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FACTORS ASSOCIATED WITH CONIOTHYRIUM CANKER OF *EUCALYPTUS* IN SOUTH AFRICA

By

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DECLERATION

I, the undersigned, hereby declare that the thesis submitted herewith for the degree, Philisophiae doctoriae, to the University of the Orange Free State, contains my own independent work. This work has hitherto not been submitted for any degree at any other university of faculty.

Leonel Merwe van Zyl
July 1999

Dedicated to my family

A PHILOSOPHER'S life is spent in discovering that, of the half-dozen truths he knew when a child, such an one is a lie, as the world states it in set terms; and then, after a weary lapse of years, and plenty of hard thinking, it becomes a truth again after all, as he happens to newly consider it and view it in a different relation with the others.

A Soul's Tragedy, Part II.

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PREFACE

The production of *Eucalyptus* is of considerable economic importance to the South African forestry industry. More than 50 % of the annual timber produced is derived from a range of *Eucalyptus* species, hybrids and clones. Exotic plants, established in monoculture are often susceptible to infection by various pathogens and *Eucalyptus* trees are no exception. A number of diseases have, thus, been reported to affect *Eucalyptus* propagation in this country and elsewhere.

In September 1988, a new and devastating *Eucalyptus* stem canker disease was observed for the first time in the Zululand forestry region of KwaZulu-Natal. The causal agent was identified as a species of *Coniothyrium*. Five *Coniothyrium* species are known to be associated with leaf diseases of *Eucalyptus*. However, the species associated with stem cankers in South Africa was considered to be unique. The fungal pathogen was, therefore, described as *Coniothyrium zuluense* due to its origin and occurrence in the Zululand forestry area.

Since the discovery of Coniothyrium canker in South Africa, it has become important to have an effective management strategy against this disease. Currently, the most reliable method of reducing losses due to this disease is through the planting of disease resistant species and clones of *Eucalyptus*. In order to effectively manage Coniothyrium canker, it is essential to gain knowledge regarding the biology, as well as the population characteristics of the pathogen. Information pertaining to these characteristics will make it possible to predict the relative durability of selected disease resistant clones.

Very little is currently known about *Coniothyrium zuluense* or the disease that it causes. This thesis represents the first in a series of studies involving various aspects of the population biology and factors influencing development of Coniothyrium canker. Each chapter has been written as an individual entity, although a close relationship exists between research represented in each of these units. A degree of repetition between chapters has been unavoidable.

As an introduction, the thesis commences with a literature review on important aspects of the genus *Coniothyrium*. Firstly, the taxonomic uncertainties and problems linked to the genus are considered. Furthermore, the role of *Coniothyrium* in plant pathology is discussed. The main focus of this review considers the occurrence of *Coniothyrium* species associated with *Eucalyptus* trees, either as saprophytes or parasites. Specific attention is given to *C. zuluense* and the likely impact that the disease might have in South Africa. A list of *Coniothyrium* species causing disease, as well as their ecological importance is also presented.

Since the discovery of Coniothyrium canker in South Africa, considerable effort has been expended on obtaining knowledge of this pathogen. During surveys, we collected a large number of *C. zuluense* isolates from severely infected *Eucalyptus* species and clones. In chapter two, I consider variability in morphology, cultural and virulence characteristics of *C. zuluense*. The primary goal here was to consider possible variability in the population structure of the pathogen. A high degree of variability in pathogenicity would be indicative of a genetically diverse population and *vice versa*.

In chapter three, the population diversity of *C. zuluense* is investigated. The diversity of a pathogen population is indicative of the durability in resistance of selected disease resistant clones. Data pertaining to genetic diversity also reflects on the mode of reproduction, as well as the possible origin of the pathogen. More diverse populations are, thus, more likely to overcome disease resistance in selected clones and it would be more likely that the pathogen originated locally.

Coniothyrium zuluense is known only in South Africa. In 1996, however, a *Coniothyrium* sp. causing similar disease symptoms on an *E. camaldulensis* clone in Thailand, was observed. In chapter four, the phylogenetic relationships between the Thailand *Coniothyrium* species and *C. zuluense* is investigated using molecular techniques. Molecular evidence was needed to determine the identity of the *Coniothyrium* species from Thailand and to show its relatedness to *C. zuluense*. Morphological and pathogenicity tests on the *Coniothyrium* sp. from Thailand were also conducted to support molecular data.

No information is available regarding the biology and factors influencing disease development in *C. zuluense*. During disease surveys, it was noted that bacteria commonly exude from necrotic cankers on severely infected *Eucalyptus* clones. Isolations from cankers have shown that two bacteria commonly occur, together with *C. zuluense*. In chapter five, I consider the identity of these two bacteria using a diagnostic nutrient utilisation method (Biolog's Microplate technique) together with DNA sequences. Pathogenicity tests on Granny Smith apples, as well as on a susceptible *E. grandis* clone were also conducted to investigate the importance of both bacteria in disease development.

In chapter six, levels of polygalacturonase (PG) activity in *C. zuluense* isolates varying in pathogenicity to a susceptible *E. grandis* clone, and in two bacterial species, are determined. PG is considered to be the first cell wall-degrading enzyme produced during plant-pathogen interactions and has been identified as a determining factor in disease development for both fungal and bacterial plant pathogens. The results obtained would give an indication of the possible relationship between *C. zuluense* and the two bacteria that accompany it in nature.

The activation of resistance genes, as well as pathogenesis-related proteins, has been linked positively to disease resistance in various fungal and bacterial pathogens. In a previous study, it was shown that significant differences in disease resistance of two *E. grandis* clones (ZG 14 and TAG 5) to *C. zuluense* infection, was evident. In chapter seven, the presence of such genes is investigated. Possible differences between disease resistance in the two *E. grandis* clones are also consistent. The justification for undertaking this study was to determine whether molecular markers to screen clones for disease susceptibility or resistance might emerge. Such markers would accelerate breeding for improved disease resistant *Eucalyptus* clones.

This thesis expands our knowledge of *C. zuluense* and factors influencing its pathogenicity. It is my sincere hope that the research encompassed in this document will contribute towards an increased knowledge pertaining to *C. zuluense* and also towards the improvement of *Eucalyptus* propagation in South Africa.

CHAPTER 1

The genus *Coniothyrium* in plant pathology, with special reference to species that cause disease on *Eucalyptus*

1.0 INTRODUCTION

Species of *Eucalyptus* L' Heritier are of considerable economic importance, both in Australia where they are native, and in many other countries, where they have been successfully introduced for plantation development. Not only do they represent a major timber resource, but these trees are also used for distillates, tannins, essential oils, nectar, pollen, the production of rayon and viscose, as well as for firewood (Poynton, 1979; Turnbull, 1991). In South Africa, more than 50 % of timber production annually is derived from various *Eucalyptus* species, of which the most important is *E. grandis* Hill ex Maid. (Anonymous, 1995). *Eucalyptus* species in South Africa are managed on a medium-length to short rotation for the production of sawlogs, telephone and transmission poles, mining timber, rough building and fencing materials (Poynton, 1979). The greatest production of industrial eucalypt wood, however, is for the pulp and paper industry and mainly in the form of bleached kraft pulp (Turnbull, 1991).

Where exotic trees are established in plantation monocultures, they are more threatened by pathogens than in natural forests. In South Africa, a number of diseases have been reported on various species and clones of *Eucalyptus* and these cause serious economic losses. Cryphonectria canker, caused by *Cryphonectria cubensis* (Bruner) Hodges, is one of the most serious *Eucalyptus* canker diseases in South Africa (Wingfield *et al.*, 1989). Other stem and root diseases include Botryosphaeria canker caused by *Botryosphaeria dothidea* (Mong.:Fr.) Ces & De Not (Smith *et al.*, 1994) and Pythium and Phytophthora root rot (Linde *et al.*, 1994).

A serious stem canker disease, apparently unknown elsewhere in the world, was first observed in the Zululand forestry region of KwaZulu-Natal, in September of 1988 on a single clone of *E. grandis* (Wingfield *et al.*, 1997). It has subsequently become widespread in the area and occurs, not only on a wide range of *E. grandis* clones, but also on hybrids of this and other species (Coutinho *et al.*, 1997; Wingfield *et al.*, 1997). The causal fungus was identified as *Coniothyrium zuluense* Wingfield, Crous & Coutinho (Wingfield *et al.*, 1997).

Coniothyrium zuluense is of considerable concern to the South African forestry industry, as well as to other forestry groups elsewhere in the world. Its impact on forestry has necessitated investigations on strategies to reduce losses. The aim of this review is to summarise relevant knowledge pertaining to *C. zuluense*. Particular attention is also given to taxonomic problems linked to the genus *Coniothyrium* Corda, as well as the importance of other *Coniothyrium* species previously described as *Eucalyptus* pathogens.

2.0 *CONIOTHYRIUM* CORDA

Coniothyrium is one of the oldest genera in the Coelomycetes and also one of the largest (Reisinger *et al.*, 1977). The genus includes 800 described species that vary considerably in pycnidium structure, conidium and conidiophore morphology (Sutton, 1980). *Coniothyrium* was first described in 1821 as *Clisosporium* Fr. (Fries, 1823), and was subsequently changed to *Coniothyrium* in 1840 (Corda, 1840). However, in 1859 the name was changed to *Monoplodia* Westd. and in 1917 to *Asteropsis* Frag.. During 1923 it was renamed as *Coniothyrinula* Petrak (Petrak, 1923), but was later transferred back to, *Coniothyrium* Corda.

Sutton (1971b) stated that various authors preferred the conservation of the genus *Coniothyrium* Sacc. (type species *C. fuckelii* Sacc.), rather than *Clisosporium* Fr. or *Coniothyrium* Corda. This was in contrast to the published proposal in the *International Code* of 1935, which stated that *Coniothyrium* Corda emend. Sacc. (type species, *C. diplodiella* (Speg.) Sacc.) should be conserved, rather than using earlier homonyms. Subsequently, *Coniothyrium* Corda, lectotype species *C.*

palmarum Corda, was conserved against *Clisosporium* Fr. (type species *C. lignorum* Fr.), and published in the *International Code*, 1956.

Sutton (1971b) stated that the selection of *C. palmarum* as lectotype species for the genus *Coniothyrium*, was unfortunate. *Coniothyrium palmarum* is characterised by annellidic conidiogenous cells, thus, restricting the generic name to a limited number of species. The majority of species described in *Coniothyrium* are, however, similar to *C. fuckelii* in having phialidic conidiogenous cells. Therefore, they are incorrectly placed in the genus *Coniothyrium*. Sutton (1971b, 1980), therefore, proposed that many species currently described in *Coniothyrium*, should be accommodated in *Microsphaeropsis* Höhn. *Microsphaeropsis*, type species *M. olivacea* Höhn, is congeneric with *C. fuckelii* and, thus, provides a more suitable generic place for many *Coniothyrium* species.

Minter *et al.* (1982, 1983a, b) proposed a re-definition of the stages of conidiogenesis. They concluded that all conidia previously described as "annello-conidia", and most conidia described as "phialo-conidia" are all holoblastic and that it is no longer appropriate to distinguish between phialides and annellides in most instances (Minter *et al.*, 1982, 1983a, b). It is, thus, no longer necessary to separate the genera *Coniothyrium* and *Microsphaeropsis*. Taxonomic mycologists, however, have not changed their approach to identify fungal isolates and it is clear that revision is needed to determine which of the 800 described taxa should be retained in *Coniothyrium* and which of these should be accommodated in *Microsphaeropsis*. Ideally mycologists should incorporate molecular techniques, such as sequence analysis together with traditional morphological and ultra-structural studies in making such a decision. This would be extremely difficult as cultures are not available for most taxa in question.

3.0 IMPORTANCE OF THE GENUS *CONIOTHYRIUM* IN PATHOLOGY

Species of *Coniothyrium* are known to survive either as saprophytes, hyperparasites of various plant pathogens, human pathogens, or as plant pathogens on a wide range of plant hosts (Tables 1 and 2). *Coniothyrium* is best known for species such as *C. fuckelii* and *C. minitans* Campbell, which are well known pathogens, saprophytes, and hyperparasites of plants, animals including humans and insects (Tables 1 and 2). The main focus of this review is, however, on the importance of *Coniothyrium* spp. as pathogens of *Eucalyptus*.

3.1 Coniothyrium fuckelii: A plant and human pathogen

Coniothyrium fuckelii is considered to be the anamorph of the ascomycete *Leptosphaeria coniothyrium* (Fuckel.) Sacc. (Sutton, 1971a). This fungus is known as a serious plant pathogen of various plants (Table 1). Its primary hosts are *Rosa* Thunb. and *Rubus* L. species, on which it causes graft canker (Sweets *et al.*, 1982; Muthaiyan *et al.*, 1992) and cane blight (Williamson & Jennings, 1992), respectively. The fungus has also been reported as a hyperparasite of nematodes (Clovis & Nolan, 1983), as well as a human pathogen (Kiehn *et al.*, 1987).

Kiehn *et al.* (1987) diagnosed *C. fuckelii* as the causal agent of a liver infection in a patient suffering from “acute myelogenous leukemia”. In vitro antifungal testing suggested susceptibility to both amphotericin B and ketoconazole. After several weeks, however, the patient refused further treatment and later died. An autopsy was refused. A second report of human infection with *C. fuckelii* was reported by Schell (unpublished data), where a “cutaneous phaeohyphomycosis” was described in a 14 year old girl. The “erythematous plaque” was treated with ketoconazole with no effect. The lesion was then surgically removed.

3.2 Coniothyrium minitans: A fungal biocontrol agent

Coniothyrium minitans is a sclerotial mycoparasite of *Sclerotinia sclerotiorum* (Lib.) de Bary (Adams, 1990; Whipps *et al.*, 1991; Wipps & Gerlagh, 1992; Tu, 1997). Infection of *S. sclerotiorum* by the hyperparasite results in the destruction of hyphal cells (Huang & Kokko, 1987, 1988; Huang & Kozub, 1991; Whipps & Gerlagh, 1992; Tu, 1997) and sclerotial tissues (Huang & Kokko, 1987; Whipps *et al.,* 1991; Gerlagh *et al.*, 1996; McLaren *et al.*, 1996). Several studies have revealed that the mode of hyperparasitism of *C. minitans* on hyphal cells, involves the direct penetration of the host hyphae and the degradation of host cell walls (Adams, 1990; Whipps & Gerlagh, 1992; Tu, 1997).

Sclerotinia sclerotiorum is the causal agent of white mold, also known as sclerotinia rot and sclerotinia wilt, on a wide range of hosts and has a world-wide distribution on numerous field crops and vegetables (Huang & Kokko, 1987; McLaren *et al.*, 1994; McQuilken & Whipps, 1995; Tu, 1997). Most of the biocontrol studies involving *C. minitans* have been concerned with its use as an inoculant applied either to foliage (Harrison & Stewart, 1988; Gerlagh *et al.*, 1996, 1999) or, more frequently, to soil for the control of sclerotia forming pathogens (Whipps, 1987; Budge & Whipps, 1991; Whipps *et al.*, 1992; Whipps *et al.*, 1993; McLaren *et al.*, 1996). Studies have, however, also indicated that *C. minitans* is important in natural biological control of *S. sclerotiorum* in the field (Adams, 1990; McLaren *et al.*, 1994; Tu, 1997). It has been shown that when *C. minitans* is applied to soil as a solid-substrate inoculum, it can infect sclerotia of *S. sclerotiorum* year-round and effectively reduce their number and viability (Budge *et al.*, 1995; Gerlagh *et al.*, 1996, 1999).

Biocontrol measures using *C. minitans* against the white mold fungus (*S. sclerotiorum*) has been extensively studied (see Table 2). Many of the emerging results from this study, however, have yet to be practically applied. This is mainly due to the fact that biocontrol agents are subjected to strict registration guidelines. Another major problem regarding the use of *C. minitans*, lies in the quantity of solid-substrate preparations that are required for effective control (Whipps & Gerlagh, 1992).

3.3 Pathogens or saprophytes of Eucalyptus

To date, 11 *Coniothyrium* species have been described on *Eucalyptus*. Six of these are referred to as "true" *Coniothyrium* species, characterised by annellidic conidiogenous cells (Sutton, 1980). Four species, previously described in *Coniothyrium* have since been re-described, and are now accommodated in the genus *Microsphaeropsis* (Sutton, 1971b, 1980). This genus is currently used for species similar to *Coniothyrium*, but with phialidic conidiogenous cells (Sutton, 1971b, 1980). The fifth *Coniothyrium* sp. was re-described and is currently accommodated in the genus *Fairmaniella* Petrak & Syd. (Sutton, 1980). Morphological characteristics, as well as disease symptoms of fungi formerly described in *Coniothyrium*, are presented in Tables 3A and 3B.

3.3.1 Microsphaeropsis

Most of the *Microsphaeropsis* spp. formerly described in *Coniothyrium*, occur as saprophytes on eucalypts (Sutton, 1974, 1980). The type species, *Microsphaeropsis olivacea* (Bonord: Höhn) Sutton (Basionym, *C. olivaceum* Bonord. apud. Fuckel.) occurs as a saprophyte on *E. ficifolia* Fr. Muell. and has been reported from Australia, India and the USA (Sutton, 1980; Sinclair *et al.*, 1987). Similarly, *Microsphaeropsis eucalypti* (Fragoso) Sutton (Basionym, *C. olivaceum* Bonord *var. eucalypti* Fragoso), as well as *M. globulosa* (Camara) Sutton (Basionyms, *C. globulosum* Camara; *C. olivaceum* Bonord *var. eucalypti* Fragoso; *M. eucalypti* (Fragoso) Sutton; *C. eucalypti* Fragoso), are apparently of no significance to the *Eucalyptus* industry, in that they occur as saprophytes on old leaves of *E. globulus* Labill in Portugal (Sutton, 1971b).

The only *Microsphaeropsis* sp. causing disease on *Eucalyptus* species, is *Microsphaeropsis callista* (H Syd.) (Basionym, *C. callistum* H Syd.) (Sutton, 1971b). This fungus was reported from Australia as a pathogen on living leaves of *E. haemastoma* Sm. causing separate, circular to irregular shaped lesions up to 5 mm in diam. (Sutton, 1971b). Leaf spots sometimes coalesce (Sutton, 1971b). Disease symptoms are similar on both sides of the leaf with raised edges separated from healthy tissue by brown to purplish brown lines surrounded by diffuse halos of brown to purplish brown discolouration (Sutton, 1971b; Cabral, 1985). This pathogen is, however, not considered to be of great economic importance in Australia (Sutton, 1971b).

3.3.2 Fairmaniella

The genus *Fairmaniella* is monotypic with *F. leprosa* (Fairm.) Petrak & Syd. (Synonyms, *C. leprosum* Fairman; *Melanconium eucalypticola* Hansford) as the only species (Sutton 1971b, 1980). *Fairmaniella leprosa* causes disease symptoms that vary for different *Eucalyptus* species (Sutton, 1971b, 1980). This fungal pathogen causes lesions on leaves and shoots of *E. fasciculosa* in Australia (Sutton, 1971b, 1980; Swart, 1988), as well as *E. globulus* from Chile (Sutton, 1971b, 1980; Wingfield *et al.*, 1995). Lesion diam. varies between 3 - and 15-(20) mm and is typically circular to elliptical or irregular in shape. The upper surfaces of lesions are mottled pale to medium brown and surrounded by slightly raised ridges (Sutton, 1971b, 1980). The central region of the lower surface is characterised by grayish brown discolouration (Sutton, 1971b). Lesions on leaves of *E. citriodora* Hook., collected from Zambia, have been shown to vary between 1 - and 7 mm in diam.. Lesions ranged from minute circular flecks to larger irregular lesions that were medium brown, paler in the centre with distinct dark brown raised edges (Sutton, 1971b, 1980). Similar symptoms were also reported from *E. robusta* Sm. in Hawaii and from two unknown *Eucalyptus* species in New Zealand (Sutton, 1971b, 1980).

Fairmaniella leprosa has also been reported from South Africa, where it was found to cause distinct, round, cork-like lesions on leaves of *E. globulus* in the Franschhoek and Stellenbosch areas of the Western Cape Province (Crous *et al.*, 1989a, b, c). Lesions occur 4 m above the ground on mature, older leaves (Crous *et al.*, 1989a, b, c). This pathogen is, however, not considered to be of great economic importance in South Africa, due to its limited host range and distribution (Crous *et al.*, 1989a, b, c).

3.3.3 Coniothyrium senso stricto

Only six of the *Coniothyrium* spp. associated with *Eucalyptus* trees remains in the genus *Coniothyrium*. This is due to Sutton's proposal (1980) that species producing conidia from phialides should be accommodated in either *Microsphaeropsis* or *Fairmaniella*. Differences in morphology, as well as in disease symptoms associated with these *Coniothyrium* species are presented in Tables 4A and 4B.

Coniothyrium ahmadii Sutton (synonym, *Coniothyrium eucalypti* Ahmad.) and *C. kallangurense* Sutton & Alcorn, are not considered to be of any economic importance to the *Eucalyptus* forestry industry. *Coniothyrium ahmadii* occurs on twigs and branches of *Eucalyptus* species in Pakistan (Sutton, 1974, 1980). Its importance as a pathogen is, however, not known. *Coniothyrium kallangurense* is a saprophyte on leaves of *E. microcorys* F. Muell. in Australia (Sutton, 1975).

In 1971, a leaf disease on *E. leptophylla* in Australia was ascribed to *C. eucalypticola* Sutton (Sutton, 1971b). This disease was characterised by circular to elliptical lesions, ranging between 2 – 10 mm in diam.. Pale brown, slightly raised edges were evident due to the pronounced exudation of conidial masses spreading over the leaf surfaces (Sutton, 1971b). Subsequently, Swart (1986) distinguished two additional *Coniothyrium* species as pathogens on *Eucalyptus*, *C. parvum* Swart and *C. ovatum* Swart. *Coniothyrium parvum* causes necrotic leaf spots on *E. melliodora* A. Cunn. ex Schau. and *E. regnans* F. Muell. in Australia. Lesions vary between 1 – 1.5 mm in diam. (Swart, 1986). *Coniothyrium ovatum* causes necrotic leaf spots (1 mm in diam.) on three *Eucalyptus* species, *E. dives* Schau., *E. macrorhyncha* F. Muell. Ex Benth. and *E. obliqua* L'Herit. (Swart, 1986). These species are, however, of no economic importance.

During 1988, Crous *et al.* reported that *C. ovatum* is the causal agent of leaf spots on *E. cladocalyx* F. Muell. and *E. lehmannii* (Preiss ex Schau.) in South Africa. Leaf spots occur mainly on the lower branches of mature trees, and on young coppice undergrowth, causing a prominent discolouration of the upper surface of juvenile leaves (Crous *et al.*, 1988). The leaf spots are irregular and dispersed randomly over the leaves. They are dark purple to almost black in the middle, changing to purplish-brown towards the edges (Crous *et al.*, 1988). The pathogen is, however, not considered to be of any significance to the local forestry industry due to the insignificance of susceptible species.

Coniothyrium zuluense Wingfield, Crous & Coutinho has recently been described as the causal agent of a devastating *Eucalyptus* stem canker disease in South Africa (Wingfield *et al.*, 1997). Comparison of this pathogen with previously described *Coniothyrium* species from *Eucalyptus*, suggested that the species is new to Science (Wingfield *et al.*, 1997). The following section of this review will summarise all relevant information known about *C. zuluense*.

4.0 CONIOTHYRIUM CANKER OF *EUCALYPTUS*

Coniothyrium canker was first observed in the Zululand forestry region of KwaZulu-Natal Province in South Africa in September 1988, where it occurred on a single clone of *E. grandis* (Wingfield *et al.*, 1997). Since its discovery, the pathogen has become widespread and affects various *Eucalyptus* species, clones and hybrids. This disease has rapidly become one of the most serious problems affecting the *Eucalyptus* forestry in South Africa.

4.1 Morphological and diagnostic characteristics

According to Wingfield *et al.* (1997) mycelium of *C. zuluense* is situated internally within the host tissue and is medium to dark brown in colour. The mycelium is branched, septate, thick-walled and smooth to verruculose, ranging between 1.5 – 3 µm in diam.. Pycnidia occur as single or aggregated structures. They are typically intra- or sub-epidermal, globose to depressed ranging between 60 – 120 µm in width and 60 – 80 µm in height. Pycnidial walls are composed of two to three layers of dark brown *textura angularis*. Conidiogenous cells are characteristically annellidic, pale brown, smooth, doliiform to reniform in shape, ranging between 4 - 8 x 2.5 - 3.5 µm in size (Fig. 1A). *Conidia* are medium brown, thick-walled, smooth to verruculose and broadly ellipsoidal (Fig. 1B). Apices of conidia are obtuse and bases sub-truncate to bluntly rounded ranging between (4 -) 4.5 – 5 (- 6) x 2 - 2.5 (- 3.5) µm in size.

The fungus is extremely slow growing on artificial media (Wingfield *et al.*, 1997). Average colony diam. after 21 days on Potato Dextrose Agar (PDA) is 40.5 mm at 30 °C. The slow growing nature of *C. zuluense*, has been attributed to its biotrophic nature (Coutinho *et al.*, 1997; Wingfield *et al.*, 1997). Optimal growth temperature is found to be 30 °C, although *C. zuluense* is able to grow at temperatures, ranging from 15 to 30 °C (Coutinho *et al.*, 1997; Wingfield *et al.*, 1997).

When grown on PDA at 30 °C, it was observed that colonies are irregular, pale olivaceous, with an outer olivaceous grey band of mycelium that is characterised by a pale, mouse grey, margin (Fig. 2A). According to Wingfield *et al.* (1997) colony margins tend to be smoother at lower temperatures. They also observed that when colonies are viewed from below, four bands of colour are evident. The outer two bands are olivaceous with the third band greenish-black and the forth band in the centre of the colonies is rust coloured (Fig. 2B).

4.2 Symptoms and Damage

Initial infections occur on young, green stem tissue during the growing season (Coutinho *et al.*, 1997; Wingfield *et al.*, 1997). This gives rise to small (2 – 5 mm diam.), discrete, necrotic lesions on the stem (Fig. 3). These small lesions coalesce to form large necrotic patches (Fig. 4) (Coutinho *et al.*, 1997; Wingfield *et al.*, 1997). These patches give rise to spindle-shaped swellings that are often cracked and exude copious amounts of red / brown kino (Fig. 5 and 6). This is especially evident in highly susceptible *Eucalyptus* species, clones and hybrids (Coutinho *et al.*, 1997; Wingfield *et al.*, 1997).

Severely susceptible *Eucalyptus* clones are characterised by the development of a series of stem cankers along the entire stem (Fig. 7). Cankers coalesce causing large zones of dead cambium that causes the underlying xylem to dry out. The dried wood cracks and checks (Fig. 8). Epicormic shoots or branches are often observed in highly susceptible stands (Fig. 9). This is due to the partial girdling of stems by the cankers (Coutinho *et al.*, 1997; Wingfield *et al.*, 1997). Epicormic branches subsequently also become diseased and die at their apices. It was also reported that in an extremely susceptible *E. grandis* clone (ZG 14), top die-back occurred due to the girdling effect of cankers, resulting in a loss of height growth (Fig. 10) (Wingfield *et al.*, 1997).

4.3 Distribution and host range

Infection by *C. zuluense* is most severe in the Zululand forestry region of KwaZulu-Natal (Coutinho *et al.*, 1997; Wingfield *et al.*, 1997). This region is typified by a sub-tropical climate. Field reports show that the fungus also occurs in the Mpumalanga Province of South Africa (Wingfield *et al.*, 1997). All indications are, however, that the disease is substantially less severe in those areas with temperate climates. The distribution is, therefore, probably limited to sub-tropical climates that are apparently required for growth and spread of the pathogen (Coutinho *et al.*, 1997; Wingfield *et al.*, 1997).

Coniothyrium zuluense was first reported on a single *E. grandis* clone (Wingfield *et al.*, 1997). Since its discovery, the disease has become common and damaging in all *E. grandis* stands derived from seed, as well as many other *E. grandis* clones (Wingfield *et al.*, 1997). In addition, hybrid clones of *E. grandis* with *E. urophylla* S.T. Blake and *E. camaldulensis* Dehnh. previously believed to be disease resistant, have started to show signs of infection (Coutinho *et al.*, 1997; Wingfield *et al.*, 1997).

Although *Eucalyptus* species are the only known hosts of *C. zuluense*, the disease is not known in Australia, where most of the *Eucalyptus* species are indigenous. According to Wingfield *et al.* (1997), this might suggest that *C. zuluense* is native to South Africa. They proposed that the fungus might occur on native *Myrtaceae* in South Africa and that it could have developed the capacity to infect *Eucalyptus* species. This view was based on similar findings with *Eucalyptus* rust caused by *Puccinia psidii* Winter (Ferreira, 1981; Coutinho *et al.*, 1998). The latter fungus is not known in Australia, but is common and damaging in South and Central America, where it apparently originated from native *Myrtaceae.*

4.4 Dispersal and Infection

The distribution of Coniothyrium canker is probably determined by humid conditions needed for the growth and spread of the pathogen (Coutinho *et al.*, 1997; Wingfield *et al.*, 1997). The incidence of cankers in plantations varies greatly, depending upon climatic conditions and *Eucalyptus* species, clones and hybrids planted (Coutinho *et al.*, 1997; Wingfield *et al.*, 1997). Infection is strongly favoured by relatively high rainfall and temperatures above 25 °C (Wingfield *et al.*, 1997). This lowers the potential for serious damage to *Eucalyptus* species in other parts of South Africa with low rainfall and temperatures.

Very little is known about the biology of *C. zuluense* (Coutinho *et al.*, 1997; Wingfield *et al.*, 1997). It has, however, been shown that once conidia germinate, the germ tubes infect the stems directly through the epidermis of the young tissue (Wingfield, unpublished data). The means by which conidia are spread is, however, still unknown. It has been proposed that conidia are dispersed during rain and by wind which is typical of most pycnidial Coelomycetes (Wingfield *et al.*, 1997). Conidia, suspended in rainwater, flowing down stems might provide opportunities for secondary infections lower down on stems (Coutinho *et al.*, 1997, Wingfield *et al.*, 1997).

4.5 Host susceptibility

Variation in resistance to Coniothyrium canker exists within and among *Eucalyptus* species (Coutinho *et al.*, 1997; Wingfield *et al.*, 1997). Various *E. grandis* clones currently available for planting are highly susceptible (Coutinho *et al.*, 1997; Wingfield *et al.*, 1997). However, certain *E. grandis* clones are moderately resistant to *C. zuluense* infection (Coutinho *et al.*, 1997; Wingfield *et al.*, 1997). Some hybrid clones of *E. grandis* with *E. urophylla* S. T. Blake, *E. camaldulensis* or *E. nitens* (Deane et Maid.) Maid. are highly resistant to *C. zuluense* infection. These hybrid clones would, therefore, be excellent choices for planting in high hazard areas.

There is considerable inter- and intraspecific variation in susceptibility to *C. zuluense*. This may reflect differences in provenances of *E. grandis* that vary in their relative susceptibility, or to the low virulence of the pathogen. However, the threat of *C. zuluense* to South African forestry is dependent on the susceptibility of *Eucalyptus* species, clones and hybrids planted. *Eucalyptus grandis*, is extensively planted in South Africa and is highly susceptible to this pathogen (Wingfield *et al.*, 1997). It is, therefore, important for the South African Forest Industry not to plant clones susceptible to *C. zuluense* in areas where this pathogen is likely to be problematic. For this reason, clones and hybrids should be screened for susceptibility to the pathogen.

4.6 Management strategies

Currently, the most reliable management strategy to reduce the impact of Coniothyrium canker, is by selecting clones and hybrids that show disease resistance (Coutinho *et al.*, 1997; Wingfield *et al.*, 1997). However, there are indications that clones previously believed to be resistant to infection are beginning to show signs of infection (Wingfield *et al.*, 1997). This is an indication that virulence in the pathogen is changing (Coutinho *et al.*, 1997; Wingfield *et al.*, 1997).

Wingfield *et al.* (1997) were not able to find a sexual state for *C. zuluense* and suggested that the fungus probably propagate asexually. If this is true, one should expect that the fungus would have difficulties adapting to environmental changes, such as the introduction of disease resistant clones. Knowledge regarding the population structure of *C. zuluense* in South Africa is, therefore, of crucial importance for programmes aimed at reducing the impact of this disease. The amount of genetic diversity within the population of *C. zuluense* would also provide some insight into the origin of the fungus.

5.0 CONCLUSIONS

Taxonomic problems with the genus *Coniothyrium* have resulted in considerable confusion for many taxonomists. Sutton's studies (Sutton, 1971b, 1980) resulted in a more precise concept for the genus, limiting species of *Coniothyrium* to only those producing conidia from annellides. The position of the more than 800 species that have been described in the genus remains uncertain and must await further study.

Only six of the previously described 11 *Coniothyrium* species known from *Eucalyptus* species produce conidia from annellides. The rest of the species have been accommodated in either *Microsphaeropsis* or *Fairmaniella*. It is currently fairly easy to establish whether newly collected *Eucalyptus* fungi belonging in *Coniothyrium,* differ from other species known on this host.

All *Coniothyrium* species on *Eucalyptus*, are either saprophytic or weak leaf-spotting pathogens. This is in sharp contrast to the recently described *Eucalyptus* stem canker pathogen, *C. zuluense*. This pathogen has caused extensive losses in plantation forestry in the Zululand areas of the KwaZulu-Natal Province, South Africa.

Coniothyrium zuluense has already caused considerable damage and it has the potential to cause serious losses in the future. Very little is known about this fungus, and research is needed to reduce its economic impact. The only long-term control strategy for this disease is by breeding and selection of disease resistant trees. However, in order to capitalise on disease resistance, knowledge regarding the population structure of *C. zuluense* in South Africa would be useful. Information regarding the genetic composition of the pathogen, together with programmes aimed at screening various *Eucalyptus* clones, species and hybrids for disease resistance are needed to successfully manage the disease in future.

Very little is currently known about *C. zuluense* in South Africa. It is hoped that studies contained in this thesis will contribute towards our understanding of the pathogen as a whole. This will be relevant, not only to South Africa but also to other countries where *Eucalyptus* is grown. If the pathogen is not of Australian origin, it might also threaten native Myrtaceae in that country.

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Table 1. List of *Coniothyrium* species occurring as pathogens on different plant hosts.

***Coniothyrium* spp.**	**Host**	**Symptoms**	**References**
C. clematidis-rectae Petrak	*Clematis* Jacq. spp.	Withering	Slavekoorde, 1974; Howells, 1993
C. concentricum (Desm.) Sacc.	*Yucca filamentosa* L.	Parasitic effect	Crisan *et al.*, 1980
C. conorum Sacc. & Roum.	*Picea ajanensis* (Lindl. et Gord.) Fisch. ex Carr	De-foliage	Man'ko & Azbukina, 1992
C. diplodiella (Spegazzini) Sacc.	*Vitis* Engelm. spp.	White rot	Tanaka *et al.*, 1977; Abelentsev, 1980
		Vine-cluster drying-off	Belli *et al.*, 1970; Bisiach & Battino-Viterbo, 1973; He *et al.*, 1991
		Italia grapevine rachis dieback	Conte *et al.*, 1984
		Grape stalk necrosis	Shin *et al.*, 1984
	Artabotrys hexapetalous L.	White rot	Locci & Quaroni, 1972; Shreemali, 1973
C. ficicola	Ornamental plants	Leaf blight	Dayakar-Yadav & Rao, 1978
C. fragariae (Oudem.) Sutton	*Fragaria* Duch. spp.	Blight	Kovacs, 1969; Jarvis & Hargreaves, 1972
C. fuckelii Sacc	*Malus* Borkh. spp.	Blister canker & bark necrosis	Koganezawa & Sakuma, 1980
	Bambusa balcooa Roxb.	Dieback & blight	Rahman & Khisha, 1981; Rahman *et al.*, 1983
	Rubus L. spp.	Cankers	Ellis *et al.*, 1984
	Corchorus capsularis L.	Leaf spot	Ali & Saikia, 1991
	Juniperus communis L.	Leaf and shoot death	Humphreys-Jones, 1977a
	Prunus persica (L.) Batsch	Dieback	Zaharia & Rafaila, 1977
	Persimmon L. spp.	Leaf spot	Agarwala & Kondal, 1971
	Populus L. spp.	Sooty moulds	Bao *et al.*, 1992
	Prunus cornuta L.	Leaf spot	Ram, 1979
	Quercus myrsinaefolia Thunb.	Greyish leaf blight	Kaneko, 1982
	Rosa rugosa Thunb.	Blight	Muthaiyan *et al.*, 1992
	Rosa spp.	Stem cankers	Sweets *et al.*, 1982
		Blight	Dishon *et al.*, 1978
	Thuya orientalis L.	Leaf & shoot death	Humphreys-Jones, 1980
	Rubus idaeus L.	Cane blight	Williamson & Hargreaves, 1981; Jennings, 1982; Baudry *et al.*, 1993; Williamson *et al.*, 1983

***Coniothyrium* spp.**	**Host**	**Symptoms**	**References**
C. fuckelii	*Rubus idaeus*	Cane blight	Williamson *et al.*, 1984; Williamson & Jennings, 1992
C. hellebori Cooke & Massee	*Helleborus* L. spp.	Leaf spot	Fox, 1994
C. phyllachorae Maubl.	*Zea mays* L.	Tarspot	Hock *et al.*, 1992a, b
C. olivaceum Bonorden	*Citrus* L. spp.	Soil sickness	Reddy *et al.*, 1983
C. pini	*Pinus pinea* L..; *P. halepensis* Miller; *P. brutia* Ten.	Leaf blight & dieback	Bellar & Bayaa, 1993
C. piricola Poteb., cf	*Plumeria* L. spp.	Leaf blight	Rafaila & Dinulescu, 1977
C. pyrinum (Sacc.) J. Sheld	*Malus* spp.	Leaf spot	Kondal & Agarwala, 1974
C. prunicolum	*Plumeria* spp.	Spots on leaves, shoots & fruits	Glits, 1984
C. quercinum (Bonorden) Sacc.	*Quercus petraea* (Mattuschka) Liebl.	Inhibit respiration (high SO_2)	Newsham *et al.*, 1992
C. viburni	*Viburnum burkwoodii* Thunb.	Leaf blotch	Humphreys-Jones, 1977b
C. wernsdorffiae Laubert	*Rosa* spp.	Fire blight	Protsenko & Chelyshkina, 1973; Semina *et al.*, 1982; Semina *et al.*, 1991
Coniothyrium sp. [a]	*Atriplex versicaria* Hew. ex Benth.	Dieback	Cother & Gilbert, 1994
"	*Cupressus* Miller spp.	Lesions on stems	Luisi & Triggiani, 1977
"	*Cupressus* spp.	Lesions on stems	Motta & Saponaro, 1982
"	*Picea abies* L.	Powdery mildew	Venn, 1983
"	*Populus trichocarpa*	Branch cankers	Shirnina, 1983
"	*Protea repens* (L.) L.	Tip blight	Serfontein & Knox-Davies, 1990
"	*Rosa* spp.	Stem cankers	Vargas *et al.*, 1990
"	*Simmondsia chinensis* (Link.) C. Schneider	Leaf blight	Young & Alcorn, 1981

[a] = Not specified

Table 2. *Coniothyrium* species used as biological control agents.

***Coniothyrium* spp.**	**Host**	**Hyperparasitism**	**Reference**
C. fuckelii Sacc.	*Globodera rostochiensis* (nematode)	Mycoparasite	Clovis & Nolan, 1983
C. minitans Campbell	*Sclerotinia sclerotiorum* (Lib.) de Bary	Mycoparasite	Voros, 1969; Jones & Johnson, 1970; Ghaffar, 1972; Huang & Hoes, 1976; Huang, 1977, 1979; Trutmann *et al.*, 1980; Huang, 1981; Trutmann *et al.*, 1982; Chaban & Yakubova, 1983; Fedulova, 1983; Grisenko *et al.*, 1983; Huang, 1983; Phillips & Price, 1983; Tribe, 1984; Tu, 1984; Monaco, 1989; Phillips, 1989; Adams, 1990; Whipps & Budge, 1990; Budge & Whipps, 1991; Huang & Kozub, 1991; Whipps *et al.*, 1991; Lueth *et al.*, 1992; Sesan & Csep, 1992; Whipps *et al.*, 1992; Whipps & Gerlagh, 1992; Verdam *et al.*, 1993; Sandys-Winsch *et al.*, 1993; Whipps, 1993; Whipps *et al.*, 1993; Whipps & Budge, 1993; McLaren *et al.*, 1994
	Botrytis cinerea Pers.:Fr	Mycoparasite	Sesan & Tica, 1990
	Sclerotium cepivorum Berk	Mycoparasite	Ahmed & Tribe, 1977; Oliveira *et al.*, 1984
C. olivaceum Bonorden	*Sclerotinia sclerotiorum*	Mycoparasite	Ivancia, 1992
C. sporulosum (W. Gams & Domsch)	*Alternaria alternata* (Fr.:Fr.) Keissler	Mycoparasite	Turhan, 1993
"	*Rhizoctonia solani* Kühn	Mycoparasite	Turhan, 1990

Table 3A. Morphological characteristics of species of *Coniothyrium*, *Microsphaeropsis* and *Fairmaniella* on *Eucalyptus*.[a]

	C. palmarum	*C. fuckelii*	*M. callista*	*M. eucalypti*	*M. globulosa*	*M. olivacea*	*F. leprosa*
Conidiomata							
Pycnidia/Acervuli	Pycnidia	pycnidia	pycnidia	pycnidia	pycnidia	Pycnidia	acervuli
Size (μm diam.)	150	115-370	150-200	100	100	200-300	100-200
Shape	Globose	sub-globose	sphaerical - globose	sphaerical - globose	sphaerical - globose	Globose	-
Occurrence	sub-stomatal	sub-stomatal	sub-epidermal	sub-epidermal	sub-epidermal	Sub-epidermal	sub-stomatal
Ostiole	circular / central	circular / central	circular / not protruding	papillate / not protruding	papillate / not protruding	Circular	-
Pycnidial walls	Thick	thick	thick	thick	thick	Thin	thick
Conidiogenous cells							
Annellidic / Phialidic	Annellidic	phialidic	phialidic	phialidic	phialidic	Phialidic	phialidic
Shape	doliiform – cylindrical	ampulliform	doliiform – ampulliform	doliiform – ampulliform	doliiform – ampulliform	Doliiform – ampulliform	ampulliform
Sizes (μm)	5-12 x 3-5	4-4.5 x 5-6	4-7 x 2.5-4	4-9 x 3-5	4-9 x 3-5	4-6 x 3-4	3-5 x 2.5-3.5
Colour	hyaline to pale brown	hyaline	hyaline	hyaline	hyaline	Hyaline	hyaline
Conidia							
Colour	Brown	medium brown	brown	dark brown	dark brown	Pale brown	pale brown
Thickness	thick-walled	thick-walled	thick-walled	thick-walled	thick-walled	Thin-walled	thick-walled
Septate	0-1 euseptate	Aseptate	aseptate	aseptate	aseptate	Aseptate	aseptate
Size (μm diam.)	6-8.5 x 4.5	2-2.6 x 3.6-4.5	8.8 x 4.5-5.5	6-7 x 5-6	6-7 x 5-6	4.8 x 2.5-5	3-5 x 2.5-3.5
Shape	Cylindrical; spherical; eliptical; clavate	elliptical	elliptical; obtuse apex; truncate base	globose; sub-globose-pyriform; truncate base	globose; sub-globose-pyriform; truncate base	Oval – ellipsoidal	elliptical / obovate
[a]References	**Sutton, 1980**	**Sutton, 1971b**	**Sutton, 1971b**	**Sutton, 1971b**	**Sutton, 1971b**	**Sutton, 1980**	**Sutton, 1971b**

Table 3B. Symptoms and hosts of species of *Microsphaeropsis* and *Fairmaniella* associated with *Eucalyptus* spp.[a]

	M. callista	***M. eucalypti***	***M. globulosa***	***M. olivacea***	***F. leprosa***
Hosts	*E. haemastoma*	*E. globulus*	*E. globulus*	*E. ficifolia*	*E. fasciculosa* *E. globulus* *E. citriodora* *E. robusta*
Geographic distribution	Australia	Portugal	Portugal	Australia, India & USA	Australia, Chile, Hawaii, Zambia, New Zealand, South Africa
Symptoms	lesions on leaves	saprophyte on leaves	saprophyte on leaves	Saprophyte on leaves	lesions on leaves and shoots
Size (mm diam.)	1-5	-	-	-	1-20
[a]**References**	**Sutton, 1971b;** **Cabral, 1985**	**Sutton, 1971b;** **Sutton, 1974**	**Sutton, 1971b**	**Sutton, 1980**	**Sutton, 1971b;** **Crous *et al.*, 1989a,b**

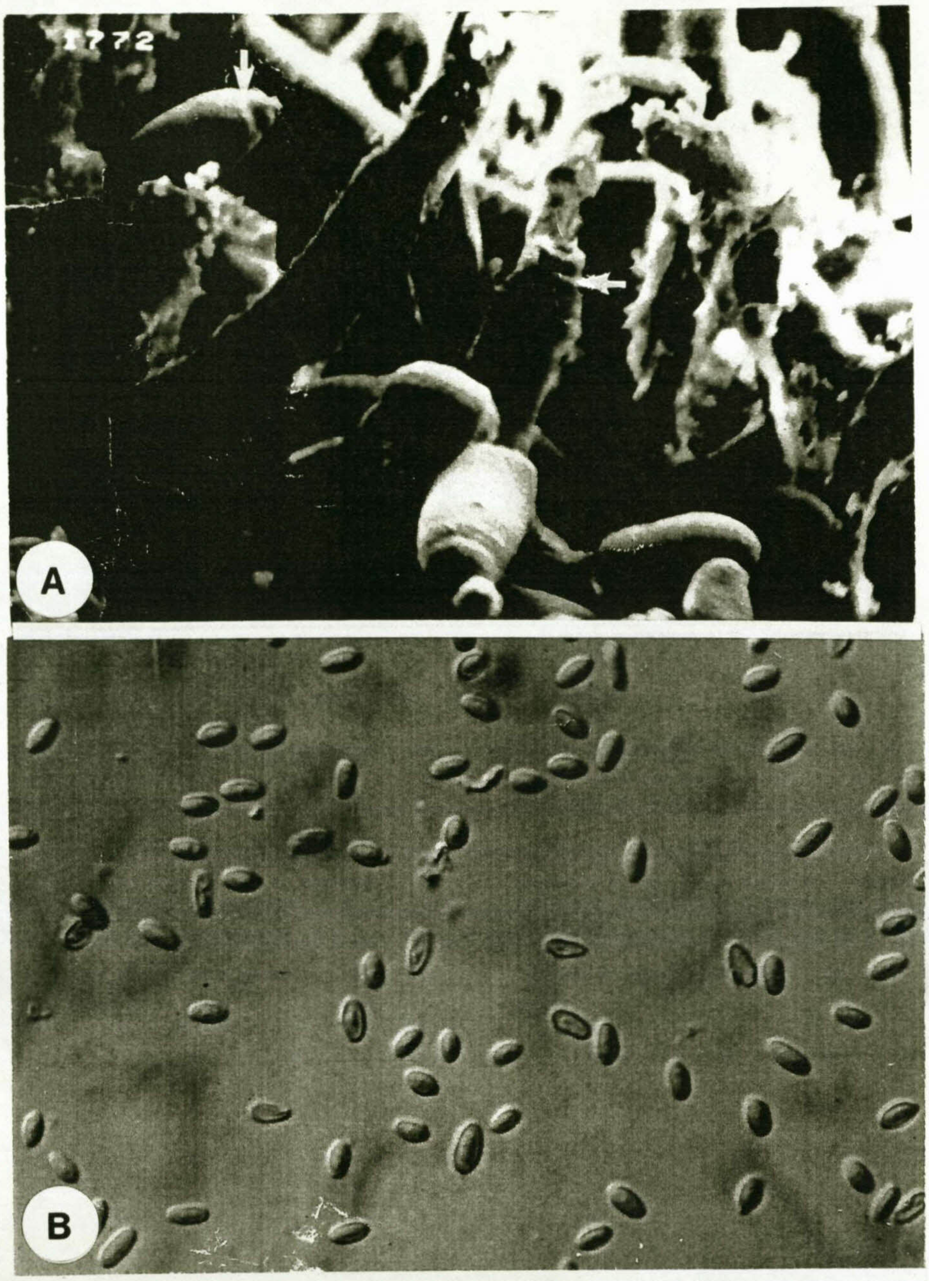
Table 4A. Comparison of conidiomata of different species of *Coniothyrium* from *Eucalyptus*. [a]

	C. palmarum	*C. fuckelii*	*C. ahmadii*	*C. eucalypticola*	*C. kallangurense*	*C. ovatum*	*C. parvum*	*C. zuluense*
Conidiomata								
Pycnidia	pycnidia	pycnidia	pycnidia	pycnidia	pycnidia	pycnidia	pycnidia	pycnidia
Size (μm diam.)	150	115-370	100	50	250	40-70	40-70	60-120
Shape	globose	sub-globose	globose	globose	globose	globose / depressed	globose / depressed	globose / depressed
Occurrence	sub-stomatal	sub-stomatal	sub-stomatal	sub-stomatal	superficially	sub-stomatal	sub-stomatal	intra or sub-epidermal
Ostiole	circular & central	circular & central	circular & central	circular & central	circular & central	circular & central	circular & central	circular & central
Pycnidial walls	thick	thick	thick	thick	thick	thick	thick	thick
Conidiogenous cells								
Annellidic / Phiallidic	annellidic	phiallidic	annellidic	annellidic	annellidic	annellidic	annellidic	annellidic
Form	doliiform – cylindrical	ampulliform	doliiform - cylindrical	doliiform - cylindrical	doliiform – cylindrical	short-flask shape	short-flask shape	doliiform to reniform
Sizes (μm)	5-12 x 3-5	4-4.5 x 5-6	6-7 x 3.5-4.4	2.5-7 x 4-6	5-14 x 3-5.5	4-5 x 4-7	3 x 6	4-8 x 2.5-3.5
Colour	hyaline to pale brown	hyaline	hyaline to pale brown	hyaline to pale brown	hyaline to pale brown	hyaline to pale brown	hyaline to pale brown	medium brown
Conidia								
Colour	brown	medium brown	pale brown	medium brown	dark brown	dark brown	dark brown	medium brown
Thickness	thick-walled	thick-walled	thick-walled	thick-walled	thin-walled	thin-walled	thin-walled	thick-walled
Septate	0-1 euseptate	aseptate	aseptate	aseptate	aseptate	aseptate	aseptate	aseptate
Size (μm diam.)	6-8.5 x 4.5	2-2.6 x 3.6-4.5	6–7 x 3.5-4.5	8.5–10 x 6-7.5	4-7 x 2.5-5	7-11 x 3-4.5	4.5-6 x 2-3.5	4.5-5 x 2-2.5
Form	cylindrical; spherical; eliptical; clavate	elliptical	elliptical	elliptical	pyriform	clavate	clavate	broadly ellipsoidal
[a]**References**	**Sutton, 1980**	**Sutton, 1971b**	**Sutton, 1974**	**Sutton, 1971b**	**Sutton, 1975**	**Swart, 1986**	**Swart, 1986**	**Wingfield *et al*., 1997**

Table 4B. Comparison of symptoms and hosts of species of *Coniothyrium* ex *Eucalyptus*. ^{a}

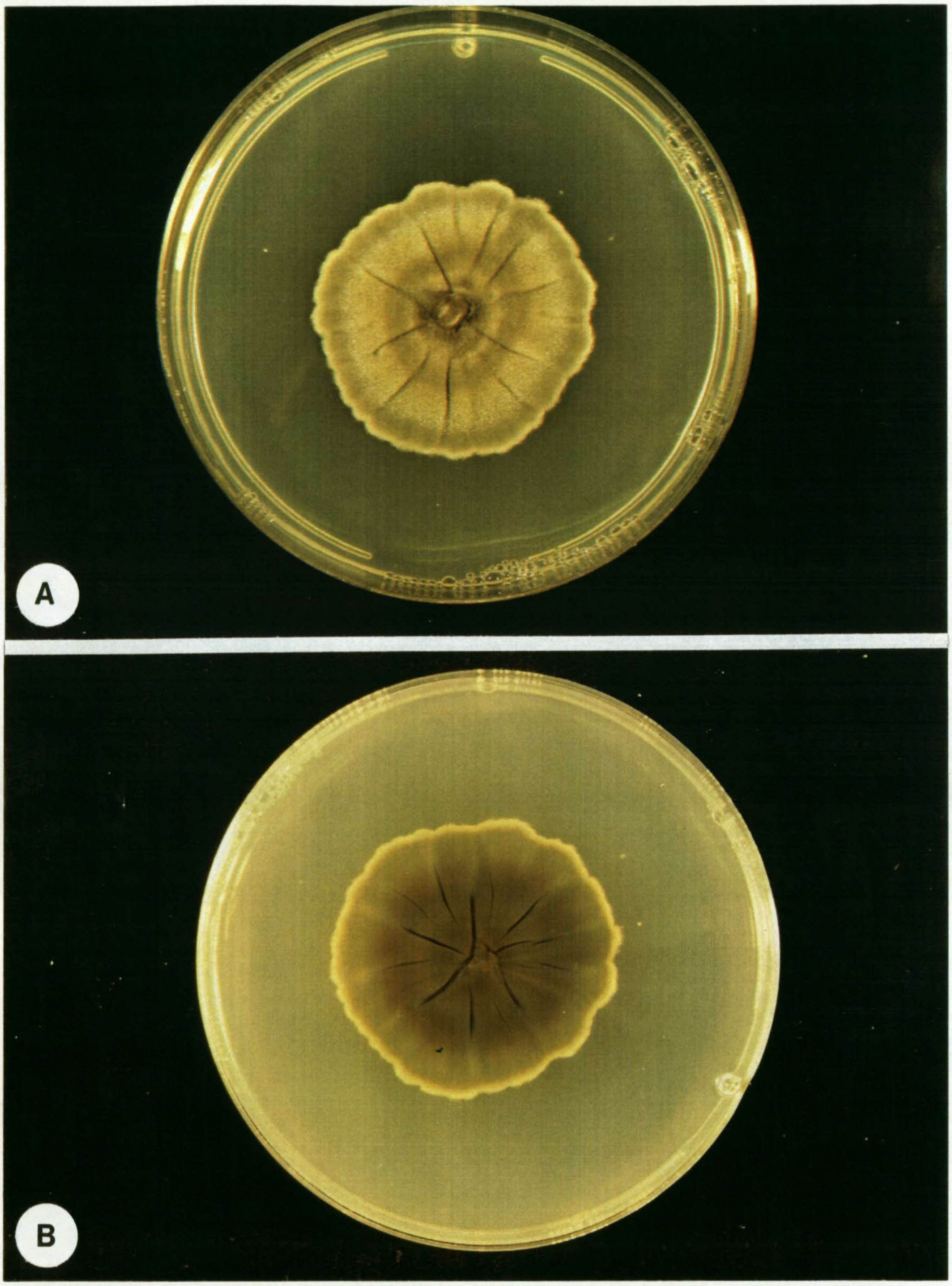
	C. ahmadii	***C. eucalypticola***	***C. kallangurense***	***C. ovatum***	***C. parvum***	***C. zuluense***
Hosts	*Eucalyptus* spp.	*E. leptophylla*	*E. microcorys*	*E. dives* *E. macrorhyncha* *E. obliqua* *E. cladocalyx* *E.lehmannii*	*E. melliodora* *E. regnans*	*E. grandis* and various *E. grandis* hybrid clones
Geographic distribution	Pakistan	Australia	Australia	Australia South Africa	Australia	South Africa
Symptoms	branches	leaf spots	saprophyte	necrotic leaf spots	necrotic leaf spots	necrotic stem cankers
Size (mm)	-	2-10	-	1	1-1.5	varies
^{a}**References**	**Sutton, 1974**	**Sutton, 1971b**	**Sutton, 1975**	**Swart, 1986** **Crous *et al.*, 1988**	**Swart, 1986**	**Wingfield *et al.*, 1997** **Coutinho *et al.*, 1997**

Fig. 1. Conidiogenous cells and conidia of *Coniothyrium zuluense*. (A) Characteristic annellidic conidiogenous cell (bar = 4 µm). (B) Single-celled ovoid conidia (bar = 10 µm).



(from: Wingfield *et al.*, 1997)

Fig. 2. Colour characteristics of single-conidial *Coniothyrium zuluense* cultures, grown on Potato Dextrose Agar (PDA). (A) Colonies viewed from above are irregular, pale olivaceous with an outer olivaceous grey band of mycelium. (B) Colonies viewed from below possessed a rust colour centre with an olivaceous fringe.


A
B

Figures 7 - 10. Symptoms associated with Coniothyrium canker on *Eucalyptus grandis* in South Africa.

Fig. 7. Severely infected trees ultimately develop a series of stem cankers along the stems.

Fig. 8. In susceptible trees cankers coalesce and form large cracks along the stems.

Fig. 9. Epicormic shoots produced from cankered stem, indicate partial girdling due to cankers.

Fig. 10. Top die-back due to the girdling effect of cankers.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Figures 3 - 6. Symptoms associated with Coniothyrium canker on *Eucalyptus grandis* in South Africa.

Fig. 3. Initial infections are visible as discrete necrotic lesions on young green stem tissue.

Fig. 4. Small necrotic lesions coalesce to form large necrotic patches.

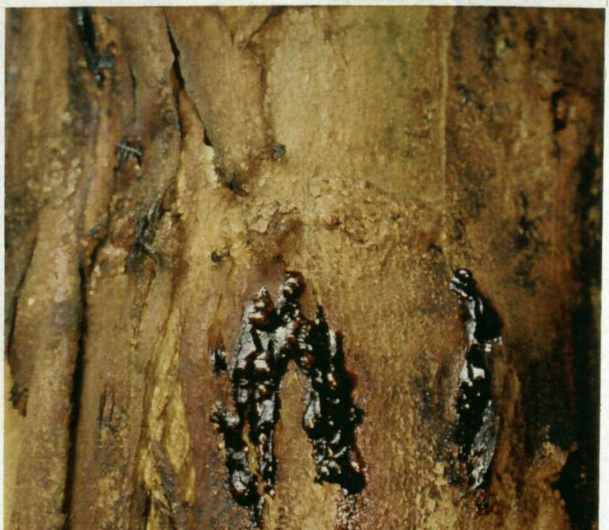
Fig. 5. Spindle-shaped malformation on severely infected trees.

Fig. 6. Necrotic cankers are often cracked and exude copius amounts of red/brown kino.


3


4


5


6

CHAPTER 2

Morphological, cultural and pathogenic characteristics of *Coniothyrium zuluense* isolates from different plantation regions in South Africa

Coniothyrium canker, caused by *Coniothyrium zuluense*, is a serious stem canker disease of *Eucalyptus* species in sub-tropical regions of South Africa. This disease is typified by necrotic bark lesions that coalesce to form large kino-impregnated cankers along the stems. The strategy currently used to manage *Coniothyrium* canker in plantations is to deploy *Eucalyptus* species or clones that are resistant to the disease. Considerable success has already been achieved in this regard, but the long-term durability of resistance is of concern. Thus, forest managers are interested in the genetic diversity of the pathogen and its potential to overcome disease resistance in planting stock. In this study, 344 isolates of *C. zuluense* from different plantation regions in South Africa were compared on the basis of colony colour, conidial morphology, growth characteristics on agar and virulence to a susceptible *E. grandis* clone. Conidia of all *C. zuluense* isolates measured were similar in size and shape. The fungus is slow growing in culture, which is indicative of its apparent biotrophic habit, with optimum growth observed at 30 °C. Isolates of *C. zuluense* displayed considerable variation in colony colour and pathogenicity in inoculated trials. Variation in morphology and pathogenicity amongst isolates suggests that *C. zuluense* has been present in South Africa for an extended period of time, or that it is changing rapidly due to strong directional selection pressures.

