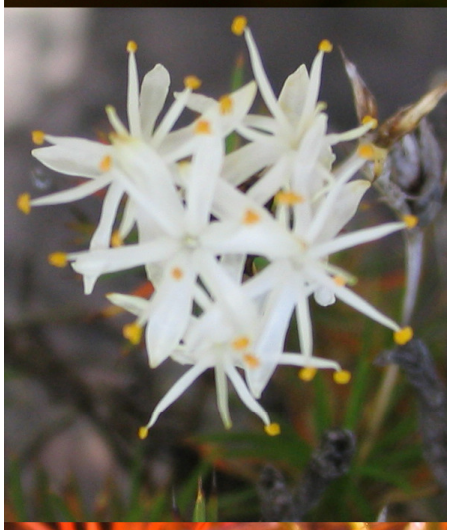
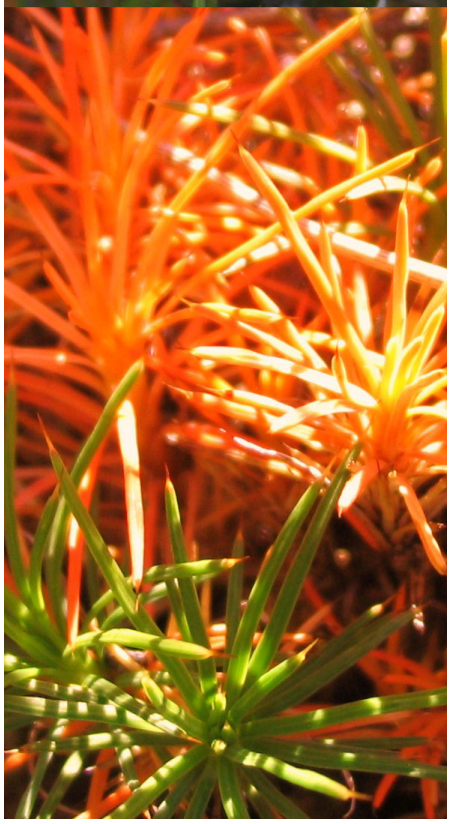
Borya mirabilis

steps in the recovery of a critically endangered Australian native plant





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A thesis submitted to the School of Applied Sciences, RMIT University in fulfilment of the requirements for Doctor of Philosophy, June 2008.

Declaration

Except where due acknowledgement has been made this work including all photographic material is my own. The work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of this thesis is the result of work which has been carried out since the official commencement date of my Doctor of Philosophy in March 2004, any editorial work, paid or unpaid, carried out by a third party has been acknowledged; ethics procedures and guidelines have been followed where required.

Parts of this thesis have been submitted for formal publication in National and International Journals.

Noushka H Reiter 22/06/2008

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Dad you were an absolute inspiration, this would have given you a chuckle.

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Summary

Borya mirabilis is one of the world's most critically endangered plants. The research in this thesis has illuminated key aspects of: its reproductive biology; interspecies and intraspecies molecular relationships, mycorrhizal status, tissue culture potential and disease threats. Each of these aspects has fundamental management implications for the active management of *B. mirabilis*.

Floral observations of *B. mirabilis* and related species affirmed the uniqueness of the Boryaceae amongst the Asparagales. *B. mirabilis* had an unusually high number of floral abnormalities compared with other species of *Borya* observed. *B. mirabilis* is fly-pollinated. Pollen of *Borya* species showed little difference in the characteristics of mature pollen between species, with viable pollen being prolate and unicolpate with a single colpa-style aperture and a unique patterning of the pila. The structural immaturity of *B. mirabilis* pollen correlated with evidence from pollen growth experiments, where *B. mirabilis* pollen had extremely low germination rates, with those grains that did germinate being slow to do so and with slow-growing pollen tubes compared to those of fertile *Borya* species. Examination of the ovules of *B. mirabilis* showed that morphologically they were viable compared to viable *Borya* species. The field population of *B. mirabilis* was crossed, with one seed produced (the first recorded seed for this species). Cross-pollination using the pollen of the closely related *B. constricta* and *B. sphaerocephala* with *B. mirabilis* ovules proved unsuccessful. Examination of the chromosome number of *B. mirabilis* showed that it had approximately 66 chromosomes and is probably hexaploid, relative to the diploid number of 26 in *B. constricta*. This may explain its low fertility.

Interspecies and intraspecies relationships of the Boryaceae and *Borya mirabilis* were investigated using sequences of chloroplast and nuclear DNA. The closest similarities to *B. mirabilis* were *B. constricta* and *B.*

sphaerocephala. *B. mirabilis* may have emerged from alloploidy of these species in the past. Because of the consistent similarities of *B. mirabilis* and *B. constricta* chloroplast sequences, it is proposed that both shared a common ancestor with a chromosome number of 2n=22. A malfunction n meiosis may have resulted in ovules with 2n=44. The high similarity of the nuclear ribosomal ITS region DNA suggests that the nuclear DNA was derived from *B. sphaerocephela*. *B. mirabilis* may be an allopolyploid, from fertilisation of a diploid ovule of *B. constricta* with haploid pollen of *B. sphaerocephala*, resulting in a reproductively isolated polyploidy of low fertility. The wild population of *B. mirabilis* was determined to have a small amount of genetic variation. The genetic variation in the field population was not fully reflected in the *ex-situ* population.

An effective means of micro-propagation of *B. nitida* for use in *B. mirabilis* has been established, providing an effective means of mass production of the species. The research has determined: a suitable explant (shoot tips) for regeneration; an effective means of reducing contamination in tissue culture (PPM); what medium is required to micro-propagate the species (LMHM); an appropriate gelling agent (Phytagel); and a practical method for inducing roots on the shoots grown in tissue culture.

B. mirabilis has been established as mycorrhizal. The predominant mycorrhizal association is a nodular arbuscular mycorrhiza, present in the form of coils in root nodules over wetter months and as spores in these nodules over dryer months. A significant increase in the health of the *ex-situ* population of *B. mirabilis* was recorded after addition of soil containing fine roots of the wild population. Of the plants associated with the wild population, *Callitris rhomboidea* had the most morphologically similar vesicular arbuscular mycorrhizal relationship. But molecular identification was not achieved due to recalcitrance of DNA in PCR attempts.

Potential translocation sites for some of the *ex-situ* population of *B. mirabilis* were examined for *Phytophthora* infestation. Reid's Lookout and

Mackey's Peak were infected with *P. cinnamomi*. Vegetation at Mackey's Peak displayed characteristic infection symptoms, resulted in isolates of *P. cinnamomi* from baiting and would directly receive runoff from both the walking track and the existing infested *B .mirabilis* site. At the Reid's Lookout site, both walking track and proposed translocation site were infested with *P. cinnamomi*, yet did not display the associated symptoms in the vegetation. The Pine Plantation translocation site was uninfected at the level of sampling undertaken. Its vegetation did not display any characteristic infection symptoms and was not isolated when soil samples were baited. It was therefore chosen for translocation and so far the plants are healthy and actively growing.

This research has provided critical knowledge to aid the recovery team in its current and future endeavours to manage this species and bring it back from the brink of extinction.

1. Introduction

1.1. 'The Problem'

Borya mirabilis Churchill is one of the world's most critically endangered plants and is in imminent danger of extinction. Only 70 ramets (shoots) from seven discrete colonies (plants) remain, all located within an area of 60 metres by 20 metres in the Grampians National Park in Victoria. The condition of the remaining plants was declining by 2000 and has worsened since then, with the notorious plant dieback pathogen *Phytophthora cinnamomi* Rands now isolated from the site. *B. mirabilis* is likely to become extinct without active and immediate preventative management.

At the commencement of this research project, little was known of many of the fundamental aspects of the species including the floral biology, genetic variability, tissue culture ability, mycorrhiza status, or the presence of pathogens in the *ex-situ* population or potential translocation sites. These gaps in the knowledge of this species were hampering the Recovery Team's efforts to manage this species and rescue it from the brink of extinction.

1.2. Aim and Scope

The specific aims of this project were to use systematic methods:

1 ***to improve sexual propagation of Borya mirabilis by:***

- hand-pollination involving all colonies in the wild at normal flowering time;
- depositing pollen closer to ovules;
- having a fuller understanding of the state of the plants' reproductive structures;
- attempting to produce hybrid seed with the phylogenetically closest species.

2 ***to improve vegetative propagation of Borya mirabilis by:***

- using innovative explants and media for tissue culture;

- improving media and cultural conditions to maintain cuttings and deflasked material;
- investigating the mycorrhizal associations of *B. mirabilis* and associated flora;
- testing mycorrhizal propagules for their effect on survival, rooting and growth *ex-situ*.

3 ***to improve translocation of Borya mirabilis by:***

- acquiring a fuller knowledge of the genetic diversity of the *ex-situ* population and the *ex-situ* collection so as to properly represent this in translocation sites;
- having a fuller understanding of the presence of pathogens in the *ex-situ* population and proposed translocation sites.

Fulfilment of these aims would achieve the short-term goals of increasing the population and spread of this species and would ensure its continued or improved genetic diversity. These are vital goals in the path to recovery and establishing self-sustaining secure populations for the future, both *in-situ* and *ex-situ*.

1.3. Overview of Study

The first two chapters give an overview of the thesis and background information on *B. mirabilis*. Chapters 3 to 7 are research chapters each with specific literature relevant to that area of research presented as an introduction to the relevant chapter. The floral biology of *B. mirabilis,* including pollen and ovule structure and viability, ability to produce seed, the presence of pollinators and chromosomes is investigated in Chapter 3. Chapter 4 investigates the molecular phylogeny of the Boryaceae and the population genetic structure of *B. mirabilis*. Chapter 5 explores methods of producing *B. mirabilis* through tissue culture. Chapter 6 investigates the mycorrhizal status of *B. mirabilis* in the field and the benefits of introducing mycorrhiza to the *ex-situ* plant population. Chapter 7 discusses the presence of known pathogens especially *P. cinnamomi,* in the *ex-situ* collection and

proposed translocation sites. Chapter 8 evaluates the success of each of these avenues of research and draws management conclusions from these.

2. Background

2.1. History of the Boryaceae

Borya Labill. species were first noticed on rocky outcrops in early expeditions in the 1800's and the genus was first officially recognized in 1805, in Western Australia, by the French botanist Labillardière (Hopper *et al*. 1997). *Borya* species have been discovered more recently in the Northern Territory, Queensland and Victoria. The genus *Borya* was first thought to be a member of the family Liliaceae but in 1984 was put into a separate family, Anthericaceae, by Keighery (1984). *Borya* species were more recently put into their own family, the Boryaceae, which they share with one other monotypic genus (*Alania* Endl.). The family was confirmed as a unique monophyletic clade amongst the Asparagales based on *rbcL* research by Chase *et al*. (1996).

Bremer (2000) put the relationship of Boryaceae within the monocots into perspective using DNA sequence data of the plastid *rbcL* gene in his study on the early Cretaceous lineages of monocotyledonous flowering plants. Bremer placed the Boryaceae as a sister group to the Orchidaceae. While it is currently accepted that the Boryaceae belong to the Asparagales, their relationships to other families within the clade are widely argued and form part of the research presented in this thesis.

2.2. Distribution of the Boryaceae

The genus *Borya* consists of 13 species (Table 2-1), distributed widely throughout areas of reasonably high but seasonal rainfall (Figure 2-1). *Borya* species are found predominantly on rock outcrops, but can be found amongst the grassy under-storey of open woodlands in Western Australia with *Eucalypt* or *Callitris* over-storeys. The majority of the family is found in the south-west corner of Western Australia.

Table 2-1: Known *Borya* species and states of Australia in which they are found, asterisked indicates threatened species.

Species	*Distribution*
*Borya mirabilis** Churchill.	Victoria
Borya inopinata P.I. Forst. and E.J. Thomps.	Queensland
Borya septentrionalis F. Muell.	Queensland
Borya constricta Churchill.	Western Australia
Borya laciniata Churchill.	Western Australia
*Borya longiscapa** Churchill.	Western Australia
Borya nitida Labill.	Western Australia
Borya scirpoidea Lindl.	Western Australia
Borya sphaerocephala R.Br.	Western Australia
Borya stenophylla Barrett ined	Western Australia
Borya subulata C.A. Gardner.	Western Australia
Borya sp. *Wheatbelt* (A.S. George 16470) WA Herbarium.	Western Australia
*Borya jabirabela** Churchill.	Western Australia/Northern Territory

Three of the 13 species of *Borya* are currently endangered (asterisked). Two of these, *B. longiscapa* and *B. jabirabela,* grow in Western Australia along with the majority of *Borya* species. *B. mirabilis,* the topic of this thesis, is found in the Grampians National Park (Gariwerd) approximately 3 hours' drive north-west of Melbourne, Victoria. Although several accounts of *B. mirabilis* have been recorded from both the Wonderland Range and Victoria Range, there is currently only one known site in the Wonderland Range. Despite organised community searches on foot and by helicopter it remains the sole authenticated site. *B. mirabilis* is one of the world's most critically endangered plants (*sensu* IUCN, 1994).

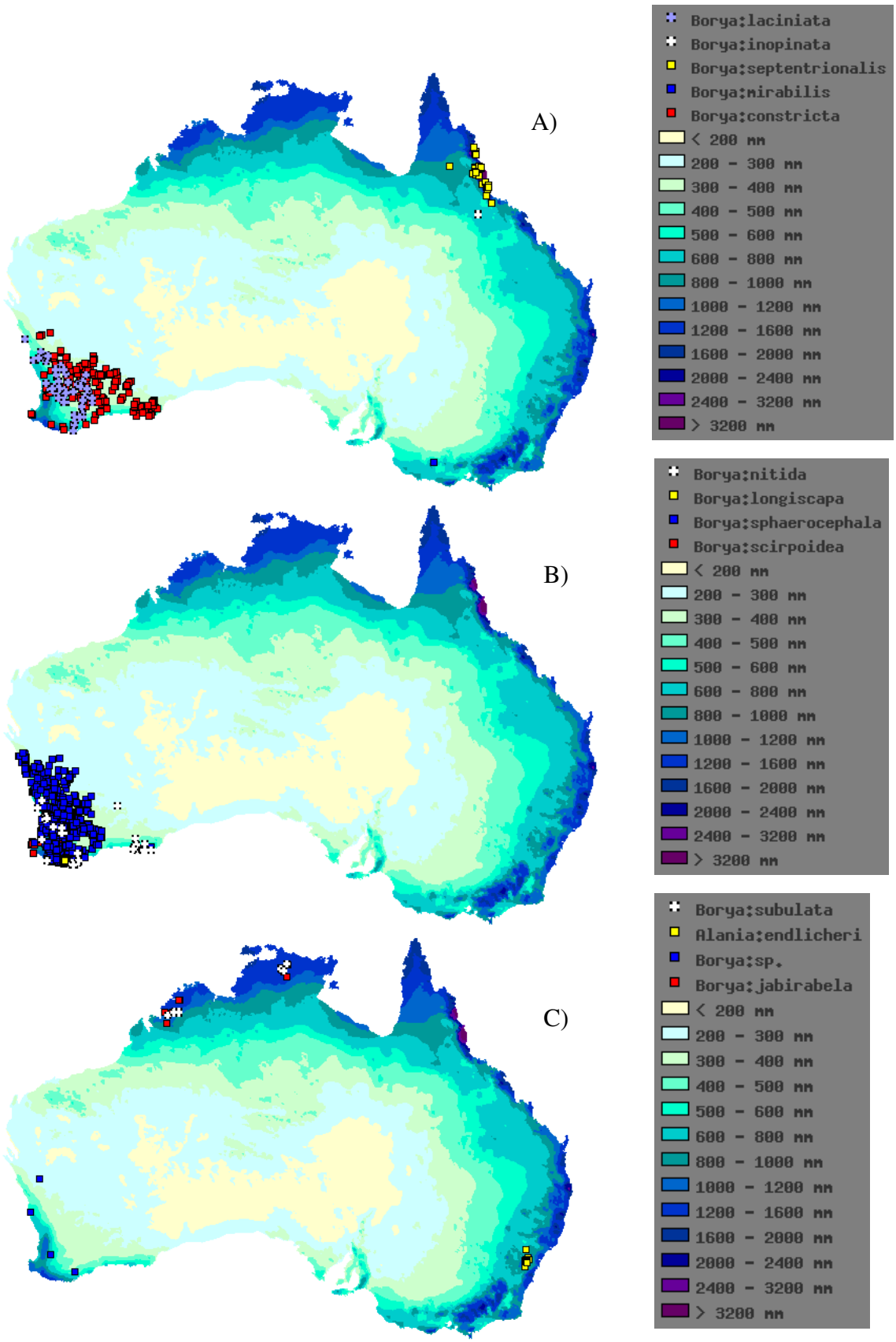

Figure 2-1: Approximate distribution map of all known species of the Boryaceae; distribution compiled from known data on the Boryaceae from herbarium records obtained from all Australian state herbaria. A) distribution and annual rainfall for *Borya mirabilis, B. inopinata, B. septentrionalis, B. constricta and B. laciniata.* B) distribution and annual rainfall for *B. longiscapa*, *B. nitida, B. scirpoidea* and *B. sphaerocephala*. C) distribution and annual rainfall for *B. subulata*, *Alania endlicheri*, *Borya sp.* and *B. jabirabela*.

2.3. Physiology of *Borya* species

B. mirabilis is desiccation-tolerant, like all others of this genus (Gaff and Churchill, 1976). Desiccation-tolerant plants occupy a niche left by other plants, as the environmental fluctuations in these microclimates are too severe for many other plants to grow. Despite their adaptations to live in extreme conditions, the Boryaceae are also found in areas of relatively adequate and reliable rainfall (Figures 2-2 and 2-3).

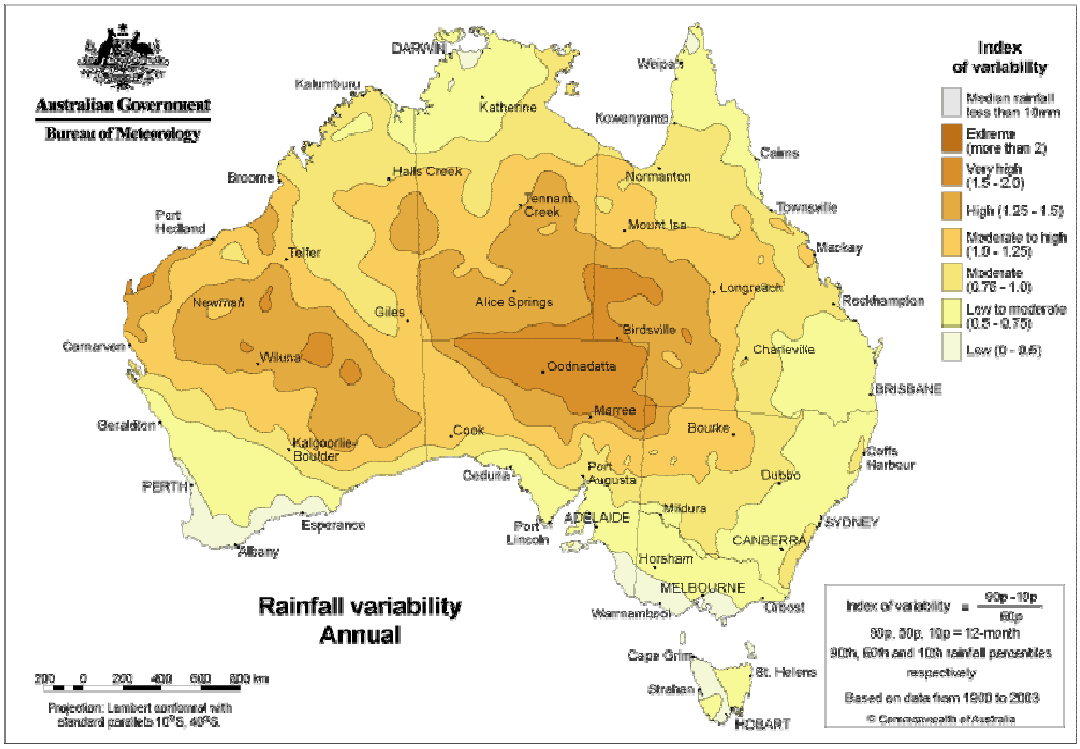

Figure 2-2: Map of Australian rainfall variability; areas in which the Boryaceae are located have low to moderate rainfall variability. Map compiled by the Australian Bureau of Meteorology (http://www.bom.gov.au/climate/).

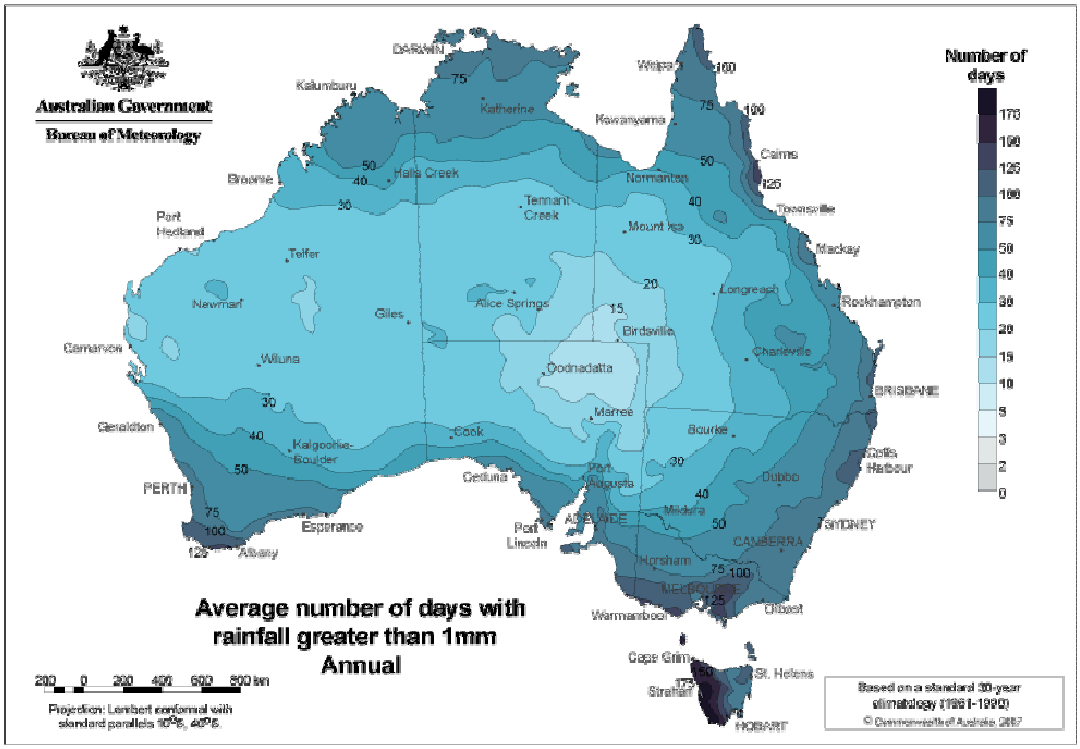

Figure 2-3: Map of Australian average rainfall; areas in which the Boryaceae are located receive 40 days or more of greater than 1 mm rainfall. Map compiled by the Australian Bureau of Meteorology (http://www.bom.gov.au/climate/).

Desiccation-tolerant vascular plants are rare, and have a particular affinity for rocky outcrops and inselbergs, which are islands of steep-sided, often granitic rock (Porembski and Barthlott, 2000), like that of Marichetti Rock in Western Australia (Figure 2-4). There are two forms of desiccation-tolerant vascular plants: homoiochlorophyllous (these keep most of their chlorophyll and thylakoids when dehydrated) and poikilochlorophyllous (these lose most of their chlorophyll and thylakoids when dehydrated) (Tuba *et al*. 1998). The poikilochlorophyllous plants which include *Borya* species are restricted to monocotyledons (Bewley and Krochklo, 1982). Fewer yet of these species have another curious adaptive feature, the velamen radicle (a spongy multi-layered epidermis covering aerial roots, like that in epiphytic orchids), which aids with water absorption and which the *Borya* species share with other mat-forming desiccation-tolerant species (Porembski and Barthlott, 2000).

Figure 2-4: Marichetti rock near Newdegate in Western Australia, an example of an inselberg of particular importance for *Borya* species. The orange ground vegetation surrounding the rock consists mostly of *Borya* species (indicated by the red arrow).

Inselbergs are paramount to the survival of desiccation-tolerant plants and their importance has been emphasized in literature since Hambler (1961). The majority of desiccation-tolerant plants are found in Western Australia, Africa and South America. Although Gaff (1987) proposed that Australia's desiccation-tolerant species are less tolerant of water loss than their colleagues on other continents, possibly due to a shorter time for adaptation, *B. nitida* is one of the most desiccation-tolerant species in existence, with the ability to survive the loss of over 94% of its water and occurs in Western Australia along with many other species of the same genus. Porembski and Barthlott (2000) propose that the ancestors of today's resurrection plants occurred in similar habitats. These habitats and their inhabitants are threatened due to climatic changes and loss of habitat through grazing and farming.

Borya species are typical of the majority of desiccation-tolerant vascular plants, generally forming mats on inselbergs (Porembski and Barthlott,

2000). Although it is widely thought that *Borya* species prefer rock outcrops, members of the genus grow well on flat sandy soils in Western Australia with a grassy under-storey, and either a *Eucalyptus* or *Casuarina* over-storey (Figures 2-5 and 2-6).

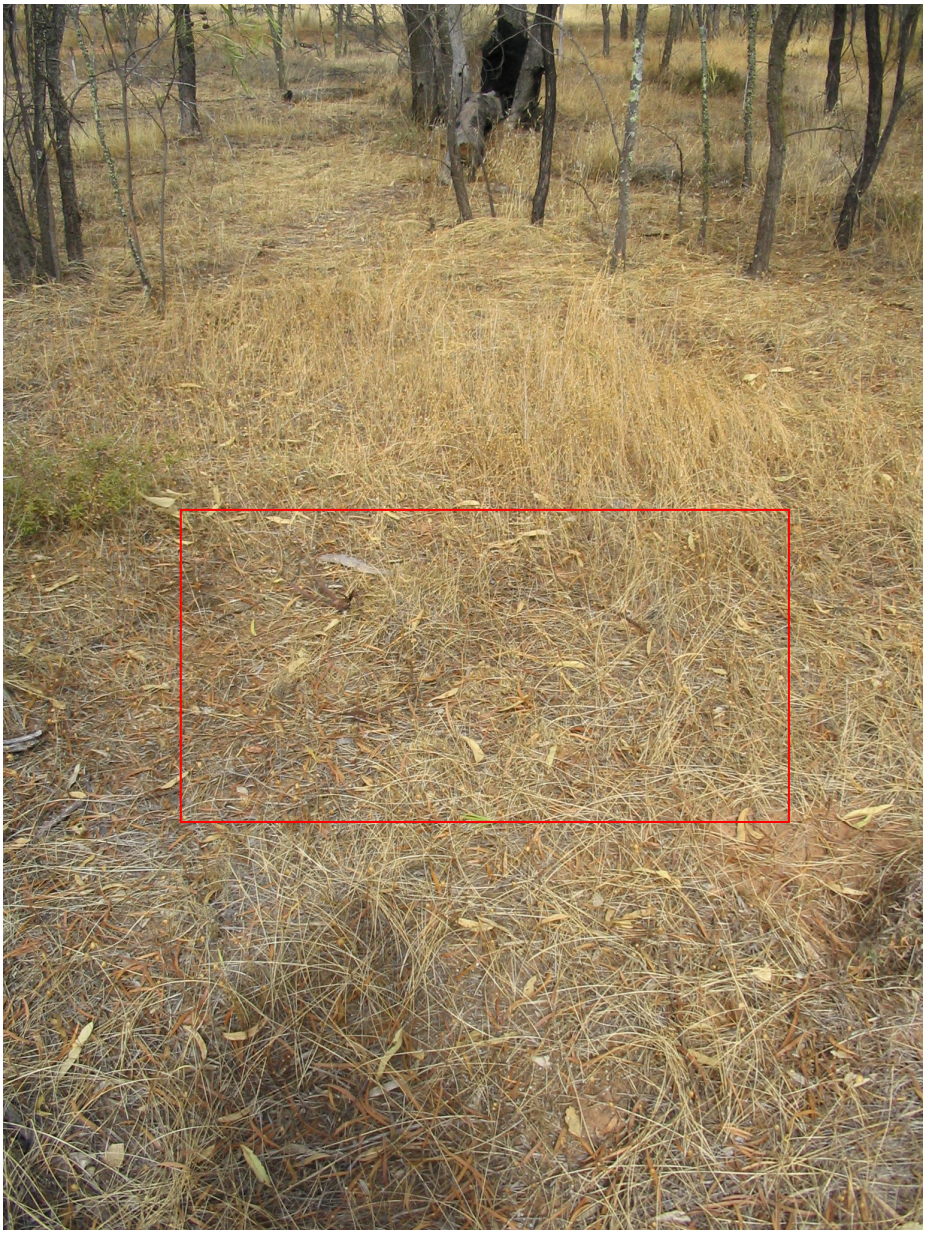

Figure 2-5: Open *Casuarina* and *Eucalyptus* woodland with grassy understorey, near Beverley in Western Australia. Typical habitat of some *Borya* species, with red box indicating the location of *Borya sphaerocephala* plants amongst the grass.

Figure 2-6: *Borya sphaerocephala* growing amongst native grasses, *Casuarina* and *Eucalyptus* leaf litter. This is a close-up of the previous figure, with red arrows indicating *B. sphaerocephala* plants.

Borya species, like many other resurrection plants, are able to survive a state of dehydration for months and possibly years (Gaff, 1987). The process in which the leaves of resurrection plants shrink without damage to the tissue is known as cytorrhysis. *Boryas* take advantage of this either by desiccating or shedding their leaves, and thus survive dry, hot summer months.

Studies on *B. nitida* suggest that chlorophyll is lost with the degeneration of chloroplast thylakoids (Gaff and Churchill, 1976). Rehydration then triggers the reformation of the chloroplast lamellae, causing re-greening (Gaff and Churchill, 1976). This capacity for re-greening is greater in older than younger leaves, as they have greater resources (Hetherington and Smillie, 1982). Dehydrating leaves undergo a series of colour changes, going from green to a brilliant shade of orange and finally a straw-like color. With sufficient rainfall, the plants slowly spring back to life, re-greening from the

bases of their leaves (Gaff, 1987). Several weeks after adequate rain, the plants begin to bud, putting forth a shoot from the apex of each stalk. These buds hold an inflorescence of from four to 12 flowers, which separate slightly from the bud before the small white flowers open.

2.4. Distribution and description of *Borya mirabilis*

B. mirabilis was first discovered in 1924 by C.W. Dalton and is located in the Greater Grampians Bioregion in Victoria. The Mackey's Peak population located within the Wonderland Range of the park was first thought to be an isolated occurrence of the Western Australian *B. nitida*. This opinion quickly changed based on morphological evidence by Churchill (1985, 1987), who defined it as a unique species distinguished by its bracteoles, which are longer than its floral bracts. The species was presumed extinct, as it was not sighted in the field for many years. The Mackey's Peak population was rediscovered in 1983 by a school teacher, who organized a systematic search of Mackey's Peak with his students. Although the population was rediscovered on the same peak, it was in a different location from that originally recorded. There are various unconfirmed reports of another population located elsewhere within the Victoria Range.

B. mirabilis is a perennial herb with multiple shoots (ramets) (Figure 2-7) growing to 15 cm in height. Its stems are a brown-grey color and are covered in old leaf sheaths which develop as the plant grows and have the appearance of scales. Stems can be multi-branched and some have aerial roots with the appearance of dark black or olive-green wire. Leaves are small, linear and spiky, with a detachable spike, and are from 10-16 mm long and 0.5 mm wide. Growth of new shoots and inflorescences is terminal. Flowers are white, born on inflorescences, and take up to 4 months to mature. Flowers are born on scapes 3-7 cm long in an ovoid head containing 4-12 flowers (Churchill, 1985; Reiter personal observations). The plant enters its desiccation state after its late spring flowering (Figure 2-8) and re-greens with milder, wetter weather at the end of summer (Figure 2-9). The species does not produce seed (Churchill, 1987). *B. mirabilis*, like the

majority of *Borya* species, is pseudostemmed (the stems have a type of secondary thickening) (Porembski and Barhtlott, 2000). *B. mirabilis* also has adventitious roots.

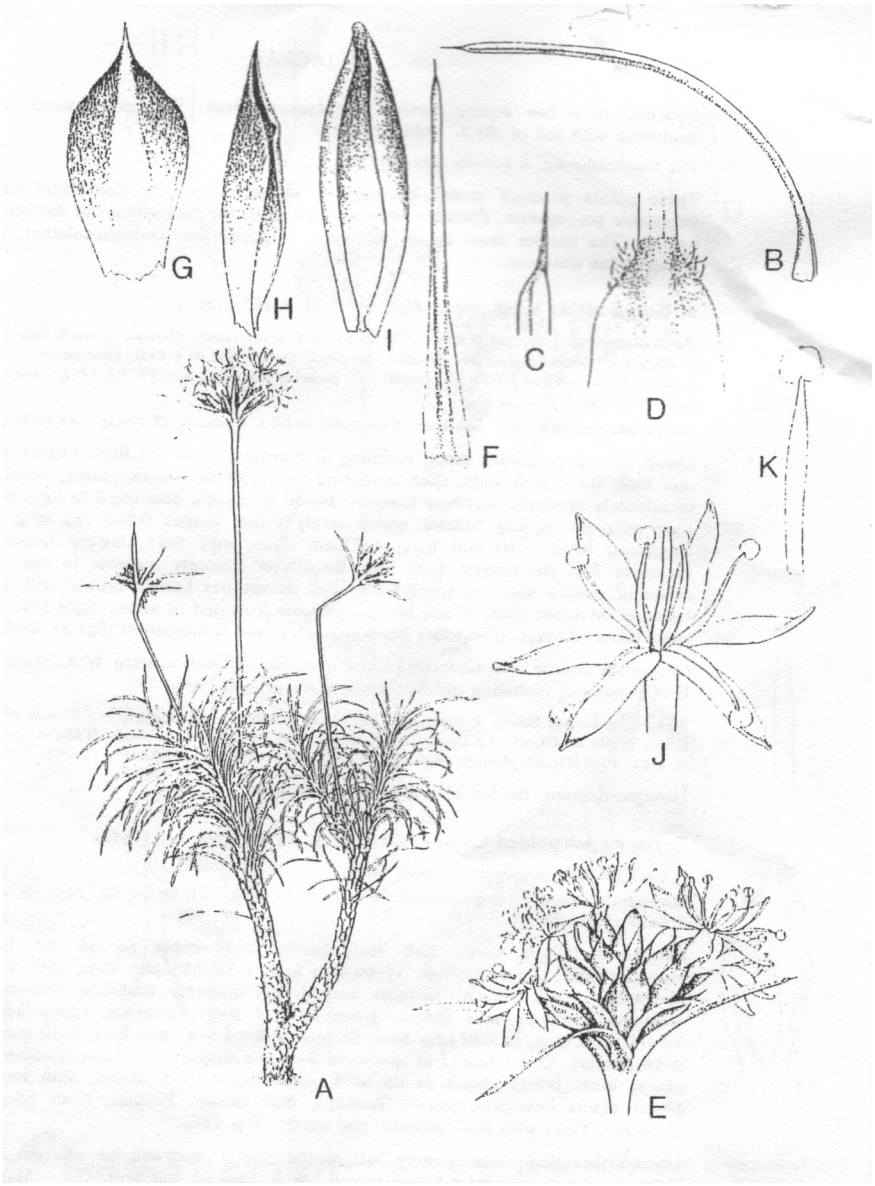

Figure 2-7: From Flora of Australia (Churchill, 1987). Floral biology of *Borya mirabilis* A. fertile shoots X 1: B, leaf with basal sheath X 3; C, leaf tip X 8; D, leaf base X 8; H. flower bract X 8; I, flower bracteole X 8; J, flower X 4; K. stamen X 8 (A-K, cultured Royal Botanic Gardens, Melbourne), Drawn by A. Podwyszynski.

Figure 2-8: Colony 5 of *Borya mirabilis* entering resurrection mode in the last week of October 2004 (prior to the Mt Lubra fires).

Figure 2-9: Colony 5 of *Borya mirabilis* re-greening in June 2004 (prior to the Mt Lubra fires).

The plants are located in an area of sprawling terraced rock outcrops of ferruginous sandstone and sandy loam soil ranging from 1 cm to 1 m deep (Kohout and Coates, 2007). According to the Action Statement released by the Department of Sustainability and Environment in 2003, the Mackey's Peak population covered an area of 60 m by 20 m and consisted of 7 colonies, containing approximately 70 ramets (Figure 2-10). The plants form irregular clumps of ramets within the colonies and individual 'plants' (= clumps of ramets) within the colonies have been tagged and monitored bi-annually by Parks Victoria over the last decade. Of these 7 colonies, colony 1 and 3 were in extremely poor health in 2005 at the initiation of the current research project. The associated plant species on this site (Table 2-2) include common trees (*Eucalyptus alaticaulis*, *Callitris rhomboidea*), shrubs (*Leptospermum scoparium*, *Grevillea aquifolium*), grasses (*Austrodanthonia setacea*) and herbs (*Drosera whittakeri*).

Figure 2-10: Site of *Borya mirabilis in-situ* population numbers 1- 7 indicating the five surviving colonies of *B. mirabilis* located in the Grampians National Park in a 60 m by 20 m area (sourced from Recovery Team information 2004). Each of these colonies contains several 'plants' and each plant numerous ramets.

Table 2-2: Species associated with the naturally occurring populations of *Borya mirabilis* as listed in the Action Statement by Coates *et al*. (2003).

Dominant species	***Associated species***	***Annual herbs***	***Rare species***	***Other***
Calytrix tetragona	*Austrodanthonia setacea*	*Centrolepis aristata*	*Austrostipa hemipogon*	Bryophytes
Dodonaea viscosa subsp. *spatulata*	*Callitris rhomboidea*	*Centrolepis strigosa*		Lichens
Grevillea aquifolium	*Eucalyptus alaticaulis*	*Drosera peltata* subsp. *auriculata*		
Kunzea parvifolia	*Gonocarpus mezianus*	*Drosera whittakeri* subsp. *aberrans*		
Melaleuca decussata	*Leptospermum scoparium* J	*Siloxerus multiflorus*		
	Lepidosperma viscidum			
	Phyllanthus hirtellus			
	Thryptomene calycinia			

The Mt Lubra fires of 2006 took place in the middle of the research in this thesis, completely leveling the entire site and incinerating all the *B. mirabilis* plants (Figures 2-10 and 2-11).The plants on the natural site were thus temporarily extinct. Charcoal deposits on the site suggest the area has been burnt previously, however, the response of *Borya* plants to fire had never been studied or documented previously. Since 2006, the plants have regenerated post-fire from underground tissue and the monitoring results from Parks Victoria show that 50% of the plants had regenerated 6 months after the fire. This figure has dropped slightly in the intervening period; 43% regeneration was observed in the most recent monitoring, in November 2007 (Figure 2-12). Importantly, two of the original seven colonies have not regenerated post-fire; one of these was possibly dead prior to the fire and the other colony had been in declining health over the last decade and was infested with *P. cinnamomi*.

Figure 2-11: *Borya mirabilis* site, outlined in red and lacking any over-storey after the Mt Lubra fires in 2006.

Figure 2-12: Colony 5, 6 weeks after the Mt Lubra fires of March 2006. The red box outlines the area in which Colony 5 previously covered.

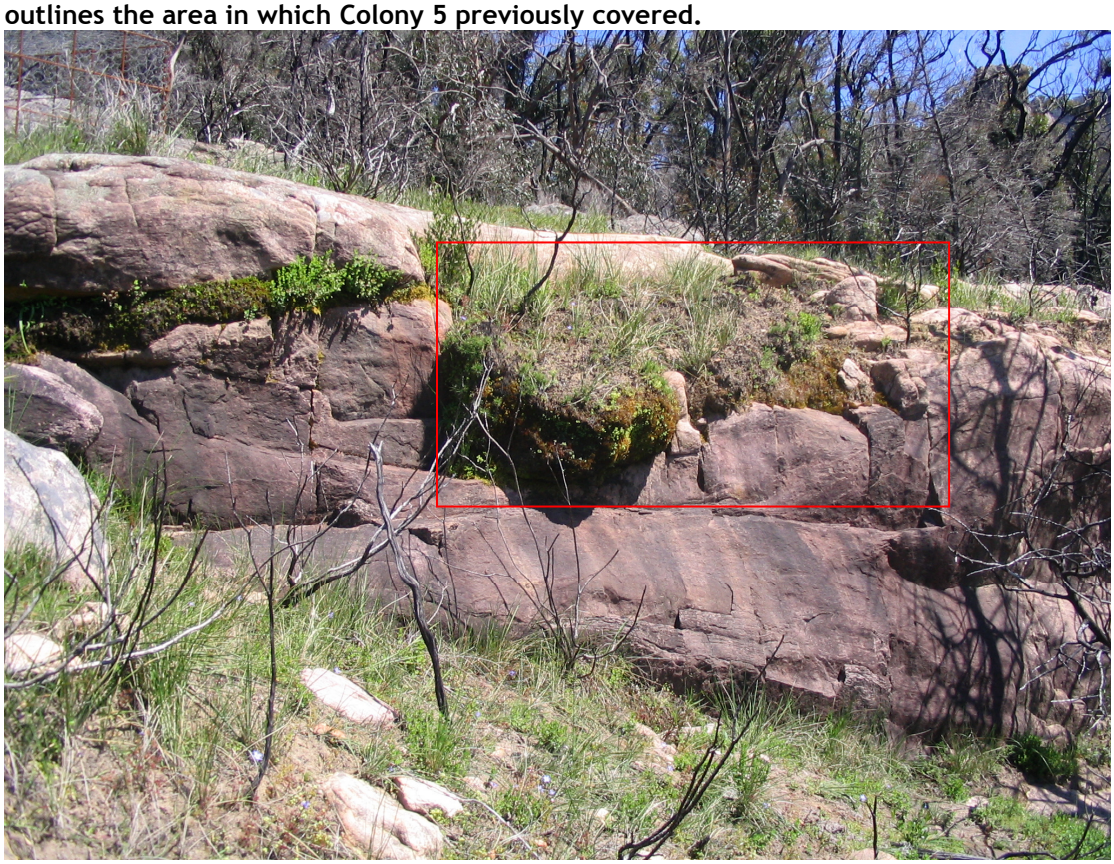

Figure 2-13: Colony 5 in September 2007 recovering from the Mt Lubra fires and under heavy competition from other post-fire regenerating plant species, outlined in the red box.

2.4.1. Management of *Borya mirabilis*

B. mirabilis is listed as endangered species nationally under the Environment Protection and Biodiversity Conservation Act 1999. It meets three of the five criteria, in that:

- the distribution of the species is low and precarious;
- the number of the mature individuals is low;
- the number of total individuals is low.

In Victoria, *B. mirabilis* is listed as a threatened taxon under the Flora and Fauna Guarantee Act 1988. A recovery plan (Appendix 2-1) was written by Fiona Coates in 2001 (Coates *et al*. 2002) and a further draft recovery plan was written in 2007 (Kohout and Coates, 2007).

The Recovery team still struggles to propagate plants and implement some of the original plans. An *ex-situ* population established from cuttings at the Royal Botanic Gardens, Melbourne is of particular importance to the conservation of the species.

One of the critical areas of management of the *B. mirabilis* Recovery Team is the successful establishment of new translocation sites. Three major requirements in the successful establishment of translocation sites in the short term as outlined by Vallee *et al*. (2004) are:

- the management and control of threats;
- having a suitable number of individuals within a population to survive environmental fluctuations;
- the ability to reproduce and recruit on their own.

A lack of basic knowledge on this species' growth requirements is hampering the efforts being made to recover *B. mirabilis*. The research presented in this thesis will begin the investigation into these areas, including: the molecular taxonomy of the species; population genetic structure; floral biology; tissue culture; mycorrhizal associations and the requirements for translocation.

At the outset of this research little was known of these mentioned topics and in many cases research had never been attempted previously. When this

research was initiated in 2004, *B. mirabilis* was thought not to produce seed (Churchill, 1987). Population analysis had been attempted in earlier studies; both allozyme (Y. Fripp and S. Cropper, La Trobe University, unpubl. data) and RAPD genetic analysis (Coates *et al*. 2002) had been carried out on the population previously. Allozyme analysis described Colony 1 as unique but RAPD analysis using 20 primer pairs found no differences between the colonies.

Although no one had witnessed pollination with *B. mirabilis* previously, Keigherley (1984), in his study of Western Australia *Borya* species, reported that they are pollinated by flies. He also reported that the roots are mycorrhizal, although no further details of these studies have been published. Coates *et al*. (2002) investigated post pollination events in *ex-situ B. mirabilis* plants and concluded that the pollen tubes did not reach the ovaries. The methods used in this study did not elucidate the cause of the apparent infertility, as the study was only a preliminary investigation of the biology of the species. If *B. mirabilis* has lost the ability and pollinator to produce seed, it is little more than a living relict in the field.

2.4.2. Known threats to *Borya mirabilis*

Phytophthora cinnamomi

Death of shrubs on the Mackey's Peak site over the past decade and a general decline in the health of the plants, have been of concern and a focus area of the original Recovery Plan. The shrub deaths and decline in plant health were attributed to changes in the climatic conditions over the last decade and the prevailing drought in the region (Coates *et al. 2002*), rather than to pathogens such as *P. cinnamomi*.

The field population was, however, tested by Reiter *et al*. (2004) in 2001 and Colony 1 infected with *P. cinnamomi*. The area was then further mapped for the presence and distribution of *P. cinnamomi* on the site in a grid (68 m by 36 m) from which 5 out of 153 samples tested positive for *P. cinnamomi* (Reiter, 2002), with these positive samples distributed throughout the site. It is likely therefore that the decline in the health of the vegetation of the area is caused by *P. cinnamomi* (listed as a nationally threatening process) and the

effects of the pathogen on the vegetation have likely been exacerbated by the drought over the last decade.

Erosion

Erosion has been of concern on the site particularly since the Mt Lubra fires of 2006.

Lack of recruitment

There is a lack of recruitment on the site, through both seed and vegetative reproduction.

2.4.3. Active management of *Borya mirabilis*

The field population is monitored either biannually or quarterly by Parks Victoria staff to assess the health and growth of the populations. Regular aerial and ground surveys are instigated and conducted by Parks Victoria staff and community groups. Large tracts of the Grampians National Park have now been surveyed. Unfortunately no other population of *B. mirabilis* has been found to date. A small *ex-situ* collection at the RBG has been established for some time from cuttings. At the initiation of this research, the general health of the plants was of concern.

In the field, two colonies have been caged, colonies 4 and 7. Prior to the fires they were caged with shade cloth. When the fire passed through the shade cloth melted and encrusted over the plants and surrounding area (Figure 2-14). Post-fire the colonies have been caged with wire mesh instead (Figure 2-15). Jute matting and plastic stepping stones have been installed after the 2006 fire to minimize erosion and degradation of the site when monitoring is undertaken (Figure 2-16). Hygiene measures have been initiated on the site to reduce impact on the site and spread of *P. cinnamomi*. Plants in the field population have been mulched with *Casuarina* leaves in an effort to increase soil microbes and sprayed with phosphonate until the fires, to minimize the effect of *P. cinnamomi* on the plants. Recommendations to re-route the walking track and fence the entire site (Reiter, 2002) have been made. A post-and-wire fence was installed along the walking track, but it is only a visual deterrent and did not keep people or animals out. This fence was destroyed during the fires along with the walking track, which is now closed and is not planned to reopen.

Figure 2-14: *Borya mirabilis* population that had been covered with shade cloth pre-Mt Lubra fire, resulting in melted mesh over the plants.

Figure 2-15: Stepping stones on *Borya mirabilis* site to minimise erosion.

Figure 2-16: Installation of matting post-Mt Lubra fire to minimise erosion on *Borya mirabilis* field site.

B. mirabilis is unique as a species, in that it is geographically isolated and restricted and has a physiological ability to resurrect its leaves. The lack of recruitment and low numbers both in the *ex-situ* and field population make this species extremely susceptible to anomalies such as wildfire, and these events pose a real risk of extinction to the species. Although this species is being actively managed, some of these management decisions have taken place without key areas of knowledge (which is understandable both in an economic and management sense). The resulting management without sound knowledge could have long-term implications for the survival of the species.

To achieve the main goals of translocation and ultimate survival of the species, key areas that need to be addressed, and which are explored in the following five chapters are:

- Why is seed set absent (Chapter 3)?
- What are the relationships of *B. mirabilis* plants to one another and with other *Borya* spp. (Chapter 4)?
- Can tissue culture be used to improve vegetative propagation (Chapter 5)?

- Is *B. mirabilis* mycorrhizal? Can the addition of mycorrhizal propagules improve the health of the *ex-situ* population (Chapter 6)?
- How can we minimize pathogen associated problems in translocation (Chapter 7)?

3. Floral Biology

3.1. Introduction

Reproductive biology has an important place in the management of endangered plants. To establish successful reproduction in endangered species, knowledge is often required of the entire life history. This information may take many years to gather and has been the source of long term research in many endangered species (Weekley and Race, 2000). Self-incompatibility poses a serious problem and can, without aggressive intervention, doom a species to extinction (Weller, 1994).

A key area of knowledge in the recovery of any threatened species is its reproductive biology. This is a core component in developing any recovery program (Weller, 1994). Several areas for illumination include:

- Floral biology and flowering phenology: What are the arrangements of the floral parts within the flower? What is the spatial and temporal separation of the male and female organs within the flowers (Anderson *et al*. 2001, Evans *et al*. 2003)? When are the male and female organs within the flower mature and receptive (Evans *et al*. 2003)? Are the pollen and ovules developing correctly and viable?
- Pollination: What insects/mammals visit the flowers (Hai-Qin *et al*. 2003, Osunkoya, 1999)? Did they contact male and female parts of the flowers (Evans *et al*. 2003), for how long did they visit and how many flowers did they visit (Evans *et al*. 2003)? Were there any smell or nectar rewards associated with the flowers?
- Mating system: Does the plant have spontaneous pollination (Evans *et al*. 2003)? Are the plants able to reproduce asexually (Evans *et al*. 2003)? Are the flowers self-compatible (Wallace, 2003; Weller, 1994; Weekley and Race, 2000; Evans *et al*. 2003)? Are the plants able to outcross? Are the flowers successfully pollinated when left alone (Evans *et al*. 2003)?

These areas have proved fundamental in how to manage the endangered population, as can be seen in the case studies below.

- Seed production limited due to deficiencies in pollinator abundance, a widespread phenomenon (Bierzychudek, 1981; Lamont *et al*. 1993; Bond, 1994; Steiner and Whitehead, 1996; Mawdsley *et al*. 1998; Larson and Barrett, 2000; Spira, 2001), e.g. in the endangered orchid *Changniena amoena* (Hai-Qin *et al*. 2003)
- Mating patterns determining genetic variation within and between populations (Hamrick *et al*. 1991; Ellstrand and Elam, 1993; Hamrick and Godt, 1996)
- Self-incompatibility, e.g. in the Mauna Key Silversword (Walker and Powell, 1999)
- Reduced reproductive success, e.g. in the endangered plant *Arnica montana*, due to the lack of cross-compatible pollen, possibly due to a reduced set of S alleles (Kahmen and Poschlod, 2000)
- Determination of which populations or plants of a species should be protected, e.g. in *Cordylanthus palmatus* (Fleishman *et al*. 2001)
- Low seed survival, e.g. in the endangered *Aster kantoensis* (Kuramoto *et al*. 1995).

3.1.1. Borya mirabilis

Many of the aspects of the documented biology of *B. mirabilis* have been outlined in the previous chapters. For clarity, it is worth reminding the reader that there is only one known natural population (*in-situ*) of *B. mirabilis* in the wild, which is located in an area 60 m by 20 m on Mackeys Peak in the Grampians National Park (Figure 3-1). This *in-situ* population consisted prior to the Mt Lubra fires of seven colonies (five of which were known to be alive), each colony containing several plants consisting of up to 100 ramets (Figure 3-2). There is also an *ex-situ* population of *B. mirabilis* (approximately 20 'plants') located at the RBG Melbourne which has been sourced from cuttings of the *in-situ* population, but the details from which colony and plant they were taken have been lost.

Figure 3-1: Site of *Borya mirabilis in-situ* population numbers 1- 7 indicating the five surviving colonies of *B. mirabilis* located in the Grampians National Park in a 60 m by 20 m area (sourced from recovery team information) each of these colonies contains several plants and each plant numerous ramets.

Figure 3-2: *Borya mirabilis* 'plant' 1 (of 13 'plants' in Colony 5) consisting of approximately 125 ramets within Colony 5 of the *in-situ* population (Figure 1) in the Grampians National Park.

Currently little is known of the reproductive biology of *B. mirabilis*. Preliminary floral structure has been documented the Flora of Australia (Churchill, 1987), yet no recruitment or seed set have been observed in either the *in-situ* or *ex-situ* population. It has therefore been assumed that *B. mirabilis* is self-incompatible (Coates *et al*. 2002). This was a very preliminary

study in which *ex-situ* plants showed that pollen germinated on the stigma but pollen tubes did not progress past the end of the style. This study however, neither compared the pollination and fertilisation of *B. mirabilis* to that of other species in the genus nor did appropriate crossing, with knowledge of the spatial as well as the temporal separation of the female and male reproductive parts.

Translocation of some of the *ex-situ* rooted shoot cuttings from the RBG of *B. mirabilis* was undertaken in 2005 as part of the actions undertaken by the Recovery Team. The translocation was undertaken because the threat to the survival of the only known population of *B. mirabilis* was considered too great. The translocation was undertaken without some fundamental knowledge on the floral biology of the species or the genus in general, which is a key area of knowledge when conducting pre-translocation assessments (Vallee *et al*. 2004). It was therefore necessary to begin this study of *B. mirabilis* by examining various aspects of its floral biology in order to understand the role that this plays in the species' predicament.

The aim of this chapter was to understand if the lack of recruitment and seed seen in the field with *B. mirabilis* is a result of lack of pollinator, self-incompatibility, pollen inadequacy or ovule inadequacy.

3.2. Methods

3.2.1. Floral observations

3.2.1.1. Ex-situ population observations

This study used plants of the *ex-situ* populations of *B. mirabilis* at the Royal Botanic Gardens Melbourne (RBG). Flowers were observed every day after opening until decomposed. Between 3 p.m. and 4 p.m., ten flowers were observed as to when:

- anthers dehisced
- the stigma was receptive
- nectaries were present
- flowers had a sweet smell

- the flowers stayed open.

3.2.1.2. Comparison of floral structure of ex-situ populations of B. mirabilis and B. sphaerocephala

The floral structure of 100 flowers of *B. mirabilis* and 100 flowers of *B. sphaerocephala* were observed. Number of petals, number of anthers, stigma length and unusual protuberances were particularly noted.

3.2.2. *Ex-situ* study of pollen viability

3.2.2.1. Light microscope observations

Actively dehiscing anthers of *B. mirabilis*, *B. constricta*, *B. nitida* and *B. sphaerocephala* were removed from the flowers, tapped on a microscope slide with a drop of water and covered with a cover slip. One hundred pollen grains from each of three different flowers were examined for each species and the characteristic shapes of the pollen grains were photographed.

3.2.2.2. Fluorescein diacetate (FDA) staining

The FDA stain (Widholm, 1972) was prepared by dissolving 0.025 g of FDA in 5 ml of absolute acetone and then mixing this slowly dropwise with 5 ml of distilled water to avoid precipitation. The mixture was filtered and stored at 4ºC wrapped in aluminium foil.

B. mirabilis and *B. constricta* flowers were taken from the RBG and kept on ice in a Petri dish until examined, within two hours of removal. Anthers actively liberating pollen were tapped over a microscope slide containing a drop of FDA. 500 pollen grains were examined with a fluorescence microscope and the presence/absence of fluorescence was recorded.

3.2.2.3. Scanning electron microscope (SEM) study

Pollen collected as above from actively dehiscing anthers of *B. mirabilis*, *B. constricta*, *B. nitida* and *B. sphaerocephala* was smeared on double-sided sticky tape on aluminium stubs and sputter-coated with gold with a SPI-Module™ sputter-coater. The pollen was viewed and images recorded digitally using a Jeol JSM-35CF Scanning Electron Microscope at 15 kV.

3.2.2.4. Pollen tube growth

Pollen collected as above from actively dehiscing anthers of *B. mirabilis, B. constricta* and *B. nitida* was dusted onto separate plastic Petri dishes, each containing one of four agar/sucrose concentrations (5 g/L of agar and one of 0%, 5%, 10% or 20% sucrose). The experiment was replicated four times. Petri dishes were randomized and placed on a tray at room temperature (18ºC) under natural light. Pollen tube growth was examined with a light microscope and 100 random pollen grains from each Petri dish were scored for pollen tube growth at 4 h, 24 h and 48 h.

3.2.3. *Ex-situ* study of ovule viability

Key stages in development of the flower were noted from observations and 10 flowers from each treatment (Table 3-1) were fixed in 4% glutaraldehyde.

Table 3-1: *B. mirabilis* flower treatments used to examine ovule viability.

Treatment	*Key Stage*	*Pollination*
1	Anther out, flower closed	Not pollinated
2	Flower open	Not pollinated
3	Flower open, stigma receptive	Pollinated for 2 h
4	Flower open, stigma receptive plus 2 days	Pollinated for 2 h and 2 days
5	Flower collapsed	Pollinated since stigma receptive (7+ days)

In addition, 10 open, receptive flowers of *B. constricta, B. nitida* and *B. sphaerocephela* were processed in the same way for comparison, once in 2005 at the RBG and again in 2007 at RMIT University.

3.2.3.1. Processing

First schedule

Flowers were fixed whole in 4% formaldehyde and kept at 4ºC until processed. Individual flowers were placed in cassettes with sponge to stop the flowers floating away and processed in a Shandon Hypercentre with the following schedule: 30 minutes in each of the following solutions: 70% alcohol, 80% alcohol, 90% alcohol, 95% alcohol, absolute alcohol (3x), xylene (3x), wax with vacuum on (2x).

Second schedule

This was as above with the schedule reduced to 5 minutes in each solution, as damage to the specimens was observed with the first schedule.

3.2.3.2. Embedding

Flowers were placed and set on the bottom of the cassette before the cassettes were filled with molten wax and placed on a cooling block to set before being removed and trimmed. The blocks were sectioned 3 µm thick with a Leica Rotary Microtome (Model RM2135). Serial wax sections were floated onto a water bath and lifted onto glass slides. The slides were dried on a warm plate in racks and placed in a 60ºC oven for 30 minutes to fix the sections onto the slide before staining.

3.2.3.3. Staining: Haematoxylin and Eosin Progressive

Haematoxylin stain

1 g of haematoxylin was added to 0.2 g of sodium iodate, 50 g of potassium aluminium sulphate and 1000 ml of distilled water. This was dissolved overnight and 50 g of chloral hydrate and 1 g of citric acid was added.

Scotts tapwater

The Scotts tap water substitute was prepared by adding 2 g of potassium bicarbonate and 20 g of magnesium sulphate to 1000 ml of distilled water.

Acid alcohol

The acid alcohol was made by adding 0.5 ml of concentrated hydrochloric acid to 100 ml of 70% alcohol.

Procedure

The sections were de-waxed in xylene for 5 minutes and hydrated through graded alcohols to water, immersed in Mayer's haematoxylin for 3 minutes and briefly rinsed in tap water, followed by 1 minute in Scott's tap water. The sections were then immersed in 1% aqueous eosin for 2 minutes, rinsed in tap water and dehydrated through an alcohol series, cleared in xylene and mounted in DPX. The slides were examined with a light microscope.

3.2.4. *In-situ* and *ex-situ* fertility

3.2.4.1. Presence of pollinators

On four occasions over 2 years, possible pollinators were observed (approximately 20 h observation) at the original field site of *Borya mirabilis* in the Grampians National Park in the first week of November in 2004 and 2005. When observed, potential pollinators were photographed.

3.2.4.2. Borya nitida ex-situ pollinations compared to Borya mirabilis field pollinations

Five pollination treatments were carried out with both the field population of *B. mirabilis* in The Grampians National Park and *ex-situ* population of *B. nitida* located at RMIT University. Treatments and replicates are listed in Table 3-2.

Table 3-2: Pollination treatments and replicates undertaken with *ex-situ* collection of *Borya nitida* and naturally occurring field population of *Borya mirabilis*.

Treatment	**B. mirabilis** *No. replicates*	**B. nitida** *No. replicates*
1. Flowers with nothing done to them	60	30
2. Flowers and pollen from same plant	60	32
3. Flowers and pollen from same plant with stigmas shortened by half	210	Not attempted due to number of flowers available
4. Crosses between plants from different colonies for *B. mirabilis* and different populations for *B. nitida*	210	54
5. Crosses between plants from different colonies for *B. mirabilis* or plants from different populations for *B. nitida,* stigmas shortened by half	120	Not attempted due to number of flowers available

The field population of *B. mirabilis* at the time of pollinating in 2005 consisted of five separate colonies each divided into individual 'plants' (Figure 3-1). An assumption based on previous RAPDs analysis (Coates *et al*. 2002) was that plants within a colony were genetically identical and so the individual plant within a colony was not recorded in the pollinations. At the time of pollinating, several hundred flower stalks had been produced, providing several thousand individual flowers. Unfortunately these flowers were only produced on three of the five colonies (Colony 7, Colony 5 and Colony 3, Figure 3-1), one of which only produced a handful of flower stalks, thus limiting the cross-pollinations undertaken in the field. The previous year's flowering produced only seven flower stalks from one colony and the following year (post-fire) also only produced a handful of flower stalks.

From the previous observations of the flowers, pollinations were undertaken between 10 a.m. and 2 p.m. between flowers that were actively dehiscing pollen from anthers and flowers that had a moist stigma. Forceps were used to excise anthers and dab pollen onto the receptive stigmas until a golden coat of pollen was seen on the stigma. Flower heads were covered with an

empty Liptons Tea bag (dyed green with food colouring for camouflage purposes), shortened to fit the flower head, and sealed with Micropore Tape™ Surgical and Medical Products Pty Ltd. Control flowers were bagged with their anthers excised as for the other treatments but before pollination would take place. Black permanent marker was used to label the Micropore Tape™. Flowers and bags were left on the plants for approximately 2 months (Figure 3-3), at which stage the *Borya* plants had begun their dormant phase. Bags were opened and flower remains were examined using a dissecting microscope for the presence of seed.

Figure 3-3: *In-situ Borya mirabilis* colony seven plants one-three after pollination.

3.2.4.3. *Ex-situ crosses of Borya mirabilis with B. constricta and B. sphaerocephala*

Actively dehiscing anthers from *B. constricta* and *B. sphaerocephala* were used to pollinate 30 flowers of *B. mirabilis* plants. Thirty flowers were also pollinated with *B. mirabilis* pollen as a control (Table 3-3).

Table 3-3: *Ex-situ* pollinations of *Borya mirabilis* with *B. constricta* and *B. sphaerocephala*.

Borya *sp. ovaries*	Borya *sp. pollen*	*No. crosses*
B. mirabilis	*B. mirabilis*	30
B. mirabilis	*B. constricta*	30
B. mirabilis	*B. sphaerocephela*	30

3.2.5. Chromosome analysis

The end centimetre of *B. mirabilis* and *B. nitida* downward-facing roots with white tips were removed, placed in a tube containing a saturated solution of paradichlorobenzene and left for 24 h in the fridge at 4ºC. Roots were fixed in acetic alcohol (glacial acetic acid 1 part: absolute ethanol 3 parts) for a further 24 h, placed in 70% ethanol and stored at -20ºC. The roots were softened and cleared in 60ºC HCl for approximately 5 min in a water bath. Root tips were excised, placed on a slide with a drop of orcein and tapped with a small metal rod until the tissue formed a cell suspension, covered with a coverslip and gently warmed over flame. The slide was placed between several thick pieces of adsorbent tissue and pressed firmly. The slides were examined with a light microscope for the presence and number of chromosomes.

3.3. Results

3.3.1. Floral observations

3.3.1.1. Ex-situ population observations

The inflorescences of *B. mirabilis* in the *ex-situ* collection opened acropetally on Day 1 (Figure 3-4A) and each flower opened on Day 2 or Day 3 by exerting the tips of the anthers, which had begun to dehisce, through the tips of the separating petals (Figure 3-4B). When flowers opened fully, within a day pollen was scattered over the entire flower, including the stigma which was not receptive at this stage (Figure 3-4C). By the time the stigma was receptive at Day 4, and from Day 5 onwards, the anthers had completely shed their pollen (Figure 3-4D and Figure 3-5A). When the stigma became receptive, it was covered in a sticky secretion (Figure 3-4D) to which the pollen attached well. The stigma was observed to be papillate (Figure 3-5B).

From the time individual flowers within an inflorescence opened until the time they collapsed took approximately 10 days. Flowers within an inflorescence opened acropetally, with inflorescences in the *ex-situ* population lasting as long as a month. Over 70% of the flowers had anther tips protruding through closed petals and shedding pollen 1-2 days before the flower opened fully. In over 90% of the flowers stigmas became receptive between 4 and 5 days (Table 3-4). The receptive stigmas lasted approximately 4 days, although stigmas remained erect even when the rest of the flower had collapsed at 10-11 days, with over 90% of the anthers finishing shedding pollen before or just as the stigma became receptive. Nectar production was variable, occurring randomly throughout the 10 days the flowers were open, at days 3, 5 and 10 (Table 3-4). Nectar was produced from petaloid nectaries (Figure 3-5C and Figure 3-5D).

Figure 3-4: ***Borya mirabilis*****: flower bud closed (A); in florescence opening acropetally, day 1 (Table 3-1), flower bud, anthers dehiscing prior to flower opening fully or stigma being presented (B); day 2-3 (Table 3-1), flower open, anthers dehiscing pollen over entire flower including stigma (C); day 4-5 (Table 3- 1) stigma receptive as seen by stigmatic secretions (D).**

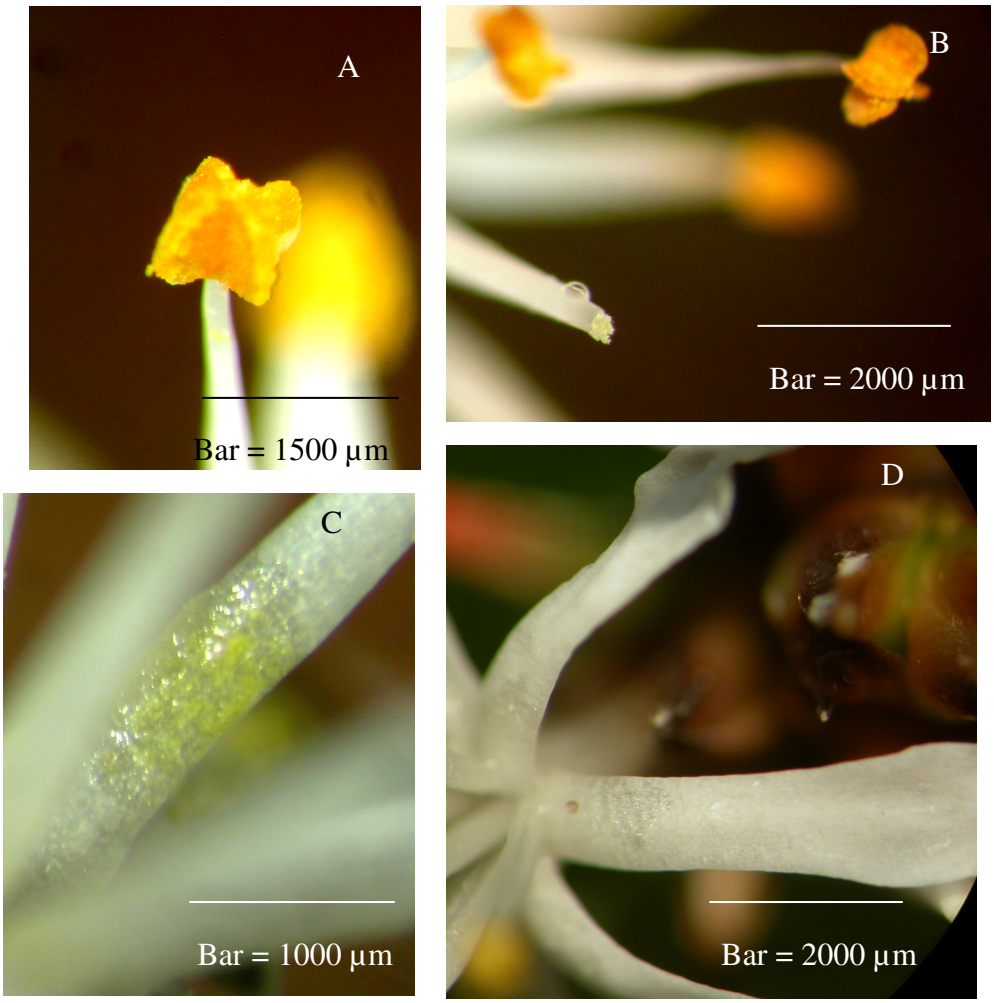

Figure 3-5: *Borya mirabilis*: anthers having dehisced their pollen completely at the same time as stigma becomes receptive, day 5 and 6 onwards (Table 3-1) (A); papillae present at the end of the stigma (B); floral nectaries, secretions trapping fresh pollen (C); floral nectaries (D).

Table 3-4: Floral observations of *Borya mirabilis* flower development in the *ex-situ* population.

Flower	*Day*												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
1	3*	3*	3*,5	3,4	4,6	4,6	4,6	4,6	4,6	7	7,8	7,8	7,8
2	1	3*	3*,5	3,4	4,6	4,6	4,6	4,6	4,6	7	7,8	7,8	7,8
3	1	2	3, 5	3,4,5	4,6	4,6	4,6	4,6	7	7,8	7,8	7,8	7,8
4	1	2	2	3	3*,4,6	6,4	6,4	6,4	4,5,6	7, 8	7,8	7,8	7,8
5	1	2	2	3	3	3*,4,6	6,4	6,4	6,4	4,5,6	7, 8	7,8	7,8
6	1	2	3	3	3,4,5	4,6	4,6	4,6	4,6	4,6	7	7,8	7,8
7	1	3	3	3	3,4, 5	4,6	4,6	4,6	4,6	4,6	7	7,8	7,8
8	1	2	2	3	3*,4,6	6,4	6,4	6,4	4,5,6	7, 8	7,8	7,8	7,8
9	1	2	3	3	3,4, 5	4,6	4,6	4,6	4,6	4,6	7	7,8	7,8
10	1	2	2	3	3*,4,6	6,4	6,4	6,4	4,5,6	7, 8	7,8	7,8	7,8
11	1	2	3	3	3, 4, 5	4,6	4,6	4,6	4,6	4,6	7	7,8	7,8

1= Flower closed
2= Flower closed, anthers out, anthers dehiscing
3= Flower opened ,anthers dehiscing
3*= Flower opened (anthers finished dehiscing)
4= Stigma receptive
5= Nectaries wet and pleasant smelling
6= Anthers dry
7= Flower collapsed
8= Stigma erect

3.3.1.2. Comparison of floral structures in ex-situ population of B. mirabilis and B. sphaerocephala

Several floral irregularities were noted in the infertile *B. mirabilis* but not in the fertile *B. sphaerocephala*, a closely related species (Table 3-5). These included, 5-14% of flowers with fewer than six petals or anthers (Figure 3-6A), flowers with unusual protuberances from petals or filaments (Figure 3-6B and Figure 3-6C), 5% of flowers opening from extended leafy peduncles (Figure 3-6D) or unusually short styles (by half the length).

Table 3-5: Flower structure of *B. mirabilis* and *B. sphaerocephala* taken from observations of 100 flowers of each species.

Species	*% of flowers with less than six petals*	*% of flowers with less than six stamens*	*% flowers with unusual protuberances from petals or filaments*	*% of flower heads opening from extended leafy peduncles*	*% flowers with unusually short styles*
B. sphaerocephala	0	0	0	0	0
B. mirabilis	12	14	6	5	10

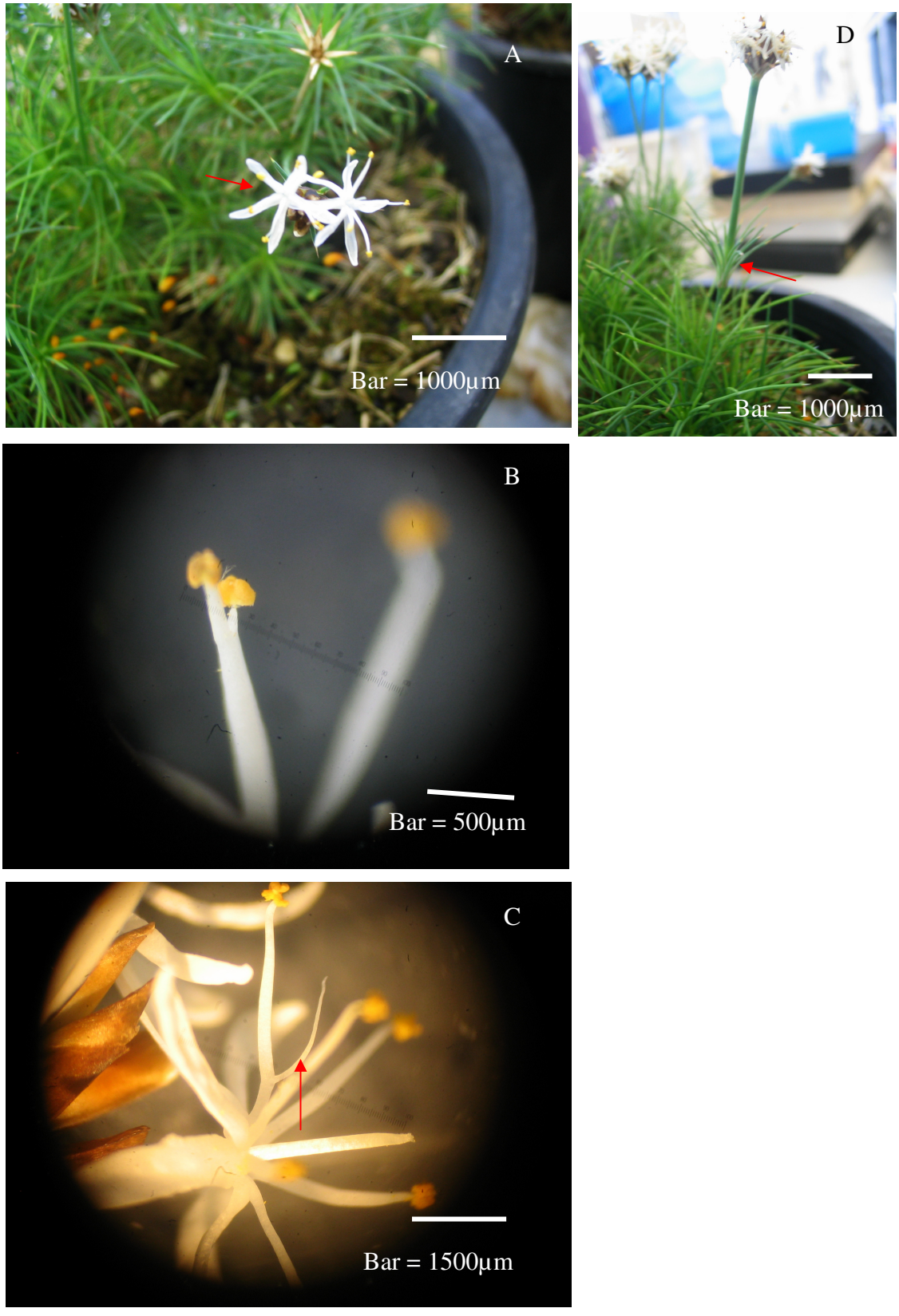

Figure 3-6: Red arrow pointing to *Borya mirabilis* flower with: 5 petals and anthers (A), filament with double anthers (B); stamen protuberance (C); and floral stem of *B. mirabilis* producing a shoot, producing a flower head (normally floral stems produce just flower heads) (D).

3.3.2. *Ex-situ* study of pollen viability

3.3.2.1. Light microscope

Most pollen grains of *B. mirabilis* appeared irregular and thin-walled, collapsing in water (Figure 3-7A), whereas most pollen grains of *B. nitida* (Figure 3-7B) and *B. constricta* (Figure 3-7C) appeared ovoid, thick-walled, highly textured and with a deep grove on one side.

3.3.2.2. Fluorescein diacetate (FDA)

Only 46% of pollen grains of the infertile *B. mirabilis* fluoresced with FDA (Table 3-6, Figure 3-8), indicating relatively poor viability. Pollen of *B. mirabilis* appeared mis-shaped and collapsed, whereas 92% of the pollen of the fertile *B. constricta* fluoresced with FDA and appeared prolate and plump.

Table 3-6: Pollen viability of *Borya mirabilis* and *B. constricta* when stained with FDA, flowers at day 2-3 (Table 3-4).

Species	*Number of pollen grains examined*	*Number of viable pollen*	*% Viable pollen*
B. mirabilis	500	232	46
B. constricta	500	460	92

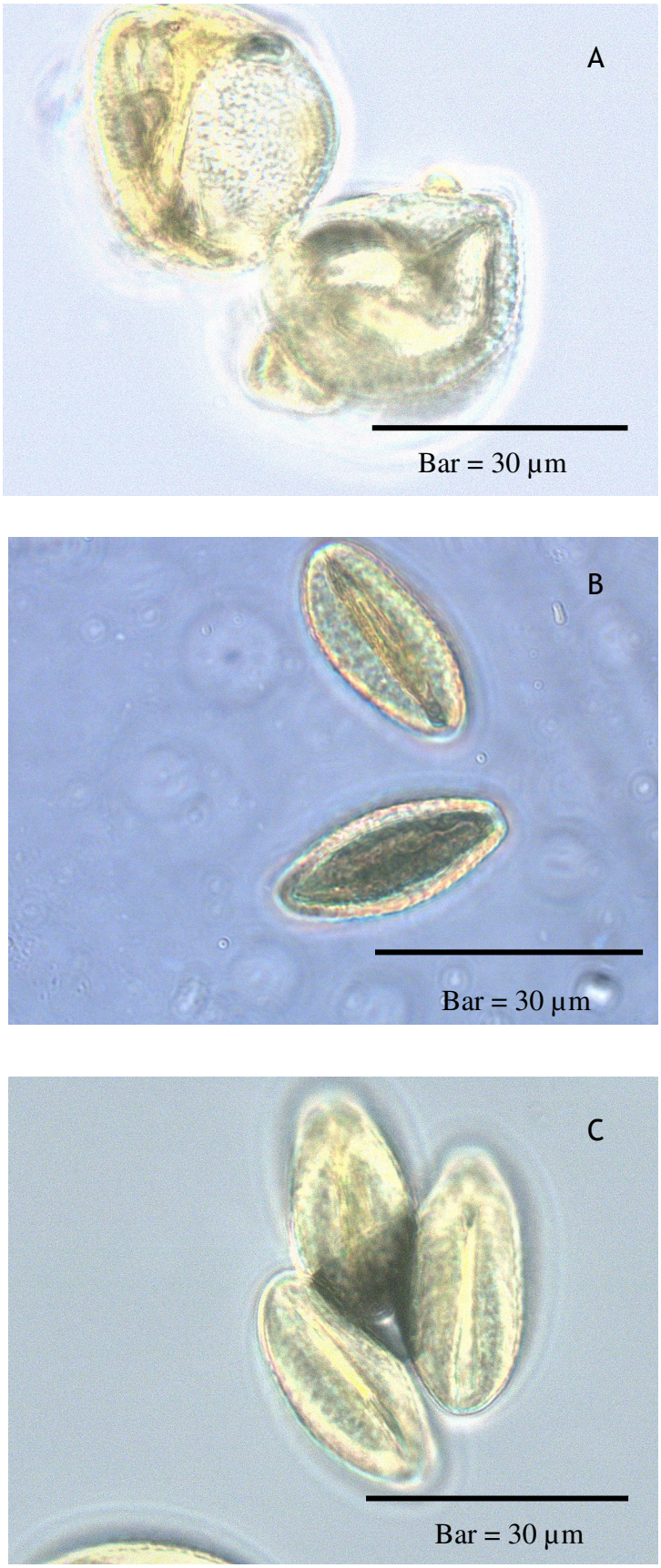

Figure 3-7: Pollen of *Borya mirabilis* (A), *B. nitida* (B) and *B. constricta* (C) mounted in water viewed with the light microscope. Note that pollen of *B. mirabilis* is collapsed compared to that of the fertile *B. constricta* and *B. nitida*, which are unicolpateate, prolate and plump.

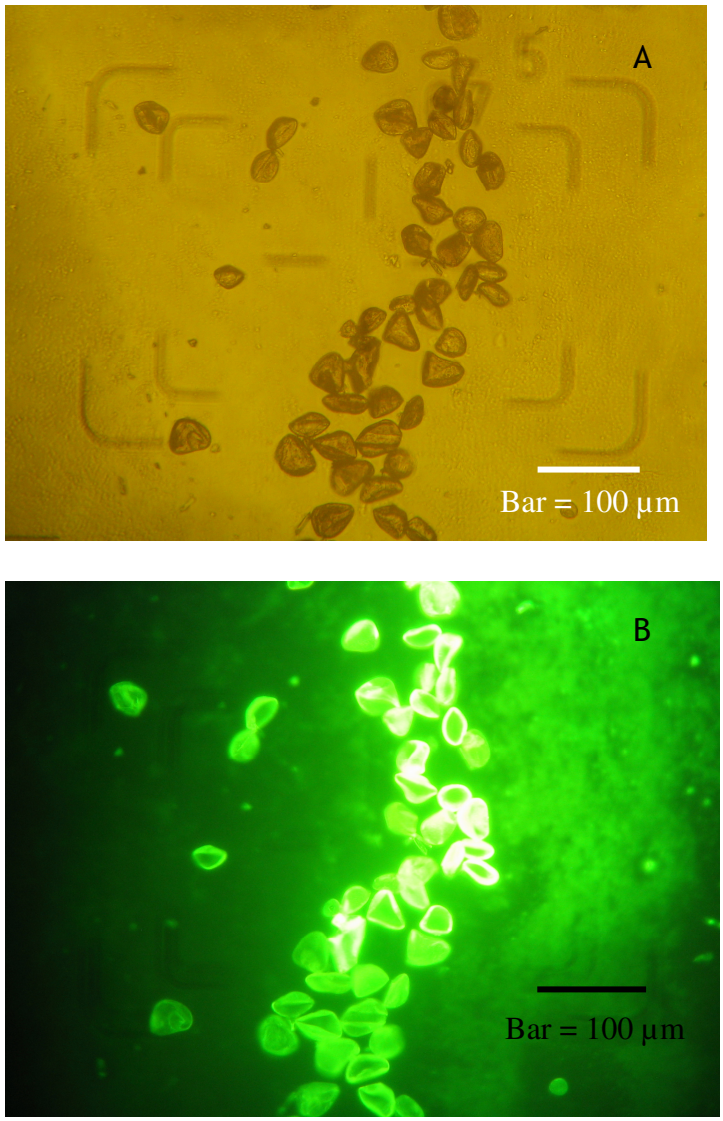

Figure 3-8: Day 2-3 *Borya mirabilis* pollen as seen with light microscope in white light mounted in fluorescein diacetate (A) and in epi-fluorescence (B). Pollen that is viable glows bright green when exposed to ultraviolet light.

3.3.2.3. Scanning Electron Microscope

The difference in appearance of the pollen between the infertile *B. mirabilis* and the fertile *B. constricta*, *B. nitida* and *B. sphaerocephala* was shown more clearly by SEM. Of the fertile *Borya* species, 92-97% of pollen was ovoid, thick-walled and monocolpate (Figures 3-9, Figure 3-10 and Figure 3-11), whereas 98.5% of *B. mirabilis* pollen was irregularly shaped, thin-walled and collapsed (Table 3-7 and Figure 3-12).

The prolate pollen was unicolpate with a single colpa-style aperture running most of the length of the pollen grain. The length of the functional pollen grains between *Borya* species varied from 20-35 µm and the width from 11-20 µm. The sculpturing of the lipoid material covering the pila in all species was arranged in a characteristic reticulate pattern, while differing in the denseness and thickness of patterning between species and between prolate and misshapen pollen.

Table 3-7: Pollen structure of *Borya* species observed with scanning electron microscope.

