# Reintroduction of locally extinct vertebrates impacts arid soil fungal communities

Laurence J. Clarke[1,2,3], Laura S. Weyrich[1], Alan Cooper[1]

1. Australian Centre for Ancient DNA, University of Adelaide, Adelaide, SA 5005 Australia

2. Australian Antarctic Division, Channel Highway, Kingston, Tasmania 7050, Australia

3. Antarctic Climate & Ecosystems Cooperative Research Centre, University of Tasmania, Private Bag 80, Hobart, Tasmania 7001, Australia.

Keywords: Arid Recovery, coextinction, coprophilous, ecological interaction, ecosystem restoration, internal transcribed spacer I (ITS1).

Corresponding author: Laurence J. Clarke

Australian Antarctic Division, Channel Highway, Kingston, Tasmania 7050, Australia

Fax: +61 3 6232 3288

E-mail: laurence.clarke@utas.edu.au

Running title: Reintroduced vertebrates impact soil fungi.

**Abstract**

Introduced species have contributed to extinction of native vertebrates in many parts of the world. Changes to vertebrate assemblages are also likely to alter microbial communities through coextinction of some taxa and the introduction of others. Many attempts to restore degraded habitats involve removal of exotic vertebrates (livestock and feral animals) and reintroduction of locally extinct species, but the impact of such reintroductions on microbial communities is largely unknown. We used high-throughput DNA sequencing of the fungal internal transcribed spacer I (ITS1) region to examine whether replacing exotic vertebrates with reintroduced native vertebrates led to changes in soil fungal communities at a reserve in arid central Australia. Soil fungal diversity was significantly different between dune and swale (interdune) habitats. Fungal communities also differed significantly between sites with exotic or reintroduced native vertebrates after controlling for the effect of habitat. Several fungal operational taxonomic units (OTUs) found exclusively inside the reserve were present in scats from reintroduced native vertebrates, providing a direct link between the vertebrate assemblage and soil microbial communities. Our results show that changes to vertebrate assemblages through local extinctions and the invasion of exotic species can alter soil fungal communities. If local extinction of one or several species results in the coextinction of microbial taxa, the full complement of ecological interactions may never be restored.

## Introduction

Current global extinction rates are higher than the background rate estimated from the fossil record (Barnosky *et al.* 2011). However, the full ecological impacts of extinction remain unknown due to the number and complexity of potential species interactions. Species extinctions are expected to result in the loss of other species that depend on them (coextinction, Dunn *et al.* 2009). Indeed, models predict that coextinction, particularly of mutualists and parasites, may be the most common form of species loss (Dunn *et al.* 2009; Koh *et al.* 2004). However, the potential for coextinction of microorganisms remains largely unexplored.

Australia has been a hotspot of extinction in the last 200 years since European colonization. Ground dwelling mammals in the critical weight range (Burbidge & McKenzie 1989) of 35 g – 5.5 kg have largely disappeared from arid and semi-arid Australia south of the tropics (Johnson 2006). In many parts of Australia, livestock (e.g. sheep and cattle) and feral animals (e.g. European rabbits (*Oryctolagus cuniculus*), foxes (*Vulpes vulpes*), and cats (*Felis catus*)) have replaced the native mammalian fauna. Although the impact of these exotic species on Australian fauna and flora is well catalogued (e.g. Burbidge & McKenzie 1989; Cooke 2012; Johnson 2006; Read & Bowen 2001), the effect of changing the terrestrial vertebrate assemblage (replacing native mammals with exotic species) on microorganisms, including fungi and bacteria, is unknown.

Exotic or introduced vertebrates have different diets and foraging strategies compared to native Australian vertebrates, and thus are unlikely to provide the ecological services with which Australian fungal communities have co-evolved. Mycophagous vertebrates may play

65 an important role in creating, maintaining and enhancing fungal communities through the
66 dispersal of spores in scats (Claridge 2002). Fungi form a major dietary component of many
67 native Australian vertebrates (Claridge & May 1994), but not exotic pest species (cats,
68 rabbits, foxes) or domestic livestock (cattle, sheep), although exotic rodents and pigs are
69 known to disperse fungal spores (Claridge 2002; Vernes & McGrath 2009). Native and exotic
70 herbivores often have different foraging habits (e.g. Robley *et al.* 2001; Ryan *et al.* 2003), and
71 selective herbivory can also alter plant community composition that in turn impacts the
72 abundance and diversity of mycorrhizal fungi (Gehring *et al.* 2002). Indeed, exclusion of
73 terrestrial vertebrates led to a decline in mycorrhizal diversity in a rainforest soil, with
74 implications for ecosystem health (Gehring *et al.* 2002). Fungi are essential for ecosystem
75 functioning due to their roles in nutrient cycling (Read & Perez-Moreno 2003; van der Wal *et*
76 *al.* 2013), and the ability of mycorrhizal species to facilitate nutrient uptake by plants,
77 particularly important in the nutrient-poor soils of arid central Australia (Brundrett 2009). In
78 addition, bioturbation (digging, burrowing, and foraging) by vertebrates can influence fungal
79 communities by increasing soil turnover, altering soil structure and chemistry, and increasing
80 water infiltration (reviewed by Fleming *et al.* 2014). Burrows and foraging pits also capture
81 organic matter, increasing habitat heterogeneity and the number of niches for fungal
82 colonization (Garkaklis *et al.* 2000; James & Eldridge 2007). James & Eldridge (2007)
83 demonstrated that greater bilbies (*Macrotis lagotis*) and burrowing bettongs (*Bettongia*
84 *lesueur*) excavated significantly more soil in an arid landscape than rabbits and goannas, and
85 showed that reintroduced native mammals help create fertile patches.
86
87 Ecological restoration projects that incorporate reintroductions of native vertebrates, such as
88 'Arid Recovery' in central Australia, can provide insight into the ecological impacts of

species extinction or displacement (James & Eldridge 2007). In this study, we used high-throughput DNA sequencing (HTS) to investigate whether reintroduction of native terrestrial vertebrates has changed the soil fungal community in the Arid Recovery reserve. We hypothesized that fungal communities would differ between areas with only native vertebrates present (inside the reserve) and areas dominated by exotic vertebrates. The presence of dune and swale habitats at the reserve also allowed us to investigate whether vertebrate assemblage has a similar impact on fungal communities in different soil types. Lastly, we characterized coprophilous fungal communities of reintroduced native vertebrates to further explore the link between the vertebrate assemblage and soil fungi inside the Arid Recovery reserve.

