Genetic population structure and fungicide resistance of *Botrytis cinerea* in pear orchards in the Western Cape of South Africa

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*Botrytis cinerea* isolates from pear blossoms (*Pyrus communis*) in South Africa were collected from four orchards in two production areas in the Western Cape. The cryptic species status based on vegetative-incompatibility alleles of the Bc-\emph{bc}b gene indicated that all the isolates belonged to *B. cinerea*. A microsatellite analysis of *B. cinerea* populations was performed to assess the genetic population structure. Total gene diversity (\(H\)) was high, with a mean of 0.69 across all populations. Some genotype flow was evident between orchards as indicated by the spread of microsatellite multilocus genotypes, in agreement with the moderate, but significant population differentiation among orchards (mean \(\Phi_{PT} = 0.118, P = 0.001\)). Index of association analyses (\(I_{A}\) and \(F_{r}\)) suggest that the populations reproduce mostly asexually, even though mating type distribution did not differ significantly from a 1:1 ratio, suggesting frequency-dependent selection. Isolates resistant to benomyl were evident in one orchard only. This orchard was also significantly differentiated from all other populations, suggesting infrequent localized selection for benomyl resistance. Overall, the findings of this study highlight the dangers of a mixed reproduction system, and stress the importance of regularly monitoring fungicide resistance levels towards developing more efficient management practices.

**Keywords:** benomyl, *Botrytis cinerea*, iprodione, linkage disequilibrium, microsatellite, population genetics

**Introduction**

Calyx-end decay of pears (*Pyrus communis*) caused by *Botrytis cinerea* (telemorph *Botryotinia fuckeliana*) results in considerable economic losses each year in South Africa. Calyx-end decay results from early season infection of pear blossoms. It was found that following blossom infection, decay invariably spreads from the mesocarp tissue, which adjoins the sepal in a radial manner, into the vascular bundles (Dekock & Holz, 1992). This led to the suggestion that postharvest control of grey mould could be achieved by fungicide application at full bloom and/or 75% petal fall. This is to be followed 1 month later with another application when the floral tubes start to close (Dekock & Holz, 1992).

Propagules of *B. cinerea* (e.g. sclerotia, mycelium and asexual spores) can be either soilborne, airborne, or found on plant litter (Lennox \textit{et al.}, 2003) where sclerotia often overwinter in crop debris (Williamson \textit{et al.}, 2007) within the orchard. Sclerotia that germinate during early spring produce asexual spores, which can be dispersed as wet or dry conidia and serve as inoculum for fruit (Lennox \textit{et al.}, 2003; Spotts & Serdani, 2006) or blossom infection. Therefore, orchard sanitation is crucial as a preventative measure to limit inoculum build-up (Spotts & Serdani, 2006).

*Botrytis cinerea* has been divided into two phylogenetic clades (Fournier \textit{et al.}, 2005), groups I and II. This division was recently reinforced with the description of group I as a separate species from group II, based on several species concepts (phylogenetic, biological and ecological) (Walker \textit{et al.}, 2011). Group I is known as *B. pseudocinerea* and group II as *B. cinerea*. Since the abolishment of Article 59 and the dual nomenclature for fungi of the International Code of Nomenclature for Algae, Fungi and Plants (ICN), the use of the asexual genus name *Botrytis* has been proposed (Rossman, 2014). Based on population genetic analyses, no gene flow was found between the two species with high within-species diversity (Walker \textit{et al.}, 2011). *Botrytis cinerea* is the most prevalent species and displays high levels of genetic variation along with sexual recombination, as illustrated through the use of microsatellite markers (Fournier & Giraud, 2008; Karchani-Balma \textit{et al.}, 2008; Vaczy \textit{et al.}, 2008). Isolation of *B. pseudocinerea* is sporadic outside France (Walker \textit{et al.}, 2011) and is apparently subject to temporal succession, being most abundant in spring on floral debris in grapevines (Walker \textit{et al.}, 2011).

Transposable elements are often used to distinguish between isolates of *B. cinerea*. The transposable elements (TEs) that have been characterized are Boty, a 6 kb
long-terminal-repeat retrotransposon, and Flipper, a 1842 bp Fot1-like transposable element (Diolez et al., 1995; Levis et al., 1997). On the basis of the presence or absence of these TEs, Giraud et al. (1997) found B. cinerea could be divided into two sympatric species, transposa and vacuma, although these TEs alone are not sufficient for complete separation of B. cinerea from B. pseudocinerea (Albertini et al., 2002; Fournier et al., 2005). Transposa contains the transposable elements Boty and Flipper whereas vacuma contains neither transposable element. In grapevine pathology studies in France, isolates taken from flowering parts in vineyards were found to be predominantly of the vacuma type (Giraud et al., 1997, 1999). It was suggested that this is an indication of greater saprophytic capability, explaining the prevalence of vacuma isolates on senescing floral parts. Thus, these observations supported the possibility of genetic differentiation between transposon types (Martinez et al., 2005).

The resistance levels of B. cinerea from South African pear orchards towards the fungicides benomyl and iprodione are currently unknown. Benomyl and iprodione are registered as full bloom and 75% petal-drop sprays in South Africa. However, in practice, benomyl is rarely used in orchards and iprodione is used in postharvest applications only. Knowledge of the population dynamics of B. cinerea, together with the application of effective fungicides, will aid in the integrated management of calyx-end decay of pears.

