

# Molecular Phylogeny of the Plant Pathogenic Genus *Botrytis* and the Evolution of Host Specificity

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The cosmopolitan genus *Botrytis* contains 22 recognized species and one hybrid. The current classification is largely based on morphological characters and, to a minor extent, on physiology and host range. In this study, a classification of the genus was constructed based on DNA sequence data of three nuclear protein-coding genes (*RPB2*, *G3PDH*, and *HSP60*) and compared with the traditional classification. Sexual reproduction and the host range, important fitness traits, were traced in the tree and used for the identification of major evolutionary events during speciation. The phylogenetic analysis corroborated the classical species delineation. In addition, the hybrid status of *B. allii* (*B. byssoidea* × *B. aclada*) was confirmed. Both individual gene trees and combined trees show that the genus *Botrytis* can be divided into two clades, radiating after the separation of *Botrytis* from other *Sclerotiniaceae* genera. Clade 1 contains four species that all colonize exclusively eudicot hosts, whereas clade 2 contains 18 species that are pathogenic on either eudicot (3) or monocot (15) hosts. A comparison of *Botrytis* and angiosperm phylogenies shows that cospeciation of pathogens and their hosts have not occurred during their respective evolution. Rather, we propose that host shifts have occurred during *Botrytis* speciation, possibly by the acquisition of novel pathogenicity factors. Loss of sexual reproduction has occurred at least three times and is supposed to be a consequence of negative selection.

## Introduction

Fungi of the genus *Botrytis* Persoon are important pathogens of many agronomically important crops, such as grapevine, tomato, bulb flowers, and ornamental crops (Jarvis 1977). *Botrytis* diseases appear primarily as blossom blights and fruit rots but also as leaf spots and bulb rots in the field and in stored products. *Botrytis* species are necrotrophs, inducing host-cell death resulting in progressive decay of infected plant tissue. The pathogen produces abundantly sporulating gray mycelium on infected tissue. Macroconidia (mitotically produced spores) can be transported by wind over long distances. *Botrytis* overwinters in the soil as mycelium in decaying plant debris and as sclerotia, melanized mycelial survival structures. Some species frequently produce a sexual teleomorphic stage in which ascospores are produced in an apothecium. When collected in nature, apothecia are found under cool weather conditions, arising from sclerotia, which have developed on decayed plant parts in moist soil. *Botrytis* and its sexual form *Botryotinia* Whetzel comprise 22 species and one hybrid (Hennebert 1973; Yohalem, Nielsen, and Nicolaisen 2003) and are classified within the family *Sclerotiniaceae* Whetzel (Inoperculate Discomycetes). Delineation of species has traditionally been based on morphological characteristics, especially macroconidium ontogeny, and species have been named based on host association. Most species have a worldwide distribution or occur wherever their host crops are grown (Jarvis 1977).

The genus *Botrytis* comprises one generalist, *B. cinerea*, infecting over 200 eudicot hosts, especially senescing or otherwise weakened or wounded plants (MacFarlane 1968). All other species are considered specialists with a narrow host range. They infect only one or a few closely related species within the same plant genus (Mansfield 1980), with the exception of *B. fabae*, which can

infect species of the genera *Vicia*, *Lens*, *Pisum*, and *Phaseolus*, all belonging to *Fabaceae* (Jarvis 1977). Specialized species occur on corolliferous monocotyledons and on members from the four eudicot families *Fabaceae*, *Ranunculaceae*, *Geraniaceae*, and *Paeniaceae* (Jarvis 1977) (table 1). Narrow host range *Botrytis* species parasitize living leaves or bulbs of their hosts but may also occur as saprophytes. In many cases, host-specific *Botrytis* species are able to cause primary lesions on a nonhost, but these primary lesions fail to expand (Prins et al. 2000).

Several specialists are able to infect members of the same plant family or the same plant species. Three species of *Botryotinia* have been described occurring on the buttercup family *Ranunculaceae*; *B. calthae* occurs on *Caltha palustris* L., *B. ranunculi* on *Ranunculus* spp., and *B. ficariorum* on *Ficaria verna*. Occasionally *B. calthae* has been observed infecting *Ficaria verna*, but otherwise they are specific to their host (Hennebert and Groves 1963). As many as seven *Botrytis* species occur on *Allium* spp., which is one of the largest genera of the petaloid monocotyledons (Friesen, Fritsch, and Blattner 2004). *Allium* includes several economically important species, such as common onion, garlic, chives, and leek, all of which are infected by *B. byssoidea*, *B. aclada*, and *B. allii*. The last species is an allodiploid hybrid based on mitotic chromosome counts, conidia size, restriction fragment length polymorphism (RFLPs), and sequence data (Nielsen and Yohalem 2001; Yohalem, Nielsen, and Nicolaisen 2003; Shirane, Masuko, and Hayashi 1989). *B. byssoidea* and *B. aclada* are considered to be its parental species (Nielsen and Yohalem 2001; Yohalem, Nielsen, and Nicolaisen 2003). Other *Botrytis* species infecting *Allium* spp. have a more restricted host range; they have become specialized on wild *Allium* spp. or infect only one or two economically important *Allium* crops.

To investigate whether closely related *Botrytis* species are pathogenic on closely related plant species, we compared phylogenies of fungi and their angiosperm hosts (APG 2003) for possible cospeciation. Parallel cladogenesis between host and pathogens would be

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**Table 1**  
**Species Recognized by Hennebert (1973), Sexual Stage, Disease Symptoms, and Typical Host Plant Species**

Species	Sexual Stage	Common Disease Name	Typical Host/Tissue Specificity	Host-Plant Species	Host-Plant Family
<i>B. cinerea</i> Pers. / <i>B. fuckeliana</i> (de Bary) Whetzel	Yes	Gray mould	Fallen leaves, fruits, and flowers	>235 plant species	Polyphagous on eudicotyledons
<i>B. fabae</i> Sardiña	No	Chocolate spot	Leaves of bean	<i>Vicia</i> spp. L., <i>Pisum</i> spp. L., <i>Lens</i> spp. L., <i>Phaseolus</i> spp. L.	<i>Fabaceae</i>
<i>B. calthae</i> Hennebert	Yes		Stem of marsh-marigold	<i>Caltha palustris</i>	<i>Ranunculaceae</i>
<i>B. ranunculi</i> Hennebert	Yes		Buttercup	<i>Ranunculus</i> spp. L.	<i>Ranunculaceae</i>
<i>B. ficariarum</i> Hennebert	Yes		Buttercup	<i>Ficaria verna</i>	<i>Ranunculaceae</i>
<i>B. pelargonii</i> Roed	Yes		Leaves of geranium	<i>Pelargonium</i> spp. L.	<i>Geraniaceae</i>
<i>B. paeoniae</i> Oud.	No	Peony blight	Stems of cultivated peonies	<i>Paeonia</i> spp. L.	<i>Paeoniaceae</i>
<i>B. hyacinthi</i> Westerd. and Beyma	No	Hyacinth fire	Leaves of hyacinth	<i>Hyacinthus</i> spp. L.	<i>Hyacinthaceae</i>
<i>B. tulipae</i> Lind	No	Tulip fire	Leaves, stems, and flowers of cultivated tulips	<i>Tulipa</i> spp. L.	<i>Liliaceae</i>
<i>B. elliptica</i> (Berk.) Cooke	Yes	Lily fire	Leaves, stems, and flowers of cultivated lilies	<i>Lilium</i> spp. L.	<i>Liliaceae</i>
<i>B. squamosa</i> Walker	Yes	Onion leaf blight	Leaves of onion	<i>Allium cepa</i>	<i>Alliaceae</i>
<i>B. aclada</i> (Fresen.) Yohalem	No	Gray-mould neck rot	Bulbs of onion, garlic, and leek	<i>Allium</i> spp. L.	<i>Alliaceae</i>
<i>B. allii</i> <sup>a</sup> (Munn) Yohalem	No	Gray-mould neck rot	Bulbs of onion, garlic, and leek	<i>Allium</i> spp. L.	<i>Alliaceae</i>
<i>B. byssoidea</i> Walker/ <i>B. allii</i> (Sawada) Yamamoto	Yes	Mycelial neck rot	Bulbs of onion, garlic, and leek	<i>Allium</i> spp. L.	<i>Alliaceae</i>
<i>B. globosa</i> Raabe	Yes	Neck rot	Wild garlic	<i>Allium ursinum</i> .	<i>Alliaceae</i>
<i>B. porri</i> Buchw.	Yes		Bulbs of garlic, leek	<i>Allium</i> spp. L.	<i>Alliaceae</i>
<i>B. sphaerosperma</i> Buchw.	Yes	Blight	Three-cornered Leek (White-flowered Onion)	<i>Allium triquetrum</i>	<i>Alliaceae</i>
<i>B. narcissicola</i> Kleb. Ex Westerd. and Beyma	Yes	Smoulder mould	Bulbs of narcissus	<i>Narcissus</i> spp. L.	<i>Amaryllidaceae</i>
<i>B. polyblastis</i> Dowson	Yes	Narcissus fire	Leaves of narcissus	<i>Narcissus</i> spp. L.	<i>Amaryllidaceae</i>
<i>B. galanthina</i> (Berk. and Br.) Sacc.	No	Blight	Snowdrop	<i>Galanthus</i> spp. L.	<i>Amaryllidaceae</i>
<i>B. convoluta</i> Whetzel and Drayton	Yes	<i>Botrytis</i> rhizome rot	Rhizomes of cultivated iris	<i>Iris</i> spp. L.	<i>Iridaceae</i>
<i>B. croci</i> Cooke and Massee	No	Crocus blight	Leaves of cultivated crocus	<i>Crocus</i> spp. L.	<i>Iridaceae</i>
<i>B. gladiolorum</i> Timm. / <i>B. draytonii</i> (Budd. and Wakef.) Seaver	Yes	Gladiolus blight	Stems of cultivated gladiolus	<i>Gladiolus</i> spp. L.	<i>Iridaceae</i>

