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Bacterial community survey of sediments at Naracoorte Caves, Australia

Eric M. Adetutu¹, Krystal Thorpe¹, Esmaeil Shahsavari¹, Steven Bourne², Xiangsheng Cao^{1,3}, Ramin Mazaheri Nezhad Fard¹, Greg Kirby¹, and Andrew S. Ball¹

Abstract:

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Bacterial diversity in sediments at UNESCO World Heritage listed Naracoorte Caves was surveyed as part of an investigation carried out in a larger study on assessing microbial communities in caves. Cave selection was based on tourist accessibility; Stick Tomato and Alexandra Cave (> 15000 annual visits) and Strawhaven Cave was used as control (no tourist access). Microbial analysis showed that *Bacillus* was the most commonly detected microbial genus by culture dependent and independent survey of tourist accessible and inaccessible areas of show (tourist accessible) and control caves. Other detected sediment bacterial groups were assigned to the Firmicutes, Actinobacteria and Proteobacteria. The survey also showed differences in bacterial diversity in caves with human access compared to the control cave with the control cave having unique microbial sequences (*Acinetobacter*, *Agromyces*, *Micrococcus* and *Streptomyces*). The show caves had higher bacterial counts, different 16S rDNA based DGGE cluster patterns and principal component groupings compared to Strawhaven. Different factors such as human access, cave use and configurations could have been responsible for the differences observed in the bacterial community cluster patterns (tourist accessible and inaccessible areas) of these caves. Cave sediments can therefore act as reservoirs of microorganisms. This might have some implications on cave conservation activities especially if these sediments harbor rock art degrading microorganisms in caves with rock art.

Keywords: caves; tourism; 16S rDNA; denaturing gradient gel electrophoresis

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INTRODUCTION

There has been a renewed focus from both scientific and economic points of view on microbial diversity in caves in recent times due to the increasing popularity of cave tourism. Tourists are attracted to caves because of their artefacts and unique features leading to substantial economic benefits for the tourism industry (Pulido-Bosch et al., 1997). These artefacts and features include rock art, Palaeolithic paintings and tools, speleothems and fossil deposits. However, changes in the caves' microclimatic conditions such as temperature, CO₂, relative humidity and the caves' microbial composition and abundance are associated with human access. These changes can lead to damages of cave features and artefacts, the very

factors attracting tourists (Russell & MacLean, 2008; Bastian et al., 2009; Stomeo et al., 2009).

Cave microorganisms range from bacterial groups such as Proteobacteria, Actinobacteria, and Firmicutes to yeasts and saprophytic fungus such as *Penicillium*, *Fusarium* and *Trichurus* (Barton & Jurado, 2007; Zhou et al., 2007; Bastian et al., 2009; Portillo et al., 2009a; Jurado et al., 2010; Adetutu et al., 2011; Vaughan et al., 2011). These microbial communities are affected by the geological nature of the caves, prevailing environmental conditions, soil or sediment factors and cave factors (configuration and use) (Ikner et al., 2007; Shapiro & Pringle, 2010; Adetutu et al., 2011). Cave microorganisms in nutrient rich or limited environments (high and low energy caves) are metabolically versatile acquiring energy from cave compounds, gases and by oxidizing metals from rocks. Through these activities, these microorganisms play critical roles in caves' biogeochemical cycles and in the formation of cave features such as stalactites and stalagmites (Barton & Jurado, 2007). This delicate microbial balance can however be disrupted by uncontrolled human access with adverse effects on the cave environment (Bastian et al., 2010).

¹School of Biological Sciences, Flinders University of South Australia, Adelaide, GPO Box 2100, Adelaide SA 5001 Australia. Corresponding author: Eric M. Adetutu (eric.adetutu@flinders.edu.au)

²Department for Environment and Heritage, P O Box 134, Naracoorte, South Australia 5271.

³College of Civil Engineering, Beijing University of Technology, Beijing, China 100124

Most scientific investigations of cave environments have been focused on cave walls leading to abundant information on microbial diversity and function on cave walls (Gurtner et al., 2000; Portillo et al., 2009b; Stomeo et al., 2009; Martinez and Asencio, 2010; Pašić et al., 2010). This is because cave microbial activities are thought to take place on caves' rocky surfaces as most caves are devoid of substantial soil or sediment layer. Cave walls also bear rock art and Palaeolithic paintings which are the main tourist attractions but are highly susceptible to microbial damage (Schabereiter-Gurner et al., 2002; Bastian et al., 2010). Cave sediment or soils (Park et al., 2000) are poorly studied and where present should also be subject to scientific investigations for a variety of reasons.

Cave sediments are usually the primary recipient of exogenous materials associated with human access to caves and can be good indicators of the impact of human access on cave environments. In addition, the movement of microorganisms would occur in a cycle between the cave walls and sediments; sediment microorganisms are therefore part of the cave's ecosystem. Cave sediments could also serve as a reservoir of microorganisms (such as rock art degrading organisms) which could complicate potential conservation work focused on walls of caves with sediments. In addition to this, bacterial surveys are usually carried out in areas of tourist access or activity in show caves with non tourist accessible caves being used as controls. Consequently, these surveys only reflect the partial bacterial diversity in these areas. However, a survey that includes samples obtained from both tourist accessible and inaccessible areas of a cave would give a more accurate and comprehensive picture of bacterial diversity.

Naracoorte Cave Park (UNESCO World Heritage Listed Site) in South Australia is a popular cave park because of its extensive fossil deposits, speleothems and accessibility for adventure caving. Importantly, some of the caves in this park have extensive sediment deposits which have not been studied in detail. These caves therefore offer a good opportunity for studying microbial diversity in cave sediments. This study was carried out as part of a large study commissioned by the Naracoorte Cave Management to assess the impact of tourism on the cave environment. This involved carrying out a survey of bacterial diversity in tourist accessible areas and inaccessible areas of selected high impact caves using culture dependent and independent techniques (PCR-DGGE-Sequencing). Assessment of the bacterial community structures in these areas will provide more information on their diversity in cave sediments and reveal any change associated with human access.

