Botrytis species on flower bulb crops: Phylogeny, genetic variation and host specificity

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General introduction and outline

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Flower bulb production in the Netherlands

The Netherlands is a renowned flower bulb producing country. Commercial flower bulb cultivation started at the end of sixteenth century in the area between Haarlem and Leiden. This region became known as 'de Bollenstreek' – the bulb district, with the town of Lisse centered in the middle. Today, much of the bulb production still takes place in this region, although over the years bulb cultivation has also spread to other regions in the Netherlands. At present, approximately nine billion flower bulbs are produced annually, of which tulip is the most widely grown bulb flower, followed by lily, narcissus, gladiolus, and hyacinth.

Flower bulb production in the Netherlands comprises an economically important activity. An annual export value of over 630 million Euros is achieved on approximately 17,000 ha (5% of the arable crop area). The average annual revenue from tulip fields is 31.000 Euros/ha and from lily fields is 63.000 Euros/ha. The production value per hectare is at least 17 times higher for flower bulbs than for arable crops. Moreover, income is generated at the regional scale by the touristic value of flowering fields.

Growing bulbs is, however, damaging to the environment. The pesticide usage per hectare in bulb-cultivation is one of the highest in Dutch agriculture, despite the attempts to reduce pesticide inputs. The pesticide use in this sector increased from 13% to 16% of the total pesticide usage in the Netherlands between 1995 and 2000, primarily due to the strong expansion of area of lily bulbs grown and the very high pesticide usage of more than 100 kg/ha in lily cultivation. Fungicide usage accounted for 35.5% of the total pesticide treated area of outdoor bulb and flower crops grown in the Netherlands in 2000. Costs of fungicide application are relatively low, and sprays are therefore applied routinely in weekly or biweekly schedules. However, the reliance on fungicides increases the risk of resistance development among target species, as has been reported for *Botrytis elliptica* in lily (Chastagner and Riley, 1990; Migheli et al., 1989).

***Botrytis* diseases of flower bulb crops**

Fungi of the genus *Botrytis* Persoon are major pathogens of many agronomically important crops, including all major flower bulb crops (Jarvis, 1977). The most important species with respect to economic damage are *B. elliptica*, *B. tulipae*, and

B. gladiolorum, each potentially causing yield losses up to 60%.

Botrytis species infecting flower bulb crops are considered specialists with a narrow host range, except *B. cinerea*, which is an opportunistic pathogen. *B. cinerea* infects only flowers and dead or senescing plant parts of bulb flowers (Table 1). In many cases host-specific *Botrytis* species are able to cause primary lesions on a non-host, but these primary lesions fail to expand (Prins et al., 2000). Furthermore, specialized species may occur as saprophytes on debris of non-host species, e.g. *B. tulipae* is able to colonize dead lily stems (van den Ende and Pennock-Vos, 1997a).

Table 1: Host specificity of *Botrytis* species in flower bulbs (after Hennebert 1973)

Species	Common Disease Name	Typical host/Tissue Specificity	Host-Plant Species
B. cinerea Pers. /*B. fuckeliana* (de Bary) Whetzel	Gray mould	Fallen leaves, and flowers	>235 plant species
B. convoluta Whetzel and Drayton	*Botrytis* rhizome rot	Rhizomes of cultivated iris	*Iris spp. L.*
B. croci Cooke and Massee	Crocus blight	Leaves of cultivated crocus	*Crocus spp. L.*
B. elliptica (Berk.) Cooke	Lily fire	Leaves, stems, and flowers of cultivated lilies	*Lilium spp. L.*
B. galanthina (Berk. and Br.) Sacc.	Blight	Snowdrop	*Galanthus spp. L.*
B. gladiolorum Timm. / *B. draytonii* (Budd. and Wakef.) Seaver	Gladiolus blight	Stems of cultivated gladiolus	*Gladiolus spp. L.*
B. hyacinthi Westerd. and Beyma	Hyacinth fire	Leaves of hyacinth	*Hyacinthus spp. L.*
B. narcissicola Kleb. ex Westerd. and Beyma	Smoulder mould	Bulbs of narcissus	*Narcissus spp. L.*
B. polyblastis Dowson	Narcissus fire	Leaves of narcissus	*Narcissus spp. L.*
B. tulipae Lind	Tulip fire	Leaves, stems, flowers, and bulbs of cultivated tulips	*Tulipa spp. L.*

All *Botrytis* species are necrotrophic pathogens that actively kill plant cells and subsequently live on dead tissue. The disease symptoms produced by *Botrytis* species in the flower bulb crops are called 'blight', 'fire', and 'bulb rot'. Infections appear as small necrotic spots as a result of lignification of epidermal cell walls, an active mechanism of resistance to fungal infection (Mansfield and Hutson, 1980). However, under conditions that are favorable to the pathogen (long wetness periods, susceptible cultivar, and critical temperature regimes) resistance fails and the necrotic lesions rapidly expand on leaves, flowers and stems, thereby producing typical 'fire' symptoms. New infections of healthy tissue occur by dispersal of macroconidia (mitotically produced spores) produced on dead infested plant tissue or by direct contact between diseased and healthy plant tissue.

Botrytis species are able to produce sclerotia on the surface of diseased hosts (Coley-Smith, 1980). Sclerotia are melanized structures that are important for the survival of *Botrytis* species. Sclerotia remain quiescent on plant debris and in soil during the over-wintering phase, which covers the period between harvest and planting. Early in the growth season, sclerotia can produce macroconidia, which function as a source of primary inoculum. Some species, like *B. tulipae* and *B. narcissicola*, may also form sclerotia on bulbs. During the storage period these sclerotia are inactive (Doornik and Bergman, 1975). However, after planting the fungus spreads over the scales and sporulates on emerging shoots (the so-called 'primaries') (Price, 1970; Doornik and Bergman, 1971). Some infected bulbs rot completely to release sclerotia in the soil (Doornik and Bergman, 1973).

A number of *Botrytis* species, including *B. elliptica*, occasionally produce a sexual stage in which ascospores (meiotically produced spores) are produced in an apothecium (the *Botryotinia* teleomorph stage) (van den Ende and Pennock, 1996). Apothecia may arise from over-wintering sclerotia in early spring. However, the importance of ascospores in the epidemiology of *Botrytis* diseases in flower bulbs is still unclear.

Taxonomy

Botrytis and its sexual form *Botryotinia* Whetzel are classified within the family *Sclerotiniaceae* Whetzel (Inoperculate Discomycetes). The species of *Botrytis*/*Botryotinia* are delimited on the basis of morphological and cultural characteristics, although host specificity is also used as discriminatory trait (Hennebert, 1973). Morphological traits such as the characteristics of conidia, mycelia and sclerotia are useful in delimiting some species. However, many species are morphologically similar. Furthermore, a key to all recognized species is not available. Some species have been distinguished based on sexual crosses between them (Bergquist and Lorbeer, 1972). However, homothallism (self-fertilization) is not uncommon in *Botrytis*, which makes it difficult to ensure if progeny had two parents. Other *Botrytis* species apparently entirely lack sexuality, which further limits the use of the biological species concept for species discrimination.

Rather than on morphological traits, species may be identified by phylogenetic analyses of variable nucleic acid sequences. In this approach, an evolutionary tree is used to model the relationships of a group of individuals. Phylogenetic species can be identified as terminal monophyletic clades. The internal transcribed spacer (ITS)

region of the ribosomal DNA repeat has been widely used for species-level discrimination of fungal species, but variation in the ITS region within *Botrytis* is low, limiting its use in this genus (Holst-Jensen et al., 1998; Nielsen et al., 2001). Protein-coding genes are more suitable in this regard because of their high content of functional information and the relatively high substitution rate in intron regions. Phylogenies based on nuclear gene sequences that encode DNA-dependent RNA polymerase subunit II (*RPB2*), and glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) have been successfully used for fungal molecular systematics (Berbee et al., 1999; Liu et al., 1999). However, due to various genetic rearrangements such as duplication or introgression, phylogenetic hypotheses based on the evolution of one gene region may not reflect the evolution of the organism investigated (Doyle, 1992; Avise and Wollenberg, 1997). Furthermore, one gene region may be insufficient to provide resolution within different parts of the phylogenetic tree. Therefore, phylogenetic analyses should ideally be based on molecular data from several independent gene regions. Morphological characters, together with DNA sequence data of multiple protein-coding genes can be used to unambiguously identify *Botrytis* species and open the way to a better understanding of the genetic diversity within the genus.

Genetic variability

Most of the knowledge on mechanisms that generate genetic variability in *Botrytis* is based on studies in *B. cinerea*. This well-studied species causes serious economical damage on many eudicot plant species, including most vegetable and fruit crops, flowers, woody ornamentals and greenhouse-grown crops.

Heterokaryosis has been shown to account for variability. The macroconidial and hyphal compartments of *B. cinerea* and other species are multinucleate, which enables nuclei within the mycelium to be genetically different. *B. cinerea* contains on average 4 - 5.1 nuclei per macroconidium, but the nuclear number may differ significantly between *Botrytis* species (Shirane et al., 1989). Evidence for the existence of heterokaryons was initially obtained by the observation of distinct morphotypes in macroconidial subcultures from the same parent (Lorbeer, 1980). In the field, fungicide resistant strains have been isolated that contained fungicide-resistant and fungicide-sensitive genotypes in the heterokaryon (Summers et al., 1984; Faretra and Pollastro, 1993; Pollastro et al., 1996). Heterokaryosis can arise either by mutation in a resident nucleus or as a result of hyphal fusions (anastomosis) followed by the

cohabitation of genetically distinct nuclei. The latter has been shown in the laboratory using auxotrophic mutants derived from the same strain (Weeds et al., 1998). However, vegetative incompatibility prevents hyphal fusions between mycelia that belong to different vegetative incompatibility groups (VCGs) (Beever and Parkes, 1993; Weeds et al., 1998). The genetic basis of vegetative incompatibility in *B. cinerea* and other *Botrytis* species in not known, but it is likely to relate to the systems found in other filamentous ascomycetous fungi (Glass et al., 2000). In these, vegetative incompatibility is determined by a series of vegetative incompatibility (*vic* or *het*) genes that exist in two or more allelic states. Strains can only fuse if the alleles at all loci are identical. The existence of a large number of VCGs may limit the extent of heterokaryon formation and thus restrict genetic variability. Beever and Weeds (2004) have identified at least 66 distinct VCGs in *B. cinerea*, implying the involvement of 7 *het* loci, assuming that the loci are biallelic.

Variations in ploidy levels among strains of *B. cinerea* are reported (Büttner et al., 1994). Based on the DNA content of individual nuclei as determined by fluorescent microscopy, strains were suggested to be haploid, diploid, or even triploid. Heteroploidy may arise from the failure of mitosis or fusion of nuclei, coupled with loss or gain of chromosomes. It is unknown whether heteroploidy in *B. cinerea* affects the growth rate, conidial production, enzyme activity or pathogenicity, as has been shown for several other fungi (Tolmsoff, 1983). Polyploidy is also reported in other *Botrytis* species. *B. allii* is suggested to be an allopolyploid that has arisen through interspecific hybridization beween *B. aclada* and *B. byssoidea* (Nielsen and Yohalem, 2001). The two putative parental species each have 16 mitotic chromosome numbers, whereas *B. allii* has 32 chromosomes (Shirane et al., 1989).

B. cinerea is able to promote and maintain genetic diversity by sexual reproduction as most strains are heterothallic (outcrossing) (Faretra et al., 1988; van der Vlugt-Bergmans et al., 1993; Beever and Parkers, 1993). The apothecia are formed after fertilization of preconditioned sclerotia with a suspension of microconidia (uninuclear spermatial cells) (Faretra and Antonacci, 1987). Compatible matings are controlled by a single mating type locus with two idiomorphs: MAT1-1 and MAT1-2 (Faretra et al., 1988). Even though *B. cinerea* is potentially capable of sexual reproduction, the apothecia are rarely found in nature (Lorbeer, 1980). The absence of a compatible partner may prevent sexual reproduction, although there is ample evidence that both mating types are equally distributed in nature (Faretra et al., 1988; Beever and Parkers, 1993; Faretra and Pollastro, 1993). Furthermore, adverse

environmental conditions may inhibit the formation of apothecia, as these structures only develop under cool weather conditions in moist soil. Several *Botrytis* species that infect bulb flowers possess a sexual stage, including *B. elliptica* and *B. gladiolorum* (teleomorph *Botryotinia draytonii*). *B. elliptica* has been shown to mate in the field and in the laboratory (van den Ende and Pennock-Vos, 1997b; van den Ende, unpublished data). *B. elliptica* is heterothallic and two idiomorphs were identified, as in *B. cinerea*. No sexual stage has so far been found for *B. tulipae*, *B. hyacinthi*, *B. galanthina* and *B. croci*. These species are therefore considered to reproduce clonally.

Pathogens that reproduce strictly asexually exist as clonal lineages, in which the variation within a clonal lineage is exclusively generated by mutation. Base substitutions or deletions may occur at low frequency during the mitotic cycle. The mutation rates are likely to be higher in fungal populations with active transposable elements (Daboussi, 1996). *B. cinerea* strains have been shown to possess at least two different transposable elements, named *Boty* and *Flipper* (Diolez et al., 1995; Levis et al., 1997a). *Flipper* is present in up to 20 copies per genome, and was confirmed to be mobile by the detection of its insertion into nitrate reductase during spontaneous mutant selection (Levis et al., 1997b). It is unknown whether transposable elements are present in *Botrytis* species infecting flower bulb crops.

Population genetic studies

Most population genetic studies in *Botrytis* have focused on *B. cinerea*. Giraud et al. (1997) studied genetic diversity of *B. cinerea* in France using a range of markers including the presence or absence of transposable elements *Boty* and *Flipper*, and restriction fragment length polymorphisms (RFLPs) of PCR amplified DNA regions. This study revealed that the *B. cinerea* population is genetically very diverse, with no indication of widespread clonal lineages and a significant role for recombination. Furthermore, *B. cinerea* appears to be composed of two subgroups, *transposa* and *vacuma* that are genetically isolated and occur in sympatry on the same host plants and in the same region (Giraud et al., 1997). *Transposa* contains the transposable elements *Boty* and *Flipper*, whereas neither of these can be amplified from strains of the *vacuma* population. Additionally, significant genetic differentiation was detected among strains from different host plants in France (Giraud et al., 1999).

The genetic variation in populations of *B. elliptica* in the USA and Taiwan has been studied using random amplified polymorphic DNA (RAPD) markers (Huang et al., 2001). The genotypic diversity was high with a total of 43 distinct genotypes

among 69 isolates. Clonal genotypes were mainly found within one field. Evidence for recombination was found by estimating the degree of nonrandom association of alleles at different loci. These data suggested the importance of sexual reproduction in determining the population structure of *B. elliptica*. No population genetic studies have been performed for other *Botrytis* species infecting flower bulb crops.

Pathogenesis of *Botrytis*

Botrytis possesses virulence factors that enable the pathogen to complete the consecutive stages of its necrotrophic lifecycle. These virulence factors include phytotoxic metabolites and proteins, as well as extracellular enzymes that decompose plant material or inactivate plant defence mechanisms. Most of the information comes from research on *B. cinerea*.

Botrytis spp. can enter the host via stomata and other natural openings (Clark and Lorbeer, 1976; Fourie and Holz, 1995; Hsieh et al., 2001). Alternatively, the invasion of host tissue can start by active penetration directly through the cuticle. Studies with antibodies against a cutinase (Salinas, 1992) and a lipase (Commenil et al., 1998) suggested that these enzymes play a role in the infection process of *B. cinerea*. A gene replacement mutant that lacked the enzyme activity of a secreted cutinase, however, did not affect in its ability to infect tomato and gerbera petals (van Kan et al., 1997). Other cutinases and lipases were suggested to be involved in active penetration (van Kan et al., 1997; Gindro and Pezet, 1999).

When the cuticle is breached, the underlying epidermal cells are killed before they are invaded by hyphae (Clark and Lorbeer, 1976). Botcinolide and botrydial are low molecular weight metabolites with a general phytotoxic activity that are secreted by *B. cinerea* (Cutler et al., 1993; Durán-Patrón et al., 2000; Colmenares et al., 2002). However, their role in pathogenesis is unknown. There is no evidence for the production of host-selective toxins (HSTs) by *B. cinerea*, which is in agreement with the broad host range of this species. HSTs are typically active only towards plants that serve as hosts for the specialized pathogens that produce them (Wolpert et al., 2002). The production of toxins conferring host specificity was reported for *B. fabae* infecting *Vicia faba* (Harrison, 1980). Other specialized *Botrytis* species may also be equipped with HSTs, but this remains to be studied.

B. cinerea produces oxalic acid, which may have direct toxic effects through acidification of the environment (Germeier et al., 1994). Furthermore, oxalic acid may be a co-factor in pathogenesis as several fungal cell wall degrading enzymes, such as

endo- and exopolygalacturonases and pectin methylesterase, are most active at low pH values (ten Have et al., 2002). The activities of many different pectinolytic and non-pectinolytic enzymes cause the breakdown of plant cell walls by which carbohydrates are released that form the major carbon source for consumption (ten Have et al., 2002). Many genes encoding cell wall degrading enzymes are present in multiple copies in *B. cinerea* and other *Botrytis* species (Hancock et al., 1964; Chilosi and Magro, 1997; Wubben et al., 1999). *B. cinerea* endopolygalacturonases are differentially expressed during pathogenesis on different hosts, which may contribute to the broad host range of this species (Wubben et al., 2000; ten Have et al., 2001).

The production of Active Oxygen Species (AOS) assists the colonization of plant tissues by *B. cinerea* (Tiedemann, 1997; Govrin and Levine, 2000). AOS are normal products of primary metabolic processes in cells, and both plant and pathogen are able to protect themselves against oxidative stress (Mayer et al., 2001; Gil-ad et al., 2000; Schouten et al., 2002b). However, *B. cinerea* is able to exploit the production of AOS to induce host cell death, which facilitates growth of necrotrophic pathogens (Tiedemann, 1997; Govrin and Levine, 2000).

Botrytis species are able to counteract plant defense compounds, such as secondary metabolites with antifungal activity, which is often crucial for successful host colonization. For example, *B. cinerea* can detoxify resveratrol, a stilbene phytoalexin from grapevine leaves using laccases (Adrian et al., 1998). In addition, *B. cinerea* is able to inactivate α-tomatine from tomato leaves using the enzyme tomatinase (Quidde et al., 1998). The pathogen also possesses non-specific detoxification mechanisms. ATP-binding cassette transporters and Major Facilitator proteins are able to excrete a spectrum of fungitoxic compounds from cells of the pathogen (de Waard, 1997; Schoonbeek et al., 2001). Specialized pathogen species may have evolved enzyme systems that can specifically degrade toxic secondary metabolites from their respective hosts. Infection of tulip bulbs and pistils by *B. tulipae* is associated with conversion of preformed tuliposides into inactive hydroxylic acids, whereas *B. cinerea* converts tuliposides into the toxic tulipalins (Schönbeck and Schroeder, 1972).

Outline of the thesis

The aim of this thesis was to provide a better understanding of *Botrytis* diseases occurring on flow bulbs crops. The thesis focuses on the interaction between *B. tulipae* and its host plant tulip, as well as the interaction between *B. elliptica* and its host plant lily. Molecular markers were developed to unambiguously identify the *Botrytis* species of interest. Furthermore, the extent of genetic variation within collections of each species was evaluated. The final aim of this thesis was to study factors that determine the host specificity of *B. tulipae* and *B. elliptica*.

Chapter two describes a classification of the genus *Botrytis* based on DNA sequence data of three nuclear protein-coding genes encoding glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*), Heat-shock Protein 60 (*HSP60*), and DNA-dependent RNA polymerase subunit II (*RPB2*). The phylogenetic analysis that encompasses all species of the genus was compared to the classical species delineation. In addition, the phylogenies of the genus *Botrytis* and their angiosperm hosts were compared in order to explore the possible occurrence of co-speciation.

Chapter three describes the development of three DNA-based molecular methods to differentiate isolates of *B. tulipae*, *B. elliptica* and *B. cinerea*. Regions of five nuclear DNA-encoding genes [*RPB2*, *G3PDH*, *HSP60*, Histone H3 (*H3*) and elongation factor 1 alpha (*EF-1a*)] and the ITS region were sequenced in order to identify polymorphisms within these regions. Furthermore, PCR-RFLP of the intergenic spacer (IGS) region and Amplified Fragment Length Polymorphism (AFLP) analysis were used. The discriminatory power of these three molecular typing methods were compared.

Chapter four investigates the genetic diversity among isolates of *B. tulipae* and *B. elliptica* from the Netherlands using AFLP markers. Isolates were intensively sampled during successive growing seasons in Lisse, which is located in one of the main bulb growing areas of the Netherlands. We set out to test for the existence of a randomly mating population of *B. elliptica* and a clonal population of *B. tulipae* by examining the genetic structures of both species within and between growing seasons.

The previous chapters focus on estimating the level of inter- and intraspecific variation within the genus *Botrytis*. Additionally, the aim of the thesis was to study the mechanisms causing host specificity. All *Botrytis* species are necrotrophic pathogens and they rely on active cell death programs in the plant for successful infection (Govrin and Levine, 2000). Within this context, we focused on Necrosis and

Ethylene-inducing Proteins (NEP) from *Botrytis* species. NEP1-like proteins (NLPs) cause active cell death in many dicot plant species (reviewed by Pemberton and Salmond, 2004; Gijzen and Nürnberger, 2006).

Chapter five describes the sequencing of two NLPs from all *Botrytis* species. Bioinformatics tools were used to predict their secondary structures and post-translational modification motifs. Furthermore, maximum likelihood methods were used to compare the evolutionary patterns of positive and purifying selection of positions within the NLP genes.

The evolutionary analyses presented in Chapter 5 triggered a hypothesis that NLP genes might be determinants of host range, i.e. that the toxicity spectrum of an NLP from a particular *Botrytis* species would enable it to specifically infect its host plant species. Chapter six describes an attempt to validate this hypothesis by performing a functional analysis of two NLPs from *B. elliptica*. Gene-replacement mutants were made for the *BeNEP1* and *BeNEP2* gene and their roles in virulence were tested. Furthermore, both NLPs were produced in *Pichia pastoris* and the necrotizing activity of each individual NLP was tested in lily and a range of other plant species.

Chapter seven provides a general discussion of the thesis and some future directions in research on the described pathosystems are suggested.

Chapter 2

Molecular phylogeny of the plant pathogenic genus *Botrytis* and the evolution of host specificity

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Key words: bayesian inference, *Botrytis*, coevolution, host shift, molecular phylogeny, necrotrophic fungus

Molecular phylogeny of the plant pathogenic genus *Botrytis* and the evolution of host specificity

Abstract

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* x *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 et al., 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 *Paeoniaceae* (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 non-host, 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. ficariarum* 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 et al., 2001). *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 polymorphisms (RFLPs) and sequence data

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

[A]= Hybrid species according to Yohalem, Nielsen, and Nicolaisen 2003.

(Shirane et al., 1989; Nielsen and Yohalem, 2001; Yohalem et al., 2003). *B. byssoidea* and *B. aclada* are considered to be its parental species (Nielsen and Yohalem, 2001; Yohalem et al., 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 indicative of ancient coevolution, a reciprocal process in which characteristics of one organism evolves 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 avirulence 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 they are invaded by the fungus (Clark and Lorbeer, 1976; van Baarlen et al., 2004b), 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 et al. (1998) analyzed nuclear ribosomal internal transcribed spacer (ITS) DNA sequences and concluded that the *Botryotinia* 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 et al., 1999; Liu et al., 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 et al. (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 et al. (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 the 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 µM of each deoxynucleoside triphosphate (Promega, Madison, WI), 0.2 pmol of each primer (Amersham Pharmacia Biotech), 2 mM $MgSO_4$ and 1.0 U of

Table 2: Isolates of *Botrytis*, origin, year of collection, and GenBank accession numbers for DNA sequences used in this study

				GenBank accession number of sequences			
Species	Collection nr.	Geographic origin	Year	ITS	*RPB2*	*HSP60*	*G3PDH*
B. aclada	PRI006[A]	-	-	AJ716295	AJ745665	AJ716051	AJ704993
	MUCL3106[B C]	USA	1961	N.D.[J]	AJ745663	AJ716049	AJ704991
	MUCL8415[B C]	Germany	1965	N.D.	AJ745664	AJ716050	AJ704992
B. allii	MUCL403[B C]	The Netherlands	1957	N.D.	AJ745666 (allele 1)	AJ716055 (allele 1)	AJ704996 (allele 1)
					AJ745667 (allele 2)	AJ716056 (allele 2)	AJ704997 (allele 2)
						AJ716057 (allele 3)	
						AJ716058 (allele 4)	
	MUCL1150[B C]	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)	
B. byssoidea	MUCL94[B C D]	USA	1923	N.D.	AJ745670	AJ716059	AJ704998
B. calthae	CBS 175.63[E]	USA	1961	AJ716302	AJ745671	AJ716060	AJ704999
	MUCL1089[B]	Belgium	1960	N.D.	AJ745672	AJ716061	AJ705000
	MUCL2830[B]	USA	1961	N.D.	AJ745673	AJ716062	AJ705001
B. convoluta	9801[F]	The Netherlands, Lisse	1998	AJ716304	AJ745679	AJ716068	AJ705007
	MUCL11595[B]	USA	1968	N.D.	AJ745680	AJ716069	AJ705008
B. cinerea	SAS56[G]	Italy	-	AJ716294	AJ745677	AJ716067	AJ705006
	SAS405[G]	Italy	-	N.D.	AJ745678	AJ716066	AJ705005
	B05.10[H]	-	1994	N.D.	AJ745674	AJ716063	AJ705002
	BC7[K]	The Netherlands	1970	N.D.	AJ745675	AJ716064	AJ705003
	MUCL87[B D]	The Netherlands	1928	N.D.	AJ745676	AJ716065	AJ705004
B. croci	MUCL436[B]	The Netherlands	1968	N.D.	AJ745681	AJ716070	AJ705009
B. elliptica	BE9714[F]	The Netherlands, Elsloo	-	AJ716300	AJ745684	AJ716073	AJ705012
	BE9610[F]	The Netherlands	-	N.D.	AJ745683	AJ716072	AJ705011
	BE0022[F]	The Netherlands, Smilde	2001	N.D.	AJ745682	AJ716071	AJ705010
B. fabae	CBS 109.57[E]	The Netherlands	1957	AJ716303	AJ745685	AJ716074	AJ705013
	MUCL98[B D]	Spain	1929	N.D.	AJ745686	AJ716075	AJ705014
B. ficariarum	CBS 176.63[D E]	Belgium	1960	AJ716296	AJ745687	AJ716076	AJ705015
	MUCL376[B]	Belgium	1957	N.D.	AJ745688	AJ716077	AJ705016
B. galanthina	MUCL435[B]	The Netherlands	1958	N.D.	AJ745689	AJ716079	AJ705018
	MUCL3204[B]	The Netherlands	1963	N.D.	AJ745690	AJ716078	AJ705017
B. gladiolorum	9701[F]	-	1997	N.D.	AJ745691	AJ716080	AJ705019

Table 2: Continued

Species	Collection nr.	Geographic origin	Year	GenBank accession number of sequences			
				ITS	*RPB2*	*HSP60*	*G3PDH*
B. gladiolorum	MUCL3865[B]	The Netherlands, Andijk	1963	N.D.	AJ745692	AJ716081	AJ705020
B. globosa	MUCL444[B]	Belgium	1958	N.D.	AJ745693	AJ716083	AJ705022
	MUCL21514[B]	UK	1963	N.D.	AJ745694	AJ716082	AJ705021
B. hyacinthi	0001[F]	The Netherlands, Lisse	1999	AJ716297	AJ745695	AJ716084	AJ705023
	MUCL442[B]	The Netherlands, Breezand	1958	N.D.	AJ745696	AJ716085	AJ705024
B. narcissicola	MUCL18857[B]	UK	1972	N.D.	AJ745698	AJ716086	AJ705025
	MUCL2120[B]	Canada	1961	N.D.	AJ745697	AJ716087	AJ705026
B. paeoniae	MUCL16084[B]	Belgium	1970	N.D.	AJ745700	AJ716089	AJ705028
	0003[I]	The Netherlands	2002	AJ716298	AJ745699	AJ716088	AJ705027
B. pelargonii	CBS 497.50[D E]	Norway	1949	AJ716290	AJ745662	AJ716046	AJ704990
	MUCL1152[B]	Norway	1960	N.D.	AJ745701	AJ716090	AJ705029
B. polyblastis	MUCL21492[B]	UK	1963	N.D.	AJ745703	AJ716092	AJ705031
	CBS287.38[D E]	UK	1938	AJ716291	AJ745702	AJ716091	AJ705030
B. porri	MUCL3234[B D]	-	1926	AJ716292	AJ745704	AJ716093	AJ705032
	MUCL3349[B]	Belgium	1963	N.D.	AJ745705	AJ716094	AJ705033
B. ranunculi	CBS178.63[D E]	USA	1963	N.D.	AJ745706	AJ716095	AJ705034
B. sphaerosperma	MUCL21481[B]	UK	1963	AJ716293	AJ745708	AJ716096	AJ705035
	MUCL21482[B]	UK	1963	N.D.	AJ745709	AJ716097	AJ705036
B. squamosa	PRI026[A]	-	-	AJ716299	AJ745707	AJ716100	AJ705039
	MUCL1107[B D]	USA	1923	N.D.	AJ745710	AJ716098	AJ705037
	MUCL9112[B]	The Netherlands	1966	N.D.	AJ745711	AJ716099	AJ705038
B. tulipae	BT9830[F]	The Netherlands	2000	AJ716301	AJ745713	AJ716102	AJ705041
	BT9001[F]	The Netherlands	2000	N.D.	AJ745712	AJ716101	AJ705040
	BT9901[F]	The Netherlands, Apeldoorn	2000	N.D.	AJ745714	AJ716103	AJ705042
B. anthophila	CBS122.26[D E]	The Netherlands	1926	AJ716305	N.D.	N.D.	N.D.
M. fructigena	9201[L]	-	1992	N.D.	AJ745715	AJ716047	AJ705043
S. sclerotiorum	484[L]	-	-	N.D.	AJ745716	AJ716048	AJ705044

[A]= Dr. P. van den Boogert, Plant Research International, Wageningen; [B]= Belgium Coordinated Collection of Microorganisms (BCCM); [C]= Yohalem et al. (2003); [D]= Type, neotype or epitype specimen; [E]= Centraalbureau voor Schimmelcultures, Fungal Biodiversity Center (CBS-KNAW); [F]= Applied Research Plant & Environment, Research Unit Flower Bulbs; [G]= Faretra et al. (1988); [H]= Büttner et al. (1994);
[I]= Applied Research Plant & Environment, Research Unit Glasshouse Horticulture; [J]= N.D. = Not Determined; [K]= Van der Vlugt-Bergmans et al. (1992); [L]= Department of Biological Farming systems, Wageningen University.

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 comparison 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 μg/ml ampicillin, 20 μg/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 to 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 *Eco*RV (Promega), whereas *RPB2* fragments were digested with *Hind*III (Promega). Subsequently, individual clones, containing different alleles, were selected for sequencing based on their restriction profiles. Clones were sequenced in both directions using SP6 and T7 primers (see Table 3).

Table 3: Primers used for PCR amplification and sequencing

Primer name	Target region	Primer sequence (5′ – 3′)	Reference or position
G3PDHfor+	*G3PDH*	gtgactgtaaaacgacggccagtATTGACATCGTCGCTGTCAACGA	790 – 817[A]
G3PDHrev+		gtgaccaggaaacagctatgaccACCCCACTCGTTGTCGTACCA	1769 – 1789[A]
HSP60for+	*HSP60*	gtgactgtaaaacgacggccagtCAACAATTGAGATTTGCCCACAAG	651 – 674[B]
HSP60rev+		gtgaccaggaaacagctatgaccGATGGATCCAGTGGTACCGAGCAT	1750 – 1776[B]
RPB2for+	*RPB2*	gtgactgtaaaacgacggccagtGATGATCGTGATCATTTCGG	Modified from Liu et al. (1999)
RPB2rev+		gtgaccaggaaacagctatgaccCCCATAGCTTGCTTACCCAT	Modified from Liu et al. (1999)
ITS1+	ITS	gtgactgtaaaacgacggccagtTCCGTAGGTGAACCTGCGG	Modified from White et al. (1990)
ITS4+		gtgaccaggaaacagctatgaccTCCTCCGCTTATTGATATGC	Modified from White et al. (1990)
M13 (-20) forward	-	TGTAAAACGACGGCCAGT	
M13 Reverse	-	CAGGAAACAGCTATGACC	
SP6	SP6 promotor	GATTTAGGTGACACTATAG	
T7	T7 promotor	TAATACGACTCACTATAGGG	

[A]= Base pair coordinates in homologous gene in *Sclerotinia sclerotiorum* (AF417110).
[B]= Base pair coordinates in homologous gene in *Coccidioides immitis* (U81786).

Table 4: Summary of the *RPB2, HSP60, G3PDH,* and combined sequence alignments

	HSP60	*G3PDH*	*RPB2*	Combined
Total/mean positions[A]	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[B]	4-8, 15-19, 183-192	none	none	1100-1104, 1111-1115, 1279-1288
Phylogenetic informative sites (substitutions/indels)	158 (157/1)	126 (125/1)	203 (201/2)	487 (483/4)

[A]= Total positions in the alignment/mean number of positions averaged across taxa.
[B]= Excluded positions due to ambiguous alignment.

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 2 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 the tree 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, 1000 replicates of random taxon-addition order, and all character transformations treated as equally likely (Fitch parsimony [Fitch, 1971]). To minimize the time spent on searching for a 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 (≥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 the 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 datasets was evaluated by comparing jackknife topologies via visual inspection. Visual inspection permits the precise location of areas of strong discordance (branches with conflicting jackknife values of at least 85% and Bayesian PP of at least 0.95) between phylogenies generated by different data sets.

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

Results

In preliminary experiments, sequence data of eight protein-coding loci and the non-coding 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 et al. (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 are included on the MP semistrict consensus tree in figure 1. The semistrict consensus tree topology of the two most-parsimonious trees (MTPs) contains 24 nodes with at least 63% jackknife and at least 95% Bayesian 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. byssoidea*.

