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FULL RESEARCH PAPER

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

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Abstract The objective of this study was to assess the genetic diversity and to infer the mode of reproduction of *Botrytis elliptica* and *B. tulipae* in the Netherlands. First, three molecular typing methods were compared for their ability to differentiate isolates of *B. tulipae*, *B. elliptica*, and *B. cinerea*. The methods compared were multilocus sequencing, restriction analysis of the ribosomal intergenic spacer (IGS) region, and amplified fragment length polymorphism (AFLP) analysis. AFLP fingerprinting provided the most efficient method to differentiate isolates within each *Botrytis* species and therefore this method was used for population analyses of *B. elliptica* and *B. tulipae*. Isolates of both species were sampled during successive growing seasons in experimental field plots in Lisse and other locations in the Netherlands. Among 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 four 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.

Keywords *Botrytis* · AFLP · Genotypes · Clonal diversity · Recombination

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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 begin

producing conidia that cause spreading leaf infections, commonly referred to as leaf blight or 'fire' (Lorbeer, Seyb, de Boer, & van den Ende, 2004). *Botrytis 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 (Doornik & Bergman, 1973). In contrast, *B. elliptica* only rarely attacks bulbs and bulb infections are not important for survival in the field (Lorbeer et al., 2004). 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).

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 & Pennock-Vos, 1997). Development of apothecia is limited to early spring when the environmental conditions are suitable. Experimentally produced sexual crossings showed that *B. elliptica* is heterothallic (outcrossing) with a diallelic compatibility system (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, Hsieh, Chastagner, & Hsiang, 2001). Most population genetic studies in *Botrytis* (reviewed by Beever & Weeds, 2004) have focused on *B. cinerea* using a variety of molecular techniques including RFLP analysis of PCR-amplified DNA regions (Giraud, Fortini, Levis, Leroux, & Brygoo, 1997), PCR detection of transposable elements (Diolez, Marches, Fortini, & Brygoo, 1995; Levis, Fortini, & Brygoo, 1997), PCR fingerprinting of the whole genome by RAPD analysis or amplified fragment length polymorphism (AFLP) analysis (Moyano, Alfonso, Gallego, Raposo, & Melgarejo, 2003), fingerprinting of repetitive sequences by microsatellite primed (MP)-PCR (Zhonghua & Michailides, 2005), PCR amplification of microsatellite loci (Fournier et al., 2002) and DNA sequencing of gene regions (Albertini, Thebaud, Fournier, & Leroux, 2002; Fournier et al., 2003).

In this study, three DNA-based methods were compared for their potential to differentiate isolates of *B. elliptica, B. tulipae* 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 most discriminatory method was then used as a tool to study the genetic diversity in field isolates of *B. tulipae* and *B. elliptica* 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.

Materials and methods

Collection of isolates

Isolates used for comparison of fingerprinting methods are listed in Table 1. Isolates of *B. tulipae* and *B. elliptica* were provided by I. Pennock-Vos and Dr. M. de Boer of Applied Plant Research (PPO) in Lisse, the Netherlands. Seven *B. cinerea* isolates have been previously used to study genetic variation by RAPD analysis (Kerssies, Bosker-van Zessen, Wagemakers, & van Kan, 1997; Van der Vlugt-Bergmans, Brandwagt, van't Klooster, Wagemakers, & van Kan, 1993). Additional isolates of *B. cinerea* were provided by Paul and Bettina Tudzynski (WWU Münster, Germany) and Paul van den Boogert (Plant Research International, Wageningen, the Netherlands).

For population analyses, *B. elliptica* and *B. tulipae* isolates were collected in Lisse and other locations in the Netherlands (Table 2). In addition, the *B. elliptica* and *B. tulipae* isolates listed in Table 1 were included. The isolates

Table 1 Sample date, sample location, and GenBank accession no. of isolates used for the comparison of three fingerprinting methods

Species	Isolate	Sample date	Location/Region	GenBank accession number of sequences						
				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.
	B05.10	1994	Germany	AJ705002[f]	AJ745674[f]	AJ716063[f]	AM233549	AM235186	AM235297	n.d.
	SAS56[c]	<1990	Italy	AJ705006[f]	AJ745677[f]	AJ716067[f]	AM233551	AM235187	AJ716294[f]	AM233400
	SAS405[c]	<1990	Italy	AJ705005[f]	AJ745678[f]	AJ716066[f]	AM233550	AM235182	AM235293	n.d.
	M14	Unknown	Unknown	AM231157	AM231315	AM232670	AM233546	AM235181	AM235299	n.d.
	BcIPO	Unknown	Unknown	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