MATERIALS AND METHODS

Sampling site and sample collection

Three caves (Alexandra, Stick Tomato and Strawhaven) were selected for investigations based on tourist access and cave configurations. Sediment samples (up to 100 g) were collected from multiple

locations in sections of the caves designated as tourist traffic route by digging 5 cm deep into the sediment. Sediment samples were also obtained from areas of the caves that were inaccessible to tourists.

The caves

Alexandra Cave has speleothems, mirror pools and straw clusters (thin stalactites) and is accessible by guided tour with up to 18,000 annual visits. Alexandra Cave has two entrances, each with a locked door and has no twilight zone. The main door opens into a 3-4 m long concrete staircase leading to the tourist track. Samples were obtained in the "dark zone" of the cave (6 m from the bottom of the staircase) in 4-5 m² grids (D1-D3) as shown in Fig. 1A. Sediment samples from tourist inaccessible areas were obtained from an alcove about 15 m away from the cave's entrance in 4-5 m² grids (ND1-ND3) shown in Fig. 1A. Multiple sediment samples (n = 12) were collected in each grid with sediments from each grid being pooled (Fig. 1A). Stick Tomato Cave is a multiple use cave (self guided tour and adventure caving) with up to 20,000 annual visits. It has two open entrances (> 3 m wide), with the entrance in the adventure caving section capturing large amounts of water and detritus during storm events. Stick Tomato Cave has twilight zone of ~20 m into the cave (Fig. 1B) and sediment samples were obtained on the cave's tourist path from about 6 m into dark zone (~26 m into the cave) in three grids. Sediment samples were also obtained from tourist inaccessible areas in the grids (ND1-ND3) shown in Fig. 1B. Multiple sediment samples were collected in 4-5 m² grids with the approximate location of each grid depending on the topography of the cave floor. Sediment samples from each grid were pooled together to represent the microbial community in that area for ease of analysis. Strawhaven is a research cave inaccessible to tourists and samples were obtained from about 12 m into the cave in areas shaded in Fig. 1C.

Plate count and microbial isolation

Sediment samples from tourist accessible and inaccessible areas were serially diluted in 0.01 M phosphate buffered saline (pH 7.4) (Sigma, St Louis, MO, USA) at 10, 100 and 1000 fold dilutions. Aliquots from each dilution series were inoculated into sterile dilute Nutrient Agar (1:100) (Oxoid Ltd, Hampshire, England) plates for the detection and enumeration of oligotrophic cave bacteria (Portillo et al., 2009a). These plates were incubated at 25 ± 1 °C for up to four weeks after which the microbial colonies on each plate were counted and expressed as the Log of the total number of colony forming units (CFU). Bacterial isolates on these plates were then grouped based on morphological characteristics, purified and subject to molecular analysis.

DNA Extraction and PCR

DNA was extracted from replicate cave sediments (0.25 g) using the PowerSoil™ DNA extraction kit (Mo Bio Laboratories Inc, Carlsbad, CA, USA) according to the manufacturer's instructions. 16S rDNA amplification was carried out using primer pair 341F GC and 518R (Muyzer et al., 1993).

