# Final report for project US64C

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## 1. Outline/Background

Biological "Soil health" is a highly complex issue. In broad terms, biologically active soils are robust and resilient to change. Microbes produce complex arrays of metabolites, each species contributing to, and interacting with a slightly different array of processes in soil. Moreover, biologically active soil may confer a number of specific functions. These potentially include plant growth enhancement, carbon sequestration, toxin degradation, mineral cycling and pathogen suppression.

In the last three years methods to measure and quantify the soil biology via profiling of the microbial community have changed dramatically. Previously, soil microbial diversity was measured by inference using techniques which include direct counts, Fatty Acid Methyl Ester (FAME) profiles, Biolog substrate utilisation, and biomass (see Schutter et al 2001). In more recent studies, molecular DNA based measures of microbial diversity and community composition have become more common. Molecular techniques too have their limitations and analyses that include both molecular and other methods may provide a more holistic view of soil biology and function.

## 2. List the project objectives and extent to which these have been achieved

Our original application had the following primary objectives.

a) *Developments of molecular methods*
Molecular methods were developed to characterise "soil health" as related to microbial biodiversity. Pre-DNA extraction storage of soil, optimisation of DNA extractions and two community profiling methods (TRFLP & ARDRA – see Section 3) were successfully tested and optimised for soils used for cotton cropping.

b) *Test the tools in the field*
The methods developed above were used to assay archaeal, bacterial and fungal community diversity and structure in cotton growing soils and soils used for cotton cropping that are currently under different land management regimes eg: fallows, rotations, pasture, organic practices and natural or regenerating soils. The molecular tools were successfully tested on field soils and soils under other management practices.

c) Design the tools for wider use
The molecular tools developed are robust and can be used to measure microbial diversity of selected microbial groups in cotton cropping soils.

## 3. Methodology

A number of methods for profiling the microbial community in soils used for cotton cropping were investigated:

*TRFLP*

Terminal restriction fragment length polymorphism (TRFLP) is a robust measure of community diversity and composition. TRFLP was used extensively in this project to assess total community diversity and changes in composition. TRFLP is inexpensive and reasonably simple to perform on large numbers of samples. TRFLP does not allow TRFs to be identified (except as "peaks") and as such it is possible to note changes in diversity and compostion but not possible to identify which species are changing within the community.

*ARDRA*

Amplified ribosomal DNA restriction analysis (ARDRA) is a clone screening approach to profiling community diversity and composition. Like TRFLP it is a robust measure of diversity and has the additional advantage of identifying (via DNA sequencing) the species which are changing in the community. ARDRA is a much more laborious and expensive technique than TRFLP.

In the project US64C we sucessfully used TRFLP and ARDRA as our primary methods to profile the microbial communities in cotton cropping and associated soils of the Namoi valley. Prior to community profiling by these methods DNA is extracted from soil. DNA extraction from soils can be difficult and quantities of DNA recovered variable. Several improvements were made to the DNA extraction method and pre-extraction storage of soil to allow more accurate profiling of soil microbial communities.

*Field sites*

We examined the microbial community at a number of field sites. They are discussed in brief below.

1. **ACRI**

Three sites at ACRI were investigated; these were: **CW** (Field 4 Cotton-Wheat rotation), **FF** (Field 4 Seven year fallow experimental plot), **ADJ** (a weedy area next to field 4). Additional collections were made from field 4: **CC** (Continuous cotton), **CV** (Cotton-vetch), **CN** (Cotton canola) though examination/profiling of these soils was more limited. Where these soils were profiled they were generally not significantly different to CW.

*Justification:*

The CW field site was regarded as typical cotton-cropping system. The ADJ site was chosen as it is directly adjacent to CW and is not cropped. Unfortunately, the ADJ site has had considerable disturbance with a history of clearing and additions of gravel/roadbase to the soil profile. The experimental FF site was an enforced 7-year fallow chosen as a site under considerable disturbance. It was hypothesized that the absence of plants, tillage, irrigation and irradition would make this a biologically depauperate site.

2. **Brigalow Park Nature Reserve.**

Much of the Narrabri cotton growing region once supported brigalow woodlands. The site was chosen as a relatively undisturbed natural site for comparison and as a potential repository of organisms of interest.

*Justification*

As the ADJ site does not provide an undisturbed control for comparison – several other sites were chosen for analysis. Brigalow Park Nature Reserve is 22km (by road) SW of Narrabri, on similar black, cracking clay soils to ACRI and is under similar rainfall and temperature regimes.

### 3. Claremont Nature Reserve

Claremont is a partially revegetated natural grassland, in the process of reversion to brigalow woodland.

*Justification*

As the ADJ site does not provide an undisturbed control for comparison – several other sites were chosen for analysis. Claremont Nature Reserve is 22km (by road) SW of Narrabri, on superifically similar black, cracking clay soils to ACRI and is under similar rainfall and temperature regimes. Its regenerating status was of interest, as it was thought it may provide some insights into the changes in microbial as a system moved from an pastural to a natural system.

### 4. Outgroup soils

These were two sites chosen on differing soil-types, but under similar abiotic conditions (eg: similar rainfall, temperatures etc). The sites were **JC**, a pilliga eucalypt woodland and **WAR**, a herbaceous field at Warrumbungles National Park. While the JC site is relatively undisturbed wooland (limited logging has occured in the area), the WAR site is a partially revegetated agricultural site. In the 1950s the WAR site was gazetted by National Parks and Wildlife and has not been cropped since this time.

*Justification*

The Jack's Creek site (JC) is ~25km (by road) SSW of Narrabri, on sandy free-draining soil. Despite differences in soil type, JC is under similar rainfall and temperature regimes to ACRI. Similarly, the Warrumbungle National Park site (WAR) was under similar rainfall and temperature regimes to ACRI. Like the JC site, the WAR site was on a different soil type, the soils at WAR being a free-draining, alluvial loam. Of particular interest, was the site history at the WAR site. The site was cleared of trees and used to grow lucerne and other crops in the past. Since being gazetted, natural vegetation has been returning, and the changes that occur during this process were of interest.

### 5. Other sites

In addition, we obtained samples from a number of other field sites in cotton cropping areas. These included **OC,** a cotton farm under organic practices, **LP1/LP2** two pasture sites, **WR1** Woodsreef Mine site, **MNR** (Midkin Nature Reserve), and four conventional cotton farms; **LR** (Lowes Rd), **SF** (Springfield), **AM** (Auscott Moree) and **BD** (Biddstrup farms) at Goondiwindi (LR, SF), Moree and Dalby, respectively. Some intial work on soil microbial diversity and composition was also conducted on at **BF**, 3-year droughted cotton farm at Bourke, NSW.

*Justification*

A second round of collections was undertaken in mid-late 2005. After differences were observed in cropping soils at ACRI it was determined that these differences should be tested in other cropping systems. Microbial diversity of these soils is partially characterised and work is ongoing.

*Soil chemical analyses*

Analyses of the chemical and physical characterisics of these sites (excluding 5.) were undertaken in collaboration with Ms. Leonie Whiffen (PhD scholarship CRC 6.2.22). It was determined from these analyses that the soil at the BNR site was most similar to the soils at ACRI used for cropping (see Table 1 in Attachment 2: *Molecular diversity of soil basidiomycetes in northern-central NSW*).

*Sampling / collection of soils*
All samples were taken with a cleaned trowel or asparagus knife (washed with ethanol between collections). Samples were taken at a depth of 5-15cm subsurface. All samples were either stored/transported at 4 C and processed expediently or stored at –50 C on dry ice and subsequently at –70 C at the University of Sydney prior to DNA extraction. The layer of organic material (if present) and the top 5 cm of soil was not collected.

## 4. Detail and discuss the results including the statistical analysis

Major achievements numbered and discussed separately:

1. *Optimised DNA extraction and pre-extraction storage of DNA.*
   DNA extraction from soil is problematic and often yields variable amounts of DNA. This is partially due to the extremely heterogeneous nature of soil and the clumped distribution of organisms around hot spots (presumably associated with organic matter) in soil. We successfully implemented and optimised DNA pooling methods designed to minimise the effect of this spatial heterogeneity of microbes in the soil. Additionally, vertisolic soils from the Narrabri area seem particularly prone to DNA degradation upon storage at 4° C. The precise reasons for this are unclear, however, storage for ~10 days resulted in very little extractable DNA. Similar phenomena were less severe in sandy soils examined, the water holding capacity of vertisols may exacerbate this problem. Storage at –20° C, slows but does not halt this process. Storage at –70° C successfully halted DNA degradation in soils. Soil may thus be stored in small aliquots at –70° C prior to DNA extraction without loss of DNA.

2. *Selection and justification of primer sets*
   The most diverse and abundant fungi in soil come from the divisions Ascomycota, Basidiomycota and Zygomycota. Primers for the basidiomycetes and ascomycetes were chosen. The diversity of Glomeromycota (Arbuscular mycorrhizal fungi), in soils used for cotton cropping, was examined by Ms. Stella Loke (PhD scholarship CRDC 65 and CCC CRC 1.3.12). The division Zygomycota is polyphyletic and as such universal primer sets do not exist for this group of fungi. Primers for Ascomycota and Basidiomycota for the Internal Transcribed Spacer (ITS) Region (ITS1, 5.8S rDNA, ITS2), laccase, endoglucanase, cellobiohydrolase and ß-1-4-glucosidase were examined for suitability. Unfortunately most fungal primer sets for functional genes yielded non-specific amplicons in cotton soils, only ITS amplicons (basidiomycete and ascomycete) were used in the final analyses of fungal diversity. For bacterial groups a number of functional and taxonomic primer sets were selected for trial in soil used for cotton production. 16S rDNA was examined for Acidobacteria (purportedly the 2nd largest group of soil bacteria), while for Proteobacteria (purportedly largest group of soil microrganisms) two genes involved in nitrate reduction (*nosZ, narG*), particulate methane monooxygenase (*pmoA*), ammonia oxidase (*amoA*) and a polyphenol oxidase were examined. For Actinobacteria, a group of bacteria known for their antibacterial/antifungal activities a chitinase gene was examined. Of the bacterial primers *nargG*, *amoA* and the polyphenol oxidase were discontinued as they amplified non-specific

products from cotton soils. A single archaeal primer (for 16S rDNA) was chosen for use in a pilot study on diversity.

Selection of groups for assessment was based on availability and suitability of primer sets for use in cotton soils. DNA extracted from soil is estimated to contain 5000-10000 genomes so non-specific amplification can be a problem, particularly with degenerate primer design. Time permitting it is possible to develop new, highly stringent primer sets for other groups of microorganisms or for specific functions eg: phosphotases, phytases, cellulases etc. However, we viewed assessment of microbial groups in soil, rather than development of new primer sets as a priority.

### 3. *PCR primer sets for archaeal, bacterial and fungal groups were optimised*

Of the primers for bacteria examined, proteobacterial nitrate reductase gene and *pmoA* and phyla specific primers for *Acidobacteria* and *Actinobacteria* were all tested and optimised for use in soil from ACRI. In addition, ITS primers specifically targeting basidiomycetes and ascomycetes were tested and optimised. A number of primers for functional genes in both bacteria and fungi were examined, many while stringent when used with pure cultures, proved non-specific when applied to the soil microbial community. The archaeal primer set (used in the pilot experiment) was also tested and optimised.

### 4. *A pilot experiment to examine changes in the archaeal community of soil at four field sites in the Narrabri area.*

TRFLP and ARDRA were used on a small part of the microbial community (Archaea) to test the techniques in field. The archaeal community was compared at ACRI and at two regeneration and one undisturbed field site at Brigalow Park Nature Reserve. Soil physical and chemical characteristics such as pH, organic matter, organic carbon, nitrogen and phosphorus were compared in collaboration with Ms. Leonie Whiffen (PhD scholarship CRC 6.2.22). A multivariate comparison developed in collaboration with Dr. Michael Stewart, School of Mathematics and Statistics, University of Sydney, was used to compare chemical and physical characteristics at the field sites. Soils from the four sites were broadly similar, although organic carbon was significantly greater at the natural sites and lower at ACRI, conversely, phosphorus and water content was significantly higher at ACRI. Archaeal diversity, as measured via a novel bootstrapped comparison of the Simpson's Index (discussed in detail in the next point) was found to be significantly higher ($P<0.01$) at ACRI than at any of the natural sites. Several of the taxa discovered are novel and probably form part of a new order/family level division within the archaeal kingdom *Euryarchaeaota*. Archaea may be useful indicators of disturbance or cropping. Complete details of results of the pilot study and the analysis method for ARDRA derived datasets, developed by Stewart and Midgley, are detailed in Attachment 1; "*Novel lineages of* Archaea *are present in semi-arid soils of eastern Australia*" (Midgley, Saleeba, Stewart & McGee) a submitted paper attached to this document.

### 5. *Development of a statistical method for comparison of TRFLP data*

Using data from the pilot experiment a method for comparing Simpson's Index, was developed tested and optimised in collaboration with Dr. Michael Stewart. For each pairwise comparison the absolute difference in the Simpson's index was used as a test statistic and a P-value was obtained by re-sampling from a pair of communities with an empirically estimated common TRF composition profile. Multiplicity was addressed using a Bonferroni adjustment. This method can be used on datasets derived from TRFLP, but not ARDRA data.

### 6. *Assessment of bacterial diversity*

Bacterial diversity in a range of soils used to grow cotton was examined using the TRFLP method. For two functional genes, nitrate reductase and particulate methane monooxygenase no significant variation was observed between any of the field sites examined regardless of management practice (nitrate reductase is shown in Fig 1). No significant differences were observed in either diversity measures, such as the Simpson's Index or species (=TRF) richness, or in the community composition of these two functional genes. Possible reasons for this include high conservation of the gene in organisms and functional redundancy. Similarly, for some taxonomic markers, such as those for the bacterial phyllum *Acidobacteria*, no significant differences in community composition or richness were observed between sites under differing land management practices. In contrast, the *Actinobacteria,* a bacterial phylum whose representatives are known for the production of antibiotics such as streptomycin and neomycin varied significantly between field sites (Fig. 1.). Cropping sites have lower diversity and different community structure within this phylum compared to natural sites.