<sup>a</sup> Hybrid species according to Yohalem, Nielsen, and Nicolaisen 2003.

indicative of ancient coevolution, a reciprocal process in which characteristics of one organism evolve in response to specific characteristics of another. The process of coevolution between pathogen and host is often based on gene-for-gene relationships, in which resistance gene alleles in the host are matched by virulence gene alleles in the pathogen (Flor 1955; Heath 1991; Thompson and Burdon 1992; Kniskern and Rausher 2001). Coevolution is more likely expected between obligate biotrophic pathogens or symbionts and their host plants than it is with necrotrophic pathogens. Obligate parasites do not kill host cells but get their nutrients either by penetrating living cells or by establishing close contact with them. Necrotrophic pathogens such as *Botrytis* need to kill host cells before the cells are invaded by the fungus (Clark and Lorbeer 1976; van Baarlen, Staats, and van Kan 2004), which might suggest absence or a low degree of coevolution. There is very limited knowledge about the phylogenetic relationships among members of the genus *Botrytis*. Holst-Jensen, Vaage, and Schumacher (1998) analyzed

nuclear ribosomal internal transcribed spacer (ITS) DNA sequences and concluded that the *Botrytinia* teleomorph along with *Botrytis* anamorphs constitute a monophyletic lineage. However, the relationships among members of the genus *Botrytis* could not be resolved because of the limited phylogenetically informative ITS sequence characters.

In this study, we made use of fragments of three single-copy nuclear DNA (nDNA) genes encoding glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*), Heat-shock Protein 60 (*HSP60*), and DNA-dependent RNA polymerase subunit II (*RPB2*). All three genes encode enzymes that are involved in basic cellular processes, and both *G3PDH* and *RPB2* evolve at moderate evolutionary rates (Smith 1989; Berbee, Pirseyedi, and Hubbard 1999; Liu, Whelen, and Hall 1999; Liu and Hall 2004). The first objective was to clarify the evolutionary history of the genus *Botrytis* and investigate whether DNA sequence data support the classical species delineation. Second, the effect of the hybrid species *B. allii* on tree structure was examined because hybrids may cause topological changes, especially

when parents are distantly related (McDade 1992). The third objective was to trace the evolution of the reproductive mode and the host-range spectrum within the genus *Botrytis*.

## Materials and Methods

### Fungal Isolates

A set of 52 isolates (table 2) was chosen to represent all the recognized *Botrytis* species. Most strains were obtained from the Belgian Coordinated Collections of Microorganisms (BCCM, Belgium, Brussels). Additional strains were obtained from the Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands) and personal collections. Single isolates of *Sclerotinia sclerotiorum* and *Monilinia fructigena* were selected as outgroups on the basis of a phylogenetic study by Holst-Jensen, Vaage, and Schumacher (1998). Strains were grown on malt agar (Oxoid) for 10 to 14 days in the dark at 18°C.

### DNA Extraction, Amplification, and Sequencing

Mycelial tissue was harvested, lyophilized, submerged in liquid nitrogen and ground into a powder. Genomic DNA was extracted from 10 to 20 mg dry tissue using the Puregene DNA isolation kit (Gentra systems Inc./Biozym systems, Landgraaf, The Netherlands) according to the manufacturer's instructions. DNA pellets were dissolved in 100 µl of TE (10mM Tris-HCl [pH 8.0], 1mM EDTA) and stored at 4°C or -20°C.

Primer combinations (table 3) were designed or modified to amplify regions of three nuclear DNA genes (*G3PDH*, *HSP60*, and *RPB2*). PCR primers to amplify the *RPB2* region that have been described previously by Liu, Whelen, and Hall (1999) were slightly modified. To amplify *G3PDH* and *HSP60*, forward and reverse primers were designed based on homologous gene sequences from GenBank and a *Botrytis cinerea* cDNA library (Genoscope, Centre National de Séquençage, France). For amplification of ITS region, primers ITS1 and ITS4 were used (White et al. 1990). To facilitate batch sequencing of different PCR products, primers were extended with M13(-20) forward primers or M13 reverse primers.

PCR amplification was carried out in a 50-µl reaction mixture that contained 10 to 50 ng of genomic DNA, 1X GeneAmp PCR buffer (PerkinElmer, Norwalk, Conn.), 200 mM of each deoxynucleoside triphosphate (Promega, Madison, Wis.), 0.2 pmol of each primer (Amersham Pharmacia Biotech), 2 mM MgSO<sub>4</sub> and 1.0 U of AmpliTaq DNA polymerase (PerkinElmer). Amplifications were carried out in a Peltier Thermal Cycler-200 (Biozym, Landgraaf, The Netherlands). The following thermocycling pattern was used to amplify *HSP60* and *RPB2* gene fragments: 94°C for 5 min (1 cycle); 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s (35 cycles), and then 72°C for 10 min (1 cycle). The same program with an annealing temperature of 64°C was used for *G3PDH* gene fragments. For amplification of the ITS region, an amplification protocol was used as described by White et al. (1990). The PCR products were separated on a 1% agarose-Tris-borate-EDTA (1X TBE) gel, containing ethidium bromide. The fragment size of the PCR product was verified by compari-

son to a 100-bp DNA Ladder (GibcoBRL). PCR products were purified using the QIAquick PCR purification kit (Qiagen) following manufacturer's instructions. Purified PCR products were sequenced (BaseClear Holding B.V., Leiden, The Netherlands) in both directions using M13(-20) forward and M13 reverse primers.

Six PCR products were cloned because the yield of amplified product was insufficient for direct sequencing: *M. fructigena* 9201 (locus *RPB2*), *B. hyacinthi* MUCL442 (loci *RPB2*, *HSP60* and *G3PDH*), *B. byssoidea* MUCL94 (locus *HSP60*), and *B. calthae* MUCL1089 (locus *G3PDH*). Furthermore, multiple alleles of allopolyploid *B. allii* strains MUCL403 and MUCL1150 were cloned as well. PCR products were extracted from 1% agarose gel using the GFX PCR purification kit (Amersham Biosciences). PCR products were then ligated into a pGemT-Easy Vector (Promega) using standard protocols and introduced into electrocompetent *Escherichia coli* DH5α cells. Transformed cells were spread on LB-plates, each containing 100 mg/ml ampicillin, 20 mg/ml X-gal, and 0.1 mM IPTG and incubated at 37°C overnight. White colonies were screened for correct insert size using gene-specific PCR primers. To distinguish between alleles of each locus, 20 positive clones of *B. allii* strains MUCL1150 and MUCL403 were selected for restriction digestion. Restriction enzymes were selected based on polymorphic sites between aligned sequences of each locus of the presumed parental species *B. aclada* (isolate MUCL8415) and *B. byssoidea* (isolate MUCL94). Predicted restriction profiles were compared with digestion patterns of cloned fragments of both parental species, as well as both *B. allii* strains. *G3PDH* and *HSP60* fragments were digested with the restriction enzyme *EcoRV* (Promega), whereas *RPB2* fragments were digested with *HindIII* (Promega). Subsequently, individual clones, containing different alleles, were selected for sequencing based on restriction profiles. Clones were sequenced in both directions using SP6 and T7 primers (see table 3).

### Phylogenetic Analysis

Automated sequence outputs were imported into Vector NTI suite 8.0 (InforMax, Inc.) for visual inspection of chromatographs and assembly of the contigs. For unresolved basepairs, the IUPAC nucleotide coding system was used. Sequences were compared between complementary strands for reading errors and aligned using ClustalX version 1.8 (Thompson et al. 1997). Alignments were visually inspected and adjusted manually when necessary. Regions of sequences with ambiguous alignment were excluded from all analyses (table 4). The full alignment containing all three loci is deposited in TREEBASE (accession number S1170). Individual sequences are deposited in GenBank under accession numbers AJ704990 to AJ705044, AJ716290 to AJ716305, AJ716046 to AJ716103, and AJ745662 to AJ745716 (see table 2).