Species	*No. pollen grains counted*	*No. irregularly shaped pollen*	*% potentially viable pollen*
B. mirabilis	518	510	1.5
B. constricta	778	24	96.9
B. nitida	574	46	91.9
B. sphaerocephela	580	22	96.2

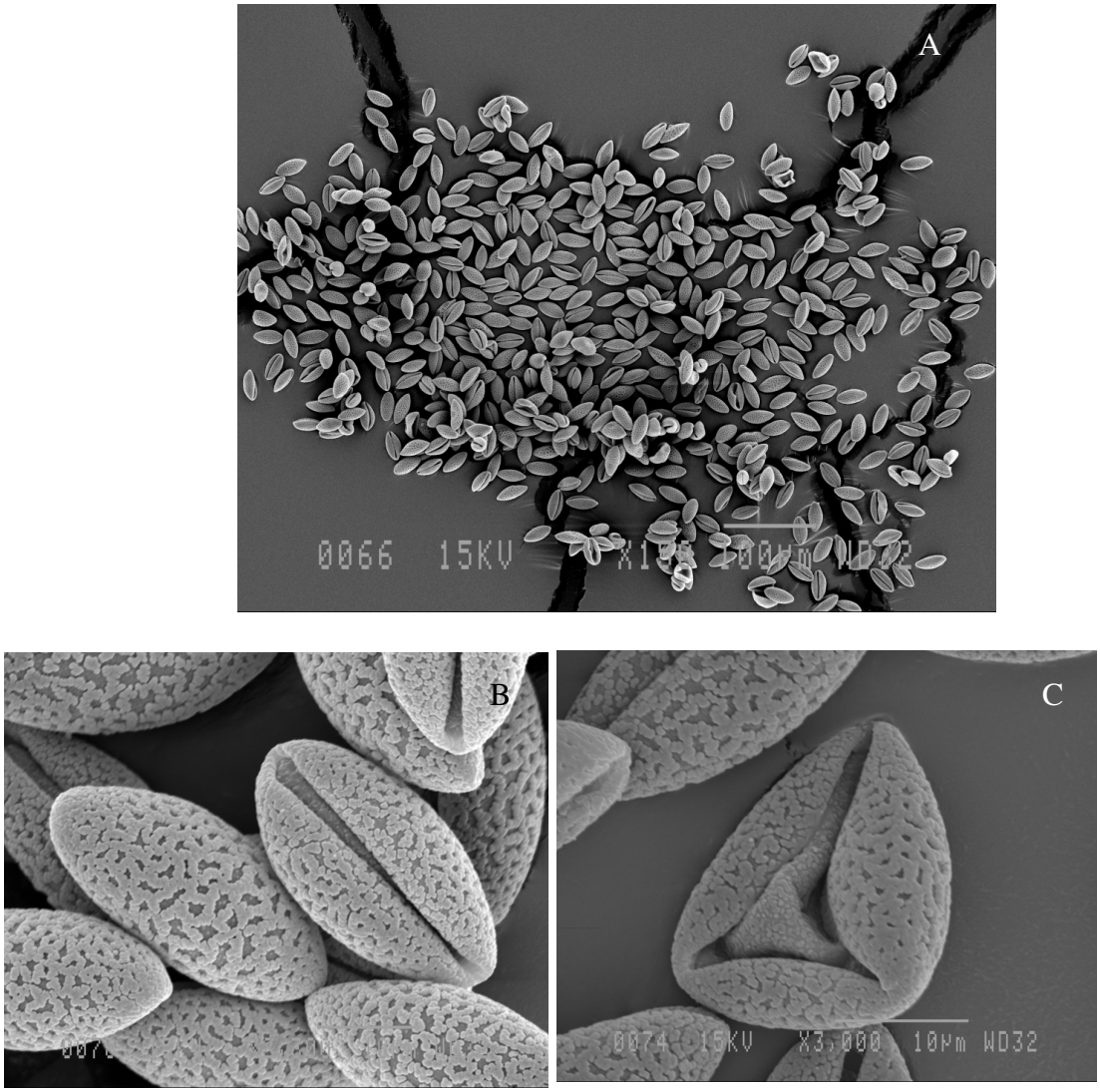

Figure 3-9: *Borya constricta* has both functional and mis-shaped pollen (A) concurrently dehisced. The functional pollen (B) is prolate and unicolpate with a single colpa-style aperture running the majority of the length of the pollen grain. The length of each pollen grain is approximately 23 µm and the width approximately 13 µm. The sculpturing of the lipoid material covering the grains (pila) is arranged in a characteristic reticulate pattern. The mis-shapen pollen (C) is variable in size, often appearing collapsed and misshapen.

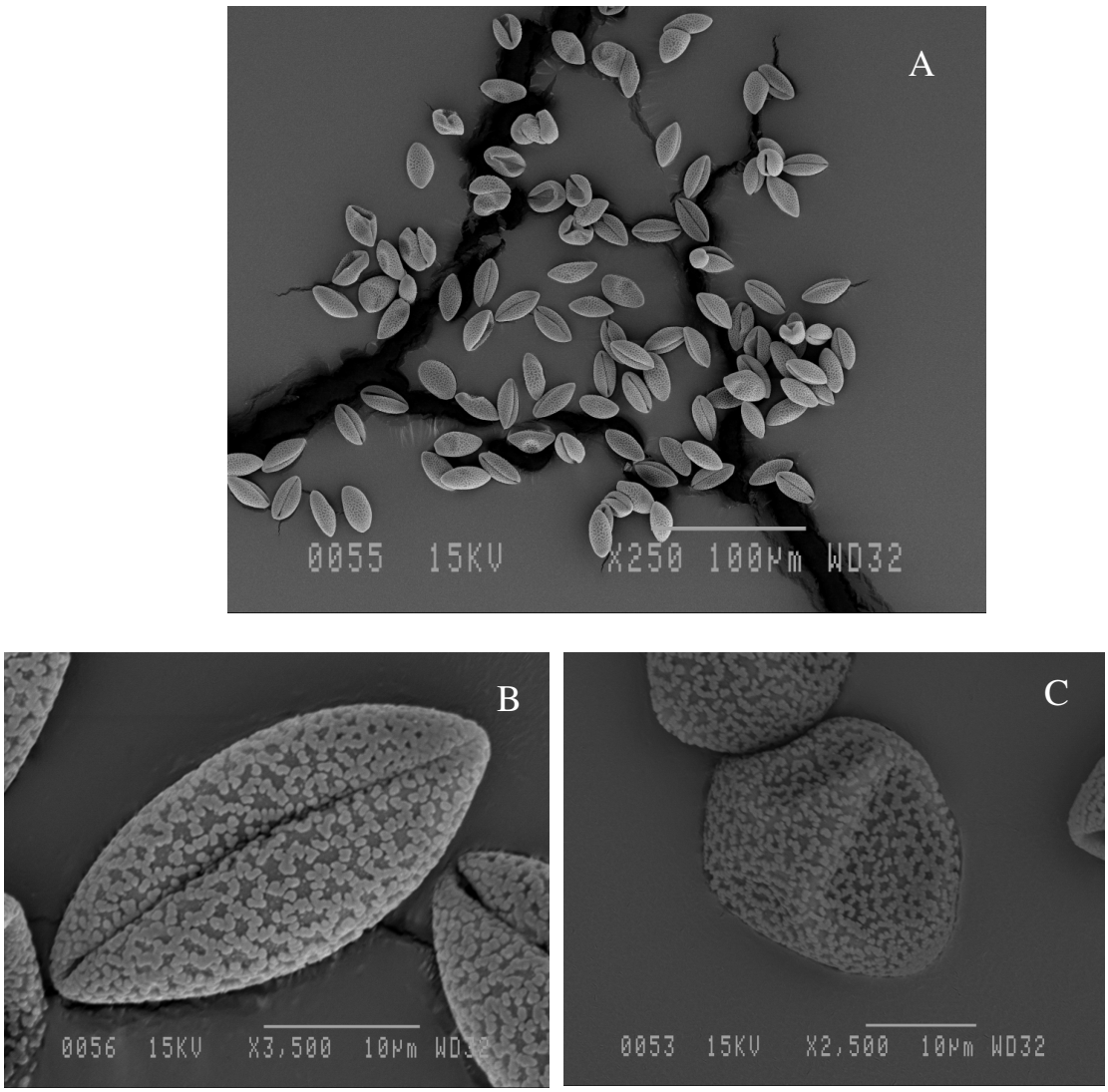

Figure 3-10: *Borya nitida* has both functional and mis-shaped pollen concurrently dehisced (A). The functional pollen (B) is prolate and unicolpate with a single colpa-style aperture running the majority of the length of the pollen grain. The length of each pollen grain is approximately 25 µm and the width approximately 13 µm. The sculpturing of the lipoid material covering the grains (pila) is arranged in a characteristic reticulate pattern. The mis-shaped pollen (C) is variable in size, often appearing collapsed.

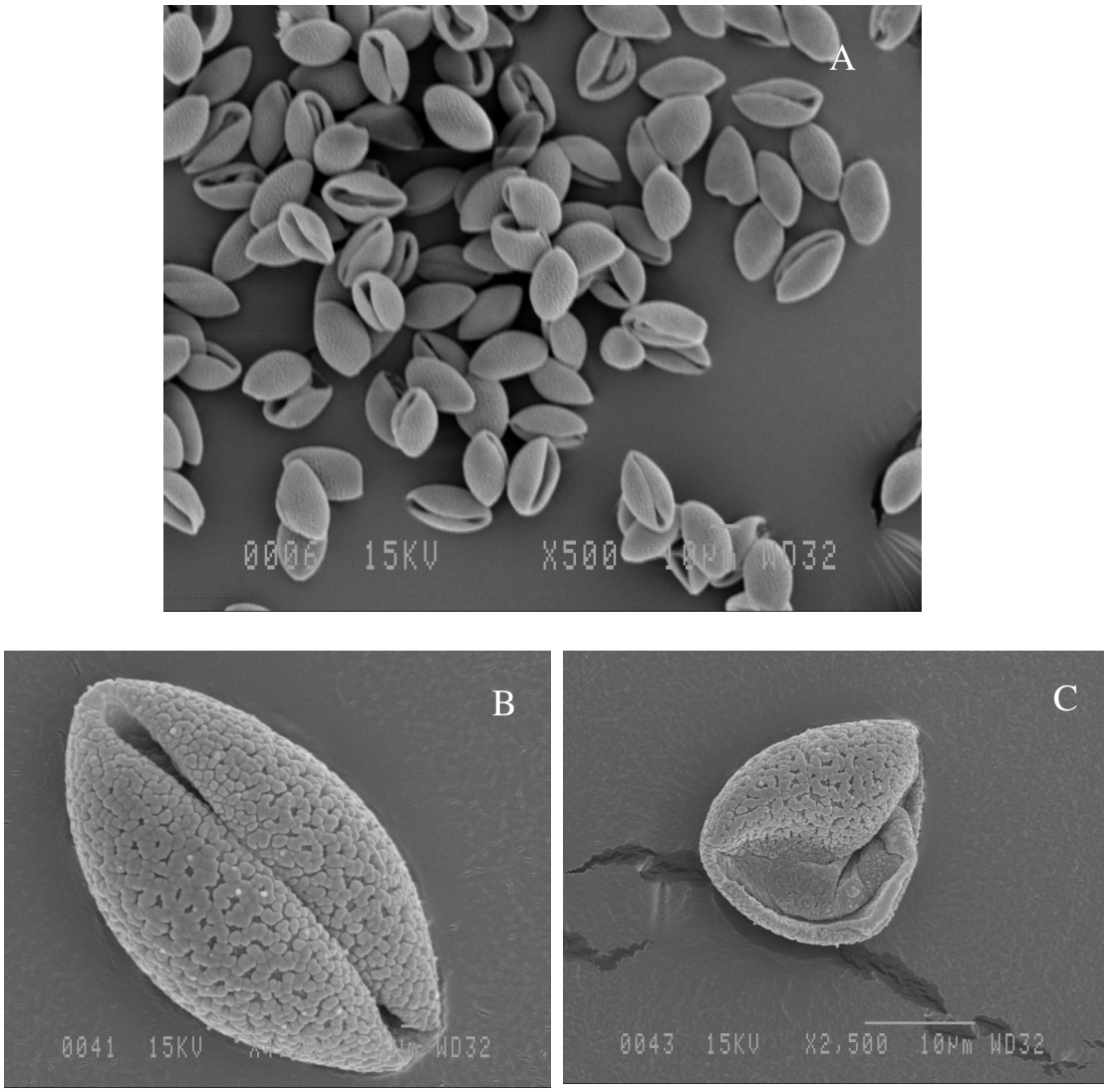

Figure 3-11: *Borya sphaerocephala* has both functional and mis-shaped pollen concurrently dehisced (A). The functional pollen (B) is prolate and unicolpate with a single colpa-style aperture running the majority of the length of the pollen grain. The length of each pollen grain is approximately 20 µm and the width approximately 11 µm. The sculpturing of the lipoid material covering the grains (pila) is arranged in a characteristic reticulate pattern. The mis-shaped pollen (C) is variable in size, often appearing collapsed.

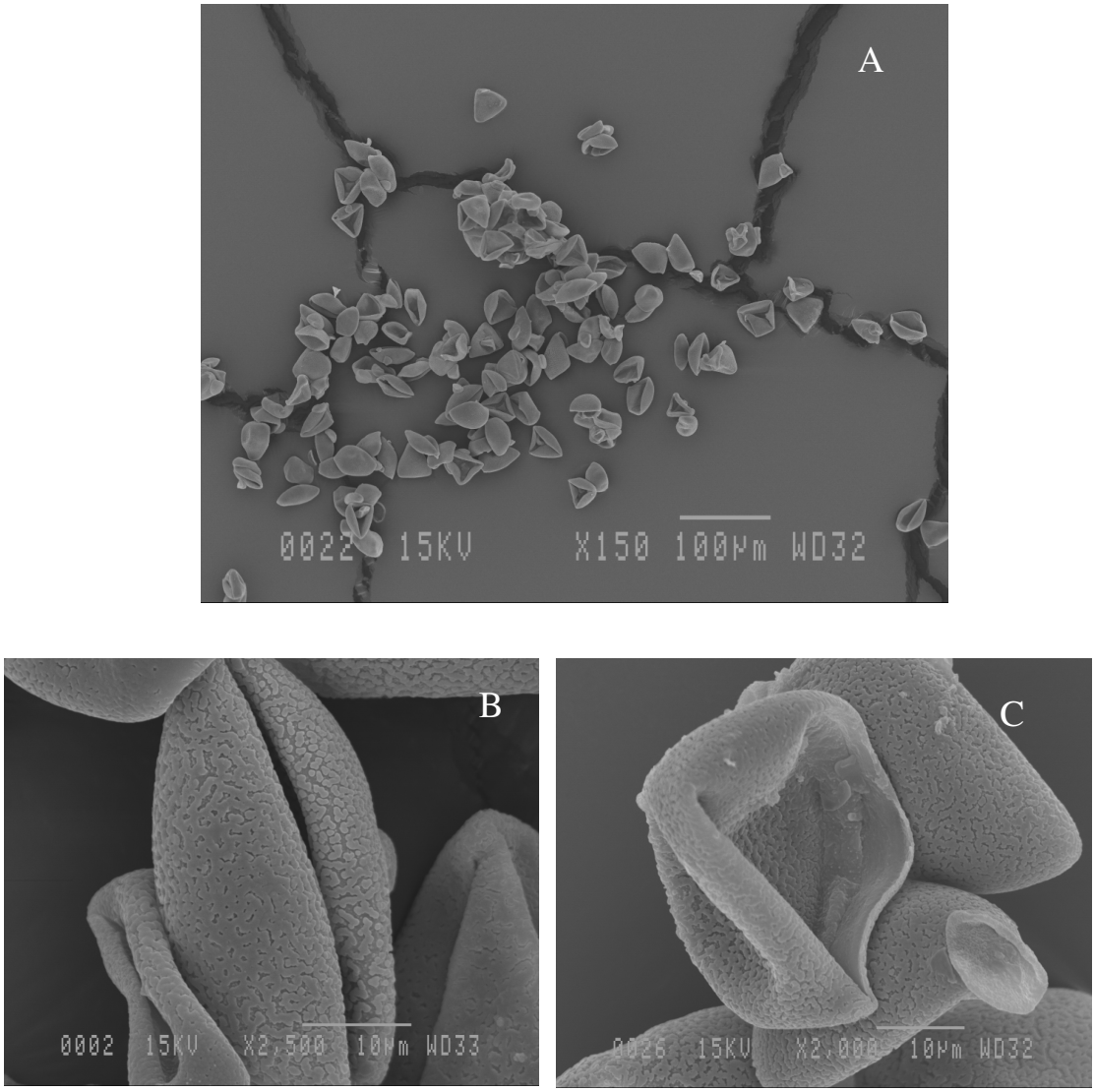

Figure 3-12: *Borya mirabilis* has both functional and mis-shaped pollen concurrently dehisced (A). The functional pollen (B) is prolate and unicolpate with a single colpa-style aperture running the majority of the length of the pollen grain. The length of each pollen grain is approximately 35 µm and the width approximately 20 µm. The sculpturing of the lipoid material covering the grains (pila) is arranged in a characteristic reticulate pattern. The mis-shaped pollen (C) is variable in size, often appearing collapsed.

3.3.2.4. Pollen tube growth

Pollen tube development varied with species, sucrose concentration and time (Table 3-8, Figures 3-13 to Figure 3-27), whereas the fertile *B. constricta* and *B. nitida* germinated at 4 h for all sucrose concentrations and had reached above 95% germination of pollen tubes by 24 h when grown in 5-20% sucrose concentrations, whereas *B. mirabilis* only developed pollen tubes after 48 h on 10% sucrose (Figures 3-25 to Figure 3-27). Neither *B. nitida* or *B. constricta* had high germination rates on 0% sucrose.

Table 3-8: Pollen tube growth of *Borya* species on different sucrose concentrations.

Species	*% sucrose concentration*	*% of pollen growing pollen tubes at 4 h*	*% of pollen growing pollen tubes at 24 h*	*% of pollen growing pollen tubes at 48 h*
B. mirabilis	0	0	0	0
B. mirabilis	5	0	0	0
B. mirabilis	10	0	0	1
B. mirabilis	20	0	0	0
B. nitida	0	20	27	27*
B. nitida	5	53	98	98*
B. nitida	10	94.98	95*	95*
B. nitida	20	42	98	98
B. constricta	0	17	20	20*
B. constricta	5	25	98	98*
B. constricta	10	50	95*	95*
B. constricta	20	22.5	95	95*

* This percent or above (difficult to tell due to massive growth of pollen tubes, making it difficult to connect tubes to pollen grains).

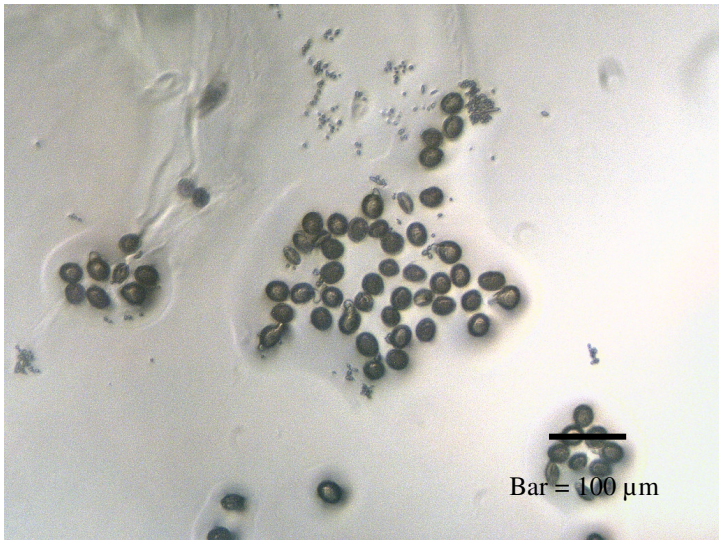

Figure 3-13: *Borya constricta* pollen on 0% sucrose for 3 h, showing little pollen tube development.

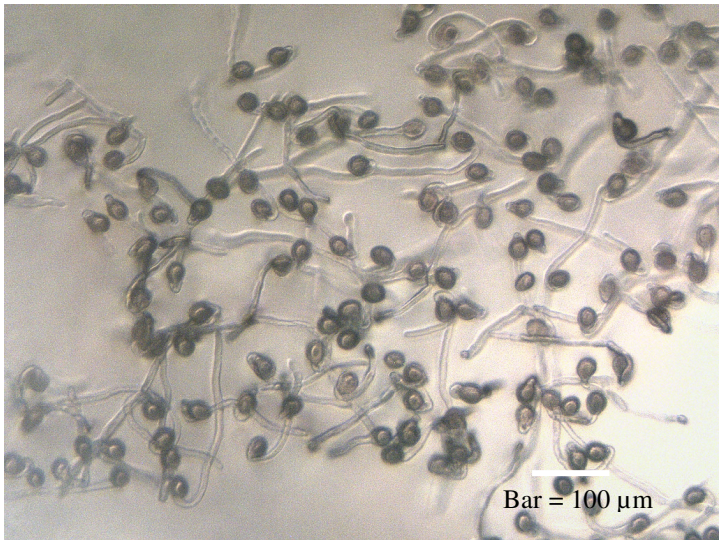

Figure 3-14: *Borya nitida* pollen on 0% sucrose after 3 h, showing extensive pollen tube development.

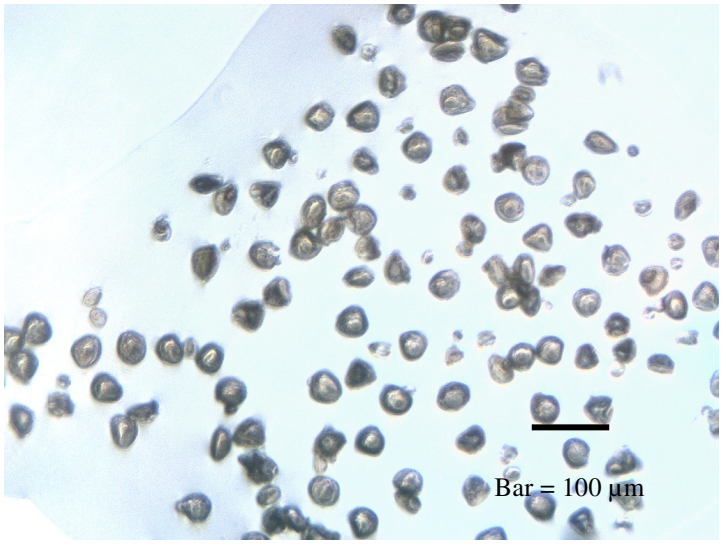

Figure 3-15: *Borya mirabilis* pollen on 0% sucrose for 3 h, showing lack of germination.

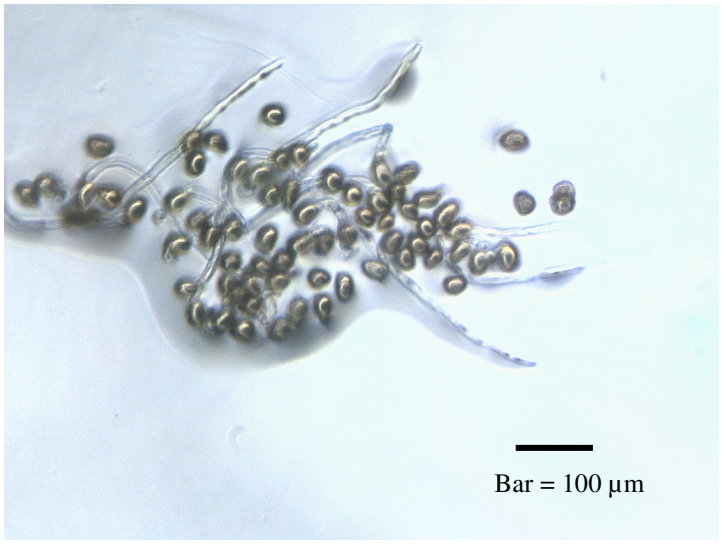

Figure 3-16: *Borya constricta* pollen on 5% sucrose for 3 h, showing pollen development.

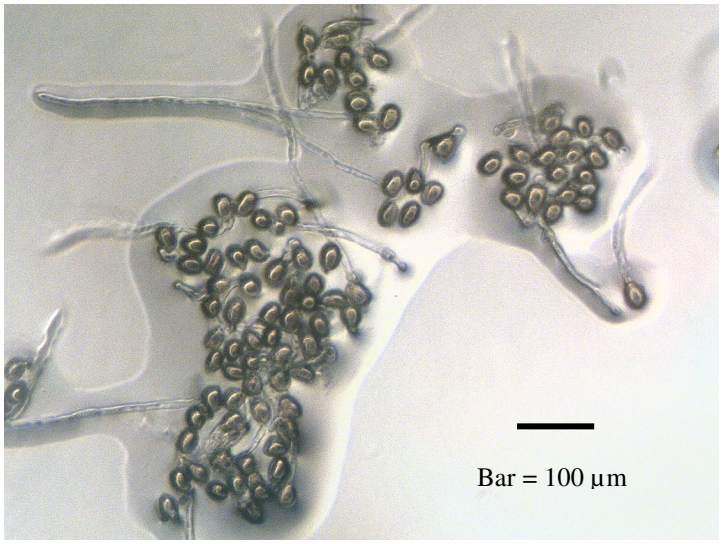

Figure 3-17: *Borya nitida* pollen on 5% sucrose for 3 h, showing extensive pollen tube development.

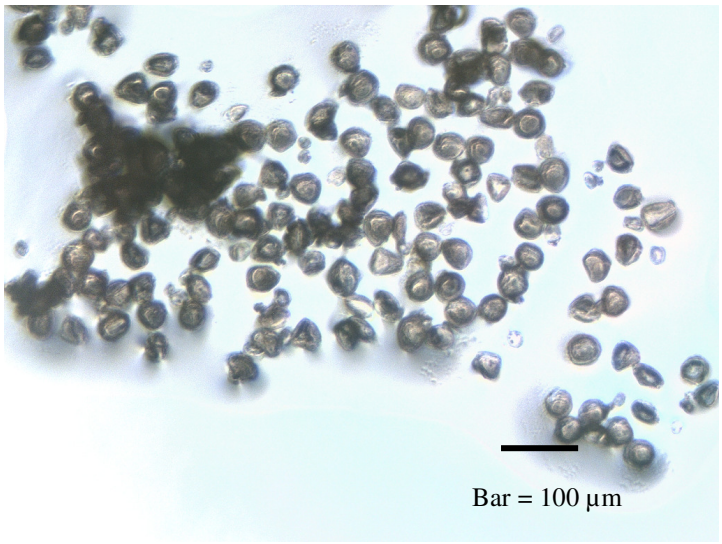

Figure 3-18: *Borya mirabilis* on 5% sucrose for 3h, showing no pollen tube development.

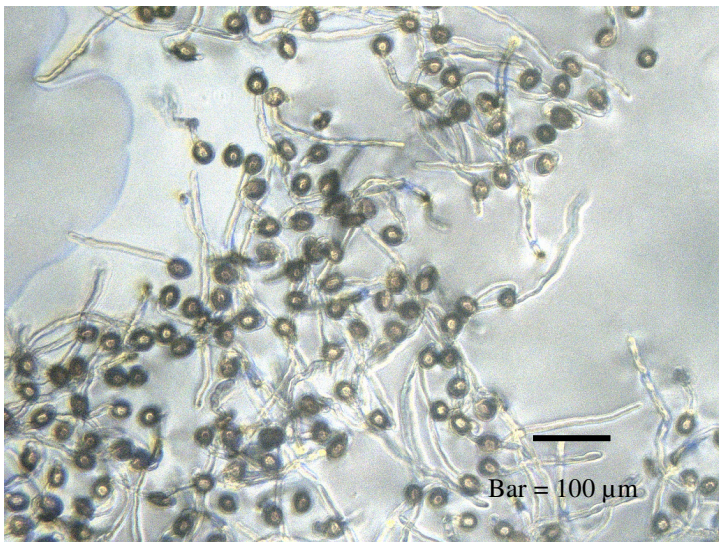

Figure 3-19: *Borya constricta* on 10% sucrose for 3 h, showing extensive pollen development.

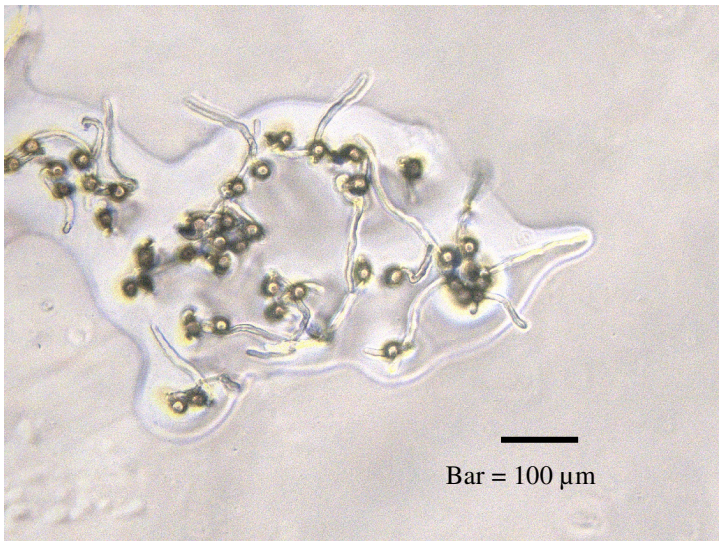

Figure 3-20: *Borya nitida* pollen on 10% sucrose for 3 h, showing extensive pollen development.

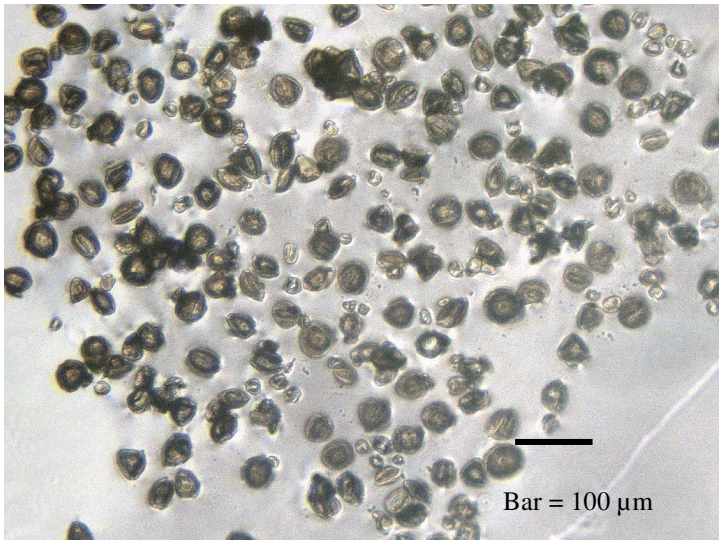

Figure 3-21: *Borya mirabilis* pollen on 10% sucrose for 3h, showing no pollen tube development.

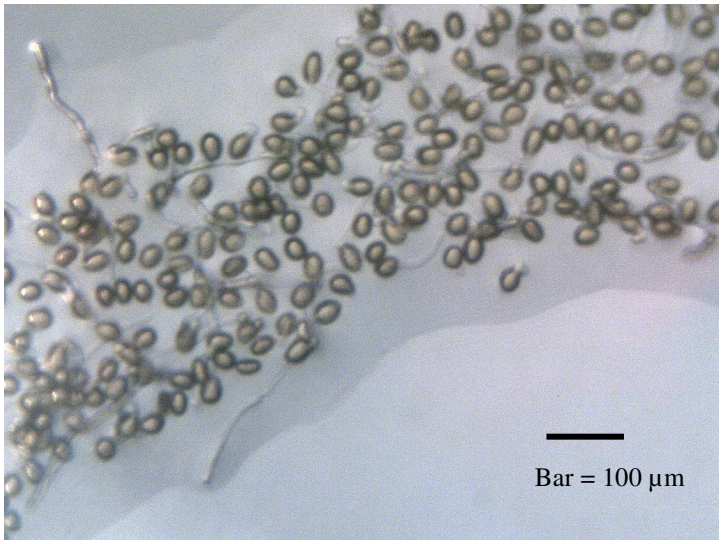

Figure 3-22: *Borya constricta* on 20% sucrose for 3 h, showing little pollen tube development.

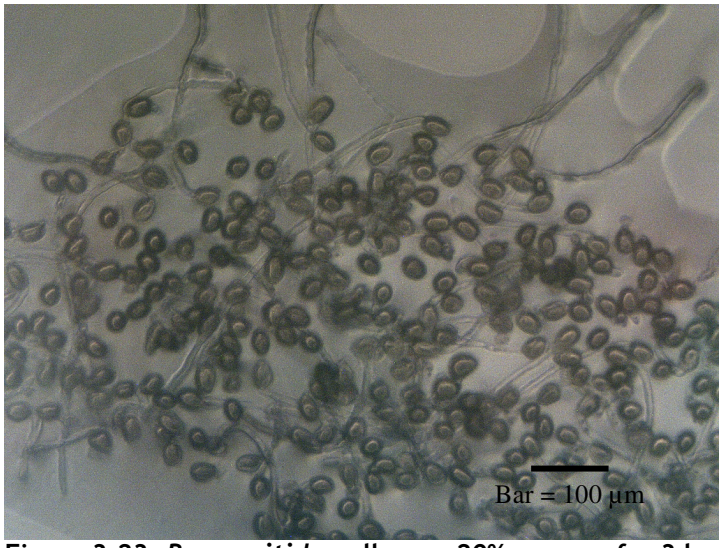

Figure 3-23: *Borya nitida* pollen on 20% sucrose for 3 h, showing pollen tube development.

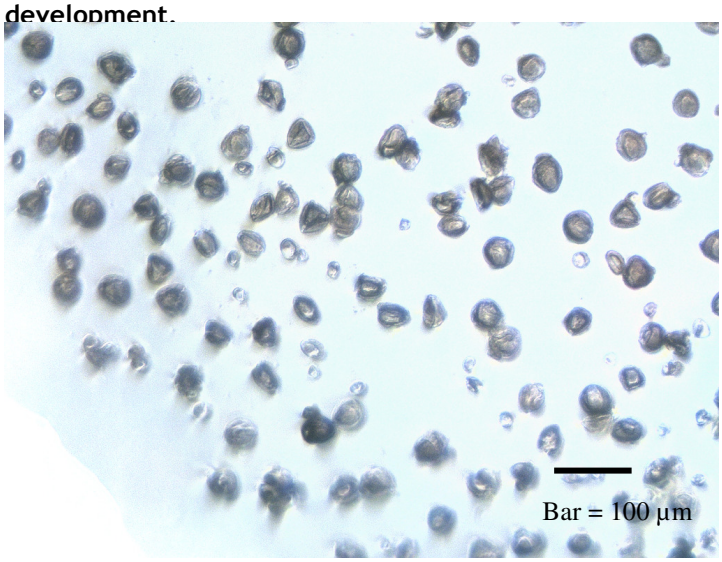

Figure 3-24: *Borya mirabilis* pollen on 20% sucrose for 3h, showing no pollen tube development.

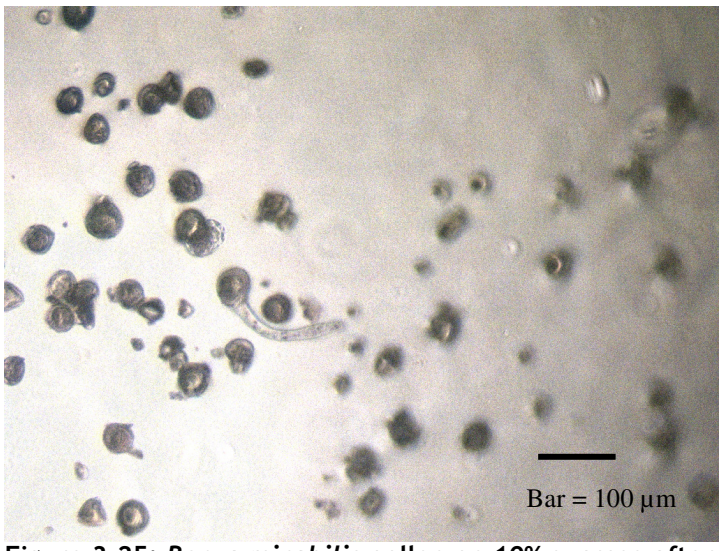

Figure 3-25: *Borya mirabilis* pollen on 10% sucrose after 48 h, showing one grain with pollen tube development.

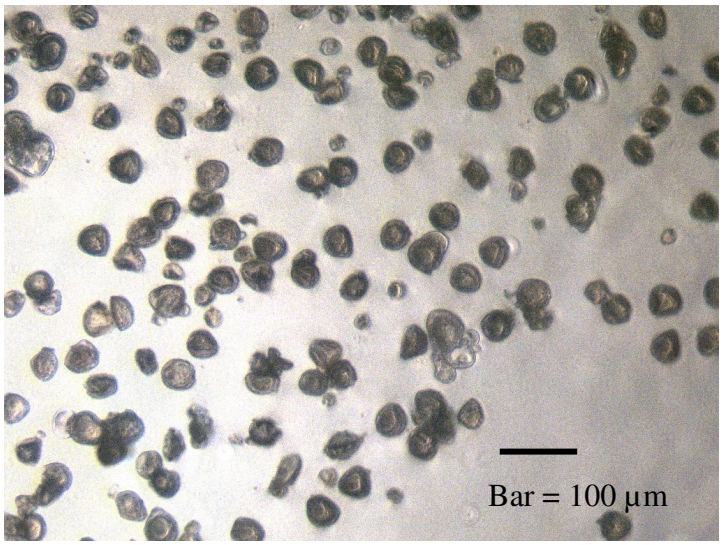

Figure 3-26: *Borya mirabilis* pollen on 10% sucrose after 48 h, showing no pollen tube development.

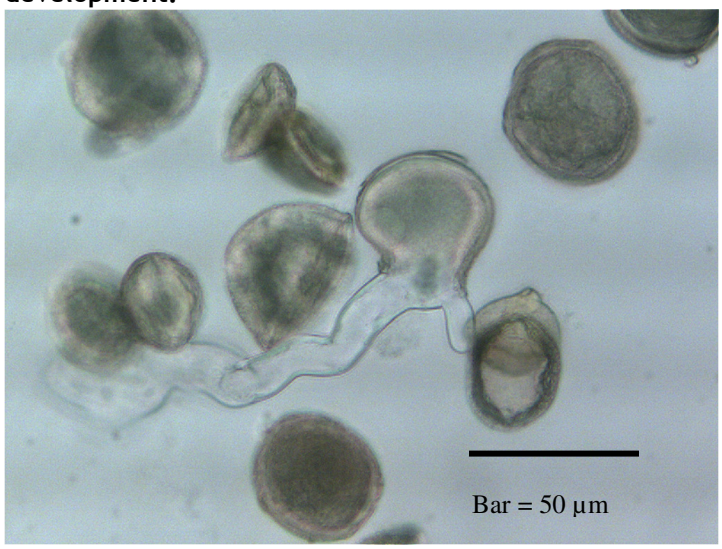

Figure 3-27: *Borya mirabilis* pollen on 10% sucrose after 48 h, showing one grain with pollen tube development.

3.3.3. *Ex-situ* study of ovule viability

The morphology of *B. mirabilis*, *B. constricta*, *B. nitida* and *B. sphaerocephala* ovules was similar in longitudinal section, displaying three distinct locules, with ovaries of each of the species displaying axile placentation (Figure 3-28, Figure 3-39, Figure 3-41 and Figure 3-44), with thick funicles, containing many ovules per locule (Figure 3-29, Figure 3-39, Figure 3-41 and Figure 3-44). In general, the development of the ovules in all species in *B. constricta*, *B. nitida* and *B. sphaerocephala* was reasonably uniform, however, that of *B. mirabilis* showed ovules in clearly different stages of development within the one ovary (Figure 3-28).The ovaries had fully developed hollow styles and ovules were in ovaries clearly divided into three breaks with axile placentation (Figure 3-36 to 3-38).

The ovules in all species of *Borya* examined appeared to develop from a single large megasporocyte as seen in *B. mirabilis* (Figure 3-30 to Figure 3-32), surrounded by three layers: nucellus, inner integument and outer integument. The ovary slowly became more vacuolated in *B. mirabilis*, *B. constricta* and *B. nitida* as the megaspore divided (Figure 3-33 to Figure 3-35, Figure 3-40 and Figure 3-42 to Figure 3-43) until the ovary consisted of an egg surrounded by two synergids at the base of a large vacuolated region, which became variably cellular towards the top half, containing one-several large nucleated cells (Figure 3-33 and Figure 3-43). In general the embryo sac in *B. nitida* ovules was more vacuolated than those of *B. mirabilis*, *B. constricta* or *B. sphaerocephala*.

3.3.3.1. Borya mirabilis ovary and ovule sections

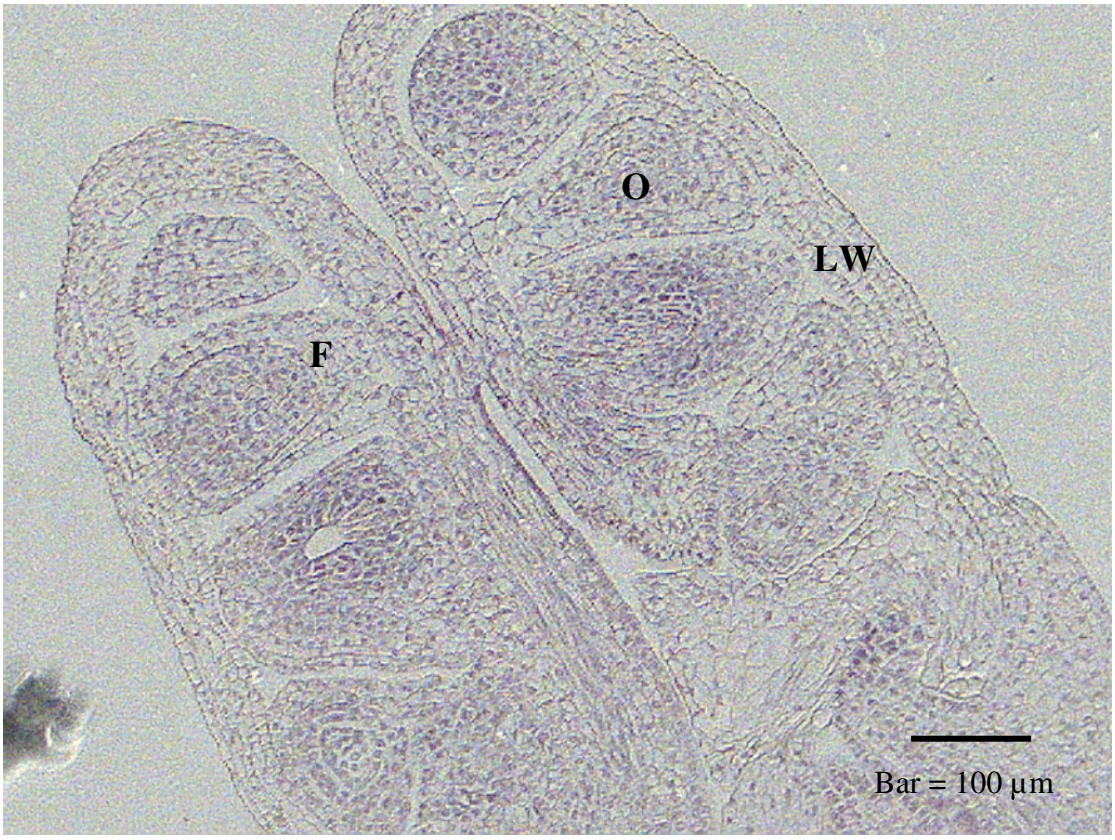

Figure 3-28: Longitudinal section of ovary of *Borya mirabilis* (Day 4) showing placentation of ovules in locules and non-uniform development of ovules. LW=locule wall, O=ovule, F=funicle.

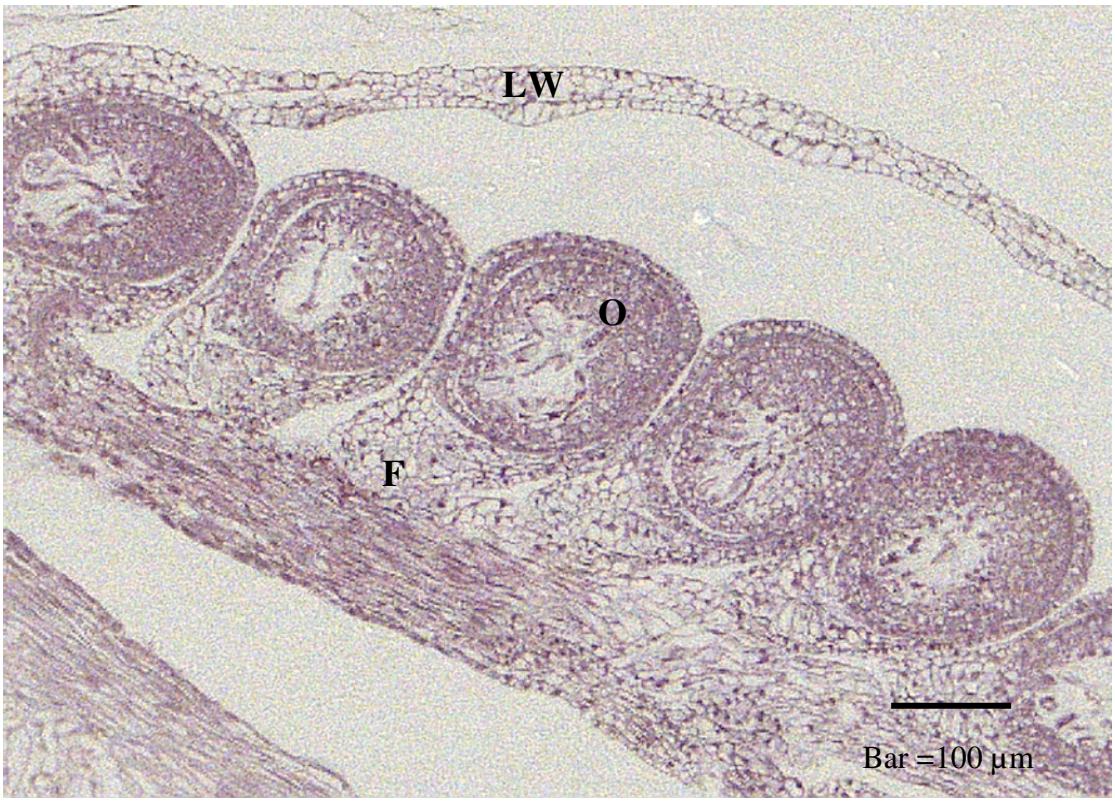

Figure 3-29: Longitudinal section of *Borya mirabilis* (Day 4) ovary. Depicts longitudinal section through one of the three locules (LW=locule wall) showing how the ovules (O) are attached to the funicles (F) and the axile placentation.

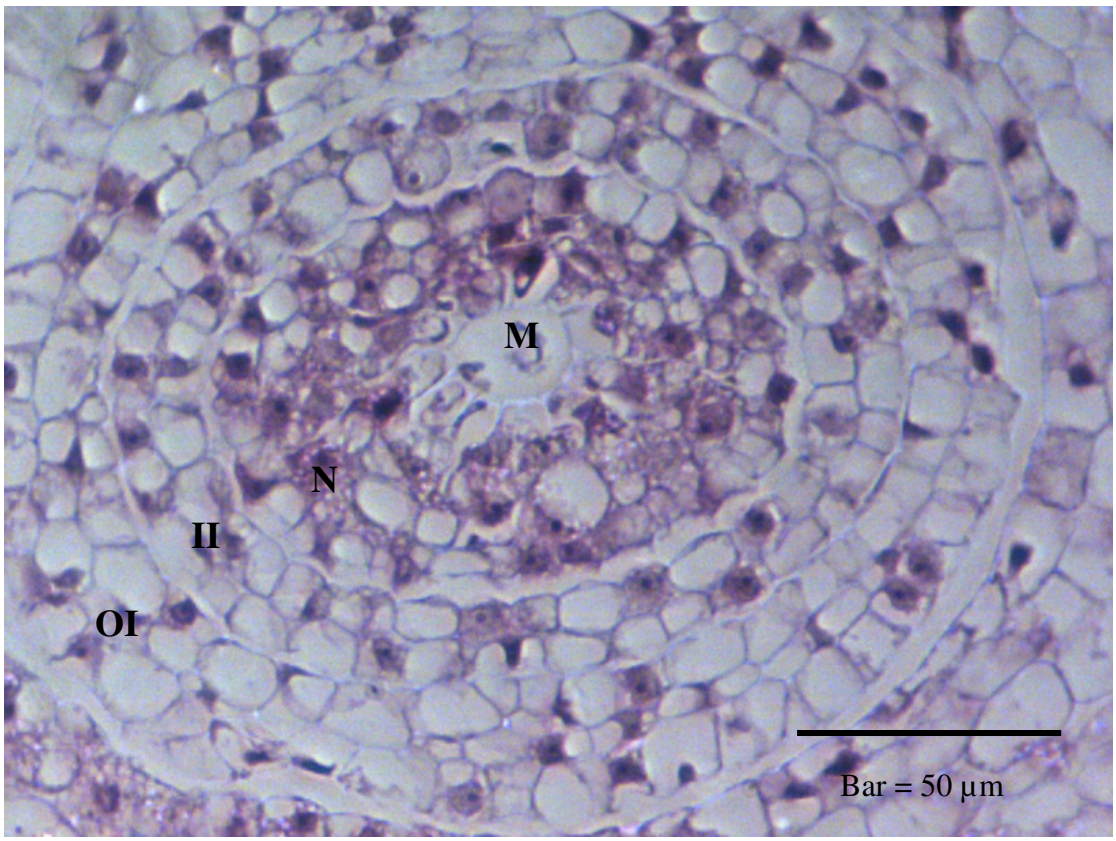

Figure 3-30: Longitudinal section of *Borya mirabilis* (Day 2), ovule containing megasporocyte (M) surrounded by small vacuole with three nutritive levels, nucellus (N), Inner Integument (II) and Outer Integument (OI).

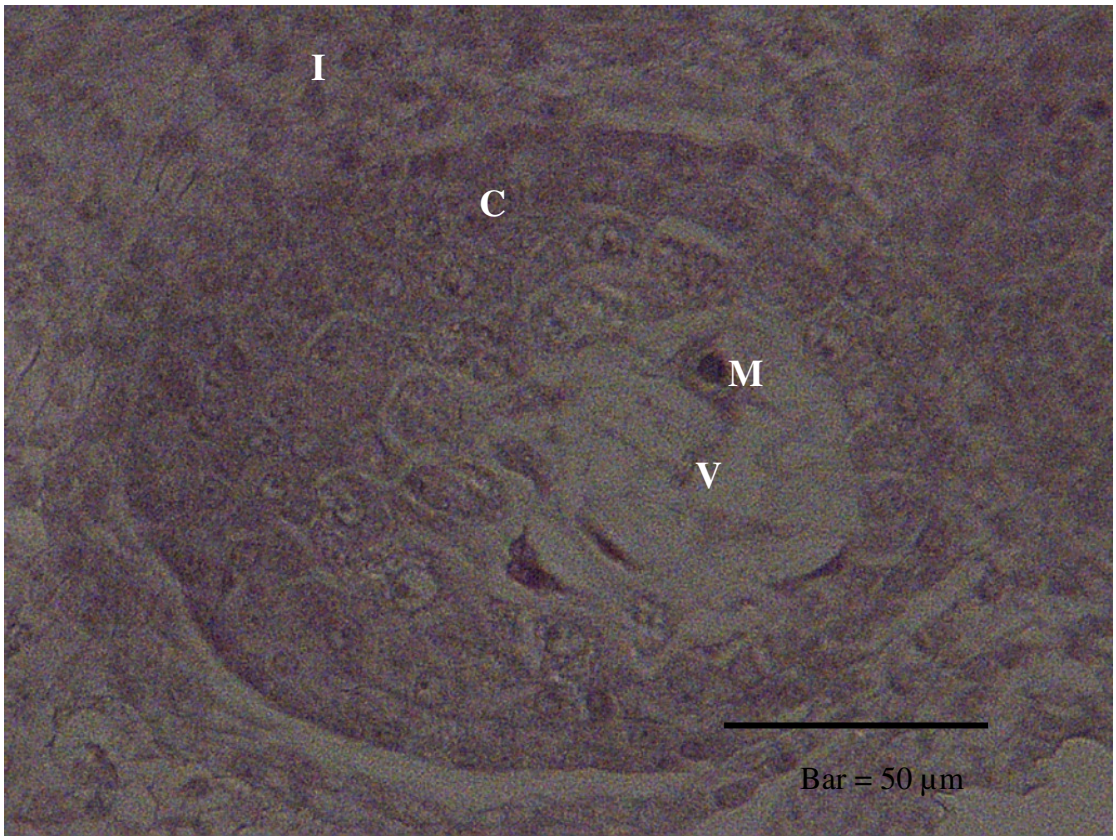

Figure 3-31: Longitudinal section of *Borya mirabilis* (Day 4) megasporocyte (M) in vacuole (V) surrounded by cellular embryo sac (C) inside ovule surrounded by inner integument (I).

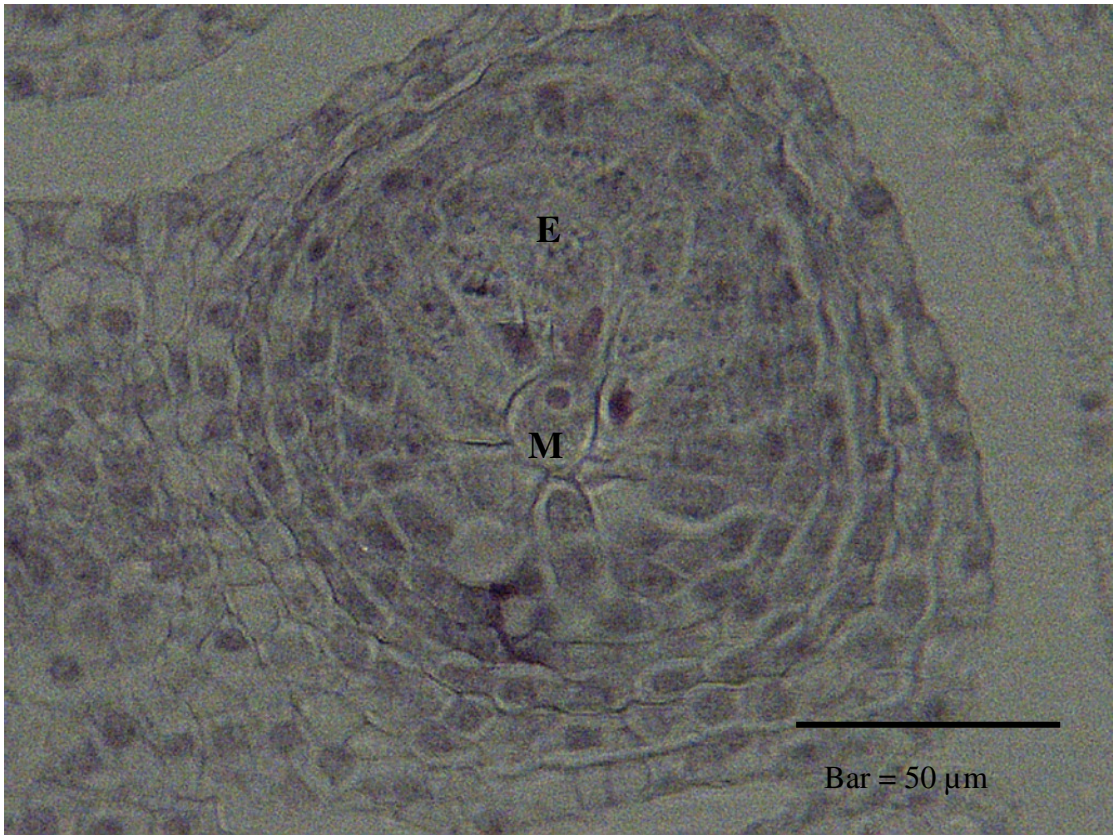

Figure 3-32: Longitudinal section of *Borya mirabilis* (Day 4) ovule displaying megasporocyte (M) surrounded by cellular embryo sac (E).

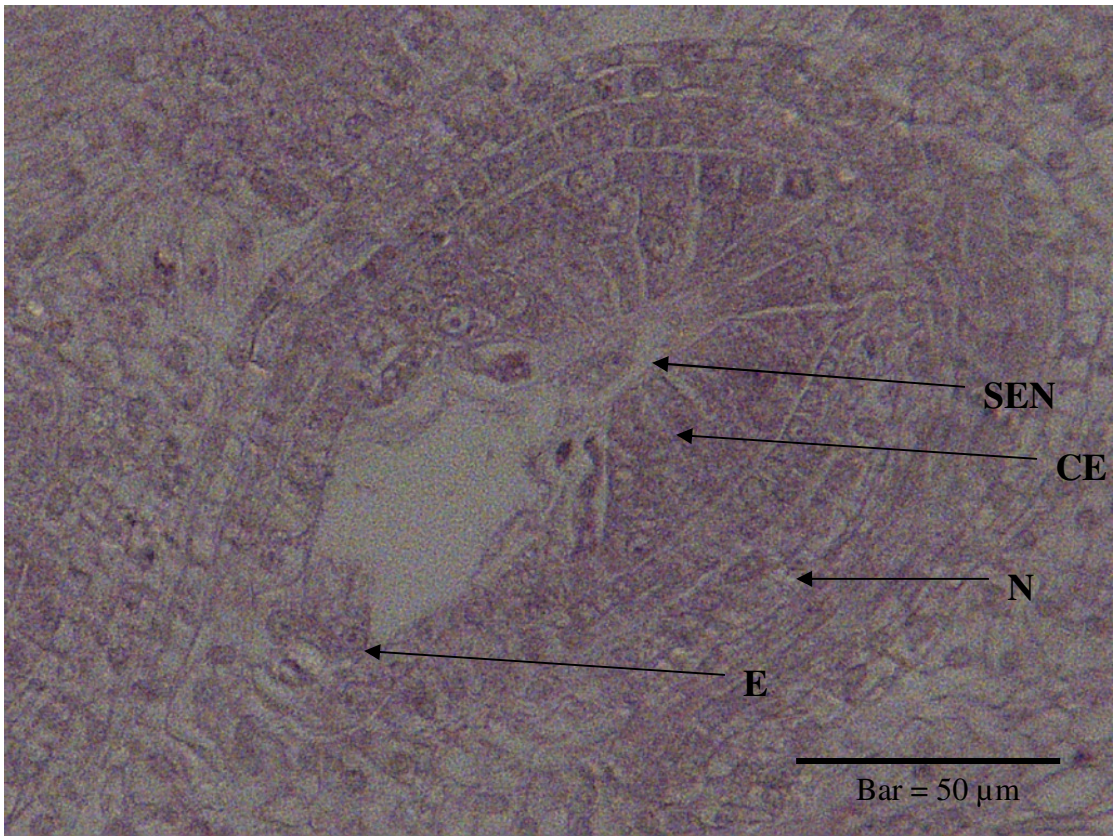

Figure 3-33: *Borya mirabilis* (Day 6), depicting arrangement of cells inside ovule, egg cell surrounded by two synergids (E), cellular embryosac (CE), secondary endosperm nucleus (SEN) and nucellus (N).

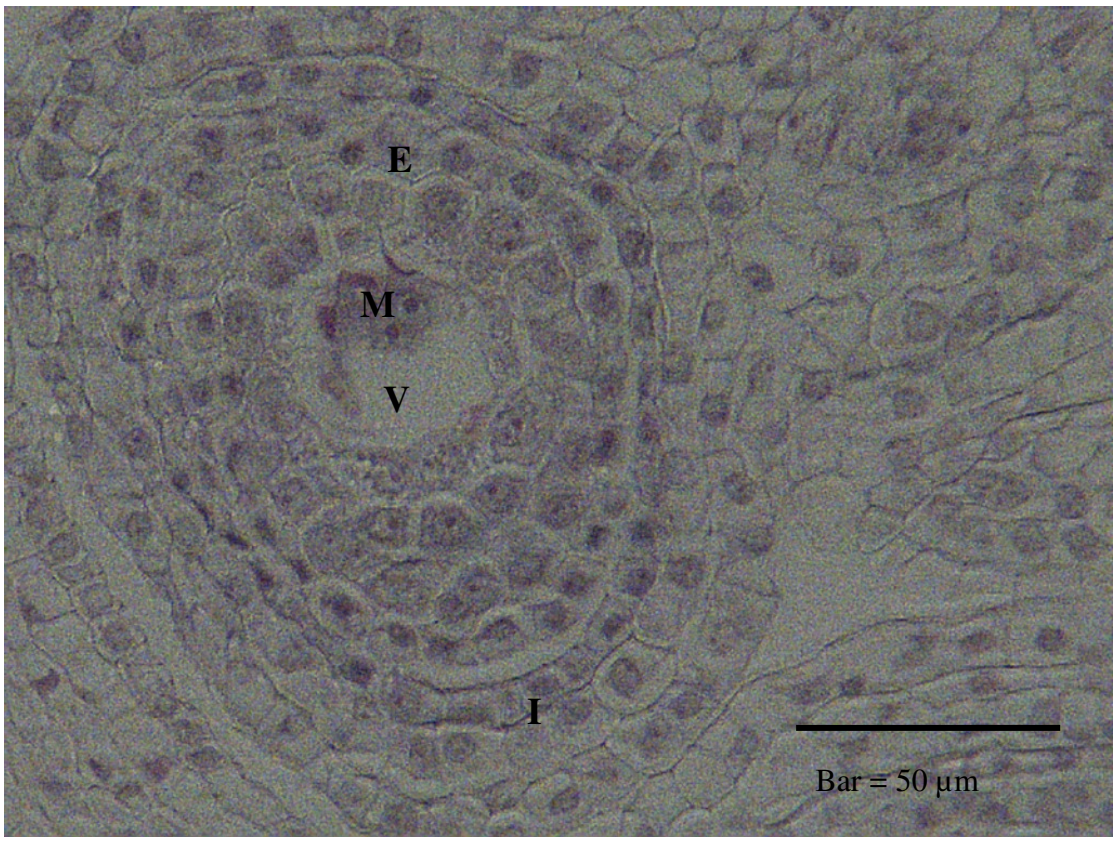

Figure 3-34: Longitudinal section of *Borya mirabilis* (Day 4) ovule depicting dividing megasporocyte (M) inside vacuole (V) surrounded by cellular embryosac (E) and integuments (I).

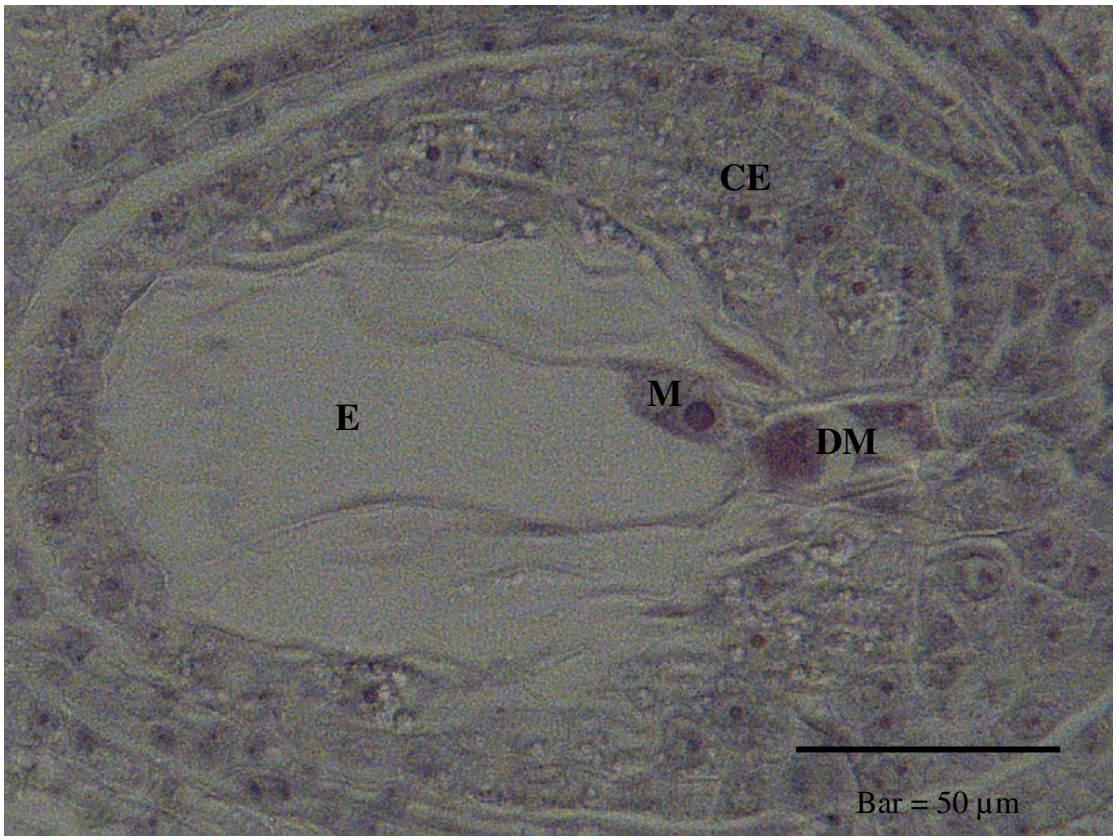

Figure 3-35: Longitudinal section of *Borya mirabilis* (Day 4) ovule, depicting megaspore (M) and dividing megaspore (DM) surrounded by vacuolated embryosac (E).

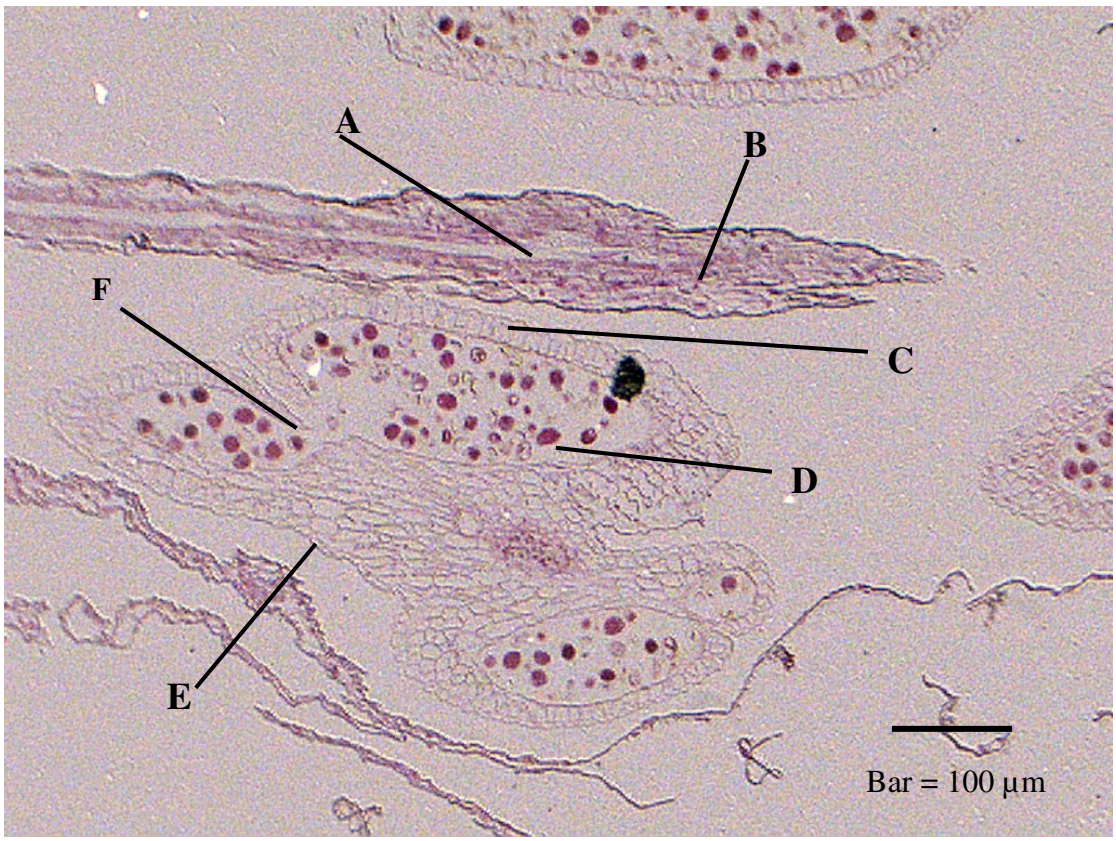

Figure 3-36: Longitudinal section through style and anthers of *Borya mirabilis* (Day2), displaying non-cellular (A) and cellular (B) regions of the style, and endothecium (C), pollen grains (D), epidermis (E) and pore (F) of the anther.

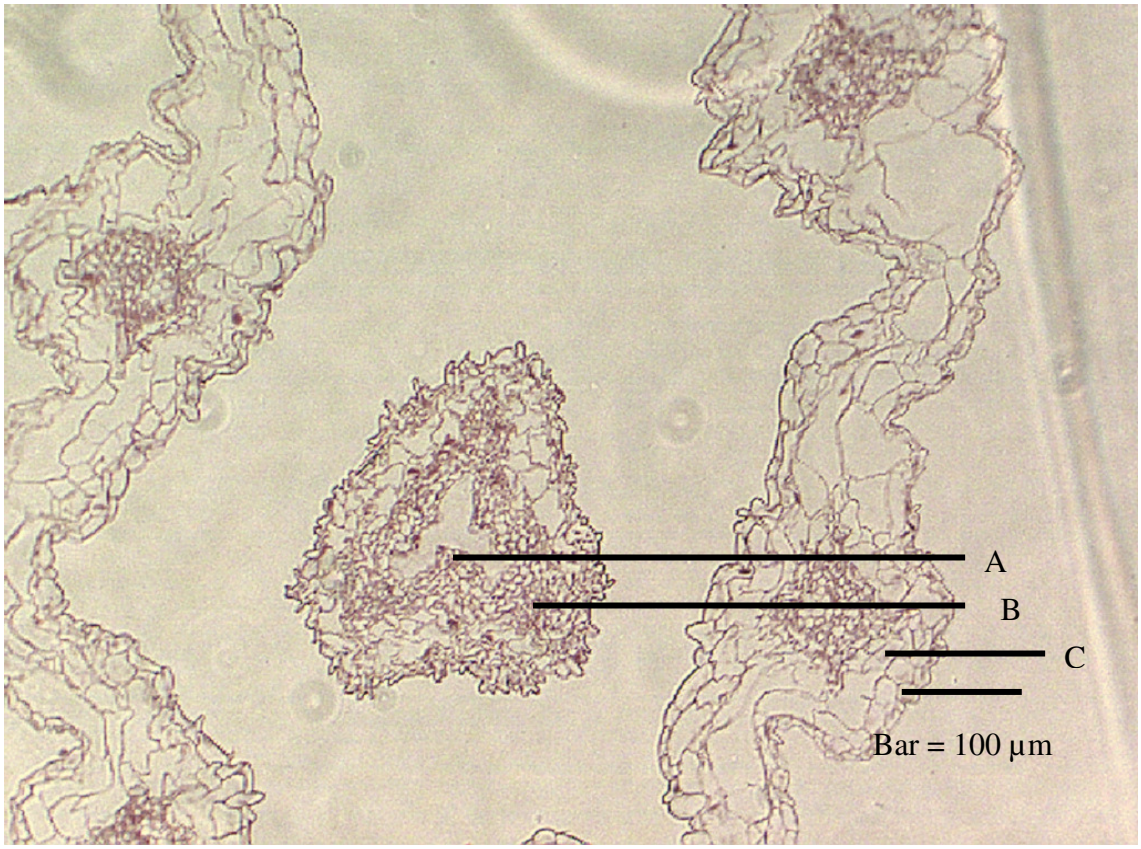

Figure 3-37: *Borya mirabilis* cross-section through tip of style, depicting hollow region (A) through centre of style, surrounded by cellular region (B), encircled by a cross section of the tepals (C).

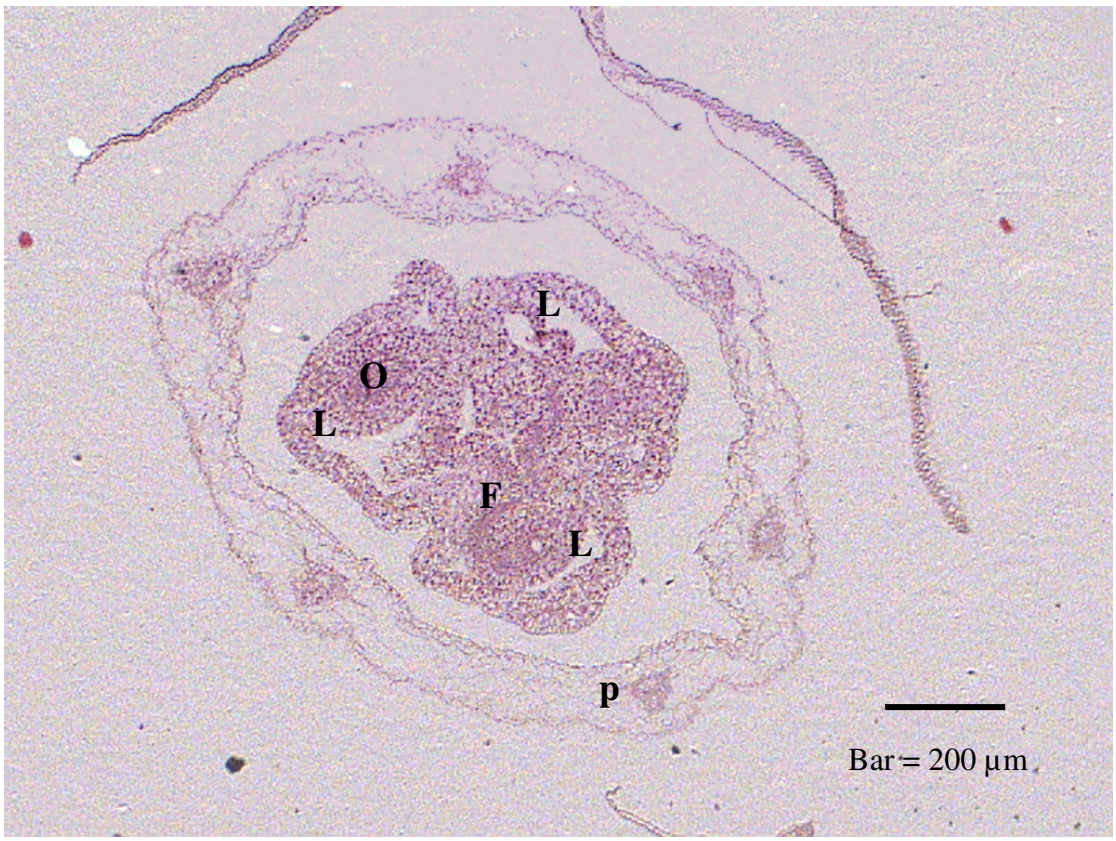

Figure 3-38: ***Borya mirabilis*** **cross-section through ovary of individual flower, depicting the three locules (L) and slicing through the ovules(O) within attached to the placenta by funicles (F), inside the tepals (P).**

3.3.3.2. Borya constricta ovary sections

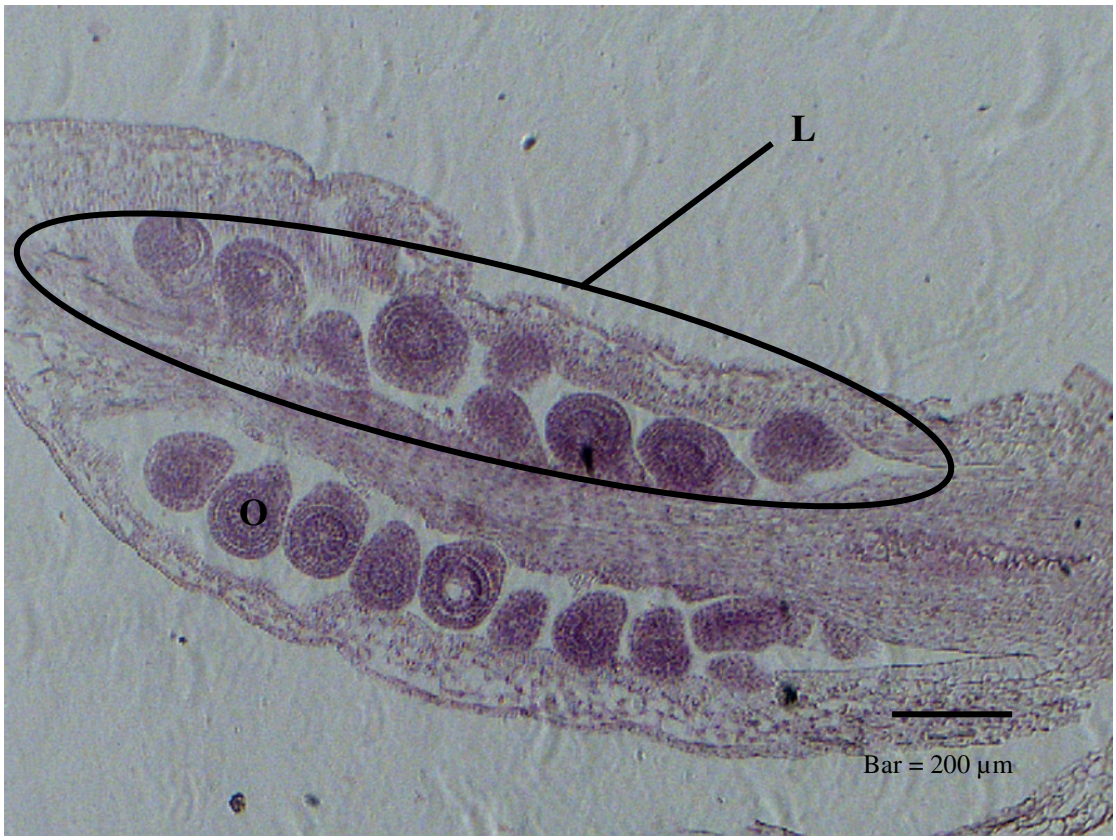

Figure 3-39: Longitudinal section of *Borya constricta* ovary (Day 4), depicting arrangement of ovules (O) inside the locules (L) and axial placentation.

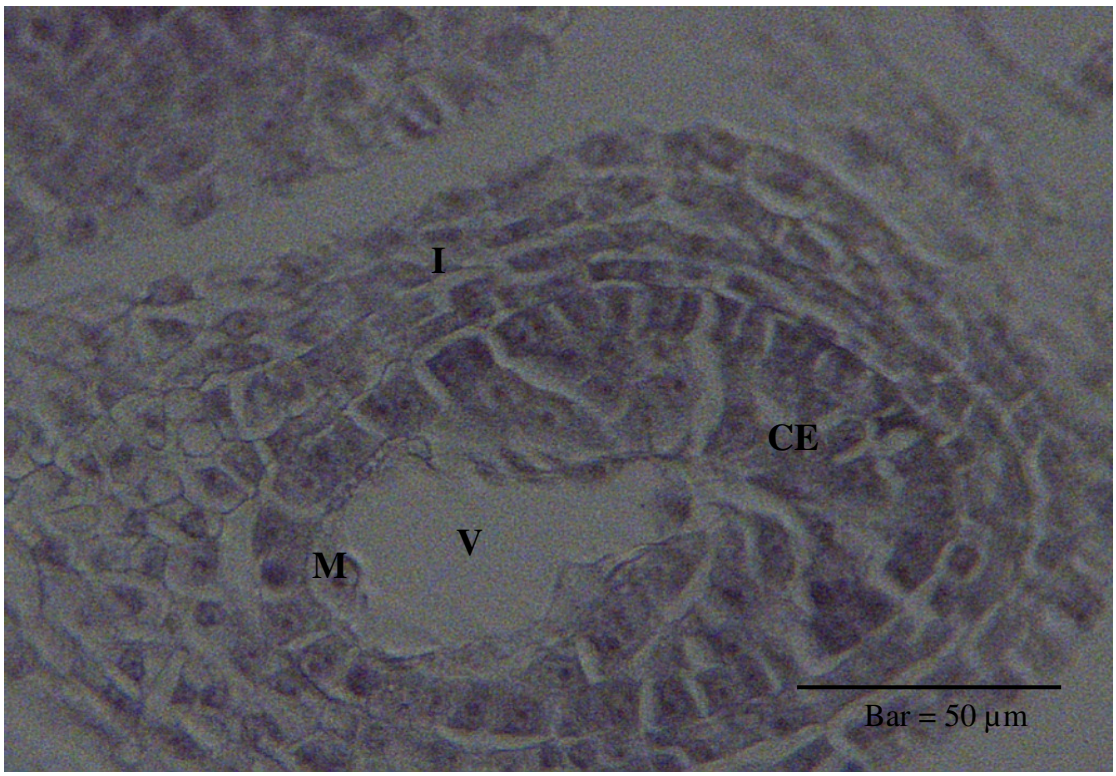

Figure 3-40: Longitudinal section of *Borya constricta* ovule (Day 4) containing megasporcyte (M) inside vacuole (V), surrounded by cellular embryosac (CE) and Integument (I).

3.3.3.3. Borya nitida ovary sections

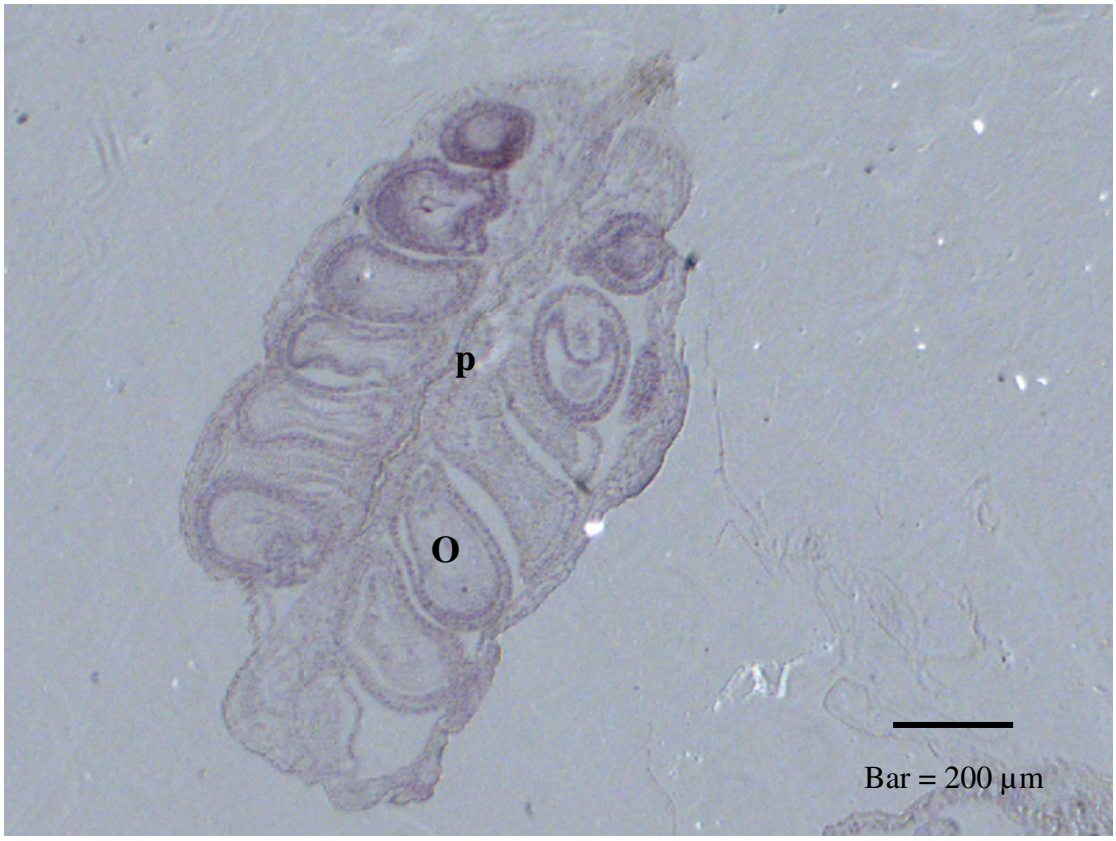

Figure 3-41: Longitudinal section of *Borya nitida* ovary (Day 4) containing large, mostly evenly matured ovules (O) on either side of the placenta (P).

