Morphological and molecular identification of fungi associated with south African apple core rot

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Abstract

Core rot is a major contributor to postharvest losses in apples worldwide. Pathogens most commonly associated with the disease are *Alternaria* spp. and *Penicillium* spp. Although both genera show specific morphological characteristics, they can be difficult to identify to species level. In this study, *Alternaria* spp. (49) and *Penicillium* spp. isolates (97), associated with pre- and post-harvest apple core rot-symptoms and isolates from potential inoculum sources were identified using molecular methods. Initially, dry core rot causing *Alternaria* spp. were identified morphologically in an average of 70% of infected fruit pre-harvest and 32% postharvest. Furthermore, 78% of mouldy core rot causing pathogens were identified as *Alternaria* spp. preharvest and 40% postharvest. Wet core rot was associated with *Penicillium* spp. in 64% of cases preharvest and 36% postharvest. Species identity of a selection of samples was confirmed using the endopolygalacturonase (endo-PG) gene, the ITS region, and the anonymous genomic regions (OPA1–3, 2–1), which resulted in the identification of *A. alternata, A. arborescens, A. dumosa, A. eureka* and *A. tenuissima*. *Penicillium* species were identified through ITS sequencing and partial beta-tubulin polymerase chain reaction – random fragment length polymorphisms (PCR-RFLP) for the samples collected from wet core rot symptoms. Phylogenetic analyses separated the *Alternaria* spp. into five clades, including three separate clades for *A. alternata, A. tenuissima* and *A. arborescens,* respectively. This is the first report of *A. eureka* and *P. polonicum* as potential core rot pathogens. Phylogenetic analysis identified *Penicillium ramulosum and P. expansum* as the most commonly occurring species associated with WCR symptoms.

Keywords

Alternaria- and *Penicillium* species identification
Multi-gene phylogeny
Postharvest pathogens

Electronic supplementary material

The online version of this article (https://doi.org/10.1007/s10658-018-1601-x) contains supplementary material, which is available to authorized users.

Introduction

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Core rot, caused by various pathogenic fungi including several *Alternaria* and *Penicillium* species, is a major post-harvest disease of apples world-wide. Control of postharvest pathogens is complicated by the fact that infection might occur at any time from bloom or during fruit development depending on the causal pathogen. Fungicides are usually registered for specific disease causing pathogens, since they have varying control efficacy on different pathogen species. Therefore species identification of the causal organisms of the disease is required. Another complication is that fungal spores, such as *Alternaria* spp., could be carried into the apple cavity by mites (*Tarsonemus* sp.). Particularly susceptible to Tarsonemus mite-vectored infections would be the open calyx varieties such as Red Delicious (van der Walt et al. 2011). Therefore fungicide application during bloom alone, might not be effective to control all core rot pathogens. Currently no fungicides are applied to control *Alternaria* sp. in South Africa, although other fungicide applications to prevent apple scab infection, such as difenoconazole are thought to also inhibit *A. alternata* (Reuveni and Sheglov 2002; Reuveni and Prusky 2007). The apple variety Starking (sport of Red Delicious) was found to be particularly susceptible to core rot, with over 20% decay caused in single consignments leading to rejection at the export destination (pers. com. Dr. Cheryl Lennox). About 12% of the total apple areas planted in South Africa in 2016 was either the apple variety Topred or Starking, which corresponds to 2947 ha of orchards (Hortgro Statistics 2016). Currently identification of fungal species that cause core rot is labour intensive and requires trained personnel and laboratory facilities. Molecular methods are promising tools for species identification, which would enable targeted control strategies. A quick, reliable identification method for fungal pathogens, would also allow monitoring of quarantine pathogens. South Africa has quarantine requirements against *Alternaria mali* Roberts, as this species does not occur in the country. Screening of quarantine organisms from imported plant material can reduce the risk of quarantine organisms causing major losses if not contained.

Core rot includes three symptoms, namely mouldy core (MC), dry core rot (DCR) and wet core rot (WCR) (Carpenter 1942). Core rot symptoms are generally restricted to inside and around the core cavity of the fruit and external symptoms are rare (Combrink and Ginsburg 1973; Reuveni et al. 2003, 2007). Mouldy core symptoms are restricted to the core cavity of the apple, whereas those of DCR infect the tissue surrounding the core cavity forming a dry, corky lesion (Carpenter 1942). Fungi associated with WCR move rapidly through the tissue causing a soft, wet rot. Niem et al. (2007) established that core rot causal organisms infect during blossom, especially infecting apple cultivars with an open calyx end (Miller 1959). Shtienberg (2012), on the other hand, found a correlation between fruit cultivar size and *Alternaria alternata* (Fr.) Keissler infection risk.

Based on morphological characteristics, fungi previously associated with core rot were identified as *Alternaria* Nees, *Aspergillus* P. Micheli ex Link, *Botryosphaeria* Ces. and De Not., *Botrytis* P. Micheli ex Pers., *Candida* Cif. and Redaelli, *Cladosporium* Link, *Colletotrichum* Corda and Deutschl., *Coniothyrium* Corda, *Epicoccum* Link, *Fusarium* Link, *Gloeosporium* Desm. and Mont., *Penicillium* Link, *Pestalotia* De Not., *Phoma* Sacc., *Trichoderma* Pers., *Rhizopus* Ehrenb., *Pleospora* Rabenh. ex Ces. and De Not., *Spencermartinsia* Phillips, Alves and Crous, *Stemphylium* Wallr., *Trichothecium* Link, *Ulocladium* Preuss and others (Taylor 1955; Ellis and Barrat 1983; Combrink et al. 1985; Spotts et al. 1988; Spotts 1990; Van der Walt et al. 2010; Gao et al. 2013). The most prevalent fungi associated with DCR and MC are *Alternaria* spp. while *Penicillium* spp. and sometimes *Fusarium* spp. are associated with WCR (Combrink and Ginsburg 1973; Combrink et al. 1985; Spotts 1990; Wenneker et al. 2016). As morphological identification of core rot fungi to species level is not always possible, since some species have very similar morphology (i.e. cryptic species), molecular characterisation is frequently used. Some fungi can be identified to species level using a single genetic locus such as the beta-tubulin gene for *Penicillium* spp. (Seifert et al. 2007), whereas other species can only be distinguished using multiple gene regions such as *A. alternata* and *A. tenuissima* Nees and T. Nees: Fr. Wiltshire (Peever et al. 2004, 2005).

The four *Alternaria* species most frequently associated with core rot in South Africa are *A. alternata, A. tenuissima, A. arborescens* E.G. Simmons and *A. infectoria* E.G. Simmons (Kang et al. 2002; Serdani et al. 2002). Only *A. infectoria* could be distinguished from the other three species using the internal transcribed spacer (ITS) as there is a lack of polymorphism in the ITS region (de Hoog and Horré 2002; Berbee et al. 2003; Andersen et al. 2009). Alternative genetic loci have been used to identify the small-spored species of *Alternaria* with varying levels of success. These gene regions include glyceraldehyde-3-phosphate dehydrogenase (gpd), translocation elongation factor 1α (tef-1α), endo-PG, mitochondrial small and large ribosomal subunits (mtSSU and mtLSU), beta-tubulin and anonymous genomic regions OPA1–3, OPA2–1 and OPA10–2 (Kusaba and Tsuge 1995; Pryor and Gilbertson 2000; Pryor and Michailides 2002; de Hoog and Horré 2002; Peever et al. 2002, 2004, 2005; Pryor and Bigelow 2003; Hong et al. 2005; Pavón et al. 2010). These sequences could identify *A. arborescens* and other *Alternaria* species but *A. alternata* and *A. tenuissima* could not be distinguished (Pryor and Michailides 2002).

