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A new plant pathogenic sterile white basidiomycete from Australia

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Abstract

A sterile white fungus was isolated from the healthy looking roots of buffalo grass (*Stenotaphrum secundatum*) grown on cleared bush land in Perth, Western Australia. The fungal strain was pathogenic on 12 plant species screened under the greenhouse conditions. The clamp connections and dolipore septa indicated that the isolate was a Basidiomycete. Mycelial features, growth rate at different temperatures, as well as pathogenicity patterns of this sterile white basidiomycete (SWB) were distinctly different from those of a strain with a similar morphology, ATCC 28344, previously described as a pathogen in Florida and Georgia (USA). All attempts to induce sporulation failed. The isolates were also compared using the nucleotide sequence analysis of the ribosomal DNA array. Approximately 1 kbp of the 5′ end of the large subunit ribosomal RNA gene, complete sequences of the small subunit ribosomal RNA gene and the entire ITS region (including ITS1, ITS2 and 5.8S gene) were sequenced for the purpose. The obtained sequences were compared with the homologous regions of other genera of Agaricales available in GenBank. Relatively low sequence similarities between the American and Australian strains, as well as the phylogenetic analysis of the studied regions has suggested that these two fungi belong to different genera. Interesting results were achieved in the case of the large subunit ribosomal DNA since this region has been widely studied for taxonomy of Basidiomycetes. The Australian strain 3034 appeared to be closely related to the genus *Campanella* and the American SWB was identified as belonging to the genus *Marasmius*, possibly to *M. graminum*. Our data suggest that the Australian strain is a novel pathogen, and is different from the American SWB isolates described to date.

Abbreviations: SWB – sterile white basidiomycete

Introduction

Mycelia Sterilia, or sterile fungi, is an artificial taxonomic group that shares the property of being defective in forming true conidia and consists of fungi of various morphological types (Hawksworth et al., 1983). Sterile isolates are common colonizers of different natural substrates. Many wood-inhabiting basidiomycetes are sterile under laboratory conditions, although produce fruiting bodies in nature (Desjardin, 1990). Sterile fungi are also common inhabitants of root cortex in agricultural crops (Sivasithamparam, 1998), but are usually neglected due to their inability to produce spores under laboratory conditions, therefore making their identification by means of classical mycological techniques difficult, meaning that their taxonomic position cannot be clarified.

Plant pathogenic sterile white basidiomycete (SWB) strain 3034, which was investigated in this study, originated from Western Australia. It was obtained during the isolations of sterile fungi from plant roots. The sterile isolates obtained were screened in root colonization experiments aiming at determining their potential to affect plant growth. One isolate proved to be very pathogenic, and was investigated further.

A pathogenic SWB fungus has been described as a causal agent of stem rot of snap bean (*Phaseolus vulgaris*) in central Florida (Howard et al., 1977), root rot of on corn (*Zea mays*), lima bean (*Phaseolus lunatus*), rye (*Secale cereale*) and many other crops in Georgia (USA) (Sumner et al., 1979; Bell and Sumner, 1984), and in Puerto Rico, where a similar fungus was reported as the causal agent of crown canker of pigeon pea (*Cajanus cajan*) (Kaiser et al., 1987). This fungus has been reported to infect crops in 16 plant genera. The fungus was called 'Sterile White Basidiomycete' (SWB) and proposed to belong to the genus *Athelia* (a sexual state of *Sclerotium*) (Howard et al., 1977). It has also been described as a soil-borne pathogen of dry, edible beans (*Phaseolus vulgaris*) in Western Nebraska (Harveson, 2002). The isolates from that study were presumably identical to those already described in Florida and Puerto Rico. Also, this was the first report of occurrence of SWB pathogens in a region with a temperate climate.

Howard et al. (1997) compared SWB to *Sclerotium rolfsii* because of its morphological resemblance. However, the SWB failed to produce any types of sclerotia in culture and its host plant range was different from that of *S. rolfsii*. There were also distinct differences in the aminopeptidase profiles of these two fungi (Sumner et al., 1979). SWB strains were also compared to *Rhizoctonia solani* – the most common plant pathogenic sterile basidiomycete. Although SWB produced monilioid cells, as does *R. solani* prior to formation of sclerotia, it had clamp connections, which *Rhizoctonia* species usually do not have. All the isolates of SWB recorded from Florida, Georgia and Puerto Rico, were apparently morphologically indistinguishable and produced similar symptoms both on the test plants in the glasshouse and in the field (Kaiser et al., 1987). Further identification of the SWB strains was not possible due to the lack of sporulation. Attempts to induce spore production or formation of sclerotia in the strains were not successful for a long time (Howard et al., 1977; Sumner, 1992). However, in 1991 in the USA, Baird et al. (1992) induced the teleomorph of one of the isolates of SWB under the laboratory conditions. Surprisingly, the fruiting bodies formed were accommodated within two different species of *Marasmius*: *M. graminum* and *M. rotula*, although they originated from a single strain. The authors however did not exclude possible contamination of the flask by spores of one of these fungi (Baird et al., 1992). They also suggested that it was most likely that fungi designated as SWB represented a complex of different species of basidiomycetes, sharing similar morphological and pathological properties of the genus *Marasmius* or other yet unknown or unreported genera (Baird et al., 1992).

The symptoms of the disease caused by the American strains of SWB are similar in most crops: black to grey cankers on hypocotyls and roots usually covered with white superficial mycelium. The fungus has also been reported as causing pre- and post-emerging damping-off on many crops (Sumner et al., 1979).

The survival of SWB in soil for a year and its wide host range suggested that it has the potential to cause root diseases in many plants that are used in crop rotation in Georgia (Sumner et al., 1979). Later work of Bell and Sumner (1984) suggested that the fungus could cause only slight to moderate root disease severity on field crops in subsequent years, due to a rapid decline in inoculum density. However, the authors stated that severe crop losses occurred in fields with favourable microhabitat where susceptible crops had previously been planted.

