 1980). In grapevine, gray mold is a major disease requiring the use of fungicides in many vine-growing regions. Disease development, based on various infection pathways, depends on a number of factors, including climatic conditions, inoculum levels, grape cluster architecture and berry susceptibility (Elmer and Michailides 2004, Holz *et al*. 2004). Moreover, the epidemiological development of *B. cinerea* may be influenced by the genetic structure of the pathogen population which can vary over time according to the grapevine developmental stage (Martinez *et al*. 2005).

Genetic diversity among isolates of *B. cinerea* has been studied using various techniques including restriction fragment length polymorphism (RFLP) (Graïa *et al*. 2001), polymerase chain reaction (PCR) (Van der Vlugt-Bergmans *et al*. 1993, Alfonso *et al*. 2000, Muñoz *et al*. 2002, Moyano *et al*. 2003), PCR-RFLP (Fournier *et al*. 2003, Giraud *et al*. 1999), random amplified polymorphic DNA (RAPD) (Kerssies *et al*. 1997, Thompson and Latorre 1999, Calpas *et al*. 2006), amplified fragment length polymorphisms (AFLP) (Giraud *et al*. 1997, Moyano *et al*. 2003), and more recently, microsatellite primed-PCR (Fournier *et al*. 2002, Ma and Michailides 2005). Detection of the pathogen by PCR methods has also been investigated as reviewed by Pezet *et al*. 2004 and Dewey and Yohalem 2004. In this context, transposable elements have also been shown to be valuable selected markers in population studies of fungal pathogens in plants, including *B. cinerea* (Diolez *et al*. 1995, Levis *et al*. 1997, Talbot 1998, Giraud *et al*. 1999, Kempken 1999, Daboussi and Capy 2003). Recent studies of DNA polymorphism of different nuclear genes of 40 isolates showed that *B. cinerea* isolates consistently clustered in two different clades, Group I and Group II, which were therefore proposed to be phylogenetic species (Fournier *et al*. 2003, 2005). Group I strains belonged exclusively to the *vacuma* (I-*vacuma*) transposable element type (TE type) and are naturally resistant to the fungicide fenhexamid. On the other hand, Group II strains included both *vacuma* (II-*vacuma*) and *transposa* (II-*transposa*) TE types which showed sensitivity or acquired resistance to fenhexamid. *Transposa* strains were characterized by the presence of two transposable elements, *Boty* (Diolez *et al*. 1995) and *Flipper* (Levis *et al*. 1997), whereas *vacuma* strains possess none of them (Giraud *et al*. 1997, 1999). In addition to the genetic differences, significant biological and phenotypic differences between the two TE types, within Group II, have been

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reported in i) mycelial growth rate, ii) aggressiveness on grape berries and iii) fungicide resistance (Martinez et al. 2003, 2005). Furthermore, II-*vacuma* strains that are mostly detected at flowering in vineyards have been hypothesized as expressing greater saprophytic capability than the II-*transposa* isolates prevailing on mature berries (Martinez et al. 2003, 2005). In Europe, Chile and California, strains containing only the *Boty* element have also been isolated from grapevine (Giraud et al. 1999, Muñoz et al. 2002, De Miccolis et al. 2003, Ma and Michailides, 2005) as well as strains harboring the *Flipper* element only (Albertini et al. 2002, De Miccolis et al. 2003). However, in these studies, isolates were characterized according to the presence of the two transposable elements, but without distinguishing between Group I and Group II. Therefore, it is unclear whether the findings on the TE-type distribution according to the host plant may have been biased particularly because the *vacuma* type included both I-*vacuma* and II-*vacuma* isolates. Furthermore, distribution of both TE types within Group II, II-*boty* and II-*flipper*, according to the sampling period within the season and the host organ has not been documented in vineyards, as for II-*vacuma* and II-*transposa*.

In population biology studies, the choice of genetic marker and technique can have substantial consequences on the analysis and interpretation of data (McDonald 1997). Concerning the transposable elements, *B. cinerea* isolates were screened initially by using dot-blot hybridization (Giraud et al. 1997). However, this method is labor-intensive and requires radioactive compounds. More recently, PCR tests have been developed by designing primers within the transposon sequences (Muñoz et al. 2002, Milicevic et al. 2004, Ma and Michailides 2005). However, PCR detection of the *Boty* transposon may be biased by the presence of variant copies of the transposon in the genome of numerous *vacuma* strains (Giraud 1998). Moreover, *Boty* is a retroelement with five genes shared by all retroelements possibly causing misleading and unspecific detection by PCR. Therefore, we considered the detection of *Flipper* as more reliable than for *Boty*. Concerning identification of the *Flipper* element, one study only mentioned that the results from PCR, by using the primer pair F300 and F1550, were consistent with those from dot blot (Muñoz et al. 2002). However, that study included only few *vacuma* (n = 8) and *Boty* (n = 8) isolates, which was not favorable to show potential inconsistencies between the two methods. Furthermore, when we used this primer pair in PCR tests (data not published) there was a possible amplification in *vacuma* strains characterized by dot blot, which is further confirmed in this study. Thus, we have designed specifically a new overlapping primer derived from a *Flipper* genomic flanking region because DNA regions flanking transposon insertion sites have been used successfully to distinguish genetically divergent populations or special forms in fungal plant pathogens (He et al., 1996, Fernandez et al., 1998, Chiocchetti et al. 1999).

