

Fungal phoenix rising from the ashes?

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Abstract: During May 2010, sporocarps of what appeared to be an *Armillaria* sp. were found in large clumps in historic Kirstenbosch Botanical Gardens on the foot of Table Mountain, Cape Town, South Africa. These sporocarps could be physically linked to the roots of unidentified dead trees and *Protea* spp. The aim of this study was to identify the *Armillaria* sp. found fruiting in Kirstenbosch. To achieve this goal isolates were made from the mycelium under the bark of dead roots linked to sporocarps. The ITS and IGS-1 regions were sequenced and compared to sequences of *Armillaria* spp. available on GenBank. Cladograms were generated using ITS sequences to determine the phylogenetic relationship of the isolates with other *Armillaria* spp. Sequence comparisons and phylogenetic analyses showed that the isolates represented *A. mellea*. They were also identical to isolates of this species previously discovered in the Company Gardens in South Africa and introduced from Europe apparently by the early Dutch Settlers. *Armillaria mellea* is alien and apparently invasive in Cape Town, fruits profusely and has the potential to spread to sensitive native forests on the foothills of the City.

Key words:

Armillaria mellea
Armillaria root rot
fungal introduction
Proteaceae

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INTRODUCTION

Species of *Armillaria* are some of the most important pathogens of woody plants in the world. These fungi have been known as tree pathogens since their first discovery by Danish botanist Martin Vahl. While the taxonomy of these *Armillaria* spp. has been controversial and widely debated over an extended period of time, application of the biological species concept (Korhonen 1978, Anderson & Ullrich 1979, Ota *et al.* 1998, Qin *et al.* 2007) and more recently DNA sequence comparisons (Coetzee *et al.* 2000a, 2003a, 2005, Gezahgne *et al.* 2004, Keča *et al.* 2006, Mwenje *et al.* 2006, Hasegawa *et al.* 2010) have resolved many problems relating to the delineation of species. At least 40 species are now recognised in *Armillaria* (Volk & Burdsall 1995, Lima *et al.* 2008, Pildain *et al.* 2010) and it is likely that other species will emerge from under-sampled areas in the future.

Armillaria root rot, the disease caused by pathogenic *Armillaria* spp. can result in serious losses to productivity in tree plantations, fruit tree orchards and in gardens (Gregory *et al.* 1991, Hood *et al.* 1991). In native forests, *Armillaria* spp. cause disease but this is most typically a natural process (Kile *et al.* 1991). Interestingly, species of these fungi exist as clones covering huge areas of land and in these situations they are considered to be amongst the largest and oldest living organisms (Gould 1992, Smith *et al.* 1992).

Only a single native *Armillaria* sp. occurs in South Africa (Coetzee *et al.* 2000a). This fungus, *A. fuscipes* is

occasionally found on native trees (Kotzé 1935, referred to as *A. mellea*). In contrast, it can be a serious pathogen in plantations of non-native *Pinus* spp. and on fruit trees planted in moist areas that have been cleared of native forest (Lundquist 1986, 1987, Coetzee *et al.* 2000a). A more intriguing *Armillaria* sp. in South Africa is *A. mellea* that was discovered in the Company (Dutch East India Company) Gardens in the centre of Cape Town (Coetzee *et al.* 2001). The fungus in that situation represents a single genetic entity that was shown to be at least 358 years old. It was most likely introduced into the city when gardens were established to provide sailors travelling to the East with fresh produce (Coetzee *et al.* 2001).

Some years after the discovery of the *A. mellea* clone in Cape Town, Armillaria root rot was found killing *Protea* plants in the historic Kirstenbosch Botanical Gardens (<sanbi.org>) on the foothills of South Africa's iconic Table Mountain (Coetzee *et al.* 2003b). The fungus in that situation was never seen fruiting but isolates were identified as those of *A. gallica*, and it was suggested that the fungus was introduced into the gardens with plants brought from Asia (Coetzee *et al.* 2003b). A few of the isolates collected on the *Protea* plants were also thought to represent *A. mellea*, but the identification was tentative and based only on RFLP comparisons, without comparison of DNA sequence data against other *Armillaria* spp.