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 due to 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 log-likelihood values –3357.89 and –3390.86; data not shown). Bayesian PP and jackknife percentages are included on the MP semistrict consensus tree in figure 2. The semi-strict consensus tree topology of the 320 MTPs contains 23 nodes with at least 63% jackknife and at least 95% Bayesian 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.

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 a RI of 0.91. Relationships among the main clades were topologically identical in MP and Bayesian consensus

Table 5: Cladistic behavior of *RPB2*, *HSP60*, *G3PDH*, and combined data sets with and without hybrid taxa

Locus	Pars.-Inf. sites	Ingroup taxa	Major clades with significant or strong support in semistrict consensus[A]	Placement of hybrids	Most-Pars. cladograms	CI	RI	Steps
G3PDH	126	48	A – – – – – G – i – – – M – O – – R S t	–	2	0.73	0.89	312
G3PDH + hybrid	129	53	A – – – – – G – i – – – M – O – – R S t	Basal to *B. aclada*/*B. byssoidea*	11	0.71	0.89	323
HSP60	158	48	A – – – e – – – – – k – M N O P – R S T	–	320	0.74	0.90	373
HSP60 + hybrid	160	56	A – – – – – – – – – – – M N O P – R S T	Basal to *B. aclada*/*B. byssoidea*	320	0.69	0.89	404
RPB2	203	48	A – – – E F G H i J – l M – O P – R S –	–	7	0.73	0.91	439
RPB2 + hybrid	205	53	A – – – e F G H i J – l M – O P – R S –	Basal to *B. aclada*/*B. byssoidea*	6	0.70	0.91	459
Combined	487	48	A b c d E F G – I – – – M – O P q R S T	–	80	0.72	0.89	1151

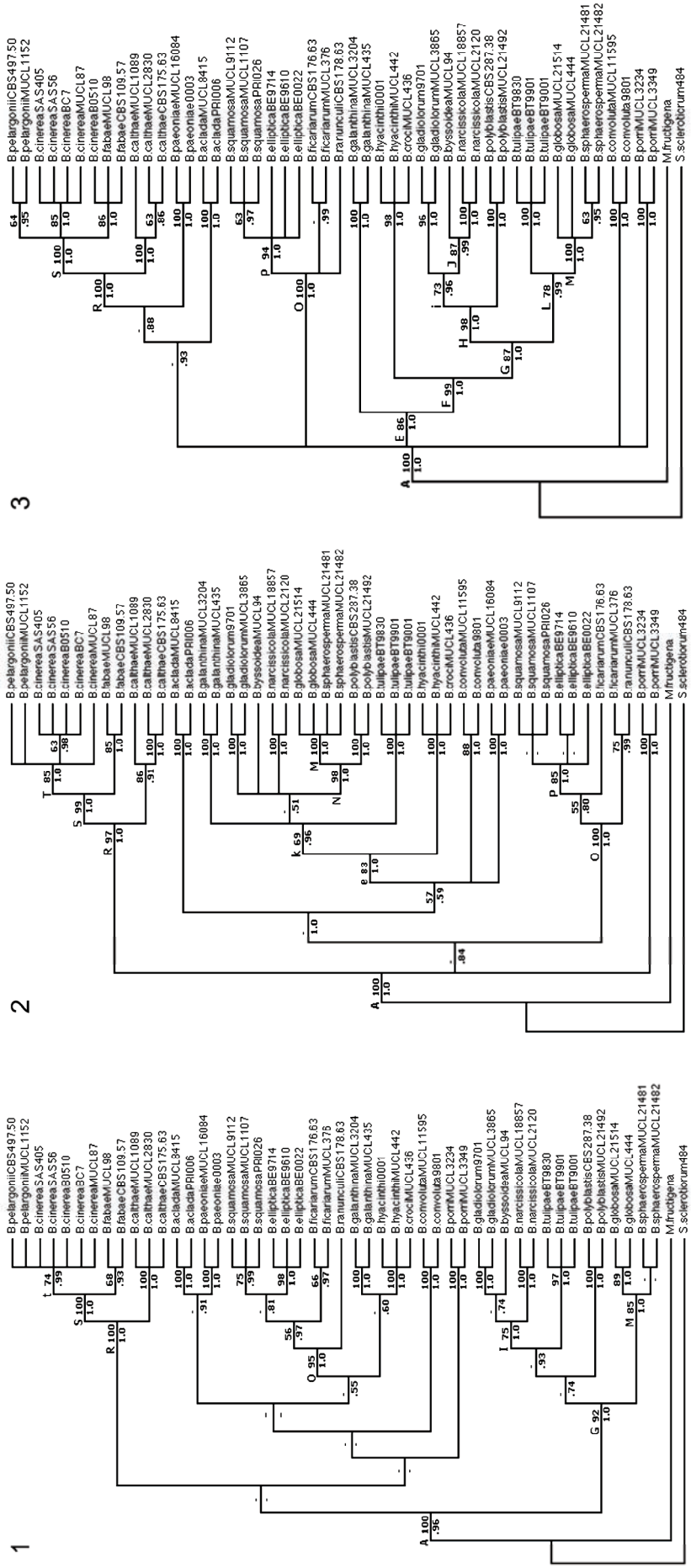
[A] Lowercase letters indicate significantly supported branches (jackknife support at least 63% and Bayesian PP at least 0.95). Capital letters indicate strongly supported branches (jackknife support at least 85% and Bayesian PP at least 0.95). Dashes indicate unsupported branches (jackknife support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

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 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% Bayesian 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 the 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, MUCL403 allele 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 [Figures 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 (Figure 3). In comparison to the *RPB2* topology, more resolution is provided by *HSP60* at the base of the tree (Figures 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 ≥0.95 Bayesian PP) between *RPB2* and *HSP60* phylogenies was evident in the placement of *B. cinerea* strain B05.10 and *B. polyblastis*.



Figures 1-3: Semistrict consensus of most-parsimonious trees based on **(1)** *G3PDH,* **(2)** *HSP60,* and **(3)** *RPB2* data. The trees were 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 Bayesian PP.

Molecular phylogeny distinguishes two major groups

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* (Figures 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 [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 (Figures 2 and 3). In contrast to the other relationships in the genus, the 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* x *B. aclada* [Yohalem et al., 2003]) was confirmed (Figure 5). The two isolates of *B. hyacinthi* did not appear to be monophyletic, because they lacked shared characters that distinguished them as a group (Figure 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 2955 bp between both species.

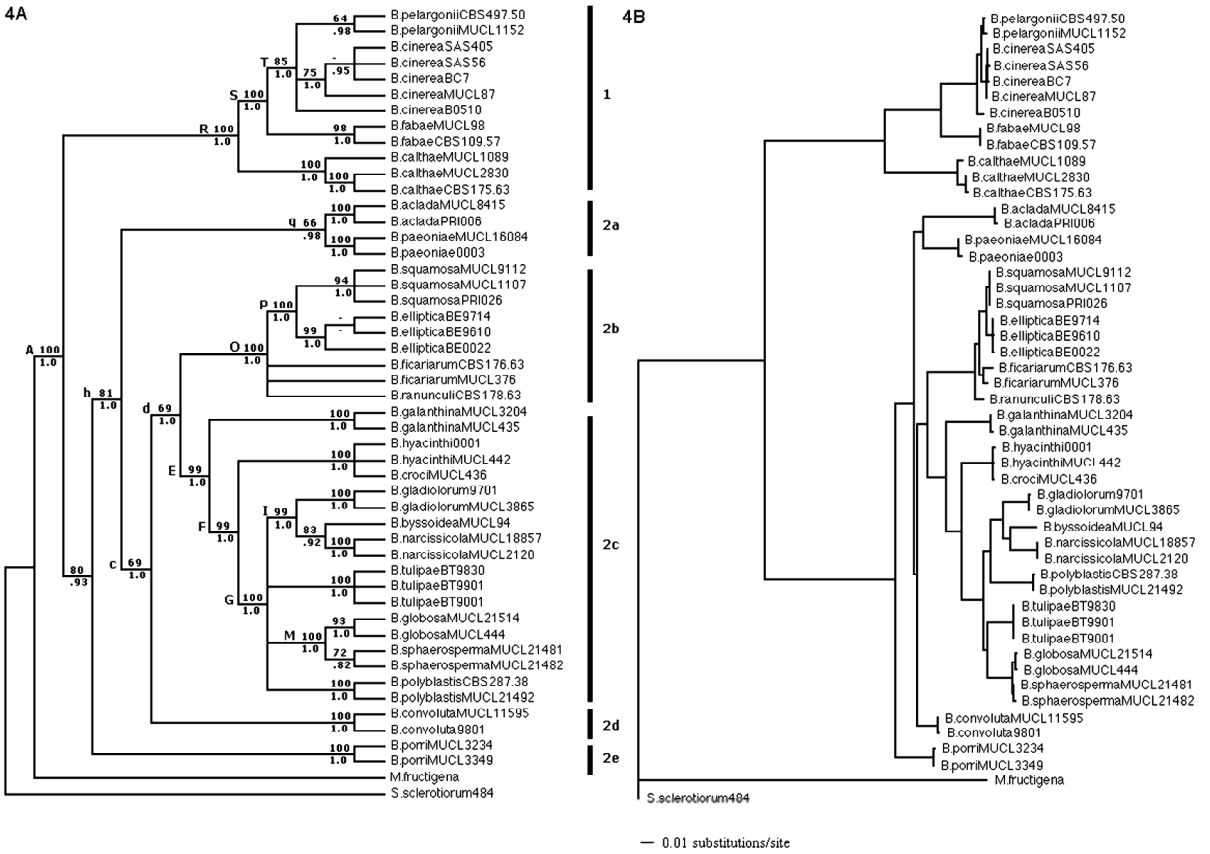


Figure 4: Molecular phylogeny for 48 *Botrytis* taxa and two outgroup species based on combined *G3DPH*, *HSP60*, and *RPB2* data. (**A**) Semistrict consensus of 80 most-parsimonious trees. Jackknife frequencies (10,000 replicates) are shown above each node; Bayesian posterior probabilities (PP) are shown below each node. Letters indicate major clades with significant or strong support (see table 5). Bars indicate the (sub)clades. (**B**) Bayesian inference showing a 50% majority-rule consensus tree from a five-million generation MCMC analysis.

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 Bayesian PP), and they were, therefore, assigned to sub-clade 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 sub-clade within clade 2, and has therefore been assigned to sub-clade 2d. Both sub-clade 2b and 2c 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 *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.

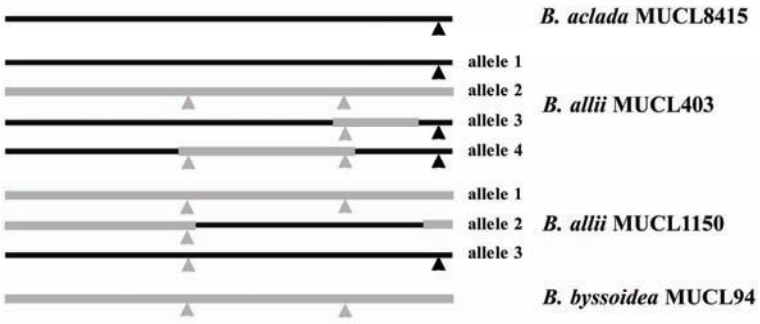


Figure 5: Schematic representation of a sequence alignment of multiple *HSP60* alleles for allopolyploid *B. allii* (strains MUCL403 and MUCL1150) and its presumed parental species *B. aclada* (isolate MUCL8415) and *B. byssoidea* (isolate MUCL94). Black and gray lines indicate stretches of sequence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

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 et al., 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 the cloned fragments of *B. allii* (strains MUCL1150 and MUCL403), digestion patterns of either the *B. aclada*-type or *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 (Figure 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 for *B. allii* strain MUCL1150. Hybrid alleles contained stretches of sequences originating from both parental alleles (Figure 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 the 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 (Figure 4A) using parsimony. Sexual reproduction has not been found in seven species, five of which parasitize monocot hosts and two parasitize a eudicot hosts (Table 1). Based on the reproductive mode, sexual reproduction was reconstructed as the ancestral condition (Figure 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 (Figures 6B and 6C). The order *Asparagales* is parasitized by 14 of the previously recognized *Botrytis* species, the order *Ranunculales* by three species, 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 (Figure 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 (Figure 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* 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 et al. (1998). The phylogenetic analyses of DNA sequence data show that the sequences from multiple isolates of the same species grouped 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 (Figure 4) and provides more resolution than individual gene phylogenies (Figures 1 to 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 et al., 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 et al., 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* or *B. byssoidea* within the *Botrytis* phylogeny. The occurrence of up to four different alleles per locus (Figure 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 which 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. alli*, 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* (Figure 4) showed that *Botrytis* species that parasitize hosts from one plant family do often not 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 (Figure 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 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 for *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; Muňoz 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.

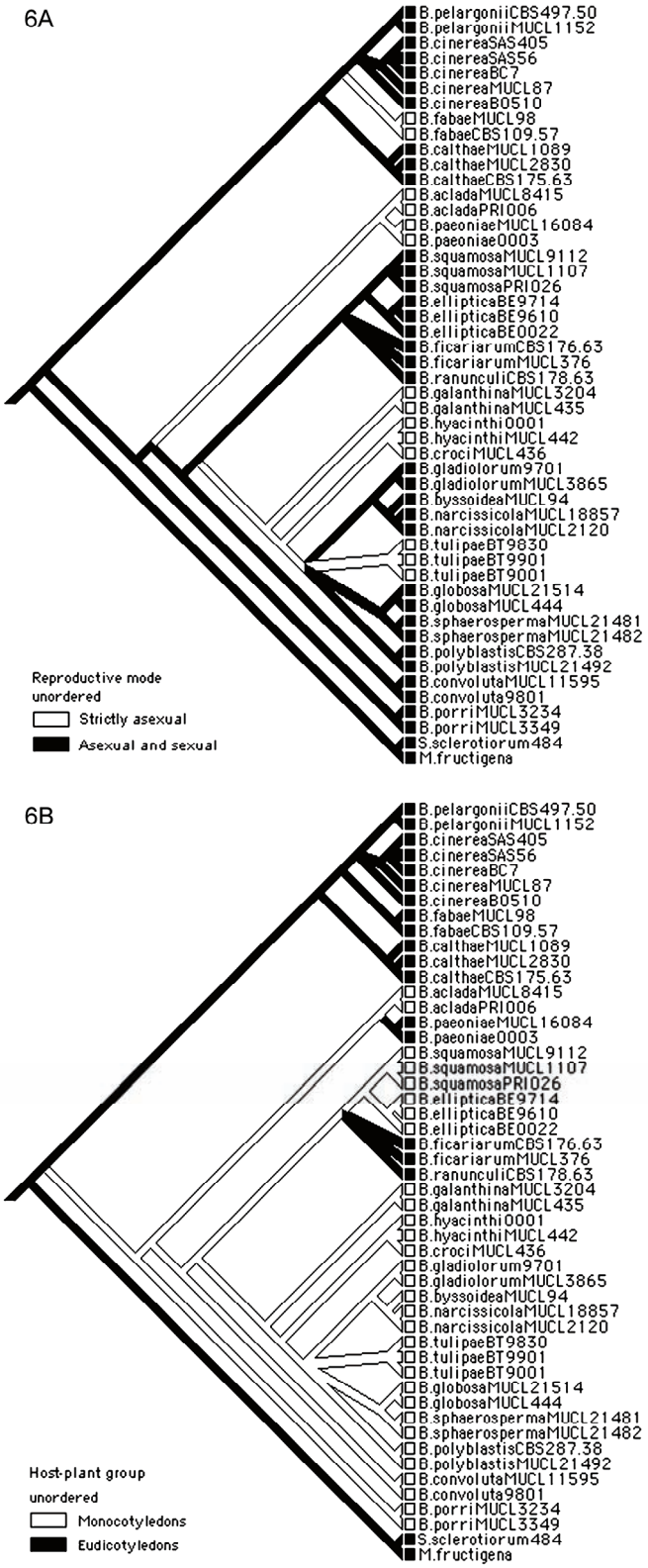


Figure 6: Reconstructions of character-state evolution mapped onto the combined molecular phylogeny (the same topology as in figure 4A) for *Botrytis* under parsimony: (**A**) reproductive mode, and (**B**) host-plant group.

Pathogenicity may have evolved from saprotrophy 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., 2002a, Rolke et al., 2004). Oxalic acid acidifies plant tissue in order 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 et al., 2004b). 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 results), 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.

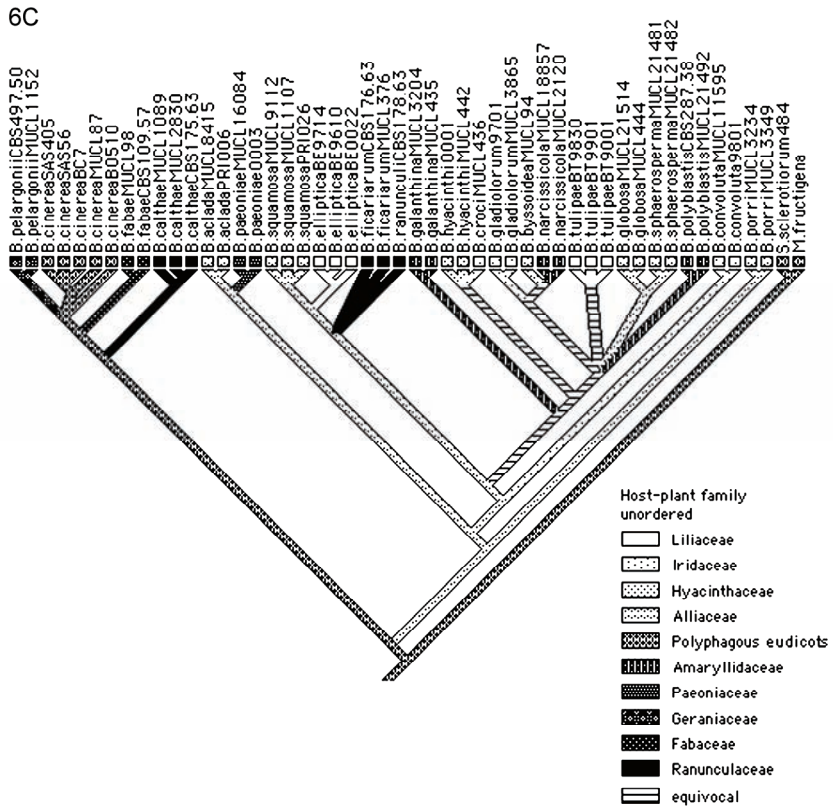


Figure 6C: Host-plant family evolution in *Botrytis* under parsimony using MacClade. The phylogeny has the same topology as in figure 4A.

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* (Figure 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 et al., 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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Chapter 3

Comparison of molecular typing methods for studying intraspecific variation in three *Botrytis* species

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To be submitted

Abbreviations: AFLP: Amplified Fragment Length Polymorphism; ITS: Internal Transcribed Spacer; IGS: Intergenic Spacer; RFLP: Restriction Fragment Length Polymorphism

Keywords: AFLP, genetic diversity, IGS, ITS, multilocus sequencing, PCR-RFLP

Comparison of molecular typing methods for studying intraspecific variation in three *Botrytis* species

Abstract

This study investigated the differentiation of isolates of *Botrytis tulipae*, *B. elliptica*, and *B. cinerea* by sequencing of six DNA regions, by restriction analysis of the ribosomal intergenic spacer region using six restriction enzymes, and by amplified fragment length polymorphism (AFLP) analysis using six different primer sets. Ten isolates of each species were tested. DNA sequencing produced 2, 7 and 9 multilocus genotypes for *B. tulipae*, *B. elliptica* and *B. cinerea*, respectively. PCR-RFLP analysis could reveal some variation in *B. cinerea* and *B. elliptica*, but generated only monomorphic patterns for *B. tulipae*. AFLP fingerprinting differentiated all isolates of *B. elliptica* and *B. cinerea* and 8 isolates of *B. tulipae*. For *B. elliptica* and *B. cinerea*, a single AFLP primer set could distinguish all ten isolates. This study indicated that the use of AFLP fingerprinting provides the most efficient method for the differentiation of *Botrytis* strains isolated from the field and for epidemiological studies.

Introduction

Botrytis spp. are necrotrophic pathogens causing pre- and postharvest diseases in many crops, such as grapevine, tomato, and bulb crops (Elad et al., 2004). The genus is divided into 22 species based on morphological characteristics, host specificity and DNA sequence information (Hennebert, 1973; Beever and Weeds, 2004; Staats et al., 2005). *Botrytis* spp. that attack flower bulbs include *B. tulipae* and *B. elliptica*, the causal agents of leaf blight in tulip and lily, respectively. Severe epidemics can cause yield losses up to 60% and growers spray weekly with protective fungicides to control disease (Lorbeer et al., 2004).

Genetic diversity in *B. tulipae* has not previously been studied, whereas for *B. elliptica*, one population study has been reported using randomly amplified polymorphic DNA (RAPD) markers (Huang et al., 2001). Most population genetic studies in *Botrytis* have focused on *B. cinerea* using a variety of molecular techniques including RFLP analysis of PCR amplified DNA regions (Giraud et al., 1997; Giraud

et al., 1999), PCR detection of transposable elements (Diolez et al., 1995; Levis et al., 1997a), PCR fingerprinting of the whole genome by RAPD analysis (Alfonso et al., 2000) or amplified fragment length polymorphism (AFLP) analysis (Moyano et al., 2003), fingerprinting of repetitive sequences by microsatellite primed (MP)-PCR (Zhonghua and Michailides, 2005), PCR amplification of microsatellite loci (Fournier et al., 2002) and DNA sequencing of gene regions (Albertini et al., 2002; Fournier et al., 2003).

Molecular typing methods should be reproducible, discriminatory, high throughput, and easy-to-use. The PCR-RFLP method has been widely used and has been shown to be discriminatory and reproducible (Giraud et al., 1997; Giraud et al., 1999). The advantage of nucleic acid sequencing is that all DNA polymorphisms in a particular region are revealed, which might have been overlooked in restriction analysis. The AFLP method (Vos et al., 1995) represents a convenient PCR-based fingerprinting technique that reveals differences in the whole genome, and AFLP has the advantage of being reproducible, rapid, and economical.

In this study, three DNA-based molecular methods were compared for their potential to differentiate isolates of *B. tulipae*, *B. elliptica* and *B. cinerea*. Ten isolates of each species were tested. Regions of five nuclear DNA-encoded genes and the internal transcribed spacer (ITS) region were sequenced in order to detect polymorphisms. Furthermore, PCR-RFLP of the ribosomal intergenic spacer (IGS) region and AFLP analysis were used. The aim of this study was to compare the discriminatory power of these three molecular typing methods. The most discriminatory method was used as a tool to study the genetic diversity in field isolates of *B. tulipae* and *B. elliptica* from the Netherlands (Chapter 4).

Materials and Methods

Collection of isolates and DNA isolation

All isolates of *B. tulipae* and *B. elliptica* were provided by I. Pennock-Vos and Dr. M. de Boer of Applied Plant Research (PPO), Flower Bulb Research Unit in Lisse, the Netherlands. The isolates of *B. tulipae* and *B. elliptica* have been sampled in different years and from several different locations in the Netherlands (Table 1). Seven *B. cinerea* isolates have been previously used to study genetic variation by RAPD analysis (van der Vlugt-Bergmans et al., 1993; Kerssies et al., 1997). Additional isolates of *B. cinerea* were kindly provided by Paul and Bettina Tudzynski (WWU

Table 1: Sample date, number of isolates, and sample location of isolates used in this study

				GenBank accession number of sequences						
Species	Isolate	Sample date	Location/Region	*G3PDH*	*RPB2*	*HSP60*	*H3*	*EF-1α*	ITS	IGS
B. tulipae	BT9601[a]	1996	The Netherlands, Lisse	AM231173	AM231330	AM232678	AM233554	AM235188	AM235309	AM233399
	BT9701[a]	1997	The Netherlands, Lisse	AM231172	AM231325	AM232681	AM233560	AM235192	n.d.	n.d.
	BT9815[a]	1998	The Netherlands, Espel	AM231169	AM231329	AM232677	AM233562	AM235191	n.d.	n.d.
	BT9806[a]	1998	The Netherlands, Lisse	AM231175	AM231328	AM232682	AM233561	AM235190	n.d.	n.d.
	BT9813[a]	1998	The Netherlands, Friesland	AM231170	AM231327	AM232680	AM233559	AM235196	AM235312	n.d.
	BT9903[a]	1999	The Netherlands, Wieringen	AM231174	AM231324	AM232676	AM233557	AM235189	AM235310	n.d.
	BT9901[a]	2000	The Netherlands, Tollebeet	AJ705042[f]	AJ745714[f]	AJ716103[f]	AM233556	AM235194	AM235313	n.d.
	BT9001[a]	2000	The Netherlands, Lisse	AJ705040[f]	AJ745712[f]	AJ716101[f]	AM233553	AM235195	AM235308	n.d.
	BT9830[a]	2000	The Netherlands, Lisse	AJ705041[f]	AJ745713[f]	AJ716102[f]	AM233558	AM235193	AJ716301[f]	n.d.
	BT0005[a]	2000	The Netherlands, Ens	AM231171	AM231326	AM232679	AM233555	AM235197	AM235311	n.d.
B. elliptica	BE9401[ab]	1994	The Netherlands, Lisse	AM231167	n.d.[g]	n.d.	AM233533	AM235200	n.d.	n.d.
	BE9605[a]	1996	The Netherlands, Lisse	AM231168	AM231318	AM232668	AM233542	AM235201	n.d.	n.d.
	BE9610[a]	1996	The Netherlands,Bergentheim	AJ705011[f]	AJ745683[f]	AJ716072[f]	AM233535	AM235204	AM235302	n.d.
	BE9612[a]	1996	The Netherlands, Anerveen	AM231164	AM231319	AM232666	AM233541	AM235205	AM235303	AM233398
	BE9623[a]	1996	The Netherlands, Wieringen	AM231165	AM231323	AM232669	AM233536	AM235198	AM235304	n.d.
	BE9714[a]	1997	The Netherlands, Elsloo	AJ705012[f]	AJ745684[f]	AJ716073[f]	AM233538	AM235206	AJ716300[f]	n.d.
	BE9732[a]	1997	The Netherlands, Lisse	AM231163	AM231321	AM232667	AM233534	AM235202	AM235305	n.d.
	BE0004[a]	2000	The Netherlands, Bant	AM231162	AM231320	AM232665	AM233539	AM235207	AM235306	n.d.
	BE0006[a]	2000	The Netherlands, Rutten	AM231166	AM231322	AM232664	AM233537	AM235203	n.d.	n.d.
	BE0022[a]	2001	The Netherlands, Smilde	AJ705010[f]	AJ745682[f]	AJ716071[f]	AM233540	AM235199	AM235307	n.d.
B. cinerea	Bc7[d]	1970	The Netherlands	AJ705003[f]	AJ745675[f]	AJ716064[f]	AM233547	AM235179	AM235296	n.d.
	Bc12[d]	1986	The Netherlands	AM231161	AM231313	AM232671	AM233552	AM235184	AM235294	n.d.
	Bc21[d]	1990	The Netherlands	AM231158	AM231317	AM232675	AM233544	AM235180	AM235301	n.d.
	7A[e]	1992	The Netherlands, Aalsmeer	AM231159	AM231316	AM232673	AM233548	AM235178	AM235298	n.d.
	8A[e]	1992	The Netherlands, Aalsmeer	AM231160	AM231314	AM232672	AM233543	AM235183	AM235295	n.d.
	B0510	1994	Germany	AJ705002[f]	AJ745674[f]	AJ716063[f]	AM233549	AM235186	AM235297	n.d.
	SAS56[c]	-	Italy	AJ705006[f]	AJ745677[f]	AJ716067[f]	AM233551	AM235187	AJ716294[f]	AM233400
	SAS405[c]	-	Italy	AJ705005[f]	AJ745678[f]	AJ716066[f]	AM233550	AM235182	AM235293	n.d.
	M14	-	-	AM231157	AM231315	AM232670	AM233546	AM235181	AM235299	n.d.
	BcIPO	-	-	AM231156	AM231312	AM232674	AM233545	AM235185	AM235300	n.d.

[a] Applied Research Plant and Environment, research Unit Flower Bulbs; [b] Kessel et al. (2001); [c] Faretra et al. (1988); [d] Van der Vlugt-Bergmans et al. (1993); [e] Kerssies et al. (1997); [f] Staats et al. (2005); [g] n.d. = not determined.

Münster, Germany) and Paul van den Boogert (Plant Research International, Wageningen, the Netherlands).

All isolates were grown on malt agar (Oxoid) for approximately 14 days at 18°C. Mycelial tissue was harvested, lyophilized, and stored at -80°C. Lyophilized mycelium (10 to 20 mg) was ground into a powder using a mortar and pestle in liquid nitrogen. Genomic DNA was extracted with the Puregene DNA isolation kit (Gentra systems Inc., Biozym systems, Landgraaf, the Netherlands) according to the manufacturer's instructions. DNA pellets were dissolved in 100µl of TE (10mM Tris-HCL [pH 8.0], 1 mM EDTA) and stored at 4°C.

Amplification and sequencing of DNA regions

DNA sequences of 7 genomic regions were obtained to screen for intraspecific sequence polymorphisms among 10 isolates of each of three *Botrytis* species. Three primer combinations, previously described by Staats et al. (2005), were used to PCR amplify and sequence regions of three nuclear DNA genes encoding glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*), Heat-shock Protein 60 (*HSP60*) and DNA-dependent RNA polymerase subunit II (*RPB2*). Primers designed by White *et al*. (1990) were used to amplify the nuclear rDNA region containing the two ITS regions (ITS1 and ITS2) and the 5.8S rRNA gene, as well as the entire intergenic spacer region (*IGS*) of the nuclear ribosomal DNA repeat unit (Table 2). PCR primers to amplify regions of the translation elongation factor 1-alpha (*EF-1α)* gene and the Histone H3 (*H3*) gene were designed based on alignment of homologous gene sequences from *Neurospora crassa* and fragments from a *Botrytis cinerea* cDNA library (Genoscope, France). PCR amplification and sequencing protocols for *G3PDH*, *RPB2* and *HSP60* were previously described (Staats et al., 2005). The same program, but with an annealing temperature of 64°C, was used for amplifying *H3* and *EF-1a* gene fragments.

The full-length *IGS* region was amplified using primers NL2F and NS1R (Table 2) and sequenced only for *B. elliptica* strain BE9612, *B. tulipae* strain BT9601, and *B. cinerea* strain SAS56. PCR amplification of the IGS region was performed in a 50-µl reaction volume and contained approximately 20 ng of template DNA, 1X Expand High Fidelity Buffer with $MgCl_2$ (Roche), 2.0 U of Expand High Fidelity enzyme mix (Roche), 0.2 pmol of each primer (Amersham Pharmacia Biotech), and 200 µM of each dNTP (Promega). Amplifications were carried out in a Peltier Thermal Cycler-200 (Biozym, Landgraaf, The Netherlands).

Table 2: Primer sets and corresponding amplification targets

Primer name	Target region	Primer sequence (5′ – 3′)	Reference
G3PDHfor+	*G3PDH*	gtgactgtaaaacgacggccagtATTGACATCGTCGCTGTCAACGA	Staats et al. (2005)
G3PDHrev+		gtgaccaggaaacagctatgaccACCCCACTCGTTGTCGTACCA	Staats et al. (2005)
HSP60for+	*HSP60*	gtgactgtaaaacgacggccagtCAACAATTGAGATTTGCCCACAAG	Staats et al. (2005)
HSP60rev+		gtgaccaggaaacagctatgaccGATGGATCCAGTGGTACCGAGCAT	Staats et al. (2005)
RPB2for+	*RPB2*	gtgactgtaaaacgacggccagtGATGATCGTGATCATTTCGG	Modified from Liu et al. (1999)
RPB2rev+		gtgaccaggaaacagctatgaccCCCATAGCTTGCTTACCCAT	Modified from Liu et al. (1999)
H3for+	*H3*	gtgactgtaaaacgacggccagtATGGCTCGTACCAAGCAAACTG	
H3rev+		gtgaccaggaaacagctatgaccCGCTCACCACGGAGACGACG	
EF1alpha-for+	*EF-1a*	gtgactgtaaaacgacggccagtCAAGTACGCATGGGTTTTGGACAA	
EF1alpha-rev+		gtgaccaggaaacagctatgaccACTTGCAAGCAATGTGAGCAGTGTG	
ITS1+	ITS	gtgactgtaaaacgacggccagtTCCGTAGGTGAACCTGCGG	Modified from White et al. (1990)
ITS4+		gtgaccaggaaacagctatgaccTCCTCCGCTTATTGATATGC	Modified from White et al. (1990)
NL2F	IGS	AATTGTGCTACACGGTCCTG	Carbone et al. (1999)
NS1R		GAGACAAGCATATGACTAC	White et al. (1990)
IGSnr1for	IGS	CACGGTCCTGTAAGTAGTAGA	
IGSnr1rev		GGGAGCTCACCGGGAACCTTA	
IGSnr3for	IGS	TCCCGGTGAGC(C/T)TTTTA	
IGSnr3rev		CATCGGCCACTAATCCACAGACTC	
M13(-20) forward	-	TGTAAAACGACGGCCAGT	
M13 Reverse	-	CAGGAAACAGCTATGACC	

The following program was used: 94°C for 2 min (1 cycle), 94°C for 15 s, 61°C for 30 s, 68°C for 2.5 min (10 cycles), 94°C for 15 s, 61°C for 30 s, 72°C for 2.5 min + 5 s cycle elongation for each successive cycle (20 cycles), and then 72°C for 7 min (1 cycle). 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 comparison to a DNA marker created by digesting Lambda DNA with E*co*RI, *Hind*III, and *Bam*HI (Promega). PCR products were purified and cloned as described by Staats et al. (2005). The clones containing the IGS region (approx. 3.5 kbp) from each of the three *Botrytis* isolates were sequenced single stranded.

