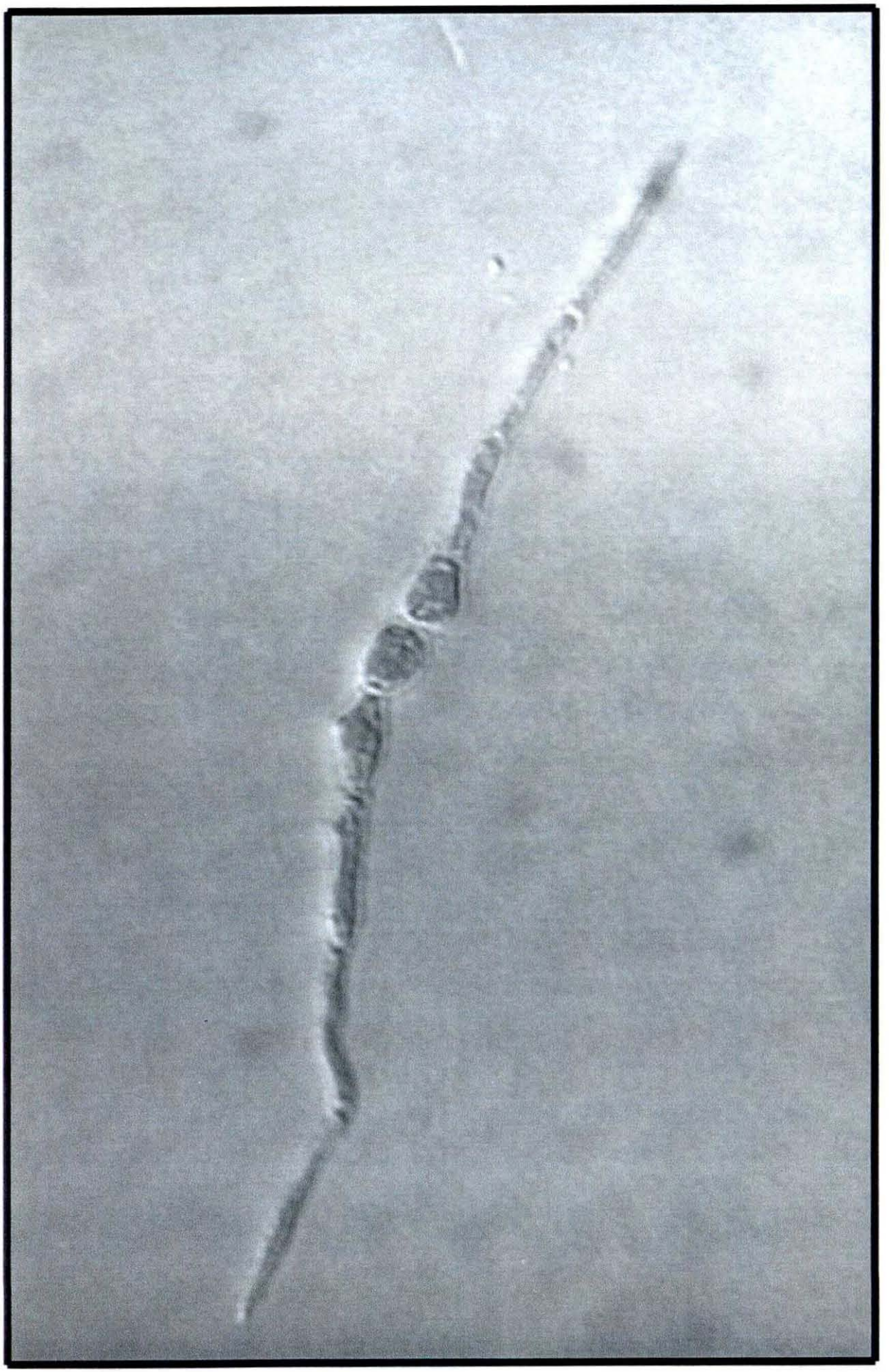
Chapter 4

A PCR-based identification method for common species of *Mycosphaerella* occurring on *Eucalyptus*



ABSTRACT

Twenty-nine species of *Mycosphaerella* are associated with Mycosphaerella leaf disease (MLD), a serious disease of natural and commercially grown *Eucalyptus* spp. worldwide. Identification of these species relies on teleomorph-anamorph characteristics, ascospore germination patterns and DNA sequence data. The most reliable of these is DNA sequence analysis but this is an expensive and time consuming approach to identification. The goal of this study was to consider the feasibility of employing restriction enzymes to distinguish between common species of *Mycosphaerella* occurring on *Eucalyptus* spp. This was achieved by identifying restriction enzyme recognition sites from DNA sequence data of the Internal Transcribed Spacer (ITS) region of the rDNA operon for 21 species. Selected enzymes were tested for their efficiency in distinguishing between species by digesting PCR amplified fragments of the ITS region. Results showed that the restriction enzyme *Hae*III is effective in distinguishing several *Mycosphaerella* species and that it also results in discrete groups for the remaining species. Using this restriction enzyme in conjunction with ascospore germination patterns was effective and reduced the need to use DNA sequencing.

INTRODUCTION

Many species belonging to the ascomycetous genus *Mycosphaerella* Johanson are found on leaves of *Eucalyptus* L'Heritier, where they are associated with a disease known as Mycosphaerella leaf disease (MLD) (Crous 1998, Carnegie & Keane 1998, Dick & Dobbie 2001). Several *Eucalyptus* spp. belonging to both the sub-genera *Symphyomyrtus* and *Monocalyptus* are recognized as being susceptible to *Mycosphaerella* spp. (Park & Keane 1982, Crous 1998). Infection results in the formation of extensive leaf spotting, which decreases the photosynthetic potential of leaves. Leaves abscise prematurely resulting in defoliation and in severe cases growth retardation and death (Beresford 1978). Some species also have the ability to infect young shoots and branches causing tree malformation (Ganapathi 1979).