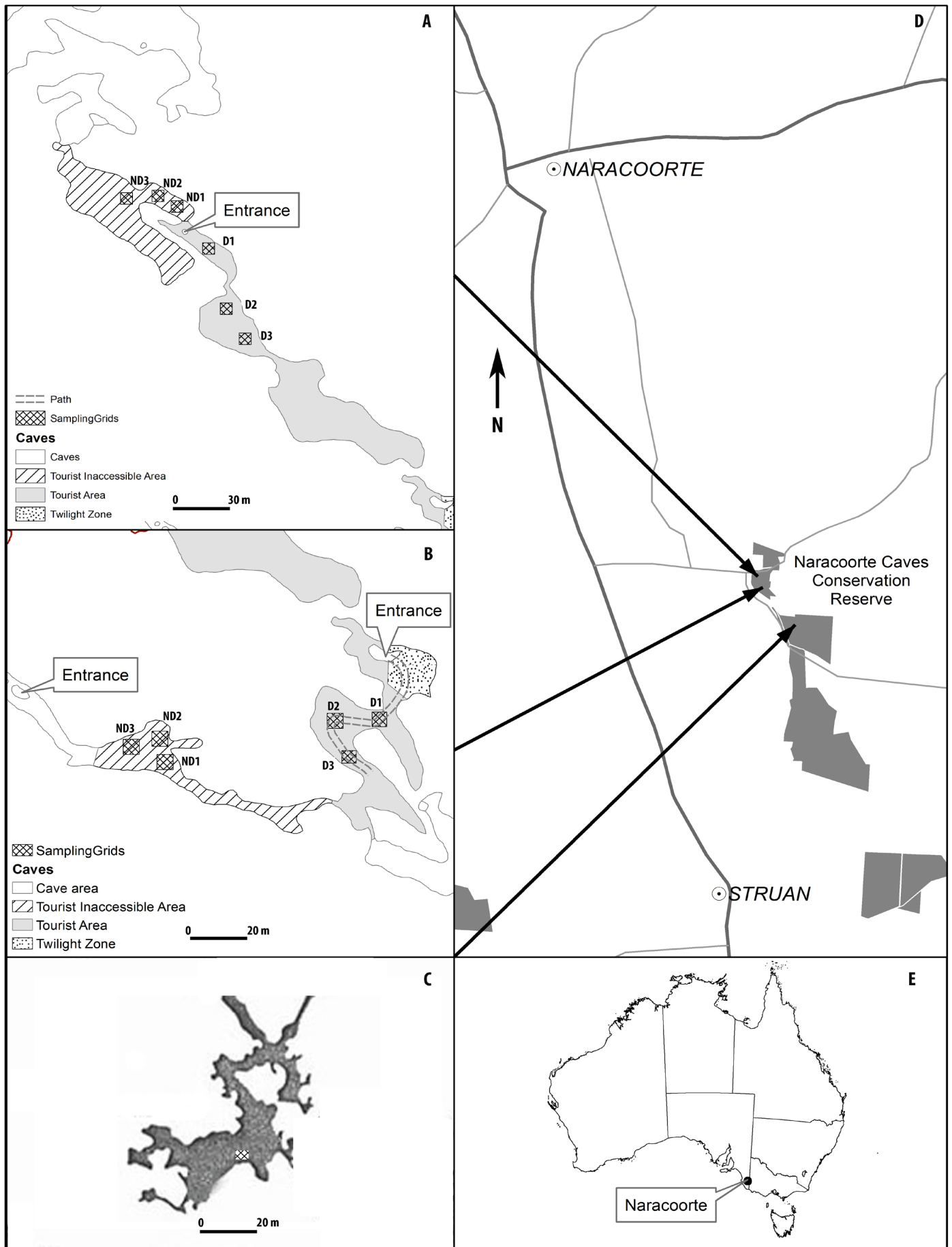


Fig. 1. A map of Naracoorte showing the caves studied and approximate areas where samples were taken. A = Alexandra Cave, B = Stick Tomato Cave, C = Strawhaven Cave, D = location of Naracoorte Caves and E = location of Naracoorte in Australia. D1-D3 = areas accessible to tourists and ND1-ND3 = areas inaccessible to tourists. Sampling grids and shaded areas (c) are not drawn to scale and are approximate sampling points.

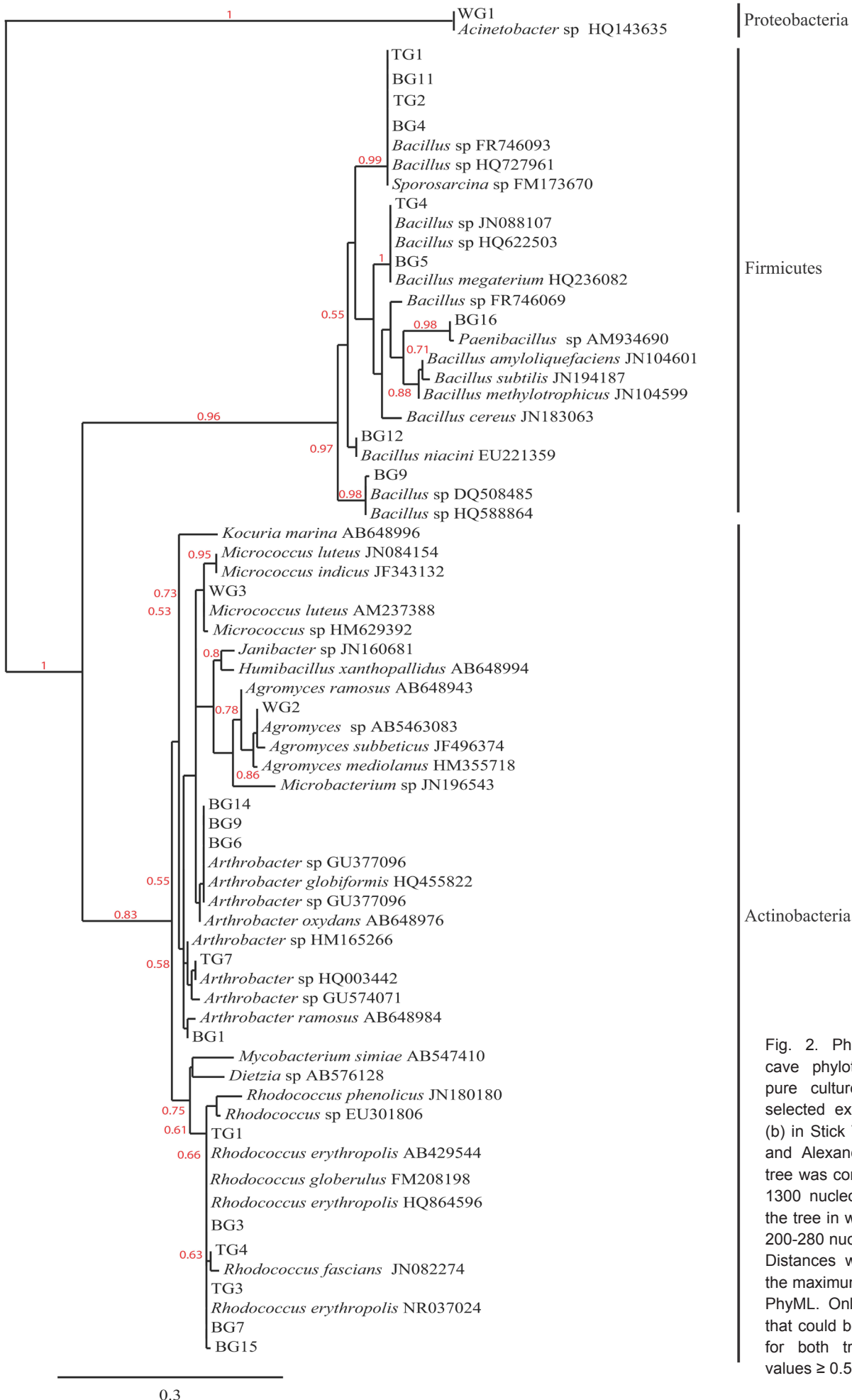


Fig. 2. Phylogenetic trees of cave phylotypes derived from pure culture isolates (a) and selected excised DGGE bands (b) in Stick Tomato, Strawhaven and Alexandra caves. For (a), tree was constructed from 1100-1300 nucleotide positions while the tree in (b) was constructed from 200-280 nucleotide positions. Distances were calculated with the maximum likelihood model in PhyML. Only partial sequences that could be aligned were used for both trees and bootstrap values ≥ 0.50 are shown.