The objective of this research was to investigate the population dynamics of B. cinerea in four pear orchards in two areas within the Western Cape of South Africa. Specifically, an investigation was made of: (i) which Botrytis species was present in pear orchards, responsible for pear blossom infections and present in weeds; (ii) the evolutionary potential of the pathogen as typified by measures of its genetic diversity, mode of reproduction and gene flow through analyses of seven microsatellite markers; (iii) whether genetic differentiation as determined with microsatellites corresponded to transposon types; (iv) the mating type distribution of the studied population; and (v) the fungicide resistance levels towards iprodione and benomyl.

Materials and methods

Botrytis cinerea sampling

In total, 3640 pear blossoms were sampled from four orchards (910 blossoms per orchard). Two orchards were in the Ceres region (8 km apart; cultivar Forelle) and two in the Grabouw region (15 km apart; cultivar Packam’s Triumph; Fig. 1). The
maximum distance between orchards (A and D) was 90 km. The cultivars sampled represent the dominant cultivar in each region. Hierarchical sampling (McDonald et al., 1999) was conducted in spring (October) 2009 to include 30 trees per orchard. In the first row, three adjacent trees were sampled, starting at the fifth tree in the row; thereafter, one tree was missed, the next tree sampled, then two trees were missed and the next tree sampled; subsequently, 20 trees were skipped before sampling a sixth and final tree from the row. The adjacent row was sampled similarly, then one row skipped, and the next row sampled. Two rows were then missed before sampling the next and then three rows were skipped before sampling the fifth and final row. The maximum distance between sampled trees within a row was 60 m (orchard C) and 45 m (orchard A, B and D). The maximum distance between sampled rows was 49.5 m (orchard B and C) and 44 m (orchard A and D; Figs S1–S4). From each tree 30 blossoms were collected, except for the 21st tree (row 4), from which 40 were taken. This was the intended intensive sampling point in each orchard. Representative samples of weeds were taken around each tree, if occurring.

**Processing of blossoms**

Thirty blossoms per tree were sterilized for 30 s in 70% ethanol and divided into two groups of 15 blossoms each. Two incubation methods were used to maximize *B. cinerea* isolation. For the first method, three blossoms were incubated in a Petri dish containing Kerssies medium (Kerssies, 1990). Blossoms placed on Kerssies medium had their petals removed and were placed upside down on the surface of the medium, i.e. with anthers and stamens touching the medium. The second method involved incubating five blossoms per plastic container containing a paper towel wetted with 2–3 mL sterile distilled water. In the case of the 21st tree (intensive sampling point) the flowers were divided into two groups (20 each). Half were placed individually on Petri dishes containing Kerssies medium, the other half were placed in groups of four per sowing point. The flowers were divided into two groups (20 and stored on potato dextrose agar (PDA) slants and in sterile dishes. In all cases, blossoms were checked for growth of *B. cinerea* for approximately 2 weeks at room temperature, after which they were left a week to ensure fresh air and growth stimulation. These were left open during the day to allow fresh air to circulate. Plant material was routinely checked and any *B. cinerea* sporulation observed was collected via single spore isolation and stored as described above.

**DNA isolation**

DNA was extracted from mycelial cultures after 2 weeks' growth on PDA Petri dishes at 25 °C. Approximately 100 mg mycelium was used to extract DNA as described by Goodwin et al. (1992). The concentration of each DNA sample was determined on an ND-1000 spectrophotometer (NanoDrop) and diluted to a final concentration of 25 ng µL⁻¹.

**Distinction of *B. cinerea* and *B. pseudocinerea***

The Neurospora crassa vegetative incompatibility locus homolog, Bc-hch, was amplified as described by Fourrier et al. (2003) using the primers 262 (5’-AACCCCTTCTGATGCTTGGA-3’) and 520L (5’-ACGGATCCGAACTAAAGTA-3’). Restriction fragment length polymorphisms (RFLPs) were generated through digestion of the 1171 bp product with the restriction enzyme *HhaI* for 90 min at 37 °C, resolved on a 2% agarose gel and visualized by ethidium bromide staining under a UV light.

**Detection of the transposable elements Boty and Flipper**

The transposable element status of the isolates was determined, using the primer pairs Boty4. The *Boty* and *Flipper* elements were detected by PCR, using primers provided by Jan van Kan, Wageningen University, Netherlands [Van Kan et al., (1998)].

**Mating type determination**

The presence/absence of the mating type genes *MAT1-1* and *MAT1-2* within each isolate was determined with PCR using primers developed by Van Kan et al. (2010). The primers HMG5 (5’-ATGTCTCTCCTCTCCTGCG-3’) and HMG3 (5’-GGAAAAGAATGTGTAAGACCTTG-3’) amplified a partial *MAT1-2* HMG gene (c. 1100 bp). The MATalpha5 (5’-ATGACGCTCCCTCTCAAAACC-3’) and MATalpha3 (5’-GGTGTTGAAGGGA CATC TTC-3’) primers were used to amplify the *MAT1-1* alpha gene (c. 1050 bp). Separate reactions were set up for each mating type locus. PCR reactions were performed in a GeneAmp PCR System 2720 thermocycler (Applied Biosystems), in a total reaction volume of 40 µL as described by Ma & Michailides (2005). The PCR conditions were as follows: an initial denaturing step of 95 °C for 3 min; 40 cycles of 40 s at 94 °C, 40 s at 67 °C and 58 °C for Boty and Flipper respectively, and 1 min at 72 °C; and a final extension at 72 °C for 10 min. PCR products were stained with ethidium bromide and visualized on a 1.5% agarose gel under UV light. Each reaction was performed in duplicate.

