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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 ACRI* adjacent sit to cotton field	S30° 12' 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 Brigalow Nature Park Reserve	S30° 24'; 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 Claremont Nature reserve	S30° 23' 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 ACRI* cotton-wheat rotation	S30° 12' 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 ACRI* 7 year fallow site	S30° 12' 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 Jacks Creek State Forest	S30° 27'; 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 Warrumbungle National Park	S31° 31'; 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)** 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 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 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 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 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 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 ϕ_i and ϕ_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_α, 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_α, 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_α^*, 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; $M^* = \max_{i \neq j} \dfrac{\left|\left(\hat{\phi}_i^* - \hat{\phi}_j^*\right) - \left(\hat{\phi}_i - \hat{\phi}_j\right)\right|}{\mathrm{SE}\left(\hat{\phi}_i^* - \hat{\phi}_j^*\right)}$;
4. Repeat, 10 000 times.

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

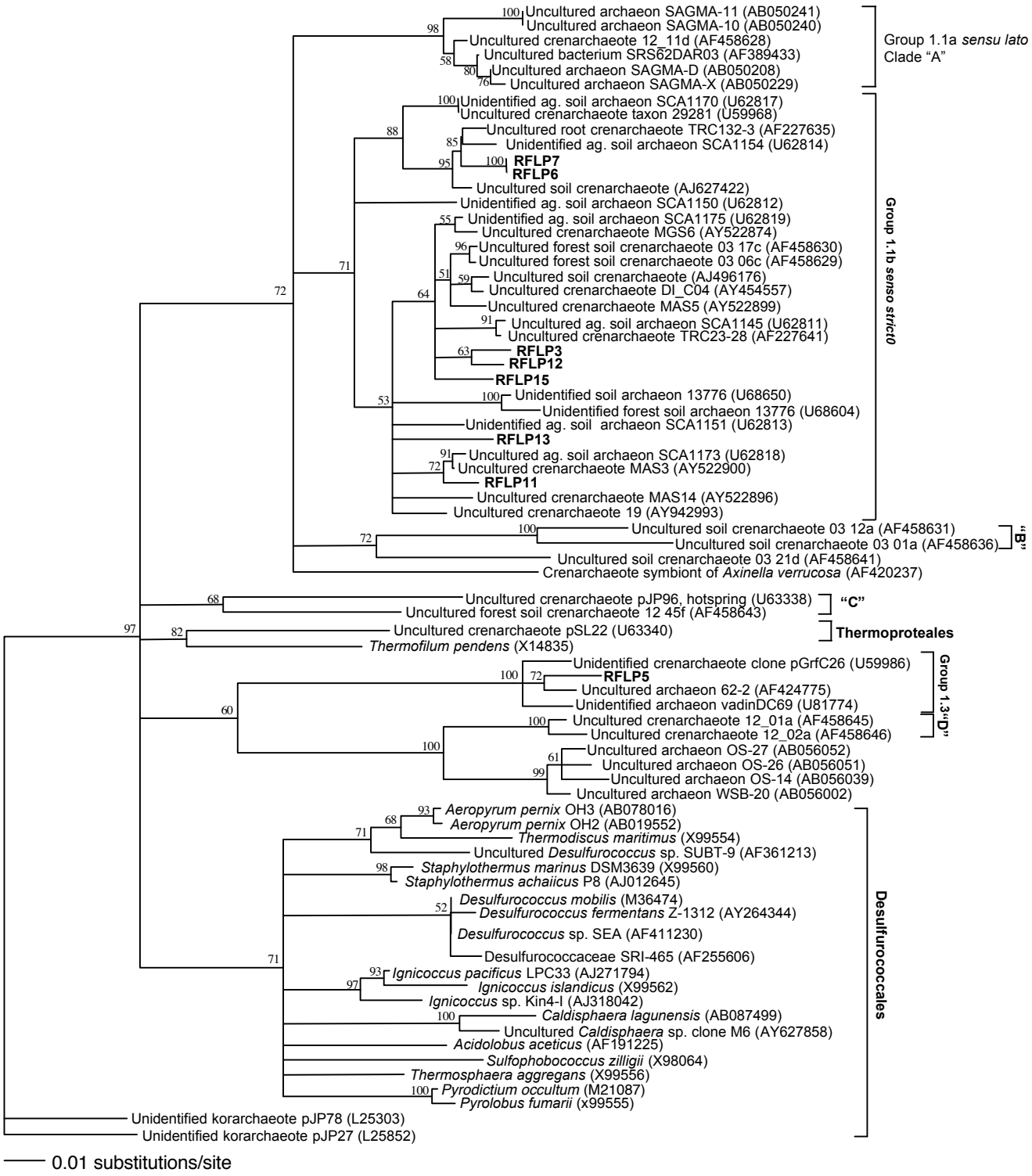
Table 4. Pair-wise differences between the Simpson's Index (1-D) for all pairs of archaeal communities (± standard error). Multiplicity adjusted P-values for all pairs were obtained using a bootstrapping method with 10 000 replicates.

