Short communication

PCR–RFLP and sequence data delineate three *Diaporthe* species associated with stone and pome fruit trees in South Africa

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Abstract

Diaporthe species include canker pathogens on a wide variety of hosts. In South Africa, Diaporthe canker of apple, pear and plum rootstocks has been attributed to *Diaporthe ambigua*. Recently, we recognized that isolates of *D. ambigua* exhibited different morphological features and thus questioned the identity of these isolates. A small set of isolates was thus chosen for comparison using DNA-based methods. Polymerase chain reaction amplification of ribosomal DNA, Restriction Fragment Length Polymorphisms and DNA sequencing revealed that isolates which had been regarded as *D. ambigua* in the past were three distinct species. These are *D. ambigua, D. perjuncta* and an unknown *Phomopsis* sp. This discovery has special relevance to research done on a dsRNA virus previously thought to occur in *D. ambigua* and now shown to infect *D. perjuncta*.

Diaporthe ambigua Nitschke is an important pathogen of *Malus domestica*, *Pyrus communis* and *Prunus salicina* in South African fruit orchards (Smit et al., 1996a,b; 1998). Infection by *D. ambigua* is associated with sunken lesions with longitudinal cracks on affected fruit trees (Smit et al., 1996a,b). The fungus rapidly kills nursery rootstocks, but also kills mature rootstocks over a longer period of time (Smit et al., 1996b).

South African isolates of *D. ambigua* from fruit trees differ both in morphology and virulence (Smit et al., 1996a). Some isolates were shown to be infected with a mycovirus (Smit et al., 1996b). Subsequent studies have characterized this virus, which is now known as *Diaporthe ambigua RNA virus* (DaRV) (Preisig et al., 2000). Since the virus-infected isolates do not sporulate in culture, morphology alone is not sufficient to correctly identify these isolates.

In order to correctly identify *D. ambigua* isolates associated with fruit tree cankers, especially those infected with DaRV, Polymerase Chain Reaction–Restriction Fragment Length Polymorphisms (PCR–RFLP) and sequencing studies were undertaken. In this study, we report that the virus-infected isolates previously thought to represent *D. ambigua* are, in fact, representative of *D. perjuncta*. The virus-free isolates used in previous studies include two different species, namely *D. ambigua* and an unknown *Phomopsis* sp. (anamorph of *Diaporthe*).

All fungal isolates used in this study were obtained from the culture collection of the Tree Pathology Co-operative Programme (TPCP), housed at the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria. These included the virus-infected *D. perjuncta* (CMW3407 and CMW5289); *D. ambigua* (CMW5287; CMW5288; CMW5587) and *Phomopsis* sp. (CMW5588). All isolates were grown on 2% potato dextrose agar (PDA) (Biolab).

Sterile apple twigs were placed in Petri dishes and 2% water–agar was added so that the twigs were half covered with agar. Agar plugs of the test strains were placed in separate Petri dishes in contact with the apple twigs and the plates were sealed with Parafilm (American National Can, IL). The plates were subsequently incubated in the dark at 25 °C for 1 week. They were then exposed to a mixture of cool-white fluorescent and near-ultraviolet light and checked regularly for sporulation (Smit et al. 1996a,b).

Isolation of chromosomal DNA was achieved using a modification of the technique described by Raeder and Broda (1985). Mycelium was grown in 2% malt extract broth (Biolab) in Erlenmeyer flasks at room temperature. The mycelium was harvested after 2 weeks and lyophilized. The lyophilized mycelium was ground to a fine powder with a pestle and mortar in liquid nitrogen. DNA was isolated using DNA extraction buffer (200 mM Tris–HCl (pH 8.5); 250 mM NaCl; 25 mM EDTA (pH 8.0); 0.5% SDS) and extracted with a 1 : 1 mixture of phenol and chloroform followed by a final chloroform extraction. DNA was precipitated with 2 volumes of 100% ethanol in the presence of 10% (v/v) of 3 M sodium acetate (pH 4.5) and pelleted by centrifugation in a bench-top centrifuge at 12 000 rpm for 30 min at 4 °C. Pelleted DNA was washed with 70% ethanol.