During recent decades, molecular tools for studies of phylogenetic relationships among fungal taxa have been developed. Sequencing data sets obtained from different genes and spacers of rDNA array at present are serving as the most popular and reliable source for identifying fungal isolates at different taxonomic levels (Bruns et al., 1991, 1992; Moncalvo et al., 2002). Nuclear Small Subunit rRNA gene (nSSU-rDNA) has been widely used for resolving phylogenetic relationships in fungi at higher taxonomic groups such as classes or orders, while this gene also contains less conserved regions that can help in classifying

isolates at the lower ranks (Bruns et al., 1992). Internal transcribed spacers (ITS) 1 and 2, on the other hand, are usually used to separate fungal isolates of different species (Sreenivasaprasad et al., 1996; Zhang et al., 1998). The first 1 Kbp of the nuclear Large Subunit (25–28S) rRNA gene (nLSU-rDNA) is widely used in phylogenetic studies within Basidiomycetes, mostly on the generic level (Moncalvo et al., 2002). Therefore using nucleotide sequences of different regions of the rDNA array may help to resolve the taxonomic position of morphologically unidentifiable isolates. Phylogenetic studies within the class of Basidiomycetes have been extensively studied during the recent years, and a considerable amount of sequencing data from several regions of rDNA (and especially the nLSU) has accumulated in GenBank.

The morphology and symptoms caused by the Australian isolate of SWB showed similarities with the descriptions of the American strains of SWB. Therefore, the aim of our study was to compare the isolates from the two countries and to investigate plant pathogenic properties of the Australian fungus. In this work, the taxonomic relatedness between the American and Australian isolates of the plant pathogenic SWB was studied by comparing their nucleotide sequences obtained from several regions of the rDNA array. The nucleotide sequences were also compared with those of the homologous regions from other taxa belonging mainly to Agaricales.

Materials and methods

Isolations

The Australian strain of SWB (isolate No 3034) was isolated in April 2000 from healthy looking roots of buffalo grass (*Stenotaphrum secundatum*), growing on cleared bush land soil in the field station of the University of Western Australia at Shenton Park, Western Australia. Roots were washed under running tap water, sterilized in 1.25% NaOCl for 1 min, rinsed in three changes of sterile distilled water, blot-dried, cut into approximately 0.25 cm long pieces and plated on 1.5% water agar amended with 85% lactic acid (1 ml added to 1 l of medium). Pure cultures were recovered and plated on Potato Dextrose Agar (PDA) (DIFCO). A reference strain of SWB (ATCC 28344) causing the stem rot of bean in Florida and deposited by Howard (Howard et al., 1977), as well the type strain of *Tetrapyrgos* (*Campanella*) *subdendrophora* (ATCC 42449), were purchased from the American Type Culture Collection (ATCC). The original strain of American SWB (ATCC 28344), isolated from bean seedling roots in Florida (Howard et al., 1977), had been kept in the ATCC under the name *Rhizoctonia* sp., designated as ''Bean-X fungus''. The fungal strains used in this study were maintained at room temperature (approx. 23 °C) on half-strength PDA slants.

Pathogenicity tests

Pathogenicity tests were performed under the greenhouse conditions with cultures of ATCC 28344 and 3034. Wheat seeds were sown in soil infested with either of these two fungi as described below. Isolates freshly recovered from the diseased wheat roots were used in all further pathogenicity tests.

To produce the inoculum, 50 g lots of rye-grass seeds were soaked in distilled water in 500 ml Pyrex® bottles, autoclaved at 121 °C for 20 min and inoculated with 5 mycelial plugs 5 mm in diameter of either of the fungi, obtained from the growing margins of 5 d old cultures grown on PDA. The flasks were incubated for 10 d at 24 °C in darkness and shaken every other day to ensure even colonization by the mycelium.

The potting mixture consisted of 50/50 (w/w) mixture of commercial garden soil (containing (v/v): 60% light peat, 25% black peat, 15% sand 0.5–4 mm; - for 1 m^3: 1.3 kg, NPK 14-7-15, 0.050 kg FTE 36, 6.0 kg limestone flour and 2.0 kg dolomite flour; Hasselfors Garden AB, Sweden) and coarse sand. To provide a low-competitive environment for the test fungi, the described substrate was steam-treated at 100 °C for 18 h, and cooled to 20 °C before the inoculum was added. Non-surface sterilized seeds were pre-germinated overnight on moist filter paper in Petri dishes (11 cm in diameter) and sown into the 8 × 8 cm VEFI pots (Larvik, Norway) filled the substrate that was previously amended with 0.5% (w/v) of inoculum. Twelve plant species belonging to five families were selected for the test (Table 1). All test plant species were inoculated with either

Table 1. Percent of emergence and disease severity indices for two isolates of SWBs on twelve crop species of plants

Plant species	Soil treatment	Emergence (%)	DSI*
Poaceae			
Avena sativa	ATCC 28344	66.6	1.0
	3034	87.5	3.0**
Triticum aestivum cv. Curry	ATCC 28344	90	1.5
	3034	56.7	2.25
Zea mays var. *saccharata* cv. Seneca Horizon	ATCC 28344	60	4.0
	3034	80	4.0
Sorghum nigrum cv. Durra	ATCC 28344	8.3	5.0
	3034	50	1.5
Hordeum vulgare cv. Sundelius	ATCC 28344	37.5	1.33
	3034	66.7	2.25
Lolium perenne cv. Herman	ATCC 28344	79.2	0.75
	3034	95.8	2.50**
Fabaceae			
Phaseolus vulgaris cv. Nerina	ATCC 28344	0.0	–
	3034	75	4.0
Pisum sativum cv. Sw. Capella	ATCC 28344	80	2.75
	3034	65	2.75
Vicia faba cv. Aurora	ATCC 28344	95	2.75
	3034	90	4.25**
Cucurbitaceae			
Cucurbita pepo cv. Batani	ATCC 28344	60	1.75
	3034	100	0.75
Brassicaceae			
Brassica rapa cv. Maskot	ATCC 28344	95	0
	3034	100	1.75**
Solanaceae			
Lycopersicon esculentum cv. Danish Export	ATCC 28344	100	0
	3034	100	1.2**

* Disease severity index (DSI) scale: 0 indicated no infection, 1 = approximately 10% of the roots lesioned, no changes evident in shoots, 2 = 25% roots with lesions, leaves exhibiting light chlorosis, 3 = 50% roots had lesions, plants wilting with extensive chlorosis, 4 = 75% of roots had lesions, plants wilted or stunted, 5 = plants dead.
** Indicates significantly different DSI values, according to the Duncan's Multiple Range Test, $P = 0.05$.

the Australian or American SWB strains. Plants, sown in pots containing no inoculum served as the control. The pot trial was set-up following a complete randomized block design. Greenhouse conditions were set at a regime of 12 h:12 h (day:night), 25 ± 3 °C and 85% relative humidity. Symptoms were scored after 4 weeks. Roots were carefully washed under running tap water; ratings were done immediately after washing. Symptoms were rated according to the scale ranging from 0 to 5, where 0 indicated no infection, 1 = approximately 10% of the roots lesioned, no changes evident in shoots, = 25% roots with lesions, leaves exhibiting light chlorosis, 3 = 50% roots had lesions, plants wilting with extensive chlorosis, 4 = 75% of roots had lesions, plants wilted or stunted, 5 = plants dead. To satisfy Koch's postulates, pure cultures of the SWBs were recovered from the diseased root tissue, using the same method as that used during the initial isolation. The experiment was carried out twice.

