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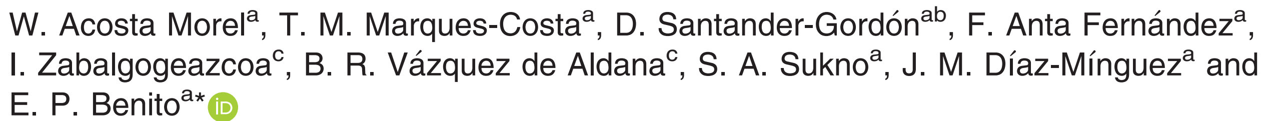
Physiological and population genetic analysis of *Botrytis* field isolates from vineyards in Castilla y León, Spain



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Grey mould is reported in the vineyards of Castilla y León, Spain, every year. However, the natural populations of the pathogen have yet to be properly characterized. Vineyards from six wine-producing areas were surveyed in 2002 and 2007, sampling from bunches of grapes with and without symptoms. A total of 283 *Botrytis* field isolates were selected for physiological and genetic analyses. *Botrytis cinerea* isolates predominated in the population, although isolates belonging to *Botrytis pseudocinerea* and *Botrytis prunorum* were also identified. These two species are recorded for the first time in Spain in this work. In addition, two isolates closely related to *Botrytis californica* were identified. Physiologically, the *B. cinerea* population is very diverse, displaying a normal distribution of aggressiveness values in *Vitis vinifera* leaves, suggesting a quantitative nature for this trait. Several isolates unable to cause infection were identified, most of them belonging to a mycelial morphotype. Population genetic analysis revealed that genotypic diversity is high and that multiple infections of the same bunch of grapes by different genotypes occur frequently. The high genotypic diversity observed, an even distribution of both mating types and the linkage disequilibrium values detected support a mixed mode of reproduction with low levels of clonality. The wine-producing area in which each isolate was collected imposed a low degree of population differentiation, an effect that does not depend solely on the geographic distances but rather on the management practices used by growers and wine producer associations.

Keywords: AFLP, aggressiveness, genetic diversity, grey mould, population structure

Introduction

One of the main pathogens of grapevine is the necrotrophic fungus *Botrytis cinerea*, the causal agent of grey mould. *Botrytis cinerea* is a filamentous, heterothallic ascomycete that can infect more than 200 mostly dicotyledonous plant species, leading to important losses in yield and quality (Williamson *et al.*, 2007). It is the best-known species and the major representative of the genus *Botrytis*, which currently comprises about 30 species. Together with *B. pseudocinerea*, *B. fabae*, *B. pelargoniae*, *B. calthae*, *B. sinoviticola* and *B. californica*, it forms a phylogenetic clade of species (clade I) that infect mostly dicotyledonous plants, well separated from a second clade grouping all other *Botrytis* species (clade II), characterized by a narrow host range and pathogenic on either eudicotyledoneous or monocotyledoneous plants (Hyde *et al.*, 2014).

Botrytis cinerea is a highly versatile microorganism. As a necrotroph, it can extract nutrients from dead or senescent plant materials, but it can also infect living tissues (van Kan, 2006). It has a wide host range and can infect any kind of plant organ or tissue at all developmental stages. Furthermore, it can also grow saprophytically. All of these factors, together with its capacity to produce large amounts of asexual spores that are easily dispersed by wind, insects and human activities, make the fungus widely distributed in the environment and difficult to control. *Botrytis cinerea* is also recognized as a highly variable microorganism. Classically, *B. cinerea* had been considered as a single species. However, recent studies have demonstrated that *B. cinerea* is actually a species complex, in which at least another cryptic species, *B. pseudocinerea*, has been found living in sympatry with *B. cinerea* (Walker *et al.*, 2011; Johnston *et al.*, 2013; Plesken *et al.*, 2015). Within the species, significant differences between individuals have been observed in natural populations with respect to vegetative growth, conidiation, sclerotium

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formation, secondary metabolism, fungicide resistance, light responses and virulence.

This morphological and physiological variation implies that the individuals within the populations are also genetically diverse. Numerous works have focused on the detection and quantification of genetic variation in natural populations of *B. cinerea*. The development of molecular techniques and markers (e.g. RAPDs, RFLPs, AFLPS, SSRs, transposons) and access to large datasets of sequence information during the last two decades have facilitated the analysis of variation. For example, determination of the presence or absence of the transposable elements Boty (Diolez *et al.*, 1995) and Flipper (Levis *et al.*, 1997) made possible the description of four types of populations: *vacuma* (strains with neither transposon), *transposa* (strains with both elements), *boty* and *flipper* (strains with one or the other element). The established subdivision proved to be of limited taxonomic value (Hahn *et al.*, 2014; Walker, 2016), but paved the way for the definition of *B. pseudocinerea* as a new species (Fournier *et al.*, 2005; Fournier & Giraud, 2008; Walker *et al.*, 2011). On the basis of analysis of molecular variation, together with the physiological characterization of field isolates in relation to host preference and fungicide resistance, a new genetic entity called *Botrytis* group S has been described (Leroch *et al.*, 2013). This genotype was found to be predominant in German strawberry fields, although it has also been reported at a lower frequency in grapevine (Johnston *et al.*, 2013; Leroch *et al.*, 2013). Whether group S represents a subpopulation adapted to a specific host or a sympatric new species in grey mould populations is still to be determined.

Numerous works have also focused on the analysis of factors that can contribute to shape the population structure (reviewed by Walker, 2016). This information is essential to understand key aspects of the biology of the microorganism in nature and may have important implications for new control strategies in agricultural systems. Geographic distance and host-specialization are major factors extensively considered in previous studies. Population differentiation of *B. cinerea* has been described at a large scale, among different continents (Isenegger *et al.*, 2008), but at smaller scales, patterns of geographical subdivision appear to be much weaker (Fournier & Giraud, 2008; Karchani-Balma *et al.*, 2008). Analysis of host-specific differentiation has offered contrasting results. Significant genetic differentiation has been found among isolates collected from grape, tomato, kiwifruit and bramble in Chile (Muñoz *et al.*, 2002), from grape and bramble in France (Fournier & Giraud, 2008), from grape, tomato, faba bean and strawberry in Tunisia (Karchani-Balma *et al.*, 2008), and also among wild hosts in the UK (Rajaguru & Shaw, 2010). However, when isolates collected from grape, kiwifruit, pea and squash in the Californian Central Valley were genetically and physiologically characterized, no significant genetic differentiation was found (Ma & Michailides, 2005). Other factors, such as time and anthropic activities, have been less investigated. In the most comprehensive study carried out so far considering simultaneously distance, host species, time and management, a weak association between population structure and geography was detected, but a clear differentiation according to the host plant of origin was shown (Walker *et al.*, 2015). Some effect associated with the cropping system (indoor vs outdoor) was also reported (Walker *et al.*, 2015).

Grapevine is a strategic crop in Castilla y León (Spain). The economic activity derived from vineyards is very important, and grapevine cultivation and wine production are strictly regulated. The producers pursue high quality wines based on autochthonous varieties. The Denominations of Origin (DOs) are geographic regions in which strict winemaking quality procedures and regulatory rules are followed. Growers and producers, under the supervision of a Regulatory Council, establish specific guidelines concerning the grapevine varieties that can be grown within the DO and the way in which they have to be managed and processed. When complying, the wines produced within the DO are given a certification that guarantees their origin and quality. Often, these regulations impose severe restrictions on the movement of plant materials within and between DOs. Currently, in Castilla y León there are nine wine-producing areas recognized as DO (Ribera del Duero, Cigales, Rueda, Toro and Arribes among them) and three more awaiting this recognition (Sierra de Salamanca being one of them). Climatic conditions in the region are considered to be Mediterranean with Atlantic influences. The summer period is dry. During September average precipitations rise to 30–35 mm (http://www.aemet/es/serviciosclimaticos).

