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Characterisation of ectomycorrhizal formation by the exotic fungus *Amanita muscaria* with *Nothofagus cunninghamii* in Victoria, Australia

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Abstract The occurrence of the exotic ectomycorrhizal fungus *Amanita muscaria* in a mixed *Nothofagus–Eucalyptus* native forest was investigated to determine if *A. muscaria* has switched hosts to form a successful association with a native tree species in a natural environment. A mycorrhizal morphotype consistently found beneath *A. muscaria* sporocarps was examined, and a range of morphological and anatomical characteristics in common with those described for ectomycorrhizae formed by *A. muscaria* on a broad range of hosts were observed. A full description is provided. The likely plant associate was determined to be *Nothofagus cunninghamii* based upon anatomy of the roots. Analysis of ITS-1 and ITS-2 regions of nuclear ribosomal DNA sequences confirmed the identities of both fungal and plant associates. These findings represent conclusive evidence of the invasion of a non-indigenous ectomycorrhizal fungus into native forest and highlight the ecological implications of this discovery.

Keywords *Amanita muscaria* · *Nothofagus cunninghamii* · Ectomycorrhizae · ITS · Host-switching · Characterisation · Australia

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Introduction

Species of *Eucalyptus* and *Pinus* are now extensively grown in areas far removed from their respective native distributions, and the success of these introductions has been primarily due to the recognition of host specificity among ectomycorrhizal (ECM) fungi (Mikola 1973), which has led to the introduction of exotic fungi with plantation trees to many regions around the world. Given time, such introductions may lead to host shifting of the fungus or the hybridisation with or out-competing of local fungi (Selosse 1997), especially when exotic plantations are commonly situated adjacent to native forests with ectomycorrhizal plant hosts. Should exotic fungi switch hosts to indigenous plants, the physiological variability displayed between different ECM associations suggests that such hosts could potentially experience changes in nutrient transfer, carbon drain, and stress and disease tolerance. The role of ECMs in natural forest ecosystems is receiving increasing attention, particularly in relation to revegetation and management of existing forests. Should exotic fungal species have the capacity to form ECM associations with native plant species, this could result in the displacement of indigenous fungi, with potential effects on the host species and more complex ecosystem functions that cannot be predicted with the current level of knowledge. As the importance and complexity of ECM communities in forest ecosystems becomes increasingly apparent, the displacement of indigenous ECM fungi represents a conservation issue deserving of more attention.

In the past, consideration of exotic fungal introductions has primarily been focussed on the transfer of pathogenic species (Wingfield et al. 2001). Recently, reports of exotic

ECM fungi occurring in the absence of their introduced hosts have generated interest. Knowledge of such host-switching events remains limited and largely unsubstantiated, however, consistent sightings of the ECM fungus *Amanita muscaria* (L.:Fr.) Pers., a native of temperate forests across the northern hemisphere, sporulating in *Nothofagus* forests of South America, New Zealand, and Tasmania have led to the assumption of a successful invasion. In South America, *A. muscaria*, *Amanita rubescens* Pers. and *Xerocomus rubellus* (Krombh.) Quél. have been recorded as shifting from *Pinus radiata* D. Don. to *Nothofagus obliqua* (Mirb.) Oerst. and *Paxillus involutus* (Batsch) Fr. to *Luma apiculata* (DC.) Burret (Valenzuela et al. 1999). A similar situation has been reported for *A. muscaria* in *Nothofagus* forests of New Zealand (Johnston and Buchanan 1997) and with *Nothofagus cunninghamii* in Tasmania (Fuhrer and Robinson 1992; Bougher 1996; Johnston et al. 1998). *Chalciporus piperatus* (Bull.) Bataille is also found with *Nothofagus* in New Zealand (Johnston and Buchanan 1997) and in Victoria (pers. observation). In each of these cases, the fungi involved were indigenous to the northern hemisphere and introduced with *P. radiata* plantations. Díez (2005) investigated the expansion of the Australian fungus *Laccaria lateritia* Malençon (described as *Laccaria fraterna*) from eucalyptus plantations into native vegetation in Spain and indicated the formation of ECMs between this fungus and the indigenous shrub *Cistus ladanifer* L., although no supporting evidence was presented. A strong argument has been presented for the consideration of *Amanita phalloides* (Vaill. ex Fr.) Link as an exotic fungus introduced to North America, where it is now well-established in western native forests forming ECM associations with numerous indigenous tree species (Pringle and Vellinga 2006; Pringle et al. 2009). So far, these instances of host shifting have been inferred from the presence of sporocarps, without any evidence of a mycorrhizal association on the new host. There have also been unconfirmed reports of *A. muscaria* occurring in association with *Eucalyptus globulus* in plantations in Victoria (Sheehan 1999) and overseas (Bougher and Syme 1998). Garrido (1988) demonstrated the capacity for *A. muscaria* to form ECM associations with *E. globulus* and nine species of *Nothofagus* (including *N. cunninghamii*) in pure culture synthesis experiments, but none of these associations were able to be formed under greenhouse conditions. Malajczuk et al. (1982) conducted similar synthesis experiments, showing that *A. muscaria* is capable of forming ECMs with at least 11 species of *Eucalyptus*, and probably most of the genus, in pure culture. How these results relate to field conditions remains to be seen.

In 1992, sporocarps of *A. muscaria* were observed in mixed *Nothofagus–Eucalyptus* forest of Noojee State Forest, Victoria, with no *P. radiata* trees or other previously recognised hosts within sight (T. May pers. comm.). Ten years later, the fungus was still fruiting at this site, and subsequent observations over the last 5 years indicate that its range is spreading slowly, suggesting that *A. muscaria* has indeed become successfully established in a mycorrhizal association with at least one of the native species in the area. In this paper, we present a detailed characterisation of this association and identification of the new host. We also discuss some of the potential implications of this phenomenon for fungal conservation, the health of native forests, and management issues for plantation forestry.