**Microsatellite amplification**

A total of 181 *B. cinerea* isolates were successfully genotyped using seven of the nine microsatellite markers developed by
analysed using the software GENEMAPPER v. 3.7 (Applied Biosystems). Data was rated in an automated single capillary genetic analyzer LIZ Size Standard (Applied Biosystems). Fragments were separated with Hi-Di formamide (Applied Biosystems) in a total volume of 25 μL, containing 1 μL DNA (25 ng μL⁻¹), 12.5 μL KAPA2G FastHotStart ReadyMix (KapaBiosystems) and the following primer (forward and reverse) concentrations for the multiplex: Bc1, 1.6 μM; Bc2, 8 μM; Bc3, 3.2 μM; Bc5, 4 μM; Bc6, 3.2 μM and Bc10, 12 μM. The single reaction had a primer concentration of 12 μM for Bc7. The primer concentrations were adjusted to ensure the optimal amplification of all the alleles. The 5’ ends of the forward primers were labelled fluorescently as follows: Bc1/Bc10, VIC; Bc3/Bc5, 6-FAM; Bc2/Bc7, PET and Bc6 with NED. Amplifications were performed using a GeneAmp PCR System 2720 thermocycler (Applied Biosystems). An initial denaturation step at 95 °C for 1 min, was followed by 30 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 15 s and elongation at 72 °C for 10 s. This was followed by a final elongation step at 60 °C for 15 min.

PCR products were diluted 1 in 15 for the multiplex reaction, and left undiluted for the single reaction. A post-PCR clean-up was performed, after which the samples were mixed in a 1:1 ratio with Hi-Di formamide (Applied Biosystems) in a total volume of 10 μL, followed by addition of 0.45 μL GeneScan-600 LIZ Size Standard (Applied Biosystems). Fragments were separated in an automated single capillary genetic analyzer ABI3730xl DNA sequencer (Applied Biosystems). Data was analysed using the software GENEMAPPER v. 3.7 (Applied Biosystems). Only one allele was amplified per locus, consistent with the haploid nature of *B. cinerea*.

**Population genetics analyses**

Clonal distribution and frequency of genotypes between and within populations was inferred based on microsatellite profile matches. To estimate the contribution of asexual reproduction to the epidemic, the clonal fraction was calculated as the occurrence and frequency of clones within a population, \( \frac{[N - G(N)]}{N} \), where \( N \) is the sample size and \( G \) is the number of multilocus genotypes (MLGs) present.

Allele-based analyses were performed on clone-corrected populations in order to remove the bias of over-representation of clones. The software GeneALEx v. 6.4 (Peakall & Smouse, 2006) was used to calculate Nei’s gene diversity (Nei, 1973) and the mean number of alleles for each population. Allele richness of each population, corrected for sample size by the rarefaction procedure, was determined in **STAT** v. 2.9.3 (Goudet, 2001). In order to compare the number of alleles and allele richness between orchards, the data were subjected to analysis of variance (ANOVA). A Fisher’s LSD was calculated at a confidence level of 95% to observe significant differences between alleles in populations of orchards using **ADDINSOFT XLSTAT** v. 2011.2.06 (www.xlstat.com).

To test for sexual recombination, linkage disequilibrium by means of an index of association \( I_{AS} \) and \( r_{AS} \) was determined after 1000 permutations in **MULTILOCUS** v. 1.3b (Agapow & Burt, 2001), using clone-corrected data from the four orchards. To further investigate the mating system of *B. cinerea* in South Africa, frequency-dependent selection of mating types was tested with a \( \chi^2 \) test to determine the goodness of fit of the observed distribution to the expected distribution of 1:1 in all populations.

Differentiation among and within populations was determined by calculating \( \Phi_{ST} \) (analogue of \( F_{ST} \)) (Wright, 1951) in pairwise comparisons, after 999 permutations (Peakall & Smouse, 2006). Both \( \Phi_{ST} \) and \( F_{ST} \) standardize the degree of genetic differentiation among populations, with scores ranging from 0 (no differentiation) to 1 (completely differentiated). To estimate the relative contribution of location (populations A and B near Ceres and populations C and D near Grabouw) on the distribution of the genetic variation observed, a hierarchical analysis of molecular variance (AMOVA) was performed and the significance of genetic variations were determined after 999 permutations in pairwise population comparisons. Lastly, to determine whether there was a significant correlation between geographic distance, expressed as the log of geographic distance in pairwise comparisons of populations, and genetic differentiation between all pairs of genotypes of subpopulations, a Mantel test (Mantel, 1967) was performed in GENALEX v. 6.4. The significance of this comparison was determined after 999 permutations. A significant negative correlation is expected if the geographic distance affects the extent of genetic differentiation between populations, which would indicate isolation by distance. Analyses were conducted in GENALEX v. 6.4.

**Screening for resistance towards iprodione and benomyl**

A total of 181 genotyped isolates were screened for resistance to iprodione and benomyl. Technical-grade iprodione (a.i. 98%; ACI Chemicals) was dissolved in acetone and commercial grade benomyl (Benomyl 500 WP; Villa Crop Protection) was suspended in sterile distilled water to make 1 g L⁻¹ stock solutions/suspensions. Mycelial growth sensitivity was determined for discriminatory concentrations of iprodione (3 mg L⁻¹) and benomyl (5 mg L⁻¹) (Fourie & Holz, 2003) on amended PDA. Where applicable, the final amount of acetone in the medium was 0.1%, including the control plates. There were three replicates per concentration per isolate.

