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Ceratocystis fimbriata infecting *Eucalyptus grandis* in Uruguay

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Abstract. Uruguay has a rapidly growing forestry industry consisting mainly of exotic *Pinus* and *Eucalyptus* spp. Recently, there have been reports of individual *E. grandis* trees wilting and dying rapidly in plantations. The aim of this investigation was to survey the dying *E. grandis* in the Rivera area of Uruguay and to determine the cause of the Eucalyptus wilt. Sap-staining symptoms were observed on recently pruned *E. grandis*. Discs of discoloured wood were cut from these pruned trees and from the stems of dying trees. These disks were stored in a moist environment to induce fungal sporulation. Ascomata, typical of a *Ceratocystis* sp., were found covering the edges of the wood where streaking symptoms occurred. Morphologically, the fungus resembles *C. fimbriata*. The internal transcribed spacer regions of the ribosomal RNA operon of the *Ceratocystis* sp. were amplified and sequenced. Sequence data confirmed placement of this fungus amongst other isolates of *C. fimbriata*. Furthermore, the sequence data showed that the Uruguay isolates are most closely related to those from diseased *Eucalyptus* spp. in Brazil, Congo and Uganda. *C. fimbriata* is a well-known pathogen of many woody plants and could constitute a serious threat to intensively managed *E. grandis* in Uruguay where the fungus was not previously known. The relationship between the pruning of *E. grandis* and infection by *C. fimbriata* will, in future, need to be evaluated.

Introduction

Uruguay has a rapidly growing forestry industry consisting mainly of exotic *Pinus* and *Eucalyptus* spp. A total of 3% of the country's land is allocated to commercial forestry and approximately 400 000 ha is used exclusively for growing *Eucalyptus* spp. The two main species used for forestry are *E. grandis* and *E. globulus*.

Eucalyptus spp. are native to Australia, Indonesia and surrounding islands (Poynton 1979). Due to the excellent quality and versatility of its wood, *Eucalyptus* spp. have become one of the world's most widely planted forestry crops (Poynton 1979; Turnbull 1991; Wingfield *et al.* 2001). In Uruguay, *Eucalyptus* plantations were initially established from seeds obtained locally and also from Australia, Chile and South Africa. Although *Eucalyptus* spp. in Uruguay have largely been removed from their natural enemies, planting these trees in an exotic location will expose them to new diseases, for which they may lack natural resistance. It is also likely that pathogens from their areas of origin will be introduced and these could result in large-scale losses due to the relatively narrow genetic base in commercial plantations (Wingfield 1999; Wingfield and Wingfield 1999).

Recently, it has been noted that individual *E. grandis* trees have been wilting and dying rapidly in plantations near Rivera in northern Uruguay. This sudden death of trees has given rise to concern, especially due to the fact that trees up to 5 years old are dying. The aim of this investigation was to determine the probable cause of the Eucalyptus wilt disease.

Methods

Collection and isolation of fungi

During April 2001, individual 1- to 5-year-old *E. grandis* trees were observed dying rapidly in the Rivera area of Northern Uruguay (Fig. 1*a*). Sections of bark and wood were sampled at approximately breast height from the stems of 21 dying trees. These sections were cut into discs, 8-cm wide, wrapped in newspaper and placed in plastic bags for 6 days at room temperature to induce fungal sporulation. Discs were then inspected using a dissection microscope.

Ascomata, typical of a *Ceratocystis* sp., were observed covering the edges of the wood where streaking symptoms occurred. Ascospore masses accumulating at the apices of the ascomatal necks were transferred to 2% MEA (malt-extract agar, Biolab) using a sterile needle. Ascospore masses from structures in cultures were repeatedly transferred to clean plates until pure cultures had been obtained. One pure culture was thus obtained for each of the 21 trees sampled. Representative cultures were lodged in the culture collection of the