Determination of the dose response

Snap beans (*Phaseolus vulgaris*) and maize (*Zea mays*) were the test plants used in this test, since they were the most susceptible to both isolates in the pathogenicity test where 0.5% of inoculum was used. For each of the test fungi, the potting mixture was amended with 0.1, 0.25, 0.5, 0.75 or 1% (w/v) of inoculum. No inoculum was added in the soil where control plants were sown. Five plants were raised per pot and five replicates were done for each plant species and for each fungus. The experiment was arranged according to a complete

randomized block design. Light, temperature and moisture conditions were the same as in the previous experiment. Results were recorded after 4 weeks.

Morphological examination

The fungal strains were studied under the light microscope after 7 days incubation on PDA plates at 22 ± 2 °C. Fluorescent staining of the dolipore septa and nuclei was performed using the Hoechst Dye 33258 (Sigma – Fluka - Aldrisch) as described by Yang et al. (1991). In an attempt to induce sporulation, both isolates were sub-cultured on PDA (DIFCO), Oatmeal Agar (OXOID), Potato-Carrot Agar (Smith and Onions 1994), Water Agar (OXOID) and Minimal Medium (Cove, 1966), and also onto PDA plates containing either dried lupine stems sterilised by propylene dioxide, and autoclaved birch toothpicks. Fungi were grown at constant illumination under the near-UV lamps (Phillips, TL20W/08) at 22 ± 2 °C for 2 months with regular checks for presence of sporulation. For measuring the hyphal diameter 3 d old cultures on PDA were used, 25 measurements of young hyphae from colony advancing margin were taken per isolate.

Temperature response

Fungal plugs, 5 mm in diameter, were cut from margins of 5 day-old cultures and placed in the middle of Petri dishes (9 cm in diam) of PDA with three replicates per isolate. Cultures were incubated in the dark, at 4, 10, 15, 20, 25, 30, 35 or 40 °C. Radii of the colonies were measured after 5 days and four readings per plate were taken.

Statistical analysis

The data obtained were subjected to analysis of variance (ANOVA) using the General Linear Model (GLM) procedure of the SAS computer software (SAS Institute Inc. Cary, NC). Mean relative DSI-values were compared using Duncan's multiple range test at $P \leq 0.05$ level.

Examination of the diseased root tissue

Roots of bean and maize showing typical lesions in the pathogenicity test were used for this purpose. In order to follow the invasion behaviour of the SWBs in the penetrated plant tissues, root segments 3 cm in length were thoroughly washed under a running tap and fixed in formaldehyde – glacial acetic acid – ethanol (FAA) solution as described by Kormanic et al. (1980). The fixed segments were cross-sectioned under the binocular, using a sharp razor blade. Obtained cross-sections were then stained in Trypan blue – lactophenol solution, examined under the light microscope and presence of the fungal hyphae in different root tissues was recorded.

Molecular characterization

To obtain fungal DNA, 1 cm^2 agar-free pieces of mycelium were removed from the PDA plates of 5 days old cultures and the extraction performed according to the method of Cenis (1992). PCR reactions were performed using PE AmpliTaq Gold PCR kit (PE Applied Biosystems), following the manufacturer's protocol. Amplification of the ITS region was achieved using ITS 1–F/ITS 4 primer pair (Gardes and Bruns, 1993; White et al., 1990, respectively). First approximately 1 Kbp of the nLSU rRNA gene were amplified in two steps using two sets of forward and reverse primer pairs Ctb-6/Tw13 and Ctw13/Tw14 (for details see http://plantbio.berkeley.edu/~bruns/picts/results/28s.jpg and http://plantbio.berkeley.edu/~bruns/primers.html). Amplification of the whole nSSU rRNA gene was achieved using NS1/NS2, NS3/NS4, NS5/NS6 and NS7/NS8 primer pairs in separate reactions (White et al., 1990). Amplifications were carried out in PE PCR thermocycler, programmed for 1 cycle of 95 °C for 3 min, and 35 cycles consisting of denaturation at 95 °C for 75 s, annealing at 50 °C for 75 s and extension at 72 °C for 2 min followed by a 10 min extension step at 72 °C. Amplified fragments were separated by electrophoresis in 1.5% LE agarose (Promega, USA) gel, stained in Tris-Borate-EDTA buffer containing 0.5 μl/ml of ethidium bromide, and visualized under UV transilluminator. The sizes of the fragments were estimated using 100 bp DNA ladder (Promega, USA). The PCR products were then purified using QiaGen PCR Purification kit (QiaGen Ltd., Crawley, UK). Direct sequencing reactions of the amplified ITS region were performed in both directions using ITS 1-F (Gardes and Bruns, 1993), ITS 2, ITS 3 and ITS 4 primers (White et al., 1990) to achieve full sequencing of

the ITS fragment. In case of the amplified portions of the LSU and the SSU rRNA genes, the same sets of primers as for PCR amplification were used. The reactions were performed using the PE Big Dye Terminator Cycle Sequencing kit 2.0v (PE Applied Biosystems, AB) according to the manufacturer's instructions. The sequencing gels were run at the Centre for Genomic Research at the Karolinska Institute in Stockholm, Sweden.

Sequences were manually proofread in the DNAStar computer software package (Lasergene, Madison, US). The ClustalW program available in the same software package was used for creating multiple sequence alignments and estimation of the percent of divergence among the sequences obtained in the current study. The BLAST search was performed through NCBI home page at http://www.ncbi.nlm.nih.gov/.

