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Pathogen profile

# *Teratosphaeria nubilosa*, a serious leaf disease pathogen of *Eucalyptus* spp. in native and introduced areas

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## SUMMARY

**Background:** *Teratosphaeria nubilosa* is a serious leaf pathogen of several *Eucalyptus* spp. This review considers the taxonomic history, epidemiology, host associations and molecular biology of *T. nubilosa*.

**Taxonomy:** Kingdom Fungi; Phylum Ascomycota; Class Dothideomycetes; Order Capnodiales; Family Teratosphaeriaceae; genus *Teratosphaeria*; species *nubilosa*.

**Identification:** Pseudothecia hypophyllous, less so amphigenous, ascomata black, globose becoming erumpent, asci aparaphysate, fasciculate, bitunicate, obovoid to ellipsoid, straight or incurved, eight-spored, ascospores hyaline, non-guttulate, thin walled, straight to slightly curved, obovoid with obtuse ends, medially one-septate, slightly constricted at the median septum, tapering to both ends, ascospore germination type F, germinating from both ends, germ tubes growing parallel to the long axis of the spore with distortion of the primary ascospore cell.

**Host range:** *Teratosphaeria nubilosa* is a primary pathogen of several *Eucalyptus* spp., including *E. botryoides*, *E. bicostata*, *E. bridgesiana*, *E. cypellocarpa*, *E. dunnii*, *E. globulus* ssp. *bicostata*, *E. globulus* ssp. *globulus*, *E. globulus* ssp. *maidenii*, *E. globulus* ssp. *pseudoglobulus*, *E. grandis*, *E. gunnii*, *E. nitens*, *E. pilularis*, *E. quadrangulata*, *E. viminalis*, *E. grandis* × *E. resinifera* and *E. urophylla* × *E. globulus*.

**Disease symptoms:** Leaf spots predominantly occur on juvenile *Eucalyptus* foliage; however, *T. nubilosa* has also recently been found on mature *Eucalyptus* foliage. Leaf spots are amphigenous, varying in size from small spots that are round to irregular. Lesions enlarge and coalesce to form larger blotches over the leaf surface. Initial lesions appear as pale-green spots surrounded by purple margins and, once mature, are generally yellow to pale brown with dark-brown raised borders.

**Useful websites:** Mycobank, http://www.mycobank.org; *Mycosphaerella* identification website, http://www.cbs.knaw.nl/mycosphaerella/BioloMICS.aspx

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## INTRODUCTION

Many species of the ascomycete genera *Mycosphaerella* and *Teratosphaeria* infect leaves of *Eucalyptus* spp., where they cause a disease broadly referred to as *Mycosphaerella* leaf disease (MLD) (Burgess *et al.*, 2007; Carnegie *et al.*, 2007; Crous, 1998; Crous *et al.*, 2004a, 2006b, 2007a,b). The predominant symptoms of MLD are leaf spots on the abaxial and/or adaxial leaf surfaces that vary in size, shape and colour (Crous, 1998). The leaf spots often enlarge and coalesce to form larger blotches across the leaf surface, reducing the photosynthetic capability of trees, which leads to premature leaf abscission (Park, 1988b; Pinkard and Mohammed, 2006). Premature defoliation reduces the growth of susceptible *Eucalyptus* species, causing a decrease in the eventual wood volume of infected trees (Carnegie and Ades, 2003; Lundquist and Purnell, 1987; Milgate *et al.*, 2005a). MLD therefore poses a continued threat to the commercial propagation of *Eucalyptus* species by forestry companies.

One of the most virulent species of *Teratosphaeria* causing disease on *Eucalyptus* is *Teratosphaeria nubilosa* (= *Mycosphaerella nubilosa*). Since it was first identified in south-eastern Australia, *T. nubilosa* has been reported from several countries in Africa and Europe, where it has become a major impediment to the continued propagation of cold-tolerant *Eucalyptus* spp. For example, the commercial propagation of *E. globulus* in South Africa was abandoned in the 1930s because of its susceptibility to *T. nubilosa* (Lundquist and Purnell, 1987). *Eucalyptus globulus* was later replaced with *E. nitens* in South Africa as a favoured cold-tolerant species grown at higher altitudes. Some provenances of *E. nitens*, particularly those from Victoria, Australia, are also

 

susceptible to infection by *T. nubilosa* during the first 2–3 years of growth. Thus, the selection of planting stock resistant to this disease has proven to be of paramount importance.

Heavy outbreaks of MLD caused by *T. nubilosa* have also occurred in Australia, including more than 95% defoliation of plantations of *E. globulus* in north-eastern New South Wales (Carnegie, 2007b). *Teratosphaeria nubilosa* was identified as the main cause of severe damage and defoliation in an *E. globulus* plantation in Tasmania (Milgate *et al.*, 2005a). In most instances, however, severe outbreaks of MLD in Australia are associated with *T. nubilosa* and *T. cryptica* occurring together (Barber *et al.*, 2008; Carnegie *et al.*, 1994; Carnegie and Ades, 2002; Park, 1988b). *Eucalyptus globulus* was replaced by *E. nitens* in north-western Tasmania as the preferred plantation species because of MLD (Mohammed *et al.*, 2003). Severe defoliation (over 75%) has been observed in young *E. globulus* plantations in the Otways in Victoria in recent years. Previously only known from juvenile and intermediate foliage (Crous, 1998; Park and Keane, 1982a), *T. nubilosa* has recently been identified from adult *Eucalyptus* foliage in eastern Australia (Kularatne *et al.*, 2004; Maxwell *et al.*, 2001), an aspect which could substantially increase the damage caused by this pathogen.

Considerable research has been conducted on *T. nubilosa* during the course of the past 25 years. Most of the early research was focused on the taxonomy, host associations and the disease caused by the pathogen. More recent studies have included aspects related to the epidemiology, phylogeny, intraspecific variation and population biology of this pathogen. The focus of this paper is to provide an overview of the literature pertaining to *T. nubilosa*. Furthermore, we present opinions regarding the status of knowledge and priorities for research on this increasingly important pathogen.

## TAXONOMIC HISTORY

*Sphaerella cryptica* and *S. nubilosa* were originally described from diseased *Eucalyptus* leaves collected near Melbourne, Australia (Cooke, 1891). These two ascomycetous fungi were to become two of the most well-recognized and important *Eucalyptus* leaf pathogens. Their placement in *Sphaerella* was incorrect and, after examination of the type material, *S. cryptica* and *S. nubilosa* were transferred to *Mycosphaerella* (Hansford, 1956).

South Africa was one of the first countries to establish *Eucalyptus* spp. in plantations for timber production. Initial surveys of *Eucalyptus* in this country led Doidge (1950) to identify *Mycosphaerella molleriana* from several *Eucalyptus* spp. Although morphologically similar, *M. molleriana* and *M. nubilosa* were shown to represent two distinct species (Crous *et al.*, 1991; Crous and Wingfield, 1996), with *M. juvenis* being a synonym of *M. nubilosa* (Crous *et al.*, 2004a; Hunter *et al.*, 2004a,b). DNA sequence comparisons for four nuclear gene regions led Hunter *et al.* (2006b) to suggest that *Mycosphaerella* was not monophyletic (Crous *et al.*, 2001b; Goodwin *et al.*, 2001). By employing nuclear large subunit sequence data for many *Mycosphaerella* species from diverse hosts, Crous *et al.* (2007a) confirmed the polyphyletic nature of *Mycosphaerella*, and revealed various species occurring on *Eucalyptus* to be more appropriately accommodated in *Teratosphaeria*. Thus, *M. nubilosa* was shown to be most appropriately accommodated in *Teratosphaeria* as *T. nubilosa*.

