A near-fatal case consistent with mushroom poisoning due to *Amanita* species

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Abstract

Although some *Amanita* species are consumed in Australia without apparent ill effect, the "death cap" mushroom, *Amanita phalloides* (Vaill.:Fr.) Link, which occurs mostly in association with broad-leaved trees such as Oak, has been responsible for several fatalities or severe mushroom poisonings in the Australian Capital Territory and Victoria. A recent near-fatal mushroom poisoning in the Sydney region suggests other Australian *Amanita* species may be involved. This recent case, occurring in a south-east Asian community on the outskirts of Sydney, may have resulted from ingestion of a native Australian species superficially similar to the paddy-straw mushroom, *Volvariella volvacea* (Bull.:Fr.) Singer which shares many features with *Amanita* species in the button stage. Although no material other than *Amanita ochrophylla* (Cooke & Massee) Cleland was recovered from the remains of a meal consumed by the patient, a new species *Amanita volvarielloides* B.J. Rees is described which was found at the site four days after the accidental poisoning. Morphological and molecular evidence is presented for this new species, to explore relationships with known causes of *Amanita* poisonings from both hemispheres.

Key words: *Amanita*, Australia, poisoning, *Allocasuarina*, LSU.

Introduction

The incidence of severe mushroom poisoning in Australia has been low (Barbato 1993). This is due in part to the innately conservative attitude towards mushroom eating of our early Anglo-Celtic forebears (Southcott 1996), and to the fact that the most poisonous mushroom *Amanita phalloides* (the "death cap"), does not occur natively in Australia. It grows in a mutually beneficial association with exotic plants such as oak, hazelnut and chestnut (Read pers. comm.) and occasionally liquid-amber, birch and beech (Cole 1993) forming a mycorrhiza with host plants, which have been introduced into Australia over many years.

With changing ethnic composition, more experimental eating habits and more wide-spread sightings of *A. phalloides* in the ACT, Tasmania, Victoria, and recently South Australia, the incidence of mushroom poisoning has increased, and several fatalities have been documented (Trim *et al.* 1999; Brine 2002). A second species, *Amanita preissii* (Fr.) Sacc., has also been implicated in mushroom poisoning (Cleland 1943; Harris & Stokes 1976; Southcott 1996). In general, however, edibility of most native Australian mushroom species of fungi is untested (Southcott 1996).

The onset of symptoms of mushroom poisoning may vary depending on the identity of the mushroom, the amount ingested, the nature of the toxic principle involved and the length of exposure to the toxin (Barbato 1993). Some *Amanita* species can produce extremely toxic cyclopeptides including amatoxins and phallotoxins (Vetter 1998). Where amanitin is the principal toxic factor, in species such as *A. phalloides*, the initial onset of symptoms may be delayed up to 24 hours followed by a symptom-free latent period of a further 24 hours before onset of hepatic and renal failure occur (Barbato 1993). If symptoms are not recognized early, this delay reduces the chance of a successful recovery from the poisoning.

Not all *Amanita* species are poisonous. Some species such as *Amanita caesarea* (Scop.:Fr.) Pers. are prized in Europe for their outstanding flavour, but in general, *Amanita* is regarded as a genus best avoided. Pockets of "expertise" exist within migrant populations in Australia, who see similarities with the mycota of their countries of origin, and a level of experimental eating occurs with largely undocumented results. Much of this information is passed onto new arrivals in semi-rural areas around Australian major cities and regular collecting trips to plantation forests of exotic species are an annual event for some ethnic groups in the autumn fungal season.

A Lao religious community in south-east Sydney had been advised by "knowledgeable locals" that *Amanita ochrophylla* (Cooke & Massee) Cleland, a fairly robust-looking and strong-smelling mushroom which grew naturally on their property, was edible and quite delicious in soup. This was prepared regularly and enjoyed with some relish and seemingly no untoward results. Visitors to the community often gathered the species from mixed *Eucalyptus*/*Allocasuarina* open woodland close to the settlement before or after religious observances. There were no exotic tree species present at the site. The father of one visiting family was in the habit of including other small white species familiar to him which were present at the site and which were regarded as particularly flavoursome. Following the preparation of soup later at their own home, the father of the family developed symptoms of mushroom poisoning and was admitted to a nearby public hospital as outlined below.