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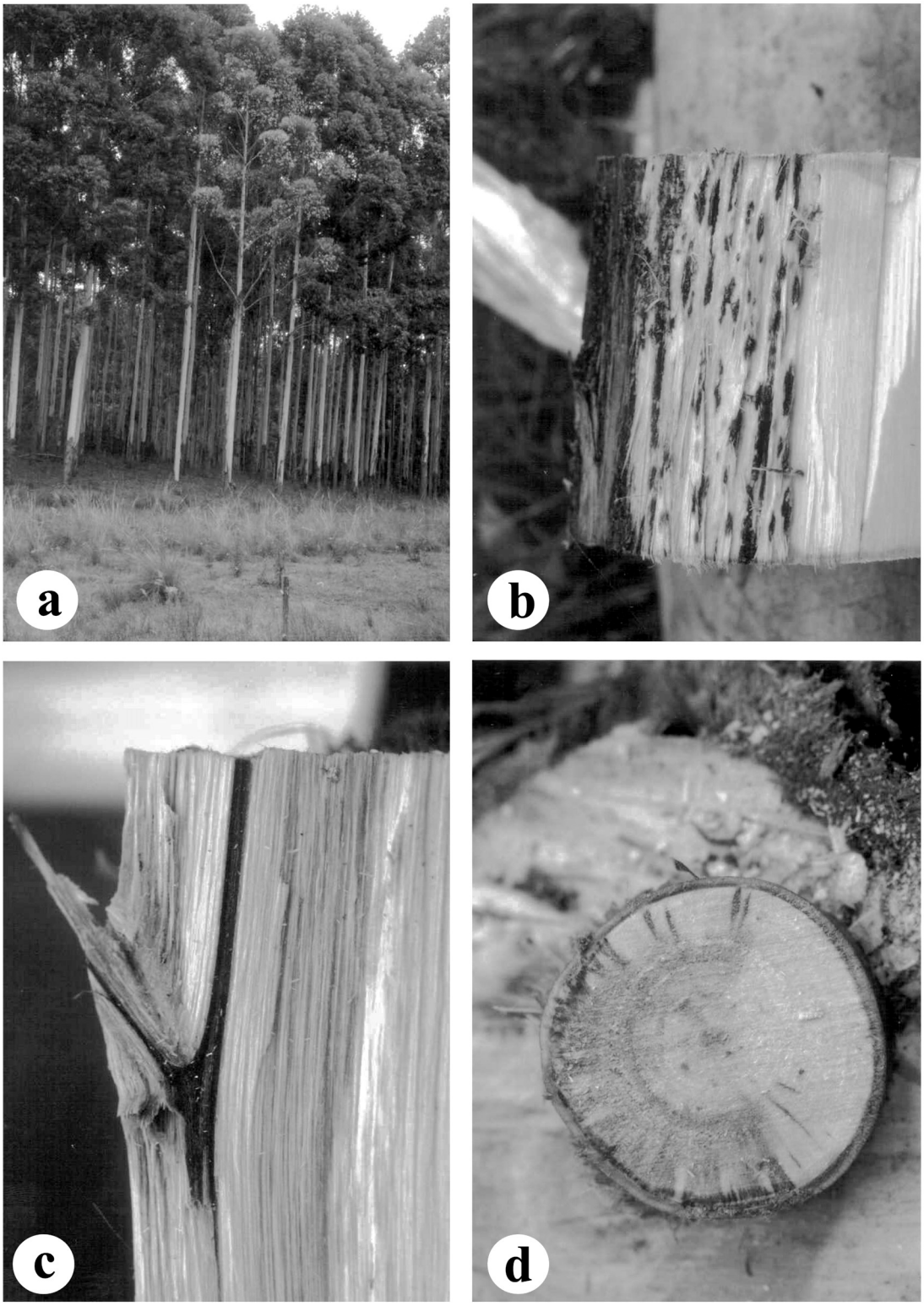

Fig. 1. Symptoms of infection of *Eucalyptus grandis* by *Ceratocystis fimbriata* in Uruguay. (*a*) Single, 5-year-old trees dying with typical wilt symptoms; (*b*) kino pockets under the bark of *E. grandis*; (*c*) discolouration of the wood at site where side branch had been pruned; (*d*) cross section through trunk of *E. grandis* showing the streaking discolouration caused by *C. fimbriata*.