Phylogenetic analysis of the obtained sequence data was performed using PAUP (Phylogenetic Analysis Using Parsimony) 4.0.b software (Swoford, 1999), using both parsimony and neighbour-joining (NJ) methods. Data sets of the studied regions were analysed separately.

The nLSU sequences obtained from the strains used in this study (ATCC 28344, ATCC 42449 and 3034), as well as those of several species of *Marasmius* and *Campanella* and *Tetrapyrgos* available from GenBank (Table 2) were also incorporated into the existing PAUP file originally containing sequences of 154 basidiomycetous taxa and available at http://www.biology.duke.edu/fungi/mycolab/default.htm (Moncalvo et al., 2000), resulting in 168 taxa in total. The file was executed with character deletion, gap treatment and outgroup selection as advised in the original publication of Moncalvo et al. (2000). In case of parsimony analysis, heuristic search was performed with 1000 random sequence addition with TBR branch swapping algorithm. In the case of NJ, the distances were calculated using Kimura 2-parameter. The clade composition of the NJ and the most parsimonious trees obtained in this study were compared with the ones of the above-mentioned publication. The support of the obtained clades in the most parsimonious tress was estimated by the bootstrap analysis with 100 sequence random addition replicates, 100 bootstrap replicates and TBR branch swapping.

For phylogenetic analysis of nSSU, additional sequences of homologous region of other basidiomycetes were downloaded from the GenBank (Table 2) and analysed as described above. Sequences of *Ganoderma australe* were chosen as the outgroup (Moncalvo et al., 2000). Robustness of internal branches was estimated by parsimony bootstrap analysis using the PAUP 4.0.b software with 1000 replications in a heuristic search with random stepwise addition with 10 replicates.

Due to a low number of complete ITS sequences of Agaricales available in the GenBank, no separate phylogenetic analysis of this region was performed. The ITS sequences obtained from 3034, ATCC 28344 and ATCC 42449 were compared with each other and the distances were calculated using MEGALIGN program within DNAStar software package.

Results

Pathogenicity test

Results of the pathogenicity tests of isolate 3034 and ATCC 28344 are shown in Table 1. No disease symptoms appeared in any of control plants. Koch's postulates were satisfied for all the plants that developed typical disease symptoms. The two fungi caused different levels of root and crown rot of inoculated plants. The early symptoms of disease appeared as chlorosis and/or necrosis of the apices of the leaves, followed by extensive necrosis of leaves and wilt of entire plants. Affected plants were usually stunted with the crown turning black. Roots were rotten or covered with black or dark-brown lesions. Sometimes, white superficial mycelia were found to cover entire roots and occasionally crowns and even portions of stems above the soil level. The development of the symptoms differed slightly between the American and Australian SWB strains. The American strain tended to be more restricted to the crown and hypocotyl area, as well as the seed, whereas the Australian isolate was damaging the entire root system (Figure 1). In case of the legumes, strain 3034 caused "root pinching" (Figure 2), a phenomenon observed in cases when epidermal and cortical tissues of the roots are macerated, with the stele still intact. These symptoms are similar to those produced by

Table 2. Sequences of the fungal isolates used in this study

Fungal species/strain	Sequenced region	GenBank accession number	Reference
3034	ITS	AY445119	This work
	SSU	AY445116	This work
	LSU	AY445113	This work
	ITS	AY445120	This work
ATCC 28344	SSU	AY445117	This work
	LSU	AY445114	This work
Tetrapyrgos (Campanella subdendrophora)	ITS	AY445121	This work
	SSU	AY445118	This work
ATCC 42449	LSU	AY445115	This work
Campanella junghuhnii	LSU	AJ406561	Langer (Unpublished)
Campanella sp.	LSU	AF261339	Moncalvo et al. (2002)
Campanella sp.	LSU	AF261340	Moncalvo et al. (2002)
Marasmius androsaceus	ITS	AF519893	Klonowska et al. (2003)
M. androsaceus	LSU	AF519891	Klonowska et al. (2003)
M. alliaceus	LSU	AJ506587	Langer (Unpublished)
M. cladophyllus	ITS	AY216475	Figueira et al. (Unpublished)
M. fulvoferrugineus	LSU	AF261584	Moncalvo et al. (2002)
M. graminum	LSU	AF291345	Weiss and Oberwinkler (2001)
M. quercophilus	LSU	AF519889	Klonowska et al. (2003)
M. rotula	LSU	AF261345	Moncalvo et al. (2002)
M. scorodonius	LSU	AF261332	Moncalvo et al. (2002)
M. scorodonius	LSU	AF261331	Moncalvo et al. (2002)
Tetrapyrgos nigripes	LSU	AF261337	Moncalvo et al. (2002)
Tetrapyrgos sp.	LSU	AF261338	Moncalvo et al. (2002)
Agaricus bisporus	SSU	L36658	Hinkle et al. (1994)
Athelia bombacina	SSU	M55638	Illingworth et al. (Unpublished)
Calvatia gigantea	SSU	AF026622	Hibbett et al. (1997)
Crucibulum laeve	SSU	AF026624	Hibbett et al. (1997)
Cyanthus striatus	SSU	AF026617	Hibbett et al. (1997)
Ganoderma australe	SSU	AF026629	Hibbett et al. (1997)
Entoloma strictius	SSU	AF287832	Hibbett, Gillbert and Donogue (2000)
Heliocybe sulcata	SSU	AF334915	Hibbett and Donoghue (2001)
Laccaria pumila	SSU	AF287838	Hibbett, Gillbert and Donogue (2000)
Lentinula lateritia	SSU	AF026596	Hibbett et al. (1997)
Leucopaxillus albissimus	SSU	AF287839	Hibbett, Gillbert and Donogue (2000)
Lepiota procera	SSU	L36659	Hinkle et al. (1994)
Limnoperdon incarnatum	SSU	AF426952	Hibbett and Binder (2001)
Ossicaulis lignatilis	SSU	AF334923	Hibbett and Donoghue (2001)
Panellus serotinus	SSU	AF026590	Hibbett et al. (1997)
Peniophora nuda	SSU	AF026586	Hibbett et al. (1997)
Pleurotus tuberregium	SSU	AF026595	Hibbett et al. (1997)
Pluteus petasatus	SSU	AF026634	Hibbett et al. (1997)
Termitomyces sp.	SSU	AB051894	Katoh et al. (Unpublished)

pathogenic *Rhizoctonia solani*. Overall, maize was the most susceptible host to both strains and the highest disease severity was observed on broad beans inoculated with the Australian isolate. Maize roots were heavily damaged by both pathogens and no healthy roots could be found in the inoculated pots. Seedlings of snap bean and sorghum were severely affected by pre-emergence damping-off by ATCC 28344 (Table 1), and the seeds in inoculated pots were totally covered by the mycelium of the fungus. Squash appeared to be the least affected by the two SWBs. Tomato and oil-seed rape showed less extensive root lesions in comparison to all other plant species screened. In our experiments, the ATCC isolate failed to cause any symptoms on tomato and oil-seed rape (Table 1).