The context of a complex population structure, associated with a high degree of genetic and phenotypic diversity, led us to further investigate PCR identification and distribution of transposable elements, particularly within

B. cinerea group II. The first objective was to compare detection of the transposable elements, in particular *Flipper*, by the dot-blot reference method and by using different PCR primers. By using new PCR primers, the second objective was to assay whether the main TE types within Group II had preferential distribution between times and/or host organs in two French vine-growing regions.

Material and Methods

M e t h o d s   o f   r e f e r e n c e : DNA extraction was performed from 4-5 d old cultures of *B. cinerea* on solid Malt Agar (MA) medium (15 g Cristomalt, Materne, France and 20 g agar per L). Mycelium and conidia were harvested by scraping, freeze-dried and then DNA was extracted as described by Möller et al. (1992). DNA quantification was performed using a spectrophotometer (UV-1605, Shimadzu, Kyoto, Japan) at $\lambda = 260$ nm. Dot blot was used as the method of reference (Giraud et al. 1997) to check and compare results from PCR tests newly developed to detect the two transposable elements, *Flipper* and *Boty*. The presence of *Boty* and *Flipper* was assessed visually according to the presence (or absence) of an autoradiographic signal following the fixation of the probe on the transposable element target. In order to distinguish Group-I and Group-II strains in the tested *B. cinerea* populations, a PCR-RFLP test was used as previously published (Fournier et al. 2003) to characterize specifically the Group-I strains. Knowing that Group-I strains lack both transposable elements (Fournier et al. 2005), strains showing a positive signal for both probes *Boty* and *Flipper* by dot blot were not tested by this PCR-RFLP test.

P r i m e r   d e s i g n : In order to distinguish the genetic TE types, two PCR primer pairs, F300-F1550 and BotyF4-BotyR4, have been used successfully to detect the two transposable elements (*Flipper* and *Boty*, respectively) and described in detail by Ma and Michailides (2005). In this study, a first primer pair was designed by using the *Flipper* sequence (GenBank accession number U74294). The FLIP primer (5' GGA CCA CCC CTC TTT TGG AC 3') was derived from the Inverted Terminal Repeat and the FLIP2 primer (5' CGG TTG TGT AAA GTG GTG CG 3') was in the transposase gene (Fig. 1). Another unexpected annealing site for FLIP2 outside the transposon was found out in this study (Fig. 1). An additional primer pair was developed which comprised FLIP2 and FABR (5' GTG CCA CCT AAG TTG AGT ACC CC 3') that corresponded to the insertion site of *Flipper*. FABR was derived from the sequence of a cloned 741 bp DNA fragment obtained by PCR amplification of a 2287 bp fragment using the FLIP2 primer (Fig. 1). This 2287 bp PCR product was obtained after genomic DNA amplification using the FLIP-FLIP2 primer pair (Fig. 1). A third primer pair, comprising FLIP3 (5' CCC CAT TTT AAG CAC TAC CT 3') and FLIP4 (5' ATC TGT TTT CGG GCT TGA TA 3'), located in the *Flipper* transposon at the 54 and 604 nucleotide position, respectively, was designed and tested on *B. cinerea* population from the Loire Valley. Lastly, two primers were designed in the *Boty* transposon, BOT1 (5' AGC CAA GGG
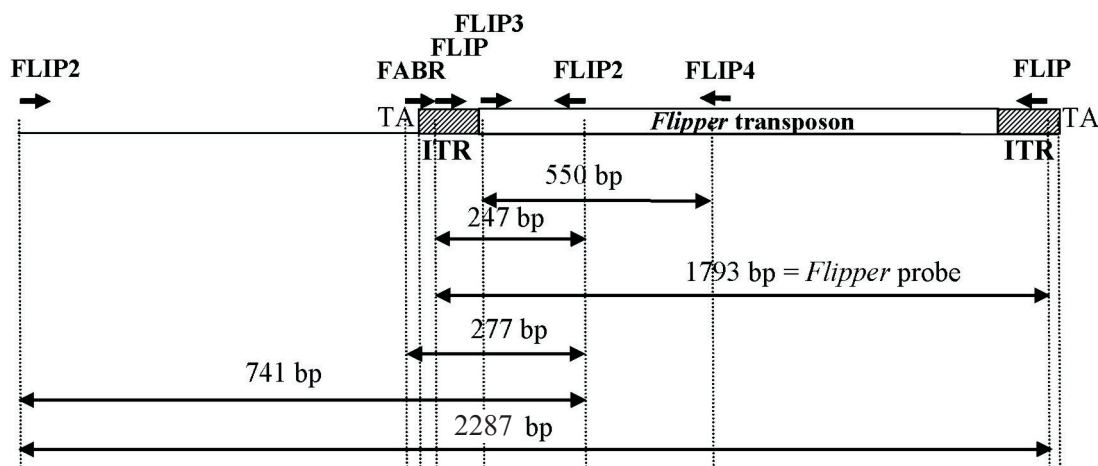
Fig. 1: Schematic representation of the *Flipper* transposable element and its genomic insertion site. The *Flipper* transposon is framed by the two ITRs (Inverted Terminal Repeats) terminated by TA nucleotides. The sites corresponding to the different primers used are represented (arrows) as well as corresponding amplification products by PCR and the cloned 741 bp fragment.