Case History

Mr LC, a 58 year old Lao man, presented to the emergency department at 0226 hrs complaining of nausea, headache, abdominal pain and diarrhoea. He

reported to the staff that he suspected some mushrooms that he had ingested 6 h previously might be involved. He was a diabetic but was fit and active, involved in boxing and regular weight training. His assessment at that time included normal vital signs and he was treated with antispasmodics (hyoscine butylbromide), antiemetics (metoclopramide), and intra-venous (IV) fluids. Blood collected revealed a very mild elevation in his liver function tests (LFT's). He was assessed as having a mushroom induced gastroenteritis and was discharged after four hours with some improvement in his symptoms.

He represented that same evening, 17 h after his initial presentation and 25 h after the initial ingestion, with ongoing diarrhoea and cramping abdominal pain. In addition he was complaining of vomiting, shortness of breath (SOB) and dizziness on standing.

He was initially assessed by a junior doctor who found him to be clammy, tachycardic, SOB with generalised abdominal tenderness. He was commenced on IV fluids. A more senior review that evening noted an abnormal chest X-Ray and admitted him as an atypical pneumonia and commenced IV antibiotics.

Overnight in the emergency department, Mr LC continued to be tachycardic and tachypnoeic, with a falling blood pressure.

On review by an Emergency Physician the following morning he was extremely unwell. He was hypotensive with an acidosis and gross derangement of his LFT's, significantly worse than his original tests. The possibility of an *Amanita* poisoning was raised and discussed with the Poisons Centre Toxicologist.

He was resuscitated with IV fluids to replace his large fluid loss, high dose penicillin and *N*-acetylcysteine for the *Amanita* poisoning, and he was transferred to the Intensive Care Unit at Royal Prince Alfred Hospital to be cared for by their Liver Unit.

He subsequently had an extremely stormy course, characterised by liver and cardiac failure. In the early stages he was not expected to survive. He was eventually discharged after one month in hospital.

A request was received by the senior author from the Liver Unit at the Royal Prince Alfred Hospital, Sydney for help in identifying mushroom fragments present in the remains of a yellow- coloured soup consumed by the patient. The patient was suffering extreme liver damage, and was not expected to survive the next 24 h. He had consumed the largest amount of the soup, while other members of the family who had also eaten the soup had suffered no ill effect. Symptoms described were consistent with amatoxin poisoning, and the involvement of species similar to *Amanita phalloides* suspected. Only pumpkin-coloured, large pieces of lamella tissue, with ellipsoid to elongate spores characteristic of *A. ochrophylla* could be found in the remains.

A visit to the most likely location at the site where the patient habitually gathered mushrooms for consumption was rewarded with the collection of *Amanita ochrophylla*, and several collections of a small-statured, white to grayish fawn-coloured *Amanita* species occurring only in association with *Allocasuarina* L. Johnson. This *Amanita* species bore a strong similarity morphologically to the button stage of the "paddy-straw" mushroom *Volvaria volvacea*. It could not be confused with *Amanita ochrophylla* juvenile fruitbodies, which were more robust and ochraceous yellow in colour.

Materials and methods

Material brought back to the lab was photographed, macro (or field) characters described, and the collection dried at 45°C for permanent storage. Colour notation used was from Kornerup & Wanscher (1986). Microscopic examination by bright field microscopy was carried out on fresh and dried material and a portion reserved for later DNA extraction and comparison with identified *Amanita* species.

DNA Extraction and amplification

Tissue (approximately 10-50 mg) was ground in extraction buffer (Carlson *et al.* 1991), then inclubated at 65°C for 30 min. Samples were extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous phase was then extracted with an equal volume of chloroform:isoamyl alcohol (24:1). An equal volume of 100% isopropanol was added to the aqueous phase and incubated at room temperature for 30 min. The sample was centrifuged for 30 min. at 12000 G and decanted. The DNA pellet was washed in 70% ethanol, air-dried and 50 µL of tris-EDTA buffer was added. One µL of DNase-free RNase A was added and the sample incubated at 37°C for 15 min.

