Chapter 6
Diversity Within and Between Species of *Botrytis*

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Abstract The genus *Botrytis* is highly diverse, with numerous species differing in terms of their biology, ecology, morphological features and host range. Progress in molecular genetics, and the development of relevant phylogenetic markers in particular, has resulted in the establishment of ≈ 30 species, a hybrid and a species complex. At least seven new species have been identified in the last decade, albeit with limited scientific support in some cases. *B. cinerea* has long been known to display broad population diversity, possibly due to intense recombination and large population sizes. The introduction of powerful markers, such as SSRs, has provided new insight into the respective contributions of the forces driving this diversity. It has recently been shown that populations may be structured, not only by the host plant as shown in preliminary studies, but also by other factors, such as cropping system, geography and fungicide applications. Evidence of recombination and gene flow, between and within compartments, has also been obtained. Finally, this chapter focuses on the biological and genetic characteristics of *Botrytis* spp. favouring their adaptation to their local environment and speciation. This information is particularly useful for improving the management of diseases on cultivated hosts.

Keywords Adaptation • Phylogeny • Population genetics • Species • Structuring factor

6.1 Introduction

Diversity is the degree of variation within a group. In population biology, diversity makes it possible to distinguish between individuals on the basis of the variation of phenotypic (morphological, physiological or behavioral characteristics) or genotypic (based upon the examination of one or several combined loci) criteria.

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Evolution is thus based on variation of the characteristics of organisms at several scales: (1) differences between individuals within a population, and/or (2) between populations and species. At the population scale, the frequencies of the different variants, characterized by their alleles at the loci considered, can be predicted by Mendel's laws in ideal conditions of Hardy-Weinberg equilibrium. Any violation of this equilibrium modifies the frequencies of the variants in populations. The evolutionary path of a given allelic variant may therefore be affected by the effects of four evolutionary forces (*i.e.* genetic drift, mutation, migration, and selection), acting in isolation or in combination, and of the mode of reproduction, which may affect the probability of particular gametes coming together during mating. In some cases, divergence between populations may be driven by ecological forces alone (ecological specialization), with the selection of specific variants in different environments. This may ultimately lead to speciation if reproductive isolation occurs. Speciation thus links microevolution (occurring within populations and species) and macroevolution (diversification of higher taxa). This chapter aims to describe the diversity occurring within the genus *Botrytis* and within individual species of this genus, and the determinants of this diversity. We will focus, in particular, on the factors underlying the partitioning of the population, with the long-term aim of adapting agricultural practices appropriately so as to decrease the impact of grey mould.

6.2 Diversity in the Genus *Botrytis*

6.2.1 General Comments

The definition of the term "species" has long been a matter of debate, mostly because it has been confused with the species criteria (or species concepts) used to delimit species. It is now widely agreed by biologists that species are segments of evolutionary lineages that evolve independently from each other, without exchanging genes any more. Speciation can thus be defined as the splitting of one species into two or more daughter species (Samadi and Barberousse 2006; De Queiroz 1998).

Up to 22 species concepts are available for species delimitation (Mayden 1997), but it is generally accepted that several of these concepts are required for the correct definition of species boundaries (Sites and Marshall 2004). Four of these concepts are recognized as being of particular utility in fungi (Giraud et al. 2008; Taylor et al. 2000): (1) the biological species concept (BSC), focusing on reproductive isolation, (2) the morphological species concept (MSC), focusing on morphological divergence, (3) the ecological species concept (ESC), focusing on adaptation to a particular ecological niche and (4) the phylogenetic species concept (PSC), and its extension, genealogical concordance phylogenetic species recognition (GCPSR), focusing on the lack of gene flow between lineages and nucleotide divergence.

6.2.2 *Taxonomy and Species Identification in the Genus* Botrytis

Botrytis spp. are Ascomycete fungi of class *Leotiomycetes*, order *Heliotiales* and family *Sclerotiniaceae*. The genus *Botrytis* was first described in 1729 by Pier Antonio Micheli, who listed it in the "*Nova Plantarum Genera*". Most of the species were eventually established by Hennebert (1973), Groves and Loveland (1953), and Beever and Weeds (2004). This genus is very closely related to *Sclerotinia*, with the proteins encoded by the genomes of *B. cinerea* and *S. sclerotiorum* displaying 83 % identity (Amselem et al. 2011). The genus *Botrytis* contains more than 30 species (Table 6.1). The life cycle of *Botrytis* spp. can be generalized as follows. There is a somatic (vegetative) stage, in which the mycelium produces asexual macroconidia, sclerotia and microconidia (spermatia). This is essentially the anamorph stage of *Botrytis*. There is then a sexual stage, during which microconidia may fertilize sclerotia to produce apothecia, in which meiosis occurs and ascospores are produced (the *Botryotinia* teleomorph stage, named by Whetzel, 1945). More details about the biology and genetics of *Botrytis* species are provided in Chaps. 2 and 3. Not all *Botrytis* spp. have known teleomorphs. Moreover, in a recent paper, Wingfield et al. (2012) pleaded the cause of a simplification of fungi taxonomy, which has become possible with the advent of molecular tools, which have made the need to split morphs obsolete. At a recent meeting (Bari, Italy, June 2013), the *Botrytis* research community unanimously decided to retain the asexual name, *Botrytis*, which is also the oldest and most widely used name, for fungi from this genus (Chap. 1). The teleomorph name should therefore no longer be used. Johnston et al. (2014) put forward the proposal for this taxonomic change and provided *Botrytis* names for the only two *Botryotinia* species lacking a *Botrytis* equivalent.

6.2.2.1 Identification on the Basis of the Morphological Species Concept

Botrytis spp. have mostly been delimited on the basis of morphological and culture characteristics (MSC species concept). All species of *Botrytis* display the common morphological feature that gave rise to the name of the genus: the botryose, or cluster-of-grape shape of the conidiophores (the conidiophores bear clusters of macroconidia that resemble clusters of grapes). The size and shape of the macroconidia have repeatedly been used as criteria for distinguishing species, as have the number, organization and size of the sclerotia and the morphology of the mycelium on artificial media (Jarvis 1977; Hennebert 1973; Zhang et al. 2010a, b; Lorenzini and Zapparoli 2014; Li et al. 2012). However, many species are morphologically similar (*e.g. B. cinerea* and *B. pseudocinerea* (Walker et al. 2011); *B. aclada* and *Botrytis* sp. B83 (Lorenzini and Zapparoli 2014)). Growing conditions may also significantly influence variation (Grindle 1979; Martinez et al. 2003). Moreover, high levels of morphological diversity may also be recognized in a single species.

Table 6.1 Some species of *Botrytis*

Botrytis sp. (anamorph)[a]	*Botryotinia* sp. (teleomorph)[e]	Mating system	Major hosts	Refs[f]
B. aclada Fresen.	–	–	*Allium*	4,7,8,16
B. allii Munn	–	–	*Allium*	4,8,16
B. anthophila Bondartsev[b,a]	–	–	*Trifolium*	4,8,12
B. byssoidea Walker[c]	–	–	*Allium*	4,7,8,16
B. calthae Hennebert	*Bt. calthae* Hennebert & Elliott	–	*Caltha*	4,6,7,8,9,25
B. caroliniana	–	–	*Rubus*	17
B. cinerea Pers.:Fr	*Bt. fuckeliana* (de Bary) Whetzel	Heterothallic	Polyphagous	4,7,8,9
B. convallariae (Kleb.) Ondřej[a]	–	–	*Convallaria*	8
B. convoluta Whetzel & Drayton	*Bt. convoluta* (Drayton) Whetzel	–	*Iris*	4,7,8,9
B. croci Cooke & Massee	–	–	*Crocus*	7,8,11
B. deweyae		Heterothallic	*Hemerocallis*	18
B. elliptica (Berk.) Cooke	? *Botryotinia* sp.	Heterothallic	*Lilium*	4,7,8,13,14
B. fabae Sardiña	*Bt. fabae* Lu & Wu	–	*Vicia*	7,8,15
B. ficariarum Hennebert	*Bt. ficariarum* Hennebert	–	*Ficaria*	6,7,8,9
B. fabiopsis	–	–	*Vicia*	19
B. galanthina (Berk. & Broome) Sacc.	–	–	*Galanthus*	4,7,8
B. gladiolorum Timmerm.	*Bt. draytonii* (Buddin & Wakef.) Seaver	–	*Gladiolus*	4,7,8,9
B. globosa Raabe	*Bt. globosa* Buchw.	Homothallic	*Allium*	1,7,8,9
B. hyacinthi Westerd. & Beyma	–	–	*Hyacinthus*	4,7,8
B. mali[a]	–	–	*Malus*	24
B. narcissicola Kleb. Ex Westerd. & Beyma	*Bt. narcissicola* (Greg.) Buchw.	–	*Narcissus*	4,7,8,9
B. paeoniae Oudem.	–	–	*Paeonia*, *Allium*	4,7,8,9
B. pelargonii Røed	*Bt. pelargonii* Røed	–	*Pelargonium*	7,8,9
B. polyblastis Dowson	*Bt. polyplastis* (Greg.) Buchw.	–	*Narcissus*	4,7,8,9
B. porri Buchw.	*Bt. porri* (Beyma) Whetzel	Homothallic	*Allium*	3,7,8,9
B. ranunculi Hennebert	*Bt. ranunculi* Hennebert & Groves	Heterothallic	*Ranunculus*	4,6,7,8,9

