Biological control of an Australian noxious weed "Angled Onion" (*Allium triquetrum* L.) using Molecular and Traditional Approaches

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Dedicated to my mum, ***Afsaneh Khodayari,***

and dad, ***Mostafa Tehranchian,***

whose support helped me get this far and

who made me try to be my best, always.

Declaration

I declare this thesis is my own work and was performed while I was enrolled fulltime for the degree of Doctor of Philosophy at the School of Applied Sciences, RMIT University. It has not been submitted for any degree or any other award. To the best of my knowledge, the content of this thesis is the result of my own work and all work performed by other, published or unpublished, has been fully acknowledged.

Parsa Tehranchian
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Summary

Angled Onion (*Allium triquetrum* L.) is one of Australia's successful weeds and is difficult to control, especially in natural habitats. It is a herbaceous bulb-forming perennial member of the *Amaryllidaceae* (previously known as *Liliaceae*) and a noxious weed in Vic, SA, NSW and WA. It has been a sleeper weed and is expanding rapidly now that rainfall has returned close to normal. It reduces understory biodiversity significantly and so affects regeneration of native flora. It forms monocultures and its allelopathic traits endanger species such as orchids, native lilies and grasses. Therefore Angled Onion needs to be considered as a high priority invasive environmental weed in Australia. Also, in agriculture, because of the strong onion odour, there are problems of milk and meat taint when it is consumed by farm animals. There are no selective herbicides and grubbing is too expensive and impossible for such vast infestations. Biological control is the only option for control. Evaluation of three pathogens (*Stromatinia cepivora* Berk., *Pectobacterium carotovorum* subsp. *carotovorum* Waldee and a novel bacterium close to *Ochrobactrum* Holmes et al. species) conducted in this study suggested that the fungus and the two bacteria are potential biological control organisms. Each was separately pathogenic and the fungus and one bacterium were highly virulent; infected plants died. This is the first attempt at biological control of *A. triquetrum* a model plant for a range of bulbous weeds in Australia.

Chapter 2 investigated the genetic diversity of *A. triquetrum* provenances from across Australia. The first aim of chapter 2 was to determine if there is genetic variation between and within provenances. The second aim was to investigate if genetic differences are due to polyploidy. *A. triquetrum* bulbs were collected from infestations throughout Australia. There was no significant variation in plant morphology except from Ararat and woodland near Westernport Bay in Victoria, which produced larger bulbs than in other sites. DNA

extracted from bulbs from each provenance was assessed for diversity by ITS-PCR, RFLPs and RAPDs, which found relatively small genetic diversity between and within provenances from across Australia. ITS sequencing and RFLP analysis suggested relatively small genetic diversity of *A. triquetrum* in Australia but with some differences in samples from Ferny Creek, Victoria, which makes it a suitable target for biological control. RAPD analysis showed three geographical groupings, one for each of South Australian and Western Australian provenances, but with Victorian provenances split in two; however, genetic variation was also observed within provenances in each state. Only diploid, not triploid plants were found in 12 of these provenances form Australia based on karyotypic studies. The relatively small genetic diversity and homogeneity within most provenances suggested that most spread was clonal, by bulbs, but within-provenance variation suggested also reproduction by seed, leading to genetic variation in seedlings.

The main aim of chapter 3 was to explore the potential of the fungus *S. cepivora* as a biological control agent to infect *A. triquetrum in vitro* (test tube trials) and *in vivo* (pot trials). *A. triquetrum* bulbs collected from infestations and cultivated *Allium* species were micropropagated to remove problems with endogenous bacteria and test-tube grown plants were inoculated with sclerotia. There was a significant difference in pathogenicity between the two Australian *S. cepivora* isolates tested. The DPI isolate was pathogenic to all provenances and cultivated *Allium* species, whereas VPRI 12439a was not pathogenic on one provenance (from Wonthaggi) because its sclerotia did not germinate *in vitro*. In the glasshouse, plants inoculated with sclerotia in sterile sand produced no white rot disease on *A. triquetrum* and cultivated *Allium* species 3 months post-inoculation, but retrieved sclerotia grew on V8 agar. By contrast, plants inoculated with mycelium showed white rot disease 3 months post-inoculation, except for Hardy's Picnic Ground, Dandenongs plants, which were not infected. This fungus was also highly virulent on

cultivated *Allium* species but was not pathogenic on a range of Australian native monocotyledons liable to be present in infested environments.

Chapter 3 also investigated genetic variation in the fungus *S. cepivora*, the causal agent of white rot disease of onion. Two local isolates, VPRI 12439a and a fresh isolate from DPI field trials were used, from the Herbarium VPRI National Collection of Fungi and the Department of Primary Industries, Victoria, respectively. Isolates were compared genetically using PCR amplification of the ITS region, RAPDs and mycelial compatibility. ITS sequences of *S. cepivora* isolates downloaded from GenBank via NCBI were used to determine the genetic diversity between Australian and international isolates. ITS sequence alignment exhibited relatively small nucleotide variations but the Australian isolates clustered together separately from the rest. RAPD analysis showed genetic variation between the local isolates but only using OPA-11 primer. Mycelia of the isolates were also incompatible, supporting the case for genetic variation between the isolates. This genetic variation was reflected in the variation in pathogenicity and virulence on *Allium* species. As neither isolate affected native Australian monocots, either or both could be used separately or together as a biocontrol agent.

In chapter 4, a soft-rotting bacterium was isolated from rotting *A. triquetrum* bulbs collected from Horsnell Gully, South Australia, after 2 months of storage at 4ºC. The bacterium was identified as *Pectobacterium carotovorum* subsp. *carotovorum* by 16S r-DNA sequencing and physiological tests. In test-tube trials, the bacterium produced severe soft rot symptoms 12 h post-inoculation and rotten young seedlings collapsed after 24 h incubation at 25ºC. Identical symptoms were observed at 15ºC and 4ºC, but with a longer aetiology. Histology of infected plants suggested that the bacterium invaded both the cortical and vascular tissue. In the glasshouse, all *A. triquetrum* provenances inoculated

with 10^8 CFU/plant showed soft rot symptoms 20 days post-inoculation but cultivated *Allium* species and Australian native grasses were not affected 3 months post-inoculation. The soft-rotting bacterium was identified from infected *A. triquetrum* bulbs and leaves in the glasshouse using PCR amplification with specific primers (Kanf *et al.* 2003) as *P. carotovorum* subsp. *carotovorum*.

In chapter 5, an endogenous bacterium was isolated from noted on surface-sterilized *A. triquetrum* bulbs and on water agar collected from the Dandenongs, Victoria. The bacterium was pathogenic on *A. triquetrum* and cultivated *Allium* species in test-tube trials, causing yellowing and necrosis of infected foliage. Histology of the infected tissue demonstrated some destroyed cortical parenchyma cells, but there were no bacterial masses in cells. Both the bacterium and *S. cepivora* were separately pathogenic and highly virulent in test-tube trials, in that infected plants died 2 months post-inoculation. The bacterium; however, inhibited the fungus from growing when tested together, though host plants still died from the bacterium alone. 16S r-DNA sequence analysis showed 97% nucleotide similarity of the bacterium to *Ochrobactrum* species but also indicated nucleotide variation of this bacterium from five known *Ochrobactrum* species. RFLP analysis of the 16 r-DNA PCR products and physiological tests suggested that the novel bacterium is closest to *Ochrobactrum rhizosphaerae* (Kämpfer *et al.* 2008), but physiological parameters did not match exactly. It is proposed that this is a new species of *Ochrobactrum* but it is not a potential biocontrol agent for *A. triquetrum* because of its lack of pathogenicity in pot trials. The isolate's repression of sclerotial germination and mycelial growth may, however, suggest that it is a potential biological control agent for white rot disease in cultivated *Allium* species.

Variation in host resistance to biocontrol agents is likely to reduce 100% success by a single agent. The fungus and *P. carotovorum* subsp. *carotovorum* both were separately pathogenic to *A. triquetrum* in the glasshouse. A combination of the both pathogens could help to control *A. triquetrum* infestations and overcome variations in susceptibility to the pathogens. *P. carotovorum* subsp. *carotovorum* is an effective biocontrol agent in wet areas where *S. cepivora* cannot germinate, whereas *S. cepivora* is effective in drier areas.

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Conference proceedings (E1) (peer-reviewed as for journal articles)

- **Tehranchian, P.,** Lawrie A.C. and Adair, R. (2010). In vitro assessment of *Stromatinia cepivora* as a potential biological control agent for angled onion (*Allium triquetrum*) in Victoria, Australia. *Papers and Proceedings of the 17th Australasian Weeds Conference,* ed. S.M. Zydenbos. (New Zealand Plant Protection Society) ISBN 978-0-86476-239-9

Conference papers presented to professional societies (N1)

- **Tehranchian. P.,** Lawrie, A.C and Adair, R. (2011). Evaluation of *Ochrobactrum* sp. as a potential bioherbicide for angled onion (*Allium triquetrum* L.) at laboratory condition. New Frontiers in Plant Pathology for Asia and Oceania, ACPP APPS conference, Darwin 2011.

- **Tehranchian. P.,** Lawrie, A.C and Adair, R. (2010). *In vitro* estimation *of Ochrobactrum anthropi* capability as a potential biological control agent for angled onion (*Allium triquetrum*) in Australia. (Poster presentation at Weeds Society of Victoria, (also RMIT University Higher Degree student's research conference 2010).

Local presentations

- **Tehranchian. P.,** Lawrie, A.C and Adair, R. (2009). *Sclerotium cepivorum* a potential biological control agent for angled onion (*Allium triquetrum*) in Australia. (Poster presentation at RMIT University Higher Degree student's research conference 2009). mams.rmit.edu.au/hphqi6q9na98z.pdf

Chapter 1

Literature Review

1.1 Weeds definition

Weeds are wild plants with a high capability for growth (Muzik 1970). Ecologically, weeds are adapted to disturbed habitats and can occur wild in the environment. Before man, disrupted habitats may have been rather rare. Many weeds do not grow in undisturbed habitats, only in association with humans (Zohary 1962). Harlan and de Wet (1965) point out various definitions of weeds by plant scientists:

Blatchley 1912 “a plant out of place or growing where it is not wanted”

Georgia 1916 “A plant that is growing where it is desired that something else shall grow”

Robbins et al. 1942 “these obnoxious plants are known as weeds”

Muenscher 1946 “those plants with harmful or objective habits characteristics which grow where they are not wanted, usually in places where it is desired that something else should grow”

1.1.1 Environmental weeds

Environmental weeds have become one of the major threats to Australian biodiversity and almost all biological communities in Australia (Adair and Groves 1998). Environmental weeds are exotic plants that have become established in natural ecosystems normally dominated by native plants (Carr 1988; Groves 1991). According to Groves (1986), the rate of invasive plant invasion has increased since European settlement in Australia (Adair and Groves 1998; Csurhes and Edwards 1998).

1.1.2 Weeds as an environmental problem in Australia

Weeds have become a serious problem in the Australian natural environment. They cause significant habitat degradation and reduce native biodiversity in addition to losses (in forests and farm productivity), emphasising the importance of and need for weed management in Australia. The spread of weeds in Australia is faster than can be controlled. According to the Australian Government (2008), alien invasive plants have significant impacts on 15% of the total flora and it is estimated that invaders are increasing by 10 species per year.

Non-native plants that have become established and are self-propagating without human assistance outside their natural range are called naturalised (Csurhes 1995; Spencer 2006). More than 2200 non-native plant species are naturalised in Australia (Csurhes and Edwards 1998), of which 1067 were listed as environmental weeds in 1994 (Swarbrick and Skarratt 1994). This number was increased significantly to 1388 in 2003 (Groves 2003). Weeds have been introduced to Australia through, horticulture and agriculture and as contaminates on transported soil, livestock and machinery. More than 65% of naturalised plants were introduced as ornamentals and only 7% in association with agriculture (Spencer 2006). Although there are significant numbers of naturalised plants in Australia, not all are considered environmental weeds, as they are restricted in natural ecosystems (Csurhes and Edwards 1998).

Degradation of biological diversity by environmental weeds can be defined in terms of genetic diversity, species diversity and ecosystem diversity. There are, however, considerable differences in the extent and impact of exotic plant invasions and human developments are a key factor that directly affect dispersion of invasions (Adair and

Groves 1998). Control of environmental weeds is usually time-consuming and expensive and eradication is only possible by catching the weeds before they become widespread. Availability of sufficient funding and labour is one of the most important complications of eradication programs (Csurhes and Edwards 1998). Eradication of some invasions in North Queensland cost more than AU$800,000 over five years (1994-1999) (Csurhes and Edwards 1998). However, there is not sufficient funding for biological control of environmental weed in comparison to farming, horticulture and forestry, and chemical control in native vegetations is costly and labour-intensive and sometimes impossible due to lack of selective herbicides.

1.2 Biology

1.2.1 Origin & distribution of Allium triquetrum L.

It is believed that *A. triquetrum* originated in western and central Mediterranian regions (Blood 2001; Parsons and Cuthbertson 1992; 2001) (Fig. 1.1). According to the United States Department of Agriculture (2008), it is a native of African and European countries that are located in the Mediterranean region. Hanelt (1986) mentioned that attempts were made to domesticate *A. triquetrum* in Algeria as a cultivated crop and ornamental plant during the first decades of the 20th century but were not successful.

Figure 1.1 Distribution of *Allium triquetrum* in native (Mediterranean) regions (Flora Italiana 2009).

A. triquetrum is not a native plant in Australia and is proclaimed as a noxious weed in parts of Australia (Walsh and Entwisle 1994; Blood 2001). It is not known when this weed migrated to Australia; however, it was naturalized in temperate regions of South Australia by 1909 (Parsons and Cuthbertson 1992). It occurs in South Australia (Government of South Australia 2005), Western Australia (Walsh *et al.* 1994), Tasmania, and New South Wales (Weeds Australia 2008) (Fig. 1.2).

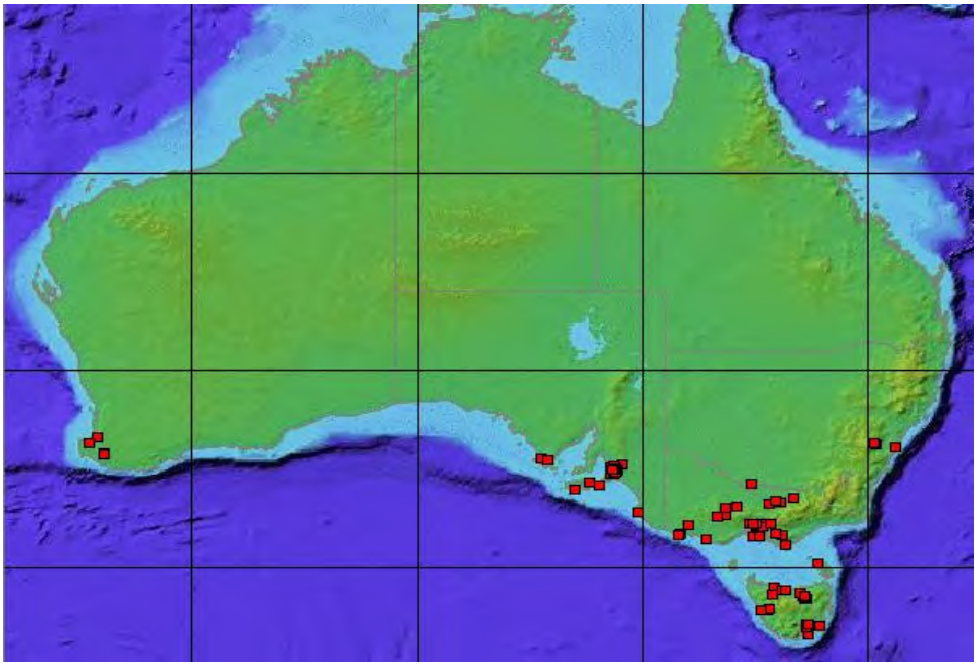

Figure 1.2 Distribution of *Allium triquetrum* in Australia (Australia's Virtual Herbarium 2008).

A. triquetrum grows in well-watered areas such as gardens, bushland, wetlands, creek banks, shaded areas, riversides, and roadsides (DPI 2008a; Parsons and Cuthbertson 1992; Weeds Australia 2008). Parsons and Cuthbertson (1992) reported that it occurs widely as colonies on the north and central coast of New South Wales and grows also on the western slopes of the Great Dividing Range and South Western Plains of NSW. In Victoria the distribution of the plant is concentrated around Melbourne and some rural cities and towns (Fig 1.3), and this state has a high potential level for infestation by *A. triquetrum*, as calculated according to climatic suitability (Fig. 1.4). In South Australia the distribution of *A. triquetrum* is clustered around Adelaide and a few other discrete well-watered areas (Fig. 1.5) and it is also distributed in the South-West corner of Western Australia (Fig. 1.6).

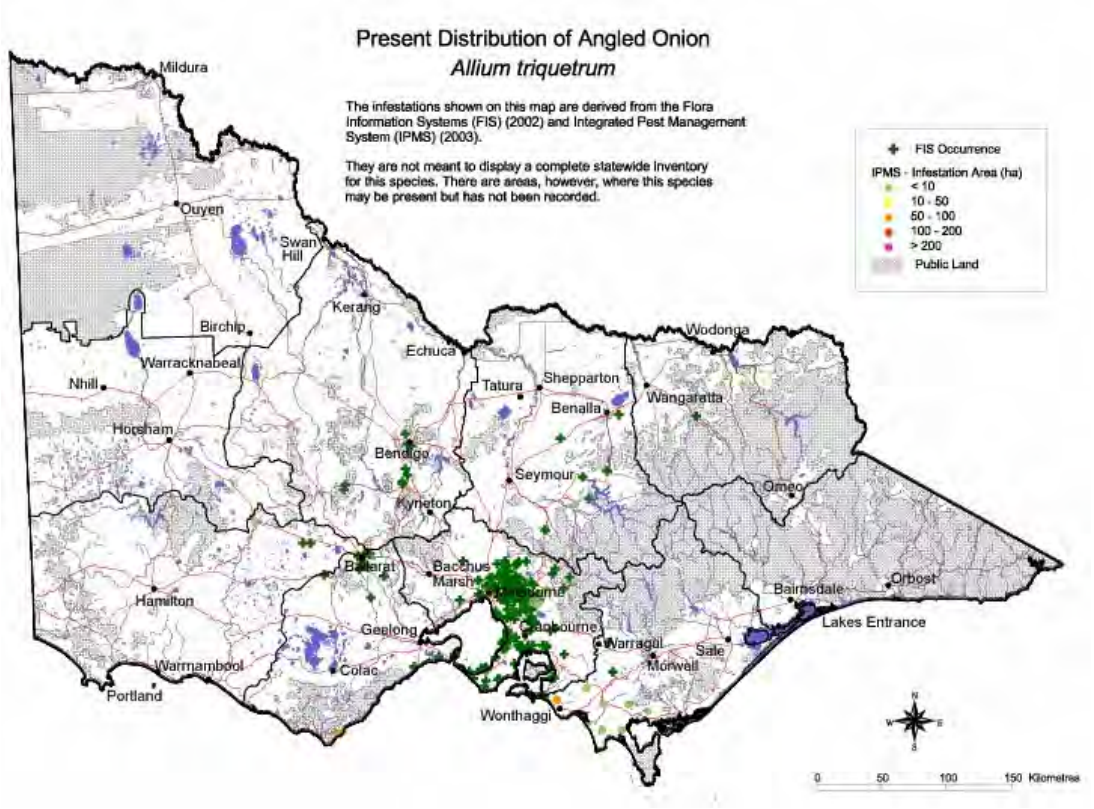

Figure 1.3 Map of present distribution of *Allium triquetrum* in the state of Victoria (DPI 2008b).

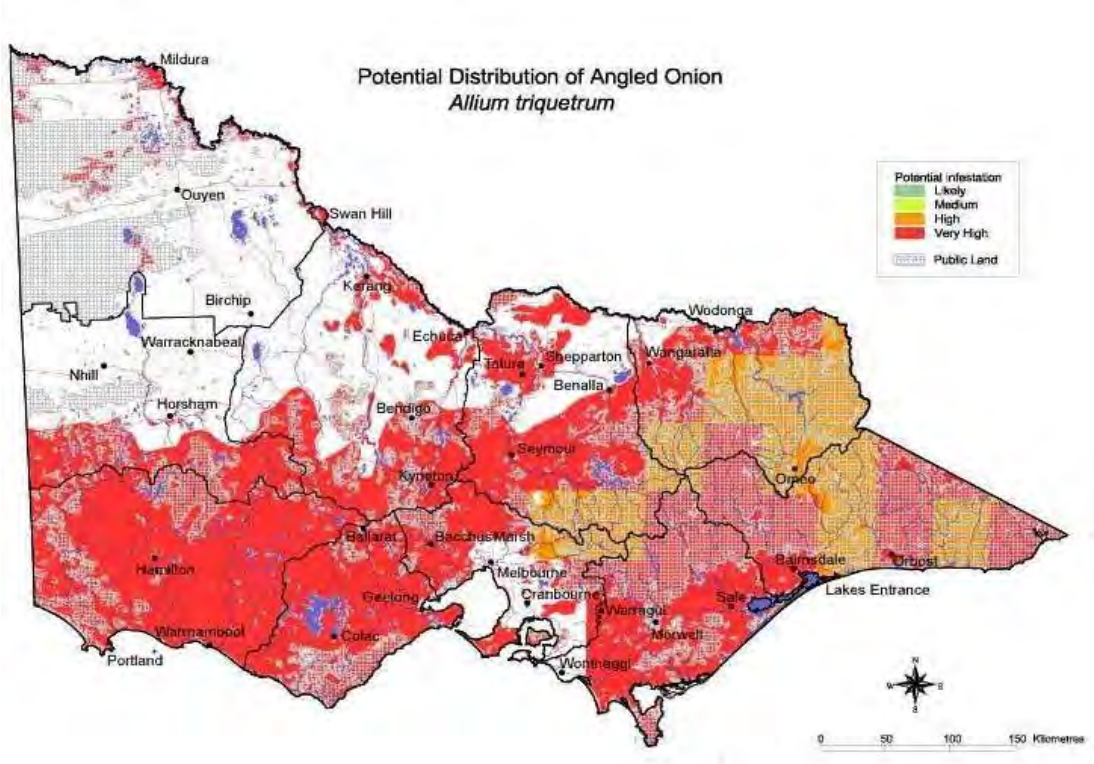

Figure 1.4 Map of potential distribution of *Allium triquetrum* in the state of Victoria, as calculated according to climatic suitability (DPI 2008c).

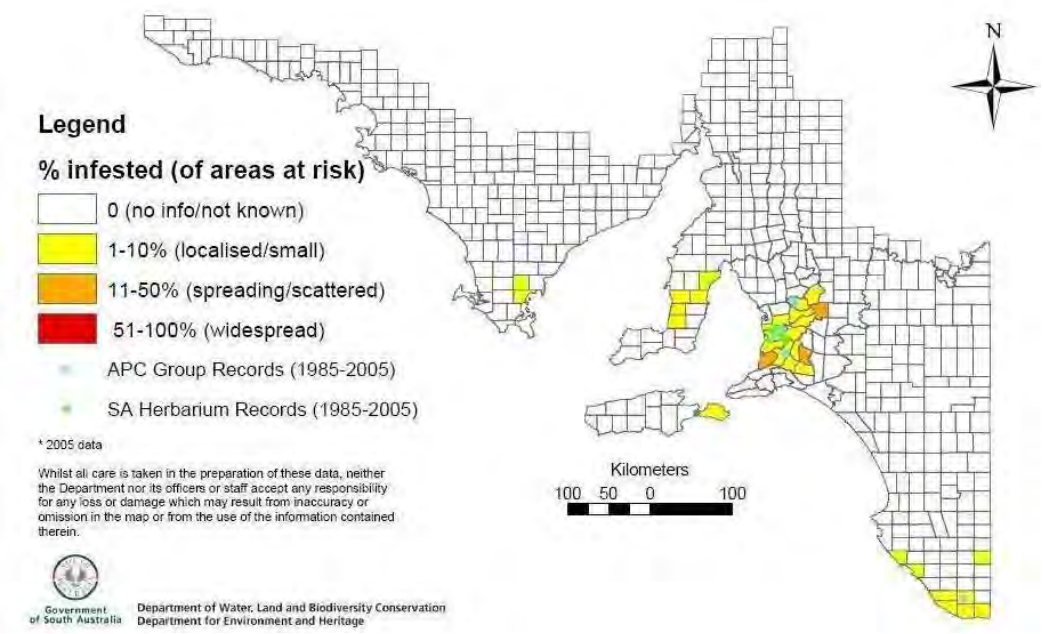

Figure 1.5 Map of distribution of *Allium triquetrum* in South Australia (Government of South Australia 2005).

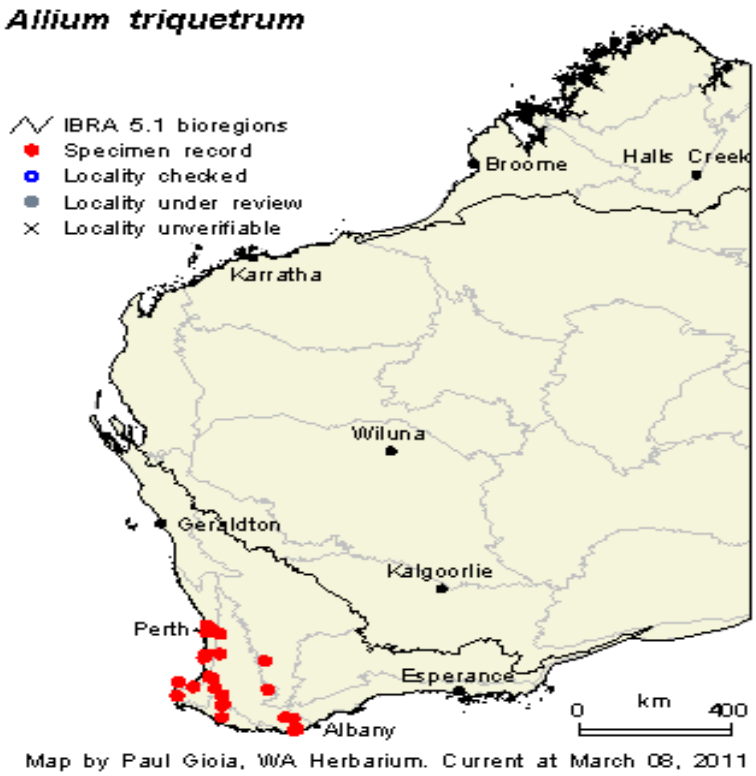

Figure 1.6 Map of distribution of *Allium triquetrum* in Western Australia (Western Australia Flora 2008).

A. triquetrum has been categorised as a noxious weed in these states of Australia, based on the risk of spread (Weeds Australia 2008) as follows:

VIC: class R ("Restricted Weeds. Plants that do not occur in Victoria, but pose an unacceptable risk of spread if they are sold or traded"), though clearly this is out of date.
SA: class 7* ("generally requiring control of the plant in part of the State").
WA: class Unass ("Plant species declared in other States and Territories that are not on the Permitted and Prohibited list, are unassessed and are prohibited until assessed via a weed risk assessment").

1.2.2 Taxonomy & classification

The botanical name of Angled Onion is ***A. triquetrum*** L. It is a monocotyledonous flowering plant (angiosperm). According to the Flora of Australia (1985) it belongs to the class ***Liliopsida***, family ***Liliaceae*** and subfamily ***Alliaceae*** (Table 1.1). However, according to the Angiosperm Phylogeny Group III system, ***A. triquetrum*** belongs to the order ***Asparagales,*** family ***Amaryllidaceae*** and subfamily ***Allioideae*** (Table 1.1). The species of the genus ***Allium*** are perennials, perennating by tunicate bulbs. ***Liliaceae*** is a large family of monocotyledons that consists of 220 genera and 3500 species (Nicholson ***et al.*** 1975). The genus ***Allium*** consists of 800 species that are centred in Asia and Russia (Nicholson ***et al.*** 1975, Friesen ***et al.*** 2006). Cultivation of ***Allium*** species is worldwide and both horticultural and ornamental species are planted far from their native ranges.

Table 1.1 Taxonomic classification of *Allium triquetrum* L. in Flora of Australia (1985) and the Angiosperm Phylogeny Group III system. (www.mobot.org/mobot/research/apweb/orders/asparagalesweb.htm#Alliaceae).

Flora of Australia (1985)	**APG III classification system**
Kingdom: *Plantae*	**Kingdom**: *Plantae*
Subkingdom: *Tracheobionta*-Vascular plants	**Clade**: Angiosperms
Superdivision: *Spermatophyta*- Seed plants	**Clade**: Monocot
Division: *Magnoliophyta*- Flowering plants	**Order**: *Asparagales*
Class: *Liliopsida*- Monocotyledons	**Family**: *Amaryllidaceae*
Subclass: *Liliidae*	**Subfamily**: *Allioideae*
Order: *Liliales*	**Genus**: *Allium*
Family: *Liliaceae*	**Species**: *Allium triquetrum* L.
Subfamily: Alliaceae	
Genus: *Allium*	
Species: *Allium triquetrum* L.	

Recognition of *A. triquetrum* is not difficult and the plant can be identified by the following distinct characteristics: the three-cornered shape of the flowering stem, the flowers with white tepals and central green stripes, and its distinctive onion odour (Parsons and Cuthbertson 1992; Walsh and Entwisle 1994). Blood (2001) has listed some species similar to *A. triquetrum* (Table 1.2); however, they are easily distinguished by lack of all three characters together.

In Australia, various *Allium* species are grown as ornamental and cultivated crops. Australia is one of the main exporters of agricultural products in the world and onion is one of the major products. Table 1.3 lists some examples of perennial and biennial common weedy, cultivated, and ornamental *Allium* species in Australia.

Table 1.2 Botanical and common name of some species which are morphologically similar to *Allium triquetrum* (Blood 2001).

Botanical name	Common name	Status
Asphodelus fistulosus L.	Onion weed	Weed
Burchardia umbellata R. Br.	Milkmaids	Endemic
Calostemma purpureum R. Br.	Garland lily	Ornamental
Leucojum aestivum L.	Snowdrop	Ornamental
Nothoscordum inodorum (Ait.) Nichols	Onion weed	Weed

Table 1.3 Common *Allium* species in Victoria, Western Australia, and South Australia (from information in Nicholson *et al.* 1975; Berrie 1977; Hyde-Wyatt and Morris 1980; Parsons 1992; Walsh and Entwisle 1994; Paczkowska 1994; eFlora- SA 2007). www.daff.gov.au/__data/assets/pdf_file/0011/.../100b-wwf-ap2.pdf

* Noxious weed listed in the Australian noxious weeds list conducted by the Australian government.

BOTANICAL NAME	COMMON NAME	CATEGORY
Allium triquetrum L. *	Angled Onion	Noxious weed
Allium vineale L. *	Crow Garlic	Noxious weed
Allium ampeloprasum L.	Wild leek	Weed
Allium neapolitanum Cirillo	Naples Onion	Weed
Allium oleraceum L.	Field Garlic	Weed
Allium paniculatum L.	Mediterranean Onion	Weed
Allium roseum L.	Rosy Garlic	Weed
Allium rotundum L.	Runder Lauch	Weed
Allium scorodoprasum L.	Sand Leek	Weed
Allium ascalonicum L.	Shallots	Cultivated
Allium canadense L.	Canadian Garlic	Cultivated
Allium cepa L.	Commercial Onion	Cultivated
Allium chinense G.Don	Rankkyo	Cultivated
Allium fistulosum L.	Welsh Onion	Cultivated
Allium porrum L.	Leek	Cultivated
Allium sativum L.	Garlic	Cultivated
Allium schoenoprasum L.	Chives	Cultivated
Allium sphaerocephalon L.	Round-headed Garlic	Cultivated
Allium tuberosum Roth	Garlic Chives	Cultivated
Allium carinatum L.	Keeled Garlic	Ornamental
Allium cernuum Roth	Lady's Leek	Ornamental
Allium giganteum Roth	Giant Onion	Ornamental
Allium nigrum L.	Black Garlic	Ornamental
Allium orientale Boiss	None	Ornamental
Allium paniculatum L.	Jewels-of-Opar	Ornamental
Allium paradoxum G.Done	Few-Flowered Leek	Ornamental

1.2.3 Description of A. triquetrum

1.2.3.1 Leaves & flowering Stem

The size of *A. triquetrum* plants varies somewhat, depending on provenance and environmental conditions. In mesophytic habitats the plant is taller and somewhat fleshy. The number of the leaves differs but each plant usually consists of 2-5 leaves and 1-3 flowering stems per bulb (Fig.1.7) (Parsons and Cuthbertson 1992; Blood 2001; DPI 2008a). The leaves are 30 to 40 cm tall and approximately 1.5 cm wide, and arise from the bulb. Leaves are green, lanceolate and have a strong onion odour when cut or crushed (Parsons and Cuthbertson 1992; Walsh & Entwisle 1994; DPI 2008a). The peduncle is triangular in section, erect, arising directly from the bulb and usually 30 to 50 cm taller than the leaves. It terminates in an umbel, each containing five to ten flowers (Parsons and Cuthbertson 1992; Walsh and Entwisle 1994; DPI 2008a).

Figure 1.7 Leaves and flowering stem of *Allium triquetrum*. The flowers are in at the tops of the peduncles.

1.2.3.2 Bulbs

A. triquetrum overwinters as a small tunicate bulb or cluster of bulblets on the basal stem plate with a cluster of white fleshy roots that do not spread deeply (Parsons and Cuthbertson 1992; Walsh and Entwisle 1994; Hussey *et al.* 1997; Blood 2001; Department Natural Resources and Environment 2001; DPI 2008a) (Fig. 1.8). Bulbs are an underground storage organ when the leaf foliage dies off and form the young plant in the next growing season. Bulbs and bulblets are usually ovoid, white-cream and 4-22 mm diameter by 0.5-2 cm long with a strong onion-like odour. The lengths of the roots from the bulbs vary with season and soil type and can extend up to 30 cm long in standard potting mix (personal observation).

Figure 1.8 Bulbs and roots of *Allium triquetrum*. Bulbs are 4-22 mm diameter and usually contain some bulblets.

1.2.3.3 Inflorescences and flowers

A. triquetrum flowers are campanulate and pendulous (Fig. 1.9) (Parsons and Cuthbertson 1992; Blood 2001). The umbel is subtended by a spathe (united bracts) and flowers are bisexual and usually five to ten per umbel. When closed bulbs are covered by “two white

papery bracts" (Parsons and Cuthbertson 1992) and the six tepals (perianth) are in two whorls of three; each tepal is white with a central green stripe. The pedicel is longer than the flower, and is usually 1 to 2.5 mm long (Parsons and Cuthbertson 1992; Walsh and Entwisle 1994; Blood 2001). Flowers are actinomorphic and the gynoecium comprises an superior ovary with three loculi, a short style and a stigma divided into three. The ovary has three united carpels, each with 1-2 ovules per loculus (Clarke and Lee 1987). Flowers are chasmogamous, i.e. pollination and fertilization occur after flowers open. García et al. (2006) reported that flowers open even when anthers are still closed. The androecium consists of six stamens, fused to the tepals at the base, of which three are inner (longer) and three are outer (shorter). This difference decreases after anthesis from 1.2 mm to approx. 0.5 mm (García *et al.* 2006). The longer stamens (inner) are almost vertical at anthesis and then bend downwards. The stamens are longer than the style and so the pollen grains on the anthers do not contact the stigma.

Figure 1.9 The parts of the *Allium triquetrum* flower. The six petals are white with a green central stripe; the pistil is in the middle of the flower, surrounded by the stamens (Clarke and Lee 1987).

A. triquetrum has several growing stages. Seeds germinate in the first year and bulbs are formed in the second and subsequent years; however, seeds are produced if flowers are

pollinated each year (Parsons and Cuthbertson 1992; Walsh and Entwisle 1994; Blood 2001 DPI; 2008a). The flowering time is late winter to early spring, although it can be altered by seasonal conditions and temperature after bud maturation significantly. Eichii *et al.* (2000) reported that bulbs stored at 5-10°C bloomed earlier than those stored at 15°C.

1.2.3.4 Sexuality

There is no evidence as to whether *A. triquetrum* is self-pollinated or cross-pollinated; however, the open flowers with closed anthers at anthesis suggest that it is cross-pollinated. The chromosome number is 2n=18 (Traub 1968). According to Balog (1979), a triploid form of the plant (3n = 27) was isolated in New Zealand and autotriploid forms were created during genetic studies by Fredrison (1969) and Rickards (1970). Typically most triploid plants are sterile; however, both fertile triploids and apomixis have been reported (Balog 1979; 1984; Strinath 1940). There is no report of polyploidy of *A. triquetrum* in Australia.

1.2.3.5 Fruits & seeds

After fertilization a green globular capsule is produced per flower. The size of the capsule differs depending on the number of seeds. Capsule size is from 4-7 cm long and 8-10 mm diameter, usually containing six black ovoid seeds (Fig. 1.10) that are 2-5 mm long (Parsons and Cuthbertson 1992; Blood 2001). Seeds are short-lived and lose viability after a year (Blood 2001). Each plant can produce up to 50 seeds per season (O'Neil 1962; Hyde-Wyatt 1980).

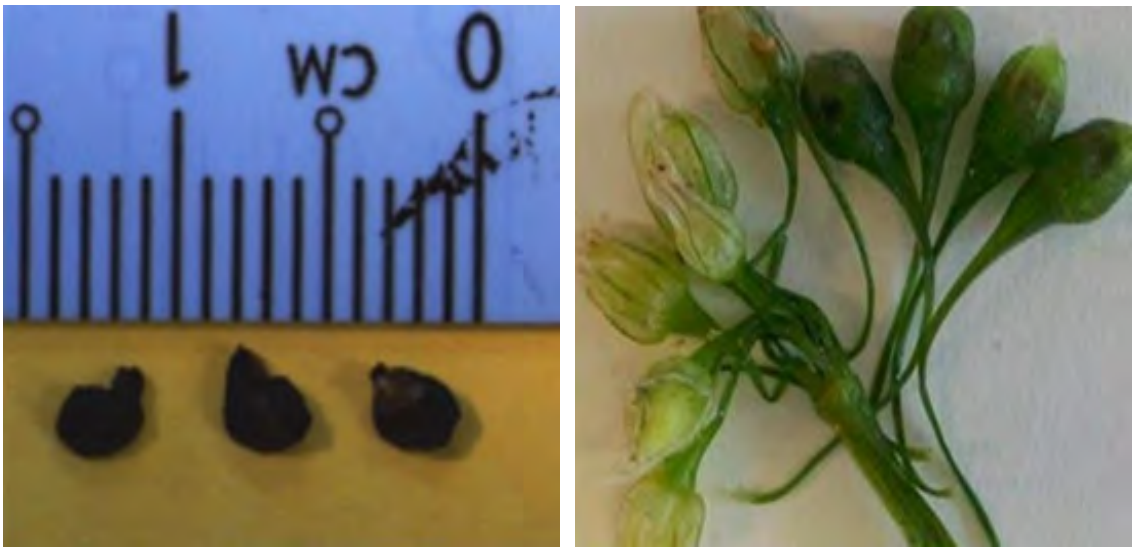

Figure 1.10 The seeds (left) and fruits (right) of *Allium triquetrum*.

1.2.4 Life cycle

A. triquetrum is a herbaceous perennial and propagates vegetatively by bulbs and seeds or a combination of both (Weeds Australia 2008). Flowers set seed and fruit is produced in winter-spring before the plant dies back in early summer. Seeds probably germinate in autumn. The growth rate of seedlings is slow through winter and early spring and two to four leaves are formed (Parsons and Cuthbertson 1992). Bulbs are formed in spring and plants die back in summer and perennate by bulbs and bulblets, which grow new foliage leaves in the next growing season, autumn (Parsons and Cuthbertson 1992; Walsh and Entwisle 1994; Blood 2001).

1.2.5 Dormancy

Both bulbs and seeds have dormancy periods, but the length of dormancy has not yet been determined. Seeds may remain dormant in the soil even when conditions are conducive to germinate and so have innate dormancy (Muzik 1970). Since seeds only germinated in this project after two months of exposure to moisture in a misting bed, it is likely that they have endogenous inhibitions that are leached out over time.

Bulbs can show dormancy but it seems to be in response to unfavourable environmental conditions because germination can be induced by favourable environmental conditions *in vitro* and glasshouse treatments (personal observation).

1.2.6 Dispersal

Dispersal of *A. triquetrum* occurs by seed, bulb and bulblet movement to new habitats where it eventually forms monocultures. Each plant produces many tiny bulblets that spread the colonies in the next growing season. Bulbs and bulblet dispersal may occur by flood and road building.

Seeds have been recorded as disseminating by: (1) wind, (2) agricultural machinery and vehicles, (3) insects, particularly ants, (4) animals (hair, wool, and faeces) (5) human activities (6) water movement and (7) soil movement (Parsons and Cuthbertson 1992; 2001; Blood 2001). Seeds mature in November-December, capsules become papery, thin and brittle, and open into three vertical segments, when the small black seeds fall out (Benson and McDougall 2002).

1.3 Weed problem

A. triquetrum is primarily a problem in natural environment (Muyt 2001; Department Natural Resources and Environment 2001). Three major problems have caused *A. triquetrum* to be categorized as noxious weed in Australia: its threat to native biodiversity (environment), its strong onion odour in milk (agriculture) and lack of effective means of control (environment) (Weeds Australia 2008; DPI 2008a).

The first problem is reduction of biodiversity in natural habitats (DPI 2008a). As a result of its rapid growth in shaded areas (Blood 2001) and its allelopathic exudates (DPI

2008a), it is invasive and forms monocultures and so is a threat to native herbaceous plants. The second problem is its strong onion odour. If farm animals consume *A. triquetrum*, milk smell and taste can be altered (Parsons and Cuthberston 1992; Weeds Australia 2008), which makes the dairy products unfit for sale.

The third problem is lack of effective control, as there is no selective or effective herbicide because they do not kill the bulbs. Hand-pulling is time-consuming and labour-intensive and hence expensive. Also according to Muyt (2001), dried plants in late summer produce fuel and because of its colonial growth there is a bushfire risk.

1.3.1 Control

1.3.1.1 Grubbing and slashing

Physical control also has its problems and hand-pulling of *A. triquetrum* is time-consuming and labour-intensive. It is impossible to remove all the bulbs and its cost makes it impracticable. It is also only effective in late winter-early spring when the soil is wet and the plants can be pulled up easily. The traditional approach for control in agricultural and bushland environments is hand-pulling and digging up the weeds roots, bulbs, etc. Slashing before flowering has been successful in restricting the spread of seeds to other regions.

1.3.1.2 Chemical control

The most common control method for *A. triquetrum* is by chemicals (herbicides). Various herbicides are recommended but none is selective. Among different herbicides recommended for this weed, most are in group I (Table 1.4) (Storrie 2007), but none kills the bulbs and they must be applied again in the next growing season.

Table 1.4 Recommended herbicides for *Allium triquetrum,* classification based on the primary action groups (MOA) (Storrie 2007).

Product name	Chemical Formula	HRAC	Mode of action		Reference
Metsulfuron methyl	C14H15N5O6S	Group B	Inhibitors of acetolactate synthase	Systemic	Environmental Bay of Plenty Council (2008)
Imazethapyr	C15H19N3O3	Group B	Inhibitors of acetolactate synthase	Systemic/contact	Pritchard (1996)
Chlorsulfuron	C12H12ClN5O4S	Group B	Inhibitors of acetolactate synthase	Systemic	Pritchard (1996)
Clorimuron ethyl	C15H15ClN4O6S	Group B	Inhibitors of acetolactate synthase	Systemic	Pritchard (1996)
Amitrole	C2H4N4	Group F	Inhibition of carotenoid biosynthesis	Systemic	Alcock (1974) and Hannay (1975)
MCPA	C9H9ClO3	Group I	Hormone analogues	Systemic	Tamar Valley Weed Strategy (2008)
2,4-D amine	C8H6Cl2O3	Group I	Hormone analogues	Systemic	Anon (1983)
2,4-D ester	C8H6Cl2O3	Group I	Hormone analogues	Systemic	Alcock (1974) and Hannay (1975)
2,2-DPA	C3H4Cl2O2	Group I	Hormone analogues	Systemic	Alcock (1974) and Hannay (1975)
Dicamba 500	C8H6Cl2O3	Group I	Hormone analogues	Systemic/contact	CHEMAG (2003)
Picloram	C6H3Cl3N2O2	Group I	Hormone analogues	Systemic	O'Neill (1962)
Glyphosate	C3H8NO5P	Group M	Inhibitors of EPSPS synthase	Systemic`	Pritchard (1996)

HRAC: Resistance Action Committee Classification System.

1.4 Biological control definition

According to Huffaker *et al.* (1971) (micro-) organisms that are destructive exclusively for weeds can be considered as potential control agents. Biological control means use of the natural enemies to reduce the host population to lower than the level that is a problem (Harley and Forno 1992; Bruzzese 1993). Debach and Rosen (1991) have stated that almost all living species have natural enemies in their native regions that regulate the population by feeding on them. Different options can be used in biological control, such as: predators, parasitoids, herbivors and pathogens. It is believed that biological control occurs naturally and is the interaction of natural enemies with pests; however, it may not occur in non-native regions or even not be adequate in its homeland. Well established successful biological control programs require determination of target hosts, biological agents and host-specificity (Sheppard 2003).

1.4.1 Importance of biological control

Biological control has been discussed for many years by practitioners in weed control programs (Impson *et al.* 2008). The focused items in this discussion are the release and interaction of agents (Briese 1997; McEvoy and Coombs 2000); host specificity and effectiveness (Hoffman and Moran 1998; Anderson *et al.* 2000; McEvoy *et al.* 2008); and safety and risk management (Denoth *et al.* 2002; Pearson and Callaway 2005). The increase in alien plant invasions around the world and the cost of control emphasises the importance of finding suitable biological control agents for weeds. For instance, weed control in infested areas was estimated to cost the livestock industry alone in Australia about $3-4.5 billion per annum and to take several years to be performed (Sinden *et al.* 2004). Control of serrated tussock in Victoria and NSW alone costs respectively $5 and $40 million per year (Nicolson *et al.* 1997; Jones and Vera 1998) and according to Aberdeen (1995), Victoria could save $35 million per year by restriction of its

dissemination to 200,000 ha where it could invade. As another example, South Africa was estimated to have 10 million hectares infested by alien plants (8% of the total area) and to need 20 years and at least US $1 million per year to control then (Versveld *et al.* 1998). Selective herbicides for alien plants are usually not available (Australian Government 2007). Therefore using natural enemies to reduce the target weed population to below the risk level that requires active control is necessary (Harley and Forno 1992; Bruzzese 1993) is highly desirable. Although biological control is useful in weed management systems, it does not eradicate weeds completely and may even increase invasions by other weeds that take over vacated niches (Callaway *et al.* 1998).

1.4.2 Biological control categories

Biological control is classified into two distinct methods: classical and inundative (Wapshere *et al.* 1989).

1.4.2.1 Classical biological control

Classical biological control is introducing a biocontrol agent from the area of endemism into the area of endemism to the invaded area to control the population of invaders and restore natural balance (Wilson 1972; Harley and Forno 1992; Waage 1992). Classical biological control was the first biological technique to be applied environmentally and agriculturally worldwide (Wapshere *et al.* 1989; Delfosse 1990). Classical biological control needs official permission from an authorized organisation such as a government (Mahr *at al.* 2008).

Host specificity is the main requirement in classical biological control programs. Only organisms that influence the target host exclusively or at most some unimportant closely

related species (Huffaker *et al.* 1971) can be considered as suitable and reliable biocontrol agents (Bruckart *et al.* 1996). Harley and Forno (1992) stated that application of a biocontrol agent that itself can be a biological risk in the future is not logical and risk assessments must be undertaken. Huffaker *et al.* (1971) believed that consideration of ecological circumstances is essential for establishment of a successful biological control program.

Advantages of classical biological control are self-perpetuating and self-disseminating biocontrol agents. Although classical biological control has been used worldwide for a long time, it does have disadvantages. Extensive phylogenetic host range testing of native flora and important crop plants is needed before any implementation to control the risk (Templeton and Smith 1977; Adams 1988; Delfosse 1990). In addition climate alteration can have a significant impact on the biocontrol agent's activities. Wapshere *et al.* (1989) and McFadyen (1991) stated that for a successful biological control program, climate modelling needs to be undertaken, as the biocontrol agent is not native to the infested areas.

1.4.2.1.1 Classical biological control history in Australia

The first attempt at classical biological control in Australia was as early as 1908, to find a biological potential agent to control prickly pear, and by 1925 a successful classical biological control program was in Australia to controlling prickly pear using *Cactoblastis cactorum* (cactus moth) (Delfosse 1990). Biological control was initiated officially in Australia in 1914 to control *Lantana camara* as an invasive noxious weed using Mexican insects. The same classical biological control program had previously been performed in Hawaii in 1902 to control *L. camara* and so is the oldest biocontrol program in Hawaii (Waage 1992). In 1973 a classical biological control program using a rust fungus was

initiated for European blackberry, which had caused widespread losses of agricultural land products and environmental degradation (Bruzzese and Hasan 1986). *Phragmidium violaceum*, a defoliating rust fungus, was released in 1991 in Victoria and South Australia and caused the expected defoliation of blackberry in higher rainfall areas but not in drier areas, in which the agent appeared to die out.

Suppression of Noogoora burr, a problematic weed for livestock industries in Northern Australia, is another example. Almost 30 new agents have been introduced in Australia for its control so far, leading to 16 agents being established species but only 4 agents showing significant impact (Page and Lacey 2006). *Puccinia xanthii*, an exotic rust fungus, was also introduced in Australia in 1970s, as a biocontrol control agent in non-tropical regions in Northern Australia (Morin *et al.* 1996; Morin *et al.* 2008). This biological control program has been only partially successful, due to environmental conditions and in variations in weed susceptibility and agents' biodiversity.

Biocontrol of *Chondrilla juncea*, Skeleton weed, a problematic weed in crops in South-eastern Australia, has been partially successful. Host-specificity tests were performed using four biological control agents from the native origin of *C. juncea* in Mediterranean regions and plants from the infested provenances in Australia. The rust fungus (*Puccinia chondrillina*) subsequently showed promising control of Skeleton weed in South-eastern Australia and its population was reduced significantly for a short time, but not to an economically important level (Hasan 1972; Caresche and Wapshere 1974; Supkoff *et al.* 1988; Wapshere *et al.* 1989).

Following these only partial successes, it was realised that environmental conditions and hidden variation in host resistance to one biocontrol agent were likely to mitigate against

success by a single agent and more recent interactions have focused on using several biocontrol agents together. Three agents for the groundsel bush, *Baccharis halimifolia*, a serious problem in South-eastern Queensland and Northern NSW (Verma *et al.* 1996) were released in Australia after they were successfully tested for similar biocontrol program in the USA, Brazil and Mexico (Julien and Griffiths 1998; Palmer *et al.* 2010). A rust fungus, *Puccinia evadens,* in combination with a stem borer, *Hellensia balanotes,* and a gall fly, *Rhopalomyia californica*, demonstrated significant control of Prickly acacia (*Acacia nilotica* subsp. *indica*), a noxious woody weed in Northern Australia (Thorp and Lynch 2000) in the 1990s. *Chiasmia assimilis*, which originated in Kenya, was established by 1997 and now causes distinctive defoliation on Prickly acacia in coastal regions (Palmer and McLennan 2006; Palmer and Witt 2006).

Phytopathogenic bacteria such as *Pseudomonas syringae* pv. *tagetis* and *Xanthomonas campestris* pv. *poannua* have been considered as potential biological agents on Canada thistle and annual bluegrass in bermudagrass respectively (Zhou and Neal 1995; Johnson and Wyes 1996; Tichich and Doll 2006).

1.4.2.2 Inundative biological control (augmentation)

The aim of inundative biological control programs is creating an epidemic disease on a weed by applying a large quantity of native organisms as a bioherbicide (Wapshere 1990; Templeton 1992). Augmentation consists of rearing and releasing biological control agents where the weed is at the level economically that needs be controlled. Usually the biological control agents in the inundative method are natural enemies that already exist in an infested area but in lower abundance than required to significantly affect the target hosts. It is important to survey the range of available (micro-) organisms that can be used to control the target host.

Inundative biological control also has its advantages and disadvantages. The main advantage of this technique over the classical method is the use of an agent that is native to the infested area and so there is no need for the long period of testing overseas and quarantine permission for importation. It usually works faster than the classical method and the chance of the biocontrol agent attacking the other species is low as it would already have been observed (Wapshere 1990; Templeton 1992). Advantages of augmentation biocontrol over chemical treatment are its selectivity and high specificity, lack of environmental contamination due to chemical residues and cheaper production (Adams 1988; Auld 1991). The main disadvantage of this technique is insufficient investment returns as a consequence of the highly specific products and the costs of efficacy and safety tests and bioherbicide registration (Templeton and Smith 1977). Integration of a biological control program with other control methods such as herbicides can be necessary and reduce chemical contamination as the potential biocontrol agents may not be effective alone to reduce the invasive population to a desirable level (Watson and Wymore 1989).

1.4.2.2.1 Mycoherbicides

Mycoherbicides are fungi applied to weeds for their biocontrol (Templeton and Smith 1977; Delfosse and Cullen 1985). The mycoherbicide formulation can be a spore or mycelium suspension applied to the target weed at a suitable time for infection, which is typically in the early stage of the target weed growth (Shrum 1982; Hasan 1988). There are several differences between mycoherbicides and classical agents. Most naturally occurring mycoherbicides use fungi that are not enough virulent enough to cause highly destructive disease and therefore need to be applied regularly to maintain damaging levels of infection.

Mycoherbicides have specific applications in the inundative control of environmental, pastoral and agricultural weeds. For instance, *Fusarium nygamai* and *F. oxysporum* are used as mycoherbicides on *Striga* spp., a parasitic weed in cereal crops, due to their highly specific mode of actions on the host plant (Boyette et al. 1993). The rust fungi that cause foliar lesions are also widely used in inundative control, e.g. in Musk thistle and Canada thistle (Baudoin *et al.* 1993; Thomas *et al.* 1994). Table 1.5 shows some of the evaluated mycoherbicides and bioherbicides since 1973.

Normally to establish a successful inundative fungus based biological control program, several steps need to be considered, as follows:

- Selection of the target weed, that needs to be controlled due to lack of selective herbicides or costly conventional methods (Wapshere *et al.* 1989).

- Identification of a suitable fungus that is highly virulent on the target weed (Delfosse 1989). Fungi are easier than other micro-organisms such as bacteria to identify and can be grown in mass under controlled conditions (Baker and Cook 1974). Taxonomic identification of the fungus to investigate the disease mechanism and its life cycle is not necessary (Hallett *et al.* 1990). Unlike in classical biological control, there is no risk of an alternative host susceptibility being altered.

- Assessment of the biocontrol agent's reproduction (Harris 1981). Potential biocontrol agents should be grown rapidly on sterile media and the stability of spores or mycelium should be assessed for storage and field applications. Items such as spore quantity produced, adhesion to the host plant and toxicity should be considered in commercialization (Hassan 1988; Wapshere *et al.* 1989; Van Dyke 1989).

- Centrifugal phylogenetic testing as for classical biological control (Wapshere *et al.* 1989) to assess the effect of the formulated mycoherbicide on the target weed, its close relatives and other important plants in ornamental and crop applications. Because the agents are native in the infested area, the biosafety risk is lower.

- Formulation of the biological agent for effective performance (Burge and Irvine 1985). Maximising the biocontrol agent's effectiveness into a cost-effective product is called formulation (Burge and Irvine 1985; Boyette *et al.* 1991; William *et al.* 1991). Formulation can protect the mycoherbicide from microbial degradation and rapid desiccation. Connick *et al.* (1990) suspended spores or mycelium in glycerol-water and so improved the agent's dispersion in spraying. The addition of a non-toxic preservative to the liquid suspension may also be necessary for long-term storage (Kenney and Couch 1981). Various 'wetters' and 'stickers' may be added to improve adhesion to the host's surface as for chemical herbicides and they must not be toxic to the fungus (Guijarro *et al.* 2008). Protection against desiccation may also involve using various clays and gels (Auld *et al.* 1988).

Table 1.5 Examples of some evaluated weed biological control agents (fungi and bacteria) and their host plants since 1973.

Biocontrol agent	Host plant	Commercialized	References
Alternaria crassa	*Datura stramonium*	No	Boyette (1986)
Alternaria eichhorniae	*Eichhornia crassipes*	No	Shabana *et al.* (1995)
Alternaria alternate	*Eichhornia crassipes*	No	Babu *et al.* 2002
Bipolaris euphorbiae	*Euphorbia heterophylla*	No	Barreto and Evans (1998)
Bipolaris sacchari	*Imperata cylindrica*	No	Yandoc *et al.* (2005)
Colletotrichum gloeosporioides f. sp. *aeschynomene*	*Aeschynomene virginica*	Yes	Smith (1986)
Corynespora cassiicola	*Lantana camara*	No	Pereira *et al.* (2003)
Drechslera sp.	*Bromus* spp.	No	Lawrie *et al.* (1998)
Drechslera gigantea	*Imperata cylindrica*	No	Yandoc *et al.* (2005)
Fusarium nygamai	*Striga hermonthica*	No	Abbasher and Sauerborn (1992)
Fusarium oxysporum	*Striga hermonthica*	No	Kroschel *at al.* (1996)
Fusarium sp.	*Alternanthera philoxeroides*	No	Tan *et al.* (2002)
Myrothecium verrucaria	*Pueraria lobata*	No	Boyette *et al.* (2002)

Myrothecium verrucaria	*Portulaca oleracea*	No	Boyette *et al.* (2007)
Myrothecium verrucaria	*Lygodium microphyllum*	No	Clarke *et al.* (2007)
Nimbya alternantherae	*Alternanthera philoxeroides*	No	Pomella *et al.* (2007)
Nigrospora oryzae	*Sporobolus fertilis*	No	Ramasamy (2008)
Phragmidium violaceum	*Rubus fruticosus*	No	Bruzzese and Hassan (1986)
Phytophthora palmivora	*Morrenia odorata*	Yes	Bowers (1986); Kenny (1986)
Pseudomonas syringae	*Ambrosia artemisiifolia*	Yes	Johnson *et al.* (1996)
Puccinia chondrillina	*Chondrilla juncea*	No	Cullen *et al.* (1973)
Puccinia carduorum	*Carduus thoermeri*	No	Baudoin *et al.* (1993)
Puccinia punctiformis	*Convolvulus arvense*	No	Thomas *et al.* (1994)
Puccinia myrysiphylli	*Asparagus asparagoides*	No	Morin and Edward (2006)
Stagonospora sp.	*Convovulus arvensis*	No	Pfirter and Defago (1998)
Ulocladium botrytis	*Orobanche* spp.	No	Muller-Stover and Kroschel (2005)
Xanthomonas campestris	*Poa annua*	Yes	Imaizumi *et al.* (1997)

1.5 Evaluation of biocontrol agents to control A. triquetrum

Existing management options for *A. triquetrum* are neither non-selective nor environmentally sustainable. Non-selective herbicides such as picloram+2,4-D and glyphosate can control *A. triquetrum* partially but cannot kill the bulbs and are lethal for native plants also. Therefore an alternative control method such as biocontrol using agents that will not harm native plants is a high priority for this environmental problem that has no currently effective long-term control. Since the bulb is the perennating part and leads to invasive monocultures, the bulbs is the preferred target. The main objective of this study is to evaluate soil-borne pathogens that already exist in Australia and are preferably specific to *Allium* species that can attack *A. triquetrum* bulbs where it has invaded natural habitats. As *A. triquetrum* is an environmental weed, it is important to find pathogens that are not pathogenic (or at least not virulent) on native plants, particularly in the family *Liliaceae*.

Cultivated *Allium* species are attacked by disease agents such as bacteria, fungi, viruses and phytoplasmas. Of these, facultative fungal and bacterial pathogens are most practically considered as biological control agents for *A. triquetrum* (Table 1.6). Soil-borne pathogens that attack the bulbs are preferable, so that these *Allium* pathogens can survive for a long time in the soil by producing sclerotia and spores or other resistant structures. Disease incidence caused by soil-borne pathogens is affected by various environmental factors. It is important to consider epidemiological factors such as temperature, humidity, organic matter, soil texture, light and rainfall for a potential biocontrol agent (Schwartz 2011). Maude (2006) categorised the pathogenic fungi of onion based on fungal growth under different temperatures *in vitro* (Table 1.6) and only those active at soil temperatures in infested sites should be considered.

Among the pathogenic fungi and bacteria of cultivated *Allium* species, those that attack bulbs in the field rather than in storage can be considered for biocontrol agents for ***A. triquetrum***. For instance, ***Alternaria porri*** (purple blotch) and ***Pyrenochaeta terrestris*** (pink rot) both occur in the field, but they do not attack the bulb and the disease severity varies with soil temperature (Hansen 1929; Fahim 1966; Walker ***et al.*** 2009). ***Leveillula taurica, Peronospora destructor*** and ***Botrytis squamosa*** cause powdery mildew, downy mildew and leaf rot and blight respectively. They also occur in the field but they only affect the leaves of susceptible plants (Hansen 1929; Lindsey ***et al.*** 2004; Walker ***et al.*** 2009) not the bulbs and the first two are obligate pathogens that would be difficult to rear and use. Fungi and bacteria that only cause onion storage disease are not suitable as biocontrol agents for ***A. triquetrum***. For instance, ***Aspergillus niger***, ***Aspergillus fumigatus***, ***Fusarium oxysporum*** f. sp. ***cepae*** and ***Pseudomonas marginalis*** pv. ***marginalis*** are only pathogenic during storage on onions grown in hot climates (Fahim 1966; Abawi and Lorbeer 1971; Ohuchi ***et al.*** 1983; Maude 1990; Walker ***et al.*** 2009). ***Botrytis*** bulb and neck rot occurs in temperate onion-growing regions frequently in cool, moist spring conditions and rot severity increases during storage (Hancock and Lorbeer 1963).

Table 1.6 Fungal pathogens and pathogenic bacteria of *Allium* species and their disease symptoms.

Pathogen	Disease symptom	Optimum (ºC)	Pathogenic only to *Allium*	Reference
Fungi				
Alternaria porri	Purple blotch (field & storage)	25	Yes	Fahim (1966)
Aspergillus niger	Black mould (storage)	31	No	Maude (1990)
Aspergillus fumigatus	Blue/green mould (storage)	37.5	No	Maude (1990)
Botrytis aclada	Bulb & neck rot (field & storage)	22-23	yes	Walker (1925)
Botrytis allii	Bulb & neck rot (storage)	20	Yes	Hancock and Lorbeer (1963)
Botrytis squamosa	Leaf rot & blight (field)	24	No	Hancock and Lorbeer (1963)
Fusarium oxysporum f.sp. *cepae*	Basal plate & root rot (field)	24-27	Yes	Abawi and Lorbeer (1971)
Leveillula taurica	Powdery mildew (field)	25-30	No	Lindsey *et al.* (2004)
Peronospora destructor	Downy mildew (field)	13	Yes	Yarwood (1943)
Pyrenochaeta terrestris	Pink rot (field)	26	No	Hansen (1929)

Stromatinia cepivora	White rot (field)	20-24	Yes	Coley-Smith (1990a)
Urocystis cepulae	Smut (field)	20	Yes	Dow and Lacy (1969)
Bacteria				
Burkholderia gladioli subsp. *alliicola* (previously known as *Pseudomonas*)	Soft rot (field & storage)	16-18	No	Yabuuchi *et al.* (1992)
Burkholderia cepacia (previously known as *Pseudomonas*)	Soft rot (field)	30-35	Yes	Yabuuchi *et al.* (1992)
Pectobacterium carotovorum subsp. *caratovorum* (previously known as *Erwinia carotovora.* pv. *carotovora*)	Soft rot (field & storage)	30	No	Dye (1969)
Pseudomonas aeruginosa	Soft rot (storage)	37	No	Cother *et al.* (1976)
Pseudomonas marginalis pv. *marginalis*	Soft rot (storage)	30-33	No	Ohuchi *et al.* (1983)
Xanthomonas axonopodis pv. *allii*	Leaf blight (field & storage)	20-24	Yes	Roumagnac *et al.* (2004)

Among all these fungi (Table 1.6), only *Stromatinia cepivora* (white rot) and *Urocystis cepulae* (onion smut) infect onions in the field severely and persist in the soil for a long time (Coley-Smith 1960; Dow and Lacy 1969). Onion smut disease occurs when the onion crops are planted and the fungal spores germinate and penetrate the fresh seeds. Lesions form on growing leaves and can spread into bulbs in the field but the fungus is an obligate pathogen and destructive disease on onion crops such as leek should not be planted in the invaded areas for 8 years (Hessayon 2009). *Stromatinia cepivora* causes white rot disease, a common and destructive disease of cultivated and ornamental *Allium* species. The fungus is a serious and fatal soil-borne pathogen of commercial *Allium* cultivars and attacks bulbs and roots in the field (Alexopoulos and Mims1979). *S. cepivora* is resistant to cold climates and survives by soil-borne sclerotia that germinate specifically in the presence of *Allium* exudates in the soil (Booer 1946). Since all *Allium* species are exotic to Australia, the fungus would not be expected to attack native plants that co-exist in natural environments, although it could be clearly not be used where it might contact cultivated *Allium* crops.

Among all fungi listed in table 1.6, only *Stromatinia cepivora* is highly specific to *Allium* exudates and can attack onion bulbs in the field. The fungus persists in the soil by forming sclerotia for 20 years after its host also died (Coley-Smith *et al.* 1990). It exists in almost all onion-growing areas in Australia such as Victoria, Eastern Australia, Queensland and Tasmania (Merriman *et al.* 1980; Wong and Hughes 1986; Mackie and McKirdy 2002; Metcalf *et al.* 2004) and so would not face quarantine restrictions. As a consequence of its reproductive system and survival in the soil, only limited applications may be needed to control *A. triquetrum* in native vegetation and it could even be used in permanent pastures not adjacent to horticultural enterprises growing *Allium* species.

Quick growth of bacteria in liquid culture and easy stabilization in frozen or dried formulations make them potential biocontrol candidates. Making artificial wounds or using surfactant during bioherbicide application can solve the problem of lack of entry into tissues (Johnson *et al.* 1996). Soft-rotting bacteria are major post-harvest and storage disease that can infect wide range of crops such as onion (Table 1.6) (Liao *et al.* 1993; Barras *et al.* 1994). Only two pathogenic bacteria, *Burkholderia cepacia* and *Xanthomonas axonopodis* pv. *allii*, are specific to onion crops. Both bacteria are soil-borne but usually remain isolated in one or more infecting bulb scales and allow the other scales remain healthy (Walker *et al.* 2009) and so could not be expected to destroy the bulbs. *Pseudomonas aeruginosa* and *P. marginalis* pv. *marginalis* can be excluded as only causing rot in storage. This leaves *Burkholderia cepacia* (specific to *Allium*), *B. gladioli* subsp. *allicola* and *Pectobacterium carotovorum* subsp. *carotovorum*, which are active in both field and storage, though better known in the latter.

1.6 Objective of this research

The aim of this project was to evaluate pathogens of *Allium* species currently in Australia as biological control agents for control of *A. triquetrum*. The potential biocontrol agents would preferably be specific to *Allium* species, not pathogenic to native plants and preferably not virulent to cultivated *Allium* species used as crops. *Stromatinia cepivora* is an imperfect fungus was selected as specific to *Allium* species and soilborne, causing white rot. During the course of this testing, rotting of stored wild-collected bulbs led to the isolation of two pathogenic bacteria: *Pectobacterium carotovorum* subsp. *carotopvorum* and a bacterium mostly closely related to *Ochrobactrum*. These were also evaluated as biological control agents. The aim was to assess the pathogenicity and virulence of the fungus and the bacteria against host provenance collections for pathogenicity, virulence and specificity. Finding strains with high virulence on *A. triquetrum* but not on native

plants or cultivated *Allium* species was a high priority. Genetic diversity of pathogens and host was analysed using molecular methods. Centrifugal Phylogenetic Testing was performed to test the specificity of the fungus and bacteria, starting with cultivated *Allium* species such as onion, garlic, chives and leek, other genera in the *Liliaceae* and then other native monocots. Eventually formulation and particular method of inoculation will be introduced for use in the field. The studies undertaken were on:

- Genetic diversity of *Allium triquetrum* provenances from across Australia using the polymerase chain reaction (PCR) and primers for the internal transcribed spacer region of rDNA (ITS), restriction fragment length polymorphism (RFLP) analysis and sequencing and randomly amplified polymorphic DNA (RAPD) (Chapter 2).

- Pathogenicity and virulence testing of *Stromatinia cepivora* on *A. triquetrum* provenances throughout Australia, cultivated *Allium* species and native plants *in vitro* and *in vivo* as well as evaluation of genetic variation between the fungus isolates within Australia and internationally (Chapter 3).

- Identification and evaluation of the pathogenicity and virulence of *Pectobacterium carotovorum* subsp. *carotovorum,* isolated from an infected *A. triquetrum* bulb collected from South Australia, as a potential biocontrol agent for *A. triquetrum* and its host-specificity *in vitro* and *in vivo* (Chapter 4).

- Identification of a novel bacterium mostly closely related with *Ochrobactrum* isolated from an infected *A. triquetrum* bulb collected from Victoria and its evaluation as a potential biocontrol agent for *A. triquetrum* and its host-specificity *in vitro* and *in vivo* (Chapter 5).

Chapter 2

Genetic diversity and molecular analysis of *Allium triquetrum* in Australia

2.1 INTRODUCTION

Before undertaking a biological control program for a weed, it is necessary to investigate its diversity to ensure that the agents are tested and active across all variants of the species.

2.1.1 Variation

Angled onion seems to have uniform morphology in Australia and its other habitats over the world, as there is no report of discontinuous morphological variation. The phenotype is the result of interaction between the genotype and the environment. Traditional morphological observation is not enough, therefore, to determine genotypic diversity in the plant populations (Wen and Hsaio 1999).

Polyploidy may result in different biotypes that may or may not be morphologically distinguishable. Cytological investigations suggested that diploid 2n = 18 (Traub 1968) and triploid 3n = 27 (Balog 1979) karyotypes exist in *A. triquetrum* but there has been no report of typical gigas characters, e.g. more robust growth.

There may also be some hidden genetic diversity, which can result in different biotypes that lead to weed management difficulties (Tranel and Wassom 2001). There is no report of Angled onion biotypes in Australia; however, several studies have found biotypes in other species, such as *Scilla peruviana,* in the family *Liliaceae* (Carmona at al. 1983). At the extreme, various degrees of genetic differences can be detected even between

individual plants of weedy species located in a single provenance (Tranel and Wassom 2001).

2.1.2 Propagation and diversity

A. triquetrum reproduces both asexually (by bulbs) and sexually (by seeds). Asexual propagation from bulbs typically leads to populations that are identical genetically to the maternal plants (Fryxell 1957). Angled onion mainly propagates by bulbs and bulblets rather than seeds, since the quantity of bulbs and bulblets at end of each season is greater than its seed production. Although genetic diversity has been reported even in clonal plants, the degree of diversity is typically much less than in exclusively sexually propagating species (Persson and Gustavsson 2001). Many studies have argued that a high incidence of asexual propagation within a plant species could also lead to significant genetic variation due to even small non-deleterious mutations being perpetuated, e.g. blackberry (Maynard Smith 1978; Bell 1982; Antonovics and Ellstrand 1984; Ellstrand and Antonovics 1985; Schmitt and Antonovics 1986; Ellstrand and Roose 1987). However, sexual reproduction remains as the main means of within-population diversity.

2.1.3 Herbicide resistance

Although herbicides are the main method of control, no herbicide resistance has been reported so far in *A. triquetrum*. Any genetic diversity within the species; however, together with the repeated use of one herbicide, could select for a biotype that is resistant to that herbicide, as has occurred in many other species (Stankiewicz et al. 2001). According to Heap (2007) more than 315 herbicide-resistant biotypes have been reported in 180 plant species and 49 weed species resistant to one or more herbicides have been reported in Australia (Heap 2007; Storrie 2007), such as biotypes of *Lolium rigidum* (Hoy 1999; Pratley *et al.* 1999) and wild radish (Cheam *et al*. 1999). Therefore a study of the genetic diversity within *A. triquetrtum* has value beyond merely biological control.

2.1.4 Measurement of variation

Chemical and biological control of successful weedy populations are highly linked to understanding of the level of a weed's biodiversity (Cavan *et al.* 2000). Genetic information, phenotypic plasticity, response to natural selection pressure and interaction of the invasive population with native biodiversity are the basic data that need to be considered for invasive plant management (Thompson 1998; Sakai *et al.* 2001). Traditional methods such as seed germination and plant morphology traits have been used to measure variation in many weeds, but cannot detect genetic variation without some morphological or physiological expression of the genes (Pappert *et al.* 2000; Sakai *et al.* 2001; Lee 2002). Molecular analysis using the polymerase chain reaction (PCR) has been conducted for several years as a reliable, powerful and productive tool for highlighting genetic diversity of plant species (Provan *et al.* 2004; Ye *et al.* 2005). PCR amplification of DNA fragments using molecular markers often results in polymorphism in the target genome and hence characterization of genetic variation.

2.1.5 Ribosomal DNA and ITS regions

Eukaryotic ribosomal DNA (rDNA) has been used for systematics and to construct phylogenetic histories of taxonomically related taxa in different organisms (Berbee and Taylor 1992; Lee and Taylor 1992). As a consequence of the high copy number of DNA in cells and repeating units in coding and non-coding regions throughout the genome, rDNA is easy to detect and use in genetic diversity studies (Beckingham 1982; O'Donnell 1992; Suh *et al.* 1993). In particular, that portion of rDNA known as the internal transcribed spacer (ITS) region has dominated biodiversity studies in plants and fungi. The ITS region lies between the 18S and 26S coding regions and comprises two internal transcribed spacers (ITS 1 and 2) with a coding 5.8S region between them (Rogers and Bendich 1987; Garber *et al.* 1988; Shivji *et al.* 1992). The region between the 18S and

5.8S regions and between the 5.8S and 26S coding regions are known as ITS1 and ITS2 respectively (Fig. 2.1) and are transcribed into RNA but that RNA is not translated into protein (Nues *et al.* 1994).

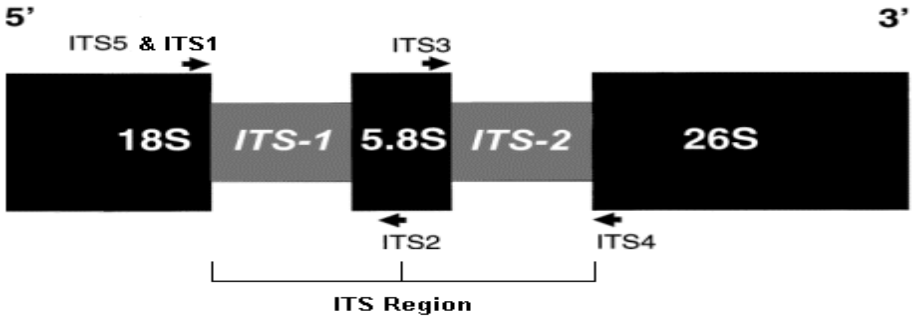

Figure 2.1 Internal transcribed spacer region 18S-26S ribosomal DNA. Arrows indicate approximate position of the White *et al.* (1990) primers used to amplify the region (Baldwin 1992).

PCR amplification and sequencing of the ITS regions of many organisms has been performed as a source of phylogenetic information and relationships since about 1990. The ITS sequences have shown differences among genera and even within a genus (White *et al.* 1990). The ITS region varies in length among organisms and generally is less than 1 kbp in size. It consists of non-coding sequences that mutate randomly without the adverse consequences associated with coding regions and so demonstrates a greater range of variation than the rDNA genes (Schaal and Learn 1988; Suh *et al.* 1993; Ritland *et al.* 1993; McLain *et al.* 1995). It is hypothesized that the ITS sequence differences between species or individuals have arisen by cumulative point mutations during evolution (Arnheim *et al.* 1980), enabling time since they split from a hypothetical ancestor to be calculated.

Restriction fragment length polymorphism (RFLP) and sequencing are two common methods used to analyse the ITS-PCR products of closely related species. The ITS regions

can be distinguished by digestion of the ITS amplicons using restriction endonucleases and size fractionation on agarose gels (Yamashita and Tashiro 2001). RFLP analysis is a common tool in almost all genotype fingerprinting and has been used widely in phylogenetic studies and genetic disease analysis (Cooly 1992; Tashiro *et al.* 1995; Krupa 1999; Arifin *et al.* 2000). The advantages of this method are detection of both coding and non-coding regions of the genome and unlimited applications due to the numerous endonucleases enzymes (McDonald and McDermott 1993). RFLP is widely employed in plant and microorganism genetic diversity studies. It provides molecular markers for the analysis of genetic relationships (Tsuge and Kobayashi 1991; Cooley 1992).

DNA sequencing to determine the order of nucleotide bases in the DNA has been approved as the best tool in genetic diversity studies and weed research (O´Hanlon *et al.* 2000). For several years sequencing was performed only for well-distinguished genes. Bioinformatics improvement and modern sequencing technology in recent years has allowed the study of highly variable regions and for sequencing to be used as a relatively easy interpretation tool. ITS sequencing using universal primers White *et al.* (1990) is a useful supportive tool for weed phylogeographical studies (Dumolinlapegue *et al.* 1997, O´Hanlon *et al.* 2000). However, a major disadvantage is that it explores variation only in a small part of the genome and hence ITS sequencing is not useful appropriate for markers that are distributed throughout the genome. There are several ITS sequences of *A. triquetrum* in GenBank and available through portals such as ANGIS (Australian National Genomic Information Service, http://biomanager.info/, now closed) or NCBI (National Centre for Biotechnology Information, http://www.ncbi.nlm.nih.gov/)

2.1.6 Randomly Amplified Polymorphic DNA (RAPD)

The randomly amplified polymorphic DNA technique was primarily developed by Williams *et al.* (1990) using arbitrary short primers of 8-12 nucleotides, with 10-mers being most popular. The technique uses short arbitrary primers to bind to and amplify short pieces of DNA across the whole template DNA and polymorphism in amplicons is detected using gel electrophoresis (Fig. 2.2).

The RAPD technique has been used extensively in plant genetic diversity and breeding systems studies. For instance, RAPD-PCR has been performed for the classification, diversity and molecular analysis of progeny from sexual and asexual reproducing cultivated *Allium* species (Wilkie *et al.* 1993; Le Thierry D'ennequin *at al.* 1997; Tanikawa *et al.* 2002). Compared with RFLP, RAPD-PCR is faster and less laborious (Williams *et al.* 1990; Welsh *et al* 1991), and there is no need for knowledge of the DNA sequences. The technique has however, been criticized by some as not necessarily being reproducible (McDonald and McDermott 1993; Fang and Roose 1997; Esselman *et al.* 1999). RAPD analysis is much used in taxonomic and cladistic studies as it screens all of the genome, rather than only selected regions, which is the limitation of DNA sequencing and RFLP (Bachmann 1997; Wolfe and Liston 1998).

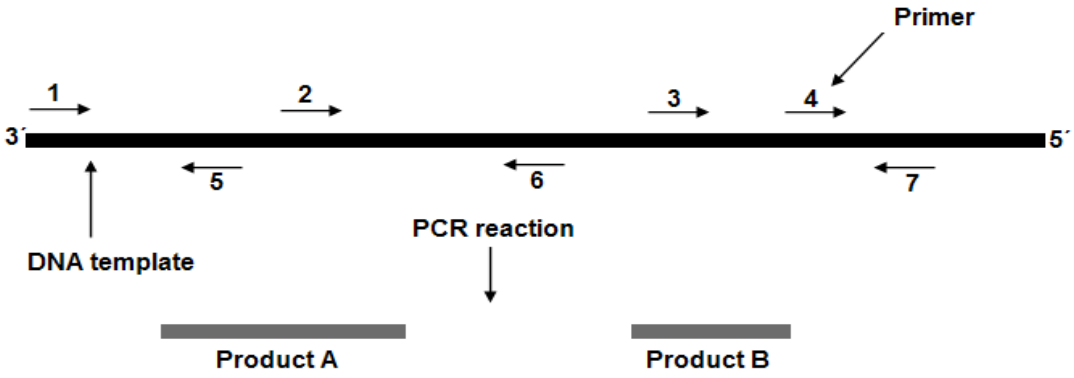

Figure 2.2 RAPD-PCR reactions containing a large DNA fragment as a template and several copies of a single primer which amplifies unknown sequences (Williams et al. 1990).

Several studies have shown that RAPD probes can screen the non-coding regions of the DNA (Penner 1996). RAPD-PCR has also some disadvantages that have been noted by several researchers: high variation in DNA amplification, scoring difficulties for non-homologous bands (Maniruzzaman *et al.* 2010), low annealing temperature of RAPD primers (template competition for amplifiable products) (Wilkerson *et al.* 1993) and amplification of some DNA fragments below the detection limits on agarose gel. Some of these RAPD-PCR problems can be overcome by optimization of the reagents and more care with gel electrophoresis (McClelland and Welsh 1994; Smith *et al.* 1994, Backeljau *et al.* 1995).

A third technique commonly used in genetic diversity studies is inter-simple sequence repeat (ISSR) based on microsatellites. Microsatellites are randomly repeated nucleotide motifs that are densely interspersed with simple sequences in eukaryotic genomes (Hamada *et al.* 1982; Tautz and Renz 1984). The levels of polymorphism and reproducibility in ISSR are greater than in RAPD-PCR (Tikunov *et al*. 2003); however, RAPD markers are more informative in genetic diversity studies.

2.2 Aims

The aim of this study was to evaluate the genetic diversity of *A. triquetrum* in Australia using ITS-RFLP, ITS sequencing and RAPDs.

2.2 MATERIALS AND METHODS

2.2.1 DNA Extraction

Plant materials were collected from 23 provenances from across Australia (Table 2.1). Three bulbs were selected randomly from each site and surface-sterilized in 1.5% NaOCl for 10 min and then rinsed three times with with sterile MilliQ water. Total DNA was

extracted and purified using a Qiagen DNeasy Mini Kit using the manufacturer's protocol (Qiagen DNeasy Plant Handbook) as described briefly below.

A maximum of 100 mg of each *A. triquetrum* bulb was extracted. The plant material was powdered under liquid nitrogen using a mortar and pestle. The frozen powdered material was transferred to an Eppendorf tube and 400 µL AP1 buffer plus 4µl of RNase A (100 mg/ml) added, mixed by vortexing and incubated for 10 min at 65ºC to lyse the cells, then 130 µl AP2 buffer was added to the lysate and incubated for 5 min on ice to precipitate proteins, polysaccharides etc. The samples were centrifuged for 5 min at 20,000 x g and the supernatant was loaded on to a QIAshredder Mini spin column to remove cell debris and precipitates and centrifuged at 20,000 x g for 2 min. The flow-through was transferred into a 1.5 volumes of AP3/E buffer and mixed by pipetting to precipitate the DNA. To bind the DNA, the mixture was pipetted into a DNeasy Mini spin column and centrifuged at 6,000 x g for 1 min. The DNA bound to the membrane was rinsed with, two lots of 500 µL AW buffer, followed by centrifugation at 6000 x g for 1 min and then at 20,000 x g for 2 min to remove the residual ethanol and dry the membrane. The DNA was eluted by soaking the membrane for 5 min with 100 µL Buffer AE and subsequently centrifuging at 6,000 x g for 1 min. This step was repeated to get the final volume, 200 µL. The DNA concentration and quality was visualised by loading 10 µL of DNA extract plus 2 µL of Fermentas loading dye (10 mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol and 60 nM EDTA) onto a 1.5% agarose gel (Agarose, Molecular Grade, BIOLINE), gel electrophoresing at 100 V for 45 min, staining with 10 mg/mL ethidium bromide and imaging using a Bio-Rad Gel Doc system. The DNA concentration was estimated by simultaneously electrophoresing 1 µL of Fermentas GeneRuler^{TM} 100 bp and comparing the brightness of staining with that of the 500 bp band containing 80 ng/0.5µg DNA.

Table 2.1 *Allium triquetrum* samples used for genetic diversity studies**.** The table shows the collection sites, state of origin and approximate latitudes and longitudes.

Provenance	**State**	**Latitude & Longitude**
Belair National Park	South Australia	S 35º00’45.44” E 138º39’24.67”
Fairview Park	South Australia	S 34º48’17” E 138º43’25”
Horsnell Gully	South Australia	S 34º55’44 ” E 138º41’59 ”
Mylor	South Australia	S 35º02’23.34” E 138º45’47.98”
Waterfall Gully	South Australia	S 35°59’56 ” E 138º38’14 ”
Ararat	Victoria	S 37º15’55.0” E 142º55’06.2”
Bendigo Creek (Bendigo)	Victoria	S 36º46’42.37” E 144º14’50.72”
Ferny Creek Recreation Area (Dandenongs)	Victoria	S 37º53’30.87” E 145º22’01.79”
Gardiner’s Creek (Glen Iris, Melbourne)	Victoria	S 37º51’45.00” E 145º03’43.24”
Hardy’s Picnic Ground near Kallista (Dandenongs)	Victoria	S 37º53’42.21” E 145º21’45.96”
Kangaroo Flat (Bendigo)	Victoria	S 36º47’19.80” E 144º14’40.17”
Kinglake National Park	Victoria	S 37º53’30.87” E 145º22’01.79”
Memorial Drive near Yellow Gum Park (Plenty Gorge Parklands)	Victoria	S 37º40’06.21” E 145º06’29.20”
Merri Creek (Reservoir, Melbourne)	Victoria	S 37º24’02.48” E 145º16’00.57”
Plenty Road (Reservoir, Melbourne)	Victoria	S 37º43’39.87” E 145º01’25.99”
Sherbrooke Picnic Ground (Dandenongs)	Victoria	S 37º53’33.87” E 145º21’54.12”
Yarra Bend Park (Studley Park)	Victoria	S 37º47’57.99” E 145º00’35.99”
Westernport Bay 1	Victoria	S 38º38’07.90” E 145º33’40.6”
Westernport Bay 2	Victoria	S 38º13’26.00” E 145º13’03.60”
White Hill (Bendigo)	Victoria	S 36º14’19.85” E 144º17’43.45”
Wonthaggi	Victoria	S 38º36’29.14” E 145º34’32.01”
Bridgetown	Western Australia	S 33º57’44.0” E 116º09’17.0”
King’s Park (Perth) *	Western Australia	S 31º57’21.5” E 115º50’25.2”

***** Kindly collected by Dr Janet Anthony and Dr Siegy Krauss, Kings Park and Botanic Garden, Western Australia.

2.2.2 ITS-PCR amplification

The entire ITS region (coding and non-coding regions) was amplified with the universal primers ITS1 (5´-TCCGTAGGTGAACCTGCGG-3´) and ITS4 (5´ TCCTCCGCTTATTGATATGC-3´) (White *et al.* 1990). Each 25 µL reaction contained 12.5 µL of PCR Green Taq Mastermix (Fermentas), 2 µL of approximately ~16 ng of genomic DNA, 0.5 µL of ITS1 (70.2295 µM), 0.5 µL of ITS4 (72.95 µM) and nuclease-free water to make the final volume up to 25 µL. A negative control reaction was prepared by adding an equal volume of nuclease-free water instead of DNA. The thermocycler cycles were based on the protocol of Provan *et al.* (2004). The primers' annealing temperatures were estimated according to Meinkoth and Wahl (1984) (T (ºC) = 4(G+C) + 2(A+T)) and checked against Tm provided by the manufacturer, Micromon (Monash University). The thermocycler was programmed for an initial denaturation at 94ºC for 10 min, 35 cycles of: 30 s denaturation at 94ºC, 1 min annealing at 51ºC and 1 min extension at 72ºC; and a final extension of 10 min at 72ºC followed by storage at 4ºC. PCR products (5 µL) were separated by electrophoresis on 1.5% agarose gel in 1 x TBE buffer (54 g/LTrisma base, 27.5 g/L Boric acid, 20 mL EDTA) at 80-90 V. Approximate amplicon size was estimated by simultaneously electrophoresing 1 µL of GeneRuler^{TM} 100 bp and products imaged using a Bio-Rad Gel Doc system using Quantity One software as before.

2.2.3 Restriction Fragment Length Polymorphism (RFLP)

ITS-PCR amplicons were sequenced initially for two *A. triquetrum* samples, one from Victoria and the other from Western Australia (Section 2.2.4). Suitable restriction enzymes were selected using restriction analysis software, Restrict (EMBOSS), Biomanager, (ANGIS: http:// www.biomanager.angis,org.au). Nine restriction endonucleases (*Taq*I, *BamH*I, *Hha*I, *Eco*RI, *Eha*I, *Msp*I, *Rsa*I, *Xho*I, *Not*I) were tested and

six (*Taq*I, *BamH*I, *Eha*I, *Msp*I, *Rsa*I, *Xho*I) that digested ITS-PCR products consistently were used for final analysis.

RFLPs were performed in 5 µl reactions containing 4 µl of the ITS-PCR product, 0.5 µl (5,000 U) endonuclease and 0.5 µl 10X buffer for each enzyme. All reactions were incubated at the optimal temperature (37ºC for all except *Taq*I, which was 65ºC) for a minimum of 3 h. The digested products were separated on 2% agarose gel by electrophoresis at 100 V along with a marker lane of 1 µl GenRuler 100 bp. Gels were stained by ethidium bromide and imaged by a Bio-RAD Gel Doc system as before. DNA extracts were grouped according to their RFLP patterns and DNA from at least one member of each RFLP group was sequenced.

2.2.4 DNA Sequencing

The ITS-PCR products were purified using a QIAGEN PCR Purification Kit according to the manufacturer's protocol as follows. The remaining intact ITS-PCR products were mixed with PB1 buffer (in a 1:5 ratio) and subsequently precipitated DNA was bound to the membrane of a QIAquick spin column and centrifuged at 10,000 x g for 1 min. To wash the DNA to remove contaminants, 750 µL ml PE buffer was added to the column and centrifuged at the same speed for 1 min. DNA was eluted from the membrane with 50 µL of EB buffer (10 mM Tris-Cl, pH 8.5).

DNA was sequenced using the Applied Biosystems BigDye Terminator v3.1 Cycle Sequencing Kit protocol. Each 20 µL reaction contained 2 µL of purified DNA as a template, 2 µL of 10X buffer, 2 µL of one primer (ITS4), 1 µL of BigDye and nuclease-free water. The thermocycler was programmed for an initial denaturation at 96ºC for 1 min and 25 cycles of: 10 s denaturation at 96ºC, 5 s annealing at 51ºC and 4 min

elongation at 60ºC.The BigDye-labelled DNA was precipitated using the ethanol-precipitation protocol (Elmer 1998). For each reaction, 20 µL of Big Dye product was added to 80 µL of a master mix (62.5 µL 95% ethanol, 3 µL 3 M sodium acetate (pH 5.2), and 14.5 µL nuclease-free water) and incubated at room temperature for 30 min, followed by 20 min centrifugation at 20,000 x g. The precipitated DNA was rinsed with 250 µL of 70% ethanol and then centrifuged for 5 min at the same speed. The supernatant was removed and the tube contents were dried in a laminar flow cabinet overnight. The products were sent to the DNA Sequencing Facility, Micromon, Monash University, for further analysis.

2.2.5 Sequence edition and alignment

The sequences was downloaded from Micromon and edited by cross-referencing between sequences using BioEdit software on ANGIS, Biomanager. Approximately 80-200 bp of the 5´ beginning and 3´end of each sequence was deleted due to unreliable signals. The closest match for each was searched in the GenBank database using the BLAST program (Altschul *et al.* 1997) provided through ANGIS and the closest matching sequences downloaded. The sequence of *Allium cepa* (NCBI accession number FJ664287.1) was downloaded from GenBank via NCBI to use as an outgroup. Sequences were aligned using the ClustalW program (Thompson *et al.* 1994). A distance matrix of sequences was constructed using the DNAdist program (Felsenstein 1989) to estimate the total branch length between two species, using the Kimura (1980) model of nucleotide substitution. A phylogenetic tree was constructed from the distance matrix data using Fitch-Margoliash and Kitsch criteria (Felsenstein 1989). Phylogeny was also estimated by neighbour-joining and UPGMA methods (average linkage clustering) (Felsenstein 1989). Phylogenetic trees were constructed from the ClustalW alignment using the program MEGA4 (Tamara et al., 2007).

2.2.6 RAPD-PCR

Sixty RAPD primers (3 sets: OPA, OPB, OPM (Operon Technologies)), each 10 nucleotides in length, were used to amplify the DNA (Table 2.2). Each 25 µL reaction comprised 12.5 µL of Green Taq Mastermix, 1 µL (0.6 µM) of each primer, 2 µL of approximately 16 ng DNA template, and nuclease-free water. A negative control reaction substituted 2 µL nuclease-free water for the DNA. A G-STORM (GT-11426) thermocycler was programmed based on the Manzanares-Dauleux *et al.* (2001) protocol for initial denaturation at 94ºC for 30 s, followed by 35 cycles of: 94ºC for 30 s, 35ºC for 1 min and 72ºC for 2 min 30 s; with a final elongation at 72 ºC for 5 min. The RAPD-PCR products were separated on 1.5% agarose gel and visualised as before. Initially all primers were used to amplify a sample from each provenance. The RAPD primers that amplified at least 3 bands were then used for all provenances, with three replicates from each provenance. PCR amplification was performed 3 times to check the reproducibility of the RAPD primers. Polymorphism of consistent bands was scored manually based on presence (1) or absence (0) of bands of different sizes for each primer and entered manually on an Excel spreadsheet. This was used to construct a similarity matrix and phylogenetic tree using hierarchical cluster analysis on the program PASW Statistics Data Editor provided by SPSS software. RAPD dendrogram was performed using single-linkage rescaled distance cluster combine, generated by SPSS Hierarchical Cluster analysis.

Table 2.2 Sixty RAPD primers (3 sets: OPA, OPB, OPM (Operon Technologies)), each 10 nucleotides in length, used for RAPD analysis.

Primers number	**Operon Technologies RAPD primers**		
	OPA Sequence 5´ to 3´	**OPB** Sequence 5´ to 3´	**OPM** Sequence 5´ to 3´
1	CAGGCCCTTC	GTTTCGCTCC	GTTGGTGGCT
2	TGCCGAGCTG	TGATCCCTGG	ACAACGCCTC
3	AGTCAGCCAC	CATCCCCCTG	GGGGGATGAG
4	AATCGGGCTG	GGACTGGAGT	GGCGGTTGTC
5	AGGGGTCTTG	TGCGCCCTTC	GGGAACGTGT
6	GGTCCCTGAC	TGCTCTGCCC	CTGGGCAACT
7	GAAACGGGTG	GGTGACGCAG	CCGTGACTCA
8	GTGACGTAGG	GTCCACACGG	TCTGTTCCCC
9	GGGTAACGCC	TGGGGGACTC	GTCTTGCGGA
10	GTGATCGCAG	CTGCTGGGAC	TCTGGCGCAC
11	CAATCGCCGT	GTAGACCCGT	GTCCACTGTG
12	TCGGCGATAG	CCTTGACGCA	GGGACGTTGG
13	CAGCACCCAC	TTCCCCCGCT	GGTGGTCAAG
14	TCTGTGCTGG	TCCGCTCTGG	AGGGTCGTTC
15	TTCCGAACCC	GGAGGGTGTT	GACCTACCAC
16	AGCCAGCGAA	TTTGCCCGGA	GTAACCAGCC
17	GACCGCTTGT	AGGGAACGAG	TCAGTCCGGG
18	AGGTGACCGT	CCACAGGAGT	CACCATCCGT
19	CAAACGTCGG	ACCCCCGAAG	CCTTCAGGCA
20	GTTGCGATCC	GGACCCTTAC	AGGTCTTGGG

2.2.7 Karyotypes of A. triquetrum

Three bulbs from each provenance were selected randomly, germinated on sterile water agar at ambient temperature and 5 cm at the ends of root tips removed for karyotyping between 10 a.m. and 1 p.m. Three pre-treatment solutions were examined to improve metaphase plate detection as follows:

1. icy water for 24 h (Mirzaghaderi 2010).
2. 0.002 M 8-hydroxyquinoline for 2-4 h at 20-22ºC (Langeland 1989).
3. 0.5% colchicine for 2-4 h at 20-22ºC (Rickards 1977).