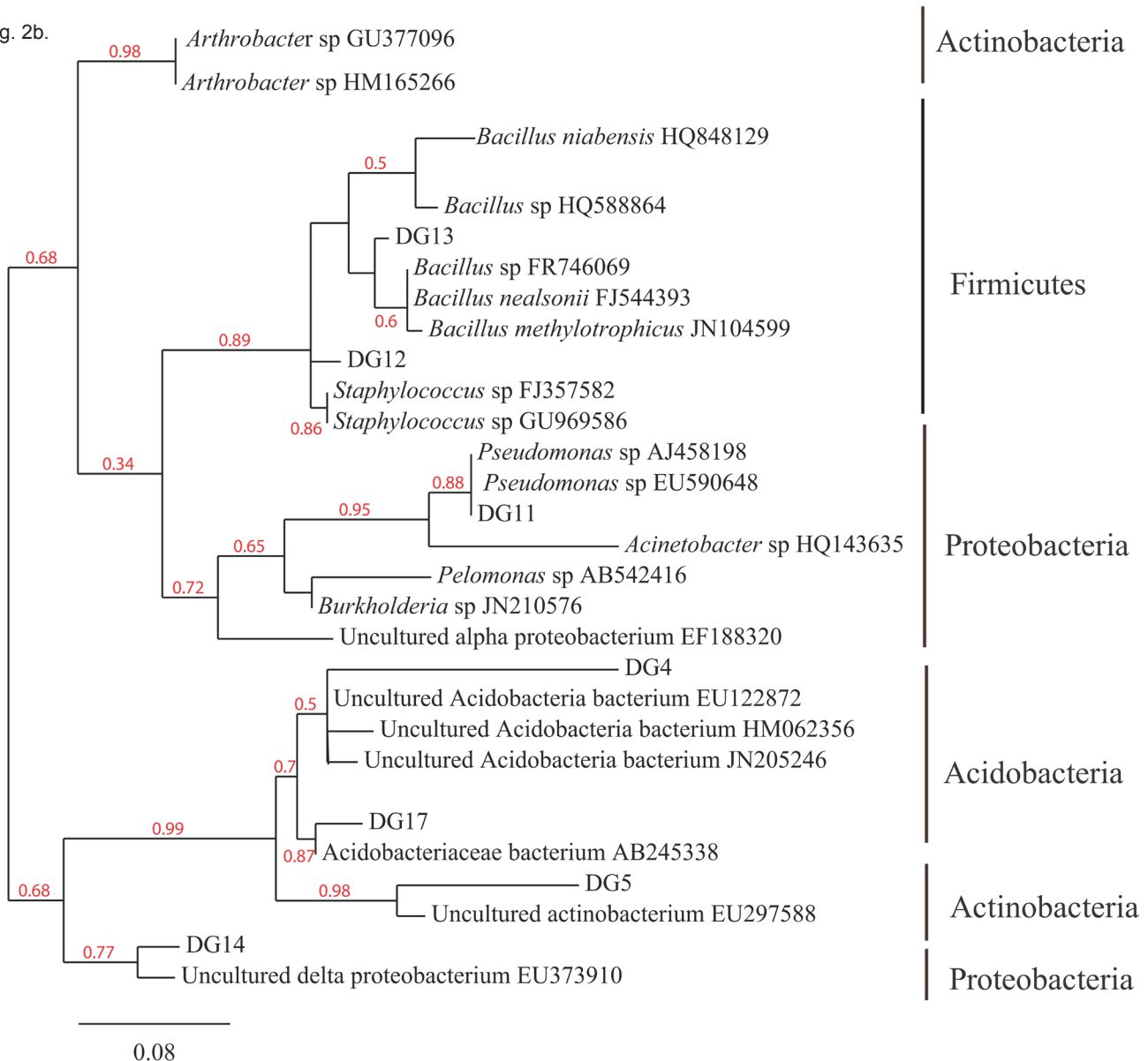
DNA was also extracted from pure bacterial cultures by flooding replicate plates with 5 ml of sterile phosphate buffer, homogenising plate cultures with sterile spreaders and transferring aliquots into sterile 1.5 ml Eppendorf tubes. Samples were pelleted by centrifugation at 12000 × g for 15 min. The supernatant was discarded and the pellet was transferred aseptically to the PowerSoil PowerBead tubes for DNA extraction. The extracted DNA (2 µl) were subjected to PCR using primers 63F and 1389R (Osborn et al., 2000). The thermocycling condition used for 63F and 1389R reactions involved an initial denaturation for 5 min at 94°C, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min and a final extension of 72°C for 10 min.

DGGE, community profiling and analysis

Denaturing Gradient Gel Electrophoresis analysis of 16S rRNA genes was carried out with 40 to 60% urea-formamide denaturant gradient (9% polyacrylamide gels), run for 20 h at 60 °C and 60 V. DGGE gels were silver stained (Girvan et al., 2003) and scanned. DGGE gels were analysed

with Phoretix 1D software (Non Linear Dynamics, Durham, USA) to generate similarity clusters using the unweighted pair group method with mathematical averages (UPGMA). Shannon index (H') was also calculated from DGGE profiles as described by Girvan et al., (2003) using the formula $H' = -\sum p_i \ln p_i$. Principal component analysis (PCA) was carried out using SPSS version 19 on the matrix data obtained from Phoretix 1D analysis (TL 120). Pareto Lorenz (PL) curves were used to estimate evenness within the microbial community. Bands were ranked from high to low based on their intensities. The normalized cumulative bands (numbers) were plotted on x-axis while their respective normalized cumulative intensities were plotted on the y-axis to draw a PL curve. Intercept was set at 20% of population (0.2 x-axis) to determine proportional cumulative intensity and perfect evenness drawn at 45° (Marzorati et al., 2008). Statistical significance was determined in samples within or between caves by either T test or analysis of variance (ANOVA) and Tukey tests on SPSS.