Plates were inoculated with 5-mm diameter plugs of mycelium (mycelium facing downward) from actively growing colony margins of 3-day-old cultures. Radial growth of colonies was measured after 36 h incubation at 22 °C in the dark. The average growth reduction was determined for each isolate ((growth of control – growth on fungicide-amended media)/growth of control × 100). Isolates were regarded as sensitive when growth reduction was 100% (Fourie & Holz, 1998). The resistance frequency, expressed as a percentage of the number of isolates tested, was then determined for *Botrytis* populations from each orchard and the population as a whole.

**Results**

A total of 181 *B. cinerea* isolates were obtained from blossoms and weeds in four pear orchards. *Botrytis cinerea* was found on all trees sampled in orchard B and D, 50% of the trees in orchard A and on 13 (43%) in orchard C. The highest number of infected flowers per tree out of 30 varied between orchards; from 3 in A, 4 in C to 8 in B and 9 in D. Orchard B and D both had high incidences (10% and 17%, respectively) of *B. cinerea* infection on blossoms sampled. Both orchards A and C had 3% blossoms infected.

Weeds were also infected with *B. cinerea*, but the incidences on these were lower than on the blossoms and they represent approximately 14% of the total isolates.
sampled. Three species were responsible for 65% of the total \textit{B. cinerea} sampled from weeds. These were: \textit{Hypo-chaeris radicata}, \textit{Plantago lanceolata} and \textit{Sonchus asper}.

Distinction of \textit{B. cinerea} and \textit{B. pseudocinerea}

All 181 isolates belonged to \textit{B. cinerea} as all yielded a 517 bp fragment after digestion of the \textit{Bc-hcb} locus with \textit{Hba}1.

Transposable element characterization

Three combinations of the transposable elements Boty and Flipper were detected in the isolates of \textit{B. cinerea}; 46 (25.4\%) of the isolates had both the Boty and Flipper elements (\textit{transposa} type), 134 (74\%) had only the Boty element (\textit{boty}-only), and one (0.6\%) had the Flipper element only (\textit{flipper}-only) (Table 1). No isolates of the \textit{vacuma} type, i.e. containing neither transposable element, were detected in this study. The incidence of \textit{transposa} and \textit{boty}-only isolates was similar in the Ceres (23 and 77\%, respectively) and Grabouw areas (28\% and 71\%, respectively). The one \textit{flipper}-only isolate was detected from orchard D. At orchard level, there was one orchard in each region that had a higher incidence of \textit{boty}-only isolates, with 84\% for orchard A and 100\% for orchard C, as well as a higher incidence of \textit{transposa} isolates (Table 1).

Mating type determination

The amplification of the \textit{MAT1-1} alpha locus yielded a product of approximately 1050 bp and the amplification of the \textit{MAT1-2} HMG locus yielded a product of approximately 1100 bp. All isolates were heterothallic, containing either the \textit{MAT1-1} or \textit{MAT1-2} allele. In total, approximately 47\% of the isolates had the \textit{MAT1-1} allele and 53\% the \textit{MAT1-2} allele (Table 2). A \(\chi^2\) test was performed to determine the goodness of fit of this distribution compared to the expected mating type ratio of 1:1, for the total population (\(\chi^2 = 0.67, P > 0.05\), d.f. = 1) and subpopulations (Table 2). The hypothesis of frequency-dependent selection, with the resultant equal distribution of \textit{MAT1-1} and \textit{MAT1-2} alleles, was thus accepted for all orchard populations including the total population (Table 2).

Table 1 Number of \textit{Botrytis cinerea} isolates of the different transposable element types from each orchard

<table>
<thead>
<tr>
<th>Orchard</th>
<th>No. of isolates</th>
<th>boty-only</th>
<th>flipper-only</th>
<th>transposa (Boty + Flipper)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>25</td>
<td>21</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>B</td>
<td>58</td>
<td>43</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>C</td>
<td>25</td>
<td>25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>73</td>
<td>45</td>
<td>1</td>
<td>27</td>
</tr>
<tr>
<td>Total</td>
<td>181</td>
<td>134</td>
<td>1</td>
<td>46</td>
</tr>
</tbody>
</table>

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Genetic diversity within populations

All microsatellites amplified were polymorphic and the number of alleles amplified per locus ranged between 7 and 21, giving sufficient discriminatory power to assess population dynamics. Microsatellites were analysed for a total of 181 isolates. Within the South African populations in pear orchards, 91 MLGs were observed. The clonal fraction was highest for orchard C (0.72) and the lowest for orchard B (0.39) (Table 3). In the total population, 66 MLGs were observed once and 25 were observed at least twice or more (Table 4). MLGs that were observed once varied between populations and ranged between 7 (orchard C) and 36 (orchard D) (Table 4).

The mean number of alleles for all loci in the total population was 6.2 (Table 3). Analyses of variance indicated a significant effect for the main effect: mean number of all alleles for all loci for orchards (\(F = 0.011\)). Orchard C (3.6) had a significantly (\(P = 0.011\)) lower number of alleles than the rest of the sampled orchards (6.9–9.1), which in turn did not differ significantly from each other. Allelic richness after rarefaction (orchard C excluded due to low sample size after clone correction) differed significantly among populations (\(P = 0.011\)) with a Fisher’s LSD test indicating that orchard D’s allele richness was significantly lower than that of orchard A and B (Table 3). Mean gene diversity (\(H\)) values were high, ranging from 0.61 (orchard D) to 0.79 (orchard B), with an overall mean of 0.69 (Table 3). Orchard B had considerably higher gene diversity compared to A and D and particularly to C. The index of association (\(I_A\)) differed significantly from zero for three of the populations (B, C and D) with values ranging from 0.46 (orchard B and D) to 2.15 (orchard A), rejecting the hypotheses for random mating and indicating strong associations between loci (Table 3). However, for orchard A the \(I_A\) did not differ significantly from zero, although the sample size was small (\(n = 10\)) (Table 3). Similarly, significant linkage disequilibrium was identified with \(\overline{F}_{\bar{d}}\) for orchards B, C, and D, with values significantly different from zero. In orchard A, as for the \(I_A\), the \(\overline{F}_{\bar{d}}\) was not significantly different from zero, showing linkage equilibrium that indicates random mating (Table 3).