The basic data set for each locus consisted of 50 taxa: 48 *Botrytis* specimens and two outgroup species. Phylogenetic analyses were conducted for each of the three loci (*RPB2*, *HSP60*, and *G3PDH*) and for a combined data matrix of all three. To assess the impact of hybrids on tree

**Table 2**  
**Isolates of *Botrytis*, Origin, Year of Collection, and GenBank Accession Numbers for DNA Sequences Used in This Study**

Species	Collection Number	Geographic Origin	Year	GenBank Accession Number of Sequences			
				ITS	<i>RPB2</i>	<i>HSP60</i>	<i>G3PDH</i>
<i>B. aclada</i>	PRI006 <sup>a</sup>	—	—	AJ716295	AJ745665	AJ716051	AJ704993
	MUCL3106 <sup>b,c</sup>	USA	1961	N.D. <sup>j</sup>	AJ745663	AJ716049	AJ704991
	MUCL8415 <sup>b,c</sup>	Germany	1965	N.D.	AJ745664	AJ716050	AJ704992
<i>B. allii</i>	MUCL403 <sup>b,c</sup>	The Netherlands	1957	N.D.	AJ745666 (allele 1)	AJ716055 (allele 1)	AJ704996 (allele 1)
					AJ745667 (allele 2)	AJ716056 (allele 2)	AJ704997 (allele 2)
	MUCL1150 <sup>b,c</sup>	Norway	1960	N.D.	AJ745668 (allele 1)	AJ716052 (allele 1)	AJ704994 (allele 2)
					AJ745669 (allele 2)	AJ716053 (allele 2)	AJ704995 (allele 1)
						AJ716054 (allele 3)	
<i>B. byssoides</i>	MUCL94 <sup>b,c,d</sup>	USA	1923	N.D.	AJ745670	AJ716059	AJ704998
<i>B. calthae</i>	CBS 175.63 <sup>e</sup>	USA	1961	AJ716302	AJ745671	AJ716060	AJ704999
	MUCL1089 <sup>b</sup>	Belgium	1960	N.D.	AJ745672	AJ716061	AJ705000
	MUCL2830 <sup>b</sup>	USA	1961	N.D.	AJ745673	AJ716062	AJ705001
<i>B. convoluta</i>	9801 <sup>f</sup>	The Netherlands, Lisse	1998	AJ716304	AJ745679	AJ716068	AJ705007
	MUCL11595 <sup>b</sup>	USA	1968	N.D.	AJ745680	AJ716069	AJ705008
<i>B. cinerea</i>	SAS56 <sup>g</sup>	Italy	—	AJ716294	AJ745677	AJ716067	AJ705006
	SAS405 <sup>g</sup>	Italy	—	N.D.	AJ745678	AJ716066	AJ705005
	B05.10 <sup>h</sup>	—	1994	N.D.	AJ745674	AJ716063	AJ705002
	BC7 <sup>i</sup>	The Netherlands	1970	N.D.	AJ745675	AJ716064	AJ705003
	MUCL87 <sup>b,d</sup>	The Netherlands	1928	N.D.	AJ745676	AJ716065	AJ705004
<i>B. croci</i>	MUCL436 <sup>b</sup>	The Netherlands	1968	N.D.	AJ745681	AJ716070	AJ705009
<i>B. elliptica</i>	BE9714 <sup>f</sup>	The Netherlands, Elsloo	—	AJ716300	AJ745684	AJ716073	AJ705012
	BE9610 <sup>f</sup>	The Netherlands	—	N.D.	AJ745683	AJ716072	AJ705011
<i>B. fabae</i>	BE0022 <sup>f</sup>	The Netherlands, Smilde	2001	N.D.	AJ745682	AJ716071	AJ705010
	CBS 109.57 <sup>e</sup>	The Netherlands	1957	AJ716303	AJ745685	AJ716074	AJ705013
<i>B. ficariorum</i>	MUCL98 <sup>b,d</sup>	Spain	1929	N.D.	AJ745686	AJ716075	AJ705014
	CBS 176.63 <sup>d,e</sup>	Belgium	1960	AJ716296	AJ745687	AJ716076	AJ705015
<i>B. galanthina</i>	MUCL376 <sup>b</sup>	Belgium	1957	N.D.	AJ745688	AJ716077	AJ705016
	MUCL435 <sup>b</sup>	The Netherlands	1958	N.D.	AJ745689	AJ716079	AJ705018
<i>B. gladiolorum</i>	MUCL3204 <sup>b</sup>	The Netherlands	1963	N.D.	AJ745690	AJ716078	AJ705017
	9701 <sup>f</sup>	—	1997	N.D.	AJ745691	AJ716080	AJ705019
	MUCL3865 <sup>b</sup>	The Netherlands, Andijk	1963	N.D.	AJ745692	AJ716081	AJ705020
<i>B. globosa</i>	MUCL444 <sup>b</sup>	Belgium	1958	N.D.	AJ745693	AJ716083	AJ705022
	MUCL21514 <sup>b</sup>	UK	1963	N.D.	AJ745694	AJ716082	AJ705021
<i>B. hyacinthi</i>	0001 <sup>f</sup>	The Netherlands, Lisse	1999	AJ716297	AJ745695	AJ716084	AJ705023
	MUCL442 <sup>b</sup>	The Netherlands, Breezand	1958	N.D.	AJ745696	AJ716085	AJ705024
<i>B. narcissicola</i>	MUCL1885 <sup>b</sup>	UK	1972	N.D.	AJ745698	AJ716086	AJ705025
	MUCL2120 <sup>b</sup>	Canada	1961	N.D.	AJ745697	AJ716087	AJ705026
<i>B. paeoniae</i>	MUCL16084 <sup>b</sup>	Belgium	1970	N.D.	AJ745700	AJ716089	AJ705028
	0003 <sup>k</sup>	The Netherlands	2002	AJ716298	AJ745699	AJ716088	AJ705027
<i>B. pelargonii</i>	CBS 497.50 <sup>d,e</sup>	Norway	1949	AJ716290	AJ745662	AJ716046	AJ704990
	MUCL1152 <sup>b</sup>	Norway	1960	N.D.	AJ745701	AJ716090	AJ705029
<i>B. polyblastis</i>	MUCL21492 <sup>b</sup>	UK	1963	N.D.	AJ745703	AJ716092	AJ705031
	CBS287.38 <sup>d,e</sup>	UK	1938	AJ716291	AJ745702	AJ716091	AJ705030
<i>B. porri</i>	MUCL3234 <sup>b,d</sup>	—	1926	AJ716292	AJ745704	AJ716093	AJ705032
	MUCL3349 <sup>b</sup>	Belgium	1963	N.D.	AJ745705	AJ716094	AJ705033
<i>B. ranunculi</i>	CBS178.63 <sup>d,e</sup>	USA	1963	N.D.	AJ745706	AJ716095	AJ705034
<i>B. sphaerosperma</i>	MUCL21481 <sup>b</sup>	UK	1963	AJ716293	AJ745708	AJ716096	AJ705035
	MUCL21482 <sup>b</sup>	UK	1963	N.D.	AJ745709	AJ716097	AJ705036
<i>B. squamosa</i>	PRI026 <sup>a</sup>	—	—	AJ716299	AJ745707	AJ716100	AJ705039
	MUCL1107 <sup>b,d</sup>	USA	1923	N.D.	AJ745710	AJ716098	AJ705037
	MUCL9112 <sup>b</sup>	The Netherlands	1966	N.D.	AJ745711	AJ716099	AJ705038
<i>B. tulipae</i>	BT9830 <sup>f</sup>	The Netherlands	2000	AJ716301	AJ745713	AJ716102	AJ705041
	BT9001 <sup>f</sup>	The Netherlands	2000	N.D.	AJ745712	AJ716101	AJ705040
	BT9901 <sup>f</sup>	The Netherlands, Apeldoorn	2000	N.D.	AJ745714	AJ716103	AJ705042
<i>B. anthophila</i>	CBS122.26 <sup>d,e</sup>	The Netherlands	1926	AJ716305	N.D.	N.D.	N.D.
<i>M. fructigena</i>	9201 <sup>l</sup>	—	1992	N.D.	AJ745715	AJ716047	AJ705043
<i>S. sclerotiorum</i>	484 <sup>l</sup>	—	—	N.D.	AJ745716	AJ716048	AJ705044

<sup>a</sup> Dr. P. van den Boogert, Plant Research International, Wageningen.<sup>b</sup> Belgium Coordinated Collection of Microorganisms (BCCM).<sup>c</sup> Yohalem, Nielsen, and Nicolaisen 2003.<sup>d</sup> Type, neotype or epitype specimen.<sup>e</sup> Centraalbureau voor Schimmelcultures, Fungal Biodiversity Center (CBS-KNAW).<sup>f</sup> Applied Research Plant and Environment, Research Unit Flower Bulbs.<sup>g</sup> Farettra, Antonacci, and Pollastro 1988.<sup>h</sup> Büttner et al. 1994.<sup>i</sup> Van der Vlugt-Bergmans et al. 1992.<sup>j</sup> N.D. = Not Determined.<sup>k</sup> Applied Research Plant and Environment, Research Unit Glasshouse Horticulture.<sup>l</sup> Department of Biological Farming systems, Wageningen University.

**Table 3**  
**Primers Used for PCR Amplification and Sequencing**

Primer Name	Target Region	Primer Sequence (5'–3')	Reference or Position
G3PDHfor+	G3PDH	gtgactgtaaacgacggccagtATTGACATCGTCGCTGTC AACGA	790–817 <sup>a</sup>
G3PDHrev+	G3PDH	gtgaccaggaaacagctatgaccACCCACTCGTTGTCTG TACCA	1769–1789 <sup>a</sup>
HSP60for+	HSP60	gtgactgtaaacgacggccagtCAACAATTGAGATTTGCC CACAAG	651–674 <sup>b</sup>
HSP60rev+	HSP60	gtgaccaggaaacagctatgaccGATGGATCCAGTGG TACCGAGCAT	1750–1776 <sup>b</sup>
RPB2for+	RPB2	gtgactgtaaacgacggccagtGATGATCGTGATCAT TCCGG	Modified from Yajuan et al. (1999)
RPB2rev+	RPB2	gtgaccaggaaacagctatgaccCCCATAGCTTGCTT ACCCAT	
ITS1+	ITS	gtgactgtaaacgacggccagtTCCGTAGTGTAACCTG CCGG	Modified from White et al. (1990)
ITS4+	ITS	gtgaccaggaaacagctatgaccTCCTCCGCTTATTG ATATGC	
M13 (–20) forward	—	TGTA AACGACGGCCAGT	
M13 Reverse	—	CAGGAAACAGCTATGACC	
SP6	SP6 promotor	GATTTAGGTGACACTATAG	
T7	T7 promotor	TAATACGACTCACTATAGGG	

<sup>a</sup> Base pair coordinates in homologous gene in *Sclerotinia sclerotiorum* (AF417110).