No fruiting bodies or sclerotia were observed on diseased plant parts or in the soil around them. During the microscopic examination of the

Figure 1. Symptoms on pea roots caused by SWB (arrows pointing disease symptoms). (a) Isolate 3034; (b) Isolate ATCC 28344.

Figure 2. Shredding of cortex (arrow) leading to root-pinching caused by the isolate 3034 on bean root.

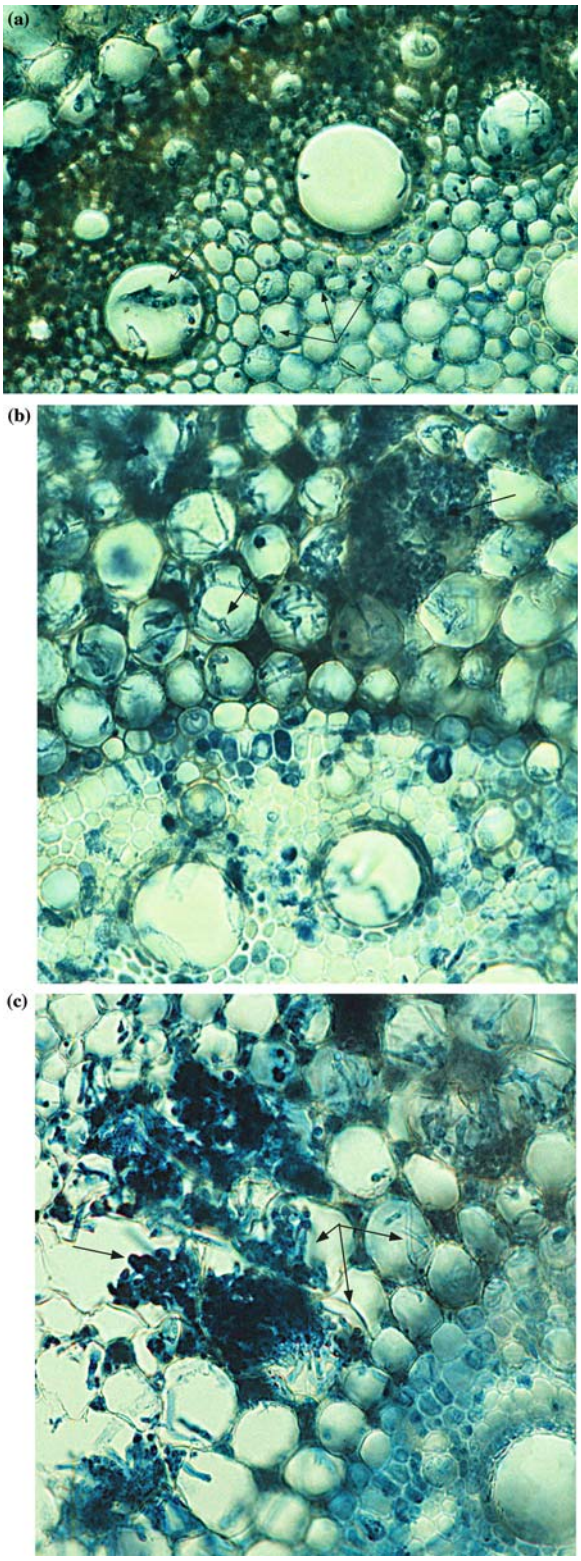

Figure 5. SWB inside maize roots (arrows pointing fungal hyphae): (a) 3034 inside stele; (b) 3034 inside the cortex; (c) ATCC 28344 inside the cortex.

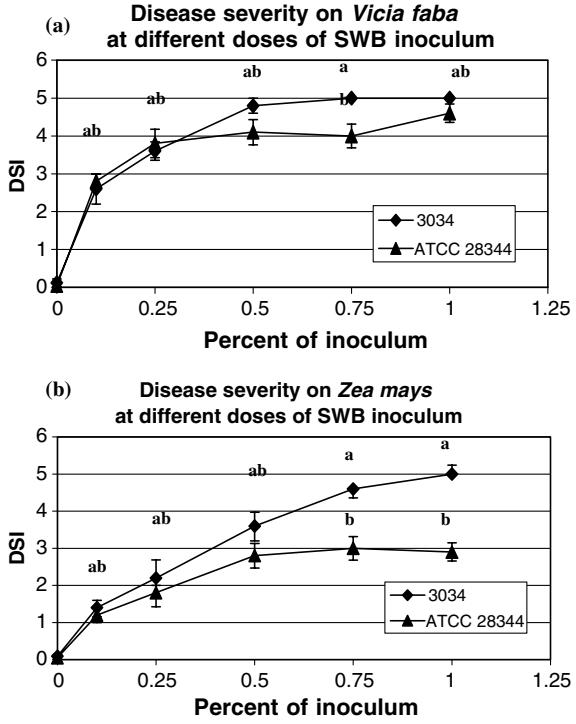

Figure 3. Disease severity of SWB 3034 and ATCC 28344 on plants inoculated with different doses of inoculum: (a) On broad bean; (b) on maize. Values labelled with the same letters are not significantly different ($P < 0.05$) according to Duncan's multiple range test.