G3PDH, *RPB2*, and *HSP60* were sequenced double stranded using M13(-20) forward and M13 reverse primers. *EF-1a* and *H3* were sequenced single stranded using only the M13(-20) forward primer. All sequence reactions were performed by BaseClear (Leiden, The Netherlands). A total of 142 new DNA sequences are deposited in GenBank under accession numbers AM231156-AM231175 (*G3PDH*), AM231312-AM231330 (*RPB2*), AM232664-AM232682 (*HSP60*), AM233533-AM233562 (*H3*), AM235178-AM235207 (*EF-1a*), AM235293-AM235313 (ITS) and AM233398-AM233400 (IGS) (Table 1).

DNA sequence chromatographs were visually inspected using Vector NTI suite 8.0 (InforMax, Inc.). Sequences were aligned using ClustalX version 1.8 (Thompson et al., 1997) and adjusted manually when necessary. Sequence alignments of the DNA regions were unambiguous with a low number of indels, except for the IGS region in which indels occurred throughout the alignment (not shown).

IGS restriction analysis

To facilitate PCR amplification of the entire IGS region, two primer sets were designed that each amplify an approximately 1500 bp fragment of the full-length IGS region and of which the amplification products do not overlap. A sequence alignment was made between the three full-length IGS sequences of *B. elliptica* strain BE9612, *B. tulipae* strain BT9601, and *B. cinerea* strain SAS56. The low level of sequence similarity, between 73% and 79%, and the large number of indels in the sequence alignment, complicated primer design. The forward primer of primer set IGSnr1 was located in the highly conserved 28S rDNA region, whereas the reverse primer was located in a small conserved region in the middle of the IGS region starting at position 1338 of the *B. cinerea* SAS56 sequence (Table 2). The fragment size

of the IGS region with primer set IGSnr1 for *B. elliptica* was approximately 170 bp larger, due to the presence of an additional sequence in this part of the IGS region. The degenerate forward primer of primer set IGSnr3 is located at position 1601-1617 of the IGS region of *B. cinerea* SAS56 and the reverse primer is located in the highly conserved 18S rDNA region (Table 2).

The PCR protocol described by Staats et al. (2005) was used for amplification of both partial IGS regions. An annealing temperature of 61°C and 56°C was used for the amplification of IGSnr1 and IGSnr3, respectively. Both IGS fragments were digested restriction enzymes *Rsa*I, *Hinc*II, *Hind*III, *Bam*HI, *Taq*I, and *Hae*III (Promega). Three 4-bp recognition restriction enzymes (*Rsa*I, *Taq*I, and *Hae*III) and one 6-bp recognition restriction enzyme (*Hinc*II) were selected based on the presence of their recognition sequence in the partial IGS regions of at least one of the three *Botrytis* species. *Bam*HI and *Hind*III were chosen based on previous studies by Giraud et al. (1997). DNA fragments were separated by electrophoresis in 1.5% agarose gels containing ethidium bromide.

AFLP analysis

The protocol of Vos et al. (1995) was used with the following modifications: Restriction digestion and adapter ligation were performed simultaneously on 250 ng of genomic DNA using 5 units of *Eco*RI (Pharmacia), 5 units of *Mse*I (New England Biolabs Inc.) and 1 unit of T4 DNA ligase (Pharmacia). Restriction fragments were ligated to *Mse*I adapters and *Eco*RI adapters (Table 2). This restriction ligation was performed in 50 µl of One-Phor-All™ buffer (Pharmacia) with 10mM ATP (Pharmacia) and 5mM dithiothreitol (Sigma) at 37°C for 6-8 hours. The ligation products were diluted 10 times in 10mM Tris-HCl (pH 8.0) and 0.1mM EDTA. Pre-amplification of the template was performed in a 50µl reaction volume using the nonselective primers *Eco*00 and *Mse*00 (Table 3). The amplified products were checked on 1.5% agarose gels. Selective PCR was performed in a 20µl reaction volume with 5µl of 10X diluted pre-amplification products, 50 ng of an *Mse*-primer with two selective nucleotides, and 5 ng of a fluorescent labeled *Eco*-primer with two selective nucleotides (Table 3). The selective primer *Eco*12 was labeled with the infrared dye IRD800 and the selective primer *Eco*13 was labeled with IRD700. All PCR reactions were performed using SuperTaq™ DNA polymerase (Sphaero-Q) in a PE9700 Thermocycler (Perkin-elmer). Products were loaded on a ready-to-use 5% polyacrylamide gel (Sequagel-6®, National Diagnostics) and run on a LI-COR 4200

DNA sequencer (Westburg). AFLP amplification products were designated according to the primer combination used and their sizes estimated by reference to the SequaMark™ 10 base ladder (Research Genetics, Huntsville, Alabama, USA).

Table 3: Sequences of AFLP primers and adapters

Primer Name	Primer Sequence
MseI adapters	5′-GACGATGAGTCCTGAG-3′
	3′-TACTCAGGACTCAT-5′
EcoRI adapters	5′-CTCGTAGACTGCGTACC-3′
	3′-CTGACGCATGGTTAA-5′
Eco00	GACTGCGTACCAATTC
Eco12	GACTGCGTACCAATTCAC
Eco13	GACTGCGTACCAATTCAG
Mse00	GATGAGTCCTGAGTAA
Mse15	GATGAGTCCTGAGTAACA
Mse16	GATGAGTCCTGAGTAACC
Mse17	GATGAGTCCTGAGTAACG
Mse18	GATGAGTCCTGAGTAACT

DNA sequences are oriented from 5′ to 3′ unless indicated otherwise

Six primer combinations were used for fingerprinting all DNA samples, i.e. primer combinations *Eco*12 + *Mse*16, *Eco*12 + *Mse*17, *Eco*13 + *Mse*15, *Eco*13 + *Mse*16, *Eco*13 + *Mse*17, and *Eco*13 + *Mse*18. DNA samples were isolated twice from independent fungal cultures and AFLP reactions were performed twice to verify the reproducibility of AFLP band patterns.

Cluster analysis

The unweighted pair group method with arithmetic mean (UPGMA) and Jukes and Cantor's (1969) evolutionary distance model were used for tree reconstruction with the combined data of five DNA regions (*EF-1α*, *H3*, *G3PDH*, *RPB2*, and *HSP60*) using MEGA version 3.1 (Kumar et al., 2004).

AFLP and IGS-RFLP bands were scored manually as binary characters. Bands at the same height were treated as one putative locus with two alleles, one allele indicating the presence of the band and the other its absence. Genetic similarities were calculated with Jaccard's similarity coefficient by NTSYS-pc version 2.02j (Applied Biostatistics Inc.). Jaccard's similarity coefficient only considers the presence of bands; absence of bands is not interpreted as a similar character between isolates. The similarity matrix was used to construct a dendrogram by the UPGMA cluster method.

Results

DNA sequence variation

DNA sequences of six genomic regions were obtained to differentiate 30 isolates from three *Botrytis* species (*B. tulipae*, *B. elliptica* and *B. cinerea*), covering over 4300 bp of sequence data per isolate (Table 4). DNA amplification products from each of the six primer sets were recovered from every isolate. However, sequences of *HSP60*, *RPB2*, and ITS were not determined for all isolates (Table 5). Five DNA regions encoding proteins and spanning one or two introns were amplified (Table 4). The positions of the introns in *G3PDH* and *RPB2* were conserved and the sequence variation was highest in the intron sequences (not shown). Sequence variation among *Botrytis* species was highest in *H3*, with approximately 12% of sites being polymorphic, and lowest in ITS and *G3PDH*, with approximately 0.2% and 5.5% of sites being polymorphic (Table 4). Indels were observed in sequence data of *HSP60*, *EF-1α*, and *H3* and were only found in the intron regions (not shown). Indels were generally conserved within species and polymorphic between species, except for one indel in *EF-1α*, which was polymorphic among *B. elliptica* strains (not shown). Additional intraspecific sequence polymorphisms were found in *HSP60*, *RPB2*, *EF-1α*, and *H3* (Table 4).

Table 4: Summary of the *G3PDH*, *HSP60*, *RPB2*, *EF-1α*, *H3* and ITS sequence alignments

	G3PDH	*HSP60*	*RPB2*	*EF-1α*	*H3*	ITS	Combined[b]
Number of exons	2	2	1	2	2	-	9
Number of introns	2	2	1	1	2	-	8
Total size	886	978	1093	448	481	445	3886
Intron size	117	84	28	55	179	-	463
Indels	0	4	0	2	5	0	11
Variable sites/Phylo-inf[a]. (all)	49/49	70/67	88/85	29/26	57/57	1/1	293/284
Variable sites/Phylo-inf. (*B. tulipae*)	0/0	0/0	0/0	1/0	0/0	0/0	1/0
Variable sites/Phylo-inf. (*B. elliptica*)	0/0	4/2	0/0	8/8	0/0	0/0	12/10
Variable sites/Phylo-inf. (*B. cinerea*)	0/0	1/0	6/0	6/3	2/1	0/0	15/4

[a] The number of variable sites and the number of potentially phylogenetically informative sites
[b] Combined *G3PDH*, *HSP60*, *RPB2*, *EF-1a*, and *H3* data

Intraspecific variability was low among the 10 *B. tulipae* strains compared to the other two species (Table 4 and 5). Only one nucleotide position was polymorphic in over 4300 bp, i.e. in the EF-1α sequence of *B. tulipae* strain BT9901. Therefore only two multilocus sequence genotypes were identified among the 10 *B. tulipae* strains (Table 5).

Table 5: Single and multilocus genotypes based on DNA sequence data of six genomic regions

Species	Isolate	*G3PDH*	*HSP60*	*RPB2*	*EF-1α*	*H3*	ITS	Multilocus[a]
B. tulipae	BT9601	A	A	A	A	A	A	A
	BT9701	A	A	A	A	A	n.d.	A
	BT9815	A	A	A	A	A	n.d.	A
	BT9806	A	A	A	A	A	n.d.	A
	BT9813	A	A	A	A	A	A	A
	BT9903	A	A	A	A	A	A	A
	BT9901	A	A	A	B	A	A	B
	BT9001	A	A	A	A	A	A	A
	BT9830	A	A	A	A	A	A	A
	BT0005	A	A	A	A	A	A	A
Distinct genotypes		1	1	1	2	1	1	2
B. elliptica	BE9401	B	n.d.[b]	n.d.	C	B	n.d.	n.d.
	BE9605	B	B	B	C	B	n.d.	C
	BE9610	B	C	B	C	B	A	D
	BE9612	B	D	B	C	B	A	E
	BE9623	B	E	B	D	B	A	F
	BE9714	B	F	B	C	B	A	G
	BE9732	B	E	B	C	B	A	H
	BE0004	B	F	B	C	B	A	F
	BE0006	B	E	B	C	B	n.d.	H
	BE0022	B	D	B	D	B	A	I
Distinct genotypes		1	5	1	2	1	1	7
B. cinerea	Bc7	C	G	C	E	C	B	J
	Bc12	C	H	C	F	D	B	K
	Bc21	C	H	C	E	E	B	L
	7A	C	H	C	E	C	B	M
	8A	C	H	C	F	E	B	N
	B0510	C	H	D	G	C	B	O
	SAS56	C	H	E	G	C	B	P
	SAS405	C	H	C	H	C	B	Q
	M14	C	H	C	E	C	B	M
	BcIPO	C	H	C	I	E	B	R
Distinct genotypes		1	2	3	5	3	1	9

[a] Multilocus genotypes are based on the combined variation from five nuclear loci (*G3PDH*, *HSP60*, *RPB2*, *EF-1a*, and *H3*); [b] n.d. = not determined

For *B. elliptica*, seven multilocus sequence genotypes were detected with 12 polymorphic sites (Table 4 and 5). *HSP60* was the most informative locus for this species, as it could distinguish 5 genotypes with 4 polymorphic sites (sites 56, 223, 779, and 897; Table 5). An additional 8 sites were polymorphic in *EF-1α* however, only two genotypes were identified with this locus (Table 5).

The level of intraspecific polymorphism was highest in *B. cinerea*. A total of 9 multilocus sequence genotypes were identified based on 15 nucleotide polymorphisms (Tables 4 and 5). Six sites were polymorphic in *RPB2* and *EF-1α*, and these loci enabled to identify 3 and 5 genotypes, respectively.

There were 293 variable sites in the combined *G3PDH*, *HSP60*, *EF-1α*, *RPB2* and *H3* data, 284 of which were potentially phylogenetically informative (Table 4). Of

the intraspecific variable sites, ten were potentially informative for *B. elliptica* and four were potentially informative for *B. cinerea* (Table 4). The UPGMA tree constructed with the combined DNA data is shown in Figure 1.

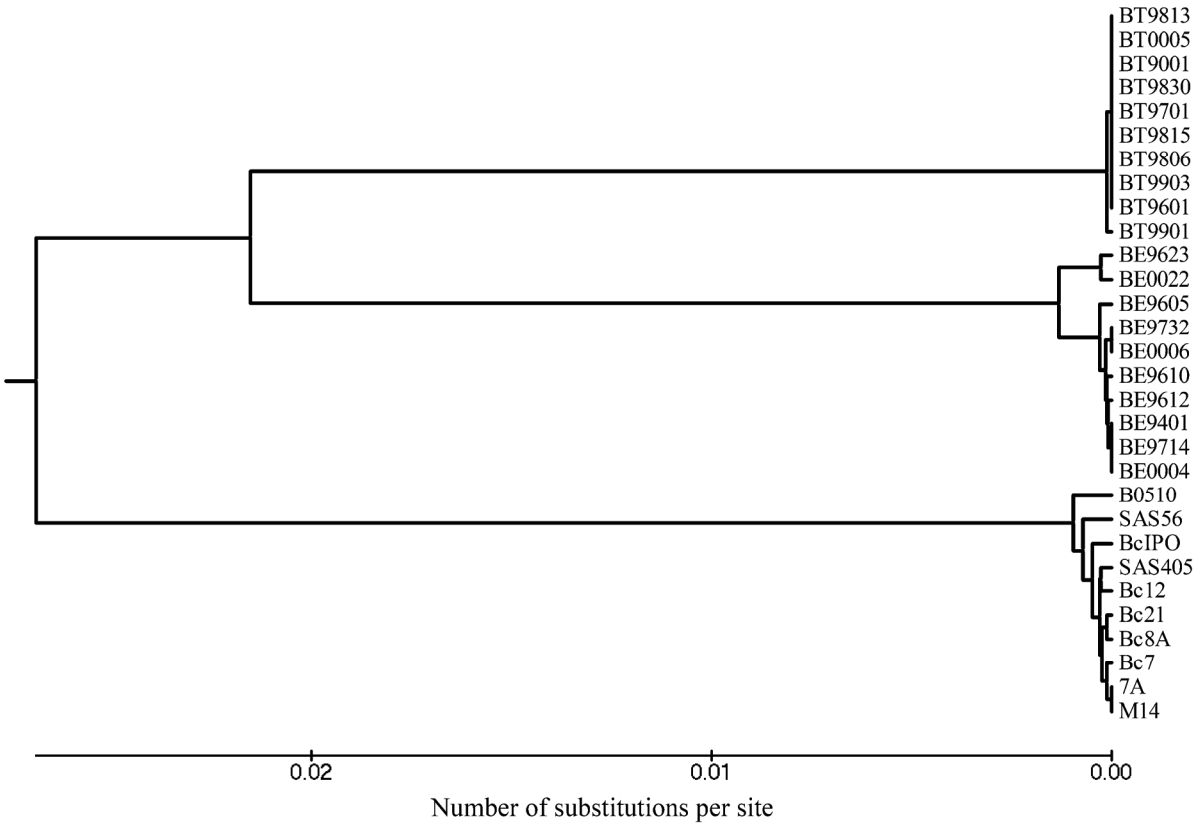

Figure 1: Phylogenetic tree with mid-point rooting for the combined DNA data (*G3PDH*, *HSP60*, *EF-1a*, *RPB2*, and *H3*) inferred from the UPGMA method

IGS restriction variation

Sequence length variation was observed between the full-length IGS regions of the three *Botrytis* species. The full-length IGS region was obtained for *B. tulipae* strain BT9601 (3418 bp), *B. elliptica* strain BE9612 (3598 bp), and *B. cinerea* strain SAS56 (3372 bp). The IGS region of *B. elliptica* strain BE9612 contains an approximately 170 bp insert. The overall sequence similarity was low, between 73-79%. Approximately 5-8% of the sequence alignments were gap characters (not shown).

Two primer sets were designed that amplified different fragments of the IGS region, such that the two partial IGS regions combined span the entire IGS region, except for an approximately 260 bp internal region. The amplification product IGSnr1 was 1460 bp, 1630 bp, and 1440 bp for *B. tulipae*, *B. elliptica*, and *B. cinerea*, respectively, and the amplification product IGSnr3 was approximately 1450 bp for all three species. Length polymorphisms were not observed among the partial IGS

Table 6: IGS-RFLP profiles

		IGSnr1						IGSnr3						
Species	Isolate	RsaI	HincII	HindIII	BamHI	TaqI	HaeIII	RsaI	HincII	HindIII	BamHI	TaqI	HaeIII	Combined
B. tulipae	BT9601	A	U[a]	U	U	A	A	A	A	U	U	A	U	A
	BT9701	A	U	U	U	A	A	A	A	U	U	A	U	A
	BT9815	A	U	U	U	A	A	A	A	U	U	A	U	A
	BT9806	A	U	U	U	A	A	A	A	U	U	A	U	A
	BT9813	A	U	U	U	A	A	A	A	U	U	A	U	A
	BT9903	A	U	U	U	A	A	A	A	U	U	A	U	A
	BT9901	A	U	U	U	A	A	A	A	U	U	A	U	A
	BT9001	A	U	U	U	A	A	A	A	U	U	A	U	A
	BT9830	A	U	U	U	A	A	A	A	U	U	A	U	A
	BT0005	A	U	U	U	A	A	A	A	U	U	A	U	A
Distinct genotypes		1	1	1	1	1	1	1	1	1	1	1	1	1
B. elliptica	BE9401	B	U	U	U	B	B	B	B	U	U	B	A	B
	BE9605	C	U	U	U	B	B	B	C	U	U	B	A	C
	BE9610	C	U	U	U	B	B	B	C	U	U	B	A	C
	BE9612	C	U	U	U	B	B	B	B	U	U	B	A	D
	BE9623	B	U	U	U	B	B	B	B	U	U	B	A	B
	BE9714	C	U	U	U	B	B	B	B	U	U	B	A	D
	BE9732	B	U	U	U	B	B	U	B	U	U	B	A	E
	BE0004	D	U	U	U	C	B	B	B	U	U	B	A	F
	BE0006	B	U	U	U	B	B	B	B	U	U	B	A	B
	BE0022	C	U	U	U	B	B	B	C	U	U	B	A	C
Distinct genotypes		3	1	1	1	2	1	2	2	1	1	1	1	5
B. cinerea	Bc7	E	A	U	A	D	C	C	U	U	U	C	B	G
	Bc12	F	A	U	U	E	C	C	U	U	U	C	B	H
	Bc21	E	A	U	U	F	D	C	U	U	U	C	B	I
	7A	E	A	U	A	F	C	C	U	U	U	C	B	J
	8A	E	A	U	U	F	C	C	U	U	U	C	B	K
	B0510	F	A	U	U	n.d.[b]	n.d.	C	U	U	n.d.	n.d.	B	n.d.
	SAS56	E	A	U	U	F	C	C	U	U	U	C	B	K
	SAS405	E	A	U	A	F	C	C	U	U	U	C	B	J
	M14	E	B	U	A	F	D	C	U	U	U	C	B	L
	BcIPO	G	A	U	A	G	C	C	U	U	U	C	B	M
Distinct genotypes		3	2	1	2	4	2	1	1	1	1	1	1	7

[a] U, uncut; [b] n.d. = not determined

amplification products of different isolates belonging to the same species. Each of the 60 PCR amplification products was subjected to restriction digestion with six different restriction enzymes, except for *B. cinerea* strain B0510 (Table 6).

No restriction enzyme digestion variants were observed among the ten *B. tulipae* isolates and no restriction enzyme recognition sequence was present for six different digestions (Table 6). In contrast, five genotypes were detected for *B. elliptica*. Restriction digestion of IGSnr1 in combination with *Rsa*I was the most informative and distinguished three RFLP profiles. Seven genotypes were detected for *B. cinerea* and most RFLP profiles were distinguished using fragment IGSnr1 in combination with restriction enzymes *Rsa*I and *Taq*I. No polymorphisms were detected for IGSnr3 in any of the *B. cinerea* isolates (Table 6). Strains of *B. cinerea* and *B. elliptica* with identical multilocus sequence profiles did not have identical combined IGS restriction profiles, for example strain BE0006 and BE9732 shared the same sequence polymorphisms, but they did not have the identical combined IGS restriction profiles (Tables 5 and 6).

The RFLP restriction profiles were combined and used to generate an UPGMA tree for the 30 *Botrytis* strains (Figure 2). Similarity between *Botrytis* species was low, between 4% and 8%. The ten isolates of *B. cinerea* are the most variable group, with 62% similarity.

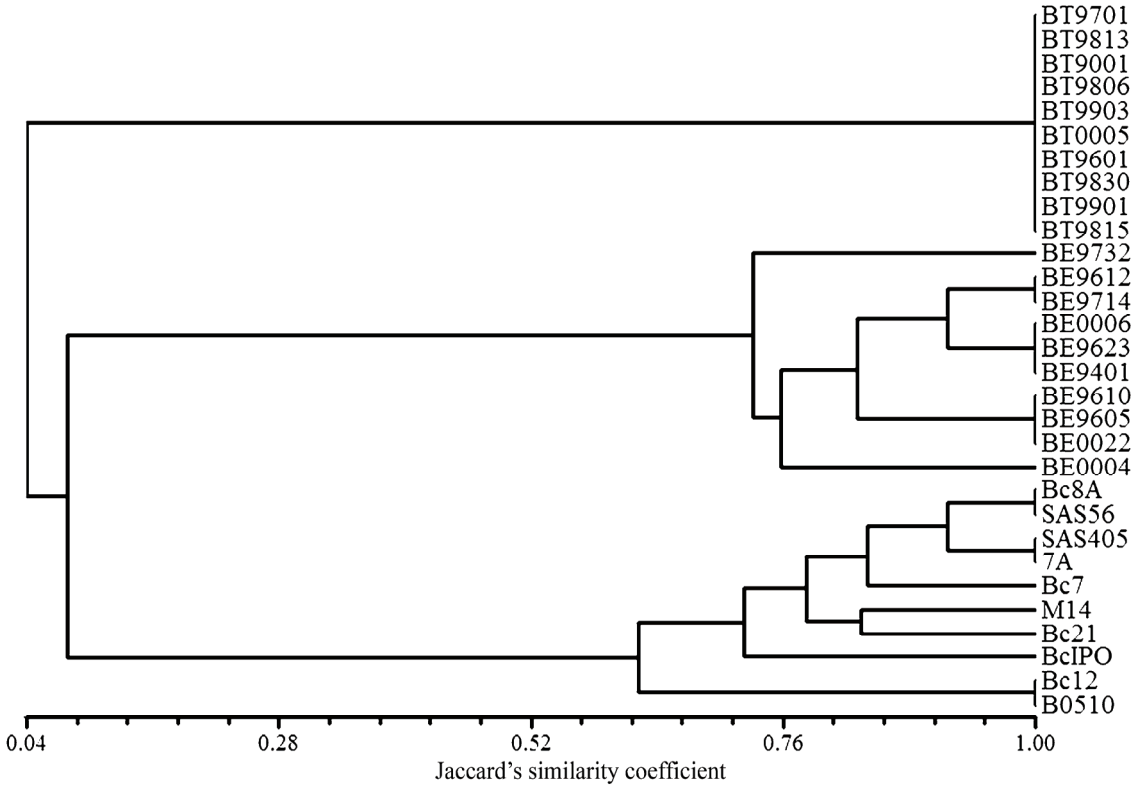

Figure 2: Jaccard's similarity relationships based on cluster analysis (UPGMA) using the combined IGS restriction data

AFLP restriction variation

The six AFLP primer combinations produced well-spaced bands in the range of approximately 100 bp to 500 bp. All bands were reproducible when fingerprints were repeated with DNA samples isolated from independent fungal cultures. The number of AFLP bands scored and the number of polymorphic bands differed depending on the primer combination and the species used (Table 7).

Table 7: Number of AFLP loci per species and per AFLP primer set

Species	*Eco*12*Mse*16	*Eco*12*Mse*17	*Eco*13*Mse*15	*Eco*13*Mse*16	*Eco*13*Mse*17	*Eco*13*Mse*18	Combined
B. tulipae	30 (4)	24 (3)	32 (8)	27 (1)	24(4)	28 (4)	165 (24)
B. elliptica	30 (5)	23 (6)	37 (7)	39 (10)	17 (6)	30 (6)	176 (40)
B. cinerea	30 (11)	16 (2)	46 (19)	42 (20)	24 (13)	46 (24)	204 (89)

The number of polymorphic loci are given in brackets.

In *B. tulipae*, the highest proportion of polymorphic loci was scored with primer set *Eco*13*Mse*15, for which 8 out of 32 loci were variable. For this species, primer set *Eco*13*Mse*16 yielded the least polymorphic pattern. Overall, approximately 14.5% (24 out of 165; Table 7) of the loci were polymorphic. In *B. elliptica*, the number of polymorphic loci found with each primer set ranged between 5 and 10. The proportion of polymorphic bands for *B. elliptica* was 22.7% (40 out of 176; Table 7). The highest proportion of polymorphic bands (44%, 89 out of 204) pooled for all six primer sets was found in *B. cinerea*, (Table 7). The number of AFLP bands shared between species ranged between ~2% and ~10%, depending on the species being compared (Table 8).

Table 8: Band sharing between species based on the combined AFLP data

Species comparisons	Total number of bands	Shared bands	% band sharing
B. tulipae v.s. *B. elliptica*	306	32	10.46
B. cinerea v.s. *B. tulipae*	333	28	8.41
B. cinerea v.s. *B. elliptica*	350	26	7.43
B. cinerea v.s. *B. tulipae* v.s. *B. elliptica*	458	9	1.97

In *B. tulipae*, eight genotypes were identified with the combined AFLP data, and the most informative primer sets was *Eco*13*Mse*15, which distinguished 5 genotypes (Table 9). All ten isolates of *B. elliptica* and ten isolates of *B. cinerea* were distinguished based on the combined AFLP data of all six primer combinations. Even with primer set *Eco*13*Mse*15 alone, all isolates of both species were discerned. All other primer sets were more informative for *B. cinerea* than for the other two species, except for primer set *Eco*12*Mse*17, which identified only three genotypes in *B. cinerea*.

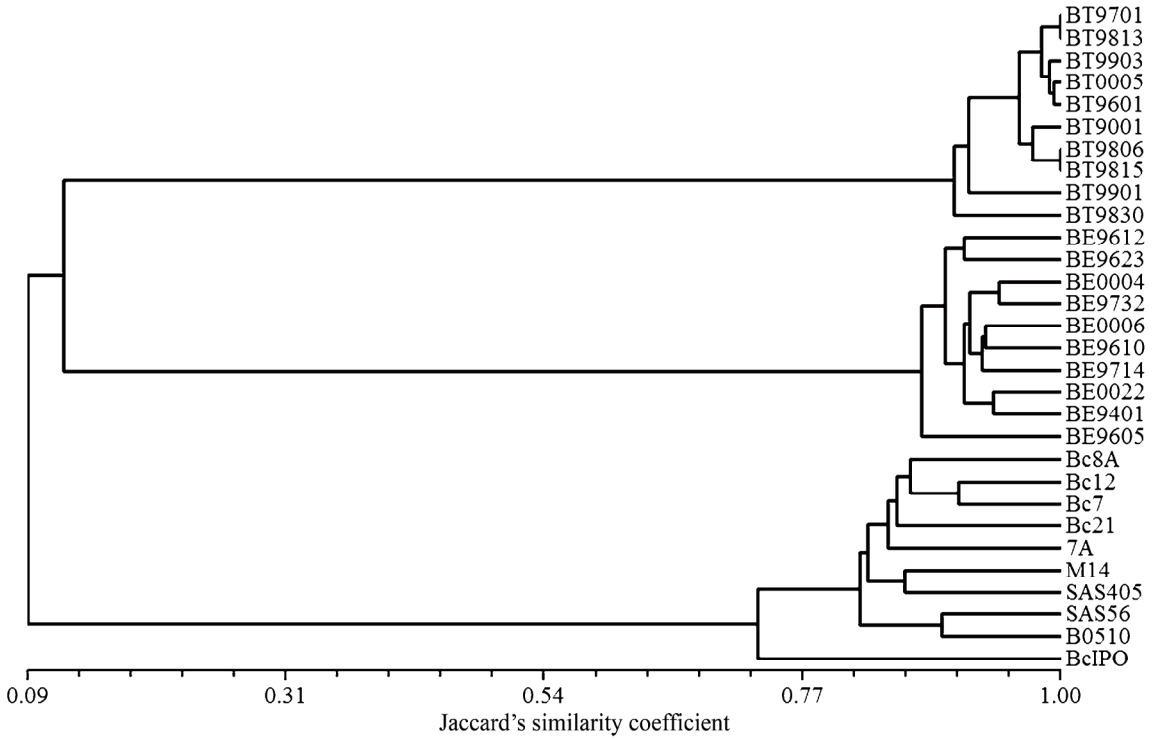

Figure 3: Jaccard's similarity relationships based on cluster analysis (UPGMA) using the combined AFLP fingerprint data

Based on the UPGMA cluster analysis similarity among species ranged between 9% and 12% (Figure 3). Within the *B. cinerea* clade, strain BcIPO was the least similar (73%) to the rest of the *B. cinerea* strains, which were 82% similar. Similarity in AFLP patterns was highest among isolates of *B. tulipae* (89%, Figure 3).

Discussion

The primary goal of this study was to compare the effectiveness of molecular markers in differentiating Dutch isolates of *B. elliptica* and *B. tulipae*, two major pathogens of flower bulb crops in the Netherlands. Ten strains of each species were selected and their genetic variation was analyzed with three different molecular marker techniques. Until now, population genetic studies in *Botrytis* have mainly focused on *B. cinerea.* Great effort has been put into DNA-based molecular approaches to differentiate strains of *B. cinerea* (e.g. Giraud et al., 1997; Fournier et al., 2002; Moyano et al., 2003). Only one study has been devoted to *B. elliptica* (Huang *et al*. 2001). We decided to include for comparison ten strains of *B. cinerea*, some of which have been studied previously by RAPD analysis (van der Vlugt-Bergmans et al., 1993; Kerssies et al., 1997). The 30 *Botrytis* isolates for this study were chosen such as to reflect a large diversity in geographic origin and year of isolation.

Table 9: AFLP profiles with six primer sets

Species	Isolate	*Eco*12*Mse*16	*Eco*12*Mse*17	*Eco*13*Mse*15	*Eco*13*Mse*16	*Eco*13*Mse*17	*Eco*13*Mse*18	Total
B. tulipae	BT9601	A	A	A	A	A	A	A
	BT9701	A	A	A	B	B	B	B
	BT9815	B	A	B	B	B	B	C
	BT9806	B	A	B	B	B	B	C
	BT9813	A	A	A	B	B	B	B
	BT9903	A	A	A	A	B	C	D
	BT9901	A	B	C	A	C	D	E
	BT9001	A	A	D	B	A	B	F
	BT9830	C	C	E	A	D	E	G
	BT0005	A	A	A	A	B	A	H
Distinct genotypes		3	3	5	2	4	5	8
B. elliptica	BE9401	D	D	F	C	E	F	I
	BE9605	E	E	G	D	F	G	J
	BE9610	F	F	H	E	G	H	K
	BE9612	G	G	I	F	H	I	L
	BE9623	H	H	J	G	I	J	M
	BE9714	I	I	K	F	G	F	N
	BE9732	J	J	L	H	J	K	O
	BE0004	K	K	M	I	K	L	P
	BE0006	J	L	N	J	L	H	Q
	BE0022	D	J	O	K	E	M	R
Distinct genotypes		8	9	10	9	8	8	10
B. cinerea	Bc7	L	M	P	L	M	N	S
	Bc12	M	M	Q	M	N	O	T
	Bc21	N	N	R	N	O	P	U
	7A	O	N	S	O	P	Q	V
	8A	M	N	T	P	Q	R	W
	B0510	P	M	U	Q	R	S	X
	SAS56	Q	M	W	R	S	T	Y
	SAS405	R	M	V	S	T	U	Z
	M14	S	O	X	T	U	V	AA
	BcIPO	T	N	Y	U	V	W	AB
Distinct genotypes		9	3	10	10	10	10	10

Three molecular marker techniques useful in population genetics were evaluated for differentiating individuals or clones of three *Botrytis* species. These included sequencing of five known gene regions, restriction variation in the IGS region of the nuclear ribosomal DNA repeat unit, and AFLP analysis, a method that provides information on variation at a large number of loci dispersed across the genome (Vos et al., 1995). This study shows that AFLP fingerprinting was superior over the two other methods in detecting genetic differences among isolates of the three *Botrytis* species analyzed. Distinct AFLP patterns were obtained for each species, and the general pattern was readily useful for species identification. All ten strains of *B. elliptica* and *B. cinerea* could be distinguished using a single AFLP primer set. In contrast, the combined AFLP fingerprint data of multiple primer sets could only distinguish 8 out of 10 *B. tulipae* strains, either resulting from biased sampling and/or primer sets, or truly reflecting a lower extent of genetic diversity in this species.

Only few disadvantages of the AFLP technique have been reported, which include problems with size homoplasy and non-independence of fragments (Majer et al., 1996; Vekemans et al., 2002). However, these problems are not exclusive for AFLP and they are negligible when AFLP-length co-dominance is rare and a large number of informative bands have been studied (Parson and Shaw 2001). The AFLP technique has been used successfully in fungi to estimate genetic diversity within species and to study life history traits (Peever et al., 2004; Zeller et al., 2004). AFLP was able to detect genetic variation where other methods failed (Kauserud et al., 2004). The use of microsatellite-derived markers would be a good alternative for high-throughput analysis of genetic variation and population differentiation in populations of *Botrytis* species because of their multiallelic nature and their ease of interpretation. We did attempt to incorporate in our studies loci that, in *B. cinerea,* contain microsatellites (Fournier et al., 2002), but the amplification of these loci from DNA of *B. tulipae* and *B. elliptica* isolates was unsuccessful using primers based on the *B. cinerea* sequence (M. Staats, Wageningen University, the Netherlands, unpubl.).