<sup>b</sup> Base pair coordinates in homologous gene in *Coccidioides immitis* (U81786).

structure, separate analyses were performed for the three DNA regions with and without hybrid taxa. Basic data sets plus five to eight hybrids were analyzed separately. All alleles of each locus of *B. allii* MUCL1150 and *B. allii* MUCL403, as well as of *B. aclada* MUCL3106 were treated as hybrid taxa. Because multiple alleles were found for hybrid taxa, combined analysis was performed with the basic data set only.

Parsimony analyses were carried out using the software package PAUP\* version 4.0b10 (Swofford 2002) on a G4 Power Macintosh. The following settings were used: heuristic search with tree bisection-reconnection (TBR) branch swapping, with MULPARS (keeping all equally most-parsimonious trees) on, 1,000 replicates of random taxon-addition order, and all character transformations treated as equally likely (Fitch parsimony [Fitch 1971]). To minimize the time spent searching large numbers of trees, a limit of five trees was set for each replicate. After completing the replicates, all trees found were then used as starting trees for another round of swapping, with no limit to the number of trees. Gaps were treated as fifth character state, and multistate characters were treated as uncertain. A jackknife analysis was carried out to assess data structure and identify significant

supported clades ( $\geq 63\%$ ). Search type was set to “fast” stepwise-addition (for individual loci) or full heuristic (for combined analysis). Jackknife values were generated by randomly deleting 37% of the characters for 10,000 replicates and using “Jac” resampling (Farris et al. 1996).

Bayesian analyses were performed using MrBayes version 3.0b4 (Ronquist and Heulsenbeck 2003). Each run consisted of four incrementally heated Markov chains run simultaneously, with heating values set to default (0.2). Default uniform priors were used for all model parameters (six substitution rates, four base frequencies, proportion of invariable sites, and alpha value of gamma distribution). Data partitions were set up for all coding and noncoding regions of each locus. *HSP60* and *G3PDH* both contained two introns and two exons. For both loci individually, the two introns were pooled, and the two exons were pooled and analyzed as two partitions. Likelihood model parameters were separately optimized during MCMC searches for each data partition. Markov chains were initiated from a random tree and run for five-million generations; samples were taken every 100th generation. Convergence among chains was monitored by examining plots of log-likelihood values. Burn-in period (i.e. lack of improvement of log-likelihood values) was evaluated visually. All samples taken before burn-in were discarded in PAUP\*, and the remaining samples were used to determine posterior probability (PP) distributions. Each run was performed twice. Bayesian 50% majority-rule consensus trees were generated in PAUP\*. Branches with at least 63% jackknife support and with at least 0.95 Bayesian PP were considered as well supported.

Concordance of combined data sets was evaluated by comparing jackknife topologies via visual inspection and partition homogeneity tests (PHT [Farris et al. 1995]) implemented with PAUP\*. Visual inspection permits the precise location of areas of strong discordance (branches conflicting with jackknife values at least 85% and Bayes PP at least 0.95) between phylogenies generated by different data sets. PHTs were carried out for all pairwise combinations of the three data sets and for all data sets combined using heuristic search (TBR) with 100 random-addition replicates and informative characters only. The null hypothesis of congruence was rejected if  $P < 0.001$  (Darlou and Lecointre 2002; Dettman, Jacobson, and Taylor 2003).

**Table 4**  
**Summary of the RPB2, HSP60, G3PDH, and Combined Sequence Alignments**

	<i>HSP60</i>	<i>G3PDH</i>	<i>RPB2</i>	Combined
Total/mean positions <sup>a</sup>	983/977	890/886	1096/1093	2969/2956
Total intron size (bp)/number of introns	88/2	121/2	31/1	240/5
Excluded positions <sup>b</sup>	4–8 15–19 183–192	none	none	1100–1104 1111–1115 1279–1288
Phylogenetic informative characters (substitutions/indels)	158 (157/1)	126 (125/1)	203 (201/2)	487 (483/4)

<sup>a</sup> Total positions in the alignment/mean number of positions averaged across taxa.

<sup>b</sup> Excluded positions caused by ambiguous alignment.

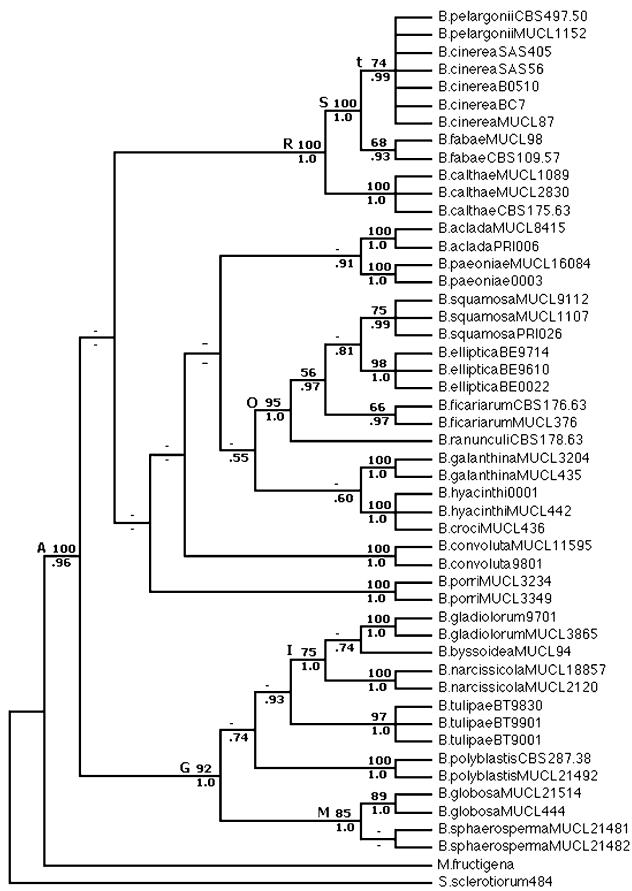


FIG. 1.—Semistrict consensus of two most-parsimonious trees based on *G3PDH* data. This tree was compatible overall in highly supported lineages to the Bayesian 50% majority-rule consensus tree. Jackknife frequencies (10,000 replicates) are shown above each node; Bayesian posterior probabilities (PP) are shown below each node. Letters indicate major clades with significant or strong support (see table 5). A dash indicates support for the branch was less than 50% jackknife and less than 0.50 PP.

Functional characters were mapped onto the molecular phylogeny using MacClade version 4 (Maddison and Maddison 2000): reproductive mode (two states; strictly asexual or asexual and sexual); host-plant group (two states; monocotyledons or eudicotyledons) and host-plant family (10 states; *Hyacinthaceae*, *Liliaceae*, *Alliaceae*, *Amaryllidaceae*, *Iridaceae*, *Paeniaceae*, *Geraniaceae*, *Ranunculaceae*, *Fabaceae*, or Polyphagous on eudicotyledons). All characters were equally weighted under parsimony. Character states from literature (Jarvis 1977, 1980; Hennebert and Groves 1963; Hennebert 1973) are listed in table 1.

## Results

In preliminary experiments, sequence data of eight protein-coding loci and the noncoding ITS region were acquired for multiple isolates of three *Botrytis* species. The three most-informative loci (*G3PDH*, *HSP60*, and *RPB2*) were used as starting point for this study. A total of 188 new sequences of 54 specimens are reported here. Sequences of protein-coding gene fragments and ITS sequences were first evaluated in a standard GenBank Blast search. Nearly all obtained sequences corresponded

with listed *Botrytis cinerea* or *Sclerotinia sclerotiorum* sequences. The ITS sequence of *B. anthophila* Bondartsev, a species reported in Jarvis (1977), was identical to *Rhizoctonia* sp. ITS sequences, and this sequence was, therefore, excluded from further analyses. An alignment of ITS characters, consisting of 453 nucleotide positions of the sequences listed here (table 2) and of sequences of *Botrytis* species reported by Holst-Jensen, Vaage, and Schumacher (1998), contained a limited number of singletons and phylogenetic informative characters (data not shown). Therefore, ITS sequence data were excluded from further analyses.

## Analysis of *G3PDH* Sequence Data and the Influence of Hybrid Taxa on Tree Structure

The basic sequence matrix (table 4) consists of 890 sites, of which 186 (21%) were variable characters and 126 (14%) were potentially parsimony informative. Analysis of *G3PDH* sequences with equal (Fitch) weights resulted in two equally most-parsimonious trees of 312 steps, with a consistency index (CI) of 0.73 and a retention index (RI) of 0.89. Relationships among the main clades were topologically identical in MP and Bayesian consensus trees (data not shown), except for some minor differences. Comparison between replicate Bayesian analyses resulted in identical majority-rule consensus trees, with only marginally lower mean log-likelihood values (−2998.75 and −3025.33). Bayesian PP and jackknife percentages of branches are included on the MP semistrict consensus tree in figure 1. The semistrict consensus tree topology of the two most-parsimonious trees (MPTs) contains 24 nodes with at least 63% jackknife and at least 95% PP support.

The *G3PDH* data set plus hybrids consists of 53 taxa, including two alleles of both *B. allii* MUCL1150 and *B. allii* MUCL403. The heuristic search yielded 11 MPTs with higher tree length and slightly lower CI ( $L = 323$ ,  $CI = 0.71$ , and  $RI = 0.89$ ). MP and Bayesian analyses resulted in almost identical trees (data not shown). The semistrict consensus of those fundamental trees recovers the same major clades as in the *G3PDH* data sets without hybrid taxa, and branch support was only marginally lower (table 5). Within these trees, one allele of *B. allii* strain MUCL1150 (allele 1) and two alleles of *B. allii* strain MUCL403 (alleles 1 and 2) are placed in a clade together with *B. aclada* (strains MUCL8415, MUCLPRI006, and MUCL3106). Allele 2 of *B. allii* strain MUCL403 is placed together with *B. bysoidea*.