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Morphology

The morphology of the *Ceratocystis* sp. was studied on the original wood and on structures produced on 2% MEA plates. Samples were prepared by mounting fungal structures on microscope slides in lactophenol, amended with cotton blue. Identification of the fungus was based on the shape and size of ascomata, ascospores and conidia, as well as colour of ascomata and hyphal ornamentation on the ascomatal bases. Colours were determined using the colour charts of Rayner (1970).

DNA extraction

For molecular analysis, single masses of ascospores were transferred from pure cultures and grown in 50 mL of malt broth for 12 days at 22ºC. Mycelial mats were freeze-dried and pulverised using liquid nitrogen. DNA was extracted using a phenol/chloroform protocol as previously described by Barnes *et al.* (2001*b*).

PCR amplification

Primers, specifically designed to amplify the internal transcribed spacer regions of the ribosomal RNA operon (White *et al*. 1990), were used to sequence the ITS1, 5.8S and ITS2 regions. The polymerase chain reaction (PCR) mixture, in a total volume of 50 µL, contained 2–10 ng DNA, 1.75 U enzyme (Expand H.F. PCR System, Roche Molecular Biochemicals), 1 × buffer containing 1.5 mM $MgCl_2$ (supplied with the enzyme), 200 nM of primers ITS1 and ITS4, and 200 µM of each dNTP. The PCR programme contained an initial denaturation step at 96ºC for 2 min, followed by 10 cycles of 20 s at 94°C, 48 s at 55ºC, and 45 s at 72ºC. A 5 s extension after each cycle with the annealing time altered to 40 s, was carried out for a further 25 cycles. A 10 min elongation step at 72ºC ended the programme. All PCR amplicons were purified using the Magic PCR Preps Purification System (Promega).

Sequencing

PCR products were amplified in both directions for sequencing using an ABI PRISM Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), according to the manufacturer's protocol. Primers ITS1 and ITS4 were used. Sequence reactions were run on polyacrylamide gel electrophoresis using an ABI PRISM 377 Autosequencer (Applied Biosystems). Sequence electropherograms were analysed using Sequence Navigator version 1.0.1 (Applied Biosystems).

Phylogeny

Forward and reverse sequences were matched and analyzed using Sequence Navigator version 1.0.1 (Applied BioSystems). Additional sequences for use in the analysis were obtained from Genbank (Table 1). Sequences were manually aligned by means of gap insertions. The phylogeny of the aligned sequences was determined using PAUP 4.0b10 (Phylogenetic Analysis Using Parsimony (Swofford 1998). All characters were given equal weight and missing data were treated as a fifth character (new state). The heuristic search option with 100 random stepwise additions and 'tree bisection reconnection' (TBR) was used as the swapping algorithm. The Mulpar option was in effect and branches collapsed if they equaled zero. Confidence levels of the branching points were determined using 1000 bootstrap replicates and length distribution of 1000 trees. *Petriella setifera* (AFO43596) was used as the outgroup and treated as a paraphyletic sister group to the ingroup. All sequences derived in this study have been deposited in Genbank (Table 1).

Results

Collection and isolation of fungi

Dead and dying *E. grandis* trees from which samples were taken, retained their leaves, suggesting that wilt had occurred rapidly (Fig. 1*a*). Small cankers on the bark of dying trees