## Materials and Methods

### *Site*

The Arid Recovery reserve is located 20 km north of Roxby Downs, central Australia (30º29'S, 136º53'E, Fig. 1). The climate is arid and rainfall is erratic, with a long-term average annual rainfall of 166 mm. Mean annual maximum and minimum temperatures are 35 ºC and 4 ºC, respectively (Olympic Dam Operations, 1994). The area contains two distinct habitat types, dunes and swales (Fig. S1). Dunes support open shrubland of sandhill wattle (*Acacia ligulata*) and narrow-leaved hopbush (*Dodonea viscosa*) on sandy topsoil (5-10% clay). The interdune swales have sandy clay topsoils (35-40% clay) dominated by chenopod shrubs (*Atriplex vesicaria* and *Maireana astrotricha*, James & Eldridge 2007).

Exotic vertebrates (cats, cattle, foxes, European rabbits) have been removed and excluded from a 60-km$^2$ area within the reserve since 2001. Four native vertebrates have been successfully reintroduced to the reserve: greater stick-nest rats (*Leporillus conditor*,

reintroduced in 1998), burrowing bettongs (*Bettongia lesueur*, 1999), greater bilbies (*Macrotis lagotis*, 2000), and western barred bandicoots (*Perameles bougainville*, 2001). The four reintroduced species have occasionally been observed outside the reserve, but are present at negligible densities compared to inside (Kylie Piper, Arid Recovery, personal communication). The terrestrial vertebrate fauna outside the reserve includes rabbits, cats, foxes, and emus (*Dromaius novaehollandiae*). Red kangaroos (*Macropus rufus*), goannas (*Varanus* sp.) as well as small mammals and reptiles occur both inside and outside the reserve (see Moseby *et al.* 2009; Read & Cunningham 2010 for more details).

***Sampling design***

Surface soil was collected from inside and outside the reserve, from both dune and swale habitats in August 2012 (Fig. 1). Each sample comprised 11 soil cores (*ca*. 100 mm depth, 28 mm diameter) collected along a 50 m transect at 5 m intervals. A minimum of six samples was collected for each combination of habitat (dune or swale) and treatment (inside or outside the reserve), for a total of 27 samples. Samples were stored at -20 ºC prior to extraction.

Scats were collected from the Main Exclosure (Fig. 1a) in May 2014. Scats deposited the previous night (based on appearance/moisture content; P. Carter, personal communication) were collected to minimize intrusion of environmental fungi. Scats were stored in paper bags on silica gel for four weeks prior to extraction.

***DNA extraction and PCR amplification***

Soil samples were freeze-dried and 400-450 g was sub-sampled for DNA extraction. DNA was extracted using a commercial service (SARDI Molecular Diagnostics, Urrbrae, SA,

Australia) (Ophel-Keller *et al.* 2008; Riley *et al.* 2010). Scats were dissected with a sterile scalpel and 10-120 mg of material isolated from the interior to ensure any fungi detected were endogenous to the scats. DNA was extracted from faecal material using the MoBio PowerSoil DNA Isolation kit as used for human faecal samples by the Human Microbiome Project (McInnes & Cutting 2010), following the manufacturer's instructions. Extraction blank controls were also carried through the soil and scat extractions. We identified the species that deposited each scat (burrowing bettong, greater bilby, or western barred bandicoot) by PCR-amplifying and Sanger sequencing *ca.* 100 bp of mitochondrial 12S rDNA using the primers 12SV5F and 12SV5R (Riaz *et al.* 2011).

We PCR-amplified part of the internal transcribed spacer I (ITS1) region from soil and scat samples using the ITS5 and 5.8S_fungi primers (Epp *et al.* 2012). The 5.8S_fungi primer was modified to include the Ion Torrent Primer A-key followed by a 7 bp multiplex identifier (MID) sequence (Meyer & Kircher 2010) at the 5' end, with the Primer P1-key followed by a 6 bp MID at the 5' end of the ITS5 primer. Each sample was amplified in triplicate in a reaction mix containing 2 mM $MgCl_2$, 1 mM dNTPs, 5 pmol each of forward and reverse primer, 2 µg bovine serum albumin (BSA), 0.5 U AmpliTaq Gold DNA polymerase in 1 x reaction buffer (Applied Biosystems, Melbourne, Australia), and 1 µL DNA extract in a total reaction volume of 10 µL. The thermal cycling protocol consisted of 94 ºC for 5 min, followed by 30 cycles of 94 ºC for 30 s, 54 ºC for 30 s and 72 ºC for 45 s, with a final extension at 72 ºC for 10 min.

PCR products were purified by polyethylene glycol (PEG)/NaCl precipitation with a final concentration of 9% (w/v) PEG, using Sera-Mag Carboxylate-Modified Magnetic Speed-

beads (Thermo Scientific, Waltham, Massachusetts, USA) as the solid phase (DeAngelis *et al.* 1995; Lundin *et al.* 2010). Separate amplicon libraries were made for the soil and scat PCR products by quantifying the purified PCR products using a Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, California, USA) and combining them in equimolar ratios. The concentrations of the pooled libraries were quantified on an Agilent 2200 TapeStation using High Sensitivity D1K ScreenTape and reagents (Agilent Technologies, Santa Clara, California, USA). Emulsion PCR, enrichment, and HTS were performed using the Ion Torrent OneTouch™ 2 and PGM™ (Life Technologies) as described in Clarke *et al*. (2014).