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


Uncultured archaeon SAGMA-11 (AB050241)
Uncultured archaeon SAGMA-10 (AB050240)
Uncultured crenarchaeote 12_11d (AF458628)
Uncultured bacterium SRS62DAR03 (AF389433)
Uncultured archaeon SAGMA-D (AB050208)
Uncultured archaeon SAGMA-X (AB050229)
Group 1.1a sensu lato
Clade "A"
Unidentified ag. soil archaeon SCA1170 (U62817)
Uncultured crenarchaeote taxon 29281 (U59968)
Uncultured root crenarchaeote TRC132-3 (AF227635)
Unidentified ag. soil archaeon SCA1154 (U62814)
RFLP7
RFLP6
Uncultured soil crenarchaeote (AJ627422)
Unidentified ag. soil archaeon SCA1150 (U62812)
Unidentified ag. soil archaeon SCA1175 (U62819)
Uncultured crenarchaeote MGS6 (AY522874)
Uncultured forest soil crenarchaeote 03 17c (AF458630)
Uncultured forest soil crenarchaeote 03 06c (AF458629)
Uncultured soil crenarchaeote (AJ496176)
Uncultured crenarchaeote DI_C04 (AY454557)
Uncultured crenarchaeote MAS5 (AY522899)
Uncultured ag. soil archaeon SCA1145 (U62811)
Uncultured crenarchaeote TRC23-28 (AF227641)
RFLP3
RFLP12
RFLP15
Unidentified soil archaeon 13776 (U68650)
Unidentified forest soil archaeon 13776 (U68604)
Unidentified ag. soil archaeon SCA1151 (U62813)
RFLP13
Uncultured ag. soil archaeon SCA1173 (U62818)
Uncultured crenarchaeote MAS3 (AY522900)
RFLP11
Uncultured crenarchaeote MAS14 (AY522896)
Uncultured crenarchaeote 19 (AY942993)
Group 1.1b senso strict0
Uncultured soil crenarchaeote 03 12a (AF458631)
Uncultured soil crenarchaeote 03 01a (AF458636)
"B"
Uncultured soil crenarchaeote 03 21d (AF458641)
Crenarchaeote symbiont of Axinella verrucosa (AF420237)
Uncultured crenarchaeote pJP96, hotspring (U63338)
Uncultured forest soil crenarchaeote 12 45f (AF458643)
"C"
Uncultured crenarchaeote pSL22 (U63340)
Thermofilum pendens (X14835)
Thermoproteales
Unidentified crenarchaeote clone pGrfC26 (U59986)
RFLP5
Uncultured archaeon 62-2 (AF424775)
Unidentified archaeon vadinDC69 (U81774)
Uncultured crenarchaeote 12_01a (AF458645)
Uncultured crenarchaeote 12_02a (AF458646)
Group 1.3"D"
Uncultured archaeon OS-27 (AB056052)
Uncultured archaeon OS-26 (AB056051)
Uncultured archaeon OS-14 (AB056039)
Uncultured archaeon WSB-20 (AB056002)
Aeropyrum pernix OH3 (AB078016)
Aeropyrum pernix OH2 (AB019552)
Thermodiscus maritimus (X99554)
Uncultured Desulfurococcus sp. SUBT-9 (AF361213)
Staphylothermus marinus DSM3639 (X99560)
Staphylothermus achaiicus P8 (AJ012645)
Desulfurococcus mobilis (M36474)
Desulfurococcus fermentans Z-1312 (AY264344)
Desulfurococcus sp. SEA (AF411230)
Desulfurococcaceae SRI-465 (AF255606)
Ignicoccus pacificus LPC33 (AJ271794)
Ignicoccus islandicus (X99562)
Ignicoccus sp. Kin4-I (AJ318042)
Caldisphaera lagunensis (AB087499)
Uncultured Caldisphaera sp. clone M6 (AY627858)
Acidolobus aceticus (AF191225)
Sulfophobococcus zilligii (X98064)
Thermosphaera aggregans (X99556)
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
Uncultured archaeon HDBW-WA16 (AB237749)
Uncultured archaeon CG-8 (AB233298)
Uncultured archaeon GZK14 (AJ576214)
Uncultured archaeon GZK24 (AJ576219)
Uncultured euryarchaeote BDC3SE12 (AY689606)
Uncultured archaeon WCHD3-02 (AF050616)
Uncultured archaeon 33-1 (AF424770)
Uncultured archaeon FenC1-16S (AJ548941)