The identification of *Mycosphaerella* spp. is complicated and relies on a combination of several techniques. The manner in which ascospores of *Mycosphaerella* spp. occurring on *Eucalyptus* spp. germinate, has been used as a characteristic by which species or groups of species can be identified (Park & Keane 1982, Crous 1998). Ascospores may germinate from one or both spore poles, may become prominently constricted and pigmented upon germination, produce secondary lateral branches or become verrucose after prolonged germination. All of these characteristics can be assembled in fourteen types of ascospore germination patterns (Crous 1998). However, some germination patterns provide insufficient information for species identification, and rather indicate groups consisting of several species (Crous 1998).

At present, 23 anamorph genera are recognized as conidial states of *Mycosphaerella* (Crous *et al.* 2000). Some researchers state that these anamorphs might indicate lineages within *Mycosphaerella* (Crous 1998). There has, however, been controversy regarding the use of anamorph associations for the identification of *Mycosphaerella* spp. Arx (1983) believed that anamorph genera should not be used to define species lineages within *Mycosphaerella* because morphological characteristics were inordinately divergent between anamorph genera. Using multiple correspondence analysis (MCA), Crous (1998) found that anamorphs reflected groups within *Mycosphaerella* spp. from *Eucalyptus*. However, subsequent DNA sequencing studies have shown *Mycosphaerella* to be monophyletic and

that some anamorph genera have evolved more than once in the genus (Crous *et al.* 1999, Crous *et al.* 2000 Crous *et al* 2001).

DNA sequencing of various regions of the genome has become increasingly useful in identifying species and lineages within *Mycosphaerella*. The ribosomal DNA operon has been targeted in many studies and has been found to be phylogenetically informative (Crous *et al.* 1999, Stewart *et al.* 1999, Crous *et al.* 2001). By employing DNA sequencing it has been possible to place certain *Mycosphaerella* spp. with no recognized anamorph associations into *Mycosphaerella* within certain anamorph clades, thus offering an idea of their asexual state (Stewart *et al.* 1999, Crous *et al.* 2001). Moreover species previously not recognised as being unique have been discovered and described.

Molecular markers have become increasingly important in identifying various fungi and have also found application within *Mycosphaerella*. Randomly amplified polymorphic DNA markers (RAPD's) have been developed and found to be effective in distinguishing between *M. cryptica* (Cooke) Hansf., *M. nubilosa* (Cooke) Hansf., *M. gregaria* Carnegie & Keane and *M. marksii* Carnegie & Keane, all important species contributing to outbreaks of MLD in Australia (Carnegie *et al.* 2001). Carlier *et al.* (1994) were able to distinguish between *M. musicola* Leach ex Mulder and *M. fijiensis* Morelet, two morphologically similar banana pathogens, using restriction fragment length polymorphisms (RFLP's). Such DNA based markers offer advantages in fungal identification, especially where data from these methods is combined with classical morphological characteristics.

Although these various methods have been developed to facilitate the identification of *Mycosphaerella* spp. on *Eucalyptus*, they have limitations. Ascospore germination patterns do not consistently yield reliable identifications and instead, may only indicate groups of species. DNA sequencing is a reliable technique to identify species but it is time consuming, expensive and inaccessible to many laboratories and forestry companies. The aim of this study was, therefore, to investigate the possibility of using restriction fragment length polymorphisms (RFLP's) to distinguish between common species of *Mycosphaerella* occurring on *Eucalyptus*. This was achieved by identifying restriction enzyme recognition sites within the sequence of the Internal Transcribed Spacer (ITS) region of the rDNA operon.

MATERIALS AND METHODS

Isolates studied

Twenty-one species of *Mycosphaerella* occurring on *Eucalyptus* spp. from various countries were used for this study (Table 1). The twenty-one species were chosen based on the availability of cultures and to a lesser extent on the common occurrence of species. All of the species used in this study are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria. All isolates were grown on 2% malt extract agar (MEA) (wt/v) (Biolab, South Africa) and incubated in a walk in incubator at 25°C under continuous cool white light.

DNA isolation

Following growth of pure cultures, mycelium was scraped directly from agar plates and used for DNA isolation. Mycelium was dried under vacuum and lypholized with liquid nitrogen. DNA was isolated using the method described by Raeder & Broda (1985), with minor modifications. The 1:1 phenol:chloroform purification step was repeated until the interphase between the two aqueous phases was clean of any cellular debris. Nucleic acids were precipitated by the addition of 10% 3M NaAc and 2 volumes of absolute ethanol and incubated at -20°C for 2 hours. DNA was further purified by washing with 70% ethanol and dried under vacuum, after which the resulting DNA pellet was resuspended in 50 µl SABAX water. RnaseA (10 µg/µl) was added to the DNA samples, and incubated at 37°C for three to four hours to digest any residual protein or RNA. DNA was visualized on a 1% agarose gel (wt/v) (Boehringer Mannheim, Germany) stained with ethidium bromide and viewed under ultra-violet light. DNA was quantified for all samples with a Beckman DU Series 60 Spectrophotometer (Beckman, Germany).