Table 2 Sample date, number of isolates, and sample location of *B. tulipae* and *B. elliptica* isolates used for population analyses

Species name	Sample date	Number of isolates	Location/province
B. tulipae	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)
B. elliptica	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)

collected in Lisse were sampled from tulip or lily plants grown in experimental field plots (45 m × 12 m) of Applied Plant Research, Flower Bulb Research Unit (PPO). *Botrytis* isolates were collected as primary necrotic spots from leaves of tulip cv. Bellona and Oriental lily cv. 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, plots were approximately 100 m away from the original location. In 2003, dry weather conditions prevented disease from developing in the tulip plot and only 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. 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. Primary infections of *B. elliptica* on lily were sampled on 9 September 2002 (collection LISSE09-09-02) and 18 August 2003 (collection 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. Two additional collections consisted of either *B. elliptica* isolates or *B. tulipae* isolates sampled in different years and different locations in the Netherlands. These isolates mainly originated from infected plant material provided by commercial bulb growers and are listed in Tables 1 and 2.

All isolates were obtained from diseased leaves by surface-sterilizing in 1% hypochlorite for 1 min, followed by a rinse in sterile distilled water. Necrotic spots of approximately 3 mm 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. Mycelial tissue was harvested, lyophilized, and DNA was extracted as described in Staats, van Baarlen, and van Kan (2005).

Amplification and sequencing of DNA regions

DNA sequences of seven genomic regions were obtained to screen for intraspecific sequence polymorphisms among 10 isolates of each of three *Botrytis* species (Table 1). Four primer combinations were used to PCR amplify and sequence regions of genes encoding glyceraldehyde-3-phosphate dehydrogenase (G3PDH), Heat-shock Protein 60 (HSP60), DNA-dependent RNA polymerase subunit II (RPB2) and the ITS regions (ITS1, ITS2, and 5.8S rDNA) as described by Staats et al. (2005). The same programme, but with an annealing temperature of 64°C, was applied for amplifying H3 and EF-1α gene fragments using primers listed in Table 3. The full-length IGS region was amplified using primers NS1R and NL2F (Carbone, Anderson, & Kohn, 1999; White, Bruns, Lee, & Taylor, 1990) and sequenced only for *B. elliptica* strain BE9612, *B. tulipae* strain BT9601, and *B. cinerea* strain SAS56 (Table 1). PCR amplification of the IGS region was performed in a 50 µl reaction volume and contained 0.2 pmol of each primer (Amersham Pharmacia Biotech), 20 ng of template DNA, 2.0 U of Expand High Fidelity enzyme mix (Roche Applied Science) and 200 µM of each dNTP (Promega). Amplifications were carried out in a Peltier Thermal Cycler-200 (Biozym, Landgraaf, the Netherlands) using the following programme: 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 gel, containing ethidium bromide and cloned as described by Staats et al. (2005). All sequences were determined by BaseClear (Leiden, the Netherlands). A total of 142 new DNA sequences are deposited in GenBank under accession numbers listed in Table 1. Sequence alignments of 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

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. Two primer sets were designed that each amplify an approximately 1500 bp fragment of the IGS region and of which the amplification products do not overlap. 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

Table 3 Primer sets and corresponding amplification targets

Primer name	Locus	Primer sequence (5′–3′)[a]
H3for+	H3	gtgactgtaaaacgacggccagtATGGCTCGTACCAAGCAAACTG
H3rev+		gtgaccaggaaacagctatgaccCGCTCACCACGGAGACGACG
EF1alpha-for+	EF-1α	gtgactgtaaaacgacggccagtCAAGTACGCATGGGTTTTGGACAA
EF1alpha-rev+		gtgaccaggaaacagctatgaccACTTGCAAGCAATGTGAGCAGTGTG
IGSnr1for	IGS	CACGGTCCTGTAAGTAGTAGA
IGSnr1rev		GGGAGCTCACCGGGAACCTTA
IGSnr3for	IGS	TCCCGGTGAGC(C/T)TTTTA
IGSnr3rev		CATCGGCCACTAATCCACAGACTC

[a] Nucleotides in lower case represent extensions of the primers that were used for sequencing

starting at position 1338 of the *B. cinerea* SAS56 sequence (Table 3). The fragment size of the IGS region with primer set IGSnr1 for *B. elliptica* was 170 bp larger, due to the presence of an additional sequence in this region. The degenerate forward primer of primer set IGSnr3 is at position 1601–1617 of the IGS region of *B. cinerea* SAS56 and the reverse primer is in the conserved 18S rDNA region (Table 3).