Materials and methods

Study site

Field work for this study was conducted on fortnightly visits to Noojee State Forest, Victoria, Australia, between April and June 2002, with additional incidental observations of sporocarp fruiting from April 2003–July 2007. This forest is formed on granitic soils and situated on the eastern perimeter of the Loch Valley in the southern Central Highlands, at an elevation of approximately 1,093 m and with a mean annual rainfall of >1,400 mm. The site (37° 47′ 08S″ and 146° 08′ 03″E) was located in regenerating *Eucalyptus* (primarily *Eucalyptus nitens* Dean & Maiden) dominated montane forest, with mature *N. cunninghamii* mainly occupying the middle canopy, surrounded by woolly tea tree (*Leptospermum lanigerum* (Sol. ex Aiton) Sm.), ericaceous shrubs, bracken (*Pteridium esculentum* (Forst.) Nakai) and several asteraceous shrubs. The ground layer was almost completely covered in mosses and lichens, with a few small grasses, lilies, and forbs. There are no records of *P. radiata* or other introduced trees being present at this site in the past, and the nearest specimens of *P. radiata* were located approximately 1,700 m west along the access road. The site is exposed to considerable disturbance, including continued logging of eucalypts in the surrounding area, and is situated amidst a camping and gravel picnic ground.

Distribution of *A. muscaria* sporocarps

Potential mycorrhizal plants at the site were mapped on a grid. Throughout the sampling period, the position of all *A. muscaria* sporocarps observed was recorded to determine their range, density and relative proximity to nearby potential host species.

Sample collection

Roots of the introduced plant associate *P. radiata* (from the nearest stand) and two potential native plant associates, *N.*

cunninghamii and *E. nitens* from the study site, were sampled for structural and mycorrhizal comparisons. A sapling of *N. cunninghamii* (approximately 3 years old) was dug up with as much of the root system as possible, placed in a bucket of water, and left to soak overnight to loosen the soil particles. Roots leading from the base of individual trees of *E. nitens* and *P. radiata* were traced outward to the fine feeder roots with ECMs. These were excised, moistened, stored in plastic bags, and returned to the lab where they were washed gently under running water. The roots were then cut into small lengths (approx. 3–4 cm lengths) and inspected for the presence of both uninfected and ECM root-tips. ECMs were removed and the different morphotypes described (Dunk 2002). Samples were stored in distilled water and kept at 4˚C.

Ten *A. muscaria* sporocarps were collected from the site and five from the distant stand of *P. radiata* (with *N. cunninghamii* co-occurring) for comparison of the attached roots and ECMs and confirmation of plant associates. A spade was used to excavate the sporocarps and the surrounding soil to a depth of approximately 10 cm in a 10-cm radius, taking care not to disturb the sporocarp. The specimens were placed in plastic bags, returned to the lab, and stored at 4°C until needed. For each sporocarp, the stipe was cut off at the base, any coarse organic matter and large soil particles and rocks removed, and the specimens soaked in distilled water overnight. A light jet of water was then used to wash the remaining soil from the roots and mycelia. The stipe bases and attached roots were then examined under a dissector microscope and all ECMs present removed. Particular attention was given to evidence of hyphal links between the sporocarp tissues and mycorrhizae. To identify the host species, details of the vascular system of the mycorrhizal roots were recorded, segments of mycorrhizal and non-mycorrhizal roots examined, and the findings compared with those for *N. cunninghamii* and *E. nitens*.

Representative specimens of *A. muscaria* morphotype mycorrhizae from the study site were sealed in 1.5-ml microtubes, frozen in liquid nitrogen, and stored at –70°C, with samples randomly selected for molecular analysis.

Additional *A. muscaria* sporocarps were collected from native forest on site for herbarium preparation and molecular study. Fresh stipe material was surface-sterilised in 70% ethanol for 1 min, transferred to a lamina flow cabinet, and the interior cut into smaller sections (approx. $5\times2\times2$ mm^2). These were frozen and stored in the same manner as the mycorrhizae.

Microscopy

Both non-mycorrhizal and ECM root samples were prepared for resin embedding by fixing in formal–acetic–alcohol for 2 h, rinsing in a 0.1 M phosphate buffer (pH 7.0), and dehydrating via an ethanol series of increasing concentrations (10%, 30%, 50%, 70%, 90%, and twice at 100%), with 10 min at each concentration. The dehydrated specimens were transferred to glass vials containing pure LR White resin (London Resin Company Ltd., London) and placed in a vacuum. After 24 h, the mycorrhizae were transferred to plastic moulds containing fresh resin and the moulds sealed with a plastic film and solidified at 60°C for 3 h. A Spencer microtome (American Optical Company, New York) was used to obtain 10-μm thick transverse and longitudinal sections of non-mycorrhizal and ECM samples. The sections were stained with 0.01% toluidine blue O (Sigma Chemical Company, St Louis, Brundett et al. 1996) for 5 min, rinsed in distilled water, and heat-dried. Permanent slides were prepared by placing on the sections a drop of histological clearing agent (Histo-Clear™, National Diagnostics, Somerville) and oil-based mounting medium (Eukitt®, O. Kindler, Freiberg), and then a glass cover slip.

Mantle preparations of fresh ECMs were stained in 0.01% toluidine blue O, mounted in distilled water, and examined immediately. ECM description followed the method and terminology of Goodman et al. (1996). Morphological characters were examined under a dissector microscope and the anatomical features described from the details apparent in the embedded sections and mantle preparations under a light microscope at ×400 and ×1,000 (oil immersion) magnification.

Molecular analysis

DNA preparation, amplification and sequencing Genomic DNA was isolated using a DNeasy Plant Mini Kit (Qiagen Inc., Valencia, Calif.), following the manufacturer's protocol. For fungal material, primers ITS1 (Gardes and Bruns 1993) and ITS 4 (White et al. 1990) were used to amplify the ITS-1 and ITS-2 regions of nuclear ribosomal DNA (nrDNA). To amplify plant nrDNA from mycorrhizae, primers S3 or S6 (Käss and Wink 1997) and 26SE (Sun et al. 1994) were used. Polymerase chain reactions (PCR) were conducted in a volume of 50 μl and contained 1.25 U QIAGEN HotStar Taq DNA Polymerase, 10 pmol of each primer, 1.5 mM $MgCl_2$, and 0.25 mM each dNTP. Amplifications were performed in an Eppendorf Mastercycler Gradient Thermal Cycler. Cycling conditions for fungal isolations consisted of 10 min activation at 95°C, followed by 30 cycles of 30 s at 94°C, 30 s at 60°C, 1 min at 72°C, 5 min at 72°C, and held at 4°C. Cycling conditions for plant isolations consisted of 10 min activation at 95°C, followed by 35 cycles of 45 s at 94°C, 45 s at 57°C, 130 s initially then increasing by 1 s/cycle at 72°C, 5 min at 72°C, and held at 4°C. Products of PCR amplification were

purified using the QIAquick® PCR Purification Kit (Qiagen Inc., Valencia, CA), then directly sequenced using the ABI Prism BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Sequencing was carried out by means of an ABI Automated 377DNA Sequencer. Sequences were assembled using Sequencher 4.1 (GeneCodes, Michigan). Sequences were aligned in ClustalX (Thompson et al. 1997), and then corrected manually in BioEdit v5.0.9 (Ibis Therapeutics 2001). Novel sequences were lodged with GenBank.