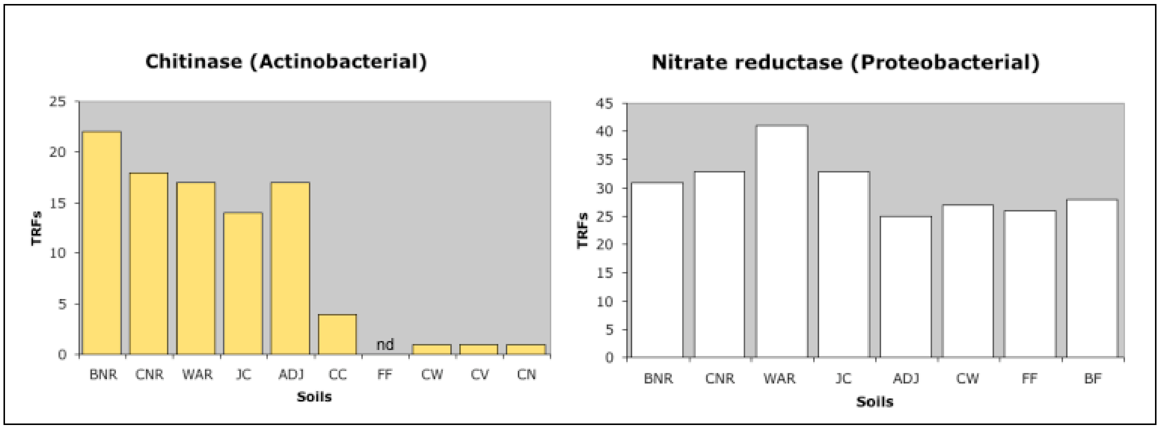

Fig. 1. Diversity of chitinase and nitrate reductase (based on Taq$\alpha$1 TRFs) for a range of soils.
Nd= not detected

### 7. *TRFLP based assessment of the fungal community*

The fungal community was profiled at a number of field sites, including soils from ACRI, a *Fov* affected cotton farm at Bourke and a number of revegetation or natural sites. Basidiomycete diversity was dramatically affected by land management practice. Basidiomycete richness was significantly higher in native vegetation sites compared to cropping sites (Fig 2.). In addition, community composition varied considerably between the natural sites, while it was more broadly similar at disturbed sites (Table 1). The same pattern was observed for ascomycetes, however, the difference in richness was greater between cropping systems and natural systems for these organisms. Some of the diversity measured in the basidiomycete community was probably due to the presence of ectomycorrhizal (ECM) fungi. Cotton and its various rotation crops are not ectomycorrhizal so the lower diversity of basidiomycete taxa can be partially explained by this possibility. In contrast most ascomycetes are not involved in ECM relationships and yet the difference is even more pronounced. It is unclear from TRFLP data (as it is currently not possible to determine the identity of peaks) what these changes may mean from a production perspective. When combined with ARDRA data, however, (discussed below #8.) it is possible to make some broad conclusions.

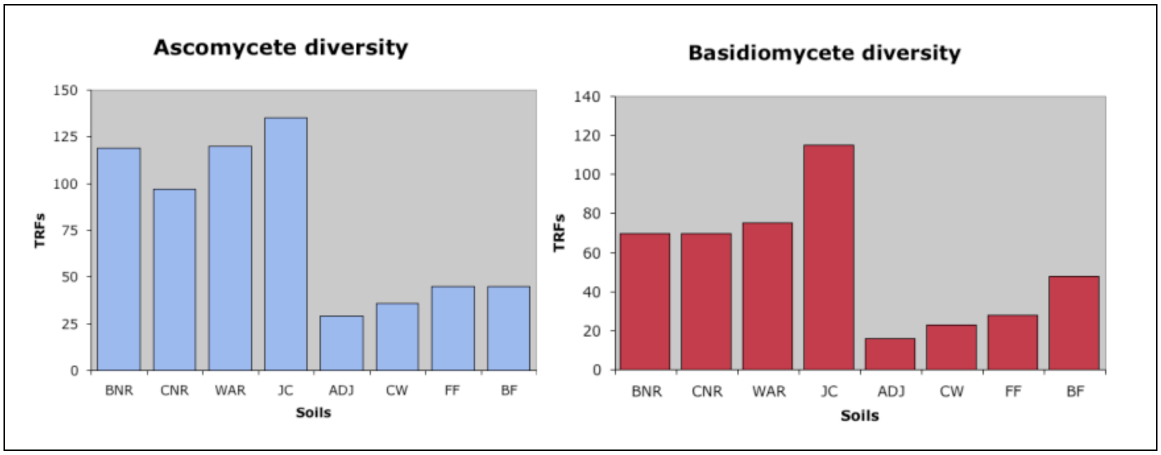

Fig. 2. Basidiomycete and ascomycete diversity (based on ITS Taqα1 TRFs)

Table 1. Pairwise percentage similarity between sites with respect to ascomycete community diversity (Basidiomycete data shows a similar pattern). Stipped "paris" show high community similarity (> 50%)

| | ADJ | BNR | CNR | CW | NF | JC | BF | WAR |
|---|---|---|---|---|---|---|---|---|
| **ADJ** | 100.0 | 19.3 | 39.5 | 69.4 | 60.0 | 18.5 | 51.0 | 22.5 |
| **BNR** | | 100.0 | 24.4 | 18.5 | 24.4 | 14.1 | 17.7 | 17.5 |
| **CNR** | | | 100.0 | 22.7 | 36.1 | 24.4 | 11.3 | 37.5 |
| **CW** | | | | 100.0 | 57.8 | 20.7 | 44.4 | 18.3 |
| **NF** | | | | | 100.0 | 27.4 | 28.9 | 30.8 |
| **JC** | | | | | | 100.0 | 11.1 | 19.3 |
| **BF** | | | | | | | 100.0 | 12.5 |
| **WAR** | | | | | | | | 100.0 |

## 8. *ARDRA based assessment of the fungal community*

ARDRA was successfully used to identify dominant members of both the basidiomycete and ascomycete community at a number of field sites under cropping and natural or regenerating vegetation.

Comparisons of the basidiomycete community suggest that cropping dramatically alters the community of common basidiomycetes in soil. Considered in the absence of the ECM taxa, the basidiomycete saprophyte community at the BNR, CNR, JC and WAR consists of taxa from the orders Agaricales, Aphyllophorales, Cantharellales, Ceratobasidales, Phallales and Trichosporonales. In contrast, the community of basidiomycetes in cotton soils consist only of *Ceratobasidium* (Rhizoctonia) spp. The life history of the *Ceratobasidium* spp. detected in cropping soils is unknown, however, they may be potentially saprophytic or pathogenic. This shift in dominance of the saprophytic community warrants further investigation and is discussed in further detail in Attachment 2 ("*Molecular diversity of soil basidiomycetes in northern-central New South Wales.* Midgley, Saleeba, Stewart and McGee").

Analyses of the common members of the ascomycete community in the same soils, suggests a broadly similar pattern to the Basidiomycota. The orders Hypocreales (which include *Fusarium* spp.) and Xylariales appear to be common at the cropping site, and are rarely detected at the non-agricultural sites (Table 2). It is also noteworthy that a number of *Penicillium* spp. appear to be common species in natural sites, however, we were unable to detect any *Penicillium* spp. in cropping systems using ARDRA. This suggests they have lower relative abundance in cropping sites than at natural sites (Table 2). The absence of more antagonistic, antibiotic producing taxa in the commonly detected ascomycete community may be a factor in absence of pathogen suppression in some cropping systems.

Taken together, data from the basidiomycete and ascomycete communities suggest cropping practices shift the fungal community towards a community dominated by *Ceratobasidium* (Rhizoctonia) spp. along with Hypocreales and Xylariales taxa. These taxa are presumably able to better withstand cropping related disturbances such as tillage, fertilisation and irrigation. The long-term production outcomes for cotton growing in this soil community remain unclear. Further studies to determine the prevalence of these taxa in other cotton cropping systems, at other times, and other under management practices would be useful in determining the potential effects for sustainable cotton production.

Subsequent culture based assessment of Trichocomaceae in cotton soils (E Cooper, DJ Midgley) suggest that Trichocomaceae can be isolated from cotton cropping soils. This presumably indicates they are relatively rare, or inactive, in soils under cotton production.

Table 2. Common ascomycetes of cotton-cropping and natural soils of the Narrabri area.

| **SITE** | **Code** | ***Closest ID*** | **Closest ID (Acc. No.)** | **% ID** | **OL (bp)** | **Tentative ID** |
|---|---|---|---|---|---|---|
| CW | Cw-A1 | *Humicola fuscoatra* | AJ279444 | 86.3 | 460 | Ascomycete #1 |
| CW | Cw-A2 | *Fusarium lateritium* | AF310980 | 78.5 | 567 | Hypocreomycetidae taxon #1 |
| CW | Cw-A3 | *Cordyceps jezoensis* | AB027365 | 84.1 | 615 | Hypocreales taxon #1 |
| CW | Cw-A4 | *Microdochium* sp. 00019 | AJ246155 | 86.4 | 405 | Xylariales taxon #1 |
| CW | Cw-A5 | *Myrothecium cinctum* | AJ302004 | 83.5 | 638 | Hypocreales taxon #4 |
| CW | Cw-A6 | Leaf litter ascomycete its31 | AF502825 | 88.2 | 524 | Xylariales taxon #2 |
| CW | Cw-A7 | *Fusarium oxysporum* FO-07 | AY928414 | 98.8 | 513 | Hypocreales, *Fusarium* sp. #1 |
| BNR | B-A1 | *Bisporella citrina* | AF335454 | 81.5 | 422 | Pezizomycotina taxon #1 |
| BNR | B-A2 | *Cordyceps* sp. 97003 | AB027375 | 66.5 | 520 | Ascomycete #2 |
| BNR | B-A3 | *Phaeococcomyces chersonesos* Ch49 | AJ507323 | 92.1 | 620 | Chaetothyriales taxon #1 |
| BNR | B-A4 | *Hortaea werneckii* ATCC 36317 | DQ168665 | 85.7 | 533 | Dothideales taxon #1 |
| BNR | B-A5 | Ericoid mycorrhizal sp. Sd2 | AF269068 | 77.3 | 625 | Helotiales taxon #1 |
| BNR | B-A6 | *Phaeococcomyces chersonesos* Ch49 | AJ507323 | 92.4 | 621 | Chaetothyriales taxon #2 |
| BNR | B-A7 | *Phoma* sp. 2 | AF218789 | 90.4 | 619 | Phoma-like ascomycete #1 |
| BNR | B-A8 | *Penicillium pimiteouiense* NRRL 26933 | AF037434 | 80.0 | 480 | Trichocomaceae taxon #1 |
| BNR | B-A9 | *Penicillium striatisporum* | AF038938 | 98.3 | 593 | Trichocomaceae – *Penicillium* sp. #1 |
| CNR | Cn-A1 | *Neosartorya stramenia* NRRL 4652 | AF459733 | 77.5 | 654 | Trichocomaceae taxon #2 |
| CNR | Cn-A2 | *Coniosporium* sp. MA4666 | AJ971447 | 78.5 | 599 | Ascomycete #3 |
| CNR | Cn-A3 | *Botryosphaeria dothidea* CBS 116742 | AY786321 | 77.2 | 591 | Ascomycete #4 |
| CNR | Cn-A4 | *Didymella cucurbitacearum* | AY293804 | 96.2 | 578 | Dothideomycetes, *Didymella* sp. #1 |
| CNR | Cn-A5 | *Didymella cucurbitacearum* | AY293804 | 80.6 | 589 | Dothideomycetes taxon #1 |
| CNR | Cn-A6 | Ericoid mycorrhizal sp. UBCS9 | AF081442 | 91.6 | 587 | Helotiales taxon #2 |
| CNR | Cn-A7 | *Penicillium striatisporum* | AF038938 | 98.5 | 593 | Trichocomaceae – *Penicillium* sp. #2 |
| CNR | Cn-A8 | *Penicillium canescens* FRR 910 | AY373901 | 100.0 | 607 | Trichocomaceae – *Penicillium* sp. #3 |
| CNR | Cn-A9 | *Massarina lacustris* | AF250831 | 83.7 | 498 | Dothideomycetes taxon #2 |
| CNR | CnA10 | *Curvularia trifolii* wb403 | AF455446 | 95.2 | 609 | Pleosporales, *Curvularia* sp. #1 |
| WAR | W-A1 | *Phoma herbarum* ATCC 12569 | AY293791 | 97.5 | 562 | Phome sp. #1 |
| WAR | W-A2 | *Myrothecium atroviride* BBA 71016 | AJ302002 | 99.0 | 615 | Hypocreales, *Myrothecium* sp. #1 |
| WAR | W-A3 | *Peziza violacea* WZ0024 | AY789366 | 91.5 | 542 | Pezizaceae taxon #1 |
| WAR | W-A4 | *Penicillium canescens* FRR 910 | AY373901 | 100.0 | 607 | Trichocomaceae – *Penicillium* sp. #4 |
| WAR | W-A5 | *Humicola fuscoatra* IDSM 63302 | AJ279444 | 87.1 | 458 | Ascomycete #5 |
| WAR | W-A6 | *Dactylella varietas* YMF1.118 | AY902805 | 79.5 | 538 | Orbiliales taxon #1 |
| WAR | W-A7 | *Phaeococcomyces chersonesos* Ch49 | AJ507323 | 91.8 | 659 | Chaetothyriales taxon #3 |
| WAR | W-A8 | *Dictyosporium toruloides* CBS 209.65 | DQ018093 | 84.7 | 555 | Ascomycete #6 |
| WAR | W-A9 | *Peziza violacea* WZ0024 | AY789366 | 91.3 | 543 | Pezizaceae taxon #2 |
| JC | J-A1 | *Phaeococcomyces chersonesos* Ch49 | AJ507323 | 94.4 | 662 | Herpotrichiellaceae taxon #1 |
| JC | J-A2 | *Penicillium verrucosum* ATCC 44407 | AY373937 | 94.9 | 610 | Trichocomaceae – *Penicillium* sp. #5 |
| JC | J-A3 | Ericoid mycorrhizal sp. UBCS9 | AF081442 | 94.0 | 589 | Helotiales taxon #3 |
| JC | J-A4 | *Glomerella lagenaria* BBA 71047 | AJ301970 | 80.0 | 582 | Pezizomycotina taxon #2 |

## 5. Objectives vs conclusions – What is the “take home” message?

5. Objectives vs conclusions – What is the “take home” message?

Initially, it was hypothesized that soil microbial communities were distributed along a hypothetical linear continuum of diversity. At one end of this continuum were “unhealthy” soils, which were expected to have lower microbial diversity, while at the other end of the continuum were "healthy" productive soils, where microbial diversity was predicted to be high. While our results partially support this hypothesis, it was an oversimplification and the real situation is a complex interaction of microbial community functions, diversity (both species richness and evenness) and community composition.

Cropping reduces the richness of microbes we could detect compared to natural systems. This reduction in richness occured regardless of the soil-type ie: cropping soils from Bourke and from Narrabri, under various rotations, both had reduced richness of microbial species compared to natural systems. Disturbances, which include cropping, also increase the similarity in the microbial communities between sites (see Table 1), although an interaction between locality is also evident. This community similarity probably occurs as microbes better adapted to disturbed environments are positively selected under tillage, irrigation and fertilisation regimes associated with cropping. In addition, cropping dramatically shifts the most abundant members of the basidiomycete and ascomycete communities away from a diverse community of saprobes (ECM fungi excluded) to a less diverse community of *Ceratobasdium* (Rhizoctonia) spp., Hypocreales taxa (including *Fusarium* spp.) and Xylariales taxa. The effects of the reduction in overall diversity and shifts in community composition for sustainable cotton production remain unclear.

It is also noteworthy, that microbial diversity in soils with diverse vegetation vary considerably between sites, and are consistently both highly diverse and dissimilar from each other. Additionally, with respect to functional diversity, some groups of microorganisms which produce compounds with antibiotic activity (eg: Trichocomaceae spp. and Actinobacteria spp.) seem to be much less common in cropping systems than in natural systems. These organisms have been variously implicated in suppression of pathogens (via direct antagonistic interactions, competition or induced resistance) and plant growth promotion via improved mineral, particularly phosphorus, cycling.

We will rigorously test the correlation between diversity of these selected groups of organisms and microbial diversity, against disease and growth of crops in our current project, thereby, linking our observations on microbial diversity and "soil health" to a tangible measure of cotton plant performance.

