Fungal hyphae of a *Coprinopsis* sp. found in a bilby *(Macrotis lagotis)* burrow spoil-mound

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Abstract

Mycelium-like material was found in soil samples collected from the spoil-mound of a bilby *(Macrotis lagotis)* burrow in central Western Australia. The presence of clamp connections indicated that it belonged to the Basidiomycota. Analysis of the ITS region of rDNA resulted in 99% similarity with sequences from *Coprinopsis spilospora* (Romagn.) Redhead, Vilgalys and Moncalvo and *Coprinus subdomesticus* Murrill and 98% with a sequence from *C. alcobae* Ortega. *Coprinopsis spilospora* was the best match to the bilby-spoil sequence, leading to the conclusion that it should be identified as *Coprinopsis* cf. *spilospora*. *Coprinopsis spilospora* has not previously been reported from Australia and has been recorded in only a few localities in Europe. This opportunistic discovery of a fungus previously unknown to be associated with bilby burrow spoil-mounds shows that there is great potential for further investigation into the interrelationship between soil modification by mammals, the prevalence of soil fungi and the influence of mammals on soil and fungi ecology in Australia.

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Introduction

Restoration of rangeland ecosystems requires an understanding of the functional relationships between animals and their environment, so that ecosystem processes can be restored (Werner, 1990). One such relationship is that between digging mammals and fungi. Globally, the burrows, leaf nests and latrines of digging mammals have been found to be associated with specific taxa and/or a high diversity of fungi (Sagara *et al.*, 1981, 1989, 1993; Hawkins, 1996; Whitford & Kay, 1999; Sagara, 2009). In Australia, fungi are an important food source for a range of native digging mammals (Claridge & May, 1994) and research has shown that mammals are important agents of fungal spore dispersal. For instance, the woylie *(Bettongia penicillata ogilbyi)* plays a critical role in dispersing ectomycorrhizal fungal spores in their scats, thereby performing an important ecosystem service, because the fungi form a symbiotic relationship with plants (Lamont *et al.*, 1985), facilitating nutrient and moisture uptake (Smith & Read, 1997).

The bilby *(Macrotis lagotis)* consumes fungi and disperses fungal spores in its scats (Gibson, 2001; Newell, 2008). It digs foraging pits and burrows and these accumulate moisture and nutrients and moderate microclimates (Eldridge & James, 2009; Chapman, 2013). It seems likely, therefore, that bilbies may also facilitate fungal proliferation and form associations with fungi. If so, bilbies, may play an important role in maintaining soil, plant and fungi interrelationships and contribute to rangeland restoration programs. Bilbies have been reintroduced into a rangeland restoration project in central Western Australia and research investigating their role in ecosystem function and restoration is underway (Miller *et al.*, 2010; Chapman, 2013). This paper reports on the discovery and identity of fungal mycelium found in the soil of a bilby burrow spoil-mound. It also discusses issues around identification of fungi from DNA sequence data.

Materials and Methods

The discovery took place at Lorna Glen (known locally as Matuwa), a former pastoral station located about 1,100 km northeast of Perth in central Western Australia. Bilbies were first reintroduced to Lorna Glen in 2007, as part of a rangeland restoration program (Morris & Dunlop, 2008; Miller *et al.*, 2010) and the burrow in this study (Fig. 1) was located at 121.35667 East, -26.21955 South (WGS84).

During a pilot study to compare soil properties, 12 soil samples were collected from the top 10 cm of the burrow spoil-mound and 12 from nearby undisturbed (inter-hummock) soil on 19 August 2011. The samples were oven dried at 60 ºC and then sieved through a 2 mm mesh before being sent for chemical analysis.

Mycelium-like material consisting of fine, pale brown cords, with soil particles attached (Fig. 2), was found in several of the burrow samples. The number of samples containing the material was not recorded and it was not found in any of the undisturbed soil samples (but not all of them were closely examined because the

Fig 1. A typical bilby burrow and spoil-mound at Lorna Glen. Bar = 50 cm.