The wide availability of sequence data in databases enabled us to rapidly design primers and sequence fragments of five protein-coding genes and their introns (Glass and Donaldson, 1995; Carbone and Kohn, 1999). Currently, primer design for use in *B. cinerea* population studies will be accelerated by the availability of annotated genome sequences of two strains of *B. cinerea*. The genome of *B. cinerea* strain B0510 has been made available by the Fungal Genome Initiative (FGI) at

http://www.broad.mit.edu/annotation/genome/botrytis_cinerea. The genome of *B. cinerea* strain T4 will become available at http://urgi.infobiogen.fr/projects/.

In our study, sufficient interspecific variability was present in the five studied protein-coding gene regions to be useful in differentiating and identifying *Botrytis* species. The *RPB2* product contained the highest number of informative sites. However, the intraspecific variability within each species was low for each species. The highest number of polymorphic sites was found among isolates of *B. cinerea.* Nine *B. cinerea* isolates could be distinguished even though only ~0.4% of the all nucleotide sites were polymorphic. The low variation in genes may need to be compensated by sequencing more loci. Alternatively, PCR products can be screened for alleles using single-strand DNA conformation polymorphism analysis (SSCP) prior to sequencing (Orita et al., 1989; Carbone et al., 1999). AFLP bands can be sequenced to confirm their identity using sequence-confirmed amplified region (SCAR) analysis, although it is difficult to find the alternative allele, unless segregation analyses of family data are conducted.

Recently, Fournier et al. (2005) have proposed that *B. cinerea* is a species complex based on the concordance among four gene genealogies. Two phylogenetic species were identified, named *B. cinerea* Group I and Group II isolates, and both groups exhibited phenotypic differences. In this study, however, we did not determine whether the *B. cinerea* strains belong to either Group I or Group II. The sequence data presented in this study may aid detailed inferences about the genetic and ecological processes that have contributed to speciation events in the *B. cinerea* complex and related species using the cohesion species concept (Templeton, 1989; Templeton, 2001). The cohesion species is defined as an evolutionary lineage or set of lineages with genetic exchangeability and/or ecological interchangeability (Templeton, 1989). Species boundaries can be statistically tested using nested clade analysis (NCA) and the procedure can incorporate morphological and/or ecological data. The method has been successfully applied to study speciation processes in the genus *Sclerotinia* (Carbone and Kohn, 2001; Phillips et al., 2002), which is closely related to *Botrytis*, as well as in the more distantly related *Magnaporthe oryzae* (Couch et al., 2005).

We have used AFLP technology in a large-scale study to analyze the genotypic diversity among field isolates of *B. elliptica* and *B. tulipae* in the Netherlands and to make inferences about their reproductive modes. The results of this population study are presented in chapter 4.

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Chapter 4

AFLP analysis of genetic diversity in populations of *Botrytis elliptica* and *Botrytis tulipae* from the Netherlands

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AFLP analysis of genetic diversity in populations of *Botrytis elliptica* and *Botrytis tulipae* from the Netherlands

Abstract

***Botrytis elliptica* and *B. tulipae* are the causal agents of leaf blight in lily and tulip, respectively. The objective of this study was to asses the genetic diversity and to infer the mode of reproduction of both *Botrytis* species in the Netherlands using amplified fragment length polymorphism (AFLP) multilocus fingerprints. Isolates were sampled during successive growing seasons in experimental field plots in Lisse and other locations in the Netherlands. Among the 174 *B. elliptica* isolates, 105 genotypes could be discriminated and 87 genotypes were found only once, reflecting high genotypic variation. Clonal genotypes were found only within growing seasons and in one location. Linkage disequilibrium analyses indicated that between 9.4% and 19.3% of the loci in clone-corrected samples were linked. The multilocus association index provided no evidence for random mating. We conclude that sexual recombination occurs in the *B. elliptica* population.**

Among the 170 *B. tulipae* isolates, 25 genotypes could be discriminated and 4 genotypes were found only once, reflecting a low genotypic variation. Clonal genotypes were frequently found in different growing seasons and different locations. Linkage disequilibrium analyses indicated that between 25.2% and 48.6% of the loci in clone-corrected samples were linked. We conclude that the *B. tulipae* population is mainly clonal with some recombination.

Introduction

The fungal pathogens *Botrytis elliptica* and *B. tulipae* are necrotrophs that can cause serious economic damage in lilies (*Lilium* spp.) and tulips (*Tulipa* spp.), respectively. Epidemics begin when overwintering sclerotia in crop debris and/or soil start producing conidia that cause spreading leaf infections, commonly referred to as leaf blight or 'fire' (Lorbeer et al., 2004). *B. tulipae* is able to infect tulip bulbs and form sclerotia on them. During bulb storage the sclerotia are dormant, but they are able to germinate and infect emerging shoots early in the growing season after planting

(Price, 1970; Doornik and Bergman, 1973). In contrast, *B. elliptica* only rarely attacks bulbs and bulb infections are not important for survival in the field (van den Ende and Pennock, 1996). Under favourable weather conditions repeated cycles of sporulation cause secondary leaf infections that result in early withering of leaves and a decrease in bulb weight. Both species are specific to their host, and although single spores may cause primary lesions on other flower bulb crops, fire symptoms never develop on a non-host under natural conditions (Prins et al., 2000).

Whether offspring are produced sexually or clonally has profound effects on patterns of genetic variation of organisms (Milgroom, 1996). Genetic recombination between different genotypes causes sexual populations to be generally more diverse than clonal populations. Nevertheless, sexual populations may have low genetic diversity as a result of genetic drift (Kauserud et al., 2004). Furthermore, some fungi infrequently reproduce sexually. Therefore, even when clonal genotypes are repeatedly recovered, recombination can still be the underlying mechanism in generating genetic variation (Maynard Smith et al., 1993). Several studies have found evidence for recombination using tests for association of alleles at different loci in fungal populations that were initially thought to be clonal (Burt et al., 1996; Kohli and Kohn, 1998; Pringle et al., 2005).

No sexual stage has so far been found for *B. tulipae* and therefore this species is considered to reproduce clonally. In contrast, apothecia of *B. elliptica* containing fertile sexual ascospores have been found in experimental field plots in the Netherlands (van den Ende and Pennock-Vos, 1997b). Development of apothecia is limited to early spring when the environmental conditions are right. Experimentally produced sexual crossings showed that *B. elliptica* is heterothallic (outcrossing) with a diallelic compatibility system (Lorbeer et al., 2004). Randomly amplified polymorphic DNA (RAPD) analysis revealed high genetic variation between isolates of *B. elliptica* from the USA and Taiwan (Huang et al., 2001). Furthermore, they detected significant gametic equilibrium among genotypes from Taiwan, suggesting that sexual reproduction is important in this population.

The goal of the present study was to assess the genetic diversity in collections of *B. elliptica* and *B. tulipae* from the Netherlands. Isolates were sampled during successive growing seasons in Lisse, which is located in the main bulb growing area of the Netherlands. We evaluated the occurrence of a randomly mating population of *B. elliptica* and a clonal population of *B. tulipae* by examining the genetic structure of both species within and between growing seasons. Amplified fragment length

polymorphisms (AFLPs) were used that have shown a high level of polymorphism and reproducibility in *Botrytis* species, including *B. elliptica* and *B. tulipae* (Moyano et al., 2003; Chapter 3). The method has been successfully applied for studies on population differentiation and/or recombination in fungi as *Tetrachaetum elegans* (Laitung et al., 2004) and *Gibberella zeae* (Zeller et al., 2004).

Materials and Methods

Collection of isolates

Isolates of *B. elliptica* and *B. tulipae* were sampled to estimate the genetic diversity in experimental field plots in Lisse and elsewhere in the Netherlands. *B. elliptica* isolates have been sampled from 14 different locations and in 8 different years (Table 1). *B. tulipae* isolates have been sampled from 8 different locations and in 8 different years (Table 1).

The isolates that were collected in Lisse were isolated from tulip or lily plants grown in experimental field plots (45m X 12m) of the Applied Plant Research, Flower Bulb Research Unit (PPO-Lisse). *Botrytis* isolates were collected as primary necrotic spots from leaves of the tulip cultivar Bellona, and the Oriental lily cultivar Stargazer. Both cultivars display moderate levels of field resistance against *Botrytis* infection. No fungicide sprays were applied to control disease. *Botrytis* isolates were collected when the first disease symptoms appeared and again at the end of the growing season before bulb harvest. In the following year, *Botrytis* isolates were again sampled when the first disease symptoms appeared, however the lily and tulip field plots were not located at exactly the same site. Due to crop rotation, experimental plots were approximately 100m away from the original location. In 2003, dry weather conditions prevented disease from developing in the tulip plot and only very few *B. tulipae* isolates were collected. Therefore, *B. tulipae* infections were again sampled in 2004. Samples of *B. elliptica* and *B. tulipae* collected in Lisse were grouped by sample date.

Primary infections of *B. elliptica* on lily were sampled on 9 September 2002 (collection LISSE09-09-02) and 18 August 2003 (LISSE18-08-03). Secondary *B. elliptica* infections were collected from the same location as collection LISSE09-09-02, at the end of the growing season on 21 October 2002 (collection LISSE21-10-02). The combined *B. elliptica* collection, LISSETOTAL, comprises isolates of LISSE09-09-02, LISSE21-10-02, and LISSE18-08-03.

Table1: Sample date, number of isolates, and sample location of *B. tulipae* and *B. elliptica* isolates used in this study

Species name	Sample date	Number of isolates	Location/province
B. elliptica	1994	1	Lisse (Zuid-Holland)
	1996	1	Lisse (Zuid-Holland)
	1996	1	Bergentheim (Overijssel)
	1996	1	Anerveen (Overijssel)
	1996	1	Wieringen (Noord-Holland)
	1997	1	Elsloo (Friesland)
	1997	1	Lisse (Zuid-Holland)
	2000	1	Bant (Flevoland)
	2000	1	Rutten (Flevoland)
	2001	1	Smilde (Drenthe)
	September 9, 2002	46	Lisse (Zuid-Holland)
	October 21, 2002	50	Lisse (Zuid-Holland)
	August 18, 2003	41	Lisse (Zuid-Holland)
	September 2004	7	Dirkshorn (Noord-Holland)
	September 2004	1	Moerstraten (Noord-Brabant)
	September 2004	3	Vledder (Drente)
	October 2004	6	Lisse (Zuid-Holland)
	October 2004	3	Schagerbrug (Noord-Holland)
	October 2004	4	´t Zand (Noord-Holland)
	October 2004	3	Anna Paulowna (Noord-Holland)
B. tulipae	1996	1	Lisse (Zuid-Holland)
	1997	1	Lisse (Zuid-Holland)
	1998	1	Espel (Flevoland)
	1998	1	Lisse (Zuid-Holland)
	1998	1	(Friesland)
	1999	1	Wieringen (Noord-Holland)
	2000	2	Lisse (Zuid-Holland)
	2000	1	Tollebeet (Flevoland)
	2000	1	Ens (Flevoland)
	April 17, 2002	41	Lisse (Zuid-Holland)
	June 18, 2002	42	Lisse (Zuid-Holland)
	Augustus 2003	13	Lisse (Zuid-Holland)
	May 2004	1	St. Maartensbrug (Noord-Holland)
	May 20, 2004	31	Lisse (Zuid-Holland)
	July 2004	23	Schagerbrug (Noord-Holland)
	July 2004	9	St. Maartensbrug (Noord-Holland)

Primary infections of *B. tulipae* on tulip were collected on 17 April 2002 (collection LISSE17-04-02) and 20 May 2004 (collection LISSE20-05-04). Secondary *B. tulipae* infections were collected from the same location as collection LISSE17-04-02, at the end of the growing season on 18 June 2002 (collection LISSE18-06-02). The combined *B. tulipae* collection, LISSETOTAL, comprises isolates of LISSE17-04-02, LISSE18-06-02, and LISSE20-05-04.

Two additional collections consisted of either *B. elliptica* isolates or *B. tulipae* isolates that were sampled in different years and different locations in the

Netherlands. These isolates mainly originated from infected plant material provided by commercial bulb growers, and listed in Table 1.

All isolates were obtained from diseased leaves by surface-sterilizing in 1% hypochlorite for 1 minute, followed by a rinse in sterile distilled water. Necrotic spots of approximately 3 mm in diameter were excised, plated onto malt extract agar (Oxoid) and cultured at 18°C. Although independent lesions were assumed to result from infection by different spores, mycelium of *Botrytis* growing at the edge of a colony was transferred at least twice to fresh agar plates in order to minimize the culturing of mixed genotypes co-occurring in a single lesion. The cultures were stored as mycelium in 75% glycerol stocks at -80°C.

Table 2: Sequences of amplified fragment length polymorphism (AFLP) primers and adapters

Primer name	Primer sequence
*Mse*I adapters	5′-GACGATGAGTCCTGAG-3′
	3′-TACTCAGGACTCAT-5′
*Eco*RI adapters	5′-CTCGTAGACTGCGTACC-3′
	3′-CTGACGCATGGTTAA-5′
*Mse*00	GATGAGTCCTGAGTAA
*Eco*00	GACTGCGTACCAATTC
*Mse*15	GATGAGTCCTGAGTAACA
*Mse*17	GATGAGTCCTGAGTAACG
*Mse*18	GATGAGTCCTGAGTAACT
*Eco*12	GACTGCGTACCAATTCAC
*Eco*13	GACTGCGTACCAATTCAG

DNA sequences are orientated from 5′ to 3′ unless indicated otherwise.

DNA extraction and AFLP analysis

Mycelial tissue was harvested, lyophilized, and DNA was extracted as described in Staats et al. (2005). AFLP analysis was performed as described in chapter 3, but using three primer combinations (*Eco*12 + *Mse*17, *Eco*13 + *Mse*15, *Eco*13 + *Mse*18) for the fingerprinting of all samples (Table 2). These primer combinations were selected because they produced fingerprints with a sufficient number of well-separated polymorphic and non-polymorphic bands in the range between 100 and 500 bp. DNA samples of 80 isolates per species were isolated twice from independent fungal cultures and fingerprinted twice to test the reproducibility of AFLP band patterns. PCR reactions were performed and products analyzed on a LI-COR 4200 DNA sequencer (Westburg) as described in chapter 3. The AFLP amplification products were designated according to the primer combination used and their sizes estimated with reference to the SequaMark™ 10 base ladder (Research Genetics, USA).

Data analysis

AFLP bands were scored manually as binary characters and bands at the same size were treated as putative unique AFLP loci. A binary matrix was constructed containing all reproducible bands and all isolates. In subsequent analyses, data was treated as haplotypic data and bands were considered as loci, with absence or presence as putative alleles. AFLP binary data was analyzed in a Bayesian analysis using a simple F81-like model implemented in MrBayes version 3.1 (Ronquist and Huelsenbeck, 2003). The analysis was performed for distinct multilocus AFLP genotypes only, with the following settings: Nchains=4, Ngen=10,000,000, Samplefreq=200, Temp=0.15, statefreqpr=fixed(equal) and coding=noabsencesites (Felsenstein, 1992). Convergence among the two independent runs was examined using Tracer version 1.3 (Rambaut and Drummond, 2005). Bayesian 50% majority-rule consensus trees with Bayesian posterior probability (PP) values were generated after discarding 25% of the samples as 'burn-in'.

Genetic similarities were calculated with Jaccard's similarity coefficient by NTSYS-pc version 2.02j (Applied Biostatistics Inc.). This coefficient only considers the presence of bands; absence of bands is not interpreted as a similar character between isolates. The similarity matrix was used to construct a dendrogram by the UPGMA cluster method. Bootstrap values were calculated for 1000 replicates with SplitsTree version 4 (Huson and Bryant, 2006). Branches with at least 70% bootstrap support and at least 95% Bayesian PP were considered as well supported.

The indices of genotypic diversity were calculated using Nei's (1987) diversity index corrected for sample size, and the Shannon-Wiener index corrected for sample size (Chao and Shen, 2003) with GENODIVE (Meirmans and Van Tienderen, 2004). TFPGA version 1.3 (Miller, 1997) was used to calculate the pair-wise measures of Rogers' modified genetic distance (Wright, 1978), and Nei's unbiased measure of genetic identity between populations (Nei, 1978). TFPGA was also used to quantify population subdivision using hierarchical *F*-statistics by calculating Weir and Cockerham's (1984) theta (θ), the equivalent of Wright's F_{st}. We interpreted the resultant θ(F_{st}) values based on Wright's (1978) qualitative guidelines of θ(F_{st}) values (θ(F_{st}) = 0-0.05 indicates no or little population differentiation, 0.05-0.15 indicates moderate differentiation, 0.15-0.25 indicates great differentiation, and >0.25 indicates very great differentiation). The 95% confidence level of θ(F_{st}) was generated using 10000 bootstrap replicates. Confidence limits around θ that did not overlap with 0 were taken as evidence of significant genetic differentiation of populations.

The multi-loci statistic of Fisher's combined probability test of genetic differentiation was estimated using Genepop DOS version 3.4 (Raymond and Rousset, 1995a; 1995b). The following settings were used: dememorization number = 1000, number of batches = 1000, number of iterations = 10000. The null hypothesis for genetic differentiation was H_0: 'the allele distribution of AFLP loci is identical across field populations/collections'.

Two-locus gametic disequilibrium was calculated using an exact test for haplotypic data (Slatkin, 1994) with ARLEQUIN version 3.0b (Excoffier et al., 2005). Since the occurrence of clonal genotypes can be confounding, inferences about recombination were also made using unique genotypes only (McDonald et al., 1994). A second test for gametic disequilibrium uses the standardised index of association ($\bar{r}_d$), which estimates overall association among fragments, and was calculated with Multilocus 1.3b (Agapow and Burt, 2001). The $\bar{r}_d$ summary statistic largely removes the dependency on the number of loci in comparison to the index of association (I_A) developed by Maynard Smith et al. (1993) and therefore $\bar{r}_d$ facilitates comparisons between studies with different numbers of loci. Moreover, $\bar{r}_d$ can be used as a relative measure of panmixis. Values of $\bar{r}_d$ differing significantly from zero indicate a deviation from gametic equilibrium. Significance testing of $\bar{r}_d$ was based on comparison of the observed value to 1000 randomized data sets to test the hypothesis of complete panmixia (Burt et al., 1996).

Results

Sampling and identification of isolates from *Botrytis* species

In early spring of 2002 and 2003, apothecia of *B. elliptica* were not detected in the field. Of every isolate collected from infected plant material the *Botrytis* species was determined based on mycelial growth, spore shape, and sclerotia size. Occasionally, isolates with the typical morphological characteristics of *B. tulipae* or *B. cinerea* were found growing out of primary necrotic lesions isolated from lily leaves. These observations are possibly due to the overlap in growing season of tulips (February-June) with lilies (May-October), and the ubiquitous presence of *B. cinerea* throughout the year. However, isolates of *B. tulipae* were never found causing spreading lesions on lily leaves, and isolates of *B. tulipae* were never isolated from infected lily leaves at the end of the growing season. On tulip leaves, isolates of *B. cinerea*, but never *B.*

elliptica, were found growing out of lesions. AFLP fingerprints were determined for 174 *B. elliptica* isolates and 170 *B. tulipae* isolates.

AFLP fingerprinting and genetic variation

For studying variation in *B. elliptica* and *B. tulipae* collections, the three AFLP primer combinations were chosen that produced up to 60 well-spaced bands per primer per species. Reproducible bands were scored only in the size range of approximately 100 bp to 500 bp. Fingerprints of amplifications on DNA isolation replicates (from independently cultured isolates) for 80 samples per species were identical. AFLP fingerprints of both species generated by the same primer combinations were clearly distinct and the number of AFLP bands scored of the same size between *B. elliptica* and *B. tulipae* was low, approximately 8% (14 of 171 loci). Separate analyses were performed on the collections of *B. elliptica* and *B. tulipae*.

Among the 174 *B. elliptica* isolates, 98 AFLP loci were scored of which 31% were polymorphic (30 out of 98; Table 3). A total of 105 multilocus AFLP genotypes could be discriminated and 87 multilocus AFLP genotypes were found only once, reflecting an overall high genotypic variation. With the exception of genotypes with identical fingerprints, most genotypes differed from each other by several fragments. Pairwise comparisons between the 105 unique AFLP fingerprints (Figure 1) showed a normal distribution, with only 21 out of 5460 pairs differing by one or two fragments. Only 2% of the comparisons were different by four or fewer fragments, which allows for the unequivocal determination of unique genotypes in most cases (Figure 1). Genotypic diversity measurements were lowest for LISSE18-08-03 and highest for THE NETHERLANDS and ranged between 73% and 99% for the field populations (Table 3). The number of clonal genotypes was limited (Figure 2A), although two genotypes were present at high frequencies, i.e. genotype h10 had a frequency of 30% (14 out of 46; Figure 2A) in collection LISSE09-09-02 and genotype h59 had a frequency of 51% (21 out of 41) in collection LISSE18-08-03, indicating the importance of clonal reproduction shortly after primary infections.

Among the 170 *B. tulipae* isolates, 88 AFLP loci were scored, which is less than for the TOTAL *B. elliptica* collection (Table 3). The percentage of polymorphic loci was lower for *B. tulipae* (24%; 21 out of 88) as compared to *B. elliptica* (31%). Approximately 26% of the 325 pairwise comparisons between the 25 unique AFLP fingerprints differed by four or fewer fragments (Figure 1). The number of distinct genotypes, the number of genotypes found once and the

Table 3: Comparison of AFLP genotype diversity in collections of *B. elliptica* and *B. tulipae* isolates

Species	Collection name	n	Loci	Polymorphic loci	Distinct genotypes	Genotypes found once	Genotypic diversity[a]	Shannon-Wiener index[b]	Shared genotypes[c]
B. elliptica	TOTAL	174	98	30	105	87	0.97	2.12	-
	LISSETOTAL	137	98	30	73	57	0.95	1.89	-
	LISSE09-09-02	46	98	29	26	19	0.90	1.52	4 (LISSE21-10-02)
	LISSE21-10-02	50	98	29	36	30	0.98	1.86	4 (LISSE09-09-02)
	LISSE18-08-03	41	98	29	15	10	0.73	1.05	0
	THE NETHERLANDS	37	98	29	32	30	0.99	2.16	0
B. tulipae	TOTAL	170	88	21	25	4	0.89	1.19	-
	LISSETOTAL	114	88	21	18	4	0.89	1.11	-
	LISSE17-04-02	41	88	21	11	3	0.88	0.99	8 (LISSE18-06-02) 4 (LISSE20-05-04) 9 (THE NETHERLANDS)
	LISSE18-06-02	42	88	21	11	2	0.86	0.98	8 (LISSE17-04-02) 5 (LISSE18-06-02) 9 (THE NETHERLANDS)
	LISSE20-05-04	31	88	21	9	5	0.79	0.87	4 (LISSE17-04-02) 5 (LISSE18-06-02) 7 (THE NETHERLANDS)
	THE NETHERLANDS	56	88	21	20	10	0.88	1.23	9 (LISSE17-04-02) 9 (LISSE18-06-02) 7 (LISSE20-05-04)

[a] Nei's (1987) diversity index corrected for sample size.
[b] Shannon-Wiener index corrected for sample size (Chao and Shen, 2003).
[c] The collection with which genotypes are shared is given within parentheses.

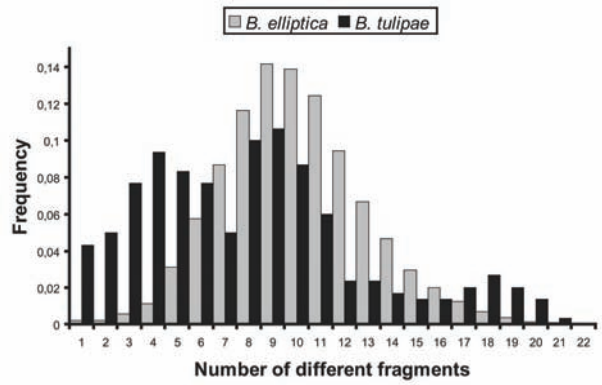

Figure 1: Observed frequency distribution of the AFLP fragments (presence or absence) in pairwise comparison of unique fingerprints of *B. elliptica* and *B. tulipae*. For *B. elliptica*, pairs of fingerprints differ from one another in 10.0 of the 98 fragments on average. For *B. tulipae*, the average was 8.0 out of 88 fragments.

measures of genotypic diversity were lower for the *B. tulipae* samples than for the *B. elliptica* samples. Levels of genotypic diversity were comparable between the four distinguished *B. tulipae* collections and ranged between 79% and 88% for the field populations. Clonal genotypes were sampled frequently in each of the *B. tulipae* collections (Figure 2B). For instance, genotype h6 made up 26.5% (45 of 170) of all *B. tulipae* isolates and was found in all four collections (not shown).

Population structure

UPGMA dendrograms with bootstrap support values and Bayesian PP were constructed to investigate clustering among the isolates (Figures 3 and 4). The dendrogram constructed with all 105 unique *B. elliptica* genotypes contained only one well supported node with 97.6% bootstrap support and 99% Bayesian PP (Figure 3) and this node supported a small clade of two genotypes. The Jaccard similarity coefficient values for the 105 genotypes ranged from 0.84 to 0.99. The cluster analysis did not group genotypes based on date of sampling (not shown).

The dendrogram constructed with all 25 distinct *B. tulipae* genotypes contained two well supported nodes (Figure 4), both supporting two clades each containing two genotypes. The similarity values ranged between 0.81 and 0.99, however the similarity values were at least 0.88 when excluding genotypes h1 and h5. There was no grouping of *B. tulipae* genotypes based on date of sampling (not shown).

When the *B. elliptica* collections were compared, the analysis of genetic differentiation revealed that most collections were significantly different, as the

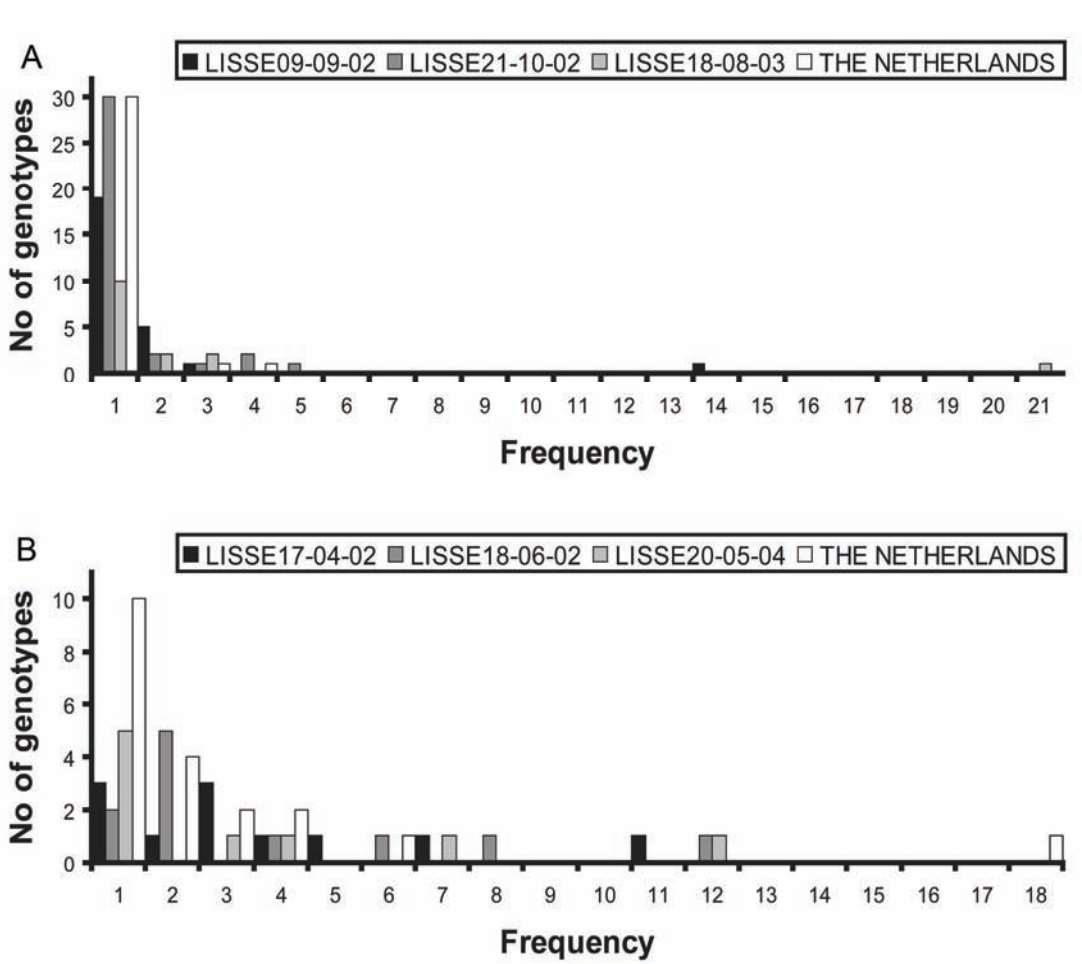

Figure 2: The number of genotypes and their frequencies for (**A**) *B. elliptica* collections and (**B**) *B. tulipae* collections

probabilities for pair-wise comparisons significantly rejected the null hypothesis stating an absence of population differentiation (P≤0.017, Table 4A). The estimates of θ(F_{st}) for comparisons between LISSE18-08-03 and all other collections were between 0.11 and 0.22, indicating that LISSE18-08-03 was moderately to greatly differentiated from all other collections.

The estimate of θ(F_{st}) for comparison between LISSETOTAL and THE NETHERLANDS was 0.04, indicating that both collections are weakly differentiated. Pair-wise comparisons of Nei's genetic identity measure showed only limited differences between the *B. elliptica* collections (Table 5A). Based on Rogers' modified genetic distance, LISSE09-09-02 and LISSE21-10-02 have the highest relatedness of all pair-wise comparisons and they were the only two collections in which identical genotypes were found (Table 3 and 5A).

Table 4: Two tests for population differentiation among (A) *B. elliptica* collections and (B) *B. tulipae* collections

Panel A	LISSE09-09-02	LISSE21-10-02	LISSE18-08-03	LISSETOTAL	THE NETHERLANDS
LISSE09-09-02	****	0.834	**<0.001**	0.107	0.056
LISSE21-10-02	0.00 (0.01, -0.01)	****	**<0.001**	**0.017**	**<0.001**
LISSE18-08-03	0.22 (0.29, 0.14)	0.22 (0.29, 0.14)	****	**<0.001**	**<0.001**
LISSETOTAL	0.01 (0.03, 0.00)	0.02 (0.03, 0.01)	0.11 (0.14, 0.07)	****	**<0.001**
THE NETHERLANDS	0.03 (0.05, 0.01)	0.05 (0.08, 0.02)	0.20 (0.29, 0.12)	0.04 (0.07, 0.01)	****
Panel B	LISSE17-04-02	LISSE18-06-02	LISSE20-05-04	LISSETOTAL	THE NETHERLANDS
LISSE17-04-02	****	0.474	**<0.001**	0.808	**<0.001**
LISSE18-06-02	0.00 (0.02, -0.01)	****	**<0.001**	0.674	0.597
LISSE20-05-04	0.10 (0.15, 0.05)	0.13 (0.18, 0.08)	****	**<0.001**	**<0.001**
LISSETOTAL	0.00 (0.00, -0.01)	0.00 (0.01, 0.00)	0.05 (0.05, 0.02)	****	**<0.001**
THE NETHERLANDS	0.03 (0.06, 0.01)	0.00 (0.01, -0.01)	0.19 (0.27, 0.10)	0.03 (0.05, 0.02)	****

Below the diagonal, genetic differentiation among collections as estimated by hierarchical *F*-statistics $\theta(F_{st})$. The 95% credibility interval (within parentheses) was derived by bootstrapping. Above the diagonal, probabilities for each pair-wise comparison using a Fisher's combined probability test for population differentiation. Numbers in bold indicate collections that are significantly different ($P<0.05$).

Table 5: Pair-wise comparisons of genetic distance and genetic identity among (A) *B. elliptica* collections and (B) *B. tulipae* collections

Panel A	LISSE09-09-02	LISSE21-10-02	LISSE18-08-03	LISSETOTAL	THE NETHERLANDS
LISSE09-09-02	****	0.0471	0.1628	0.0528	0.0684
LISSE21-10-02	0.9986	****	0.1662	0.0553	0.0835
LISSE18-08-03	0.9721	0.9708	****	0.1141	0.1584
LISSETOTAL	0.9977	0.9973	0.9865	****	0.0753
THE NETHERLANDS	0.9961	0.9935	0.9737	0.9946	****
Panel B	LISSE17-04-02	LISSE18-06-02	LISSE20-05-04	LISSETOTAL	THE NETHERLANDS
LISSE17-04-02	****	0.0430	0.1036	0.0334	0.0573
LISSE18-06-02	0.9990	****	0.1091	0.0376	0.0344
LISSE20-05-04	0.9896	0.9883	****	0.0759	0.1235
LISSETOTAL	0.9996	0.9991	0.9946	****	0.0572
THE NETHERLANDS	0.9974	0.9994	0.9846	0.9970	****

Below the diagonal, Nei's unbiased measure of genetic identity (Nei 1978) and above the diagonal, Rogers' modified genetic distance (Wright 1978).

The estimates of θ(F_{st}) were low for each pair-wise comparison among *B. tulipae* collections, except for LISSE20-05-04 compared to LISSE17-04-02, LISSE21-10-02, and THE NETHERLANDS. Estimates of θ(F_{st}) for these combinations were between 0.10 and 0.19, indicating that these collections are moderately to greatly differentiated (Table 4B). The collections LISSETOTAL and THE NETHERLANDS were only weakly differentiated (θ(F_{st})=0.03, Table 4B). Four to nine *B. tulipae* genotypes were shared between collections (Table 3). A total of thirteen genotypes were not only sampled in Lisse in the year 2002/2003, but were also found in seven other locations in the Netherlands, some of which are located 100 km apart. Furthermore, clonal genotypes have been recovered repeatedly over 7 years of sampling. Limited differences were observed for Nei's genetic identity index between the *B. tulipae* collections (Table 5B). Differences in genetic distance between the collections were also limited, although LISSE20-05-04 had the highest genetic distance to all other collections (Table 5B).