## DISTRIBUTION OF *T. NUBILOSA*

*Teratosphaeria nubilosa* has been observed causing damage in plantations in most states of Australia, including Victoria (Barber *et al.*, 2008; Carnegie *et al.*, 1994), Tasmania (Dungey *et al.*, 1997; Milgate *et al.*, 2001), Western Australia (Jackson *et al.*, 2008; Maxwell, 2004), New South Wales (Carnegie, 2007b) and South Australia (Carnegie, 2000). Herbarium records confirmed by A. J. Carnegie reveal that *T. nubilosa* was identified in Queensland on *E. globulus* in the 1960s, but significant damage has not been reported. *Teratosphaeria nubilosa* has also been identified from New Zealand (Dick, 1982; Dick and Gadgil, 1983), although *T. cryptica* is the most damaging species in this country (Dick, 1982; Dick and Gadgil, 1983; Hood *et al.*, 2002), as the main hosts planted are less susceptible to *T. nubilosa*.

Several countries in Africa have identified *T. nubilosa* from commercial *Eucalyptus* plantations. Doidge (1950) first identified *T. nubilosa* (as *M. molleriana*) from several *Eucalyptus* species in South Africa. Currently, *T. nubilosa* is widespread within South Africa on various *Eucalyptus* species and can be found in several provinces, namely Gauteng, Kwa-Zulu Natal, Limpopo, Mpumalanga, Eastern Cape and Western Cape (Crous, 1998; Crous *et al.*, 2004a; Crous and Wingfield, 1996; Hunter *et al.*, 2004a,b; G. Perez, FABI, Pretoria, South Africa, unpublished data).

Recent surveys in other African countries have also resulted in the confirmation of *T. nubilosa*. *Teratosphaeria nubilosa* has been identified from south, south-western and western Ethiopia, causing severe defoliation on *E. globulus* (Gezahgne *et al.*, 2006). *Eucalyptus* plantations in Kenya, Tanzania and Zambia have also been affected by *T. nubilosa*, where it has been identified causing defoliation of *E. globulus* (Crous *et al.*, 2004a; Hunter *et al.*, 2008).

Europe has also seen the introduction of *T. nubilosa* into its commercial *Eucalyptus* plantations. Collections of diseased *E. globulus* leaves from seven locations in Spain identified *T. nubilosa* causing leaf spots and premature defoliation (Crous *et al.*, 2004a). Similarly, *T. nubilosa* has also been identified on *E. globulus* from northern Portugal (Hunter *et al.*, 2008). More recently, *T. nubilosa* has also been identified from *E. globulus* and *E. dunnii* plantations in Uruguay (G. Perez, FABI, Pretoria, South Africa, unpublished data), and on *E. globulus* in Brazil (P. W. Crous and A. C. Alfenas, unpublished data).


**Fig. 1** Typical leaf symptoms caused by *Teratosphaeria nubilosa* on *Eucalyptus*. (A, B) Typical premature defoliation of young *Eucalyptus* trees caused by *T. nubilosa*, indicating a bottom-up defoliation pattern. (C, D) Small, round to irregular leaf spots that coalesce to form leaf blotches over the leaf surface.

## SYMPTOMATOLOGY AND MORPHOLOGY OF *T. NUBILOSA*

Leaf spots caused by *T. nubilosa* predominantly occur on juvenile and intermediate *Eucalyptus* foliage (Carnegie and Ades, 2002; Crous, 1998; Park and Keane, 1982a). However, recent studies have also identified this pathogen from adult *Eucalyptus* foliage (Kularatne *et al.*, 2004; Maxwell *et al.*, 2001). *Teratosphaeria nubilosa* leaf spots are amphigenous, varying in size from small to large spots that are round to irregular (Fig. 1C). Lesions often enlarge and coalesce to form large blotches covering the leaf surface and, in severe cases, large lesions can lead to leaf blight. Initial lesions appear as pale-green spots surrounded by purple margins and, once mature, are generally yellow to pale brown with dark-brown raised borders (Fig. 1D) (Crous, 1998; Crous *et al.*, 2004a; Park and Keane, 1982b). Defoliation of *Eucalyptus* trees tends to occur from the lower crown moving upwards (i.e. bottom-up) (Fig. 1B).

Ascomata of *T. nubilosa* are generally hypophyllous, but have also rarely been observed to occur amphigenously (Barber *et al.*, 2008; Carnegie and Ades, 2002). Ascomata are black, globose to punctiform, immersed with a papillate ostiole, becoming erumpent with age, measuring 40–90 µm in diameter. *Teratosphaeria*

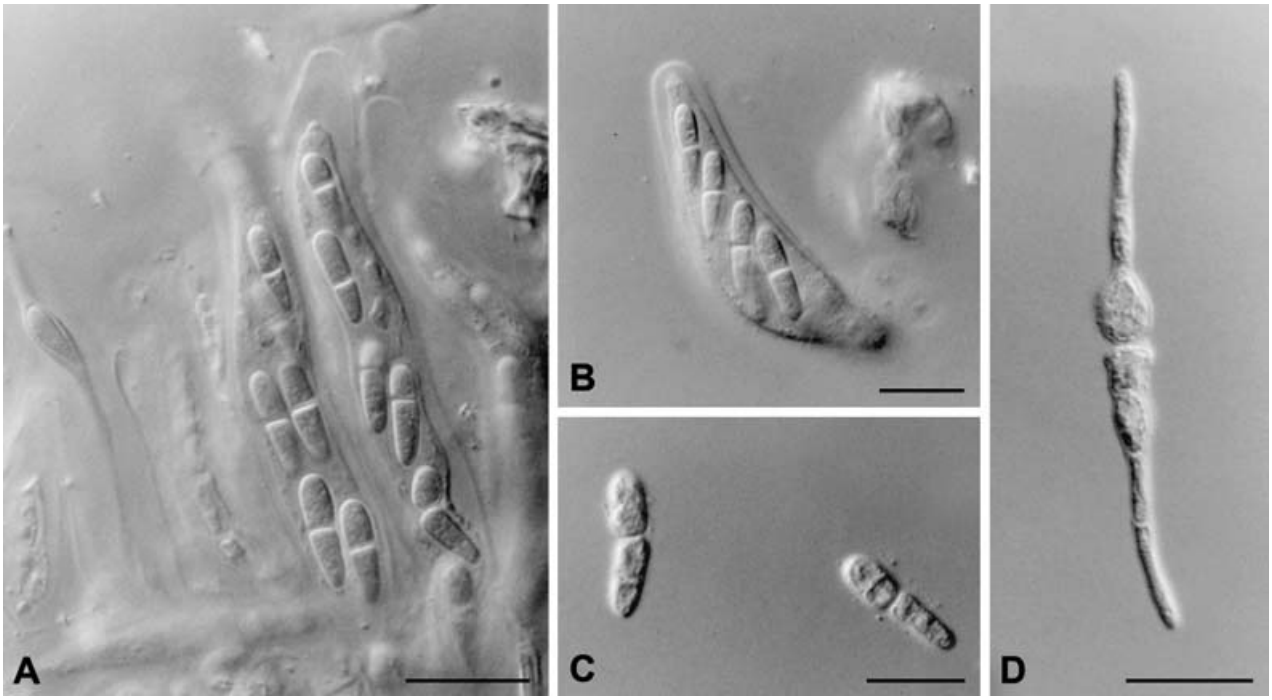


**Fig. 2** Morphological characteristics of *Teratosphaeria nubilosa*. (A, B) Eight-spored bitunicate ascii. (C) Ellipsoidal to obovoid, guttulate, one-septate ascospores. (D) Typical type F ascospore germination pattern of *T. nubilosa*. Scale bars, 10 μm.

