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Discovery of two northern hemisphere *Armillaria* species on Proteaceae in South Africa

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Armillaria root rot symptoms were observed on native *Protea* and *Leucadendron* (Proteaceae) species in Kirstenbosch Botanical Gardens in the Western Cape Province of South Africa. Intergenic spacer (IGS)-1 polymerase chain reaction (PCR) restriction fragment-length polymorphism (RFLP) profiling indicated the presence of at least two *Armillaria* species. The profiles of two isolates were identical to those of *A. mellea* s. str., originating in Europe. Phylogenetic analyses incorporating internal transcribed spacer (ITS) and IGS-1 sequence data identified the remaining isolates as closely related to *A. calvescens*, *A. gallica*, *A. jezoensis* and *A. sinapina*. These isolates displayed mating compatibility with *A. gallica*. From the RFLP profiles, sequencing results and sexual compatibility studies, it is concluded that the two species on Proteaceae in Kirstenbosch represent *A. mellea* and *A. gallica*. These are northern hemisphere fungi that have apparently been accidentally introduced into South Africa. This is the second report of *Armillaria* being introduced into South Africa. The introduction probably occurred early in the colonization of Cape Town, when potted plants from Europe were used to establish gardens.

Keywords: *Armillaria gallica*, *Armillaria mellea*, IGS, ITS, mating compatibility tests, RFLP

Introduction

The Proteaceae represent one of the most interesting and prominent families of flowering plants in the southern hemisphere. In the South-Western Cape region (fynbos biome) of South Africa alone, the family encompasses 14 genera and 330 species (Rebelo, 1995). Disease reports dating back to the beginning of the 1900s have indicated that native Proteaceae in South Africa are affected by a large number of plant pathogens, mainly host-specific. These include pathogens causing leaf speck, leaf blotch, leaf spot, shoot and stem diseases, as well as soilborne diseases (Knox-Davies *et al*., 1987; Taylor & Crous, 2000; Denman *et al*., 2003). Among the most important root-rot pathogens known on native Proteaceae is the omnivorous oomycete *Phytophthora cinnamomi*, which has an extremely wide host range on this family of plants (Von Broembsen, 1984). Recent studies have also suggested that *P. cinnamomi* has been introduced into South Africa (Linde *et al*., 1997), and this might account for the very high levels of susceptibility of Proteaceae occurring in this region.

The present study concerns dying *Protea* and *Leucadendron* plants that were encountered in planted beds of the internationally renowned Kirstenbosch Botanical Gardens in Western Cape Province, in May 2000. In South Africa, dying Proteaceae with obvious root disease are generally attributed to *P. cinnamomi* infections. In a disease report by Denman *et al*. (2000), roots of the affected plants were blackened and lacked feeder roots, typical of phytophthora root rot. Removal of the bark, however, revealed white mycelial fans in the cambial region, characteristic of the root-infecting pathogen *Armillaria*. Basidiocarps of *Armillaria* were not found in the vicinity of the infected plants, making field identification of the *Armillaria* species impossible.

Armillaria root rot is a well known problem on Proteaceae in different regions of the world. These include Australia (Porter *et al*., 1996), California (Farr *et al*., 1989), Hawaii (Laemmlen & Bega, 1974), Kenya (Denman *et al*., 2000), Madeira (Moura & Rodriques, 2001), New Zealand (Pennycook, 1989), Tanzania (Denman *et al*., 2000)

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and Zimbabwe (Masuka *et al*., 1998). In countries where this disease occurs in commercial protea cut-flower plantations, losses are of economic significance to the producers. In South Africa, armillaria root rot of Proteaceae has been reported only once, but this was on *Grevillea robusta* (Doidge, 1950), a tree species of Australian origin.

Identification of *Armillaria* species based on morphology is generally considered to be difficult. This is because basidiocarps of the fungus are short-lived and infrequently produced. Sexual compatibility tests between haploid tester strains of known identity and haploid or diploid field isolates provide an alternative to identification based on basidiocarp morphology (Korhonen, 1978; Anderson & Ullrich, 1979). These tests gained much acceptance due to their simplicity, but they are time-consuming and results are often ambiguous. This is especially true of diploid field isolates. Molecular-based identification techniques offer an effective alternative to sexual compatibility tests due to their time-efficient, relatively simple and informative nature (Harrington & Wingfield, 1995; Coetzee *et al*., 2003).