Fig. 2b.



Sequence and phylogenetic analysis

Dominant bands (numbered) on DGGE gels were aseptically excised and the DNA eluted (Patil et al., 2010). Eluted DNA was then cleaned up by repeated PCR, band excision and DGGE (using a narrower gradient range), in order to get pure bands for sequencing. Purified DGGE bands and amplicons from pure cultures PCR were sequenced and analysed as described by Adetutu et al., (2011) with the aligned sequences being submitted to BLASTN from GenBank (<http://www.ncbi.nlm.nih.gov> (see Table 1 for accession number) for generating similarity searches. Sequences from the database that matched the unknown sequence data submitted were aligned with CLUSTALW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) module and used to calculate pairwise evolutionary distances between the sequences. Phylogenetic trees were then constructed and viewed using maximum likelihood algorithm and Tree Dyné in PhyML (Dereeper et al., 2008).

RESULTS

Culture Dependent Analysis

The total viable bacterial counts in 0.01x Nutrient Agar are shown in Table 1. The disturbed (tourist accessible) regions of Stick Tomato and Alexandra Caves had consistently higher bacterial counts than their non disturbed areas (tourist inaccessible). The non disturbed areas of these caves however had a higher bacteria count than the control Strawhaven Cave. Actinomycetes putatively identified as *Streptomyces* (data not shown) were detected in Strawhaven and absent in other caves (Table 1). Culture based survey also showed that both Stick Tomato and Alexandra Caves largely contained similar bacterial genera (such as *Bacillus*) in both tourist accessible and inaccessible areas with a few exceptions. For example *Arthrobacter* sp. is only found in Stick Tomato (non disturbed) while being present in all sampling areas in Alexandra (Table 2). These genera were assigned to the Firmicutes and Actinobacteria (Table 2 and Fig. 2a). However a different set of microbial genera which included *Acinetobacter* (Proteobacteria), *Agromyces* and *Micrococcus* (Actinobacteria) species were detected in Strawhaven Cave (Table 2 and Fig.

2a). Fig. 2b however shows the phylogenetic tree of sequences from culture independent analysis.

Culture Independent Analysis

Detailed examination of the cave bacterial community cluster showed that the bacterial banding patterns in Stick Tomato and Alexandra Cave were substantially different from that of the control Strawhaven Cave (Fig. 3a). Cluster analysis (UPGMA dendrogram) also showed that the bacterial banding pattern in tourist accessible areas of Stick Tomato Cave was different from that of areas not accessible to tourists. This was in contrast to the trend observed in Alexandra Cave where the banding patterns in these two areas were similar. Principal component analysis of these caves also showed four dissimilar groups based on caves and access to tourists with tourist accessible and inaccessible areas being grouped together only in Alexandra Cave (Fig. 3b). Sequence analysis of some of the bands unique to tourist accessible areas showed that they were most similar to uncultured *Acidobacteria* and alpha proteobacteria groups (Stick Tomato) with sequences most similar to *Pelomonas* (beta proteobacteria) being unique to the inaccessible areas (Table 3). Other bacterial species detected in other caves are also shown in Table 3 with *Bacillus* being detected in all the caves irrespective of sampling area. The Phylogenetic tree showed that four different bacterial groups (Actinobacteria, Firmicutes, Proteobacteria and Acidobacteria) were detected in these selected caves at Naracoorte (Table 3 and Fig. 2b). However, only two of these groups (Firmicutes and Proteobacteria) were detected in the Strawhaven Cave (control) (Table 3). Pareto Lorenz Curve distribution patterns for caves' bacterial communities also showed distinct differences between the communities in Stick Tomato Cave (74-76%) and Alexandra Cave (55-58%) (Fig. 4). The bacterial community in Stick Tomato therefore had less evenness and different functional organization to Alexandra. Bacterial community diversity analysis using Shannon Weaver Diversity (H') only showed significant differences between Stick Tomato and Strawhaven Cave (ANOVA, $P < 0.05$) (data not shown).

Table 1. Total viable count of bacteria cells in 0.01 strength Nutrient Agar in tourist accessible and inaccessible areas of selected caves at Naracoorte.

Microorganism	Log CFU g ⁻¹ soil				
	Stick Tomato		Alexandra		Strawhaven
	D	ND	D	ND	ND
Bacteria	5.31±0.05 ^{a*}	4.71±0.07 ^{a*}	5.38±0.10 ^{b*}	4.93±0.19 ^{b*}	1.52±0.66 [*]
Actinomycetes	-	-	-	-	2.22±0.90

Note: - none detected. Values shown are means of log CFU of 3 replicate plates. Same letter with asterisk are significantly different from each other. ^{a*} and ^{b*} are significantly different from *. D- tourists accessible areas, ND- areas inaccessible to tourists.