Penicillium species isolated from apple WCR worldwide include *P. aurantiogriseum* Dierckx, *P. brevicompactum* Dierckx, *P. commune* Thom, *P. crustosum* Thom, *P. expansum* Link, and *P. solitum* Westling (Amiri and Bompeix 2005). Similarly, *P. expansum* has been reported as the causal organism for WCR in South Africa (Combrink and Ginsburg 1973; van der Walt et al. 2010). Infection was thought to occur during the diphenylamine (DPA)-emulsion treatment of pome fruit against superficial scald in the pack house (Combrink et al. 1985, 1987; Spotts et al. 1988). *Penicillium* spores could be washed into the open calyx through the fungi-contaminated DPA-emulsion as DPA lowers the surface tension of the suspension and allows the water to move through the open calyx (Combrink and Ginsburg 1973). Currently DPA is being phased out and replaced with 1-methylcyclopropene (1-MCP), a gaseous ethylene inhibitor (Kim and Xiao 2008) which does not protect against fungi and has been reported to increase the decay incidence of *P. expansum* post-harvest (Janisiewicz et al. 2003; Leverentz et al. 2003; Kim and Xiao 2008). Wet core rot infection has been suggested to take place in the orchard prior to harvest (de Kock et al. 1991; Van der Walt et al. 2010). Although detailed studies on inoculum sources have not been conducted previously.

Several other species of the genus *Penicillium,* have been identified to cause WCR. Sanderson and Spotts (1995) identified the species, such as *P. solitum* Westling, collected from their symptomatic fruit using the colony colour and morphology of the isolates. *Penicillium roquefortii* Thom has been found in field bins and from wet core rot and decay lesions on apples and pears post-harvest (Sanderson and Spotts 1995; Spotts et al. 1988). Two other *Penicillium* species, namely, *P. expansum* and *P. funiculosum* Thom were reported to cause WCR in South Africa (Combrink and Ginsburg 1973; Combrink et al. 1985; de Kock et al. 1991; Serdani et al. 1998). Van der Walt et al. (2010) identified the species of *Penicillium* found in three apple production areas of South Africa that caused pre-harvest WCR, and characterised the virulence and pathogenicity of these isolates. The species identified were *P. ramulosum* prov. Nom., *P. expansum, Penicillium* sp. (aff. *P. dendriticum* Pitt), *P. glabrum* (Wehmer) Westling, *Penicillium* sp. (aff. *P. cecidicola* Seifert, Hoekstra and Frisvad) and *P. chermesinum* Biourge.

The following gene regions have been used to identify *Penicillium* species: ITS, cytochrome c oxidase 1 (CO1) gene, and the beta-tubulin gene (Lobuglio et al. 1993, 1994; Skouboe et al. 1999; Seifert et al. 2007). Van der Walt et al. (2010) used the beta-tubulin PCR-RFLP technique with restriction enzymes *Hae*III and *Rsa*I to distinguish between *Penicillium* species isolated from core rot symptoms.

The objective of this study was to identify the *Alternaria* and *Penicillium* species sampled from symptomatic core rot fruit and inoculum sources (air, mummies and mites), using molecular methods.

Materials and methods

Core rot fungal isolations and inoculum sources

In 2009 and 2010, core rot infected apples ($N = 291$) were collected from two 'Starking' orchards ("orchard B and C") in the Koue Bokkeveld pre- and postharvest, Western Cape and fungi isolated, single spored and identified. Monospore cultures of *Alternaria* spp. ($N = 49$) and *Penicillium* spp. isolates ($N = 97$) were collected from fruit of orchard B and air samples collected from orchards B and C. In addition, single spored isolates collected from other inoculum sources (mummies and mites) from orchard B during the bloom period for both seasons were included (Supplementary Table 1).

Core rot inoculum source and fungal isolation

Air samples were collected during bloom at ten sites per orchard at 1 and 3 min intervals, with each site replicated three times. A portable air sampler (Burkard Manufacturing Co Ltd., Hertfordshire, UK, patent No. 8819423.8) was used to collect airborne conidia onto potato dextrose agar (PDA) plates. The plates were incubated for seven days at 21°C room temperature, and fungal colonies sub-cultured onto PDA before being identified using molecular methods.

Mummified fruit, overwintered from the previous season, were collected from the same orchards where the air samples were taken in 2009 and 2010. From each orchard, 10 mummies were collected from 10 trees in accordance with Hong et al. (2000). Mummies were transported to the laboratory in brown paper bags sealed in plastic bags. Mites, retrieved from mummies with a thin needle, were placed in 1 ml sterile distilled water (SDW) in a 2-ml Eppendorf tube. Glass beads were placed in the SDW solution containing the mites and vortexed for 1 min. One hundred microliter of the mite - washing water was plated out onto each of three PDA plates. Plates were incubated at room temperature for 7 days after which fungal colonies were identified using molecular methods.

Isolation of fungi associated with core rot from orchard fruit pre-harvest

In 2009 and 2010 non-symptomatic fruit were collected from the orchard floor in March, two weeks (14 d) before harvest. Commercial farms with a history of core rot were selected in the Witzenberg Valley (near Ceres). In 2009, 200 fruit and in 2010, 800 fruit were collected pre harvest (20 fruit per tree). In the laboratory, the surface sterilised (70% ethanol for 30 s) fruit was cut in half and isolations were made from the edge of core rot lesions, which were classified as either dry core, mouldy core or wet core rot.

Isolation of fungi associated with core rot on fruit at harvest

Ten replicates of 100 fruit were harvested in April 2009 and 2010, and stored under controlled atmosphere for up to seven months. Post-harvest isolations were made as described above from symptomatic fruit ($N = 126$ in 2009; $N = 176$ in 2010).

Molecular identification

DNA extraction

The *Alternaria* and *Penicillium* isolates were grown on PDA at 21°C for 4 to 7 days. DNA was extracted from resulting mycelial growth using the UltraClean® Microbial DNA Isolation kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) following the manufacturer's instructions.

PCR-RFLP of pre- and post-harvest wet core rot *Penicillium* species

Genomic DNA was extracted from 124 single-spored *Penicillium* isolates using a modified method by Lee and Taylor (1990). The beta-tubulin PCR-RFLP group of each isolate was determined by PCR amplification of a partial region of the beta-tubulin gene using primers Bt2a (5'-GGTAACCAAATCGGTGCTGCTTTC-3′) (Glass and Donaldson 1995) and PentubR (5'-GACGGACGACATCGAGAACCTG-3′) (Van der Walt et al. 2010). The PCR reaction consisted of 0.2 µM of each primer, 0.2 mM of each dNTP, 1× PCR buffer, 0.5 U BIOTAQ DNA polymerase, 0.2 mg bovine serum albumin (BSA) Fraction V, 2 µl DNA and 3 mM $MgCl_2$ in a final volume of 40 µl. Amplifications were conducted in a 2720 Applied Biosystems thermocycler, starting with an initial denaturation cycle of 5 min at 94 °C, followed by 40 cycles of 45 s at 94 °C, 45 s at 55 °C and 60 s at 72 °C, and a final extension cycle of 7 min at 72 °C.

Successfully amplified PCR products were digested using the restriction enzymes *Hae*III and *Rsa*I (Fermentas Inc., Glen Burnie, MD, USA). The *Hae*III digest reaction consisted of 1× enzyme buffer, 15 U/µl *Hae*III and 10 µl PCR product in a total volume of 20 µl. The *Rsa*I digestion reaction consisted of 1× enzyme buffer, 5 U/µl *Rsa*I and 10 µl PCR product in a total volume of 20 µl. Digests were incubated overnight at 37 °C, and 15 µl of the restriction digest products were separated along with a 50 bp DNA standard on a 3% agarose gel (Seakem®, Lonza, Rockland, ME USA) containing 0.001% of ethidium bromide. Isolates exhibiting the same restriction pattern for both enzymes were classified into the same beta-tubulin PCR-RFLP groups using the Van der Walt et al. (2010) restriction patterns as controls (Figs. 1, 2, 3, and 4).