The forestry industry in South Africa relies almost exclusively on the planting of exotic species of *Pinus* and *Eucalyptus*. These genera are planted in approximately equal proportions and about 1.5 million hectares of land is currently afforested (Anonymous, 1995). Planting of *Eucalyptus* clones is a common practice and results in large, genetically uniform stands. These plantations are at risk from damage due to pests and diseases (Wingfield, 1990; Wingfield & Kemp, 1994). The current means of reducing losses due to disease is by planting of disease resistant species and clones of *Eucalyptus* (Wingfield *et al.*, 1991). Strategies to ensure that large numbers of disease resistant clones are planted and that a high degree of genetic diversity is maintained in clonal plantations, have, therefore, been implemented (Wingfield, 1990).

Coniothyrium zuluense Wingfield, Crous & Coutinho is a serious stem canker pathogen of *Eucalyptus* trees in South Africa (Coutinho *et al.*, 1997; Van Zyl *et al.*, 1997; Wingfield *et al.*, 1997). Disease symptoms were first noted on a single clone of *Eucalyptus grandis* Hill ex Maid. at Honey Farm plantation in the Zululand region of the KwaZulu-Natal province. Since its discovery in 1988, various *Eucalyptus* species, clones, and hybrids have displayed symptoms of infection. The earliest symptoms of infection by *C. zuluense* on trees are small, discrete, necrotic lesions on the young, green bark. These lesions coalesce to form large necrotic cankers on the stems that exude copius amounts of kino. Epicormic shoots are commonly produced from stems of cankered trees, indicative of partial girdling. In severely infected clones, the tops of trees die, due to the girdling effect of the cankers resulting in loss of growth in height (Coutinho *et al.*, 1997; Van Zyl *et al.*, 1997; Wingfield *et al.*, 1997).

Coniothyrium zuluense, and the canker disease associated with it, were first observed nine years ago (Wingfield *et al.*, 1997). Currently, no information is available concerning the population characteristics of the pathogen. Knowledge regarding fungal population structures is important to programmes aimed at reducing the impact of disease, as this must affect the likely durability of disease resistant clones. The aim of this study is, therefore, to consider variability in morphological, cultural and virulence characteristics, amongst a large collection of *C. zuluense* isolates.

MATERIALS AND METHODS

Isolates and cultures

A survey of *C. zuluense* in nine *Eucalyptus* growing regions of KwaZulu-Natal was conducted during 1995 and 1996. Pieces of bark, showing characteristic disease symptoms, were collected from each tree sampled. These bark samples were incubated in Petri dishes containing moist filter paper at 30 °C to induce production of pycnidia. Conidial masses from 172 pycnidia were then washed onto the surface of agar in Petri dishes containing 2 % w/v water agar (20 g agar (Biolab); 1 l distilled H_2O) and spread across the medium surface. Plates were incubated for 19 – 24 hr at 30 °C. Single germinating conidia were then lifted from each plate with the aid of a dissecting microscope and sterile syringe needle. The germinating conidia were transferred to sterile 9 cm diam. Petri dishes containing 15 ml of 4 % w/v Potato Dextrose Agar (PDA) (24 g Potato Dextrose extract (Merck); 1 g Yeast extract (Merck); 1 g Glucose (Merck); 40 g agar (Merck); 1 l distilled H_2O) and incubated at 30 °C. Isolates produced in this manner were stored on PDA slants in screw-capped tubes at 4 °C.

Colony and conidia morphology

A total of 344 single conidial isolates of *C. zuluense* were transferred to 4 % w/v PDA plates in order to observe colony colour and growth characteristics in culture. Colony colour was rated using mycological colour charts of Rayner (1970). Spore morphology was determined by measuring the length and width of thirty conidia per pycnidium. Conidia from ten randomly selected pycnidia (each from different trees) were measured for each of the nine plantation regions sampled.

Growth studies

Growth rates and temperature requirements were determined for each of the 344 *C. zuluense* isolates collected. Isolates were transferred to PDA (five mm diam. mycelial plugs) with three plates for each temperature and isolate to be tested. Plates were incubated in the dark at temperatures ranging from 10 to 35 °C, at five-degree intervals for 30 days. Growth of isolates was determined by measuring colony diameter.

Pathogenicity tests

Pathogenicity tests were conducted on six-month-old trees of an *E. grandis* clone (ZG 14) that is known to be highly susceptible to Coniothyrium canker under natural conditions in KwaZulu-Natal. Twenty trees were inoculated with each of the 344 different single conidial isolates. Inoculations were done by removing a 10 mm diameter disc of bark from the trees at breast height, and replacing this with a PDA disc of agar bearing the fungus, or an uninoculated disc in the case of the 20 controls. Inoculation wounds were covered with masking tape to prevent desiccation of the inoculum. Lesion length was measured six weeks after inoculation. This experiment was repeated using all pathogenic isolates and 20 randomly selected non-pathogenic isolates.

RESULTS

Colony and conidial morphology

Isolates of *C. zuluense* varied considerably in colony colour (Figs 1, 2). Surface colony colour of all 344 isolates screened in this study, varied from olive grey (V$23^{''''}$b), isabella ($19^{''}$i), greenish glaucous ($33^{'''}$f) to a grayish olive ($21^{''''}$) colour.

Colonies viewed from below were either black or rust coloured with white margins. There was no predominant colony colour for isolates from any specific region.

Conidia of all isolates measured were similar in size and shape. Conidia, from lesions on trees from all nine regions sampled, were pale brown in colour, thick walled, smooth to verruculose and broadly ellipsoidal. The apices of these conidia were obtuse and the bases sub-truncate to bluntly rounded (Table 1).

Growth studies

Coniothyrium zuluense is slow growing in culture, which is indicative of its apparent biotrophic habit (Wingfield *et al.*, 1997). The fungus failed to grow at 10 °C (Fig. 3). At 15 °C the mean colony diameter for all isolates was 12.6 mm. Best growth was observed at 30 °C (61.4 mm diam.) followed by 25 °C (39.3 mm diam.), 35 °C (30.9 mm diam.) and 20°C (25.1 mm diam.), respectively.

Pathogenicity tests

Isolates of *C. zuluense* differed markedly in their relative pathogenicity (Fig. 4). Of the 344 isolates tested, 269 (78 %) appeared to be non-pathogenic. Lesion lengths for these isolates varied between 10 mm and 20 mm (Fig. 4). The remaining 75 (22 %) isolates produced lesions varying between 21 mm and 61 mm long. Lesion lengths obtained from pathogenicity trials were divided into pathogenicity ranges (Fig. 4).

Control inoculations developed no symptoms and inoculation points were covered with callus tissue (Fig. 5A). The most pathogenic isolates of *C. zuluense* gave rise to a distinct swelling of the stem tissue around the inoculation site after six weeks (Fig. 5B). Tissue surrounding the inoculation points was necrotic (Fig. 5B). These symptoms were similar to those associated with natural infections.

DISCUSSION

Morphological and cultural characteristics of *C. zuluense* presented in this study were similar to those published by Wingfield *et al.* (1997). Data regarding spore morphology and growth characteristics were also consistent with those previously published (Coutinho *et al.*, 1997; Wingfield *et al.*, 1997). Results of colony colour and pathogenicity studies, however, showed evidence of considerable variation in isolates of *C. zuluense*.

There was a wide diversity in colony colour of *C. zuluense* isolates. The majority of isolates (66 %) were olive grey which is consistent with results of Wingfield *et al.* (1997). The remaining (34 %) isolates had colony colours varying between isabella, greenish glaucous and grayish olive. It is of interest that there were no consistent patterns of colony colour in terms of origin of isolates.

A considerable degree of variation was observed in pathogenicity of the isolates tested in this study. It was particularly interesting that 78 % of all *C. zuluense* isolates were not able to cause any disease on the susceptible *E. grandis* clone. A relatively small number (22 %) of isolates were able to cause necrotic canker lesions. All isolates used had been collected and variability in pathogenicity is unlikely be limited to age of isolates.

No sexual state has been found for *C. zuluense*, despite the fact that considerable effort has been made to find such structures (Coutinho *et al.*, 1997; Wingfield *et al.*, 1997). The assumption is, therefore, made that the fungus predominantly exists in an asexual form and would thus have a limited capacity to change. Sexual reproduction combines genes in a population continually into new combinations that could subsequently lead to a rapid increase in virulence, whereas asexually reproducing fungi possess a limited number of different gene combinations (Anagnostakis & Kranz, 1987; McDonald & McDermott, 1993). The presence of spermatogonia in some cultures, however, suggest that a sexual state may occur, but has yet to be discovered (Coutinho *et al.*, 1997; Wingfield *et al.*, 1997). If it is present, it seems unlikely to occur on *Eucalyptus* which have been very carefully examined, but it could be present on a native South African plant species. The sudden appearance of the disease in South Africa, as well as diversity of colony types and pathogenicity, favours the hypothesis that *C. zuluense* originated from native plants in this country.

Eucalyptus clones that are highly susceptible to *C. zuluense* fail to grow effectively, which leads to significant losses to the South African forestry industry. A large number of clones that are currently available for planting, show susceptibility to infection (Coutinho *et al.*, 1997; Van Zyl *et al.*, 1997; Wingfield *et al.*, 1997). There is also evidence to suggest that clones previously known to be resistant, are beginning to show signs of infection (Coutinho *et al.*,1997; Wingfield *et al.*, 1997). This indicates that the virulence of the pathogen is changing. In plantation programmes, regular deployment of new resistant clones and hybrids, impose strong directional selection on pathogen populations. This is especially true for asexual reproducing fungi because they must constantly adapt to changes in their environment in order to survive (McDonald & McDermott, 1993). Such pressure might have lead to the pathogenic variation in *C. zuluense*.

Eucalyptus species are being propagated extensively outside Australia (where most of these species are native) with about 8 million hectares currently grown in plantations (Wingfield *et al.*, 1997). *Coniothyrium zuluense* is a potential threat to these plantations, particularly in areas with a tropical or sub-tropical climate and dedicated efforts are, therefore, needed to avoid the spread of this fungus to other countries. In this regard, it is important to note that control strategies can only be successful if populations, instead of individuals are targeted (McDonald & McDermott, 1993). Future research will, therefore, focus on understanding the population structure of *C. zuluense*. Such information will be valuable in understanding the evolution of the population in response to the deployment of new disease resistant clones.

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Table 1. Differences in conidial size of isolates of *Coniothyrium zuluense*, collected from nine different plantation regions in KwaZulu-Natal.

Plantation[a]	Number of isolates	Width and length of conidia (μm)[b]
Aboyni	11	2 – 3.1 (2.6) x 3.5 – 5.1 (4.5)
Fairbreeze	16	2.1 – 3.5 (2.5) x 3.5 – 5 (4.1)
Futululu	70	2.5 - 3.6 (2.8) x 3.5 – 4.5 (4.1)
Honey Farm	50	2.5 - 3 (2.6) x 3 - 5.2 (4.3)
Palm Ridge	55	2 – 3 (2.8) x 4.3 - 5.6 (4.8)
Shire	10	2.6 - 2.8 (2.7) x 3.5 –6 (4.5)
Teranera	28	2.7 - 3.3 (2.8) x 4 – 4.3 (4.1)
Teza	81	2.5 - 3.6 (2.8) x 4.5 – 4.7 (4.5)
Trust	23	2.5 - 2.8 (2.6) x 4.2 – 4.5 (4.3)

[a] Plantations in Zululand, KwaZulu-Natal Province, South Africa

[b] Each size measurement represents the range of conidial lengths (averages in parenthesis) and widths, computed from an average of 30 conidia derived from 10 randomly collected pycnidia.

Fig. 1. Differences in surface colony colour of 344 *Coniothyrium zuluense* isolates grown on PDA in Petri dishes for 30 days at 30 °C. Bars represent the number of *C. zuluense* isolates in each colour class, and colours are those of Rayner (1970).

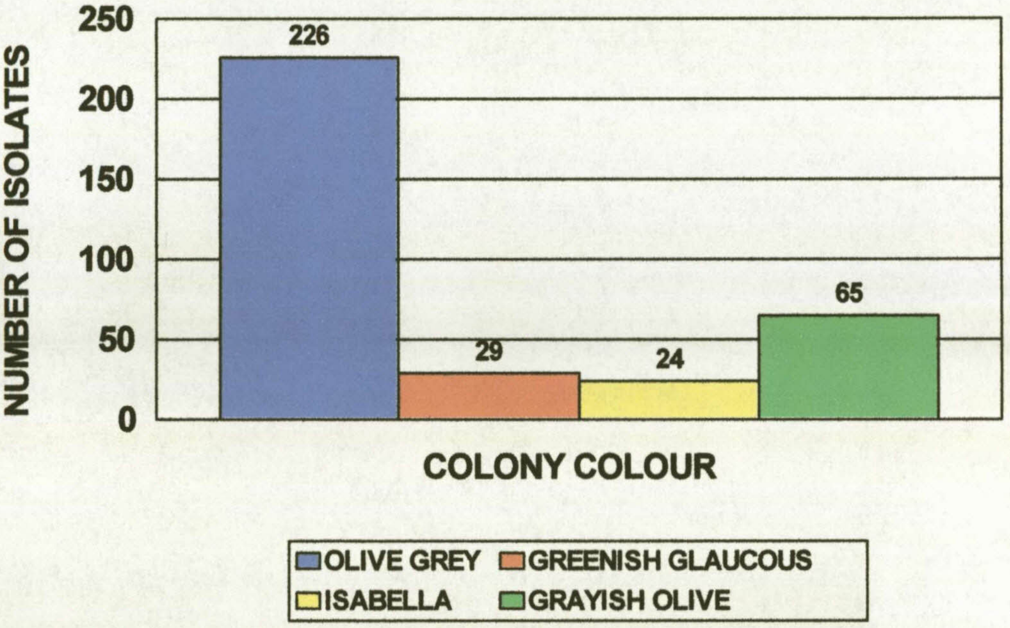

250
200
150
100
50
0
NUMBER OF ISOLATES
226
29
24
65
COLONY COLOUR
OLIVE GREY
GREENISH GLAUCOUS
ISABELLA
GRAYISH OLIVE

Fig. 2. Differences in surface colony colour of *Coniothyrium zuluense* isolates grown on PDA in Petri dishes for 30 days at 30 °C. Surface colony colour varied from (A) olive grey ($V23^{''''}b$), (C) isabella ($19^{''}i$), (E) greenish glaucous ($33^{'''}f$) to a (G) grayish olive ($21^{''''}$) colour. Colonies viewed from below (B, D, F, H) were either black or rust coloured with white margins. Colours are those of Rayner (1970).

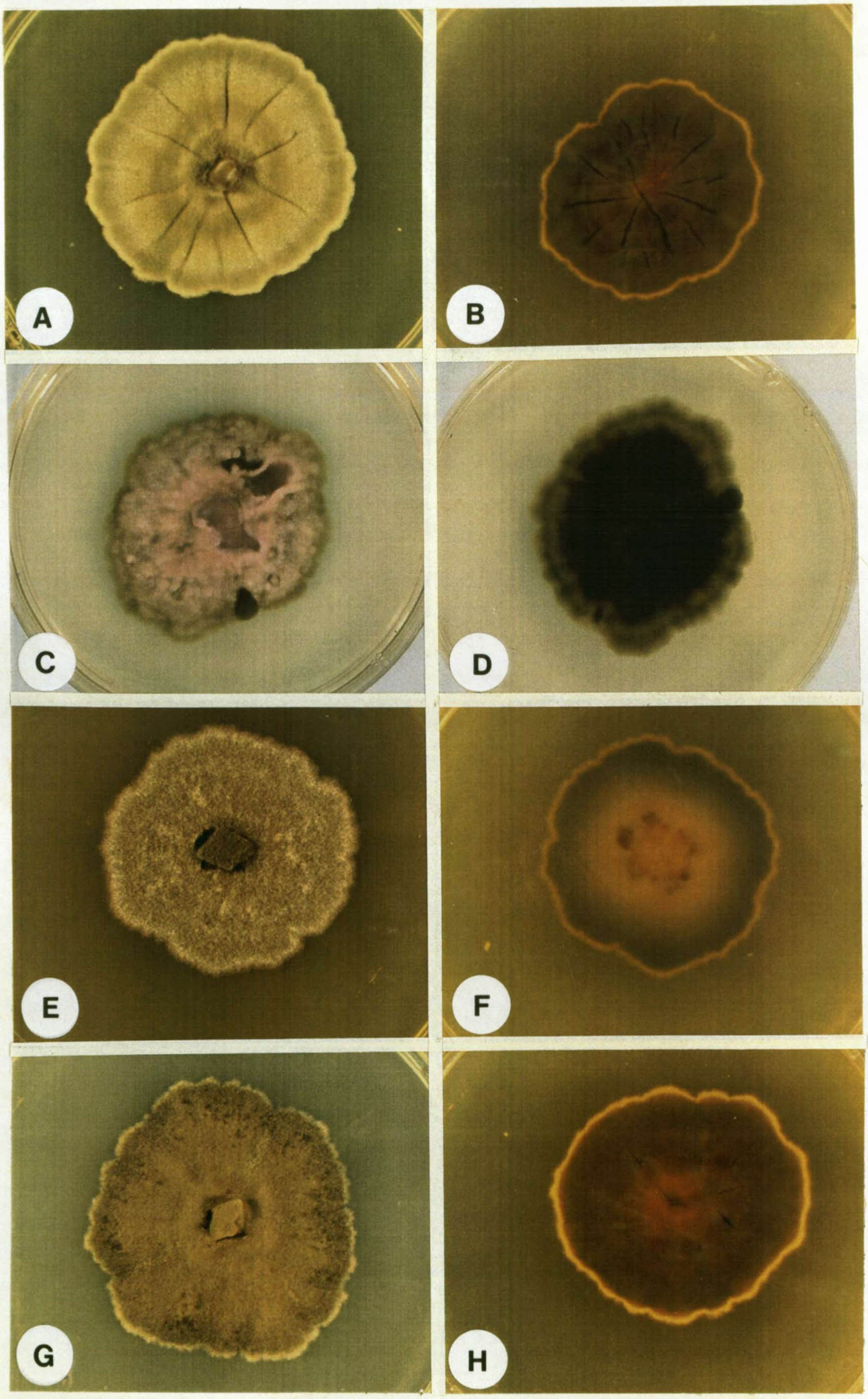

A
B
C
D
E
F
G
H

Fig. 3. Ranges of average colony diameters of 344 single conidial isolates of *Coniothyrium zuluense* after incubation for 30 days at different temperatures between 10 to 35 °C. Bars represent the minimum and maximum colony diameters for each temperature. Average colony diameters are represented by horizontal bars.

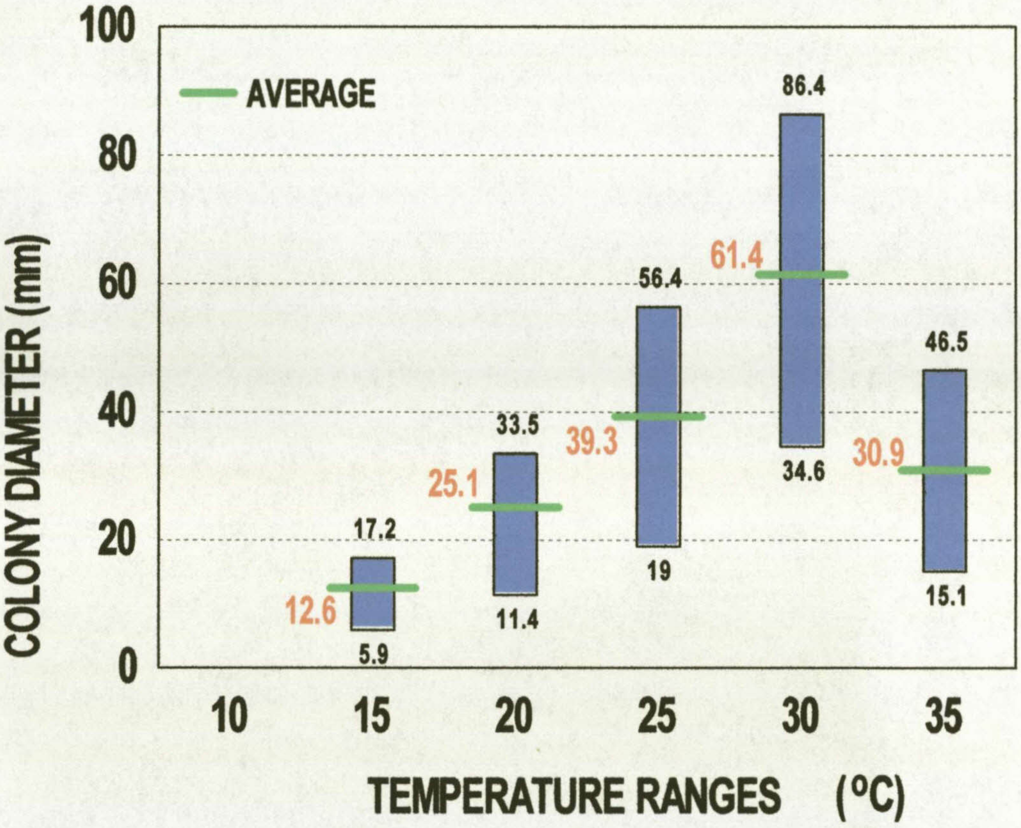

AVERAGE
COLONY DIAMETER (mm)
100
80
60
40
20
0
10
15
20
25
30
35
TEMPERATURE RANGES (°C)
17.2
12.6
5.9
33.5
25.1
11.4
56.4
39.3
19
86.4
61.4
34.6
46.5
30.9
15.1

Fig. 4. Ranges of lesion lengths associated with inoculation of 344 *Coniothyrium zuluense* isolates. Bars represent the number of *C. zuluense* isolates within each of five different lesion ranges produced after inoculation of a susceptible *Eucalyptus grandis* clone (ZG 14). Each of the 344 isolates was inoculated onto the stems of 20 trees and means computed. Different letters differ significantly (*P* = *0.05*) according to Tukey's procedure for comparison of means (CV = 24.6 %).

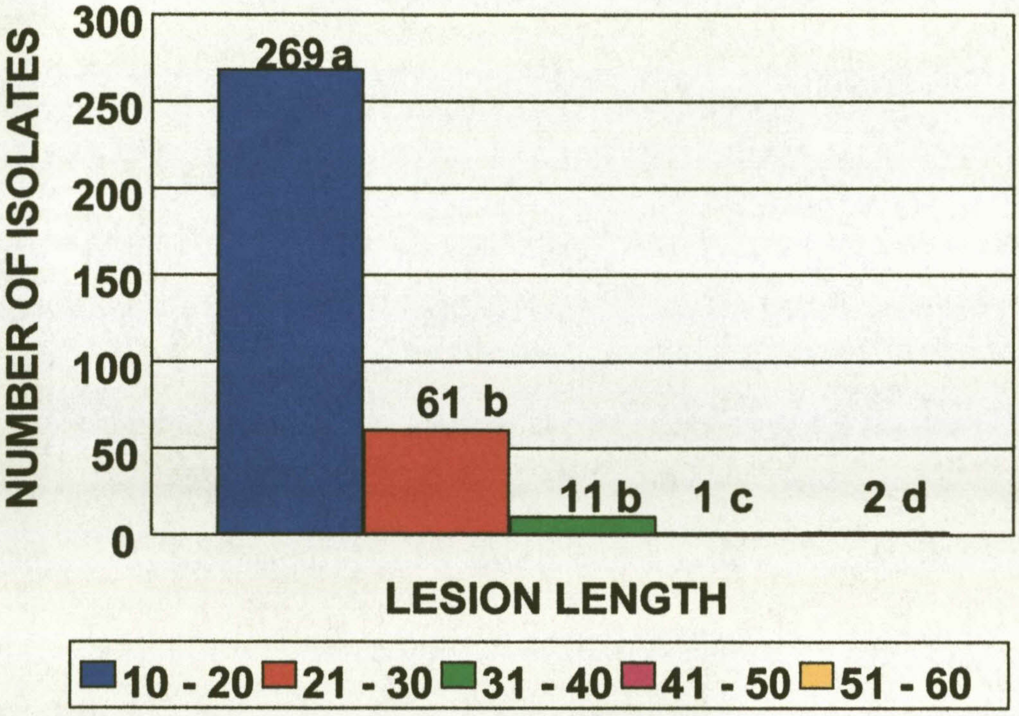
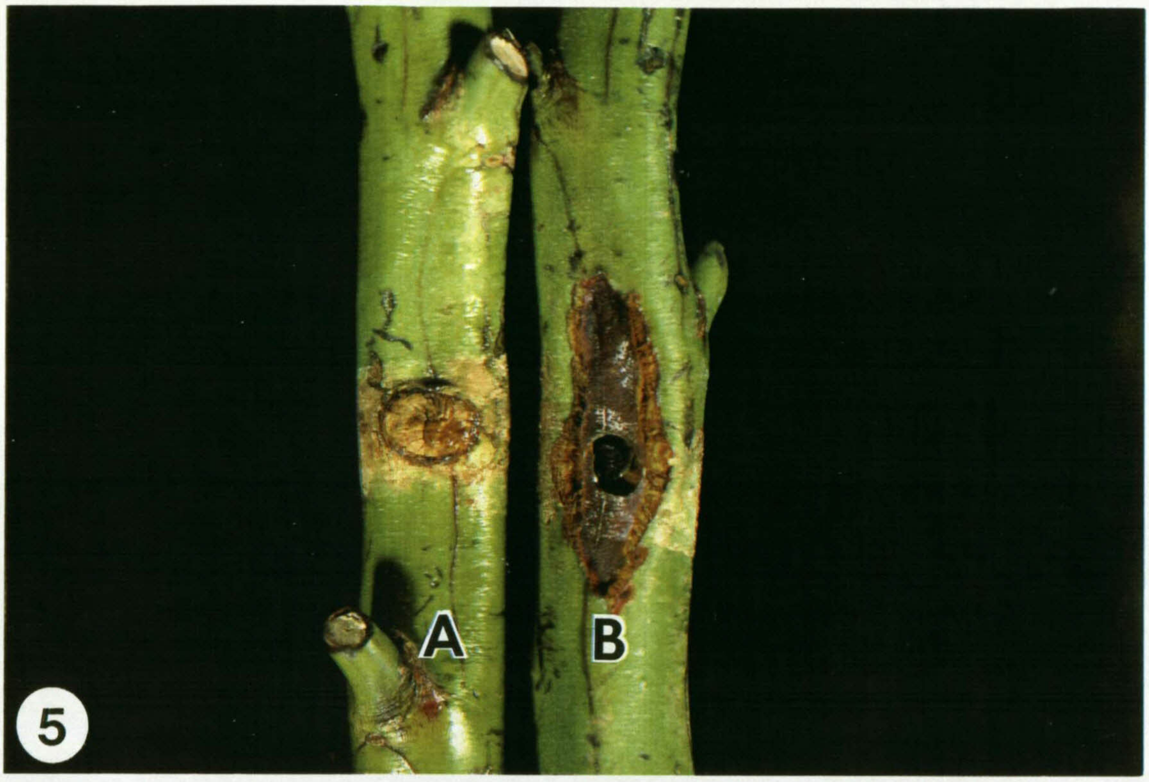

NUMBER OF ISOLATES
300
250
200
150
100
50
0
269 a
61 b
11 b
1 c
2 d
LESION LENGTH
10 - 20
21 - 30
31 - 40
41 - 50
51 - 60

Fig 5. Lesions associated with the inoculation of *Eucalyptus grandis* clone, ZG 14, with isolates of *Conithyrium zuluense*. (A) Inoculation with an avirulent isolate of *C. zuluense,* showing no lesion development. (B) Inoculation with a virulent isolate, showing extensive lesion development.


5
A
B

CHAPTER 3

Genetic variation among 108 isolates of *Eucalyptus* stem canker pathogen, *Coniothyrium zuluense*

A serious stem canker disease, caused by *Coniothyrium zuluense*, has become one of the most important diseases on *Eucalyptus* species in South Africa. Knowledge pertaining to the population structure of *C. zuluense* is crucial for selecting disease resistant clones for future planting. The durability of disease resistance in these clones will depend strongly on the genetic variability of the pathogen. The aim of this study is thus to determine the genetic diversity of the *C. zuluense* population in South Africa. In order to assess genetic variability, 108 *C. zuluense* isolates were selected based on their pathogenicity to a susceptible *Eucalyptus* clone. Isolates originated from nine different plantation regions in Zululand, KwaZulu-Natal. Amplified fragment length polymorphism (AFLP) markers were used to determine the genetic diversity and population structure of *C. zuluense*. Amplified fragments were scored as discrete characters and analysed by cluster analysis. The level of genetic diversity was relatively low, but higher than expected for an asexually reproducing pathogen. Equally low variation was evident between the different plantation regions. Genetic similarity values suggested a significant population differentiation between different plantation regions (sub-populations). Interpretation of results, thus, indicates that gene flow, together with selection, may be responsible for most of the gene diversity. It is expected that new epidemics would not be as a result of the emergence of new aggressive strains, but would rather be due to the introduction of susceptible *Eucalyptus* spp., together with environmental conditions favouring disease development.

The world-wide planting of *Eucalyptus* spp. has continued to increase with large new industrial plantations and new pulp mills emerging regularly. In South Africa, for example, commercial *Eucalyptus* accounts for more than 50% of all newly afforested areas in South Africa (Anonymous, 1995). A number of serious diseases have been reported to occur on various species and clones of *Eucalyptus* for the first time in South Africa during the course of the past decade (Linde *et al.*, 1994; Smith *et al.*, 1994; Wingfield *et al.*, 1989). Amongst these diseases, was the first report of a serious stem canker disease caused by *Coniothyrium zuluense* Wingfield, Crous & Coutinho in 1988 in KwaZulu-Natal (Wingfield *et al.*, 1997).

Coniothyrium zuluense has been described only from South Africa and is suspected to be endemic to the country (Wingfield *et al.*, 1997). A number of *Coniothyrium* species have previously been described as being pathogens of *Eucalyptus* (Sutton, 1975, 1980; Swart, 1986). These fungi are, however, only associated with leaf spots on eucalypts. It, therefore, appears that the South African *Coniothyrium* sp. associated with stem cankers, is unique. However, it is possible that the disease occurs in areas of origin of *Eucalyptus*, but in an ecologically balanced situation, thus making it inconspicuous.

Initial disease symptoms of Coniothyrium canker are visible as small necrotic lesions on young stem tissue. On clones that are highly susceptible, these lesions merge to give rise to large patches of dead, black bark that is often cracked and exudes copius amounts of kino (Coutinho *et al.*, 1997; Wingfield *et al.*, 1997). In cases of severe infection, epicormic shoots are produced on the stems around the spindle-shaped swelling of the stems and the tops of trees begin to die (Coutinho *et al.*, 1997; Wingfield *et al.*, 1997).

Currently, the most reliable management strategy to reduce the impact of Coniothyrium canker is by selecting clones and hybrids that show disease resistance (Wingfield *et al.*, 1997). Field trials have, however, shown significant variation in the susceptibility of different *Eucalyptus* clones, species and hybrids (Van Zyl *et al.*, 1997; Wingfield *et al.*, 1997). In forestry ecosystems, environmental changes, such as the deployment of new disease resistant clones and hybrids, impose strong directional selection on pathogen populations that have to adapt to such changes in the environment in order to survive (McDonald & McDermott, 1993). Control strategies must, therefore, target a population instead of an individual if they are to be effective (McDonald & McDermott, 1993).

There is little information available regarding the genetic make-up of *C. zuluense* in South Africa. No sexual structures of *C. zuluense* have been found in South Africa, indicating that the fungus reproduces only asexually (Wingfield *et al.*, 1997). This would lead to clonal lineages within a population (McDonald, 1997). Considerable variation has, however, been observed in virulence levels of *C. zuluense* isolates (Van Zyl *et al.*, 1997). The genes that are involved in host-specificity represent a small fraction of genes in the pathogen that may be subjected to host selection (Leung *et al.*, 1993). Analysis of *C. zuluense* field isolates with molecular markers would, therefore, give a more precise measure of their genetic relatedness and origin. The genetic diversity within a population will also give an indication of the level of sexual or asexual reproduction in the population (McDonald, 1997).

Knowledge of the genetic structure of *C. zuluense* will yield valuable information about the life-cycle of the fungus, as well as the durability of the resistance of different *Eucalyptus* clones. The aim of this study is, thus, to determine the population diversity of *C. zuluense* in South Africa. For this purpose, Amplified Fragment Length Polymorphism (AFLP) technology was used to test the relation between various isolates.

MATERIALS AND METHODS

Fungal isolates

Isolates of *C. zuluense* were obtained from cankers on the stems of severely infected *Eucalyptus* clones, originating from nine different plantation regions in KwaZulu-Natal (Fig. 1). Single conidial isolates were generated as previously described (Van Zyl *et al.*, 1997). A total of 108 single conidial isolates of *C. zuluense* were selected based on their pathogenicity to a susceptible *Eucalyptus grandis* clone, ZG 14 (Van Zyl *et al.*, 1997) (Table 1).

Pathogenicity characteristics for the selected isolates ranged between avirulent (-), intermediate (I) and virulent (+). Isolates were randomly selected from 344 isolates previously collected, and were composed of 43 avirulent, 42 intermediate and 23 virulent isolates. Inoculum preparation and pathogenicity tests were performed as previously described (Van Zyl *et al.*, 1997).

Molecular comparisons

DNA extractions. Nucleic acid was extracted from all 108 *Eucalyptus* stem canker isolates. Total genomic, high molecular weight DNA was extracted from all isolates grown in 250 ml of enriched Potato dextrose broth (24 g Potato Dextrose extract (Merck); 1 g Yeast extract (Merck); 1 g Glucose (Merck); 1 l distilled H_20) in 500 ml Erlenmeyer flasks. Cultures were incubated at 30 °C on rotary shakers for seven days. Mycelium was then harvested by filtration through Whatmann no. 1 filter paper and dried using several layers of paper towel. Care was taken to remove all agar-plugs. Two grams of dried mycelium was ground to a fine powder in liquid nitrogen with a mortar and pestle. Ten ml of extraction buffer (100 mM Tris-HCl, pH 8; 50 mM EDTA; 500 mM NaCl; 1.25 % SDS; 10 mM B-mercaptoethanol; 4 mM Spermidine; 1 mM Spermine; 1 mM Phenylmethylsulfonyl fluoride (PMSF)), maintained at 65 °C, was added to each isolate. The resultant slurry was transferred to a sterile centrifuge tube and stored at – 20 °C until all samples were ready for further processing.

Samples were incubated in a water bath at 65 °C for 60 min with frequent mixing. Potassium acetate (0.4 volumes of 5M stock) was added to each sample, transferred to 30 ml centrifuge tubes, and incubated on ice for 20 min. The supernatant was collected by centrifugation for 15 min at 17000 x g at 4 °C. The aqueous phase was transferred to clean 30 ml glass corex tubes and nucleic acid was precipitated by adding 0.58 volumes of ice cold isopropanol. Tubes were placed at –20 °C overnight. The precipitate was collected by centrifugation for 10 min at 5000 x g at 4 °C. Nucleic acid pellets were washed with 5 ml of 70 % ethanol and incubated overnight at 4 °C.

Pellets were collected by centrifugation at 5000 x g for 10 min and air dried in a fume hood at room temperature. Nucleic acid was re-suspended in 500 µl sterile water for 60 min at 37 °C. Nucleic acid suspensions were transferred to 1.5 ml Eppendorf tubes and centrifuged for 10 min at 10000 x g. The supernatant, containing the DNA, was collected in sterile 1.5 ml Eppendorf tubes and stored at – 20 °C. All DNA extracts were quantified by fluorometry and adjusted to a final concentration of 30 ng/µl.

Amplified Fragment Length Polymorphism's (AFLP) analysis

Restriction, ligation, and amplification were performed as described by Vos *et al.* (1995). Genomic DNA (500 ng) from each sample was incubated for 16 hours at 25 ° C in a solution containing 10 U/µl *Cfo*1 and 5 U/µl *Mse*1 (Boehringer Mannheim, USA), 1 x restriction-ligation buffer (10 mM Tris-HAc, pH7.5; 10 mM MgAc; 50 mM KAc; 5 mM DTT), 1 U/µl T4 DNA Ligase, 50 pmol/µl *Cfo*1-adaptors, 50 pmol/µl *Mse*1-adaptors (Table 2), 10 mg/ml RNase and 100 mM spermidine. The final sample volume was increased to 100 µl with sterile water. After ligation the reaction mixture was diluted 10 - fold with T.1E buffer (10 mM TRIS-HCl, pH8.0; 10 mM EDTA) and stored at –20 °C. These ligated fragments served as templates in the amplification reaction.

A pre-selective PCR (+ 1 reaction) amplification reaction was performed in 20 µl PCR reaction mix containing 5 µl of the diluted DNA, 0.5 µl of each +1 primer (10 pmol/µl) (Table 2), 100 mM Tris-HCl, pH 8.0, 15 mM $MgCl_2$, 500 mM KCl, 25 mM $MgCl_2$, 5U/µl *Taq* DNA Polymerase (Boehringer Mannheim, USA) and 250 µM of dNTP. Initial denaturation was performed at 94 °C for 1min, followed by 30 cycles of 30 sec at 94 ° C, 60 sec at 56 °C (primer annealing) and 60 sec at 72 °C (final chain elongation). The amplification PCR products were diluted 10-fold in 1 x T.1E and used as templates in the second amplification.

The second amplification (+ 3 reaction) was performed using primers derived from the first set of primers with additional nucleotides at the 3' end (Table 2). The *Cfo*1 primer was HEX fluorochrome-labeled and the *Mse*1 primer was FAM fluorochrome-labeled (AmpFISTR Profiler PCR Kit, Perkin-Elmer, Norwalk, Conn.). Fluorescent AFLPs were amplified under the following conditions: 0.2 µl of the HEX fluorochrome-labeled *Cfo*1 + 3 primer (50 pmol/µl) was added to a 20-µl PCR reaction solution containing 5 µl of the diluted + 1 pre-amplification mix, 0.4µl of the FAM fluorochrome-labeled *Mse*1 + 3 primer, 5 U/µl *Taq* DNA Polymerase (Boehringer Mannheim, USA), 10 x Buffer (100 mM Tris-HCl, pH 8.0; 15 mM $MgCl_2$; 500 mM KCl, pH8.3), 25 mM $MgCl_2$ and 250 µM of dNTP. The following temperature profile was used: 12 cycles of 30 sec at 94 °C, 30 sec at 65 °C, 60 sec at 72 °C, where the annealing temperature was subsequently reduced by 0.7 °C after each cycle. The amplification was continued for 22 cycles of 30 sec at 94 °C, 30 sec at 56 °C, and 60 sec at 72 °C. All amplification reactions were performed using a Hybaid Omnigene thermocycler (Hybaid, Middlesex, UK).

Electrophoresis and visualisation of AFLP PCR products.

PCR products (1.5µl) were combined with 3 *f*mol TAMRA fluorescent-labeled GeneScan 500 internal size standard (ABI), 1.6 µl formamide, and 0.3 µl 25 mM EDTA (pH 8.0) containing 50 mg/ml blue dextran. This mixture was heat denatured for 3 min at 95 °C, and immediately cooled on ice. Samples were loaded on a 5 % denaturing polyacrylamide gel in 1 x TBE (Tris-borate EDTA, pH 8.0) and electrophoresed for 2.5 hours at 1680 watts using the GS 36A-2400 run module. Data was processed by GeneScan Analysis software (version 2.02) to produce a gel image. PCR fragments for individual samples were automatically sized by the GeneScan software using a comparison of the mobility of the internal lane size standard to that of the sample fragment.

AFLP data analysis

Each polymorphic AFLP fragment was treated as a unit character and scored as present (1) or absent (0) in all isolates. The experiments were repeated, and only reproducible bands were scored. The index of genetic similarities (F_{ST}) of Nei & Li (1979) was used to calculate pairwise genetic distances. Genetic diversity (H_T) was calculated as $H = 1 - F_{ST}$ (Nei, 1973). Sub-population diversity, H_S, was estimated as the mean diversity of AFLP markers among regions. The diversity between regions, D_{ST}, was determined as the difference between H_T and the mean diversity among all regions (H_S). Differentiation among sub-populations is defined as the percentage of diversity between regions out of the total diversity. Unweighted Pair-Group Mean Arithmetic (UPGMA) was used for cluster analysis of the pairwise similarity matrix that generated a dendrogram representing the genetic similarity among fungal isolates. UPGMA analysis was carried out using NCSS97 (Visual Components, UK).

RESULTS

AFLP analysis. Averages of 44 fragments were obtained for each *C. zuluense* isolate, ranging from 40 to 491 bp. Polymorphic fragments were distributed across the entire size range with the major proportion (72 %) between 89 – 309 bp. The total number of fragments scored across each of the 108 *C. zuluense* isolates, was 84 fragments for primer combination 1(HEX fluorochrome-labeled *Cfo*1 + 3 primer) and 75 fragments for combination 2 (FAM fluorochrome-labeled *Mse*1 + 3 primer), of which 13 (8.2 %) were monomorphic for all isolates, while 146 (91.8 %) displayed informative polymorphism's. No differences were encountered on the AFLP profile of the control check, based on replicate lanes of DNA, which were run on each gel. DNA from a duplicate set of 20 *C. zuluense* isolates was extracted and amplified with primer combinations to test for repeatability. Less than 1 % of the bands were evaluated differently.

When NSCC97 (Visual Components, 1997) analysis was performed with each primer combination individually, UPGMA groupings were almost identical to those when both primer combinations were analysed together (data not shown). Spearman's Rank Correlation Coefficient (r_s) between genetic distances based on primer combination 1 and 2, was 34 % ($r_s = 0.34$). This low level of correlation suggests that each primer combination provided somewhat different and, therefore, complementary information. However, correlation of genetic distances for both primer combinations (Primer combination 1 and 2) with those of either Combination 1 or primer Combination 2, were 92 % ($r_s = 0.92$), respectively. Thus, each primer combination individually, would have given an approximation of the entire data set, but sufficient differences existed between combinations that both were necessary.

Genetic distance analysis. The total gene diversity, H_T, was 19.5 %, with an average within region diversity, H_S, of 18.3 %. Diversity between regions, D_{ST}, was therefore 1.2 %, which is 6.2 % of the total diversity. Thus, approximately 6.2 % of the overall gene diversity were accounted for by differences among regions. The remaining 93.9 % was attributed to variation within regions. F_{ST} values were also used as a measure of population differentiation between and within *C. zuluense* isolates collected from different plantation regions (F_{ST} = 0.195; $P < 0.01$).

Average F_{ST} values are presented in Table 3. Results indicate that significant genetic variation occurs among isolates of *C. zuluense* within most individual plantation regions (F_{ST} = 0.195; $P < 0.01$). There was, however, no significant variation in genetic similarity (GS) among fungal isolates collected from four of the nine plantation regions used in this study. Isolates collected from Aboyni (F_{ST} = 0.098, 90.2 % GS), Shire (F_{ST} = 0.034, 96.6 % GS), Teranera (F_{ST} = 0.018; 98.2 % GS) and Trust (F_{ST} = 0.089; 91.1 % GS) plantation regions showed no significant ($P < 0.01$) variation, indicative of a highly clonal population. Average genetic similarity among fungal isolates, within each individual plantation region, varied between 79.1 % (20.9 % dissimilarity) (F_{ST} = 0.288; $P < 0.01$) for Futululu and 98.2 % (1.8 % dissimilarity) (F_{ST} = 0.018, NS) for Teranera plantation (Table 3).

Significant differentiation between the nine plantation regions was clearly evident. Differentiation (F_{ST}) among the different plantations (sub-populations) is defined as the percentage of diversity between regions out of the total diversity. The similarity percentages among fungal isolates ranged from 71.2 % (F_{ST} = 0.288; $P < 0.01$) to 83.8 % (F_{ST} = 0.162; $P < 0.01$), with an average similarity of 78.8 % (F_{ST} = 0.212; $P < 0.01$) (Table 3). It was evident that isolates from the Teranera plantation region, were more genetically distant, ranging from 71.2 % (28.8 % dissimilarity) (F_{ST} = 0.288; $P < 0.01$) to 77.3 % (22.7 dissimilarity) (F_{ST} = 0.227; $P < 0.01$) similarity. There was no correlation between geographic distance and F_{ST} values (r_s = 0.34, NS).

The similarity matrix was used to cluster the data using the unweighted pair-group method, with an arithmetic average (UPGMA) algorithm. The dendrogram reflected the average genetic distance, 19.5 %, between isolates of *C. zuluense* collected from nine different plantation regions within KwaZulu-Natal (Fig. 2). What was also evident, is that no specific cluster or grouping was visible among the 108 *C. zuluense* isolates, irrespective of the fact that they varied in pathogenicity to *Eucalyptus*.

DISCUSSION

This study showed that the AFLP technique is useful for the characterisation of intraspecific variation among the *C. zuluense* population. In this study, 40 % of markers were polymorphic in a collection of 108 *C. zuluense* isolates from nine plantation regions. The large number of polymorphism's detected with AFLP analysis has the advantage that markers that appear unreliable can be discarded. This approach also has a clear advantage in terms of the proportion of the genome being analysed per reaction. Population diversity estimates are, therefore, more accurate than estimates based on few loci in the genome. The data also showed that the majority of AFLP markers segregate independently of one another and the risk of overestimating variation is low.

A pathogenicity study conducted during 1997 showed a significant variation in pathogenicity of *C. zuluense* isolates to a susceptible *E. grandis* clone (Van Zyl *et al.*, 1997). This is indicative of a diverse fungal population. Results from the present study, however, revealed that *C. zuluense* in South Africa is represented by a relatively low level of genetic diversity. AFLP analysis also indicated that no group having similar pathogenicity characteristics formed a specific cluster. This is in contrast to results obtained by Pongam *et al.* (1999) who showed with AFLP markers that isolates of *Leptosphaeria maculans* (Desmaz.) Ces. & De Not. from North Dakota, Western Canada, Georgia and the UK formed one tightly clustered AFLP group and were mostly of the same pathogenicity group. Thus, pathogenicity data alone may not reflect the true genetic variability and evolutionary history of the isolates. Isolates that are genetically distinct may have similar or identical pathogenicity patterns due to the fact that they were exposed to the same selection pressures by a common set of hosts.

The relatively low but well established level of genetic diversity in *C. zuluense* suggests that there may be a high level of asexual reproduction in the fungus. Genetic diversity values would be expected to be much higher for mainly sexual reproducing fungi (McDonald & McDermott, 1993). This conclusion is further supported by the fact that a low genetic variation was observed within each of the sampled regions. Fungi capable of only asexual reproduction have been found to have a lower degree of genetic diversity than organisms that reproduce sexually (McDonald & McDermott, 1993; Wolf & McDermott, 1994; Milgroom, 1996). The low level of genetic diversity obtained in this study is in agreement with genetic diversity values reported for the asexually reproducing fungus, *Rhynchosporium secalis* (Oud) J.J. Davis (Goodwin *et al.*, 1993). The authors estimated a genetic diversity value of between 0.16 and 0.29 in Australia, Norway and the USA (Goodwin *et al.*, 1993). Results of the current study thus also suggest that *C. zuluense* reproduces asexually, but that it has been present in South Africa for an extended period of time.

In the present study, a moderate, but significant level of geographic differentiation was found between isolates of *C. zuluense* collected from individual plantation regions. This is in contrast to other pathogens that are known to be predominantly clonal, for example, *Phytophthora infestans* (Mont.) de Bary in the Netherlands (G_{ST} = 0.06) (Fry *et al.*, 1991) and *Mycosphaerella graminicola* (Fuckel.) Schroter populations in California and Oregon (G_{ST} = 0.039) (Boeger *et al.*, 1993). In these studies, G_{ST}, is a measure of genetic differentiation, which is similar to F_{ST} (Nei, 1973). Values of G_{ST} and F_{ST} not significantly different from zero would indicate no differentiation between populations. The geographic differentiation value obtained in this study is, however, similar to levels of geographic differentiation reported for *Pyrenopeziza brassicae* B. Sutton & Rawlinson (F_{ST} = 0.16; Majer *et al.* 1998). Therefore, the relatively high F_{ST} value for *C. zuluense* also suggests that there are distinct geographic sub-populations of the fungus within the *Eucalyptus* growing areas of Zululand, KwaZulu-Natal.

Genetic variation within fungal populations is known to be influenced either by ecologically important or selective neutral variation (McDermott & McDonald, 1993; McDonald & McDermott, 1993; Anderson & Kohn, 1995; Milgroom, 1995, 1996; Milgroom & Fry, 1997). Milgroom & Fry (1997) referred to ecologically important variation as traits that affect fitness and are thought to be under selection. Asexually reproducing pathogens, such as *C. zuluense*, are forced to constantly adapt to changes in their environment in order to survive. The development of disease resistant *Eucalyptus* clones or hybrids imposes strong directional selection on the pathogen population, leading to increased numbers of individual genotypes. Thus, genetic diversity is directly influenced by selection, meaning that genotypes with the highest fitness will increase over a period of time (McDonald *et al.*, 1989).

Selective neutral variation does not affect fitness and is also known to be affected by evolutionary forces, such as mutations, mating systems, gene flow or migration and population size (McDermott & McDonald, 1993; Anderson & Kohn, 1995; Milgroom, 1995, 1996). Cluster analysis of data from the current study showed no evidence for one or two genotypes being widespread as would be expected of predominantly asexual pathogens. This would suggest that epidemics of Coniothyrium canker are the result of genetic flow of local populations, rather than the emergence of aggressive strains of *C. zuluense* that have spread throughout the Zululand forestry area. In the absence of any movement of gametes among populations, it is expected that genetic drift will lead to random changes in allele frequencies for neutral loci in different populations (Boeger & McDonald, 1991). Even if there were only limited movement of genes among populations, a correlation between genetic and geographic distance among populations should be observed (Boeger & McDonald, 1991). Results of this study showed no correlation between F_{ST} values and geographic distance and, therefore, strongly support our hypothesis that genetic flow can be considered to be the main factor contributing towards genetic variation within the *C. zuluense* population.

This study provides valuable information regarding future management strategies of Coniothyrium canker. In many situations, ecologically important variation (traits effected by selection) is relevant for disease management (Leung *et al.*, 1993; Anderson & Kohn, 1995; Milgroom & Fry, 1997). However, an understanding of ecological, as well as selective neutral variation, is necessary since it helps to predict how a pathogen population will respond to the implementation of new disease resistant clones. Population genetic data presented in this paper suggest that new epidemics would not be due to the emergence of aggressive strains (increase in virulence) spreading rapidly through the country. Outbreaks would rather be due to the introduction of new susceptible *Eucalyptus* clones, species or hybrids, together with environmental conditions that favour disease development.

Population genetic data presented in this paper indicated there is a low level of genetic variation in *C. zuluense*, suggestive of asexual reproduction. Disease outbreaks would thus be due to the introduction of susceptible *Eucalyptus* species combined with environmental conditions conductive to infection. The low, but well-established genetic variation is also suggestive that the fungus has been present in this country for an extended period of time.

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Table 1. *Coniothyrium zuluense* isolates differing in colony morphology and pathogenicity that were used in amplified fragment length polymorphism (AFLP) analysis.

	Isolate[a]	**Plantation**[b]	**Pathogenicity Grouping**[c]
1	CRY 949	Aboyni	I
2	CRY 950	“	I
3	CRY 951	“	I
4	CRY 952	“	I
5	CRY 953	“	-
6	CRY 954	“	-
7	CRY 955	Futululu	-
8	CRY 956	“	I
9	CRY 957	“	-
10	CRY 958	“	+
11	CRY 959	“	-
12	CRY 960	“	+
13	CRY 961	“	I
14	CRY 962	“	I
15	CRY 963	“	I
16	CRY 964	“	-
17	CRY 965	“	-
18	CRY 966	“	I
19	CRY 967	“	I
20	CRY 968	“	I
21	CRY 969	“	-
22	CRY 970	“	-
23	CRY 971	“	-
24	CRY 972	“	-
25	CMW 1772	Honey Farm	+
26	CMW 1778	“	I
27	CMW 2100	“	+
28	CRY 973	“	I
29	CRY 974	“	+
30	CRY 975	“	+
31	CRY 976	“	I
32	CRY 977	“	-
33	CRY 978	“	-
34	CRY 979	“	+
35	CRY 980	“	I
36	CRY 981	“	-
37	CRY 982	“	-
38	CRY 983	“	+
39	CRY 984	“	I
40	CRY 985	“	I
41	CRY 986	“	I
42	CRY 987	“	I

	Isolate[a]	Plantation[b]	Pathogenicity Grouping[c]
43	CRY 988	Palm Ridge	-
44	CRY 989	“	-
45	CRY 990	“	I
46	CRY 991	“	-
47	CRY 992	“	I
48	CRY 993	“	I
49	CRY 994	“	-
50	CRY 995	“	-
51	CRY 996	“	-
52	CRY 997	“	I
53	CRY 998	“	+
54	CRY 999	“	I
55	CRY 1000	“	I
56	CRY 1001	“	+
57	CRY 1002	“	I
58	CRY 1003	“	I
59	CRY 1004	Shire	-
60	CRY 1005	“	-
61	CRY 1006	“	-
62	CRY 1007	“	+
63	CRY 1008	Teranera	-
64	CRY 1009	“	-
65	CRY 1010	“	I
66	CRY 1011	“	+
67	CRY 1012	“	-
68	CRY 1013	“	-
69	CRY 1014	“	-
70	CRY 1015	“	-
71	CRY 1016	Teza	I
72	CRY 1017	“	I
73	CRY 1018	“	+
74	CRY 1019	“	+
75	CRY 1020	“	+
76	CRY 1021	“	+
77	CRY 1022	“	+
78	CRY 1023	“	+
79	CRY 1024	“	+
80	CRY 1025	“	+
81	CRY 1026	“	+
82	CRY 1027	“	I
83	CRY 1028	“	I
84	CRY 1029	“	-
85	CRY 1030	“	I
86	CRY 1031	“	I
87	CRY 1032	“	+
88	CRY 1033	“	-
89	CRY 1034	“	I

	Isolate[a]	**Plantation**[b]	**Pathogenicity Grouping**[c]
90	CRY 1035	Teza	-
91	CRY 1036	“	I
92	CRY 1037	“	I
93	CRY 1038	“	-
94	CRY 1039	“	I
95	CRY 1040	“	-
96	CRY 1041	“	-
97	CRY 1042	“	-
98	CRY 1043	“	I
99	CRY 1044	Trust	I
100	CRY 1045	“	I
101	CRY 1046	“	I
102	CRY 1047	“	+
103	CRY 1048	“	-
104	CRY 1049	“	-
105	CRY 1050	“	-
106	CRY 1051	Fairbreeze	-
107	CRY 1052	“	-
108	CRY 1053	“	-

[a] CRY and CMW numbers refer to *Coniothyrium zuluense* isolates used in this study.

[b] Isolates were collected from nine different *Eucalyptus* growing plantation regions in the Zululand forestry area of KwaZulu-Natal, South Africa.

[c] Pathogenicity grouping of different isolates based on the degree of virulence towards susceptible *Eucalyptus grandis* clone, ZG 14 (+ = pathogenic, - = non-pathogenic, I = intermediately pathogenic). Pathogenicity was determined in a previous study (Van Zyl *et al.*, 1997).

Table 2. Sequences of primers and adaptors used for AFLP analysis.

Name	Enzyme	Type	Sequence
CA	*Cfo*1	Adaptor	5'-GACGATGAGTCCTGAACG-3' 3'-TACTCAGGACTT-5'
MA	*Mse*1	Adaptor	5'-GACGATGAGTCCTGAG-3' 3'-TACTCAGGACTCAT-5'
C-T	*Cfo*1	Primer + 1	5'-GATGAGTCCTGAACGCT-3'
C-TCC	*Cfo*1	Primer + 3	5'-GATGAGTCCTGAACGCTCC-3'
C-TGG	*Cfo*1	Primer + 3	5'-GATGAGTCCTGAACGCTGG-3'
M-A	*Mse*1	Primer + 1	5'-GATGAGTCCTGAGTAA-3'
M-AGT	*Mse*1	Primer + 3	5'-GATGAGTCCTGAGTAAGT-3'
M-AGC	*Mse*1	Primer + 3	5'-GATGAGTCCTGAGTAAGC-3'

Table 3. Average F_{ST} values of the *Coniothyrium zuluense* population[a]. Bold numbers indicate F_{ST} is significantly different from zero at *$P < 0.01$*

	Plantations	1	2	3	4	5	6	7	8	9
1	Aboyni	0.098								
2	Futululu	**0.191**	**0.209**							
3	Honey Farm	**0.199**	**0.208**	**0.185**						
4	Palm Ridge	**0.179**	**0.203**	**0.192**	**0.170**					
5	Shire	**0.192**	**0.198**	**0.211**	**0.187**	0.034				
6	Teranera	**0.236**	**0.260**	**0.227**	**0.237**	**0.263**	0.018			
7	Teza	**0.162**	**0.200**	**0.185**	**0.156**	**0.208**	**0.215**	**0.151**		
8	Trust	**0.211**	**0.212**	**0.210**	**0.222**	**0.258**	**0.274**	**0.216**	0.089	
9	Fairbreeze	**0.175**	**0.221**	**0.214**	**0.194**	**0.222**	**0.288**	**0.185**	**0.227**	**0.186**

[a] Average F_{ST} values for *Coniothyrium zuluense* isolates were calculated among and between plantations, based on the values obtained from the F-statistic of Nei & Li (1979).