The primers ITS-1 (White *et al.* 1990) and LR7 (Moncalvo *et al*. 2000) were used to amplify the internal transcribed spacer (ITS) region and the 5' end of the 26-28S rRNA gene. Amplification conditions were as follows: each 50 µL final volume contained 5 µL of BioTaq 10 X NH_4 buffer (BioLine Co.), 2.5 µL of mM $MgCl_2$, 5 µL of a mixture of four deoxynucleotide triphosphates, each at a concentration of 2.5 mM, 1 µL of each of the primers at a concentration of 20 µM, 35 µL of sterile water, and 2.5 U of *Taq* polymerase (BioTaq, BioLine Co.). A touchdown PCR program was used, with an initial denaturation of 95°C for 5 min. The initial annealing temperature was 65°C, reducing to 45°C, with two cycles at each of the higher temperatures, followed by 30 repetitions of the final cycle. Each cycle had a denaturation step of 30 s at 95°C, and an extension step of 90 s at 72°C. PCR products were purified by spin column purification (Wizard PCR Preps, Promega) and sequenced using ABI Dye terminator cycle sequencing chemistry (Perkin Elmer Co.), using the primers ITS-1 and LR7. Only the 5' end of the 26-28S rRNA gene was used for subsequent analysis.

Sequences were assembled and edited using ContigExpress (Vector NTI Advance 10.3.0, Invitrogen Corp.) and FinchTV version 1.4.0 (Geospiza Inc.). The sequence determined in this study (Genbank accession number EU915295) was manually aligned with a sequence of *A. phalloides* (Genbank AY380359) and those from Drehmel *et al.* (1999) (TreeBASE study accession number S360) using Se-Al version 2.0a11 (Rambaut 2002). The dataset contained 1058 characters, of which 93 were excluded due to ambiguous alignment. A phylogenetic analysis was done using MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003), using a general time reversible model with gamma-distributed rate variation across sites and a proportion of invariable sites. One million generations were run, sampling every hundredth generation. The standard deviation of split frequencies was 0.007397 after 1000000 generations. Trees and branch length samples were summarised after discarding the first 25% (250000) of the samples. A maximum parsimony bootstrap analysis was done (195 parsimony informative characters) using PAUP* version 4.0b10 (Swofford 2003), with 1000 replicate heuristic analyses using 2 random addition replicates per bootstrap replicate and the tree bisection-reconnection branch swapping algorithm. The tree was rooted with *Limacella glischra* (Genbank U85301).