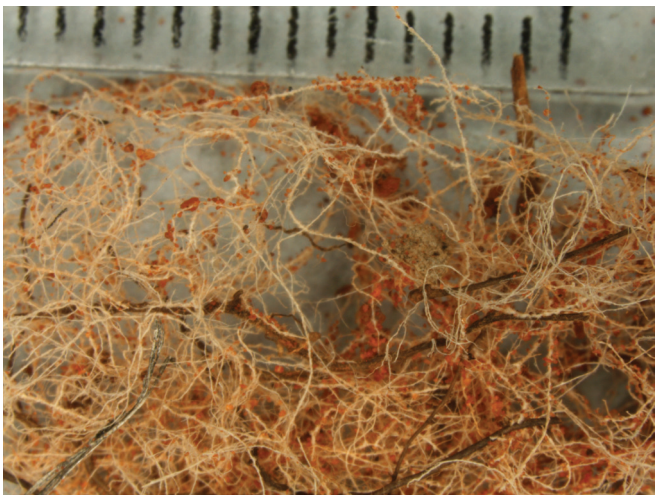

Fig 2. Mycelium and mycelial cords collected from the bilby burrow spoil-mound (scale at the top of the photograph shows 1 mm increments).

significance of the material found was unknown at the time). A sample of the material was stored in a paper seed envelope and taken to the Western Australian Herbarium for further examination on 11 October 2011. The specimen was later lodged under the accession number PERTH 08516502.

A sample of the material was sent to the Royal Botanic Gardens Melbourne for analysis of the ITS region of rDNA. Genomic DNA was isolated following a modified protocol of the EZNA Forensic DNA kit (OMEGA). Dry mycelium was first pulverised in liquid nitrogen using micropestles and incubated for one hour at 65ºC in a lysis solution including 250 µl of STL buffer, 25 µl of proteinase K and 0.8 µl of β-mercaptoethanol per sample. The procedure then followed the standard protocol for isolating DNA 'from hair, nails and feathers', except that centrifugation time was increased to at least 3 min and the final elution was done in 50 µl of buffer. The ITS regions were then amplified using the primer set ITS1-F (Gardes & Bruns, 1993) / ITS4

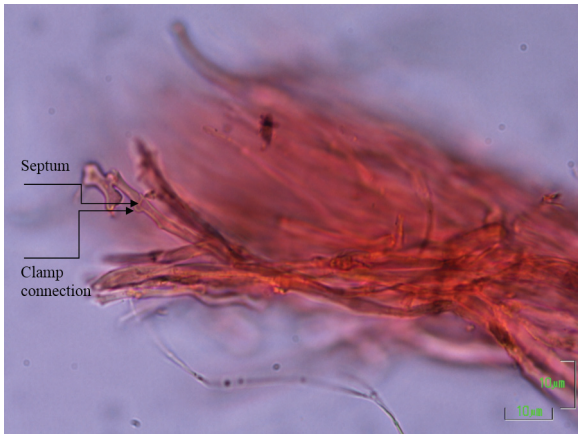

Fig 3. Photomicrograph of stained hyphae aggregated into a mycelial cord from the sample (scale shown in bottom right corner). Bar = 10 µm.

(White *et al.*, 1990). PCR mixture and thermal cycling conditions are described in Stefani *et al.* (2014). The ITS nucleotide sequence was deposited in the NCBI GenBank database under the accession KJ737436. The sequence was compared to existing sequences in Genbank using blastn (Altschul *et al.*, 1990).

Results

Hyphae with septa (cross walls) and clamp connections (Fig. 3) were identified from stained material examined using light microscopy; confirming that the material was indeed fungal mycelium. The presence of clamp connections indicated that it belonged to the Basidiomycota. The sequence (KJ737436) had 99% similarity with sequences from *Coprinopsis spilospora* (Romagn.) Redhead, Vilgalys and Moncalvo (HQ847041) [without location, but presumably from Europe] and *Coprinus subdomesticus* Murrill (HQ847038) from U.S.A., and 98% similarity with sequences from *C. alcobae* Ortega (HQ847037) [also without location but presumably from Europe] and an un-named *Coprinopsis* (JF681946) from France and another sequence of *C. spilospora* (JF907840) from Italy. The best match, with 618 of 626 base pairs being identical, was with the sequence HQ847041 of *Coprinopsis spilospora*. There were 617 base pairs identical against the *Coprinus subdomesticus* sequence. In a distance tree the two sequences of *C. spilospora* do not cluster together.