Table 2 Distribution and mating type frequencies of \textit{Botrytis cinerea} isolates

<table>
<thead>
<tr>
<th>Population</th>
<th>No. of isolates</th>
<th>MAT1-1</th>
<th>MAT1-2</th>
<th>(\chi^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orchard A</td>
<td>25</td>
<td>15</td>
<td>10</td>
<td>1.0</td>
</tr>
<tr>
<td>Orchard B</td>
<td>58</td>
<td>24</td>
<td>34</td>
<td>1.72</td>
</tr>
<tr>
<td>Orchard C</td>
<td>25</td>
<td>14</td>
<td>11</td>
<td>0.36</td>
</tr>
<tr>
<td>Orchard D</td>
<td>73</td>
<td>32</td>
<td>41</td>
<td>1.11</td>
</tr>
<tr>
<td>Total</td>
<td>181</td>
<td>85</td>
<td>96</td>
<td>0.67</td>
</tr>
</tbody>
</table>

\(\chi^2\) value based on 1:1 ratio and 1 d.f. None of the values differed significantly from a 1:1 ratio (\(P > 0.05\)).
Table 3 Genetic diversity indices of *Botrytis cinerea* populations collected from four pear orchards in South Africa

<table>
<thead>
<tr>
<th>Population</th>
<th>No. of isolates</th>
<th>No. of MLGs&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Clonal fraction</th>
<th>Mean no. of alleles per locus&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Allelic richness&lt;sup&gt;d&lt;/sup&gt;</th>
<th>I&lt;sub&gt;α&lt;/sub&gt;</th>
<th>T&lt;sub&gt;α&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orchard A</td>
<td>25</td>
<td>10</td>
<td>0.60</td>
<td>0.75 (0.04)</td>
<td>7.0 ± 1.23</td>
<td>6.96 a</td>
<td>–</td>
</tr>
<tr>
<td>Orchard B</td>
<td>58</td>
<td>35</td>
<td>0.39</td>
<td>0.79 (0.04)</td>
<td>9.1 ± 1.33</td>
<td>7.76 a</td>
<td>–</td>
</tr>
<tr>
<td>Orchard C</td>
<td>25</td>
<td>7</td>
<td>0.72</td>
<td>0.62 (0.03)</td>
<td>3.6 ± 0.61</td>
<td>Nd&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.148</td>
</tr>
<tr>
<td>Orchard D</td>
<td>73</td>
<td>39</td>
<td>0.46</td>
<td>0.61 (0.06)</td>
<td>6.9 ± 0.96</td>
<td>5.49 b</td>
<td>0.461</td>
</tr>
<tr>
<td>Total</td>
<td>181</td>
<td>91</td>
<td>0.49</td>
<td>0.69 (0.02)</td>
<td>6.2 ± 0.57</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

<sup>a</sup>MLGs, multilocus genotypes.<br>
<sup>b</sup>H<sub>α</sub>, gene diversity (Nei, 1973), standard error shown in parentheses.<br>
<sup>c</sup>Standard deviation shown.<br>
<sup>d</sup>Allelic richness values followed by the same letter do not differ significantly (<i>P</i> < 0.05) as determined with a Fisher’s LSD test.<br>
<sup>e</sup>Nd, not determined.

Table 4 Frequency distribution of *Botrytis cinerea* microsatellite multilocus genotypes (MLGs) within pear orchard populations in the Ceres and Grabouw areas of South Africa

<table>
<thead>
<tr>
<th>Population</th>
<th>No. of genotypes</th>
<th>Ceres</th>
<th>Grabouw</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of times</td>
<td>Orchard A</td>
<td>Orchard B</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>24</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>27</td>
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<sup>a</sup>Four orchard populations pooled together, only genotypes shared among populations recorded in total.

Genetic diversity and genotype/gene flow between populations

Results for the pairwise determination of population differentiation, <i>φ</i><sub><i>PT</i></sub>, yielded moderate values, with not all comparisons significant (Table 5) (orchard C excluded due to low sample size after clone correction). Comparisons of orchard D to the two other orchards yielded significant values. Moderate to high <i>φ</i><sub><i>PT</i></sub> values were obtained when orchard D was compared to orchard A (<i>φ</i><sub><i>PT</i></sub> = 0.120, <i>P</i> = 0.001) and B (<i>φ</i><sub><i>PT</i></sub> = 0.134, <i>P</i> = 0.001), signifying little genetic exchange between the populations. Other pairwise comparisons yielded non-significant values (Table 5). An *AMOVA* analysis of the *transposa* and *boty*-only populations (*flipper*-only isolate excluded) indicated very low but significant differentiation among isolates, with a <i>φ</i><sub><i>PT</i></sub> value of 0.044 (<i>P</i> = 0.001; Table 6).

Direct evidence of genotype flow was evident through the examination of MLGs shared between two or more populations. Repeated MLGs were observed several times within an orchard, especially within orchards B and D. The distribution of MLGs occurring at least three times within an orchard was followed and together with the hierarchical sampling scheme presented as schematic diagrams (Figs S1–S4). The most frequently isolated genotype (X) was isolated 27 times in total from orchards A to D (Figs S1–S4). The second most frequently isolated genotype (W) was isolated 14 times and was shared among orchards A, B and C (Figs S1–S4). Within a given orchard, MLGs of *B. cinerea* could be found up to 49.5 m apart within a row (orchard D), or 49.5 m apart across rows (orchard C). The approximate distance between the two production areas is 90 km, indicating that MLGs are able to disperse at least 90 km.