***Bioinformatics***

HTS reads for the soil and scat amplicon libraries were combined for denoising and operational taxonomic unit (OTU) picking. Amplicon Pyrosequencing Denoising Program version 1.1 (APDP) was used to process the raw reads, remove reads containing putative sequencing errors and generate a set of 'validated' sequences for further analyses (Baldwin *et al.* 2013; Bradford *et al*. 2013; Morgan *et al.* 2013). The first step in APDP was used to remove reads that did not contain an exact match to the ITS5 and 5.8S_fungi primers, as well as the forward and reverse MID tags. Reads were assigned to samples by MID tag before the primers and MID tags were trimmed from each read. In the second step, reads were clustered into taxonomic groups by a megablast search against the NCBI nucleotide (nt) database (downloaded March 2014). For each group of reads, the most abundant unique sequence was retained, as well as any sequence with >50% of the reads observed for the most abundant sequence. Three-way alignments were performed to identify and remove potential chimeric, indel, and substitution errors in each sample. Validated reads were converted into a format

184 compatible with QIIME version 1.8.0 (Caporaso *et al.* 2010) using a perl script supplied with
185 APDP for further analyses.
186
187 The 'pick_open_reference_otus.py' script was used to cluster reads and assign taxonomy
188 (Wang *et al.* 2007) against the UNITE database including global and 97% singletons (version
189 6, released 9th February 2014, http://unite.ut.ee/repository.php
190 [sh_refs_qiime_ver6_97_s_090214.fasta]) using the 'uclust' method (Edgar 2010), and to *de*
191 *novo* cluster reads with no significant hit in the database (--min_otu_size 2, --
192 suppress_align_and_tree). OTUs present in extraction blank controls were removed from the
193 data set using a series of QIIME scripts ('filter_samples_from_otu_table.py',
194 'filter_otus_from_otu_table.py' and 'biom convert'). The
195 'summarize_taxa_through_plots.py' script in QIIME was used to examine taxa associated
196 with each sample type. For OTUs not assigned taxonomy using the UNITE database, we used
197 BLAST searches (blastn algorithm) against the NCBI 'nt/nr' database to obtain putative
198 identifications. The BLAST hit with the highest bit score was retained if the taxon was
199 identified to order or higher with greater than 90% pairwise identity over at least 100 bp. The
200 observed number of species in each sample was calculated as a measure of alpha-diversity
201 based on a rarefied OTU table (17247 reads per sample). The effect of habitat, location inside
202 or outside the reserve, and their interaction on alpha-diversity in soil samples was assessed
203 using two-way ANOVA (IBM SPSS Statistics, version 21, Armonk, NY, USA).
204 Differentiation among habitats or locations was compared using Bray-Curtis and binary
205 Jaccard distances, and visualized using principal co-ordinate analysis plots
206 (beta_diversity_through_plots.py). A distance-based Redundancy Analysis (db-RDA,
207 function 'capscale') combined with an ANOVA-like permutation test (function 'anova.cca'

999 permutations) was used to assess the significance of the constraining variables habitat and location using the vegan package (Oksanen *et al.* 2013) in R version 3.0.2 (R Core Team 2013). Given the strong effect of habitat on soil fungal community (see Results), the effect of location was explored after "partialling out" habitat as a conditioning variable. Distance matrices generated in QIIME were imported into R using the qiimer package (Bittinger 2014). Using a resampling approach, we explored whether the proportion of scat OTUs also detected in soil samples exclusively inside (IN), exclusively outside (OUT), or both inside and outside the reserve (BOTH) differed from expected frequencies. We randomly sampled the observed number of soil OTUs found in scats (159) from a multinomial distribution defining the total pool of soil OTUs observed across IN, OUT and BOTH (840, in the ratio 134:96:610, see Fig. 3), with the probability of sampling an OTU weighted according to the proportion of samples it was present in for the relevant category. Sampling was repeated 5000 times, and the significance of observed scat OTU frequencies was assessed by comparing them to appropriate quantiles (alpha=0.05, two-tailed test) of the empirical distributions for each of IN, OUT and BOTH derived by resampling.

## Results

A total of 4 173 996 and 9 140 119 raw HTS reads were obtained for the soil and scat libraries, respectively. Of these, 762 476 and 1 627 086 were validated by APDP and imported into QIIME for OTU picking and further analyses.

### *Soil fungi*

We used the OTU tables generated from the APDP-validated reads to explore the influence of distinct habitats and vertebrate assemblages on soil fungal communities in the vicinity of the

Arid Recovery reserve. A total of 840 fungal OTUs were detected across the soil samples, with a greater number of fungal OTUs per sample observed in swale soil samples (224 ± 25, mean ± SD) compared to the dune habitat (182 ± 21, $F_{1,23}$=20.74, $P$<0.001, Fig. S1). There was a trend toward greater numbers of OTUs per sample inside the reserve (211 ± 29) compared to outside (195 ± 33), although this was not significant ($F_{1,23}$=2.62, $P$=0.12).

Principal co-ordinate analysis also showed strong differentiation of dune and swale communities using both Bray-Curtis ($F_{1,24}$=9.81, $P$<0.001, Fig. S2) and binary Jaccard distance measures ($F_{1,24}$=8.50, $P$<0.001, Fig. 2). We therefore analysed the effect of location (inside versus outside) on community composition after partialling out the effect of habitat. Although analyses based on Bray-Curtis distance showed no significant effect of location after accounting for the effect of habitat ($F_{1,24}$=1.21, $P$=0.19), analyses based on presence-absence data (binary Jaccard) revealed a significant difference between fungal communities inside and outside the reserve ($F_{1,24}$=1.30, $P$=0.016). These results suggest that overall community structure is similar between locations in each habitat (based on the Bray-Curtis distance), but that sites inside and outside the reserve support unique species.