Botrytis cinerea is present in all the areas where grapevines are grown in Castilla y León, although the damage it causes is often limited. However, when rain occurs soon before the harvest, and temperatures are moderate, significant losses can occur. Generally, producers do not apply preventive treatments against *B. cinerea* in the extensive vineyards of Castilla y León, but they continuously monitor the sanitary status of the vineyards in order to make crop management and harvest decisions. Although the presence of the pathogen is documented year after year (Benito *et al.*, 2008), there has been no previous study quantifying the incidence of *B. cinerea* and describing its natural populations in the vineyards in Castilla y León. There is also no report on the analysis of *B. cinerea* diversity in vineyards from other wine-producing areas in Spain and very limited information concerning *B. cinerea* populations in other crops. Only the populations from some greenhouses in two regions in Almería, in the south of Spain, were characterized using molecular markers. Those studies detected high levels of variation, and indicated that most of the genetic variation in *B. cinerea* populations was present within the subpopulations (greenhouses), with a null effect of the temporal factor and a very weak effect of the geographic factor on the genetic structure of the populations (Alfonso *et al.*, 2000; Moyano *et al.*, 2003).

Being aware of the problems derived from the infections caused by *B. cinerea* in the vineyards in Castilla y

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León, this work aimed to (i) obtain information about the physiological and genetic diversity of *B. cinerea* populations in vineyards, and (ii) assess whether the plant management practices that DOs impose, together with the geographical factor, condition the way in which genetic variation is structured in those DOs.

Materials and methods

Botrytis sampling

Botrytis isolates were obtained from bunches of grapes collected from vineyards in six wine-producing areas in Castilla y León (Fig. 1). Representative vineyards of each area were visited in 2002 and 2007 during the first 2 weeks of October (1–2 weeks before harvest). Whenever it was possible, the same vineyards were visited in both years. In 2007, several samples were also collected at the end of August (Table 1). For isolate purification, individual bunches were collected in plastic bags in the field. Once in the laboratory, three or four grapes from each bunch were placed on a potato dextrose agar (PDA) plate and the growth of microorganisms was followed daily. All the isolates that resembled *Botrytis* upon visual inspection of morphological characteristics were selected for further purification. To stimulate sporulation, isolates were grown on PDA supplemented with tomato leaf extract (25% w/v). Single-spore isolates were obtained and stored in 15% glycerol at −80 °C. Table S1 shows the list of isolates used in this work.

DNA isolation and AFLP analysis

Total genomic DNA was obtained from mycelium cultured in liquid Gamborg's B5 salts medium (AppliChem) supplemented with 10 mM sucrose and 10 mM KH_2PO_4 (pH 6.0) using the EZNA Plant DNA kit (Omega Biotek). The AFLP analysis was conducted according to the protocol of Vos *et al.* (1995) using the AFLP Plant Mapping kit for Small Plant Genomes (Applied Biosystems). Preselective amplification of the template was performed with nonselective primers *Eco*RI and *Mse*I. Selective amplification reactions were performed using a *Mse*I-primer with two selective nucleotides and a FAM 5′-labelled *Eco*RI-primer with two selective nucleotides. The amplified products were separated under denaturing conditions in an ABI Prism 310 Genetic Analyzer and using the GeneScan-500 LIZ size standard (Applied Biosystems). The electropherograms were analysed with the GeneMapper v. 4.0 software (Applied Biosystems).

To test the reproducibility of the AFLP methodology, 25 different isolates were selected, and DNA was extracted twice from independent fungal cultures. The electropherograms generated with the 50 DNA samples and the primer combination *Eco*RI-AC/*Mse*I-CA were analysed, generating a matrix in which 161 markers were scored. The genetic similarity coefficients (Dice) of the two DNA samples derived from the same isolate were always higher than 0.97 and for 18 of the 25 isolates considered, the value was 1. The value of 0.97 is used in other works (Bentley *et al.*, 2009) as the cut-off value to consider two individuals as clones, representative of the same haplotype, and this criterium was used in the present work. These results indicated that the methodology used generated highly reproducible AFLP patterns, avoiding the need to perform the entire analysis in duplicate. The primer combinations *Eco*RI-AC/*Mse*I-CA, *Eco*RI-AC/*Mse*I-CC and *Eco*RI-AC/*Mse*I-CG were used for the genetic diversity analysis of the entire population. The three primer combinations generated electropherograms with 100–200 peaks, well separated and representing fragments in the size range between 50 and 500 bp. A binary data matrix computing 388 AFLP markers (161 generated with primers *Eco*RI-AC/*Mse*I-CA, 105 with primers *Eco*RI-AC/*Mse*I-CC, and 122 with primers *Eco*RI-AC/*Mse*I-CG) was created.

Table 1 Summary of the number of *Botrytis* isolates analysed, indicating the denomination of origin (wine-producing area) where the sampled vineyards are located.

Origin	2002 October	2007 August	2007 October	Total
Arribes	8	0	59	67
Cigales	3	2	10	15
Ribera de Duero	15	6	53	74
Rueda	6	0	30	36
Sierra de Salamanca	0	0	44	44
Toro	10	4	33	47
External isolates				6
Total	42	12	229	289

Six external *B. cinerea* reference strains were included.

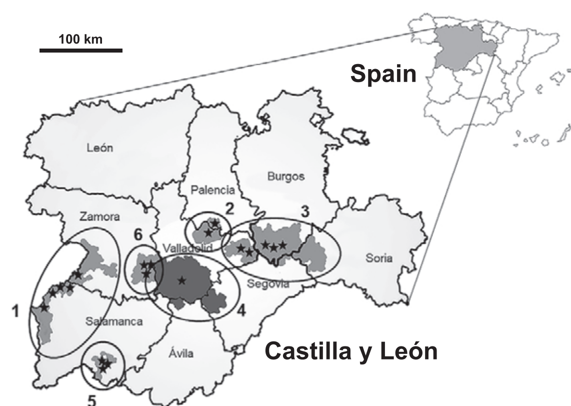

Figure 1 Map of wine-producing areas in Castilla y León, Spain, where samples of grapes were collected from representative vineyards. (1) Arribes; (2) Cigales; (3) Ribera del Duero; (4) Rueda; (5) Sierra de Francia; (6) Toro. *Location of sampled vineyards.

Phylogenetic analysis

Regions of the *G3PDH* and *HSP60* genes from 24 *Botrytis* field isolates were amplified with primer combinations G3PDH-FOR1/G3PDH-REV1 and HSP60F/HSP60R (Staats *et al.*, 2005). The concatenated partial gene sequences (794 nt of *G3PDH* and 732 nt of *HSP60*), together with reference sequences from isolates representative of 24 *Botrytis* species, including one *B. cinerea* group N isolate (B05.10) and one *B. cinerea* group S isolate (ICMP19667) (accession numbers listed in Table S2), were aligned with ClustalW using the default parameters. The alignments were examined by the maximum-likelihood (ML) method in MEGA 7.0, performing 1000 bootstrap replicates. *Sclerotinia sclerotinium* was chosen as an out-group for the phylogenetic trees.

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Detection of polymorphisms and mobile elements

Polymorphism at the *Bc-hch* locus was detected using the PCR-RFLP molecular diagnosis marker previously described (Fournier *et al.*, 2003). The genotype at the microsatellite Bc6 locus was determined by PCR (Fournier *et al.*, 2002). *Botrytis cinerea* genotypes N and S were identified on the basis of the detection by PCR of a 21-bp indel in the *Bcmrr1* gene (Leroch *et al.*, 2013). Determination of the presence or absence of Boty (Diolez *et al.*, 1995) and Flipper (Levis *et al.*, 1997) was made by PCR. To detect Boty the primer pair BotyF4 and BotyR4 was used (Ma & Michailides, 2005). The PCR primer pair F300 and F1550 was used to detect Flipper (Levis *et al.*, 1997). The genotype of isolates at the mating type locus was determined by PCR: the *MAT1-1* idiomorph was detected with primers allowing the amplification of the *MAT1-1-1* gene (MATalpha5′flank: 5′-CACACATACATCATGACGGCTCCC-3′ and MATalpha3′flank: 5′-GAGTGTGTTGATCGTGGAGCCGAG-3′), and the *MAT1-2* idiomorph with primers allowing the amplification of the *MAT1-2-1* gene (HGM5′flank: 5′-AAGATCAGACGGAG TGCATTACCTC-3′ and HGM3′flank: 5′- CTCCTTT CCATAA GTCGTAAGTCGTG-3′). Reactions contained 0.5–1.0 ng μL^{-1} genomic DNA, 200 μM dNTPs, 0.2 μM each primer, 2 mM $MgCl_2$ and 0.05 U μL^{-1} Biotools DNA polymerase (Biotools). PCR was performed using the following conditions: 95 °C for 1 min; 40 cycles of 1 min at 95 °C, 30 s at 61 °C and 1.5 min at 72 °C; and a final step at 72 °C for 5 min.