(continued)

Table 6.1 (continued)

Botrytis sp. (anamorph)[a]	*Botryotinia* sp. (teleomorph)[e]	Mating system	Major hosts	Refs[f]
B. prunorum	–	–	*Prunus*	26
B. pseudocinerea	*Bt. pseudofuckeliana*	Heterothallic	Polyphagous	20
B. ricini Buchw.[a]	*Bt. ricini* (Godfrey) Whetzel	Homothallic	*Ricinus*	4,5,7,8,9
B. sinoallii	–	–	*Allium*	21
B. sinoviticola	–	–	*Vitis*	22
Botrytis fritillarii-pallidiflori (Chen & Li) Seifert & Kohn	*Bt. fritillarii-pallidiflori* Chen & Li	–	–	10
Botrytis sp.[a]	*Sclerotinia spermophila* Noble[b]	Homothallic	*Trifolium*	4,8,9,12
Botrytis sp. B83[d]	–	–	Polyphagous	23
B. sphaerosperma Buchw.	*Bt. sphaerosperma* (Greg.) Buchw.	–	*Allium*	7,8,9
B. squamosa Walker	*Bt. squamosa* Vienn.-Bourg.	Heterothallic	*Allium*	2,4,7,8,9
B. tulipae Lind	–	–	*Tulipa*, *Allium*, *Lilium*	4,7,8,9

Modified and updated from Beever and Weeds (2004)

[a]List of species established from published data. Some species could not be recovered from ancient collections, to check their delimitation in the genus phylogeny (Staats et al. 2005; Van Kan, pers. com.) and their status is therefore potentially doubtful. *B. mali* is a herbarium specimen for which no living culture is available

[b]It has been assumed (*e.g.* Farr et al. 1989) that *Sclerotinia spermophila* is the teleomorph of *B. anthophila* but Noble (1948) discussed this possibility and concluded that further evidence was required to confirm this link

[c]It has been assumed (*e.g.* Jarvis (1980) that *Bt. allii* (Sawada) Yamam. is the teleomorph of *B. byssoidea*. However, (Kohn 1979) provided evidence that Yamamoto was wrong to conclude that Sawada's species produced a *Botrytis* anamorph and she regrouped *Sclerotinia allii* (Sawada) as *Ciborina allii* (Sawada) Kohn. Nevertheless, Yamamoto (1949), who worked on Japanese isolates, identified the anamorph as *B. byssoidea*, in which case the teleomorph he describes is that of *B. byssoidea*. However, the relationships of the Japanese fungus require further investigation (Hennebert 1963; Nielsen et al. 2001)

[d]This new entity is actually represented by only one strain collected in Italy, but the available information confirm that it almost certainly corresponds to a new species (Lorenzini and Zapparoli 2014)

[e]Teleomorph names are given wherever possible, to make it possible to refer to older studies. However, *Botrytis* has been accepted over *Botryotinia* for the genus name (Johnston et al. 2014). Therefore, only *Botrytis* (anamorph names) should be used in future publications

[f]1 = Buchwald 1953; 2 = Bergquist and Lorbeer 1972; 3 = Elliott 1964; 4 = Farr et al. 1989; 5 = Godfrey 1923; 6 = Hennebert and Groves 1963; 7 = Hennebert 1973; 8 = Jarvis 1980; 9 = Kohn 1979; 10 = Li and Chen 1987; 11 = Moore 1959; 12 = Noble 1948; 13 = Van den Ende and Pennock 1996; 14 = Van den Ende and Pennock-Vos 1997; 15 = Wu and Lu 1991; 16 = Yohalem et al. 2003; 17 = Li et al. 2012; 18 = Grant Downton et al. 2014; 19 = Zhang et al. 2010a; 20 = Walker et al. 2011; 21 = Zhang et al. 2010b; 22 = Zhou et al. 2014; 23 = Lorenzini and Zapparoli 2014; 24 = O'Gorman et al. 2008; 25 = Plesken et al. 2015; 26 = Ferrada et al. 2015

For example, in *B. cinerea*, the mycelium has been described as short, aerial or woolly, and the sclerotia have been described as organized randomly, in circles or at the edge of Petri dishes, with conidial density differing between cultures (Martinez et al. 2003). Phenotypic differences can usually be found in complete sets of ascospores from single asci, and in independent cultures established from a single ascospore or conidium from the same wild isolate. Moreover, morphotypes may not remain stable during subculture (Lorenz 1983).

6.2.2.2 Identification on the Basis of the Biological Species Concept

The use of the biological species concept (BSC), which is based on interbreeding, is limited to species for which sexual crosses can be conducted *in vitro*, however laborious. For example, the putative new species *Botrytis* sp. B83 only rarely produces sclerotia, preventing the use of BSC criteria to confirm its existence (Lorenzini and Zapparoli 2014). Respect for the mating system is another essential condition. Some species, such as *B. porri* and *B. globosa*, have been reported to be homothallic (*i.e.* self-fertile) and can produce sexual progeny in a single culture (Elliott 1964; Buchwald 1953). Others are heterothallic (*i.e.* self-sterile) and require strains with a compatible mating type to produce their progeny. Mating is governed by a mating-type locus (two alleles, MAT 1-1 and MAT 1-2; Faretra et al. 1988) in *B. cinerea*, with each locus carrying two different, non-homologous genes (Amselem et al. 2011). The use of the BSC is dependent on mating experiments and is therefore limited and has been largely restricted to elucidation of the sexual system. Nevertheless, the BSC has been successfully used to differentiate between *B. squamosa* and *B. cinerea* (Bergquist and Lorbeer 1972) and between *B. cinerea* and *B. pseudocinerea* (Walker et al. 2011).

6.2.2.3 Identification on the Basis of the Ecological Species Concept

The ESC seems to have been more successfully used for the definition of species. Indeed, most *Botrytis* spp. are host-specific or have a narrow host range. *B. fabae*, for example, infects only plants from the same botanical family *Fabaceae* (Jarvis 1977). *Botrytis* species are, therefore, usually named after their host plants (Table 6.1). Only two species have been reported to have multiple host plants from different botanical families in the wild: *B. cinerea* and *B. pseudocinerea* (Walker et al. 2011; Jarvis 1980; Fekete et al. 2012). *Botrytis* spp. can infect more than 1,400 host species, including not only wild plants, but also major ornamental, greenhouse and field crops, such as tomato, grapevine, lettuce, strawberry, tulip, rose and onion (Chap. 21). They are all necrotrophs, inducing host-cell death, leading to the progressive decay of infected plant tissue. They are also commonly found as saprophytes on dead tissues. However, there is an exception. The newly described species *B. deweyae*, which infects *Hemerocallis*, was recently shown to have an endophytic lifestyle under appropriate conditions (Grant Downton et al. 2014). *B. cinerea* may also be found as an endophytic contaminant of *Primula* and lettuce seeds

(Barnes and Shaw 2003; Sowley et al. 2010). Moreover, ecological preferences may not be governed solely by the host. *B. pseudocinerea*, which is sympatric with *B. cinerea* on the same hosts, is regularly found to be more abundant in the spring than in the autumn. It is also mostly found on dead flower parts from grapevine rather than on living berries. Finally, unlike *B. cinerea*, the distribution of this species is not uniform worldwide, or even within a particular region (Walker et al. 2011; Johnston et al. 2013). This suggests that at least some *Botrytis* species are capable of fine-niche adaptation, but this aspect requires further investigation.

6.2.2.4 Identification on the Basis of the Phylogenetic Species Concept

The PSC is undoubtedly the most widely used concept in *Botrytis* taxonomy. The phylogenetic approach to systematics has been boosted by advances in DNA sequencing and by the identification, over the last 10 years, of genes highly relevant to the resolution of *Botrytis* phylogeny. As a consequence, seven new species have come to the fore in the last four years (Table 6.1). The ITS rDNA region has been extensively used for the species-level discrimination of fungal species. Unfortunately, the number of informative sequence characters is too small in *Botrytis* for the full resolution of relationships between all species. This approach was nevertheless successfully used to infer a preliminary phylogeny of the family *Sclerotiniaceae* (Holst-Jensen et al. 1998). Universal-primed polymerase chain reaction (UP-PCR) fingerprinting, coupled with the restriction of ITS rDNA regions also led to the identification of five groups: *B. cinerea*, *B. squamosa*, *B. byssoidea* and two groups of *B. aclada* (Nielsen et al. 2001), one of which was subsequently recognized as the hybrid between *B. aclada* and *B. byssoidea* and renamed *B. allii* (Nielsen and Yohalem 2001; Yohalem et al. 2003). Moreover, a method coupling ITS rDNA sequencing and the multiplex PCR amplification of two laccase introns can identify several species from the *Sclerotiniaceae*, including *B. cinerea* (Hirschhäuser and Fröhlich 2007). Finally, the intergenic spacer (IGS) rDNA region was successfully used in preliminary studies as an RFLP marker for distinguishing between *B. cinerea* and *B. pseudocinerea* (Giraud et al. 1997).