Table 2. Summary and location of bacterial isolates detected in selected caves from Naracoorte Park (culture based isolations)

Phylotype	Cave	Division (or subdivision)	Nearest taxon	Similarity(%)
TG1	STD	Firmicutes	<i>Bacillus</i> sp HQ727966	99
TG2	STD	Firmicutes	<i>Bacillus</i> sp HQ727961	99
TG3,8,BG3	STD&STND	Actinobacteria	<i>Rhodococcuserythropolis</i> HQ864596	99
TG4	STD	Firmicutes	<i>Bacillusmegaterium</i> HQ236082	100
TG5	STD	Actinobacteria	<i>Rhodococcus</i> sp EU301806	98
TG6	STD	Firmicutes	<i>Bacillus</i> sp HQ588864	98
TG7	STND	Actinobacteria	<i>Arthrobacter</i> sp HQ003442	100
WG1	SND	Proteobacteria	<i>Acinetobacter</i> sp HQ143635	100
WG2	SND	Actinobacteria	<i>Agromyces</i> sp AB546308	100
WG3	SND	Actinobacteria	<i>Micrococcusluteus</i> AM237388	100
BG1	AD	Actinobacteria	<i>Arthrobacter</i> sp HM165266	100
BG2	AD	Firmicutes	<i>Bacillus</i> sp FR746069	99
BG4	AD	Firmicutes	<i>Bacillus</i> sp FR746093	100
BG5	AD	Firmicutes	<i>Bacillus</i> sp HQ622503	100
BG6,9	AD&AND	Actinobacteria	<i>Arthrobacter</i> sp GU377096	99-100
BG7	AND	Actinobacteria	<i>Rhodococcuserythropolis</i> NR0370241	99
BG8	AND	Firmicutes	<i>Bacillus</i> sp DQ508485	100
BG10	AND	Actinobacteria	<i>Arthrobacterglobiformis</i> EU333890	95
BG11	AND	Firmicutes	<i>Bacillus simplex</i> FJ999940	99
BG12	AND	Firmicutes	<i>Sporosarcina</i> FM173670	99
BG13	AND	Firmicutes	<i>Bacillusniacine</i> EU221359	99
BG14	AND	Actinobacteria	<i>Arthrobacterglobiformis</i> HQ455822	100
BG15	AND	Actinobacteria	<i>Rhodococcuserythropolis</i> AB429544	98
BG16	AND	Firmicutes	<i>Paenibacillus</i> sp AM934690	98

Note: STD- Stick Tomato Disturbed (tourists accessible areas), STND- Stick Tomato Non Disturbed (areas inaccessible to tourists), AD- Alexandra Disturbed (tourists accessible areas), AND- Alexandra Non Disturbed (areas inaccessible to tourists), SND-Strawhaven Non Disturbed (control; inaccessible to tourists).

Table 3. Summary and location of bands excised from 16S rRNA based DGGE profiles of selected caves from Naracoorte Park.

Phylotype	Cave	Division (or subdivision)	Nearest taxon	Similarity(%)
DG1	STND	Betaproteobacteria	<i>Pelomonas</i> sp AB542416	92
DG2	STD	Acidobacteria	Uncultured acidobacteria HM062356	98
DG3	STD	Acidobacteria	Uncultured Acidobacteria EU122872	95
DG4	STD	Acidobacteria	Uncultured Acidobacteria EU979056	97
DG5	STD & STND	Actinobacteria	Uncultured Actinobacteria EU300539	93
DG6	STD STND, AD AND	Firmicutes	<i>Bacillus</i> sp HM366466	92
DG 7	STD & STND	Firmicutes Uncultured	<i>Bacillus</i> sp EF074890	98
DG8	STD	Alphaproteobacteria	Uncultured alphaproteobacteria EF188320	99
DG9	SND	Alphaproteobacteria	<i>Sphingomonas</i> sp AB288313	100
DG10	SND	Firmicutes	<i>Bacillus</i> sp EU589406	100
DG11	SND	Gammaproteobacteria	<i>Pseudomonas</i> sp EU590648	98
DG12	SND	Firmicutes	Uncultured bacteria JF239301	98
DG 13	SND	Firmicutes	<i>Bacillus nealsonii</i> FJ544393	93
DG14	AD & AND	Deltaproteobacteria	Uncultured Deltaproteobacterium EU373910	93
DG15	AND	Bacteria	Uncultured bacteria FJ409452	90
DG16	AND	Firmicutes	Uncultured bacteria EF072331	98
DG17	AD	Firmicutes	<i>Bacillus</i> sp FJ373035	98
		Acidobacteria	Acidobacteria AB245338	98
DG18	AD & AND	Alphaproteobacteria	Uncultured alphaproteobacteria HM057723	94

Note: STD- Stick Tomato Disturbed (tourists accessible areas), STND- Stick Tomato Non Disturbed (areas inaccessible to tourists), AD- Alexandra Disturbed (tourists accessible areas), AND- Alexandra Non Disturbed (areas inaccessible to tourists), SND-Strawhaven Non Disturbed (control; inaccessible to tourists).