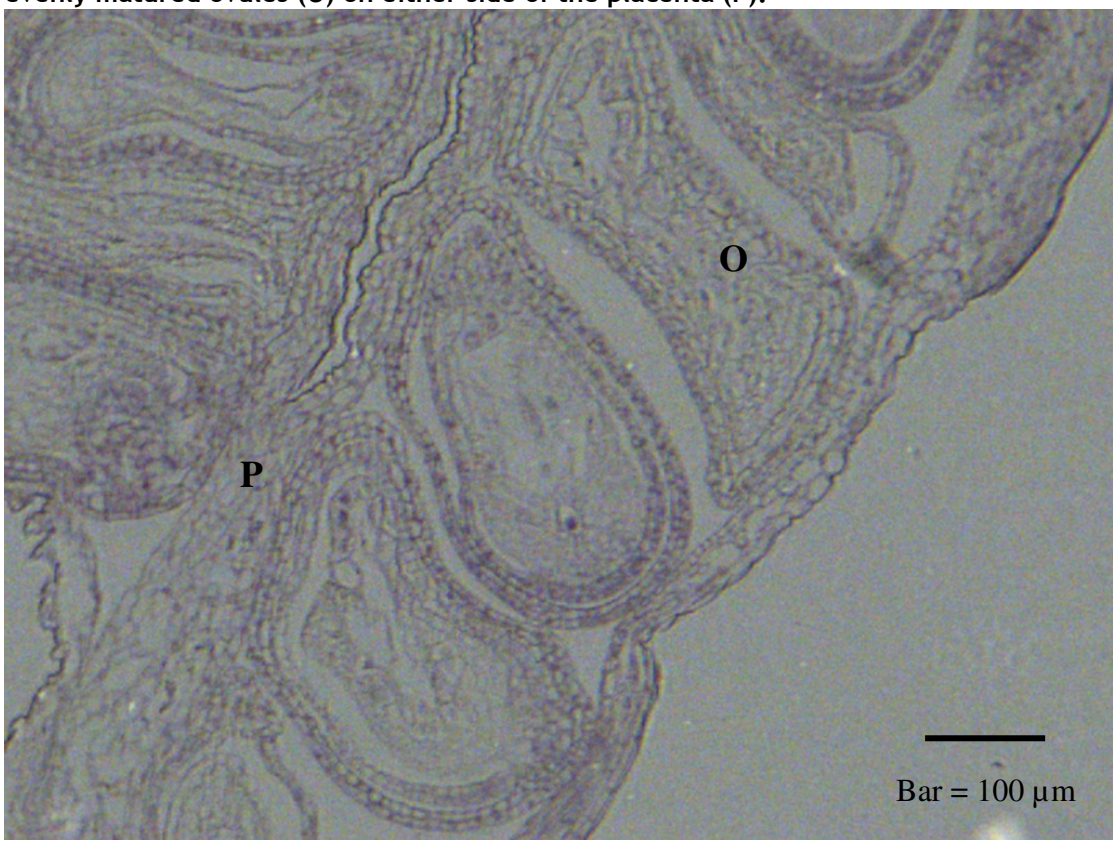

Figure 3-42: Longitudinal section of *Borya nitida* ovules (Day 4) displaying mostly uniform development of ovules (O) on either side of the placenta (P).

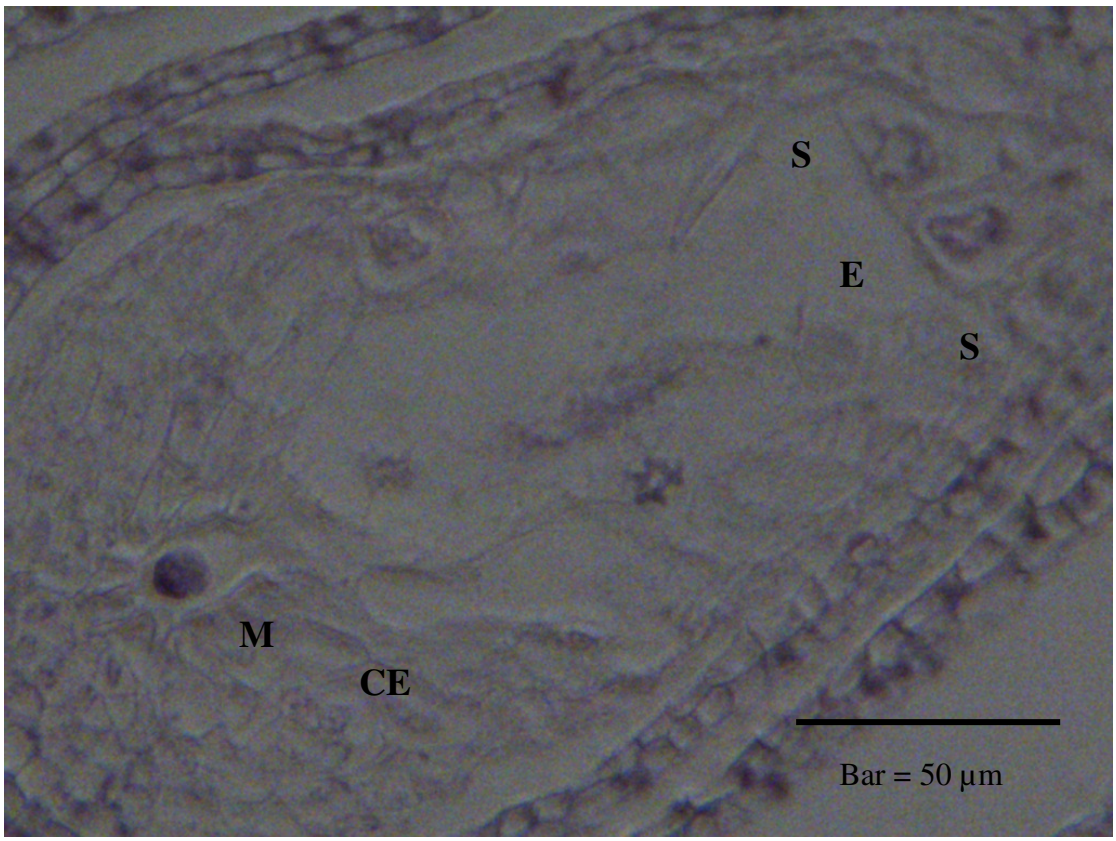

Figure 3-43: Longitudinal section through *Borya nitida* ovule containing secondary endosperm nucellus (M) surrounded by cellular embryo sac (CE) with two synergids (S) on either side of the egg (E).

3.3.3.4. Borya sphaerocephala ovary sections

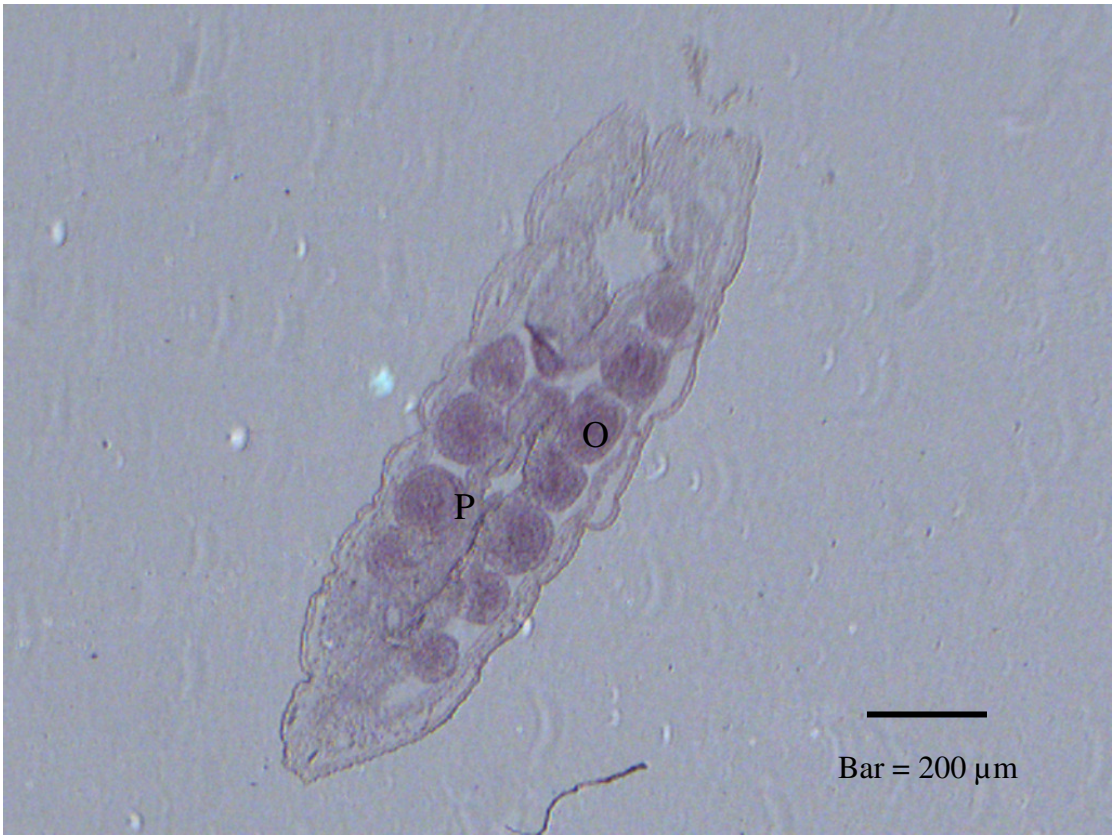

Figure 3-44: Longitudinal section through *Borya sphaerocephala* ovary (Day 4-5), depicting evenly matured ovules (O) on either side of the placenta (P).

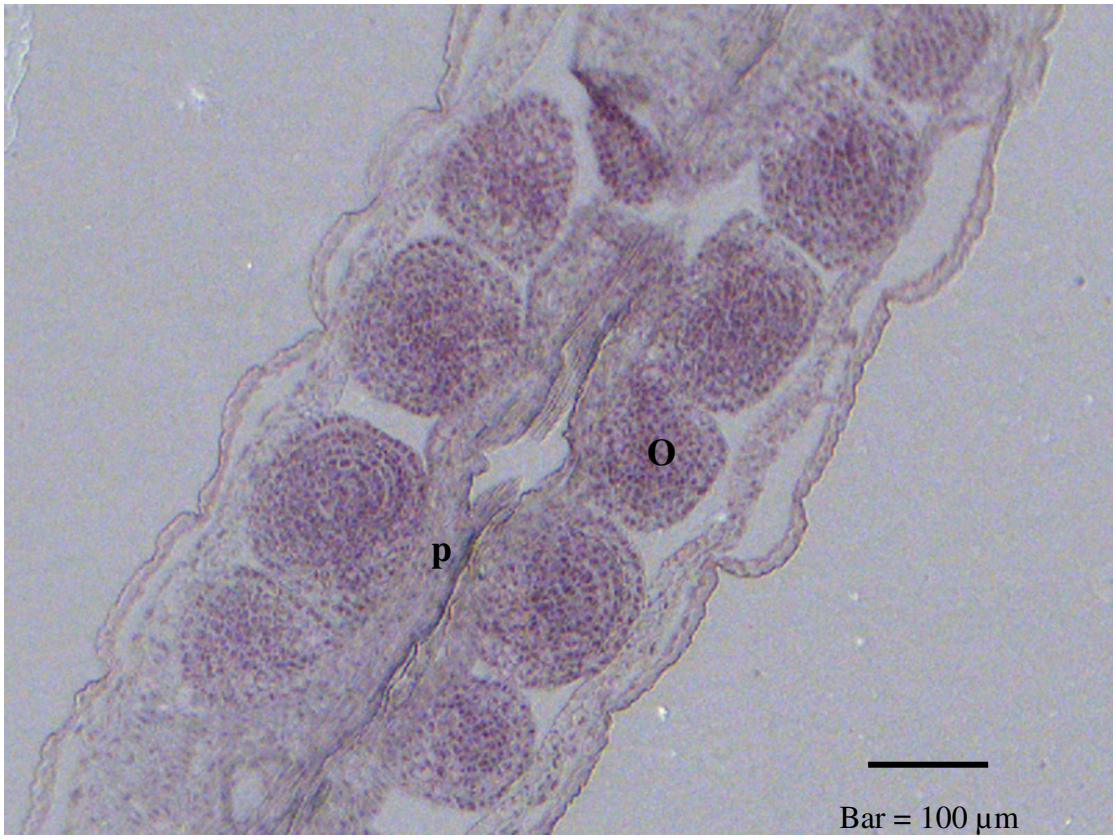

Figure 3-45: Longitudinal section through *Borya sphaerocephala* ovary (Day 4-5) depicting undifferentiated ovules (O) attached to placenta (P).

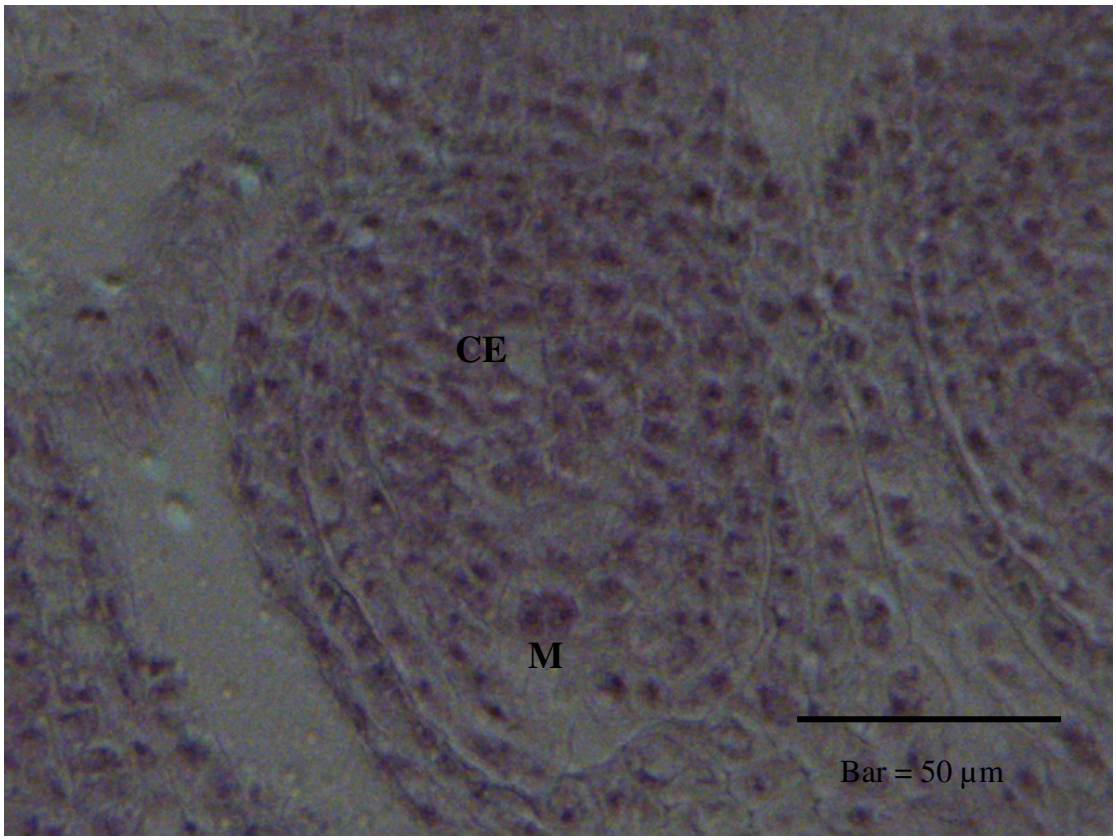

Figure 3-46: Longitudinal section through *Borya sphaerocephala* (Day 4-5) undifferentiated ovule, containing dividing megasporocyte (M) surrounded by cellular embryosac (CE).

3.3.4. *In-situ* and *ex-situ* fertility

3.3.4.1. Presence of pollinators in the naturally occurring field site of B. mirabilis

Several insects visited *B. mirabilis* flowers in the natural field site in November 2004-2005. Flies (Figures 3-47 and 3-48) and ants (Figures 3-49 to Figure 3-51) were the most common and appeared to feed on the nectar produced by the flowers.

Figure 3-47: Fly observed feeding from nectar in *Borya mirabilis* flower in *in-situ* population in November 2004.

Figure 3-48: Fly observed feeding from *Borya mirabilis in-situ* population November 2004.

Figure 3-49: Ants observed feeding from nectaries of *Borya mirabilis in-situ* population, November 2005.

Figure 3-50: Ants observed feeding from nectarines of *Borya mirabilis in-situ* population, November 2005.

Figure 3-51: Ants observed feeding from nectarines of *Borya mirabilis*, November 2005.

3.3.4.2. Borya nitida ex-situ pollinations compared to Borya mirabilis field pollinations

From 450 crosses of *B. mirabilis in-situ* in November 2005 (Table 3-9) only one seed was produced, between the ovules of Colony 5 and the pollen of Colony 7 (Figure 3-52). Three aborted seeds were produced by crossing the pollen of Colony 5 with the ovules of Colony 7 (Figure 3-53).

This was in stark contrast to pollinations undertaken with *ex-situ B. nitida*. *B. nitida* plants produced seed (Figure 3-54) with the same as well as with plants from different populations (Table 3-10), although to a lesser extent when self pollinated (same plant).

Table 3-9: Artificial pollination (hand) of *Borya mirabilis* field population in the first 2 weeks of November 2005.

Colony (C)	Date	No. of crosses	Control	Crossed with flowers on same colony	Crossed with flower of different colony*	Stigma shortened	No. seed produced
C7	12.11.05	30	Yes	No	No	No	0
C7	4.11.05	30	No	Yes	No	No	0
C7	4.11.05	30	No	Yes	No	Yes	0
C7	4.11.05	30	No	No	oC7 pC5	No	3 aborted seed
C7	5.11.05	30	No	No	oC7 pC5	Yes	0
C7	12.11.05	30	No	No	oC7 pC3	No	0
C7	12.11.05	30	No	No	oC7 pC3	Yes	0
C5	12.11.05	30	Yes	No	No	No	0
C5	4.11.05	30	No	Yes	No	No	0
C5	4.11.05	30	No	Yes	No	Yes	0
C5	4.11.05	30	No	No	oC5 pC7	No	1
C5	5.11.05	26	No	No	oC5 pC3	No	0
C5	5.11.05	30	No	No	oC5 pC7	Yes	0
C5	12.11.05	4	No	No	oC5 pC3	No	0
C5	12.11.05	30	No	No	oC5 pC3	Yes	0
C3	12.11.05	30	No	No	oC3 pC7	No	0

* Ovule (o), Pollen (p)

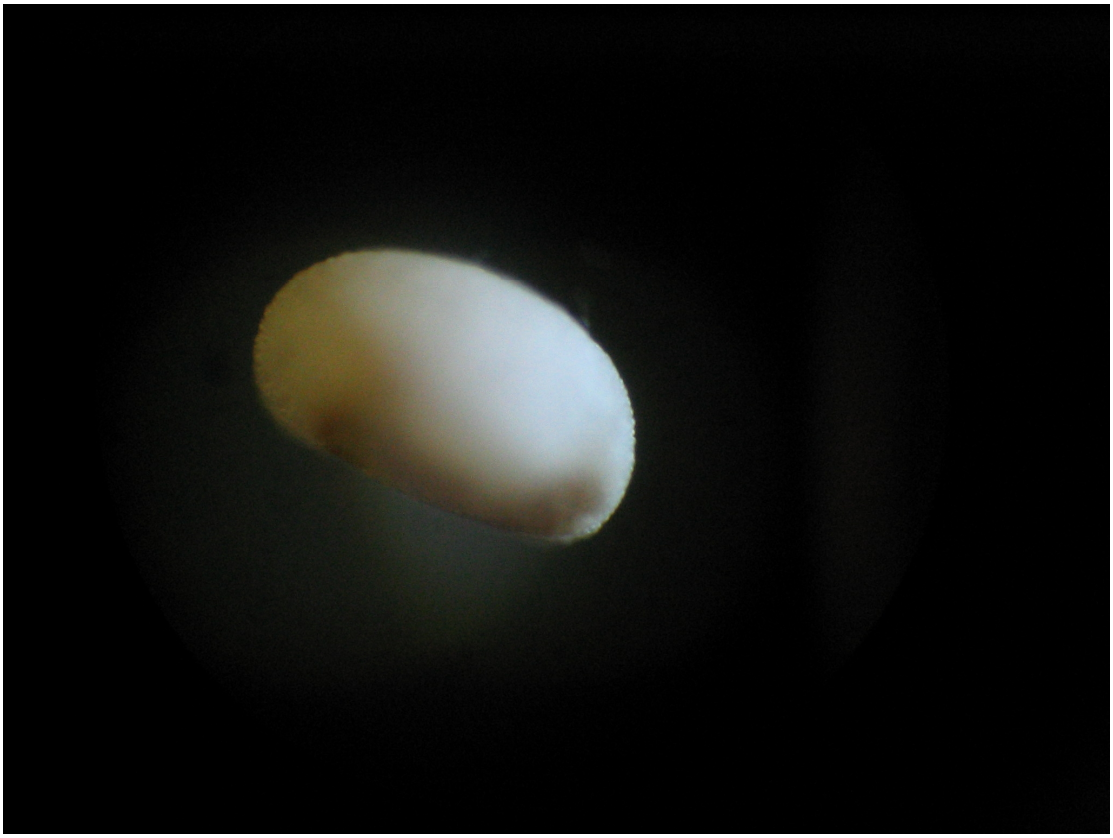

Figure 3-52: Pale seed produced from *in-situ* cross of ovule from Colony 5 and pollen of Colony 7 of *Borya mirabilis*.

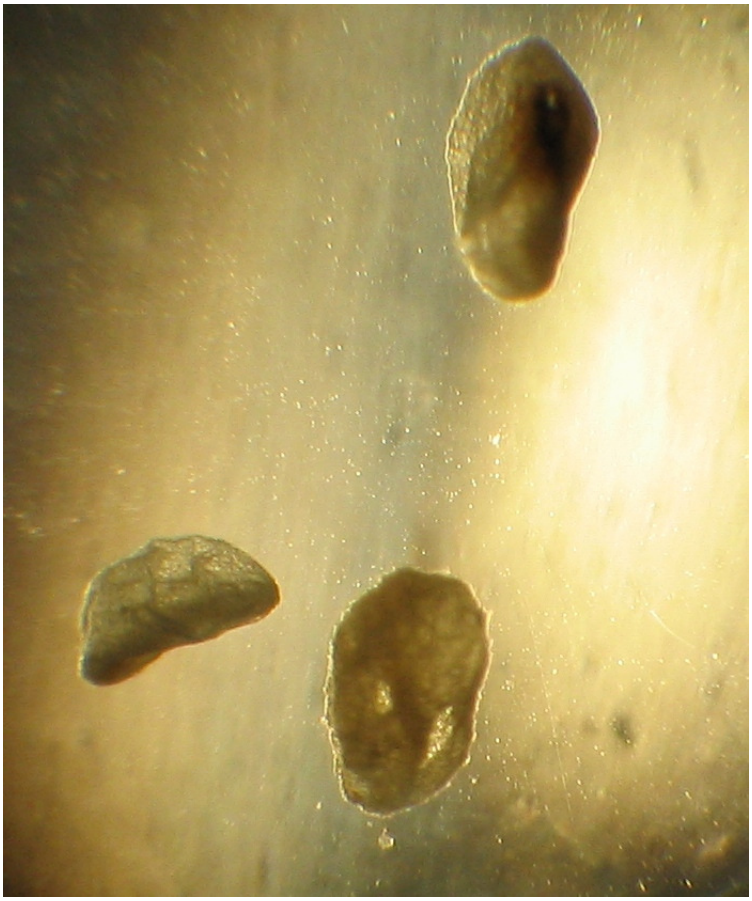

Figure 3-53: Aborted seed from *in-situ* crosses of ovules from Colony 7 and pollen from Colony 5 of *Borya mirabilis*.

Table 3-10: *Borya nitida ex-situ* pollinations in October 2005.

Plant	*No. of crosses*	*Date*	*Crossed with same plant*	*Crossed with plant from different population*	*Plant from different population*	*No. of seeds produced*
U1 (11)	2	12/10/05	Yes	No		0
	3	13/10/05	Yes	No		0
	12	15/10/05	Yes	No		4
	4	16/10/05	Yes	No		0
	8	17/10/05	Yes	No		0
	10	19/10/05	Yes	No		0
P7	2	16/10/05	No	Yes	U1(11)	*
	10	17/10/05	No	Yes	U1(11)	*
	7	19/10/05	No	Yes	P11	101
	5	20/10/05	Yes	No		0
	8	20/10/05	No	Yes	P11	82
	7	20/10/05	No	Yes	P11	101
	7	20/10/05	No	Yes	P11	143
	8	20/10/05	No	Yes	P11	179
	7	20/10/05	No	Yes	P11	103
	6	20/10/05	No	Yes	P11	85
	8	20/10/05	No	Yes	P11	103
	4	20/10/05	Yes	No		22
P11	7	17/10/05	No	Yes	P7	100
	5	19/10/05	No	Yes	P7	40
	2	20/10/05		Yes	P7	39
	1	20/10/05	No	Yes	P7	41
	1	20/10/05	No	Yes	P7	22
P9	5	19/10/05	No	Yes	P7	90
	2	20/10/05	No	Yes	P7	42
P4	5	20/10/05	Yes	No		0
	4	20/10/05	No	Yes	P7	57
	5	20/10/05	No	Yes	P7	76
P10	5	20/10/05	No	Yes	P9	70
	1	20/10/05	Yes	No		0

* missing data

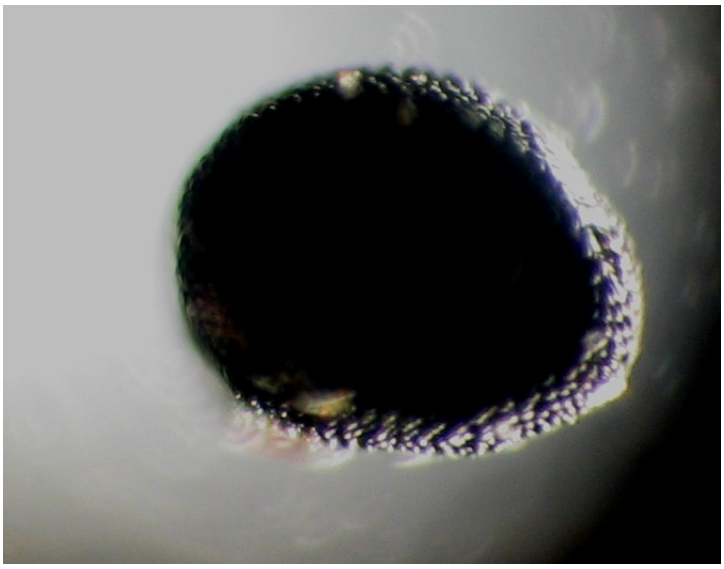

Figure 3-54: *Borya nitida* seed produced from *ex-situ* crosses in October 2005.

3.3.4.3. Ex-situ population of B. mirabilis crossed with B. constricta and B. sphaerocephala

No seed was produced by crossing *ex-situ B. sphaerocephala* pollen or *B. constricta* pollen with *ex-situ B. mirabilis* ovules. Also, no seed was produced from crossing *ex-situ B. mirabilis* pollen with *ex-situ B. mirabilis* ovules.

3.3.5. Chromosome analysis

The squashed root tips of *B. constricta* contained 26 chromosomes. The squashed root tips of *B. mirabilis* contained approximately 66 chromosomes (Figure 3-55 and 3-56).

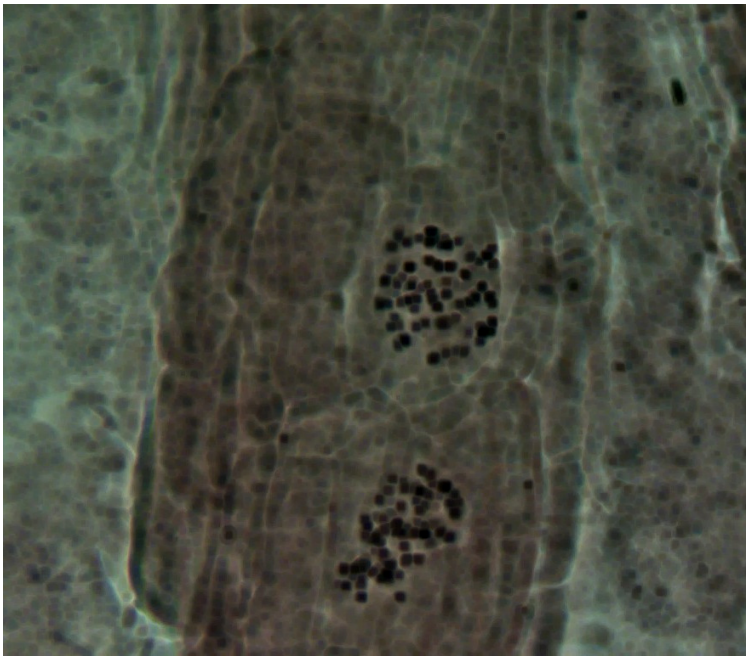

Figure 3-55: *Borya mirabilis* chromosomes.

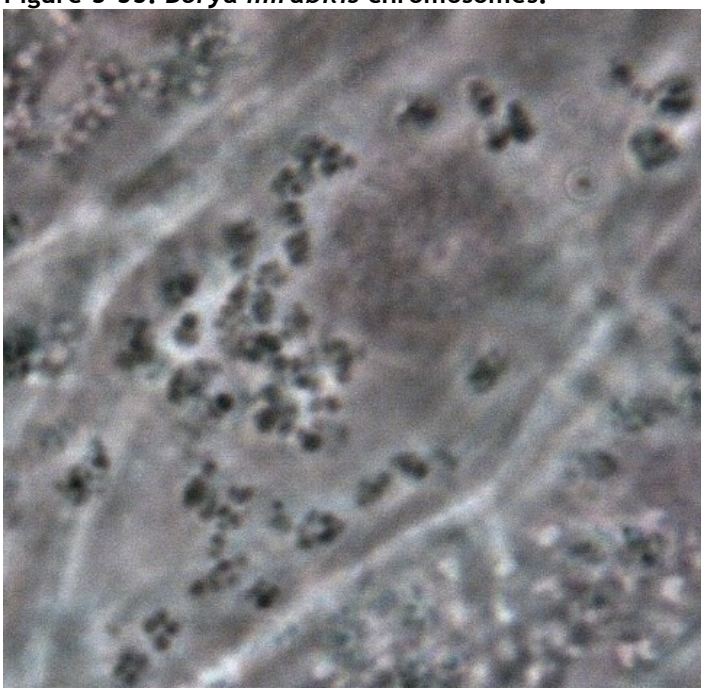

Figure 3-56: *Borya mirabilis* chromosomes.

3.4. Discussion

Infertility begets rarity. This is unfortunately the situation that has been clarified with *B. mirabilis* in this research, like that undertaken with other threatened Australian native plants (Kimpton *et al*. 2002; Burne *et al*. 2003, Gross and Caddy, 2006). At the initiation of the research presented in this chapter, there were several possible causes for the lack of recruitment and seed production seen in *B. mirabilis*. These included: pollen inviability, ovule inviability, self-incompatibility, lack of pollinator and cytological abnormalities. The findings of this chapter illustrate that a combination of these factors, including unviable pollen and, unevenly maturing ovules, possibly caused by the polyploid nature of this species, are the likely causes for the extremely low fertility in *B. mirabilis*.

The extremely high chromosome number when compared to other *Borya* species suggests that infertility seen in *B. mirabilis* is caused by polyploidy, rather than just self-incompatibility. This would explain the highly abnormal floral morphology; aberrant pollen production and viability; and the uneven maturity of ovules. Infertility caused by several sexual aberrations in threatened species is relatively common (Koshy and Jee, 2001; Gross and Caddy, 2006), with factors such as pollen viability, anthesis mechanisms and ovule viability all affecting fertility. Unfortunately the mechanisms behind infertility are often looked at in isolation, without the context of cytology and geneology. The work in this chapter combined the floral information with cytology and will aim to put this in context of the molecular phylogeny of the Boryaceae in subsequent chapters.

3.4.1. Floral observations

Observations of the flowers of *B. mirabilis* found some unique surprises. There was no difference in the length flowers remained opened un-pollinated compared to those pollinated, which is unusual as pollination is usually followed by a reduced flowering length, but was expected due to the reported lack of seed production and hence lack of pollen tube growth and

fertilisation. Uniquely amongst the Asparagales other than the orchids is the presence of petaloid nectaries in *B. mirabilis* and the other *Borya* species examined. Septal nectaries occur in the majority of the Asparagales (Vogel, 1998a) and these results contradict the study by Berardello (2007), in which he found septal nectaries in *Borya* species. The Asparagales are commonly known to have dry stigmas (Dahlgren and Clifford, 1982), whereas *B. mirabilis* has wet stigmas, particularly after the anthers have dehisced (perhaps unique amongst the Asparagales). The style in the *Borya* species examined were hollow, which is common for the higher asparagoids and most syncarpous monocots (Rudall, 2002).

The floral observations of *B. mirabilis* and related species affirm the uniqueness of the Boryaceae amongst the Asparagales. *B. mirabilis* recorded an unusually high number of reproductive abnormalities compared to the fertile *B. sphaerocephala, B. constricta and B. nitida*. This suggests that factors other than inbreeding or self-incompatibility as a cause of the lack of seed set in the species. These difficulties are likely to be the result of polyploidy. These abnormalities are prominent in the examination of the reproductive structures, particularly that of the pollen when compared to its closely related species.

3.4.2. *Ex-situ* study of pollen viability

Mature pollen of *Borya* species showed many differences in the characteristics of the mature pollen between species, with viable pollen being unicolpate and prolate with thick walls and reticulate patterning of the pila. Examination of the viability of pollen of *B. mirabilis* with FDA, like that of Coates *et al*. (2002), estimated a possible 46% of the pollen to be viable. FDA uses the integrity of the plasmalemma of the pollen as an indicator of viability (Heslop-Harrison and Heslop Harrison, 1970).

Further examination under the light and electron microscope of *Borya* pollen showed the majority (approximately 99%) of the pollen dehisced to be immature, with the reverse situation occurring in *B. nitida*, *B.*

sphaerocephala and *B. constricta*. Pollen inviability, with infertile pollen often being smaller in size, lacking in cellular content and having abnormally thick exine, like that seen in *B. mirabilis* has been reported in many other threatened species (*Grevillea rhizomatosa* Gross and Caddy, 2006; *Grevillea infecunda* Kimpton *et al*. 2002; *Grevillea althoferorum* Burne *et al*. 2003; Hescorvitch and Martin, 1989).

It is likely that the lack of fertile pollen was due to a lack of maturity at anthesis. These conclusions about immaturity of the pollen of *B. mirabilis* are based on pollen morphology, coupled with the evidence from the pollen growth experiments, where *B. mirabilis* had extremely low germination rates, slow growing pollen and deformed pollen tube growth compared to that of other *Borya* species. Small amounts of similarly deformed pollen are seen in many fertile species, with the small proportion in fertile *Borya* species examined correlating well with pollen tube growth.

Pollen infertility is characteristic of many clonally reproducing species (Warburton *et al*. 2000; Sharma, 2001; Kimpton *et al*. 2002). *B. mirabilis* is the only species in its family to have clonal reproduction as its main form of propagation. Prolonged clonal production affects population genetic structures (Honnay *et al*. 2006), with clonally producing plants often losing the capacity to evolve broad ecological tolerances (Anderson *et al*. 1994), though cloned propagation avoids the problems of infertility and makes it possible for the species to continue to expand.

3.4.3. *Ex-situ* study of ovule viability

Examination of the structure of *B. mirabilis*, *B. sphaerocephala*, *B. constricta* and *B. nitida* ovules confirmed that the ovules appeared to be viable. *B. mirabilis* was distinct in generally having non-uniform development of the ovules. Gynoecial malformations have been important limiters to fertility, as seen in the threatened *Ulmus minor* (Lopez *et al*. 2003). This does not seem to be the case with *B. mirabilis*, although the uneven maturity of the ovules could potentially cause extremely low seed

set. Developmentally it appears that *Borya* species ovules mature from the division of the initial megasporocyte into two megaspores, one of which undergoes further division to become the egg and synergids, the other which remains surrounded by a degrading cellular embryo sac and possibly undergoes division at a later stage, not witnessed here. At no stage of pollination was disruption of the ovaries by pollen tubes seen, which would be a result of the lack of pollen viability combined with the viable pollen being slow to develop. Although ovules seem slow to develop, at least a proportion seemed to be viable, with clearly delimited egg cells, synergids and endosperm nuclei, awaiting fertilisation.

3.4.4. *In-situ* and *ex-situ* fertility

It is unlikely that pollination limitations are the cause of infertility of *B. mirabilis,* as seen with other threatened species such as *Thelymitra epipactoides* (Cropper *et al.* 1989) and *Banksia godii* (Lamont *et al*. 1993). Pollinator observations in the field population suggests that many potential pollinators are available. When the *B. mirabilis* field population plants are in flower they are a virtual hum of activity, being frequently visited by flies and small black ants. Reports on the Western Australian *Borya* species suggest that they are fly-pollinated (Keighery, 1984) and this is also possible for *B. mirabilis*, as flowers attracted flies as well as ants.

The hand pollinations carried out in the field and *ex-situ* populations of *Borya mirabilis* produced only one seed, from the field population. This, however, is extremely important as it is the first seed of this species ever produced artificially and the first recorded seed for the species. While the production of only one seed confirms that there is very low fertility, it demonstrates that it is worth persevering with attempts to hand-pollinate the species. The production of seed and aborted seed is similar to the situation seen in the threatened *Coptis teeta* (Pandit and Babu, 2003). The production of both mature and aborted seed from crosses between Colony 5 and Colony 7 in the field population suggests that there may be as yet un-identified genetic diversity within the field population of *B. mirabilis* and

this could be further exploited on these colonies in the future, with efforts at hand pollination to be focused .

With the fertile *B. nitida,* seed was produced from crossing between plants as well as within a plant, although the latter to a lesser extent. This suggests that the infertility seen in *B. mirabilis* may not be due to self incompatibility, as self incompatibility does not occur in *B. nitida* (although self fertility was very low compared to other crosses and suggests that if to little difference between ovule and pollen occurs, dramatically induced fertility ensues). Pollinations, using the pollen of the closely related *B. constricta* and *B. sphaerocephala* on *B. mirabilis* plants, proved unsuccessful. This was later shown to be the likely consequence of chromosomal difference, with *B. mirabilis* possessing approximately three times the chromosome number of the other species. It is unlikely, therefore, that further attempts to introduce hybrid vigour would be successful. If future attempts are made to introduce hybrid vigour reduction of the chromosomes of *B. mirabilis* by producing haploid plants in tissue culture would first need to be attempted, this alone may aid the infertility issues within the species.

3.4.5. Chromosome analysis

A polyloid plant is one with two or more sets of homologous chromosomes. Polyploid simply means a number of ids, an id being a unit of germplasm (Weissman, 1892). Polyploid plants are extremely common. In the relatively short period since the Cretaceous era angiosperms have gone through several polyploid events, these events being a means of adaptation and speciation (Ohno, 1970). *Arabidopsis thaliana* is estimated to have gone through three such doublings of chromosome number over geological time (Henry *et al.* 2005). Therefore discovering that *B. mirabilis* is polyploid does not hold a lot of meaning in itself; it is only when the ploidy level of its closest fertile relatives, combined with sound knowledge on the species, reproductive biology that this comes into context.

Many studies on endangered species limit research to structural aberrations; several studies take this concept further and look at the causes behind the structural aberrations encountered, the cause often being a ploidy event. Examples of these holistic studies have been seen in research on: *Abronia macrocarpa,* a polyploid with a long history of isolation (Williamson and Werth, 1999); *Rutidesis leptorrhynchoides,* a self incompatible autoployploid (Brown and Bledsoe, 2000); *Swainsona recta,* a tetraploid (Buza *et al*. 2000); and *Spartina X towsendii,* a fertile hybrid (Thompson *et al*. 1991).

3.4.6. Conclusions

The evaluation of the mechanism of infertility in *B. mirabilis* has helped unravel the evolutionary pathway this species has undertaken towards sterility. Lack of fertility in *B. mirabilis* is probably due to a combination of reasons including ovule fertility, pollen inviability and polyploidy. The evaluation of the precise causes of infertility will guide future practical interventions with this threatened species, with a sound knowledge of what is worthwhile implementing in management actions. Consequences of these investigations are that random field pollinations are an inefficient use of resources but that more precise pollinations between plants in Colony 5 and Colony 7, may produce more seed. It is vitally important to represent all plants in translocation sites so that all pollinations that can occur naturally in the field are possible. The small amount of seed set suggests that a small but critically important amount of genetic variation is present amongst the plants in the field, a finding which substantiates the need to investigate the genetic variability in the field and in *ex-situ* populations in the next chapter. As a means of propagating this species it would therefore be more effective to undertake clonal propagation particularly tissue culture/micropropagation.

The findings in this chapter open further questions about the nature of *B. mirabilis*. If *B. mirabilis* is a polyploid, is it a polyploid of one of the common Western Australian species or of a now extinct diploid ancestor? Is

B. mirabilis a unique species of its own? Is it an allopolyloid arising from hybridization of other *Borya* species? If so, what are the progenitors of the species? These questions are addressed by using molecular techniques in the following chapter.

4. Molecular Phylogeny of the Boryaceae

4.1. Introduction

One of the priorities of the *Borya mirabilis* Recovery Team is achieving healthy self-sustaining translocated populations, sourced from *ex-situ* plants grown (currently from cuttings) in the *ex-situ* population at the Royal Botanic Gardens Melbourne (RBG). The objectives of all translocation programs should be to support the conservation of the target species, and to establish or maintain one or more self-sustaining populations surviving in both the short and long term.

The following chapter investigates the relationships amongst the field population and *ex-situ* collection and determines their genetic variability. It is important to understand the genetics of a population in order to adequately represent the genetic diversity in translocation sites and preserve any ability the population has to adapt and change. Adequate representation of a population also reduces the risk of inbreeding.

As outlined by Vallee *et al*. (2004), research into the genetics of a species is essential when:

- the status of a taxon is debatable (the status of *B. mirabilis* as a separate species is debatable);
- the origin of the material to be translocated is unknown (*B. mirabilis* cuttings at RBG have lost their original labels);
- selecting which natural population from which to take source material (there is only one natural population of *B. mirabilis* but this may be genetically diverse);
- clonal reproduction of a species is extensive (each plant of *B. mirabilis* has many ramets, which are now possibly isolated from their parents).

B. mirabilis is listed as a separate species from *B. nitida* in Western Australia. The morphological differences between the two species are only

slight and their separate status has been the subject of debate. This research investigates if *B. mirabilis* is a separate species on molecular evidence. This molecular research also sets out to differentiate between species of the genus *Borya* and elucidate their relationships to one another.

Chapter 2 pointed out the extremely low fertility of *B. mirabilis*. One possible avenue of re-introducing fertility into the population would be to cross *B. mirabilis* with its closest relative and then backcross repeatedly with *B. mirabilis* to produce fertile species but with the genetics of *B. mirabilis*, as done to introduce new traits to crop species. This chapter therefore investigates the relationship of *B. mirabilis* to other *Borya* species and determines its closest relative; in addition it examines the variation within the current population of *B. mirabilis* and the origins of the *ex-situ* plant material currently available for translocation.

4.1.1. Relationships amongst the Boryaceae

The Boryaceae consists of two genera: *Borya* and *Alania* (a monotypic genus). The Boryaceae are well supported as a unique clade amongst the Asparagales in the monocots (Källersjö *et al*. 1998). The relationships among the species in the genus *Borya* have not been described using molecular methods in the literature to date and species relationships are not yet known.

Several papers have been published on the molecular relationships amongst the monocots, including the Boryaceae.

- In Bremer (2000), the Boryaceae, Orchidaceae and Lanariaceae cluster together in a clade on the basis of *rbcL* chloroplast DNA sequences.
- Janssen and Bremer (2004), placed the Boryaceae as a sister group to the Orchidaceae and the rest of the Asparagales when investigating *rbcL* sequences.
- Chase *et al*. (2000) placed the Boryaceae as unresolved at the base of the Asparagales (based on *rbcL* chloroplast sequences, *atpB* sequences and 18S rDNA sequences).

- On *matK* chloroplast DNA sequence information (Hilu *et al*. 2003) the Boryaceae were placed as a sister group to the Orchidaceae in the lower Asparagales within the astelioid clade, but there was less than 50% jackknife support.
- Fay *et al*. (2000) using *rbcL*, *atpB* and *trnL-F* regions, placed the Boryaceae as a sister group to the astelioid clade, which was a sister group to the core Asparagales.
- Davis and Stevenson, (2004) placed the Orchidaceae as a sister clade to the rest of the Asparagales and Boryaceae, within a clade containing *Blandfordia* and *Astelia* that was basal, and sister clade to the rest of the Asparagales; again the branch supporting this clade had low jackknife and bootstrap support (less than 75 BP).

All these studies differ in the placement of the sister groups of the Boryaceae and in the clade in which the Boryaceae resides. This suggests that the relationships amongst the Asparagales are more complex than encompassed by those regions that have been examined. Until larger data sets from differing regions are combined and larger sampling of the Boryaceae is included, the relationship of the Boryaceae within the Asparagales will remain unclear.

4.1.1.1. Useful molecular regions

There are several well known useful regions used for examining species relationships at the molecular level. Several useful molecular regions, including those which are used in this chapter, have been outlined below (Figure 4-1).

The plastid genome is useful to look at the family and subfamily level loci. This has many benefits (Olmstead and Palmer, 1994), including the fact that it does not amplify fungal or insect DNA. A combination of non-coding chloroplast regions has proved useful in determining relationships amongst

plants at both the genus (Gernandt *et al*. 2005) and species level (Smith *et al*. 2006).

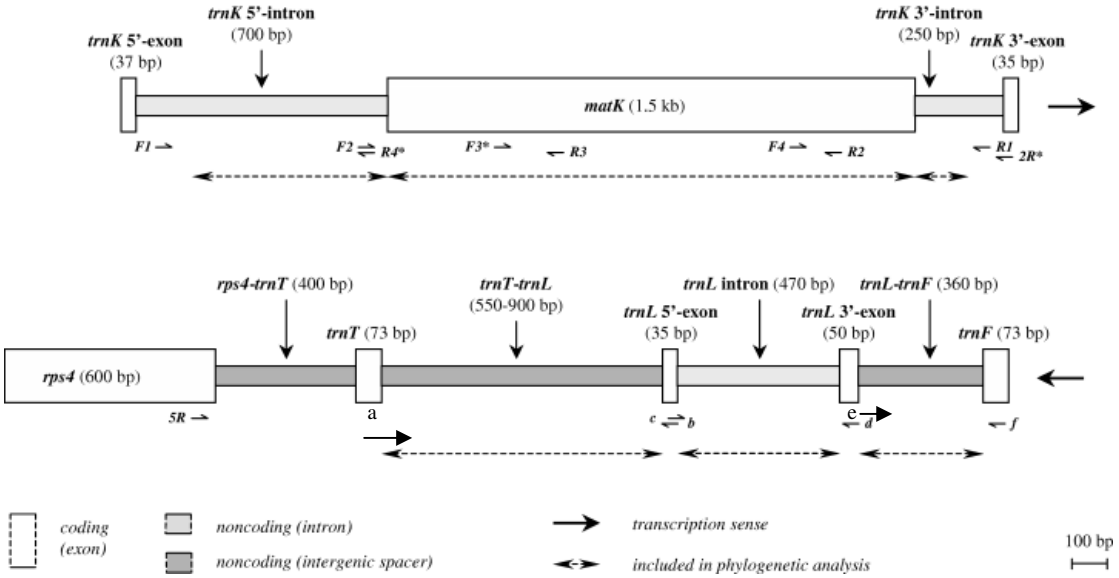

Figure 4-1: Schematic representation of the chloroplast regions used in this study (Sauquet *et al*. 2003). a-b show the positions of the trn T-L region primers, c-d show the position of the of the trnL intron region primers and e-f show the position of the trnL-F region primers.

Ribosomal DNA (rDNA) contains both genes and spacers, which makes it suitable for investigations at different taxonomic levels. Significant differences are found in the spacer regions between species, while not apparent in the coding regions. The 18S rDNA is not useful for distinguishing below tribal level. To look at intraspecies relationships, one must look at the ITS (internal transcribed spacer) region of genomic DNA (Bateman *et al*. 2003; Clements *et al*. 2002; Hillis and Dixon, 1991).

Typically the ITS regions that are used are ITS2 and ITS2, with a variety of primers devised by White *et al*. (1990) and subsequent authors (Figure 4-2). The drawbacks of using ITS primers are that they can amplify contamination from insects and fungi, and therefore prove difficult if endophytes are present in plant material.

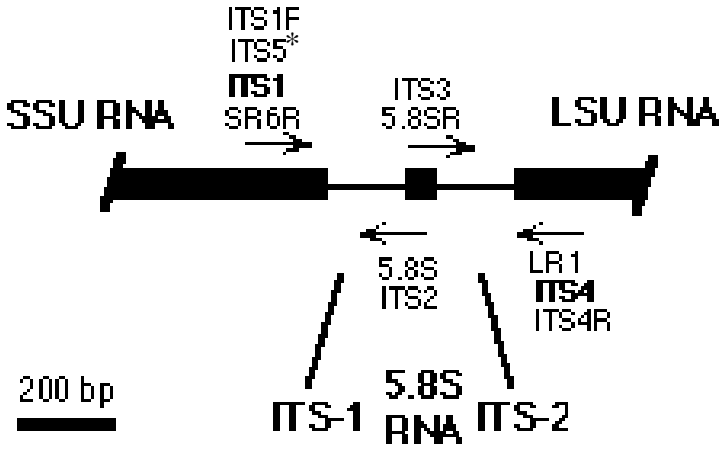

Figure 4-2: Internal transcribed spacer (ITS) region primers (Vingalys Lab Duke University, www.biology.duke**.edu/fungi/mycolab/**primers**).**

Another option to avoid contamination is to use plant-specific ITS primers or, once several sequences are obtained, to develop *Borya*-specific primers. Some plant-specific primers have already been developed for the Orchidaceae and these may be of use in *Borya* due to their sister relationship. A plant-specific primer set has been developed for *Dendrobium* spp. (Tsai *et al*. 2004), among others, and it should be possible to develop specific primers for *Borya* spp.

Examining a combination of chloroplast and genomic DNA can often prove informative as to the evolutionary development of particular species. Chloroplast DNA is mostly maternally inherited while genomic DNA is inherited through both parents. Incongruence between chloroplast DNA and genomic DNA can often resolve hybridization and therefore speciation events. Two main reasons for incongruence between chloroplast and genomic DNA have been proposed. The first is division of an ancestor that contained many chloroplast types (Riesberg and Wendel, 1993; Wendel *et al*. 1994) and the second and probably most likely explanation is hybridization followed by the exclusion of the maternal genome (Soltis and Kuzoff, 1995).

4.1.2. Relationships within *Borya mirabilis*

Understanding the genetics of a population is critical to endangered species management. It allows for adequate preservation of the genetics of a species now, for their future evolution and, if understood early enough in the

degradation of a species, may help prevent a situation where a species suffers from inbreeding depression. Endangered species are prone to inbreeding and genetic drift (Schemske *et al*. 1985; Barrett and Kohn, 1991; Elltrand and Elam, 1993). The general premise is to limit the chance of inbreeding and maintain genetic variation (Hedrick and Miller, 1992). This will preserve some ability to adapt to change in the environment, whether from a new pathogen or degradation of the natural habitat, as seen with *B. mirabilis*.

Unfortunately, there are no precise figures available to determine the size of population sufficient to maintain this diversity and estimates range from 500 (Franklin, 1980) to 5000 (Lynch and Lande, 1998) breeding individuals. The *B. mirabilis* population already falls well short of these numbers. Genetic analysis does not often form part of key conservation goals in the recovery of a species, largely due to the additional expense and expertise that would be required for limited funded recovery programs. The major questions are how then to proceed and what is the best method?

The relationships of the plants forming the only known wild population of *B. mirabilis* have been described in two separate studies, which produced contradictory results. The first study used isozymes (Fripp and Cropper, La Trobe University unpublished), which are heavily dependent on the state of metabolism of the plant and have been poor measures of genetic diversity in other plants (Hoffmann and Parsons, 1991; Gray, 1997), and the second study used a RAPD (randomly amplified polymorphic DNA) analysis (Coates *et al*. 2002). Isozymes results suggested that four of the five clumps were identical, whereas all clumps were found to be identical using RAPD analysis. The latter study that suggested that they were identical used 20 Operon primers. In the RAPDS on *B. mirabilis* (Coates *et al*. 2002) the following primers were used (Liz James, pers. comm.): OPB 1-4, OPB 8-9, OPB 11-14, OPB 20, OPA 1-2, OPA 5, OPA 7-12 and OPA 15-20 (Operon Technologies).

These partly contradictory results were based on only limited testing using different plant material at different times. They also used relatively small

numbers of isozymes or primers, fewer than have been used in other studies where results from the two methods have agreed. For example in the endangered *Aldrovanda vesiculosa*, both RAPD and allozyme research (Adamec and Tichy, 1997) were conducted, but gave complementary results **(**Maldonado *et al*. 2003). One hundred and fifty one RAPD primers and 15 loci of seven enzymatic systems were used. The genetic diversity of *B. mirabilis* therefore needs to be re-examined, as knowledge of its genetic diversity is an important part of recovery plans and may determine from which plants it is most desirable to propagate, to conserve the remaining biodiversity.

DNA-based methods of testing for genetic diversity, especially PCR (polymerase chain reaction) based methods, have the advantages of requiring only small amounts of tissue (100 mg is sufficient) and of being insensitive to metabolic state. These methods have been widely used (Innis *et al*. 1990) in biodiversity and phylogenetic studies. For example, a study in the endangered *Ophiopogon xylorrhizus* compared its genetic diversity to that of its closely related but common progenitor *Ophiopogon intermedius* (He *et al*. 2000) and showed that the widespread common species was able to maintain higher levels of genetic diversity than the endangered species.

There are several ways to determine the genetic variation within a population of plants using molecular methods. The most commonly used of these techniques, RAPD and ISSR, are discussed below.

- RAPD (Random Amplified Polymorphic DNA) analysis is effective for population and intra-species level diversity estimates. It has been used in profiling other endangered species populations such as *Iliamna corei* (Stewart and Porter, 1995) where 940 primers (Operon Technologies) were used to determine clonal identity in asexually reproducing plants. Advantages of RAPDs include more markers being available than with allozymes; being more discriminating than AFLP's; markers not being focused on a family of sequences or gene products like allozymes; and surveying the entire genome (Stewart and Porter, 1995). RAPDs are a dominant marker system (Williams *et al*. 1990) and are widely used to estimate genetic diversity (Auge *et al*. 2001;

Moya *et al*. 2001; Belaj *et al*. 2002; Lanteri *et al*. 2004; Geleta *et al*. 2007) and genetic structure (Pluess and Stocklin 2004; Besnard *et al*. 2007) of clonal plants.

- ISSR (Inter Simple Sequence Repeats) are effective for population genetic studies such as that performed on the endangered species *Changium smyrnioides* (Qiu *et al*. 2004). The results from this study were compatible with a study previously conducted on the same plant with allozymes and RAPD. ISSR can reveal more polymorphic fragments per primer than RAPDs (Qian *et al*. 2001) and has greater reproducibility due to more stringent annealing temperatures (Ge and Sun, 1999; Camacho and Liston, 2001).

4.2. Methods

4.2.1. Interspecies relationships in the Boryaceae: analysis of chloroplast and nuclear DNA

Plant material was collected fresh and stored at -20ºC with silica beads until use or, where fresh material could not be acquired, herbarium specimens were obtained (Table 4-1 to Table 4-3). Botanists in Queensland, the Northern Territory and Western Australia were contacted for aid in collecting samples of *Borya* species. In addition, a personal expedition of the south-west of Western Australia was undertaken. Permits for Victoria and Western Australia for collection of *Borya* plant material were obtained from Parks Victoria and Conservation and Land Management (CALM), Western Australia. Records of confirmed and unconfirmed locations of *B. constricta*, *B. sphaerocephala*, *B. longiscapa*, *B. scirpoidea* and *B. laciniata* were obtained for the south-west corner of Western Australia (the most *Borya*-rich region of Australia). These sites were then located using GPS and *Borya* species in these locations were sampled for subsequent identification at the relevant state Herbaria and use in analysis of the interspecies relationships of the Boryaceae. All plant samples used and collectors are listed in Table 4.1. For each species DNA was extracted from 100 mg samples of plant leaves using Plant DNA Easy Mini Kits (Qiagen), including the extra centrifugation step, following the manufacturer's instructions. Sterile-DNA free sand was used to aid the grinding of the material. Extracts were stored at -20ºC.

Table 4-1: Species samples (as listed next to cladograms), collection sites and GPS references.

Sample	*Species*	*Collector*	*Collection site*	*GPS reference*
1	*B. constricta* Churchill	W. Archer 2005	Mt Ridley WA	33º17'36"S 122º07'06"E
2	*B. constricta*	W. Archer 2005	WA	33º17'S 122º07'E
3	*B. septentrionalis* Muell	J.R. Clarkson 30/07/04	Wandarin QLD	17º22'S 145º47'E
4	*B. constricta*	N.H. Reiter January 2005	East of Newdegate, Marchetti Rock WA	33º3'S 119º16'E
5	*B. constricta*	N.H. Reiter January 2005	East of Newdegate, Marchetti Rock WA	33º3'S 119º16'E
6	*B. constricta*	N.H. Reiter January 2005	East of Newdegate, Marchetti Rock WA	33º3'S 119º16'E
7	*B. constricta*	W. Archer 2005	Mt Ridley WA	33º17'36"S 122º07'06"E
8	*B. sphaerocephala* Sprengel	N.H. Reiter January 2005	Beverley airfields WA	32º7'49.6"S 116º56'23.300"E
9	*B. constricta*	N.H. Reiter January 2005	Newdegate WA	33º3'S 119º16'E
10	*B. constricta*	N.H. Reiter January 2005	Newdegate WA	33º3'S 119º16'E
11	*B. constricta*	W. Archer 2005	Mt Ridley WA	33º17'48"S 122º07'34ºE
12	*B. sphaerocephala*	N.H. Reiter January 2005	Corner of Soldiers Rd Gnowangerup WA	33º56'18"S 117º57'17"E
13	*Laxmania grandiflora* subsp. *grandiflora*	W. Archer 2005	12 km SW of Mt Ridley WA	33º17'S 122º07'E
14	*B. constricta*	W. Archer 2005	Mt Coobaninya WA	33º01'16"S 123º20'27"E
15	*B. constricta*	N.H. Reiter January 2005	East of Newdegate, Marchetti Rock WA	33º3'S 119º16'E
16	*B. septentrionalis*	J.R. Clarkson 30/07/04	Herbeton QLD	17º22'51"S 145º21'07"E
17	*B. septentrionalis*	J.R. Clarkson 31/07/04	Lambshead QLD	17º01'00"S 145º38'05"E
18	*B. septentrionalis*	J.R. Clarkson 02/08/04	Walsh's Pyramid QLD	17º07'26"S 145º47'49"E

* indicates samples from herbarium material

Table 4-2: Species samples, collection sites and GPS references.

Sample	*Species*	*Collector*	*Collection site*	*GPS reference*
19	*B. constricta*	N.H. Reiter January 2005	East of Newdegate, Marchetti Rock WA	33º3'S 119º16'E
20	*B. longiscapa* Churchill	N.H. Reiter January 2005	Albany WA	34º51' 17.7"S 117º18'05.5"E
21	*B. constricta*	W. Archer 2005	Mt Buraminya WA	33º01'10"S 123º20'27"E
22	*B. constricta*	W. Archer 2005	Clyde Hill WA	33º21'15"S 123º 00' 00"E
23	*B. constricta*	W. Archer 2005	Mt Buraminya WA	33º07'14"S 123º17'41"E
24	*B. constricta*	W. Archer 2005	Breeboorina Rock WA	33º05'52"S 123º19'41"E
25	*B. constricta*	W. Archer 2005	Mt Buraminya WA	33º07'14"S 123º17'41"E
26	*B. constricta*	W. Archer 2005	Mt Buraminya WA	33º08'15" S 123º16'14"E
27*	*B. laciniata* Churchill	Royal Botanic Gardens Melbourne Accession: 2014122	Cullimbin Water Reserve WA	30º 52' S 117º 15' E
28*	*B. scirpoidea* Lindley	Royal Botanic Gardens Melbourne Accession : 657215	6.7 km N of Palgarnup WA	34º 52'S 117º15'E
29*	*B. sp.* 'Wheatbelt'	Royal Botanic Gardens Melbourne Accession: 112575	Marchetti Rock, E of Newdegate WA	34º07'05"S 116º12'02"E
30*	*B. jabirabela* Churchill	Royal Botanic Gardens Melbourne Accession: 718256	West Kimberley Wren Gorge WA	33º03'S 119º06'E
31*	*B. subulata* Gardner	Royal Botanic Gardens Melbourne Accession: 227797	Kimberley WA	16º01'S 125º14'E
32	*B. stenophylla* M.D. Barrett ined.	M.D. Barrett 2006	Beverley Springs Stream WA	15º59'15"S 125º44'18"E
33	*B. nitida* Labill.	W. Archer 2005	Esperance WA	**
34	*B. mirabilis* Churchill	N.H. Reiter June 2005	Grampians National Park Wonderland Range Vic	**

* indicates samples from Herbarium material ** GPS reference not available

Table 4-3: Species samples, collection sites and GPS references.

Sample	*Species*	*Collector*	*Collection site*	*GPS reference*
35*	*B. inopinata*	Isotype Austrobaileya 4:597 (1997) Herbarium number AQ 601425	Mt Stewart Range 'Allandale' QLD	20º19'27"S 145º30'36"E
36*	*Alania endlicheri* Kunth	Royal Botanic Gardens Melbourne	Blue Mountains, 4 km from Kurrrajong Heights NSW	33º31'S 150º36'E

* indicates samples from herbarium material ** GPS reference not available

4.2.1.1. Primers

The Mat K region of the chloroplast was amplified using the primers listed in Table 4-4.Three regions between the *trnT* and *trnF* genes of the chloroplast (including the *trnT-L* and *trnL-F* intergenic spacers and *trnL* intron) were amplified in separate reactions using the primers (Taberlet *et al*. 1991; Table 4-4). The ITS1- 5.8S-ITS2 region of the genomic DNA was amplified using the primers: ITS1 and ITS4 (Table 4-4) and the ITS1- 5.8S region of the genomic DNA was amplified using the primers: ITS2 (White *et al*. 1990) and ITS5* (Table 4-4).

Table 4-4: Primers used in interspecies analysis of the Boryaceae.

Region	*Forward Primer 5'-3'*	*Reverse Primer 5'-3'*
Chloroplast MatK1	AAC TAG TCG GAT CGA GTA G	TCT GGA GTC TTT CTT GAG
Chloroplast MatK2	TMT TCA TCA GAA TAA GAG T	CGT TCT GAC CAT ATT GCA
Chloroplast trnL-F	CGA AAT CGG TAG ACG CTA	GGG GAT AGA GGG ACT TGA
Chloroplast trnL intron	GGT TCA AGT CCC TCT ATC CC	ATT TGA ACT GGT GAC ACG
Chloroplast trnT-L	CAT TAC AAA TGC GAT GCT CT	TCT ACC GAT TTC GCC ATA TC
Nuclear ITS1-5.8S-ITS2	TCC GTA GGT GAA CCT GCG G	TCC TCC GCT TAT TGA TAT GC
Nuclear ITS1- 5.8S	GCTGCGTTCTTCATCGATGC	GGA AGG AGA AGT CGT AAC AAG G

4.2.1.2. Product amplification

The chloroplast DNA PCR reactions contained 2 µl of extract, 12.5 µl of GoTaq® Green Master Mix (Promega Pty Ltd), 0.5 µl of each primer and 9.5 µl of sterile MilliQ water (dH_2O). The chloroplast PCR cycle was one cycle of 95ºC for 15 min, followed by 30 cycles of: 94ºC for 30 s, 57ºC for 30 s, 71ºC for 1 min and a final extension of 72ºC for 5 min before cooling and storing at 4ºC. The ITS PCR reactions contained 2 µl of extract, 12.5 µl of GoTaq® Green Master Mix, 0.5 µl of each primer and 9.5 µl of dH_2O. The ITS region PCR cycle was run using a touchdown cycle at 95ºC for 2 min, followed by 8 cycles of: 94ºC for 30 s, 55ºC (-1ºC for each cycle) for 1 min and 72ºC for 2 min, followed by 25 cycles of: 94ºC for 30 s, 47ºC for 1 min and 72ºC for 2 min, with a final extension of 72ºC for 5 min before cooling and storing at 4ºC.

4.2.1.3. Product separation and sequencing

All PCR products (chloroplast and nuclear) were visualised on a 1.4% agarose gel using TBE buffer (242 g Tris buffer;14.61 g EDTA; 57 ml of acetic acid made up to 1 L with distilled water, pH 5.2). The first and last lanes were loaded with 2 µL GeneRulerTM 100 bp DNA Ladder Plus (Fermentas, Australia), run at 90 V and post-stained for 15 min with ethidium bromide (600 µL of 10 mg/mL ethidium bromide) for 10 min, de-stained with running water for 30 min. Images were viewed with a Chromato-Vue® UV transilluminator (Model TM 36, Ultra-violet Product, Inc.) and the image recorded using the Geldoc® Quantity One program (Biorad Laboratories, Hercles, CA).

All PCR products obtained were purified and sequenced using Big Dye Terminator V3.1 (Applied Biosystems). PCR products were purified using a Qiaquick PCR Purification Kit (Qiagen) following the manufacturer's protocol. The Big Dye PCR sequencing reactions followed the Applied Biosystems protocol with the purified PCR product in the following 20 µl reaction: reaction mix 1 µl, buffer 3.5 µl, 1 µl of 5 µM forward or reverse primer (Table 4-4), 1 µl of template and 13.5 µl of dH_2O. Sequencing reactions were run with the following PCR cycle: 96ºC for 1 min followed by 25 cycles of: 96ºC for

10 s, 50ºC for 5 s and 60ºC for 4 min with a holding temperature of 4ºC. Sequencing reactions were precipitated using the Big Dye Terminator V 3.1 (Applied Biosystems) ethanol/sodium acetate precipitation in microcentrifuge tubes as advised by Applied Biosystems, the DNA was vacuum-dried and sent for sequencing. Sequences were analysed on an ABI 377 automated sequencer, using standard dye-terminator chemistry (Micromon, Monash University, Clayton, Victoria).

Sequence analysis

All clear sequences (nuclear and chloroplast) that were obtained were edited and aligned using BioEdit version 9.0 (Hall, 1999). Particularly short or ill-aligning sequences were disregarded in some analyses. Outgroups from the Asparagales other than the Boryaceae were sourced from Genbank where available at the time and Genbank accession numbers are listed next to these sequences. The evolutionary history was inferred using two methods as outlined below (for both nuclear and chloroplast DNA). Phylogenetic analyses were conducted in MEGA Version 4 (Tamura *et al*. 2007) with 1000 bootstraps. Cladograms from both methods are provided for comparison.

- The Unweighted Pair Group Method Analysis (UPGMA) method (Sneath and Sokal, 1973), in which the bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1993) is taken to represent the evolutionary history of the taxa analysed (Felsenstein, 1993). Branches corresponding to partitions reproduced in less than 50% of the bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1993). The trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*. 2004) and are in the units of the number of base substitutions per site. All positions containing

alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option).

- Maximum Parsimony Analysis (MPA) (Eck and Dayhoff, 1966), in which the bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed (Felsenstein, 1993). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1993). The MP tree was obtained using the Close-Neighbour-Interchange algorithm (Nei and Kumar, 2000) with search level 3 (Felsenstein, 1993; Nei and Kumar, 2000) in which the initial trees were obtained with the random addition of sequences (10 replicates). The tree is drawn to scale, with branch lengths calculated using the average pathway method (Nei and Kumar, 2000) and are in units of the number of changes over the whole sequence. All alignment gaps were treated as missing data.

4.2.2. *Borya mirabilis* population RAPD analysis

Field samples of fresh green leaf tips were collected on 10th October 2007 from 19 plants in the field (Table 4-5 and Table 4-6). Sampling of the five plants in the field was limited to those plants that had recovered sufficiently from the Mt Lubra fire of 2006; this resulted in four of the five remaining colonies being sampled (Colony 1 had not re-sprouted after the Mt Lubra fire) and all plants within these colonies that had three or more healthy ramets were sampled. The eight mature plants of the *ex-situ* population at the RBG (Table 4-7), the origins of which were unknown, were also sampled. All further cuttings at the RBG have been taken from these eight mature plants but these cuttings could not be sampled due to their small size (the precise plants from which the cuttings were taken are unknown).

All samples were placed in sterile tubes, placed on ice and stored at -20ºC until the DNA was extracted. Each sample was cleaned with 70% ethanol, rinsed with sterile distilled water and then 100 mg of each sample was extracted using a DNA Easy Plant Mini Kit (Qiagen) following the manufacturer's instructions, including the extra centrifugation step. Samples were run on a 1.4% agarose gel for 2 h, stained with ethidium bromide and visualised as before to determine DNA concentration. DNA concentration was equilibrated to 5 ng/µl before use in PCR (to ensure each sample had approximately the same concentration of DNA).

All DNA extracts were stored at -20ºC and all PCR reactions were stored at -4ºC after amplification. PCR reactions were mixed on ice in a laminar flow cabinet to avoid contamination. One sample was run with all 60 OPA, OPB and OPM primers listed in Table 4-8 to find those primers that would be used for RAPDs analysis. Those primers that gave up to seven clear bands were used with all extracts, giving a total of 104 scorable bands (Table 4-8). Each PCR reaction contained 2 µl of extract, 12.5 µl of GoTaq® Green Master Mix, 2 µl of each primer and 8.5 µl of dH_2O. The PCR cycle was 94ºC for 2 min followed by 45 cycles of 94ºC for 30 s, 34ºC for 30 s, 72ºC for 1 min with a final

extension of 72ºC for 5 min. Each reaction for each primer was run on the same gel with molecular weight ladders at both ends. Each 1.5 % agarose gel was run at 90 V with 10 µl aliquots of PCR reaction; the same size combs were used for each gel. Gels were post stained with ethidium bromide for 10 min, de-stained with running water for 30 min and images were recorded on a BioRad Geldoc as before.

Table 4-5: Status of *Borya mirabilis* population (from Parks Victoria Recovery Team monitoring) and representatives that were sampled as having >3 healthy crowns.

Colony / Plant	*Sampled for RAPDS*	*1 August 2006 Alive (crowns)*	*19 August 2007 Alive (crowns)*	*Colony / 'Plant'*	*Sampled for RAPDS*	*1 August 2006 Alive (crowns)*	*19 August 2007 Alive (crowns)*
1/1	No (dead)	Yes (1)	No	5/2	Yes	Yes (54)	Yes (48)
1/2	No (dead)	Plant removed	Plant removed	5/3	Yes	Yes (9)	Yes (18)
1/4	No (dead)	Yes (1)	No	5/4	No (dead)	No	No
1/6	No (dead)	No	No	5/5	Not found	Yes(2)	Yes (1)
1/7	No (dead)	Not found	No	5/6	Yes	Yes(40)	Yes (41)
1/8	No (dead)	No	No	5/7	Yes	Yes (7)	Yes(8)
1/10	No (dead)	Not found	No	5/8	No	Yes (15)	Yes (17)
2/1	No (dead)	No	No	5/9	No	Yes (15)	Yes (9)
2/2	Yes	Yes (9)	Yes (6)	5/10	No	Yes (8)	Yes (4)
2/3	No	Yes (2)	Yes (2)	5/11	No (dead)	No	No
2/4	Yes	Yes (5)	Yes (6)	5/12	No (dead)	No	No
2/5	No	Yes (2)	Yes (1)	5/13	No (dead)	No	No
2/10	No (dead)	No	No	7/1	Yes	Yes (2)	Yes (3)
2/13	No (dead)	No	No	7/2	No	Yes (3)	Yes (1)
2/14	Yes	Yes (9)	Yes (12)	7/3	No	Yes (6)	Yes (4)

Table 4-6: Status of *Borya mirabilis* population (from Parks Victoria Recovery Team monitoring) and representatives that were sampled as having >3 healthy crowns.

Colony / Plant	*Sampled for RAPDS*	*1 August 2006 Alive (crowns)*	*19 August 2007 Alive (crowns)*	*Colony / 'Plant'*	*Sampled for RAPDS*	*1 August 2006 Alive (crowns)*	*19 August 2007 Alive (crowns)*
2/15	No (dead)	Yes (1)	No	7/4	Yes	Yes (41)	Yes (55)
2/17	No (dead)	No	No	7/5	Yes	Yes (123)	Yes (115)
2/18	No	No	Yes (1)	7/6	No	No	Yes (1)
2/19	No	Yes (6)	Yes (1)	7/7	No (plant caged)	Yes (40)	Yes (36)
2/20	No (dead)	No	No	7/8	Yes	Yes (74)	Yes (76)
3/1	No (dead)	No	No	7/9	Yes	Yes (38)	Yes (23)
3/2	No (dead)	No	No	7/10	Yes	Yes (40)	Yes (56)
3/3	Yes	Yes (10)	Yes (7)	7/11	No (dead)	Yes (1)	No
3/4	No (dead)	No	No	7/12	Yes	Yes (7)	Yes (3)
3/5	No (dead)	No	No	7/13	Yes	Yes (35)	Yes (42)
3/6	No (dead)	Yes (2)	Not monitored	7/14	Yes	Yes (26)	Yes (23)
5/1	Yes	Yes(125)	Yes (110)	7/15	Yes	Yes (29)	Yes (26)

Table 4-7: Representatives of the *ex-situ* Royal Botanic Gardens population that were sampled.

Sample	*Botanic Gardens Accession Number*
1	070685/009
2	070685/008
3	991061/001
4	991057/001
5	070685/001
6	000893/003
7	070685/007
8	991064/001

Table 4-8: Operon RAPD Primers used for the population study of *Borya mirabilis*.

OPA primers screened	*Sequence 5' to 3'*	*OPB primers screened*	*Sequence 5' to 3'*	*OPM primers screened*	*Sequence 5' to 3'*
A-01	CAGGCCCTTC	B-01	GTTTCGCTCC	M-01*	GTTGGTGGCT
A-02*	TGCCGAGCTG	B-02*	TGATCCCTGG	M-02*	ACAACGCCTC
A-03	AGTCAGCCAC	B-03	CATCCCCCTG	M-03*	GGGGGATGAG
A-04*	AATCGGGCTG	B-04*	GGACTGGAGT	M-04*	GGCGGTTGTC
A-05	AGGGGTCTTG	B-05*	TGCGCCCTTC	M-05*	GGGAACGTGT
A-06	GGTCCCTGAC	B-06*	TGCTCTGCCC	M-06*	CTGGGCAACT
A-07	GAAACGGGTG	B-07*	GGTGACGCAG	M-07	CCGTGACTCA
A-08	GTGACGTAGG	B-08*	GTCCACACGG	M-08	TCTGTTCCCC
A-09*	GGGTAACGCC	B-09	TGGGGGACTC	M-09	GTCTTGCGGA
A-10	GTGATCGCAG	B-10	CTGCTGGGAC	M-10*	TCTGGCGCAC
A-11*	CAATCGCCGT	B-11*	GTAGACCCGT	M-11	GTCCACTGTG
A-12	TCGGCGATAG	B-12*	CCTTGACGCA	M-12*	GGGACGTTGG
A-13	CAGCACCCAC	B-13*	TTCCCCCGCT	M-13	GGTGGTCAAG
A-14	TCTGTGCTGG	B-14*	TCCGCTCTGG	M-14	AGGGTCGTTC
A-15	TTCCGAACCC	B-15*	GGAGGGTGTT	M-15	GACCTACCAC
A-16*	AGCCAGCGAA	B-16	TTTGCCCGGA	M-16	GTAACCAGCC
A-17	GACCGCTTGT	B-17*	AGGGAACGAG	M-17	TCAGTCCGGG
A-18*	AGGTGACCGT	B-18	CCACAGCAGT	M-18	CACCATCCGT
A-19	CAAACGTCGG	B-19	ACCCCCGAAG	M-19	CCTTCAGGCA
A-20	GTTGCGATCC	B-20*	GGACCCTTAC	M-20	AGGTCTTGGG

*Indicates those primers which were then run for the population analysis having reproducible banding patterns.

4.3. Results

4.3.1. Interspecies relationships in the Boryaceae: analysis of chloroplast and nuclear DNA

4.3.1.1. Analysis of the Mat K region

Clear single 900-1000bp band products were obtained with Mat K1 primers from most of the species sampled (Table 4-1 to Table 4-3) on gel electrophoresis (Figure 4-3). Mixed unclear signals were obtained for over 80% of the samples for the Mat K region when sequenced and therefore did not provide enough information to undergo phylogenetic analysis. The Mat K2 primers for this region, despite repeated attempts, did not amplify DNA from more than a few species (Figure 4-4) and no further work was undertaken on this region.

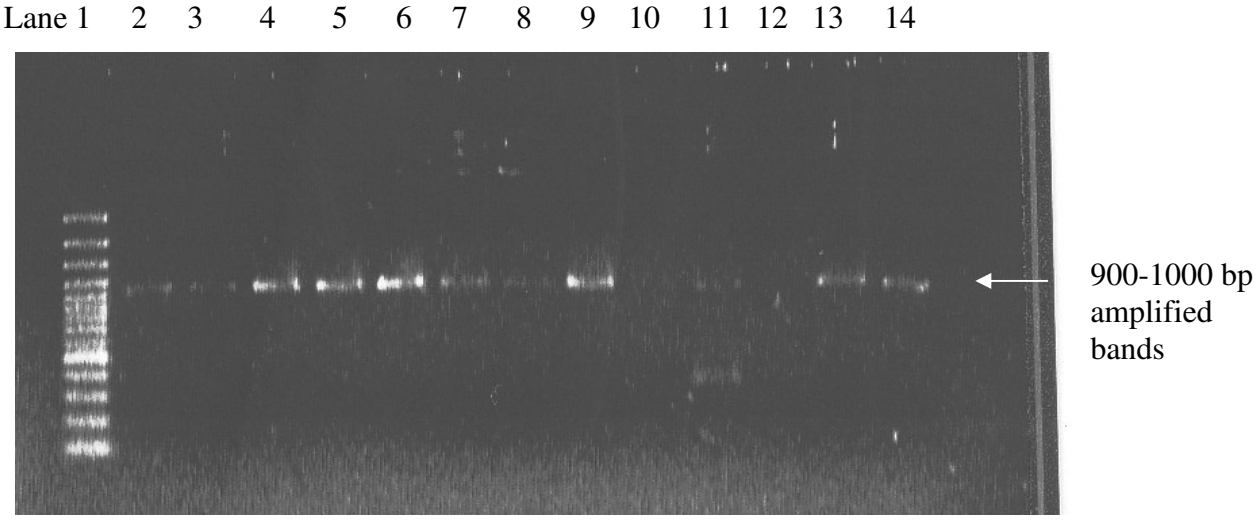

Figure 4-3: 900-1000bp bands amplified with Mat K1 primers and *Borya* extracts (Table 4-1 to Table 4-3), left-right Lane 1-14: molecular weight marker, *B. constricta* 1, *B. constricta* 2, *B. septentrionalis* 3, *B. constricta* 4, *B. constricta* 5, *B. constricta* 6, *B. constricta* 7, *B. sphaerocephala* 8, *B. constricta* 9, *B. constricta* 10, *B. constricta* 11, *B. sphaerocephela* 12, *Laxmania grandiflora* 13.

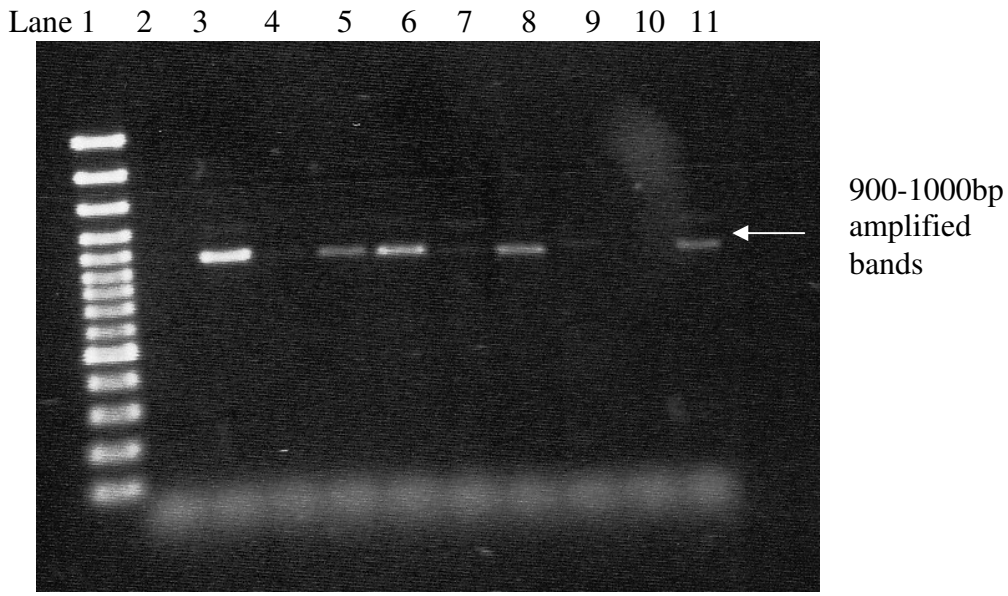

Figure 4-4: 900-100 bp bands amplified with MatK2 primers and *Borya* extracts (Table 4-1 to 4-3), left-right Lane 1-11: molecular weight marker, *B. constricta* 1, *B. constricta* 2, *B. septentrionalis* 3, *B. constricta* 4, *B. constricta* 5, *B. constricta* 6, *B. constricta* 7, *B. sphaerocephala* 8, *B. constricta* 9, *B. constricta* 10.

4.3.1.2. Analysis of the chloroplast trnL-F intergenic spacer

Clear 500-600 bp single bands (products) were obtained (e.g. Figure 4-5) using trnL-F chloroplast primers to amplify the species sampled (Table 4-1 to Table 4-3). In the alignment of the trnL-F intergenic spacer region there was a total of 591 positions in the final data set, of which 350 were phylogenetically informative. MPA analysis (Figure 4-6) and UPGMA analysis (Figure 4-7) show the consensus trees inferred from 1000 replicates.

The MPA method (Figure 4-6) showed the Boryaceae as a weakly supported clade, bootstrap=45 (BP=45) amongst the Asparagales but did not strongly support any other clade of the Asparagales as a sister group to the Boryaceae. There were three subclades with 44-62% support. *B. mirabilis* in the MPA method was grouped in a subclade with 13 of the 17 samples of *B. constricta* but this had a bootstrap value of less than 50. The other four samples of *B. constricta* constituted a subclade with *B. laciniata*, *B. jabirabela*, *B. inopinata* and *B. stenophylla* (but with only 62% bootstrap support). *B. sphaerocephela* and *B. septentrionalis* formed a separate subclade with 52% support, with *B. longiscapa* as an out-group.

The UPGMA method of analysis (Figure 4-7) did not support the Boryaceae as a single coherent clade amongst the Asparagales and did not resolve any relationships amongst the Asparagales, but did group samples of six species together with 100% fidelity: *B. mirabilis,* with 13 samples of *B. constricta* and *B. subulata* and then these with *B. sp.* 'Wheatbelt', *B. sphaerocephela* and *B. laciniata*. Analysis of the Boryaceae alone, without an out-group from amongst the Asparagales (Figure 4-8) provided greater bootstrap and branch length support for individual species. *B. mirabilis* was grouped in a strong clade (BP=95) with five other species of *Borya*, again including *B. constricta*. *B. nitida* supposedly the closest relative of *B. mirabilis,* was the most distant species in the genus.