*nubilosa* asci are bitunicate with a multilayered endotunica, ellipsoidal to obclavate, straight or curved, subsessile, eight-spored, 30–60 × 9–18 μm. Ascospores are two- to three-seriate, oblique, ellipsoidal to obovoid, usually straight in the ascus, colourless, smooth, guttulate, one-septate, not or slightly constricted at the median septum, widest in the middle of the upper cell, tapering more strongly towards the lower end, (11–)13–16 × 3–3.5(–4.5) μm surrounded by a non-persistent mucous sheath (Fig. 2) (Crous, 1998; Crous *et al.*, 2004a, 2007a).

Ascospore germination patterns have been used as diagnostic features for various *Mycosphaerella* spp. on *Eucalyptus* (Crous, 1998; Park and Keane, 1982a). Examination of fresh *T. nubilosa* ascospores that had been allowed to germinate for 24 h and longer showed that *T. nubilosa* has a typical type F pattern (Crous *et al.*, 2004a), germinating from both ends with germ tubes growing parallel to the long axis of the spore, with prominent distortion of the original ascospore. However, recent collections of *T. nubilosa* from northern New South Wales, Australia, have resulted in ascospores that produce multiple germ tubes and, as such, do not exhibit the classical type F form (Carnegie, 2007a).

## INFECTION PROCESS AND EPIDEMIOLOGY

Knowledge of the infection process and disease development in species of *Mycosphaerella* and *Teratosphaeria* on *Eucalyptus* is largely based on studies of *T. cryptica* and *T. nubilosa* (Park, 1988a,b; Park and Keane, 1982a,b). These studies have investigated the ability of these pathogens to infect *Eucalyptus* leaves both *in situ* and *in vitro*. Apart from the infection processes followed by these pathogens, various environmental conditions have also been investigated in order to understand how they affect the ability of *T. nubilosa* and *T. cryptica* to infect *Eucalyptus*.

Both ascospores and conidia in species of *Mycosphaerella* and *Teratosphaeria* can initiate disease on *Eucalyptus* leaves. Ascospores, however, act as the primary source of inoculum for the majority of these, whereas some infect primarily by means of conidia (Crous *et al.*, 2007a; Park, 1988b; Park and Keane, 1987; Wingfield *et al.*, 1996). The source of ascospores is predominantly from attached infected leaves, or from fallen overwintered leaf litter (Park and Keane, 1987). Within the leaf tissue, ascogonia of *Mycosphaerella* and *Teratosphaeria* species have been shown to remain viable for several months, thereby providing sufficient inoculum for successive infection cycles (Cheah and Hartill, 1987; Park, 1988b; Park and Keane, 1982b, 1987).

The infection of *Eucalyptus* leaves by *T. nubilosa* predominantly occurs during the vegetative period of the host during the summer and autumn months (Cheah and Hartill, 1987; Ganapathi, 1979). Park (1988a) showed that young expanding leaves of *E. globulus* (less than 46 days old), were particularly susceptible to *T. nubilosa*. As *Eucalyptus* leaves age, they become progressively more resistant to infection as a result of the deposition of resistant compounds (Park, 1988a).

A few hours after ascospores have been deposited onto leaf surfaces, they germinate to form germ tubes. The germ tubes of *T. nubilosa* will often branch and enter several stomata (Park and Keane, 1982b). The germination of *T. nubilosa* ascospores on the *Eucalyptus* leaf surface occurs between 3 and 30 °C, with an optimum temperature of 20 °C (Park, 1988a; Park and Keane, 1982b). Infection of the *Eucalyptus* leaf surface by the spores of *Mycosphaerella* and *Teratosphaeria* spp. may be direct or indirect. Direct penetration involves the penetration of the leaf through the leaf cuticle. This is achieved by the ability of the fungal spore to form an infection peg at the end of a germination tube. Through turgor pressure, the infection peg is able to penetrate the cuticle and gain access to the interior of the leaf. However, in indirect penetration, the fungal spore is unable to produce an infection peg and instead relies on natural openings in the leaf surface, such as stomata, to gain access to the leaf interior. *Teratosphaeria nubilosa* employs an indirect penetration strategy, where infection occurs through the stomata and germ tubes produce hyphal swellings within the stomatal pores and substomatal cavities (Park and Keane, 1982b).

The levels of moisture in the environment play an important part in the ability of *T. nubilosa* to infect *Eucalyptus* leaves. In an inoculation experiment, Park (1988b) found that premature leaf defoliation and large lesions were caused by *T. nubilosa* on leaves of *E. globulus* ssp. *globulus* when plants were exposed to a wetting period of 5–7 days. He also showed that the severity of disease increased with longer periods of leaf wetness.

After penetrating a leaf, fungal hyphae grow along the vascular bundles and colonize the leaf tissue, becoming established throughout the leaf. Following chlorosis, hyphae grow intercellularly throughout the spongy mesophyll and eventually aggregate in the substomatal cavities (Park and Keane, 1982b). These hyphal aggregates then develop into immature ascomata with trichogynes (Park and Keane, 1982b).

Ganapathi (1979) described the development of the ascomata of *T. cryptica* (as *M. nubilosa*) in detail. His studies showed that the ascocarp initials comprise a group of cells. Developing ascomata have the appearance of stromata with the presence of trichogynes, which grow towards the stomatal apex. During ascogonial development, the stroma matures and breaks through the host surface. The developing trichogynes grow through the top of the stroma and are fertilized by spermatia. Spermatia are formed in a gelatinous matrix that seeps from the spermatogonial ostiole onto the leaf surface (Ganapathi and Corbin, 1979). After fertilization, ascogonia mature through successive developmental steps from asci and ascospores. Mature ascogonia of *Teratosphaeria* and *Mycosphaerella* spp. generally have large, thick, elongated cells impregnated with melanin that form the outer layers of the ascocarp wall (Niyo *et al*., 1986). Although cells making up the inner ascocarp walls generally contain lower melanin levels than those of the outer ascogonial walls, similar cellular organelles are observed in both cell types (Niyo *et al*., 1986).

The liberation of ascospores of *Mycosphaerella* and *Teratosphaeria* spp. is dependent on moisture. For example, ascospores of *T. cryptica* are discharged when the relative humidity is greater than 95% and not when it is below 90% (Park and Keane, 1982b). Rainfall acts as the main stimulus for the release of ascospores from mature ascomata, and longer periods of rainfall lead to the discharge of greater numbers of spores (Park, 1988b). The discharge of ascospores continues in the presence of sufficient moisture and relative humidity until the asci within the ascomata are exhausted of ascospores (Cheah and Hartill, 1987).