*AMOVA* revealed that 88% of the total variation was distributed within the populations, 9% between them and only 3% among the two regions with a total <i>φ</i><sub><i>PR</i></sub> value of 0.118 (<i>P</i> = 0.001; Table 6). Of this 0.027 (<i>φ</i><sub><i>RT</i></sub>) was due to the among-region component and 0.094 (<i>φ</i><sub><i>PR</i></sub>) was due to the among-population within region component. All the population differentiation measures were significant (Table 6). Because all isolates sampled from the cultivar Forelle came from Ceres and all those sampled from Packham’s Triumph came from Grabouw, it was impossible to separate the effect of cultivar from that of region in this study. Although the <i>φ</i><sub><i>RT</i></sub> measure for the among-region effect was significant, the contribution of this to the total differentiation was very low, as indicated by the 3% variation between regions. This indicates that there is very little effect of region/cultivar on population differentiation. A Mantel test showed no significant negative correlation between geographic
distance and population differentiation, indicating no isolation by distance ($y = 0.803x + 3.872, R^2 = 0.126, P = 0.315$).

Screening for resistance towards iprodione and benomyl

**Iprodione**
The four populations sampled yielded a very low incidence of resistance towards iprodione. Only three resistant isolates were found in the Ceres area, one from orchard A and two from orchard B. The percentage reduction in growth of these three isolates ranged between 65% and 77% and they were subsequently classified as ultra low level resistant (Beever & Parkes, 1993; Fourie & Holz, 1998, 2003). These three isolates only amounted to 2% resistance in the total population. In the Grabouw area there was no incidence of resistance towards iprodione.

**Benomyl**
Growth reduction values of the 181 isolates clearly grouped into two distinct groups: 77.0–100% and 0–11.1%, which were designated as sensitive and resistant, respectively. Isolates from three of the four orchards had negligible frequencies of resistance towards benomyl, ranging from 0 to 4%. However, orchard D had a 78% incidence of resistant isolates (Fig. 2). Out of the 46 MLGs that occurred within orchard D, 35 were resistant (76%). One isolate from orchard A was resistant to both iprodione and benomyl.

**Combined characteristics of the most frequently isolated MLGs**
Three or more isolates were recovered for 11 MLGs, which represent 83 of the isolates. Table 7 provides a summary of these MLGs together with their transposable element status, mating type and fungicide resistance towards iprodione and benomyl. Furthermore the spatial distribution of these MLGs is given within orchards A to D in Figures S1 to S4. All isolates of the same MLG showed the same resistance pattern towards benomyl, being either sensitive or resistant. Isolates with different mating types were found in most MLGs (7 of 11), and 4 of 11 MLGs had isolates with different transposon elements (Table 7). This suggests that these microsatellite loci are not discriminatory enough to distinguish among clones. Multiple isolates of benomyl resistant MLGs were found exclusively in orchard D (J, M, N, O) (Fig. S4).

**Discussion**
This is the first study to fully evaluate the natural occurrence of *B. cinerea* on blossoms in pear orchards in South Africa. All of the isolates sampled in this present study belonged to *B. cinerea*, according to the RFLP patterns of the Bc-hch locus (Fournier et al., 2003, 2005). This finding is in agreement with findings from studies in other countries, where this species is also predominant (Fournier & Giraud, 2008; Vaczy et al., 2008; Esterio et al., 2011).

The extent of blossom infection varied between orchards with one orchard per region (orchards B and D) having a higher incidence than the others (more than 10%). Microclimatic conditions may play a role, as these two orchards are next to mountains, where rainfall is

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**Table 6** Analysis of molecular variance (AMOVA) with geographic origin and transposable element (TE) types (transposa and boty-only) as grouping factors

<table>
<thead>
<tr>
<th>Source</th>
<th>d.f.</th>
<th>SS</th>
<th>MS</th>
<th>Est.Var.</th>
<th>%</th>
<th>Statistic</th>
<th>Value</th>
<th>P</th>
</tr>
</thead>
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<td>Between regions</td>
<td>1</td>
<td>15.006</td>
<td>15.006</td>
<td>0.080</td>
<td>3</td>
<td>0.027</td>
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<tr>
<td>Between populations</td>
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<td>12.811</td>
<td>6.406</td>
<td>0.274</td>
<td>9</td>
<td>0.094</td>
<td>0.001</td>
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<tr>
<td>Within populations</td>
<td>87</td>
<td>230.666</td>
<td>2.651</td>
<td>2.651</td>
<td>88</td>
<td>0.118</td>
<td>0.001</td>
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<tr>
<td>Total</td>
<td>90</td>
<td>258.484</td>
<td>2.651</td>
<td>3.006</td>
<td></td>
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<tr>
<td>Between TE types</td>
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<td>8.061</td>
<td>8.061</td>
<td>0.129</td>
<td>4</td>
<td></td>
<td></td>
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<tr>
<td>Within TE types</td>
<td>88</td>
<td>247.506</td>
<td>2.813</td>
<td>2.813</td>
<td>96</td>
<td></td>
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<tr>
<td>Total</td>
<td>89</td>
<td>255.567</td>
<td>2.813</td>
<td>2.942</td>
<td></td>
<td>0.044</td>
<td>0.001</td>
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</table>

*aSS, sum of squares.*

*bMS, mean square.*

*cEstimated variance.*

*dPercentage of total variation.*

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*Figure 2* Incidence of resistance of 181 *Botrytis cinerea* isolates in four orchards (A–D) towards 5 mg L$^{-1}$ benomyl.
typically higher, assisting infection. Orchard practices could also contribute to *B. cinerea* incidence. Orchards B and D were the two orchards that had the most vegetation underneath the trees, although this was not necessarily reflected in the incidence of *B. cinerea* obtained from weeds in these orchards. In the case of orchard D, only two weed samples revealed latent *B. cinerea* infections.