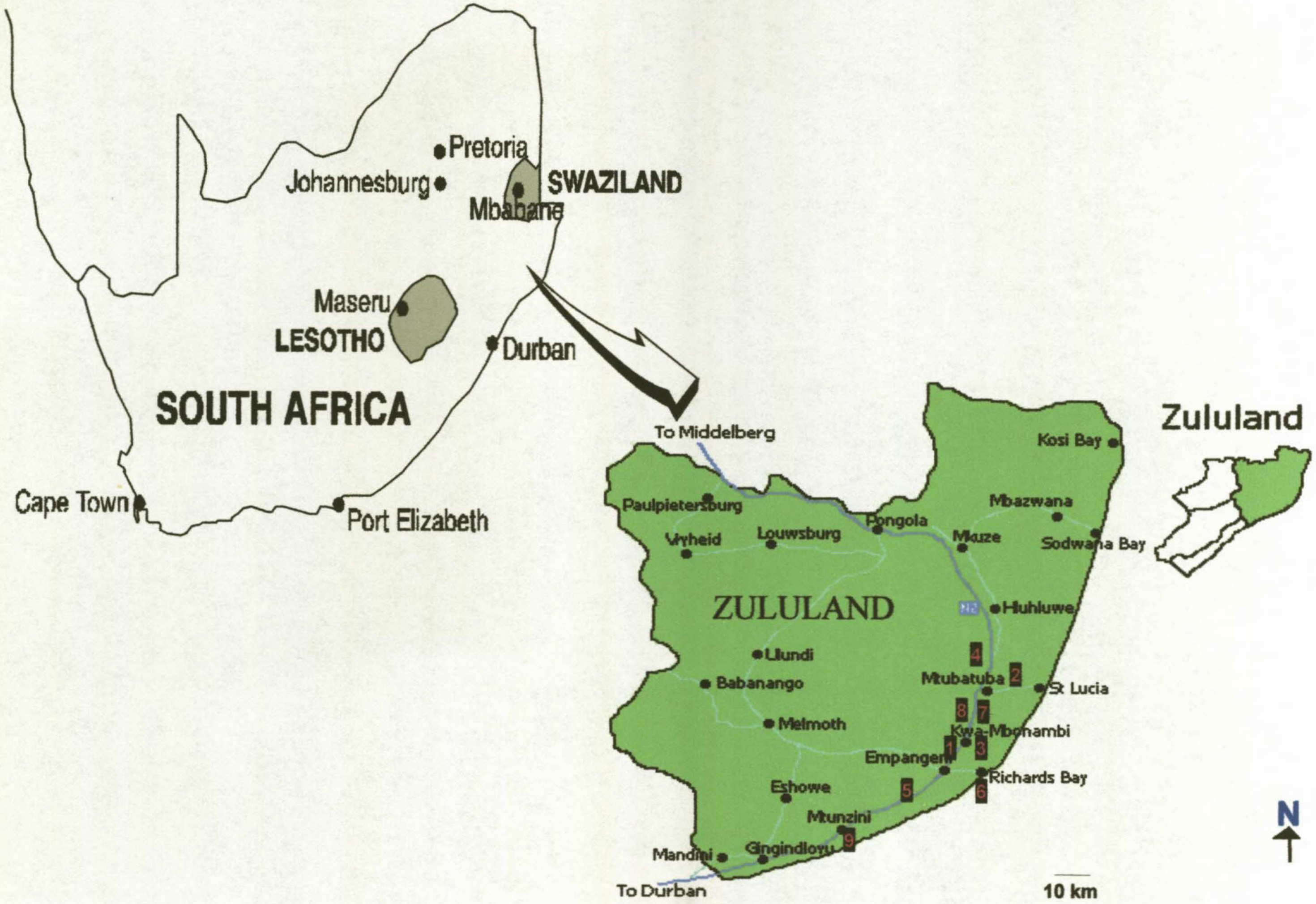
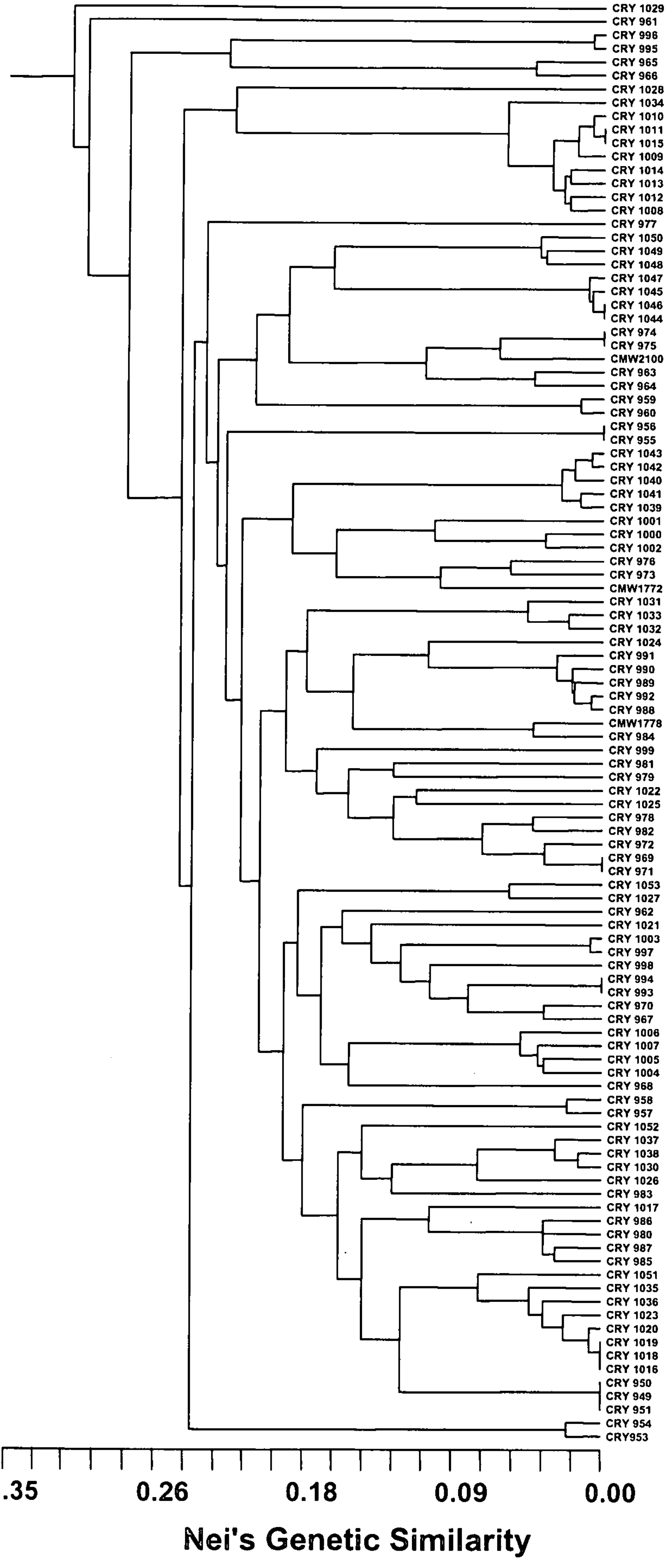

Pretoria
Johannesburg
SWAZILAND
Mbabane
Maseru
LESOTHO
Durban
SOUTH AFRICA
Cape Town
Port Elizabeth
To Middelberg
Zululand
Kosi Bay
Paulpietersburg
Mbazwana
Pongola
Vryheid
Louwsburg
Mkuze
Sodwana Bay
ZULULAND
N2
Hluhluwe
4
Ulundi
2
Mtubatuba
Babanango
St Lucia
8
7
Melmoth
Kwa-Mbonambi
1
3
Empangeni
Richards Bay
Eshowe
5
6
Mtunzini
N
9
Mandini
Gingindlovu
To Durban
10 km

Fig. 2. Dendrogram of 108 *Coniothyrium zuluense* isolates based on AFLP data using UPGMA cluster analysis of pairwise distance data. The scale represents genetic distance obtained using the equation of Nei & Li (1979).


CRY 1029
CRY 961
CRY 996
CRY 995
CRY 965
CRY 966
CRY 1028
CRY 1034
CRY 1010
CRY 1011
CRY 1015
CRY 1009
CRY 1014
CRY 1013
CRY 1012
CRY 1008
CRY 977
CRY 1050
CRY 1049
CRY 1048
CRY 1047
CRY 1045
CRY 1046
CRY 1044
CRY 974
CRY 975
CMW2100
CRY 963
CRY 964
CRY 959
CRY 960
CRY 956
CRY 955
CRY 1043
CRY 1042
CRY 1040
CRY 1041
CRY 1039
CRY 1001
CRY 1000
CRY 1002
CRY 976
CRY 973
CMW1772
CRY 1031
CRY 1033
CRY 1032
CRY 1024
CRY 991
CRY 990
CRY 989
CRY 992
CRY 988
CMW1778
CRY 984
CRY 999
CRY 981
CRY 979
CRY 1022
CRY 1025
CRY 978
CRY 982
CRY 972
CRY 969
CRY 971
CRY 1053
CRY 1027
CRY 962
CRY 1021
CRY 1003
CRY 997
CRY 998
CRY 994
CRY 993
CRY 970
CRY 967
CRY 1006
CRY 1007
CRY 1005
CRY 1004
CRY 968
CRY 958
CRY 957
CRY 1052
CRY 1037
CRY 1038
CRY 1030
CRY 1026
CRY 983
CRY 1017
CRY 986
CRY 980
CRY 987
CRY 985
CRY 1051
CRY 1035
CRY 1036
CRY 1023
CRY 1020
CRY 1019
CRY 1018
CRY 1016
CRY 950
CRY 949
CRY 951
CRY 954
CRY953
0.35
0.26
0.18
0.09
0.00
Nei's Genetic Similarity

CHAPTER 4

Morphological and molecular relatedness of geographically diverse isolates of *Coniothyrium zuluense* from South Africa and Thailand

Coniothyrium canker, caused by *Coniothyrium zuluense*, is a serious stem canker disease of *Eucalyptus* species in sub-tropical parts of South Africa. A *Coniothyrium* sp. associated with similar symptoms on *E. camaldulensis* was observed in 1996 in Thailand. It was previously thought that *C. zuluense* was restricted to South Africa. The aim of this study is, thus, to compare South African isolates of *C. zuluense* with isolates of the *Coniothyrium* sp. from Thailand at the morphological and molecular level. Results of morphological comparisons indicate that the South African and Thailand isolates are the same. This was further confirmed when all *Coniothyrium* isolates associated with stem cankers on *Eucalyptus* spp. grouped together in a single major clade for both rDNA sequence data and AFLP analysis. This clade was distant from isolates of other *Coniothyrium* spp. included for comparative purposes. Although the *Coniothyrium* isolates from South Africa and Thailand resided in two separate clades, these were closely related and, we believe that the isolates from Thailand represent *C. zuluense*. This is, thus, the first record of the important *Eucalyptus* stem canker pathogen, *C. zuluense,* outside South Africa.

Eucalyptus species are native to Australia, but approximately 8 million hectares of *Eucalyptus* plantations have been established, mostly in tropical and sub-tropical countries of the world (Anonymous, 1995). The success of *Eucalyptus* propagation, however, is often hampered by their susceptibility to fungal diseases. These diseases include both stem, root and leaf diseases, and have been shown to cause considerable economic losses on various *Eucalyptus* species, clones and hybrids (Park & Keane, 1984; Florence *et al.*, 1986; Hodges *et al.*, 1986; Ferreira, 1989; Conradie *et al.*, 1990; Linde *et al.*, 1994; Smith *et al.*, 1994; Crous & Wingfield, 1994, 1996).

Coniothyrium canker caused by the recently described *Coniothyrium zuluense* Wingfield, Crous & Coutinho, is a serious *Eucalyptus* stem canker pathogen from South Africa (Wingfield *et al.*, 1997). This fungus was first reported in 1988 in an isolated area in Zululand, KwaZulu-Natal, on a single clone of *E. grandis* Hill: Maid. The earliest signs of infection by *C. zuluense* on trees are small, discrete, necrotic lesions on the young, green bark (Coutinho *et al.*, 1997; Wingfield *et al.*, 1997). These lesions coalesce to form large necrotic patches on the stems from which copius amounts of red kino exude (Coutinho *et al.*, 1997; Wingfield *et al.*, 1997). Epicormic shoots are commonly produced in the cankered areas, indicative of partial girdling of the stems. In severely infected clones, the tops of trees die, due to the girdling effect (Coutinho *et al.*, 1997; Wingfield *et al.*, 1997).

Since its discovery in 1988, *C. zuluense* has become widespread throughout *Eucalyptus* growing areas of Zululand and occurs on a wide range of *E. grandis* clones and hybrids, as well as other species of *Eucalyptus* (Coutinho *et al.*, 1997; Wingfield *et al.*, 1997). An intensive disease survey was undertaken during 1995 and 1996, that lead to the discovery that *C. zuluense* isolates differ considerably in surface colony colour and pathogenicity to a susceptible *E. grandis* clone (Van Zyl *et al.*, 1997). It was, therefore, hypothesised that more than one species of *Coniothyrium* might be responsible for disease in South Africa.

A number of *Coniothyrium* spp. have been reported as pathogens of *Eucalyptus* leaves in the past. In Australia, *C. eucalypticola* Sutton, *C. kallangurence* Sutton & Alkorn, *C. ovatum* Swart and *C. parvum* Swart are associated with leaf spot on various *Eucalyptus* spp. (Sutton, 1975, 1980; Swart, 1986). *Coniothyrium ahmadii* Sutton has been isolated from eucalypt leaf spots in Pakistan (Sutton, 1974). The only report of a pathogenic *Coniothyrium* sp. from South Africa, prior to the discovery of *C. zuluense*, was of leaf spot caused by *C. ovatum* (Crous *et al.*, 1988). In this case, *C. ovatum* was isolated from leaf spots occurring mainly on the lower branches of mature *E. cladocalyx* F. Muell. and *E. lehmannii* Preiss:Schauer trees in Stellenbosch, Western Cape province.

During 1996, a survey of diseases of eucalypt plantations in the Sinai area of Thailand was undertaken. This led to the discovery of a serious stem canker disease of *E. camaldulensis* Dehnh. Disease symptoms were found to be very similar to those caused by *C. zuluense* from South Africa. *Coniothyrium zuluense* is, however, only known from South Africa and was hypothesised to be endemic to this country (Coutinho *et al.*, 1997; Wingfield *et al.*, 1997). Based on superficial morphological and cultural characteristics, the Thailand isolates were identified as representing a species of *Coniothyrium*. The aim of this study is, therefore, to compare isolates of *C. zuluense* with those of the *Coniothyrium* sp. from Thailand, based on morphological comparisons, rDNA sequence data and Amplified Fragment Length Polymorphism (AFLP) analysis.

MATERIALS AND METHODS

Isolates

Six single conidial isolates of a *Coniothyrium* sp. were collected from severely infected *E. camaldulensis* trees from the Sinai region of Thailand (Table 1). Isolations were made from segments of symptomatic material that were placed in humidity chambers to induce the formation of fungal fruiting bodies. Single conidial isolates were obtained as described by Van Zyl *et al.* (1997). Each isolate originated from a different tree.

Nine single conidial isolates of *C. zuluense* were collected from nine *Eucalyptus* plantation regions in Zululand, KwaZulu-Natal, South Africa (Table 1). These isolates were chosen to be compared with those from Thailand, based on their differences in surface colony colour and pathogenicity to a susceptible *E. grandis* clone (Van Zyl *et al.*, 1997). Colony colour characteristics of these isolates varied between olive grey ($V23^{\mathrm{IIIIb}}$), greenish glaucous (33^{IIIf}), isabella (19^{II}I) and grayish olive (21^{IIII}) (Van Zyl *et al.*, 1997). The selected isolates represented three non-pathogenic, four isolates of intermediate pathogenicity and two isolates with high levels of pathogenicity.

Three *Coniothyrium* spp. other than *C. zuluense* were included for comparative purposes (Table 1). These included *C. ovatum* Swart, a leaf-spotting pathogen isolated from *E. diversicolor* in Stellenbosch, Western Cape, South Africa; *C. palmarum* Corda (CBS 758.73), the lectotype species of *Coniothyrium* and *C. fuckelii* Sacc. (CBS 132.26) that causes stem cankers on various *Rosa* Thunb. and *Rubus* L. species. *Massarina corni* Sh. (CBS 496.64) was included as an outgroup (Table 1). All isolates were grown on Petri dishes containing 15 ml of an enriched 4 % w/v Potato Dextrose Agar (PDA) (24 g Potato Dextrose extract (Difco); 1 g Yeast extract (Difco); 1 g Glucose (Difco); 40 g agar (Difco); 1 l distilled H_20). Plates were then incubated at 30 °C for 10 days. Isolates are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, and will be deposited in other recognised international culture collections.

Morphological comparisons

Colony and conidial morphology. *Coniothyrium* isolates from Thailand were grown on PDA (39 g PDA (Difco); 1 l distilled H_2O) at 30 °C. Measurements (length and width) using a Zeiss Axioskop light microscope were made of mature conidia (thirty from each structure). Colony colour was rated using mycological colour charts of Rayner (1970). Colony colour and conidial measurements for the Thailand isolates were compared with those of *C. zuluense* isolates associated with *Eucalyptus* stem canker in South Africa (Van Zyl *et al.*, 1997).

Growth studies. Growth rates and temperature requirements for the South African and Thailand isolates were determined on PDA. The PDA plates were inoculated with 5 mm mycelial plugs removed from the margins of actively growing colonies and placed, face down, at the centre of the plates. Plates were maintained at temperatures ranging from 10 to 35 °C, at five degree intervals in the dark for 30 days. A total of 3 plates were incubated for each isolate at each temperature. Two diameter measurements were obtained from each colony, perpendicular to each other. A total of 6 measurements were thus taken at each temperature for every isolate studied, and the mean growth computed.

Greenhouse pathogenicity trials

All six Thailand and nine South African isolates were used in greenhouse inoculation trials. Isolates were grown on enriched 4 % PDA for two weeks prior to inoculation. Pathogenicity tests were conducted on six-month-old *E. grandis* trees of the clone ZG 14, which is known to be highly susceptible to Coniothyrium canker under natural conditions (Wingfield *et al.*, 1997). Twenty trees were inoculated for each isolate tested. A small wound (10 mm diameter) was made on the stem of each tree by removing the bark and exposing the cambium. Mycelial plugs, of similar size, overgrown with the test fungi, were placed into each wound with the mycelium facing the cambium. Inoculation wounds were covered with masking tape to prevent desiccation of the inoculum. For control inoculations, sterile PDA plugs were used and inserted into wounds on the stems of five trees. Mean lesion lengths were assessed after six weeks and statistical differences for each isolate were determined using Tukey's studentised range test ($P = 0.05$). The experiment was repeated once.

Molecular comparisons

DNA extractions. Nucleic acid was extracted from all *Eucalyptus* stem canker isolates, as well as, isolates included in the study for comparative purposes (Table 1). Total genomic, high molecular weight DNA was extracted from all isolates by culturing them in 250 ml of enriched Potato dextrose broth (24 g Potato Dextrose extract (Merck); 1 g Yeast extract (Merck); 1 g Glucose (Merck); 1 l distilled H_20) in

500 ml Erlenmeyer flasks. Cultures were incubated at 30 °C on rotary shakers for seven days. Mycelium was then harvested by filtration through Whatmann no. 1 filter paper and lyophilised.

DNA was isolated using the technique of Raeder & Broda (1985) with some amendments. One gram of dried mycelium was ground to a fine powder in liquid nitrogen with a mortar and pestle. Ten ml of extraction buffer (100 mM Tris-HCl, pH 8.0; 50 mM EDTA, pH 8.0; 500 mM NaCl; 1.25 % SDS; 10 mM B-mercaptoethanol; 4 mM Spermidine; 1 mM Spermine; 1 mM Phenylmethylsulfonyl fluoride (PMSF)), maintained at 65 °C, was added to each isolate and incubated in a water bath at 65 ° C for 60 min with frequent mixing. The aqueous phase was collected after centrifugation and phenol/chloroform (1:1 phenol to chloroform) extractions were performed until the interface was completely clean. Precipitation of the nucleic acids was done using 3 M NaAc (0.1 v/v) and isopropanol (0,6 v/v) followed by overnight incubation at – 20 °C. After centrifugation, to harvest the nucleic acids, and washing with 70 % EtOH, the pellet was re-suspended in 200 μl sterile water. One μl of RNaseA (10 mg / ml) was added to the re-suspended sample and left at 37 °C overnight to degrade all RNA in the sample. All DNA extracts were quantified by fluorometry and adjusted to a final concentration of 30 ng/μl.

DNA sequence comparisons

Polymerase Chain Reaction. The ITS1 and ITS2, as well as the 5.8S gene of the ribosomal RNA operon were amplified using the polymerase chain reaction (PCR) (Saiki *et al.*, 1988). Amplifications were performed using ITS primers ITS 1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5'-GCTGCGTTCTTCATCGATGC-3') (White *et al.*, 1990). The PCR reaction mixture (100 μl) included 2 units of *Taq* DNA Polymerase (Boehringer Mannheim, Germany), 10 x reaction buffer (Boehringer), 4.5 mM $MgCl_2$ (Boehringer), 250 mM dNTPs and 0.5 μl of each primer (100 pM). Amplification reactions were done in a Hybaid Omnigene Temperature Cycler (Hybaid, Middlesex, U.K.). Denaturation was performed at 95 °C for 30 sec, followed by primer annealing at 55 °C for 45 sec. Chain elongation took place at 72 °C for 2 min. These steps were repeated for 35 cycles. Final chain elongation took place at

72 °C for 5 min. PCR products were electrophoresed in 1.5 % agarose gels, stained with ethidium bromide, and visualised using UV light. Amplification reactions were done in duplicate.

DNA sequencing and data analysis. All PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN, Germany). PCR products were sequenced in both directions using the Big Dye Cycle Sequencing kit with *Amplitaq®* DNA Polymerase, FS (Perkin-Elmer, Warrington, UK) on a ABI PRISM^{TM} 377 DNA Autosequencer (Perkin-Elmer). Primers ITS 1 and ITS 4 were used in the sequence reaction. The nucleotide sequences were manually aligned. Phylogenetic relationships among species were determined using the Heuristic search option in PAUP (Phylogenetic Analysis Using Parsimony), with gaps treated as missing data (Swofford, 1985). Confidence intervals were determined using DNA BOOTSTRAP analysis (Bootstrap confidence intervals on DNA parsimony) (1000 replicates) (Felsenstein, 1993).

Amplified Fragment Length Polymorphism (AFLP) analysis

Restriction, ligation, and amplification were performed as described by Vos *et al.* (1995). Genomic DNA (500 ng) from each sample was incubated for 16 hours at 25 ° C in a solution containing 10 U/µl *Cfo*1 and 5 U/µl *Mse*1 (Boehringer Mannheim, USA), 1 x restriction-ligation buffer (10 mM Tris-HAc, pH7.5; 10 mM MgAc; 50 mM KAc; 5 mM DTT), 1 U/µl T4 DNA Ligase, 50 pmol/µl *Cfo*1-adaptors, 50 pmol/µl *Mse*1-adaptors (Table 2), 10 mg/ml RNaseA and 100 mM spermidine. The final sample volume was increased to 100 µl with sterile water. After ligation the reaction mixture was diluted 10 - fold with T.1E buffer (10 mM TRIS-HCl, pH8.0; 10 mM EDTA) and stored at –20 °C. These ligated fragments served as templates in the amplification reaction.

A pre-selective PCR (+ 1 reaction) amplification reaction was performed in 20 µl PCR reaction mix containing 5 µl of the diluted DNA, 0.5 µl of each +1 primer (10 pmol/µl) (Table 2), 100 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$, 500 mM KCl, 25 mM $MgCl_2$, 5U/µl *Taq* DNA Polymerase (Boehringer Mannheim, USA) and 250 µM of dNTP. Initial denaturation was performed at 94 °C for 1min, followed by 30 cycles of 30 sec at 94 °C, 60 sec at 56 °C (primer annealing) and 60 sec at 72 °C (final chain elongation). The amplification PCR products were diluted 10-fold in 1 x T.1E buffer and used as templates in the second amplification.

The second amplification (+ 3 reaction) was performed using primers derived from the first set of primers, with additional nucleotides at the 3' end (Table 2). The *Cfo*1 primer was HEX fluorochrome-labeled and the *Mse*1 primer was FAM fluorochrome-labeled (AmpFlSTR Profiler PCR Kit, Perkin-Elmer, Norwalk, Conn.). Fluorescent AFLPs were amplified under the following conditions: 0.2 µl of the HEX fluorochrome-labeled *Cfo*1 + 3 primer (50 pmol/µl) was added to a 20-µl PCR reaction solution containing 5 µl of the diluted + 1 pre-amplification mix, 0.4µl of the FAM fluorochrome-labeled *Mse*1 + 3 primer, 5 U/µl *Taq* DNA Polymerase (Boehringer Mannheim, USA), 10 x Buffer (100 mM Tris-HCl, pH 8.0; 15 mM $MgCl_2$; 500 mM KCl, pH8.3), 25 mM $MgCl_2$ and 250 µM of dNTP. The following temperature profile was used: 12 cycles of 30 sec at 94 °C, 30 sec at 65 °C, 60 sec at 72 °C, where the annealing temperature was subsequently reduced by 0.7 °C after each cycle. The amplification was continued for 22 cycles of 30 sec at 94 °C, 30 sec at 56 °C, and 60 sec at 72 °C. All amplification reactions were performed using a Hybaid Omnigene thermocycler (Hybaid, Middlesex, UK).

Electrophoresis and visualisation of AFLP PCR products. PCR products (1.5µl) were combined with 3 fmol TAMRA fluorescent-labeled GeneScan 500 internal size standard (ABI), 1.6 µl formamide, and 0.3 µl 25 mM EDTA (pH 8.0) containing 50 mg/ml blue dextran. This mixture was heat denatured for 3 min at 95 °C, and immediately cooled on ice. Samples were loaded on a 5 % denaturing polyacrylamide gel in 1 x TBE (Tris-borate EDTA, pH 8.0) and electrophoresed for 2.5 hours at 1680 watts using the GS 36A-2400 run module. Data were processed by GeneScan Analysis software (version 2.02) to produce a gel image. PCR fragments for individual samples were automatically sized by the GeneScan software using a comparison of the mobility of the internal lane size standard to that of the sample fragment.

AFLP data analysis. Each polymorphic AFLP fragment was treated as a unit character and scored as present (1) or absent (0) across all isolates. The experiments were repeated, and only reproducible bands were scored. The index of genetic similarities was calculated according following the Nei & Li (1979) definition of similarity. Unweighted pair-group mean arithmetic (UPGMA) was used for cluster analysis of the pairwise similarity matrix that generated a dendrogram representing the genetic similarity among fungal isolates. UPGMA analysis was carried out using NCSS97 (Visual Components, UK).

RESULTS

Morphological comparisons

Colony and conidial morphology. Conidia of the Thailand *Coniothyrium* isolates investigated were 4.1 – 5.2 μm long and 2.5 – 3.5 μm wide (Table 3) whereas, conidia of *C. zuluense* from South Africa were 3.5 – 5.6 μm long and 2.1 – 3.6 μm wide (Van Zyl *et al.*, 1997). All conidia used in this study were thick walled, smooth and broadly ellipsoidal. The apices were obtuse and the bases sub-truncate too bluntly rounded.

The colour of the *Coniothyrium* isolates from Thailand were all a grayish olive ($21^{''''}$) colour (Table 3). South African isolates of *C. zuluense* vary from an olive grey ($V23^{''''}b$), isabella ($19^{''}i$), greenish glaucous ($33^{'''}f$) to a grayish olive ($21^{''''}$) colour (Table 3) (Van Zyl *et al.*, 1997). All South African and Thailand isolates viewed from below were black or rust coloured with white margins.

Growth studies. In growth studies, isolates of the *Coniothyrium* spp. from Thailand and *C. zuluense* had growth optima at 30 °C (Table 4). Thailand isolates failed to grow at 10 °C and 15 °C. *Coniothyrium zuluense* isolates were, however, able to grow at 15 °C, although very slowly.

Pathogenicity tests. Results showed that none of the Thailand isolates, screened for pathogenicity, were able to cause disease (Table 5). Results were consistent between repetitions. Significant differences in lesion development were, however, evident among *C. zuluense* isolates from South Africa. Those isolates previously defined as having high levels of pathogenicity produced significantly larger lesions ($P = 0.05$) than those isolates having intermediate and low levels of pathogenicity (Table 5). These isolates also differed significantly among each other in their capacity to cause disease. There was no significant difference ($P = 0.05$) between lesion lengths for the isolates of intermediate pathogenicity. Significant differences in lesion development were, however, observed for isolates previously described as having intermediate and low levels of pathogenicity. Isolates previously defined as being non-pathogenic produced no lesions in this study. No symptoms developed on trees inoculated as controls (Table 5). The inoculated pathogen was consistently re-isolated from the lesions on inoculated trees and never from control trees.

Molecular comparisons

DNA sequence comparisons. Sequence data were manually aligned by inserting gaps (Fig. 1). Alignment of the DNA sequence data within the ITS 1 and ITS 2 regions proved to be variable between all the different species studied. A Heuristic search from the aligned DNA sequence data (573 characters) of the ITS 1, 5.8S, and ITS 2 regions of the rRNA operon, produced one most parsimonious tree (Fig. 2) of 458 steps (CI = 0.930, HI = 0.070, RI = 0.934). A thousand replicate bootstrap analyses were done to ascertain the confidence intervals of the branch points of the tree. Phylogenetic analysis was done using midpoint rooting. Three major clades emerged. All the *Coniothyrium* isolates associated with stem cankers on *Eucalyptus* grouped together in a single major clade. This clade appeared clearly distant from isolates of other *Coniothyrium* species included for comparative purposes. Bootstrap analysis showed that the branch point separating *Eucalyptus* stem canker isolates from the other *Coniothyrium* species, had a confidence interval of 100 %.

Isolates representing *C. zuluense* and the *Coniothyrium* sp. from Thailand could be sub-divided into two distinct sub-groups. The one group included *C. zuluense* isolates from South Africa (91 % bootstrap value). The second sub-clade included the *Coniothyrium* isolates from Thailand (99 % bootstrap value). *Coniothyrium palmarum* formed a single, strongly supported (100 % bootstrap value) clade. The three remaining *Coniothyrium* spp. grouped together in a third clade (99 % bootstrap value). This clade was sub-divided into two distinct sub-groups. *Massarina corni* and *C. ovatum* grouped together (100 % bootstrap value) with *C. fuckelii*, but in a separate though strongly supported (99 % bootstrap value) sub-group.

AFLP analysis. A total of 177 scorable AFLP markers were included in this study. Of these, 9 (5.1 %) were monomorphic for all the species in the study while 168 (94.9 %) displayed informative polymorphism's. Fragment size ranged from 40 to 499 bp. A similarity matrix based on the similarity coefficient of Nei & Li (1979) was produced using all 177 (polymorphic and monomorphic) fragments (Table 6). Pairwise genetic similarity revealed an average similarity of 82.3 % (17.7 % dissimilar) among isolates of the *Coniothyrium* sp. from Thailand and 77.6 % similarity (22.4 % dissimilar) among the *C. zuluense* isolates. The average percentage similarity shared between isolates of *C. zuluense* from South Africa and those of the *Coniothyrium* sp. from Thailand was 76.1 % (23.9 % dissimilar). Genetic similarity ranged from 61.7 % to 86.2 %. UPGMA analysis of the similarity matrix yielded a dendrogram (Fig. 3) which grouped the South African and Thailand isolates together in a single clade.

UPGMA analysis of the similarity matrix grouped all of the *Coniothyrium* isolates associated with stem cankers on *Eucalyptus* separate from the four species included for comparative purposes. This is in agreement with results obtained for sequence data. Average percentage similarity between the *Eucalyptus* stem canker pathogens and the other *Coniothyrium* spp. was 11.2 % for *C. fuckelii*, 18,2 % for *C. palmarum*, 37.3 % for *M. corni* and 43.4 % for *C. ovatum*.

DISCUSSION

In this study, morphological comparisons, pathogenicity tests and molecular comparisons strongly support the view that the *Coniothyrium* sp. from Thailand is the same as *C. zuluense*, which causes *Eucalyptus* stem canker in South Africa. This is also the first report of *C. zuluense* outside South Africa.

Based on morphological comparisons, the *Coniothyrium* sp. from Thailand is virtually indistinguishable to *C. zuluense* from South Africa. Conidial measurements of the Thailand isolates were within size ranges published for *C. zuluense* (Van Zyl *et al.*, 1997; Wingfield *et al.*, 1997). Thailand isolates, however, differed from *C. zuluense* in that they were a grayish olive colour and failed to grow at 15 °C. This was in contrast to characteristics published for *C. zuluense* from South Africa (Van Zyl *et al.*, 1997). Isolates of the latter fungus varied between an olive grey, greenish glaucous, isabella or grayish olive colour and were able to grow at temperatures ranging from 15 to 35 °C (Van Zyl *et al.*, 1997). Optimal growth temperature for both *Coniothyrium* species was, 30 °C. Although some differences in colony colour and temperature requirement for growth were observed, we do not believe that these are sufficient to separate the isolates, from the two regions, into different taxa.

Pathogenicity tests on young trees in the greenhouse showed that the Thailand *Coniothyrium* sp. was not pathogenic to *E. grandis*. Its role in tree disease in Thailand is, therefore, uncertain. However, only a small number of isolates from Thailand were available for study. Pathogenicity studies conducted in South Africa during 1997 (Van *Zyl et al.*, 1997), showed that only 22 % of 344 *C. zuluense* isolates collected, were able to cause lesions on a susceptible *E. grandis* clone (Van *Zyl et al.*, 1997). Most of these isolates (78 %) collected from severely infected trees in the field were, thus, not able to cause disease. In the future, we would, however, hope to collect a sufficient number of isolates from Thailand and to conduct pathogenicity tests on established trees in that country. Such tests will expand our understanding of the role that *C. zuluense* has in *Eucalyptus* disease in Thailand.

Phylogenetic analysis of sequence data from the ribosomal RNA operon, confirmed that the Thailand isolates and *C. zuluense* are the same. The ribosomal RNA operon is well known to be an extremely useful source of genetic data for taxonomic comparisons at species level (Blanz & Unseld, 1986; White *et al.*, 1990; Kurtzman, 1992; Wingfield & Wingfield, 1993; Mitchell *et al.*, 1995; Wingfield *et al.*, 1996a, b; Witthuhn *et al.*, 1998). Data analysis of the present study showed that the South African and Thailand isolates produced a single clade, separate from the other related *Coniothyrium* spp. used for comparative purposes. However, *Coniothyrium* isolates from the two regions formed distinct sub-groups within this major clade. This might suggest that *C. zuluense* in Thailand is in the process of diverging away from *C. zuluense* in South Africa, due to environmental influences.

Genetic similarity between Thailand isolates and *C. zuluense*, as determined by AFLP analysis, confirmed that they are the same. AFLP analysis is a novel PCR fingerprinting technique which selectively amplifies DNA fragments, corresponding to unique positions on the genome (Zabeau & Vos, 1993; Vos *et al.*, 1995). This technique has previously been shown to be extremely useful in determining genetic similarities between different fungal populations (Majer *et al.*, 1996; Majer *et al.*, 1998; Pongam *et al.*, 1999). Results of the current study showed that percentage genetic similarity values between South African and Thailand genotypes, ranged from 61.7 % to 86.2 %, with an average similarity of 76.1 %. Data, thus, suggest that genetic differences between the Thailand isolates and *C. zuluense* are evident, but that they clearly share a common origin.

This report represents the first record of *C. zuluense* outside South Africa. It is, however, intriguing to consider the possible origin of *C. zuluense* on *Eucalyptus* species. Wingfield *et al.* (1997) suggested that the fungus is native to South Africa. This hypothesis was based on the fact that the disease is not known elsewhere in the world, especially in Australia where *Eucalyptus* is native. They suggested that the fungus might have originated from native Myrtaceae, and had developed the capacity to infect *Eucalyptus*. This is similar to the situation with *Eucalyptus* rust, caused by *Puccinia psidii* Winter, which is not known in Australia, but is common and damaging in South and Central America where it apparently originated from native Myrtaceae (Coutinho *et al.*, 1998). Results of the present study indicate that *C. zuluense* occurs elsewhere in the world. Surveys to find possible alternative hosts, both native and introduced must, therefore, be conducted in sub-tropical and tropical *Eucalyptus* growing areas.

South African *C. zuluense* isolates display considerable variation in colony colour and virulence (Van Zyl *et al.*, 1997). Large variations in virulence are widely associated with diverse pathogen populations that are influenced by a number of factors, including the capacity for sexual reproduction in the fungus. Organisms capable of sexual reproduction have higher genetic diversity than those reproducing only asexually (McDonald & McDermott, 1993; Wolf & McDermott, 1994; Milgroom, 1996). *Coniothyrium zuluense* is known to reproduce only asexually (Wingfield *et al.*, 1997), thus, the large variation in virulence was not expected. One hypothesis has been that more than one species of *Coniothyrium* might be responsible for disease in South Africa. Sequencing results in this study have, however, shown that only a single, yet highly variable species of *Coniothyrium*, is responsible for cankers on *Eucalyptus* species in South Africa.

The discovery of *C. zuluense* outside South Africa is important and shows that the fungus is more widespread than previously believed (Wingfield *et al.*, 1997). Surveys for this disease in other subtropical and tropical *Eucalyptus* growing areas of the world are required to provide information regarding its origin. Furthermore, pathogenicity tests with a wider range of isolates should be undertaken in Thailand. Such tests will allow us to compare the susceptibility of different *Eucalyptus* spp., clones and hybrids. The ultimate aim will be to avoid the disease, which could be achieved through selection of disease resistant planting stock.

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Table 1. List of fungal isolates used in DNA sequence comparisons.

Species	Isolate no.[a]	Origin	Collector
Coniothyrium zuluense	CRY 957	Zululand, KZN, South Africa[b]	L.M. van Zyl
“	CRY 964	“	“
“	CRY 1000	“	“
“	CRY 1017	“	“
“	CRY 1023	“	“
“	CRY 1047	“	“
“	CRY 1049	“	“
“	CRY 1056	“	“
“	CRY 1057	“	“
C. ovatum	CO 1	Western Cape, South Africa	L.M. van Zyl & M.J. Wingfield
Coniothyrium sp.	CMW 5231	Thailand	“
“	CMW 5232	“	“
“	CMW 5233	“	“
“	CMW 5234	“	“
“	CMW 5235	“	“
“	CMW 5236	“	“
C. palmarum	CBS 758.73	Israel	Y. Pinkas
C. fuckelii	CBS 132.26	Netherlands	F. Laibach
Massarina corni	CBS 496.64	-	-

[a] Isolates are maintained in the culture collection of the Tree Pathology Co-operative Programme, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria. CBS refers to the Centraal Bureau voor Schimmelcultures, Baarn, Netherlands.

[b] KZN refers to the KwaZulu-Natal Province in South Africa.

Table 2. Sequences of primers and adaptors used for AFLP analysis.

Name	**Enzyme**	**Type**	**Sequence**
CA	*Cfo*1	Adaptor	5'-GACGATGAGTCCTGAACG-3' 3'-TACTCAGGACTT-5'
MA	*Mse*1	Adaptor	5'-GACGATGAGTCCTGAG-3' 3'-TACTCAGGACTCAT-5'
C-T	*Cfo*1	Primer + 1	5'-GATGAGTCCTGAACGCT-3'
C-TCC	*Cfo*1	Primer + 3	5'-GATGAGTCCTGAACGCTCC-3'
C-TGG	*Cfo*1	Primer + 3	5'-GATGAGTCCTGAACGCTGG-3'
M-A	*Mse*1	Primer + 1	5'-GATGAGTCCTGAGTAA-3'
M-AGT	*Mse*1	Primer + 3	5'-GATGAGTCCTGAGTAAGT-3'
M-AGC	*Mse*1	Primer + 3	5'-GATGAGTCCTGAGTAAGC-3'

Table 3. Comparison of important morphological characteristics of *Coniothyrium zuluense* and a *Coniothyrium* sp. from Thailand

		Morphological characteristics			
Fungus	**Isolate no.**	**Colony colour[a]**	**Conidium length[b]**	**Conidium width**	**Source of data**
C. zuluense	CRY 1017	21^{IIII}	4.6 – 4.7 (4.5)	2.6 – 3.1 (2.8)	Van Zyl *et al.*, 1997
"	CRY 1023	33^{IIIf}	4.5 – 4.7 (4.5)	2.6 – 3.6 (2.8)	"
"	CRY 1056	V23^{IIIIb}	4.5 – 4.6 (4.5)	2.5 – 2.9 (2.7)	"
"	CRY 1047	"	4.2 – 4.5 (4.3)	2.5 – 2.8 (2.6)	"
"	CRY 1049	19^{II}I	4.1 – 4.3 (4.2)	2.7 – 2.8 (2.5)	"
"	CRY 957	V23^{IIIIb}	3.5 – 4.5 (4.1)	2.5 – 3.6 (2.8)	"
"	CRY 964	33^{IIIf}	3.5 – 4.2 (4.0)	2.5 – 2.9 (2.6)	"
"	CRY 1000	21^{IIII}	4.3 – 5.6 (4.8)	2.1 – 3.2 (2.8)	"
"	CRY 1057	33^{IIIf}	4.3 – 5.2 (4.8)	2.4 – 3.5 (2.7)	"
Coniothyrium sp.	CMW 5231	21^{IIII}	4.2 – 4.5 (4.3)	2.5 – 2.8 (2.5)	This study
"	CMW 5232	"	4.1 – 4.4 (4.3)	2.5 – 2.8 (2.5)	"
"	CMW 5233	"	4.4 – 4.7 (4.5)	2.6 – 3.2 (2.8)	"
"	CMW 5234	"	4.4 – 4.8 (4.5)	2.5 – 3.5 (2.8)	"
"	CMW 5235	"	4.5 – 5.2 (4.8)	2.6 – 3.1 (2.8)	"
"	CMW 5236	"	4.4 – 4.8 (4.5)	2.5 – 2.8 (2.8)	"

[a] Colour classes are those published by Rayner (1970). Colour codes represent the following: olive grey (V23^{IIIIb}), greenish glaucous (33^{IIIf}), isabella (19^{II}I) and grayish olive (21^{IIII}).

[b] All measurements are listed in μm. Values are the mean of 30 measurements.

Table 4. Growth of *Coniothyrium zuluense* isolates compared with those of the *Coniothyrium* sp. from Thailand.

		Growth Studies[a] (average colony diameter (mm))					
		Temperature (°C)					
Fungus	**Isolate number**	**10**	**15**	**20**	**25**	**30**	**35**
Coniothyrium zuluense	CRY 1017	0 a[bc]	7.5 b	16.5 a	25.4 a	39.4 a	16.2 a
“	CRY 1023	0 a	14.2 b	26.4 bc	42.3 bc	66.8 cd	35.6 c
“	CRY 1056	0 a	11.7 b	23.8 ab	36.5 b	59.6 bc	27.9 bc
“	CRY 1047	0 a	11.8 b	24.1 ab	34.5 ab	53.8 b	26.5 b
“	CRY 1049	0 a	15.2 bc	30.9 c	50.5 d	75.4 e	40.1 d
“	CRY 957	0 a	15.4 bc	30.2 c	53.6 d	80.1 e	40.1 d
“	CRY 964	0 a	8.9 b	17.5 a	27.8 a	43.6 a	23.5 b
“	CRY 1000	0 a	9.8 b	20.1 a	32.1 ab	50.6 b	24.5 b
“	CRY 1057	0 a	10.4 b	19.8 a	28.4 a	42.1 a	19.8 ab
Coniothyrium sp.	CMW 5231	0 a	0 a	27.4 bc	38.7 bc	62.4 c	15.8 a
“	CMW 5232	0 a	0 a	23.5 ab	35.8 b	58.4 bc	14.4 a
“	CMW 5233	0 a	0 a	28.4 bc	41.6 c	66.5 cd	16.9 a
“	CMW 5234	0 a	0 a	23.3 ab	40.2 c	58.9 bc	14.8 a
“	CMW 5235	0 a	0 a	24.0 ab	41.5 c	62.4 c	15.7 a
“	CMW 5236	0 a	0 a	24.2 ab	38.2 bc	59.9 bc	14.8 a

[a] Growth was measured after incubating cultures for 30 days in the darkness.

[b] Each value represents an average of six measurements.

[c] Each value with a different letter differs significantly at $P = 0.05$ from the others for that specific temperature range (CV = 13.45 %).

Table 5. Lesion lengths associated with inoculations using *Coniothyrium* isolates from South Africa and Thailand.

Fungus	**Isolate number**	**Pathogenicity**[a,b] **Lesion length (mm)**
Coniothyrium zuluense	CRY 1017	23.6 b[c]
"	CRY 1023	56.4 e
"	CRY 1056	10 a
"	CRY 1047	37.6 d
"	CRY 1049	10 a
"	CRY 957	17.8 b
"	CRY 964	10 a
"	CRY 1000	27.8 c
"	CRY 1057	23.1 b
Coniothyrium sp.	CMW 5231	10 a
"	CMW 5232	10 a
"	CMW 5233	10 a
"	CMW 5234	10 a
"	CMW 5235	10 a
"	CMW 5236	10 a
	CONTROL	10 a

[a] Six-month-old trees of a susceptible *Eucalyptus grandis* clone (ZG 14) were inoculated under glass-house conditions.

[b] Each value is an average of 20 measurements. CV = 11.3 %

[c] Values followed by different letters differ significantly at $P = 0.05$.

Table 6. Pairwise distance matrix based on the F-statistic of Nei & Li (1979) converted to a percentage, i.e. %S*ij* = 1-*Sij*

CMW 5232	100																		
CMW 5233	91.27	100																	
CMW 5236	74.96	68.75	100																
CMW 5235	82.17	74.96	83.82	100															
CMW 5234	83.82	78.7	88.43	86.94	100														
CMW 5231	91.96	86.18	71.95	77.79	81.33	100													
CRY 1017	82.17	82.17	72.97	82.17	78.7	79.59	100												
CRY 1023	80.47	76.86	70.91	78.7	76.86	75.92	85.41	100											
CRY 1056	71.95	71.95	62.93	73.98	69.84	70.91	77.79	71.95	100										
CRY 1049	79.59	69.84	60.4	69.84	71.95	76.86	73.98	69.84	61.68	100									
CRY 1047	82.17	80.47	68.75	72.97	72.97	77.79	88.43	80.47	71.95	83	100								
CRY 1000	79.59	75.92	60.4	81.33	65.34	76.86	89.16	81.33	78.7	66.5	83	100							
CRY 1057	85.41	76.86	78.7	82.17	85.41	75.92	86.94	85.41	71.95	67.64	80.47	77.79	100						
CRY 964	76.86	72.97	64.15	70.91	72.97	79.59	78.7	76.86	67.64	79.59	82.17	69.84	74.96	100					
CRY 957	77.79	69.84	71.95	79.59	79.59	78.7	81.33	83	68.75	68.75	73.98	78.7	79.59	75.92	100				
M .corni	38.08	29.49	19.26	48.82	29.49	36.06	38.08	41.91	47.17	27.11	24.62	53.47	33.96	41.91	43.72	100			
C.ovatum	48.82	38.08	24.62	51.96	38.08	47.17	45.47	41.91	47.17	40.03	33.96	56.35	38.08	45.47	43.72	95.23	100		
C.fuckelii	24.62	-21.27	-1.15	19.26	6.57	10.04	13.29	6.57	10.04	10.04	29.49	16.36	13.29	-1.15	16.36	-506	-506	100	
C.palmarum	2.85	-15.44	-5.48	2.85	10.04	6.57	-5.48	2.85	-56.2	-21.3	-27.9	-21.3	2.85	-27.9	-10.2	-506	-431	-506	100

Fig. 1. Aligned sequences of the ITS 1 and ITS 2 regions, as well as the conserved 5.8S RNA gene. CMW numbers represent the Thailand *Coniothyrium* isolates while CRY numbers refer to *Coniothyrium zuluense* isolates from South Africa. Sequence data generated for *C. palmarum*, *C. fuckelii* and *Massarina corni* were included for comparative purposes. N indicates unknown bases and a dash indicates a gap in the sequence inserted to achieve the alignment.

```
CRY 1056        CTCCCAACCC CCCATCGAA- --TTTTCCAA ACCATGTTGC GCCTCGGGGG -CGACCCGGC CATCGCGC-C GGTGGCCCCC GGTGGACCCC TCCAACTCTG
CRY 1023        CTCCCAACCC CCCATCGAA- --TTTTCCAA ACCATGTTGC GCCTCGGGGG -CGACCCGGC CATCGCGC-C GGTGGCCCCC GGTGGACCCC TCCAACTCTG
CRY 1057        CTCCCAACCC CCCATCGAA- --TTTTCCAA ACCATGTTGC GCCTCGGGGG -CGACCCGGC CATCGCGC-C GGTGGCCCCC GGTGGACCCC TCCAACTCTG
CRY 1000        CTCCCAACCC CCCATCGAA- --TTTTCCAA ACCATGTTGC GCCTCGGGGG -CGACCCGGC CATCGCGC-C GGTGGCCCCC GGTGGACCCC TCCAACTCTG
CRY 1047        CTCCCAACCC CCCATCGAA- --TTTTCCAA ACCATGTTGC GCCTCGGGGG -CGACCCGGC CATCGCGC-C GGTGGCCCCC GGTGGACCCC TCCAACTCTG
CRY 1049        CTCCCAACCC CCCATCGAA- --TTTTCCAA ACCATGTTGC GCCTCGGGGG -CGACCCGGC CATCGCGC-C GGTGGCCCCC GGTGGACCCC TCCAACTCTG
CRY 957         CTCCCAACCC CCCATCGAA- --TTTTCCAA ACCATGTTGC GCCTCGGGGG -CGACCCGGC CATCGCGC-C GGTGGCCCCC GGTGGACCCC TCCAACTCTG
CRY 964         CTCCCAACCC CCCATCGAA- --TTTTCCAA ACCATGTTGC GCCTCGGGGG -CGACCCGGC CATCGCGC-C GGTGGCCCCC GGTGGACCCC TCCAACTCTG
CMW 5231        CTCCCAACCC CCCAT-G--- --TTTTCC-A A-CCATGTTG -CCTCGGGGG -CGACCCGGC CATCGCGGGC CGGGGCCCCC GGTGGACCCC TCCAACTCTG
CMW 5234        CTCCCAACCC CCCAT-G--- --TTTTCC-A A-CCATGTTG -CCTCGGGGG -CGACCCGGC CATCGCGGGC CGGGGCCCCC GGTGGACCCC TCCAACTCTG
CMW 5235        CTCCCAACCC CCCAT-G--- --TTTTCC-A A-CCATGTTG -CCTCGGGGG -CGACCCGGC CATCGCGGGC CGGGGCCCCC GGTGGACCCC TCCAACTCTG
CMW 5236        CTCCCAACCC CCCAT-G--- --TTTTCC-A A-CCATGTTG -CCTCGGGGG -CGACCCGGC CATCGCGGGC CGGGGCCCCC GGTGGACCCC TCCAACTCTG
CMW 5232        CTCCCAACCC CCCAT-G--- --TTTTCC-A A-CCATGTTG -CCTCGGGGG -CGACCCGGC CATCGCGGGC CGGGGCCCCC GGTGGACCCC TCCAACTCTG
CMW 5233        CTCCCAACCC CCCAT-G--- --TTTTCC-A A-CCATGTTG -CCTCGGGGG -CGACCCGGC CATCGCGGGC CGGGGCCCCC GGTGGACCCC TCCAACTCTG
C. ovatum       GCGGCGGCCT TAAATCGCGC CAAATGGC-A CCCTTTGTTT ACGGAGTACC G-TTCGTTTC C-T------- ---------C GGTGGG---- ----------
M. corni        GCGGCGGCCT TAAATCGCGC CAAATGGC-A CCCTTTGTTT ACGGAGTACC G-TTCGTTTC C-T------- ---------C GGTGGG---- ----------
C. palmarum     TGACCCGCCC TGTCT-G--- --AATATATA CCCC-TGTTT ATTGCGTACT ACTT-GTTTC CTT------- ---------- GGTGGG---- ----------
C. fuckelii     TCACGCGCCG CAT-TCCTGC ---AT-CCT- ----TTTTTT ACGAAGCACC -TTTCGTTTC CTTCGGCGGG CAACCTGCC- GCTGGAA-CT T--AACAAAA


                       110        120        130        140        150        160        170        180        190        200
CRY 1017        CATCT-TTGC GTCT-GAG-- -T------CA ---CAAAAT- ---------- --------TA AA-T------ ------CAA- --TCAAAACT TTCAACAACG
CRY 1056        CATCT-TTGC GTCT-GAG-- -T------CA ---CAAAAT- ---------- --------TA AA-T------ ------CAA- --TCAAAACT TTCAACAACG
CRY 1023        CATCT-TTGC GTCT-GAG-- -T------CA ---CAAAAT- ---------- --------TA AA-T------ ------CAA- --TCAAAACT TTCAACAACG
CRY 1057        CATCT-TTGC GTCT-GAG-- -T------CA ---CAAAAT- ---------- --------TA AA-T------ ------CAA- --TCAAAACT TTCAACAACG
CRY 1000        CATCT-TTGC GTCT-GAG-- -T------CA ---CAAAAT- ---------- --------TA AA-T------ ------CAA- --TCAAAACT TTCAACAACG
CRY 1047        CATCT-TTGC GTCT-GAG-- -T------CA ---CAAAAT- ---------- --------TA AA-T------ ------CAA- --TCAAAACT TTCAACAACG
CRY 1049        CATCT-TTGC GTCT-GAG-- -T------CA ---CAAAAT- ---------- --------TA AA-T------ ------CAA- --TCAAAACT TTCAACAACG
CRY 957         CATCT-TTGC GTCT-GAG-- -T------CA ---CAAAAT- ---------- --------TA AA-T------ ------CAA- --TCAAAACT TTCAACAACG
CRY 964         CATCT-TTGC GTCT-GAG-- -T------CA ---CAAAAT- ---------- --------TA AA-T------ ------CAA- --TCAAAACT TTCAACAACG
CMW 5231        CATCTCTTGC GTCT-GAG-- -T------CA ---CAAAA-- ---------- --------TA AAAT------ ------CAA- --TCAAAACT TTCAACAACG
CMW 5234        CATCTCTTGC GTCT-GAG-- -T------CA ---CAAAA-- ---------- --------TA AAAT------ ------CAA- --TCAAAACT TTCAACAACG
CMW 5235        CATCTCTTGC GTCT-GAG-- -T------CA ---CAAAA-- ---------- --------TA AAAT------ ------CAA- --TCAAAACT TTCAACAACG
CMW 5236        CATCTCTTGC GTCT-GAG-- -T------CA ---CAAAA-- ---------- --------TA AAAT------ ------CAA- --TCAAAACT TTCAACAACG
CMW 5232        CATCTCTTGC GTCT-GAG-- -T------CA ---CAAAA-- ---------- --------TA AAAT------ ------CAA- --TCAAAACT TTCAACAACG
CMW 5233        CATCTCTTGC GTCT-GAG-- -T------CA ---CAAAA-- ---------- --------TA AAAT------ ------CAA- --TCAAAACT TTCAACAACG
C. ovatum       -----CTTGC CT-----GCC ATGACCCCCA ACCCAAAACC C-TTATGTAG -TAGCCAGTA ACCTTCAGTA AGTAAACAAA A-TCAAAACT TTCAACAACG
M. corni        -----CTTGC CT-----GCC ATGAGGACCA ACCCAAAACC C-TT-TGTAG -TAGC-AGTA ACCTTCAGTA AGAAACAATA --TCAAAACT TTCAACAACG
C. palmarum     -----CTTGC C-C----GCC AAAAGGACAC CTATAAAACC TCTTGTAATT ---GC-AGTC AGCGTCAGAA AAACTTAATA ATTCAAAACT TTCAACAACG
C. fuckelii     CCTTTTTTGC ATCTAGCAT- -TACCTGTTC TGATAAAAAA CAATCGTTA- ---------- ---------- ---------- ---CAAAACT TTCAACAACG
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CRY 1056         GATCTCTTGG TTCTGGCATC GATGAAGAAC GCAGCGAAAT GCGATAAGTA ATGTGAATTG CAGAATTCAG TGAATCATCG AATCTTTGAA CGCACATTGC
CRY 1023         GATCTCTTGG TTCTGGCATC GATGAAGAAC GCAGCGAAAT GCGATAAGTA ATGTGAATTG CAGAATTCAG TGAATCATCG AATCTTTGAA CGCACATTGC
CRY 1057         GATCTCTTGG TTCTGGCATC GATGAAGAAC GCAGCGAAAT GCGATAAGTA ATGTGAATTG CAGAATTCAG TGAATCATCG AATCTTTGAA CGCACATTGC
CRY 1000         GATCTCTTGG TTCTGGCATC GATGAAGAAC GCAGCGAAAT GCGATAAGTA ATGTGAATTG CAGAATTCAG TGAATCATCG AATCTTTGAA CGCACATTGC
CRY 1047         GATCTCTTGG TTCTGGCATC GATGAAGAAC GCAGCGAAAT GCGATAAGTA ATGTGAATTG CAGAATTCAG TGAATCATCG AATCTTTGAA CGCACATTGC
CRY 1049         GATCTCTTGG TTCTGGCATC GATGAAGAAC GCAGCGAAAT GCGATAAGTA ATGTGAATTG CAGAATTCAG TGAATCATCG AATCTTTGAA CGCACATTGC
CRY 957          GATCTCTTGG TTCTGGCATC GATGAAGAAC GCAGCGAAAT GCGATAAGTA ATGTGAATTG CAGAATTCAG TGAATCATCG AATCTTTGAA CGCACATTGC
CRY 964          GATCTCTTGG TTCTGGCATC GATGAAGAAC GCAGCGAAAT GCGATAAGTA ATGTGAATTG CAGAATTCAG TGAATCATCG AATCTTTGAA CGCACATTGC
CMW 5231         GATCTCTTGG TTCTGGCATC GATGAAGAAC GCAGCGAAAT GCGATAAGTA ATGTGAATTG CAGAATTCAG TGAATCATCG AATCTTTGAA CGCACATTGC
CMW 5234         GATCTCTTGG TTCTGGCATC GATGAAGAAC GCAGCGAAAT GCGATAAGTA ATGTGAATTG CAGAATTCAG TGAATCATCG AATCTTTGAA CGCACATTGC
CMW 5235         GATCTCTTGG TTCTGGCATC GATGAAGAAC GCAGCGAAAT GCGATAAGTA ATGTGAATTG CAGAATTCAG TGAATCATCG AATCTTTGAA CGCACATTGC
CMW 5236         GATCTCTTGG TTCTGGCATC GATGAAGAAC GCAGCGAAAT GCGATAAGTA ATGTGAATTG CAGAATTCAG TGAATCATCG AATCTTTGAA CGCACATTGC
CMW 5232         GATCTCTTGG TTCTGGCATC GATGAAGAAC GCAGCGAAAT GCGATAAGTA ATGTGAATTG CAGAATTCAG TGAATCATCG AATCTTTGAA CGCACATTGC
CMW 5233         GATCTCTTGG TTCTGGCATC GATGAAGAAC GCAGCGAAAT GCGATAAGTA ATGTGAATTG CAGAATTCAG TGAATCATCG AATCTTTGAA CGCACATTGC
C. ovatum        GATCTCTTGG TTCTGGCATC GATGAAGAAC GCAGCGAAAT GCGATAAGTA GTGTGAATTG CAGAATTCAG TGAATCATCG AATCTTTGAA CGCACATTGC
M. corni         GATCTCTTGG TTCTGGCATC GATGAAGAAC GCAGCGAAAT GCGATAAGTA GTGTGAATTG CAGAATTCAG TGAATCATCG AATCTTTGAA CGCACATTGC
C. palmarum      GATCTCTTGG TTCTGGCATC GATGAAGAAC GCAGCGAAAT GCGATAAGTA GTGTGAATTG CAGAATTCAG TGAATCATCG AATCTTTGAA CGCACATTGC
C. fuckelii      GATCTCTTGG TTCTGGCATC GATGAAGAAC GCAGCGAAAT GCGATAAGTA GTGTGAATTG CAGAATTCAG TGAATCATCG AATCTTTGAA CGCACATTGC


                        310        320        330        340        350        360        370        380        390        400
CRY 1017         GCCCT-CTGG TATTCCGGAG GGCATGCCTG TTCGAGCGTC ATT-ACACCA --CTCCAGCC -TCGCTGGGT ATTGGGCGCC GCGGCCTCC- GCGCGCCTTA
CRY 1056         GCCCT-CTGG TATTCCGGAG GGCATGCCTG TTCGAGCGTC ATT-ACACCA --CTCCAGCC -TCGCTGGGT ATTGGGCGCC GCGGCCTCC- GCGCGCCTTA
CRY 1023         GCCCT-CTGG TATTCCGGAG GGCATGCCTG TTCGAGCGTC ATT-ACACCA --CTCCAGCC -TCGCTGGGT ATTGGGCGCC GCGGCCTCC- GCGCGCCTTA
CRY 1057         GCCCT-CTGG TATTCCGGAG GGCATGCCTG TTCGAGCGTC ATT-ACACCA --CTCCAGCC -TCGCTGGGT ATTGGGCGCC GCGGCCTCC- GCGCGCCTTA
CRY 1000         GCCCT-CTGG TATTCCGGAG GGCATGCCTG TTCGAGCGTC ATT-ACACCA --CTCCAGCC -TCGCTGGGT ATTGGGCGCC GCGGCCTCC- GCGCGCCTTA
CRY 1047         GCCCT-CTGG TATTCCGGAG GGCATGCCTG TTCGAGCGTC ATT-ACACCA --CTCCAGCC -TCGCTGGGT ATTGGGCGCC GCGGCCTCC- GCGCGCCTTA
CRY 1049         GCCCT-CTGG TATTCCGGAG GGCATGCCTG TTCGAGCGTC ATT-ACACCA --CTCCAGCC -TCGCTGGGT ATTGGGCGCC GCGGCCTCC- GCGCGCCTTA
CRY 957          GCCCT-CTGG TATTCCGGAG GGCATGCCTG TTCGAGCGTC ATT-ACACCA --CTCCAGCC -TCGCTGGGT ATTGGGCGCC GCGGCCTCC- GCGCGCCTTA
CRY 964          GCCCT-CTGG TATTCCGGAG GGCATGCCTG TTCGAGCGTC ATT-ACACCA --CTCCAGCC -TCGCTGGGT ATTGGGCGCC GCGGCCTCC- GCGCGCCTTA
CMW 5231         GCCCT-CTGG TATTCCGGAG GGCATGCCTG TTCGAGCGTC ATT-ACACCA --CTCCAGCC -TCGCTGGGT ATTGGGCGCC GCGGCCTCC- GCGCGCCTTA
CMW 5234         GCCCT-CTGG TATTCCGGAG GGCATGCCTG TTCGAGCGTC ATT-ACACCA --CTCCAGCC -TCGCTGGGT ATTGGGCGCC GCGGCCTCC- GCGCGCCTTA
CMW 5235         GCCCT-CTGG TATTCCGGAG GGCATGCCTG TTCGAGCGTC ATT-ACACCA --CTCCAGCC -TCGCTGGGT ATTGGGCGCC GCGGCCTCC- GCGCGCCTTA
CMW 5236         GCCCT-CTGG TATTCCGGAG GGCATGCCTG TTCGAGCGTC ATT-ACACCA --CTCCAGCC -TCGCTGGGT ATTGGGCGCC GCGGCCTCC- GCGCGCCTTA
CMW 5232         GCCCT-CTGG TATTCCGGAG GGCATGCCTG TTCGAGCGTC ATT-ACACCA --CTCCAGCC -TCGCTGGGT ATTGGGCGCC GCGGCCTCC- GCGCGCCTTA
CMW 5233         GCCCT-CTGG TATTCCGGAG GGCATGCCTG TTCGAGCGTC ATT-ACACCA --CTCCAGCC -TCGCTGGGT ATTGGGCGCC GCGGCCTCC- GCGCGCCTTA
C. ovatum        GCCCTTC-GG TATTCCGTTG GGCATGCCTG TTCGAGCGTC ATTTAATCAA --CTCAAGCC CTGCTT-GGT GT-GGGTGTT T--G-TTCC- GCC-----TC
M. corni         GCCCTTC-GG TATTCCGTTG GGCATGCCTG TTCGAGCGTC ATTTAATCAA ---TCAAGCC CTGCTT-GGT GT-GGGTGTT T--G-TTCC- GCC-----TC
C. palmarum      GCCCTTC-GG TATTCCGTGG GGCATGCCTG TTCGAGCGTC ATTT-GTACC --CTCAAGCT TTGCTT-GGT GTTGGGTGTT T--G--TCCC GTG----TTA
C. fuckelii      GCCCTTC-GG TATTCCATGG GGCATGCCTG TTCGAGCGTC ATT--CTACA CCCTCAAGCT CTGCTT-GGT GTTGGGCGTC T--G--TCCC GCC----TTC
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CRY 1056          AATGTCTCCG GCCGAGCCGA CCGTCTCCAA GCGTTGTGGC ACAA-CTGTT TCGCTTTCGG GA-CCGGTCC ---GGC-GAC GCGCCGTTAA ACCCTTTCAC
CRY 1023          AATGTCTCCG GCCGAGCCGA CCGTCTCCAA GCGTTGTGGC ACAA-CTGTT TCGCTTTCGG GA-CCGGTCC ---GGC-GAC GCGCCGTTAA ACCCTTTCAC
CRY 1057          AATGTCTCCG GCCGAGCCGA CCGTCTCCAA GCGTTGTGGC ACAA-CTGTT TCGCTTTCGG GA-CCGGTCC ---GGC-GAC GCGCCGTTAA ACCCTTTCAC
CRY 1000          AATGTCTCCG GCCGAGCCGA CCGTCTCCAA GCGTTGTGGC ACAA-CTGTT TCGCTTTCGG GA-CCGGTCC ---GGC-GAC GCGCCGTTAA ACCCTTTCAC
CRY 1047          AATGTCTCCG GCCGAGCCGA CCGTCTCCAA GCGTTGTGGC ACAA-CTGTT TCGCTTTCGG GA-CCGGTCC ---GGC-GAC GCGCCGTTAA ACCCTTTCAC
CRY 1049          AATGTCTCCG GCCGAGCCGA CCGTCTCCAA GCGTTGTGGC ACAA-CTGTT TCGCTTTCGG GA-CCGGTCC ---GGC-GAC GCGCCGTTAA ACCCTTTCAC
CRY 957           AATGTCTCCG GCCGAGCCGA CCGTCTCCAA GCGTTGTGGC ACAA-CTGTT TCGCTTTCGG GA-CCGGTCC ---GGC-GAC GCGCCGTTAA ACCCTTTCAC
CRY 964           AATGTCTCCG GCCGAGCCGA CCGTCTCCAA GCGTTGTGGC ACAA-CTGTT TCGCTTTCGG GA-CCGGTCC ---GGC-GAC GCGCCGTTAA ACCCTTTCAC
CMW 5231          A-TGTCTCCG GCCGAGCCGA CCGTCTCCAA GCGTTGTGGC ACAA-CTGTT TCGCTTTCGG GA-CCGGTCC ---GGCAGAC GCGCCGTTAA ACCCTTTCAC
CMW 5234          A-TGTCTCCG GCCGAGCCGA CCGTCTCCAA GCGTTGTGGC ACAA-CTGTT TCGCTTTCGG GA-CCGGTCC ---GGCAGAC GCGCCGTTAA ACCCTTTCAC
CMW 5235          A-TGTCTCCG GCCGAGCCGA CCGTCTCCAA GCGTTGTGGC ACAA-CTGTT TCGCTTTCGG GA-CCGGTCC ---GGCAGAC GCGCCGTTAA ACCCTTTCAC
CMW 5236          A-TGTCTCCG GCCGAGCCGA CCGTCTCCAA GCGTTGTGGC ACAA-CTGTT TCGCTTTCGG GA-CCGGTCC ---GGCAGAC GCGCCGTTAA ACCCTTTCAC
CMW 5232          A-TGTCTCCG GCCGAGCCGA CCGTCTCCAA GCGTTGTGGC ACAA-CTGTT TCGCTTTCGG GA-CCGGTCC ---GGCAGAC GCGCCGTTAA ACCCTTTCAC
CMW 5233          A-TGTCTCCG GCCGAGCCGA CCGTCTCCAA GCGTTGTGGC ACAA-CTGTT TCGCTTTCGG GA-CCGGTCC ---GGCAGAC GCGCCGTTAA ACCCTTTCAC
C. ovatum         AGCGCGT--G GA-------- ----CTC--- -------GCC TCAAA-T-T- -CCATT--GG CAGCCGGTAT GTTGGC---- ---------- -----TTC--
M. corni          AGCGCGT--G GA-------- ----CTC--- -------GCC TCAAA-T-T- -CCATT--GG CAGCCGGTAT GTTGGC---- ---------- -----TTC--
C. palmarum       --TGCGT--G GACTCGCC-- -----T---- ---------- TAAAGC---- --GATT--GG CAGCCGGCAT ATTGGCCGTG GAGCAGCAGT ACA--TTCAG
C. fuckelii       -GCGCG-C-G GACTCGCCCC AAAT-TCATT GGCAGCGG-- TCC------- ----TT--GC C------TCC TCTCGCGCAG CACAA-TTGC GTCTGCGGGG


                         510        520        530        540        550        560        570
CRY 1017          CAAAGGTTGA CG-------- ----TCG-GA T-CAAGTAGG GATACCACGC TGAACTTAAG CATATCAATA AGC
CRY 1056          CAAAGGTTGA CG-------- ----TCG-GA T-CAAGTAGG GATACCACGC TGAACTTAAG CATATCAATA AGC
CRY 1023          CAAAGGTTGA CG-------- ----TCG-GA T-CAAGTAGG GATACCACGC TGAACTTAAG CATATCAATA AGC
CRY 1057          CAAAAGTTGA CC-------- ----TCG-GA T-CAAGTAGG GATACCACGC TGAACTTAAG CATATCAATA AGC
CRY 1000          CAAAAGTTGA CC-------- ----TCG-GA T-CAAGTAGG GATACCACGC TGAACTTAAG CATATCAATA AGC
CRY 1047          CAAAGGTTGA CC-------- ----TCG-GA T-CAAGTAGG GATACCACGC TGAACTTAAG CATATCAATA AGC
CRY 1049          CAAAGGTTGA CC-------- ----TCG-GA T-CAAGTAGG GATACCACGC TGAACTTAAG CATATCAATA AGC
CRY 957           CAAAGGTTGA CC-------- ----TCG-GA T-CAAGTAGG GATACCACGC TGAACTTAAG CATATCAATA AGC
CRY 964           CAAAGGTTGA CC-------- ----TCG-GA T-CAAGTAGG GATACCACGC TGAACTTAAG CATATCAATA AGC
CMW 5231          CAAAGGTTGA CC-------- ----TCG-GA T-CATGTAGG GATACCACGC TGAACTTAAG CATATCAATA AGC
CMW 5234          CAAAGGTTGA CC-------- ----TCG-GA T-CATGTAGG GATACCACGC TGAACTTAAG CATATCAATA AGC
CMW 5235          CAAAGGTTGA CC-------- ----TCG-GA T-CATGTAGG GATACCACGC TGAACTTAAG CATATCAATA AGC
CMW 5236          CAAAGGTTGA CC-------- ----TCG-GA T-CATGTAGG GATACCACGC TGAACTTAAG CATATCAATA AGC
CMW 5232          CAAAAGTTGA CC-------- ----TCG-GA T-CATGTAGG GATACCACGC TGAACTTAAG CATATCAATA AGC
CMW 5233          CAAAGGTTGA CC-------- ----TCG-GA T-CATGTAGG GATACCACGC TGAACTTAAG CATATCAATA AGC
C. ovatum         -----GT-GC GCAGCACATT G-CGTCGCGA TTC-T---GG CAGACCTC-C TCCCATTAAG C-TCCTTTCT AGA
M. corni          -----GT-GC GCAGCACATT G-CGTCGCGA TTC-T---GG CAGACCTC-C TCCCATTAAG C-TCCTTTCT AGT
C. palmarum       CTCTC-TACA CCATAAAGTT GGCAT-CC-A T-CTT-T--- ---------- -GAA------ ---CCNNNNN NNN
C. fuckelii       GGGCG-TGGC CCGCGTCCAC GAAGCAACAT TACGTCTTT- ---------- -GAA------ ---CCNNNNN NNN
```