Data analysis

Fungal taxa Analyses of sequences were conducted using *Amanita ceciliae* (Berk. & Broome) Bas and *Amanita melleiceps* Hongo as outgroup taxa, 55 *A. muscaria* sequences from a broad geographic range after Geml et al. (2008), and three novel sequences. The novel sequences included one sporocarp and two ECM root-tips from the study site.

Plant taxa Sequences of two mycorrhizal isolates and one non-mycorrhizal root from the study site were compared with 19 taxa representing a broad cross-section of potential native and introduced plant hosts (Table 1).

Heuristic searches for the most parsimonious trees were made using PAUP* 4.0b10 (Swofford 2002). Uninformative characters were excluded from the analysis, and gaps were treated as missing data. One thousand random addition sequence replicates were conducted using tree-bisection-connection (TBR) branch swapping and MULTREES on. Nodal supports were tested by bootstrapping of 400 replicates with the heuristic search option (TBR and MULTREES off), including groups compatible with 50% majority rule consensus, with 20 random addition sequences.

Results

Seventeen sporocarps identified as *A. muscaria* (Fig. 1) were found in the absence of their exotic hosts over a 3-month period in 2002. Four indigenous plants (*Acacia dealbata*, *E. nitens*, *L. lanigerum,* and *N. cunninghamii*) known to be at least capable of forming ECMs under laboratory conditions (Warcup 1980) were present in the immediate area (Fig. 2). However, *A. dealbata* and *L. lanigerum* are unlikely candidates as *A. muscaria* sporocarps were fruiting well beyond the root zone of individual plants, and none of the mycorrhizal root-tips examined had the structural morphology of these two taxa

ECM roots of the *N. cunninghamii* sapling were easily distinguishable by the presence of a mantle. Nine distinct morphotypes were established on the basis of colour, branching pattern and emanating hyphal elements. A brief examination of these morphotypes was conducted in order to gain a general idea of the morphology of *N. cunninghamii* ECMs, however, only one morphotype consistent with *A. muscaria*-type mycorrhizae was examined further for this study. These white *A. muscaria*-type mycorrhizae were found on approximately 22% of the root-tips examined.

A white ECM found attached to the roots beneath six of the ten *A. muscaria* sporocarps from the study site was consistently the most abundant morphotype observed (approximately 65–80% of root-tips/sporocarp). Comparison of this morphotype with a white morphotype collected from under *A. muscaria* sporocarps growing with *P. radiata* (Fig. 3) revealed similarities. Only one other white morphotype was found at the study site, however, this possessed a mass of emanating hyphae and a translucent mantle, neither features characteristic of known *A. muscaria* associations, and the morphotype was not further examined in this study.

Description of *A. muscaria* mycorrhizas on *N. cunninghamii*

The mycorrhizae (Fig. 4a–d) are typically a monopodial pyramidal system that is variably branched to form a dense cluster or an open arrangement. Unbranched, terminal examples (Fig. 4d) also occur frequently. The straight to bent or slightly tortuous tips are white, becoming a pale, creamy brown with bruising, and have a fine, felt-like texture. Ranging from 0.20 to 1.65 mm in length and 0.17–0.28 mm wide, the tips are usually around 1 mm long and 0.2 mm wide. White mycelial strands 25–100 μm in diameter extend from the mantle surface at restricted points (Fig. 4d). The *rhizomorphs* are composed of compact, smooth-undifferentiated hyphae of variable width (1.0–6.5 μm; Fig. 5e). These *hyphae* are hyaline and thin-walled, and often possess fine granular cell contents. Septa are common and unclamped. Hyphal junctions are rare, and no anastomoses were observed. True emanating hyphae are absent, however, short cystidia-like hyphae (Fig. 5f) are present, though rare. These hyphae are varied in form, ranging from single celled fusiform projections (3–12 μm long and 2–3 μm wide), to bent, septate hyphae of several cells (15–40 μm long and 1.5–2 μm wide) that narrow towards the tip or end in bulbous cells (to 5 μm in diameter). No clamps connections were observed. The *mantle* is of medium thickness (8–35 μm; Figs. 4e–g and 5b), with an outer layer of dense net prosenchyma (Fig. 5c). The inner layer, though mostly of non-interlocking irregular

Table 1 Plant taxa analysed

Taxon	GenBank accession number
Alnus rubra	AY352321
Betula papyrifera	AY761126
B. albosinensis	AY761099
Casuarina equisitifolia	U42515
Eucalyptus grandis	AF390472
E. pachyphylla	AF390473
E. camaldulensis 1	AF190363
E. camaldulensis 2	AF058473
E. obliqua	AF058484
E. tindaliae	AF390534
Fagus sylvatica subsp. *hohenackeriana*	AF456990
F. sylvatica var *atropunicea*	AF457005
Leptospermum scoparium	AY772398
N. cunninghamii	U96868
N. glauca	U96866
N. menziesii	U96869
N. mooreii	U96870
N. obliqua	U96867
Nothofagus sp. (5029rb)	AF480092
N. cunninghamii-ECM (Tasmania)	EU236719
N. cunninghamii root (Tasmania)	EU236720
Quercus alba	AF098419

synenchyma, occasionally consists of elongated or slightly interlocking cells (Fig. 6d). The septate hyphae of the outer mantle are generally 2–4 μm in diameter, however, cells located at the frequent hyphal junctions are often swollen (up to 10 μm). A well-developed Hartig Net (Fig. 4e–h) composed of beaded hyphae 2–6 μm wide is present between the radially elongated epidermal cells, terminating at the outer limit of the cortex region in which the cells have thickened walls and stain heavily with toluidine blue O. The Hartig Net does not penetrate between the epidermal and cortical cells, is never more than one cell thick, and forms a palmettes arrangement of opposed branched hyphae (Fig. 4h). No Hartig Net was observed in the main axis of the mycorrhiza, and here, the epidermal cells show no elongation (Fig. 5a). No intracellular penetration was observed.