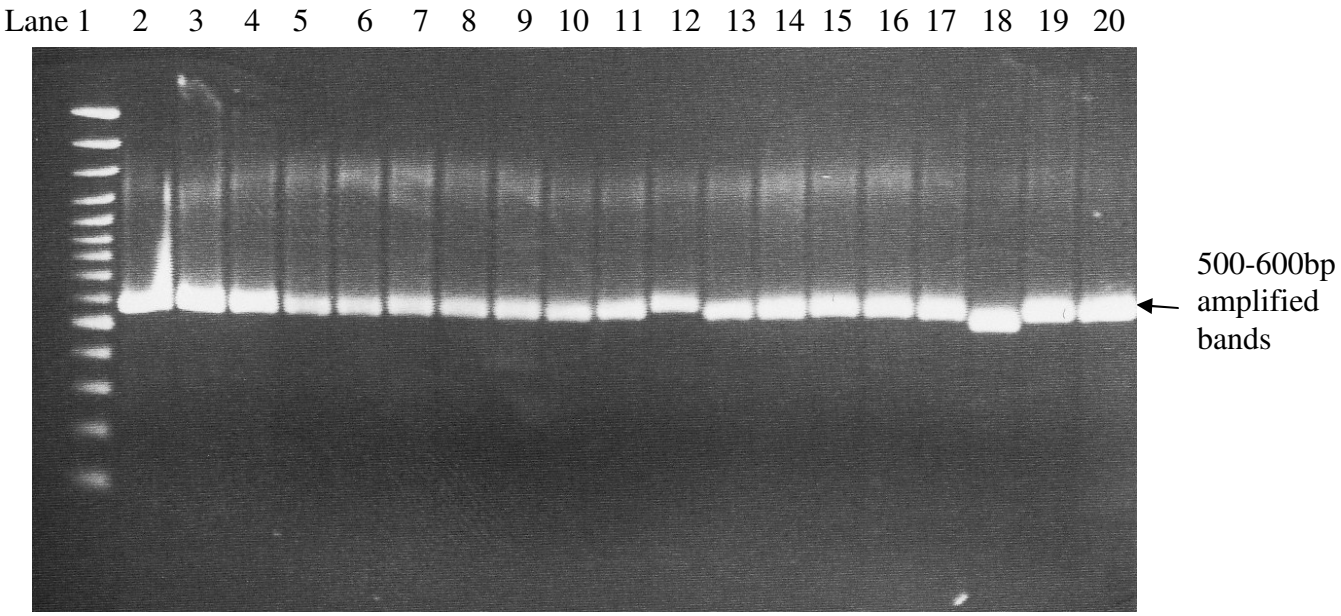

Figure 4-5: 500-600 bp bands amplified with trnL-F primers and *Borya* extracts (Table 4-1 to Table 4-3), left-right Lane 1-20: molecular weight marker, *B. constricta* 1, *B. constricta* 2, *B. septentrionalis* 3, *B. constricta* 4, *B. constricta* 5, *B. constricta* 6, *B. constricta* 7, *B. sphaerocephala* 8, *B. constricta* 9, *B. constricta* 10, *B. constricta* 11, *B. sphaerocephela* 12, *Laxmania grandiflora* 13, *B. constricta* 14, *B. constricta* 15, *B. septentrionalis* 16, *B. septentrionalis* 17, *B. septentrionalis* 18, *B. constricta* 19.

7

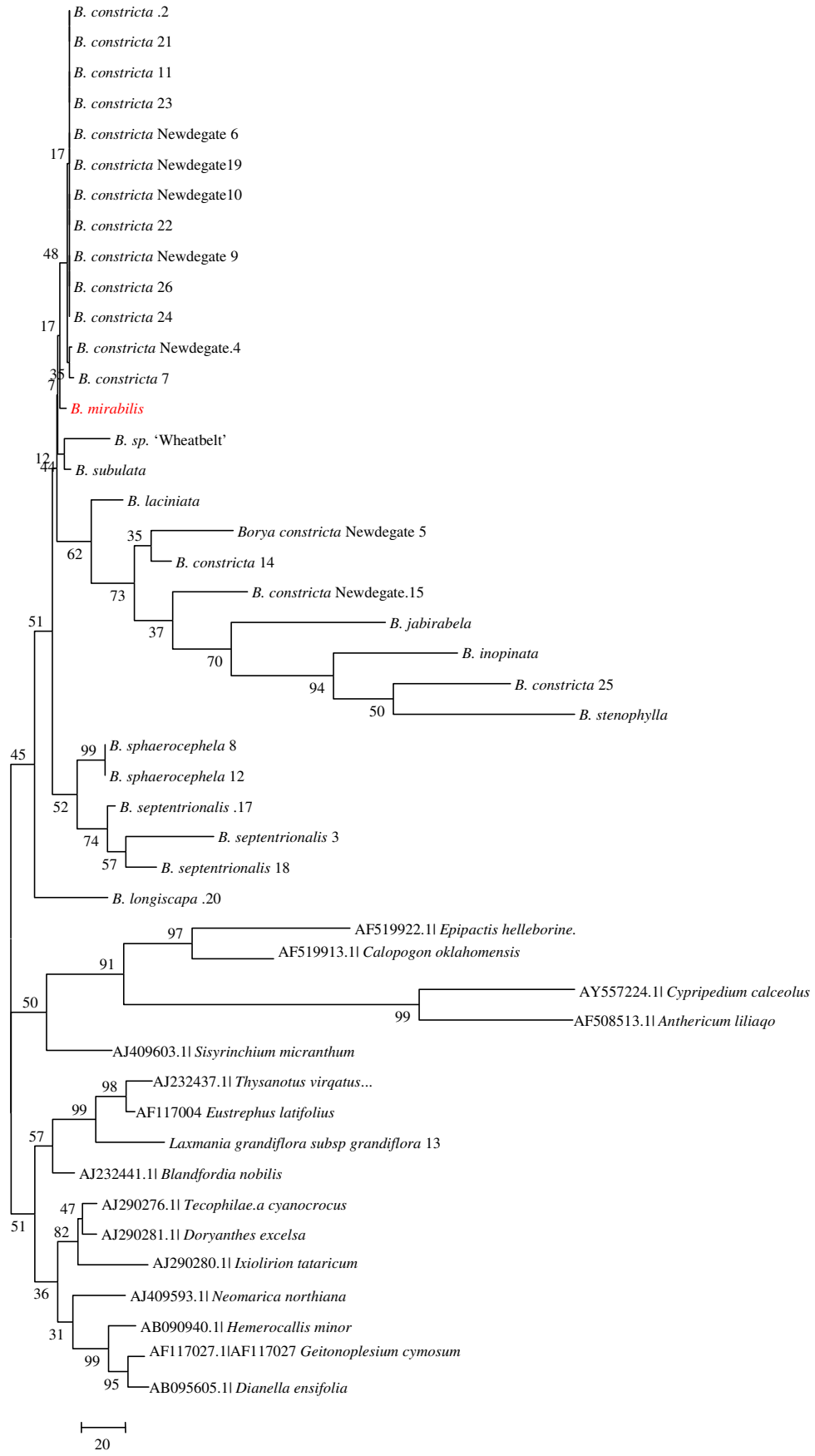

Figure 4-6: Evolutionary relationships of 46 taxa within the Asparagales including 30 samples from the genus *Borya* and one sample of *Laxmania grandiflora* a species outside the Asparagales with a similar growth form to *Borya* spp., based on chloroplast DNA amplified by trnL-F primers. The evolutionary history was inferred using the Maximum Parsimony method (Eck and Dayhoff, 1966). There was a total of 591 positions in the final dataset, out of which 350 were parsimony-informative. Phylogenetic analyses were conducted in MEGA Version 4 (Tamura *et al.* 2007). Numbers after species refer to samples of the same species as listed in Table 4-1 to 4-3. Sequences obtained from Genbank have accession numbers listed before the species.

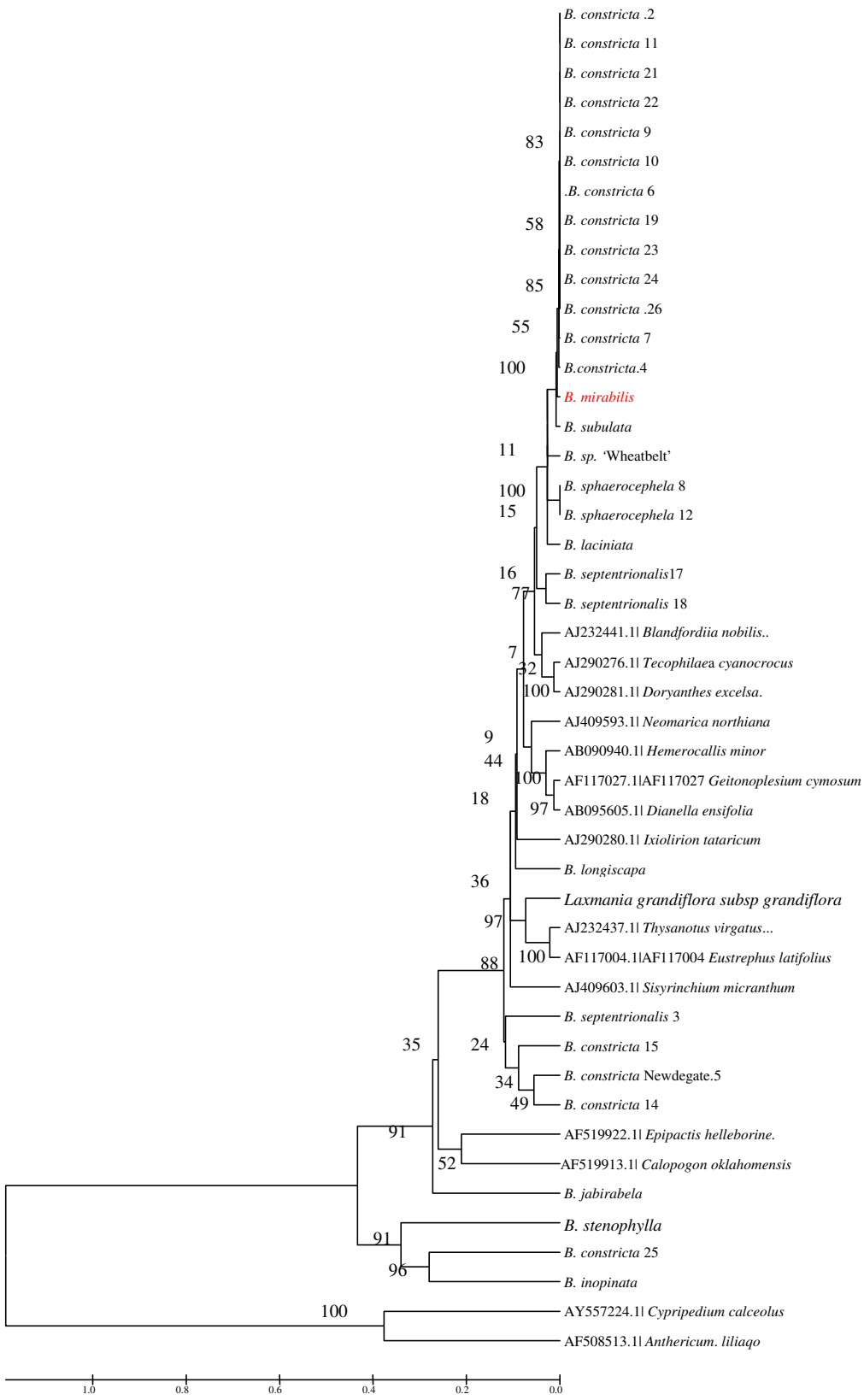

Figure 4-7: Evolutionary relationships of 46 taxa within the Asparagales including 30 samples from the genus *Borya* and one sample of *Laxmania grandiflora* a species outside the Asparagales with a similar growth form to *Borya* spp., based on chloroplast DNA amplified by trnL-F primers. The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). There were a total of 591 positions in the final dataset. Phylogenetic analyses were conducted in MEGA Version 4 (Tamura *et al.* 2007). Numbers after species refer to samples of the same species as listed in Table 4-1 to 4-3. Sequences obtained from Genbank have accession numbers listed before the species.

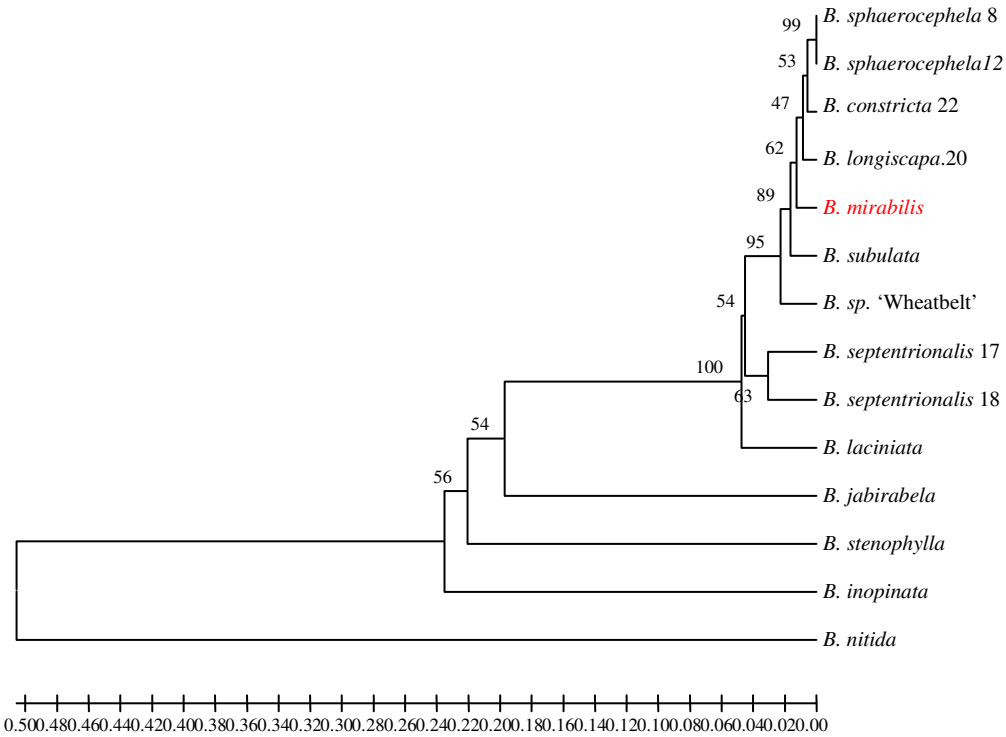

Figure 4-8: Evolutionary relationships of 14 taxa within the Boryaceae constructed using the chloroplast region trnL-F. The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). There was a total of 498 positions in the final dataset. Phylogenetic analyses were conducted in MEGA Version 4 (Tamura *et al*. 2007). Numbers after species refer to samples of the same species as listed in Table 4-1 to 4-3.

4.3.1.3. Analysis of the chloroplast trnL intron region

Clear 400-500 bp single band products (Figure 4-9) were obtained using trnL intron chloroplast primers to amplify the DNA of the species sampled (Table 4-1 to Table 4-3). In the alignment of the trnL intron, flanked by two short tRNA-encoding exons there was a total of 443 positions in the final data set, of which 213 were phylogenetically informative. MPA (Figure 4-10) and UPGMA (Figure 4-11) show the consensus trees inferred from 1000 replicates. The percentage of the replicate trees in which the associated taxa group together are shown next to the branches.

Both MPA (Figure 4-10) and UPGMA analysis (Figure 4-11) of 45 taxa, including 30 *Borya* spp. Samples, had strongly supported bootstrap values (BP=99, BP=100) for the Boryaceae as a unique clade amongst the Asparagales with the exception of *B. scirpoidea* in the UPGMA analysis. All other relationships amongst the Boryaceae were at most weakly supported (BP<65), with samples of the same species from different populations scattered among other species. An exception was the UPGMA analysis, which moderately (BP=86) supported the grouping of two of the *Borya constricta* samples. Within the Asparagales, the genera *Nypa*, *Trithrinax*, *Calamus*, *Phytelepha*, and *Euterpe* were placed as a sister clade (BP=100) to the remaining Asparagales including the Boryaceae. *Doryanthes, Hemerocallis* and *Dianella* which was a weakly supported as a sister clade to the Boryaceae; and *Tecophilaea*, *Blandfordia*, *Cypripedia*, *Epipactus* and *Calopogon* weakly supported (BP=38) as a sister group to the above.

Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

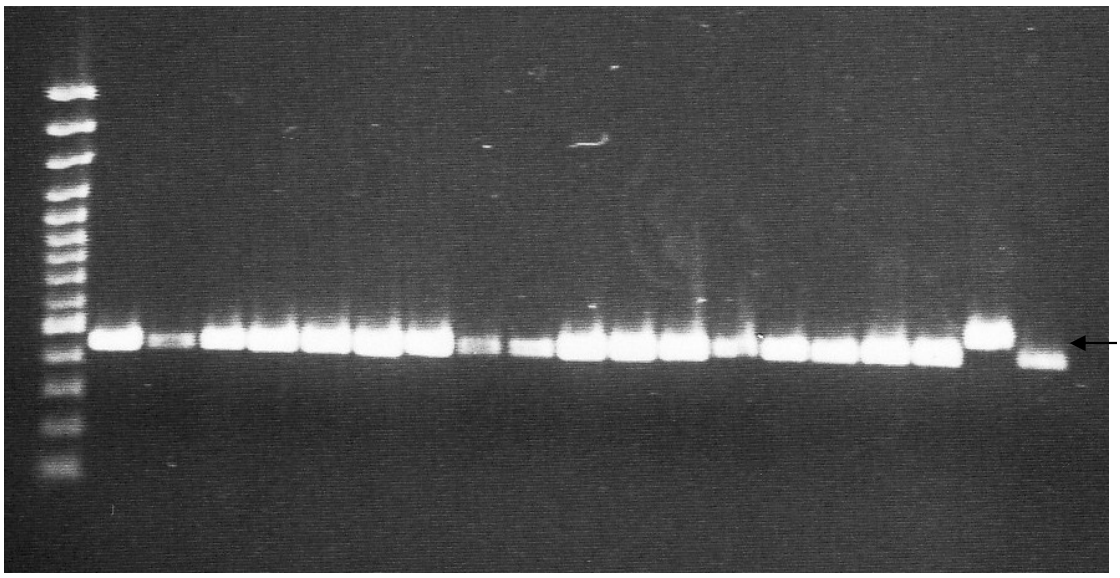

Figure 4-9: 400-500 bp bands amplified with trnL intron primers and *Borya* extracts (Table 4-1 to Table 4-3), left-right Lane 1-20: molecular weight marker, *B. constricta* 1, *B. constricta* 2, *B. septentrionalis* 3, *B. constricta* 4, *B. constricta* 5, *B. constricta* 6, *B. constricta* 7, *B. sphaerocephala* 8, *B. constricta* 9, *B. constricta* 10, *B. constricta* 11, *B. sphaerocephela* 12, *Laxmania grandiflora* 13, *B. constricta* 14, *B. constricta* 15, *B. septentrionalis* 16, *B. septentrionalis* 17, *B. septentrionalis* 18, *B. constricta* 19 .

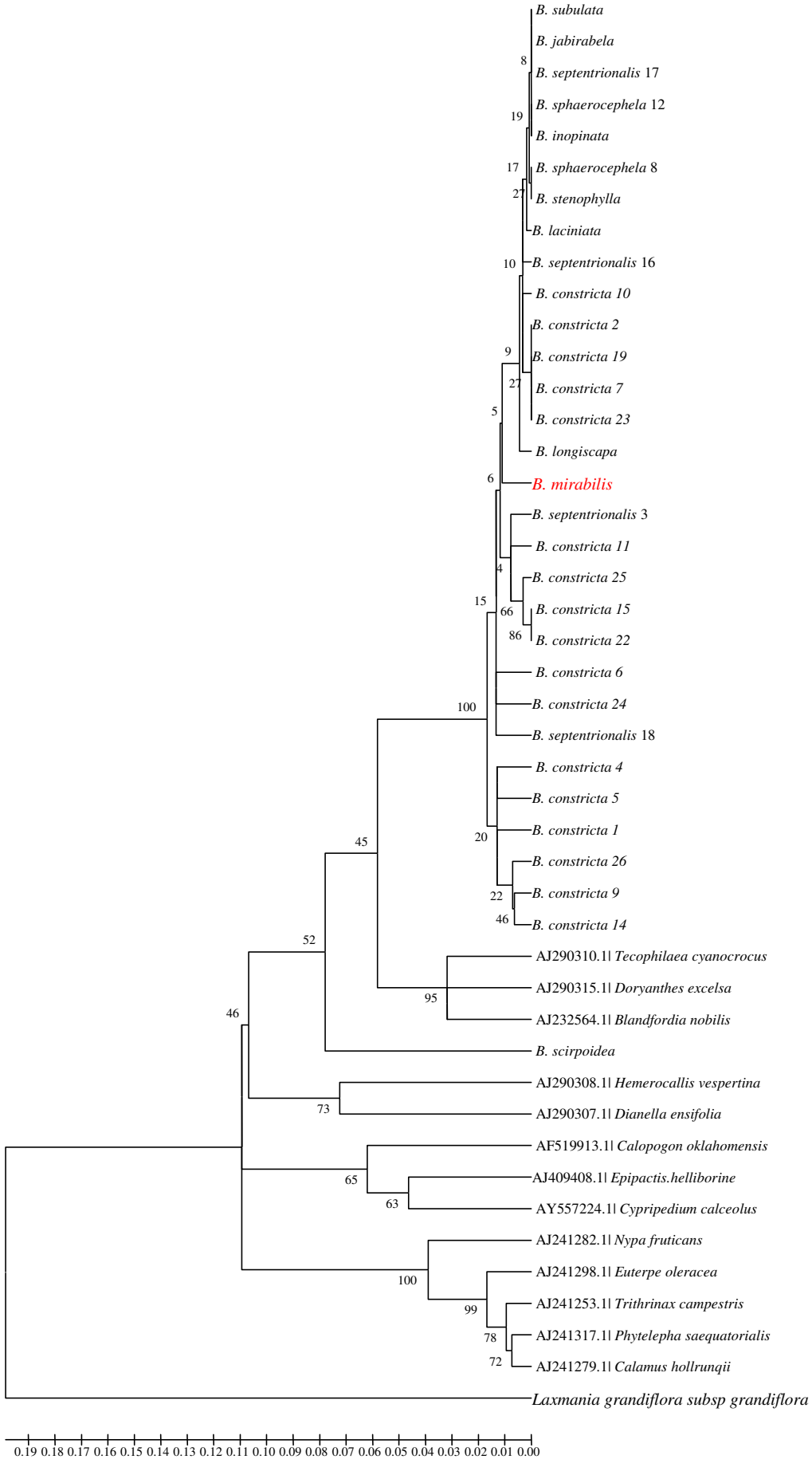

Figure 4-10: Evolutionary relationships of 45 taxa based on the trnL intron of chloroplast DNA. The evolutionary history was inferred using the Maximum Parsimony method (Eck and Dayhoff, 1966). There was a total of 443 positions in the final dataset, out of which 213 were parsimony-informative. Phylogenetic analyses were conducted in MEGA Version 4 (Tamura *et al.* 2007). Numbers after species refer to the samples of the same species as listed in Table 4-1 to 4-3. Sequences obtained from Genbank have accession numbers listed before the species.

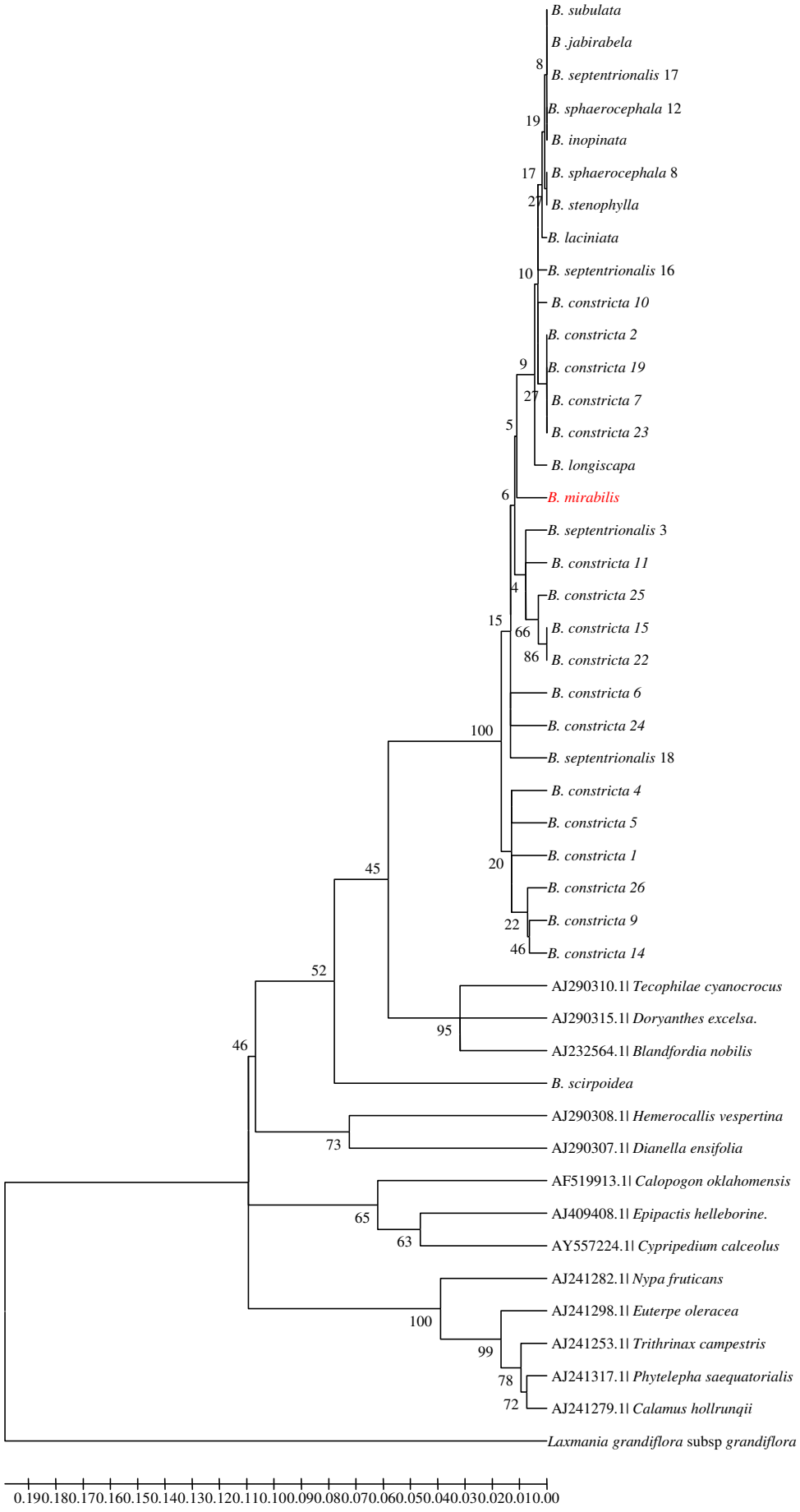

Figure 4-11: Evolutionary relationships of 45 taxa based on the trnL intron of chloroplast DNA. The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). There were a total of 443 positions in the final dataset. Phylogenetic analyses were conducted in MEGA Version 4 (Tamura *et al.* 2007). Numbers after species refer to the samples of the same species as listed in Table 4-1 to 4-3. Sequences obtained from Genbank have accession numbers listed before the species.

4.3.1.4. Analysis of the chloroplast trnT-trnL intergenic spacer region

Clear 700-800 bp single products (Figure 4-12) were obtained using trnT-trnL intergenic spacer chloroplast primers to amplify the species sampled (Table 4-1 to Table 4-3). There was a total of 736 positions in the final dataset, out of which 215 were parsimony-informative. Both MPA (Figure 4-13) and UPGMA (Figure 4-14) showed (1) the Boryaceae as a strongly supported unique clade (BP=99, BP=100 respectively), (2) *B. mirabilis* again as most closely similar to *B. constricta*, and (3) *A. endlicheri* as within the *Borya* clade. When only *Borya* species were analysed (Figure 4-15), *B. mirabilis* again was most closely similar to *B. constricta*. UPGMA analysis supports the separation of *B. jabirabela* and *B. stenophylla* into separate species.

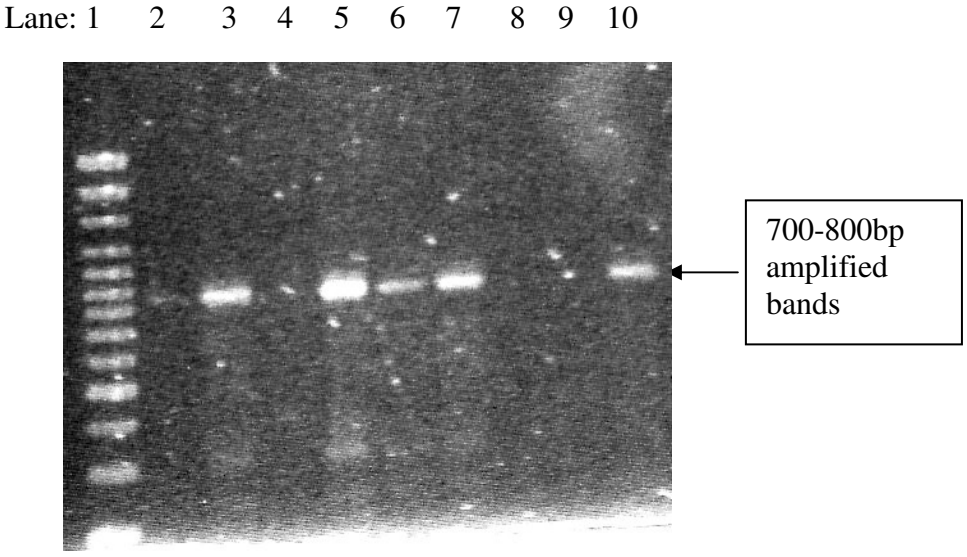

Figure 4-12: 700-800bp bands amplified with trnT-trnL chloroplast primers and *Borya* extracts (Table 4-1 to Table 4-3), left-right Lane 1-10: molecular weight marker, *B. constricta* 1, *B. constricta* 2, *B. septentrionalis* 3, *B. constricta* 4, *B. constricta* 5, *B. constricta* 6, *B. constricta* 7, *B. sphaerocephala* 8, *B. constricta* 9.

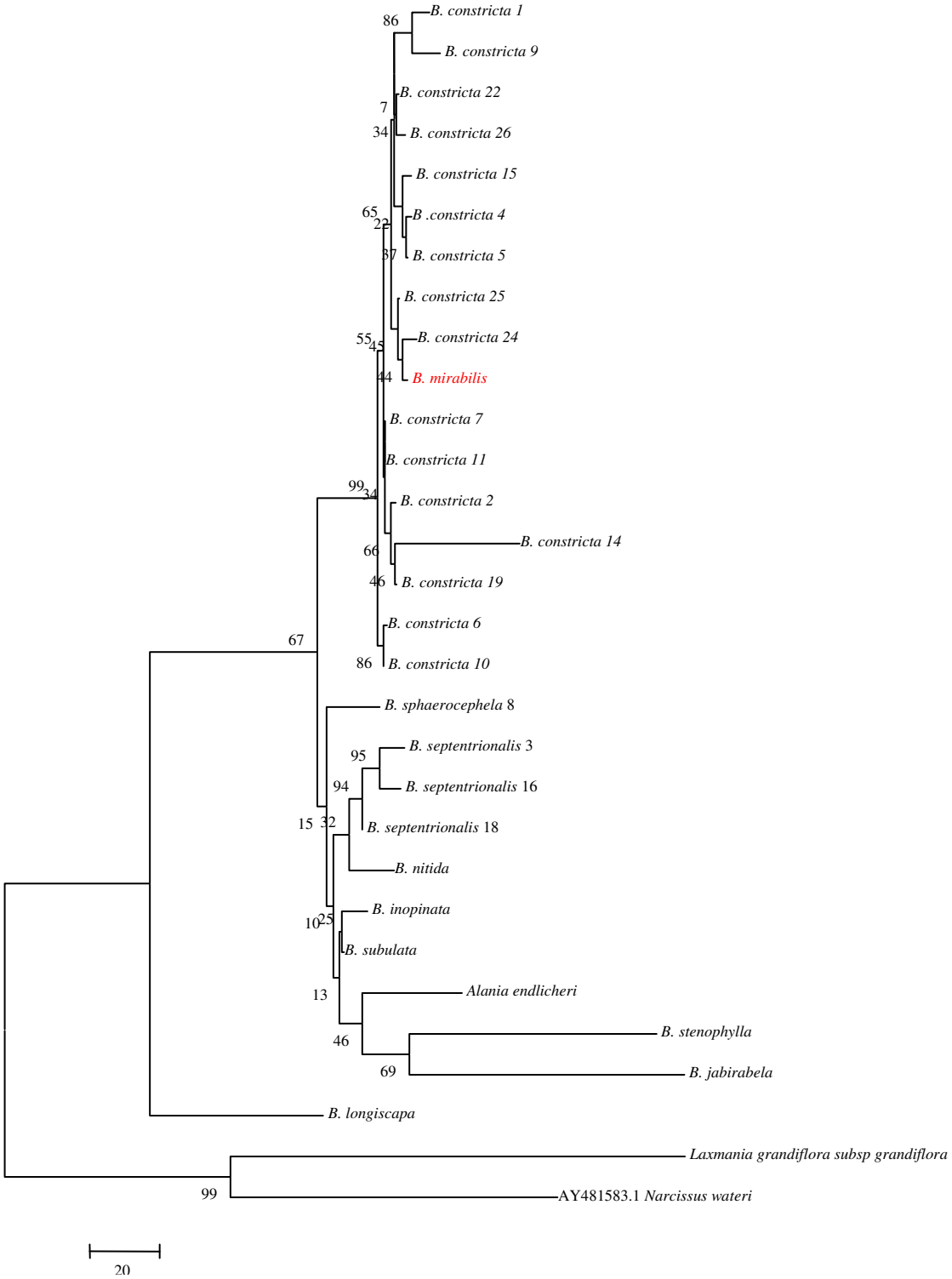

Figure 4-13: Evolutionary relationships of 30 taxa based on the TrnT-L region of chloroplast DNA. The evolutionary history was inferred using the Maximum Parsimony method (Eck and Dayhoff, 1966). There was a total of 736 positions in the final dataset, out of which 215 were parsimony-informative. Phylogenetic analyses were conducted in MEGA Version 4 (Tamura *et al.* 2007). Numbers after species refer to the sample where multiple samples of the same species were used as listed in Table 4-1 to Table 4-3. Sequences obtained from Genbank have accession numbers listed before the species.

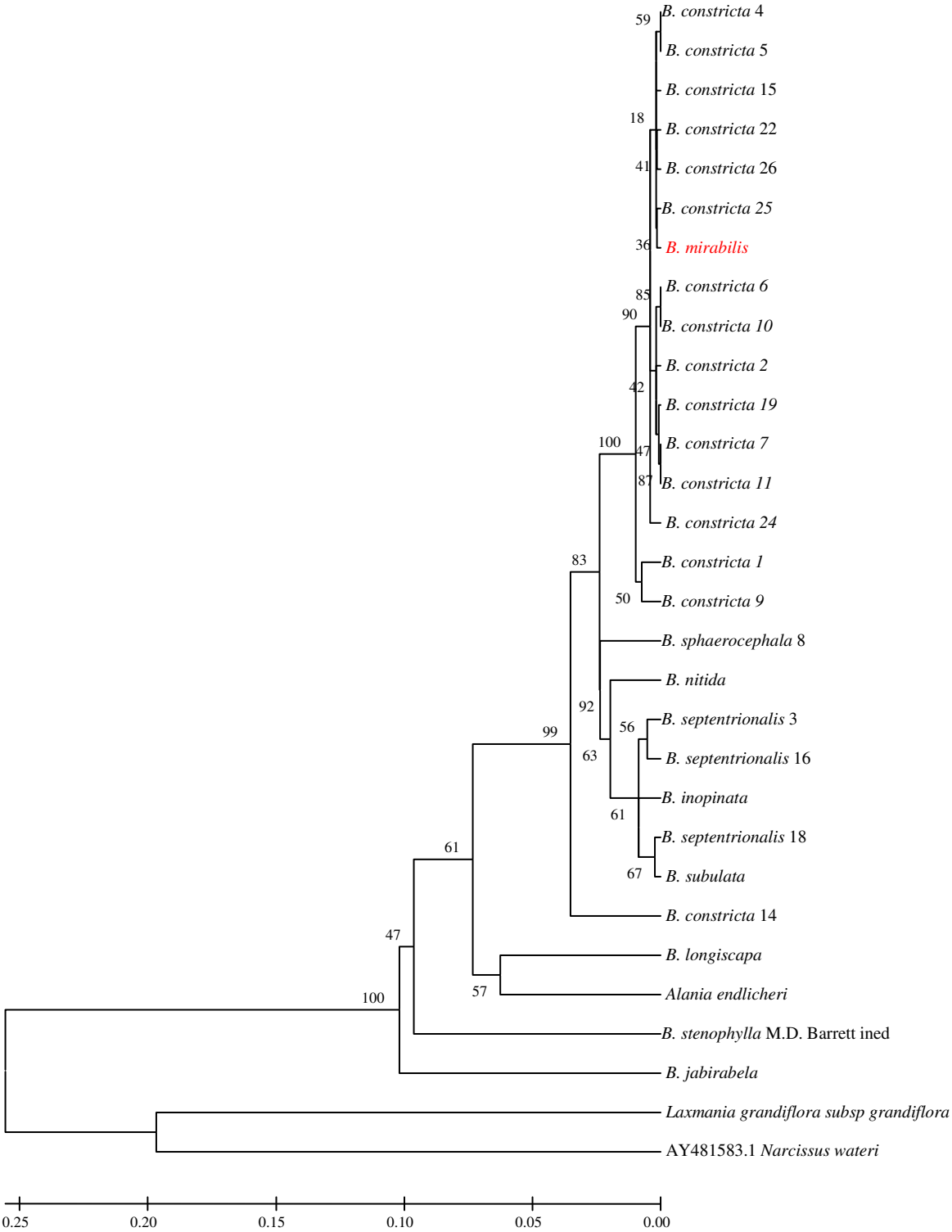

Figure 4-14: Evolutionary relationships of 30 taxa based on the TrnT-L reion of chloroplast DNA. The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). There was a total of 736 positions in the final dataset. Phylogenetic analyses were conducted in MEGA Version 4 (Tamura *et al*. 2007). Numbers after species refer to the sample when multiple samples of the same species were used as listed in Table 4-1 to 4-3. Numbers before species refer to Genbank accessions were used.

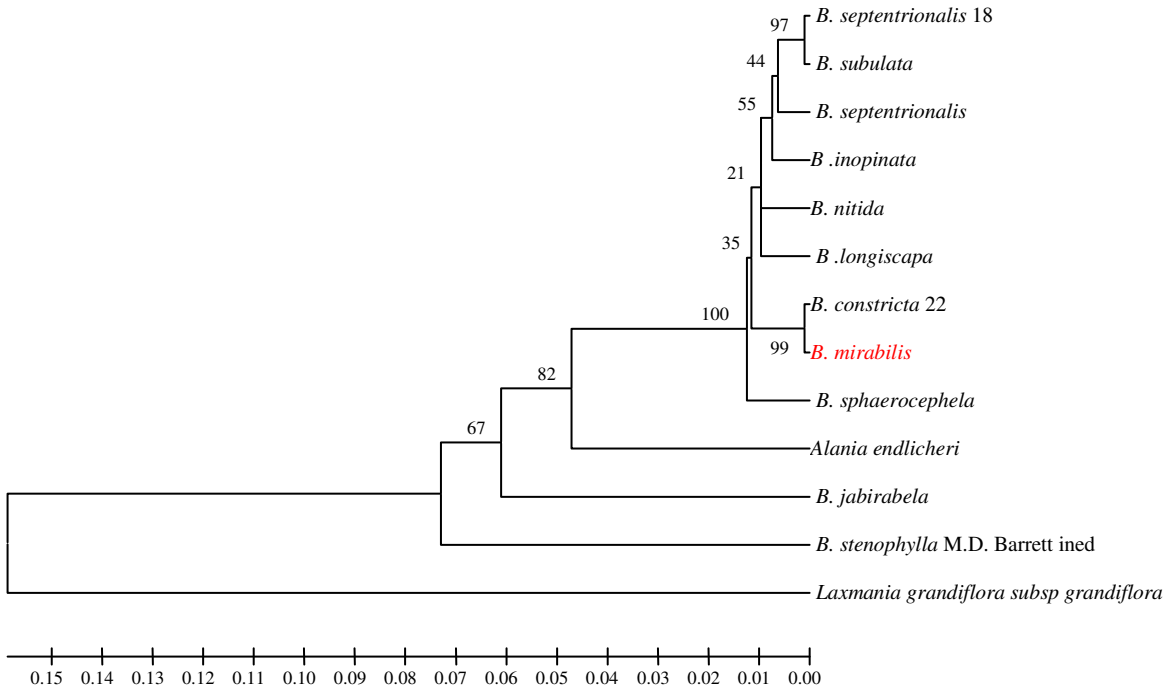

Figure 4-15: Evolutionary relationships of 13 taxa based on the TrnT-L region of chloroplast DNA. The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). There were a total of 739 positions in the final dataset. Phylogenetic analyses were conducted in MEGA Version 4 (Tamura *et al*. 2007). Numbers after species refer to the sample when multiple samples of the same species were used as listed in Table 4-1 to 4-3.

4.3.1.5. Position of the Boryaceae within the monocots (trnT-F sequences)

MegaBlast results on one sample from each species within the Boryaceae for the three chloroplast regions sequenced showed closest similarity to those from several different families within the Monocots (Table 4-9 to Table 4-11). The trnL-F region sequences showed closest similarity of *Borya* to species from the Tecophilaeaceae or the Arecaceae. The trnL intron region sequences consistently showed closest similarity of *Borya* to species of the Doryanthaceae within the Asparagales. The trnT-L sequences showed most similarity of *Borya* with the Arecaceae.

Table 4-9: MegaBlast (highly similar sequences) results for a selection of sequenced species for each chloroplast region used in the phylogenetic analysis. Numbers after species refer to samples as listed in Table 4-1 to Table 4-3.

Species	*Region*	*Top Blast result*	*Query coverage*	*Max Identity*	*Family*
B. sphaerocephala 8	**trnL-F**	*Itaya amicorum* AJ404890.1	99%	92%	Arecaceae
B. sphaerocephala 8	trnL intron	*Doryanthes excelsa* AJ290315.1	96%	91%	Doryanthaceae
B. sphaerocephala 8	trnT-L	*Nypa fruticans* AY145339	48%	86%	Arecaceae
B. sphaerocephala 12	**trnL-F**	*Itaya amicorum* Aj404890.1	99%	92%	Arecaceae
B. sphaerocephala 12	trnL intron	*Doryanthes excelsa* AJ290315.1	98%	91%	Doryanthaceae
B. sphaerocephala 12	trnT-L	Not available			
B. septentrionalis 16	**trnL-F**	*Zephyra elegans*	97%	92%	Tecophilaeaceae
B. septentrionalis 16	trnL intron	*Doryanthes excelsa* AJ290315.1	99%	91%	Doryanthaceae
B. septentrionalis 16	trnT-L	*Nypa fruticans* AY145339.1	49%	85%	Arecaceae
B. septentrionalis 17	**trnL-F**	*Tecophilaea cyanocrocus* AJ290276.1	100%	89%	Tecophilaeaceae
B. septentrionalis 17	trnL intron	*Doryanthes excelsa* AJ290315.1	98%	91%	Doryanthaceae
B. septentrionalis 17	trnT-L	No significant similarities			
B. septentrionalis 18	**trnL-F**	*Tecophilaea cyanocrocus* AJ290276.1	100%	87%	Tecophilaeaceae
B. septentrionalis 18	trnL intron	*Doryanthes excelsa* AJ290315.1	98%	89%	Doryanthaceae
B. septentrionalis 18	trnT-L	*Nypa fruticans* AY145339.1	48%	86%	Arecaceae
B. longiscapa 20	**trnL-F**	*Bulbophyllum variegatum* EF200125.1	99%	86%	Orchidaceae
B. longiscapa 20	trnL intron	*Doryanthes excelsa* AJ290315.1	97%	91%	Doryanthaceae
B. longiscapa 20	trnT-L	*Nypa fruticans* AY145339.1	48%	86%	Arecaceae

Table 4-10: MegaBlast (highly similar sequences) results for a selection of sequenced species for each chloroplast region used in the phylogenetic analysis. Numbers after species refer to samples as listed in Table 4-1 to Table 4-3.

Species	*Region*	*Top Blast result*	*Query coverage*	*Max Identity*	*Family*
B. constricta 22	**trnL-F**	*Tecophilaea cyanocrocus* AJ290276.1	98%	92%	Tecophilaeaceae
B. constricta 22	trnL intron	*Doryanthes excelsa* AJ290315.1	100%	90%	Doryanthaceae
B. constricta 22	trnT-L	*Nypa fruticans* AY145339.1	46%	85%	Arecaceae
B. laciniata	**trnL-F**	*Tecophilaea cyanocrocus* AJ290276.1	95%	86%	Tecophilaeaceae
B. laciniata	trnL intron	Doryanthes excelsa AJ290315.1	98%	91%	Doryanthaceae
B. laciniata	trnT-L	Not available			
B. scirpoidea	**trnL-F**	Not available			
B. scirpoidea	trnL intron	*Doryanthes excelsa* AJ290315.1	88%	85%	Doryanthaceae
B. scirpoidea	trnT-L	Not available			
B. sp. 'Wheatbelt'	**trnL-F**	*Itaya amicorum* Aj404890.1	97%	89%	Arecaceae
B. sp. 'Wheatbelt'	trnL intron	*Doryanthes excelsa* AJ290315.1	86%	90%	Doryanthaceae
B. sp. 'Wheatbelt'	trnT-L	Not available			
B. jabirabela	**trnL-F**	No significant similarity found			
B. jabirabela	trnL intron	*Doryanthes excelsa* AJ290315.1	98%	91%	Doryanthaceae
B. jabirabela	trnT-L	*Lilium henryi* AB064566.1	9%	88%	Liliaceae
B. subulata	**trnL-F**	*Tecophilaea cyanocrocus* AJ290276.1	100%	92%	Tecophilaeaceae
B. subulata	trnL intron	*Doryanthes excelsa* AJ290315.1	98%	92%	Doryanthaceae
B. subulata	trnT-L	*Nypa fruticans* AY145339.1	48%	86%	Arecaceae
B. stenophylla	**trnL-F**	*Kabuyea hostifolia* AJ290278.1	50%	84%	Tecophilaeaceae
B. stenophylla	trnL intron	*Doryanthes excelsa* AJ290315.1	98%	91%	Doryanthaceae
B. stenophylla	trnT-L	No significant similarity found			

Table 4-11: MegaBlast (highly similar sequences) results for a selection of sequenced species for each chloroplast region used in the phylogenetic analysis. Numbers after species refer to samples as listed in Table 4-1 to Table 4-3.

Species	*Region*	*Top Blast result*	*Query coverage*	*Max Identity*	*Family*
B. nitida	**trnL-F**	No significant similarity found Blastn *Cerastium argenteum* Ay521299.1	23%	73%	Caryophyllaceae
B. nitida	trnL intron	No significant similarity found			
B. nitida	trnT-L	*Philactis zinniodes* AY215971.1	91%	93%	Asteraceae
B. mirabilis	**trnL-F**	*Tecophilaea cyanocrocus* AJ290276.1	97%	92%	Tecophilaeaceae
B. mirabilis	trnL intron	*Doryanthes excelsa* AJ290315.1	99%	88%	Doryanthaceae
B. mirabilis	trnT-L	*Nypa fruticans* AY145339.1	47%	85%	Arecaceae
A. endlicheri	**trnL-F**				
A. endlicheri	trnL intron				
A. endlicheri	trnT-L	*Orontium aquaticum* AY145338	63%	85%	Arecaceae

4.3.1.6. Combined analysis of the chloroplast trnT-F region

The trnT-trnF alignment for the Boryaceae consisted of three adjacent non-coding fragments, the trnT-trnL intergenic spacer, the trnL intron (flanked by two short tRNA-encoding exons), and the trnL-trnF intergenic spacer. The alignment had a total of 1772 positions in the final dataset, of which 303 were considered to be parsimony-informative.

The combined chloroplast regions of 15 taxa analysed using the MPA and UPGMA method (Figure 4-16 and Figure 4-17) placed *B. constricta* as the closest species to *B. mirabilis*. The relationships between the two species

remains unresolved, with *B. mirabilis* and *B. constricta* strongly grouped in as a single clade in both analyses (BP=94 MPA, BP=87 UPGMA).

MPA analysis grouped a moderately strong clade (BP=94) clade containing three samples of *B. constricta* and *B. mirabilis*. Another strong clade consisted of *B. sp.* 'Wheatbelt' and *B. nitida* (BP=100) grouped closely together with *B. scirpoidea* (BP=99). Two further strong groups consisted of a clade with *B. stenophylla* and *B. inopinata* (BP=99), both of which were strongly grouped in a clade with *B. jabirabela*.

UPGMA (Figure 4-17) also grouped three samples of *B. constricta*, *B. mirabilis* with the addition of *B. subulata* in a clade as more closely related to one other than to any other taxon (BP=99). These three species and four other species: *B. sphaerocephala*, *B. septentrionalis*, *B. longiscapa* and *B. laciniata* were strongly supported (BP=100) in a larger clade. *B. sp.* 'Wheatbelt' and *B. nitida* also formed a strong but separate clade of their own. The four remaining *Borya* spp. did not strongly associate with any of the above clades.

Both analyses support *B. stenophylla* M.D. Barrett ined as a unique species (BP=100 UPGMA, BP= 99 MPA). UPGMA analysis placed *B. stenophylla* as a sister taxon to all other *Borya* spp. except *B. nitida* and *B. sp.* 'Wheatbelt', which form their own distinct clade (BP=100). Maximum Parsimony analysis grouped *B. stenophylla* with *B. inopinata* (BP=99) both species distant from other *Borya* spp. Both UPGMA and MPA analysis closely linked *B. mirabilis* to *B. constricta* and far from *B. nitida,* which was consistently linked with *B. sp.* 'Wheatbelt'.

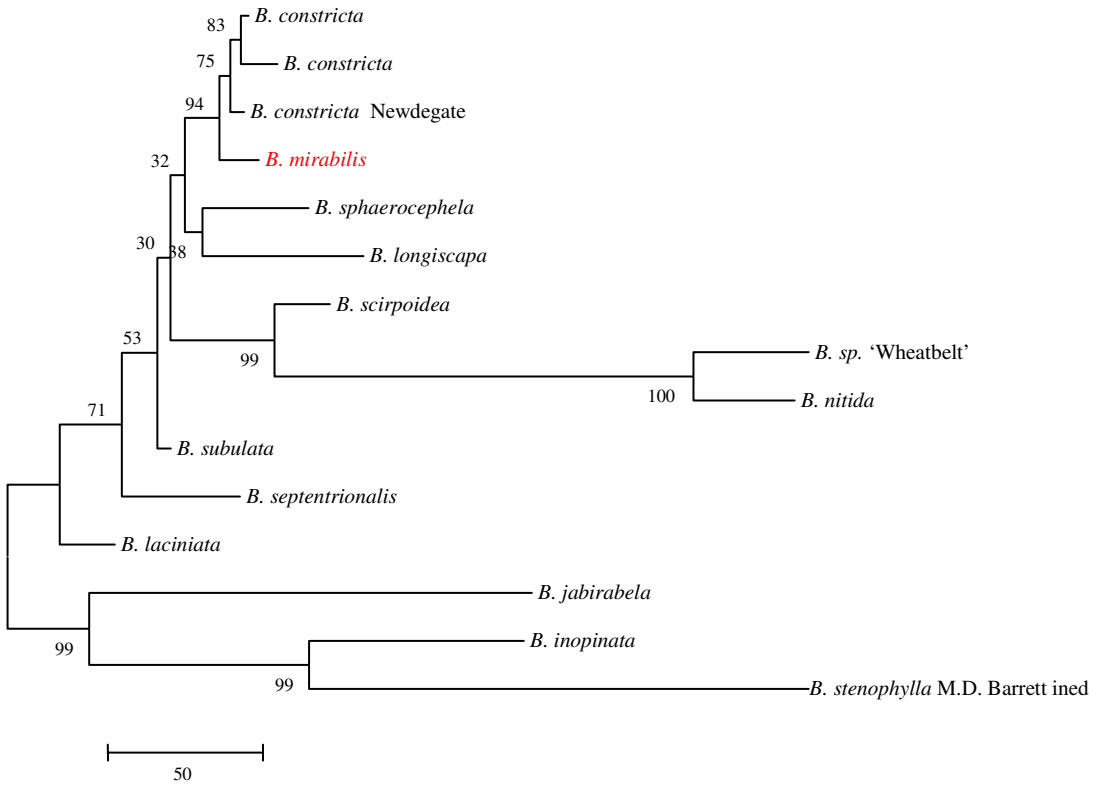

Figure 4-16: Evolutionary relationships of 15 taxa based on the combined analysis of the trnT-L intergenic spacer, trnL-F intergenic spacer and trnL intron of chloroplast DNA. The evolutionary history was inferred using the Maximum Parsimony method (Eck and Dayhoff, 1966). There was a total of 1772 positions in the final dataset, out of which 303 were parsimony-informative. Phylogenetic analyses were conducted in MEGA Version 4 (Tamura *et al*. 2007).

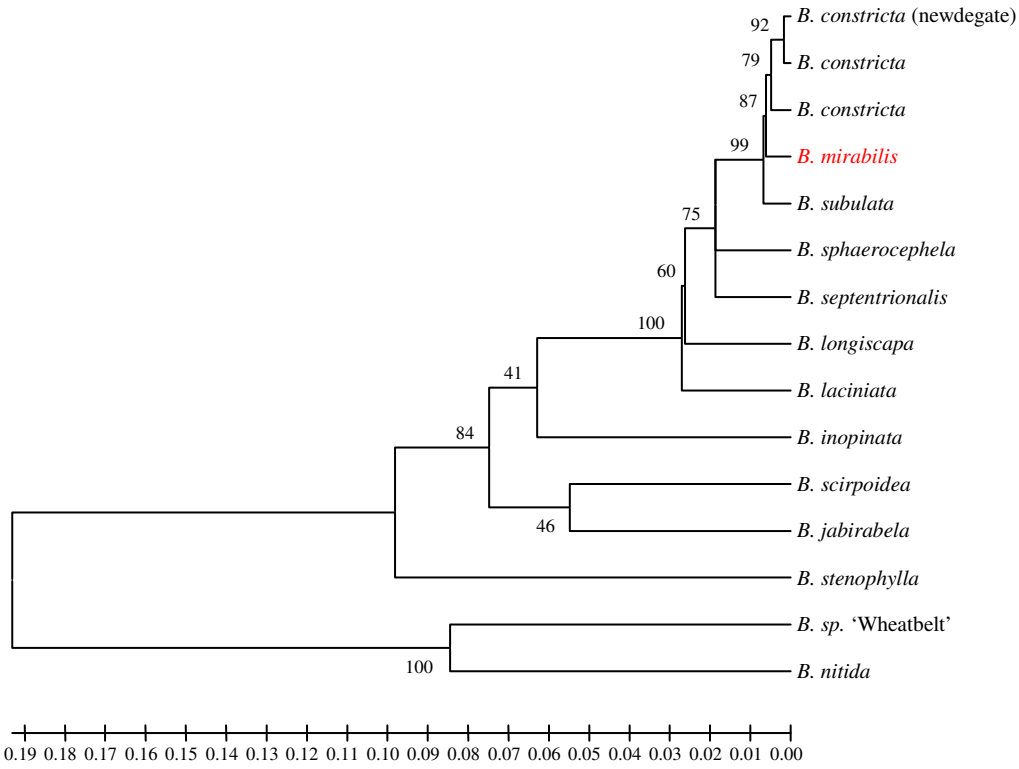

Figure 4-17: Evolutionary relationships of 15 taxa based on the combined analysis of the trnT-L intergenic spacer, trnL-F intergenic spacer and trnL intron of chloroplast DNA. The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). There was a total of 1772 positions in the final dataset. Phylogenetic analyses were conducted in MEGA Version 4 (Tamura *et al*. 2007).

4.3.1.7. Analysis of the ITS 1-4 region of genomic DNA

Clear single bands where obtained for only 8 species with primers ITS1 and ITS4 (Figure 4-18). Of the products that were obtained, five were sequenced and sequences were fungal. Using Blast searches the following fungi were identified: *Sacrostroma* (ascomycetes), Ascomycete sp., uncultured soil fungus, *Davidiella tassiana* (Mycosphaerellaceae) and *Cladosporium sp.* (Mycosphaerellaceae).

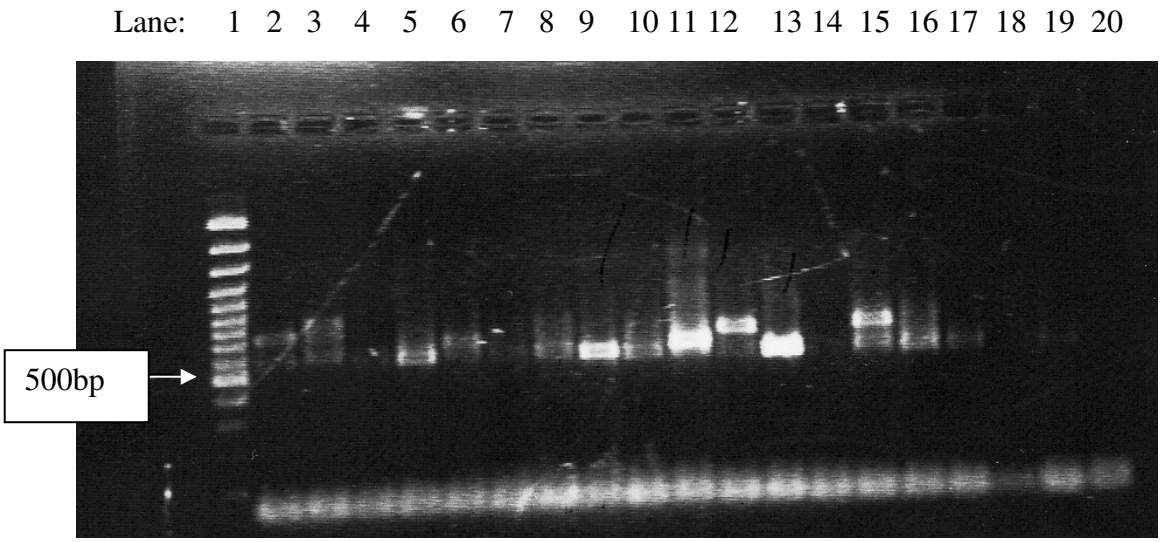

Figure 4-18: 500-900 bp variable and multiple bands were amplified with ITS 1-4 primers and *Borya* extracts (Table 4-1 to Table 4-3), left-right Lane 1-20: molecular weight marker, *B. constricta* 1, *B. constricta* 2, *B. septentrionalis* 3, *B. constricta* 4, *B. constricta* 5, *B. constricta* 6, *B. constricta* 7, *B. sphaerocephala* 8, *B. constricta* 9, *B. constricta* 10, *B. constricta* 11, *B. sphaerocephela* 12, *Laxmania grandiflora* 13, *B. constricta* 14, *B. constricta* 15, *B. septentrionalis* 16, *B. septentrionalis* 17, *B. septentrionalis* 18, *B. constricta* 19 .

4.3.1.8. Analysis of the ITS 2-5 genomic DNA region

A single product was obtained for five species: *B. mirabilis*, *B. sphaerocephela*, *B. nitida*, *B. constricta* and *B. septentrionalis*, but not from the majority of species (Figure 4-20). In the ITS 2-5 region alignment, there was a total of 727 positions in the final dataset, out of which 35 were parsimony-informative. MPA (Figure 4-21) and UPGMA (Figure 4-22) provided moderate to strong branch support for all species (BP= 82-100) analyzed.

In both analyses, *B. sphaerocephala* was resolved as the closest species among the five analyzed to *B. mirabilis* (BP= 82 MPA, BP=87 UPGMA) and in both analyses the branch separating *B. mirabilis* and *B. sphaerocephala* from the other species was highly supported (BP=100). Both analyses highly supported the grouping of *B. septentrionalis* with *B. constricta* (BP=97 MPA, BP=99 UPGMA). The methods of analysis differ in the placement of *B. nitida'* with UPGMA placing this species as an out-group to the other taxa, while MPA placed the species as a sister group to *B. mirabilis* and *B. sphaerocephala*.

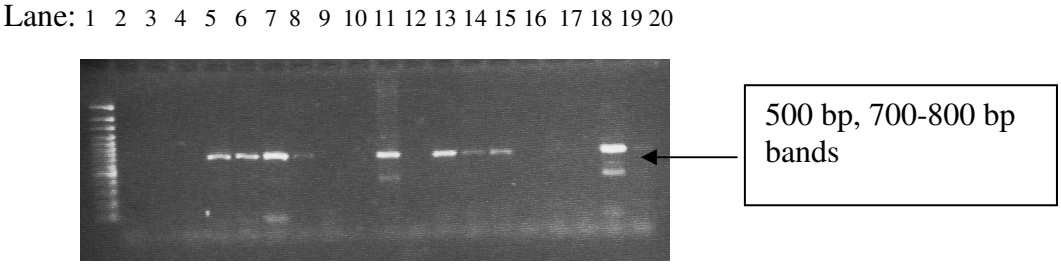

Figure 4-19: 500-800 bp variable and multiple bands were amplified with ITS 2-5* primers and *Borya* extracts (Table 4-1 to Table 4-3), left-right Lane 1-20: molecular weight marker, *B. constricta* 1, *B. constricta* 2, *B. septentrionalis* 3, *B. constricta* 4, *B. constricta* 5, *B. constricta* 6, *B. constricta* 7, *B. sphaerocephala* 8, *B. constricta* 9, *B. constricta* 10, *B. constricta* 11, *B. sphaerocephela* 12, *Laxmania grandiflora* 13, *B. constricta* 14, *B. constricta* 15, *B. septentrionalis* 16, *B. septentrionalis* 17, *B. septentrionalis* 18, *B. constricta* 19.

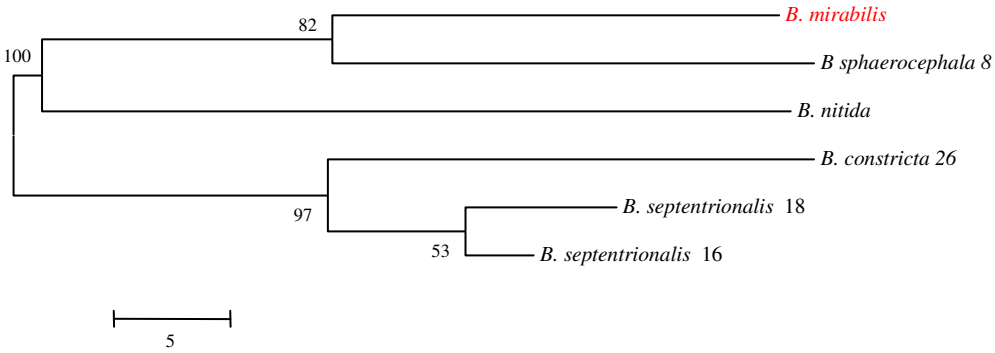

Figure 4-20: Evolutionary relationships of 6 taxa with ITS2-5* primers. The evolutionary history was inferred using the Maximum Parsimony method (Eck and Dayhoff, 1966). In the ITS2-5 region alignment there was a total of 727 positions in the final dataset, out of which 35 were parsimony-informative. Phylogenetic analyses were conducted in MEGA Version 4 (Tamura *et al*. 2007). Numbers after species refer to samples of the same species as listed in Table 4-1 to Table 4-3.

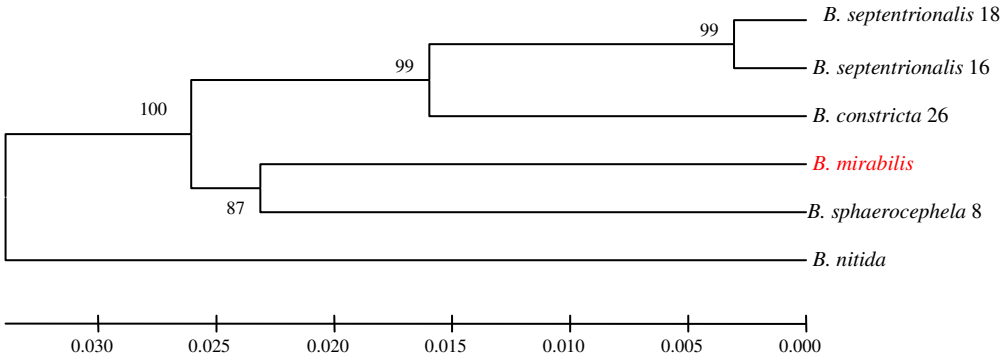

Figure 4-21: Evolutionary relationships of 6 taxa with ITS2-5* primers. The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). There was a total of 669 positions in the final dataset. Phylogenetic analyses were conducted in MEGA Version 4 (Tamura *et al*. 2007). Numbers after species refer to samples of the same species as listed in Table 4-1 to Table 4-3.

4.3.2. *Borya mirabilis* population analysis

Clear bright extracts were obtained for each of the 19 samples (Figure 4-22) obtained from the *B. mirabilis* field population and each of the eight samples obtained from the *B. mirabilis ex-situ* population (Figue 4-22). Each of the Operon primers that were selected after screening produced consistent banding patterns (Figure 4-23 to Figure 4-53). Of the primers run with all extracts (30 primers), OPA 4 (Figure 4-24), OPA 11 (Figure 4-26) and OPB 13 (Figure 4-38) showed the most variation in banding patterns between samples. The remaining primers showed little or no variation between samples.

Dendograms based on Nei's (1978) Genetic distance (Table 4-12 to Table 4-14) using UPGMA of *B. mirabilis* RBG *ex-situ* population and the *in-situ* population with each of the 19 *in-situ* samples (colony/plant) and 8 *ex-situ* samples analysed as an individual or as groups (Figure 4-53 and 4-54) showed differences between individual plants within a colony and between colonies in the wild population. Differences between individual plants in the *ex-situ* population were also seen; however, the amount of genetic variation in the wild was not reflected in the *ex-situ* population. The analysis as individuals (Figure 4-53) grouped Field Colony 2/plant 2 and Field Colony 5/plant 5 as different from all other plant samples. The differences seen in the *in-situ* population were not seen in the *ex-situ* population. The plants from the RBG reflected only the genetics of a small number of the colonies in the wild (Figure 4-53) with the *ex-situ* plants grouped with plants from Colony 7 or Colony 3. Colony 2 and Colony 5 did not group with any plant from the *ex-situ* population.

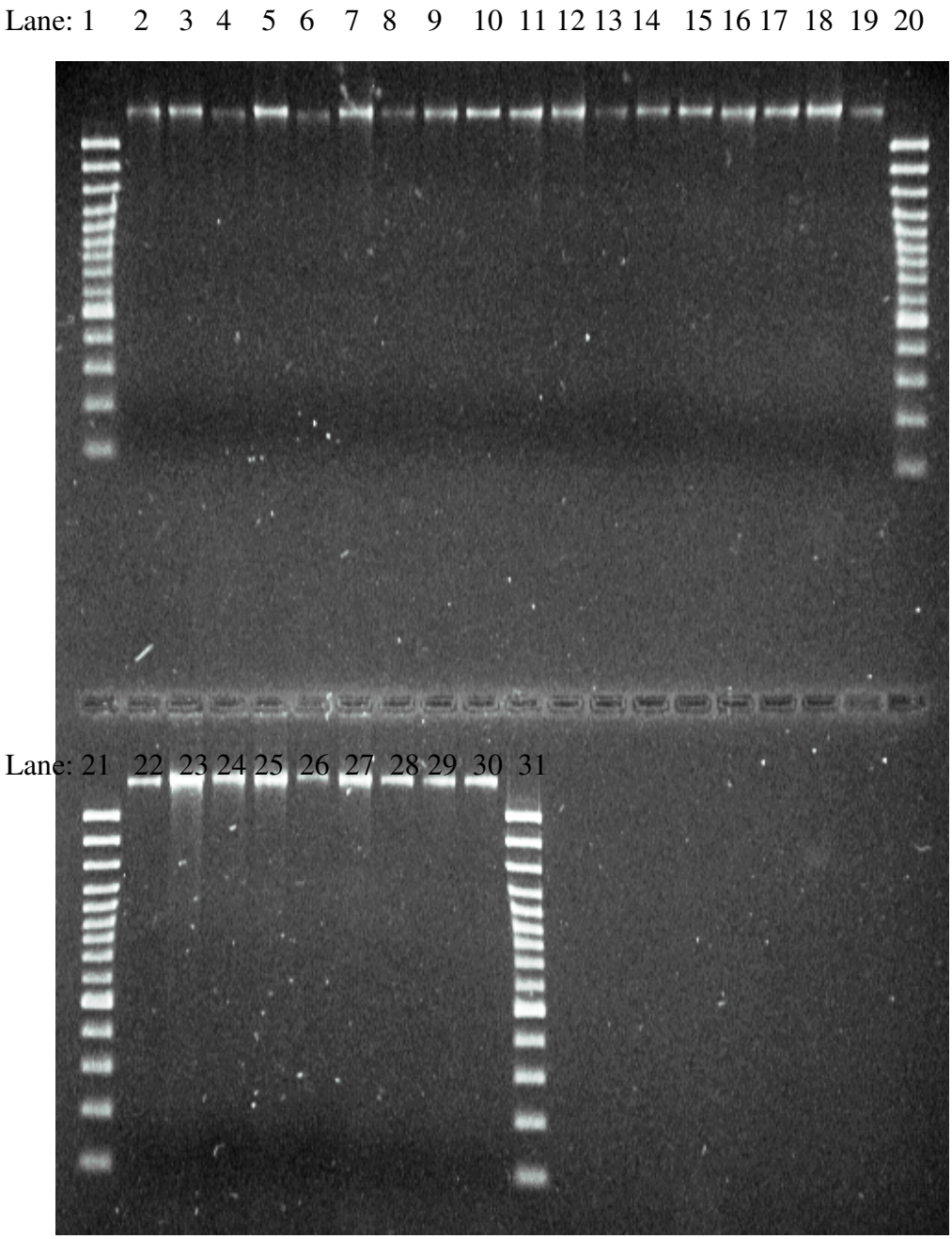

Figure 4-22: DNA Extracts of samples used for *Borya mirabilis* RAPD population analysis prior to dilution. Lane 1-31: molecular weight marker, 2/2 field, 2/4 field, 2/14 field, 3/3 field, 5/1 field, 5/2 field, 5/3 field, 5/6 field, 5/7 field, 7/1 field, 7/4 field, 7/5 field, 7/8.2 field, 7/9 field, 7/10 field, 7/12 field, 7/13 field, 7/14 field, molecular weight marker, molecular weight marker 7/15 field, 991064/001 RBG, 070685/009 RBG, 070685/008 RBG, 991061/001 RBG, 991057/001 RBG, 070685/001 RBG, 000893/003 RBG, 070685/007 RBG.

Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

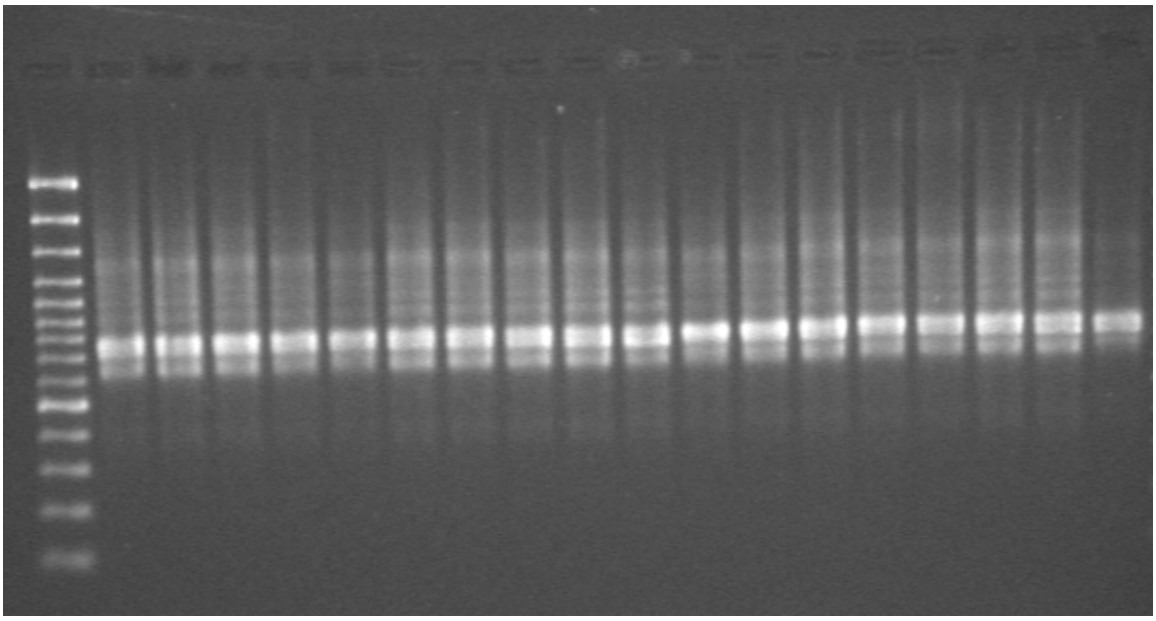

Figure 4-23: OPA 2 DNA products of samples used for *Borya mirabilis* RAPD population analysis prior to dilution. Lane 1-19: molecular weight marker, 2/2 field, 2/4 field, 2/14 field, 3/3 field, 5/1 field, 5/2 field, 5/3 field, 5/6 field, 5/7 field, 7/1 field, 7/4 field, 7/5 field, 7/8.2 field, 7/9 field, 7/10 field, 7/12 field, 7/13 field, 7/14 field.

Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

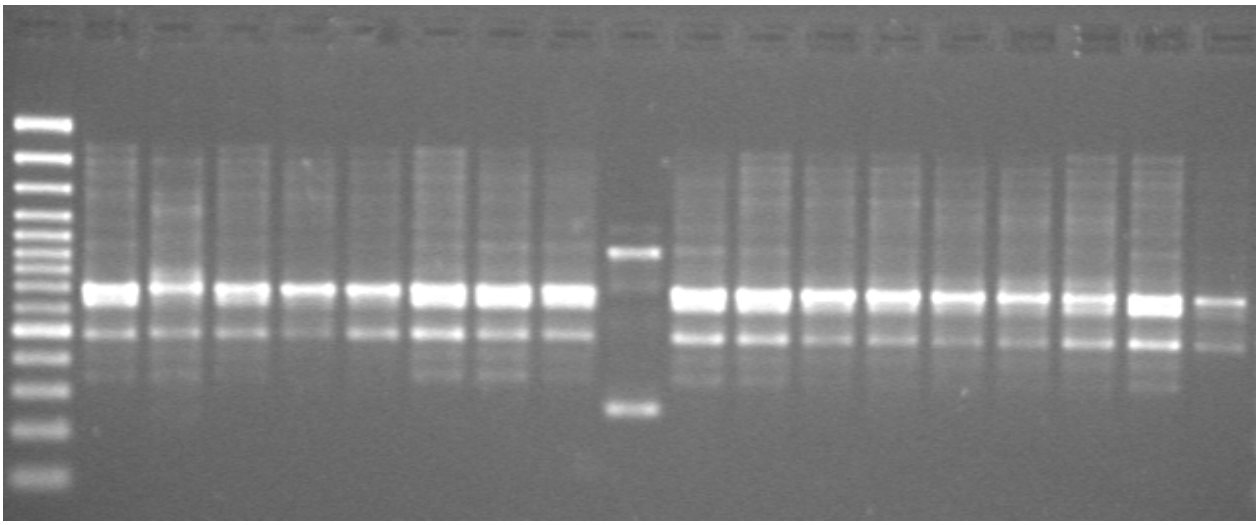

Figure 4-24: OPA 4 DNA products of samples used for *Borya mirabilis* RAPD population analysis prior to dilution. Lane 1-19: molecular weight marker, 2/2 field, 2/4 field, 2/14 field, 3/3 field, 5/1 field, 5/2 field, 5/3 field, 5/6 field, 5/7 field, 7/1 field, 7/4 field, 7/5 field, 7/8.2 field, 7/9 field, 7/10 field, 7/12 field, 7/13 field, 7/14 field.

Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

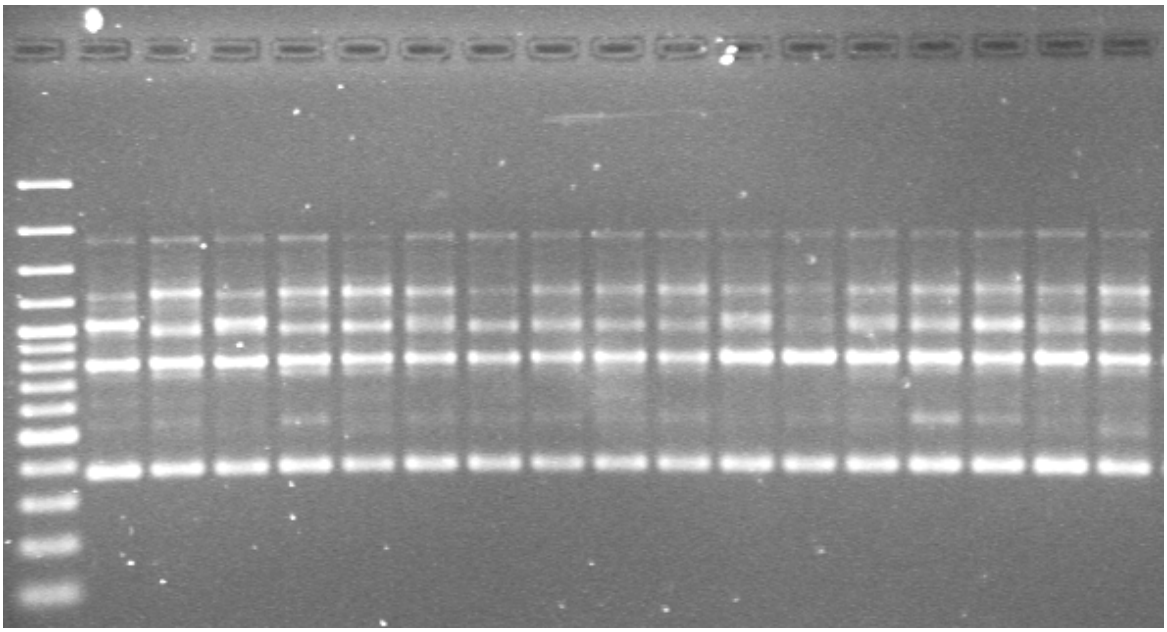

Figure 4-25: OPA 9 DNA products of samples used for *Borya mirabilis* RAPD population analysis prior to dilution. Lane 1-18: molecular weight marker, 2/2 field, 2/4 field, 2/14 field, 3/3 field, 5/1 field, 5/2 field, 5/3 field, 5/6 field, 5/7 field, 7/1 field, 7/4 field, 7/5 field, 7/8.2 field, 7/9 field, 7/10 field, 7/12 field, 7/13 field.

Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

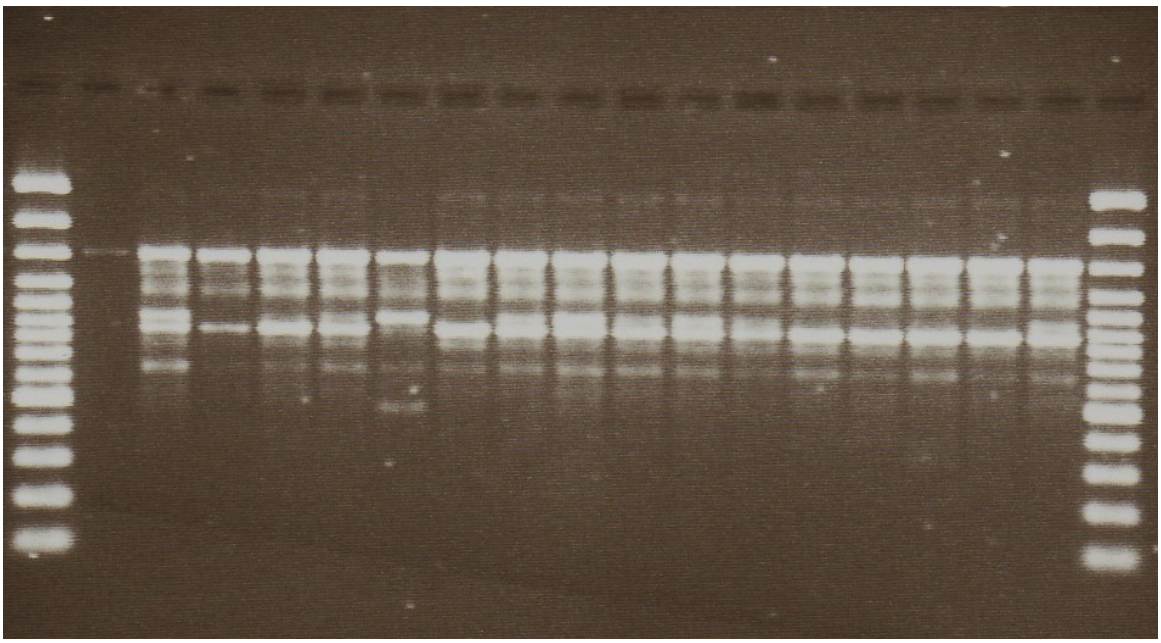

Figure 4-26: OPA 11 DNA products of samples used for *Borya mirabilis* RAPD population analysis prior to dilution. Lane 1-19: molecular weight marker, 2/2 field, 2/4 field, 2/14 field, 3/3 field, 5/1 field, 5/2 field, 5/3 field, 5/6 field, 5/7 field, 7/1 field, 7/4 field, 7/5 field, 7/8.2 field, 7/9 field, 7/10 field, 7/12 field, 7/13 field, 7/14 field.

Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

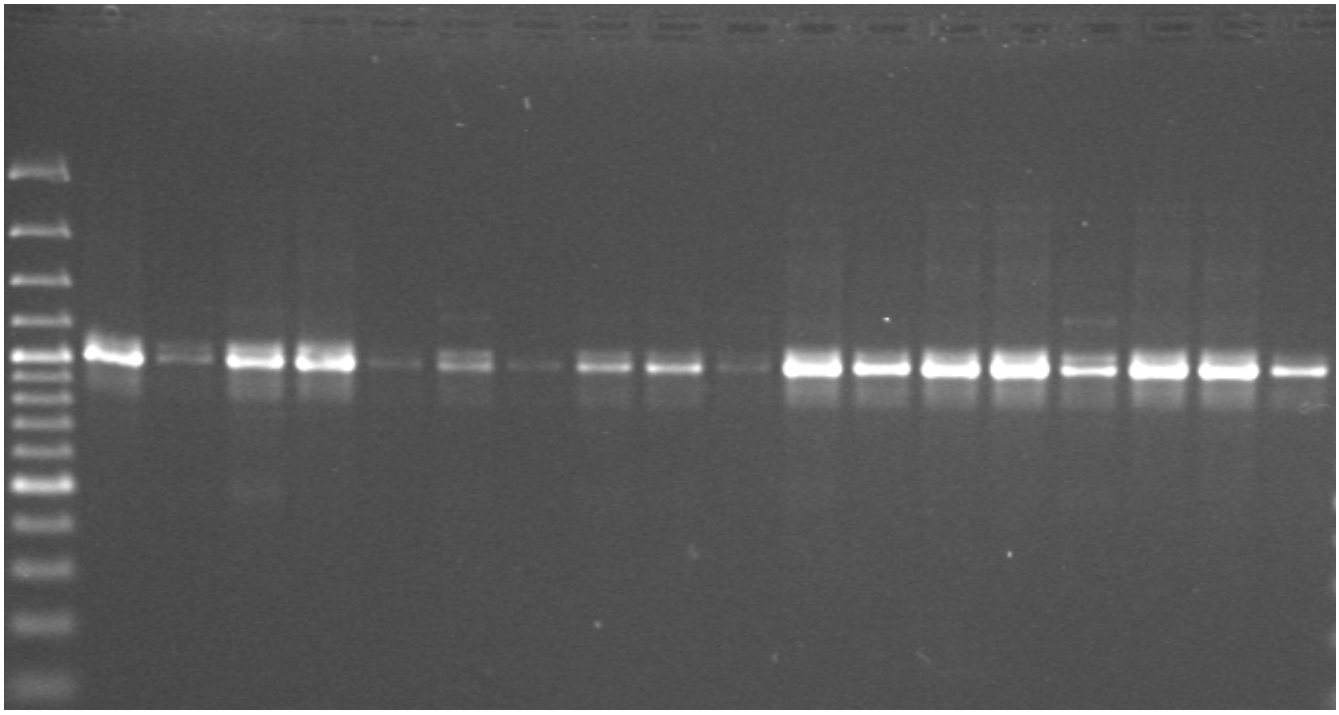

Figure 4-27: OPA 18 DNA products of samples used for *Borya mirabilis* RAPD population analysis prior to dilution. Lane 1-19: molecular weight marker, 2/2 field, 2/4 field, 2/14 field, 3/3 field, 5/1 field, 5/2 field, 5/3 field, 5/6 field, 5/7 field, 7/1 field, 7/4 field, 7/5 field, 7/8.2 field, 7/9 field, 7/10 field, 7/12 field, 7/13 field, 7/14 field.

Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

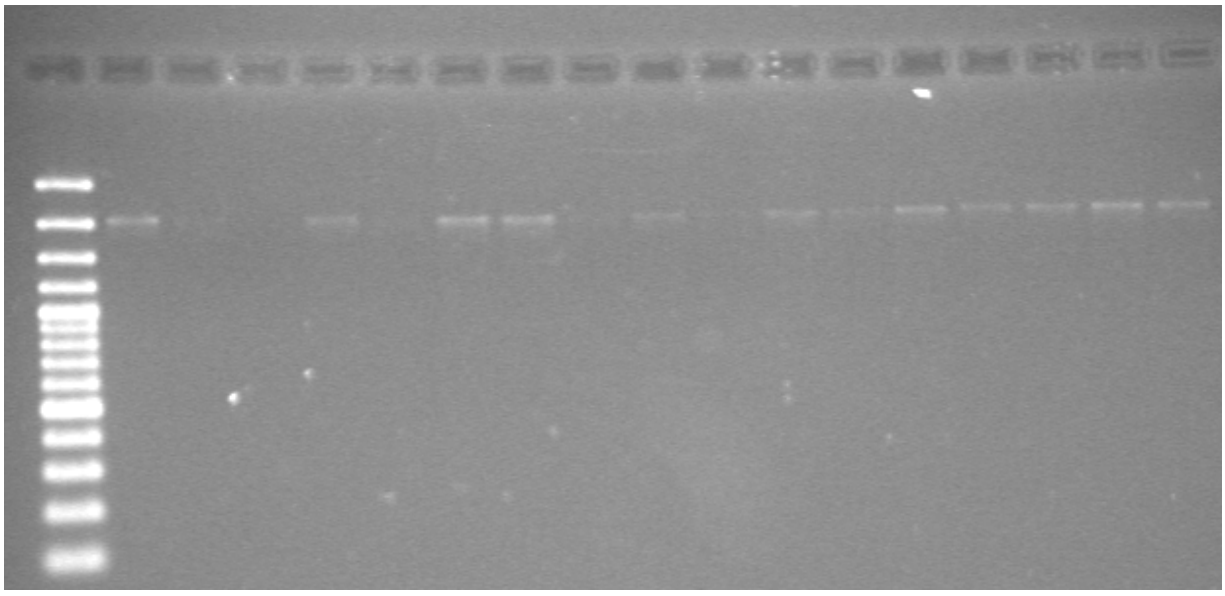

Figure 4-28: OPA 16 DNA products of samples used for *Borya mirabilis* RAPD population analysis prior to dilution. Lane 1-18: molecular weight marker, 2/2 field, 2/4 field, 2/14 field, 3/3 field, 5/1 field, 5/2 field, 5/3 field, 5/6 field, 5/7 field, 7/1 field, 7/4 field, 7/5 field, 7/8.2 field, 7/9 field, 7/10 field, 7/12 field, 7/13 field.

Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

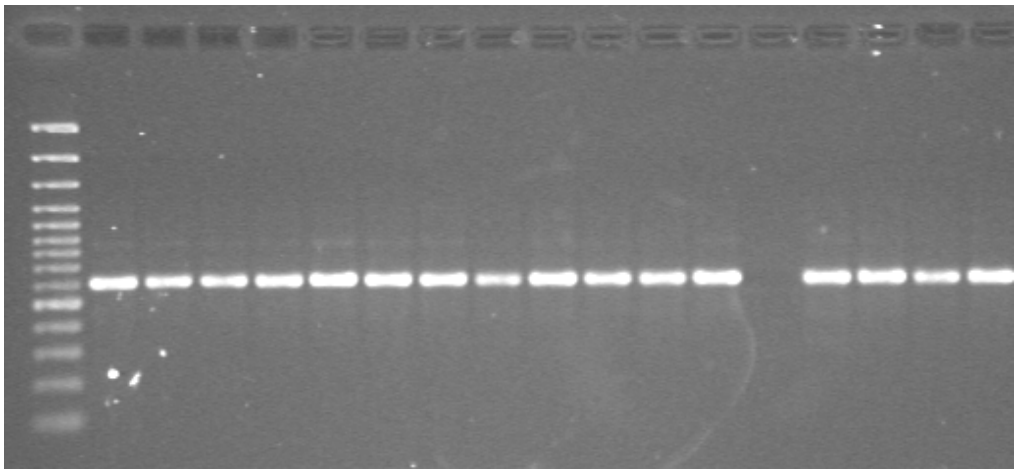

Figure 4-29: OPB 11 DNA products of samples used for *Borya mirabilis* RAPD population analysis prior to dilution. Lane 1-18: molecular weight marker, 2/2 field, 2/4 field, 2/14 field, 3/3 field, 5/1 field, 5/2 field, 5/3 field, 5/6 field, 5/7 field, 7/1 field, 7/4 field, 7/5 field, 7/8.2 field, 7/9 field, 7/10 field, 7/12 field, 7/13 field.

Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

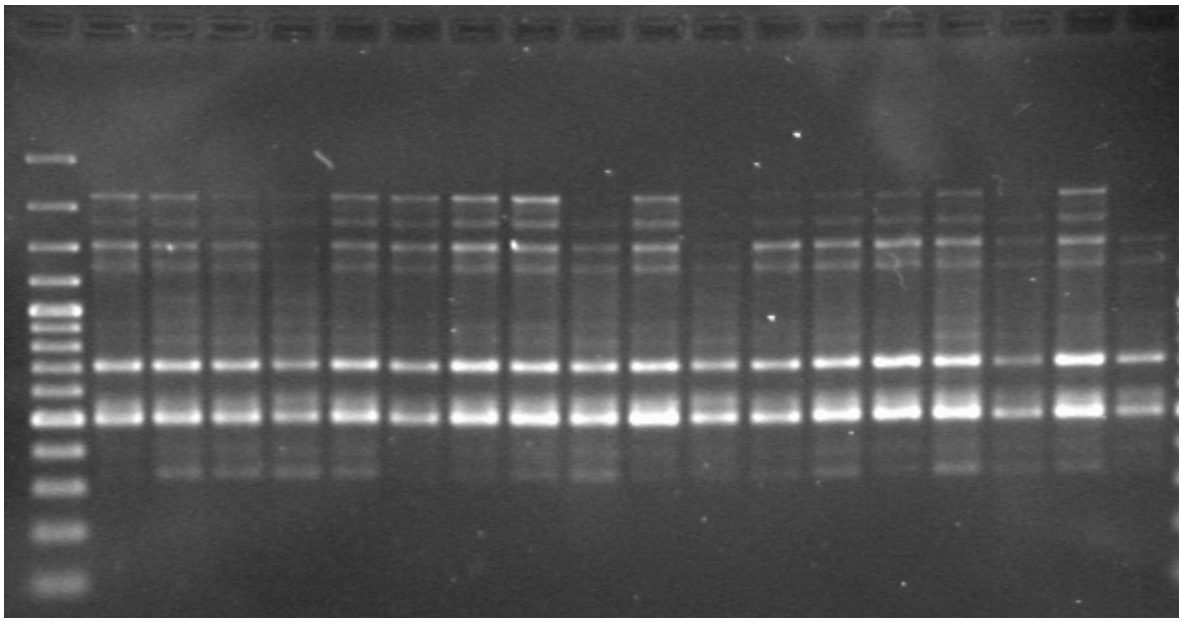

Figure 4-30: OPB 2 DNA products of samples used for *Borya mirabilis* RAPD population analysis prior to dilution. Lane 1-19: molecular weight marker, 2/2 field, 2/4 field, 2/14 field, 3/3 field, 5/1 field, 5/2 field, 5/3 field, 5/6 field, 5/7 field, 7/1 field, 7/4 field, 7/5 field, 7/8.2 field, 7/9 field, 7/10 field, 7/12 field, 7/13 field, 7/14 field.

Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

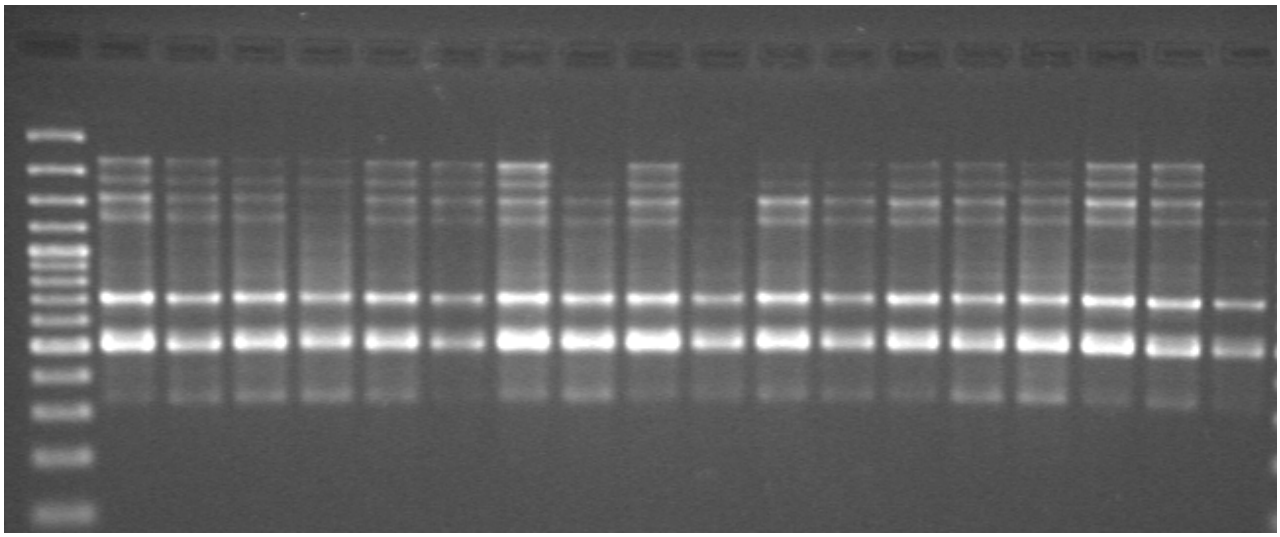

Figure 4-31: OPB 2 DNA products of samples used for *Borya mirabilis* RAPD population analysis prior to dilution. Lane 1-19: molecular weight marker, 2/2 field, 2/4 field, 2/14 field, 3/3 field, 5/1 field, 5/2 field, 5/3 field, 5/6 field, 5/7 field, 7/1 field, 7/4 field, 7/5 field, 7/8.2 field, 7/9 field, 7/10 field, 7/12 field, 7/13 field, 7/14 field.

Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

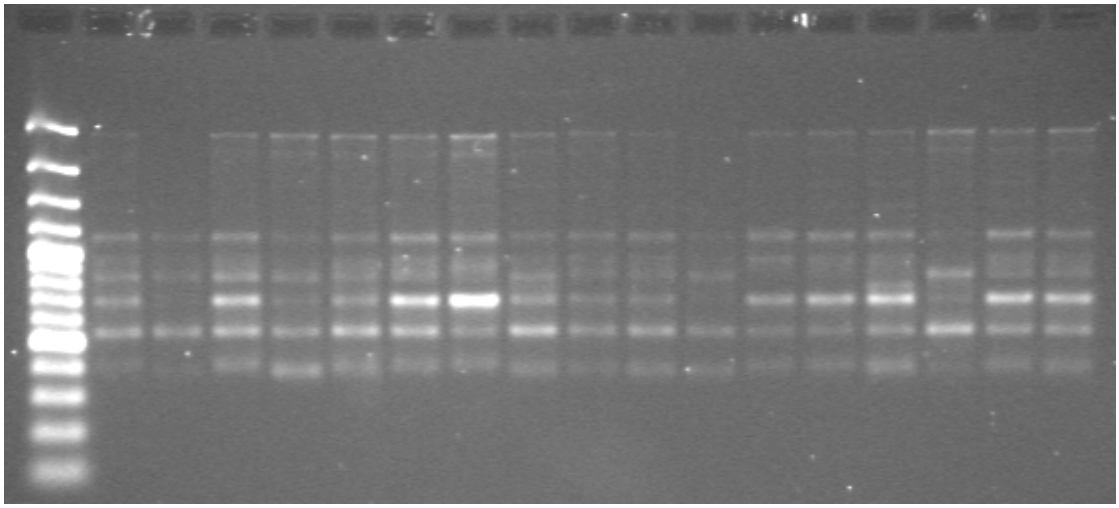

Figure 4-32: OPB 4 DNA products of samples used for *Borya mirabilis* RAPD population analysis prior to dilution. Lane 1-18: molecular weight marker, 2/2 field, 2/4 field, 2/14 field, 3/3 field, 5/1 field, 5/2 field, 5/3 field, 5/6 field, 5/7 field, 7/1 field, 7/4 field, 7/5 field, 7/8.2 field, 7/9 field, 7/10 field, 7/12 field, 7/13 field, 7/14 field.

Lane: 1 2 3 4 5 6 7 8 9 10 11 12

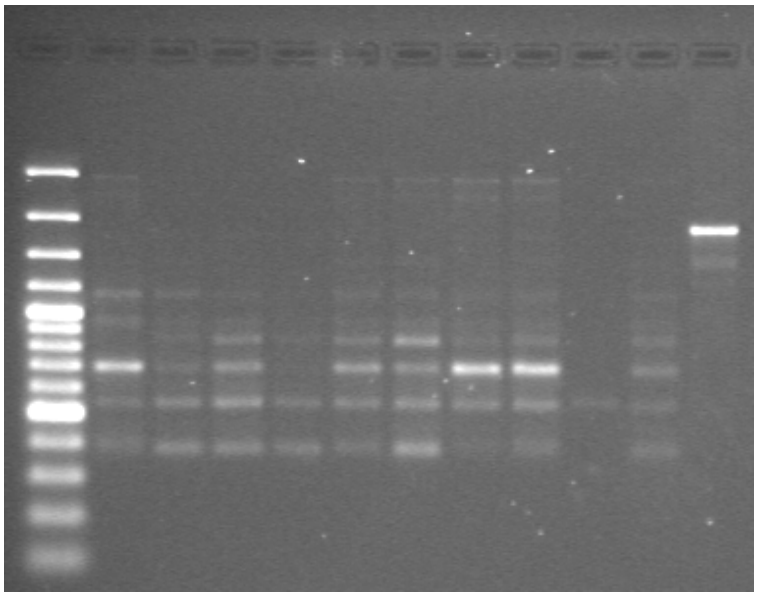

Figure 4-33: OPB 4 2nd gel row of DNA products of samples used for *Borya mirabilis* RAPD population analysis prior to dilution. Lane 1-12: molecular weight marker, 7/15 field, 070685/009 RBG, 070685/008 RBG, 991061/001 RBG, 991057/001 RBG, 070685/001 RBG, 000893/003 RBG, 070685/007 RBG, *B. constricta*, 991064/001 RBG, *B. sphaerocephala*.

Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

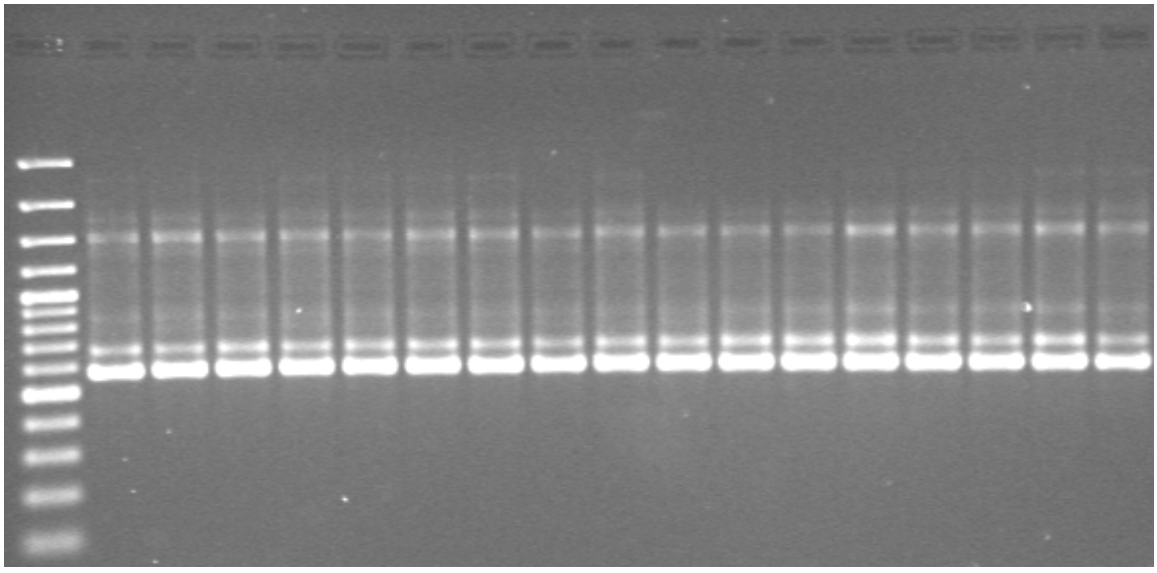

Figure 4-34: OPB 6 DNA products of samples used for *Borya mirabilis* RAPD population analysis prior to dilution. Lane 1-18: molecular weight marker, 2/2 field, 2/4 field, 2/14 field, 3/3 field, 5/1 field, 5/2 field, 5/3 field, 5/6 field, 5/7 field, 7/1 field, 7/4 field, 7/5 field, 7/8.2 field, 7/9 field, 7/10 field, 7/12 field, 7/13 field.

Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

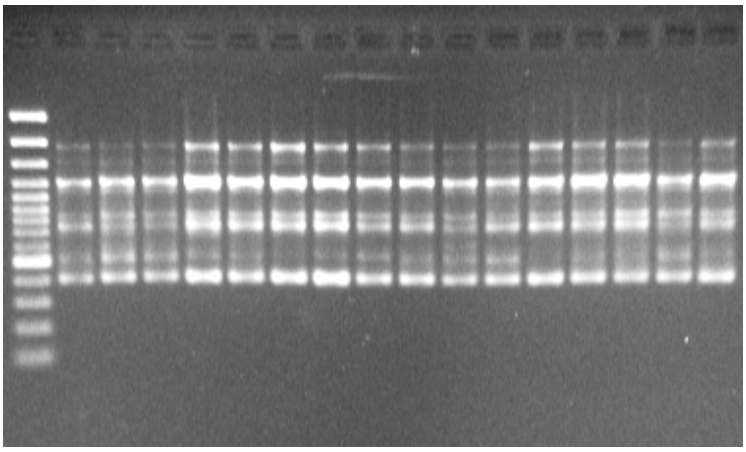

Figure 4-35: OPB 7 DNA products of samples used for *Borya mirabilis* RAPD population analysis prior to dilution. Lane 1-17: molecular weight marker, 2/2 field, 2/4 field, 2/14 field, 3/3 field, 5/1 field, 5/2 field, 5/3 field, 5/6 field, 5/7 field, 7/1 field, 7/4 field, 7/5 field, 7/8.2 field, 7/9 field, 7/10 field, 7/12 field.

Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

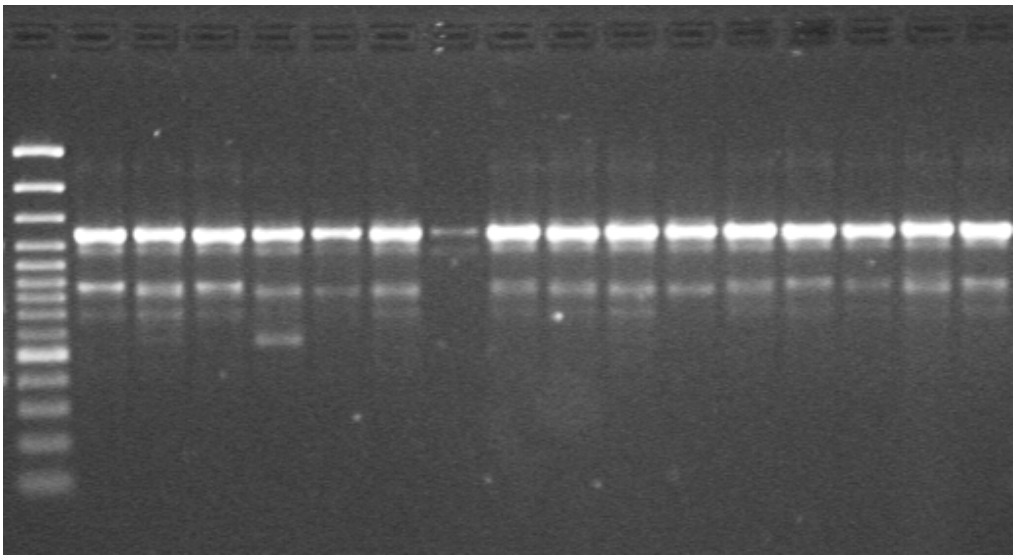

Figure 4-36: OPB 8 DNA products of samples used for *Borya mirabilis* RAPD population analysis prior to dilution. Lane 1-17: molecular weight marker, 2/2 field, 2/4 field, 2/14 field, 3/3 field, 5/1 field, 5/2 field, 5/3 field, 5/6 field, 5/7 field, 7/1 field, 7/4 field, 7/5 field, 7/8.2 field, 7/9 field, 7/10 field, 7/12 field.

Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

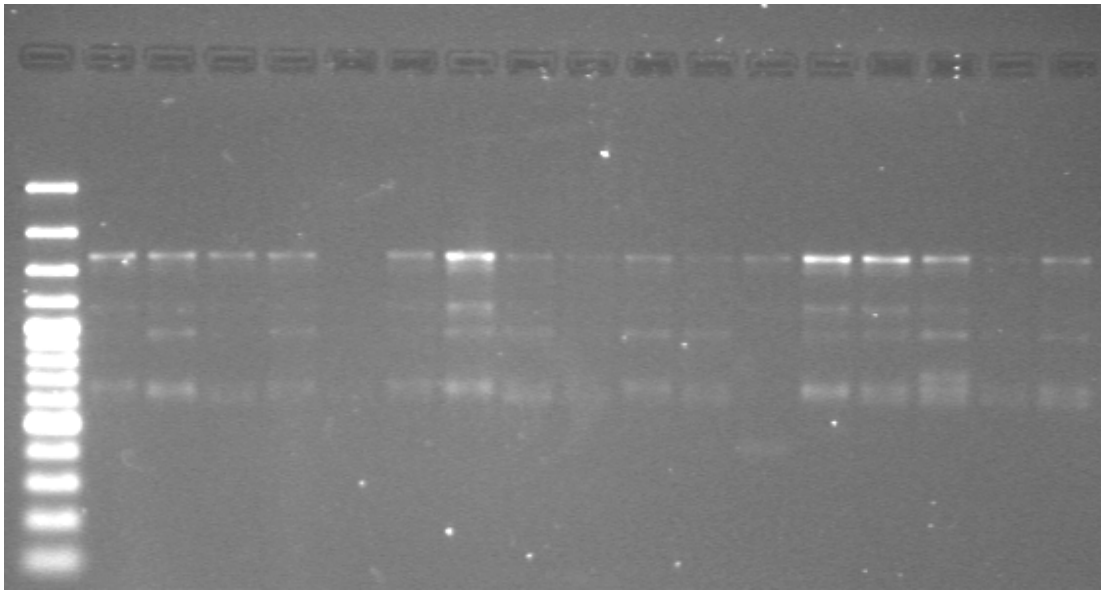

Figure 4-37: OPB 12 DNA products of samples used for *Borya mirabilis* RAPD population analysis prior to dilution. Lane 1-18: molecular weight marker, 2/2 field, 2/4 field, 2/14 field, 3/3 field, 5/1 field, 5/2 field, 5/3 field, 5/6 field, 5/7 field, 7/1 field, 7/4 field, 7/5 field, 7/8.2 field, 7/9 field, 7/10 field, 7/12 field, 7/13 field.

Lane : 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

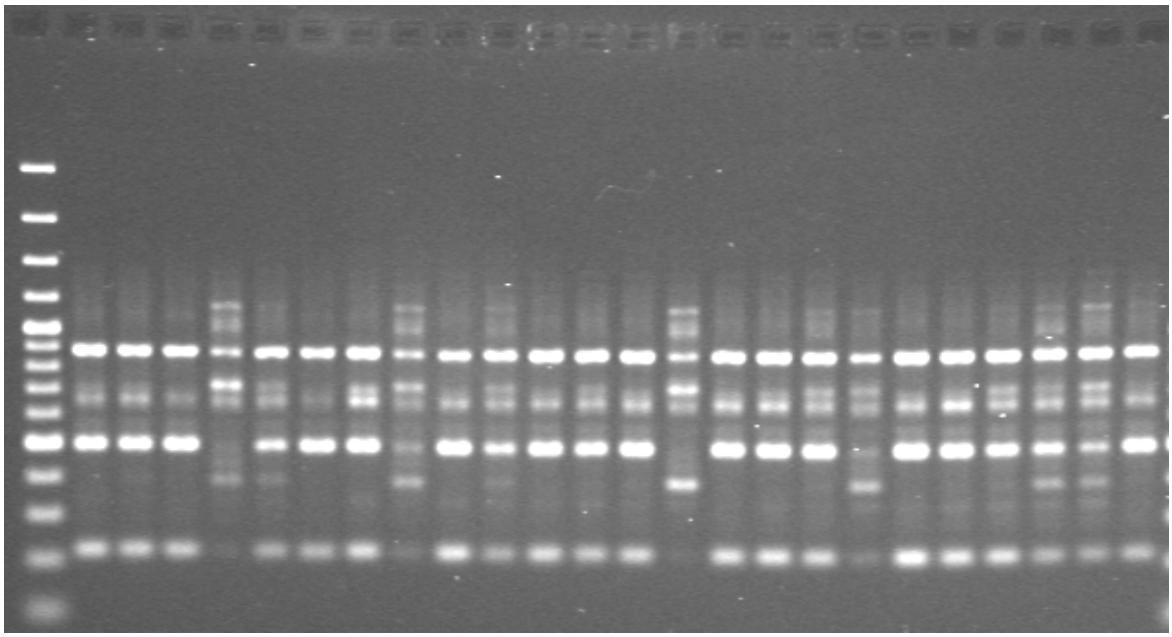

Figure 4-38: OPB 13 DNA products of samples used for *Borya mirabilis* RAPD population analysis prior to dilution. Lane 1-25: molecular weight marker, 2/2 field, 2/4 field, 2/14 field, 3/3 field, 5/1 field, 5/2 field, 5/3 field, 5/6 field, 5/7 field, 7/1 field, 7/4 field, 7/5 field, 7/8.2 field, 7/9 field, 7/10 field, 7/12 field, 7/13 field, 7/14 field, 7/15 field, 991064/001 RBG, 070685/009 RBG, 070685/008 RBG, 991061/001 RBG, 991057/001 RBG.

Lane: 1 2 3 4 5 6 7

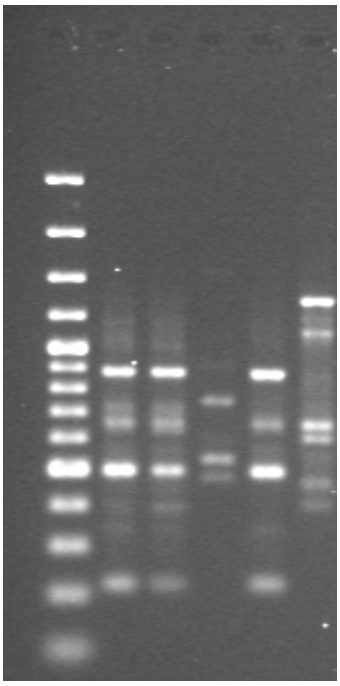

Figure 4-39: OPB 13 2nd gel row of DNA products of samples used for *Borya mirabilis* RAPD population analysis prior to dilution. Lane 1-7: molecular weight marker, 000893/003 RBG, 070685/007 RBG, *B. constricta*, 991064/001 RBG, *B. sphaerocephala*.

Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

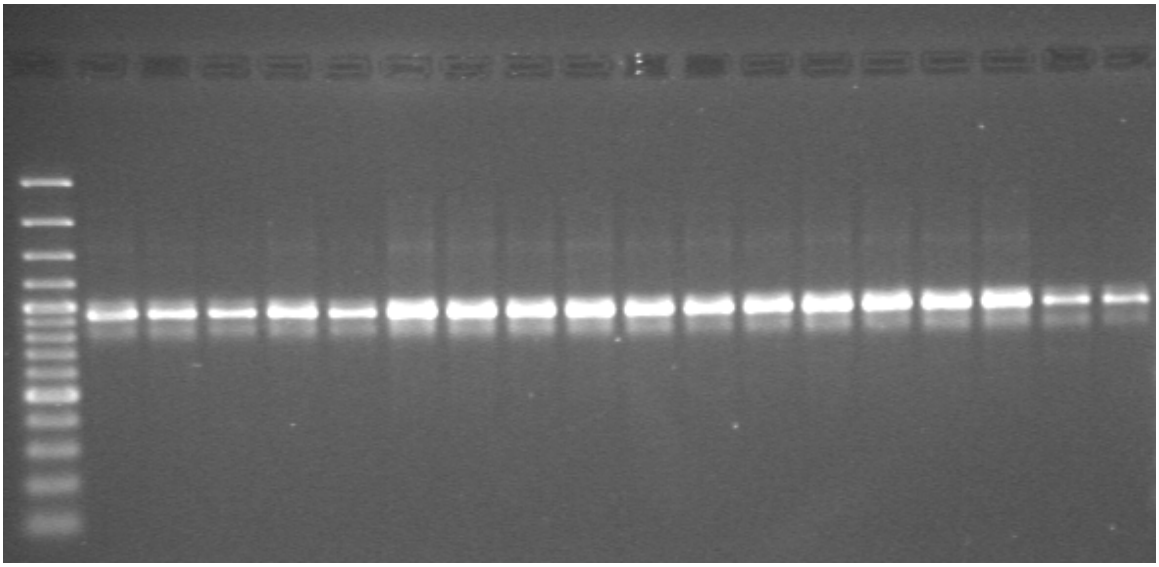

Figure 4-40: OPB 14 DNA products of samples used for *Borya mirabilis* RAPD population analysis prior to dilution. Lane 1-31: molecular weight marker, 2/2 field, 2/4 field, 2/14 field, 3/3 field, 5/1 field, 5/2 field, 5/3 field, 5/6 field, 5/7 field, 7/1 field, 7/4 field, 7/5 field, 7/8.2 field, 7/9 field, 7/10 field, 7/12 field, 7/13 field, 7/14 field.

Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

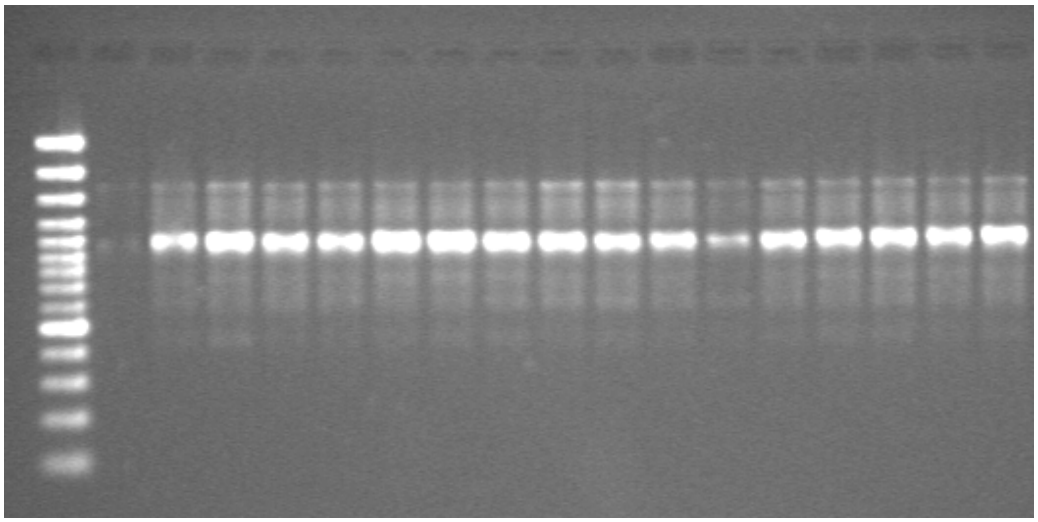

Figure 4-41: OPB 15 DNA products of samples used for *Borya mirabilis* RAPD population analysis prior to dilution. Lane 1-18: molecular weight marker, 2/2 field, 2/4 field, 2/14 field, 3/3 field, 5/1 field, 5/2 field, 5/3 field, 5/6 field, 5/7 field, 7/1 field, 7/4 field, 7/5 field, 7/8.2 field, 7/9 field, 7/10 field, 7/12 field, 7/13 field.

Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

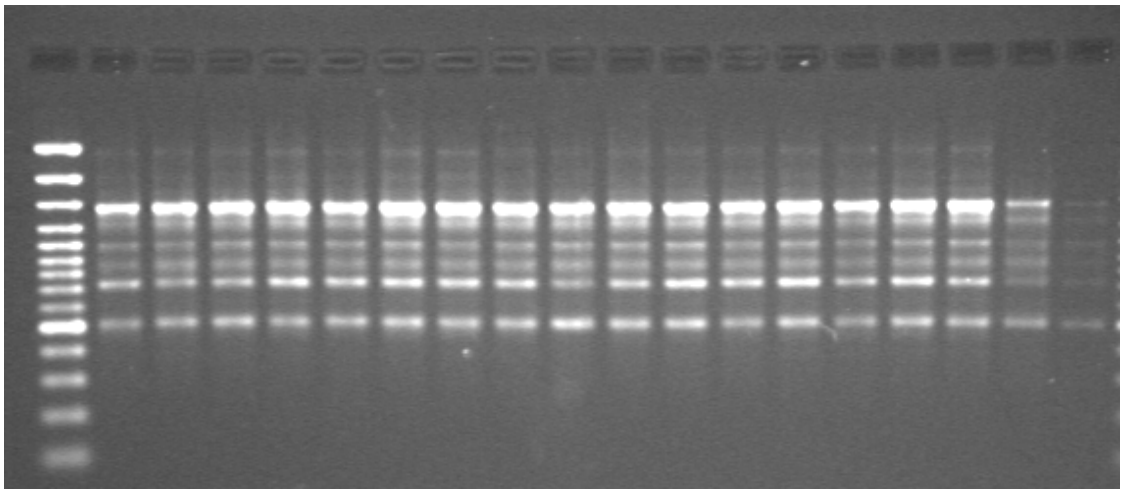

Figure 4-42: OPB 17 DNA products of samples used for *Borya mirabilis* RAPD population analysis prior to dilution. Lane 1-19: molecular weight marker, 2/2 field, 2/4 field, 2/14 field, 3/3 field, 5/1 field, 5/2 field, 5/3 field, 5/6 field, 5/7 field, 7/1 field, 7/4 field, 7/5 field, 7/8.2 field, 7/9 field, 7/10 field, 7/12 field, 7/13 field, 7/14 field.

Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

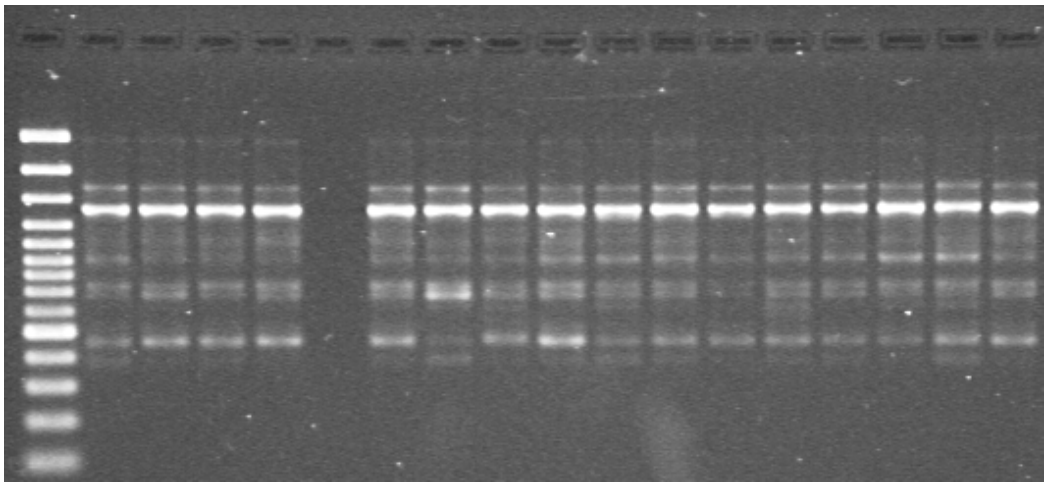

Figure 4-43: OPB 20 DNA products of samples used for *Borya mirabilis* RAPD population analysis prior to dilution. Lane 1-18: molecular weight marker, 2/2 field, 2/4 field, 2/14 field, 3/3 field, 5/1 field, 5/2 field, 5/3 field, 5/6 field, 5/7 field, 7/1 field, 7/4 field, 7/5 field, 7/8.2 field, 7/9 field, 7/10 field, 7/12 field, 7/13 field.

Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13

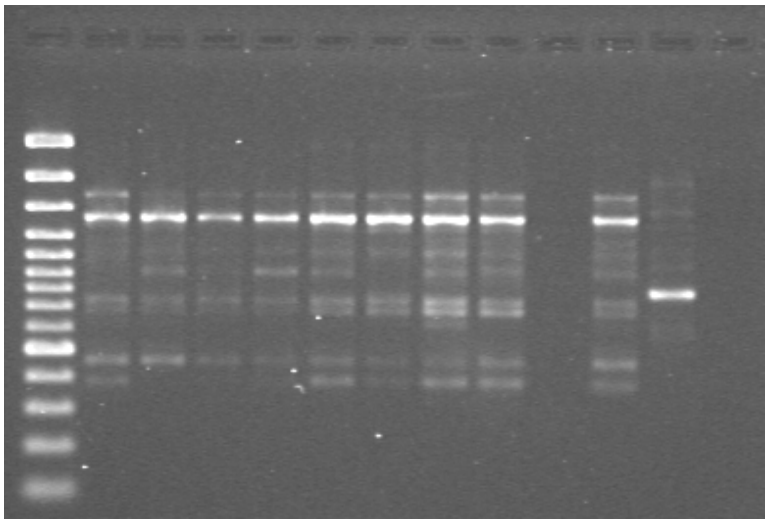

Figure 4-44: OPB 20 2nd gel row of DNA products of samples used for *Borya mirabilis* RAPD population analysis prior to dilution. Lane 1-13: molecular weight marker, 7/15 field, 070685/009 RBG, 070685/008 RBG, 991061/001 RBG, 991057/001 RBG, 070685/001 RBG, 000893/003 RBG, 070685/007 RBG, *B. constricta*, 991064/001 RBG, *B. sphaerocephala*.

Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

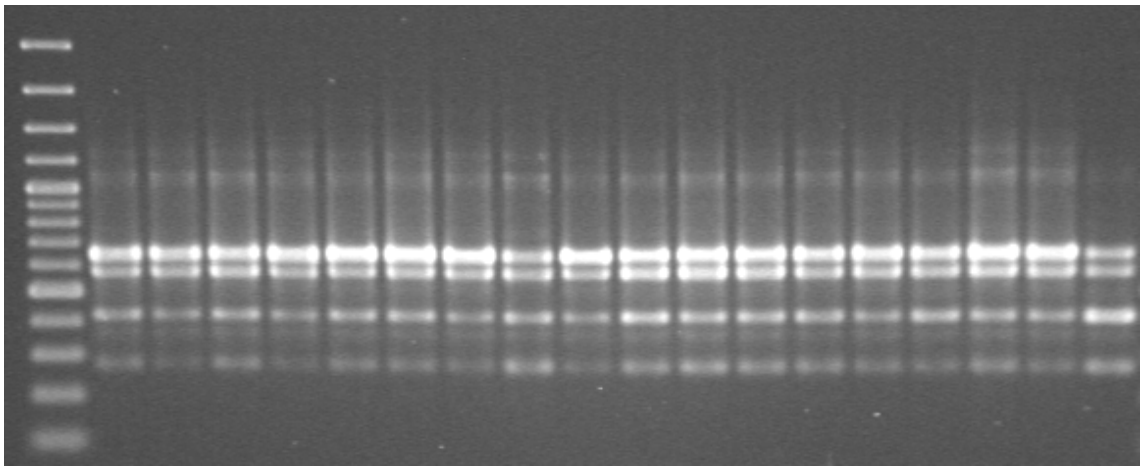

Figure 4-45: OPM 1 DNA products of samples used for *Borya mirabilis* RAPD population analysis prior to dilution. Lane 1-19: molecular weight marker, 2/2 field, 2/4 field, 2/14 field, 3/3 field, 5/1 field, 5/2 field, 5/3 field, 5/6 field, 5/7 field, 7/1 field, 7/4 field, 7/5 field, 7/8.2 field, 7/9 field, 7/10 field, 7/12 field, 7/13 field, 7/14 field.

Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

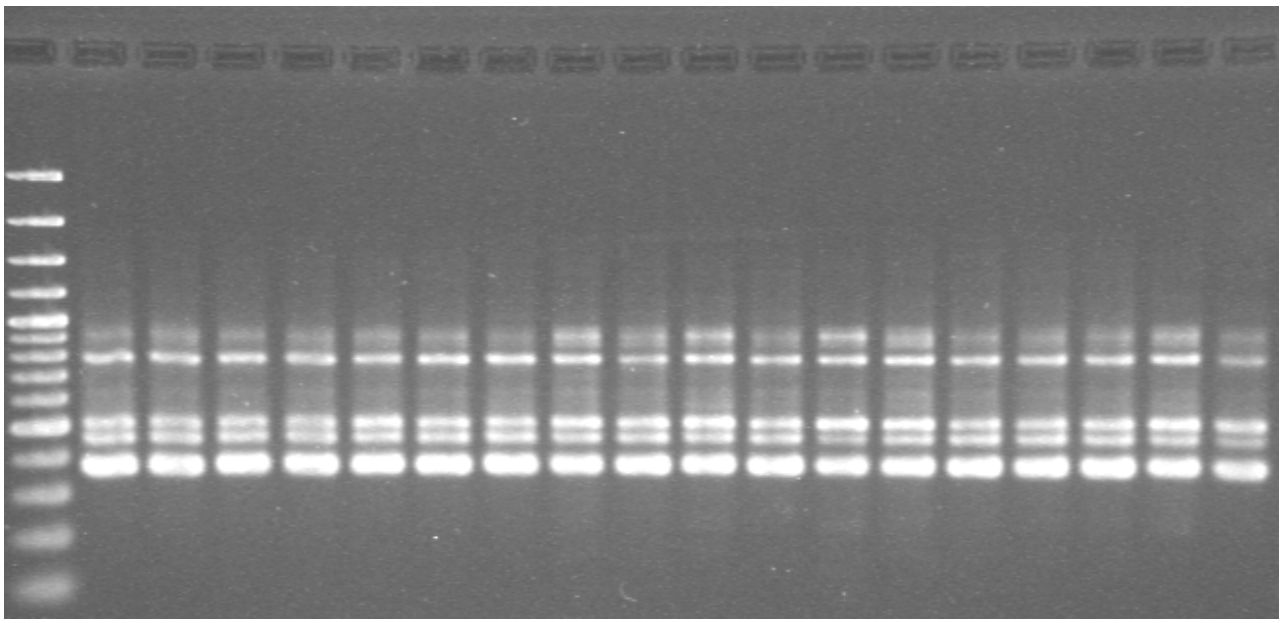

Figure 4-46: OPM 2 DNA products of samples used for *Borya mirabilis* RAPD population analysis prior to dilution. Lane 1-19: molecular weight marker, 2/2 field, 2/4 field, 2/14 field, 3/3 field, 5/1 field, 5/2 field, 5/3 field, 5/6 field, 5/7 field, 7/1 field, 7/4 field, 7/5 field, 7/8.2 field, 7/9 field, 7/10 field, 7/12 field, 7/13 field, 7/14 field.

Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

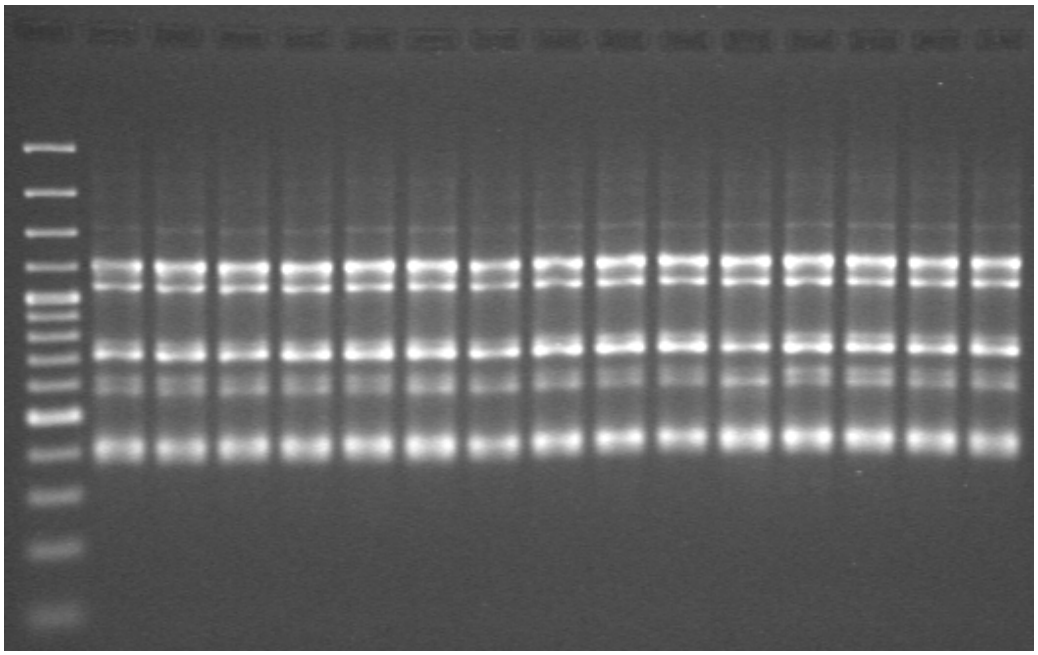

Figure 4-47: OPM 3 DNA products of samples used for *Borya mirabilis* RAPD population analysis prior to dilution. Lane 1-16: molecular weight marker, 2/2 field, 2/4 field, 2/14 field, 3/3 field, 5/1 field, 5/2 field, 5/3 field, 5/6 field, 5/7 field, 7/1 field, 7/4 field, 7/5 field, 7/8.2 field, 7/9 field, 7/10 field.

Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

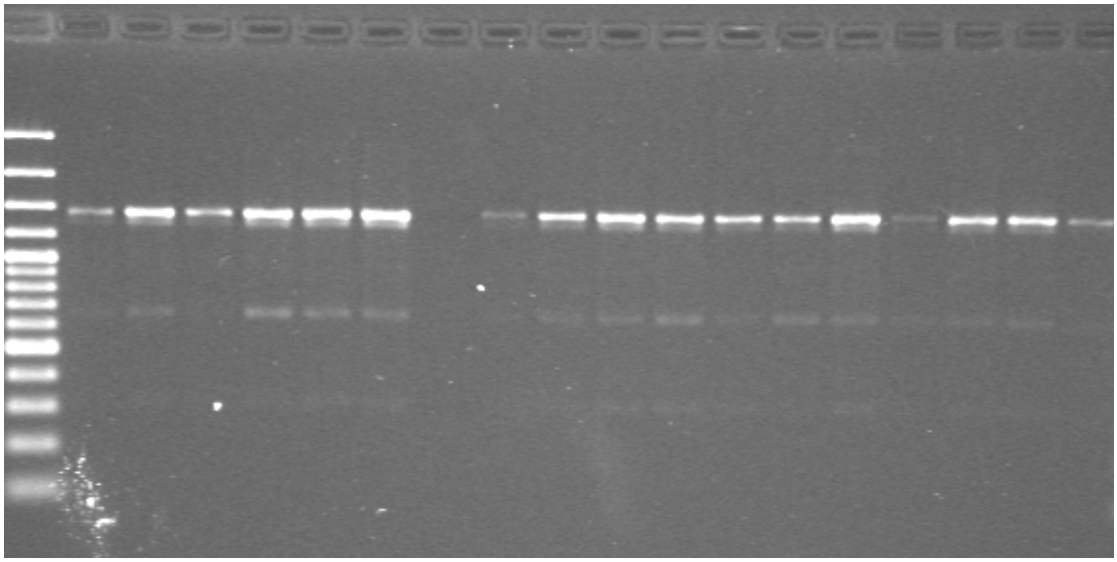

Figure 4-48: OPM 4 DNA products of samples used for *Borya mirabilis* RAPD population analysis prior to dilution. Lane 1-19: molecular weight marker, 2/2 field, 2/4 field, 2/14 field, 3/3 field, 5/1 field, 5/2 field, 5/3 field, 5/6 field, 5/7 field, 7/1 field, 7/4 field, 7/5 field, 7/8.2 field, 7/9 field, 7/10 field, 7/12 field, 7/13 field, 7/14 field.

Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

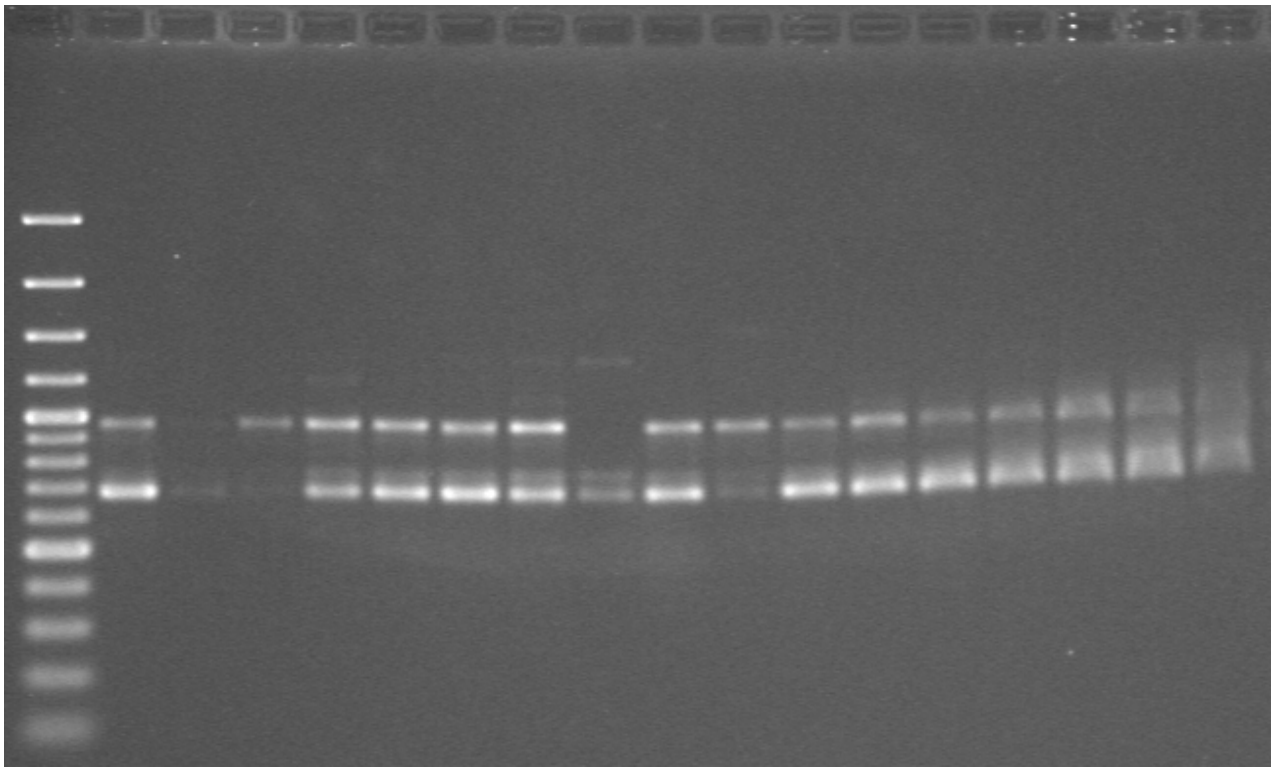

Figure 4-49: OPM 5 DNA products of samples used for *Borya mirabilis* RAPD population analysis prior to dilution. Lane 1-18: molecular weight marker, 2/2 field, 2/4 field, 2/14 field, 3/3 field, 5/1 field, 5/2 field, 5/3 field, 5/6 field, 5/7 field, 7/1 field, 7/4 field, 7/5 field, 7/8.2 field, 7/9 field, 7/10 field, 7/12 field, 7/13 field.

Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14

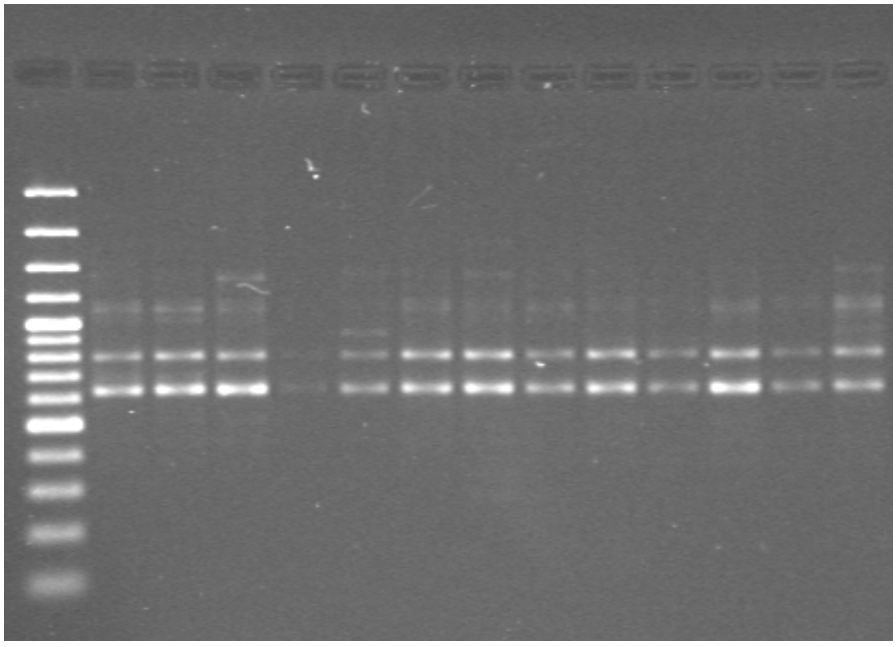

Figure 4-50: OPM 6 DNA products of samples used for *Borya mirabilis* RAPD population analysis prior to dilution. Lane 1-14: molecular weight marker, 2/2 field, 2/4 field, 2/14 field, 3/3 field, 5/1 field, 5/2 field, 5/3 field, 5/6 field, 5/7 field, 7/1 field, 7/4 field, 7/5 field, 7/8.2 field.

Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

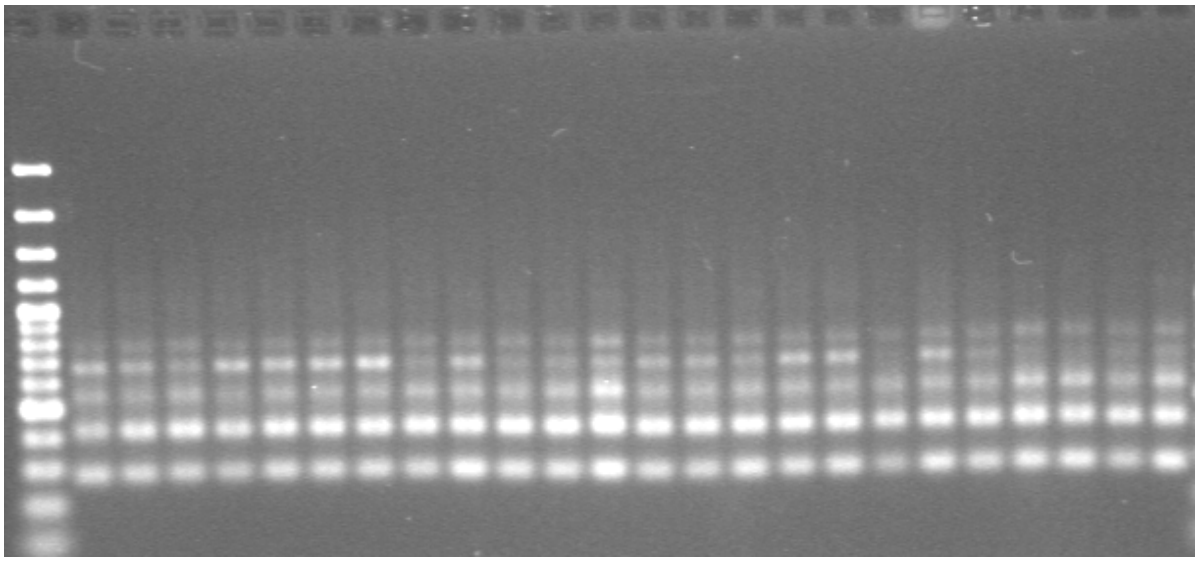

Figure 4-51: OPM 10 DNA products of samples used for *Borya mirabilis* RAPD population analysis prior to dilution. Lane 1-25: molecular weight marker, 2/2 field, 2/4 field, 2/14 field, 3/3 field, 5/1 field, 5/2 field, 5/3 field, 5/6 field, 5/7 field, 7/1 field, 7/4 field, 7/5 field, 7/8.2 field, 7/9 field, 7/10 field, 7/12 field, 7/13 field, 7/14 field, 7/15 field, 991064/001 RBG, 070685/009 RBG, 070685/008 RBG, 991061/001 RBG, 991057/001 RBG.

Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

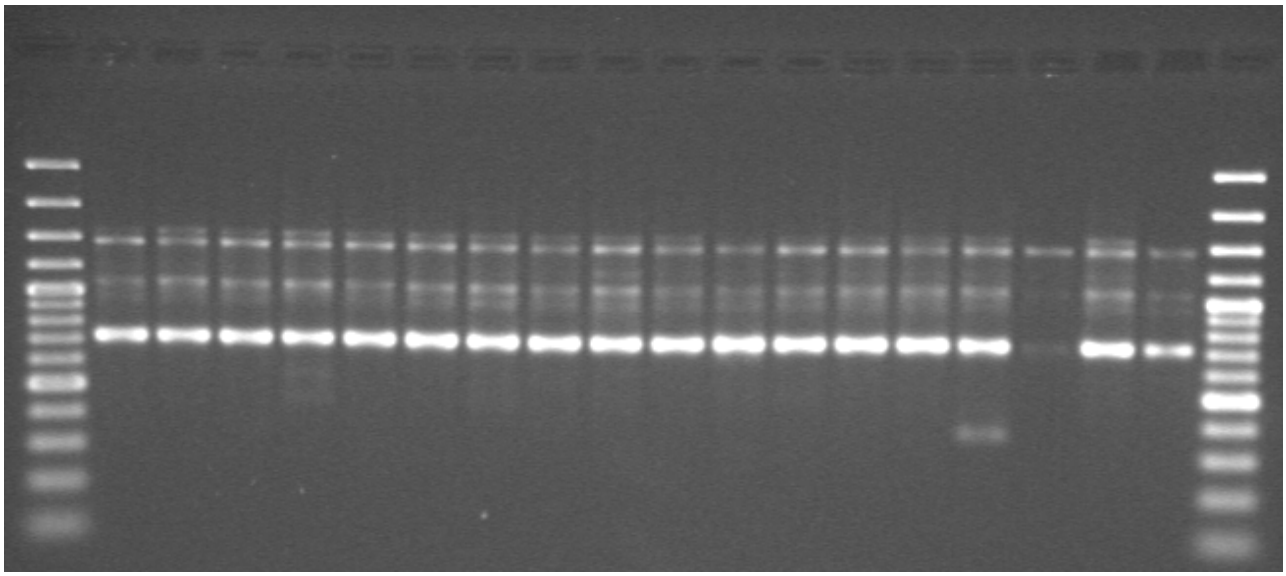

Figure 4-52: OPM 12 DNA products of samples used for *Borya mirabilis* RAPD population analysis prior to dilution. Lane 1-20: molecular weight marker, 2/2 field, 2/4 field, 2/14 field, 3/3 field, 5/1 field, 5/2 field, 5/3 field, 5/6 field, 5/7 field, 7/1 field, 7/4 field, 7/5 field, 7/8.2 field, 7/9 field, 7/10 field, 7/12 field, 7/13 field, 7/14 field, molecular weight marker.

Table 4-12: Nei's Unbiased Measures of Genetic Identity and Genetic distance (Nei, 1972), genetic identity above diagonal and genetic distance below diagonal.

Pop/ID	*Field 2/2*	*Field 2/4*	*Field 2/14*	*Field 3/3*	*Field 5/1*	*Field 5/2*	*Field 5/3*	*Field 5/6*	*Field 5/7*	*Field 7/1*	*Field 7/4*	*Field 7/5*
Field 2/2	****	0.9417	0.9612	0.9029	0.9223	0.9515	0.932	0.8932	0.9029	0.9029	0.9417	0.9417
Field 2/4	0.06	****	0.9806	0.9417	0.9806	0.9903	0.9709	0.9515	0.9612	0.9612	1	1
Field 2/14	0.0396	0.0196	****	0.9417	0.9612	0.9903	0.9709	0.932	0.9417	0.9417	0.9806	0.9806
Field 3/3	0.1021	0.06	0.06	****	0.9612	0.932	0.9515	0.9709	0.9029	0.9806	0.9417	0.9417
Field 5/1	0.0809	0.0196	0.0396	0.0396	****	0.9709	0.9709	0.9709	0.9417	0.9806	0.9806	0.9806
Field 5/2	0.0498	0.0098	0.0098	0.0704	0.0296	****	0.9612	0.9417	0.9515	0.9515	0.9903	0.9903
Field 5/3	0.0704	0.0296	0.0296	0.0498	0.0296	0.0396	****	0.9417	0.932	0.9515	0.9709	0.9709
Field 5/6	0.1129	0.0498	0.0704	0.0296	0.0296	0.06	0.06	****	0.9126	0.9903	0.9515	0.9515
Field 5/7	0.1021	0.0396	0.06	0.1021	0.06	0.0498	0.0704	0.0914	****	0.9223	0.9612	0.9612
Field 7/1	0.1021	0.0396	0.06	0.0196	0.0196	0.0498	0.0498	0.0098	0.0809	****	0.9612	0.9612
Field 7/4	0.06	0	0.0196	0.06	0.0196	0.0098	0.0296	0.0498	0.0396	0.0396	****	1
Field 7/5	0.06	0	0.0196	0.06	0.0196	0.0098	0.0296	0.0498	0.0396	0.0396	0	****
Field 7/8	0.0498	0.0098	0.0098	0.0498	0.0296	0.0196	0.0196	0.06	0.0498	0.0498	0.0098	0.0098
Field 7/9	0.1129	0.0704	0.0704	0.0098	0.0498	0.0809	0.06	0.0396	0.1129	0.0296	0.0704	0.0704
Field 7/10	0.06	0.0196	0.0196	0.06	0.0396	0.0296	0.0296	0.0704	0.06	0.06	0.0196	0.0196
Field 7/12	0.0498	0.0098	0.0098	0.0498	0.0296	0.0196	0.0196	0.06	0.0498	0.0498	0.0098	0.0098
Field 7/13	0.0914	0.0296	0.0498	0.0498	0.0498	0.0396	0.0396	0.0396	0.0704	0.0296	0.0296	0.0296
Field 7/14	0.0809	0.0396	0.0396	0.0396	0.0396	0.0296	0.0704	0.0296	0.0809	0.0196	0.0396	0.0396
Field 7/15	0.0498	0.0098	0.0098	0.0498	0.0296	0.0196	0.0196	0.06	0.0498	0.0498	0.0098	0.0098
070685/009	0.06	0	0.0196	0.06	0.0196	0.0098	0.0296	0.0498	0.0396	0.0396	0	0
070685/008	0.06	0	0.0196	0.06	0.0196	0.0098	0.0296	0.0498	0.0396	0.0396	0	0
991061/001	0.0809	0.0396	0.0396	0.0196	0.0396	0.0498	0.0498	0.0296	0.0809	0.0196	0.0396	0.0396
991057/001	0.0809	0.0396	0.0396	0.0196	0.0396	0.0498	0.0498	0.0296	0.0809	0.0196	0.0396	0.0396
070685/001	0.0809	0.0396	0.0396	0.0196	0.0396	0.0498	0.0296	0.0296	0.0809	0.0196	0.0396	0.0396
000893/003	0.06	0	0.0196	0.06	0.0196	0.0098	0.0296	0.0498	0.0396	0.0396	0	0
070685/007	0.06	0	0.0196	0.06	0.0196	0.0098	0.0296	0.0498	0.0396	0.0396	0	0
991064/001	0.06	0	0.0196	0.06	0.0196	0.0098	0.0296	0.0498	0.0396	0.0396	0	0

Table 4-13: Nei's Unbiased Measures of Genetic Identity and Genetic distance (Nei, 1972) continued, genetic identity above diagonal and genetic distance below diagonal.

`Pop/ID	Field 7/8	Field 7/9	Field 7/10	Field 7/12	Field 7/13	Field 7/14	Field 7/15	)70685/009	)70685/008	)91061/001	)91057/001	)70685/001	)00893/003	)70685/007	)91064/001
Field 2/2	0.9515	0.8932	0.9417	0.9515	0.9126	0.9223	0.9515	0.9417	0.9417	0.9223	0.9223	0.9223	0.9417	0.9417	0.9417
Field 2/4	0.9903	0.932	0.9806	0.9903	0.9709	0.9612	0.9903	1	1	0.9612	0.9612	0.9612	1	1	1
Field 2/14	0.9903	0.932	0.9806	0.9903	0.9515	0.9612	0.9903	0.9806	0.9806	0.9612	0.9612	0.9612	0.9806	0.9806	0.9806
Field 3/3	0.9515	0.9903	0.9417	0.9515	0.9515	0.9612	0.9515	0.9417	0.9417	0.9806	0.9806	0.9806	0.9417	0.9417	0.9417
Field 5/1	0.9709	0.9515	0.9612	0.9709	0.9515	0.9612	0.9709	0.9806	0.9806	0.9612	0.9612	0.9612	0.9806	0.9806	0.9806
Field 5/2	0.9806	0.9223	0.9709	0.9806	0.9612	0.9709	0.9806	0.9903	0.9903	0.9515	0.9515	0.9515	0.9903	0.9903	0.9903
Field 5/3	0.9806	0.9417	0.9709	0.9806	0.9612	0.932	0.9806	0.9709	0.9709	0.9515	0.9515	0.9709	0.9709	0.9709	0.9709
Field 5/6	0.9417	0.9612	0.932	0.9417	0.9612	0.9709	0.9417	0.9515	0.9515	0.9709	0.9709	0.9709	0.9515	0.9515	0.9515
Field 5/7	0.9515	0.8932	0.9417	0.9515	0.932	0.9223	0.9515	0.9612	0.9612	0.9223	0.9223	0.9223	0.9612	0.9612	0.9612
Field 7/1	0.9515	0.9709	0.9417	0.9515	0.9709	0.9806	0.9515	0.9612	0.9612	0.9806	0.9806	0.9806	0.9612	0.9612	0.9612
Field 7/4	0.9903	0.932	0.9806	0.9903	0.9709	0.9612	0.9903	1	1	0.9612	0.9612	0.9612	1	1	1
Field 7/5	0.9903	0.932	0.9806	0.9903	0.9709	0.9612	0.9903	1	1	0.9612	0.9612	0.9612	1	1	1
Field 7/8	****	0.9417	0.9903	1	0.9612	0.9515	1	0.9903	0.9903	0.9709	0.9709	0.9709	0.9903	0.9903	0.9903
Field 7/9	0.06	****	0.9515	0.9417	0.9417	0.9515	0.9417	0.932	0.932	0.9709	0.9709	0.9709	0.932	0.932	0.932
Field 7/10	0.0098	0.0498	****	0.9903	0.9515	0.9417	0.9903	0.9806	0.9806	0.9612	0.9612	0.9612	0.9806	0.9806	0.9806
Field 7/12	0	0.06	0.0098	****	0.9612	0.9515	1	0.9903	0.9903	0.9709	0.9709	0.9709	0.9903	0.9903	0.9903
Field 7/13	0.0396	0.06	0.0498	0.0396	****	0.9709	0.9612	0.9709	0.9709	0.9709	0.9709	0.9709	0.9709	0.9709	0.9709
Field 7/14	0.0498	0.0498	0.06	0.0498	0.0296	****	0.9515	0.9612	0.9612	0.9806	0.9806	0.9612	0.9612	0.9612	
Field 7/15	0	0.06	0.0098	0	0.0396	0.0498	****	0.9903	0.9903	0.9709	0.9709	0.9709	0.9903	0.9903	)91064/001
070685/009	0.0098	0.0704	0.0196	0.0098	0.0296	0.0396	0.0098	****	1	0.9612	0.9612	0.9612	1	1	0.9612
070685/008	0.0098	0.0704	0.0196	0.0098	0.0296	0.0396	0.0098	0	****	0.9612	0.9612	0.9612	1	1	0.9903
991061/001	0.0296	0.0296	0.0396	0.0296	0.0296	0.0196	0.0296	0.0396	0.0396	****	1	0.9806	0.9612	0.9612	1
991057/001	0.0296	0.0296	0.0396	0.0296	0.0296	0.0196	0.0296	0.0396	0.0396	0	****	0.9806	0.9612	0.9612	1
070685/001	0.0296	0.0296	0.0396	0.0296	0.0296	0.0396	0.0296	0.0396	0.0396	0.0196	0.0196	****	0.9612	0.9612	0.9612
000893/003	0.0098	0.0704	0.0196	0.0098	0.0296	0.0396	0.0098	0	0	0.0396	0.0396	0.0396	****	1	0.9612
070685/007	0.0098	0.0704	0.0196	0.0098	0.0296	0.0396	0.0098	0	0	0.0396	0.0396	0.0396	0	****	0.9612
991064/001	0.0098	0.0704	0.0196	0.0098	0.0296	0.0396	0.0098	0	0	0.0396	0.0396	0.0396	0	0	1

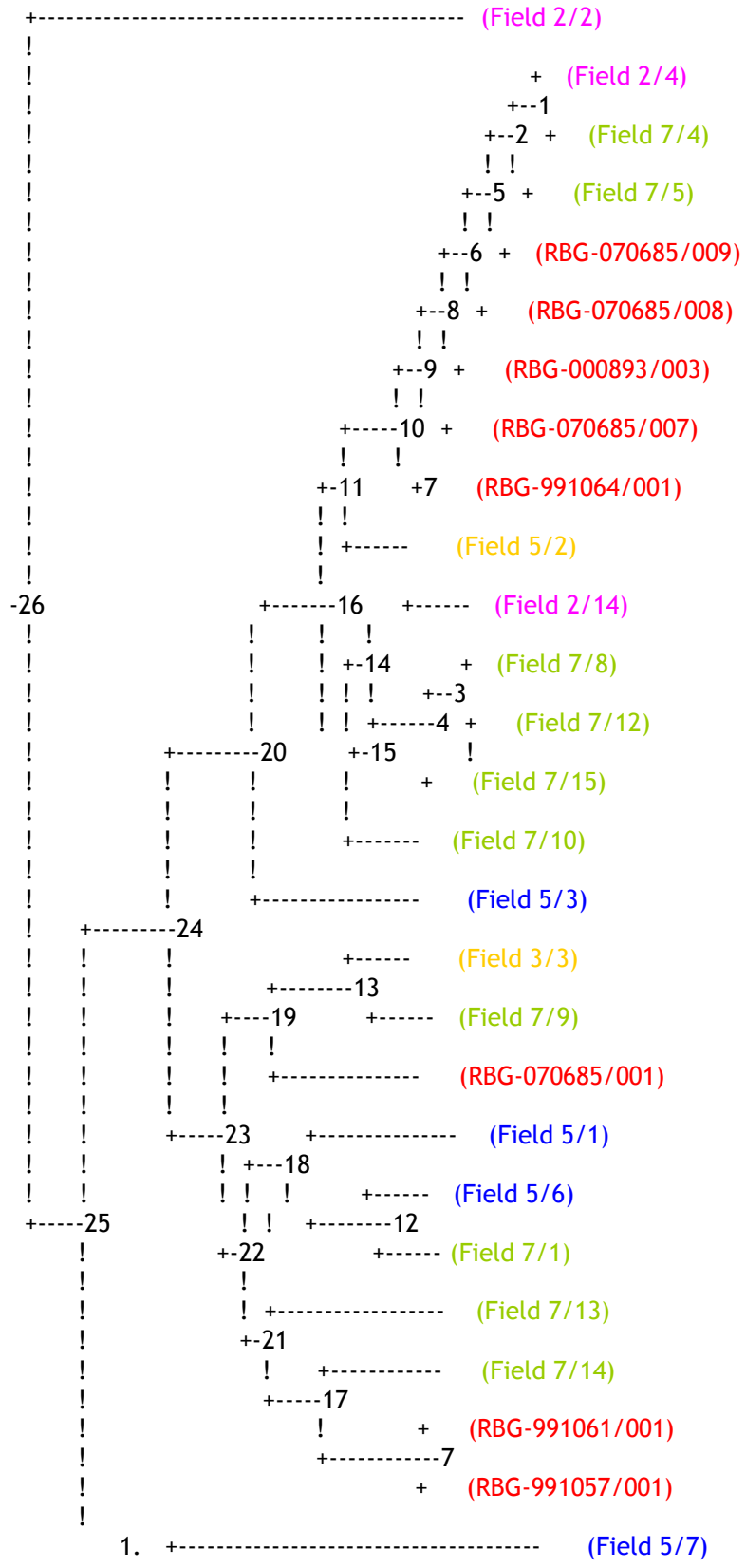

Figure 4-53: Dendrogram based on Nei's (1978) genetic distance using UPGMA, modified from NEIGHBOR procedure of PHYLIP (Version 3.5) for *Borya mirabilis ex-situ* Population and *in-situ* population with each of the 19 *in-situ* samples (Field Colony/plant) and 8 *ex-situ* samples (RBG – Accession Number) analysed as an individual using Popgen32 analysis for dominant markers. Numbers refer to order of amalgamation.

1972), genetic identity above diagonal and genetic distance below diagonal.

Samples	*Colony 2*	*Colony 3*	*Colony 5*	*Colony 7*	*070685/009*	*070685/008*	*991061/001*	*991057/001*	*070685/001*	*000893/003*	*070685/007*	*991064/001*
Colony 2	****	0.9309	0.9735	0.9806	0.9739	0.9739	0.9506	0.9506	0.9506	0.9739	0.9739	0.9739
Colony 3	0.0716	****	0.9513	0.966	0.9417	0.9417	0.9806	0.9806	0.9806	0.9417	0.9417	0.9417
Colony 5	0.0268	0.0499	****	0.9906	0.986	0.986	0.9639	0.9639	0.9667	0.986	0.986	0.986
Colony 7	0.0196	0.0346	0.0095	****	0.9907	0.9907	0.9774	0.9774	0.9762	0.9907	0.9907	0.9907
070685/009	0.0265	0.06	0.014	0.0093	****	1	0.9612	0.9612	0.9612	1	1	1
070685/008	0.0265	0.06	0.014	0.0093	0	****	0.9612	0.9612	0.9612	1	1	1
991061/001	0.0507	0.0196	0.0368	0.0229	0.0396	0.0396	****	1	0.9806	0.9612	0.9612	0.9612
991057/001	0.0507	0.0196	0.0368	0.0229	0.0396	0.0396	0	****	0.9806	0.9612	0.9612	0.9612
070685/001	0.0507	0.0196	0.0339	0.0241	0.0396	0.0396	0.0196	0.0196	****	0.9612	0.9612	0.9612
000893/003	0.0265	0.06	0.014	0.0093	0	0	0.0396	0.0396	0.0396	****	1	1
070685/007	0.0265	0.06	0.014	0.0093	0	0	0.0396	0.0396	0.0396	0	****	1
991064/001	0.0265	0.06	0.014	0.0093	0	0	0.0396	0.0396	0.0396	0	0	****

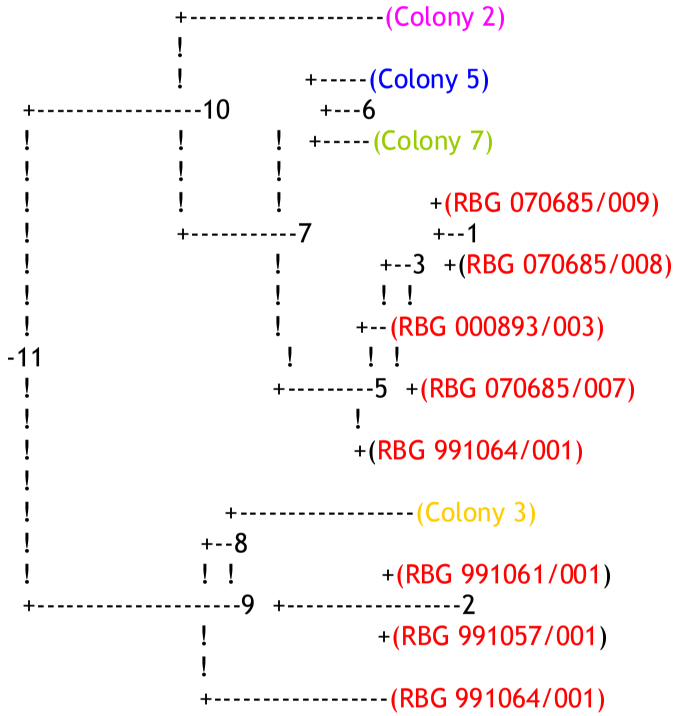

Figure 4-54: Dendrogram based on Nei's (1978) genetic distance method using UPGMA, modified from NEIGHBOR procedure of PHYLIP (Version 3.5) of *Borya mirabilis ex-situ* and *in-situ* populations with each of the 19 field samples (*in-situ*) grouped into their four field colonies and analysed as four separate 'populations' (Colony number) and the 8 *ex-situ* (RBG accession number) plant samples analysed as individuals using Popgen32 analysis for dominant markers. Numbers in dendogram refer to order of amalgamation.

4.4. Discussion

The major achievement of this chapter has been to confirm *Borya mirabilis* as a separate species amongst the Boryaceae. Analysis of the DNA, both chloroplast (maternal) and ITS (parental) has built on the knowledge gained by chromosomal analysis in the previous chapter and suggests that the polyploid nature of *B. mirabilis* has possibly arisen through the ancestral hybridization of *B. constricta* (as the maternal parent) and *B. sphaerocephala* (as the paternal parent). *B. constricta* and *B. sphaerocephela* are the closest related species amongst the Boryaceae to *B. mirabilis*. One of the major achievements of this chapter with immediate practical implications for the management of *B. mirabilis* is the discovery of genetic variability in the field population of *B. mirabilis,* albeit at a low level. Molecular analysis has also shown that the *ex-situ* collection is likely to be mostly derived from Colony 7. Other colonies are under represented in the *ex-situ* collection and consequently the translocation site.

4.4.1. Interspecies relationships in the Boryaceae: analysis of chloroplast and nuclear DNA

4.4.1.1. Chloroplast vs. ITS DNA analysis

The genomic composition of *B. mirabilis* was closer to that of *B. sphaerocephala,* while the composition of the chloroplast DNA suggested that *B. mirabilis* was closest to *B. constricta*. Genomic sequences compared 727 positions from only six samples due to difficulties in amplifying and obtaining sequences that were not fungal. Out of 727 positions only 35 were parsimony-informative. The combined chloroplast regions compared 1772 positions, out of which 303 were parsimony-informative, using representatives from the majority of the Boryaceae, and therefore provided a stronger and more complete analysis. The chloroplast regions had more phylogenetically informative indels than the ITS region, which is traditionally used to look at differences between species, in this case four times greater.

4.4.1.2. Chloroplast evidence- closest relative to *B. mirabilis*

Chloroplast sequences from *B. constricta* constantly grouped with those from *B. mirabilis* both in the MPA and UPGMA analysis in all three chloroplast regions examined. *B. constricta* is therefore the closest maternal relative to *B. mirabilis*. *B. mirabilis* has its greatest affinity with the south Western Australian species. This suggests that *B. mirabilis* may have shared a common ancestor with those species from the south-west corner of Western Australia rather than with species from New South Wales, Queensland or the Northern Territory.

The closest relative of *B. mirabilis* is unlikely to be *B. nitida*, as *B. nitida* consistently grouped distantly from *B. mirabilis*. Morphologically there is very little difference between *B. mirabilis* and *B. nitida*. The only physical difference is the length of the bracteoles. On *B. mirabilis* they are longer than the floral bracts, whereas on *B. nitida* they are not. *B. mirabilis* and *B. nitida* were thought to be the same species until separated by Churchill (1987). This molecular evidence confirms they are separate species and that their similar morphology is a case of convergent evolution.

The reconstructed phylogeny of the Boryaceae does not strongly correlate with geographical distance between species. It also suggests that a common ancestor of the *Borya* spp. may have been widespread and that a combination of geographic isolation in the past by water, and islandisation through intense farming practices in the last 200 years, has led to *B. mirabilis* forming a distinct and reproductively repressed species. Phylogenetic analysis largely separates the northern Australian species, *B. jabirabela*, *B. stenophylla* and *B. inopinata*, while *B. laciniata,* a south-western re-sprouting species, comes out amongst the northern Australian species.

B. stenophylla is supported as a separate species of *Borya* from *B. jabirabela* in both the individual and combined chloroplast regions using both methods of analysis. This strongly supports the recent naming of the new species.

There appears to be some evidence for the disjunction within *B. constricta*. Of the many *B. constricta* samples analysed, some did not consistently group together. The samples that often appear to group differently are also slightly different morphologically, yet still fall within the broad classification of *B. constricta* outlined in the flora of Australia. There were two distinct forms of *B. constricta* used in the analysis. Those from Newdegate were smaller and did not fit into the size descriptions used for the species in the Flora of Australia (Churchill, 1987). This particular area around Newdegate has several different *Borya* species growing together, including *B. sp.* 'Wheatbelt', *B. constricta* and *B. sphaerocephala* and there is therefore the potential for hybridization.

The placement of the Boryaceae within the Asparagales remains unresolved. Based on the information from the TrnL intron (the other regions require sequence information for more families within the Asparagales), the closest sister taxa to the majority of the Boryaceae are the *Tecophiaea*, *Doryanthes* and *Blandfordia*.

Methodology

The differences between the results of the analysis of the different methods (UPGMA and Maximum Parsimony) can be explained by the methods that they use to group the data. Each method used here may be equally valid and the differences between the two sets of data may be explained by the algorithms used in their analysis.

Combining the analysis of the data of the three chloroplast regions provided greater resolution than any one data s*et al*one as it differentiated between individual species and grouped individuals of the same species together. This approach has also been successful in studies, of other species including *Diuris* (Smith, 2006), *Genista* (Pardo *et al*. 2004), *Microseris douglasii* (Roelofs and Bachmann, 1997) and within the monocotyledons generally (Tamura *et al*. 2004).

ITS evidence

The molecular evidence from the ITS genomic DNA region differs from the chloroplast region, which places *B. constricta* as the closest relative to *B. mirabilis* (and does not resolve *B. mirabilis* as a unique species), whereas the ITS region places *B. sphaerocephala* as the closest relative f *B. mirabilis*. This can be explained by the separate inheritance from the two regions. The chloroplast region only tracks the relationships among *Borya* spp. on the maternal line, whereas the ITS region tracks both parental lines. There are two possible explanations for the incongruence between the two data sets, as mentioned in the introduction. The first is division of an ancestor that contained many chloroplast types (Riesberg and Wendel, 1993; Wendel *et al*. 1994) and the second and probably most likely explanation is hybridization followed by the exclusion of one maternal genome (Soltis and Kuzoff, 1995).

B. mirabilis possibly arose as an ancestral hybrid from *B. constricta* and *B. sphaerocephala*, *B. constricta* being the ovular parent and *B. sphaerocephala* being the pollen parent**.** The exceptionally low fertility investigated in the previous chapter in theory may have been overcome by hybridizing to introduce sexual vigor back into *B. mirabilis*. If this was to be pursued, *B. constricta* and *B. sphaerocephala* would be the logical choices as being the possible progenitors of *B. mirabilis*. The polyploid nature of *B. mirabilis* however, would render the compatibility of *B. mirabilis* with either species extremely unlikely, as indeed cross-pollination failed between these two species (Chapter 3). Reducing chromosome numbers to produce haploid versions of *B. mirabilis* from pollen or ovule cultures might result in successful pollination.

The preferential amplification of fungal DNA by ITS1-4 primers indicates that contamination of *Borya* leaves with fungi from the environment is common, which is not surprising as the plants are poikilochlorophyllous and leaves can re-green and in some cases be several years old. Leaves which have re-hydrated and dehydrated may have been kept on the individual plants for many years, thereby increasing the chance of the leaves becoming

contaminated. Using young leaves from non-dehydrated plants may reduce the problem of fungal contamination.

4.4.2. *Borya mirabilis* population analysis

The low genetic variation detected amongst *B. mirabilis* (less than 10%) in the field supports the two previous studies undertaken on the species (Coates *et al*. 2002; Y. Fripp and S. Cropper, La Trobe University unpublished). The small differences that have been detected are probably due to the increased sampling and increased number of primers used in this study. Unfortunately the genetic variation between Colony 1 and the other four colonies, as described by Fripp and Cropper, could not be investigated as the fires of 2005 destroyed Colony 1, located 50 m away from the other colonies, and it has not recovered.