## 6. Addressing the Corporations three outputs: Economic, Environmental and Social

a) *Economic*

The research conducted in this project demonstrated that populations of selected groups of microbes are altered by land management practice including cropping. Some beneficial microbes (eg Actinobacteria and *Penicillium* as producers of antibiotics) appear to be depleted and some potential pathogens (eg *Fusarium oxysporum* and *Rhizoctonia*) are more readily detected in soils that are cropped. The implications for production of cotton are unknown, however, optimisation of the microbial community in cropping systems may lead to significant improvments in crop yields. For example, if it is possible to amend cultivated soils or inoculate cotton seedlings with beneficial groups of microbes, improved seedling growth may result due to suppression of pathogens. Diverse microbes are known to be involved with important processes such as solubilising minerals such as phosphate, and cycling carbon (beneficial if cycling is slowed). These processes remain to be examined.

b) *Environmental*

Manipulation of microbial systems potentially has great environmental benefits. Optimisation of cropping systems with respect to microbial biodiversity may lead to a reduction in use of pesticides and fertilisers. Additionally, it may lead to a more sustainable cropping system with a stable organic carbon pool.

c) *Social*

Clearer understanding of soil biology may lead to more sustainable methods of production. Common concerns with cotton production, including use of pesticides and fetilisers, may be modified through manipulation of microbes in soil.

## 7. Summary with respect to:

a) *technical advances acheived (eg: commercially significant developments, patents or granted licenses)*

We have determined that for single time points cropping significantly alters the microbial flora of the soil. Additionally, we determined that different management practices have differing effects on this microbial community. The implications for production of cotton are at present unclear, however, optimisation of this microbial community for the most effective mycorrhizal species, phosphate solubilising organisms and organisms involved in suppression of cotton pathogens, for example, may have very significant economic implications for the cotton industry.

b) *other information developed (eg: significant developments in methodology, equiptment design)*

DNA extraction methodology from cotton soils has been significantly improved. In addition, methods for pre-DNA extraction storage of soil have been optimised. This is critically important as problems with DNA extraction cause downstream applications to be affected. In addition, over 200 sequences of microorganisms from soils used for cotton cropping will be made publically available on the GenBank nucleotide database. These are an important resource for researchers in the cotton industry examing microbial diversity in soils.

c) *changes to the IP register*

At present we do not see the need for any changes to the IP register.

## 8. Detail a plan for the activities or other steps that may be taken:

a) *to further develop or to exploit the project technology*

A direct test of the correlations between microbial biodiversity, presence of seedling pathogens (such as black root rot *Thielaviopsis basicola*) and plant growth would be a natural extension of this work.

b) *for the future presentation and dissemination of the project outcomes*

Information obtained on the microbiology of soils used for cotton cropping is being diseminated to the scientific and cotton community via scientific publications. Additionally, genetic information from microorganisms which occur in cotton growing soils will or has been lodged in the publically accessible GenBank nucleotide database. We propose to submit a report to the Cotton Conference 2006, and will initiate discussions with extension personnel in the near future.

c) *for future research*
Significant progress has been made towards a better understanding of the microbiology of soils used to grow cotton. Such an understanding allows a solid foundation on which a number of future research programmes might be developed. We are pursuing one line of research in our current project, CRC71 – Microbial Biodiversity for Soil Health, regarding the correlation between microbial diversity, disease suppression and plant growth. Similarly, research into optimisation of the VAM-cotton relationship, and further correlations between microbial diversity and other factors such as organic carbon levels and phosphorus availability would provide important information to further our long term goal of sustainability in cotton production.

## 9. Publication Plan

Several publications in preparation from the research. We have experienced some recent IT problems with our current bioinformatics The problems have now been resolved and we will be submitting papers in early 2006.

1. *Phylogenetically diverse consortia of Archaea are present in cotton-growing, natural and revegetated soils in a semi-arid region of eastern Australia*
   (authors: DJ. Midgley, JA. Saleeba, MI Stewart and PA. McGee)
   Status: submitted to Canadian Journal of Microbiology
2. *Molecular diversity of soil basidiomycetes in northern-central New South Wales*
   (authors: DJ. Midgley, JA. Saleeba, MI Stewart and PA. McGee)
   Status: draft paper, for submission to Australian Journal of Botany
3. Culture dependant and culture independent assessment of ascomycete communities in soils of eastern Australia.
   (authors: DJ Midgley, JA Saleeba K Donohoe and PA McGee)
   Status: in preparation.

## 10. Online resources developed during the project

No specific online resources were developed during this project. Genetic information (ribosomal sequences) from a variety of soil bacteria, Archaea and fungi from cotton-growing soils have or will be made available via the Genbank nucleotide database at:

http://www.ncbi.nih.gov/

## 11. Impact of results and conclusions of the research for the cotton industry. Potential costs and benefits to the Australian cotton industry or the Australian community

Soil health is a complex issue. It is not a simple continuum from “healthy” to “unhealthy”. We now have a better understanding of the microbiology of soils used to grow cotton. For some bacterial groups cropping does not seem to influence diversity or community composition. For other bacterial groups, such as Actinobacteria, cropping significantly alters both species richness and community composition. Similarly, for both major fungal groups (basidiomycetes and ascomycetes) cropping dramatically alters both species richness and community composition. In particular, the observation of fewer antibiotic-producing microbes in cropping soils is of concern. Antibiotic producing organisms, such as fungi from the genera *Aspergillus, Penicillium* and bacteria from the phyla Actinobacteria have been demonstrated to induce suppression to diseases such as those caused by *Pythium* spp., *Rhizoctonia* spp., *Verticillium* spp. and *Fusarium oxysporum*. However, manipulation of microbial biodiversity is now at an exciting stage. It may be possible to manipulate or inoculate groups of microbes in cropping systems to reduce seedling disease and thereby improve crop yields. Indeed, we envisage management of microbial processes leading to new approaches to increasing soil carbon, better managing mineral availability and improving efficiency of mineral application.

Improved management and manipulation of soil microbes has significant potential benefit both in economic and environmental terms for the cotton industry and cropping industries more generally.

**Short communication for Australian Journal of Botany**

# Molecular diversity of soil basidiomycetes in northern-central New South Wales

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Abridged running head: Soil basidiomycetes of northern-central NSW

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## Introduction

Vertisolic soils of northern-central New South Wales (Service) are amongst Australia's most productive, yielding economically important crops, including cotton (*Gossypium hirsutum* L.) and wheat (*Tritcium aestivum* L.). Additionally, these soils host remnant stands of brigalow (*Acacia harpophylla* F. Muell. ex Benth) woodland and its associated vegetation communities. More than 90 % of brigalow woodlands have been cleared and the vegetation community is currently listed as endangered in the Australian *Environment Protection and Biodiversity Conservation Act 1999*. Despite this acknowledged economic and ecological importance, virtually nothing is known of fungal diversity of the soils of this region. To date, there are no collections of fungal fruiting bodies from this region, nor molecular based below-ground assessments of microbial diversity have been reported from these soils.

Composition and diversity of fungal communities may be an important determinant of functions of soil (Doran and Zeiss 2000; Hill *et al.,* 2000). Basidiomycetes are important components of the soil fungal community and include many saprotrophic, mycorrhizal and pathogenic taxa (Dighton 2003). Basidiomycetes play critically important roles in below-ground carbon and mineral cycling, plant growth and they underpin ecosystem function (Christensen 1989; Dighton and Boddy, 1989; Dighton 2003). Understanding the diversity and structure of fungal communities is therefore essential for developing sustainable agricultural practices, and regenerating vegetation at disturbed sites.

The current study is part of a program to clarify changes in fungal diversity and the impact of the differences on functional attributes of soil. The aims of the current study were thus to, identify the dominant members of the basidiomycete community and investigate differences in fungal community composition at seven field sites, under different land use practices, in the semi-arid brigalow bioregion of northern-central NSW. In order to overcome acknowledged problems in culturing some basidiomycete taxa, the present study used amplified ribosomal DNA restriction analysis (ARDRA) to profile basidiomycete communities.

## Materials and Methods

### *Field sites and soil sampling*

Soil samples were collected at seven 750 $m^2$ field sites in northern-central New South Wales (Service) in April, 2004 (Table 1). Twelve ca. 500 g soil samples were randomly collected using a cleaned trowel at each 750 $m^2$ field site and kept at 4 °C in transit to the laboratory prior to soil DNA extraction. In all cases samples were taken from a depth of 5 – 15 cm.

### *DNA extraction from soil*

DNA was extracted from 0.5 g soil samples using a method modified from Yan and Vancov (unpublished, but detailed here) *ca.* 0.5 g of soil was placed into 2.0 ml tubes with 0.8 g of 0.8 – 1.0 mm sterile acid washed ceramic beads (Saint-Gobain ZirPro, Le Pontet, France), 0.3 g 100 μm glass beads (Daintree Scientific, St Helens, Australia), 900 μl 0.1 M $NaPO_4$ and 110 μl of a 50 mM disodium EDTA, 100 mM hydroxymethyl aminomethane, 1% sodium dodecyl sulphate, 1% polyvinylpyrolidone-40 and 0.5% Extran® MA03 solution (Merck and Co, Inc. Whitehouse Station, USA). Samples were homogenised at 5.5 $ms^{-1}$ for 30 s. in a FastPrep® Instrument (Bio101 Inc. La Jolla, USA). The tubes were then placed on ice for 5 min prior to centrifugation at 12, 500 g for 20 min at 4 °C. 700 μl of aqueous supernatant was mixed with 125 μl of 7.5 M potassium acetate. The tubes were incubated at 4 °C for 1 h then centrifuged at 12,500 g for 20 min at 4 °C. A half volume of 20% w/v polyethyleneglycol 6000; 2.5 M NaCl was added to the supernatant. Nucleic acids were then precipitated by incubation at 4 °C followed by centrifugation at 12,500 g for 20 mins at 4 °C. The pellet was washed overnight in 95 % ethanol at 4 °C, centrifuged at 12,500 g for 20 min 4 °C, air dried and resuspended in 50 μl of sterile Milli-Q® water which contained 1/200th volume of 10 mg ml$^{-1}$ RNase A (Sigma, St. Louis, USA). The three replicate 500 mg soil DNA extractions performed for each (of the 12) individual soil sample were then combined prior to PCR.

*PCR amplification*

The rDNA internal transcribed spacer (ITS) region was amplified in 25 μl reaction volumes, each containing 1X Reaction Buffer (67 mM hyroxymethyl aminomethane-HCl, 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100 v/v, 0.2 mg l$^{-1}$ gelatin) (Fisher Biotec, West Perth, Australia), 1.5 mM $MgCl_2$, 25 pmol each of the primers ITS1F and ITS4B (Gardes and Bruns, 1993), 100 mM of dATP, dCTP, dGTP and gTTP (Fisher Biotec), 0.7 units of Taq DNA polymerase (Fisher Biotec) and *ca.* 25 ng of sample DNA. Amplifications were performed in a Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany) with a four minute melt at 94 °C proceeding 28 cycles of 94 °C for 45 s, 50 °C for 45 s and 72 °C for 45 s, followed by an 8 minute extension at 72 °C. Amplification products were electrophoresed in 1x SB gels containing 1.5% agarose (Brody and Kern 2004) stained with ethidium bromide and visualised under UV light.

### *Amplified ribosomal DNA restriction analysis (ARDRA)*

Twelve replicate PCR-products from each field site were pooled and purified by gel extraction using the QIAquick gel extraction kit (Qiagen, Doncaster, UK). Approximately 100 ng of purified pooled PCR product was ligated in the pDrive vector (Qiagen) following the manufacturers directions and subsequently cloned into competent E. coli DH5α. Thirty clones for each field site were sorted into Taqα1 (New England Biolabs Inc., Ipswich, USA) restriction fragment length polymorphism (RFLP) types and a representative clone of each RFLP-type was chosen at random for DNA sequencing. Sequencing reactions were performed by Macrogen Inc. (Seoul, Korea).

### *Sequence analysis*

Each ITS sequences was divided into ITS1, ITS2 and 5.8S regions and then BLAST searches (Altschul et al., 1990) against the GenBank nucleotide database were conducted on each region, using the BLAST program at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). For ITS1 and ITS2, matches under 80% identity over less than 80% of the sequence submitted were regarded as below acceptable confidence levels and were not reported. One sequence for each RFLP-type was submitted to the GenBank nucleotide database under the following accession numbers DQ672270-DQ627336.

### *Soil analyses*

Soil percentage water content was determined by weighing *ca* 5 g sub-samples from each of the 12 replicates for each site, prior to and after 12 h incubation in an oven at

95 ºC. Percentage organic matter was determined for the samples dried previously for water content, by determining loss on ignition after 2 hours at 360 ºC followed by 4 hours at 475 ºC in a muffle furnace. Soil organic carbon, pH (in deionised water and $CaCl_2$), nitrate (mg $L^{-1}$) and total nitrogen were measured for three randomly selected replicates for each site and were conducted by Incitec Pivot Laboratories (Werribee, Australia). Physical and chemical parameters were compared for soil from the seven field sites in a multivariate nonparametric, multiplicity adjusted fashion.

## Results

All ITS amplicons were basidiomycete in origin and ranged in length from *ca.* 515 – 750 bp. For all field sites, except F, all 12 soil samples yielded strong amplication products that were cloned, pooled and subjected to ARDRA followed by DNA sequencing. PCR of soil DNA from the F site produced only faint amplification products inconsistently. Cloning and DNA sequencing demonstrated that the most common basidiomycete taxon present in soil F was a basidiomycetous yeast (Table 2; data not shown). Due to problems with consistency of amplification basidiomycetes from F soil were not further characterised. From the 180 clones examined 67 basidiomycete RFLP-types were identified. Amongst the 30 clones examined at each site the B field site had the greatest richness of RFLP-types (15), while the A and Cn field sites both had 13 RFLP-types. Ten RFLP-types were detected at both the J and W field sites, and five RFLP-types were detected at the Cw field site. Most sites had similar patterns of relative abundance, with 1 - 2 common RFLP-types and a greater number of rarer RFLP-types (Table 2).

RFLP-types with affinities to ectomycorrhizal (ECM) fungi were detected at the A, B, Cn, J and W field sites (Table 2). These putatively ECM RFLP-types had affinities to the Thelephorales (B2, B9, B11, Cn1, J3, J6, J7 and W3), *Pisolithus* (Cn4 and J9), Cantharellales (Cn10 and J1), Geastreales (W8) and Lycoperdales (B7). RFLP-types with affinities to ECM fungi were not detected at the Cw field site. RFLP-types with affinities to the Ceratobasidaceae were also commonly detected at the A, B, Cn, Cw and W field sites. Along with filamentous fungi, RFLP-types with affinities to basidiomycete yeasts were also detected (Cn5, J2, J10 and F1). More than 25 % of all RFLP-types could not be reliably identified at a level below the class Homobasidiomycotes (Table 2.). Almost all RFLP-types were limited to single field sites, although the J and Cn field sites shared 2 RFLP-types (Cantharellales taxon #1 and *Cryptococcus* sp. #1).