A preliminary report on the discovery of armillaria root rot on Proteaceae in the Kirstenbosch Botanical Gardens of South Africa was published by Denman *et al*. (2000). This report discussed general taxonomy, epidemiology and distribution of *Armillaria* on Proteaceae. The species causing the disease, however, could not be identified at the time. The aim of the present study was to identify the species on affected *Protea* and *Leucadendron* species in Kirstenbosch Botanical Gardens, based on intergenic spacer (IGS)-1 restriction fragment-length polymorphism (RFLP) comparisons, their phylogenetic relationships using IGS-1 and internal transcribed spacer (ITS) sequence data, and their sexual compatibility with other species of *Armillaria*.

Materials and methods

Isolation and cultivation of fungal isolates

Small pieces of white mycelium were removed from below the bark on the roots of dead and dying *Leucadendron* and *Protea* species from Kirstenbosch Botanical Gardens (33°59′ S, 18°26′ E, altitude 89 m) and placed on selective dichloran–benomyl–streptomycin (DBS) medium (Harrington *et al*., 1992). Isolates were then incubated in the dark at 24°C for 2 weeks. Rhizomorph tips that developed from the primary cultures were transferred to DBS medium and further incubated under the same conditions. This procedure was repeated until pure cultures were obtained. Cultures were maintained on malt extract yeast agar (MYA): 10 g L^{-1} malt extract, 2 g L^{-1} yeast extract, 15 g L^{-1} agar (Biolab, Midrand, Johannesburg, Republic of South Africa).

DNA extractions

Cultures were grown in liquid MY medium (10 g L^{-1} malt extract, 2 g L^{-1} yeast extract) for 4 weeks in the dark at 24°C. Mycelium was harvested using a sterilized metal strainer, frozen at −70°C for 20 min and lyophilized. The dry mycelium was ground to a fine powder in liquid nitrogen and stored at −70°C. DNA extraction from the powdered mycelium was performed using the method described by Coetzee *et al*. (2000b).

PCR amplification

The ITS regions ITS-1 and ITS-2 and the 5·8S gene, situated between the small and large subunits of the ribosomal DNA operon, were amplified using the primer set ITS1/ITS4 (White *et al*., 1990). The intergenic spacer region 1 (IGS-1) of the rRNA operon was amplified using primer sets P-1 (Hsiau, 1996) and 5S2B (Coetzee *et al*., 2000a), and CLR12R (Veldman *et al*., 1981) and O-1 (Duchesne & Anderson, 1990). Primers P-1 and O-1 were alternatively used to obtain IGS-1 PCR fragments for RFLP comparison with profiles published by Coetzee *et al*. (2001). The PCR reaction mixture (50 μL, final volume) included dNTP (200 μM of each), *Taq* DNA polymerase (2·5 U) (Roche Diagnostics, Mannheim, Germany), *Taq* DNA polymerase buffer containing $MgCl_2$ (supplied with the enzyme), additional $MgCl_2$ (2·5 mM), 0·1 μM of each primer, and approximately 80 ng of template DNA. The thermocycling (Perkin Elmer 9600) conditions were an initial denaturation at 94°C for 2 min, followed by 30 cycles at 58°C (ITS amplification) or 64°C (IGS-1 amplification) for 30 s (annealing), 72°C for 30 s (elongation) and 94°C for 30 s (denaturation). A final elongation was allowed for 7 min at 72°C. The quality and sizes of ITS and IGS-1 PCR products were determined on an agarose gel (10 g L^{-1} agarose) stained with ethidium bromide and visualized under UV illumination.

Restriction enzyme digestion of the PCR products

The IGS-1 amplicons were digested with the restriction endonuclease *Alu*I. Ten units of restriction enzyme were added to the amplified PCR fragments within the PCR reaction mix (20 μL) and incubated at 37°C for 6 h. Resulting restriction fragments were separated on 25 g L^{-1} agarose gel stained with ethidium bromide and visualized under UV illumination. Both CLR12R/O-1 and P-1/O-1 RFLP profiles were compared with those previously observed for *Armillaria* species from North America, Europe and Asia.

DNA sequencing and sequence analysis

Sequences for the ITS and IGS-1 DNA regions were determined using an ABI PRISM 377 DNA automated sequencer. The ITS regions were sequenced in both directions using primers ITS1 and ITS4 and internal primers CS2B and CS3B (Coetzee *et al*., 2000b). The IGS-1 region was sequenced using primers CLR12R and O-1 as well as internal primers MCO-2 and MCO-2R (Coetzee *et al*., 2000b). The sequence reactions were carried out using an ABI PRISM Dye Terminator Cycle Sequencing Ready

Table 1 List of internal transcribed spacer (ITS) and intergenic spacer (IGS)-1 sequence data obtained for isolates of *Armillaria* spp. from GenBank for phylogenetic analyses