More than 200 OTUs were found exclusively either inside or outside the reserve (Fig. 3). Many reads in all four combinations of habitat and location were not assigned taxonomy (mean = 17.8%) or could not be identified below kingdom level using the UNITE database (mean = 38.2%). When unidentified reads were excluded, Ascomycota represented the majority of reads in all samples (mean ± SD, 94 ± 4%), followed by Basidiomycota (5 ± 4%) consistent with the relative abundance of fungal phyla in other dry environments (Porras-Alfaro *et al.* 2011; Timling *et al.* 2014). However, unidentified reads could play a vital role in

fungal soil ecology. We therefore used BLAST searches to explore the taxonomic affinity of 24 OTUs typically found either inside or outside the reserve and that were present in four or more samples (Table 1), as OTUs present in multiple samples are less likely to represent stochastic sampling effects (see Supporting Information). Of these, eight (33.3%) OTUs were found exclusively in swale samples, while all other OTUs were shared between habitat types. Although putative IDs were obtained for only 14 OTUs, six of these (43%) were similar (≥93% ID) to known coprophilous taxa, including *Sporormia*, *Preussia*, and *Cercophora* sp., or to OTUs isolated from mammalian herbivore dung (Herrera *et al.* 2011). Other OTUs found predominantly either inside or outside the reserve were identified as saprotrophs, endophytes, and dematiaceous species associated with arid environments (e.g. *Embellisia* sp.).

*Fungi present in scats*

We characterized coprophilous fungal communities of reintroduced native vertebrates to further explore the link between the vertebrate assemblage and soil fungal communities inside the Arid Recovery reserve. The majority of the scats collected were from burrowing bettongs (15/18, 83.3%, Table S1), which reflects the relative abundance of this species within the reserve. Fungal profiles from western barred bandicoot and greater bilby scats were the most similar to the soil samples (Fig. 4), presumably due to the presence of soil within scats from these species (L.J. Clarke, personal observation). However, OTU profiles from scats were distinct from soil samples, and were often dominated by *Preussia* sp. (mean ± SD, 56 ± 29% reads per scat). The 18 scats assessed yielded 191 OTUs. Of these, 159 were also found in soil samples, with significantly more OTUs from the scats found only in soil samples from inside the reserve than expected by chance (21 OTUs, $P<0.05$, Fig. 3). Of the 21 OTUs, six were identified as coprophilous, three as endophytes and six as soil fungi (Table 2). Although

10 scat OTUs were also detected in soil samples exclusively outside the reserve, this did not differ from the expected frequency ($P$>0.10), with each of these OTUs typically present in only 1-2 samples (mean ± SD, 1.5 ± 0.7).

## Discussion

Although the negative impact of exotic species on the Australian flora and fauna is well documented, the effect of changing vertebrate assemblages on the microbiota has not been characterized to date despite their critical role in maintaining ecosystem health (Brundrett 2009; Read & Perez-Moreno 2003; van der Wal *et al.* 2013). Our results suggest that changes to terrestrial vertebrate assemblages in arid environments can directly lead to changes in soil fungal communities. Several fungal OTUs were detected exclusively either inside or outside the Arid Recovery reserve, including several identified as coprophilous taxa. Analysing fungal diversity in scats from reintroduced native vertebrates revealed the presence of many fungal OTUs found in the soil samples, including several found only inside the reserve, providing a direct link between the reintroduced species and soil fungal communities. Although Gehring *et al.* (2002) demonstrated that excluding terrestrial vertebrates altered arbuscular myccorhizal fungal communities in a rainforest, our study is the first to demonstrate that replacing an exotic terrestrial vertebrate assemblage with native species can also impact soil fungal communities in arid zones. This suggests that reintroduction of native vertebrates can potentially contribute to ecosystem restoration by altering soil fungal communities.

We found soil fungal communities differed between sites with reintroduced native vertebrates and areas dominated by exotic vertebrates with presence-absence (binary Jaccard) but not

abundance-based (Bray-Curtis) distance measures. DNA is subject to taphonomy in arid soils and is likely to skew the observed abundance of some taxa when using a HTS approach (e.g. Adler *et al.* 2013). Unweighted (presence-absence) distance measures are potentially more reliable in such circumstances (Adler *et al.* 2013). We acknowledge that our sampling represents a single time point, and that the soil fungal community composition is likely to change following substantial rainfall events, for example. However, we suspect that rainfall is more likely to alter the relative abundance rather than presence-absence of fungal taxa, thus we feel the observed difference in soil fungi inside and outside the reserve would be robust to temporal sampling. Similarly, dune and swale fungal communities were distinct with both presence-absence and abundance-based distance measures, hence should also be robust to changes in abundance due to rainfall.

The detection of several OTUs exclusively inside the Arid Recovery reserve and in scats from reintroduced vertebrates suggests loss of terrestrial vertebrates through range contraction or extinction could lead to coextinction of microbial taxa such as fungi. Gehring *et al.* (2002) found a decrease in arbuscular myccorhizal fungal species richness on exclusion of vertebrates from rainforest plots, suggesting some fungal taxa had become locally extinct with the loss of vertebrates. We also found a trend towards increased numbers of fungal OTUs inside the reserve compared to outside, suggesting habitats with reintroduced native vertebrates could support greater fungal diversity. Our relatively small survey of scat fungi, with only three scats from species other than burrowing bettongs, showed 21.6% (149/690) of fungal OTUs from inside the reserve were associated with native scats, including 15.7% (21/134) of OTUs found exclusively inside the reserve, demonstrating the close association between the vertebrate and soil fungal communities. If further research were to confirm that

these fungi do not associate with exotic vertebrates and are in fact dependent on native vertebrates, e.g. for dispersal, they would be at risk of coextinction if these vertebrates become locally extinct. As coextinction is predicted to be the most common form of species loss (Dunn *et al.* 2009; Koh *et al.* 2004), it is likely that microbial taxa other than fungi became extinct with the loss of many native mammals from large tracts of arid and semi-arid Australia (Johnson 2006). Similar studies using bacterial 16S or eukaryotic 18S markers would reveal whether altering terrestrial vertebrate assemblages has similar impacts on the broader soil eukaryotic or prokaryotic community.