## Discussion

Data from the present study indicate that diverse communities of basidiomycetes are present in soils of northern-central NSW. This is the first report of fungal diversity in soils of northern-central NSW. Interestingly, *ca.* 25 % of RFLP-types could not be identified beyond class Homobasidiomycetes, suggesting that a number of RFLP-types observed represent as yet undescribed fungal genera or species.

The abundance of RFLP-types with affinities to the Thelephoraceae, most of which are probably ectomycorrhizal *Tomentella* spp. is intriguing. To date, only ten species of *Tomentella* have been described in Australia (Cunningham 1963; Agerer and Bougher 2001). *Tomentella* species produce inconspicuous resupinate sporocarps and are difficult to detect in surveys of sporocarps and may be underrepresented in collections even if common (Agerer and Bougher 2001). In a molecular based fungal survey of soils of the Sydney region, Chen and Cairney (2002) detected seven *Tomentella*-like RFLP-types. Comparisons of ITS1 and ITS2 from the *Tomentella*-like taxa detected in the present study and those detected by Chen and Cairney (2002), however, suggest the Sydney RFLP-types are not closely related to those from northern-central NSW (data not shown). If ITS RFLP-types are indicative of putative species in the genus *Tomentella*, then a number of, as yet undescribed, *Tomentella* species may occur in northern-central NSW.

Other ECM fungi detected included two *Pisolithus* species, a putative *Clavulina*-like fungus and putative Lycoperdonales and Gaestreales taxa all of which are probably ECM. Interestingly, RFLP-type A5 has relatively high sequence identity to a Ceratobasidaceae sp. previously implicated in dual orchid-ECM symbiosis (Bidartondo et al., 2004). Closely related members of the Ceratobasidaceae, however, have orchid mycorrhizal, saprophyticand pathogenic life histories (Ogoshi, 1987) and, as such the role of RFLP-type A5 in the field is unclear.

Despite the limited scale of sampling in the present study, some general comparisons between sites under differing land management can be made. Differences between the A, B, Cn and Cw sites, all of which are on the same soil type, suggest vegetation and land use dramatically alter basidiomycete community composition. ECM fungi are absent from the Cw site, as cotton and wheat are not ECM plant hosts. It is interesting, however, that the A, B and Cn sites also harbour a relatively diverse community of putatively saprophytic RFLP-types (from the orders Agaricales, Aphyllophorales, Cantharellales, Ceratobasidales, Phallales and Trichosporonales). In contrast, all RFLP-types at Cw were from the order Ceratobasidales. This shift to a saprophyte community dominated by *Ceratobasidium* spp. presumably reflects the ability of *Ceratobasidium* spp. to persist in agricultural soils despite disturbances such as tillage, irrigation and other cropping practices. If this shift in dominance occurs in other cotton cropping soils then the prevalence of *Ceratobasidium* spp., and/or the absence of other common saprophytic basidiomycetes, may have implications for sustainable cotton production. Further studies of basidiomycete communities in other cotton cropping systems, and pathogenicity testing of *Ceratobasidium* isolates may provide insights into functional implications of the presence of *Ceratobasidium* spp. at the Cw site.

Diversity and composition, assayed by molecular based surveys are prone to primer and PCR bias (Anderson and Cairney, 2004). Consequently some common fungi may not be represented if they have a lower affinity to the primer selected. In addition, fungal communities are dynamic, and change with time, thus this survey provides indicative data for one time point only. These limitations aside, data from the current study illustrates that basidiomycete communities in northern-central NSW are both diverse and poorly characterised. In addition, marked changes in basidiomycete community structure occur under differing land management regimes and/or vegetation. Improved understanding of functioning of fungal communities in these soils may lead to improved sustainability in agriculture and to improvements in methods of regeneration of natural vegetation communities.

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**Table 1. Field sites, soil type, physical & chemical descriptors.**

| Site | Location | Soil type | Vegetation | Land use | pH in $CaCl_2$ & ( $dH_20$ ) | OC % | OM % | $NO_3^{-2}$ mg $L^{-1}$ | % N | % P | K Meq 100g $^{-1}$ | Ca Meq 100g $^{-1}$ | Mg Meq 100g $^{-1}$ | Na Meq 100g $^{-1}$ | Cl mg kg $^{-1}$ | CEC Meq 100g $^{-1}$ | % WC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A**<br>ACRI* adjacent sit to cotton field | S30° 12’<br>E149° 35’ | Grey vertisol | Grasses, *Salix* sp., weedy herbaceous species. | Area directly next to Cw, not cropped. | 7.6 (6.9) | 1.3 ± 0.22 | 5.5 ± 0.44 | 6.9 ± 0.14 | 0.1 ± 0.02 | 0.05 ± <0.01 | 1.4 ± 0.28 | 13.7 ± 1.2 | 6.3 ± 1.0 | 0.06 ± 0.01 | 10 ± <0.01 | 21.5 ± 1.6 | 7.7 ± 0.77 |
| **B**<br>Brigalow Nature Park Reserve | S30° 24’;<br>E149° 35’ | Grey vertisol | Mature brigalow woodland community. | Nature reserve | 6.6 (7.3) | 2.4 ± 0.57 | 7.4 ± 0.99 | 2.9 ± 0.78 | 0.2 ± 0.08 | 0.03 ± 0.01 | 1.1 ± 0.17 | 19.0 ± 5.0 | 6.8 ± 2.2 | 1.1 ± 0.49 | 42 ± 17.3 | 28.0 ± 7.3 | 6.6 ± 0.48 |
| **Cn**<br>Claremont Nature reserve | S30° 23’<br>E149° 34’ | Grey vertisol | Grassland, regenerating Brigalow woodland | Nature reserve | 5.1 (6.3) | 0.8 ± 0.07 | 6.7 ± 0.49 | 1.1 ± 0.06 | 0.1 ± 0.03 | 0.02 ± <0.01 | 0.4 ± 0.03 | 6.7 ± 0.3 | 6.7 ± 0.3 | 1.0 ± 0.12 | 10 ± <0.01 | 14.9 ± 1.7 | 5.0 ± 0.22 |
| **Cw**<br>ACRI* cotton-wheat rotation | S30° 12’<br>E149° 35’ | Grey vertisol | Cotton (Summer) Wheat (Winter) | Agricultural (25+ yrs) | 8.2 (7.6) | 0.9 ± 0.06 | 5.6 ± 0.33 | 1.7 ± 0.32 | < 0.1 ± 0.01 | 0.04 ± <0.01 | 1.4 ± 0.08 | 21.0 ± 1.9 | 8.8 ± 0.3 | 0.41 ± 0.06 | 10 ± <0.01 | 32.0 ± 1.7 | 10.4 ± 0.29 |
| **F**<br>ACRI* 7 year fallow site | S30° 12’<br>E149° 35’ | Grey vertisol | Plants removed for 7 years | Experimental plot (no plants 7 years) | 8.2 (7.6) | 0.7 ± 0.01 | 5.1 ± 0.05 | 25.3 ± 8.37 | < 0.1 ± 0.01 | 0.05 ± <0.01 | 1.3 ± 0.03 | 21.0 ± 0.3 | 8.4 ± 0.3 | 0.77 ± 0.03 | 15 ± 4.6 | 32.0 ± 0.3 | 11.4 ± 0.55 |
| **J**<br>Jacks Creek State Forest | S30° 27’;<br>E149° 43’ | Sandy | Pilliga eucalypt woodland (trees & understorey shrubs) | State forest | 5.2 (4.2) | 1.2 ± 0.31 | 5.0 ± 0.31 | 4.2 ± 0.05 | < 0.1 ± 0.01 | 0.02 ± <0.01 | 0.3 ± 0.06 | 0.3 ± <0.1 | 1.2 ± 0.3 | 0.06 ± 0.02 | 10 ± <0.01 | 2.8 ± 0.4 | 2.1 ± 0.11 |
| **W**<br>Warrumbungle National Park | S31° 31’;<br>E149° 6’ | Alluvial loam | Herbaceous weedy plain. Treeless. | National Park, intense rabbit and macropod grazing. | 6.3 (7.0) | 2.0 ± 0.32 | 5.9 ± 0.57 | 2.4 ± 0.72 | 0.1 ± 0.05 | 0.05 ± 0.01 | 1.4 ± <0.01 | 0.2 ± <0.1 | 9.2 ± 0.16 | 0.02 ± <0.01 | 10 ± <0.01 | 12.4 ± 0.3 | 1.7 ± 0.13 |

OC = Organic carbon, OM = Organic matter, CEC = cation exchange capacity, WC = Water content

**Table 2.** Putative identities of basidiomycete fungi from differing field sites in northern central New South Wales, Australia determined via BLASTN searches between 5.8s, ITS1 and ITS2 regions of cloned sequences and those in the GenBank nucelotide database.