Table 1. *Ceratocystis* isolates selected for sequence comparisons in this study

Isolate	Country	Genbank accession no.	Culture no.[A]	Host	Collector
C. fimbriata	Italy	AF264903	CMW 2242	*Platanus* sp.	A. Panconesi
C. fimbriata	Switzerland	AF395681	CMW 1896	*Platanus* sp.	O. Petrini
C. fimbriata	Brazil	AF395683	CMW 4903	*Eucalyptus* sp.	A. Alfenas
C. fimbriata	Uruguay[B]	AF453438	CMW 7383	*E. grandis*	M. J. Wingfield
C. fimbriata	Uruguay[B]	AF453439	CMW 7387	*E. grandis*	M. J. Wingfield
C. fimbriata	Uruguay[B]	AF453440	CMW 7389	*E. grandis*	M. J. Wingfield
C. fimbriata	Colombia	AF395688	CMW 4829	*Citrus* sp.	B. L. Castro
C. fimbriata	Colombia	AF395689	CMW 4835	*Coffea* sp.	B. L. Castro
C. fimbriata	Colombia	AF395691	CMW 4844	*Coffea* sp.	B. L. Castro
C. fimbriata	Colombia	AF395692	CMW 4824	*Coffea* sp.	B. L. Castro
C. fimbriata	Congo	AF395684	CMW 4793	*Eucalyptus clone*	J. Roux
C. fimbriata	Uganda	AF395687	CMW 5312	*E. grandis*	J. Roux
C. albofundus	South Africa	AF264910	CMW 2148	*Acacia mearnsii*	M. J. Morris
C. albofundus	South Africa	AF043605	CMW 2475	*A. mearnsii*	S. McLennan
C. coerulescens	Germany	U75615	CBS 140.37	*Picea abies*	T. Rohde
C. fagacearum	USA	AF043598	CMW 2651	*Quercus* sp.	F. Paula
C. eucalypti	Australia	U75627	CMW 3254	*Eucalyptus sieberi*	G. Kile
C. virescens	USA	AF043603	CMW 0460	*Quercus* sp.	—
C. moniliformis	South Africa	AF043597	CMW 3782	*Erythrina* sp.	G. Zhao
Petriella setifera	Kenya	AF043596	ATCC 26490	*Rock hyrax*	—

[A]CMW represents cultures from the culture collections of the Forestry and Agricultural Biotechnology Institute, ATCC from the American Type Culture Collection and CBS from Centraalbureau voor Schimmelcultures.
[B]Isolates sequenced in this study.