Importantly, a small amount of genetic variation has been detected both between colonies (Colony 3 and all other colonies) and amongst plants within a colony (Plant 2 Colony 2 and Plant 5 Colony 7 differing from other plants within their colonies). This corroborates with the ability to produce seed in the previous chapter (between Colony 5 and 7) and if the genetics of the population is not represented in future translocation sites, this may impact on the potential of these sites to produce seed in the future. The knowledge acquired in the research in this chapter therefore should influence the way in which the *ex-situ* population and the translocation of this species is handled. Currently the *ex-situ* population is not labelled as to the source of the material in the wild, although it was in the past, and there has been no attempt to reproduce the diversity that is seen in the original population in the current translocation site, with almost all of the *ex-situ* collection resembling Colony 7. Parks Victoria Management should keep track of any further cuttings taken from the wild population and attempt to reproduce the genetic variation of this population in the translocation site. The *ex-situ* collection at the RBG should be augmented over time to reflect more adequately the diversity in the field, as this could have long lasting consequences on the ability of any translocated populations to evolve.

4.4.3. Conclusion

The analysis of chloroplast regions and the nuclear regions of genomic DNA inferred that the closest relatives to the endangered *B. mirabilis* are *B. sphaerocephala* (nuclear) and *B. constricta* (chloroplast) and not *B. nitida, of* which *B. mirabilis* was originally thought to be a disjunctive population. Due to the failure of cross pollinations between *B. constricta* and *B. sphaerocephala* in the previous chapter, probably due to large differences in chromosome number, the best and possibly only possibility for producing large numbers of plants for reintroduction is through tissue culture, which is explored in the following chapter.

The population analysis of *B. mirabilis* found only small differences between colonies after screening 60 RAPD primers and analyzing 30. Importantly, all plants within a colony were not genetically identical and there were differences between plants within a colony, as well as between colonies. These genetic differences in the field population are currently not represented in the RBG population and therefore potential translocation sites: this situation needs to be addressed urgently to avoid hindering evolutionary development of the species (Madsen *et al*. 1996).

5. Tissue Culture

5.1. Introduction

Active management is one of the management options available to threatened species that have only a small number of remaining plants or populations and that may not benefit sufficiently through habitat improvement or protection. One of the more high-risk active management options is augmentation of the existing population or the establishment of new populations through translocation. Underpinning the success of these options is the establishment of appropriate numbers of plants in an *ex-situ* population. This can be achieved in a number of ways: from seed where available, through cuttings of the plants for vegetative propagation or through tissue culture. Any of these interventions has the potential, if inappropriately implemented, to affect the genetic structure and hence the evolution of the species (Cropper, 1993).

B. mirabilis has little capacity to reproduce by seed. There has been no obvious vegetative regeneration in the field. The plants have so far been propagated by conventional shoot tip cuttings at the Royal Botanic Gardens in Melbourne, but this is an extremely slow process due to the naturally slow growth rates of these plants and the fastidious nature of the cuttings in nursery culture. The *ex-situ* population currently stands at approximately 20 plants.

A Recovery Plan has been prepared for *B. mirabilis* and the slow process of translocation via cutting propagation has been underway for the last 3 years, with two small-scale translocations at the same site successful so far. Previously tissue culture has been attempted on buds of *B. mirabilis* (private communication, RBG and Vitrotec); however, both attempts proved unsuccessful due to inadequate tissue amounts and contamination.

Full implementation of the Recovery Plan requires effective *ex-situ* propagation techniques to be developed. The research presented here used

the closely related species *B. nitida* as a model for screening effective media for the propagation of *B. mirabilis*, due to the scarcity of *B. mirabilis* material for explants.

Tissue culture is an excellent means to reproduce many plants of a species. This is of particular interest in extremely slow-growing plants such as CAM plants (Malda *et al*. 1999) and, in this case, *Borya* spp. The genus *Borya* has not been successfully cultured before; therefore fundamental questions needed to be investigated before a successful culture technique and medium can be developed, namely:

- What explant is suitable for regeneration?
- What is the best method of surface-sterilising the tissue before culture?
- What levels of nutrients are required to sustain and produce healthy explants (the main categories of interest here include macronutrients, micronutrients, growth factors and hormones)?
- What preference does the explant have for gelling agent: agar or Phytagel™?

Other resurrection plants have been tissue cultured successfully. As physiologically these plants have similarities to *Borya*, aspects of their tissue culture are of interest. Culturing has been successful in *Ramonda myconi* (Toth *et al*. 2004) and *Craterostigma plantagineum* (Toldi *et al*. 2002), both desiccation-tolerant plants. Although the strength of base nutrients in each was vastly different, some similarities existed. Picloram was good for induction of callus and significant increases in explant survival were obtained when glutathione was added to the medium and then the medium was stabilised with K-phosphate buffer; this was attributed to the long incubation periods between sub-culturing required for these plants (Toth *et al*. 2004). Activated charcoal is often used to prevent hyperhydricity, and improve growth. Mostly it absorbs inhibitory substances such as phenolics that are produced by the explant during culture (Theander and Nelson, 1988; Teixeira *et al*. 1994).

This is not the first attempt to cultivate endangered plant species through tissue culture. Successful productions of endangered species in tissue culture include: *Mammillaria sanangelensis* (Rubluo *et al*. 1993), *Leuchtenbergia principis* (Starling, 1985), *Aztekium ritteri* (Rodriguez *et al*. 2000), *Obregonia denegrii*, *Valeriana walllichii* (Banerjee *et al*. 1998) and *Coryphantha minima* (Malda *et al*. 1999) as well as many endangered orchid species (Dixon, 1994; Fay,1992).

5.1.1. Aim

The aim of this research was therefore to provide a means for relatively fast mass production of *Borya* species.

5.2. Methods

5.2.1. General method

After a preliminary attempt to micropropagate *B. mirabilis* in culture, research focused on micropropagation of shoot tips of *B. nitida* (a common *Borya* species) in solidified media. Using *B. nitida* enabled systematic, replicated screening of many media, a process which required large numbers of explants that were not available from *B. mirabilis*. As *B. nitida* was also thought at the time to be the closest taxonomically to *B. mirabilis*, it seemed a good model to use.

5.2.2. Plant material

Whole bare-rooted *B. nitida* specimens were obtained from near Esperance in Western Australia and potted in a mixture of 3 parts perlite: 1 part Seymour grit: 1 part peatmoss and watered thoroughly with Seasol® (Seasol International Pty Ltd, Bayswater, Victoria, Australia). Initially the plants were revived under spray misting in a shaded area of a glasshouse with a temperature of 15-25°C and natural lighting, later being moved to a non-misted area and watered twice weekly. Six months later, material was

taken for micro-propagation. All shoot-tip explants used for tissue culture of *B. mirabilis* were taken from mature plants of *B. mirabilis* from the *ex-situ* population at the Royal Botanic Gardens Melbourne.

5.2.3. Explants and surface-sterilization

For each of the experiments outlined in this chapter unless stated otherwise. The explants consisted of shoot tips (approximately 1-2 cm long) of *B. nitida* (Section 5.2.6-Section 5.2.7), removed from glasshouse-grown Western Australia specimens, cross sections of stems and flower buds of *B. nitida* (Section 5.2.7) and flower buds (Section 5.2.7) and shoot tips (Section 5.2.4) of *B. mirabilis* plants the latter kept at the Botanic Gardens, Melbourne. All explants were washed in a sieve for 10 minutes under running tap water. The tissue was shaken for 10 minutes in 1% Tween 20 and then drained and rinsed over a sieve with distilled water until no froth remained. The tissue was surface-sterilized for 1 minute in 70% ethanol, rinsed with sterile distilled water, shaken for a further 10 minutes in 1.56% NaOCl and washed in three changes of sterile distilled water in a laminar flow cabinet. The tissue was aseptically plated out cut end down, into test-tubes of the various media. Explants from different specimens were randomised across treatments unless stated otherwise.

5.2.4. *Borya mirabilis* trial

Shoot tips and unopened buds of *B. mirabilis* (obtained from the *ex-situ* population) were tested in liquid Murashigee and Skoog (MS), (1962) media with various supplements (Table 5-1). The trial took place in 2005 and was abandoned in favour of use of *B. nitida* (the common species) in a more statistically sound reproducible situation. Containers used were 2 L flasks joined to a pump which provided aeration of the medium (Figure 5-1). The explants were kept at 25°C with a 12 h photoperiod at 270 µmol m^{-2} s^{-1}.

Table 5-1: *Borya mirabilis* trial, media hormones and explants.

Media	*Hormones*	*Explant Tissue*
MS 20 g/L sucrose	0.2 mg/L IAA, 2.0 mg/L BA	One shoot of *B. mirabilis*
MS 40 g/L sucrose	0.2 mg/L IAA, 2.0 mg/L BA	One shoot *B. mirabilis*
MS 40 g/L sucrose	0.2 mg/L IAA, 0.2 mg/L BA	One shoot of *B. mirabilis*
MS with 50 mL coconut milk 40 g/L sucrose	0.2 mg/L IAA, 2.0mg/L BA	One shoot of *B. mirabilis*
MS 50 mL coconut milk 40 g/L sucrose	0.2 mg/L IAA, 2.0 mg/L BA	One shoot of *B. mirabilis*
MS 1% activated charcoal 40 g/L sucrose	0.2 mg/L IAA, 2.0 mg/L BA	Three flower buds of *B. mirabilis*
MS 40 g/L sucrose	0.2 mg/L IAA, 2.0 mg/L BA	Three flower buds of *B. mirabilis*
MS with 100 mL coconut milk 40 g/L sucrose	0.2 mg/L IAA, 2.0 mg/L BA	Three flower buds of *B. mirabilis*
MS 40 g/L sucrose	0.3 mg/L IAA, 2.0 mg/L BA	Three shoots of *B. mirabilis*
MS 40 g/L sucrose	0.3 mg/L IAA, 2.0 mg/L BA	Three shoots of *B. mirabilis*

Figure 5-1: Liquid tissue culture of aerated glass jars, joined by sterilised tubing which provides sterile air through micropore filters.

5.2.5. *Borya nitida* trial

B. nitida leaves were tested as suitable explants, with leaf sections (tip; middle; base) and whole leaves being used. Each of these explants was tested on MS medium or MS medium with the addition of glutathione. Each of these media was present in either glass or plastic Petri dishes. Ten replicates of each combination were scored for percentage of green leaf remaining after 2 months.

5.2.6. Control of contamination and gelling agent

5.2.6.1. PPM

Thirty 1-2 cm shoot tips were harvested from *B. nitida*, placed into 500 mL of sterile water containing 2 mL of Plant Preservative Mixture (PPM)(Plant Preservative Mixture, Austratec, Kilsyth, Victoria, Australia) and shaken at ambient temperature for 1 week prior to washing and surface-sterilising as described above. Another 30 shoot tips were treated exactly the same but

without 1 week on the rotator with PPM. The plants were placed in test-tubes 150 mm x 25 mm (Austratec, Kilsyth, Australia) in the optimal medium determined below (LMHM) and assigned randomised places on racks. The tissue was scored visually for the presence or absence of contamination at 6 weeks. The PPM protocol was used in all subsequent treatments.

5.2.6.2. Agar versus Phytagel

Sixty 1-2 cm shoot tips were harvested from *B. nitida*. The plants were placed in test-tubes in the optimal medium determined below (LMHM), half using agar as a setting agent and half using Phytagel (Sigma), and assigned randomized places on racks. The tissue was scored visually for the presence or absence of contamination at 6 weeks. Phytagel was used as the setting agent in all subsequent experiments.

5.2.7. Medium optimization

The de Fossard (1976) matrix of 81 media, replicated four times, was used to screen shoot tip (*B. nitida*) and flower bud (*B. mirabilis*) explants. Each of the four factors: 1. Minerals, 2. Auxins, 3. Cytokinins and 4. Growth factors, amino acids and sucrose (referred to as sucrose + growth factors), was used at three concentrations (low, medium and high), giving a matrix of 81 media, concentrations of which are outlined in Table 5-3 to 5-7. The original Auxin component was altered to include only IAA (indole acetic acid), IBA (indole butyric acid) and NAA (naphthalene acetic acid) at equivalent concentrations (Table 5-5). For *B. nitida* shoot tips, there were no other additions. For *B. nitida* and *B. mirabilis* flower buds and stem sections, 150 ml/l of coconut milk was added.

Due to the substantial amount of tissue required, shoot tips were taken on two occasions, allowing 2 months for the stock plants to recover and produce new explant material; these are referred to as Date 1 and Date 2 in the analysis. The explants were randomised over treatments on each date and survivors were subcultured at 6 weeks.

The explants were placed in 20 mL of medium with Phytagel (Sigma, Sydney Australia) in glass test-tubes and randomised for location in racks. The explants were kept at 25°C with a 12 h photoperiod at 270 µmol m^{-2} s^{-1}. Plant growth and health were described according to the categories in Table 5-2 prior to subculturing and at 6 and 12 weeks.

Table 5-2: Health rating for *Borya* micropropagation.

Score	*Health*
5	Tissue predominantly green, new growth
4	Tissue predominately green
3	New growth in centre, majority growth brown
2	Occasional leaf green predominantly brown
1	Brown/ black dead tissue

Table 5-3: Concentrations of minerals in the broad spectrum experiment media (de Fossard, 1976).

Constituents	*Concentration Range*		
	Low	**Medium**	**High**
Macronutrients elements (mM)			
NH_4NO_3	5	10	20
KNO_3	-	10	20
KH_2PO_4	0.1	-	-
NaH_2PO_4	-	1	2
KCl	1.9	-	-
$CaCl_2$	1	2	3
$MgSO_4$	0.5	1.5	3
Micronutrient elements (µM)			
H_3BO_3	10	50	150
$MnSO_4$	10	50	100
$ZnSO_4$	1	20	40
$CuSO_4$	0.01	0.1	1.5
Na_2MoO_4	0.01	0.1	1.0
$CoCl_2$	0.1	0.5	1.0
KI	0.5	2.5	5.0
$FeSO_4$	10	50	100
Na_2EDTA	10	50	100
Na_2SO_4	40	450	650

Table 5-4: Balance sheet for mineral ions in the broad spectrum media (de Fossard, 1976).

Ions	*Concentration Range*		
	Low	**Medium**	**High**
(mM)			
NO_3^-	5	20	40
NH_4^+	5	10	20
Total N	10	30	60
PO_4^{3-}	0.1	1	2
K^+	2.0	10.0	20.0
Ca^{2+}	1	2	3
Mg^{2+}	0.5	1.5	3
Cl^-	3.9	4.001	6.0
(µM)			
Fe^{2+}	10	50	100
SO_4^{2-}	561.	2,070	3,891
Na^+	100	2,000	3,502
B^+	10	50	150
Mn^{2+}	10	50	100
Zn^{2+}	1	20	40
Cu^{2+}	0.01	0.1	1.5
Mo^+	0.01	0.1	1.0
Co^{2+}	0.1	0.5	1.0
I^-	0.5	2.5	5.0

Table 5-5: Constituents of the Auxin component of the broad spectrum media (de Fossard, 1976).

Constituents	*Concentration (µM)*		
	Low	**Medium**	**High**
IAA	0.1	1	10
IBA	0.1	1	10
NAA	0.1	1	10

Table 5-6: Constituents of the Cytokinin component of the broad spectrum media (de Fossard, 1976).

Constituents	*Concentration (µM)*		
	Low	**Medium**	**High**
Kinetin	0.1	1	10
BAP	0.1	1	10

Table 5-7: Constituents of the sucrose plus growth factors (plus amino acids) component of the broad spectrum media (de Fossard, 1976).

Constituents	*Concentration*		
	Low	**Medium**	**High**
Carbon source (mM)			
Sucrose	6	60	120
Growth factors (µM)			
Inositol	100	300	600
Nicotinic acid	4	20	40
Pyridoxine HCl	0.6	3	6
Thiamine HCl	0.1	2	40
Biotin	0.04	0.2	1
D-Ca-Pantothenate	0.2	1	5
Riboflavin	0.1	1	10
Ascorbic acid	0.1	1	10
Choline chloride	0.1	1	10
Amino acids (µM)			
L-cysteine. HCl	10	60	120
Glycine	0.5	5	50

5.2.8. Effect of plant source

To examine if growth differences were due to the plant from which the explant was sourced, ten shoot tips were taken from five different plants of *B. nitida*, pre-treated as above and placed onto the optimum medium LMHM. They were randomised and scored as before except that the explants were subcultured at 6 weeks and assessed at 8 weeks.

5.2.9. Root induction

5.2.9.1. Rooting *in-vitro*

Effect of water agar

Forty *B. nitida* shoot tips were grown on optimal LMHM medium for 8 weeks. Twenty explants were subcultured onto water agar and 20 were subcultured on to LMHM medium. Tissue was assessed for health and root formation at 6 weeks after subculturing.

Effect of auxin level

One hundred *B. nitida* shoot tips were grown on LMHM for approximately 8 weeks and subcultured once at 4 weeks. The tissue was subcultured onto five media based on the optimal medium LMHM but with exponentially increasing levels of auxins. There were 20 replicates of each of the following five auxin concentrations: 1 µM auxins (control) (as per broad spectrum media), 2.5 µM, 5 µM, 25 µM, and 100 µM.

Effect of mineral level

Sixty-four *B. nitida* shoot tips were grown for 8 weeks and subcultured at 5 weeks on LMHM, after which the tissue was divided into four treatments of 16 replicates: quarter-strength mineral salts, half-strength mineral salts, full-strength mineral salts (all without hormones) and a control of LMHM. The tissue was assessed for root development and increase in height after 8 weeks on the new treatments.

5.2.9.2. Rooting *ex-vitro*

Twenty *B. nitida* explants were grown for 4 months in tissue culture with LMHM and potted up into 'forestry tubes' (pots 4 cm diameter x 20 cm deep) containing equal parts perlite, peatmoss and Seymour grit. The bases of the explants were dipped in a concentrated slurry of equal parts of IAA, NAA and IBA. The pots were then placed to a quarter of their depth in gravel on heat beds under constant mist for 10 days, reducing the misting so that the tissue was kept moist for 3 months. Tissue was removed from the pots and examined for root growth.

5.2.10. Data analysis

Data were checked for normality. If the data conformed to normality, parametric tests of ANOVA and regression were used to analyse the data. If data did not conform to normality, they were analysed using the non-parametric Kruskal-Wallis test. Data were analysed using the statistical package Minitab™, Version 15.0.

5.3. Results

5.3.1. Plant material

Both *B. nitida* and *B. mirabilis* re-sprouted shoots from severed tips within three months of removing explant material (Figure 5-2).

Figure 5-2: Re-sprouting of *Borya nitida* after shoot tips were taken for use as explants for micro-propagation.

5.3.2. *Borya mirabilis* trial

Shoot tip explants produced lateral shoots in liquid culture but buds did not (Table 5-8, Figure 5-3 and Figure 5-4). Explants stayed alive for approximately 6 months, sub-culturing once a month, after which time material either senesced or died. Removing newly developed side shoots from explants caused them to senesce.

Table 5-8: Summary of trial liquid culture of *Borya mirabilis*.

Media	*Hormones*	*Explant Tissue*	*Result*
MS 20 g/L sucrose	0.2 mg/L IAA, 2.0 mg/L BA	One shoot of *B. mirabilis*	Never grew, remained green for several months
MS 40 g/L sucrose	0.2 mg/L IAA, 2.0 mg/L BA	One shoot *B. mirabilis*	Grew side shoots after several months
MS 40 g/L sucrose	0.2 mg/L IAA, 0.2 mg/L BA	One shoot of *B. mirabilis*	Died
MS with 50 mL coconut milk 40 g/L sucrose	0.2 mg/L IAA, 2.0 mg/L BA	One shoot of *B. mirabilis*	Formed side shoots
MS 50 mL coconut milk 40 g/L sucrose	0.2 mg/L IAA, 2.0 mg/L BA	One shoot of *B. mirabilis*	Formed new side shoot and died on transferring into MS without coconut milk
MS 1% activated charcoal 40 g/L sucrose	0.2 mg/L IAA, 2.0 mg/L BA	Three buds of *B. mirabilis*	Died
MS with 40 g/L sucrose	0.2 mg/L IAA, 2.0 mg/L BA	Three buds of *B. mirabilis*	After three months sepals elongated and turned green, buds turned into flowers. No further growth
MS with 100 mL coconut milk 40 g/L sucrose	0.2 mg/L IAA, 2.0 mg/L BA	Three buds of *B. mirabilis*	After three months sepals elongated and turned green, buds turned into flowers. No further growth.
MS 40 g/L sucrose	0.3 mg/L IAA, 2.0 mg/L BA	Three shoots of *B. mirabilis*	After several months developed side shoots. When side shoots removed to develop further, all original explants died
MS 40 g/L sucrose	0.3 mg/L IAA, 2.0 mg/L BA	Three shoots of *B. mirabilis*	After several months developed side shoots. Once side shoots grown and explant reached approximately 6 months, explants died.

MS = Murashigee and Skoog (1962)
IAA= Indole acetic acid
BA= Benzylaminopurine

Figure 5-3: *Borya mirabilis* shoot growing and forming side shoots in liquid culture of MS with
40 g/L sucrose, 0.3 mg/L IAA and 2.0 mg/L BA.

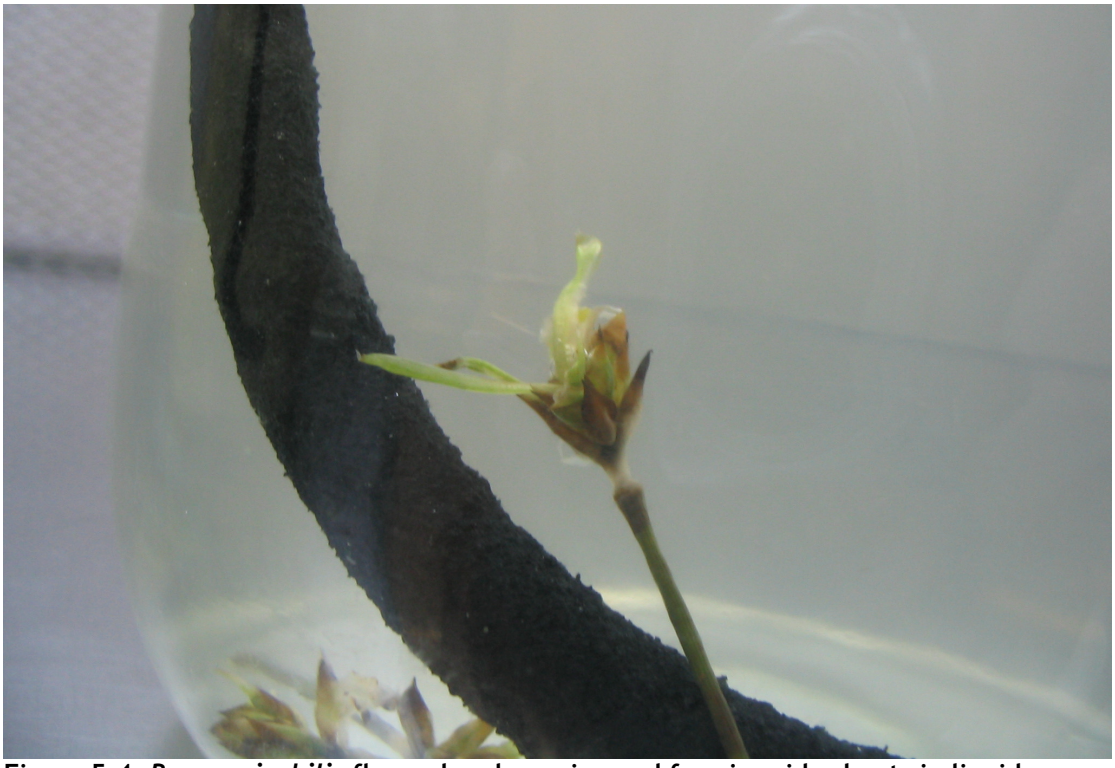

Figure 5-4: *Borya mirabilis* flower head growing and forming side shoots in liquid culture of MS with 40 g/L sucrose, 0.2 mg/L IAA and 2.0 mg/L BA.

5.3.3. *Borya nitida* trial

Overall whole leaves fared better as explants than tips, middle or base leaf sections (Figure 5-5). No benefit was produced by adding glutathione to the media. No benefit was found by using glass Petri dishes instead of plastic Petri dishes. While leaves from some treatments stayed green for long periods of time, no growth was recorded from any treatment.

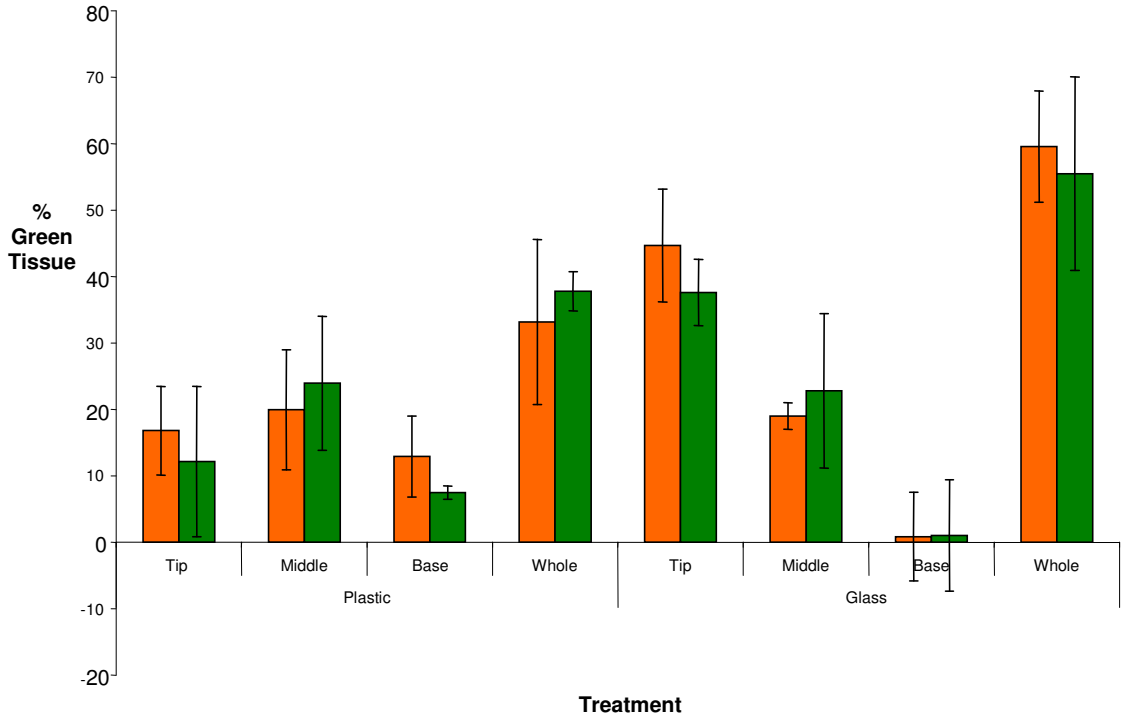

Figure 5-5: Effect of container and leaf explant type on explant health as measured by % green tissue of *Borya nitida* leaf explants exposed to 0.4 MS media (Murishigee and Skoog, 1962) with (orange) or without glutathione (green) in either glass or plastic Petri dishes. Columns represent the proportion of green material after 2 months, with error bars representing 2X the standard error of each treatment.

Control of contamination and gelling agent

5.3.3.1. PPM

Adding PPM significantly reduced contamination (ANOVA, $F=841$, $P<0.001$, Appendix 5-1). No contamination was observed in treatments where explants were exposed to PPM for one week prior to culturing, whereas without PPM treatment, 97% of explants were contaminated with fungi or bacteria. PPM had no apparent detrimental effect on the tissue.

5.3.3.2. Agar versus Phytagel

There was a significant difference in contamination between tissue in Phytagel and that in agar (Figure 5-6). Phytagel had significantly less contamination (ANOVA, p=0.014, F=6.37, Appendix 5-2) than agar. There was also a significant difference in the health of the explants between the two gelling agents (ANOVA, p=0.002, F=10.02 Appendix 5-3). The health of the explants was significantly greater in Phytagel (Figure 5-7).

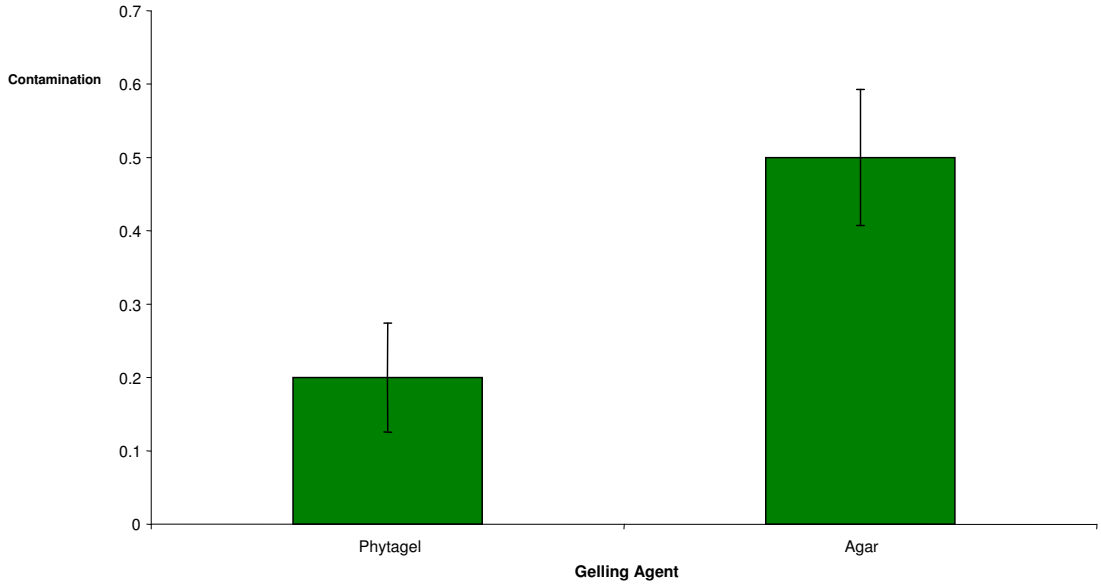

Figure 5-6: Effect of gelling agent on contamination.

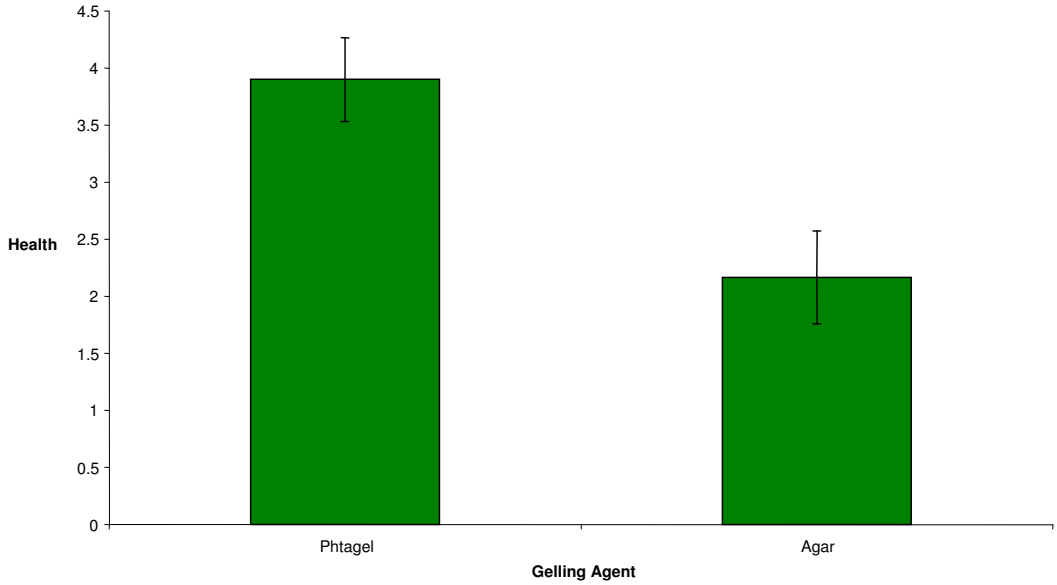

Figure 5-7: Health of explants with the gelling agent Phytagel and agar. Health rating determined from Table 5-2.

5.3.4. Effect of explant

Only shoot tip explants survived and grew, whereas no growth was observed over 3 months for either flower buds or stem sections. The source (plant) of the explant had a significant effect on the health of the explant at 8 weeks (ANOVA, $F=8.66$, $P<0.001$) (Figure 5-8), with explants from plant 9 and 11 significantly greater in health than the others.

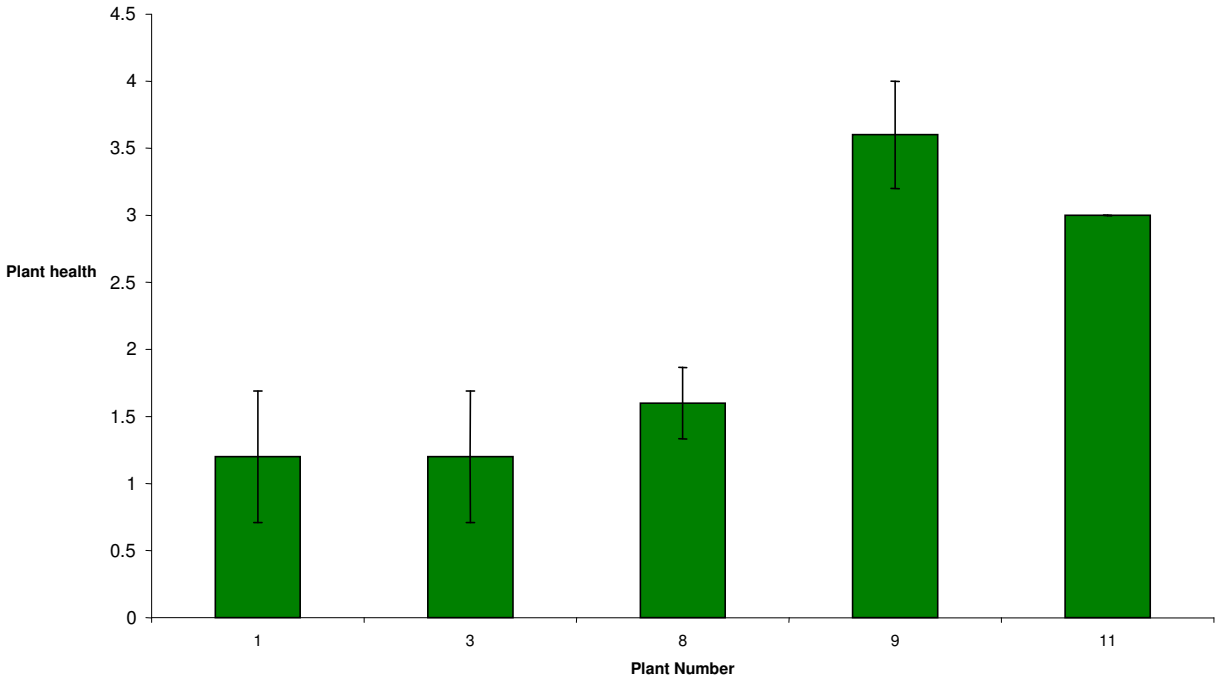

Figure 5-8: Differences in health of shoots in culture between explants of individual *Borya nitida* plants. Health rating determined from Table 5-2.

5.3.5. Medium optimisation

5.3.5.1. *Borya mirabilis* buds and *Borya nitida* buds and stem sections

No growth was observed over 3 months for either buds or stem sections.

5.3.5.2. *Borya nitida* shoots

Shoot growth at 6 weeks

AT 6 weeks, explant heath varied significantly across media (ANOVA, $F=20.90$, $p<0.001$) (Figure 5-9 to Figure 5-11). Multiple regression analysis showed (Table 5-9) that either low or medium minerals and either low or medium sucrose + growth factors significantly improved explant health compared with high minerals and sucrose + growth factors (ANOVA, $F=14.87$, $P<0.001$). Neither cytokinins nor auxins had a significant effect (Table 5-10). Explants put into culture on date 1 were also significantly healthier than those on date 2 (Table 5-9). There was no significant interaction between

any factors (ANOVA = $p>0.05$, Table 5-10). Mortality was included in the data analysed.

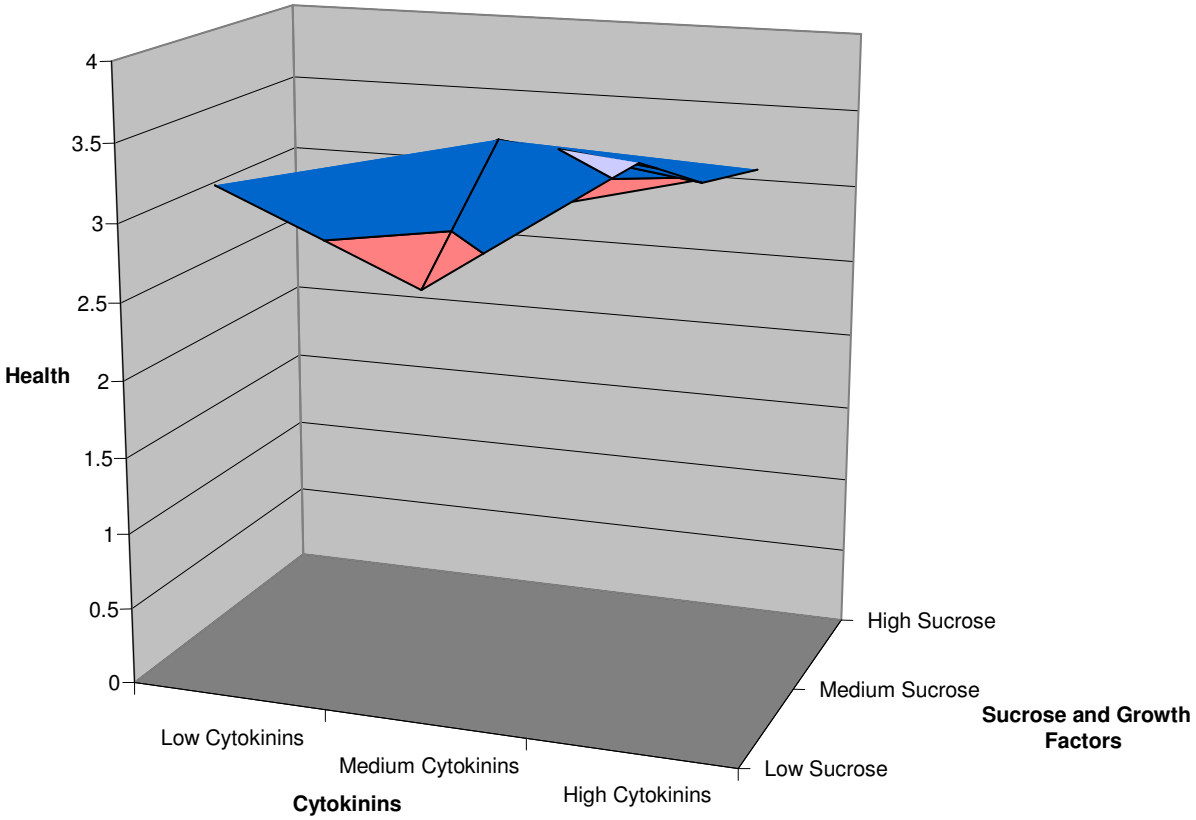

Figure 5-9: The effects of cytokinins, sucrose + growth factors with low minerals on health of *Borya nitida* shoot tip explants at 6 weeks, including mortality.

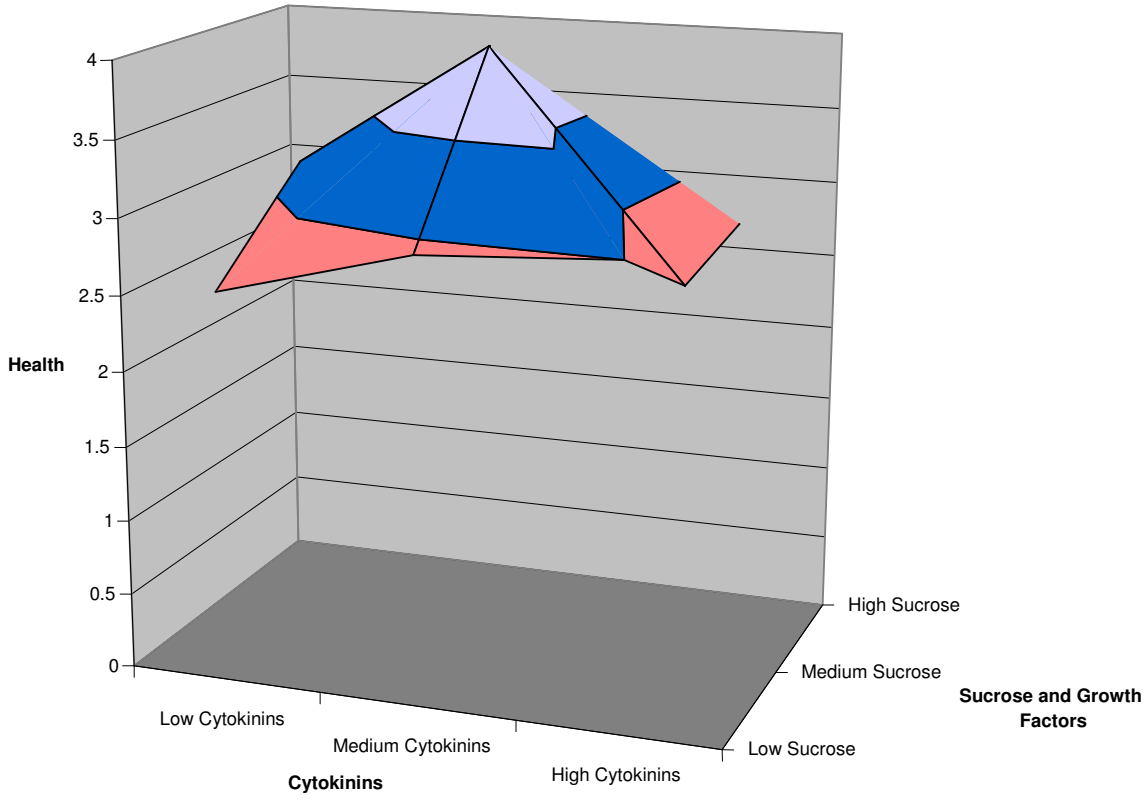

Figure 5-10: The effects of cytokinins, sucrose + growth factors with medium minerals on health of *Borya nitida* shoot tip explants at 6 weeks, including mortality.

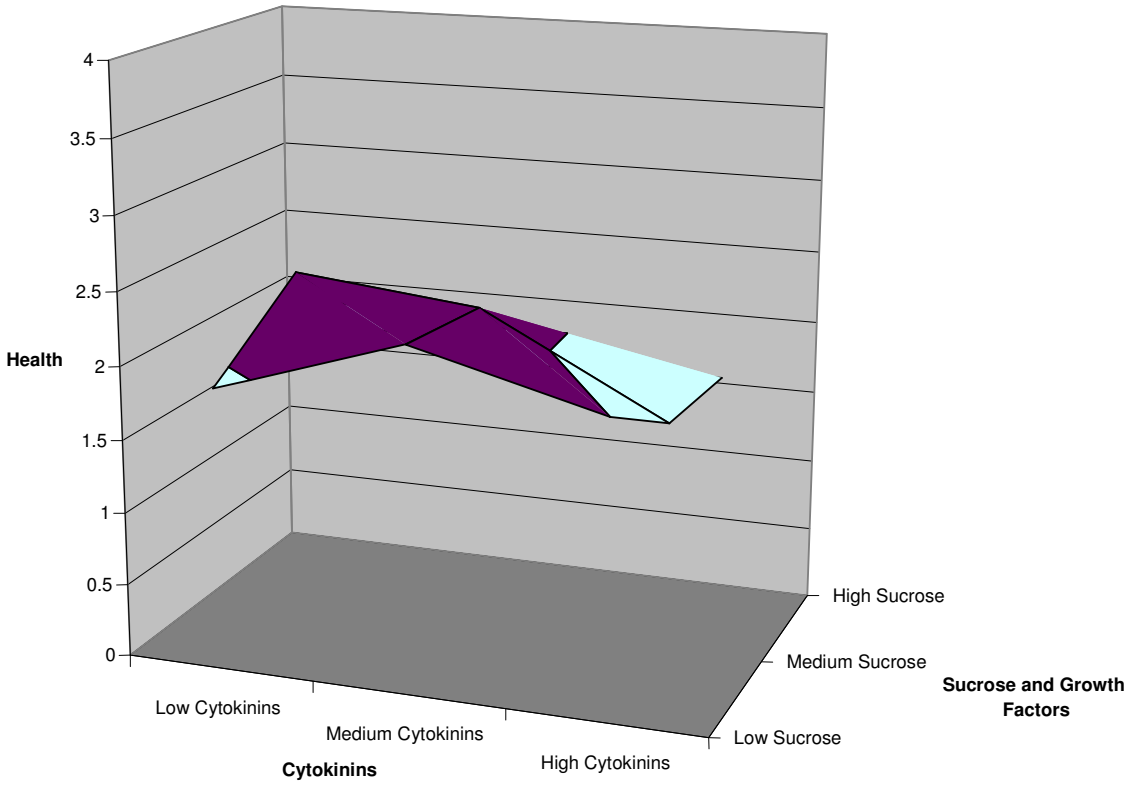

Figure 5-11: The effects of cytokinins, sucrose + growth factors with high minerals on health of *Borya nitida* shoot tip explants at 6 weeks, including mortality.

Table 5-9: Regression equation data for health of *Borya nitida* shoots at 6 weeks in de Fossard's, (1976) matrix media compaed with high levels of all factors.

Predictor	*Coef*	*SE Coef*	*T*	*P*
Constant	1.1625	0.1972	5.9	<0.001*
Low minerals	1.1111	0.1526	7.28	<0.001*
Medium minerals	1.0155	0.153	6.64	<0.001*
Date 1	0.9218	0.1248	7.39	<0.001*
Low sucrose	0.3889	0.1526	2.55	0.011*
Medium sucrose	0.4877	0.153	3.19	0.002*
Low cytokinin	-0.0648	0.1526	-0.42	0.671
Medium cytokinin	0.0803	0.153	0.52	0.6
Low auxin	0.1204	0.1526	0.79	0.431
Medium auxin	-0.0771	0.153	-0.5	0.614

S = 1.122 R-Sq = 30.0% R-Sq (adj) = 27.9% * Significant

Table 5-10: Analysis of variance table for explant health, at 6 weeks.

Source	*DF*	*Seq SS*	*Adj SS*	*Adj MS*	*F*	*P*
Sucrose and Growth Factors	2	13.827	15.437	7.718	4.95	0.008*
Cytokinins	2	0.515	0.965	0.483	0.31	0.734
Auxins	2	2.044	2.623	1.312	0.84	0.432
Minerals	2	87.089	84.825	42.413	27.2	<0.001*
Sucrose and Cytokinins	4	11.677	11.543	2.886	1.85	0.12
Sucrose and Auxins	4	3.38	3.536	0.884	0.57	0.687
Sucrose and Minerals	4	3.122	3.23	0.808	0.52	0.723
Cytokinins and Auxins	4	5.931	6.314	1.579	1.01	0.401
Cytokinins and Minerals	4	9.356	9.313	2.328	1.49	0.205
Auxins and Minerals	4	3.812	3.517	0.879	0.56	0.689
Sucrose and Cytokinins and Auxins	8	4.204	4.506	0.563	0.36	0.94
Sucrose and Cytokinins and Minerals	8	12.328	12.343	1.543	0.99	0.445
Sucrose and Auxins and Minerals	8	4.256	3.982	0.498	0.32	0.958
Cytokinins and Auxins and Minerals	8	9.754	9.931	1.241	0.8	0.606
Other interactions	16	16.389	16.389	1.024	0.66	0.835
Error	262	408.55	408.55	1.559		
Total	342	596.233				

Shoot growth at 12 weeks

The 12 week data were analysed for interactions between the chemicals and the explant. While Cytokinins and Minerals had a significant effect on the health of the explant they also had a significant interaction with each other, (Table 5-12, Figures 5-12, Figure 5-13) in that the health varied with cytokinin level but the extent of the response varied with mineral level. Surviving explants were healthy after 3 months and had tripled in length (Figure 5-14).

Explant health at 12 weeks was bimodal and so was analysed using non-parametric tests. As before, the presence of low or medium minerals significantly improved explant health (Kruskal-Wallis, $H = 17.45$, $P < 0.001$) (Figure 5-12). The presence of cytokinins also significantly improved explant health (Kruskal-Wallis $H = 14.05$, $P = 0.001$) (Figure 5-12). Auxins had no effect (Kruskal-Wallis, $H = 0.84$, $P = 0.656$), nor did sucrose + growth factors (Kruskal-Wallis $H = 0.98$ DF = 2 $P = 0.613$).

When dead explants were removed, data for survivors at 12 weeks conformed to a normal distribution and so were analysed using parametric tests. The regression equation for significant factors ($P<0.05$) at 12 weeks, excluding mortality, was: Health = 3.46 + 0.496 Medium Cytokinins - 0.668 Low Sucrose - 0.688 Date 1 (ANOVA, $F=2.49$, $p<0.019$).

Sucrose + Growth Factors had a significant effect on the health of the surviving explants (ANOVA, $F= 3.28$, $P=0.045$) (Figure 5-13). Explants put into culture on date 1 were also significantly healthier than those on date 2. None of the other factors had a significant effect on health of the survivors: auxins (ANOVA, $F=0.66$, $P=0.523$), cytokinins (ANOVA, $F=1.90$, $P=0.159$) or minerals (ANOVA, $F= 0.54$, $P=0.584$). From this, it was concluded that the optimal medium was LMHM (Low minerals, Medium Auxins, High Cytokinins and Medium Growth Factors + Sucrose). Two shoot tips of *B. mirabilis* from potted plants, grown from shoot-tip cuttings at the Royal Botanic Gardens, Melbourne, tested on these media survived and grew well.

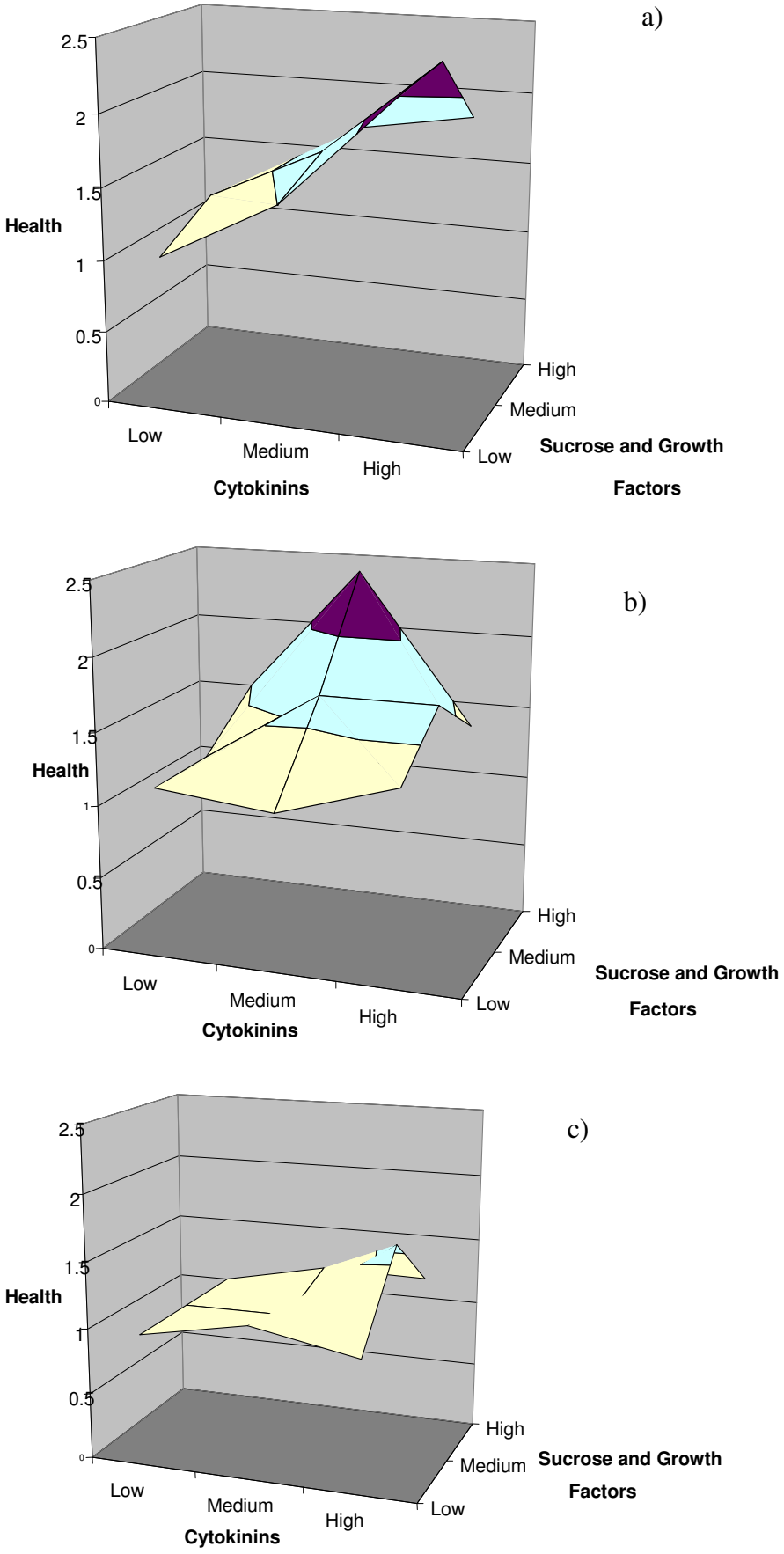

Figure 5-12: The effects of Cytokinins, Sucrose + Growth Factors and (a) low, (b) medium and (c) high Minerals on Health of *Borya nitida* shoot tip explants at 12 weeks, including mortality.

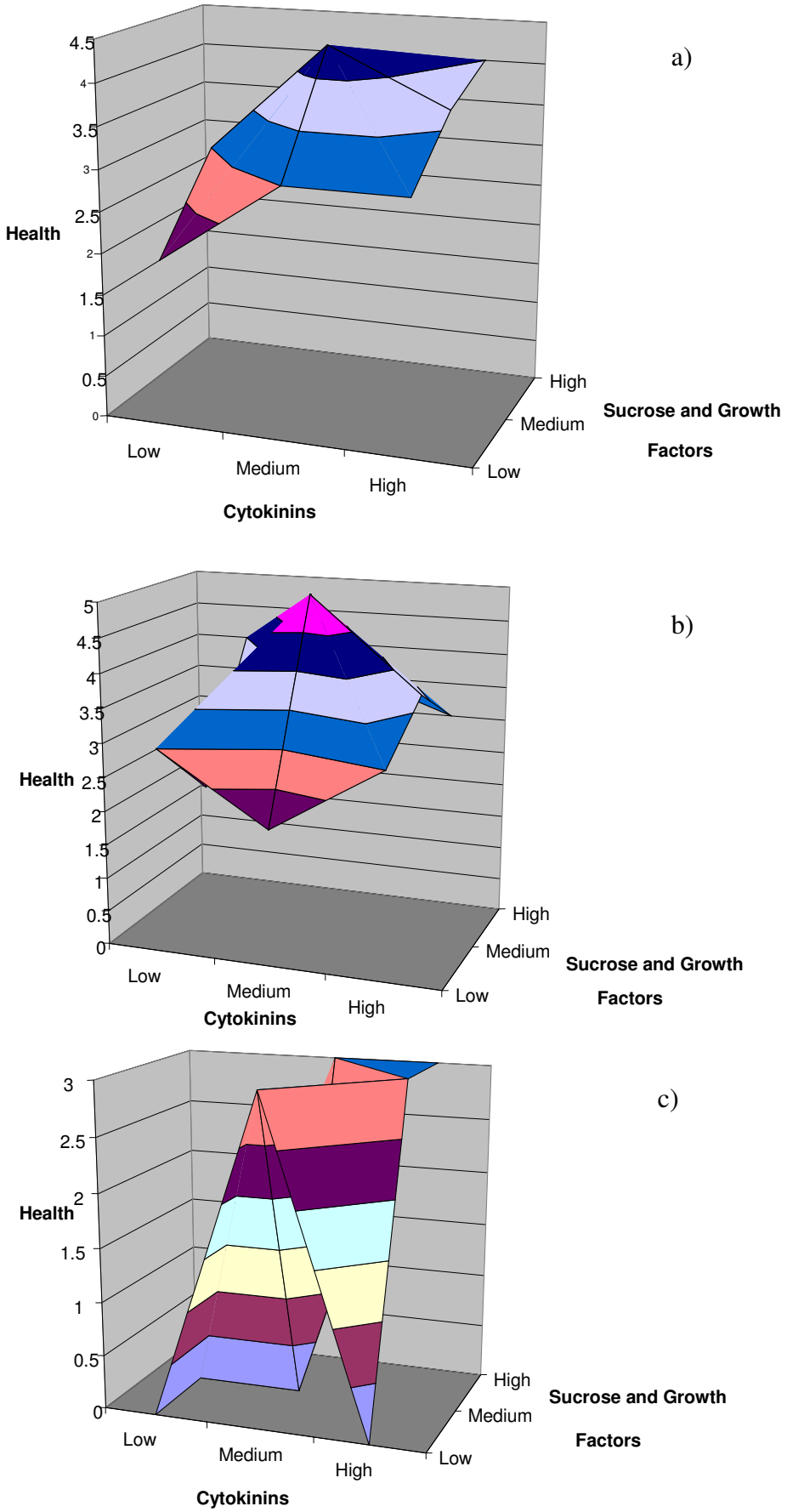

Figure 5-13: The effects of Cytokinins, Sucrose + Growth Factors and (a) low, (b) medium and (c) high Minerals on Health of *Borya nitida* shoot tip explants at 12 weeks, excluding mortality.

Table 5-11: Data from regression equation at 12 weeks excluding mortality.

Predictor	*Coef*	*SE Coef*	*T*	*P*
Constant	3.4566	0.4703	7.35	<0.001*
Low minerals	0.1772	0.4259	0.42	0.679
Medium minerals	0.1607	0.4292	0.37	0.71
Low auxins	0.2058	0.2802	0.73	0.466
Medium auxins	0.474	0.3015	1.57	0.122
Low cytokinins	-0.3848	0.3544	-1.09	0.283
Medium cytokinins	0.4965	0.2835	1.75	0.086
Low sucrose	-0.6679	0.3012	-2.22	0.031*
Medium sucrose	0.1181	0.2807	0.42	0.676
Date 1	-0.6882	0.28	-2.46	0.017*

S = 0.8742 R-Sq = 30.5% R-Sq(adj) = 18.2%

Table 5-12: ANOVA table from linear regression analysis at 12 weeks.

Source	*DF*	*Seq SS*	*Adj SS*	*Adj MS*	*F*	*P*
Sucrose	2	2.645	2.4177	1.2088	1.37	0.255
Cytokinin	2	13.1906	11.8396	5.9198	6.73	0.001*
Auxins	2	0.3313	0.161	0.0805	0.09	0.913
Minerals	2	16.3072	15.3812	7.6906	8.74	<0.001*
Sucrose*Cytokinin	4	6.5188	7.218	1.8045	2.05	0.088
Sucrose*Auxins	4	2.1227	1.4187	0.3547	0.4	0.806
Sucrose*Minerals	4	4.1959	4.6645	1.1661	1.33	0.261
Cytokinin*Auxins	4	1.1244	0.8054	0.2013	0.23	0.922
Cytokinin*Minerals	4	9.9846	9.6434	2.4108	2.74	0.029*
Auxins*Minerals	4	4.5372	4.3126	1.0781	1.23	0.300
Sucrose*Cytokinin*Auxins	8	5.3752	4.447	0.5559	0.63	0.751
Sucrose*Cytokinin*Minerals	8	4.4413	4.7672	0.5959	0.68	0.711
Sucrose*Auxins*Minerals	8	3.3639	2.9606	0.3701	0.42	0.908
Cytokinin*Auxins*Minerals	8	13.7872	13.2201	1.6525	1.88	0.064
Sucrose*Cytokinin*Auxins* Minerals	16	18.5653	18.5653	1.1603	1.32	0.185
Error	260	228.7	228.7	0.8796		
Total	340	335.1906				

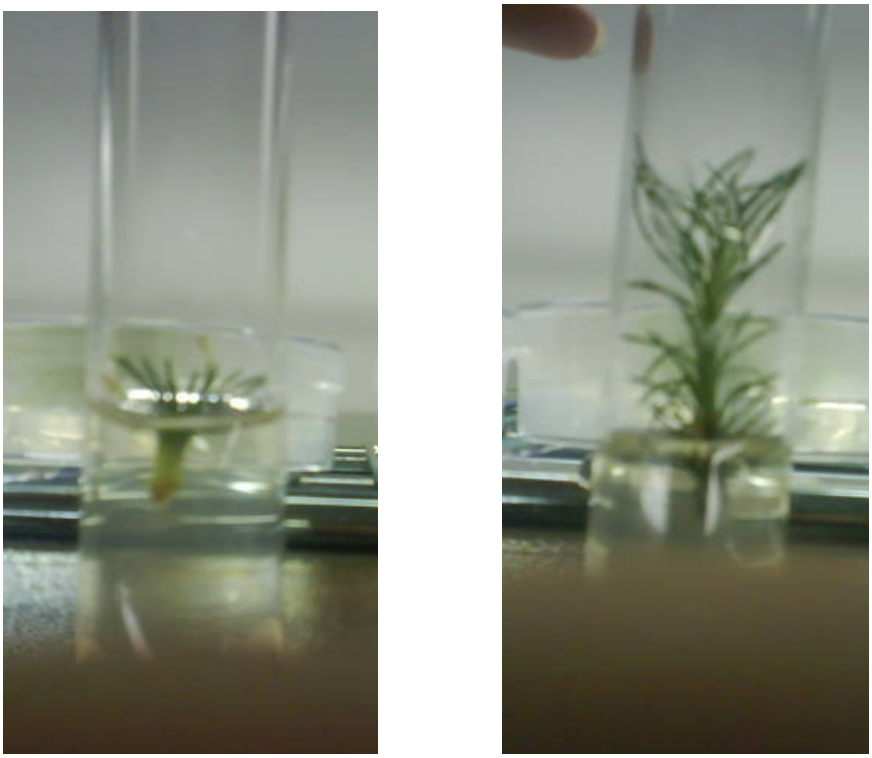

Figure 5-14: Left: *Borya nitida* initial explant size and right; *B. nitida* explant after growing in LMHM medium for 4 months.

5.3.6. Root induction in *Borya nitida*

Effect of water agar

After 1 month, half of the shoots were dead; after 2 months all explants were dead and there was no indication of root growth *in vitro*.

Effect of auxin concentration

There was no sign of root induction after a further 2 months in culture; after 6 months the tissue began to senesce *in vitro*.

Effect of nutrient reduction

No root growth was observed *in vitro*. The average increase in height of explants grown in the optimal control group was 2.1 cm over 8 weeks (Figure 5-15). There was no significant difference between the different treatments in shoot growth (ANOVA, F=2.72, P=0.052).

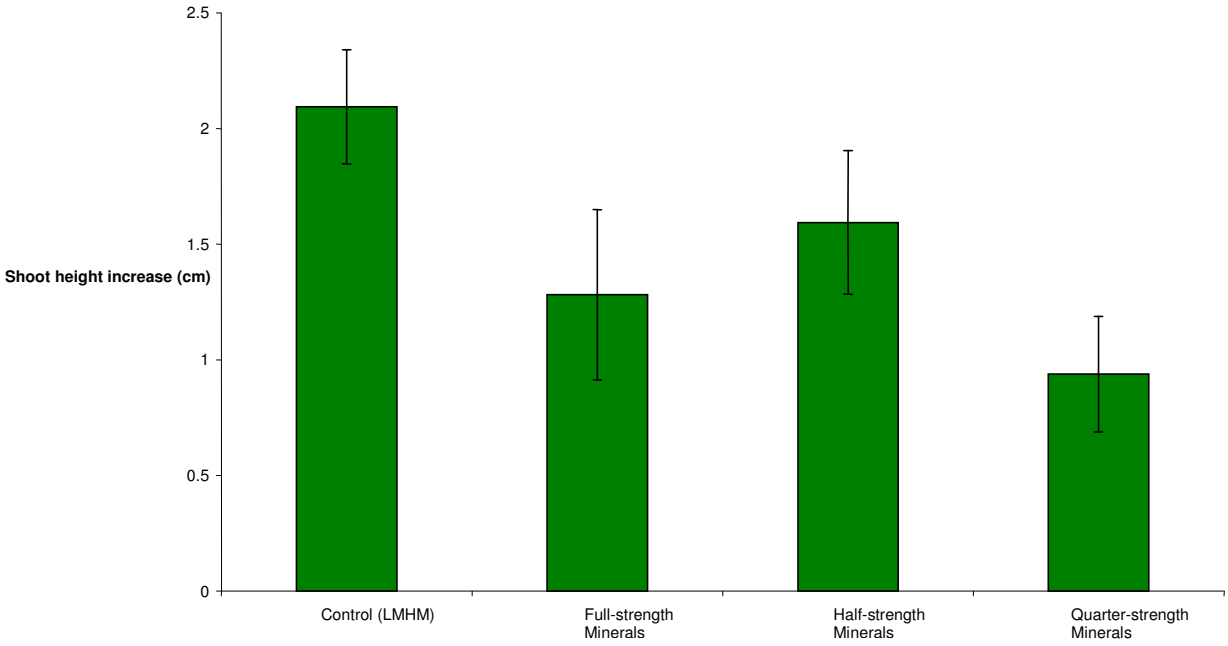

Figure 5-15: Mean shoot increase from initial height after 8 weeks.

5.3.6.1. Root induction *ex-vitro*

Seven of the 20 tissue cultured shoots (35%) produced root systems (Figure 5-16 and 5-17) and became healthy plants after 3 months. The remaining plants browned after about 1 month and slowly died off.

Figure 5-16: Micro-propagated shoots of *Borya nitida* under misters for root formation.

Figure 5-17: Micropropagated shoot of *Borya nitida* 3 months after potting up. Bar indicates initial explant size prior to shoot elongation *in vitro* and exposure to auxins and potting up in perlite, Seymour git and peatmoss *ex vitro*.

5.4. Discussion

This is the first published information on tissue culture of any *Borya* species or member of the Boryaceae. The research presented here has answered fundamental questions about the establishment of an effective means of micro-propagation of *B. nitida* and provided a more rapid means of propagating this difficult resurrection species than by cuttings. This research has determined the most suitable explant for regeneration, provided a reliable means of reducing the contamination *in vitro*, optimised media for explant survival and growth and developed an efficient system for induction of roots. It also seems likely to be effective for the critically endangered *B. mirabilis* and possibly other *Borya* species.

5.4.1. Explant

Only explants of shoot tips, not of flower buds or stem sections, could be established in culture. Shoot tips are also the only effective form of propagation for many other threatened species (Jusaitis and Sorenson, 1994). Further work needs to be undertaken to establish tissue culture as a viable means of producing more plants. Taking shoot tips from the stock *ex-situ* plants of *B. nitida* has the added benefit of encouraging the plants to re-sprout directly beneath the area from which the tissue was taken. This allows more shoots than would otherwise be produced to be harvested for further explant trials. For the critically endangered *B. mirabilis*, this method is likely to result in few long-term effects on the existing *ex-situ* population and the micropropagated plants will be able to reproduce the genetic variation in the only known wild population of the plant. Further trials are needed with more shoots to confirm the suitability of shoot-tips as an explant for *B. mirabilis*.

The significant variation in shoot tip response from genetically different individuals would, along with the date of harvest, account for some of the intrinsic variation in the data. Similar individual-based differences have been seen in tissue culture of other species, e.g. *Doryanthes excelsa* (Dimech *et al*. 2007) and *Epacris impressa* (Thompson, 1986; Anthony et *al*.

2000). Whilst this might present problems for a genetically diverse species, it does not for *B. mirabilis* because it has low genetic variation (Coates *et al*. 2002). Nevertheless, the dominance of Colony 7-derived plants in the *ex-situ* population at the RBG suggests that it may be easier to propagate and so may be more successful in this micropropagation system. This should be investigated as a first priority.

5.4.2. Contamination

An effective means of reducing contamination was found in the form of pre-treatment with PPM, a proprietary product. PPM is a heat-stable preservative/biocide and effectively eliminated contamination from fungi and bacteria. This is extremely important, as *B. mirabilis* material is limited and previous tissue culture experiments have been hampered by contamination. Similar contamination problems have been encountered in the Epacridaceae (Anthony *et al*. 2000), in which successful propagation was reliant upon pre-treatment with broad-spectrum fungicides and antibiotics of plants that were a source of shoot tip cuttings. PPM can potentially be used to control infection even with plants from the wild, where it would not be possible to use fungicides and antibiotics.

5.4.3. Medium

Different medium ingredients were important at different stages in the processes of establishment and further growth. In the initial 6 weeks, low nutrients were significantly better at keeping and maintaining explant health than high nutrients, while levels of hormones had no effect. The growth of shoot tips of woody plants in low levels of minerals and toxicity to the tissue in high levels of minerals has been a long-standing problem in using traditional media such as MS (Murashige and Skoog, 1962) in the propagation of Australian plants and is one of the reasons why de Fossard (1976) formulated the mini broad-spectrum approach. Many Australian soils, exemplified by those in the Grampians, are nutrient-poor. Probably as a consequence of the soils in which they naturally occur, some Australian plants, including *Borya*, find high-nutrient media toxic.

This sensitivity to high levels of nutrients can also be seen in the few published reports of tissue culture in Australian genera in related families of the monocotyledons, e.g. the threatened *Diuris purdii* (Orchidaceae; Collins and Dixon, 1992) and *Sowerbaea multicaulis* (Lomaceae; Rossetto *et al.* 1992), in which shoots and embryos prefer low mineral media.

During the next 6 weeks, however, a surprisingly high cytokinin concentration was needed to reduce the mortality rate of the tissue. Cytokinins in general control regeneration in monocotyledons (Zheng *et al.* 1999), but the large concentrations of cytokinins used here (10 µM) are relatively uncommon in the tissue culture of Australian plants, with usual concentrations varying from 0-1 µM. There have, however, been reports of other monocotyledonous species requiring high cytokinins, with shoots in the Iridaceae requiring 1 mg/L (Boltenkov and Zarembo, 2005), orchid inflorescences requiring 10 µM BA (Collins and Dixon, 1992) and *Eriostemon australis* requiring 30 µM BA (Plummer and de Fossard, 1981). Auxin concentration had no significant effect on the health of the explants at any stage and did not induce rooting at the concentrations and auxin types tested. Although it is possible that a different auxin, combination or concentration could have affected the explants, it appears unlikely. Limited trials with the necessarily restricted material of *B. mirabilis* indicated that it behaved like *B. nitida* and the strategy of using a common species to screen media for an endangered species in the same genus may have merit for other species.

5.4.4. Rooting

A reliable means of inducing roots (in 35% of shoot tips) was only found when the micro-propagated shoots were grown in a soil-based potting medium over bottom heat. Many other native Australian plants have proved extremely difficult to root *in vitro,* including *Epacris impressa* (Thompson, 1986; Anthony *et al.* 2000). *In-vivo* rooting has added benefits to the entire micropropagation process (Taji and Williams, 1996), as shown in *Grevillea scapigera* (Bunn and Dixon, 1992), such as stronger root systems and less damage to the explants when deflasking.

5.4.5. Conclusion

This work has answered some of the basic questions required to have a successful micropropagation system set up for *Borya* species. Using the common species *B. nitida* has proved an effective means for developing a protocol to micropropagate the endangered *B. mirabilis*. This method of dealing with the propagation of threatened species can be seen in other threatened Australian species such as *Lomatia tasmanica* (Proteaceae: Cambecedes and Balmer, 1995). Future work on this species should determine more precisely the nutrient requirements of *B. mirabilis*. This protocol can now be used to mass-propagate this species, a fundamental requirement in its recovery plan. Further work is needed to establish protocols for shoot multiplication.

6. The mycorrhizal associations of *Borya mirabilis*

Underpinning the successful establishment and growth of an *ex-situ* population and translocation is adequate knowledge of the ecology of the plant species. As has been eloquently illustrated with research into the establishment and translocation of terrestrial orchids within Australia, knowledge of plant ecology includes the understanding of their mycorrhizal nature and the ecology and the diversity of the mycorrhizal partner. In many cases, without this knowledge successful *ex-situ* reproduction and translocation is extremely hampered if not impossible. Investigating and identifying the mycorrhizal associations of *B. mirabilis* and the subsequent provision for these associations in the *ex-situ* environment may allow for better cultivation and successful translocation of this species.

When considering the conservation of an endangered plant, the investigation of its biology should not be limited to the environment above the soil, but should also try to preserve, capture and translocate the associated diversity below the soil surface. In the guideline for translocation of threatened plants in Australia (Vallee *et al*. 2004) it is recommended that "details of any potential inter-relationships between the taxon and other taxa, including mycorrhiza" be collated; this information should also be sought when identifying the appropriate propagation methodology.

6.1. Types of mycorrhiza

There are four main groups of mycorrhizal fungi: orchid mycorrhiza (basidiomycetes) and specific to the relationships with orchids; ericoid mycorrhiza (ascomycetes), which form relationships only with ericoid plants; ectomycorrhiza (ECM) (basidiomycetes and ascomycetes), which may form associations with woody plants; and arbuscular mycorrhiza (AM) (glomalean fungi), which are more ubiquitous and non-specific. A summary of the main features of these mycorrhizas is presented in Table 6-1. There are also minor types of mycorrhiza like lobelioid mycorrhiza not relevant to *Boryas* and therefore not referred to here. This introduction will focus on

ectomycorrhiza and AM, as these are the only two described mycorrhiza likely to be found in *Borya* species.

Table 6-1: Distinguishing features of the main types of mycorrhiza, modified from Harley and Smith (1983).

Character	AM	ECM	Ericoid	Orchid
Hyphae septate	±	+	+	+
Hyphae intracellular	+	-	+	+
Hyphae in coils, intracellular	+	-	+	+
Arbuscules present	+	-	-	-
Vesicles present	±	-	-	-
Mantle present, extra-cellular	-	+	-	-
Hartig net present, extra-cellular	-	+	-	-
Plants	Vascular plants	Gymnosperms and angiosperms	Ericales	Orchidaceae
Fungi	Glomeromycota (Schubler *et al.* 2002)	Basidiomycetes, ascomycetes and zygomycetes	Ascomycetes and some basidiomycetes (Selosse *et al.* 2007)	Basidiomycetes

6.1.1. Arbuscular mycorrhiza

The fungi forming arbuscular mycorrhiza (AM) belong to the Glomeromycota (Schubler, 2002). They are extremely ancient organisms that are only known to produce asexually (Hijri and Sanders, 2005). The first fossil evidence of AM dates to 400 million years ago (Remy *et al*. 1994). They form relationships with most groups of higher plants and form associations with 60% of terrestrial plants (Trappe, 1987).

The main interaction between AM and plants is a resource exchange of carbohydrates for phosphate, as outlined in Bucking and Shachar-Hill (2005), although there can be many other benefits. They are considered generalists and have the ability to connect different plant species through their mycelium (Newman, 1988). Their ability to confer benefits and to inoculate plant species differs (Hass and Krikun, 1985; Johnson, 1993). Preliminary research suggests that multiple AM inocula offer greater benefits than single infections (Edathil *et al*. 1996) and that AM offer greater benefits in nutrient-poor soils (Abbott and Robson, 1982).

AM are obligate and can not be grown asymbiotically. In general they have a preferred niche for an environment and their associated species (Klironomos *et al*. 1993) and thus particular AM communities are to be found (Helgason *et al*. 1998). AM have an effect on plant species' co-existence (van der Heijden *et al*. 2003) and diversity (Grime *et al*. 1987, Klironomos *et al*. 2000).

The amount and infection of AM decreases with depth once past the main rooting zone (10-30 cm) (Kabir *et al*. 1998; Rillig and Field, 2003). Deeper soil layers contain different abundance and diversity of infection (Oehl *et al*. 2005); a decline in colonisation below 10 cm has been found in most studies (Powers *et al*. 2005; Cooke *et al*. 1993; Brown and Bledsoe, 1996 and Ingleby *et al*. 1997).

AM fungi are biotrophs or at least colonise roots biotrophically. They provide a direct link between plant roots and the soil (Smith and Read, 1997). Depending on the fungal taxa present (van der Heijden *et al*. 2003), benefits to the host plant include:

- Increased phosphorus (George *et al*. 1992; Titus and Leps, 2000)
- Increased nitrogen, magnesium and iron (Fitter and Merryweather, 1992; Barea and Jeffries, 1995; Varma 1995), zinc and copper (Marschner, 1995)
- Increased resistance to stress, pathogens, heavy metals, acidity and salinity (Pfetffer and Bloss, 1988; Dodd *et al*. 1990; George *et al*. 1992; Barea and Jeffries, 1995; Marschner, 1995)
- Changing compounds in the soil to an easily absorbed form (Leyval and Berthelin, 1989; Knight *et al*. 1989; Marschner and Dell, 1994).
- Increased plant size (Grime *et al*. 1987; Smith and Read, 1997; Bohrer *et al*. 2003; van der Heijden *et al*. 2003).

AM fungi can be broken up into three morphological types, Arum (Figure 6-1 A) and Paris (Figure 6-2 B) types as described in Figure 6-2, and a third type known as *Rhizophagus* or *Endogone* mycorrhiza (Figure 6-3). This third group of AM fungi is associated with plants that form nodular structures on their roots (Saxton, 1930).

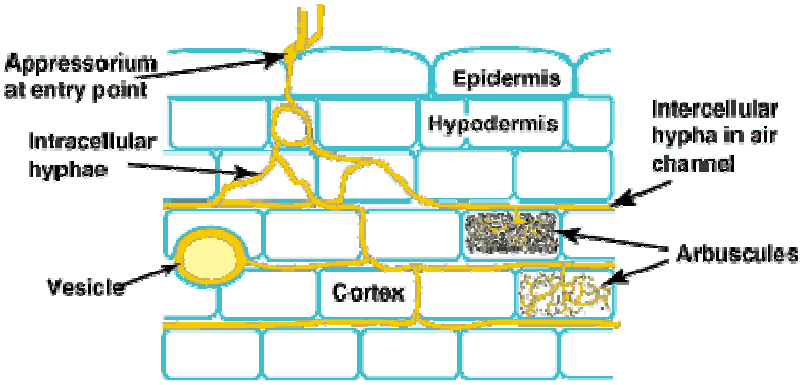

Figure 6-1: Schematic representation of arbuscular mycorrhiza symbiosis in plant roots (Brundrett *et al*. 1996).

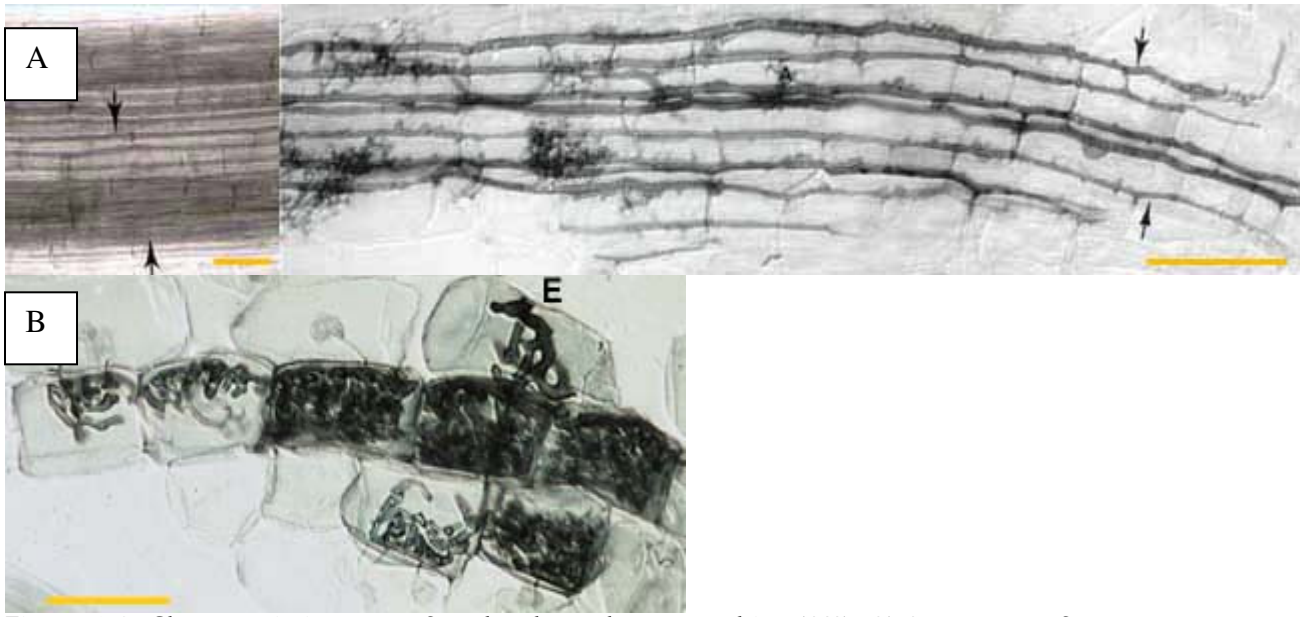

Figure 6-2: Characteristic types of each arbuscular mycorrhiza (AM); A) Arum type of AM, B) Paris type of VAM (Brundrett *et al*. 1996).

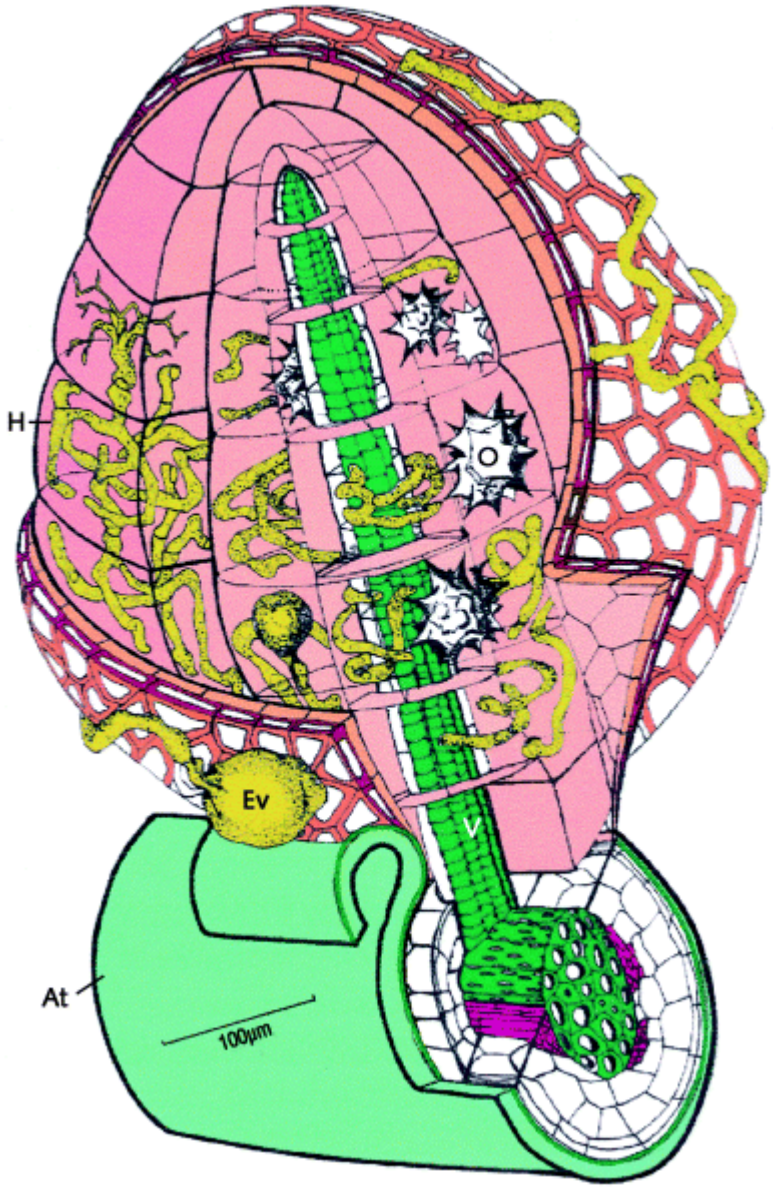

Figure 6-3: Diagram of a typical mycorrhizal nodule from *Gymnostoma* (from Duhoux *et al*. 2001). O, crystals of calcium oxalate, At adjacent root, H extra-radical fungal hyphae, Ev extra-radical hyphae with vesicle.