During May 2010, sporocarps of what appeared to be an *Armillaria* sp. were found in large clumps in the upper corner

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Fig. 1. *Armillaria* root rot in Kirstenbosch Botanical Gardens. **A.** Native woody shrubs and forest deeper in the valleys common on the Cape Peninsula. **B, C.** Clusters of fruiting bodies found on a stump. **D–G.** Robust fruiting bodies of *Armillaria* sp. showing a yellow cap, prominent annulus and stipe tapering down to the base. **H.** Rhizomorphs produced in culture.

of Kirstenbosch Botanical Gardens and close to Rycroft's Gate. These sporocarps could be physically linked to the roots of unidentified dead trees and *Protea* spp. (Fig. 1B, C). Upon removal of the bark from the dead roots, sheets of white mycelium typical of *Armillaria* root rot were found. The aim of this study was to identify the *Armillaria* sp. found fruiting in Kirstenbosch using data that were not available at the time of the discovery of *Armillaria* root rot in Cape Town (Coetzee *et al.* 2003b).

MATERIALS AND METHODS

Isolates

Isolation and purification of isolates followed the methods outlined in Coetzee *et al.* (2003). Cultures were maintained on malt extract yeast agar (MYA) (15 g/L malt extract, 2 g/L yeast extract, 15 g/L agar). Cultures are stored in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria and with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands.

Molecular methods

Isolates for DNA extractions were grown at 25 °C in the dark for 3 wk in conical flasks containing liquid malt extract yeast (MY). Mycelium was harvested using a tea strainer, freeze-dried and lyophilised. DNA extractions followed the protocol of Coetzee *et al.* (2000b). A NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) was used to quantify the DNA. The IGS-1 and ITS regions of the rDNA operon were amplified using primer pairs P-1 / O-1 and ITS-1 / ITS-4, respectively. PCR reaction mixture and conditions were the same as those published by Coetzee *et al.* (2000b), except that FastStart Tag DNA polymerase was used instead of an Expand High Fidelity PCR System. The PCR products were purified prior to sequencing using a MSB® Spin PCRapace kit (Invitex, Germany). DNA sequences for the IGS-1 and ITS-1 regions were obtained in both directions using the same primers employed for their amplification. The sequence reactions were carried out using an ABI PRISM Dye Terminator Cycle Sequencing Ready kit with AmpliTaq DNA Polymerases FS (Applied Biosystems) following the manufacturer's instructions. Chromatographs were analysed and contigs assembled in CLC Main Workbench v. 5.7 (CLC bio, Denmark).

DNA sequence comparisons and phylogenetic analyses

DNA sequences were compared against those available in the NCBI GenBank database using a BlastN search. IGS-1 and ITS sequences generated in this study were aligned against those of isolates CMW 3975 and CMW 3978 available on GenBank and originating from the Company Gardens in Cape Town. This was done to determine nucleotide variation between the isolates from the Company Gardens and those from Kirstenbosch Botanical Gardens.

Phylogenetic analysis was conducted with a sub-set of the ITS-1 dataset generated by Coetzee *et al.* (2003). The dataset was amended with DNA sequences for *A. fuscipes* from South Africa and *A. mellea* from Europe, Asia, western USA, eastern USA and the Company Gardens, South Africa. Sequences were re-aligned using MAFFT v. 6 (Katoh & Toh 2008). Cladograms were generated using a heuristic tree search algorithm in PAUP v. 4 with branch swapping set to TBR and random addition of sequences (10 replicates). Trees were rooted to *A. fuscipes*. Bootstrap analysis (1000 replicates) was done to gain support for the grouping of taxa using the same settings as above but with addition of sequences set to nearest.

RESULTS

Isolates

The macro-morphology of basidiocarps produced by the fungus was similar to that described for *A. mellea* (Watling *et al.* 1982) (Fig. 1D–G). The cap colour of the basidiocarps was distinctly yellow and they had thick annuli and stipes tapering towards the base. The basidiocarps also had a caespitose growth habit typical of *A. mellea*.

Two isolates (CMW 36264 and CMW 36265) were retrieved from infected roots and these produced rhizomorphs typical of *Armillaria* spp. in culture (Fig. 1H). The rhizomorphs displayed a dichotomous growth habit and were produced in abundance. White aerial mycelium was observed on the surface of the rhizomorphs at areas that had grown out of the medium.