CTC AAG ATG A 3') and BOT2 (5' TAC GCT CGT TGT GGT GAA GT 3'). All the primers were synthesized by Isoprim S.A. Toulouse, France.

Specific PCR amplification: For the F300-F1550 primer pair, the PCR amplification reactions were performed as described by Ma and Michailides (2005). For the other new primer pairs which were developed, amplification reactions were performed in 50 µl of a reagent mixture containing 200 µM of each dNTP (MBI Fermentas, Vilnius, Lithuania), 2 mM MgCl₂, 67 mM Tris-HCl (pH 8.8 at 25 °C), 16 mM (NH₄)₂SO₄, 0.01 % Tween-20 (w/v), 0.2 µM of each primer, 40 ng of genomic DNA and 0.15 U of SilverStar DNA Polymerase (Eurogentec S. A., Seraing, Belgium). Amplification was performed on a Crocodile III DNA thermal cycler (Appligene S.A., Oncor®) programmed according to the primers employed. The PCR conditions are summarized in Tab. 1. The PCR products were separated in 1 % (w/v) agarose gels in 0.5 X TBE buffer (0.045 M Tris-borate, 0.001M EDTA).

Cloning and sequencing of a PCR DNA fragment: PCR products of 741 bp and 2287 bp (see above) were gel purified in 1 % (w/v) agarose gel and purified using a DNA purification kit (UltraClean™, MO BIO Laboratories, Inc.). The purified DNA fragment of 741 bp was cloned in *Escherichia coli* XL1 Blue (Stratagene, La Jolla, USA.) using the pGEM-T vector system kit (Promega Corporation, Madison, USA). Sequencing was performed by MWG-Biotech France SA on both strands by an extension of the specific primers, T7 and SP6, present in the pGEM-T vector. Sequences were analyzed using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) to determine homologies with sequences registered in GenBank. The sequence of the 741 bp has been deposited in GenBank (accession number AY302536).

Comparative testing of *B. cinerea* isolates from vineyards: In order to develop, validate and compare the above-described PCR methods, a large number of *B. cinerea* mono-conidial isolates (*n* = 597) were sampled between 1998 and 2002 from conventionally managed vineyards in Bordeaux and Loire Valley (Tab. 2). All the isolates from Bordeaux collected in 2000 (n = 407) were used to compare detection of the *Flipper* transposable element by dot blot and by different PCR tests using the primer pairs FLIP-FLIP2 and FLIP2-FABR. In order to check the F300-F1550 primer pair, 95 Bordeaux isolates were selected among those sampled in 2000. This subset comprised 62 II-*transposa*, 16 II-*vacuma*, 15 II-*boty* and 2 II-*flipper* isolates as characterized beforehand by the dot-blot reference method. Isolates from the Loire valley (n = 127) allowed comparison between the PCR tests only. In each vineyard, gray mold lesions were sampled in a subplot (ca. 40 vines x 10 rows) in which no fungicide with activity against *Botrytis* had been applied. All isolates were collected arbitrarily, using sterilized forceps, from grapevine tissue showing typical gray mold symptoms as scattered as possible (noble rot excluded). At different host developmental stages, isolates originated from blossoms, grape berries and leaves and, during the winter, from scle-

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PCR specifications and conditions

Primers	First denaturation step	Second step				Final elongation step
		Number of cycles	Denaturation	Annealing	Elongation	
FLIP-FLIP2		37	30s at 95 °C	1 min at 64 °C	2 min 30 s at 72 °C	
FLIP2-FABR		37	30s at 95 °C	1 min at 69 °C	1 min at 72 °C	
FLIP3-FLIP4	10 s at 94 °C	35	30s at 95 °C	30 s at 60 °C	1 min at 72 °C	5 min at 72 °C
BOT1-BOT2	10 s at 94 °C	40	30s at 95 °C	30 s at 60 °C	1 min at 72 °C	6 min at 72 °C

Table 2

Botrytis cinerea isolates collected from Bordeaux vineyards[a] in 1999 and 2000 and from vineyards in the
Loire valley[b] in 2002

Location; cultivar	Winter Woody canes	Bloom[c] Blossom	Leaf	Veraison[c] Berry	Leaf	Harvest Berry	Leaf
1999							
Pessac-Léognan; Sémillon	0	0	0	1	0	30	0
Médoc, St-Julien; Merlot	0	11	2 (1)	2	0	3	9
Sauternes; Sémillon	0	2	0	2	0	0	0
Total	0	13	3	5	0	33	9
2000							
Pessac-Léognan; Sémillon	9	2	18	15	16	7	19
Médoc, St-Julien; Merlot	30	17 (3)	17 (3)	30	17 (1)	19	21
Sauternes; Sémillon	16	17 (2)	18 (1)	38	8 (1)	37	25
Total	55	41	57	83	43	63	65
2002							
Layon; Chenin	0	12	9	2	0	15	0
Landreau; Melon	0	20	24	4	0	10	31
Total	0	32	33	6	0	25	31

[a] The strains originated from Bordeaux vineyards were characterized by dot blot and by PCR with the primers
    pairs FLIP-FLIP2 and FLIP2-FABR.
[b] The strains from Loire Valley were characterized by PCR only with the primers pairs FLIP-FLIP2,
    FLIP2-FABR, FLIP3-FLIP4 and BOT1-BOT2.
[c] The numbers of Group-I strains are indicated in parenthesis.

rotia at the surface of woody canes (Tab. 2). After incubation of infected tissues or sclerotia in a humid chamber at 20 °C, the growing fungus was transferred to solid MA medium. After mono-conidial isolation, isolates were put in storage (5 °C).