## Analysis of *HSP60* Sequence Data and the Influence of Hybrid Taxa on Tree Structure

The basic sequence matrix consists of 983 characters, from which 20 positions were removed because of ambiguity in the alignment (table 4). Of the remaining 963 characters, 225 (23.4%) were variable, and 158 (16.4%) were potentially phylogenetically informative. The Fitch parsimony recovered 320 shortest trees, each 373 steps long ( $CI = 0.74$ ,  $RI = 0.90$ ). The Bayesian tree was consistent with the consensus of these trees, and separate Bayesian analyses resulted in identical trees (mean

**Table 5**  
**Cladistic Behavior of *RPB2*, *HSP60*, *G3PDH*, and Combined Data Sets with and Without Hybrid Taxa**

Locus	Number of		Number of		Placement of Hybrids	Number of		CI	RI	Steps				
	Parsimony-Informative Characters	Number of Ingroup Taxa	Major Clades with Significant or Strong Support Recovered in Semistrict Consensus <sup>a</sup>	Most-Parsimonious Cladograms										
<i>G3PDH</i>	126	48	A	—	—	—	—	0.730	0.89	312				
<i>G3PDH</i> + hybrid	129	53	A	—	—	—	—	0.710	0.89	323				
<i>HSP60</i>	158	48	A	—	—	—	—	0.740	0.90	373				
<i>HSP60</i> + hybrid	160	56	A	—	—	—	—	0.690	0.89	404				
<i>RPB2</i>	203	48	A	—	—	—	—	0.730	0.91	439				
<i>RPB2</i> + hybrid	205	53	A	—	—	—	—	0.700	0.91	459				
Combined	487	48	A	b	c	d	E	F	G	—	80	0.720	0.89	1,151

<sup>a</sup> Lowercase letters indicate significantly supported branches (jackknife branch support at least 63% and Bayesian PP at least 0.95). Capital letters indicate strongly supported branches (jackknife branch support at least 85% and Bayesian PP at least 0.95). Dashes indicate unsupported branches (jackknife branch support less than 50% and Bayesian PP less than 0.95). Clades that, in addition to wild terminals, include hybrids are also considered recovered.

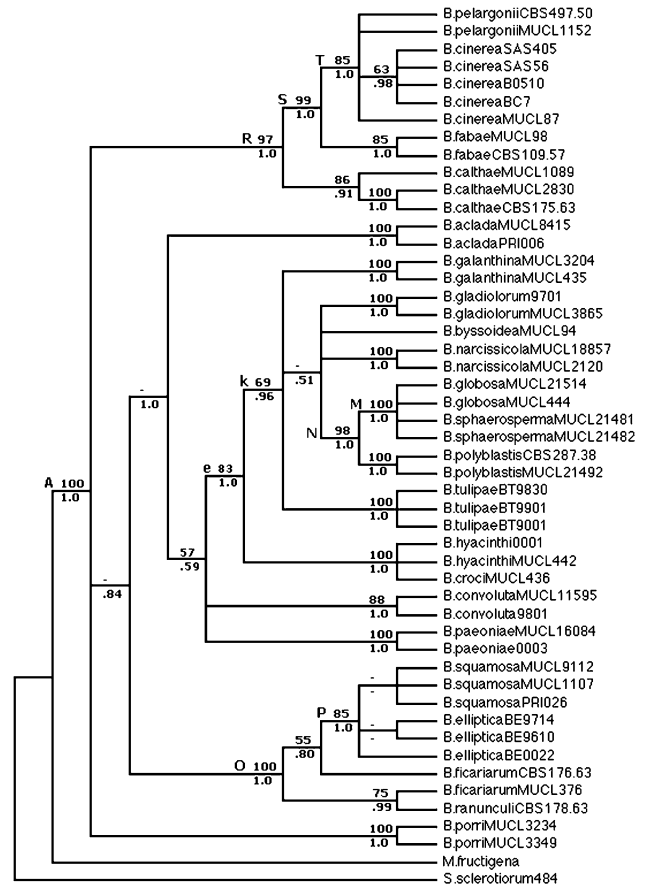


Fig. 2.—Semistrict consensus of 320 most-parsimonious trees based on *HSP60* data. This tree was compatible overall in highly supported lineages to the Bayesian 50% majority-rule consensus tree. Jackknife frequencies (10,000 replicates) are shown above each node; Bayesian posterior probabilities (PP) are shown below each node. Letters indicate major clades with significant or strong support (see table 5). A dash indicates support for the branch was less than 50% jackknife and less than 0.50 PP.

log-likelihood values  $-3357.89$  and  $-3390.86$  [data not shown]). Bayesian PP and jackknife percentages of branches are included on the MP semistrict consensus tree in figure 2. The semistrict consensus tree topology of the 320 MTPs contains 23 nodes with at least 63% jackknife and at least 95% PP support.

The *HSP60* data set plus hybrids consist of 58 taxa, including three alleles of *B. allii* strain MUCL1150 and four alleles of *B. allii* strain MUCL403. Fitch analysis gave 320 trees of 404 steps, with CI = 0.69 and RI = 0.89. Hybrid taxa had strong influence on tree structure in both MP and Bayesian analyses, resulting in the collapse of (sub)clades e, and k (table 5). MP and Bayesian analyses resulted in almost identical topologies (data not shown); however, there are also important differences. Bayesian analysis strongly supported (100% PP) a large clade containing members of clade e, *B. convoluta*, *B. paeoniae*, and a subclade (98% PP) placing hybrids as basal members of their derived parents *B. byssoidea* and *B. aclada*. This clade is highly supported by Bayesian posterior probabilities but is not present at all in the jackknife tree.

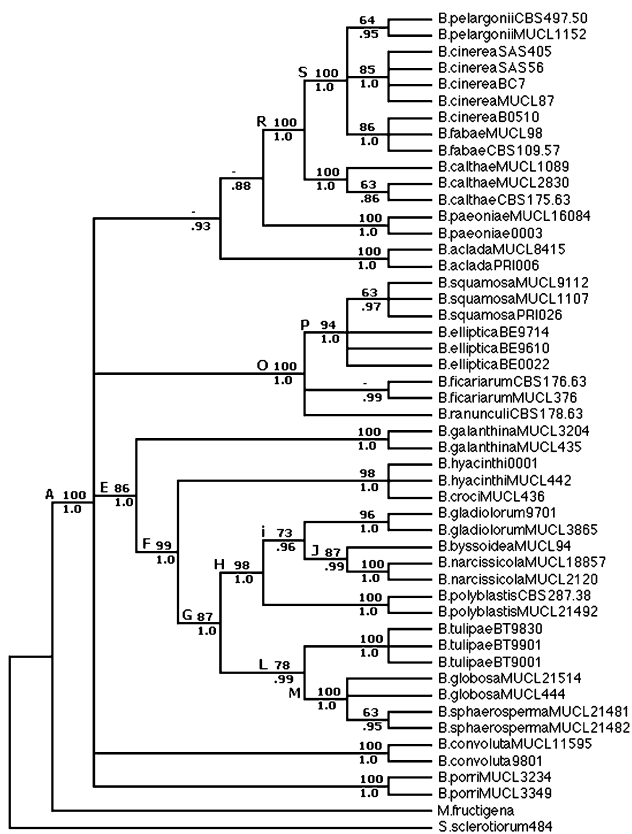


FIG. 3.—Semistrict consensus of seven most-parsimonious trees based on *RPB2* data. This tree was compatible overall in highly supported lineages to the Bayesian 50% majority-rule consensus tree. Jackknife frequencies (10,000 replicates) are shown above each node; Bayesian posterior probabilities (PP) are shown below each node. Letters indicate major clades with significant or strong support (see table 5). A dash indicates support for the branch was less than 50% jackknife and less than 0.50 PP.

#### Analysis of *RPB2* Sequence Data and the Influence of Hybrid Taxa on Tree Structure

The basic sequence matrix consists of 1096 positions, of which 274 (25%) were variable and 203 (18.5%) were potentially parsimony informative (table 4). Analysis of *RPB2* sequences with equal (Fitch) weights resulted in seven equally most-parsimonious trees of 439 steps, with a CI of 0.73 and an RI of 0.91. Relationships among the main clades were topologically identical in MP and Bayesian consensus trees (data not shown). Comparison between replicate Bayesian analyses resulted in identical majority-rule consensus trees, with only marginally lower mean log-likelihood values (−3955.80 and −3970.45; not shown). Bayesian PP and jackknife percentages of branches are included on the MP semistrict consensus tree in figure 3. Of the three individual regions, *RPB2* provided the highest number of significantly supported nodes (26 nodes with at least 63% jackknife and at least 95% PP support), which are mainly located at the terminal branches. The *RPB2* topology contains the three main (sub)clades (R, O, and E) that also occur in the combined analysis (table 5), although relationships among these clades remained unresolved.