DNA sequence comparisons and phylogenetic analysis

The IGS-1 and ITS DNA sequences of isolates from Kirstenbosch were most similar to sequences of *A. mellea* in GenBank. Comparisons of IGS-1 and ITS sequences revealed the absence of nucleotide variation between isolates from Kirstenbosch and *A. mellea* from the Company Gardens.

The ITS dataset included 925 characters of which 183 characters were parsimoniously informative. A heuristic search generated 6 trees with tree lengths of 250 steps (Fig. 2). The consistency index was 0.864, and retention index 0.941. The isolates from Kirstenbosch formed a monophyletic group with *A. mellea* from the Company Gardens with strong bootstrap support and together these were placed in a clade that included sequences of *A. mellea* from Europe (99 % bootstrap support).

DISCUSSION

Sporocarps linked to infected roots from which cultures were made in this study were morphologically similar to those of *A. mellea* previously found in the Cape Town city centre. DNA sequence comparisons also showed that the cultures were those of *A. mellea* and the sequences were identical to those from the Company Gardens. Although vegetative compatibility tests were not done to test whether these represent the same clone as those in the City Centre, there was no IGS-1 or ITS nucleotide variation between isolates from the two locations and they most likely are the same.

There are three possible means of introduction of *A. mellea* into Kirstenbosch Botanical Gardens, via air-dispersed basidiospores, on infected plant material or on infested wood mulch. *Armillaria mellea* in the Company Gardens fruits profusely every year at the onset of the first rains in autumn. Although the fungus clone is entirely surrounded by roads and buildings, the basidiospore cloud is likely to easily spread within the city and at least up the foothills of Table Mountain, on which Kirstenbosch is situated. While *A. mellea* might have been introduced into Kirstenbosch separately to that of the clone found in the Company Gardens and as *A. gallica* must have been, it would perhaps more easily have spread to this nearby location via basidiospores. One further possible route of introduction to consider relates to the cultivation practices used in the garden. Flowerbeds and paths are frequently covered with wood and bark mulch. As *Armillaria* spp. are common wood rotting fungi, it is possible that *A. mellea* was introduced into the Gardens through this substrate.