Fig. 1

Phylogeny of *Alternaria* species based on the internal transcribed spacer of ribosomal RNA region. The tree presents one of ten equally parsimonious trees of a heuristic search (TL = 248, CI = 0.734, RI = 0.875, RC = 0.642; 91 parsimony informative characters). The reference sequences for *A. alternata, A. arborescens, A. longipes* and *A. tenuissima* grouped together in one clade. The phylogenetic tree was rooted with the reference isolates *Pleospora tarda* and *Paradendryphiella salina.* Branch lengths are proportional to the inferred amount of evolutionary change and the scale represents 5.0 nucleotide substitutions per site

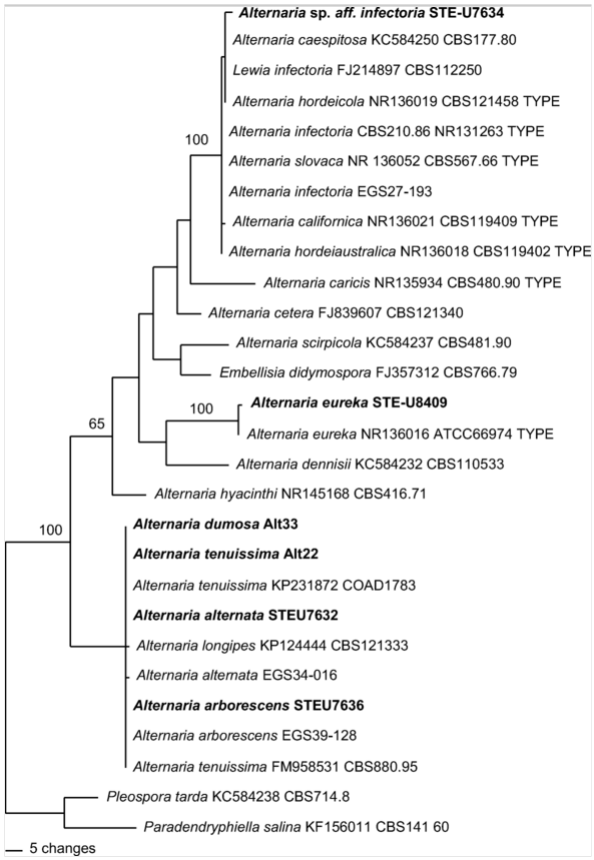


Fig. 2

Phylogeny of *Alternaria* species sampled from apple and apple orchard air samples based on the combined sequences of the endopolygalacturonase gene (endoPG), OPA1–3 and OPA2–1 regions. The tree presents one of two equally parsimonious trees of a heuristic search. Numbers within the tree represents the bootstrap values followed by probability values in brackets. Bootstrap values lower than 60% are not shown. Branch lengths are proportional to the inferred amount of evolutionary change and the scale represents 5.0 nucleotide substitutions per site. TL = 204, CI = 0.760, RI = 0.918. Reference sequences (EGS) in bold print represent type strains and isolates from citrus as published by Peever et al. (2004 and 2005). Numbers in columns with header (endoPG, OPA1–3, OPA2–1) indicate clade designations of sequences in individual loci's phylogenetic analysis (incongruences between phylogenies are indicated by hatched boxes)

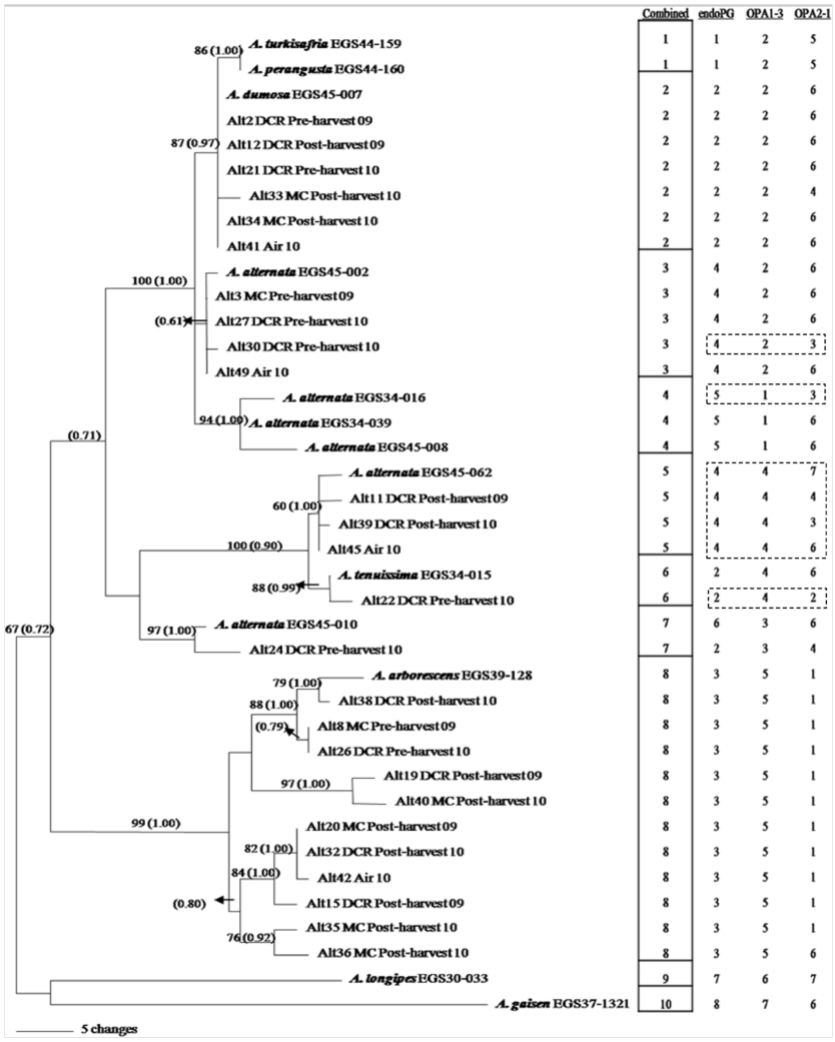


Fig. 3

The phylogenetic tree obtained from one of 830 parsimony analysis trees from the ITS region (including Genbank entries of the same genetic region from *Penicillium expansum, P. cecidicola, P. funiculosum, P. dendriticum, P. chermesinum, P.* sp. (aff. *Dendriticum* and *cecidicola*), *P. paneum, P. solitum, P. crustosum, P. brevicompactum, P. novae-zeelandiae, P. glabrum, P. rugulosum, P. ramulosum* and *Clonostachys rogersoniana*). The scale bar indicates the number of base changes per 100 nucleotide positions in the parsimony analysis, with parsimony bootstrap values of 1000 replicates indicated above and below the branches respectively. Bootstrap values lower than 60% are not shown

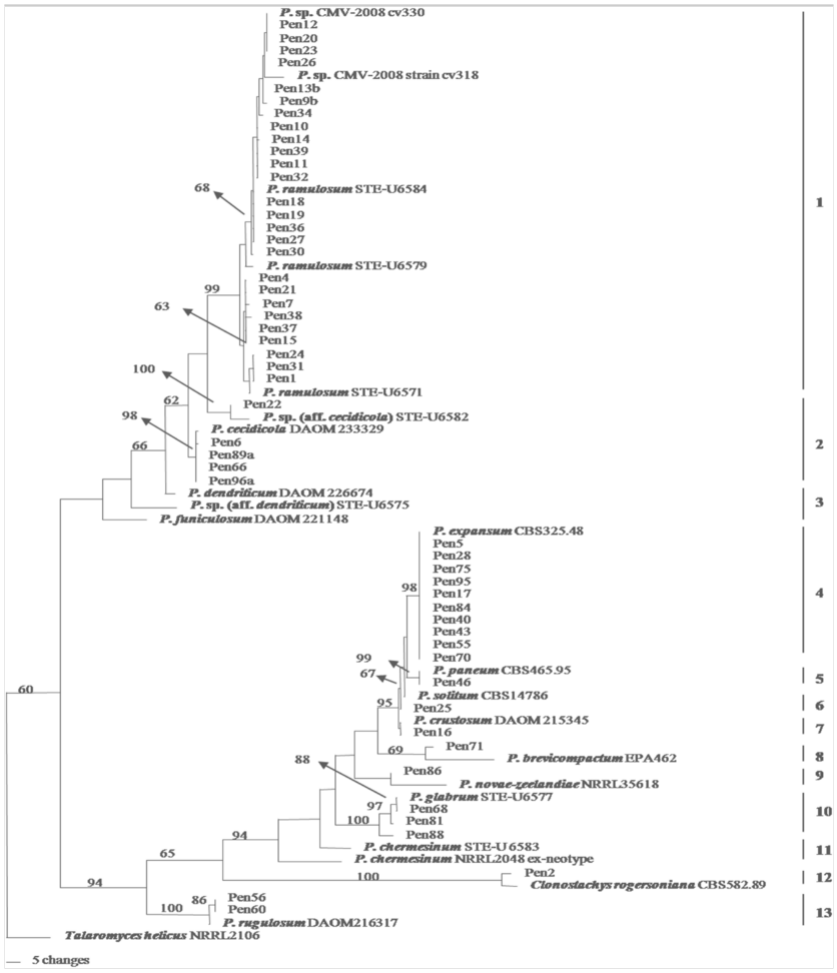
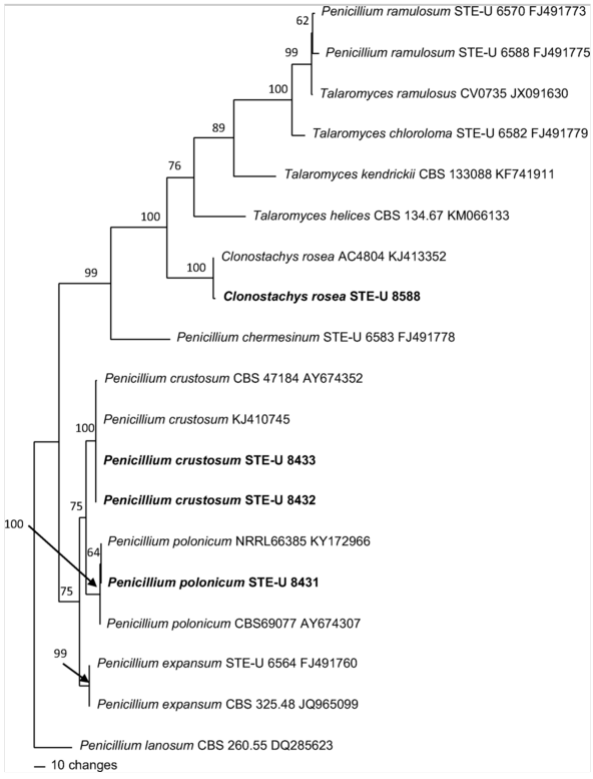