Park and Keane (1982b) found that the optimum temperature for ascospore discharge in *T. nubilosa* was 25 °C and, in field conditions in south-eastern Australia, the release of *T. nubilosa* ascospores occurred between 5 and 15 °C. These spores can be ejected up to a distance of 12–15 mm above the ascomata. This allows the spores to be wind dispersed for considerable distances (Park and Keane, 1982b).

## HOST RANGE AND SUSCEPTIBILITY

Increased surveys of *Eucalyptus* plantations in various parts of the world during the course of the past two decades have led to a substantially increased number of reported *Eucalyptus* hosts for *T. nubilosa*. This pathogen now has a relatively wide host range in the *Eucalyptus* subgenus *Symphyomyrtus* series *Viminalis* and *Resiniferae* (Carnegie, 2007a; Carnegie and Keane, 1994, Crous, 1998, Jackson *et al*., 2005; Park and Keane, 1982a,b). To date, this pathogen has been isolated from *E. botryoides*, *E. bridgesiana*, *E. cypellocarpa*, *E. dunnii*, *E. globulus* ssp. *bicostata*, ssp. *globulus*, ssp. *maidennii* and ssp. *pseudoglobulus*, *E. grandis*, *E. gunnii*, *E. nitens*, *E. quadrangulata*, *E. viminalis*, *E. grandis* × *E. resinifera* and *E. urophylla* × *E. globulus* (Carnegie and Keane, 1994; Crous, 1998; Crous *et al*., 2004a; Hunter *et al*., 2004a,b; Jackson *et al*., 2005; Park and Keane, 1982a). However, some species, such as *E. globulus*, are considerably more susceptible than others.

Numerous studies have reported wide variation in susceptibility to MLD amongst *Eucalyptus* species, provenances or families (Carnegie and Ades, 2005; Carnegie *et al*., 1994, 1998, 2004; Dungey *et al*., 1997; Hood *et al*., 2002; Milgate *et al*., 2005a; Purnell and Lundquist, 1986; Wilcox, 1982). In an assessment of MLD on the juvenile foliage of 14 species of *Eucalyptus*, Carnegie *et al*. (1998) reported that *E. globulus*, *E. nitens* and *E. cypellocarpa* had significantly more disease than all other species. Wide variation has been reported at the provenance level for both *E. globulus* and *E. nitens*.

During initial provenance trials of *E. nitens* in South Africa, Purnell and Lundquist (1986) observed that New South Wales provenances of *E. nitens* outperformed Victorian provenances.

They found that New South Wales provenances exhibited higher average levels of height, diameter at breast height and volume than Victorian provenances. New South Wales provenances also showed higher survival and lower MLD than Victorian provenances (Lundquist and Purnell, 1987). Purnell and Lundquist (1986) therefore suggested that provenances to be planted in South Africa should be selected on the basis of growth rate, stem form, wood quality and disease resistance. Carnegie *et al.* (1998) also reported significant variation in susceptibility to MLD amongst provenances of *E. nitens*, planted in Victoria, Australia, although there was little disease on the adult foliage of these trees assessed several years later, and no variation amongst provenances (Carnegie and Ades, 2005).

Carnegie *et al.* (1994) investigated the variation within several provenances of the four *E. globulus* subspecies, and determined that *E. globulus* ssp. *globulus* provenances (two from Tasmania and one from Victoria), together with one *E. globulus* spp. *bicostata* provenance from Victoria, were significantly more susceptible to MLD than an *E. globulus* ssp. *pseudoglobulus* provenance from Victoria and an *E. globulus* ssp. *maidenii* provenance from New South Wales. Dungey *et al.* (1997) reported that King Island, Tasmania, provenances of *E. globulus* ssp. *globulus* were less susceptible than *E. globulus* ssp. *globulus* provenances from Taranna, Tasmania. However, Dungey *et al.* (1997) reported that a single, unknown provenance of *E. globulus* ssp. *bicostata* showed relative resistance to MLD, and Carnegie (2007b) reported that *E. globulus* ssp. *maidenii* was highly susceptible to *T. nubilosa* in northern New South Wales, contrasting with the earlier observations by Carnegie *et al.* (1994), most probably as a result of the differences in provenances between these studies. Significant variation at the family level has also been observed for *E. globulus* (Carnegie and Ades, 2005; Dungey *et al.*, 1997; Milgate *et al.*, 2005a).

The use of hybrids in *Eucalyptus* forestry has gained importance over the past couple of decades (Denison and Kietzka, 1993; Lee, 2007). However, hybrids can often be more susceptible to pests and diseases than their respective parents. First-generation progeny of *E. globulus* and *E. nitens* hybrids exhibit higher levels of susceptibility to MLD than their respective parents (Carnegie and Ades, 2002; Dungey *et al.*, 1997).

Genetic parameters for resistance to MLD have been calculated for several species. Narrow-sense heritability estimates of severity on juvenile foliage (caused by *T. cryptica*) for *E. nitens* range from 0.12 to 0.21 (Dungey *et al.*, 1997). For *E. globulus*, heritability for MLD on juvenile foliage (caused by both *T. nubilosa* and *T. cryptica*) ranges from 0.12 to 0.36, and from 0.17 to 0.36 for *T. cryptica* on adult foliage (Carnegie and Ades, 2005; Dungey *et al.*, 1997). For defoliation of the juvenile crown, heritability estimates range from 0.28 to 0.48 (Carnegie and Ades, 2005). Milgate *et al.* (2005a) reported heritability of 0.60 for the severity of *T. nubilosa* on *E. globulus*, similar to that reported for disease severity on *E. globulus* × *E. nitens* hybrids (0.51; Dungey *et al.*, 1997).

Variation in resistance to infection amongst provenances of *Eucalyptus* spp. has been shown at the histological level. Smith *et al.* (2006) showed that the *E. nitens* provenance (Ebor) from northern New South Wales was more resistant than the *E. nitens* provenance (Tallaganda) from southern New South Wales. This was a result of the higher degree of packed parenchyma cells within leaves of more resistant provenances that were able to divide more quickly and form an impenetrable necrophylactic periderm (Smith *et al.*, 2006).

## *T. NUBILOSA* INTRASPECIFIC VARIATION

The internal transcribed spacer (ITS) region of the rDNA operon has traditionally been targeted for DNA sequence comparisons in *Mycosphaerella* species. Studies on *T. nubilosa* using sequences for the ITS region have identified variation within this taxon, resolving it into two distinct phylogenetic clades (Crous *et al.*, 2004a; Maxwell, 2004). Hunter (2007) termed these two clades ITS lineage 1 and ITS lineage 2, respectively (Fig. 3). ITS lineage 1 accommodates isolates of *T. nubilosa* from New Zealand, south-eastern Australia (Tasmania and Victoria) and south-western Australia (Western Australia), whereas ITS lineage 2 accommodates isolates from a broader geographical area, including south-eastern Australia (Victoria), eastern Australia (New South Wales), south-western Australia (Western Australia), Tanzania, South Africa, Ethiopia, Spain, Portugal and Kenya (Hunter, 2007; Maxwell, 2004). ITS lineage 1 of *T. nubilosa* can be distinguished from ITS lineage 2 by four fixed base-pair polymorphisms, two transitions and two transversions, occurring at nucleotide positions 13, 37, 255 and 334, respectively, of the ITS gene region (Hunter, 2007).