The root tips were fixed subsequently using Farmer's solution (Snow 1963) (3:1 95% ethanol/glacial acetic acid) for a minimum of 24 h at 4ºC. The fixed root tips were rinsed in 95% ethanol for 10 min, stained with HCl-carmine (30 g/L carmine and 20 mL/L concentrated HCl in 80% ethanol) for 48 h at room temperature, rinsed with 45% glacial acetic acid for 2 min and then rinsed three times with 70%, then 95%, ethanol. The tips were stored in 75% ethanol at 4ºC for future studies. The meristematic cells were hydrolysed with 1N HCl at ambient temperature for 8-10 min. The terminal 1 mm was squashed on a glass side in a drop of MilliQ water using a blunt-end probe, covered with a coverslip, squashed again and then fixed in DPX Mountant.

Slides were observed and photographed on a Leica DM2500 compound microscope with epifluorescence using x1000, x1500 and x2000 magnification. The metaphase plates and chromosome images were edited using Microsoft Office Picture Manager and chromosome number was counted. For the study of karyotype variation, 10 metaphase plates were screened per provenance.

2.3 RESULTS

2.3.1 ITS region PCR amplification products

The ITS primers amplified a single product of about 800 bp for DNA from all provenances throughout Australia, except from Bridgestone from Western Australia. Replicates 2 and 3 from Bridgestone (WA) amplified a slightly smaller product of about 790 bp (Fig. 2.3, gel 4). The ITS-PCR was repeated for these samples but always showed the same result.

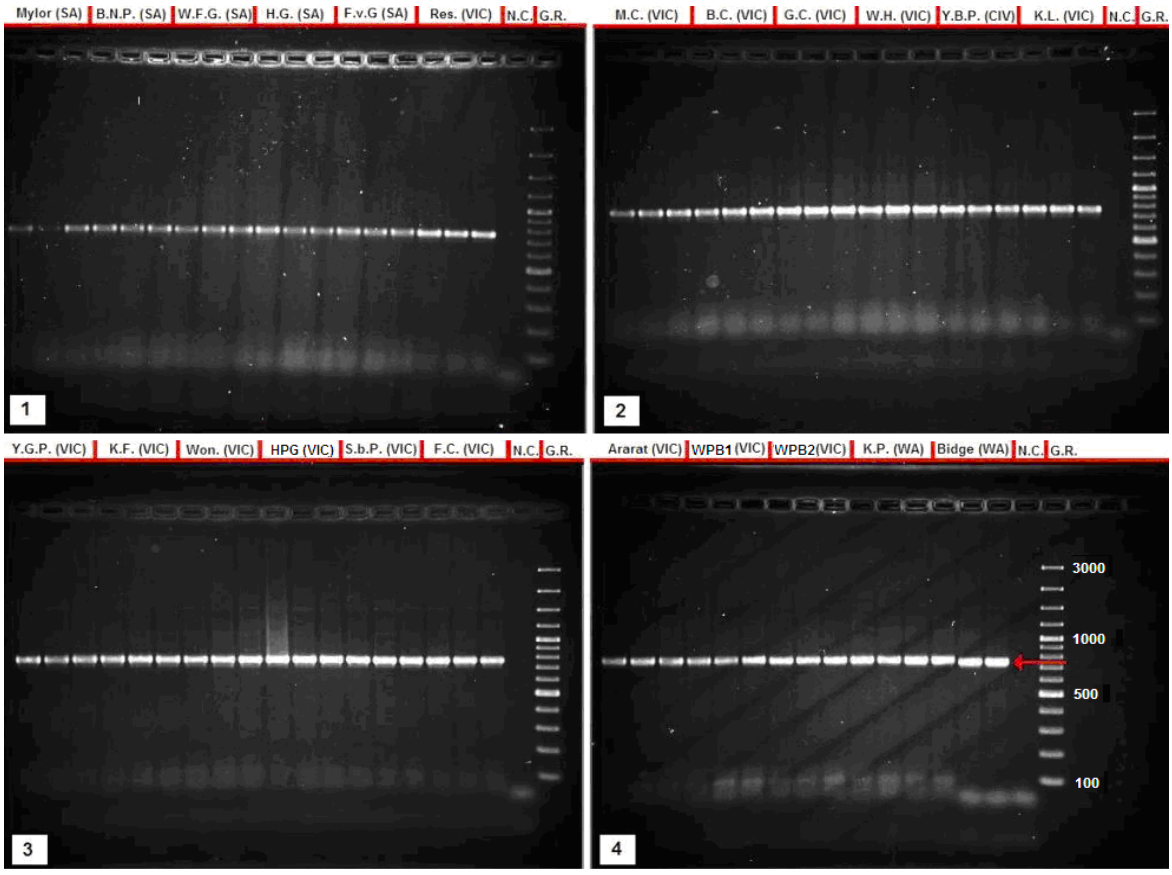

Figure 2.3 Amplification of the of *Allium triquetrum* samples from across Australia using ITS1 and ITS4 primers. The red arrow indicates 790 bp products of Bridgestone replicates 2 and 3. Lanes are (Left to Right), **Gel 1**: 1-3 Mylor (SA), 4-6 Belair National Park (SA), 7-9 Waterfall Gully (SA), 10-12 Horsnell Gully (SA), 13-15 Fairview (SA), 16-18 Plenty Road, Reservoir (VIC), 19 Negative control (N.C.), 20 GeneRuler (G.R.). **Gel 2**: 1-3 Merri Creek (VIC), 4-6 Bendigo Creek (VIC), 7-9 Gardiner's Creek (VIC), 10-12 White Hill (VIC), 13-15 Yarra Bend Park (VIC), 16-18 Kinglake National Park (VIC), 19 Negative control (N.C.), 20 GeneRuler (G.R.). **Gel 3**: 1-3 Yellow Gum Park (VIC), 4-6 Kangaroo Flat (VIC), 7-9 Wonthaggi (VIC), 10-12 Hardy's Picnic Ground, Dandenongs (VIC), 13-15 Sherbrooke Picnic Ground, Dandenongs (VIC), 16-18 Ferny Creek Area, Dandenongs (VIC), 19 Negative control (N.C.), 20 GeneRuler (G.R.). **Gel 4**: 1-3 Ararat (VIC), 4-6 Westernport Bay 1 (VIC), 7-9 Westernport Bay 2 (VIC), 10-12 Kings Park (WA), 13-15 Bridgestone (WA), 16 Negative control (N.C.), 17 GeneRuler (G.R.).

2.3.2 ITS-RFLP analysis

Provenances demonstrated almost identical digestion products with all enzymes (Table 2.3, Fig. 2.4-2.7). Some exceptions were noted as follows. With *Taq*I (Fig. 2.4), the 270 bp product was absent in all samples from Ferny Creek (VIC), Westernport Bay 2 (VIC), Plenty Road, Reservoir (VIC) and replicates 2 and 3 from Bridgestone (WA) (Fig. 2.4). For the Bridgestone (WA) replicates 2 and 3 the digested products of 260 and 240 bps was observed instead of 270 and 250 bp. With *Rsa*I (Fig. 2.5), some partly undigested 800 bp products were observed in samples from Westernport Bay 1 and 2 and Wonthaggi.

With *Xho*I (Fig. 2.6), the 650 bp fragment was absent in all Westernport Bay 2 samples and replicates 2 and 3 from Bridgestone (WA); Bridgestone replicates 2 and 3 had slightly smaller products of 640, 590, and 160 bp. Some partially undigested products of about 800 bp were observed in samples from all provenances except for Bridgestone samples 2 and 3. Products of 400 and 350 bp were also observed in Westernport Bay 1 replicates 1-2 and Westernport Bay 2 replicate 2.

With *Hha*I (Fig. 2.7), samples from Westernport Bay 2 (VIC), Ferny Creek (VIC), Sherbrooke Picnic Ground (VIC) and Bridgestone (WA) differed from other samples. The 290 bp fragment was absent from Sherbrooke Picnic Ground sample 2, Ferny Creek samples 1 and 3, Westernport Bay 2 samples 1 and 3 and Bridgestone samples 2 and 3; however, fragments of 280, 180 and 140 bps were observed in Bridgestone samples 2 and 3.

Table 2.3 RFLP analysis of approximately 800 bp ITS-PCR products of *Allium triquetrum* digested by six restriction enzymes.

Restriction enzyme	**Digestion**	**Digestion products sizes (bp)**
*Taq*I	+	270, 260, 250, 240, 180,100
*BamH*I	-	
*Hha*I/ *Cfo*I	+	400, 290, 280, 200, 190, 170, 150,140,80
*EcoR*I	-	
*Eha*I	+	250, 200, 170, 150
*Msp*I	+	600, 500, 350
*Rsa*I	+	500, 290, 190
*Xho*I	+	650, 640, 600, 590, 500, 400, 350, 160
*Not*I	-	

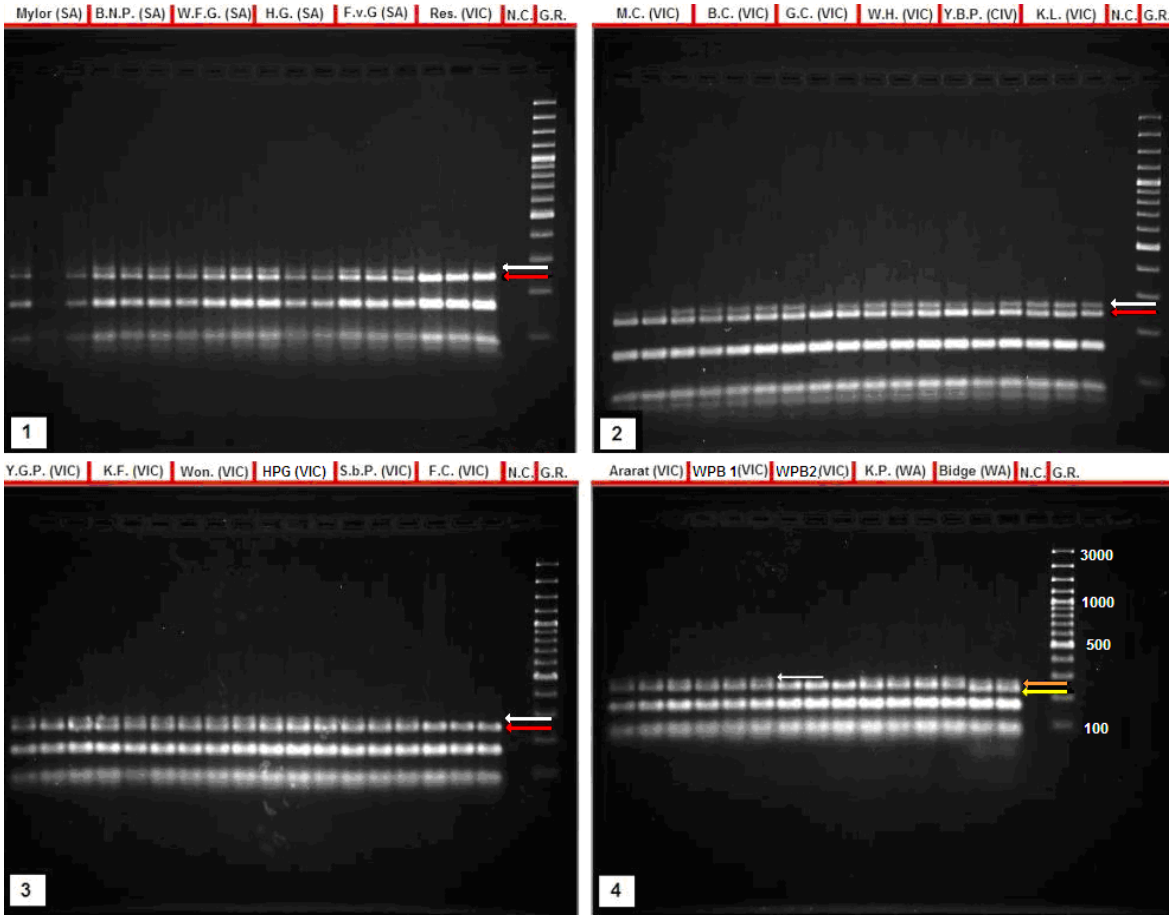

Figure 2.4 Digestion of the ITS products using *Taq*I. The white arrows demonstrate 270 bp, the yellow arrow indicates 260 bp, the red arrows indicate 250 bp, and the orange arrow shows 240 bp products. Lanes are (Left to Right), **Gel 1**: 1-3 Mylor (SA), 4-6 Belair National Park (SA), 7-9 Waterfall Gully (SA), 10-12 Horsnell Gully (SA), 13-15 Fairview (SA), 16-18 Plenty Road, Reservoir (VIC), 19 Negative control (N.C.), 20 GeneRuler (G.R.). **Gel 2**: 1-3 Merri Creek (VIC), 4-6 Bendigo Creek (VIC), 7-9 Gardiner's Creek (VIC), 10-12 White Hill (VIC), 13-15 Yarra Bend Park (VIC), 16-18 Kinglake National Park (VIC), 19 Negative control (N.C.), 20 GeneRuler (G.R.). **Gel 3**: 1-3 Yellow Gum Park (VIC), 4-6 Kangaroo Flat (VIC), 7-9 Wonthaggi (VIC), 10-12 Hardy's Picnic Ground, Dandenongs (VIC), 13-15 Sherbrook Picnic Ground, Dandenongs (VIC), 16-18 Ferny Creek Area, Dandenongs (VIC), 19 Negative control (N.C.), 20 GeneRuler (G.R.). **Gel 4**: 1-3 Ararat (VIC), 4-6 Westernport Bay 1 (VIC), 7-9 Westernport Bay 2 (VIC), 10-12 Kings Park (WA), 13-15 Bridgestone (WA), 16 Negative control (N.C.), 17 GeneRuler (G.R.).

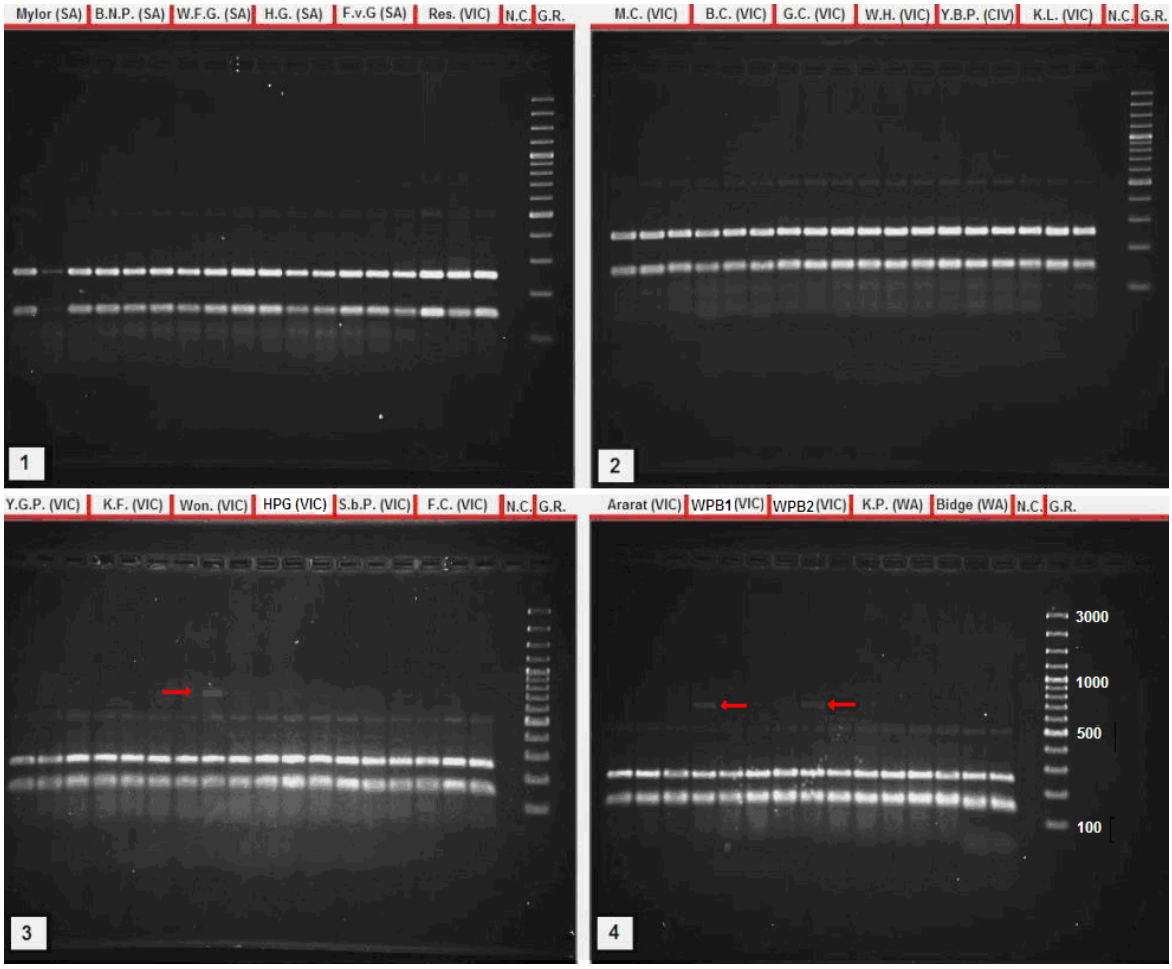

Figure 2.5 Digestion of ITS products using the *Rsa*I. Red arrows indicate 800 bp undigested products. Lanes are (Left to Right), **Gel 1**: 1-3 Mylor (SA), 4-6 Belair National Park (SA), 7-9 Waterfall Gully (SA), 10-12 Horsnell Gully (SA), 13-15 Fairview (SA), 16-18 Plenty Road Reservoir (VIC), 19 Negative control (N.C.), 20 GeneRuler (G.R.). **Gel 2**: 1-3 Merri Creek (VIC), 4-6 Bendigo Creek (VIC), 7-9 Gardiner's Creek (VIC), 10-12 White Hill (VIC), 13-15 Yarra Bend Park (VIC), 16-18 Kinglake National Park (VIC), 19 Negative control (N.C.), 20 GeneRuler (G.R.). **Gel 3**: 1-3 Yellow Gum Park (VIC), 4-6 Kangaroo Flat (VIC), 7-9 Wonthaggi (VIC), 10-12 Hardy's Picnic Ground, Dandenongs (VIC), 13-15 Sherbrooke Picnic Ground, Dandenongs (VIC), 16-18 Ferny Creek Area, Dandenongs (VIC), 19 Negative control (N.C.), 20 GeneRuler (G.R.). **Gel 4**: 1-3 Ararat (VIC), 4-6 Westernport Bay 1 (VIC), 7-9 Westernport Bay 2 (VIC), 10-12 Kings Park (WA), 13-15 Bridgestone (WA), 16 Negative control (N.C.), 17 GeneRuler (G.R.).

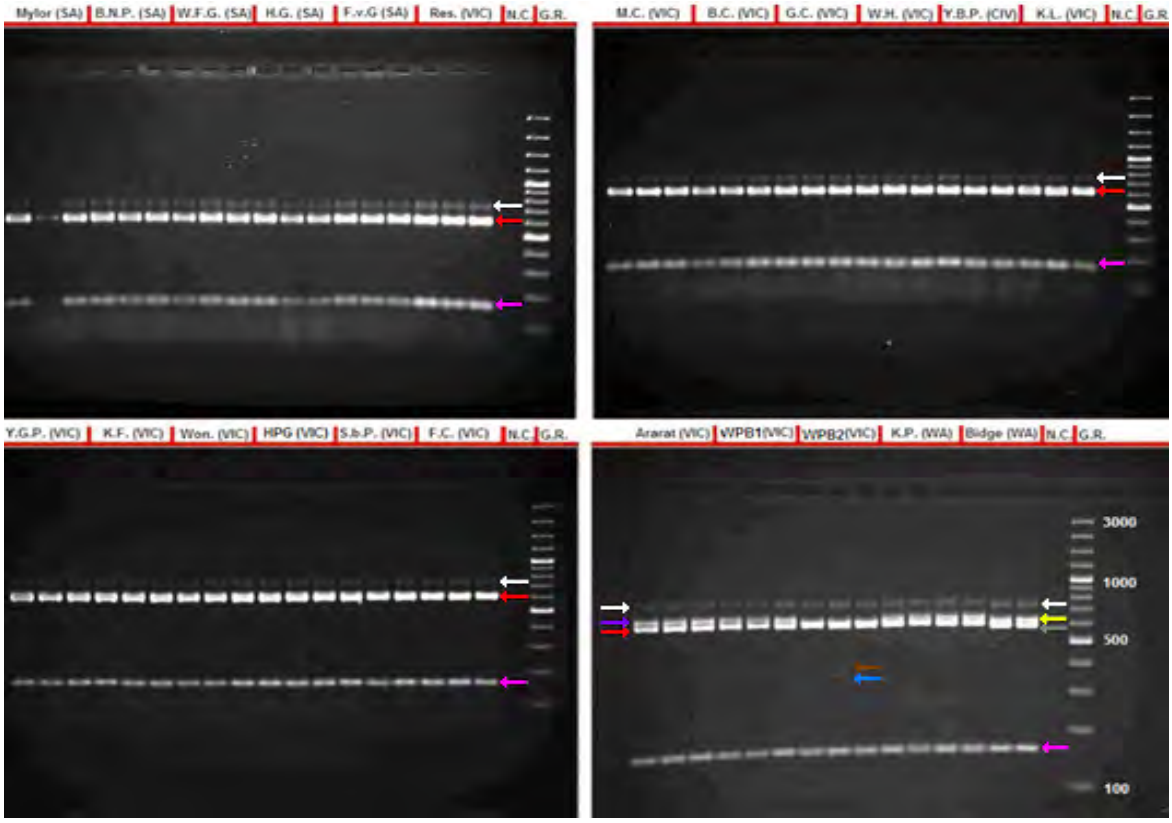

Figure 2.6 Digestion of ITS products using *Xho*I. White arrows indicate ~800 bp undigested prodcts, purple arrows 650 bp, yellow arrows 640 bp, red arrows 600 bp, gray arrows 590, brown arrows 400 bp, blue arrows 350 and pink arrows 160 bp. Lanes are (Left to Right), **Gel 1**: 1-3 Mylor (SA), 4-6 Belair National Park (SA), 7-9 Waterfall Gully (SA), 10-12 Horsnell Gully (SA), 13-15 Fairview (SA), 16-18 Plenty Road Reservoir (VIC), 19 Negative control (N.C.), 20 GeneRuler (G.R.). **Gel 2**: 1-3 Merri Creek (VIC), 4-6 Bendigo Creek (VIC), 7-9 Gardiner's Creek (VIC), 10-12 White Hill (VIC), 13-15 Yarra Bend Park (VIC), 16-18 Kinglake National Park (VIC), 19 Negative control (N.C.), 20 GeneRuler (G.R.). **Gel 3**: 1-3 Yellow Gum Park (VIC), 4-6 Kangaroo Flat (VIC), 7-9 Wonthaggi (VIC), 10-12 Hardy's Picnic Ground, Dandenongs (VIC), 13-15 Sherbrooke Picnic Ground, Dandenongs (VIC), 16-18 Ferny Creek Area, Dandenongs (VIC), 19 Negative control (N.C.), 20 GeneRuler (G.R.). **Gel 4**: 1-3 Ararat (VIC), 4-6 Westernport Bay 1 (VIC), 7-9 Westernport Bay 2 (VIC), 10-12 Kings Park (WA), 13-15 Bridgestone (WA), 16 Negative control (N.C.), 17 GeneRuler (G.R.).

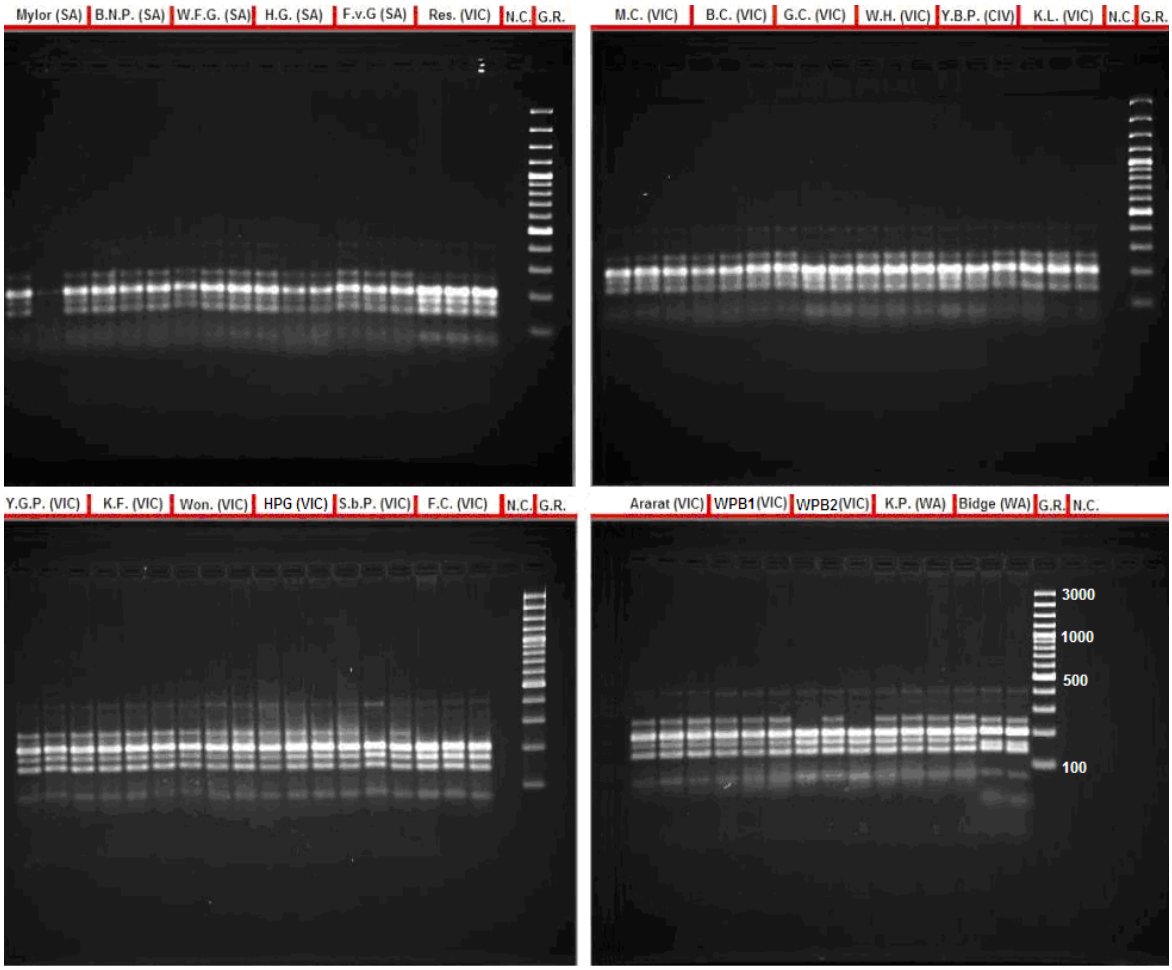

Figure 2.7 Digestion of ITS products using *Hha*I. Lanes are (Left to Right), **Gel 1**: 1-3 Maylor (SA), 4-6 Belair National Park (SA), 7-9 Waterfall Gully (SA), 10-12 Horsnell Gully (SA), 13-15 Fairview (SA), 16-18 Plenty Road, Reservoir (VIC), 19 Negative control (N.C.), 20 GeneRuler (G.R.). **Gel 2**: 1-3 Merri Creek (VIC), 4-6 Bendigo Creek (VIC), 7-9 Gardiner's Creek (VIC), 10-12 White Hill (VIC), 13-15 Yarra Bend Park (VIC), 16-18 Kinglake National Park (VIC), 19 Negative control (N.C.), 20 GeneRuler (G.R.). **Gel 3**: 1-3 Yellow Gum Park (VIC), 4-6 Kangaroo Flat (VIC), 7-9 Wonthaggi (VIC), 10-12 Hardy's Picnic Ground, Dandenongs (VIC), 13-15 Sherbrooke Picnic Ground, Dandenongs (VIC), 16-18 Ferny Creek Area, Dandenongs (VIC), 19 Negative control (N.C.), 20 GeneRuler (G.R.). **Gel 4**: 1-3 Ararat (VIC), 4-6 Westernport Bay 1 (VIC), 7-9 Westernport Bay 2 (VIC), 10-12 Kings Park (WA), 13-15 Bridgestone (WA), 16 Negative control (N.C.), 17 GeneRuler (G.R.).

2.3.3 Sequence analysis

Sequences of 717-1128 bases were obtained from sequencing with ITS4. After deletion of the start and end of each sequence after Clustal W alignment, the resulting 718 bp sequences showed small nucleotide variations among provenances (Fig. 2.8); differences were observed between the provenances, and within the Sherbrooke provenance.

The distance matrix indicated that values different provenances ranged from (0-0.3511) (Table 2.4). The most different sequences were from Bridgestone sample 2. Mylor 1 (SA) and Kings Park1 (WA) were more closely similar to each other than to the rest of the samples. The greatest distances were observed with the outgroup, *A. cepa*.

Phylogenetic trees obtained with UPGMA and Neighbour-Joining analysis in MEGA4 indicated two main clusters (Fig. 2.9, 2.10). With UPGAMA all *A. triquetrum* sequences were clustered together and separated from the outgroup sequence for *A. cepa* (Fig 2.9, 2.10). Only the Sherbrooke sequences 2 had 69% bootstrap values from the remaining sequences (Fig. 2.9). With the Neighbour-Joining method, sequences from Sherbrooke samples 2 and 3 were separated as a separate clade with bootstrap value of 73% (Fig. 2.10) and Mylor 1 (SA) and Kings Park1 (WA) were separated to a clade with bootstrap value of 99%. Bridgestone and Westernport Bay samples each were separated as a separate clade with bootstrap values of 55% and 51% respectively.

```
        1         11        21        31        41        51
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FC1     CCGCCTGAACTGAGGTCATGGTCCGATGTTGCAAAATGTGCAAGCATCGTTATGGGTATT
WPB2.2  CCGCCTGA-CTGAGGTCATGGTCCGATGTTGCAAAATGTGCAAGCATCGTTATGGGTATT
Sb2     TCGCCTGA-CGGAGGTCATGGTCCGATGTTGCAAAATGTGCAAGCATCGTTATGGGTATT
Sb3     TGTCTCGA-GTGAGGTCATGGTCCGATGTTGCAAAATGTGCAAGCATCGTTATGGGTATT
Sb1     CCGCCTGA-CTGAGGTCATGGTCCGATGTTGCAAAATGTGCAAGCATCGTTATGGGTATT
WPB2.1  CCGCTGAC-CTGAGGTCATGGTCCGATGTTGCAAAATGTGCAAGCATCGTTATGGGTATT
WPB2.3  CCGCCTGA-CTGAGGTCATGGTCCGATGTTGCAAAATGTGCAAGCATCGTTATGGGTATT
BS1     TCCGGTGACTTGAGGTCATGGTCCGATGTTGCAAAATGTGCAAGCATCGTTATGGGTATT
BS3     CCGCCTGACT-GAGGTCATGGTCCGATGTTGCAAAATGTGCAAGCATCGTTATGGGTATT
Myl     CCGCCTGACT-GAGGTCATGGTCCGATGTTGCAAAATGTGCAAGCATCGTTATGGGTATT
KP1     CCGTCTGACT-GAGGTC-TGGTCCGATGTTGCAAAATGTGCAAGCATCGTTATGGGTATT
BS2     CCGACTGACT-GAGGTCATGGTCCGATGTTGCAAAATGTGCAAGCATCGTTATGGGTATT
A.cepa  CCACCTGATCTGAGGTCATGTTCCGAGCTTGCAACTAAGGCAAACGTCGAAAATGGTTTC

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FC1     ACTTCCCGTAAGC-CTTTGCAAGGAGTCAGGTACGCAGCTGGAGGCTTTATGCATGCGCA
WPB2.2  ACTTCCCGTAAGC-CTTTGCAAGGAGTCAGGTACGCAGCTGGAGGCTTTATGCATGCGCA
Sb2     ACTTCCCGCAAGC-CTTTGCAAGGAGTCAGGTACGCAGCTGGAGGCTTTATGCATGCGCA
Sb3     ACTTCCCGCAAGC-CTTTGCAAGGAGTCAGGTACGCAGCTGGAGGCTTTATGCATGCGCA
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WPB2.1  ACTTCCCGTAAGC-CTTTGCAAGGAGTCAGGTACGCAGCTGGAGGCTTTATGCATGCGCA
WPB2.3  ACTTCCCGTAAGC-CTTTGCAAGGAGTCAGGTACGCAGCTGGAGGCTTTATGCATGCGCA
BS1     ACTTCCCGTAAGC-CTTTGCAAGGAGTCAGGTACGCAGCTGGAGGCTTTATGCATGCGCA
BS3     ACTTCCCGTAAGC-CTTTGCAAGGAGTCAGGTACGCAGCTGGAGGCTTTATGCATGCGCA
Myl     ACTTCCCGTAAGC-CTTTGCAAGGAGTCAGGTACGCAGCTGGAGGCTTTATGCATGCGCA
KP1     ACTTCCCGTAAGC-CTTTGCAAGGAGTCAGGTACGCAGCTGGAGGCTTTATGCATGCGCA
BS2     ACTTCCCGTAAGC-CTTTGCAAGGAGTCAGGTACGCAGCTGGAGGCTTTATGCATGCGCA
A.cepa  AGTTATTGTTACATCGTTCGTAGGACTCCTGGACGCAGTTAGAGACAT----CGTGTGTT

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FC1     TAACATATCCACCATTCGCCGCACGCAAAACCTAGCAACGGCCATCACTCAAACCCGCCG
WPB2.2  TAACATATCCACCATTCGCCGCACGCAAAACCTAGCAACGGCCATCACTCAAACCCGCCG
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WPB2.1  TAACATATCCACCATTCGCCGCACGCAAAACCTAGCAACGGCCATCACTCAAACCCGCCG
WPB2.3  TAACATATCCACCATTCGCCGCACGCAAAACCTAGCAACGGCCATCACTCAAACCCGCCG
BS1     TAACATATCCACCATTCGCCGCACGCAAAACCTAGCAACGGCCATCACTCAAACCCGCCG
BS3     TAACATATCCACCATTCGCCGCACGCAAAACCTAGCAACGGCCATCACTCAAACCCGCCG
Myl     TAACATATCCACCATTCGCCGCACGCAAAACCTAGCAACGGCCATCACTCAAACCCGCCG
KP1     TAACATATCCACCATTCGCCGCACGCAAAACCTAGCAACGGCCATCACTCAAACCCGCCG
BS2     TAACATATCCACCATTCGCCGCACGCAAAACCTAGCAACGGCCATCACTCAAACCCGCCG
A.cepa  AACTCGATACACCATTCGCCGCGCGTAGA-CCTAACGACAACATTCACTTAAACCAACCG
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FC1     CGCATCGAAAGCACGAACGGTCAATATCCACATCCGCACACACCGGTACCCACATAGCGG
WPB2.2  CGCATCGAAAGCACGAAGGGTCAATATCCACATCCGCACACACCGGTACCCACATAGCGG
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Sb1     CGCATCGAAAGCACGAAGGGTCAATATCCACATCCGCACACACCGGTACCCACATAGCGG
WPB2.1  CGCATCGAAAGCACGAAGGGTCAATATCCACATCCGCACACACCGGTACCCACATAGCGG
WPB2.3  CGCATCGAAAGCACGAAGGGTCAATATCCACATCCGCACACACCGGTACCCACATAGCGG
BS1     CGCATCGAAAGCACGAAGGGTCAATATCCACATCCGCACACACCGGTACCCACATAGCGG
BS3     CGCATCGAAAGCACGAAGGGTCAATATCCACATCCGCACACACCGGTACCCACATAGCGG
Myl     CGCATCGAAAGCACGAAGGGTCAATATCCACATCCGCACACACCGGTACCCACATAGCGG
KP1     CGCATCGAAAGCACGAAGGGTCAATATCCACATCCGCACACACCGGTACCCACATAGCGG
BS2     CGCATCGAAAGCACGAAGGGTCAATATCCACATCCGCACACACCGGTACCCACATAGCGG
A.cepa  CACAATTAAGGCACGGAGGGTCAATCTCCACATCCGC---CATC---ACTCACAGTATGT

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FC1     TTTAAGTTGGGCAGTTGTTTGGAGTGACGCAAGGCGTGACGCCCAAGCAGACGTGCCCTT
WPB2.2  TTTAAGTTGGGCAGTTGTTTGGAGTGACGCAAGGCGTGACGCCCAAGCAGACGTGCCCTT
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Myl     TTTAAGTTGGGCAGTTGTTTGGAGTGACGCAAGGCGTGACGCCCAAGCAGACGTGCCCTT
KP1     TTTAAGTTGGGCAGTTGTTTGGAGTGACGCAAGGCGTGACGCCCAAGCAGACGTGCCCTT
BS2     TTTAAGTTGGGCAGTTGTTTGGAGTGACGCAAGGCGTGACGCCCAAGCAGACGTGCCCTT
A.cepa  TTACAGTAGGTG-GATGGTTAGAATGACGCAAGGCATGACGCCCAAACAGACGTGCTCTC

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FC1     GACCTGATGGTCTAAGGCGCAACTTGCATTCAAAGACTCGATGATTCACGGGATTCTGCA
WPB2.2  GACCTGATGGTCTAAGGCGCAACTTGCATTCAAAGACTCGATGATTCACGGGATTCTGCA
Sb2     GACCTGATGGTCTAAGGCGCAACTTGCATTCAAAGACTCGATGATTCACGGGATTCTGCA
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Myl     GACCTGATGGTCTAAGGCGCAACTTGCATTCAAAGACTCGATGATTCACGGGATTCTGCA
KP1     GACCTGATGGTCTAAGGCGCAACTTGCATTCAAAGACTCGATGATTCACGGGATTCTGCA
BS2     GACCTGATGGGCTAAGGCGCAACTTGCATTCAAAGACTCGATGATTCACGGGATTCTGCA
A.cepa  AACCTAATGGCCTCGAGCGCAACTTGCATTCAAAGACTCGATGGTTCACGGGATTCTGCA
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FC1     ATTCACACCAAGTATCGCATTTTGCTACGTTCTTCATCGACGCGAGAGCCTAGATATCCG
WPB2.2  ATTCACACCAAGTATCGCATTTTGCTACGTTCTTCATCGACGCGAGAGCCTAGATATCCG
Sb2     ATTCACACCAAGTATCGCATTTTGCTACGTTCTTCATCGACGCGAGAGCCTAGATATCCG
Sb3     ATTCACACCAAGTATCGCATTTTGCTACGTTCTTCATCGACGCGAGAGCCTAGATATCCG
Sb1     ATTCACACCAAGTATCGCATTTTGCTACGTTCTTCATCGACGCGAGAGCCTAGATATCCG
WPB2.1  ATTCACACCAAGTATCGCATTTTGCTACGTTCTTCATCGACGCGAGAGCCTAGATATCCG
WPB2.3  ATTCACACCAAGTATCGCATTTTGCTACGTTCTTCATCGACGCGAGAGCCTAGATATCCG
BS1     ATTCACACCAAGTATCGGATTTTGCTACGTTCTTCATCGACGCGAGAGCCTAGATATCCG
BS3     ATTCACACCAAGTATCGCATTTTGCTACGTTCTTCATCGACGCGAGAGCCTAGATATCCG
Myl     ATTCACACCAAGTATCGCATTTTGCTACGTTCTTCATCGACGCGAGAGCCTAGATATCCG
KP1     ATTCACACCAAGTATCGCATTTTGCTACGTTCTTCATCGACGCGAGAGCCTAGATATCCG
BS2     ATTCACACCAAGTATCGCATTTTGCTACGTTCTTCATCGACGCGAGAGCCTAGATATCCG
A.cepa  ATTCACACCAAGTGTCGCATTTCGCTACGTTCTTCATCGACACGAGAGCCAAGATATCCA

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FC1     TTGCCAAGAGTCATGCATATTCTCAAACCTAGTAAGAGGATTACACCGTACCAGAT-AAA
WPB2.2  TTGCCAAGAGTCATGCATATTCTCAAACCTAGTAAGAGGATTACACCGTACCAGAT-AAA
Sb2     TTGCCAAGAGTCATGCATATTCTCAAACCTAGTAAGAGGATTACACCGGACCAGAT-AAA
Sb3     TTGCCAAGAGTCATGCATATTCTCAAACCTAGTAAGAGGATTACACCGGGGGGGAG-AAA
Sb1     TTGCCAAGAGTCATGCATATTCTCAAACCTAGTAAGAGGATTACACCGTACCAGAT-AAA
WPB2.1  TTGCCAAGAGTCATGCATATTCTCAAACCTAGTAAGAGGATTACACCGTACCAGAT-AAA
WPB2.3  TTGCCAAGAGTCATGCATATTCTCAAACCTAGTAAGAGGATTACACCGTACCAGAT-AAA
BS1     TTGCCAAGAGTCATGCATATTCTCAAACCTAGTAAGAGGATTACACCGAACCAGAT-AAA
BS3     TTGCCAAGAGTCATGCATATTCTCAAACCTAGTAAGAGGATTACACCGAACCAGAT-AAA
Myl     TTGCCAAGAGTCATGCATATTCTCAAACCTAGTAAGAGGATTACACCGTACCAGAT-AAA
KP1     TTGCCAAGAGTCATGCATATTCTCAAACCTAGTAAGAGGATTACACCGTACCAGAT-AAA
BS2     TTGCCAAGAGTCATGCATATTCTCAAACCTAGTAAGAGGATTACACCGAACCAGAT-AAA
A.cepa  TTGCCAGGAGTCATTCAGACGCTCA--C-T-------GGAATAACACGAAGCACATCAAA

        481       491       501       511       521       531
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FC1     GCAACGGGGG-CACTCCGACACCAACCATCCTTGGCGCAGACAACGCCGGGGTCTTATTC
WPB2.2  GCAACGGTG--CACTCCGACACCAACCATCCTTGGCGCAGACAACGCCGGGGTCTTATTC
Sb2     GCAACGGTG--CACTCCGACACCAACCATCCTTGGCGCAGACAACGCCGGGGTCTTATTC
Sb3     GCAACGGTG--CACTCCGACACCAACCATCCTTGGCGCAGACAACGCCGGGGTCTTATTC
Sb1     GCAACGGTG--CACTCCGACACCAACCATCCTTGGCGCAGACAACGCCGGGGTCTTATTC
WPB2.1  GCAACGGTG--CACTCCGACACCAACCATCCTTGGCGCAGACAACGCCGGGGTCTTATTC
WPB2.3  GCAACGGTG--CACTCCGACACCAACCATCCTTGGCGCAGACAACGCCGGGGTCTTATTC
BS1     GCAACGGTG--CACTCCGACACCAACCATCCTTGGCGCAGACAACGCCGGGGTCTTATTC
BS3     GCAACGGTG--GACTCCGACACCAACCATCCTTGGCGCAGACAACGCCGGGGTCTTATTC
Myl     GCAACGGTG--CACTCCGACACCAACCATCCTTGGCGCAGACAACGCCGGGGTCTTATTC
KP1     GCAACGGTG--CACTCCGACACCAACCATCCTTGGCGCAGACAACGCCGGGGTCTTATTC
BS2     GCAACGGTG--CACTCCGACACCAACCATCCTTGGCGCAGACAACGCCGGGGTCTTATTC
A.cepa  ATGATGGCAAGCTCTCCAACAACAACTGTCCTTGGCACAAACCGTGCCGGTTTCTTAGTT
```

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        541       551       561       571       581       591
YBP1    CCGTTCTCCTGCTTTGCCTCGAGTATCGCAAGAA-GAATGCGGCAAAAGGACATCCATCT
FC1     CCGTTCTCCTGCTTTGCCTCGAGTATCGCAAGAA-GAATGCGGCAAAAGGACATCCATCT
WPB2.2  CCGTTCTCCTGCTTTGCCTCGAGTATCGCAAGAA-GAATGCGGCAAAAGGACATCCATCT
Sb2     CCGTTCTCCTGCTTTGCCTCGAGTATCGCAAGAA-GAATGCGGCAAAAGGACATCCATCT
Sb3     CCGTTCTCCTGCTTTGCCTCGAGTATCGCAAGAA-GAATGCGGCAAAAGGACATCCATCT
Sb1     CCGTTCTCCTGCTTTGCCTCGAGTATCGCAAGAA-GAATGCGGCAAAAGGACATCCATCT
WPB2.1  CCGTTCTCCTGCTTTGCCTCGAGTATCGCAAGAA-GAATGCGGCAAAAGGACATCCATCT
WPB2.3  CCGTTCTCCTGCTTTGCCTCGAGTATCGCAAGAA-GAATGCGGCAAAAGGACATCCATCT
BS1     CCGTTCTCCTGCTTTGCCTCGAGTATCGCAAGAA-GAATGCGGCAAAAGGACATCCATCT
BS3     CCGTTCTCCTGCTTTGCCTCGAGTATCGCAAGAA-GAATGCGGCAAAAGGACATCCATCT
Myl     CCGTTCTCCTGCTTTGCCTCGAGTATCGCAAGAA-GAATGCGGCAAAAGGACATCCATCT
KP1     CCGTTCTCCTGCTTTGCCTCGAGTATCGCAAGAA-GAATGCGGCAAAAGGACATCCATCT
BS2     CCGTTCTCCTGCTTTGCCTCGAGTATCGCAAGAA-GAATGCGGCAAAAGGACATCCATCT
A.cepa  CTACTCTACATCTTA-CTTCAATTGAAGCAAACATGAAGGCAGCAAATG-AAACCCATCC

        601       611       621       631       641       651
YBP1    ATAGAGCAGAATTGC-GGTTGCAAAACTAACTCCTCCTCGACGGGTGTAATGCTACAAGT
FC1     ATAGAGCAGAATTGC-AGTTGCAAAACTAACTCCTCCTCGACGGGTGTAATGCTACAAGT
WPB2.2  ATAGAGCAGAATTGC-GGTTGCAAAACTAACTCCTCCTCGACGGGTGTAATGCTACAAGT
Sb2     ATAGAGCAGAATTGC-AGTTGCAAAACTAACTCCTCCTCGACGGGTGTAATGCTACAAGT
Sb3     ATAGAGCAGAATTGC-AGTTGCAAAACTAACTCCTCCTCGACGGGTGTAATGCTACAAGT
Sb1     ATAGAGCAGAATTGC-AGTTGCAAAACTAACTCCTCCTCGACGGGTGTAATGCTACAAGT
WPB2.1  ATAGAGCAGAATTGC-GGTTGCAAAACTAACTCCTCCTCGACGGGTGTAATGCTACAAGT
WPB2.3  ATAGAGCAGAATTGC-GGTTGCAAAACTAACTCCTCCTCGACGGGTGTAATGCTACAAGT
BS1     ATAGAGCAGAGTTGC-AGTTGCAAAACTAACTCCTCCTCGACGGGGGTAATGCTACAAGT
BS3     ATAGAGCAGAGTTGC-AGTTGCAAAACTAACTCCTCCTCGACGGGTGTAATGCTACAAGT
Myl     ATAGAGCAGAGTTGC-AGTTGCAAAACTAACTCCTCCTCGACGGGTGTAATGCTACAAGT
KP1     ATAGAGCAGAGTTGC-AGTTGCAAAACTAACTCCTCCTCGACGGGTGTAATGCTACAAGT
BS2     ATAGAGCAGAGTTGC-AGTTGCAAAACTAACTCCTCCTCGACGGGTGTAATGCTACAAGT
A.cepa  AAACAACGCAAGTGCTAACCGCACAAATACGTAGTTCTCGACGGGTATGAG--TACAATT