Species	GenBank no.	Origin	Reference
ITS data set			
A. borealis	U54808	Finland	Chillali *et al.* (1998)
	U54809	France	Chillali *et al.* (1998)
	AJ250052	Finland	O. Schmidt *et al.*, Hamburg University D-21031, unpublished
A. cepistipes	U54810	France	Chillali *et al.* (1998)
	U54811	France	Chillali *et al.* (1998)
	AJ250053	Poland	Schmidt *et al.*, unpublished
A. gallica	U54812	France	Chillali *et al.* (1998)
	U54814	France	Chillali *et al.* (1998)
	AJ250054	Italy	Schmidt *et al.*, unpublished
A. ostoyae	U54813	France	Chillali *et al.* (1998)
	U54815	France	Chillali *et al.* (1998)
	U54816	France	Chillali *et al.* (1998)
A. sinapina	AF169646	NY, USA	Coetzee *et al.* (2000b)
A. tabescens	U54821	France	Chillali *et al.* (1998)
	U54822	Italy	Chillali *et al.* (1998)
IGS-1 data set			
A. borealis	AF243055	Finland	Anderson & Stasovski (1992)
	AF243056	Munich, Germany	Anderson & Stasovski (1992)
A. calvescens	AF243070	VT, USA	Anderson & Stasovski (1992)
	AF243071	VT, USA	Anderson & Stasovski (1992)
A. cepistipes	AF243067	France	Anderson & Stasovski (1992)
	AF243068	Helsinki, Finland	Anderson & Stasovski (1992)
	AF243069	Tampere, Finland	Anderson & Stasovski (1992)
	D89919	Mie, Japan	Terashima *et al.* (1998[a])
A. gemina	AF243053	VT, USA	Anderson & Stasovski (1992)
	AF243054	VT, USA	Anderson & Stasovski (1992)
A. jezoensis	D89921	Hokkaido, Japan	Terashima *et al.* (1998[a])
A. gallica	AF243064	MI, USA	Anderson & Stasovski (1992)
	AF243066	VT, USA	Anderson & Stasovski (1992)
	D89920	Hokkaido, Japan	Terashima *et al.* (1998[a])
A. nabsnona	AF243059	ID, USA	Anderson & Stasovski (1992)
	AF243060	BC, Canada	Anderson & Stasovski (1992)
NABS X	AF243061	BC, Canada	Anderson & Stasovski (1992)
	AF243062	ID, USA	Anderson & Stasovski (1992)
A. ostoyae	AF243051	Denmark	Anderson & Stasovski (1992)
	AF243052	Michigan, USA	Anderson & Stasovski (1992)
A. sinapina	AF243057	BC, Canada	Anderson & Stasovski (1992)
	AF243058	NY, USA	Anderson & Stasovski (1992)
	D89925	Hokkaido, Japan	Terashima *et al.* (1998[a])
A. singula	D89926	Hokkaido, Japan	Terashima *et al.* (1998[a])

Reaction Kit with AmpliTaq DNA Polymerases FS (Applied Biosystems, Johannesburg, RSA) according to the manufacturer's directions.

The ITS and IGS-1 sequence data available for *Armillaria* spp. were obtained from GenBank (Table 1). These sequences were aligned against ITS and IGS-1 sequences for isolates CMW7202 and CMW7204 [GenBank accession numbers AY190247 and AY190248 (ITS); AY190245 and AY190246 (IGS-1)] from Kirstenbosch Botanical Gardens using CLUSTAL W (Thompson *et al.*, 1994) and manually adjusted. Missing and ambiguously aligned regions were excluded before analysing the ITS and IGS-1 data sets. Distance and parsimony phylogenetic analyses were performed to determine the identity and phylogenetic relationships between the isolates from Kirstenbosch and other *Armillaria* species. Distance analyses were based on the neighbour-joining search algorithm using the BioNJ method and the HKY85 nucleotide substitution model (Hasegawa *et al.*, 1985). Phylogenetic trees were generated in parsimony analysis by using the heuristic search method with TBR (tree bisection reconnection) branch swapping, MULPARS active, random addition of sequences (100 replicates) and gaps treated as missing data. Bootstrap analysis (1000 replicates) was carried out to determine the support at each branching point on the phylogenetic trees. Trees generated from the ITS and IGS-1 data sets were rooted with *A. tabescens* as monophyletic sister outgroup.

Diploid–haploid compatibility tests

Pairings between diploid isolates from Kirstenbosch Botanical Gardens were made with *Armillaria* haploid tester strains to confirm the results emerging from DNA-based identification. Compatible reactions between the diploid isolates and the haploid tester strains were determined using the methods of Rizzo & Harrington (1992). In this study, mycelial plugs of two representative diploid cultures from Kirstenbosch Botanical Gardens (CMW7202 and CMW7204) were paired in all combinations with North American and European haploid tester strains. Paired cultures were incubated for 4 weeks at 24°C in the dark prior to evaluating the results.