Fungicide resistance

To identify fenhexamid resistant isolates, a test on malt extract agar (MEA) plates containing 5 mg L^{-1} of fenhexamid was performed (Plesken *et al.*, 2015). To do this, 5 mm agar plugs taken from the border of fungal colonies actively growing on MEA plates were placed on MEA plates amended with the fungicide. The radial growth was recorded 72 h later.

Isolate aggressiveness

The ability of isolates, representative of the different *Botrytis* species identified in this study, to infect *V. vinifera* 'Tempranillo' leaves and table grapes was evaluated using inoculation tests based on mycelial agar plugs. Detached leaves taken from plants grown outdoors were inserted by the petiole into floral foam. Table grapes were placed into 0.5% sodium hypochlorite for 2 min, rinsed with sterile water three times and finally allowed to air dry in a laminar flow hood. Fruits were placed on 120 mm square Petri dishes without lids and attached using double-sided tape. Fruits were arranged sideways in order that their widest part (the equator) was uppermost. Leaves and fruits (at the equator) were wounded with a sterile needle (2 mm deep). Subsequently, 5 mm agar plugs containing fresh mycelium from the edge of fungal colonies actively growing on MEA plates were placed on top of the wound with the mycelium side down. The inoculated materials were incubated in closed plastic boxes, to maintain high humidity conditions, at 22 °C with a 16 h photoperiod. Aggressiveness of isolates on leaves was evaluated by measuring the diameter of lesions at 96 h post-inoculation (hpi). Isolates that did not expand from the inoculation site were considered to be unable to cause any lesion in *Vitis* leaves. They were given a lesion diameter value of 5 mm, which is the diameter of the mycelium plug used as inoculum and represents the lower limit of the range of aggressiveness considered in the evaluations. Aggressiveness on grapes was estimated 120 hpi by following the scale described by Saito *et al.* (2016), which quantifies disease severity on each fruit as follows: 0 = no symptoms; 1 = <25% of surface area decayed; 2 = 25–49% area decayed; 3 = 50–75% area decayed; 4 = >75% area decayed. In each experiment, six inoculations of leaves and six of grapes were evaluated for each isolate and the experiment was repeated three times.

In addition, estimates of aggressiveness on *V. vinifera* leaves of the entire *B. cinerea* field isolate collection were obtained. This was performed as described above, but in nonwounded leaves. The ability of isolates to infect *Prunus domestica* 'Claudia' green leaves and fruits was also evaluated. Inoculations and quantification of aggressiveness were performed as described for *V. vinifera*.

Crosses and determination of morphotypes

Crosses were attempted following the methodology described by Faretra & Antonacci (1987). PDA cultures of isolates were kept for 20 days at 22 °C under continuous light, and morphotypes were then visually characterized as mycelial, conidial or sclerotial-conidial.

Population genetics analysis

Calculation of the Dice's genetic similarity coefficients, generation of the derived UPGMA dendrogram and principal component analysis (PCA) were performed using NTSYS-PC v. 2.11W software. Individuals with a genetic similarity coefficient greater than 0.97 were considered clones.

For differentiation analysis, the whole population was divided into subpopulations, according to the DO where the isolates were collected: (i) Arribes, (ii) Cigales, (iii) Ribera del Duero, (iv) Rueda, (v) Sierra de Salamanca and (vi) Toro. The population was also analysed according to the transposon type of the isolates. The analyses were performed using both the original matrix and the matrix without clones (clone-corrected matrix).

Percentage of polymorphic loci, Nei's genetic diversity statistics and the coefficients of genetic differentiation (G_{ST}) were calculated with POPGENE v. 1.32 software. The genotypic diversity index, G, was calculated using the formula: $G = 1/[\sum fx(x/n)^2]$, were fx is the number of genotypes observed x times in the population and n is the number of isolates analysed. The normalized genotypic diversity was calculated as G/N, were N is the number of haplotypes. It reaches a maximum value of 1 when the number of haplotypes equals the number of isolates. The clonal fraction, FC, was calculated using the formula FC = [1 − (unique genotypes/total genotypes)] × 100. POPGENE v. 1.32 was also used to compute Nei's genetic identity index between each pair of subpopulations in all possible combinations, and to generate the derived unweighted pair group method with arithmetic mean (UPGMA) dendrogram based on the calculated genetic distances between populations. Pearson's correlation coefficient between genetic and geographical distances was calculated with IBM SPSS STATISTICS v. 23. ARLEQUIN v. 3.0 was used for analysis of molecular variation (AMOVA) to determine the distribution of variance components between populations and within populations. Linkage disequilibrium (RBARD algorithm) for the entire population and for each of the six populations according to the DO factor was calculated with MULTILOCUS v. 1.3b, and compared with data generated from 1000 randomizations with the expectation of panmixis. Mating type ratios for each of the populations were tested with χ^2 against an expected 1:1 ratio.

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Results

Botrytis isolates from vineyards of Castilla y León

Representative vineyards from six wine-producing areas in Castilla y León (Fig. 1) were visited in 2002 and 2007. In the 2002 survey, only bunches showing grey mould symptoms in the field were considered for isolate purification. From the collection generated, 42 isolates, each derived from a different grape bunch, were selected for physiological and genetic characterization. In 2007 grape bunches with and without symptoms were considered. From the collection of *Botrytis* isolates of that year, 241 isolates obtained from 134 bunches (75 with symptoms and 59 symptomless) were selected for further analyses (Table 1). Sixty-two of these isolates were obtained from different bunches and 179 isolates were derived from bunches from which multiple isolates (either 2, 3, 4 or 5) had been obtained. No *Botrytis* isolate was recovered from 31 additional symptomless bunches (Table 2).

DNA-based identification and characterization

A PCA revealed a large and diverse group of isolates in which the *B. cinerea* reference strains were included and occupied distant positions (Fig. 2). In addition, a clearly differentiated group composed of seven isolates was identified. A few individuals were found between the two groups. An UPGMA analysis grouped the population in two clades, one clustering the group of seven isolates, and another one with all the other isolates (the dendrogram, with additional physiological and genetic information about the isolates, is shown in Fig. S1). Because the AFLP analysis did not provide information about the taxonomic identity of the isolates, this information was combined with that derived from the analysis of the sequence of two nuclear genes, *G3PDH* and *HSP60* (GenBank accession numbers listed in Table 3), and that generated from PCR-based markers, to differentiate species and genotypes. In the phylogenetic analysis carried out (Fig. 3), the seven isolates that formed a clade in both the PCA and the dendrogram were grouped with a strong bootstrap value together with *B. prunorum*. Isolate B209 grouped unambiguously with *B. pseudocinerea*, and isolates B568 and B572 appeared to represent an undescribed *Botrytis* species closely related to *B. californica* and *B. sinoviticola*. Isolates B381, B329 and B380 clustered with the *B. cinerea* strains (Fig. 3), forming part of a clade more closely related to *B. pelargonii* and to *B. fabae*. These results confirmed that the large population identified in the PCA and in the AFLP dendrogram is composed of *B. cinerea* isolates. A PCR-RFLP analysis of the *Bc-hch* gene confirmed that isolates B209, B568 and B572 were not *B. cinerea* because a *hch-1* allele was amplified in the three samples (Fig. S2a; Fournier *et al.*, 2003). It was possible to differentiate isolate B209 from isolates B568 and B572 by genotyping them at the Bc6 microsatellite locus (Fig. S2b; Fournier *et al.*, 2002; Walker *et al.*, 2011). Taken together, and in the absence of specific molecular discrimination markers, these observations indicated that isolates B568 and B572 were *B. californica* isolates or represented isolates belonging to a distinct genetic entity closely related to *B. californica* and to *B. sinoviticola*.