        661       671       681       691       701       711
YBP1    TCTCAATCTCTTGAAGAGGGACTTGACAATGATCCTTCCGCAGGTT-CACCTTACGGAA
FC1     TCTCAATCTCTTGAAGAGGGACTTGACAATGATCCTTCCGCAGGTT-CACCT-ACGGAA
WPB2.2  TCTCAATCTCTTGAAGAGGGACTTGACAATGATCCTTCCGCAGGCCACCCCTTACGGAA
Sb2     TCTCAATCTCTTGAAGAGGGACTTGACAATGATCCTTCCGCAGGTT-CACCCTACGGAA
Sb3     TCTCAATCTCTTGAAGAGGGACTTGACAATGATCCTTCCGCAGGTT-CACCCTACGGAA
Sb1     TCTCAATCTCTTGAAGAGGGACTTGACAATGATCCTTCCGCAGGTT-ACCCTTACGGAA
WPB2.1  TCTCAATCTCTTGAAGAGGGACTTGACAATGATCCTTCCGCAGGTC-ACCCCTACGGAA
WPB2.3  TCTCAATCTCTTGAAGAGGGACTTGACAATGATCCTTCCGCAGGCACACCTCTACGGAA
BS1     TCTCAATCTCTTGAAGAGGGACTTGACAATGATCCTTCCTCAGGTT-CCCCAAACGGAA
BS3     TCTCAATCTCTTGAAGAGGGACTTGACAATGATCCTTCCTCAGGTT-CCCCAAACGGAA
Myl     TCTCAATCTCTTGAAGAGGGACTTGACAATGATCCTTCCGAGGGCC-CCC-AAAAGGGA
KP1     TCTCAATCTCTTGAAGAGGGACTTGACAATGATCCTTCCGAGGGCC-CCC-AAAAGGAA
BS2     TCTCAATCTCTTGAAGAGGGACTTGACAATGATCCTTCCGCAGGTT-CAC-ATACGGAA
A.cepa  TCACAGTTGTTCGAGAAGGAACTCTACAATGATCCTTCCGCAGGTT-CAC-CTACGGAA
```

Figure 2.8 Clustal W sequence alignment of the ITS sequences of *Allium triquetrum* from across Australia. Sequences were edited after alignment by deletion of 3' start and 5' end of sequences using BioEdit, Biomanager, ANGIS. Provenances are abbreviated as follows:
Victoria: Ferny Creek samples 1 (FC), Yarra Bend Park sample 1 (YBP), Westernport Bay 2 samples 1-3 (WPB) and Sherbrooke Picnic Ground samples 1-3 (Sb), Southern Australia: Mylor sample 1 (My l), Western Australia: Bridgestone (BS) and Kings Park sample 1 (KP1).

Table 2.4 Distance matrix of ITS sequences of *Allium triquetrum* across Australia and the outgroup species, *Allium cepa*. Standard error calculation was based on bootstrap values (%) from 1000 bootstrap replicates generated by the DNAdist program, Biomanager, ANGIS.

Sample	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
1- Yarra Bend Park	0.0													
2- Ferny Creek 1	0.0099	0.0												
3- Westernport Bay 2.2	0.0099	0.0085	0.0											
4- Sherbrooke 2	0.0142	0.01	0.0128	0.0										
5- Sherbrooke 3	0.0273	0.023	0.0258	0.0157	0.0									
6- Sherbrooke 1	0.0099	0.0057	0.0056	0.0099	0.023	0.0								
7- Westernport Bay 2.3	0.0171	0.0157	0.0099	0.0171	0.0259	0.0099	0.0							
8- Bridgestone 1	0.0142	0.0128	0.0071	0.0142	0.0274	0.0071	0.0099	0.0						
9- Bridgestone 3	0.0289	0.0245	0.0245	0.023	0.0319	0.0216	0.0289	0.0274	0.0					
10- Mylor1	0.0201	0.0158	0.0157	0.0157	0.0289	0.0128	0.0215	0.0186	0.0114	0.0				
11- Westernport Bay 2.1	0.023	0.0186	0.0128	0.0201	0.0334	0.0157	0.0215	0.0157	0.023	0.0143	0.0			
12- Kings Park1	0.023	0.0187	0.0128	0.0201	0.0334	0.0157	0.0216	0.0158	0.0216	0.0143	0.0028	0.0		
13- Bridgestone 2	0.0157	0.0129	0.0143	0.0114	0.0245	0.0114	0.0201	0.0157	0.0143	0.0071	0.0157	0.0143	0.0	
14- *Allium Cepa*	0.3511	0.3565	0.3585	0.3534	0.3689	0.3544	0.367	0.3595	0.3754	0.3652	0.377	0.3778	0.3567	0.0

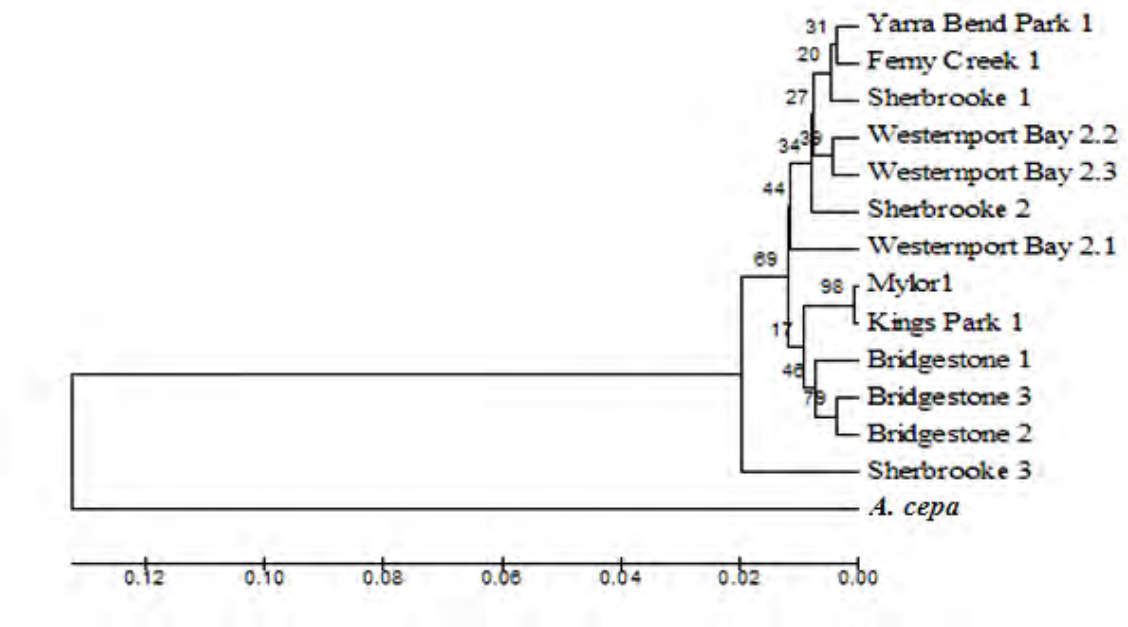

Figure 2.9 Bootstrap consensus phylogenetic tree obtained from UPGMA analysis of ITS sequences of *Allium triquetrum* provenances and outgroup *Allium cepa*. Numbers on branches are bootstrap values (%) from 1000 bootstrap replicates.

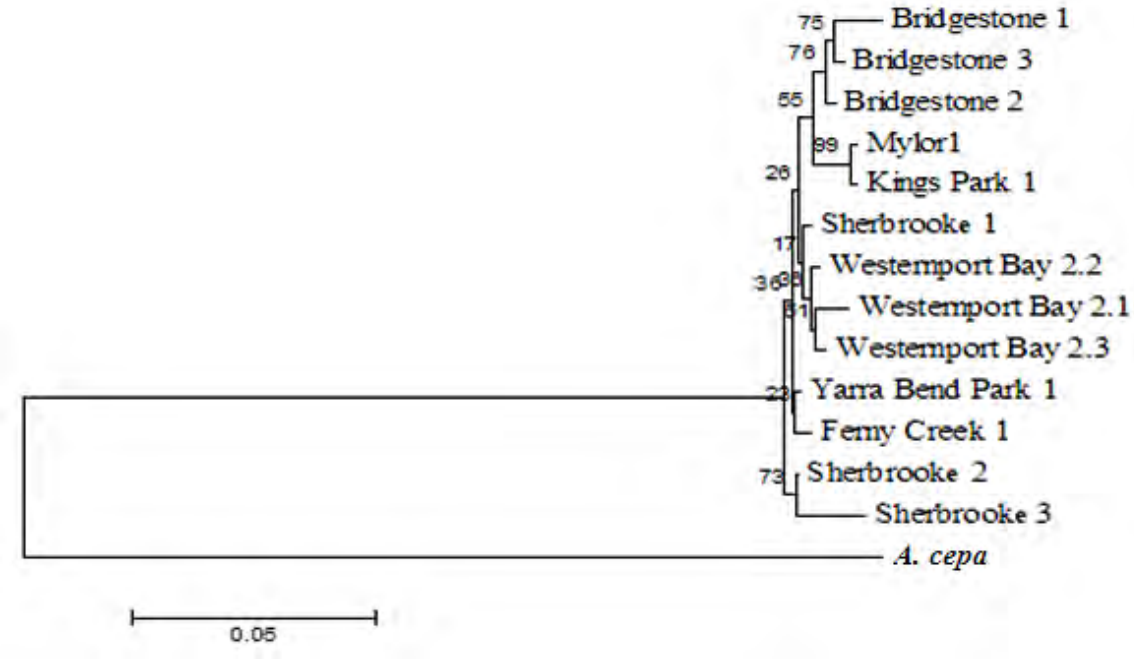

Figure 2.10 Bootstrap consensus phylogenetic tree generated from Neighbour-Joining analysis of ITS sequences of *Allium triquetrum* provenances and outgroup *Allium cepa*. Numbers on branches are bootstrap values (%) from 1000 bootstrap replicates.

2.3.4 RAPD-PCR analysis

Of over sixty 10-mer RAPD primers from Operon kits OPA, OPB and OPM, only 19 primers produced reproducible clear bands for all provenances (Table 2.5). Of these, 7 primers produced strong polymorphic consistent bands. OPB-01 (Fig. 2.11), OPB-04 (Fig. 2.12), OPB-05 (Fig. 2.13), OPM-02 (Fig. 2.14), OPM-05 (Fig. 2.15) and OPM-06 (Fig. 2.16) and OPM-12 (Fig. 2.17) produced a total of 14-20 strong clear bands of 200-3000 bp that showed polymorphism. Polymorphism occurred between provenances, e.g. Westernport Bay 1 and 2 (VIC) (Fig. 2.11), as well as within provenances e.g. Ferny Creek Area (VIC) (Fig. 2.13).

Some primers produced bands unique to few provenances, for instance the OPB-01 primer amplified a faint unique band at 2800 bp for Reservoir 1 and Ferny Creek Area 1, whereas no bands were observed for the other samples at this size (Fig. 2.11). The Mylor replicate 2 amplified some faint products using OPB-01 (Fig. 2.11) and OPB-04 (Fig. 2.12) but there was no reactivity with OPB-05 (Fig. 2.13).

The distance matrix (Table 2.6) and RAPD nearest-neighbour dendrogram (Fig. 2.18) generated by SPSS Hierarchical Cluster analysis divided 69 clusters into three main clades. Differences were observed between and within provenances, though most samples from each provenances clustered together. Clade 1 comprised Ararat and Westernport Bay 2 samples, clade 2 comprised the two Western Australian provenances and Clade 3 comprised the rest of the provenances from Victoria and all provenances from South Australia, all at a distance of 25. Within this, sample 2 from Mylor was separated from all other samples. The rest of the provenances in Victoria separated into two clades, one for Ferny Creek samples and the others, at a distance of 22. The rest of the provenances from Victoria and South Australia separated into Reservoir and others at distance 18 and then the rest of the provenances separated into two clades at a distance 17, one for Victorian

provenances alone and the others for both Victoria and South Australian provenances. A clade at distance of 15 later split Victorian from South Australian samples (Fig. 2.19).

Table 2.5 Polymorphism detected using RAPD primers from Operon kits (OPA-OPB-OPM) among *Allium triquetrum* provenances throughout Australia. + : polymorphism, - : no polymorphism, N/A : no reaction.

Primer number	Polymorphism		
	OPA	**OPB**	**OPM**
1	-	+	+
2	N/A	+	+
3	-	+	+
4	-	+	+
5	N/A	+	+
6	N/A	+	+
7	N/A	+	+
8	N/A	N/A	N/A
9	+	-	-
10	-	-	-
11	-	-	-
12	+	-	+
13	-	-	N/A
14	N/A	-	N/A
15	N/A	+	N/A
16	N/A	N/A	-
17	N/A	+	-
18	N/A	-	-
19	N/A	N/A	N/A
20	-	-	-

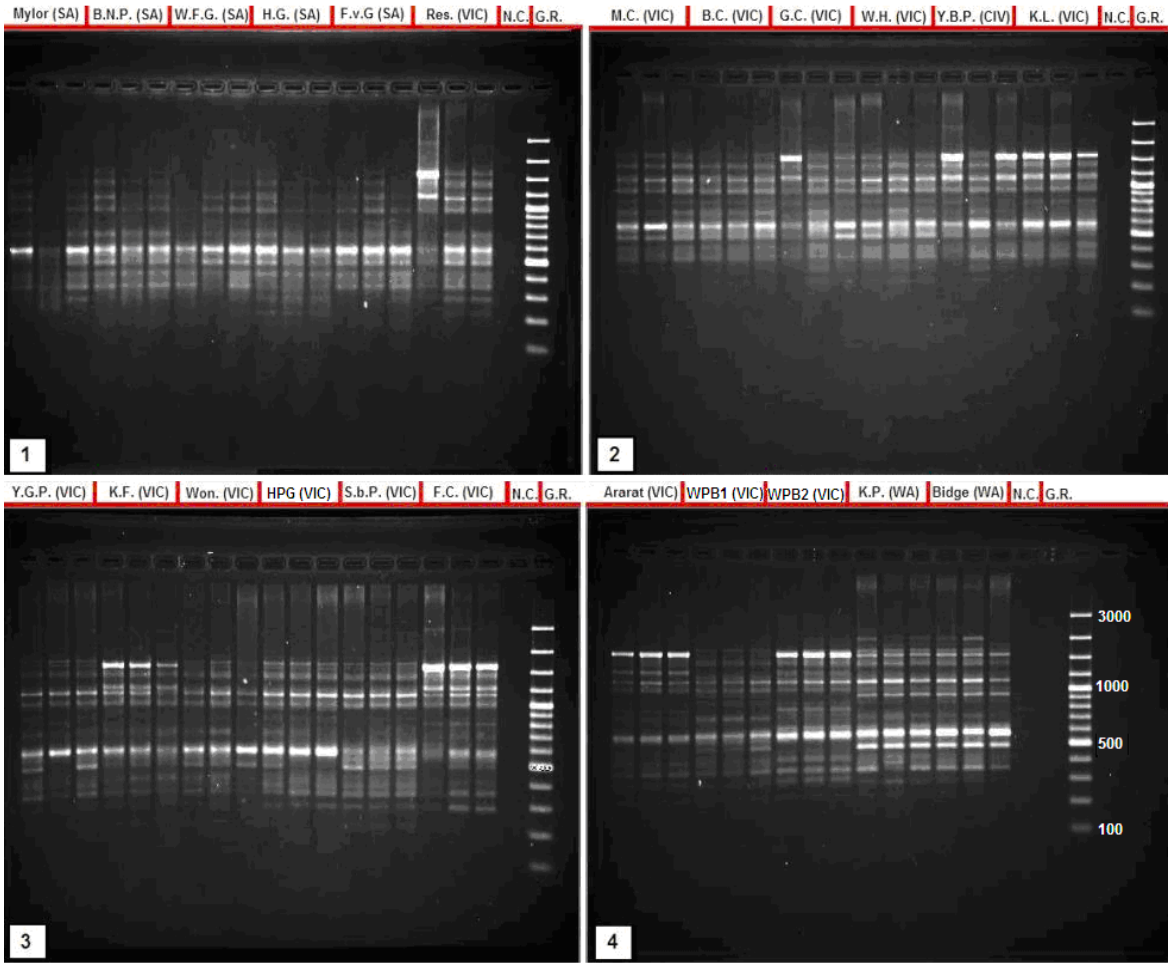

Figure 2.11 RAPD-PCR products from DNA of three samples from *Allium triquetrum* provenances amplified with OPB-01. Three replicate samples are shown for each provenance. Lanes are: **Gel 1**: 1-3 Mylor (SA), 4-6 Belair National Park (SA), 7-9 Waterfall Gully (SA), 10-12 Horsnell Gully (SA), 13-15 Fairview (SA), 16-18 Plenty Road, Reservoir (VIC), 19 Negative control (N.C.), 20 GeneRuler (G.R.). **Gel 2**: 1-3 Merri Creek (VIC), 4-6 Bendigo Creek (VIC), 7-9 Gardiner's Creek (VIC), 10-12 White Hill (VIC), 13-15 Yarra Bend Park (VIC), 16-18 Kinglake National Park (VIC), 19 Negative control (N.C.), 20 GeneRuler (G.R.). **Gel 3**: 1-3 Yellow Gum Park (VIC), 4-6 Kangaroo Flat (VIC), 7-9 Wonthaggi (VIC), 10-12 Hardy's Picnic Ground, Dandenong (VIC), 13-15 Sherbrooke Picnic Ground, Dandenongs (VIC), 16-18 Ferny Creek Area, Dandenongs (VIC), 19 Negative control (N.C.), 20 GeneRuler (G.R.). **Gel 4**: 1-3 Ararat (VIC), 4-6 Westernport Bay 1 (VIC), (7-9) Westernport Bay 2 (VIC), 10-12 Kings Park (WA), 13-15 Bridgestone (WA), 16 Negative control (N.C.), 17 GeneRuler (G.R.).

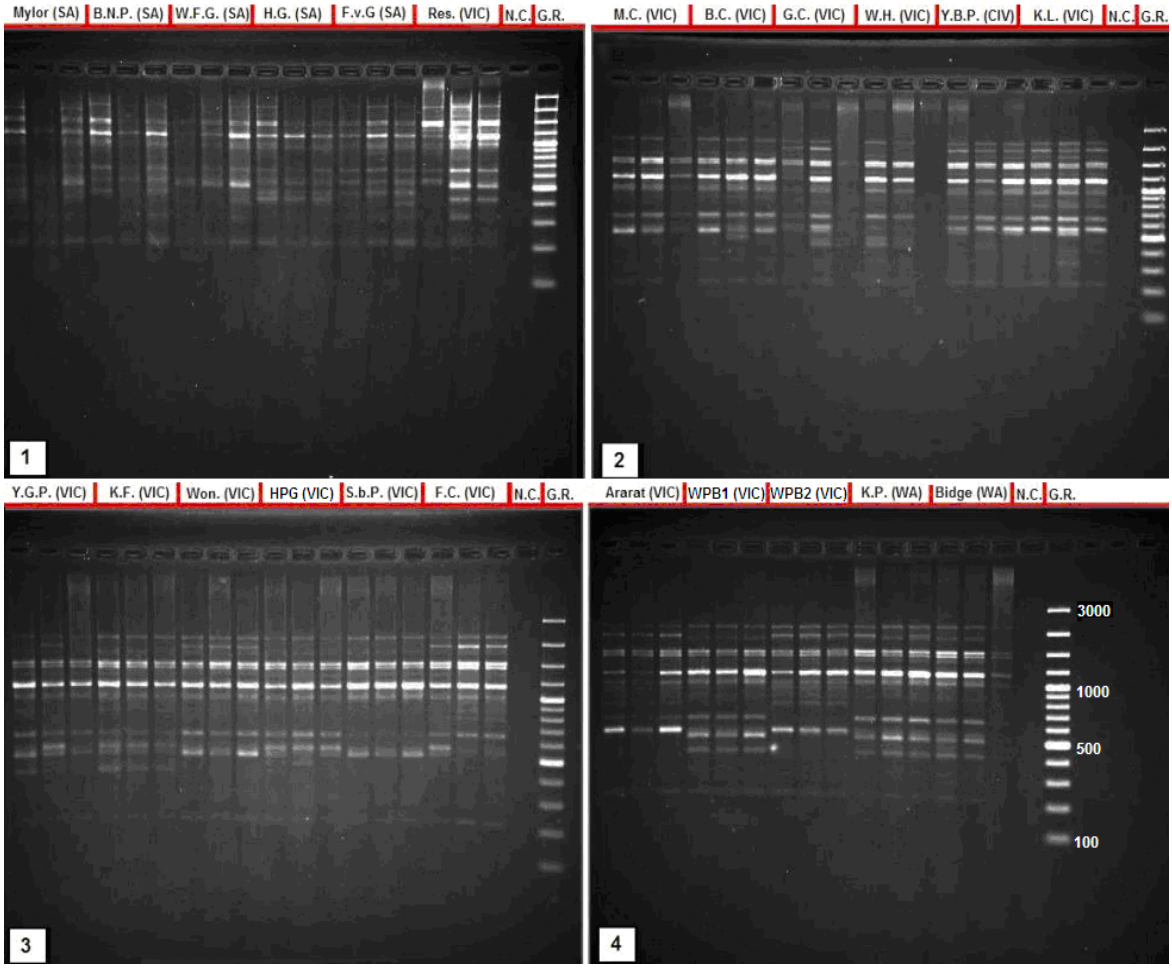

Figure 2.12 RAPD-PCR products from DNA of three samples from *Allium triquetrum* provenances amplified with OPB-04. Three replicate samples are shown for each provenance. Lanes are: **Gel 1**: 1-3 Mylor (SA), 4-6 Belair National Park (SA), 7-9 Waterfall Gully (SA), 10-12 Horsnell Gully (SA), 13-15 Fairview (SA), 16-18 Plenty Road, Reservoir (VIC), 19 Negative control (N.C.), 20 GeneRuler (G.R.). **Gel 2**: 1-3 Merri Creek (VIC), 4-6 Bendigo Creek (VIC), 7-9 Gardiner's Creek (VIC), 10-12 White Hill (VIC), 13-15 Yarra Bend Park (VIC), 16-18 Kinglake National Park (VIC), 19 Negative control (N.C.), 20 GeneRuler (G.R.). **Gel 3**: 1-3 Yellow Gum Park (VIC), 4-6 Kangaroo Flat (VIC), 7-9 Wonthaggi (VIC), 10-12 Hardy's Picnic Ground, Dandenongs (VIC), 13-15 Sherbrooke Picnic Ground, Dandenongs (VIC), 16-18 Ferny Creek Area, Dandenongs (VIC), 19 Negative control (N.C.), 20 GeneRuler (G.R.). **Gel 4**: 1-3 Ararat (VIC), 4-6 Westernport Bay 1 (VIC), 7-9 Westernport Bay 2 (VIC), 10-12 Kings Park (WA), 13-15 Bridgestone (WA), 16 Negative control (N.C.), 17 GeneRuler (G.R.).

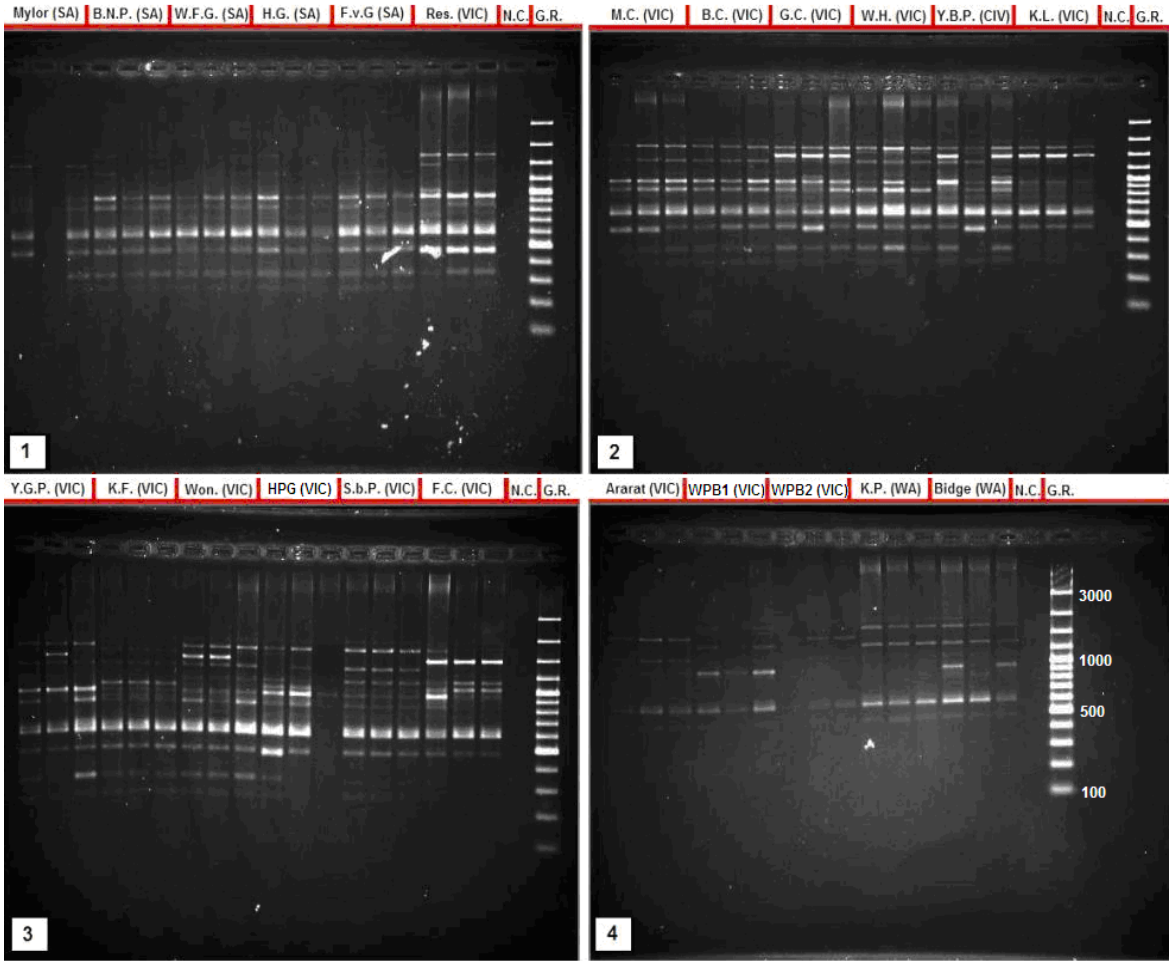

Figure 2.13 RAPD-PCR products from DNA of three samples from *Allium triquetrum* provenances amplified with OPB-05. Three replicate samples are shown for each provenance. Lanes are: **Gel 1**: 1-3 Mylor (SA), 4-6 Belair National Park (SA), 7-9 Waterfall Gully (SA), 10-12 Horsnell Gully (SA), 13-15 Fairview (SA), 16-18 Plenty Road, Reservoir (VIC), 19 Negative control (N.C.), 20 GeneRuler (G.R.). **Gel 2**: 1-3 Merri Creek (VIC), 4-6 Bendigo Creek (VIC), 7-9 Gardiner's Creek (VIC), 10-12 White Hill (VIC), 13-15 Yarra Bend Park (VIC), 16-18 Kinglake National Park (VIC), 19 Negative control (N.C.), 20 GeneRuler (G.R.). **Gel 3**: 1-3 Yellow Gum Park (VIC), 4-6 Kangaroo Flat (VIC), 7-9 Wonthaggi (VIC), 10-12 Hardy's Picnic Ground, Dandenongs (VIC), 13-15 Sherbrooke Picnic Ground, Dandenongs (VIC), Ferny Creek, Dandenongs (VIC), 19 Negative control (N.C.), 20 GeneRuler (G.R.). **Gel 4**: 1-3 Ararat (VIC), 4-6 Westernport Bay 1 (VIC), 7-9 Westernport Bay 2 (VIC), 10-12 Kings Park (WA), 13-15 Bridgestone (WA), 16 Negative control (N.C.), 17 GeneRuler (G.R.).

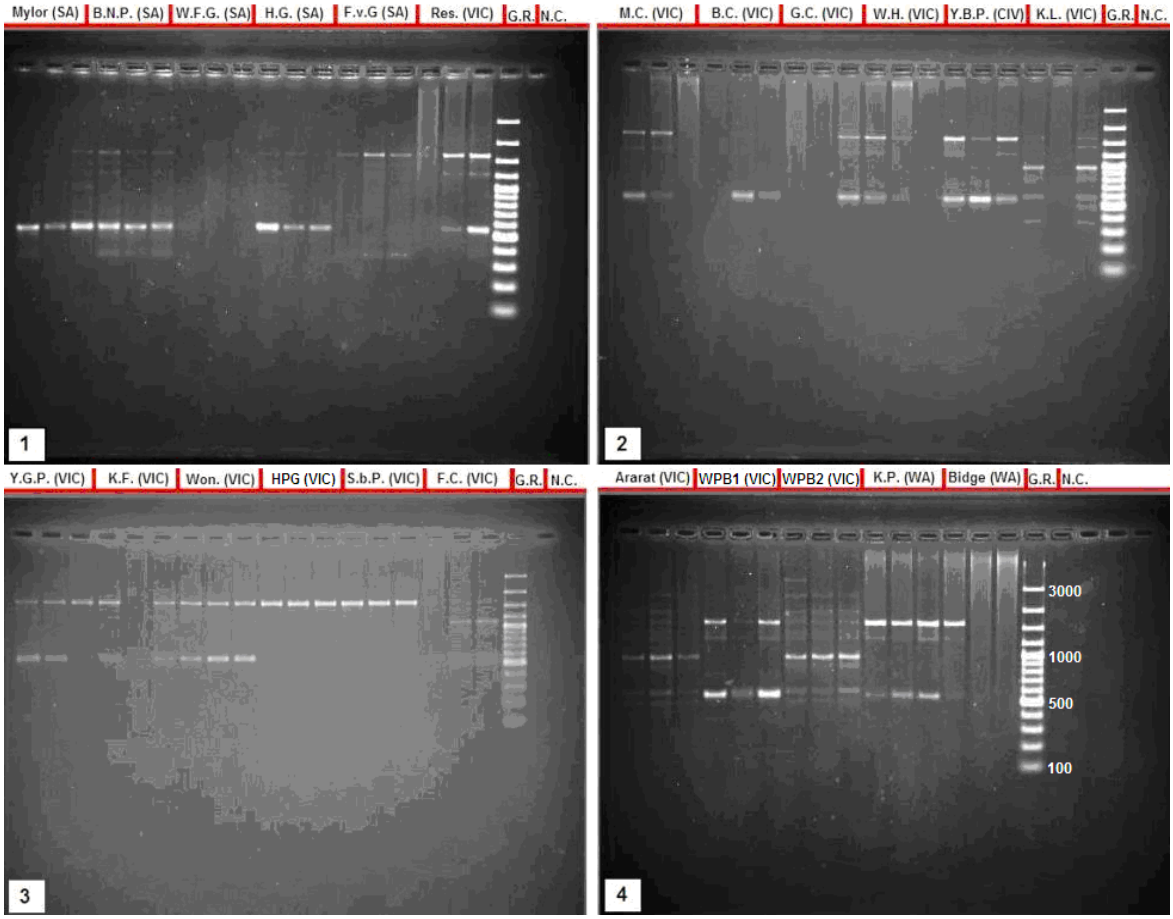

Figure 2.14 RAPD-PCR products from DNA of three samples from *Allium triquetrum* provenances amplified with OPM-02. Three replicate samples are shown for each provenance. Lanes are: **Gel 1**: (1-3) Mylor (SA), (4-6) Belair National Park (SA), (7-9) Waterfall Gully (SA), (10-12) Horsnell Gully (SA), (13-15) Fairview (SA), (16-18) Plenty Road, Reservoir (VIC), (19) Negative control (N.C.), (20) GeneRuler (G.R.). **Gel 2**: (1-3) Merri Creek (VIC), (4-6) Bendigo Creek (VIC), (7-9) Gardiner's Creek (VIC), (10-12) White Hill (VIC), (13-15) Yarra Bend Park (VIC), (16-18) Kinglake National Park (VIC), (19) Negative control (N.C.), (20) GeneRuler (G.R.). **Gel 3**: (1-3) Yellow Gum Park (VIC), (4-6) Kangaroo Flat (VIC), (7-9) Wonthaggi (VIC), (10-12) Hardy's Picnic Ground, Dandenongs (VIC), (13-15) Sherbrooke Picnic Ground, Dandenongs (VIC), 16-18 Ferny Creek Area, Dandenongs (VIC), (19) Negative control (N.C.), (20) GeneRuler (G.R.). **Gel 4**: (1-3) Ararat (VIC), (4-6) Westernport Bay 1 (VIC), (7-9) Westernport Bay 2 (VIC), (10-12) Kings Park (WA), (13-15) Bridgestone (WA), (16) Negative control (N.C.), (17) GeneRuler (G.R.).

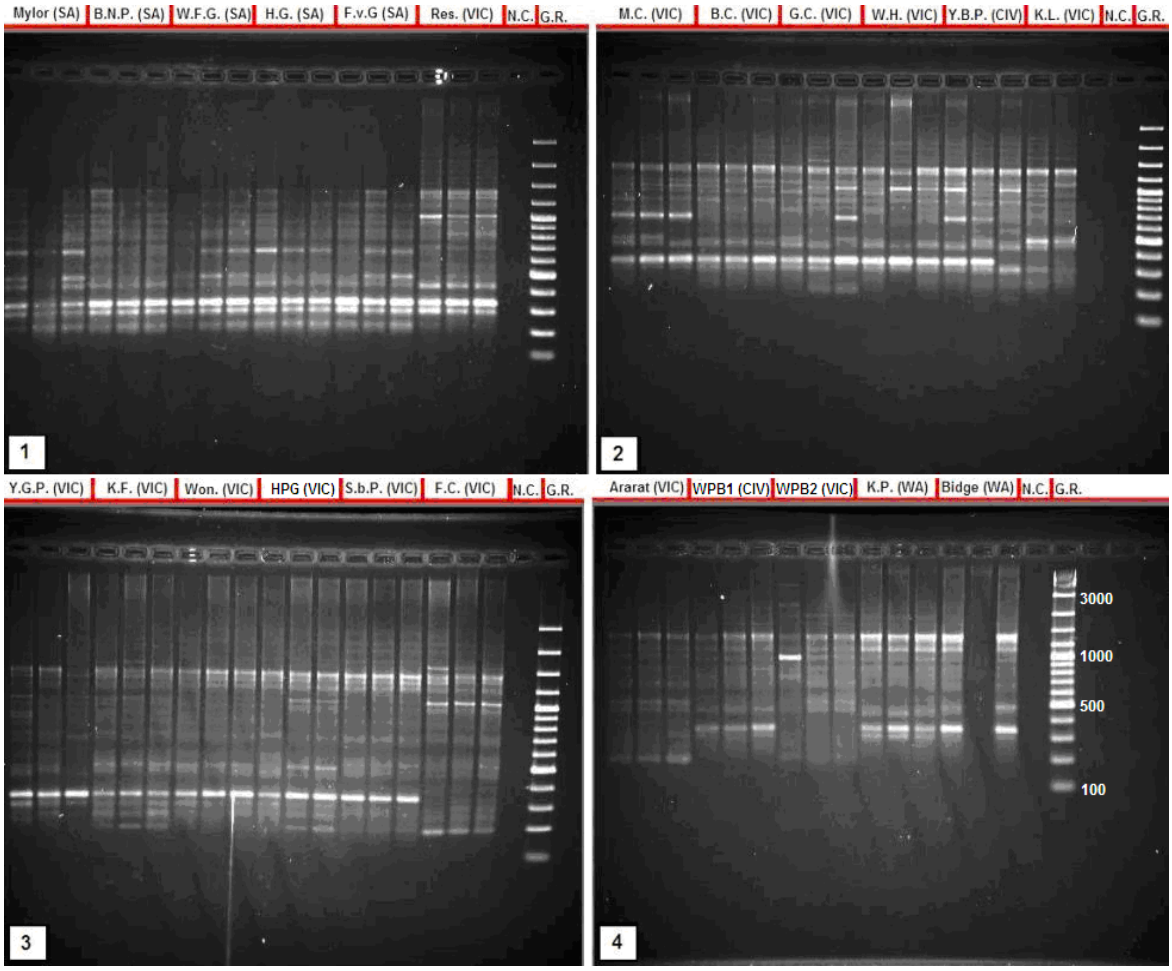

Figure 2.15 RAPD-PCR products from DNA of three samples from *Allium triquetrum* provenances amplified with OPM-05. Three replicate samples are shown for each provenance. Lanes are: **Gel 1**: 1-3 Mylor (SA), 4-6 Belair National Park (SA), 7-9 Waterfall Gully (SA), 10-12 Horsnell Gully (SA), 13-15 Fairview (SA), 16-18 Plenty Road, Reservoir (VIC), 19 Negative control (N.C.), 20 GeneRuler (G.R.). **Gel 2**: 1-3 Merri Creek (VIC), 4-6 Bendigo Creek (VIC), 7-9 Gardiner's Creek (VIC), 10-12 White Hill (VIC), 13-15 Yarra Bend Park (VIC), 16-18 Kinglake National Park (VIC), 19 Negative control (N.C.), 20 GeneRuler (G.R.). **Gel 3**: 1-3 Yellow Gum Park (VIC), 4-6 Kangaroo Flat (VIC), 7-9 Wonthaggi (VIC), 10-12 Hardy's Picnic Ground, Dandenongs (VIC), 13-15 Sherbrook Picnic Ground, Dandenongs (VIC), 16-18 Ferny Creek Area, Dandenongs (VIC), 19 Negative control (N.C.), 20 GeneRuler (G.R.). **Gel 4**: 1-3 Ararat (VIC), 4-6 Westernport Bay 1 (VIC), 7-9 Westernport Bay 2 (VIC), 10-12 Kings Park (WA), 13-15 Bridgestone (WA), 16 Negative control (N.C.), 17 GeneRuler (G.R.).

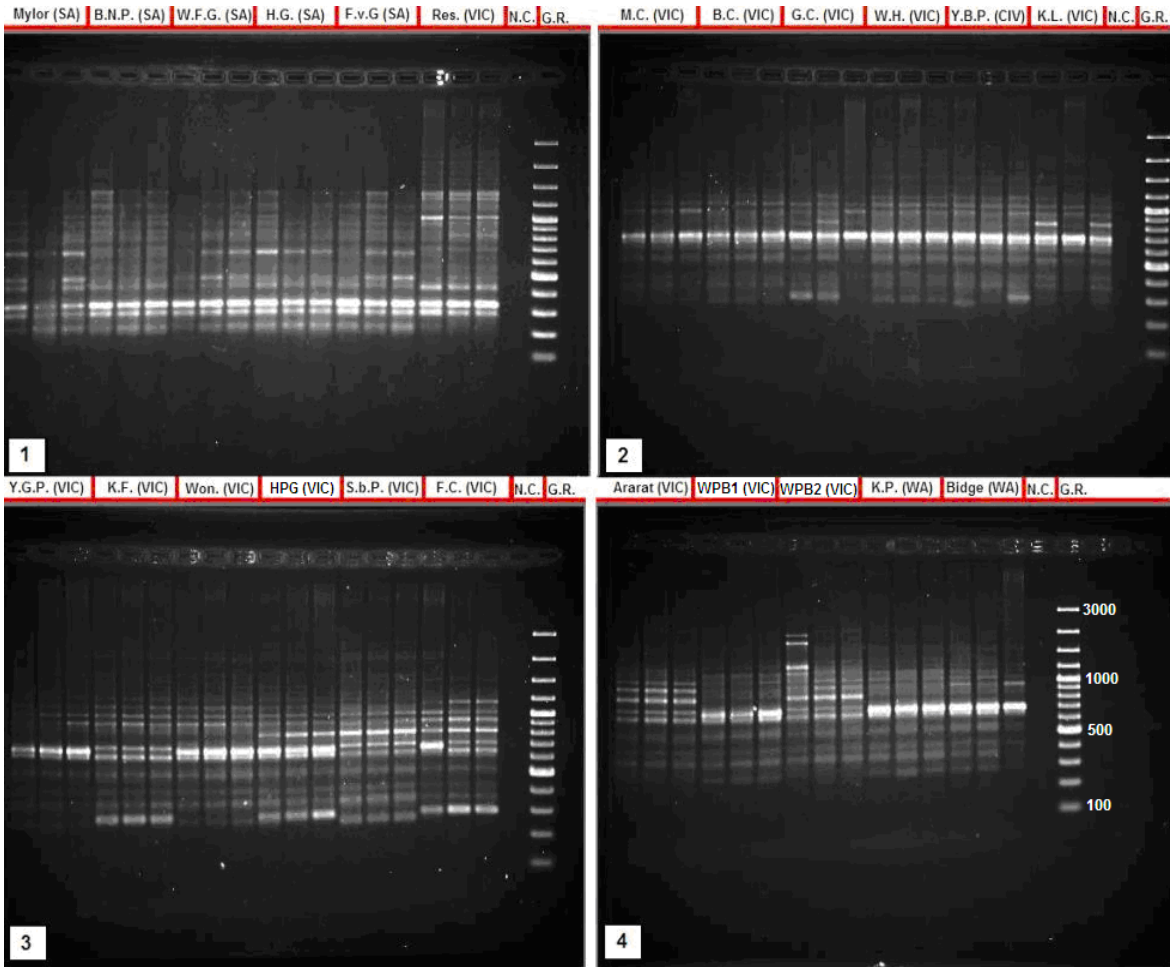

Figure 2.16 RAPD-PCR products from DNA of three samples from *Allium triquetrum* provenances amplified with OPM-06. Three replicate samples are shown for each provenance. Lanes are: **Gel 1**: 1-3 Mylor (SA), 4-6 Belair National Park (SA), 7-9 Waterfall Gully (SA), 10-12 Horsnell Gully (SA), 13-15 Fairview (SA), 16-18 Plenty Road, Reservoir (VIC), 19 Negative control (N.C.), 20 GeneRuler (G.R.). **Gel 2**: 1-3 Merri Creek (VIC), 4-6 Bendigo Creek (VIC), 7-9 Gardiner's Creek (VIC), 10-12 White Hill (VIC), 13-15 Yarra Bend Park (VIC), 16-18 Kinglake National Park (VIC), 19 Negative control (N.C.), 20 GeneRuler (G.R.). **Gel 3**: 1-3 Yellow Gum Park (VIC), 4-6 Kangaroo Flat (VIC), 7-9 Wonthaggi (VIC), 10-12 Hardy's Picnic Ground, Dandenongs (VIC), 13-15 Sherbrooke Picnic Ground, Dandenongs (VIC), 16-18 Ferny Creek, Dandenongs (VIC), 19 Negative control (N.C.), 20 GeneRuler (G.R.). **Gel 4**: 1-3 Ararat (VIC), 4-6 Westernport Bay 1 (VIC), 7-9 Westernport Bay 2 (VIC), 10-12 Kings Park (WA), 13-15 Bridgestone (WA), 16 Negative control (N.C.), 17 GeneRuler (G.R.).

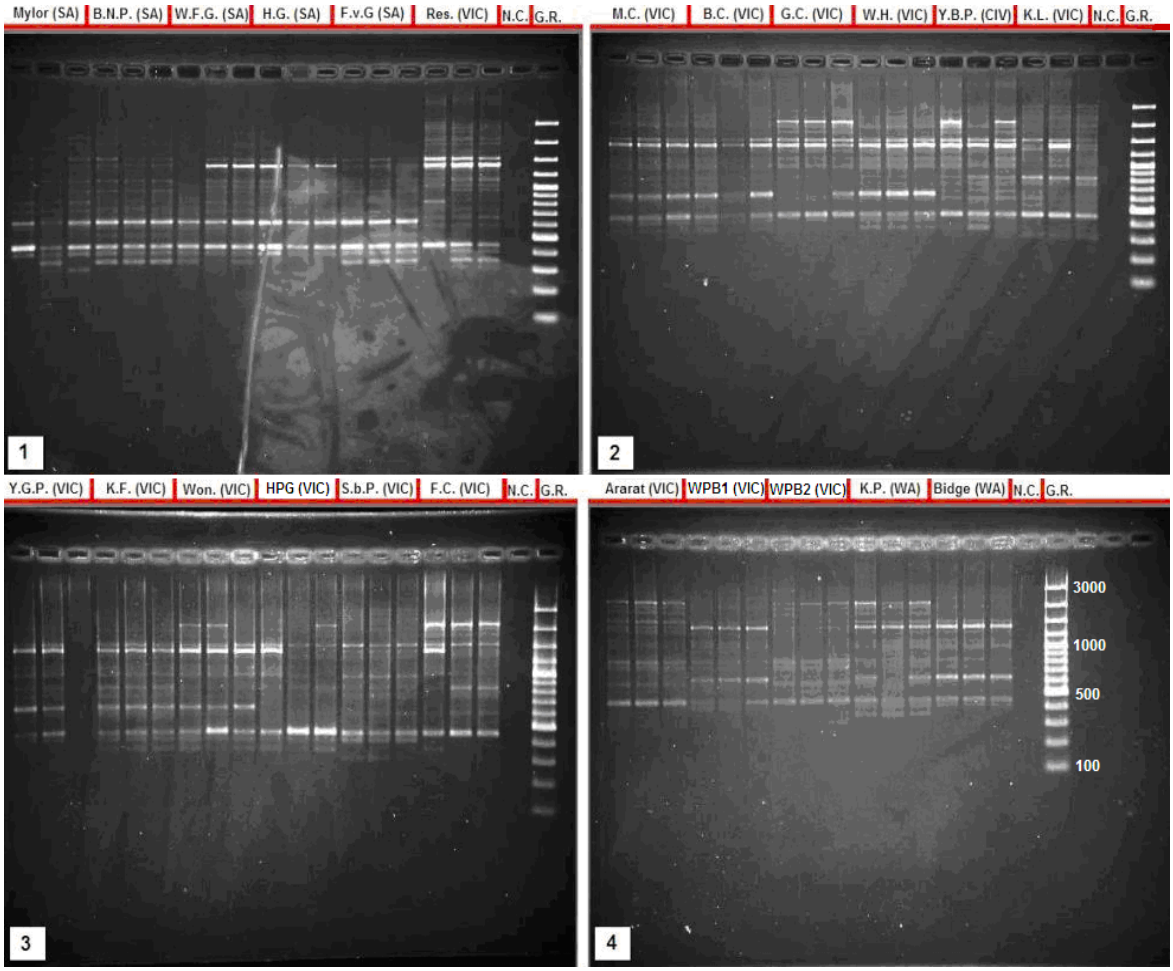

Figure 2.17 RAPD-PCR products from DNA of three samples from *Allium triquetrum* provenances amplified with OPM-12. Three replicate samples are shown for each provenance. Lanes are: **Gel 1**: 1-3 Mylor (SA), 4-6 Belair National Park (SA), 7-9 Waterfall Gully (SA), 10-12 Horsnell Gully (SA), 13-15 Fairview (SA), 16-18 Plenty Road, Reservoir (VIC), 19 Negative control (N.C.), 20 GeneRuler (G.R.). **Gel 2**: 1-3 Merri Creek VIC, 4-6 Bendigo Creek (VIC), 7-9 Gardiner's Creek (VIC), 10-12 White Hill (VIC), 13-15 Yarra Bend Park (VIC), 16-18 Kinglake National Park (VIC), 19 Negative control (N.C.), 20 GeneRuler (G.R.). **Gel 3**: 1-3 Yellow Gum Park (VIC), 4-6 Kangaroo Flat (VIC), 7-9 Wonthaggi (VIC), 10-12 Hardy's Picnic Ground, Dandenongs (VIC), 13-15 Sherbrooke Picnic Ground, Dandenongs (VIC), 16-18 Ferny Creek, Dandenongs (VIC), 19 Negative control (N.C.), 20 GeneRuler (G.R.). **Gel 4**: 1-3 Ararat (VIC), 4-6 Westernport Bay 1 (VIC), 7-9 Westernport Bay 2 (VIC), 10-12 Kings Park (WA), 13-15 Bridgestone (WA), 16 Negative control (N.C.), 17 GeneRuler (G.R.).

Table 2.6 Distance matrix of RAPD-PCR analysis of *Allium triquetrum* provenances with the outgroup species *Allium cepa* generated by SPSS Hierarchical Cluster analysis

Case	1:Maylor1	2:Maylor2	3:Maylor3	4:BN1	5:BN2	6:BN3	7:Wfal1	8:Wfal2	9:Wfal3	10:Hors1	11:Hors2	12:Hors3	13:Fview1	14:Fview2	15:Fview3	16:Rese1	17:Reser2	18:Reser3	19:MCr1	20:MCr2	21:MCr3	22:Bend1	23:Bend2	24:Bend3
1:Maylor1	.000	33.000	10.000	12.000	13.000	13.000	23.000	14.000	13.000	13.000	13.000	13.000	13.000	13.000	16.000	37.000	31.000	31.000	32.000	31.000	31.000	32.000	28.000	30.000
2:Maylor2	33.000	.000	39.000	39.000	38.000	42.000	28.000	37.000	40.000	38.000	32.000	34.000	40.000	40.000	39.000	60.000	56.000	58.000	51.000	50.000	52.000	51.000	47.000	49.000
3:Maylor3	10.000	39.000	.000	12.000	13.000	11.000	25.000	16.000	13.000	9.000	11.000	9.000	15.000	13.000	14.000	35.000	27.000	27.000	28.000	29.000	31.000	30.000	28.000	28.000
4:BN1	12.000	39.000	12.000	.000	3.000	3.000	23.000	16.000	13.000	13.000	15.000	13.000	9.000	7.000	12.000	33.000	27.000	25.000	22.000	21.000	23.000	28.000	26.000	26.000
5:BN2	13.000	38.000	13.000	3.000	.000	4.000	22.000	15.000	14.000	14.000	16.000	16.000	10.000	8.000	15.000	34.000	26.000	24.000	23.000	22.000	24.000	29.000	27.000	27.000
6:BN3	13.000	42.000	11.000	3.000	4.000	.000	26.000	17.000	12.000	12.000	16.000	14.000	8.000	6.000	11.000	34.000	24.000	24.000	19.000	20.000	24.000	25.000	23.000	23.000
7:Wfal1	23.000	28.000	25.000	23.000	22.000	26.000	.000	17.000	20.000	24.000	18.000	20.000	24.000	26.000	23.000	46.000	44.000	44.000	37.000	36.000	34.000	35.000	33.000	35.000
8:Wfal2	14.000	37.000	16.000	16.000	15.000	17.000	17.000	.000	7.000	11.000	13.000	13.000	13.000	15.000	16.000	31.000	33.000	33.000	30.000	29.000	25.000	26.000	28.000	28.000
9:Wfal3	13.000	40.000	13.000	13.000	14.000	12.000	20.000	7.000	.000	8.000	12.000	10.000	8.000	10.000	9.000	32.000	28.000	30.000	27.000	26.000	24.000	23.000	27.000	25.000
10:Hors1	13.000	38.000	9.000	13.000	14.000	12.000	24.000	11.000	8.000	.000	6.000	4.000	12.000	12.000	11.000	34.000	26.000	26.000	25.000	26.000	28.000	25.000	23.000	23.000
11:Hors2	13.000	32.000	11.000	15.000	16.000	16.000	18.000	13.000	12.000	6.000	.000	2.000	14.000	16.000	9.000	38.000	32.000	32.000	29.000	30.000	30.000	27.000	25.000	25.000
12:Hors3	13.000	34.000	9.000	13.000	16.000	14.000	20.000	13.000	10.000	4.000	2.000	.000	12.000	14.000	7.000	36.000	30.000	30.000	27.000	28.000	28.000	25.000	25.000	23.000
13:Fview1	13.000	40.000	15.000	9.000	10.000	8.000	24.000	13.000	8.000	12.000	14.000	12.000	.000	4.000	7.000	30.000	28.000	28.000	21.000	20.000	20.000	19.000	21.000	19.000
14:Fview2	13.000	40.000	13.000	7.000	8.000	6.000	26.000	15.000	10.000	12.000	16.000	14.000	4.000	.000	7.000	34.000	30.000	30.000	23.000	22.000	24.000	23.000	23.000	23.000
15:Fview3	16.000	39.000	14.000	12.000	15.000	11.000	23.000	16.000	9.000	11.000	9.000	7.000	7.000	7.000	.000	37.000	31.000	31.000	26.000	27.000	27.000	24.000	26.000	24.000
16:Rese1	37.000	60.000	35.000	33.000	34.000	34.000	46.000	31.000	32.000	34.000	38.000	36.000	30.000	34.000	37.000	.000	20.000	20.000	31.000	30.000	26.000	31.000	35.000	33.000
17:Reser2	31.000	56.000	27.000	27.000	26.000	24.000	44.000	33.000	28.000	26.000	32.000	30.000	28.000	30.000	31.000	20.000	.000	4.000	27.000	28.000	32.000	31.000	29.000	29.000
18:Reser3	31.000	58.000	27.000	25.000	24.000	24.000	44.000	33.000	30.000	26.000	32.000	30.000	28.000	30.000	31.000	20.000	4.000	.000	29.000	30.000	32.000	33.000	31.000	31.000
19:MCr1	32.000	51.000	28.000	22.000	23.000	19.000	37.000	30.000	27.000	25.000	29.000	27.000	21.000	23.000	26.000	31.000	27.000	29.000	.000	1.000	9.000	12.000	12.000	10.000
20:MCr2	31.000	50.000	29.000	21.000	22.000	20.000	36.000	29.000	26.000	26.000	30.000	28.000	20.000	22.000	27.000	30.000	28.000	30.000	1.000	.000	8.000	13.000	13.000	11.000
21:MCr3	31.000	52.000	31.000	23.000	24.000	24.000	34.000	25.000	24.000	28.000	30.000	28.000	20.000	24.000	27.000	26.000	32.000	32.000	9.000	8.000	.000	9.000	15.000	11.000
22:Bend1	32.000	51.000	30.000	28.000	29.000	25.000	35.000	26.000	23.000	25.000	27.000	25.000	19.000	23.000	24.000	31.000	31.000	33.000	12.000	13.000	9.000	.000	6.000	2.000
23:Bend2	28.000	47.000	28.000	26.000	27.000	23.000	33.000	28.000	27.000	23.000	25.000	25.000	21.000	23.000	26.000	35.000	29.000	31.000	12.000	13.000	15.000	6.000	.000	4.000
24:Bend3	30.000	49.000	28.000	26.000	27.000	23.000	35.000	28.000	25.000	23.000	25.000	23.000	19.000	23.000	24.000	33.000	29.000	31.000	10.000	11.000	11.000	2.000	4.000	.000
25:GC1	37.000	52.000	37.000	35.000	36.000	36.000	40.000	33.000	32.000	36.000	38.000	36.000	28.000	32.000	35.000	24.000	32.000	34.000	23.000	22.000	16.000	17.000	23.000	19.000
26:GC2	39.000	58.000	37.000	37.000	38.000	34.000	48.000	37.000	32.000	34.000	36.000	34.000	28.000	32.000	33.000	26.000	28.000	32.000	23.000	24.000	24.000	17.000	21.000	19.000
27:GC3	39.000	54.000	35.000	29.000	28.000	30.000	40.000	37.000	36.000	34.000	36.000	36.000	30.000	32.000	37.000	28.000	26.000	26.000	21.000	20.000	20.000	25.000	25.000	23.000
28:WHi1	32.000	53.000	30.000	24.000	25.000	21.000	39.000	30.000	27.000	23.000	27.000	27.000	23.000	25.000	28.000	33.000	25.000	27.000	12.000	13.000	15.000	10.000	6.000	8.000
29:WHi2	33.000	54.000	31.000	25.000	26.000	22.000	40.000	29.000	24.000	24.000	28.000	26.000	20.000	24.000	25.000	28.000	24.000	26.000	11.000	12.000	10.000	7.000	9.000	7.000
30:WHi3	37.000	50.000	35.000	29.000	28.000	28.000	36.000	29.000	28.000	28.000	30.000	30.000	26.000	30.000	31.000	30.000	30.000	30.000	19.000	20.000	12.000	13.000	19.000	15.000
31:YBP1	31.000	54.000	31.000	25.000	26.000	26.000	42.000	35.000	34.000	32.000	36.000	34.000	26.000	26.000	33.000	26.000	26.000	26.000	17.000	16.000	16.000	17.000	17.000	15.000
32:YBP2	31.000	54.000	29.000	27.000	28.000	26.000	46.000	37.000	32.000	26.000	32.000	30.000	28.000	28.000	31.000	34.000	24.000	24.000	15.000	16.000	22.000	19.000	19.000	17.000
33:YBP3	35.000	54.000	37.000	31.000	30.000	32.000	46.000	39.000	40.000	38.000	42.000	40.000	32.000	32.000	39.000	24.000	28.000	28.000	25.000	24.000	24.000	25.000	25.000	23.000
34:KL1	33.000	56.000	37.000	35.000	34.000	36.000	50.000	41.000	42.000	40.000	42.000	40.000	36.000	36.000	39.000	30.000	32.000	32.000	33.000	32.000	32.000	33.000	35.000	31.000
35:KL2	34.000	55.000	40.000	38.000	37.000	37.000	47.000	34.000	35.000	39.000	39.000	39.000	33.000	37.000	38.000	25.000	29.000	33.000	30.000	29.000	27.000	26.000	30.000	28.000
36:KL3	38.000	53.000	44.000	42.000	41.000	43.000	45.000	42.000	47.000	45.000	45.000	45.000	43.000	41.000	44.000	39.000	43.000	43.000	44.000	43.000	41.000	42.000	42.000	40.000
37:YGP1	42.000	55.000	38.000	36.000	35.000	35.000	51.000	40.000	39.000	35.000	41.000	39.000	39.000	39.000	42.000	33.000	29.000	29.000	34.000	35.000	37.000	36.000	36.000	36.000
38:YGP2	43.000	52.000	43.000	39.000	40.000	38.000	54.000	45.000	44.000	38.000	44.000	42.000	40.000	40.000	45.000	36.000	36.000	38.000	33.000	34.000	36.000	35.000	35.000	35.000
39:YGP3	46.000	55.000	44.000	44.000	43.000	43.000	41.000	44.000	45.000	41.000	47.000	45.000	43.000	43.000	48.000	47.000	45.000	45.000	46.000	47.000	49.000	44.000	42.000	44.000
40:KF1	43.000	56.000	39.000	37.000	36.000	36.000	50.000	41.000	38.000	36.000	42.000	40.000	38.000	38.000	41.000	36.000	32.000	32.000	35.000	36.000	38.000	35.000	35.000	35.000
41:KF2	42.000	55.000	40.000	40.000	39.000	39.000	45.000	36.000	33.000	35.000	39.000	37.000	35.000	39.000	38.000	33.000	33.000	33.000	36.000	37.000	35.000	30.000	34.000	32.000
42:KF3	42.000	55.000	38.000	36.000	35.000	35.000	49.000	40.000	37.000	35.000	41.000	39.000	37.000	37.000	40.000	37.000	33.000	33.000	34.000	35.000	37.000	34.000	34.000	34.000
43:Won1	41.000	52.000	37.000	33.000	34.000	32.000	48.000	41.000	40.000	40.000	44.000	42.000	36.000	36.000	41.000	32.000	30.000	32.000	31.000	32.000	34.000	35.000	35.000	35.000
44:Won2	42.000	53.000	38.000	34.000	35.000	33.000	49.000	42.000	41.000	41.000	45.000	43.000	37.000	37.000	42.000	31.000	31.000	33.000	32.000	33.000	35.000	36.000	36.000	36.000
45:Won3	43.000	50.000	43.000	35.000	34.000	36.000	48.000	39.000	40.000	42.000	48.000	46.000	38.000	38.000	45.000	32.000	32.000	34.000	33.000	32.000	34.000	37.000	37.000	37.000
46:Har1	46.000	61.000	42.000	38.000	37.000	37.000	53.000	44.000	41.000	41.000	47.000	45.000	41.000	41.000	44.000	33.000	33.000	33.000	32.000	33.000	35.000	36.000	36.000	36.000
47:Har2	42.000	55.000	40.000	34.000	33.000	33.000	49.000	42.000	41.000	41.000	45.000	43.000	37.000	37.000	42.000	35.000	33.000	33.000	30.000	31.000	33.000	34.000	34.000	34.000
48:Har3	45.000	52.000	41.000	35.000	36.000	36.000	50.000	45.000	44.000	44.000	46.000	44.000	40.000	40.000	43.000	38.000	36.000	36.000	31.000	32.000	34.000	35.000	35.000	35.000
49:Sherb1	43.000	52.000	39.000	39.000	38.000	38.000	50.000	43.000	46.000	40.000	44.000	42.000	42.000	42.000	47.000	32.000	32.000	32.000	33.000	34.000	40.000	37.000	35.000	35.000
50:Sherb2	43.000	52.000	39.000	39.000	38.000	38.000	50.000	43.000	46.000	40.000	44.000	42.000	42.000	42.000	47.000	32.000	32.000	32.000	33.000	34.000	40.000	37.000	35.000	35.000
51:Sherb3	43.000	52.000	39.000	39.000	38.000	38.000	50.000	43.000	46.000	40.000	44.000	42.000	42.000	42.000	47.000	32.000	32.000	32.000	33.000	34.000	40.000	37.000	35.000	35.000
52:FCr1	50.000	61.000	50.000	44.000	47.000	45.000	53.000	50.000	49.000	49.000	49.000	47.000	45.000	49.000	48.000	29.000	39.000	41.000	36.000	37.000	39.000	40.000	42.000	40.000
53:FCr2	49.000	58.000	47.000	43.000	46.000	44.000	56.000	51.000	52.000	48.000	48.000	46.000	46.000	48.000	49.000	30.000	40.000	42.000	39.000	40.000	44.000	45.000	45.000	43.000
54:FCr3	47.000	58.000	47.000	41.000	44.000	44.000	54.000	49.000	50.000	48.000	48.000	46.000	44.000	46.000	49.000	28.000	40.000	40.000	41.000	40.000	44.000	47.000	47.000	45.000
55:Ararat 1	47.000	48.000	43.000	45.000	48.000	46.000	54.000	49.000	50.000	44.000	42.000	40.000	48.000	48.000	45.000	52.000	52.000	54.000	39.000	40.000	42.000	39.000	41.000	37.000
56:Ararat 2	47.000	48.000	43.000	45.000	48.000	46.000	54.000	49.000	50.000	44.000	42.000	40.000	48.000	48.000	45.000	52.000	52.000	54.000	39.000	40.000	42.000	39.000	41.000	37.000
57:Ararat 3	47.000	48.000	43.000	45.000	48.000	46.000	54.000	49.000	50.000	44.000	42.000	40.000	48.000	48.000	45.000	52.000	52.000	54.000	39.000	40.000	42.000	39.000	41.000	37.000
58:SMar1.1	40.000	39.000	40.000	42.000	41.000	39.000	43.000	40.000	39.000	39.000	39.000	37.000	43.000	43.000	40.000	49.000	43.000	47.000	40.000	41.000	43.000	40.000	40.000	40.000
59:SMar1.2	41.000	38.000	41.000	41.000	42.000	40.000	42.000	41.000	40.000	40.000	38.000	36.000	44.000	44.000	39.000	50.000	44.000	48.000	41.000	42.000	44.000	41.000	41.000	41.000
60:SMar1.3	41.000	40.000	41.000	43.000	42.000	40.000	44.000	41.000	40.000	40.000	40.000	38.000	44.000	44.000	41.000	48.000	42.000	46.000	41.000	42.000	44.000	41.000	41.000	41.000
61:SMar2.1	57.000	54.000	55.000	57.000	60.000	58.000	66.000	59.000	60.000	56.000	56.000	54.000	58.000	58.000	57.000	54.000	52.000	54.000	47.000	48.000	50.000	47.000	51.000	47.000
62:SMar2.2	54.000	55.000	52.000	54.000	57.000	55.000	65.000	56.000	57.000	53.000	53.000	51.000	55.000	55.000	54.000	51.000	49.000	51.000	44.000	45.000	47.000	46.000	50.000	46.000
63:SMar2.3	53.000	54.000	51.000	53.000	56.000	54.000	64.000	55.000	56.000	52.000	52.000	50.000	54.000	54.000	53.000	52.000	50.000	52.000	43.000	44.000	46.000	45.000	49.000	45.000
64:KP1	44.000	45.000	44.000	42.000	41.000	41.000	47.000	42.000	43.000	43.000	43.000	43.000	45.000	47.000	46.000	39.000	35.000	37.000	40.000	41.000	41.000	42.000	42.000	42.000
65:KP2	44.000	45.000	44.000	42.000	41.000	39.000	47.000	42.000	41.000	43.000	43.000	43.000	43.000	45.000	44.000	41.000	35.000	39.000	38.000	39.000	41.000	40.000	40.000	40.000
66:KP3	44.000	45.000	44.000	42.000	41.000	39.000	47.000	40.000	41.000	43.000	43.000	43.000	43.000	45.000	44.000	39.000	33.000	37.000	38.000	39.000	41.000	40.000	40.000	40.000
67:Bridg1	36.000	43.000	40.000	40.000	39.000	37.000	41.000	36.000	37.000	37.000	37.000	37.000	39.000	41.000	40.000	41.000	37.000	41.000	36.000	37.000	39.000	36.000	34.000	36.000
68:Bridg2	44.000	45.000	48.000	48.000	47.000	45.000	43.000	40.000	41.000	43.000	43.000	43.000	45.000	47.000	44.000	45.000	43.000	47.000	44.000	45.000	43.000	42.000	42.000	44.000
69:Bridg3	41.000	42.000	43.000	41.000	40.000	40.000	40.000	33.000	38.000	38.000	38.000	38.000	38.000	42.000	41.000	40.000	44.000	44.000	35.000	36.000	32.000	33.000	35.000	35.000

Squared Euclidean Distance

25:GC1	26:GC2	27:GC3	28:WHi1	29:WHi2	30:WHi3	31:YBP1	32:YBP2	33:YBP3	34:KL1	35:KL2	36:KL3	37:YGP1	38:YGP2	39:YGP3	40:KF1	41:KF2	42:KF3	43:Won1	44:Won2	45:Won3	46:Har1	47:Har2	48:Har3	49:Sherb1
37.000	39.000	39.000	32.000	33.000	37.000	31.000	31.000	35.000	33.000	34.000	38.000	42.000	43.000	46.000	43.000	42.000	42.000	41.000	42.000	43.000	46.000	42.000	45.000	43.000
52.000	58.000	54.000	53.000	54.000	50.000	54.000	54.000	54.000	56.000	55.000	53.000	55.000	52.000	55.000	56.000	55.000	55.000	52.000	53.000	50.000	61.000	55.000	52.000	52.000
37.000	37.000	35.000	30.000	31.000	35.000	31.000	29.000	37.000	37.000	40.000	44.000	38.000	43.000	44.000	39.000	40.000	38.000	37.000	38.000	43.000	42.000	40.000	41.000	39.000
35.000	37.000	29.000	24.000	25.000	29.000	25.000	27.000	31.000	35.000	38.000	42.000	36.000	39.000	44.000	37.000	40.000	36.000	33.000	34.000	35.000	38.000	34.000	35.000	39.000
36.000	38.000	28.000	25.000	26.000	28.000	26.000	28.000	30.000	34.000	37.000	41.000	35.000	40.000	43.000	36.000	39.000	35.000	34.000	35.000	34.000	37.000	33.000	36.000	38.000
36.000	34.000	30.000	21.000	22.000	28.000	26.000	26.000	32.000	36.000	37.000	43.000	35.000	38.000	43.000	36.000	39.000	35.000	32.000	33.000	36.000	37.000	33.000	36.000	38.000
40.000	48.000	40.000	39.000	40.000	36.000	42.000	46.000	46.000	50.000	47.000	45.000	51.000	54.000	41.000	50.000	45.000	49.000	48.000	49.000	48.000	53.000	49.000	50.000	50.000
33.000	37.000	37.000	30.000	29.000	29.000	35.000	37.000	39.000	41.000	34.000	42.000	40.000	45.000	44.000	41.000	36.000	40.000	41.000	42.000	39.000	44.000	42.000	45.000	43.000
32.000	32.000	36.000	27.000	24.000	28.000	34.000	32.000	40.000	42.000	35.000	47.000	39.000	44.000	45.000	38.000	33.000	37.000	40.000	41.000	40.000	41.000	41.000	44.000	46.000
36.000	34.000	34.000	23.000	24.000	28.000	32.000	26.000	38.000	40.000	39.000	45.000	35.000	38.000	41.000	36.000	35.000	35.000	40.000	41.000	42.000	41.000	41.000	44.000	40.000
38.000	36.000	36.000	27.000	28.000	30.000	36.000	32.000	42.000	42.000	39.000	45.000	41.000	44.000	47.000	42.000	39.000	41.000	44.000	45.000	48.000	47.000	45.000	46.000	44.000
36.000	34.000	36.000	27.000	26.000	30.000	34.000	30.000	40.000	40.000	39.000	45.000	39.000	42.000	45.000	40.000	37.000	39.000	42.000	43.000	46.000	45.000	43.000	44.000	42.000
28.000	28.000	30.000	23.000	20.000	26.000	26.000	28.000	32.000	36.000	33.000	43.000	39.000	40.000	43.000	38.000	35.000	37.000	36.000	37.000	38.000	41.000	37.000	40.000	42.000
32.000	32.000	32.000	25.000	24.000	30.000	26.000	28.000	32.000	36.000	37.000	41.000	39.000	40.000	43.000	38.000	39.000	37.000	36.000	37.000	38.000	41.000	37.000	40.000	42.000
35.000	33.000	37.000	28.000	25.000	31.000	33.000	31.000	39.000	39.000	38.000	44.000	42.000	45.000	48.000	41.000	38.000	40.000	41.000	42.000	45.000	44.000	42.000	43.000	47.000
24.000	26.000	28.000	33.000	28.000	30.000	26.000	34.000	24.000	30.000	25.000	39.000	33.000	36.000	47.000	36.000	33.000	37.000	32.000	31.000	32.000	33.000	35.000	38.000	32.000
32.000	28.000	26.000	25.000	24.000	30.000	26.000	24.000	28.000	32.000	29.000	43.000	29.000	36.000	45.000	32.000	33.000	33.000	30.000	31.000	32.000	33.000	33.000	36.000	32.000
34.000	32.000	26.000	27.000	26.000	30.000	26.000	24.000	28.000	32.000	33.000	43.000	29.000	38.000	45.000	32.000	33.000	33.000	32.000	33.000	34.000	33.000	33.000	36.000	32.000
23.000	23.000	21.000	12.000	11.000	19.000	17.000	15.000	25.000	33.000	30.000	44.000	34.000	33.000	46.000	35.000	36.000	34.000	31.000	32.000	33.000	32.000	30.000	31.000	33.000
22.000	24.000	20.000	13.000	12.000	20.000	16.000	16.000	24.000	32.000	29.000	43.000	35.000	34.000	47.000	36.000	37.000	35.000	32.000	33.000	32.000	33.000	31.000	32.000	34.000
16.000	24.000	20.000	15.000	10.000	12.000	16.000	22.000	24.000	32.000	27.000	41.000	37.000	36.000	49.000	38.000	35.000	37.000	34.000	35.000	34.000	35.000	33.000	34.000	40.000
17.000	17.000	25.000	10.000	7.000	13.000	17.000	19.000	25.000	33.000	26.000	42.000	36.000	35.000	44.000	35.000	30.000	34.000	35.000	36.000	37.000	36.000	34.000	35.000	37.000
23.000	21.000	25.000	6.000	9.000	19.000	17.000	19.000	25.000	35.000	30.000	42.000	36.000	35.000	42.000	35.000	34.000	34.000	35.000	36.000	37.000	36.000	34.000	35.000	35.000
19.000	19.000	23.000	8.000	7.000	15.000	15.000	17.000	23.000	31.000	28.000	40.000	36.000	35.000	44.000	35.000	32.000	34.000	35.000	36.000	37.000	36.000	34.000	35.000	35.000
.000	18.000	16.000	27.000	24.000	20.000	12.000	22.000	18.000	26.000	21.000	39.000	43.000	38.000	53.000	38.000	33.000	37.000	36.000	35.000	36.000	41.000	39.000	40.000	42.000
18.000	.000	28.000	21.000	20.000	28.000	20.000	20.000	22.000	34.000	27.000	43.000	33.000	30.000	43.000	26.000	21.000	25.000	30.000	31.000	34.000	29.000	29.000	32.000	36.000
16.000	28.000	.000	23.000	26.000	16.000	18.000	26.000	24.000	30.000	29.000	45.000	41.000	40.000	55.000	38.000	39.000	39.000	36.000	37.000	36.000	37.000	35.000	36.000	34.000
27.000	21.000	23.000	.000	5.000	17.000	19.000	17.000	27.000	33.000	28.000	42.000	32.000	31.000	44.000	31.000	32.000	30.000	35.000	36.000	35.000	30.000	30.000	31.000	37.000
24.000	20.000	26.000	5.000	.000	14.000	20.000	18.000	28.000	34.000	29.000	43.000	31.000	30.000	43.000	32.000	31.000	31.000	34.000	35.000	34.000	31.000	31.000	32.000	38.000
20.000	28.000	16.000	17.000	14.000	.000	24.000	24.000	32.000	40.000	35.000	51.000	35.000	36.000	47.000	38.000	35.000	37.000	36.000	37.000	36.000	37.000	37.000	38.000	40.000
12.000	20.000	18.000	19.000	20.000	24.000	.000	12.000	8.000	22.000	25.000	33.000	35.000	32.000	47.000	32.000	35.000	31.000	28.000	27.000	30.000	35.000	33.000	34.000	36.000
22.000	20.000	26.000	17.000	18.000	24.000	12.000	.000	20.000	26.000	31.000	41.000	29.000	28.000	43.000	28.000	31.000	27.000	32.000	33.000	32.000	31.000	33.000	34.000	34.000
18.000	22.000	24.000	27.000	28.000	32.000	8.000	20.000	.000	20.000	23.000	31.000	35.000	34.000	47.000	28.000	31.000	29.000	30.000	29.000	30.000	31.000	29.000	32.000	34.000
26.000	34.000	30.000	33.000	34.000	40.000	22.000	26.000	20.000	.000	9.000	17.000	47.000	44.000	57.000	42.000	43.000	43.000	48.000	47.000	44.000	43.000	41.000	44.000	44.000
18.000	22.000	24.000	27.000	28.000	32.000	8.000	20.000	.000	20.000	23.000	31.000	35.000	34.000	47.000	28.000	31.000	29.000	30.000	29.000	30.000	31.000	29.000	32.000	34.000
26.000	34.000	30.000	33.000	34.000	40.000	22.000	26.000	20.000	.000	9.000	17.000	47.000	44.000	57.000	42.000	43.000	43.000	48.000	47.000	44.000	43.000	41.000	44.000	44.000
21.000	27.000	29.000	28.000	29.000	35.000	25.000	31.000	23.000	9.000	.000	24.000	46.000	43.000	58.000	41.000	38.000	42.000	47.000	46.000	43.000	42.000	40.000	43.000	45.000
39.000	43.000	45.000	42.000	43.000	51.000	33.000	41.000	31.000	17.000	24.000	.000	54.000	47.000	54.000	51.000	50.000	52.000	53.000	52.000	51.000	52.000	48.000	51.000	53.000
43.000	33.000	41.000	32.000	31.000	35.000	35.000	29.000	35.000	47.000	46.000	54.000	.000	11.000	18.000	13.000	18.000	12.000	9.000	10.000	7.000	14.000	20.000	23.000	17.000
38.000	30.000	40.000	31.000	30.000	36.000	32.000	28.000	34.000	44.000	43.000	47.000	11.000	.000	21.000	22.000	27.000	21.000	14.000	15.000	12.000	21.000	23.000	26.000	22.000
53.000	43.000	55.000	44.000	43.000	47.000	47.000	43.000	47.000	57.000	58.000	54.000	18.000	21.000	.000	29.000	32.000	28.000	25.000	26.000	23.000	26.000	30.000	33.000	27.000
38.000	26.000	38.000	31.000	32.000	38.000	32.000	28.000	28.000	42.000	41.000	51.000	13.000	22.000	29.000	.000	5.000	1.000	18.000	17.000	18.000	11.000	15.000	22.000	24.000
33.000	21.000	39.000	32.000	31.000	35.000	35.000	31.000	31.000	43.000	38.000	50.000	18.000	27.000	32.000	5.000	.000	6.000	23.000	22.000	23.000	16.000	18.000	25.000	29.000
37.000	25.000	39.000	30.000	31.000	37.000	31.000	27.000	29.000	43.000	42.000	52.000	12.000	21.000	28.000	1.000	6.000	.000	17.000	16.000	17.000	12.000	16.000	23.000	25.000
36.000	30.000	36.000	35.000	34.000	36.000	28.000	32.000	30.000	48.000	47.000	53.000	9.000	14.000	25.000	18.000	23.000	17.000	.000	1.000	6.000	19.000	19.000	22.000	20.000
35.000	31.000	37.000	36.000	35.000	37.000	27.000	33.000	29.000	47.000	46.000	52.000	10.000	15.000	26.000	17.000	22.000	16.000	1.000	.000	7.000	20.000	20.000	23.000	21.000
36.000	34.000	36.000	35.000	34.000	36.000	30.000	32.000	30.000	44.000	43.000	51.000	7.000	12.000	23.000	18.000	23.000	17.000	6.000	7.000	.000	17.000	21.000	24.000	18.000
41.000	29.000	37.000	30.000	31.000	37.000	35.000	31.000	31.000	43.000	42.000	52.000	14.000	21.000	26.000	11.000	16.000	12.000	19.000	20.000	17.000	.000	6.000	11.000	19.000
39.000	29.000	35.000	30.000	31.000	37.000	33.000	33.000	29.000	41.000	40.000	48.000	20.000	23.000	30.000	15.000	18.000	16.000	19.000	20.000	21.000	6.000	.000	7.000	21.000
40.000	32.000	36.000	31.000	32.000	38.000	34.000	34.000	32.000	44.000	43.000	51.000	23.000	26.000	33.000	22.000	25.000	23.000	22.000	23.000	24.000	11.000	7.000	.000	24.000
42.000	36.000	34.000	37.000	38.000	40.000	36.000	34.000	34.000	44.000	45.000	53.000	17.000	22.000	27.000	24.000	29.000	25.000	20.000	21.000	18.000	19.000	21.000	24.000	.000
42.000	36.000	34.000	37.000	38.000	40.000	36.000	34.000	34.000	44.000	45.000	53.000	17.000	22.000	27.000	24.000	29.000	25.000	20.000	21.000	18.000	19.000	21.000	24.000	.000
42.000	36.000	34.000	37.000	38.000	40.000	36.000	34.000	34.000	44.000	45.000	53.000	17.000	22.000	27.000	24.000	29.000	25.000	20.000	21.000	18.000	19.000	21.000	24.000	.000
33.000	27.000	33.000	40.000	41.000	43.000	35.000	39.000	33.000	35.000	34.000	42.000	40.000	31.000	44.000	35.000	32.000	36.000	33.000	32.000	35.000	30.000	28.000	29.000	31.000
38.000	32.000	34.000	43.000	44.000	46.000	36.000	40.000	36.000	36.000	39.000	43.000	35.000	30.000	43.000	34.000	37.000	35.000	30.000	29.000	32.000	31.000	33.000	34.000	26.000
40.000	34.000	36.000	45.000	46.000	48.000	36.000	42.000	34.000	38.000	41.000	43.000	35.000	34.000	43.000	34.000	37.000	35.000	30.000	29.000	32.000	31.000	33.000	34.000	28.000
48.000	44.000	46.000	41.000	42.000	46.000	42.000	40.000	46.000	44.000	47.000	49.000	49.000	46.000	59.000	48.000	51.000	47.000	46.000	47.000	50.000	47.000	45.000	40.000	44.000
48.000	44.000	46.000	41.000	42.000	46.000	42.000	40.000	46.000	44.000	47.000	49.000	49.000	46.000	59.000	48.000	51.000	47.000	46.000	47.000	50.000	47.000	45.000	40.000	44.000
48.000	44.000	46.000	41.000	42.000	46.000	42.000	40.000	46.000	44.000	47.000	49.000	49.000	46.000	59.000	48.000	51.000	47.000	46.000	47.000	50.000	47.000	45.000	40.000	44.000
47.000	43.000	51.000	44.000	43.000	45.000	43.000	39.000	45.000	53.000	50.000	60.000	32.000	41.000	46.000	39.000	38.000	38.000	35.000	36.000	35.000	44.000	44.000	43.000	37.000
48.000	44.000	52.000	45.000	44.000	46.000	44.000	40.000	46.000	54.000	51.000	61.000	33.000	42.000	47.000	40.000	39.000	39.000	36.000	37.000	36.000	45.000	45.000	42.000	38.000
46.000	42.000	50.000	45.000	44.000	46.000	42.000	40.000	44.000	52.000	49.000	59.000	31.000	40.000	45.000	38.000	37.000	37.000	34.000	35.000	34.000	43.000	43.000	44.000	36.000
52.000	46.000	50.000	51.000	48.000	52.000	50.000	46.000	54.000	48.000	49.000	45.000	53.000	46.000	61.000	56.000	57.000	55.000	50.000	51.000	54.000	55.000	53.000	46.000	50.000
51.000	45.000	47.000	50.000	47.000	51.000	47.000	43.000	49.000	45.000	46.000	46.000	54.000	49.000	64.000	51.000	54.000	52.000	51.000	52.000	55.000	52.000	50.000	45.000	47.000
50.000	44.000	48.000	49.000	46.000	50.000	46.000	42.000	50.000	46.000	47.000	47.000	53.000	48.000	63.000	52.000	55.000	51.000	50.000	51.000	54.000	53.000	51.000	46.000	48.000
45.000	43.000	45.000	42.000	43.000	43.000	41.000	39.000	41.000	49.000	46.000	54.000	34.000	41.000	44.000	41.000	40.000	42.000	33.000	34.000	35.000	34.000	34.000	33.000	35.000
47.000	43.000	47.000	40.000	41.000	43.000	43.000	41.000	43.000	51.000	46.000	56.000	36.000	43.000	44.000	43.000	42.000	44.000	35.000	36.000	37.000	34.000	34.000	33.000	35.000
45.000	41.000	45.000	40.000	41.000	43.000	41.000	39.000	41.000	49.000	44.000	54.000	34.000	41.000	48.000	41.000	40.000	42.000	33.000	34.000	35.000	36.000	36.000	35.000	35.000
43.000	39.000	45.000	38.000	39.000	41.000	39.000	35.000	39.000	45.000	40.000	52.000	34.000	41.000	44.000	37.000	36.000	38.000	37.000	38.000	37.000	36.000	36.000	37.000	33.000
51.000	45.000	55.000	46.000	43.000	47.000	51.000	49.000	49.000	55.000	46.000	42.000	38.000	41.000	42.000	45.000	40.000	46.000	41.000	42.000	41.000	42.000	42.000	43.000	43.000
38.000	40.000	42.000	39.000	38.000	34.000	40.000	38.000	42.000	50.000	43.000	55.000	33.000	42.000	43.000	38.000	35.000	37.000	36.000	37.000	36.000	39.000	37.000	38.000	36.000

50:Sherb2	51:Sherb3	52:FCr1	53:FCr2	54:FCr3	55:Ararat 1	56:Ararat 2	57:Ararat 3	58:SMar1.1	59:SMar1.2	60:SMar1.3	61:SMar2.1	62:SMar2.2	63:SMar2.3	64:KP1	65:KP2	66:KP3	67:Bridg1	68:Bridg2	69:Bridg3
43.000	43.000	50.000	49.000	47.000	47.000	47.000	47.000	40.000	41.000	41.000	57.000	54.000	53.000	44.000	44.000	44.000	36.000	44.000	41.000
52.000	52.000	61.000	58.000	58.000	48.000	48.000	48.000	39.000	38.000	40.000	54.000	55.000	54.000	45.000	45.000	45.000	43.000	45.000	42.000
39.000	39.000	50.000	47.000	47.000	43.000	43.000	43.000	40.000	41.000	41.000	55.000	52.000	51.000	44.000	44.000	44.000	40.000	48.000	43.000
39.000	39.000	44.000	43.000	41.000	45.000	45.000	45.000	42.000	41.000	43.000	57.000	54.000	53.000	42.000	42.000	42.000	40.000	48.000	41.000
38.000	38.000	47.000	46.000	44.000	48.000	48.000	48.000	41.000	42.000	42.000	60.000	57.000	56.000	41.000	41.000	41.000	39.000	47.000	40.000
38.000	38.000	45.000	44.000	44.000	46.000	46.000	46.000	39.000	40.000	40.000	58.000	55.000	54.000	41.000	39.000	39.000	37.000	45.000	40.000
50.000	50.000	53.000	56.000	54.000	54.000	54.000	54.000	43.000	42.000	44.000	66.000	65.000	64.000	47.000	47.000	47.000	41.000	43.000	40.000
43.000	43.000	50.000	51.000	49.000	49.000	49.000	49.000	40.000	41.000	41.000	59.000	56.000	55.000	42.000	42.000	40.000	36.000	40.000	33.000
46.000	46.000	49.000	52.000	50.000	50.000	50.000	50.000	39.000	40.000	40.000	60.000	57.000	56.000	43.000	41.000	41.000	37.000	41.000	38.000
40.000	40.000	49.000	48.000	48.000	44.000	44.000	44.000	39.000	40.000	40.000	56.000	53.000	52.000	43.000	43.000	43.000	37.000	43.000	38.000
44.000	44.000	49.000	48.000	48.000	42.000	42.000	42.000	39.000	38.000	40.000	56.000	53.000	52.000	43.000	43.000	43.000	37.000	43.000	38.000
42.000	42.000	47.000	46.000	46.000	40.000	40.000	40.000	37.000	36.000	38.000	54.000	51.000	50.000	43.000	43.000	43.000	37.000	43.000	38.000
42.000	42.000	45.000	46.000	44.000	48.000	48.000	48.000	43.000	44.000	44.000	58.000	55.000	54.000	45.000	43.000	43.000	39.000	45.000	38.000
42.000	42.000	49.000	48.000	46.000	48.000	48.000	48.000	43.000	44.000	44.000	58.000	55.000	54.000	47.000	45.000	45.000	41.000	47.000	42.000
47.000	47.000	48.000	49.000	49.000	45.000	45.000	45.000	40.000	39.000	41.000	57.000	54.000	53.000	46.000	44.000	44.000	40.000	44.000	41.000
32.000	32.000	29.000	30.000	28.000	52.000	52.000	52.000	49.000	50.000	48.000	54.000	51.000	52.000	39.000	41.000	39.000	41.000	45.000	40.000
32.000	32.000	39.000	40.000	40.000	52.000	52.000	52.000	43.000	44.000	42.000	52.000	49.000	50.000	35.000	35.000	33.000	37.000	43.000	44.000
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28.000	28.000	15.000	4.000	.000	38.000	38.000	38.000	49.000	48.000	48.000	44.000	41.000	42.000	39.000	41.000	39.000	41.000	47.000	46.000
44.000	44.000	41.000	36.000	38.000	.000	.000	.000	33.000	32.000	34.000	20.000	15.000	14.000	35.000	35.000	35.000	35.000	47.000	34.000
44.000	44.000	41.000	36.000	38.000	.000	.000	.000	33.000	32.000	34.000	20.000	15.000	14.000	35.000	35.000	35.000	35.000	47.000	34.000
44.000	44.000	41.000	36.000	38.000	.000	.000	.000	33.000	32.000	34.000	20.000	15.000	14.000	35.000	35.000	35.000	35.000	47.000	34.000
37.000	37.000	54.000	49.000	49.000	33.000	33.000	33.000	.000	1.000	1.000	45.000	42.000	41.000	22.000	22.000	20.000	12.000	26.000	17.000
38.000	38.000	53.000	48.000	48.000	32.000	32.000	32.000	1.000	.000	2.000	44.000	41.000	40.000	23.000	23.000	21.000	13.000	27.000	18.000
36.000	36.000	53.000	48.000	48.000	34.000	34.000	34.000	1.000	2.000	.000	46.000	43.000	42.000	23.000	23.000	21.000	13.000	27.000	18.000
50.000	50.000	47.000	42.000	44.000	20.000	20.000	20.000	45.000	44.000	46.000	.000	7.000	6.000	45.000	47.000	45.000	47.000	45.000	46.000
47.000	47.000	44.000	39.000	41.000	15.000	15.000	15.000	42.000	41.000	43.000	7.000	.000	1.000	40.000	42.000	40.000	40.000	44.000	41.000
48.000	48.000	45.000	40.000	42.000	14.000	14.000	14.000	41.000	40.000	42.000	6.000	1.000	.000	41.000	43.000	41.000	41.000	45.000	40.000
35.000	35.000	38.000	39.000	39.000	35.000	35.000	35.000	22.000	23.000	23.000	45.000	40.000	41.000	.000	2.000	4.000	12.000	22.000	19.000
35.000	35.000	40.000	41.000	41.000	35.000	35.000	35.000	22.000	23.000	23.000	47.000	42.000	43.000	2.000	.000	4.000	12.000	22.000	21.000
35.000	35.000	42.000	39.000	39.000	35.000	35.000	35.000	20.000	21.000	21.000	45.000	40.000	41.000	4.000	4.000	.000	10.000	20.000	19.000
33.000	33.000	46.000	41.000	41.000	35.000	35.000	35.000	12.000	13.000	13.000	47.000	40.000	41.000	12.000	12.000	10.000	.000	16.000	11.000
43.000	43.000	50.000	47.000	47.000	47.000	47.000	47.000	26.000	27.000	27.000	45.000	44.000	45.000	22.000	22.000	20.000	16.000	.000	21.000
36.000	36.000	49.000	46.000	46.000	34.000	34.000	34.000	17.000	18.000	18.000	46.000	41.000	40.000	19.000	21.000	19.000	11.000	21.000	.000

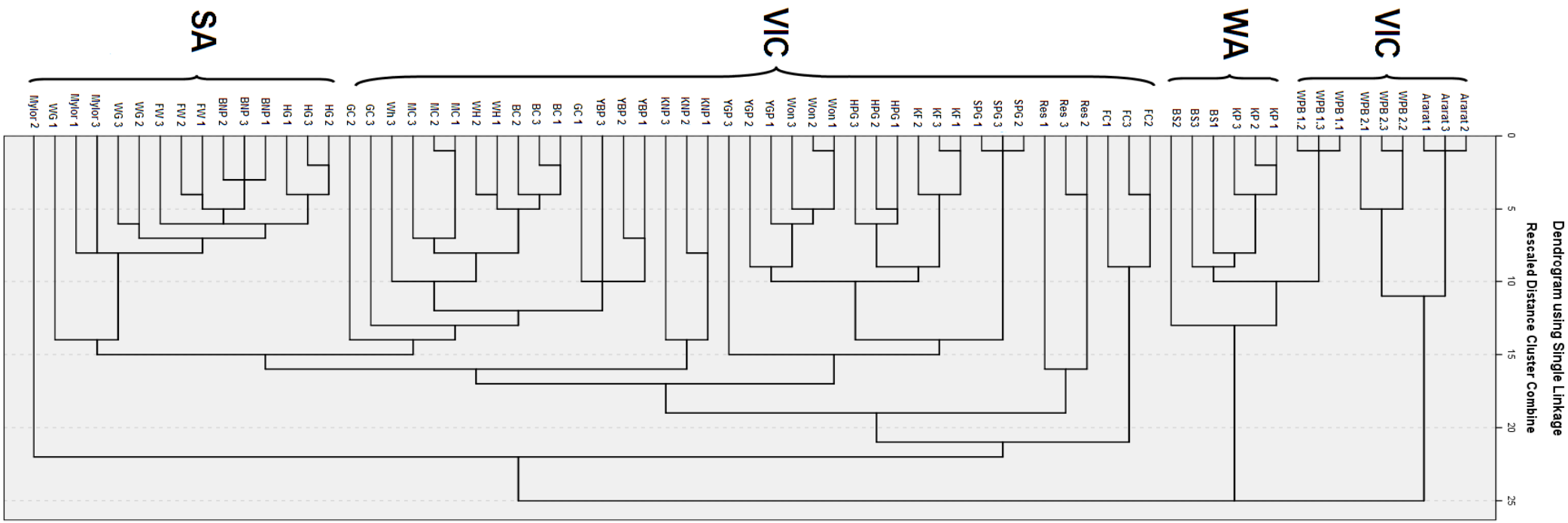
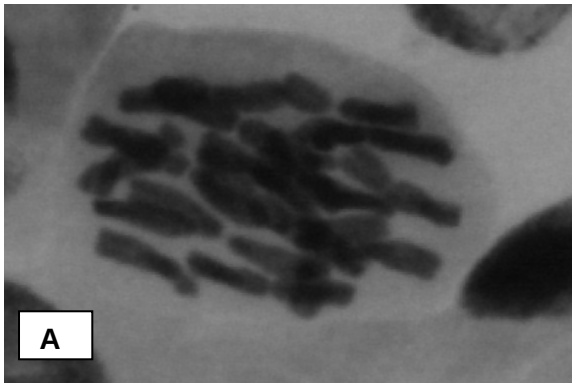


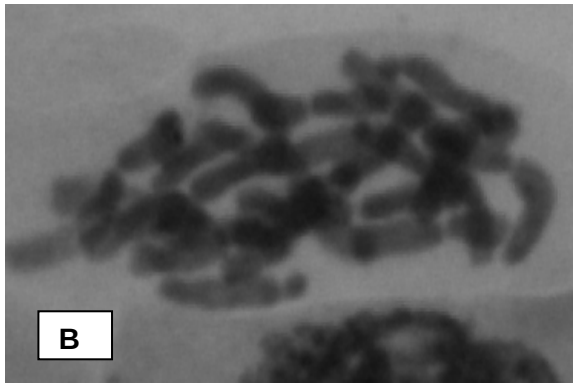
Figure 2.18 RAPD dendrogram using single-linkage rescaled distance cluster combine, generated by SPSS Hierarchical Cluster analysis, of *Allium triquetrum* provenances throughout Australia. Three replicates from each provenance were used for RAPD-PCR analysis. Provenances are as follows: Victoria: Ararat, Reservoir (Res), Ferny Creek (FC), Sherbrooke Picnic Ground (SPG), Yarra Bend Park (YBP), Westernport Bay 1 (WPB1), Westernport Bay 2 (WPB 2), Yellow Gum Park (YGP), Gardiner's Creek (GC), Merri Creek (MC), Wonthaggi (Won), Bendigo Creek (BC), Kangaroo Flat (KF), Kinglake National Park (KNP), Hardy's Picnic Ground (HPG) and White Hill (WH). South Australia: Mylor, Belair National Park (BNP), Waterfall Gully (WG), Horsnell Gully (HG) and Fairview (FW). Western Australia: Bridgestone (BS) and Kings Park (KP).

2.3.5 Karyotyping of A. triquetrum

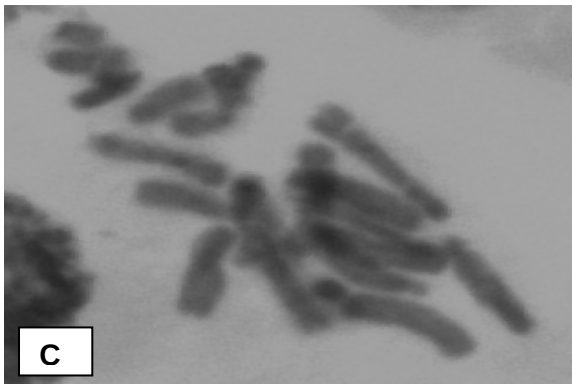
Clear metaphase plates were obtained using 0.05% colchicine pre-treatment for 2 h at room temperature. Cells were diploid (2n=18) for all provenances (Fig. 2.18). Metacentric, submetacentric and acrocentric chromosomes were observed.



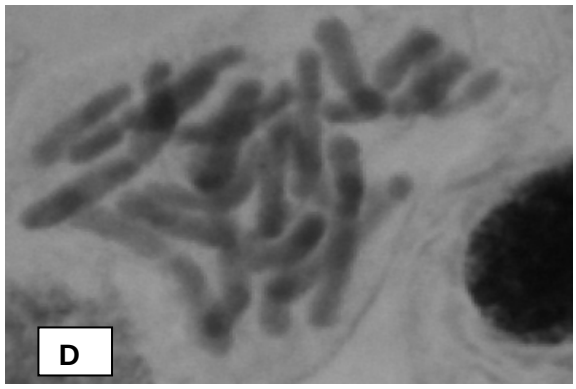
Waterfall Gully (SA)



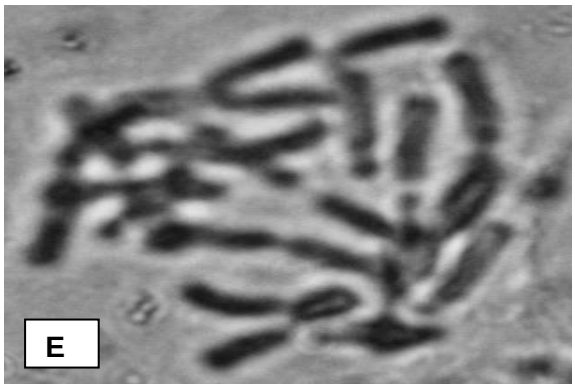
Mylor (SA)



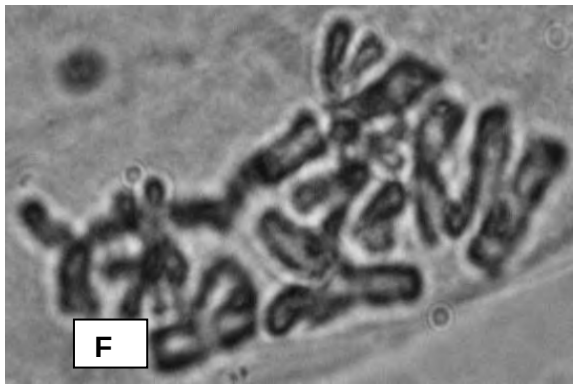
Horsnell Gully (SA)



Belair National Park (SA)



Yellow Gum Park (VIC)



Yarra Bend Park (VIC)

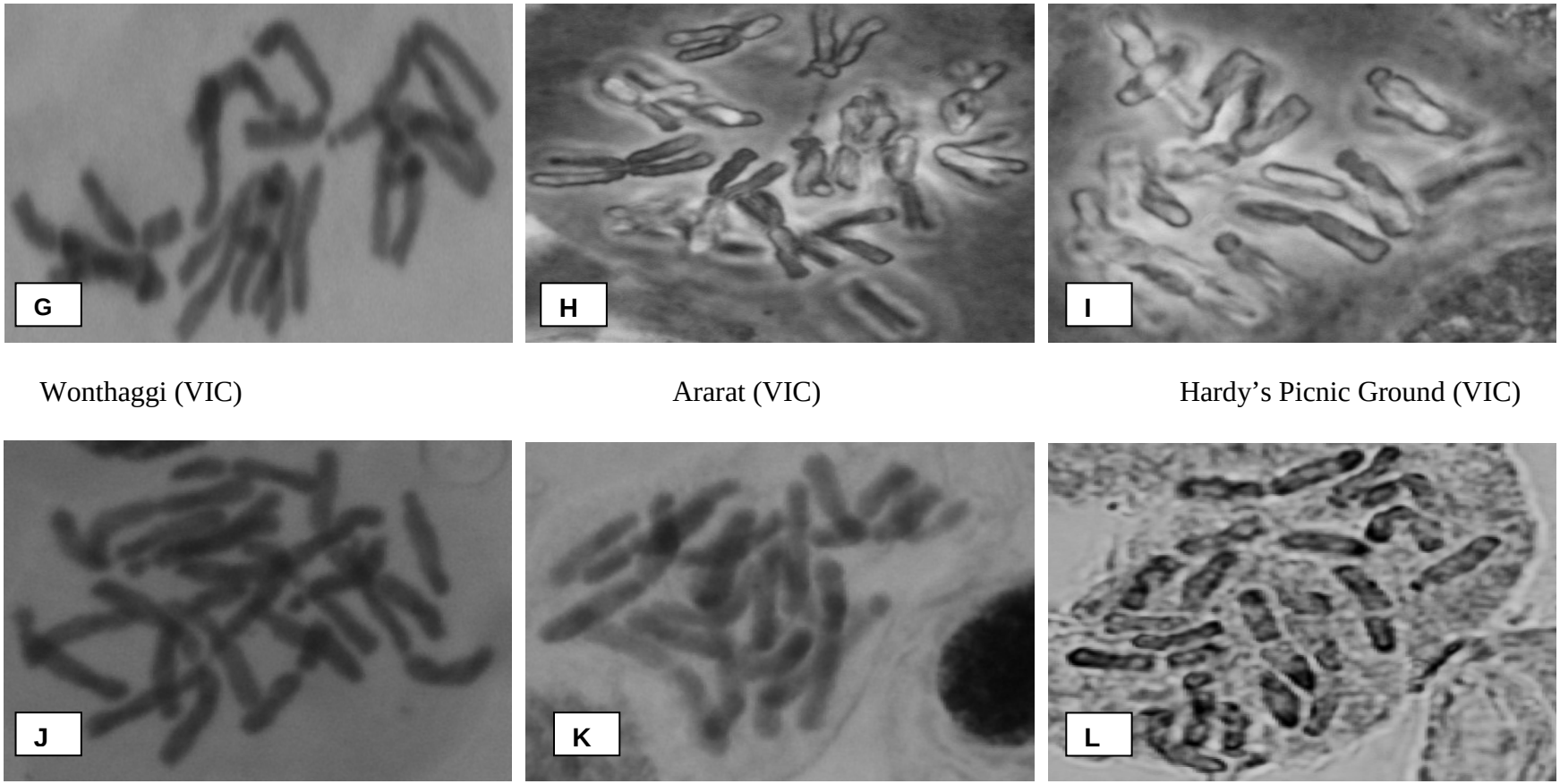


Figure 2.19 Mitotic metaphase of *Allium triquetrum* cells stained by HCl-carmine and photographed with a Leica DM2500 compound microscope at x2000 magnification. Images are labelled as follows: A: Waterfall Gully (SA), B: Mylor (SA), C: Horsnell Gully (SA), D: Belair National Park (SA), E: Yellow Gum Park (VIC), F: Yarra Bend Park (VIC), G: Wonthaggi (VIC), H: Ararat (VIC), I: Hardy's Picnic Ground (VIC), J: Bendigo Creek (VIC), K: King's Park (WA), L: Bridgestone (WA).

2.4 DISCUSSION

Genetic analysis of *A. triquetrum* provenances across Australia suggested that the degree of variation was relatively small, as would be expected for a species that spread within provenances mainly by clonal propagation from bulbs. This makes it a suitable target for biological control as large genetic variation may result in genotypes varying in susceptibility to biocontrol agents and reduce the effectiveness of biocontrol of the invasive species. The variation was mainly confirmed to a few provenances and suggests that either there were different incursions from overseas or that seed production has produced new genotypes. It also suggests that any biological control agents must be screened on the provenances that show the extremes of genetic variation to confirm the agent is equally effective on all genotypes.

2.4.1 ITS analysis

The ITS products were ~800 bp for all provenances except Bridgestone sample 2 and 3 (~790 bp). This may be due to deletions near the start of the ITS1 region or the end of the ITS4 region, since they were not observed in the 718 bp edited sequences used for alignment and analysis or the RFLP analysis. Using primers for the ITS1 and ITS2 regions separately (White *et al.* 1990) may elucidate where these occur. These two Bridgestone samples should be included in any screening of biological control agents to ensure adequate testing

The RFLP result also indicated differences between and within provenances based on digested ITS-PCR products, e.g. Westernport Bay 2, Reservoir and Bridgestone. Observation of fragments with the same size as the original ITS products suggested that the ITS-PCR products were not always digested completely. It was unlikely to be because of insufficient restriction endonuclease in the RFLP reaction, as other enzymes digested

completely. Failure of some restriction enzymes to digest completely has been noted previously (Nusaibah *et al.* 2011). It was also unlikely to be because of heterocaryons because most of the products digested (Hibbett 1992). RFLP analysis is a useful tool to detect genetic variations in different species (Krupa 1999; Arifin et al 2000; Yamashita and Tashiro 2001). It is useful to find specific genes on a chromosome and understanding the genetic models for the plant's breeding system (Farooq and Azam 2002). RFLP was a useful tool here to screen the genetic diversity in *A. triquetrum* ITS-products, and to select samples to be sequenced but was not comprehensive enough to show all the genetic diversity. Also, as the ITS-PCR products are used as a template for RFLP analysis, RFLP analysis only showed differences in a small part of the genome.

The 85% similarity of the ITS sequences suggests that *A. triquetrum* in Australia is the same species but has different genotypes. The Bridgestone samples, for example, had a few deletions when sequences were aligned. Most nucleotide differences in the ITS sequences between provenances are the result of single-base substitution rather than large deletions or inserts, indicating random variation. The only group with significant genetic diversity (23%) was from Sherbrooke (2-3) in Victoria where two of the three samples were genetically diverse from one another (73%) as well as from samples from other provenances. This suggests either that there was more than one genetically different incursion or that sexual reproduction coupled with inherent genetic variation with this provenance led to genetically diverse progeny. The presence of genetically dissimilar individuals from such a small sample number suggests some degree of sexual reproduction by seeds rather than high mutagenesis in somatic cloning by bulbs. This suggests, in turn, that these populations may have initially been established from seeds rather than bulbs and continue to reproduce actively by seeds as well as bulbs. This also

emphasises the importance of ensuring that the small black seeds do not leave infested sites.

Previous studies on some plant species have ascertained that even small variations in the ITS region can lead to differences within species as subspecies (Hsiao *et al.* 1995; Ainouche and Bayer 1997). Morphological characterization and ecological studies are complementary to molecular phylogeny and have traditionally been essential for differentiation of taxonomic categories (Inden and Asahira 1990; Ainouche, Misset and Huon 1996; Wako *et al.* 2009). The ITS sequence is a small part of the genome which is less than 1 kbp. To determine the genetic diversity more completely, other molecular techniques that screen the whole genome are needed. Despite the universal use and the popularity of ITS-PCR data in taxonomic and genetic diversity studies, Álvarez and Wendel (2003) concluded that its unpredictable evolutionary performance diminished the efficacy of this sequence in phylogenetic analysis.

2.4.2 RAPD-PCR

RAPD analysis showed genetic diversity between and within *A. triquetrum* provenances across Australia. Welsh and McClelland (1990) proposed that, since a large part of the genome can be screened by RAPD analysis, even small genetic variation is disclosed. This technique has been used successfully for phylogeographic and classification studies in many taxa (Gabrielson *et al.* 1997; Friesen *et al.* 1999). Geographical grouping of the plant samples into the Australian states suggests perhaps single origins for infestations in South and Western Australia but multiple incursions or a single genetically heterogeneous incursion in Victoria. The greatest variation between and within provenances was observed in DNA samples from Victoria. This might be because more provenances were sampled in Victoria than in South and Western Australia. The RAPD dendrogram

separated the provenances Ararat and Westernport Bay 2 together in one of the three main clades and morphologically they produced larger bulbs than other provenances, suggesting that both should be included in screening any potential biological control agent.

The genotype differences between provenances throughout Australia based on RAPD analysis suggest that *A.triquetrum* distribution is at least partly by seeds rather than only bulbs and bulblets. If the plants reproduce only clonally in asexual propagation by bulbs and bulblets, no genetic diversity would be expected, especially within provenances (Persson and Gustavsson 2001). Environmental factors such as soil fertility and ecological aspects can select for some specific genetically diverse progeny (Cavan *et al.* 2000). Natural selection under different environmental conditions even at the same site might therefore have resulted in genetic variation even within one provenance, and different conditions in different provenance may have led to selection of the most suitable genotype for each. *A. triquetrum* seeds were dormant *in vitro* but germinated after 2 months under constant misting in the glasshouse, whereas bulbs grew up rapidly after a light cold shock (chapter 3), therefore the chance of asexual reproduction by bulbs and bulblets is likely to be greater than seeds. Further analysis of single bulbs and seedlings within and between provenances would test the degree to which genetic variation is the result of seedlings.

The sensitivity of RAPD-PCR and screening of the whole genome suggests that RAPD analysis was a more useful tool than sequencing the ITS region to show genotypic variation between and within provenances of *A. triquetrum*, as noted in other species (Hadrys *et al.* 1992; Baumel *et al.* 2001; Persson and Gustavsson 2001). The main problem with RAPD-PCR is reproducibility of the amplicons and it is essential to have a consistent protocol to obtain reproducible bands (Rajput *et al.* 2006). In this study RAPD-PCR was performed three times using a single thermocycler (G-STORM 2) thermocycler

and seven primers showed consistent banding patterns. Failure to obtain the same banding pattern using the other thermocycler of the same model and age showed how important it was to maintain consistency. DNA purity is also a critical factor to obtain consistent results. Once reactive RAPD primers had been selected, only one DNA sample (Mylor 2) did not react with some RAPD primers. Low reproducibility may also reflect DNA damage (Uzonur *et al.* 2007) but was not noted in this study.

2.4.3 Karyotype analysis

Only the diploid (2n=18) number of chromosomes were observed in this study suggesting that triploid forms (3n = 27) (Balog 1979) do not exist in Australia, though more collections would be required for certainty. Balog (1979) isolated a triploid form of *A. triquetrum* from a wild population in New Zealand but it was not clear how the triploid formed or was found in the wild. Of 23 provenances used for RAPD analysis, bulbs from 12 infestations across Australia were all diploid. The samples from Ararat and Westernport Bay 2, which were clustered together in the RAPD dendrogram separate from the rest of Victorian provenances, had larger bulbs that could have indicated *gigas* characters, which tend to be associated with polyploidy; however, the Ararat sample was diploid. If seeds are a source of variation, it might be better to employ seeds for karyotypic studies, thereby giving an enhanced overview of ploidy variation.

2.4.4 Conclusions and further research

The low variation in both ITS-PCR products and RFLP analysis suggested that *A. triquetrum* in Australia is the same species but of different closely similar genotypes, as would be expected from a plant with a narrow genetic base that reproduces mainly clonally by bulbs rather than seeds. There was no distinction in chromosome number of *A. triquetrum*. RAPD-PCR is a useful bio-monitoring tool in genetic diversity studies but

there are problems in of consistency of protocol using this technique (Pambuli 1996). Using other molecular technique such as Inter-simple sequence repeats (ISSR) could be helpful to investigate further the genetic diversity of *A. triquetrum*. More sample collections from other infestations and further analysis of seedlings would show better the degree of diversity between and within provenances. In conclusion, *A. triquetrum* genetic diversity is an important factor in biological control program of this noxious weed and its variation in genotype must be used for adequate screeing of potential biocontrol agents to ensure successful biocontrol in all provenances.

Chapter 3

Evaluation of *Stromatinia cepivora* as biological control agent

3.1 *Stromatinia cepivora* - a potential biocontrol agent

3.1.1 Taxonomy and classification

Stromatinia cepivora (Berk.) Whetzel was previously known by the synonym *Sclerotium cepivorum* Berk. (http://www.indexfungorum.org/Names/Names.asp). The fungus is classified as phylum: Ascomycota, class: Leotiomycetes, order: Helotiales, family: *Sclerotiniaceae* and genus: *Stromatinia* with no known sexual stage (Whetzel 1945) (Table 3.1).

Table 3.1 Classification of *Stromatinia cepivora* Whetzel 1945 (http://www.indexfungorum.org/Names/Names.asp).

Kingdom: *Fungi*

Phylum: Ascomycota

Class: Leotiomycetes

Order: Helotiales

Family: Sclerotiniaceae

Genus: *Stromatinia*

Species: *Stromatinia cepivora* (Berk.) Whetzel

3.2 Disease

3.2.1 Host specificity and life cycle

S. cepivora is a serious soil-borne pathogen of commercial *Allium* crops (Alexopoulos and Mims 1979) such as *Allium cepa* (onion), *Allium cepa* var. *aggregatum* (shallot), *Allium sativum* (garlic), *Allium tuberosum* (garlic chives), *Allium schoenoprasum* (chives), *Allium porrum* (leek) and *Allium fistulosum* (spring onion). It reproduces asexually by forming small spherical black sclerotia (Fig. 3.1) (masses of hardened mycelium) on white hyphae (Agrios 2005). The fungus is resistant to cold climates and survives between hosts as sclerotia (Booer 1946), which can persist in the soil for up to 20 years in the absence of the host plant (Coley-Smith & Holt 1966; Coley-Smith 1979; Coley-Smith et al. 1990) and remain dormant in soil until stimulated by *Allium* root exudates. Sclerotia germinate in cool temperatures with soil moisture at field capacity and mycelium develops in the soil. Once the hyphae contact the host plant, appresoria form, the fungus penetrates the host tissue and later sclerotia form on the completely decayed plants and fall into the soil (Crowe 2008). The fungus also forms unicellular microconidia (Fig. 3.2) and their germination on onion leaves has been reported but did not lead to infection of the host plants (Gindro and L'Hoste 1997). Other *Sclerotium* species propagate sexually by basidiospores (*Sclerotium sclerotiorum*) or ascospores (*Sclerotium oryzae*), or asexually by conidia (*Sclerotium bataticola*) (Barnett and Hunter 1972; Agrios 2005).

Figure 3.1 Sclerotia and white mycelium of *Stromatinia cepivora* developing on stem and bulb of garlic.

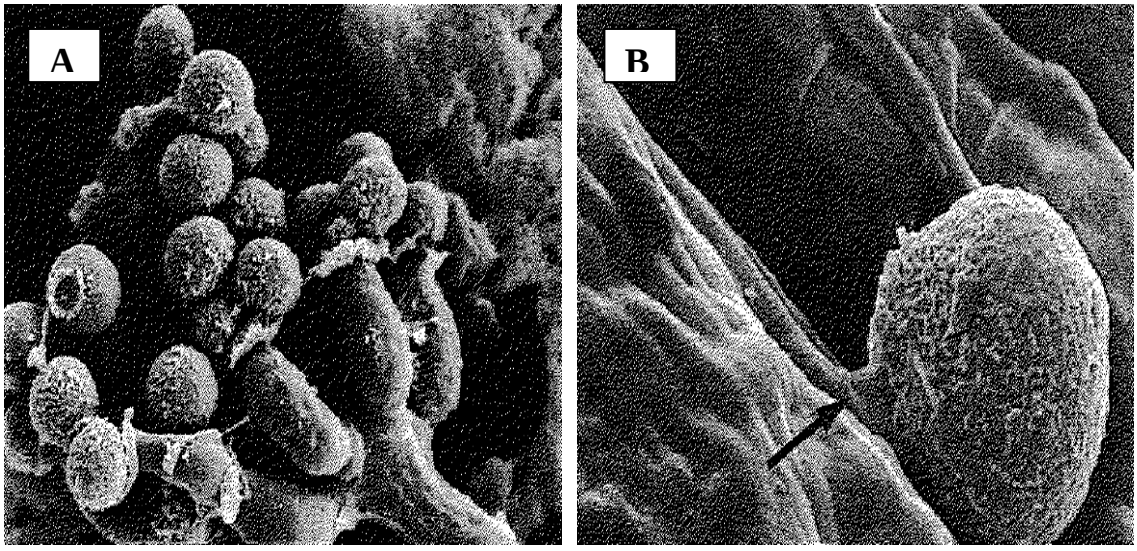


Figure 3.2 Microconidial production and germination of *Stromatinia cepivora* by scanning electron microscopy. A: a cluster of microconidia on a microconidiophore, 4 weeks after inoculation on potato dextrose agar. B: a germinated microconidium 8 days after inoculation on an onion leaf (Gindro and L'Hoste 1997).

3.2.2 Disease symptoms and infection process

S. cepivora infect young seedlings of cultivated *Allium* species severely. The disease usually occurs on young foliage, bulbs and roots in the field. In the soil, *Allium* root exudates seem needed to stimulate sclerotia to germinate in the soil even though sclerotia germinate readily on sterile media in the laboratory (Coley-Smith and Holt 1966; Allen

and Young 1968; Coley-Smith *et al.* 1990). The disease symptoms usually become visible only when root and crown infection is well stablished (Koike *et al.* 2007) and infected plants exhibit premature yellowing of the foliage and leaf tips (Anwar Haq *et al.* 2003), followed by collapse of the older leaves and eventually necrosis of the whole plant (Abd-El-Razik *et al.* 1973; Mackie and McKridy 2002). The pathogen can be transferred plant-to-plant easily and one infected host plant is enough to spread the disease to 40 adjacent plants (Crowe and Hall 1980a). Infected *Allium* plants can be pulled out easily in the field in advanced stages of disease, when the bulbs and bulblets are covered by white mycelium and sclerotia and become soft and rotted.

The hyphae penetrate the host bulb and root epidermis and cortex and develop intracellularly into cortical parenchyma (Abd-El-Razik *et al.* 1973; Crowe and Hall 1980a). Metcalf (2004) reported that *S. cepivora* in Tasmania caused extensive infection of cortical tissues of the onion root, although the epidermal and stele tissues remained healthy.

3.2.3 Distribution

S. cepivora is one of the most important widespread and destructive diseases of cultivated *Allium* species (Schwartz & Mohan 1995). It is distributed worldwide in different regions of Asia, Africa, and Europe, America, Australia and New Zealand (Crowe *et al.* 1980; Entwistle, 1990; Schwartz & Mohan 1995; Pinto *et al.* 1998; Zewide *et al.* 2007a,b). Infestation of the fungus appears during garlic harvesting in California, USA (Crowe and Hall 1980a,b). Numerous losses of 1-100% in onion crops have been reported in Mexico (Perez-Moreno *et al.* 1998) and garlic yield was significantly reduced in Ethiopia (Zewide *et al.* 2007a) and Spain (María *et al.* 1993).

About 5300 ha of Australian agricultural land is infested by *S. cepivora*, mainly in Victoria; of 2500 ha of Allium-growing areas, almost 2000 ha was affected by the fungus in 1979 (Merriman *et al.* 1979). It is widespread in Eastern Australia (Merriman *et al.* 1980; Mackie and McKirdy 2002) and in Queensland the fungus grows on onions in winter, which is the favourable time for sclerotial germination (Wong and Hughes 1986). In Tasmania also the commercial onion industry has been greatly affected and yield declines in late winter as a result of disease (Metcalf *et al.* 2004). Abnormally large sclerotia were detected in Tasmania 1996; these were 0.5 cm diameter (Metcalf 1997). This size of sclerotia has not been observed elsewhere in Australia. It has not been detected so far in Western Australia, probably due to inter-state quarantine (Mackie and McKirdy 2002). The fungus was detected in Canterbury, New Zealand, in 1930 and significant damage to onion crops was reported by 1960 in major onion-growing areas in New Zealand (Beeson 1960).

3.2.4 Factors affecting disease

Development of disease is the result of the interaction of three components: virulence of the pathogen, environmental conditions and susceptibility of the host (Agrios 2005). Temperature, soil depth, pH, soil moisture, soil texture, stimulating materials, and interactions with various other microorganisms are factors that alter the germination of sclerotia of *S. cepivora* (Cook *et al.* 1975; Leggett *et al.* 1983; Smith *et al.* 1989; Alexander and Stewart 1994; Gindro and L' Hoste 1997).

3.2.4.1 Depth of Soil

Mycelial growth of *S. cepivora* occurs in two stages. In the primary stage, mycelium is produced by sclerotial germination and grows 1-2 cm in the soil. In the secondary stage, mycelium spreads from the infected host plant to adjacent plants and eventually forms

sclerotia on all parts (Crowe and Hall 1980b). Vertical dissemination of sclerotia has been reported in different depths of soil in onion-growing areas. Crowe and Hall (1980b) showed sclerotial germination and white rot on garlic cloves (Californian Late) as deep as 30 cm in a glass-walled box containing air-dried soil. The soil was inoculated with 1000 sclerotia at various depths before planting garlic. Usually most fungal activity was 1-3 mm around the germinated sclerotia. The number of germinated sclerotia declined with increase in soil depth, probably because there were fewer roots in the lower zone and the limited mycelium from sclerotia could not reach them (Rayan and Kavanaugh 1977).

3.2.4.2 Soil temperature

S. cepivora demonstrated greatest infection on salad onion during late spring to summer and minimal infection in late autumn and early winter under field conditions, matching the growth of the host (Entwistle and Munasinghe 1979). The optimum temperature for sclerotia to germinate is 20ºC, but germination has been reported from near zero up to 35ºC (Asthana 1946) and sclerotial germination declined rapidly in winter (Leggett *et al.* 1983). Variation in temperature across 4-20ºC does not have a large influence on sclerotial and microconidial production but sclerotial production was slower at 4ºC (Gindro and L' Hoste 1997). Some other species of the *Sclerotiniaceae* family such as *Botrytis fabae* have also demonstrated greater microconidia production at low temperature and in high soil moisture (Harrison and Hargreaves 1977). The sclerotial number declined significantly when temperature was increased to 35ºC (Vannacci *et al.* 1988). Adams (1987) showed that sclerotial viability decreased by 50% at 50ºC for 48 min *in vitro*. Similar observations were reported by Porter and Merriman (1983), in which 50ºC for 6 hours/day reduced sclerotial viability by 50% after 7 days and by 100% after 14 days.

3.2.4.3 Soil moisture

Soil moisture plays a critical role in white rot disease incidence. High soil moisture leads to a decline in sclerotial number and germination (Cook *et al.* 1975; Coley-Smith 1990), but it did not affect microconidial production (Harrison 1979). Gindro and L' Hoste (1997) stated that in high soil moisture microconidia of *S. cepivora* were responsible for the fungus' survival but there is no report of white rot disease by microconidia *in vivo*. No sclerotia formed on PDA cultures immersed in water, where a cushion-like hyphal growth covered the medium and the water surface (Gindro and L' Hoste 1997). Like sclerotia of *Sclerotium rolfsii*, which remain dormant at field capacity (Bandara 1980), long-term flooding in the field reduces the sclerotia number of *S. cepivora* or sclerotia lose their viability by (Cook *et al.* 1975; Coley-Smith 1990).

3.2.4.4 Soil texture and pH

Soil texture and pH also affect sclerotial viability (Adams 1975; Alexander and Stewart 1994). *S. cepivora* occurs in clay loam, silty clay, muck soil and Bagshot sand (Leggett *et al.* 1983; Alexander and Stewart 1994; Harper and Stewart 2000; Harper *et al.* 2002). A combination of soil type and potential biocontrol agents such as *Trichoderma pseudokoningii* affects the survival of sclerotia. Up to 80% of the sclerotia of *S. cepivora* were degraded by *T. pseudokoningii* in silty clay soil (Clarkson *et al.* 2002) but only 60% in other soil types (Clarkson *et al.* 2004). Leggett *et al.* (1983) reported that the high organic material of Muck soil may be responsible for the reduction in sclerotial viability of *S. cepivora,* which was reduced to 26.8% after only 4 months.

3.2.4.5 Factors affecting dormancy

Coley-Smith and Elser (1983) stated that the sclerotia of *S. cepivora* can be dormant in the soil for up to 20 years in the absence of the host plants. Sclerotia normally undergo dormancy of 1-3 months in the field, during which they do not show any response to *Allium* species, but germinate on sterile medium (Coley-Smith 1960). Two forms of dormancy, constitutive and exogenous, occur in *S. cepivora*. Constitutive dormancy can be expressed as a physiological inability of the sclerotia to germinate that can be overcome by chemical stimulation or long-term incubation in non-sterile soil (Sussman and Halvorson 1966; McLean *et al.* 2005). Exogenous dormancy occurs in unfavourable conditions for the sclerotia to germinate. Insufficient endogenous nutrients in many fungal propagules and high microbial competition in nutritionally rich soil may lead to failure in germination in soil (Ko and Lockwood 1967; Bristow and Lockwood 1975). Sclerotia of *S. cepivora* do not need the exogenous nutrition to germinate and so antibiosis may be the main reason for their long dormancy (Allen and Young 1968). In the field, *Allium* root exudates are responsible for sclerotial germination of *S. cepivora*. A component of primary *Allium* root exudate, S-alk(en)yl-L-cysteine sulphoxides diallyl disulphide, is broken down by microbial activity to 1-propyl- and 2-propenyl (diallyl) disulphide (Fig. 3.3). The breakdown products stimulate the sclerotia to germinate in the soil (Coley-Smith 1960; Coley-Smith and Holt 1966; Elser and Coley-Smith 1983). In the absence of the host plant, sclerotial dormancy can be overcome by burial of sclerotia in unsterile soil for 5 weeks in laboratory conditions, but the reason for this dormancy breakdown is unknown (Crown *et al.* 1980). Sclerotia produced on plates of media by the fungus, however, germinate readily when transferred to new media.

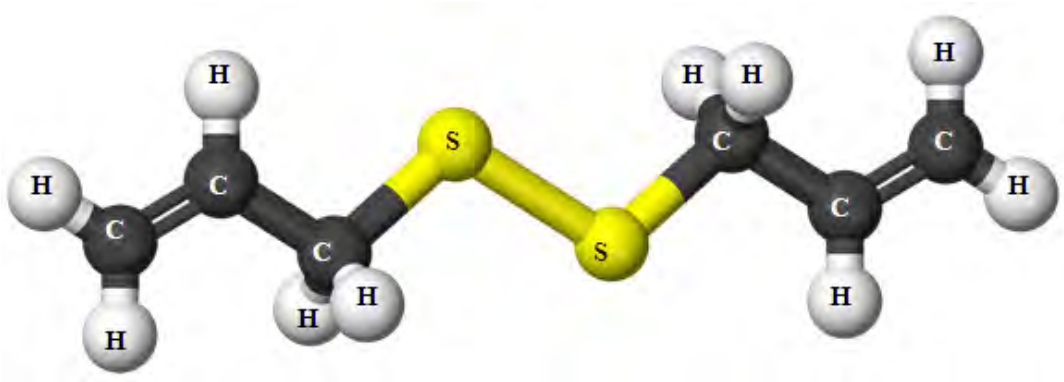


Figure 3.3 Chemical structure of diallyl disulphide in *Allium* root exudates, known as a stimulating factor for sclerotial germination in *Stromatinia cepivora* (www.garlic-central.com/allicin-chemistry.html)

3.3 Control of *S. cepivora*

Introduction of a serious horticultural crop pathogen to control weedy *Allium* species would need serious consideration as to how it could be controlled or eliminated if it presents too great a threat to other species. The following section, therefore, deals briefly with control methods for *S. cepivora* in *Allium* crops.

3.3.1 Mechanical control including crop rotation, flooding and artificial germination of sclerotia

As *Allium* white rot disease is a recurrent problem for cultivated *Allium* species and causes significant annual yield reductions, management of the pathogen is vital. Control of diseases caused by sclerotial fungi is very difficult due to the high sclerotial production in soil and their dormancy; therefore the disease management needs special controlling strategies (Zewdie 2007 a,b). Crop rotation, flooding, stimulation of sclerotial germination, and soil solarisation are methods that have been tested for controlling *S. cepivora* instead of chemical use.

3.3.1.1 Soil solarisation

Soil solarisation (covering the soil surface by thick black polythene during the hot season) is an effective way to control most soil-borne fungi in less than 4 months (Katan 1981) and is one of the most satisfactory ways to control *Allium* white rot. Significant reduction of sclerotial number in a highly infested area in Spain has been achieved by soil solarisation after 11 weeks (Basallote-Ureba and Melero-Vara 1993). An increase in temperature (up to 35ºC) reduced the sclerotial population significantly in the field (Porter & Merriman 1985; Coley-Smith 1987; Vannacci *et al.* 1988; Satour *et al.* 1989; Basallote-Ureba and Melero-Vara 1993; Melero *et al.* 2000). However, although the sclerotial inoculum was decreased in the top 10 cm depth of artificially inoculated soil in Victoria, Australia, the disease level was not changed (Porter and Merriman 1985). This was not the experience in other studies; Basallote-Ureba and Melero-Vara (1993) recorded that disease incidence decreased to ~25% in garlic crops 2.5 months after soil solarisation in Southern Spain and garlic yields increased up to 331%. The incidence of disease and sclerotia density increased after planting during the second year up to the level where it was uneconomic to grow the crop due to losses. Soil solarisation also increased the garlic yield up to 152% in Mexico, decreased sclerotial viability by up to 84% and decreased disease incidence to 22% (Ulacio-Osorio *et al.* 2006). Desiccation may also play a role, as germination of sclerotia declined when they were air-dried before burial (Smith 1972).

3.3.1.2 Stimulation of sclerotial germination

Artificial stimulation in order to germinate sclerotia in the absence of the host can reduce the inoculum in soil (Coley-Smith & Parfitt 1986; Entwistle 1990; Mc Donald & Hovius 1998), as the lifetime of hyphae from germinated sclerotia in the absence of *Allium* species would be short. Coley-Smith and King (1969) reported that *Allium* exudates such as monosulphides and disulphides component, or allyl radicals, could be used to stimulate

sclerotial germination. Sclerotial stimulation by onion oil reduced the sclerotia number from 187 to 87/kg in South Werribee, Victoria, Australia and a 5% solution of onion oil in water reduced sclerotial number by up to 91% in the top 10 cm depth of the field soil (Merriman *et al.* 1980).

3.3.1.3 Flooding

Flooding is an effective strategy to reduce sclerotial number in soils and disease in the following crop. For example, winter flooding in muck soils resulted in a reduction of 80% in sclerotial number in eastern Canada (Banks and Edgington 1989). In British Columbia, winter flooding reduced sclerotial survival significantly (Leggett and Rahe 1985). Banks and Edgington (1989) showed that flooding reduced the sclerotial population by 11% in young sclerotia and 23% in aged sclerotia, although disease was still present when *Allium* species were planted in the field. Crow and Carlson (1995) reported that single-season flooding in Tulelake, California, USA, was a potential means of management to reduce the sclerotial number up to 96%, but a subsequent increase in sclerotial population was observed in late summer and early fall. Flooding after the crop has been harvested may eradicate the sclerotia for the next planting season, as Crow and Carlson (1995) showed two seasons of flooding degraded the sclerotia completely at low cost relative to fumigants.

3.3.1.4 Crop rotation

Crop rotation in fields infested by sclerotia-forming fungi can reduce the disease incidence and sclerotial density in soil. There are few reports on the effect of crop rotation to control *S. cepivora* but sclerotia of *Sclerotium rolfsii* were reduced by up to 62% in peanut by crop rotation with cultivated *Allium* (Zeidan *et al.* 1986) and similar effects may occur

with *S. cepivora*. Planting crops such as carrot (non-host) rather than *Allium* species in the infested area resulted in a decline in the population of sclerotia within one year (Banks and Edgington 1989). Although crop rotation reduced sclerotial number, the remaining sclerotia in the onion-growing field were sufficient for the disease to occur in the next growing season. Coley-Smith (1960) stated that crop rotation was an ineffective management technique for the *Allium* white rot disease, as the sclerotia are dormant in soil for up to 20 years in the absence of the host plant. This is, however, a desirable characteristic for a biocontrol agent, as it gives long-term control against seedlings of *A. triquetrum*.

3.4 Chemical control

Both contact and systematic fungicides reduce white rot disease significantly (Table 3.2) (Zewide *et al.* 2007a). Fungicides such as vinclozolin and iprodione are members of the imide group of the dicarboximides class of fungicides that control *Allium* white rot disease to the desired level, but microbial degradation decreases their effectiveness (Melero-Vara *et al.* 2000). Vinclozolin and iprodione showed 75-95% control of white rot at 45 and 75 days after application as soil and seed treatment. Tebuconazole also demonstrated a significant control of the *S. cepivora* (Melero-Vara *et al.* 2000; De Dennis 2001). The disease incidence in garlic was reduced by 40-44% and yield increased by 43-73% by using tebuconazole in Ethiopia (Zewide *et al.* 2007b). Systemic fungicides such as benomyl and tebuconazole are more effective than contact fungicides in crop protection and are effective for longer times (Fullerton *et al.* 1995; Melero-Vara *et al.* 2000; De Dennis 2001; Zewide *et al.* 2007b). Virtual elimination of the disease on onion crops has been reported in the UK by combination treatments of tebuconazole as a fungicide and *Trichoderma viride* as a biocontrol agent (Clarkson *et al.* 2006).

Table 3.2 Fungicides recommended for controlling *Allium* white rot based on the Fungicide Resistance Action Committee Code, primary mode of action and chemical group (FRAC code list 2009).

Product name	Chemical formula	Chemical group	FRAC	Mode of action	Reference
Benomyl	$C_{14}H_{18}N_4O_3$	Benzimidazoles	B1	Mitosis and cell division	Maloy and Machtmes (1974)
Boscalid	$C_{18}H_{12}Cl_2N_2O$	Succinate dehydrogenase inhibitors	C2	Respiration	Carlson and Kirby (2005)
Fludioxonil	$C_{12}H_6F_2N_2O_2$	Phenylpyrroles	E2	Signal transduction	Carlson and Kirby (2005)
Iprodione	$C_{13}H_{13}Cl_2N_3O_3$	Dicarboximides	E3	Signal transduction	Wicks and Philp (1985)
Procymidone	$C_{13}H_{11}Cl_2NO_2$	Dicarboximides	E3	Signal transduction	Duff *et al.* (2001)
Vinclozolin	$C_{12}H_9Cl_2NO_3$	Dicarboximides	E3	Signal transduction	Wicks and Philp (1985)
Dicloran	$C_6H_4Cl_2N_2O_2$	Aromatic hydrocarbons	F3	Lipids and membrane synthasis	Fletcher and Knight (1971)
Tebuconazole	$C_{16}H_{22}ClN_3O$	Triazoles	G2	Sterol biosynthesis in membranes	Dennis (1997)
Captan	$C_9H_8Cl_3NO_2S$	Phthalimides	M	Multi-site contact activity	Zewide *et al.* (2007a)
Mancozeb	$C_4H_6N_2S_4Mn.$ $C_4H_6N_2S_4Zn$	Dithiocarbamates and relatives	M	Multi-site contact activity	Zewide *et al.* (2007a)
Thiram	$C_6H_{12}N_2S_4$	Dithiocarbamates and relatives	M	Multi-site contact activity	Zewide *et al.* (2007a)

FRAC: Fungicide Resistance Action Committee Code

One of the best methods to control the disease is pre-treatment of the seeds by an effective fungicide. Coating onion seeds with procymidone controlled *S. cepivora* during the first 8 weeks of seedling growth, though its effectiveness decreased afterwards (Porter *et al.* 1991; Fullerton & Stewart 1991; Kay & Stewart 1994). Onion seed treatment by dicloran, even at low concentrations, eliminated increases in white rot disease (Fletcher & Knight 1971). Although partial disease control can be achieved, chemicals are seldom used because of their high cost. For instance, pre-sterilization of soil by fumigants such as vapam and methyl bromide in the past reduced the sclerotial population and decreased white rot disease incidence by up to 90%, but such treatments are uneconomic (Merriman & Sutherland 1978).

3.5 Biological control of *S. cepivora*

Biological control of *S. cepivora* has been attempted previously. Several fungi and bacteria inhibit either sclerotial germination or mycelial growth. In the fungi, the first attempt to use biological control was performed in 1969 using *Penicillium nigricans* (Gaffar 1969). This inhibited germination of sclerotia of *S. cepivora* due to its toxic exudates *in vitro* and consistent disease control was obtained in garden loam. Some other *Penicillium* species, such as *P. jeanselmei, P. notatum* and *P. lanosum* inhibited the mycelial growth of *S. cepivora* significantly on media (Ghaffar 1969; Moubasher *et al.* 1970; Harrison & Stewart 1988). Species of *Trichoderma, Fusarium, Aspergillus, Paecilomyces, Stachybotrys* and *Penicillium* competed with *S. cepivora* in artificial media (Moubasher *et al.* 1970). Key and Stewart (1994) showed that *Chaetomium globosum* reduced the disease incidence significantly, by 74-78%. The application of *Coniothyrium minitans* as a pre-treatment, seed coating or soil dusting protected cultivated *Allium* species from white rot in a glasshouse experiment as well as or better than the fungicide calomel (Cook *et al.* 1975; Ahmed and Tribe 1977; Smith *et al.* 1989; Alexander and

Stewart 1994). *Trichoderma* species such as *T. viride*, *T. pseudokoningii* and *T harzianum* demonstrated high control of *Allium* white rot in Petri dish trials. High sclerotial degradation in silty clay soil was achieved after applying *T. viride* and *T. pseudokoningii* in infested areas (De Oliveria *et al.* 1984; Clarkson *et al.* 2004).

The major antagonistic bacteria to *S. cepivora* are the common soil-borne bacteria *Bacillus* species. In 1971 Coley-Smith and Dickinson showed that sclerotial exudates contained stimulatory carbohydrates such as trehalose, glucose and mannitol that caused bacterial growth on media. Wong and Hughes (1986) demonstrated that 80% of bacteria recovered from fields infested by *S. cepivora* in Queensland belonged to *Bacillus* species. Isolation of *Bacillus* species from surface-sterilized un-germinated sclerotia suggested that sclerotial exudates affected bacterial activity in soil and resulted in reduction of sclerotial germination (Dickinson and Coley-Smith 1970; Coley-Smith and Dickinson 1971; Utkhade and Rahe 1982; Wong and Hughes 1986). By contrast, *Pseudomonas* and *Erwinia* species were the most antagonistic bacteria isolated from onion roots in Queensland onion fields (Wong and Hughes 1986).

3.6 Biological control of *A. triquetrum*

In considering the use of such a virulent and persistent pathogen as *S. cepivora* in the environment, it is important to assess the impact of any strain of fungus used on other species of *Allium* (especially those cultivated as crops) and other important plants could potentially be affected by it. The same procedures therefore have to be followed as for a new pathogen introduced to a non-endemic area. Ideally, the strain selected would be highly virulent on *A. triquetrum* and relatively mild on cultivated *Allium* species, but it must not be pathogenic to endemic flora.

3.6.1 Pathogenicity tests

Pathogenicity testing is the selection of the most effective strain of the pathogen used as a biocontrol agent to control the target weed and its testing on a wide range of provenances, close relatives and other important plants. In a biological control program, pathogenicity testing plays a critical role and is necessary to determine the agent's ability to cause disease (pathogenicity) and virulence. Yoder (1980) stated that virulence is that portion of the pathogenicity that measures the severity of the disease caused by the pathogen. There are many factors involved in virulence of the agent on the target weed, such as genes coding pectinolytic enzymes (Babalola 2010) that degrade the plant cell wall and toxic materials excreted by the pathogen (Tsuge and Kobayashi 1991).In classical biological control programs, as the pathogen is not native in the area which the weed grows, environmental conditions must be appropriate for the pathogen to exhibit the disease symptoms (Zeng and Qiang 2002).

3.6.2 Host specificity testing

Host specificity is one of the most important steps of a successful biological control program. To establish a classical biological control, selection of a biocontrol agent that demonstrates specificity to the target weed is essential. Wapshere (1974) stated that the strategy of evaluating potential biocontrol agents has two major components: "Centrifugal phylogenetic testing and a safeguard against failure of the centrifugal method". In centrifugal phylogenetic testing, the biocontrol agent is tested on closely related species of the target weed. The reliability of the centrifugal phylogenetic testing can be assessed by testing the biocontrol agent on closely related cultivated species to the target weed, and cultivated plants infected by closely related microorganism to the biocontrol agent (Wapshere 1974).

3.6.3 Aim

In this chapter pathogenicity of *S. cepivora* isolates was assessed on *A. triquetrum* provenances, cultivated *Allium* species and native monocots *in vitro* and *in vivo*. Assessing the level of genetic diversity of both pathogen and the target weed is seen as beneficial to achieve a successful biological control program. Genetic diversity of *Allium triquetrum* from across Australia was shown in chapter 2. As *S. cepivora* reproduces asexually, large genetic diversity of the fungus is not expected. There has been no extensive genetic diversity study of *S. cepivora* undertaken in Australia; however, RAPD analysis and mycelial compatibility studies have shown differences between *S. cepivora* isolates in different countries. In this chapter, genetic variation of the fungus isolates was also investigated using molecular and traditional approaches.

3.7 Materials and Methods

3.7.1 A. triquetrum Sample Collection

A. triquetrum was collected as flowering plants with bulbs from five infested sites: (Yarra Bend Park, Yellow Gum Park, Bendigo Creek, Kangaroo Flat and Wonthaggi) in Victoria (Fig. 3.5-3.9). The plants were potted up and grown on in a RMIT University glasshouse until the end of the growing season (Fig. 3.4). Mature seeds were collected when the capsules became papery and dried. Bulbs were removed from pots after the plants died back washed in tap water and dried at room temperature for 24-48 h, then stored at 4ºC in paper bags. Seeds and bulbs were used for seed germination and pathogenicity tests.

Figure 3.4 Potted *Allium triquetrum* of different provenances in the glasshouse.

Figure 3.5 Area infested by *Allium triquetrum* located at Wonthaggi, Victoria.

Figure 3.6 Area infested by *Allium triquetrum* located at Hardy's Picnic Ground, Dandenongs, Victoria.

Figure 3.7 Area infested by *Allium triquetrum* located at Kangaroo Flat, Bendigo, Victoria.

Figure 3.8 Area infested by *Allium triquetrum* located at Horsnell Gully, South Australia.

Figure 3.9 Area infested by *Allium triquetrum* located at Waterfall Gully, South Australia.

3.7.2 S. cepivora cultures

Two *S. cepivora* isolates were used for genetic diversity and pathogenicity testing (Table 3.3). The fungal cultures were received from the Department of Primary Industries (Knoxfield). The first isolate (VPRI 12439 a) was from the Herbarium VPRI National Collection of Fungi. The second *S. cepivora* culture was isolated from infected garlic by Dr Oscar Villalta – DPI Victoria. As the primary fungal culture was contaminated, mainly by *Penicillium* sp., sclerotia of *S. cepivora* were surface sterilized with 70% ethanol for 30 s and 2% NaOCl for 1 min, washed for 1 min in sterile Milli Q water and cultured on 90 cm diameter Petri plates containing 20 ml of fresh potato dextrose agar at 25 ºC for 15 days. Clean sclerotia were stored at -80 ºC in 65% glycerol and used to grow cultures as needed. Fresh sclerotia were used for DNA extraction, mycelial compatibility and pathogenicity studies.

Table 3.3 *Stromatinia cepivora* cultures and the host plant from which they were isolated.

Name	Isolate NO.	Host	Isolation date	Provider
S. cepivora	VPRI 12439 a	*Allium vineale* L.	18 Sep 1984	Herbarium VPRI DPI Victoria
S. cepivora	N/A	*Allium sativum* L.	3 May 2010	Dr. Oscar Villalta – DPI Victoria

3.7.3 A. triquetrum seed germination trials

Mature seeds from 5 provenances in victoria (Fig. 3.13) were soaked in a beaker filled with tap water and only those seeds that sank were used for germination assays. Twenty seeds were surface sterilized by 75% ethanol for 1 min, 2 % NaOCl with 0.1% Tween 20 for 10 min and washed for 5 min three times in sterile MilliQ water. Twenty seeds from each provenance were incubated for 24 h at 4ºC before any treatment. Seed germination

was assessed on sterile moist filter or water agar in glass Petri plates and incubated at 25ºC using three treatments as follows:

In-vitro treatments:

1- Distilled water
2- 100 ppm gibberellic acid
3- 500 ppm gibberellic acid
4- *A. triquetrum* root exudates, assessed by placing seeds close to *A. triquetrum* roots on water agar Petri plates.

In-vivo trial:

Seeds were sown in 24 x 28 cm plastic trays using autoclaved soil and misted for 2 months in a glasshouse in the RMIT University glasshouse with temperature of 15-25ºC night/day and 60-80% humidity.

3.7.4 Pathogenicity testing

3.7.4.1 A. triquetrum tissue culture

Initial attempts to germinate seeds failed to produce axenic seedlings for pathogenicity testing. Also, initial attempts to surface-sterilize and grow bulbs in test-tubes containing water agar failed due to contamination by endogenous deleterious bacteria (similar to *Ochrobactrum* sp. based on DNA sequencing and physiological tests) (Chapter 5). *A. triquetrum* tissue culture was performed to obtain axenic seedlings for *in vitro* pathogenicity testing. The central plate, young leaves and meristems from within the bulbs were micropropagated in Murashige and Skoog (MS) medium (Dixon 1985) and resulted in axenic seedlings.

3.7.4.1.1 Micropropagation

Surface-sterilised bulbs were cut vertically without damaging the central young meristem using a sterile scalpel. The young parts of the plant, consisting of the shoot apex and part of the basal stem were removed and cultured vertically in polypropylene tubes (diameter 27 mm x length 80 mm) containing Murashige and Skoog (MS) medium (Smith 2000) with 1-naphthaleneacetic acid (NAA) (0.24 mg/L) and 6-γ-γ-(dimethylallylamino)-purine (2iP) (0.5 mg/L). Tubes were incubated at 25°C in a 16 h photoperiod with 270 moles m^{-1} s^{-1} provided by 'Fluora' fluorescent lamps (Fig. 3.10). Cultures were checked each week and contaminated tubes were eliminated. Micropropagated plants (5-6 cm length) were subcultured into water agar in test tubes (diameter 25 mm x length 14.5 cm) and used for pathogenicity testing.

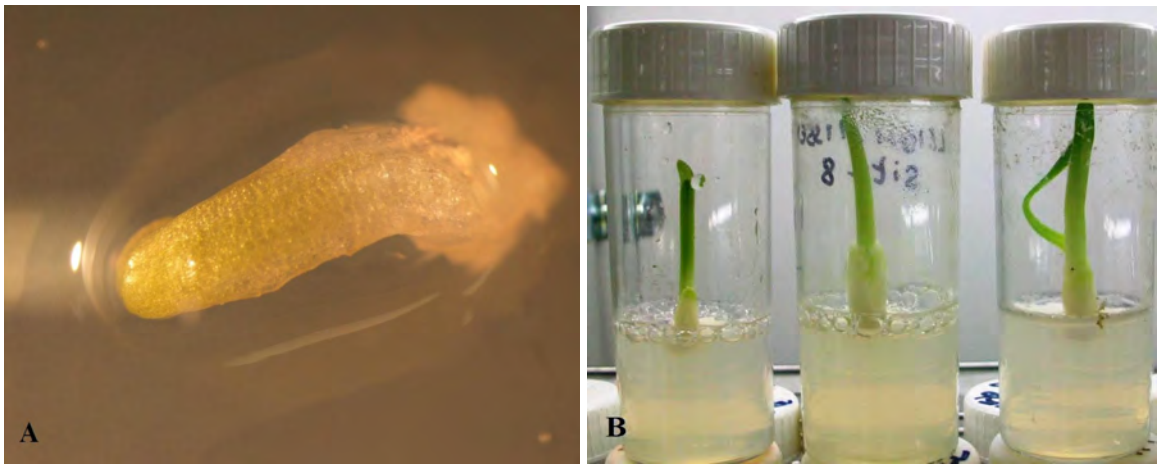


Figure 3.10 *Allium triquetrum* explant (A) and micropropagated seedlings on MS medium.

3.8.4.2 In vitro pathogenicity testing of S. cepivora on A. triquetrum provenances.

Test-tube-grown *A. triquetrum* was used for the *in-vitro* pathogenicity testing of *S. cepivora* isolates (VPRI 12439a and DPI isolate). Sclerotia of both isolates were harvested from 15-day-old cultures on potato dextrose agar (PDA) plates. To remove any accessible carbon sources required for fungal growth, sclerotia were washed for 5 min in sterile

MilliQ water and sub-cultured on water agar aseptically for 2 days before use. Test-tube-grown plants were inoculated by placing a block of water agar containing 30 sclerotia close to the seedling's collar region (Fig. 3.11). The experiment was replicated four times for each provenance and test-tubes were incubated at 25ºC as before in a completely randomized block design. Test-tube-grown plants were screened for white rot disease symptoms with a disease ranking scale to determine the pathogenicity and virulence level of *S. cepivora*. Pathogenicity was scored as of seven stages:

1- No infection
2- Sclerotia germinated
3- Hyphae developed
4- Hyphae covering the agar surface
5- Hyphae climbing the plant/foliage yellowing
6- Sclerotia production/chlorosis
7- Plants dead/necrosis

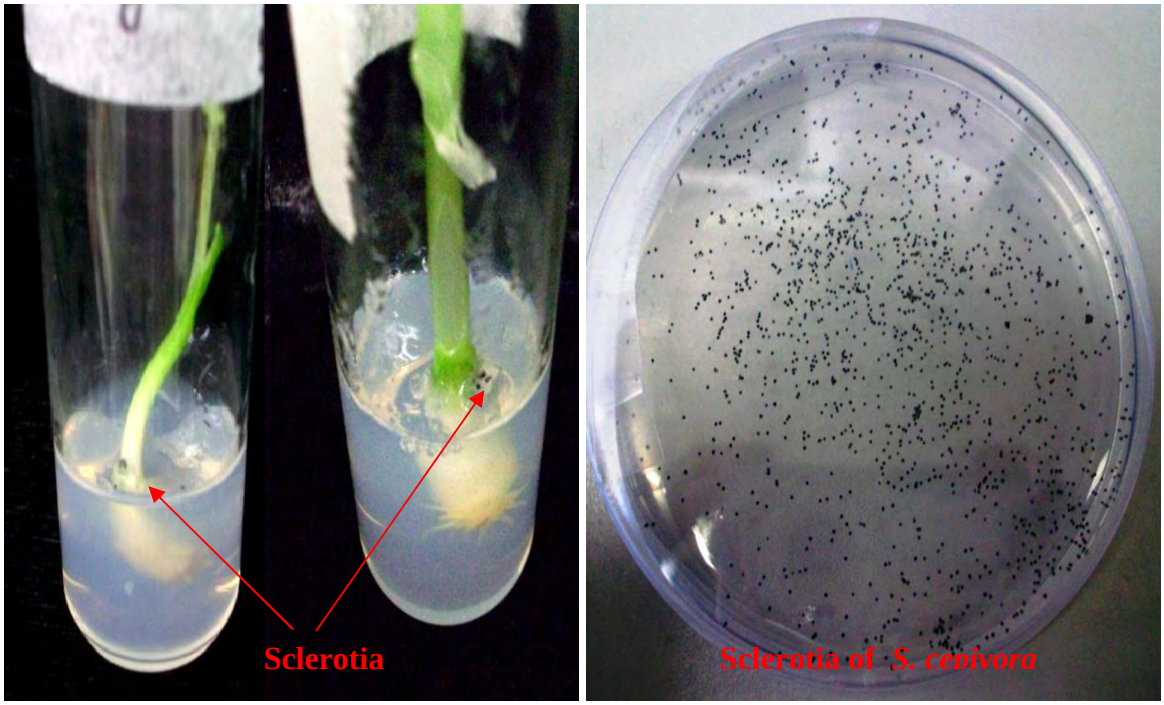


Figure 3.11 Test-tube-grown *Allium triquetrum* (left) inoculated by 30 sclerotia from cultures on water agar (right).

3.7.4.3 In vivo pathogenicity testing of S. cepivora on potted A. triquetrum.

A. triquetrum bulbs stored at 4ºC were potted using sterile washed river sand and left in the RMIT University glasshouse with temperature of 15-25ºC night/day and 60-80% humidity until they sprouted. Only uniformly sized plants were used for pathogenicity testing. Plants were inoculated with two kinds of inoculum: 100 sclerotia and mycelium of the *S. cepivora* DPI isolate. In the first trial, the plants were inoculated by a block of water agar containing 100 sclerotia close to the plant's collar region under the sand surface. In the second trial, one 5 mm diameter fresh mycelial block from PDA cultures on PDA was used per plant. There were three replicates for each provenance arranged in a randomized complete block design. Control plants had no inoculation. To decrease evaporation, aerial contamination and avoid algal growth, the sand surface was covered with sterile polythene beads. Plants were irrigated regularly every 2 days and fertilized every 2 weeks using modified Hoagland nutrient solution (Taiz and Zeiger 2002). Pathogenicity was assessed as of seven stages:

1- No infection
2- Yellowing of older leaf tips
3- Wilting of younger leaves
4- Hyphal growth on root base
5- White hyphae growth on bulb and collar region
6- Sclerotia production/leaf chlorosis
7- Plants dead /necrosis

3.7.5 Centrifugal phylogenetic testing (Host specificity)

3.7.5.1 Pathogenicity testing of S. cepivora on test-tube-grown cultivated Allium species

Seeds of cultivated *Allium* species (Table 3.4) were surface sterilized with 70% ethanol for 1 min, 2% NaOCl with 0.1% Tween 20 for 10 min and rinsed 1 min three times with sterile MilliQ water. Seeds were dried using sterile filter paper and plated on water agar in the dark for a week at 25ºC. Germinated seedlings were subcultured into test tubes containing water agar and incubated at 25 ºC for another week with a 16 h photoperiod as for *A. triquetrum*. The uniformly sized seedlings were used for pathogenicity testing and seedlings were inoculated with 30 sclerotia on 4 mm diameter water agar blocks. There were three replicates for each *Allium* species and plants were incubated at 25ºC in a completely randomized block design as for *A. triquetrum*. The disease incidence and pathogenicity were assessed as explained in Section 3.7.4.2. Data were analysed in a randomized complete block design using SPSS statistical software.

Table 3.4 Cultivated *Allium* species used for pathogenicity testing of *Stromatinia cepivora* in test tube trials.

Common name	**Botanical name**	**Provider**
Chives	*Allium schoenoprasum* L.	Yates Australia
Garlic Chives	*Allium tuberosum* L.	Yates Australia
Leek	*Allium porrum* L.	Mr. Fothergill's Seeds Pty Ltd.
Onion Cream Gold	*Allium cepa* L.	Mr. Fothergill's Seeds Pty Ltd.
Onion Hunter River White	*Allium cepa* L.	Yates Australia
Onion Red Rossa	*Allium cepa* L.	Hortico (Aust.) Pty Ltd.
Onion Sweet Red	*Allium cepa* L. var. *cepa*	Yates Australia
Pickling Onion – Paris Silverskin	*Allium cepa* L.	Mr. Fothergill's Seeds Pty Ltd.
Shallot	*Allium cepa* L.	Mr. Fothergill's Seeds Pty Ltd.
Spring Onion Straight leaf	*Allium cepa* L. var. *cepa*	Yates Australia
Spring Onion White Lisbon	*Allium fistulosum* L.	Mr. Fothergill's Seeds Pty Ltd.

3.7.5.2 Pathogenicity of S. cepivora on potted cultivated Allium species

Cultivated *Allium* seeds (Table 3.4) were surface sterilized and germinated as outlined in Section 3.3.7.1. The 3-4 cm long seedlings were transferred into the pots filled with sterile sand and grown on in a glasshouse for 3 weeks. Young seedlings 12-15 cm tall were inoculated with 100 sclerotia and mycelium of *S. cepivora* DPI isolate separately and plants were irrigated and fertilized as explained in Section 3.8.4.3. Control pots were un-inoculated. There were three replicates for each *Allium* species in a completely randomized block design. Pathogenicity was scored as in Section 3.7.4.3.

3.7.5.3 Pathogenicity of S. cepivora on Australian native monocots

Nine Australian native monocots were purchased form the Kuranga Native Nursery, Melbourne, Victoria (Table 3.5). The potted plants were in the plastic tubes - 50mm square x 120mm high, filled by non-sterile potting mix. This experiment was replicated three times and plants were inoculated, irrigated and fertilized per 3.7.4.3 in the glasshouse. Disease incidence and pathogenicity were ranked as mentioned in 3.7.4.3.

Table 3.5 Australian native monocots used for host specificity trials ***in vivo.*** Taxa are according to the Flora of Victoria (Walsh and Entwisle 1994).

Common name	**Botanical name**	**Family**
Black Anther Flax-Lily	*Dianella revoluta* R.Br.	*Liliaceae*
Bulbine Lily	*Bulbine bulbosa* (R.Br.) Haw.	*Liliaceae*
Chocolate Lily	*Arthropodium strictum* R.Br.	*Liliaceae*
Yellow Rush Lily	*Tricoryne elatior* R.Br.	*Liliaceae*
Purple-Flag	*Patersonia occidentalis* R.Br.	*Iridaceae*
Clustered Wallaby-Grass	*Austrodanthonia racemosa* R.Br.	*Poaceae*
Weeping Grass	*Microlaena stipoides* (Labill.) R.Br.	*Poaceae*
Wiry Tussock-Grass	*Poa sieberiana* Spreng.	*Poaceae*
Spiny-Headed Mat-Rush	*Lomandra longifolia* Labill.	*Xamthorrhoeaceae*

3.7.6 Effect of low temperature on sclerotia of S. cepivora germination

Low temperature stimulates sclerotia to germinate and white rot disease is greater in cooler than warmer temperatures (Coley-Smith 1990a). Sclerotia of *S. cepivora* did not germinate after three months inoculation on *A. triquetrum* and cultivated *Allium* species in the glasshouse. To test if the temperature of 15-25ºC in the glasshouse inhibited the sclerotial germination and therefore white rot disease, potted chives in sterile sand were inoculated with 100 sclerotia and mycelium separately and incubated at 15ºC in a 16 h photoperiod provided by 'Fluora' tubes. Plants were irrigated and fertilized and disease incidence was scored as in Section 3.7.4.3.

3.7.7 S. cepivora molecular analysis

3.7.7.1 DNA extraction

DNA was extracted using a QIAGEN DNeasy Mini Kit from 150 mg fresh sclerotia harvested from PDA plates and ground in liquid nitrogen using a pestle and mortar. The extraction procedure was as explained in Section 2.2.1. DNA concentration and quality was detected by electrophoresis of 10 µL DNA in 1.5 % agarose gel with 1 µL of GeneRuler^{TM} 100 bp and. DNA concentration was estimated (ng/µL) based on band brightness compared with the GeneRuler bands as before. The DNA extracts were stored at -20 ºC and used for ITS region analysis, RAPD-PCR and DNA sequencing.

3.7.7.2 ITS region amplification

The ITS regions of *S. cepivora* isolates (DPI and VPRI 12439a) were amplified using ITS1 and ITS4 primers in 25 µL reactions containing 20 ng/µL of the extracted genomic DNA as explained in Section 2.2.2. A G-STORM thermocycler was programmed based on the Provan *et al.* (2004) protocol. The PCR products were separated in 1.5% agarose gel

along with 1 µL of GeneRuler^{TM} 100 bp, stained with ethidium bromide and visualized using a Biorad Gel Doc system as for *A. triquetrum*.

3.7.7.3 ITS-PCR products sequencing

The ITS-PCR products were purified using a QIAGEN PCR Purification Kit as for *A. triquetrum*. Sequencing reactions and precipitation were performed based on the BigDye Terminator v3.1Cycle Sequencing Kit protocol as in Section 2.2.4 and the reaction tubes were sent to the Micromon Sequencing Facility at the Monash University for further analysis as before. ITS sequences (Internal Transcribed Spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence) of ten *S. cepivora* isolates with different countries of origin were downloaded from GenBank through the NCBI webpage for sequence alignment and nucleotide variation analysis (Table 3.6).

Table 3.6 ITS sequence of *Stromatinia cepivora* isolates and *Sclerotium rolfsii* (outgroup) used for sequence alignment.

Fungal species	Origin	Host plant	Strain No.	NCBI No.
S. cepivora	Australia	*Allium sativum*	N/A (DPI, VIC)	N/A
S. cepivora	Australia	*Allium vineale*	VPRI 12439a	N/A
S. cepivora	Canada, Ontario	*Allium* sp.	ATCC 66452	Z99683.1
S. rolfsii	China	*Arachis hypogaea*	ATCC 39291	JF819727.1
S. cepivora	Egypt	N/A	CBS 271.30	FJ231399.1
S. cepivora	England	*Allium cepa*	CBS 320.65	FJ231401.1
S. cepivora	England	*Allium cepa*	CBS 321.65	FJ231402.1
S. cepivora	Holland	*Allium* sp.	1878.S=LMK1	Z99682.1
S. cepivora	Netherlands	*Allium* sp.	CBS 276.93	FJ231400.1
S. cepivora	Netherlands	*Allium cepa*	CBS 189.82	FJ231398.1
S. cepivora	Netherlands	N/A	CBS 342.47	FJ231403.1
S. cepivora	New Jersey, USA	*Allium* sp.	1889.S=LMK71	Z99681.1

3.7.7.4 Sequencing edition and alignment

The sequences were edited using BioEdit software in ANGIS as for ***A. triquetrum*** in Chapter 2. Approximately 100-180 bp of the 5´ start and 3´end of the sequence (DPI and VPRI 12439a) were edited using the chromatogram to remove unreliable readouts. ClastulW (accurate), in ANGIS BioManager was used to determine the sequence similarities of ***S. cepivora*** isolates. The ClastulW result was edited to equalise the nucleotide numbers and the file was converted to MEGA4 to make a phylogenetic tree as before. A distance matrix was constructed using DNAdist (Jukes and Cantor 1969; Kimura 1980; Jin and Nei 1990). A phylogentic tree was formed in MEGA4 by neighbour-joining and UPGMA methods (Felsenstein 1989) using the ClustalW alignment.

3.7.7.5 RAPD-PCR

Extracted DNA of *S. cepivora* isolates were used for RAPD-PCR analysis. Sixty 10-mer RAPD primers (3 sets: OPA, OPB, OPM) were used for the PCR amplification as in Section 2.2.6. The RAPD-PCR amplification was repeated three times using a G-STORM thermal cycler to determine the RAPD-PCR product reproducibility. The PCR machine was programmed based on as in section 2.2.6 and the amplicons were fractioned in 1.5% agarose gel along with 1 µL of GeneRuler^{TM} 100 bp and visualized in a Bio-RAD Gel Doc system as previously.

3.7.8 Traditional approach

3.7.8.1 Mycelial compatibility grouping

A mycelial compatibility test for Australian *S. cepivora* isolates (DPI and VPRI 12439a) was performed by culturing isolates on PDA plates to determine any relationship between

isolates. Initially 6 sclerotia of each isolate were cultured on a fresh PDA plate and incubated in the dark at 25ºC for 7 days. Mycelial blocks 5 mm diameter were subcultured and paired 5 cm apart on PDA in 90 mm diameter Petri plates and incubated at 25ºC for 7 days. The pairing was performed twice. Incompatibility was assessed on the basis of the presence of an interaction zone and failure of the two isolates to fuse (Kohli *et al.* 1992, Earnshaw and Boland 1997).

3.7.8.2 Effect of media on mycelial growth and sclerotia production of S. cepivora isolates

Growth media have been used for differentiating amongst strains of *S. cepivora* maintaining on growth characteristics (Coley-Smith and Holt 1966; Coley-Smith 1979). The aim of this experiment was to investigate mycelial growth and sclerotia production of the isolates on a range of media to differentiate between the isolates phenotypically. *S. cepivora* isolates (Table 3.3) were grown initially on PDA for a week and 5mm mycelial blocks of *S. cepivora* isolates were sub-cultured on six media with three replicates each (Table 3.7) and incubated at 25ºC in a 16 h photoperiod for 3 weeks in the light as for test-tubes. Mycelial growth rate was assessed after a week by measuring the diameter of the mycelial growth. Sclerotia production was assessed after 3 weeks incubation by harvesting sclerotia from individual plates, drying for 24 h at 50ºC and weighing them. Data on mycelial growth and sclerotia production were analysed using a split-split plot design using SPSS statistical software.

Table 3.7 Media types used for mycelial growth and sclerotia production of *Stromatinia cepivora*.

Symbol	Medium name	Contents	g/L
GEA	Garlic extract agar	Garlic extract	100 mL
		Glucose	5 g
		Agar	20 g
IMA	Indian mustard extract agar	Indian mustard extract	100 mL
		Glucose	5 g
		Agar	20 g
MSA	Mineral salt agar	KH_2PO_4	3 g
		$MgCl_2.6\ H_2O$	0.5 g
		$CaCl_2.2\ H_2O$	0.25 g
		NH_4Cl	0.2 g
		Agar	20 g
PDA	Potato dextrose agar	Potato extract	100 mL
		Glucose	5 g
		Agar	20 g
SEA	Soil extract agar	$NaNO_3$	1 g
		Glucose	10 g
		Agar	25 g
		Soil extract	1 L
V8A	V8 juice agar	V8 juice	200 mL
		$CaCO_3$	2 g
		Agar	20 g

3.7.8.3 Effect of smoke water on mycelial growth and sclerotia production of S. cepivora

Post-fire soil environments support a variety of soil microbial activities that are antagonistic to forest pathogens (Reaves *et al.* 1990). According to DPC (2003), the past 1996-2009 drought years have made Victoria a fire-prone state. Smoke water inhibits the growth of several soil-borne fungi e.g. *Rhizoctonia solani* (Zagory and Parmeter 1984). Most of Kinglake National Park was burnt during the February 2009 bushfire and some of the biggest *A. triquetrum* infestations are next to Kinglake National Park. Therefore smoke water may have effect on sclerotia germination and mycelial growth of *S. cepivora.* The effect of smoke water on the mycelial growth and sclerotia production of *S. cepivora*

isolates was assessed using PDA containing different smoked water concentrations. Smoked water used in this experiment was Regen-2000 smoked water (water, wood smoke, Emulsifier and food colour 129) provided by Grayson, Tecnica, Pty. Ltd. Mycelium of *S. cepivora* isolates was cultured on PDA at 25°C for a week and 5 mm diameter mycelial blocks were sub-cultured on PDA Petri plates containing different concentrations of smoked water. The media were prepared by adding 0, 25, 50, 75, 100 and 200 mL of Regen-2000 smoked water in 1 L of potato extract. This experiment was performed with three replicates for each treatment in a split-split plot design. Plates were incubated at25ºC for 3 weeks in the light as for test-tubes. The effect on mycelial growth was observed after a week and sclerotia production after 3 weeks as mentioned in Section 3.7.8.2.

3.8 Results

3.8.1 A. triquetrum seed germination trials

A. triquetrum seed germination varied from 0-80% under different dormancy-breaking treatments. Seeds collected from five provenances in Victoria did not germinate using moisturized filter paper and 500 ppm gibberellic acid. Only 20% of seeds germinated using 100 ppm gibberellic acid after two months. There was no seed germination on the water agar plates containing *A. triquetrum* roots. However; 80% of seeds in every provenance individually germinated in sterile potting mix misted in the glasshouse for two months (Fig. 3.12).

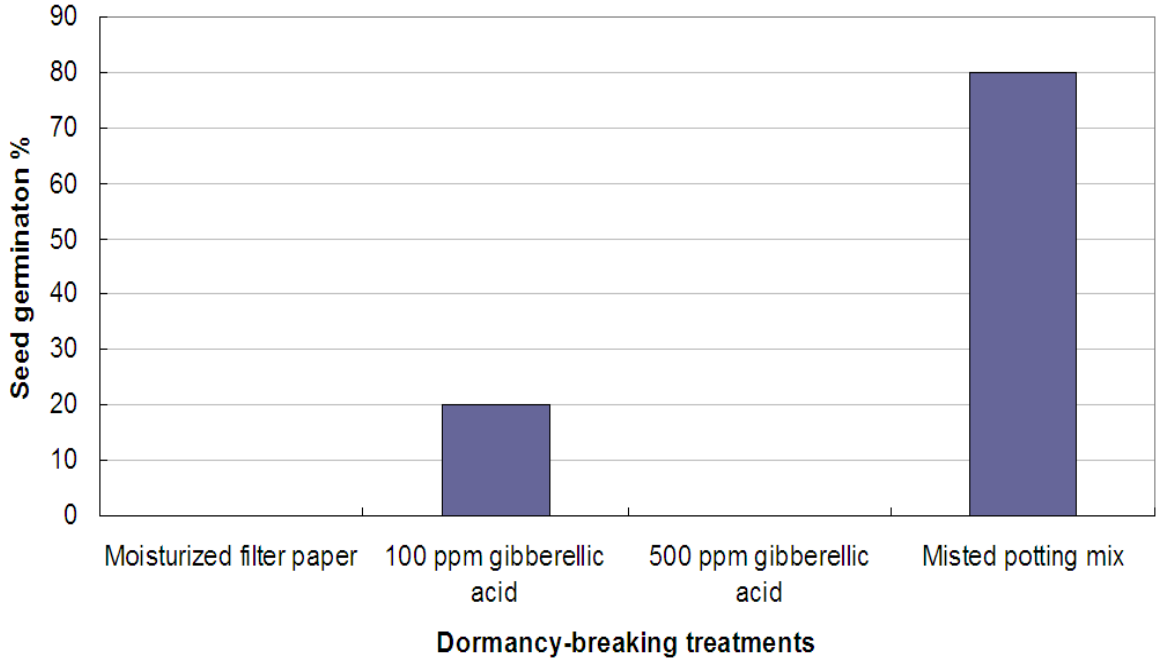


Figure 3.12 *Allium triquetrum* seed germination using 4 dormancy-breaking treatments.

3.8.2 S. cepivora pathogenicity on test-tube-grown A. triquetrum

3.8.2.1 Comparison of pathogenicity of S. cepivora isolates on A. triquetrum

All test-tube-grown *A. triquetrum* from all five provenances (Yarra Bend Park, Yellow Gum Park, Bendigo Creek, Kangaroo Flat and Wonthaggi) were killed by the DPI isolate after 4 weeks and sclerotia were observed on the dead plants (Fig. 3.13, 3.14). VPRI 12439a was pathogenic on only four provenances and not on plants from Wonthaggi, where only one replicate out of four was infected (Fig. 3.13, 3.14). Un-germinated sclerotia from the other tubes were subcultured on PDA and sclerotial germination was observed after one week's incubation at 25ºC. Data analysis in a split plot design showed significant differences between isolates and between provenances and that there was a significant interaction between isolate and provenance (Table 3.8). The experiment was conducted three times with the same result and so the result is replicable. As the *S. cepivora* isolate from DPI was highly virulent on all provenances in Victoria, it was used for further testing on all provenances from across Australia.

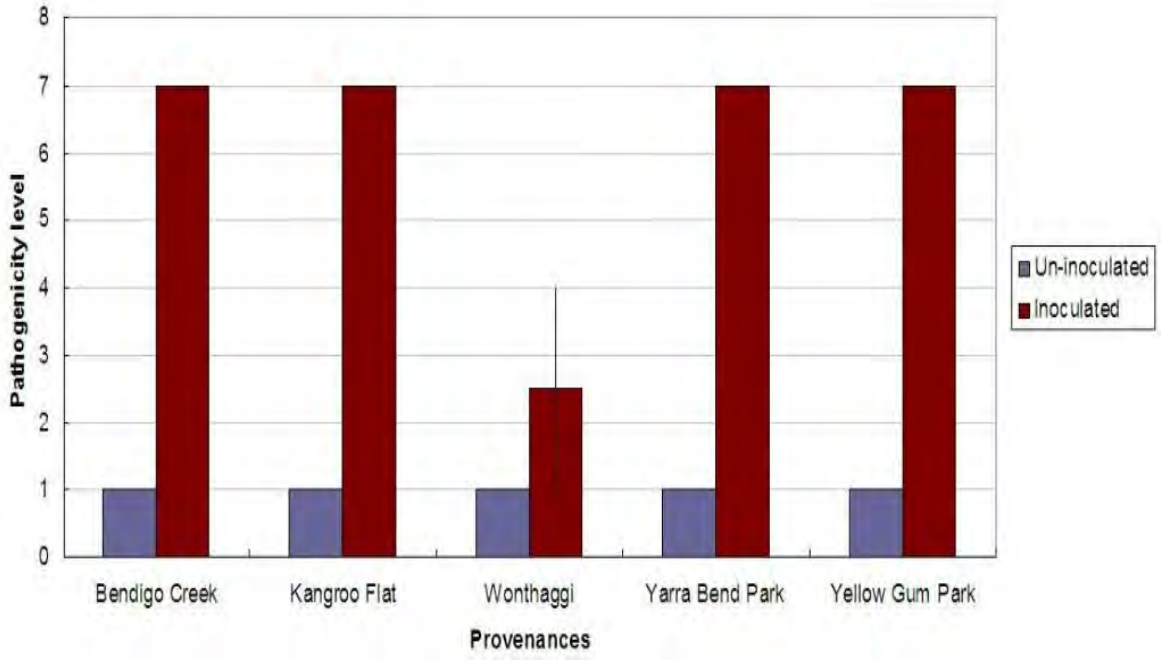


Figure 3.13 Pathogenicity of *Stromatinia cepivora* VPRI 12439a on test-tube-grown *Allium triquetrum* from five provenances in Victoria (Bars=2 x standard error).

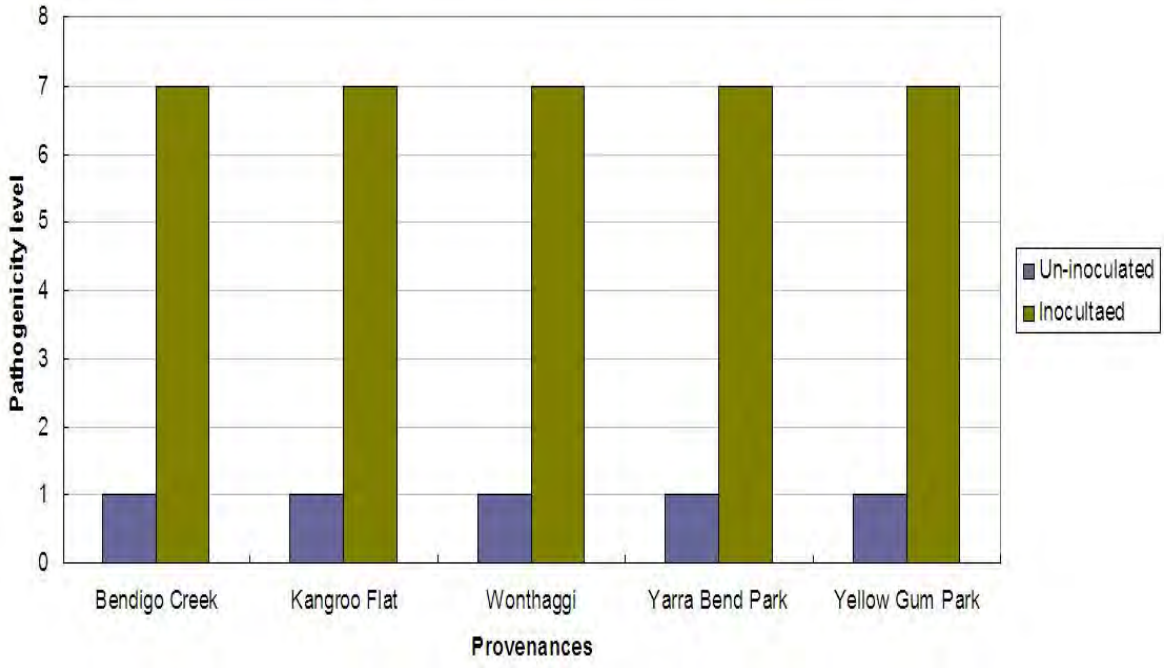


Figure 3.14 Pathogenicity of the *Stromatinia cepivora* DPI on test-tube-grown *Allium triquetrum* from five provenances in Victoria (Bars=2 x standard error).

Table 3.8 Analysis of pathogenicity of *Stromatinia cepivora* isolates on *Allium triquetrum* provenances in a split plot design using SPSS software.

Tests of Between-Subjects Effects

Dependent Variable: pathogenicity

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	78.300[a]	15	5.220	5.800	.000
Intercept	1716.100	1	1716.100	1906.778	.000
Replicates	2.700	3	.900	1.000	.410
Isolates	8.100	1	8.100	9.000	.006
Replicates * Isolates	2.700	3	.900	1.000	.410
Provenances	32.400	4	8.100	9.000	.000
Isolates * Provenances	32.400	4	8.100	9.000	.000
Error	21.600	24	.900		
Total	1816.000	40			
Corrected Total	99.900	39			

a. R Squared = .784 (Adjusted R Squared = .649)

3.8.2.2 Pathogenicity of S. cepivora DPI isolate on test-tube-grown A. triquetrum provenances from across Australia.

Sclerotia germinated 4-8 days after inoculation in all provenances and the first disease symptoms were observed after 15 days. Hyphae covered the agar surface in the test-tube and climbed the *A. triquetrum* seedlings, followed by chlorosis, necrosis and death of plants (Fig. 3.15). Control plants without fungal inoculation remained healthy. Almost all replicates demonstrated sclerotia production (Stage 7) on dead leaves after 30 days inoculation; however, sclerotia did not germinate on one replicate from Bendigo Creek (VIC), Horsnell Gully (SA) and Mylor (SA) (Fig. 3.16). Only *S. cepivora* was re-isolated from the infected plants in the test-tubes on V8 agar plates. Data analysis indicated that

there was no significant difference in pathogenicity and virulence of *S. cepivora* DPI on provenances from across Australia (Table 3.9).

Figure 3.15 Test-tube-grown *Allium triquetrum* from different provenances infected by *Stromatinia cepivora* DPI isolate.

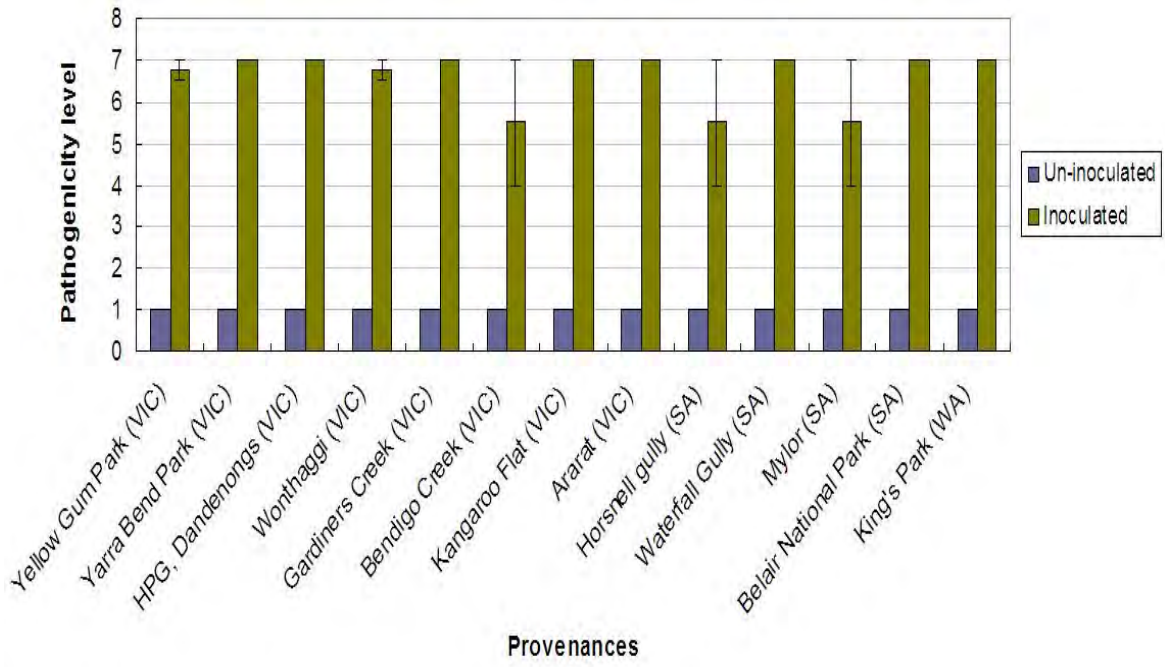


Figure 3.16 Pathogenicity of *Stromatinia cepivora* DPI isolate on test-tube-grown *Allium triquetrum* provenances from across Australia. HPG: Hardy's Picnic Ground (Bars=2 x standard error).

Table 3.9 Analysis of pathogenicity of *Stromatinia cepivora* DPI isolate on test-tube-grown *Allium triquetrum* infected provenances from across Australia in randomized complete block design using SPSS software.

Tests of Between-Subjects Effects

Dependent Variable: Pathogenicity

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	39.962[a]	15	2.664	1.538	.143
Intercept	2275.692	1	2275.692	1314.033	.000
Replicates	20.154	3	6.718	3.879	.017
Provenances	19.808	12	1.651	.953	.508
Error	62.346	36	1.732		
Total	2378.000	52			
Corrected Total	102.308	51			

a. R Squared = .391 (Adjusted R Squared = .137)

3.8.3 Pathogenicity of S. cepivora DPI on seedlings of cultivated Allium species grown in polycarbonate tubes

Seedlings of all cultivated *Allium* were affected by the *S. cepivora* DPI isolate. The disease symptoms were observed after 7 days, with browning of leaves followed by necrosis and death of the seedlings after 14 days (Fig. 3.17). Pathogenicity of the fungus varied on different species. Onion River White, Onion Cream Gold and Chives were highly infected (Stage 5) week after inoculation. Spring Onion White Lisbon was not affected by the fungus after 7 days, however; plants were infected and dead 14 days after inoculation. The control plants remained healthy under the same environmental conditions. There was no variation within the infected treatments (all died) and so it was not possible to test it statistically, as the difference between the control and inoculated treatments was qualitative rather than quantitative.

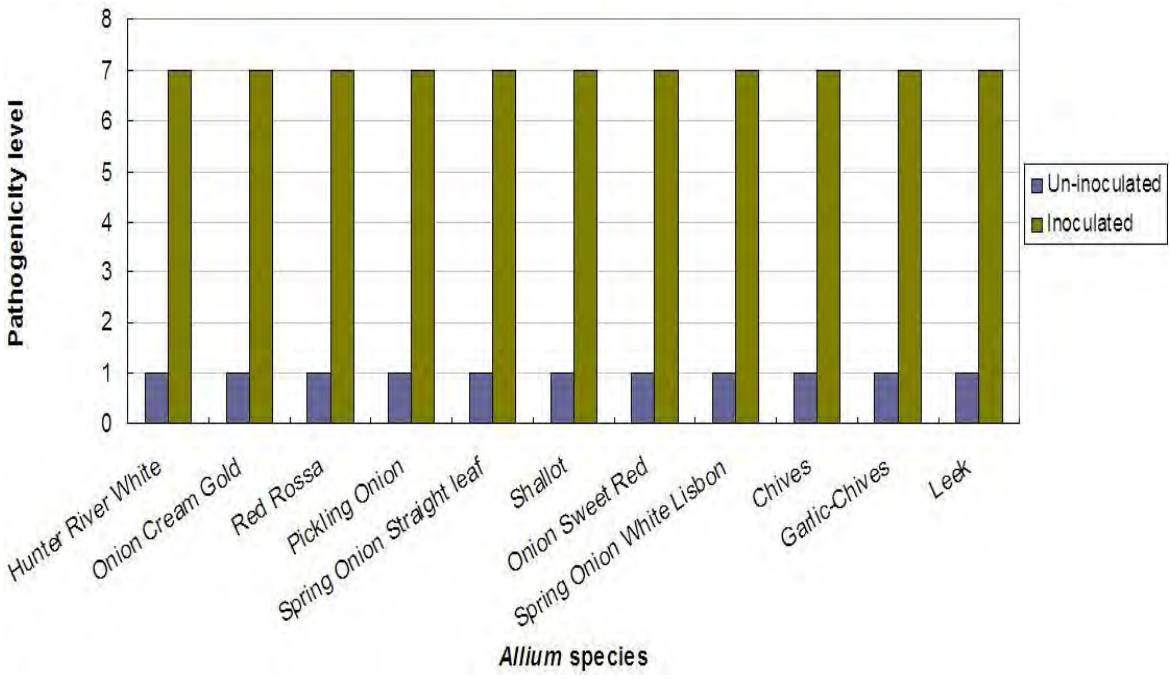


Figure 3.17 Pathogenicity of *Stromatinia cepivora* DPI isolate on cultivated polypropylene-tube-grown *Allium* seedlings (Bars=2 x standard error).

3.8.4 Pathogenicity of S. cepivora DPI on potted A. triquetrum provenances from across Australia in the glasshouse.

Inoculation using sclerotia resulted in no disease symptoms after 3 months and all plants remained healthy. Inoculated plants were pulled out and un-germinated sclerotia were collected. Surface-sterilized sclerotia plated on V8 agar germinated and produced hyphal growth after 7 days incubation at 25ºC and sclerotia were produced after 2 weeks.

Inoculation using mycelium resulted in white rot disease symptoms 30 days after inoculation in the glasshouse as yellowing of leaves and shrunken collar regions. Necrosis and death was observed in the third month after inoculation (Fig. 3.18). Pathogenicity of the fungus varied between provenances and varied from stage 1 (no infection) to stage 7 (plant death) (Fig. 3.19). No disease was recorded for Hardy's Picnic Ground, Dandenongs provenance. For the plants from Kangaroo Flat (VIC), Ararat (VIC) and Waterfall Gully (SA) only one of three plants was infected and they showed less than

maximal virulence levels. Control plants without fungal inoculation remained healthy under the same environmental conditions. Sclerotia were produced on infected plants (Fig. 3.20). Data analysis indicated a significant difference in pathogenicity and virulence of *S. cepivora* among provenances from across Australia (Table 3.10).

S. cepivora was re-isolated from infected plants in the glasshouse on V8 agar along with *Fusarium* sp., *Bispora* sp. and *Botrytis* sp. On surface-sterilisation and plating of control uninfected *A. triquetrum,* the same other fungal species were identified but not *S. cepivora*. To test the pathogenicity of these other re-isolated fungi on *A. triquetrum*, three replicate pots of *A. triquetrum* were inoculated with mycelial blocks of each fungal species and grown on in the glasshouse. Only *Botrytis* sp. caused die back of *A. triquetrum* leaves but it did not rot the bulbs. The results suggested that only *S. cepivora* caused white rot disease on potted *A. triquetrum* plants in the glasshouse.

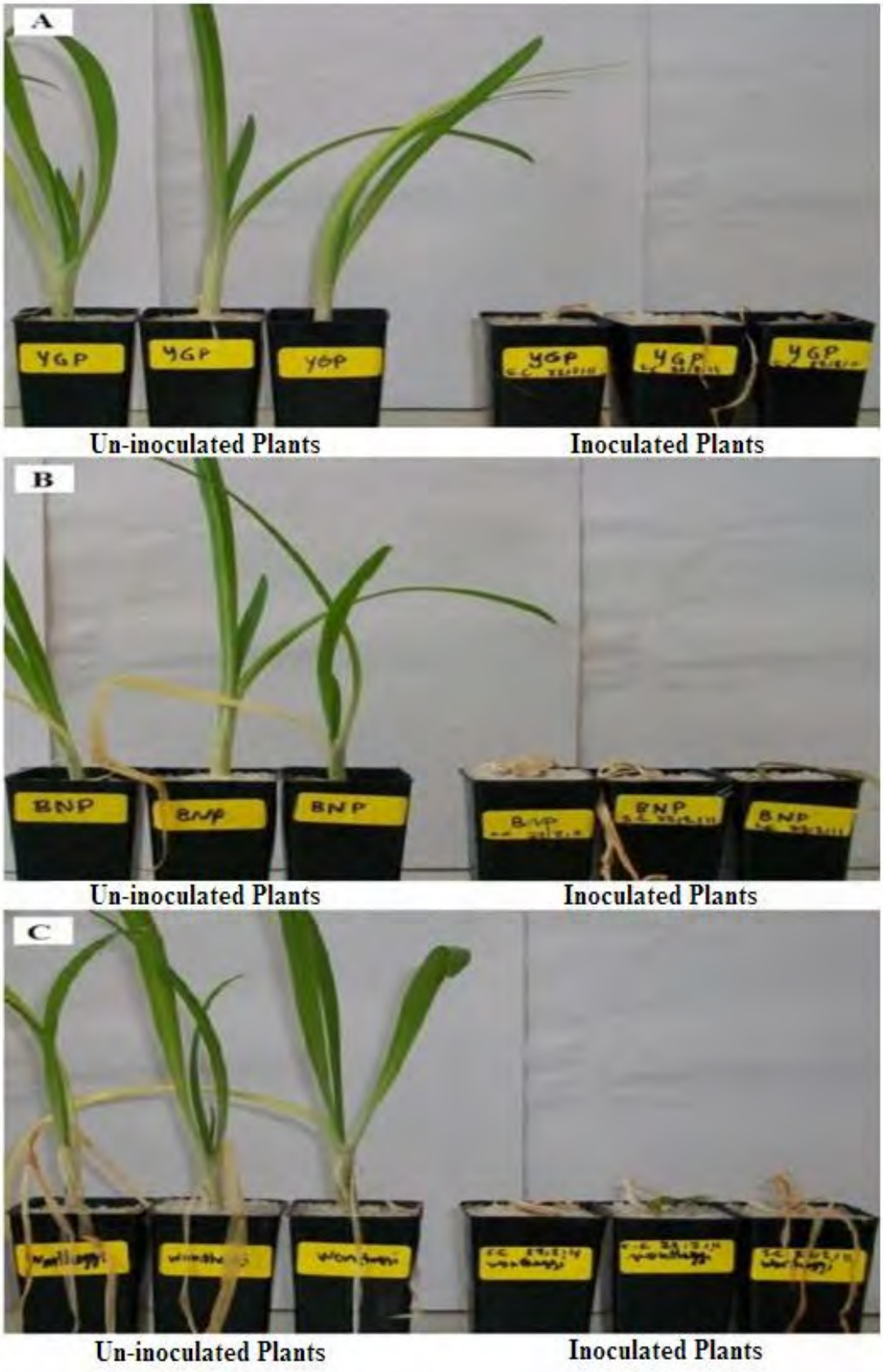


Figure 3.18 Pathogenicity of *Stromatinia cepivora* on potted *Allium triquetrum* provenances in the glasshouse. A: Yellow Gum Park (VIC), B: Belair National Park (SA), C: Wonthaggi (VIC).

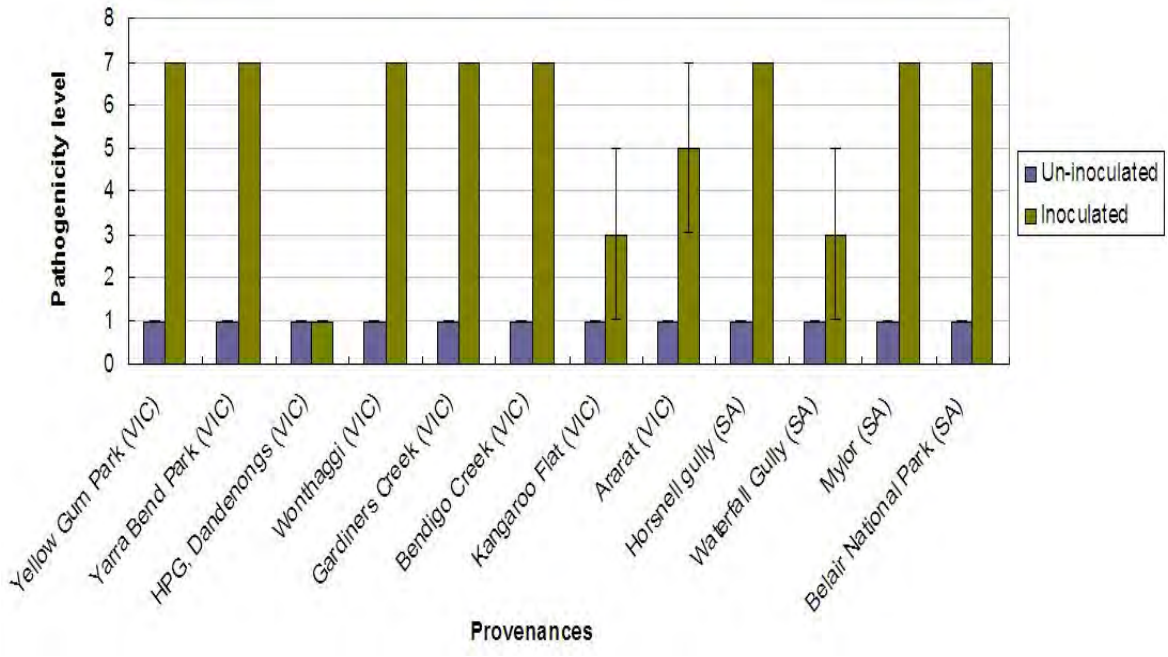


Figure 3.19 Pathogenicity of *Stromatinia cepivora* DPI isolate on *Allium triquetrum* from across Australia in the glasshouse (Bars=2 x standard error).

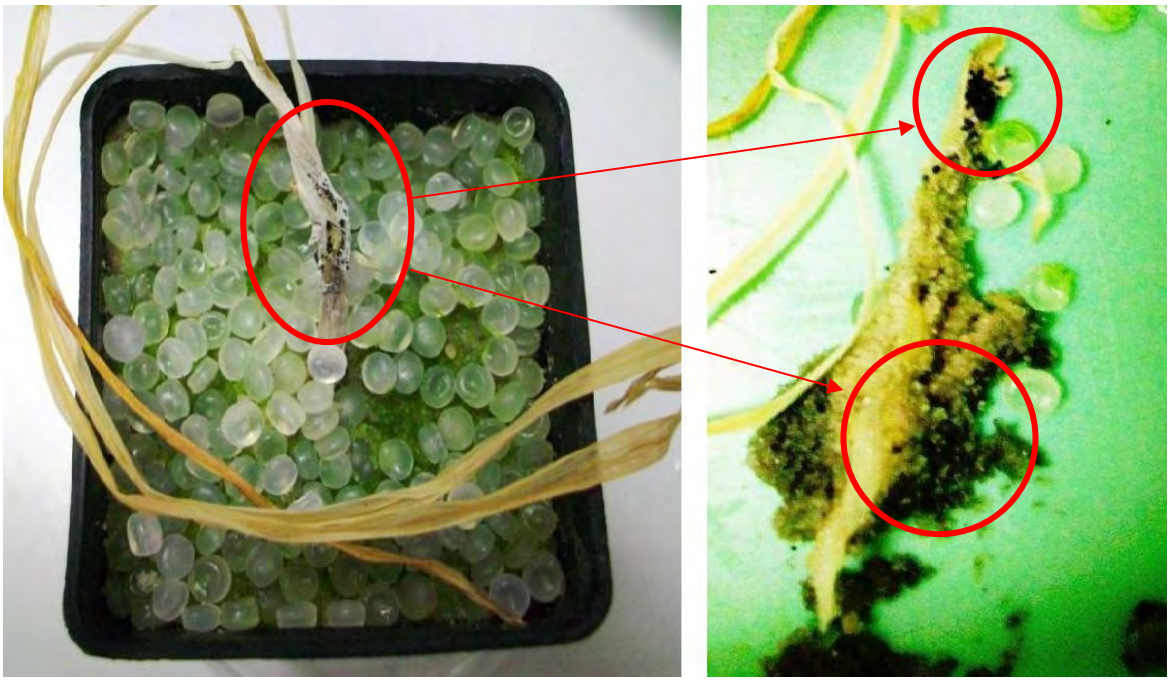


Figure 3.20 Sclerotia of *Stromatinia cepivora* produced on infected *Allium triquetrum* in the glasshouse.

Table 3.10 Analysis of pathogenicity of *Stromatinia cepivora* DPI isolate on *Allium triquetrum* provenances from across Australia in a randomized complete block design using SPSS software.

Tests of Between-Subjects Effects

Dependent Variable: Pathogenicity

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	154.000[a]	13	11.846	3.723	.003
Intercept	1156.000	1	1156.000	363.314	.000
Replicates	2.000	2	1.000	.314	.734
Provenances	152.000	11	13.818	4.343	.002
Error	70.000	22	3.182		
Total	1380.000	36			
Corrected Total	224.000	35			

a. R Squared = .688 (Adjusted R Squared = .503)

3.8.5 Pathogenicity of S. cepivora DPI on cultivated Allium species in pot trials in the glasshouse.

With sclerotia as inoculum, no sclerotial germination was observed on inoculated plants 3 months after-inoculation. Sclerotia collected from pots, surface-sterilized and plated out on V8 agar germinated after 2 weeks incubation at 25ºC. With mycelium as inoculum, white rot disease symptoms were observed almost on all species inoculated (Fig. 3.21). Pathogenicity was observed for all *A. cepa* cultivars such as Onion Cream Gold, Onion Hunter White, Onion Pickling, Onion Red Rossa and Onion Sweet Red (Fig. 3.22). The disease symptoms were identical to those described in Section 3.8.4 and control plants remained healthy after 3 months in the glasshouse. Sclerotia were produced on infected bulbs and observed when they were pulled out from the pots. Only *S. cepivora* was re-isolated from the infected plants on V8 agar plates. Statistical analysis of pathogenicity data indicated significant differences between treated and control plants. However; there

was no significant difference in pathogenicity and virulence of the fungus on cultivated *Allium* species (Table 3.11).

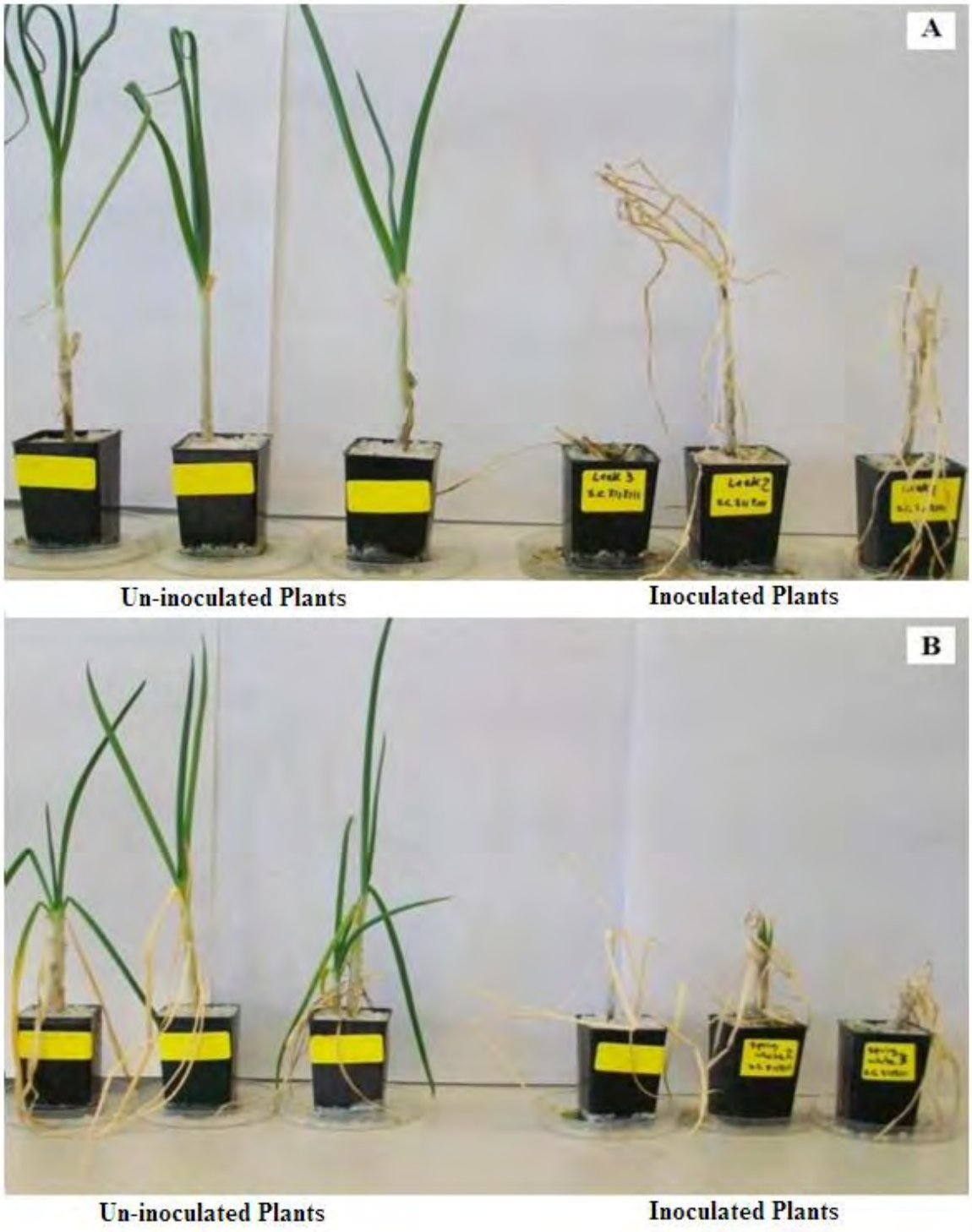


Figure 3.21 Pathogenicity of *Stromatinia cepivora* on cultivated *Allium* species in the glasshouse after 3 months. A: Leek (*Allium porrum*), B: Spring Onion White Lisbon (*Allium fistulosum*).

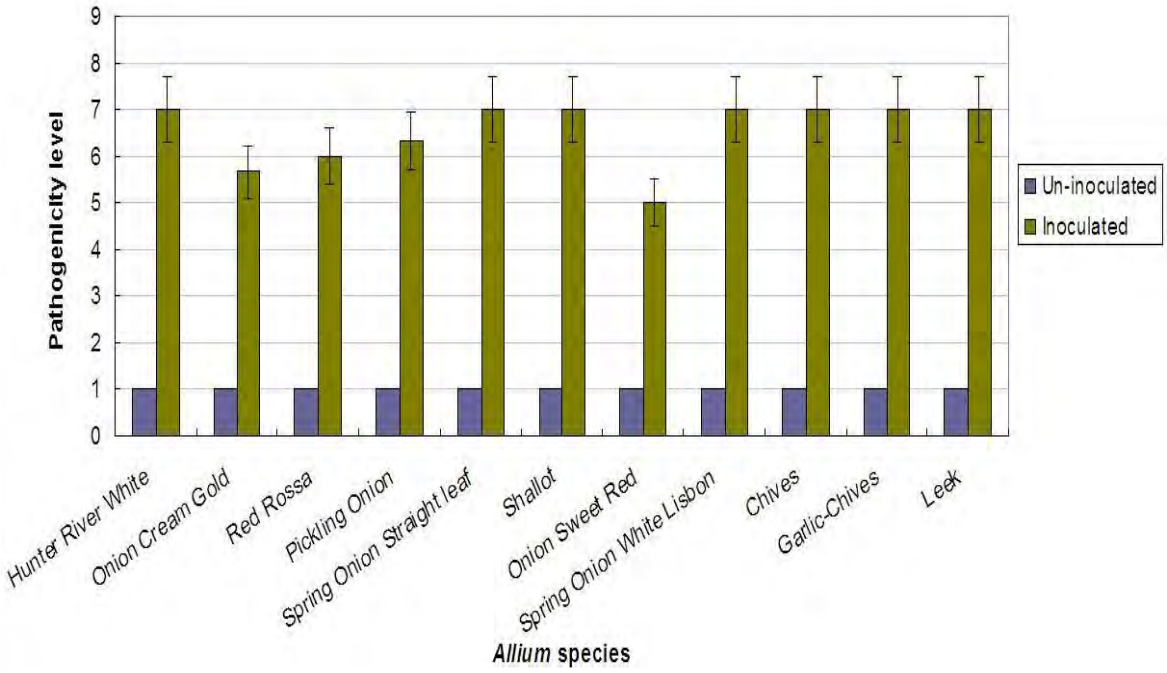


Figure 3.22 Pathogenicity of *Stromatinai cepivora* DPI isolate (mycelium) on cultivated *Allium* species in the glasshouse (Bars=2 x standard error).

Table 3.11 Analysis of pathogenicity of *Stromatinia cepivora* DPI isolate (mycelium) on cultivated *Allium* species in a randomized complete block design using SPSS software.

Tests of Between-Subjects Effects

Dependent Variable: Pathogenicity

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	20.485[a]	12	1.707	2.493	.034
Intercept	1413.818	1	1413.818	2064.425	.000
Replicates	5.636	2	2.818	4.115	.032
Allium species	14.848	10	1.485	2.168	.068
Error	13.697	20	.685		
Total	1448.000	33			
Corrected Total	34.182	32			

a. R Squared = .599 (Adjusted R Squared = .359)

3.8.6 Pathogenicity of S. cepivora DPI isolate on Australian native monocots in the glasshouse.

No white rot disease symptoms were observed on Australian native grasses inoculated with sclerotia and mycelium of *S. cepivora* 3 months after inoculation. *A. triquetrum,* used as a positive control, indicated infection caused by *S. cepivora* mycelium (Fig. 3.23). All inoculated native plants along with control treatments were scored as stage 1 and *A. triquetrum* was scored as stage 7. Data analysis showed significant differences between inoculated native plants and *A. triquetrum* but no difference among inoculated native plants. Un-germinated sclerotia collected from inoculated pots, surface-sterilised and plated out as before, germinated and produced mycelium and sclerotia. Attempts at fungal re-isolation failed from the plants inoculated by *S. cepivora* mycelium. Only common soil contaminants such as *Alternaria* sp., *Penicillium* sp. and *Bispora* sp. were re-isolated from both inoculated and control plants in the glasshouse.

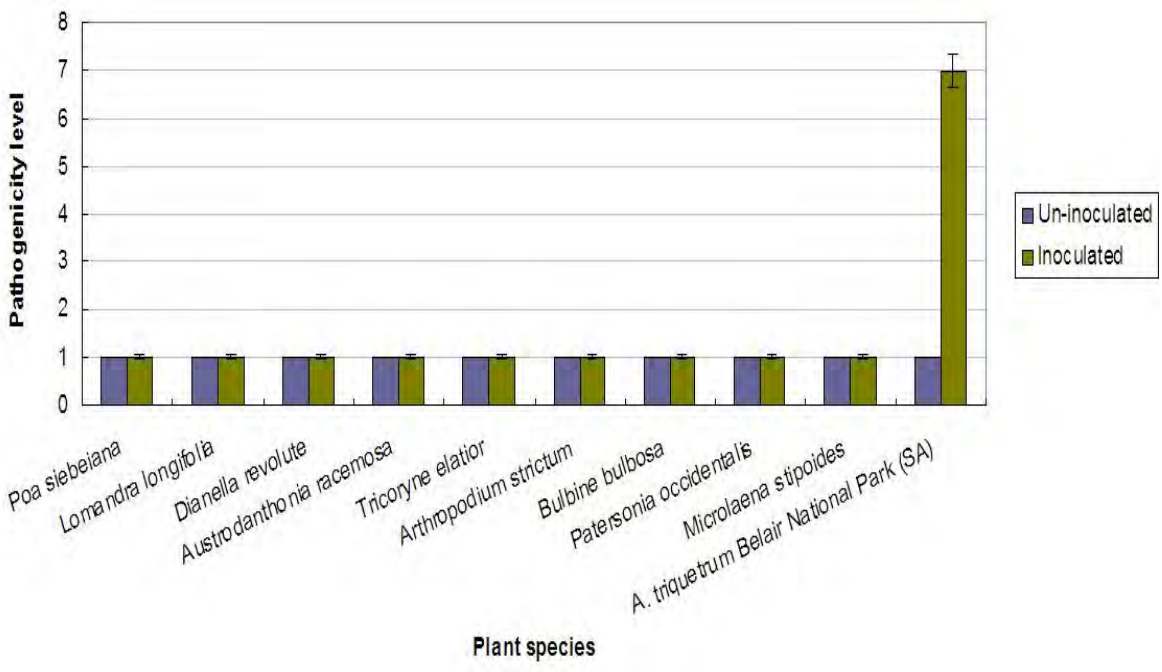


Figure 3.23 Pathogenicity of *Stromatinia cepivora* mycelium on Australian native monocots in the glasshouse. Infected *Allium triquetrum* plants were used as positive controls (Bars=2 x standard error).

3.8.7 Effect of low temperature on sclerotia of S. cepivora germination

No white rot disease symptoms were noticed on chives inoculated with 100 sclerotia of *S. cepivora* at 15ºC. Both control and inoculated plants with sclerotia remained healthy 3 months after inoculation (Fig. 3.24). Chives inoculated by *S. cepivora* mycelium exhibited white rot disease at 15ºC (Fig. 3.25) and only *S. cepivora* was re-isolated from infected plants on V8 agar plates.

Figure 3.24 Effect of 15°C on pathogenicity of *Stromatinia cepivopra* on chives inoculated with sclerotia or mycelium.

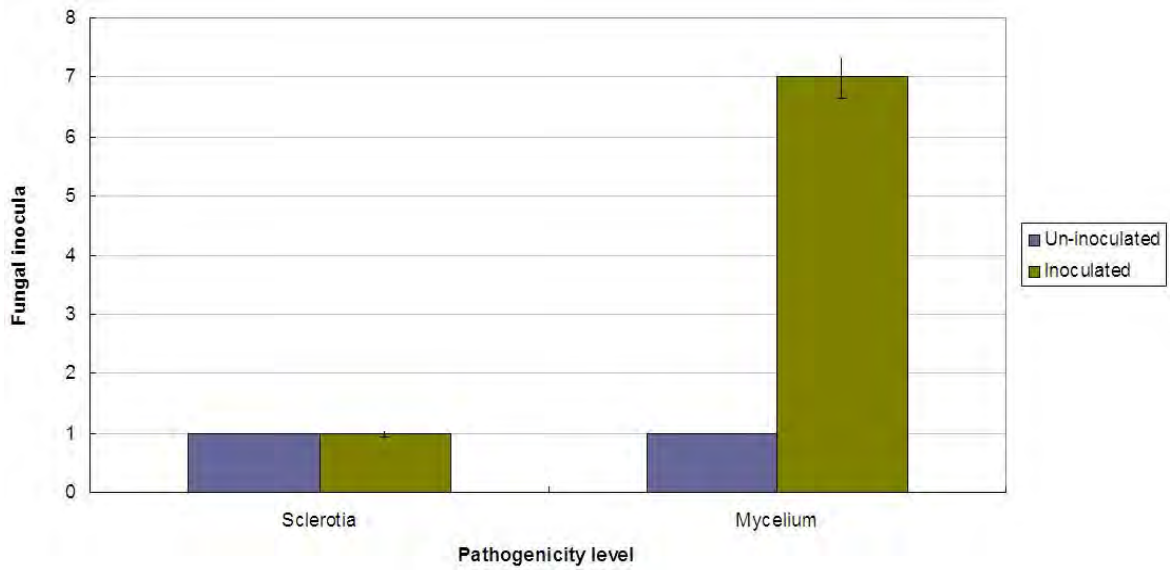


Figure 3.25 Pathogenicity of *Stromatinia cepivora* on chives at 15 ºC using sclerotia and mycelium (Bars=2 x standard error).

3.8.8 S. cepivora genetic diversity

3.8.8.1 Molecular analysis

3.8.8.1.1 ITS-PCR and sequencing

The ITS region was amplified and a single product of approximately 550 bp was observed for both *S. cepivora* isolates in 1.5% agarose gel. Alignment of sequences produced using ITS4 (reverse) primer showed variation in only 5 different nucleotides between DPI and VPRI 12439a (Fig. 3.26). The alignment of trimmed sequences of *S. cepivora* isolates with *S. rolfsii* showed no nucleotide variation between DPI and VPRI 12439a, but some variation between Australian *S. cepivora* isolates and isolates from other countries (Fig.3.27).

The distance matrix demonstrated that the *S. cepivora* isolates were relatively similar (Table 3.12). The ITS sequences of FJ231403.1, DPI and VPRI 12439a showed nucleotide differences from the other isolates. The DPI and VPR 12439a isolates were more closely similar to each other than to the rest of the isolates. The greatest distance was observed with the outgroup *S. rolfsii*.

Dendrograms produced with UPGMA and Neighbour-Joining analysis in MEGA4 indicated the two main clusters. All *S. cepivora* isolates were clustered together and separate from *S. rolfsii* (Fig. 3.28). In the UPGMA method, DPI and VPRI 12439a formed a clade with a low bootstrap value of 38%. FJ231403.1 from the Netherlands was different from the other isolates at a 77% bootstrap value. A clade of 84% confidence was formed with the remaining *S. cepivora* sequences, from FJ231398.1, FJ231399.1, FJ231400.1, FJ231401.1, FJ2313402.1, Z99681.1, Z99682.1 and Z99683.1.

The Neighbour-Joining phylogenetic tree also separated *S. cepivora* isolates and *S. rolfsii* (Fig. 3.29). FJ231403.1 was again different from the other *S. cepivora* isolates and bunched off into a separate clade of 60% bootstrap value. An 80% bootstrap value clade was formed separating DPI and VPRI 12439a sequences from the remainder of the sequences, for FJ231398.1, FJ231399.1, FJ231400.1, FJ231401.1, FJ2313402.1, Z99681.1, Z99682.1 and Z99683.1. Based on the branch lengths, DPI and VPRI 12439a were the least closely allied groups among all sequences.