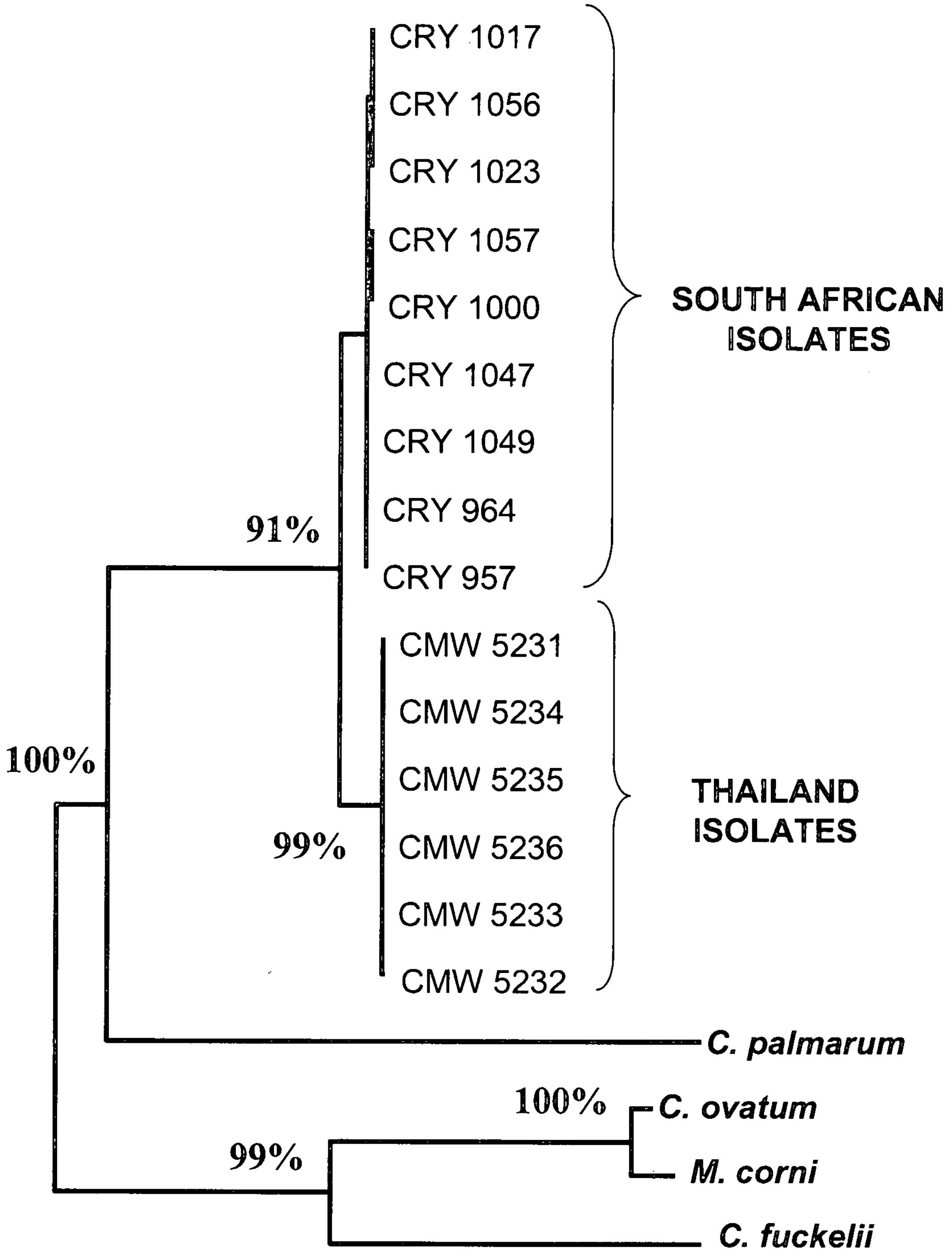
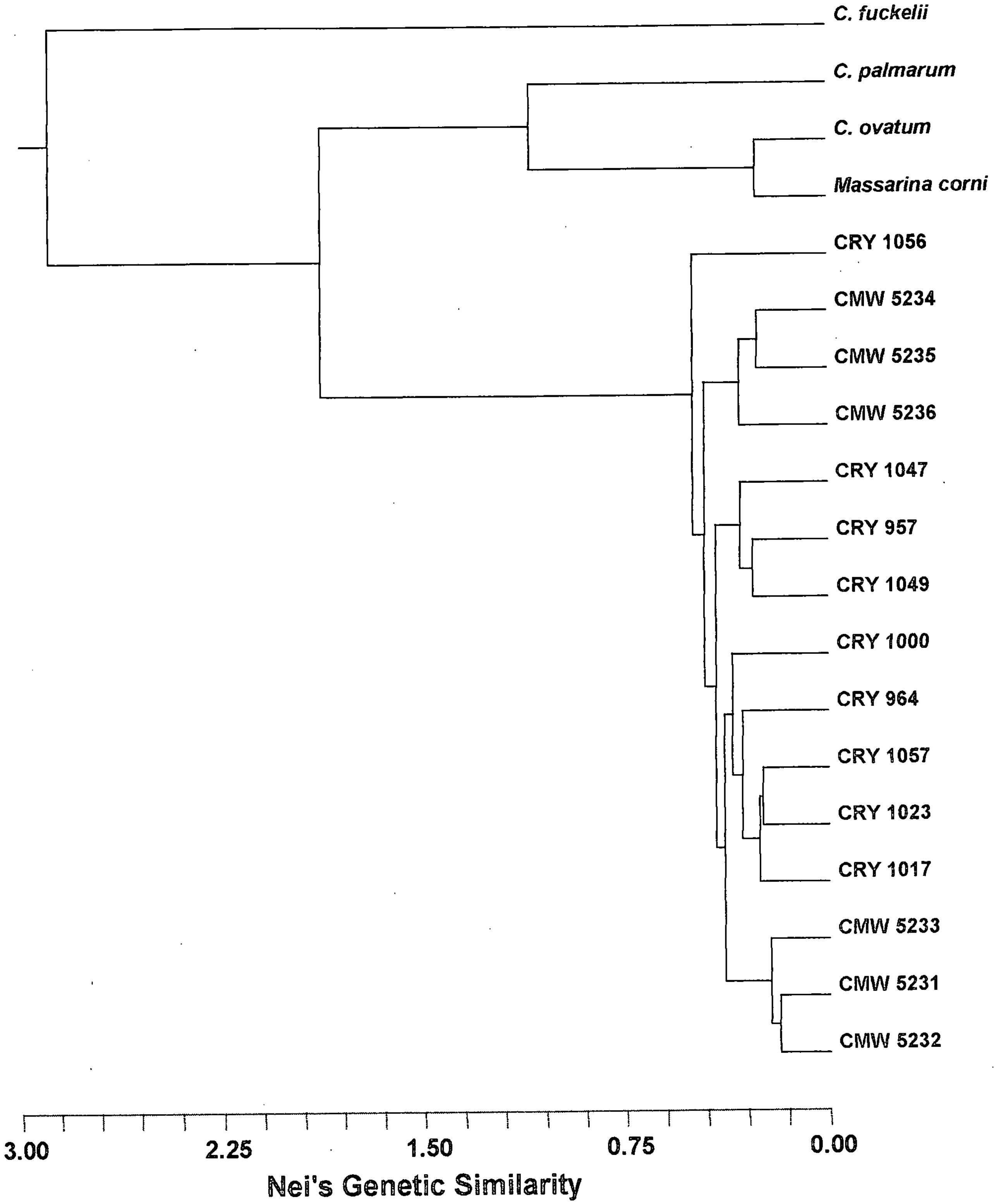

CRY 1017
CRY 1056
CRY 1023
CRY 1057
CRY 1000
CRY 1047
CRY 1049
CRY 964
CRY 957
SOUTH AFRICAN ISOLATES
91%
CMW 5231
CMW 5234
CMW 5235
CMW 5236
CMW 5233
CMW 5232
THAILAND ISOLATES
100%
99%
C. palmarum
100%
C. ovatum
M. corni
99%
C. fuckelii

Fig. 3. Dendrogram generated of *Coniothyrium zuluense* isolates from South Africa (CRY numbers) and Thailand (CMW numbers), together with four related species [*C. palmarum* (CBS 758.73), *C. fuckelii* (CBS 132.26), *Massarina corni* (CBS 496.64) and *C. ovatum*], based on AFLP data using UPGMA cluster analysis of pairwise distance data. The scale represents genetic similarity obtained using the equation of Nei & Li (1979).


C. fuckelii
C. palmarum
C. ovatum
Massarina corni
CRY 1056
CMW 5234
CMW 5235
CMW 5236
CRY 1047
CRY 957
CRY 1049
CRY 1000
CRY 964
CRY 1057
CRY 1023
CRY 1017
CMW 5233
CMW 5231
CMW 5232
3.00
2.25
1.50
0.75
0.00
Nei's Genetic Similarity

Appendix 1. Input data matrix used for UPGMA analysis of 177 AFLP markers^{a}

Thai4.3

10000000100100000001010000100110100100000000010000000011100110101000

01001001001000101000000000010100000010000000000000000000100001010010

10000100000000000000000001000100000000000

Thai7.5

10011000100100000001010001100110100100000000010000010011010100101010

01001001001000111000000100010100000010000000010010000000100001010010

10000100000000000010000001000100000000000

Thai14.5

00010010000110000010110001101110110110000010010000001011000110001000

01001001001100011100101010010100110100000000000000010000010001010010

10001000000000000000000001001100000000000

Thai11.5

00000010000110000000010001110100100000000010011000001001000110101000

11001001001100011000100010010010010000000000000000000000000001010010

10000000000000000000000001001100000000000

Thai10.1

10000010000110000000010001100110110100000010010000001011000110001000

11101001001000111100100010010100010000000000000000011000000001010010

10001000000000000000000001000100000000000

Thai1.3

10100001000100000000101000110011010010000000001000000001100011110101

01001001001001101100000000010100000011000000000000100000000001010010

10000100000000000000000000000100000000000

Teza1.4

00101000000010000000010001100110100000000100010000001001000100101010

01011001001000011010110110010100001010000000000000000000100001010100

10001000000000000000000001000100000000000

Teza3.2

000111001101000000000100011101101000001010000100000010110001101010 00

010110110010000110101100101000010000000000000000001000000001010000

10010100000000000000000000001000000000000

Teza18.3

00001001000000000000001000111010010010000001001100000101100010000101 0

01011001010100011001010100010000001010000000001000011000101001010010

10001110000000100000000000000100100000000

Trus4.1

00000000000100001001010000100100000100000000010000001011001110001000

01000100001001101010100100011100000010100000000000001000000000010100

10000000010000000000000000000100000000010

Trus1.13

00000000100100001001010001101110100000000100010000000101100010000101 0

01010001001000011010010100011100000000000000000000000000100000010100

10000000010000000000000000000100000000000

PR8.2

01100000100100000000010001110100100000000100010000000001000110101010

01011001010100011010010110010010001010000000000000000000100011010100

10000100000000000000000000000100000000000

PR10.3

00000100100110000000001000110011010000000000000100001010111001101010 00

01101001001000011010110010010100001000000000000000001000010000111001 0

10001010000000000000000010001000000000000

Fut6.2

00100101001000000001010001100110100100000000010000001011000110101010

01001001001010011010100000011100000000010000010000000100000100001010 0

10000000110000000000000000000100100000010

Fut2.2

01100100000000000000010001100110110000000010010000000100100011010100 0

11110011001001011010001010010100001010000000000000000100001000101001 0

10000100100000000000000000000100000000000

Massarina corni (CBS 496.64)

000001000000001000000110111101010101010010111111000100111001101011100100100011010001101100101111001000101001000101000100100100010101010010000000000000100100010100000001011100000

C. ovatum

100000000000001000000110111001010001010010111111000000111011101011100100100011010001101000101111001000101001000101000100100100010101010010000000000000100000010100000001011100000

C. fuckelii (CBS 132.26)

010000010000110110000101001000001110001100100100100000000001101110010101000010100000100101000011000110010000000010110011001010000001100100000001010110010101100110110000000010101

C. palmarum (CBS 758.73)

000000010100100001001100001000001010010111001101011101100101010010101110101000011010101010101001010001010000111000000110110000000010110010110011000100100100000100100001000100100

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[a]Each text string shows the name of different *Coniothyrium zuluense* isolates together with related species, as well as, the presence (1) and absence (0) of AFLP markers.

CHAPTER 5

A synergistic relationship between the *Eucalyptus* canker pathogen, *Coniothyrium zuluense,* and two *Pantoea* species

Coniothyrium zuluense is the causal agent of a serious *Eucalyptus* stem canker disease in South Africa. Despite this fact, very little is known about its biology. Bacteria commonly exude from necrotic cankers or severely infected *Eucalyptus* clones in plantations. Isolation from cankers has shown that two bacterial species commonly occur, together with *C. zuluense*. The objectives of this study were to identify these bacteria and to consider whether they influence pathogenicity of *C. zuluense*. Bacteria were identified using the Biolog identification system, as well as by 16S rRNA gene sequence. Sequence data were then compared to those of related *Enterobacteriaceae*. Combined and individual inoculation studies, using both bacterial and fungal isolates, were conducted on fresh Granny Smith apples, as well as on a susceptible *Eucalyptus grandis* clone. We constructed a phylogenetic tree based on 16S rDNA sequence data and found that one bacterium is *Pantoea ananatis* pv. *ananatis* and the other appears to be probably an undescribed *Pantoea* species. Combined fungal and bacterial inoculations resulted in a significant increase in pathogenicity as opposed to individual inoculations. Results indicate a synergistic interaction between *C. zuluense* and the two *Pantoea* species in disease development.

Coniothyrium zuluense Wingfield, Crous & Coutinho was first reported in South Africa by Wingfield *et al.* (1997). The disease, Coniothyrium canker, poses a serious threat to the local forestry industry where *Eucalyptus* species are cultivated extensively in plantations. The South African forestry industry depends largely on vegetatively propagated *Eucalyptus* species and, therefore, these genetically uniform stands are at risk from diseases such as Coniothyrium canker (Wingfield *et al.*, 1997). Strategies to ensure that large numbers of different clones are planted and that a high degree of genetic diversity is maintained in clonal plantations, have thus been implemented.

Initial *C. zuluense* infections occur on the young, green stem tissue during the peak of the growing season. It has been shown that once conidia germinate, the germ tubes infect the stems directly through the epidermis of the young tissue (Wingfield *et al.*, unpublished data). Small necrotic lesions are then formed that coalesce to form large necrotic spindle-shaped cankers (Coutinho *et al.*, 1997; Wingfield *et al.*, 1997). It is thought that conidia are dispersed through rain that flows down the stem and thus ultimately leads to the development of a series of stems cankers representing annual infection events (Coutinho *et al.*, 1997; Wingfield *et al.*, 1997).

Considerable variation in resistance to Coniothyrium canker exists within and among *Eucalyptus* species (Coutinho *et al.*, 1997; Wingfield *et al.*, 1997). Many *E. grandis* Hill ex. Maid. clones currently available for planting show susceptibility to infection. Some hybrid clones of *E. grandis* with *E. urophylla* S. T. Blake, *E. camaldulensis* Dehnh. or *E. nitens* (Deane et Maid.) Maid. are highly resistant to *C. zuluense* infection. Recent evidence, however, suggests that disease resistant clones are beginning to show signs of infection (Wingfield *et al.*, 1997). This is unexpected as *C. zuluense* in South Africa is represented by a uniform population structure, indicative of an asexually reproducing fungus (Van Zyl *et al.*, unpublished data). Generally this would suggest that the capacity of the pathogen to adapt and overcome tree disease resistance mechanisms, would be limited.

In a study undertaken to collect isolates of *C. zuluense* from severely infected *Eucalyptus* clones, it was found that bacteria commonly exude from cankers. Two bacterial species were also frequently isolated together with single conidia of *C.*

zuluense. The objectives of this study are, therefore, to identify the bacterial species and to consider their possible role in lesion development.

MATERIALS AND METHODS

Bacterial and fungal isolates

Bacterial masses exuding from cankers on severely infected *Eucalyptus* clones were collected (Fig. 1). It was evident from isolations that two bacterial species were present. Bacteria with colony morphologies similar to those obtained from Coniothyrium cankers were also frequently isolated in association with single conidia of *C. zuluense* (Fig. 2). Ten isolates of each bacterial species were used for further investigation (Table 1).

Six *C. zuluense* isolates were used in this study (Table 2). Fungal isolates (CRY 1016, CRY 1023, CRY 1054, CRY 1055, CMW 1778, CMW 2100) were selected based on their association with the two bacterial species (Table 2). Choice of these *Coniothyrium* isolates was also based on the fact that they represent a range of levels of pathogenicity including non-pathogenic, intermediately pathogenic and highly pathogenic, as determined in a previous study (Van Zyl *et al.*, 1997). All fungal and bacterial isolates are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria.

Identification of bacteria

Gram negative microplate technique. Pure bacterial cultures were obtained by streaking isolates on the Biolog Universal Growth Medium (BUGM) (Biolog, CA). Once pure cultures had been obtained, characteristics, such as Gram stain and colony morphology, were determined. Identification was carried out using the Biolog Gram negative microplate technique (GN MicroPlate^{TM}, Biolog, CA), together with Biolog's MicroLog^{TM} 1, MicroLog 2, or MicroLog 3 computer software programmes (Biolog, CA).

DNA sequence comparisons. Bacterial isolates (one isolate for each bacterium) were grown overnight in 5 ml of LB broth (10 g Tryptone (Merck); 5 g Yeast Extract (Merck); 5 g NaCl (Merck); 1 l distilled H_20) and pelleted by centrifugation. Nucleic acid was extracted and purified as described by Hauben *et al.* (1997). DNA pellets were dried and suspended in 100 µl H_20. Nucleic acid extracts were quantified by fluorometry and adjusted to a final concentration of 30 ng / µl.

Two primers were used for amplification of 16S rDNA (Table 2). Bacterial DNA was added to a solution (100 µl) containing 2 units of *Taq* DNA polymerase (Boehringer Mannheim, Germany), 10 x reaction buffer (Boehringer), 1.5 mM $MgCl_2$ (Boehringer), 20 mM dNTPs and 0.5 µl of each primer (100 pM). Amplification reactions were done in a Hybaid Omnigene Temperature Cycler (Hybaid, Middlesex, U.K.). The samples were processed through an initial extensive denaturation step consisting of 94 °C for 2 min. This was followed by 30 amplification cycles consisting of 1 min at 92 °C, 30 sec of annealing at 54 °C, and 1 min of primer extension at 72 °C. Final chain elongation took place at 72 °C for 10 min. PCR products were electrophoresed in 1.5 % agarose gels, stained with ethidium bromide, and visualised using UV light. Amplification reactions were done in duplicate.

PCR products were purified using a QIAquick gel extraction kit (Qiagene GmgH, Hilden, Germany). Purified products were then sequenced in both directions using the Big Dye Cycle Sequencing kit with *Amplitaq*® DNA polymerase, FS (Perkin-Elmer, Warrington, UK) on a ABI PRISM^{TM} 377 DNA automatic sequencer (Perkin-Elmer). Nearly complete 16S rDNA sequences were determined by constructing internal primers (Table 3). Nucleotide sequences were manually aligned.

In order to consider the relationship between the bacterial species with related species in the family *Enterobacteriaceae*, 16S rDNA sequence data for phytopathogenic species of the genera *Erwinia*, *Pantoea*, *Pectobacterium* and *Brenneria* were obtained from GenBank (Table 4). All of these species were previously known as members of the genus *Erwinia* (Hauben *et al.*, 1998).

Relationships between species were determined by the neighbour-joining method of Satou & Nei (1987) using the neighbour-joining program of PAUP 4.0 (Phylogenetic Analysis Using Parsimony) (Swofford, 1993). Trees were rooted to two outgroup taxa, *Buchnera aphidicola* (L18927) and *Proteus vulgaris* (J01874), as previously shown by Hauben *et al.* (1998). The stability of the relationships was assessed using the BOOTSTRAP (Bootstrap confidence intervals on DNA parsimony) method (1000 replicates) (Felsenstein, 1993).

Pathogenicity tests

On apples. Granny Smith apples were inoculated with 10 isolates of each of the two bacterial species. For each isolate, five apples were inoculated by removing a 5 mm diameter disk of tissue, and replacing this with discs of agar bearing similar amounts of bacteria (3.4×10^7 CFU / ml). All wounds were closed with masking tape to prevent desiccation of the inoculum and wounds. Apples were then incubated at 25 °C for 7 days and lesion diameters were measured. Means were tested for significance using Tukey's procedure (ANOVA analysis, NCSS97). The isolate of each of the two bacterial species producing the largest lesions was then selected for further study.

Granny Smith apples were inoculated with pure bacteria-free *C. zuluense* isolates and each of the bacterial isolates selected in preliminary screening. At first, apples were inoculated with each of six *C. zuluense* isolates that were free of bacteria. Similarly, both of the pre-selected pathogenic isolates of the two bacterial species were inoculated individually. One *C. zuluense* isolate that had been associated with bacteria at the time of isolation was then selected from non-pathogenic and intermediate pathogenic isolates, as well as isolates showing high pathogenicity. An additional pathogenic isolate of *C. zuluense* was also included. Each of these four *C. zuluense* isolates, CRY 1055, CRY 1016, CRY 1023 and CMW 2100, were then inoculated alone and in combination with each of the two bacterial species. In addition to this, each of these fungal isolates was inoculated together with both bacterial species. In each case, five apples were inoculated for each treatment. Inoculations were done by removing a 5 mm diameter disk of tissue, and replacing these with individual discs of agar bearing either fungal or bacterial isolates. Sterile uninoculated agar discs were used to inoculate five apples to serve as control treatments. All wounds were closed with masking tape to prevent desiccation of the inoculum and wounds. Apples were then incubated at 25 °C for 7 days and lesion diameters were measured. Means were tested for significance using Tukey's procedure (ANOVA analysis, NCSS97). The entire experiment was repeated once.

On trees. *Coniothyrium zuluense* and the two bacterial species were inoculated into young green stem tissue on a susceptible one-year-old *E. grandis* clone (ZG 14) in the Zululand forestry region, KwaZulu-Natal. Treatments were the same as those on apples where six *C. zuluense* isolates, as well as the two *Pantoea* species were initially inoculated alone. The selected four *C. zuluense* isolates (same as those selected for the apple experiment) were then inoculated in combination with each of the two bacteria, followed by the inoculation of each fungal isolate together with both *Pantoea* species. Twenty trees were inoculated for each treatment. Inoculations were done by removing a 10 mm diameter disc of bark from trees at breast height, and replacing this with individual discs of agar bearing either fungal or bacterial growth, respectively. Sterile uninoculated agar discs were used to inoculate twenty trees to serve as control treatments. Inoculation wounds were covered with masking tape to prevent desiccation of the inoculum. Lesion lengths were measured six weeks after inoculation. Means were tested for significance using Tukey's procedure (ANOVA analysis, NCSS97). The entire experiment was repeated once.

RESULTS

Identification of bacteria

Gram negative microplate technique. Two bacterial species were isolated from surface lesions in the field, as well as occurring together with single conidial isolates of *C. zuluense*. These species were characterised by Gram negative straight rods varying between 0.5 – 1.0 μm in width and 1.0 - 3.0 μm in length. Computer analysis of the data using the Biolog Gram negative microplate technique showed that both bacteria were *Enterobacteriaceae*. One bacterium was identified as *Erwinia ananas* with a Biolog similarity index ranging between 0.540 and 0.543 after 24 hours. Colonies of this bacterium have a typically yellow pigment, domed, shining, and mucoid after 2 or 3 days at 27 °C. Biolog analysis indicated that the other bacterium was also a species of *Erwinia*. However, it was not possible to identify a specific taxon for it. Colonies are characteristically white with a slight yellow pigment and mucoid after 2 or 3 days at 27 °C.

DNA sequence comparisons. Direct sequencing of the PCR-amplified 16S rDNAs, allowed us to determine a continuous stretch of 1478 base pairs (bp) for the unknown *Erwinia* species and 1472 bp for *E. ananas*. Manual alignment of 16S rDNA sequences gave rise to a total alignment length of 1470 bp (Fig. 3).

The 16S rDNA sequences of the suspected *Erwinia* species were compared with 16S rDNA sequences of other related *Enterobacteriaceae* (genera *Erwinia*, *Pantoea*, *Pectobacterium* and *Brenneria*) obtained from GenBank. The levels of 16S rDNA sequence similarity for species used in this study, ranged from 91 % to 99.9 %. Sequence similarity of the bacterium tentatively identified as *E. ananas* was 99.9 % homologous to *Pantoea ananatis* pv. *ananatis*, followed by *P. ananatis* pv. *uredovora* (99.8 %) and *P. agglomerans* (99 %). 16S rDNA sequence similarity of the unknown *Erwinia* species was found to be 98 % homologous to *Pantoea stewartii* subspecies *stewartii*, followed by *Erwinia psidii* (96.6 %). The levels of 16S rDNA sequence similarity are shown in Table 5.

A phylogenetic tree was constructed using distance matrix data obtained from the 16S rDNA sequences and the neighbour-joining method (Fig. 4). The tree was based on 1470 pairwise aligned sequence similarities. The 16S rDNA sequences of both *Erwinia* species from *C. zuluense* cankers clustered together with species of *Pantoea* (Fig. 4). Phylogenetic analysis indicated that the purported *E. ananas* isolates from South Africa are closely related to *P. ananatis* pv. *ananatis* (synonym *E. ananas*), with a more distant relatedness to *P. ananatis* pv. *uredovora* (synonym, *E. uredovora*) followed by *P. agglomerans* (synonym, *E. herbicola*). Sequence data, thus supported results obtained using the Biolog identification system in that this bacterium associated with *C. zuluense*, is *P. ananatis* pv. *ananatis*.

Phylogenetic analysis indicated a close relationship between the unknown *Erwinia* species from *C. zuluense* cankers and that of phytopathogenic bacterium, *P. stewartii* subspecies *stewartii* (synonym *E. stewartii*). A more distant phylogenetic relationship was evident to *E. psidii, E. tracheiphila* and *E. mollotivora*, respectively. It is, thus evident that the unknown *Erwinia* species from South Africa is most closely related to *P. stewartii* subspecies *stewartii*. However, based on a somewhat low level of similarity, this bacterium most probably represents a new species of *Pantoea*.

Pathogenicity tests

On apples. All bacterial isolates were able to cause tissue maceration (Table 6). No significant differences (*P = 0.05*) in lesion diameter were evident among isolates of the same bacterial species. Significant differences in lesion diameters were, however, evident between isolates of the two different bacteria (Table 6). Based on this, we selected one isolate of each bacterial species producing the largest lesions (Table 6, highlighted in bold). These isolates were then used for further investigations of a possible synergistic effect between bacteria and *C. zuluense*.

Inoculations of individual *C. zuluense* isolates showed that little tissue maceration was evident when inoculated into Granny Smith apples (Table 7; Fig. 5A). No significant differences (*P = 0.05*) were evident between *C. zuluense* isolates varying in their pathogenicity to a susceptible *E. grandis* clone. Both *Pantoea* species, however, caused severe tissue maceration that significantly (*P = 0.05*) distinguished them from *C. zuluense* isolates (Table 7; Fig. 5B). *Pantoea ananatis* pv. *ananatis* produced significantly larger lesions than the unknown *Pantoea* species (Table 7).

Significant differences (*P = 0.05*) in lesion diameter were evident when each of the four selected *C. zuluense* isolates were inoculated in combination with each of the two *Pantoea* species (Table 7; Fig. 5C). Inoculation of the non-pathogenic or intermediately pathogenic *C. zuluense* isolates (CRY 1055 and CRY 1016) in combination with either *P. ananatis* pv. *ananatis* or the unknown *Pantoea* species, produced significantly larger lesions compared with those using these fungi alone.

This enhanced effect was especially obvious when pathogenic *C. zuluense* isolates (CMW 2100 and CRY 1023) were inoculated in combination with either *P. ananatis* pv. *ananatis* or the unknown *Pantoea* sp. (Table 7).

Inoculation results where each of the four selected *C. zuluense* isolates were inoculated together with both *Pantoea* species, showed a significant ($P = 0.05$) increase in lesion development as compared with those obtained when the fungi were inoculated alone or in combination with each of the two *Pantoea* species (Table 7). The only exception was observed when non-pathogenic *C. zuluense* isolate, CRY 1055, showed no increase in lesion length when inoculated in combination with both bacteria (Table 7).

On trees. All *C. zuluense* isolates were able to produce lesions on a susceptible *E. grandis* clone (ZG 14) when inoculated on their own and in the absence of bacteria (Table 7; Fig. 6B). Neither of the *Pantoea* species were able to cause lesions alone (Table 7; Fig. 6A). Using ANOVA, it was possible to identify significant differences ($P = 0.05$) among lesion lengths produced by different *C. zuluense* isolates (Table 7). Thus, previously designated pathogenic *C. zuluense* isolates produced significantly larger lesions than those of the selected intermediate and non-pathogenic isolates. Lesion lengths were the greatest for isolate, CRY 1023 which differed significantly from isolate CMW 2100 (Table 7).

When *Coniothyrium* isolates were inoculated in combination with either *P. ananatis* pv. *ananatis* or the unknown *Pantoea* species, significantly larger lesions developed ($P = 0.05$), compared to inoculations where the fungus was used alone (Table 7). Results showed that *P. ananatis* pv. *ananatis* inoculated in combination with each of the *C. zuluense* isolates led to a significant increase in lesion size (Table 7). Similar results were obtained when the unknown *Pantoea* species was used, although the synergistic effect was less obvious (Table 7). The inoculation of both *Pantoea* species together with *C. zuluense* produced lesions that were significantly larger ($P = 0.05$) than when *C. zuluense* isolates were inoculated alone or in combination with either *P. ananatis* pv. *ananatis* or the unknown *Pantoea* sp. (Table 7, Fig. 6C).

Results obtained from inoculating the susceptible *E. grandis* clone, ZG 14, correlated significantly ($r_s = 0.74$) with data obtained after inoculating Granny Smith apples. The main difference between the two inoculation trials was that both *Pantoea* species were unable to cause disease symptoms on the susceptible *Eucalyptus* clone when inoculated alone (Table 7; Fig. 6).

DISCUSSION

In this study, we have shown that bacteria found exuding from cankers caused by *C. zuluense* and in association with single isolated conidia, represent two species of *Pantoea*. Most Gram-negative bacteria in the *Enterobacteriaceae* colonise the apoplast of plants and produce a range of symptoms on virtually all crop plants (Alfano & Collmer, 1996). Phytopathogenic species of *Brenneria, Enterobacter*, *Erwinia*, *Pantoea*, and *Pectobacterium* are particularly well known for causing blights, cankers, die back, leaf spots, wilts and soft-rots on a wide range of plant species (Starr & Chatterjee, 1972; Perombelon, 1980; Hauben *et al*., 1998). It is, therefore, not surprising that the two *Pantoea* species reported in this study are associated with disease.

Analysis of Biolog data enabled us to identify one of the two bacterial species. It was, however, not possible to identify a specific taxon for the second bacterium. Identification of this bacterium was made possible by sequencing the 16S rRNA gene. One bacterium was identified *as P. ananatis* pv. *ananatis* (synonym, *Erwinia ananas*) and the other has shown to be closely related to *P. stewartii* subsp. *stewartii*. Analysis of 16S rDNA sequences has previously been used to successfully determine phylogenetic, as well as, inter- and intrageneric relationships between bacterial species (Bereswill *et al*., 1995; Leblond-Bourget *et al*., 1996; Hauben *et al*., 1997; Kwon *et al*., 1997; Hauben *et al*., 1998; Kim *et al*., 1999). This is due to the existence of conserved regions for all bacterial genera within the 16S rRNA gene, together with smaller parts that are variable to accurately determine phylogenies (Massol-Deya, 1995; Hauben *et al*., 1997; Kwon *et al*., 1997; Hauben *et al*., 1998; Kim *et al*., 1999).

Pantoea ananatis pv. *ananatis* is the causal agent of brown rot of pineapple (*Ananas comosus* Schult. f.) fruitlets (Fahy & Persley, 1983; Mergaert *et al*., 1993). It has also previously been reported to occur on sugar cane (*Saccharum* L. hybrids) (Elliott, 1951; Fahy & Persley, 1983). As far as we are aware, this is the first report of this bacterial pathogen from South Africa. The second unknown *Pantoea* sp. was shown to be closely related to *P. stewartii* subsp. *stewartii* (synonym, *Erwinia stewartii*), however, nucleotide differences of the 16S rDNA sequences indicated that this bacterium is probably new to science. *Pantoea stewartii* subsp. *stewartii* is the causative agent of Stewart's bacterial wilt of maize (*Zea mays* L.) and sweet corn (*Zea mays* var. *rugosa* L.) (Fahy & Persley, 1983; Mergaert *et al*., 1993). Further biochemical, genotypic, as well as pathogenicity characteristics are needed before this South African species can be fully identified.

Both *Pantoea* species were able to cause significant tissue maceration on Granny Smith apples when inoculated alone. In contrast to this, no lesion development was evident when each of the two *Pantoea* species was inoculated into a susceptible *E. grandis* clone. Similarly, pure bacteria-free *C. zuluense* isolates produced almost no tissue maceration on Granny Smith apples, whereas, significant lesions were produced when they were inoculated into a susceptible *E. grandis* clone (ZG 14). However, inoculation of *C. zuluense* isolates in combination with either *P. ananatis* pv. *ananatis* or the unknown *Pantoea* species, or both these species on Granny Smith apples, showed an average of a 307 % increase in lesion diameter relative to the inoculation of fungal isolates alone. This was also true for field inoculations of a susceptible *E. grandis* clone (ZG 14) where a significant increase (43 %) in lesion length was observed. Results of this study, therefore, indicate a strong synergistic interaction between *C. zuluense* and both *Pantoea* species.

Synergism refers to a simultaneous effect of two organisms or environmental factors acting together, producing a change greater than either could alone (Hawksworth *et al*., 1995). Results of this study indicate a strong synergistic effect between *C. zuluense* and both *Pantoea* species. Similar synergistic interactions between fungi and bacteria have been reported previously. Toro *et al*., (1996) showed that combined fungal and bacterial inoculations of the mycorrhizal fungus, *Glomus mosseae* (Nicol & Gerd) Gerd & Trappe, and species of rhizosphere calciumphosphate solubilizing bacteria, *Bacillus* and *Pseudomonas*, resulted in a 283 % increase in growth of *Pueraria phasealoides* (Kudzu) relative to the uninoculated controls. Similarly, Hodrova *et al.* (1995) showed that co-culture inoculations of anaerobic rumen fungus, *Orpinomyces joyonii*, together with two bacteria species, *Megasphaera elsdenii* and *Eubacterium limosum*, resulted in an increase of between 7.96 % to 10.12 % of microcrystalline cellulose degradation.

In a previous study, Van Zyl *et al.* (1997), showed a very large variation in pathogenicity among *C. zuluense* isolates. It was found that 78 % of a large collection of *C. zuluense* isolates (344 isolates in total) were not able to cause lesions when inoculated into a susceptible *E. grandis* clone (ZG 14). At that stage, we were unable to explain this phenomenon in that all isolates were collected from severely infected *Eucalyptus* species and clones. However, the discovery of a synergistic interaction between *C. zuluense* and both *Pantoea* species in the present study, might provide a possible explanation for this anomaly. All *C. zuluense* isolates were purified of all bacteria prior to pathogenicity studies in the field. This might be the reason why such a large variation in virulence was evident. In the presence of the bacteria, many non-pathogenic *C. zuluense* isolates might have resulted in disease development. This question deserves further study.

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Table 1. Isolates of the two *Pantoea* species found in association with *Coniothyrium zuluense*.

Bacterial species	**Strain no.**[a]	**Plantations of origin in KwaZulu-Natal**
Pantoea ananatis pv. *ananatis*	Ea1	Fairbreeze
"	Ea2	Futululu
"	Ea3	Teza
"	Ea4	Palm Ridge
"	Ea5	Trust
"	Ea6	Honey Farm
"	Ea7	Teranera
"	Ea8	Aboyni
"	Ea9	Palm Ridge
"	Ea10	Teza
Unknown *Pantoea* species	uE1	Futululu
"	uE2	Palm Ridge
"	uE3	Teza
"	uE4	Teza
"	uE5	Honey Farm
"	uE6	Shire
"	uE7	Teranera
"	uE8	Palm Ridge
"	uE9	Honey Farm
"	uE10	Teza

[a] Bacterial strains are stored in the culture collection of Dr. K-H Riedl, Department of Microbiology and Biochemistry, University of the Orange Free State, Bloemfontein.

Table 2. List of *Coniothyrium zuluense* isolates used in this study.

Culture number[a]	**Origin**[b]	**Pathogenicity to an *Eucalyptus grandis* clone (ZG 14)**[c]
CRY 1055	Zululand, KZN	non-pathogenic
CRY 1054	"	non-pathogenic
CRY 1016	"	intermediately pathogenic
CRY 1023	"	pathogenic
CMW 1778	"	intermediately pathogenic
CMW 2100	"	pathogenic

[a] CMW and CRY numbers represent *C. zuluense* isolates maintained in the culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

[b] All isolates were collected from diseased *Eucalyptus* species, clones and hybrids in South Africa. KZN refers to the KwaZulu-Natal Province.

[c] Pathogenicity of relevant isolates was determined in a previous study (Van Zyl *et al.*,1997).

Table 3: Primer sequences for the 16S rRNA gene amplification.

Primer	Sequence (5’-3’)	Target region	Reference
PA	AGA GTT TGA TCC TGG CTC AG	1.5 kb DNA fragment from 16S rDNA region	Massol-Deya *et al.*, 1995
PH	AAG GAG GTG ATC CAG CCG CA	“	“
OT1	GAA GAA GGC CTT CGG GTT G	Internal	Current study
OT2	CAC GAC ACG AGC TGA CGA C	Internal	Current study

Table 4: List of bacterial strains considered in this study together with the GenBank accession numbers for their 16S rDNA sequence.

Species or subspecies	Synonyms	GenBank accession numbers
Pantoea ananatis pv. *ananatis*	*Erwinia ananas*	U80196
Pantoea ananatis pv. *uredovora*	*Erwinia uredovora*	U80209
Pantoea agglomerans	*Erwinia herbicola*	U80202
Pantoea agglomerans	*Erwinia milletiae*	U80183; AB004757
Pantoea stewartii subspecies *stewartii*	*Erwinia stewartii*	U80208
Erwinia tracheiphila	-	Y13250
Erwinia psidii	-	Z96085
Erwinia mallotivora	-	AJ233414; Z96084
Erwinia persicinus	-	AJ001190; Z96086U80205
Erwinia rhapontici	-	AJ233417; Z96087U80206
Erwinia amylovora	-	Z96088; AJ233410AJ010485; U80195
Erwinia pyrifoliae	-	AJ009930
Pectobacterium cypripedii	*Erwinia cypripedii*	Z96094
Pectobacterium chrysanthemi	*Erwinia chrysanthemi*	AJ233412
Pectobacterium carotovorum subspecies *carotovorum*	*Erwinia carotovora* subspecies *carotovora*	Z96089
Pectobacterium carotovorum subspecies *odoriferum*	*Erwinia carotovora* subspecies *odoriferum*	AJ223407
Pectobacterium carotovorum subspecies *wasabiae*	*Erwinia carotovora* subspecies *wasabiae*	AJ223408
Pectobacterium carotovorum subspecies *betavasculorum*	*Erwinia carotovora* subspecies *betavasculorum*	U80198
Pectobacterium carotovorum subspecies *atrosepticum*	*Erwinia carotovora* subspecies *atrosepticum*	Z96090
Pectobacterium cacticidum	*Erwinia cacticida*	Z96092
Brenneria salicis	*Erwinia salicis*	Z96097
Brenneria rubrifaciens	*Erwinia rubrifaciens*	AJ233418
Brenneria paradisiaca	*Erwinia paradisiaca*	Z96096
Brenneria nigrifluens	*Erwinia nigrifluens*	AJ233415
Brenneria alni	*Erwinia alni*	AJ233409

Table 5: Percentages of sequence similarity based on 16S rDNA sequences.

	Organism	% 16S rDNA sequence similarity															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	***Erwinia ananas*, Zululand, KZN, SA[a]**	100															
2	**Unknown *Erwinia* species, Zululand, KZN, SA[a]**	96.7	100														
3	*Pantoea ananatis* pv. *ananatis* (U80196)	**99.9**[b]	96.5	100													
4	*Pantoea ananatis* pv. *uredovora* (U80209)	**99.8**	96.5	99.9	100												
5	*Pantoea agglomerans* (U80202)	**99.0**	96.4	99.0	98.9	100											
6	*Pantoea agglomerans* (AB004757)	**98.8**	96.6	98.7	98.6	99.0	100										
7	*Pantoea agglomerans* (U80183)	**98.2**	96.2	98.1	98.0	98.8	99.3	100									
8	*Pantoea stewartii* subspecies *stewartii* (U80208)	97.4	**98.0**	97.4	97.4	97.1	96.9	96.5	100								
9	*Erwinia amylovora* (Z96088)	95.4	95.8	95.3	95.2	95.2	95.5	95.1	95.9	100							
10	*Erwinia tracheiphila* (Y13250)	95.7	95.1	95.6	95.5	95.6	95.3	95.2	95.4	94.8	100						
11	*Erwinia psidii* (Z96085)	95.4	96.6	95.2	95.2	95.2	95.0	94.8	96.6	95.3	96.0	100					
12	*Erwinia mallotivora* (Z96084)	94.5	96.1	94.4	94.3	94.4	94.9	94.7	95.9	96.3	94.8	96.8	100				
13	*Pectobacterium carotovorum* subspecies *carotovorum* (Z96089)	95.2	96.3	95.1	95.0	95.1	95.5	95.4	95.4	95.2	95.0	95.2	94.7	100			
14	*Brenneria salicis* (Z96097)	93.3	92.7	93.3	93.3	92.9	92.8	92.8	92.5	93.2	91.1	91.4	92.9	94.3	100		
15	*Proteus vulgaris* (J01874)	91.1	91.6	91.1	91.0	91.6	91.4	91.2	91.0	91.4	90.1	91.0	91.7	92.0	91.6	100	
16	*Buchnera aphidicola* (L18927)	86.8	86.5	86.7	86.6	86.3	86.5	86.6	86.4	86.8	85.4	87.3	86.2	86.8	85.8	85.1	100

[a]The sequences of both South African *Erwinia* species were determined in this study; the other sequences were obtained from the GenBank database.

[b] The strains correspond with the strains in bold in Fig. 4.

Table 6. Lesions produced on Granny Smith apples inoculated with each of the two *Pantoea* species

Bacterial species	**Strain no.**[a]	**Lesion diameter (mm)**[b]
Pantoea ananatis pv. *ananatis*	Ea1	23.5 b
"	**Ea2**	**26.1 b**
"	Ea3	24.6 b
"	Ea4	23.6 b
"	Ea5	25.6 b
"	Ea6	25.3 b
"	Ea7	24.9 b
"	Ea8	23.5 b
"	Ea9	24.1 b
"	Ea10	25.4 b
Unknown *Pantoea* species	UE1	15.5 a
"	UE2	15.7 a
"	UE3	16.3 a
"	UE4	14.9 a
"	UE5	16.2 a
"	UE6	16.5 a
"	**UE7**	**18.2 a**
"	UE8	16.3 a
"	UE9	16.1 a
"	UE10	16.8 a

[a] One isolate of each bacterial species (bold) was selected for further inoculation.

[b] Values are the means of two duplicate sets of five inoculated apples. CV = 10.3 %. Values followed by the same letter are not significantly different at *P = 0.05*.

Table 7. Comparison of pathogenicity after inoculation with *Coniothyrium zuluense* and two *Pantoea* species on apples and *Eucalyptus* trees.

Isolates[a]	Pathogenicity study			
	Apples[b] Lesion diam. (mm)		Trees[c] Lesion length (mm)	
	Exp. #1	Exp. #2	Exp. #1	Exp. #2
Control	5.0 a[d]	5.0 a[d]	10.0 a[e]	10.0 a[e]
CRY 1055	5.9 a	5.0 a	10.0 a	10.0 a
CRY 1054	5.0 a	5.0 a	10.0 a	10.1 a
CMW 1778	7.2 a	8.1 b	27.2 cd	26.5 de
CRY 1016	7.0 a	7.6 b	27.1 cd	27.9 de
CMW 2100	11.1 b	10.8 c	37.2 ef	36.8 fg
CRY 1023	13.0 b	13.4 d	59.2 i	57.3 j
P. a. pv. *ananatis* (Strain Ea2)	25.9 d	23.9 fg	10.7 a	10.0 a
Pantoea sp. (Strain uE7)	18.1 c	19.4 e	10.1 a	10.0 a
CRY 1055 + *P.a.* pv. *ananatis*	25.8 d	25.3 gh	18.0 b	20.5 c
CRY 1016 + *P.a.* pv. *ananatis*	31.4 ef	29.8 i	34.1 e	35.8 f
CMW 2100 + *P.a.* pv. *ananatis*	37.2 g	39.6 k	47.4 h	46.3 i
CRY 1023 + *P.a.* pv. *ananatis*	40.5 h	40.0 k	71.6 k	72.0 l
CRY 1055 + *Pantoea* species	20.6 c	22.4 f	14.8 b	15.8 b
CRY 1016 + *Pantoea* species	23.4 d	22.9 f	29.7 d	28.5 e
CMW 2100 + *Pantoea* species	30.7 e	32.8 j	41.0 fg	41.8 h
CRY 1023 + *Pantoea* species	31.8 ef	32.8 j	64.9 j	63.4 k
CRY 1055 + *P. a.* pv. *ananatis* + *Pantoea* sp.	25.7 d	26.5 h	24.8 c	24.0 cd
CRY 1016 + *P. a.* pv. *ananatis* + *Pantoea* sp.	33.9 f	33.4 j	42.4 g	40.6 gh
CMW 2100 + *P. a.* pv. *ananatis* + *Pantoea* sp.	40.0 h	40.9 k	61.0 j	58.9 j
CRY 1023 + *P. a.* pv. *ananatis* + *Pantoea* sp.	45.5 i	45.0 l	82.8 l	80.7 m

[a] CRY and CMW numbers refer to *C. zuluense* isolates used in this study. Two *Pantoea* species, *P. ananatis* pv. *ananatis* and an unknown *Pantoea* species, are isolated in association with *C. zuluense*. All isolates were collected from severely infected *Eucalyptus* trees in KwaZulu-Natal.

[b] Fresh Granny Smith apples were used in this pathogenicity study.

[c] A susceptible *E. grandis* clone, ZG 14, was used for field conditions.

[d] Values are the means of five inoculated apples (Exp. #1: CV = 11.3 %) (Exp. #2: CV = 10.5 %). Values followed by the same letter are not significantly different at *P = 0.05*. Exp. # 1 and 2 represents two separate inoculation studies.

[e] Values are the means of twenty inoculated trees (Exp. #1: CV = 18.5 %) (Exp. #2: CV = 16.7 %). Values followed by the same letter are not significantly different at *P = 0.05*. Exp. # 1 and 2 represents two separate inoculation studies.


1


2

Fig. 3. Aligned nucleotide sequences data for the 16S rDNA of both South African *Erwinia* species, as well as species of the genera *Pantoea*, *Erwinia*, *Pectobacterium*, *Brenneria*, *Proteus* and *Buchnera* (Table 3). All sequences, except those of the two South African species were obtained from the GenBank database (Table 3). Gaps that were inserted due to alignment are indicated by a dash (-). N indicates unknown bases.