| Clone – RA$^{++}$ (Acc. No) | 5.8S closest match | ID \| OL* | ITS1 closest match | ID \| OL* | ITS2 closest match | ID \| OL* | Tentative ID |
|---|---|---|---|---|---|---|---|
| A1 – 6.7 (DQ672270) | AY636057 *Pleurotus salmoneostramineus* | 97 \| 155 | MUC | - | MUC | - | Homobasidiomycete #1 |
| A2 – 16.7 (DQ672275) | AY854072 *Collybia tuberosa* | 99 \|155 | AY969369 Uncultured basidiomycete | 92 \| 246 | AY969369 Uncultured basidiomycete | 92 \| 203 | Agaricales #1 |
| A3 – 20.0 (DQ672276) | AY969798 Uncultured basidiomycete | 99 \| 153 | MUC | - | MUC | - | Basidiomycete #1 |
| A4 – 3.3 (DQ672277) | AY854072 *Collybia tuberosa* | 100 \| 155 | AF407006 *Rhizoctonia* sp. TBR TBR39 [1] | 97 \| 245 | AF407008 *Rhizoctonia* sp. TBR TBR41 [1] | 97 \| 205 | Agaricales #2 |
| A5 – 3.3 (DQ672278) | DQ452073 *Ceratobasidium* sp. | 99 \| 155 | AY634163 Uncultured ectomycorrhiza [2] | 95 \| 202 | AY634163 Uncultured ectomycorrhiza [2] | 93 \| 260 | *Ceratobasidium* sp. #1 Probable ECM taxon |
| A6 – 6.7 (DQ672279) | AY969404 Uncultured basidiomycete | 99 \| 156 | MUC | - | DQ421268 Uncultured soil fungus | 87\| 239 | Basidiomycete #2 |
| A7 – 3.3 (DQ672280) | AY654738 *Sphaerobolus* sp. | 98 \|156 | MUC | - | MUC | - | Phallales sp. #1 |
| A8 – 3.3 (DQ672281) | AY854066 *Coprinus comatus* | 100 \| 155 | MUC | - | AY176368 *Lepiota farinolens* | 84 \| 247 | Agaricales #3 |
| A9 – 20.0 (DQ672282) | DQ452073 *Ceratobasidium* sp. RDLT-2006a | 100 \| 155 | DQ102434 *Ceratobasidium* sp. AG-F | 96 \| 218 | DQ102433 *Ceratobasidium* sp. AG-F | 99 \| 271 | *Ceratobasidium* sp. #2 |
| A10 – 6.7 (DQ672271) | AY854072 *Collybia tuberosa* | 100 \| 155 | AF407008 *Rhizoctonia* sp. TBR TBR41 [1] | 92 \| 248 | AF407008 *Rhizoctonia* sp. TBR TBR41 [1] | 90 \| 205 | Agaricales #4 |
| A11 – 3.3 (DQ672272) | AY456375 *Geastrum* sp. NC-8353 | 98 \| 156 | MUC | - | MUC | - | Homobasidiomycete #2 |
| A12 – 3.3 (DQ672273) | AY593868 *Rigidoporus ulmarius* | 99 \| 154 | MUC | - | MUC | - | Homobasidiomycete #3 |
| A13 – 3.3 (DQ672274) | AF291270 *Auricularia fuscosuccinea* | 99 \| 155 | MUC | - | MUC | - | Aphyllophorales #1 |
| B1 – 3.3 (DQ672283) | AY650256 *Sphaerobolus iowensis* “SS99” | 99 \| 156 | ITS1 not available | - | MUC | - | Homobasidiomycete #4 |
| B2 – 3.3 (DQ672290) | DQ195591 Thelephorales sp. A.Becerra 08 | 100 \| 156 | AJ581540 Uncultured *Tomentella* sp. | 87 \| 218 | AY748876 Uncultured ectomycorrhiza [3] | 92 \| 262 | Thelephoraceae #1 Probable ECM taxon |
| B3 – 16.7 (DQ672291) | AY842958 *Crinipellis* sp. | 100 \| 155 | AY916711 *Marasmius* sp. MCA1577 | 90 \| 197 | AY445120 Tricholomataceae sp. | 89 \| 226 | Tricholomataceae sp. #1 |
| B4 – 20.0 (DQ672292) | AY650256 *Sphaerobolus iowensis* “SS99” | 99 \| 156 | AY558797 *Radiigera fuscogleba* “TK166” | 93 \| 174 | MUC | - | Geastrales #1 |
| B5 – 6.7 (DQ672293) | AY854078 *Ramaria rubella* “AFTOL-ID 724” | 97 \| 156 | MUC | - | MUC | - | Homobasidiomycete #5 |
| B6 – 3.3 (DQ672294) | AY703913 *Agaricus tollocanensis* “CA235” | 100 \| 153 | MUC | - | MUC | - | Homobasidiomycete #6 |
| B7 – 16.7 (DQ672295) | AY854075 *Lycoperdon pyriforme* | 100 \| 155 | MUC | - | DQ421107 Uncultured soil fungus “53-39” | 92 \| 249 | Lycoperdales #1 Probable ECM taxon |
| B8 – 6.7 (DQ672296) | AY703913 *Agaricus tollocanensis* “CA235” | 99 \| 154 | MUC | - | MUC | - | Agaricales #5 |
| B9 – 3.3 (DQ672297) | AF461574 Uncultured fungus “RFLP13” | 97 \| 155 | AF461574 Uncultured fungus “RFLP13” | 94 \| 215 | AF461568 Uncultured fungus “RFLP7” | 91 \| 257 | Thelephoraceae #2 Probable ECM taxon |
| B10 – 3.3 (AQ672284) | AY230244 *Thelephora terrestris* “DGC-8(1)” | 98 \| 156 | AY874381 Uncultured *Tomentella* ectomycorrhiza | | ITS2 not available | - | Thelephoraceae #3 Probable ECM taxon |
| B11- 3.3 (DQ672285) | AY310859 Uncultured ectomycor. fungus “TAM2055” | 98 \| 154 | AF461574 Uncultured fungus “RFLP13” | 93 \| 215 | AF461568 Uncultured fungus “RFLP7” | 92 \| 257 | Thelephoraceae #4 Probable ECM taxon |
| B12 – 3.3 (DQ672286) | AY805606 *Ceratobasidium* sp. olrim559 | 98 \| 155 | AY643805 Fungal sp. PO1 | 96 \| 195 | AY643805 Fungal sp. PO1 | 96 \| 261 | *Ceratobasidium* sp. #3 |
| B13 – 3.3 (DQ672287) | AY854078 *Ramaria rubella* “AFTOL-ID 724” | 97 \| 156 | MUC | - | MUC | - | Homobasidiomycete #7 |
| B14 – 3.3 (DQ672288) | AY805606 *Ceratobasidium* sp. “olrim559” | 100 \| 155 | AY643805 Fungal sp. PO1 | 95 \| 187 | AY643805 Fungal sp. PO1 | 96 \| 261 | *Ceratobasidium* sp. #4 |
| B15 - 3.3 (DQ672289) | AY456375 *Geastrum* sp. “NC-8353” | 100 \| 156 | MUC | - | MUC | - | Homobasidiomycete #8 |
| Cn1 – 40 (DQ672298) | AY635177 *Tomentella stuposa* | 98 \| 156 | AJ633588 Uncultured ectomycorrhizal fungus [3] | 88 \| 215 | AY351622 Uncultured ectomycorrhizal fungus [3] | 92 \| 211 | Thelephoraceae #5 Probable ECM taxon |
| Cn2 – 3.3 (DQ672303) | DQ233769 Uncultured ectomycorrhizal fungus | 98 \| 155 | MUC | - | MUC | - | Homobasidiomycete #9 |
| Cn3 – 3.3 (DQ672304) | AY842958 *Crinipellis* sp. | 99 \| 154 | MUC | - | AY627833 Epacris root associate EP57 | 87 \| 333 | Agaricales #6 |
| Cn4 – 10.0 (DQ672305) | AY318748 *Pisolithus* sp. | 99 \| 156 | AF374677 *Pisolithus tinctorius* | 95 \| 186 | AF374676 *Pisolithus tinctorius* | 93 \| 252 | *Pisolithus* sp. #1 Probable ECM taxon |
| Cn5 – 3.3 (DQ672306) | DQ420874 Uncultured soil fungus “9b38” | 99 \| 155 | DQ420874 Uncultured soil fungus “9b38” | 96 \| 121 | DQ420874 Uncultured soil fungus “9b38” | 99 \| 198 | *Cryptococcus* sp. #1 **(+J)** |
| Cn6 – 3.3 (DQ672307) | DQ452073 *Ceratobasidium* sp. RDLT-2006a | 100\| 155 | DQ421057 Uncultured soil fungus | 98 \| 240 | DQ421057 Uncultured soil fungus | 99 \| 270 | *Ceratobasidium* sp. #5 |
| Cn7 – 6.7 (DQ672308) | AY805626 Basidiomycete sp. olrim499 | 98 \| 154 | MUC | - | MUC | - | Basidiomycete #3 |
| Cn8 – 13.3 (DQ672309) | AY969374 Uncultured basidiomycete | 98 \| 155 | MUC | - | MUC | - | Homobasidiomycete #10 |
| Cn9 – 3.3 (DQ672310) | AY970290 Uncultured basidiomycete | 98 \| 155 | MUC | - | MUC | - | Homobasidiomycete #11 |
| Cn10 – 3.3 (DQ672299) | AM161513 Uncultured ectomycorrhizal fungus | 98 \| 155 | AY730686 Uncultured ectomycorrhizal fungus | 88 \| 219 | DQ233811 Uncultured ectomycorrhizal | 93 \| 272 | Cantharellales #1 **(+J)** Probable ECM taxon |
| Cn11 – 3.3 (DQ672300) | DQ452073 *Ceratobasidium* sp. RDLT-2006a | 100 \| 155 | MUC | - | DQ102402 *Ceratobasidium* sp. AG-G | 91 \| 260 | *Ceratobasidium* sp. #6 |
| Cn12 – 3.3 (DQ672301) | DQ452073 *Ceratobasidium* sp. RDLT-2006a | 100 \| 153 | MUC | - | DQ421057 Uncultured soil fungus | 99 \| 270 | *Ceratobasidium* sp. #7 |
| Cn13 – 3.3 (DQ672302) | DQ233797 Uncultured ectomycorrhizal fungus | 98 \| 155 | AJ633588 Uncultured ectomycorrhizal fungus [3] | 89 \| 215 | AY228348 *Clitopilus prunulus* | 88 \| 235 | Homobasidiomycete #12 Probable ECM taxon |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Cw1 – 6.7 (DQ672311) | DQ45207 *Ceratobasidium* sp. RDLT-2006a | 99 \| 155 | DQ45207 *Ceratobasidium* sp. RDLT-2006a | 96 \| 200 | DQ278934 *Ceratobasidium* sp. CBS137.82 | 94 \| 276 | *Ceratobasidium* sp. #8 |
| Cw2 – 40.0 (DQ672312) | DQ102415 *Ceratobasidium* sp. AG-A | 100 \| 155 | DQ278934 *Ceratobasidium* sp. CBS137.82 | 94 \| 269 | DQ279055. Ceratobasidium sp. FJ31.4 | 98 \| 179 | *Ceratobasidium* sp. #9 |
| Cw3 – 46.7 (DQ672313) | DQ452073 *Ceratobasidium* sp. RDLT-2006a | 98 \| 155 | AJ427404 *Ceratobasidium ramicola* | 98 \| 189 | DQ279055. Ceratobasidium sp. FJ31.4 | 97 \| 270 | *Ceratobasidium* sp. #10 |
| Cw4 – 3.3 (DQ672314) | AY634163 Uncultured ectomycorrhiza [2] | 100 \| 155 | DQ452073 *Ceratobasidium* sp. RDLT-2006a | 96 \| 199 | DQ279051 *Ceratobasidium* sp. SJ08 | 97 \| 269 | *Ceratobasidium* sp. #11 |
| Cw5 – 3.3 (DQ672315) | DQ452073 *Ceratobasidium* sp. RDLT-2006a | 100 \| 155 | DQ452073 *Ceratobasidium* sp. RDLT-2006a | 95 \| 200 | DQ279051 *Ceratobasidium* sp. SJ08 | 98 \| 269 | *Ceratobasidium* sp. #12 |
| FF1 (DQ672316) | AF444493 *Rhodosporidium paludigenum* “CBS6567” | 100 \| 155 | MUC | - | AY969375 Uncultured basidiomycete | 87 \| 271 | Trichosporonales #1 |
| J1 – 3.3 (DQ672317) | AM161513 Uncultured ectomycorrhiza | 98 \| 155 | AY730686 Uncultured ectomycorrhizal fungus | 88 \| 219 | DQ233811 Uncultured ectomycorrhizal fungus | 93 \| 272 | Cantharellales #1 **(+Cn)**<br>Probable ECM taxon |
| J2 – 20.0 (DQ672319) | DQ069015 *Cryptococcus podzolicus* “aurim727” | 99 \|155 | AF461670 Uncultured fungus RFLP109 | 100 \| 120 | AF444321 *Cryptococcus podzolicus* “CBS6819” | 99 \| 201 | *Cryptococcus* sp. #2 |
| J3 – 10.0 (DQ672320) | DQ195591 Thelephorales sp. A.Becerra 08 | 100 \| 155 | AJ581540 Uncultured *Tomentella* sp. | 87 \| 219 | AY748876 Uncultured ectomycorrhiza [3] | 92 \| 262 | Thelephoraceae #6<br>Probable ECM taxon |
| J4 – 3.3 (DQ672321) | DQ069015 *Cryptococcus podzolicus* “aurim727” | 100 \| 155 | AJ581036 *Cryptococcus podzolicus* | 99 \| 120 | AF444321 *Cryptococcus podzolicus* “CBS6819” | 99 \| 201 | *Cryptococcus* sp. #3 |
| J5 – 3.3 (DQ672322) | AY969518 Uncultured basidiomycete | 100 \| 155 | ITS1 not available | - | DQ420877 Uncultured soil fungus “138-40” | 98 \| 256 | Basidiomycete #5 |
| J6 – 46.7 (DQ672323) | DQ195591 Thelephorales sp. A.Becerra 08 | 100 \| 156 | MUC [4] | - | AY748876 Uncultured ectomycorrhiza [3] | 91 \| 262 | Thelephoraceae #7<br>Probable ECM taxon |
| J7 – 3.3 (DQ672324) | DQ195591 Thelephorales sp. A.Becerra 08 | 99 \| 156 | AJ581540 Uncultured *Tomentella* sp. | 87 \| 218 | AY748876 Uncultured ectomycorrhiza [3] | 92 \| 262 | Thelephoraceae #8<br>Probable ECM taxon |
| J8 – 3.3 (DQ672325) | AY854066 *Coprinus comatus* | 100 \| 155 | MUC [5] | - | MUC [5] | - | Psathyrellaceae #1 |
| J9 – 3.3 (DQ672326) | AF416589 *Pisolithus* sp. KN6 | 100 \| 156 | AF374675 *Pisolithus tinctorius* “MH731” | 100 \| 182 | AF374670 *Pisolithus tinctorius* “MH132” | 99 \| 253 | *Pisolithus* sp. #2<br>Probable ECM taxon |
| J10 – 3.3 (DQ672318) | DQ420874 Uncultured soil fungus “9b38” | 99 \| 155 | DQ420874 Uncultured soil fungus “9b38” | 96 \| 121 | DQ420874 Uncultured soil fungus “9b38” | 99 \| 198 | *Cryptococcus* sp. #1 **(+Cn)** |
| W1 – 3.3 (DQ672327) | AY969894 Uncultured basidiomycete | 99 \| 153 | MUC | - | MUC | - | Homobasidiomycete #13 |
| W2 – 16.7 (DQ672329) | DQ234537 *Basidioradulum radula* “AFTOL-ID 451” | 100 \| 155 | AY613915 *Limonomyces roseipellis* “Auckland” | 100 \| 207 | MUC | - | *Laetisaria* sp. #1 [7] |
| W3 – 33.3 (DQ672330) | AY635177 *Tomentella stuposa* | 98 \| 156 | AJ633588 Uncultured ectomycorrhizal fungus [3] | 89 \| 215 | AY351622 Uncultured ectomycorrhizal fungus [3] | 93 \| 259 | Thelephoraceae #9<br>Probable ECM taxon |
| W4 – 3.3 (DQ672331) | DQ234537 *Basidioradulum radula* “AFTOL-ID 451” | 100 \| 151 | AY613915 *Limonomyces roseipellis* “Auckland” | 100 \| 207 | MUC | - | *Laetisaria* sp. #2 [7] |
| W5 – 3.3 (DQ672332) | DQ421268 Uncultured soil fungus “9b36” | 99 \| 155 | DQ421268 Uncultured soil fungus “9b36” | 95 \| 170 | DQ421268 Uncultured soil fungus “9b36” | 97 \| 233 | Aphyllophorales #2 |
| W6 – 6.7 (DQ672333) | DQ192182 *Galerina marginata* “AFTOL-ID 465” | 99 \| 155 | DQ421094 Uncultured soil fungus “111-6” | 97 \| 263 | DQ421094 Uncultured soil fungus “111-6” | 98 \| 252 | Psathyrellaceae #2 |
| W7 – 20.0 (DQ672334) | AY521250 *Coprinellus verrucispermus* | 100 \| 155 | MUC [6] | - | MUC [6] | - | Agaricales #7 |
| W8 – 3.3 (DQ672335) | AY456375 *Geastrum* sp. “NC-8353” | 98 \| 153 | MUC | - | MUC [7] | - | Geastrales #1 |
| W9 - 3.3 (DQ672336) | DQ233769 Uncultured ectomycorrhizal fungus “908” | 100 \| 155 | AY613915 *Limonomyces roseipellis* “Auckland” | 100 \| 203 | MUC | - | *Laetisaria* sp. #3 [7] |
| W10 – 6.7 (DQ672328) | DQ452073 *Ceratobasidium* sp. RDLT-2006a | 99 \| 155 | AY927356 *Rhizoctonia* sp. R70 | 99 \| 206 | DQ279056 *Ceratobasidium* sp. AG-K | 98 \| 275 | *Ceratobasidium* sp. #13 |

# Novel soil lineages of *Archaea* are present in semi-arid soils of eastern Australia

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## Abstract

The diversity of *Archaea* was studied in vertisolic and loam soils of a semi-arid region in Australia. Sampling was undertaken at an agricultural site, two grassland environments and a brigalow (*Acacia harpophylla*) woodland. Archaeal community structure was profiled using Amplified Ribosomal DNA Restriction Analysis (ARDRA) combined with rDNA sequencing of an example of each RFLP-type. Sequence comparison and phylogenetic analysis demonstrated that both crenarchaeotal and euryarchaeotal *Archaea* were present at oxic depths in the soil, at all field sites. Along with previously described soil archaeal lineages, novel soil lineages and the deeply divergent Pendant-33 group of *Euryarchaeota* were also detected. A novel statistical method for comparing ARDRA derived data is demonstrated and implemented using the archaeal communities from the four field sites. Archaeal diversity, as measured by this method, was significantly higher in the agricultural site than at either of the grassland sites or the brigalow woodland.

Keywords: Archaeal communities, cotton, Euryarchaeota, Crenarchaeota, soil

## Introduction

Limited molecular studies of *Archaea* indicate that a considerable diversity of nonthermophilic taxa occur in soils (Bintrim *et al.,* 1997; Borneman and Triplett 1997; Jurgens *et al.,* 1997; Buckley *et al.,* 1998; Chelius and Triplett 2001; Nicol *et al.,* 2003; Ochsenreiter *et al.,* 2003). Molecular probing estimates archaeal communities contribute *ca* 1% to total prokaryotic activity in soil (Buckley *et al.,* 1998; Sandaa *et al.,* 1999; Ochsenreiter *et al.,*2003), making them a significant, though poorly understood component of the soil microbiota. The majority of archaeal rDNA sequences identified from oxic soils belong within the kingdom *Crenarchaeota* (Bintrim *et al.,*1997; Jurgens, Lindstrom *et al.,*1997; Nicol *et al.,*2003). Within this kingdom a number of phylogenetically distinct assemblages of non-thermophilic crenarchaeotes have been identified (major groups are numbered 1.1a, 1.1b, 1.1c, 1.2, 1.3a, 1.3b; after (DeLong 1998; Jurgens *et al.,* 2000; Ochsenreiter *et al.,* 2003). Assemblage 1.1b appears to be the most widespread, and has been identified in almost all soils examined to date (Jurgens *et al.,*1997; DeLong 1998; Jurgens and Saano 1999; Pesaro and Widmer 2002; Ochsenreiter et *al.,*2003). Sequences from groups 1.1a have also been recovered from the soil environment, though not from oxic soils (Ochsenreiter et *al.,*2003). Organisms from groups 1.1c and 1.3 are most often found in anaerobic environments (Ochsenreiter et *al.,*2003), but have been infrequently detected in oxic soils (Jurgens et al., 1997; Nicol et al., 2005). Group 1.2 have not yet been detected from soils and may be limited to aquatic sediments, marine and thermophilic environments. Euryarchaeota are often found in anoxic environments such as freshwater sediments and rice paddy soils (Großkopf *et al.,* 1998; Wachinger *et al.,* 2000; Ochsenreiter et *al.,* 2003), however, sequences with affinities to the euryarchaeal order *Thermoplasmatales* have been infrequently detected in oxic soils (Pesaro and Widmer 2002). Members of the other archaeal kingdoms, *Korarchaeota* (Barns *et al.,* 1994; Barns *et al.,* 1996; Pesaro and Widmer 2002) and *Nanoarchaeota* (Huber *et al.,* 2002; Huber *et al.,* 2003) remain unrecorded in soil, possibly because studies conducted to date used PCR primers that do not amplify known representatives of either kingdom (Huber *et al.,* 2002; Baker *et al.,* 2003).