Gametic disequilibrium

To estimate whether random mating has occurred among isolates in the collections of *B. elliptica* and *B. tulipae*, two tests for associations between pairs of loci were used. In a clonal species, loci would be expected to be inherited together without recombination. As a result, different AFLP loci are observed together more often than expected under random mating, which is referred to as gametic disequilibrium.

In the *B. elliptica* collections, the percentage of pairs of loci with significant association ranged from 16.7% to 50.2% for comparisons that included all isolates (Table 6). When analyzing only distinct genotypes, the exact test showed a 2-4 fold decrease in the number of significantly linked loci, indicating the importance of censoring clonemates. The standardized index of association ($\bar{r}_d$), a measure of multilocus gametic disequilibrium, differed significantly from 0 for all collections (Table 6), which allowed the null hypothesis of gametic equilibrium to be rejected. The $\bar{r}_d$ for LISSE21-10-02 with clone-correction was close to zero ($\bar{r}_d$ = 0.01; Table 6) with a significance value of 0.016, indicating only a weak departure from gametic equilibrium.

For *B. tulipae*, the exact test showed significant gametic disequilibrium for 93.3% of loci pairs for the TOTAL collection (Table 7). When excluding clonemates, the percentage of linked loci was approximately two times lower. For the seasonal collections, the proportion of significantly linked loci ranged from 25% to 49%, which

is 2.5 to 5 times higher than for the *B. elliptica* collections. Based on the $\bar{r}_d$ coefficient, there was no evidence for significant gametic equilibrium, i.e. observed values of $\bar{r}_d$ in the *B. tulipae* collections deviate from zero, which would be the value if each AFLP locus would have recombined at random (Table 7). In comparison to *B. elliptica*, values of $\bar{r}_d$ for the collections of *B. tulipae* isolates were between 2.7 and 44 times higher, when excluding clonemates.

Discussion

In this study, we used AFLP markers to examine the genetic structures of field populations of *B. elliptica* and *B. tulipae* sampled in field plots in Lisse and elsewhere in the Netherlands. AFLP is a powerful method for studying genetic variation in these species (Chapter 3). The application of AFLP fingerprinting was sensitive enough for obtaining distinguishable banding patterns of 105 unique *B. elliptica* genotypes and 25 unique *B. tulipae* genotypes. The total number of bands and the proportion of polymorphic fragments generated by the AFLP method was slightly lower for *B. tulipae* (24%) compared to *B. elliptica* (31%). The band sharing between both *Botrytis* species with the same primer combination was low, and patterns of both species were easily distinguishable. The AFLP method thus provides a powerful tool for diagnostics to study both species, besides DNA sequence data (Staats et al., 2005).

This study revealed a genetic diversity of up to 99% for the *B. elliptica* field population, in agreement with a previous study of *B. elliptica* isolates collected from several regions in the USA and Taiwan, using RAPD markers (Huang et al., 2001). Interestingly, levels of genotypic diversity of *B. elliptica* in our study are as high as reported for *B. cinerea*, a species that infects multiple host plants and reproduces sexually in the field (Faretra et al., 1988; Giraud et al., 1997; Alfonso et al., 2000). It should be noted that it is difficult to compare levels of genetic diversity obtained with different markers and different sample sizes. Only unbiased estimates of genotypic diversity can be used for direct comparisons between studies (Grünwald et al., 2003).

Based on the genotypic diversity, our results suggest that *B. elliptica* in Lisse may have an epidemic population structure, which is characterized by the occurrence of a few successful clonal genotypes in an overall recombining population (Maynard Smith et al., 1993). A small number of clonal genotypes were indeed found

Table 6: Linkage disequilibrium in different collection of *B. elliptica* isolates

	Two-locus gametic disequilibrium			multilocus gametic disequilibrium ($\bar{r}_d$)	
Collection name	No. of pairwise comparisons[a]	Total sample[b]	Clone-Corrected sample[b]	Total sample[c]	Clone-Corrected sample[c]
TOTAL	465	43.0	19.3	0.04	0.02
LISSE09-09-02	435	29.6	9.9	0.07	0.02
LISSE21-10-02	435	18.2	10.8	0.02	0.01
LISSE18-08-03	435	50.2	13.1	0.20	0.06
LISSETOTAL	465	42.1	17.0	0.05	0.02
THE NETHERLANDS	435	16.7	9.4	0.02	0.02

[a] The number of pairwise comparisons of polymorphic pairs of loci is the same for the total sample and the clone-corrected sample.
[b] Exact test of linkage disequilibrium between all pairs of polymorphic loci based on 100.000 Markov chain steps (Slatkin 1994). Given are the percentages of pairwise comparisons that were in significant associations at P=0.05.
[c] The test of significance for the standardized index of association (Agapow and Burt 2001) was calculated with 1000 replicates and $P \geq 0.05$. All values differ significantly from 0, indicating a departure from linkage equilibrium for all collections.

Table 7: Linkage disequilibrium in different collections of *B. tulipae* isolates

	Two-locus gametic disequilibrium			multilocus gametic disequilibrium ($\bar{r}_d$)	
Collection name	No. of pairwise comparisons[a]	Total sample[b]	Clone-Corrected sample[b]	Total sample[c]	Clone-Corrected sample[c]
TOTAL	231	93.3	42.9	0.36	0.19
LISSE17-04-02	231	74.3	25.2	0.47	0.35
LISSE18-06-02	231	85.2	48.6	0.50	0.44
LISSE20-05-04	231	38.1	25.2	0.31	0.19
LISSETOTAL	231	87.1	37.1	0.40	0.26
THE NETHERLANDS	231	61.4	34.8	0.25	0.16

For notes a and b see table 6.
[c] All values differ significantly from 0, indicating a departure from linkage equilibrium for all collections.

in high frequency during the infection season. However, genotypes sampled in Lisse in 2002 were not found again among isolates collected in 2003 or in the collection of isolates from different years and locations in the Netherlands, suggesting that *B. elliptica* genotypes found in one year are replaced by new genotypes the next. We did not detect apothecia in early spring in Lisse, but *B. elliptica* does have the potential to produce viable sexual offspring in the field (van den Ende and Pennock-Vos, 1997b; Lorbeer et al., 2004). Significant linkage disequilibrium between loci was detected in 19.3% of the pairs of loci for the entire collection (Table 6), which is consistent with some extent of genetic exchange and recombination. Even when the collection was subdivided by date the proportions of linked loci were higher than expected under panmixis. Indeed, multilocus association was observed in each of the clone-corrected collections, thus providing no evidence for random mating of *B. elliptica* in the natural populations sampled in this study.

The *B. elliptica* population from Lisse is differentiated from a collection of Dutch field isolates. Additional studies are needed to assess the level of gene flow and genotype flow between Lisse and other lily growing regions in the Netherlands. Huang et al. (2001) detected one clonal genotype in the USA isolated from fields, as far as 450 km apart, and they proposed this to result from transport of infected bulb material. Airborne macroconidia of *B. cinerea* are usually deposited close to the inoculum source and they are short-lived (Holz et al., 2004). Macroconidia of *B. elliptica* are substantially larger than those of *B. cinerea* and therefore, long-distance migration of *B. elliptica* via dispersal of airborne macroconidia is probably limited. It is unknown whether ascospores of *B. elliptica* are able to spread over long distances and how long they remain viable.

In conclusion, our study supports the hypothesis that recombination occurs in *B. elliptica* populations in the Netherlands, as anticipated from the discovery of apothecia in the field (van den Ende and Pennock-Vos, 1997b). Unlike Huang et al. (2001), we found no evidence for occurrence of random mating. Multilocus linkage disequilibrium can be caused by epistatic selection, gene flow, drift and physical linkage of AFLP loci (Tibayrenc, 1995; Milgroom, 1996). Huang et al. (2001) suggested the possible occurrence of cultivar specialization among *B. elliptica* isolates in Taiwan. If this is the case for the Dutch *B. elliptica* population, it may have resulted in non-random associations that may have masked the detection of random mating.

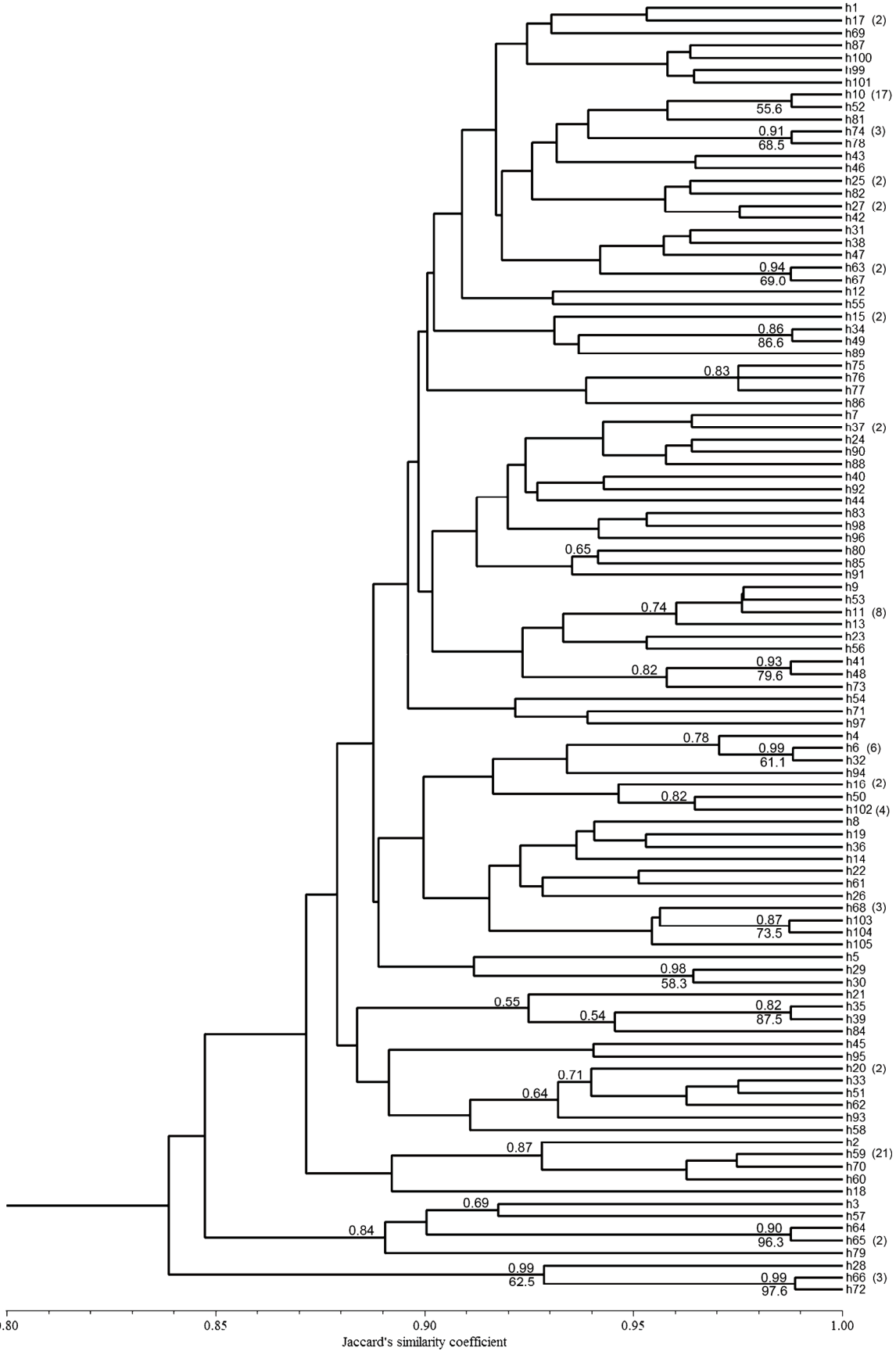

Figure 3: Jaccard's similarity relationships based on cluster analysis (UPGMA) using the AFLP fingerprinting data of 105 *B. elliptica* genotypes (numbered h1-h105). Genotype frequencies higher than one found among the 174 isolates are shown in brackets besides the genotype name. Bayesian PP values above 0.50 are shown above each branch. Bootstrap values higher than 50% are shown below each branch.

The overall genetic diversity in *B. tulipae* was lower than for *B. elliptica*. The repeated recovery of identical *B. tulipae* genotypes among isolates sampled in different years and in different geographic regions in the Netherlands suggests clonality. *B. tulipae* apothecia have never been found over many years of study in the Netherlands (van den Ende, personal communication).

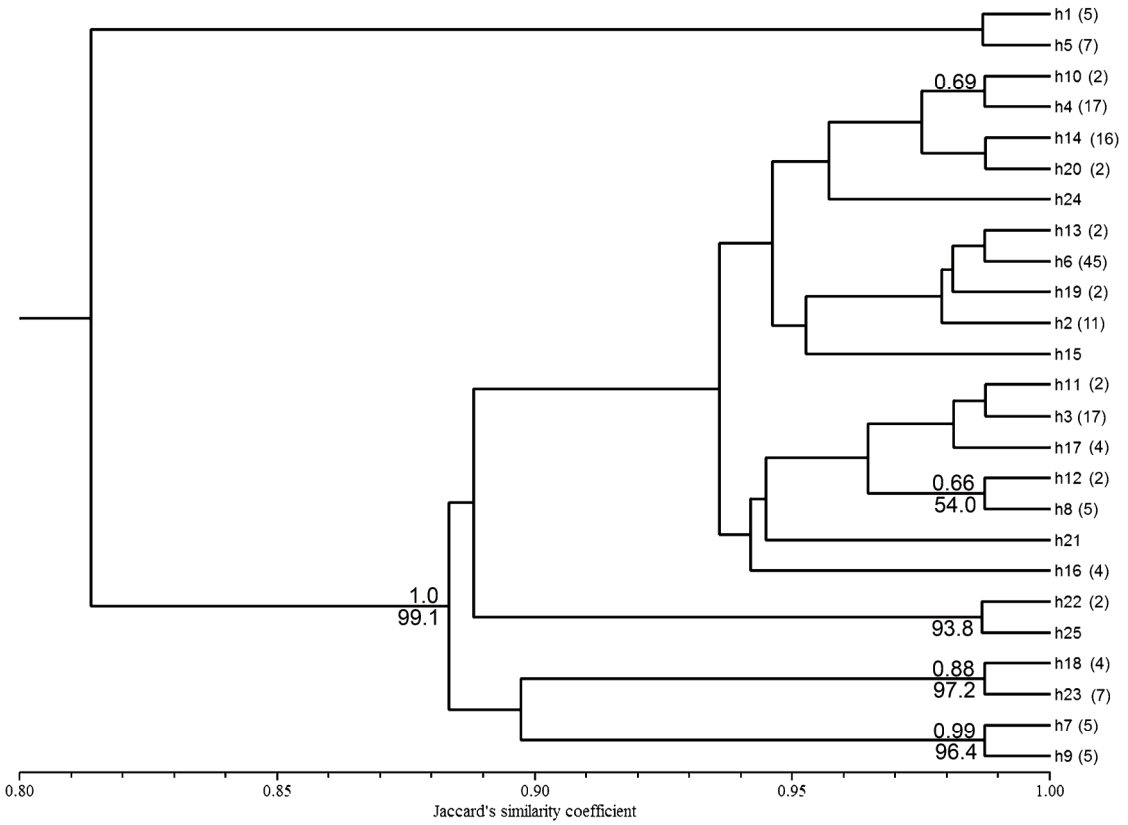

Figure 4: Jaccard's similarity relationships based on cluster analysis (UPGMA) using the AFLP fingerprinting data of 25 *B. tulipae* genotypes (numbered h1-h25). Genotype frequencies higher than one found among the 170 isolates are shown in brackets besides the genotype name. Bayesian PP values above 0.50 are shown above each branch. Bootstrap values higher than 50% are shown below each branch.

In addition, we have not observed apothecia or apothecial primordia in crossing experiments with multiple *B. tulipae* isolates (unpublished data). The sclerotia of *B. tulipae* are very small (1-2 mm) and they may not contain enough nutrients to support apothecial development. The sclerotia of *B. tulipae* appear to function exclusively as survival structures and are an important source of asexual propagules (Doornik and Bergman, 1973).

Clonal genotypes of *B. tulipae* may have been transported via infected tulip bulbs to different regions. Epidemics of *B. tulipae* are able to start from over-wintering sclerotia in crop debris or soil, or by latent infections in bulbs. In view of

the long crop rotation schemes and the observation that sclerotia cannot survive in soil for more than two growing seasons, the most probable primary sources of inoculum are the latent infections in bulbs (Price, 1970; Coley-Smith and Javed, 1972).

Additional evidence for clonality of *B. tulipae* was provided by strong association across loci in multilocus tests. Furthermore, the distribution of differences observed among pairs of fingerprints (Figure 1) is at least partially consistent with diversity being generated by mutations within clonal lineages, because a large fraction of DNA fingerprints differ by only few fragments (Milgroom et al., 1992). Besides mutation, activity of transposable elements may have contributed in generating diversity in clones (Daboussi and Capy, 2003). However, the results of tests for pairwise comparisons among loci were ambiguous. There was strong evidence that linkage disequilibrium is caused by linkage between the loci in each of the clone-corrected field populations, however the percentages of linked loci are lower than expected for a strictly clonal organism. Our results do not exclude some level of recombination that would result in generating new genotypes. In a natural population of *Cryphonectria parasitica*, parasexual recombination has been reported to occur even between vegetatively incompatible individuals (McGuire et al., 2005). The presence of heterokaryons in natural populations of *S. sclerotiorum* (Kohli and Kohn, 1998) and *B. cinerea* (Faretra et al., 1988; Pollastro et al., 1996) provides a possibility for mitotic recombination. Unfortunately, knowledge about heterokaryosis and mycelial (in)compatibility in *B. tulipae* is lacking.

In conclusion, *B. tulipae* appears to have a mainly clonal population structure, as evidenced by the low genotypic diversity, repeated recovery of clonal genotypes over long distances and in different years, and strong multilocus association. Whether recombination truly occurs in *B. tulipae* might be studied by comparison of multiple gene genealogies (Carbone and Kohn, 2004) and additional markers.

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Chapter 5

Positive selection in phytotoxic protein-encoding genes of *Botrytis* species

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Abbreviations: BEB: Bayes Empirical Bayes; HK: housekeeping; NEP: necrosis and ethylene-inducing protein; NLP: NEP1-like protein; PP: posterior probability

Key words: adaptive evolution, diversifying selection, host-selective toxin, necrotrophic fungus, NEP1-like protein, NLP

Positive selection in phytotoxic protein-encoding genes of *Botrytis* species

Abstract

Evolutionary patterns of sequence divergence were analyzed in genes from the fungal genus *Botrytis* (Ascomycota), encoding phytotoxic proteins homologous to a Necrosis and Ethylene-inducing Protein from *Fusarium oxysporum*. Fragments of two paralogous genes (designated *NEP1* and *NEP2*) were amplified from all known *Botrytis* species and sequenced. *NEP1* sequences of two *Botrytis* species contain premature stop codons, indicating that they may be non-functional. Both paralogs of all species encode proteins with a remarkably similar predicted secondary structure, however, they contain different types of post-translational modification motifs, which are conserved across the genus. While both *NEP* genes are, overall, under purifying selection, we identified a number of amino acids under positive selection based on inference using maximum likelihood models. Positively selected amino acids in NEP1 were not under selection in corresponding positions in NEP2. The biological significance of positively selected residues and the role of NEP proteins in pathogenesis remain to be resolved.

Introduction

A Necrosis and Ethylene-inducing Protein (NEP1) produced by the fungal plant pathogen *Fusarium oxysporum* was first described by Bailey (1995). NEP1-like proteins (NLPs) constitute a family of secreted, phytotoxic proteins (reviewed by Pemberton and Salmond, 2004; Gijzen and Nürnberger, 2006) that elicit cell death and defense responses in a large number of dicotyledonous plants (Bailey, 1995; Koch et al., 1998; Fellbrich et al., 2002; Qutob et al., 2002). NLP genes are found in phylogenetically very diverse microorganisms including oomycetes (Koch et al., 1998; Qutob et al., 2002), true fungi (Bailey 1995; Galagan et al., 2003), gram-positive bacteria (Redenbach et al., 1996; Takami et al., 1999) and gram-negative bacteria (Jores et al., 2003; Mattinen et al., 2004; Pemberton et al., 2005). So far, no NLP homologs are found in the animal, plant or Archaea kingdoms. It has been argued that a common feature shared by microorganisms possessing NLPs, is their

association with plants and a necrotrophic lifestyle in a part of their life cycle (Mattinen et al., 2004). Indeed, NLPs act as virulence factors for the soft-rotting bacteria *Erwinia carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica* (Mattinen et al., 2004; Pemberton et al., 2005) and transformation with NEP1 from *Fusarium oxysporum* of the fungus *Colletotrichum coccodes* increased the virulence and broadened the host range of this plant pathogen (Amsellem et al., 2002). Furthermore, expression of a NLP in *Phytophthora sojae*, NPP1, was associated with the necrotrophic phase of the infection (Qutob et al., 2002). A gene family encoding a different type of phytotoxic proteins, unrelated in sequence to NLPs, was recently shown to undergo diversifying selection in the potato late blight pathogen *Phytophthora infestans* (Liu et al., 2005).

Positive, diversifying selection is an important evolutionary force that accelerates divergence between homologous proteins (Swanson et al., 2001). Among the proteins identified to be under positive selection are immune-response and defense-related genes (Bishop, 2005; Nielsen et al., 2005; Wang et al., 2003), and toxin protein genes (Duda and Palumbi, 2000; Liu et al., 2005). Positive selection can be identified if higher non-synonymous (amino acid-changing) substitution rates (d_N) than synonymous (silent) substitution rates (d_S) are measured at the codon level, i.e., the ratio $\omega = d_N/d_S > 1$ (e.g. Yang and Bielawski, 2000; but see Kosakovsky Pond and Muse, 2005; Wyckoff et al., 2005). When ω equals 1 or is lower than 1, neutral evolution and purifying selection can be inferred, respectively. When knowledge of functional domains of the protein is unavailable, and in a background of purifying selection, maximum likelihood (ML) methods that allow for heterogeneous ω ratios among sites were shown to be powerful in detecting positive selection (Nielsen and Yang, 1998; Yang et al., 2000; Bishop, 2005). Most amino acid residues are expected to be highly conserved and adaptive evolution most likely affects only a few sites (Gillespie, 1991; Cooper et al., 2003). These types of analyses have thus far only rarely been performed across fungal species (Johannesson et al., 2004).

The fungal genus *Botrytis* (Ascomycota) comprises 22 plant pathogenic species. *Botrytis* diseases cause serious yield losses in many agronomically important crops, such as grapevine, tomato, bulb flowers, and ornamental crops (Jarvis, 1977; Elad et al., 2004). Many *Botrytis* species are specialists with a narrow host range and they infect only one or a few related species within the same plant genus (Mansfield, 1980). A recent classification of the genus based on a phylogenetic tree derived from partial sequences of three housekeeping genes corroborated the classical species

delineation (Staats et al., 2005). Comparison of *Botrytis* and angiosperm phylogenies showed no evidence of cospeciation of pathogens and their hosts. Rather, the incongruence between the phylogenies of pathogens and hosts was explained by the occurrence of frequent host shifts (Staats et al., 2005). The production of host-specific toxins that confer the ability to kill and invade specific plants may have contributed to speciation in *Botrytis*, for instance, host specificity conferring toxins were reported for *B. fabae* infecting *Vicia faba* (Harrison, 1980) and for *B. elliptica* infecting lily (van Baarlen et al., 2004b). *B. cinerea* produces several non-specific phytotoxic metabolites, in agreement with the broad host range of this species (Collado et al., 2000; Colmenares et al., 2002; Siewers et al., 2005). Yet, the molecular mechanism underlying host specificity of *Botrytis* spp. remains to be unraveled.

An interesting hypothesis to explore is whether individual *Botrytis* species produce distinct NLPs with differential phytotoxic activity towards various plant species, such that the toxicity of an NLP may determine the host range of the *Botrytis* species that produces this protein. As a first step towards testing this hypothesis, the objective of this study was to determine the sequences of NLPs in all *Botrytis* species and compare patterns of putative positive and purifying selection of positions within the NLP genes. To this end, we sequenced and compared two NLP gene regions from all known species of the genus *Botrytis*. Analyses were conducted with the purpose to identify amino acid residues under positive selection. This paper provides the first evolutionary analysis of a protein family from an entire genus of plant pathogenic fungi.

Materials and Methods

Amplification and sequencing of NEP sequences

DNA sequence data of two NLP genes (*NEP1* and *NEP2*) from all known 22 *Botrytis* species are included here. The fungal strain origins, culturing method and DNA extraction protocol were previously described in Staats et al. (2005).

A Blast search of the genomic sequence of *B. cinerea* B05.10 (Syngenta Biotechnology Inc., Research Triangle Park, NC, USA) yielded two sequences with significant similarity to *NEP1* of *F. oxysporum* (Bailey, 1995; Accession No. AAC97382). The *B. cinerea NEP* sequence with highest similarity to the *NEP1* sequences of *F. oxysporum* will be referred to as *BcNEP1*, the other as *BcNEP2*. Two primer combinations (Table 1), which are located in the predicted promoter and

predicted terminator regions of both *BcNEP1* and *BcNEP2*, were designed to amplify full-length *NEP1* and *NEP2* homologs of *B. elliptica* strain BE9401 (Kessel et al., 2001), *B. tulipae* strain BT0005, *B. fabae* strain MUCL98 and *B. pelargonii* strain MUCL1152. Primer combination NEP1(-207)for-NEP1(+1124)rev was designed to amplify an 1331 bp fragment containing the entire *NEP1* region (Table 1). Primer combination NEP2(-200)for-NEP2(+1147)rev was designed to amplify an 1347 bp fragment containing the entire *NEP2* region.

Table 1: Primers used for PCR amplification

Primer Name	Target Region	Primer Sequence (5′-3′)	Position[a]
NEP1(-207)for	NEP1	CACCTTGTGGGAGATTGTATGGGTGGATATACATC	-207
NEP1(+1124)rev	NEP1	GGTCACCTAATTTTGGCTTTCAGGGTC	+1124
NEP2(-200)for	NEP2	GAACTTTGAATAGTGGGCAGTTGGG	-200
NEP2(+1147)rev	NEP2	GAGTTTCAGGTATATTCGTTTGGTGGA	+1147
NEP1for	NEP1	gtgactgtaaaacgacggccagtCCAACGCAAAATTCCTTTCTATCC	+11
NEP1revA	NEP1	gtgaccaggaaacagctatgaccCTTGGCGAGGTTGTTGTTGAAGTT	+842
NEP1revB	NEP1	gtgaccaggaaacagctatgaccGTTGGCGAAGTTGTGGTCATTGAA	+807
NEP2forD	NEP2	gtgactgtaaaacgacggccagtTTGCCTTCTCAAAATCATTACAGC	+5
NEP2revD	NEP2	gtgaccaggaaacagctatgaccTCTAGAAAGTAGCCTTCGCAAGAT	+846
NEP2forE	NEP2	gtgactgtaaaacgacggccagtTCATCATGGTTGCCTTCTCAAGAT	-5
NEP2revE	NEP2	gtgaccaggaaacagctatgaccAAGTAGCAGCTGCAAGATTGTTTG	+840
NEP2forF	NEP2	gtgactgtaaaacgacggccagtTTCGGTCTTGGCATCTACAGTCAT	+36

[a] Base pair coordinates in homologous gene of *B. cinerea* strain B0510 (GenBank Accession Nos. DQ211824 and DQ211825)

Based on a pairwise sequence comparison between the five full-length gene sequences, five new primer sets were designed that amplify either partial *NEP1* gene regions or partial *NEP2* gene regions (Table 1). Primer combination NEP1for-NEP1revA was used to amplify a *NEP1* fragment (831 bp) of *Botrytis* species belonging to clade 1 (Staats et al., 2005). Primer combination NEP1for-NEP1revB was used to amplify a *NEP1* fragment (796 bp) of *Botrytis* species belonging to clade 2, except for *B. porri* strain MUCL3234 for which primer set NEP1for-NEP1revA was used. Primer combination NEP2forD-NEP2revD was used to amplify a *NEP2* fragment (841 bp) of *Botrytis* species belonging to clade 1. Primer combination NEP2forE-NEP2revE was used to amplify a *NEP2* fragment (845 bp) of *Botrytis* species belonging to clade 2, except for *B. calthae* strain MUCL2830 for which primer set NEP2forF-NEP2revD (810 bp) was used. The PCR amplification protocol was as described by Staats et al. (2005), but an annealing temperature of 50°C in stead of 64°C was used to amplify all fragments.

Multiple sequences were obtained for most of the 22 *Botrytis* species; *NEP1* fragments were obtained from 44 *Botrytis* isolates, whereas *NEP2* fragments were obtained from 36 *Botrytis* isolates. The data set for each gene region used in this study consisted of one gene fragment for each of the 22 *Botrytis* species (Figure 1).

Most PCR products were directly sequenced, however 33 PCR products were cloned using the DNA sequencing protocol and DNA cloning protocol as previously described by Staats et al., (2005). The *NEP1* loci of the following 15 *Botrytis* strains were cloned: *B. tulipae* Bt0005, *B. elliptica* Be0004, *B. elliptica* Be9610, *B. elliptica* BE9401, *B. fabae* MUCL98, *B. fabae* MUCL7923, *B. pelargonii* MUCL1152, *B. calthae* MUCL2830, *B. byssoidae* MUCL94, *B. polyblastis* CBS287.38, *B. globosa* MUCL21514, *B. sphaerosperma* MUCL21481, *B. sphaerosperma* MUCL21482, *B. porri* MUCL3234, and *B. aclada* MUCL8415. The *NEP2* loci of the following 18 *Botrytis* strains were cloned: *B. tulipae* BT0005, *B.cinerea* B0510, *B. elliptica* BE0004, *B. elliptica* BE9401, *B. fabae* MUCL98, *B. fabae* MUCL7923, *B. pelargonii* MUCL1152, *B. aclada* MUCL8415, *B. ranunculi* CBS178.63, *B. hyacinthi* 0001, *B. croci* MUCL436, *B. byssoidae* MUCL94, *B. polyblastis* CBS 287.38, *B. paeoniae* 0003, *B. ficariarum* CBS176.63, *B. squamosa* MUCL1107, *B. convoluta* MUCL11595, and *B. calthae* MUCL2830. All DNA fragments were sequenced in both directions. Vector NTI suite 8.0 (InforMax, Inc.) was used to visually inspect chromatographs, align and annotate DNA sequences. PCR primer sequences were excluded from the phylogenetic and evolutionary analyses. GenBank accession numbers of the 80 partial and complete *NEP1* and *NEP2* gene sequences are DQ211822 to DQ211834, AM087089 to AM087091, and AM087025 to AM087088. *NEP1* and *NEP2* sequence alignments are deposited at the EMBL Nucleotide sequence database (Webin-Align Accession Nos. ALIGN_000946 and ALIGN_000947).

Phylogenetic analysis

Bayesian analyses of phylogeny were performed using MrBayes version 3.1 (Ronquist and Huelsenbeck, 2003). Sequence alignments of *NEP* gene regions were unambiguous and indels occurred in intron regions only. Gaps and missing characters were treated as missing data. Data partitions were set up for the coding regions of each gene region (*NEP1* and *NEP2*), i.e. excluding the intron regions. Subsequently, Modeltest 3.7 (Posada and Crandall, 1998) was used to determine the best-fitting model of molecular evolution for each data partition as established by hierarchical Likelihood Ratio Tests (hLRTs). Because MrBayes does not allow for the

TrN model (Tamura and Nei, 1993) to be implemented, we used the next more complex model available in the program using nst=6, which is equivalent to the General Time Reversible (GTR) model (Rodriguez et al., 1990). MrBayes was set to perform two simultaneous and independent runs starting from random trees with the following MCMC parameter settings; Nchains=4, Ngen=8,000,000 and Samplefreq=200. Convergence among the two independent runs was examined using the average standard deviation of split frequencies, which typically dropped to less than 1% towards the end of the run. Bayesian 50% majority-rule consensus trees were generated after discarding 25% of the samples as "burn-in".

A jackknife analysis was carried out using PAUP version 4.0b10 (Swofford, 2002) to assess data structure and identify significant supported clades (≥63%). Search type was set to "fast" stepwise addition. Jackknife values were generated by randomly deleting 37% of the characters for 10,000 replicates and using "Jac" resampling (Farris et al., 1996). Concordance of between-data set branching patterns was evaluated by comparing jackknife values and Bayesian posterior probability (PP) values. Branches with at least 63% jackknife support and with at least 0.95 PP were considered to be weakly supported. Branches conflicting between different phylogenies with at least 85% jackknife and with at least 0.95 PP indicate areas of strong discordance.

Data mining

The *Botrytis* NEP1 and NEP2 proteins and the sequence of *Phytophthora parasitica* NPP1 (TrEMBL entry Q9AT28) were submitted to the PredictProtein META-PP server (Rost et al., 2003). For identifying conserved motifs, the output from the PredictProtein and MyHits server (Falquet et al., 2002) was compared. Secondary structure predictions were generated using JPred (Cuff and Barton, 1999) and Ps*i*-Pred (McGuffin et al., 2000) server output. To search for interacting amino acid substitutions, the NEP1 and NEP2 multiple alignments were analyzed using the on-line CRASP server (Afonnikov and Kolchanov, 2004). Homology searches at the higher taxonomic superfamily level were conducted using hidden Markov model search algorithms (Gough et al., 2001) provided via the Superfamily server (Madera et al., 2004).

Protein sequences were aligned in CLUSTAL-W (Thompson et al., 1994) and the aligned output was converted into a format displaying biochemical properties of the amino acids using CHROMA (Goodstadt and Ponting, 2001). The conserved domains

and secondary structure information was plotted onto the alignments. Statistical tests for detecting of gene conversion between *NEP1* and *NEP2* were performed using GENECONV 1.81 (Sawyer, 1989).