```
             1         11        21        31        41        51
DPI          ACTACGTTCAGGACCCAACGGCGCCGCCACTGATTTTATTAGTCTGCCATTGCTGACATG
VPRI 12439a ACTACGTTCAGGACCCAACGGCGCCGCCACTGATTTTAG-AGTCTGCCATTGCTGACATG

             61        71        81        91        101       111
DPI          GACTCAATACCAAGCTGAGCTTGAGGGTTGAAATGACGCTCGAACAGGCATGCCCCCCGG
VPRI 12439a GACTCAATACCAAGCTGAGCTTGAGGGTTGAAATGACGCTCGAACAGGCATGCCCCCCGG

             121       131       141       151       161       171
DPI          AATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCA
VPRI 12439a AATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCA

             181       191       201       211       221       231
DPI          CATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTT
VPRI 12439a CATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTT

             241       251       261       271       281       291
DPI          GAAAGTTTTAACTATTATATAGTACTCAGACGACATTAATAAAAAGAGTTTTGATATTCT
VPRI 12439a GAAAGTTTTAACTATTATATAGTACTCAGACGACATTAATAAAAAGAGTTTTGATATTCT

             301       311       321       331       341       351
DPI          CTGGCGAGCATACGAGGCCCCAAAGGGCAGCTCGCCAAAGCAACAAAGTAATAATACACA
VPRI 12439a CTGGCGAGCATACGAGGCCCCAAAGGGCAGCTCGCCAAAGCAACAAAGTAATAATACACA

             361       371       381       391       401       411
DPI          AGGGTGGGAGGTCTACCCTTTCGGGCATGAACTCTGTAATGATCCTTCCGCAGGTTCACC
VPRI 12439a AGGGTGGGAGGTCTACCCTTTCGGGCATGAACTCTGTAATGATCCTTCCGCAGGTTCACC

             421       431       441       451       461       471
DPI          TACGGAAAAGGATCATTACAGAGTTCATGCC-GAAAGGGTAGACCTCCC-CCCTTGTGTA
VPRI 12439a TACGGATAAGGATCATTACAGAGTTCATGCCCGAAAGGGTAGACCTCCCACCCTTGTGTA

             481       491
DPI           TTATTACTTTGTTGCTTTGGC
VPRI 12439a   TTATTACTTTGTTGCTTTGGC
```

Figure 3.26 Sequence alignment of ITS-PCR products of *Stromatinia cepivora* Australian isolates. Sequences were edited by deletion of start and end sequences using BioEdit in BioManager, ANGIS (http://www.angis.org.au).

```
             1         11        21        31        41        51
DPI          CATTACA-GAGTTCATGCC-GA--------AAGGGTAGACCTCCC-CCCTTGTGTATTAT
VPRI 12439a  CATTACA-GAGTTCATGCCCGA--------AAGGGTAGACCTCCCACCCTTGTGTATTAT
FJ231399.1   CATTACA-GAGTTCATGCCCGA--------AAGGGTAGACCTCCCACCCTTGTGTATTAT
FJ231400.1   CATTACA-GAGTTCATGCCCGA--------AAGGGTAGACCTCCCACCCTTGTGTATTAT
FJ231401.1   CATTACA-GAGTTCATGCCCGA--------AAGGGTAGACCTCCCACCCTTGTGTATTAT
Z99681.1     CATTACA-GAGTTCATGCCCGA--------AAGGGTAGACCTCCCACCCTTGTGTATTAT
Z99682.1     CATTACA-GAGTTCATGCCCGA--------AAGGGTAGACCTCCCACCCTTGTGTATTAT
Z99683.1     CATTACA-GAGTTCATGCCCGA--------AAGGGTAGACCTCCCACCCTTGTGTATTAT
FJ231402.1   CATTACA-GAGTTCATGCCCGA--------AAGGGTAGACCTCCCACCCTTGTGTATTAT
FJ231398.1   CATTACA-GAGTTCATGCCCGA--------AAGGGTAGACCTCCCACCCTTGTGTATTAT
FJ231403.1   CATTACA-GAGTTCATGCCCGA--------AAGGGTAGACCTCCCACCCTTGTGTATTAT
S.rolfsii    -ATTGCATGTGCACACTCTGGAGCTATATAATATATACACCTGTGAACCAACTGTAGTCA

             61        71        81        91        101       111
DPI          T---ACTTTGTTGCTTTGGCGAGCTGCC-TTTGGGGCCTCGTATGCTCGCCAGAGA-ATA
VPRI 12439a  T---ACTTTGTTGCTTTGGCGAGCTGCC-TTTGGGGCCTCGTATGCTCTCCAGAGA-ATA
FJ231399.1   T---ACTTTGTTGCTTTGGCGAGCTGCCCTTTGGGGCCTCGTATGCTCGCCAGAGA-ATA
FJ231400.1   T---ACTTTGTTGCTTTGGCGAGCTGCCCTTTGGGGCCTCGTATGCTCGCCAGAGA-ATA
FJ231401.1   T---ACTTTGTTGCTTTGGCGAGCTGCCCTTTGGGGCCTCGTATGCTCGCCAGAGA-ATA
Z99681.1.g   T---ACTTTGTTGCTTTGGCGAGCTGCC-TTTGGGGCCTCGTATGCTCGCCAGAGA-ATA
Z99682.1.g   T---ACTTTGTTGCTTTGGCGAGCTGCC-TTTGGGGCCTCGTATGCTCGCCAGAGA-ATA
Z99683.1.g   T---ACTTTGTTGCTTTGGCGAGCTGCC-TTTGGGGCCTCGTATGCTCGCCAGAGA-ATA
FJ231402.1   T---ACTTTGTTGCTTTGGCGAGCTGCCCTTTGGGGCCTCGTATGCTCGCCAGAGA-ATA
FJ231398.1   T---ACTTTGTTGCTTTGGCGAGCTGCCCTTTGGGGCCTCGTATGCTCGCCAGAGA-ATA
FJ231403.1   T---ACTTTGTTGCTTTGGCGAGCTGCCCTTTGGGGCCTCGTACGTTCGCCAGAGA-ATA
S.rolfsii    GGAGAAATCCTAACTATGATTACCCTATATAACTCTTATTGTATGTTACATAGAACGATT

             121       131       141       151       161       171
DPI          TCAAA---CTCTTTTTATTAATGTCGTCTGAGTACTATATAATAGTTTAAAACTTTTCA-
VPRI 12439a  TCAAAA--CTCTTTTTATTAATGTCGTC-GAGTACTATATAATAGTT-AAAACTTTTCA-
FJ231399.1   TCAAAA--CTCTTTTTATTAATGTCGTCTGAGTACTATATAATAGTT-AAAACTTTCAAC
FJ231400.1   TCAAAA--CTCTTTTTATTAATGTCGTCTGAGTACTATATAATAGTT-AAAACTTTCAAC
FJ231401.1   TCAAAA--CTCTTTTTATTAATGTCGTCTGAGTACTATATAATAGTT-AAAACTTTCAAC
Z99681.1.g   TCAAAA--CTCTTTTTATTAATGTCGTCTGAGTACTATATAATAGTT-AAAACTTTCAAC
Z99682.1.g   TCAAAA--CTCTTTTTATTAATGTCGTCTGAGTACTATATAATAGTT-AAAACTTTCAAC
Z99683.1.g   TCAAAA--CTCTTTTTATTAATGTCGTCTGAGTACTATATAATAGTT-AAAACTTTCAAC
FJ231402.1   TCAAAA--CTCTTTTTATTAATGTCGTCTGAGTACTATATAATAGTT-AAAACTTTCAAC
FJ231398.1   TCAAAA--CTCTTTTTATTAATGTCGTCTGAGTACTATATAATAGTT-AAAACTTTCAAC
FJ231403.1   TCAAAA--CTCTTTTTATTAATGTCGTCTGAGTACTATATAATAGTT-AAAACTTTCAAC
S.rolfsii    TCATATTGAAACTTTGTTTTCTGACAAGTTTCTCTTAATTAAAAATATACAACTTTCAAC