According to the GCPCSR, as discussed for fungi by Taylor et al. (2000), the best way to demonstrate a lack of gene flow between evolutionary lineages (*i.e.* the definition of a phylogenetic species) is to compare the phylogenies obtained for several independent loci: barriers to gene flow (or species limits) correspond to the nodes from which independent phylogenies become incongruent. The identification of relevant protein-coding genes has greatly improved the resolution of the *Botrytis* phylogeny. Three genes, in particular, are used: the glyceraldehyde 3-phosphate dehydrogenase (*G3PDH*), heat shock protein 60 (*HSP60*) and DNA-dependent RNA polymerase subunit II (*RPB2*) genes. Staats et al. (2005) combined these genes in a comprehensive phylogenetic analysis of the whole genus. The combined analysis of all three loci provides greater resolution than individual gene phylogenies. As a result, these authors were able to confirm the morphological and host plant-based classification of the *Botrytis* genus and divided the genus into two well separated clades. The first of these clades contains only five species (*B. cinerea*,

B. fabae, *B. pelargonii*, *B. calthae*, and *B. pseudocinerea*, added after further investigation) that infect only eudicot plants, whereas the second clade contains all the other species, infecting either eudicot or monocot plants. Interestingly, the host and pathogen topologies were incongruent (particularly for the species infecting the same host, *Allium*, clustered in different clades), suggesting regular host shifts rather than co-speciation. Alternatively, host range may have been underestimated for some species currently thought to be strict single-host pathogens. This study also confirmed the hybrid nature of *B. allii*. Finally, these authors investigated the mode of reproduction in each species. Their results suggested that sexual reproduction had been lost at least three times during the evolution of the genus, due to negative selection. This work probably paved the way for the delimitation of many new species (see next section and Li et al. 2012; Lorenzini and Zapparoli 2014; Zhang et al. 2010a, b; Grant Downton et al. 2014). An updated phylogenetic tree, adapted from the work of Staats et al. (2005) and including these new taxa is presented in Fig. 6.1.

In other studies, one or several of these genes have been used in combination with other genes for the identification of other species. The Funybase phylogenetic database containing 1:1 orthologs from 30 fungal genomes of the highest informative value at the desired taxonomic level, can be considered a good source of genes useful for phylogenetic analyses (Marthey et al. 2008). For example, Walker et al. (2011) used the ATP-dependent RNA helicase gene (*MS547*) to help determine the systematic position of *B. pseudocinerea* in the genus. Andrew et al. (2012) also refined the phylogeny of the *Sclerotiniaceae*, by combining *G3PDH* and *HSP60* topologies with that for the calmodulin gene. Khan et al. (2013) recently distinguished between species causing neck rot disease on onion in New Zealand, using a combination of data for the *ITS* and *IGS* regions and the *G3PDH* sequence, which greatly improved the molecular identification of species. Interestingly, sequences established with the *G3PDH* and β-tubulin genes confirmed the existence of *B. mali* from herbarium apple specimens (O'Gorman et al. 2008), following the initial approximate and, therefore, dubious description of this species (Ruehle 1931). The findings for these genes suggest that this species would probably cluster with *B. paeoniae*. Finally, in addition to the previous markers, two genes encoding the necrosis and ethylene-inducing proteins, *NEP1* and *NEP2*, were found to be highly relevant for establishment of the phylogeny of *Botrytis* phylogeny (Staats et al. 2007a) and for refining the systematic positions of new species (Lorenzini and Zapparoli 2014; Li et al. 2012; Zhang et al. 2010a). The trees established with these genes were entirely congruent with the concatened trees established by Staats et al. (2005), with the exception of *B. gladiolorum*, which was located in a different position in the *NEP2* tree. The *NEP1* and *NEP2* genes are involved in interactions with the plant and have been shown to have evolved under positive selection (Staats et al. 2007a). Caution is therefore required in their use and their neutrality should be checked before their use for phylogenetic purposes, particularly if they are to be used for dating.

Recent developments in molecular phylogenetics have generated a set of relevant genes for delimiting *Botrytis* spp. They have been shown to be of significant interest

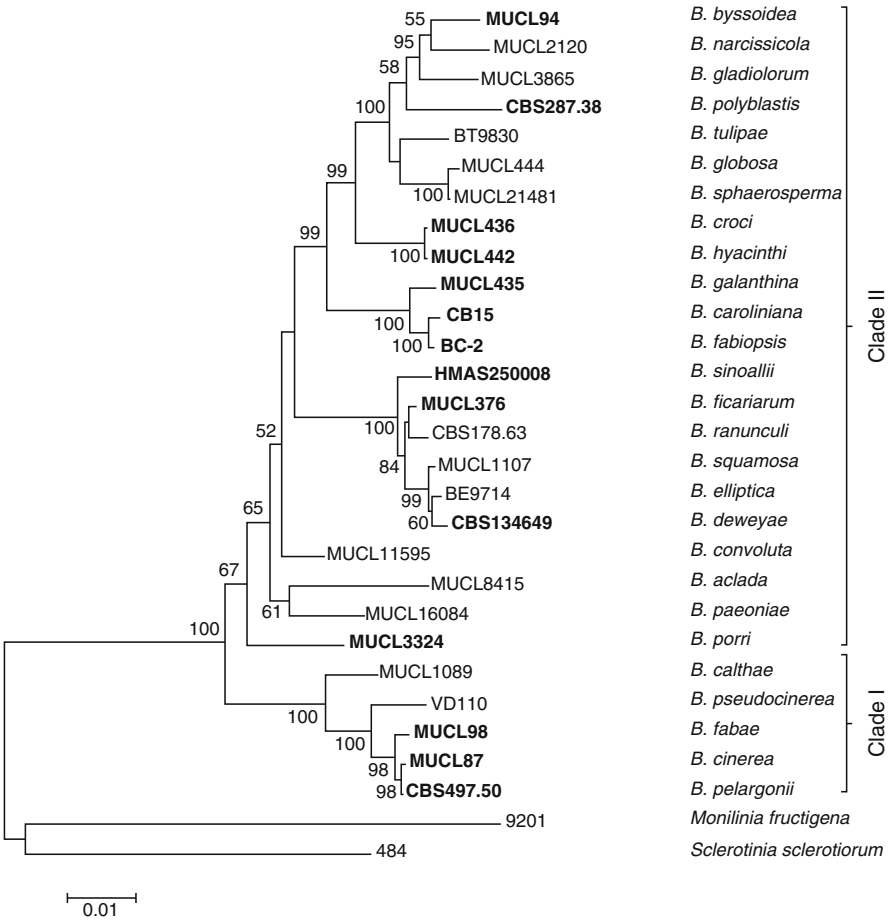

Fig. 6.1 Updated phylogenetic tree of *Botrytis* sp., based on concatenated sequences of the G3PDH, HSP60 and RPB2 genes (From Hyde et al. 2014). The tree was constructed with the maximum likelihood method. Some species from Table 6.1 could not be integrated in this tree, due to a lack of published sequences or an inability to obtain fresh cultures from collections for the establishment of these sequences

in at least one phylogenetic study and it is, therefore, difficult to rank them. However, it has now been established that the most appropriate approach for correctly delimiting distinct evolutionary lineages (*i.e.* for placing species boundaries) is to use several independent genes and to evaluate the congruence of their phylogenies. Unfortunately, the congruence of gene topologies is only rarely quantified, although at least one useful congruence index has been published (de Vienne et al. 2007, 2009). Moreover, given these limitations, new species should not be delimited exclusively on the basis of molecular phylogenetic data (a tempting approach as it would minimize the amount of laboratory work required), but on the basis of a body of evidence accumulated from as many of the species concepts as possible.