Gene regions other than the ITS rDNA operon have been considered for the possible variation that their sequences might reflect for isolates of *T. nubilosa*. However, only a limited number of alternative loci have been studied, and these have shown little phylogenetic signal for variation within this species. Some variation within the β-tubulin (Bt) 2 gene region has been observed, where isolates of *T. nubilosa* can be distinguished from each other on the basis of the presence of three base-pair insertions at nucleotide position 191 (ACA/XXX) and a transition mutation at nucleotide position 215 (A/G) (Hunter, 2007). Although the translation elongation factor-1α (EF-1α) gene region has also been used to compare isolates of *T. nubilosa*, it appears that this gene region is conserved within the taxon, showing no polymorphisms (Hunter, 2007).

Variation within *T. nubilosa* has also been observed at the phenotypic level. This has notably been seen in patterns of germination in the fungus. Although *T. nubilosa* most typically has an F-type ascospore germination, a recent study has shown that specimens from northern New South Wales in Australia produce

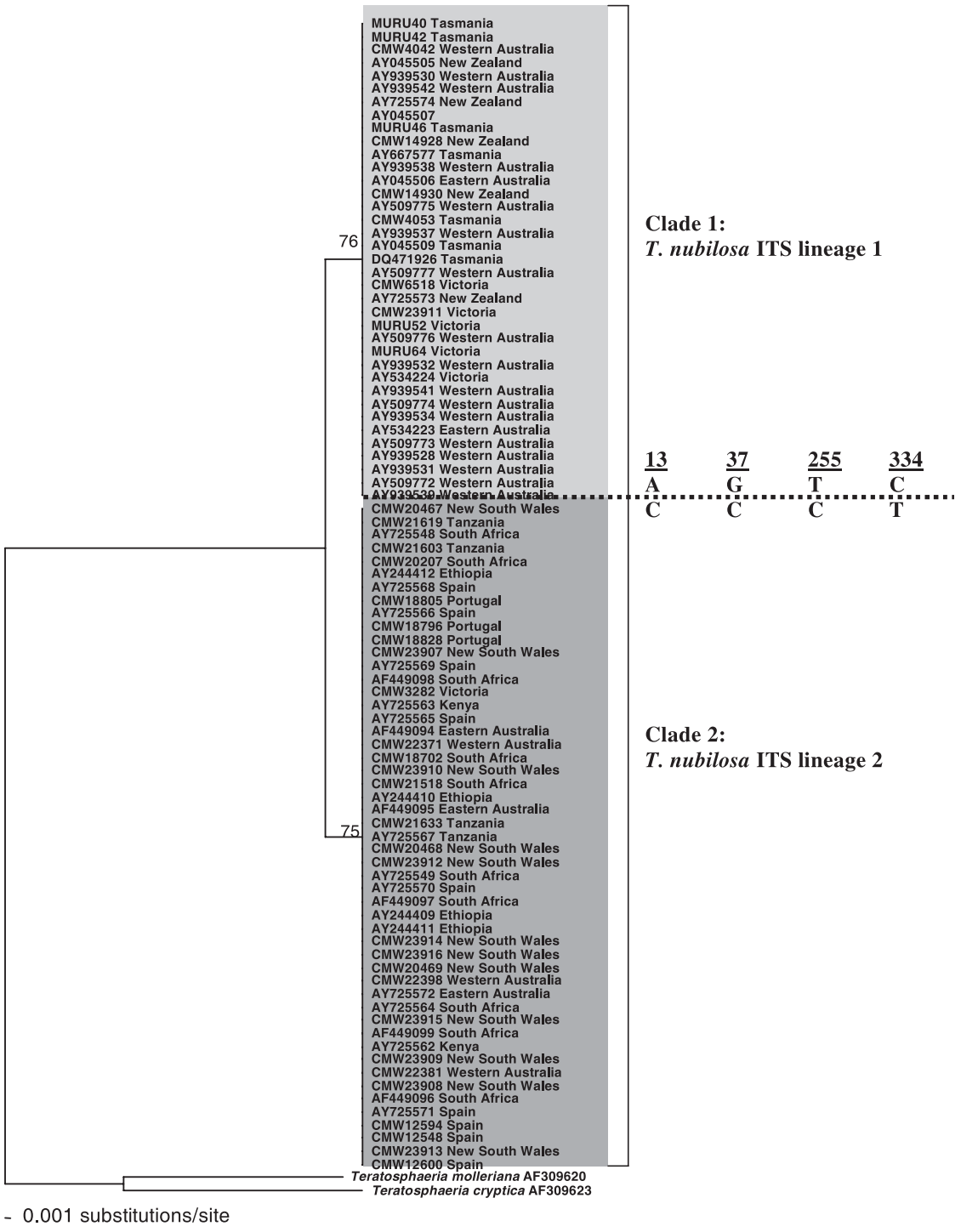


**Fig. 3** Neighbour-joining phylogram obtained from a distance analysis using the Hasegawa–Kishino–Yano (HKY) substitution model on internal transcribed spacer (ITS) sequence data of *Teratosphaeria nubilosa* isolates. Bootstrap values after 1000 replicates are shown above the branches. *Teratosphaeria molleriana* and *T. cryptica* were used as outgroups. Fixed sequence polymorphisms, separating clade 1 from clade 2, and their base-pair positions are indicated at the broken line.

multiple germ tubes on germination (Carnegie, 2007a), as well as typical type F germination.

Culture morphology is another plastic character in *T. nubilosa*. Early studies of this species in Australia by Park and Keane (1982b) showed two distinct culture morphologies. In one of these, the mycelium is black and submerged, producing dark-green aerial hyphae. In the other culture form, the mycelium is submerged, with white to olive green aerial mycelium (Park and

Keane, 1982b). Similar findings were noted by Hunter (2007), who found that the ex-epitype isolate of *T. nubilosa* (isolated in Victoria, south-eastern Australia) produced a colony morphology identical to the second type described by Park and Keane (1982b). However, two other colony morphologies were noted by Hunter (2007) for isolates from Victoria. In one of these colonies, uneven and irregular margins showing folding and sectoring with predominant white mycelial tufts occurred as extensive aerial mycelium. These colonies were pale greenish grey on the surface and pale olivaceous grey on the reverse. In the second type, colonies grew extremely slowly and produced irregular margins, extensive folding and convolutions with sparse aerial mycelium and submerged mycelium. These colonies were olivaceous grey on the surface and iron-grey on the reverse (Hunter, 2007). In addition, cultures of *T. nubilosa* from northern New South Wales grew similar in colour to the second type described by Park and Keane (1982b), but much more slowly, with a somewhat raised appearance (A. J. Carnegie, unpublished data).

## POPULATION BIOLOGY

Several studies on the population biology of species of *Mycosphaerella* occurring on cereal hosts have been published over the past several years. However, very little research into the population biology of *Mycosphaerella* and *Teratosphaeria* species occurring on *Eucalyptus* has been undertaken, apart from two studies into the population biology of *T. cryptica* and *T. nubilosa* (Hunter, 2007; Hunter *et al.*, 2008; Maxwell, 2004; Milgate *et al.*, 2005b).