               181       191       201       211       221
PT.ITS4_DP     CAAGGATCTCTTGGTTCTGGCATC-ATGAAGAA-GCAGCGAAAATG
PT.ITS4_VP     AACGGATCTCT-GGTTCTGGCATC-ATGAAAAAAGCAGCGAAAATG
FJ231399.1     AACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAA-TG
FJ231400.1     AACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAA-TG
FJ231401.1     AACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAA-TG
Z99681.1.g     AACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAA-TG
Z99682.1.g     AACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAA-TG
Z99683.1.g     AACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAA-TG
FJ231402.1     AACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAA-TG
FJ231398.1     AACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAA-TG
FJ231403.1     AACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAA-TG
S.rolfsii      AACGGATCTCTTGGCTCTTGCATCGATGAAGAACGCAGCGAAA-TG
```

Figure 3.27 Sequence alignment of ITS-PCR products of *Stromatinia cepivora* isolates (Table 3.2). Sequences were edited by deletion of start and end sequences using BioEdit in BioManager, ANGIS (http://www.angis.org.au).

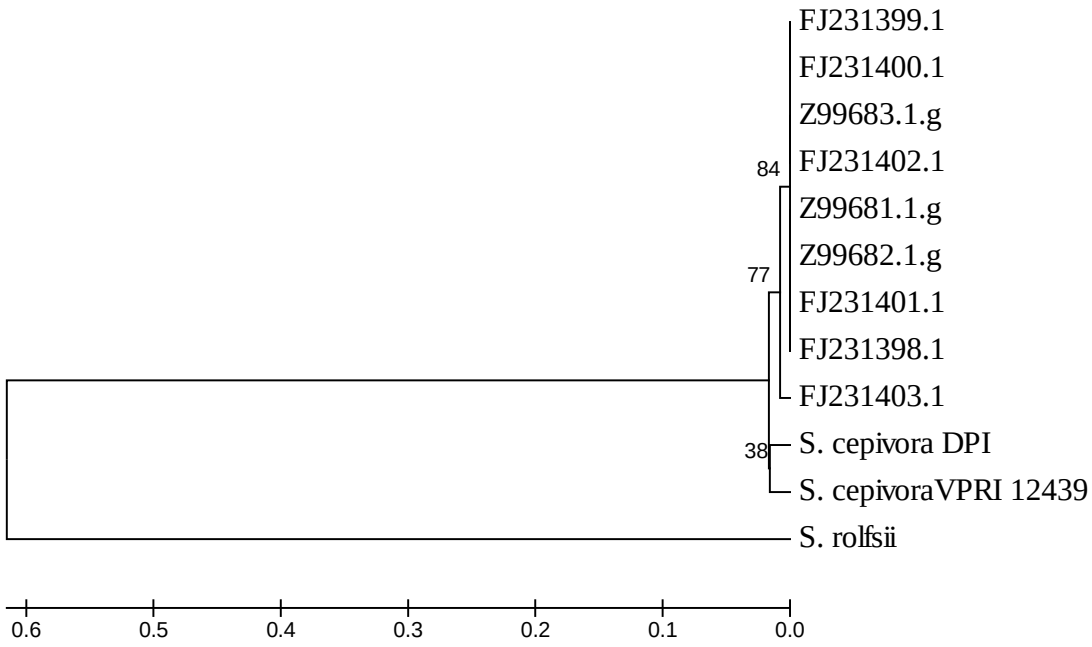


Figure 3.28 Bootstrap consensus phylogenetic tree obtained from UPGMA analysis of sequences of *Stromatinia cepivora* isolates and *Sclerotium rolfsii*. Numbers on clades demonstrate bootstrap value (%) from 1000 bootstrap replicates.

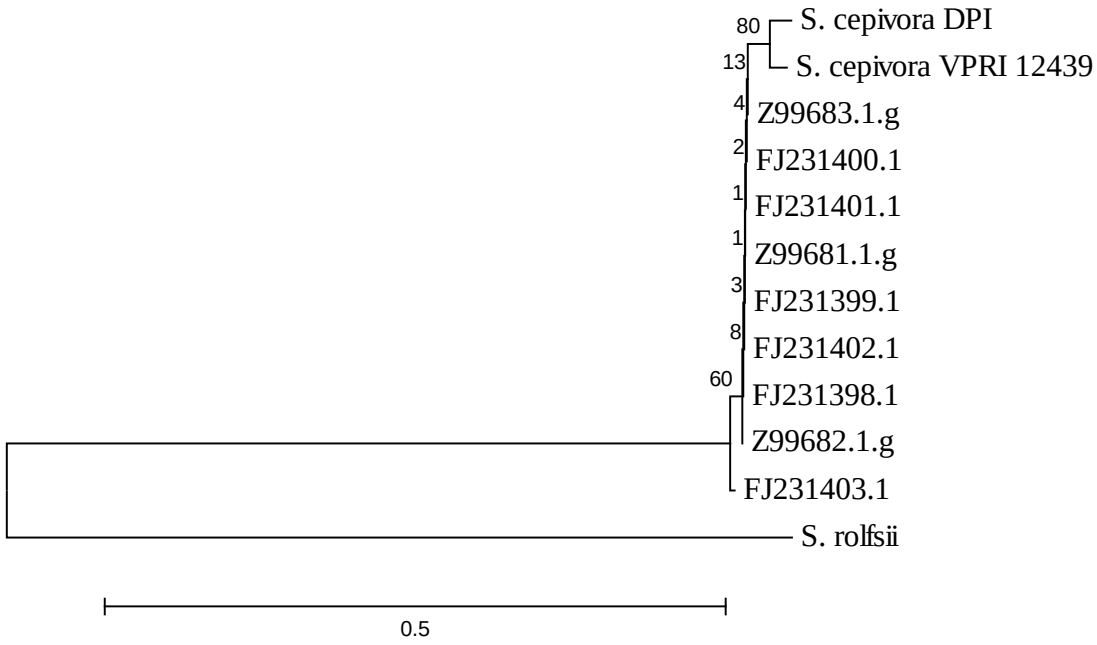


Figure 3.29 Bootstrap consensus phylogenetic tree generated from Neighbour-Joining analysis of nucleotide sequences of *Stromatinia cepivora* isolates and out-group *Sclerotium rolfsii*. Numbers on each branch demonstrate bootstrap values (%) from 1000 bootstrap replicates.

Table 3.12 Distance matrix of ITS sequences of *Stromatinia cepivora* isolates with the out-group species *Sclerotium rolfsii*. Standard error calculation was based on bootstrap values (%) from 1000 bootstrap replicates.

DPI	0.0											
VPRI 12439a	0.0203	0.0										
FJ231399.1	0.0202	0.0251	0.0									
FJ231400.1	0.0202	0.0251	0.0	0.0								
FJ231401.1	0.0202	0.0251	0.0	0.0	0.0							
Z99681.1	0.0202	0.0251	0.0	0.0	0.0	0.0						
Z99682.1	0.0202	0.0251	0.0	0.0	0.0	0.0	0.0					
Z99683.1	0.0202	0.0251	0.0	0.0	0.0	0.0	0.0	0.0				
FJ231402.1	0.0202	0.0251	0.0	0.0	0.0	0.0	0.0	0.0	0.0			
FJ231398.1	0.0202	0.0251	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		
FJ231403.1	0.0305	0.0353	0.0096	0.0096	0.0096	0.0097	0.0097	0.0097	0.0096	0.0096	0.0	
JF819727.1	0.07798	0.8	0.7233	0.7233	0.7233	0.712	0.712	0.712	0.7233	0.7233	0.7233	0.0
	DPI	**VPRI 12439a**	**FJ231399.1**	**FJ231400.1**	**FJ231401.1**	**Z99681.1**	**Z99682.1**	**Z99683.1**	**FJ231402.1**	**FJ231398.1**	**FJ231403.1**	**JF819727.1**

3.8.8.1.2 RAPD-PCR analysis

Of over sixty 10-mer RAPD primers from Operon kits OPA, OPB and OPM, only 24 primers produced clear amplicons with at least three bands on 1.5% agarose gel for DPI and VPRI 12439a isolates (Fig. 3.30). These primers produced 1-15 strong consistent bands of 150-3000 bps (Fig. 3.30) for both isolates and RAPD-PCR profiles were reproducible.

Polymorphism was assessed on presence and absence of RAPD products. Little genetic variation was observed between DPI and VPRI 12439a isolates using RAPD primers. Only one primer did not result in the same amplicons (Fig. 3.30). Polymorphism was only observed with the OPA-11 primer, which produced three amplicons of 2500, 1100 and 600 bp for the DPI isolate but more for the VPRI 12439a isolate (Fig. 3.30). There was some doubt about this, however, since the DPI isolate DNA extract amplified relatively faint bands with several RAPD primers compared with VPRI 12439a and the lack of fainter bands might have been due to lack of sensitivity in the detection method. No proximity matrix or RAPD dendrogram could be generated by SPSS Hierarchical Cluster analysis as only two isolates of *S. cepivora* were used.

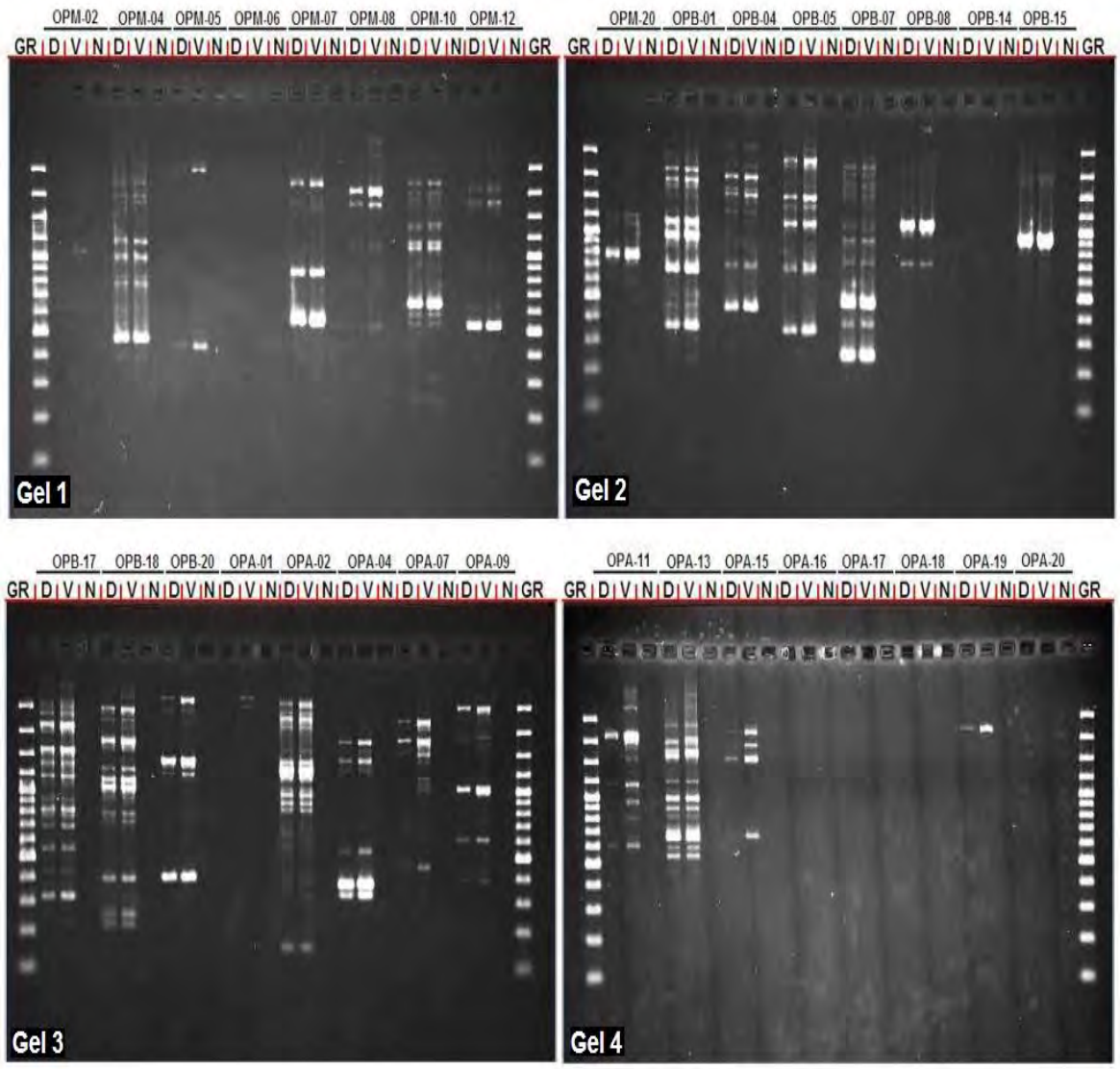


Figure 3.30 RAPD-PCR products and polymorphism of Australian *Stromatinia cepivora* isolates (DPI and VPRI 12439a) amplified with 32 RAPD primers. Symbols are: D: *S. cepivora* DPI isolates, V: *S. cepivora* VPRI 12439°, N: Blank (no DNA), GR: GeneRuler^{TM} 100 bp.

Primers used:

- Gel 1: OPM-02, POM-04, OPM-05, OPM-07, OPM-08, OPM-10, OPM-12
- Gel 2:OPM-20, OPB-01, OPB-04, OPB-05, OPB-07, OPB-08, OPB-14, OPB-15
- Gel3: OPB-17, POB-18, OPA-01, OPA-04, OPA-07, OPA-09
- Gel4:OPA-11, OPA-13, OPA-15, OPA-16, OPA-17, OPA-18, OPA-19, OPA-20

3.8.8.2 Traditional approaches

3.8.8.2.1 Mycelial compatibility

Mycelial incompatibility was observed between DPI and VPRI 12439a as a clear line at the interaction zone (Earnshaw and Boland 1997). The DPI isolate showed faster growth than VPRI 12439a (Fig. 3.31). VPRI 12439a produced few sclerotia only around the mycelial block, whereas the DPI isolate produced abundant sclerotia all over its growth. The same result was obtained in repeated pairings.

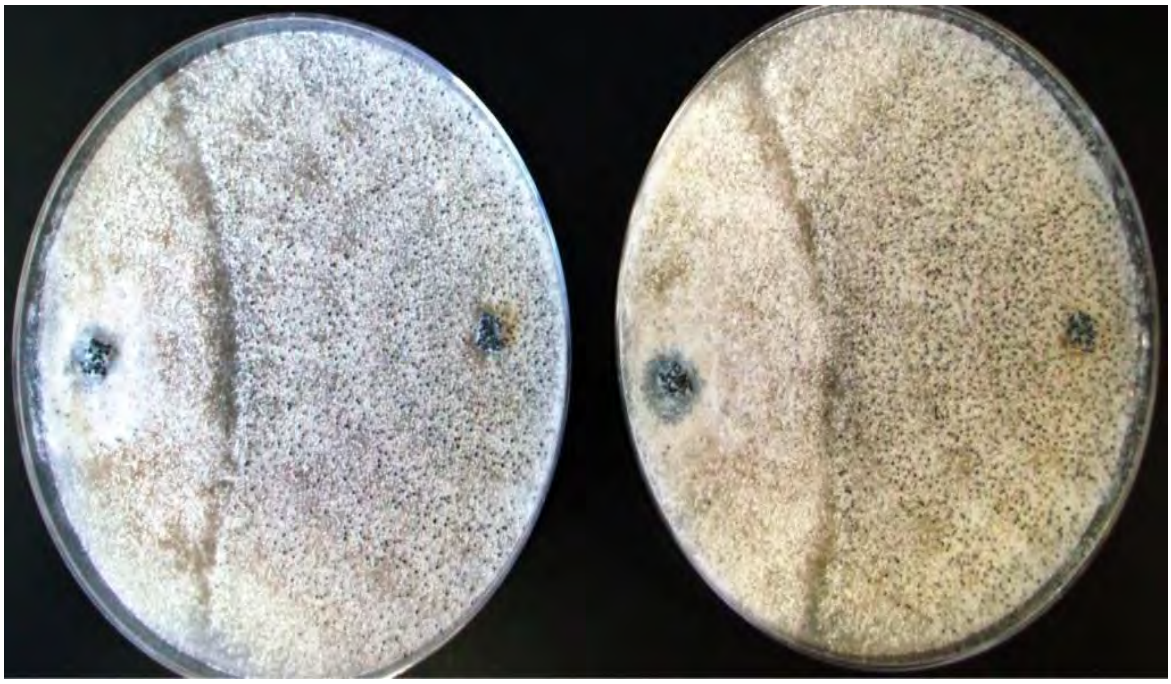


Figure 3.31 Pairing of Australian *Stromatinia cepivora* isolates on PDA for mycelial compatibility test between the isolates. Mycelial blocks of DPI (right inoculum) and VPRI 12439a (left inoculum) were subcultured on PDA in duplicate. The incompatible reaction can be observed as a clear line at the interaction zone.

3.8.8.2.2 Effect of different media on the mycelial growth and sclerotia production of S. cepivora isolates

Mycelial growth and sclerotia production were significantly different among media and between *S. cepivora* isolates (Table 3.13, 3.14). The DPI isolate produced greater mycelial growth (Fig.3.32) on almost all media than the VPRI 12439a isolate. Fresh PDA showed

the greatest mycelial growth among media, for the DPI isolate, but several other media were equivalent with the VPRI isolate. Medium affected sclerotia production significantly. SEA and MSA culture resulted in no sclerotia (Fig. 3.34). The DPI isolate produced the greatest number of sclerotia on IMA, whereas for the VPRI isolate IMA was one of the poorest media for sclerotia production. All other media produced sclerotia but with less difference between the isolates for the DPI isolate.

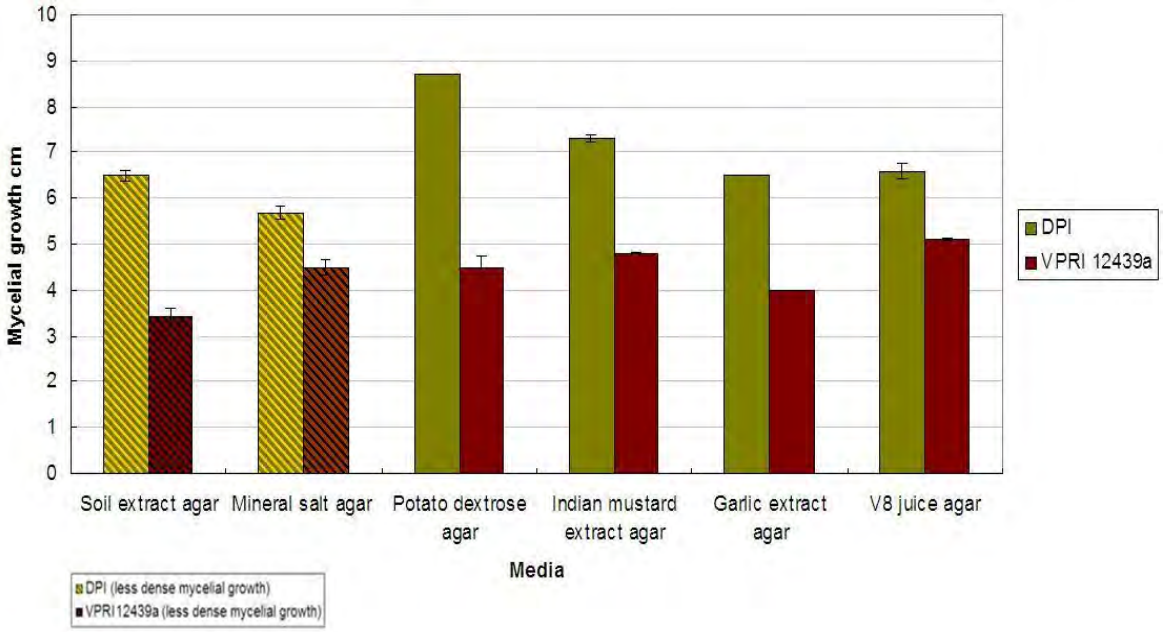


Figure 3.32 Effect of different media on mycelial growth of *Stromatinia cepivora* isolates (Bars=2 x standard error).

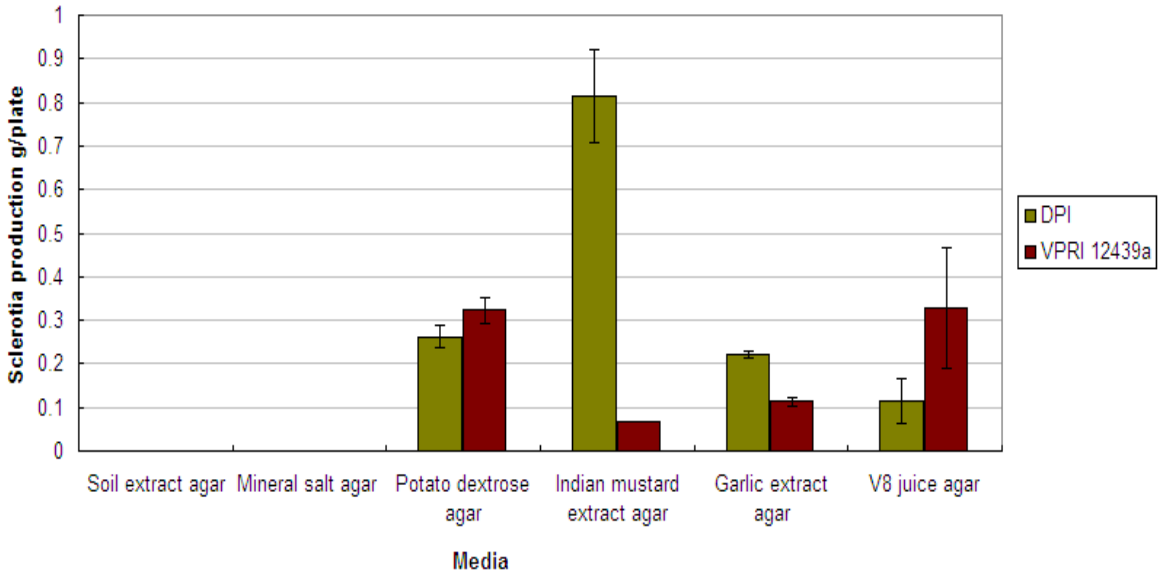


Figure 3.33 Effect of different media on sclerotia production of *Stromatinia cepivora* isolates (Bars=2 x standard error).

Table 3.13 Analysis of mycelial growth of *Stromatinia cepivora* isolates on different media in a split plot design using SPSS software.

Tests of Between-Subjects Effects

Dependent Variable: Mycelium

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	73.371[a]	15	4.891	79.89	.000
Intercept	1148.080	1	1148.080	18517.419	.000
Replicates	.264	2	.132	2.130	.051
Isolate	50.647	1	50.647	816.887	.000
Replicates * Isolate	.124	2	.062		.000
Media	12.918	5	2.584	41.677	.000
Isolate * Media	9.418	5	1.884	30.387	.000
Error	.859	20	.043		
Total	1222.310	36			
Corrected Total	74.230	35			

a. R Squared = .988 (Adjusted R Squared = .980)

Table 3.14 Analysis of sclerotia production of *Stromatinia cepivora* isolates on different media in a split plot design using SPSS software.

Tests of Between-Subjects Effects

Dependent Variable: Sclerotia

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.842[a]	15	.123	14.003	.000
Intercept	1.256	1	1.256	143.266	.000
Replicates	.032	2	.016	1.837	.185
Isolate	.084	1	.084	9.536	.006
Replicates * Isolate	.001	2	.000	.045	.956
Media	.881	5	.176	20.100	.000
Isolate * Media	.844	5	.169	19.250	.000
Error	.175	20	.009		
Total	3.274	36			
Corrected Total	2.017	35			

a. R Squared = .913 (Adjusted R Squared = .848)

3.8.8.2.3 Effect of smoke water on mycelial growth and sclerotia production of S. cepivora

Smoke water significantly reduced mycelial growth of *S. cepivopra* isolates (Figure 3.34) (Table 3.15, 3.16). No mycelial growth was observed on PDA with the greatest smoke water concentration. Mycelial growth was observed after 4 days on the next greatest concentration. The DPI isolate had greater growth on control plates without smoke water than the VPRI isolate, but then declined with concentration. By contrast, VPRI 12439a was stimulated by the lowest concentration of smoke water and then declined. Sclerotia production declined with smoke water concentration but only for VPRI 12439a and not evenly (Fig. 3.35).

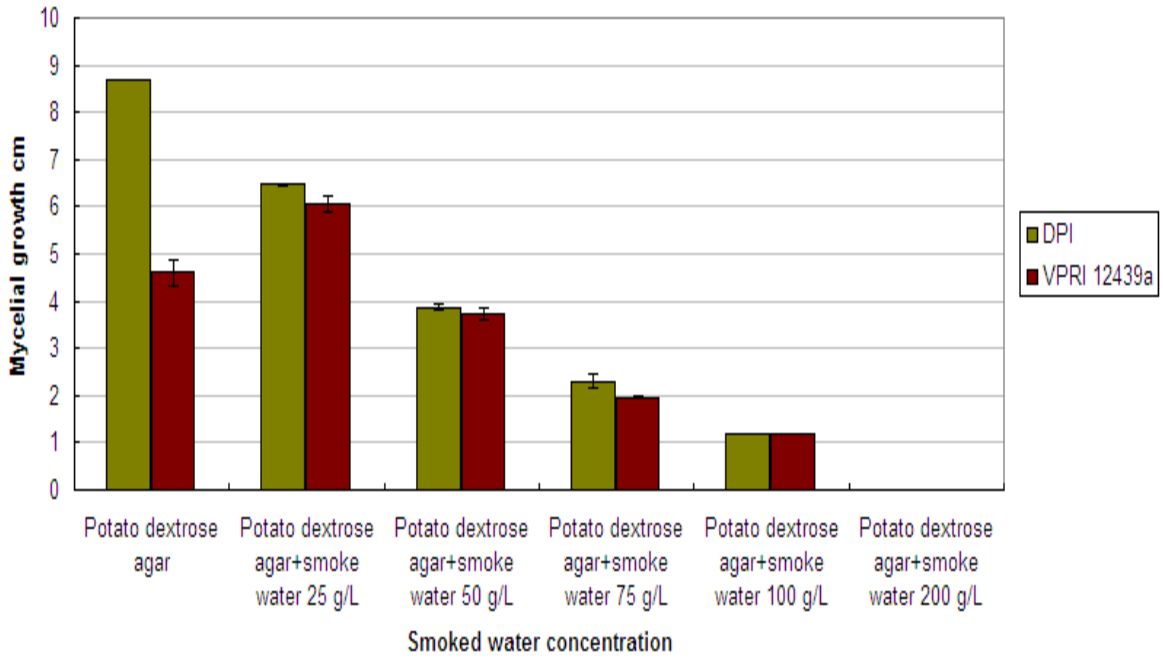


Figure 3.34 Effect of different concentrations of smoke water on mycelial growth of *Stromatinia cepivora* isolates (Bars=2 x standard error).

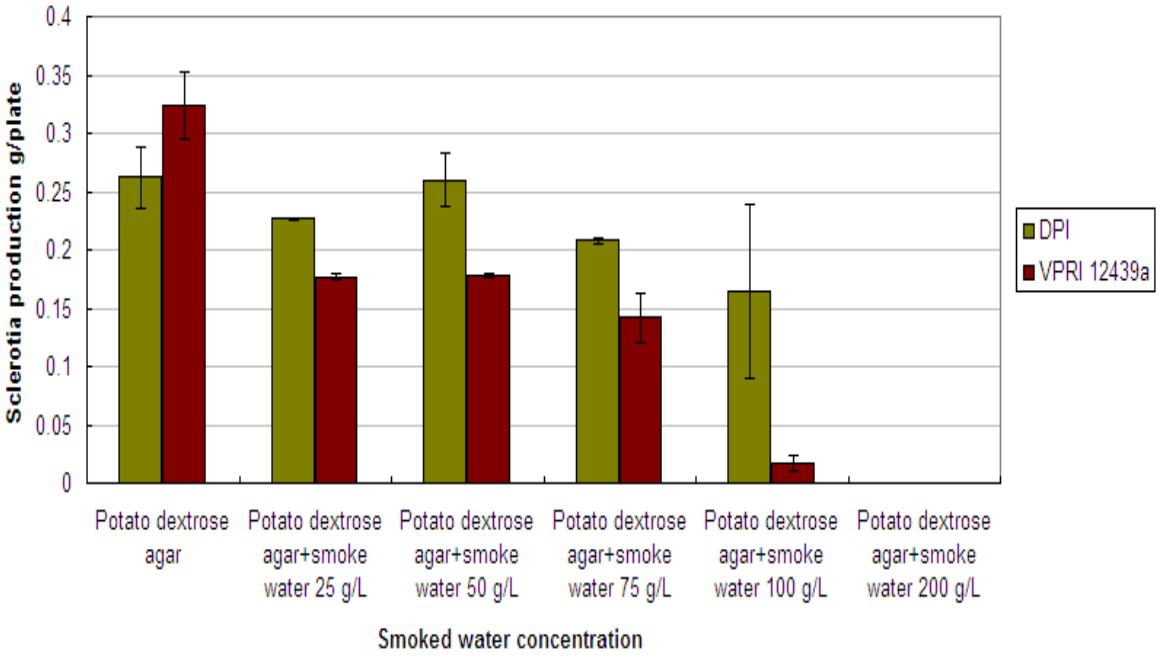


Figure 3.35 Effect of different concentration of smoked water PDA on sclerotia production of *Stromatinia cepivora* isolates (Bars=2 x standard error).

Table 3.15 Analysis of mycelial growth of *Stromatinia cepivora* isolates on different concentration of smoke water-PDA in a split plot design using SPSS software.

Tests of Between-Subjects Effects

Dependent Variable: mycelium

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	247.246[a]	15	16.483	401.483	.000
Intercept	402.003	1	402.003	9791.671	.000
Replicates	.002	2	.001	.020	.980
Isolate	6.167	1	6.167	150.210	.000
Replicates * Isolate	.051	2	.025	.616	.550
Media	221.546	5	44.309	1079.249	.000
Isolate * Media	19.481	5	3.896	94.903	.000
Error	.821	20	.041		
Total	650.070	36			
Corrected Total	248.068	35			

a. R Squared = .997 (Adjusted R Squared = .994)

Table 3.16 Analysis of sclerotia production of *Stromatinia cepivora* isolates on different concentration of smoke water-PDA in a split plot design using SPSS software.

Tests of Between-Subjects Effects

Dependent Variable: Sclerotia

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.393[a]	15	.026	14.929	.000
Intercept	.961	1	.961	547.089	.000
Replicates	.007	2	.003	1.903	.175
Isolate	.020	1	.020	11.502	.003
Replicates * Isolate	.007	2	.004	2.101	.149
Media	.321	5	.064	36.586	.000
Isolate * Media	.038	5	.008	4.299	.008
Error	.035	20	.002		
Total	1.389	36			
Corrected Total	.428	35			

a. R Squared = .918 (Adjusted R Squared = .857)

3.9 DISCUSSION

This is the first report showing white rot infection of *A. triquetrum* by *S. cepivora*. Since all *Allium* species are exotic to Australia, *S. cepivora* thus does have potential to control *A. triquetrum* in bushland, provided it is specific to *Allium*. It was not pathogenic on a range of endemic monocots found in infested areas, although more testing would be desirable, and field trials would be the next step. It also may have limited applications in permanent pasture to control problems with livestock milk and meat taint caused by *A. triquetrum*, provided these are distant from *Allium*-growing areas, as the isolates tested were also virulent pathogens on them. The main objective of this research was to find a *S. cepivora* isolate that is pathogenic to all *A. triquetrum* provenances and less pathogenic to cultivated *Allium* species. DPI isolate was pathogenic to all provenances by comparison with VPRI 12439a *in vitro* and almost *in vivo*. It therefore seemed luckily to fit the desired behaviour and no further isolates were sought.

3.9.1 Pathogenicity testing in vitro

The DPI strain was highly pathogenic to all provenances of *A. triquetrum* from across Australia and so would be preferred as a biological control agent, whereas strain VPRI 12439a was fatal to only four of the five provenances tested, even under axenic conditions inside test-tubes. The mycelium from both isolates covered all areas of the plants, whereas in the field it appears only on roots and crowns (DPI 2008). This foliar invasion is probably caused by the axenic, high humidity conditions inside the test-tubes, since normally the fungus invades only roots and bulbs (Abd-El-Razik et al. 1973). The exception to effectiveness of *S. cepivora* on *A. triquetrum* was 3/4 of the Wonthaggi plants, which showed no sign of infection with VPRI 12439a, despite lack of genetic difference by RAPD-PCR. This suggests that this one strain alone would not be effective in biological control. Sclerotia did not germinate and no mycelium grew. In white rot disease in cultivated *Allium* species, sclerotial germination is stimulated by S-alkyl-L-cysteine sulphoxides in exudates from the host plant roots (Coley-Smith and Holt 1966, Coley-Smith et al. 1990). Esler and Coley-Smith (1984) stated that resistance to the fungus within the genus *Allium* was due to lack of ability to stimulate sclerotia to germinate. Plants from this provenance may not exude this chemical in sufficient quantities or they may exude a similar compound that is not recognised by the sclerotia. Another possibility is that the Wonthaggi plants still harboured the inhibitory endogenous bacterium (Chapter 5), despite being micropropagated and appearing uncontaminated on MS medium. The bacteria may have prevented sclerotia germinating in tests *in vitro* and so endogenous infection in the field may limit control by *S. cepivora*.

3.9.2 Pathogenicity testing in vivo

While the results of *in vitro* pathogenicity testing were promising, pot trials under more exacting conditions were performed to indicate more realistically what the effect of the

fungus might be in infestations. The lack of white rot disease symptoms 3 months post-inoculation on *A. triquetrum* provenances and cultivated *Allium* species inoculated by sclerotia raised the question of whether or not the sclerotia were viable. Under sterile conditions, sclerotia do not need specific materials produced by *Allium* roots and germinate without an "external supply of nutrient" (Coley-Smith *et al.* 1967). The ready germination of surface-sterilised sclerotia collected from inoculated pots suggests that sclerotia were viable but did not germinate in the pots in the presence of the host plants. Coley-Smith *et al.* (1990) stated that sclerotia remain dormant in the soil until they have been stimulated by the host root exudates. The primary *Allium* root exudates, S-alk(en)yl-L-cysteine sulphoxides diallyl disulphide, stimulate sclerotia of *S. cepivora* to germinate, produce hyphae and subsequently penetrate the host tissues, but only indirectly and in non-sterile soil (Coley-Smith 1960; Coley-Smith and Holt 1966; Elser and Coley-Smith 1983). In non-sterile soil, microbes break down the S-alk(en)yl-L-cysteine sulphoxides diallyl disulphide into to 1-propyl- and 2-propenyl (diallyl) disulphide and it is these breakdown products that stimulate the sclerotia to germinate (Coley-Smith 1960; Coley-Smith and Holt 1966; Elser and Coley-Smith 1983). Since the plants were grown in sterile sand, the lack of microbes may have interfered with this process.

Coley-Smith *et al.* (1967) proposed that *Allium* exudates cause antibiotic responses around the roots and so reduce microbial activity and provide suitable conditions for sclerotia germination but it appears that microbial activity in non-sterile soil has a number of significant other effects on sclerotial germination (King and Coley-Smith 1969). Dickinson and Coley-Smith (1970) indicated that the addition of sclerotia to soil directly increased the microbial development there in, perhaps because sclerotia of *S. cepivora* exude stimulatory carbohydrates such as trehalose, glucose and mannitol, which cause bacterial sporulation on media (Coley-Smith and Dickinson 1971). Although the experiment was conducted using sterile sand, microorganisms are likely to have been

transferred though non-sterile water during irrigation of the pots. Bacterial growth (Gram + and Gram -) was noticed on plated sclerotia collected from inoculated pots. These may have been inhibitory to sclerotial germination in the soil but did not inhibit germination *in vitro*. The question of why viable sclerotia did not germinate in the pots is quite complex and further experimentation would be required to solve the problem, but it is not likely to be a problem in non-sterile soil in field trials and so was not pursued further. It might, however, be of interest in a program attempting to prevent sclerotial germination in cultivated *Allium* species.

One of the possible reasons for lack of sclerotial germination was temperature. Entwistle (1990) categorized the *Allium* species disease incidence based on temperature. Severe pathogenicity of *S. cepivora* in lower temperatures has been reported on cultivated *Allium* species (Crowe and Hall 1980a). Inoculation of potted chives at 15ºC still showed no sclerotial germination or white rot 3 months post-inoculation and so no difference from results at 15-25°C in the glasshouse. Under the same conditions, death of plants inoculated by mycelium was recorded. These results suggested that, if temperature affected sclerotial germination, it was not the main factor in lack of sclerotial germination.

The results of the pathogenicity testing of *A. triquetrum*, cultivated *Allium* species and native Australian monocots using mycelium of the fungus indicated that the strains tested were pathogenic and virulent on all *Allium* species tested but not on plants of other (native Australian) monocots tested, as no white rot or other disease symptoms were observed on Australian native plants 3 months post-inoculation. This suggests that the fungus would not attack these species when used as a biological control for *A. triquetrum*, though it would have been desirable not to have cultivated *Allium* species infected. With cultivated *Allium* species, there was no significant difference in pathogenicity of the fungus to

cultivated species and almost all plants were killed 3 months after inoculation in the glasshouse.

However; significant difference were observed between *A. triquetrum* provenances under the same environmental conditions. For instance, no disease symptoms were observed on any of the Hardy's Picnic Ground, Dandenongs, replicates, although all test-tube-grown plants died. It is not known if the mycelium of *S. cepivora* was not able to penetrate the host tissues or the plants were resistant to the fungus. Although there is only a small sign of resistance to white rot in *A. caeruleum* (Adams and Papavizas 1971), it seems that germinated sclerotia can infect species of *Allium* and four other members of the Liliaceae/Amaryllidaceae (Esler and Coley-Smith 1984). Only three replicates from Hardy's Picnic Ground, Dandenongs, were tested *in vivo*. Further work could involve more samples from this provenance for pathogenicity testing; however, none of the samples were affected even using mycelium, and therefore it seems likely that it has some degree of resistance. If so, it might be useful as a source of resistance genes and a resistant model plant in plant breeding.

3.9.3 S. cepivora genetic diversity

Pathogenicity variation suggested possible genotypic or phenotypic differences among Australian isolates, although the fungus lacks a sexual phase and genetic diversity between isolates was not expected (Brix and Zinkernagel 1992). Genetic differences along with environmental conditions may affect traits such as fungicide resistance and pathogenicity (Tyson *et al.* 2001; Pérez-Moreno *et al.* 2002). In this study similar ITS sequences of *S. cepivora* isolates from Netherlands, England, Holland, Canada, USA, Egypt, China and Australia suggested relatively small genetic diversity of the fungus over the world and the few nucleotide variations in the sequences seemed to be the result of single base-pair

mutations (Arnheim *et al.* 1980; Hsiao *et al.* 1994; Hsiao *et al*. 1999). The identical ITS-PCR products and the sequences of Australian *S. cepivora* isolates suggested that DPI and VPRI 12439a are identical; however, they were different in pathogenicity. Even small variations in the ITS region may reflect differences between species and subspecies and pathogenicity, as with *Ophiostoma ulmi* (Hsiao *et al.* 1995; Ainouche and Bayer 1997).

Although the ITS sequence analysis is popular in taxonomic studies, it consists of non-coding regains and the variation may not be reflected in functionality. Due to its limited size, which is less than 1 kbp, it screens only a small part of the whole genome. As both Australian isolates used in this study were collected from Victoria, it is impossible to conclude that there is no genetic diversity of the fungus within Australia. If further isolates had been obtained, the small variation in ITS sequences of this fungus around the world makes it seem unlikely that large variation will be found.

The RAPD-PCR profiles also did not demonstrate genetic variation of the Australian isolates; however; Tyson *et al.* (2001) and Pérez-Moreno *et al.* (2002) reported geographical grouping of *S. cepivora* isolates using RAPD-PCR. As the fungus lacks a sexual phase, genetic diversity between strains is not expected (Brix and Zinkernagel 1992). Almost all 24 RAPD primers amplified the same banding patterns for both fungal isolates except OPA-11. With OPA-11 three distinctive bands were amplified for DPI isolate where VPRI 12439a showed 11 bands. Variation in band intensity of the DPI isolate can be due to its different DNA concentration. Similar results have been documented previously for other species by Crowhurst *et al.* (1991) and Goodwin and Annis (1991). The RAPD-PCR result indicates high genetic similarity between DPI and VPRI 12439a but application of more RAPD primers such as OPG, OPK, and OPP or microsatellites in ISSR (Inter-Sequence Simple Repeats) may find further variation. The global geographic grouping of *S. cepivora* isolates was recorded based on RAPD analysis

in previous studies. Tyson *et al.* (2001) demonstrated six genetic groups of the fungus from Australia, Brazil, Canada, Netherlands, Spain, Switzerland, Germany and United Kingdom. Almost all isolates from the same origin clustered together on the parsimony tree based on single band differences (Tyson *et al.* 2001) and not genetic variation was observed between four Australian isolates using RAPD primers. Pérez-Moreno *et al.* (2002) also showed the genetic dissimilarity of *S. cepivora* from Spain, Mexico and Venezuela.

RAPD analysis is a useful PCR-based techniques used in biodiversity studies as a large part of the genome can be screened (Welsh and McClelland 1990). Like the ITS sequences uniformity, RAPD analysis did not demonstrate clear genetic diversity between the Australian isolates, but only two isolates from Australia were used in this study. Pérez-Moreno *et al.* (2002) showed that in several cases the genetic variation of the fungus collected from a single field was greater than differences among isolates collected from different states in Mexico. It is documented that genetic variation detected by RAPD-PCR can reflect the pathogenicity and virulence of pathogens such as *Fusarium solani* f. sp. *curcurbitae* (Crowhurst *et al.* 1991), *Fusarium oxysporum* f. sp. *pisi* (Grajal-Martin *et al.* 1993) and *Leptosphaeria maculans* (Goodwin and Annis 1991). Therefore more fungal collections from different infested regions in Australia and other countries and their examination by RAPDs or ISSR could help to determine genetic variation in the fungus accurately; however, attempts to import the fungus through Australian quarantine were subject to protracted delay and were abandoned as it would not be possible to test their pathogenicity or use them in *A. triquetrum* natural habitats.

3.9.4 Traditional approaches

3.9.4.1 Mycelial compatibility

Mycelial compatibility grouping and pathogenicity testing of the fungal strains suggested that they are only minimally genetically different. Mycelial incompatibility is associated with variation in DNA level and multiple loci (Kohn *et al.* 1990; Liu and Milgroom 1996; Couch and Kohn 2000). Grouping of plant pathogenic fungi based on mycelial compatibility has been reported in a wide range of Ascomycetes such as *Sclerotinia sclerotiorum* (Kohn *et al.* 1990), *Fusarium oxysporum* (Harveson and Rush 1997) and *Phomopsis subordinaria* (Meijer *et al.* 1994). In this study, mycelia of DPI and VPRI 12439a isolates were incompatible. This result validated the genetic heterogeneity of the fungus in Australia that was demonstrated in RAPD-PCR analysis and pathogenicity testing. Somatic phenotype diversity of *S. cepivora* has been demonstrated in previous studies (Earnshaw and Boland 1997; Tyson *et al.* 2001); however; Tayson *et al.* (2001) reported that the isolates from Australia, New Zealand and England were compatible with each other and the fungus may have been transferred from England to Australia and New Zealand. Therefore this study is the first report of genetic diversity of *S. cepivora* isolates based on mycelial incompatibility in Australia. Mycelial compatibility is a useful method to reflect the genetic diversity in *S. cepivora* population, however; it cannot detect the degree of variation (Earnshaw and Boland 1997).

3.9.4.2 Media assessment and effect of smoke water on fungal growth

Mycelial growth and sclerotia production on a range of media showed phenotypic variation between DPI and VPRI 12439a isolates. Sclerotia germination and mycelial growth of the fungus is directly related to carbohydrates in sterile media (Papavizas 1970). The less dense mycelial growth and lack of sclerotia production of both isolates on mineral salt agar and soil extract agar with no extra glucose suggested that simple carbon sources stimulate sclerotial germination and soil extract is more likely to have complex

carbon source. Antifungal activities of Indian mustard seed oil and garlic juice have been reported on many plant pathogenic fungi and bacteria (Sitara *et al.* 2008; Charimbu *et al.* 2009) but not in *S. cepivora*, as the DPI isolate showed greater mycelial growth and sclerotial production on Indian mustard agar and garlic extract agar.

Smoke water did not affect sclerotial germination and only inhibited the mycelial growth of the fungus at greater concentrations; therefore there is unlikely to be a problem using the fungus in infestations such as areas near Kinglake National Park that was burnt in 2009. Wolkowskaja and Lapszin (1962) stated that smoke solution has an inhibitory effect on mycelial growth of wide range of fungal species.

3.9.5 Mycoherbicide development and further research

Releasing a virulent pathogen for cultivated *Allium* species into bushland or pasture is controversial and any field release would require safeguards against spread to areas suitable for the production of cultivated *Allium* species, such as onions, leeks and garlic, before *S. cepivora* could be introduced as a biological control agent. Experiments are currently under way in collaboration with Parks Victoria to field-test the DPI isolate in environmental *A. triquetrum* infestations in Victoria. Although no white rot disease symptoms were observed on treated native monocots in the glasshouse, other native Australian members of the *Liliaceae/amaryllidaceae* must be pot-tested as a priority to ensure that the fungus is specific to *Allium* species in the field. Testing of further provenances and strains is justified, not only to find the most effective combinations of plant provenance and fungal strain, but also to establish the host specificity (Muller-Stover and Kroschel 2005, Morin *et al.* 2006).

3.9.6 Conclusion

S. cepivora is a promising biocontrol agent for *A. triquetrum* provenances in Australia. The DPI isolate was pathogenic and virulent almost on all provenances *in vitro* and *in vivo* and it could be a potential bioherbicide to control the weed in infestations. However; pathogenicity variation on the potted plants in the glasshouse e.g. Hardy's Picnic Ground, suggested that more than one isolate may be needed to achieve successful biocontrol of all provenances. Genotypic and phenotypic variations between Australian isolates based on pathogenicity testing, molecular analysis, mycelial compatibility and media assessment suggested that DPI and VPRI 12439a are different and genetic variation exists in *S. cepivora* in Australia.

Chapter 4

Evaluation of *Pectobacterium carotovorum* as a potential biocontrol agent for *Allium triquetrum*

4.1 INTRODUCTION

This chapter reviews and tests an isolate of *Pectobacterium carotovorum* ssp. *carotovorum* as a biological control agent for *A. triquetrum*. The rationale for this is that soft rot of some bulbs was noted in storage and this bacterium isolated and tested. *A. triquetrum* plants collected from South Australia were potted up and grown on in a RMIT University glasshouse. Bulbs were collected after seasonal dieback, washed with tap water, dried at room temperature for 24 h and stored at 4ºC in paper bags for future use in pathogenicity testing.

Soft rot disease symptoms were observed after 2 months' storage on a few bulbs collected from Horsnell Gully (SA) in 2010. The disease was observed as rotted yellow smelly bulbs that emitted cellular debris and bacterial masses on pressing the infected bulbs. A bacterium was isolated from the infected bulbs on nutrient agar and physiological and molecular analysis suggested that *Pectobacterium carotovorum* ssp. *carotovorum* caused the soft rot disease. This suggested that the bacterium should be evaluated as a potential biocontrol agent for *A. triquetrum* in Australia, as the plant occurs in damp woodlands, wetlands and shaded areas, which would be suitable conditions for the bacteria to grow. The remainder of this section therefore reviews bacterial soft rots with a particular focus on *Pectobacterium* species. Many pathogenic fungal species have been studied as potential agents for the management of various weed species (Pereira *et al.* 2003; Yandoc

et al. 2005). However; no pathogenic bacterium has yet been recorded as an effective agent for control of invasive plant species.

Bacterial soft rot is a common destructive disease of wide range of plants such as vegetables, fleshly fruits and ornamentals worldwide (Liao *et al.* 1993; Barras *et al.* 1994). Soft rot disease caused by several soil-borne bacterial species affects crops in the field, garden, greenhouse and storage. Soft rots infect the host parenchyma tissues by producing tissue-macerating enzymes. Only a few bacterial species cause soft rot in living plant tissues (Pérombelon and Kelman 1987; Pérombelon 2002). These include some Gram-positive and Gram-negative bacterial species such as species of *Erwinia*, *Pseudomonas*, *Clostridium* and *Flavobacterium* (Dowson 1941; Lund 1979; Pérombelon 1980; Pennycook 1989; Wright and Hale 1992; Wright *et al.* 1993; Wright and Triggs 2005). Lack of host specificity is the main characteristic of the soft rots and one crop can be affected by several pathovars or species.

Soft rot disease is an important disease of commercial onions worldwide (Wright 1993). The causative bacteria are common in the environment and can be isolated from infected tissues in the soil as well as healthy foliage. Soft-rotting agents can infect onion in any growth stage, from seeds to stored bulbs (Hale *et al.* 1992). Of the bacterial species and pathovars cause soft rot in onions, species of *Erwinia*, *Pectobacterium* and *Pseudomonas* are the most common pectolytic species with *Allium* (Cother and Sivasithamparam 1983).

4.1.1 Erwinia

Pectobacterium was known previously as *Erwinia. Erwinia* sp. is divided into three main groups: *E. amylovora*, *E. herbicola* and *E. carotovora* (Dye 1983; Cother and Sivasithamparam 1983). *Erwinia* spp. cause a wide range of disease symptoms in plants

and survive between hosts on infected plant tissues or in insect (Leach 1964). The *E. amylovora* group are plant pathogens causing wilts or dry-necrotic symptoms on plants and so do not produce pectic enzymes or yellow pigments (Dye 1983). The *E. herbicola* group are common epiphytes and opportunistic pathogens on several plants. They produce yellow water-insoluble pigments and their disease symptoms are in association with other phytopathogenic bacteria (Gibbins 1978). The *E. carotovora* (known as *Pectobacterium carotovorum*) group is highly pectolytic and produces macerating enzymes causing soft rot in plants (Dye 1983; Cother and Sivasithamparam 1983).

4.1.2 Pectobacterium carotovorum cause soft rots in plants

4.1.2.1 Taxonomy and classification

Pectobacterium carotovorum Waldee 1945 was known previously as *Erwinia carotovora* (Jones 1901) (Winslow *et al.* 1920) and is a member of the family *Enterobacteriaceae* (Table 4.1). Dye (1983) described the bacterium as a Gram-negative motile rod (0.5-1 x 1-3 µm) that forms single or short chains of cells. It is facultatively anaerobic and its optimum growth temperature is 27-30ºC. *P. carotovorum* is oxidative negative and catalase-positive, produces acid by fermentation and assimilates a wide range of carbon sources such as glucose, sucrose, galactose, fructose and α–methyl glucoside (Dye 1983). *P. carotovorum* has three major important sub-species causing soft rots in plants (Table 4.2): *P. carotovorum* subsp. *carotovorum* authority (Jones 1901; Bergey *et al.* 1923), *P. carotovorum* subsp. *atrosepticum* authority (Van Hall 1902) and *P. chrysanthemi* authority (Burkholder *et al.* 1953).

Table 4.1 Classification of *Pectobacterium carotovorum* (Waldee 1945).

Domain: *Bacteria*

Phylum: *Proteobacteria*

Class: *Gamma Proteobacteria*

Order: *Enterobacteriales*

Family: *Enterobacteriaceae*

Genus: *Pectobacterium* Waldee

Species: *Pectobacterium carotovorum* Waldee 1945

4.1.2.2 Isolation and identification of P. carotovorum

Pectobactertium species can be isolated from rotted plant tissues such as stems, tubers and bulbs. There are many selective media designed for isolation of *Pectobactertium* species. The most commonly used medium is modified crystal violet pectate (CVP) designed by Cuppels and Kelman (1975), which contains crystal violet. The addition of manganous sulphate (0.8 g/L) to CVP medium can inhibit pectolytic *Pseudomonas* (O'Neill and Logan 1975).

Colonies of all *P. carotovorum* subspecies are greyish-white to creamy-white on most media. Dye (1983) described *P. carotovorum* colonies on agar after 24 h as smooth, round, slightly raised and glistening. Some colonies produce dark blue pigment after 5-10 days in incubation at 22-27ºC on yeast extract-dextrose-calcium carbonate agar (YDC) (Bradbury 1977). A 'fried-egg' appearance can be observed in some colonies on PDA after 3-6 days. Table 4.3 indicates the physiological and biochemical tests used to differentiate *P. carotovorum* subsp. *carotovorum* from other *Pectobacterium* species.

Table 4.2 Examples of soft rot disease caused by *Pectobacterium* species and their host plants (vegetables, fleshy fruits and ornamentals).

Disease name and host plant	Bacterial agent	Reference
Barn rot of tobacco	*P. carotovorum* subsp. *carotovorum*	Holdeman and Borkhulder (1956)
Soft rot and blackleg of potato stem	*P. carotovorum* subsp. *carotovorum*	Stanghellini and Meneley (1975)
Soft rot of bunching onion	*P. carotovorum* subsp. *carotovorum*	Halfeld-Vieira and Nechet (2008)
Soft rot of Calla lily	*P. carotovorum* subsp. *carotovorum*	Ni *et al.* (2010)
Soft rot of celery	*P. carotovorum* subsp. *carotovorum*	Wimalajeewa (1976)
Soft rot of Chinese cabbage	*P. carotovorum* subsp. *carotovorum*	Mew *et al.* (1976)
Soft rot of lily bulb	*P. carotovorum* subsp. *carotovorum*	Sang *et al.* (2003)
Stem rot of tomato	*P. carotovorum* subsp. *carotovorum*	Hibar *et al.* (2007)
Root rot of sugar-beet	*P. carotovorum* subsp. *atroseptica*	De Mendonca and Stanghellini (1979)
Soft rot and blackleg of potato	*P. carotovorum* subsp. *atroseptica*	Powelson (1980)
Foot rot of rice	*P. chrysanthemi* (*Dickeya* sp.)	Goto (1979)
Fruit collapse of pineapple	*P. chrysanthemi* (*Dickeya* sp.)	Lim and Lowings (1978)
Soft rot of onion	*P. chrysanthemi* (*Dickeya* sp.)	Palacio-Bielsa et al. (2006)
Soft rot of orchids	*P. chrysanthemi* (*Dickeya* sp.)	Abdullah and Kadzimin (1993)
Stalk rot of maize	*P. chrysanthemi* (*Dickeya* sp.)	Hoppe and Kelman (1969)
Stalk rot of corn	*P. chrysanthemi* (*Dickeya* sp.)	Abdulah (1982)
Soft rot of ginger	*P. chrysanthemi* (*Dickeya* sp.)	Stirling (2002)

Table 4.3 Physiological and biochemical tests used for identification of *Pectobacterium carotovorum* subsp. *carotovorum* (Pérombelon 1973; Lelliott 1974; Dickey 1979; Dye 1983). R: resistance ≥ 23, S: sensitive ≤ 13.

Physiological tests	***P. carotovorum* subsp. *carotovorum***	***P. carotovorum* subsp. *atroseptica***	***P. chrysanthemi***
Blue pigment on PDA	-	-	V
Growth at 37ºC	+	-	+
Reducing substances from sucrose	-	+	**V**
Acid production in 7 days:	----------	----------	----------
Maltose	**V**	+	-
α–methyl glucoside	-	+	-
Lactose	+	+	-
Trehalose	+	+	+
Palatinose	-	+	-
Dulcitol	-	-	-
Utilization of malonate	-	-	+
Utilization of tartrate	-	-	+
Indole production	-	-	+
Growth in 5% NaCl	+	+	**V**
Lecithinase	-	-	+
Phosphatase	-	-	+
Erythromycin sensitivity *	**R**	**R**	**S**
Optimum temperature	**28-30**	**27**	**34-37**
Minimum & maximum temp.	**6-42**	**3-35**	**6->45**

* 15µg/mL
V: Variable

4.1.3 Distribution

The *P. carotovorum* group is distributed widely geographically and its soft rot disease has been reported on different crops in the field and storage (Pérombelon 2002). Three soft rotting *P. carotovorum* strains are specific to a number of crops. *P. carotovorum* subsp.

carotovorum is widely distributed in the temperate and tropical zones (Toth *et al.* 2003; Charkowski 2006) and has the broadest host range. The disease caused by *P. carotovorum* subsp. *carotvorum* during storage has been reported mainly on potato, tomato and onion; however, it is also reported on field crops such as rice, corn and sugar beet in the field. *P. carotovorum* subsp. *antroseptica* is mostly pathogenic on potatoes and occasionally on closely related species in cool climates, mainly in Russia (Fig. 4.1) (Dickey 1979). *P. chrysanthemi* is distributed mainly in tropical and sub-tropical areas and is a common pathogen in glasshouse-grown crops (Fig. 4.2). However; *P. chrysanthemi* is widely distributed in European areas causing blackleg of potatoes (Toth *et al.* 2011). It has been reported in field-grown crops such as maize and rice in the filed (Hoppe and Kelman 1969; Goto 1979).

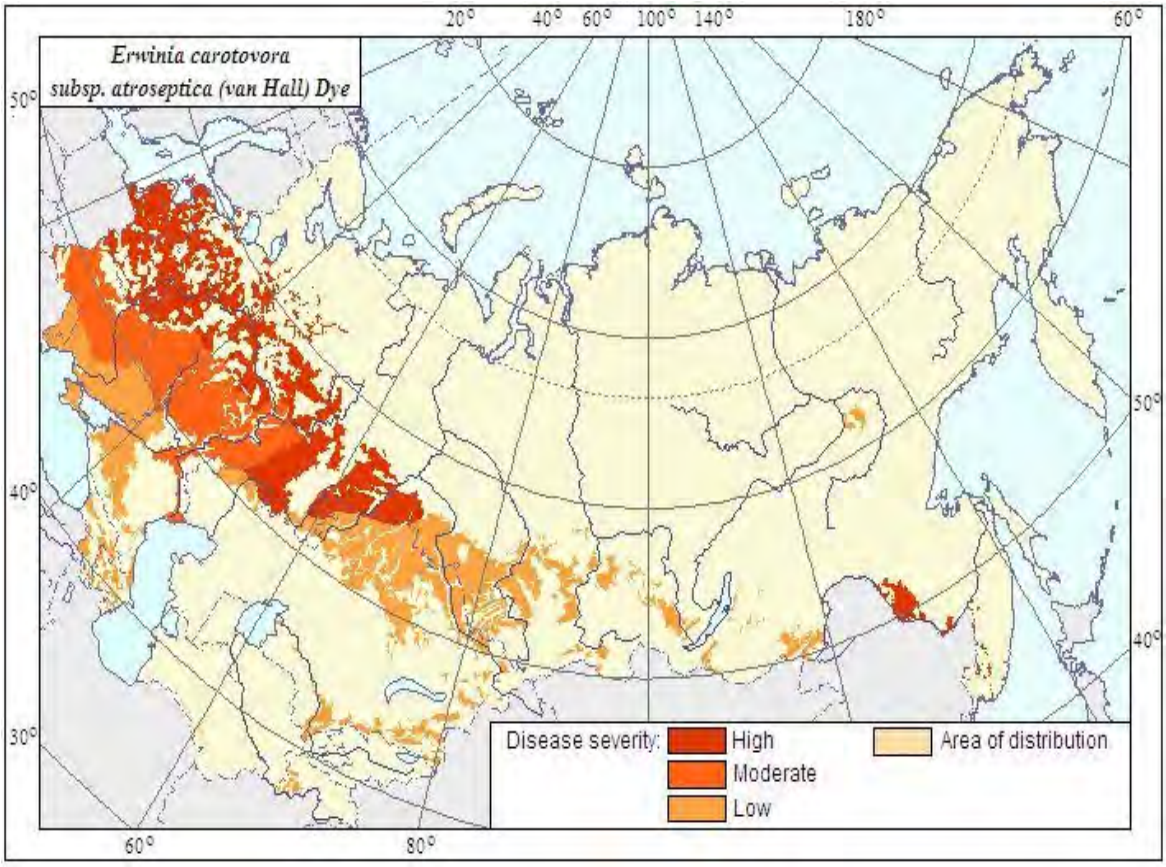


Figure 4.1 *Pectobacterium carotovorum* subsp. *antroseptica* distribution and zone of severity of blackleg (Lazarer and Saulich 2007).

Figure 4.2 Geographical distributions of *Pectobacterium chrysanthemi* disease (Ollie Martin from EPPO Date Sheet 2011).

4.1.4 Disease symptom and infection process

The bacterial soft rot disease symptoms caused by *Pectobacterium sp.* are similar on most crops. The most significant crop loss caused by the *P. carotovorum* group was observed on potato tubers (Lund 1979). Disease is common in wet soil and lesions usually form on the underground tissues with symptoms of maceration and rotting of parenchymatous tissues (Pérombelon 1980). It then spreads up to the shoots and fleshy tissues in most of the crops in the field and in storage. The disease symptoms on decaying *Allium* species are mushy, watery and smelly pale-brown bulbs (Babadoost 1990). The infected bulbs look 'cooked' from the inside and the slimy matter which is the bacterial mass and cellular debris is emitted if pressed (Fig. 4.3) (Babadoost 1990).

The soft rot bacteria enter the host plant tissues initially through wounds and naturally occurring cracks in the field or storage and survive between hosts on the infected tissues (Pérombelon 1980). They can also infect the plant tissues through the presence of other disease-producing agents such as fungal pathogens (Meneley and Stanghellini 1975;

Babadoost 1990). Moisture is an essential factor for bacterial enzymatic activity and penetration of the host plant tissues (OBI and Umezurike 1981). At field capacity, healthy tissues can be infected too (Meneley and Stanghellini 1975). The soft rot bacteria multiply in the infected plants by digesting the cell wall and middle lamella and the infected plants collapse suddenly. Nematodes and onion maggot are the main agents transferring the disease from infected tissues to the adjacent plants in the field (Delahaut and Stevenson 2004).

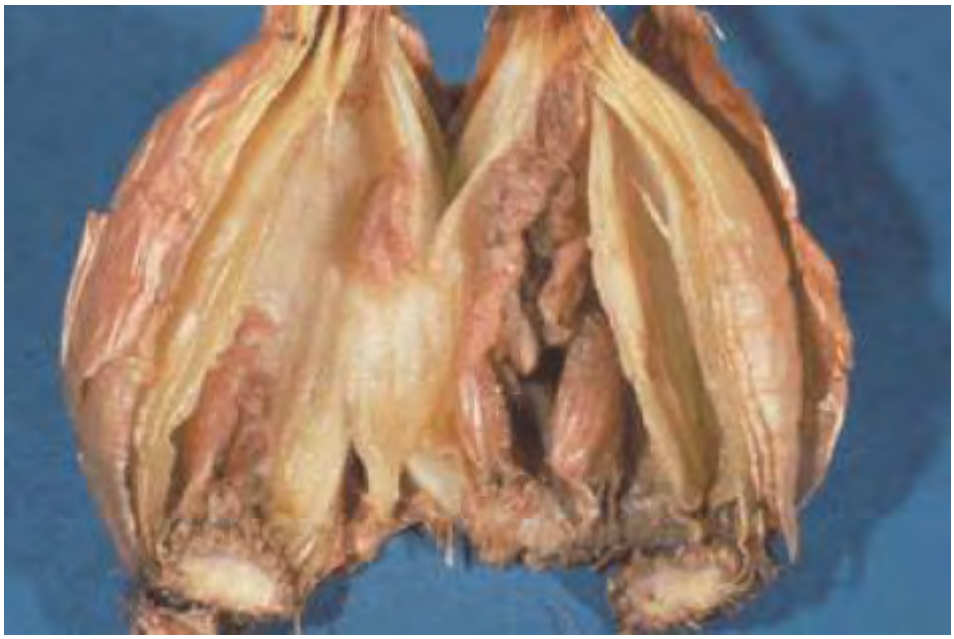


Figure 4.3 Soft rot disease of green onion (*Allium cepa*) caused by *Pectobacterium carotovorum* (Babadoost 1990).

4.1.4.1 Environmental factors involved in disease development

There are many factors involved in crop storage rot caused by soft rotting bacteria such as *P. carotovorum*. Anaerobic condition along with high humidity and optimal temperature for bacterial growth initiate rotting in storage (Cromarty and Easton 1973; Kelman *et al.* 1978). However; it is documented that *P. carotovorum* growth is higher under aerobic condition *in vitro* (Wells 1974). Under favourable condition only few numbers of soft rotting bacterial cells are sufficient to initiate the infection, however; the host plant

decaying time is relatively dependent to the optimal temperature (Pérombelon 1973; De Boer *et al.* 1978). Graham (1979) stated that the soft rot disease incidence on potato tubers in temperate region occurs in cooler temperature and average rainfall, however; high humidity is an essential factor in warmer regions. Temperature is also one of the effective factors in pathogenicity of the soft rotting bacteria in the field and storage. Many studies have been performed to investigate the relationship between temperature and pathogenicity in soft rotting bacteria. *P. carotovorum* suhsp. *carotovorum* and *P. carotovorum* subsp. *atrosepticum* are typically pathogenic on host crops under 15ºC (Graham and Dowson 1960; Lund and Nicholls 1970; Erinle 1975). Pérombelon *et al.* (1979) showed that the pathogenicity of these two strains declined at higher temperatures, by contrast with other pectolytic species such as *Bacillus* spp.

4.1.5 Enzymatic activity

Different enzymes are involved in cell wall digestion by the soft rot bacteria. Biochemical studies showed that the pectinases are the main reason for maceration of plant tissues (Basham and Bateman 1975 a, b). *P. carotovorum* produces different depolymerizing extracellular enzymes such as phospholipases, pectinases, xylanases, cellulases and proteases (Collmer and Keen 1986; Kotoujansky 1987).

Alberghina *et al.* (1973) stated that enzymatic activity of *P. carotovorum* is directly correlated with high humidity and water potential of the host plant tissues. A similar conclusion was reached by Tanaka and Kikumoto (1976) in the study of the relationship between free water and soft rot in Chinese cabbage caused by *P. carotovorum*. In high moisture, the host cells' turgidity increases, which causes to the lenticels and phelloderm cells (Pérombelon and Lowe 1975) where the bacteria are located. Therefore solutes leak

from between the lenticels to the cortex and this leads to favourable conditions for bacterial penetration into the cortical tissues (Pérombelon 1980).

Endo-pectate lyase, endo-polygalacturonase and endo-pectin lyase are three known pectolytic enzymes produced by wide range of micro-organisms such as *Aspergillus japanicus*, *Pseudomonas cepacia* and *Bacillus polymyxa* (Barras 1994). Chesson (1980) stated that endo-pectate lyase is associated with bacterial species rather than with other micro-organisms. Endo-pectate lyases macerate the plant cell walls' insoluble pectins, resulting in cell separation in the host tissues. Ishii (1976) stated that the optimum pH for activity of the pectolytic enzymes was 5.0 in almost all plant tissues.

4.1.6 Soft rot disease control

There is no particular chemical treatment to control soft rot disease in the field or storage. Soft rot disease caused by fungi can be controlled partially using some fungicides such as dichlozoline, benomyl (MaCoy and Machtmes 1974) and basamid, bromafume and benlate (Merriman and Sutherland 1978) used to control white rot disease caused by *S. cepivora*.

Environmental conditions such as high humidity, poorly drained soil and shade are factors that affect bacterial soft rot disease (Babadoost 1990). There are many recommendations to control the presence of free water to control soft rot disease incidence in the field and storage. As the soft rot bacteria infect plants through wounds, it is necessary to keep plants undamaged in the field pre- and post-harvest. Insects such as onion maggot cause wounds and transfer bacteria from infected sites to the healthy plant (Delahaut and Stevenson 2004); therefore pre- and post- harvest insecticide application can help to reduce spread of soft rot disease. In the onion industry, it is important to harvest the crops after the neck

areas have dried. The dried neck prevents bacterial movement from infected leaves and soft rot disease occurrence during storage. Washing vegetables and fruits using water containing chlorine can reduce soft rot disease incidence during shipment and storage (Babadoost 1990).

Biological control is another method to control plants' bacterial disease (Alivizatos and Pantazis 1992; Ozaktan et al. 1999). Several antagonistic bacteria and fungi have been tested as biological controls for soft rot diseases caused by *P. chrysanthemi* on greenhouse grown crops in Turkey but only partial disease control was achieved (Aysan *et al.* 2003).

4.1.7 Aim

The aim of this chapter was to identify the soft-rotting bacterium and evaluate its pathogenicity on *A. triquetrum* provenances from throughout Australia. Histological study of the infected *A. triquetrum* bulb was performed to determine the plant tissues' susceptibility to the pathogen. Centrifugal phylogenic testing was performed on cultivated *Allium* species and closely related Australian native monocots.

4.2 MATERIALS AND METHODS

4.2.1 Origin, isolation and storage

The rotted *A. triquetrum* bulbs were observed initially during harvest at the RMIT University glasshouse. The soft-rotting bacterium was isolated from infected bulbs collated from Horsnell Gully, South Australia in 2010 after 2 months' storage at 4ºC. Small amounts of the rotted bulb were suspended in 0.9% sterile saline and streak dilution of the solute was performed on Nutrient agar (NA) (Oxoid) plates and modified trypticase soy agar (TSA) selective medium (Grant and Holt 1976). The plates were incubated at 30ºC for 24 h and single colonies were Gram-stained (Gram 1884).

Grant and Holt (1976)'s selective TSA medium for *Pseudomonas* species contained 9 µg/mL basic fuchsin, 0.09% cycloheximide, 0.014% tetrazolium chloride (TTC), 10 µg/mL of nitrofurantoin and 23 µm of nalidixic acid. However; the modified TSA used in this study contained 19 µg/mL of basic fuchsin and 0.014% TTC to remove the Gram-positive bacteria and fungi. Basic fuchsin is an inhibitor for Gram-positive bacteria and TTC is an antifungal agent (Grant and Holt 1976). Pure cultures of the unknown pathogen were stored at -80ºC in 65% glycerol containing 0.1 M $MgSO_4$, 0.025 M Tris (pH 8) and 22.5 mL MilliQ water (Bacterial Glycerol Stocks, Van Dyke Lab, University of North Carolina

(http://www.protocol-online.org/prot/Microbiology/Bacteria/Bacteria_Culture/index.html).

4.2.2 DNA Extraction

The bacterial DNA was extracted by the phenol/chloroform method based on (Jones and Bartlet (1990) and modified DNA extraction method by using DNA binding columns from a QIAGEN DNeasy Plant Mini Kit. For DNA extraction a single loopful of pure culture from – 80 ºC was streak cultured on an NA plate and incubated at 30ºC for 24-48 h.

4.3.2.1 Phenol/chloroform DNA extraction

Two loopfuls of bacterium were suspended aseptically in 50 mL BD Falcon Tubes containing 9.4 mL TE buffer and 0.1mL of 0.5 M EDTA (pH: 8), 50 µL of proteinase K (supplier) and 0.5 mL of 10% SDS were added subsequently and mixed by vortexing. TE buffer, EDTA and SDS are detergents that digest the bacterial cell wall and proteinase K degrades the proteins. The suspension was incubated for 24 h at 37ºC to allow time for bacterial cell wall digestion and proteinase K activation. CTAB (in full)-nucleic acid precipitation was performed by adding 1.8 mL of 5 M NaCl and 1.5 mL 10% CTAB (in full)/ 0.7 M NaCl to the solution and mixing followed by 1 h incubation at 65ºC to remove

the cell debris, denatured protein and the polysaccharides/CTAB complex. Subsequently 5 mL of chloroform/isoamyl alcohol (24:1) was added to the solution and centrifuged for 15 min at 4500 state g instead at 4ºC. After centrifugation a white interface was visible between two solutions. The supernatant containing the DNA was transferred to a new 50 mL Falcon Tube and 5 mL of phenol/chloroform/isoamyl alcohol (25:25:1) was added into the solution. The tube was centrifuged once again at 4500 state g instead for 15 min at 4ºC to remove the remaining CTAB precipitate. The supernatant was transferred to a new 50 mL Falcon Tube and 0.6 volume of 100% isopropanol was added. The tube was vortexed gently until a stringy white DNA strand was visible.

The DNA was fished out using sealed glass Pasteur pipette and transferred to a microfuge tube containing 70% to remove the remaining CTAB and phenol. The tube was centrifuged at 14000 x g instead for 1 min to precipitate the DNA and ethanol was removed. The extracted DNA was dried at 70ºC for 3 min using a heating block and resuspended in 500-1000 µL of Nuclease Free Water (Promega) until it dissolved. To remove degraded DNA (visible on 1.5% agarose gel as a bright smear on gel electrophoresis) from the extract) a QIAGEN PCR Purification Kit was used as explained in Section 2.2.4.

4.2.2.2 Boiling + QIAGEN DNeasy Mini Kit

One full loop of bacterium was suspended in a microfuge tube containing 400 µL of API lysis buffer provided with the QIAGEN Plant DNeasy MiniKit. The bacterial suspension was boiled for 5 min at 100ºC using a heating block, cooled for 5 min in ice, 4 µL of RNase was added and incubated at 65ºC for 10 min for RNase inactivation. For the next step, the extraction procedure was performed according to the QIAGEN DNeasy MiniKit protocol as explained in Section 2.2.1.

4.2.3 DNA concentration estimation

The DNA concentration was estimated by loading 10 µL of DNA solution containing 2 µL of gel loading dye (composition) into a 1.5% agarose gel along with 1 µL of Fermentas GeneRuler^{TM} 100 bp. The gel was electrophoresed at 100 volts and subsequently stained with ethidium bromide and imaged by a Bio-Rad Gel Doc system. The DNA concentration was estimated based on the bands' brightness compared with the fractioned GeneRuler.

4.2.4 16S-rDNA PCR amplification

16S ribosomal DNA (rDNA) was amplified using fD1 and rP2 primers (Weisburg *et al.* 1991) as follows:

- fD1: CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG
- rP2: CCCGGGATCCAAGCTTACGGCTACCTTGTTACGACTT

The PCR reaction was performed in 25 µL containing 12.5 µL Green Taq Mastermix (Fermentas), ~16 ng of genomic DNA, 1µL of each 0.62 µM primer and nuclease-free water. A thermocycler was programmed based on the Weisburg *et al.* (1991) protocol as: initial denaturation at 94ºC for 10 min; followed by 35 cycles of: 95ºC for 2 min, 51ºC for 30 s, and 72ºC for 4 min; with a final elongation at 72ºC for 7 min. The 16S-rDNA PCR product was fractioned on 1.5% agarose gel and visualized as in Section 4.2.3. The amplicon size was estimated by simultaneous electrophoresis of 1 µL of Fermentas GeneRuler^{TM} 100 bp.

4.2.5 16S-rDNA PCR purification and sequencing

The 16S-rDNA PCR products were purified by a QIAGEN PCR Purification Kit as explained in Section 2.2.4. The purified PCR products were used for sequencing and the sequencing reaction was performed in a 20 µL volume based on the BigDye Terminator v3.1 protocol. The sequencing reaction contained 2 µL of purified double-stranded DNA as a template, 2 µL of 10X buffer, 2 µL of rP2 (reverse) primer and 1 µL of BigDye. To make the final volume up to 20 µL, NFW was added. The thermocycler was programmed for initial denaturation at 96 ºC for 1 min and 25 cycles of: 10 s denaturation at 96ºC, 5 s annealing at 51ºC, and 4 min elongation at 60ºC. The ethanol precipitation was performed as explained in Section 2.2.4 and the tube was dried at room temperature overnight and sent to the Micromon Sequencing Facility at Monash University for analysis.

4.2.6 Physiological tests

The bacterial physiological characterisation was performed for 10 discrete colonies on the 10^{-6} NA plate from serial dilution of the culture at –80ºC, using the API 20NE identification system for non-fastidious, non-enteric Gram-negative rods. Carbohydrate assimilation and acid production tests were also performed using Ayer's medium (Fahy and Persley 1983). Erythromycin antibiotic susceptibility testing was performed using Oxoid erythromycin antibiotic disks; a lawn culture of the bacterium with turbidity equivalent to McFarland 0.5 was made on Müller-Hinton agar and a 15 µg/ml antibiotic disk was applied.

The API 20NE strip is a combination of 8 conventional and 12 assimilation tests:

- Conventional tests:

  reduction of nitrates to nitrites, reduction of nitrates to nitrogen, indole production (tryptophan), fermentation of glucose, arginine dihydrolase, urease, hydrolysis (ß-glucosidae) (escullin), hydrolysis (protease) (gelatin) and ß-galactosidase (para-nitrogenphenyl- ß-D-galactopyranosidase).

- Assimilation tests:

  Assimilation of glucose, arabinose, manose, manitol, n-acetyl-glucosamine, maltose, potassium gluconate, capric acid, adipic acid, malate, trisodium citrate and phenylacetic acid. The bacterium was suspended in 0.9% sterile saline and its turbidity was adjusted to McFarland 0.5. The strip was inoculated according to the manufacturer's protocol. Kovac's oxidase test was performed using Oxidase reagent (Oxoid).

4.2.7 In vitro pathogenicity of P. carotovorum on test-tube-grown A. triquetrum

A loopful of pure culture of the bacterium from -80ºC was sub-cultured in LB Broth medium (Difco) and incubated at 30ºC in a Thermoline Scientific Shaker Incubator for 24 h at 140 rpm. The bacterial culture was centrifuged at 4500 x g for 10 min at 4ºC in 50 µL Falcon tubes. The supernatant was removed and the bacterial cells were resuspended in sterile MilliQ water. The concentration was adjusted to 1 x 10^8 bacterial cells/mL by serial dilution and used for pathogenicity testing.

A. triquetrum was micropropagated as explained in Section 3.7.4.1.1 and test-tube-grown plants in water agar were used for *in vitro* pathogenicity testing. Test-tube-grown plants were inoculated by injection of 1 ml (10^8 cells) of bacterial suspension into the water agar.

There were three replicate tubes for each provenance and the control had no bacteria. The test-tubes were incubated at 4ºC, 15ºC or 25ºC in a completely randomized design. The soft rot disease symptoms were scored with a combined bacterial growth/disease ranking scale of six stages as follows:

1. No infection
2. Water agar turbidity
3. Bacterial clumps in water agar
4. Wilting of the upper leaves
5. Green to yellow slimy masses on the agar surface
6. Dead plants/dissolved collar region/bulb

4.2.8 Histological study of infected A. triquetrum cells

Infected test-tube-grown plants were fixed in 5% formaldehyde solution for molecular biology (Sigma) overnight at room temperature and subsequently transferred into 70% ethanol and stored at 4 ºC. The collar region of the infected plants was sliced into 4 mm diameter pieces and placed into tissue cassettes. The fixed plant tissues were processed using Leica ASP 200S tissue processor programmed for soft tissues based on the manufacturer's protocol (Table 4.4). Tissue was embedded using a Shandon Histocentrie 3, (Thermo Scientific). The processed tissues were embedded in blocks of molten paraffin wax with the surface to be cut facing down in the block (Ruzin 1999) and allowed to solidify (Fig. 4.4).

The embedded tissues were sectioned 3-4 µm thick and fixed on the glass slides at 50ºC for an hour. The specimens were soaked in m-xylene (Sigma-99%) for 5 min to remove excessive paraffin wax from the sectioned tissues and subsequently rinsed in 90%, 70% and 50% for 5 min each (Ruzin 1999). These sections were Gram-stained and fixed in

DPX Mountant for examination. The sections were photographed on a Leica DM2500 compound microscope.

Table 4.4 The soft tissue processing program used in the Leica ASP 200S tissue processor.

Reagents	Station	Duration (min)	Temperature
Neutral buffered formalin	1	20	N/A
Ethanol 70%	2	10	N/A
Ethanol 90%	3	10	N/A
Ethanol absolute	4	10	N/A
Ethanol absolute	5	20	N/A
Xylene	6	10	37
Xylene	7	10	37
Xylene	8	15	37
Paraffin wax	Wax I	10	62
Paraffin wax	Wax II	10	62
Paraffin wax	Wax III	20	62

Figure 4.4 Embedding samples in paraffin wax.

4.2.9 In vivo pathogenicity testing of P. carotovorum on potted A. triquetrum provenances from across Australia in the glasshouse.

Potted *A. triquetrum* plants were prepared in a RMIT glasshouse under 60-80% humidity and 15-25ºC temperature as explained in Section 3.7.4.3. The inoculum was prepared as explained in Section 4.2.7. Inoculation was performed twice 30 days apart with 1 ml containing 10^8 cells each time to ensure retention of enough bacterial cells in the pots and the bacterial suspension culture was released on the collar region of *A. triquetrum* using a micropipette. Plants were irrigated from the base every 2 days and fertilised every 2 weeks using modified Hoagland nutrient solution (Taiz and Zeiger 2002). There were three replicate pots for each provenance arranged in a randomized complete block design. Three control pots without bacterial inoculation were used for each provenance. The soft rot disease symptoms on infected plants were scored with a disease ranking scale of six stages as follows:

1. No infection
2. Host tissue softness
3. Wilting of the upper leaves
4. Mushy and watery leaves
5. Green to yellow slimy masses of leaves on the soil
6. Dead plants/dissolved collar region/bulb

4.2.10 In vivo pathogenicity testing of P. carotovorum on cultivated Allium species in the glasshouse.

Seedlings of seven cultivated *Allium* species or cultivars were purchased from a plant nursery and 10-15 seedlings for each species were re-potted in sterile potting mix and grown on in a RMIT glasshouse for 7 days before inoculation. The plants were inoculated, irrigated and fertilized as explained in Section 4.2.9. This experiment had three replicate

pots for each *Allium* species in a completely randomized design. Control pots were un-inoculated as before. Pathogenicity was scored as in Section 4.2.9. The *Allium* species used in this study were: Chives (*Allium schoenoprasum* L.), Garlic Chives (*Allium tuberosum* L.), Spring Onion (*Allium cepa* L.), Leek (*Allium porrum* L.), Red Onion (*Allium cepa* L.), Brown Onion (*Allium cepa* L.) and White Onion (*Allium cepa* L.).

4.2.11 In vivo pathogenicity testing of P. carotovorum on related Australian native monocots.

Seedlings of seven Australian native monocot species were purchased form Kuranga Native Nursery, Melbourne, Victoria (Table 4.5) in plastic tubes 50mm square x 120 mm high, containing non-sterile potting mix and sand and left in the tubes for the experiment to avoid re-potting damage. There were three replicate pots for each species and plants were inoculated, irrigated and fertilized as in Section 4.2.9 along with *A. triquetrum* as a positive control. Control pots were un-inoculated. Pathogenicity was scored as in Section 4.2.9.

Table 4.5 Australian native plants used for host specificity trials *in vivo*.

Common name	Botanical name	Family
Black Anther Flax-Lily	*Dianella revolute* R.Br.	*Liliaceae*
Bulbine Lily	*Bulbine bulbosa* (R.Br.) Haw.	*Liliaceae*
Chocolate Lily	*Arthropodium strictum* R.Br.	*Liliaceae*
Yellow Rush Lily	*Tricoryne elatior* R.Br.	*Liliaceae*
Clustered Wallaby-Grass	*Austrodanthonia racemosa* R.Br.	*Poaceae*
Large Tussock-Grass	*Poa labillardieri* Spreng.	*Poaceae*
Spiny-Headed Mat-Rush	*Lomandra longifolia* Labill.	*Xamthorrhoeaceae*

4.2.12 Pectobacterium carotovorum identification from infected plant tissues using specific primer

As a Blast search of the 16S-rDNA sequence of the isolated bacterium showed that the closest matches were all *P. carotovorum* ssp. *carotovorum*, the DNA was also amplification using specific primers for *P. carotovorum* subsp. *carotovorum* designed by Kang *et al.* (2003) from a URP-PCR fingerprinting-derived polymorphic band to identify the bacterium from infected *A. triquetrum* plants in Section 4.2.9.

The specific primers used in this study were:

- Reverse primer: EXPCCR (5'-GCCGTAATTGCCTACCTGCTTAAG-3')
- Forward primer: EXPCCF (5'-GAACTTCGCACCGCCGACCTTCTA-3')

The PCR reaction was performed in 25 µL reaction volume containing 12.5 µL of Green Taq Mastermix, 1 µL (0.6 µM) of each primer, ~16 ng DNA template, and nuclease-free water. A G-STORM thermocycler was programmed according to Kang *et al.* (2003)'s protocol for initial denaturation at 94ºC for 4 min followed by 30 cycles of: 94ºC for 1 min, 60ºC for 1 min, and 72ºC for 2 min; with a final elongation at 72ºC for 7 min. The negative control had 2µL of NFW instead of DNA extract. The specificity of the primer was checked by simultaneous PCR amplification of 16 bacterial species (Table 4.6) and the genomic DNA of the bacteria was extracted as explained in Section 4.2.2.2.

Table 4.6 Bacterial isolates used to check specificity of *Pectobacterium carotovorum* specific primers.

Species	**Family**	**Collection number**
Aeromonas hydrophila Chester 1901	*Vibrionaceae*	RMIT Uni collection 8/3
Azotobacter sp. Beijerinck 1901	*Azotobacteraceae*	RMIT Uni collection 7/1
Enterobacter aerogenes Hormaeche & Edwards 1960	*Enterobacteriaceae*	ATCC 13048
Enterobacter cloacae Jordan 1890	*Enterobacteriaceae*	ATCC 23355
Enterobacter agglomerans Ewing & Fife 1972	*Enterobacteriaceae*	RMIT Uni collection 100/3
Hafnia alvei Moller 1954	*Enterobacteriaceae*	RMIT Uni collection 142/1-1
Providencia alcalifaciens Ewing 1962	*Enterobacteriaceae*	RMIT Uni collection 282/2
Pseudomonas aeruginosa (Schroeter 1972) Migula 1900	*Pseudomonadaceae*	ATCC 27853
Pseudomonas fluorescens Migula 1895	*Pseudomonadaceae*	RMIT Uni collection 283/2
Pseudomonas oleovorans Lee & Chadler 1941	*Pseudomonadaceae*	ATCC 29347
pseudomonas syringae pv. phaseolicola (Burkholder) Young *et al.* 1978	*Pseudomonadaceae*	RMIT Uni collection 283/5
Providencia stuartii Buttiavx *et al.* 1954	*Enterobacteriaceae*	RMIT Uni collection 282/1
Rhizobium trifolii (Dangeard 1926)	*Rhizobiaceae*	RMIT Uni collection 320/2
Shigella sonnei (Levine 1920) Weldin 1927	*Enterobacteriaceae*	ATCC 25931
Stenotrophomonas maltophilia Hugh 1981	*Xanthomonadaceae*	ATCC 17672
Ochrobactrum anthropi Holmes *et al.* 1988	*Brucellaceae*	CCM 999

'RMIT Uni collection' refers to isolates received as authentic cultures from experts on those organisms and used routinely in teaching activities in microbiology.

4.3 RESULTS

4.3.1 16S-rDNA amplification and sequencing

On amplification of the 16S-rDNA of the bacterium, a band of 1500 bp was observed on 1.5% agarose gel. This produced a 16S region sequence of 1100 bp and 100 bp of the 5´ beginning of the sequences was deleted due to low quality sequence (Fig. 4.5). A Blast search in GenBank through NCBI showed that the best match was *Pectobacterium carotovorum* subsp. *carotovorum* (Accession no. AF373185.1) (Maximum identity: 98%, E value: 0.0).