PCR Amplification

Isolated DNA (50–90 ng) was used as a template for the Polymerase Chain Reaction (PCR). The Internal Transcribed Spacer (ITS) region of the rDNA operon, was targeted for amplification using primers ITS 1 (5'- TCC GTA GGT GAA CCT GCG G –3') and

LR1 (5'- GGT TGG TTT CTT TTC CT –3') (White *et al.* 1990). The ITS 1 and ITS 2 regions including the 5.8S gene were amplified. DNA was amplified in a 50 µl reaction volume containing PCR buffer (10 mM Tris-HCL, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3) (Roche Diagnostics, South Africa), 2.5 mM of each dNTP (dATP, dTTP, dCTP and dGTP) (Roche Diagnostics, South Africa), 0.2 µM of primers ITS1 and LR1 (MWG Biotech, Germany) and 2.5 U Taq DNA polymerase (Roche Diagnostics, South Africa). SABAX water was used to achieve the total volume of 50 µl.

PCR reactions were carried out using an Eppendorf Mastercycler gradient PCR machine (Eppendorf Scientific, Germany). PCR reaction conditions consisted of an initial denaturation temperature of 96°C for 2 min. Following this, 40 cycles of template denaturation for 30 s at 94°C, primer annealing for 30 s at 53°C and chain elongation for 2 min at 75°C were carried out with a final elongation at 75°C for 7 min. A negative control using water and no template DNA and a positive control containing DNA of a *Mycosphaerella* sp. was used for each reaction. PCR products were visualized in ethidium bromide stained 2% agarose gels and viewed under ultra-violet light. Sizes of PCR products were determined against a 100 bp molecular weight marker XIV (Roche Diagnostics, South Africa).

DNA restriction and electrophoresis

DNA sequence data from the Internal Transcribed Spacer region (ITS) of the rDNA operon was evaluated for the presence of restriction enzyme recognition sites using Webcutter v2.0 (Heimann 1997). DNA sequence data for the large subunit (LSU), the 5.8S gene and the small subunit (SSU) together with the ITS1 and ITS2 regions of the rDNA operon have been previously published for all species tested during this study (Crous *et al.* 2001).

Restriction analysis of the amplified ITS region was conducted using the restriction enzyme selected with Webcutter (v 2.0). The restriction enzyme (2 Units) was added directly to 20 µl unpurified PCR amplification products. These reaction mixtures were placed in a hot water bath at 37°C for 3 to 4 hours to allow for digestion. Following restriction enzyme digestion, digested fragments were electrophoresed in agarose gels containing TBE [89 mM Tris, 89 mM boric acid, 2 mM EDTA (pH 8)] buffer for fragment

size determination. Fragments were run against a 100 bp molecular marker for size estimation. For electrophoresis, 2% agarose gels were run at 100V for 2h. Agarose gels were stained with ethidium bromide and visualized under ultra-violet light.

Taxonomic differentiation

All the isolates used in this study were evaluated for differences based on ascospore germination patterns. Ascospore germination patterns, as outlined by Crous (1998) were used to differentiate species that could not be individually identified with the use a restriction enzyme.

RESULTS

DNA isolation and PCR amplification

DNA isolation resulted in high concentrations of DNA (50–90 ng/µl) for all isolates used in this study. Strong amplification products of the ITS region were obtained using primers ITS 1 and LR1. Amplification products for all fungal isolates tested were approximately 600 bp in size.

DNA restriction

Using computer based software, Webcutter (v2.0), several restriction enzymes were evaluated for recognition sites within DNA sequences of the ITS region. From observed restriction maps, the enzyme *Hae*III [GG'CC] (Roche Molecular Biochemicals, South Africa) was chosen for further restriction digests. Restriction digestion of amplified ITS-PCR fragments gave different banding profiles for most of the isolates tested. However, not all isolates could be resolved to species level, but rather formed groups with similar banding profiles (Figure 1).

Of the 21 species digested with *Hae*III, eleven groups could be distinguished (Table 2). The first group consisted of *M. flexuosa* Crous & M. J. Wingf. (CMW 5224) and *M. juvenis* Crous & M. J. Wingf. (CMW 4937). This group showed a banding pattern of two

fragments, for *M. flexuosa* these were at 261 bp and 85 bp and for *M. juvenis* they were 262 bp and 87 bp. The second group was comprised of *M. ellipsoidea* Crous & M. J. Wingf. (CMW 5165) and *M. endophytica* Crous & H. Smith (CMW 5225). Digests of this group resulted in the generation of 3 fragments of 112 bp, 75 bp and 337 bp. Group 3 was characterized by *M. lateralis* Crous & M. J. Wingf. (CMW 5164) with 2 fragments of 175 bp and 247 bp. *M. colombiensis* Crous & M. J. Wingf. (CMW 4944) resided in a group of its own (group 4), and had a banding pattern of 2 fragments of 312 bp and 88 bp.

Group 5 consisted of 4 species, namely, *M. crystallina* (CMW 3042) Crous & M. J. Wingf., *M. heimioides* Crous & M. J. Wingf. (CMW 3046), *M. heimii* Crous (CMW 4942) and *M. irregulariramosa* Crous & M. J. Wingf. (CMW 4943). Restriction digests of this group resulted in the generation of 3 fragments. For *M. crystallina* and *M. heimii* the three fragments were 74 bp, 127 bp and 320 bp. For *M. heimioides* the restriction fragments were slightly larger, 74 bp, 128 bp and 318bp. The fragments for *M. irregulariramosa* were also slightly different to those of the other three species in that their sizes were 74 bp, 128 bp and 312 bp. Three *Mycosphaerella* spp., *M. molleriana* (Thüm.) Lindau, *M. cryptica* and *M. nubilosa* (Cooke) Hansf. made up Group six. Restriction digests resulted in the generation of three fragments. For *M. nubilosa* (CMW 3282) these fragments are 99 bp, 143 bp and 253 bp. For *M. molleriana* (CMW 4940) they are 99 bp, 142 bp and 227 bp and for *M. cryptica* (CMW 2732) they are 143 bp and 277 bp. Group seven contained *M. marksii* Carnegie and Keane (CMW 5150), which produces a restriction profile of 409 bp and 76 bp upon enzyme digestion.