Hunter *et al.* (2006a) developed 10 polymorphic microsatellite markers for *T. nubilosa sensu stricto*. These markers were used to study the movement and population biology of several *T. nubilosa* populations at different hierarchical levels from five different countries, including Australia, Portugal, Spain, South Africa and Tanzania. The population parameters that were investigated included gene diversity, genotypic diversity, population differentiation and gene flow.

The centre of origin of *T. nubilosa* has not been confirmed. This pathogen was originally identified from south-eastern Australia near Melbourne in the state of Victoria (Cooke, 1891). By examining the gene diversity of a *T. nubilosa* population collected in New South Wales, eastern Australia, Hunter *et al.* (2008) were able to show that this population had a significantly higher gene diversity (0.506) when compared with the gene diversity of *T. nubilosa* populations collected in South Africa, which exhibited moderate to high diversity ranging from 0.149 to 0.250. These data substantiated the hypothesis that eastern Australia is indeed the centre of origin for *T. nubilosa*.

A large number of multilocus haplotypes (MLHs) were observed from the various *T. nubilosa* populations collected (Hunter *et al.*, 2008). In total, 68 MLHs were observed from all populations from the five countries. A total of 42 MLHs were detected in the South African populations from the various hierarchical levels and, interestingly, one of these was the only one found from the Portuguese and Spanish populations, whereas another South African MLH was the only one detected in a *T. nubilosa* population from Tanzania (Hunter, 2007; Hunter *et al.*, 2008). A further finding from this study was that the 10 MLHs of *T. nubilosa* identified from Western Australian (TN59–TN68) and the 16 MLHs identified from New South Wales (TN43–TN58) were unique to these two populations, and did not occur in any of the other countries in which populations were collected (Figs 4 and 5).

Gene flow between *T. nubilosa* populations has also been examined. Hunter *et al.* (2008) observed a high rate of gene flow between *T. nubilosa* populations collected at three plantations in South Africa. However, very little gene flow was observed between

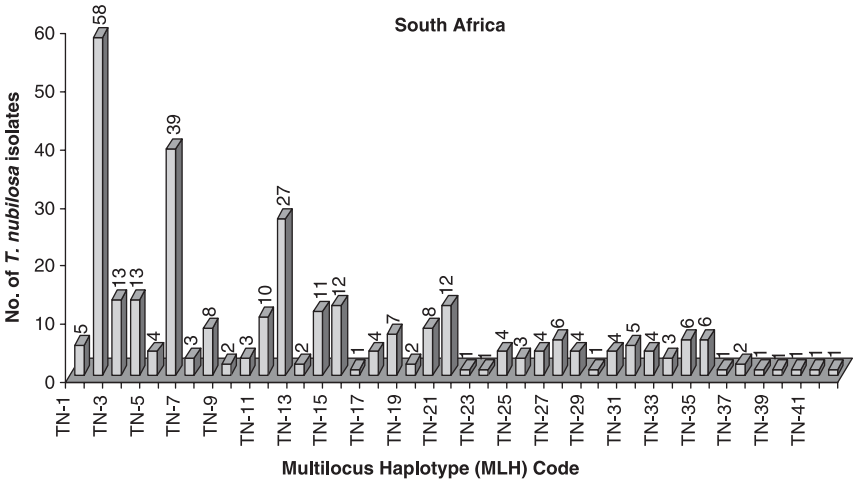


**Fig. 4** Total number of multilocus haplotypes (MLHs) of *Teratosphaeria nubilosa* isolated from *Eucalyptus nitens* in three provinces of South Africa (TN1–TN42) (Hunter *et al.*, 2008).

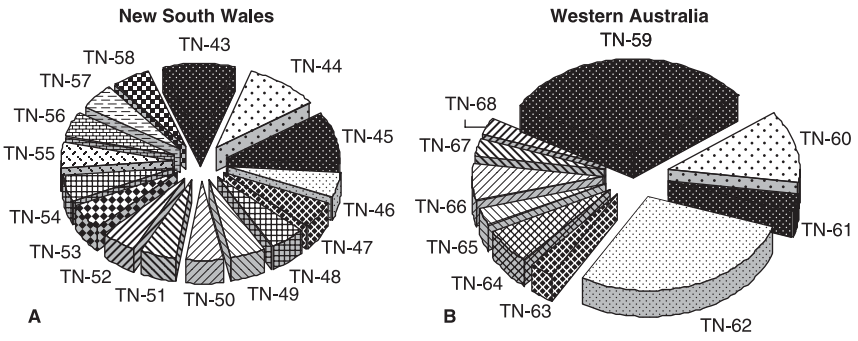


**Fig. 5** Multilocus haplotypes (MLHs) of *Teratosphaeria nubilosa* isolated from Australia: (A) 16 MLHs of *T. nubilosa* (TN43–TN58) isolated from New South Wales, eastern Australia; (B) 10 MLHs of *T. nubilosa* (TN59–TN68) isolated from Western Australia (Hunter *et al.*, 2008).

the New South Wales and Western Australian *T. nubilosa* populations examined, and a low level of gene flow occurred between the Western Australian and South African *T. nubilosa* populations. From all of these data, Hunter *et al.* (2008) hypothesized that *T. nubilosa* is native to eastern Australia and that it was introduced into South Africa from Australia. Once in South Africa, *T. nubilosa* then spread into other countries of Africa, such as Tanzania, and finally into Europe (Fig. 6).

## IDENTIFICATION TECHNIQUES

Classical taxonomic techniques are able to distinguish many of the species of *Mycosphaerella* and *Teratosphaeria* from *Eucalyptus*. The main basis is ascospore morphology, germination pattern, cultural characteristics and anamorph associations (Crous, 1998; Park and Keane, 1982a), which have been used to describe many species (Carnegie and Keane, 1994, 1998; Crous, 1998; Crous *et al.*, 1993; Park and Keane, 1982a, 1984). These techniques are still able to distinguish species, but molecular analysis is now routinely used to confirm morphological identifications and to identify cryptic species.

Randomly amplified polymorphic DNA (RAPD) is effective in distinguishing between morphologically closely related *Mycosphaerella* and *Teratosphaeria* spp. Carnegie *et al.* (2001) employed RAPDs (Table 1) to effectively distinguish between *T. cryptica*, *M. gregaria*, *T. nubilosa* and *M. marksii*. DNA-based methods, such as RAPD, which can distinguish between species based on differences in DNA sequence, are particularly advantageous. The reliability and standardization of RAPD across laboratories, however, are problematic and represent the major disadvantages of this technique.

Species-specific DNA primers are effective for distinguishing between morphologically similar species of *Mycosphaerella* and *Teratosphaeria* occurring on *Eucalyptus*. Maxwell *et al.* (2005) developed species-specific primers for *T. cryptica*, *M. lateralis*, *M. marksii*, *T. nubilosa* (Table 1) and *T. parva*. Similarly, Glen *et al.* (2007) were also able to develop species-specific primers for *T. cryptica*, *T. nubilosa* and *M. tasmaniensis* (Table 1). All of these species are known to occur in Australia and several other countries in which they cause MLD (Crous, 1998). The developed primers were used to amplify DNA from the selected *Mycosphaerella* spp. and from diseased *Eucalyptus* leaves infected with these specific *Mycosphaerella* and *Teratosphaeria* spp. In the case of *T. nubilosa*, it is interesting that the species-specific primers developed by Maxwell *et al.* (2005) and Glen *et al.* (2007) were able to detect the presence of *T. nubilosa* in *Eucalyptus* leaves that showed hardly any symptoms of *T. nubilosa* infection. This technique provides a useful tool in quarantine facilities and in nurseries, where it is necessary to detect *Mycosphaerella* spp. *in planta*. Kularatne *et al.* (2004) developed a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique for the effective identification of *T. nubilosa* (Table 1) and *T. cryptica*. This technique also allowed for these species to be detected directly from infected *Eucalyptus* leaf tissue.