The primers ITS1 (5′-TCCGTAGGTGAACCTG-CGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATA-TGC-3′) (White et al., 1990) were used to amplify and sequence fragments of ca. 500 bp from the DNA of each fungal isolate (Figure 1). The primers amplify the sequences of ITS1-2 and 5.8S rRNA gene. The sequences have been deposited in the EMBL nucleotide sequence database as CMW3407 (AJ458385), CMW5289 (AJ458386), CMW5287 (AJ458389), CMW5288 (AJ458387), CMW5587 (AJ458388) and CMW5588 (AJ458390). A total of 10 taxa were included in the analysis. These included three recently published reference taxa, namely *D. perjuncta* (STE-U2655) (AF230744), *D. ambigua* (STE-U2657) (AF230767) and a *Phomopsis* sp. (STE-U2680) (AF230766) (Mostert et al., 2001).

Amplification conditions for PCR were 1 cycle at 96 °C for 2 min, 35 cycles at 94 °C for 30 s, 64 °C for 30 s and 68 °C for 2 min followed by a final elongation step at 68 °C for 10 min. The reaction mixture consisted of ca. 5 ng DNA, 0.3 µM each primer, 0.2 mM dNTPs (each), 1 × Expand buffer and 2.5–5 U Expand *Taq* polymerase (Roche Molecular Biochemicals). The
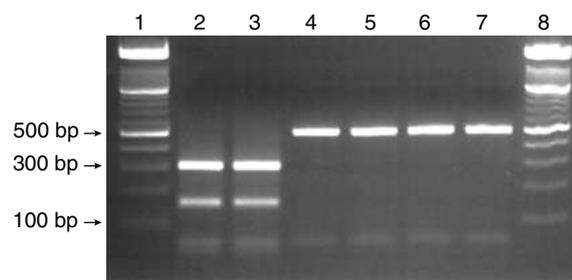

Figure 1. RFLP technique to distinguish isolates of *D. perjuncta*, *Phomopsis* sp. and *D. ambigua* used in this study. *Mse* I-digested PCR products amplified from the ITS region of the rDNA gene operon using the primer pair ITS1/ITS4. Lane 1: 100 bp DNA molecular weight marker; Lane 2: *D. perjuncta* (CMW3407); Lane 3: *D. perjuncta* (CMW5289); Lane 4: *Phomopsis* sp. (CMW5588); Lane 5: *D. ambigua* (CMW5587); Lane 6: *D. ambigua* (CMW5287); Lane 7: *D. ambigua* (CMW5288) and Lane 8: 100 bp DNA molecular weight marker. The enzyme *Mse* I has an additional restriction site in the sequences of *D. perjuncta* isolates that makes the RFLP profiles of these virus-infected isolates different from the virus-free isolates. The DNA restriction fragments were separated on a 2% agarose gel stained with ethidium bromide.

amplified PCR products were separated on 1% agarose gel in 1× Tris-acetate/EDTA buffer.

Purified PCR products were analyzed for RFLPs using *Mse* I. Additionally, their nucleotide sequences were determined and aligned using ClustalW and Phylogenetic Analysis Using Parsimony (PAUP) Version 4.0b1 (Swofford, 1998). The sequences were compared with deposited DNA sequences in GenBank using blastn.

A heuristic search from the aligned sequences produced four most parsimonious trees of 178 steps (Figure 2). The trees did not differ in topology. *Cryphonectria cubensis* was used as an outgroup. Of the 508 characters of sequence, 374 were constant. Of the remaining characters, 53 variable characters were parsimony-uninformative while 81 were parsimony-informative. The trees were evaluated with 1000 bootstrap replications and decay indices for clade stability. The phylogenetic tree topology showed three different clades.