Discussion

Coprinopsis belongs to the *Psathyrella*ceae, and was formerly included in the genus *Coprinus* (now restricted to a few species within the Agaricaceae). *Coprinopsis* and other segregates of *Coprinus* in a broad sense were originally distinguished on molecular and morphological grounds by Redhead *et al.* (2001). *Coprinopsis* has been found to be monophyletic in subsequent studies,

although the position of several species of *Psathyrella* that fall within *Coprinopsis* in some analyses has not yet been resolved (Padamsee *et al.*, 2008). Although new combinations into *Coprinopsis* have not been made yet for *Coprinus subdomesticus* and *C. alcobae*, these two species, along with *Coprinopsis spilospora*, cluster together in a clade deep within *Coprinopsis* in the molecular tree of Nagy *et al.* (2012, supplementary Figure 2). Further work is obviously required on species limits in *Coprinopsis spilospora* and related species, due to different sequences under this name not clustering together. Nevertheless, it is clear that the Australian material represents a species of *Coprinopsis*. *Coprinopsis spilospora* HQ847641 is the closest match and therefore this name is applied in a general sense to the Australian material.

Morphologically, *Coprinopsis* is characterised by a pileipellis that forms a cutis and lacks pileocystidia (Redhead *et al.*, 2001; Keirle *et al.*, 2004). There are about 12 species of *Coprinopsis* reported from Australia (May *et al.*, 2013), including *Coprinopsis atramentaria* (Bull.) Redhead, Vilgalys and Moncalvo, *C. austrophlyctidospora* (Fukiharu) and *C. stangliana* (Enderle, Bender and Gröger) Redhead, Vilgalys and Moncalvo; all known as fruit-bodies. *Coprinopsis spilospora*, which was the best match to the bilby-spoil sequence, has not previously been reported from Australia. According to Uljé and Noordeloos (1997, as *Coprinus*), it is very rare and occurs in only a few localities in Europe; usually on bare, gravelly-calcareous soil, in moss beds in deciduous forests and sometimes on burnt soil. Fruit-bodies of *C. spilospora* have a pileus up to 40 mm in diameter, white at first, then grey or pale brown, with a felt-like veil. Microscopically, the veil is made up of very distinctive elements that are thick-walled and highly branched with terminal long, hair-like endings (Uljé & Noordeloos, 1997).

The ITS region is used as a 'barcode' for fungi, because the variation of the ITS is usually such that there is one, or a few, distinct sequences for each species, that do not occur in other species (Schoch *et al.*, 2012). However, there is usually some variation of the ITS within a species. The percentage difference that characterises different species varies across groups of fungi, and there is no universal 'cut-off point' for species. Stefani *et al.* (2014) showed that a 2.0% difference in the ITS characterised species of *Cortinarius* and Sheedy *et al.* (2013) found that 1.0% difference characterised species of *Laccaria*. Therefore, a similarity of 99% is strongly suggestive of identity, but not conclusive. On this basis, the material from the bilby spoil-mound should be identified as *Coprinopsis* cf. spilospora. It will be of interest to survey around bilby burrow spoil-mounds at times conducive to fruit-body formation, such as after rain, in order to obtain *Coprinopsis* fruit-bodies for examination of morphology to assist with confirmation of the species identification.

This opportunistic discovery of a fungus previously unknown to be associated with bilby burrow spoil-mounds shows that there is great potential for further investigation into the interrelationship between soil modification by mammals, the prevalence of soil fungi and the influence of mammals on soil and fungal ecology in arid Australia. This will add to our understanding of the role of mammals in maintaining ecosystem health and their potential to facilitate the restoration of former rangelands.

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