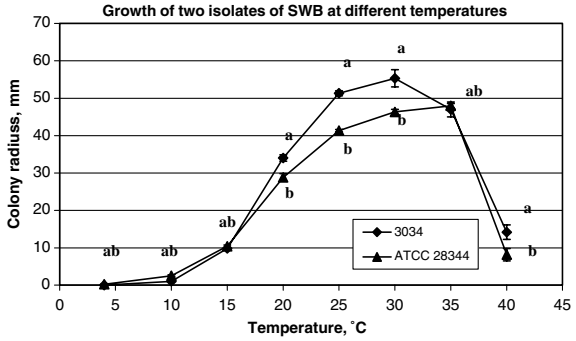

Figure 4. Growth of two isolates of SWB at different temperatures. Values labelled with the same letters are not significantly different ($P < 0.05$) according to Duncan's multiple range test.

diseased root tissue, no sporulation was observed on the plant parts colonized by both the SWBs.

Dose response

Initial symptoms on plants that were infected by No 3034 were evident 5 days after sowing (2 days after emergence). At higher concentrations of inoculum (0.75 and 1%) most of the maize and broad bean seedlings were dead 7 d after sowing. Symptoms caused by ATCC 28344 were first evident on maize (d 7), and then on beans (d 10). Both strains were pathogenic to both crops even at the lowest inoculum level tested. Broad bean appeared to be more susceptible than maize to both pathogens. Data comparisons and the level of statistical significance are shown in Figure 3.

Morphological examination

The appearances of white colonies of the American and Australian strains were noticeably different. The American isolate had denser mycelium mostly immersed in the agar, whereas the Australian strain produced much looser mycelium that was mainly aerial. Both had dolipore septa, binucleate hyphae, clamp connections, produced mycelial strands and monilioid cells. Young cultures of ATCC 28344 had thicker hyphae (4.1 ± 0.4 μm) than 3034 (3.3 ± 0.4 μm).

Temperature response

In general, the American strain grew faster at lower temperatures, while the Australian isolate grew better at temperatures higher than 20 °C (Figure 4). Mycelia of both fungi were denser within the range of 25–35 °C. Neither of the fungi grew at temperatures below 10 °C, but were viable at 40 °C.

Examination of the diseased root tissue

Hyphae of both isolates on roots of both plant species examined were easily detected by the fixing and staining method used. In both plants, hyphae of ATCC 28344 were present mostly in the cortical tissue of the roots, and never penetrated the stele. On the other hand, hyphae of isolate 3034 were present not only in the cortex, but also within the tissues of the stele, including vascular system (Figure 5). Hyphae of the American SWB isolate were present even in the unaffected tissues around the lesion, whereas hyphae of the Australian isolate extended up to the border of the dead and healthy tissues. The Australian isolate destroyed the cortex of the diseased roots with a thick layer of fungal hyphae covering the stele.

PCR-amplification and sequence analysis

Amplification of all fragments resulted in single bands; no significant size variation was detected among the studied isolates for the nLSU and nSSU sequences, however the ITS sequences of 3034 were 80 bp longer than the ones of ATCC 28344 and *T. subdendrophora* reference strain. The nSSU sequences accounted for 1.8 kbp out of the total of 3.55 kbp sequenced, ITS fragments comprised about 750 bp, and the final 1 kbp belonged to the nLSU sequences. For phylogenetic analysis, 1.7 kbp of the nSSU, 650 bp of the ITS region and 940 bp of nLSU were used, omitting the dubious sequences at the ends of the regions. Sequence similarity matrices are shown in the Table 3.

BLAST search against sequences contained in GenBank revealed a high similarity between ATCC 28344 isolate and *Marasmius graminum* based on nLSU-rRNA gene. Sequences of nSSU gene or the ITS region for this species are not yet available in the database, but according to the BLAST, the highest hits to those sequences were given by other species of *Marasmius*. The Australian 3034 isolate had the highest similarity of nLSU sequences to *T. subdendrophora* and *Campanella* sp., however data of the sequences of other rDNA regions for any of *Tetrapyrgos* or *Campanella* species did not exist in the GenBank. Therefore, the type isolate of *T. subdendrophora* was obtained from the ATCC and sequenced for the homologous regions. Based on sequencing data from the ITS region, it was concluded that ATCC 28344, *T. subdendrophora* and the isolate 3034 were clearly different. However, at present very few sequences are present in GenBank for the regions of Basidiomycetes other than nLSU that could be used for comparison in this study. At the time of the analysis, there were no *Marasmius* sequences available for the whole ITS fragment including ITS1, 5.8S and ITS2, either for nSSU.

Table 3. Percent sequence similarity among the analysed regions for the three isolates used in this study

Region	Isolate	3034	ATCC 28344
ITS region	3034	–	–
	ATCC 28344	77.1	–
	C. subdendrophora	86.5	76.2
ITS1	3034	–	–
	ATCC 28344	73.1	–
	C. subdendrophora	79.5	68.8
5.8S	3034	–	–
	ATCC 28344	98.1	–
	C. subdendrophora	97.5	99.5
ITS2	3034	–	–
	ATCC 28344	56.4	–
	C. subdendrophora	64.1	72.3
SSU	3034	–	–
	ATCC 28344	98.8	–
	C. subdendrophora	99.2	98.1
LSU	3034	–	–
	ATCC 28344	94.1	–
	C. subdendrophora	96.2	93.8

Similar to the results of Moncalvo et al. (2000), most of the variation within the nLSU sequences was evident in the regions corresponding to domains D1 (positions 126–138) and D2 (475–492 and 635–686). Sequences of the ITS regions of all three isolates when compared together harboured the highest amount of variable sites, as compared to other regions, and the level of similarity among the three sequenced isolates was relatively low, partially due to the length differences in the ITS2 region (Table 3). Analysis of the 5.8S gene has revealed that the isolates had nearly identical sequences. However four base pairs located closely to the 3′ end of the gene were in disagreement: all isolates differed at positions 121–122; *T. subdendrophora* and ATCC 28344 were different from 3034 isolate at positions 135–136). Analysis of 5.8S sequences of 18 other taxa of Basidiomycetes confirmed that these two sites are among the most variable within this overall conserved gene and vary among representatives of different genera (data not shown).