S t a t i s t i c a l   a n a l y s e s : The S+ software package (Statistical Sciences, 1993) and StatBox 6.6 software package (Grimmersoft ©) were used for all statistical analyses. In the Bordeaux vineyards, the relative isolation frequencies were calculated on a per-site basis and within each of the main genetic types identified by dot blot and PCR. These frequency data were analyzed by ANOVA procedures using a randomized block factorial design with two main factors. The first factor was the developmental period including two levels: bloom or veraison to harvest. As second factor, the host organ comprised also two levels: leaves or fructiferous organs (flowers and berries). Three blocks were used corresponding to the three experimental sites near Bordeaux (Tab. 2). The means were compared at $P = 0.05$ using Newman and Keuls's test. Isolation data from the two sites in the Loire Valley were pooled and overall relative frequencies were calculated within each of the main genetic types identified by the PCR tests. The frequency distributions according to the isolate origin (host organ X developmental stage) were compared between the genetic types using a Chi-square test at $P = 0.05$.

Results

I d e n t i f i c a t i o n   o f   g e n e t i c   t y p e s   b y   d o t   b l o t   a n d   P C R - R F L P : The dot blot reference method applied on isolates from Bordeaux vineyards in 1999 and 2000 (n = 470) showed marked differences in transposable element distribution within the whole sampled population (Tab. 3). The *Boty* element (B+ profiles) was detected with a high frequency (81.1 %), whereas the *Flipper* element (F+ profiles) was detected at lower frequency (60.9 %). Only in 1.1 % of strains (B-F+) was the presence of the *Flipper* element not associated with the *Boty* element. As for the genetic group identification by PCR-RFLP, 11 isolates (2.3 %) were identified as Group-I strains and every isolate was of the *vacuma* TE type (Tab. 3). In Group-II, the II-*transposa* strains harbouring both transposable elements (B+F+) were predominant, that is 59.8 % for both years. The II-*vacuma* strains (B-F-) were detected relatively frequently (15.5 %) as well as the II-*boty* strains (B+F-) which reached an average of 21.3 %. Lastly, few isolates only were characterized as II-*flipper* (1.1 %).

C o m p a r i s o n   b e t w e e n   d o t   b l o t   a n d   P C R   p r o f i l e s   w i t h   p r i m e r s   d e s i g n e d   w i t h i n   t h e   *F l i p p e r*   t r a n s p o s a b l e   e l e m e n t : By using the F300-F1550 primer pair, PCR amplified the expected 1250 bp fragment in all the strains characterized by dot blot as either II-*transposa* or II-*flipper* (Tab. 4). Within these TE types, this led to a maximal concordance rate of 100 % (frequency of strains characterized similarly by dot blot and PCR). However, in II-*vacuma* and II-*boty*, the *Flipper* element was detected by PCR in 3 and 10 isolates, respectively (concordance rates of 81.2 % and 33.3 %, respectively).

The two primers, FLIP and FLIP2, designed within the *Flipper* element (Fig. 1) gave rise to five electrophoretic profiles (Tab. 4). In addition to two expected fragments of 247 bp and 1793 bp according to the *Flipper* sequence, a

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Dot blot distribution profiles in *B. cinerea* groups (Group-I and Group-II differentiated by PCR-RFLP) from Bordeaux vineyards

Genetic type designation	Dot-blot profiles[a]	Group I[b]		Group II[b]		Group I[c] 1999-2000 (%)	Group II[c] 1999-2000 (%)
		1999	2000	1999	2000		
vacuma	B- F-	1	10	2	71	2.3	15.5
flipper	B- F+			1	4		1.1
boty	B+ F-			20	80		21.3
transposa	B+ F+			39	242		59.8
Total		1	10	62	397	2.3	97.7

[a] Autoradiographic signal assessed visually for both probe *Boty* (B) and *Flipper* (F):
(-) absence of signal ; (+) presence of signal.
[b] No. isolates according to the dot blot profile and the year.
[c]. Overall frequency (%) for both years.