6.2.3 *Recently Discovered* Botrytis *Species*

B. fabiopsis was first collected from 35 locations in Hubei Province (Central China), between 2006 and 2009. It was sympatric with *B. cinerea* and *B. fabae* on broad bean (*Vicia faba*) (Zhang et al. 2010a). It was the second most frequently identified species in this sampling campaign after *B. cinerea*, accounting for 36.4 % of the strains. It may therefore have a significant epidemiological impact on this crop. The "chocolate spot" symptoms were similar to those usually observed with the other two species. Host range was not investigated further. This fungus formed pale grey colonies with short aerial mycelia and produced grey to black sclerotia in concentric rings on PDA medium. It differed from *B. cinerea* and *B. fabae* in terms of sclerotium production and the size of its conidia. Phylogenetic analysis with *G3PDH*, *HSP60*, *RPB2*, *NEP1*, and *NEP2* sequences placed this species in clade II of the most recent phylogeny (Staats et al. 2005), well separated from *B. cinerea* and *B. fabae* (clade I). Instead, it was found to be a sister species to *B. galanthina*, which infects snowdrop (*Galanthus nivalis*).

B. caroliniana was first isolated in 2010 from infected blackberries (*Rubus fruticosus*) on several farms in South Carolina, USA (Li et al. 2012). It was sympatric with *B. cinerea*. It was shown to be pathogenic on broad bean leaves and tomato in laboratory conditions, and to produce lesions, without conidiation, on apple, pear, orange lemon, table grape, and raspberry (Schnabel and Li, pers. com.). Phylogenetic analysis with *G3PDH*, *HSP60*, *RPB2*, *NEP1* and *NEP2* sequences showed this species to be most closely related to *B. fabiopsis* (infecting broad bean) and to *B. galanthina* (infecting snowdrop), and to belong to clade II of the most recent phylogeny (Staats et al. 2005). This finding contrasts with what would be expected on the basis of host plant data, which would suggest phylogenetic relatedness to *B. cinerea*. It forms white to pale grey colonies with a short, tufted aerial mycelium and produces black sclerotia on PDA at 20 °C. Its conidia are similar to those of *B. cinerea*, but smaller than those of *B. fabiopsis* and *B. galanthina*. Little is known about its ecological requirements, and its frequency in *Botrytis* populations from blackberry. It is therefore difficult to draw any firm conclusions about its relevance for blackberry production.

B. deweyae was isolated in 2009 from daylilies (*Hemerocallis* hybrids) in the UK, as the causal agent of "spring sickness", an emerging disease of this ornamental plant. It produces slightly fluffy, whitish to pale brown colonies on artificial medium, but its principal characteristic is the absence of macroconidial conidiation, particularly on plants. Two alleles were detected at the mating-type locus homologous to that of *B. cinerea*, suggesting heterothallism. This new species is genetically related to *B. elliptica* (*ITS*, *G3PDH*, *NEP1*), which can also affect daylilies. This suggests that the emergent pathogen *B. deweyae* may have arisen from *B. elliptica* after a host shift. The distribution and abundance of this species in populations are unknown, as only a few specimens have been isolated. Given its morphological features, the absence of macroconidia and the nature of disease development, *B. deweyae* may be an endophyte undergoing the transition to a more aggressive pathogenic state (Grant Downton et al. 2014).

B. sinoalli was first collected in 2006 from green onion (*Allium fistulosum*) and then from other *Allium* crops at seven sites in Hubei Province (Central China; Zhang et al. 2010b). Within populations, it was found in association with *B. cinerea*, *B. squamosa*, *B. porri*, or *B. byssoidea*, but accounted for only 2.2 % of the population. This species produces white mycelia and abundant small sclerotia and can be distinguished from the other species on the basis of its conidium production on living host tissues, but not on PDA. Phylogenetic analysis with the *G3PDH*, *HSP60*, and *RPB2* genes showed that *B. sinoallii* formed a single lineage within clade II that was closely related to *B. squamosa*, but only distantly related to other species growing on *Allium*, including *B. cinerea*, *B. porri*, *B. aclada*, *B. allii*, *B. byssoidea*, *B. globosa* and *B. sphaerosperma.*

B. pseudocinerea is a new cryptic species recently distinguished from *B. cinerea* (Walker et al. 2011). It is described in more detail below, in the section on the *Botrytis* species complex.

B. sinoviticola was isolated between 2004 and 2012 from *Botrytis*-infected table grapes collected in two Chinese provinces, but little is known about its relevance in natural populations (Zhou et al. 2014). It produces whitish colonies and abundant small sclerotia. Its conidia appear to be thinner than those produced by *B. cinerea* and they carry characteristic villiform appendages on their surface. Phylogenetic analysis (*G3PDH*, *HSP60*, and *RPB2*) placed this species in a single lineage of clade I, separate from *B. cinerea* and *B. pseudocinerea*, although closely related to these species. *B. sinoviticola* has an intermediate level of sensitivity to the fungicide fenhexamid, between those of *B. cinerea* (sensitive) and *B. pseudocinerea* (naturally resistant). However, resistance to the full range of modes of action of fungicides, as in *B. pseudocinerea* (as described in Leroux et al. 1999), has not been assessed. Finally, the inoculation of grapevine leaves with mycelium showed this species to be less virulent than *B. cinerea* and *B. pseudocinerea*, suggesting that it may also occur on other plants.

A new species was recently collected from withered grapes (*Vitis vinifera*) in a fruit drying room in Valle dei Laghi (Trentino, Italy) (Lorenzini and Zapparoli 2014). This species is currently represented by a single strain, *Botrytis* sp. B83, and further collections and analyses are thus required to confirm the status of this new entity and to identify its diagnostic features. The morphology of *Botrytis* sp. B83 colonies on PDA was different from that of *B. cinerea*, which usually produces colonies of various shades of grey. *Botrytis* sp. B83 instead produces a moderately deep and floccose aerial white-cream mycelium on PDA. Sclerotia are produced rarely, and only under specific conditions. More generally, several characters indicate that this strain is different from but closely related to *B. aclada* in clade II, as confirmed by phylogenetic analysis with *G3PDH*, *HSP60*, *RPB2*, *NEP1*, and *NEP2* sequences. Conversely, its host range indicates that *Botrytis* sp. B83 behaves like *B. cinerea*, acting as a pathogen of many different plant hosts exclusively infected with grey mould (*i.e.* grapevine, green bean, kiwifruit, pepper, cucumber, strawberry, hortensia, blackcurrant, sage, and tomato). Its presence in a fruit drying room may therefore be incidental.

At last, *B. prunorum* was recently isolated from asymptomatic flowers of Japanese plums (*Prunus salicina*) in the Central Valley of Chile (Ferrada et al. 2015). They were supposed to cause blossom blight, together with *B. cinerea*. The authors described low-sporulating isolates, developing a white cottony mycelium and scarce conidiation on several media. Sclerotia were obtained only in restricted conditions. Molecular analysis with *G3PDH*, *HSP60*, *RPB2*, *NEP1*, and *NEP2* sequences established this entity as a new species within clade II, related to *B. paeoniae* and *B. aclada*. No further description was provided about the ecology and host range of this new species.

In conclusion, given the large number of newly established species, mostly identified through the use of recently developed relevant markers, *Botrytis* taxonomy seems to be at an early stage. Many more species may remain undiscovered, some parasitic but others endophytic, and therefore much harder to detect (Van Kan et al. 2014). For example, six 'new' *Botrytis* spp. from *Centaurea stoebe* (*Asteraceae*) have been identified as endophytes and their further characterization is planned in the near future (Shipunov et al. 2008). One of these species is very closely related to some *B. pseudocinerea* strains from a specific New-Zealand clade (P. Johnston, pers. com.). A foliar pathogen of *Hosta* that is closely related to *B. tulipae* is also currently awaiting full identification (Laundon 1978). In addition, the quality of new species descriptions is highly heterogeneous. Biological and ecological characteristics are often not recorded and few studies describe the mode of reproduction of the new species and provide intra- and interspecific crossing data (BSC). Moreover, population surveys are also often required to delimit geographical and host ranges. Recently developed tools should also prove useful for refining the descriptions of long-known species, such as *B. calthae* (Plesken et al. 2015; Hennebert and Groves 1963).

6.2.4 The Botrytis *Species Complex*

Cryptic species have identical morphological features but can be distinguished on the basis of their molecular sequences. They often inhabit the same environment. Thus, cryptic fungal species often parasitize the same host, produce similar symptoms and, therefore, form what is called a species complex. In the genus *Botrytis*, several species may correspond to this description. *B. pelargonii* and *B. cinerea* produce identical symptoms on *Pelargonium*; molecular phylogenetics studies do not support the separation of these two species (Staats et al. 2005, 2007a; Walker et al. 2011) and the existence of *B. pelargonii* is therefore doubtful.