Group 8 was characterized by four species, namely, *M. africana* Crous & M. J. Wingf., *M. keniensis* Crous & T. Coutinho, *M. parkii* Crous & M. J. Wingf. and *M. gregaria* Carnegie & Keane. These species showed banding profiles that were very similar, although their restriction fragments did differ slightly in size from each other. Restriction fragments for *M. africana* (CMW 4945) and *M. keniensis* (CMW 5147) were very similar. Those of *M. africana* were 117 bp and 341 bp, and for *M. keniensis* they were 118 bp and 341 bp. For *M. parkii* (CMW 3358) restriction fragments were 76 bp and 464 bp. Group 9 was characterized by one species, namely, *M. walkeri* R. F. Park & Keane (CMW 9477) that produced two bands upon digestion with *Hae*III. These two bands had sizes of 74 bp and 320 bp. Restriction group 10 was also represented by one species, *M. tasmaniensis* Crous & M. J. Wingf (CMW 5005). This group had two restriction fragments of 77 bp and 259

bp. A new species of *Mycosphaerella*, *M. fori* G. Hunter, Crous & M. J. Wingf (CMW 9095) isolated from leaves of *E. grandis* during MLD surveys in South Africa made up group 11 with three restriction fragments of sizes 103 bp, 76 bp and 349 bp.

Taxonomic differentiation

Although several species could not initially be identified to species level they formed smaller groups consisting of fewer species. Species within these groups could be further distinguished based on ascospore germination patterns as outlined by Crous (1998) (Table 2). *M. juvenis* and *M. flexuosa* (Group 1) have different ascospore germination patterns. *M. flexuosa* produces a Type K ascospore germination pattern while *M. juvenis* produces a Type F germination pattern (Crous 1998). *M. heimioides* and *M. heimii* can be distinguished from *M. crystallina* and *M. irregulariramosa* (Group 5) due to their specific ascospore germination. *M. heimioides* produces ascospore germination patterns of Type M, while *M. heimii* shows a Type C ascospore germination pattern. Group 6, containing *M. cryptica*, *M. molleriana* and *M. nubilosa* is slightly more difficult. *M. nubilosa* and *M. molleriana* have very similar ascospore germination patterns, namely, a Type C. However, *M. cryptica* can easily be separated from *M. nubilosa* and *M. molleriana* by producing a unique Type A ascospore germination pattern.

DISCUSSION

In this study we have shown that the restriction enzyme *Hae*III can be used to reliably distinguish between several species of *Mycosphaerella* from *Eucalyptus*. This enzyme was selected because it had the greatest number of recognition sites within the ITS region and thus a greater range of polymorphisms between the isolates used in this study. Not all species that were tested could be identified to species level. However, the remaining species resided in small groups of two to five species of *Mycosphaerella*. Species in these groups, could then be identified using ascospore germination patterns.

Mycosphaerella flexuosa and *M. juvenis* formed banding patterns that placed them together in group one. These two species could, however, be distinguished from each other based on ascospore germination patterns and the production of an anamorph. *M. juvenis* has a characteristic Type F germination pattern where ascospores germinate with parallel germ

tubes with a distinct swelling and distortion of the ascospore (Crous 1998). In contrast, *M. flexuosa* has a Type K ascospore germination pattern where spores germinate from both spore poles and germ tubes characteristically grow in a geniculate fashion across the agar surface (Crous 1998). *M. juvenis* readily produces its *Uwebraunia* anamorph, *Uwebraunia juvenis* Crous & M. J. Wingf in axenic culture while *M. flexuosa* has no recognized asexual state (Crous & Wingfield 1996, Crous 1998). *M. juvenis* is known to occur in South Africa, whereas *M. flexuosa* has been identified only from Colombia, South America (Crous & Wingfield 1996, Crous 1998). Thus, *Hae* III profiles together with ascospore germination patterns, anamorph associations and geographic distribution can differentiate between these two *Mycosphaerella* spp.

Mycosphaerella ellipsoidea and *M. endophytica* showed the same *Hae*III banding patterns. Both of these species are known only to occur in South Africa and they both show the same type of ascospore germination pattern (Type I) (Crous & Wingfield 1996, Crous 1998). They do, however, have distinct asexual states with *M. ellipsoidea* producing an *Uwebraunia* anamorph while *M. endophytica* forms a *Pseudocercospora* anamorph (Crous & Wingfield 1996, Crous 1998). RFLP's in combination with morphological characteristics thus allows for rapid identification.

Mycosphaerella crystallina, *M. heimioides*, *M. heimii* and *M. irregulariramosa* are recognized as being part of the *M. heimii* complex (Crous *et al.* 2000, Crous *et al.* 2001) and grouped together in one *Hae*III RFLP group. All four species in this complex have anamorphs within the same anamorph genus, *Pseudocercospora*, showing similar conidial pigmentation and shape. They also have similar cultural characteristics and as such, are thought to represent varieties of *M. heimii* (Crous 1998, Crous *et al.* 2001). From DNA sequence data it has been shown that all of these species group phylogenetically closely together in a discrete *Pseudocercospora* clade (Crous *et al.* 2001). These species do, however, have different ascospore germination patterns and dimensions (Crous & Swart 1995, Crous & Wingfield 1997, Crous 1998). Results of this study thus, support the view that these fungi are related and are logically treated as a species complex.