There are a number of potential ecological interactions between vertebrates and fungi, including mycophagy, dispersal and creating niches for fungal growth (Fleming *et al.* 2014). Fungi may be used as a food resource by vertebrates or consumed incidentally. For example, reintroduced vertebrates are likely to consume endophytic fungi in plant material (Herrera *et al.* 2011) or incidentally consume fungi with soil during foraging (Newell 2008). Morphological analysis of scats has demonstrated mycophagy by reintroduced vertebrates at the Arid Recovery reserve; greater bilby scats contained up to 46% fungal spores by volume (Bice & Moseby 2008; Newell 2008). Regardless of whether fungi are used as a food resource or consumed incidentally with plant material or soil, our results suggest reintroduced vertebrates are contributing to dispersal of soil fungi in their scats. Interestingly, the detection of two coprophilous OTUs predominantly in soil from outside the reserve (Table 1) suggests exotic vertebrate assemblages are also influencing soil fungal communities in arid central Australia. Comparing fungal taxa in scats of native and exotic terrestrial vertebrates could be used to explore whether these taxa are functionally equivalent in terms of fungal dispersal. For example, scats from native wallabies and exotic black rats contained significantly

352 different suites of fungal taxa, hence these vertebrates could play complementary roles in
353 fungal dispersal (Vernes & McGrath 2009). As well as dispersal through mycophagy,
354 vertebrates could contribute to dispersal of fungi by disturbing soil during digging,
355 burrowing, and foraging. Burrows and foraging pits can in turn capture organic matter and
356 create niches for fungal colonization (Garkaklis *et al.* 2000; James & Eldridge 2007). It is
357 possible that comparing fungal communities in burrows and pits created by exotic and native
358 vertebrates could indicate a greater impact of vertebrate assemblage than the broad habitat
359 surveys performed in this study.
360
361 Ecological interactions between reintroduced native mammals and other taxa are likely to
362 have broader repercussions for the ecosystem into the future. At the time of sampling, the four
363 native mammals had been present at Arid Recovery for just over a decade. Although we did
364 not detect a difference in the overall fungal communities inside and outside the reserve (i.e.
365 using distance measures based on abundance), our results did identify fungal species in the
366 native scat that were also present inside the reserve. This indicates that the wholesale
367 replacement of vertebrate assemblages, as has occurred in many parts of the world, can
368 change the total composition of microbial communities. Furthermore, it is likely that the
369 impact of altered vertebrate assemblages will increase with time. Reintroduction of native
370 vertebrates has improved recruitment of three native shrubs (*Senna artemisioides* and two
371 *Acacia* species) inside the Arid Recovery reserve (Munro *et al.* 2009). As these plants
372 typically form arbuscular mycorrhizal associations (Brundrett 2008), future changes to plant
373 communities inside the reserve are likely to lead to further changes in fungal communities.
374 Changes to the fungal community may in turn alter important ecosystem processes such as
375 nutrient cycling (Read & Perez-Moreno 2003; van der Wal *et al.* 2013). This study thus

represents a first step in understanding the repercussions of local extinctions and reintroductions on microbial communities.

**Acknowledgements**

Thanks to Arid Recovery for allowing us to access and sample sites at the reserve. Jennifer Young helped collect soil samples, Perri Carter (Arid Recovery) and participants in the Port Lincoln High School Indigenous Ranger Cadetship program collected scats. Steve Delean and John McKinlay provided advice on experimental design and statistical analysis. Matthew Morgan assisted with processing HTS reads with APDP. We thank the ARC for funding and ACAD members for helpful comments and assistance.

**References**

Adler CJ, Dobney K, Weyrich LS, *et al.* (2013) Sequencing ancient calcified dental plaque shows changes in oral microbiota with dietary shifts of the Neolithic and Industrial revolutions. *Nature Genetics* **45**, 450-455.

Anderson MJ (2005) PERMANOVA: a FORTRAN computer program for permutational multivariate analysis of variance. Department of Statistics, University of Auckland, New Zealand.

Baldwin DS, Colloff MJ, Rees GN, *et al.* (2013) Impacts of inundation and drought on eukaryote biodiversity in semi-arid floodplain soils. *Molecular Ecology* **22**, 1746-1758.

Barnosky AD, Matzke N, Tomiya S, *et al.* (2011) Has the Earth's sixth mass extinction already arrived? *Nature* **471**, 51-57.

Bice J, Moseby K (2008) Diets of the re-introduced greater bilby (*Macrotis lagotis*) and burrowing bettong (*Bettongia lesueur*) in the Arid Recovery reserve, northern South Australia. *Australian Mammalogy* **30**, 1-12.

Bittinger K (2014) qiimer: Work with QIIME Output Files in R. R package version 0.9.2.

Bradford TM, Morgan MJ, Lorenz Z, *et al.* (2013) Microeukaryote community composition assessed by pyrosequencing is associated with light availability and phytoplankton primary production along a lowland river. *Freshwater Biology* **58**, 2401-2413.

Brundrett MC (2008) *Australian plants.* mycorrhizas.info

Brundrett MC (2009) Mycorrhizal associations and other means of nutrition of vascular plants: understanding the global diversity of host plants by resolving conflicting information and developing reliable means of diagnosis. *Plant and Soil* **320**, 37-77.