Fig. 1 Field photographs showing: **a** *A. muscaria* fruiting in the vicinity of *N. cunninghamii* and *E. nitens*, and **b** *A. muscaria* at the base of *N. cunninghamii*

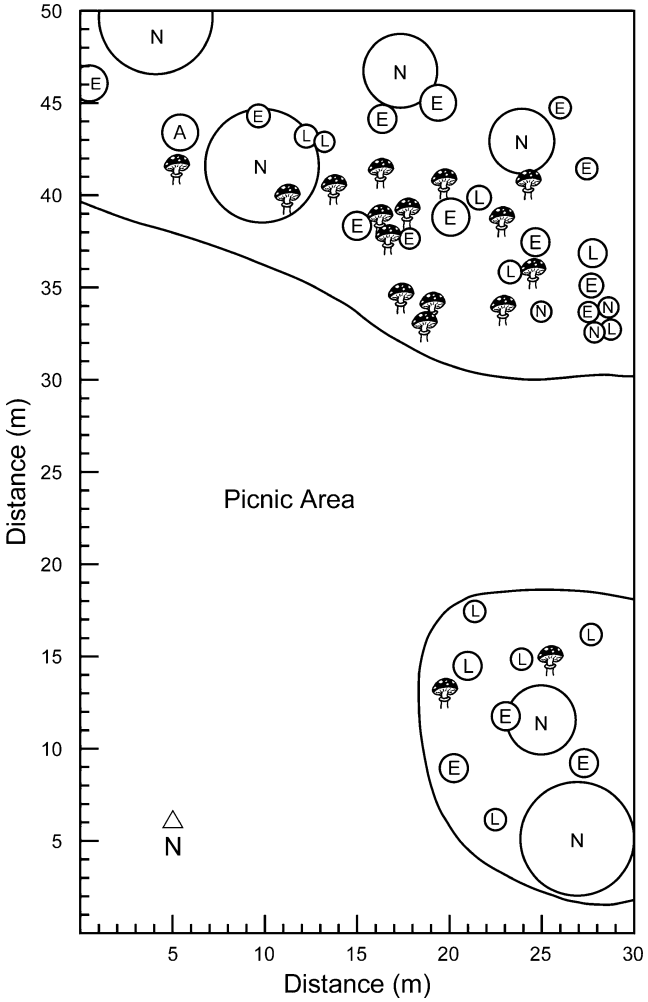

Fig. 2 A vegetation map of the area in which sporocarps of *A. muscaria* (n=17) were found, showing their position relative to nearby potential hosts. Picnic area is gravelled. Symbols: *A. muscaria* sporocarp, *A Acacia dealbata*, *E E. nitens*, *L Leptospermum lanigerum*, *N N. cunninghamii*. The *circles* represent an approximate canopy cover of individual plants

DNA analysis

Fungal The alignment of fungal ITS sequences consisted of 672 characters, of which 617 were constant and 55 were parsimony informative. Analyses resulted in 31,002 trees of 89 steps and a reasonably high degree of consistency (consistency index=0.693, retention index=0.630). The novel sequences of the white mycorrhizae type from *Nothofagus–Eucalyptus* forest are shown to definitely be those of *A. muscaria* (Fig. 6). The six main phylogeographic subclades recovered within *A. muscaria* are the same as those of Geml et al. (2008). Both the sporocarp sequence and root-tip sequences (three) from the study site appear in a well-supported clade (bootstrap 81%) representing Eurasia, Alaska, and the Pacific NW Coast of North America (clades II and II/A of Geml et al. 2008). The Pacific Northwest subclade II/A (*P. radiata* origin) is supported as distinct (96% bootstrap) from subclade II. Subclade II habitat types are a mixture of deciduous and coniferous forests (see Table 2 from Geml et al. 2008).

Plant The alignment of plant sequences from mycorrhizal and non-mycorrhizal roots consisted of 253 parsimony informative characters. Analyses resulted in 647 most parsimonious trees of length 691 (consistency index=0.7845; retention index=0.7562). Sequences of the *A muscaria*-type mycorrhizal root-tips appear in a single clade with *N. cunninghamii*, with bootstrap support of 74% (Fig. 7), confirming the plant associate as *N. cunninghamii*.

Discussion

The only other fungal species in Australia that produces sporocarps resembling those of *A. muscaria* is *Amanita xanthocephala* (Berk.) Reid and Hilton, which has a much smaller, paler pileus with fewer, less persistent and more yellowish volva remnants and a poorly developed annulus that is only present on immature specimens (Grgurinovic 1997). It is likely that earlier records of *A. muscaria* in Australia in the nineteenth and early twentieth centuries and more recent anecdotal reports of sightings in association with *Eucalyptus* are due to confusion with this species. In

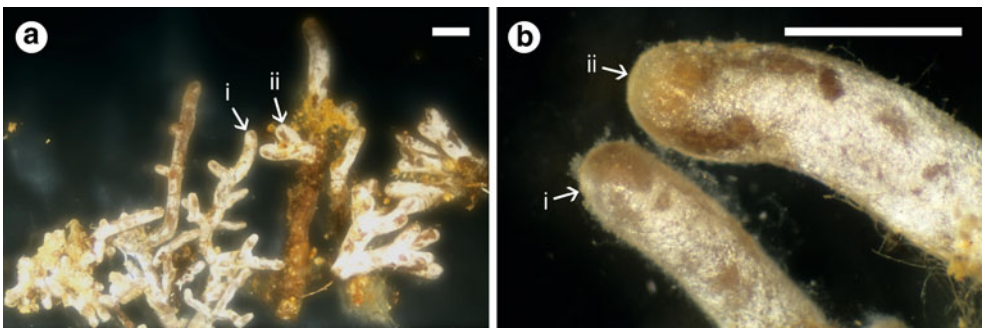

Fig. 3 Comparison of (i) white ectomycorrhizal morphotype consistently present beneath *A. muscaria* sporocarps from *Nothofagus–Eucalyptus* study site with those (ii) formed by *A. muscaria* on *P. radiata*. **a** Gross morphology of the mycorrhizal systems isolated from the field. **b** Close-up of individual tips showing relative size and similarities in colour and texture. *Scale bars*, 1 mm