6.1.2. Ectomycorrhiza

Over 5,000 known species of fungi cause ectomycorrhizal symbiosis (Molina *et al*. 2002). This type of symbiosis spans all the true fungi; however, species primarily come from the ascomycetes and basidiomycetes (Buscot *et al*. 2000). This type of symbiosis dominates boreal and temperate forests (Wiemken and Boller, 2002), particularly forests with trees and shrubs from Betulaceae, Pinaceae, Fagaceae, Salicaceae and Dipterocarpaceae (Read, 1991).

In this type of symbiosis the fungus encases the fine roots of the plant involved (Figure 6-4 and Figure 6-5) and provides nutrients and water in exchange for carbon, akin to the symbiotic relationship of AM. The relationship also induces various physical changes in the roots of their hosts, including a lack of root hairs and formation of short roots.

It is suggested that ectomycorrhizal fungi were originally saprotrophic and indeed many species still retain the ability to switch between modes of nutrition. Although a handful of ectomycorrhizal fungi can be grown and kept in pure culture, the majority have proved difficult grow in this manner (Buscot *et al*. 2000).

Another unique ability of ectomycorrhizal fungi is to link different species in the field via their mycelial connections underground; in some cases one plant may be able to support another by supplying nutrients via their shared symbiont (Simard *et al*. 1997). Ectomycorrhiza also have succession stages along with their associated species, changing the associated species as plants age (Gardner and Malajczuk, 1985).

Benefits include:

- Increased nutrients (Smith and Read,1997), e.g. phosphate from bedrock
- Linking plants to rocks
- Acquiring nitrogen from protein-containing material, and increasing nitrogen uptake (Read *et al*. 1989)
- Increasing ammonium uptake (Rygiewicz *et al*. 1984)
- Potential to clean up persistent organic pollutants (Meharg and Cairney, 2000)
- Increasing phosphorus uptake (Brunner, 2001).

Figure 6-4: Characteristic ectomycorrhiza (Brundrett *et al*. 1996).

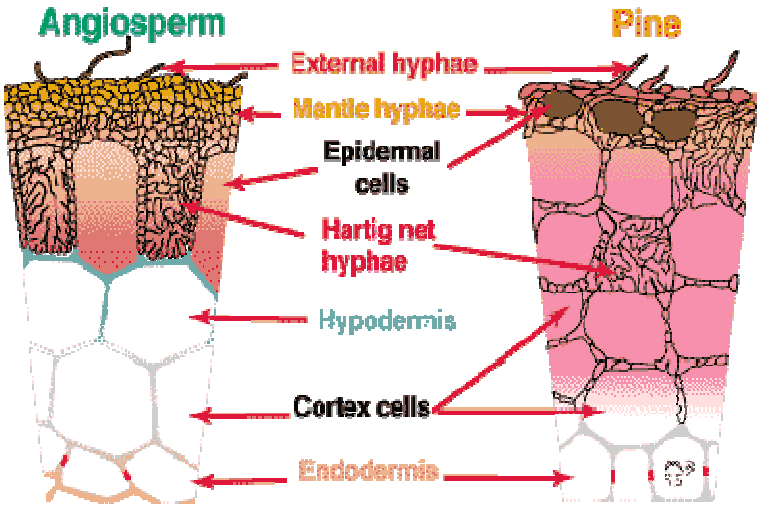

Figure 6-5: Schematic representation of ectomycorrhizal infection in plant roots (Brundrett *et al*. 1996).

6.1.3. Methods of Identification

The identification of mycorrhiza poses certain problems. AM do not produce any fruiting structures and individual parts can only be obtained through spore sieving, whereas ECM do form fruiting structures (though some only rarely) and these may be contaminated by insects or other fungi. In most instances a direct connection with the plant roots can not be obtained, only that the organisms were found in the same area.

Using the percentage of root colonisation is considered the most accurate method when studying the Glomaceae and Acaulosporaceae. Soil hyphal length is the better method for Gigasporaceae (Hart and Reader, 2002).

6.1.4. Molecular identification

The majority of molecular identification of mycorrhizas uses ribosomal DNA (Redecker *et al*. 2003) (Figure 6-6). Approaches to molecular identification can differ between ECM and AM, as suggested by Redecker *et al*. (2003). AM has a low percentage colonization per root mass, whereas that of ECM is generally high. AM are also likely to have multiple colonies whereas ECM usually have single colonies on the plant roots. It is very common to have a mix of mycorrhizae on plant roots as well as other fungi and when using material from the field, specific primers form the basis of a commonsense approach.

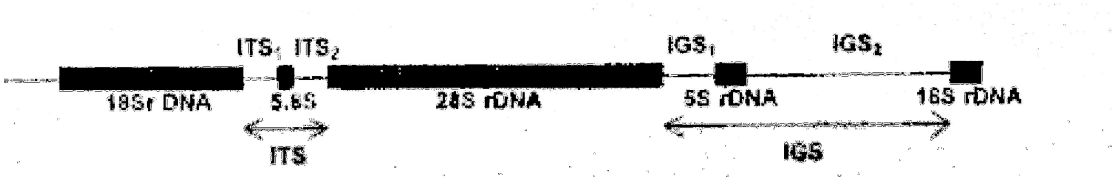

Figure 6-6: Diagram of rDNA, thin lines representing highly variable regions, thick lines representing highly conserved regions.

Employing the universal primers ITS1 and ITS4 (White *et al*. 1990) is therefore redundant, as these would not only amplify contaminants too, but if amplified directly from plant roots, which is the ideal, they would also amplify plant DNA. Using the fungal-specific primers ITS1F and ITS4B (Gardes and Bruns, 1993) (Table 6-2) is a possibility; however, in previous studies (Methvyn *et al*. 2000) amplification of mycorrhiza using these primers is not ideal due to amplification of non-mycorrhizal fungi and inability of primers to amplify all mycorrhizal fungi (Horton and Bruns, 2001). An effective approach was used by Puttsepp *et al*. (2004) using nested PCR, after using basidiomycete-specific primers for the first amplification and universal primers for the second.

The best option available for identifying mycorrhizal fungi within plant roots is to use specific primers; these have already been developed for the main types of mycorrhiza. After morphological examination of the roots, it should be possible to narrow down the primer sets to be used to target a particular

group of mycorrhiza. Specific primers include VANS1 (Table 6-2), designed by Simon *et al*. (1993) although this is not well conserved in all groups of AM (Clapp *et al*. 1999; Redecker *et al*. 2000). Van Tuinen *et al*. (1998) designed specific primers for VAM species, KjØller and Rosendahl (2001) designed species specific primers for *Glomus*, Helgason *et al*. (1999) designed primers AM1 that amplify most glomalean fungi and Redecker (2000) designed group specific primers for five groups of AM (Table 6-2).

AM contain multiple nuclei within their mycelium. These nuclei contain different DNA and spores of the same species can have different sequences. Due to these anomalies, molecular identification is currently useful only to identify sequence groups within AM (Redecker *et al*. 2003) largely due to heterogeneity in nuclei, undefined species concepts and the polymorphic nature of marker genes.

Table 6-2: Specific primers for arbuscular mycorhiza (AM) and ectomycorrhiza (ECM).

Primer type	Primer pair sequence	Mycorrhiza type
ECM	ITS1-F 5' –CTTGGTCATTTAGAGGAAGTAA-3' ITS4B 5'-CAGGAGACTTGTACACGGTCCAG-3'	Plant, basidiomycetes and ascomycetes (Gardes and Bruns, 1993)
	NS11 5' –GATTGAATGGCTTAGTGAGG-3' 58A2R 5' –CTGCGTTCTTCATCGAT-3'	ITS1 region of ectomycorrhizas (Martin and Rygiewicz, 2005)
	58A2F 5' –ATCGATGAAGAACGCAG-3 NLB4 5' –GGATTCTCACCCTCTATGAC-3	ITS2 region of ectomycorrhizas (Martin and Rygiewicz, 2005)
AM	VANS1 5'GTCTAGTATAATCGTTATACAGG-3' NS21 5'AATATACGCTATTGGAGCTGG-3'	Amplification of AM fungi (Simon *et al.* 1993)
	VANS1 5'GTCTAGTATAATCGTTATACAGG-3' VAGIGA 5'-TCACCAAGGGAAACCCGAAGG-3'	*Gigaspora* spp. (Simon *et al.*, 1993)
	VANS 1 5'GTCTAGTATAATCGTTATACAGG-3' VAACAU 5'-TGATTCACCAATGGGAAACCCC-3'	*Acaulospora* spp. (Simon *et al.*, 1993)
	VANS1 5'GTCTAGTATAATCGTTATACAGG-3' VAGLO 5'-CAAGGGAATCGGTTGCCCGAT-3'	*Glomus* spp. (Simon *et al.*, 1993)

6.1.5. Morphological methods of identification

Trap cultures are the generally accepted method of identifying AM communities. The cultures trap spores, which are then identified under the microscope. However, when using this method it is important to maintain a lengthy sampling period (i.e. 36 months) as AM require particular times to spore (Ferrol *et al.* 2004). Unfortunately spores of glomalean fungi can look very similar to spores of other non-mycorrhizal fungi (Redecker *et al.* 2000).

Accurate identification of glomalean fungi requires growing pot cultures and observing the different developmental stages (Morton, 1993). The only part of the life cycle of AM that can be studied in the absence of plants (Brundrett and Juniper, 1995) is spore germination into early hyphae.

Borya species have been reported as mycorrhizal (Keighery, 1984) and it is possible that they rely on these associations for a proportion of their nutrition. As the *ex-situ* plants have been grown from shoot-tip cuttings without access to field soil, they are not expected to have mycorrhizas. The rate of growth is slow and the health variable. It is possible that the *ex-situ* collection would benefit from inoculation with appropriate mycorrhizal fungi. The aim of this chapter was to find out if *B. mirabilis* was mycorrhizal naturally and if this was shared with the surrounding plant species and to assess the efforts of inoculation of *ex-situ* plants with mycorrhizal propagules.

The following questions were addressed in the mycorrhizal research on *B. mirabilis*.

1) Is the plant mycorrhizal and if so to what extent?

2) Can the mycorrhiza provide a beneficial relationship to the *ex-situ* plants when grown in conjunction?

3) Do any other associated species in the field share the same mycorrhizal associations so as to provide a source of inoculation and an indication of what habitats would be most suitable for translocation of the species?

6.2. Methods

6.2.1. *Borya mirabilis* field sampling

Root samples from *B. mirabilis* were collected in the first month of each season over a 12 month period (2004-2005) from the one known field population (Figure 6-7 and Figure 6-8). Three root samples were collected from at least three different colonies each season. Roots were obtained by carefully digging approximately 10 cm below the soil at the base of the plants. Samples were placed in 50% ethanol and taken back to the laboratory for clearing and staining. Root samples were gently rinsed and placed in 10% KOH, warmed to 90°C for a period of 2 hours and left to cool and clear for 30 minutes before being rinsed twice with distilled water for 5 minutes. The roots were placed in warm lactofuchsin (McLean and Lawrie, 1996) for 5 minutes, cleared in three changes of warm glycerol and stored in 50% glycerol for examination. Thirty root samples were randomly chosen from each processed clump and observed with a light microscope. The percent mycorrhizal infection of each of these roots was determined by determining the total root area using a micrometer and dividing this by the area infected with mycorrhiza and multiplying by 100. Morphology, type of mycorrhiza present and percentage infection were recorded.

Figure 6-7: *Borya mirabilis* field site from which roots of *B. mirabilis* and associated species were removed seasonally.

Figure 6-8: *Borya mirabilis* going through resurrection changes in autumn when sampling for mycorrhizal associations.

6.2.2. Mycorrhizal pot trials

Root samples of *B. mirabilis* taken from the field site were used to inoculate three pots of *B. mirabilis* (*ex-situ* population from the Royal Botanic Gardens, Melbourne (RBG)). The plants were treated with an all-purpose fungicide (Benlate) and furalaxyl prior to inoculation (the *ex-situ* plants grown from cuttings had been examined for pathogens and mycorrhizal associations in the next chapter and were found not to be mycorrhizal) and repotted in sterile potting media with mycorrhizal inoculum. Precautions against airborne re-infection were achieved by layering the field soil around the root zone of the potted plants with sterile potting mix above and below the field soil (collected from 10 cm below the soil line at the base of *B. mirabilis* plants in June), with a layer of sterile polyethylene beads over the surface and a sealed sterilised plastic watering tube inserted to two-thirds the depth of the pot so that the plants could be watered with sterile water without washing down airborne contamination (Figure 6-9). The pots were left for 3 months to infect roots.

Subsequently, the roots and soil of these plants were used to inoculate the remaining half of the *ex-situ* population, with 11 plants being inoculated and 11 left as control. Both treatments were housed in a quarantine greenhouse at the RBG and in all other respects were treated equally. The plants were monitored once a month for 6 months and number of shoots and general health recorded. At the conclusion of the trial, root samples from all 22 plants were cleared, stained and viewed for the presence/absence and type of mycorrhiza.

Figure 6-9: *Borya mirabilis ex-situ* plants inoculated with field soil and roots from the *Borya mirabilis* field population.

6.2.3. Associated species - mycorrhizal examination

A list of associated species previously prepared by an ecological study conducted on the site (Coates, 2000) was used as a guide to the associated species to be sampled (Table 6-3). During winter 2005, three root samples from each of three individual plants from each species associated with *B. mirabilis* in the field were removed. Vouchers of each plant species were identified by staff at the RBG to ensure correct naming of plants. Roots were stored in 50% ethanol, cleared and stained as for *B. mirabilis*, with the exception of tougher roots requiring longer clearing times depending on the species, and examined for the presence of mycorrhiza. Thirty slides from each species were examined and type of mycorrhizal infection recorded. The roots of plants appearing to be ectomycorrhizal were embedded in wax and cross-sectioned to observe any internal structures (refer to section 3.2.3.2 and 3.2.3.3 for methodology).

Table 6-3: Associated plant species within the field population site of *B. mirabilis*.

Dominant species	Associated species	Annual herbs	Rare species	Other
Calytrix tetragona Labill.	*Austrodanthonia setacea* (R.Br.) H.P. Linder	*Centrolepis aristata* (R.Br.) Roem. & Schult **.	*Austrostipa hemipogon* (Benth.) S.W.L. Jacobs & J. Everett **	Bryophytes**
Dodonaea viscosa subsp. *spatulata* (Sm.) J.G.West	*Acacia stricta** Andrews (Willd)	*Centrolepis strigosa* (R.Br.) Roem. & Schult. **		Lichens**
Grevillea aquifolium Lindl.	*Callitris rhomboidea* R.Br. ex Rich. & A. Rich.	*Drosera peltata* subsp. *auriculata* (Planch.) B.J. Conn		
Kunzea parvifolia Schauer.	*Eucalyptus alaticaulis* R.J. Watson & Ladiges	*Drosera whittakeri* subsp. *aberrans* Lowrie & Carlquist		
Melaleuca decussata R.Br.	*Gonocarpus mezianus* (Schindl.) Orchard	*Siloxerus multiflorus* Nees **		
	*Grevillia alpina** Lindl			
	Leptospermum scoparium J.R. Forst. & G.Forst.			
	Lepidosperma viscidum R.Br.			
	Leucopogon virgatus (Labill.) R.Br.			
	Phyllanthus hirtellus F. Muell. ex Mull.Arg			
	**Thelymitra* spp.			
	* *Themeda triandra* Forssk.			
	*Thryptomene calycina (*Lindl.) Stapf.			

* Additional plants found to be associated when sampling, not listed in (Coates, 2000).

** Plants not sampled.

6.2.4. Mycorrhiza-molecular identification

At the same time as samples were collected for microscopic examination, parallel root samples were taken for molecular analysis of the mycorrhiza of each species. These samples were stored in McCartney bottles with silica gel and kept at -20°C until used.

DNA was extracted from five species with abundant AM, including *B. mirabilis*. The species chosen were *Dodonaea viscosa*, *Callitris rhomboidea*, *Thelymitra* sp. and *Themeda triandra*. DNA was extracted for each species from 100 mg samples of roots using a Plant DNeasy Mini Kit (Qiagen), including the extra centrifugation step, following the manufacturer's instructions. Extracts were stored at -20ºC.

Separate samples of DNA extracts were purified in three different ways in an attempt to provide extracts that would amplify with the fungal primers. The three different methods used were a Qiagen DNA Purification Kit (Qiagen, Clifton Hill, VIC), CTAB (Doyle, 1991) and using Bio-Spin polypropylene columns (Bio-Rad Laboratories, North Ryde, NSW) filled with sepharose CL-6B (Fluka, Castle Hill, NSW) (Cullen and Hirsch, 1998).

The primers used to amplify the root extracts were VANS1 (5'GTC TAG TAT AAT CGT TAT ACA GG-3') and NS21 (5'AAT ATA CGC TAT TGG AGC TGG-3') for AM fungi (Simon *et al.* 1992) and VANS1 (5'GTC TAG TAT AAT CGT TAT ACA GG-3') and VAGLO (5'-CAA GGG AAT CGG TTG CCC GAT-3') for *Glomus* spp. (Simon *et al.* 1993). The PCR reactions contained 2 µl of extract, 12.5 µl of GoTaq® Green Master Mix (Promega Pty Ltd), 0.5 µl of each primer and 9.5 µl of dH_2O.Samples were run on a PCR cycle of 94ºC for 3 min, followed by 30 cycles of: 94ºC for 30 s, 58ºC for 1 min and 72ºC for 60 s with a final extension of 72ºC for 10 min. Samples were kept at 4ºC. All PCR products were run on a 1.4% agarose gel post-stained with ethidium bromide as in Chapter 4.

6.3. Results

6.3.1. *Borya mirabilis* field sampling

The roots of *B. mirabilis* were unique in structure, containing small nodules along the lengths of the lateral roots (Figure 6-10). These nodules often would appear on top of each other as off-shoots along the lengths of the roots. Arbuscular mycorrhiza were contained inside cells within these nodules.

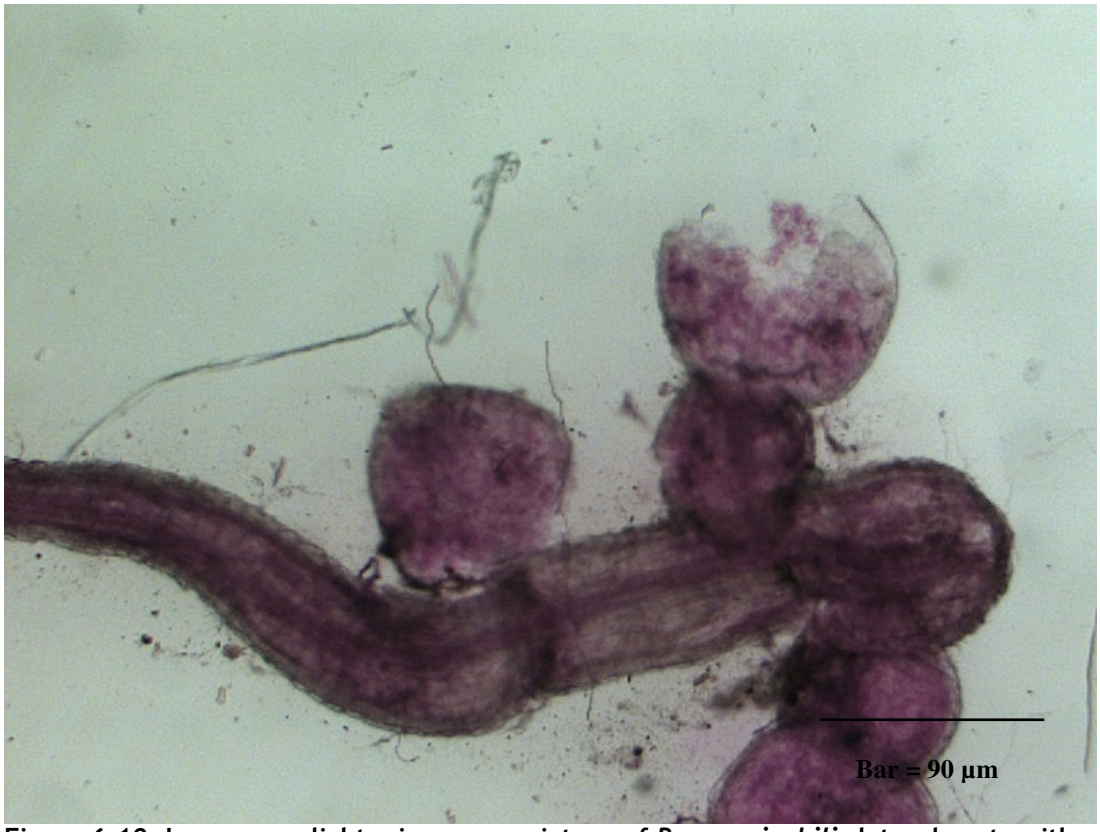

Figure 6-10: Low power light microscope picture of *Borya mirabilis* lateral roots with nodular offshoots containing mycorrhiza stained with lactofuchsin.

The roots of *B. mirabilis* had predominantly arbuscular mycorrhiza (Figure 6-11) as well as scattered ectomycorrhiza (Figure 6-12). The arbuscular mycorrhiza appeared as spores contained within the nodules on the roots in summer (Figure 6-13) and as Paris-type coils inside the nodule cells in winter (Figures 6-14 to 6-15).

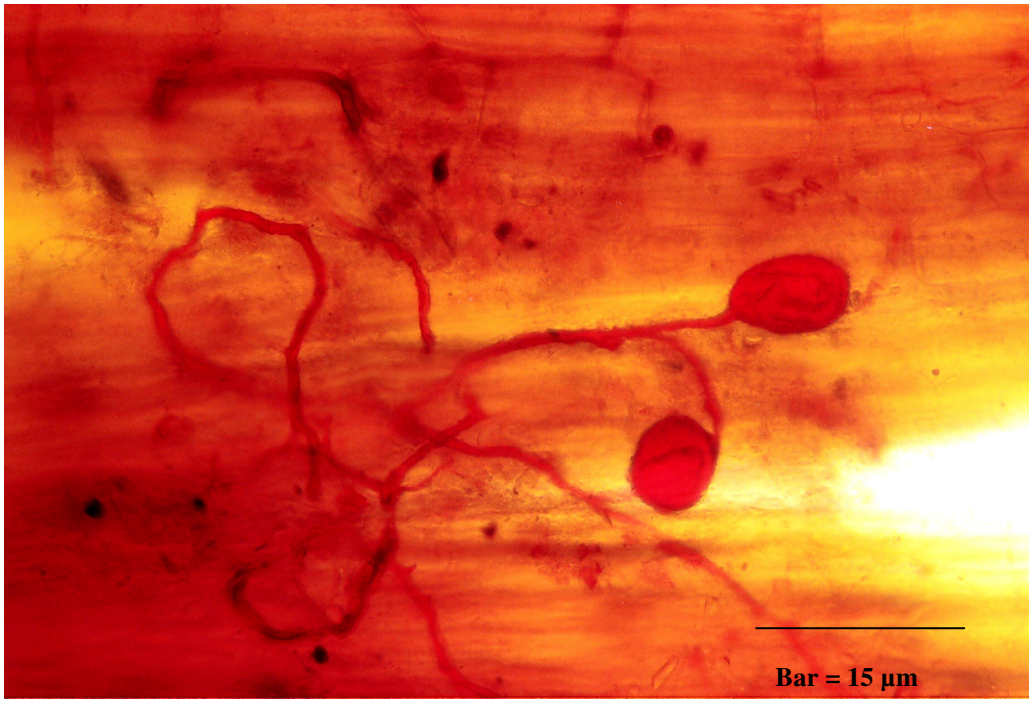

Figure 6-11: Arbuscular mycorrhiza in June within main root section of *Borya mirabilis*, stained with lactofuchsin.

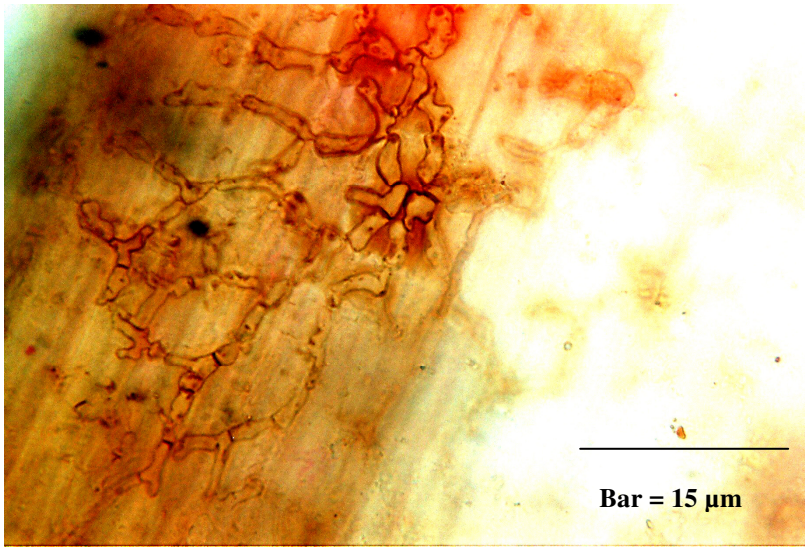

Figure 6-12: Ectomycorrhiza in June of *Cenoccoccum* type growing on surface of roots of *Borya mirabilis*, stained with lactofuchsin.

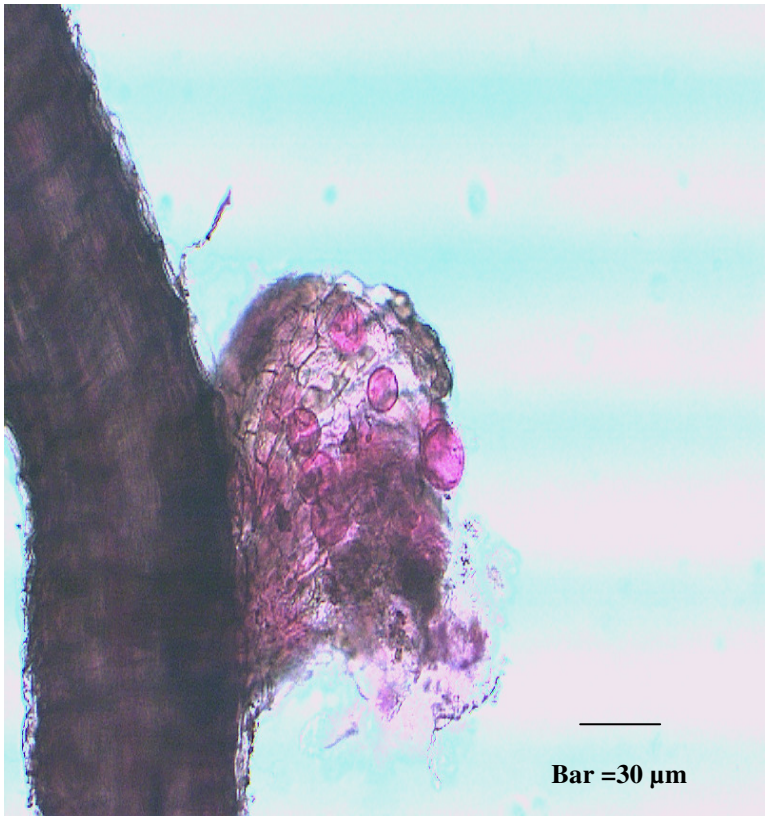

Figure 6-13: *Borya mirabilis* root with nodule containing spores over summer, stained with lactofuchsin.

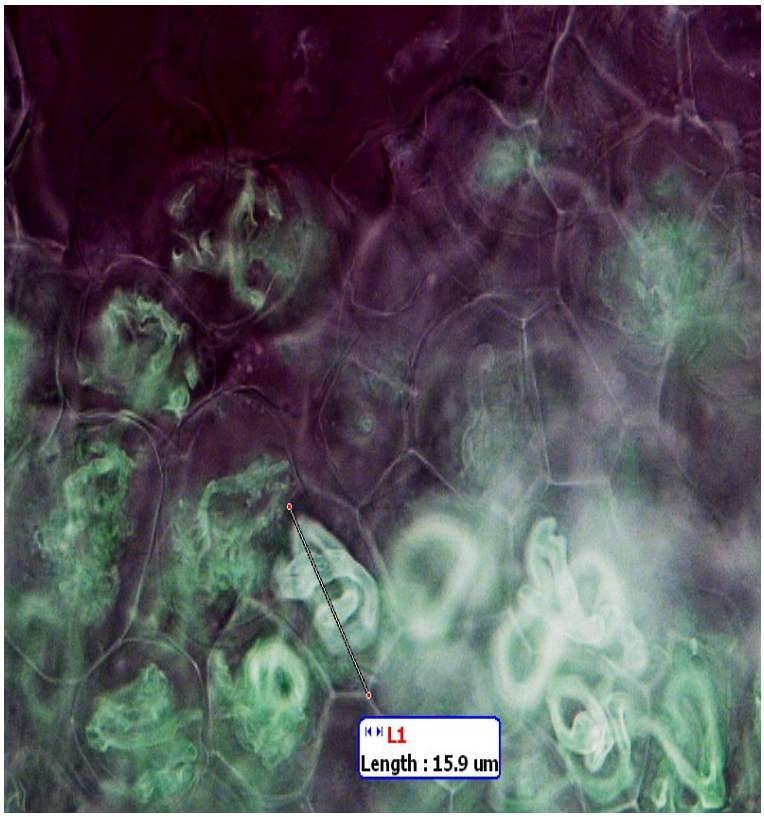

Figure 6-14: Inside a *Borya mirabilis* nodule containing Paris-type arbuscular mycorrhizal coils in winter, stained with lactofuchsin viewed with ultraviolet light.

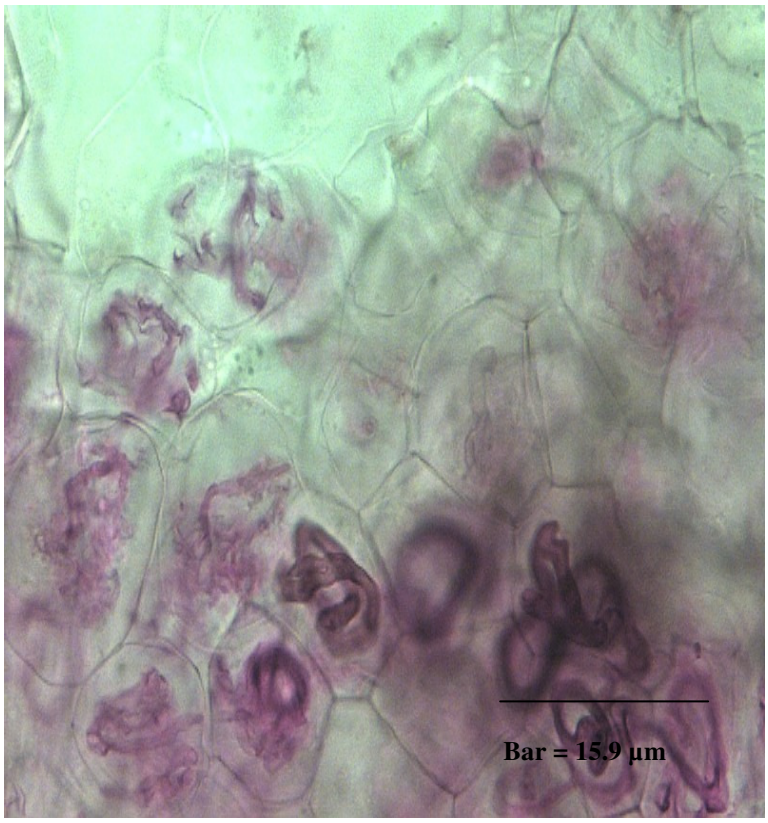

Figure 6-15: Inside a *Borya mirabilis* nodule containing Paris-type arbuscular mycorrhizal coils in winter, stained with lactofuchsin.

Both infection and structures observed varied with season (Table 4). Total infection was greatest in winter and spring (when coils were dominant) and almost absent during the dormant period of summer, when spores were dominant (Figure 6-13). Roots contained predominantly arbuscular mycorrhiza in all seasons, with more variation in the amount of AM than ECM.

Table 6-4: Mycorrhizal associations of *Borya mirabilis* over one year, showing average infection of 30 roots from each season and the type of arbuscular mycorrhizal infection.

Season	Mean total % infection	Mean % infection AM	Mean % infection ECM	AM structures
Summer	0.8 ± 0.24	0.43	0.37	Spores
Autumn	7.8 ± 0.88	4.6	3.2	Spores/coils
Winter	17.6 ± 0.87	14.9	2.7	Coils
Spring	12.4 ± 0.92	12	0.4	Coils

6.3.2. Mycorrhizal pot trials

The shoot number increase of the inoculated plants was greater than those left un-inoculated (Figure 4) (ANOVA, F= 3.63, P = 0.07).

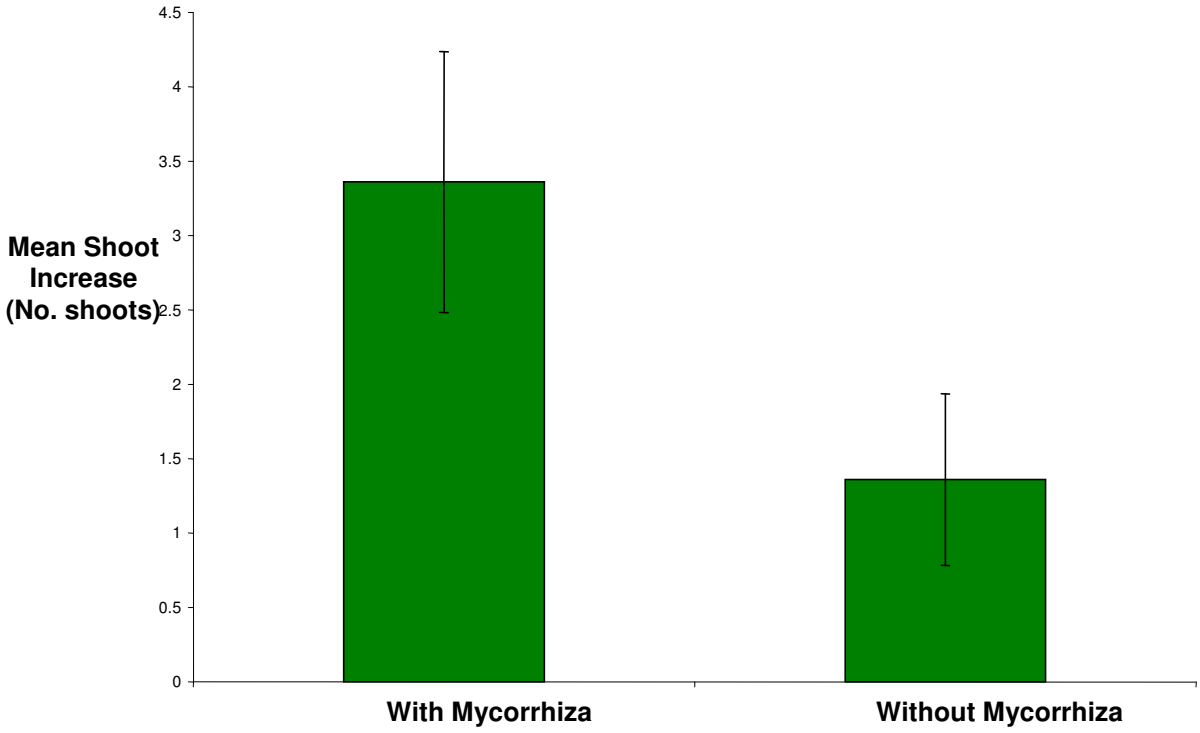

Figure 6-16: Difference in mean shoot increase between *Borya mirabilis* plants inoculated with mycorrhiza and those without over 6 months. Bars refer to 2X standard error of the mean of each treatment.

6.3.3. Associated species - mycorrhizal examination

Table 6-5 outlines the presence or absence of each type of mycorrhizal association found with each associated plant sampled from the *B. mirabilis* field site. Figures 6-17 to 6-31 show pictures of the various mycorrhizal associations found on the associated plants. Thirteen of the nineteen associated species also had vesicular arbuscular mycorrhizal associations, 12 had ectomycorrhizal associations and 11 of these species had both ectomycorrhizal associations and abuscular mycorrhizal associations. Of these associated species, *Callitris rhomboidea* displayed the most morphologically similar mycorrhizal association. Also of interest were *Acacia stricta*, *Kunzea parvifolia*, *Thelymitra* sp. and *Themeda triandra*, as they displayed arbuscular mycorrhiza in the majority of slides examined.

Table 6-5: Species associated with *Borya mirabilis* and the presence (P) of each type of mycorrhizal association, the dominant type represented in bold.

Species	*ECM*	*AM*	*Ericoid*	*None*	*Orchid*	*Proteoid Root*
Acacia stricta	*P*	***P***				
Austrodanthonia setacea				***P***		
Callitris rhomboidea		***P***				
Calytrix tetragona	*P*	*P*				
Dodonaea viscosa	*P*	***P***				
Drosera sp.				***P***		
Eucalyptus alaticaulis	***P***	*P*				
Gonocarpus mezianus	*P*	*P*				
Grevillea aquifolium						***P***
Grevillea alpina	***P***	*P*				
Kunzea parvifolia	*P*	*P*				
Lepidosperma viscosa				***P***		
Leptospermum scoparium	*P*	***P***				
Leucopogon virgatus			***P***			
Melaleuca decussata	*P*					
Phyllanthus hirtellus	***P***	*P*				
Thelymitra sp.		***P***			*P*	
Themeda triandra		***P***				
Thryptomene calycina	***P***	*P*				

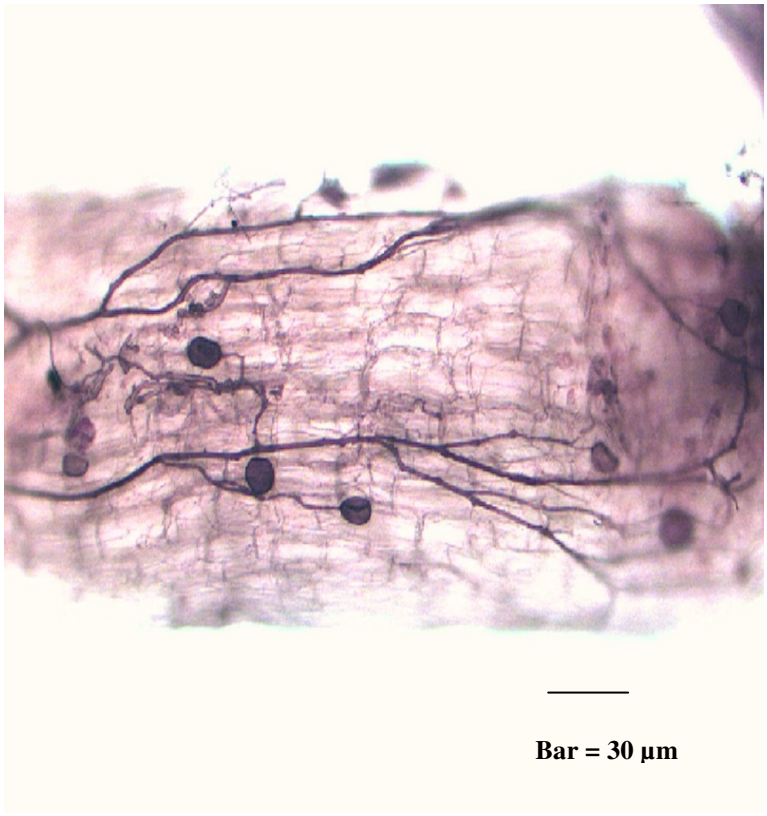

Figure 6-17: Arbuscular mycorrhiza on field roots of *Acacia stricta* from *Borya mirabilis* field site, stained with lactofuchsin.

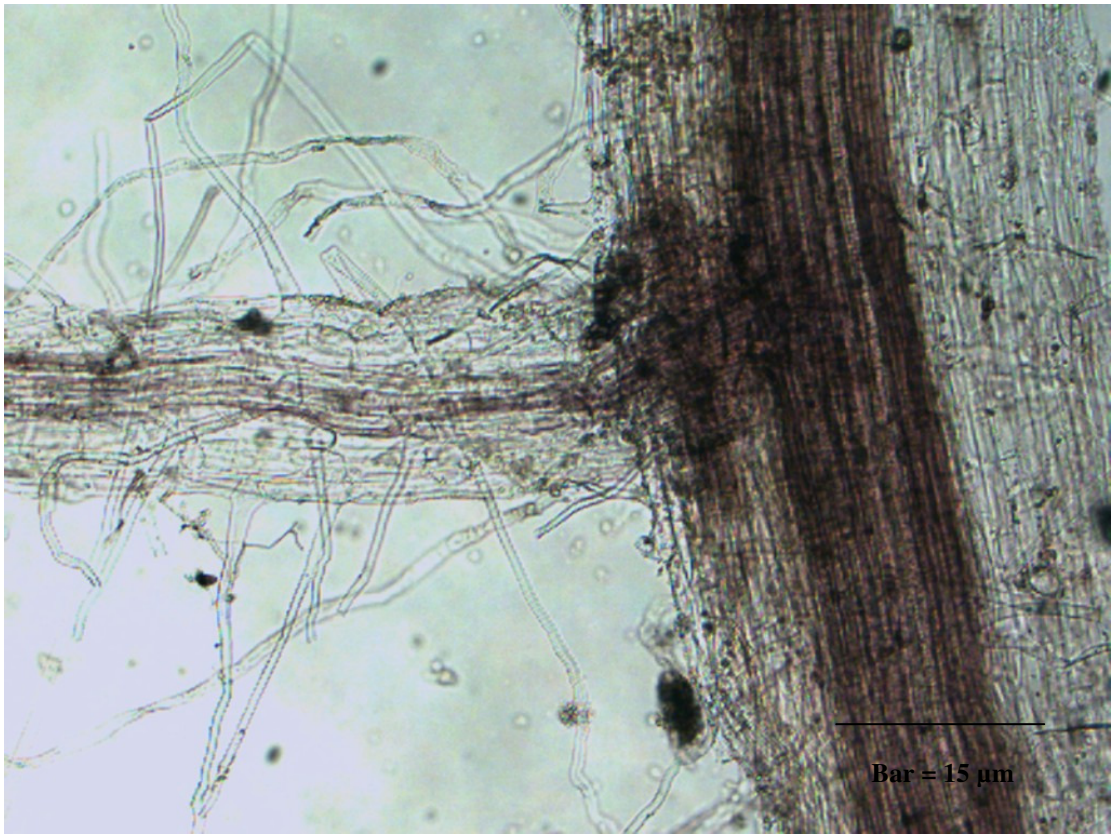

Figure 6-18: Root hairs of *Acacia setacea* roots, stained with lactofuchsin.

Figure 6-19: Paris-type arbuscular mycorrhiza in *Callitris rhomboidea* roots associated with *Borya mirabilis*, stained with lactofuchsin.

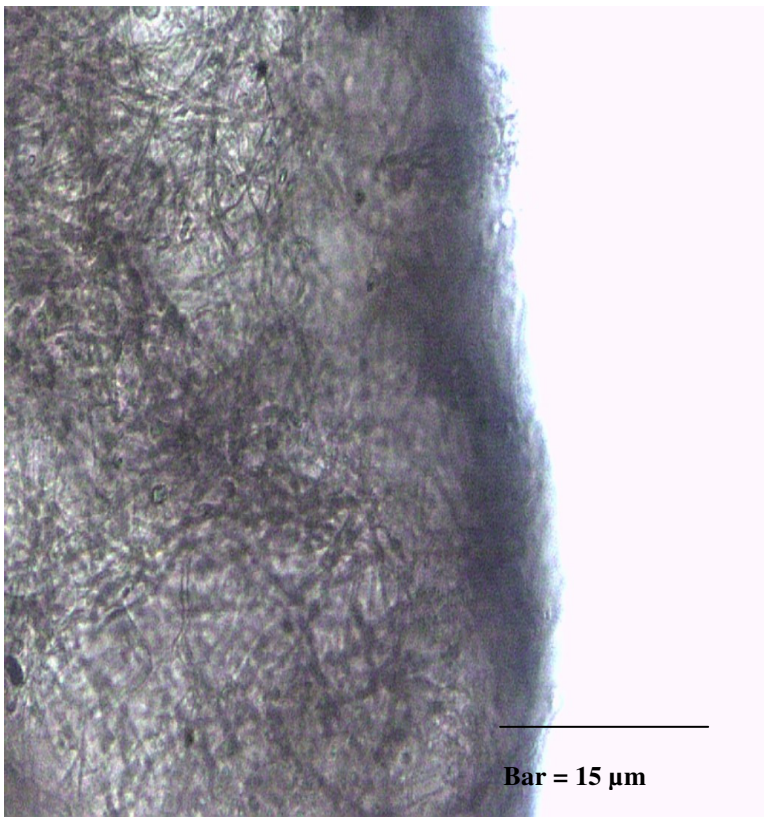

Figure 6-20: Ectomycorrhiza on *Eucalyptus alaticaulis* roots at *Borya mirabilis* field site, stained with lactofuchsin.

Figure 6-21: Ectomycorrhiza on lateral roots of *Grevillea alpina*, stained with lactofuchsin, as shown by hyphae over the surface of the lateral root.

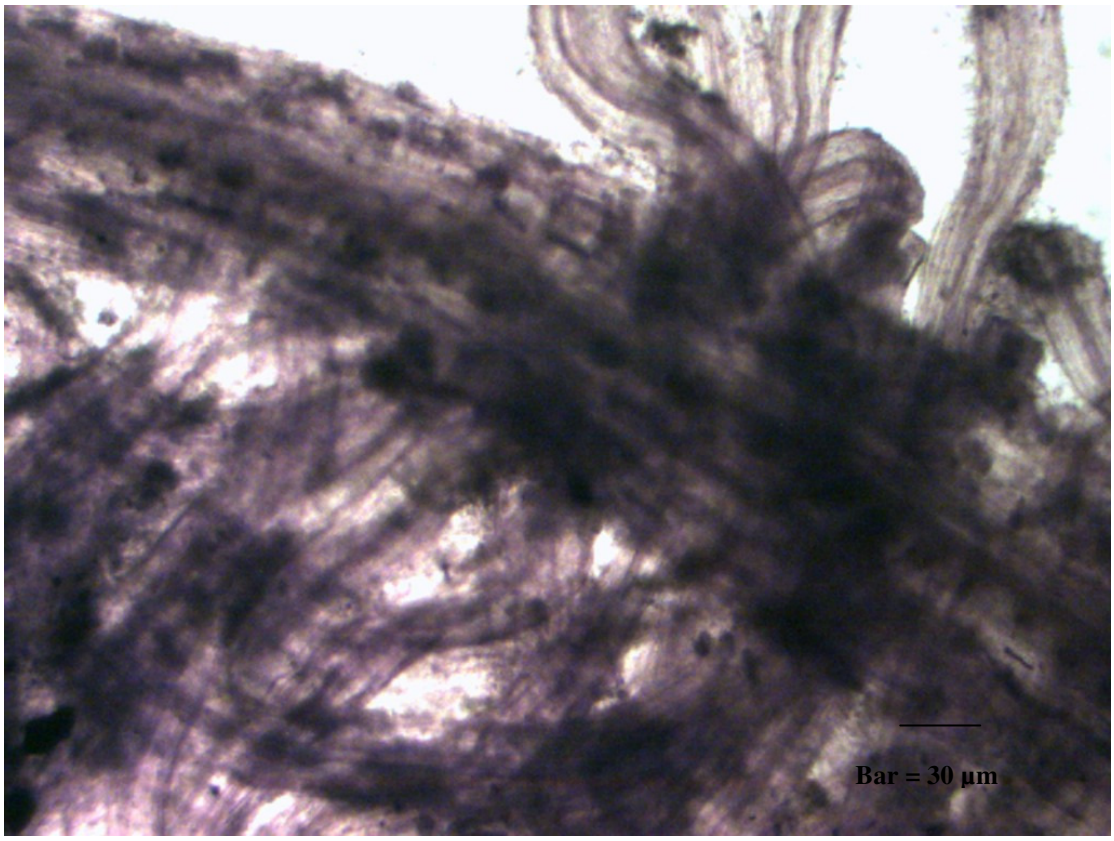

Figure 6-22: Proteoid roots of *Grevillea aquifolium*, stained with lactofuchsin**.**

Figure 6-23: Ericoid mycorrhiza on *Leucopogon virgatus* roots, stained with lactofuchsin.

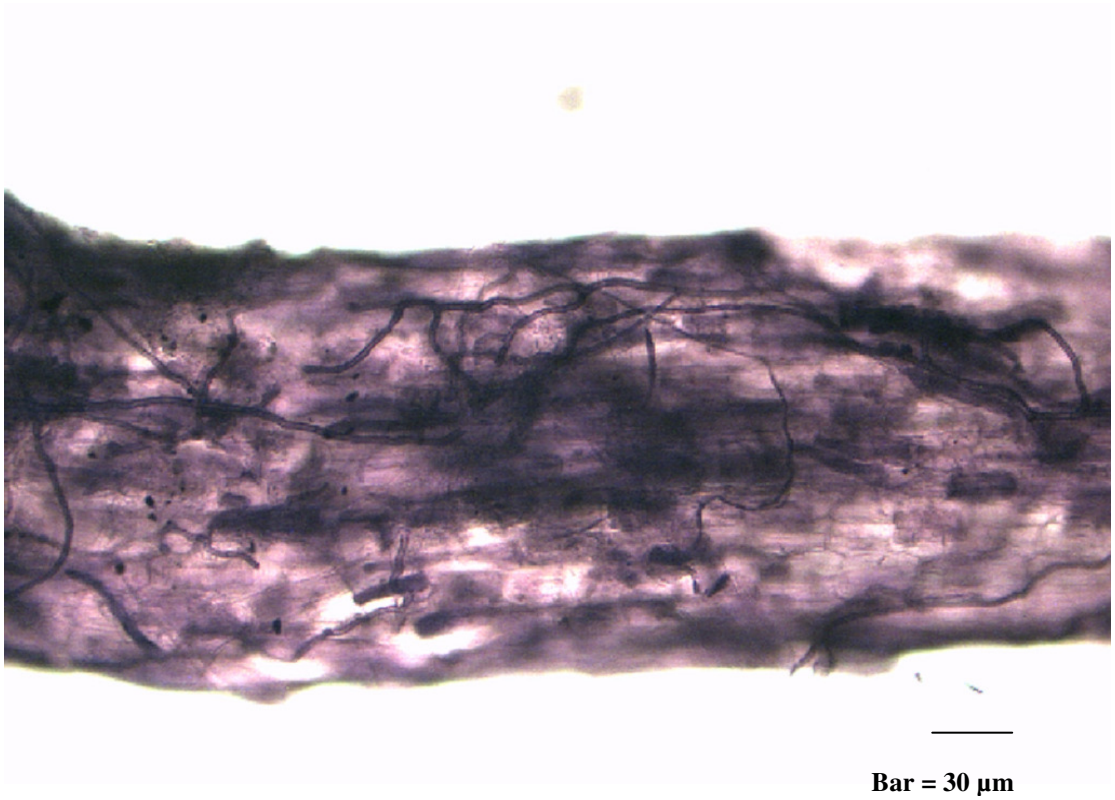

Figure 6-24: Thick external hyphae on *Melaleuca decussata* roots no sheath apparent, stained with lactofuchsin.

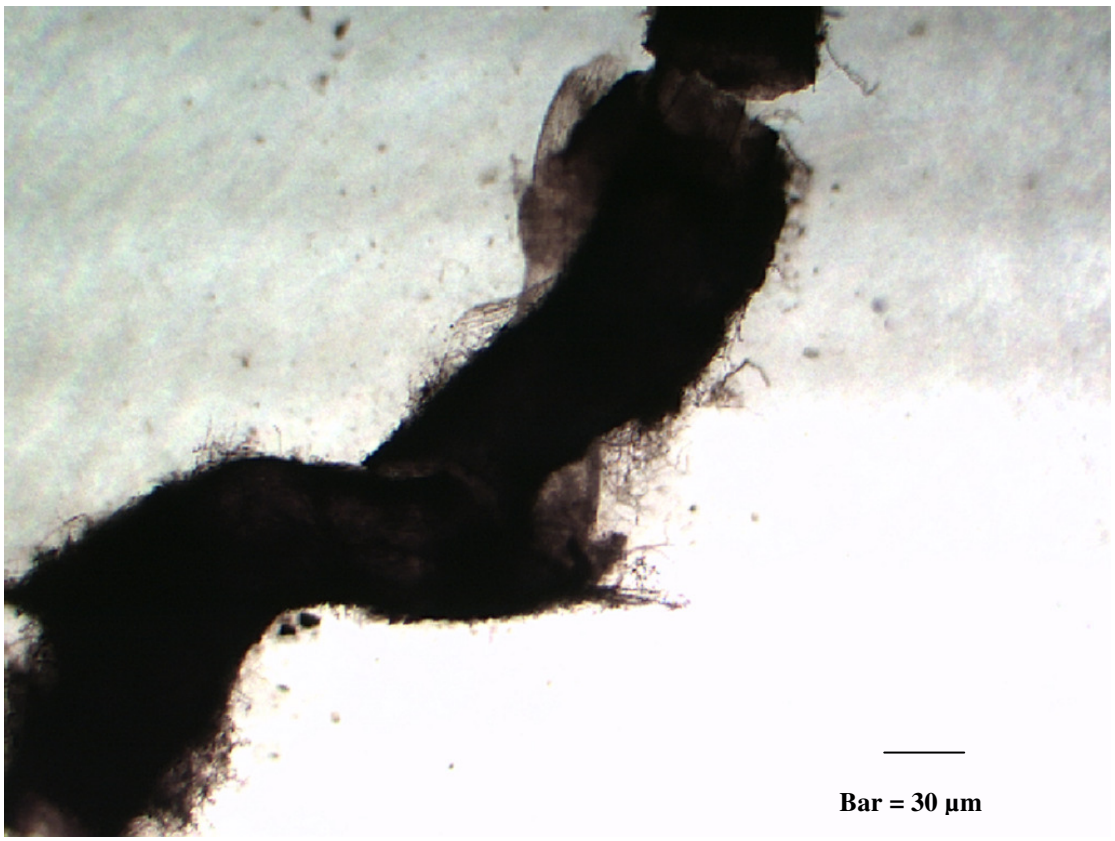

Figure 6-25: Ectomycorrhiza on *Phyllanthus hirtellus* roots, stained with lactofuchsin.

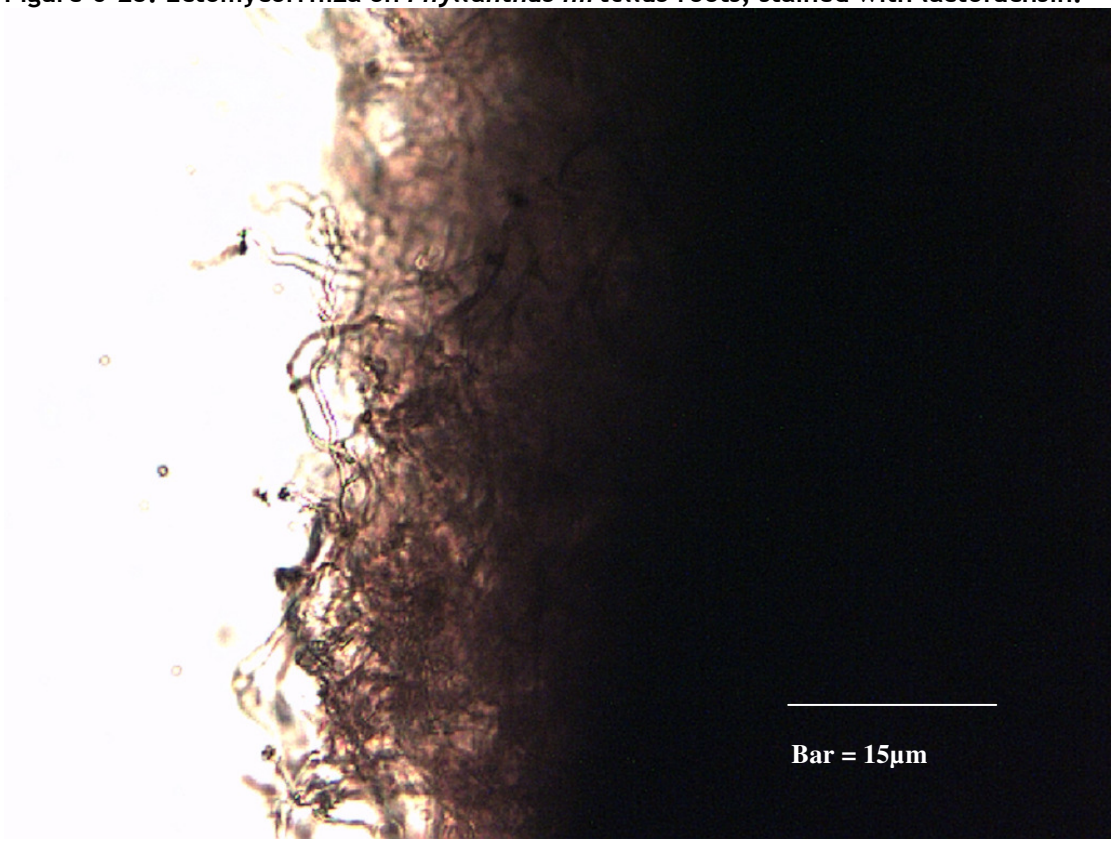

Figure 6-26: Dense ectomycorrhizae on *Phyllanthus hirtellus* roots, stained with lactofuchsin.

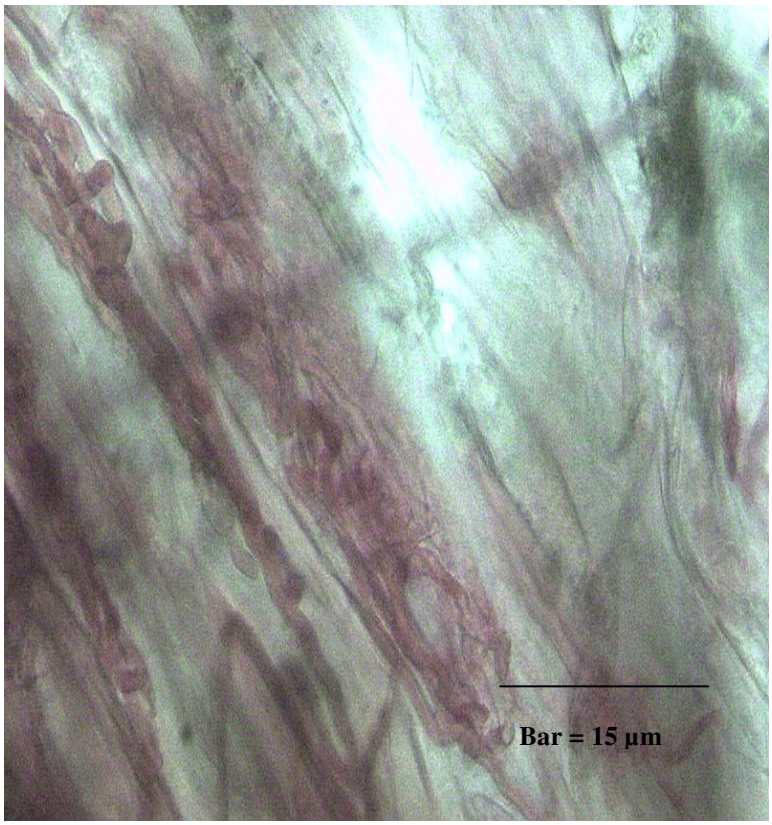

Figure 6-27: Arbuscular mycorrhiza type mycorrhiza within *Thelymitra* sp. roots associated with *Borya mirabilis*, stained with lactofuchsin, thick endophytic hyphae with no pelotons present.

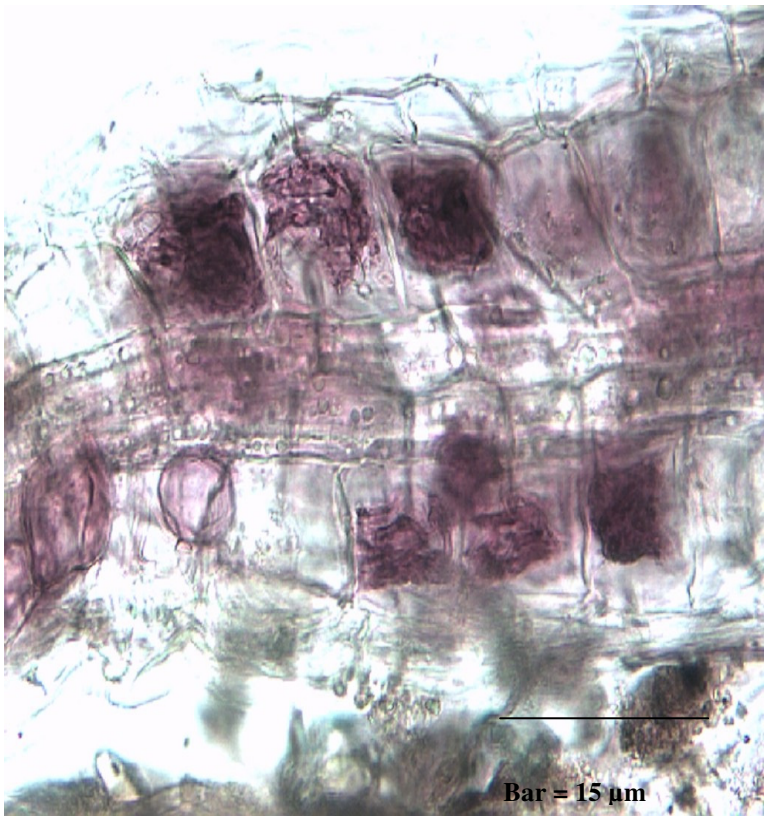

Figure 6-28: Arbuscular mycorrhiza in *Themeda triandra* roots associated with *Borya mirabilis*, stained with lactofuchsin.

Figure 6-29: Arbuscular mycorrhiza in *Themeda triandra* roots associated with *Borya mirabilis*.

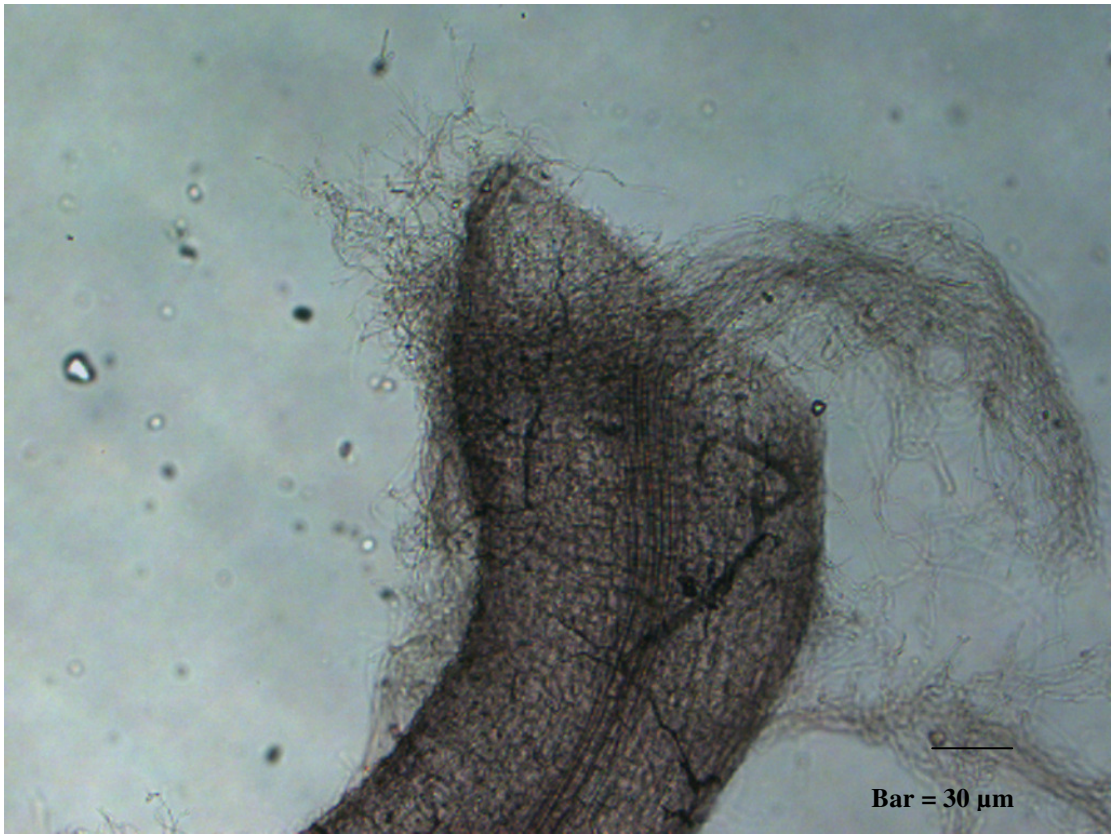

Figure 6-30: Ectomycorrhiza on *Thryptomene calycina* roots, stained with lactofuchsin.

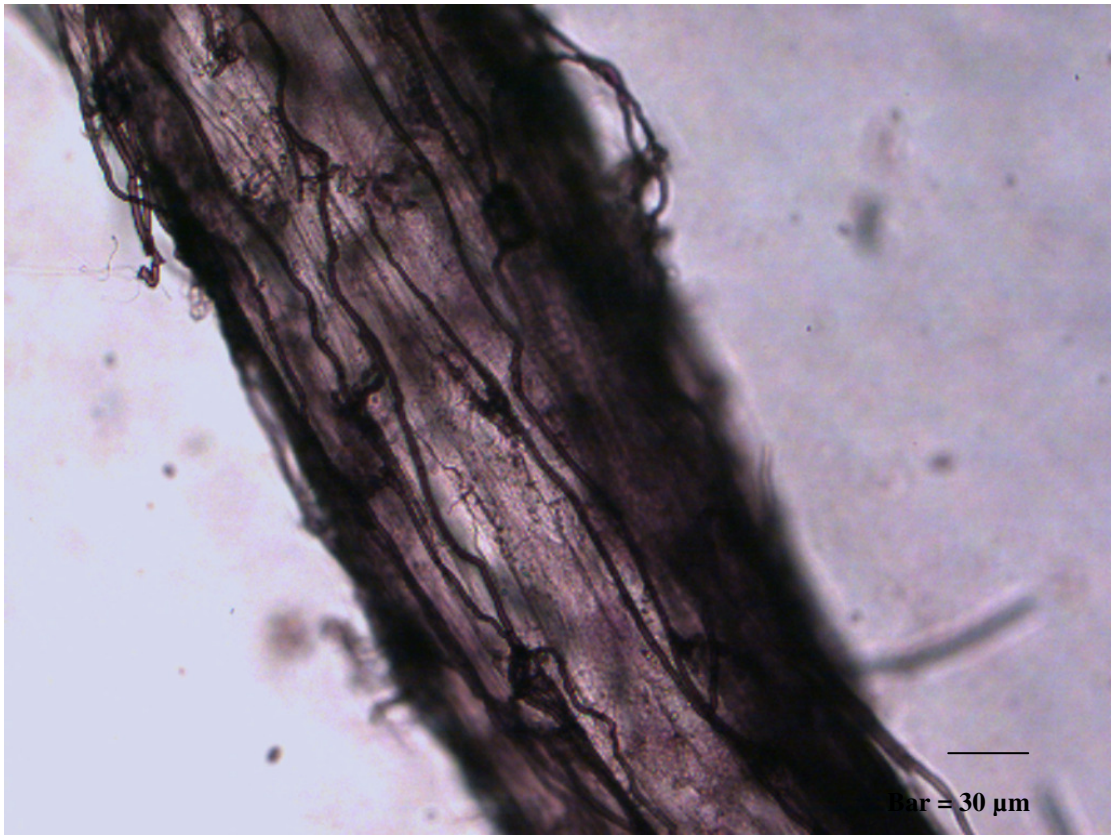

Figure 6-31: Thick external hyphae on *Thryptomene calycina*, stained with lactofuchsin.

6.3.4. Mycorrhiza - molecular identification

Clear extracts were obtained for each of the five species (Figure 6-30) and appeared to be of good quality, without smearing. No amplification product was obtained using any of the three primer sets, despite several methods of purifying unwanted substances out of the extract, and reducing the annealing temperature to 50°C.

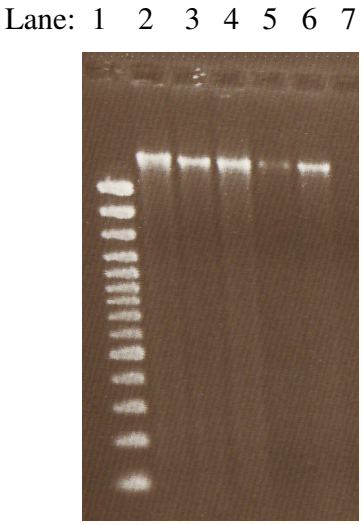

Figure 6-32: DNA extracts of roots containing AM fungi, Lanes 1-6 (left to right): Molecular weight marker (GeneRuler 1 kb), DNA extracts from *Thelymitra* sp., *Callitris rhomboidea*, *Borya mirabilis*, *Themeda triandra* and *Dodonaea viscosa*.

6.4. Discussion

The research in this chapter has demonstrated for the first time that *B. mirabilis* has a significant mycorrhizal association and that it is typical of nodular vesicular-arbuscular mycorrhizal associations. Furthermore these mycorrhiza were beneficial to the growth of *ex-situ B. mirabilis* plants. Several plants associated with the natural population of *B. mirabilis* also had arbuscular mycorrhizal associations with similar morphology, the most similar being that of *C. rhomboidea* showing Paris-type coils, suggesting that it could provide a reservoir of fungi for *B. mirabilis*.

6.4.1. *Borya mirabilis* field sampling

The morphological analysis of *B. mirabilis* roots over all seasons demonstrated *B. mirabilis* to have a predominantly AM association, with the mycorrhizal mostly occurring within the small nodules of the lateral roots. This is the first report of seasonal changes in the AM structures associated with *Borya* species or nodulated AM species. It would therefore be interesting to see if these same seasonal changes occur in other *Borya* species and if the morphological seasonal changes still occurred if plants were not allowed to go through their desiccation-tolerant phase in summer, in *ex-situ* conditions.

No mycorrhizal studies on *Borya* species have been published; however, Keigherley (1984) refers to Western Australian *Borya* species as being both ectomycorrhizal and arbuscular mycorrhizal. No mention of the nodular formations on the *Borya* roots or the AM association residing inside them has been mentioned in the literature.

AM fungi, usually belonging to the Glomales, are associated with plant roots, forming mycorrhizal nodules (Huguenin, 1969). These nodule-inhabiting mycorrhiza are associated with many species in the Podocarpaceae (Saxton, 1930; Morrison, 1963), Araucariaceae, Phyllocladaceae, Casuarinaceae and Caesalpiniodeae in the legumes (Béreau and Garbaye, 1994), This type of AM mycorrhiza is characterized by the cortex of the root being colonized, but

without any association in the endodermis. The cortex is also surrounded by a periderm which contains several layers of dead cells (Duhoux *et al*. 2001); this is unlike normal AM associations, which only infect unsuberised roots (Duhoux *et al*. 2001).

The nodular roots of *B. mirabilis* do not have determinate development, unlike most nodular mycorrhizal associations (Duhoux *et al*. 2001). In *B. mirabilis* a new nodule can form from the tip of old nodules, leading to an elongated nodular lateral root like a string of pearls. *Gymnostoma nodiflorum* (Casuarinaceae) (Duhoux, 2001) is one of the few plant species with nodular mycorrhizal roots that also has this ability (to reactivate the apical meristem to extend its lateral roots).

6.4.2. Benefits of mycorrhiza

The addition of nodule-inhabiting AM fungi from the field site of *B. mirabilis* to the *ex-situ* population improved plant growth. Benefits including increased growth, health and nutrient uptake of *Endogone* mycorrhiza (nodule-inhabiting) have been documented since the 1950's (Baylis, 1959) and thus the improved growth of the *ex-situ* population was expected.

Arbuscular mycorrhiza cannot currently be cultured asymbiotically. They require host roots to remain viable. This poses difficulties for how to enhance the recovery of the *B. mirabilis* population, not only in the *ex-situ* collection, but also in the reintroduction sites. For the *ex-situ* population, it is suggested that the mycorrhiza from the field be introduced and kept in labelled pots, and that the roots from these plants are then used to inoculate cuttings as they become available. In this way, those plants translocated to external sites will already be inoculated with beneficial mycorrhiza that will presumably aid their establishment and growth.

6.4.3. Associated species - mycorrhizal examination

As collection of endangered plant roots, especially from the field site, which is infected with *P. cinnamomi*, is not a viable means of introducing the

mycorrhiza to *ex-situ* populations, the associated plants were examined. The associated mosses on the field site were not examined, as mycorrhizal associations have never been observed in mosses (Read *et al*. 2000; Davey and Currah, 2006). Several seasonal species and the other threatened plant species on the site were also not examined due to low numbers of these species on the site.

The most morphologically similar mycorrhiza of the associated species was that of *C. rhomboidea*. The AM of *C. rhomboidea* have not been described previously; however, Pattinson *et al*. (2004) mentions the species as exclusively AM based on G. S. Pattinson's, unpublished data. Other *Callitris* species are well known for being mycorrhizal with AM. In particular, *C. glaucophylla* has AM mycorrhiza that is morphologically similar to that found on *C. rhomboidea* (Dickson *et al*. 2007). This suggests that *C. rhomboidea* could be used as a source of inoculum for pots and translocation sites, though trials are needed with *ex-situ* plants to see if they are beneficial. It may also be vital to have *C. rhomboidea* as a reservoir on translocation sites providing inoculation for *B. mirabilis*, depending on the outcome of *ex-situ* trials using *C. rhomboidea* as inoculum.

The majority of mycorrhizal associations from the *B. mirabilis* field site correlated with reports of known mycorrhizal associations (Wang and Qiu, 2006; Brundrett, 2008). There were several unexpected results, in particular that of *Grevillea alpina* (Proteaceae) which would be expected to have proteoid roots and thus no mycorrhiza, however, it is not alone in the Proteaceae in having an AM association, these associations also being recorded in *Banksia ericifolia* and *Telopea speciosissima* (Wang and Qiu, 2006). *Thelymitra* species are known to contain orchid mycorrhiza (Brundrett, 2008) but the roots also appeared to contain AM mycorrhizas; one other orchid, *Phaius mishmensis* also contained AM in its roots (Wang and Qiu, 2006). The *Thelymitra* species on site occur amongst the *B. mirabilis* plants and it is therefore not surprising that they may share some

of the same fungal associations, even if they are not proven to be beneficial.

6.4.4. Mycorrhiza - molecular identification

Molecular identification of the fungi forming the nodular mycorrhiza of *B. mirabilis* and the AM associations of four other associated field species of *B. mirabilis* was unsuccessful under the conditions used. While DNA was extracted from those species including *B. mirabilis* with similar mycorrhizal associations, the DNA proved un-reactive under the conditions trialled. Three different methods were trialled to remove impurities; however, the DNA was still unreactive with the primers tested (White *et al*. 1990). As the DNA was unreactive with the ITS1-4 universal primers as well as the AM primers it is unlikely that this is due to the primers, as the universal primers should have amplified the plant DNA. Further methods of purification to render the DNA reactive should be tested.

Future work on the nodular mycorrhizal associations of *B. mirabilis* should more precisely determine the structure of the AM fungi inside the nodules and should attempt to identify the mycorrhiza by careful washing and removal of the nodules before extraction, preferably with fresh material which may increase the initial concentration of fungi in the extract compared to plant DNA. The AM fungal DNA should then be amplified using the fungal primer AM1 and the universal eukaryotic primer NS31 as amplification was successfully achieved using these primers with nodular mycorrhiza from the Glomalean mycorrhiza growing in root nodules of *Gymnostoma* (Duhoux *et al*. 2001).

With the aid of molecular identification, the arbuscular mycorrhiza that was associated with the species in the field and was beneficial in the *ex-situ* collection could be identified. Furthermore, it could be tested to see if it is common to other associated species in particular *C. rhomboidea* also containing arbuscular mycorrhizal associations. If this was the case, then these non-endangered species' roots could be used for further fresh

inoculations of the mycorrhiza from the existing field site to the *ex-situ* population at the Botanic Gardens.

7. *Borya mirabilis* translocation-pathogen assessment

7.1. Introduction

Of concern when managing an endangered species are the threats that will directly affect the continued existence of the species. *B. mirabilis* is susceptible to *P. cinnamomi* (Reiter *et al*. 2004), a notorious plant pathogen That reduces plant health and commonly results in plant death. The risk of infection with *P. cinnamomi* is the most significant threat facing the translocation sites of *B. mirabilis*.

The 2007 draft Recovery Plan (Kohout and Coates in prep) for *B. mirabilis* lists the protection of plants from infection by *P. cinnamomi* as a specific objective, in particular the protection of both the wild and translocated population sites. The current strategy to manage the risk posed by *P. cinnamomi* includes: implementation of hygiene practices to reduce infection and spread of the pathogen, providing signage to inform the public of the pathogens presence and help the public avoid inadvertent spread, mulching of the wild site to increase beneficial microbes, spraying the plants with Foli-R-Fos® (phosphate) to increase the plants' defense responses against the pathogen and monitoring the pathogen within the field site.

Any potential translocation site for *B. mirabilis* thus needs to be screened for the presence of *P. cinnamomi*. Once a suitable pathogen-free site exists, precautions need to be undertaken to prevent the infestation of this site with pathogens inadvertently through the translocation of *B. mirabilis*. The following work was conducted within the greater context of translocation of some of the *ex-situ* population of *B. mirabilis* back into the wild. Of particular importance to this was the assessment of the *ex-situ* population and possible translocation sites for pathogens. Particulars of translocation precautions are outlined in the guide for the translocation of threatened plants in Australia (Vallee *et al*. 2004). In particular, Section 6.3.1 deals with the risk of disease transfer (from nurseries and elsewhere) into the

wild. Section 5.3.2 deals with using a industry-accredited nursery and in particular states that “Plants not raised under hygienic conditions in accredited nurseries should under no circumstances be used in a translocation program”. Section 5.2 details proper phytosanitary guidelines, including the regular inspection of the *ex-situ* population for pests and diseases and the use of fungicides where necessary to manage these diseases. In relation to selecting a source site for a translocation, Section 3.5.3 suggests seeing if any other threats are currently present and if there is a risk from a potential threat such as *P. cinnamomi*.

7.1.1. *Phytophthora cinnamomi*

P. cinnamomi belongs to the Kingdom Chromista Rands. It is a member of the oomycetes, which are closely related to Chrysophyta (golden brown algae). Characteristics include ovoid sporangia (Figure 7-1), coralloid mycelium (Figure 7-2), and globose chlamydospores (Figure 7-3). *P. cinnamomi* is heterothallic (i.e. produces compatible male and female gametes) and is capable of asexual reproduction. It has A1 and A2 mating types (A2 mating type is predominant worldwide) and oospores form when the two types pair (Zentmyer, 1979) (Figure 7-4). The mating types can be distinguished by electrophoretic migration patterns on gels of isocitrate dehydrogenase. The predominant mating type in Australia is A2 and is rare, largely resulting in asexual reproduction, via zoospores and chlamydospores (May and Simpson, 1997).

P. cinnamomi, although parasitic, does have saprophytic abilities and can survive for as long as 6 years in substrates such as gravel that have low soil flora (Zentmyer and Mircetich, 1965). *P. cinnamomi* has little ability to compete with other soil microbes and exists in the soil by being harboured in plant roots (Shea, 1979).

Figure 7-1: Non-papillate sporangia in *Phytophthora cinnamomi*.

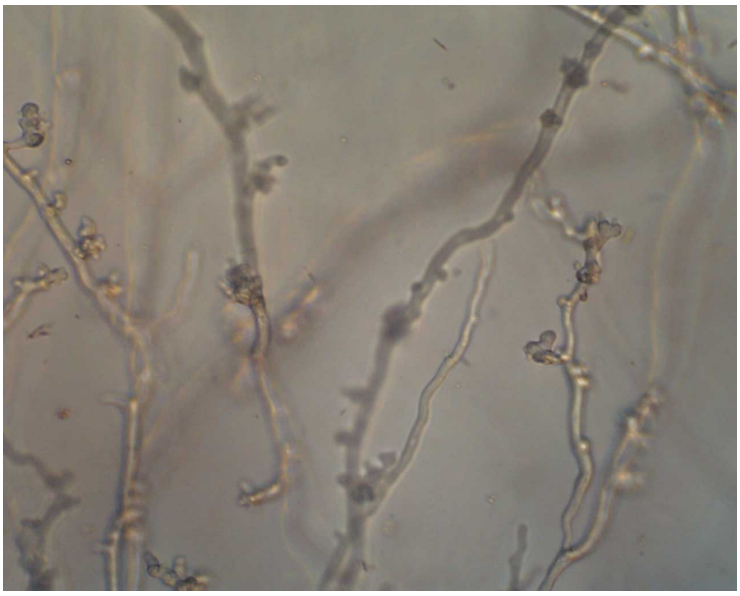

Figure 7-2: Coralloid mycelium in *Phytophthora cinnamomi*.

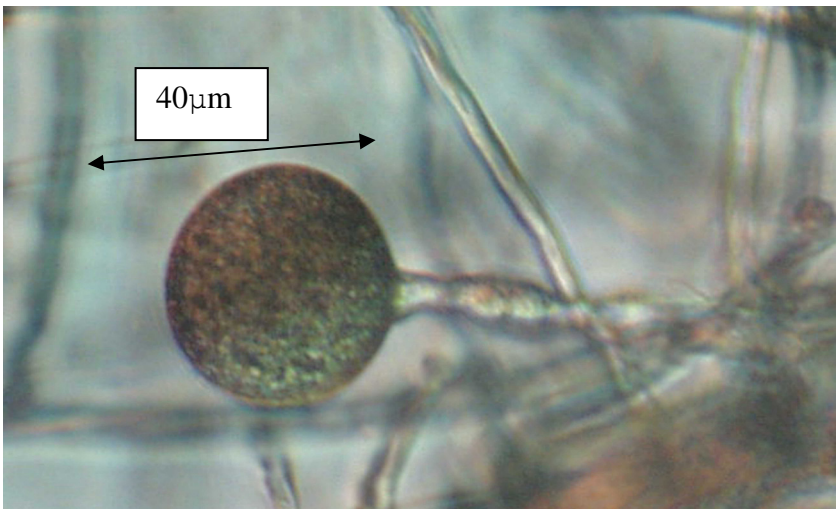

Figure 7-3: Globose chlamydospore in *Phytophthora cinnamomi*.

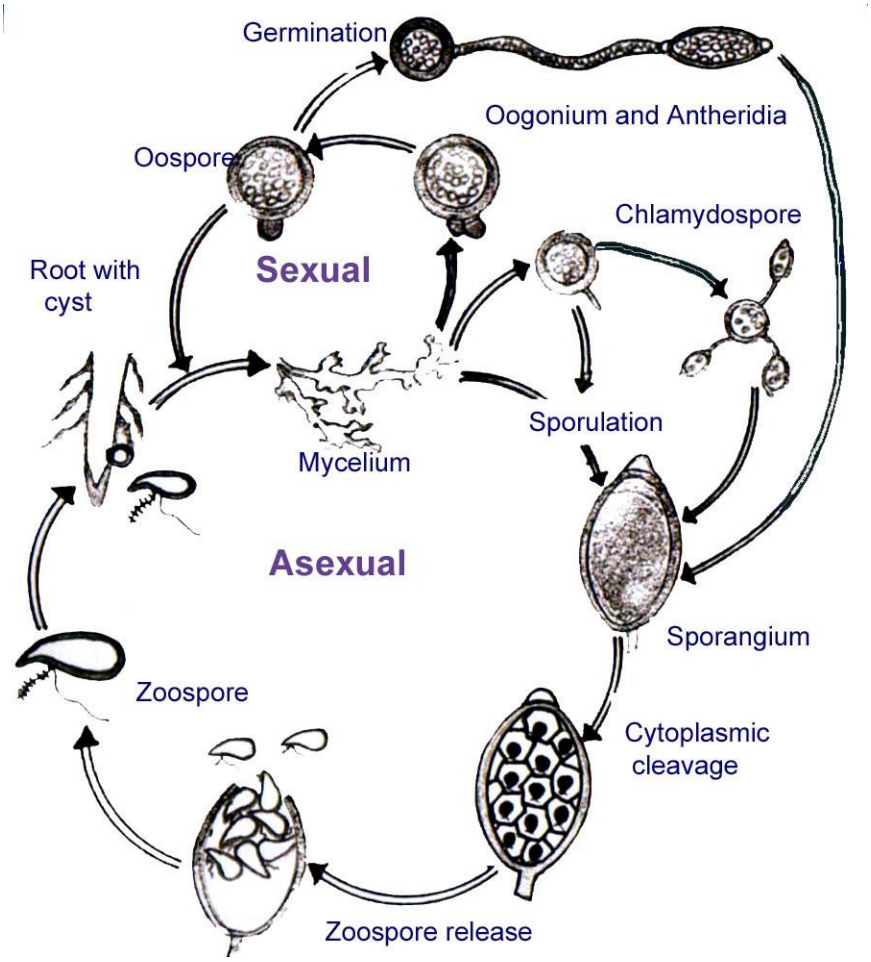

Figure 7-4: Life cycle of *Phytophthora cinnamomi* (Hardham, 1999).