In comparison to bacterial ecology and physiology, the community ecology and functional roles of *Archaea* in soil remain poorly characterised. This deficiency is made worse by the current lack of soil *Archaea* in culture from which physiological information may be derived. Despite these limiting factors, recent studies have demonstrated that diverse archaeal communities can be detected in bulk soil (Pesaro and Widmer 2002; Ochsenreiter et *al.,* 2003; Sliwinski and Goodman 2004), the rhizosphere (Nicol *et al.,* 2003; Sliwinski and Goodman 2004) and within plant roots (Simon *et al.,* 2000; Chelius and Triplett 2001).

Our limited understanding of archaeal community ecology is compounded further by the geographical limitation of field sites sampled. Presently, the majority of soil *Archaea* have been identified in the soils of Europe and North America. Australia has a unique geology and is geographically isolated which has resulted in many endemically Australian microorganisms. To our knowledge, no previous studies have examined archaeal communities in Australian oxic soils.

In eastern Australia, grey cracking clay soils (vertisols) are important both economically, for production of cotton (*Gossypium hirsutum* L.) and other crops, and ecologically as they host remnant stands of endangered brigalow (*Acacia harpophylla* F. Muell. ex Benth) woodland and its associated vegetation communities. While knowledge of plant and animal communities in this region is substantial, virtually nothing is known regarding the microbial diversity of the soils of this region.

In order to partially redress these deficiencies, the aims of the work described in the current study were to determine the presence and diversity of *Archaea* in both cotton cropping, grassland and woodland clay soils in eastern Australia and to compare the diversity and composition of archaeal communities with differing land management practices. As nonthermophilic *Archaea* are recalcitrant to isolation and maintenance in axenic culture, archaeal diversity and community structure were profiled using amplified ribosomal DNA restriction analysis (ARDRA). ARDRA has been used for examination of archaeal communities in a range of similar environments including forest and peatland soils (Pesaro and Widmer 2002; Basiliko *et al.,* 2003) and compost systems (Dees and Ghiorse 2001).

## Materials and Methods

### *Field sites and soil sampling*

Soil samples were collected at four 750 $m^2$ field sites in northwestern New South Wales (NSW) (Table 1). The cotton-wheat rotation field site at Australian Cotton Research

Institute (ACRI) has used for cropping, and subject to various agricultural practices for at least 26 years. The Brigalow Park Nature Reserve (BNR) field site was subject to some selective clearing and grazing in the 1950s, while Claremont Nature Reserve (CNR) and Warrumbungle National Park (WAR) field sites were more thoroughly cleared for agriculture and grazing in the 1950s. In particular, the WAR field site was used for growing lucerne (*Medicago sativa* L.) and oats (*Avena sativa* L.) and may have been subject to some fertiliser application. Grazing pressure by native herbivores and rabbits, along with incursions by stock animals, have presumably maintained the grassland environments at the CNR and WAR field sites. The BNR, CNR and WAR field sites were either gazetted (BNR & WAR) or have been managed (CNR) as nature reserves by the National Parks and Wildlife Service of NSW since the mid 1980s. Twelve *ca.* 500 g soil samples were randomly collected using a trowel at each 750 $m^2$ field site and kept at 4 °C in transit to the laboratory and prior to soil DNA extraction. In all cases samples were taken from a depth of 5 – 15 cm.

*DNA extraction from soil*

DNA was extracted from 0.5 g soil samples using a method modified from Yan and Vancov (unpublished, but detailed here). Approximately 0.5 g of soil was placed into 2.0 ml tubes with 0.8 g of 0.8 – 1.0 mm sterile acid washed ceramic beads (Saint-Gobain ZirPro, Le Pontet, France), 0.3 g 100 μm glass beads (Daintree Scientific, St Helens, Australia), 900 μl 0.1 M $NaPO_4$ and 110 μl of a 50 mM disodium EDTA, 100 mM hydroxymethyl aminomethane, 1% sodium dodecyl sulphate, 1% polyvinylpyrolidone-40 and 0.5% Extran® MA03 solution (Merck and Co, Inc. Whitehouse Station, USA).

Samples were homogenised at 5.5 $ms^{-1}$ for 30 s. in a FastPrep® Instrument (Bio101 Inc. La Jolla, USA). The tubes were then placed on ice for 5 min prior to centrifugation at 12, 500 *g* for 20 min at 4 °C. 700 µl of aqueous supernatant was mixed with 125 µl of 7.5 M potassium acetate. The tubes were incubated at 4 °C for 1 h then centrifuged at 12,500 *g* for 20 min at 4 °C. A half volume of 20% w/v polyethyleneglycol 6000, 2.5 M NaCl was added to the supernatant. Nucleic acids were then precipitated by incubation at 4 °C followed by centrifugation at 12,500 *g* for 20 mins at 4 °C. The pellet was washed overnight in 95 % ethanol at 4 °C, centrifuged at 12,500 *g* for 20 min 4 °C, air dried and resuspended in 50 µl of sterile Milli-Q® water which contained 1/200th volume of 10 mg $ml^{-1}$ RNase A (Sigma, St. Louis, USA). The three replicate 500 mg soil DNA extractions for performed for each individual soil sample, these were then combined and the DNA concentration assessed by electrophoresis.

*PCR amplification*

A 650 bp region of the 16S rDNA was amplified in 25 µl reaction volumes, each containing 1X Reaction Buffer (67 mM hyroxymethyl aminomethane-HCl, 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100 v/v, 0.2 mg $l^{-1}$ gelatin) (Fisher Biotec, West Perth, Australia), 1.5 mM $MgCl_2$, 25 pmol each of the primers A571F and UA1204R (Baker *et al.,* 2003), 100 mM of dATP, dCTP, dGTP and gTTP (Fisher Biotec), 0.7 units of *Taq* DNA polymerase (Fisher Biotec) and 25 ng of sample DNA. Amplifications were performed in a Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany) with a four minute melt at 94 °C proceeding 28 cycles of 94 °C for 45 s, 60.5 °C for 45 s and 72 °C for 45 s, followed by an 8 minute extension at 72 °C. Amplification products were electrophoresed in 1x SB gels containing 1.5% agarose (Brody and Kern 2004) stained with ethidium bromide and visualised under UV light.

*ARDRA*

Twelve replicate PCR-products from each field site were pooled and purified by gel extraction using the QIAquick gel extraction kit (Qiagen, Doncaster, UK). Approximately 100 ng of purified pooled PCR product was ligated in the pDrive vector (Qiagen) following the manufacturers directions and subsequently cloned into competent *E. coli* DH5α. Five hundred clones were screened and sorted into *Taqα1* (New England Biolabs Inc., Ipswich, USA) restriction fragment length polymorphism (RFLP) types and a representative clone of each RFLP-type was chosen at random for DNA sequencing. Sequencing reactions were performed by Macrogen Inc. (Seoul, Korea).

*Sequence analysis*

One sequence for each RFLP-type was submitted to the GenBank nucleotide database under the following accession numbers. Accession codes AY940176 – AY940191 were assigned to archaeal partial 16S rDNA sequences from RFLP-types I-XVI, while accession codes AY944505 – AY944535 were assigned to the unidentified, non-ribosomal sequences obtained. Each sequence was then analysed using the FASTA program version 3.4 (Pearson and Lipman 1988) to find closest matches in the Genbank nucleotide database and report expectation probabilites (E-values). For phylogenetic analysis, *ca.* 300 bp of DNA sequence from each RFLP-type were aligned with sequences of soil Archaea obtained from public databases, using the ClustalW 1.4

(Thompson *et al.,* 1994) accessory application in BioEdit 7.0.4 (Hall 1999). Alignments were then verified visually and corrected manually where required. Neighbour joining phylogenetic tress and associated bootstrap values were generated using PAUP 4.0 (Beta 10) (Swofford 1998).

*Soil analyses*

Electrical conductivity was measured for six randomly selected replicates from each soil using a TDScan20 electrical conductivity meter (Eutech Cybernetics, Singapore) on dried and sieved soil that was resuspended in 5 volumes of $dH_2O$. Soil:water mixtures were shaken for 1 h at 60 rpm on an orbital shaker and allowed to settle for 15 min prior to measurement of EC. Soil percentage water content was determined by weighing *ca* 5 g sub-samples from each of the 12 replicates for each site, prior to and after 12 h incubation in an oven at 95 ºC. Percentage organic matter was determined for the samples dried previously for water content, by determining loss on ignition after 2 hours at 360 ºC followed by 4 hours at 475 ºC in a muffle furnace. Soil organic carbon, pH (in deionised water and $CaCl_2$), nitrate (mg $L^{-1}$) and total nitrogen were measured for three randomly selected replicates for each site and were conducted by Incitec Pivot Laboratories (Werribee, Australia).

*Statistical analysis*

Archaeal biodiversity, as measured by the Simpson's Index (1-D), was compared for the four communities. For each pair $(i, j)$, a $t$-statistic of the form

$$T_{ij} = \frac{\left|\hat{\phi}_i - \hat{\phi}_j\right|}{\mathrm{SE}\left(\hat{\phi}_i - \hat{\phi}_j\right)}$$

was computed, and two sets of multiplicity-adjusted P-values were obtained by comparing each $T_{ij}$ to the upper percentage points of a reference distribution which was a bootstrapped version of $\max_{i \neq j} T_{ij}$. Physical and chemical parameters were compared for soil from the four field sites in a multivariate nonparametric, multiplicity adjusted fashion.

## Results

All soils were broadly similar in their chemical and physical attributes (Table 1). It is noteworthy, however, that the pH of soils at CNR was lower than that at CW ($P < 0.05$), significantly greater total nitrogen was present in soils at BNR than in CW ($P < 0.05$) and that soil moisture was significantly higher at CW than at any other field sites examined ($P < 0.05$).

Amplicons from the A571F and UA1204R primer set (Baker et al., 2003) ranged in size from *ca* 400-850 bp, although most amplification products were 600-750 bp in length. Forty-seven RFLP-types were identified from 125 clones examined from each field site. Sixty-eight percent of all clones, which spanned 31 RFLP-types, showed < 65 % sequence identity with entries in the GenBank nucleotide database or had E-values of 0.05 or greater or both. Given that the ribosomal genes are highly conserved, these sequences were regarded as non-ribosomal and excluded from further analysis.

Of the RFLP-types with archaeal phylogenetic affinities, 50 % had affinities with crenarchaeotal sequences while 50 % of RFLP-types were determined to have euryarchaeotal affinities (Table 2). All crenarchaeal RFLP-types had high sequence identity (94 – 99%) with known *Crenarchaeota* (Table 2) and, with the exception of RFLP-type V, had closest matches to other soil *Crenarchaeota*. RFLP-type V had closest sequence identity (99%) with an archaeal clone isolated from municipal wastewater sludge (Williams, 2001).

With the exception of RFLP-types II and VIII, all rDNA sequences with euryarchaeotal affinities had highest sequence identity (95 – 96 %) with two euryarchaeotes (AJ631245 and AJ631247) detected in cold sulphidic springs in Germany (Rudolph *et al.,* 2004). RFLP-type II had closest sequence identity (89 %) with a euryarchaeote detected in deep-sea marine sediment (Bowman *et al.,* 2000). RFLP-type VIII had closest sequence identity (99 %) with an archaeon (AB237749) detected in deep sub-surface groundwater (Table 2).

Only RFLP-type III was detected at all field sites (Table 3). This RFLP-type was very frequently detected at the WAR, CNR and BNR field sites, comprising 54 %, 62 % and 81 % respectively of archaeal clones detected at each field site, while only poorly represented (3.5 % of archaeal clones) at the CW field site (Table 3). RFLP-types VI and VIII were detected at three field sites, while RFLP-types I, IV and V were detected at two field sites. The majority of archaeal RFLP-types (II, VII, IX, X, XI, XII, XIII, XIV, XV, XVI) were detected at single field sites (Table 3).

The greatest archaeal taxon richness (11 RFLP-types) was observed at the CW field site, while richness at the WAR (6 RFLP-types), CNR (5 RFLP-types) and BNR (4 RFLP-types) field sites was lower. Patterns of relative abundance of RFLP-types at each field site were broadly similar at BNR, CNR and WAR where sites were characterised by a single common RFLP-type and a large number of less common types (Table 3). The pattern observed at the CW field site was different and a more even distribution was observed.

In the crenarchaeal phylogenetic analysis, RFLP-types III, VI, VII, XI, XII, XIII and XV clustered with sequences from group 1.1b (Fig. 1). RFLP-type V, however, clustered with sequences previously assigned to group 1.3 (Ochsenreiter *et al.,* 2003). No sequences with affinities to crenarchaeal groups 1.1a or 1.2 were detected in the soils examined. Phylogenetic trees largely supported the division of soil *Crenarchaeota* into lineages as previously described by DeLong (1998), Jurgens *et al.,* (2000) and Ochsenreiter *et al*., (2003). Most RFLP-types with euryarchaeotal affinities (I, IV, IX, X, XIV and XVI) clustered on a single long branch that grouped with sequences from the euryarchaeotal order *Thermoplasmatales* and a range of other unidentified *Archaea* (Fig 2). RFLP-type VIII also clustered within a group neighbouring the *Thermoplasmatales*, however, in a different position to the other RFLP-types (Fig 2). RFLP-type II clustered within a deeply divergent, but well supported clade with other uncultivated members of the marine sediment group denoted as ‘Pendant-33’ (Fig 2). No sequences with affinities to either *Korarchaeota* or *Nanoarcheota* were observed.

Diversity was analysed using a bootstrap method. The observed counts of each taxon at community $i$ were modelled as having a multinomial distribution, which depended on the sample size $n_i$ as well as the (theoretical) relative abundances of each taxon in the whole community, denoted $\mathbf{p}_i = (p_{i1}, \ldots, p_{iS})$; also denoted by $\hat{\mathbf{p}}_i = (\hat{p}_{i1}, \ldots, \hat{p}_{iS})$ the corresponding observed relative abundances in the sample. Exact P-values could hypothetically be obtained by comparing each $T_{ij}$ to the upper percentage points of the distribution of the random variable

$$M = \max_{i \neq j} \frac{\left| \left( \hat{\phi}_i - \hat{\phi}_j \right) - \left( \phi_i - \phi_j \right) \right|}{\mathrm{SE}\left( \hat{\phi}_i - \hat{\phi}_j \right)},$$

if it were available; here $\phi_i$ and $\phi_j$ (functions of $\mathbf{p}_i$ and $\mathbf{p}_j$) are the theoretical Simpson's indices for communities $i$ and $j$, whereas where $\hat{\phi}_i$ and $\hat{\phi}_j$ (functions of $\hat{\mathbf{p}}_i$ and $\hat{\mathbf{p}}_j$) are the observed Simpson's indices for the samples from communities $i$ and $j$. The upper percentage points $t_\alpha$, for $0 \leq \alpha \leq 1$, of the distribution of $M$ are ultimately only functions of the sample sizes $n_1, \ldots, n_k$ and the theoretical abundances $\mathbf{p}_1, \ldots, \mathbf{p}_k$:

$$t_\alpha = f_\alpha \left( n_1, \ldots, n_k; \mathbf{p}_1, \ldots, \mathbf{p}_k \right);$$

Since these theoretical abundances are unknown, these exact upper percentage points are not available. The bootstrap method presented here approximates the unknown $t_\alpha$, by replacing $\mathbf{p}_1, \ldots, \mathbf{p}_k$ in the above expression by estimates of them, that is using upper percentage points given by

$$t_\alpha^* = f_\alpha \left( n_1, \ldots, n_k; \hat{\mathbf{p}}_1, \ldots, \hat{\mathbf{p}}_k \right)$$

The Monte Carlo simulation procedure for obtaining an approximation to $t_{\alpha}^{*}$, for each $0 \le \alpha \le 1$, is as follows:

1. Simulate data from communities whose theoretical relative abundances are equal to the actual observed relative abundances relative abundances $\hat{\mathbf{p}}_1,\ldots,\hat{\mathbf{p}}_k$;
2. Compute estimates $\hat{\phi}_i^{*}$, differences $\hat{\phi}_i^{*} - \hat{\phi}_j^{*}$ and standard errors based on the simulated data.
3. Compute the quantity; $M^{*} = \max\limits_{i \ne j} \dfrac{\left|\left(\hat{\phi}_i^{*} - \hat{\phi}_j^{*}\right) - \left(\hat{\phi}_i - \hat{\phi}_j\right)\right|}{\mathrm{SE}\left(\hat{\phi}_i^{*} - \hat{\phi}_j^{*}\right)}$;
4. Repeat, 10 000 times.