An interesting result was that *M. molleriana*, *M. nubilosa* and *M. cryptica* grouped together, in one RFLP group. There has, in the past, been speculation that *M. molleriana* and *M. nubilosa* may represent a single species (Crous *et al.* 1990). However, subsequent

studies have shown that *M. molleriana* and *M. nubilosa* represent distinct species (Crous & Wingfield 1997, Crous 1998, Crous *et al.* 2001). *M. molleriana* and *M. nubilosa* have similar ascospore germination patterns, however, *M. molleriana* produces a characteristic *Colletogloeopsis* anamorph, where there is no known anamorph for *M. nubilosa* (Crous 1998). The ascospore germination pattern of *M. cryptica* is characteristically different from *M. molleriana* and *M. nubilosa* and, it forms a characteristic *Colletogloeopsis* anamorph. DNA sequence data, have shown that *M. cryptica* falls phylogenetically closest to *M. molleriana,* within a clade distinguished by *Colletogloeopsis* Crous et M. J. Wingf. anamorphs (Crous & Wingfield 1997, Crous *et al.* 2001). These three species of *Mycosphaerella* represent three of the main causal agents of disease and ease of identification is important. Using a combination of RFLP patterns, ascospore germination and anamorph associations they can be distinguished from other *Mycosphaerella* spp. causing MLD on *Eucalyptus*.

Isolates of *Mycosphaerella* residing in RFLP group eight included *M. africana*, *M. keniensis*, *M. parkii* and *M. gregaria*. Restriction fragments of these species showed slight differences in size, however, they were more similar to each other than to isolates in any other group. For the purpose of this study we chose not to separate them into single groups based on slight base pair differences. The grouping of these species was unexpected as all of these species are morphologically different from each other. They are all characterized by different ascospore germination patterns, which aids in distinguishing them from each other. *M. parkii,* is the only species in this group that has a known anamorph namely, *Stenella parkii* Crous & Alfenas (Crous *et al.* 1993, Crous 1998). Further investigation into the use of other enzymes to distinguish between members of this group will need to be conducted.

Restriction digests with *Hae* III allowed for the distinction of six species residing in discrete groups i.e., *M. lateralis*, *M. colombiensis*, *M. marksii*, *M. walkeri*, *M. tasmaniensis* and *M. fori*. Some of these species are recognized as the most important causal agents of MLD and this simple identification protocol will be useful to researchers working with these species. *M. lateralis*, *M. marksii*, *M. walkeri* and *M. tasmaniensis* contribute to outbreaks of MLD in Australia and as such, this RFLP approach will be useful for distinguishing between these species (Park & Keane 1984, Crous 1998, Crous *et al.* 1998, Maxwell *et al.* 1999, Milgate *et al.* 2001). *M. lateralis*, *M. marksii* and *M. fori* occur in

South Africa (Crous & Wingfield 1996, Crous 1998) and restriction digests using *Hae* III will be useful in future surveys to determine their occurrence and spread within this country.

There are an unusually large number of *Mycosphaerella* spp. that occur on *Eucalyptus* leaves (Crous 1998, Carnegie & Keane 1998) and their identification is problematic for mycologists, pathologists and foresters. Although desirable, it would have been unrealistic to find a single restriction enzyme or character that could allow identification of individual species. The use of *Hae* III in combination with morphological characteristics does, however, enable identification of 21 species of *Mycosphaerella* on *Eucalyptus*. It will also reduce the need to use expensive sequencing methods to identify these fungi.

This study represents the first attempt to distinguish between a large number of *Mycosphaerella* spp. rapidly using RFLP analysis. It should form a foundation for future RFLP studies aimed at identifying the *Mycosphaerella* spp. from *Eucalyptus*. At present, sequenced data for other genes are not available for this group of fungi. However, in the future, it might be possible to use RFLP's based on sequences from other regions of the genome to refine the identification protocols presented here.

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Table 1: Isolates of *Mycosphaerella* used for RFLP analysis in this study.

Culture Nr.	Identity	Host	Origin	Date of Isolation	Collector
CMW 4945	*M. africana*	*E. viminalis*	South Africa	1994	P. W. Crous
CMW 4944	*M. colombiensis*	*E. urophylla*	Colombia	1995	M. J. Wingfield
CMW 3279	*M. cryptica*	*E. globulus*	Australia	1994	A. Carnegie
CMW 3042	*M. crystallina*	*E. bicostata*	South Africa	1994	M. J. Wingfield
CMW 5165	*M. ellipsoidea*	*E. cladocalyx*	South Africa	1994	P. W. Crous
CMW 5225	*M. endophytica*	*Eucalyptus* sp.	South Africa	1995	P. W. Crous
CMW 5224	*M. flexuosa*	*E. globulus*	Colombia	1995	M. J. Wingfield
CMW 9095	*M. fori*	*E. grandis*	South Africa	2000	G. C. Hunter
CMW9476	*M. gracilis*	*E. urophylla*	Indonesia	1996	M. J. Wingfield
CMW 4942	*M. heimii*	*Eucalyptus* sp.	Madagascar	1994	P. W. Crous
CMW 3046	*M. heimioides*	*Eucalyptus* sp.	Indonesia	1996	M. J. Wingfield
CMW 4943	*M. irregulariramosa*	*E. saligna*	South Africa	1996	M. J. Wingfield
CMW 4937	*M. juvenis*	*E. grandis*	South Africa	1995	M. J. Wingfield
CMW5147	*M. keniensis*	*E. grandis*	Kenya	1995	T. Coutinho
CMW 5164	*M. lateralis*	*E. globulus*	Zambia	1995	T. Coutinho
CMW 5150	*M. marksii*	*E. botryoides*	Australia	Unknown	P. W. Crous
CMW 4940	*M. molleriana*	*E. globulus*	Portugal	1995	S. McRae
CMW 3282	*M. nubilosa*	*E. globulus*	Australia	1994	A. Carnegie
CMW 3358	*M. parkii*	*E. grandis*	Brazil	1990	M. J. Wingfield
CMW 5005	*M. tasmaniensis*	*E. nitens*	Tasmania, Australia	1996	M. J. Wingfield
CMW9477	*M. walkeri*	*E. bicostata*	Uruguay	1999	M. J. Wingfield