Fig. 4

The phylogenetic tree obtained from one of 4 parsimony analysis trees from the beta tubulin region, including Genbank entries of the same genetic region from *Clonostachys rosea, Penicillium chermesinum, P. crustosum, P. expansum, P. lanosum, P. polonicum,* and *Talaromyces ramulosus*. The scale bar indicates the number of base changes per 100 nucleotide positions in the parsimony analysis, with parsimony bootstrap values of 1000 replicates indicated above and below the branches respectively. Bootstrap values lower than 60% are not shown. Isolates from the current study in bold



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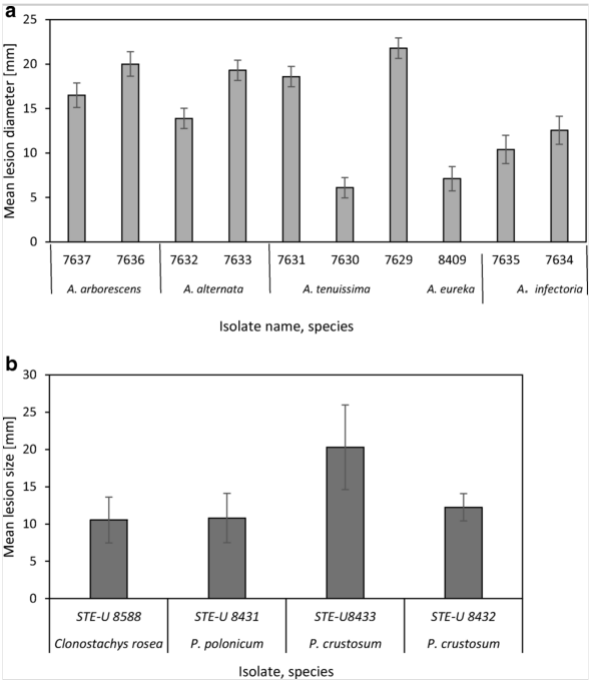
PCR amplification and sequencing

To identify individual *Alternaria* species, four genetic loci (endoPG, ITS, OPA1–3, OPA2–1) were used for sequence analysis. Samples were amplified in an Applied Biosystems 2720 Thermal cycler. The endoPG gene was amplified using primers PG3 (5'-TACCATGGTTCTTTCCGA-3′) and PG2B (5'-GAGAATTCRCARTCRTCYTGRTT-3′) as described in Isshiki et al. (2001) with the following programme: initial denaturation step for 2 min at 95 °C, 35 cycles of 1 min at 95 °C, 1 min at 50 °C, 1 min at 72 °C, and extension for 5 min at 72 °C. All PCR reactions were performed using a PCR machine (2700 Applied Biosystems) and the reaction mixture contained 1.5 mM $MgCl_2$, 1× reaction buffer, 2.5 mM dNTP, 0.5 µM of each primer, 2.5 U Bioline *Taq* polymerase and 1 µl of fungal DNA in a final volume of 25 µl. The *Penicillium*. ITS region was amplified using the primers V9G (5'-TTACGTCCCTGCCCTTTGTA-3′) and Ls266 (5'GCATTCCCAAACAACTCGACTC-3′) (de Hoog and van den Ende 1998; White et al. 1990), The ITS programme consisted of an initial denaturation step for 5 min at 94 °C, 5 cycles of 30 s at 94 °C, 40 s at 55 °C and 1 min at 72 °C, 5 cycles of 30 s at 94 °C, 40 s at 54 °C and 1 min at 72 °C, 25 cycles of 30 s at 94 °C, 40 s at 53 °C and 1 min at 72 °C, and extension for 5 min at 72 °C. The ITS PCR reaction contained 1.5 mM $MgCl_2$, 0.05 mg DMSO, 1× NH buffer, 1 mM dNTP, 10 µM of each primer, 5 U *Taq* polymerase and 1 µl (25 ng–100 ng) of fungal DNA in a final volume of 25 µl. A partial beta tubulin sequence amplified as described above was sequence analysed using primers Bt2a and PentubR (van der Walt et al. 2010) from a selected number of *Penicillium* sp. isolates to confirm species identity. After PCR amplification the products were confirmed by separation on a 1% agarose gel. PCR primers used for the OPA1–3 and OPA2–1 regions were OPA1–3 L (5'-AGGCCCTTCCAATCCAT-3′) and OPA1–3Rb (5'-AGCCACATGCTCTGGTTAGC-3′), and OPA2–1 L (5'-TGCCGAGCTGTCAGATAATTG-3′) and OPA2–1R (5'-GCCGAGCTGGTGGAGAGAGT-3′), respectively. As described in Peever et al. (2004) the following programme was used: an initial denaturation step for 1 min at 94 °C, 35 cycles of 20 s at 94 °C, 20 s at 56 °C, 20 s at 72 °C, and extension for 5 min at 72 °C. PCR reagents used were1x reaction buffer, 0.2 mM of each primer, 200 mM dNTP, 2.5 mM $MgCl_2$, 25 ng–100 ng of DNA and 1 unit of Bioline Taq polymerase. Sequencing reactions were conducted using the same primers as for the PCR amplification, using 1 µl PCR product in a 10 µl reaction, consisting of distilled water, 10 µM primer, 1× sequence mix (BigDye) and 5× buffer. The sequence programme followed an initial denaturation of 1 min at 95 °C, 30 cycles of denaturation for 10 s at 95 °C, and annealing for 5 s at 50 °C and 4 min extension at 60 °C. The sequencing product was filtered through a Sephadex filled filter plate and centrifuged at 910 rpm. Samples were analysed on an ABI Prism 3700 genetic analyser (Applied Biosystems, Foster City, CA), and forward and reverse sequences were assembled with the programme Geneious Pro 5.3.3 (Biomatters Ltd., Auckland, New Zealand). Representative isolates' sequences and cultures were submitted to Genbank (accession KX868611- KX868653; Supplementary Tables 2 and 3) or to the Stellenbosch University culture collection at the Plant Pathology department (STE-U 7629, STE-U 7630, STE-U7631, STE-U 7632, STE-U 7633, STE-U 7634, STE-U 7635, STE-U 7636, STE-U 7637, STE-U 8409, STE-U 8431-STE-U 8432, STE-U8433, STE-U 8588; Fig. 5).