Within *B. cinerea*, a subdivision into two distinct genetic groups has long been proposed, based on the presence or absence of the transposable elements *Boty* (Diolez et al. 1995) and *Flipper* (Levis et al. 1997). The presence/absence of these two elements has been used to describe four transposon types in populations: *vacuma* (strains with neither of these elements), *transposa* (strains with both elements), *Boty* and *Flipper* (strains with one or other of the two elements). For a

limited number of strains, this subdivision coincided with two sympatric species, "group I" (only *vacuma*) or "group II" (the other three types), but this pattern was subsequently shown to be invalid in independent studies of larger datasets. The identification of "group I" as a new species was corroborated by the polymorphism of many genes: *Bc-hch* (encoding the homolog of the *Neurospora crassa* het-c vegetative incompatibility locus; Fournier et al. 2003), *cyp51* (encoding the eburicol 14α-demethylase, target of azole fungicides; Albertini et al. 2002), *erg27* (encoding the 3-cetoreductase, target of the fungicide fenhexamid; Albertini and Leroux 2004), *sdhA*/*B*/*C*/*D* (genes encoding the four subunits of succinate dehydrogenase; Leroux et al. 2010), for example. All these loci display many fixed polymorphisms within groups I and II, and an absence of shared polymorphisms between groups I and II, strongly suggesting an ancient divergence, with a complete absence of gene flow between these two groups. This was also confirmed by population genetics studies based on PCR-RFLP markers (Giraud et al. 1997) or SSRs (Walker et al. 2011). This body of evidence led to the establishment of *Botrytis* "group I" as the new species *B. pseudocinerea* (Walker et al. 2011; Fournier and Giraud 2008; Fournier et al. 2005). The classification based upon transposable elements became obsolete, because some *transposa* strains were found in *B. pseudocinerea*, albeit at low frequencies (Walker et al. 2011; Johnston et al. 2013; Fekete et al. 2012).

Several phylogenetic studies using *Bc-hch*, *cyp51*, *63-R* and/or *β-tub* (Fournier et al. 2005; Johnston et al. 2013), or *G3PDH*, *HSP60* and/or *MS547* (Walker et al. 2011; Johnston et al. 2013) clearly identified strains from groups I and II as belonging to different species within clade I of the *Botrytis* phylogeny. However, these studies showed that these species were not sisters, because *B. cinerea* was closer to *B. fabae* than to *B. pseudocinerea*. Indeed, a molecular clock analysis demonstrated that these two species diverged between 7 and 18 million years ago. Until recently, *B. pseudocinerea* had been detected only in European countries (Walker et al. 2011; Fekete et al. 2012) and was thought to originate from this part of the world. However, strains have recently been found in the Southern hemisphere, in Chile, New Zealand and South Africa (Johnston et al. 2013; Wessels et al. 2013; Munoz et al. 2015) and also in Central China on tomato (Li et al. 2014) and on blueberry in North America (Saito et al. 2014), suggesting that human activities may have mediated the migration of this species. Interestingly, two clades were resolved within *B. pseudocinerea* samples from New Zealand, only one of which has been reported in European vineyards, although this clade contained only a small number of strains (Johnston et al. 2013). *B. cinerea* and *B. pseudocinerea* have identical morphological features. Mating between these two species generates no progeny or a sterile progeny, suggesting the possible existence of a reproductive barrier based on prezygotic isolation. Moreover, the two species have different patterns of susceptibility to fungicides. Among many other fungicide markers, *B. pseudocinerea* is naturally resistant to fenhexamid and hypersensitive to morpholines (Albertini et al. 2002; Leroux et al. 1999). Both are polyphagous (Plesken et al. 2015; Giraud et al. 1999; Walker et al. 2011) and they are sympatric on the same hosts, but *B. pseudocinerea* is more abundant on dead flower parts in the spring (Johnston et al. 2013; Walker et al. 2011).

Recent studies of German strawberry fields identified a predominant new entity called *Botrytis* group S (for strawberry). These strains are present on other crops, such as grapevine, but at a lower frequency (Walker unpublished data; Leroch et al. 2013; Johnston et al. 2013). They have a morphology similar to that of *B. cinerea*. They display a 21-bp deletion in a transcription factor-encoding gene, *mrr1*, which is routinely used for their identification. Some group S strains express an original multidrug-resistant phenotype (MDR1h), conferring a higher resistance factor than the previously described MDR1 phenotype (Leroux and Walker 2013; Kretschmer et al. 2009), due to an additional 3-bp deletion in the same *mrr1* gene (Leroch et al. 2013). The phylogenetic tree established with *mrr1*, *MS547*, *FG1020*, *HSP60*, and *NEP2* did not clearly resolve this clade, strains from group S being grouped together with *B. cinerea* or considered a different species more closely related to *B. fabae*, depending on the gene considered (Johnston et al. 2013; Leroch et al. 2013). More information, from the sequencing of these genomes, is required for firm conclusions to be drawn about whether this new entity can be considered to be a separate species. Population genetics studies may also help to determine whether *Botrytis* group S corresponds to a subpopulation particularly adapted to strawberry and other plants or whether it is a sympatric new species present in grey mould populations.

6.3 Diversity of *B. cinerea* Populations

6.3.1 General Comments

B. cinerea is the *Botrytis* species for which by far the most information on diversity at the population level has been provided. In association with taxonomists (see previous section), diversity was first assessed and quantified together with morphological features in populations. Isolates were classified by several authors and quantified in populations as being of a "mycelial type", with various categories, based upon the absence of sclerotia, conidiation and the appearance of the mycelium on synthetic medium, or of a "sclerotial type", based on the number, size and organization of sclerotia in Petri dishes (Martinez et al. 2003; Mirzaei et al. 2009). Diversity is also evident if metabolic criteria are considered. For example, the production of secondary metabolites may differ between strains (Chap. 15). In particular, only a very small number of strains in populations produce the pink polyketide bikaverin pigment, due to the acquisition of a fully functional six-gene cluster possibly by horizontal gene transfer from *Fusarium* sp., probably before the divergence of the genus, this cluster being partially inactivated in most strains (Schumacher et al. 2013). Finally, diversity in acquired resistance to fungicides may also be observed in populations, with strains displaying adaptation to most of the available modes of action of fungicides and the selection of original resistance mechanisms (Chap. 10).

Over the last two decades, diversity within populations has mostly been assessed with a number of neutral molecular markers. Population genetics studies have flourished over the last decade (Table 6.2) and have helped to improve our knowledge of *B. cinerea* populations in various environments, to identify the factors shaping population structure and to understand the relationships between the various compartments. Outdoor populations are often shown to have high levels of genic and genotypic diversity, consistent with the large size of these populations, strong gene flow and regular recombination events. Haplotypic diversity may be low in some greenhouse populations, due to the predominance of selected genotypes. Diversity indices estimated with SSR markers were recently compiled and compared for various situations (Leyronas et al. 2015a). Genetic diversity has also been observed at a very fine scale: lesions from a single grapevine plant have been shown to be caused by different haplotypes, and up to five haplotypes have been distinguished from a single lesion on a berry (Giraud et al. 1997). Finally, population genetics studies may also provide information about the mode of reproduction at work in populations. Some indoor tomato populations favor clonal reproduction (Walker et al. 2015), but regular recombination, consistent with cryptic sexual reproduction, is observed in outdoor populations, as shown by the low proportion of clones and low level of linkage disequilibrium (Fournier and Giraud 2008; Giraud et al. 1997, 1999; Walker et al. 2015; Vaczy et al. 2008). Nevertheless, a key exception has been reported, for outdoor populations of rooibos seedlings in the Western Cape, South Africa, which displayed higher clonal fractions, accompanied by disequilibrium of mating type ratios (Wessels et al. 2013).

6.3.2 Molecular Markers Available for Population Genetics

Some studies have used random amplified polymorphic DNA (RAPD) to assess genetic diversity in populations (Table 6.2), mostly because this technique has been shown to be a powerful tool for genetic analysis and has been applied to a wide range of organisms. Commercial random decamer primers are easily obtained, making it relatively easy to gain rapid access to this technique. However, Moyano et al. (2003) showed that these markers were less polymorphic than amplified-fragment length polymorphism (AFLP) markers for *B. cinerea* (Vos et al. 1995). Surprisingly, AFLP analyses have been carried out in only a small number of studies (Table 6.2). In addition to these multi-locus techniques, various studies have used restriction fragment length polymorphism after PCR (PCR-RFLP) to measure diversity, because this single-locus technique is more reproducible and precise for the estimation of genetic parameters (high levels of polymorphism). Many genes have been used for this technique: the IGS, nitrate reductase, ATP synthase, ADP-ATP translocase (Giraud et al. 1997; Baraldi et al. 2002; Munoz et al. 2002, 2010; Giraud et al. 1999; Kretschmer and Hahn 2008) and *Bc-hch* (Fournier et al. 2003) genes.