CMW = Culture collection of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa

Table 2: Identification key to *Mycosphaerella* species using *Hae*III restriction enzyme, anamorph association and ascospore germination

Culture No.	Teleomorph	Anamorph	Hosts	Geographic distribution	Ascospore germination (Type)	Restriction group (G)
CMW 4945	*M. africana*	Unknown	*E. deanei*, *E. globulus*, *E. grandis*, *E. radiata*, *E. viminalis*	Colombia, Portugal, South Africa, Zambia	G	G8
CMW 4944	*M. colombiensis*	*Pseudocercospora colombiensis*	*E. urophylla*	Colombia	J	G4
CMW 3279	*M. cryptica*	*Colletogloeopsis nubilosum*	*Eucalyptus* spp.	Australia, New Zealand, Chile	A	G6
CMW 3042	*M. crystallina*	*Pseudocercospora crystallina*	*E. bicostata*, *E. grandis*× *camaldulensis*	South Africa	I	G5
CMW 5165	*M. ellipsoidea*	*Uwebraunia ellipsoidea*	*E. cladocalyx*	South Africa	I	G2
CMW 5225	*M. endophytica*	*Pseudocercosporella endophytica*	*E. grandis*, *Eucalyptus* spp.	South Africa	I	G2
CMW 5224	*M. flexuosa*	Unknown	*E. globulus*	Colombia	K	G1
CMW 9095	*M. fori*	*Pseudocercospora fori*	*E. grandis*	South Africa	C	G11
CMW9576	*M. gracilis*	*Pseudocercospora gracilis*	*E. globulus*, *E. urophylla*	Indonesia	B	G8
CMW 4942	*M. heimii*	*Pseudocercospora heimii*	*E. obliqua*, *E. urophylla*, *Eucalyptus* spp.	Madagascar, Indonesia	C	G5
CMW 3046	*M. heimioides*	*Pseudocercospora heimioides*	*Eucalyptus* spp.	Indonesia	M	G5
CMW 4943	*M. irregulariramosa*	*Pseudocercospora irregulariramosa*	*E. saligna*	South Africa	I	G5
CMW 4937	*M. juvenis*	*Uwebraunia juvenis*	*E. globulus*, *E. grandis*, *E. nitens*	Kenya, South Africa, Tanzania, Zambia	F	G1
CMW5147	*M. keniensis*	Unknown	*E. grandis*	Kenya	J	G8

CMW 5164	*M. lateralis*	*Uwebraunia lateralis*	*E. globulus*, *E. grandis*× *saligna*, *E. saligna*, *E. nitens*	South Africa, Zambia, Australia	I	G3
CMW 5150	*M. marksii*	Unknown	*Eucalyptus* spp.	Australia, Indonesia, Portugal, South Africa, Uruguay	B	G7
CMW 4940	*M. molleriana*	*Colletogloeopsis molleriana*	*E. globulus*	California (USA), Portugal	C	G6
CMW 3282	*M. nubilosa*	Unknown	*Eucalyptus* spp.	Australia, New Zealand, South Africa	C	G6
CMW 3358	*M. parkii*	*Stenella parkii*	*E. grandis*, *E. saligna*, *E. globulus*	Brazil, Colombia, Indonesia	D	G8
CMW 5005	*M. tasmaniensis*	*Mycovellosiella tasmaniensis*	*E. nitens*	Tasmania, Australia	I	G10
CMW9477	*M. walkeri*	*Sonderhenia eucalypticola*	*Eucalyptus* spp.	Australia, Chile, New Zealand, Colombia, Ecuador, Portugal	C	G9

Figure 1: *Hae*III restriction fragments of amplified ITS products of various *Mycosphaerella* species used during this study. Restriction fragments visualized on a 2% agarose gel stained with ethidium bromide. A 100 bp molecular marker was run together with restriction fragments for fragment size estimation.