	10	20	30	40	50	60	70	80
Buchnera aphidicola	AACACATGCA	AGTCGAGCGG	CAGCGAAAAG	AAAGCTTGCT	TTCTTGTCGG	-CGAGCGGCA	AACGGGTGAG	TAATATCTGG
Proteus vulgaris	AACACATGCA	AGTCGAGCGG	TAACAGGAGA	AA-GCTTGCT	TTCTTGCTGA	-CGAGCGGCG	GACGGGTGAG	TAATGTATGG
***Erwinia ananas*, Zululand, KZN, SA**	**AACACATGCA**	**AGTCGGACGG**	**TAGCACAGA-**	**GA-GCTTGCT**	**CTC-GGGTGA**	**-CGAGTGGCG**	**GACGGGTGAG**	**TAATGTCTGG**
Pantoea a. pv. *ananatis*	AACACATGCA	AGTCGGACGG	TAGCACAGA-	GA-GCTTGCT	CTC-GGGTGA	-CGAGTGGCG	GACGGGTGAG	TAATGTCTGG
Pantoea a. pv. *uredovora*	AACACATGCA	AGTCGGACGG	TAGCACAGA-	GA-GCTTGCT	CTC-GTGTGA	-CGAGTGGCG	GACGGGTGAG	TAATGTCTGG
Pantoea agglomerans (U80202)	AACACATGCA	AGTCGGACGG	TAGCACAGA-	GA-GCTTGCT	CTC-GGGTGA	-CGAGTGGCG	GACGGGTGAG	TAATGTCTGG
Pantoea agglomerans (AB004757)	AACACATGCA	AGTCGGACGG	TAGCACAGA-	GA-GCTTGCT	CTC-GGGTGA	-CGAGTGGCG	GACGGGTGAG	TAATGTCTGG
Pantoea agglomerans (U80183)	AACACATGCA	AGTCGGACGG	TAGCACAGAG	GA-GCTTGCT	CTCTGGGTGA	-CGAGTGGCG	GACGGGTGAG	TAATGTCTGG
Unknown *Erwinia* sp.	**AACACATGCA**	**AGTCGAGCGG**	**TAGCACAGAG**	**GA-GCTTGCT**	**CTC-GCCGGA**	**-CGAGTGGCG**	**GACGGGTGAG**	**TAATGTCTGG**
Pantoea s. subsp. *stewartii*	AACACATGCA	AGTCGGACGG	TAGCACAGAG	GA-GCTTGCT	CTC-GGGTGA	-CGAGTGGCG	GACGGGTGAG	TAATGTCTGG
Erwinia tracheiphila	AACACATGCA	AGTCGGACGG	TAGCACAGAA	GA-GCGTGCT	CTTTGGGTGA	-CGAGTGGCG	GACGGGTGAG	TAATGTCTGG
Erwinia psidii	AACACATGCA	AGTCGAACGG	TAGCGGGAAG	AA-GCTTGCT	TCTTTGCCGA	-CGAGTGGCG	GACGGGTGAG	TAATGTCTGG
Erwinia mallotivora (AJ233414)	AACACATGCA	AGTCGAACGG	TAGCACAGGG	GA-GCTTGCT	CCCTGGGTGA	-CGAGTGGCG	GACGGGTGAG	TAATGTCTGG
Erwinia mallotivora (Z96084)	AACACATCCA	AGTCGAACGG	TAGCACAGGG	GA-GCTTCCT	CCCTGGGTGA	-CGAGTGGCG	GACGGGTGAG	TAATGTCTGG
Erwinia persicinus (AJ001190)	AACACATGCA	AGTCGAACGG	TAGCACAGA-	GA-GCTTGCT	CTC-GGGTGA	-CGAGTGGCG	GACGGGTGAG	TAATGTCTGG
Erwinia persicinus (Z96086)	AACACATGCA	AGTCGAACGG	TAGCACAGA-	GA-GCTTGCT	CTC-GGGTGA	-CGAGTGGCG	GACGGGTGAG	TAATGTCTGG
Erwinia persicinus (U80205)	AACACATGCA	AGTCGAACGG	TAGCACAGA-	GA-GCTTGCT	CTC-GTGTGA	T-GAGTGGCG	GACGGGTGAG	TAATGTCTGG
Erwinia rhapontici (AJ233417)	AACACATGCA	AGTCGAACGG	TAGCACAGAG	GA-GCTTGCT	CCTTGGGTGA	-CGAGTGGCG	GACGGGTGAG	TAATGCCTGG
Erwinia rhapontici (Z96087)	AACACATGCA	AGTCGAACGG	TAGCACAGAG	GA-GCTTGCT	CCTTGGGTGA	-CGAGTGGCG	GACGGGTGAG	TAATGCCTGG
Erwinia rhapontici (U80206)	AACACATGCA	AGTCGAACGG	TAGCACAGAG	GA-GCTTGCT	CTCTGGGTGA	-CGAGTGGCG	GACGGGTGAG	TAATGCCTGG
Erwinia amylovora (Z96088)	AACACATGCA	AGTCGAACGG	TAGCACAGA-	GA-GCTTGCT	CNT-GGGTGA	-CGAGTGGCG	GACGGGTGAG	TAATGTCTGG
Erwinia amylovora (AJ233410)	AACACATGCA	AGTCGAACGG	TAGCACAGA-	GA-GCTTGCT	CTT-GGGTGA	-CGAGTGGCG	GACGGGTGAG	TAATGTCTGG
Erwinia amylovora (AJ010485)	AACACATGCA	AGTCGAACGG	TAGCACAGA-	GA-GCTTGCT	CTT-GGGTGA	-CGAGTGGCG	GACGGGTGAG	TAATGTCTGG
Erwinia amylovora (U80195)	AACACATGCA	AGTCGAACGG	TAGCACAGA-	GA-GCTTGCT	CTT-GGGTGA	-CGAGTGGCG	GACGGGTGAG	TAATGTCTGG
Erwinia pyrifoliae	AACACATGCA	AGTCGAACGG	TAGCACAGA-	GA-GCTTGCT	CTC-GGGTGA	-CGAGTGGCG	GACGGGTGAG	TAATGTCTGG
Pectobacterium cypripedii	AACACATGCA	AGTCGGACGG	TAGCACAGNG	GA-GCTTNCT	CCCTGGGTGA	-CGAGNGGCG	GACGGGTGAG	TAATNNCTGG
Pectobacterium chrysanthemi	AACACATGCA	AGTCGGGCGG	TAGCACAAGG	GA-GCTTGCT	CCC-GGGTGA	-CGAGCGGCG	GACGGGTGAG	TAATGTCTGG
Pectobacterium c. subsp. *carotovorum*	AACACATGCA	AGTCGAGCGG	TAGCACAGAG	GA-GCTTGCT	CCTTGGGTGA	-CGAGCGGCG	GACGGGTGAG	TAATGTCTGG
Pectobacterium c. subsp. *wasabiae*	AACACATGCA	AGTCGAGCGG	TAGCACAGGA	GA-GCTTGCT	CTCTGGGTGA	-CGAGCGGCG	GACGGGTGAG	TAATGTCTGG
Pectobacterium c. subsp. *betavasculorum*	AACACATGCA	AGTCGAGCGG	CAGCGGGAAG	TA-GCTTGCT	ACTTTGCCGG	-CGAGCGGTG	GACGGGTGAG	TAATGTCTGG
Pectobacterium c. subsp. *atrosepticum*	AACACATGCA	AGTCGAGCGG	TAGCACAGAA	GA-GCTTGCT	CTTTGGGTGA	-CGAGCGGCG	GACGGGTGAG	TAATGTCTGG
Pectobacterium c. subsp. *odoriferum*	AACACATGCA	AGTCGAGCGG	TAGCACAAGA	GA-GCTTGCT	CTCTGGGTGA	-CGAGCGGCG	GACGGGTGAG	TAATGTCTGG
Pectobacterium cacticidum	AACACATGCA	AGTCGAGCGG	TAACACAGAG	GA-GCTTGCT	NTC-GGGTGA	-CGAGCGGCG	GACGGGTGAG	TAATGTCTGG
Brenneria salicis	AACACATGCA	AGTCGGGCGG	TAGCACAGAG	GA-GCTTGCT	CCTTGGGTGA	-CGAGCGGCG	GACGGGTGAG	TAAAGTCTGG
Brenneria rubrifaciens	AACACATGCA	AGTCGAGCGG	CAGCGGGAAG	TA-GCTTGCT	ACTTTGCCGG	-CGAGCGGCG	GACGGGTGAG	TAATGTCTGG
Brenneria nigrifluens	AACACATGCA	AGTCGAGCGG	TAGCACAGAG	GA-GCTTGCT	CCTTGGGTGA	-CGAGCGGCG	GACGGGTGAG	TAATGTCTGG
Brenneria alni	AACACATGCA	AGTCGGGCGG	TAGCACAGGG	GA-GCTTGCT	TCCT-GGTGA	ACGAGCGGCG	GACGGGTGAG	TAAAGTCTGG
Brenneria paradisiaca	AACACATGCA	AGTCGAGCGG	CAGCGGGGGG	AA-GCTTGCT	TCCCCGCCGG	-CGAGCGGCG	GACGGGTGAG	TAATGTCTGG

	90	100	110	120	130	140	150	160
Buchnera aphidicola	GGAT-CTGCC	CAAAAGAGGG	GGATAACTAC	TAGAAATGGT	AGCTAATACC	GCATAAAGTT	GAAAAACCAA	AGTGGGGGAC
Proteus vulgaris	GGAT-CTGCC	-GATAGAGGG	GGATAACTAC	TGGAAACGGT	GGCTAATACC	GCATGACGTC	TACGGACCAA	AGCAGGGGTT
***Erwinia ananas*, Zululand, KZN, SA**	**GGA-TCTGCC**	**CGATAGAGGG**	**GGATAACCAC**	**TGGAAACGGT**	**GGCTAATACC**	**GCATAACGTC**	**GCAAGACCAA**	**AGAGGGGGAC**
Pantoea a. pv. *ananatis*	GGA-TCTGCC	CGATAGAGGG	GGATAACCAC	TGGAAACGGT	GGCTAATACC	GCATAACGTC	GCAAGACCAA	AGAGGGGGAC
Pantoea a. pv. *uredovora*	GGA-TCTGCC	CGATAGAGGG	GGATAACCAC	TGGAAACGGT	GGCTAATACC	GCATAACGTC	GCAAGACCAA	AGAGGGGGAC
Pantoea agglomerans (U80202)	GGA-TCTGCC	CGATAGAGGG	GGATAACCAC	TGGAAACGGT	GGCTAATACC	GCATAACGTC	GCAAGACCAA	AGAGGGGGAC
Pantoea agglomerans (AB004757)	GGA-TCTGCC	CGATAGAGGG	GGATAACCAC	TGGAAACGGT	GGCTAATACC	GCATAACGTC	GCAAGACCAA	AGAGGGGGAC
Pantoea agglomerans (U80183)	GGA-TCTGCC	CGATAGAGGG	GGATAACCAC	TGGAAACGGT	GGCTAATACC	GCATAACGTC	GCAAGACCAA	AGAGGGGGAC
Unknown *Erwinia* sp.	**GAAA-CTGCC**	**CGATGGAGGG**	**GGATAACTAC**	**TGGAAACGGT**	**AGCTAATACC**	**GCATAACGTC**	**GCAAGACCAA**	**AGTGGGGGAC**
Pantoea s. subsp. *stewartii*	GAAA-CTGCC	CGATGGAGGG	GGATAACTAC	TGGAAACGGT	AGCTAATACC	GCATAACGTC	GCAAGACCAA	AGTGGGGGAC
Erwinia tracheiphila	GAAA-CTGCC	TGATGGCGGG	GGATAACCAC	TGGAAACGGT	GGCTAATACC	GCATAATCTC	GCAAGAGCAA	AGAGGGGGAC
Erwinia psidii	GAAA-CTGCC	CGATGGAGGG	GGATAACTAC	TGGAAACGGT	AGCTAATACC	GCATAACGTC	GCAAGACCAA	AGTGGGGGAC
Erwinia mallotivora (AJ233414)	GAAA-CTGCC	CGATGGAGGG	GGATAACTAC	TGGAAACGGT	AGCTAATACC	GCATAACGTC	TTCGGACCAA	AGTGGGGGAC
Erwinia mallotivora (Z96084)	GAAC-CTGCC	CGATGGAGGG	GGATANCTAC	TGGAAACGGT	AGCTAATACC	NCATANCGTC	TTCGGACCAA	AGTGGGGGAC
Erwinia persicinus (AJ001190)	GAAA-CTGCC	CGATGGAGGG	GGATAACTAC	TGGAAACGGT	AGCTAATACC	GCATAACGTC	TTCGGACCAA	AGTGGGGGAC
Erwinia persicinus (Z96086)	GAAA-CTGCC	CGATGGAGGG	GGATAACTAC	TGGAAACGGT	AGCTAATACC	GCATAACGTC	TTCGGACCAA	AGTGGGGGAC
Erwinia persicinus (U80205)	GAAA-CTGCC	CGATGGAGGG	GGATAACTAC	TGGAAACGGT	AGCTAATACC	GCATAACGTC	TTCGGACCAA	AGTGGGGGAC
Erwinia rhapontici (AJ233417)	GAAA-CTGCC	CGATGGAGGG	GGATAACTAC	TGGAAACGGT	AGCTAATACC	GCATAACGTC	TTCGGACCAA	AGTGGGGGAC
Erwinia rhapontici (Z96087)	GAAA-CTGCC	CGATGGAGGG	GGATAACTAC	TGGAAACGGT	AGCTAATACC	GCATAACGTC	TTCGGACCAA	AGTGGGGGAC
Erwinia rhapontici (U80206)	GAAA-CTGCC	CGATGGAGGG	GGATAACTAC	TGGAAACGGT	AGCTAATACC	GCATAACGTC	TTCGGACCAA	AGTGGGGGAC
Erwinia amylovora (Z96088)	GAAA-CTGCC	CGATGGAGGG	GGATAACTAC	TGGAAACGGT	AGCTAATACC	GCATAACGTC	TACGGACCAA	AGTGGGGGAC
Erwinia amylovora (AJ233410)	GAAA-CTGCC	CGATGGAGGG	GGATAACTAC	TGGAAACGGT	AGCTAATACC	GCATAACGTC	TACGGACCAA	AGTGGGGGAC
Erwinia amylovora (AJ010485)	GAAA-CTGCC	CGATGGAGGG	GGATAACTAC	TGGAAACGGT	AGCTAATACC	GCATAACGTC	TACGGACCAA	AGTGGGGGAC
Erwinia amylovora (U80195)	GAAATCTGCC	CGATGGAGGG	GGATAACTAC	TGGAAACGGT	AGCTAATACC	GCATAACGTC	TACGGACCAA	AGTGGGGGAC
Erwinia pyrifoliae	GAAA-CTGCC	CGATGGAGGG	GGATAACTAC	TGGAAACGGT	AGCTAATACC	GCATAACGTC	TACGGACCAA	AGTGGGGGAC
Pectobacterium cypripedii	GNA-TCTGCC	TGATGGNGGG	GGATAACTAC	TGGAAACGGT	AGCTAATACC	GCATACCGTC	TNCGGNNCAA	AGTGGGGGAC
Pectobacterium chrysanthemi	GAAA-CTGCC	TGATGGAGGG	GGATAACTAC	TGGAAACGGT	AGCTAATACC	GCATAACGTC	TTCGGACCAA	AGAGGGGGAC
Pectobacterium c. subsp. *carotovorum*	GAAA-CTGCC	TGATGGAGGG	GGATAACTAC	TGGAAACGGT	AGCTAATACC	GCATAACCTC	GCAAGAGCAA	AGAGGGGGAC
Pectobacterium c. subsp. *wasabiae*	GAAA-CTGCC	TGATGGAGGG	GGATAACTAC	TGGAAACGGT	AGCTAATACC	GCATAACGTC	TTCGGACCAA	AGAGGGGGAC
Pectobacterium c. subsp. *betavasculorum*	GAAA-CTGCC	TGATGGAGGG	GGATAACTAC	TGGAAACGGT	AGCTAATACC	GCATAACGTC	TTCGGACCAA	AGAGGGGGAC
Pectobacterium c. subsp. *atrosepticum*	GAAA-CTGCC	TGATGGAGGG	GGATAACTAC	TGGAAACGGT	AGCTAATACC	GCATAACGTC	TTCGGACCAA	AGAGGGGGAC
Pectobacterium c. subsp. *odoriferum*	GAAA-CTGCC	TGATGGAGGG	GGATAACTAC	TGGAAACGGT	AGCTAATACC	GCATAACCTC	GCAAGAGCAA	AGAGGGGGAC
Pectobacterium cacticidum	GAAA-CTGCC	TGATGGAGGG	GGATAACTAC	TGGAAACGGT	AGCTAATACC	GCATAATGTC	GCAAGACCAA	AGAGGGGGAC
Brenneria salicis	GGA-TCTGCC	TGATGGAGGG	GGATAACTAC	TGGAAACGGT	AGCTAATACC	GCATGACGTC	TTCGGACCAA	AGTGGGGGAC
Brenneria rubrifaciens	GGA-TCTGCC	TGATGGAGGG	GGATAACCAC	TGGAAACGGT	GGCTAATACC	GCATGACGTC	GCAAGACCAA	AGTGGGGGAC
Brenneria nigrifluens	GAAA-CTGCC	TGATGGAGGG	GGATAACTAC	TGGAAACGGT	AGCTAATACC	GCATAACCTC	GCAAGAGCAA	AGTGGGGGAC
Brenneria alni	GAAA-CTGCC	TGATGGAGGG	GGATAACTAC	TGGAAACGGT	AGCTAATACC	GCATAATGTC	TTCGGACCAA	AGTGGGGGAC
Brenneria paradisiaca	GAAA-CTGCC	TGATGGAGGG	GGATAACTAC	TGGAAACGGT	AGCTAATACC	GCATAACGTC	TTCGGACCAA	AGTGGGGGCT

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                                                170        180        190        200        210        220        230        240
Buchnera aphidicola                      CTTTTTTAAA GGCCTCATGC TTT-TGGATG AACCC-AGAC GAGATTAGCT TGTTGGTAAG GTAAAAGCTT ACCAAGGCAA
Proteus vulgaris                         CTTCGGACCT TGCGCTATCG GATGAA---- --CCC-ATAT GGGATTAGCT AGTAGGTGAG GTAATGGCTC ACCTAGGCGA
Erwinia ananas, Zululand, KZN, SA        CTTCGGGCCT CTCACTATCG GAT------G AACCC-AGAT GGGATTAGCT AGTAGGCGGG GTAACGGCCC ACCTAGGCGA
Pantoea a. pv. ananatis                  CTTCGGGCCT CTCACTATCG GAT------G AACCC-AGAT GGGATTAGCT AGTAGGCGGG GTAACGGCCC ACCTAGGCGA
Pantoea a. pv. uredovora                 CTTCGGGCCT CTCACTATCG GAT------G AACCC-AGAT GGGATTAGCT AGTAGGCGGG GTAACGGCCC ACCTAGGCGA
Pantoea agglomerans (U80202)             CTTCGGGCCT CTCACTATCG GAT------G AACCC-AGAT GGGATTAGCT AGTAGGCGGG GTAATGGCCC ACCTAGGCGA
Pantoea agglomerans (AB004757)           CTTCGGGCCT CTCACTATCG GAT------G AACCC-AGAT GGGATTAGCT AGTAGGCGGG GTAATGGCCC ACCTAGGCGA
Pantoea agglomerans (U80183)             CTTCGGGCCT CTCACTATCG GAT------G AACCC-AGAT GGGATTAGCT AGTAGGCGGG GTAATGGCCC ACCTAGGCGA
Unknown Erwinia sp.                      CTTCGGGCCT CACACCATCG GATGTG---- --CCC-AGAT GGGATTAGCT AGTAGGCGGG GTAATGGCCC ACCTAGGCGA
Pantoea s. subsp. stewartii              CTCCGGGCCT CACACCATCG GATGTG---- --CCC-AGAT GGGATTAGCT AGTAGGCGGG GTAACGGCCC ACCTAGGCGA
Erwinia tracheiphila                     CTTATGGCCT CTTGCCATCG GATGTG---- --CCC-AGAT GGGATTAGCT GGCAGGTAGG GTAACGGCCT ACCTGGGCGA
Erwinia psidii                           CTTAGGGCCT CACACCATCG GATGTG---- --CCC-AGAT GGGATTAGCT TGTTGGTGGG GTAAAAGCTC ACCAAGGCGA
Erwinia mallotivora (AJ233414)           CTTCGGGCCT CACACCATCG GATGTG---- --CCC-AGAT GGGATTAGCT GGTTGGTGAG GTAACGGCTC ACCAAGGCGA
Erwinia mallotivora (Z96084)             CTTCGGGCCT CACACCATCG GATGTG---- --CCC-AGAT GGGATTAGCT GGTTGGTGAG GTAACGGCTC ACCAAGGCGA
Erwinia persicinus (AJ001190)            CTTCGGGCCT CACACCATCG GATGTG---- --CCC-AGAT GGGATTAGCT AGTAGGTGGG GTAACGGCTC ACCTAGGCGA
Erwinia persicinus (Z96086)              CTTCGGGCCT CACACCATCG GATGTG---- --CCC-AGAT GGGATTAGCT AGTAGGTGGG GTAACGGCTC ACCTAGGCGA
Erwinia persicinus (U80205)              CTTCGGGCCT CACACCATCG GATGTG---- --CCC-AGAT GGGATTAGCT AGTAGGTGGG GTAACGGCTC ACCTAGGCGA
Erwinia rhapontici (AJ233417)            CTTCGGGCCT CACACCATCG GATGTG---- --CCC-AGGT GGGATTAGCT AGTAGGTGGG GTAATGGCTC ACCTAGGCGA
Erwinia rhapontici (Z96087)              CTTCGGGCCT CACACCATCG GATGTG---- --CCC-AGGT GGGATTAGCT AGTAGGTGGG GTAATGGCTC ACCTAGGCGA
Erwinia rhapontici (U80206)              CTTCGGGCCT CACACCATCG GATGTG---- --CCC-AGGT GGGATTAGCT AGTAGGTGGG GTAATGGCTC ACCTAGGCGA
Erwinia amylovora (Z96088)               CTTCGGGCCT CACACCATCG GATGTG---- --CCC-AGAT GGGATTAGCT GGTAGGTGGG GTAANGGCTC ACCTAGGCGA
Erwinia amylovora (AJ233410)             CTTCGGGCCT CACACCATCG GATGTG---- --CCC-AGAT GGGATTAGCT GGTAGGTGGG GTAACGGCTC ACCTAGGCGA
Erwinia amylovora (AJ010485)             CTTCGGGCCT CACACCATCG GATGTG---- --CCC-AGAT GGGATTAGCT AGTAGGTGGG GTAACGGCTC ACCTAGGCGA
Erwinia amylovora (U80195)               CTTCGGGCCT CACACCATCG GATGTG---- --CCC-AGAT GGGATTAGCT GGTAGGTGAG GTAATGGCTC ACCTAGGCGA
Erwinia pyrifoliae                       CTTCGGGCCT CACACCATCG GATGTG---- --CCC-AGAT GGGATTAGCT GGTAGGTGGG GTAACGGCTC ACCTAGGCGA
Pectobacterium cypripedii                CTTCGGGCCT CATGCCATCN GATGTG---- --CCC-AGAT GGGATTAGCT AGTAGGTGGG GTAAAGGCTC ACCTAGGCGA
Pectobacterium chrysanthemi              CTTCGGGCCT CTTGCCATCG GATGTG---- --CCC-AGAT GGGATTAGCT AGTAGGTGGG GTAAAGGCTC ACCTAGGCGA
Pectobacterium c. subsp. carotovorum     CTTNGGGCCT CTCGCCATCA GATGTG---- --CCC-AGAT GGGATTAGCT AGTAGGTGAG GTAATGGCTC ACCTAGGCGA
Pectobacterium c. subsp. wasabiae        CTTCGGGCCT CTTGCCATCG GATGTG---- --CCC-AGAT GGGATTAGCT AGTAGGTGAG GTAATGGCTC ACCTAGGCGA
Pectobacterium c. subsp. betavasculorum  CTTCGGGCCT CTTGCCATCG GATGTG---- --GCCCAGAT GGGATTAGCT AGTAGGCGGG GTAATGGCCC ACCTAGGCGA
Pectobacterium c. subsp. atrosepticum     CTTCGGGCCT CTTGCCATCA GATGTG---- --CCC-AGAT GGGATTAGCT AGTAGGCGGG GTAATGGCCC ACCTAGGCGA
Pectobacterium c. subsp. odoriferum      CTTCGGGCCT CTCGCCATCA GATGTG---- --CCC-AGAT GGGATTAGCT AGTAGGTGAG GTAATGGCTC ACCTAGGCGA
Pectobacterium cacticidum                CTTAGGGCCT CTTGCCATCG GATGTG---- --CCC-AGAT GGGATTAGCT AGTAGGTGGG GTAAAGGCTC ACCTAGGCGA
Brenneria salicis                        CTTCGGGCCT CACGCCATGA GAT------G AACCC-AGAT GGGATTAGCT GGTAGGTGAG GTAACGGCTC ACCTAGGCGA
Brenneria rubrifaciens                   CTTCGGGCCT CACGCCATCG GAT------G AACCC-AGAT GGGATTAGCT AGTAGGCGGG GTAATGGCCC ACCTAGGCGA
Brenneria nigrifluens                    CTTATGGCCT CACGCCATCG GATGTG---- --CCC-AGAT GGGATTAGCT GGTAGGTGGG GTAAAGGCTC ACCTAGGCGA
Brenneria alni                           CTTAGGGCCT CACGCCATCG GATGTG---- --CCC-AGAT GGGATTAGCT GGTAGGTGGG GTAAAGGCTC ACCTAGGCGA
Brenneria paradisiaca                    CTTCGGACCT CATGCCATCG GATGTG---- --CCC-AGAT GGGATTAGCT AGTAGGCGGG GTAAAGGCCC ACCTAGGCGA
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	250	260	270	280	290	300	310	320
Buchnera aphidicola	CGATCTCTAG	CTGGTCTGAG	AGGATAACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTC
Proteus vulgaris	CGATCTCTAG	CTGGTCTGAG	AGGATGATCA	GCCACACTGG	GACTGAGACA	CGGCCCAGAC	TCCTACGGGA	GGCAGCAGTG
***Erwinia ananas*, Zululand, KZN, SA**	**CGATCCCTAG**	**CTGGTCTGAG**	**AGGATGACCA**	**GCCACACTGG**	**AACTGAGACA**	**CGGTCCAGAC**	**TCCTACGGGA**	**GGCAGCAGTG**
Pantoea a. pv. *ananatis*	CGATCCCTAG	CTGGTCTGAG	AGGATGACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTG
Pantoea a. pv. *uredovora*	CGATCCCTAG	CTGGTCTGAG	AGGATGACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTG
Pantoea agglomerans (U80202)	CGATCCCTAG	CTGGTCTGAG	AGGATGACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTG
Pantoea agglomerans (AB004757)	CGATCCCTAG	CTGGTCTGAG	AGGATGACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTG
Pantoea agglomerans (U80183)	CGATCCCTAG	CTGGTCTGAG	GGGATGACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TC-TACGGGA	GGCAGCAGTG
Unknown *Erwinia* sp.	**CGATCCCTAG**	**CTGGTCTGAG**	**AGGATGACCA**	**GCCACACTGG**	**AACTGAGACA**	**CGGTCCAGAC**	**TCCTACGGGA**	**GGCAGCAGTG**
Pantoea s. subsp. *stewartii*	CGATCCCTAG	CTGGTCTGAG	AGGATGACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTG
Erwinia tracheiphila	CGATCCCTAG	CTGGTCTGAG	AGGATGACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTG
Erwinia psidii	CGATCCCTAG	CTGGTCTGAG	AGGATGACCA	GCCACACTGG	AACTGAGA-A	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTG
Erwinia mallotivora (AJ233414)	CGATCCCTAG	CTGGTCTGAG	AGGATGACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTG
Erwinia mallotivora (Z96084)	CGATCCCTAG	CTGGTCTGAG	AGGATGACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTG
Erwinia persicinus (AJ001190)	CGATCCCTAG	CTGGTCTGAG	AGGATGACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTG
Erwinia persicinus (Z96086)	CGATCCCTAG	CTGGTCTGAG	AGGATGACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTG
Erwinia persicinus (U80205)	CGATCCCTAG	CTGGTCTGAG	AGGATGACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTG
Erwinia rhapontici (AJ233417)	CGATCCCTAG	CTGGTCTGAG	AGGATGACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTG
Erwinia rhapontici (Z96087)	CGATCCCTAG	CTGGTCTGAG	AGGATGACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTG
Erwinia rhapontici (U80206)	CGATCCCTAG	CTGGTCTGAG	AGGATGACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTG
Erwinia amylovora (Z96088)	CGATCCCTAG	CTGGTCTGAG	AGGATGACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTG
Erwinia amylovora (AJ233410)	CGATCCCTAG	CTGGTCTGAG	AGGATGACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTG
Erwinia amylovora (AJ010485)	CGATCCCTAG	CTGGTCTGAG	AGGATGACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTG
Erwinia amylovora (U80195)	CGATCCCTAG	CTGGTCTGAG	AGGATGACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTG
Erwinia pyrifoliae	CGATCCCTAG	CTGGTCTGAG	AGGATGACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTG
Pectobacterium cypripedii	CGATCCCTAG	CTGGTCTGAG	AGGATGACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTG
Pectobacterium chrysanthemi	CGATCCCTAG	CTGGTCTGAG	AGGATGACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTG
Pectobacterium c. subsp. *carotovorum*	CGATCCCTAG	CTGGTCTGAG	AGGATGACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTG
Pectobacterium c. subsp. *wasabiae*	CGATCCCTAG	CTGGTCTGAG	AGGATGACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTG
Pectobacterium c. subsp. *betavasculorum*	CGATCCCTAG	CTGGTCTGAG	AGGATGACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTG
Pectobacterium c. subsp. *atrosepticum*	CGATCCCTAG	CTGGTCTGAG	AGGATGACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTG
Pectobacterium c. subsp. *odoriferum*	CGATCCCTAG	CTGGTCTGAG	AGGATGACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTG
Pectobacterium cacticidum	CGATCCCTAG	CTGGTCTGAG	AGGATGACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTG
Brenneria salicis	CGATCCCTAG	CTGGTCTGAG	AGGATGACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTG
Brenneria rubrifaciens	CGATCCCTAG	CTGGTCTGAG	AGGATGACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTG
Brenneria nigrifluens	CGATCCCTAG	CTGGTCTGAG	AGGATGACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTG
Brenneria alni	CGATCCCTAG	CTGGTCTGAG	AGGATGACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTG
Brenneria paradisiaca	CGATCCCTAG	CTGGTCTGAG	AGGATGACCA	GCCACACTGG	AACTGAGACA	CGGTCCAGAC	TCCTACGGGA	GGCAGCAGTG

	330	340	350	360	370	380	390	400
Buchnera aphidicola	GGGAATATTG	CACAATGGGC	GAAAGCCTG-	ATGCAGCTAT	GCCGCGTGTA	TGAAGAAGGC	CTTAGGGTTG	TAAAGTACTT
Proteus vulgaris	GGCAATATTG	CACAATGGGC	GCAAGCCTG-	ATGCAGCCAT	GCCGCGTGTA	TGAAGAAGGC	CTTAGGGTTG	TAAAGTACTT
***Erwinia ananas*, Zululand, KZN, SA**	**GGGAATATTG**	**CACAATGGGC**	**GCAAGCCTG-**	**ATGCAGCCAT**	**GCCGCGTGTA**	**TGAAGAAGGC**	**CTTCGGGTTG**	**TAAAGTACTT**
Pantoea a. pv. *ananatis*	GGGAATATTG	CACAATGGGC	GCAAGCCTG-	ATGCAGCCAT	GCCGCGTGTA	TGAAGAAGGC	CTTCGGGTTG	TAAAGTACTT
Pantoea a. pv. *uredovora*	GGGAATATTG	CACAATGGGC	GCAAGCCTG-	ATGCAGCCAT	GCCGCGTGTA	TGAAGAAGGC	CTTCGGGTTG	TAAAGTACTT
Pantoea agglomerans (U80202)	GGGAATATTG	CACAATGGGC	GCAAGCCTG-	ATGCAGCCAT	GCCGCGTGTA	TGAAGAAGGC	CTTCGGGTTG	TAAAGTACTT
Pantoea agglomerans (AB004757)	GGGAATATTG	CACAATGGGC	GCAAGCCTG-	ATGCAGCCAT	GCCGCGTGTA	TGAAGAAGGC	CTTCGGGTTG	TAAAGTACTT
Pantoea agglomerans (U80183)	GGGAATATTG	CACAATGGGC	GCAAGCCTG-	ATGCAGCCAT	GCCGCGTGTA	TGAAGAAGGC	CTTCGGGTTG	TAAAGTACTT
Unknown *Erwinia* sp.	**GGGAATATTG**	**CACAATGGGC**	**GCAAGCCTG-**	**ATGCAGCCAT**	**GCCGCGTGTA**	**TGAAGAAGGC**	**CTTCGGGTTG**	**TAAAGTACTT**
Pantoea s. subsp. *stewartii*	GGGAATATTG	CACAATGGGC	GCAAGCCTG-	ATGCAGCCAT	GCCGCGTGTA	TGAAGAAGGC	CTTCGGGTTG	TAAAGTACTT
Erwinia tracheiphila	GGGAATATTG	CACAATGGGC	GCAAGCCTG-	ATGCAGCCAT	GCCGCGTGTA	TGAAGAAGGC	CTTCGGGTTG	TAAAGTACTT
Erwinia psidii	GGGAATATTG	CACAATGGGC	GCAAGCCTG-	ATGCANCCAT	GCCGCGTGTA	TGAAGAAGGC	CTTCGGGTTG	TAAAGTACTT
Erwinia mallotivora (AJ233414)	GGGAATATTG	CACAATGGGC	GCAAGCCTG-	ATGCAGCCAT	GCCGCGTGTA	TGAAGAAGGC	CTTCGGGTTG	TAAAGTACTT
Erwinia mallotivora (Z96084)	GGGAATATTG	CACAATGGGC	GCNAGCCTG-	ATGCAGCCAT	GCCGCGTGTA	NGAAGAAGGC	CTTCGGGTTG	TAAAGNACTT
Erwinia persicinus (AJ001190)	GGGAATATTG	CACAATGGGC	GCAAGCCTG-	ATGCAGCCAT	GCCGCGTGTA	TGAAGAAGGC	CTTCGGGTTG	TAAAGTACTT
Erwinia persicinus (Z96086)	GGGAATATTG	CACAATGGGC	GCAAGCCTG-	ATGCAGCCAT	GCCGCGTGTA	TGAAGAAGGC	CTTCGGGTTG	TAAAGTACTT
Erwinia persicinus (U80205)	GGGAATATTG	CACAATGGGC	GCAAGCCTG-	ATGCAGCCAT	GCCGCGTGTA	TGAAGAAGGC	CTTCGGGTTG	TAAAGTACTT
Erwinia rhapontici (AJ233417)	GGGAATATTG	CACAATGGGC	GCAAGCCTG-	ATGCAGCCAT	GCCGCGTGTA	TGAAGAAGGC	CTTCGGGTTG	TAAAGTACTT
Erwinia rhapontici (Z96087)	GGGAATATTG	CACAATGGGC	GCAAGCCTG-	ATGCAGCCAT	GCCGCGTGTA	TGAAGAAGGC	CTTCGGGTTG	TAAAGTACTT
Erwinia rhapontici (U80206)	GGGAATATTG	CACAATGGGC	GCAAGCCTG-	ATGCAGCCAT	GCCGCGTGTA	TGAAGAAGGC	CTTCGGGTTG	TAAAGTACTT
Erwinia amylovora (Z96088)	GGGAATATTG	CACAATGGGC	GCAAGCCTG-	ATGCAGCCAT	GCCGCGTGTA	TGAAGAAGGC	CTTCGGGTTG	TAAAGTACTT
Erwinia amylovora (AJ233410)	GGGAATATTG	CACAATGGGC	GCAAGCCTG-	ATGCAGCCAT	GCCGCGTGTA	TGAAGAAGGC	CTTCGGGTTG	TAAAGTACTT
Erwinia amylovora (AJ010485)	GGGAATATTG	CACAATGGGC	GCAAGCCTG-	ATGCAGCCAT	GCCGCGTGTA	TGAAGAAGGC	CTTCGGGTTG	TAAAGTACTT
Erwinia amylovora (U80195)	GGGAATATTG	CACAATGGGC	GCAAGCCTG-	ATGCAGCCAT	GCCGCGTGTA	TGAAGAAGGC	CTTCGGGTTG	TAAAGTACTT
Erwinia pyrifoliae	GGGAATATTG	CACAATGGGC	GCAAGC-TG-	ATGCAGCCAT	GCCGCGTGTA	TGAAGAAGGC	CTTCGGGTTG	TAAAGTACTT
Pectobacterium cypripedii	GGGAATATTG	CACAATGGNC	GNAAGCCTG-	ATGCAGCCAT	GCCGCGTGTG	TGAAGAAGGC	CTTCGGGTTG	TAAAGCACTT
Pectobacterium chrysanthemi	GGGAATATTG	CACAATGGGC	GCAAGCCTG-	ATGCAGCCAT	GCCGCGTGTG	TGAAGAAGGC	CTTCGGGTTG	TAAAGCACTT
Pectobacterium c. subsp. *carotovorum*	GGGAATATTG	CACAATGGGC	GCAAGCCTG-	ATGCAGCCAT	GCCGCGTGTG	TGAAGAAGGC	CTTCGGGTTG	TAAAGCACTT
Pectobacterium c. subsp. *wasabiae*	GGGAATATTG	CACAATGGGN	GCAAGCCTG-	ATGCAGCCAT	GCCGCGTGTG	TGAAGAAGGC	CTTCGGGTTG	TAAAGCACTT
Pectobacterium c. subsp. *betavasculorum*	GGGAATATTG	CACAATGGGC	GCAAGCCTG-	ATGCAGCCAT	GCCGCGTGTG	TGAAGAAGGC	CTTCGGGTTG	TAAAGCACTT
Pectobacterium c. subsp. *atrosepticum*	GGGAATATTG	CACAATGGGC	GCAAGCCTG-	ATGCAGCCAT	GCCGCGTGTG	TGAAGAAGGC	CTTCGGGTTG	TAAAGCNCTT
Pectobacterium c. subsp. *odoriferum*	GGGAATATTG	CACAATGGGC	GCAAGCCTG-	ATGCAGCCAT	GCCGCGTGTG	TGAAGAAGGC	CTTCGGGTTG	TAAAGCACTT
Pectobacterium cacticidum	GGGAATATTG	CACAATGGGC	GCAAGCCTG-	ATGCAGCCAT	GCCGCGTGTG	TGAAGAAGGC	CTTCGGGTTG	TAAAGCACTT
Brenneria salicis	GGGAATATTG	CACAATGGGG	GAAACCCTG-	ATGCAGCCAT	GCCGCGTGTG	TGAAGAAGGC	CTTCGGGTTG	TAAAGCACTT
Brenneria rubrifaciens	GGGAATATTG	CACAATGGGG	GAAACCCTG-	ATGCAGCCAT	GCCGCGTGTG	TGAAGAAGGC	CTTCGGGTTG	TAAAGCACTT
Brenneria nigrifluens	GGGAATATTG	CACAATGGGG	GAAACCCTG-	ATGCAGCCAT	GCCGCGTGTG	TGAAGAAGGC	CTTCGGGTTG	TAAAGCACTT
Brenneria alni	GGGAATATTG	CACAATGGGG	GAAACCCTG-	ATGCAGCCAT	GCCGCGTGTG	TGAAGAAGGC	CTTCGGGTTG	TAAAGCACTT
Brenneria paradisiaca	GGGAATATTG	CACAATGGGG	GAAACCCTG-	ATGCAGCCAT	GCCGCGTGTG	TGAAGAAGGC	CTTCGGGTTG	TAAAGCACTT

	410	420	430	440	450	460	470	480
Buchnera aphidicola	TCAGCGGGGA	GGAAAAAATT	---AAAACTA	ATAA--TTTT	A-TTTGTGA	CGTTACCCGC	AGAAGAAGCA	CCGGCTAACT
Proteus vulgaris	TCAGCGGGGA	GGAAGGTGAT	---AAAGTTA	ATA-CCTTTG	TCAA--TTGA	CGTTACCCGC	AGAAGAAGCA	CCGGCTAACT
***Erwinia ananas*, Zululand, KZN, SA**	**TCAGCGGGGA**	**GGAAGGCGAT**	**GT---GGTTA**	**ATAACCGCAT**	**T-GA--TTGA**	**CGTTACCCGC**	**AGAAGAAGCA**	**CCGGCTAACT**
Pantoea a. pv. *ananatis*	TCAGCGGGGA	GGAAGGCGAT	GT---GGTTA	ATAACCGCAT	T-GA--TTGA	CGTTACCCGC	AGAAGAAGCA	CCGGCTAACT
Pantoea a. pv. *uredovora*	TCAGCGGGGA	GGAAGGCGAT	GT---GGTTA	ATAACCGCAT	T-GA--TTGA	CGTTACCCGC	AGAAGAAGCA	CCGGCTAACT
Pantoea agglomerans (U80202)	TCAGCGGGGA	GGAAGGCGAC	G----GGTTA	ATAACCCT-G	TCGA--TTGA	CGTTACCCGC	AGAAGAAGCA	CCGGCTAACT
Pantoea agglomerans (AB004757)	TCAGCGGGGA	GGAAGGCGAT	GG---GGTTA	ATAACCGC-G	TCGA--TTGA	CGTTACCCGC	AGAAGAAGCA	CCGGCTAACT
Pantoea agglomerans (U80183)	TCAGCGGGGA	GGAAGGCGAC	G----GGTTA	ATAACCCT-G	TCGA--TTGA	CGTTACCCGC	AGAAGAAGCA	CCGGCTAACT
Unknown *Erwinia* sp.	**TCAGCGAGGA**	**GGAAGGC--T**	**GGTG-GGTTA**	**ATAACCTGA-**	**TC-A-ATTGA**	**CGTTACCCGC**	**AGAAGAAGCA**	**CCGGCTAACT**
Pantoea s. subsp. *stewartii*	TCAGCGGGGA	GGAAGG---T	GGTGAGGTTA	ATAACCTCA-	TC-A-ATTGA	CATTACCCGC	AGAAGAAGCA	CCGGCTAACT
Erwinia tracheiphila	TCAGCGGGGA	GGAAGG-GAC	GCT--GGTTA	ATAACCAGCG	TC-A--TTGA	TGTTACCCGC	ANAANAAGCA	CCGGCTAACT
Erwinia psidii	TCAGCGGGGA	GGAAGG---T	GGTGAGGTTA	ATAACCTTA-	TC-A-ATTGA	CGTTACCCGC	AGAAGAAGCA	CCGGCTAACT
Erwinia mallotivora (AJ233414)	TCAGCGGGGA	GGAAGG---T	GGTGGGGTTA	ATAACCTTA-	TC-A-ATTGA	CGTTACCCGC	AGAAGAAGCA	CCGGCTAACT
Erwinia mallotivora (Z96084)	TCAGCGGGGA	GGAAGG---T	GGTGGGGTTA	ATAACCTTA-	TC-A-ATTGA	CGTTACCCGC	AGAAGAAGCA	CCGGCTAACT
Erwinia persicinus (AJ001190)	TCAGTGGGGA	GGAAGGCGA-	-TGAA-GTTA	ATAACTTCG-	TCGA--TTGA	CGTTACCCGC	AGAAGAAGCA	CCGGCTAACT
Erwinia persicinus (Z96086)	TCAGTGGGGA	GGAAGGCGA-	-TGAA-GTTA	ATAACTTCG-	TCGA--TTGA	CNTTACCCGC	AGAAGAAGCA	CCGGCTAACT
Erwinia persicinus (U80205)	TCAGTGGGGA	GGAAGGCGA-	-TGAA-GTTA	ATAACTTCG-	TCGA--TTGA	CGTTACCCGC	AGAAGAAGCA	CCGGCTAACT
Erwinia rhapontici (AJ233417)	TCAGTGGGGA	GGAAGGCGA-	-TGA-GGTTA	ATAGCCTCG-	TCGA--TTGA	CGTTACCCGC	AGAAGAAGCA	CCGGCTAACT
Erwinia rhapontici (Z96087)	TCAGTGGGGA	GGAAGGCGA-	-TGA-GGTTA	ATAGCCTCG-	NCGA--TTGA	CGTTACCCGC	AGAAGAAGCA	CCGGCTAACT
Erwinia rhapontici (U80206)	TCAGTGGGGA	GGAAGGCGA-	-TGA-GGTTA	ATAGCCTCG-	TCGA--TTGA	CGTTACCCGC	AGAAGAAGCA	CCGGCTAACT
Erwinia amylovora (Z96088)	TCAGCGGGGA	GGAAGGGGAA	--GA-GGTTA	ATAACCTCC-	TCCA--TTGA	CGTTACCCGC	AGAAGAAGCA	CCGGCTAACT
Erwinia amylovora (AJ233410)	TCAGCGGGGA	GGAAGGGGAA	--GA-GGTTA	ATAACCTCC-	TCCA--TTGA	CGTTACCCGC	AGAAGAAGCA	CCGGCTAACT
Erwinia amylovora (AJ010485)	TCAGCGGGGA	GGAAGGGGAA	--GA-GGTTA	ATAACCTTT-	TCCA--TTGA	CGTTACCCGC	AGAAGAAGCA	CCGGCTAACT
Erwinia amylovora (U80195)	TCAGCGGGGA	GGAAGGGGAA	--GA-GGTTA	ATAACCTTT-	TCCA--TTGA	CGTTACCCGC	AGAAGAAGCA	CCGGCTAACT
Erwinia pyrifoliae	TCAGCGGGGA	GGAAGGGGGA	---AAGGTTA	ATAACCTTTT	TC-A--TTGA	CGTTACCCGC	AGAAGAAGCA	CCGGCTAACT
Pectobacterium cypripedii	TCAGCGGGGA	AGAAGGGGA-	-TAA-GGTTA	ATACCCTTG-	TNNA--TTGA	CGTTACCCGC	AGAAGAAGCA	CCGGCTAACT
Pectobacterium chrysanthemi	TCAGCGGGGA	GGAAGGGAA-	--CAAGGTTA	ATACCTTTGT	TC-A--TTGA	CGTTACCCGC	AGAAGAAGCA	CCGGCTAACT
Pectobacterium c. subsp. *carotovorum*	TCAGCGAGGA	GGAAGGCGG-	-T-AAGGTTA	ATAACCTTA-	TCGA--TTGA	CGTTACTCGC	AGAANAAGCA	CCGGCTAACT
Pectobacterium c. subsp. *wasabiae*	TCAGCGGGGA	GGAAGGCAG-	-T-AAGGTTA	ATAACCTTG-	CTGA--TTGA	CGTTACCCGC	AGAAGAAGCA	CCGGCTAACT
Pectobacterium c. subsp. *betavasculorum*	TCAGCGGGGA	GGAAGGCAG-	-T-AAGGCTA	ATAACCTTA-	TTGA--TTGA	CGTTACCCGC	AGAAGAAGCA	CCGGCTAACT
Pectobacterium c. subsp. *atrosepticum*	TCNGCGGGGA	GGAAGGCAG-	-T-AAGGTTA	ATAACCTTG-	CTGA--TTGA	CGTTACCCGC	AGAAGAAGCA	CCGGCTAACT
Pectobacterium c. subsp. *odoriferum*	TCAGCGAGGA	GGAAGGCAG-	-T-CNTGTTA	ATANCACGG-	NTGA--TTGA	CGTTACTCGC	AGAAGAAGCA	CCGGCTAACT
Pectobacterium cacticidum	TCAGCGGGGA	GGAAGGCN--	-TGAAAGCGA	ATACCTTTCA	T-GA--TTGA	CNTTACCCGC	AGAANAAGCA	CCGGCTAACT
Brenneria salicis	TCAGCGGGGA	GGAAGGCGA-	--TAAACTTA	ATAAGTTTGT	T-GA--TTGA	CGTTACCCGC	AGAAGAAGCA	CCGGCTAACT
Brenneria rubrifaciens	TCAGCGGGGA	GGAAGGGGA-	--AAGGTTTA	AGAGACTTTT	TC-A--TTGA	CGTTACCCGC	AGAAGAAGCA	CCGGCTAACT
Brenneria nigrifluens	TCAGCGGGGA	GGAAGGCAA-	--CAAGCTTA	ATAATCTTGT	TC-A--TTGA	CGTTACCCGC	AGAAGAAGCA	CCGGCTAACT
Brenneria alni	TCAGCGAGGA	GGAAGGCGG-	--TAAGGTTA	ATAACCTTA-	TCGA--TTGA	CGTTACTCGC	AGAAGAAGCA	CCGGCTAACT
Brenneria paradisiaca	TCAGCGGGGA	GGAAGGGGAC	----AGGCTT	AATACGTCTG	TTCA--TTGA	CGTTACCCGC	AGAAGAAGCA	CCGGCTAACT

	490	500	510	520	530	540	550	560
Buchnera aphidicola	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCGAGCGTT	AATCAGAATT	ACTGGGCGTA	AAGAGCACGT	AGGTGGTTTT
Proteus vulgaris	CCGTGCCAGC	AGCCGCGCTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATT	ACTGGGCGTA	AAGCGCACGC	AGGCGG-CAA
***Erwinia ananas*, Zululand, KZN, SA**	**CCGTGCCAGC**	**AGCCGCGGTA**	**ATACGGAGGG**	**TGCAAGCGTT**	**AATCGGAATT**	**ACTGGGCGTA**	**AAGCGCACGC**	**AGGCGGTCTG**
Pantoea a. pv. *ananatis*	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATT	ACTGGGCGTA	AAGCGCACGC	AGGCGGTCTG
Pantoea a. pv. *uredovora*	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATT	ACTGGGCGTA	AAGCGCACGC	AGGCGGTCTG
Pantoea agglomerans (U80202)	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATT	ACTGGGCGTA	AAGCGCACGC	AGGCGGTCTG
Pantoea agglomerans (AB004757)	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATT	ACTGGGCGTA	AAGCGCACGC	AGGCGGTCTG
Pantoea agglomerans (U80183)	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATT	ACTGGGCGTA	AAGCGCACGC	AGGCGGTCTG
Unknown *Erwinia* sp.	**CCGTGCCAGC**	**AGCCGCGGTA**	**ATACGGAGGG**	**TGCAAGCGTT**	**AATCGGAATT**	**ACTGGGCGTA**	**AAGCGCACGC**	**AGGCGGTCTG**
Pantoea s. subsp. *stewartii*	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATT	ACTGGGCGTA	AAGCGCACGC	AGGCGGTCTG
Erwinia tracheiphila	CCGTGCCANC	ANCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATT	ACTGGGCGTA	AAGCGCACGC	AGGCGGTCTG
Erwinia psidii	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATT	ACTGGGCGTA	AAGCGCACGC	AGGCGGTCTG
Erwinia mallotivora (AJ233414)	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATT	ACTGGGCGTA	AAGCGCACGC	AGGCGGTCTG
Erwinia mallotivora (Z96084)	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATT	ACTGGGCGTA	AAGCGCACGC	AGGCGGTCTG
Erwinia persicinus (AJ001190)	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATT	ACTGGGCGTA	AAGCGCACGC	AGGCGGTCTG
Erwinia persicinus (Z96086)	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATT	ACTGGGCGTA	AAGCGCACGC	AGGCGGTCTG
Erwinia persicinus (U80205)	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATT	ACTGGGCGTA	AAGCGCACGC	AGGCGGTCTG
Erwinia rhapontici (AJ233417)	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATT	ACTGGGCGTA	AAGCGCACGC	AGGCGGTCTG
Erwinia rhapontici (Z96087)	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATT	ACTGGGCGTA	AAGCGCACGC	AGGCGGTCTG
Erwinia rhapontici (U80206)	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATT	ACTGGGCGTA	AAGCGCACGC	AGGCGGTCTG
Erwinia amylovora (Z96088)	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATT	ACTGGGCGTA	AAGCGCACGC	AGGCGGTCTG
Erwinia amylovora (AJ233410)	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATT	ACTGGGCGTA	AAGCGCACGC	AGGCGGTCTG
Erwinia amylovora (AJ010485)	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATT	ACTGGGCGTA	AAGCGCACGC	AGGCGGTCTG
Erwinia amylovora (U80195)	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATT	ACTGGGCGTA	AAGCGCACGC	AGGCGGTCTG
Erwinia pyrifoliae	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATT	ACTGGGCGTA	AAGCGCACGC	AGGCGGTCTG
Pectobacterium cypripedii	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATN	ACTGGGCGTA	AAGCGCACGC	AGGCGGTCTG
Pectobacterium chrysanthemi	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATG	ACTGGGCGTA	AAGCGCACGC	AGGCGGTCTG
Pectobacterium c. subsp. *carotovorum*	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATG	ACTGGGCGTA	AAGCGCACGC	AGGCGGTCTG
Pectobacterium c. subsp. *wasabiae*	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATG	ACTGGGCGTA	AAGCGCACGC	AGGCGGTCTG
Pectobacterium c. subsp. *betavasculorum*	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATG	ACTGGGCGTA	AAGCGCACGC	AGGCGGTCTG
Pectobacterium c. subsp. *atrosepticum*	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATG	ACTGGGCGTA	AAGCGCACGC	AGGCGGTCTG
Pectobacterium c. subsp. *odoriferum*	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATG	ACTGGGCGTA	AAGCGCACGC	AGGCGGTCTG
Pectobacterium cacticidum	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATG	ACTGGGCGTA	AAGCGCACGC	AGGCGGTTTG
Brenneria salicis	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATG	ACTGGGCGTA	AAGCGCACGC	AGGCGGTGTG
Brenneria rubrifaciens	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATG	ACTGGGCGTA	AAGCGCACGC	AGGCGGTCTG
Brenneria nigrifluens	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATG	ACTGGGCGTA	AAGCGCACGC	AGGCGGTCTG
Brenneria alni	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATG	ACTGGGCGTA	AAGCGCACGC	AGGCGGTCTG
Brenneria paradisiaca	CCGTGCCAGC	AGCCGCGGTA	ATACGGAGGG	TGCAAGCGTT	AATCGGAATG	ACTGGGCGTA	AAGCGCACGC	AGGCGGTCTG

	570	580	590	600	610	620	630	640
Buchnera aphidicola	TTAAGTCAGA	TGTGAAATCC	CTAGGCTTAA	CCTAGGAACT	GCATTTGAAA	CTGAAATGCT	AGAGTATCGT	AGAGGGAGGT
Proteus vulgaris	TTAAGTCAGA	TGTGAAAGCC	CCGAGCTCAA	CTTGGGAACT	GCATCTGAAA	CTGGCTGGCT	AGAGTCTTGT	AGAGGGGGGT
***Erwinia ananas*, Zululand, KZN, SA**	**TTAAGTCAGA**	**TGTGAAATCC**	**CCGGGCTTAA**	**CCTGGGAACT**	**GCATTTGAAA**	**CTGGCAGGCT**	**TGAGTCTCGT**	**AGAGGGGGGT**
Pantoea a. pv. *ananatis*	TTAAGTCAGA	TGTGAAATCC	CCGGGCTTAA	CCTGGGAACT	GCATTTGAAA	CTGGCAGGCT	TGAGTCTCGT	AGAGGGGGGT
Pantoea a. pv. *uredovora*	TTAAGTCAGA	TGTGAAATCC	CCGGGCTTAA	CCTGGGAACT	GCATTTGAAA	CTGGCAGGCT	TGAGTCTCGT	AGAGGGGGGT
Pantoea agglomerans (U80202)	TTAAGTCAGA	TGTGAAATCC	CCGGGCTTAA	CCTGGGAACT	GCATTTGAAA	CTGGCAGGCT	TGAGTCTTGT	AGAGGGGGGT
Pantoea agglomerans (AB004757)	TTAAGTCAGA	TGTGAAATCC	CCGGGCTTAA	CCTGGGAACT	GCATTTGAAA	CTGGCAGGCT	TGAGTCTTGT	AGAGGGGGGT
Pantoea agglomerans (U80183)	TTAAGTCAGA	TGTGAAATCC	CCGGGCTTAA	CCTGGGAACT	GCATTTGAAA	CTGGCAGGCT	TGAGTCTTGT	AGAGGGGGGT
Unknown *Erwinia* sp.	**TTAAGTCAGA**	**TGTGAAATCC**	**CCGGGCTTAA**	**CCTGGGAACT**	**GCATTTGAAA**	**CTGGCAGGCT**	**TGAGTCTCGT**	**AGAGGGGGGT**
Pantoea s. subsp. *stewartii*	TTAAGTCAGA	TGTGAAATCC	CCGGGCTTAA	CCTGGGAACT	GCATTTGAAA	CTGGCAGGCT	TGAGTCTCGT	AGAGGGGGGT
Erwinia tracheiphila	TTAAGTCAGA	TGTGAAATCC	CCGGGCTTAA	CCTGGGAACT	GCATTTGAAA	CTGGCAGGCT	GGAGTCTTGT	AGAGGGGGGT
Erwinia psidii	TTAAGTCAGA	TGTGAAATCC	CCGGGCTTAA	CCTGGGAACT	GCATTTGAAA	CTGGCAGGCT	TGAGTCTTGT	AGAGGGGGGT
Erwinia mallotivora (AJ233414)	TTAAGTCAGA	TGTGAAATCC	CCGGGCTTAA	CCTGGGAACT	GCATTTGAAA	CTGGCAGGCT	TGAGTCTTGT	AGAGGGGGGT
Erwinia mallotivora (Z96084)	TTAAGTCAGA	TGTGAAATCC	CCGGGCTTAA	CCTGGGAACT	GCATTTGAAA	CTGGCAGGCT	TGAGTCTTGT	AGAGGGGGGT
Erwinia persicinus (AJ001190)	TCAAGTCGGA	TGTGAAATCC	CCGGGCTCAA	CCTGGGAACT	GCATTCGAAA	CTGGCAGGCT	AGAGTCTTGT	AGAGGGGGGT
Erwinia persicinus (Z96086)	TCAAGTCGGA	TGTGAAATCC	CCGGGCTCAA	CCTGGGAACT	GCATTCGAAA	CTGGCANGCT	AGAGTCTTGT	AGAGGGGGGT
Erwinia persicinus (U80205)	TCAAGTCGGA	TGTGAAATCC	CCGGGCTCAA	CCTGGGAACT	GCATTCGAAA	CTGGCAGGCT	AGAGTCTTGT	AGAGGGGGGT
Erwinia rhapontici (AJ233417)	TCAAGTCGGA	TGTGAAATCC	CCGGGCTCAA	CCTGGGAACT	GCATTCGAAA	CTGGCAGACT	AGAGTCTTGT	AGAGGGGGGT
Erwinia rhapontici (Z96087)	TCAAGTCGGA	TGTGAAATCC	CCGGGCTCAA	CCTGGGAACT	GCATTCGAAA	CTGGCAGACT	AGAGTCTTGT	AGAGGGGGGT
Erwinia rhapontici (U80206)	TCAAGTCGGA	TGTGAAATCC	CCGGGCTCAA	CCTGGGAACT	GCATTCGAAA	CTGGCAGACT	AGAGTCTTGT	AGAGGGGGGT
Erwinia amylovora (Z96088)	TCAAGTCGGA	TGTGAAATCC	CCGGGCTTAA	CCTGGGAACT	GCATTCGANA	CTGGCAGGCT	AGAGTCTCGT	NGAGGGGGGT
Erwinia amylovora (AJ233410)	TCAAGTCGGA	TGTGAAATCC	CCGGGCTTAA	CCTGGGAACT	GCATTCGAAA	CTGGCAGGCT	AGAGTCTCGT	AGAGGGGGGT
Erwinia amylovora (AJ010485)	TCAAGTCGGA	TGTGAAATCC	CCGGGCTTAA	CCTGGGAACT	GCATTCGAAA	CTGGCAGGCT	AGAGTCTCGT	AGAGGGGGGT
Erwinia amylovora (U80195)	TCAAGTCGGA	TGTGAAATCC	CCGGGCTTAA	CCTGGGAACT	GCATTCGAAA	CTGGCAGGCT	AGAGTCTCGT	AGAGGGGGGT
Erwinia pyrifoliae	TCAAGTCGGA	TGTGAAATCC	CCGGGCTTAA	CCTGGGAACT	GCATTCGAAA	CTGGCAGGCT	AGAGTCTCGT	AGAGGGGGGT
Pectobacterium cypripedii	TNAAGTTGGA	TGTGAAATCC	CCGGGCTTAA	CCTGGGAACT	GCATTCAAAA	CTGACAGGCT	AGAGTCTCGT	AGAGGGGGGT
Pectobacterium chrysanthemi	TTAAGTTGGA	TGTGAAATCC	CCGGGCTTAA	CCTGGGAACT	GCATTCAAAA	CTGACAGGCT	AGAGTCTCGT	AGAGGGGGGT
Pectobacterium c. subsp. *carotovorum*	TTAAGTTGGA	TGTGAAATCC	CCGGGCTTAA	CCTGGGAACT	GCATTCAAAA	CTGACAGGCT	AGAGTCTTGT	AGAGGGGGGT
Pectobacterium c. subsp. *wasabiae*	TTAAGTTGGA	TGTGAAATCC	CCGGGCTTAA	CCTGGGAACT	GCATTCAAAA	CTGACAGGCT	AGAGTCTTGT	AGAGGGGGGT
Pectobacterium c. subsp. *betavasculorum*	TTAAGTTGGA	TGTGAAATCC	CCGGGCTTAA	CCTGGGAACT	GCATTCAAAA	CTGACAGGCT	AGAGTCTTGT	AGAGGGGGGT
Pectobacterium c. subsp. *atrosepticum*	TTAAGTTGGA	TGTGAAATCC	CCGGGCTTAA	CCTGGGAACT	GCATTCAAAA	CTGACAGGCT	AGAGTCTTGT	AGAGGGGGGT
Pectobacterium c. subsp. *odoriferum*	TTAAGTTGGA	TGTGAAATCC	CCGGGCTTAA	CCTGGGAACT	GCATTCAAAA	CTGACAGGCT	AGAGTCTTGT	AGAGGGGGGT
Pectobacterium cacticidum	TTAAGTTGGA	TGTGAAATCC	CCGGGCTTAA	CCTGGGAACT	GCATTCAAAA	CTGACAGGCT	AGAGTCTTGT	AGAGGGGGGT
Brenneria salicis	TTAAGTTGGA	TGTGAAATCC	CCGGGCTCAA	CCCGGGAACA	GCATTCAAAA	CTGACAGGCT	AGAGTCTCGT	AGAGGGGGGT
Brenneria rubrifaciens	TTAAGTTGGA	TGTGAAATCC	CCGGGCTTAA	CCCGGGAACT	GCATTCAAAA	CTGACAGGCT	GGAGTCTCGT	AGAGGGGGGT
Brenneria nigrifluens	TTAAGTTGGA	TGTGAAATCC	CCGGGCTTAA	CCTGGGAACT	GCATTCAAAA	CTGACAGGCT	AGAGTCTCGT	AGAGGGGGGT
Brenneria alni	TTAAGTTGGA	TGTGAAATCC	CCGGGCTTAA	CCTGGGAACT	GCATTCAAAA	CTGACAGGCT	AGAGTCTTGT	AGAGGGGGGT
Brenneria paradisiaca	TTAAGTTGGA	TGTGAAATCC	CCGGGCTTAA	CCTGGGAACT	GCATTCAAAA	CTGACAGGCT	AGAGTCTCGT	AGAGGGGGGT