411 Burbidge AA, McKenzie NL (1989) Patterns in the modern decline of Western Australia's
412 vertebrate fauna: causes and conservation implications. *Biological Conservation* **50**,
413 143-198.
414 Caporaso JG, Kuczynski J, Stombaugh J, *et al.* (2010) QIIME allows analysis of high-
415 throughput community sequencing data. *Nat Meth* **7**, 335-336.
416 Claridge AW (2002) Ecological role of hypogeous mycorrhizal fungi in Australian forests
417 and woodlands. *Plant and Soil* **244**, 291-305.
418 Claridge AW, May TW (1994) Mycophagy among Australian mammals. *Australian Journal*
419 *of Ecology* **19**, 251-275.
420 Clarke LJ, Soubrier J, Weyrich LS, Cooper A (2014) Environmental metabarcodes for
421 insects: *in silico* PCR reveals potential for taxonomic bias. *Molecular Ecology*
422 *Resources*, doi: 10.1111/1755-0998.12265.
423 Cooke BD (2012) Rabbits: manageable environmental pests or participants in new Australian
424 ecosystems? *Wildlife Research* **39**, 279-289.
425 DeAngelis MM, Wang DG, Hawkins TL (1995) Solid-phase reversible immobilization for the
426 isolation of PCR products. *Nucleic Acids Research* **23**, 4742-4743.
427 Dunn RR, Harris NC, Colwell RK, Koh LP, Sodhi NS (2009) The sixth mass coextinction:
428 are most endangered species parasites and mutualists? *Proceedings of the Royal*
429 *Society of London Series B: Biological Sciences* **276**, 3037-3045.
430 Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST.
431 *Bioinformatics* **26**, 2460-2461.
432 Epp LS, Boessenkool S, Bellemain EP, *et al.* (2012) New environmental metabarcodes for
433 analysing soil DNA: potential for studying past and present ecosystems. *Molecular*
434 *Ecology* **21**, 1821-1833.
435 Fleming PA, Anderson H, Prendergast AS, *et al.* (2014) Is the loss of Australian digging
436 mammals contributing to a deterioration in ecosystem function? *Mammal Review* **44**,
437 94-108.
438 Garkaklis MJ, Bradley JS, Wooller RD (2000) Digging by vertebrates as an activity
439 promoting the development of water-repellent patches in sub-surface soil. *Journal of*
440 *Arid Environments* **45**, 35-42.
441 Gehring CA, Wolf JE, Theimer TC (2002) Terrestrial vertebrates promote arbuscular
442 mycorrhizal fungal diversity and inoculum potential in a rain forest soil. *Ecology*
443 *Letters* **5**, 540-548.
444 Herrera J, Poudel R, Khidir HH (2011) Molecular characterization of coprophilous fungal
445 communities reveals sequences related to root-associated fungal endophytes.
446 *Microbial Ecology* **61**, 239-244.
447 Jacobson KM (1997) Moisture and substrate stability determine VA-mycorrhizal fungal
448 community distribution and structure in an arid grassland. *Journal of Arid*
449 *Environments* **35**, 59-75.
450 James AI, Eldridge DJ (2007) Reintroduction of fossorial native mammals and potential
451 impacts on ecosystem processes in an Australian desert landscape. *Biological*
452 *Conservation* **138**, 351-359.
453 Johnson C (2006) *Australia's mammal extinctions: a 50 000 year history* Cambridge
454 University Press, Port Melbourne, Australia.
455 Koh LP, Dunn RR, Sodhi NS, *et al.* (2004) Species coextinctions and the biodiversity crisis.
456 *Science* **305**, 1632-1634.
457 Kõljalg U, Nilsson RH, Abarenkov K, *et al.* (2013) Towards a unified paradigm for sequence-
458 based identification of fungi. *Molecular Ecology* **22**, 5271-5277.

Lundin S, Stranneheim H, Pettersson E, Klevebring D, Lundeberg J (2010) Increased throughput by parallelization of library preparation for massive sequencing. *PLoS ONE* **5**, e10029.
McInnes P, Cutting M (2010) *Manual of procedures for Human Microbiome Project: Core microbiome sampling Protocol A, HMP Protocol # 07-001, version number: 12.0.* Available at http://www.hmpdacc.org/doc/HMP_MOP_Version12_0_072910.pdf
Meyer M, Kircher M (2010) Illumina sequencing library preparation for highly multiplexed target capture and sequencing. *Cold Spring Harbor Protocols* doi:10.1101/pdb.prot5448.
Morgan MJ, Chariton AA, Hartley DM, Court LN, Hardy CM (2013) Improved inference of taxonomic richness from environmental DNA. *PLoS ONE* **8**, e71974.
Moseby KE, Hill BM, Read JL (2009) Arid Recovery – A comparison of reptile and small mammal populations inside and outside a large rabbit, cat and fox-proof exclosure in arid South Australia. *Austral Ecology* **34**, 156-169.
Munro NT, Moseby KE, Read JL (2009) The effects of browsing by feral and re-introduced native herbivores on seedling survivorship in the Australian rangelands. *The Rangeland Journal* **31**, 417-426.
Newell J (2008) *The Role of the Reintroduction of Greater Bilbies (Macrotis lagotis) and Burrowing Bettongs (Bettongia lesueur) in the Ecological Restoration of an Arid Ecosystem: Foraging Diggings, Diet, and Soil Seed Banks* Ph.D., University of Adelaide.
Oksanen J, Blanchet FG, Kindt R, *et al.* (2013) vegan: Community Ecology Package. R package version 2.0-10.
Ophel-Keller K, McKay A, Hartley D, Curran H, Curran J (2008) Development of a routine DNA-based testing service for soilborne diseases in Australia. *Australasian Plant Pathology* **37**, 243-253.
Porras-Alfaro A, Herrera J, Natvig DO, Lipinski K, Sinsabaugh RL (2011) Diversity and distribution of soil fungal communities in a semiarid grassland. *Mycologia* **103**, 10-21.
R Core Team (2013) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
Read DJ, Perez-Moreno J (2003) Mycorrhizas and nutrient cycling in ecosystems – a journey towards relevance? *New Phytologist* **157**, 475-492.
Read J, Bowen Z (2001) Population dynamics, diet and aspects of the biology of feral cats and foxes in arid South Australia. *Wildlife Research* **28**, 195-203.
Read JL, Cunningham R (2010) Relative impacts of cattle grazing and feral animals on an Australian arid zone reptile and small mammal assemblage. *Austral Ecology* **35**, 314-324.
Riaz T, Shehzad W, Viari A, *et al.* (2011) ecoPrimers: inference of new DNA barcode markers from whole genome sequence analysis. *Nucleic Acids Research* **39**, e145.
Riley IT, Wiebkin S, Hartley D, McKay AC (2010) Quantification of roots and seeds in soil with real-time PCR. *Plant and Soil* **331**, 151-163.
Robley AJ, Short J, Bradley S (2001) Dietary overlap between the burrowing bettong (*Bettongia lesueur*) and the European rabbit (*Oryctolagus cuniculus*) in semi-arid coastal Western Australia. *Wildlife Research* **28**, 341-349.
Ryan SA, Moseby KE, Paton DC (2003) Comparative foraging preferences of the greater stick-nest rat (*Leporillus conditor*) and the European rabbit (Oryctolagus cuniculus): implications for regeneration of arid lands. *Australian Mammalogy* **25**, 135-146.