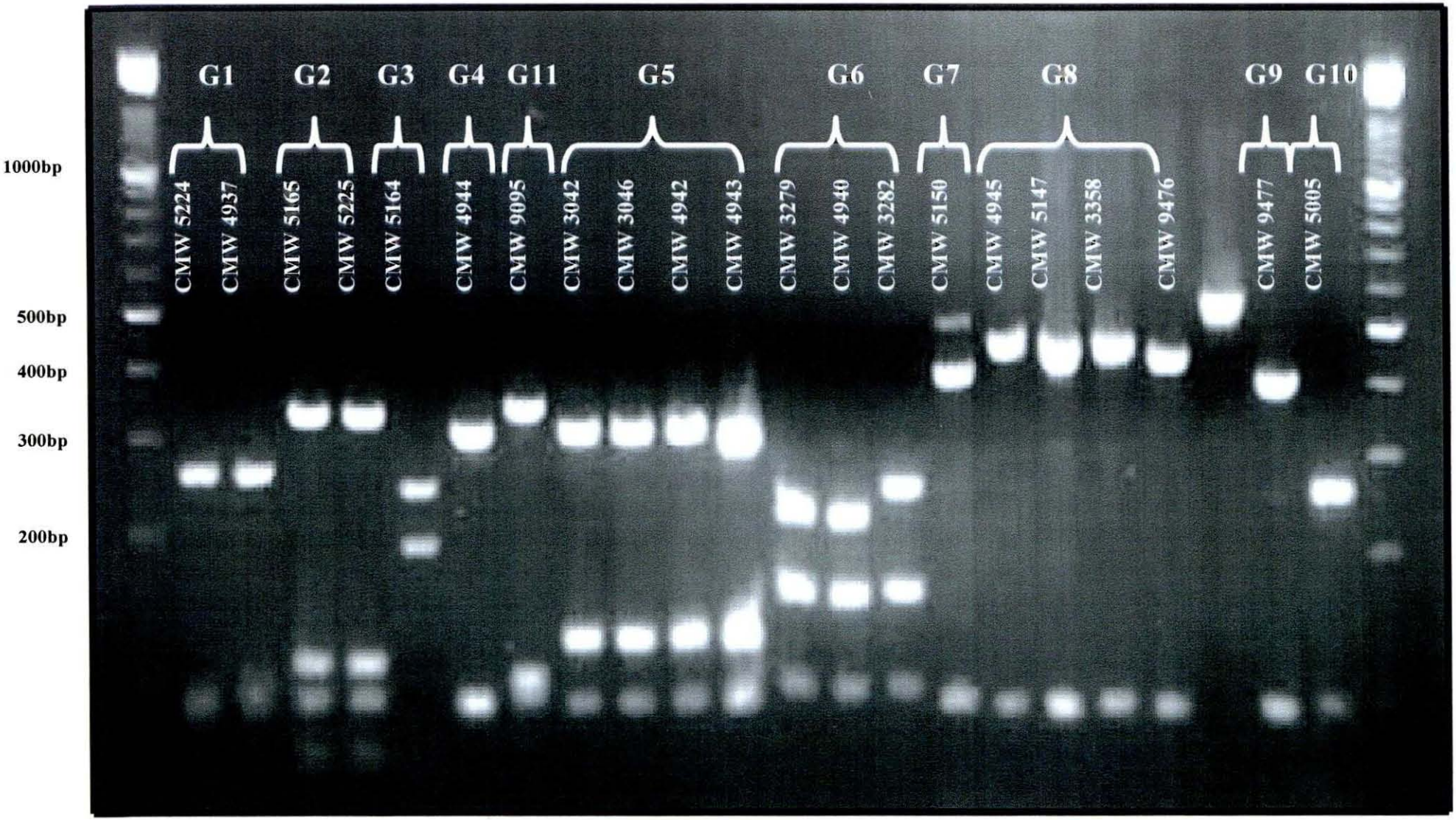
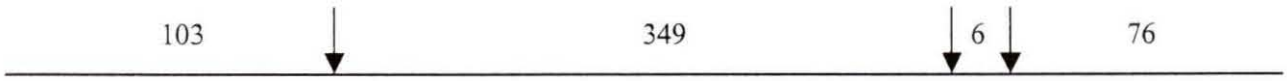
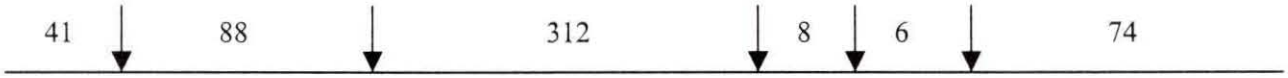


Figure 2: Restriction maps of *Mycosphaerella* species used during this study. Arrows indicate sites where the restriction enzyme *Hae* III cuts. Sizes of restriction fragments are indicated above lines.