Weeds differed in disease incidence at regional and orchard level but overall *B. cinerea* was recovered at low frequencies from weeds. More extensive sampling of weeds would be necessary to verify which weed species are statistically most prone to harbour *B. cinerea* infections. Minimizing vegetation as well as dead organic matter in the orchard as part of an integrated management strategy has been shown to be important to limit the spread of *B. cinerea* in orchards (Spotts & Serdani, 2006).

In the studied population no isolates of the *vacuma* transposon type were detected. This result was unexpected as *vacuma*, with their enhanced saprophytic abilities (Giraud *et al.*, 1999; Martinez *et al.*, 2005), would

<table>
<thead>
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<th>Table 7 (continued)</th>
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<tr>
<td><strong>Fungicide resistance</strong></td>
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<tr>
<td>MLG</td>
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<tr>
<td>------</td>
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<td>B/7/9</td>
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<td>B/12/5(c)</td>
</tr>
<tr>
<td>B/12/9(f)</td>
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<tr>
<td>B/12/9(c)</td>
</tr>
</tbody>
</table>

\(a\)B, Boty; F, Flipper.  
\(b\)S, sensitive; R, resistant.  
\(c\)Isolates from weeds beneath respective trees.  
\(d\)Isolate from a weed on the periphery of the orchard.
have been the prime candidates for blossom infection in pear orchards; in similar studies of *B. cinerea* in vineyards, *vacuma* strains dominate in the early season (Giraud et al., 1997, 1999; Martinez et al., 2005). However, all three other transposon types were detected, with the majority of isolates (74%) of the *boty*-only type and the remainder of the *transposa* type (25%), apart from one isolate, which only had the Flipper transposable element. The presence of *flipper*-only isolates was not observed in California (Ma & Michailides, 2005) or Chile (Munoz et al., 2002) and, similar to the present study, was strongly under-represented in France (Albertini et al., 2002) and Hungary (Vaczy et al., 2008). However, in Bangladesh a high incidence of *flipper*-only isolates was observed (Isenegger et al., 2008). The high incidence of *boty*-only isolates observed in the present study agrees with the results of an analysis of a population from India and Nepal (72% *boty*-only) (Isenegger et al., 2008). Pathogenicity studies with grapevine isolates have shown that *transposa* isolates were more virulent on grape berries than *vacuma* isolates (Martinez et al., 2005). However, in another study, no significant differences were found in virulence between *vacuma*, *transposa*, *boty*-only and *flipper*-only isolates from grapes, tested on grape leaves and berries (Vercesi et al., 2014). Pathogenicity studies of *B. cinerea* transposon groups from pears will contribute to the understanding of the practical relevance of transposon types.

Comparing the molecular variance of the *transposa* and *boty*-only isolates, the low \(\Phi_{ST}\) value of 0.044 (\(P = 0.001\)) suggests that there is little, but significant population differentiation. Previous studies (Ma & Michailides, 2005; Isenegger et al., 2008) have also shown that microsatellite variation does not conform to transposon types, which could explain the low differentiation seen between the transposon-type populations in the present study.

The genetic diversity observed in *B. cinerea* has long been thought to be due to heterokaryosis (Beever & Weeds, 2007) and aneuploidy (Faretra & Pollastro, 1996). Sexual recombination has not been considered as a contributor, because apothecia are rarely observed in the field (Giraud et al., 1997). Apothecia have never been observed in pear orchards in South Africa. Only indirect indications of sexual recombination were found within populations of *B. cinerea* in France (Fournier & Giraud, 2008), Tunisia (Karchani-Balma et al., 2008) and Hungary (Vaczy et al., 2008) on various hosts. In agreement with population studies conducted on *B. cinerea* in different locations (Fournier & Giraud, 2008; Isenegger et al., 2008; Karchani-Balma et al., 2008; Vaczy et al., 2008), the results of the present investigation showed a high genetic diversity for the 181 isolates genotyped in South Africa. Despite this, measures of the \(I_S\) and \(F_S\) consistently showed linkage disequilibrium (non-random mating) in three populations (B, C and D). However, according to these analyses, orchard A may be undergoing random mating, although the sample size is too small after clone correction to draw a definite conclusion.

Using mating type primers, De Miccolis Angelini et al. (2016) found either MAT1-1 or MAT1-2 type *B. cinerea* isolates. In this study both mating types are present in pear orchards in the Western Cape. The observed mating type ratio did not differ significantly from the expected 1:1 distribution (random mating) for the total population and subpopulations and is in agreement with results on other hosts (Beever & Parkes, 1993; Delcan & Melgar-ejo, 2002). Taking this into account together with the high genetic diversity, the observed linkage disequilibrium could be explained by predominantly asexual reproduction with infrequent sexual reproduction. Thus, in summary, *B. cinerea* populations in pear orchards in South Africa have the potential to recombine genetic material through sexual reproduction, and to keep advantageous mutations in the gene pool through asexual reproduction. The implication of this represents one of the most important findings of this study.