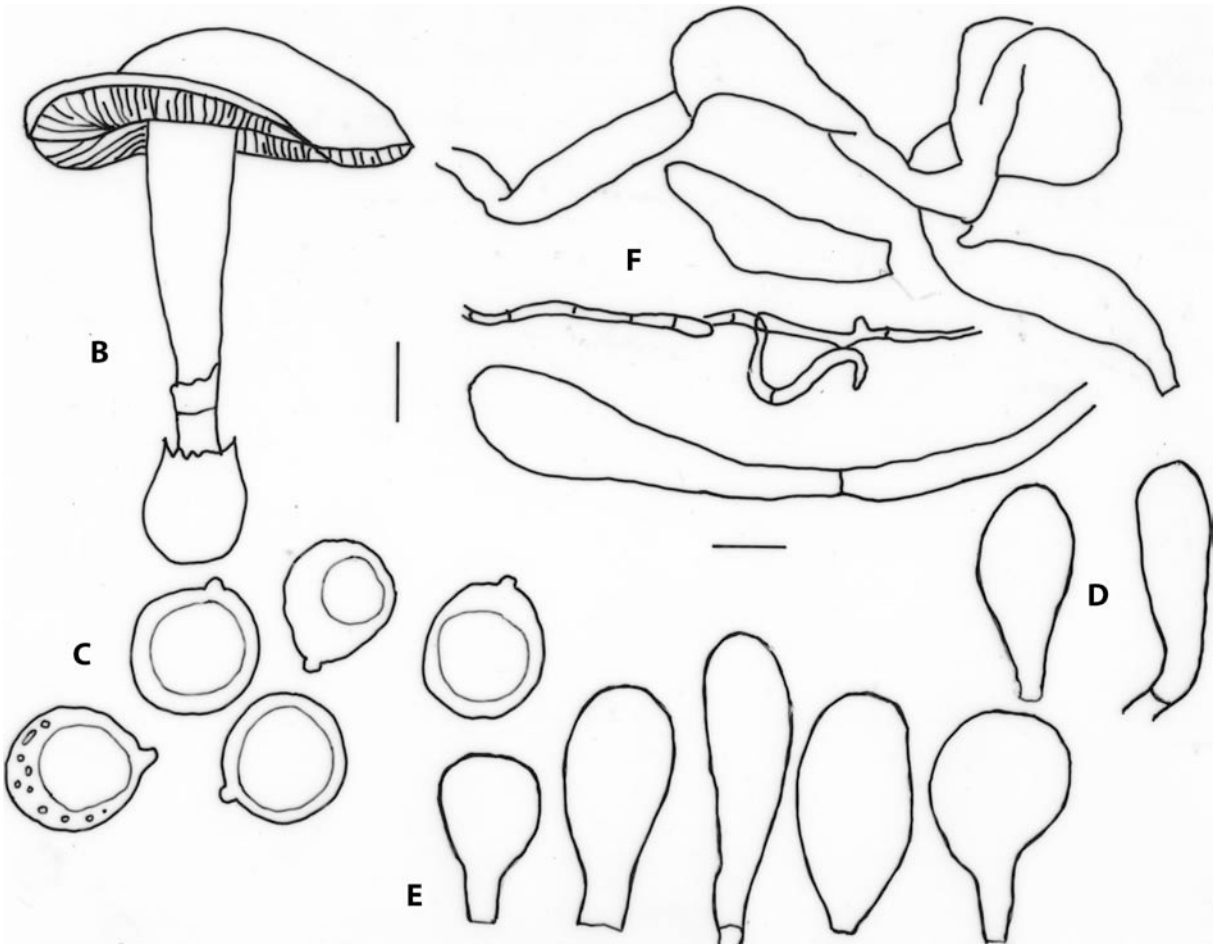

Fig. 1 *Amanita volvarielloides* B.J. Rees UNSW 03/29. **A.** Photograph of the holotype. **B.** Habit. **C.** Basidiospores. **D.** Marginal cells. **E.** Facial cells. **F.** Pileal surface universal velar remains. Scale bar = 10 mm for A and B, 5 µm for C, 10 µm for D and E, and 40 µm for F.