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Concordance rates between dot blot and PCR methods based on the distribution of strain number according to PCR profiles within the genetic TE types (isolates from Bordeaux in 2000)

Genetic TE types[a]	PCR profiles[b] (F300-F1550)		Concordance rate[c]	PCR profiles[b] (FLIP - FLIP2)					Concordance rate[d]	PCR profiles[b] (FLIP2 - FABR)		Concordance rate[c]
	No	1250 bp		No	247 bp	1793 bp	247 + 1793 bp	247 + 1793 + 2287 bp		No	277 bp	
Group I (I-*vacuma*)	na[e]	na	na	3	5	0	3	0	100 %	11	0	100 %
II-*vacuma*	13	3	81.2 %	3	20	4	25	19	73.2 %	44	27	62 %
II-*transposa*	0	62	100 %	0	4	0	4	234	96.7 %	13	229	94.6 %
II-*flipper*	0	2	100 %	0	0	0	1	3	75 %	1	3	75 %
II-*boty*	5	10	33.3 %	1	15	0	14	49	38 %	30	49	38 %

[a] The transposable element (TE) types are defined within Groups I and II by using dot blot results as in Tab. 3; [b] Correspond to the different fragments obtained using the primer pairs indicated in parentheses; [c] Within II-*transposa* and II-*flipper* types, the concordance rate corresponded to the percentage of isolates showing the expected fragment (1250bp for F300-F1550 and 277 bp for FLIP2 – FABR). Within Group I, II-*vacuma* and II-*boty* types, the concordance rate was calculated as the percentage of isolates showing no fragment (no); [d] Within II-*transposa* and II-*flipper* types, the concordance rate corresponded to the percentage of isolates showing all the 3 fragments of 247, 1793, and 2287 bp. Within Group I, II-*vacuma* and II-*boty* types, the concordance rate was calculated as: 100 - (percentage of isolates showing all the 3 fragments of 247, 1793 and 2287 pb); [e] Not assessed.

third unexpected fragment of 2287 bp was detected, which was always associated with both previous fragments. The absence of the fragment of 741 bp showed that FLIP2 did not self-amplify because, most probably, the fragment of 247 bp was preferentially amplified (Fig. 1). By considering the full PCR profile showing all three fragments, the concordance rate reached 96.7 % and 75.0 % for II-*transposa* and II-*flipper* types, respectively (Tab. 4). Conversely, within Group-I and II-*vacuma*, the relative infrequency of the 3-fragment profile led to a concordance rate reaching 100 % and 73.2 %, respectively (Tab. 4). In the II-*boty* type, detection of the *Flipper* element by dot blot and PCR showed conflicting results as indicated by a concordance rate of *ca.* 38 % (Tab. 4).

Because of the close connection between the presence of the 2287 bp fragment and the *Flipper* detection by dot blot, the 5' extremity of this fragment of 741 bp was cloned and sequenced. The sequence (GenBank accession number AY302536) comprised the 5' end of *Flipper* (267 bp) and a genomic DNA region flanking the *Flipper* sequence (Figs 1 and 2). This genomic DNA region was tested using Blastx (Altschul *et al.* 1997) over B05-10 strain genome (http.//www.broad.mit.edu/annotation/genome/botrytis_cinerea/Home.html). The sequence showed identity with a small protein of 44 amino acids (BC1G_13360.1). This protein presented no homology with public data base proteins.

Comparison between dot blot and PCR using a new primer designed in a *Flipper* insertion site: The sequenced 741 bp fragment was used to generate a new primer (FABR) overlapping the 5' extremity of *Flipper* and its genomic insertion site. The FABR primer, combined with the FLIP2 primer, amplified an expected 277 bp fragment. In Group-I, the absence of the 277 bp PCR fragment was totally consistent with the dot blot results, *i.e.* concordance rate of 100 % (Tab. 4). Most of the II-*transposa* isolates were char-

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          FLIP 2 primer ────▶
  1   CGGTTGTGTA AAGTGGTGCG GTATAAGAGA AATCTAAGCG GAGCGAATGA AGATATATCT

 61   TTAAGAGACA ATAAAGTGAT GTGCAGTTGG ACTATGAAAT GAAGCCTTGT TACTGGATAT

121   TGATTCACTT CACATTTCGC AACGCATCAT CACCATACCT GAGTTTCTAC ATCAGTATTG

181   ATCAAACATT ATTTGAGAAA GATTGGAATC GGGAAAAAAC ATGTCTTGAG GTCTTACAGC

241   GGATGAGTAG TCGTTGATTC AAAATACTTT CAAAAATGGT TAGAAAGAAG TATGAGAGTC

301   AAGTACAAGT CTTTGTTTGT TTAGTTGCCA AGAAGTCTTT TTGGTTACCA TTGAATTGGG

361   TTAGTTTCCA TTCGAGCTAC CAGCTCGAAT CTCTACTTCT AGATGCTTGA CGGCTCAGGG

                                                     FABR primer ────▶
421   TTATCAGGGT TAGAAAACTT ATTATTTGAC GGCGAGATTT CGTT STGCCA CCTA AGTTGA

                            FLIP primer ────▶
481   GTACCCC ACT TTC GGACCAC CCCTCTTTTG GAC CACCTAA AATCTTACCCC ATTTTAAGC

541   ACTACCTCAC AACTTCATCT TTAATAAATC AACAACCACA TATTCAATTGA TATAAGATT

601   TTAATACATT ATCAATTAAG CTATGACAAA GCCTTATACT GAAGATGATAT CGCTGCAGC

661   ACTTTTTGCG ATTGCAGGAG GCATGTCTAT GCGTAAGGCT TGCTCAGAATA TGGTATTCC

              ◀──── FLIP2 primer
721   C CGCACCACT TTACACAACC G
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Fig. 2: Nucleotic sequence of the 741 bp fragment (GenBank accession number AY302536) framed by sequence of the FLIP2 primer. The nucleotides in bold type correspond to the *Flipper* sequence. The unknown sequence is in normal type. This 741 bp DNA fragment was obtained by PCR amplification of a 2287 bp fragment using the FLIP2 primer.