Table 6.2 *Botrytis* spp. studies assessing diversity with various molecular markers

Botrytis species and reference	Host	Region/ country	Sample size (no. of isolates)	Neutral markers				Structuring factor exploited[a]						
				RAPD	RFLP/ AFLP	TE	SSR	Year	Season	Geography	Host plant	Cropping system	Species delim.	Other
B. cinerea														
Van Der Vlugt-Bergmans et al. (1993)	Various hosts	The Netherlands	10	●										
Giraud et al. (1997)	Grapevine	Champagne/ France	356		●	●			●	●			●	
Kerssies et al. (1997)	Rose, *Gerbera*	The Netherlands	30	●				●	●	●		●		
Choi et al. (1998)	9 different hosts	Korea	34	●				●		●	●			
Giraud et al. (1999)	21 wild hosts	Champagne/ France	107		●	●				●	●		●	
Thompson and Latorre (1999)	7 hosts, including grapevine	Chile	29	●							●			
Alfonso et al. (2000)	6 greenhouse crops, air	Almeria/ Spain	185	●					●	●		●		
Yourman et al. (2000)	Various ornamentals	South Carolina/USA	56	●										●[e]
Baraldi et al. (2002)	Kiwi fruit	Italy	90		●									●[c]

Munoz et al. (2002)	Grapevine, tomato, kiwi fruit, blueberry	Chile	58	●	●	●					●			
Rigotti et al. (2002)	Strawberry and others	Switzerland	34	●									●	
Fournier et al. (2003)	Various	France, Germany, Japan	132		●	●							●	
Moyano et al. (2003)	Unknown greenhouse crops, air	Almeria/ Spain	44	●	●[b]							●		●[d]
Topolovec-Pintaric et al. (2004)	Grapevine	Croatia	30			●								
Ma and Michailides (2005)	Fig, grapevine, kiwi fruit, pea, squash	California/ USA	234			●	●			●	●			
Martinez et al. (2005)	Grapevine	Bordeaux area/France	741			●			●					●[e,f]
Calpas et al. (2006)	20 plants in greenhouse, including tomato	Alberta/ Canada	91	●						●	●	●		
Isenegger et al. (2008b)	Chickpea	Bangladesh	146				●			●				

(continued)

Table 6.2 (continued)

Botrytis species and reference	Host	Region/ country	Sample size (no. of isolates)	Neutral markers				Structuring factor exploited[a]						
				RAPD	RFLP/ AFLP	TE	SSR	Year	Season	Geography	Host plant	Cropping system	Species delim.	Other
Isenegger et al. (2008a)	Chickpea, lentil, grape and various flowers	Bangladesh, Australia	266			●	●			●				
Karchani-Balma et al. (2008)	Tomato, grapevine, strawberry, bean, others	Tunisia	153			●				●	●	●		
Kretschmer and Hahn (2008)	Grapevine	Germany	209		●	●			●					
Martinez et al. (2008)	Grapevine	France	1,172			●			●					●[f]
Vaczy et al. (2008)	Grapevine	Eger area/ Hungary	109			●	●	●		●				
Decognet et al. (2009)	Greenhouse tomato, air	Provence/ France	897				●					●		
Mirzaei et al. (2009)	36 wild or cultivated plants	Iran	44	●					●	●	●			
Tanovic et al. (2009)	Blackberry, strawberry, raspberry	Serbia	26				●				●			
Munoz et al. (2010)	Grapevine	Argentina	35		●	●					●			

Rajaguru and Shaw (2010)	Strawberry, blackberry, dandelion, primrose	Southern England	243				●			●	●			
Valiuskaite et al. (2010)	Several horticultural plants	Lithuania	18		●[b]					●	●			
Esterio et al. (2011)	Grapevine	Chile	457	●		●			●	●				●[f]
Walker et al. (2011)	Grapevine, blackberry	Champagne/ France	1,088				●				●		●	
Fekete et al. (2012)	Rapeseed, strawberry, raspberry	Hungary	92			●	●						●	
Kuzmanovska et al. (2012)	Tomato	Macedonia	123			●				●				
Samuel et al. (2012)	Strawberry, grapevine, apple, tomato	Greece	334			●					●			
Asadollahi et al. (2013)	Strawberry, raspberry	Hungary	490		●	●	●	●	●		●			
Fournier et al. (2013)	Grapevine	France	164				●			●				●[g]
Leroch et al. (2013)	Grapevine, strawberry	Germany	323		●						●			
Johnston et al. (2013)	Grapevine	New Zealand	1,331			●			●	●				

(continued)

Table 6.2 (continued)

Botrytis species and reference	Host	Region/ country	Sample size (no. of isolates)	Neutral markers				Structuring factor exploited[a]						
				RAPD	RFLP/ AFLP	TE	SSR	Year	Season	Geography	Host plant	Cropping system	Species delim.	Other
Munoz and Campos (2013)	Pine, eucalyptus	Bio-Bio/Chile	55		●					●				
Wessels et al. (2013)	Rooibos	South Africa	207				●			●				
Bardin et al. (2014)	Greenhouse tomato, air	Southern France	712				●			●				
Kumari et al. (2014)	Chikpea, marigold and others	India, Nepal	79	●		●		●			●			
Adjebli et al. (2015)	Greenhouse tomato	Algeria	174				●					●		
Leyronas et al. (2015a)	Greenhouse tomato and lettuce	Provence/ France	86				●				●			
Walker et al. (2015)	Grapevine, blackberry, tomato, litter	France	3,546				●	●	●	●	●	●		

Leyronas et al. (2015b)	Air	Provence/ France	683				●						●[h]	*B. elliptica* and *B. tulipae*
Huang et al. (2001)	Lilies	USA, Taiwan	69	●						●	●			
Staats et al. (2007b)	Tulip, lily	The Netherlands	344		●[b]				●		●			
Botrytis species from onion														
Nielsen et al. (2001)	Onion	Denmark	51		●								●	
Valiuskaite et al. (2010)	Several horticultural plants, including onion	Lithuania	18		●[b]				●	●			●	

[a]Factor identified by the authors as possibly structuring their dataset and therefore tested in their analysis
[b]Studies using AFLP markers
[c]Adaptation to storage conditions (cold)
[d]Molecular epidemiology in greenhouses; redistribution of genotypes by the air in the greenhouse
[e]Influence of fungicide treatment
[f]Adaptation to the host organ
[g]Differentiation between noble rot and grey mould
[h]Structure of airborne populations

The transposable elements (TEs) *Boty* and *Flipper* (Sect. 2.4) are probably the most widely used markers in population studies (Table 6.2). Indeed, they were among the first markers to be identified and are rapid and convenient to use in any laboratory. However, even though the subdivision between *vacuma* and *transposa* led to the discovery of *B. pseudocinerea*, new results from genome sequencing have called into question the utility of these markers for population studies. TEs are thought to account for 0.4–0.6 % of the *B. cinerea* genome and are highly diverse (Chap. 3; Amselem et al. 2011). A single genome may contain not only various numbers of full-length copies of TEs, but also truncated copies or, for retrotransposons, solo-LTRs, resulting from recombination between the LTRs of ancestral TE copies. Consequently, diversity studies based on TE classification may principally reflect transposition dynamics within a genome, rather than exclusively population processes, as expected for canonical nuclear "neutral" markers. Moreover, several techniques are available for detecting *Boty* and *Flipper*: dot-blot hybridization (Giraud et al. 1997) and PCR detection with transposon-specific primers. Several pairs of primers are available for PCR detection. Some flank the transposon whereas others partially overlap it (Munoz et al. 2002; Ma and Michailides 2005; Martinez et al. 2008; Kretschmer and Hahn 2008; Johnston et al. 2013). These partially overlapping primers detect transposon copies in a specific genomic environment. The results obtained for a given strain may therefore differ according to the technique used, making comparisons of results difficult, as demonstrated by some reported unsatisfactory concordance rates (Martinez et al. 2008).

More recent studies have been based on the use of microsatellite or single sequence repeat (SSR) markers. These markers are thought to be neutral, and it is easy to test for departure from expectations for neutrality. They can be combined in multiplex PCR and are often highly polymorphic. This technique is easy to use and highly reproducible. A set of nine SSRs, named Bc1 to Bc10 (Fournier et al. 2002), has been used in all but one study. Bc9 and Bc10 may be linked, as they were isolated from the same clone. This SSR set has been shown to be highly discriminating for use in population studies (Karchani-Balma et al. 2008). Another set of 16 MP-PCR markers amplifying microsatellite motifs was also developed for the analysis Californian populations (Ma and Michailides 2005).

6.3.3 *Confirmation of Species Boundaries Through the Use of Population Genetics Markers*

In addition to phylogenetic genes, population genetics markers have also proved useful for the confirmation and recognition of species boundaries, because some of these markers may have specific ('private') alleles for some species and/or highly contrasting frequencies of shared alleles in natural populations. This property has been used to identify fungi, including several *Botrytis* spp., from symptomless strawberries by RAPD fingerprinting (Rigotti et al. 2002). However, it has chiefly

been used to confirm the existence of a barrier to gene flow between *B. cinerea* and *B. pseudocinerea* in populations, with either RFLP (Fekete et al. 2012; Fournier et al. 2003; Giraud et al. 1997, 1999) or SSR markers (Walker et al. 2011; Fekete et al. 2012). Allelic diversity was found to be lower at locus Bc4 and a private allele (size 86 bp) was identified at locus Bc6 in *B. pseudocinerea*, in a study based on SSR markers (Walker et al. 2011; Walker unpublished). These differences confirmed the delimitation of the two species after population genetics analysis, with indices of differentiation between the two species increasing and significantly greater than those usually observed between populations from the same species. Bayesian analysis with no prior assumptions about the species of the strain unambiguously assigned individuals into two distinct, independent clusters, confirming the absence of gene flow between them (Walker et al. 2011). Population genetics may thus constitute a powerful tool for the discovery of new cryptic species, but the taxonomic status of these entities as species must subsequently be confirmed by phylogenic approaches (see above). The specificity of the available SSR markers should nevertheless be checked in *Botrytis* sp.