	650	660	670	680	690	700	710	720
Buchnera aphidicola	AGAATTCTAG	GTGTAGCGGT	GAAATGCGTA	GATATCTGGA	GGAATACCCG	TGGCGAAAGC	GGCCTCCTAA	ACGAATACTG
Proteus vulgaris	AGAATTCCAC	GTGTAGCGGT	GAAATGCGTA	GAGATGTGGA	GGAATACCGG	TGGCGAAGGC	GGCCCCCTGG	ACAAAGACTG
***Erwinia ananas*, Zululand, KZN, SA**	**AGAATTCCAG**	**GTGTAGCGGT**	**GAAATGCGTA**	**GAGATCTGGA**	**GGAATACCGG**	**TGGCGAAGGC**	**GGCCCCCTGG**	**ACGAAGACTG**
Pantoea a. pv. *ananatis*	AGAATTCCAG	GTGTAGCGGT	GAAATGCGTA	GAGATCTGGA	GGAATACCGG	TGGCGAAGGC	GGCCCCCTGG	ACGAAGACTG
Pantoea a. pv. *uredovora*	AGAATTCCAG	GTGTAGCGGT	GAAATGCGTA	GAGATCTGGA	GGAATACCGG	TGGCGAAGGC	GGCCCCCTGG	ACGAAGACTG
Pantoea agglomerans (U80202)	AGAATTCCAG	GTGTAGCGGT	GAAATGCGTA	GAGATCTGGA	GGAATACCGG	TGGCGAAGGC	GGCCCCCTGG	ACAAAGACTG
Pantoea agglomerans (AB004757)	AGAATTCCAG	GTGTAGCGGT	GAAATGCGTA	GAGATCTGGA	GGAATACCGG	TGGCGAAGGC	GGCCCCCTGG	ACAAAGACTG
Pantoea agglomerans (U80183)	AGAATTCCAG	GTGTAGCGGT	GAAATGCGTA	GAGATCTGGA	GGAATACCGG	TGGCGAAGGC	GGCCCCCTGG	ACAAAGACTG
Unknown *Erwinia* sp.	**AGAATTCCAG**	**GTGTAGCGGT**	**GAAATGCGTA**	**GAGATCTGGA**	**GGAATACCGG**	**TGGCGAAGGC**	**GGCCCCCTGG**	**ACGAAGACTG**
Pantoea s. subsp. *stewartii*	AGAATTCCAG	GTGTAGCGGT	GAAATGCGTA	GAGATCTGGA	GGAATACCGG	TGGCGAAGGC	GGTCCCCTGG	ACGAAGACTG
Erwinia tracheiphila	GGAATTCCAG	GTGTANCNGT	GAAATGCGTA	NAGATCTGGA	GGAATACCGG	TGGCGAAGGC	GGCCCCCTGG	ACAAAGACTG
Erwinia psidii	GGAATTCCAG	GTGTAGCGGT	GAAATGCGTA	GAGATCTGGA	GGAATACCGG	TGGCGAAGGC	GGCCCCCTGG	ACAAAGACTG
Erwinia mallotivora (AJ233414)	AGAATTCCAG	GTGTAGCGGT	GAAATGCGTA	GAGATCTGGA	GGAATACCGG	TGGCGAAGGC	GGCCCCCTGG	ACAAAGACTG
Erwinia mallotivora (Z96084)	AGAATTCCAG	GTGTAGCGGT	GAAATGCGTA	GAGATCTGGA	GGAATACCGG	TGGCGAAGGC	GGCCCCCTGG	ACAAAGACTG
Erwinia persicinus (AJ001190)	AGAATTCCAG	GTGTAGCGGT	GAAATGCGTA	GAGATCTGGA	GGAATACCGG	TGGCGAAGGC	GGCCCCCTGG	ACAAAGACTG
Erwinia persicinus (Z96086)	AGAATTCCAG	GTGTAGCGGT	GAAATGCGTA	GAGATCTGGA	GGAATACCGG	TGGCGAAGGC	GGCCCCCTGG	ACAAAGACTG
Erwinia persicinus (U80205)	AGAATTCCAG	GTGTAGCGGT	GAAATGCGTA	GAGATCTGGA	GGAATACCGG	TGGCGAAGGC	GGCCCCCTGG	ACAAAGACTG
Erwinia rhapontici (AJ233417)	AGAATTCCAG	GTGTAGCGGT	GAAATGCGTA	GAGATCTGGA	GGAATACCGG	TGGCGAAGGC	GGCCCCCTGG	ACAAAGACTG
Erwinia rhapontici (Z96087)	AGAATTCCAG	GTGTAGCGGT	GAAATGCGTA	GAGATCTGGA	GGAATACCGG	TGGCGAAGGC	GGCCCCCTGG	ACAAAGACTG
Erwinia rhapontici (U80206)	AGAATTCCAG	GTGTAGCGGT	GAAATGCGTA	GAGATCTGGA	GGAATACCGG	TGGCGAAGGC	GGCCCCCTGG	ACAAAGACTG
Erwinia amylovora (Z96088)	AGAATTCCAG	GTGTAGCGGT	GAAATGCGTA	GAGATCTGGG	AGGATAC-GG	TGGCGAAGGC	GGCCCCCTGG	ACGAAGACTG
Erwinia amylovora (AJ233410)	AGAATTCCAG	GTGTAGCGGT	GAAATGCGTA	GAGATCTGGA	GGAATACCGG	TGGCGAAGGC	GGCCCCCTGG	ACGAAGACTG
Erwinia amylovora (AJ010485)	AGAATTCCAG	GTGTAGCGGT	GAAATGCGTA	GAGATCTGGA	GGAATACCGG	TGGCGAAGGC	GGCCCCCTGG	ACGAAGACTG
Erwinia amylovora (U80195)	AGAATTCCAG	GTGTAGCGGT	GAAATGCGTA	GAGATCTGGA	GGAATACCGG	TGGCGAAGGC	GGCCCCCTGG	ACGAAGACTG
Erwinia pyrifoliae	AGAATTCCAG	GTGTAGCGGT	GAAATGCGTA	GAGATCTGGA	GGAATACCGG	TGGCGAAGGC	GGCCCCCTGG	ACGAAGACTG
Pectobacterium cypripedii	AGAATTCCAG	GTGTAGCGGT	GAAATGCGTA	GAGATCTGGA	GGAATACCGG	TGGCGAAGGC	GGCCCCCTGG	ACGAAGACTG
Pectobacterium chrysanthemi	AGAATTCCAG	GTGTAGCGGT	GAAATGCGTA	GAGATCTGGA	GGAATACCGG	TGGCGAAGGC	GGCCCCCTGG	ACGAAGACTG
Pectobacterium c. subsp. *carotovorum*	AGAATTCCAG	GTGTAGCGGT	GAAATGCGTA	GAGATCTGGA	GGAATACCGG	TGGCGAAGGC	GGCCCCCTGG	ACAAANACTG
Pectobacterium c. subsp. *wasabiae*	AGAATTCCAG	GTGTANCGGT	GAAATGCGTA	NAGATCTGGA	GGAATACCGG	TGGCGAAGGC	GGCCCCCTGG	ACAAAGACTG
Pectobacterium c. subsp. *betavasculorum*	AGAATTCCAG	GTGTAGCGGT	GAAATGCGTA	GAGATCTGGA	GGAATACCGG	TGGCGAAGGC	GGCCCCCTGG	ACAAAGACTG
Pectobacterium c. subsp. *atrosepticum*	AGAATTCCAG	GTGTAGCGGT	GAAATGCGTA	GAGATCTGGA	GGAATACCGG	TGGCGAAGGC	GGCCCCCTGG	ACAAAGACTG
Pectobacterium c. subsp. *odoriferum*	AGAATTCCAG	GTGTAGCGGT	GAAATGCGTA	GAGATCTGGA	GGAATACCGG	TGGCGAAGGC	GGCCCCCTGG	ACAAAGACTG
Pectobacterium cacticidum	AGAATTCCAG	GTGTANCGGT	GAAATGCGTA	GAGATCTGGA	GGAATACCGG	TGGCGAAGGC	GGCCCCCTGG	ACAAAGACTG
Brenneria salicis	AGAATTCCAG	GTGTAGCGGT	GAAATGCGTA	GAGATCTGGA	GGAATACCGG	TGGCGAAGGC	GGCCCCCTGG	ACGAAGACTG
Brenneria rubrifaciens	AGAATTCCAG	GTGTAGCGGT	GAAATGCGTA	GAGATCTGGA	GGAATACCGG	TGGCGAAGGC	GGCCCCCTGG	ACGAAGACTG
Brenneria nigrifluens	AGAATTCCAG	GTGTAGCGGT	GAAATGCGTA	GAGATCTGGA	GGAATACCGG	TGGCGAAGGC	GGGCCCCTGG	ACGAAGACTG
Brenneria alni	AGAATTCCAG	GTGTAGCGGT	GAAATGCGTA	GAGATCTGGA	GGAATACCGG	TGGCGAAGGC	GGCCCCCTGG	ACAAAGACTG
Brenneria paradisiaca	AGAATTCCAG	GTGTAGCGGT	GAAATGCGTA	GAGATCTGGA	GGAATACCGG	TGGCGAAGGC	GGCCCCCTGG	ACGAAGACTG

	730	740	750	760	770	780	790	800
Buchnera aphidicola	ACACTGAGGT	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	GTCCATGCCG	TAAACGATGT	CGACTTGGAG
Proteus vulgaris	ACGCTCAGGT	GCGAAAGCGT	GGGGACCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCTG	TAAACGATGT	CGATTTAGAG
***Erwinia ananas*, Zululand, KZN, SA**	**ACGCTCAGGT**	**GCGAAAGCGT**	**GGGGAGCAAA**	**CAGGATTAGA**	**TACCCTGGTA**	**GTCCACGCCG**	**TAAACGATGT**	**CGACTTGGAG**
Pantoea a. pv. *ananatis*	ACGCTCAGGT	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCCG	TAAACGATGT	CGACTTGGAG
Pantoea a. pv. *uredovora*	ACGCTCAGGT	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCCG	TAAACGATGT	CGACTTGGAG
Pantoea agglomerans (U80202)	ACGCTCAGGT	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCCG	TAGACGATGT	CGACTTGGAG
Pantoea agglomerans (AB004757)	ACGCTCAGGT	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCCG	TAAACGATGT	CGACTTGGAG
Pantoea agglomerans (U80183)	ACGCTCAGGT	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCCG	TAAACGATGT	CGACTTGGAG
Unknown *Erwinia* sp.	**ACGCTCAGGT**	**GCGAAAGCGT**	**GGGGAGCAAA**	**CAGGATTAGA**	**TACCCTGGTA**	**GTCCACGCCG**	**TAAACGATGT**	**CGATTTGGAG**
Pantoea s. subsp. *stewartii*	ACGCTCAGGT	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCCG	TAAACGATGT	CGACTTGGAG
Erwinia tracheiphila	ACGCTCATGT	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCTG	TAAACGATGT	CGATTTGGAG
Erwinia psidii	ACGCTCAGGT	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCCG	TAAACGATGT	CGATTTGGAG
Erwinia mallotivora (AJ233414)	ACGCTCAGGT	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCCG	TAAACGATGT	CGATTTGGAG
Erwinia mallotivora (Z96084)	ACGCTCAGGT	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCCG	TAAACGATGT	CGATTTGGAG
Erwinia persicinus (AJ001190)	ACGCTCAGGT	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCCG	TAAACGATGT	CGACTTGGAG
Erwinia persicinus (Z96086)	ACGCTCAGGT	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCCG	TAAACGATGT	CGACTTGGAG
Erwinia persicinus (U80205)	ACGCTCAGGT	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCCG	TAAACGATGT	CGACTTGGAG
Erwinia rhapontici (AJ233417)	ACGCTCAGGT	GCCAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCCG	TAAACGATGT	CGACTTGGAG
Erwinia rhapontici (Z96087)	ACGCTCAGGT	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCCG	TAAACGATGT	CGACTTGGAG
Erwinia rhapontici (U80206)	ACGCTCAGGT	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCCG	TAAACGATGT	CGACTTGGAG
Erwinia amylovora (Z96088)	ACGCTCAGGT	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCCG	TAAACGATGT	CGACTTGGAG
Erwinia amylovora (AJ233410)	ACGCTCAGGT	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCCG	TAAACGATGT	CGACTTGGAG
Erwinia amylovora (AJ010485)	ACGCTCAGGT	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCCG	TAAACGATGT	CGACTTGGAG
Erwinia amylovora (U80195)	ACGCTCAGGT	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCCG	TAAACGATGT	CGACTTGGAG
Erwinia pyrifoliae	ACGCTCAGGT	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCCG	TAAACGATGT	CGACTTGGAG
Pectobacterium cypripedii	ACGCTCAGGT	GCGAAAGCGT	GGGGANCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCTG	TAAACGATGT	CGACTTGGAG
Pectobacterium chrysanthemi	ACGCTCAGGT	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCTG	TAAACGATGT	CGATTTGGAG
Pectobacterium c. subsp. *carotovorum*	ACGCTCAGGT	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCTG	TAAACGATGT	CGATTTGGAG
Pectobacterium c. subsp. *wasabiae*	ACGCTCAGGT	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCTG	TAAACGATGT	CGACTTGGAG
Pectobacterium c. subsp. *betavasculorum*	ACGCTCAGGT	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCTG	TAAACGATGT	CGACTTGGAG
Pectobacterium c. subsp. *atrosepticum*	ACGCTCAGGT	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCTG	TAAACGATGT	CGACTTGGAG
Pectobacterium c. subsp. *odoriferum*	ACGCTCAGGT	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCTG	TAAACGATGT	CGATTTGGAG
Pectobacterium cacticidum	ACGCTCAGGT	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCTG	TAAACGATGT	CNACTTGGAG
Brenneria salicis	ACGCTCAGGT	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCCG	TAAACGATGT	CGACTTGGAG
Brenneria rubrifaciens	ACGCTCAGGT	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	TTCCACGCCG	TAAACGATGT	CGACTTGGAG
Brenneria nigrifluens	ACGCTCAGGT	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCCG	TAAACGATGT	CGACTTGGAG
Brenneria alni	ACGCTCAGGT	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCTG	TAAACGATGT	CGACTTGAAG
Brenneria paradisiaca	ACGCTCAGGT	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA	GTCCACGCTG	TAAACGATGT	CGATTTGGAG

	810	820	830	840	850	860	870	880
Buchnera aphidicola	GTTGTTTCCA	AGAGAAGTGA	CTTCCGAAGC	TAACGCGTTA	AGTCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GCTAAAACTC
Proteus vulgaris	GTTGTGGTCT	TGAACTGTGG	CTTCTGCAGC	TAACGCGTTA	AATCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC
***Erwinia ananas*, Zululand, KZN, SA**	**GTTGTTCCCT**	**TGAGGAGTGG**	**CTTCCGGAGC**	**TAACGCGTTA**	**AGTCGACCGC**	**CTGGGGAGTA**	**CGGCCGCAAG**	**GTTAAAACTC**
Pantoea a. pv. *ananatis*	GTTGTTCCCT	TGAGGAGTGG	CTTCCGGAGC	TAACGCGTTA	AGTCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC
Pantoea a. pv. *uredovora*	GTTGTTCCCT	TGAGGAGTGG	CTTCCGGAGC	TAACGCGTTA	AGTCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC
Pantoea agglomerans (U80202)	GTTGTTCCCT	TGAGGAGTGG	CTTCCGGAGC	TAACGCGTTA	AGTCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC
Pantoea agglomerans (AB004757)	GTTGTTCCCT	TGAGGAGTGG	CTTCCGGAGC	TAACGCGTTA	AGTCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC
Pantoea agglomerans (U80183)	GTTGTTCCCT	TGAGGAGTGG	CTTCCGGAGC	TAACGCGTTA	AGTCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC
Unknown *Erwinia* sp.	**GTTGTTCCCT**	**TGAGGCGTGG**	**CTTCCGGAGC**	**TAACGCGTTA**	**AATCGACCGC**	**CTGGGGAGTA**	**CGGCCGCAAG**	**GTTAAAACTC**
Pantoea s. subsp. *stewartii*	GTTGTTCCCT	TGAGGAGTGG	CTTCCGGAGC	TAACGCGTTA	AGTCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC
Erwinia tracheiphila	GTTGTGCCCT	TGAGGTGTGG	CTTCCGTAGC	TAACGCGTTA	AATCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC
Erwinia psidii	GTTGTGCCCT	TGAGGCGTGG	CTTCCGTAGC	TAACGCGTTA	AATCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC
Erwinia mallotivora (AJ233414)	GTTGTGCCCT	TGAGGCGTGG	CTTCCGTAGC	TAACGCGTTA	AATCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC
Erwinia mallotivora (Z96084)	GTTGTGCCCT	TGAGGCGTGG	NTTCNNTAGN	TAACGCGTTA	AATCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC
Erwinia persicinus (AJ001190)	GTTGTGCCCT	TGAGGCGTGG	CTTCCGGAGC	TAACGCGTTA	AGTCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC
Erwinia persicinus (Z96086)	GTTGTGCCCT	TGAGGCGTGG	CTTCCGGAGC	TAACGCGTTA	AGTCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC
Erwinia persicinus (U80205)	GTTGTGCCCT	TGAGGCGTGG	CTTCCGGAGC	TAACGCGTTA	AGTCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC
Erwinia rhapontici (AJ233417)	GTTGTGCCCT	TGAGGCGTGG	CTTCCGGAGC	TAACGCGTTA	AGTCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC
Erwinia rhapontici (Z96087)	GTTGTGCCCT	TGAGGCGTGG	NTTCCGGAGC	TAACGCGTTA	AGTCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC
Erwinia rhapontici (U80206)	GTTGTGCCCT	TGAGGCGTGG	CTTCCGGAGC	TAACGCGTTA	AGTCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC
Erwinia amylovora (Z96088)	GTTGTTCCCC	TGAGGAGTGG	NTTCNGGAGC	TAACGCGTTA	AGTCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC
Erwinia amylovora (AJ233410)	GCTGTTCCCC	TGAGGAGTGG	CTTCCGGAGC	TAACGCGTTA	AGTCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC
Erwinia amylovora (AJ010485)	GCTGTTCCCC	TGAGGAGTGG	CTTCCGGAGC	TAACGCGTTA	AGTCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC
Erwinia amylovora (U80195)	GCTGTTCCCT	TGAGGAGTGG	CTTCCGGAGC	TAACGCGTTA	AGTCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC
Erwinia pyrifoliae	GCTGTTCCCC	TGAGGAGTGG	CTTCCGGAGC	TAACGCGTTA	AGTCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC
Pectobacterium cypripedii	GTTGTGCCCT	TGAGGCGTGA	CTTCCGGAGC	TAACGCGTTA	AGTCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTNAAACTC
Pectobacterium chrysanthemi	GTTGTGCCCT	TGAGGCGTGG	CTTCCGGAGC	TAACGCGTTA	AATCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC
Pectobacterium c. subsp. *carotovorum*	GTTGTGCCCT	TGAGGCGTGG	CTTCCGGAGC	TAACGCGTTA	AATCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC
Pectobacterium c. subsp. *wasabiae*	GTTGTGCCCT	TGAGGCGTGG	CTTCCGGAGC	TAACGCGTTA	AGTCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC
Pectobacterium c. subsp. *betavasculorum*	GTTGTGCCCT	TGAGGCGTGG	CTTCCGGAGC	TAACGCGTTA	AGTCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC
Pectobacterium c. subsp. *atrosepticum*	GTTGTGCCCT	TGAGGCGTGG	CTTCCGGAGC	TAACGCGTTA	AGTCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC
Pectobacterium c. subsp. *odoriferum*	GTTGTGCCCT	TGAGGCGTGG	CTTCCGGAGC	TAACGCGTTA	AATCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC
Pectobacterium cacticidum	GTTGTGCCCT	AAAAGCGTGG	CTTCCGGAGC	TAACGCGTTA	AGTCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC
Brenneria salicis	GCTGTGGTCT	TGAACCGTGG	CTTCCGGAGC	TAACGCGTTA	AGTCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC
Brenneria rubrifaciens	GCTGTGGTCC	AGAACCGTGG	CTTCCGGAGC	TAACGCGTTA	AGTCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC
Brenneria nigrifluens	GCTGTGGTCT	TGAACCGTGG	CTTCCGGAGC	TAACGCGTTA	AGTCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC
Brenneria alni	GTTGTGGCCT	TGAGCCGTGG	CTTTCGGAGC	AAACGCGTTA	AGTCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC
Brenneria paradisiaca	GTTGTGGTCT	TGAACCGTGG	CTTCCGGAGC	TAACGCGTTA	AATCGACCGC	CTGGGGAGTA	CGGCCGCAAG	GTTAAAACTC

	890	900	910	920	930	940	950	960
Buchnera aphidicola	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GATGCAACGC	GAAAAACCTT	ACCTGGTCTT
Proteus vulgaris	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GATGCAACGC	GAAGAACCTT	ACCTACTCTT
***Erwinia ananas*, Zululand, KZN, SA**	**AAATGAATTG**	**ACGGGGGCCC**	**GCACAAGCGG**	**TGGAGCATGT**	**GGTTTAATTC**	**GATGCAACGC**	**GAAGAACCTT**	**ACCTACTCTT**
Pantoea a. pv. *ananatis*	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATAT	GGTTTAATTC	GATGCAACGC	GAAGAACCTT	ACCTACTCTT
Pantoea a. pv. *uredovora*	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATAT	GGTTTAATTC	GATGCAACGC	GAAGAACCTT	ACCTACTCTT
Pantoea agglomerans (U80202)	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GATGCAACGC	GAAGAACCTT	ACCTACTCTT
Pantoea agglomerans (AB004757)	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GATGCAACGC	GAAGAACCTT	ACCTACTCTT
Pantoea agglomerans (U80183)	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GATGCAACGC	GAAGAACCTT	ACCTACTCTT
Unknown *Erwinia* sp.	**AAATGAATTG**	**ACGGGGGCCC**	**GCACAAGCGG**	**TGGAGCATGT**	**GGTTTAATTC**	**GATGCAACGC**	**GAAGAACCTT**	**ACCTACTCTT**
Pantoea s. subsp. *stewartii*	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GATGCAACGC	GAAGAACCTT	ACCTACTCTT
Erwinia tracheiphila	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTN	GATGCAACGC	GAAGAACCTT	ACCTGGCCTT
Erwinia psidii	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GATGCAACGC	GAAGAACCTT	ACCTGGCCTT
Erwinia mallotivora (AJ233414)	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GATGCAACGC	GAAGAACCTT	ACCTGGCCTT
Erwinia mallotivora (Z96084)	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GATGCAACGG	GAAGAACCTT	ACCTGGCCTT
Erwinia persicinus (AJ001190)	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GATGCAACGC	GAAGAACCTT	ACCTGGCCTT
Erwinia persicinus (Z96086)	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GATGCAACGC	GAAGAACCTT	ACCTGGCCTT
Erwinia persicinus (U80205)	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GATGCAACGC	GAAGAACCTT	ACCTGGCCTT
Erwinia rhapontici (AJ233417)	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GATGCAACGC	GAAGAACCTT	ACCTGGCCTT
Erwinia rhapontici (Z96087)	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GATGCAACGC	GAAGAACCTT	ACCTGGCCTT
Erwinia rhapontici (U80206)	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GATGCAACGC	GAAGAACCTT	ACCTGGCCTT
Erwinia amylovora (Z96088)	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GATGCAACGC	GAAGAACCTT	ACCTGGCCTT
Erwinia amylovora (AJ233410)	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GATGCAACGC	GAAGAACCTT	ACCTGGCCTT
Erwinia amylovora (AJ010485)	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GATGCAACGC	GAAGAACCTT	ACCTGGCCTT
Erwinia amylovora (U80195)	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GATGCAACGC	GAAGAACCTT	ACCTGGCCTT
Erwinia pyrifoliae	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GATGCAACGC	GAAGAACCTT	ACCTGGCCTT
Pectobacterium cypripedii	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GATGCAACGC	GAAGAACCTT	ACCTGCTCTT
Pectobacterium chrysanthemi	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GATGCAACGC	GAAGAACCTT	ACCTACTCTT
Pectobacterium c. subsp. *carotovorum*	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GATGCAACGC	GAAGAACCTT	ACCTACTCTT
Pectobacterium c. subsp. *wasabiae*	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GATGCAACGC	GAAGAACCTT	ACCTACTCTT
Pectobacterium c. subsp. *betavasculorum*	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GATGCAACGC	GAAGAACCTT	ACCTACTCTT
Pectobacterium c. subsp. *atrosepticum*	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GATGCAACGC	GAAGAACCTT	ACCTACTCTT
Pectobacterium c. subsp. *odoriferum*	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GATGCAACGC	GAAGAACCTT	ACCTACTCTT
Pectobacterium cacticidum	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GATGCAACGC	GAAGAACCTT	ACTAATTCTT
Brenneria salicis	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GATGCAACGC	GAAGAACCTT	ACCTACTCTT
Brenneria rubrifaciens	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GATGCAACGC	GAAGAACCTT	ACCTACTCTT
Brenneria nigrifluens	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GATGCAACGC	GAAGAACCTT	ACCTACTCTT
Brenneria alni	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GATGCAACGC	GAAGAACCTT	ACCTACTCTT
Brenneria paradisiaca	AAATGAATTG	ACGGGGGCCC	GCACAAGCGG	TGGAGCATGT	GGTTTAATTC	GATGCAACGC	GAAGAACCTT	ACCTACTCTT

	970	980	990	1000	1010	1020	1030	1040
Buchnera aphidicola	GACATCCACA	GAA-TT----	T--TTT-AGA	AATAAAAAAG	TGCCTTCGGG	AACTGTGAGA	CAGGTGCTGC	ATGGCTGTCG
Proteus vulgaris	GACATCCAGC	GAA-------	TCCTTT-AGA	GATAGAGGAG	TGCCTTCGGG	AACGCTGAGA	CAGGTGCTGC	ATGGCTGTCG
***Erwinia ananas*, Zululand, KZN, SA**	**GACATCCAGA**	**GAACTTAGC-**	**-------AGA**	**GATGCTTTGG**	**TGCCTTCGGG**	**AACTGTGAGA**	**CAGGTGCTGC**	**ATGGCTGTCG**
Pantoea a. pv. *ananatis*	GACATCCAGA	GAACTTAGC-	-------AGA	GATGCTTTGG	TGCCTTCGGG	AACTCTGAGA	CAGGTGCTGC	ATGGCTGTCG
Pantoea a. pv. *uredovora*	GACATCCAGA	GAACTTAGC-	-------AGA	GATGCTTTGG	TGCCTTCGGG	AACTCTGAGA	CAGGTGCTGC	ATGGCTGTCG
Pantoea agglomerans (U80202)	GACATCCAGC	GAACTTAGC-	-------AGA	GATGCTTTGG	TGCCTTCGGG	AACGCTGAGA	CAGGTGCTGC	ATGGCTGTCG
Pantoea agglomerans (AB004757)	GACATCCACG	GAA-TTTGGC	-------AGA	GATGCCTTAG	TGCCTTCGGG	AACCGTGAGA	CAGGTGCTGC	ATGGCTGTCG
Pantoea agglomerans (U80183)	GACATCCACG	GAA-TTTGGC	-------AGA	GATGCCTTAG	TGCCTTCGGG	GACCGTGAGA	CAGGTGCTGC	ATGGCTGTCG
Unknown *Erwinia* sp.	**GACATCCACG**	**GAACTTAGCC**	**AG-T---AGA**	**GATGCCTTGG**	**TGCCTTCGGG**	**AACGGTGAGA**	**CAGGTGCTGC**	**ATGGCTGTCG**
Pantoea s. subsp. *stewartii*	GACATCCAGC	GAACTTGGC-	-------AGA	GATGCCTTGG	TGCCTTCGGG	AACGCTGAGA	CAGGTGCTGC	ATGGCTGTCG
Erwinia tracheiphila	GACATCCACA	GAACTTAGC-	-------ACA	GATGCTTTGG	TGCCTTCGGG	AGCTGTGAGA	CAGGTGCTGC	ATGGCTGTCG
Erwinia psidii	GACATCCACA	GAACTTAGC-	-------AGA	GATGCTTTGG	TGC-TTCGGG	AACTGTGAGA	CAGGTGCTGC	ATGGCTGTCG
Erwinia mallotivora (AJ233414)	GACATCCACG	GAA------G	ACCT--CAGA	GATGGGGTTG	TGCCTTCGGG	AACCGTGAGA	CAGGTGCTGC	ATGGCTGTCG
Erwinia mallotivora (Z96084)	GACATCCACG	GAA------G	ACCT--CAGA	GATGGGGTTG	TGCCTTCGGG	AACCGTGAGA	CAGGTGCTGC	ATGGCTGTCG
Erwinia persicinus (AJ001190)	GACATCCACG	GAA-TTCGGC	-------AGA	GATGCCTTAG	TGCCTTCGGG	AACCGTGAGA	CAGGTGCTGC	ATGGCTGTCG
Erwinia persicinus (Z96086)	GACATCCACG	GAA-TTCGGC	-------AGA	GATGCCTTAG	TGCCTTCGGG	AACCGTGAGA	CAGGTGCTGC	ATGGCTGTCG
Erwinia persicinus (U80205)	GACATCCACG	GAA-TTTGGC	-------AGA	GATGCCTTAG	TGCCTTCGGG	AACCGTGAGA	CAGGTGCTGC	ATGGCTGTCG
Erwinia rhapontici (AJ233417)	GACATCCACG	GAA-TTCGGC	-------AGA	GATGCCTTAG	TGCCTTCGGG	AACCGTGAGA	CAGGTGCTGC	ATGGCTGTCG
Erwinia rhapontici (Z96087)	GACATCCACG	GAA-TTCGGC	-------AGA	GATGCCTTAG	TGCCTTCGGG	AACCGTGAGA	CAGGTGCTGC	ATGGCTGTCG
Erwinia rhapontici (U80206)	GACATCCACG	GAA-TTCGGC	-------AGA	GATGCCTTAG	TGCCTTCGGG	AACCGTGAGA	CAGGTGCTGC	ATGGCTGTCG
Erwinia amylovora (Z96088)	GACATCCACG	GAA-TT----	--CT-GCAGA	GATGCGAATG	TGCCTTCGGG	AACCGTGAGA	CAGGTGCTGC	ATGGCTGTCG
Erwinia amylovora (AJ233410)	GACATCCACG	GAA-TT----	--CT-GCAGA	GATGCGGAAG	TGCCTTCGGG	AACCGTGAGA	CAGGTGCTGC	ATGGCTGTCG
Erwinia amylovora (AJ010485)	GACATCCACG	GAA-TT----	--CT-GCAGA	GATGCGGAAG	TGCCTTCGGG	AACCGTGAGA	CAGGTGCTGC	ATGGCTGTCG
Erwinia amylovora (U80195)	GACATCCACG	GAA-TT----	--CT-GCAGA	GATGCGGAAG	TGCCTTCGGG	AACCGTGAGA	CAGGTGCTGC	ATGGCTGTCG
Erwinia pyrifoliae	GACATCCACG	GAA-TTTTGC	-------AGA	GATGCGGAAG	TGC-TTCGGG	AACCGTGAGA	CAGGTGCTGC	ATGGCTGTCG
Pectobacterium cypripedii	GACATCCAGA	GAA-TT----	--CT-GCAGA	GATGCGNTNG	TGCCTTCGGG	ACCTCTGAGA	CAGGTGCTGC	ATGGCTGTCG
Pectobacterium chrysanthemi	GACATCCAGA	GAA------G	-CCT-GCAGA	GATGCGGGTG	TGCCTTCGGG	AGCTCTGAGA	CAGGTGCTGC	ATGGCTGTCG
Pectobacterium c. subsp. *carotovorum*	GACATCCACA	GAA-TTTGG-	---T---AGA	GATACCTTAG	TGCCTTCGGG	AACTGTGAGA	CAGGTGCTGC	ATGGCTGTCG
Pectobacterium c. subsp. *wasabiae*	GACATCCACA	GAA-TTCGG-	---T---AGA	GATACCTTAG	TGCCTTCGGG	AACTGTGAGA	CAGGTGCTGC	ATGGCTGTCG
Pectobacterium c. subsp. *betavasculorum*	GACATCCACA	GAA-TTTGGC	-------AGA	GATGCCTTAG	TGCCTTCGGG	AACTGTGAGA	CAGGTGCTGC	ATGGCTGTCG
Pectobacterium c. subsp. *atrosepticum*	GACATCCAGA	GAA-TTTGGC	-------AGA	GATGCCTTAG	TGCCTTCGGG	AACTCTGAGA	CAGGTGCTGC	ATGGCTGTCG
Pectobacterium c. subsp. *odoriferum*	GACATCCAGA	GAA-TTAGC-	---T---AGA	GATAGCTGAG	TGCCTTCGGG	AACTCTGAGA	CAGGTGCTGC	ATGGCTGTCG
Pectobacterium cacticidum	GACATCCACA	GAA---GGC	---TTT-AGA	GATAGAGCTG	TGTCTTCGGA	AACTGTGAGA	CAGGTGCTGC	ATGGCTGTCG
Brenneria salicis	GACATCCAGA	GAA------G	AC-TGT-AGA	GATACGGTTG	TGCCTTCGGG	AGCTCTGAGA	CAGGTGCTGC	ATGGCTGTCG
Brenneria rubrifaciens	GACATCCAGA	GAA------G	AC-T-TCAGA	GATGAGGTTG	TGCCTTAGGG	AGCTCTGAGA	CAGGTGCTGC	ATGGCTGTCG
Brenneria nigrifluens	GACATCCTCA	GAA----GAG	AC-T---GGA	GACAGTCTTG	TGCCTTCGGG	AACTGAGAGA	CAGGTGCTGC	ATGGCTGTCG
Brenneria alni	GACATCCTCA	GAA----GAG	AC-T---GGA	GATAGTTTTG	TGCCTTCGGG	AACTGAGAGA	CAGGTGCTGC	ATGGCTGTCG
Brenneria paradisiaca	GACATC--CA	GAG---A-AG	AC-T-GCAGA	GATGCGGTTG	TGCCTTCGGG	AGCTCTGAGA	CAGGTGCTGC	ATGGCTGTCG

	1050	1060	1070	1080	1090	1100	1110	1120
Buchnera aphidicola	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCCCT	GTTGCCAGCG	GTTC--GGCC
Proteus vulgaris	TCAGCTCGTT	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTTT	GTTGCCAGCG	CGTAATGG-C
***Erwinia ananas*, Zululand, KZN, SA**	**TCAGCTCGT-**	**GTTGTGAAAT**	**GTTGGGTTAA**	**GTCCCGCAAC**	**GAGCGCAACC**	**CTTATCCTTT**	**GTTGCCAGCG**	**ATTC--GGTC**
Pantoea a. pv. *ananatis*	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTTT	GTTGCCAGCG	ATTC--GGTC
Pantoea a. pv. *uredovora*	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTTT	GTTGCCAGCG	ATTC--GGTC
Pantoea agglomerans (U80202)	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTTT	GTTGCCAGCG	ATTC--GGTC
Pantoea agglomerans (AB004757)	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTTT	GTTGCCAGCG	ATTC--GGTC
Pantoea agglomerans (U80183)	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTTT	GTTGCCAGCG	ATTC--GGTC
Unknown *Erwinia* sp.	**TCAGCTCGT-**	**GTTGTGAAAT**	**GTTGGGTTAA**	**GTCCCGCAAC**	**GAGCGCAACC**	**CTTATCCTTT**	**GTTGCCAGCG**	**ATTC--GGTC**
Pantoea s. subsp. *stewartii*	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTTT	GTTGCCAGCG	ATTC--GGTC
Erwinia tracheiphila	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTTT	GTTGCCAGCG	ATTT--GGTC
Erwinia psidii	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTTT	GTTGCCATCG	ATTC--GGTC
Erwinia mallotivora (AJ233414)	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTTT	GTTGCCATCG	ATTC--GGTC
Erwinia mallotivora (Z96084)	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTTT	GTTGCCATCG	ATTC--GGTC
Erwinia persicinus (AJ001190)	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTTT	GTTGCCAGCA	CGTAATGGT-
Erwinia persicinus (Z96086)	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTTT	GTTGCCAGCA	CGTAATGGT-
Erwinia persicinus (U80205)	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTTT	GTTGCCAGCA	CGTAATGGT-
Erwinia rhapontici (AJ233417)	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTTT	GTTGCCAGCA	CGTAATGGT-
Erwinia rhapontici (Z96087)	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTTT	GTTGCCAGCA	CGTAATGGT-
Erwinia rhapontici (U80206)	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTTT	GTTGCCAGCG	AGTAAT-GTC
Erwinia amylovora (Z96088)	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTTT	GTTGCCAGCG	ATTC--GGTC
Erwinia amylovora (AJ233410)	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTTT	GTTGCCAGCG	ATTC--GGTC
Erwinia amylovora (AJ010485)	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTTT	GTTGCCAGCG	ATTC--GGTC
Erwinia amylovora (U80195)	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTTT	GTTGCCAGCG	ATTC--GGTC
Erwinia pyrifoliae	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTTT	GTTGCCAGCG	ATTC--GGTC
Pectobacterium cypripedii	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTNTCCTCT	GTTGCCAGCA	CGTCATGGG-
Pectobacterium chrysanthemi	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTCT	GTTGCCAGCA	CGTTATGGT-
Pectobacterium c. subsp. *carotovorum*	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTTT	GTTGCCAGCG	ATTC--GGTC
Pectobacterium c. subsp. *wasabiae*	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTTT	GTTGCCAGCA	AGTAA-TGTC
Pectobacterium c. subsp. *betavasculorum*	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTTT	GTTGCCAGCG	ATTC--GGTC
Pectobacterium c. subsp. *atrosepticum*	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTTT	GTTGCCAGCG	CGTAATGG-C
Pectobacterium c. subsp. *odoriferum*	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTTT	GTTGCCAGCG	ATTC--GGTC
Pectobacterium cacticidum	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTTT	GTTGCCAGCG	ATTA--GGTC
Brenneria salicis	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTTT	GTTGCCAGCA	CGTAATGGT-
Brenneria rubrifaciens	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTTT	GTTGCCAGCG	ATTC--GGTC
Brenneria nigrifluens	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTTT	GTTGCCAGCG	ATTC--GGTC
Brenneria alni	TCAGCTCGT-	GTTGTGAAAT	GTTGGGTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTTT	GTTGCCAGCA	CGTAATGGT-
Brenneria paradisiaca	TCAGCTCGT-	GTTGTGAAAT	GTTGGNTTAA	GTCCCGCAAC	GAGCGCAACC	CTTATCCTTT	GTTGCCAGCA	CGTGATGGT-

	1130	1140	1150	1160	1170	1180	1190	1200
Buchnera aphidicola	GGGAACTCAG	AGGAGACTGC	CGGTT-ATAA	ACCGGAGGAA	GGTGGGGACG	ACGTCAAGTC	--ATCATGGC	CCTTACGACC
Proteus vulgaris	GGGAACTCAA	AGGAGACTGC	CGGTG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTTAAGTC	GTATCATGGC	CCTTACGAGT
***Erwinia ananas*, Zululand, KZN, SA**	**GGGAACTCAA**	**AGGAGACTGC**	**CGGTG-ATAA**	**ACCGGAGGAA**	**GGTGGGGATG**	**ACGTCAAGTC**	**--ATCATGGC**	**CCTTACGAGT**
Pantoea a. pv. *ananatis*	GGGAACTCAA	AGGAGACTGC	CGGTG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ATCATGGC	CCTTACGAGT
Pantoea a. pv. *uredovora*	GGGAACTCAA	AGGAGACTGC	CGGTG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ATCATGGC	CCTTACGAGT
Pantoea agglomerans (U80202)	GGGAACTCAA	AGGAGACTGC	CGGTG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ATCATGGC	CCTTACGAGT
Pantoea agglomerans (AB004757)	GGGAACTCAA	AGGAGACTGC	CGGTG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ATCATGGC	CCTTACGAGT
Pantoea agglomerans (U80183)	GGGAACTCAA	AGGAGACTGC	CGGTG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ATCATGGC	CCTTACGAGT
Unknown *Erwinia* sp.	**GGGAACTCAA**	**AGGAGACTGC**	**CGGTG-ATAA**	**ACCGGAGGAA**	**GGTGGGGATG**	**ACGTCAAGTC**	**--ATCATGGC**	**CCTTACGAGT**
Pantoea s. subsp. *stewartii*	GGGAACTCAA	AGGAGACTGC	CGGTG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ATCATGGC	CCTTACGAGT
Erwinia tracheiphila	GGGAACTCAA	AGGAGACTGC	CGGTG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ATCATGGC	CCTTACGGCC
Erwinia psidii	GGGAACTCAA	AGGAGACTGC	CGGTG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ATCATGGC	CCTTACGGCC
Erwinia mallotivora (AJ233414)	GGGAACTCAA	AGGAGACTGC	CGGTG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ATCATGGC	CCTTACGGCC
Erwinia mallotivora (Z96084)	GGGAACTCAA	AGGAGACTGC	CGGTG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ATCATGGC	CCTTACGGCC
Erwinia persicinus (AJ001190)	GGGAACTCAA	AGGAGACTGC	CGGTGCATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ATCATGGC	CCTTACGGCC
Erwinia persicinus (Z96086)	GGGAACTCAA	AGGAGACTGC	CGGTG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ATCATGGC	CCTTACGGCC
Erwinia persicinus (U80205)	GGGAACTCAA	AGGAGACTGC	CGGTG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ATCATGGC	CCTTACGGCC
Erwinia rhapontici (AJ233417)	GGGAACTCAA	AGGAGACTGC	CGGTG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ATCATGGC	CCTTACGGCC
Erwinia rhapontici (Z96087)	GGGAACTCAA	AGGAGACTGC	CGGTG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ATCATGGC	CCTTACGGCC
Erwinia rhapontici (U80206)	GGGAACTCAA	AGGAGACTGC	CGGTG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ATCATGGC	CCTTACGGCC
Erwinia amylovora (Z96088)	GGGAACTCAA	AGGAGACTGC	CGGTG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ATCATGGC	CCTTACGGCC
Erwinia amylovora (AJ233410)	GGGAACTCAA	AGGAGACTGC	CGGTG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ATCATGGC	CCTTACGGCC
Erwinia amylovora (AJ010485)	GGGAACTCAA	AGGAGACTGC	CGGTG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ATCATGGC	CCTTACGGCC
Erwinia amylovora (U80195)	GGGAACTCAA	AGGAGACTGC	CGGTG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ATCATGGC	CCTTACGGCC
Erwinia pyrifoliae	GGGAACTCAA	AGGAGACTGC	CGGTG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ATCATGGC	CCTTACGGCC
Pectobacterium cypripedii	GGGAACTCAA	GGGNGACTGC	CGGNG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ACCATGGC	CCTTACGAGN
Pectobacterium chrysanthemi	GGGAACTCAG	GGGAGACTGC	CGGTG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ATCATGGC	CCTTACGAGT
Pectobacterium c. subsp. *carotovorum*	GGGAACTCAA	AGGAGACTGC	CGGTG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ATCATGGC	CCTTACGAGT
Pectobacterium c. subsp. *wasabiae*	GGGAACTCAA	AGGAGACTGC	CGGTG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ATCATGGC	CCTTACGAGT
Pectobacterium c. subsp. *betavasculorum*	GGGAACTCAA	AGGAGACTGC	CGGTG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ATCATGGC	CCTTACGAGT
Pectobacterium c. subsp. *atrosepticum*	GGGAACTCAA	AGGAGACTGC	CGGTG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ATCATGGC	CCTTACGAGT
Pectobacterium c. subsp. *odoriferum*	GGGAACTCAA	AGGAGACTGC	CGGTG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ATCATGGC	CCTTACGAGT
Pectobacterium cacticidum	GGGAACTCAA	AGGAGACTGC	CGGTG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ATCATGGC	CCTTACGAGT
Brenneria salicis	GGGAACTCAA	AGGAGACTGC	CGGTG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ATCATGGC	CCTTACGAGT
Brenneria rubrifaciens	GGGAACTCAA	AGGAGACTGC	CGGTG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ATCATGGC	CCTTACGAGT
Brenneria nigrifluens	GGGAACTCAA	AGGAGACTGC	CGGTG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ATCATGGC	CCTTACGAGT
Brenneria alni	GGGAACTCAA	AGGAGACTGC	CGGTG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ATCATGGC	CCTTACGAGT
Brenneria paradisiaca	GGGAACTCAA	GGGAGACTGC	CGGTG-ATAA	ACCGGAGGAA	GGTGGGGATG	ACGTCAAGTC	--ATCATGGC	CCTTACGAGT

	1210	1220	1230	1240	1250	1260	1270	1280
Buchnera aphidicola	AGGGCTACAC	ACGTGCTACA	ATGGTTTATA	CAAAGAGAAG	CAAATCTGTA	AAGA-CAAGC	AAACCTCATA	AAGTAAATCG
Proteus vulgaris	AGGGCTACAC	ACGTGCTACA	ATGGCAGATA	CAAAGAGAAG	CGA-CCTCGC	GAGAGCAAGC	GGAACTCATA	AAGTCTGTCG
***Erwinia ananas*, Zululand, KZN, SA**	**AGGGCTACAC**	**ACGTGCTACA**	**ATGGCGCATA**	**CAAAGAGAAG**	**CGA-CCTCGC**	**GAGAGCAAGC**	**GGACCTCATA**	**AAGTGCGTCG**
Pantoea a. pv. *ananatis*	AGGGCTACAC	ACGTGCTACA	ATGGCGCATA	CAAAGAGAAG	CGA-CCTCGC	GAGAGCAAGC	GGACCTCATA	AAGTGCGTCG
Pantoea a. pv. *uredovora*	AGGGCTACAC	ACGTGCTACA	ATGGCGCATA	CAAAGAGAAG	CGA-CCTCGC	GAGAGCAAGC	GGACCTCATA	AAGTGCGTCG
Pantoea agglomerans (U80202)	AGGGCTACAC	ACGTGCTACA	ATGGCGCATA	CAAAGAGAAG	CGA-CCTCGC	GAGAGCAAGC	GGACCTCACA	AAGTGCGTCG
Pantoea agglomerans (AB004757)	AGGGCTACAC	ACGTGCTACA	ATGGCGCATA	CAAAGAGAAG	CGA-CCTCGC	GAGAGCAAGC	GGACCTCACA	AAGTGCGTCG
Pantoea agglomerans (U80183)	AGGGCTACAC	ACGTGCTACA	ATGGCGCATA	CAAAGAGAAG	CAA-CCTCGC	GAGAGCAAGC	GGACCTCACA	AAGTGCGTCG
Unknown *Erwinia* sp.	**AGGGCTACAC**	**ACGTGCTACA**	**ATGGCGCATA**	**CAAAGAGAAG**	**CGA-CCTCGC**	**GAGAGCAAGC**	**GGACCTCATA**	**AAGTGCGTCG**
Pantoea s. subsp. *stewartii*	AGGGCTACAC	ACGTGCTACA	ATGGCGCATA	CAAAGAGAAG	CGA-CCTCGC	GAGAGCAAGC	GGACCTCATA	AAGTGCGTCG
Erwinia tracheiphila	ANGGCTACAC	ACGTGCTACA	ATGGCGCATA	CAAAGAGAAG	CGA-CCTCGC	GAGAGCATGC	GGACCTCATA	AAGTGCGTCG
Erwinia psidii	AGGGCTACAC	ACGTGCTACA	ATGGCGCATA	CAAAGAGAAG	CGA-CCTCGC	GAGAGCAAGC	GGACCTCATA	AAGTGCGTCG
Erwinia mallotivora (AJ233414)	AGGGCTACAC	ACGTGCTACA	ATGGCGCATA	CAAAGAGAAG	CGA-CCTCGC	GAGAGCAAGC	GGATCTCATA	AAGTGCGTCG
Erwinia mallotivora (Z96084)	AGGGCTACAC	ACGTGCTACA	ATGGCGCATA	CAAAGAGAAG	CGA-CCTCGC	GAGAGCAAGC	GGATCTCATA	AAGTGCGTCG
Erwinia persicinus (AJ001190)	AGGGCTACAC	ACGTGCTACA	ATGGCGCATA	CAAAGAGAAG	CGA-CCTCGC	GAGAGCAAGC	GGACCTCATA	AAGTGCGTCG
Erwinia persicinus (Z96086)	AGGGCTACAC	ACGTGCTACA	ATGGCGCATA	CAAAGAGAAG	CGA-CCTCGC	GAGAGCAAGC	GGACCTCATA	AAGTGCGTCG
Erwinia persicinus (U80205)	AGGGCTACAC	ACGTGCTACA	ATGGCGCATA	CAAAGAGAAG	CGA-CCTCGC	GAGAGCAAGC	GGACCTCATA	AAGTGCGTCG
Erwinia rhapontici (AJ233417)	AGGGCTACAC	ACGTGCTACA	ATGGCGCATA	CAAAGAGAAG	CGA-CCTCGC	GAGAGCAAGC	GGACCTCATA	AAGTGCGTCG
Erwinia rhapontici (Z96087)	AGGGCTACAC	ACGTGCTACA	ATGGCGCATA	CAAAGAGAAG	CGA-CCTCGC	GAGAGCAAGC	GGACCTCATA	AAGTGCGTCG
Erwinia rhapontici (U80206)	AGGGCTACAC	ACGTGCTACA	ATGGCGCATA	CAAAGAGAAG	CGA-CCTCGC	GAGAGCAAGC	GGACCTCATA	AAGTGCGTCG
Erwinia amylovora (Z96088)	AGGGCTACAC	ACGTGCTACA	ATGGCGCATA	CAAAGAGAAG	CGA-CCTCGC	GAGAGCAAGC	GGACCTCATA	AAGTGCGTCG
Erwinia amylovora (AJ233410)	AGGGCTACAC	ACGTGCTACA	ATGGCGCATA	CAAAGAGAAG	CGA-CCTCGC	GAGAGCAAGC	GGACCTCATA	AAGTGCGTCG
Erwinia amylovora (AJ010485)	AGGGCTACAC	ACGTGCTACA	ATGGCGCATA	CAAAGAGAAG	CGA-CCTCGC	GAGAGCAAGC	GGACCTCATA	AAGTGCGTCG
Erwinia amylovora (U80195)	AGGGCTACAC	ACGTGCTACA	ATGGCGCATA	CAAAGAGAAG	CGA-CCTCGC	GAGAGCAAGC	GGACCTCATA	AAGTGCGTCG
Erwinia pyrifoliae	AGGGCTACAC	ACGTGCTACA	ATGGCGCATA	CAAAGAGAAG	CGA-CCTCGC	GAGAGCAAGC	GGACCTCATA	AAGTGCGTCG
Pectobacterium cypripedii	AGGGCTACAC	ACGTGCTACA	ATGGCGNATA	CAGAGAGATG	CGA-CCTCGC	GAGAGCAAGC	GGACCTCATA	AAGNGCGTCG
Pectobacterium chrysanthemi	AGGGCTACAC	ACGTGCTACA	ATGGCGCATA	CAAAGAGAAG	CGA-CCTCGC	GAGAGCAAGC	GGACCTCATA	AAGTGCGTCG
Pectobacterium c. subsp. *carotovorum*	AGGGCTACAC	ACGTGCTACA	ATGGCGTATA	CAAAGAGAAG	CGA-CCTCGC	GAGAGCAAGC	GGACCTCATA	AAGTACGTCG
Pectobacterium c. subsp. *wasabiae*	AGGGCTACAC	ACGTGCTACA	ATGGCGTATA	CAAAGAGAAG	CGA-CCTCGC	GAGAGCAAGC	GGACCTCATA	AAGTACGTCG
Pectobacterium c. subsp. *betavasculorum*	AGGGCTACAC	ACGTGCTACA	ATGGCGTATA	CAAAGAGAAG	CGA-CCTCGC	GAGAGCAAGC	GGACCTCATA	AAGTACGTCG
Pectobacterium c. subsp. *atrosepticum*	AGGGCTACAC	ACGTGCTACA	ATGGCGTATA	CAAAGAGAAG	CGAA-CTCGC	GAGAGCCAGC	GGACCTCATA	AAGTACGTCG
Pectobacterium c. subsp. *odoriferum*	AGGGCTACAC	ACGTGCTACA	ATGGCGTATA	CAAAGAGAAG	CGA-CCTCGC	GAGAGCAAGC	GGACCTCATA	AAGTACGTCG
Pectobacterium cacticidum	AGGGCTACAC	ACGTGCTACA	ATGGCGTATA	CAAAGAGAAG	CGAGCCT-GC	GAGGGTGAGC	GGACCTCATA	AAGTACGTCG
Brenneria salicis	AGGGCTACAC	ACGTGCTACA	ATGGCGCATA	CAAAGAGAAG	CGAGCCT-GC	GAGGGTGAGC	GGACCTCATA	AAGTGCGTCG
Brenneria rubrifaciens	AGGGCTACAC	ACGTGCTACA	ATGGCGCATA	CAAAGAGAAG	CGAGCCT-GC	GAGGGTGAGC	GGACCTCATA	AAGTGCGTCG
Brenneria nigrifluens	AGGGCTACAC	ACGTGCTACA	ATGGCGCATA	CAAAGAGAAG	CGAACTT-GC	GAGAGTAAGC	GGACCTCATA	AAGTGCGTCG
Brenneria alni	AGGGCTACAC	ACGTGCTACA	ATGGCGCATA	CAAAGAGAAG	CGAGCCT-GC	GAGGGTGAGC	GGACCTCATA	AAGTGCGTCG
Brenneria paradisiaca	AGGGCTACAC	ACGTGCTACA	ATGGCGCATA	CAAAGAGAAG	CGA-CCTCGC	GAGAGCAAGC	GGATCTCATA	AAGTGCGTCG