The *RPB2* data set plus hybrids consisted of 55 taxa, including two alleles of both *B. allii* strain MUCL1150 and *B. allii* strain MUCL403. The heuristic search yielded six MPTs with higher tree length and slightly lower CI ( $L = 459$ ,  $CI = 0.70$ , and  $RI = 0.91$ ). MP and Bayesian analyses resulted in topologically identical trees (data not shown). The semistrict consensus of those fundamental trees recovers the same major clades as the basic data set (table 5). However, one extra clade was recovered, although with low support (<50% jackknife and 94% PP), consisting of clade O, *B. aclada* (MUCL8415, MUCLPRI006, and MUCL3106), and *B. allii* (MUCL1150 allele 1 and MUCL403 alleles 1 and 2). Allele 2 of *B. allii* strain MUCL1150 was placed together with *B. byssoidea*. Furthermore, as a result of hybrid taxa, jackknife branch support for nodes E and G decreased from strongly supported (86% and 87%, respectively) to well supported (73% and 78%, respectively).

#### Assessment of Between-Data Sets Congruence

The *G3PDH* tree was largely congruent with the *RPB2* tree; in both trees, there is good support for three main (sub)clades (R, O, and G [figs. 1 and 3]), but arrangements within each clade could not be clearly resolved. At the base of the tree, some resolution is provided; however, branches indicating relationships between clades are not supported (fig. 3). In comparison to the *RPB2* topology, more resolution is provided by *HSP60* at the base of the tree (figs. 2 and 3). Contrary to the *RPB2* tree, the positions of *B. galanthina* and *B. hyacinthi*/*B. croci* have been exchanged, as well as of *B. polyblastis* and *B. tulipae*. As with *RPB2*, *HSP60* placed *B. porri* as part of a basal trichotomy. Jackknife topologies were used to test whether the three data sets have different underlying phylogenetic histories. Strong incongruence (>85% jackknife and  $\geq 0.95$  PP) between *RPB2* and *HSP60* phylogenies was evident in the placement of *B. cinerea* strain B05.10 and *B. polyblastis*. None of the strongly supported clades was incongruent between the other data sets (*RPB2* versus *G3PDH* and *G3PDH* versus *HSP60*). The PHT was used as an alternative method to determine whether each possible combination of loci differs significantly in phylogenetic structure. No significant conflict was detected for all pairwise comparisons of each locus (*RPB2*–*HSP60*, *RPB2*–*G3PDH*, and *HSP60*–*G3PDH*;  $0.007 \geq P \geq 0.001$ ) and the three combined ( $P = 0.001$ ).

#### Molecular Phylogeny Distinguishes Two Major Groups

Because no significant between-data set incongruence was detected and because the individual jackknife topologies produced nearly identical results, the three regions were combined directly. Analysis of this combined matrix resulted in 80 trees of 1,151 steps ( $CI = 0.72$  and  $RI = 0.89$ ). The trees were 27 steps longer than the combined number of steps from the *RPB2*, *HSP60*, and *G3PDH* trees (table 5). The Bayesian tree was consistent with the MP tree, but as with the *RPB2* MP tree, *B. polyblastis* was strongly placed (Bayesian PP = 0.99) as a sister taxa to *B. narcissicola*, *B. gladiolorum*, and *B. byssoidea* (see figures



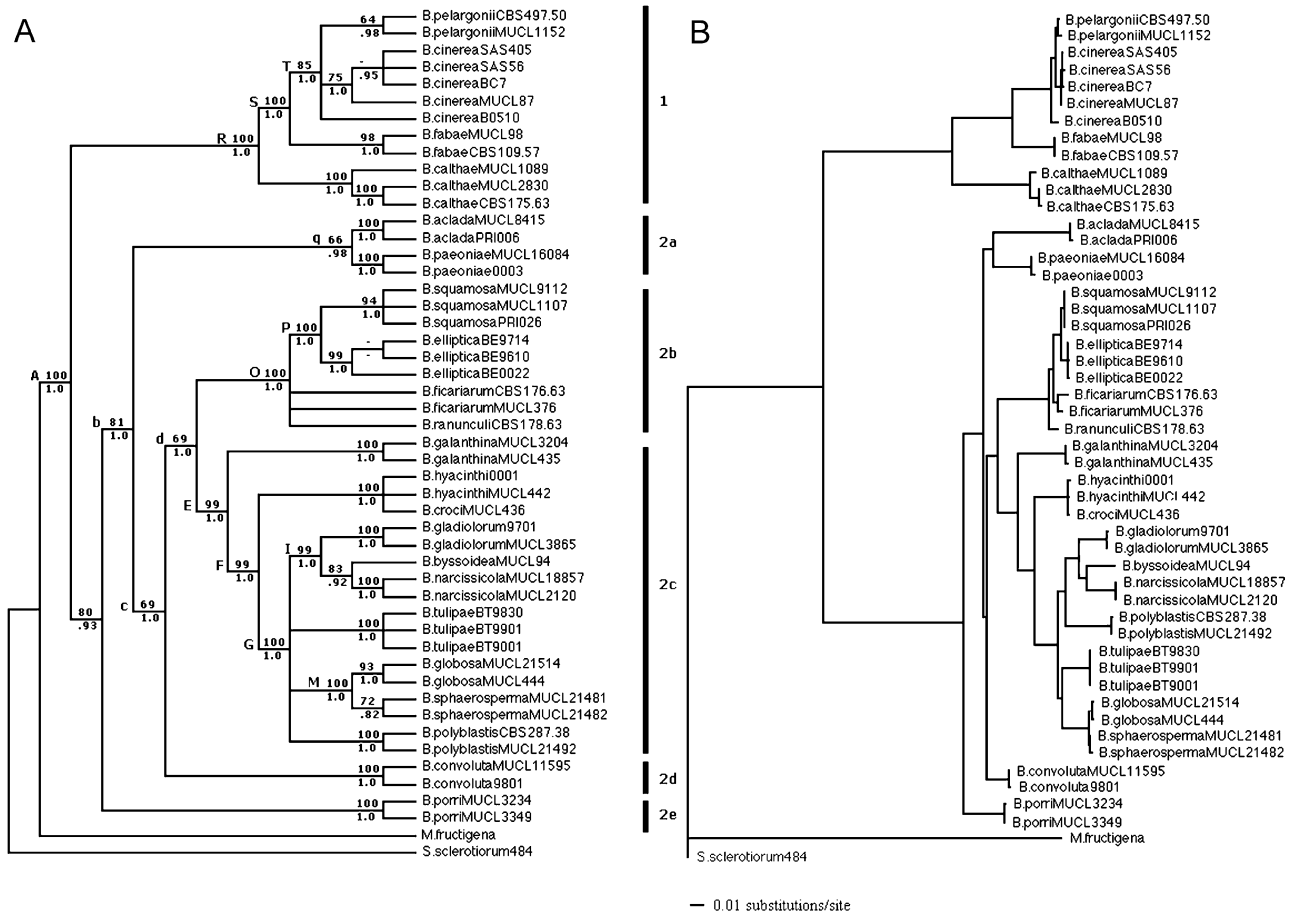


FIG. 4.—Molecular phylogeny for 48 *Botrytis* taxa and two outgroup species based on combined *G3PDH*, *HSP60*, and *RPB2* data. (A) Semistrict consensus of 80 most-parsimonious trees. Jackknife frequencies (10,000 replicates) are shown above each node; Bayesian posterior probabilities (PP) are shown below each node. Letters indicate major clades with significant or strong support (see table 5). A dash indicates the support for the branch was less than 50% jackknife and less than 0.50 PP. Bars indicate the (sub)clades. (B) Bayesian inference showing a 50% majority-rule consensus tree (mean log-likelihood =  $-10389.27$ ) from a five-million generation MCMC analysis.

3, 4A, and 4B). Furthermore, *B. tulipae* was placed basal to the node tying *B. globosa* and *B. sphaerosperma*. The relative branching order of ingroup species and mean log-likelihood values were comparable among two replicate Bayesian analyses ( $-10389.27$  and  $-10402.63$  [data not shown]). In the combined molecular phylogeny, the overall number of supported nodes was greater (26) than for the trees of each separate analysis. Also, most nodes had greater support, especially at the spine, and most were supported strongly. The exception involved the positions of the *B. cinerea* B05.10 and *B. polyblastis*, which were poorly resolved as a result of discordant positions of both species in separate analyses (see figures 2 and 3). In contrast to other relationships in the genus, placement of *B. polyblastis* and *B. tulipae* was unresolved.

Multiple isolates of the same species usually clustered together, and their gene sequences were identical or varied at fewer sites within species in the aligned sequence positions than between species. Molecular phylogenetic analysis of the DNA sequences supports the traditional morphological species classification. In addition, the hybrid status of *B. allii* (*B. byssoidea*  $\times$  *B. aclada* [Yohalem, Nielsen, and Nicolaisen 2003]) was confirmed (fig. 5). The

two isolates of *B. hyacinthi* did not appear to be monophyletic, because they lacked shared characters that distinguished them as a group (fig. 4B).

Based upon the semistrict consensus tree, two main clades were identified in the genus *Botrytis*, and clade 2 was divided into five subclades, as within this clade strongly supported clusters of species could be identified. The base of the tree is characterized by a deep split leading to clade 1 and clade 2. Clade 1 consists of the species *B. calthae*, *B. fabae*, *B. cinerea*, and *B. pelargonii* and is separated from clade 2 by a relatively long branch. The internode separating *B. pelargonii* from *B. cinerea* was very short, reflecting the high amount of interspecific sequence similarity between both species. There was only one unique polymorphism on a total of 2,955 bp between both species.