The PCR protocol of 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 with restriction enzymes *Rsa*I, *Hinc*II, *Hind*III, *Bam*HI, *Taq*I, and *Hae*III (Promega). DNA fragments were separated by electrophoresis in 1.5% agarose gels.

AFLP analysis

The AFLP 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 in 50 µl of One-Phor-All^{TM} buffer (Pharmacia) with 10 mM ATP (Pharmacia) and 5 mM DTT (Sigma) at 37°C for 6–8 h. The ligation products were diluted 10 times in 10 mM Tris–HCl (pH 8.0) and 0.1 mM EDTA. Pre-amplification of the template was performed in a 50 µl reaction volume using non-selective primers *Eco*RI and *Mse*I (Vos et al., 1995). The amplified products were checked on 1.5% agarose gels. Selective PCR was performed in a 20 µl reaction volume with 5 µl of 10× diluted pre-amplification products, 50 ng of an *Mse*I-primer with two selective nucleotides, and 5 ng of a fluorescent labelled *Eco*RI-primer with two selective nucleotides. Selective primer *Eco*RI + AC was labelled with the infrared dye IRD800 and selective primer *Eco*RI + AG was labelled with IRD700. All PCR reactions were performed using SuperTaq^{TM} 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 a Sequa-Mark^{TM} 10 base ladder (Research Genetics, Huntsville, Alabama, USA).

Six primers combinations were used for initial tests of the AFLP method on the 30 *Botrytis* isolates (Table 1). The primer combinations were *Eco*RI + AC/*Mse*I + CC, *Eco*RI + AC/*Mse*I + CG, *Eco*RI + AG/*Mse*I + CA, *Eco*RI + AG/*Mse*I + CC, *Eco*RI + AG/*Mse*I + CG and *Eco* RI + AG/*Mse*I + CT (Vos et al., 1995).

Three AFLP primer combinations (*Eco*RI + AC/*Mse*I + CG, *Eco*RI + AG/*Mse*I + CA, and *Eco*RI + AG/*Mse*I + CT) were used for the analyses of *B. elliptica* and *B. tulipae* populations (Table 2). These primer combinations produced fingerprints with a sufficient number of well-separated polymorphic and non-polymorphic bands in the size range between 100 bp 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.

Population genetic analysis

Amplified fragment length polymorphism bands were scored manually as binary characters and bands at the same size were treated as putative unique loci. A binary matrix was constructed containing all reproducible bands and all isolates. In subsequent analyses, data were treated as haplotypic data and bands were considered as loci, with absence or presence as alleles. 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 & Bryant, 2006). Branches with at least 70% bootstrap support were considered as well supported. Two-locus gametic disequilibrium was

calculated using an exact test for haplotypic data with ARLEQUIN version 3.0b (Excoffier, Laval, & Schneider, 2005). The calculation is based on distribution of allelic mismatches between pairs of genotypes over all loci. Since the occurrence of clonal genotypes can be confounding, inferences about recombination were also made using unique genotypes only. 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 & Burt, 2001). The $\bar{r}_d$ summary statistic largely avoids dependency on the number of loci in comparison to the index of association (I_A) (Maynard Smith, Smith, O'Rourke & Spratt, 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. 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, Carter, Koenig, White, & Taylor, 1996).

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 1). Intraspecific variability was low among the 10 *B. tulipae* strains compared to the other two species. Only one nucleotide position was polymorphic in over 4300 bp, i.e., in the EF-1α sequence of *B. tulipae* strain BT9901 (not shown). Therefore, only two multilocus sequence genotypes were identified among the 10 *B. tulipae* strains. For *B. elliptica*, seven multilocus sequence genotypes were detected with 12 polymorphic sites (not shown). HSP60 was the most informative locus for this species, as it could distinguish five genotypes with four polymorphic sites. An additional eight sites were polymorphic in EF-1α, however; only two genotypes were identified that contain this locus (not shown). The level of intraspecific polymorphism was highest in *B. cinerea*. A total of nine multilocus sequence genotypes were identified based on 15 nucleotide polymorphisms. Six sites were polymorphic in RPB2 and EF-1α, and these loci enabled identification of three and five genotypes, respectively (not shown).