acterized consistently by PCR and dot-blot as shown by a concordance rate of 94.6 %. Although few II-*flipper* strains were tested, the concordance rate was also high (75 %). In II-*vacuma*, the concordance rate was only of 62 % because of strains showing the 277 bp fragment. In the II-*Boty* type, the dot-blot and PCR methods showed a low concordance rate of 38.0 % (Tab. 4).

Comparison between PCR profiles based on the different primers tested: When considering all the isolates sampled near Bordeaux in 2000 (Tab. 5), irrespective of the genetic group or TE type, the comparison between the two newly developed PCR tests led to an overall concordance rate of 91.9 % (374 of 407). By combining the results from dot-blot and PCR, all the Group-I strains were characterized consistently by the absence of the *Flipper* element. Within Group-II, most of the isolates were categorized into four main genetic TE types according to the frequency distribution: II-*transposa* PCR+ (n = 226), II-*boty* PCR+ (n = 46), II-*vacuma* PCR- (n = 41), and II-*boty* PCR- (n = 27). Except for the II-*boty* type which was split into two classes due to the PCR results, in II-*transposa* PCR+ and II-*vacuma* PCR-, the isolates were identified consistently and unambiguously by using the dot blot and PCR results.

Application in Bordeaux and Loire valley vineyards: In vineyards near Bordeaux and in the Loire valley, the PCR methods were tested in order to assess the frequency distribution patterns of the main genetic TE types. The Group II isolates from Bordeaux were analyzed on the basis of the four most frequently sampled TE types, as previously defined (Tab. 5). The relative isolation frequencies depended significantly on both the host organ and the developmental stage, except for the II-*boty* PCR+ type (Tab. 6). Within the II-*transposa* PCR+ type, the isolation frequency was significantly higher in reproductive organs (23.6 %) than in leaves (17.9 %) and higher at veraison to harvest (23.3 %) than at bloom (18.2 %).

The greatest rate of isolation in II-*vacuma PCR-* was observed from leaves at bloom (45.2 %), and the other rates (average of 10.9 %) were not significantly different. As for II-*boty PCR-*, the frequencies were significantly higher on leaves (27 %) than on reproductive organs (9.1 %) and higher towards the end of the season (25 %) than at bloom (11.1 %).

In the Loire valley ($n = 127$), the isolation frequencies were analyzed within the main TE types identified by PCR within Group-II. The frequency distributions were significantly influenced by the host organ and the developmental period during the season (chi-square test = 17.16, df = 9, $P = 0.046$). Significant contributions to the chi-square at $P = 0.05$ showed that II-*transposa* isolates originated mostly (32.3 %) from fructiferous organs at the end of the season (veraison to harvest). Conversely, II-*boty*-type strains showed the lowest frequency for this origin (4.4 %) and were more frequent in leaves than in fructiferous organs. The II-*vacuma* isolates were mostly isolated at flowering from leaves or inflorescences.

Discussion

In this study, the two main genetic groups, Group-I and Group-II, and the different TE types were distinguished by using two methods of reference: dot blot (Giraud *et al.* 1997) and PCR-RFLP (Fournier *et al.* 2005). The resulting characterization of 470 isolates from Bordeaux vineyards confirmed that all Group-I strains did not harbor any of the two transposable elements, *Flipper* and *Boty* (Albertini *et al.* 2002, Fournier *et al.* 2003). The Group-I isolates were detected rarely in these vineyards, comprising only 2.3 % of the entire population sampled in 1999 and 2000. Within Group-II, which included most of the isolates (97.7 %), the dot-blot results allowed us to distinguish between four TE types, *i.e.* II-*transposa*, II-*vacuma*, II-*boty*

T a b l e 5

Distribution of the 2287 and 277 bp PCR fragments using the primers pairs FLIP-FLIP2 and FLIP2-FABR, respectively, in the *B. cinerea* population from Bordeaux vineyards in 2000

Genetic Identification [a]	PCR profiles [b]				Concordance rate [c]
	- 2287 bp - 277 bp	- 2287 bp + 277 bp	+ 2287 bp - 277 bp	+2287 bp + 277 bp	
GroupI (I-*vacuma*)	11	0	0	0	100.0 %
II-*transposa*	5	3	8	226[(y)]	95.5 %
II-*vacuma*	41[(w)]	11	3	16	80.3 %
II-*boty*	27[(x)]	3	3	46[(z)]	92.4 %
II-*flipper*	0	1	1	2	50.0 %

[a] Identification of the genetic group by PCR-RFLP and by dot-blot for the TE type.

[b] In parenthesis are letters corresponding to the four main classes within Group-II according to the dot blot and PCR results and named as followed: [(w)] II-*vacuma* PCR-; [(x)] II-*boty* PCR-; [(y)] II-*transposa* PCR+, [(z)] II-*boty* PCR+.