Fig. 5

Mean lesion diameter of (A) five *Alternaria* spp. in pathogenicity tests after 10 d incubation (mean of two trials). Isolates used were STE-U 8409 (*A. eureka*) STE-U 7632 (*A. alternata*), STE-U 7634 (*A. infectoria*) and STE-U7636 (*A. arborescens*) and (B) one *Clonostachys* sp. and three *Penicillium* spp. mean lesion size in pathogenicity test. Isolates used were STE-U 8588 (*Clonostachys rosea,* PEN05), STE-U 8431 (*Penicillium polonicum,* PEN06), STE-U 8433 (*P. crustosum*, PEN16), and STE-U 8432 (*P. crustosum*, PEN18). Vertical bars denote standard error



AQ3

Phylogenetic analysis and partition homogeneity test

ITS sequences of *Alternaria* reference strains and representative isolates from the trials were aligned using the software programme CLUSTAL W (Thompson et al. 1994). *Alternaria* sequences from the datasets endoPG, OPA1–3 and OPA2–1 were aligned using MAFFT sequence alignment programme version 6 (Katoh and Toh 2008) followed by manual adjustments of the alignments in Sequence Alignment Editor v. 2.0a11 (Rambaut 2002). Maximum parsimony analysis as well as Bayesian analysis was conducted on the *Alternaria* sequence alignments using PAUP* (Phylogenetic Analysis Using Parsimony) v.4.0b10. The analysis was performed using the heuristic search option with 100 random taxon additions. Tree bisection and reconstruction (TBR) was used as the branch swapping algorithm with the option of saving no more than 10 trees with a score greater or equal to five (Harrison and Langdale 2006). Bootstrap support values were calculated from 1000 heurisitic search replicates and 100 random taxon additions. Bootstrap values below 60% were not included for each genetic locus' phylogenetic analysis. Other measures calculated for the parsimony analysis include tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency index (RC) values.

Topologies of the resulting phylograms were compared using incongruence length difference (ILD) tests (Farris et al. 1994) to determine the suitability of combining the endoPG, OPA1–3 and OPA2–1 data sets. ILD tests were implemented in PAUP* (referred to as "partition homogeneity tests" in PAUP*) with invariant characters removed and 1000 randomized partitions. Tested data partitions included: (i) endoPG, OPA1–3 and OPA2–1; (ii) endoPG and OPA1–3, (iii) endoPG and OPA2–1, and (iv) OPA1–3 and OPA2–1. Data partitions were considered significantly different at $P < 0.05$ (Swofford 2002). Phylogenetic trees were uploaded to TreeBASE (submission ID 19918). Genbank reference sequences used are listed in Supplementary Tables 2 and 3.

Pathogenicity tests

Inoculum was prepared as described in Serdani et al. (2002) by incubating sterile toothpicks (32 mm × 2 mm) and autoclaved 5 times, lastly in potato dextrose broth (PDB) with mycelial plugs of *Alternaria* spp. or *Penicillium* spp. isolates growing on PDA for two weeks at 21°C under high relative humidity (85 to 90%). Ten 'Starking' apples were surface sterilised using 70% ethanol and wounded using the inoculated toothpicks near the calyx end (about 2 cm away), or a sterile toothpick as a non-inoculated control. Fruit was incubated at 23°C in humidity chambers for 5 d (for *Penicillium* spp.) and 10 d (for *Alternaria* spp.). Re-isolation from the edge of the lesion was done to confirm pathogenicity of the tested isolates, and fulfil Koch's postulates. Analysis of variance of pathogenicity data was conducted using Statistica v. 13.6. Fisher's least significant difference was used to determine significant differences between isolates.

Results

Genera identified from the symptomatic core rot fruit and inoculum sources included *Alternaria, Aspergillus, Botrytis, Cladosporium, Epicoccum, Fusarium, Gloeosporium, Penicillium, Stemphylium, Trichoderma, Ulocladium,* and *Verticillium* (data not shown). Fungal isolates, which were not initially classified as *Alternaria* spp. or *Penicillium* spp. are from now on referred to as "other", while isolates which could not be identified are "unidentified".

Preharvest isolations

Dry and mouldy core rot symptoms preharvest

In 2009 fungal isolates collected from 63 infected apples were identified morphologically to genus level (Table 1; Suppl. Table 1). In eight instances *Alternaria* species were co-isolated with *Penicillium* species from DCR or MC symptoms (Suppl. Table 1). *Alternaria* spp. were most frequently isolated from DCR (53%) and MC (66%) symptoms followed by *Penicillium* spp. with 37 and 26%, respectively. In 2010, 79 fungi were isolated from 800 symptomless fruit sampled (Suppl. Table 1) and identified as either *Alternaria* (87% DCR and 89% MC) or *Penicillium* species (13% DCR and 11% MC).

Table 1

Incidence of core rot fungal genera (number of isolates with percentage in brackets) in one Koue Bokkeveld orchard from symptomatic dry core rot (DCR), mouldy core (MC) or wet core rot (WCR) infected apples

Season	Pathogen*	Source**	DCR	MC	WCR
2009	*Alternaria* spp.	PRE	10 (53%)	23 (66%)	0
	Penicillium spp.	PRE	7 (37%)	9 (26%)	9 (100%)
	other	PRE	2 (11%)	3 (9%)	0
	unidentified	PRE	0	0	0
2009	*Alternaria* spp.	POST	13 (57%)	44 (53%)	2 (10%)
	Penicillium spp.	POST	1 (4%)	9 (11%)	13 (62%)
	other	POST	4 (17%)	6 (7%)	3 (14%)
	unidentified	POST	5 (22%)	23 (28%)	3 (14%)
2010	*Alternaria* spp.	PRE	45 (87%)	24 (89%)	8 (62%)
	Penicillium spp.	PRE	7 (13%)	3 (11%)	5 (38%)
	other	PRE	0	0	0
	unidentified	PRE	0	0	0
2010	*Alternaria* spp.	POST	6 (8%)	19 (27%)	1 (3%)
	Penicillium spp.	POST	8 (11%)	5 (7%)	3 (9%)
	other	POST	33 (46%)	12 (17%)	17 (50%)
	unidentified	POST	25 (35%)	34 (49%)	13 (38%)
Total	*Alternaria* spp.	PRE + POST	74 (45%)	110 (51%)	11 (14%)
	Penicillium spp	PRE + POST	23 (14%)	26 (12%)	30 (39%)

*Pathogens were morphologically identified either as *Alternaria* sp. or *Penicillium* sp., other species were grouped as "other", nonviable hyphae were categorised "unidentified"

**Source of core rot pathogen: either from apples collected from the orchard preharvest (PRE) or postharvest (POST)

Wet core rot preharvest

In 2009, from the 200 sampled symptomless apples, nine isolates were identified as *Penicillium* spp. (100%). *Alternaria* species were not isolated. In 2010, eight out of a total of 13 isolates collected from 800 symptomless apples were identified as *Alternaria* species (62%) while the remaining isolates were *Penicillium* species (5; 38%).

Post-harvest isolations

Dry and mouldy core rot postharvest

In 2009, a total of 126 isolates from core rot infected apples were identified in the 1000 sampled symptomless apples. *Alternaria* species comprised the majority of the isolates for DCR and MC with 13 (57%) and 44 (53%) respectively. A single *Penicillium* isolate was associated with DCR (4%) and nine isolates (11%) with MC. Four DCR and six MC isolates were grouped as 'other' while 28 isolates were unidentified. In 2010, 33 DCR (46%) and 12 MC (17%) isolates out of 142 isolates collected from infected apples, were predominantly associated with other fungal genera. Six DCR (8%) and 19 MC isolates (27%) were identified as *Alternaria.* Eight DCR and five MC isolates were identified as *Penicillium* spp., respectively. A further 25 isolates caused DCR (35%) and 34 isolates caused MC (49%) and were unidentified.

Wet core rot postharvest

In 2009, 13 *Penicillium* spp. (62%) and two *Alternaria* spp. (10%) isolates were identified from WCR symptoms. Three isolates (14%) were identified as "other" (meaning other fungal species, not *Alternaria* spp. or *Penicillium* spp.) and a further three (14%) were unidentified due to nonviable hyphae. In 2010, three isolates (9%) were identified as *Penicillium* spp., while only one isolate (3%) was identified as an *Alternaria* spp. The other isolations (50%) were morphologically identified as the following: *Botryosphaeria, Botrytis, Epicoccum, Fusarium, Gliocladium, Phoma, Phomopsis, Ulocladium* and *Verticillium* (data not shown). Thirteen isolates (38%) were unidentified.

Inoculum sources

Penicillium species were identified from samples taken from the air, mummies and mites and a subset of 36 isolates were identified using molecular methods. *Alternaria* species were identified from air samples only (data not shown), and a subset of nine samples were included.