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, *B. elliptica* strain BE9612, and *B. cinerea* strain SAS56 (Table 1). Two primer sets were designed that amplified different fragments of the IGS region, such that the two partial IGS regions combined span the locus, except for an approximately 260 bp internal region. The amplification product IGSnr1 was 1460, 1630, 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 amplification products of different isolates belonging to the same species. Each of the 60 PCR amplification products was subjected to digestion with six different restriction enzymes, except for *B. cinerea* strain B05.10. No restriction digest variants were observed among the 10 *B. tulipae* isolates and no restriction sites were present for six different enzymes (not shown). In contrast, five genotypes were detected for *B. elliptica* (Table 4). 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 (Table 4). No polymorphisms were detected for IGSnr3 in any of the *B. cinerea* isolates.

AFLP variation

The six AFLP primer combinations produced well-spaced bands in the range of approximately 100–500 bp. All bands were reproducible when fingerprints were repeated with DNA samples

Table 4 IGS-RFLP profiles of *B.elliptica* and *B. cinerea*[a]

Species	Isolate	IGSnr1[b]					IGSnr3[c]				Combined
		*Rsa*I	*Hin*cII	*Bam*HI	*Taq*I	*Hae*III	*Rsa*I	*Hin*cII	*Taq*I	*Hae*III	
B. elliptica	BE9401	A	U[d]	U	A	A	A	A	A	A	A
	BE9605	B	U	U	A	A	A	B	A	A	B
	BE9610	B	U	U	A	A	A	B	A	A	B
	BE9612	B	U	U	A	A	A	A	A	A	C
	BE9623	A	U	U	A	A	A	A	A	A	A
	BE9714	B	U	U	A	A	A	A	A	A	C
	BE9732	A	U	U	A	A	U	A	A	A	D
	BE0004	C	U	U	B	A	A	A	A	A	E
	BE0006	A	U	U	A	A	A	A	A	A	A
	BE0022	B	U	U	A	A	A	B	A	A	B
Distinct genotypes		3	1	1	2	1	2	2	1	1	5
B. cinerea	Bc7	D	A	A	C	B	B	U	B	A	F
	Bc12	E	A	U	D	B	B	U	B	A	G
	Bc21	D	A	U	E	C	B	U	B	A	H
	7A	D	A	A	E	B	B	U	B	A	I
	8A	D	A	U	E	B	B	U	B	A	J
	SAS56	D	A	U	E	B	B	U	B	A	J
	SAS405	D	A	A	E	B	B	U	B	A	I
	M14	D	B	A	E	C	B	U	B	A	K
	BcIPO	F	A	A	F	B	B	U	B	A	L
Distinct genotypes		3	2	2	4	2	1	1	1	1	7

[a] *B. tulipae* displayed only monomorphic restriction profiles and is not shown; [b] none of the IGSnr1 fragments was digested by *Hin*dIII, not shown; [c] none of the IGSnr3 fragments were digested by either *Hin*dIII or *Bam*HI, not shown; [d] U, uncut

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 (not shown). Among the 10 *B. tulipae* isolates, eight genotypes were identified with the combined AFLP data, and the most informative primer sets was *Eco*RI + AG/*Mse*I + CA, which distinguished five genotypes (Table 5). All 10 isolates of *B. elliptica* and 10 isolates of *B. cinerea* were distinguished based on the combined AFLP data of all six primer combinations (Table 5). Even with primer set *Eco*RI + AG/*Mse*I + CA alone, all isolates of both species were differentiated. All other primer sets were more informative for *B. cinerea* than for the other two species, except for primer set *Eco*RI + AC/*Mse*I + CG, which identified only three genotypes in *B. cinerea* (Table 5).

Population genetic analyses

For studying variation in *B. elliptica* and *B. tulipae* collections, three AFLP primer combinations were chosen that produced up to 60 well-spaced bands per primer per species. 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). Among the 170 *B. tulipae* isolates, 88 AFLP loci were scored, of which 24% were polymorphic (21 out of 88; Table 6). Approximately 26% of the 325 pairwise comparisons between the 25 unique AFLP fingerprints differed by four or fewer fragments (Fig. 1). The number of distinct genotypes, the number of genotypes found once and the 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 and in each of the *B. tulipae* collec-

Table 5 AFLP profiles with six primer combinations

Species	Isolate	*Eco*RI + AC/ *Mse*I + CC	*Eco*RI + AC/ *Mse*I + CG	*Eco*RI + AG/ *Mse*I + CA	*Eco*RI + AG/ *Mse*I + CC	*Eco*RI + AG/ *Mse*I + CG	*Eco*RI + AG/ *Mse*I + CT	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

tions (Fig. 2A). For instance, genotype h6 made up 26.5% (45 of 170) of all *B. tulipae* isolates and was found in all four collections.