Phylogenetic analysis

The tree based on the nLSU-rDNA sequences gave the most reliable result due to the highest number of sequenced taxa used for comparison. The topology of the obtained consensus parsimony tree and NJ tree was similar to the trees published by Moncalvo et al. (2000) (Figure 6). Several phylogenetic groups described in Moncalvo et al. (i.e. G, R, Y and F) were not supported on our trees due to the simplified analysis method used, but the rest of 28 groups were still present in the trees generated in this study. The overall topology of the phylogenetic group "C", which contained our sequenced isolates, was unaffected by addition of new *Marasmius*, *Tetrapyrgos* and *Campanella* sequences to the original data file or

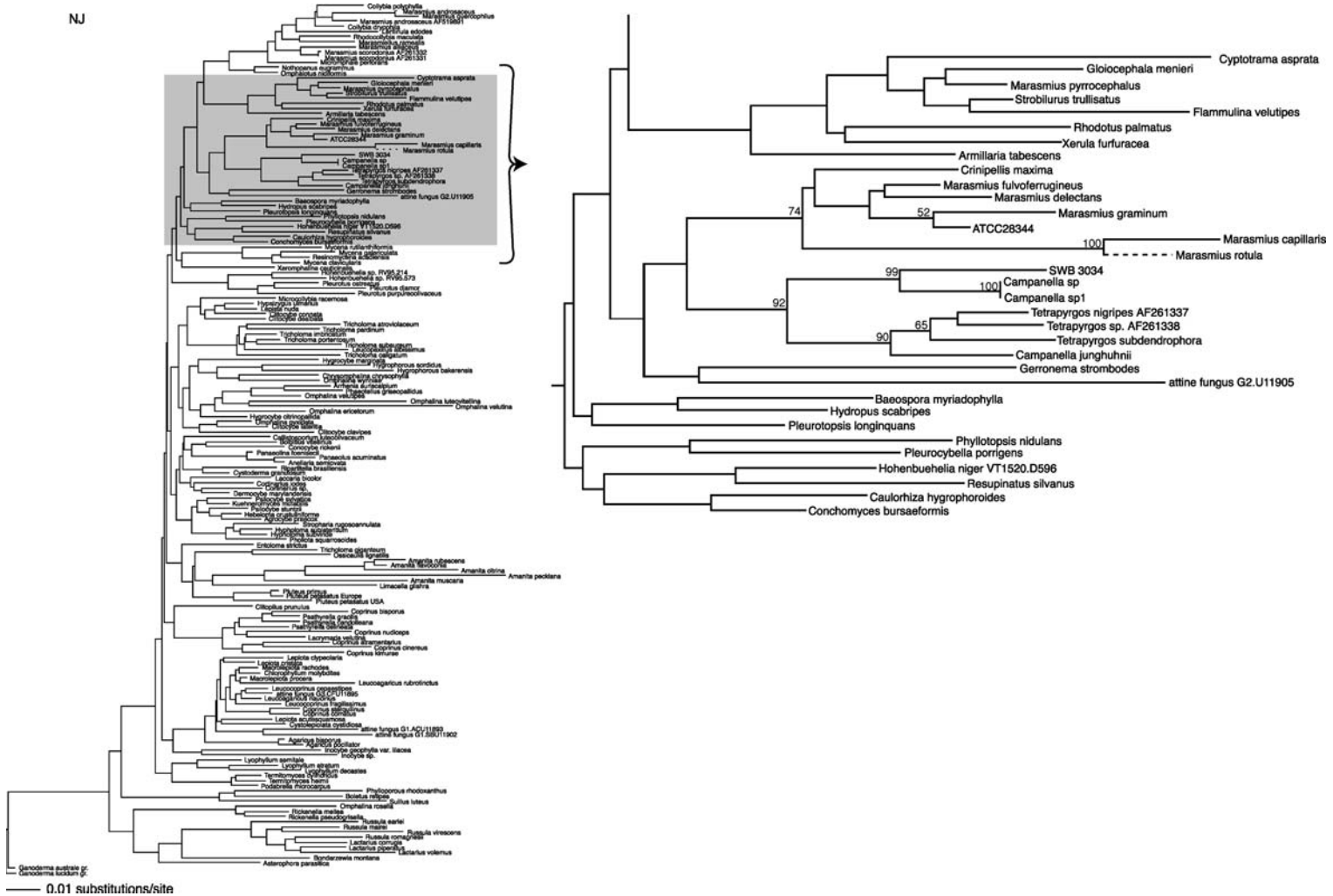

Figure 6. Phylogenetic tree of the LSU region, 164 taxa of Basidiomycetes used for the analysis. Numbers on the branches correspond to bootstrap values higher than 50%.

by the choice of the method of analysis. As expected from the results obtained during the sequence similarity comparison, ATCC 28344 and 3034 grouped in different clades. The isolate 3034 fell into the cluster containing several species of *Tetrapyrgos* and *Campanella* with 91% bootstrap support (Figure 6), grouping together with two isolates identified as *Campanella* sp., which was supported by 100% bootstrap value and was a sister clade to 90% supported clade containing *T. subdendrophora* and *C. junghuhnii*. The ATCC 28344 isolate grouped with one of the clades of the genus *Marasmius*, which was placed closely to *M. graminum*, but not to *M. rotula* (Figure 6), but this clade, however, was not supported by the bootstrap analysis (52%). Similar to the study of Moncalvo et al., the genus *Marasmius* was also polyphyletic in our trees, and ATCC 28344 was into the monophyletic clade of species of *Marasmius* possessing broom cells, within phylogenetic group "C" (Moncalvo et al., 2000).

The SSU tree was constructed using only 21 taxa. Two equally parsimonious trees were obtained. Similar to the LSU tree, the American (ATCC 28344) and Australian (3034) SWB isolates did not group together (trees not shown). 3034 isolate formed a common clade with *T. subdendrophora* ATCC 42449 with 100% bootstrap support. Since no *Marasmius* sequences of SSU were available in the GenBank at the moment when the search was performed, ATCC 28344 isolate grouped closely with *Lentinula lateritia*, however this clade did not receive support by the bootstrap analysis (tree not shown).

Discussion

To our knowledge, this is the first report of a pathogenic fungus belonging to the SWB complex isolated from grass in the Australian continent. The data obtained in our experiments suggest that the fungus is a serious potential pathogen, the pathogenicity of which is not restricted just to a specific host. The fact that the Australian strain of a sterile pathogenic basidiomycete was isolated from apparently healthy roots of buffalo grass might not necessarily mean that the fungus is not pathogenic on this particular species. The plant roots could harbour the fungus in its latent phase. It is possible that the fungus resident in the bushland soil has the potential to cause serious damage to turf or crop species brought into cultivation at the site.