Positive selection analysis

To test for amino acids under positive selection in the two gene regions, codon-substitution models were fitted to the data using the maximum likelihood implementation in the CODEML program in the PAML package (version 3.14; Yang, 1997). We used the site models M0, M1a, M2a, M7, and M8 of Nielsen and Yang (1998), Wong et al. (2004), and Yang et al. (2005) that use a statistical distribution to describe the random variation in ω (=d_N/d_s) among sites. Model M0 assumes constant ω rates across sites. Model M1a (neutral) assumes two site categories; the proportion of sites with ω rates lower than 1 (purifying selection; p_0 and ω_0) and the proportion of sites with ω rates equal to 1 (neutral selection; p_1 and ω_1). In addition to the site classes mentioned for M1a, the Model M2a (PositiveSelection) assumes a third category of sites; the proportion of sites with ω rates higher than 1 (positive selection; p_2 and ω_2). Model M7(Beta) assumes a beta distribution between 0 and 1 depending on the substitution type parameters p and q. In addition to these parameters, model M8 (Beta&ω) estimates an extra class of sites from the data; a proportion of sites with ω (p_1 and ω). Only models M2a and M8 can detect sites under positive selection. The following settings were used; Model=0, NSsites=0, 1, 2, 7, 8, and CodonFreq=2 with the branch lengths estimated under M0 used as initial values (fix_blength=1). Bayesian 50% majority-rule consensus trees of each gene region with branches with support values of at least 0.95 PP and with at least 63% support for the same nodes in a MP jackknife analysis were used as user trees. Codons positions in the alignment with missing or ambiguous data (? and N characters) were completely deleted for all taxa.

As *NEP1* genes of *B. sphaerosperma* and *B. globosa* were considered to be non-functional, analysis of positive selection was done with and without these sequences. Two pairs of models, M1a ('Nearly-Neutral') versus M2a ('PositiveSelection'), and M7 ('Beta') versus M8 ('Beta&ω') were compared using Likelihood Ratio Tests (LRT). The null models (M1a and M7) use distributions that do not allow for sites at which ω>1, whereas the alternative model does. Twice the difference in log likelihood ratio between the null model and the alternative model was compared with a chi-squared (χ^2) distribution with two degrees of freedom. The Bayes empirical Bayes (BEB)

approach of Yang et al. (2005) was then used to identify amino acids under positive selection by calculating the PPs that a particular amino acid belongs to a given selection class (neutral, deleterious or advantageous). Sites with high posterior probability (PP>95%) coming from the class with ω>1 are inferred to be under positive selection.

Codon usage bias

Variation in the rate of synonymous substitution among genes may be related to codon use (Sharp and Li, 1986; Sharp, 1991). Therefore, several parameters related to codon usage bias for each gene region, such as the codon bias index (Morton, 1993), G+C content at second and third positions as well as overall, and the effective number of codons (Wright, 1990) were estimated using DnaSP version 4.10.4 (Rozas et al., 2003).

Results

Alignment of *Botrytis NEP* gene sequences

Two homologs of NEP1 from the plant pathogen *Fusarium oxysporum* (Bailey, 1995) were retrieved from the *B. cinerea* genome sequence in a BLAST search. The homolog with highest sequence similarity (63%) to the *F. oxysporum* NEP1 sequence is named BcNEP1 here. A second predicted protein with lower sequence similarity (54%) was termed BcNEP2. All 22 *Botrytis* species that have been described previously (Staats et al., 2005) contain at least two NEP genes. Complete *NEP1* and *NEP2* locus sequences were obtained from five *Botrytis* species and partial sequences from the remaining 17 species. All 22 *Botrytis* NEP proteins contain an NPP1 domain (Pfam Accession No. PF05630; InterPro No. IPR008701).

The alignment of the 22 *NEP1* DNA sequences consists of 754 sites and includes 114 sites in two introns present in all sequences (Table 2). No length variation among the coding regions was observed. A total of 219 (34.2%) sites are variable in the exon regions and 67 (58.8%) intron sites are variable. The predicted amino acid sequences consist of 213 amino acid residues of which 68 (31.9%) are variable. The pairwise sequence similarity of the partial *NEP1* sequence was 82%-100% at the DNA level, and 88%-100% at the amino acid level. The amino acid alignment encompasses part of the predicted signal sequence and the major part of the mature protein. Two cysteine residues are completely conserved (positions 50

and 212), whereas one cysteine residue (position 76) is found in all but two species and one cysteine residue (position 123) is found only in *B. gladiolorum*. The largest single block of conserved residues is between positions 183 and 196. Two stop codons are present in the *NEP1* sequences of both *B. sphaerosperma* and *B. globosa* (positions 121 and 156), and therefore they may represent pseudogenes. In these species, one stop codon is located in the conserved GHRHDWE motif in the central region of the protein. This motif is conserved in all known NLP sequences except for *Neurospora crassa* (Pemberton and Salmond, 2004). Intraspecific polymorphic sites were detected in *B. cinerea* (nine sites, five sequences), *B. pelargonii* (one site, two sequences), *B. elliptica* (five sites, five sequences), *B. ficariarum* (11 sites, two sequences), and in *B. galanthina* (two sites, two sequences) (data not shown). In each of these within-species comparisons one polymorphic site was non-synonymous.

Table 2: Summary of *NEP1* and *NEP2* sequence alignments of 22 *Botrytis* species

	NEP1	NEP2
Number of exons	3	3
Number of introns	2	2
Number of sites (exon + intron)	754	802
Number of sites (exon)	640	687
Number of sites (intron)	114	115
Number of variable sites (exon)	219 (34.2%)	214 (31.1%)
Number of variable sites (intron)	67 (58.8%)	59 (51.3%)
Number of codons	213	228
Number of variable codons	68 (31.9%)	62 (27.2%)
Phylogenetic informative sites (exon)	127 (19.8%)	136 (19.8%)
ENC[a]	44.67	51.28
CBI[b]	0.49	0.37
CG content at coding positions	0.52	0.50
CG content at second coding positions	0.47	0.55
CG content at third coding positions	0.53	0.39

[a] Effective number of codons averaged across all taxa (Wright, 1990)
[b] Codon bias index (Morton, 1993)

The alignment of the 22 *NEP2* DNA sequences consists of 802 sites, which includes 115 sites of the two introns present in all sequences (Table 2). A total of 214 (31.1%) sites are variable in the exon regions and 59 (51.3%) variable sites are present in both introns. The predicted amino acid sequence consists of 228 amino acid residues of which 62 (27.2%) are variable. The pairwise sequence similarity of the partial *NEP2* sequences was 82%-100% at the DNA level, and 86%-100% at the amino acid level. The amino acid alignment encompasses part of the predicted signal sequence and the major part of the mature protein. The NEP2 amino acid sequence contains four conserved cysteine residues (positions 61, 87, 150, and 159). The largest

single block of conserved residues is between position 106 and 120. The GHRHDWE motif of NEP2 is not entirely conserved among all *Botrytis* species; both in *B. croci* and *B. hyacinthi* the aspartic acid (D; position 127) residue is changed into a glutamic acid (E) residue. Polymorphic sites were detected in *NEP2* sequences between isolates of the same *Botrytis* species; three in *B. convoluta* (two sequences), one in *B. polyblastis* (two sequences), five in *B. elliptica* (five sequences), four in *B. squamosa* (two sequences), and one in *B. cinerea* (three sequences) data not shown). In each of these within-species comparisons, except for *B. cinerea*, one or two polymorphic sites were non-synonymous. The pairwise sequence similarity between the partial *NEP1* and *NEP2* sequences was 49%-54% at the DNA level, and 51%-55% at the amino acid level.

***NEP* and combined housekeeping gene-based trees have congruent topologies**

The *NEP1* data set consists of 640 positions, of which 127 (19.8%) were potentially parsimony-informative (Table 2). The *NEP1* Bayesian consensus topology (Figure 1A) contains 10 nodes with at least 95% PP support corresponding with 63% support for the same nodes in a MP jackknife analysis. The *NEP1* tree topology was largely congruent with the *NEP2* topology (Figure 1B) and it was not in conflict with the combined *Botrytis* housekeeping gene phylogeny (Staats et al., 2005).

The *NEP2* data set consists of 687 positions, of which 136 (19.8%) were potentially parsimony-informative. The *NEP2* Bayesian 50% majority-rule consensus topology (Figure 1B) contains 10 nodes with at least 95% PP support corresponding with 63% support for the same nodes in a MP jackknife analysis. The placement of *B. gladiolorum* 9701 in the *NEP2* topology was in conflict (1.0 PP and 97% Jackknife) with the combined *Botrytis* housekeeping gene phylogeny of Staats et al. (2005) where it grouped as sister to *B. byssoidea* and *B. narcissicola*.

A neighbor-joining tree with bootstrap analysis of the combined *NEP* gene sequences resulted in a tree topology comprising well-supported *NEP1* and *NEP2* clades (not shown), indicating that these genes are paralogs. This was also supported by the fact that GENECONV analysis did not infer any recombination between *NEP1* and *NEP2* (not shown). Most probably, both genes originate from a duplication event that predates the origin of the genus *Botrytis*, as evidenced by the presence of both *NEP1* and *NEP2* in all *Botrytis* species and their largely congruent topologies to a *Botrytis* species tree (Staats et al., 2005).

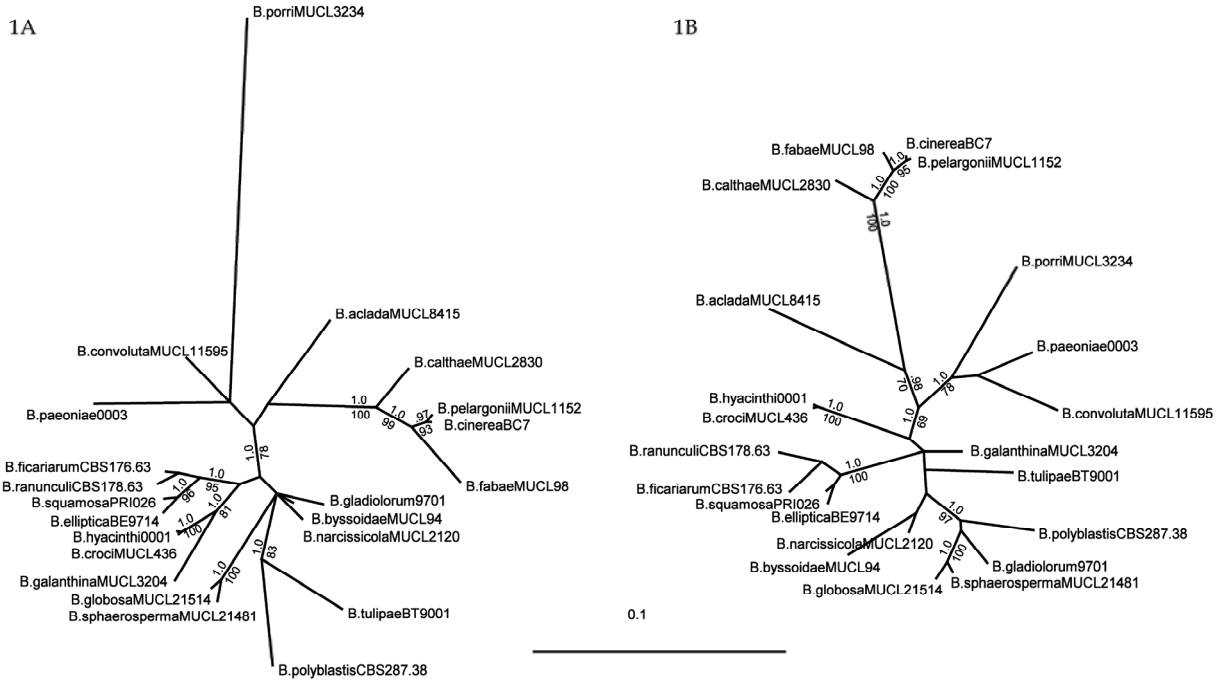


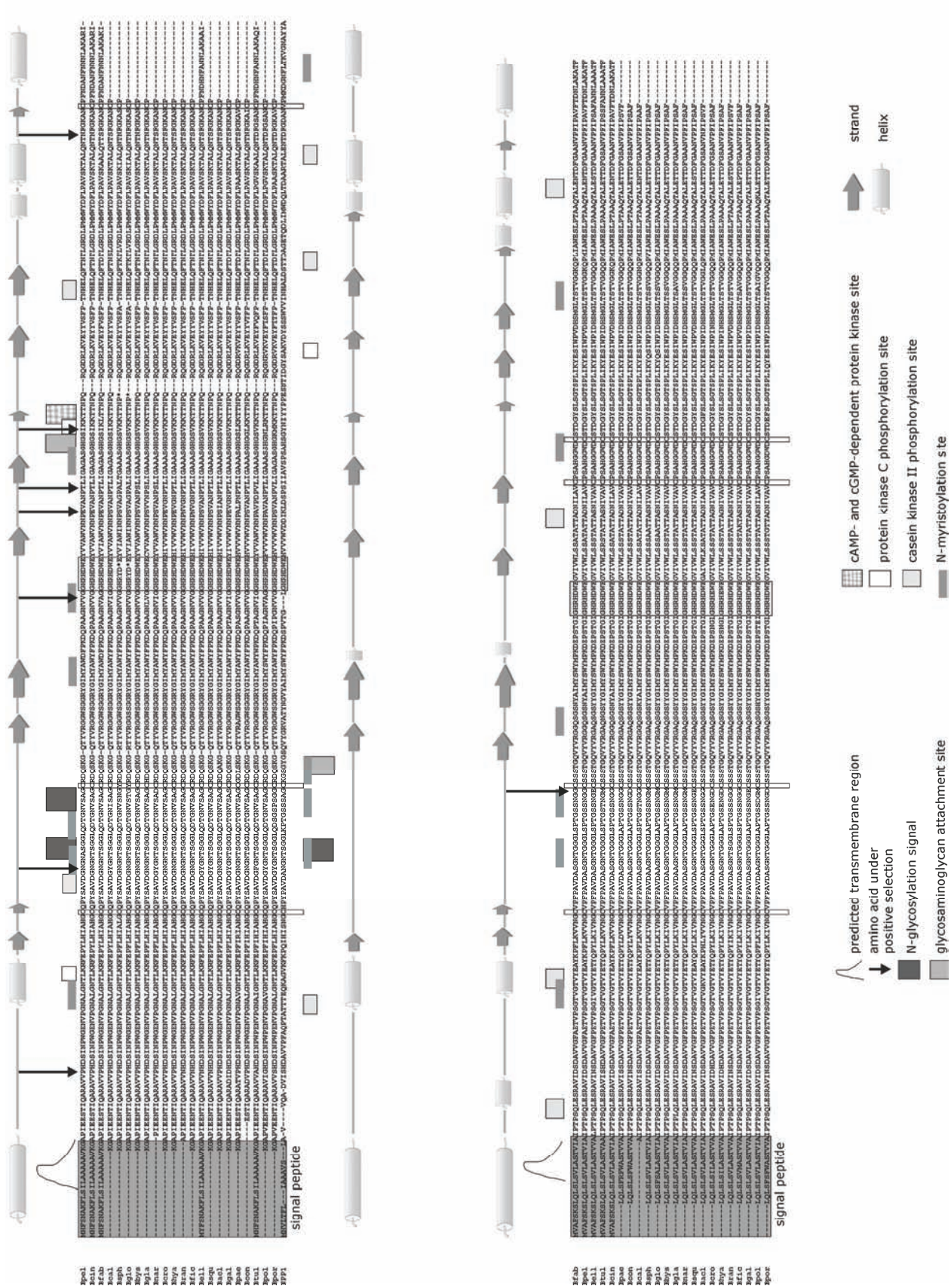
Figure 1: Bayesian inference showing unrooted phylograms of the (**A**) *NEP1* coding region and (**B**) *NEP2* coding region of 22 *Botrytis* species. Support values of at least 0.95 PP (above node) and at least 63% jackknife (below node) are shown along each branch. Branches are drawn in proportion to estimates of their lengths in substitutions/site.

Conservation of structural motifs in NEP1 and NEP2

All five NEP proteins from *Botrytis* species of which the complete *NEP* locus was sequenced were predicted to contain a signal peptide (Figure 2). The cleavage site in *B. cinerea* NEP1 was located between amino acid residues 20 and 21 (VKG-AP) as predicted by the SIGNALP 3.0 server (Bendtsen et al., 2004), while the cleavage site in *B. cinerea* NEP2 was between amino acid residues 21 and 22 (VIA-IP). The signal peptide cleavage sites predicted in the fully sequenced gene regions are conserved in the partial sequences of NEP1 and NEP2 from the remaining species.

NEP1 proteins contain three conserved cysteine residues, whereas NEP2 contains four. The first two cysteines of NEP1 are predicted to form an internal disulphide bridge (Vullo and Frasconi, 2004 as implemented in PredictProtein). In NEP2, the two most N-terminal cysteines and the two most C-terminal cysteines are predicted to form internal disulphide bonds. Both NEP1 and NEP2 proteins contain

Figure 2 (next page): Representation of secondary structure, conserved motifs and amino acids under positive selection mapped onto the multiple alignments of *Botrytis* NEP1 and NEP2 proteins. Arrows indicate the position of positively selected sites. NPP1 = Necrosis-inducing protein NPP1 of *Phytophthora parasitica* (TrEMBL entry Q9AT28).


NEP1
NPP1
NEP2
signal peptide
signal peptide
predicted transmembrane region
amino acid under positive selection
N-glycosylation signal
glycosaminoglycan attachment site
cAMP- and cGMP-dependent protein kinase site
protein kinase C phosphorylation site
casein kinase II phosphorylation site
N-myristoylation site
strand
helix

the conserved sequence GHRHDWE (Pemberton and Salmond, 2004) except for NEP1 of *B. sphaerosperma* and *B. globosa* (Figure 2). These species also lack the second conserved cysteine and contain premature stop codons in *NEP1*. Both in *P. parasitica* NPP1 and *Botrytis* NEP1, the regions surrounding the conserved GHRHDWE motif contain several hydrophobic residues, while the conserved motif itself contains both positively charged and polar residues. Both the N- and C-terminal regions contain helices and are predicted to harbor transmembrane regions. The central region contains several strands which might be part of beta-sheets in a tertiary structure. Unfortunately, the NPP1 domain and *Botrytis* NEP proteins do not bear any significant homology to proteins of which the tertiary structure is deposited in the SWISS-PDB database or in the 3D-modeling databases accessible through the 3D-search option in a META-PP search provided by the PredictProtein server (CUBIC, Columbia University, USA).

NEP1 proteins contain six different types of posttranslational modification motifs, including *N*-glycosylation signals, potential phosphorylation sites and *N*-myristoylation sites, which are highly conserved in all 22 *Botrytis* species (Figure 2). In contrast, NEP2 proteins contain two types of posttranslational modification motifs, i.e. *N*-myristoylation and casein kinase II phosphorylation sites and these are also conserved in all 22 *Botrytis* species (Figure 2). In spite of the protein sequence differences, the overall predicted secondary structure of *Botrytis* NEP1 and NEP2 proteins is comparable, with N- and C-termini harboring predicted transmembrane regions and helices and a central part containing several strands. The approximate locations of these strands in NEP1 and NEP2 are similar, in concordance with their CLUSTAL-W profile alignment (Figure 2). In order to compare the NEP1 and NEP2 proteins more extensively the *Botrytis* NEP1 and NEP2 multiple alignments were analyzed separately in CRASP (Afonnikov and Kolchanov, 2004) and compared for several characteristics including volume, hydrophobicity, exposed residues, and interacting substitutions. Evolutionary theory predicts that substitutions at given sites may promote second-site substitutions in order to maintain an evolved protein function (Afonnikov and Kolchanov, 2004). Coordinated substitutions may bear relevance to retaining protein structure and function. For all characteristics, NEP1 proteins contained significantly more interacting amino acid substitutions compared to NEP2 proteins at ratios from 2:1 to 10:1 depending on the characteristic (not shown).

Amino acid residues in NEP1 and NEP2 are under positive selection.

The proportion of variable codons in the NEP1 and NEP2 sequences ranged from 27.2 to 31.9% (Table 2), whereas the proportion of variable codons in three housekeeping genes (Staats et al., 2005) only ranged between 1.3 and 4.5% (not shown). This suggested that higher non-synonymous substitution rates occur in NEP genes than in housekeeping genes and led us to conduct a more detailed evolutionary analysis for identifying codons under positive selection. Assessment of positive selection per residue was performed at the species-level, i.e., including the sequence of one isolate per species. Including multiple sequences per species in the analysis did not influence the number or position of residues under positive selection (not shown).

The log likelihood values and parameter estimates under models of variable ω ratios among sites for the *NEP1* and *NEP2* gene regions are listed in Tables 3 and 4. The one-ratio model (M0) assumes the same ratio for all sites and fits the data more poorly than any of the other models for all data sets. Parameter estimates from model M7 suggest a U-shape for the ω distribution, which indicates that most sites are either highly conserved with the non-synonymous/synonymous substitution rate ratio ($\omega = d_N/d_S$) close to 0 or nearly neutral with ω close to 1, while few sites are in between.

For *NEP1*, analyses of positive selection were performed with and without the *B. sphaerosperma* and *B. globosa NEP1* sequences to asses the effect of the two putative pseudogenes on the analyses (Table 3). Model M8 was best-fitting for both analyses, as the log likelihood values were highest (-2640.27 and -2792.72). Parameter estimates of ω, d_N and d_S were slightly higher for the analyses that included the two putative pseudogenes. *NEP1* gene regions are moderately conserved (ω=0.297), although slightly less conserved when the putative pseudogenes were included (ω=0.321; Table 3). The LRT statistics showed that selection models M2a and M8 detect positive selection in both data sets (Table 5). The BEB approach under model M2a suggested one positively selected site with $P(\omega>1)>0.95$, while model M8 indicated four and seven sites for analyses with and without putative pseudogenes, respectively. Applying a 99% threshold in model M8 identifies only site 132E.

The *NEP2* gene regions are moderately conserved, with an average ω ratio of 0.228 for the best-fitting model M2a (Table 4). The average d_N and d_S values under model M2a are 0.335 and 1.470, respectively. Parameter estimates and LRTs suggest the presence of sites under positive selection (Table 5). Model M2a and M8 both

Table 3: Likelihood values and parameter estimates for the *NEP1* gene regions

Model code	ℓ[b]	d_N / d_S = ω	Estimates of parameters	Positively selected sites[c]
M0 (one-ratio)	-2763.02	0.392/1.511 = 0.259	ω=0.259	Not allowed
M1a (nearlyNeutral)	-2646.41	0.382/1.637 = 0.233	ω_0=0.030(ω_1=1), p_0=0.791(p_1=0.209)	Not allowed
M2a (PositiveSelection)	-2641.33	0.452/1.464 = 0.309	ω_0=0.035(ω_1=1), ω_2= 2.403, p_0=0.792, p_1=0.155, p_2=0.053	132E
M7 (Beta)	-2649.12	0.371/1.680 = 0.221	p=0.685, q=0.241	Not allowed
M8 (Beta&ω)	-2640.27	0.445/1.495 = 0.297	p_0=0.903(p_1=0.097), p=0.143, q=1.043, ω=1.966	17P, 59N, 114A, **132E**, 137T, 149I, 206S
M0 (one-ratio)[a]	-2905.10	0.442/1.554 = 0.284	ω=0.284	Not allowed
M1a (nearlyNeutral)[a]	-2799.93	0.432/1.667 = 0.259	ω_0=0.048(ω_1=1), p_0=0.778(p_1=0.222)	Not allowed
M2a (PositiveSelection)[a]	-2794.94	0.503/1.496 = 0.336	ω_0=0.055(ω_1=1), ω_2= 2.357, p_0=0.780, p_1=0.165, p_2=0.054	132E
M7 (Beta)[a]	-2802.77	0.423/1.722 = 0.246	p=0.114, q=0.349	Not allowed
M8 (Beta&ω)[a]	-2792.72	0.493/1.537 = 0.321	p_0=0.899(p_1=0.110), p=0.256, q=1.669, ω=1.869	17P, **132E**, 137T, 206S

[a] Analyses of positive selection including the two *NEP1* sequences of *B. sphaerosperma* MUCL21481 and *B. globosa* MUCL21514

[b] Log likelihood value

[c] Amino acid sites inferred to be under positive selection with PP>95%. Sites with PP>99% are in bold

Table 4: Likelihood values and parameter estimates for the *NEP2* gene regions

Model code	ℓ	d_N / d_S = ω	Estimates of parameters	Positively selected sites
M0 (one-ratio)	-2791.04	0.288/1.485 = 0.194	ω=0.194	Not allowed
M1a (nearlyNeutral)	-2718.08	0.296/1.532 = 0.193	ω_0=0.047(ω_1=1), p_0=0.846(p_1=0.154)	Not allowed
M2a (PositiveSelection)	-2709.27	0.335/1.470 = 0.228	ω_0=0.048(ω_1=1), ω_2= 7.405, p_0=0.843, p_1=0.152, p_2=0.005	**86D**
M7 (Beta)	-2722.19	0.296/1.542 = 0.192	p=0.114, q=0.479	Not allowed
M8 (Beta&ω)	-2712.34	0.326/1.492 = 0.218	p_0=0.995(p_1=0.005), p=1.129, q=0.573, ω=7.147	**86D**

suggest that 0.5% of the codons are under positive selection with $\omega = 7.405$ and $\omega_2 = 7.147$, respectively. Using the BEB approach for models M2a and M8, site 86D was identified as positively selected with PP>0.99.

Amino acid sites in the *NEP1* and *NEP2* gene regions inferred to be under positive selection were not polymorphic between isolates of the same *Botrytis* species (not shown). In addition, their state changes appear not to correlate with the combined *Botrytis* housekeeping genes phylogeny (Staats et al., 2005; not shown).

Table 5: Likelihood ratio statistics ($\Delta\ell$) for tests of positive selection[a]

Gene region	M2a vs. M1a[b]	M8 vs. M7
NEP1	10.16***	17.70***
NEP1[c]	9.98**	20.10***
NEP2	17.62***	9.85**

[a] Likelihood ratio test: $2\Delta L=2(\ln L_{\text{alternative hypothesis}}-\ln L_{\text{null hypothesis}})$. χ^2-distribution with 2 df.
[b] **Significant at 1% level, ***Significant at 0.1% level
[c] LRT statistic including the two NEP1 sequences of *B. sphaerosperma* MUCL21481 and *B. globosa* MUCL21514

Codon usage bias

Several parameters related to codon usage bias were estimated to check whether synonymous mutations are selectively neutral. The codon bias index (Morton, 1993) is a measure for the deviation from the equal use of synonymous codons. CBI values range from 0 (uniform use of synonymous codons) to 1 (maximum codon bias). CBI values were intermediate between uniform use of synonymous codons and maximum codon bias for both gene regions (Table 2). Additionally, the effective number of codons (ENC) were calculated, which may range from 20 (only one codon is used for each amino acid; i.e., the codon bias is maximum) to 61 (all synonymous codons for each amino acid are equally used; i.e., no codon bias). ENC values were intermediate (Table 2). C+G content at the second and third codon position was comparable to the overall C+G content of the total gene region, and ranged between 39.0% and 55.0%, values similar to those in *N. crassa* and *Magnaporthe grisea* (Galagan et al., 2003; Dean et al., 2005).

Discussion

Here, we report the presence in the fungal genus *Botrytis* of genes encoding proteins with high sequence similarity to NLPs, of which the protein NEP1 from the fungus *Fusarium oxysporum f.sp erythroxyli* was the first described (Bailey, 1995). Two NLP paralogs were detected in all 22 *Botrytis* species. The availability of the entire genome sequence of *B. cinerea* enables us to conclude that at least this species does not appear to contain any additional paralogs. Also in *B. tulipae* and *B. elliptica* there appear to be no additional homologous sequences, as evidenced by low stringency Southern hybridization (A. Schouten, unpublished). We conducted molecular phylogenetic analyses and investigated the sequence evolution of NLPs.

Protein sequence alignments showed high overall similarity between the two NEP paralogs. The overall predicted secondary structure is conserved and signal peptides are present in both NLPs, however the presence and distribution of conserved motifs is different between the paralogs. The motif GHRHDWE located in the central region of both NEP proteins was conserved in all *Botrytis* species, except for the *B. sphaerosperma* and *B. globosa NEP1* genes, in which stop codons are present. The *NEP1* genes in these two species may thus represent pseudogenes, which is further supported by the absence of one conserved cysteine residue close to the N-terminus. The two N-terminal cysteine residues in NEP1 are predicted to form an internal di-sulphide bridge. Both N-terminal cysteine residues of NPP1 of *Phytophthora parasitica* are essential for biological activity (Fellbrich et al., 2002).

Some evolutionary changes in *NEP* genes are driven by positive selection

The average ω ratio indicates that *NEP* genes are moderately conserved and overall under purifying selection, probably as a result of structural and functional constraints. A proportion of amino acid sites are under positive selection, based on inference using models that allow for variable selection pressures among sites (Nielsen and Yang 1998; Yang et al., 2000; Yang et al., 2005). Up to seven positively selected sites were identified for NEP1. Analysis of positive selection that included the two putative *NEP1* pseudogenes detected four positively selected sites. Pseudogenes are not subjected to functional constraints and evolve under neutral selection, thereby reducing the overall number of positively selected sites. One positively selected site was detected for NEP2. Interestingly, amino acid sites under positive selection in NEP1 were not under selection in the corresponding position of

NEP2. In NEP1, two amino acid sites inferred to be under positive selection mapped to conserved *N*-myristoylation sites, and one mapped to a conserved protein kinase C phosphorylation site (Figure 2). The other four positively selected sites mapped outside the posttranslational modification motifs and outside the predicted secondary structure elements.

No correlation was detected between state changes at positively selected amino acid sites and *Botrytis* species host range. For example, the six *Botrytis* species that are pathogens of *Allium* species did not all contain the same amino acid residues at the positions under positive selection, except for amino acid site 132 in NEP1. Furthermore, the amino acid characters did not cluster monophyletically, which indicates that some state changes have evolved via reversals (not shown). In addition, no apparent pattern was detected for the amino acid substitutions with respect to their biochemical properties (not shown). The functional effects of selected amino acid replacements on NEP protein activity remain to be validated by analysis of natural or synthetic variants, e.g. as described for polygalacturonase inhibiting proteins (Bishop, 2005).

The presence of two *NEP* gene copies may have adaptive significance

The differences in the NEP1 and NEP2 amino acid sequence and distribution of motifs between the two paralogs (Figure 2) suggest that both proteins have diverged to such extent that they may have evolved different functions in *Botrytis*. The presence of both *NEP* genes fits within several evolutionary theories that have recently been grouped into a novel model, the "adaptive radiation model" (Francino, 2005). This model postulates that under a certain selection pressure new gene functions evolve after amplification of an existing gene with some level of preadaptation for its new function. This is followed by competition among gene copies for that new function in which deleterious and adaptive mutations exert influence on the paralogs. Deleterious mutations will turn some paralogs into pseudogenes. Eventually, pseudogenes will be lost from the optimal genotype, but this genotype will still contain the original locus and a paralog that has been fully adapted to a new function in response to selection pressure. There is some evidence suggesting that *Botrytis* NEP gene paralogs fulfill these predictions. The presence in NEP1 and NEP2 proteins of conserved cysteine residues, distinct types of protein modification motifs and coordinated amino acid substitutions suggests that NEP1 and NEP2 possess distinct properties and hence exert different, yet possibly

(partially) redundant, biological functions. Furthermore, the protein sequence shows both conserved residues and motifs (under purifying selection), as well as amino acids under positive selection. Finally, the loss of conserved cysteine residues and the occurrence of premature stop codons in the NEP1 sequences of *B. sphaerosperma* and *B. globosa* (Figure 2) suggest that these sequences are pseudogenes. Redundancy or functional diversity of NLP genes may be common in fungal and oomycete pathogens; e.g. *Phytophthora sojae* contains three NLP paralogs of which only one was expressed during soybean infection (Qutob et al., 2002).

NEP1 protein function may be conserved in microbes

The fact that NLPs occur in diverse microbes, ranging from free-living bacteria to plant parasitic oomycetes (Gijzen and Nürnberger, 2006), suggests a general function for microbes which remains to be unraveled. NLPs contain a secretory signal sequence (Pemberton and Salmond, 2004) and can be isolated in significant amounts from the growth media (Bailey, 1995), indicating that their biological function is in the outer environment of the microbe. The current lack of knowledge about functional domains (Fellbrich at al., 2002), cellular targeting or protein binding motifs in the protein complicates the revelation of the actual function of NLP (Pemberton and Salmond, 2004). Although the conserved seven amino acid motif GHRHDWE is only present in NLPs, we found a similar sequence with a single substitution (GHRHDWV) in chloramphenicol acetyltransferases from various bacteria (BLAST-P search for short, nearly exact matches). Yet, these proteins as a whole lack significant homology to NLPs (not shown). The relevance of this finding remains unclear.

It is well established that several members of the NLP family may induce cell death in dicotyledonous plants, but not in any monocotyledons tested (Bailey, 1995; Veit et al., 2001; Fellbrich et al., 2002). Necrotrophic plant pathogens, such as *Botrytis*, may benefit from recognition of NLPs by plants. Necrotrophs need to kill host tissue to obtain nutrients from the dead cells (van Kan, 2006). More importantly, successful infection by necrotrophs often requires host participation in the form of active cell death programs in the plant (Govrin and Levine, 2000; Wolpert et al., 2002; van Baarlen et al., 2004b). Transgenic plants compromised in cell death pathways were resistant to several necrotrophic pathogens, including *B. cinerea* (Dickman et al., 2001). Necrotrophic plant pathogens may have modified an ancestral NLP to promote plant cell death.

In order to test the significance of adaptive evolution of NEP proteins, biological data are clearly needed. Currently, we are characterizing both NEP proteins in the species *B. cinerea* (infecting eudicots) and *B. elliptica* (infecting the monocot lily), by constructing gene disruption mutants and performing heterologous protein production using the yeast *Pichia pastoris*. By studying *NEP*-deficient mutants under diverse conditions including plant infection, we aim to find out whether the gene products are essential, either for *in vitro* growth or for parasitic growth. Proteins expressed in *P. pastoris* can be tested for their ability to induce symptoms in host and nonhost plants. These results will be the subject of future papers.