The large set of *B. cinerea* isolates was further investigated in order to determine if members of the two *B. cinerea* genotypes, S and N, could be identified. To do this, the presence (S genotype) or absence (N genotype) of a 21-bp indel in the *Bcmrr1* gene was analysed by

Table 2 Summary of the number per bunch and genetic similarity of *Botrytis* isolates recovered from bunches of grapes sampled in 2002 and 2007.

Isolates per bunch	2002 No. bunches	2007 No. bunches	2007 No. bunches[a]	Average genetic similarity (± SD)[b]
0	—	31	—	—
1	42	62	—	—
2	—	49	49	0.883 (0.080)
3	—	12	11	0.879 (0.068)
4	—	10	9	0.847 (0.079)
5	—	1	1	0.876 (0.077)
Total	42	165	—	—

[a]Number of bunches from which more than one *B. cinerea* isolate were recovered in 2007 and analysed in this study.

[b]Average genetic similarity based on Dice's genetic similarity coefficients between *B. cinerea* isolates purified from the same bunch.

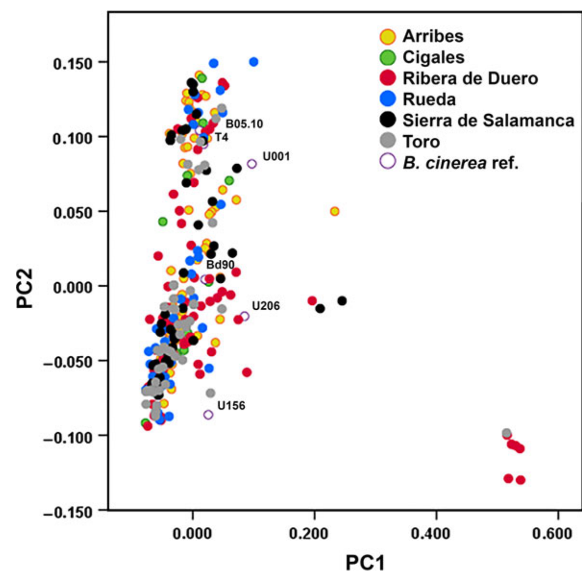

Figure 2 Principal component analysis based on Dice's genetic similarity coefficients derived from AFLP data of *Botrytis* isolates from different wine-producing areas in Castilla y León. Scatter plot shows principal component 1 (PC1) versus principal component 2 (PC2). Coloured circles indicate the wine-producing areas sampled, as shown in the key. Empty circles represent *B. cinerea* external reference isolates.

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Table 3 GenBank accession numbers of the *G3PDH* and *HSP60* sequences determined from *Botrytis* field isolates.

Isolate	Species	GenBank accession number	
		HSP60	*G3PDH*
B65	*B. cinerea*	MH260415	MH277450
B206	*B. cinerea* group S	MH260416	MH277451
B207	*B. prunorum*	MH260417	MH277441
B209	*B. pseudocinerea*	MH277440	MH277439
B211	*B. prunorum*	MH277421	MH277441
B217	*B. cinerea* group S	MH260419	MH277452
B249	*B. cinerea* group S	MH260420	MH277453
B251	*B. cinerea* group S	MH277429	MH277454
B329	*B. cinerea*	MH277430	MH277455
B351	*B. cinerea*	MH277422	MH277443
B371	*B. cinerea*	MH277431	MH277456
B380	*B. cinerea*	MH277432	MH277457
B381	*B. cinerea*	MH277433	MH277458
B400	*B. prunorum*	MH277423	MH277444
B425	*B. prunorum*	MH277424	MH277445
B444	*B. cinerea*	MH277434	MH277459
B446	*B. prunorum*	MH277425	MH277446
B448	*B. cinerea*	MH277435	MH277460
B459	*B. cinerea*	MH277436	MH277461
B509	*B. prunorum*	MH277426	MH277447
B555	*B. cinerea* group S	MH277437	MH277462
B568	Related to *B. californica*	MH277427	MH277448
B572	Related to *B. californica*	MH277428	MH277449
B585	*B. cinerea* group S	MH277438	MH277463

PCR (Leroch *et al.*, 2013). Most isolates were N genotype; only six isolates (B206, B217, B249, B251, B555, B585) were identified as S genotype (Fig. S2c). The sequences of the *G3PDH* and *HSP60* genes of the six S isolates were determined together with the sequences of three additional N isolates, B65, B448 and B459. As expected, all these isolates clustered together in the same group as the *B. cinerea* reference strains and strains B329, B380 and B381. Within this group there was no clustering of the S isolates. In the AFLP tree, isolate B247 was found to be closely related to isolate B251 and isolate B206 to B217, but, when considering the similarities between the six members of this genotype, they were found to be dispersed in the lower part of the dendrogram (Fig. S1). Inspection of the AFLP dendrogram and of the PCA plot indicated that there was no obvious clustering according to the wine-producing area where the isolates were collected.

Physiological characterization of *Botrytis* field isolates

As isolates belonging to three different species, in addition to *B. cinerea* isolates, were identified in the collection of field isolates, physiological differences between species were evaluated first. To this end, isolates identified as closely related to *B. prunorum* and those identified as related to *B. californica* and *B. sinoviticola*, together with the *B. pseudocinerea* isolate B209, three *B. cinerea* isolates (two N isolates, B448 and B547, and one S isolate, B249), and the *B. cinerea* reference strain B05.10, were analysed for their capacity to infect *V. vinifera* leaves and grapes. Only the *B. cinerea* and *B. pseudocinerea* isolates infected *V. vinifera* leaf tissue. The other two groups of isolates were unable to cause lesions in wounded leaves and did not expand at all from the inoculation site (Fig. 4a). Thus, the *B. cinerea* and the *B. pseudocinerea* isolates were physiologically differentiated from the rest of the isolates being analysed.

All the isolates caused symptoms on inoculated wounded grapes, although the *B. prunorum* and the *B. californica* related isolates displayed a more limited capacity to colonize the berries (Fig. 4b).

The same set of isolates was inoculated in *P. domestica* leaves and fruits. The isolates related to *B. californica* and the *B. prunorum* isolates infected *P. domestica* leaves and behaved similarly, but they were all less aggressive than the *B. cinerea* and the *B. pseudocinerea* isolates (Fig. S3a). Interestingly, the *B. prunorum* isolates were on average as aggressive as the *B. cinerea* isolates in fruits while the isolates related to *B. californica* behaved as weak colonizers (Fig. S3b).

Botrytis pseudocinerea is reported to exhibit a high level of natural resistance to fenhexamid (Fournier *et al.*, 2003; Walker *et al.*, 2011; Plesken *et al.*, 2015). For this reason, isolates representative of the different *Botrytis* species identified in this study were investigated for their resistance to fenhexamid. As shown in Figure 5a, only isolate B209 was resistant to fenhexamid, providing further evidence of its nature as *B. pseudocinerea*.

Data on the aggressiveness in *V. vinifera* leaves was also obtained for 271 of the 273 *B. cinerea* field isolates investigated. Large variation in aggressiveness was observed in the population. In the box plot shown in Figure 6a the mean value is close to the median, the interquartile range is small, and the two whiskers are almost the same length. This indicates a normal distribution of values, with few isolates being either highly aggressive or poorly aggressive, and most isolates displaying intermediate values of aggressiveness. Interestingly, only six isolates were completely unable to spread from the initial inoculation site (isolate B459 is a representative individual of this group in Fig. 6b,c). These isolates can be considered *B. cinerea* nonaggressive natural variants.

Variation in morphotypes, mating type and transposons

The *B. cinerea* field isolate population was also diverse in relation to morphotypes. When cultured on PDA at 22 °C under permanent artificial light conditions, three morphotypes were identified (Fig. 5b): the most abundant was the conidial type (215 isolates) followed by the sclerotial-conidial type (50 isolates). The mycelial type was rare (six isolates). No isolate producing only sclerotia was observed (Table 4; Fig. S1).