```
   1 TGGCCCGGTGACCTCGGGTCTGTGCGCCCTCCCGAGGATTAAGCATACCTACTTCTTTTG
  61 CAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTA
 121 GCATTCTGATCTACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAA
 181 TCCGGACTACGACGTACTTTATGAGGTCCGCTTGCTCTCGCGAGGTCGCTTCTCTTTGTA
 241 TACGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCATC
 301 CCCACCTTCCTCCGGTTTATCACCGGCAGTCTCCTTTGAGTTCCCGACCGAATCGCTGGC
 361 AACAAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCT
 421 GACGACAGCCATGCAGCACCTGTCTCAGAGTTCCCGAAGGCACTCAGCTATCTCTAGCTA
 481 ATTCTCTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGC
 541 TCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCC
 601 CCAGGCGGTCGATTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAA
 661 ATCGACATCGTTTACAGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTT
 721 TCGCACCTGAGCGTCAGTCTTTGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGA
 781 TCTCTACGCATTTCACCGCTACACCTGGAATTCTACCCCCCTCTACAAGACTCTAGCCTG
 841 TCAGTTTTGAATGCAGTTCCCAGGTTAAGCCCGGGGATTTCACATCCAACTTAACAGACC
 901 GCCTGCGTGCGCTTTACGCCCAGTCATTCCGATTAACGCTTGCACCCTCCGTATTACCGC
 961 GGCTGCTGGCACGGAGTTAGCCCGGTGCTTCTTCTGCGAGTAACGTCATCGATAGTTATT
1021 AACCTTACCGCCTTCCTCCTCGCTGAAAGTGCTTACAACCCGAAAGGCCTTCTTCCACAC
1081 ACACGCCGGCATGGCTGCATC
```

Figure 4.5 Sequence of 16S-rDNA product of the *Pectobacterium carotovorum* subsp. *carotovorum* isolate from *A. triquetrum* bulbs collected from Horsnell Gully, South Australia in 2010.

4.3.2 Physiological characterisation of the P. carotovorum subsp. carotovorum isolate

Gram staining and hanging drops showed that the bacterium was Gram-negative and motile. Serial dilution of the culture stored at –80ºC showed 10 discrete colonies on the 10^{-6} NA plate. All 10 colonies were streaked on NA plates for molecular and physiological

tests. The PCR amplification of all 10 colonies demonstrated a 600 bp band using *P. carotovorum* subsp. *carotovorum*-specific primers (Kang *et al.* 2003). Physiological tests were performed for all colonies using API 20 NE and Ayer's medium showed no difference between the colonies (Table 4.7). The fermentation tests in Ayer's medium showed that the bacteria could ferment lactose and could neither assimilate nor ferment maltose. The bacterium was resistant to erythromycin.

Table 4.7 Physiological and antibiotic susceptibility tests performed on *Pectobacterium carotovorum* colonies collected from *Allium triquetrum* infected bulb showed the same results.

No.	Tests	Active ingredients	Reactions/enzymes	Result
1	NO_3		Reduction of nitrates to nitrites	+
2		Potassium nitrate	Reduction of nitrates to nitrogen	+
3	TRP	L-tryptophan	Indole production	-
4	GLU	D-glucose	Fermentation (glucose)	+
5	LAC	Lactose	Fermentation (lactose)	+
6	MAL	Maltose	Fermentation (maltose)	-
7	ADH	L-arginine	Arginine dihydrolase	-
8	URE	Urea	Urease	-
9	ESC	Esculin ferric citrate	Hydrolysis (ß-glucosidase) (esclin)	+
10	GEL	Gelatine (bovine origin)	Hydrolysis (protease) (gelatine)	+
11	PNPG	4-nitrophenyl-ßD-galactopyranoside	ß-galactopyranoside (para-nitrophenyl-ß-D-galactopyranosidase)	+
12	GLU	D-glucose	Assimilation (glucose)	+
13	ARA	L-arabinose	Assimilation (arabinose)	+
14	MNE	D-mannose	Assimilation (mannose)	+
15	MAN	D-mannitol	Assimilation (mannitol)	+
16	NAG	N-acetyl-glucosamine	Assimilation (N-acetyl-glucosamine)	+
17	MAL	D-Maltose	Assimilation (maltose)	-
18	GNT	Potassium gluconate	Assimilation (potassium gluconate)	+
19	CAP	Capric acid	Assimilation (capric acid)	-
20	ADI	Adipic acid	Assimilation (adipic acid)	-
21	MLT	Malic acid	Assimilation (malate)	+
22	CIT	Trisodium citrate	Assimilation (trisodium citrate)	+
23	PAC	Phenylacetic acid	Assimilation (phenylacetic acid)	-
24	OX	Oxidase reagent	Cytochrome oxidase	-
25	ERT.	Erythromycin	Erythromycin susceptibility	R

4.3.3 Pathogenicity of the P. carotovorum subsp. carotovorum isolate on test-tube-grown A. triquetrum

The bacterium was pathogenic and highly virulent for plants from all provenances tested from across Australia and wilting of leaves as the first disease symptom was observed after only 24 h at 25ºC (Fig. 4.6). Pathogenicity of the bacterium was also pathogenic at 15°C and 4ºC after 7 days and 2 months respectively. The water agar colour changed to pale yellow and bacterial clumps were observed. Infected plants collapsed from the top and the rotted leaves became attached to the test tube. After 48 h incubation at 25ºC, green or yellow masses of leaves were collapsed on the agar surface (Fig. 4.7). The collar region of the test-tube-grown *A. triquetrum* was dissolved in all almost samples while the control plants without bacterial inoculation remained healthy. The bacterium showed almost the same pathogenicity level for all plants (Stage 6). One replicate of Kangaroo Flat (Bendigo, VIC) was scored as 5 for pathogenicity level as the top leaf was healthy but the plant had collapsed from the collar region. Only *P. carotovorum* was re-isolated from the infected plants on NA plates. Statistical analysis based on randomized complete block design indicated no significant difference between pathogenicity of *P. carotovorum* among provenances from across Australia (Table 4.8) but significant differences between control and inoculated plants.

Figure 4.6 Pathogenicity of the *P. carotovorum* subsp. *carotovorum* isolate on the test-tube-grown *Allium triquetrum* from Fairview Park, South Australia at 25 ºC.

Figure 4.7 Pathogenicity of the *P. carotovorum* subsp. *carotovorum* isolate on the test-tube grown *Allium triquetrum* from Kangaroo Flat, Bendigo, VIC at 4 ºC.

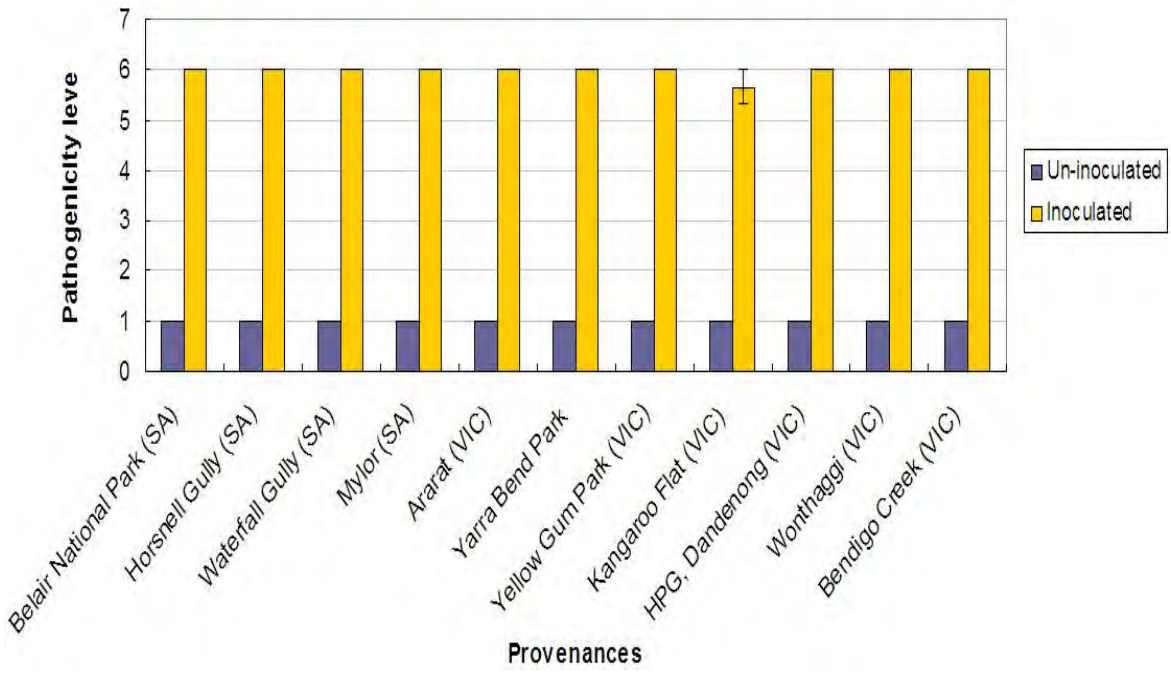


Figure 4.8 Pathogenicity of the *Pectobacterium carotovorum* subsp. *carotovorum* isolate on the test-tube-grown *Allium triquetrum* plants from provenances throughout Australia. HPG: Hardy's Picnic Ground (Bars=2 x standard error).

Table 4.8 Analysis of pathogenicity of *Pectobacterium carotovorum* subsp. *carotovorum* on inoculated *Allium triquetrum* provenances from across Australia in a randomized complete block design using SPSS software.

Tests of Between-Subjects Effects

Dependent Variable: Pathogenicity

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.361[a]	13	.028	1.000	.483
Intercept	1284.028	1	1284.028	46225.000	.000
Replicates	.056	2	.028	1.000	.384
Provenances	.306	11	.028	1.000	.477
Error	.611	22	.028		
Total	1285.000	36			
Corrected Total	.972	35			

a. R Squared = .371 (Adjusted R Squared = .000)

4.3.4 Histology of infected A. triquetrum bulbs

Bacterial cells were observed in infected tissues collected after 12 h and 24 h inoculation. The plant samples were very rotted after 24 h inoculation and were difficult to process to show sections through recognisable tissues. There was no bacterial infection in control treatments and cells were intact (Fig. 4.9). In infected *A. triquetrum* collar regions, the bacterium was observed intercellularly between the cortical parenchyma cells and some in the vascular tissues (Fig 4.10). The epidermal cells remained intact in some sections. Largely intact cortical parenchyma cells were observed in samples collected after 12 h but masses of bacterial cells were visible adjacent to the lamellae of cell walls (Fig 4.10). The cortical parenchyma cells invaded by the bacterium were collapsed in samples collected after 24 h and vascular tissues were also destroyed (Fig. 4.11).

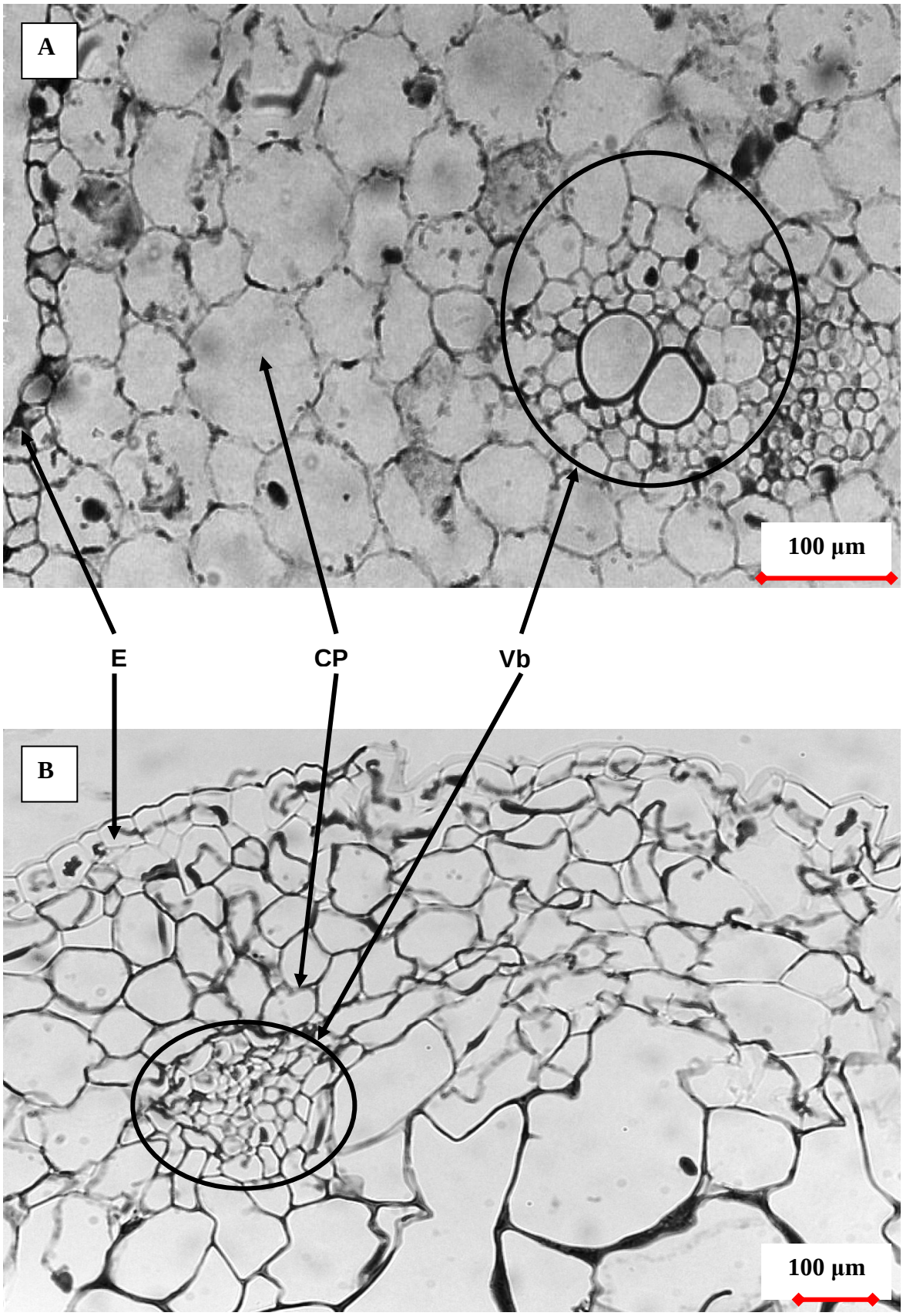


Figure 4.9 Transverse section of uninoculated *Allium triquetrum* collar region cells. E: epidermis, CP: cortical parenchyma and Vb: vascular bundle.

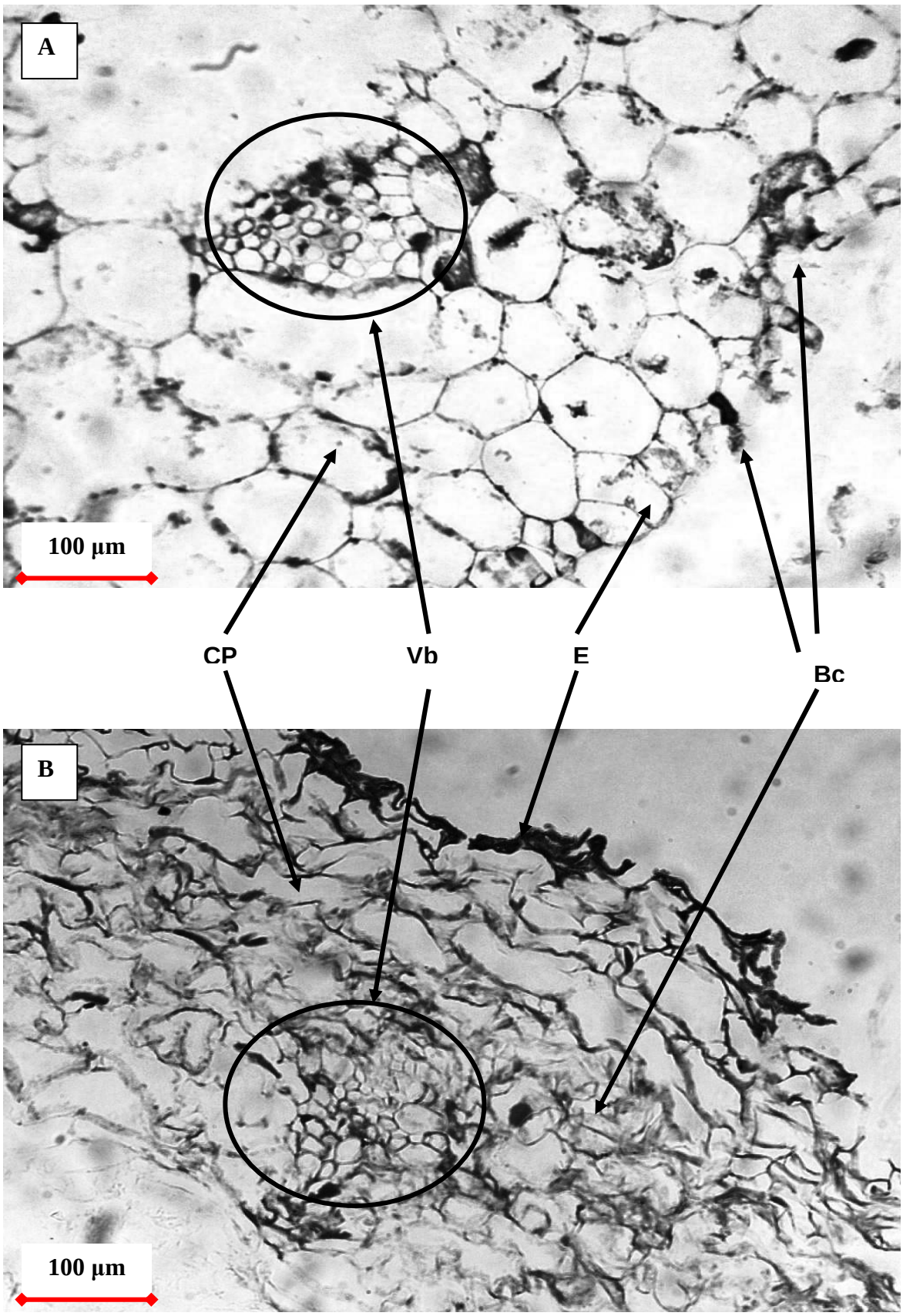


Figure 4.10 Transverse section of inoculated *Allium triquetrum* collar region cells 12 h (A) and 24 h (B). E: epidermis, CP: cortical parenchyma, Vb: vascular bundle and Bc: bacteria.

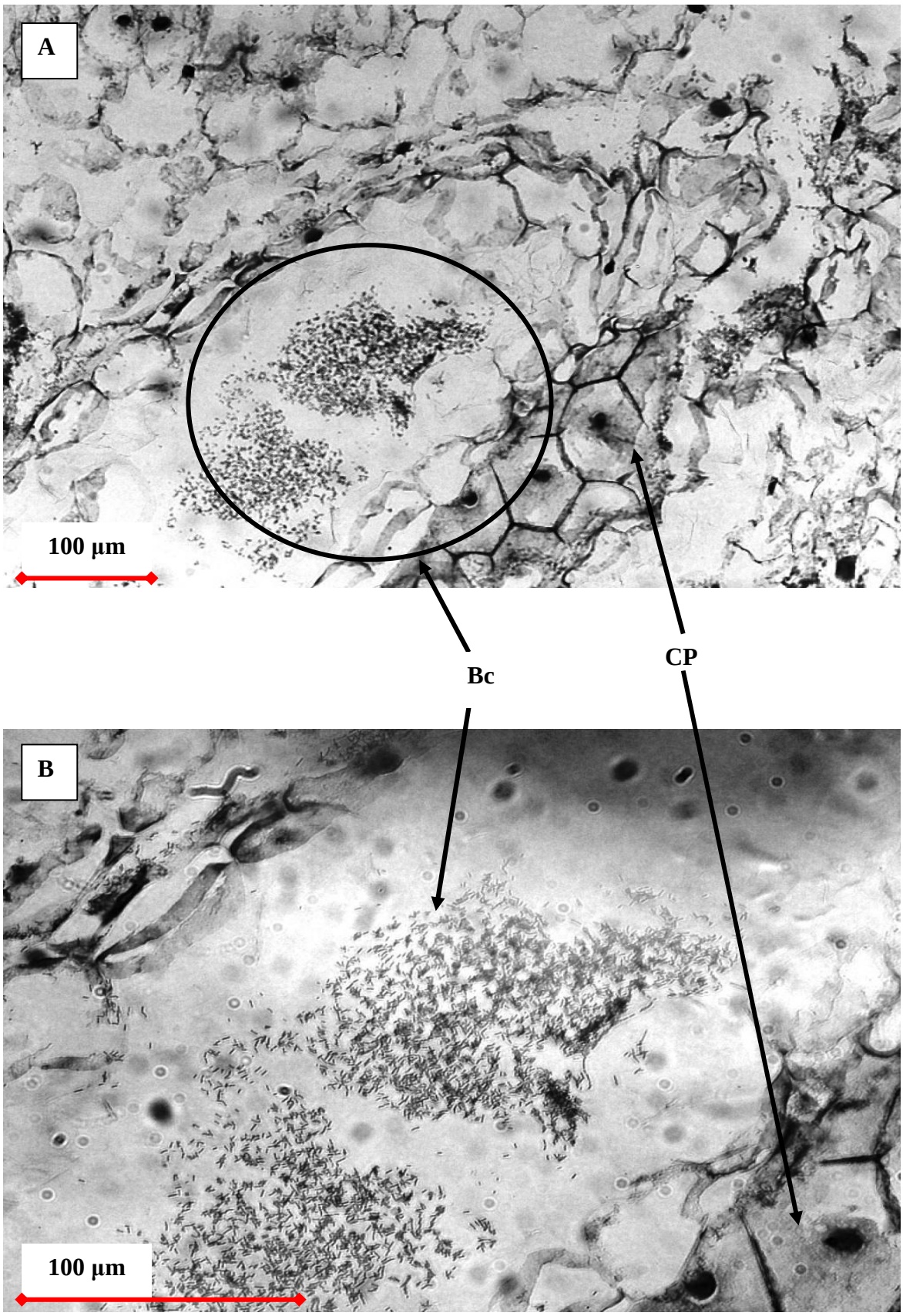


Figure 4.11 *Pectobacterium carotovorum* subsp. *carotovorum* in dissolved cortical parenchyma cells of *Allium triquetum* bulb. CP: cortical parenchyma and Bc: bacteria.

4.3.5 Pathogenicity of the P. carotovorum subsp. carotovorum isolate on potted A. triquetrum in the glasshouse

The bacterium was pathogenic and virulent on almost all provenances from across Australia in the RMIT glasshouse. The pathogenicity scored for most of the *A. triquetrum* plants was stages 4-6 (Fig. 4.12). The first disease symptoms were observed after 2 weeks as wilted collapsed older leaves. Infected plants collapsed from the collar region and rotted from the basal stem (Fig. 4.13). Death was observed after 20 days as dried collapsed leaves and rotted bulbs in the sand. Control plants without bacterial inoculation remained healthy. Various pathogenicity levels were scored on plants from Waterfall Gully (SA), Mylor (SA), Ararat (VIC), Yarra Bend Park (VIC), Wonthaggi (VIC), Bendigo Creek (VIC) and Yellow Gum Park (VIC). Mylor (SA) replicates showed the least symptoms among provenances. Plants from Belair National Park (SA), Horsnell Gully (SA), Gardiner's Creek (VIC), Kangaroo Flat (VIC) and Hardy's Picnic Ground, Dandenongs (VIC) scored 6 for all replicates. Statistical analysis based on randomized complete block design indicated significant differences among replicates but not among provenances (Table 4.9). This was because plants either became infected and collapsed or did not become infected and were healthy, as shown with the three inoculated replicates from Mylor (Fig. 4.13B).

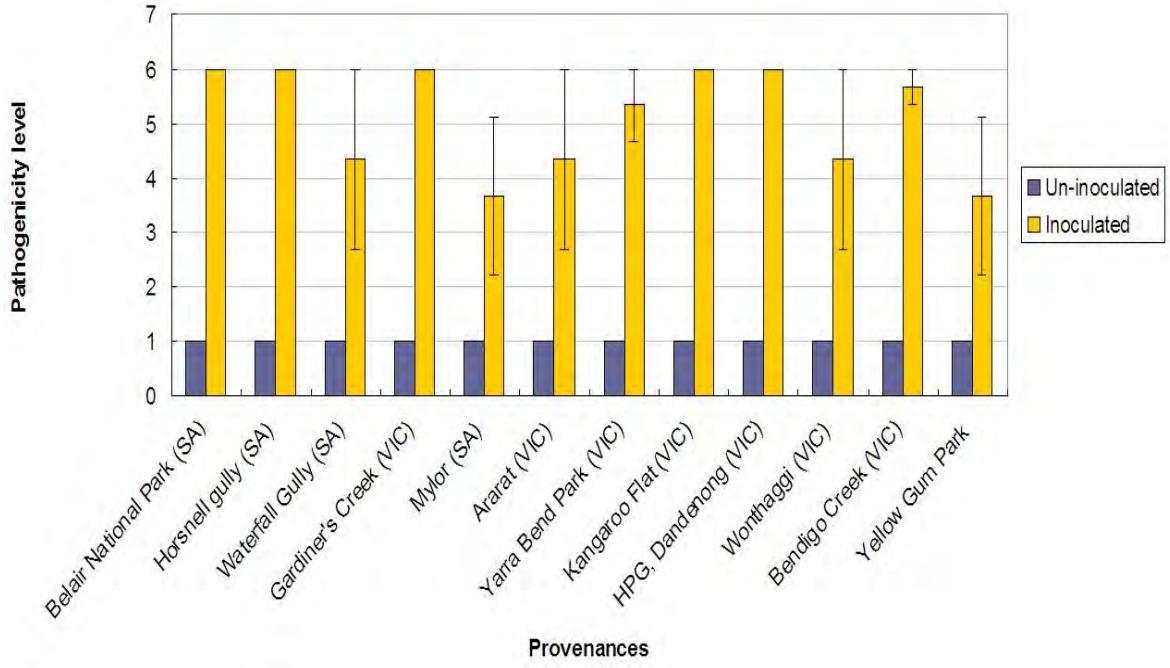


Figure 4.12 Pathogenicity of the *Pectobacterium carotovorum* subsp. *carotovorum* isolate on potted *Allium triquetrum* provenances throughout Australia in the glasshouse. HPG: Hardy's Picnic Ground (Bars=2 x standard error).

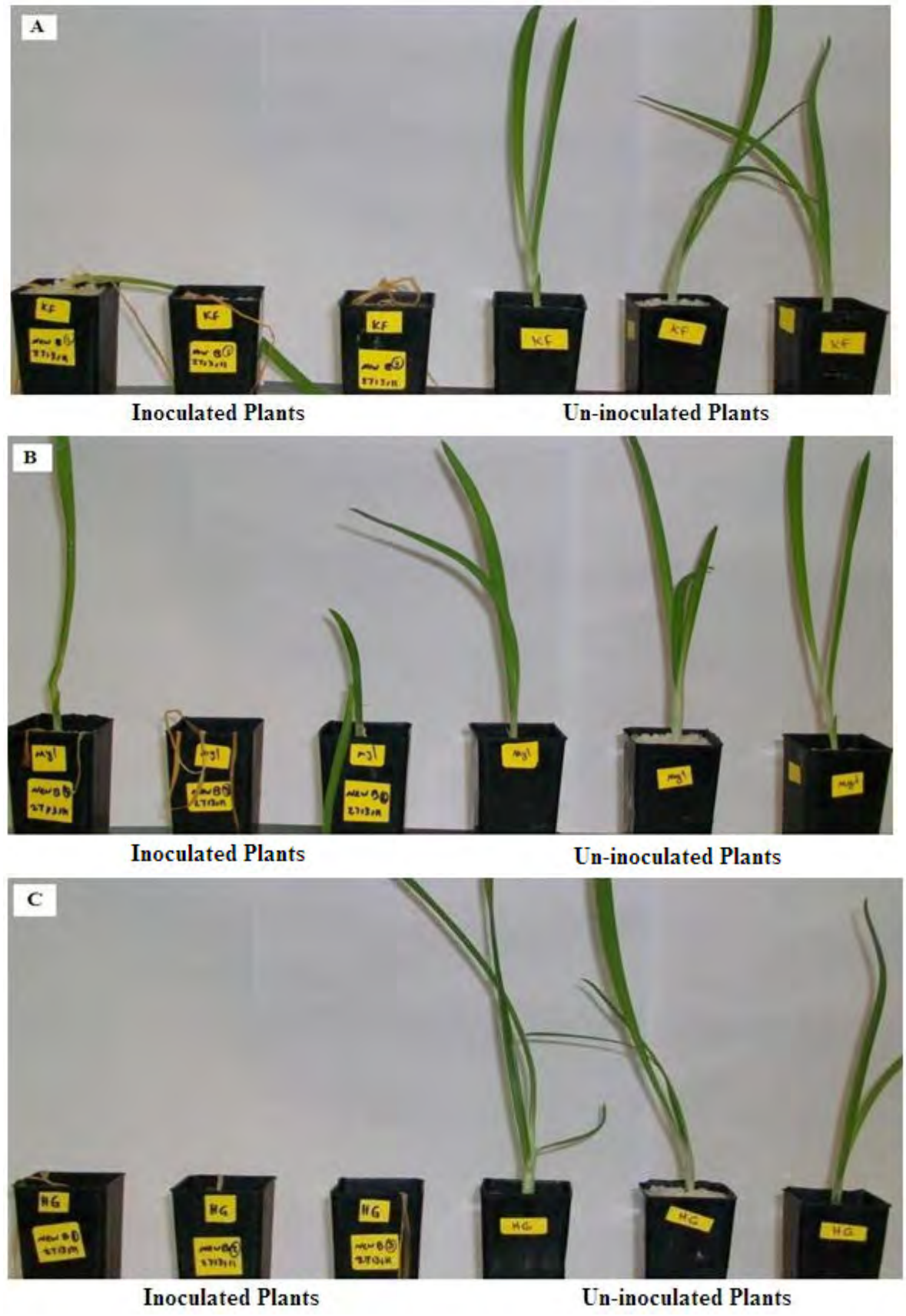


Figure 4.13 Pathogenicity of the *Pectobacterium carotovorum* subsp. *carotovorum* isolate on the potted *Allium triquetrum* from across Australia in the glasshouse. A: Kangaroo Flat (VIC), B: Mylor (SA). C: Horsnell Gully (SA).

Table 4.9 Analysis of pathogenicity of the *Pectobacterium carotovorum* subsp. *carotovorum* isolate on potted *Allium triquetrum* provenances from across Australia in the glasshouse based on a randomized complete block design using SPSS software.

Tests of Between-Subjects Effects

Dependent Variable: Pathogenicity

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	68.444[a]	13	5.265	2.817	.016
Intercept	940.444	1	940.444	503.265	.000
Replicates	37.556	2	18.778	10.049	.001
Provenances	30.889	11	2.808	1.503	.200
Error	41.111	22	1.869		
Total	1050.000	36			
Corrected Total	109.556	35			

a. R Squared = .625 (Adjusted R Squared = .403)

4.3.6 Identification of the bacterium from the infected A. triquetrum

Only the bacterium isolated from rotted bulbs of *A. triquetrum* produced a band at 600 bp as expected on 1.5 % agarose gel (Fig. 4.14) when tested with the *P. carotovorum* subsp. *carotovorum* specific primers (EXPCCF and EXPCCR). Almost all plants inoculated with the bacterium, even with different degrees of virulence levels, produced a band at 600 bp (Fig. 4.15).

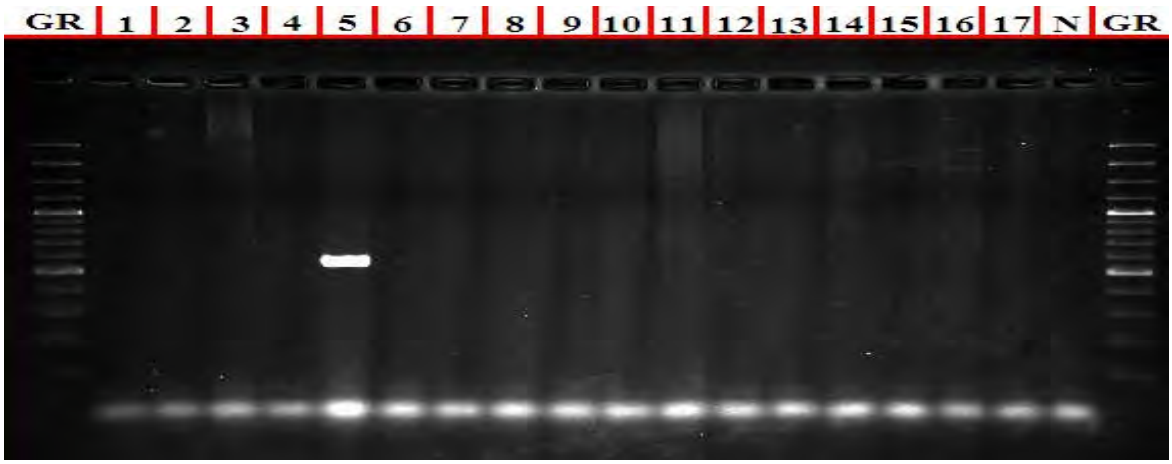


Figure 4.14 Specificity of *Pectobacterium carotovorum* subsp. *carotovorum* specific primers EXPCCF and EXPCCR among 16 bacterial species. Lane numbers (left-right): 1-*Aeromonas hydrophila,* 2-*Azotobacter* sp., 3- *Enterobacter aerogenes,* 4- *Enterobacter cloacae,* 5- *Pectobacterium carotovorum* subsp. *carotovorum* (isolate from *A. triquetrum),* 6- *Enterobacter agglomerans,* 7- *Hafnia alvei,* 8- *Providencia alcalifaciens,* 9-*Pseudomonas aeruginosa,* 10- *Pseudomonas fluorescens,* 11- *Pseudomonas olerovorans,* 12- *Pseudomonas phasicola,* 13- *Providencia incostans,* 14- *Rhizobium trifolii,* 15-*Shigella sonnei,* 16- *Stenotrophomonas maltophilia,* 17- *Ochrobacterum anthropi.* GR: GeneRuler^{TM} 100 bp and N: negative control.

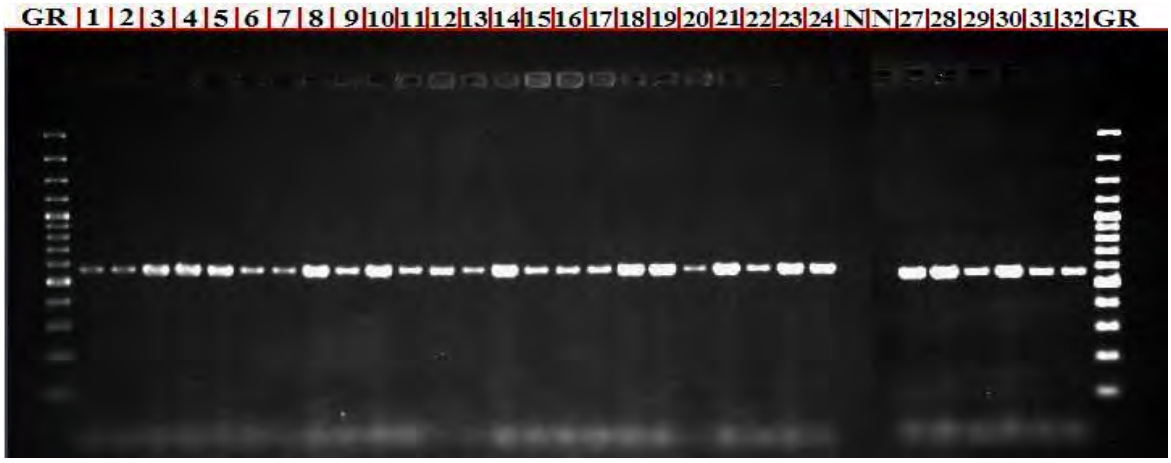
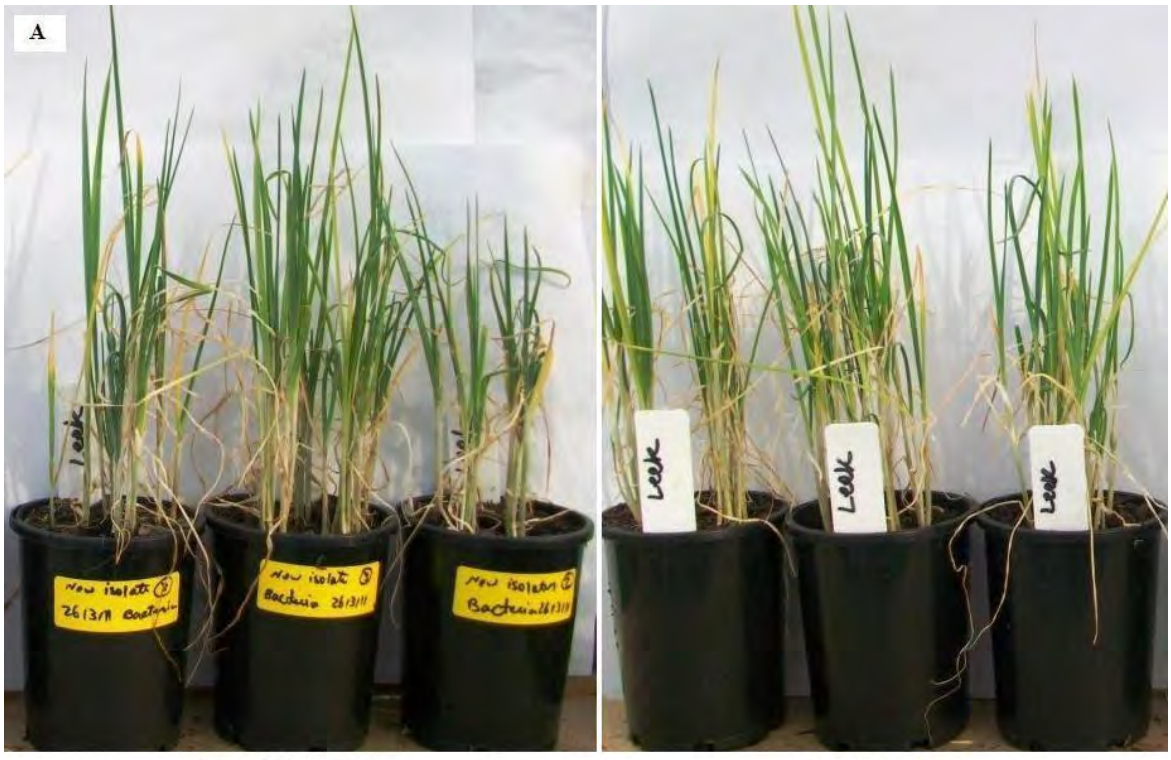


Figure 4.15 Identification of the *Pectobacterium carotovorum* subsp. *carotovorum* isolate from infected *A. triquetrum* in the glasshouse using *P. carotovorum* subsp. *carotovorum* specific primers (EXPCCF and EXPCCR). Lane umbers (left-right): 1-3 Mylor (SA), 4-6 Belair National Park (SA), 7-9 Horsnell gully (SA), 10-12 Gardiner's Creek VIC, (13-15) Waterfall Gully (SA), 16-18 Ararat (VIC), 19-21 Yarra Bend Park (VIC), 22-24 Bendigo Creek (VIC), 27-29 Hardy's Picnic Ground, Dandenongs (VIC), 30-32 Wonthaggi (VIC). GR: GeneRuler^{TM} 100 bp and N: negative control.

4.3.7 Pathogenicity of P. carotovorum subsp. carotovorum isolate on cultivated Allium species in the glasshouse.

No disease symptoms were observed on cultivated *Allium* species in the glasshouse after 3 months (Fig. 4.16), although all treated cultivated plants were wounded artificially during re-potting in the glasshouse one week previously. The pathogenicity level of the bacterium was scored as stage 1 (no infection) for all cultivated *Allium* species. All replicates of the *A. triquetrum* plants from Belair National Park (SA) used as positive controls were infected after 7 days and death was observed after 2 weeks as dissolved bulbs (Fig. 4.16, 4.17). The untreated control pots of *A. triquetrum* and cultivated species remained healthy and were also scored as stage 1. Root systems and bulbs of the cultivated *Allium* species showed no infection 3 months after inoculation and plants produced healthy roots 10-15 cm long (Fig. 4.18). Statistical analysis of the pathogenicity level of the bacterium based on randomized complete block design indicated significant difference between infected *A. triquetrum* and cultivated species but no distinction between cultivated *Allium* species.



Inoculated plants | Control plants

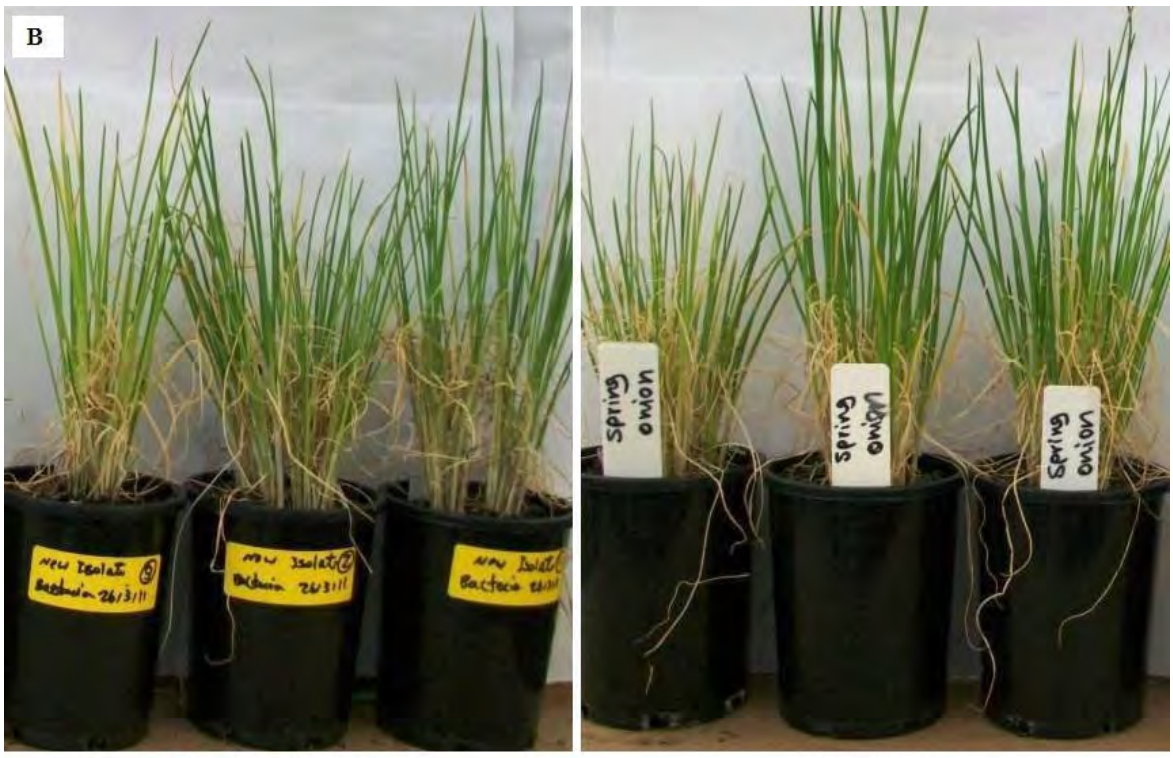


Figure 4.16 Pathogenicity of the *Pectobacterium carotovorum* subsp. *carotovorum* isolate on cultivated *Allium* species (A: Leek and B: Spring Onion) and *Allium triquetrum* (C) in the glasshouse.

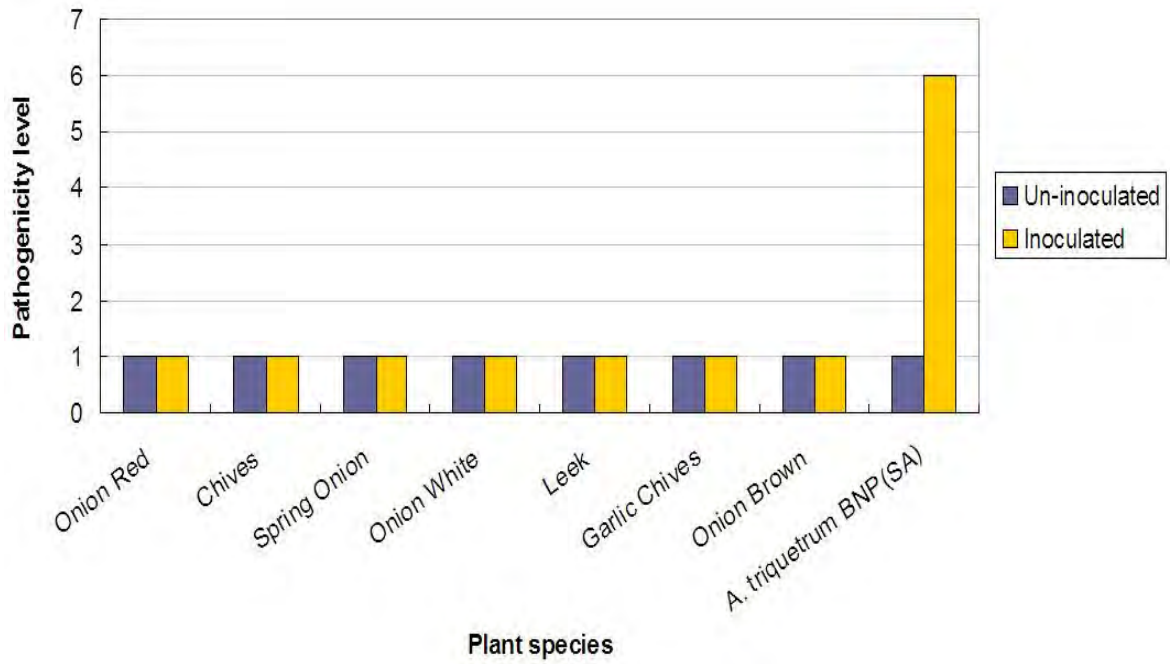


Figure 4.17 Pathogenicity of the *Pectobacterium carotovorum* subsp. *carotovorum* isolate on cultivated *Allium* species in the glasshouse. *Allium triquetrum* from Belair National Park (SA) provenance was used as the positive control (Bars=2 x standard error).

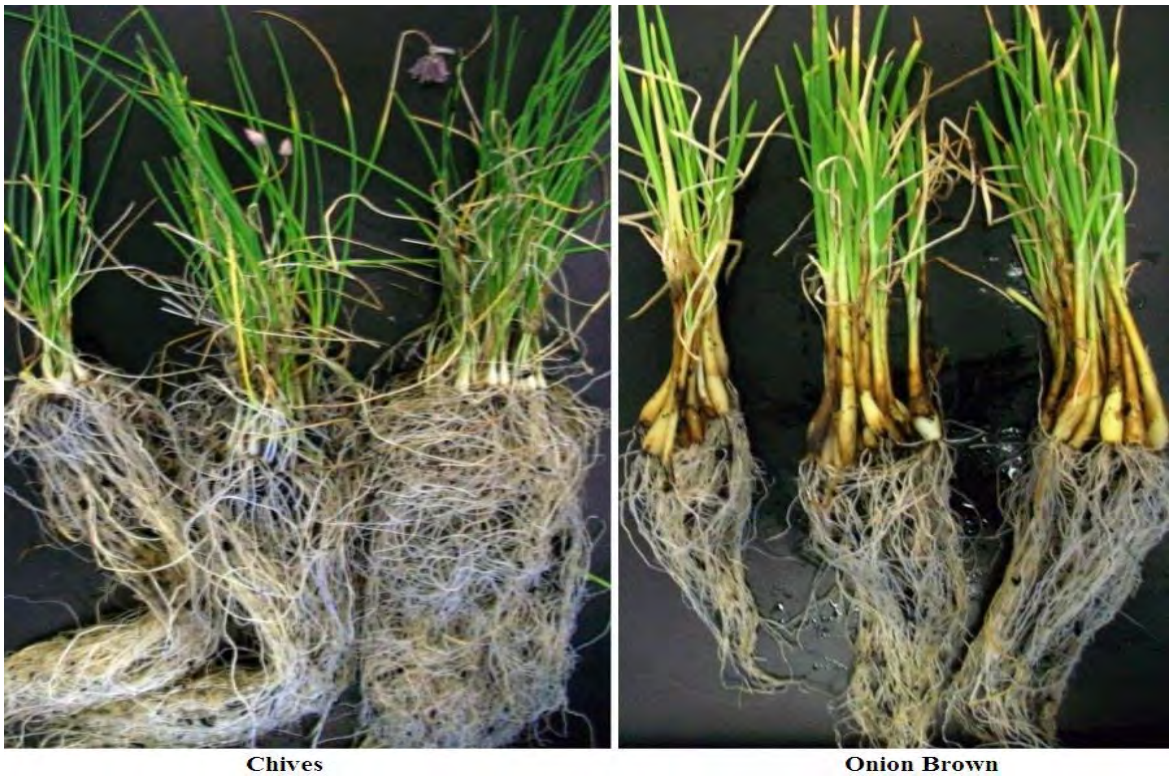


Figure 4.18 Healthy root systems of Chives and Brown Onion inoculated by the *P. carotovorum* subsp. *carotovorum* isolate 2 months after inoculation in the glasshouse.

4.3.8 Pathogenicity the P. carotovorum subsp. carotovorum isolate on Australian native monocots

There was no infection of Australian native monocots 3 months after inoculation by the *P. carotovorum* subsp. *carotovorum* isolate in the glasshouse (Fig. 4.19). However; death of *A. triquetrum* was observed 2 weeks after with *P. carotovorum*. The pathogenicity level was scored as stage one (no infection) for all inoculated native plants and stage six (complete death) for infected *A. triquetrum* plants (Fig. 4.20). The control plants remained healthy after three month and scored as stage one. Statistical analysis based on randomized complete block design indicated significant difference between *A. triquetrum* samples and native species, however; no difference was observed between native species.

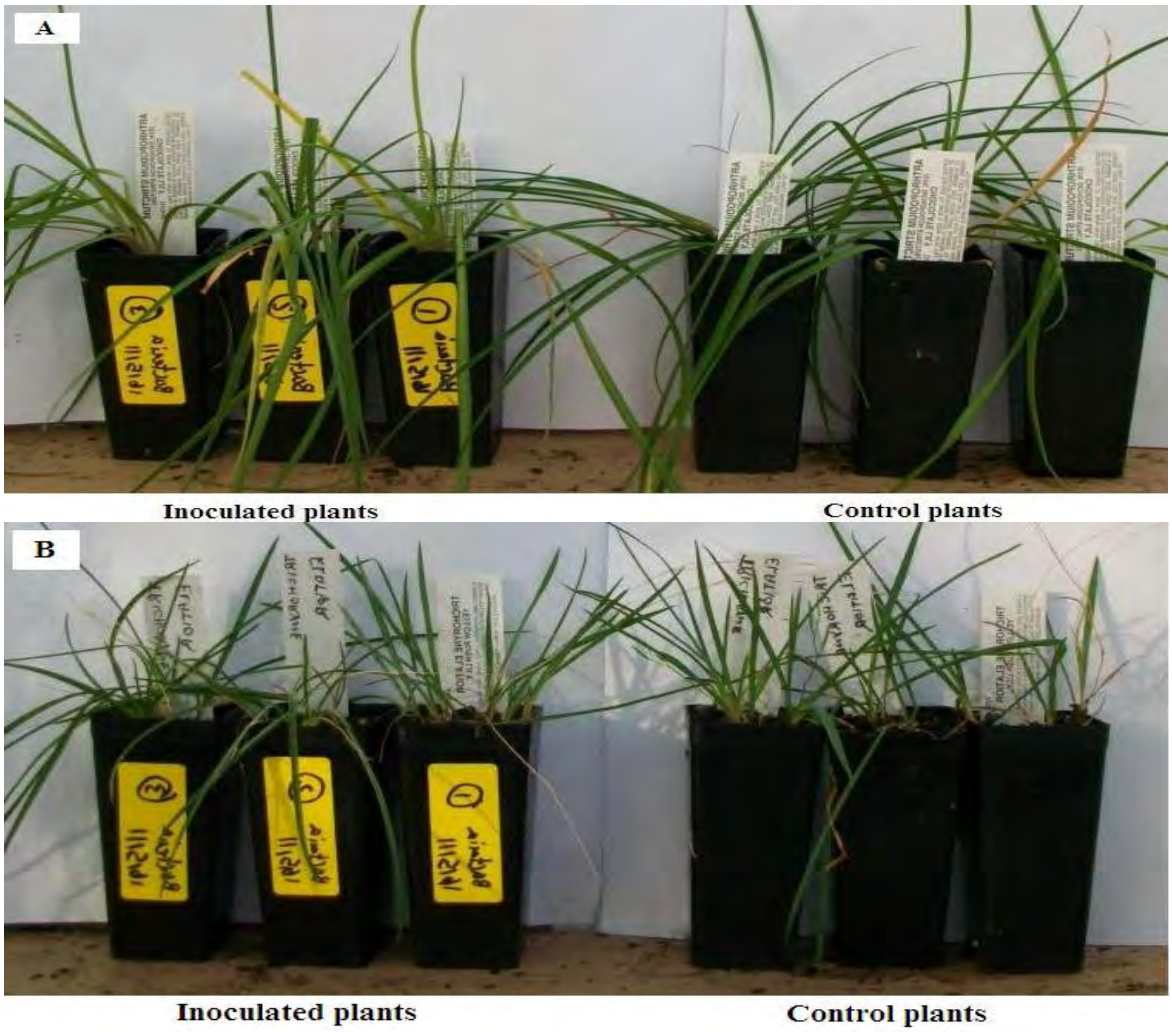


Figure 4.19 Pathogenicity of the *Pectobacterium carotovorum* subsp. *carotovorum* isolate on Australian native monocots in glasshouse (A: Chocolate Lily (*Arthropodium strictum* R. Br.), B: Yellow Rush Lily (*Tricoryne elatior*).

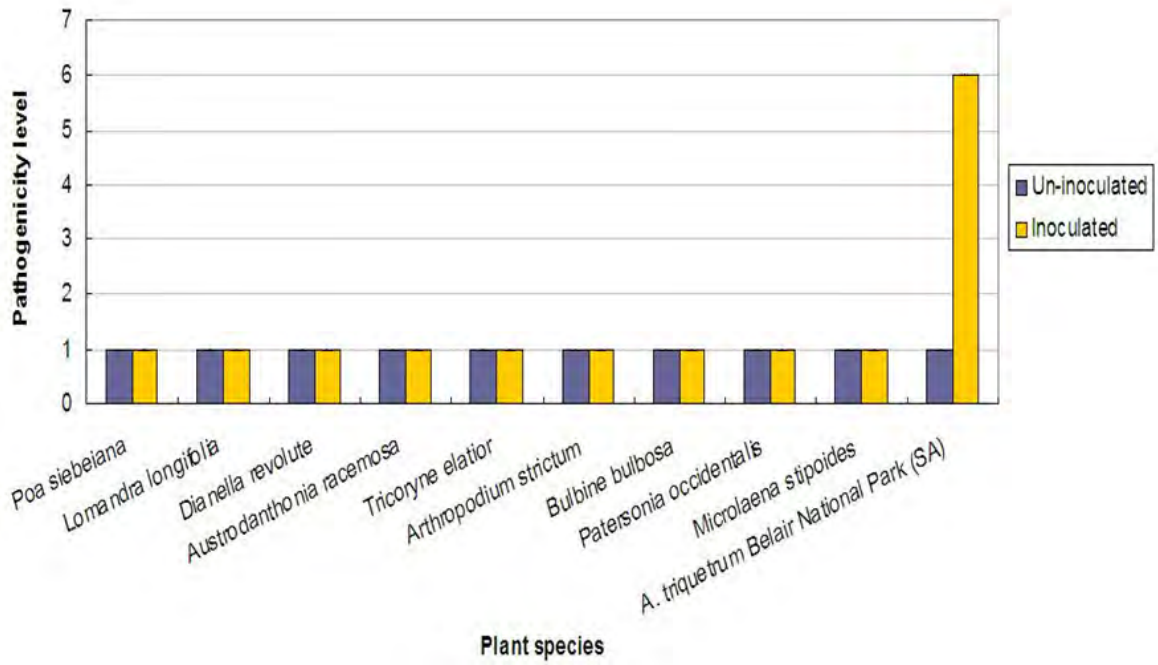


Figure 4.20 Pathogenicity of the *Pectobacterium carotovorum* subsp. *carotovorum* isolate on Australian native species in the glasshouse. *Allium triquetrum* from the Belair National Park (SA) provenance was used as the positive control (Bars=2 x standard error).

4.4 DISCUSSION

Pectobacterium carotovorum subsp. *carotovorum* has not previously been considered as a potential biological control agent for *A. triquetrum* in Australia, yet. The pathogenicity testing results for both test-tube-grown and potted *A. triquetrum* indicated that this strain of the bacterium was highly virulent on *A. triquetrum in vitro* in 24 h and *in vivo* in 2 weeks. Similar disease symptoms were observed in the field in some provenances in Victoria and in retrospect it would have been good to collect these and isolate from them. Another point in favour of this isolate as a biocontrol agent is that there was no infection of cultivated *Allium* species, unlike *S. cepivora* in Chapter 3, or native Australian monocots that would be liable to grow in the same habitats.

4.4.1 Molecular and physiological analysis

The isolation of pure cultures, 16S-rDNA sequencing and positive 600 bp amplicon with *P. carotovorum* subsp. *carotovorum* specific primers (Kang *et al.* 2003) suggests that the symptoms in the bulbs were caused by *P. carotovorum* subsp. *carotovorum.* This was confirmed by the physiological and biochemical characterization based on the API 20NE and other tests (Pérombelon 1973; Lelliott 1974; Dickey 1979; Dye 1983). The lactose, maltose and erythromycin reactions in particular suggested that the bacterium is *P. carotovorum* subsp. *carotovorum* rather than *P. chrysanthemi, as P. carotovorum* subsp. *carotovorum* is fermentative for lactose, variable for maltose and is resistant to erythromycin but *P. chrysanthemi* is not fermentative for both carbohydrates and is sensitive to erythromycin. *P. carotovorum* subspecies *carotovorum* and *P. carotovorum* subspecies *atroseptica* are very similar according to physiological tests. However; the isolated bacterium grew at 37 ºC and *P. carotovorum* subsp. *atroseptica* cannot. Thus there seems little doubt that the bacterium isolated from collapsed and rotted *A. triquetrum* bulbs at 4°C during storage was *P. carotovorum* subsp. *carotovorum*. This is well known as a storage rot, including of onions (Palacio-Bielsa *et al.* 2006).

4.4.2 Pathogenicity of P. carotovorum subsp. carotovorum on A. triquetrum

The *P. carotovorum* subsp. *carotovorum* isolate's pathogenicity both *in vitro* and *in vivo* was very rapid - only 24 h incubation in test-tubes at 25ºC and 2 weeks in pots at 15-25°C in the glasshouse. Pathogenicity of *P. carotovorum* on stored *Allium* species such as bunching onion has been reported in previous studies (Dye 1969; Hale *et al.* 1992; Halfeld-Vieira and Nechet 2008). A further point in favour of the use of this bacterium is that there was no variation in mortality among provenances *in vitro* except plants from Kangaroo Flat (Bendigo, VIC). Only one replicate of *A. triquetrum* from this provenance exhibited healthy green leaves after 24 h incubation but a highly infected collar region was

observed at 24 h and the plant collapsed after 48 h incubation. Thus there seemed no inherent resistance to infection under conditions favouring the bacterium.

According to Dye (1983), the optimum temperature for *P. carotovorum* subsp. *carotovorum* growth is 28-30ºC. The longer times taken until symptoms of infection were observed at 15ºC and 4ºC (7 days and 2 months) incubation respectively suggests that temperate affected how the host plant defence system reacts against pathogens, or the pathogenicity and virulence of pathogens (Colhoun 1964; Bell 1981). In some other weedy species, plant resistance to pathogens has varied as temperature changed (Mayama *et al.* 1975; Wang *et al.* 2009). This pathogenicity at temperatures less than the optimum suggested that the bacterium can be pathogenic in cool and cold environments similar to those experienced during growth of *A. triquetrum* in the field and so is a good attribute for a biological control agent.

Pathogenicity of the bacterium varied between *A. triquetrum* provenances in the more exacting conditions for the pathogen in the glasshouse, unlike the uniformity seen in test-tubes, where conditions favoured the pathogen greatly. The Mylor (SA) provenance had the least infection among all provenances and two replicates out of three remained healthy. This could either because of innate genetically acquired resistance in the plants or a defect in virulence in the bacterium; or it could be disease escape. Genetic diversity of *A. triquetrum* between and within provenances was demonstrated in chapter 2 and one of the Mylor replicates clustered separately from other South Australian and Victorian provenances in the RAPD dendrogram. Therefore genetic variation within this provenance could be the reason for lack of susceptibility to the bacterium. Disease escape is also a possibility, in that bacteria may have been washed below the rooting zone in the pots and so not been present at a sufficiently high dose to cause disease. This is unlikely, as 2×10^8

cells were inoculated per pot and this was sufficient to kill all replicates of most other provenances. It may also be a function of the numbers of replicates used; only three replicates were used in the glasshouse trials. This number of replicates may have been too low to cover for occasional disease escape and still show the pathogenicity of the bacterium in statistical analysis. However; small numbers of replicates is typical in weed management studies (Auld *et al.* 1989; Auld 1993).

The lack of soft rot disease symptoms in cultivated *Allium* species in particular and native Australian monocots is a good feature for a biological control agent under the same conditions as those that produced soft rot in *A. triquetrum. P. carotovorum* is pathogenic on a wide range of vegetables and crops producing fleshy tissues, bulbs and tubers (Babadoost 1990). The lack of infection in other *Allium* species, in particular, raises the question of this strain of *P. carotovorum* subsp. *carotovorum* being specifically pathogenic to *A. triquetrum*. *P. carotovorum* has many subspecies with different ranges of hosts, e.g *P. carotovorum* subsp. *atrosepticum* on potato, and it is not impossible that this is the case, though more *Allium* species would need to be tested to find out. Centrifugal phylogenetic testing is the most important factor for assessing biological control agent and release the pathogen into a new environment (Muller-Stover and Kroschel 2005 ; Morin et al. 2006). The native plants used in this study grow in *A. triquetrum*-infested habitats but they are not closely related to this weed. For instance, Chocolate Lily (*Arthropodium strictum* R. Br.) belongs to the family *Liliaceae sensu lato* was the most related native species used for pathogenicity testing, as there are no native species in the *Alliaceae sensu stricto* in Australia. The lack of soft-rot symptoms observed in root system and leaves of native Chocolate Lily and other *Liliaceae* 3 months after inoculation by the bacterium suggests that it is unlikely that more distant relatives would be infected and that was the

case, though more monocots and some dominant dicots in infested habitats should be tested on the precautionary principle.

According to Pérombelon (1980) the soft rot bacteria infect the host tissue from wounds and natural cracks occurred in the field or during storage. Although the cultivated *Allium* species used in the host specificity study were artificially wounded during re-potting in the glasshouse, the lack of soft rot symptoms 3 months after inoculation under the same conditions in which *A. triquetrum* plants were decayed after only 14 days suggests that the plants were relatively incapable of becoming infected in these conditions., The inoculation techniques and conditions such as potting mix, irrigation and inocula concentration might be the possible reason that the soft rot disease incidence did not occur on cultivated *Allium* species. Regular irrigation may wash off the bacterial cells out of the pots, however; numerous extensive roots of the plants should be able to contact the bacterial cells during irrigation. It is unlikely that they escaped disease as the bacterial inoculation was performed twice and the second inoculation was done a month after the first inoculation. The plants may become infected as they become dormant or are stored and this should be tested. As *P. carotovorum* subsp. *carotovorum* is known as a storage pathogen for many crops (Wright and Triggs 2005), it might not produce nay soft-rotting symptoms on culivated *Allium* species in the glasshouse.

4.4.3 Histology of infected tissues

Pectobacterium carotovorum subsp. *carotovorum* is a paranchymatous pathogen in *A. triquetrum* as shown by the bacteria being present intercellularly in the cortical parenchyma cells and vascular bundles. The cortical parenchyma cell walls showed evidence of rupture and vascular bundles showed evidence of damage, probably due to the bacterial enzymatic activity. Delahaut and Stevenson (2004) stated that pectolytic bacteria

digest the middle lamella of host plant cell walls, which causes the cells to be pulled apart. It seems that the bacterium enters the cortex through the epidermis and multiplies between and in the infected cells. Its enzyme production ruptures the cell wall and the bacteria continue to destroy the other intact cells. As the bacterium were even seen in the vascular-bundles, they could move up through vascular tissues and this may be why the upper leaves collapsed 12 h after inoculation. As the bacterium travels through the vascular tissues, it can be distributed quickly to the mesophyll of young leaves and cause collapse. In this study only the infected collar region of test-tube-grown *A. triquetrum* were used for microtomy and light microscopy and it was not possible to section infected leaves due to highly softened tissues and low leaf thickness. A further study of the infected tissue at different times after inoculation would test this hypothesis.

4.4.4 Inoculum

The dose of 10^8 bacterial cells was highly effective and it was sufficient to cause soft rot on *A. triquetrum in vitro* and *in vivo*. However; the effective dose may differ in different environment conditions (especially field conditions), age and size of plants and application method (Klein and Auld 1995). Increase in inoculum may improve biocontrol efficacy (Imaizumi *et al.* 1997; Klein and Auld 1995) but may not be necessary if the first-infected bulbs ooze further bacteria and infect others, as happens in storage. Further research needs to be performed to determine the least effective dose in different conditions and the best inoculation method for field use. In this regard, the observation that rotted bulbs were observed at some sites suggests that there may already be an inoculum at some sites and its augmentation is needed to cause significant disease for biological control.

4.4.5 Conclusion

The chance observation of rotted bulbs in storage in one provenance of *A. triquetrum* led to the isolation of a pure culture of a bacterium identified as *P. carotovorum* subsp. *carotovorum*. Koch's postulates were fulfilled as the bacterium caused soft rot and killed plants of *A. triquetrum* both *in vitro* in test-tubes and *in vivo* in pots in the glasshouse, even at relatively cool temperatures. In test tubes where the bacterium was favoured, there was no difference in pathogenicity and all plants were killed within 24 h, whereas in pots, some replicate plants from a few provenances were not killed and plants did not die until 2 weeks after inoculation. Bacteria destroyed the cortical parenchyma and even entered the vascular system, leading to a sudden collapse in the foliage leaves. The bacterium did not infect cultivated *Allium* species or native Australian monocots in pot trials and so it is possible that this strain is specifically pathogenic to *A. triquetrum*. The pathogenicity of the bacterium and its high virulence on the weed coupled with its apparent lack of pathogenicity to other plants tested suggests that this is a potential biological control agent for *A. triquetrum* in Australia.

Chapter 5

Evaluation of a bacterium close to *Ochrobactrum* sp. as a potential biocontrol agent for *Allium triquetrum*

5.1 INTRODUCTION

Angled Onion (*Allium triquetrum* L.*)* is a noxious weed in Australia. It invades periodically wet habitats and reduces biodiversity of native flora. Biological control offers the only long-term cure and the fungus *Stromatinia cepivora* Berk. (Chapter 3) and the bacterium *Pectobacterium carotovorum* subsp. *carotovorum* (chapter 4) have previously been shown to be pathogenic and virulent. However; their use can be problematic because they are also virulent pathogens of stored cultivated *Allium* species, e.g. garlic, onions. A bacterium was isolated from shrunken bulbs collected from the Dandenongs, Victoria after 2 months storage at 4ºC. It caused problems in growing surface-sterilised bulbs, as it appeared to be endogenous, making it necessary to strip down the bulbs to only the young parts to grow axenically. The aim of this chapter was to assess the pathogenicity and virulence of this bacterium as a potential biological control agent for *A. triquetrum*. The bacterium was identified as close to *Ochrobactrum* species using physiological tests and sequencing of the 16S r-DNA of the bacterial genome. The content that follows therefore describes the known species and their roles and how the bacterium was tested for pathogenicity and virulence on *A. triquetrum* plants in test-tube and glasshouse trials.

Isolation of *Ochrobactrum* species from agricultural soil and crops has been reported previously (Lebuhn *et al.* 2000; Bathe *et al.* 2006). At least some of the species have only been found on one plant; for instance, *O. tritici* was only isolated from wheat root (Lebuhn *et al.* 2000) and isolation of only *O. anthropi* from tea rhizosphere has been reported

(Chakraborty *et al.* 2009). There is no report on pathogenicity of *Ochrobactrum* species on plant species; however; four species of this genus, *O. anthropi*, *O. intermedium, O. haematophilum* and *O. pseudogrignonense,* are known as rare clinical pathogens (Holmes *et al.* 1988; Cieslak *et al.* 1996; Swings *et al.* 2006; Kämpfer *et al.* 2007).

5.1.1 Taxonomy and classification of Ochrobactrum sp.

The *Ochrobactrum* genus is a member of the family *Brucellaceae* (Table 5.1). It belongs to the Class *Alphaproteobacteria* and Order *Rhizobiales*. Holmes and Dawson (1983) described the bacterium initially as Gram-negative motile rods (1-1.5 µm in length) with rounded ends and parallel sides, usually occurring singly. It is obligately aerobic and its optimum growth temperature is 20-37 ºC. *Ochrobactrum* is oxidase- and catalase-positive and it does not hydrolyse insulin, gelatin and DNA. It does not produce amino acids with indole groups but utilises a range of amino acids, carbohydrates and organic acids (Holmes *et al.* 1988). *Ochrobactrum* species produce acid by assimilation of different carbon sources such as adonitol, sucrose and lactose. *Ochrobactrum* is the closest genus to *Brucella* which is a very important homogeneous antigenic group (Díaz *et al.* 1966). To date, the genus *Ochrobactrum* contains 12 species isolated as plant- endophytic bacteria and human clinical pathogens.

Table 5.1 Taxonomy and classification of *Ochrobactrum* genus (Holmes et al. 1988).

Domain: Bacteria

Phylum: Proteobacteria

Class: Alphaproteobacteria

Order: Rhizobiales

Family: *Brucellaceae*

Genus: *Ochrobactrum*

5.1.2 Growth conditions and identification of Ochrobactrum species

Ochrobactrum species can be grown on wide range of media such as nutrient agar (NA), tryptone soy agar (TSA) and yeast mannitol agar (YMA) at 25-37 ºC for 24-48 h (Holmes *et al.* 1983; Velasco *et al.* 1998; Kämpfer *et al.* 2003; Zurdo-Piñeiro *et al.* 2007; Scholz *et al.* 2008a). There is no selective media for isolation of *Ochrobactrum* species. Cell morphology differs between *Ochrobactrum* species and colony colours and textures vary from white to beige and shiny- to mucoid (e.g. *O. oryzae, O. cytisi* and *O. anthropi*) to beige and mucoid to translucent (e.g. *O. gallinifaecis, O. lupini* and *O. pseudogrignonense*). Cells are non-spore-forming Gram-negative rods and motility varies from non-motile to highly motile by means of 1-3 polar or subpolar flagella (Velasco *et al.* 1998; Lebuhn *et al.* 2000; Kämpfer *et al.* 2003). The colonies are visible on the agar medium after 24 h as smooth to mucoid, round, slightly raised and glistening to translucent. *Ochrobactrum* species do not produce pigmented colonies and growth rate is variable.

Taxonomical classification of micro-organisms depends on morphological, physiological and molecular variations between different species. *Ochrobactrum* species are differentiated by physiological and biochemical tests and fatty acid production (Table 5.2). Molecular analysis of different species based on 16S r-DNA PCR amplification and sequencing (Lebuhn *et al.* 2000), REP-PCR and ARDRA (Tripathi *et al.* 2006) and DNA-DNA hybridization Zurdo-Piñeiro *et al.* 2007) has been performed. Specific primers are other useful tools used to discriminate between various species at the species and subspecies level. Specific primers targeting the *recA* and *rrs* genes were designed to detect *O. anthropi* from *O. intermedium* and *Brucella* species (Scholz *et al.* 2008a,b), as *Brucella* is a closely related clinically pathogenic species to *Ochrobactrum,* with similar clinical symptoms.

5.1.3 Ochrobactrum species

Currently the genus *Ochrobacterum* consists of 12 species: *Ochrobactrum anthropi* (Holmes *et al.* 1988), *Ochrobacterum intermedium* (Velasco *et al.* 1998), *Ochrobacterum tritici* (Lebuhn *et al.* 2000), *Ochrobactrum grignonense* (Lebuhn *et al.* 2000), *Ochrobactrum gallinifaecis* (Kämpfer *et al.* 2003), *Ochrobactrum lupini* (Trujillo *et al.* 2005), *Ochrobactrum oryzae* (Tripathi *et al.* 2006), *Ochrobactrum cytisi* (Zurdo-Piñeiro *et al.* 2007), *Ochrobactrum haematophilum* (Kämpfer *et al.* 2007), *Ochrobactrum pseudogrignonense* (Kämpfer *et al.* 2007), *Ochrobactrum rhizosphaerae* (Kämpfer *et al.* 2008) and *Ochrobactrum thiophenivorans* (Kämpfer *et al.* 2008).

Ochrobactrum anthropi and *O. intermedium* have recently been categorised as opportunistic human pathogens infecting immunocompromised patients (Mahmood *et al.* 2000; Apisarnthanarak *et al.* 2005; Kämpfer *et al.* 2007; Scholz *et al.* 2008 ab). *Brucella* is the closest genus to *Ochrobactrum* and has sometimes been misidentified as *O. intermedium* (Velasco *et al.* 1998; Lebuhn *et al.* 2000; Lebuhn *et al.* 2006). *O. haematophilum* and *Ochrobactrum pseudogrignonense* have also been isolated from clinical specimens between 1992 and 2000 (Kämpfer *et al.* 2007).

Ochrobacterum tritici and *O. grignonense* were originally isolated in France from the roots of wheat cultivars (*Triticum aestivum* L.) and agricultural soil respectively (Lebuhn *et al.* 2000). These two bacteria were characterised on the basis of genotypic and phenotypic characters, by contrast with *O. anthropi* and *O. intermedium*.

Ochrobactrum gallinifaecis was isolated from chicken faeces by Kämpfer *et al.* (2003). The 16S-rDNA sequence analysis indicated that the bacterium was 95.6%, 95% and 94.6% similar to *O. tritici*, *O. grignonense* and *O. anthorpi* respectively. Physiological and biochemical variations between known *Ochrobactrum* species and the isolated bacterium

from chicken faeces suggested a new species. The main difference of *O. gallinifaecis* by contrast with the other species of this genus is non-motile cells.

Ochrobactrum lupini was isolated from nodules of *Lupinus honoratus* (Trujillo *et al.* 2005). The non-rhizobial bacterium reinfected the *Lupinus* plants and formed nodules. The 16S-rDNA sequence of the bacterium was 99.8% similar to *O. anthropi*. The plasmid sequence analysis of the bacterium indicated *nodD* and *nifH* genes responsible for nodulation and nitrogen fixation. However; the genus *Ochrobactrum* does not contain nitrogen-fixing species (Trujillo *et al.* 2005) and it seemed to have acquired part or all of a Sym plasmid from rhizobia and expressed these.

Ochrobactrum oryzea was originally isolated from paddy rice (*Oryza sativa*) seeds and tillers in northern India on fresh nitrogen-free semi solid medium (Tripathi *et al.* 2006). Genotypic similarity of the bacterium to *Ochrobactrum* species based on amplified ribosomal DNA restriction analysis (ARDRA) and repetitive element PCR fingerprinting (REP-PCR) suggested that it belonged to *Ochrobactrum*. The 16S-rDNA sequencing of the bacterium indicated 96.8% similarity to *O. gallinifaecis*. However; clear differences in fatty acid methyl ester patterns of *O. oryzaea* differentiated it from other *Ochrobactrum* species.

Ochrobactrum cytisi was isolated from nodules of *Cytisus scoparius* in Spain (Zurdo-Piñeiro *et al.* 2007). The bacterium 16S r-DNA sequence was 100 and 99% similar to *O. anthropi* and *O. lupini* respectively. The bacterium was named as a novel species of *Ochrobactrum* based on the results of DNA-DNA hybridization, phenotypic and fatty acid differences. Presumably it, like *O. lupini*, had acquired plasmid-borne genes from rhizobia.

Ochrobactrum rhizosphaerae and *O. thiophenivorans* were isolated from potato rhizosphere in Austria and wastewater in Germany respectively (Kämpfer *et al.* 2008). Phylogenetic analysis of *rrs* and *recA* sequences showed that *O. rhizosphaeraeis* and *O. thiophenivorans* are close to *O. grignonense* and *O. pseudogrignonense* respectively. However; morphological and physiological differences were observed and new species created.

5.1.4 Aim

The aims of the experiments in this chapter were:

- Identification of the semi-soft-rotting bacterial agent on *A. triquetrum* bulbs by physiological and biochemical tests and 16S r-DNA sequencing.
- Comparison of the bacterium with known *Ochrobactrum* species using RAPD and RFLP analysis.
- Pathogenicity testing of the bacterium on *A. triquetrum* provenances *in vitro* and *in vivo*.
- Interaction of the bacterium and *Stromatinia cepivora* in laboratory conditions.
- Centrifugal phylogenetic testing of the bacterium on cultivated *Allium* species and related Australian native monocots.
- Histological studies of the infected *A. triquetrum* bulbs.

Table 5.2 Physiological and biochemical tests used for identification of *Ochrobactrum* species (Trujillo *et al.* 2004; Kämpfer *et al.* 2008). Strains are: 1: *O lupini*, 2: *O. anthropi*, 3: *O. grignonense*, 4: *O. tritici*, 5: *O. intermedium*, 6: *O. gallinifaecis*, 7: *O. rhizosphaerae*, 8: *O. thiophenivorans*, 9: *O. oryzae*, 10: *O. pseudogrignonense*, 11: *O. haematophilum*. +: positive, -: negative, (+): weakly positive and ND: no data available.

Physiological and biochemical tests	***Ochrobactrum* species**										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Hydrolysis of:											
p-NP phenylphosphonate	-	-	-	(+)	-	-	+	(+)	ND	(+)	-
$_{L}$-glutamate-γ-3-carboxy pNA	+	(+)	-	(+)	+	-	-	-	ND	-	-
$_{L}$-Proline pNA	+	+	+	(+)	(+)	+	+	+	ND	+	+
Assimilation of :											
$_{D}$-fructose, myo-inositol, $_{D}$-sorbitol, $_{DL}$-3-hydroxybutyrate	+	+	+	+	+	-	+	+	+	+	+
$_{L}$-rhamnose	+	+	+	+	+	-	+	-	+	+	+
Cis-aconitate	-	+	+	+	+	-	+	-	+	+	+
Citrate	+	+	+	+	+	-	+	-	-	+	+
4-aminobutyrate, $_{β}$-alanine	+	+	+	+	+	(+)	+	+	ND	+	+
Maltose	+	+	-	+	+	-	+	-	+	-	+
$_{D}$-gluconate	(+)	+	+	+	+	+	-	+	+	+	+
N-acetyl-D-glucosamine	+	+	-	+	+	-	(+)	(+)	+	-	+
Adonitol	+	+	-	+	+	-	+	-	+	+	+
Sucrose, trehalose	+	+	-	+	-	-	+	-	+	+	+
Maltitol	+	+	-	-	-	-	+	-	ND	-	+
Trans-aconitate	-	-	-	-	(+)	-	+	-	ND	+	+
Cellobiose	-	+	-	-	+	-	+	-	ND	-	-
N-acetyl-$_{D}$-galactosamine	+	(+)	+	+	+	-	+	+	ND	+	-
Suberate	-	-	-	+	-	-	-	-	ND	-	-
L-aspartate	+	+	+	-	+	+	+	+	+	+	+
4-hydroxybenzoate	+	(+)	-	+	(+)	-	-	-	ND	-	+
L-histidine	+	+	+	(+)	+	+	+	-	+	+	+
L-leucine	+	+	+	(+)	+	-	+	-	+	+	+

5.2 MATERIALS AND METHODS

5.2.1 Origin and isolation of the bacterium

The bacterium was isolated from *A. triquetrum* bulbs collected from Hardy's Picnic Ground, Dandenongs, Victoria and grown on in a RMIT glasshouse until the plants died back naturally, when they were removed from the pots, washed, dried and stored as in Chapter 4. Semi-soft rot symptoms were observed in some bulbs after 2 months storage at 4ºC. During karyotypic studies of *A. triquetrum* (Chapter 2), the bulbs were surface-sterilized using 70% ethanol for 5 min and 1.5% NaOCl for 10 min, rinsed with sterile MilliQ water and cultured on water agar at room temperature to develop roots. Bacterial infection was observed after 48 h as slimy white bacterial masses as the basal plate contacted the water agar surface. A loopful of bacteria was suspended in 0.9% sterile saline and sub-cultured on Nutrient agar (NA) (Oxoid) plates and modified trypticase soy agar (TSA) selective medium (Grant and Holt 1976) (Fig. 5.1). Two types of bacteria were observed on NA as small and large colonies. Gram staining showed Gram-negative rods for small colonies and Gram-positive rods for large colonies. The Gram-positive bacteria were identified as *Bacillus* sp. based on morphology. Only the small colonies grew on TSA (Gram-negative-specific medium) modified medium as a pure culture. This latter bacterium was stored at -80ºC in 65% glycerol and used for further analysis.

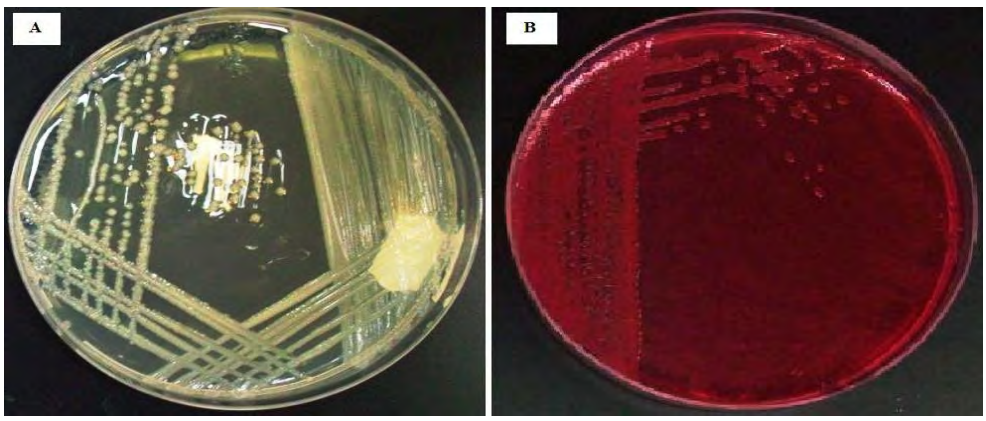


Figure 5.1 Isolated bacterium close to *Ochrobactrum* from infected *A. triquetrum* bulbs collected from Hardy's Picnic Ground, Dandenons (VIC). A: Nutrient agar, B: TSA modified medium.

5.2.2 DNA extraction and PCR amplification of 16S r-DNA

The bacterial genomic DNA was extracted based on a modified extraction method using QIAGEN DNeasy Plant Mini Kit in Section 4.3.2.2. The DNA concentration and quality was estimated by electrophoresis of 1.5% agarose gel in TBE buffer along with 1 μL of Fermentas GeneRuler^{TM} 100 bp. The gel was stained in ethidium bromide and bands visualised and photographed using a Bio-Rad Gel Doc system. The freshly extracted DNA was used for 16S r-DNA PCR amplification and sequenced. On the basis of the sequencing result, the bacterium was identified as close to *Ochrobactrum* sp. Therefore five known *Ochrobactrum* species were purchased from the Czech Collection of Microorganisms (CCM), Masaryk University, Czech Republic (Table 5.3). The bacterial DNA was extracted from them as before and used for RAPD and RFLP analysis.

Table 5.3 *Ochrobactrum* species used in RAPD and RFLP analysis provided by the Czech Collection of Microorganisms (CCM). The species marked * are classified in biohazard group 2.

Species	**Isolated from**	**CCM No.**	**Batch No.**
Ochrobactrum anthropi	Clinical specimens	999*	01121988273
Ochrobactrum grignonense	Agricultural soil	7180	1703200411709
Ochrobactrum intermedium	Clinical specimens	7036*	2605200210034
Ochrobactrum rhizosphaerae	Potato rhizosphere	7493	0711200715728
Ochrobactrum tritici	Wheat cultivars	7181	1703200411708

5.2.3 16S r-DNA PCR amplification and sequencing

16S ribosomal DNA (rDNA) was amplified using fD1 and rP2 primers as in Chapter 4 (Weisburg *et al.* 1991). The PCR reaction was performed in 25 μL containing ~16 ng of genomic DNA as explained in Section 4.2.4. A G-STORM thermal cycler was

programmed based on Weisburg *et al.* (1991) as mentioned in Section 4.3.4. The 1500 bp PCR product was purified using a QIAGEN PCR Purification Kit as explained in Section 4.2.5 and used for DNA sequencing. The sequencing reaction was prepared according to the Big Dye Terminator v3.1 protocol as explained in Section 4.2.5 using rP2 universal primer and sent to the Micromon Sequencing Facility at Monash University.

5.2.4 RFLP analysis

The 16S r-DNA PCR products of the bacterium and *Ochrobactrum* species were digested by restriction enzymes (Table 5.4). RFLP reactions were performed in 5 µl containing 4 µl of the PCR products, 0.5 µl 5,000 U restriction enzyme, and 0.5 µl 10X buffer for each enzyme as described in Chapter 2. All reactions were incubated at the optimal temperature for a minimum of 3 h and all 5 µl of the digested products was fractioned in 2% agarose gel along with 1 µl of the GeneRuler^{TM} 100 bp. Gels were stained in ethidium bromide and imaged by Bio-RAD Gel Doc system.

Table 5.4 Endonuclease restriction enzymes used for RFLP studies.

Restriction enzyme	Optimal activity	Restriction enzyme	Optimal activity
1- *Taq* I	65	8- *HinF*I	37
2- *BamH* I	37	9- *Nhe* I	37
3- *Hha* I	37	10- *Eco* 32	37
4- *EcoR* I	37	11- *BstU*I	60
5- *Rsa* I	37	12- *Xba* I	37
6- *Xho* I	37	13- *Hind* III	37
7- *Not* I	37	14- *Tru9* I	65

5.2.5 Sequencing analysis

The bacterium sequence was edited and searched for closest matches in the non-redundant nucleotide database in Genbank using the NCBI BLAST program. The 16S rDNA sequences of *Ochrobactrum* species (Table 5.3) were downloaded from the GenBank

nucleotide database to be used for sequence alignment. Sequence alignment was performed using ClustalW (accurate) program in Biomanager (ANGIS). The aligned sequences were trimmed at the ends using the Edit program, Biomanager, so that all were the same length. A distance matrix was formed using maximum parsimony in the program DNAdist (Jukes and Cantor 1969) and the results were displayed as a bootstrapped (1000 bootstraps) phylogenetic tree in Mega4 using UPGMA and Neighbour joining methods.

5.2.6 RAPD-PCR

RAPD-PCR amplification of the bacterium and *Ochrobactrum* species were conducted using 60 10-mer RAPD primers (3 sets: OPA, OPB, OPM) (Operon Technologies). RAPD PCR reactions were performed in 25 µL containing ~16 ng of genomic DNA as explained in Section 2.2.6. The RAPD-PCR amplification was replicated three times using the G-STORM thermocycler programmed as explained in Section 2.2.6. The amplicons were fractioned in 1.5 % agarose gel along with 1 µL of GeneRuler^{TM} 100 bp and visualized on a Bio-Rad Gel Doc system.

5.2.7 Physiological and biochemical tests

The bacterial cells were Gram-stained and motility was investigated using the hanging drop method. Physiological and biochemical characteristics of the bacterium and the five authentic *Ochrobactrum* species were tested using an API 20NE kit and oxidise test as in Chapter 4. The API 20NE set up was carried out for the bacterium as in Section 4.3.6.

5.2.8 Pathogenicity testing of the bacterium on test-tube-grown A. triquetrum from across Australia

A. triquetrum was micropropagated in water agar as explained in Section 3.7.4.1.1 Fresh bacterial cultures was prepared by sub-culturing of the bacterium from -80ºC stock into

LB Broth. The bacterial culture was incubated at 30ºC in a Thermoline Scientific Shaker Incubator for 48 h. The cell concentration was adjusted to 1 x 10^8 ml^{-1} as explained in Section 4.2.7. The test-tube-grown plants were inoculated with the bacterium and incubated at 25ºC in a 16 h photoperiod for 2 months as in Section 4.2.7. Control plants were not inoculated. There were three replicate tubes per treatment in a completely randomised design. The plants were scored on a disease ranking scale as follows:

1. No infection
2. Water agar turbidity
3. Bacterial clumps in water agar
4. Wilting of the upper leaves
5. Discoloration of leaves
6. Dead plants/necrosis

5.2.9 Pathogenicity testing of the bacterium on test-tube-grown cultivated Allium seedlings

Cultivated *Allium* seedlings (Table 3.4) were grown up as explained in Section 3.7.5.1 and transferred into test-tubes with water agar. Micropropagation of garlic (*Allium sativum*) was also performed by the same methods as in Section 3.7.4.1.1 The test-tube grown plants were inoculated with the bacterium and incubated at 25ºC in a 16 h photoperiod as in Section 4.2.7. Control tubes were not inoculated. There were three replicate tubes per treatment. Inoculated plants were arranged in a completely randomized and scored as in Section 4.2.7.

5.2.10 Interaction of the bacterium and S. cepivora

In the first experiment, test-tube-grown *A. triquetrum* plants from provenances in Victoria were pre-inoculated with the isolated bacterium as explained in Section 4.2.7. To test the

interaction of the bacterium and *S. cepivora*, plants were re-inoculated using 30 sclerotia on blocks of water agar and incubated at 25ºC for 2 months. Test-tube-grown plants were also inoculated with the pathogens separately and un-inoculated tubes were used as control treatments. In the second experiment, the isolated bacterium was lawn-cultured on V8 juice agar. *S. cepivora* (sclerotia and mycelium), *Alternaria* sp., *Aspergillus* sp., *Penicillium* sp. and *Fusarium* sp. then cultured on pre-treated V8 agar with the bacterium 48 h after initial inoculation and incubated at 25ºC for 2 weeks. The control plates had fungi only. There were three replicates for each treatment. The fungal growth was scored for the first experiment 2 months and for the second experiment 2 weeks post-inoculation respectively.

5.2.11 Pathogenicity testing of the bacterium on A. triquetrum provenances from across Australia, cultivated Allium species and Australian native monocots

Potted *A. triquetrum*, cultivated *Allium* species and native Australian plants were prepared as explained in Sections 3.7.4.1, 3.7.5.2 and 3.7.5.3 respectively. An inoculum of 1 mL per pot containing 10^8 cells of the bacterial culture was prepared as explained in Section 4.2.7. Plants were inoculated in the glasshouse by dispersing the bacterium on the collar regions of the plants. There were three replicate pots per treatment in a completely randomized design and inoculation was performed twice for each experiment. The second inoculation was carried out a month after the first inoculation when no symptoms were apparent. Plants were kept in the glasshouse for 4 months and the soft rot disease symptoms were scored based on the disease ranking scale in Section 4.2.9.

5.2.12 Histological study of the infected A. triquetrum in vitro

Infected *A. triquetrum* showing disease symptoms after 30 days inoculation were used for histological studies. The infected test-tube-grown *A. triquetrum* was fixed in 5%

formaldehyde solution overnight. The infected tissues, mainly collar regions, were processed on a LEICA ASP 200S tissue processor as in Chapter 4 (Table 4.4). The processed tissues were paraffin wax-embedded on a Shandon Histocentrie 3 (Thermo Scientific) and sectioned 3-4 µm thick. Specimens were fixed on the glass slides based on the Ruzin (1999) protocol as explained in Section 4.3.8 and Gram stained. The sections were photographed on a Leica DM2500 compound microscope using 350X magnification.

5.3 RESULTS

5.3.1 16S r-DNA PCR amplification and sequencing

An amplicon of 1500 bp of the 16S rDNA was produced using fD1 and rP2 primers. The length of the sequenced product was 1199 nucleotides and 60 bases at the 5´ start and 3´ end of the sequence were deleted due to low quality sequence. The nucleotide Blast search indicated *Ochrobactrum* species as the best match in GenBank (Maximum identity: 96%, E value: 0.0). This sequence was aligned with other 16S rDNA sequences for *Ochrobactrum* species and *Bacillus cereus* (used as an outgroup) (Table 5.5) downloaded from GenBank. Nucleotide variation was observed as substitutions, deletions and insertions between the bacterium and other *Ochrobactrum* species and the outgroup *Bacillus cereus* (Fig. 5.2). Relatively small variation was observed between *Ochrobactrum* species and the isolated bacterium. The greatest distances were observed between the outgroup *Bacillus cereus* sequence and all others (Table 5.6).

The UPGMA phylogenetic tree (Fig. 5.3 A) indicated three main clusters. All *Ochrobactrum* species and the isolated bacterium sequences were clustered together and separated from the outgroup *Bacillus cereus.* The isolated bacterium was shown as a separate cluster for *Ochrobactrum* spp. with bootstrap value of 99%. Almost all other

species of *Ochrobactrum* species clustered together. Within these *O. tritici* and *O. anthropi* clustered together at 99% bootstrap value.

The Neighbour-Joining tree demonstrated a similar profile in which the *B. cereus* was separated in the first step as an outgroup from the isolated bacterium and *Ochrobactrum* sequences (Fig. 5.3 B). Almost all *Ochrobactrum* species were clustered separately from the other species. The isolated bacterium was separated with *O. rhizosphaerae* from other species but with a bootstrap value of only 35%, but it was closest to a tight clade of *O. tritici* and *O. anthropi* species were clustered together with bootstrap values of 91%.

Table 5.5 16S r-DNA sequences of *Ochrobactrum* and *Bacillus* species used for sequence alignment.

Bacteria	Origin	Isolate	NCBI No.
1- *Ochrobactrum tritici*	Korea	TG23	AF508091.1
2- *Ochrobactrum tritici*	Korea	TG161	AF508092.1
3- *Ochrobactrum tritici*	Korea	TJ3	AF508089.1
4- *Ochrobactrum tritici*	China	Pyd-1	EU999218.1
5- *Ochrobactrum tritici*	China	AN4	GU345782.1
1- *Ochrobactrum anthropi*	France	CLF19	AF526523.2
2- *Ochrobactrum anthropi*	France	ADV16	AF526520.1
3- *Ochrobactrum anthropi*	France	ADV8	AF526518.1
1- *Ochrobactrum intermedium*	India	13.9	HQ696468.1
2- *Ochrobactrum intermedium*	India	S-5	FJ159425.1
3- *Ochrobactrum intermedium*	China	DSQ5	HM217123.1
4- *Ochrobactrum intermedium*	India	ABA-229	HM480366.1
1- *Ochrobactrum grignonense*	India	IHB B 1375	GU186118.1
2- *Ochrobactrum grignonense*	China	d131	FJ950668.1
3- *Ochrobactrum grignonense*	China	c278	FJ950566.1
4- *Ochrobactrum grignonense*	China	c114	FJ950557.1
5- *Ochrobactrum grignonense*	China	c232	FJ950547
1- *Ochrobactrum rhizosphaerae*	Germany	PR17	AM490632.1
B.c: *Bacillus cereus*	China	D3-1	HQ731045.1
IB: Bacterium isolated from infected *A. triquetrum* bulb	Australia	N/A	N/A

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      1         11        21        31        41        51
tri.3 CCCGCGTTGGATTAGC-TAGTTGGTGAGGTAAAGGCTCACCAAGGCGACGAT-CCATAGC
tri.4 CCCGCGTTGGATTAGC-TAGTTGGTGAGGTAAAGGCTCACCAAGGCGACGAT-CCATAGC
tri.2 CCCGCGTTGGATTAGC-TAGTTGGTGAGGTAAAGGCTCACCAAGGCGACGAT-CCATAGC
tri.1 CCCGCGTTGGATTAGC-TAGTTGGTGAGGTAAAGGCTCACCAAGGCGACGAT-CCATAGC
ant.2 CCCGCGTTGGATTAGC-TAGTTGGTGAGGTAAAGGCTCACCAAGGCGACGAT-CCATAGC
tri.5 CCCGCGTTGGATTAGC-TAGTTGGTGAGGTAAAGGCTCACCAAGGCGACGAT-CCATAGC
int.1 CCCGCGTTGGATTAGC-TAGTTGGTGGGGTAAAGGCCCACCAAGGCGACGAT-CCATAGC
int.4 CCCGCGTTGGATTAGC-TAGTTGGTGGGGTAAAGGCCCACCAAGGCGACGAT-CCATAGC
int.2 CCCGCGTTGGATTAGC-TAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGAT-CCATAGC
int.3 CCCGCGTTGGATTAGC-TAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGAT-CCATAGC
ant.4 CCCGCGTTGGATTAGC-TAGTTGGTGAGGTAAAGGCTCACCAAGGCGACGAT-CCATAGC
gri.1 CCCGCGTTGGATTAGC-TAGTTGGTAGGGTAATGGCCCACCAAGGCGACGAT-CCATAGC
rhi.1 CCCGCGTTGGATTAGC-TAGTTGGTGAGGTAAAGGCTCACCAAGGCGACGAT-CCATAGC
ant.1 CCCGCGTTGGATTAGC-TAGTTGGTGAGGTAAAGGCTCACCAAGGCGACGAT-CCATAGC
gri.2 CCCATGTTGGATTAGC-TAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGAT-CCATAGC
gri.4 CCCATGTTGGATTAGCCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATTCCATAGC
gri.5 CCCATGTTGGATTAAC-TAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGAT-CCATAGC
gri.3 CCCATGTTGGATTAGC-TAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGAT-CCATAGC
IB    TCCGAGT-GGAT-AGC-TAGTGGATGAG-TAATG--TTCGCA--GCGACGAT--CAT-GC
B.c   CCCGCGTCGCATTAGC-TAGTTGGTGAGGTAACGGCTCACCAAGGCAACGAT-GCGTAGC
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      61        71        81        91        101       111
tri.3 TGGTCTGAGAGGATGAT-CAGCCACAC--TGGGACTGAGACACGGCCCAGACTCCTACGG
tri.4 TGGTCTGAGAGGATGAT-CAGCCACAC--TGGGACTGAGACACGGCCCAGACTCCTACGG
tri.2 TGGTCTGAGAGGATGAT-CAGCCACAC--TGGGACTGAGACACGGCCCAGACTCCTACGG
tri.1 TGGTCTGAGAGGATGAT-CAGCCACAC--TGGGACTGAGACACGGCCCAGACTCCTACGG
ant.2 TGGTCTGAGAGGATGAT-CAGCCACAC--TGGGACTGAGACACGGCCCAGACTCCTACGG
tri.5 TGGTCTGAGAGGATGAT-CAGCCACAC--TGGGACTGAGACACGGCCCAGACTCCTACGG
int.1 TGGTCTGAGAGGATGAT-CAGCCACAC--TGGGACTGAGACACGGCCCAGACTCCTACGG
int.4 TGGTCTGAGAGGATGAT-CAGCCACAC--TGGGACTGAGACACGGCCCAGACTCCTACGG
int.2 TGGTCTGAGAGGATGAT-CAGCCACAC--TGGGACTGAGACACGGCCCAGACTCCTACGG
int.3 TGGTCTGAGAGGATGAT-CAGCCACAC--TGGGACTGAGACACGGCCCAGACTCCTACGG
ant.4 TGGTCTGAGAGGATGAT-CAGCCACAC--TGGGACTGAGACACGGCCCAGACTCCTACGG
gri.1 TGGTCTGAGAGGATGAT-CAGCCACAC--TGGGACTGAGACACGGCCCAGACTCCTACGG
rhi.1 TGGTCTGAGAGGATGAT-CAGCCACAC--TGGGACTGAGACACGGCCCAGACTCCTACGG
ant.1 TGGTCTGAGAGGATGAT-CAGCCACAC--TGGGACTGAGACACGGCCCAGACTCCTACGG
gri.2 TGGTCTGAGAGGATGAT-CAGCCACAC--TGGGACTGAGACACGGCCCAGACTCCTACGG
gri.4 TGGTCTGAGAGGATGATTCAGCCACACCATGGGACTGAGACACGGCCCAGACTCCTACGG
gri.5 TGGTCTGAGAGGATGAT-CAGCCACAC--TGGGACTGAGACACGGCCCAGACTCCTACGG
gri.3 TGGTCTGAGAGGATGAT-CAGCCACAC--TGGGACTGAGACACGGCCCAGACTCCTACGG
IB    TG-TC-GAGAGCATCAT-CAGC-ACA---TGGGACTGAGACACGTCCCAGATTC-TACGG
B.c   CGACCTGAGAGGGTGAT-CGGCCACAC--TGGGACTGAGACACGGCCCAGACTCCTACGG
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      121       131       141       151       161       171
tri.3 GAGGCAG-CAGTGGGGAATATTGGACAATGGGCGCAAGCCTGAT-CCAGCCA-TGCCGCG
tri.4 GAGGCAG-CAGTGGGGAATATTGGACAATGGGCGCAAGCCTGAT-CCAGCCA-TGCCGCG
tri.2 GAGGCAG-CAGTGGGGAATATTGGACAATGGGCGCAAGCCTGAT-CCAGCCA-TGCCGCG
tri.1 GAGGCAG-CAGTGGGGAATATTGGACAATGGGCGCAAGCCTGAT-CCAGCCA-TGCCGCG
ant.2 GAGGCAG-CAGTGGGGAATATTGGACAATGGGCGCAAGCCTGAT-CCAGCCA-TGCCGCG
tri.5 GAGGCAG-CAGTGGGGAATATTGGACAATGGGCGCAAGCCTGAT-CCAGCCA-TGCCGCG
int.1 GAGGCAG-CAGTGGGGAATATTGGACAATGGGCGCAAGCCTGAT-CCAGCCA-TGCCGCG
int.4 GAGGCAG-CAGTGGGGAATATTGGACAATGGGCGCAAGCCTGAT-CCAGCCA-TGCCGCG
int.2 GAGGCAG-CAGTGGGGAATATTGGACAATGGGCGCAAGCCTGAT-CCAGCCA-TGCCGCG
int.3 GAGGCAG-CAGTGGGGAATATTGGACAATGGGCGCAAGCCTGAT-CCAGCCA-TGCCGCG
ant.4 GAGGCAG-CAGTGGGGAATATTGGACAATGGGCGCAAGCCTGAT-CCAGCCA-TGCCGCG
gri.1 GAGGCAG-CAGTGGGGAATATTGGACAATGGGCGCAAGCCTGAT-CCAGCCA-TGCCGCG
rhi.1 GAGGCAG-CAGTGGGGAATATTGGACAATGGGCGCAAGCCTGAT-CCAGCCA-TGCCGCG
ant.1 GAGGCAG-CAGTGGGGAATATTGGACAATGGGCGCAAGCCTGAT-CCAGCCA-TGCCGCG
gri.2 GAGGCAG-CAGTGGGGAATATTGGACAATGGGCGCAAGCCTGAT-CCAGCCA-TGCCGCG
gri.4 GAGGCAGGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATTCCAGCCAATGCCGCG
gri.5 GAGGCAG-CAGTGGGGAATATTGGACAATGGGCGCAAGCCTGAT-CCAGCCA-TGCCGCG
gri.3 GAGGCAG-CAGTGGGGAATATTGGACAATGGGCGCAAGCCTGAT-CCAGCCA-TGCCGCG
IB    GAGGCAG-CAGTGGGCA-TACTCGACA--TGGCGCA--GCTGAT-CCAGCAT--G-CGCG
B.c   GAGGCAG-CAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGAC-GGAGCAA-CGCCGCG
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      181       191       201       211       221       231
tri.3 TGAGTGATGAAGGCCCTAGGGTTGTAAAGCTC-TTTC----------ACCGGTG-AAG--
tri.4 TGAGTGATGAAGGCCCTAGGGTTGTAAAGCTC-TTTC----------ACCGGTG-AAG--
tri.2 TGAGTGATGAAGGCCCTAGGGTTGTAAAGCTC-TTTC----------ACCGGTG-AAG--
tri.1 TGAGTGATGAAGGCCCTAGGGTTGTAAAGCTC-TTTC----------ACCGGTG-AAG--
ant.2 TGAGTGATGAAGGCCCTAGGGTTGTAAAGCTC-TTTC----------ACCGGTG-AAG--
tri.5 TGAGTGATGAAGGCCCTAGGGTTGTAAAGCTC-TTTC----------ACCGGTG-AAG--
int.1 TGAGTGATGAAGGCCCTAGGGTTGTAAAGCTC-TTTC----------ACCGGTG-AAG--
int.4 TGAGTGATGAAGGCCCTAGGGTTGTAAAGCTC-TTTC----------ACCGGTG-AAG--
int.2 TGAGTGATGAAGGCCCTAGGGTTGTAAAGCTC-TTTC----------ACCGGTG-AAG--
int.3 TGAGTGATGAAGGCCCTAGGGTTGTAAAGCTC-TTTC----------ACCGGTG-AAG--
ant.4 TGAGTGATGAAGGCCCTAGGGTTGTAAAGCTC-TTTC----------ACCGGTG-AAG--
gri.1 TGAGTGATGAAGGCCCTAGGGTTGTAAAGCTC-TTTC----------ACCGGTG-AAG--
rhi.1 TGAGTGATGAAGGCCCTAGGGTTGTAAAGCTC-TTTC----------ACCGGTG-AAG--
ant.1 TGAGTGATGAAGGCCCTAGGGTTGTAAAGCTC-TTTC----------ACCGGTG-AAG--
gri.2 TGAGTGATGAAGGTCTTAGGATTGTAAAGCTC-TTTC----------ACCGGTG-AAG--
gri.4 TGAGTGATGAAGGTCTTAGGATTGTAAAGCTCCTTTC----------ACCGGTG-AAG--
gri.5 TGAGTGATGAAGGTCTTAGGATTGTAAAGCTC-TTTC----------ACCGGTG-AAG--
gri.3 TGAGTGATGAAGGTCTTAGGATTGTAAAGGCTCTTTC----------ACCGGTGGAAG--
IB    TGAGTGATGAAGTC--TAGGAT-GTAAAGCTC--TTC----------AGCG-TG-AAG--
B.c   TGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTT
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      241       251       261       271       281       291
tri.3 --ATAA----------TGACGGT-AACCGGAG-AAGAAGCCCCGGCTAACTTCGTGC-CA
tri.4 --ATAA----------TGACGGT-AACCGGAG-AAGAAGCCCCGGCTAACTTCGTGC-CA
tri.2 --ATAA----------TGACGGT-AACCGGAG-AAGAAGCCCCGGCTAACTTCGTGC-CA
tri.1 --ATAA----------TGACGGT-AACCGGAG-AAGAAGCCCCGGCTAACTTCGTGC-CA
ant.2 --ATAA----------TGACGGT-AACCGGAG-AAGAAGCCCCGGCTAACTTCGTGC-CA
tri.5 --ATAA----------TGACGGT-AACCGGAG-AAGAAGCCCCGGCTAACTTCGTGC-CA
int.1 --ATAA----------TGACGGT-AACCGGAG-AAGAAGCCCCGGCTAACTTCGTGC-CA
int.4 --ATAA----------TGACGGT-AACCGGAG-AAGAAGCCCCGGCTAACTTCGTGC-CA
int.2 --ATAA----------TGACGGT-AACCGGAG-AAGAAGCCCCGGCTAACTTCGTGC-CA
int.3 --ATAA----------TGACGGT-AACCGGAG-AAGAAGCCCCGGCTAACTTCGTGC-CA
ant.4 --ATAA----------TGACGGT-AACCGGAG-AAGAAGCCCCGGCTAACTTCGTGC-CA
gri.1 --ATAA----------TGACGGT-AACCGGAG-AAGAAGCCCCGGCTAACTTCGTGC-CA
rhi.1 --ATAA----------TGACGGT-AACCGGAG-AAGAAGCCCCGGCTAACTTCGTGC-CA
ant.1 --ATAA----------TGACGGT-AACCGGAG-AAGAAGCCCCGGCTAACTTCGTGC-CA
gri.2 --ATAA----------TGACGGT-AACCGGAGGAAGAAGCCCCGGCTAACTTCGTGCGCA
gri.4 --ATAA----------TGACGGT-AACCGGAGAAGGAAGCCCCGGCTAACTTCGTGC-CA
gri.5 --ATAA----------TGACGGT-AACCGGAGAAG-AAGCCCCGGCTAACTTCGTGC-CA
gri.3 --ATAA----------TGACGGTCAACCGGAGAAGAAGCCCCGGGCTAACTTCGTGC-CA
IB    --ATAA----------TGACG-T--ACCAGAAGAAGAAGCCCCGACTA-CTTCGTGC-CA
B.c   GAATAAGCTGGCACCTTGACGGT--ACCTAACCAGAAAGCCACGGCTAACTACGTGC-CA
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      301       311       321       331       341       351
tri.3 GCAGCCGCG---GTAATAC-GAAGGGGGCTA-GCG-TTGTTCGGATTTACTGGGCG-TAA
tri.4 GCAGCCGCG---GTAATAC-GAAGGGGGCTA-GCG-TTGTTCGGATTTACTGGGCG-TAA
tri.2 GCAGCCGCG---GTAATAC-GAAGGGGGCTA-GCG-TTGTTCGGATTTACTGGGCG-TAA
tri.1 GCAGCCGCG---GTAATAC-GAAGGGGGCTA-GCG-TTGTTCGGATTTACTGGGCG-TAA
ant.2 GCAGCCGCG---GTAATAC-GAAGGGGGCTA-GCG-TTGTTCGGATTTACTGGGCG-TAA
tri.5 GCAGCCGCG---GTAATAC-GAAGGGGGCTA-GCG-TTGTTCGGATTTACTGGGCG-TAA
int.1 GCAGCCGCG---GTAATAC-GAAGGGGGCTA-GCG-TTGTTCGGATTTACTGGGCG-TAA
int.4 GCAGCCGCG---GTAATAC-GAAGGGGGCTA-GCG-TTGTTCGGATTTACTGGGCG-TAA
int.2 GCAGCCGCG---GTAATAC-GAAGGGGGCTA-GCG-TTGTTCGGATTTACTGGGCG-TAA
int.3 GCAGCCGCG---GTAATAC-GAAGGGGGCTA-GCG-TTGTTCGGATTTACTGGGCG-TAA
ant.4 GCAGCCGCG---GTAATAC-GAAGGGGGCTA-GCG-TTGTTCGGATTTACTGGGCG-TAA
gri.1 GCAGCCGCG---GTAATAC-GAAGGGGGCTA-GCG-TTGTTCGGATTTACTGGGCG-TAA
rhi.1 GCAGCCGCG---GTAATAC-GAAGGGGGCTA-GCG-TTGTTCGGATTTACTGGGCG-TAA
ant.1 GCAGCCGCG---GTAATAC-GAAGGGGGCTA-GCG-TTGTTCGGATTTACTGGGCG-TAA
gri.2 GCAGCCGCGTTTGTAATACAGAAGGGGGCTAAGCGATTGTTCGGATTTACTGGGCGATAA
gri.4 GCAGCCGCG---GTAATAC-GAAGGGGGCTA-GCG-TTGTTCGGATTTACTGGGCG-TAA
gri.5 GCAGCCGCG---GTAATAC-GAAGGGGGCTA-GCG-TTGTTCGGATTTACTGGGCG-TAA
gri.3 GCAGCCGCG---GTAATAC-GAAGGGGGCTA-GCG-TTGTTCGGATTTACTGGGCG-TAA
IB    GCAGCCGCG---GTAATAC-GAAGGGGGCTA-GCG-TTGTTCGGATTTACTGGGCG-TAA
B.c   GCAGCCGCG---GTAATAC-GTAGGTGGCAA-GCG-TTATCCGGAATTATTGGGCG-TAA
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      361       371       381       391       401       411
tri.3 AGCGCAC-GTAGGCGGACTTTTAA-GTCAGGGGTGAA-ATCC-CGGGGCTCAACCCCGGA
tri.4 AGCGCAC-GTAGGCGGACTTTTAA-GTCAGGGGTGAA-ATCC-CGGGGCTCAACCCCGGA
tri.2 AGCGCAC-GTAGGCGGACTTTTAA-GTCAGGGGTGAA-ATCC-CGGGGCTCAACCCCGGA
tri.1 AGCGCAC-GTAGGCGGACTTTTAA-GTCAGGGGTGAA-ATCC-CGGGGCTCAACCCCGGA
ant.2 AGCGCAC-GTAGGCGGACTTTTAA-GTCAGGGGTGAA-ATCC-CGGGGCTCAACCCCGGA
tri.5 AGCGCAC-GTAGGCGGACTTTTAA-GTCAGGGGTGAA-ATCC-CGGGGCTCAACCCCGGA
int.1 AGCGCAC-GTAGGCGGACTAATAA-GTCAGGGGTGAA-ATCC-CGGGGCTCAACCCCGGA
int.4 AGCGCAC-GTAGGCGGACTAATAA-GTCAGGGGTGAA-ATCC-CGGGGCTCAACCCCGGA
int.2 AGCGCAC-GTAGGCGGGCTAATAA-GTCAGGGGTGAA-ATCC-CGGGGCTCAACCCCGGA
int.3 AGCGCAC-GTAGGCGGGCTAATAA-GTCAGGGGTGAA-ATCC-CGGGGCTCAACCCCGGA
ant.4 AGCGCAC-GTAGGCGGACTTTTAA-GTCAGGGGTGAA-ATCC-CGGGGCTCAACCCCGGA
gri.1 AGCGCAC-GTAGGCGGACTTTTAA-GTCAGGGGTGAA-ATCC-CAGAGCTCAACTCTGGA
rhi.1 AGCGCAC-GTAGGCGGATTTTTAA-GTCAGGGGTGAA-ATCC-CGGGGCTCAACCCCGGA
ant.1 AGCGCAC-GTAGGCGGACTTTTAA-GTCAGGGGTGAA-ATCC-CGGGGCTCAACCCCGGA
gri.2 AGCGCACAGTAGGCGGACTTTTAATGTCAGGGGTGAATATCC-CAGAGCTCAACTCTGGA
gri.4 AGCGCAC-GTAGGCGGACTTTTAA-GTCAGGGGTGAA-ATCC-CAGAGCTCAACTCTGGA
gri.5 AGCGCAC-GTAGGCGGACTTTTAA-GTCAGGGGTGAA-ATCC-CAGAGCTCAACTCTGGA
gri.3 AGCGCAC-GTAGGCGGACTTTTAA-GTCAGGGGTGAA-ATCCTCAGAGCTCAACTCTGGA
IB    AGCGCAT-GTAGGCGGACTTTTAA-GTCAGGGGTGAA-ATCC-CGGGGCTCAACCCCGGA
B.c   AGCGCGC-GCAGGTGGTTTCTTAA-GTCTGATGTGAA-AGCC-CACGGCTCAACCGTGGA
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      421       431       441       451       461       471
tri.3 ACTGCCTTT-GATACTGGAAGTCT-TGAGTATGGTAGAGGTGAGTGG-AATTCCGAGTGT
tri.4 ACTGCCTTT-GATACTGGAAGTCT-TGAGTATGGTAGAGGTGAGTGG-AATTCCGAGTGT
tri.2 ACTGCCTTT-GATACTGGAAGTCT-TGAGTATGGTAGAGGTGAGTGG-AATTCCGAGTGT
tri.1 ACTGCCTTT-GATACTGGAAGTCT-TGAGTATGGTAGAGGTGAGTGG-AATTCCGAGTGT
ant.2 ACTGCCTTT-GATACTGGAAGTCT-TGAGTATGGTAGAGGTGAGTGG-AATTCCGAGTGT
tri.5 ACTGCCTTT-GATACTGGAAGTCT-TGAGTATGGTAGAGGTGAGTGG-AATTCCGAGTGT
int.1 ACTGCCTTT-GATACTGTTAGTCT-TGAGTATGGAAGAGGTGAGTGG-AATTCCGAGTGT
int.4 ACTGCCTTT-GATACTGTTAGTCT-TGAGTATGGAAGAGGTGAGTGG-AATTCCGAGTGT
int.2 ACTGCCTTT-GATACTGTTAGTCT-TGAGTATGGTAGAGGTGAGTGG-AATTCCGAGTGT
int.3 ACTGCCTTT-GATACTGTTAGTCT-TGAGTATGGTAGAGGTGAGTGG-AATTCCGAGTGT
ant.4 ACTGCCTTT-GATACTGGAAGTCT-TGAGTATGGTAGAGGTGAGTGG-AATTCCGAGTGT
gri.1 ACTGCCTTT-GATACTGGAAGTCT-TGAGTATGGTAGAGGTGAGTGG-AATTCCGAGTGT
rhi.1 ACTGCCTTT-GATACTGGAAGTCT-TGAGTATGGTAGAGGTGAGTGG-AATTCCGAGTGT
ant.1 ACTGCCTTT-GATACTGGAAGTCT-TGAGTATGGTAGAGGTGAGTGG-AATTCCGAGTGT
gri.2 ACTGCCTTT-GATACTGGAAGTCTCTGAGTATGGAAGAGGTGAGTGGGAATTCCGAGTGT
gri.4 ACTGCCTTTTGATACTGGAAGTCT-TGAGTATGGAAGAGGTGAGTGG-AATTCCGAGTGT
gri.5 ACTGCCTTT-GATACTGGAAGTCT-TGAGTATGGAAGAGGTGAGTGG-AATTCCGAGTGT
gri.3 ACTGCCTTT-GATACTGGAAGTCT-TGAGTATGGAAGAGGTGAGTGG-AATTCCGAGTGT
IB    ACTGCCTTT-GATACTGGAAGTCT-TGAGTATGGTAGAGGTGAGTGG-AATTCCGAGTGT
B.c   GG-GTCATTGGAAACTGGGAGACT-TGAGTGCAGAAGAGGAAAGTGG-AATTCCATGTGT
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      481       491       501       511       521       531
tri.3 AGAGGTGAAATT-CGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGACC
tri.4 AGAGGTGAAATT-CGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGACC
tri.2 AGAGGTGAAATT-CGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGACC
tri.1 AGAGGTGAAATT-CGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGACC
ant.2 AGAGGTGAAATT-CGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGACC
tri.5 AGAGGTGAAATT-CGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGACC
int.1 AGAGGTGAAATT-CGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGTCC
int.4 AGAGGTGAAATT-CGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGTCC
int.2 AGAGGTGAAATT-CGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGACC
int.3 AGAGGTGAAATT-CGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGACC
ant.4 AGAGGTGAAATT-CGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGACC
gri.1 AGAGGTGAAATT-CGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGACC
rhi.1 AGAGGTGAAATT-CGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGACC
ant.1 AGAGGTGAAATT-CGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGACC
gri.2 AGAGGTGAAATT-CGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGTCC
gri.4 AGAGGTGAAATTTCGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGTCC
gri.5 AGAGGTGAAATT-CGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGTCC
gri.3 AGAGGTGAAATT-CGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGTCC
IB    AGAGGTGAAATT-CGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGACC
B.c   AGCGGTGAAATG-CGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCT
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      541       551       561       571       581       591
tri.3 ATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGG-ATTAGATACCCTGGTAGT
tri.4 ATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGG-ATTAGATACCCTGGTAGT
tri.2 ATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGG-ATTAGATACCCTGGTAGT
tri.1 ATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGG-ATTAGATACCCTGGTAGT
ant.2 ATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGG-ATTAGATACCCTGGTAGT
tri.5 ATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGG-ATTAGATACCCTGGTAGT
int.1 ATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGG-ATTAGATACCCTGGTAGT
int.4 ATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGG-ATTAGATACCCTGGTAGT
int.2 ATTACTGACGCTGAGGTGCGAAAGCGTGCGGAGCAAACAGG-ATTAGATACCCTGGTAGT
int.3 ATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGG-ATTAGATACCCTGGTAGT
ant.4 ATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGG-ATTAGATACCCTGGTAGT
gri.1 ATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGG-ATTAGATACCCTGGTAGT
rhi.1 ATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGG-ATTAGATACCCTGGTAGT
ant.1 ATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGG-ATTAGATACCCTGGTAGT
gri.2 ATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGGATTAGATACCCTGGTAGT
gri.4 ATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGG-ATTAGATACCCTGGTAGT
gri.5 ATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGG-ATTAGATACCCTGGTAGT
gri.3 ATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGG-ATTAGATACCCTGGTAGT
IB    ATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGG-ATTAGATACCTTGGTAGT
B.c   GTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGG-ATTAGATACCCTGGTAGT
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      601       611       621       631       641       651
tri.3 CCACGCCGTAAACGATGAATGTTA-GCCGTTG-GGGAGTTTACTCTTCGGTGGCGCAGCT
tri.4 CCACGCCGTAAACGATGAATGTTA-GCCGTTG-GGGAGTTTACTCTTCGGTGGCGCAGCT
tri.2 CCACGCCGTAAACGATGAATGTTA-GCCGTTG-GGGAGTTTACTCTTCGGTGGCGCAGCT
tri.1 CCACGCCGTAAACGATGAATGTTA-GCCGTTG-GGGAGTTTACTCTTCGGTGGCGCAGCT
ant.2 CCACGCCGTAAACGATGAATGTTA-GCCGTTG-GGGAGTTTACTCTTCGGTGGCGCAGCT
tri.5 CCACGCCGTAAACGATGAATGTTA-GCCGTTG-GGGAGTTTACTCTTCGGTGGCGCAGCT
int.1 CCACGCCGTAAACGATGAATGTTA-GCCGTCG-GGGTGTTTACACTTCGGTGGCGCAGCT
int.4 CCACGCCGTAAACGATGAATGTTA-GCCGTCG-GGGTGTTTACACTTCGGTGGCGCAGCT
int.2 CCACGCCGTAAACGATGAATGTTA-GCCGTTG-GGGAGTTTACTCTTCGGTGGCGCAGCT
int.3 CCACGCCGTAAACGATGAATGTTA-GCCGTTG-GGGAGTTTACTCTTCGGTGGCGCAGCT
ant.4 CCACGCCGTAAACGATGAATGTTA-GCCGTTG-GGGAGTTTACTCTTCGGTGGCGCAGCT
gri.1 CCACGCCGTAAACGATGAATGTTA-GCCGTCG-GGGAGTTTACACTTCGGTGGCGCAGCT
rhi.1 CCACGCCGTAAACGATGAATGTTA-GCCGTCG-GGGGGTTTACCTTTCGGTGGCGCAGCT
ant.1 CCACGCCGTAAACGATGAATGTTA-GCCGTTG-GGGAGTTTACTCTTCGGTGGCGCAGCT
gri.2 CCACGCCGTAAACGATGAATGTTA-GCCGTCG-GGGTGTTTACACTTCGGTGGCGCAGCT
gri.4 CCACGCCGTAAACGATGAATGTTA-GCCGTCG-GGGTGTTTACACTTCGGTGGCGCAGCT
gri.5 CCACGCCGTAAACGATGAATGTTATGCCGTCG-GGGTGTTTACACTTCGGTGGCGCAGCT
gri.3 CCACGCCGTAAACGATGAATGTTA-GCCGTCG-GGGTGTTTACACTTCGGTGGCGCAGTT
IB    CCACGCCGTAAACGATGAATGTTA-GCCGTCG-GGGAGTTTACTCTTCGGTGGCGCAGCT
B.c   CCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTT
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      661
tri.3 AACGCAT
tri.4 AACGCAT
tri.2 AACGCAT
tri.1 AACGCAT
ant.2 AACGCAT
tri.5 AACGCAT
int.1 AACGCAT
int.4 AACGCAT
int.2 AACGCAT
int.3 AACGCAT
ant.4 AACGCAT
gri.1 AACGCAT
rhi.1 AACGCAT
ant.1 AACGCAT
gri.2 AACGCAT
gri.4 AACGCAT
gri.5 AACGCAT
gri.3 AACGCAT
IB    AACGCAT
B.c   AACGCAT
```

Figure 5.2 Sequence alignments of 16S rDNA sequences of the bacterium and strains of *Ochrobactrum* and *Bacillus* species. Sequences were edited by deletion of 3' beginnings and 5' ends of sequences using BioManager in ANGIS (http://www.angis.org.au). Sequences are labelled as: int: *O. intermedium*, ant: *O. anthropi*, rhi: *O. rhizosphaerae*, tri: *O. tritici*, gri: *O. grignonense*, B. c: *Bacillus cereus* and IB: isolated bacterium from infected *A. triquetrum* bulb.