Results

Fungal isolations and cultures

Eighteen isolates were obtained from dead and dying Proteaceae in Kirstenbosch Botanical Gardens. Infected plants included *Leucadendron argenteum*, *L. gandogeri*, *L. grandiflorum*, *Protea longifolia*, *P. eximia* and *P. scolymocephala*. Isolates obtained from these plants are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa. Representative isolates have also been deposited at the Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands.

PCR amplification and RFLP analysis

The IGS-1 and ITS regions were successfully amplified for all the isolates. PCR amplification with primer set P-1/5S2B yielded no amplicons for any of the isolates included in this study. The IGS-1 amplicon size (using primers CLR12R/O-1) was 900 bp for all isolates, with the exception of CMW7206 and CMW7207 which had an IGS-1 amplicon of 800 bp. The ITS region was 800 bp in size for all the isolates in this study.

Two different IGS-1/RFLP profiles were obtained for the isolates from Kirstenbosch Botanical Gardens and they were therefore separated into two groups. The first group consisted of isolates CMW7206 and CMW7207 and had fragment sizes of 215, 175 and 150 bp after digestion of their P-1/O-1 amplicons with *Alu*I. These fragment sizes were identical to those of *A. mellea* s. str. (Coetzee *et al*., 2001).

Isolates in the second group showed RFLP profiles with fragment sizes of 427 (417–441), 236 (233–238) and 183 (180–185) bp after digestion of their CLR12R/O-1 amplicons. Exact fragment sizes were calculated by mapping the restriction sites on the IGS-1 sequence data, and yielded values of 397, 233 and 182 bp. These RFLP fragment sizes were closest to those published for *A. sinapina* (White *et al*., 1998), *A. calvescens* (Kim *et al*., 2001), *A. jezoensis* (Terashima *et al*., 1998b) and *A. gallica* (Harrington & Wingfield, 1995; Banik *et al*., 1996; White *et al*., 1998). The identity of isolates in this group could not therefore be determined by means of their RFLP profiles. Two representative isolates (CMW7202 and CMW7204) were subsequently chosen from this group and subjected to further investigation.

DNA sequence analysis

IGS-1 sequence data

The number of characters included in the IGS-1 data set after alignment was 750. The number of characters included in distance analysis was 483 after exclusion of missing and ambiguously aligned regions, and 147 parsimony informative characters were included in parsimony analysis. Trees generated after heuristic and neighbour-joining analyses grouped the Kirstenbosch isolates, CMW7202 and CMW7204, within a clade that included *A. cepistipes*, *A. jezoensis*, *A. sinapina* and *A. singula* from Japan (51% bootstrap support for the NJ tree and 61% for the MP tree) (Fig. 1). These two Kirstenbosch isolates formed a strongly supported (97% bootstrap support for the NJ tree; 83% for the MP tree) monophyletic group with *A. sinapina* and *A. cepistipes* within the Japanese clade.

ITS sequence data

The ITS data set included 899 characters after alignment by inserting gaps. There were 715 characters included in distance analysis. Parsimony analysis was based on 31 parsimony informative characters. Neighbour-joining trees generated from the ITS data placed the Kirstenbosch isolates (CMW7202 and CMW7204) as sister group to *A. gallica* (U54812) with a bootstrap support of 71% (Fig. 2a). These isolates from Kirstenbosch formed a monophyletic group with *A. gallica* (U54812) in parsimony analysis, supported by a 59% bootstrap value. The Kirstenbosch isolates (CMW7202 and CMW7204), together with *A. gallica* (U54812), formed a sister group with other representative isolates of this species, although this relationship did not have strong bootstrap support (Fig. 2b).

Diploid–haploid compatibility tests

The choice of haploid tester strains used in the mating study was based on the close phylogenetic relationship between the Kirstenbosch isolates (CMW7202 and CMW7204) and *A. cepistipes*, *A. sinapina* and *A. gallica*. *Armillaria gemina*, which is not closely related to the Kirstenbosch isolates, was included as negative control. Tester strains of *A. gallica* (CMW3163 and CMW6902) were sexually compatible with the Kirstenbosch isolates (CMW7202 and CMW7204), and their culture morphology changed to brown pigmented and depressed mycelia, indicating successful diploidization (Fig. 3a). Subcultures made from three different areas from this diploidized tester retained the overall diploid culture morphology but some white aerial mycelium was observed (Fig. 3b). No sexually compatible interaction was observed between the Kirstenbosch isolates and the haploid tester strains of