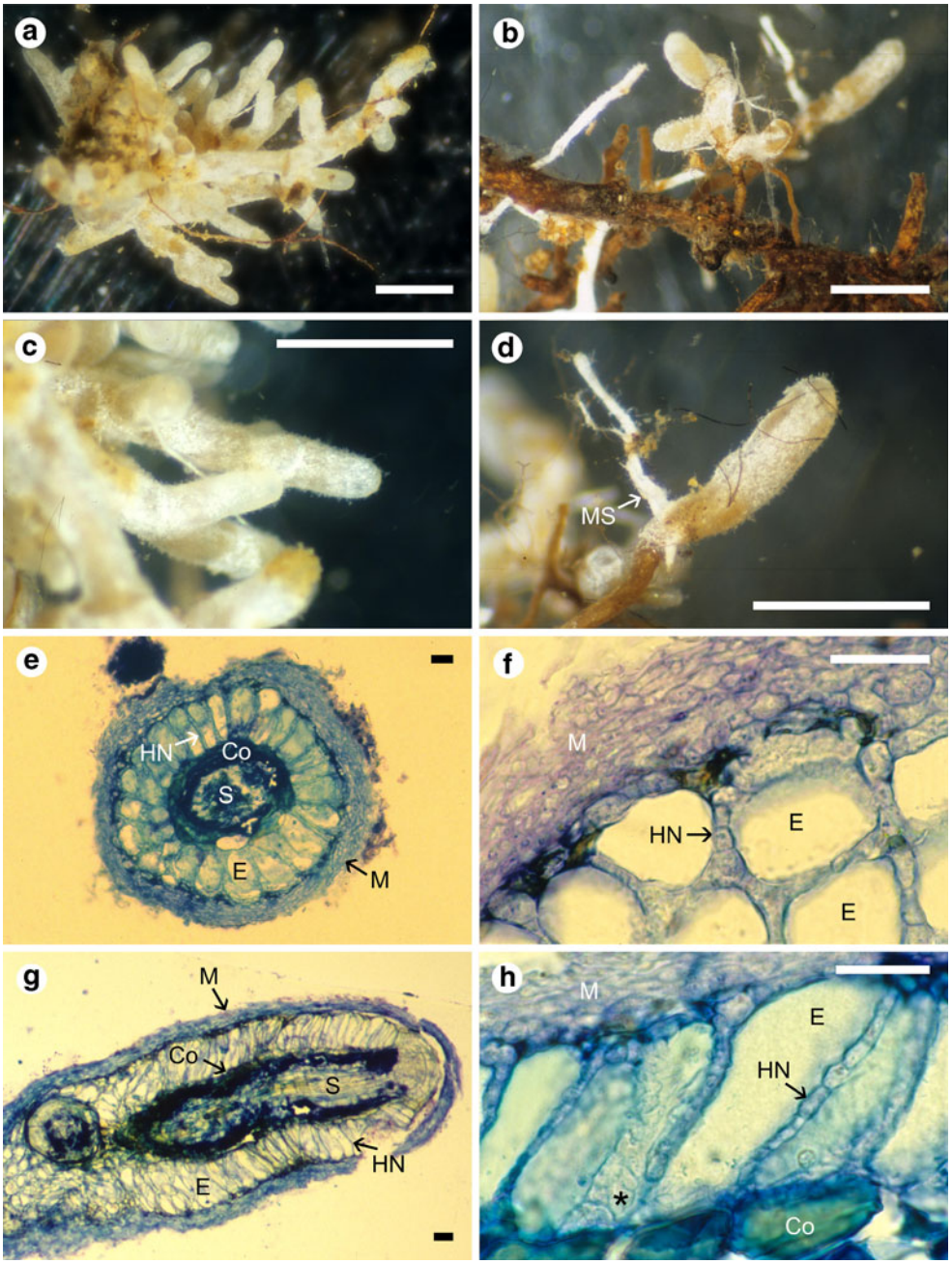

Fig. 4 *A. muscaria* associated with *N. cunninghamii*. **a** Gross morphology of a frequently branching specimen forming a dense, monopodial pyramidal arrangement. **b** A young, somewhat irregular system. **c** Close-up of the tips. **d** Close-up of a terminal, unbranched specimen showing the felt-like texture (with bruising) and the width of the enlarged tip relative to the uninfected root segment from which it arises. A mycelial strand projecting from the mantle at a restricted point is also visible. **e**, **f** Transverse sections showing the moderately thick mantle, the presence of a well-formed Hartig Net of beaded hyphae between the radially elongated epidermal cells. The cortical cells are heavily stained with thickened walls. **g** Longitudinal section showing the absence of a Hartig Net toward the tip apex where the mantle has become separated from the epidermis. The Hartig Net is evident by the dark staining between the epidermal cells. **h** Longitudinal section demonstrating the angling of the elongated epidermal cells towards the apex, pulled by the mantle and Hartig Net as the root grows. The palmettes arrangement of opposed branched hyphae of the Hartig Net is also visible. Symbols: *Co* cortex, *E* epidermis, *H* Hartig Net, *M* mantle, *MS* mycelial strand, *S* stele, *asterisk* arrangement of hyphae in the Hartig Net. *Scale bars*: **a**–**d** 1 mm; **e**–**h** 20 μm

many instances, an exotic tree has been found within 5–20 m of the site of fruiting (pers. com. T May, T Lebel). Pringle and Vellinga (2006) found similar limitations for earlier records in study of *A. phalloides*.

The survey of *A. muscaria* sporocarp production relative to the location of potential hosts at the study site revealed *E. nitens* and *N. cunninghamii* to be the likeliest plant host candidates. The continued fruiting of *A. muscaria* over a period of 15 years (1998–2006), number of sporocarps found in a small area (approx. 50 m^2) over a short time frame (10 weeks), and predominance of the mycorrhizas on roots, demonstrates that the fungus is well-established at the study site. Whether the presence of *A. muscaria* at this site is the result of one isolated case of host-switching or represents repeated instances of this phenomenon cannot be inferred from this study. Nor can we be certain that the observed distribution is a result of a single or multiple infection events, as we did not collect data to study genets (Bagley and Orlovich 2004). Although sporocarps were found in two distinct locations separated by approximately 10 m of compacted soil, this distance could easily be breached by the roots of nearby trees, and it is quite possible that the individuals here arose from vegetative propagation and are, or were once, connected by hyphal links. The apparent aggregation of some *A. muscaria* sporocarps around the base of several *E. nitens* trees and