Molecular identification of the *Alternaria* and *Penicillium* species

The 49 *Alternaria* spp. isolates selected for species identification included 10 isolates from pre- and post-harvest symptomatic fruit, from both seasons, and nine isolates from the air inoculum. *Alternaria* spp. were neither isolated from apple mummies nor from mites associated with the mummies. The 97 *Penicillium* isolates selected for molecular species identification included 10 isolates from pre- and post-harvest symptomatic fruit and 10 isolates each from the mummies and mites for each season. Ten *Penicillium* isolates, collected from air inoculum in orchard B in 2009 were included. As the trees in orchard B were removed in 2010, seven *Penicillium* isolates from the air inoculum from an alternative 'Starking' orchard located within a 10 km radius in the same valley were included ("orchard C").

Fallen fruit were collected preharvest and postharvest for the seasons 2009 and 2010. Isolations were made from infected tissue of fruit with WCR symptoms. Nine and five *Penicillium* spp. samples were collected pre-harvest and 13 and three samples were collected post-harvest in 2009 and 2010, respectively.

Alternaria species associated with DRC and MC

Isolates Alt10 (pre-harvest collection 2009) and Alt16 (STE-U 8409; post-harvest collection 2009) were identified through a Genbank Basic Local Alignment Search Tool search for nucleotides (BLASTn) (Clark et al. 2016) and phylogenetic analysis as *A. infectoria* and *A. eureka* Simmons respectively (Fig. 1; Suppl. Table 2). The *Alternaria*

species, *A. alternata, A. arborescens* and *A. tenuissima* could not be separated using sequencing of the ITS region (Fig. 1), thus phylogenetic (parsimony) analysis was conducted on each of the following genetic loci: endoPG, OPA1–3 and OPA2–1. The sequences obtained from GenBank (Suppl. Table 2) are representative type strains of *A. alternata* EGS34–016 and *A. perangusta* EGS44–160 being ex-type strains and *A. arborescens* EGS39–128 and *A. dumosa* EGS45–007 being holotype strains (Simmons 2007).

A partition homogeneity test (PHT) was performed on the invariant characters of the three combined datasets (endoPG, OPA1–3 and OPA2–1) as well as the combination of individual genetic loci with another locus (endoPG and OPA1–3, endoPG and OPA2–1, and OPA1–3 and OP2–1). Each of the combinations gave a *P* value of 0.001 in the PHT. Parsimony and Bayesian analysis were conducted on the combined dataset.

The phylogeny of the combined datasets resulted in 10 clades, each containing the sequence of a GenBank reference isolate (Fig. 2). Two equally parsimonious trees were recovered. Significant Bayesian probability values (>60%) are shown together with the most likelihood (bootstrap) values of the parsimony analysis. Clade 1 consists of the reference strains *A. turkisafria* EGS 44–159 and *A. perangusta* EGS 44–160. Clade 2 contains the reference strain *A. dumosa* EGS 45–007, Clade 3 contains the reference strain *A. alternata* EGS 45–002, and Clade 4 contains the ex-type reference strain *A. alternata* EGS 34–016 as well as the reference isolates *A. alternata* EGS 34–039 and EGS 45–008. Clades 5, 6 and 7 contained the reference strains *A. alternata* EGS 45–062, *A. tenuissima* EGS 34–015 and *A. alternata* EGS 45–010, respectively. Clade 8 contains the reference strain *A. arborescens* EGS 39–128. The outgroup sequences were *A. gaisen* EGS 37–1321 (Clade 9) and *A. longipes* EGS 30–033 (Clade 10). The representative strains and the isolates identified as *A. infectoria* and *E. eureka* could not be sequenced for the endoPG region or for the anonymous region datasets, OPA1–3 and OPA2–1, due to a lack of PCR amplification with the primers used.

The *Alternaria* species identified morphologically could not be separated into distinct clades by using phylogenetic analysis of the endoPG region alone. This analysis separated the isolates into three species-groups, group one constituted *A. arborescens* and *A. dumosa,* group two *A. alternata,* and group three *A. tenuissima.* For the pre-harvest season's sample collections (2009, 2010) and the air inoculum the species-groups *A. dumosa/A. tenuissima* had the highest occurrence with 13 out of 21 samples, while the *A. arborescens* species-group occurred most frequently for both post-harvest seasons with eight out of 16 tested samples.

The genetic region, OPA1–3, could not distinguish between *A. alternata* and *A. tenuissima* nor could *A. alternata* be distinguished from various other reference sequences. *Alternaria arborescens,* which occurred most frequently post-harvest, was the only species-group that could be distinguished from the other reference sequences with the genetic region, OPA2–1. *Alternaria alternata* clustered with reference sequences including *A. tenuissima, A. dumosa* and *A. gaisen.* However, isolates could not be separated from each other in this clade.

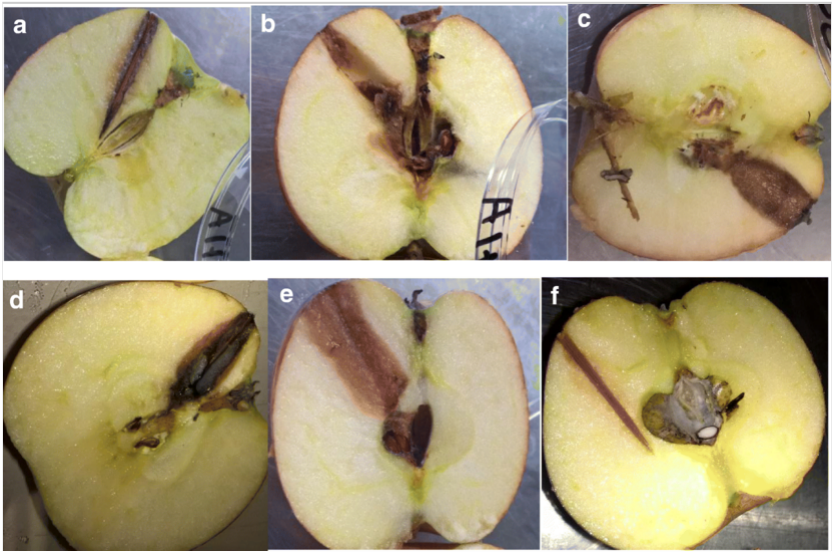
The three genetic regions were combined in a multi-gene phylogeny which could distinguish between the various *Alternaria* species. *Alternaria arborescens* occurred frequently in the post-harvest subset of samples. *Alternaria alternata* and *A. tenuissima* could be separated into distinct clades, with *A. alternata* occurring in all the sources more frequently during pre-harvest. *Alternaria dumosa* was most frequently detected from the aerial samples. Only one isolate from the 2010 pre-harvest collection was identified as *A. tenuissima.*

Pathogenicity of *Alternaria* species

Five days after inoculation the species that appeared the most virulent on 'Starking' apples was *A. arborescens* with an average lesion size of 11.03 mm (±2.79, 95% CI). The species *A. alternata*, *A. infectoria* and *A. eureka* had a smaller average lesion size of 9.71 mm (±2.79), 8.74 mm (±3.14), and 6.78 mm (±2.72), respectively (data not shown). After 10 d incubation there was no significant difference between *A. alternata* (13.90 mm, ±2.79), *A. infectoria* (12.56 mm, ±3.14), *A. arborescens* (20.01 mm, ±2.72) and two of the three tested *A. tenuissima* isolates (20.2 mm, ±2.26) had significantly larger lesions. While *A. eureka* and one of the *A. tenuissima* isolates appeared less virulent (7.11 mm, ±2.72; Fig. 4). While *A. arborescens, A. alternata,* and *A. infectoria* often colonised the core of the inoculated apples, *A. eureka* was pathogenic in the flesh of the apple, but frequently did not colonise the core (Fig. 6). Occasionally a dry corky lesion surrounding the core was observed for *A. eureka* inoculated apples. *Alternaria alternata* produced classic DCR symptoms of dry, corky lesions, while *A. arborescens* presented brown, spongy lesions, and *A. infectoria* lesions appeared dark brown to black. Re-isolation from the edge of the lesion confirmed pathogenicity of the tested isolates thereby fulfilling Koch's postulates.