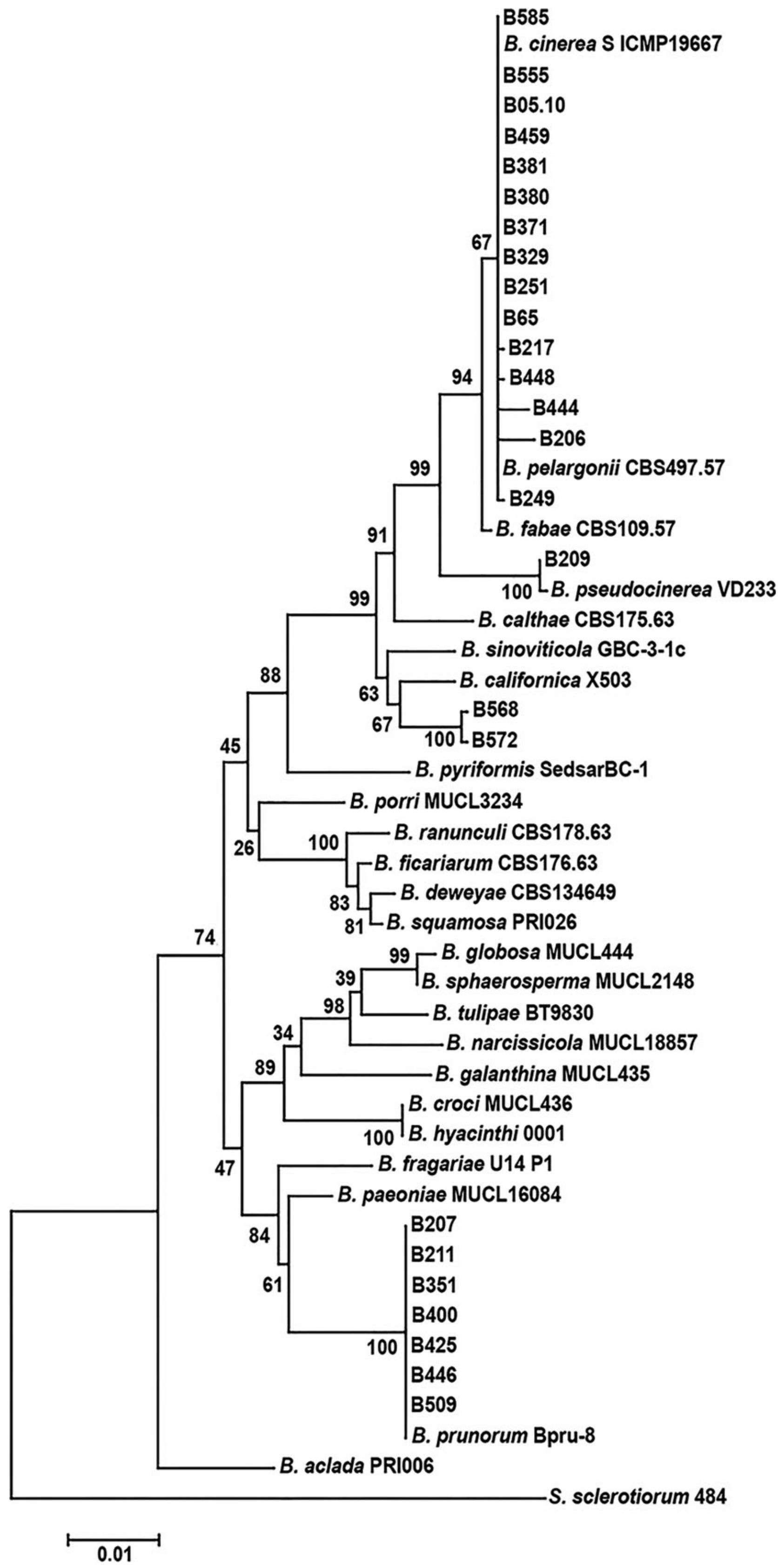

Figure 3 Unweighted pair group method with arithmetic mean dendrogram of selected *Botrytis* isolates from the field isolates collected in this study and of reference isolates of different species of the genus, based on concatenated partial sequences of the *HSP60* and *G3PDH* genes. *Sclerotinia sclerotiorum* was used as the out-group.

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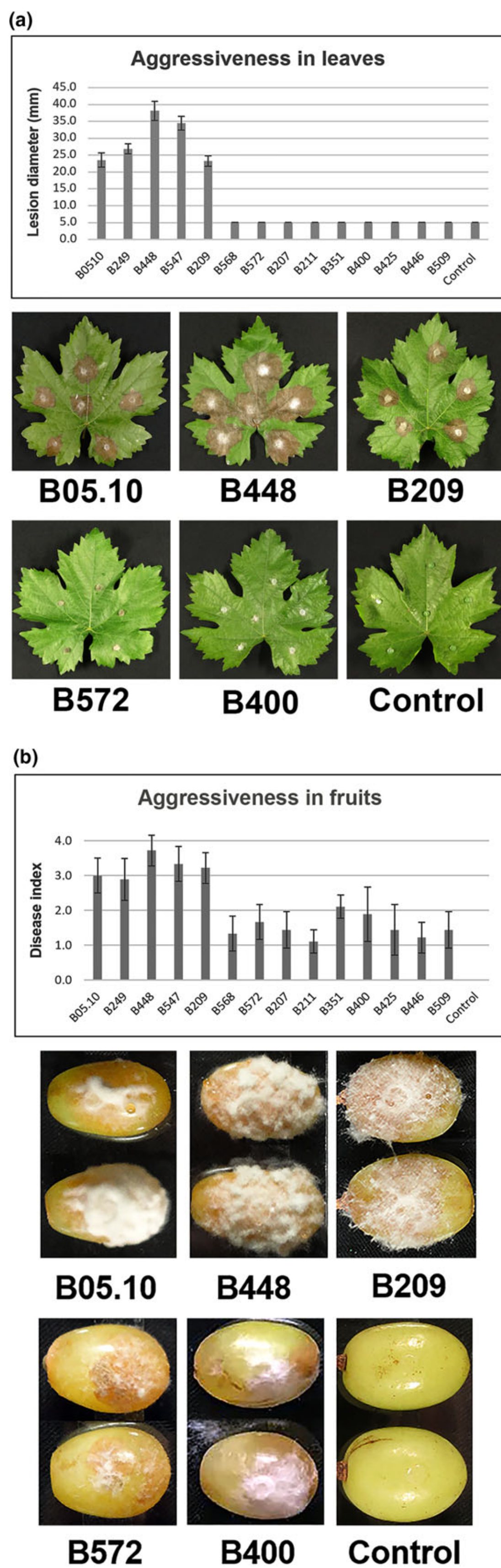

Figure 4 Evaluation of aggressiveness in *Vitis vinifera* leaves (a) and table grapes (b) of the isolates identified as *Botrytis pseudocinerea*, *B. prunorum* and related to *B. californica*, together with selected *B. cinerea* isolates. The bars indicate mean values from three independent experiments. Standard deviations are shown. Wound-inoculated leaves and fruits were incubated at 22 °C with a 16 h photoperiod for 96 and 120 h, respectively. Images of leaves and fruits inoculated with representative isolates are shown.

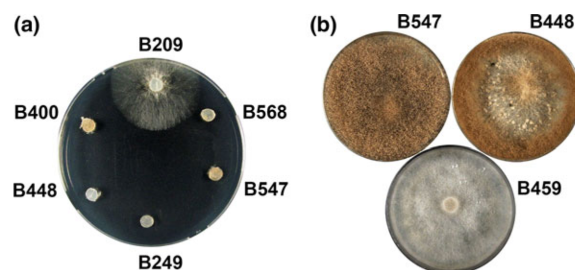

Figure 5 (a) Evaluation of resistance to fenhexamid in isolates representative of the different *Botrytis* species identified in this study. (b) Isolates representative of the different morphotypes identified within the population of *Botrytis cinerea* field isolates: B547, conidial; B448, conidial-sclerotial; B459, mycelial.

The distribution of sexual types in the experimental population was investigated by determining the mating type idiomorph in each isolate by PCR. Both mating types appeared to be present in similar numbers, 122 isolates of mating type 1-1 and 126 of mating type 1-2. Both idiomorphs were detected in a small number of isolates (12), and the mating type could not be determined for 13 isolates (Table 4; Fig. S1). In the whole population, as well as in the six DO subpopulations, no significant deviation from the 1:1 ratio was found for the two mating types.