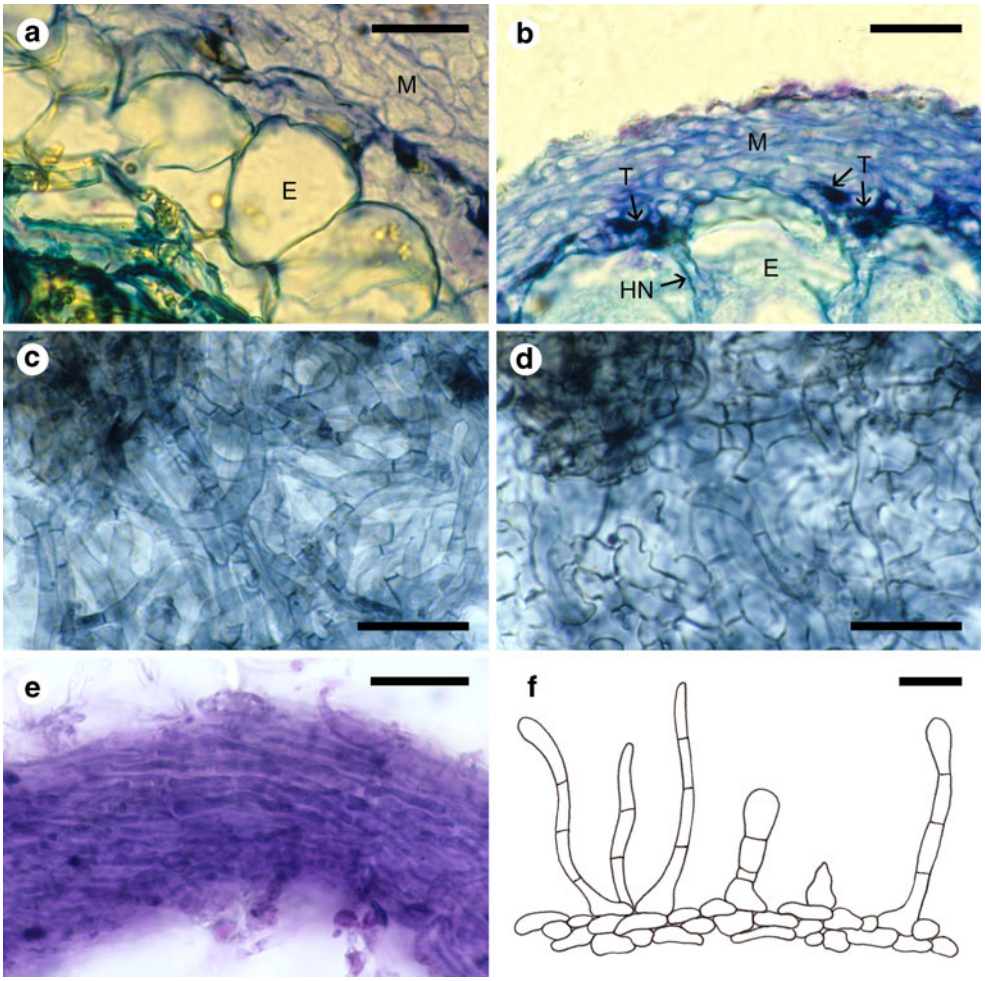

Fig. 5 *A. muscaria* associated with *N. cunninghamii*. **a** Transverse section of the main axis showing the absence of a Hartig Net between the epidermal cells, which have not undergone radial elongation. **b** Transverse section showing tannin deposits between the mantle and epidermis. **c** The outer mantle layer of net prosenchyma. **d** The inner mantle layer of net synenchyma with some slightly interlocking cells. **e** The smooth-undifferentiated hyphae of a mycelial strand. **f** Line-drawing of the various forms of cystidia-like hyphae projecting from the mantle surface, as seen in transverse section. Symbols: *E* epidermis, *M* mantle, *T* tannins. *Scale bars* **a**–**e** 20 μm; **f** 5 μm

various shrubs, where bark and leaf litter had accumulated, would appear to suggest a likely association. However, sporocarp formation is the result of nutrient 'pooling' by the whole mycorrhizal system and tends to be initiated in patches of high nutrient availability (Chilvers 1968). As a result, sporocarps may form at a considerable distance from their respective mycorrhizae. Also, soil moisture levels are likely to be higher where organic matter has accumulated, which would enhance ECM formation and sporocarp production, and the bases of the *E. nitens* trees were well within the likely range of *N. cunninghamii* roots. Therefore, the proximity of sporocarps to *E. nitens* plants may be a property of the soil organic layer and the particular plant species merely incidental.

The distribution of sporocarps in 2002 and subsequent 6 years shows that *A. muscaria* is still relatively confined to the original site, although there does appear to be a slow extension of localised range of the fungus (unpub. data). More extensive sampling of mycorrhizal roots is required to confirm this and to determine the extent of the invasion.

Characterisation of *A. muscaria* mycorrhizae on *N. cunninghamii*

A number of distinct morphological and anatomical features were found to be congruent with previous descriptions of *A. muscaria* associations. Foremost of these is the presence of short, variously formed cystidia-like hyphae projecting from the mantle surface. Ingleby et al. (1990) described similar hyphal elements for *A. muscaria* on *Betula pendula* Roth. and suggested that these may be indicative of *Amanita* species in general, with particular details of their shape and dimensions possibly distinguishing *A. muscaria*. Those recorded here were often longer, but otherwise appear to be identical. The thickness of the mantle is within the ranges previously given (e.g. Chu-Chou and Grace 1983; Garrido 1988; Ingleby et al. 1990) and, more importantly, the structure of the outer and inner mantle layers is consistent with that found on *B. pendula*. (Ingleby et al. 1990) Other descriptions of mantle layers vary. This character is open to a certain degree of interpretation, with room for overlap between categories