Table 5.6 Distance matrix of 16S r-DNA sequences of the bacterium and *Ochrobactrum* species with the outgroup species *Bacillus cereus*. Standard error calculation was based on bootstrap values (%) from 1000 bootstrap replicates.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
tri.3	**0.0**																			
tri.4	**0.0**	**0.0**																		
tri.2	**0.0**	**0.0**	**0.0**																	
tri.1	**0.0**	**0.0**	**0.0**	**0.0**																
ant.2	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**															
tri.5	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**														
int.1	**0.0183**	**0.0183**	**0.0183**	**0.0183**	**0.0183**	**0.0183**	**0.0**													
int.4	**0.0183**	**0.0183**	**0.0183**	**0.0183**	**0.0183**	**0.0183**	**0.0**	**0.0**												
int.2	**0.0783**	**0.0149**	**0.0149**	**0.0149**	**0.0149**	**0.0149**	**0.0132**	**0.0132**	**0.0**											
int.3	**0.0149**	**0.0132**	**0.0132**	**0.0132**	**0.0132**	**0.0132**	**0.0116**	**0.0116**	**0.0016**	**0.0**										
ant.4	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0183**	**0.0183**	**0.0149**	**0.0132**	**0.0**									
gri.1	**0.0165**	**0.0165**	**0.0165**	**0.0165**	**0.0165**	**0.0165**	**0.0217**	**0.0217**	**0.025**	**0.0233**	**0.0165**	**0.0**								
rhi.1	**0.0082**	**0.0082**	**0.0082**	**0.0082**	**0.0082**	**0.0082**	**0.02**	**0.02**	**0.0233**	**0.0216**	**0.0082**	**0.0199**	**0.0**							
ant.1	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0183**	**0.089**	**0.0149**	**0.0132**	**0.0**	**0.0165**	**0.0082**	**0.0**						
gri.2	**0.0283**	**0.0283**	**0.0283**	**0.0283**	**0.0283**	**0.0283**	**0.0233**	**0.0233**	**0.0336**	**0.0318**	**0.0283**	**0.0182**	**0.03**	**0.0283**	**0.0**					
gri.4	**0.03**	**0.03**	**0.03**	**0.03**	**0.03**	**0.03**	**0.0249**	**0.0249**	**0.0353**	**0.0336**	**0.03**	**0.0199**	**0.0317**	**0.03**	**0.0033**	**0.0**				
gri.5	**0.0318**	**0.0183**	**0.0318**	**0.0318**	**0.0318**	**0.0318**	**0.0267**	**0.0267**	**0.0371**	**0.0353**	**0.0318**	**0.0216**	**0.0335**	**0.0318**	**0.0049**	**0.0016**	**0.0**			
gri.3	**0.0438**	**0.0438**	**0.0438**	**0.0438**	**0.0438**	**0.0438**	**0.0386**	**0.0386**	**0.0493**	**0.0475**	**0.0438**	**0.0335**	**0.0455**	**0.0438**	**0.0165**	**0.0132**	**0.0132**	**0.0**		
IB	**0.0502**	**0.0502**	**0.0502**	**0.0502**	**0.0502**	**0.0502**	**0.0676**	**0.0676**	**0.0636**	**0.0616**	**0.0502**	**0.0613**	**0.0558**	**0.0502**	**0.0671**	**0.0709**	**0.0729**	**0.0864**	**0.0**	
B.c	**0.02106**	**0.2106**	**0.2106**	**0.2106**	**0.2106**	**0.2106**	**0.2133**	**0.2133**	**0.2251**	**0.2226**	**0.2106**	**0.2171**	**0.2084**	**0.2106**	**0.2186**	**0.2202**	**0.2188**	**0.2298**	**0.2533**	**0.0**
	tri.3	**tri.4**	**tri.2**	**tri.1**	**ant.2**	**tri.5**	**int.1**	**int.4**	**int.2**	**int.3**	**ant.4**	**gri.1**	**rhi.1**	**ant.1**	**gri.2**	**gri.4**	**gri.5**	**gri.3**	**IB**	**B.c**

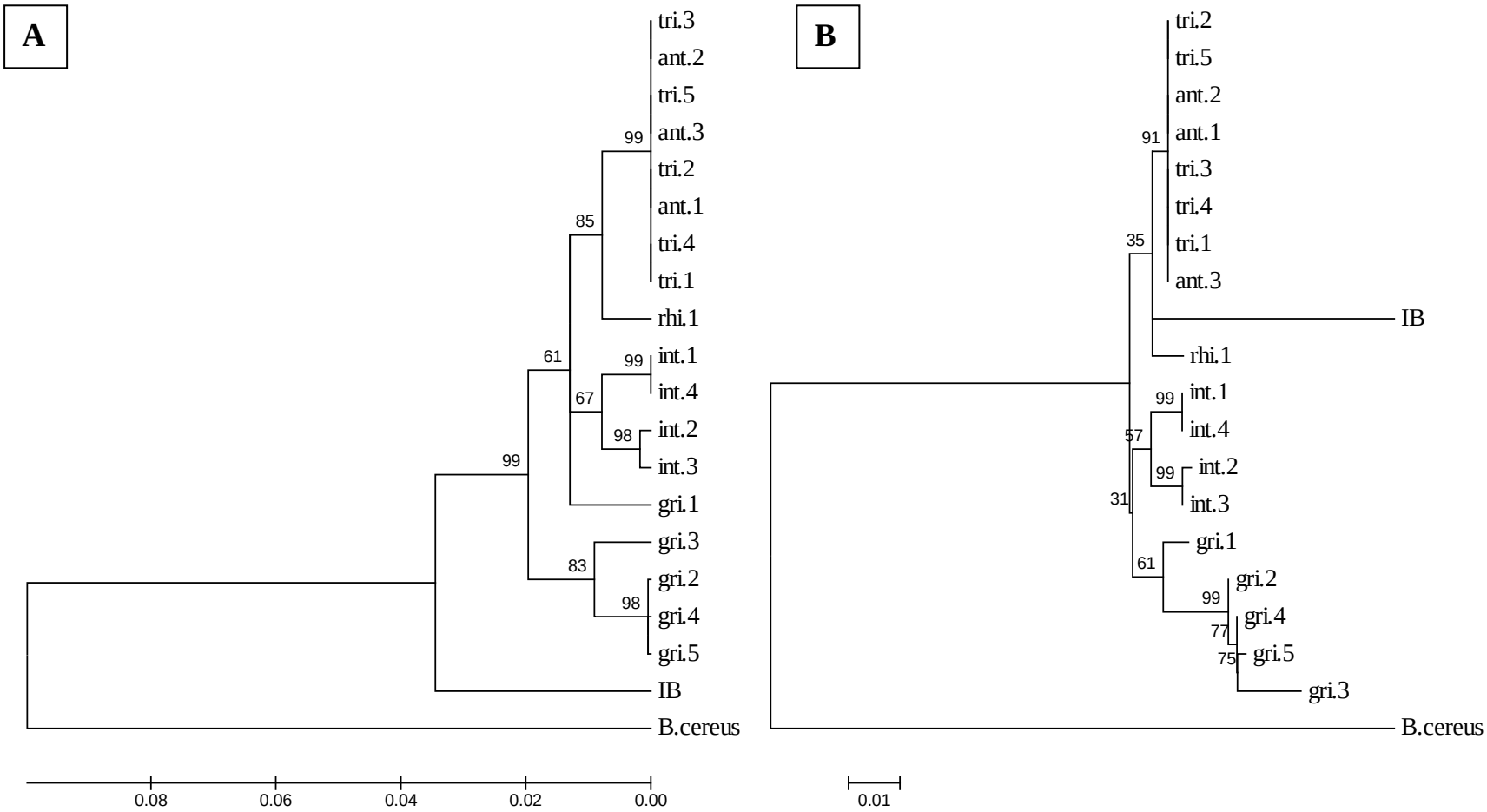

Figure 5.3 Bootstrap consensus phylogenetic tree obtained from UPGMA (A) and Neighbour-Joining (B) analysis for nucleotide sequences of the isolated bacterium, *Ochrobactrum* species isolates and ***Bacillus cereus.*** Numbers on clades demonstrate bootstrap value (%) from 1000 bootstrap replicates in Mega4. Sequences are labelled as: int: ***O. intermedium,*** ant: ***O. anthropi***, rhi: ***O. rhizosphaerae,*** tri: ***O. tritici***, gri: ***O. grignonense,*** B. c: ***Bacillus cereus*** and IB: isolated bacterium from infected ***A. triquetrum*** bulb.

5.3.2 RFLP analysis

The 16S r-DNA PCR products of the bacterium and five *Ochrobactrum* species were digested by only seven restriction enzymes (Fig. 5.4). All bacteria showed identical digested products with *Xho*I and *EcoR*I. Polymorphism was observed between *Ochrobactrum* species digested by *Hin*F1, *Bsu*I, *RSa*I, *Hha*I and *EcoR*I. Identical banding patterns were observed in the bacterium and *O. rhizosphaerae* using all restriction enzymes except *Taq*I. In *Taq* I the isolated bacterium showed a band at 550 bp that was not present in any of the *Ochrobactrum* species; however; a sum of the digested bands suggested that the band at 550 bp was a product of partial digestion. The isolated bacterium did not produce a pattern identical to any one *Ochrobactrum* species with all endonucleases but produced a pattern that resembled at least one species with *Hinf*1, *Nhe*1 and *Rsa*1. The RFLP dendrogram (single linkage rescaled distance cluster combine) generated based on proximity matrix using SPSS Hierarchical Cluster analysis (Fig. 5.5) showed two main clades where *O. intermedium* was clustered separately from the others. *O. grignonense* and *O. rhizosphaerae* indicated identical RFLP profiles, as did *O. tritici* and *O. anthropi*. The closest results (7%) to the isolated bacterium were *O. rhizosphaerae* and *O. tritici*.

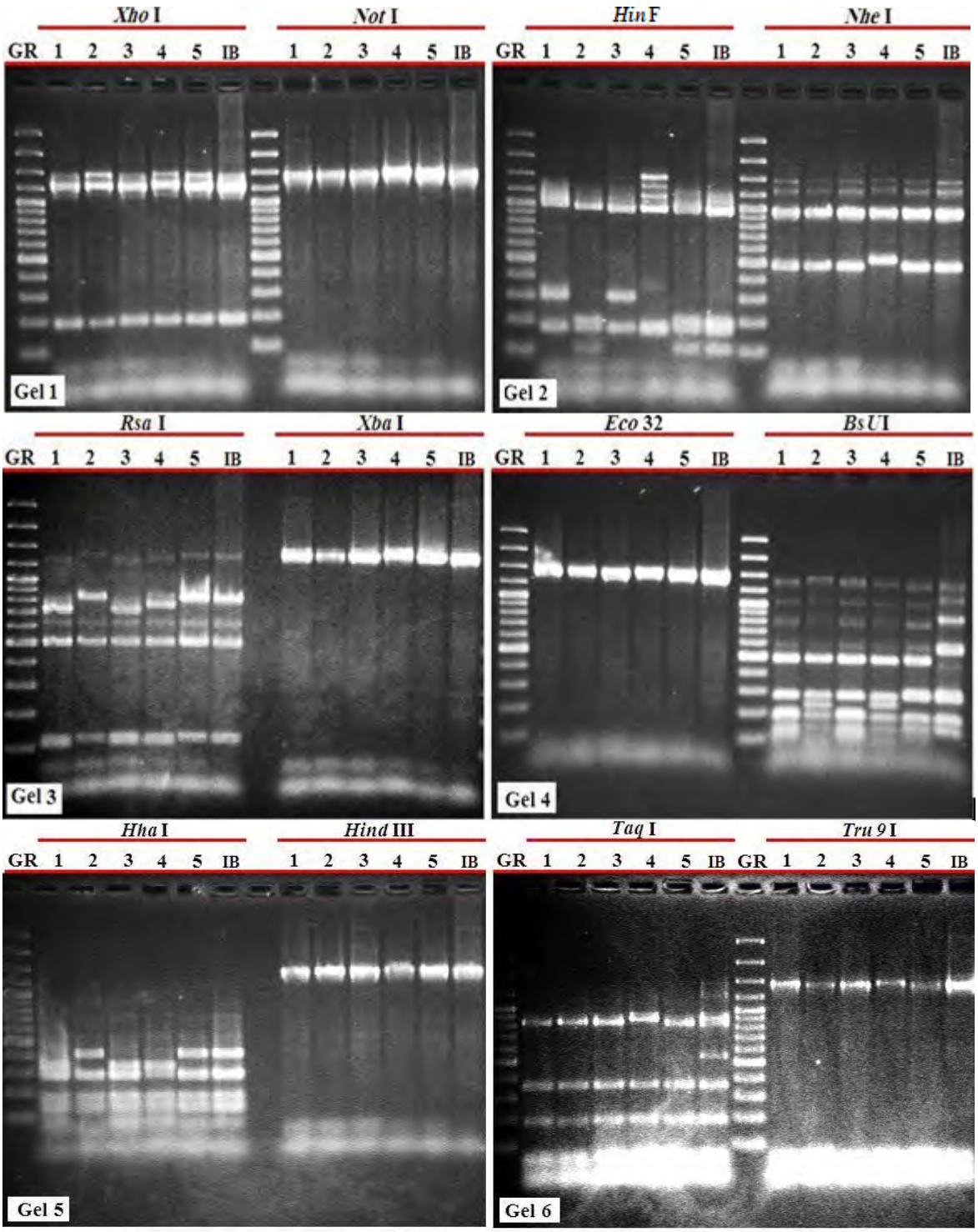

Figure 5.4 Restriction digestions of 16S r-DNA PCR products of the bacterium and *Ochrobactrum* species. Restriction enzyme used are: Gel1: *Xho*I and *Not*I, Gel2: *Hin*F I and *Nhe*I, Gel3: *Rsa*I and *Xba*I, Gel 4 *Eco*32 and *BsU* I, Gel 5: *Hha*I and *Hind*III, Gel 6: *Taq*I and *Tru9*I. Lanes: 1: *O. anthropi*, 2: *O. grignonense*, 3: *O. tritici*, 4: *O. intermedium*, 5: *O. rhizosphaerae*, IB: the bacterium and GR: GeneRuler^{TM} 100 bp.

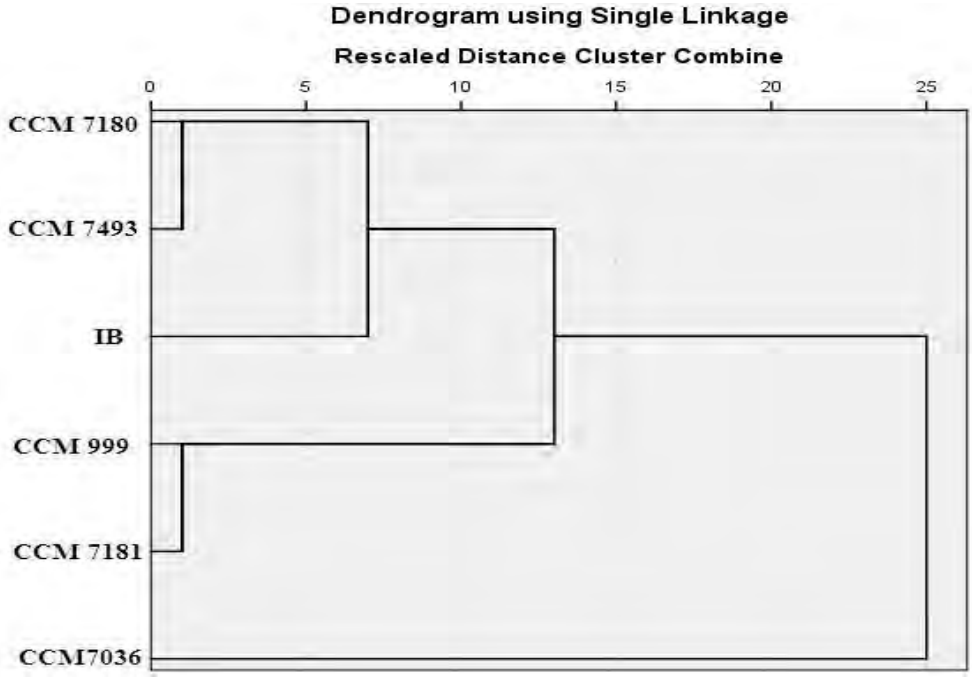

Figure 5.5 RFLP dendrogram of the bacterium and *Ochrobactrum* species generated by SPSS Hierarchical Cluster analysis. CCM 7180: *Ochrobactrum grignonense,* CCM 7493: *Ochrobactrum rhizosphaerae*, CCM 999: *Ochrobactrum anthropi*, CCM 7181: *Ochrobactrum tritici*, CCM 7036: *Ochrobactrum intermedium*, IB: the bacterium.

5.3.3 RAPD analysis

Of the 60 RAPD primers from Operon kits OPA, OPB and OPM, only 18 primers amplified consistent patterns for the bacterium and *Ochrobactrum* species (Fig. 5.6). Polymorphism was observed between *Ochrobactrum* species and the bacterium. The isolated bacterium had unique profiles with OPA-02, OPA-04, OPA-09, OPA-11, OPB-01, OPB-05, OPB-06, OPB-07, OPB-11, OPM-01 and OPM-10. OPA-05, OPA-11, OPM-03, OPM-04, OPM-07, OPM-12 did not amplify any products for the isolated bacterium but polymorphism was observed among *Ochrobactrum* species.

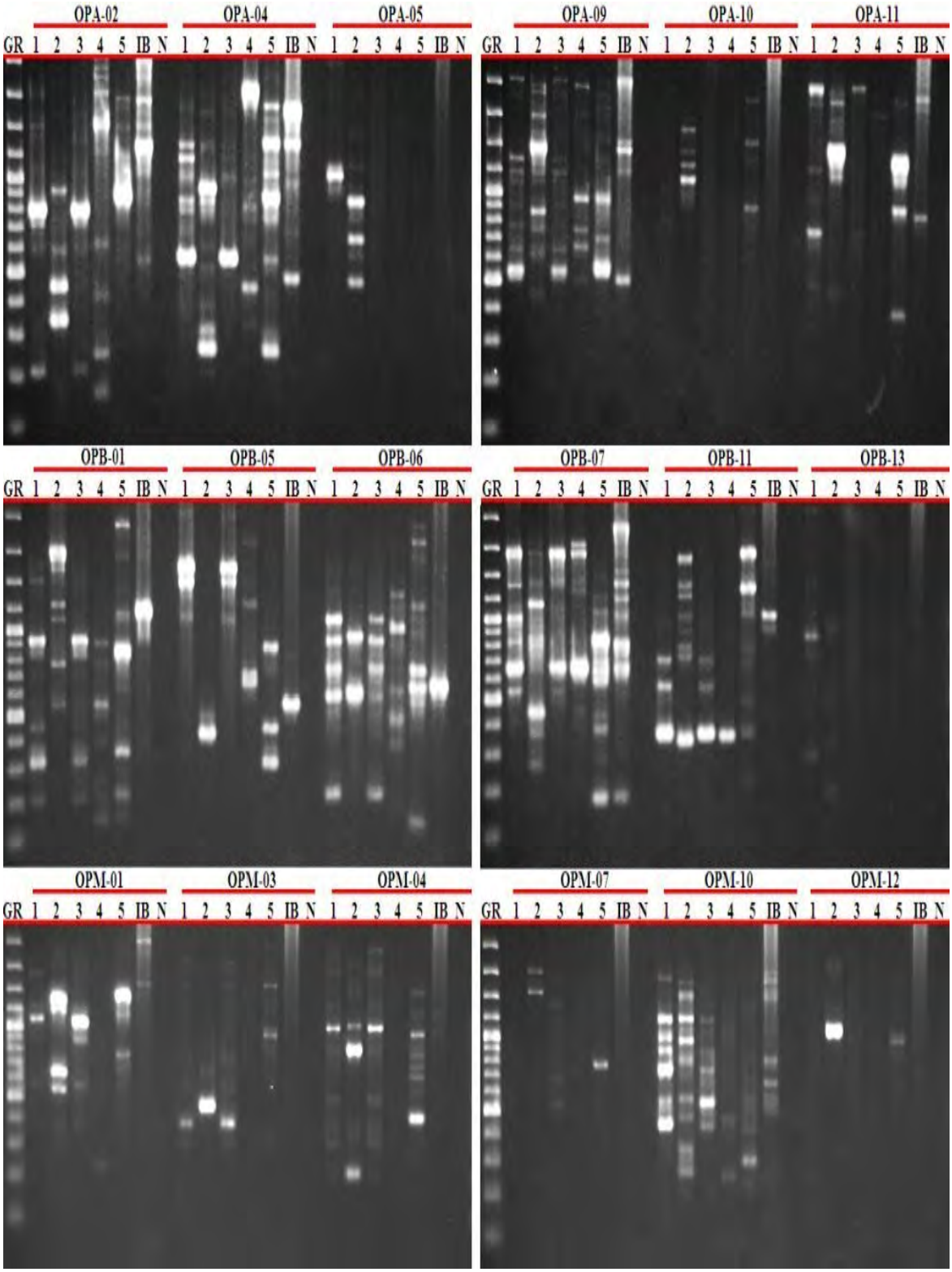

Figure 5.6 RAPD-PCR products and polymorphism of the bacterium and *Ochrobactrum* species using 18 RAPD primers. Lanes: 1: *O. anthropi*, 2: *O. grignonense*, 3: *O. tritici*, 4: *O. intermedium*, 5: *O. rhizosphaerae*, IB: the bacterium, N: negative control and GR: GeneRuler^{TM} 100 bp.

5.3.4 Physiological characterization

Based on physiological tests performed by API 20 NE, the bacterium was similar to the five *Ochrobactrum* species (Table 5.3) used in this study. The bacterium was oxidase positive and could assimilate glucose, arabinose, mannose, N-acetyl-glucosamine, malate and trisodium citrate (Table 5.7). The closest physiological profile to the bacterium was *O. rhizosphaerae*. The only difference observed between *O. rhizosphaerae* and the bacterium was on assimilation of maltose that was negative for the bacterium.

Table 5.7 Physiological and biochemical characterisation of the bacterium and five known *Ochrobactrum* species using API 20 NE. Bacteria are labelled as: IB: isolated bacterium from infected *A. triquetrum* bulb, 7180: *O. grignonense*, 7493: *O. rhizosphaerae*, 7181: *O. tritici*, 999: *O. anthropi*, 7036: *O. intermedium*.

		Physiological characteristics					
No.	**Reactions/enzymes**	**IB**	**7180**	**7493**	**7181**	**999**	**7036**
1	Reduction of nitrates to nitrites	-	+	-	-	-	-
2	Reduction of nitrates to nitrogen	-	+	-	+	+	+
3	Indole production	-	-	-	-	-	-
4	Fermentation (Glucose)	-	-	-	-	-	-
5	Arginine dihydrolase	-	-	-	-	-	-
6	Urease	-	-	-	+	+	-
7	Hydrolysis (ß-glucosidase) (Esclin)	-	-	-	-	-	-
8	Hydrolysis (protease) (Gelatine)	-	-	-	-	-	-
9	ß-galactopyranoside (Para-Nitrophenyl-ßD-galactopyranosidase)	-	-	-	-	-	-
10	Assimilation (Glucose)	+	+	+	+	+	+
11	Assimilation (Arabinose)	+	+	+	+	+	+
12	Assimilation (Mannose)	+	+	+	+	+	+
13	Assimilation (Mannitol)	-	-	-	-	-	-
14	Assimilation (N-acetyl-glucosamine)	+	+	+	+	+	+
15	Assimilation (Maltose)	-	-	+	+	+	+
16	Assimilation (Potassium gluconate)	-	-	-	+	-	-
17	Assimilation (Capric acid)	-	+	-	-	+	+
18	Assimilation (Adipic acid)	-	-	-	-	-	-
19	Assimilation (Malate)	+	+	+	+	+	-
20	Assimilation (Trisodium citrate)	+	+	+	+	+	+
21	Assimilation (Phenylacetic acid)	-	-	-	-	-	-
24	Cytochrome oxidase	+	+	+	+	+	+

5.3.5 Pathogenicity testing of the bacterium on test-tube-grown A. triquetrum

Various pathogenicity levels of the bacterium were observed on test-tube-grown *A. triquetrum* provenances from across Australia (Fig. 5.7). The first disease symptoms were observed 30 days after inoculation at 25ºC as discoloration and soft rot of the older leaves. A pale yellow colour in water agar and bacterial clumps around the plant bases and roots were observed 15 days post-inoculation. Infected plants were discoloured and died after 2 months incubation (Fig. 5.8). Slight decay in the collar region was observed almost in all samples but un-inoculated plants remained healthy and green. Infected plants were scored between stages 2 for Gardiner's Creek to stage 6 for Bendigo Creek. Death was recorded in inoculated plants from Waterfall Gully and Mylor in South Australia and Ararat, Dandenongs, Wonthaggi and Bendigo Creek in Victoria. Only the original bacterium was re-isolated from the infected plants on NA plates. Statistical analysis based on a randomized complete block design indicated significant differences in pathogenicity of the isolated bacterium on different provenances such as Gardiner's Creek (VIC) and Waterfall Gully (SA) (Table 5.8).

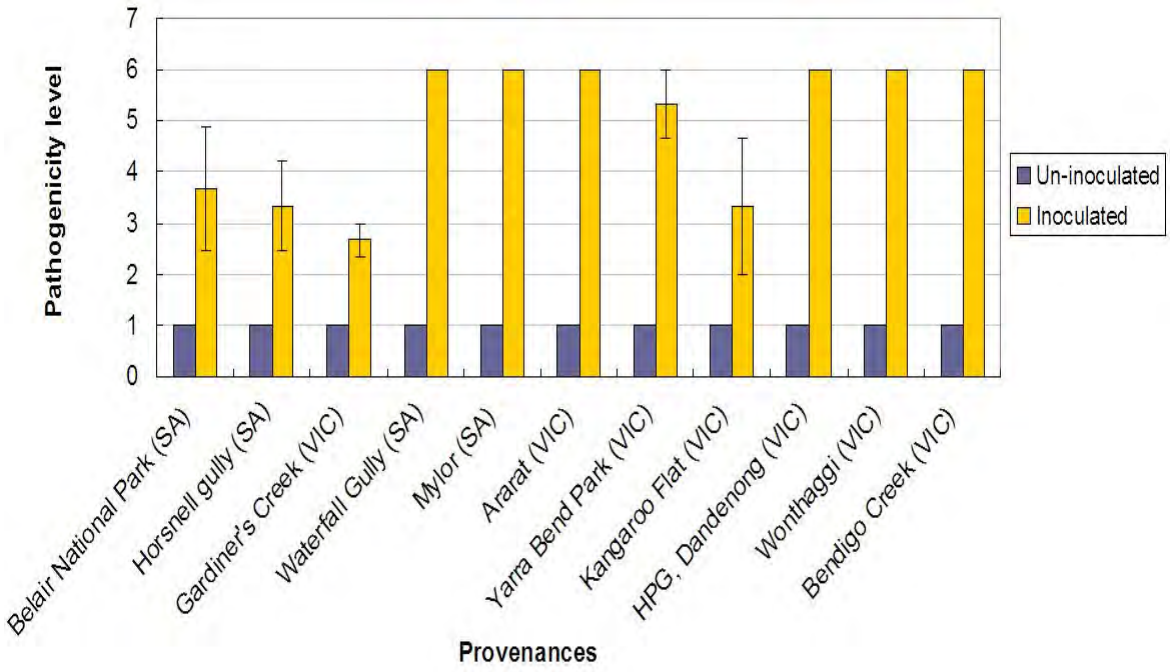

Figure 5.7 Pathogenicity of the isolated bacterium on the test-tube-grown *Allium triquetrum* provenances from Victoria and South Australia. HPG: Hardy's Picnic Ground (Bars=2 x standard error).

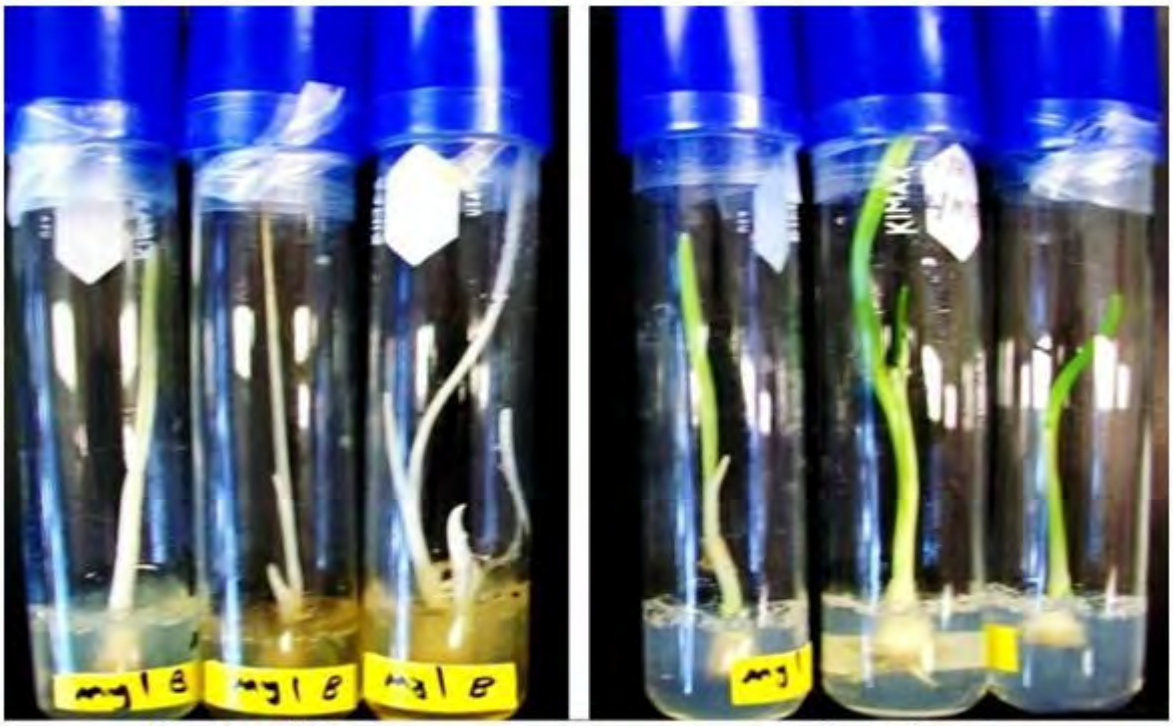

Figure 5.8 Pathogenicity of the isolated bacterium on the test-tube-grown *Allium triquetrum* from Mylor, South Australia at 25ºC, 2 months post-inoculation.

Table 5.8 Analysis of pathogenicity of the isolated bacterium on *Allium triquetrum* provenances from across Australia in a randomized complete block design using SPSS software.

Tests of Between-Subjects Effects

Dependent Variable: Pathogenicity

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	141.091[a]	12	11.758	5.215	.001
Intercept	981.818	1	981.818	435.484	.000
Replicates	16.909	2	8.455	3.750	.041
Provenances	124.182	10	12.418	5.508	.001
Error	45.091	20	2.255		
Total	1168.000	33			
Corrected Total	186.182	32			

a. R Squared = .758 (Adjusted R Squared = .613)

5.3.6 Histology of the infected A. triquetrum bulbs caused by the isolated bacterium

As the soft rot disease caused by the isolated bacterium was not severe and only discoloration of the leaves occurred, there was no difficulty in processing the infected plant tissues. Only a few bacterial cells were observed in infected cortical parenchyma tissues collected 30 days post-inoculation, though cells were disrupted (Fig. 5.9). There was no bacterial infection observed in the control treatments and cells remained intact. Disrupted cells in cortical parenchyma had much of the cell walls visible. There was no disruption or bacteria observed in epidermal cells and vascular tissues.

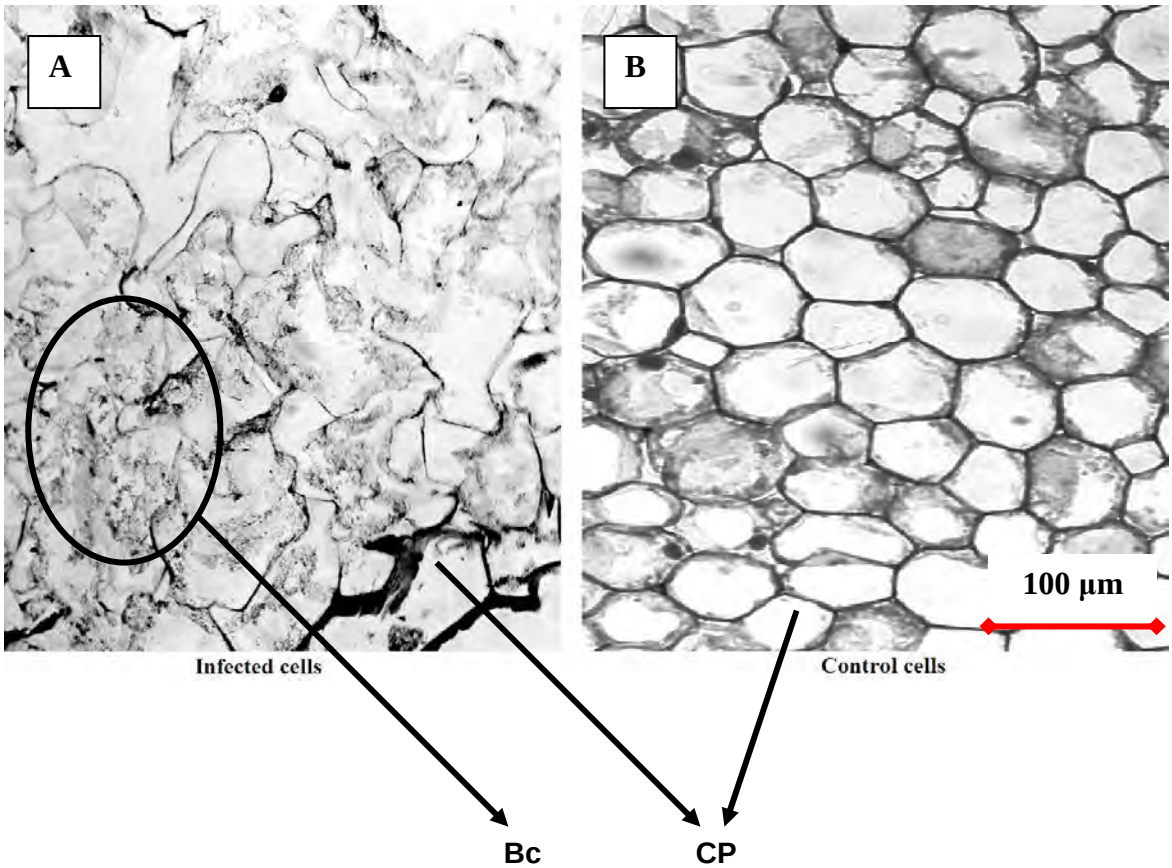

Figure 5.9 Transverse section of the *Allium triquetrum* cortical parenchyma in the collar region 30 days after inoculation. A: inoculated treatment, B: control treatment, CP: cortical parenchyma, Bc: bacteria.

5.3.7 Interaction of the isolated bacterium and S. cepivora

In test-tube trials, both the bacterium and the fungus were separately pathogenic and virulent on *A. triquetrum* provenances; infected plants died. However; the bacterium inhibited the the sclerotia from growing when tested together, though host plants still died (Fig. 5.10). Sclerotia retrieved from the tubes and surface-sterilized germinated readily and so were still viable. The control plants without any treatments remained healthy. Statistical analysis based on a split plot design showed differences with isolates (inocula) but no difference between adding bacteria alone or with the fungus (Table 5.9). No significant difference was observed in virulence of the fungus or bacterium among provenances.

In the second experiment, sclerotia and mycelium of *S. cepivora* isolates (DPI, VPRI 12439a) did not grow on the medium pre-treated with the bacterium, but did grow and produce sclerotia on control plates after 3 weeks. However; mycelial growth of the other fungal species with the isolated bacterium was recorded (Fig. 5.11), though their growth varied. For instance, *Penicillum* sp. and *Fusarium* sp. grew more on the pre-treated V8 agar, but *Aspergillus* sp. and *Alternaria* sp. grew less (Fig. 5.12). All fungi grew on the control plates without bacteria and almost covered the medium surface after 2 weeks incubation. Statistical analysis of the fungal growth data performed in completely randomised block design indicated a significant difference between *S. cepivora* and the other fungi used in this experiment (Table 5.10).

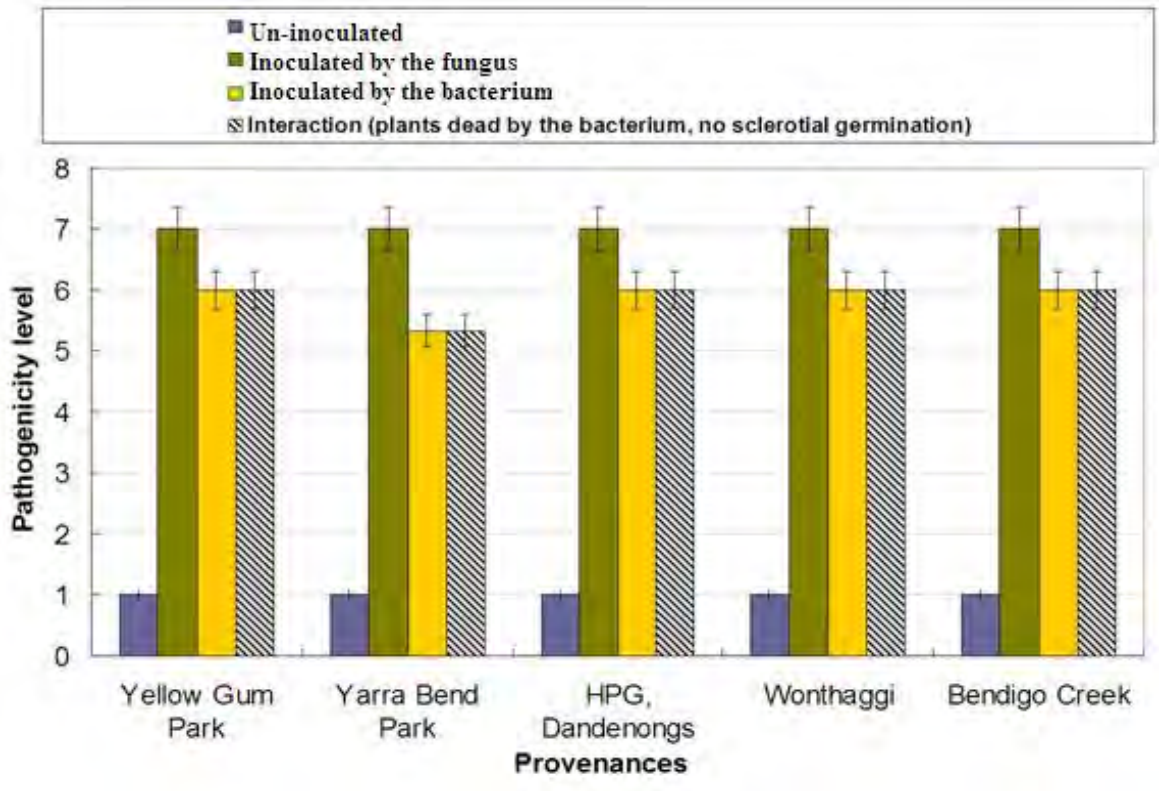

Figure 5.10 Interaction of the isolated bacterium and *Stromatinia cepivora* on test-tube-grown *Allium triquetrum* provenances from Victoria. HPG: HPG: Hardy's Picnic Ground (Bars=2 x standard error).

Table 5.9 Analysis of interaction of the isolated bacterium and *Stromatinia cepivora* on test-tube-grown *Alium triquetrum* provenances in a split plot design using SPSS software.

Tests of Between-Subjects Effects

Dependent Variable: pathogenicity

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	173.333[a]	13	13.333	16.000	.000
Intercept	333.333	1	333.333	400.000	.000
Replicates	1.667	2	.833	1.000	.390
Isolates	163.333	1	163.333	196.000	.000
Replicates * Isolates	1.667	2	.833	1.000	.390
Provenances	3.333	4	.833	1.000	.436
Isolates * Provenances	3.333	4	.833	1.000	.436
Error	13.333	16	.833		
Total	520.000	30			
Corrected Total	186.667	29			

a. R Squared = .929 (Adjusted R Squared = .871)

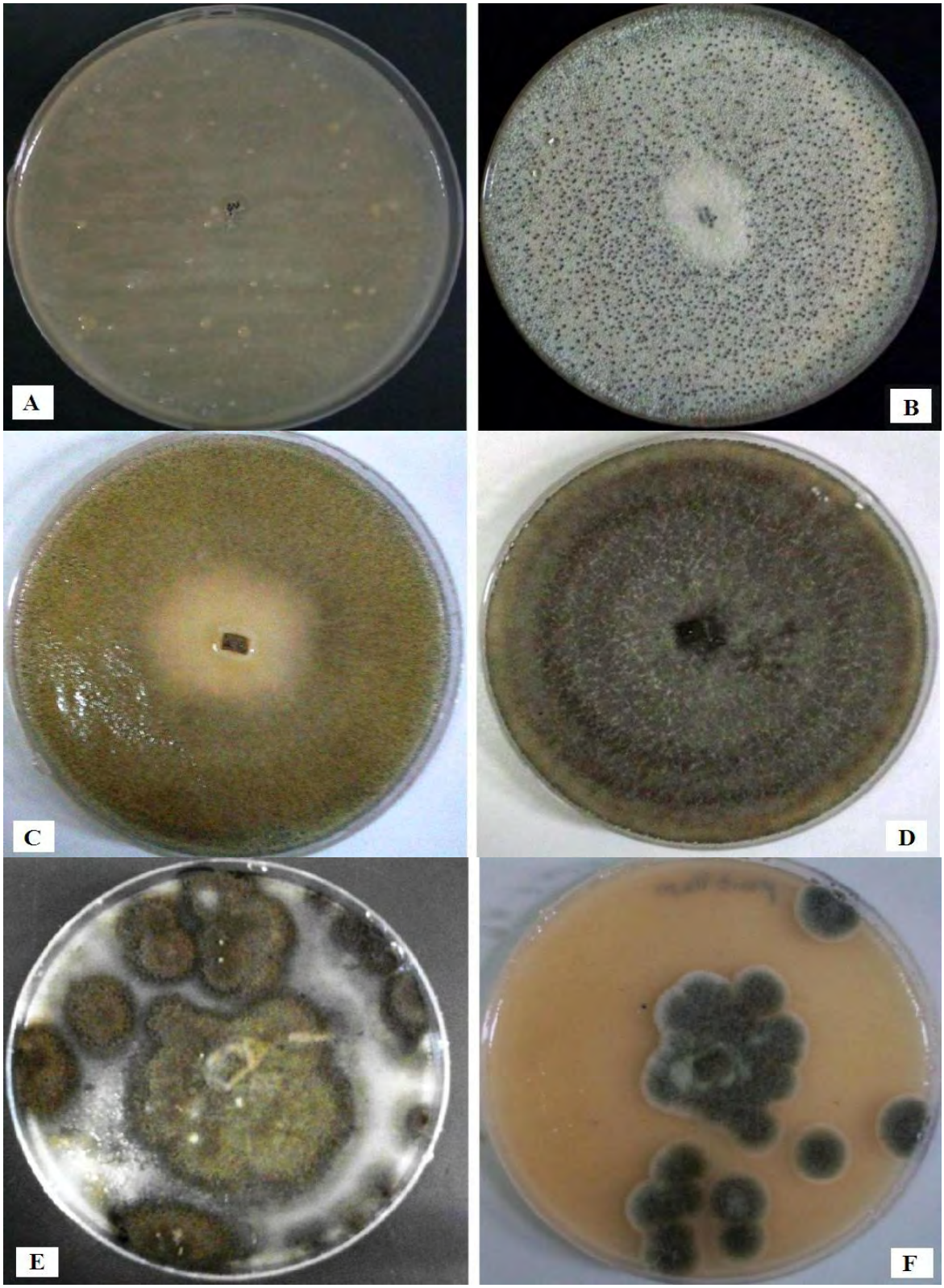

Figure 5.11 Interaction of the isolated bacterium on fungal growth using pre-treated V8 agar. A-B: *Stromatinia cepivora* on pre-treated (A) and control plate (B), C-D: *Alternaria* sp. on pre-treated (C) and control plate (D), E-D: *Penicillium* sp. on pre-treated (E) and control plate (F).

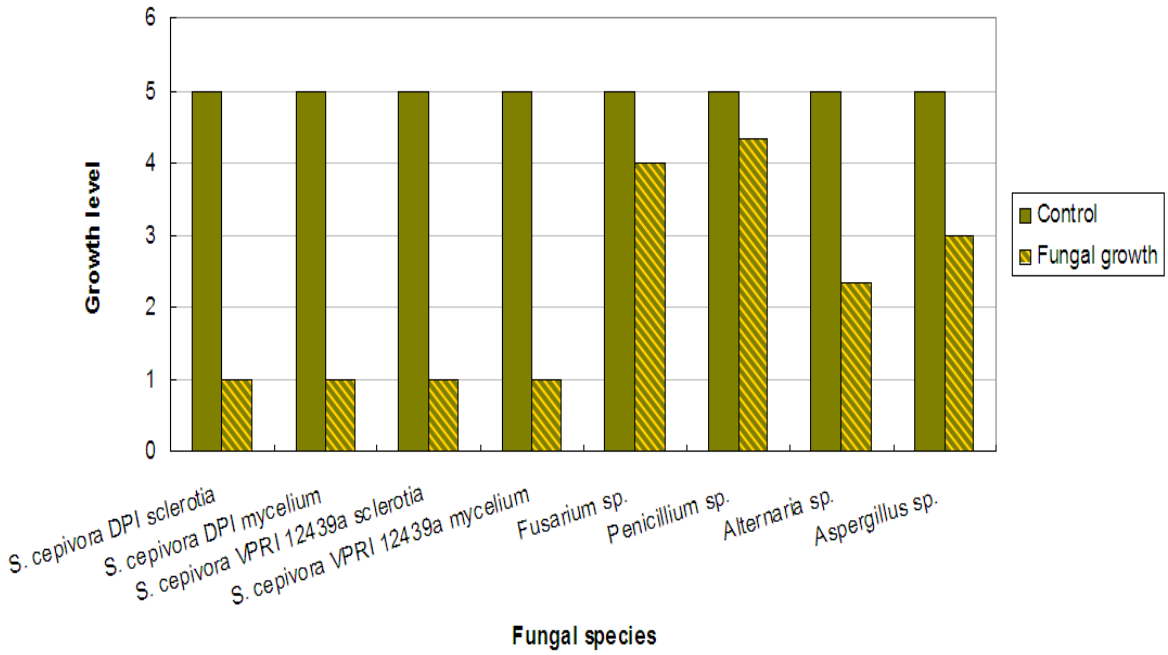

Figure 5.12 Sclerotia and mycelial growth of *S. cepivora* and mycelial growth of other fungi on pre-treated and control V8 agar with the isolated bacterium.

Table 5.10 Analysis of sclerotila germination and mycelial growth of *Stromatinia cepivora* isolates and mycelial growth of other fungal species on pre-treated vs control V8 agar in completely randomized block design using SPSS software.

Tests of Between-Subjects Effects

Dependent Variable: Growth

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	42.958[a]	9	4.773	66.824	.000
Intercept	117.042	1	117.042	1638.583	.000
Replicates	.333	2	.167	2.333	.133
Fungi	42.625	7	6.089	85.250	.000
Error	1.000	14	.071		
Total	161.000	24			
Corrected Total	43.958	23			

a. R Squared = .977 (Adjusted R Squared = .963)

5.3.8 Pathogenicity of the isolated bacterium on the test-tube-grown cultivated Allium seedlings

Seedling of all cultivated *Allium* species were infected by the isolated bacterium (Fig. 5.13). Disease symptoms were noticed after 15 days as discolouration of the leaf tips followed by chlorosis, necrosis and death of the seedlings 27 days post-inoculation (Fig. 5.14). Similar results were achieved in a parallel experiment on micropropagated garlic (Fig. 5.15), in which disease symptoms were observed with only 10^4 cells. The control plants remained healthy. On plating the infected plants, only one bacterium was re-isolated on NA plates and was identified as the original inoculum using API 20NE. There was no significant difference in pathogenicity of the isolated bacterium among cultivated *Allium* species but a significant difference was observed between treatments and controls.

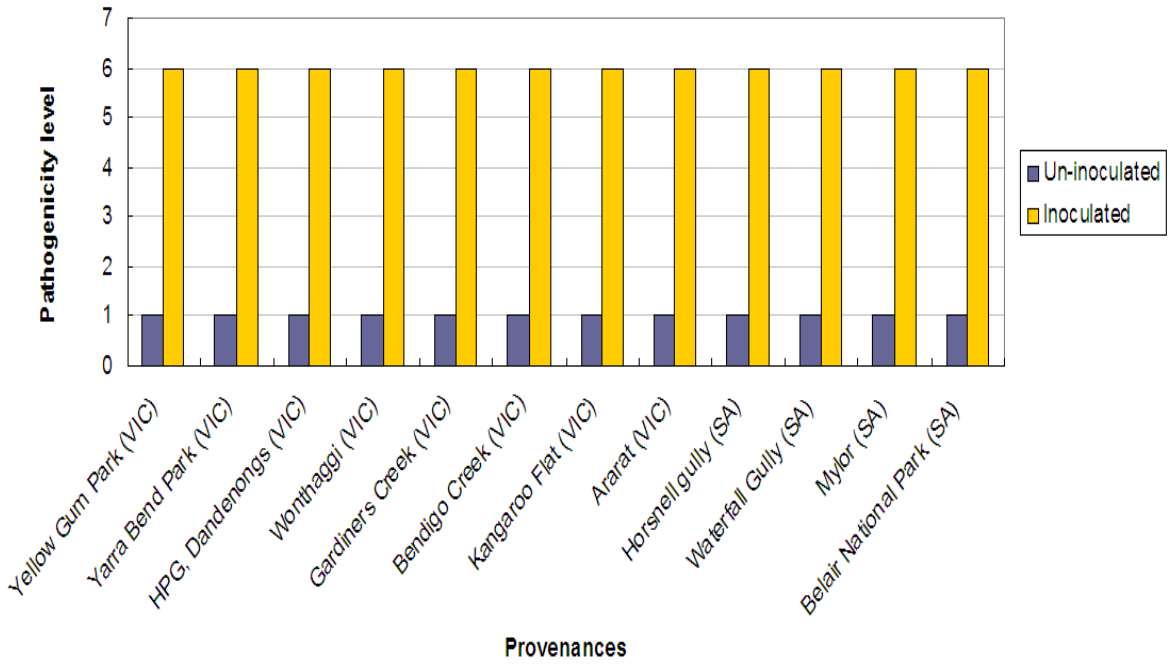

Figure 5.13 Pathogenicity of the isolated bacterium on the test-tube-grown cultivated *Allium* species at 25ºC, 2 months post-inoculation.

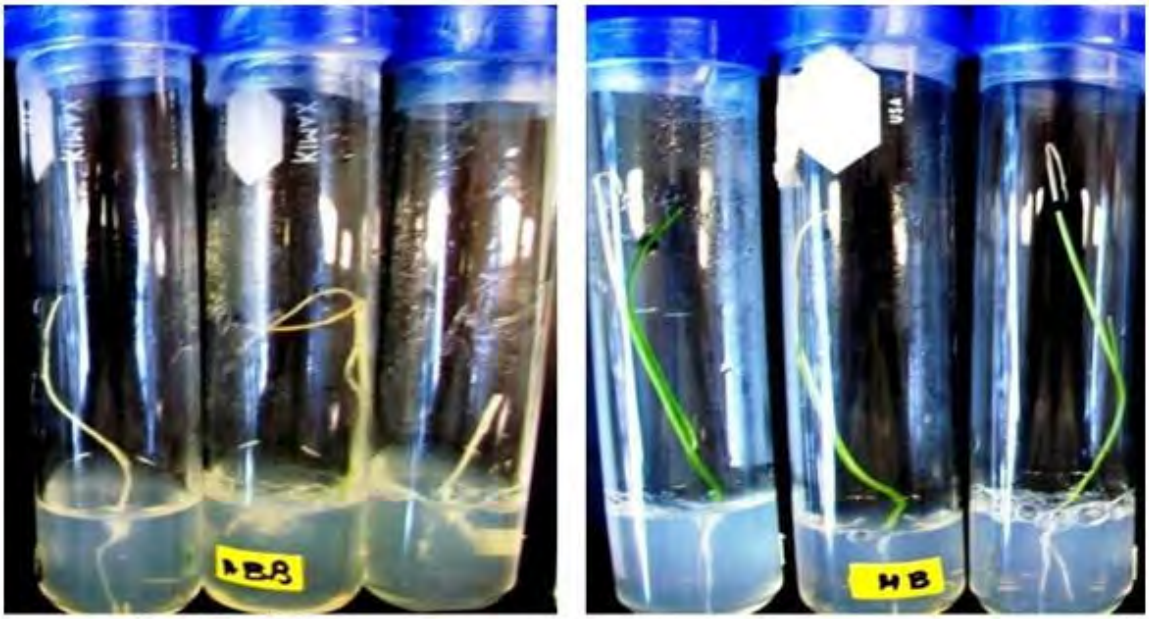

Figure 5.14 Pathogenicity of the isolated bacterium on test-tube-grown Hunter Brown Onion (*Allium cepa*) seedlings at 25ºC, 2 months post-inoculation.

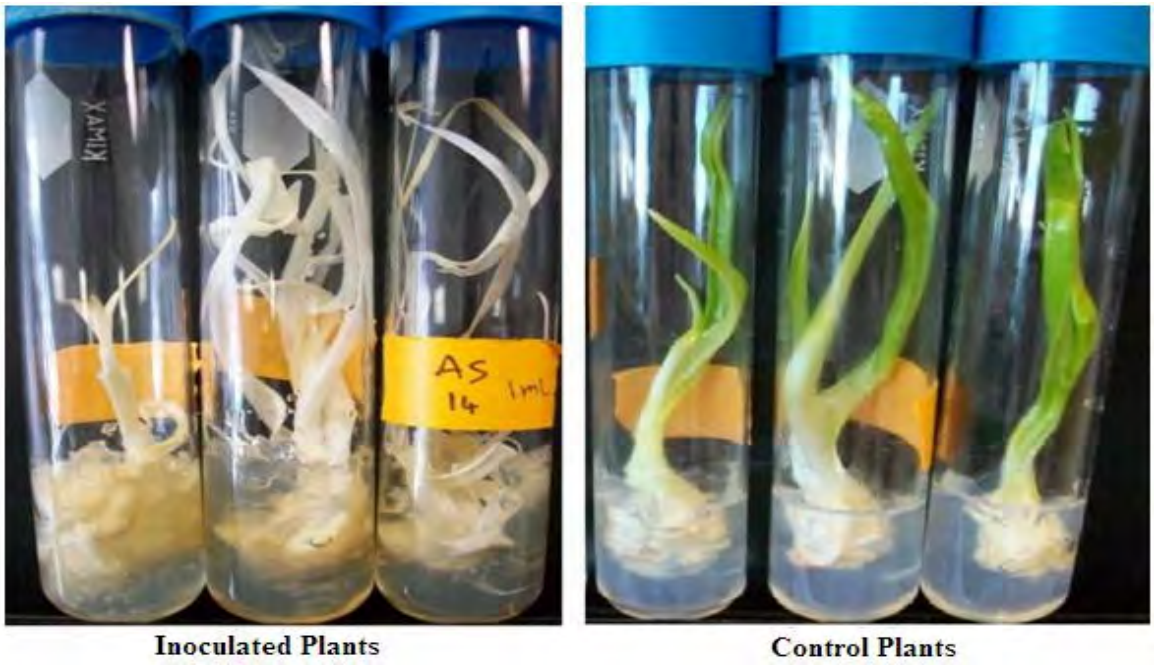

Figure 5.15 Pathogenicity of the isolated bacterium on test-tube-grown garlic (*Allium sativum*) at 25 ºC, 2 months post-inoculation.

5.3.9 Pathogenicity of the isolated bacterium on the potted A. triquetrum, cultivated Allium species and Australian native plants in the glasshouse

No disease symptoms were noticed on *A. triquetrum* (Fig. 4.16), cultivated *Allium* species (Fig. 5.17) and native monocots (Fig. 5.18) in the glasshouse almost 4 months after inoculation. There was no significant difference in growth between treated and control plants. The pathogenicity level of the bacterium was scored as stage one (no infection) for all inoculated plants. Root systems of the *A. triquetrum* plants and cultivated *Allium* species were healthy and no infection was observed. Only Gram-negative, oxidase-positive rods were sub-cultured on NA and identified using an API 20NE kit and DNA sequencing. All bacteria were identified as species of *Pseudomonas* and *Xanthomonas*, which are commonly associated with plant roots (Schippers *et al.* 1987; Latour *et al.* 1996).

Figure 5.16 Appearance of *A. triquetrum* plants from Ararat provenance, Victoria, 3 months post-inoculation with the isolated bacterium in the glasshouse.

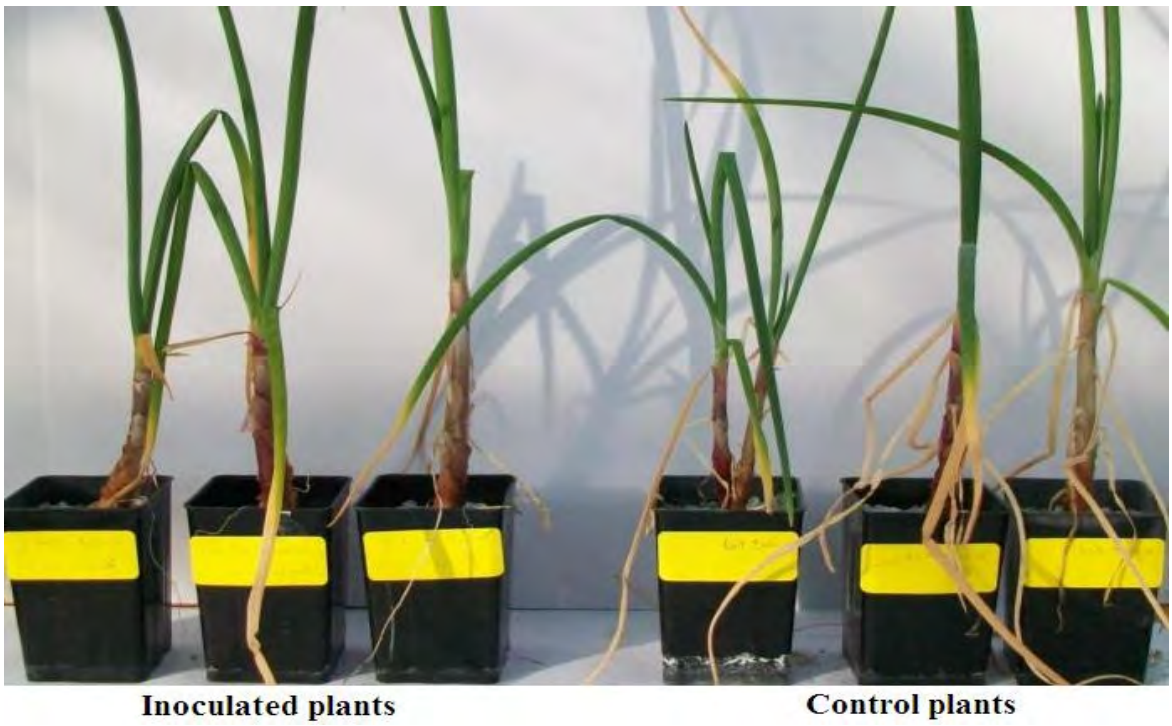

Figure 5.17 Appearance of Sweet Red Onion (*Allium cepa*) 3 months post-inoculation with the isolated bacterium in the glasshouse.

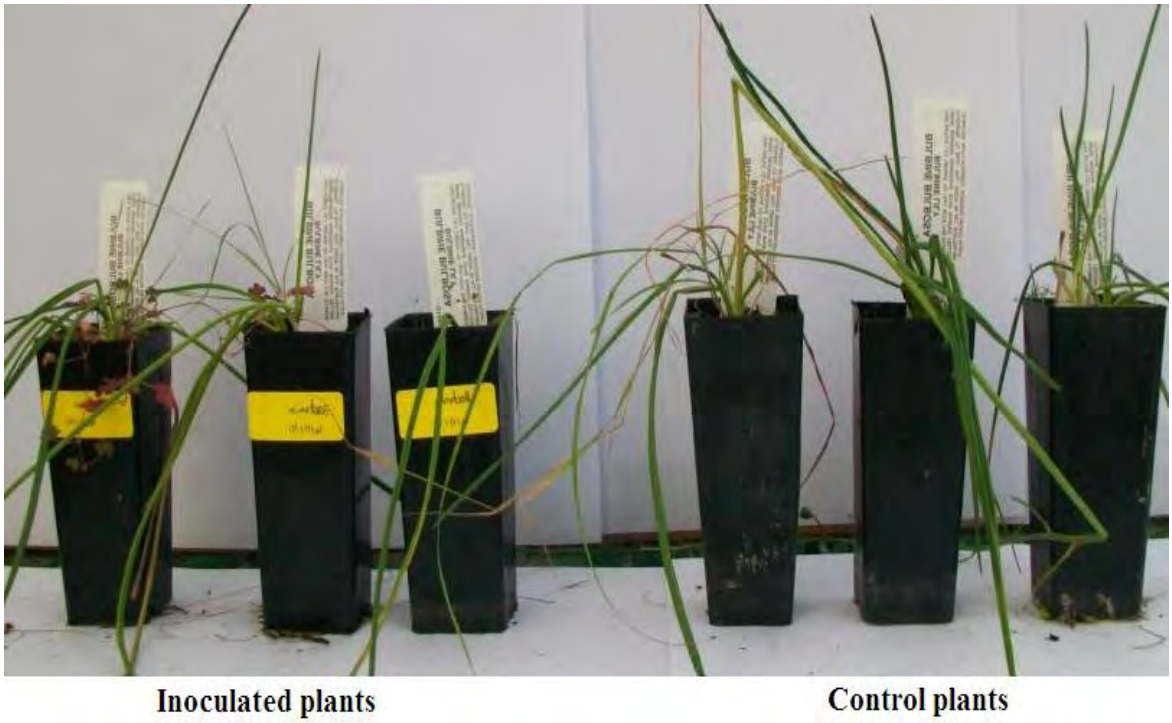

Figure 5.18 Appearance of Bulbine Lily (*Bulbine bulbosa* R. Br. Haw.) 3 months post-inoculation with the isolated bacterium in the glasshouse.

5.4 DISCUSSION

The isolated bacterium from shrunken *A. triquetrum* bulbs stored at 4°C collected from the Hardy's Picnic Ground, Dandenong provenance in Victoria was closest molecularly to species in the genus *Ochrobactrum* (96% nucleotide similarity to *Ochrobactrum* species in a Blast search). Molecular (16S rDNA sequencing and RFLP) and physiological testing (API NE) against known *Ochrobactrum* species showed that it did not match the sequences of the tested strains of known species and it is suggested that the isolated bacterium is a novel species of *Ochrobactrum*, though this would require further testing against a wider range of authentic strains of *Ochrobactrum* species. Its role in *A. triquetrum* is difficult to say, but it may be a weak pathogen or an endophyte. It was pathogenic and virulent *in vitro* but not *in vivo* in all *A. triquetrum* provenances and cultivated *Allium* species tested. The disease symptoms *in vitro* were chlorosis, necrosis and death but the lack of any of these in potted plants and the failure to re-isolate the bacterium from the inoculated plants suggests that it is not a promising biological control agent.

5.4.1 Identification

Analysis of the bacterium 16S r-DNA sequence suggested that the closest match for the isolated bacterium is in the genus *Ochrobactrum*. Sequence alignment results indicated minor nucleotide differences between the five known *Ochrobactrum* species compared with those between them and the isolated bacterium. Sequence alignment in Neighbour-Joining analysis suggested that the isolated bacterium was close to *O. rhizosphaerae* and *O. tritici* but its position was much less clear with UPGMA analysis. Phylogenetic analysis of the 16S r-RNA along with physiological and biochemical tests have been promising tools for establishing new species in general and in the genus *Ochrobactrum* in particular. For instance, *O. lupini* (Trujillo *et al.* 2005), *O. oryzae* (Tripathi *et al.* 2006)

and *O. cytisi* (Zurdo-Piñeiro *et al.* 2007) were established as new species of this genus on the basis of nucleotide variation in 16S r-RNA gene sequences and physiological tests.

Restriction digestion of the 16S r-DNA PCR products and the cluster analysis also suggested *O. rhizosphaerae* and *O. tritici* as the closest neighbours to the isolated bacterium, but the fingerprints produced were unique to the bacterium and reflected small variation in the sequence alignment. RFLP analysis is used commonly in genotype fingerprinting, phylogenetic studies and disease analysis (Cooly 1992; Tashiro *et al.* 1995; Krupa 1999; Arifin *et al.* 2000) and proved sufficient to differentiate among the strains of the known *Ochrobactrum* species. The large differences in RAPD-PCR banding patterns suggested that the isolated bacterium was not close to any known *Ochrobactrum* species.

Physiological and biochemical tests suggested, like the RFLP results and sequence alignment, that the isolated bacterium was closest to *O. rhizosphaerae* and the only difference was failure of the bacterium to assimilate maltose. Physiological differences between bacteria in utilization of various carbon sources have been used in many studies (Velasco *et al.* 1998; Lebuhn *et al.* 2000; Kämpfer *et al.* 2007; Kämpfer *et al.* 2008) and the API 20 E and API 20 NE kits are popular diagnostic tests used in studies related to *Ochrobactrum* species (Bathe *et al.* 2006; Swings *et al.* 2006).

Taken together, these results suggest that the isolated bacterium could be a new species of the genus *Ochrobactrum*, all of which are associated with plant roots or soil. Further molecular and physiological tests with a wider range of strains of the known species of *Ochrobactrum* would be needed to validate this, but it seems clear that the isolated bacterium is unique. Percentage of cellular fatty acid compositions is another diagnostic tool used for identification and differentiation between *Ochrobactrum* species (Jarvis *et al.*

1996; Tighe *et al.* 2000; Trujillo *et al.* 2005; Zurdo-Piñeiro *et al.* 2007; Kämpfer *et al.* 2007) and may help to identify the isolated bacterium as a new species. Further analysis such as plasmid DNA screening for related genes in *Ochrobactrum* species can be helpful to well in order to categorise this as a novel bacterium.

5.4.2 In vitro pathogenicity of the isolated bacterium on test-tube-grown A. triquetrum and cultivated Allium species

The variation in pathogenicity of the isolated bacterium in test-tube-grown *A. triquetrum* suggests that if used as a biological control agent, further testing on a wider range of provenances would be required. Gardiner's Creek samples from Victoria showed the least susceptibility and plants from the Hardy's Picnic Ground, Dandenongs provenance from which it was isolated showed one of the greatest. This variation in different provenances has also been reported in studies on many other weeds (Oehrens 1977; Bruzzese and Hasan 1986; Carnegie *at al.* 1994). The bacterium also showed the same disease symptoms and even killed test-tube-grown cultivated *Allium* species. The greater susceptibility of garlic than other species was unexpected but may indicate lack of innate resistance. Garlic is a sterile plant that is propagated by vegetatively whereas all other species would be expected to vary in resistance because of their ability to reproduce by seeds. *In vitro* susceptibility, however, is not a good guide to behaviour under the more difficult conditions for a pathogen of pot and field trials.

Histological studies indicated crushed cortical parenchyma cells in inoculated but not un-inoculated treatments and few bacterial cells were observed, which was surprising by contrast with *Pectobacterium carotovorum* subsp. *carotovorum* (Chapter 4) and so did not demonstrate typical soft rot disease symptoms. As the infected plants were not severely rotted but discoloured, they may have been affected by toxins produced by the isolated

bacterium that could damage photosynthetic cells. Further analysis of effect of the isolated bacterium exudates and their analysis using high performance liquid chromatography (HPLC) might help in understanding the isolated bacterium physiology and solve how inoculation resulted in rapid leaf chlorosis.

Although potted *A. triquetrum*, cultivated *Allium* species and Australian native plants in the glasshouse were inoculated twice by the isolated bacterium, no disease symptoms were noticed after almost 4 months, suggesting that either the dose was inadequate or the bacterium was incapable of causing infection in pots. The dose of 10^8 cells was effective in *P. carotovorum* subsp. *carotovorum* in Chapter 4 under the same test conditions. Failure to re-isolate the bacterium from the inoculated plants suggested that the bacterium may have leached out during irrigation, yet this did not happen with *P. carotovorum* subsp. *carotovorum*. The isolation of *Pseudomonas* and *Xanthomonas* species, which are common bacteria associated with *Allium* roots (Schippers *et al.* 1987; Latour *et al.* 1996) suggests that microbial competition may not have allowed the bacterium to multiply and infect the host plants (Bell *et al.* 1990; Thomas and Wimpenny 1996; Bunchanan and Bagi 1999). Further research would be needed to investigate the cause, but was not pursued further in this study because the main aim was to find biological control agents for *A. triquetrum* and this seemed less promising than either *S. cepivora* or *P. carotovorum* subsp. c*arotovorum*.

5.4.3 Interaction of the isolated bacterium and Stromatinia cepivora

Bacteria may be potentially effective and suitable biocontrol agents in wet areas, where *S. cepivora* sclerotia do not germinate. Both the bacterium and *S. cepivora* were separately pathogenic and virulent and so their compatibility was tested for simultaneous application. The inhibition of sclerotial germination in test-tube experiments was unexpected.

Similarly, the inhibition of mycelial growth of *S. cepivora* and some other common soil fungi showed that the bacterial possibly exuded a specific antifungal compound. Its effect on inhibiting sclerotial germination makes it potentially useful as a biocontrol of white rot disease in cultivated *Allium* species, provided that the bacterium does not itself cause disease in the field.

As no disease was observed in the glasshouse trials, it is not possible to conclude that the bacterium has the potential to be developed as a bioherbicide for *A. triquetrum*. The test-tube-grown plants would be more susceptible due to high humidity and lack of nutrition. There is no record of pathogenicity of *Ochrobactrum* sp. on plant species but endophytic species such as *O. oryzae*, *O. lupini*, *O. rhizosphaerae* and *O. cytisi* have been reported in previous studies (Tripathi *et al.* 2006; Trujillo *et al.* 2005; Zurdo-Piñeiro *et al.* 2007; Kämpfer *et al.* 2008). As the bacterium inhibited the germination of sclerotia of *S. cepivora*, it should be evaluated as a biocontrol agent for *Allium* white rot disease. Biological control of *S. cepivora* has been shown previously using antagonistic fungal and bacterial species. For instance, Wong and Hughes (1986) showed antagonistic activity of the common soil-borne bacteria *Bacillus* spp. on *S. cepivora*. Therefore further studies on interaction of the isolated bacterium and *S. cepivor*a in glasshouse trials can help to achieve better understanding of the inhibition of the bacterium on the fungus and its potential use.

Chapter 6

General Discussion

Research on biological control of *A. triquetrum* began in mid-2008 at the School of Applied Sciences, RMIT University, in collaboration with the Department of Primary Industries, Frankston, Victoria. There was no report of biological control of this Australian noxious weed and no research was conducted on the genetic diversity of *A. triquetrum* over the Australian states. The aim of the project was initially to evaluate *S. cepivora,* a fungal pathogen causing white rot disease of cultivated *Allium* species, as a biocontrol agent to control the weed in infestations. It was not known whether the fungi would be pathogenic on this weed or if genetic differences between fungal strains or plant provenances would affect the pathogenicity and virulence. During this study two soft rotting bacteria isolated from infected *A. triquetrum* bulbs were also evaluated as biocontrol agents in the wetter areas where sclerotia of the fungus are reported as not germinating.

6.1 Major achievements in this project

This is the first report of biological control of *A. triquetrum* and it is also the first report on genetic diversity of both the plants and the strains of the pathogens in Australia. Although the initial aim of the project has been achieved, there are still many unanswered questions. The major achievements of this biological control program were:

1. Genetic diversity assessment of *Allium triquetrum* between and within provenances and geographical groupings of the weed in Australia.
2. Genetic variation assessment of *Stromatinia cepivora* strains within Australia and between Australia and other countries.

3. Pathogenicity assessment of *S. cepivora* DPI strain on almost all provenances from across Australia by comparison with strain VPRI 12439a.
4. Evaluation of pathogenicity of *Pectobacterium carotovorum* subsp. *carotovorum* on *A. triquetrum* provenances from across Australia.
5. Evaluation of pathogenicity and identification of an endophytic bacterium isolated from infected *A. triquetrum* bulbs close to *Ochrobactrum* species.

6.2 Allium triquetrum genetic diversity

A. triquetrum plants from infestations throughout Australia were tested based on taxonomically useful ITS (Hsiao *et al.* 1995; Ainouche and Bayer 1997) sequences and RFLP analysis (Yamashita and Tashiro 2001) that indicated relatively small nucleotide variations between provenances. However; RAPD-PCR amplification, which screens almost whole genome, suggested biotypes of *A. triquetrum* in Australia when plants were karyotypically uniform. The genetic diversity between and within provenances was documented, particularly in Victoria where more samples were collected. There was no significant phenotypic variation between provenances but plants in wetter areas were taller with larger bulbs, such as from Westernport Bay (VIC) and Ararat (VIC). The RAPD dendrogram indicated geographical grouping of *A. triquetrum* provenances across Australian states. It is not known if genetic differences all correspond to minor phenotypic differences but the Westernport Bay and Ararat samples were clustered together separately from other provenances. Further research on morphological traits using molecular techniques to find related genetic markers may answer this question.

As genetic diversity exists in *A. triquetrum* in Australia, it may be reflected in different responses of this weed to biocontrol agents, but the results suggest relatively small genetic diversity, making it a suitable biological control target. It is not possible to conclude that

the minor genetic differences within provenances exhibited by RAPD analysis are due to genetic variation between samples or DNA contaminated with endophytic microorganisms. This hypothesis was suggested during the course of this study when bacterial DNA was detected using specific bacterial primers from extracted DNA of inoculated plants in pathogenicity testing and only axenic central parts of the bulbs should be tested in future.

6.3 Genetic diversity of S. cepivora

This is the first report of genetic variation of *S. cepivora* in Australia using RAPD primers, though only two isolates, both from Victoria were used due to problems with state quarantine. *S. cepivora* biodiversity assessment has not been performed in Australia and only a few Australian isolates were tested by Tyson *et al.* (2001) to determine the variability of the fungus population in New Zealand. Similar ITS sequences of *S. cepivora* domestic and international strains used in this study suggested small genetic variation in this fungus. Due to the fungus biology and breeding system, no genetic diversity was expected as the fungus re-produces asexually (Kay and Stewart 1994; Harper and Stewart 2000). RAPD-PCR of the Australian strains showed polymorphism in only one out of 60 RAPD primes. The result is reliable as the DNA was extracted from the pure cultures. Mycelial incompatibility between Australian strains complemented the RAPD analysis as incompatibility reflects the genetic diversity between strains (Kohn *et al.* 1990; Liu and Milgroom 1996). Differences in pathogenicity of *S. cepivora* strains on *A. triquetrum* provenances also indicated that genetic diversity of the fungus does exist in Australia.

One of the main factors affecting biological control programs is the biodiversity of the biocontrol agent, which may be reflected in their pathogenicity and virulence (Ross *et al.* 2000; Schroer *et al.* 2008). Due to Australian quarantine, *S. cepivora* strains from other

countries could not be imported through AQIS and so DNA sequences available in GenBank were used to show that the Australian isolates clustered together apart from the rest. Increase in the number of *S. cepivora* strains analysed from Australia and genetic analysis of domestic and international strains using RAPD or ISSR techniques would provide more information about the genetic diversity of this asexually propagating fungus, but it seems relatively small, though important in pathogenicity

6.4 Pathogenicity of S. cepivora on A. triquetrum provenances

The results achieved in this study demonstrated that there was a difference in pathogenicity and virulence of *S. cepivora* isolates on test-tube-grown plants in that only the DPI fresh isolate was pathogenic to Wonthaggi provenance. These results reflect the genetic diversity of both biocontrol agent and the host plants.

There was significant difference in pathogenicity of *S. cepivora* using sclerotia and mycelium as inocula. Sclerotia did not germinate in sterile sand possibly because microbial activities are needed to convert the inhibitory *Allium* exudate to a stimulatory compound for sclerotia germination in unsterile soil (King and Coley-Smith 1969). Mycelium of *S. cepivora* was virulent on almost all *A. triquetrum* provenances and cultivated *Allium* species. There was no significant difference in pathogenicity of the fungus among cultivated species. Although the fungus infected all test-tube-grown plants from Hardy's Picnic Ground, Dandenongs *in vitro*, no white rot disease symptoms were observed on potted plants from the same provenance in the glasshouse, suggesting that the combination of innate resistance and more exacting conditions for the pathogen resulted in lack of pathogenesis. RAPD analysis also showed genetic diversity even within provenances and this may explain the lack of pathogenicity in some replicates. It would be

necessary to carry this investigation using further fungal strains particularly on different provenances from Western Australia that were not used for pathogenicity testing *in vivo*.

S. cepivora is a specific pathogen for the genus *Allium* and a few related species in the super-family *Liliaceae* (Alexopoulos and Mims1979) and seems ideal as a biological control, as there are no native Australian *Allium* species and none even in the sub-family *Alliaceae*. The *Allium* root volatile exudates salkyl-L-cysteine sulphoxides stimulate sclerotia of the fungus to germinate in soil and they cannot be stimulated by other plant species. There are no records of pathogenicity of *S. cepivora* mycelium on other sub-families in the *Liliaceae/ Amaryllidaceae*. The pathogenicity results of the fungus on common Australian native monocots, even in the Liliaceae, demonstrated no disease with either sclerotia or mycelium and all inoculated plants remained healthy 3 months post-inoculation. These native plants were selected for pathogenicity testing as they grow in *A. triquetrum* infested habitats. Further host-specificity and field trials need to be carried out for other related Australian native plants to develop the fungus as a potential mycoherbicide.

6.5 Evaluation of P. carotovorum subsp. carotovorum as a potential bioherbicide for A. triquetrum provenances from across Australia.

This is the first report of pathogenicity of *P. carotovorum* subsp. *carotovorum* on *A. triquetrum* in Australia. The soft rotting bacterium was isolated from infected *A. triquetrum* bulbs collected from Horsnell Gully, South Australia in 2010. The soft rot disease symptoms were observed after 2 months; storage of bulbs at 4ºC. The bacterium was identified on the basis of physiological tests and molecular analysis of the 16S r-DNA sequence. The bacterium had high virulence to test-tube-grown *A. triquetrum* from across Australia at 4-25 ºC. In test-tube trials conducted at 25ºC, death of the plants was observed

24 h post-inoculation and the collar regions were totally rotted. Histological studies demonstrated the bacterium as a cortical parenchymatous pathogen which could cross the vascular tissues and may use this to spread rapidly to the top leaves 12 h post-inoculation. Bacterial cells were observed clearly in the infected cortical parenchyma cells using wax embedding and microtomy.

Promising *in vitro* pathogenicity results were also observed on potted *A. triquetrum* in the glasshouse, where all plants were infected and killed by the bacterium 20 days after inoculation. The bacterium infected all provenances of *A. triquetrum*. A good indication for a biological control role is that there was no soft rot disease symptoms observed on cultivated *Allium* species or Australian native monocots 3 months post-inoculation in the glasshouse. Pathogenicity of *P. carotovorum* on *Allium* species such as bunching onion in the field has been reported in previous studies (Dye 1969; Hale *et al.* 1992; Halfeld-Vieira and Nechet 2008). However; *P. carotovorum* is better known as a storage pathogen for many crops (Wright and Triggs 2005). Further host-specificity tests would be necessary on different crops such as potato (Lund 1979) and tomato (Hibar *et al.* 2007) before it could be used as a biological control agent.

P. carotovorum and *S. cepivora* can be also complementary, as the sclerotia of the fungus do not germinate in very wet soil and the bacterium proliferates best in inundated soil, and so both drier and wetter areas with the weed are covered. Since *A. triquetrum* occurs in seasonally wet areas and can survive inundation, this should ensure control of the weed above and below the ground all year round, thus giving a good chance of effective control. Interaction tests of both pathogens inoculated simultaneously would be necessary in the glasshouse, as microbial activity in general inhibits sclerotia germination in unsterile soil (King and Coley-Smith 1969). The question arises as to why no disease symptoms were

observed in the glasshouse but they may have been observed if the bulbs had been left in the pots and failed to sprout the following year. It may also be that the overwhelming inoculum applied was similar to the technique used in inundative biological control and changed a minor to a major pathogen as it overcame the host plant's defences. It is also important to take into consideration the risk of the bacterium escaping into agricultural fields as some provenances are in riversides.

6.6 Identification of the novel endophytic bacterium isolated from A. triquetrum bulbs collected from Hardy's Picnic Ground, Dandenongs, Victoria.

An endogenous bacterium was isolated from shrunken bulbs collected from the Dandenongs. The bacterium was identified as close to *Ochrobactrum* species using physiological tests and sequencing of the 16S region of the bacterial genome. A Blast in GenBank indicated the closest match was 97% nucleotide similarity to *Ochrobactrum* species. Sequence alignments in ClustalW between known *Ochrobactrum* species and the isolated bacterium exhibited similarity of the bacterium to *O. rhizosphaerae and O. tritici*. Physiological characterisation of the bacterium, however, along with five known *Ochrobactrum* species using an API 20NE kit demonstrated that the bacterium was similar to *O. rhizosphaerae* (Kämpfer *et al.* 2008) and a similar result was achieved in RFLP analysis of the 16S r-DNA PCR products of the isolated bacterium and five known *Ochrobactrum* species, though the isolated bacterium had unique profiles in RFLP. The isolated bacterium indicated pathogenicity of test-tube-grown *A. triquetrum* provenances and cultivated *Allium* seedlings *in vitro*. The disease symptoms of yellowing, discolouring and rotten collar regions were observed in test-tube grown plants 2 months post-inoculation but bacterial cells were rarely observed in histology of infected tissues even though cortical parenchyma cells were crushed. Pathogenicity of the novel bacterium was not observed on potted plants of any *Allium* species in the glasshouse and it seems

unlikely that increase in inoculum concentration may improve biocontrol efficacy (Imaizumi *et al.* 1997; Klein and Auld 1995), as inoculation was performed twice *in vivo* and inoculated bacteria were not recovered from plants. No *Ochrobactrum* species are recorded as pathogenic on plants and only endophytic species have been identified in this genus (Tripathi *et al.* 2006; Trujillo *et al.* 2005; Zurdo-Piñeiro *et al.* 2007; Kämpfer *et al.* 2008). It seems that the novel bacterium is endogenous as it was also isolated from surface-sterilised bulbs, necessitating time-consuming micropropagation to obtain axenic *A. triquetrum* for testing (Tehranchian *et al.* 2010). Importantly, this study showed that the bacterium inhibited the *S. cepivora* sclerotial germination and mycelial growth and the combination inoculum resulted in death of test-tube-grown *A. triquetrum*, but not by the fungus. Similar results were observed in media pre-treated by the bacterium in which mycelial growth of several other fungi was inhibited as well as sclerotial germination and mycelial growth of *S. cepivora*.

As the novel bacterium is not pathogenic on cultivated *Allium* species in pot trials and inhibited *S. cepivora*, further research needs to be undertaken to assess the inhibition level of the novel bacterium *in vivo*. The bacteria prevented sclerotia germinating in tests *in vitro* and may be useful in controlling sclerotial germination and hence white rot disease in cultivated *Allium* species. Biological control of *S. cepivora* has been reported in previous studies using microorganisms such as *Bacillus* spp. (Dickinson and Coley-Smith 1970; Utkhede and Rahe 1982; Wong and Hughes 1986), *Penicillium nigricans* (Ghaffar 1969) and *Trichoderma* spp (ABD-EL-Razik *et al.* 1973; De Oliveria *et al.* 1984; Kay and Stewart 1994; Clarkson et al. 2006). This may add a new microorganism, provided that it is not pathogenic itself to cultivated *Allium* species in the field.

6.7 Further research

Managing invasive weeds consumes a large proportion of available funding each year for land managers of natural habitats. Weeds prevent the regeneration and establishment of native vegetation and alter the habitat for native fauna, especially after disturbance associated with fire, flood and road building. Some are brought in by visitors, on tyres and footwear. Several popular peri-urban parks now have a wide selection of weeds along all walking tracks and roads. These are damaging to the native flora and fauna that the parks are meant to conserve, and especially those weeds that reduce biodiversity by establishing dominant monocultures cause a great risk to the native flora and fauna. *Allium triquetrum* is such a weed in the ground layer.

Many reserves controlled by Parks Victoria list *A. triquetrum* as a weed and in each of these reserves the management plan details annual costs for its control. Control by grubbing is laborious, expensive and ultimately ineffective because some bulbs always escape and seedlings re-establish. Control by spot application of herbicide is only effective with isolated plants but not with the monocultures currently existing in reserves such as the Dandenong Ranges National Park and Kinglake National Park, where widespread application of non-selective herbicide would be ineffective because it does not prevent regeneration from bulbs. Parks authorities have no effective means of control and many have given up trying to tackle it - as an example, one of the WA specimens used in this research was sent by the Director of the Kings Park Botanic Garden in Perth from their grounds. Concerted action along with local authorities controlling roadsides and other public lands is needed to have any impact on the widespread infestation in peri-urban areas and re-invasion is common even if herbicide is applied. This expenditure each year competes with and reduces funding for conservation of threatened species such as orchids and native grasses used as food by native animals, species that this weed threatens directly

because it forms monocultures in the ground layer. The plant meets all the requirements for biological control as the only effective means of tackling the problem.

Further research would be necessary to formulate a field-tested product containing the *S. cepivora*, *P. carotovorum* subsp. *carotovorum* or both for application on *A. triquetrum* infestations. It would also be useful to produce a fact sheet to go with the product and to distribute this widely throughout local councils, shires and natural resources management offices in Victoria. The results and the fact sheet need to be distributed to similar relevant authorities in other infested states offering to send the agents for trial there if they are successful in field trials currently about to commence with Parks Victoria funding in some of the infestations reported here.