Among the 174 *B. elliptica* isolates, 98 AFLP loci were scored of which 31% were polymorphic (30 out of 98; Table 6). 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 (Fig. 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 (Fig. 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 6). The number of clonal genotypes was limited (Fig. 2B), although two genotypes were present at high frequencies, i.e., genotype h10 had a frequency of 30% (14 out of 46; Fig. 2B) 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.

Table 6 Comparison of AFLP genotype diversity in collections of *B. elliptica* and *B. tulipae* isolates

Species	Collection[a]	*n*	Loci	Polymorphic loci	Distinct genotypes	Genotypes found once	Genotypic diversity[b]	Shannon-Wiener index[c]	Shared genotypes[d]
B. tulipae	Total	170	88	21	25	4	.89	1.19	–
	LISSE17-04-02	41	88	21	11	3	.88	.99	8 (LISSE18-06-02) 4 (LISSE20-05-04) 9 (Netherlands)
	LISSE18-06-02	42	88	21	11	2	.86	.98	8 (LISSE17-04-02) 5 (LISSE18-06-02) 9 (Netherlands)
	LISSE20-05-04	31	88	21	9	5	.79	.87	4 (LISSE17-04-02) 5 (LISSE18-06-02) 7 (Netherlands)
	LISSE total	114	88	21	18	4	.89	1.11	–
	Netherlands	56	88	21	20	10	.88	1.23	9 (LISSE17-04-02) 9 (LISSE18-06-02) 7 (LISSE20-05-04)
B. elliptica	Total	174	98	30	105	87	.97	2.12	–
	LISSE09-09-02	46	98	29	26	19	.90	1.52	4 (LISSE21-10-02)
	LISSE21-10-02	50	98	29	36	30	.98	1.86	4 (LISSE09-09-02)
	LISSE18-08-03	41	98	29	15	10	.73	1.05	0
	LISSE total	137	98	30	73	57	.95	1.89	–
	Netherlands	37	98	29	32	30	.99	2.16	0

[a] The collection name is coded according to sampling date (see Table 1)

[b] Nei's (1987) diversity index corrected for sample size

[c] Shannon-Wiener index corrected for sample size (Chao & Shen, 2003)

[d] The collection with which genotypes are shared is given within parentheses

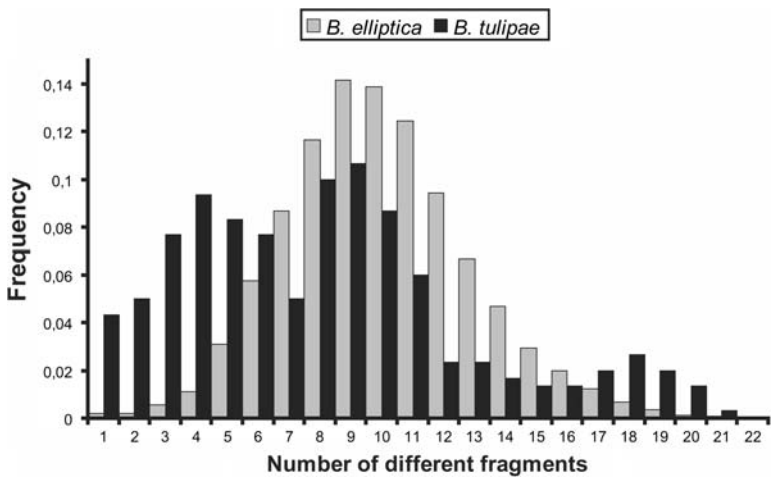

Fig. 1 Observed frequency distribution of AFLP fragments (presence or absence) in pairwise comparison of unique fingerprints of *B. elliptica* and *B. tulipae*. For *B. elliptica*, pairs of fingerprints differ from one another in 10 of the 98 fragments on average. For *B. tulipae*, the average was 8 out of 88 fragments