Regarding transposons, Boty alone was detected in 176 isolates (*boty* type), and both transposons were found in 96 isolates (*tranposa* type; Table 4; Fig. S1). Only one *flipper* isolate was found and no *vacuma* isolate was identified. The six DO subpopulations showed a similar distribution of the transposon types. The *B. pseudocinerea* B209 isolate belonged to the *boty* type (Fig. S1). In the group of isolates related to *B. prunorum*, the *transposa* and the *boty* types were found. In the group of *B. californica* related isolates, one, B572, was shown to be of the *boty* type while the other, B568, was found to be of the *flipper* type (Fig. S1).

Intra- and interspecific crosses

Crosses were made between three field isolates and the reference strain SAS56 of *B. cinerea* and isolates representative of the three other species identified in the analysis. As shown in Table 5, crosses were successful only in combinations involving the reference strain SAS56 and field isolates identified as *B. cinerea*, corroborating that

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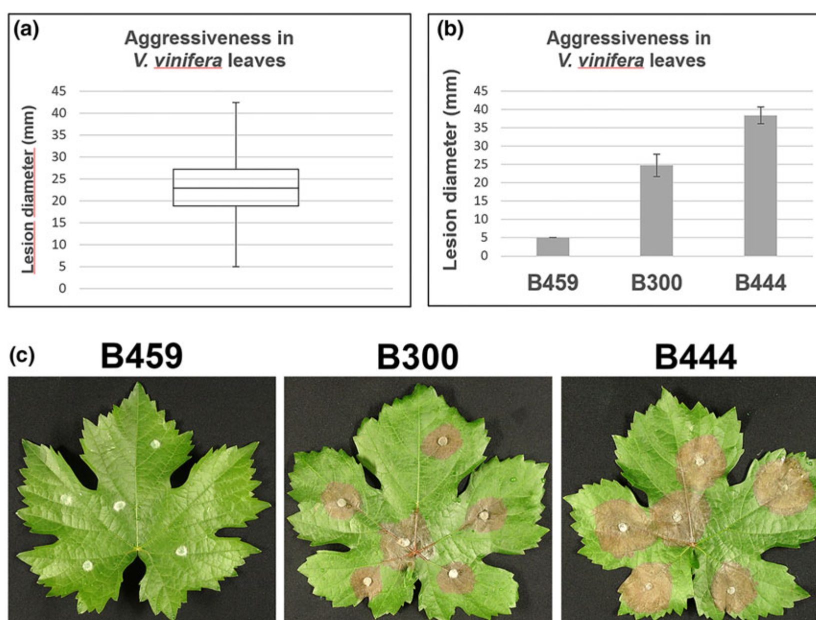

Figure 6 (a) Box plot summarizing the distribution of the phenotype 'Aggressiveness in *Vitis vinifera* leaves' in the population of *Botrytis cinerea* field isolates analysed. (b) Aggressiveness of representative isolates of *B. cinerea* in *V. vinifera* leaves: B459 is a nonaggressive isolate, B300 is a medium-aggressiveness isolate and B444 is a highly aggressive isolate. Inoculated leaves were incubated at 22 °C with a 16 h photoperiod for 96 h. (c) Phenotypes of leaves inoculated with isolates B459, B300 and B444.

Table 4 Number of *Botrytis cinerea* field isolates in each population collected from vineyards in different wine-producing areas, classified according to their mating type, morphotype, aggressiveness and transposon content.

		Mating type				Morphotype				Aggressiveness			Transposons		
Denomination of origin (DO)	Isolates	Mat1-1	Mat1-2	Both	ND	C	SC	M	ND	Aggr.	Nonaggr.	ND	Boty	Flipper	Both
Arribes	67	31	31	1	4	45	21	1	—	67	—	—	42	1	24
Cigales	15	10	3	1	1	13	2	—	—	15	—	—	12	—	3
Ribera del Duero	67	28	31	4	4	57	5	3	2	63	3	—	40	—	27
Rueda	36	11	20	5	—	29	7	—	—	34	1	—	26	—	10
Sierra de Salamanca	42	21	17	1	3	34	7	1	—	40	1	1	27	—	15
Toro	46	21	24	—	1	37	8	1	—	44	1	1	29	—	17
Total	273	122	126	12	13	215	50	6	2	265	6	2	176	1	96

ND, not determined.

isolates B448, B459 and B65 are indeed *B. cinerea* isolates and the others are not. Fruiting bodies were never observed in combinations that included isolates identified by molecular criteria as different species.

Genotypic diversity within the *B. cinerea* field isolates population

To quantify the genetic diversity in *B. cinerea* natural populations from Castilla y León vineyards, and to investigate the way in which this diversity is structured, a population genetic analysis was made. For this purpose, the seven isolates related to *B. prunorum*, the two isolates related to *B. californica* and the *B. pseudocinerea* isolate, together with the six *B. cinerea* external reference strains, were excluded from the AFLP dataset. The matrix derived was used to calculate the genetic similarity coefficients between each pair of isolates and the genetic diversity indices for the entire population and for the six DO subpopulations.

Considering clones as those individuals with a genetic similarity coefficient >0.97, 254 haplotypes were identified in the population including 273 isolates (Table 6): two haplotypes were shared by three individuals each, and 15 haplotypes by two individuals each. Two hundred and thirty-seven haplotypes were unique. These values determine a low clonal fraction (CF = 6.69%) and a high genotypic diversity (G = 236.6; G/N = 0.93). High values of genotypic diversity were also estimated for the six subpopulations considered: it was maximal in Cigales and Rueda, where clones were not detected, and minimal in Sierra de Salamanca, the subpopulation where a higher CF was detected (13.88%). In all cases, the individuals representing the same haplotype were purified from the same bunch of grapes (Fig. S1; Table S1). However, it is interesting to note that these bunches represent only 17 out 70 bunches from which more than one *B. cinerea* isolate was purified and analysed. In all the other cases the isolates derived from the same bunch were different. The average genetic similarity among

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Table 5 Crosses attempted between selected *Botrytis cinerea* field isolates and representative isolates of the three other *Botrytis* species identified in this study.

	Female							
Male	SAS56	B448	B459	B65	B209	B568	B446	B351
SAS56 (1.1)								
B448 (1.1)				+	–			
B459 (1.2)	+	+						
B65 (1.2)		+					–	–
B209 (1.2)		–				–		–
B568 (both)		–						
B446 (1.1)				–				
B351 (1.1)				–				

SAS56 (Faretra *et al.*, 1988) was used as the reference *B. cinerea* strain. +, successful cross; –, fruiting bodies not observed. The mating type idiomorph identified in each isolate is indicated in parentheses. *Botrytis cinerea* isolates are indicated by light grey shading. Self-fertilization crosses (dark grey shading) were not attempted.

isolates from bunches from which several isolates were purified ranged from 0.847 (bunches with four isolates) to 0.883 (bunches with two isolates; Table 2). Taken together, these observations indicate that the genotypic diversity of the *B. cinerea* field population is very high, and multiple infections of the same bunch by genetically different individuals occur frequently.

Botrytis cinerea field isolates population structure

For the total population, 87.63% of the 388 AFLP markers computed were polymorphic (Table 6). In the six DO subpopulations the amount of polymorphic markers ranged from 32.99% in Cigales to 68.56% in Arribes. Nei's gene diversity estimations showed similar values for the six subpopulations, with the smallest value (0.0895) occurring at the Cigales subpopulation. Maintaining just a representative of each haplotype and generating the clone-corrected populations had little impact on Nei's gene diversity indices.

Regarding population differentiation, the G_{ST} values obtained (0.0619 in the original population and 0.0605 in the clone-corrected population) are indicative of low genetic differentiation between subpopulations (Table 7). The estimated number of migrants per generation (*Nm*) (7.58 for the original population and 7.76 for the clone-corrected population) was high, indicating that it is possible to detect a significant movement of individuals between populations, a factor that undoubtedly contributes to limit the divergence of subpopulations. The effect that the presence/absence of transposons in the different individuals could have in structuring the population was also considered. As only one *flipper* type isolate and no *vacuma* types isolates were identified, the analysis took into consideration only two populations, *transposa* and *boty* types. A null differentiation effect was detected (Table 7). Analysis of molecular variance (AMOVA) in the clone-corrected population for the factor 'DO' indicated that most of the genetic variation occurred within populations (95.29%) whereas genetic variation among populations was low (4.71%; Table 8), corroborating a low differentiation level of populations. When the factor 'transposon type' was considered, the amount of variation found among populations was much lower.