Chapter 7

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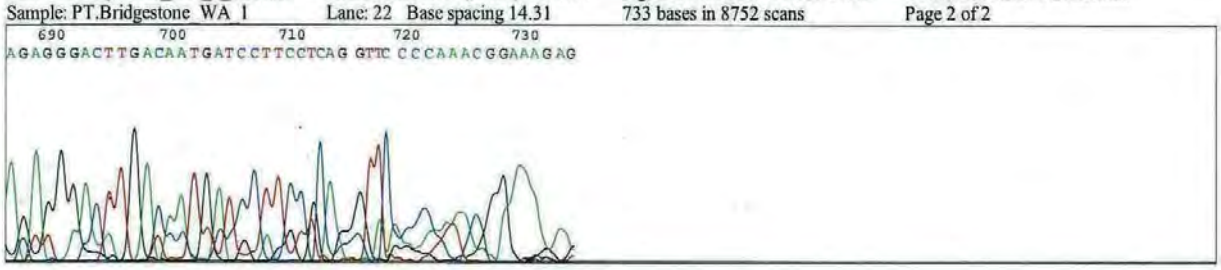
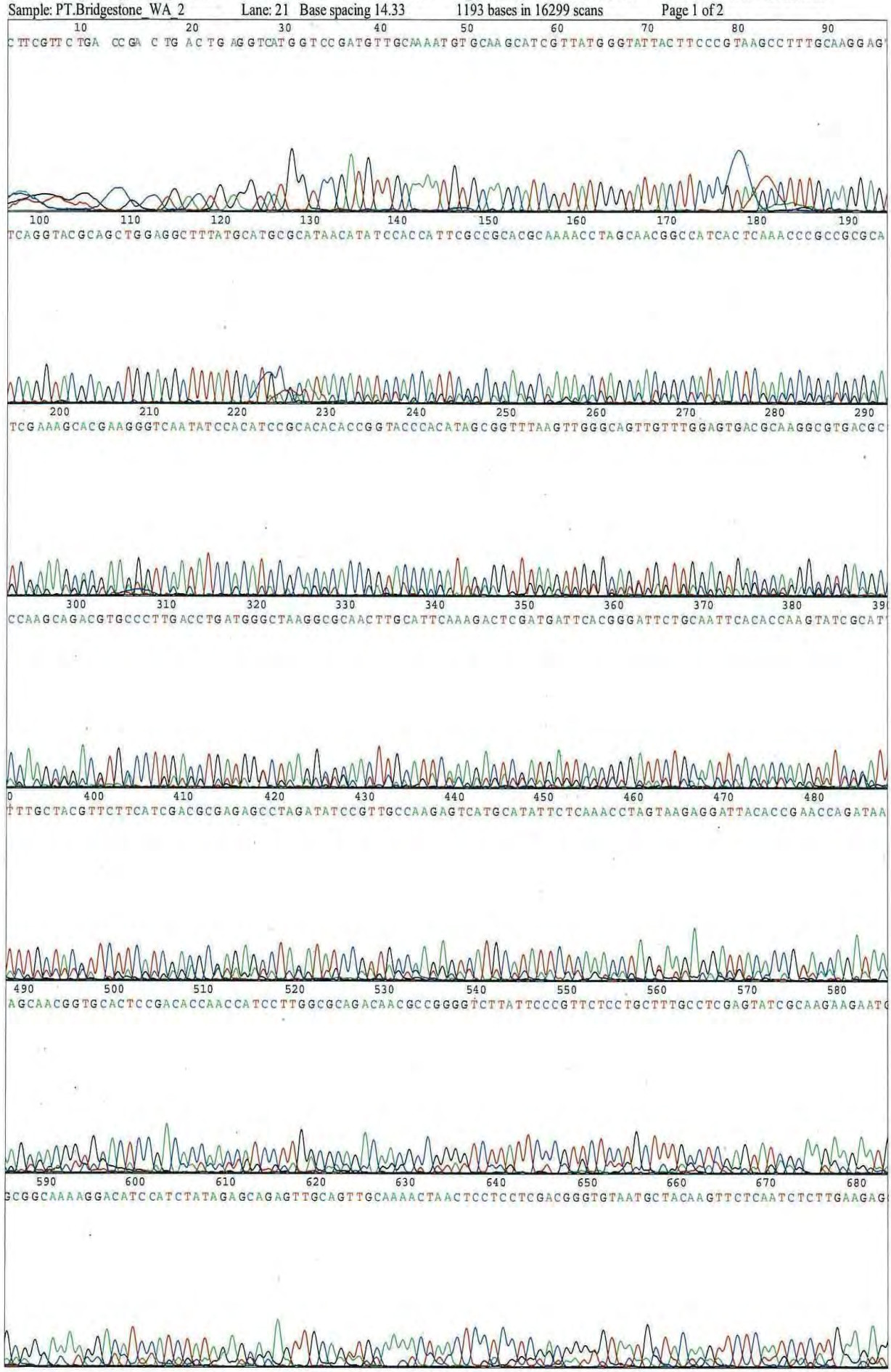
Appendix


File: PT.Bridgestone_WA_1_C05.ab1
Run Ended: Dec 9, 2010, 14:00:54
Signal G:928 A:894 T:670 C:748
Comment: Parsa Tehranchian
Sample: PT.Bridgestone_WA_1
Lane: 22 Base spacing 14.31
733 bases in 8752 scans
Page 1 of 2

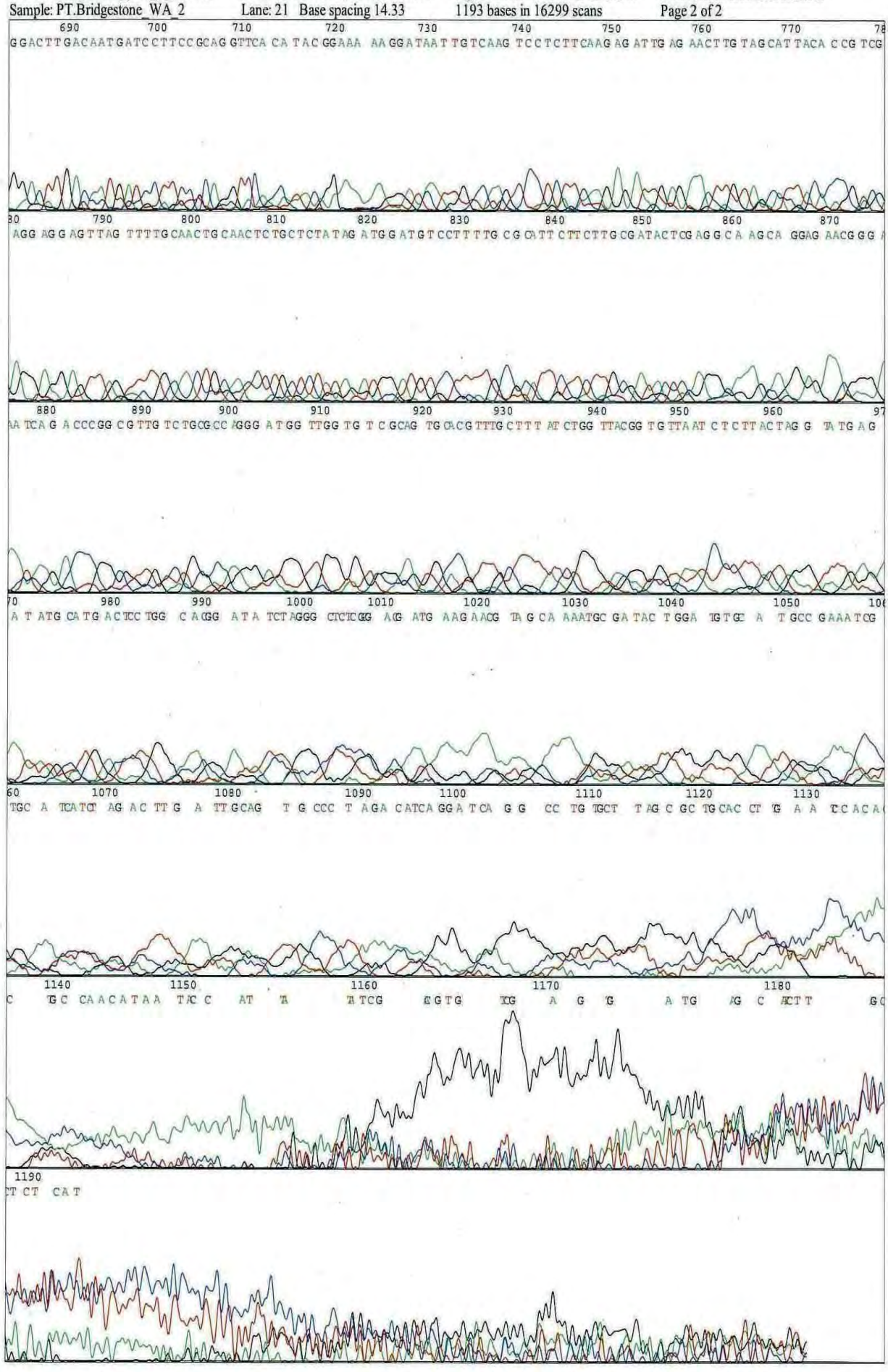
File: PT.Bridgestone_WA_1_C05.ab1 Run Ended: Dec 9, 2010, 14:00:54 Signal G:928 A:894 T:670 C:748 Comment: Parsa Tehranchian
Sample: PT.Bridgestone_WA_1 Lane: 22 Base spacing 14.31 733 bases in 8752 scans Page 2 of 2



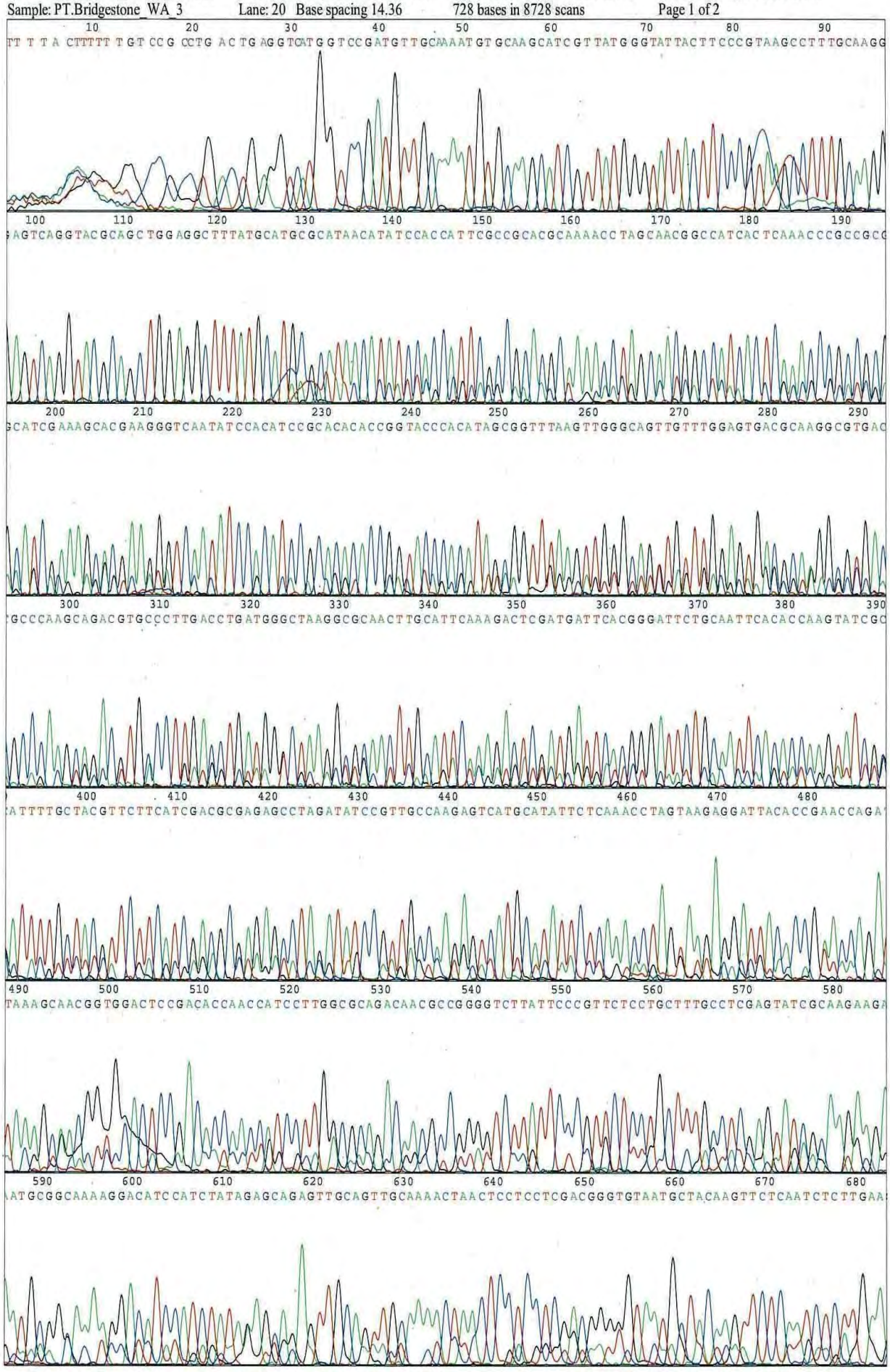
File: PT.Bridgestone_WA_2_D05.ab1 Run Ended: Dec 9, 2010, 14:00:54 Signal G:467 A:424 T:316 C:347 Comment: Parsa Tehranchian
Sample: PT.Bridgestone_WA_2 Lane: 21 Base spacing 14.33 1193 bases in 16299 scans Page 1 of 2



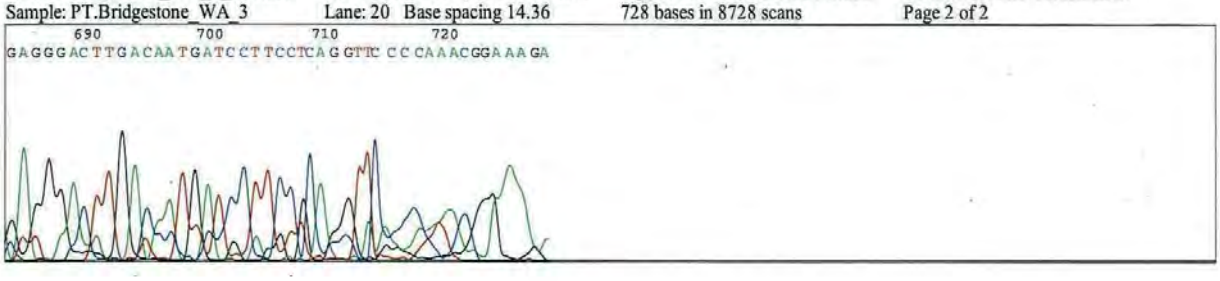
File: PT.Bridgestone_WA_2_D05.ab1 Run Ended: Dec 9, 2010, 14:00:54 Signal G:467 A:424 T:316 C:347 Comment: Parsa Tehranchian
Sample: PT.Bridgestone_WA_2 Lane: 21 Base spacing 14.33 1193 bases in 16299 scans Page 2 of 2



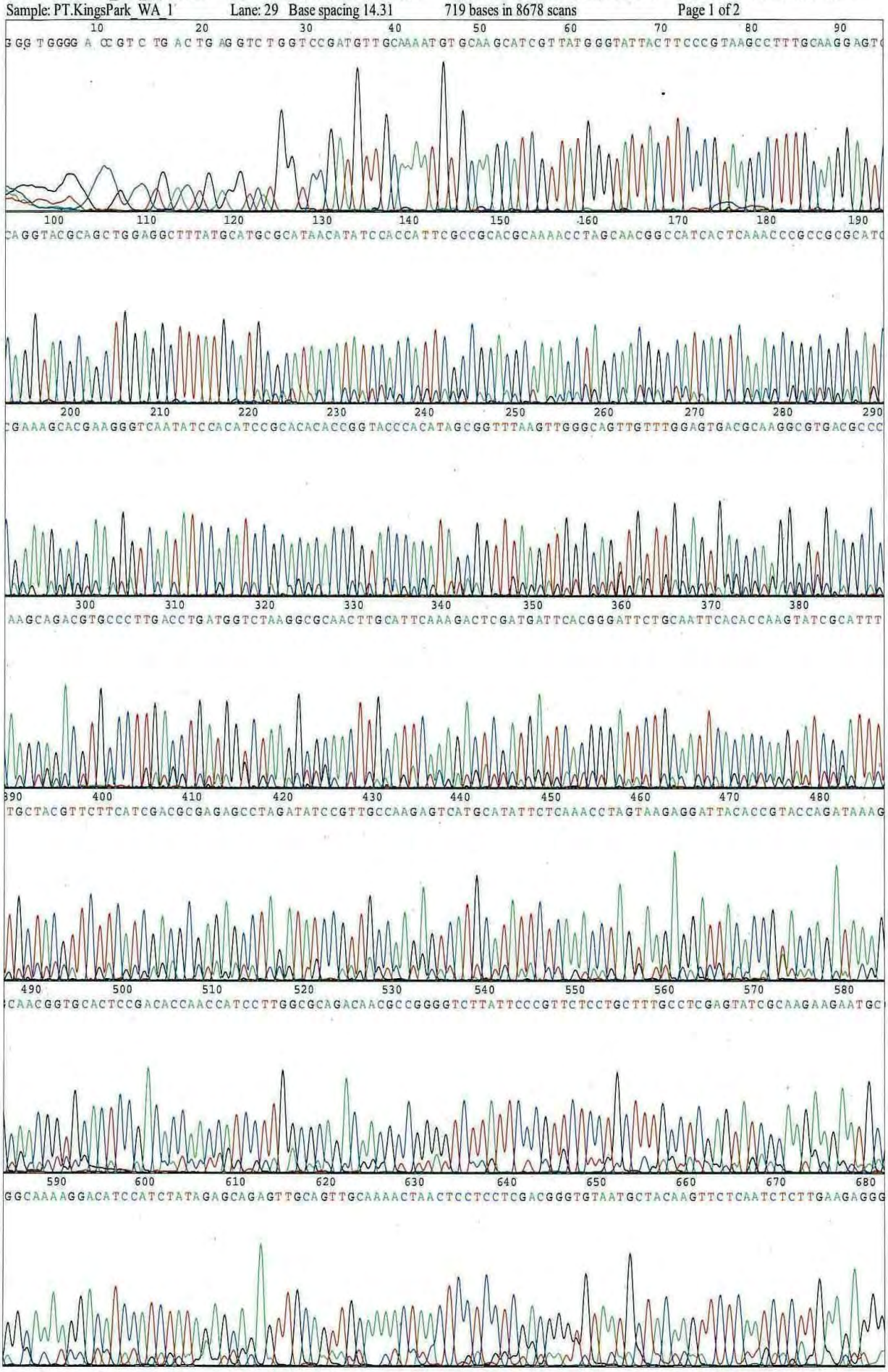
File: PT.Bridgestone_WA_3_E05.ab1 Run Ended: Dec 9, 2010, 14:00:54 Signal G:695 A:720 T:564 C:633 Comment: Parsa Tehranchian
Sample: PT.Bridgestone_WA_3 Lane: 20 Base spacing 14.36 728 bases in 8728 scans Page 1 of 2



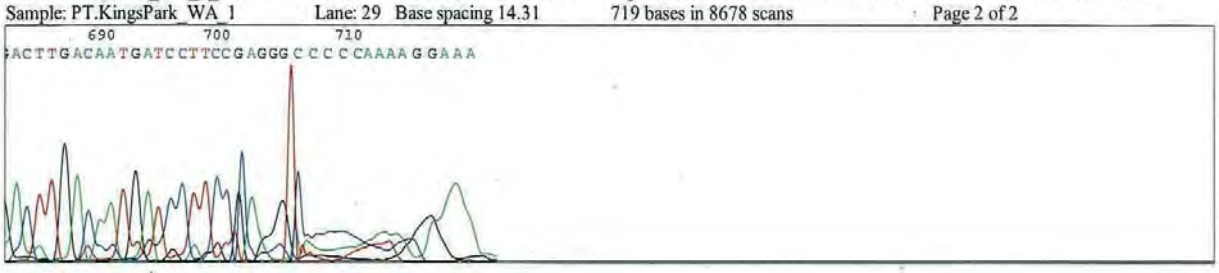
File: PT.Bridgestone_WA_3_E05.ab1 Run Ended: Dec 9, 2010, 14:00:54 Signal G:695 A:720 T:564 C:633 Comment: Parsa Tehranchian
Sample: PT.Bridgestone_WA_3 Lane: 20 Base spacing 14.36 728 bases in 8728 scans Page 2 of 2



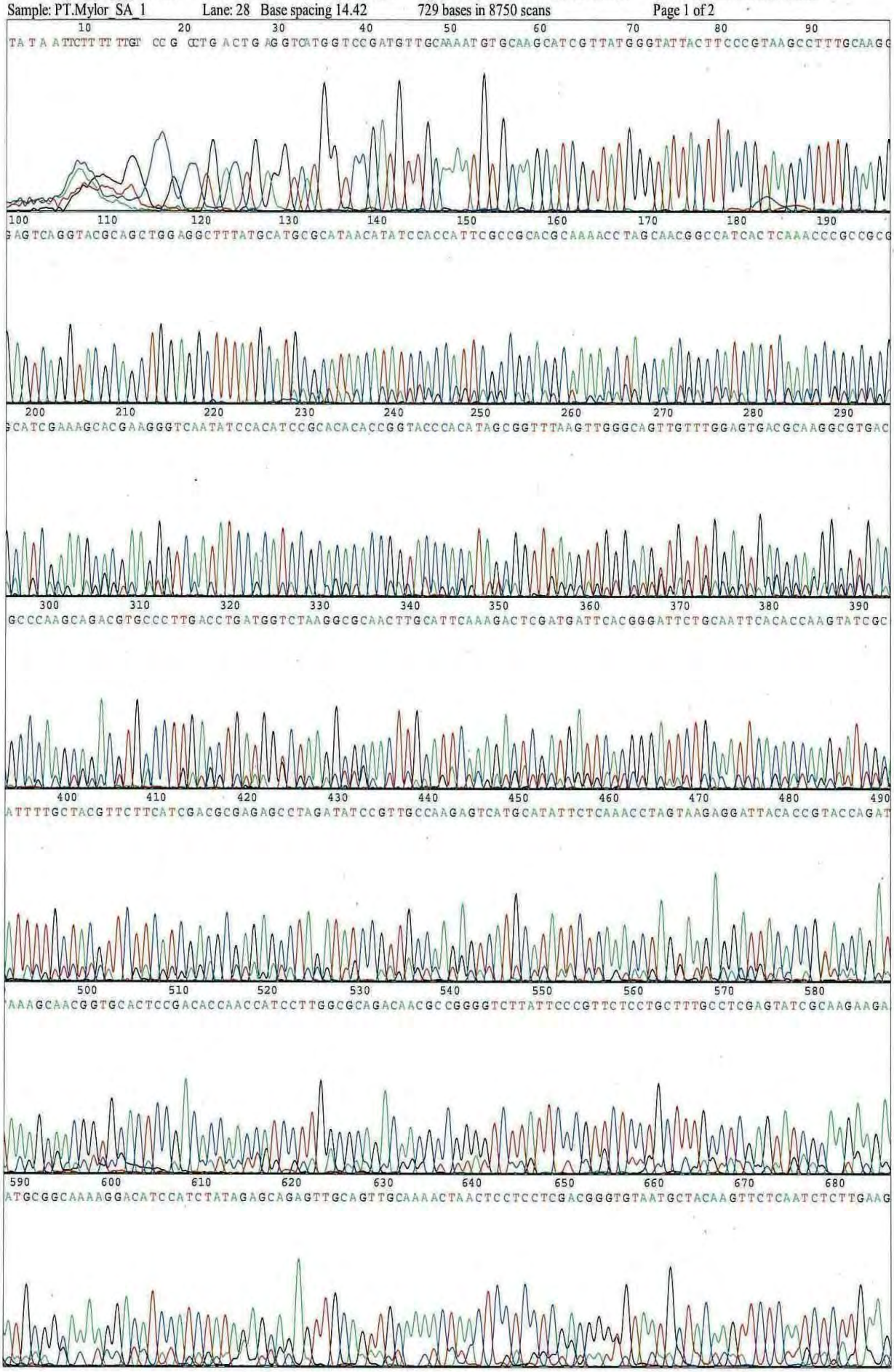
File: PT.KingsPark_WA_1_D07.ab1 Run Ended: Dec 9, 2010, 14:00:54 Signal G:2341 A:2139 T:1704 C:2146 Comment: Parsa Tehranchian
Sample: PT.KingsPark_WA_1 Lane: 29 Base spacing 14.31 719 bases in 8678 scans Page 1 of 2



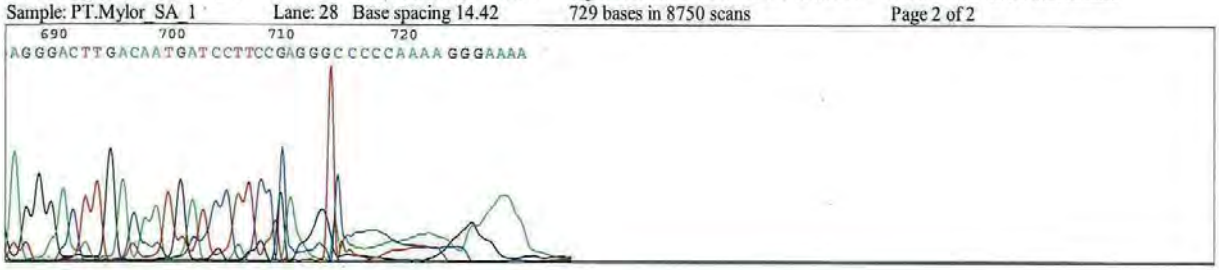
File: PT.KingsPark_WA_1_D07.ab1 Run Ended: Dec 9, 2010, 14:00:54 Signal G:2341 A:2139 T:1704 C:2146 Comment: Parsa Tehranchian
Sample: PT.KingsPark_WA_1 Lane: 29 Base spacing 14.31 719 bases in 8678 scans Page 2 of 2

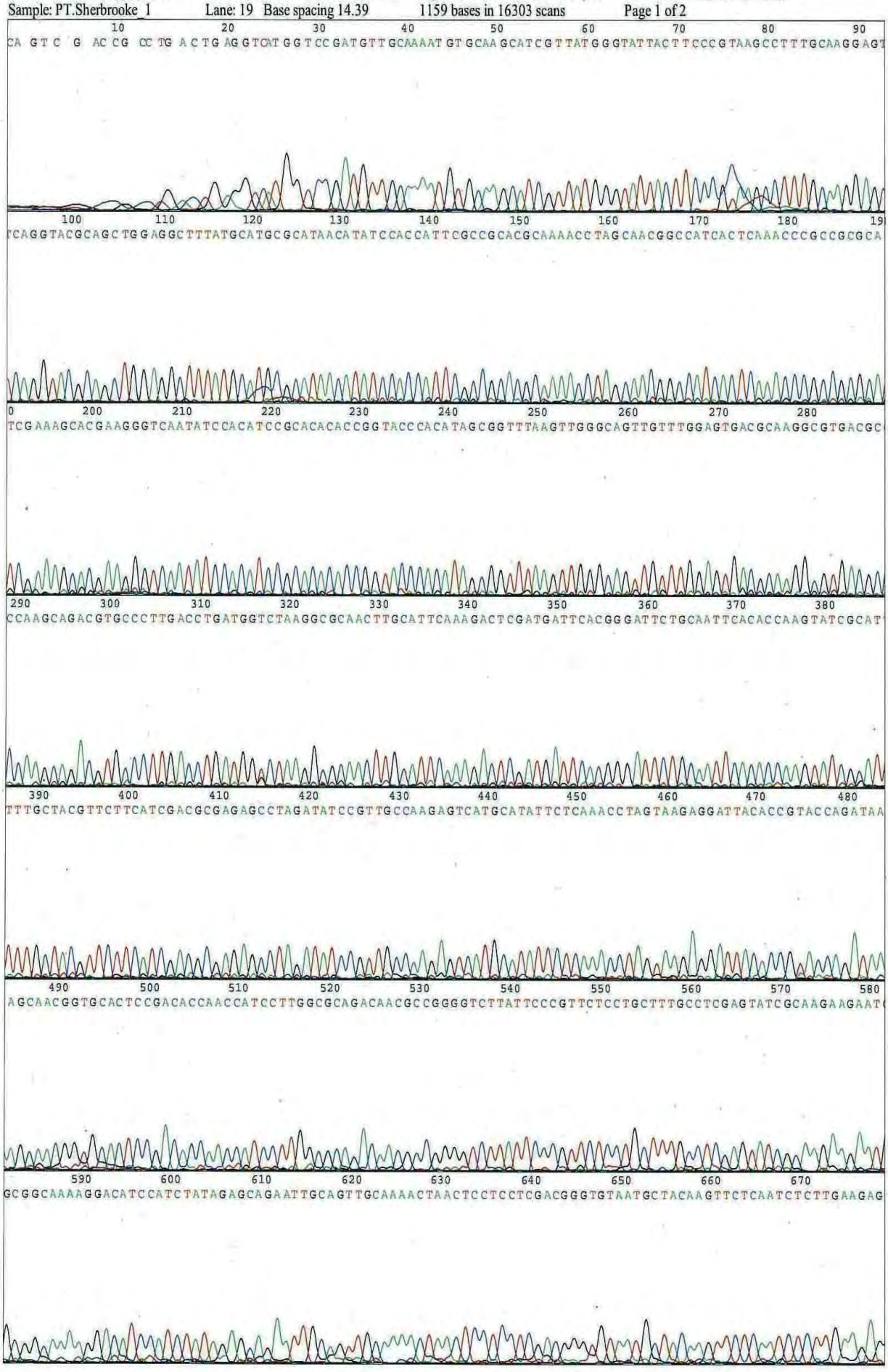


File: PT.Mylor_SA_1_E07.ab1 Run Ended: Dec 9, 2010, 14:00:54 Signal G:3057 A:2854 T:2268 C:2738 Comment: Parsa Tehranchian
Sample: PT.Mylor_SA_1 Lane: 28 Base spacing 14.42 729 bases in 8750 scans Page 1 of 2

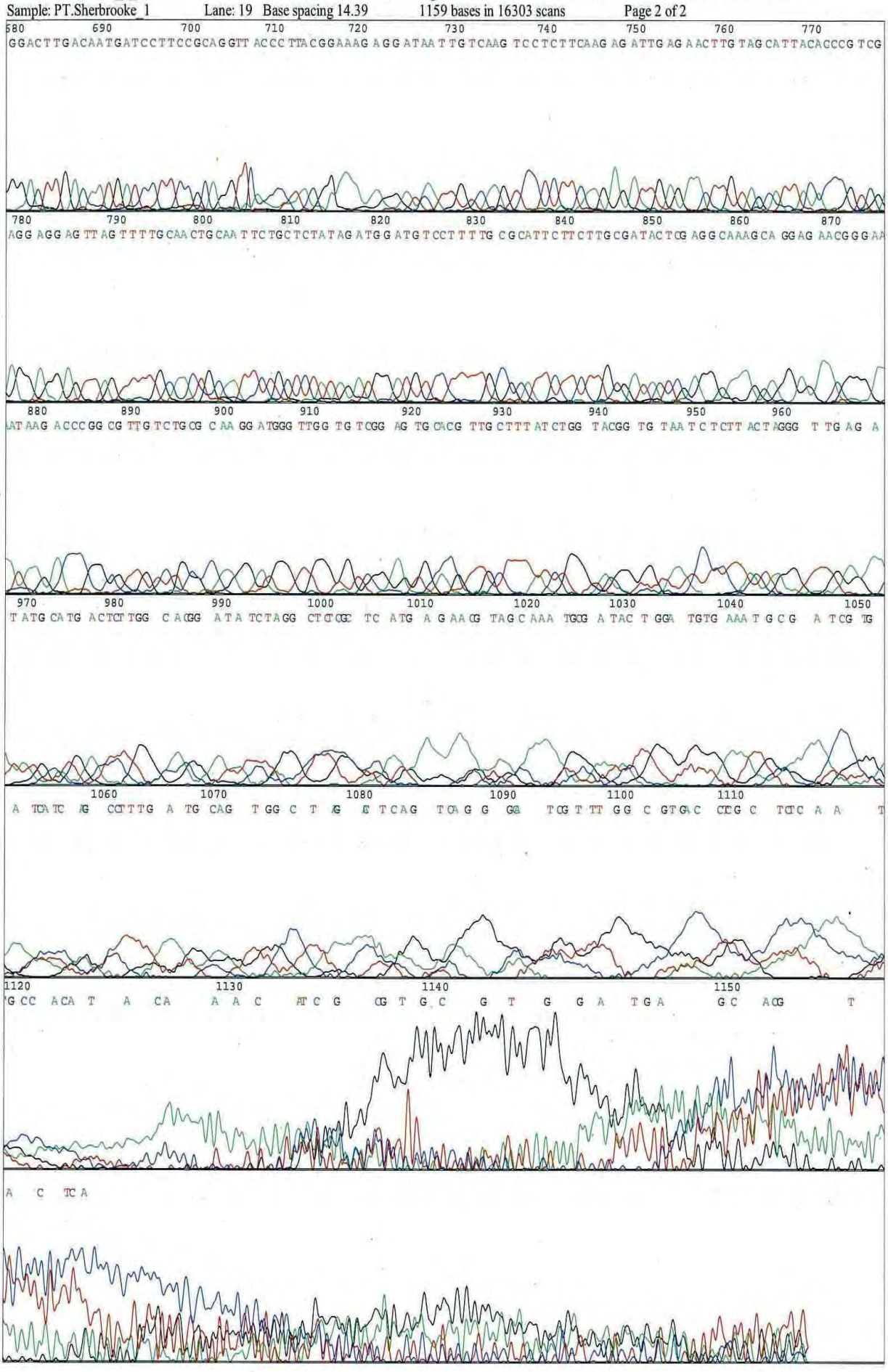


File: PT.Mylor_SA_1_E07.ab1 Run Ended: Dec 9, 2010, 14:00:54 Signal G:3057 A:2854 T:2268 C:2738 Comment: Parsa Tehranchian
Sample: PT.Mylor_SA_1 Lane: 28 Base spacing 14.42 729 bases in 8750 scans Page 2 of 2

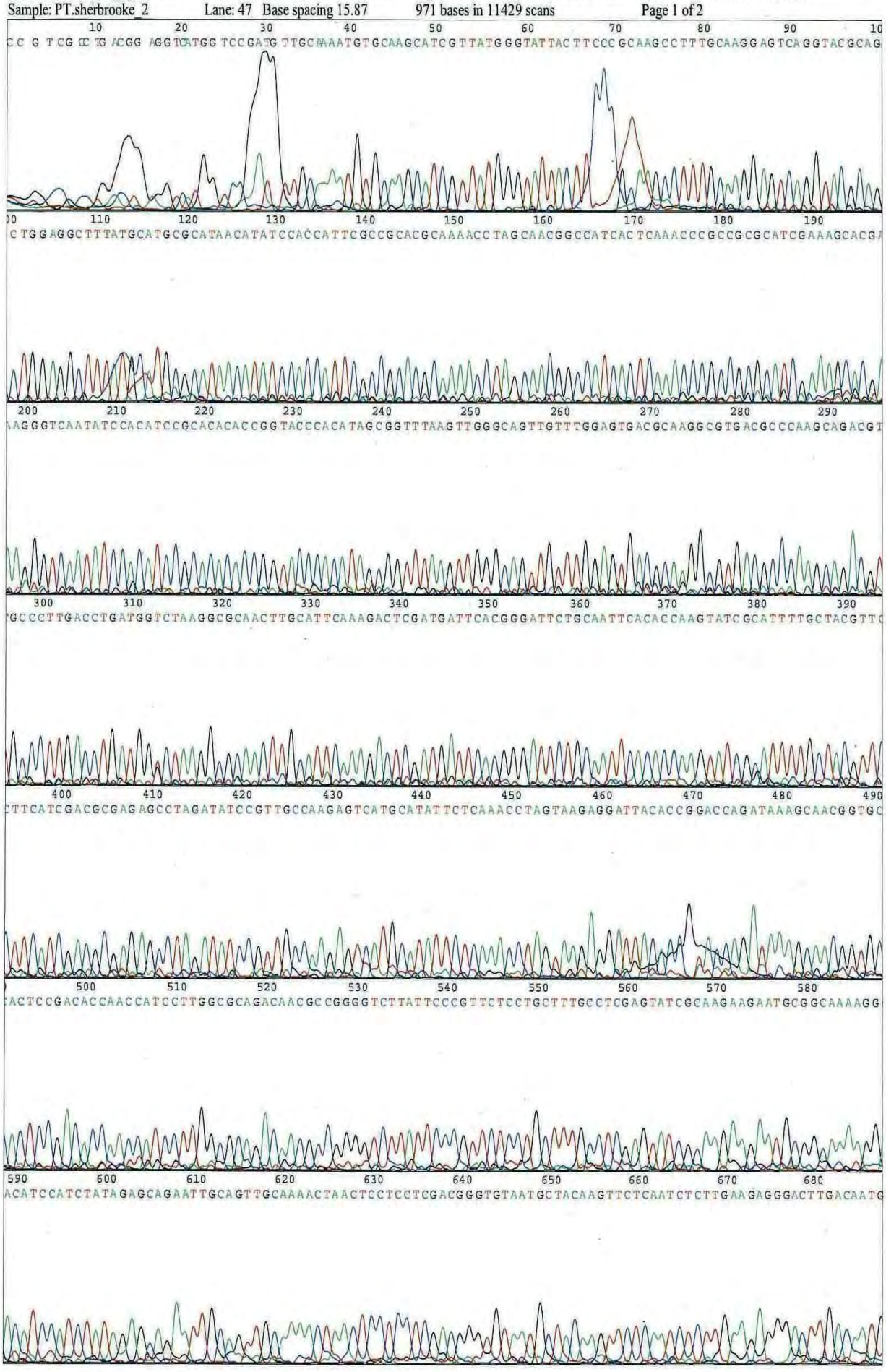



File: PT.Sherbrooke_1_F05.ab1 Run Ended: Dec 9, 2010, 14:00:54 Signal G:540 A:486 T:378 C:410 Comment: Parsa Tehranchian
Sample: PT.Sherbrooke_1 Lane: 19 Base spacing 14.39 1159 bases in 16303 scans Page 1 of 2

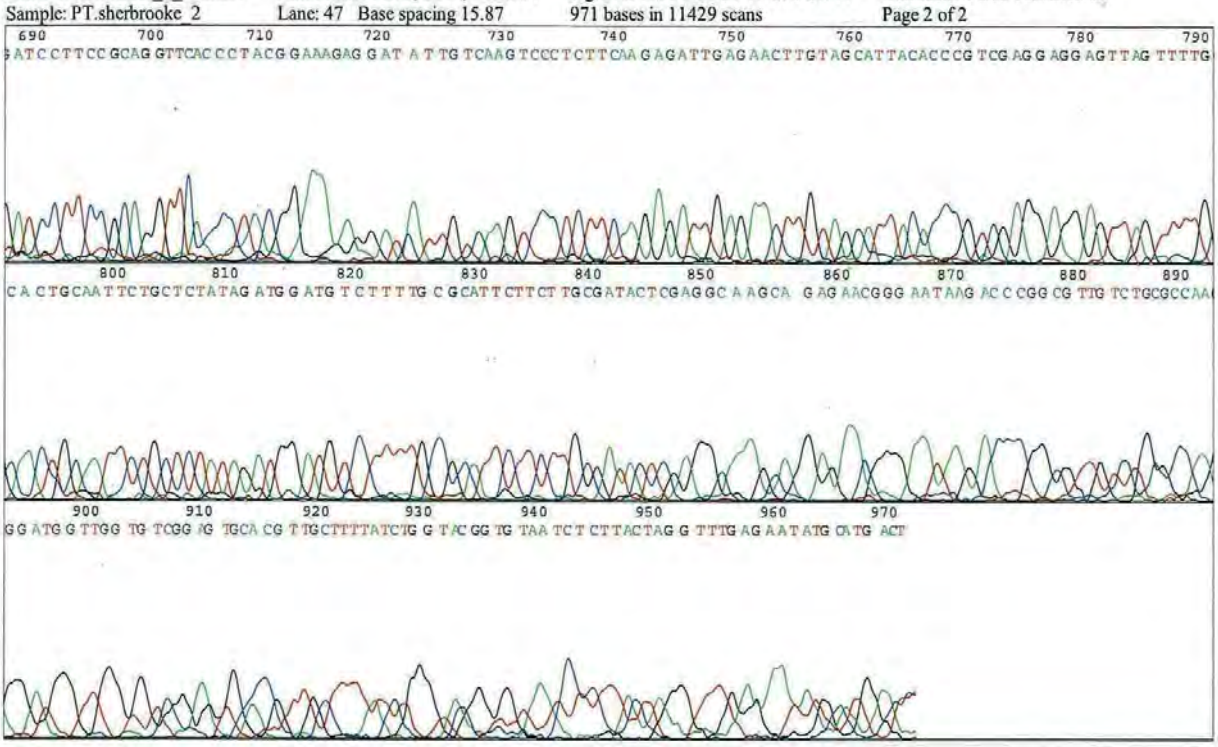
File: PT.Sherbrooke_1_F05.ab1 Run Ended: Dec 9, 2010, 14:00:54 Signal G:540 A:486 T:378 C:410 Comment: Parsa Tehranchian
Sample: PT.Sherbrooke_1 Lane: 19 Base spacing 14.39 1159 bases in 16303 scans Page 2 of 2



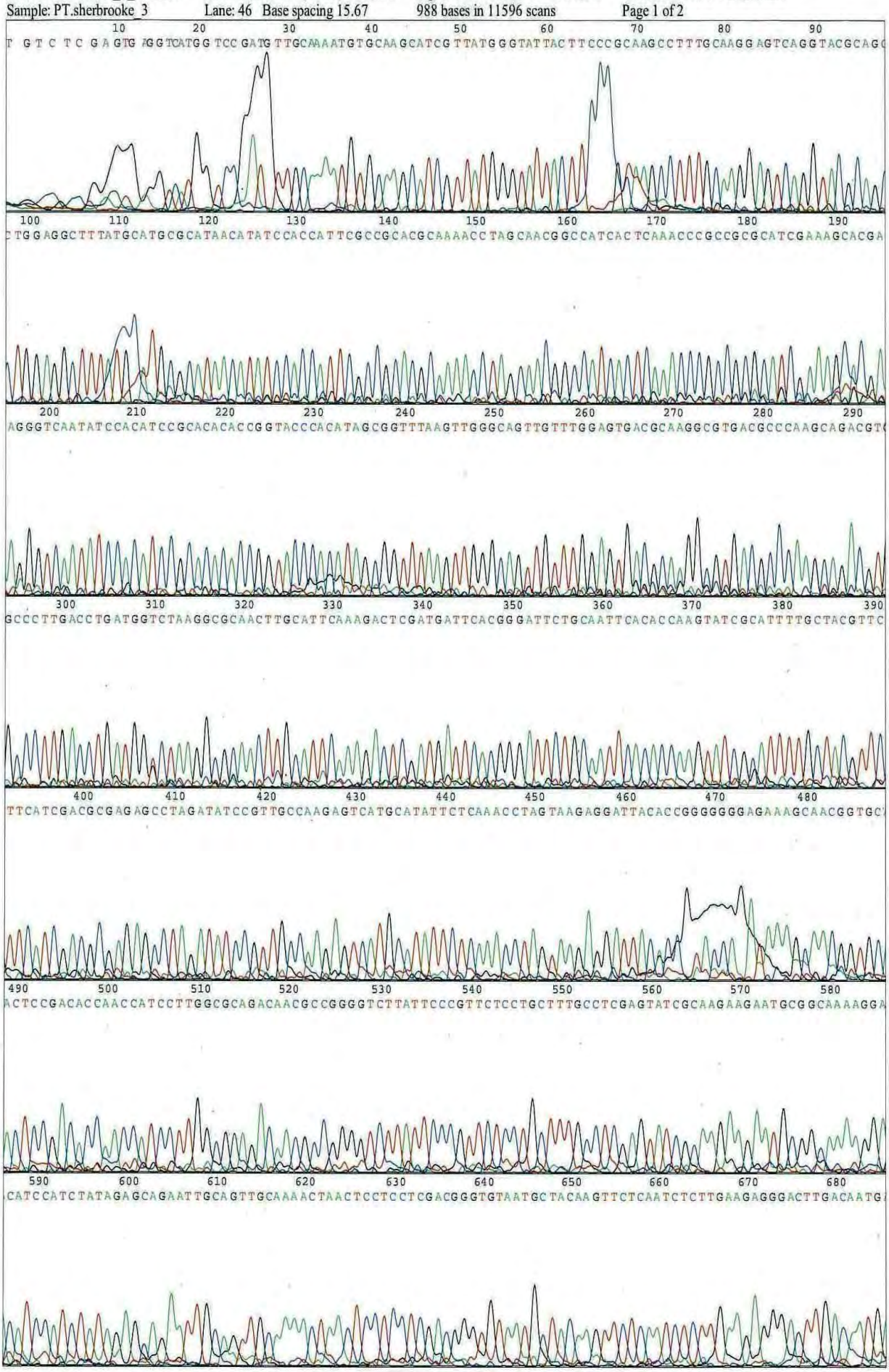
File: PT.sherbrooke_2_B12.ab1 Run Ended: Feb 3, 2011, 12:38:54 Signal G:1223 A:1287 T:1070 C:944 Comment: Parsa Tehranchian
Sample: PT.sherbrooke_2 Lane: 47 Base spacing 15.87 971 bases in 11429 scans Page 1 of 2



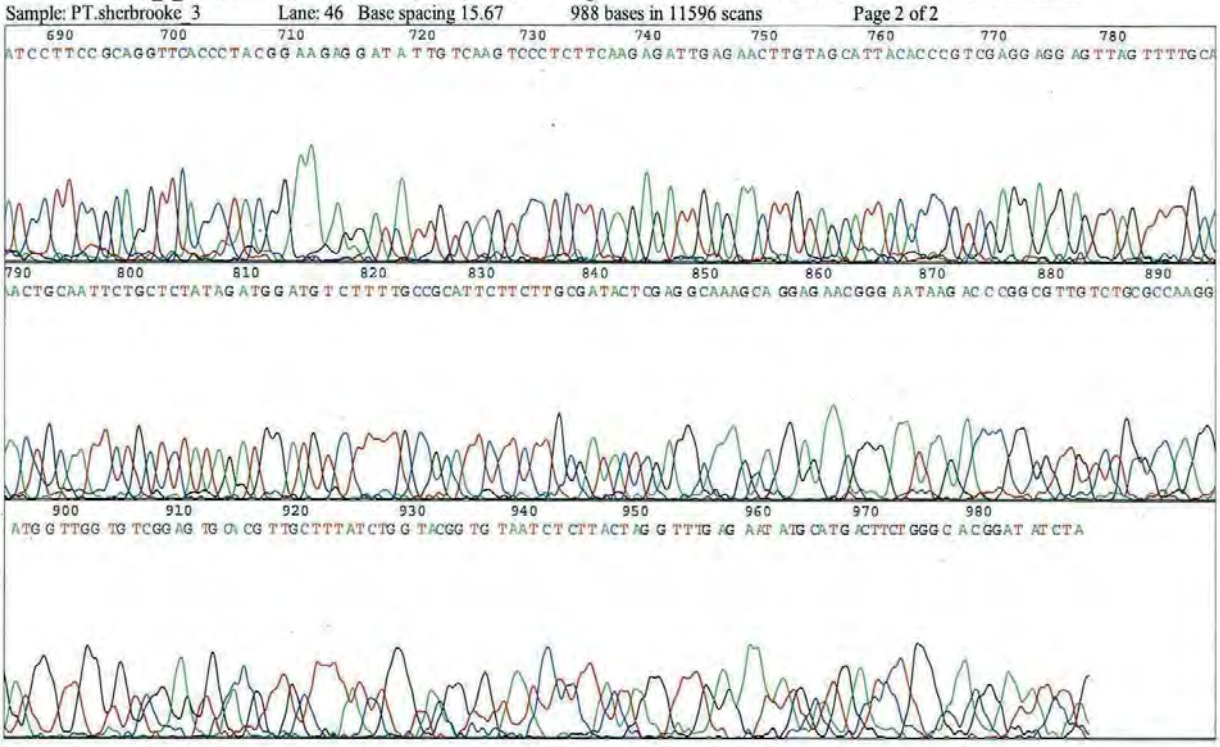
File: PT.sherbrooke_2_B12.ab1 Run Ended: Feb 3, 2011, 12:38:54 Signal G:1223 A:1287 T:1070 C:944 Comment: Parsa Tehranchian
Sample: PT.sherbrooke_2 Lane: 47 Base spacing 15.87 971 bases in 11429 scans Page 2 of 2



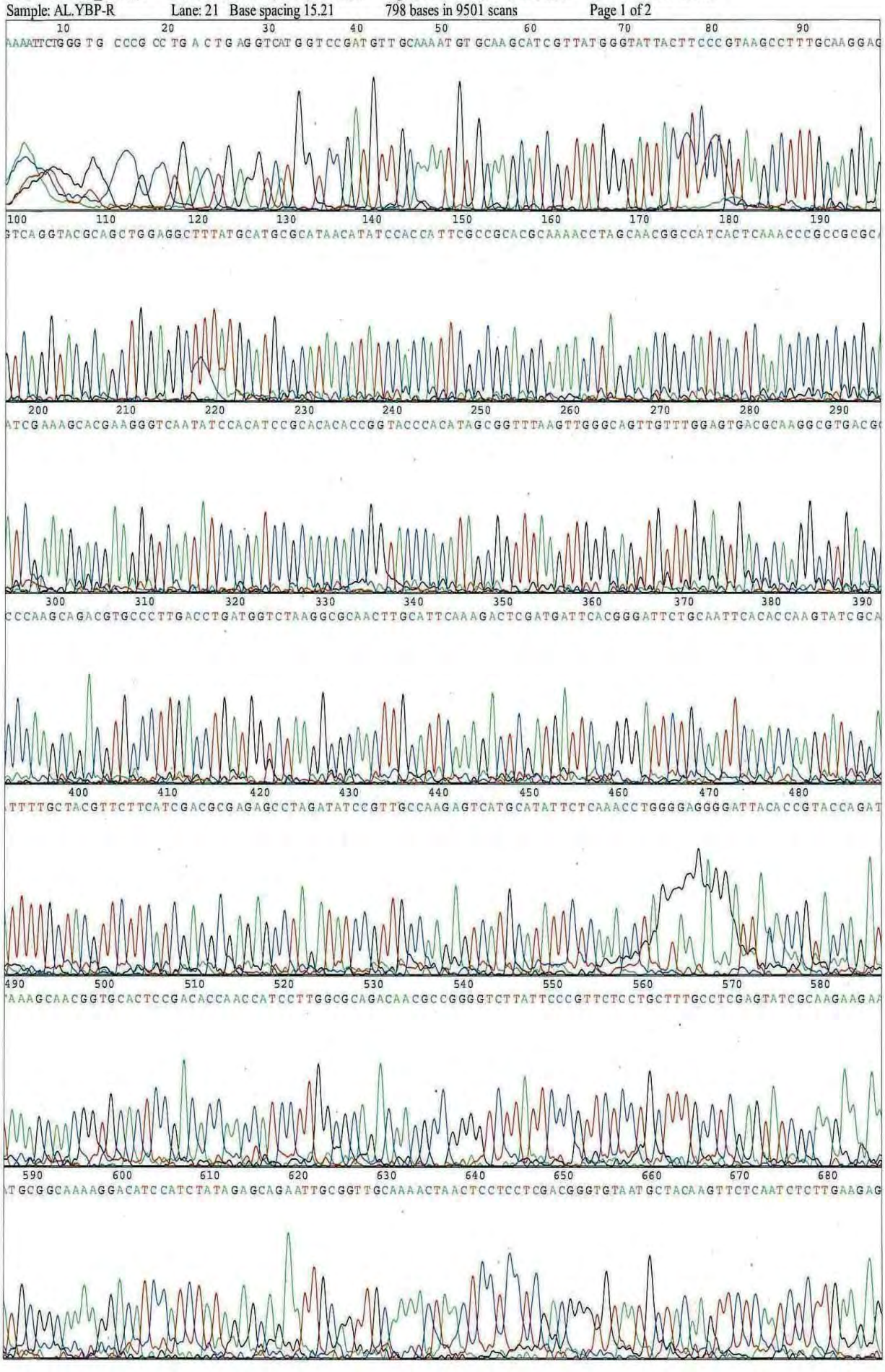
File: PT.sherbrooke_3_C12.ab1 Run Ended: Feb 3, 2011, 12:38:54 Signal G:778 A:907 T:772 C:620 Comment: Parsa Tehranchian
Sample: PT.sherbrooke_3 Lane: 46 Base spacing 15.67 988 bases in 11596 scans Page 1 of 2



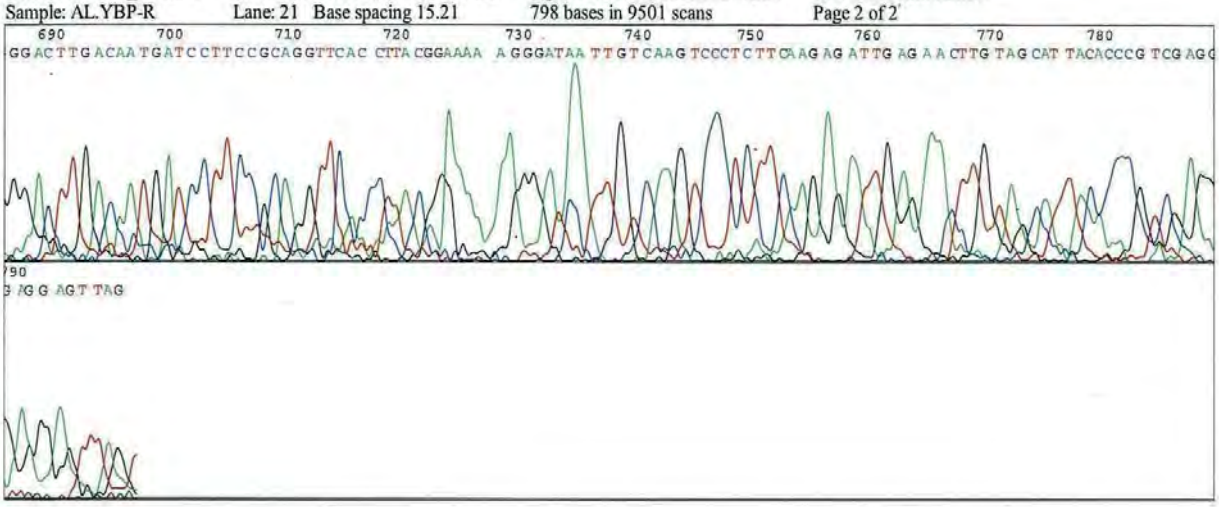
File: PT.sherbrooke_3_C12.ab1 Run Ended: Feb 3, 2011, 12:38:54 Signal G:778 A:907 T:772 C:620 Comment: Parsa Tehranchian
Sample: PT.sherbrooke_3 Lane: 46 Base spacing 15.67 988 bases in 11596 scans Page 2 of 2



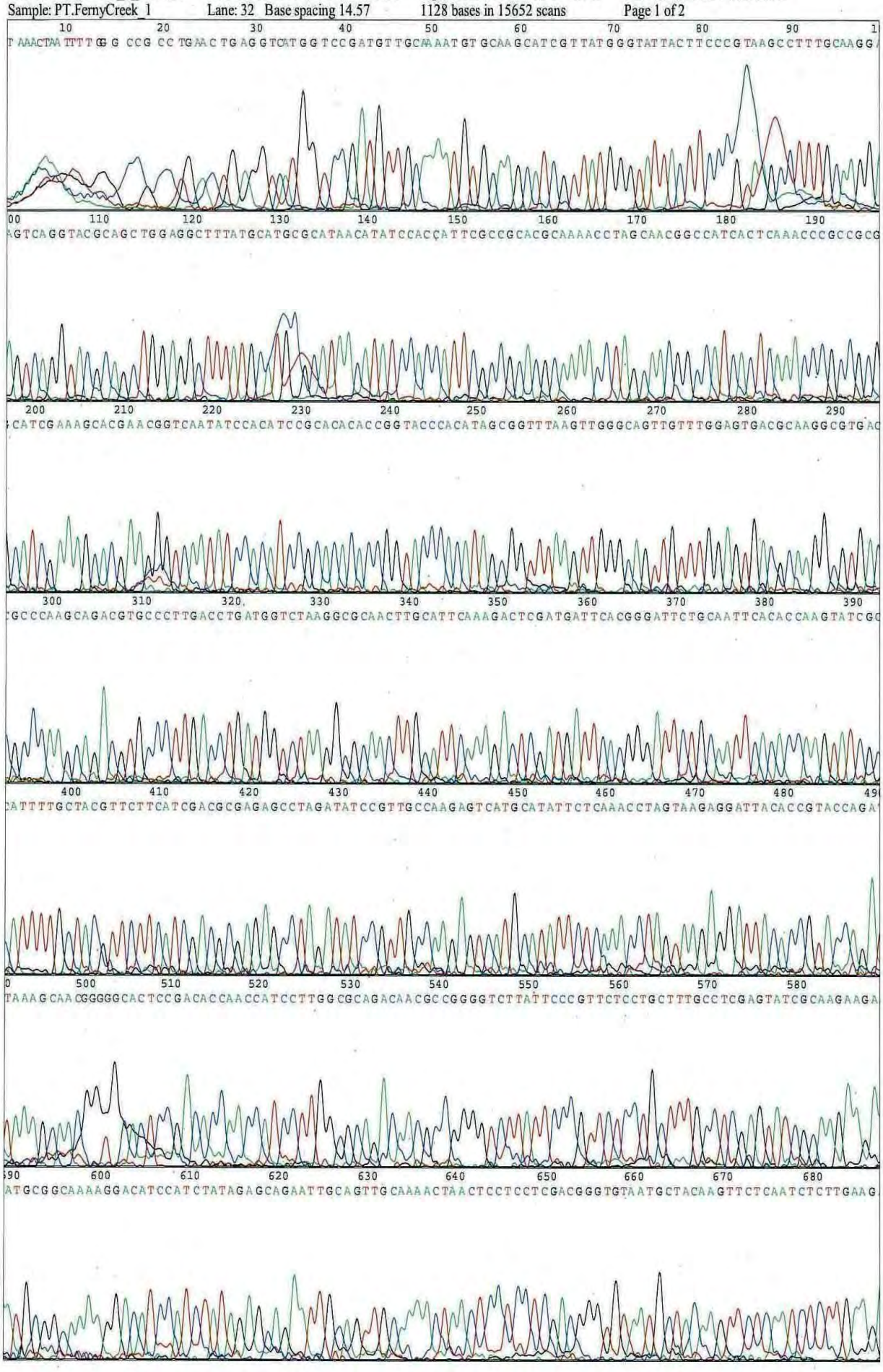
File: AL.YBP-R_D06.ab1 Run Ended: Mar 4, 2010, 22:33:35 Signal G:524 A:533 T:553 C:530 Comment: Ann Lawrie
Sample: AL.YBP-R Lane: 21 Base spacing 15.21 798 bases in 9501 scans Page 1 of 2



File: AL.YBP-R_D06.ab1 Run Ended: Mar 4, 2010, 22:33:35 Signal G:524 A:533 T:553 C:530 Comment: Ann Lawrie
Sample: AL.YBP-R Lane: 21 Base spacing 15.21 798 bases in 9501 scans Page 2 of 2



File: PT.FernyCreek_1_A07.ab1 Run Ended: Dec 9, 2010, 14:00:54 Signal G:287 A:249 T:203 C:141 Comment: Parsa Tehranchian
Sample: PT.FernyCreek_1 Lane: 32 Base spacing 14.57 1128 bases in 15652 scans Page 1 of 2



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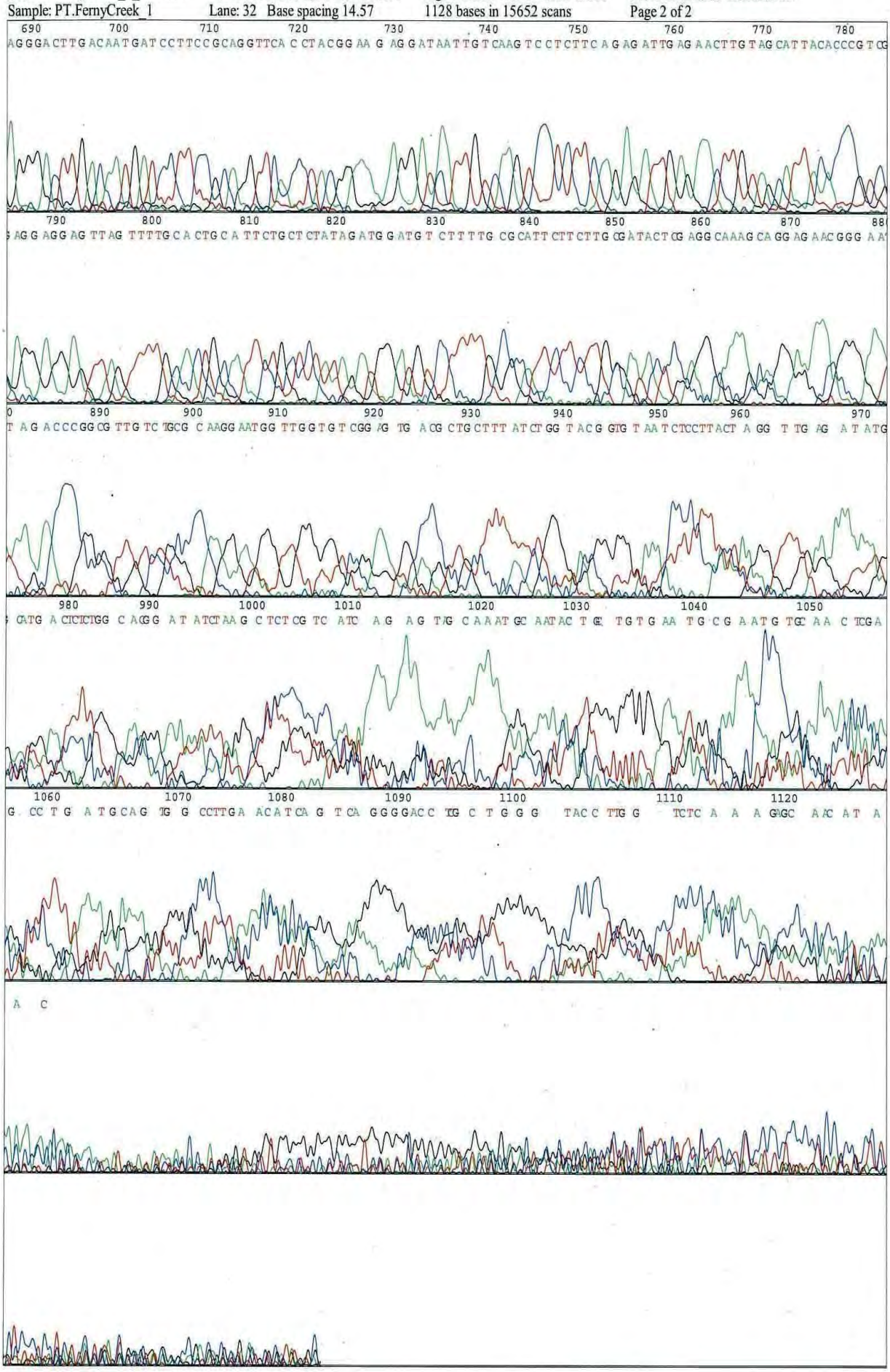
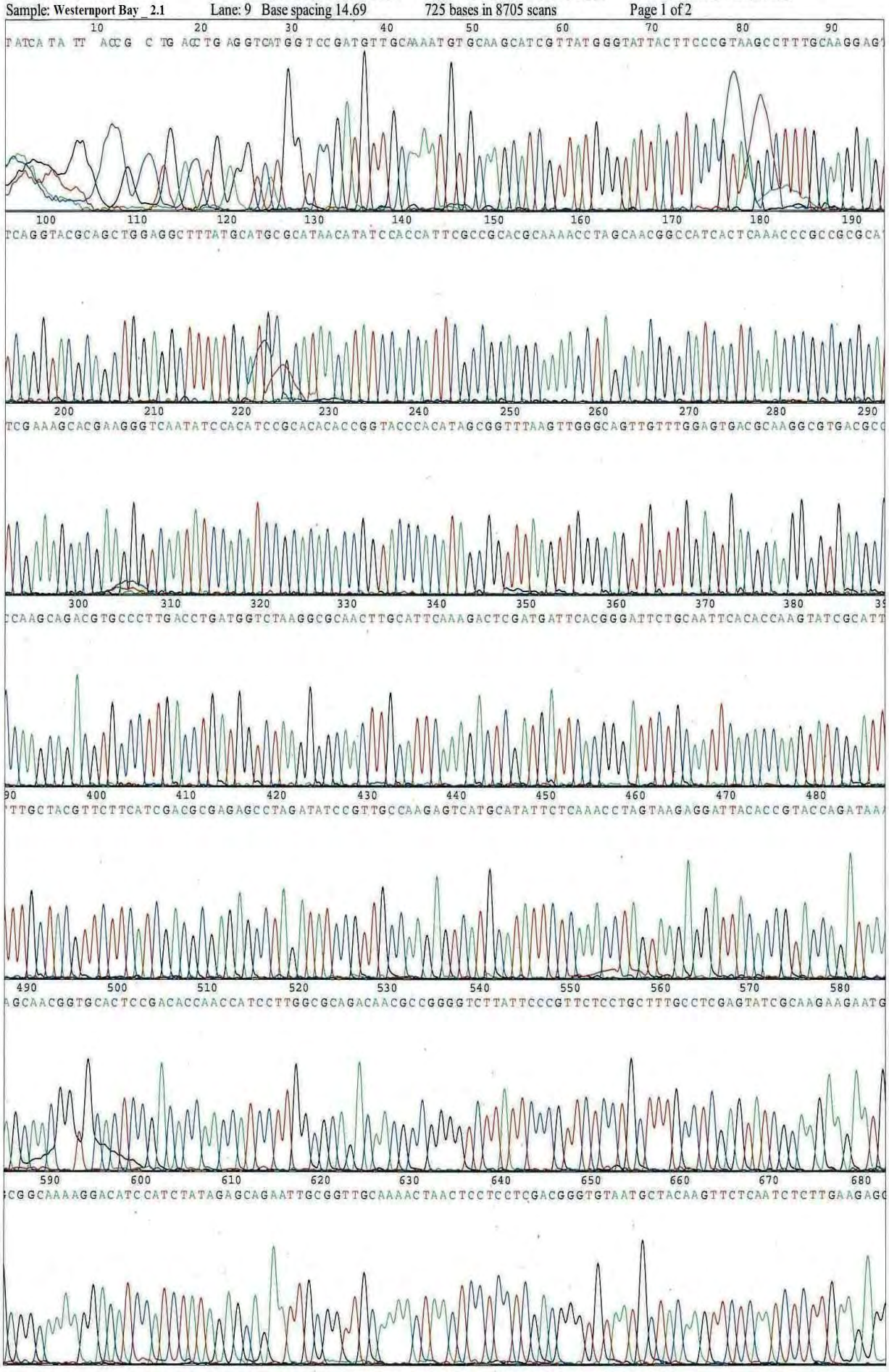
Sample: PT.FernyCreek_1 Lane: 32 Base spacing 14.57 1128 bases in 15652 scans Page 2 of 2

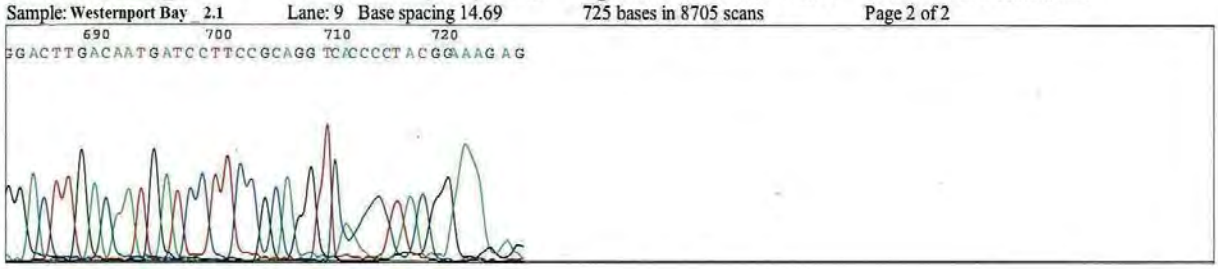


File: Westernport Bay _ 2.1 Run Ended: Dec 9, 2010, 14:00:54 Signal G:740 A:698 T:581 C:651 Comment: Parsa Tehranchian
Sample: Westernport Bay _ 2.1 Lane: 9 Base spacing 14.69 725 bases in 8705 scans Page 1 of 2

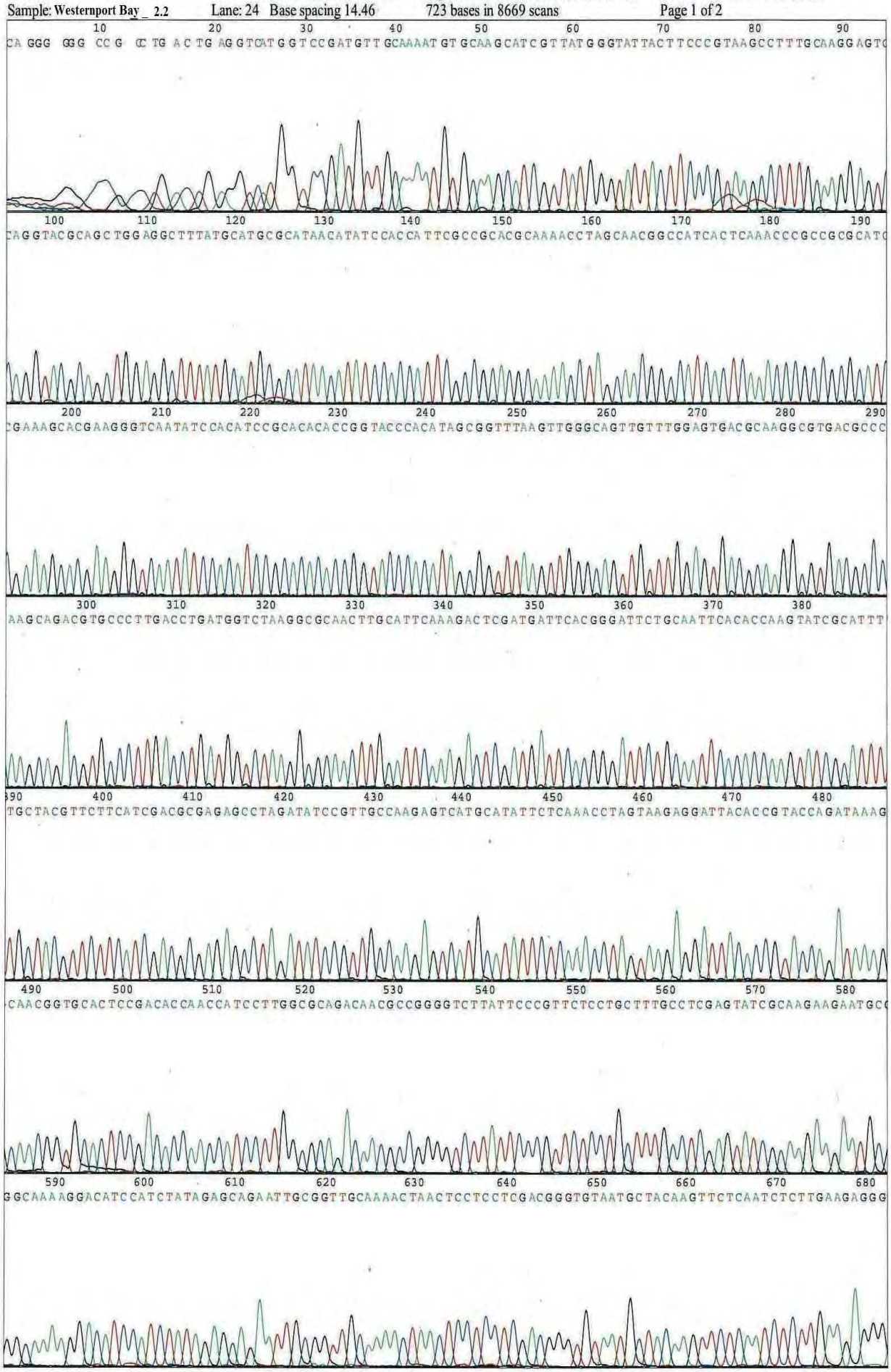


File: Westernport Bay _ 2.1 | Run Ended: Dec 9, 2010, 14:00:54 | Signal G:740 A:698 T:581 C:651 | Comment: Parsa Tehranchian

Sample: Westernport Bay _ 2.1 | Lane: 9 Base spacing 14.69 | 725 bases in 8705 scans | Page 2 of 2

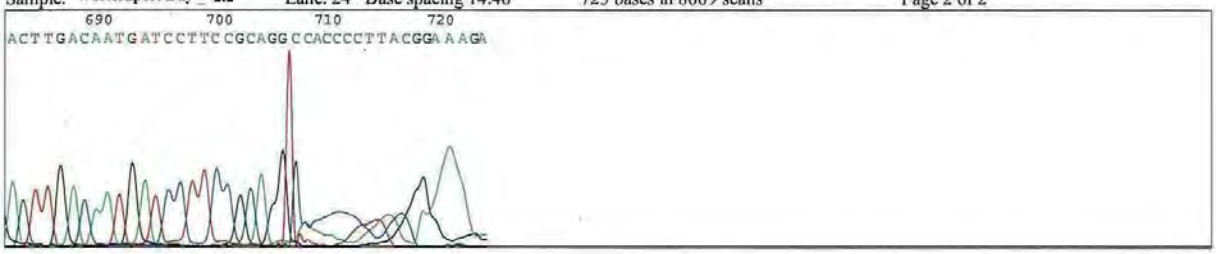


File: Westernport Bay _ 2.2 | Run Ended: Dec 9, 2010, 14:00:54 | Signal G:1474 A:1425 T:1148 C:1402 | Comment: Parsa Tehranchian
Sample: Westernport Bay _ 2.2 | Lane: 24 Base spacing 14.46 | 723 bases in 8669 scans | Page 1 of 2

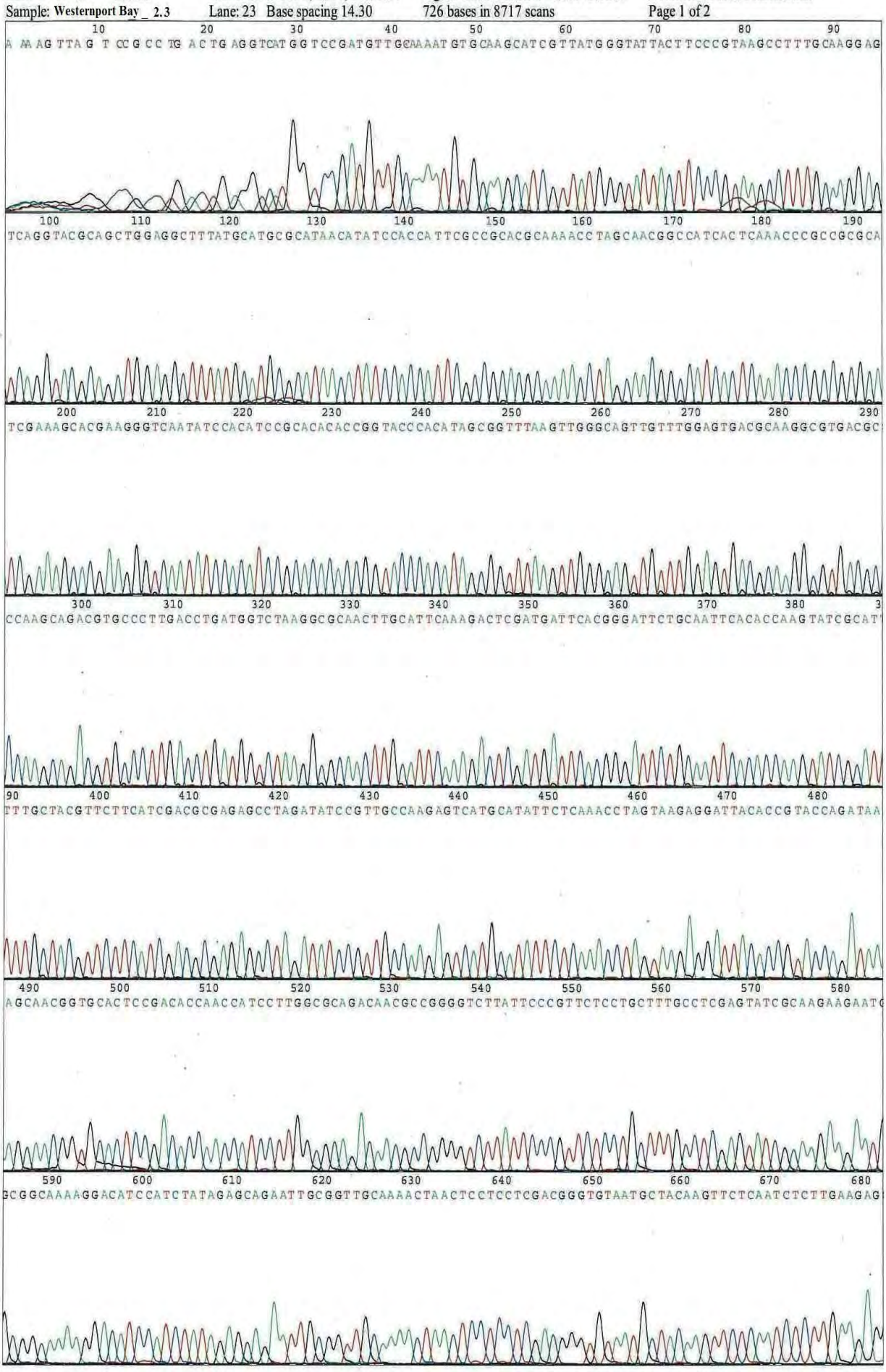


File: **Westernport Bay 2.2** Run Ended: Dec 9, 2010, 14:00:54 Signal G:1474 A:1425 T:1148 C:1402 Comment: Parsa Tehranchian

Sample: **Westernport Bay _ 2.2** Lane: 24 Base spacing 14.46 723 bases in 8669 scans Page 2 of 2

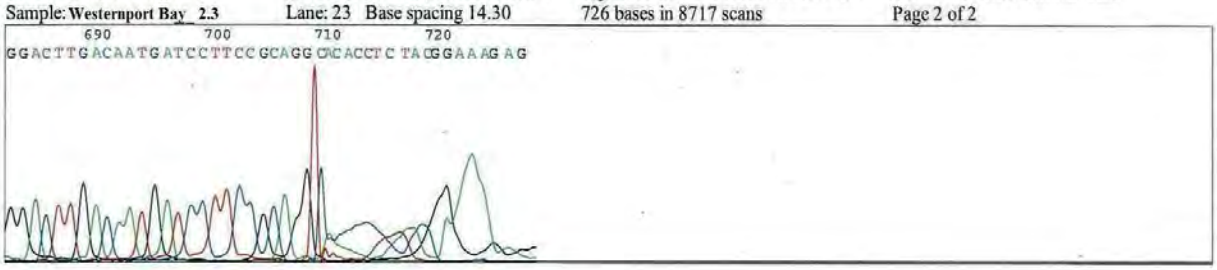


File: Westernport Bay _ 2.3 Run Ended: Dec 9, 2010, 14:00:54 Signal G:1224 A:1295 T:999 C:1207 Comment: Parsa Tehranchian
Sample: Westernport Bay _ 2.3 Lane: 23 Base spacing 14.30 726 bases in 8717 scans Page 1 of 2

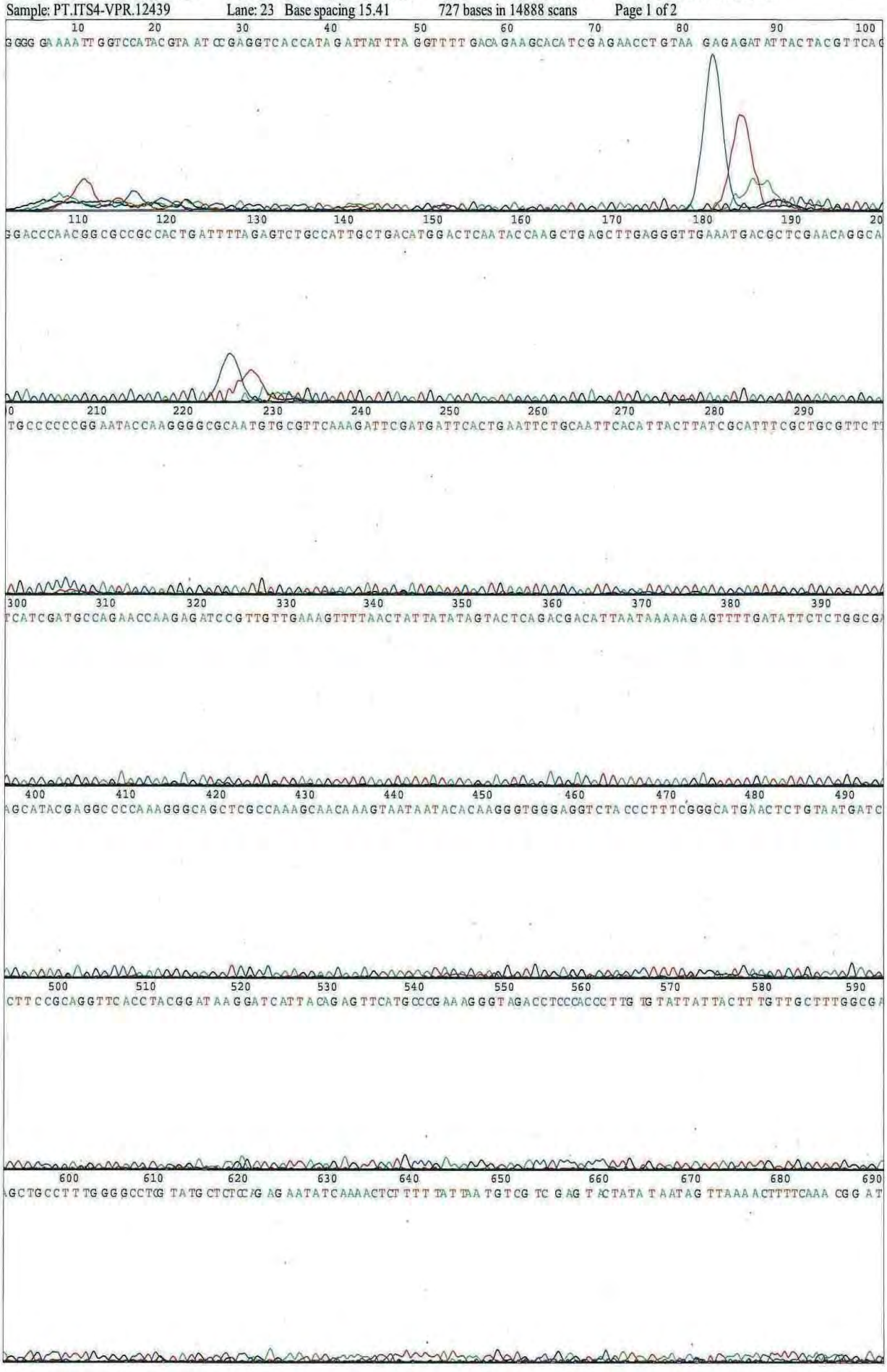


File: **Westernport Bay_ 2.3** Run Ended: Dec 9, 2010, 14:00:54 Signal G:1224 A:1295 T:999 C:1207 Comment: Parsa Tehranchian

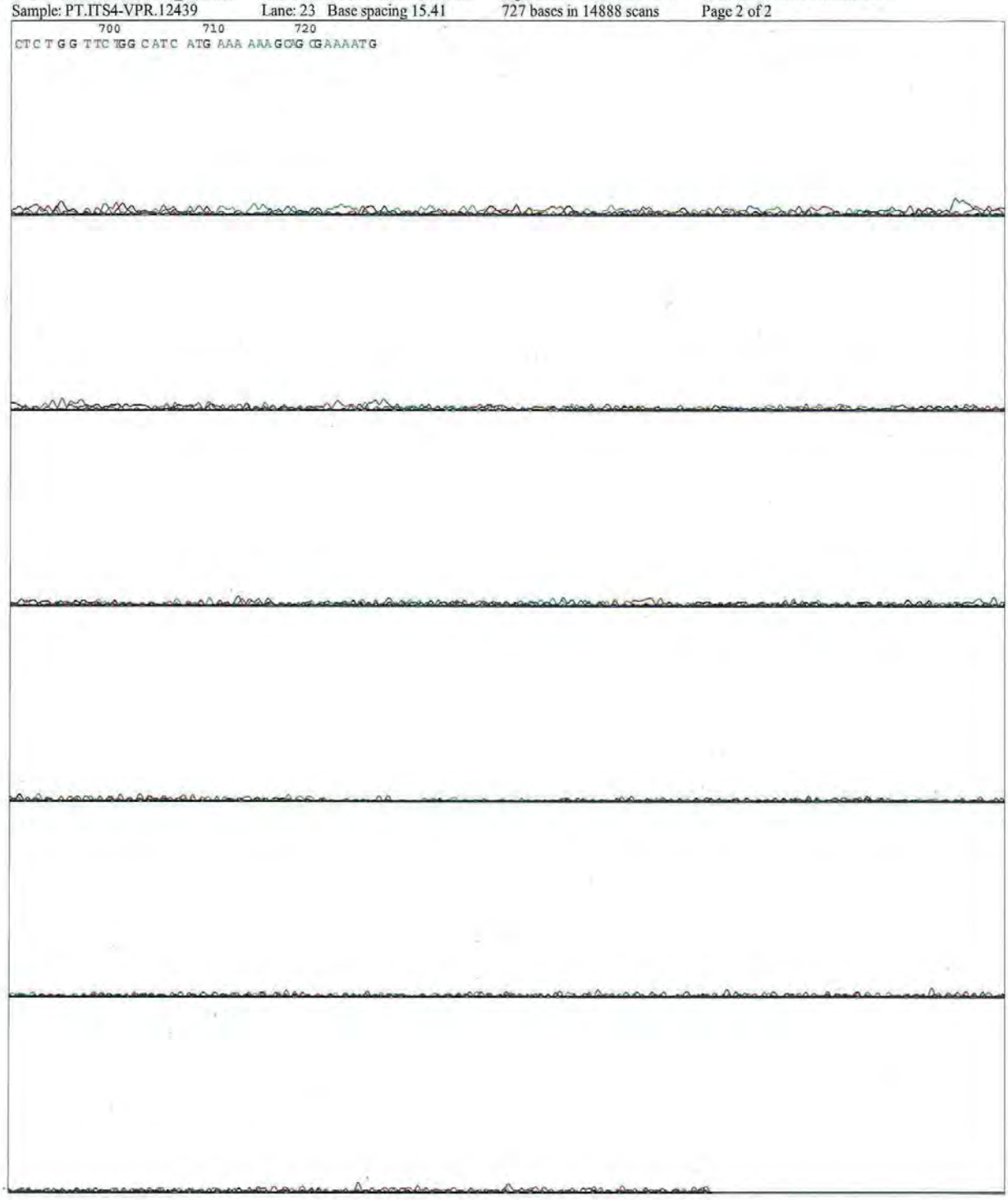
Sample: **Westernport Bay_ 2.3** Lane: 23 Base spacing 14.30 726 bases in 8717 scans Page 2 of 2



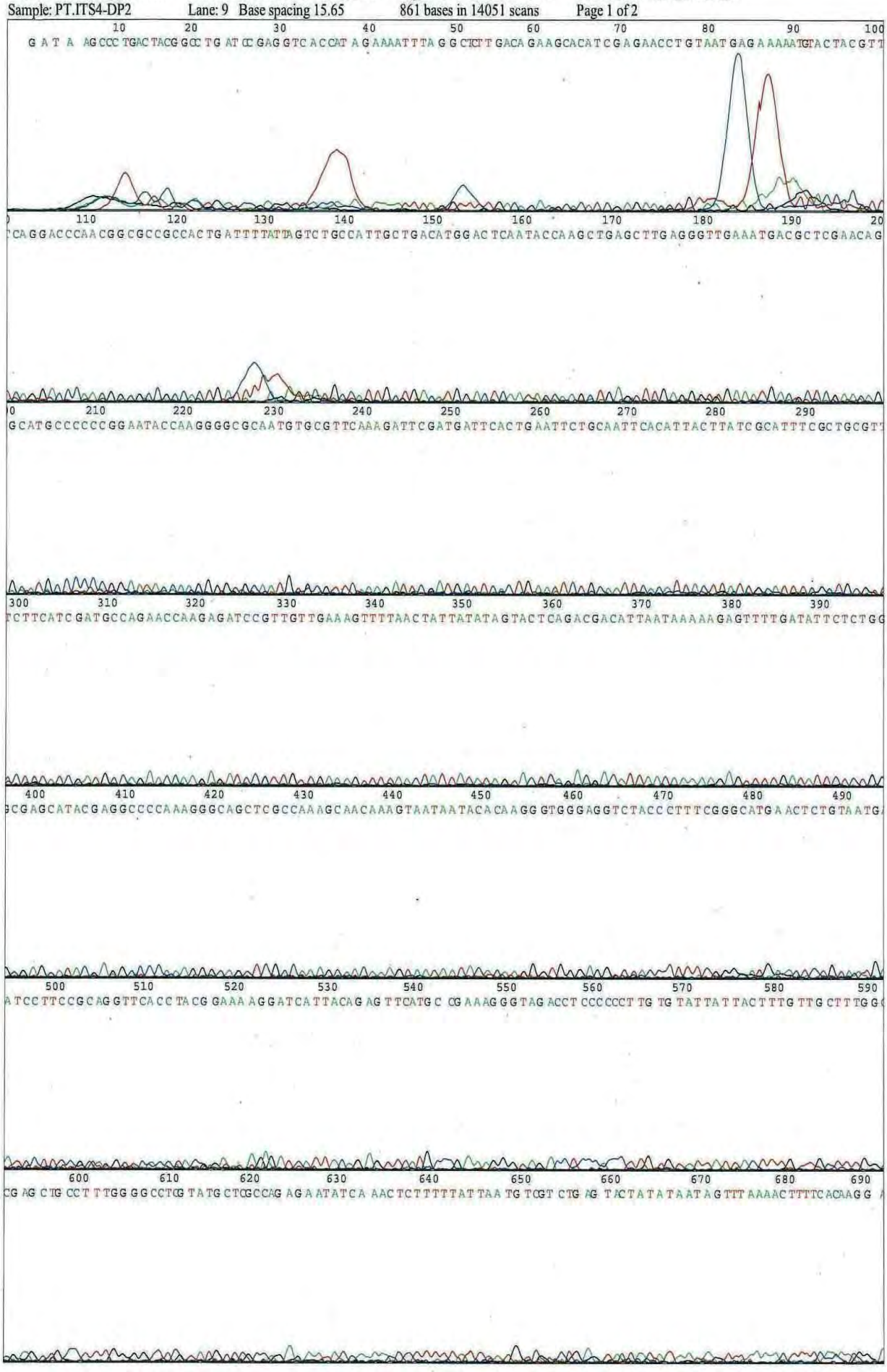
File: PT.ITS4-VPR.12439_B06.ab1 Run Ended: Jun 10, 2010, 0:15:50 Signal G:70 A:72 T:72 C:74 Comment: Parsa Tehranchian
Sample: PT.ITS4-VPR.12439 Lane: 23 Base spacing 15.41 727 bases in 14888 scans Page 1 of 2



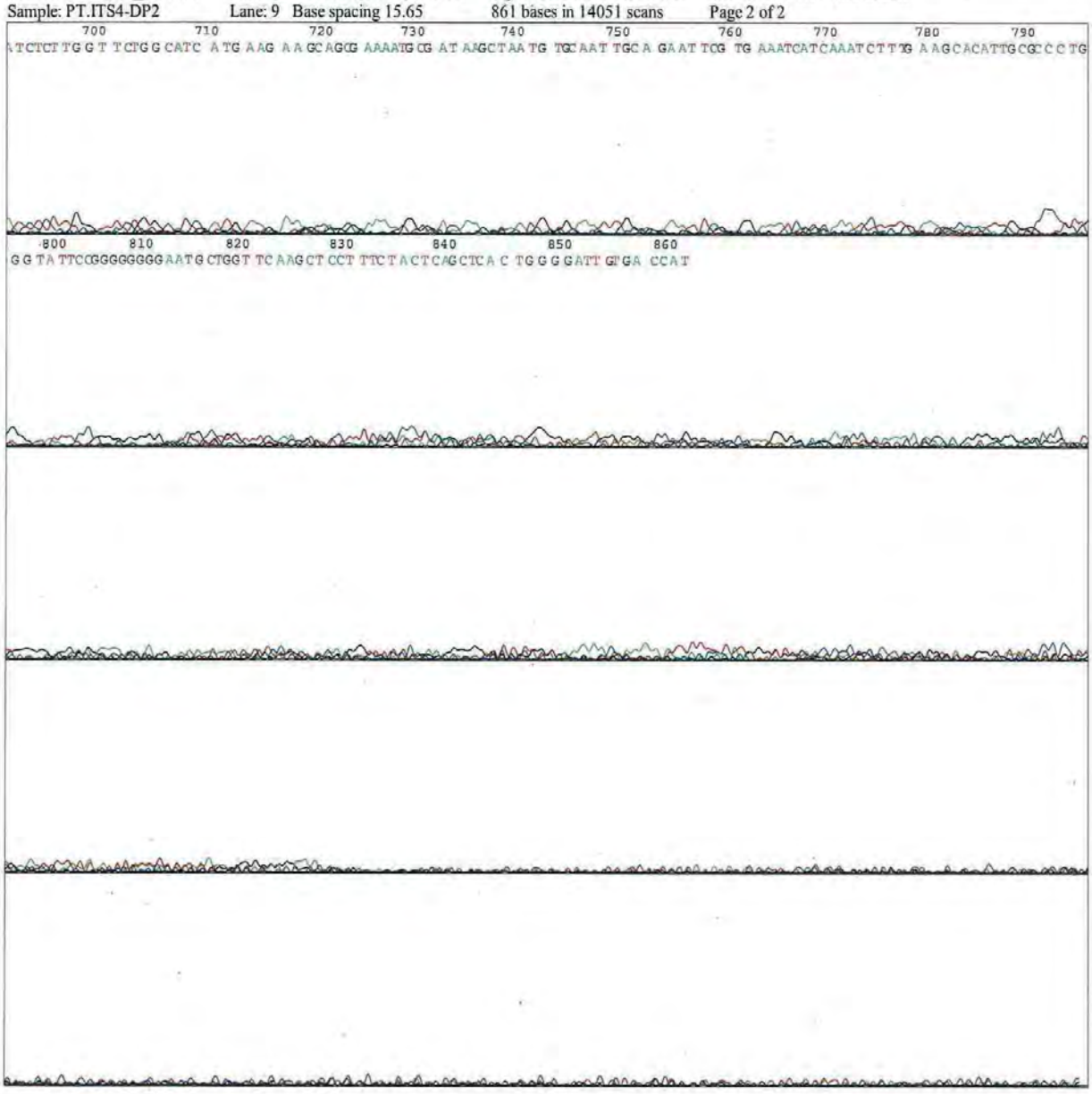
File: PT.ITS4-VPR.12439_B06.ab1 Run Ended: Jun 10, 2010, 0:15:50 Signal G:70 A:72 T:72 C:74 Comment: Parsa Tehranchian
Sample: PT.ITS4-VPR.12439 Lane: 23 Base spacing 15.41 727 bases in 14888 scans Page 2 of 2



File: PT.ITS4-DP2_H04.ab1 Run Ended: Jun 10, 2010, 0:15:50 Signal G:79 A:72 T:81 C:75 Comment: Parsa Tehranchian
Sample: PT.ITS4-DP2 Lane: 9 Base spacing 15.65 861 bases in 14051 scans Page 1 of 2

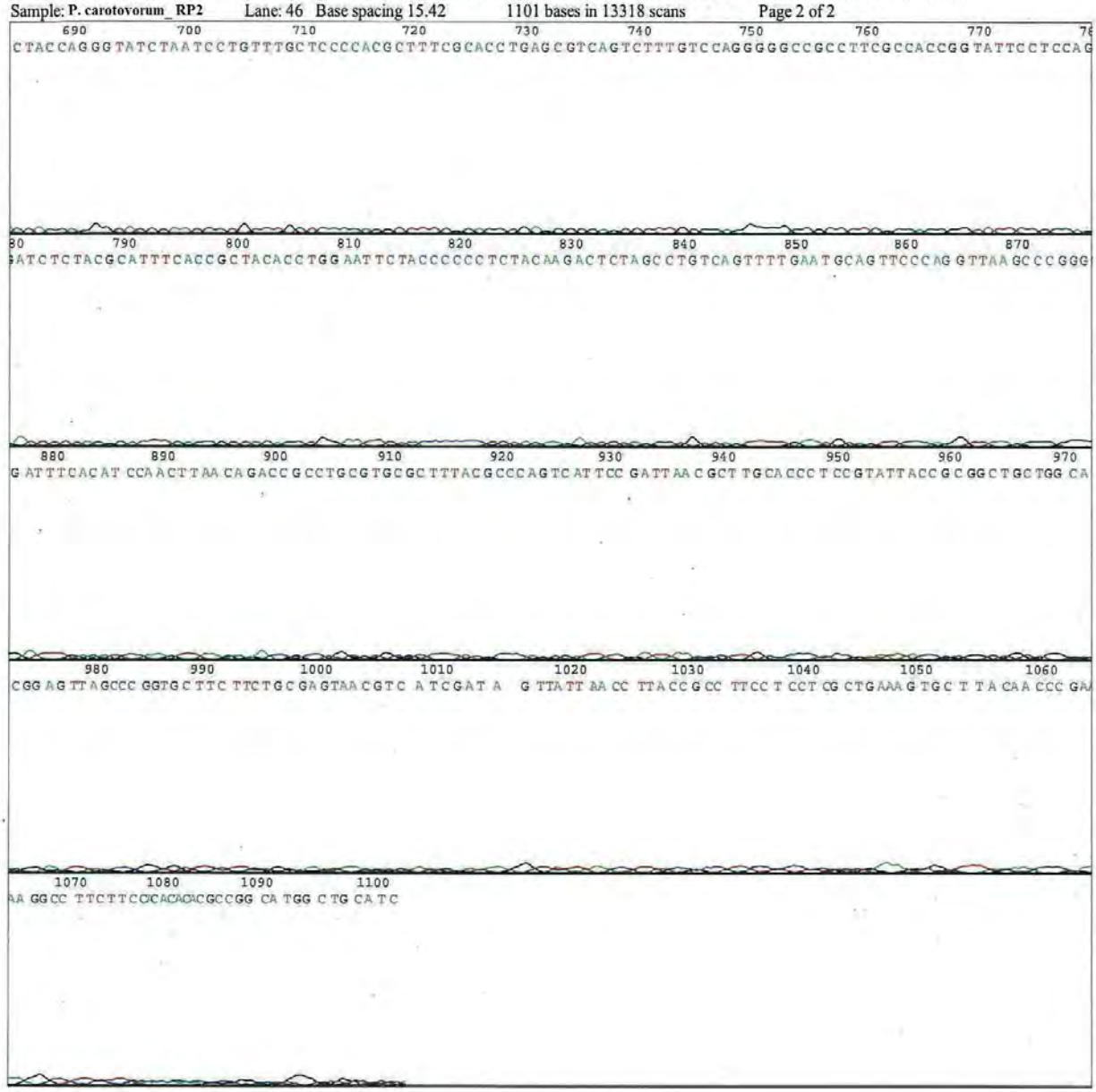


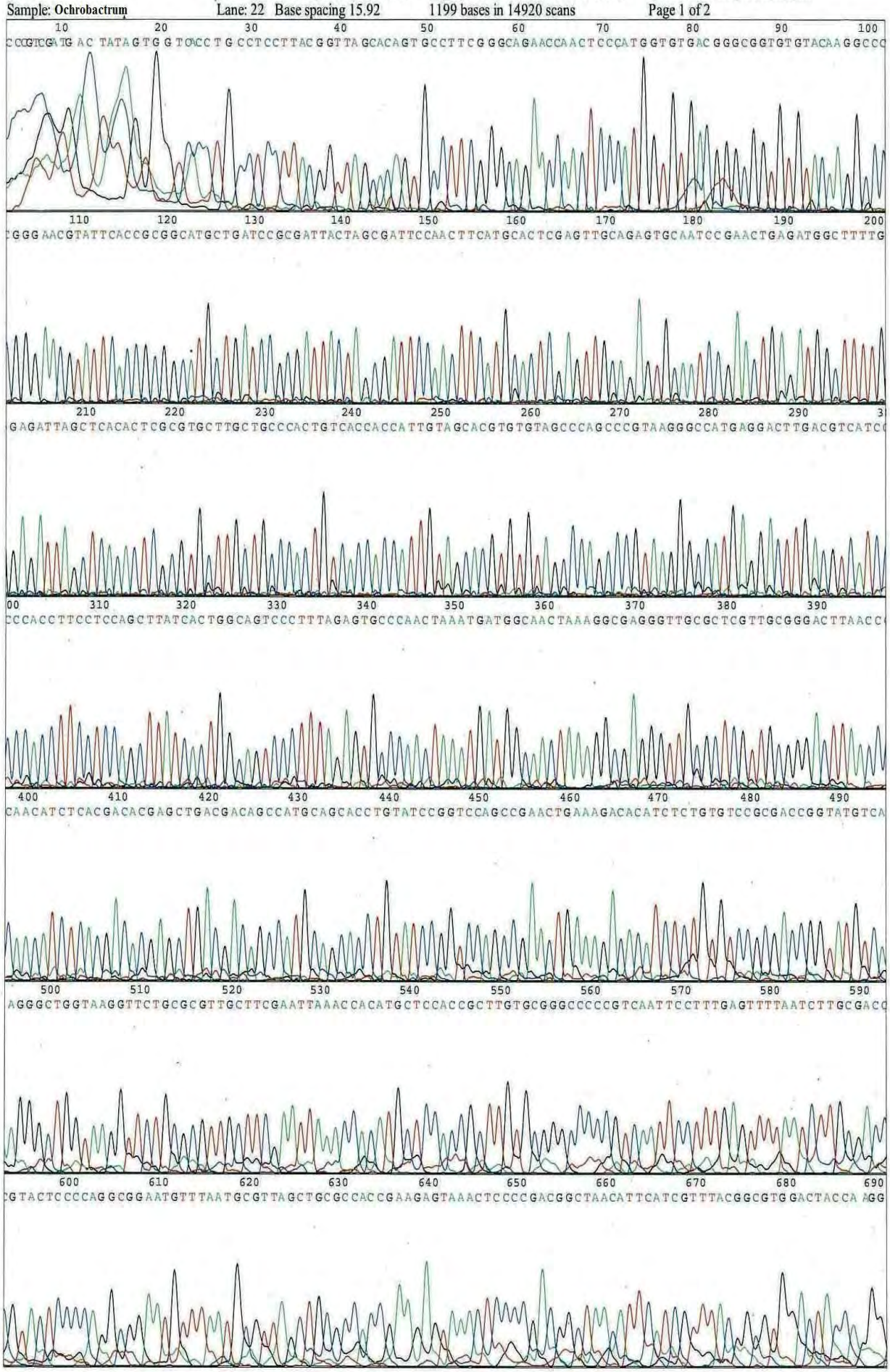
File: PT.ITS4-DP2_H04.ab1 Run Ended: Jun 10, 2010, 0:15:50 Signal G:79 A:72 T:81 C:75 Comment: Parsa Tehranchian
Sample: PT.ITS4-DP2 Lane: 9 Base spacing 15.65 861 bases in 14051 scans Page 2 of 2



File: P. carotovorum _RP2 Run Ended: Jun 3, 2011, 12:52:42 Signal G:309 A:338 T:357 C:354 Comment: Parsa Techranchian
Sample: P. carotovorum _RP2 Lane: 46 Base spacing 15.42 1101 bases in 13318 scans Page 1 of 2

File: P. carotovorum _RP2 Run Ended: Jun 3, 2011, 12:52:42 Signal G:309 A:338 T:357 C:354 Comment: Parsa Techranchian
Sample: P. carotovorum_ RP2 Lane: 46 Base spacing 15.42 1101 bases in 13318 scans Page 2 of 2




File: Ochrobactrum
Sample: Ochrobactrum
Run Ended: Jun 3, 2010, 16:29:36
Lane: 22 Base spacing 15.92
Signal G:1095 A:1048 T:961 C:899
1199 bases in 14920 scans
Comment: Parsa Tehranchian
Page 1 of 2

File: Ochrobactrum | Run Ended: Jun 3, 2010, 16:29:36 | Signal G:1095 A:1048 T:961 C:899 | Comment: Parsa Tehranchian

Sample: Ochrobactrum | Lane: 22 Base spacing 15.92 | 1199 bases in 14920 scans | Page 2 of 2