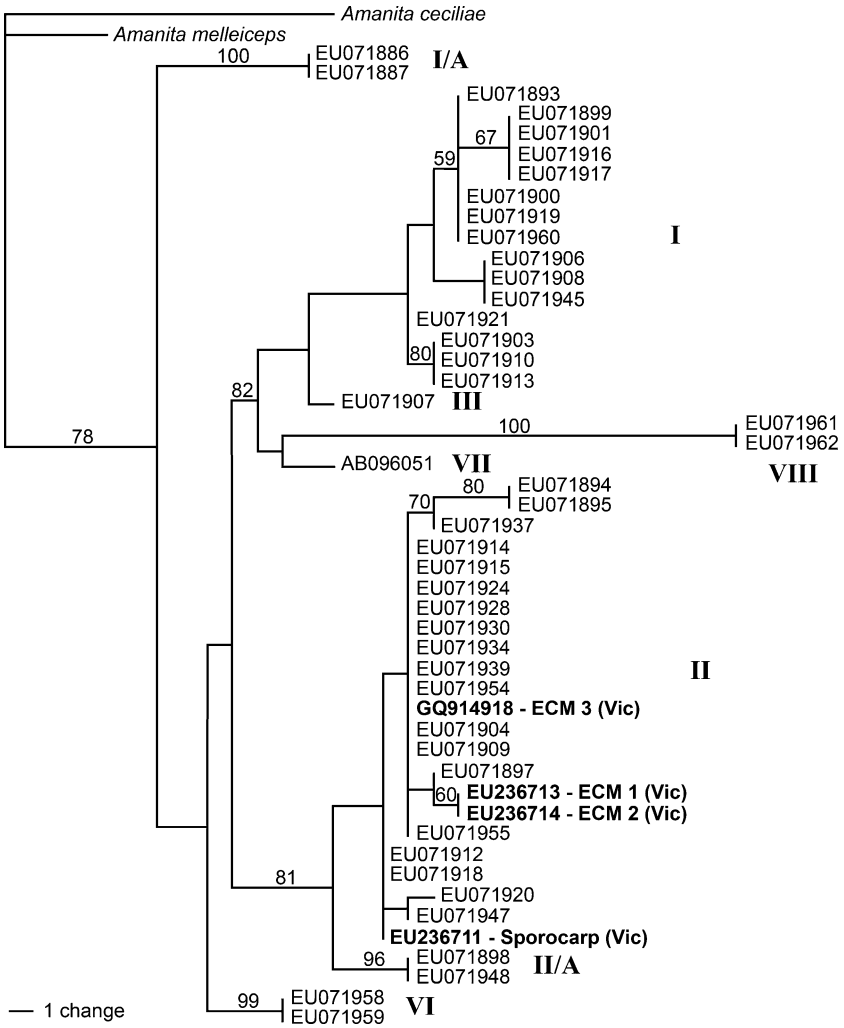

Fig. 6 One of 31,002 trees (length=1,028 steps, CI=0.7103, RI=0.6955) showing the relationship between the ITS sequences of *A. muscaria*-like mycorrhizae (labelled ECM 1–3) isolated from *Nothofagus–Eucalyptus* forest in Victoria, a sporocarp of *A. muscaria* from the same site, and *A. muscaria* sequences from GenBank. Bootstrap values (400 replicates) >50% are given above the branches

and no universally accepted terminology. Also, the descriptions often fail to specify the region of the mantle examined. Garrido (1988) reported the presence of clamp connections on hyphae of the mantle and Hartig Net on ECM associations between *A. muscaria* and *N. obliqua*, but these have not been observed in other studies and were not detected here. The width, arrangement and lack of differentiation of the mycelial strand hyphae, and the presence of granular cytoplasm within these hyphae are all useful additional traits for identification purposes (Chilvers 1968; Goodman et al. 1996) and are also consistent with the findings of Ingleby et al. (1990). The final and most immediately recognisable similarity between mycorrhizal root-tips and known *A. muscaria* associations is the bright white colour that turns pale brown with bruising.

Dissimilarities between the relationship described here and previous characterisations of *A. muscaria* associations, such as the branching pattern, the size of the tips and the structure and penetration of the Hartig Net, are all features linked to the response of the particular host species and are also differences that can potentially be attributed to environmental influences (Egger 1995; Peter et al. 2001; Wurzburger et al. 2001). Therefore, these are not necessarily contradictions. Rather, they may simply represent natural variation, highlighting both the complexity of ECMs as symbioses and the difficulties inherent in their study.

The dark staining of the cortical cells may be due to the accumulation of phenolic compounds (Malajczuk et al. 1982); this feature and the thickening of the cortical cell walls and the presence of tannins between the mantle and epidermis are host responses and have been described for other species of *Nothofagus* (Morrison 1956; Mejstrik 1972; Garrido 1988) and on *Eucalyptus* (Chilvers and Pryor 1965; Chilvers 2000).

Host-switching

Evidence for host shifting within lineages of ectomycorrhizal fungi is becoming more widely established as in-depth phylogenies of plant hosts and associated fungi are

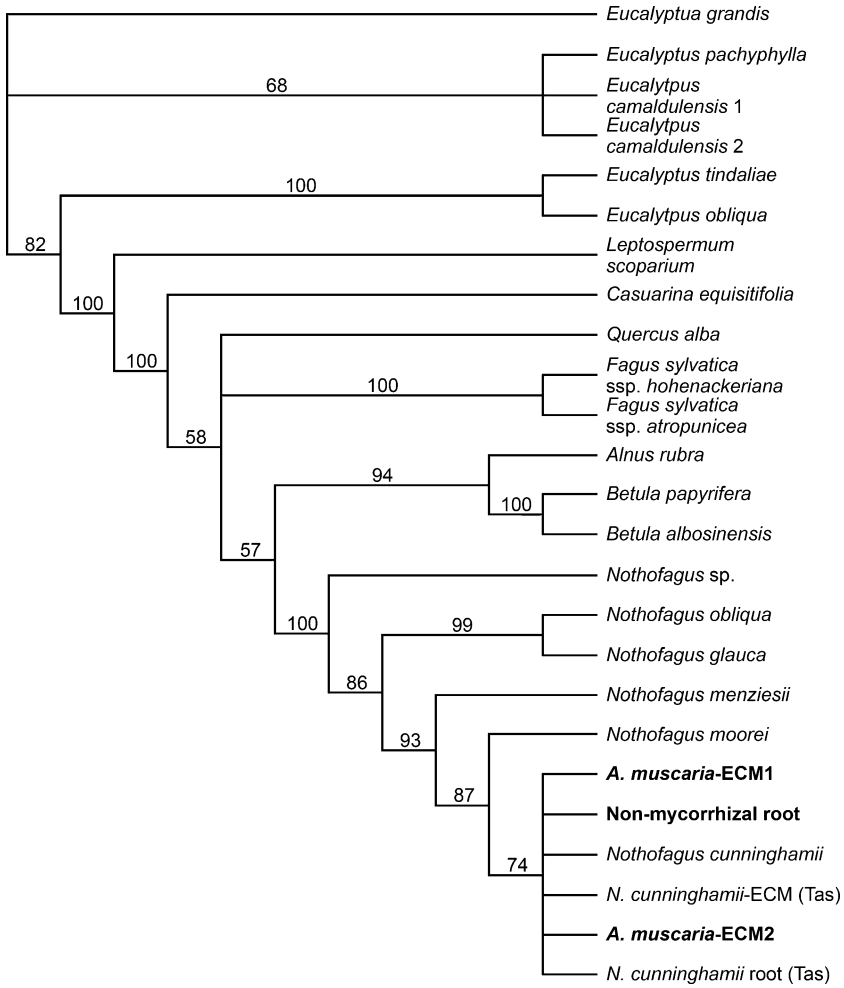

Fig. 7 One of 647 trees (length =691 steps, CI=0.7845, RI= 0.7562) obtained from a comparison of ITS sequences from a range of known broad-leafed hosts, potential indigenous hosts (including *Eucalyptus* and *Nothofagus*), two (ECM1, ECM2) *A. muscaria*-like mycorrhizal root-tips and a *N. cunninghamii* root sample (non-mycorrhizal root) from the *Nothofagus–Eucalyptus* study site in Victoria. Bootstrap values (400 replicates) >50% are given above the branches