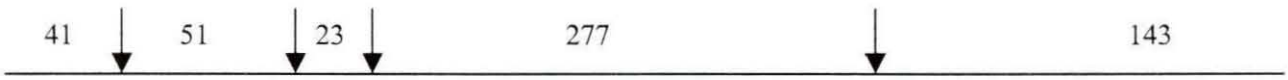
M. africana



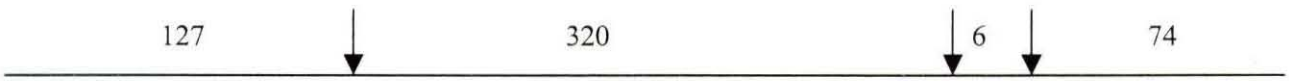
M. colombiensis



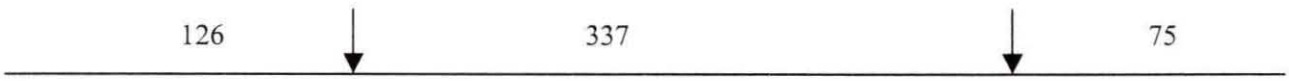
M. cryptica



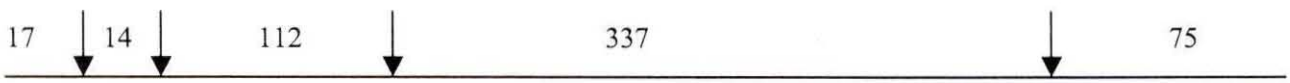
M. crystallina

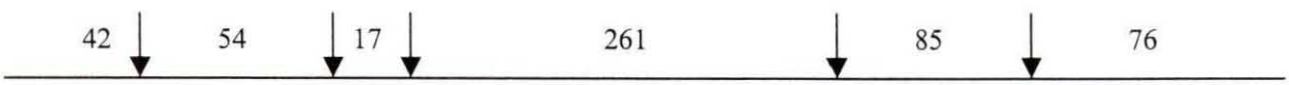


M. ellipsoidea

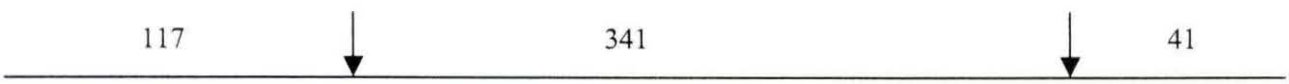


M. endophytica

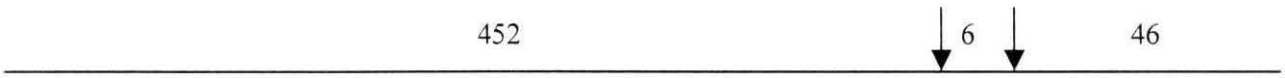




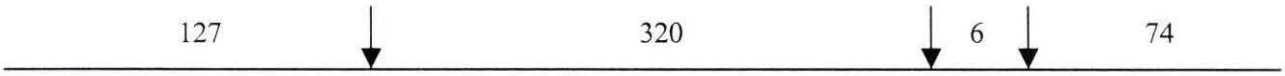
M. fori



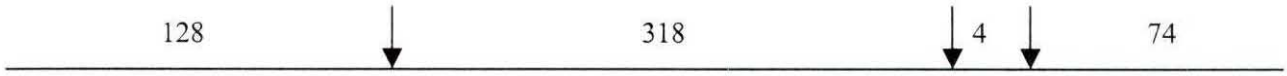
M. gracilis



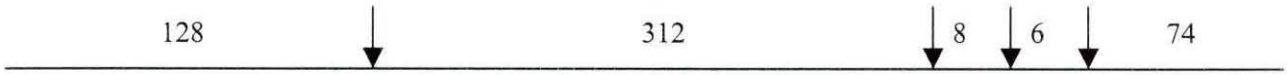
M. heimii



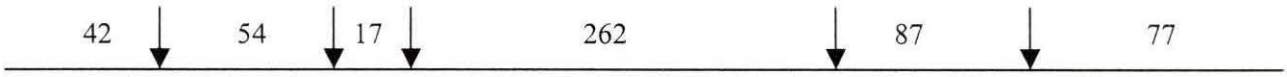
M. heimioides



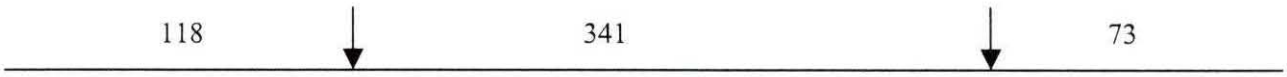
M. irregulariramosa



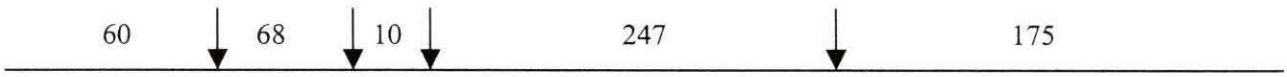
M. juvenis



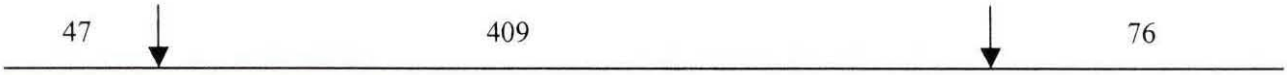
M. keniensis



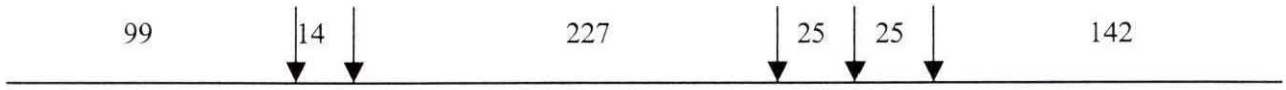
M. lateralis



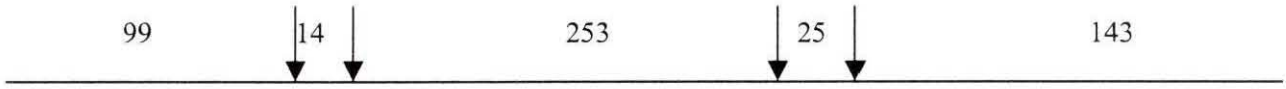
M. marksii



M. molleriana

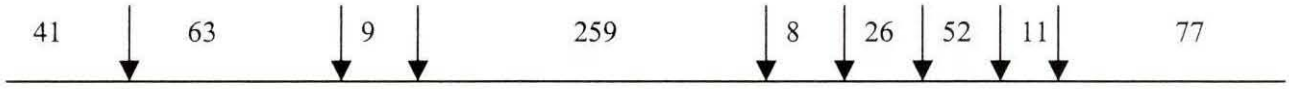


M. nubilosa



M. parkii

M. tasmaniensis



M. walkeri