Cluster analyses

UPGMA dendrograms with bootstrap support values were constructed to investigate clustering among isolates (Figs. 3 and 4). The dendrogram constructed with all 25 distinct *B. tulipae* genotypes contained four well supported nodes (Fig. 3), each containing two genotypes. The similarity values ranged between .81 and .99; however the similarity values were at least .88

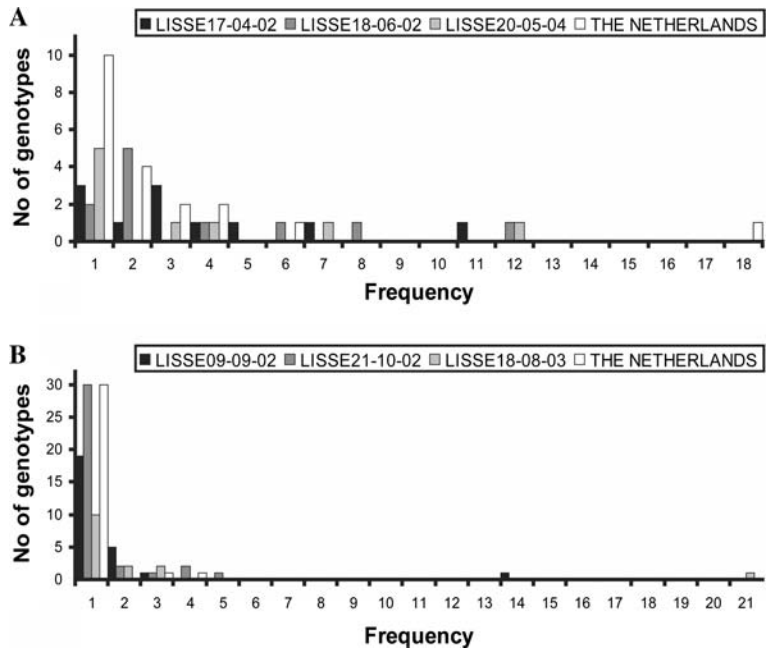

Fig. 2 The number of genotypes and their frequencies for (**A**) *B. tulipae* collections and (**B**) *B. elliptica* collections

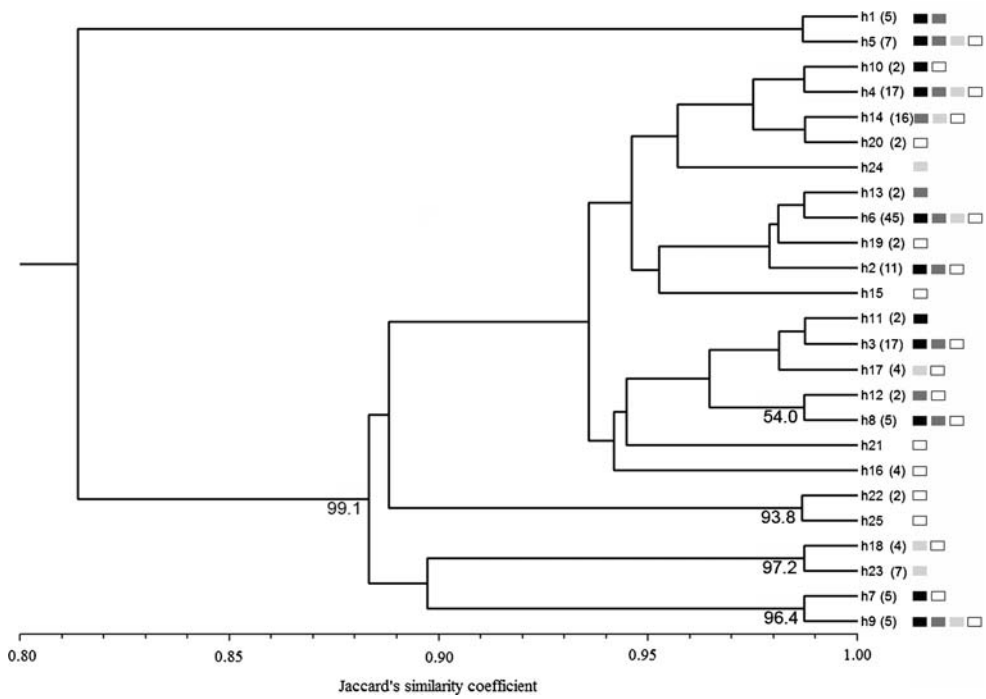

Fig. 3 Jaccard's similarity relationships based on cluster analysis (UPGMA) using AFLP fingerprinting data of 25 *B. tulipae* genotypes (numbered h1–h25). Coloured square indicates collection name: black—LISSE17-04-02, dark grey—LISSE18-06-02, light grey—LISSE20-05-04, white—the Netherlands. Genotype frequencies among the 170 isolates, when higher than one, are shown in brackets behind the genotype code. Bootstrap values higher than 50% are shown below each branch