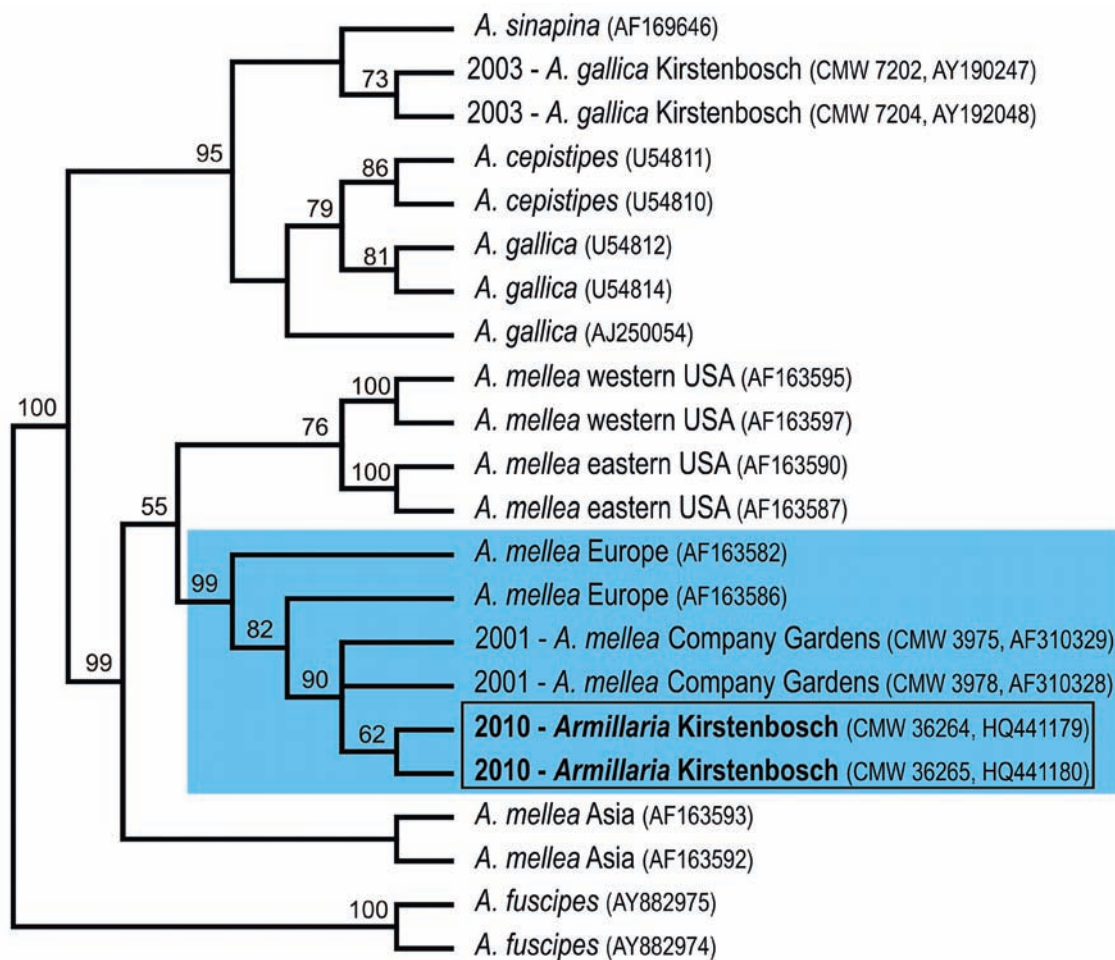


Fig. 2. Cladogram generated from ITS DNA sequence data. Bootstrap values are indicated above the tree branches. The year during which isolates from Kirstenbosch and the Company Gardens were reported are indicated next to the taxon name. Isolates obtained during this study are shown in the rectangle.

Armillaria sporocarps have not previously been found in Kirstenbosch. This may simply be related to the fact that they are ephemeral and have not been present when mycologists or plant pathologists might have been visiting the botanical garden. When these sporocarps were discovered, they were relatively widespread and all were morphologically similar. The infected roots from which isolates were made were also from a number of locations, none of which had been associated with the infection by *A. gallica*. It is possible that *A. gallica* also fruits in the garden, but at a different time to *A. mellea*, or it is less prone to fruiting. Regular observations will be needed to resolve this question.

Peripheral surveys of the native forest on the foothills of Table Mountain and that extending out of the Kirstenbosch Botanical Gardens have not revealed evidence of *Armillaria* root rot. The fact that *A. mellea* is able to fruit profusely in the gardens suggests that it may spread to native forests in the vicinity (Fig. 1A) and more careful surveys should be undertaken to determine whether this is already occurring. Certainly this invasive alien fungus has the capacity to result in serious disease problems in the native environment as has been true with the introduced invasive *Phytophthora cinnamomi* on *Leucodendron argenteum* (Silver Trees) in

Kirstenbosch (van Wyk 1973, Linde *et al.* 1997). This potential risk to Kirstenbosch and the native forest associated with it deserve consideration.

The Dutchman Jan Van Riebeeck was the founder and first commander of Cape Town between 1652 and 1662. One of his tasks was to establish a vegetable and fruit garden to provide ships of the Dutch East India Company sailing between The Netherlands and East Asia with fresh produce and to offset serious problems due to vitamin C deficiency [for a fascinating account of the ship's surgeons of the Dutch East India Company see Bruijn (2009)]. This is the origin of the Company Gardens and the historic avenue of oak (*Quercus robur*) trees that line Government Avenue, the death of which prompted the discovery of *A. mellea* in Cape Town (Coetzee *et al.* 2001). At the time of this discovery, popular press took an interest in the problem (fabinet.up.ac.za/tcp/news) and referred to the tragic death of historic trees as "Van Riebeeck's curse". The appearance of *A. mellea* fruiting profusely in Kirstenbosch, another historic garden of great national importance, suggests that the fungal "Van Riebeeck's curse" remains not only present but is growing in importance. It further illustrates the devastating impact that invasive alien pathogens can have on natural woody ecosystems many years after their introduction.

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