produced (Den Bakker et al. 2004; Hibbett et al. 2000; Li et al. 2009; Rochet et al. 2011). The conditions under which these shifts are suggested to have occurred, changing climate affecting plant distributions, is occurring now in Australia (Pittock 2003), and the consequences are not likely to be confined to Australia. However, none of these studies have examined invasive ECM taxa, and the interactions that might affect the degree of invasiveness or potential for host-switching.

Analysis of molecular data matched the sporocarp and the fungal component of the described mycorrhizae from the Victorian *Nothofagus–Eucalyptus* site to known sequences of *A. muscaria*. Similarly, investigation of host nrDNA clearly indicated that the plant was *N. cunninghamii* and not other nearby plant species. Therefore, the combination of mycorrhizal characteristics and molecular data provide strong evidence that *A. muscaria* is capable of forming a functional ECM association with *N. cunninghamii* in field conditions.

Confirmation that an exotic ECM fungus has successfully adapted to its new environment in the absence of the host with which it was introduced highlights some of the limitations of current knowledge in ECM ecology. *A. muscaria* was deliberately introduced with *P. radiata* in Australia in the 1920s to the 1940s and to the Victorian site in the 1960s', and probably accidentally with other introduced plant hosts (e.g. oaks, poplar and birch) during the same time frames (Cleland 1924; Kessel 1923; Kessel and Stoat 1936; S Marsh pers.com). This means that host-switching between *Pinus* to *Nothofagus* has been achieved in a relatively short time, raising concerns for the future. We need to know how many times this host switch has occurred: Is the fungus spreading in native vegetation, and if so, how quickly, and perhaps, most importantly, will *A. muscaria* again switch hosts and become established on *Eucalyptus* spp.?

The initial processes of introduction of *A. muscaria* (as mycelium and spores from established plantations), disturbance (such as logging and road construction) and invasion of damaged native vegetation involved in typical biological invasions are apparent in this situation (Mitchell et al. 2006; Richardson et al. 2000a, b; Richardson 1998; Simberloff and Von Holle 1999). The distribution and density of sporocarps, predominance on roots and persistence over

time, provide evidence that *A. muscaria* is competitive when established in the field. Sporocarp production observed at the site studied here indicate that *A. muscaria* is able to persist and thrive in native vegetation in Australia, in parallel with observations of its spread in New Zealand (Orlovich and Cairney 2004). Sawyer et al. (2001) showed that *A. muscaria* exhibits significant longevity in pine plantations. This longevity is supported by monitoring of the spread of *A. muscaria* in *Nothofagus* forests of New Zealand's southern island over the last 10 years (Johnston and Buchanan 2007). So far, our observations are that this invasion remains restricted to localised areas that have been exposed to some form of disturbance, and the fungus has not yet spread far to nearby undisturbed vegetation. Fungi as a whole have been almost exclusively overlooked as invaders, especially those species involved in mutualisms (where the focus has been solely on their role as facilitators of or barriers to plant invasions, e.g. Richardson et al. 2000a, b), and the concept of an ECM fungal 'weed' is relatively novel and worthy of note.

The vegetation at the Victorian study site consists of mixed *Nothofagus–Eucalyptus* forest, with the roots of individual *Eucalyptus* trees surrounded and in close proximity to the invasion zone. Importantly, *A. muscaria* is known to exhibit a particularly broad host range (Molina and Trappe 1982), with its reputation as a generalist extending to successful associations formed in synthesis experiments with numerous and taxonomically diverse host genera (Garrido 1988; Molina and Trappe 1982) including *Eucalyptus* spp. (Garrido 1988; Malajczuk et al. 1982). In New Zealand, Ridley (1991) recorded *A. muscaria* in association with ornamental plantings of *Eucalytpus ficifolia* and *Eucalytpus pauciflora*; *E. ficifolia* is endemic to the south-west of Western Australia and is widely planted throughout southern Australian. The recent discovery of *A. muscaria* (on an exotic host) in the south-west of Western Australia is therefore cause for some concern (Robinson 2010). Thus there is a potential threat of *A. muscaria* establishing itself on *Eucalyptus*.

The immediate effect of invasions on indigenous fungal species is likely to be twofold. Firstly, a change in the availability of nutrients, and secondly, native ECM species are likely to be displaced from the host's roots, with unknown consequences for ECM community function (Dunk 2002). Further research is needed to address the many broader implications of the host-switching documented here, including the impact on native fungal diversity and conservation, the effect on *Nothofagus* community health (a rare and threatened plant community in Victoria) including nutrient and water relations, interactions with mycophagous animals, the potential for host-switches by other introduced mycorrhizal fungi and the continued location of pine plantations adjacent to native forest, in particular, *Nothofagus*.

In a review of the risks and benefits associated with biotic exchange between Australian *Eucalyptus* plantations and adjacent native forests, the transfer of ECM fungi was considered only in terms of the potential benefit that local species may confer on plantation productivity, despite the finding that 13 species present in the plantation were not found in nearby forests (Strauss 2001). Orlovich and Cairney (2004) have, however, raised many questions about the potential consequences of fungal invaders, with particular emphasis on the impact and spread of *A. muscaria*, whilst Díez (2005) has voiced similar concerns about the spread of ECM fungi from eucalypt plantations and highlighted the unique role of exotic ECM fungi in facilitating the establishment and spread of invasive plants. Clearly, any further research into the use of ECM inoculations to increase productivity in plantation forestry needs to address the issue of host-switching, as the threat to native fungal diversity and associated plant community health is real. The fact that the consequences of this phenomenon are unknown only serves to emphasise the importance of continued monitoring and investigation, and *A. muscaria* may prove to be a valuable model for understanding less conspicuous occurrences.

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