Timling I, Walker DA, Nusbaum C, Lennon NJ, Taylor DL (2014) Rich and cold: diversity, distribution and drivers of fungal communities in patterned-ground ecosystems of the North American Arctic. *Molecular Ecology* **23**, 3258-3272.

van der Wal A, Geydan TD, Kuyper TW, de Boer W (2013) A thready affair: linking fungal diversity and community dynamics to terrestrial decomposition processes. *FEMS Microbiology Reviews* **37**, 477-494.

Vernes K, McGrath K (2009) Are introduced black rats (*Rattus rattus*) a functional replacement for mycophagous native rodents in fragmented forests? *Fungal Ecology* **2**, 145-148.

Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology* **73**, 5261-5267.

## Data accessibility

High throughput sequencing reads (FASTQ) for the soil and scat libraries and the OTU table from the 'pick_open_reference_otus.py' script (including soil and scat OTUs) are available from Dryad Digital Repository, doi:10.5061/dryad.g1q85.

## Author contributions

L.C. conducted laboratory work and led the analysis and writing. All authors contributed to designing the study and editing the manuscript.

## Figure legends

**Figure 1.** Location of the Arid Recovery reserve in central Australia, and map of fence lines and sampling sites inside and outside the reserve (a). Representative dune (b) and swale (c) habitat at the Arid Recovery reserve. Photo credits: Arid Recovery.

**Figure 2.** Principle co-ordinate analysis (PCoA) plots using binary Jaccard distance of soil fungal communities in the vicinity of the Arid Recovery reserve, central Australia. The equivalent PCoA plots using Bray-Curtis distance are available in the Supporting Information.

**Figure 3.** Area-proportional Venn diagram comparing OTU richness in soil samples from inside the Arid Recovery reserve, outside the reserve and in scats of mammals reintroduced to the reserve.

**Figure 4.** Principle co-ordinate analysis (PCoA) plots using binary Jaccard distance of fungal communities from scat of reintroduced native mammals and soil in the vicinity of the Arid Recovery reserve, central Australia.

**Table 1.** Distribution and putative identities based on BLAST results using the NCBI nr/nt database for soil fungal OTUs found predominantly either inside or outside the Arid Recovery reserve. Accession numbers, percentage pairwise identity and alignment length, name and taxonomic classification of the most similar sequence identified to order or lower are listed. The species hypothesis (SH, Kõljalg *et al.* 2013) is provided where available. Only OTUs present in four or more samples are listed. *Top BLAST hit.

| OTU | Putative ID | Distribution | | | | % identity | Alignment length (bp) | Accession | Phylum | Order | SH | Notes |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Inside | | Outside | | | | | | | | |
| | | Swale | Dune | Swale | Dune | | | | | | | |
| **Inside** | | | | | | | | | | | | |
| 316 | Unidentified fungus | 6 | 4 | 0 | 0 | - | - | - | - | - | - | |
| 816 | Unidentified fungus | 5 | 3 | 2 | 0 | - | - | - | - | - | - | |
| 824 | Pleosporales sp. | 6 | 2 | 1 | 1 | 99 | 165 | GU911103.1 | Ascomycota | Pleosporales | - | Coprophilous |
| 473 | Unidentified fungus | 6 | 2 | 0 | 1 | - | - | - | - | - | - | |
| 337 | *Keissleriella trichophoric* | 6 | 1 | 1 | 0 | 96 | 165 | KJ869113.1 | Ascomycota | Pleosporales | - | Endophyte |
| 860 | *Sporormia subticinensis* | 3 | 3 | 1 | 0 | 93 | 164 | AY943051.1 | Ascomycota | Pleosporales | - | Coprophilous |
| 703 | Pleosporales sp.* | 4 | 2 | 0 | 0 | 94 | 175 | JF449883.1 | Ascomycota | Pleosporales | - | European beech leaf litter |
| 186 | *Embellisia* sp. | 3 | 2 | 0 | 1 | 98 | 196 | JN578612.1 | Ascomycota | Pleosporales | - | Endophytic, dematiaceous |
| 327 | *Geastrum minimum** | 5 | 0 | 1 | 0 | 90 | 207 | EU784238.1 | Basidiomycota | Geastrales | - | Saprotroph, earthstar mushroom |
| 395 | Unidentified fungus | 5 | 0 | 1 | 0 | - | - | - | - | - | - | |
| 697 | Unidentified fungus | 5 | 0 | 1 | 0 | - | - | - | - | - | - | |
| 522 | Unidentified fungus | 4 | 1 | 0 | 1 | - | - | - | - | - | - | |
| 737 | *Preussia* sp.* | 1 | 4 | 1 | 0 | 99 | 182 | JN418774.1 | Ascomycota | Pleosporales | - | Coprophilous, endophyte |
| 460 | Unidentified fungus | 4 | 1 | 0 | 0 | - | - | - | - | - | - | |
| 18 | *Thelebolus* sp. | 2 | 2 | 0 | 0 | 100 | 195 | AB916508.1 | Ascomycota | Thelebolales | SH017132.06F | Psycrophilic fungi from feather |
| 793 | Capnodiales sp. | 4 | 0 | 0 | 0 | 93 | 183 | GU910741.1 | Ascomycota | Capnodiales | - | Coprophilous |
| 471 | Unidentified fungus | 4 | 0 | 0 | 0 | - | - | - | - | - | - | |
| 651 | *Fimetariella rabenhorstii* | 4 | 0 | 0 | 0 | 94 | 200 | JX421715.1 | Ascomycota | Sordariales | - | Endophyte |
| 864 | Unidentified fungus | 3 | 1 | 0 | 0 | - | - | - | - | - | - | |
| **Outside** | | | | | | | | | | | | |
| 455 | *Preussia* sp.* | 1 | 0 | 3 | 2 | 93 | 169 | JN418771.1 | Ascomycota | Pleosporales | - | Coprophilous, endophyte |
| 27 | *Peziza polaripapulata* | 0 | 0 | 4 | 0 | 93 | 104 | JF908570.1 | Ascomycota | Pezizales | SH020131.06F | Saprotroph |
| 680 | *Tetracladium* sp. | 0 | 0 | 2 | 2 | 99 | 205 | KC785565.1 | Ascomycota | Mitosporic Ascomycota | - | Saprotroph, endophyte |
| 733 | *Cercophora caudata** | 0 | 0 | 1 | 3 | 95 | 170 | AY999135.1 | Ascomycota | Sordariales | - | Coprophilous |
| 822 | Unidentified fungus | 0 | 0 | 4 | 0 | - | - | - | - | - | - | |