DNA sequencing comparison for various DNA gene regions and phylogenetic inference has become the most commonly used approach to confirm the morphological diagnosis of *Mycosphaerella* and *Teratosphaeria* spp. occurring on *Eucalyptus* spp. The ITS rDNA operon was the first and is the most commonly used gene region to identify these fungi (e.g. Arzanlou *et al.*, 2008; Crous *et al.*, 2000, 2001a,b, 2006a; Hunter *et al.*, 2004a,b). However, the ITS gene region does not always provide sufficient resolution to distinguish between *Mycosphaerella* spp. and their anamorphs (Verkley and Starink-Willemse, 2004). Therefore, several other nuclear gene regions, such as Actin (ACT), EF-1$\alpha$ and Bt, have also recently been used to consider species' boundaries and cryptic taxa within *Mycosphaerella* (Crous *et al.*, 2006b, 2007a; Hunter *et al.*, 2006b). It is certain that the generation of greater numbers of DNA sequence data sets of various nuclear and mitochondrial gene regions, and the combination of these data sets, will contribute substantially to our understanding of species' concepts within *Mycosphaerella* and *Teratosphaeria* in the future. They should also provide a more refined understanding of teleomorph and anamorph morphologies that are phylogenetically informative.

**Fig. 6** World map indicating the present distribution of *Teratosphaeria nubilosa* (black shaded areas). Arrows indicate the proposed pathway of gene flow and movement of *T. nubilosa* from Australia into South Africa and from South Africa into other countries of Africa and, finally, to Europe.

## DISEASE MANAGEMENT

The management of MLD is difficult because commercial *Eucalyptus* plantations cover thousands of hectares (over 451 000 ha of *E. globulus* in Australia alone; Parsons *et al.*, 2006). This, combined with the fact that *T. nubilosa* produces wind-dispersed ascospores (Park and Keane, 1982b), complicates efforts to control the disease. Nonetheless, various management strategies have been used for the control of MLD and, in combination, they can lead to a decrease in losses caused by *T. nubilosa*.

The most effective means to deal with diseases of plantation-grown trees is by deploying resistant planting stock. The selection of less susceptible *Eucalyptus* species has been used to combat MLD in several countries following severe epidemics during early plantings. For example, *E. nitens* has replaced the more susceptible *E. globulus* in South Africa (Lundquist and Purnell, 1987) and north-western Tasmania, Australia (Mohammed *et al.*, 2003). Early plantations in New Zealand, using *E. delegatensis* and *E. regnans*, were severely damaged by MLD (*T. cryptica*), which led to the use of more resistant species, such as *E. nitens*.

Provenance variation has been utilized to further reduce the impact of MLD. New South Wales provenances of *E. nitens* have been selected for planting over Victorian provenances in South Africa (Purnell and Lundquist, 1986). Several authors have recommended the use of the inherent resistance in *E. globulus* for planting in high-risk areas for MLD in south-eastern Australia (Carnegie *et al.*, 1994; Milgate *et al.*, 2005a).

Hybrids between susceptible and resistant *Eucalyptus* spp. have been very useful in avoiding damage caused by various diseases. Hybrids between *Eucalyptus* spp., such as *E. globulus* (susceptible to *T. nubilosa*), and distantly related species, such as *E. grandis* (tolerant to *T. nubilosa*), are only now emerging from breeding programmes. These hybrids between *E. globulus* and *E. grandis* are displaying very high levels of resistance to MLD in Uruguay (M. J. Wingfield, unpublished data). This is in contrast with the parent *E. globulus* which is often severely affected. However, although hybrids provide an exciting opportunity to avoid MLD caused by *T. nubilosa*, there is also cause to be cautious. For example, Dungey *et al.* (1997) and Carnegie and Ades (2002) found that F1 *E. globulus* × *E. nitens* hybrids were more susceptible to MLD than either parents of the cross. Carnegie and Ades (2002) further suggested that such *Eucalyptus* hybrids should not be used in environments where MLD is severe. This enhanced susceptibility may be related to the fact that the parent trees both carried genes for susceptibility to infection. Similarly, in New Zealand, hybrids of *E. regnans* × *E. delegatensis* were more severely damaged by MLD (*T. cryptica*) (Wilcox, 1982). The caveat is that hybridization between species can result in a complex suite of traits, and the testing of progeny carefully prior to commercial deployment is an essential prerequisite.

Available moisture levels play an important part in the development of ascomata and ascospores. Knowing the optimal amount of leaf moisture necessary for the development and maturation of ascomata and ascospores of *Mycosphaerella* and *Teratosphaeria* spp., the frequency and length of watering in nursery systems can be adjusted to decrease the levels of inoculum (Mondal and Timmer, 2002). Furthermore, any dead or decomposing leaf material present in a nursery or plantation acts as an

**Table 1** Sequences of primers that have been used in molecular biology techniques in the identification and study of *Teratosphaeria nubilosa*.

| Primer | Usage | Sequence (5′–3′) | Annealing temperature | Product size (bp) | Polymorphisms | Reference |
|---|---|---|---|---|---|---|
| OPW-06 | RAPD | AGGCCCGATG | 40 | 250–2000 | 8–11* | Carnegie *et al.* (2001) |
| OPV-08 | RAPD | GGACGGCGTT | 40 | 250–2000 | 8–11* | |
| OPV-18 | RAPD | TGGTGGCGTT | 40 | 250–2000 | 8–11* | |
| OPV-19 | RAPD | GGGTGTGCAG | 40 | 250–2000 | 8–11* | |
| OPX-01 | RAPD | CTGGGCACGA | 40 | 250–2000 | 8–11* | |
| OPX-07 | RAPD | GAGCCAGGCT | 40 | 250–2000 | 8–11* | |
| MNF | SSP | CGTCGGAGTAATACAACC | 50 | 199 | N/A | Kularatne *et al.* (2004) |
| MNR | | AGGCTGGAGTGGTGAAATG | | | | |
| MnubF | SSP | CAACCCCATGTTTTCCCACCACG | 62 | 395 | N/A | Glen *et al.* (2007) |
| MnubR | | CGCCAGACCGGTCCCCGTC | | | | |
| MN1F | SSP | GCGCCAGCCCGACCTCC | 57 | 404 | N/A | Maxwell *et al.* (2005) |
| MN1R | | GGTCCCCGTCAGCGAAACAGT | 56 | | | |
| MN-1 | SSR | TCCTGAAATGAGTGCAGACG | 60 | 257–271 | $(AG)_{10}(TG)_{10}$† | Hunter *et al.* (2006a) |
| | | TCCTCATCCTCTGTGGAACC | | | | |
| MN-2 | SSR | CATTGCTTCGGCGGTTATAG | 60 | 182–266 | $(ACT)_8$59bp$(AC)_{11}$† | |
| | | ATGCACGAAGTCGTTGTTTG | | | | |
| MN-3 | SSR | GACTCAACCGTCGTCGAAAC | 60 | 306–320 | $(AC)_{13}$† | |
| | | CGAACTGAATCCGCTGTGTA | | | | |
| MN-4 | SSR | TGTCACAAGACTTTGGATTGC | 60 | 137–165 | $(ATTGTGG)_{10}$† | |
| | | CCACCACAATCTCCTCACAA | | | | |
| MN-7 | SSR | CGCCTCACAGTTACACATGG | 60 | 377–395 | $(TGTA)_6$† | |
| | | CGAAAGGCTGAGGCTGAA | | | | |
| MN-8 | SSR | TTCTATATACTATATTCTATTTAGG | 53 | 202–322 | $(CTCTCTATA)_{20}$† | |
| | | ATATACTATATCTAAAAGAGGTAG | | | | |
| MN-9 | SSR | CGAATGGGCTATCAGAAACG | 60 | 211–221 | $(CT)_{20}$† | |
| | | ACAGGGCAAGGACCTCGTAT | | | | |
| MN-10 | SSR | ACACCTCGAAATCGCTCATC | 60 | 136–144 | $(TC)_{11}$† | |
| | | TAGCTCTGTGCTGCCTTTGA | | | | |
| MN-11 | SSR | CTCACCAGTGCCGTCTAGGT | 60 | 193–223 | $(TTGGTG)_5$† | |
| | | GGAAATCCTGCCCTAACCTC | | | | |
| MN-14 | SSR | TCGACTACCGTAGGGGACTACT | 60 | 100–112 | $(AC)_{13}$† | |
| | | ATGCACGAAGTCGTTGTTTG | | | | |