*B. aclada* and *B. paeoniae* are less closely related to other taxa grouping at a basal position of clade 2 (81% jackknife and 1.0 PP), and they were, therefore, assigned to subclade 2a. Individual gene trees did not support this clade, although both species always grouped closely together. *B. convoluta* could not unambiguously be placed in either subclade within clade 2 and has, therefore, been assigned to subclade 2d. Both subclade 2b and subclade 2c

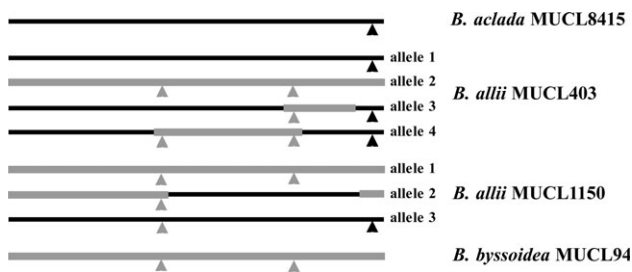


FIG. 5.—Schematic representation of sequence alignment of multiple *HSP60* alleles for allopolyloid *B. allii* (strains MUCL403 and MUCL1150) and its presumed parental species *B. aclada* (isolate MUCL8415) and *B. byssoidea* (isolate MUCL94). Black and gray lines indicate stretches of sequence polymorphisms of *B. aclada* MUCL8415 and *B. byssoidea* MUCL94, respectively. Black and gray arrows indicate *EcoRV* restriction endonuclease cleavage sites.

were defined based on high support values (100/1.0 and 99/1.0, respectively) in combined analysis and the consistent support in separate analyses. The relationships between *B. ranunculi* and *B. ficariarum* in subclade 2b and between *B. hyacinthi* and *B. croci* in subclade 2c were unresolved. Although *B. porri* is closely related, there was no support for its placement in either subclade within clade 2 in combined and separate nDNA analyses. *B. porri* was, therefore, placed in a separate clade, designated as subclade 2e.

#### The Allodiploid Hybrid *B. allii* May Contain Up to Four Different Alleles

To confirm earlier reports about the hybrid status of *B. allii* (Nielsen and Yohalem 2001; Yohalem, Nielsen, and Nicolaisen 2003) restriction profiles and sequence data of cloned fragments were compared with those of its parental species *B. aclada* and *B. byssoidea*. Clones of *B. aclada* strain MUCL8415 and *B. byssoidea* strain MUCL94 could be distinguished based on the unique digestion patterns among the two species. Among cloned fragments of *B. allii* (strains MUCL1150 and MUCL403), digestion patterns of either the *B. aclada*-type or the *B. byssoidea*-type (data not shown) were detected for all three loci, indicating the presence of the two parental alleles. However, in case of *HSP60*, divergent patterns were also detected. Fragment sizes could be explained by the presence of three restriction sites (fig. 5), of which two sites are present in *B. byssoidea* and one is present in *B. aclada*. Alleles were sequenced to verify this interpretation. For *HSP60*, four alleles were identified for *B. allii* strain MUCL403 and three alleles were identified for *B. allii* strain MUCL1150. Hybrid alleles contained stretches of sequences originating from both parental alleles (fig. 5). For the *G3PDH* and *RPB2* genes, hybrid alleles have also been found despite identical restriction patterns in both parental alleles.

The *HSP60* locus of *B. aclada* strain MUCL3106 was identical to *B. byssoidea* except for two polymorphic sites, whereas both *RPB2* and *G3PDH* were identical to *B. aclada* strain MUCL8415. Because fragments of *B. aclada* strain MUCL3106 were directly sequenced, allelic variants have not been detected for each locus.

#### Loss of Sexual Reproduction Has Evolved Three Times

Two important fitness traits, reproductive mode and host-range spectrum, were expressed as multistate “characters” and optimized on the cladogram (fig. 4A) using parsimony. Sexual reproduction has not been found in seven species, five of which parasitize monocot hosts and two parasitize eudicot hosts (table 1). Based on reproductive mode, sexual reproduction was reconstructed as the ancestral condition (fig. 6A). Strict asexual reproduction was inferred to be a homoplasious trait, and loss of sex appears to have occurred three times. Two groups, containing two and four strictly asexual reproducing *Botrytis* species are present in the tree (*B. aclada*/*B. paeoniae* and *B. hyacinthi*/*B. croci*/*B. galanthina*/*B. tulipae*). Within clade 1, the asexual species *B. fabae* separates from a clade predominated by sexual reproduction. There is indication for a reversal from strictly asexual reproduction to sexual reproduction of six taxa in clade 2c.

#### Host and Pathogen Phylogenies Are Not Congruent

To assess the possible occurrence of cospeciation patterns between *Botrytis* species and their hosts, the distribution of host-plant family or angiosperm group was mapped on the cladogram (fig. 6B and C). The order *Asparagales* is parasitized by 14 of the previously recognized *Botrytis* species, the order *Ranunculales* by three species, and the *Liliales* by two species. Three eudicot orders contain one specialist *Botrytis* species, and these orders can be parasitized by the generalist *B. cinerea* as well. *Botrytis* species parasitizing either eudicot or monocot hosts do not tend to cluster together (fig. 6B). Within clade 2, transitions from pathogens infecting monocot hosts to eudicot hosts seem to have occurred twice. Furthermore, *Botrytis* species parasitizing hosts from the same plant family appear to be unrelated (fig. 6C). The best example of incongruency occurs in the host-plant family *Ranunculaceae*, containing three *Botrytis* species, of which *B. calthae* clusters in clade 1, and the species *B. ficariarum*/*B. ranunculi* cluster in clade 2b.

#### Discussion

##### DNA Sequence Data Support the Morphological Species Delineation

In the present study, we conducted molecular phylogenetic analyses of 22 *Botrytis* species and one hybrid. Our analysis provides the first comprehensive phylogenetic study of the entire genus. Our analysis further supports monophyly of the genus *Botrytis*, as has previously been proposed by Holst-Jensen, Vaage, and Schumacher 1997. The phylogenetic analyses of DNA sequence data show that the sequences from multiple isolates of the same species group together and, therefore, support the classical *Botrytis* species status (Hennebert 1973; Jarvis 1977). Morphological characters, together with DNA sequence information, can be used to identify *Botrytis* species; for instance, in a diagnostic key of *Botrytis* species. Such a key may be a potentially powerful tool for diagnostics of this important group of plant pathogens.

The combined analysis of all three loci supported two main clades (fig. 4) and provides more resolution than individual gene phylogenies (fig. 1–3). This result shows the value of conducting phylogenetic analysis on multiple genes, which is an established tool to address complex phylogenetic questions (Rokas et al. 2003; Soltis, Soltis, and Chase 1999; O'Donnell et al. 1998).

The taxonomic status of *B. croci* and *B. hyacinthi* remains uncertain; both species exhibit high sequence similarity, which makes the separation into distinct species questionable. In this study, data of *B. croci* were based on a single isolate, so additional sequencing is needed to resolve its status as a separate species.

Reports about the hybrid status of *B. allii* (Nielsen and Yohalem 2001; Yohalem, Nielsen, and Nicolaisen 2003) were confirmed. Interspecific hybrid species were identified by the presence of multiple *HSP60*, *RPB2*, and *G3PDH* gene copies that consistently grouped with *B. aclada* and *B. byssoidea* within the *Botrytis* phylogeny. The occurrence of up to four different alleles per locus (fig. 5) is indicative of a diploid, dikaryotic genome. However, higher ploidy levels or aneuploidy in these isolates cannot be excluded. The selection of alleles for sequence analysis was based on polymorphic digestion patterns in individual clones of amplified loci; additional alleles that were nonpolymorphic in restriction sites may have escaped our attention. The mixture of parental and recombinant alleles indicates that reciprocal recombination and gene conversion have occurred several times during diversification within the hybrid *B. allii*. These hybrids were placed as basal members of the clade that includes their parents (table 5). Major topological change was caused by hybrid sequences in the *HSP60* phylogeny, resulting in the collapse of the portion of the cladogram that included the hybrid's parents. In case of *RPB2* and *G3PDH*, the cladistic relationships remained largely unchanged, causing only a slight decrease in support values in branches leading to the hybrid's parents. Except for *B. allii*, none of the 22 *Botrytis* species appeared to be hybrids, nor was there any indication from sequence data or host-plant range that they arose by hybridization.

#### Incongruence of Host and Pathogen Phylogenies Does Not Support Cospeciation

Comparison of the phylogenies of the angiosperm families (APG 2003) and *Botrytis* (fig. 4) showed that *Botrytis* species that parasitize hosts from one plant family do not often cluster together. A prominent example is found within the eudicot family *Ranunculaceae*, where *B. calthae* on the one hand and *B. ficariarium*/*B. ranunculi* on the other hand cluster apart in the *Botrytis* clades 1 and 2, respectively (fig. 6C). Also, the six *Botrytis* species infecting *Alliaceae* are widely dispersed over clade 2. Conversely, the related species *B. aclada* and *B. paeoniae*, clustering in clade 2a, are pathogens of monocot and eudicot hosts, respectively. Thus, there is no evidence for cospeciation of *Botrytis* species and their hosts. The observed lack of congruence between pathogen and host trees could be explained by host shifts. Host plants occupying similar habitats and growing during the same