	1290	1300	1310	1320	1330	1340	1350	1360
Buchnera aphidicola	TAGTCCGGAC	TGGAGTCTGC	AACTCGACTC	CACGAAGTCG	GAATCGCTAG	TAATCGTGGA	TCA-GAATGC	CACGGTGAAT
Proteus vulgaris	TAGTCCGGAT	TGGAGTCTGC	AACTCGACTC	CATGAAGTCG	GAATCGCTAG	TAATCGTAGA	TCA-GAATGC	TACGGTGAAT
***Erwinia ananas*, Zululand, KZN, SA**	**TAGTCCGGAT**	**CGGAGTCTGC**	**AACTCGACTC**	**CGTGAAGTCG**	**GAATCGCTAG**	**TAATCGTGGA**	**TCA-GAATGC**	**CACGGTGAAT**
Pantoea a. pv. *ananatis*	TAGTCCGGAT	CGGAGTCTGC	AACTCGACTC	CGTGAAGTCG	GAATCGCTAG	TAATCGTGGA	TCA-GAATGC	CACGGTGAAT
Pantoea a. pv. *uredovora*	TAGTCCGGAT	CGGAGTCTGC	AACTCGACTC	CGTGAAGTCG	GAATCGCTAG	TAATCGTGGA	TCA-GAATGC	CACGGTGAAT
Pantoea agglomerans (U80202)	TAGTCCGGAT	CGGAGTCTGC	AACTCGACTC	CGTGAAGTCG	GAATCGCTAG	TAATCGTGGA	TCA-GAATGC	CACGGTGAAT
Pantoea agglomerans (AB004757)	TAGTCCGGAT	CGGAGTCTGC	AACTCGACTC	CGTGAAGTCG	GAATCGCTAG	TAATCGTGGA	TCA-GAATGC	CACGGTGAAT
Pantoea agglomerans (U80183)	TAGTCCGGAT	CGGAGTCTGC	AACTCGACTC	CGTGAAGTCG	GAATCGCTAG	TAATCGTGGA	TCA-GAATGC	CACGGTGAAT
Unknown *Erwinia* sp.	**TAGTCCGGAT**	**TGGAGTCTGC**	**AACTCGACTC**	**CATGAAGTCG**	**GAATCGCTAG**	**TAATCGTAGA**	**TCA-GAATGC**	**TACGGTGAAT**
Pantoea s. subsp. *stewartii*	TAGTCCGGAT	CGGAGTCTGC	AACTCGACTC	CGTGAAGTCG	GAATCGCTAG	TAATCGTGGA	TCA-GAATGC	CACGGTGAAT
Erwinia tracheiphila	TAGTCCGGAT	CGGAGTCTGC	AACTCGACTC	CGTGAAGTCG	GAATCGCTAG	TAATCGTGGA	TCA-GAATGC	CACGGTGAAT
Erwinia psidii	TAGTCCGGAT	CGGAGTCTGC	AACTCGACTC	CGTGAAGTCG	GAATCGCTAG	TAATCGTGGA	TCA-GAATGC	CACGGTGAAT
Erwinia mallotivora (AJ233414)	TAGTCCGGAT	CGGAGTCTGC	AACTCGACTC	CGTGAAGTCG	GAATCGCTAG	TAATCGTGGA	TCA-GAATGC	CACGGTGAAT
Erwinia mallotivora (Z96084)	TAGTCCGGAT	CGGAGTCTGC	AACTCGACTC	CGTGAAGTCG	GAATCGCTAG	TAATCGTGGA	TCA-GAATGC	CACGGTGAAT
Erwinia persicinus (AJ001190)	TAGTCCGGAT	CGGAGTCTGC	AACTCGACTC	CGTGAAGTCG	GAATCGCTAN	TAATCGTAGA	TCAAGAATGC	TACGGTGAAT
Erwinia persicinus (Z96086)	TAGTCCGGAT	CGGAGTCTGC	AACTCGACTC	CGTGAAGTCG	GAATCGCTAG	TAATCGTAGA	TCA-GAATGC	TACGGTGAAT
Erwinia persicinus (U80205)	TAGTCCGGAT	CGGAGTCTGC	AACTCGACTC	CGTGAAGTCG	GAATCGCTAG	TAATCGTAGA	TCA-GAATGC	TACGGTGAAT
Erwinia rhapontici (AJ233417)	TAGTCCGGAT	CGGAGTCTGC	AACTCGACTC	CGTGAAGTCG	GAATCGCTAG	TAATCGTAGA	TCA-GAATGC	TACGGTGAAT
Erwinia rhapontici (Z96087)	TAGTCCGGAT	CGGAGTCTGC	AACTCGACTC	CGTGAAGTCG	GAATCGCTAG	TAATCGTAGA	TCA-GAATGC	TACGGTGAAT
Erwinia rhapontici (U80206)	TAGTCCGGAT	CGGAGTCTGC	AACTCGACTC	CGTGAAGTCG	GAATCGCTAG	TAATCGTAGA	TCA-GAATGC	TACGGTGAAT
Erwinia amylovora (Z96088)	TAGTCCGGAT	CGGAGTCTGC	AACTCGACTC	CGTGAAGTCG	GAATCGCTAG	TAATCGTAGA	TCA-GAATGC	TACGGTGAAT
Erwinia amylovora (AJ233410)	TAGTCCGGAT	CGGAGTCTGC	AACTCGACTC	CGTGAAGTCG	GAATCGCTAG	TAATCGTAGA	TCA-GAATGC	TACGGTGAAT
Erwinia amylovora (AJ010485)	TAGTCCGGAT	CGGAGTCTGC	AACTCGACTC	CGTGAAGTCG	GAATCGCTAG	TAATCGTAGA	TCA-GAATGC	TACGGTGAAT
Erwinia amylovora (U80195)	TAGTCCGGAT	CGGAGTCTGC	AACTCGACTC	CGTGAAGTCG	GAATCGCTAG	TAATCGTAGA	TCA-GAATGC	TACGGTGAAT
Erwinia pyrifoliae	TAGTCCGGAT	CGGAGTCTGC	AACTCGACTC	CGTGAAGTCG	GAATCGCTAG	TAATCGTAGA	TCA-GAATGC	TACGGTGAAT
Pectobacterium cypripedii	NAGTCCGGAT	TGGAGTCTGC	AACTCGACTC	CATGAAGTCG	GAATCGCTAG	TAATCGTAGA	TCA-GAATGG	TACGGTGAAT
Pectobacterium chrysanthemi	TAGTCCGGAT	TGGAGTCTGC	AACTCGACTC	CATGAAGTCG	GAATCGCTAG	TAATCGTAGA	TCA-GAATGC	TACGGTGAAT
Pectobacterium c. subsp. *carotovorum*	TAGTCCGGAT	TGGAGTCTGC	AACTCGACTC	CATGAAGTCG	GAATCGCTAG	TAATCGTAGA	TCA-NAATGC	TACGGTGAAT
Pectobacterium c. subsp. *wasabiae*	TAGTCCGGAT	TGGAGTCTGC	AACTCGACTC	CATGAAGTCG	GAATCGCTAG	TAATCGTAGA	TCA-GAATGC	TACGGTGAAT
Pectobacterium c. subsp. *betavasculorum*	TAGTCCGGAT	TGGAGTCTGC	AACTCGACTC	CATGAAGTCG	GAATCGCTAG	TAATCGTAGA	TCA-GAATGC	TACGGTGAAT
Pectobacterium c. subsp. *atrosepticum*	TAGTCCGGAT	TGGAGTCTGC	AACTCGACTC	CATGAAGTCG	GAATCGCTAG	TAATCGTAGA	TCA-GAATGC	TACGGTGAAT
Pectobacterium c. subsp. *odoriferum*	TAGTCCGGAT	TGGAGTCTGC	AACTCGACTC	CATGAAGTCG	GAATCGCTAG	TAATCGTAGA	TCA-GAATGC	TACGGTGAAT
Pectobacterium cacticidum	TAGTCCGGAT	TGGAGTCTGC	AACTCGACTC	CATGAAGTCG	GAATCGCTAG	TAATCGTANA	TCA-GAATGC	TACGGTGAAT
Brenneria salicis	TAGTCCGGAT	TGGAGTCTGC	AACTCGACTC	CATGAAGTCG	GAATCGCTAG	TAATCGTAGA	TCA-GAATGC	TACGGTGAAT
Brenneria rubrifaciens	TAGTCCGGAT	TGGAGTCTGC	AACTCGACTC	CATGAAGTCG	GAATCGCTAG	TAATCGTAGA	TCA-GAATGC	TACGGTGAAT
Brenneria nigrifluens	TAGTCCGGAT	TGGAGTCTGC	AACTCGACTC	CATGAAGTCG	GAATCGCTAG	TAATCGTAGA	TCA-GAATGC	TACGGTGAAT
Brenneria alni	TAGTCCGGAT	TGGAGTCTGC	AACTCGACTC	CATGAAGTCG	GAATCGCTAG	TAATCGTGGA	TCA-GAATGC	CACGGTGAAT
Brenneria paradisiaca	TAGTCCGGAT	TGGAGTCTGC	AACTCGACTC	CATGAAGTCG	GAATCGCTAG	TAATCGCGGA	TCA-GAATGC	CGCGGTGAAT

	1370	1380	1390	1400	1410	1420	1430	1440
Buchnera aphidicola	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAAGAAGC	AGATTTCCTA	ACCAC-GAAA
Proteus vulgaris	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGCTTG	CAAAAGAAGT	AGGTAGCTTA	ACCTTCGGGA
***Erwinia ananas*, Zululand, KZN, SA**	**ACGTTCCCGG**	**GCCTTGTACA**	**CACCGCCCGT**	**CACACCATGG**	**GAGTGGGTTG**	**CAAAAGAAGT**	**AGGTAGCTTA**	**ACCTTCGGGA**
Pantoea a. pv. *ananatis*	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAAGAAGT	AGGTAGCTTA	ACCTTCGGGA
Pantoea a. pv. *uredovora*	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAAGAAGT	AGGTAGCTTA	ACCTTCGGGA
Pantoea agglomerans (U80202)	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAAGAAGT	AGGTAGCTTA	ACCTTCGGGA
Pantoea agglomerans (AB004757)	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAAGAAGT	AGGTAGCTTA	ACCTTCGGGA
Pantoea agglomerans (U80183)	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAAGAAGT	AGGTAGCTTA	ACCTTCGGGA
Unknown *Erwinia* sp.	**ACGTTCCCGG**	**GCCTTGTACA**	**CACCGCCCGT**	**CACACCATGG**	**GAGTGGGTTG**	**CAAAAGAAGT**	**AGGTAGCTTA**	**ACCTTCGGGA**
Pantoea s. subsp. *stewartii*	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAAGAAGT	AGGTAGCTTA	ACCCTCGGGA
Erwinia tracheiphila	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAAGAAGT	ANGTANCTTA	ACCTTCGGGA
Erwinia psidii	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAAGAAGT	AGGTAGCTTA	ACCTTCGGGA
Erwinia mallotivora (AJ233414)	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAAGAAGT	AGGTAGCTTA	ACCTTCGGGA
Erwinia mallotivora (Z96084)	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAAGAAGT	AGGTAGCTTA	ACCTTCGGGA
Erwinia persicinus (AJ001190)	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAAGAAGT	AGGTAGCTTA	ACCTTCGGGA
Erwinia persicinus (Z96086)	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAAGAAGT	AGGTAGCTTA	ACCTTCGGGA
Erwinia persicinus (U80205)	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAAGAAGT	AGGTAGCTTA	ACCTTCGGGA
Erwinia rhapontici (AJ233417)	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAAGAAGT	AGGTAGCTTA	ACCTTCGGGA
Erwinia rhapontici (Z96087)	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAAGAAGT	AGGTAGCTTA	ACCTTCGGGA
Erwinia rhapontici (U80206)	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAAGAAGT	AGGTAGCTTA	ACCTTCGGGA
Erwinia amylovora (Z96088)	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAAGAAGT	AGGTAGCTTA	ACCTTCGGGA
Erwinia amylovora (AJ233410)	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAAGAAGT	AGGTAGCTTA	ACCTTCGGGA
Erwinia amylovora (AJ010485)	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAAGAAGT	AGGTAGCTTA	AC-TTCGGGA
Erwinia amylovora (U80195)	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAAGAAGT	AGGTAGCTTA	ACCTTCGGGA
Erwinia pyrifoliae	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAAGAAGT	AGGTAGCTTA	AC-TTCGGGA
Pectobacterium cypripedii	NCGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAANGAAGT	AGGTAGCTTA	ACCNTCGGGA
Pectobacterium chrysanthemi	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAAGAAGT	AGGTAGCTTA	ACCTTCGGGA
Pectobacterium c. subsp. *carotovorum*	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAAGAAGT	AGGTAGCTTA	ACCTTCGGGA
Pectobacterium c. subsp. *wasabiae*	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAAGAAGT	AGGTAGCTTA	ACCTTCGGGA
Pectobacterium c. subsp. *betavasculorum*	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAAGAAGT	AGGTAGCTTA	ACCTTCGGGA
Pectobacterium c. subsp. *atrosepticum*	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAAGAAGT	AGGTAGCTTA	ACCTTCGGGA
Pectobacterium c. subsp. *odoriferum*	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAAGAAGT	AGGTAGCTTA	ACCTTCGGGA
Pectobacterium cacticidum	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAANAAGT	AGGTAGCTTA	ACCTTCGGGG
Brenneria salicis	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAAGAAGT	AGGTAGCTTA	ACCTTAGGGG
Brenneria rubrifaciens	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAAGAAGT	AGGTAGCTTA	ACCTTCGGGG
Brenneria nigrifluens	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAAGAAGT	AGGTAGCTTA	ACCTTAGGGG
Brenneria alni	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAAGAAGT	AGGTAGCTTA	ACCTCAGGGG
Brenneria paradisiaca	ACGTTCCCGG	GCCTTGTACA	CACCGCCCGT	CACACCATGG	GAGTGGGTTG	CAAAAGAAGT	AGGTAGCTTA	AC-TTCGGGA

	1450	1460	1470	
Buchnera aphidicola	GTGGAAGGCG	-TCTACCACT	TTGTGATTCA	TGA
Proteus vulgaris	G-----GGCG	CT-TACCACT	TTGTGATTCA	TGA
***Erwinia ananas*, Zululand, KZN, SA**	**G-----GGCG**	**CT-TACCACT**	**TTGTGATTCA**	**TGA**
Pantoea a. pv. *ananatis*	G-----GGCG	CT-TACCACT	TTGTGATTCA	TGA
Pantoea a. pv. *uredovora*	G-----GGCG	CT-TACCACT	TTGTGATTCA	TGA
Pantoea agglomerans (U80202)	G-----GGCG	CT-TACCACT	TTGTGATTCA	TGA
Pantoea agglomerans (AB004757)	G-----GGCG	CT-TACCACT	TTGTGATTCA	TGA
Pantoea agglomerans (U80183)	G-----GGCG	CT-TACCACT	TTGTGATTCA	TGA
Unknown *Erwinia* sp.	**G-----GGCG**	**CT-TACCACT**	**TTGTGATTCA**	**TGA**
Pantoea s. subsp. *stewartii*	G-----GGCG	CT-TACTACT	TTGTGATTCA	TGA
Erwinia tracheiphila	G-----GGCG	CT-TACCACT	TTGTGATTCA	TNA
Erwinia psidii	G-----GGCG	CT-TACCACT	TTGTGATTCA	TGA
Erwinia mallotivora (AJ233414)	G-----GGCG	CT-TACCACT	TTGTGATTCA	TGA
Erwinia mallotivora (Z96084)	G-----GGCG	CT-TACCACT	TTGTGATTCA	TGA
Erwinia persicinus (AJ001190)	G-----GGCG	CT-TACCACT	TTGTGATTCA	TGA
Erwinia persicinus (Z96086)	G-----GGCG	CT-TACCACT	TTGTGATTCA	TGA
Erwinia persicinus (U80205)	G-----GGCG	CT-TACCACT	TTGTGATTCA	TGA
Erwinia rhapontici (AJ233417)	G-----GGCG	CT-TACCACT	TTGTGATTCA	TGA
Erwinia rhapontici (Z96087)	G-----GGCG	CT-TACCACT	TTGTGATTCA	TGA
Erwinia rhapontici (U80206)	G-----GGCG	CT-TACCACT	TTGTGATTCA	TGA
Erwinia amylovora (Z96088)	G-----GGCG	CT-TACCACT	TTGTGATTCA	TGA
Erwinia amylovora (AJ233410)	G-----GGCG	CT-TACCACT	TTGTGATTCA	TGA
Erwinia amylovora (AJ010485)	G-----GGCG	CT-TACCACT	TTGTGATTCA	TGA
Erwinia amylovora (U80195)	G-----GGCG	CT-TACCACT	TTGTGATTCA	TGA
Erwinia pyrifoliae	G-----GGCG	CT-TACCACT	TTGTGATTCA	TGA
Pectobacterium cypripedii	G-----GGCG	CT-TACCACT	TTGTGATTCA	TGA
Pectobacterium chrysanthemi	G-----GGCG	CT-TACCACT	TTGTGATTCA	TGA
Pectobacterium c. subsp. *carotovorum*	G-----GGCG	CT-TACCACT	TTGTGATTCA	TGA
Pectobacterium c. subsp. *wasabiae*	G-----GGCG	CT-TACCACT	TTGTGATTCA	TGA
Pectobacterium c. subsp. *betavasculorum*	G-----GGCG	CT-TACCACT	TTGTGATTCA	TGA
Pectobacterium c. subsp. *atrosepticum*	G-----GGCG	CT-TACCACT	TTGTGATTCA	TGA
Pectobacterium c. subsp. *odoriferum*	G-----GGCG	CT-TACCACT	TTGTGATTCA	TGA
Pectobacterium cacticidum	G-----GGCG	CT-TACCACT	TTGTGATTCA	TGA
Brenneria salicis	G-----GGCG	CT-TACCACT	TTGTGATTCA	TGA
Brenneria rubrifaciens	G-----GGCG	CT-TACCACT	TTGTGATTCA	TGA
Brenneria nigrifluens	G-----GGCG	CT-TACCACT	TTGTGATTCA	TGA
Brenneria alni	G-----GGCG	CT-TACCACT	TTGTGATTCA	TGA
Brenneria paradisiaca	G-----GGCG	CT-TACCACT	TTGTGATTCA	TGA

Fig. 4. Dendrogram based on 16S rDNA sequences, showing the phylogenetic relationships of both South African *Erwinia* species with species of the genera *Pantoea*, *Erwinia*, *Pectobacterium* and *Brenneria*. The tree is rooted with *Proteus vulgaris* and *Buchnera aphidicola* and was constructed by using the neighbour-joining method and bootstrap values calculated from 1000 trees. The tree length = 1161 steps, CI = 0.498, HI = 0.502 and RI = 0.766. Bootstrap values are given above each branching point.

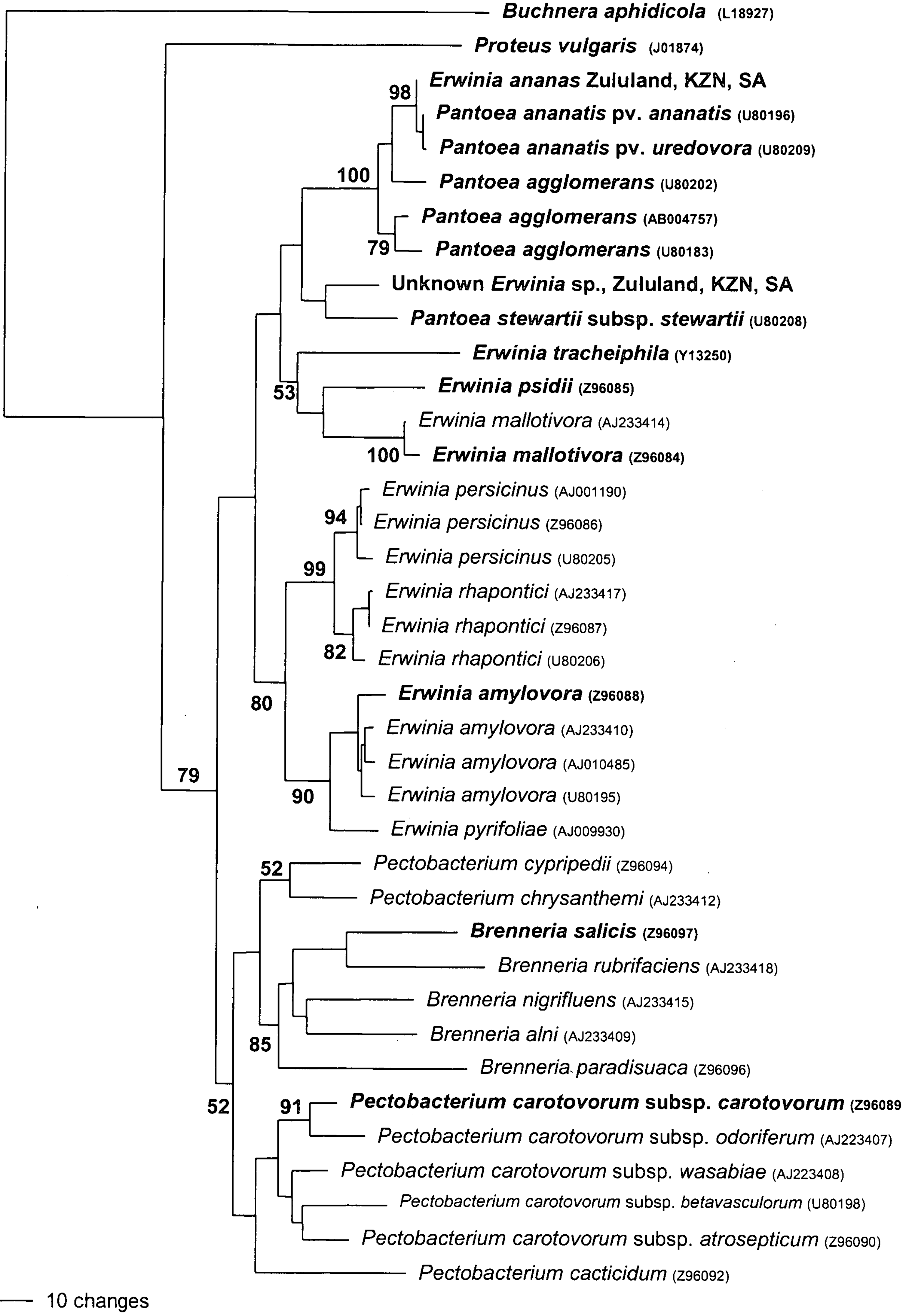
Buchnera aphidicola (L18927)
Proteus vulgaris (J01874)
98
Erwinia ananas Zululand, KZN, SA
Pantoea ananatis pv. ananatis (U80196)
Pantoea ananatis pv. uredovora (U80209)
100
Pantoea agglomerans (U80202)
Pantoea agglomerans (AB004757)
79
Pantoea agglomerans (U80183)
Unknown Erwinia sp., Zululand, KZN, SA
Pantoea stewartii subsp. stewartii (U80208)
Erwinia tracheiphila (Y13250)
53
Erwinia psidii (Z96085)
Erwinia mallotivora (AJ233414)
100
Erwinia mallotivora (Z96084)
Erwinia persicinus (AJ001190)
94
Erwinia persicinus (Z96086)
Erwinia persicinus (U80205)
99
Erwinia rhapontici (AJ233417)
Erwinia rhapontici (Z96087)
82
Erwinia rhapontici (U80206)
Erwinia amylovora (Z96088)
80
Erwinia amylovora (AJ233410)
79
Erwinia amylovora (AJ010485)
90
Erwinia amylovora (U80195)
Erwinia pyrifoliae (AJ009930)
52
Pectobacterium cypripedii (Z96094)
Pectobacterium chrysanthemi (AJ233412)
Brenneria salicis (Z96097)
Brenneria rubrifaciens (AJ233418)
Brenneria nigrifluens (AJ233415)
Brenneria alni (AJ233409)
85
Brenneria paradisuaca (Z96096)
52
91
Pectobacterium carotovorum subsp. carotovorum (Z96089
Pectobacterium carotovorum subsp. odoriferum (AJ223407)
Pectobacterium carotovorum subsp. wasabiae (AJ223408)
Pectobacterium carotovorum subsp. betavasculorum (U80198)
Pectobacterium carotovorum subsp. atrosepticum (Z96090)
Pectobacterium cacticidum (Z96092)
10 changes

Fig. 5. Necrotic lesion development on fresh Granny Smith apples after inoculation with *C. zuluense* (CRY and CMW numbers) and both *Pantoea* species (*P. ananatis* pv. *ananatis* and an unknown *Pantoea* sp.). (A - D) Insignificant lesion development was observed when apples were inoculated with *C. zuluense* isolates (A = CRY 1055; B = CRY 1016; C = CMW 2100; D = CRY 1023). (E - F) Bacteria species caused significant tissue maceration when inoculated alone (E = unknown *Pantoea* sp.; F = *P. ananatis* pv. *ananatis*). (G - H) Inoculation of both *Pantoea* species in association with *C. zuluense* resulted in significantly larger lesions (G = *Pantoea* sp. + *P. ananatis* pv. *ananatis* + CMW 2100; H = *Pantoea* sp. + *P. ananatis* pv. *ananatis* + CRY 1023).

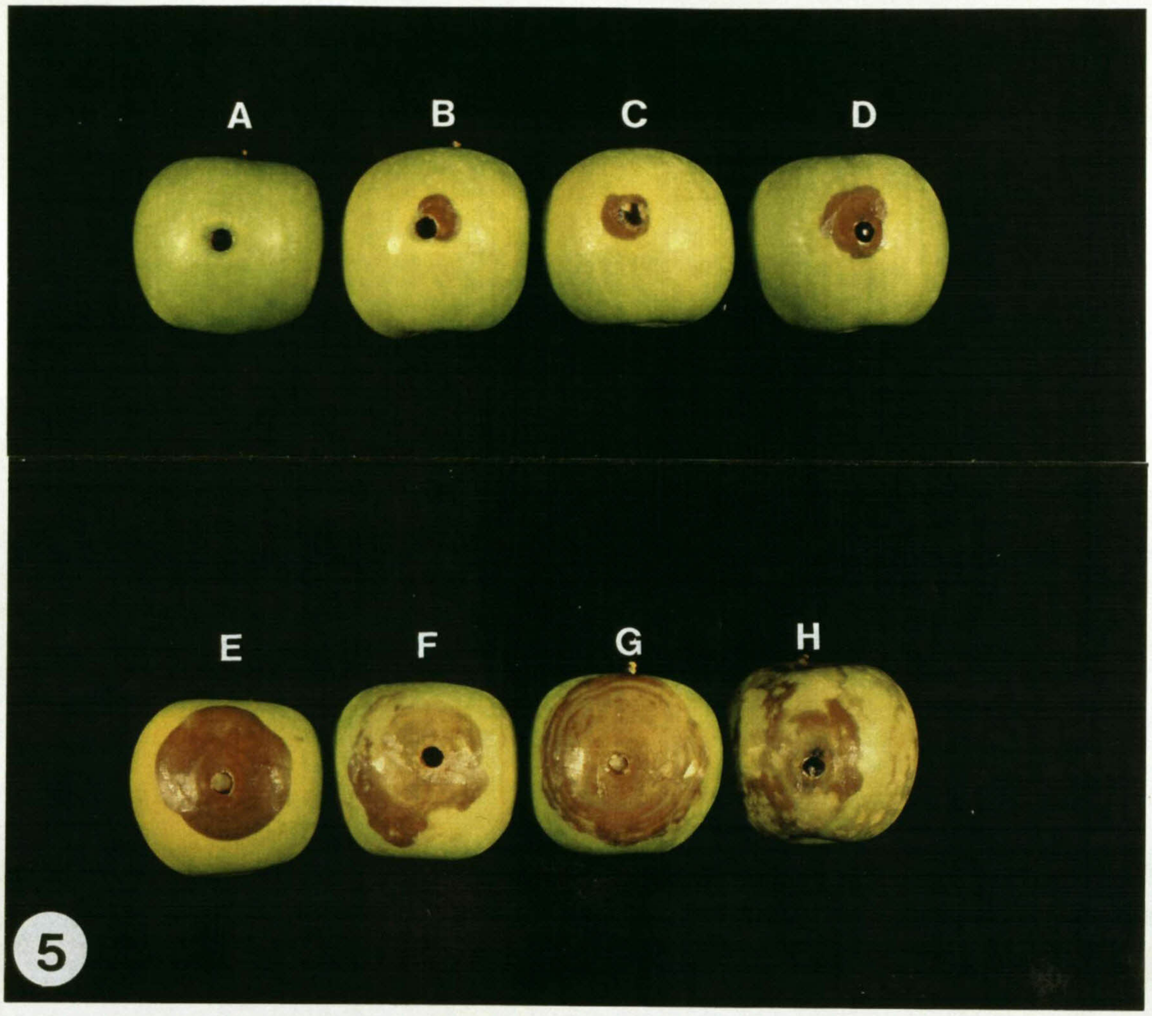
A
B
C
D
E
F
G
H
5

Fig. 6. Lesion development on a susceptible *Eucalyptus grandis* clone (ZG 14) inoculated with isolates of *C. zuluense* and both *Pantoea* species (A) No lesion development was observed after inoculations using each of the *Pantoea* species, respectively (B) Canker development after inoculation with virulent isolates of *C. zuluense*. (C) Significantly larger lesions were observed with inoculation of *C. zuluense* in combination with both *Pantoea* species.


A
B
C

CHAPTER 6

Polygalacturonase production by the *Eucalyptus* canker pathogen, *Coniothyrium zuluense*, and two *Pantoea* species

The *Eucalyptus* stem canker pathogen, *Coniothyrium zuluense*, has been shown to be associated with two bacterial species, *Pantoea ananatis* pv. *ananatis* and an unknown *Pantoea* species. A significant increase in pathogenicity occurs when these bacteria are inoculated in combination with *C. zuluense*. This suggests that a synergistic interaction may possibly exist between these micro-organisms. Pathogenicity in some fungi has been shown to be correlated with the enzyme, polygalacturonase (PG). In this study we determined the ability to produce PG for pathogenic and non-pathogenic *C. zuluense* isolates, as well as both *Pantoea* species. The level of PG production by these micro-organisms was estimated by cup-plate and reducing sugars assays. A significant correlation was found between PG activity and pathogenicity of *C. zuluense*. Experimental assays demonstrated that levels of PG activity for both *Pantoea* species were significantly higher than those obtained for *C. zuluense* isolates. As PG is the first enzyme produced during disease development, it is hypothesised that the two *Pantoea* species may play a significant role in the development of Coniothyrium canker. Production of PG might also be used as an assay to evaluate pathogenicity in different isolates of *C. zuluense*.

The biotrophic canker pathogen, *Coniothyrium zuluense* Wingfield, Crous & Coutinho, is the causal agent of a serious *Eucalyptus* stem canker disease in South Africa (Wingfield *et al.*, 1997). Wingfield *et al.* (unpublished data) showed that once conidia of this fungus germinate, the germ tubes infect the stem directly through the epidermis of young stem tissue. To accomplish this, a number of cell-wall-degrading enzymes are needed to enable penetration and colonisation of the plant tissue, as well as to release the nutrients necessary for growth (Walton, 1994; Alghisi & Favaron, 1995; Annis & Goodwin, 1997). Enzymes that are known to be involved in this process include pectic enzymes, cellulases, arabinases, xylanases and galactanases (Alghisi & Favaron, 1995; Annis & Goodwin, 1997). Of these enzymes, only the pectin-degrading group of enzymes have been positively linked to pathogenesis (Barras *et al.*, 1994; Alghisi & Favaron, 1995; Kombrink & Somssich, 1995; Hugouvieux-Cotte-Pattat *et al.*, 1996).

Pectic enzymes are produced in large quantities by many plant-associated micro-organisms and are important requirements for colonisation of plant tissue (Cooper, 1983; Collmer & Keen, 1986; Barras *et al.*, 1994; Alghisi & Favaron, 1995; Alfano & Collmer, 1996; Hugouvieux-Cotte-Pattat *et al.*, 1996; Annis & Goodwin, 1997). Pectin is the major component of the primary plant cell wall and middle lamellae. The depolymerisation of pectin is, therefore, essential for further cell wall breakdown by other cell-wall degrading enzymes (Karr & Albersheim, 1970). Polygalacturonase (E.C. 3.2.1.15) (PG) is the main component of the pectic enzyme complex (Collmer & Keen, 1986; Collmer *et al.*, 1988). PG is the first hydrolytic enzyme produced by many plant pathogens and is a determining factor in pathogenicity for bacterial and fungal pathogens (Barras *et al.*, 1994; Alghisi & Favaron, 1995; Hugouvieux-Cotte-Pattat *et al.*, 1996; Annis & Goodwin, 1997).

The role that polygalacturonase production plays in bacteria, differs significantly between bacterial species. An endo-polygalacturonase defective mutant of *Pectobacterium carotovorum* subsp. *carotovorum* (synonym, *Erwinia carotovora* subsp. *carotovora*), that maintained pectate-lyase and exo-polygalacturonase activities, kept its virulence on tissues of different plants (Willis *et al.*, 1987). Similarly, the endo-polygalacturonase of *P. solanacearum* pv. *tomato* is not required to cause tomato disease, although it accelerates its development (Denny *et al.*, 1990). On the contrary, mutation in the single polygalacturonase-encoding gene of *Agrobacterium tumefaciens*, eliminates polygalacturonase activity and substantially decreases its virulence (Rodriquez-Palenzuela *et al.*, 1991). It is, therefore, evident that endo - and exo - polygalacturonase production is important in diseases caused by bacteria.

There are relatively few studies on the role of fungal polygalacturonases in plant disease. Most studies have considered the ability of purified enzymes to reproduce disease symptoms (Benhamou *et al.*, 1991; Favaron *et al.*, 1993). Convincing evidence of polygalacturonase involvement, however, was obtained using *Aspergillus flavus* Link.. Low-virulence strains lacking a major endo-polygalacturonase, caused a reduction of disease symptoms in developing cotton bolls (Cleveland & Cotty, 1991, Brown *et al.*, 1992). Similarly, mutants of *Fusarium oxysporum* f.sp. *lycopersici* Schlechtend.:Fr. [(Sacc.) W.C. Snyder & H.N. Hans.] lacking polygalacturonase activity had reduced virulence on tomato (Mann, 1962). In contrast, a strain of *Cochliobolus carbonum* Nelson in which the gene for endo-polygalacturonase had been disrupted, was unaffected for pathogenicity on maize (Scott-Craig *et al.*, 1990). The importance of the contribution of fungal polygalacturonases towards pathogenesis must be considered case by case. However, this enzyme together with the rest of the depolymerases is essential for complete disease development.

Coniothyrium canker has been shown to be associated with two *Pantoea* species, *P. ananatis* pv. *ananatis* and an unknown *Pantoea* species (Van Zyl *et al.*, chapter 5). A significant increase in pathogenicity to a susceptible *E. grandis* clone was observed when fungal and bacterial isolates were inoculated in combination (Van Zyl *et al.*, chapter 5). It is known that pathogenicity of fungal and bacterial plant pathogens is directly influenced by the production of polygalacturonase (Yang *et al.*, 1992; Barras *et al.*, 1994; Le Cam *et al.*, 1994; Alghisi & Favaron, 1995; Alfano & Collmer, 1996; Annis & Goodwin, 1997). The objective of this study was, therefore, to screen strains of *C. zuluense* and the two *Pantoea* species for PG activity and to link this with pathogenicity data presented in a previous study (Van Zyl *et al.*, chapter 5).

MATERIALS AND METHODS

Fungal and bacterial isolates

Van Zyl *et al.* (Chapter 5) observed a possible synergistic interaction between *C. zuluense* and both *Pantoea* species, *Pantoea ananatis* pv. *ananatis* and an unknown species closely related to *Pantoea stewartii* subsp. *stewartii*. Based on this, we have selected six *C. zuluense* isolates to be used in this study (Table 1). Pathogenicity characteristics of pure bacteria-free *C. zuluense* isolates varied between non-pathogenic, intermediately pathogenic and highly pathogenic as determined in a previous study (Van Zyl *et al.*, 1997). It was also shown that relative pathogenicity of selected *C. zuluense* isolates was significantly increased when fungal and bacterial isolates were inoculated in combination with each other (Van Zyl *et al.*, Chapter 5).

One strain of each *Pantoea* sp. was included in this study (Table 1). Van Zyl *et al.* (chapter 5) showed that significant differences in tissue maceration of Granny Smith apples were evident between strains of each of the two *Pantoea* species (ten strains for each species). No significant tissue maceration was, however, found among strains of each bacterial species (Van Zyl *et al.*, chapter 5). For the purpose of this study we have, therefore, selected one strain for each *Pantoea* species, producing most severe tissue maceration (Table 1).

All fungal and bacterial isolates used in this study were collected from the Zululand forestry region in the KwaZulu-Natal province of South Africa and are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria.

Detection of PG in C. zuluense

Polygalacturonase production was induced by growing isolates of *C. zuluense* in a sterile liquid minimum salts medium (2.0 g NH_4NO_3; 1.0 g KH_2PO_4; 0.1 g $MgSO_4$; 0.5 g yeast extract (Merck); 1.0 g NaOH; 3.0 g $_{DL}$–malic; 1 l distilled H_2O) supplemented with 0.5 % w/v sodium polygalacturonic acid (PGA) (Sigma Chemical Company, St. Louis, MO) as a carbon source (Errampalli & Kohn, 1995). Five, 4 mm plugs of mycelium, from actively growing areas of 7-day-old *C. zuluense* colonies were inoculated in 100 ml of liquid medium and incubated for 10 days on a shaker incubator at 25 °C in the dark. Mycelium was harvested by filtration through Whatman no. 113 filter paper, using a Buchner funnel and suction filtration system. Samples were collected each day for 10 days, supernatant was filter sterilised (0.22 μm disposable syringe filters, Millipore, US) and stored at 4 °C. All samples were assayed for PG activity in triplicate.

Detection of PG in Pantoea spp.

Culture filtrates were prepared by growing *Pantoea* species in Errampalli & Kohn's (1995) minimum salts medium with PGA as a carbon source. Bacteria were rinsed from a 24-hr nutrient agar slant culture and diluted according to the method described by Varvaro (1987). One ml of the suspension was then added to 100 ml of the minimum salts medium in 250 ml Erlenmeyer flasks. Isolates were incubated at 25 °C for 10 days. Samples were collected each day, bacteria were removed by filtration through 0.22 μm disposable syringe filters (Millipore, US), and the filtrate stored at 4 °C. All samples were then assayed for PG activity in triplicate.

Polygalacturonase assay

Cup-plate assay. Polygalacturonase activity was visualised using a modified agarose diffusion assay (Cup-plate method) described by Dingle *et al.* (1953). Assay medium contained 0.5 % ammonium oxalate, 0.2 % sodium azide, and 1.0 % Type II agarose (Sigma Chemical Co.) dissolved in 100 ml of 0.2 M phosphate buffer (1 M K_2HPO_4; 1 M KH_2PO_4; 1 l distilled H_2O, adjusted to pH 5.3). PGA (0.01 %) was used as substrate. The medium was heated to dissolve PGA and agarose, then transferred to Petri plates (20 ml per plate). A no. 1 cork borer was used to punch five wells, 4.1 mm in diameter and 2.5 cm apart, in the solidified medium. Each well was filled (30 μl) with standard, control, or unknown (filtrate) solutions. Petri dishes were incubated overnight at 30 °C. The gel was developed after incubation by flooding the assay plate with 10 ml of 0.05 % ruthenium red (Sigma Chemical Co.) for 2 hr at 25 °C. Excess dye was removed by washing the plate several times with ddH_2O. A clear area around the well (Fig. 1) indicated PG activity. Two diameter measurements (at right angles) of the cleared areas were taken for each well from duplicate plates and averages recorded. Each isolate was tested three times. The supernatant showing the highest activity (largest rings) was then assayed using the reduced end-group analysis procedure, in order to determine a more accurate enzyme activity.

A standard curve (ring diameter vs. concentration of standard) was prepared using polygalacturonase, poly [1,4-α-D-galacturonide] glycanohydrolase: EC 3.2.1.15 (Sigma Chemical Co.) at the following concentrations: 5.0, 0.5, 0.05, 0.005, and 0.0005 mg / ml in dH_2O. The lyophilised polygalacturonase had an activity of 1440 units (U)/mg (Sigma Chemical Co.). Pectolytic activity was expressed as Units / ml.

Reducing sugar assay. Polygalacturonase activity was determined by measuring the release of reducing-end groups by enzymatic hydrolysis of PGA. The reducing-end groups were measured by the method of York *et al.* (1985). Galacturonic acid was used as the standard for the assays. Activity of polygalacturonase was measured by incubating 50 μl of the sample to be assayed, for 60 min at 30 °C, in 1 ml of a solution containing 0.25 % w/v PGA and 40 mM sodium-acetate (pH 5.0).

The reaction was terminated by addition of 1.5 ml of PAHBAH reagent (p-Hydroxybenzoic acid hydrazide (Fw 152.2), Sigma #H 9882). PAHBAH reagent was freshly prepared each time, by mixing 4 volumes of 0.5 M NaOH with 1 volume of 5 % PAHBAH in 0.5 M HCl. The tubes containing the samples were boiled for 10 min, cooled and the absorbence was read at $A_{410\ \mathrm{nm}}$. One unit of PG activity (RGU) was defined as the amount of enzyme producing 1 μmol of reducing group per minute at 30 °C with 0.5 % PGA in 40 mM of sodium acetate buffer, pH 5. Experiment was done in triplicate. Means were tested for significance according to Tukey's procedure for comparison of means (ANOVA analysis, NCSS97).

RESULTS

Detection of PG

All *C. zuluense* isolates, as well as the two *Pantoea* species produced polygalacturonase in the presence of polygalacturonic acid (PGA) in a minimal salts medium. *Coniothyrium zuluense* isolates reached maximum PG activity after 5 days of incubation. Both *Pantoea* species reached maximum PG activity 3 days after inoculation (Fig. 2).

Cup-plate assay. Using ANOVA (NCSS97), it was possible to identify significant differences ($P = 0.05$) between *C. zuluense* isolates that differ in their pathogenicity to a susceptible *E. grandis* clone (Table 2). The most pathogenic isolates, CMW 2100 and CRY 1023, showed significantly higher levels of PG activity (0.243 U/ml, $P = 0.05$; 0.249 U/ml, $P = 0.05$, respectively) than intermediate and non-pathogenic isolates (Table 2). There was no significant difference ($P = 0.05$, not significant, NS) between PG activity of intermediate (CMW 1778 and CRY 1016) and non-pathogenic (CRY 1055 and CRY 1054) isolates (Table 2).

A significant difference of PG activity ($P = 0.05$) between isolates of *C. zuluense* and those of both *Pantoea* species, was evident (Table 2). *Pantoea ananatis* pv. *ananatis* produced significantly higher PG activity (0.418 U/ml; $P = 0.05$) than the isolate of the unknown *Pantoea* species (0.348 U/ml; $P = 0.05$) (Table 2). Levels of

PG activity from both *Pantoea* species were, however, significantly higher when compared with PG levels measured for the *C. zuluense* isolates (Table 2).

Reducing sugar assay. The amount of reducing groups released (depolymerisation of PGA) showed a significant correlation of 96 % (r = 0.96) with levels of PG activity obtained in the cup-plate assay procedure. The depolymerisation of PGA differed significantly (*P = 0.05*) between PG activity of pathogenic, intermediate and non-pathogenic *C. zuluense* isolates. PG activity of the pathogenic isolates (CMW 2100 and CRY 1023) was significantly higher (*P = 0.05*) than that of the intermediate and non-pathogenic isolates (Table 2). No significant difference was, however, evident between PG activity of pathogenic isolates, CMW 2100 (0.136 μmol ml^{-1} min^{-1}; NS) and CRY 1023 (0.14 μmol ml^{-1} min^{-1}; NS). A significant difference (*P = 0.05*) in the level of depolymerisation of PGA was observed between intermediate (CMW 1778 and CRY 1016) and non-pathogenic (CRY 1055 and CRY 1054) isolates of *C. zuluense* (Table 2).

Data indicated that the depolymerisation of PGA was more rapid and more extensive for the two *Pantoea* species than that of *C. zuluense* isolates. There was a significant difference in the ability of *P. ananatis* pv. *ananatis* (0.36 μmol ml^{-1} min^{-1}; *P = 0.05*) to depolymerise PGA, compared to that of the unknown *Pantoea* species (0.31 μmol ml^{-1} min^{-1}; *P = 0.05*) (Table 2). Isolates of *C. zuluense* had significantly lower levels of PG activity (*P = 0,05*) than the two *Pantoea* species, thus, significantly reducing the ability of *C. zuluense* to depolymerise PGA (Table 2).

DISCUSSION

Results of this study showed that isolates of *C. zuluense* were able to produce PG. The level of PG activity was, however, low in comparison to levels of PG activity reported for other fungal pathogens (Collmer & Keen, 1986; Le Cam *et al.*, 1994). PG activity levels for *C. zuluense* were, however, similar to those reported for the mycorrhizal fungus, *Glomus versiforme* (Karst.) Berchin (Peretto *et al.*, 1995). These authors concluded that biotrophic fungi are characterised by low and regulated production of cell wall-degrading enzymes, particularly cellulases and pectinolytic enzymes. *Coniothyrium zuluense* is a biotrophic fungus and low PG activity levels are, perhaps not surprising.

There was a positive correlation between levels of PG activity and the pathogenicity of *C. zuluense* isolates. Non-pathogenic *C. zuluense* isolates were characterised by low levels of PG activity and *vice versa*. Similar results were reported for the necrotrophic pathogen, *Mycocentrospora acerina* (Hartig) Deighton (Le Cam *et al.*, 1994). These authors reported a positive correlation between levels of PG activity and the aggressiveness of different *M. acerina* isolates to carrot roots. Results of the current study, therefore, indicate that levels of PG production might play a role in the pathogenicity of *C. zuluense* isolates.

Both species of *Pantoea* that are associated with Coniothyrium canker in South Africa were able to produce high levels of polygalacturonase. The production of PG is, however, characteristic of soft rot bacteria belonging to the genus *Pectobacterium*. Species of *Pectobacterium* are particularly well known for their production and secretion of a variety of pectolytic enzymes (He & Collmer, 1990; Collmer & Keen, 1986; Barras *et al.*, 1994; Hugouvieux-Cotte-Pattat *et al.*, 1996). Both *Pantoea* species, used in this study, are not known to be associated with soft rot. However, the relatively high levels of PG activity produced by these two bacteria are in agreement with those reported for PG production by other non-soft rot bacteria (Basham *et al.*, 1985; Rodriquez-Palenzuela *et al.*, 1991; Longland *et al.*, 1992; Magro *et al.*, 1994; Herlache *et al.*, 1997).

High levels of PG activity were observed for both *P. ananatis* pv. *ananatis* and the unknown *Pantoea* species. This suggests that PG activity might play a significant role in disease development. Pathogenicity results in a previous study (Van Zyl *et al.*, chapter 5) showed that both *Pantoea* species were able to cause severe tissue maceration on Granny Smith apples, suggestive of a positive correlation between levels of PG activity and the ability to cause disease. This is in agreement with results obtained by Herlache *et al.* (1997) who showed that levels of PG activity produced by *Agrobacterium vitis*, *Pectobacterium carotovorum* subsp. *carotovorum* (synonym, *Erwinia carotovora*) and *Ralstonia solanacearum* are positively correlated with their respective ability to cause plant tissue maceration. Similar results were also reported for PGs produced by *Pseudomonas syringae* pv. *glycinea* (Magro *et al.*, 1994).

Levels of PG activity were significantly higher for both *Pantoea* species, *P. ananatis* pv. *ananatis* and the unknown *Pantoea* species, compared to PG activity levels of *C. zuluense* isolates. Pathogenicity data from a previous study, using the same isolates, showed a significant increase (43 %) in the pathogenicity of the fungus when it was inoculated in combination with both bacteria species (Van Zyl *et al.*, chapter 5). It was, therefore, concluded that a synergistic interaction occurs between these micro-organisms. Results of this study, thus, support this hypothesis. It is, therefore, possible that both *Pantoea* species, each capable of producing relatively high levels of PG activity, might actively contribute to the acceleration of tissue maceration. *Coniothyrium zuluense* isolates are characterised by low levels of PG activity, suggestive of a limited ability to cause tissue maceration. It is, however, known that bacteria are able to alter the rates of cellulose activity and wood colonisation of decay fungi and simultaneously help provide growth factors and soluble nitrogen (Shortle *et al.*, 1978). The extensive breakdown of cell walls due to the action of PG from both *Pantoea* species could, therefore, be important in improving the ability of *C. zuluense* to colonise wood.

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Table 1. List of *Coniothyrium zuluense* isolates, as well as the two *Pantoea* species from *Eucalyptus* clones, used in this study.

Culture number[a]	**Association with bacteria spp.**[b]	**Pathogenicity**[c]
CRY 1055	*P. a.* pv. *ananatis* and a *Pantoea* sp.	non-pathogenic
CRY 1054	"	non-pathogenic
CRY 1016	"	Intermediately pathogenic
CRY 1023	"	pathogenic
CMW 1778	"	Intermediately pathogenic
CMW 2100	"	pathogenic
P. a. pv. ananatis[d]	_	pathogenic
Unknown *Pantoea* sp.	_	pathogenic

[a] CMW and CRY numbers refer to *C. zuluense* isolates. All isolates were collected from diseased *Eucalyptus* species, clones and hybrids in the Zululand forestry region of KwaZulu-Natal.

[b] Isolates selected for this study were isolated in association with two bacterial species, *Pantoea ananatis* pv. *ananatis* and an unknown *Pantoea* sp.

[c] Pathogenicity of pure bacteria-free *C. zuluense* isolates to a susceptible *E. grandis* clone (ZG 14) was determined in a previous study (Van Zyl *et al.*, 1997). Pathogenicity of both *Pantoea* species to Granny Smith apples was determined in a previous study (Van Zyl *et al.*, chapter 5).

[d] *P. a.* pv. *ananatis* represents *Pantoea ananatis* pv. *ananatis*.

Table 2. Comparison of polygalacturonase (PG) activity between isolates of *Coniothyrium zuluense* and two *Pantoea* species.

	Polygalacturonase activity [a]		
Isolates	**Cup-plate**[b] **(units / ml)**	**Reducing sugars released**[c] **(units / ml / min)**	**Degree of pathogenicity of isolates**[e]
CRY 1055	0.1160 a[d]	0.071 a	non-pathogenic
CRY 1054	0.1240 a	0.052 a	non-pathogenic
CRY 1016	0.1960 ab	0.1106 b	intermediate
CMW 1778	0.1880 ab	0.1082 b	intermediate
CMW 1200	0.2730 c	0.1355 c	pathogenic
CRY 1023	0.2860 c	0.1395 c	pathogenic
P. ananatis. pv. *ananatis*	0.4680 e	0.3596 e	pathogenic
Unknown *Pantoea* sp.	0.3980 d	0.3070 d	pathogenic
PG (EC 3.2.1.15)	0.5236 f	0.4248 f	_

[a] PG production was induced when isolates were grown in a liquid minimal salts medium, supplemented with polygalacturonic acid (PGA) as the sole carbon source.

[b] Polygalacturonase activity (units / ml) was determined according to the cup-plate assay procedure of Dingle *et al.* (1953).

[c] Reducing sugar assays for polygalacturonase activity was adapted from the method described by York *et al.* (1985).

[d] Values are the mean of three repetitions. Within columns, values followed by different letters differ significantly at $P = 0.05$.

[e] Pathogenicity of pure bacteria-free *C. zuluense* isolates was determined in a previous study (Van Zyl *et al.*, 1997). Pathogenicity of *P. ananatis* pv. *ananatis* and the unknown *Pantoea* species to Granny Smith apples was determined in a previous study (Van Zyl *et al.*, chapter 5)

Fig. 1. Polygalacturonase (PG) activity using the 'cup-plate' method described by Dingle *et al.* (1953). A clear zone surrounding the well indicates polygalacturonase activity. Different letters represent PG activity produced by *C. zuluense* isolates (CRY and CMW numbers) or the two *Pantoea* species. (A) CRY 1055, (B) Unknown *Pantoea* sp., (C) CRY 1054, (D) CRY 1016, (E) *P. ananatis* pv. *ananatis*, (F) Commercial polygalacturonase from *Aspergillus niger* (Sigma # P3429, Sigma Chemical Co.), (G) CRY 1023 and (H) CMW 2100.

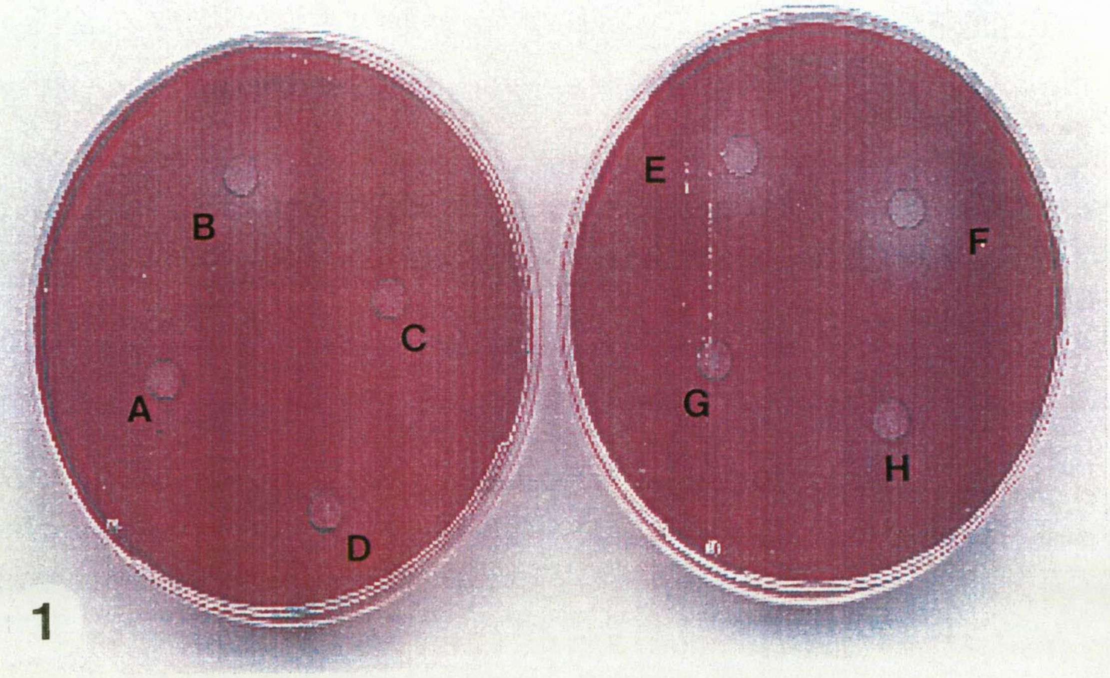
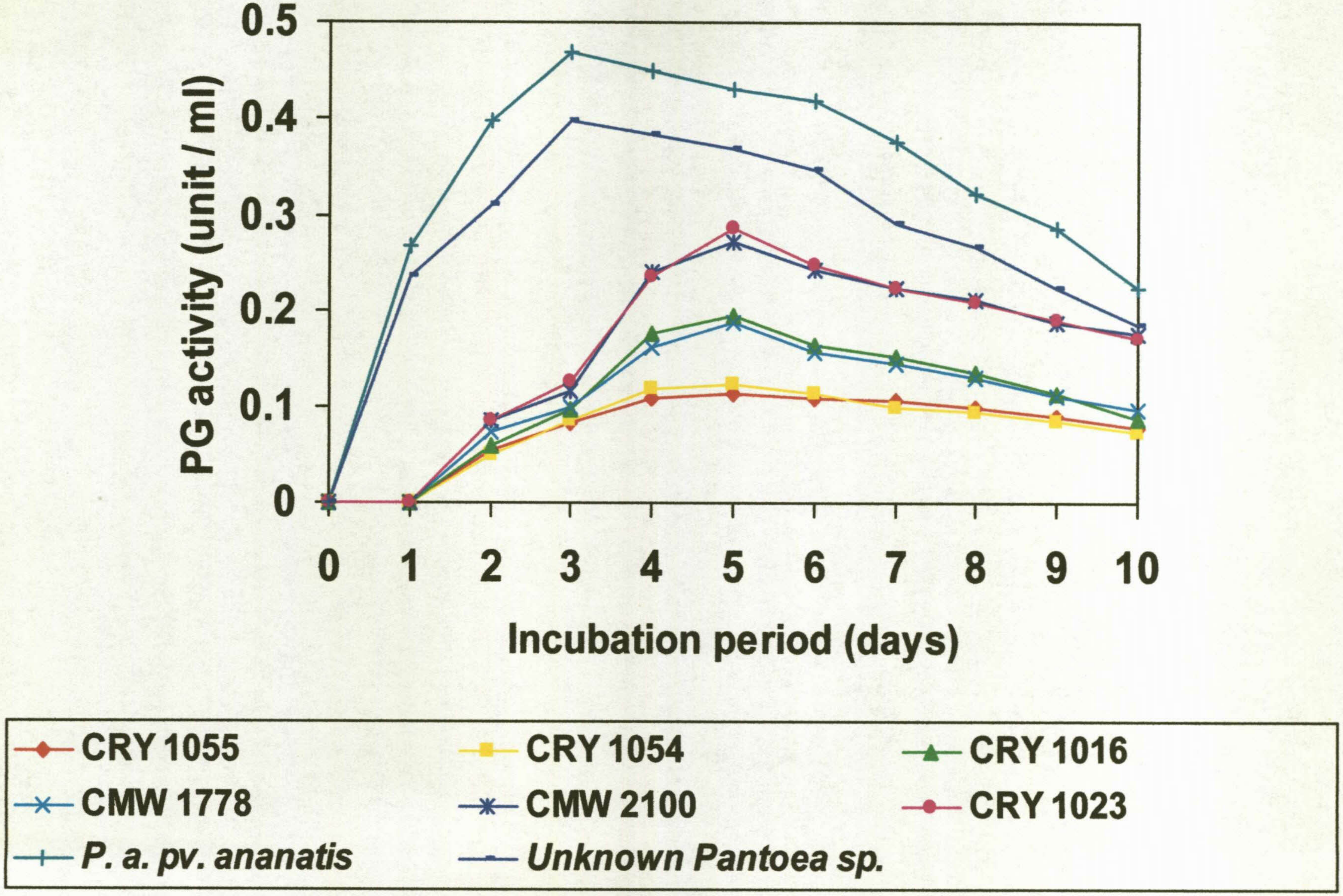

B
C
A
D
E
F
G
H
1

Fig. 2. Polygalacturonase (PG) activity produced by six *Coniothyrium zuluense* and two *Pantoea* spp. (*P. ananatis* pv. *ananatis* and an unknown *Pantoea* sp. closely related to *P. stewartii* subsp. *stewartii*). PG activity was measured over a period of 10 days on minimal salts medium supplemented with polygalacturonic acid (PGA). Polygalacturonase activity (units / ml) was determined according to the cup-plate assay procedure of Dingle *et al.* (1953). *Coniothyrium zuluense* isolates reached maximum PG activity after five days of incubation. The *Pantoea* spp. used in this study reached maximum PG activity three days after inoculation.


PG activity (unit / ml)
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
5
6
7
8
9
10
Incubation period (days)
CRY 1055
CRY 1054
CRY 1016
CMW 1778
CMW 2100
CRY 1023
P. a. pv. ananatis
Unknown Pantoea sp.

CHAPTER 7

Partial cloning of a possible disease resistance gene from two *Eucalyptus grandis* clones

Pathogen-produced cell wall-degrading enzymes play a key role in activating plant defence responses. Most inducible defence responses are the result of transcriptional activation of genes. Various plant resistance (*R*) genes, as well as pathogenesis-related proteins, such as polygalacturonase inhibiting proteins (PGIPs), have been linked with resistance to various fungal and bacterial pathogens. The objective of this study is to determine whether such genes are present in two *Eucalyptus grandis* clones differing in their response to infection by *Eucalyptus* stem canker pathogen, *Coniothyrium zuluense*. Clone TAG 5 is known to be resistant and clone ZG 14 is extremely susceptible to this pathogen. Degenerate primers for nucleotide sequences of the pear PGIP gene were used. Amplification resulted in a range of fragments of different size, with a major fragment of about 600 bp. Each major PCR fragment was sub-cloned and sequenced. The nucleotide sequences for TAG 5 and ZG 14, comprised of 556 bp and 555 bp, respectively. Suggested amino acid sequences indicated the existence of a single open reading frame for clone TAG 5. A shift in reading frame of this gene, however, was observed in the susceptible *Eucalyptus* clone, ZG 14. Homology analysis suggests that the partially sequenced *E. grandis* gene showed very low homology to PGIPs and is most similar to a gene hypothesised to be associated with resistance in *Arabidopsis thaliana*.

Coniothyrium zuluense Wingfield, Crous & Coutinho is an important *Eucalyptus* stem canker pathogen in South Africa. The disease was initially reported to occur on a single *E. grandis* clone, but has since become widespread and currently affects various *Eucalyptus* species, clones and hybrids (Coutinho *et al.*, 1997; Wingfield *et al.*, 1997). The rapid spread of this fungal pathogen throughout South Africa is of considerable concern to local forestry industries and strategies to manage the impact of this disease are currently being investigated.

The most reliable management strategy used to reduce the impact of *Eucalyptus* diseases is by selecting clones and hybrids that show disease resistance. Field trials have clearly indicated variation in the susceptibility of different *Eucalyptus* clones to *C. zuluense* infection (Wingfield *et al.*, 1997). However, previously selected disease resistant clones are beginning to show signs of infection, indicating that virulence of the pathogen is changing (Coutinho *et al.*, 1997; Wingfield *et al.*, 1997).

Virulence of plant pathogens to their hosts, has been shown to be influenced by the production of several cell-wall-degrading enzymes (Barras *et al.*, 1994; Alghisi & Favaron, 1995; Kombrink & Somssich, 1995; Alfano & Collmer, 1996; Hugouvieux-Cotte-Pattat *et al.*, 1996). A study previously conducted in our laboratory showed that the degree of pathogenicity of *C. zuluense* to a susceptible *Eucalyptus* clone, was positively linked to the levels of polygalacturonase (PG) produced by isolates of the fungus (Van Zyl *et al.*, chapter 6). Polygalacturonase belongs to the pectic enzyme group and is considered to be a determining factor in fungal pathogenicity (Alghisi & Favaron, 1995; Annis & Goodwin, 1997).

Pectic enzymes are not only known to be important to pathogens in breaching plant cell wall defence, but they are essential in the process of detection by which plants detect the presence of pathogens (De Lorenzo *et al.*, 1994; Walton, 1994; Barras *et al.*, 1994; Alghisi & Favaron, 1995; Kombrink & Somssich, 1995; Hugouvieux-Cotte-Pattat *et al.*, 1996; Protsenko, 1996). There is also a growing body of evidence that microbial enzymes that hydrolyse the pectic substances in plant cell walls, generate fragments that activate the defence system (De Lorenzo *et al.*, 1994; Walton, 1994; Alghisi & Favaron, 1995; Kombrink & Somssich, 1995; Annis & Goodwin, 1997). These hydrolytic enzymes are the "pre-elicitors" that release "true" elicitors from plant cell walls (De Lorenzo *et al.*, 1994; Walton, 1994; Alghisi & Favaron, 1995; Protsenko, 1995; Annis & Goodwin, 1997). True elicitors are biologically active oligosaccharides produced as a result of endogenous microbial enzyme hydrolysis on larger, inactive polysaccharides (De Lorenzo *et al.*, 1994; Walton, 1994; Alghisi & Favaron, 1995). Induction of disease resistance in plants due to the transcriptional activation of the corresponding genes, is closely associated with the release of active oligogalacturonides (De Lorenzo *et al.*, 1994; Walton, 1994; Alghisi & Favaron, 1995; Kombrink & Somssich, 1995). The physiological mechanism by which these processes are activated is, however, not clear.