Table 6 Summary of statistics comparing populations of *Botrytis cinerea* from different wine-producing areas in Castilla y León, Spain.

Population	n^a	N^b	PL (%)[c]	h^d	H^e	G^f	G/N^g	CF (%)[h]	$\bar{r}_d{}^i$ ($P < 0.01$)
Arribes	67	62	68.56	0.1099	0.1119	56.82	0.91	6.45	0.02880
Cigales	15	15	32.99	0.0895	0.0895	15.00	1	0	0.02881
Ribera del Duero	67	63	57.73	0.1032	0.1045	59.85	0.95	6.35	0.03059
Rueda	36	36	47.42	0.1006	0.1006	36.00	1	0	0.02243
Sierra de Francia	42	36	51.29	0.1034	0.1064	31.50	0.88	13.88	0.03289
Toro	46	42	52.58	0.0991	0.1016	39.18	0.93	9.52	0.01955
Total	273	254	87.63	0.1096	0.111	236.6	0.93	6.69	0.01799

[a]Number of isolates analysed.
[b]Number of isolates adjusted for clonal haplotypes (Dice's coefficient of similarity >0.97).
[c]Proportion of polymorphic markers.
[d]Nei's gene diversity.
[e]Nei's gene diversity adjusted for clonal haplotypes.
[f]Genotypic diversity.
[g]Normalized genotypic diversity.
[h]Clonal fraction.
[i]Linkage disequilibrium.

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The low degree of differentiation detected when considering the factor 'DO of collection' was further characterized by computing Nei's genetic identity for each pair of subpopulations. These genetic distance values were low, ranging from 0.0035 for Ribera del Duero–Toro to 0.0135 for Sierra de Salamanca–Ribera del Duero. In an UPGMA dendrogram based on Nei's genetic identity values (Fig. 7) two clades could be identified. The first one includes the Arribes and the Sierra de Salamanca subpopulations, and the second one clusters the other four subpopulations. This grouping suggests some geographical effect on the genetic relationship between the *B. cinerea* subpopulations because Arribes and Sierra de Salamanca are located in the southwest of Castilla y León, in areas topographically and environmentally different from the other four wine-producing areas located in the central region. To characterize this effect more precisely, a correlation analysis of the genetic distances and the lineal geographic distances estimated from the geographic centre of each wine-producing area was carried out (Fig. 8). When the six subpopulations were considered, Pearson's correlation coefficient between the two variables was 0.695, suggesting the existence of a positive relationship between genetic and geographic distances. When only the four DOs located in the central area of Castilla y León were considered, the correlation coefficient was lower and negative (−0.455). Therefore, at a local scale, geographic distances explain poorly the genetic differences displayed by the subpopulations from the different DOs, but at a greater scale, geographical distance affects the genetic structure of *B. cinerea* populations.

Linkage disequilibrium analysis

To estimate whether random mating had occurred in the *B. cinerea* population, a linkage disequilibrium analysis was carried out. The standardized index of association ($\bar{r}_d$) was significantly different ($P < 0.01$) from the expectation of random mating ($\bar{r}_d = 0$) for all the populations (clone-corrected; Table 6), indicating that the null hypothesis of random mating can be rejected. However, all the values were low ($\bar{r}_d < 0.25$), reflecting a low level of clonality.

Discussion

Grey mould is a ubiquitous disease in vineyards worldwide, including the wine-producing areas of Castilla y León. Most isolates obtained from grape bunches in this study were identified as *B. cinerea*. Although with differences in aggressiveness, the majority were able to infect *V. vinifera* leaves. In addition, 10 isolates belonging to three other *Botrytis* species were identified. A phylogenetic analysis and its resistance to fenhexamid indicated that isolate B209 belongs to *B. pseudocinerea* (Fournier *et al.*, 2003; Walker *et al.*, 2011; Plesken *et al.*, 2015). Interestingly, this was the only one of the 10 non-*B. cinerea* isolates identified that could infect *V. vinifera* leaves, indicating a fundamental physiological difference of this isolate from the other nine.

Botrytis pseudocinerea was formally described as a species in 2011 (Walker *et al.*, 2011) and is considered a cryptic species living in sympatry with *B. cinerea* in the same hosts (Walker *et al.*, 2011; Johnston *et al.*, 2013; Plesken *et al.*, 2015). This species had not been reported previously in Spain. Only one isolate of this species was recovered in the current survey. If this is representative of its frequency in the natural populations considered, *B. pseudocinerea* does not seem to have an important role as a causal agent of grey mould in the vineyards of Castilla y León. However, *B. pseudocinerea* appears to be more abundant on dead flower remains and in spring (Walker *et al.*, 2011; Johnston *et al.*, 2013; Plesken *et al.*, 2015), and the present survey was made at the end of August and mostly in October, collecting only bunches of grapes for purification of isolates.

Phylogenetic analysis clustered the other nine non-*B. cinerea* isolates into two groups. Seven isolates were identified as *B. prunorum*, a species described as living in sympatry with *B. cinerea* on plums in the Central Valley of Chile (Ferrada *et al.*, 2016). Additional support for this classification came from the physiological characterization of these seven isolates. They appeared to be less aggressive in leaves than the *B. cinerea* isolates, but infected plum fruits efficiently. This represents the first report of *B. prunorum* in Spain. The two remaining isolates were grouped in a clade that includes *B. californica*, recently described as a cryptic species sympatric with *B. cinerea* on blueberries and table grapes in California (Saito *et al.*, 2016), and *B. sinoviticola*, isolated from *Botrytis*-infected table grapes in two Chinese provinces (Zhou *et al.*, 2014). However, the bootstrap value observed makes the taxonomic identity of these isolates

Table 7 G_{ST} statistics in the original and in the clone-corrected *Botrytis cinerea* field isolates population subdivided according to the denomination of origin (DO) and transposon type.

Factor	G_{ST}	*Nm*
DO		
Original population	0.0619	7.58
Clone-corrected population	0.0605	7.76
Transposon		
Original population	0.0076	65.40
Clone-corrected population	0.0075	65.96

Table 8 Analysis of molecular variation among and within populations of *Botrytis cinerea* field isolates from vineyards in different denominations of origin (DO) in Castilla y León, Spain.

Factor	Source of variation	d.f.	Variance component	% of variation
DO	Among populations	5	1.02793	4.71
	Within populations	248	20.79704	95.29
	Total	253	21.82497	
Transposon type	Among populations	1	0.14190	0.65
	Within populations	251	21.54632	99.35
	Total	252	21.68822	

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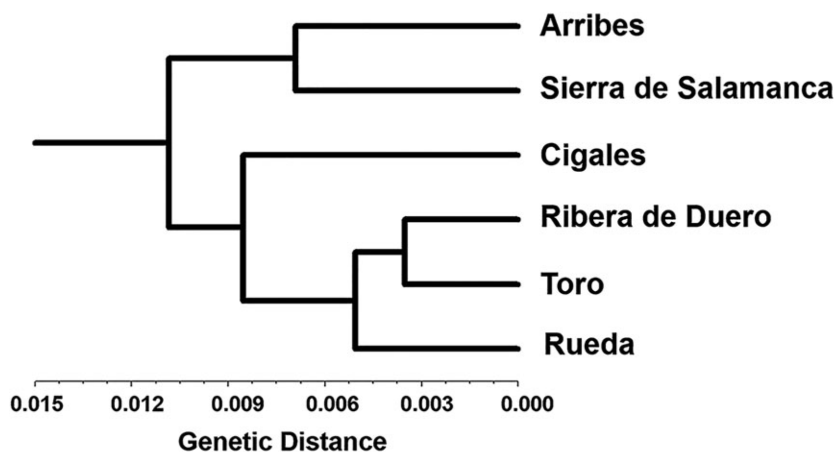

Figure 7 Unweighted pair group method with arithmetic mean dendrogram generated with Nei's genetic distance coefficients between populations of *Botrytis cinerea* from vineyards in different wine-producing regions (Denominations of Origin) in Castilla y León.