$t_{\alpha}^{*}$, for each $0 \le \alpha \le 1$, is then approximated by the upper $\alpha$ quantile of the simulated $M^{*}$ values, e.g. $t_{0.05}^{*}$ is approximated by the 500th largest $M^{*}$ value.

Archaeal biodiversity, as measured by the Simpson's Index was significantly higher (P <0.01) in at the cotton-wheat field site than at any of the other field sites (Table 4). In contrast, archaeal biodiversity was not significantly different (P>0.05) between soils at CNR, WAR or BNR (Table 4). The accuracy of the bootstrap method used in the present study was examined using an iterated version, which was found to be very accurate, even for the small sample size in the present study.

## Discussion

Data from the present study indicate that phylogenetically diverse consortia of *Archaea* are present in Australian soils. This is the first report of *Archaea* in oxic soils from the

Australian continent. Previous studies in the northern hemisphere have detected predominately crenarchaeal taxa in soils (Bintrim *et al.,*1997; Borneman and Triplett 1997; Jurgens *et al.,*1997; Buckley *et al.,*1998; Chelius and Triplett 2001; Nicol *et al.,*2003). In the present study, however, fifty percent of taxa detected were euryarchaeal in origin.

*Euryarchaeota* are often found associated with anaerobic environments (Kudo *et al.,* 1997; GroBkopf *et al.,*1998; Newberry *et al.,* 2004). At presumably oxic soil depths, euryarcheotes had been previously detected only once in boreal forest soils (Pesaro and Widmer, 2002). The present study extends that range to include semi-arid woodland, grassland and agricultural soils at oxic depths. Interestingly, four of the eight euryarchaeotal RFLP-types from the present study were detected exclusively at the CW field site at the Australian Cotton Research Institute (ACRI). The CW site is routinely irrigated and is thus significantly moister than the non-agricultural sites examined. This irrigation may create more anoxic microhabitats in the soil in which anaerobic taxa may occur. Further studies would be required to test this hypothesis.

Six of the euryarchaeotal RFLP-types in this study clustered together on a single long branch within a well-supported clade that contained the *Thermoplasmatales*, group “E” forest soil euryarchaeotes (Pesaro and Widmer, 2002) and a range of uncultured organisms including RFLP-type VIII. Six RFLP-types shared greatest sequence identity with two organisms (AJ631245 and AJ631247) detected in cold sulphidic springs in Eschenlöhe, Germany (Rudolph et al., 2004). Unfortunately, due to differences in the region of 16S rDNA amplified in the present study and by Rudolph et al., (2004), the two archaeons from cold sulphidic springs (AJ631245 and AJ631247) could not be included in the phylogenetic analyses presented here. Despite this, the close identity between AJ631245, AJ631247 and RFLP-types I, IV, IX, X, XIV and XVI suggest they probably form part of a single group (denoted in the present study as the Eschenlöhe-ACRI group). Based on 16S rDNA sequence the Eschenlöhe-ACRI group appears to be considerably different to both the known Thermoplasmatales, group "E" forest soil euryarchaeotes and other organisms within this clade. The low bootstrap support within this clade renders relationships between known Thermoplasmatales, the Eschenlöhe-ACRI group, with group "E" forest soil euryarchaeotes and organisms more closely related to RFLP VIII, difficult to resolve.

RFLP-type II shared the greatest sequence identity (89%) with an uncultured archaeal isolate (AF424536) from Antarctic shelf sediments (Bowman *et al.,* 2003). Phylogenetically, this RFLP-type clusters with the deeply diverged Pendant-33 group of *Archaea*. Included in this group are a range of extremeophile isolates primarily from deep-sea, Antarctic freshwater or hydrothermal vent habitats. Sequence analysis by Bowman *et al.,* (2000) suggested a phylogenetic position for the Pendant-33 assemblage, equidistant from the euryarchaeotal orders *Thermoplasmatales*, *Methanomicrobiales* and *Halobacteriales*, but remote from other *Euryarchaeota* or *Crenarchaeota* (Bowman *et al.,* 2000). The detection of RFLP-type II at the CNR field site is the first time, to our knowledge, that a member of this poorly characterised group has been detected in soil.

Most crenarchaeal RFLP-types (except RFLP-type V) from the present study had high sequence identity to, and clustered with, previously detected group 1.1b *Crenarchaeota*. Group 1.1b appears to be very widely distributed in soils, having been detected in a number of soils in North America, Europe and Asia (Bintrim *et al.,*1997; Buckley *et al.,*1998; Sandaa *et al.,*1999; Kim *et al.,* 2000; Ochsenreiter et *al.,*2003) and now Australia. Moreover, RFLP-type III was detected at all field sites (Table 3, Figure 1), further demonstrating the ubiquity of this group in soils.

Interestingly, RFLP-type V grouped with other uncultured crenarchaeotes from group 1.3. Most 1.3 isolates have been previously detected in anoxic environments such as freshwater sediments or wetland soils (Hershberger *et al.,* 1996; Ochsenreiter et *al.,*2003; Utsumi *et al.,* 2003). If it is presumed this organism, like other 1.3 crenarchaeotes, is anaerobic, then this taxon may be found inside heavy clay aggregates or in other anoxic microhabitats at our field sites. Alternatively, RFLP-type V may represent an aerobic or at least oxygen tolerant taxon within this group. In oxic soils this group has been previously described from 50-100 cm sub-surface, a presumably anoxic depth, in a boreal forest site in Europe (Pesaro and Widmer 2002).

The bootstrap method used to compare diversity in this study yielded similar P-values for pairs of sites with similar diversity to the method proposed by Rogers and Hsu (2001). When pairs of field sites with very different diversity indices were compared, however, the method proposed by Rogers and Hsu (2001) gave much smaller P-values (up to 10 times smaller) than bootstrap method, indicating that although conservative in large samples, the Rogers and Hsu (2001) method is actually anti-conservative with data of small sample size.

Significant differences in archaeal biodiversity were observed between the field sites by using this method. In the four soils sampled, greatest biodiversity, as measured by the Simpson's Index, was present at the CW field site. Three of the field sites (CW, CNR, & BNR) have vertisolic soils and all are located in the Narrabri area. Prior to agricultural development, the soils were presumably physically and chemically similar. Differences among the archaeal communities between these sites may therefore be a result of soil management practices or changes to plant community structure. The greater diversity at the CW field site may indicate that cropping soils have greater archaeal diversity than soils in less disturbed grassland or woodland sites. It is noteworthy, however, that this may only apply to the one time point sampled, in the field sites examined. Further studies are required to rigorously test this possibility. Land management practice has been previously shown to alter archaeal community structure in improved and unimproved pasture soils in the UK (Nicol *et al.,*2003), though biodiversity of *Archaea per se* was not compared. If soil archaeal communities are consistently more diverse in disturbed soils, they may represent a useful group of so called 'indicator' organisms for the assessment of disturbance in soils.

Of the 47 RFLP-types, 31 RFLP-types were not ribosomal DNA. It seems likely that these RFLP-types represent amplification of poorly conserved regions from as yet unstudied organisms. Sequence identities between these sequences were low (data not shown) suggesting a range of templates were amplified. The primer set used in the current study was proposed by Baker *et al.,* (2003) and has been used to sucessfully amplify crenarchaeal sequences from microbial communities in thermal springs (Baker, Smith *et al.,*2003; Baker and Cowan 2004). The amplification of non-ribosomal sequences in the present study is presumably a function of both the degeneracies in the primers and the much greater diversity of templates in soil compared to that in a thermal spring. Additionally, *Archaea* are less common in soil, compared to their relatively high representation in the microbial community in thermal springs. For these reasons it would seem that this primer set is not the most optimal for profiling of soil archaeal communities. Conversely, the increased percentage of Euryarchaeota detected in the present study may be a result of less crenarchaeal bias in the Baker et al., (2003) primer set.

In conclusion, the findings of the current study indicate a large phylogenetic range of *Archaea* are present in semi-arid vertisolic and loam soils of eastern Australia. Further, cultivation and cropping may influence archaeal community structure. Examination of soils under a range of edaphic stresses and manipulative experiments, may improve understanding of the importance of disturbance on the diversity of *Archaea*.

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**Table 1.** Field site locations and soil properties.

| Field site | Soil-type | pH in $CaCl_2$ & ($dH_2O$) | EC $\mu S$ $cm^{-1}$ | %WC | %SOC | %ORM | $NO^{3-}$ mg $L^{-1}$ | %N | Vegetation | Location | Land use |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Cotton-wheat rotation (CW) | Grey vertisol | 7.6 (8.3) | 48 (± 7) | 9.6 (± 0.3) | 0.94 (± 0.06) | 5.57 (± 0.33) | 1.73 (± 0.32) | 0.07 (± 0.01) | Cotton-wheat rotation | S30° 12’ E149° 35’ | Heavily cropped > 25 years, agricultural |
| Claremont Nature Reserve (CNR) | Grey vertisol | 5.1 (6.3) | 27 (± 10) | 5.0 (± 0.2) | 0.84 (± 0.07) | 6.72 (± 0.49) | 1.06 (± 0.06) | 0.11 (± 0.03) | Mainly native grasses | S30° 23’ E149° 34’ | Regenerating grazing/agricultural land, cleared in the 1950s |
| Brigalow Park Nature Reserve (BNR) | Grey vertisol | 6.6 (7.3) | 52 (± 13) | 6.6 (± 0.5) | 2.43 (± 0.57) | 7.39 (± 0.99) | 2.87 (± 0.78) | 0.23 (± 0.08) | Mature brigalow with mixed understorey plants | S30° 24’; E149° 35’ | Mature brigalow woodland, some limited clearing in the 1950s |
| Warrumbungle National Park (WAR) | Alluvial loam | 6.3 (7.0) | 48 (± 8) | 1.7 (± 0.1) | 1.97 (± 0.32) | 5.87 (± 0.57) | 2.43 (± 0.72) | 0.15 (± 0.05) | Mixed herbaceous plants | S31° 31’; E149° 6’ | Regenerating grazing/agricultural land, cleared in the 1950s |

EC = mean electrical conductivity ± standard error (SE), %WC = mean percentage water content (±SE), %SOC = mean percentage organic carbon (±SE), %ORM = mean percentage organic matter (±SE), %N = mean percentage nitrogen (±SE).

**Table 2.** Comparison of representative sequences from RFLP-types with GenBank nucleotide database entries.

| Archaeal RFLP-types (accession code) | Closest GenBank database match | GenBank accession code | Affinity | Identity (%) | Nucleotide overlap (bp) |
|---|---|---|---|---|---|
| **I** (AY940176) | Uncultured archaeon 'HSWK56' | AJ631247 | Euryarchaeota | 95.6 | 547 |
| **II** (AY940177) | Uncultured euryarchaeote 'MERTZ_21CM_297' | AF424536 | Euryarchaeota | 88.9 | 553 |
| **III** (AY940178) | Uncultured soil crenarchaeote | AJ496176 | Crenarchaeota | 97.9 | 642 |
| **IV** (AY940179) | Uncultured archaeon 'HSWK20' | AJ631245 | Euryarchaeota | 96.5 | 550 |
| **V** (AY940180) | Uncultured archaeon 62-2 | AF424775 | Crenarchaeota | 98.4 | 646 |
| **VI** (AY940181) | Unidentified archaeon 'SCA1154' | U62814 | Crenarchaeota | 98.6 | 653 |
| **VII** (AY940182) | Unidentified archaeon 'SCA1154' | U62814 | Crenarchaeota | 94.1 | 657 |
| **VIII** (AY940183) | Uncultured archaeon 'HDBW-WA16' | AB237749 | Euryarchaeota | 98.9 | 651 |
| **IX** (AY940184) | Uncultured archaeon 'HSWK20' | AJ631245 | Euryarchaeota | 96.7 | 533 |
| **X** (AY940185) | Uncultured archaeon 'HSWK20' | AJ631245 | Euryarchaeota | 96.8 | 547 |
| **XI** (AY940186) | Uncultured crenarchaeote 'MAS3' | AY522900 | Crenarchaeota | 99.2 | 636 |
| **XII** (AY940187) | Uncultured soil crenarchaeote | AJ496176 | Crenarchaeota | 98.3 | 654 |
| **XIII** (AY940188) | Uncultured crenarchaeote '19' | AY942993 | Crenarchaeota | 98.9 | 654 |
| **XIV** (AY940189) | Uncultured archaeon clone 'HSWK20' | AJ631245 | Euryarchaeota | 97.8 | 550 |
| **XV** (AY940190) | Uncultured crenarchaeote clone 'MGS6' | AY522874 | Crenarchaeota | 98.5 | 655 |
| **XVI** (AY940191) | Uncultured archaeon clone 'HSWK20' | AJ631245 | Euryarchaeota | 96.7 | 533 |

**Table 3.** Distribution and *Taq*α1restriction fragment sizes of archaeal RFLP-types.

| **RFLP-type** | **Fragment sizes (bp)** | **Number of clones in which fragment sizes were observed** | | | |
|---|---|---|---|---|---|
| | | **CW** | **WAR** | **CNR** | **BNR** |
| I | 379, 189, 43, 25, 17 | **7** | - | **1** | - |
| II | 389, 294 | - | - | **2** | - |
| III | 471, 99, 72 | **1** | **20** | **41** | **21** |
| IV | 543, 51, 43, 17 | **3** | **3** | - | - |
| V | 537, 109 | **3** | - | **13** | - |
| VI | 470, 183 | **3** | **1** | - | **1** |
| VII | 470, 115, 72 | - | **1** | - | - |
| VIII | 650, 4 | **7** | **11** | **9** | - |
| IX | 506, 88, 43, 17 | **2** | - | - | - |
| X | 569, 85 | **1** | - | - | - |
| XI | 376, 165, 94 | - | - | - | **3** |
| XII | 471, 111, 72 | - | - | - | **1** |
| XIII | 415, 229, 104, 83, 46 | **4** | - | - | - |
| XIV | 353, 189, 43, 26, 17 | **3** | - | - | - |
| XV | 471, 184 | - | **1** | - | - |
| XVI | 594, 43, 17 | **1** | - | - | - |

“-“ = Not detected. CW = Cotton wheat rotation, WAR = Warrumbungle National Park, CNR = Claremont Nature Reserve, BNR = Brigalow Park Nature Reserve

**Table 4.** Pair-wise differences between the Simpson's Index (1-D) for all pairs of archaeal communities (± standard error). Multiplicity adjusted P-values for all pairs were obtained using a bootstrapping method with 10 000 replicates.