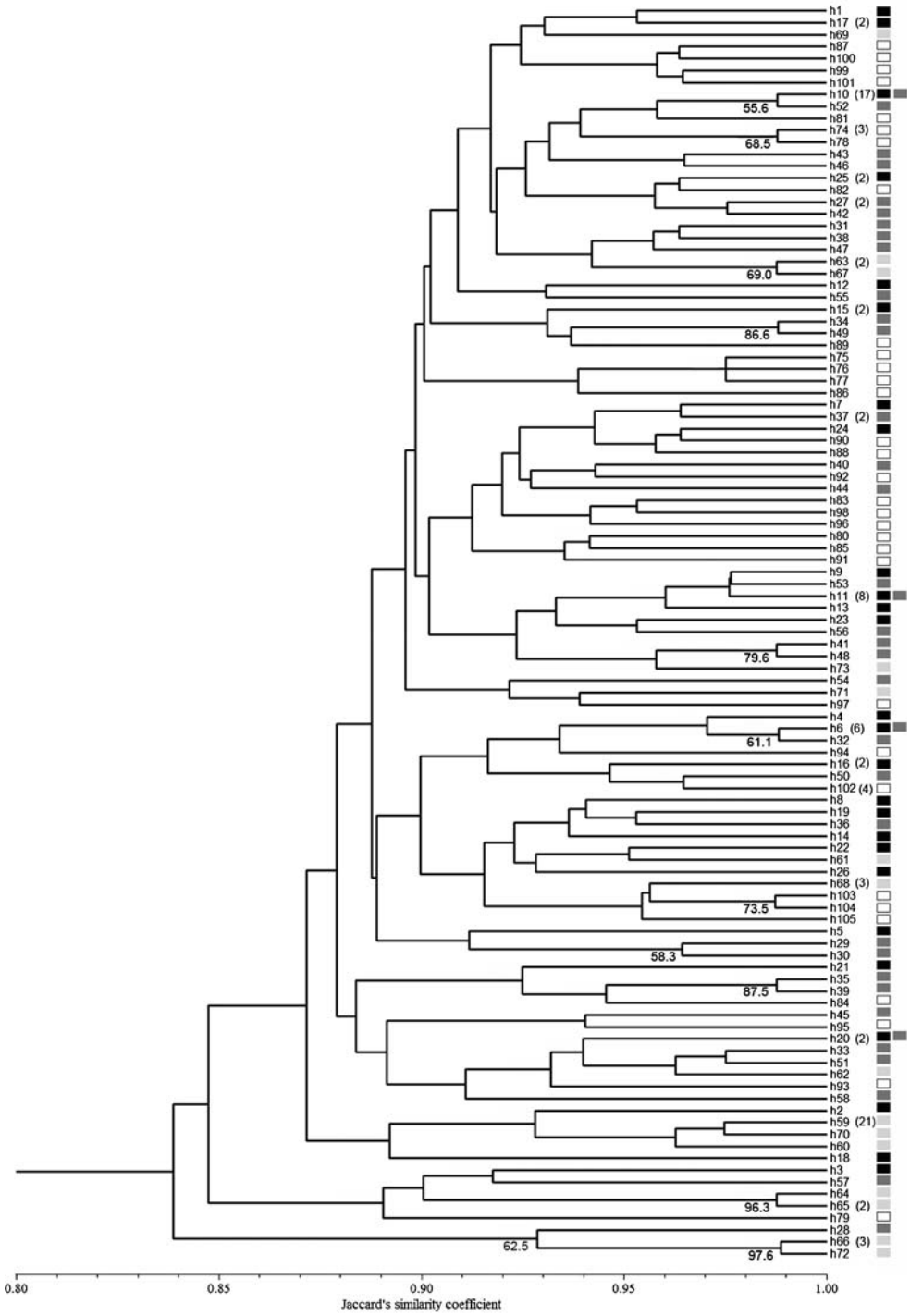

Fig. 4 Jaccard's similarity relationships based on cluster analysis (UPGMA) using the AFLP fingerprinting data of 105 *B. elliptica* genotypes (numbered h1–h105). Coloured square indicates collection name: black—LISSE09-09-02, dark grey—LISSE21-10-02, light grey—LISSE18-08-03, white—the Netherlands. Genotype frequencies higher than one found among the 174 isolates are shown in brackets besides the genotype name. Bootstrap values higher than 50% are shown below each branch