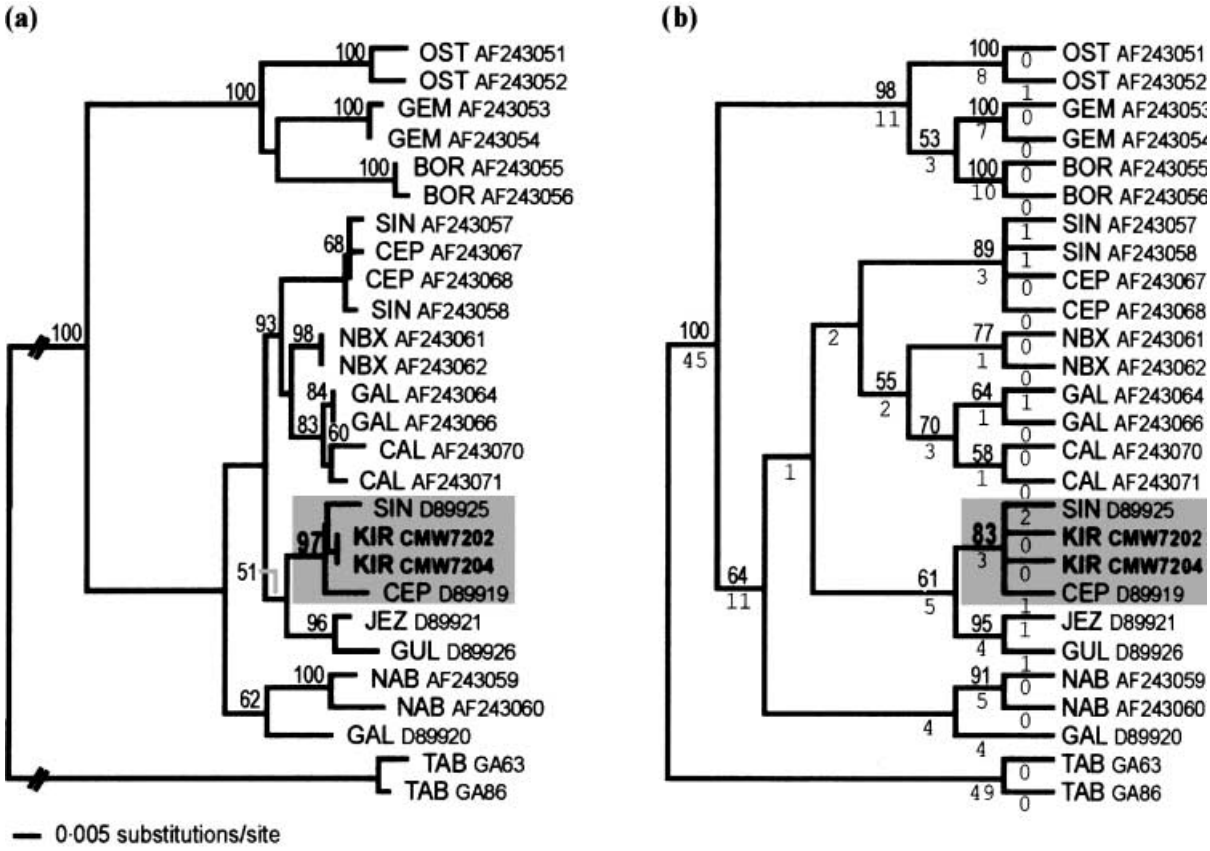

Figure 1 Phylogenetic trees generated based on IGS-1 sequence data. Bootstrap values are indicated above the tree branches; grey blocks indicate grouping of the *Armillaria* isolates from Kirstenbosch with *A. sinapina* and *A. cepistipes* from Japan. (a) Neighbour-joining tree generated from the data matrix. Branch lengths and scale bar below the tree correspond to distances measured as the proportion of nucleotide substitutions between sequences. (b) One of 12 most parsimonious trees with branch lengths indicated below the branches. Tree length (TL) = 193 steps; consistency index (CI) = 0·839; retention index (RI) = 0·915. Abbreviations: OST, *A. ostoyae*; GEM, *A. gemina*; BOR, *A. borealis*; SIN, *A. sinapina*; CEP, *A. cepistipes*; NBX, NABS X; GAL, *A. gallica*; CAL, *A. calvescens*; JEZ, *A. jezoensis*; GUL, *A. singula*; NAB, *A. nabsnona*; TAB, *A. tabescens*; KIR, *Armillaria* isolates from Kirstenbosch.