Population differentiation was low to moderate among populations, indicating that the studied populations are not considerably influenced by geographic location or pear cultivar studied. Significant, but moderate \(\Phi_{ST}\) values were found only in comparisons of orchard D to orchards A and B. This is most probably due to selection, as population D is the only population with high levels of benomyl resistance. Furthermore, genotypic diversity measures were similarly low for orchards A, B and D, but were substantially lower for orchard C, indicating the presence of clonal lineages within orchards. The presence of clonal genotypes within populations is consistent with the presence of asexual spores that can spread readily within a field (Jarvis, 1980). This occurs through gusty winds or rain splash dispersal (Jarvis, 1980) and may spread over a distance of 6.5 km (Ma & Michailides, 2005). Natural dispersal between orchards A and B, which are geographically closest (approximately 8 km apart), most probably resulted in low population differentiation and shared MLGs. The two orchards furthest from each other (A and D, 90 km apart) were moderately differentiated yet still shared MLGs. Thus, MLGs shared by orchards A, B and C serve as an indication that MLG exchange between orchards/regions up to 90 km apart is possible, most probably occurring over several generations through intermediate orchards and hosts in a stepwise manner.

Whether the shared MLGs are identical by descent or identical in state, in other words if it is in actual fact due to microsatellite size homoplasy, remains an open question. However, with such a small sampling radius it is unlikely that these populations are evolving independently. MLGs shared between orchards, the lack of isolation by distance and the low population differentiation between some orchards, could also suggest that dispersal is aided by anthropogenic activities. The most probable human mediated transport is with managed bee hives. *Monilinia vaccinii-corymbosi*, responsible for mummy berry disease of blueberry (*Vaccinium ashei*), has been shown to be vectored by pollinators such as honey bees (Batra & Batra, 1985). Dispersal of *B. cinerea* through insect vectors (e.g. mites and bees) needs to be investi-
gated in pear orchards. Another means by which B. cinerea may spread and survive between orchards is on secondary host plants. Between Grabouw and Ceres lies a large wine grape production area in Rawsonville, Worcester and Slanghoek where both grape berries and weeds could serve as hosts for B. cinerea. This would imply a stepping stone model of migration (Kimura & Weiss, 1964). Further sampling would be necessary to establish whether this is possible.

Fungicide sensitivity testing showed low frequencies of resistance towards iprodione, and low frequencies of resistance towards benomyl except in orchard D. The dicarboximide iprodione is registered as a full bloom and/or 75% petal-drop spray in South Africa, but is applied only when previous years’ calyx-end decay incidences necessitate it (pers. comm. with technical advisors). In practice, it is used in the postharvest treatment of the fruit, and the recommendation to farmers is not to use the same active ingredient as in preharvest. The records for the orchards in the Ceres region, from which the isolates resistant to iprodione were obtained, did not indicate any dicarboximide sprays. However, iprodione is registered and used for the control of B. cinerea on stone fruit trees and onions in the Ceres region, from where iprodione resistant inoculum might have migrated.

Resistance towards benomyl was low except in orchard D where an almost 80% incidence of resistance was observed. Benomyl is registered as a full bloom and/or 75% petal drop spray in South Africa, and legislation enforced its application until 40 years ago. A minority of farmers today still apply benomyl once per season at full bloom and/or 75% petal drop. The records for orchard D showed that benomyl was applied until at least 2009, indicating prolonged selection pressure for benzimidazole resistance in this orchard. This could explain the observation that isolates in MLGs J, M, N and O, occurring exclusively within orchard D, were all resistant to benomyl. The mechanism of resistance toward benzimidazoles is due to point mutations within the β-tubulin gene BenA (Yarden & Katan, 1993). Four mutations have been reported in B. cinerea (E198A, E198K, E198V and F200Y), and each of these mutations can have different levels of fitness penalties (Walker et al., 2013). The underlying basis for the resistance toward benomyl was not investigated in the present study and so it is impossible to infer possible fitness penalties for the observed resistance; however, these MLGs appear to be persistent. The resistance towards benomyl highlights the inherent danger of a mixed reproduction system present in the orchard environment. Resistance possibly arose in one or two individuals through chance mutations, and these would then have been spread to different MLGs in the population through recurring sexual reproduction, and then proliferated through asexual reproduction. The presence of MLGs with a low level of resistance in the adjacent orchard C suggests a movement of resistant MLGs from orchard D to C. It is unlikely that resistance developed independently in orchard C, because there was no recent recorded use of benomyl.

In conclusion, the studied populations of B. cinerea occurring in pear orchards in the Ceres and Grabouw area in the Western Cape of South Africa are characterized by high levels of gene diversity and a predominantly asexual reproduction system. However, the spread of MLGs shows there is population admixture to a large extent, and the studied population represents a large panmictic population. This, together with no isolation by distance, suggests that the South African B. cinerea populations in pear orchards are most probably dispersed through anthropogenic activities. The level of linkage disequilibrium may contribute to keeping well-adapted combinations of genes together through asexual reproduction and, together with high levels of gene diversity and the possibility of sexual reproduction, suggests a mixed reproduction system. The potential loss of chemical control is illustrated by the prolonged selection for benomyl resistance in orchard D. Integrated use of multiple chemical classes should be encouraged in this orchard, and ideally complete cessation of benomyl application. The findings of this study stress the importance of integrated disease management at a regional level with constant assessment of fungicide resistance levels. Collaboration between producers and industry is essential to aid in the overall sustainable management of this disease. Knowledge of the population genetics and resistance levels of B. cinerea during blossoming in pear orchards may, in future, aid the development of more integrated management strategies.

Acknowledgements

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References