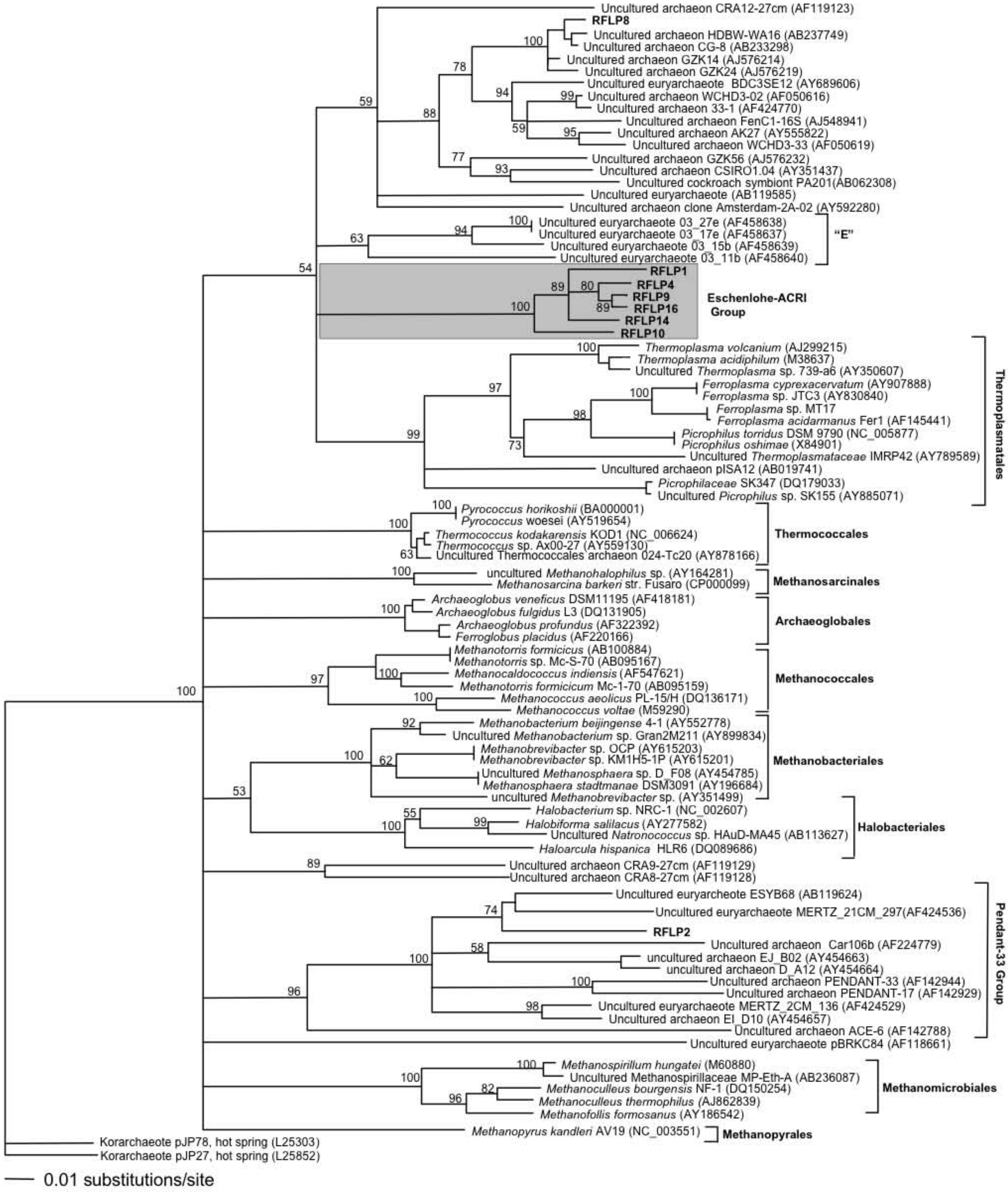
| **Site:** | **WAR** | **CNR** | **BNR** |
|---|---|---|---|
| **CW** | 0.271 ± 0.096<br>(P<0.01) | 0.290 ± 0.092<br>(P<0.01) | 0.510 ± 0.148<br>(P<0.01) |
| **WAR** | - | 0.019 ± 0.118<br>(P>0.05) | 0.239 ± 0.148<br>(P>0.05) |
| **CNR** | - | - | 0.219 ± 0.170<br>(P>0.05) |

CW = Cotton wheat rotation, WAR = Warrumbungle National Park, CNR = Claremont Nature Reserve, BNR = Brigalow Park Nature Reserve.

**Figure 1.** Neighbour-joining phylogram of partial 16S rDNA sequences from crenarchaeal RFLP-types (bold) with cultured and uncultivated members of the *Crenarchaeota*. Two Korarchaea were used as outgroup taxa. Brackets denote major clades. Bootstrap values > 80% are shown.

**Figure 2.** Neighbour-joining phylogram of partial 16S rDNA sequences from euryarchaeal RFLP-types (bold) with cultured and uncultivated members of the *Euryarchaeota*. Two Korarchaea were used as outgroup taxa. Brackets denote major clades. Bootstrap values > 80% are shown.


100
Uncultured archaeon SAGMA-11 (AB050241)
Uncultured archaeon SAGMA-10 (AB050240)
98
Uncultured crenarchaeote 12_11d (AF458628)
58
Uncultured bacterium SRS62DAR03 (AF389433)
80
Uncultured archaeon SAGMA-D (AB050208)
76
Uncultured archaeon SAGMA-X (AB050229)
Group 1.1a *sensu lato*
Clade "A"
100
Unidentified ag. soil archaeon SCA1170 (U62817)
Uncultured crenarchaeote taxon 29281 (U59968)
88
Uncultured root crenarchaeote TRC132-3 (AF227635)
85
Unidentified ag. soil archaeon SCA1154 (U62814)
95
100
**RFLP7**
**RFLP6**
Uncultured soil crenarchaeote (AJ627422)
Unidentified ag. soil archaeon SCA1150 (U62812)
55
Unidentified ag. soil archaeon SCA1175 (U62819)
Uncultured crenarchaeote MGS6 (AY522874)
96
Uncultured forest soil crenarchaeote 03 17c (AF458630)
Uncultured forest soil crenarchaeote 03 06c (AF458629)
71
51
59
Uncultured soil crenarchaeote (AJ496176)
Uncultured crenarchaeote DI_C04 (AY454557)
72
64
Uncultured crenarchaeote MAS5 (AY522899)
91
Uncultured ag. soil archaeon SCA1145 (U62811)
Uncultured crenarchaeote TRC23-28 (AF227641)
63
**RFLP3**
**RFLP12**
**RFLP15**
100
Unidentified soil archaeon 13776 (U68650)
53
Unidentified forest soil archaeon 13776 (U68604)
Unidentified ag. soil archaeon SCA1151 (U62813)
**RFLP13**
91
Uncultured ag. soil archaeon SCA1173 (U62818)
72
Uncultured crenarchaeote MAS3 (AY522900)
**RFLP11**
Uncultured crenarchaeote MAS14 (AY522896)
Uncultured crenarchaeote 19 (AY942993)
**Group 1.1b *senso strict0***
100
Uncultured soil crenarchaeote 03 12a (AF458631)
72
Uncultured soil crenarchaeote 03 01a (AF458636)
**"B"**
Uncultured soil crenarchaeote 03 21d (AF458641)
Crenarchaeote symbiont of *Axinella verrucosa* (AF420237)
68
Uncultured crenarchaeote pJP96, hotspring (U63338)
Uncultured forest soil crenarchaeote 12 45f (AF458643)
**"C"**
97
82
Uncultured crenarchaeote pSL22 (U63340)
**Thermoproteales**
*Thermofilum pendens* (X14835)
Unidentified crenarchaeote clone pGrfC26 (U59986)
100
72
**RFLP5**
Uncultured archaeon 62-2 (AF424775)
60
Unidentified archaeon vadinDC69 (U81774)
**Group 1.3**
100
Uncultured crenarchaeote 12_01a (AF458645)
Uncultured crenarchaeote 12_02a (AF458646)
**"D"**
100
Uncultured archaeon OS-27 (AB056052)
61
Uncultured archaeon OS-26 (AB056051)
99
Uncultured archaeon OS-14 (AB056039)
Uncultured archaeon WSB-20 (AB056002)
93
*Aeropyrum pernix* OH3 (AB078016)
68
*Aeropyrum pernix* OH2 (AB019552)
71
*Thermodiscus maritimus* (X99554)
Uncultured *Desulfurococcus* sp. SUBT-9 (AF361213)
98
*Staphylothermus marinus* DSM3639 (X99560)
*Staphylothermus achaiicus* P8 (AJ012645)
*Desulfurococcus mobilis* (M36474)
52
*Desulfurococcus fermentans* Z-1312 (AY264344)
*Desulfurococcus* sp. SEA (AF411230)
Desulfurococcaceae SRI-465 (AF255606)
71
93
*Ignicoccus pacificus* LPC33 (AJ271794)
97
*Ignicoccus islandicus* (X99562)
*Ignicoccus* sp. Kin4-I (AJ318042)
100
*Caldisphaera lagunensis* (AB087499)
Uncultured *Caldisphaera* sp. clone M6 (AY627858)
*Acidolobus aceticus* (AF191225)
*Sulfophobococcus zilligii* (X98064)
*Thermosphaera aggregans* (X99556)
100
*Pyrodictium occultum* (M21087)
*Pyrolobus fumarii* (x99555)
**Desulfurococcales**
Unidentified korarchaeote pJP78 (L25303)
Unidentified korarchaeote pJP27 (L25852)
0.01 substitutions/site


Uncultured archaeon CRA12-27cm (AF119123)
RFLP8
100
Uncultured archaeon HDBW-WA16 (AB237749)
Uncultured archaeon CG-8 (AB233298)
78
Uncultured archaeon GZK14 (AJ576214)
Uncultured archaeon GZK24 (AJ576219)
59
Uncultured euryarchaeote BDC3SE12 (AY689606)
94
99
Uncultured archaeon WCHD3-02 (AF050616)
88
Uncultured archaeon 33-1 (AF424770)
Uncultured archaeon FenC1-16S (AJ548941)
59
95
Uncultured archaeon AK27 (AY555822)
Uncultured archaeon WCHD3-33 (AF050619)
77
Uncultured archaeon GZK56 (AJ576232)
93
Uncultured archaeon CSIRO1.04 (AY351437)
Uncultured cockroach symbiont PA201(AB062308)
Uncultured euryarchaeote (AB119585)
Uncultured archaeon clone Amsterdam-2A-02 (AY592280)
100
Uncultured euryarchaeote 03_27e (AF458638)
94
Uncultured euryarchaeote 03_17e (AF458637)
63
Uncultured euryarchaeote 03_15b (AF458639)
"E"
Uncultured euryarchaeote 03_11b (AF458640)
54
RFLP1
89
80
RFLP4
RFLP9
Eschenlohe-ACRI Group
100
89
RFLP16
RFLP14
RFLP10
100
Thermoplasma volcanium (AJ299215)
Thermoplasma acidiphilum (M38637)
Uncultured Thermoplasma sp. 739-a6 (AY350607)
97
Ferroplasma cyprexacervatum (AY907888)
100
Ferroplasma sp. JTC3 (AY830840)
Ferroplasma sp. MT17
98
Ferroplasma acidarmanus Fer1 (AF145441)
Thermoplasmatales
99
Picrophilus torridus DSM 9790 (NC_005877)
Picrophilus oshimae (X84901)
73
Uncultured Thermoplasmataceae IMRP42 (AY789589)
Uncultured archaeon pISA12 (AB019741)
Picrophilaceae SK347 (DQ179033)
Uncultured Picrophilus sp. SK155 (AY885071)
100
Pyrococcus horikoshii (BA000001)
100
Pyrococcus woesei (AY519654)
Thermococcus kodakarensis KOD1 (NC_006624)
Thermococcales
Thermococcus sp. Ax00-27 (AY559130)
63
Uncultured Thermococcales archaeon 024-Tc20 (AY878166)
100
uncultured Methanohalophilus sp. (AY164281)
Methanosarcinales
Methanosarcina barkeri str. Fusaro (CP000099)
Archaeoglobus veneficus DSM11195 (AF418181)
100
Archaeoglobus fulgidus L3 (DQ131905)
Archaeoglobales
Archaeoglobus profundus (AF322392)
Ferroglobus placidus (AF220166)
Methanotorris formicicus (AB100884)
Methanotorris sp. Mc-S-70 (AB095167)
100
Methanocaldococcus indiensis (AF547621)
97
Methanococcales
Methanotorris formicicum Mc-1-70 (AB095159)
100
100
Methanococcus aeolicus PL-15/H (DQ136171)
Methanococcus voltae (M59290)
92
Methanobacterium beijingense 4-1 (AY552778)
Uncultured Methanobacterium sp. Gran2M211 (AY899834)
100
Methanobrevibacter sp. OCP (AY615203)
62
Methanobrevibacter sp. KM1H5-1P (AY615201)
Methanobacteriales
Uncultured Methanosphaera sp. D_F08 (AY454785)
Methanosphaera stadtmanae DSM3091 (AY196684)
53
uncultured Methanobrevibacter sp. (AY351499)
55
Halobacterium sp. NRC-1 (NC_002607)
99
Halobiforma salilacus (AY277582)
100
Uncultured Natronococcus sp. HAuD-MA45 (AB113627)
Halobacteriales
Haloarcula hispanica HLR6 (DQ089686)
89
Uncultured archaeon CRA9-27cm (AF119129)
Uncultured archaeon CRA8-27cm (AF119128)
Uncultured euryarcheote ESYB68 (AB119624)
74
Uncultured euryarchaeote MERTZ_21CM_297(AF424536)
RFLP2
58
Uncultured archaeon Car106b (AF224779)
100
uncultured archaeon EJ_B02 (AY454663)
uncultured archaeon D_A12 (AY454664)
Pendant-33 Group
96
100
Uncultured archaeon PENDANT-33 (AF142944)
Uncultured archaeon PENDANT-17 (AF142929)
98
Uncultured euryarchaeote MERTZ_2CM_136 (AF424529)
Uncultured archaeon EI_D10 (AY454657)
Uncultured archaeon ACE-6 (AF142788)
Uncultured euryarchaeote pBRKC84 (AF118661)
100
Methanospirillum hungatei (M60880)
100
Uncultured Methanospirillaceae MP-Eth-A (AB236087)
82
Methanoculleus bourgensis NF-1 (DQ150254)
Methanomicrobiales
96
Methanoculleus thermophilus (AJ862839)
Methanofollis formosanus (AY186542)
Methanopyrus kandleri AV19 (NC_003551)
Methanopyrales
Korarchaeote pJP78, hot spring (L25303)
Korarchaeote pJP27, hot spring (L25852)
0.01 substitutions/site