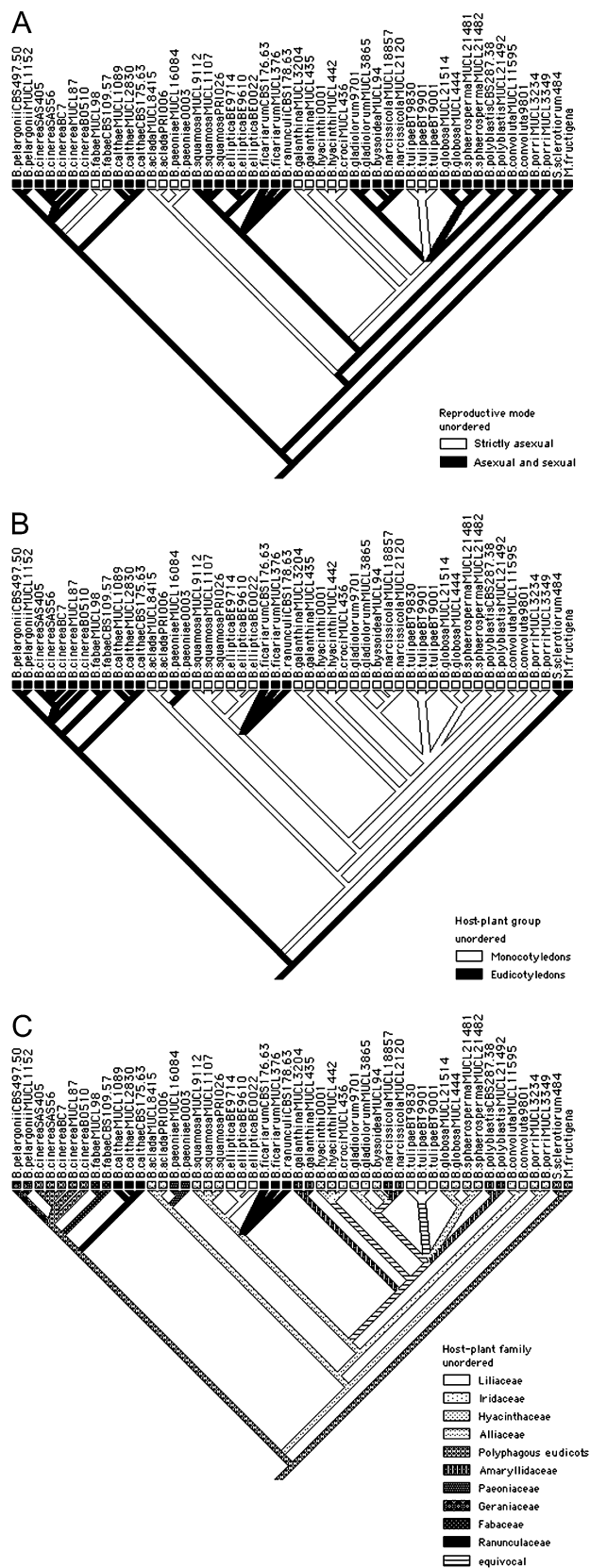


FIG. 6.—Reconstructions of character-state evolution mapped onto the combined molecular phylogeny (the same topology as in figure 4A) for *Botrytis* under parsimony: (A) reproductive mode, (B) host-plant group, and (C) host-plant family.

period of year offer possibilities for host shifting (Roy 2001; Nikoh and Fukatsu 2000). Knowledge of the distribution of genetic variation would have aided finding centers of origin of *Botrytis* species, and data on genetic/geographical associations would allow us to perform nested clade analyses to better delineate species based on sequence data. Unfortunately, detailed knowledge on the geographic distribution, centers of origin, and fossil data of *Botrytis* species are lacking. In addition, information on population dynamics and genetic variation is lacking for most *Botrytis* species. Studies on genetic variation have thus far only been performed with small population sizes (Van der Vlugt-Bergmans et al. 1993; Muñoz et al. 2002; Huang et al. 2001), with one exception (Giraud et al. 1997).

The ability of *Botrytis* species to infect living host plants may result from a combination of at least four factors (see below): (1) possession of pathogenicity factors (e.g., toxins and cell-wall degrading enzymes) that confer the ability to kill and invade plant tissue (reviewed by Prins et al. [2000]), (2) the ability to avoid or counteract plant resistance mechanisms, (3) the ability to survive outside host-plant tissue under less favorable (e.g., low humidity, UV irradiation) conditions, and (4) the ability to reproduce and disperse.

#### Pathogenicity May Have Evolved from Saprotrrophy via Acquisition of Pathogenicity Factors

In the genera *Cochliobolus* (Turgeon 2000) and *Alternaria* (Thomma 2003), the production of host-specific toxins is an important factor that distinguishes the necrotrophic pathogenic species from their saprophytic relatives. Such toxins mediate the currently described host-parasite specificity. It was proposed that plant pathogenic *Cochliobolus* species evolved from saprophytic or weakly pathogenic ancestors by the acquisition of genes involved in host-specific toxin production, possibly by horizontal gene transfer (Turgeon 2000).

Could plant pathogenic *Botrytis* species have evolved from saprophytic ancestors? Like saprophytic fungi, *Botrytis* species produce oxalic acid, extracellular cell-wall degrading enzymes (ten Have et al. 2002; Wubben et al. 1999), and oxidases (e.g., Edlich et al. 1989; Schouten et al. 2002; Rolke et al. 2004). Oxalic acid acidifies plant tissue to facilitate cell-wall hydrolysis (Dutton and Evans, 1996). However, in the case of *Botrytis*, cell-wall degradation is preceded by host-cell killing. Earlier studies of *Botrytis* diseases (reviewed by Brown [1965]) had already demonstrated that killing of plant cells and tissue maceration were distinct processes, likely with different causal agents. Brown (1965) proposed the involvement of a colloidal toxin, possibly an enzyme with protease or lipase activity. This hypothesis was further supported by reports that *B. cinerea* secretes a phospholipase able to induce cytoplasmic leakage (Shepard and Pitt 1976). The production of toxins conferring host specificity was reported for *B. fabae* infecting *Vicia faba* (Harrison 1980) and *B. elliptica* infecting lily (van Baarlen, Staats, and van Kan 2004).

Additional factors mediating host-parasite specificity are those that help to overcome plant resistance. The tulip

secondary metabolite, tulipalin A, is toxic to all *Botrytis* species, except *B. tulipae* (Schönbeck and Schlösser 1976). *B. tulipae* is the only *Botrytis* species able to infect tulip and it neutralizes tulipalin A enzymatically (our unpublished data), suggesting a causal relation between host specificity and defense compound detoxification. We propose that *Botrytis* speciation was driven by host shifts, resulting from the acquisition of pathogenicity genes that confer novel or wider host specificity.

#### Loss of Sexual Reproduction in *Botrytis* Species May Result from Negative Selection

Parasitic sclerotiniaceous fungi differ from their saprophytic conspecifics in their ability to survive in the absence of hosts by means of sclerotia, whereas saprophytes can only survive relatively mild adverse periods as mycelium in decayed host tissues (discussed in Willetts [1997]). Furthermore, the sclerotia serve as maternal parent in the production of apothecia, containing the sexual ascospores.

Sexual reproduction has at least three times been lost in *Botrytis* (fig. 6A). It is most parsimonious to assume that sexual reproduction was the ancestral form of reproduction of early *Botrytis* species. *Botryotinia* teleomorphs are generally found on decaying plant material in shaded locations with high humidity. The warm, humid climates that prevailed during the origin of present-day angiosperm *Botrytis* hosts were favorable for this form of reproduction (Willetts 1997). In drier climates with more intense solar radiation, the thin-walled, easily dehydrated ascospores are possibly not an efficient manner of dispersal. The thick-walled, dehydration-tolerant and UV-tolerant macroconidia likely evolved as an adaptation to low humidity and higher UV irradiation, conferring the capacity to *Botrytis* species to infect the aerial parts of plants. We propose that loss of sexual reproduction in *Botrytis* may reflect habitat adaptation. In present-day drier habitats and under conditions of low humidity, ascospores are not produced, and only the asexual macroconidia are formed. This condition may result in negative selection against sexual reproduction. Under conditions adverse for sexual spore production and dispersal, investment in the production of the energy-costly teleomorph (Willetts 1997) will be wasted and selected against, leading to loss of sexual reproduction. It should be noted that microconidia, the “male” spermatial gametes, and sclerotia are still formed in exclusively asexually reproducing *Botrytis* species. Although the capacity to produce macroconidia confers a selective advantage in less humid habitats, the majority of *Sclerotiniaceae* spp. do not produce macroconidia.

#### Natural Interspecific Hybrids May Accelerate the Origin of Novel Species

The occurrence of natural hybrids in the group of *Botrytis* species causing onion neck rot and the resulting gene flow poses a difficulty for the maintenance of species identity. Nielsen and Yohalem (2001) suggested that polyploidization may have been the result of parasexual hybridization of *B. aclada* and *B. byssoidea* coinfecting

onions. Alternatively, hybrid offspring may have been the result of interspecific sexual crosses (review by Schardl and Craven [2003]). Of the two parental species, only *B. byssoidea* has a sexual stage (table 1). During the hybridization events leading to the hybrid *B. allii*, the sclerotia of *B. byssoidea* have likely functioned as recipient for the spermatia (microconidia) produced by the presumed asexual *B. aclada*. At present, it is unknown whether the resulting hybrid offspring may only propagate vegetatively or whether backcrosses and crosses between hybrid offspring are possible. Repeated backcrossing to the parents may not be possible because of unequal chromosome number (Futuyma 1986). The hybridization event could be of recent origin because few mutations have accumulated in hybrid sequences, as was suggested previously by Nielsen and Yohalem (2001). Moreover, the observed differences in nucleotide sequence may originate from intraspecific variation present in both parental populations. There is no evidence that the hybridization events have led to host-range expansion within the *B. byssoidea-aclada-allii* complex, and hybrids do not exhibit increased pathogenicity to onion (Yohalem, Nielsen, and Nicolaisen 2003). The combination of interspecific sex, gene flow, and ploidy cycling gives ample opportunity for novel genotypes to arise, eventually differentiating into novel species (Kondrashov 1997).

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