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Chapter 6

Functional analysis of NLP genes from *Botrytis elliptica*

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Functional analysis of NLP genes from *Botrytis elliptica*

Abstract

We functionally analyzed two NLP genes from *Botrytis elliptica* (a specialist pathogen of lily), encoding proteins homologous to the Necrosis and Ethylene-inducing Protein NEP1 from *Fusarium oxysporum*. Single gene-replacement mutants were made for *BeNEP1* and *BeNEP2*, providing the first example of transformation and successful targeted mutagenesis in this fungus. The virulence of both mutants on lily leaves was not affected. *BeNEP1* and *BeNEP2* were individually expressed in the yeast *Pichia pastoris*, and the necrosis-inducing activity was tested by infiltration of both proteins into leaves of several monocots and eudicots. Necrotic symptoms developed on the eudicots tobacco, *Nicotiana benthamiana* and *Arabidopsis thaliana*, and cell death was induced in tomato cell suspensions. No necrotic symptoms developed on the leaves of the monocots rice, maize, and lily. These results support the hypothesis that the necrosis-inducing activity of NLPs is limited to eudicots. We conclude that NLPs are not essential virulence factors and that they do not function as host-selective toxins for *B. elliptica*.

Introduction

Nep1-like proteins (NLPs) comprise a family of secreted proteins produced by bacteria, fungi and oomycetes (reviewed by Pemberton and Salmond, 2004; Gijzen and Nürnberger, 2006). NLPs have been named after the Necrosis and Ethylene-inducing Protein (NEP1) purified from culture filtrate of *Fusarium oxysporum* f.sp. *erythroxyli* (Bailey, 1995). Infiltration of eudicots with NLPs induces programmed cell death and general stress responses (Keates et al., 2003; Bailey et al., 2005; Bae et al., 2006). Monocotyledons are considered not to respond to the currently described NLPs (Bailey, 1995; Veit et al., 2001; Fellbrich et al., 2002).

NLPs are involved in virulence in some pathosystems, but not in others. The disruption of *NEP1* had no effect on the pathogenicity of *F. oxysporum* towards its host plant, *Erythroxylum coca* var. *coca* (Bailey et al., 2002). Yet, a transgenic strain of

Colletotrichum coccodes, containing *NEP1* of *F. oxysporum*, was not only hypervirulent on its host *Abutilon*, but was also virulent on several non-host plants (Amsellem et al., 2002). NLP-deficient mutants of the soft-rotting bacteria *Erwinia carotovora* subsp. *carotovora* were reduced in virulence on potato tuber, but not on potato stems (Mattinen et al., 2004; Pemberton et al., 2005), whereas NLP-deficient mutants of *E. carotovora* subsp. *atroseptica* were reduced in virulence on both tubers and stems of potato (Pemberton et al., 2005).

The genus *Botrytis* contains 22 necrotrophic plant pathogens, which generally display a narrow host range within monocot or eudicot orders, with the exception of the wide host range pathogen *B. cinerea* (Beever and Weeds, 2004; Staats et al., 2005). *Botrytis* species require the ability to induce active cell death in their hosts in order to be pathogenic (Harrison, 1980; Govrin and Levine, 2000; van Baarlen et al., 2004b; van Kan, 2006). One of the specialized *Botrytis* species is *B. elliptica*, a pathogen of lily. Culture filtrate of *B. elliptica* contains an unidentified protein with specific phytotoxic activity towards lily (van Baarlen et al., 2004b). This protein meets the criteria of a Host-Specific Toxin ([HST]; Wolpert et al., 2002). Purification and characterization of this proteinaceous HST was thus far unsuccessful (van Baarlen and van Kan, unpublished).

All *Botrytis* species contain two NLP paralogs that have undergone considerable sequence divergence (Staats et al., in press). Detailed analysis of amino acid substitution patterns indicated that specific positions are under positive, diversifying selection (Staats et al., in press). We considered the possibility that individual *Botrytis* species produce distinct NLPs with differential phytotoxic activity towards various plant species, such that the toxicity of an NLP may determine the host range of the *Botrytis* species that produces this protein. We postulated that the previously detected HST produced by *B. elliptica* (van Baarlen et al., 2004b) might be a NLP. This hypothesis was tested, both by comparing the virulence of single, *BeNEP1* or *BeNEP2* gene-replacement mutants to that of the wild-type strain and by determining the necrosis-inducing activity of the two NLP proteins from *B. elliptica* in leaves of its host (lily) and several non-host plants.

Materials and Methods

Fungal growth conditions and genomic DNA isolation

For sporulation, *B. elliptica* wild-type stain Be9401 and transformants were cultured on 4% (w/v) potato dextrose agar (PDA; Oxoid) amended with 0.6% (w/v) purified agar (Oxoid) and 20% (w/v) freshly pulped tomato leaves. Cultures were grown for two weeks at 18°C under continuous light from an 18 W black-light and an 18W cool-white fluorescent tube. A conidial suspension of each strain was prepared in sterile tap water and filtered through a sterile 5 ml pipette tip containing glasswool. Conidia were washed twice by centrifuging the suspension at 1000 rpm and rinsing with sterile tap water. The conidial suspension was stored in 75% (v/v) glycerol at -80°C.

For DNA isolation, wild-type strain Be9401 and mutants were grown on malt agar (Oxoid) for two weeks in the dark at 18°C. Mycelium was harvested, lyophilized, submerged in liquid nitrogen and ground into a powder. Genomic DNA was extracted from 10 to 20 mg tissue using a Puregene DNA isolation kit (Gentra systems Inc.) 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.

Growth conditions of tomato cell suspensions

The tomato (*Lycopersicon esculentum* cv. Moneymaker, line Msk8) cell suspension was grown in a medium consisting of 4.4 g/L MS medium at pH 5.7 plus vitamins (Duchefa), 3% (w/v) sucrose, 5.4 µM naphtyl acetic acid (NAA) and 1 µM 6-benzyladenine. Cell suspensions were grown in 50 ml volumes in 300 ml glass Erlenmeyer flasks in the dark at 24 °C, on an orbital shaker at 125 rpm. Five ml of cell suspension was transferred weekly to fresh medium.

Gene replacement strategy of *BeNEP1* and *BeNEP2*

The gene replacement strategy described by Kars et al. (2005) was used to generate *B. elliptica* mutants that lack either *BeNEP1* or *BeNEP2*. The strategy uses a single-step overlap-extension PCR (SOE-PCR) to fuse two gene-specific fragments of the target gene to a selection marker. Amplification of the upstream *BeNEP1* target-gene fragment, NEP1-5BE (552 bp), was performed with primers NEP1-5forBE and NEP1-5revBE-SOE (Table 1). Amplification of the downstream *BeNEP1* target-gene fragment, NEP1-3BE (490 bp), was performed with primers NEP1-3forBE-SOE and NEP1-3revBE. Primers NEP1-5forBE and NEP1-3revBE are located just outside the

open reading frame (ORF) of *BeNEP1*. Primers NEP1-5revBE-SOE and NEP1-3forBE-SOE contained the primer extensions complementary to the selection marker cassette, which are required for SOE-PCR. The same strategy was used for the amplification of the upstream and downstream *BeNEP2* target-gene fragments, which were 504 bp and 428 bp, respectively, with *BeNEP2* specific primers (Table 1). The amplification reaction was carried out in a reaction volume of 50 µl using 1 U AmpliTaq DNA polymerase (PerkinElmer), 1X GeneAmp PCR buffer, 0.2 mM of each dNTP, 2 mM $MgCl_2$, 1 µm of each primer (Amersham Pharmacia Biotech), and 10-50 ng DNA. The following PCR conditions were used: 1 cycle of 94°C for 5 min; 35 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 90 s; followed by a final extension at 72°C for 5 min.

An *hph* selection marker cassette (2.7 kb) conferring resistance to hygromycin B (Genbank accession AJ439603) was used in the gene replacement procedure. The selection marker cassette was amplified with primers cassette-5 (20) and cassette-3 (21) (Table 1). The amplification was carried out in a reaction volume of 50 µl using 2.6 U Expand High Fidelity polymerase (Roche Diagnostics) with 1X Expand High Fidelity buffer containing $MgCl_2$, 0.4 µm of each primer (Amersham Pharmacia Biotech), 0.2 mM of each dNTP, and 50-100 ng plasmid DNA. The PCR conditions were: 1 cycle of 94°C for 2 min; 10 cycles of 94°C for 15 sec, 50°C for 30 sec and 72°C for 2 min; 20 cycles of 94°C for 15 sec, 50°C for 30 sec and 72°C for 2 min increasing with 5 s per cycle, followed by a final extension at 72°C for 7 min.

The two target-gene fragments of *BeNEP1* or *BeNEP2* were fused to the *hph* selection marker cassette by SOE-PCR in a single PCR amplification step. Primers NEP1-5NforBE and NEP1-3NrevBE, located nested onto the target-gene fragments, were used to fuse the *hph* cassette to the two target-gene fragments of *BeNEP1*. Primers NEP2-5NforBe and NEP2-3NrevBE were used to fuse the two *BeNEP2* target-gene fragments to the *hph* cassette. The total gene replacement constructs for *BeNEP1* and *BeNEP2* were 3742 bp and 3632 bp, respectively. The amplification reaction was carried out in a reaction volume of 50 µl with the same Expand-PCR reaction mixture as described above, except 20 ng of each of the three templates were used per reaction. The PCR conditions were: 1 cycle of 94°C for 2 min; 10 cycles of 94°C for 15 sec, 50°C for 30 sec and 68°C for 3 min; 20 cycles of 94°C for 15 sec, 50°C for 30 sec and 68°C for 3 min increasing with 5 s per cycle, followed by a final extension at 68°C for 7 min. The amplified gene replacement constructs were extracted from 1% agrarose gel using the GFX PCR purification kit (Amersham

Pharmacia Biotech). PCR products were then ligated into pGem®-T Easy Vector (Promega) using standard protocol and introduced into JM109 competent cells.

***Botrytis elliptica* transformation**

Macroconidia of *B. elliptica* strain Be9401 were inoculated in 1% malt extract liquid culture (Difco Laboratories) and incubated for 24 h at 20°C and 180 rpm. The young mycelium was isolated by filtration (22.4 µm filter) and washed twice with 1 volume of KC solution (0.6M KCl and 50mM $CaCl_2$). The mycelial suspension was then incubated with lysing enzymes from *Trichoderma harzianum* (5 mg/ml Glucanex®; Sigma-Aldrich Chemie) for approximately 1.5 h at 25°C in an orbital shaker at 180 rpm. Mycelial fragments were separated from the protoplasts by filtration over a 22.4 µm nylon gauze and washed twice with 1 volume of KC solution. Protoplasts were transformed with 4-5 µg DNA (gene replacement construct), regenerated and selected on Hygromycin B as described by ten Have et al. (1998). Emerging colonies were transferred to malt extract agar plates containing hygromycin B (100 µg/ml). Hyphal tips were then excised and transferred to malt agar plates without selection medium. Subsequently, hyphal tips were again transferred to malt agar plates with selection medium. Colonies were then grown on PDA with pulped tomato leaves to produce macroconidia, followed by growth of single spore cultures on malt agar. Genomic DNA was isolated for PCR screening and Southern hybridization.

PCR screening of the transformants

Initial screening of the *B. elliptica* transformants was by PCR. The screening for correct orientation and integration of the *BeNEP1* gene replacement construct was done essentially as described by Kars et al. (2005) using primers screen5-int and NEP1-5forBE, and screen3-int and NEP1-3revBE (Table 1). Primers NEP1-5forBE and NEP1-3revBE were used to check the putative BeNEP1 transformants for presence or absence of the wild-type *NEP1* gene. An amplification reaction with primers NEP1-5forBE and NEP1-5revBE-SOE was used as a positive control. The same strategy was applied to screen for putative *BeNEP2* mutants using the *BeNEP2* specific set of primers. PCR amplifications were carried out using the same ingredients and conditions as described above for the target-gene fragments.

RNA extraction and cloning of *BeNEP1* and *BeNEP2* cDNAs

Leaves of the Arabidopsis *pad3* mutant (Zhou et al., 1999) colonized by *B. elliptica* strain Be9401 were used to obtain high-quality RNA preparations, using the protocol of Eggermont et al. (1996) with minor modifications. cDNA of *BeNEP1* and *BeNEP2* mRNA was generated by using the One-Step RT-PCR kit (Invitrogen) according to the manufacturer's protocol, using a pre-denaturation cycle of 45 °C, 30 min, followed by 94 °C, 2 min, and a final extension cycle of 68 °C, 10 min. The RT-PCR primer combinations were based on the genomic sequence of both genes (Table 1). Both forward primers begin at the ATG start codon and are preceded by an *Eco*RI restriction site. Both reverse primers are located 53 bases downstream the TAA-stop codon and contain a *Not*1 restriction site at their 5'-ends.

The reverse transcription reaction followed by amplification yielded DNA fragments of approximately 750 bp, which were cloned in the pCR4-TOPO vector (Invitrogen), transformed into *E. coli* TOP10-cells (Invitrogen) according to the manufacturer's recommendations. Plasmids were verified by sequencing (Baseclear) to ensure that the PCR steps had not caused undesired mutations.

Heterologous expression of *BeNEP1* and *BeNEP2* in *Pichia pastoris*

The *BeNEP1* and *BeNEP2* cDNAs were PCR-amplified from the pCR4-TOPO vector using the RT-PCR primers listed in table 1. The resulting fragments were digested with *Eco*RI and *Not*I, purified on a GFX column (Amersham Pharmacia Biotech) and cloned into the *Eco*RI/*Not*I-digested expression vector pPIC3.5 (Invitrogen). Both constructs were verified by sequencing prior to transformation into *P. pastoris* strain GS115 using electroporation. Transformants were analyzed by growing 5 ml batch cultures in 6-wells Bio-One test plates (Greiner) in BMM (100 mM potassium phosphate [pH 6.0], 1.34% Yeast nitrogen base [YNB; Difco Laboratories], 4×10^{-5}% biotin), containing 5% methanol to induce the Aox1-promoter-mediated expression. The culture filtrates were filter-sterilized and concentrated via a Microcon YC10 filter unit (Amicon) by centrifugation according to manufacturer's recommendations. The filter was washed with 10 mM potassium phosphate (pH 6.0) and protein contents analyzed by polyacrylamide gel electrophoresis.

High quantities of *B. elliptica* NEP1 protein were produced in a Bioflo 3000 fermentor (New Brunswick Scientific), applying the protocol described by van den Burg et al. (2001). Approximately 3 liters of culture filtrate containing BeNEP1 was harvested, sterilized over a 0.45 µm filter and stored in aliquots at -20 °C. An Amicon

Ultrafiltration Cell with an YM-3 filter and 10 mM potassium phosphate (pH 7.0) was used to desalt and concentrate the culture filtrate to a 20-fold concentrated protein solution. This solution was further purified in a BioRad Econocolumn (1.5 x 10 cm) loaded with Streamline SP XL (Amersham Pharmacia Biotech) equilibrated with 3 column volumes 10 mM potassium phosphate (pH 7.0). The column was loaded with 17 ml concentrated protein solution and rinsed with two volumes 10 mM potassium phosphate (pH 7.0). Bound proteins were recovered from the column by eluting with increasing salt concentrations in three steps: one column volume 0.25 M NaCl, one column volume 0.5 M NaCl and one column volume 1 M NaCl (10 mM potassium phosphate [pH 7.0]). The eluted protein fractions were desalted and concentrated using Amicon Ultra-15 PGCL Centrifugal filters. The purified BeNEP1 protein was finally dissolved in 10 mM potassium phosphate (pH 7.0) and stored in 1 ml aliquots of 100 or 200 µg/ml at –80 °C.

Gel electrophoresis and protein quantification

Protein concentrations were determined using the BCA protein assay kit (Pierce) according to the manufacturer's protocol. Proteins were size-separated by polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970) using the Mini Protean II system (BioRad) in combination with 12% Tris-HCl Ready gels (BioRad) and visualized by colloidal Coomassie Brilliant Blue staining (Neuhoff et al., 1988). Precision Plus Protein standards (BioRad) served as a reference for molecular masses.

Testing the biological activity of heterologously expressed protein

Samples containing crude or purified *B. elliptica* NEP proteins were injected into leaves of three eudicots and three monocots, using a 10 ml syringe without a needle. The eudicots tested were tobacco (*Nicotiana tabacum* cv. Samsun), *N. benthamiana* and Arabidopsis Col-0 (NASC, The European Arabidopsis Stock Centre). The monocots tested were lily (cvs. Stargazer and Vivaldi), rice (cv. Nippon-Bare) and maize. Effects of NEP proteins were also tested on tomato Msk8 cell suspensions; 40 µl of protein was added to 4 ml of a 5-days-old cell culture and incubated in the dark at 24°C, on an orbital shaker at 125 rpm.

Table 1: PCR and RT-PCR primers used in this study

Target gene	Primer name	Primer sequence (5′- 3′)[ab]
BeNEP1	NEP1-5forBE	TCGAGTGATGATAGCAAAATA
	NEP1-5revBE-SOE	tactaacagatatcaagcttGACCTCCAGACCAGCCACCTC
	NEP1-3forBE-SOE	gggtaccgagctcgaattcCCGCCGCATCTGGTCACG
	NEP1-3revBE	GACTTTGGACAGAGTAGTGTAGAA
	NEP1-5NforBE	ATATAAGTATCGACGATGTTCTCT
	NEP1-3NrevBE	AATTTCATTACAGATTCTTT
BeNEP2	NEP2-5forBE	ACCGAAGATTAGAAATGTAGTTAT
	NEP2-5revBE-SOE	tactaacagatatcaagcttCCTCGGACGTATACTTGACC
	NEP2-3forBE-SOE	gggtaccgagctcgaattcGCCCTCAACCGGTATTGGTCAC
	NEP2-3revBE	CACAGGGAAAAGCCAGAATACTCC
	NEP2-5NforBE	AATCCTCCATATTCTTTCCTGTTG
	NEP2-3NrevBE	TCCCAGAGTATAGCCCCCAGTATT
Selection marker	cassette-5 (20)	GAATTCGAGCTCGGTACCC
	cassette-3 (21)	AAGCTTGATATCTGTTAGTA
	screen5-int	GCTTGTTATTGCCAGTGCCGCTTC
	screen3-int	GCACCTCGGACGCAAATCTTGACC
BeNEP1 (RT-PCR)	BeNEP1+1-for-Pp	CCGGAATTC**ATG**TACTTCTCCAACGCA
	BeNEP1+1024-rev-Pp	ATAAGAATGCGGCCGC**CTA**GATTGCAGCCTTGGCGAGGTTG
BeNEP2 (RT-PCR)	BeNEP2+1-for-Pp	CCGGAATTC**ATG**GTTGCCTTCTCAAGATC
	BeNEP2+823-rev-Pp	ATAAGAATGCGGCCGC**CTA**GAAAGTAGCAGCTGCAAGATTGTT

[a] Lowercase letters indicate primer extensions complementary to the selection marker cassette, which are required for single-step overlap-extension PCR.

[b] Underlined are the *Not*I and *Eco*RI restriction sites; start and reverse-complement stop codons are in boldface.

Results

Two sequences, each encoding a protein homologous to the necrosis- and ethylene-inducing protein (NEP) from *F. oxysporum* (Bailey, 1995), were previously amplified from *B. elliptica* strain Be9401 and sequenced (Staats et al., in press). The homolog with highest similarity (64%) to the *F. oxysporum* NEP1 protein was named BeNEP1, whereas the second homolog (with sequence similarity of 54%) was named BeNEP2. Low-stringency Southern hybridization indicated that the *B. elliptica* genome does not contain additional NEP homologous sequences (A. Schouten and E. Dekkers, unpublished). GenBank accession numbers of the full-length *BeNEP1* and *BeNEP2* sequences are AM261028 and AM261029, respectively.

Single gene-replacement mutants were made for *BeNEP1* and *BeNEP2* using the protocol described by Kars et al. (2005). Protoplasts of *B. elliptica* were transformed with a gene replacement construct containing a hygromycin B selection marker cassette flanked by two target gene fragments, which was produced by SOE-PCR. A total of 60 and 40 transformants were obtained for *BeNEP1* and *BeNEP2* in three experiments, respectively. This number was substantially lower than the numbers routinely obtained with *B. cinerea* (our unpublished results). This is the first report of successful transformation of *B. elliptica*. Homologous recombinants were identified by PCR screening according to the strategy described by Kars et al. (2005), using primer combinations that partially spanned the putative recombination sites at both ends of the gene replacement construct. In addition, the recombinants were screened by Southern hybridization according to the protocol in ten Have et al. (1998). The genomic DNA was digested with *Hind*III and blots were hybridized with *BeNEP1*, *BeNEP2*, or *hph* probes (Figure 1).

Three independent *BeNEP1* mutants and one *BeNEP2* mutant were obtained. Judged from the Southern hybridizations, the mutants Δ*BeNEP1*-43366 and Δ*BeNEP2*-6011 appeared to have undergone correct gene replacement without further integration, whereas mutants Δ*BeNEP1*-31298 and Δ*BeNEP1*-2241 contained an additional band of 5.0-kb and 3.3-kb respectively, probably resulting from additional ectopic insertion (Figure 1). Several additional transformants had undergone correct homologous recombination either at the *BeNEP1* or *BeNEP2* locus, however, Southern hybridization indicated that they also contained nuclei with the wild-type locus and therefore were heterokaryotic, as also reported for *B. cinerea* (van Kan et al., 1997).

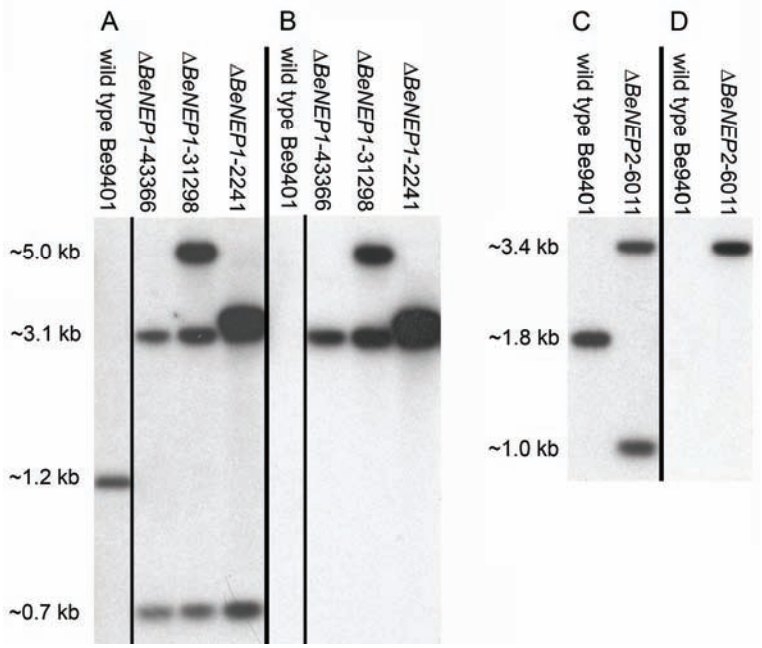

Figure 1: Southern hybridization with gene-specific probes and the selection marker probe to confirm gene replacement in the *B. elliptica* mutants. Genomic DNA was digested with *Hind*III. The genomic DNA of three Δ*BeNEP1* mutants was hybridized with (**A**) *BeNEP1* and (**B**) *hph*. The genomic DNA of one Δ*BeNEP2* mutants was hybridized with (**C**) *BeNEP2* and (**D**) *hph*.

In spite of repeated attempts these heterokaryons could not be resolved into pure, homokaryotic mutants. All three Δ*BeNEP1* mutants were morphologically indistinguishable from the wild-type recipient strain. Mutant Δ*BeNEP2*-6011, however, produced approximately 10^4 times less spores than the wild-type strain or the Δ*BeNEP1* mutant strains (not shown). Furthermore, the radial growth rate of Δ*BeNEP2* on malt agar plates was 30% reduced compared to the wild-type recipient strain (not shown).

The virulence of the three Δ*BeNEP1* mutants and one Δ*BeNEP2* mutant, as well as the wild-type recipient strain Be9401 were tested on lily leaves (cv. Stargazer). Detached leaves from plants that had initiated flowering were inoculated on the abaxial side with *B. elliptica* spore suspension (~10^3 conidia in 3-5µl of half-strength potato dextrose broth). For the wild-type and the four mutant strains (Δ*BeNEP1*-43366, Δ*BeNEP1*-31298, Δ*BeNEP1*-2241, and Δ*BeNEP2*-6011), all inoculation droplets led to development of small water-soaked spots within 36-48 hours post inoculation. These spots developed into spreading lesions concurrent with dark brown or black necrotic lesions and mycelium development (Figure 2). The disease symptoms of the four mutants were similar to the wild-type strain (Figure 2) and their radial lesion sizes were not significantly different (Table 2).

Table 2: Average radial lesion size of wild-type, Δ*BeNEP1*- and Δ*BeNEP2*-deficient strains on lily leaves at 3, 4, and 5 days post inoculation (dpi)

		Radial lesion size ± SD (mm)		
	n	3 dpi[a]	4 dpi[a]	5 dpi[a]
Wild-type	48	7.1 ± 2.1	11.8 ± 2.2	16.9 ± 1.9
Δ*BeNEP1*-43366	12	7.3 ± 1.4	13.1 ± 1.5	18.4 ± 1.6
Δ*BeNEP1*-31298	12	6.4 ± 1.6	12.5 ± 1.6	17.3 ± 1.2
Δ*BeNEP1*-2241	12	6.9 ± 1.5	11.9 ± 1.5	16.8 ± 1.8
Δ*BeNEP2*-6011	12	7.7 ± 1.8	12.7 ± 1.1	17.5 ± 2.1

n = total number of lesions.

[a] = No statistical significant differences in radial lesion size of mutants compared to the wild-type recipient strain. Statistical analyses were performed in SPSS version 12.0.0 (SPSS Inc.). All comparisons of lesions size met the assumption of normality (Kolmogorov Normality test) and homogeneity of variances (Levene test).

For the heterologous expression of both NLPs in *Pichia pastoris*, cDNA fragments of *BeNEP1* and *BeNEP2* were obtained by RT-PCR from *B. elliptica*-infected leaves of the Arabidopsis camalexin-deficient mutant *pad3* (Zhou et al., 1999), which is susceptible to *B. elliptica* (van Baarlen et al., in press). In this way, the difficult mRNA extraction from infected lily leaves could be circumvented. The cDNA fragments were then cloned into the vector pPIC3.5 and transformed into the yeast *P. pastoris*. Expression of BeNEP1 and BeNEP2 protein in batch cultures was induced by addition of methanol to the medium. In the culture filtrate of BeNEP1-expressing yeast, one predominant band of the expected size of 25 kDa was observed while in the BeNEP2-expressing yeast, two bands were observed, both around the expected size of ca. 25 kDa (not shown). The yeast cells transformed with an empty vector did not produce any detectable proteins in the range of 15-35 kDa (not shown).

The yields of the proteins in batch culture were 10-50 μg/ml for BeNEP1 and 300-1000 μg/ml for BeNEP2. BeNEP1 was also produced on large scale in a fermentor and purified using anion exchange chromatography. The biological activity and phytotoxicity of protein samples was determined by infiltration into plant leaves and addition to tomato (*L. esculentum* cv. Moneymaker, line Msk8) cell suspensions.

BeNEP1 (1-10 μg/ml) and BeNEP2 (10-100 μg/ml) were infiltrated into three eudicot plants (*N. tabacum* cv. Samsun, *N. benthamiana* and Arabidopsis Col-0) and three monocot plants (lily cv. Stargazer and Vivaldi, rice cv. Nippon-Bare and maize). Both proteins were infiltrated separately and as a mixture, in the latter case each protein at half the concentration. Neither BeNEP1 and BeNEP2, nor a mixture of both proteins, showed any detectable phytotoxic activity in maize, rice or lily (not shown), even though the lily cultivar used was sensitive to the *B. elliptica* PCD

inducing protein fraction (van Baarlen et al., 2004b). By contrast, the BeNEP proteins induced strong necrotic symptoms in all tested eudicot plants: Arabidopsis, *N. tabacum* (not shown) and *N. benthamiana* (Figure 3). Symptoms were visible within one day after infiltration for BeNEP1 and two days after infiltration for BeNEP2, whereas infiltration of protein samples of yeast cells transformed with an empty vector did not cause necrotic symptoms in any of the tested plants at any time.

In vitro tests of BeNEP1 and BeNEP2 were performed by studying the responses of tomato Msk8 cell suspensions to the same BeNEP protein concentrations as infiltrated into leaves. Protein was added to aliquots of the cell suspension, normally showing an off-white color. Cell suspensions exposed to BeNEP1 protein turned orange within 2-4 hours; cell suspensions exposed to BeNEP2 protein turned a lighter shade of orange/pink within 6-8 hours (not shown). Cells exposed to equivalent amounts of culture filtrates of methanol-induced control yeast (transformed with empty vector) did not change in color and continued growing until the medium was exhausted (not shown).

A fluorescent vital staining technique employing the cell-permeable blue fluorochrome DAPI simultaneously with the red propidium iodide (PI), which cannot enter living cells, enables to distinguish living from dead plant cells in an epifluorescence microscope (e.g. van Baarlen et al., 2004b). Using this combined DAPI-PI stain, it appeared that nearly all cells in control-treated, off-white cell suspensions were alive whereas cells of BeNEP-treated suspensions were mostly dead (Figure 4). Results with BeNEP2 were essentially similar, however, 10 times higher BeNEP2 concentrations were needed for responses to be observed, as compared to BeNEP1 and responses were slower (not shown).

Discussion

This is the first study where NLPs from a pathogen of a monocot host plant are characterized. The gene replacement of *BeNEP1* and *BeNEP2* did not affect the virulence of *B. elliptica* on lily leaves. In addition, the *B. elliptica* NEP proteins were not toxic to lily or the other monocots tested. However, BeNEP1 and BeNEP2 were toxic to all tested eudicots. This finding is in agreement with the necrosis-inducing activity reported for other NLPs, i.e. NLPs from *F. oxysporum* (Bailey, 1995), *Pythium aphanidermatum* (Veit et al., 2001), and *Phytophthora parasitica* (Fellbrich et al., 2002).

In the present study, the necrotic response induced in eudicots depended on

Figure 2: Disease symptoms caused by Δ*BeNEP1*-31298 (top left) and Δ*BeNEP2*-6011 (top right) mutants and wild-type recipient strain Be9401 (bottom left and right) on lily leaves, 5 dpi.

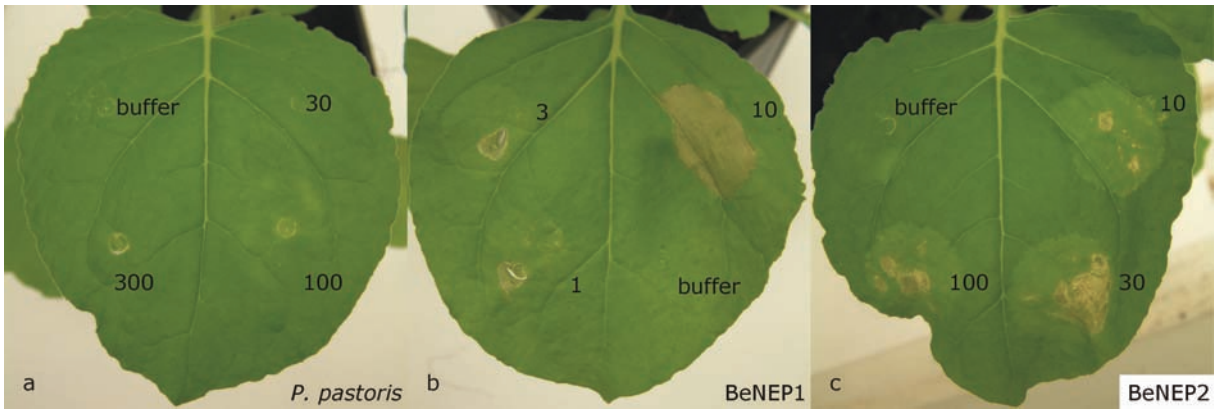

Figure 3: Symptoms in *N. benthamiana* after infiltration of culture filtrates of *P. pastoris* containing (**a**) an empty pPIC3.5 vector, (**b**) pPIC3.5-BeNEP1 or (**c**) pPIC3.5-BeNEP2. Numbers on the leaves indicate the infiltrated protein concentration in microgram. Photographs were taken at 2 dpi, except for figure 3b, which was taken at 1 dpi.

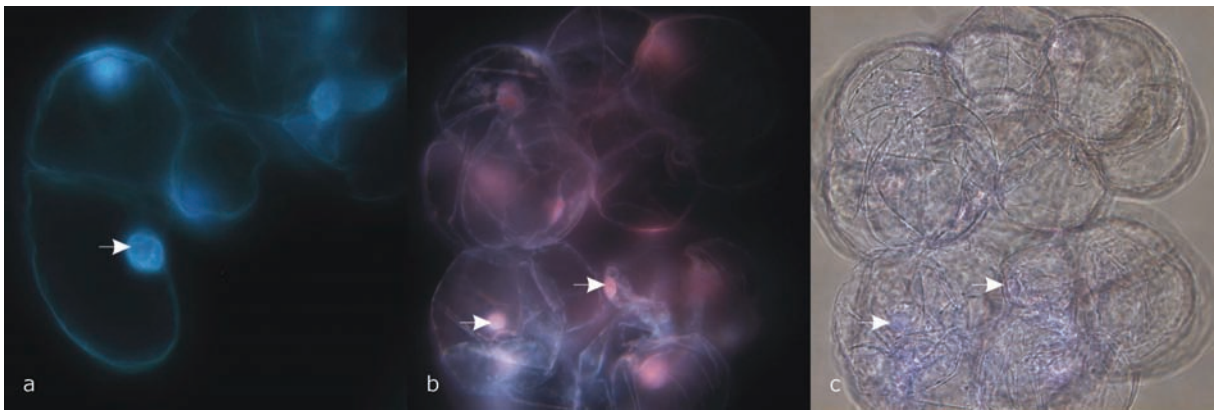

Figure 4: Vital staining of tomato Msk8 suspension cells at 6 hours post addition of culture filtrate of *P. pastoris* transformed with (**a**) an empty pPIC3.5 vector or (**b**) pPIC3.5-BeNEP1. Cells displaying blue nuclei in panel (**a**) were alive and excluded the red PI dye while allowing the blue DAPI to enter. The pink/red stained nuclei in panel (**b**) were not able to exclude the PI dye, due to membrane damage, indicative of cell death. Image in panel (**c**) is the bright-field image of panel (**b**). Arrows indicate nuclei. Results with BeNEP2 were essentially similar.

the BeNEP protein that was infiltrated. BeNEP1 induced a continuous area of dry necrosis, whereas BeNEP2 induced a patchy pattern of necrosis, with areas of green cells interspersed with areas of dry necrosis. Furthermore, plant responses to BeNEP1 were visible approximately one day earlier than responses to BeNEP2. Similar differences in plant responses were observed after infiltration of NEP1 and NEP2 from *Botrytis cinerea*, a species that infects a broad range of eudicots (Schouten et al., unpublished).

