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Phylogenetic diversity of fungal communities in areas accessible and not accessible to tourists in Naracoorte Caves

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Abstract: The fungal diversity in areas accessible and not accessible to tourists at UNESCO World Heritage-listed Naracoorte Caves was investigated with culture-dependent and culture-independent techniques for assistance in cave management protocol development. The caves were selected based on tourist numbers and configurations: Stick Tomato (open, high numbers), Alexandra (lockable openings, high numbers) and Strawhaven (control; no access). Culture-based survey revealed Ascomycota dominance irrespective of sampling area with Microascales (*Trichurus* sp.) being most frequently isolated. Some Hypocreales-like sequences belonging to *Fusarium* sp., *Trichoderma* sp. and *Neonectria* sp. (Stick Tomato) were cultured only from areas not accessible to tourists. These orders also were detected by DGGE assay irrespective of sampling area. The predominance of Ascomycota (especially Microascales) suggested their important ecological roles in these caves. Culture-independent analysis showed higher Shannon fungal diversity values (from ITS-based DGGE profiles) in tourist-accessible areas of these caves than in inaccessible areas with the fungal community banding patterns being substantially different in Stick Tomato Cave. Further investigations are needed to determine the cause of the differences in the fungal communities of Stick Tomato Cave, although cave-related factors such as use, configuration and sediment heterogeneity might have contributed to these differences.

Key words: DGGE, environmental factors, ITS regions, tourism

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INTRODUCTION

Naracoorte Caves National Park covers 600 hectares in southeastern South Australia with a 307-hectare section of the park being inscribed by United Nations Educational, Scientific and Cultural Organization (UNESCO) as a World Heritage Site in 1994. The park has 26 known caves formed in tertiary limestone of the East Naracoorte Dune. These caves have a high natural and scientific value due to more than 100 fossil sites providing a comprehensive fossil and climate record of at least the past 500 000 y (Reed and Bourne 2000).

Cave environments such as those found in Naracoorte have different microbial groups (bacteria and fungi) with microbial diversity being affected by the energy status of the caves (whether energy rich or poor) (Groth et al. 1999, Jurado et al. 2008). Cave microorganisms are found in a variety of places, such as walls, sediments, air, surface and underground water samples, where they occupy different ecological niches (Novakova 2009, Portillo et al. 2008). Cave microorganisms also mediate both constructive (calcification and crystal growth) and destructive (substrate etching and breakdown) processes in caves (Barton and Northup 2007).

The presence of speleothems, fossils and other archaeological elements has spurred interests in cave environments not just from a scientific viewpoint but also from an economic one. An increasing number of caves now are open to tourists, bringing in substantial profits to the local economy and governments (Calaforra et al. 2003, Pulido-Bosch et al. 1997). Unfortunately this growing access has the potential of causing irreversible damage to the cave environment. Studies conducted on the anthropological effects on cave environments suggest tourism can cause changes in microbial composition, ecosystem and microclimatic conditions (Baker and Genty 1998, Calaforra et al. 2003, Dupont et al. 2007, Pulido-Bosch et al. 1997, Shapiro and Pringle 2010). These changes may

adversely affect the rate of corrosion of speleothems, rocks and cave walls and paintings.

There are a number of scientific reports on general cave microflora, their roles in the deterioration of cave structures (Stomeo et al. 2009), the effect of tourism on the deterioration of cave structures (Russell and MacLean 2008) and paleogeological investigations (Forbes and Bestland 2007). However most of these reports are predominantly on the bacterial community despite the fact that cave fungi are crucial to the food chain, contribute to biomass and can damage structures. In addition few reports specifically investigate cave microbial diversity by comparing the microbial community structure in tourist-accessible areas with areas not accessible to tourists within the same cave ecosystem. Without such reports it is difficult to comprehensively evaluate the cave fungal diversity and assess any human effect on their distribution before developing and applying management protocols. Therefore the aim of this study was to carry out a survey of the fungal community in selected caves in Naracoorte by assessing community structures and diversity with a combination of culture-dependent and culture-independent techniques (PCR-DGGE-sequencing). This was carried out on samples obtained from selected areas accessible and inaccessible to tourists in selected caves.