[c] The concordance rate between the two PCR methods was calculated as the cumulated frequency (%) of strains showing either the absence of both expected fragments (− 2287 − 277) or presence of both expected fragments (+ 2287 + 277).

T a b l e 6

Analyses of variance of the isolation ratio according to the grapevine developmental stage (bloom *vs* veraison to harvest) and organ (blossom or berries *vs* leaves) within the main TE types in Bordeaux vineyards in 2000

Source of variation[(a)]	df	Mean square	*F* value	*P*
ANOVA in II-*transposa PCR+*				
Developmental stage	1	78.14	8.03	0.029
Organ	1	98.63	10.13	0.019
Interaction	1	8.04	0.83	0.401
ANOVA in II-*vacuma PCR-*				
Developmental stage	1	856.21	13.27	0.011
Organ	1	1332.25	20.65	0.004
Interaction	1	536.81	8.32	0.027
ANOVA in II-*Boty PCR-*				
Developmental stage	1	576.71	7.95	0.030
Organ	1	961.25	13.25	0.011
Interaction	1	55.28	0.76	0.42
ANOVA in II-*Boty PCR+*				
Developmental stage	1	1496.10	2.20	0.186
Organ	1	868.39	1.28	0.302
Interaction	1	1503.25	2.22	0.185

[(a)] The four main genetic TE types were defined by using dot-blot and both PCR tests (Tab. 5). Three blocks were used for the analysis corresponding to the three vineyards sampled.

and II-*flipper*. In the Bordeaux vineyards, the frequency distribution of the Group-II isolates according to the different TE types was consistent with previous results from different grapevine-growing countries. First, the predominance of the II-*transposa* type (59.8 %) was also shown clearly in California, Italy, Croatia and Chile (MUÑOZ *et al*. 2002, DE MICCOLIS ANGELINI *et al*. 2003, MA and MICHAILIDES 2005). Second, the II-*vacuma* strains were a minority (15.5 %), which was also comparable to the previous studies showing *vacuma* isolates comprising 9 % of the popu-

lation in Chile, 21 % in Italy and Croatia, and 0 % in the Californian vineyards. However, in these studies, *vacuma* isolates were characterized without distinguishing between Group-I and Group-II. Third, isolates harboring either the *Boty* element only or the *Flipper* element only have also been detected in this study as in previous ones (GIRAUD *et al*. 1999, ALBERTINI *et al*. 2002, MUÑOZ *et al*. 2002, DE MICCOLIS ANGELINI *et al*. 2003, MILICEVIC *et al*. 2004, MA and MICHAILIDES 2005). The *Boty* element, due to its presence in both II-*transposa* and II-*boty* types, was widely distrib-

uted (81.1 %) through the sampled Bordeaux populations. Similarly, these two genetic types, together, comprised more than 75 % of the whole *B. cinerea* population in the previous studies (Muñoz *et al.* 2002, De Miccolis Angelini *et al.* 2003, Ma and Michailides 2005). Lastly, the presence of the *Flipper* element only, without *Boty*, was detected in very few strains, i.e. 1.1 % of II-*flipper* isolates. The infrequency of this TE type was also noticeable in Italy and Croatia, where these isolates amounted to 3.8 % only of the sampled populations, as in California where they were not detected (De Miccolis Angelini *et al.* 2003; Ma and Michailides 2005).

Different specific primers of the *Flipper* element were designed, tested and checked with dot blot results. Both primer pairs designed within the *Flipper* transposable element, F300-F1550 and FLIP-FLIP2, led to high concordance rates between PCR and dot blot (from 96.7 % to 100 %) in the I-*vacuma* and II-*transposa* types. A similar trend was noticeable in II-*flipper*, but the number of isolates (n < 5) was insufficient to interpret definitely the results. However, the concordance rates were lower in II-*vacuma* (73.2 % and 81.2 %) and different PCR products were detected showing the potential presence of the *Flipper* element in many isolates (Tab. 4). In II-*boty*, the discrepancy between dot blot and PCR was very marked: concordance rates of 33.3 % and 38.0 %. Therefore, these results may be used to challenge the initial definition of these two last TE types. The discordant results between PCR and dot blot may result from the greater sensitivity of the PCR method in detecting the transposon *Flipper* than the dot blot (Li *et al.* 1998). This has also been noticed for the *Boty* element which was not detected by dot blot in *vacuma* strains, but was by PCR (Giraud 1998). The sensitivity of dot blot depends on the efficiency of the probe hybridization and/or the copy number of each transposon in the *B. cinerea* strain. As for the *Flipper* element, probe hybridization may be affected because of the presence of more or less complete forms, exhibiting mutations, which have been revealed by sequencing *Flipper* copies in K1 *vacuma* strain (C. Levis and Y. Brygoo, personal communication). These mutations may be attributed to a RIP (repeat induced point mutation) process leading to the inactivation of transposable elements, as observed in *Neurospora crassa* (Windhofer *et al.* 2000), *Podospora anserina* (Graïa *et al.* 2001) and *Magnaporthe grisea* (Ken-ichi *et al.* 2002), and that is directed against foreign and/or repeated sequences. In this connection, the RIP process may also have affected the PCR results by altering one or more primer sites. This was substantiated by the results with the primer pair FLIP-FLIP2 showing that not all PCR products were generated according to the isolate considered (Tab. 4). For example, a fairly large number of II-*vacuma* and II-*boty* isolates showed an incomplete profile with the 247bp PCR fragment only. Because the RIP process occurs during meiosis, it can be hypothesized that sexual reproduction would occur in the populations tested from vineyards leading to different patterns of distribution of the PCR products. In support of this, a potential occurrence of sexual recombination within Group-II has been suggested by Fournier