when excluding genotypes h1 and h5. There was no grouping of *B. tulipae* genotypes based on date of sampling (Fig. 3). The dendrogram constructed with all 105 unique *B. elliptica* genotypes contained six well supported nodes (Fig. 4), each containing two genotypes. The Jaccard similarity coefficient values for the 105 genotypes ranged from .84 to .99. The cluster analysis did not group genotypes based on date of sampling (Fig. 4).

Gametic disequilibrium

To estimate whether random mating had occurred among isolates in the collections of *B. tulipae* and *B. elliptica*, 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. tulipae* collections, 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 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 7). 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 7), 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 ($\bar{r}_d$ = .01; Table 7) with a significance value of .016, indicating only a weak departure from

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

Species	Collection name	Two-locus gametic disequilibrium			Multilocus gametic disequilibrium ($\bar{r}_d$)	
		Number of pairwise comparisons[a]	Total sample[b]	Clone-corrected sample[b]	Distinct genotypes[c]	Genotypes found once[c]
B. tulipae	TOTAL	231	93.3	42.9	.36	.19
	LISSE17-04-02	231	74.3	25.2	.47	.35
	LISSE18-06-02	231	85.2	48.6	.50	.44
	LISSE20-05-04	231	38.1	25.2	.31	.19
	LISSE total	231	87.1	37.1	.40	.26
	NETHERLANDS	231	61.4	34.8	.25	.16
B. elliptica	TOTAL	465	43.0	19.3	.04	.02
	LISSE09-09-02	435	29.6	9.9	.07	.02
	LISSE21-10-02	435	18.2	10.8	.02	.01
	LISSE18-08-03	435	50.2	13.1	.20	.06
	LISSE total	465	42.1	17.0	.05	.02
	NETHERLANDS	435	16.7	9.4	.02	.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. Given are the percentages of pairwise comparisons that were in significant associations at $P = .05$

[c] The test of significance for the standardized index of association (Agapow & Burt, 2001) was calculated with 1000 replicates and $P \geq .05$. Values that differ significantly from 0 indicate a departure from linkage equilibrium

gametic equilibrium. 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

Amplified fragment length polymorphism fingerprinting was shown to be superior over two other methods (multilocus sequencing and IGS-RFLP) in detecting genetic differences among isolates of the three *Botrytis* species analyzed. Therefore, 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. The application of AFLP fingerprinting was sensitive enough for distinguishing between 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 distinguish between 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, Antonacci, & Pollastro, 1988; Giraud et al., 1997). 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 comparison between studies (Grünwald, Goodwin, Milgroom, & Fry, 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 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 (Lorbeer et al., 2004; Van den Ende & Pennock-Vos, 1997). Significant linkage disequilibrium between loci was detected in 19.3% of the pairs of loci for the entire collection (Table 7), which is consistent with some extent of genetic exchange and recombination. Even when the collection was subdivided by season 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.

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 that this resulted 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, Coertze, & Williamson, 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 on sclerotia in lily stem debris in the field (Van den Ende & Pennock-Vos, 1997). Unlike Huang et al. (2001), we found no evidence for the occurrence of random mating. Multilocus linkage disequilibrium can be caused by epistatic selection, gene flow, drift and physical linkage of AFLP loci (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.

The overall genetic diversity in *B. tulipae* was lower than in *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. *Botrytis tulipae* apothecia have never been found over many years of study in the Netherlands (Van den Ende, personal communication). 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 & 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 (Coley-Smith & 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 (Fig. 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, Lipari, & Powell, 1992). Besides mutation, activity of transposable elements may have contributed in generating diversity in clones (Daboussi & 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 *B. cinerea* (Faretra et al., 1988; Pollastro, Faretra, Canio, & De Guido, 1996) provides a possibility for mitotic recombination. Unfortunately, knowledge about heterokaryosis or 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 & Kohn, 2004) and additional markers.

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