Pathogen derived elicitors are recognised by specific plant target receptors, encoded by major disease resistance genes (De Lorenzo *et al.*, 1994; Walton, 1994; Kombrink & Somssich, 1995). If recognised, plants protect themselves by activating various defence mechanisms that include preformed, as well as induced defence responses (Walton, 1994; Alghisi & Favaron, 1995; Kombrink & Somssich, 1995). The synthesis of several disease resistance (*R*) gene products (Martin *et al.*, 1993; Bent *et al.*, 1994; Jones *et al.*, 1994; Mindrinos *et al.*, 1994; Whitham *et al.*, 1994; Song *et al.*, 1995; Salmeron *et al.*, 1996), as well as pathogenesis-related proteins, such as polygalacturonase inhibiting proteins (PGIPs) (Abu-Goukh *et al.*, 1983; Lafitte *et al.*, 1984; Salvi *et al.*, 1990; Bergmann *et al.*, 1994; Gao & Shain, 1995; Caprari *et al.*, 1996), have been positively correlated with increased resistance to pathogen invasion.

Results from various studies have shown that PGIPs have a high degree of sequence similarity to various disease resistance gene products (Jones *et al.*, 1994; Steinmayr *et al.*, 1994). This suggests a possible evolutionary conservation of these proteins. The objective of this preliminary study is to determine whether PGIP or related plant resistance genes are present in two *E. grandis* clones, ZG 14 and TAG 5, differing in their susceptibility to *C. zuluense*.

MATERIALS AND METHODS

Plant material and DNA isolation

Nucleic acid was isolated from *E. grandis* clones, ZG 14 and TAG 5. DNA extracts were prepared using a modified rapid DNA isolation method (Edwards *et al.*, 1991). Fresh leaves were cut into small sections and crushed to a powder in liquid nitrogen. Five ml of a pre-heated CTAB isolation buffer (5 % Cytyltrimethylammonium bromide (CTAB) (Sigma Co., USA); 1.4 M NaCl; 0.2 % (v/v) 2-Mercaptoethanol; 20 mM Tris-HCl, pH 8.0; 1 % (w/v) polyvinylpyrolidone (PVP)) was subsequently added to the frozen tissue. This mixture was incubated at 60 °C for one hour and mixed by inversion every 5 min. One volume of chloroform:isoamyl-alcohol (24:1) was added before mixing vigorously. The extract was centrifuged at 12000 x g for 15 min and the aqueous phase subsequently removed to a new tube containing 900 μl ice-cold 100 % ethanol. After 5 min, the spooled DNA was removed (Micheli *et al.*, 1994) using a sterile pipette tip and washed in 500 μl buffer [76 % v/v ethanol; 10 mM ammonium acetate]. The DNA was air-dried, dissolved in 100 μl sterile water and its concentration determined by using a fluorometer. All DNA samples were diluted to 2.5 ng / μl and stored at -20 °C.

PCR procedure

Oligonucleotides used as primers were synthesised (Applied Biosystems) on the basis of a published sequence of *Pyrus communis* L. cv Bartlett PGIP gene (Stotz *et al.*, 1993) and have the following sequences: Primer 1 (5' –G GAA TTC AAY CCN GAY GAY AAR AAR GT- 3'), primer 2 (5' –GC TCT AGA TCD ATN GAN GTR AAR TCC AT- 3'), and primer 3 (5' –G GAA TTC CAR ATH AAR AAR GCN TTY GG- 3'). Degeneracies are indicated using conventional nucleotide codes and are primarily in the third base position. Polymerase chain reactions from diluted genomic DNA (1:50) were carried out in a reaction volume of 100 μl with 1 unit of Super*Taq* DNA Polymerase (Applied Biotechnologies), 10 X *Taq* PCR buffer (Applied

Biotechnologies), 3 pmol of each primer 1 and 2, 0.25 mM dNTPs and 1.5 mM $MgCl_2$. Amplification conditions were for 40 cycles at 94 °C for 1 min., 48 °C for 2 min., 65 °C for 3 min. and 1 cycle of 72 °C for 5 min. Reactions were carried out in a Hybaid Omnigene Temperature Cycler (Hybaid, Middlesex, U.K.). The identity of the PCR product was verified by amplifying 150 pg of DNA from the first PCR in a second PCR, utilising primers 2 and 3. The reaction conditions were the same as described above, although 25 cycles were used. Amplified products were analysed on 1.5 % agarose gels.

Sequencing

PCR products were extracted from agarose gels and purified using QIAquick Gel Extraction and QIAquick PCR Purification Kits (QIAGEN Inc., USA), respectively. PCR fragments were then cloned using the pCR-Script^{TM} Amp SK (+) Cloning Vector System as described in the pCR-Script^{TM} Amp SK (+) Cloning Vector Systems technical manual (Stratagene, San Diego, CA). Screening for positive colonies containing the insert was done by PCR using plasmid-specific primers M13U (5' – GTA AAA CGA CGG CCA GFT- 3') and M13R (5' –GGA AAC AGC TAT GAC CAT G- 3') (pCR-Script^{TM} Amp SK (+) Cloning Kit, Stratagene, San Diego, CA). Cloned products were precipitated and purified as described above and sequenced using primers M13U and M13R.

PCR products were sequenced using the Big Dye Cycle Sequencing kit with *Amplitaq®* DNA Polymerase, FS (Perkin-Elmer, Warrington, UK) on a ABI PRISM™ 377 DNA Autosequencer (Perkin-Elmer). Phylogenetic relationships from full-length amino acid sequences of different PGIP and resistance (*R*) genes were obtained from the GenBank database (Table 1). Amino acid sequences were aligned using the Clustal (release 6.7) program (Higgins & Sharp, 1988). PAUP version 3.1.1 (Phylogenetic Analysis Using Parsimony) (Swofford, 1993) was used to analyse the sequence data by executing Heuristic searches. Gaps were treated as missing data (Swofford, 1993). A 1000 replicate bootstrap analysis was performed to assess the confidence intervals of the branch points (Felsenstein, 1993).

RESULTS

Polymerase chain reaction analysis carried out on genomic DNA of both *Eucalyptus* clones (ZG 14 and TAG 5), resulted in a range of amplification fragments, with one major fragment of about 600 bp. Amplified products were cloned and sequenced. However, in order to avoid incorporation of errors due to *Taq* DNA Polymerase activity, nucleotide sequencing was also performed directly on the product amplified from genomic DNA. This yielded the same nucleotide sequence as for the sub-cloned products.

Nucleotide sequence data for amplified PCR products of both *E. grandis* clones, TAG 5 and ZG 14, comprised 556 bp and 555 bp, respectively (Fig. 1). Amino acid sequences indicated the existence of a single open reading frame for clone TAG 5 (Fig. 2). However, a shift in reading frame was observed for susceptible *E. grandis* clone, ZG 14 (Fig. 2). A Heuristic search option, with no branch swapping, from the aligned amino acid sequence data (Fig. 3) produced one most parsimonious tree of 1551 steps (Fig. 4). Values for the Consistency Index (CI), Retention Index (RI) and Homoplasy Index (HI) were 0.879, 0.121, and 0.773, respectively.

Analysis showed that PGIPs grouped separately from most resistance genes (Fig. 4). Resistance gene, *FIL 2* of *Anthirrhinum majus* L., however, grouped closest to PGIPs. *FIL 2* formed a sister group to PGIPs of *Phaseolus vulgaris* L. and *Glycine max* (L.) Merr. with a confidence interval at the branching point of 55 %. Resistance gene, *CF-9* isolated from *Lycopersicon pimpinellifolium* (Jusl.) Mill., also grouped closer to PGIPs as compared to most other resistance genes used in this study (61 % confidence interval).

Two clades were obtained from Heuristic searches of amino acid sequence data of the different plant resistance genes (100 % confidence interval) (Fig. 4). The one clade contained representative resistance genes isolated from *Brassica napus* L. (*1A* and *9N*), *Arabidopsis thaliana* (L.) Heynh. (*RPM1* and *RPS2*) and *Lycopersicon esculentum* Mill. (PRF). The second clade was comprised of two unknown resistance genes isolated from *A. thaliana* (*R*), the partially sequenced *E. grandis* (*R*) gene, as well as resistance gene *N,* isolated from *Nicotiana glutinosa* L. (Fig. 4). The function of both genes isolated from *A. thaliana* is currently unknown, however, amino acid sequence analysis revealed similarities to other known plant resistance genes. The partially sequenced *E. grandis* gene grouped closest to the newly isolated *A. thaliana* genes (100 % confidence interval) with tobacco *N* (isolated from *Nicotiana glutinosa*) basal to them.

DISCUSSION

In this preliminary study, a possible pathogen resistance gene from *E. grandis* was partially cloned, sequenced and characterised. Phylogenetic analysis showed a distant relationship between the sequence data of the gene obtained from *E. grandis* and sequence data of the pathogenesis-related protein (PGIP) from *Pyrus communis.* This was unexpected, since primers used in this study were derived from published *P. communis* PGIP sequences. Instead, PAUP analysis indicated a stronger similarity between sequence data from *E. grandis* and that of disease resistance genes in other plants. These genes are known to be very similar to PGIPs in that they all possess leucine-rich repeats (Jones *et al.*, 1994; Steinmayr *et al.*, 1994; Kombrink & Somssich, 1995).

Preliminary results from the current study indicated the existence of a possible plant disease resistance gene in *E. grandis* that is closely related to two unknown *Arabidopsis thaliana* resistance genes obtained from GenBank. No information is available regarding their role in pathogenesis. It was, however, stated that these resistance genes are similar to the tobacco mosaic virus (TMV) resistance gene *N*, isolated from *Nicotiana glutinosa* (Whitham *et al.*, 1994). PAUP analysis of results supported the view that these two genes are closely related.

A deletion of a single nucleotide that causes an interruption in the open-reading frame was detected in the partially sequenced *E. grandis* gene in clone ZG 14, which is also susceptible to Coniothyrium canker. This was, however, not true for the more disease resistant *E. grandis* clone TAG 5, in which the amino acid sequence was complete. Similar findings have been reported for the *Arabidopsis RPM1* gene

(Grant *et al*., 1995). Grant *et al.* (1995) identified a single nucleotide deletion that caused a frame shift and resulted in a loss of function. This finding may provide a partial explanation for disease susceptibility of *E. grandis* clone ZG 14. However, gene isolation procedures such as positional cloning and transposon tagging are needed to determine the role that this gene might play in disease resistance.

The cloning and characterisation of part of a possible *E. grandis* disease resistance gene makes it possible to design primers to sequence the rest of the gene. The complete sequences should provide further information on the relationship to other resistance (*R*) genes and a more complete view of their role in fungal pathogenesis. Results of the present study could, furthermore, provide valuable information for the development of molecular markers to screen clones that are susceptible to Coniothyrium canker for similar open reading frame interruptions. Such a process could significantly speed up breeding for improved disease resistant *Eucalyptus* clones.

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Table 1. Phylogenetic relatedness of the partially characterised *E. grandis* gene was done using full-length amino acid sequences of different PGIPs and plant resistance genes accessed from the GenBank databases.

Gene	Plant species	GenBank accession numbers
PGIP	*Actinidia deliciosa* Siebold & Zucc.	CAA88846
PGIP	*Citrus unshiu* Marcovitch	BAA31841
PGIP	*Fortunella margarita* Swingle	BAA34814
PGIP	*Glycine max*	CAA55081
PGIP	*Phaseolus vulgaris*	P35334
PGIP	*Poncirus trifoliata* (L.) Raf	BAA34813
PGIP[a]	*Pyres communis*	Q05091; AAA33865
FIL2	*Antirrhinum majus*	CAA54303
RPM1	*Arabidopsis thaliana*	CAA61131
RPS2	*Arabidopsis thaliana*	AAA50236
Unknown *R*	*Arabidopsis thaliana*	AC0023544.4; AC0072606
1A	*Brassica napus*	AAC99464
9N	*Brassica napus*	AAC99466
Prf[b]	*Lycopersicon esculentum*	AAC49408
Cf-9	*Lycopersicon pimpinellifolium*	CAA05274
N	*Nicotiana glutinosa*	AAA50763

Fig. 1. Aligned nucleotide sequences of the partial *Eucalyptus grandis* gene from two *E. grandis* clones, TAG 5 and ZG 14. The nucleotide sequences of TAG 5 and ZG 14 comprises 556 bp and 555 bp, respectively. Frame shift is indicated in (⁞).

```
                10         20         30         40         50
TAG5      GGAATTCAAC CCTGAATAAC AAGAAGGTCG AGAGGGAGAC CACTAGTGCA   [50]
ZG14      GGAATTCAAT CCTGGATGAT AAGAAGGTCG AGAGGGAAAC AACTAGTG-A   [49]

                60         70         80         90        100
TAG5      CGGGTTGAGG TACGTCACCA AGGTGCTGGT GTGCTTTTTG GTGGGGACTC   [100]
ZG14      CAGGTTGAGG TACTTCACCA AGGTGCTGGT GTGCTTTTTG GTGGGGACTC   [99]

               110        120        130        140        150
TAG5      TACTGTGGTT GATCAAAACA CTGATCATCA AGGTGCTGGC ATGGTCGTTC   [150]
ZG14      TACTGTGGTT GATCAAAACA CTGATCATCA AGGTGCTGGC ATGGTCGTTC   [149]

               160        170        180        190        200
TAG5      CATGTGAGCA CCTACTTTGA ACGGATCCAG AAGTCTCTGT TCAATCAGTA   [200]
ZG14      CATGTGAGCA CCTACTTTGA ACGGATCCAG AATTCTCTGT TCAATCAGTA   [199]

               210        220        230        240        250
TAG5      CGTGATCGAG ACGTTGTCGG GTCCCCCTGT GATTGAAATT TGGAGGAGAC   [250]
ZG14      CGTGATCGAA ACGTTGTCGG GTCCCCCTGT GATTGAAATT TGGAGGAGAC   [249]

               260        270        280        290        300
TAG5      AGGAAGAGGA GGAGGAGATT GCATGTGATC TCCAGACTTT ACAGAAAGCA   [300]
ZG14      AGGAAGAGGA GGAGGAGATT GCATGTGATC TCCAGACTTT ACAGAAAGCA   [299]

               310        320        330        340        350
TAG5      GAAGTGACTG TGCGGCCAGA TTTAAGGGCG GCGGCTTTTC CAACTGAAAG   [350]
ZG14      GGCGTGACTG TGCCGCCAGA TTTAAGGGCG GCAGCTTTTC CAACTGAAAG   [349]

               360        370        380        390        400
TAG5      TGGGATGGTA GTTGGGAGCG GAGGAGGGCT TCAGAGAAGT CCCCGAGAGA   [400]
ZG14      TGGGATGGTA GTTGGGAGCG GAGGAGGGCT TCAGAGAAGT CCCCGAGAGA   [399]

               410        420        430        440        450
TAG5      AGAACACCAA GCTCTTTCCA GGGCTGTCGG GAAACAGCGA GGGAGGGATC   [450]
ZG14      AGAACACCAA GCTCTCTCCA GGGCTGTCGG GAAACAGCGA GGGAGGGATC   [449]

               460        470        480        490        500
TAG5      ACAATGGAGC ACTTGCAAAA GATGAATCCC AAAAATGTGT TTGCCTGGAA   [500]
ZG14      ACAATGGAGC ACTTGCAAAA GATGAATCCC AAAAATGTGT TTGCCTGGAA   [499]

               510        520        530        540        550
TAG5      TATGAAAAGA TTGATTAATG TCGTGCGGCA TGGACTTCAC TCCATAGATT   [550]
ZG14      TATGAAAAGA TTGATTAATA TCATGCGGCA TGGACTTCAC TCAATCGATT   [549]


TAG5      TAGAGC   [556]
ZG14      TAGAGC   [555]
```

Fig. 2. Amino acid sequences derived from nucleotide sequences of the partial *Eucalyptus grandis* gene. Two *E. grandis* clones were used, disease resistant clone TAG 5, and susceptible clone ZG 14. PC Gene analysis indicated an interruption in the open reading frame of clone ZG 14, compared to the uninterrupted open-reading frame of TAG 5.

TAG5:

RRSRGRRTVHGLRYVTKVLVCFLVGTLLWLIKTLIIKVLAWSFHVSTYFERIQKSLFN
QYVIETLSGPPVIEIWRRQEEEEEIACDLQTLQKAEVTVRPDLRAAAFPTESGMVVG
SGGGLQRSPREKNTKLFPGLSGNSEGGITMEHLQKMNPKNVFAWNMKRLINVVRH
GLHSIDLE

ZG 14:

RRSRGKQTVTG-

Fig. 3. Comparisons of amino acid sequence data for PGIPs and plant resistance genes isolated from several plant species. All sequences, except that of the partially characterised *E. grandis* gene, were obtained from the GenBank database. Amino acid sequences were aligned using the Clustal (release 6.7) program (Higgins & Sharp, 1988). Gaps that were inserted due to alignment are indicated by a dash (-).

```
                                             10         20         30         40         50
PGIP - Fortunella margarita          ---------- ---------- ---------- ---------- ----------
PGIP - Citrus unshiu                 ---------- ---------- ---------- ---------- ----------
PGIP - Poncirus trifoliata           ---------- ---------- ---------- ---------- ----------
PGIP - Pyrus communis                ---------- ---------- ---------- ---------- ----------
PGIP - Pyrus communis                ---------- ---------- ---------- ---------- ----------
PGIP - Actinidia deliciosa           ---------- ---------- ---------- ---------- ----------
FIL2 - Antirrhinum majus             ---------- ---------- ---------- ---------- ----------
PGIP - Phaseolus vulgaris            ---------- ---------- ---------- -----MTQFN ----------
PGIP - Glycine max                   ---------- ---------- ---------- ---------- ----------
CF-9 - Lycopersicon pimpinellifolium LSSNSLTGPI PSNISGLQNL ECLYLSSNHL NGSIPSWIFS ----------
1A - Brassica napus                  LVERCQGLPL AIASLGSMMS TKRLESEWKQ VYNSLNWELN ----------
9N - Brassica napus                  LLERCQGLPL AIASLGSMMS TKRLESEWKQ VYNSLNWELN ----------
RPM1 - Arabidopsis thaliana          LVERCQGLPL AIASLGSMMS TKKFESEWKK VYSTLNWELN ----------
Unknown R - Arabidopsis thaliana     ---------- ---------- --LFDKKVEK ETQSD--VLL ----------
Unknown R - Arabidopsis thaliana     ---------- ---------- --LFDEKVAK AANTK--ALR ----------
Eucalyptus grandis                   ---------- ---------N SILDDKKVER ETNSDR--LR ----------
PRF - Lycopersicon esculentum        AVAIEAESAV CLHYEDNMNN NSREINQVLQ FLTVTFWLIK SEGNLMDLLK
RPS2 - Arabidopsis thaliana          TTMMEQVLEF LSEEEERGII GVYGPGGVGK TTLMQSINNE LITKG-----
N - Nicotiana glutinosa              LIQDMGKYIV NFQKDPGERS RLWLAKEVEE VMSNNTGTMA MEAIWVS---

                                             60         70         80         90        100
PGIP - Fortunella margarita          ---------- -------MSN TSLLSLFFFL CLCISPSLS- ----DLCN--
PGIP - Citrus unshiu                 ---------- -------MSN TSLLSLFFFL CLCISPSLS- ----DLCN--
PGIP - Poncirus trifoliata           ---------- -------MSN TSLLSLFFFL SLFTSLSLS- ----DLCN--
PGIP - Pyrus communis                ---------- -----MELKF STFLSLTLLF SSVLNPALS- ----DLCN--
PGIP - Pyrus communis                ---------- -----MELKF STFLSLTLLF SSVLNPALS- ----DLCN--
PGIP - Actinidia deliciosa           ---------- -------MKS TTAISLLLFL S-LLSPSLS- ----DRCN--
FIL2 - Antirrhinum majus             ---------- -------MKI T-FLLVLSLL ALFSQPFLSQ ---AERCH--
PGIP - Phaseolus vulgaris            ---------- -----IPVTM SSSLSIILVI LVSLRTALS- ----ELCN--
PGIP - Glycine max                   ---------- ---------- ---------- ---------- ----ELCN--
CF-9 - Lycopersicon pimpinellifolium ---------- -----LPSLV ELDLSNNTFS GKIQEFKSKT ---LSAVT--
1A - Brassica napus                  ---------- -----NNLEL KVVRSILLLS FSDLPYPLKR C--FLYCCLF
9N - Brassica napus                  ---------- -----NNLEL KVVRSILSLS FSDLPYPLKR C--FLYCCMF
RPM1 - Arabidopsis thaliana          ---------- -----NNHEL KIVRSIMFLS FNDLPYPLKR C--FLYCSLF
Unknown R - Arabidopsis thaliana     ---------- -----LMSKI LVCFLLSTVL WLIKTLVVK- ---VLASS--
Unknown R - Arabidopsis thaliana     ---------- -----VVTKI FVCLLVGFLL WLVKTLLVK- ---VLASS--
Eucalyptus grandis                   ---------- -----YFTKV LVCFLVGTLL WLIKTLIIK- ---VLAWS--
PRF - Lycopersicon esculentum        HKSTLGNQVL DLIESAHEEL ILLRSILMDL LRKKLYRLDD L--LMHAEVT
RPS2 - Arabidopsis thaliana          ------HQYD VLIWVQMSRE FGECTIQQAV GARLGLSWDE K--ETGENRA
N - Nicotiana glutinosa              -----SYSST LRFSNQAVKN MKRLRVFNMG RSSTHYAIDY LPNNLRCFVC

                                            110        120        130        140        150
PGIP - Fortunella margarita          ---------- ---------- -----PNDKK VLLKFK---- ----------
PGIP - Citrus unshiu                 ---------- ---------- -----PNDKK VLLKFK---- ----------
PGIP - Poncirus trifoliata           ---------- ---------- -----PNDKR VLLNFK---- ----------
PGIP - Pyrus communis                ---------- ---------- -----PDDKK VLLQIK---- ----------
PGIP - Pyrus communis                ---------- ---------- -----PDDKK VLLQIK---- ----------
PGIP - Actinidia deliciosa           ---------- ---------- -----PNDKK VLLRIK---- ----------
FIL2 - Antirrhinum majus             ---------- ---------- -----PQDKR VLLKIK---- ----------
PGIP - Phaseolus vulgaris            ---------- ---------- -----PQDKQ ALLQIK---- ----------
PGIP - Glycine max                   ---------- ---------- -----PQDKQ TLLQIK---- ----------
CF-9 - Lycopersicon pimpinellifolium ---------- ---------- -----LKQNK LKGRIP---- ----------
1A - Brassica napus                  PVNYRMKR-- -KKLVRMWMA QRFVEPIRGV KAEEVA---- ----------
9N - Brassica napus                  PVNYRMKR-- -KRLVRMWMA QRFVEPIRGV KAEEVA---- ----------
RPM1 - Arabidopsis thaliana          PVNYRMKR-- -KRLIRMWMA QRFVEPIRGV KAEEVA---- ----------
Unknown R - Arabidopsis thaliana     ---------- ---------- -----FHVST YFDRIQ---- ----------
Unknown R - Arabidopsis thaliana     ---------- ---------- -----FHMST YFDRIQ---- ----------
Eucalyptus grandis                   ---------- ---------- -----FHVST YFERIQ---- ----------
PRF - Lycopersicon esculentum        AKRLAIFSGS CYEYFMNGSS TEKMRPLLSD FLQEIESVKV EFRNVCLQVL
RPS2 - Arabidopsis thaliana          LKIYRALR-- ---------- QKRFLLLLDD VWEEIDLEKT G---------
N - Nicotiana glutinosa              TNYPWESFP- ---------- --STFELKML VHLQLR---- ----------
```

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                                                   160        170        180        190        200
PGIP - Fortunella margarita          ---------- ---------- ---------- ---------- ----------
PGIP - Citrus unshiu                 ---------- ---------- ---------- ---------- ----------
PGIP - Poncirus trifoliata           ---------- ---------- ---------- ---------- ----------
PGIP - Pyrus communis                ---------- ---------- ---------- ---------- ----------
PGIP - Pyrus communis                ---------- ---------- ---------- ---------- ----------
PGIP - Actinidia deliciosa           ---------- ---------- ---------- ---------- ----------
FIL2 - Antirrhinum majus             ---------- ---------- ---------- ---------- ----------
PGIP - Phaseolus vulgaris            ---------- ---------- ---------- ---------- ----------
PGIP - Glycine max                   ---------- ---------- ---------- ---------- ----------
CF-9 - Lycopersicon pimpinellifolium ---------- ---------- ---------- ---------- ----------
1A - Brassica napus                  ---------- ---------- ---------- ---------- ----------
9N - Brassica napus                  ---------- ---------- ---------- ---------- ----------
RPM1 - Arabidopsis thaliana          ---------- ---------- ---------- ---------- ----------
Unknown R - Arabidopsis thaliana     ---------- ---------- ---------- ---------- ----------
Unknown R - Arabidopsis thaliana     ---------- ---------- ---------- ---------- ----------
Eucalyptus grandis                   ---------- ---------- ---------- ---------- ----------
PRF -  Lycopersicon esculentum       DISPFSLTDG EGLVNFLLKN QAKVPNDDAV SSDGSLEDAS STEKMGLPSD
RPS2 - Arabidopsis thaliana          ---------- ---------- ---------- ---------- ----------
N - Nicotiana glutinosa              ---------- ---------- ---------- ---------- ----------

                                            210        220        230        240        250
PGIP - Fortunella margarita          ---------- ---------- ---------- ---KALNNPY VLA-------
PGIP - Citrus unshiu                 ---------- ---------- ---------- ---KSLNNPY VLA-------
PGIP - Poncirus trifoliata           ---------- ---------- ---------- ---KALNNPY VLA-------
PGIP - Pyrus communis                ---------- ---------- ---------- ---KAFGDPY VLA-------
PGIP - Pyrus communis                ---------- ---------- ---------- ---KAFGDPY VLA-------
PGIP - Actinidia deliciosa           ---------- ---------- ---------- ---QALNNPY LLA-------
FIL2 - Antirrhinum majus             ---------- ---------- ---------- ---KAFNNPY HLA-------
PGIP - Phaseolus vulgaris            ---------- ---------- ---------- ---KDLGNPT TLS-------
PGIP - Glycine max                   ---------- ---------- ---------- ---KELGNPT TLS-------
CF-9 - Lycopersicon pimpinellifolium ---------- ---------- ---------- ---NSLLNQK NLQ-------
1A - Brassica napus                  ---------- ---------- ---------- ---DGYLNEL VYRNM-----
9N - Brassica napus                  ---------- ---------- ---------- ---DGYLNEL VYRNM-----
RPM1 - Arabidopsis thaliana          ---------- ---------- ---------- ---DSYLNEL VYRNM-----
Unknown R - Arabidopsis thaliana     ---------- ---------- ---------- ---EALFHHY LIET------
Unknown R - Arabidopsis thaliana     ---------- ---------- ---------- ---ESLFTQY VIET------
Eucalyptus grandis                   ---------- ---------- ---------- ---NSLFNQY VIET------
PRF - Lycopersicon esculentum        FLREIESVEI KEARKLYDQV LDATHCETSK TDGKSFINIM LTQQDKLPDY
RPS2 - Arabidopsis thaliana          ---------- -VPRPDRENK CKVMFTTRSI ALCNNMGAEY KLRVEFLEKK
N - Nicotiana glutinosa              ---------- ---------- ---------- --HNSLRHLW TETKHLPSLR

                                            260        270        280        290        300
PGIP - Fortunella margarita          ---------- ---------- ---------- ---------- ----------
PGIP - Citrus unshiu                 ---------- ---------- ---------- ---------- ----------
PGIP - Poncirus trifoliata           ---------- ---------- ---------- ---------- ----------
PGIP - Pyrus communis                ---------- ---------- ---------- ---------- ----------
PGIP - Pyrus communis                ---------- ---------- ---------- ---------- ----------
PGIP - Actinidia deliciosa           ---------- ---------- ---------- ---------- ----------
FIL2 - Antirrhinum majus             ---------- ---------- ---------- ---------- ----------
PGIP - Phaseolus vulgaris            ---------- ---------- ---------- ---------- ----------
PGIP - Glycine max                   ---------- ---------- ---------- ---------- ----------
CF-9 - Lycopersicon pimpinellifolium ---------- ---------- ---------- ---------- ----------
1A - Brassica napus                  ---------- ---------- ---------- ---------- ----------
9N - Brassica napus                  ---------- ---------- ---------- ---------- ----------
RPM1 - Arabidopsis thaliana          ---------- ---------- ---------- ---------- ----------
Unknown R - Arabidopsis thaliana     ---------- ---------- ---------- ---------- ----------
Unknown R - Arabidopsis thaliana     ---------- ---------- ---------- ---------- ----------
Eucalyptus grandis                   ---------- ---------- ---------- ---------- ----------
PRF - Lycopersicon esculentum        DAGSVSYLLN QISVVKDKLL HIGSLLVDIV QYRNMHIELT DLAERVQDKN
RPS2 - Arabidopsis thaliana          HAWELFCS-- ---------- ---------- ---------- ----------
N - Nicotiana glutinosa              ---------- ---------- ---------- ---------- ----------
```

```
                                            310        320        330        340        350
PGIP - Fortunella margarita          ---------- --SW------ ---------- ---------- --------NP
PGIP - Citrus unshiu                 ---------- --SW------ ---------- ---------- --------NP
PGIP - Poncirus trifoliata           ---------- --SW------ ---------- ---------- --------NP
PGIP - Pyrus communis                ---------- --SW------ ---------- ---------- --------KS
PGIP - Pyrus communis                ---------- --SW------ ---------- ---------- --------KS
PGIP - Actinidia deliciosa           ---------- --SW------ ---------- ---------- --------NP
FIL2 - Antirrhinum majus             ---------- --SW------ ---------- ---------- --------IP
PGIP - Phaseolus vulgaris            ---------- --SW------ ---------- ---------- --------LP
PGIP - Glycine max                   ---------- --SW------ ---------- ---------- --------HP
CF-9 - Lycopersicon pimpinellifolium ---------- --LL------ ---------- ---------- --------LL
1A - Brassica napus                  --------LQ VILW------ ---------- ---------- --------NP
9N - Brassica napus                  --------LQ VILW------ ---------- ---------- --------NP
RPM1 - Arabidopsis thaliana          --------LQ VILW------ ---------- ---------- --------NP
Unknown R - Arabidopsis thaliana     ---------- --LS------ ---------- ---------- --------GP
Unknown R - Arabidopsis thaliana     ---------- --LS------ ---------- ---------- --------GP
Eucalyptus grandis                   ---------- --LS------ ---------- ---------- --------GP
PRF - Lycopersicon esculentum        YICFFSVKGY IPAWYYTLYL SDVKQLLKFV EAEVKIICLK VPDSSSYSFP
RPS2 - Arabidopsis thaliana          ---------- -KVWRKDLLE SSSIRRLAEI IVSKCG---- ---G----LP
N - Nicotiana glutinosa              --------RI DLSWSKR--- ------LTR- ---------- --------TP

                                            360        370        380        390        400
PGIP - Fortunella margarita          KTD------- ---------- ---------- ---------- ----------
PGIP - Citrus unshiu                 KTD------- ---------- ---------- ---------- ----------
PGIP - Poncirus trifoliata           KTD------- ---------- ---------- ---------- ----------
PGIP - Pyrus communis                DTD------- ---------- ---------- ---------- ----------
PGIP - Pyrus communis                DTD------- ---------- ---------- ---------- ----------
PGIP - Actinidia deliciosa           DND------- ---------- ---------- ---------- ----------
FIL2 - Antirrhinum majus             DTD------- ---------- ---------- ---------- ----------
PGIP - Phaseolus vulgaris            TTD------- ---------- ---------- ---------- ----------
PGIP - Glycine max                   KTD------- ---------- ---------- ---------- ----------
CF-9 - Lycopersicon pimpinellifolium SHN------- ---------- ---------- ---------- ----------
1A - Brassica napus                  FGR------- ---------- ---------- ---------- ----------
9N - Brassica napus                  FGR------- ---------- ---------- ---------- ----------
RPM1 - Arabidopsis thaliana          FGR------- ---------- ---------- ---------- ----------
Unknown R - Arabidopsis thaliana     PML------- ---------- ---------- ---------- ----------
Unknown R - Arabidopsis thaliana     P-L------- ---------- ---------- ---------- ----------
Eucalyptus grandis                   PV-------- ---------- ---------- ---------- ----------
PRF - Lycopersicon esculentum        KTNGLGYLNC FLGKLEELLR SKLDLIIDLK HQIESVKEGL LCLRSFIDHF
RPS2 - Arabidopsis thaliana          LALITLG--- ---------- ---------- ---------- ---------G
N - Nicotiana glutinosa              DFTG------ ---------- ---------- ---------- ---------M

                                            410        420        430        440        450
PGIP - Fortunella margarita          CCD--WYCVT CDLTTN---- ---------- ---------- -------RIN
PGIP - Citrus unshiu                 CCD--WYCVT CDLTTN---- ---------- ---------- -------RIN
PGIP - Poncirus trifoliata           CCD--WYCVT CDLTTN---- ---------- ---------- -------RIN
PGIP - Pyrus communis                CCD--WYCVT CDSTTN---- ---------- ---------- -------RIN
PGIP - Pyrus communis                CCD--WYCVT CDSTTN---- ---------- ---------- -------RIN
PGIP - Actinidia deliciosa           CCD--WYNVD CDLTTN---- ---------- ---------- -------RII
FIL2 - Antirrhinum majus             CCS--WYVVE CDRTTN---- ---------- ---------- -------RIN
PGIP - Phaseolus vulgaris            CCNRTWLGVL CDTDTQT--- ---------- ---------- ------YRVN
PGIP - Glycine max                   CCNNSWVGVS CDTVTPT--- ---------- ---------- ------YRVD
CF-9 - Lycopersicon pimpinellifolium NISGHISSAI CNLKT----- ---------- ---------- --------LI
1A - Brassica napus                  PKVFKMHDVI REIALSI--- ---------- ---------- ------SKAE
9N - Brassica napus                  PKVFKMHDVI REIALSI--- ---------- ---------- ------SKAE
RPM1 - _Arabidopsis thaliana         PKAFKMHDVI WEIALSV--- ---------- ---------- ------SKLE
Unknown R - Arabidopsis thaliana     -EL-SRIEEE EDRTQDE--- ---------- ---------- ------IYKM
Unknown R - _Arabidopsis thaliana    IEIQKNEEEE E-RISVE--- ---------- ---------- ------VKKF
Eucalyptus grandis                   IEIWRRQEEE EE-IACD--- ---------- ---------- ------LQTL
PRF - Lycopersicon esculentum        SESYDEHDEA CGLIARVSVM AYKAEYVIDS CLAYSHPLWY KVLWISEVLE
RPS2 - Arabidopsis thaliana          AMAHRETEEE WIHASEVLTR FPAEMKG--- ---------- ----MNYVFA
N - Nicotiana glutinosa              PNLEYVNLYQ CSNLEEVHHS LG-------- ---------- ----CCSKVI
```

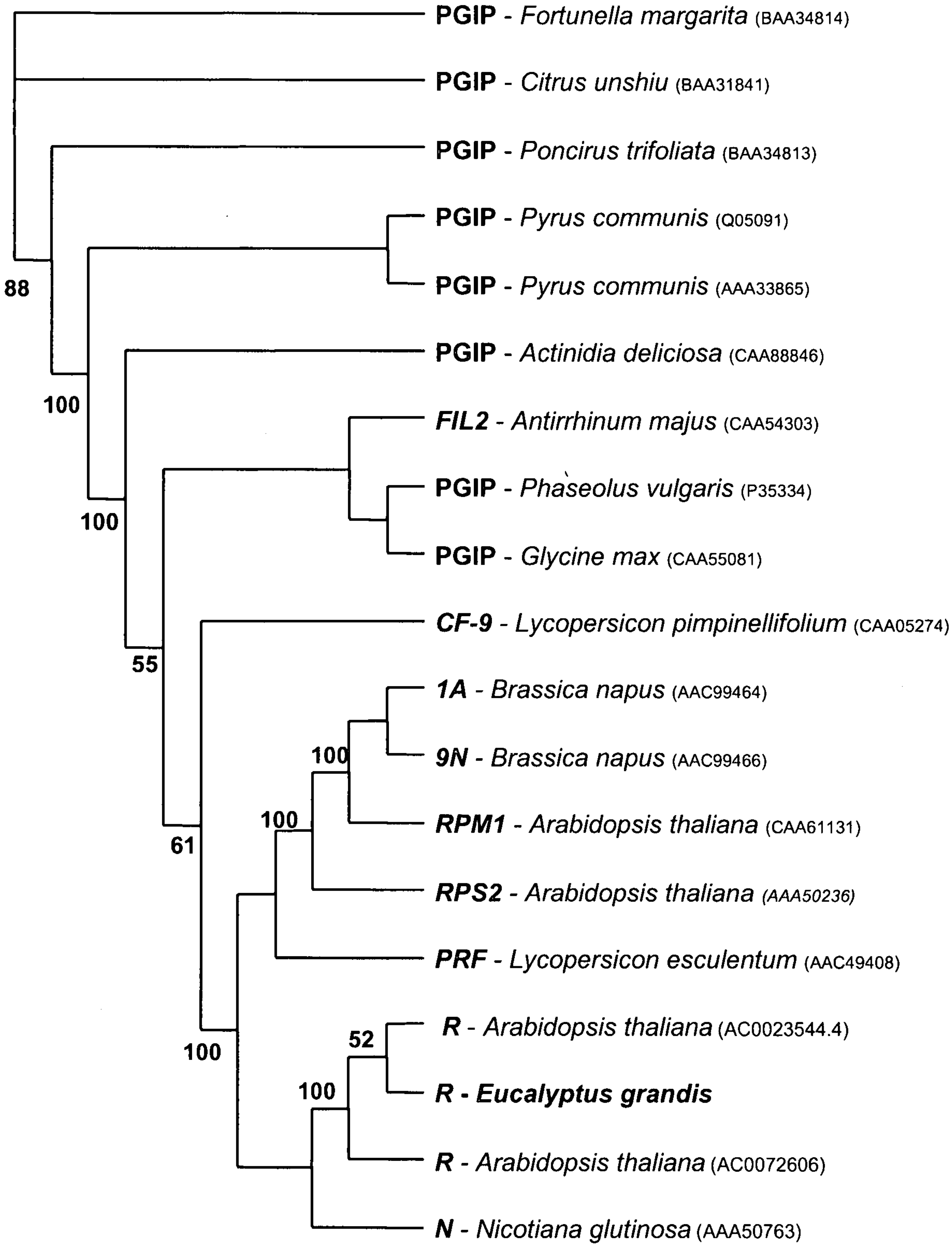
```
                                            460        470        480        490        500
PGIP - Fortunella margarita          SLTIFAGDLP G-----QIPP EVGD------ --LPYLDTLM FHKLPSLTGP
PGIP - Citrus unshiu                 SLTIFAGDLP G-----QIPP EVGD------ --LPYLETLM FHKLPSLTGP
PGIP - Poncirus trifoliata           SLTIFAGDLP G-----QIPP EVGD------ --LPYLETLM FHKLPSLTGP
PGIP - Pyrus communis                SLTIFAGQVS G-----QIPA LVGD------ --LPYLETLE FHKQPNLTGP
PGIP - Pyrus communis                SLTIFAGQVS G-----QIPA LVGD------ --LPYLETLE FHKQPNLTGP
PGIP - Actinidia deliciosa           ALTIFSGNIS G-----QIPA AVGD------ --LPYLQTLI FRKLSNLTGQ
FIL2 - Antirrhinum majus             DFHLFSASVS G-----QIPE TIAE------ --LPFLESLM FRKITNLTGT
PGIP - Phaseolus vulgaris            NLDLSGHNLP KP---YPIPS SLAN------ --LPYLNFLY IGGINNLVGP
PGIP - Glycine max                   NLDLSELNLR KP---YPIPP SVGS------ --LPCLKFLY ITNNPNIVGT
CF-9 - Lycopersicon pimpinellifolium LLDLGSNNLE G-----TIPQ CVVER----- --NEYLSHLD LSK-NRLSGT
1A - Brassica napus                  RFCDVNGDDD D----DDDAE TAEDHGTRHL CIQKEMRSGT LRRTNLHTLL
9N - Brassica napus                  RFCDVNGDDD D----DD-AE TAEDHGTRHL CIQKEMRSGT VRRTNLHTLL
RPM1 - Arabidopsis thaliana          RFCDVYNDDS DG---DDAAE TMENYGSRHL CIQKEMTPDS IRATNLHSLL
Unknown R - Arabidopsis thaliana     QK-GGADLSP E-LCSAAFPQ EKSGSTMN-- --MKFSPIIP KTGSDN----
Unknown R - Arabidopsis thaliana     QNPGGVEIQS G----AQKSP MKTGKSP--- ---FLSHVLS NGGGGG-GE-
Eucalyptus grandis                   QKAG-VTVPP D-LRAAAFPT E-SGMVVGSG GGLQRSPREK NTKLSPGLSG
PRF - Lycopersicon esculentum        NIKLVNKVVG ETCERRNIEV TVHEVAKTTT YVAPSFSAYT QRANEEMEGF
RPS2 - Arabidopsis thaliana          LLKFSYDNLE SDLLRSCFLY CALFPEEHSI EIEQLVEYWV GEGFLTSSHG
N - Nicotiana glutinosa              GLYLNDCKSL KRFPCVNVES LEYLGLRSCD SLEKLPEIYG RMKPEIQIHM

                                            510        520        530        540        550
PGIP - Fortunella margarita          IQP------- AIAKLKNLKT LRISWT---- ---------- ----------
PGIP - Citrus unshiu                 IQP------- AIAKLKNLKT LRISWT---- ---------- ----------
PGIP - Poncirus trifoliata           IQP------- AIAKLKNLKM LRISWT---- ---------- ----------
PGIP - Pyrus communis                IQP------- AIAKLKGLKS LRLSWT---- ---------- ----------
PGIP - Pyrus communis                IQP------- AIAKLKGLKS LRLSWT---- ---------- ----------
PGIP - Actinidia deliciosa           IPS------- AISKLSNLKM VRLSWT---- ---------- ----------
FIL2 - Antirrhinum majus             IPH------- AITRLTRLRS LTISWT---- ---------- ----------
PGIP - Phaseolus vulgaris            IPP------- AIAKLTQLHY LYITHT---- ---------- ----------
PGIP - Glycine max                   IPT------- TITKLTKLRE LNIRYT---- ---------- ----------
CF-9 - Lycopersicon pimpinellifolium INT------- TFSVGNILRV ISLHGN---- ---------- ----------
1A - Brassica napus                  VCT--KHSIE LPPSLKLLRA LDLEGS---- ---------- ----------
9N - Brassica napus                  VCT--KHSIE LPPSLKLLRA LDLEGS---- ---------- ----------
RPM1 - Arabidopsis thaliana          VCSSAKHKME LLPSLNLLRA LDLEDS---- ---------- ----------
Unknown R - Arabidopsis thaliana     ---------- ---GITMDDL HKMNQK---- ---------- ----------
Unknown R - Arabidopsis thaliana     NK-------- ---GITIDSL HKLNPK---- ---------- ----------
Eucalyptus grandis                   NSEG------ ---GITMEHL QKMNPK---- ---------- ----------
PRF - Lycopersicon esculentum        QDTIDELKDK LLGGSPELDV ISIVGMPGLG KTTLAKKIYN DPEVTSRFDV
RPS2 - Arabidopsis thaliana          VNTIYKG-YF LIGDLKAACL LETGDEKTQV KMHN------ ----------
N - Nicotiana glutinosa              QGSGIRELPS SIFQYKTHVT KLLLWN---- ---------- ----------

                                            560        570        580        590        600
PGIP - Fortunella margarita          ---------- ---------- ---------- -----NISGP VPDFISQLTN
PGIP - Citrus unshiu                 ---------- ---------- ---------- -----NISGP VPDFISQLTN
PGIP - Poncirus trifoliata           ---------- ---------- ---------- -----NISGP VPDFISQLTN
PGIP - Pyrus communis                ---------- ---------- ---------- -----NLSGS VPDFLSQLKN
PGIP - Pyrus communis                ---------- ---------- ---------- -----NLSGS VPDFLSQLKN
PGIP - Actinidia deliciosa           ---------- ---------- ---------- -----NLSGP VPSFFSQLKN
FIL2 - Antirrhinum majus             ---------- ---------- ---------- -----NISGP VPAFLSELKN
PGIP - Phaseolus vulgaris            ---------- ---------- ---------- -----NVSGA IPDFLSQIKT
PGIP - Glycine max                   ---------- ---------- ---------- -----NISGQ IPHFLSQIKA
CF-9 - Lycopersicon pimpinellifolium ---------- ---------- ---------- -----KLTGK VPRSMINCKY
1A - Brassica napus                  ---------- ---------- ---------- -----GVT-K LPDFLVTLFN
9N - Brassica napus                  ---------- ---------- ---------- -----GIS-K LPEILVTLFN
RPM1 - Arabidopsis thaliana          ---------- ---------- ---------- -----SIS-K LPDCLVTMFN
Unknown R - Arabidopsis thaliana     ---------- ---------- ---------- -----NVSAW NMKRLMKIVR
Unknown R - Arabidopsis thaliana     ---------- ---------- ---------- -----NVSAW KMKRLMNIIR
Eucalyptus grandis                   ---------- ---------- ---------- -----NVFAW NMKRLINIMR
PRF - Lycopersicon esculentum        HAQCVVTQLY SWRELLLTIL NDVLEPSDRN EKEDGEIADE LRRFLLTKRF
RPS2 - Arabidopsis thaliana          ---------- ---------- ---------- --VVRSFALW MASEQGTYKE
N - Nicotiana glutinosa              ---------- ---------- ---------- ----MKNLVA LPSSICRLKS
```

	610	620	630	640	650
PGIP - *Fortunella margarita*	LTFLE-----	----------	----------	--LSFNNLSG	-----TIPGS
PGIP - *Citrus unshiu*	LTFLE-----	----------	----------	--LSFNNLSG	-----TIPGS
PGIP - *Poncirus trifoliata*	LTFLE-----	----------	----------	--LSFNNLSG	-----TIPSS
PGIP - *Pyrus communis*	LTFLD-----	----------	----------	--LSFNNLTG	-----AIPSS
PGIP - *Pyrus communis*	LTFLD-----	----------	----------	--LSFNNLTG	-----AIPSS
PGIP - *Actinidia deliciosa*	LTFLD-----	----------	----------	--LSFNDLTG	-----SIPSS
FIL2 - *Antirrhinum majus*	LTSLD-----	----------	----------	--LSFNNLSG	-----SIPPS
PGIP - *Phaseolus_vulgaris*	LVTLD-----	----------	----------	--FSYNALSG	-----TLPPS
PGIP - *Glycine max*	LGFLD-----	----------	----------	--LSNNKLSG	-----NLPSW
CF-9 - *Lycopersicon pimpinellifolium*	LTLLD-----	----------	----------	--LGNNMLND	-----TFPNW
1A - *Brassica napus*	LKYLN-----	----------	----------	--LSKTEVK-	-----ELPRD
9N - *Brassica napus*	LKYLN-----	----------	----------	--LSKTEVK-	-----ELPRD
RPM1 - Arabidopsis thaliana	LKYLN-----	----------	----------	--LSKTQVK-	-----ELPKN
Unknown *R* - Arabidopsis thaliana	NVSLS-----	----------	----------	--T-LDEQAL	QNTC-EDEST
Unknown *R* - Arabidopsis thaliana	NGSLT-----	----------	----------	--T-LDEQLQ	DPSL-DDDKG
Eucalyptus grandis	**HGLHS-----**	**----------**	**----------**	**--IDL-----**	**----------**
PRF - *Lycopersicon esculentum*	LILIDDVWDY	KVWDNLCMCF	SDVSNRSRII	LTTRLNDVAE	YVKCESDPHH
RPS2 - *Arabidopsis thaliana*	LILVEPSMG-	----------	--------HT	EAPKAENWRQ	ALVISLLDNR
N - *Nicotiana glutinosa*	LVSLS-----	----------	----------	--VSGCSKLE	-----SLPEE

Fig. 4. Phylogram generated with PAUP analysis based on comparisons of amino acid sequence data from PGIPs and plant resistance genes. All sequence data, except those of the partially characterised *E. grandis* gene, were obtained from the GenBank. GenBank accession numbers are indicated in brackets. The tree length = 1551 steps, CI = 0.879, HI = 0.121 and RI = 0.773. Bootstrap values (1000 replicates) are given at the branching points.


PGIP - *Fortunella margarita* (BAA34814)
PGIP - *Citrus unshiu* (BAA31841)
PGIP - *Poncirus trifoliata* (BAA34813)
PGIP - *Pyrus communis* (Q05091)
PGIP - *Pyrus communis* (AAA33865)
PGIP - *Actinidia deliciosa* (CAA88846)
FIL2 - *Antirrhinum majus* (CAA54303)
PGIP - *Phaseolus vulgaris* (P35334)
PGIP - *Glycine max* (CAA55081)
CF-9 - *Lycopersicon pimpinellifolium* (CAA05274)
1A - *Brassica napus* (AAC99464)
9N - *Brassica napus* (AAC99466)
RPM1 - *Arabidopsis thaliana* (CAA61131)
RPS2 - *Arabidopsis thaliana* *(AAA50236)*
PRF - *Lycopersicon esculentum* (AAC49408)
R - *Arabidopsis thaliana* (AC0023544.4)
R* - *Eucalyptus grandis
R - *Arabidopsis thaliana* (AC0072606)
N - *Nicotiana glutinosa* (AAA50763)
88
100
100
55
61
100
100
100
52
100

SUMMARY

In chapter one of this thesis, the literature pertaining to the genus *Coniothyrium* and its importance in plant pathology, is reviewed. Special attention is given to *Coniothyrium* species associated with *Eucalyptus* but the focus is on *Eucalyptus* stem canker pathogen, *C. zuluense. Coniothyrium zuluense* is an important pathogen in South Africa and has, since its discovery, become widespread throughout plantation areas of KwaZulu-Natal. The current means for reducing the impact of this disease is to plant disease resistant species and clones of *Eucalyptus*. It is evident from this review that very little information is available pertaining to the biology, reproductive system, or the population structure *of C. zuluense.* Such information is essential for managing the disease successfully in the future.

The strategy currently used to reduce the impact of *Coniothyrium* canker in plantations is to deploy *Eucalyptus* species or clones that are resistant to the disease. Considerable success has already been achieved in this regard, but the long-term durability of resistance is of concern. Results of the study represented in chapter two showed that there is considerable variation in colony colour and pathogenicity of a large collection (344) of *C. zuluense* isolates. Conidial morphology and growth requirements are, however, similar for all isolates tested. The considerable variation in pathogenicity indicates that *C. zuluense* has been present in South Africa for an extended period of time, or that virulence is changing rapidly due to strong directional selection pressure.

Results of the taxonomic and pathogenicity studies in chapter two, suggest that the *C. zuluense* population is well established. In chapter three, the population diversity of 108 *C. zuluense* isolates, differing in their pathogenicity to a susceptible *Eucalyptus* clone, was investigated using Amplified Fragment Length Polymorphism (AFLP) technology. Results indicated that the level of genetic diversity is relatively low, but higher than expected for an asexually reproducing pathogen. Genetic similarity values also indicated a significant population differentiation between different plantation regions (sub-

populations), suggesting that gene flow, together with selection, might be responsible for most of the gene diversity. New epidemics would, therefore, not be as a result of the emergence of new aggressive strains, but would rather be due to the introduction of susceptible *Eucalyptus* species, together with environmental conditions favouring disease development.

A *Coniothyrium* species associated with similar symptoms to those associated with *C. zuluense* in South Africa was observed on *E. camaldulensis* in Thailand in 1996. It was previously thought that *C. zuluense* was restricted to South Africa. In chapter four, I show using morphological and molecular comparisons, as well as pathogenicity studies, that *C. zuluense* and the *Coniothyrium* sp. from Thailand are the same organism. This is, thus, the first record of this important *Eucalyptus* stem canker pathogen, *C. zuluense,* outside South Africa.

Bacteria commonly exude from necrotic cankers on severely infected *Eucalyptus* clones in plantations. In chapter five, it was shown that bacteria associated with Coniothyrium canker in the field are species of the genus *Pantoea.* These species were identified based on 16S rDNA sequence data as *P. ananatis* pv. *ananatis* and a species closely related to *P. stewartii* subsp. *stewartii.* It was also shown that a synergistic interaction between *C. zuluense* and both *Pantoea* species exists. Inoculation studies, using both *Pantoea* species together with *C. zuluense* isolates, resulted in a significant increase in pathogenicity as opposed to inoculations where the bacterial and fungal isolates were used alone. Future studies should consider the presence or absence of both bacteria species in disease development in Thailand.

During plant-pathogen interactions, pathogens are known to produce cell wall-degrading enzymes, in particular pectin degrading enzymes. Polygalacturonase (PG) is the first enzyme produced during such interactions and is known to be a determining factor in pathogenicity. Chapter six showed that *C. zuluense* isolates and both *Pantoea* species, *P. ananatis* pv. *ananatis* and an unknown *Pantoea* sp., produce PG. Experimental assays show that levels of PG activity for both *Pantoea* spp. are significantly higher than those obtained for *C. zuluense* isolates. As PG is the first enzyme produced during disease development it is hypothesised that the two *Pantoea* species might play a significant role in the development of Coniothyrium canker. Production of PG could also be used as an assay to evaluate pathogenicity in different isolates of *C. zuluense*.

Pathogen-produced cell wall-degrading enzymes play a key role in activating plant defence responses. Most inducible defence responses are the result of transcriptional activation of genes. Various plant resistance (*R*) genes, as well as pathogenesis-related proteins, such as polygalacturonase inhibiting proteins (PGIPs), have been linked with resistance to various fungal and bacterial pathogens. In chapter seven, a partially sequenced resistance gene from disease resistant *E. grandis* clone, TAG 5, was shown to be similar to a gene associated with a disease resistance gene in *Arabidopsis thaliana*. The most exciting aspect of this study was, however, the discovery of a shift in reading frame of this gene for the susceptible *Eucalyptus* clone, ZG 14. The complete sequence of this gene should provide a more complete view of its importance in disease resistance. Screening for similar interruptions in the open reading frame of various commercially available *Eucalyptus* clones could significantly speed up breeding programmes aimed at producing improved disease resistant clones.

OPSOMMING

Die literatuur met betrekking tot die belang van die genus *Coniothyrium* in plant patologie is in hoofstuk 1 in heroonskou geneem. Spesifieke aandag is gegee aan die *Eucalyptus* stamkankerpatogeen, *Coniothyrium zuluense*. Uit die oorsig is dit duidelik dat daar 'n tekort aan informasie rakende die basiese biologie van die fungus soos voortplantings meganisme en populasie diversiteit bestaan. Hierdie inligting is van kardinale belang met die oog op die ontwikkeling van siekte beheerprogramme.

Die strategie wat tans gebruik word om verspreiding van die siekte te beheer, is deur die seleksie van siekte weerstandbiedende *Eucalyptus* klone. Bogenoemde praktyk is suksesvol, alhoewel daar kommer bestaan oor die langdurigheid van weerstand in geselekteerde klone. Resultate van hoofstuk twee het getoon dat daar 'n aansienlike variasie in die populasie van *C. zuluense* is met betrekking tot koloniekleur en patogenisiteit. Spoor morfologie en groei temperatuur voorkeure was egter dieselfde vir al die isolate (344 isolate) bestudeer. Die vlak van variasie in patogenisiteit kan moontlik daarop dui dat *C. zuluense* al 'n geruime tyd in Suid Afrika voorkom, of dat 'n verandering in virulensie bespoedig word as gevolg van seleksie druk.

Resultate van hoofstuk twee dui daarop dat die populasie van *C. zuluense* in Suid Afrika relatief divers is. In hoofstuk drie is daar, dus, gefokus op die bepaling van die populasie diversiteit van 108 *C. zuluensie* isolate met behulp van "Amplified Fragment Length Polymorphism". Geselekteerde isolate het verskil ten opsigte van hul patogenisiteit tot 'n vatbare *Eucalyptus* kloon. Resultate het daarop gedui dat die vlak van genetiese diversiteit relatief laag was, maar hoër as wat verwag is van 'n patogeen wat nie-seksueel voortplant. Genetiese similariteitswaardes het ook aangedui dat betekenisvolle differensiasie tussen plantasie gebiede (sub-populasies) van die patogeen voorkom. Dit is 'n aanduiding dat genetiese vloei, tesame met seleksie, moontlik vir die geen diversiteit verantwoordelik is. Uitbreek van nuwe epidemies sal dus nie veroorsaak word deur nuwe aggressiewe rasse van die patogeen nie, maar eerder deur die aanplanting van vatbare *Eucalyptus* klone, tesame met gunstige omgewings faktore.

Coniothyrium kanker was voorheen net bekend in Suid Afrika. Gedurende 1996, is siekte simtome, soortgelyk aan die van *C. zuluense* in Suid Afrika, opgemerk op 'n *E. camaldulensis* kloon in Thailand. In hoofstuk vier is deur middel van morfologiese-, molekulêre- en patogenisiteits-studies bewys dat die *Coniothyrium* spesie van Thailand en *C. zuluense*, dieselfde organisme is. Dit is dus die eerste aanmelding van hierdie gevreesde siekte buite Suid Afrika.

Bakterieë word alledaags opgemerk waar dit uit stam kankers van uiters vatbare *Eucalyptus* klone vloei. Resultate van hoofstuk vyf het getoon dat twee bakterie spesies van die genus *Pantoea* met Coniothyrium kanker geassosieer is. Identifikasie is gedoen deur die basispaar opeenvolging van die 16S rDNA geen van beide bakterieë te vergelyk met die basispaar opeenvolging van 16S rDNA gene van verwante spesies. Een bakterium is geïdentifiseer as *P. ananatis* pv. *ananatis* en die ander spesie as naby verwant aan *P. stewartii* subsp. *stewartii.* Uit die resultate was dit ook duidelik dat daar 'n sinergistiese verwantskap tussen *C. zuluense* en beide *Pantoea* spesies bestaan. Patogenisiteits-studies het getoon dat 'n betekenisvol grooter letsels geproduseer is wanneer *C. zuluense* isolate in kombinasie met beide *Pantoea* spesies in bome geinokuleer is.

Plant patogene is bekend vir die produksie van verskeie ensieme om plantselwande af te breek, spesifiek pektien-afbreekende-ensieme. Die pektiese ensiem, polygalacturonase (PG), is bekend as die eerste ensiem wat gedurende plant-patogeen interaksies geproduseer word, en word beskou as 'n bepalende faktor in siekte vorming. Resultate uit hoofstuk ses toon dat *C. zuluense* en beide *Pantoea* spesies, *P. ananatis* pv. *ananatis* en die onbekende *Pantoea* spesie, PG kan produseer. Polygalacturonase aktiwiteit vir beide *Pantoea* spesies was betekenisvol hoër in vergelyking met die van *C. zuluense.* Dit bleik uit die resultate dat PG produksie van beide *Pantoea* spesies moontlik 'n noemenswaardige rol kan speel in letsel vorming van Coniothyrium canker.

Plantselwand-afbrekende-ensieme speel ook 'n noemenswaardige rol in die aktivering van die plant se weerstandsmeganismes. Die aktivering van verskeie PG inhiberings-proteine (PGIPs), is voorheen direk gekoppel aan verhoogde weerstand teen fungus- and bakteriesiektes. In hoofstuk sewe is gevind dat 'n weerstandsgeen wat naby verwant is aan 'n weerstandsgeen in *Arabidopsis thaliana*, in 'n siekte-tolerante *E. grandis* kloon, TAG 5, teenwoordig is. Die *E. grandis* kloon, ZG 14, wat bekend is vir vatbaarheid vir verskeie bosbou verwante siektes, het egter 'n leesraam verskuiwing in die bogenoemde geen gehad. Dit kan 'n moontlike verduideliking verskaf vir die vatbaarheid van die spesifieke kloon vir siektes. Laasgenoemde resultate kan gebruik word vir die vinnige identifisering van soortgelyke leesraam verskuiwings in klonale materiaal, wat tot die uitskakeling van sulke vatbare klone in die bosbou bedryf kan lei.