7.1.2. Dispersal and transfer

Zoospores are the major form of dispersal, and when weather conditions are extremely wet, *P. cinnamomi* is able to disperse through mycelium as well (Zentmyer, 1980). Chlamydospores provide the pathogen with a long-term source of inoculum. Humans have spread the pathogen via transfer of soil in the timber industry, road making, wildflower collecting, bushwalking, four-wheel driving and forest management (Turton, 2005).

7.1.3. Symptoms and method of infection

Primary infection of roots is just behind the zone of elongation (Hardham, 2001). The pathogen enters easily through broken roots and also the stem, when splashback allows it to land on susceptible material. Primary infection

causes necrosis, root rot and sometimes cankers. Secondary symptoms resulting from water and nutrient deficiency include chlorosis, wilting, microphylly, epicormic shooting and dieback (Weste and Marks, 1987).

Symptoms are similar to those of other facultative plant pathogens and typically become apparent in the understorey 10 years or more before becoming noticeable in the overstorey (Weste, 2003). *P. cinnamomi* affects the majority of Australia's native vegetation with approximately 85% of Australias vegetation being susceptible with most of the Proteaceae, Epacridaceae and Fabaceae being effected.

Zoospores are chemotactically attracted to roots (Carlile, 1983). They adhere to them (encysting in 15-30 min), and germinate to form a germ tube, which enters root tissue. The pathogen penetrates along anticlinal walls at the junction of two epidermal cells (Hardham, 2001). Penetration is both enzymatic and mechanical. The pathogen can then penetrate all cells, including those in the xylem and phloem. Sporangia form on the root surface within 24 hours (Weste *et al*. 1982). In susceptible species, lesions form and extend along the root into secondary tissue. In the field, resistant species' lesions are contained (Phillips and Weste, 1984). The pathogen primarily infects the fine root systems of plants (Marks and Toppett, 1978). Moist soil conditions over summer yield rapid lesion extension in host plants (Tippett and Hill, 1984).

7.1.4. Methods of detection

In the field, visual detection of infection symptoms in susceptible species is undertaken to determine the presence and spread of the disease, though it is not diagnostic. One of the best-known indicator species is *Xanthorrhoea australis* (Aberton *et al*. 2001). Aerial mapping is helpful to determine the extent of the spread of disease in sites that are infected, but is of little diagnostic help, ground-truthing being essential to verify *P. cinnamomi* infection.

Morphological detection involves baiting soil with susceptible plant tissue (Chee and Newhook, 1965) using soil samples that contain fine roots and incubating soil, water and baits at 25ºC for 3-5 days. Baits are examined microscopically after 3-5 days for characteristic sporangial formation and plated onto selective agar. Many types of susceptible baits can be used, including lupins (Aryantha *et al*. 2000), *Rhododendron* leaves (Bulletin, 2004), pineapple pieces, avocado, pear (Chen and Zentmyer, 1970), *Eucalyptus sieberi* cotyledons (Dobrowolski and O'Brien, 1993) and *Pimelea ferruginea* leaves (McDougal, 2002). *In-situ* baiting with *Banksia grandis* is more effective than *ex-situ* soil and root baiting (McDougal *et al*. 2002). Spring and Autumn are better for baiting due to the generally warm and wet conditions, which favour the production of zoospores and thus the spread of the pathogen.

Drenth and Irwin (2001) have developed molecular methods of detection and identification of *Phytophthora* species, including *P. cinnamomi*. When implemented this technique (Figure 7-5) efficiently decreases detection times and increases accuracy.

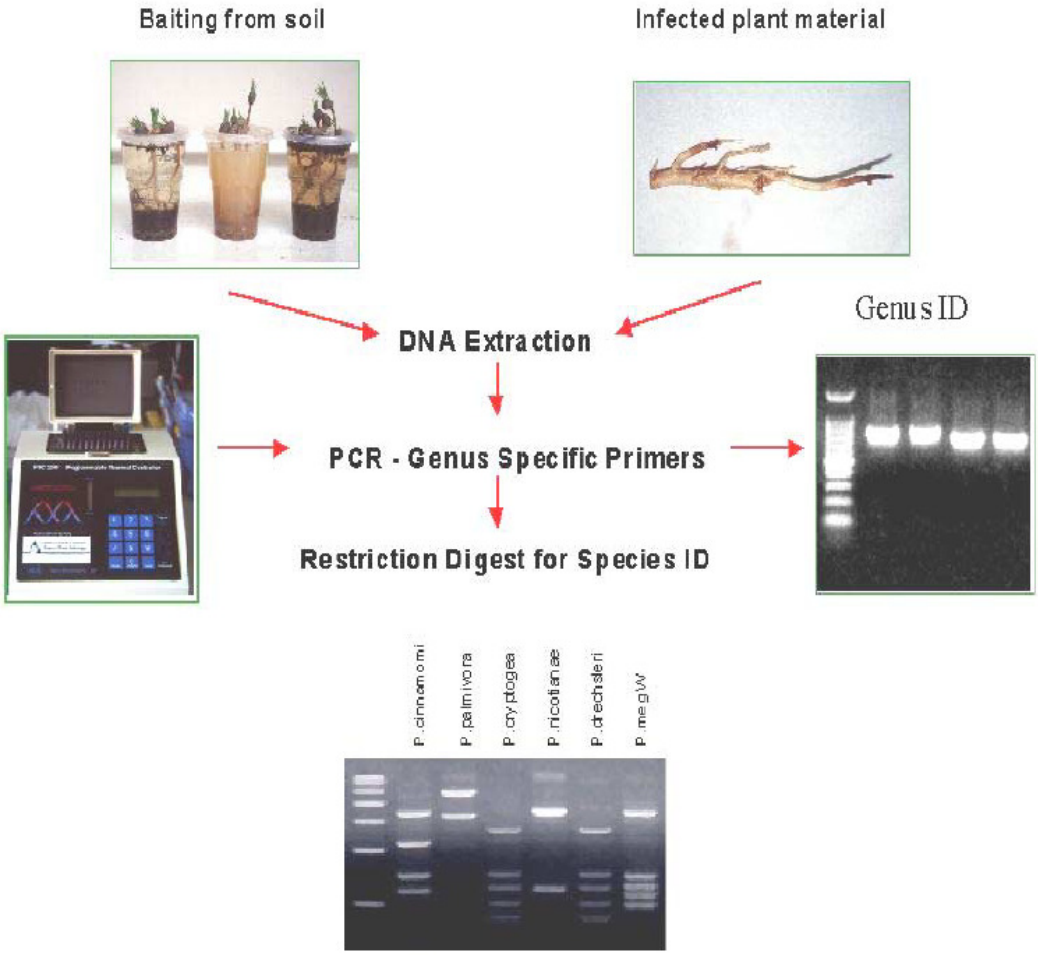

Figure 7-5: Molecular methods of detecting *Phytophthora cinnamomi* (Drenth and Irwin, 2001).

B. mirabilis is susceptible to *P. cinnamomi* (Reiter *et al*. 2004) and infected plants lose leaves, wilt and eventually die from root necrosis (Figure 7-6). It is imperative that:

- *Ex-situ* plants do not carry infection from the nursery to the translocation site
- Translocation sites are free of pathogens that might affect translocated plants.

Figure 7-6: *Borya mirabilis* field site, healthy *Borya mirabilis* (A) and *B. mirabilis* infected with *Phytophthora cinnamomi* (B).

Since *B. mirabilis* is very susceptible to *P. cinnamomi* and *P. cinnamomi* is now in the only field site, it is imperative that it does not infect any translocation populations. This means ensuring that plants are pathogen-free in the *ex-situ* collection and ensures that translocation sites are *P. cinnamomi* free.

The aims of this chapter were therefore to:

- Test *ex-situ* plants at the RBG for the presence of pathogens, especially *P. cinnamomi,* which might explain the relatively poor growth and decline.
- Ensure that *ex-situ* plants are pathogen-free prior to their translocation.
- Ensure that *ex-situ* plants are translocated to sites free of pathogens, particularly *P. cinnamomi*.

7.2. Methods

7.2.1. *Phytophthora* testing of *ex-situ Borya mirabilis* population

Healthy intact leaves were removed from *Pimelea ferruginea* Labill. plants. These leaves were surface-sterilised in 70% ethanol for 1 minute and rinsed in deionised water for 1 minute. Leaves were placed into plastic cups that contained approximately 50 g of soil (containing fine roots) from each of the 22 plants within the *ex-situ* collection and filled with deionised water 1-2 cm above the soil (Figure 7-7). The cups and baits were left for a period of 3-5 days at 25ºC. The baits were removed, surface-sterilised with 70% ethanol for a few seconds, rinsed in sterile deionised water for a few seconds, blotted dry on sterile absorbent paper and aseptically placed onto selective agar (Appendix 7-1) for isolation of *Phytophthora* spp. The plates (Figure 7-8) were examined daily for the presence of pathogens over the course of a week (Reiter, 2002). The testing was conducted in March 2004.

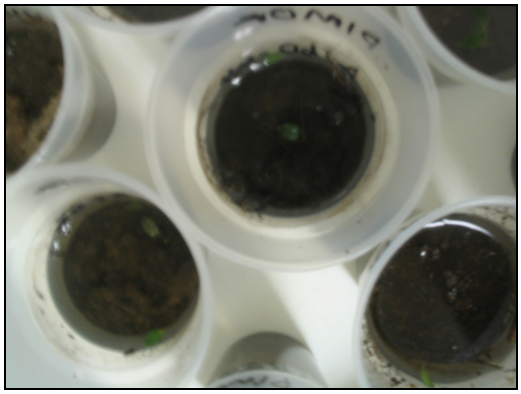

Figure 7-7: Baiting of 50 g of field soil from each sample taken for testing in plastic cups with sterile distilled water and the baits, *Pimelea ferruginea* leaves, which are attractive to the zoospores of *P. cinnamomi* to swim to, adhere and infect.

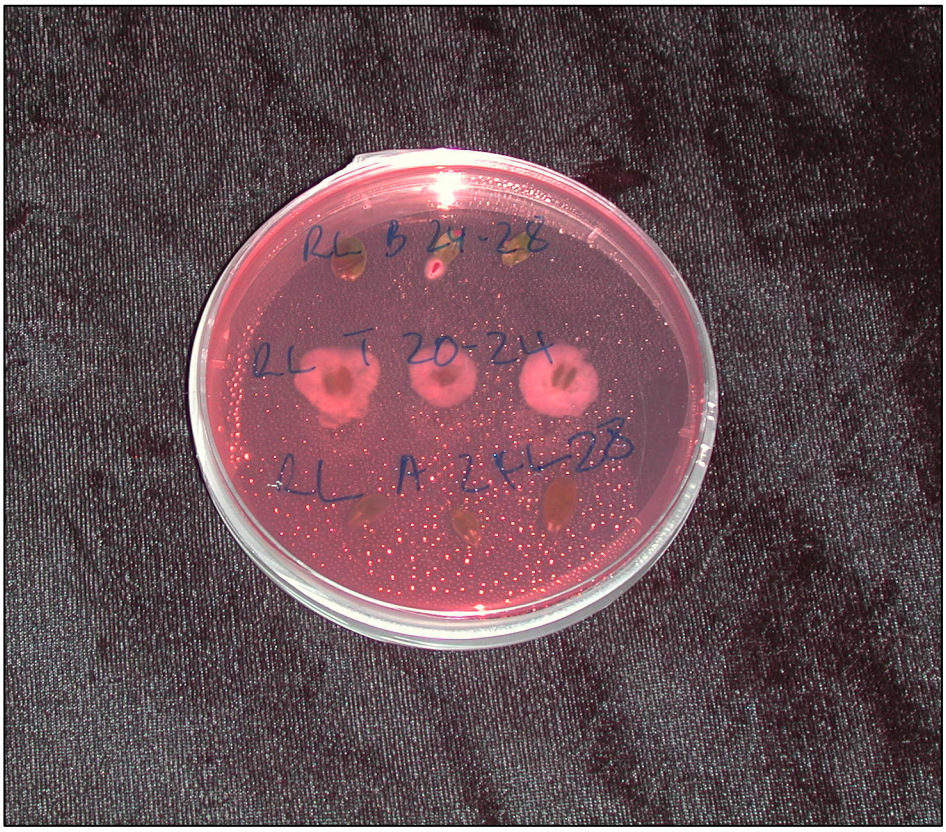

Figure 7-8: Mycelial growth of *Phytophthora cinnamomi* from baits (*Pimelea ferruginea* leaves) after being plated for 3 days onto *Phytophthora* selective medium.

7.2.1.1. *Borya mirabilis ex-situ* population pathogen treatment

All *B. mirabilis* plants were removed from the Botanic Gardens and transferred to the Greenhouses at Bundoora RMIT because of restriction on fungicide treatment at the RBG. Two trial plants were root drenched with 1.5 g/L of Benlate® (active ingredient benomyl) to kill soil borne fungi. This was done by placing pots in deep-sided tubs and filling these tubs with the above concentration of Benlate. Plants were soaked in their pots to prevent further damage. The plants and pots were immersed in the chemical suspension for 30 minutes. All chemical handling was undertaken in a fume hood. The pots were allowed to drain and were placed in a glasshouse for a period of 10 days to monitor the effects of the chemical. Each pot was also drenched with Fongarid® as per manufacturer's instructions to kill oomycetes.

7.2.2. Testing of potential translocation sites for *Phytophthora*

Three proposed *B. mirabilis* translocation sites: Mackey's Peak (Figure 7-9), Pine Plantation (Figure 7-10) and Reid's Lookout (Figure 7-11) were chosen by the Recovery Team on the basis of soil, slope, altitude, aspect and vegetation being a close approximation to the natural field site of *B. mirabilis*.

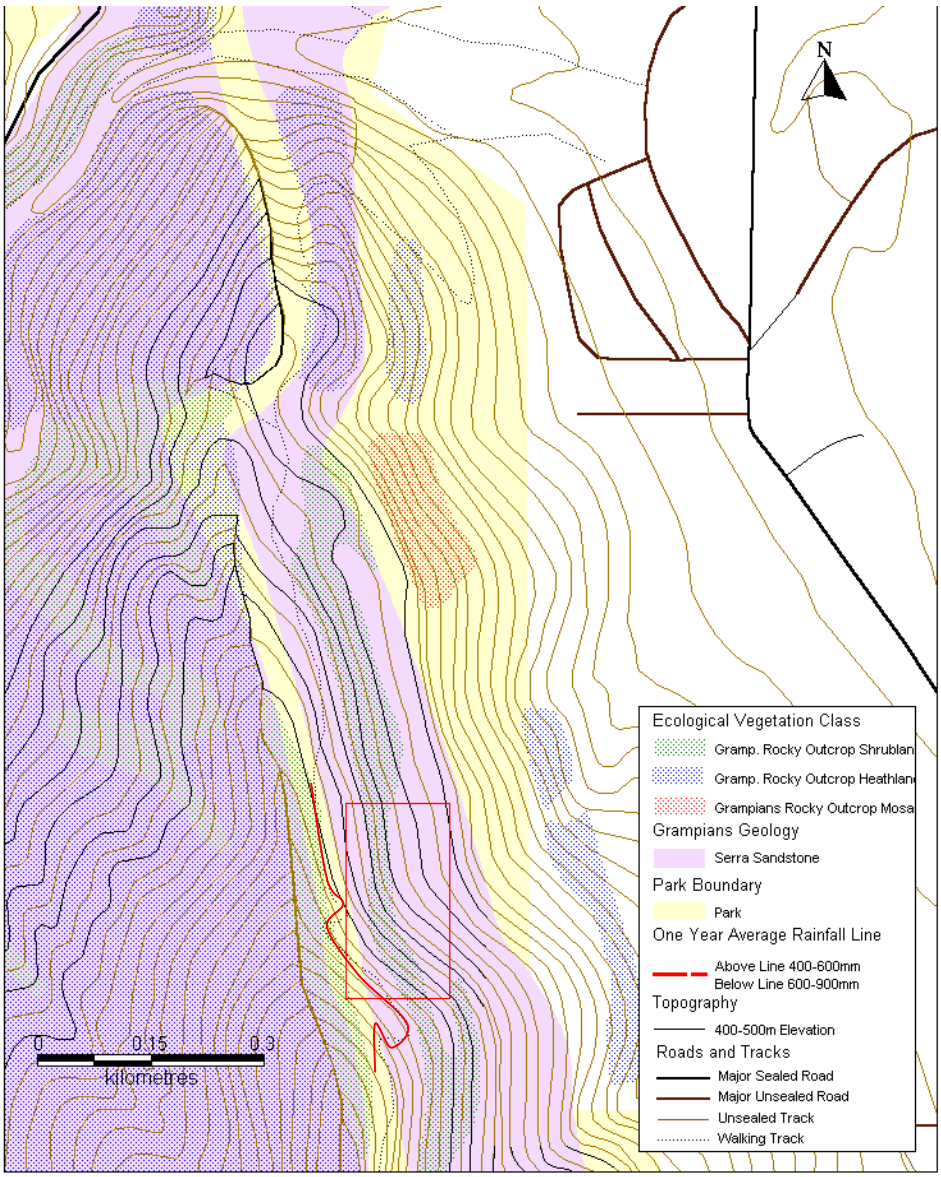

Figure 7-9: Mackeys' Peak potential translocation site. The red box indicates area of proposed translocation site sampled for *Phytophthora* and red line indicates area of track sampled for *Phytophthora*.

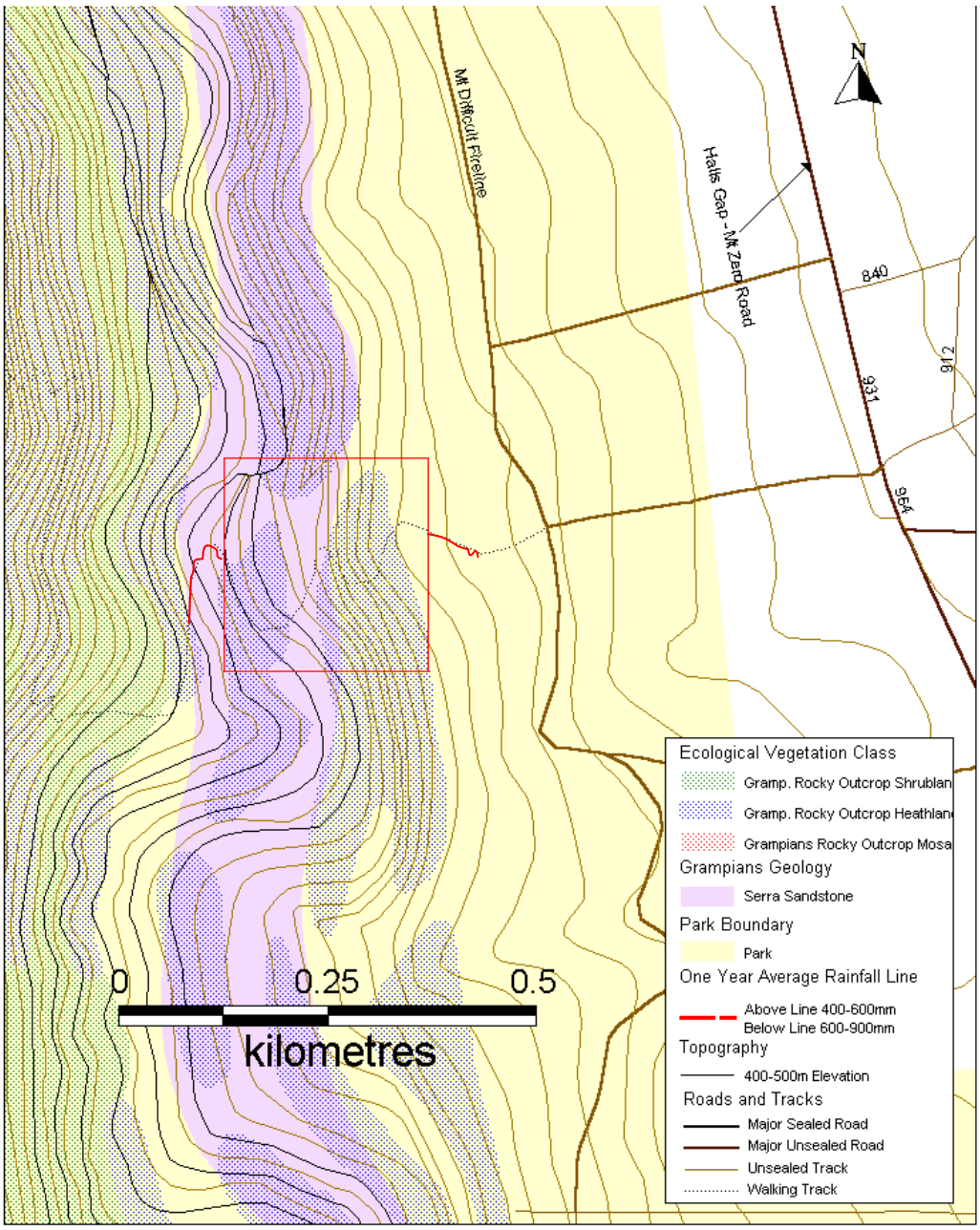

Figure 7-10: Pine Plantation potential translocation site. The red box indicates area of proposed translocation site sampled for *Phytophthora* and red line indicates area of track sampled for *Phytophthora*.

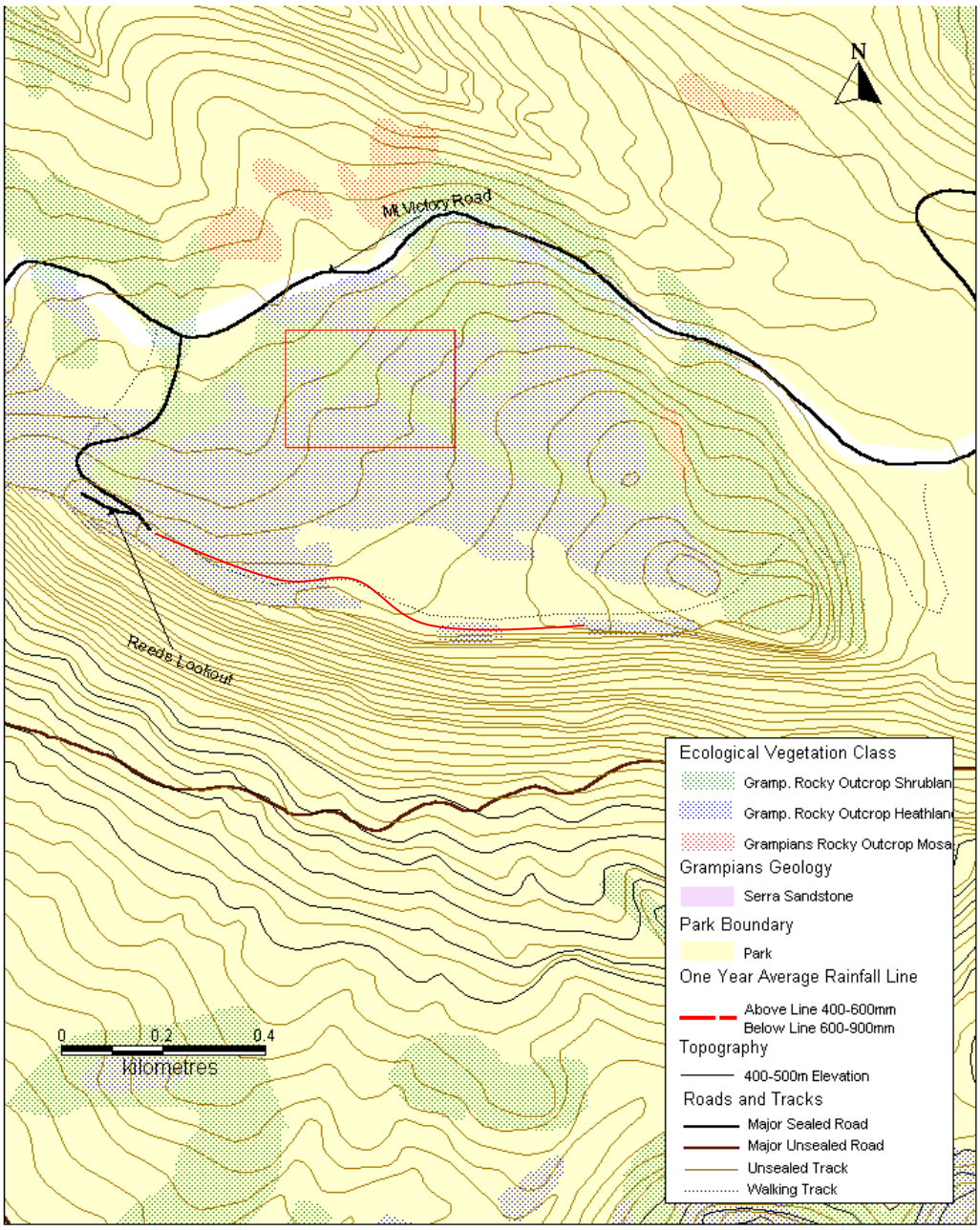

Figure 7-11: Reid's Lookout potential translocation site. The red box indicates area of proposed translocation site sampled for *Phytophthora* and red line indicates area of track sampled for *Phytophthora*.

7.2.2.1. Ground-truthing

Each site was visually assessed for disease including the presence of: chlorosis, wilting, microphylly, epicormic shooting and dieback in susceptible vegetation. Prior to sampling, photographs were taken of the location.

7.2.2.2. Soil Sampling

Mackey's Peak

The section of the Mackey's Peak Track sampled was 30 m above and 30 m below the proposed translocation site (red boxed area, Figure 7-9). Samples were taken every 4 m along this transect, resulting in 16 samples in total from the track. The potential translocation site (Figure 7-9) of 20 m by 28 m was divided into 16 m^2 quadrats, with susceptible species' roots being sampled from within each quadrat, resulting in 35 samples.

Pine Plantation

The potential translocation site (Figure 7-10), an area of 24 m by 28 m, was divided into 16 m^2 quadrats, resulting in 41 samples (one quadrat not sampled due to the incline). The closed walking track (Figure 7-10) was sampled every 5 m for 45 m on either side of the potential translocation site, resulting in 19 samples.

Reid's Lookout

From the start of Reid's Lookout walking track (Figure 7-11), soil was sampled every 4 m for 64 m, resulting in 17 samples over the 64 m transect along the track. The potential translocation site (red boxed, area Figure 7-11) was divided into quadrats of 4 m by 5 m, with a total of 35 samples taken from susceptible species' roots within these quadrats

Before entering each site and upon exiting, boots were cleaned with a stiff brush and Phytoclean® solution. Once sites were divided into quadrats or transects, a single soil sample within each quadrat was taken, consisting of approximately one trowelful of fine roots and soil, and was placed in a labelled sealed plastic bag. Between collecting each soil sample, the trowel was wiped down with either 70% ethanol or Phytoclean, and dried with tissue paper. Soil analysis was performed as in Section 7.2.1.

7.2.3. Molecular identification of *Phytophthora* isolates

Mycelium was scraped from pure cultures of *P. cinnamomi* that had been previously isolated from samples taken in Section 7.2.2.2. DNA was extracted following the manufacturer's instructions (Qiagen) using DNeasy Plant Mini Kit. DNA was amplified

using the primers DC6 (Cook *et al*. 1983) (5'-GCT ATT TAG TTA AAA AGC AGA-3') specific for Peronosporales and Pythiales and PhtophR (Lee and Taylor, 1992) (5'GAG GGA CTT TTG GGT AAT CA-3') specific for the *Phytophthora* genus. The 25 µl PCR reactions contained: 2 µl of extract, 12.5 µl of Green Master Mix (Go Taq®), 0.5 µl of each primer and 9.5 µl of sterile MilliQ purified water. The PCR was run at 95°C for 2 min, 30 cycles at 94°C for 30 s, 55°C for 45 s and 72°C for 1 min, followed by 72°C for 10 min. The PCR products were run on a 1.4% agarose gel and stained with ethidium bromide.

There was no *P. cinnamomi* registered on Genbank with these primers and therefore two of these isolates RLT12-16 and RLT 16-20 were sequenced using ITS1 and ITS4 (White *et al*. 1990) primers.

Purified DNA was analyzed for both forward and reverse sequences on an ABI 377 automated sequencer (Micromon, Monash University, Clayton Campus) as in Chapter 4. Sequences were edited using Bio Edit Version 3.0.

7.3. Results

7.3.1. *Phytophthora* testing of *ex-situ Borya mirabilis* population

Two fungal species were isolated that did not appear to be ubiquitous soil fungi. These were subcultured aseptically and kept for identification. One of the unknowns was identified as *Phytophthora nicotiana* (Figure 7-12), another as an ascomycete (for which further identification was not undertaken).

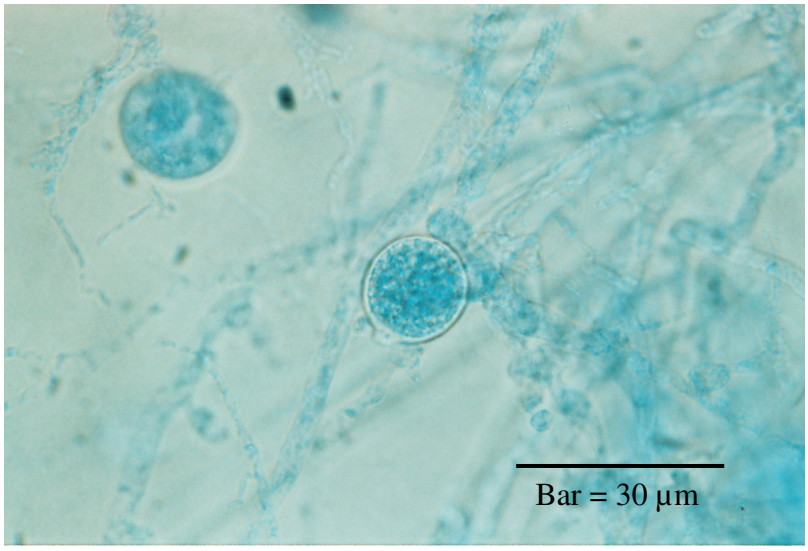

Figure 7-12: *Phytophthora* species reproducing, with antheridia and oogonium, isolated from Royal Botanic Gardens *ex-situ* population roots.

7.3.1.1. *Borya mirabilis ex-situ* population pathogen treatment

No deleterious effects were seen with the two trial plants after 10 days (Figure 7-13 and Figure 7-14). All of the *ex-situ* population (remaining 20 plants) was then drenched (Figure 7-15) as in Section 7.2.1.1, left for a period of 1 week and returned to the RBG.

Figure 7-13: *Borya mirabilis* plants 13 and 2 from the *ex-situ* population at the Royal Botanic Gardens Melbourne, prior to treatment with Benlate and Fongarid.

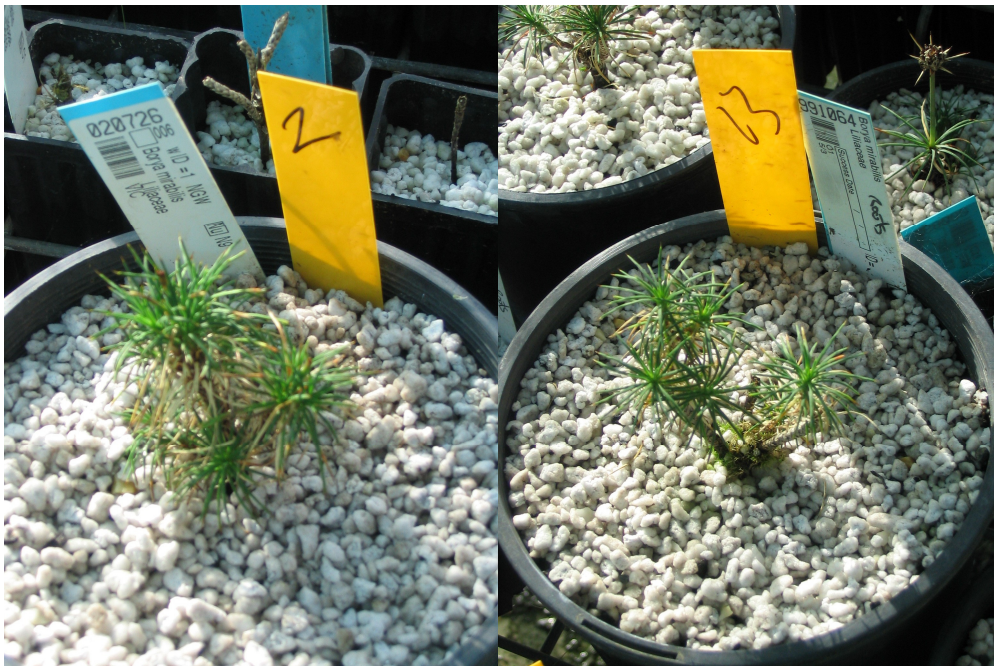

Figure 7-14: *Borya mirabilis ex-situ* plants 13 and 2 from the Royal Botanic Gardens Melbourne, 10 days after treatment with Benlate and Fungarid.

7.3.2. Testing of potential translocation sites for *Phytophthora*

7.3.2.1. Ground-truthing

Mackey's Peak

This site had noticeably sick and dying vegetation, including chlorotic *Grevillea aquifolium* and *Eucalyptus* spp. (Figure 7-15). The site was in close proximity to known *P. cinnamomi* infestations.

Figure 7-15: Mackey's Peak proposed translocation site

Pine Plantation

This site appeared disease-free, although somewhat windswept (Figure 7-16).

Figure 7-16: Pine Plantation potential translocation site.

This site appeared free of symptoms in the vegetation, with no discernible plants dead or dying (Figure 7-17). The vegetation appeared disease-free although windswept.

Figure 7-17: Reid's Lookout potential translocation site.

7.3.2.2. Soil Sampling

Mackey's Peak

Soil and root analysis revealed of the 12 samples collected from the track (Appendix 7.6), 1 tested positive for *P. cinnamomi* and all 41 samples collected from the site (Appendix 7.7) tested negative.

Pine Plantation

Soil and root analysis revealed the 19 samples collected on the track (Appendix 7.8) and the 41 samples collected from the site (Appendix 7.9) as negative for *P. cinnamomi*.

Reid's Lookout

Soil and root analysis revealed, of the 17 samples collected on the track, 9 tested positive (Appendix 7.4) for *P. cinnamomi* and of the 36 samples collected from the site (Appendix 7.5), 4 tested positive for *P. cinnamomi*.

7.3.3. Molecular identification of *Phytophthora* isolates

All samples identified morphologically as *P. cinnamomi* were amplified and displayed a 600 bp fragment thought to be *Phytophthora*-specific (Figure 7-18), those identified morphologically as *Pythium* did not. Isolates RLT 12-16 and RLT 16-20 from Reid's Lookout were 99% identical to *P. cinnamomi* (Table 7-1).

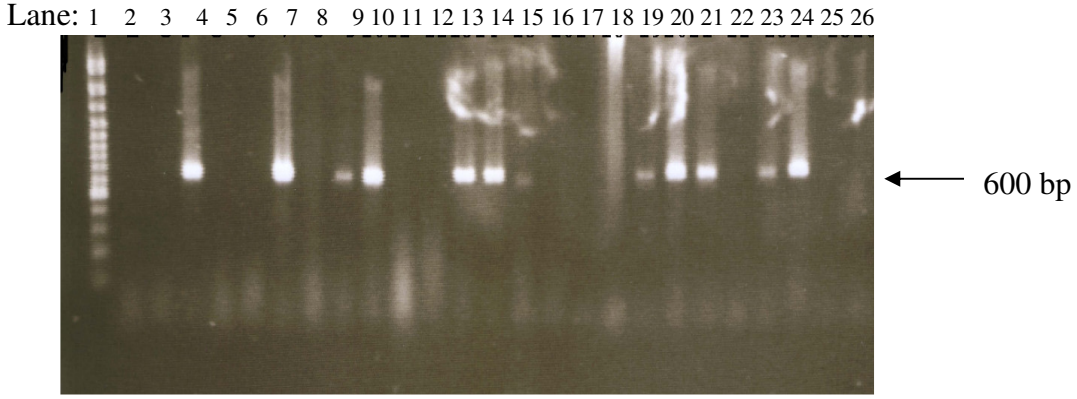

Figure 7-18: *Phytophthora cinnamomi isolates with* positive results amplifying a 600 bp fragment. Lane 1-26 (left to right): Molecular weight marker (GeneRuler, 1kb) , *Pythium* sp., *Pythium* sp., *Phytophthora* sp., *Pythum* sp., *Pythium* sp., *Phytophthora* sp., *Pythium* sp., *Phytophthora* sp., *Phytophthora* sp., *Pythium* sp., *Pythium* sp., *Phytophthora* sp., *Phytophthora* sp., *Phytophthora* sp., *Pythium* sp., *Pythium* sp., *Pythium* sp., *Phytophthora* sp., *Phytophthora* sp., *Phytophthora* sp., *Pythium* sp., *Phytophthora* sp., *Phytophthora* sp., *Pythium* sp., *Pythium* sp.

7-1: Isolates sequenced using ITS1 and ITS4.

Sample	Identity NCBI/Blast
RLT 12-16	*Phytophthora cinnamomi* 99% Max identity, E-value 0.0
RLT 16-20	*Phytophthora cinnamomi* 91% Max identity, E-value 0.0

7.4. Discussion

This chapter has shown that it is necessary to take all precautions recommended in the translocation guidelines for threatened species (Vallee *et al*. 2004), as failure to do the testing recommended in the translocation guidelines in this case would have led to introduction of *P. nicotiana* into the Grampians National Park. Reid's Lookout and Mackey's Peak sites were infected with *P. cinnamomi*. The Pine Plantation translocation site was uninfected at the level of sampling undertaken. If translocation of the plants had taken place at either Reid's Lookout or Mackey's Peak it is likely that the translocated *B. mirabilis* species would have become infected with *P. cinnamomi,* a pathogen which we already know *B. mirabilis* to be susceptible. As a precaution, any future translocation material should be assessed for pathogens and treated similarly to avoid introducing disease to the chosen *Phytophthora*-free translocation site.

7.4.1. *Ex-situ* population

The *ex-situ* population appear visually to have improved considerably in health since being treated for root pathogens. The fungicide an oomycete treatment, combined with a more aerated potting medium and additions of mycorrhiza (previous chapter), has seen the population flourish. Regular assessment of the *ex-situ* population health is required to maintain a healthy population, and any decline in the health of the population should be taken as a sign of possible infection. Treatment with furalaxyl should not affect the AM mycorrhiza as furalaxyl is active only with species of *Phytophthora* and *Pythium,* both of which are likely to be less of a problem in well-drained medium.

7.4.2. Translocation sites

The Mackey's Peak walking track and the proposed translocation site displayed symptoms of *P. cinnamomi* and it was subsequently isolated from the walking track. The translocation site would directly receive runoff from

both the walking track and the existing *P. cinnamomi* infected *B. mirabilis* site; thus translocation is not recommended. *P. cinnamomi* is likely to be the cause of the decline in the vegetation in the area.

The Reid's Lookout site, both walking track and proposed translocation site, was infected with *P. cinnamomi*, yet interestingly did not display the associated symptoms in the vegetation. Despite this, molecular identification confirmed the positive morphological identification in both the walking track and the proposed translocation site as *Phytophthora* (from specific primer banding patterns) and sequencing of two of the walking track isolates, were identified as *P. cinnamomi,* therefore translocation is not recommended. It is recommended that the Balconies walking track (the walking track that was tested at Reid's Lookout) be closed. It is a risk to uninfected vegetation within the park and should be quarantined. If allowed to remain open quarantine and hygiene procedures need to be implemented to mitigate the effects of *P. cinnamomi*. Adequate disinfectant foot baths and washing stations for vehicles and warning signs for park visitors need to be installed and rigorous protocols and educational information need to be in place. *P. cinnamomi* is listed as a Key Threatening Process under the under the Environment Protection and Biodiversity Conservation Act.1999 and as such should be taken seriously in species management.

This leaves three plausible scenarios for the Reid's Lookout site. The first is that these sites are newly infested and the pathogen has not yet had time to damage a noticeable amount of vegetation. The second is that the isolates at these sites are not very virulent and are able to co-exist with the vegetation without causing noticeable signs. The third is that plants may have disease escape due to altitude. *P. cinnamomi* was reported not to cause serious disease at altitudes above 600 m (Pratt and Heather, 1973) although elsewhere in the Grampians (Mt William) it can be seen to be causing chlorosis and necrosis in *Banksia saxicola* displaying necrotic disease symptoms above 600 m and soil baiting from roots has isolated *P. cinnamomi* (Weste *et al*. 2004).

The Pine Plantation site did not display any symptoms in the vegetation and did not provide any positive isolates when soil samples were analyzed and so is suitable for translocation. It is therefore imperative that this site be kept clean. One way of implementing this is to fence the area. The one opening into the site could then have a foot scrub installed, where footwear can be scrubbed and disinfected before entering the site. It is recommended to do this both upon leaving the walking track and upon entering the translocation site. Alternatively a spot could be designated where people change their shoes on one side of a mat and walk across the mat to the other side where they put clean shoes on. These precautions have been in place in many sites throughout Australia, particularly in Western Australia (Shea, 1979). Modification of the area to create an environment that does not favour the pathogen as proposed by Shea (1979) including: rerouting or modifications to walking tacks, modification of drainage patterns and organic amendments in the soil to increase *P. cinnamomi* antagonistic microbes may also be a feasible and less visually offensive option.

7.5. Outcomes

The Pine Plantation site was subsequently chosen as the best translocation site for the *ex-situ* cuttings of *B. mirabilis* to be translocated into the wild. The translocation took place in June 2004 and has been supplemented with additional plants in 2006, with further additions planned for 2008-2009. The plants have been growing successfully since being translocated and disease symptoms are absent at the site.

8. General conclusions and management implications

B. mirabilis is one of the world's most critically endangered plants. The research in this thesis has illuminated key aspects of: its reproductive biology; interspecies and intraspecies molecular relationships, tissue culture potential, mycorrhizal status and disease threats. Each of these aspects has fundamental management implications for the active management of *B. mirabilis*.

8.1. Floral biology

Examination of the floral biology of *B. mirabilis* and some of its close relatives confirms that seed production for *B. mirabilis* is achievable but extremely challenging. The impediments to breeding, including extreme pollen infertility, are in all probability due to the polyploid nature of the species.

Examination of the pollen of *Borya* species showed little difference in the characteristics of the pollen between species. The most striking difference between species was that over 95% of *B. mirabilis* pollen was not viable. The structural immaturity of the pollen corroborated with the evidence from the pollen growth experiments where *B. mirabilis* had low germination rates and slow and deformed pollen tube growth.

Field and *ex-situ* populations of *B. mirabilis* were extensively crossed with only one seed produced between Colony 5 and Colony 7. *B. mirabilis*'s infertility is unlikely to be caused by lack of pollinators, with flowers being regularly visited by flies and ants. Further pollinations using the pollen of the closely related *B. constricta* and *B. sphaerocephala* on *B. mirabilis* plants proved unsuccessful, which was expected due to the large differences in chromosome numbers between the species.

Examination of the chromosomes of *B. mirabilis* has found it to be polyploid, containing approximately three times the number of chromosomes than its close relatives. This corroborates with the observations of the reproductive structures including: an unusually high number of floral abnormalities, pollen infertility, uneven maturity of ovules, extremely low seed production and inability to hybridize with its closest relatives.

8.1.1. Management implications

Further crosses in the field are unlikely to generate significant quantities of seed. Further crosses should be systematically conducted, reflecting the genetic differences found between plants in Chapter 4. A log book of crosses should be kept with the aim of finding those plants within the population which are able to breed. Further crosses with *B. constricta* and *B. sphaerocephela* at this stage should be discontinued due to chromosomal differences.

8.2. Interspecies and intraspecies relationships of the Boryaceae

Interspecies and intraspecies relationships of the Boryaceae and *Borya mirabilis* were investigated. It was determined that the closest relations to *B. mirabilis* are *B. sphaerocephela* and *B. constricta* and that *B. mirabilis* is likely to have emerged from the ancestral hybridisation of these species. The wild population of *B. mirabilis* was determined to have a small amount of genetic variation. The variation in the field population was not reflected in the *ex-situ* population.

8.2.1. Management implications

The *ex-situ* population should be tagged appropriately to represent the genetic variability in this study. Representatives of the genetic diversity in the field population not currently represented in the *ex-situ* population should be sought and propagated. The translocation sites should then be altered, to adequately represent the variation found in the naturally occurring population.

8.2.2. Further work

Future work in this area could determine the placement of the Boryaceae within the Asparagales, using the sequences in this study combined with new sequences of these regions from other taxa in the Asparagales.

Future testing of the individuals within the translocated population may not be necessary (as this is expensive) if efforts are made to introduce all the known diversity from the original population.

If tissue culture work is attempted from pollen or ovules and produces plants with compatible chromosome levels, then future work to introduce hybrid vigour into the population should be attempted but may not be necessary.

8.3. Tissue Culture

The research on tissue culture presented here has answered some fundamental questions in the establishment of an effective means of micro-propagation of *B. nitida* for further testing in *B. mirabilis*. The research has provided an effective means of mass production of the species. The research has determined: a suitable explant for regeneration, an effective means of reducing the contamination in tissue culture, what levels of nutrients are required to micro-propagate the species, a preference for Phytagel over agar and a practical method for inducing roots on the shoots grown in culture (although this should be improved). Further research is needed to determine if this can be used with other *Borya* species and resurrection plants.

8.3.1. Management implications

As an effective means of decontamination has been found, and shoot-tip explants 2 cm long from the plants results in plants re-sprouting, it may be feasible to take tissue from the wild population to more adequately represent the molecular diversity without an overly detrimental effect on the wild plants. Micro-propagation of *B. mirabilis* should be undertaken

from the *ex-situ* population to mass-produce plants for further translocations. Further refinement of *ex-vitro* rooting techniques and media are needed.

8.4. Mycorrhizal associations

B. mirabilis has been determined to be mycorrhizal. The predominant mycorrhizal association is that of nodular arbuscular mycorrhizas, present in the form of coils in root nodules over wetter months and as spores in these nodules over drier months. A significant increase in the health of the *ex-situ* population of *B. mirabilis* was recorded after addition of soil containing fine roots of the wild population. Of the plants associated with the wild population, *C. rhomboidea* had the most morphologically similar vesicular arbuscular mycorrhizal relationship and may act as a reservoir of fungi for *B. mirabilis*. It may be important to ensure that this species exists or is planted in future translocation sites.

8.4.1. Management implications:

The arbuscular mycorrhiza needs to be transferred to all *ex-situ* plants at the Royal Botanic Gardens Melbourne and these plants then need to be treated before translocation with furalaxyl (this is only active against oomycetes, e.g. *Phytophthora* and *Pythium* and so should not affect the vesicular arbuscular mycorrhiza on the roots). It may be wise to position translocation plants near *C. rhomboidea* plants in the field; however, functional verification of the morphological similarity in the mycorrhizas of *B. mirabilis* and *C. rhomboidea* needs to be clarified through inoculation and molecular identification.

Future work should determine molecular identification of the mycorrhiza and if any benefit can be gained by the addition of fungal propagules to the micro-propagated plants in root induction.

If *C. rhomboidea* is shown to be a source of mycorrhizal inoculum for *B. mirabilis*, introduction of these plants into translocation sites should be

considered. *C. rhomboidea* if verified as a reservoir of mycorrhizas for *B. mirabilis* could then be used to re-introduce mycorrhizal propagules to the *ex-situ* collection, although the benefits of this would also need to be verified by *ex-situ* inoculations.

8.5. Pathogen detection

The pine plantation site out of the three potential translocation sites tested was chosen for the translocation of *B. mirabilis*. Of the three sites tested it was the only site that had not tested positive for *P. cinnamomi* and also did not display symptoms of disease in the vegetation. The *ex-situ* population harboured *P. nicotiana*.

8.5.1. Management implications

The pine plantation site out of the three sites tested is the only suitable site for translocation. Due to the prevalence of *P. cinnamomi* in the park, any future translocation sites must be tested for the presence of *P. cinnamomi* as the visual assessment as shown in this thesis is not reliable. In addition to pathogen detection potential translocation sites should be viewed, taking into account climate change and the ability of the species to survive in even drier conditions.

The *ex-situ* population and current and future translocation site should be assessed at intervals to determine if they have become infected with *Phytophthora* and then treated accordingly: the *ex-situ* population with furalaxyl drenches and the translocation sites with Phosphonate. Phosphonate spray as is currently undertaken with the *P. cinnamomi* infected natural population of *B. mirabilis* is an effective means of controlling the effect of *P. cinnamomi* on native Australian vegetation, it increases the plants defence responses (Guest and Grant, 1991) rather than acting directly on the pathogen. This combined with mulching and appropriate drainage of sites (Shea, 1979) will minimize the damage caused by *P. cinnamomi* to the vegetation. Any use of the *ex-situ* collection for translocations should be preceded by a precautionary treatment of those plants with fungicide and Phytophthoracides to reduce the risk of

introducing disease into the pine plantation site.

The research has provided critical insight into the sexual propagation of *B. mirabilis* and the impediments with which it faces, provided an alternative method for propagation through tissue culture, elucidated the mycorrhizal nature of these plants, investigated the population structure of the species and relationships within the family and has investigated the presence of pathogens both in proposed translocation sites and the *ex-situ* collection. This work has provided critical knowledge for the current and future management of this critically endangered species.

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10. APPENDIX

11. Appendix 2-1: Recovery Plan for *Borya mirabilis*

12. Grampians Pincushion Lily (*Borya mirabilis* Churchill) Recovery Plan 2001-2005

Fiona Coates
La Trobe University School of Botany, February 2000

12.1. Contents

12.2. Acknowledgments

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Abbreviations: PV = Parks Victoria; ESP = Endangered Species Program, Environment Australia, Canberra; LTU = La Trobe University; RBG = Royal Botanic Gardens, Melbourne; NRE = Department of Natural Resources and Environment.

12.3. Summary

12.3.1. Current species status

Endangered Species Protection Act 1992: Listed on Schedule 1 (Vulnerable, to be updated to Endangered)

Victorian Flora and Fauna Guarantee Act 1988 Listed on schedule 2

ESAC Priority A

ROTAP (Briggs and Leigh 1996): 2ECit

IUCN (1994) CR

ANZECC (1999) E

12.3.2. Habitat requirements and limiting factors

Borya mirabilis is a narrow endemic lily and resurrection plant restricted to low open shrubland on a single rocky outcrop in the Wonderland Range, Grampians National Park, western Victoria. The only known population consists of approximately 70 ramets distributed between five colonies within

an area of approximately 60 m by 20 m. Its habitat requirements include adequate moisture availability during autumn and winter, and substrate stability.

Die-back, including leaf shedding has recently been observed. Major current threats include soil drying caused by burrowing and digging by rabbits, as well as general soil disturbance and erosion, failure to annually set seed, and small population size. Its pre European distribution is not known, but fire may have contributed to its current restricted distribution (Churchill 1985).

12.3.3. Recovery Goals

The **long term goal** is to prevent extinction or further decline in numbers of *B. mirabilis*, by maintaining and augmenting the population, and by managing existing habitat for the future self sustainability of the species.

Within the life span of the Recovery Plan, the **short term goals** of recovery are:

1. Significantly reduce the impact of immediate threats.

2. Increase the size of the existing population.

3. Attempt to extend the range of *B. mirabilis*.

4. Establish a genetically representative *ex-situ* collection.

12.3.4. Recovery Criteria

The criteria for assessing the achievement of these objectives are:

1. Establishment of a comprehensive biological, ecological and horticultural knowledge base.

2. A decrease in disturbance and damage to the site where the population occurs.

3. A measurable increase in the population's productivity and resurrection potential.

4. Completion of a comprehensive search.

5. Maintain plants in cultivation and translocated plants in the wild.

12.3.5. Recovery Actions

1. Prevent further soil disturbance.

2. Measure plant health against habitat management

3. Search for new sites

4. Establish an *ex-situ* collection.

5. Translocation.

6. Prepare Action Statement and Critical Habitat Determination under the *Flora and Fauna Guarantee Act* 1988.

12.3.6. Estimated Cost of Recovery Actions

Year	2001	2002	2003	2004	2005
TC	$49,450	$54,350	$14,800	$33,200	$33,200

12.3.7. Biodiversity Benefits

Management of the site will contribute to habitat viability and integrity, species richness and protection of other threatened flora associated with *B. mirabilis*. Implementation of the Recovery Plan will contribute to achieving the objectives of the *Flora and Fauna Guarantee Act* 1988, by managing potentially threatening processes.

12.4. 1 INTRODUCTION

12.4.1. 1.1 Species description

B. mirabilis is a clump forming, herbaceous lily up to about 15 cm high. It has brown erect or ascending stems, covered with the remains of leaf sheaths which have the appearance of fine scales, and on their lower parts support aerial roots. Leaves are spiky, linear, 10-16 mm long, and approximately 0.5 mm wide, their base tapering gradually from the persistent sheath. Growth of new shoots and inflorescences is terminal. Flowers are white, consisting of borne on scapes 3-7 cm long, ovoid with 4-12 flowers surrounded by several sharp, involucral bracts (Churchill 1985, 1987; Cropper 1993; Conran 1994).

12.4.2. 1.2 Taxonomy and nomenclature

Borya mirabilis was recognised by Churchill (1985; 1987) as a narrow Victorian endemic, having been previously included under *B. nitida* Labill., a species restricted to the southwest of Western Australia. *B. mirabilis* is distinguished by its bracteoles being longer than the floral bracts.

Nomenclature used throughout the Recovery Plan follows Cameron *et al*. (1999).

12.4.3. 1.3 Distribution

Borya Labill. is a genus of about 10 species, mainly confined to southwestern Australia but with one species known from northern Queensland and one from northern Western Australia and the Northern Territory (Churchill 1985). In Victoria, in occurs in the Greater Grampians Bioregion (DNRE 1997), but has only been recorded with certainty from a single population in the Wonderland Range, Grampians National Park. There is one unconfirmed report of another population in the Victoria Range (S. Cropper pers. comm.), but otherwise no firm evidence of a previously more widespread distribution.

C. W. Dalton collected the first *B. mirabilis* specimens in 1924 from the Grampians National Park but the site was thought to have become extinct (Gaff and Churchill 1976) until 1983, when it was re-discovered (Cropper 1994). Its location was made known to the National Parks and Wildlife Service in 1992, but otherwise is largely kept confidential.

12.4.4. 1.4 Habitat

The site supporting *B. mirabilis* in the Wonderland Range consists of low open shrubland on a ferruginous sandstone outcrop consisting of a series of rocky terraces (Cropper 1994). Colonies are distributed over an area approximately 60 m x 20 m. Soils are seasonally moist by virtue of seepage, which has also caused erosion of the bedrock and contributed to the accumulation of fine sandy loam soil, a relatively rare occurrence in rocky environments which may have favoured the persistence of the population at this site. Soil depth ranges from just a few centimetres, up to one metre. Slopes supporting *B. mirabilis* colonies are about 15^0 on average, facing northeast. Charcoal particles distributed across the site suggest that it has been burnt in the past, although there are no records of fire.

Dominant species are *Grevillea aquifolium*, *Kunzea parvifolia*, *Calytrix tetragona*, *Melaleuca decussata* and *Dodonaea viscosa* spp. *spatulata*. Associated species include *Lepidosperma viscosa*, *Gonocarpos mezianus*, *Phyllanthus hirtellus*, *Leptospermum scoparium* and *Austrodanthonia setacea*. *Callitris rhomboidea* and *Eucalyptus alaticaulis* are occasional emergents. A number of annual herbs appear in spring, associated with bryophyte and lichen communities, including *Siloxerus multiflora*, *Drosera whittakeri* subsp. *aberrans*, *D. peltata* subsp. *auriculata*, *Centrolepis strigosa*, and *C. aristata*.

One other rare species (Cameron *et al*. 1999), *Austrostipa hemipogon* has been recorded from the site.

12.4.5. 1.5 Life History

Borya mirabilis is a perennial herb. Plants flower between September and October, although no fruit or seed development have been observed. Plants gradually change from green to orange over summer, until fully desiccated by February, although a return to moist conditions can reverse this process (D. Handscombe, PV, pers. comm.). Further discolouration to a yellowish straw like appearance continues until early autumn, when leaves rehydrate and gradually become green once more. Leaves may also be shed during the desiccation period. Buds probably develop in June, although the complete life history of the species has not been observed.

12.4.6. 1.6 Ecology and Biology

B. mirabilis is a resurrection plant, having the ability to tolerate desiccation over summer and rehydrate after the onset of autumn rains. However, disturbance to soils or litter cover, or unseasonal weather patterns which restrict or disrupt moisture levels may in turn disrupt physiological processes critical to revival.

In general, resurrection plants such as *Borya* are unable to recover from a quiescent state which extends beyond a couple of years, after which time a proportion of cells fail to regenerate and ultimately the plant dies (D. Gaff, Monash University, pers. comm). Resurrection plants tend to be poor competitors, depending on shallow soils where more deep rooted, vigorous species are unable to establish.

Reproduction is vegetative, presumably by adventitious roots which occur on stems, or by separation of sections of the colony. On more vertical sections of rock, *B. mirabilis* colonies appear to be part of lithoseral stages, where bare rock is colonised by algae and crustose lichens, followed by bryophytes, herbs and geophytes which facilitate soil accumulation, and subsequent colonisation by more deep rooted or woody species. At a critical stage, when the substrate is no longer able to support their weight, parts or the whole of the colonising community will break away.

The response of *B. mirabilis* to fire is unclear, although the site does appear to have been burnt at some time in the past.

B. mirabilis is likely to be insect pollinated. However, the population is thought to consist of only two widely separated genotypes and plants may be unable to breed because of genetic restrictions (Cropper 1994).

12.4.7. 1.7 Population structure and condition

Approximately 70 ramets are distributed within five colonies over an area approximately 60 m x 20 m, although all except one colony occur within 20 m of each other. Ramets in each colony appear as irregular shaped 'clumps', but are not always easy to separate into individual plants.

Leaf shedding and a gradual decline in the ability of plants to produce new growth or resurrect fully from a desiccated state has been observed by Parks Victoria Rangers since 1996 (D. Handscombe, J. Read, PV, pers. comm.). Although some Western Australian *Borya* species are known to avoid drought by shedding leaves during periods of stress (Churchill 1987), this has not been observed in *B. mirabilis* and the current cause of leaf loss is unknown. Some basal resprouting was observed in early summer, 1997, but seemed to be restricted to plants growing in deeper soils protected from disturbance and moisture loss, beneath shrubs and in crevices between boulders.

Plants confined to more open sites are generally in poor health and often rooted in raised mounds of soil, suggesting that there has been a significant amount of erosion of soil within the population. There are a number of dead shrubs at the site, although the species affected suggest that this is most likely a result of drought or natural senescence, rather than infection by fungal pathogens such as *Phytophthora cinnamomi*.

Thus, it is likely that the current observed decline can be attributed to inadequate moisture supplies throughout autumn and winter since 1996, exacerbated by additional soil drying related to habitat disturbance. As a consequence, plants may be dessicated for too long a period and have been unable to fully recover, or the period and degree of rehydration between long spells of dryness is inadequate to sustain them in the following season.

12.4.8. 1.8 Propagation potential

B. mirabilis can be cultivated from cuttings but appears to require a reasonably specific watering regime, including wet and dry periods (N. Walsh, RBG, pers. comm.).

A large collection of Australian *Borya* spp., including *B. mirabilis*, was maintained at the Royal Botanic Gardens, Melbourne, but has declined in recent years. There is some anecdotal evidence of a private collection of *B. mirabilis* grown from material collected in the Victoria Range by Dr Ross McDonald (S. Cropper, pers. comm.).

Three to four plants were also held in cultivation at Monash University, established from a single plant donated by David Churchill in the early 1980s. During their cultivation, it was apparent that well watered, flourishing plants gradually lost their ability to desiccate and rehydrate. However, had they been given sufficient water stress so that the green appearance of leaves diminished gradually before full air drying, resurrection may have been more successful (Dr Don Gaff, Botany Department, pers. comm.). Plants also tended to get long and leggy in cultivation and probably need occasional pruning (D. Gaff, pers. comm.).

12.4.9. 1.9 Reasons for listing

The single, small population prompted listing as an Endangered species under the *Endangered Species Protection Act* 1992. In its recommendation for listing as a threatened taxon on Schedule 2 of the *Flora and Fauna Guarantee Act* 1988, the Scientific Advisory Committee determined that *B. mirabilis* is very rare in terms of abundance and distribution, is significantly prone to future threats which are likely to result in extinction and is possibly one of the most endangered plant species in Australia.

12.4.10. 1.10 Threats

The site is prone to erosion due to slope and soil characteristics, and hydrology of the area (S. Cropper, Department of Natural Resources and Environment file 89/2950-1). However, this is likely to have been exacerbated in recent periods by prolonged drought, sometimes followed by sudden episodes of unseasonal heavy rainfall in western Victoria in 1997 and 1998.

Other factors contributing to soil disturbance and erosion are digging by animals, in particular rabbits, but also echidnas; trampling by black wallabies which have increased in numbers in the Grampians in recent years (P. Menkhorst, NRE, pers. comm.), and possibly damage by goats.

Vegetation at the site may be coming more open with dying back of associated shrubs, with the consequence of reduced shading and increased soil drying.

The Grampians is an extremely popular National Park, and the site is close to areas used by rock climbers and bush walkers. Broken glass at the site suggests site visits, and therefore trampling, has occurred in the past. Illegal collection of plants, or damage to the site from naturalists also threaten the long term survival of the population.

Wildfire may threaten the population's persistence (Churchill 1987), although there is evidence that the site has been burnt in the past. However, fire fighting activities including use of retardant and raking are potential threats.

The population lacks genetic variability and fails to set seed, and its small size and single occurrence suggests it is particularly vulnerable to extinction. Neither the biology nor ecology of *B. mirabilis* or other members of the genus is particularly well understood, so that population management may be hampered by lack of knowledge.

12.4.11. 1.11 Existing conservation measures

Ground searches have been conducted by Field Naturalists and National Parks Officers (S. Cropper pers. comm.), and searches to identify broadly

similar habitat were conducted by helicopter in 1993. Three distinct areas were identified. The feasibility of using fine resolution remote sensing techniques to locate other populations or similar habitat was investigated in 1993 (Cropper 1994).

A large *ex-situ* collection of *Borya* spp., including *B. mirabilis* was established at the Royal Botanic Gardens, Melbourne by David Churchill in the 1970s and 1980s. These were included with wild material in an allozyme analysis of *Borya*, conducted at La Trobe University in 1992 (Dr Yvonne Fripp), to determine the genetic variation within the population (Cropper 1994).

The walking track near to the existing population will realigned by parks Victoria and the location of the site is kept confidential to reduce the likelihood of illegal collection and site damage.

Funding was received from Environment Australia to implement interim recovery actions in 1999, to be completed in mid 2000. Work includes identifying *B. mirabilis* habitat by comparing its floristic and environmental attributes with adjacent habitat to assess its potential to support translocated plants, and to determine whether the population might have been larger in the past. Permanent transects have also been established through individual plants to measure the proportion of resurrected or new shoots. Identification of life history stages and an assessment of population health is also underway.

12.5. 2 Strategy for Recovery

The strategy for recovery of *B. mirabilis* will interpret ecological, biological, genetic and horticultural information to manage existing habitat, establish an *ex-situ* collection, augment the existing population with cultivated plants, search for new populations and identify habitat suitable for translocation.

12.5.1. 2.1 Community Involvement

Community participation will be sought during the *ex-situ* phases of recovery, by encouraging individuals or groups with demonstrated expertise to assist with propagation and cultivation.

12.5.2. 2.2 Recovery goals

The **long term goal** is to prevent extinction or further decline in numbers of *B. mirabilis*, by maintaining and augmenting the population, and by managing existing habitat for the future self sustainability of the species.

Within the life span of the Recovery Plan, the **short term goals** of recovery are:

1. Significantly reduce the impact of immediate threats.

2. Increase the size of the existing population.

3. Attempt to extend the range of *B. mirabilis*.

4. Establish a genetically representative *ex-situ* collection.

12.5.3. 2.3 Recovery Criteria

The criteria for assessing the achievement of these objectives are:

1. Establishment of a comprehensive biological, ecological and horticultural knowledge base.

2. A decrease in disturbance and damage to the site where the population occurs.

3. A measurable increase in the population's productivity and resurrection potential.

4. Completion of a comprehensive search.

5. Maintain plants in cultivation and translocated plants in the wild.

12.5.4. 2.4 Recovery Actions

1. Prevent further soil disturbance.

2. Measure plant health against habitat management

3. Search for new sites

4. Establish an *ex-situ* collection.

5. Translocation.

6. Prepare FFG Action Statement and Critical Habitat Determination

12.6. 3 RECOVERY ACTIONS

12.6.1. 3.1 Prevent further soil disturbance

- 3.1.1 Control pests

- 3.1.2 Habitat restoration
- 3.1.3 Prepare a fire response plan

12.6.1.1. *3.1.1 Control pests*

There is a need to investigate methods for destroying rabbit populations within and nearby the *B. mirabilis* site, while preventing further soil disturbance. An intensive program might include establishing baiting stations. Rocky terrain and the need to maintain site security will preclude fencing. A survey for feral goats should also be conducted within the Wonderland Range, with a view to controlling animals. A survey to determine whether utilisation of the site by native fauna is a significant threat to the population should also be undertaken. Baiting should be carried out quarterly and surveys should be undertaken annually.

Funds are required to contract a zoologist to carry out the work. Parks Victoria will implement pest control, provide materials, prepare the project brief, engage and manage contractor, and assist with surveys where required.

Year	2001	2002	2003	2004	2005
TC	$3,050	$1,350	$1,350	$1,350	$1,350

12.6.1.2. *3.1.2 Habitat restoration*

Remedial work at the site should commence immediately, to prevent further soil loss and to minimise further soil drying. Measures to reduce erosion should be investigated and trialled. These might include mulching around *Borya* plants using litter from taxa known to occur at the site, translocated bryophyte mats or translocated soil which has been suitably tested for physical and chemical properties and screened for *Phytophthera cinnamomi*. Work should be maintained annually.

Funds are required to contract a specialist horticulturalist such as the Royal Botanic Gardens to carry out the work and conduct follow up monitoring. Parks Victoria will prepare the project brief, manage the work and assist where required.

Year	2001	2002	2003	2004	2005
TC	$9,800	$6,000	$6,000	$6,000	$6,000

12.6.1.3. *3.1.3 Prepare a fire response plan*

Fire control guidelines should be prepared and circulated to fire crews prior to planned burns, and in the event of unplanned fires, so that the risk of accidental damage to the population is reduced. These should include

guidance on the use of retardant, use of rakes and clearing of vegetation for fire breaks. The location of the *B. mirabilis* population should also be made known to fire crews and its significance explained.

Parks Victoria will prepare and implement the guidelines, update maps for inclusion in operations folders and liaise with NRE to include guidelines in the Horsham District Fire Plan..

Year	2001	2002	2003	2004	2005
TC	$2,000	$ 0	$ 0	$ 0	$ 0

12.6.2. 3.2 Measure plant health against habitat management

The physiological performance of individual plants should be assessed indirectly each year by comparing the proportions of new or resurrected shoots with dead material in autumn, spring and early summer. A method has been developed using transects divided into 20 x 1 cm intervals permanently placed through each ramet in the population. The number of 1 cm intervals with a living or dead shoot are recorded and ratios calculated.

Funds are required to contract a botanist to collect and analyse the data, and to report on the efficacy of recovery actions. Parks Victoria will prepare the project brief and manage the contract.

Year	2001	2002	2003	2004	2005
TC	$2,200	$2,200	$2,200	$2,200	$2,200

12.6.3. 3.3 Search for new sites and potential habitat

Risk of extinction would be significantly reduced if the range of *B. mirabilis* was extended either as existing populations or as translocated populations to suitable sites. New populations are most likely to be seen in late summer, when plants have dessicated and turned bright orange, so that they are easily identified from a distance.

12.6.3.1. *3.3.1 Survey design*

Broad areas which might contain suitable *Borya* habitat were identified by air and searches for unknown populations carried out in 1993 (NRE File 89\19-2950\1; S. Cropper pers. comm.). However, owing to wet, windy conditions, flight time was limited. Furthermore, plants in the Wonderland Range were known to have rehydrated and turned green after unseasonal

wet weather, so that any other existing populations were unlikely to have been seen from the air. A predictive analysis using recent (1998/9) vegetation data derived from EVC mapping, combined with recent (1999) geological maps and existing knowledge should be undertaken and potential search sites digitised.

Funds are required to contract a specialist geographer to identify sites and digitise mapping. Parks Victoria will assist wih site identification, prepare the project brief and manage the work.

Year	2001	2002	2003	2004	2005
TC	$7,500	$0	$0	$0	$0

12.6.3.2. *3.3.2 Conduct searches*

Ground searches of identified areas should be conducted in late February/March, after dry weather when plants are most likely to be seen. Volunteer botanists with helicopter access to targeted areas, owing to steep terrain and the likelihood of hot weather, will be the most efficient approach.

Searching will be carried out by Parks Victoria Rangers, RBG, Universities and DNRE Botanists. Parks Victoria Rangers will plan and manage the search and co-ordinate logistical support. Funds are sought to contribute to helicopter hire for 6 hours and to cover transport and accommodation costs of 10 specialist volunteers.

Year	2001	2002	2003	2004	2005
TC	$0	$21,300	$0	$0	$0

12.6.4. 3.4 Establish an *ex-situ* collection

- 3.4.1 Collect representative samples
- 3.4.2 Develop propagating techniques
- 3.4.3 Investigate breeding system

12.6.4.1. *3.4.1 Collect representative samples*

A representative *ex-situ* collection should be established using material collected in late autumn or winter from all plants in the population. Where possible, three cuttings should be taken from each of five plants in each colony, spread over two years to account for losses and to minimise the impact on the population. Use of healthy shoots is likely to yield the best results. Cuttings will be propagated at the Royal Botanic Gardens.

The Royal Botanic Gardens, Melbourne will be responsible for collection. Parks Victoria will liaise with horticulturalists where necessary.

Year	2001	2002	2003	2004	2005
TC	$1,500	$1,500	$0	$0	$0

12.6.4.2. *3.4.2 Develop propagating techniques*

There is a need to establish *ex-situ* populations, for use in genetic work, translocation and inclusion into living collections in State and National Botanic Gardens in Melbourne, Perth and Canberra, to safeguard the species in the event of any unforeseen destruction of the wild population.

Borya species have been successfully cultivated in the past but collections have diminished and horticultural expertise lost. Preliminary trials suggest that cuttings may be difficult to strike. Development and documentation of methods and cultivating techniques are needed, including tissue culture. Watering should be carried out to determine a method of maintaining a suitable physical environment so that plants retain their ability to resurrect.

Funds are sought to contract the Royal Botanic Gardens, Melbourne to cultivate an *ex-situ* population. Parks Victoria will prepare the project brief and manage the work.

Year	2001	2002	2003	2004	2005
TC	$11,000	$11,000	$0	$0	$0

12.6.4.3. *3.4.3 Investigate breeding system*

There is a need to determine the population's genetic structure and breeding system, and to test for self-incompatibility to produce a representative collection in cultivation.

The degree of genetic diversity within the existing populations is not clear, although it has been suggested that there are only two genotypes present (Cropper 1993). However, no viable seed has been collected from plants. *B. mirabilis* plants held in cultivation in close proximity to other *Borya* species have set seed, probably as a result of hybridisation (D. Gaff, Monash University, pers. comm.).

Necessary work includes allozyme analysis using a representative number of samples across the population and screening a range of enzymes, followed by confirmation using a DNA based technique such as Amplified Fragment Length Polymorphism (AFLP), if necessary. Interpretation of results will indicate the current state of the breeding system, which should be confirmed by examination of floral development, stigma function and ovule

formation in both wild and cultivated populations, and tests for pollen viability using the fluorochromatic reaction. Manual cross pollination trials between and within genotypes will determine whether self incompatibility is preventing seed set. This will determine whether hand pollination of wild plants is worthwhile in the future.

Funds are sought to contract a conservation geneticist to undertake the work. The Royal Botanic Gardens will provide laboratory facilities. Funding in 2002 will only be necessary if AFLP is used.

Year	2001	2002	2003	2004	2005
TC	$5,150	$5,750	$0	$0	$0

12.6.5. 3.5 Translocation

- 3.5.1 Augment the existing population
- 3.5.2 Translocate cultivated plants to a new site
- 3.5.3 After care and monitoring

12.6.5.1. *3.5.1 Augment the existing population*

Site environmental and floristic survey results (in progress) will be analysed to determine suitable sites for translocating plants. Numbers of plants to be translocated will depend on the extent of available habitat, and cultivation success.

Funds are required to contract the Royal Botanic Gardens to prepare the site, transport and establish plants. Parks Victoria will manage the work and assist where required.

Year	2001	2002	2003	2004	2005
TC	$0	$0	$0	$7,800	$7,800

12.6.5.2. *3.5.2 Translocate cultivated plants to a new site*

If a suitable site is found, plants should be translocated on a trial basis, using suitable site preparation and translocation methods.

Funds are required to contract the Royal Botanic Gardens, Melbourne to carry out the work, managed by Parks Victoria.

Year	2001	2002	2003	2004	2005
TC	$0	$0	$0	$7,800	$7,800

12.6.5.3. *3.5.3 After care and monitoring.*

Survivorship and life history stages of translocated plants will be recorded at critical stages following translocation, with the aim of modifying management if necessary.

Funds are required to contract the Royal Botanic Gardens, Melbourne to carry out the work. Parks Victoria will manage the work and assist where required.

Year	2001	2002	2003	2004	2005
TC	$0	$0	$0	$2,800	$2,800

3.6 Revise FFG Action Statement and prepare Critical Habitat Determination

An Action Statement and Critical Habitat Determination should be prepared under the Victorian *Flora and Fauna Guarantee Act* 1988 to maximise the species' protection.

The Department of Natural Resources and Environment will be responsible for the carrying out and publishing the work, in consultation with Parks Victoria.

Year	2001	2002	2003	2004	2005
TC	$2,000	$0	$0	$0	$0

3.7 Manage Recovery Plan Implementation

Consultation in the form of Recovery Team meetings should take place twice a year, to evaluate recovery progress, and review and amend the Recovery Plan where necessary. The Recovery Team will include Rangers, NRE staff, RBG staff and local naturalists.

Parks Victoria will manage Recovery and facilitate Recovery Team meetings.

Year	2001	2002	2003	2004	2005
TC	$5,250	$5,250	$5,250	$5,250	$5,250

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12.8. 5 APPENDICES

12.8.1. 5.1 Appendix 1 Summary of Recovery Objectives and Actions

Action 1 Prevent further soil disturbance

1.1 Control pests

1.2 Habitat restoration

1.3 Prepare a fire response plan

Action 2 Measure plant health against habitat management

Action 3 Search for new sites

3.1 Survey design

3.2 Conduct searches

Action 4 Establish an *ex-situ* collection

4.1 Collect representative samples

4.2 Develop propagating techniques

4.3 Investigate breeding system

Action 5 Translocation

5.1 Augment the existing population

5.2 Translocate cultivated plants to a new site

5.3 After care and monitoring

Action 6 Revise FFG Action Statement and prepare Critical Habitat Determination

Action 7 Manage Recovery Plan implementation

12.8.2. 5.2 Appendix 2 Implementation Schedule

Task	Description	Priority	Feasibility	Resp Party	Cost					
					2001	2002	2003	2004	2005	Tota
1	*Prevent further soil disturbance*									
1.1	Control pests	1	100	PV/Contractor	3.05	1.35	1.35	1.35	1.35	8.4
1.2	Habitat restoration.	1	80	PV/Contractor	9.8	6.0	6.0	6.0	6.0	33.
1.3	Prepare a fire plan	1	100	PV	2.0	0	0	0	0	2.(
2	*Measure plant health*	1	80	PV/Contractor	2.2	2.2	2.2	2.2	2.2	11.
3	*Search for new sites*	1	10							
3.1	Design survey	1	100	PV/Contractor	7.5	0	0	0	0	7.5
3.2	Conduct searches	1	100	PV/Volunteers	0	21.3	0	0	0	21.
4	*Ex-situ cultivation*									
4.1	Collect representative samples	1	100	PV/RBG	1.5	1.5	0	0	0	3.(
4.2	Develop propagation techniques	2	70	PV/RBG	11.0	11.0	0	0	0	22.
4.3	Investigate breeding system	2	70	PV/RBG	5.15	5.75	0	0	0	10.
5	*Translocation*									
5.1	Augment the existing population	2	50	PV/RBG	0	0	0	7.8	7.8	15.
5.2	Translocate to a new site	2	20	PV/RBG	0	0	0	7.8	7.8	15.
5.3	After care and monitoring	2	100	PV/RBG	0	0	0	2.8	2.8	5.(
6	*Revise FFG Action Statement*	1	100	DNRE	2.0	0	0	0	0	2.(
7	*Manage implementation*	1	100	PV	5.25	5.25	5.25	5.25	5.25	26.2
	Totals ($000s)				**49.45**	**54.35**	**14.8**	**33.2**	**33.2**	**18**

Citation: Coates, F. (2000) Recovery Plan 2001-2005 - *Borya mirabilis* Churchill (Grampians Pincushion Lily). School of Botany, La Trobe University, Victoria.

A Recovery Plan prepared under the Commonwealth Environment Protection and biodiversity Conservation Act 1999.

Appendix 5-1: ANOVA t results for LMHM media with or without the addition of PPM for reducing contamination of explants.

Source	*DF*	*SS*	*MS*	*F*	*P*
PPM added	1	14.0167	14.0167	841.00	0.000
Error	58	0.9667	0.0167		
Total	59	14.9833			

S= 0.1291 R-Sq=93.55% R-Sq (adj) =93.44%

Appendix 5-2: ANOVA t results for LMHM media with agar or Phytagel gelling agent for reducing contamination of explants.

Source	*DF*	*SS*	*MS*	*F*	*P*
Gelling agent	1	1.350	1.350	6.37	0.014
Error	58	12.300	0.212		
Total	59	13.650			

S= 0.4605 R-Sq=9.89% R-Sq (adj) =8.34%

Appendix 5-3: ANOVA t results for LMHM media with agar or Phytagel gelling agent effecting explant health.

Source	*DF*	*SS*	*MS*	*F*	*P*
Gelling agent	1	45.07	45.07	10.02	0.002
Error	58	260.87	4.50		
Total	59	305.93			

S= 2.121 R-Sq=14.73% R-Sq (adj) =13.26%

Appendix 7-1: *Phytophthora* selective media (Reiter, 2002).

Ingredients	*Quantity*
Malt Extract	15 g
Agar	20 g
Deionised water	1 L

Autoclave at 121°C for 20 min and allow to cool to 45°C and add antibiotics.

Ingredients	*Quantity*
Mycostatin (Nilstat)	1 mL
Hymexazol (as tachiarin)	50 mg
Rifamicin #	10 mg
Ampicillin^	250 mg
Rose of bengal	30 mg

Dissolve in 70% ethanol
^ Dissolve in 10 mL of sterile deionised water

Appendix 7-4: 'Pine Plantation' *Borya mirabilis* translocation site.

Quadrate	Positive	Negative	Bacterial contamination
1A 0-4	0	1	
1A 4-8	0	1	1
1A 8-12	0	1	
1A 12-16	0	1	
1A 16-20	0	1	
1B 0-4	0	1	
1B 4-8	0	1	
1B 8-12	0	1	
1B 12-16	0	1	
1B 16-20	0	1	
2A 0-4	0	1	
2A 4-8	0	1	
2A 8-12	0	1	
2B 0-4	0	1	
2B 4-8	0	1	
2B 8-12	0	1	
3A 0-5	0	1	
3A 5-10	0	1	
3A 10-15	0	1	
3A 15-20	0	1	
3A 20-25	0	1	
3B 0-5	0	1	
3B 5-10	0	1	
3B 10-15	0	1	
3B 15-20	0	1	
3B 20-25	0	1	
4A 0-5	0	1	
4A 5-10	0	1	
4A 10-15	0	1	1
4A 15-20	0	1	
4A 20-25	0	1	
4B 0-5	0	1	
4B 5-10	0	1	

Quadrate	*Positive*	*Negative*	*Bacterial contamination*
4B 10-15	0	1	
4B 15-20	0	1	
4B 20-25	0	1	
4C 0-5	0	1	
4C 5-10	0	1	
4C 10-15	0	1	
4C 15-20	0	1	
4C 20-25	0	1	
T1 -15-10	0	1	
T1 -10-5	0	1	
T1 -5 0	0	1	
T1 0-5	0	1	
T1 5-10	0	1	
T1 10-15	0	1	1
T1 15-20	0	1	1
T1 20-25	0	1	
T1 25-30	0	1	
T1 30-35	0	1	1
T1 35-40	0	1	
T1 40-45	0	1	
T1 45-50	0	1	
T2 0-5	0	1	
T2 5-10	0	1	
T2 10-15	0	1	
T2 15-20	0	1	
T2 20-25	0	1	
T2 25-30	0	1	

Appendix 7-5: 'Mackey's Peak' *Borya mirabilis* translocation site soil analysis.

Quadrate	Positive	Negative	Bacterial complications
A 0-4	0	1	
A 4-8	0	1	
A 8-12	0	1	
A 12-16	0	1	
A 16-20	0	1	
A 20-24	0	1	
A 24-28	0	1	
B 0-4	0	1	
B 4-8	0	1	
B 8-12	0	1	1
B 12-16	0	1	
B 16-20	0	1	
B 20-24	0	1	
B 24-28	0	1	
C 0-4	0	1	
C 4-8	0	1	
C 8-12	0	1	
C 12-16	0	1	
C 16-20	0	1	1
C 20-24	0	1	
C 24-28	0	1	
D 0-4	0	1	
D 4-8	0	1	
D 8-12	0	1	
D 12-16	0	1	
D 16-20	0	1	
D 20-24	0	1	
E 0-4	0	1	1
E 4-8	0	1	
E 8-12	0	1	
E 12-16	0	1	
E 16-20	0	1	

Quadrate	*Positive*	*Negative*	*Bacterial complications*
E 20-24	0	1	
E 24-28	0	1	
F 0-4	0	1	
F 4-8	0	1	
F 8-12	0	1	
F 12-16	0	1	
F 16-20	0	1	
F 20-24	0	1	
F 24-28	0	1	
T1 0-5	0	1	
T1 5-10	0	1	
T1 10-15	0	1	
T1 15-20	1	0	
T1 20-25	0	1	
T1 25-30	0	1	
T1 30-35	0	1	
T1 35-40	0	1	
T1 40-45	0	1	
T1 45-50	0	1	
T1 50-55	0	1	
T1 55-60	0	1	

Appendix 7-6: 'Reid's Lookout' *Borya mirabilis* translocation site soil analysis.

Quadrate	Positive	Negative	Bacterial complications
A 0-4	1	0	
A 4-8	0	1	
A 8-12	0	1	
A 12-16	0	1	
A 16-20	0	1	
A 20-24	0	1	
A 24-28	1	0	
A 28-32	0	1	
A 32-36	0	1	
B 0-4	0	1	
B 4-8	1	0	
B 8-12	0	1	
B 12-16	1	0	
B 16-20	0	1	
B 20-24	0	1	
B 24-28	0	1	
B 28-32	0	1	
B 32-36	0	1	
C 0-4	0	1	
C 4-8	0	1	
C 8-12	0	1	
C 12-16	0	1	
C 16-20	0	1	
C 20-24	0	1	
C 24-28	0	1	
C 28-32	0	1	
C 32-36	0	1	
D 0-4	0	1	
D 4-8	0	1	
D 8-12	0	1	
D 12-16	0	1	
D 16-20	0	1	
D 20-24	0	1	

Quadrate	*Positive*	*Negative*	*Bacterial complications*
D 24-28	0	1	
D 28-32	0	1	
D 32-36	0	1	
T 0-4	0	1	
T 4-8	1	0	
T 8-12	1	0	
T 12-16	1	0	
T 16-20	1	0	
T 20-24	1	0	
T 24-28	1	0	
T 28-32	1	0	
T 32- 36	1	0	
T 36-40	0	1	
T 40-44	1	0	
T 44-48	0	1	
T 48-32	0	1	
T 32-36	0	1	
T 36-40	0	1	
T 40-44	0	1	
T 44-48	0	1	