One of the isolates used in this study and previously identified as *D. ambigua* (CMW5588) grouped with a *Phomopsis* sp. (STE-U2680) (100% bootstrap). Three isolates, *D. ambigua* (CMW5288, 5587, 5287) grouped together with a reference strain of this fungus (STE-U2657). Two isolates, believed to be *D. ambigua*
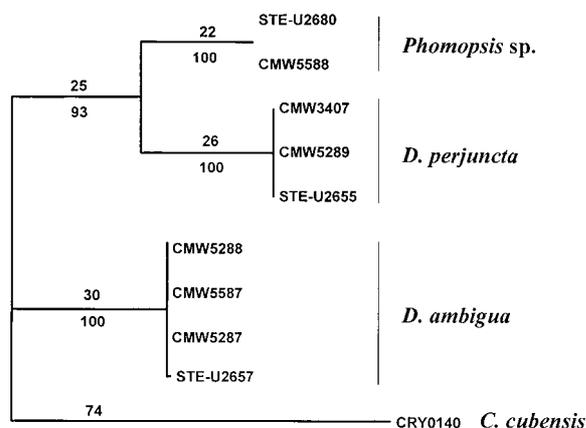
Figure 2. Phylogenetic relatedness of *D. perjuncta, Phomopsis* sp. and the different *D. ambigua* isolates used in this study. This is one of four most parsimonious trees (tree length = 178, CI = 0.949, RI = 0.958, RC = 0.910 and HI = 0.051) resulting from maximum parsimony analysis of the aligned ITS sequence data of the respective isolates. Bootstrap values are indicated below the branches while the number of base substitutions are indicated above the branches. The virus-infected *D. perjuncta* isolates (CMW3407 and CMW5289) form a clade of their own together with the reference *D. perjuncta* (STE-U2655). The tree is rooted to *C. cubensis*. The *D. ambigua* isolates (CMW5288, CMW5587 and CMW5287) and the *Phomopsis* sp. isolate (CMW5588) were those used in transfection studies.

(CMW3407 and CMW5289), and from which DaRV was isolated and characterized (Preisig et al., 2000), grouped together with *D. perjuncta* (STE-U2655).

Restriction digests of the ITS region amplicons using *Mse* I produced unique restriction fragment patterns that distinguished the virus-infected isolates shown to be *D. perjuncta* (CMW3407 and CMW5289) from the virus-free *D. ambigua* (CMW5587, 5288 and 5287) and *Phomopsis* sp. (CMW5588) isolates (Figure 1). Aligned sequences showed that the virus-infected isolates have an additional *Mse* I site (T↓TAA) approximately at position 150 from the 5′ end of the ITS region amplicons. Therefore, the RFLPs using *Mse* I resulted in two bands (ca. 150 bp and 350 bp) in the case of *D. perjuncta* isolates, while the other three isolates had only one detectable band.

The virus-free and virus-infected isolates used in this study had all been initially identified as *D. ambigua* (Smit et al., 1996a,b). This study preceded DNA sequencing techniques now commonly used in fungal taxonomy. With the exception of *Phomopsis* sp. that originated from peach, all the isolates originated from

apple. In this study, we have shown that DaRV did not originate from *D. ambigua* but from *D. perjuncta*. This implies that the acronym DaRV previously provided for the virus might be somewhat misleading. However, it could still be used, with Da reflecting two letters in the genus name *Diaporthe*. Certain characteristics of the virus-infected *D. perjuncta* isolate such as reduced growth rate and virulence might, therefore, be species-specific and not necessarily due to infection by DaRV as assumed in previous studies (Smit et al., 1996b; Preisig et al., 2000).

Comparison of the virus-free and virus-infected isolates show some morphological differences. The virus-infected isolates display morphological characteristics such as altered colony morphology and suppressed sporulation, which have been observed in hypoviras-infected *Cryphonectria parasitica* and *C. cubensis* (Anagnostakis, 1982; Chen et al., 1996; van Heerden et al., 2001). This suggests that the virus has potential as a biological control agent to control *D. perjuncta*, which is a pathogen of grapevine (Mostert et al., 2001; Melanson et al., 2002). Although *D. perjuncta* is not regarded as an important pathogen in South African vineyards (Mostert et al., 2001), it has proved to be significant in Australian vineyards (Melanson et al., 2002). In Australia, the fungus is regarded to play a major role in bud loss of grapevines, even though mites and adverse weather conditions may also contribute in this regard. However, since the fungus reproduces sexually, the development of DaRV as a biological control agent for *D. perjuncta* may be difficult to deploy in practice. This is because mycoviruses are transmitted during hyphal anastomosis between a virus-infected and a virus-free isolate. Sexual reproduction gives rise to different vegetative incompatibility groups (VCGs) which are known to form a barrier to virus spread within a fungal population (Anagnostakis, 1977).

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