The results obtained with the American isolate ATCC 28344 in greenhouse pathogenicity tests were slightly different from those of Kaiser et al. (1987). In our experiments, the fungus caused pre-emergence damping-off on *Phaseolus* beans and sorghum, whereas data obtained by Kaiser and his co-workers showed that this isolate showed a low level of pathogenicity on these crops, and the germination rates of inoculated seeds were higher. In our trials, squash planted in soil infested with ATCC 28344 showed lower emergence than described before, but tomato plants were not affected by this strain. This may be explained by the fact that we used different cultivars of these plants. It has been shown previously that different cultivars of snap bean responded differently to SWB infection (Howard et al., 1977). In a disease screening with an SWB from pigeon pea, incidence of infected plants was found to be lowest with tomato (Kaiser et al., 1987). In their screen, Kaiser et al. (1987) used a different strain of SWB originating from pigeon pea in Puerto Rico (SWB-PR) that caused similar symptoms on the pigeon pea and caused the same level of disease severity as the ATCC 28344 isolate from Florida that was also used in our study. Anastomosis was observed between hyphae of these two isolates, and they there indistinguishable morphologically, which would suggest that they belonged to the same species (Kaiser et al., 1987). The slight differences between performances of the American strain used in our study and in the study of Kaiser et al. may be explained by a certain degree of variation between the two isolates of SWB. In our studies, we did not observe any anastomosis between hyphae of 3034 and ATCC 28344 (data not shown).

Previous work has shown that the isolates of SWB obtained in various studies in America were presumed to belong to the same species (Howard et al., 1977; Sumner et al., 1979; Kaiser et al., 1987). However, the discovery of two different kinds of sporulation in one of the isolates of SWB in the United States (resembling those of *Marasmius graminum* and *M. rotula*) suggested that SWB might be a complex of mycelia of different, possibly yet unknown taxa of basidiomycetes (Baird et al., 1992). Our experimental data including the sequence analysis of the rDNA array

have clearly indicated that there are considerable differences between the Australian and American strains of the SWB, therefore supporting the above-mentioned assumption of Baird and his colleagues.

First of all, the isolates have distinctly different morphology. Besides, the Australian strain has a wider host range, than the American strain tested. It is also more pathogenic, even at lower doses of inoculum. Differences in morphological features as well as in the inoculum potential and pathogenicity profiles suggest that we are dealing with two different fungi. However, differences in aggressiveness and pathogenicity profiles between the Australian and American strains may be due to the fact that the isolate ATCC 28344 was stored frozen for many years and could probably have deteriorated in culture, whereas 3034 is a relatively fresh isolate. We tried to minimize such possibility by setting up the pathogenicity tests using the mycelia that were freshly re-isolated from experimentally infected plant roots.

Both fungi showed different infection strategies on the affected plant roots. The fact that the Australian isolate was present only in the diseased root tissues and not far from the margin with the healthy tissues of plant (Figure 5), suggests that isolate 3034 may possess stronger enzymatic activity than the American one. Microscopic examination of the lesions revealed that the root-pinching symptoms that occur in the case of 3034 are due to the shredding of the cortical tissues caused by the macerating enzymes produced by the fungus in the invaded area. Further investigations of the enzymatic activity possessed by these two different isolates of SWB are currently being carried out. The more severe and rapid wilting symptoms caused by the Australian SWB could probably be explained by the fact that the fungus was able to penetrate the stele, whereas the American strain was restricted to the root cortex.

The sequence analysis of the rDNA array performed on the American and Australian SWB isolates confirmed the results inferred from the morphological and pathogenicity data that these two fungi represent different genera of Basidiomycetes. Phylogenetic analysis of the nLSU gave the most significant results. As shown on the phylogenetic tree of this region (Figure 6), the isolate ATCC 28344 does belongs to the genus *Marasmius*, as suggested by Baird et al. (1992). Many species of *Marasmius* are plant pathogens (Baird et al., 1992). *Marasmius graminum*, which was the closest match to ATCC 28344 according to the nLSU sequences, is known to cause diseases of wheat and other grain crops (Singer, 1975). Several other forms of this genus, such as *M. inoderma*, *M. graminis* var. *brevispora* and *M. sacchari* var. *hawaiiensis* are pathogens of maize (Sabet et al., 1968; Pont, 1973).

According to the BLAST search and subsequent phylogenetic analysis, the nucleotide sequence of the LSU region of ATCC 28344 had the highest similarity to *M. graminum*, but not to *M. rotula* (Figure 6). However, the clade containing ATCC 28344 and *M. graminum* received very low bootstrap support (52%), therefore it cannot be completely trusted. Sequences of SSU and ITS regions were not available in the GenBank for any of these two species of *Marasmius*.

The genus *Marasmius* appeared to be polyphyletic according to the nLSU sequence analysis (Moncalvo et al., 2000). As it can be seen from the Figure 6, isolates of *Marasmius* fall into three different phylogenetic groups. This is a part of the problem of generic concept of *Marasmius* that has been a matter of discussions in studies based on morphological analysis (Gilliam, 1976). Relationships between different species of *Marasmius*, as well as several others closely related genera, remain to be resolved. Further sequencing of the type strains of several species of *Marasmius* should be performed to clarify the phylogenetic position of ATCC 28344.

On all reconstructed phylogenetic trees inferred from the nLSU, strain 3034 grouped closer to *Tetrapyrgos* and *Campanella* spp., than to *Marasmius*. However, analysis of the 5.8S gene suggested that strain 3034 might belong to a different genus than *Tetrapyrgos* or *Campanella*. Strain 3034 differed from *T. subdendrophora* by four nucleotides. Similarly, the ITS2 region of 3034 was longer, than it is in *T. subdendrophora*. The phylogenetic identity of the 3034 strain thus remains unresolved, but according to the phylogenetic analysis of nSSU and nLSU regions, it is closely related to *Tetrapyrgos* or *Campanella*.

In their paper in 1992, Baird et al. stated that: "The SWB fungi may apparently consist of a complex of basidiomycetes with similar cultural characters. These basidiomycetes may all belong only to the genus *Marasmius* or may consist of

several as of yet unreported genera.'' Our study is a good confirmation of this statement. In this paper we prove identification of ATCC 28344 as *Marasmius* sp., (possibly as *M. graminum*) and demonstrate close relatedness of the Australian plant pathogenic SWB isolate to the genera *Tetrapyrgos* and *Campanella*.

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