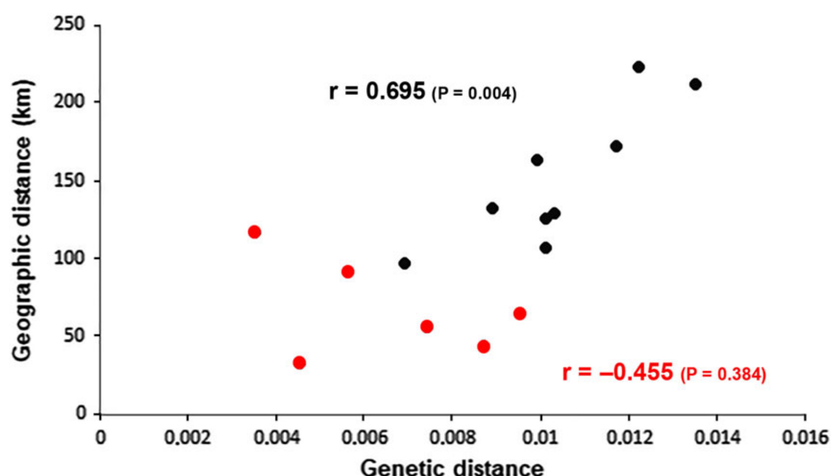

Figure 8 Correlation between genetic and geographic distance among field populations of *Botrytis cinerea* from vineyards in Castilla y León. Correlation values in black were calculated considering all the populations. Values in red were calculated considering only the four populations from the central area of Castilla y León (red dots).

uncertain. Physiologically, the two isolates behaved as weak pathogens in both *P. domestica* leaves and fruits. These nine isolates, although unable to infect *V. vinifera* leaves, caused decay of grapes. They might represent spillover individuals belonging to a genetic group specialized on another host, a situation that is reported frequently (Fournier & Giraud, 2008; Leyronas *et al.*, 2015; Walker *et al.*, 2015). The low frequency with which they have been isolated from vineyards and the reduced severity of symptoms they produce in table grapes makes it unlikely that they can have a relevant impact on grey mould development in the field.

All the other field isolates characterized in this work belong to *B. cinerea*. Physiologically, this population was highly diverse. Most isolates displayed a conidial type in the absence (more frequently) or in the presence of sclerotia, while a few isolates appeared to be of the mycelial type. Both mating types were found, being equally represented in the population. When tested on *V. vinifera* leaves, aggressiveness followed a normal distribution. This is the distribution expected for a complex, quantitative, multigenic character such as the ability of a generalistic necrotroph like *B. cinerea* to infect host tissues (Amselem *et al.*, 2011). Interestingly, a number of isolates unable to cause lesions were identified in the present analysis. These can be considered nonpathogenic natural variants that deserve further attention, as they can provide valuable information about the genetic factors involved in aggressiveness. Their characterization is currently underway in the laboratory.

The population was also very diverse genetically. Most isolates were of the N type, but a few were group S isolates, making this the first description of the *B. cinerea* S genotype in Spain. The six S isolates were obtained at four different DOs and they do not form a genetically uniform clade according to the AFLP analysis. Notably, physiological differences were also observed among the S isolates, with isolate B217 being of the mycelial morphotype and nonaggressive. It has not yet been evaluated if S isolates occur in strawberry or other crops in Spain, as the current analysis was restricted to vineyards of Castilla y León. Nevertheless, the results indicate that, although at low frequencies, the *B. cinerea* S genotype is widely distributed. In its original description in Germany, the S genotype was restricted to strawberry (Leroch *et al.*, 2013), but this may be the result of the wide

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B. cinerea population becoming selected by host-related factors or by differences in local management of fungicide use, as suggested previously (Johnston *et al.*, 2013).

High values of normalized genotypic diversity and low values of clonal fraction characterize the populations investigated. Only a few haplotypes (17 out of 254) were not unique and those were found only in two or three individuals. In all cases, the members of the same haplotype were purified from the same bunch of grapes. They probably derived from an individual that multiplied asexually nearby. This is as expected for *B. cinerea* infections, given the capacity of the fungus to sporulate profusely on infected tissues. However, it is interesting to note that in 75% of the bunches from which more than one isolate was recovered, the individuals had different genotypes. Therefore, multiple infections occur frequently in the vineyards visited, a situation previously reported for vineyards in France (Giraud *et al.*, 1997). If only bunches from which one isolate was recovered had been considered, as is often done, then the genotypic diversity detected would have been maximal.

Low levels of clonality and high levels of genotypic diversity are considered to indicate recombination acting in the population (Beever & Weeds, 2004). Furthermore, the presence of the two mating types distributed in equal frequencies supports the occurrence of sexual reproduction (Giraud *et al.*, 1997; Beever & Weeds, 2004). Nevertheless, linkage disequilibrium, indicating that full panmixis does not occur, was detected. Therefore, taking all the above evidence together, a mixed mode of reproduction in the *B. cinerea* populations in the vineyards of Castilla y León can be inferred.

This study aimed to determine the effect of anthropic activities deriving from the existence of DOs. Differentiation coefficients, as well as the AMOVA, indicated a low differentiation among DOs. As the DOs are restricted to geographic areas, this possible anthropic effect can be considered linked and perhaps partially dependent on the geographic effect. For a fungus that produces large amounts of spores, easily dispersed by several means, with a wide host range and adapted to grow saprophytically in different substrates, the free movement of genotypes would be expected in large open-air cropping systems and in the absence of major natural elements that might prevent dispersal. That would imply a high correlation between genetic distances and geographic distances. In agreement with this reasoning, population genetic differentiation has been observed at the continental scale or when important geographic barriers are present, with little or no differentiation at the national or regional scales (reviewed by Walker, 2016). The analysis performed in Castilla y León vineyards indicates that when the six wine-producing areas are considered, a moderate correlation is observed between genetic and geographic distances; however, when only the four areas located at the central region of Castilla y León are considered, the correlation is low and negative. Therefore, at a regional scale, geography can explain part of the genetic difference between populations, but at a more local scale its effect is not relevant. It is possible that the management practices that the growers follow in order to elaborate and commercialize wines at each DO explain at least part of this differentiation. These practices condition the way in which the harvest is carried out and the movement of the harvested products within the DOs, probably limiting the free dispersion of the pathogen. It is interesting to note that the four DOs located in the central area of Castilla y León, Cigales, Ribera del Duero, Rueda and Toro, were established many years ago, with a prolonged and consolidated activity over time. The other two areas of production are of recent creation, with more limited activity.

In summary, this work provides a description of the physiological and genetic diversity of the *B. cinerea* natural populations in the vineyards of Castilla y León. Information about basic aspects of its biology can be derived, which may help the growers and producers to understand the behaviour of the fungus in the field and in relation to cultural and management practices. Furthermore, natural variants of *B. cinerea* were identified and their characterization will contribute to genetic dissection of the interaction between the fungus and its host.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. Complete unweighted pair group method with arithmetic mean (UPGMA) dendrogram based on Dice's coefficients of similarity derived from the AFLP data of the *Botrytis* field isolates characterized in this study. Physiological and genetic information is provided for each isolate.

Figure S2. Molecular markers used for *Botryti*s species and genotype discrimination. (a) PCR-RFLP analysis of the *Bc-hch* gene (Fournier *et al*., 2003) in the indicated isolates. (b) Genotype at the Bc6 locus (Fournier *et al*., 2002) in the indicated isolates. (c) Determination of the presence/absence of a 21-bp indel in the *Bcmrr*1 gene (Leroch *et al*., 2013) in the indicated isolates.

Figure S3. Evaluation of aggressiveness in *Prunus domestica* leaves (a) and fruits (b) of the isolates identified as *Botrytis pseudocinerea*, *B. prunorum* and related to *B. californica*, together with selected *B. cinerea* isolates. Wounded inoculated leaves and fruits were incubated at 22 °C with a 16 h photoperiod for 96 and 120 h, respectively. Images of leaves and fruits inoculated with representative isolates are shown.

Table S1. Field isolates analysed in this work.

Table S2. GenBank accession numbers of sequences retrieved from NCBI and used for building the phylogenetic tree.

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