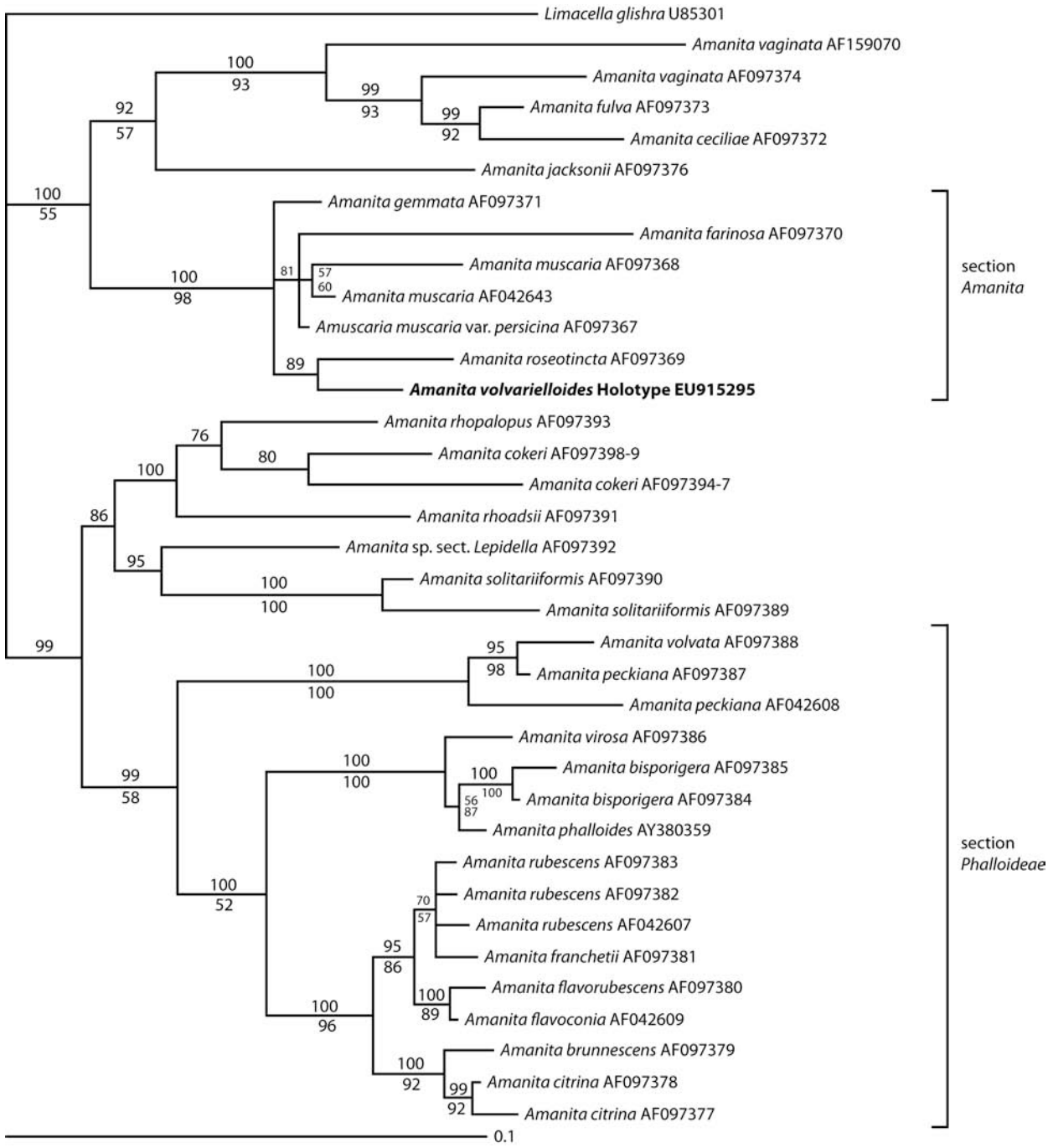

Fig. 2 Bayesian tree from the analysis of the nuclear rRNA gene sequences. Bayesian posterior probabilities are given above the branches and maximum parsimony bootstrap values are given below the branches. Sections *Amanita* and *Phalloideae* are indicated following Drehmel *et al.* 1999.

Results

The following description of a species collected from open *Eucalyptus* and *Allocasuarina* woodland where the patient was in the habit of collecting fungi for later consumption was arrived at by morphological examination.

Amanita volvarielloides B.J. Rees sp. nov. (Fig. 1).

MycoBank MB513335.

Australia. New South Wales. Wedderburn. On private property, on sandy soil under *Allocasuarina* sp., B. J. Rees *et al.*, 2.iv.2003. Holotype UNSW 2003/29.

Pileus maturus 45–55 mm diametro, late convexus exiguo umbone, pallido-ochraceus vel dilutus griseo-brunneus, laevis vel radialiter fibrillosus reliquis veli albo-gossypinis sicut paginam in areis magnis adsunt. Lamellae adnexae, tenues, confertae interventationibus iuxta stipem et carnem pilei. Margo valde laceratus et facies pulveraceae. Stipes sine annulo, albus vel squalidus griseo-flaveus super base amplificata in prominenti laxa saccata volva involuta; apex squamatus vel pruinosus. Basidiosporae globosae vel subglobosae, 8.7–10.3 × 7.8–9.0 µm, Q = 1.1, albae, leviter amyloideae. Basidia late clavata, 28–30 × 10–13 µm. Cellulae marginales 25–30 x 10–13 µm sphaero-pedunculatae vel vesiculatae; cellulae faciales 23–38 × 12–13 µm similes sed majores. Fibulae in cellulis margine supero volvae adsunt. Holotypus: Australia. New South Wales. Wedderburn, private property. On sandy soil under *Allocasuarina* in mixed

Eucalyptus/*Allocasuarina* open woodland, *B.J.Rees et al*., 2.iv.2003, UNSW 03/29 hic designatus.