6.3.4 Geography as a Structuring Factor

Studies of geographic distance as a factor potentially structuring population diversity are justified by the ability of pathogens to disperse gradually over space, due to their biological features and the properties of their spores and the presence of natural elements (*e.g.* mountain chains, oceans or rivers) that may prevent dispersal. This has important consequences for disease propagation. Indeed, this factor was explored in 51 % of the papers reviewed here (Table 6.2). The effect of geographic distance is mostly dependent on the scale studied. Very little, or no differentiation has been reported at the regional (Kerssies et al. 1997; Giraud et al. 1999; Alfonso et al. 2000; Moyano et al. 2003; Ma and Michailides 2005; Calpas et al. 2006; Vaczy et al. 2008; Rajaguru and Shaw 2010; Wessels et al. 2013), and national scales (Choi et al. 1998; Isenegger et al. 2008b; Valiuskaite et al. 2010; Walker et al. 2015; Fournier and Giraud 2008; Munoz et al. 2010; Mirzaei et al. 2009; Esterio et al. 2011). By contrast, differentiation has been reported for some Tunisian populations separated by the Great Dorsal, a mountain chain constituting a geographic barrier to gene flow (Karchani-Balma et al. 2008). Differentiation is observed at the continental scale, between populations collected on different hosts in Australia and South Asia (Bangladesh, Nepal, India) (Isenegger et al. 2008a). High levels of genetic differentiation have also been observed between grapevine populations in France and Argentina (Munoz et al. 2010). These findings suggest that effective population sizes are large and that migration rates are high at the regional and national scales. This intense migration may also be facilitated by the large number of hosts that can potentially be contaminated, constituting as many transient reservoirs within an agricultural or natural landscape.

6.3.5 Host as a Structuring Factor

Adaptation to the host is a key issue for generalist pathogens such as *B. cinerea*, because it sets crucial levels for disease management (role of different hosts as within- *vs*. between-crop reservoirs or in the diffusion of fungicide resistance alleles, for example). Indeed, wild (*e.g.* Giraud et al. 1999; Rajaguru and Shaw 2010) or cultivated (*e.g.* Leyronas et al. 2015a; Karchani-Balma et al. 2008) host plants were found to have been studied as a structuring factor in 42 % of the population studies available (Table 6.2). Studies carried out with TE markers have shown that *transposa* strains predominate in populations collected from grapes, strawberries and tomatoes, whereas *vacuma* strains dominate *B. cinerea* populations from kiwi fruit and apples (Esterio et al. 2011; Johnston et al. 2013; Martinez et al. 2005; Munoz et al. 2002; Samuel et al. 2012). However, there is currently no clear explanation for these observations. Moreover, in studies based on the use of SSRs, differentiation indices or analysis recognize the host as the most powerful structuring factor for populations, ahead of geography (*e.g.* Fournier and Giraud 2008; Walker et al. 2015). Thus, populations collected from the same host in distant regions may be more genetically similar than populations collected from different hosts within the same region. An exception has been reported, for Californian populations, for which no genetic differentiation was observed between populations collected from different host plants (various fruit spp.) (Ma and Michailides 2005). However, this study used an original set of markers, ruling out comparisons with other studies. Similarly, no differentiation was observed between populations collected from grapevine and those collected from the surrounding litter (dead and decaying plant tissues lying on the ground) (Walker et al. 2015).

The coexistence in sympatry of subdivided populations (*i.e.* with restricted gene exchange) developing on different hosts in agricultural and natural environments suggests an effect of ecological divergence due to adaptation to the host. Indeed, fungal life cycles are unusual in that there is no migration between development on a host and reproduction: a given individual can reproduce with another individual that developed on the same host. Thus, the probability of a particular individual mating is dependent solely on its genotype, which favours or disfavours its development on a particular host. Specialization (*i.e.*, selection for genotypes with the best fitness on one host) therefore acts as a "magic trait" (Gavrilets 2004) in some fungi, pleiotropically allowing both adaptation to the host and reproductive isolation, thus facilitating sympatric divergence (Giraud et al. 2008).

Many studies highlighting the existence of sympatric subdivided *B. cinerea* populations on different hosts, thus suggesting ecological adaptation to the host, also showed that ecological divergence was not "complete", with some gene flow continuing between the different demes. Indeed, significant proportions of spillover individuals (*i.e.* individuals collected on one host but belonging to a genetic group specialized on another host) are regularly recorded (Fournier and Giraud 2008; Leyronas et al. 2015a; Walker et al. 2015). This may be because host adaptation in *B. cinerea* is mostly based on quantitative, rather than in "all or nothing" mechanisms.

Thus, gene flow and mating can still occur between host-specialized sub-populations and individuals are able to infect several host plants, although differences in aggressiveness may be recorded. As discussed by Anderson et al. (2004), ecological divergence, even if incomplete, should be accompanied by a trade-off in the aggressiveness of the pathogen on a different host (*i.e.* a local adaptation pattern for quantitative traits). This issue has seldom been addressed and further investigations are warranted. Leyronas et al. (2015a) showed that the host specialization of *B. cinerea* on tomato and lettuce grown in the same greenhouses was not always accompanied by significant measurable differences in aggressiveness. This may reflect the large, diverse and sophisticated biochemical arsenal of *B. cinerea*, with numerous alternative pathways deployed during the various steps of penetration, development, and decomposition processes (Chap. 12; Amselem et al. 2011; Van Kan 2006). This would allow basal growth to occur on a large range of hosts, with very small quantitative differences.

Moreover, although host adaptation may be incomplete in *B. cinerea*, recent studies have suggested that it may operate at a very fine scale. Indeed, Walker et al. (2015) demonstrated the surprising subdivision of grapevine-adapted populations into three genetic clusters co-existing on this host and only partially subsisting over time, due to recurrent recombination between them and/or with strains from clusters specialized on other hosts. The reasons for this fine-scale adaptation are unclear, but may involve specific host tissues, organs or elements of host physiology. Indeed, preliminary studies have shown that the frequencies of *vacuma* and *transposa* isolates differ significantly as a function of grapevine phenological stage and organ (Martinez et al. 2005, 2008), however, these markers may not always be relevant (see discussion above). Moreover, this clustering may reflect differences in the lifestyle of *B. cinerea*, as it has been suggested that this species can display both facultative cryptic endophytic behavior and classical necrotrophic behavior (Van Kan et al. 2014).

6.3.6 Cropping System as a Structuring Factor

The cropping system may significantly affect the genetics and dynamics of *Botrytis* populations. Indeed, many *B. cinerea* hosts are not cultivated outdoors, where gene flow is thought to be affected by host adaptation and geography. Instead, they are grown indoors, in greenhouses. The diversity of populations sampled from the air, within greenhouses or outdoors, has been shown to be high (Decognet et al. 2009; Kerssies et al. 1997; Bardin et al. 2014), consistent with the hypothesis that substantial amounts of inoculum from the external environment frequently find their way into the greenhouse (Chap. 7). Nevertheless, a comprehensive SSR-based study assessing diversity in populations collected from various ecological niches demonstrated that isolates collected from greenhouse tomatoes in several French regions clustered together and displayed a high degree of differentiation from isolates collected outdoors on other crops. This suggests that, together with host specialization, cropping

system could be a powerful structuring factor (Walker et al. 2015). The proportion of clonal genotypes in populations was low or rising, depending on the greenhouse considered and, particularly, its technological equipment and the prophylaxis measures implemented (*i.e.* the efforts made to limit gene flow from the outside).