RAPD, randomly amplified polymorphic DNA; SSP, species-specific primer; SSR, simple sequence repeat/microsatellite marker.

*Number of scoreable polymorphic amplicons used to identify *Teratosphaeria nubilosa* with RAPD.

†Core microstallite region amplified with microsatellite primers and exhibiting size polymorphisms in repeat number.

inoculum source, as ascomata are capable of developing in such material (Park and Keane, 1987). It is therefore beneficial to remove any such material from a nursery system. Overhead irrigation mechanisms should also be avoided in nurseries, as water accumulates on leaf surfaces and stimulates the production of ascospores. Alternative drip irrigation or hydroponic systems for irrigation of nursery stock should be used, thereby avoiding the high humidity levels and the periods of water accumulation on leaf surfaces.

Fungicide applications can provide a means to control the development of *T. nubilosa*. In a nursery environment, this method is most feasible because of the smaller size of the *Eucalyptus* seedlings and the growth tunnels in which they are housed. Furthermore, it has been suggested that the cost of fungicide applications can be reduced by spraying during the vegetative period of the host (Park, 1988b). Carnegie and Ades (2003) showed that spraying both a protectant and systemic fungicide significantly reduced the development of MLD on juvenile and adult foliage of *E. globulus* in the field. Disease forecasting systems can also be used to determine the most appropriate time for fungicide application (Jacome *et al.*, 1991). However, once deployed into the field and at a plantation level, applications are unlikely to be economically viable for forestry companies (Carnegie and Ades, 2003). They are also detrimental to the environment and are typically prohibited by groups that certify forest operations, such as the Forestry Stewardship Council (FSC, http://www.fsc.org/).

Carnegie (2007b) proposed three strategies for the management of foliar fungi in *Eucalyptus* plantations: (i) risk-site mapping, i.e. identifying high-risk sites for disease and deploying

more tolerant genotypes into these areas; (ii) tree resistance, as discussed above; and (iii) increasing tree tolerance and recovery, i.e. reducing the impact of defoliation events by ensuring that trees are growing optimally, either through correct site selection or remedial fertilization following a defoliation event.

## CONCLUSIONS AND FUTURE PERSPECTIVES

*Mycosphaerella* and *Teratosphaeria* spp. were traditionally regarded as host-specific fungi. However, recent studies have identified several species of *Mycosphaerella* and *Teratosphaeria* that have undergone host jumps and are now able to infect new susceptible hosts. For example, *M. citri*, a recognized pathogen of *Citrus*, has been identified from leaves of *Acacia mangium* in Thailand, and several other plant hosts, such as *Musa* sp., *Aeglopsis* spp., *Fortunella* and *Poncirus* (Crous *et al*., 2004b). *Mycosphaerella communis* is a *Eucalyptus* pathogen that has also been found on *Protea* spp. (Crous *et al*., 2004a). With increased surveys, there is no doubt that more *Mycosphaerella* and *Teratosphaeria* spp. will be found on a larger number of hosts. Similarly, *T. nubilosa*, which has hitherto only been known from *Eucalyptus* spp., has been isolated from *Acacia* in Thailand (Crous and Groenewald, 2005). The full implications of this finding is not yet known and, until it has been established whether *T. nubilosa* can also cause disease on hosts other than *Eucalyptus*, it must be accepted that it can also act as a facultative saprobe on leaf spots caused by other species of *Mycosphaerella* and *Teratosphaeria*.

Over the past few years many fungal genomes have been sequenced. Genomic sequencing is becoming more affordable, and several important plant pathogenic fungi are candidates for future genomic sequencing. The genomes of various ascomycete fungi have also been sequenced, including species of *Mycosphaerella*, such as *M. graminicola* (http://genome.jgi-psf.org/Mycgr1/Mycgr1.home.html) and *M. fijiensis* (http://genome.jgi-psf.org/Mycfi1/Mycfi1.home.html), two important plant pathogens. Considering the importance of *T. nubilosa* as a severe pathogen of *Eucalyptus* spp., it would represent a good candidate for complete genomic sequencing. A genome sequence of *T. nubilosa* would offer several advantages. Virulence genes could be relatively easily mapped, and such information would aid in the selection and breeding of resistant or tolerant *Eucalyptus* species and clones. Furthermore, many more microsatellite regions could be easily located and targeted, thereby increasing the number of markers to be used in population studies of this important pathogen.

Leaf lesions caused by species of *Mycosphaerella* and *Teratosphaeria* generally show the presence of more than one species within a lesion. It is possible, therefore, that mycelium of different *Mycosphaerella* and *Teratosphaeria* species may come into contact within a lesion, leading to hyphal anastomosis and the exchange of genetic material. Through this process, hybrids between different species may be formed. No research has thus far been undertaken to identify hybrids within *Mycosphaerella* or *Teratosphaeria* species occurring on *Eucalyptus,* although multigene DNA sequence data suggest that there may be evidence of hybridization events on this host.

The movement of infected plant material between countries and continents is increasing rapidly (Wingfield *et al*., 2001). Therefore, many fungal pathogens will most probably be introduced into new environments (Wingfield, 1999). Quarantine measures should consequently be strictly implemented and updated to reduce the risk of fungal pathogens, such as *T. nubilosa*, being introduced into new environments. For example, *T. nubilosa* has recently been identified in South America (G. Perez, FABI, Pretoria, South Africa, unpublished data), an area from which it was previously unknown. Therefore, it is important that *Mycosphaerella* and *Teratosphaeria* spp. be incorporated into quarantine regulations and actionable lists.

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