**Table 2.** Distribution and putative identities based on BLAST results using the NCBI nr/nt database for fungal OTUs found exclusively inside the Arid Recovery reserve and in scats of native mammals reintroduced to the reserve. Accession numbers, percentage pairwise identity and alignment length, name and taxonomic classification of the most similar sequence identified to order or lower are listed. The species hypothesis (SH, Kõljalg *et al.* 2013) is provided where available. *Top BLAST hit.

| OTU | Putative ID | Distribution | | | | Species (no. scats) | % identity | Alignment length (bp) | Accession | Phylum | Order | SH | Notes |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Inside | | Outside | | | | | | | | | |
| | | Swale | Dune | Swale | Dune | | | | | | | | |
| 460 | Unidentified fungus | 4 | 1 | 0 | 0 | Bandicoot (1) | - | - | - | - | - | - | |
| 18 | *Thelebolus* sp.* | 2 | 2 | 0 | 0 | Bettong (11) | 100 | 195 | AB916508.1 | Ascomycota | Thelebolales | SH017132.06FU | Psycrophilic fungi from feather |
| 105 | *Rhizopus oryzae** | 0 | 3 | 0 | 0 | Bettong (1) | 100 | 225 | KF225032.1 | Incertae sedis | Mucorales | SH026234.06FU | Saprotrophic |
| 153 | *Aspergillus* sp.* | 1 | 2 | 0 | 0 | Bettong (1) | 99 | 175 | KF923733.1 | Ascomycota | Eurotiales | SH034792.06FU | Saprotroph, endophyte |
| 262 | *Cryptococcus antarcticus* | 2 | 0 | 0 | 0 | Bettong (12) | 93 | 192 | JX681815.1 | Basidiomycota | Filobasidiales | - | Soil fungi |
| 352 | Thelebolales sp. | 0 | 2 | 0 | 0 | Bettong (9) | 99 | 172 | GU910336.1 | Ascomycota | Thelebolales | - | Coprophilous |
| 814 | Pleosporales sp.* | 2 | 0 | 0 | 0 | Bettong (4) | 100 | 139 | AY546016.1 | Ascomycota | Pleosporales | - | Endophytic |
| 324 | *Thelebolus* sp.* | 1 | 1 | 0 | 0 | Bettong (2) | 99 | 195 | AB916508.1 | Ascomycota | Thelebolales | - | Psycrophilic fungi from feather |
| 36 | *Cryptococcus albidus** | 1 | 1 | 0 | 0 | Bettong (2) | 100 | 187 | KC254020.1 | Basidiomycota | Filobasidiales | SH030427.06FU | Soil fungi |
| 756 | Unidentified fungus | 0 | 2 | 0 | 0 | Bettong (1) | - | - | - | - | - | - | |
| 701 | *Fusarium* sp. | 2 | 0 | 0 | 0 | Bilby (1) | 97 | 182 | KJ472205.1 | Ascomycota | Hypocreales | - | Soil fungi, saprotroph |
| 12 | Pleosporales sp. | 0 | 1 | 0 | 0 | Bettong (13) | 97 | 201 | KC438394.1 | Ascomycota | Pleosporales | SH026554.06FU | Coprophilous |
| 23 | Pleosporales sp.* | 0 | 1 | 0 | 0 | Bettong (3) | 100 | 191 | KC438389.1 | Ascomycota | Pleosporales | SH014099.06FU | Coprophilous |
| 71 | *Chaetomium* sp. | 1 | 0 | 0 | 0 | Bandicoot, bettong (2) | 99 | 199 | EU750691.1 | Ascomycota | Sordariales | SH038047.06FU | Soil fungi, saprotroph, dematiaceous |
| 560 | *Stilbella fimetaria** | 1 | 0 | 0 | 0 | Bandicoot (1) | 99 | 197 | AY952467.1 | Ascomycota | Hypocreales | - | Coprophilous |
| 313 | *Sporormiella megalospora* | 1 | 0 | 0 | 0 | Bandicoot (1) | 100 | 201 | GQ203785.1 | Ascomycota | Pleosporales | - | Coprophilous |
| 844 | *Phoma* sp. | 1 | 0 | 0 | 0 | Bettong (1) | 93 | 234 | JQ247366.1 | Ascomycota | Mitosporic Ascomycota | - | Soil fungi |
| 286 | Unidentified fungus | 1 | 0 | 0 | 0 | Bettong (1) | - | - | - | - | - | - | |
| 35 | *Preussia* sp.* | 1 | 0 | 0 | 0 | Bettong (1) | 100 | 176 | JX624309.1 | Ascomycota | Pleosporales | SH000617.06FU | Coprophilous, endophyte |
| 815 | Unidentified fungus | 0 | 1 | 0 | 0 | Bettong (1) | - | - | - | - | - | - | |
| 129 | *Trichosporon* sp.* | 0 | 1 | 0 | 0 | Bilby (1) | 100 | 150 | KC254108.1 | Basidiomycota | Tremellales | SH020706.06FU | Soil fungi |