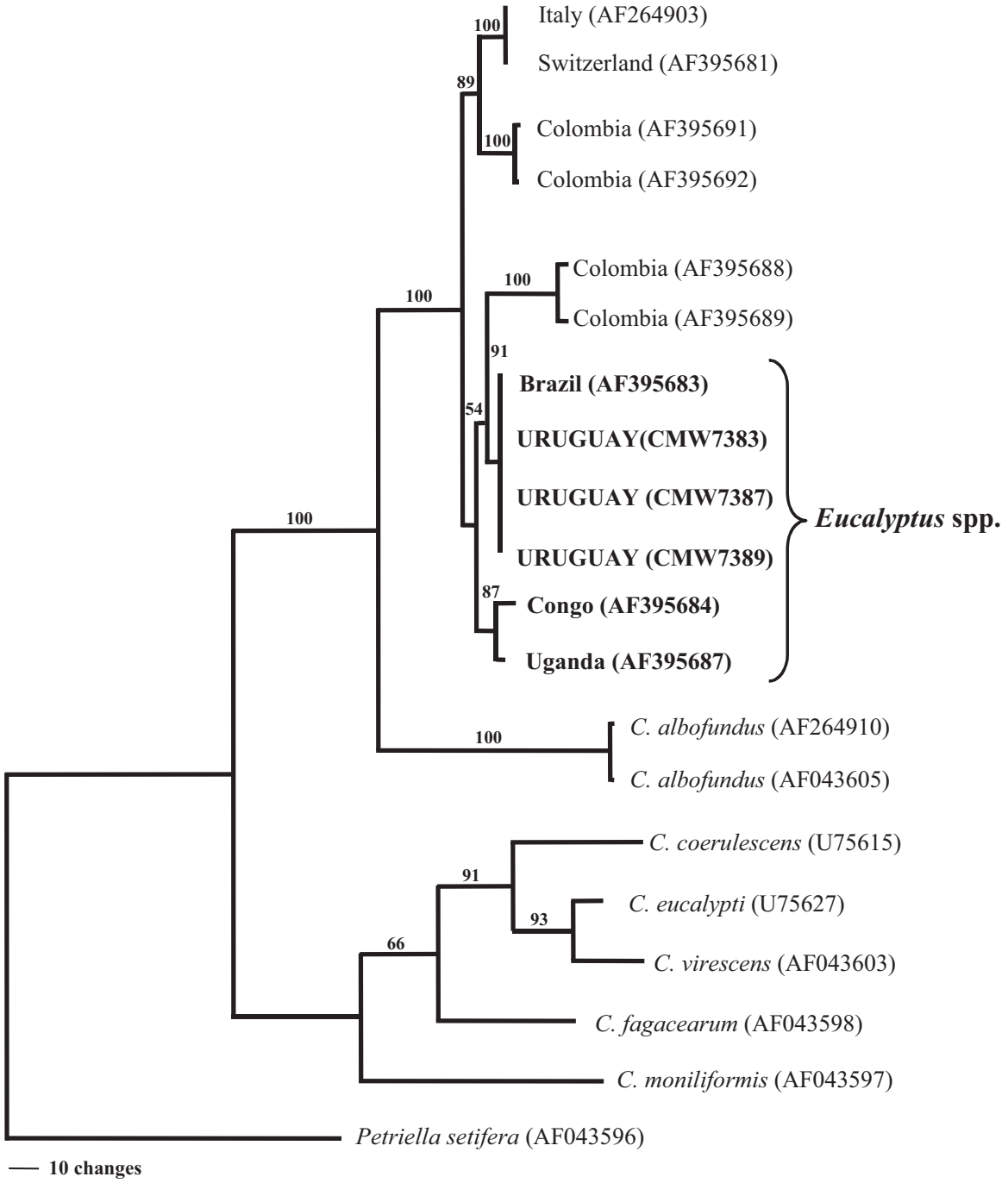

Fig. 2. Phylogenetic tree based on sequence data from the ITS regions of the rRNA operon. The phylogram was obtained using the heuristic search option with random stepwise addition and tree bisection reconnection in PAUP 4.0b10. Bootstrap values are indicated above the branches. *Petriella setifera* was used as the outgroup. *Ceratocystis fimbriata* from Uruguay is placed within the larger clade typified by *C. fimbriata* and identical to the isolate from Brazil. The isolates also group within a larger clade with those from Congo and Uganda, all collected from diseased *Eucalyptus* species.

were also observed. Removal of the bark revealed extensive kino production in the cambium (Fig. 1*b*). Dissection of the stems showed a streaking pattern of discolouration of the wood, emerging specifically from recent pruning sites (Fig. 1*c* and *d*).

Incubation of wood discs from dying trees resulted in the relatively rapid production of ascomata. Isolations were easily made from spore masses on the ascomatal necks. A total of 21 isolates was obtained from the trees sampled.

Morphology

Cultures were olivacious brown (21"K) in colour and produced a fruity aroma. Ascomata were produced both on wood pieces and on agar plates. The globose bases of the ascomata were black with long necks ending in ostiolar hyphae. The ostiolar hyphae extended from the outer layer of the neck cells and were mostly divergent. Ascospores were typically hat-shaped. Cylindrical and barrel shaped conidia were observed. Morphologically, the fungus is identical to descriptions previously given for *Ceratocystis fimbriata* (Hunt 1956; Upadhyay 1981).

Phylogeny

Sequencing of the ITS1 and ITS2 regions resulted in a DNA sequence of 570 bp long. Manual alignment of the sequences for representative isolates used in this study generated a total of 510 characters. Of these characters, 174 were constant, 106 parsimony-uninformative and 230 parsimony-informative. One most parsimonious tree (Fig. 2), with a length of 773, was produced. The consistency index (CI), retention index (RI) and g1 values for the tree were 0.79, 0.79 and –1.14, respectively.