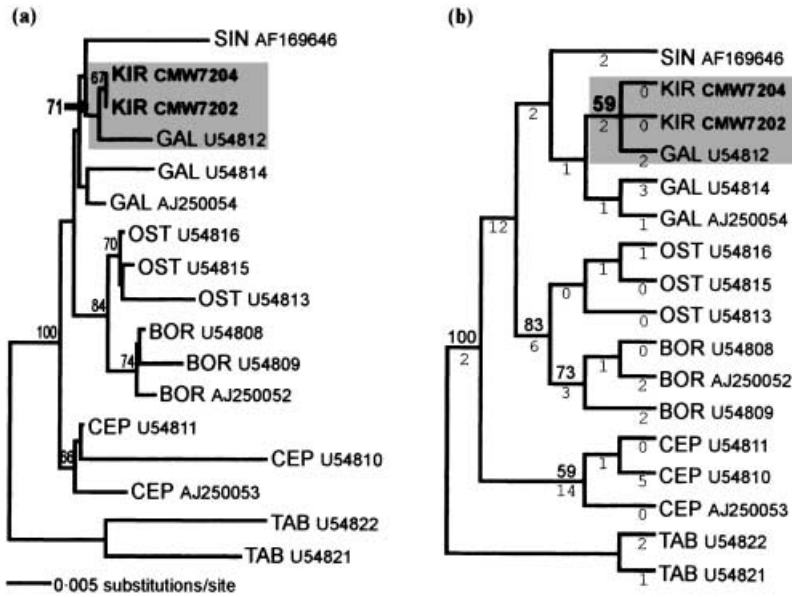

Figure 2 Phylogenetic trees generated based on ITS sequence data. Bootstrap values are indicated above the tree branches; grey blocks indicate grouping of the *Armillaria* isolates from Kirstenbosch with *A. gallica*. (a) Neighbour-joining tree generated from the data matrix. Branch lengths and scale bar correspond to the distance as a proportion of nucleotide substitutions between sequences. (b) One of 138 most parsimonious trees with *TL* = 51 steps, *CI* = 0·667 and *RI* = 0·757. Branch lengths are indicated below the branches. Abbreviations as in Fig. 1.