The results presented in this study thus reject the hypothesis that NLPs are essential virulence factors for *B. elliptica*, nor do they act as HSTs for *B. elliptica* in the interaction with lily. Therefore, the secreted *B. elliptica* protein(s) that induce programmed cell death in lily (van Baarlen et al., 2004b) still remain to be identified.

Both NLPs from *B. elliptica* can be active and show the familiar necrotic responses after infiltration in eudicots. This may indicate that *B. elliptica* has the option to colonize eudicot hosts, although this has never been observed in nature. The ability to colonize monocots seems to be a feature of certain *Botrytis* species, possibly acquired during the evolution of the species (Staats et al., 2005) allowing them to occupy a novel niche and avoid competition with other (*Botrytis*) species. Further study of the mode of action and localization of NLPs in eudicots might help to understand their lack of toxicity in monocots. A study on this matter, concerning *B. cinerea* NEP1 and NEP2 proteins, will be published elsewhere.

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Chapter 7

General discussion

General discussion

This thesis focuses on *Botrytis* diseases of flower bulb crops with emphasis on the population genetics and pathogenicity of *B. tulipae* and *B. elliptica*. The latter species are host-specific and they are able to cause severe yield losses in tulip and lily, respectively (Chapter 1). This chapter summarizes and discusses the main results of the previous chapters, which had the following objectives: to identify the *Botrytis* species of interest by developing molecular markers (Chapter 2); to evaluate the extent of genetic variation within populations of each species (Chapter 3 & 4); to reveal factors that determine host specificity of *B. tulipae* and *B. elliptica* (Chapter 5 & 6). In addition, this chapter addresses potential areas for future research.

Evolution and phylogeny of *Botrytis*

The genus *Botrytis* contains multiple species that are able to infect flower bulbs crops, of which many species are morphologically similar (Chapter 1). DNA sequence data provided useful additional characters to infer species boundaries. Phylogenetic analyses based on a multi-gene dataset (*RPB2*, *G3PDH*, and *HSP60*) corroborated the morphological species classification (Chapter 2). In addition, the hybrid status of *B. allii* was confirmed (Yohalem et al., 2003). The DNA sequence data have been successfully used by van Doorn et al. (2004) to develop PCR-based tests for the detection of *Botrytis* diseases in flower bulbs crops.

Phylogenetic analysis based on the concatenated sequences provided a highly resolved tree with more resolution than single-gene phylogenies (Chapter 2, figure 4). However, not all species relationships were well-resolved due to incongruence between single-gene analyses. Both analytical and biological factors may cause conflict between gene and species trees (Huelsenbeck, 1995; Maddison, 1997; Graybeal, 1998). Incomplete lineage sorting may account for the incongruent placement of *B. cinerea* strain B05.10 between single-gene phylogenies, as this strain has retained the ancestral polymorphisms of *B. fabae* in the *RPB2* gene region, but not in *G3PDH* and *HSP60*. Analysis of more gene regions may overrule the conflicting signals present between different genes and increase phylogenetic resolution (Soltis et al., 1999; Rokas et al., 2003; Mattern, 2004). The actin, β-tubulin, and translation elongation factor EF-1α encoding genes are widely used in fungal phylogenetic studies (Groenewald et al., 2001; Voigt and Wostemeyer, 2001; Helgason et al., 2003). In addition, the moderately conserved Nep1-like protein (NLP)-encoding genes can be used (Chapter 5).

The genus *Botrytis* is divided into two major clades that have evolved after the division from other *Sclerotiniaceae* genera (Chapter 2, figure 4). Although no common ancestor was found connecting both clades, our data support monophyly of the genus *Botrytis*. Moreover, monophyly was supported by comprehensive phylogenetic studies of the family *Sclerotiniaceae* based on internal transcribed spacer (ITS) sequences (Holst-Jensen et al., 1998; 2004). The use of protein-coding genes, as *RPB2*, *HSP60* and *G3PDH*, will allow for a more detailed study to the connection between *Botrytis* and the closely related genera *Dumontinia*, *Grovesinia*, *Monilinia* and *Sclerotinia*.

The genus *Botrytis* may comprise additional species that have not yet been discovered and which may phylogenetically connect the two *Botrytis* clades. Indeed, additional host-specific species have been reported, but they are not recognized by Hennebert (1973) as these species are poorly characterized. The following species remain to be described: *B. convallariae* (Kleb.) Ondřej on *Convallaria* spp. (monocot, *Ruscaceae*, lily-of-the-valley), *B. ricini* Buchw. on *Ricinus communis* (eudicot, *Acalypheae*, castor bean), *B. aconitincola* (Schumacher and Holst-Jensen, 1997) on *Aconitum septentrionale* (eudicot, *Ranunculaceae*, monkshood) and *B. anthophila* Bondartsev on *Trifolium* spp. (eudicot, *Fabaceae*, clover). Strain CBS122.26 of the latter species, however, appeared to be a species of *Rhizoctonia* based on ITS sequence data (Chapter 2).

Analyses of the patterns of association reflect the occurrence of host jumps but not of cospeciation, as there was little phylogenetic congruence between *Botrytis* species and their angiosperm hosts. For example, *B. elliptica* and *B. tulipae* are not closely related, yet they are both able to infect plants from the monocot family *Liliaceae* (Chapter 2, figure 6C). The mechanism behind the host shifts is unknown. The genera *Alternaria* and *Cochliobolus* contain species that produce a variety of host-selective toxins (HSTs) and as a result have different host ranges (reviewed by Wolpert et al., 2002). The production of HSTs have also been reported for *B. fabae* and *B. elliptica* (Harrison, 1980; van Baarlen et al., 2004b). Additional factors mediating host-parasite specificity that may have facilitated host shifts are those involved in defence compound detoxification, e.g. the detoxification of tulipalin A by *B. tulipae* (discussed below).

Population studies of *B. tulipae* and *B. elliptica*

At the onset of this thesis, the genetic diversity in *B. tulipae* had not been studied previously, whereas one population genetic study had been reported for *B. elliptica* (Huang et al., 2001). In contrast, many studies have focused on *B. cinerea* using a variety of DNA-based genotyping techniques (e.g. Giraud et al., 1997; Giraud et al., 1999; Zhonghua and Michailides, 2005). Three of those techniques were evaluated for their effectiveness to differentiate isolates of *B. elliptica*, *B. tulipae*, and *B. cinerea* (Chapter 3). PCR-RFLP analysis of the IGS region was the least discriminatory method, followed by DNA sequencing of five gene regions (*G3PDH*, *RPB2*, *HSP60*, *H3*, and *EF-1a*) and the internal transcribed spacer region (ITS). The AFLP fingerprinting technique appeared the most discriminatory method, and it was therefore used to assess the genotypic diversity among field isolates of *B. elliptica* and *B. tulipae* collected in the Netherlands (Chapter 4).

An epidemic population structure was found for *B. elliptica*, as was observed by Huang et al. (2001) for *B. elliptica* collections from the USA and Taiwan. Among 174 *B. elliptica* isolates, 105 multilocus AFLP genotypes could be discriminated and 87 genotypes were found only once, reflecting high genotypic diversity. In addition, the levels of genetic disequilibrium were low, which indicates that genetic recombination occurs in the field, as anticipated from the discovery of apothecia in Lisse and the report that *B. elliptica* shows outcrossing (van den Ende and Pennock-Vos, 1997b; Lorbeer et al., 2004). The multilocus association index provided, however, no evidence for random mating.

B. tulipae has mainly a clonal population structure, as evidenced by the low genotypic diversity, repeated recovery of clonal genotypes over long distances and in different years, and strong multilocus associations (Chapter 4). However, the percentages of linked loci are lower than expected for a strictly clonal organism. Therefore, it is possible that some level of recombination would have resulted in generating new genotypes.

Additional evidence for clonality or random mating can be provided by studying associations between AFLP fingerprints and additional DNA markers (e.g. RAPDs or microsatellites) or mycelial compatibility groups (MCGs). For example, evidence for clonality of *Sclerotinia sclerotiorum* was provided by the strong association between RFLPs and MCGs (Kohn et al., 1991). Unfortunately, knowledge about heterokayosis and mycelial (in)compatibility in *B. tulipae* and *B. elliptica* is lacking. Alternatively, a comparison of multiple gene genealogies allows the mode of

reproduction to be inferred (Koufopanou et al., 1996; Carbone and Kohn, 2004).

NEP1-like proteins (NLPs): molecular evolution and pathogenicity

NLPs are phytotoxic proteins that are able to induce diverse defence-related responses including active oxygen and ethylene production, callose apposition, necrosis and programmed cell death (Fellbrich et al., 2002; Keates et al., 2003; Bae et al., 2006). However, the function of NLPs, apart from the induction of plant defence mechanisms, for the organisms that possess them is unknown (reviewed by Gijzen and Nürnberger, 2006). The absence of NLP-encoding genes from plant genomes implies that the proteins are potentially of immunological relevance by acting as pathogen-associated molecular patterns (PAMPs). The necrotic response to NLPs may reflect a defence mechanism resulting from the perception by the plant of NLPs as a non-self molecule. Such a response may be suitable to restrict infection by biotrophic pathogens, but on the other hand it may be exploited by pathogens with a necrotrophic lifecycle. However, there is increasing evidence suggesting that NLPs are directly toxic to plant cells by disturbing cuticle integrity, thylakoid membranes and altering membrane permeability (Jennings et al., 2001; Keates et al., 2003).

Two NLP-encoding genes, designated *NEP1* and *NEP2*, were present in all known *Botrytis* species (Chapter 5). The predicted secondary structure is generally conserved between NEP1 and NEP2 proteins, despite the low sequence similarity between them. Nevertheless, both NLPs may have different functions, as they are predicted to contain different types of posttranslational modification motifs.

The *Botrytis* NLPs have evolved at higher evolutionary rates than three housekeeping genes (*RPB2*, *HSP60*, and *G3PDH*) in the same species (Chapter 5). Analysis of amino acid substitution rates were conducted using maximum likelihood-based models that have also been used to identify codons under diversifying selection in genes involved in defence systems or immunity, and toxin protein genes (Bishop et al., 2005; Liu et al., 2005; Nielsen et al., 2005). These analyses indicated that most of the protein encoding regions are under purifying selection, but the analysis also identified specific positions under diversifying selection. Seven residues in NEP1 and one residue in NEP2 appeared to be under diversifying selection. The biological significance of the selection pressure on these amino acid substitutions remains to be validated. The selection pressure may have acted on NLPs to promote plant cell death in different hosts, such that the toxic activity of a particular NLP may determine the host range of *Botrytis* species.

In chapter 6, we examined whether NLPs could act as virulence factors for *B. elliptica*. Gene replacement mutant strains of *BeNEP1* and *BeNEP2* showed normal virulence on lily leaves. In addition, both *B. elliptica* NLPs that were produced in the yeast *Pichia pastoris* were not toxic to monocots, including lily (Chapter 6). Therefore, our results reject the hypothesis that NLPs are essential virulence factors for *B. elliptica*. Furthermore, NLPs are not the proteinaceous factors that act as Host-Selective Toxins in the *B. elliptica*-lily pathosystem (van Baarlen et al., 2004b).

The NLP proteins of *B. elliptica* were able to induce necrotic symptoms in non-host eudicots, thereby supporting the reported necrosis-inducing activity of other NLPs towards eudicots only (Bailey, 1995; Veit et al., 2001; Fellbrich et al., 2002). Interestingly, monocots were also not affected by NEP2 of *B. tulipae*, and NEP1 and NEP2 of *B. cinerea* (Schouten et al., 2006; van Baarlen, unpublished). Possibly, NLPs are recognised only by dedicated receptor(s) that occur on the surface of the cell membrane in eudicots.

It would be interesting to investigate whether NLPs do contribute to virulence of *Botrytis* species on eudicots. If so, NLP genes in specialist pathogens of monocots may not have a biological function at all. In that case, NLPs of monocot pathogens are expected to be selected against during evolution. Indeed, putative NEP1 pseudogenes were detected in *B. sphaerosperma* and *B. globosa*, two pathogens of the monocot plant genus *Allium* (Chapter 5). Alternatively, one could envisage that NLPs have a yet unidentified general function besides their cell death-inducing activity in eudicots. Indeed, *BeNEP1* and *BeNEP2* were transcriptionally active in the wild-type strain during leaf infection of *Arabidopsis thaliana*. However, it is unknown whether both genes are also expressed in the natural host lily. Time-course RNA hybridization studies will provide a better understanding of the expression of the *BeNEP1* and *BeNEP2* genes at various stages of the life and disease cycle. Furthermore, the recent discovery of new classes of NLPs fused to additional domains, suggests that they may have multiple and varied functions. Expressed genes from *Phytophthora soja*, *P. ramorum*, and *Hyaloperonospora parasitica* encode NLPs containing an additional glutamine and proline-rich hydrophylic domain (Gijzen and Nürnberger, 2006). Interestingly, a gene with an NLP domain fused to a ricin B domain was present in the *Bacillus thuringiensis* serovar *israelensis* genome (GenBank accession EAO54154). The functions of these new classes of NLPs remain to be investigated.

Plant defense compounds as mediators of *Botrytis* non-host resistance.

The production of phytoalexins by plants is one of the induced defense mechanisms to prevent the development of pathogens. Other defense mechanisms include the physical reinforcement of cell walls through production of callose and lignin, the generation of active oxygen species and nitric oxide, and the expression of pathogenesis-related proteins (van Loon, 1999; Heath, 2002; Bhattacharjee, 2005; Delledonne, 2005). The role of phytoalexins in relation to *B. cinerea* has been studied in various plant species (reviewed by van Baarlen et al., 2004a). The best studied class of phytoalexins are the stilbenes, such as resveratrol, viniferin and pterostilbene from grape, which may accumulate to toxic levels in response to infection by *B. cinerea* (Jeandet et al., 1995; Jeandet et al., 2002).

Phytoalexins are also produced in several flower bulb crops. In narcissus, phytoalexins are involved in resistance against non-host *Botrytis* species, but not against the host-specialized species *B. narcissicola*. Inoculation of narcissus bulbs with *B. cinerea* induces the synthesis of flavan phytoalexins (Coxon et al., 1980). *B. narcissicola* is equally sensitive to the phytoalexins as *B. cinerea* (O'Neill and Mansfield, 1982). However, in the compatible interaction of narcissus and *B. narcissicola* accumulation of phytoalexins and cell wall modifications do not occur. Apparently, *B. narcissicola* is able to suppress these mechanisms of resistance or break down the phytoalexins (O'Neill and Mansfield, 1982). The cell wall modification response was also absent in lily and tulip cultivars during compatible interactions, but not during incompatible interactions with *B. cinerea*, *B. tulipae*, and *B. elliptica* (van Baarlen et al., 2004a).

In the bulbs of *Lilium maximowcii* several chlorine-containing phytoalexins and yurinelide are produced upon attack by *Fusarium oxysporum* f. sp. *lilii* (Monde et al., 1992; Monde et al., 1998). It is unknown whether these compounds are also produced in the interaction with *Botrytis*. We have performed experiments to extract fungitoxic compounds from lily leaves that were challenged with *B. cinerea* and *B. elliptica*, and from unchallenged leaves, but we were not able to identify any metabolites with antifungal activity on thin-layer chromatographs (M. Staats, unpublished results).

Tulip plants and various other liliiflorous plants contain tulipalins (Slob et al., 1975). Tulipalins are unsaturated lactones with fungitoxic activity that are stored in non-toxic glycosylated forms (tuliposides) in mesophyll cells (Schönbeck and Schlösser, 1976; Rutter et al., 1977). The white skin of tulip bulbs and pistils contain high concentrations of tuliposide A (Bergman, 1966; van Rossum et al., 1998). The

tulipalins are released upon pathogen attack, which can be spontaneous as a result of a pH shift (the tuliposide is unstable above pH 5.5) or by enzymatic conversion (Beijersbergen and Lemmers, 1972). *B. tulipae* is more tolerant to tulipalins than *B. cinerea*. Furthermore, *B. cinerea* converts tuliposides into toxic lactones, whereas non-toxic hydroxylic acids are formed by *B. tulipae* (Schönbeck and Schroeder, 1972). *B. tulipae* may contain an enzyme that is able to detoxify tulipalins. Indeed, a protein extract from *B. tulipae* was able to neutralize tulipalin A, whereas extracts from *B. cinerea* and *B. elliptica* were not (P. van Baarlen and M. Staats, unpublished results). Enzymes with lactone-hydrolyzing activity have been detected in several organisms (Shimizu et al., 2001). For example, a lactonohydrolase of *Fusarium oxysporum* catalyzes the stereoselective hydrolysis of D-pantoyl lactone (Shimizu et al., 1992; Kobayashi et al., 1998). *B. tulipae* may also possess a lactonohydrolase with specific activity towards tulipalin. Such an enzyme might act as a host specificity determinant for *B. tulipae*. Indeed, the *B. cinerea* genome sequence contains a gene with homology to the *F. oxysporum* lactonohydrolase gene, but this gene remains to be characterized. It remains to be determined whether a homolog of such a gene is present in *B. tulipae*.

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Summary

Fungi of the genus *Botrytis* (teleomorph *Botryotinia*) can cause serious damage in a large range of ornamental crops. Except for *B. cinerea*, *Botrytis* species that are pathogenic on flower bulb crops are host-specific, i.e. each species is able to infect only one or a few closely related host species. This thesis mainly focuses on the economically important species *B. elliptica* and *B. tulipae*, the causal agents of leaf blight in lily and tulip, respectively.

Molecular markers were developed that allowed for the unambiguous identification of the *Botrytis* species of interest and to study their intraspecific variation (Chapter 2, 3 & 4). Chapter two describes a classification of the genus *Botrytis* based on DNA sequence data of three protein-coding genes (*RPB2*, *G3PDH* and *HSP60*). The phylogenetic analysis that encompassed all 22 species of the genus corroborated the classical species delineation. In addition, the hybrid status of *B. allii* (*B. byssoidea* X *B. aclada*) was confirmed. A comparison of *Botrytis* and angiosperm phylogenies showed that cospeciation of pathogens and their hosts have not occurred. Rather, it is proposed that host shifts have occurred during *Botrytis* speciation.

Chapter three describes three molecular typing methods to differentiate isolates of *B. tulipae*, *B. elliptica* and *B. cinerea*. Restriction analysis of the intergenic spacer (IGS) region was the least discriminatory, followed by DNA sequencing of five gene regions (*G3PDH*, *RPB2*, *HSP60*, *H3* and *EF-1a*) and the internal transcribed spacer (ITS) region. Amplified fragment length polymorphism (AFLP) analysis appeared the most discriminatory method. It was, therefore, used to assess the genotypic diversity among field isolates of *B. elliptica* and *B. tulipae* from the Netherlands (Chapter 4). In addition, analyses were performed to infer their modes of reproduction. Isolates of both species were sampled during successive growth seasons from experimental field plots in Lisse and from several other locations in the Netherlands. The genotypic diversity for *B. elliptica* was high and clonal genotypes were found only within growing seasons. Linkage disequilibrium analyses strongly supported the occurrence of genetic recombination in the field, most likely as a result of sexual reproduction. Multilocus association analyses, however, provided no evidence for random mating.

B. tulipae has a mainly clonal population structure, as evidenced by the low genotypic diversity, the repeated recovery of clonal genotypes over long distances

and over different years, and the strong multilocus associations. However, the level of linkage disequilibrium is lower than expected for a strictly clonal organism. Therefore, it is possible that some level of recombination would have resulted in generating new genotypes.

In chapters 5 and 6 the roles of Nep1-like proteins (NLPs) as determinants of host specificity are investigated. Two NLP-encoding genes, designated *NEP1* and *NEP2*, were present in all *Botrytis* species (Chapter 5). Both NLPs may have different functions as their sequence similarity is low and because they contain different types of posttranslational modification motifs. While both NLPs are moderately conserved and are overall under purifying selection, a number of amino acid residues were predicted to be under positive selection based on inferences using maximum likelihood models. The functional effects of these amino acid replacements on the NEP protein activity remain to be resolved.

Chapter 6 describes the functional characterization of *NLP* genes from *B. elliptica*. Mutants of *B. elliptica* in which *BeNEP1* or *BeNEP2* was replaced showed normal virulence on lily leaves. In addition, the *B. elliptica* NLPs that were produced in the yeast *Pichia pastoris* were not toxic to monocots, including lily. These results show that NLPs are not essential virulence factors and do not function as host-selective toxins for *B. elliptica*.

Chapter 7 presents a general discussion of the thesis and provides future directions to study the evolution, life cycles and pathogenicity of *Botrytis* species on flower bulb crops.

Samenvatting

Schimmels van het geslacht *Botrytis* (teleomorph *Botryotinia*) kunnen ernstige schade veroorzaken in een groot aantal siergewassen. Met uitzondering van *B. cinerea* zijn alle *Botrytis* soorten die pathogeen zijn op bloembolgewassen waardplantspecifiek, d.w.z. elke soort is in staat om slechts één of meer nauw verwante waardplantsoorten te infecteren. Dit proefschrift richt zich voornamelijk op de economisch belangrijke soorten *B. elliptica* en *B. tulipae*, de veroorzakers van 'vuur' in respectievelijk lelie en tulp.

Om een eenduidige identificatie van *Botrytis* soorten mogelijk te maken en om hun intraspecifieke variatie te bestuderen, zijn moleculaire merkers ontwikkeld (Hoofdstukken 2, 3 & 4). Hoofdstuk 2 beschrijft een indeling van het geslacht *Botrytis* op basis van DNA sequentie data van drie eiwitcoderende genen (*RPB2*, *G3PDH* en *HSP60*). De fylogenetische analyse die alle 22 soorten van het geslacht *Botrytis* omvat, bevestigde de klassieke soortindeling. Daarnaast werd de hybride status van *B. allii* (*B. byssoidea* X *B. aclada*) bevestigd. Een vergelijking tussen de fylogenieën van *Botrytis* en angiospermen toonde aan dat co-speciatie van pathogenen en hun gastheren niet is voorgekomen. Mogelijk hebben er gedurende de *Botrytis* soortvorming sprongsgewijze veranderingen in de waardreeks plaatsgevonden.

Hoofdstuk 3 beschrijft drie moleculaire identificatiemethoden om onderscheid te maken tussen isolaten van *B. tulipae*, *B. elliptica* en *B. cinerea*. Restrictieanalyse van de intergenic spacer (IGS) regio had het laagst onderscheidend vermogen, gevolgd door DNA sequencing van vijf gen regio's (*G3PDH*, *RPB2*, *HSP60*, *H3* en *EF-1a*) en de internal transcribed spacer (ITS) regio. Amplified fragment length polymorphism (AFLP) analyse had het hoogst onderscheidend vermogen en daarom werd deze methode gebruikt om de genotypische diversiteit te bepalen van veldisolaten van *B. elliptica* en *B. tulipae* (Hoofdstuk 4). Bovendien zijn er aan de hand van de AFLP data analyses uitgevoerd om te bepalen of deze soorten zich seksueel of aseksueel voortplanten. Isolaten werden bemonsterd gedurende opeenvolgende groeiseizoenen in proefvelden in Lisse en op verscheidene andere locaties in Nederland. De genotypische diversiteit van *B. elliptica* was hoog en klonen werden alleen binnen groeiseizoenen gevonden. 'Linkage disequilibrium' analyse toonde aan dat er genetische recombinatie in het veld heeft plaatsgevonden, die waarschijnlijk het gevolg is van seksuele reproductie. Er was echter geen bewijs voor een 'random mating populatie' voor *B. elliptica*.

B. tulipae heeft een hoofdzakelijk klonale populatiestructuur zoals blijkt uit de lage genotypische diversiteit, de herhaalde bemonstering van klonen over grote afstanden en over verschillende jaren, en de sterke multilocus associaties. Echter, de hoeveelheid linkage disequilibrium is lager dan verwacht mag worden voor een strikt klonaal organisme. Het is daarom niet uit te sluiten dat recombinatie binnen de *B. tulipae* populatie heeft geleid tot het ontstaan van nieuwe genotypes.

In hoofdstukken 5 en 6 wordt onderzocht of Nep1-like proteins (NLPs) belangrijke factoren zijn voor waardplantspecificiteit. Twee *NLP* coderende genen, genaamd *NEP1* en *NEP2*, waren aanwezig in alle *Botrytis* soorten (hoofdstuk 5). Beide NLPs zouden verschillende functies kunnen hebben aangezien hun sequentie overeenkomsten laag zijn en omdat ze verschillende sequentiemotieven bevatten voor posttranslationele modificaties. Beide NLPs zijn matig geconserveerd en staan onder 'purifying selection'. Echter, een aantal aminozuurresiduen staan, op basis van voorspellingen met maximum likelihood modellen, onder positieve selectie. De gevolgen van deze aminozuurveranderingen op de NEP eiwitactiviteit moeten nog worden onderzocht.

Hoofdstuk 6 beschrijft de functionele karakterisering van *NLP* genen van *B. elliptica*. Mutanten van *B. elliptica* waarin *BeNEP1* of *BeNEP2* was uitgeschakeld, vertoonden normale virulentie op leliebladeren. Bovendien waren de *B. elliptica* NLPs, die waren geproduceerd in de gist *Pichia pastoris*, niet toxisch voor monocotylen, inclusief lelie. Deze resultaten tonen aan dat NLPs geen essentiële virulentie factoren zijn en dat ze niet functioneren als waardplant-specifieke toxines voor *B. elliptica*.

Hoofdstuk 7 geeft een algemene discussie van het proefschrift en geeft de richting aan voor vervolgonderzoek naar de evolutie, levenscycli en pathogeniciteit van *Botrytis* soorten op bloembolgewassen.

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Martijn Staats

Curriculum Vitae

Martijn Staats werd op 1 juni 1977 geboren te Utrecht. In 1996 behaalde hij zijn VWO-diploma aan het Isendoorn College te Warnsveld. Hierna is hij Plantenveredeling en Gewasbescherming gaan studeren aan de Wageningen Universiteit met als specialisatie Ecologische Gewasbescherming. Zijn eerste afstudeervak werd uitgevoerd bij het Laboratorium voor Nematologie van de Wageningen Universiteit onder begeleiding van Dr. ir. J. Helder en ing. S. J. J. van den Elsen. Binnen dit afstudeervak is onderzoek gedaan naar de fylogenetische verwantschappen tussen soorten van de Orde Aphelenchida op basis van het 18S rDNA. Zijn tweede afstudeervak heeft hij uitgevoerd bij het Laboratorium voor Fytopathologie van de Wageningen universiteit onder begeleiding van Dr. J. M. Raaijmakers en ir. M. Bergsma-Vlami. Hier heeft hij onderzoek gedaan naar de genetische diversiteit en de waardplantspecialisatie van 2,4-diacetylphloroglucinol producerende *Pseudomonas* spp. Zijn studie aan de Wageningen Universiteit werd in augustus 2001 afgerond. Van januari 2002 tot maart 2006 was hij als AIO werkzaam bij het Laboratorium voor Fytopathologie van de Wageningen Universiteit onder begeleiding van Dr. J. A. L. van Kan en Dr. ir. P. van Baarlen. Het onderzoek werd gefinancierd door STW en was getiteld "*Botrytis* diseases in flower bulbs: molecular diagnostics and host specificity". De resultaten van het promotieonderzoek staan beschreven in dit proefschrift. Sinds september 2006 is hij werkzaam als onderzoeker moleculaire fytopathologie bij Enza Zaden.

List of publications

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Staats M, van Baarlen P, Schouten A and van Kan JAL (In press) Functional analysis of NLP genes from *Botrytis elliptica*. Molecular Plant Pathology

Staats M, van Baarlen P, Schouten A, van Kan JAL and Bakker FT (In press) Positive selection in phytotoxic protein-encoding genes of *Botrytis* species. Fungal Genetics and Biology

Staats M, van Baarlen P and van Kan JAL (In press) AFLP analysis of genetic diversity in populations of *Botrytis elliptica* and *Botrytis tulipae*. European Journal of Plant Pathology

Staats M, van Baarlen P and van Kan JAL (To be submitted) Comparison of molecular typing methods for studying intraspecific variation in three *Botrytis* species.

Stergiopoulos I, Groenewald M, Staats M, Lindhout P, Crous PW and de Wit PJGM (In press) Mating-type genes and the genetic structure of a world-wide collection of the tomato pathogen *Cladosporium fulvum*. Fungal Genetics and Biology

van Baarlen P, Staats M and van Kan JAL (2004) Induction of programmed cell death in lily by the fungal pathogen *Botrytis elliptica*. Molecular Plant Pathology 5: 559-574

van Baarlen P, Woltering EJ, Staats M and van Kan JAL (In press) Histochemical and genetic analysis of host and non-host interactions of Arabidopsis with three *Botrytis* species: an important role for cell death control. Molecular Plant Pathology

Education Statement of the Graduate School
Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: Martijn Staats
Date: 15 January 2007
Group: Phytopathology, Wageningen University

1) Start-up phase	date
► **First presentation of your project**	
Botrytis diseases in flower bulbs: molecular diagnostics and host specificity	14 Jun 2002
► **Laboratory use of isotopes**	
Safe handeling with radioactive materials and sources	Nov 2002
Subtotal Start-up Phase	*3.0 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
Utrecht University	27 Mar 2003
Vrije Universiteit Amsterdam	03 Jun 2004
► **EPS theme symposia**	
Theme 2 'Interactions between Plants and Biotic Agents', Wageningen University	12 Dec 2003
Theme 2 'Interactions between Plants and Biotic Agents', Utrecht University	17 Sep 2004
Theme 4 'Genome plasticity', Wageningen University	09 Dec 2004
► **NWO Lunteren days and other National Platforms**	
Annual Lunteren meeting Experimental Plant Sciences	15-16 Apr 2002
Annual Lunteren meeting Experimental Plant Sciences	07-08 Apr 2003
► **Seminars (series), workshops and symposia**	
Willie Commelin Scholten dag, Utrecht University	30 Jan 2003
Duits-Nederlandse Botrytis workshop, Wageningen	30 Jun 2003
Duits-Nederlandse Botrytis workshop, Münster, Germany	17 Nov 2003
Symposium 'Evolutionary consequences of life without sex' UvA, Amsterdam	24 Nov 2003
CBS/Wageningen Phytopathology symposium, Utrecht	2003
Willie Commelin Scholten dag, Utrecht University	22 Jan 2004
KNPV voorjaarsvergadering, Wageningen	24 Mar 2004
Current Themes in Ecology "Experimental Evolution", Wageningen	02 Apr 2004
Current Themes in Ecology "Ecological and Evolutionary Genomics", Wageningen	29 Apr 2005
Duits-Nederlandse Botrytis workshop, Kaiserslautern, Germany	06-07 Jun 2005
NVvM Sectie Mycologie, Utrecht	Nov 2005
► **International symposia and congresses**	
7th International Mycological Congress (ICM7), Oslo, Norway	11-17 Aug 2002
XIII International Botrytis symposium, Antalya, Turkey	25-31 Oct 2004
10th Congress European Society for Evolutionary Biology (ESEB), Krakow, Poland	15-20 Aug 2005
1st Botrytis Genome Workshop, Kaiserslautern, Germany	050-8 Oct 2005
► **Presentations**	
7th International Mycological Congress (ICM7), Oslo, Norway (poster presentation)	11-17 Aug 2002
Duits-Nederlandse Botrytis workshop, Münster, Germany	17 Nov 2003
KNPV voorjaarsvergadering, Wageningen (oral presentation)	24 Mar 2004
In the Tracks of Evolution, Wageningen (oral presentation)	26 Mar 2004
XIII International Botrytis symposium, Antalya, Turkey (oral presentation)	25-31 Oct 2004
Duits-Nederlandse Botrytis workshop, Kaiserslautern, Germany (oral presentation)	06-07 Jun 2005
10th Congress European Society for Evolutionary Biology (ESEB), Krakow, Poland (poster presentation)	15-20 Aug 2005
1st Botrytis Genome Workshop, Kaiserslautern, Germany (oral presentation)	05-08 Oct 2005
NVvM Sectie Mycologie, Utrecht (oral presentation)	Nov 2005
In the Tracks of Evolution, AFLP meeting, Wageningen (oral presentation)	Dec 2005
► **IAB interview**	04 Jun 2004
Subtotal Scientific Exposure	*20.9 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
Autumn School "Disease Resistance in Plants"	14-16 Oct 2002
EPS PhD Course "Molecular Phylogenies; reconstruction and interpretation"	04-07 Nov 2003
► **Journal club**	
Participant in literature discussion group "In the Tracks of Evolution"	2002-2005
Subtotal In-Depth Studies	*5.1 credits**

4) Personal development	date
► **Skill training courses**	
Working with Endnote, Wageningen University	2004
Scientific Art Work, Wageningen University	2004
Scientific Writing, Language Centre Wageningen	28 Mar-23 May 2004
Subtotal Personal Development	*2.7 credits**

TOTAL NUMBER OF CREDIT POINTS*	**31,7**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 credits

** A credit represents a normative study load of 28 hours of study*

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