The *Ceratocystis* sp. collected from symptomatic *E. grandis* trees in Uruguay resided in the clade typified by *C. fimbriata*, with a bootstrap value of 100% (Fig. 2). Our results show that the Uruguay isolates are most closely related to isolates from Brazil, South America. In terms of host range, *C. fimbriata* isolates from *E. grandis* in Uruguay grouped together in a larger clade encompassing all the isolates collected from *Eucalyptus* in Brazil, Republic of Congo and Uganda (Table 1, Fig. 2).

Discussion

This study represents the first report of the important wilt pathogen, *Ceratocystis fimbriata,* from Uruguay. *C. fimbriata* is well known for causing wilt diseases on many woody hosts (Kile 1993). However, the fungus has been recognised only recently as a pathogen of *Eucalyptus* spp. These reports have emerged from Brazil (Laia *et al.* 1999; Roux *et al.* 1999), Republic of Congo (Roux *et al.* 1999) and Uganda (Roux *et al.* 2001). The only other *Ceratocystis* spp. known to infect living *Eucalyptus* trees are *C. eucalypti* (Kile *et al.* 1996) and *C. pirilliformis* (Barnes *et al.* 2003), but these fungi are not known as pathogens. In Uruguay, *C. fimbriata* is associated with wood stain and rapid death of *E. grandis.* This is a serious disease that appears to result from infection of recent pruning wounds.

Pruning of *E. grandis* is an important silvicultural practice where *Eucalyptus* is used to produce solid timber products. It is applied two or three times in the first 5 years of an approximately 15 year rotation, decreasing branch knots and increasing wood quality and value (Beadle 1999). In Uruguay, pruning occurs all year round using pruning saws, leaving trees open to infection through wounds. Wounds are essential for infection by *C. fimbriata* (Moller and DeVay 1968; Kile *et al.* 1993) and pruning provides an ideal opportunity for this pathogen. Opportunistic sap-feeding insects, mainly flies (Diptera) and picnic beetles (Nitidulidae) (Crone and Bachelder 1961; Moller and DeVay 1968; Hinds 1972), have been shown to be the main vectors of *C. fimbriata,* moving the fungus between freshly made wounds on trees. Although insects are probably the primary agents of dispersal in Uruguay, other means of spread of this fungus are possible. Since the primary source of wounds on *E. grandis* trees in Uruguay is due to pruning, infected pruning saws or other similar equipment (Teviotdale 1991) could contribute to the spread of the fungus from diseased to healthy trees.

Pathogenicity tests conducted using a range of *C. fimbriata* isolates have shown that isolates of this fungus tend to be host specific (Pontis 1951; Leather 1966). Sequencing data from the present study grouped the Uruguay isolates from *E. grandis* in a clade, together with other isolates from *Eucalyptus* spp. This provides added evidence for the presence of host-specific groups in the pathogen (Barnes *et al.* 2001*a*). These results also support the view, based on host and geographic location (Barnes *et al.* 2001*a*), that *C. fimbriata* represents a species complex (Webster and Butler 1967). Furthermore, the Uruguay isolates in this study were most closely related to those from Brazil. The fact that these countries are geographically adjacent to each other might suggest that outbreaks of the disease in the two areas are related.

The extent of the disease outbreak in Uruguay is low and until now, damage has been marginal. In one year, approximately 10% of pruned trees in a stand are infected. Only young trees have been affected and only 2- to 5-year-old trees are pruned. Symptoms of this disease start to appear between 1 and 6 months after pruning has taken place. All evidence from this study suggests that infections by *C. fimbriata* have occurred shortly after pruning.

Although *E. globulus* is widely planted in Uruguay, there have been no reports of a similar disease on this species. *E. globulus* is not pruned in Uruguay and is, therefore, not exposed to infection. The particular area in which the disease has occurred has relatively high temperatures and is also not suitable for planting *E. globulus.* Nothing is currently known regarding the influence of site on disease development. The disease problem has been relatively limited in extent but it is considered potentially important by forestry companies. In future, strategies to avoid infection of pruning wounds by *C. fimbriata* will need to be developed.

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