Pileus 45–55 mm wide at maturity, circular to oval, low convex with a slight umbo; light ochraceous to pale, greyish fawn with a vaguely metallic sheen (K&W 5A3-5B3 nearest); slightly shiny, dry, not hygrophanous, smooth to vaguely radially fibrillose with some small striations present in one older, very dry basidome only; white velar remains forming flat sheets over large areas of the surface. Lamellae adnexed, thin, moderately close, creamy white, irregular, with intervenations near to pileus and stipe, with white particulate faces and ragged concolorous margins, 2 series of lamellulae, most nearly meeting the stipe with occasional short ones at margin. Stipe 35–40 mm x 8–10 mm, central to slightly eccentric, creamy white, separating easily from pileus, slightly flattened, tapering to a small, enlarged base, apex covered in small scales; hollow and filled with soft white fibres; with no apparent annulus, but with dirty, grayish yellow, universal velar remains appressed to the stipe just above a well-developed saccate, wrinkled volva.

Basidiospores [30/3/2], 8.7–10.3 × 7.8–9.0 µm, Q=1.01–1.25, consistently sub-globose to globose, with a very prominent apiculus, cytoplasm granular with a large central circular guttule, weakly amyloid. Basidia 28–30 × 10–13 µm, clavate, no clamps obvious at base, four-spored, with sterigmata to 5 µm in length, sub-basidial cell not especially swollen. Marginal lamella cells 25–30 × 10–13 µm, broadly clavate. Facial lamella cells 23–38 × 12–13 µm, broadly clavate to sphaeropedunculate, slightly larger than the marginal ones, all hyaline. Universal velar remains on pileus surface composed of narrow, segmented hyphae 3.5–4.2 µm wide, broken up into short cells, together with long chains of elongate, septate, much wider, filamentous hyphae containing swollen intercalary and clavate to crescent-shaped terminal cells 8.5–14.5 µm wide. No free cells present. Scales at stipe apex consisting of hyphae, similar to the long, broad, filamentous pileal surface hyphae, but with only occasional free, narrowly clavate cells. Cells at the upper free edge of the saccate volva interwoven, and extremely long, like pileus universal velar cells, but with thicker walls and abundant clamp connections.

Amanita volvarielloides has small-statured, grey to fawn fruitbodies, superficially resembling the paddy-straw mushroom *Volvariella volvacea* in the button stage. No annulus is present, but universal velar remains on the stipe near the saccate volva may be confused with an annulus. The lamellae are covered in powdery remains on the faces and margins, and have intervenations close to the stipe. Spores are globose to subglobose and no truly free cells are present in the membranous velar remains.

The presence of large, membranous, flat velar remains on the pileus surface is reminiscent of *Amanita phalloides*, but the stature and colour of the fruitbodies, and the shape and size of the velar cells are inconsistent with that species identity. *A. volvarielloides* can not be easily confused with *A. preissii* in which there are no clamp connections, more elongate spores and a "free limb" volva (Wood 1997). Other similar species, such as *Amanita murina* (Cooke & Massee) Sacc. and *Amanita murinaster* Wood, seem to be closely related, but the absence of a persistent annulus, the presence of a well-developed and consistently free, saccate volva with numerous clamp connections, and the characteristic shapes of the pileal velar cells differentiate *A. volvarielloides* from either of those species. *Amanita austrophalloides* Wood is also similar, but spores are narrower in that species.

Phylogenetic analysis

Phylogenetic analysis (Fig. 2) indicated that *A. volvarielloides* is related to *A. roseotincta* (Bayesian posterior probability 0.89). These species are in a clade along with *A. muscaria*, *A. farinosa*, and *A. gemmata* that corresponds to subgenus *Amanita*, section *Amanita* of Drehmel *et al.* (1999). This clade is well supported by Bayesian posterior probability (1.00) and parsimony bootstrap (98%) values. The position of *A. farinosa* (subsection *Ovigerae*), being nested within other members of this clade (all subsection *Amanita*), renders subsection *Amanita* paraphyletic, however relationships within the clade are poorly supported. *A. volvarielloides* is not closely related to *A. phalloides*, which is related to *A. bisporigera* and *A. virosa* (section *Phalloideae*, subsection *Phalloideae* of Drehmel *et al*. 1999).

Toxicity testing

Toxicity testing of samples from parts of three different fruit bodies of *A. volvarielloides* by Electrospray Liquid chromatography/Mass spectrometry (LC/MS) was found to be "inconclusive, as the samples were probably too small for detection of any known masses of the common *Amanita* toxins. However that doesn't mean that the toxins were not present" (Barrow pers. comm.).