Similarly, high levels of diversity were also detected by RAPD analysis in greenhouses from the Almeria region in south-eastern Spain, but the comparison may have been biased by the collection of only a small number of strains per greenhouse (Alfonso et al. 2000; Moyano et al. 2003). However, in this region of intensive indoor cropping concentrated in a limited area and over a short time period, the diversity of the crops grown, together with the associated epidemics and poor prophylaxis (Alfonso et al. 2000), may favor the regular migration of genotypes within and between greenhouses. Considerable genetic diversity was also detected in tomato populations from northern Algerian greenhouses, in which it was suggested that recombination might occur (Adjebli et al. 2015). By contrast, in some tomato greenhouses in south-eastern France, a limited number of genotypes, or even a single well adapted genotype, may colonize the whole greenhouse. This highlights the importance of the secondary inoculum produced within the crop during long growing seasons and suggests that the polycyclic development of grey mould epidemics may occur in such situations (Bardin et al. 2014; Decognet et al. 2009). It may also explain why some genotypes collected on various indoor hosts could be grouped together, after RAPD analysis, according to the greenhouse of origin (Calpas et al. 2006). Finally, as host specialization is thought to be limited, successive crops (*e.g.* tomato and lettuce) may be infected by endogenous inoculum, maintaining the epidemic throughout the year. Nevertheless, differences in cropping practices for these two hosts may result in differences in the epidemiological features of the disease and may account for the more frequent production of secondary inoculum on tomato than on lettuce, resulting in lower levels of genotypic diversity on this host (Leyronas et al. 2015a).

6.3.7 Fungicides as a Structuring Factor

Fungicides may exert an intense selection pressure on populations, logically leading to an increase in the frequency of resistance within populations. However, little is known about their ability to shape population structure. A first set of studies (Table 6.2) investigated whether resistant strains clustered together in analyses with neutral markers. Resistant strains collected in Bordeaux vineyards (France) tended to be of the *transposa* type (Martinez et al. 2005), although the limitations of this marker must be borne in mind, as discussed previously. Yourman et al. (2000) found that all isolates from greenhouses in South Carolina, USA were different, but observed some clustering according to fungicide sensitivity, whereas Moyano et al. (2003) barely detected this pattern in Spanish populations collected in Almerian greenhouses. This suggests that, in some situations, resistance is selected in a limited number of neutral genetic backgrounds that recombine poorly with other genetic backgrounds.

A second set of studies (Table 6.2) investigated whether fungicides could induce the differentiation of populations subjected to various spraying programs. Fungicide treatments were found to have no effect on neutral subdivision, in Chilean populations collected from table grapes and subjected to RAPD analysis (Esterio et al. 2011) or in populations from Champagne vineyards subjected to SSR analysis (Walker and Fournier 2014). Moreover, in this last study, diversity (measured as gene diversity) and the mode of reproduction (measured as clonal richness), were similar in treated and untreated plots. Nevertheless, significant differences in allelic richness and private allele richness between the untreated and treated plots were observed for two of the three sites studied. This is consistent with greater genetic drift, leading to the loss of alleles, particularly for the rarest alleles, in populations that regularly shrink due to fungicide applications (bottlenecks). Moreover, as expected, resistance frequency was significantly higher in treated plots, but only for loci under contemporary selection, not for loci selected by ancient modes of action. Interestingly, the frequency of resistance decreased at all sites, for all four loci, probably due to the combined effect of winter migration and negative selective pressure (resistance cost). Fungicide-mediated selection was also demonstrated by the detection of clines (patterns of resistance organized into a spatial gradient between the treated and untreated plots), especially at vintage time for loci under contemporary selection (Walker and Fournier 2014). Resistance frequencies could therefore be used to model the evolution of these traits over a period of years and to provide estimates of the magnitude of the evolutionary forces at work in vineyards (*e.g.* migration, positive selection, negative selection of resistance cost).

6.3.8 *Other Hypotheses Relating to the Structuring of Populations*

Three additional studies explored the differentiation of the population according to additional factors. Baraldi et al. (2002) detected no genetic differentiation in RFLP analyses of isolates from kiwi fruit that had and had not been stored in the cold, despite the demonstration of a clear adaptation to cold in cold-stored populations. Fournier et al. (2013) used microsatellite genotyping and clustering methods to determine whether isolates sampled from plants displaying grey mould and isolates from plants displaying noble rot symptoms in three French regions belonged to genetically differentiated populations. The inferred population structure matched geography rather than the type of symptom. Noble rot symptoms therefore do not seem to be caused by a specific *B. cinerea* population, depending instead essentially on microclimatic conditions, with implications for the production of sweet wines. At last, Leyronas et al. (2015b) identified eight strongly differentiated genetic clusters from *B. cinerea* populations collected from the air, in southern France. Cluster abundance showed temporal variation and was linked to the season, the climatic parameters and the origin of air masses. *B. pseudocinerea* strains were also isolated and their frequency varied over time, tending to be greater in winter. The relationship between airborne and terrestrial clusters remains to establish but may help

understanding the emergence of grey mould epidemics on crops, as well as the distribution of fungicide resistant alleles in populations from distinct compartments.

In conclusion, considerable amount of information about population genetics has been obtained for *B. cinerea* over the last decade, due to sophisticated marker use and data analysis. In the future, efforts should be made to harmonize the sampling and analysis of isolates in different situations, to improve the comparability of results and to synergize knowledge acquisition by different research groups. Some studies are clearly based on a limited number of isolates (Table 6.2), and poor or false information may be extracted from populations of fewer than 20 or 30 individuals, particularly if these individuals are collected from different hosts or sites. Sampling schemes should dissociate structuring factors, to measure their respective impacts and to prevent situations such as the comparison of populations from tomatoes grown in a greenhouse with populations from outdoor crops or the comparison of populations from one host with those from another host in another country, for example.

6.4 Diversity the Populations of Other Botrytis spp.

Much less is known about the population genetics and genetic diversity of other species of *Botrytis* than about *B. cinerea*.

6.4.1 Botrytis elliptica *and* B. tulipae

Population genetics studies have been carried out on *B. elliptica* with 69 isolates from Taiwan and the USA and RAPD markers (Huang et al. 2001). In total, 43 unique haplotypes were identified, falling into two clusters according to country of origin. Further investigations compared the abilities of three molecular typing methods (multi-locus sequencing, restriction analysis of IGS region, AFLP) to assess variability within *B. cinerea*, *B. elliptica*, and *B. tulipae*. AFLP analysis was found to be the most effective method and was therefore used for the subsequent population genetics studies. In total, 105 genotypes were identified for 174 *B. elliptica* isolates sampled from lilies in the Netherlands. Linkage disequilibrium scores were low and clonal genotypes were detected only within the growing season, at a single site (Staats et al. 2007b). These results suggest that sexual reproduction plays a significant role in determining population diversity in this species, although apothecia have been observed in the field only in the Netherlands, with few details reported (Van den Ende and Pennock-Vos 1997).

By contrast, only 25 genotypes could be distinguished for 170 *B. tulipae* isolates, and clonal genotypes were frequently found in different growing seasons and different locations from the Netherlands. Higher linkage disequilibrium indices confirmed that the *B. tulipae* population was mostly clonal, with some recombination (Staats et al. 2007b).

6.4.2 Botrytis *Species from Onion*

Morphological mutants of *B. squamosa* have been recovered by chemical mutagenesis, and mutants resistant to the fungicide botran have also been obtained, with this resistance segregating as a single gene (Bergquist and Lorbeer 1972). Variation has been investigated in a number of onion-associated species, by UP-PCR (Nielsen et al. 2001). *B. squamosa* was found to be highly diverse, with 10 of the 11 isolates studied having unique haplotypes, consistent with the known heterothallic sexual reproduction of this species (Carisse et al. 2011). By contrast, *B. aclada* and *B. allii* displayed little variation, consistent with a high degree of clonality and the absence of known teleomorphs for these species. Only three isolates of *B. byssoidea* were examined. All were identical, despite originating from different sites in the USA, the Netherlands and the UK, suggesting that the populations of this species may be mostly clonal (Nielsen et al. 2001).

6.4.3 Botrytis pseudocinerea

The formal description of this species was accompanied by a population study (Walker et al. 2011). Indeed, sympatric populations of *B. cinerea* and *B. pseudocinerea* from Champagne vineyards clustered separately, demonstrating the lack of gene flow between these two species. Genotypic diversity was high, of the same order of magnitude as in *B. cinerea*; the clonal fraction was small, and weak linkage disequilibrium was observed, suggesting that recombination regularly occurs in *B. pseudocinerea* populations, through sexual reproduction. The two species are thought to have similar population genetics, but the influence of possible structuring factors has not yet been evaluated in *B. pseudocinerea*.

6.5 Conclusions

Our knowledge of *Botrytis* diversity has greatly increased over the last decade, following the development of relevant techniques and markers and the sequencing of two *B. cinerea* genomes. This knowledge is not only of academic interest, but may also be very useful for disease management. Delimiting species causing similar symptoms and characterizing their biological features and epidemiological importance will undoubtedly contribute to the appropriate adaptation of control strategies. Accurate and complete characterization is thus required, together with depositions in international collections and a relevant multi-criterion determination tool covering all known species. Identifying barriers to gene flow between the various population compartments of *B. cinerea* in natural and agricultural environments should facilitate their manipulation, improving prophylaxis, host deployment or appropriate antifungal compound applications, for example. Finally, although *B. cinerea* is

the model species within this genus, similar developments, particularly in population genetics, would also be useful to improve disease control on major crops affected by other *Botrytis* species.

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