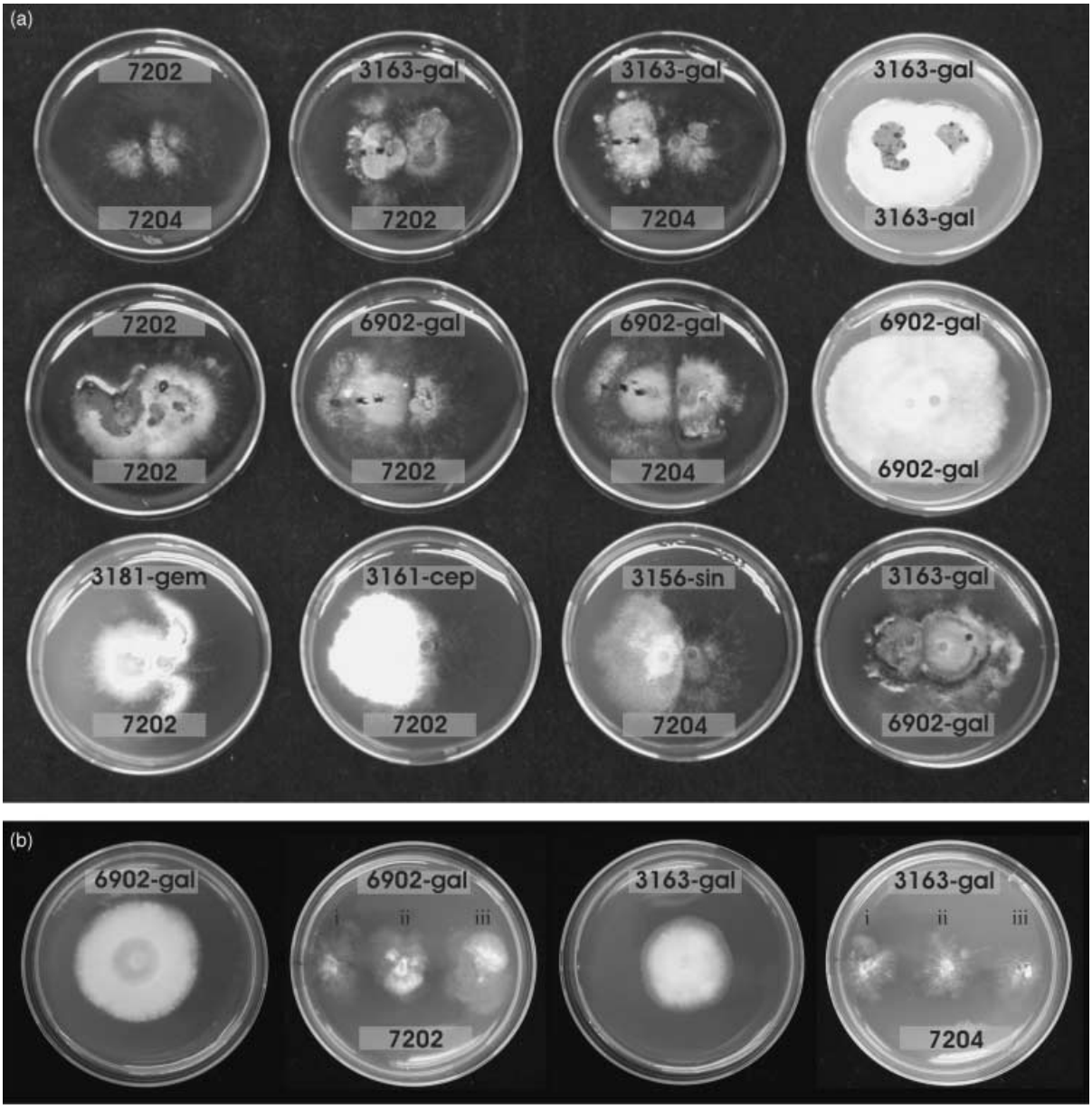

Figure 3 Sexual compatibility tests. (a) Interaction between tester strains and isolates from Kirstenbosch. Numbers at the top and bottom of the Petri dish pertain to the inoculum at the left and right, respectively. (b) Morphology of secondary cultures made from the putative diploidized haploid tester strain. Inoculum at the left (i) was taken from the point behind the interaction between the tester and the isolate from Kirstenbosch Botanical Gardens; middle inoculum (ii) was taken from a point away from the area of interaction; inoculum at the right (iii) originates from the periphery of the tester culture.

A. sinapina (CMW3156), *A. cepistipes* (CMW3161) and *A. gemina* (CMW3181) (Fig. 3a). The tester strains of these species retained their typical haploid white and fluffy aerial mycelium when paired with the diploid isolates from Kirstenbosch. After pairing, the culture morphology of the two haploid *A. gallica* tester strains was transformed from white and fluffy abundant aerial mycelium to crustose depressed pigmented mycelium, indicating successful diploidization (Fig. 3a).

Discussion

Results of this study have shown that two species of *Armillaria* are implicated in the death of *Protea* and *Leucadendron* species in the historically and internationally important Kirstenbosch Botanical Gardens of South Africa. These species, *A. mellea* and *A. gallica*, are both known to be native to the northern hemisphere, so have clearly been introduced into the gardens. This finding is

intriguing, although not without precedent in the Cape Province of South Africa. It was recently shown that *A. mellea* s. str. was introduced into the Dutch East India Company Gardens in the centre of Cape Town approximately 300 years ago (Coetzee *et al*., 2001). This was probably with citrus plants brought from Europe to provide a source of vitamin C for sailors. In the present study, an additional northern hemisphere species, *A. gallica*, was identified. It is reasonable to assume that this species was introduced during the early settlement of Cape Town, as it is known that potted plants were introduced into the area during this period.

The original expectation was that the *Armillaria* species in Kirstenbosch would represent an African species. Primer set P-1/5S2B was previously reported to amplify the IGS-1 region of African *Armillaria* species, therefore it was initially utilized (Coetzee *et al*., 2000a). However, PCR amplification of the IGS-1 region for all Kirstenbosch isolates, using this primer set, produced negative results. In contrast, primer set CLR12R/O-1 resulted in successful amplification of the IGS-1 region in all isolates. Primer set P-1/5S2B amplifies the IGS-1 region only when the 5S gene is inverted in relation to the other genes in the rRNA operon, as was found in native African *Armillaria* species (Coetzee *et al*., 2000a). Negative results provided us with early evidence that a non-African *Armillaria* species was present in Kirstenbosch Botanical Gardens.

The PCR-RFLP method developed by Harrington & Wingfield (1995) for rapid identification of *Armillaria* species was used in a preliminary analysis to determine the identity of the species present in Kirstenbosch Botanical Gardens. Two different IGS-1/RFLP profiles were observed for the isolates. Profiles obtained for two of the isolates were identical to *A. mellea* s. str. from Europe, as well as to those from the Company Gardens (Coetzee *et al*., 2001). These two isolates therefore represent *A. mellea* s. str. This is the second report of the fungus in a cultivated national heritage garden of South Africa.