Discussion

The patient's case history was clearly in accord with the known symptoms of amatoxin containing mushroom poisoning (Bresinsky & Besl 1990; Barbato 1993), in which the initial onset of gastrointestinal symptoms is delayed until 6–24 hours after ingestion, after which there is usually, but not always, a following "latent period" of a further 24 hours before the onset of hepatic or renal failure. *Amanita phalloides* is the species most often implicated in fatalities, but this species was not found in the "mushroom soup" or at the site. In *Amanita volvarielloides,* the presence of globose spores, a well-developed, saccate volva and filamentous velar remains on the pileus surface tended to suggest that it might also belong in Section *Phalloideae* of subgenus *Lepidella* of *Amanita*, but these characters are not confined to that Section alone and may also be found in other sections of sub-genus *Lepidella* or in sub-genus *Amanita* (Drehmel *et al.* 1999).

Molecular evidence indicates a closer relationship of *A. volvarielloides* with other members of Section *Amanita* (Fig. 2), which includes *Amanita gemmata* (L.:Fr.) Gillet, a known cause of mushroom poisoning (Bresinsky & Besl 1990). In this section of *Amanita*, symptoms of mushroom poisoning are usually due to the presence of muscimol, ibotenic acid or their derivatives, but the nature and onset of symptoms are usually not so delayed as for amatoxin poisoning, and the prognosis

much more favourable and usually dependant on the amount of fungus ingested (Barbato 1993).

The inclusion of other species such as *A. gemmata* and independently *A. toxica* (Lazo) Garrido & Bresinski known to occur in association with *Eucalyptus* in Chile in the Southern Hemisphere in this section (Bresinsky & Besl 1990), both of which are capable of causing serious or possibly even fatal symptoms, raises concerns that other members of section *Amanita* such as *A. volvarielloides* cannot be totally eliminated as a possible cause of this nearly-fatal poisoning. So little is known about the toxicology of Australian *Amanita* species in general, that unexplained records of poisonings due to species occurring in *Allocasuarina/Eucalyptus* woodland in the apparent absence of *A. phalloides* need to be investigated with an open mind. A similar case of poisoning resulted from ingestion of an *Amanita* species found growing in association with *Allocasuarina* sp. in North Queensland (Fechner pers. comm.), but no details of the collection have been published.

The more simple conclusion from the observations outlined, is that although *Amanita volvarielloides* is a new species found in association with *Allocasuarina* species in Australia, it probably was not the cause of the "phalloidin syndrome" experienced by the patient, and that another species may have been present at the site when the offending soup constituents were gathered some days earlier. The possibility exists, that although the patient would have displayed the same initial response as others to the ingestion of the poisonous toxins (Leal pers. comm.), his subsequent stormy history through to recovery, may have been intensified by his diabetic condition in response to the disturbances in electrolyte balance and fluid loss.

Other cases of poisoning due to confusion with the occurrence of "Paddy Straw" look-alike species have been documented before from Australia, New Zealand and the USA (Trim *et al*. 1999). *Amanita phalloides* has been recorded as growing in association with *Eucalyptus* and *Acacia* spp. in East Africa (Pegler 1977), and it is of great concern that if *A. phalloides* is able to adapt to form mycorrhizal relationships with other hosts in Australia such as *Eucalyptus,* its distribution in Australia and the risk of fatalities will be greatly increased. The association of *Amanita* species with *Eucalyptus* species outside Australia has been recorded (May and Wood 1997) and of *A. muscaria* with *Nothofagus* species in Tasmania (Lebel *et al*. unpublished).

The patient was fortunate to have survived the experience, thanks to the vigilance of the Emergency staff of Campbelltown Hospital and the institution of appropriate treatment, which varies for the type of mushroom involved. This is the reason why early identification of the mushroom ingested is so important. The incident received widespread publicity in the Campbelltown area especially among ethnic groups used to consuming the "paddy-straw" mushroom, and Mr LC has declared that "he will not be gathering wild mushrooms any more in Australia". Excellent, detailed summaries of the principal features of the "death cap" are available on websites compiled by Heino Lepp *et al.* (2003), and Trim *et al.* (1999) concerning increased sightings of the "death cap" in Australia.

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