Fig. 6

Alternaria spp. isolates in pathogenicity test on 'Starking' apples. Isolates used were **a** STE-U8409 (*A. eureka*) showing brown lesion only extending into flesh, **b** STE-U8409 (*A. eureka*) lesion extending into core, **c** STE-U7632 (*A. alternata*) with dry, corky core colonisation, **d** STE-U7634 (*A. infectoria*) with black core rot, and **e** STE-U7636 (*A. arborescens*) with spongy brown lesion, **f** negative control with sterile toothpick



Penicillium spp. associated with pre-and post-harvest wet core rot

The identification of *Penicillium* species from pre- (fallen fruit) and post-harvest WCR were undertaken using the partial beta-tubulin PCR-RFLP method and comparing the isolates' banding patterns with the identified species of Van der Walt et al. (2010). The following *Pencillium* species were identified from WCR isolations: *P. expansum, P.* species (aff. *dendriticum*), *P. ramulosum, P. rugulosum, P. chermesinum, P. glabrum* and *P.* species (aff. *cecidicola*). Of the 60 samples collected pre-harvest and the 63 samples post-harvest, 13.33 and 7.94% could not be identified to species level using the beta-tubulin PCR-RFLP, some of which were further characterised using ITS and/or beta tubulin sequencing (provided they were successfully single spored).

The two most frequently isolated *Penicillium* species from WCR were *P. expansum* and *P. ramulosum* with *P. ramulosum* having the highest incidence pre-harvest and *P. expansum* the highest incidence post-harvest. *Penicillium expansum* incidence increased from 15% pre-harvest to 52% post-harvest, while *P. ramulosum* incidence decreased from 50 to 29% pre- to post-harvest. *Penicillium glabrum* was only isolated pre-harvest and *P. rugulosum* occurred only during post-harvest. *Penicillium* sp. (aff. *cecidicola*) occurred at low frequencies preharvest in 2010 (Table 2).

Table 2

The population distribution (%) of the identified *Penicillium* isolates for each collection source using beta-tubulin PCR-RFLP

Species name	Pre-harvest 2009	Pre-harvest 2010	Post-harvest 2009	Post-harvest 2010	Mummies 2009	Mummies 2010	Mites 2009	Mites 2010	Air A* 2010	Air B* 2010
n	9	10	13	3	10	10	10	10	11	8
Clonostachys sp.	1 (11%)	0	0	0	0	0	0	0	0	0
P. brevicompactum	0	0	0	0	0	0	0	1 (10%)	0	0
P. cecidicola	1 (11%)	0	0	0	0	0	1 (10%)	0	1 (9%)	1 (13%)
P. crustosum	0	0	1 (8%)	0	0	0	0	0	0	0
P. expansum	2 (22%)	1 (10%)	2 (15%)	1 (33%)	7 (70%)	6 (60%)	4 (40%)	4 (40%)	7 (45%)	7 (88%)
P. glabrum	0	0	0	0	0	0	1 (10%)	0	2 (18%)	0
P. novae-zeelandiae	0	0	0	0	0	0	0	0	1 (9%)	0
P. paneum	0	0	0	0	1 (10%)	0	0	0	0	0
P. ramulosum	4 (44%)	6 (60%)	8 (61%)	2 (66%)	0	0	0	0	0	0
P. rugulosum	0	0	0	0	0	2 (20%)	0	0	0	0
P. solitum	0	1 (10%)	0	0	0	0	0	0	0	0
P. sp. (aff. *cecidicola*)	0	1 (10%)	0	0	0	0	0	0	0	0
Other *Penicillium* sp.	1 (11%)	1 (10%)	2 (15%)	0	2 (20%)	2 (20%)	4 (40%)	5 (50%)	2 (18%)	0

*Samples were obtained from two orchards in the Ceres region (A and B), while all other samples pre and postharvest were collected from orchard B

The ITS gene tree divided the references and eight *Penicillium* sequences into 13 clades. The majority of the *Penicillium* isolates were identified as *P. ramulosum* (Clade 1) and *P. expansum* (Clade 4). Forty-two *Penicillium* isolates' ITS sequences were identical to the *P. expansum* Genbank reference sequence AB479309 (only ten isolates represented in the phylogenetic tree; Fig. 3). Other isolates were identified as *P.* sp. *(*aff. *brevicompactum), P.* sp. (aff. *cecidicola*), *P. crustosum, P. glabrum, P.* sp. *(*aff. *glabrum), P. novae-zeelandiae, P. paneum, P. rugulosum* and *P. solitum.* Two isolates, initially classified as *Penicillium* sp., clustered with very good bootstrap support (100%), as *Clonostachys* sp. (Clade 12). *Talaromyces helicus* was used as outgroup to root the phylogenetic ITS tree (Fig. 3). Partial beta tubulin sequence analysis identified isolate STE-U 8431 as *Penicillium polonicum* Zaleski, isolate STE-U 8432 and STE-U 8433 as *P. crustosum*, and STE-U 8588 was identified as *Clonostachys rosea* (Link) Schroers, Samuels, Seifert & W. Gams (Fig. 4). The *P. polonicum* isolate and the *C. rosea* isolate were shown to be less or equally pathogenic in comparison to the two tested *P. crustosum* isolates in the 'Starking' pathogenicity test (Fig. 5).

The population distribution of *Penicillium* sp. was determined from each collection pre- and post-harvest as well as for each of the inoculum sources, air, mites and mummies (Table 2). *Penicillium expansum* was the only species that occurred consistently in all seasons and tested inoculum sources and *P. ramulosum* was detected in symptomatic fruit pre- and post-harvest, but not in potential inoculum sources (Table 2). Small percentages of the other species occurred throughout the collection sources (Table 2).

Discussion

Fungi isolated from pre- and post-harvest core rot symptomatic fruit and inoculum sources were identified to genus level using morphological characteristics. These included the two most frequently isolated fungi from core rot symptomatic fruit namely the genera *Alternaria* and *Penicillium.* It was not possible to identify these fungi to species level using only morphological characteristics, thus molecular tools were implemented to assist in the identification of the *Alternaria* and *Penicillium* species. Phylogenetic studies have shown that the Alternaria complex contains seven sections (Woudenberg et al. 2013, 2015). Eleven phylogenetic species clades and one (*Alternaria alternata*) species now comprise the genus *Alternaria*. Earlier studies have attempted to distinguish between small-spored *Alternaria* species such as *A. alternata, A. tenuissima* or *A. arborescens* within new genetic regions, but without success (Peever et al. 2004, 2005; Andrew et al. 2009). In this study, *Alternaria* spp. were identified as *A. eureka, A. dumosa, A. infectoria,* and the other three common small-spored *Alternaria* species (*A. alternata, A. arborescens and A. tenuissima*) were also present. Only the first three were identified using the ITS locus, since small-spored *Alternaria* species are difficult to distinguish using common "barcoding loci". Although *Alternaria infectoria* could be distinguished from species of the *A. alternata* complex using ITS sequence analysis due to a 26 bp insert (de Hoog and Horré 2002). *Alternaria eureka*, a species not previously identified as a core rot pathogen, was identified from the 2009 post-harvest collection in this study and confirmed to be pathogenic on 'Starking' apples.

The combined genetic loci endoPG, OPA1–3 and OPA2–1 further enabled species identification of *A. arborescens, A. alternata, A. dumosa* and *A. tenuissima.* Peever et al. (2004) reported a partition homogeneity test (PHT) performed on the combination of the loci into a single phylogeny resulted in a significant *P* value of 0.002. Combination of the datasets was therefore controversial, since Cunningham (1997) stated that the accuracy of the phylogenetic analysis increases with an increase in P value (above 0.01), and if the P value of the PHT is lower than 0.001 the combined data suffers. Due to this controversy about the multi-gene phylogeny with the P value of 0.001 observed in this study, the datasets were analysed separately to establish whether each of the genetic loci could distinguish between the *Alternaria* sp. observed from apple core rot as well as other small-spored *Alternaria* sp. reference isolates. The separate datasets, endoPG and OPA1–3 gave well defined clades, separating *A. arborescens* from *A. alternata.* In the endoPG dataset *A. tenuissima* clustered together with the reference isolate *A. dumosa,* a species reported on citrus (Fazlikhani and Soleimani 2013). Various *A. alternata* reference sequences separated into different clades of the phylogenetic tree (Fig. 2). In the OPA1–3 dataset the *A. tenuissima* reference isolate clustered together with the *A. alternata* EGS45–062 reference sequence in a well-supported clade. The OPA2–1 dataset distinguished between *A. arborescens* and *A. tenuissima / A. alternata* but the bootstrap values were not well supported and the reference isolates *A. gaisen, A. dumosa, A. tenuissima* and various *A. alternata* isolates grouped together to form one clade. *Alternaria arborescens* was consistently detected between the three analyses for each of the collection sources.