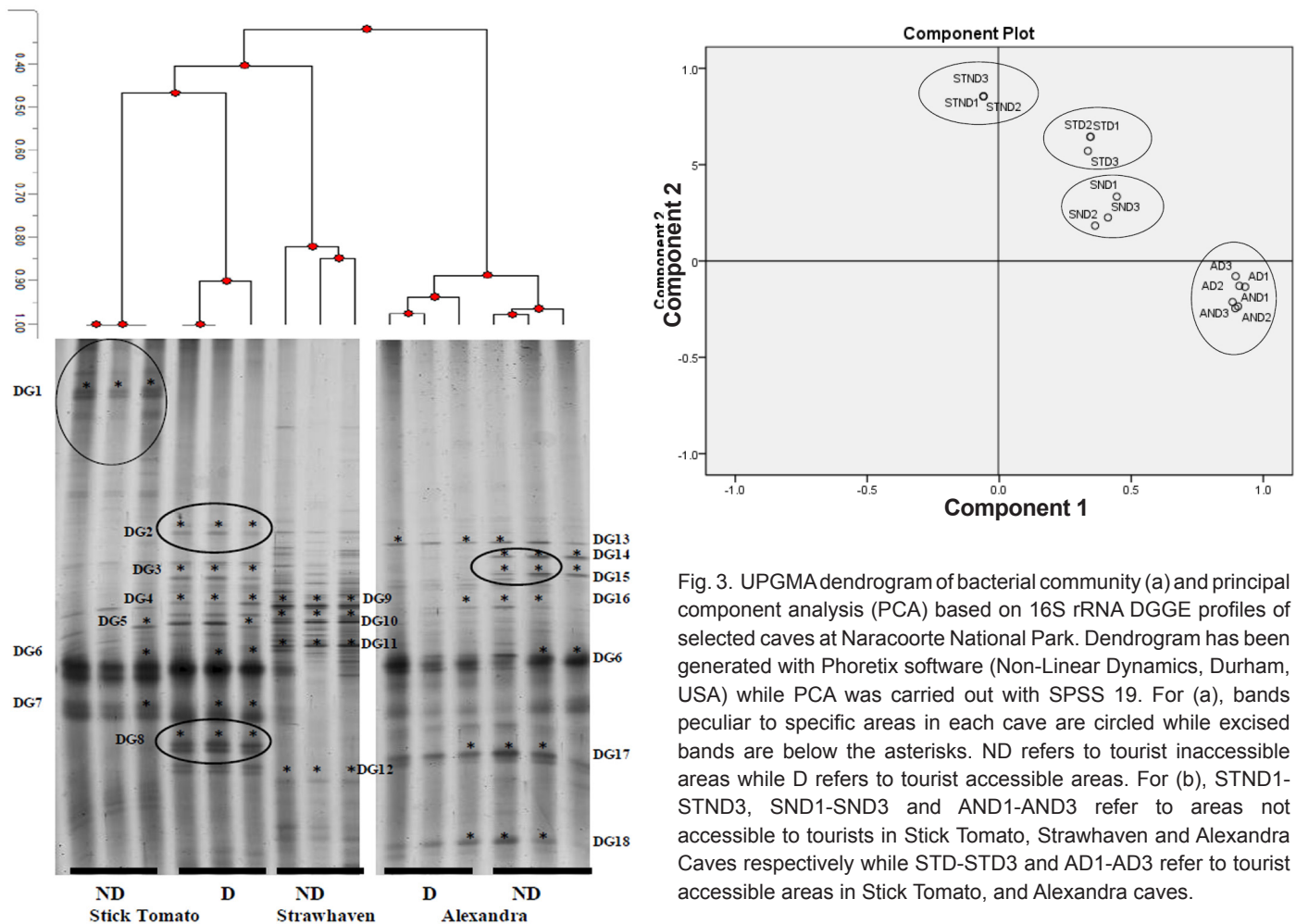


Fig. 3. UPGMA dendrogram of bacterial community (a) and principal component analysis (PCA) based on 16S rRNA DGGE profiles of selected caves at Naracoorte National Park. Dendrogram has been generated with Phoretix software (Non-Linear Dynamics, Durham, USA) while PCA was carried out with SPSS 19. For (a), bands peculiar to specific areas in each cave are circled while excised bands are below the asterisks. ND refers to tourist inaccessible areas while D refers to tourist accessible areas. For (b), STND1-STND3, SND1-SND3 and AND1-AND3 refer to areas not accessible to tourists in Stick Tomato, Strawhaven and Alexandra Caves respectively while STD-STD3 and AD1-AD3 refer to tourist accessible areas in Stick Tomato, and Alexandra caves.

DISCUSSION

Culture independent and dependent survey

The most commonly detected bacterial group in tourist and non tourist accessible areas of show and control caves by culture dependent and independent (except in Strawhaven) was Firmicutes. Although Firmicutes (*Bacillus* sp) along with *Escherichia coli* and *Staphylococcus* have been considered as indicators of human impact on caves (Lavoie & Northup, 2006), their detection in the community profiles of all the caves suggested that they were indigenous to these caves. *Bacillus* sp have been isolated in caves (Fernandez-Cortes et al., 2011; Urzi et al., 2010) and their dispersal throughout the cave ecosystem is enhanced by their abilities to produce spores. Phylogenetic trees of other DGGE sequences showed that these can be grouped under Acidobacteria, Actinobacteria and Proteobacteria and similar groups have also been detected on cave walls (Schabereiter-Gurtner et al., 2002; 2004; Ikner et al., 2007; Urzi et al., 2010). Cave walls, cave animals, insects and human visitors (Chelius & Moore, 2004; Portillo et al., 2009b) are therefore potential sources of bacterial species in cave sediments.

Bacteria play important roles in the biogeochemical cycles of caves (Zhou et al., 2007) and have been implicated in the deterioration of cave features. Some members of the Proteobacteria group are associated with the deterioration of

Palaeolithic paintings (Portillo et al., 2009b) while some Firmicutes (*Bacillus*) are involved in calcite precipitation (stalactites formation) in caves (Baskar et al., 2006). Calcite precipitation can damage parietal markings (such as prehistoric human markings) on moonmilk reducing their touristic potential. The detection of bacterial species which are members of these groups in cave sediments at Naracoorte means that sediments can potentially act as reservoirs. The abilities of these detected groups to degrade cave artefacts (painting) were not assessed in this study because none of the caves investigated in this study had rock art. This finding would probably be more important in caves with paintings where much of the microbial investigations have been on cave walls with a view of preserving such paintings (Portillo et al., 2009b; Stomeo et al., 2009). Eliminating microbial groups (especially when found to be rock art degraders) from the wall might only bring limited success as the floor (and sediments when present) could be reservoirs of these microbial groups.