et al. (2002) and Munoz *et al.* (2002) considered the *Boty* isolates as possibly resulting from crosses between *vacuma* and *transposa* strains. Furthermore, this would be in accordance with the numerous VCGs found in the *B. cinerea* populations which result also, presumably, from sexual recombination (Beever and Weeds 2004). However, the existence of a sexual stage of *B. cinerea* in fields is questionable because the discovery of apothecia has been reported very rarely, notably in vineyards (Pezet *et al.* 2004). This applies also to the Bordeaux region as well as other grapevine growing regions (Ma and Michailides 2005) where apothecia of *Botryotinia fuckeliana* have never been found on the ground in vineyards.

A new overlapping PCR primer (FABR) was designed specifically and derived from a *Flipper* genomic flanking region. The sequence of the 2287 bp overlapping fragment revealed the systematic presence of a *Flipper* copy fixed in a *transposa* genome specific location. By using the FLIP2-FABR primer pair, the results were similar to those obtained with the primer pairs designed within the *Flipper* element. The PCR and dot-blot results were consistent in Group I and II-*transposa* (concordance rate of 100 % and 94.6 %, respectively). In contrast, the concordance rates were lower in II-*vacuma* (62 %) and, particularly in II-*boty* (38 %). As above-discussed, these discordant identifications may be caused by differences in sensitivity between the two methods, but also by the presence of sequence variants of the *Flipper* element (RIP process). When RIPping of the transposable elements has likely occurred as postulated, this would generate transposon remnants with different levels of sequence identity to the active, prototype transposon. This may result in a broad, nearly continuous spectrum of PCR efficiency and hybridization intensity among the isolates. This last hypothesis requires further investigation based on sequence analyses. Nevertheless, by combining the results from different methods tested (Tab. 5), the great majority of isolates were identified similarly and unambiguously and distributed into four main categories within Group-II: II-*transposa* PCR+ (n = 226), II-*vacuma* PCR- (n = 41), II-*boty* PCR+ (n = 46), and II-*boty* PCR- (n = 27). The first two TE types can be considered as those showing biological and ecological differences (Martinez *et al.* 2003, 2005).

In the two grapevine growing regions considered, significant differences were demonstrated in the spatial and temporal distribution of the main TE types within Group II. The Bordeaux isolates were analyzed according to 4 main types defined more assuredly by combining results from dot blot and PCR. Similarly, in the Loire Valley, the identification of populations was strengthened by comparing different PCR tests. A similar pattern of distribution was evidenced in both regions. The II-*transposa* strains were isolated mostly from reproductive organs towards the end of the season, whereas the II-*vacuma* isolates were more frequent at bloom than later in the season. These findings concur with previous studies in vineyards in different countries, although in these studies the *vacuma* type included both Group-I and Group-II isolates (De Miccolis Angelini *et al.* 2003, Giraud *et al.* 1997, Ma and Michai-

LIDES 2005). Furthermore, a specific distribution pattern of the II-*boty* type was found out showing that the isolates originated mostly from leaves in both grapevine growing regions considered. However, this result was not consistent with findings in Italia and Croatia where *boty* isolates were prevalent on fruit, *i.e.* grape berries and strawberries (DE MICCOLIS ANGELINI *et al.* 2003). The discrepancy may be due to differences in i) PCR identification of the *boty* isolates which is dependent upon to the primer pair used as shown clearly in our data (II-*boty* PCR+ *vs* II-*boty* PCR-) and/or ii) frequency distribution according to the organ between different hosts plant (strawberry *vs* grapevine) which was not indicated in the previous study. This last hypothesis requires further investigation.

Our investigations have established that, when combined with dot blot results, the newly designed PCR primers have constituted complementary tools to better delineate the genetic TE types within the Group-II populations. On this basis, significant differences in the frequency distribution according to the developmental stage and the host organ of grapevine have been shown between the main TE types: II-*transposa*, II-*vacuma* and II-*boty*. At the same time, potential genetic exchanges within these Group-II populations have been corroborated in connection with the associated process of RIPping of the transposable elements. It was then difficult to develop and achieve a reliable PCR test allowing, by itself, to identify definitely the TE types within Group II populations of *B. cinerea*, even by using an overlapping primer. Similarly, the reliability of the PCR test (F300-F1550) published by MUÑOZ *et al.* (2002) to identify the *Flipper* element has been questioned for the same reasons. Thus, further studies of population differentiation in *B. cinerea*, particularly within Group II, should rely not only on PCR data but combine different marker data including also that from dot blot, microsatellite and/or sequence data.

Acknowledgements

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