Armillaria mellea s. str. is one of the most aggressive species in the genus, and tends not to be host-specific (Gregory *et al*., 1991). This fungus is restricted to the northern hemisphere, and its occurrence in the Company Gardens of Cape Town is the only previously recorded exception (Coetzee *et al*., 2001). One possible explanation for the presence of *A. mellea* s. str. in the Kirstenbosch Botanical Gardens is that it has spread from the Company Gardens, where it sporulates profusely. These areas are only a few kilometres apart, and spread could have occurred via basidiospores. If this is the case, it would suggest that other occurrences of this European fungus might be encountered in the Cape Peninsula in the future. An alternative explanation is that the fungus was introduced independently into Kirstenbosch with plants from the northern hemisphere. Additional isolates and genetic fingerprinting will be necessary to resolve this intriguing question.

The remaining 16 isolates from Kirstenbosch Botanical Gardens had identical IGS-1/RFLP profiles, suggesting that they all represent the same species. The identity of these isolates, however, was uncertain due to the similarity between their RFLP profiles and previously reported IGS-1/RFLP profiles of *A. calvescens*, *A. gallica*, *A. sinapina* and *A. jezoensis* (Harrington & Wingfield, 1995; Banik *et al*., 1996; Terashima *et al*., 1998b; White *et al*., 1998; Kim *et al*., 2001). Phylogenetic trees generated from IGS-1 sequence data indicated that these isolates are closely related to *A. sinapina* and *A. cepistipes* from Japan. Terashima *et al*. (1998a) found that *A. sinapina*, *A. cepistipes*, *A. singula* and *A. jezoensis* from Japan grouped together in a strongly supported subclade (Japanese clade), which formed part of a major clade including *A. sinapina*, *A. cepistipes* and other *Armillaria* species from Europe and North America. In the present study, *Armillaria* isolates from Kirstenbosch, other than those representing *A. mellea*, grouped within this Asian clade. They are clearly of the same geographical lineage, and it is therefore believed that they were introduced into Kirstenbosch Botanical Gardens from Japan.

Neighbour-joining and parsimony trees generated from ITS sequence data indicated that the *Armillaria* isolates from Kirstenbosch are closely related to *A. gallica*, and might represent this species. This relationship, however, was not reflected in the IGS-1 phylogenetic trees. This discrepancy between ITS and IGS-1 data might be explained by the fact that GenBank contains IGS-1 sequence data for only one Japanese *A. gallica* isolate, and intraspecific variation could have rendered this isolate unrepresentative of other Japanese *A. gallica* isolates. In the current study, as well as that of Terashima *et al*. (1998a), this isolate grouped with *A. nabsnona* from North America and not with other *Armillaria* species within the Asian clade, as might have been expected. Discounting IGS-1 data, the results from ITS phylogenetic analyses strongly suggest that the Kirstenbosch isolates represent *A. gallica*.

In order to confirm the identity of the majority of isolates from Kirstenbosch Botanical Gardens as *A. gallica*, sexual compatibility tests were conducted. When two representative isolates were paired with haploid tester strains of *A. gallica*, a strong positive interaction emerged. These results indicate that the predominant *Armillaria* species in Kirstenbosch Botanical Gardens represents *A. gallica*.

Armillaria gallica has previously been reported from areas at low altitudes and at southern latitudes in North America, Europe and Japan (Guillaumin *et al*., 1989; Harrington & Rizzo, 1993; Ota *et al*., 1998). This species is considered to be a weak pathogen, but can act as secondary agent in mortality of hosts affected by biotic and abiotic stress (Rishbeth, 1982; Gregory, 1985). It is known that the *Protea* and *Leucadendron* species in Kirstenbosch Botanical Gardens are continually subjected to infection by *Phytophthora cinnamomi* (H. Jamieson, Kirstenbosch Botanical Gardens, South Africa, personal communication), and this could have provided the necessary predisposition to favour infection by *A. gallica*.

All evidence available to us suggests that *A. gallica* in Kirstenbosch Botanical Gardens originated in Asia. Although this garden was formally established in 1913, its history goes back to the early 1800s, when it was utilized

as farmland. Chestnuts, oaks, fruit trees and vines were planted and cultivated at various stages on this land during that period. The introduction of *Armillaria* species would have been most likely to occur via soil with plants. Although the movement of potted plants has been restricted for many years, it is likely that they would have been introduced during the early establishment of Cape Town.

The discovery of *A. mellea* in the Company Gardens in Cape Town was considered most unusual (Coetzee *et al*., 2001). This was particularly because root-inhabiting basidiomycetes are not generally considered to be common invaders of new areas. Results of the present study suggest that such introductions during the early European colonization of South Africa might have been much more common than previously realized. There are clearly fascinating discoveries to be made in this area of research, and many lessons to be learned in terms of the global distribution of pathogens and quarantine procedures.

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