Culture based techniques (despite their limitations) have been used to study cave microorganisms (Stomeo et al., 2009; Campbell et al., 2011; Adetutu et al., 2011). Combining culture based data with data from culture independent techniques such as DGGE (which sometimes detect only the top 1% population) (Muyzer et al., 1993) can give a more detailed picture of microbial diversity in cave sediments. A good example of this is Alexandra Cave in which *Bacillus*, *Acidobacteria*, *uncultured*

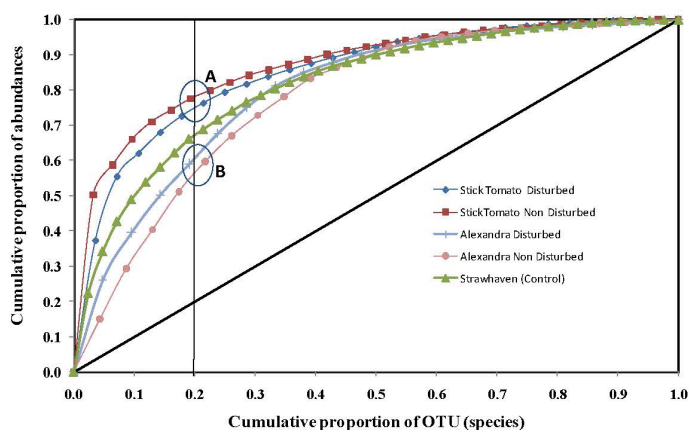


Fig. 4. Pareto-Lorenz distribution curves derived from 16S rRNA genes based DGGE profiles of areas accessible (disturbed) and inaccessible (non-disturbed) in selected caves at Naracoorte Park. The vertical line is plotted at 0.2 x-axis in order to compare different Pareto values while the 45 degree diagonal line represents the perfect evenness of a community. Letters A and B indicate different ranges of Pareto values observed at each cave.

Alpha proteobacteria and *Delta proteobacteria* were detected on the caves' DGGE profiles. Additional genera such as *Rhodococcus* and *Arthrobacter* were observed in culture based assays. A similar trend was observed in Stick Tomato Cave. Phylogenetic analysis however, indicated that these bacterial species largely belonged to similar taxa (Firmicutes, Actinobacteria, Proteobacteria and Acinetobacteria) showing some agreement between culture dependent and independent assays. Different microbial genera (*Acinetobacter*, *Agromyces*, *Micrococcus* and *Streptomyces*) were also isolated in the control cave (Strawhaven) compared to the two show caves. The observed differences in the species between the tourist accessible caves and the control cave might be related to edaphic (sediment) factors, cave use and human access.

Tourist accessible and inaccessible areas

In addition to differences in bacterial species, the cluster analysis and principal component analysis groupings of the show caves were also substantially different from that of Strawhaven Cave. The bacterial counts in Stick Tomato and Alexandra Caves were significantly higher than that of Strawhaven ($P < 0.05$) with the bacterial diversity (H') of Stick Tomato Cave being significantly different from Strawhaven's (data not shown). Changes in bacterial community composition and number have been associated with tourism (Ikner et al., 2007; Shapiro & Pringle, 2010). Higher ratios of coliform to total bacterial count have also been linked to anthropogenic pollution of show caves (Mulec et al., 2012). Tourists can bring in exogenous microorganisms from the outside environment and other caves along with substances such as hair, lint, dead skin cells and skin oils (potential nutrients for cave microorganisms) (Barton, 2006) causing changes in microbial communities. The presence of exogenous (foreign) organic matter in caves has been shown to alter caves' bacterial

abundance and diversity compromising the integrity of the indigenous bacterial community (Chelius et al., 2009). Consequently, the differences between the two show caves and control cave (Strawhaven) might be associated with human access, although other environmental factors such as sediment microbial community heterogeneity could have played some roles in this.

The observed differences in the Pareto-Lorenz (PL) distribution curves of Stick Tomato Cave and Alexandra Cave showed that the bacterial community's evenness differed between caves. The higher "within cave variation" (cluster patterns and principal component groupings) between tourist accessible and inaccessible areas of Stick Tomato as compared to Alexandra also suggested that other factors such as cave use and configurations were affecting bacterial diversity. The airflow system in caves (which was not investigated in this study) can also influence bacterial distribution in caves. The airflow system is affected by cave volume, differences in temperature and pressure between the cave and outside environment and also by the orientation of cave openings (Pflitsch & Piasecki, 2003).

Stick Tomato as an open access cave (without doors) will have a higher input of exogenous materials, possibly different temperature gradient and airflow pattern compared to a closed cave (with doors) like Alexandra. These factors could have affected the spread of cave bacteria in caves. Consequently, it is possible that convective air circulation (Fernandez-Cortes et al., 2006) common in closed caves like Alexandra resulted in a more even spread of bacteria within the cave environment and might explain the similarity in bacterial cluster patterns in the different areas of this cave.

CONCLUSION

This study has shown that the most commonly detected bacterial genus in most tourist accessible and inaccessible sediment samples of show and control caves was *Bacillus* (Firmicutes). Although there were differences in the bacteria genera detected in these caves, phylogenetic analysis indicated they belonged to four different groups (Firmicutes, Proteobacteria, Acidobacteria and Actinobacteria). Differences were also observed in the bacterial communities of the tourist accessible show caves compared to that of the control cave. Detection of bacterial groups (commonly found on walls) in sediments suggests that sediments can act as a reservoir of these organisms which might have some implications on cave conservation activities. Differences in the bacterial diversity in show caves have also highlighted the role other factors such as cave use and cave configurations might play in cave bacteria distribution. Further investigations are required with more high impact and multiple activities caves with different management regimes to validate this. In addition, the potential differences in airflow between caves with (closed) and without doors (opened) need to be further investigated especially with respect to bacterial distribution. Changes in microbial diversity can be caused by human access but cave management protocols (for example by constructing doors) may help in controlling the entry of exogenous

substances and microorganisms into the caves. The benefits of such actions will have to be evaluated in terms of their practicality and effects on the caves microclimatic environment and conservation.

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