Uncultured archaeon AK27 (AY555822)
Uncultured archaeon WCHD3-33 (AF050619)
Uncultured archaeon GZK56 (AJ576232)
Uncultured archaeon CSIRO1.04 (AY351437)
Uncultured cockroach symbiont PA201(AB062308)
Uncultured euryarchaeote (AB119585)
Uncultured archaeon clone Amsterdam-2A-02 (AY592280)
Uncultured euryarchaeote 03_27e (AF458638)
Uncultured euryarchaeote 03_17e (AF458637)
Uncultured euryarchaeote 03_15b (AF458639)
Uncultured euryarchaeote 03_11b (AF458640)
"E"
RFLP1
RFLP4
RFLP9
RFLP16
RFLP14
RFLP10
Eschenlohe-ACRI Group
Thermoplasma volcanium (AJ299215)
Thermoplasma acidiphilum (M38637)
Uncultured Thermoplasma sp. 739-a6 (AY350607)
Ferroplasma cyprexacervatum (AY907888)
Ferroplasma sp. JTC3 (AY830840)
Ferroplasma sp. MT17
Ferroplasma acidarmanus Fer1 (AF145441)
Picrophilus torridus DSM 9790 (NC_005877)
Picrophilus oshimae (X84901)
Uncultured Thermoplasmataceae IMRP42 (AY789589)
Uncultured archaeon pISA12 (AB019741)
Picrophilaceae SK347 (DQ179033)
Uncultured Picrophilus sp. SK155 (AY885071)
Thermoplasmatales
Pyrococcus horikoshii (BA000001)
Pyrococcus woesei (AY519654)
Thermococcus kodakarensis KOD1 (NC_006624)
Thermococcus sp. Ax00-27 (AY559130)
Uncultured Thermococcales archaeon 024-Tc20 (AY878166)
Thermococcales
uncultured Methanohalophilus sp. (AY164281)
Methanosarcina barkeri str. Fusaro (CP000099)
Methanosarcinales
Archaeoglobus veneficus DSM11195 (AF418181)
Archaeoglobus fulgidus L3 (DQ131905)
Archaeoglobus profundus (AF322392)
Ferroglobus placidus (AF220166)
Archaeoglobales
Methanotorris formicicus (AB100884)
Methanotorris sp. Mc-S-70 (AB095167)
Methanocaldococcus indiensis (AF547621)
Methanotorris formicicum Mc-1-70 (AB095159)
Methanococcus aeolicus PL-15/H (DQ136171)
Methanococcus voltae (M59290)
Methanococcales
Methanobacterium beijingense 4-1 (AY552778)
Uncultured Methanobacterium sp. Gran2M211 (AY899834)
Methanobrevibacter sp. OCP (AY615203)
Methanobrevibacter sp. KM1H5-1P (AY615201)
Uncultured Methanosphaera sp. D_F08 (AY454785)
Methanosphaera stadtmanae DSM3091 (AY196684)
uncultured Methanobrevibacter sp. (AY351499)
Methanobacteriales
Halobacterium sp. NRC-1 (NC_002607)
Halobiforma salilacus (AY277582)
Uncultured Natronococcus sp. HAuD-MA45 (AB113627)
Haloarcula hispanica HLR6 (DQ089686)
Halobacteriales
Uncultured archaeon CRA9-27cm (AF119129)
Uncultured archaeon CRA8-27cm (AF119128)
Uncultured euryarcheote ESYB68 (AB119624)
Uncultured euryarchaeote MERTZ_21CM_297(AF424536)
RFLP2
Uncultured archaeon Car106b (AF224779)
uncultured archaeon EJ_B02 (AY454663)
uncultured archaeon D_A12 (AY454664)
Uncultured archaeon PENDANT-33 (AF142944)
Uncultured archaeon PENDANT-17 (AF142929)
Uncultured euryarchaeote MERTZ_2CM_136 (AF424529)
Uncultured archaeon EI_D10 (AY454657)
Uncultured archaeon ACE-6 (AF142788)
Pendant-33 Group
Uncultured euryarchaeote pBRKC84 (AF118661)
Methanospirillum hungatei (M60880)
Uncultured Methanospirillaceae MP-Eth-A (AB236087)
Methanoculleus bourgensis NF-1 (DQ150254)
Methanoculleus thermophilus (AJ862839)
Methanofollis formosanus (AY186542)
Methanomicrobiales
Methanopyrus kandleri AV19 (NC_003551)
Methanopyrales
Korarchaeote pJP78, hot spring (L25303)
Korarchaeote pJP27, hot spring (L25852)
0.01 substitutions/site