The phylogenetic results of endoPG and OPA1–3 can be compared with one another to establish which genetic loci distinguishes better between *A. arborescens, A. tenuissima* and *A. alternata.* EndoPG had higher values for the consistency, retention and rescaled consistency indexes, but had lower tree length values and parsimony-informative characters compared to OPA1–3. EndoPG is a known virulence factor, and therefore most likely under selection pressure due to host-pathogen interactions, whereas OPA1–3 is an anonymous region. OPA1–3 was found to be more variable between the species, which increases its chance to separate the species from one another, but variation within the species also occurred. This results in isolates from the same species grouping in different clades of the phylogenetic tree. The combined dataset has low bootstrap values causing certain clades to collapse. This decreases the ability of the phylogenetic tree to distinguish between specific *Alternaria* species except for *A. arborescens.* The analysis using ITS and multilocus phylogeny of the pre- harvest isolates identified the following species *A. infectoria*, *A. alternata*, *A. arborescens*, *A. dumosa* and *A. tenuissima,* Whereas *A. infectoria* and *A. tenuissima* were not detected in postharvest samples. In the combined analysis the air inoculum samples were identified as *A. alternata, A. arborescens* and *A. dumosa* (Fig. 2). Previously only *A. arborescens, A. infectoria, A. tenuissima* and unidentified *Alternaria* sp. had been reported from South African Alternaria dry core rot on apples (Kang et al. 2002; Serdani et al. 2002). Serdani et al. (2002) also concluded that *A. tenuissima* was the most prevalent species to cause DCR in South Africa. Interestingly a study of core rot pathogens from China identified *A. alternata, A. arborescens*, *A. infectoria* and *A. tenuissima* (Gao et al. 2013). The same species groups were found to infect citrus and walnut (Peever et al. 2004, 2005; Hong et al. 2006). *Alternaria tenuissima* has also been reported to cause dry core rot on apples in the USA (Kou et al. 2014). The *A. eureka* isolate (STE-U 8409, Alt16), that had been isolated from postharvest DCR symptomatic fruit, was confirmed to be pathogenic on 'Starking' apples, but did not cause core rot symptoms as often as the other tested species *A. arborescens, A. alternata* and *A. infectoria* under the tested conditions. Further investigations into the frequency of occurrence of this pathogen in a larger number of orchards would be required to establish the relevance of this new core rot pathogen.

To prevent decay caused by *A. alternata* and related fungal species, integrated control recommendations (together with orchard fungicide applications and breeding efforts) include the disinfection of wooden picking bins, careful handling of fruit to prevent wounds, rapid postharvest removal of field heat from fruit, and addition of chlorine to the

dump tank (Volk et al. 2015).

The two main species isolated, from both pre- and post-harvest WCR, were *P. expansum* and *P. ramulosum. Penicillium expansum* occurred less frequently than *P. ramulosum* pre- and post-harvest. *Penicillium expansum* was distributed through all of the collection sources, with high incidence in each of the inoculum sources, its pathogenicity is well known (van der Walt et al. 2010; Villanova et al. 2012). *Penicillium ramulosum* occurred in samples from the pre- and post-harvest collection, but did not occur in any other inoculum source. The other species causing WCR preharvest were *P*. sp. (aff. *brevicompactum)*, *P*. sp. (aff. *cecidicola*), and *P. polonicum* while *P. crustosum* was found to cause WCR post-harvest. Van der Walt et al. (2010) isolated *P. ramulosum and P. expansum* frequently from pre-harvest WCR symptoms. Their and our results suggests that prior to harvest the fruit is infected with *P. ramulosum* and some of the fruit drop prematurely due to the infection of the fungi. The infected fruit that does not drop prematurely ripens normally and is harvested with the rest of the orchard. These fungi remain latent until after storage, when favourable conditions increase the infection rate. *Penicillium expansum*, an opportunistic fungus infecting citrus fruits, apples, pears and cherries post-harvest through small wounds is the main causal organism of 'blue mould'. Blue mould produces the toxin patulin that is harmful in apple juice and apple products. Patulin is produced by the fungus when it rots the host. (Sanderson and Spotts 1995; Pitt and Hocking 1997; Morales et al. 2007).
AQ4

Other *Penicillium* species confirmed through sequence analysis were identified as *P. crustosum, P. glabrum, P. novae-zeelandiae, P. paneum, P. polonicum, P. rugulosum, P. solitum, P.* sp. (*aff. brevicompactum*)*, P.* sp. (aff. *cecidicola*), and *P.* sp. (aff. *dendriticum*). Similarly, *P. glabrum, P.*sp. (aff. *cecidicola*) and *P.* sp. (aff. *dendriticum*) were identified in the study by van der Walt et al. (2010) in South African wet core rot samples. Van der Walt furthermore identified *P. chermesinum,* which was not detected in the current study. Gao et al. (2013) identified *P. chrysogenum* Thom, *P. expansum, P. paneum, P. viridicatum* associated with WCR symptoms on Fuji apples in China. *Penicillium polonicum* has previously been shown to cause storage rot on onions (Çakir and Maden 2015). This is the first report of *P. polonicum* pathogenic on apple. *Clonostachys rosea* is a biocontrol agent for soil borne pathogens, but has been described as weak pathogen on fruit and causes potato dry rot (Theron and Holz 1991; Pratella and Mari 1993). This is the first report of *C. rosea* as pathogen on apple. Weak colonisation capability on apples has been reported for other fungi, used as biocontrol agents on other crops, possibly due to cold sensitivity of the pathogens, which could prevent losses though decay (Conway 1983; Pratella and Mari 1993). This should be tested in larger studies with virulence tests involving multiple isolates of the reported species that were associated with core rot in this study.

Several mycotoxin producing *Penicillium* species were detected in this study, i.e. *P. crustosum,* which has frequently been described as apple pathogen (Frisvad and Samson 2004; Vico et al., 2014; Rharmitt et al. 2016). In South African pears and apples *P. expansum*, followed by *P. crustosum* were amongst the most pathogenic *Penicillium* species causing postharvest decay on apples (Louw and Korsten 2014). *Penicillium novae-zeelandiae* was isolated from the air inoculum. This species has previously been described as endophyte from *Vitis vinifera* L. and *Quercus suber* L. (Serra et al. 2008; Houbraken et al. 2011) and produces patulin in culture (Alfaro et al. 2003). There has been no previous mention of this species in association with apple core rot or any other plant disease. As pathogenicity tests were not done using this species its pathogenicity on apples is currently unknown. *Penicillium paneum* and *P. rugulosum* were detected in one and two samples of apple mummies, respectively, and no pathogenicity tests were conducted to verify if this species would cause core rot on apples. The occurrence of *P. solitum* preharvest causing blue mould of apple has previously been shown (Sanderson and Spotts 1995; Pianzzola et al. 2004).
AQ5
AQ6

Combrink et al. (1985) identified *P. funiculosum* as the main *Penicillium* species associated with core rot in South Africa, however none of the isolates from this study had sequence similarity to *P. funiculosum.* This could either be due to a too small sample size, the fungus not occurring in the sampled orchards or isolates previously identified morphologically as *P. funiculosum* are now, using molecular techniques, identifying as different *Penicillium* species.

Previous studies on WCR established that DPA, which was used to prevent superficial scald, reduced the water tension of the flume water, which could have allowed contaminated water to flow into the core regions of susceptible cultivars contributing to an increase in core rot infection (Combrink and Ginsburg 1973; Combrink et al. 1985; Spotts et al. 1988).

Wet core rot is not exclusively caused by *P. expansum* as previously reported, but through a wide range of different species, including *P. ramulosum* pre-harvest. Although the incidence of the *Penicillium* species that occurred in both pre- and post-harvest isolations differed, a link could be established between the species that occurred in both pre- and post-harvest WCR. Managing this link between pre- and post-harvest can be problematic. An integrated management strategy should be implemented where chemical control should be used in combination with sanitation practices, both in the orchard and in the pack house, to reduce the inoculum build-up of the same species.

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Electronic supplementary material

ESM 1	ESM 2	ESM 3
(PDF 292 kb)	(DOCX 29 kb)	(DOCX 28 kb)

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AQ7

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