MATERIALS AND METHODS

Sampling sites and sample collection.—Three caves within Naracoorte Caves National Park were selected for investigations. Selection was based on access to tourists and cave configuration. The caves chosen were Alexandra, Stick Tomato and Strawhaven. Sediment samples (up to 100 g) were collected from multiple locations in Jun 2008 in sections open to tourists and those not open to tourists by digging 5 cm into the ground.

Alexandra.—This cave (up to 18 000 annual visits) has two entrances, each with a locked door and is accessible only by guided tours of its speleothems. Because this cave has a door it therefore lacks a twilight zone. The door opened into a 3–4 m long descending concrete staircase leading to the tourist track. No outside light reached the beginning of the tourist track, and samples were obtained in the dark zone (starting from 6 m from the bottom of the staircase) in 4–5 m^2 grids (D1–D3) (FIG. 1A). Multiple sediment samples were collected in each grid and pooled (FIG. 1A). Samples for areas not visited by tourists were obtained in an alcove starting ~ 15 m from the entrance in 4–5 m^2 grids (ND1–ND3) (FIG. 1A).

Stick Tomato.—This is a multi-use cave for self-guided tour and adventure caving with up to 20 000 annual visits. The cave has two entrances greater than 3 m across and contains some bat guano with the entrance in the adventure caving section capturing large amounts of water and detritus during storms. The twilight zone extends approximately 20 m from the entrance (FIG. 1B). Samples were obtained for Stick Tomato Cave on tourist path starting from about 6 m into dark zone (~ 26 m from entrance). Multiple sediment samples were collected in each of the three 4–5 m^2 grids with the approximate location of each grid (depending on the topography of the cave floor) as illustrated (FIG. 1B). Multiple sediment samples for areas not accessible to tourists were obtained in grids ND1–ND3 (FIG. 1B). Sediment samples from each grid were pooled to represent the microbial community in that area for ease of analysis.

Strawhaven.—This is a research cave that is not accessible to tourists and receives up to four annual visits. Multiple sediment samples were collected by Naracoorte Cave Management staff about 12 m into the dark zone (FIG. 1C).

Determination of physicochemical characteristics of cave sediments.—Moisture content, pH and organic matter of sediments were determined in replicate samples with standard methods.

Isolation of fungal isolates from sediment samples.—One gram of sediments from each replicate tourist-accessible and inaccessible samples from the three caves was homogenized and serially diluted in sterile 0.01 M phosphate buffered saline (pH 7.4) (Sigma, St Louis, Missouri). Inoculations from the 10-, 100- and 1000-fold dilutions were carried out in potato dextrose agar (Oxoid Ltd, Hampshire, UK), Czapek dox agar (Oxoid Ltd, Hampshire, UK), selective Fusarium agar (Burgess et al. 1988), malt extract agar (Oxoid Ltd, Hampshire, UK) and V8 agar (200 mL V8 juice, 2 g/L $CaCO_3$, 20 g/L agar in 1 L distilled water) plates with tetracycline (0.05 g/L) for fungal isolation. Replicate plates were used for each dilution and media type and plates were incubated at 25 ± 1 C for up to 18 d.

DNA extraction from cave sediments and microbial isolates.—DNA was extracted from sediments (0.25 g) and pure fungal cultures with the PowerSoil™ DNA extraction kit (MoBio Laboratories Inc., Carlsbad, California) according to the manufacturers' instructions. DNA was extracted from pure fungal cultures by flooding replicate plates with 5 mL sterile phosphate buffer, homogenizing plate cultures with sterile spreaders and transferring aliquots to sterile 1.5 mL Eppendorf tubes. Samples were pelleted by centrifugation at 12 000× *g* for 15 min. The supernatant was discarded and the pellet was transferred aseptically to the PowerSoil PowerBead tubes for DNA extraction. All extracted DNA were stored at −20 C pending further use.

PCR amplification.—PCR was carried out for fungal isolates with ITS1 and 4 (internal transcribed spacer regions) primers and the thermocycling conditions described by Anderson and Parkin (2007). PCR was performed in 50 μL reactions. These contained nuclease-free water (27.75 μL), 2 μL (2 ng) purified DNA extract from sediments or bacterial isolate, 2 μL each forward and reverse primers (10 pmole/μL), 10 μL 10× Taq DNA Polymerase buffer, 1 μL 10 mM dNTP (Promega, Madison, Wisconsin), 5 μL 25 mM $MgCl_2$ and 1 unit Taq DNA polymerase (Promega,

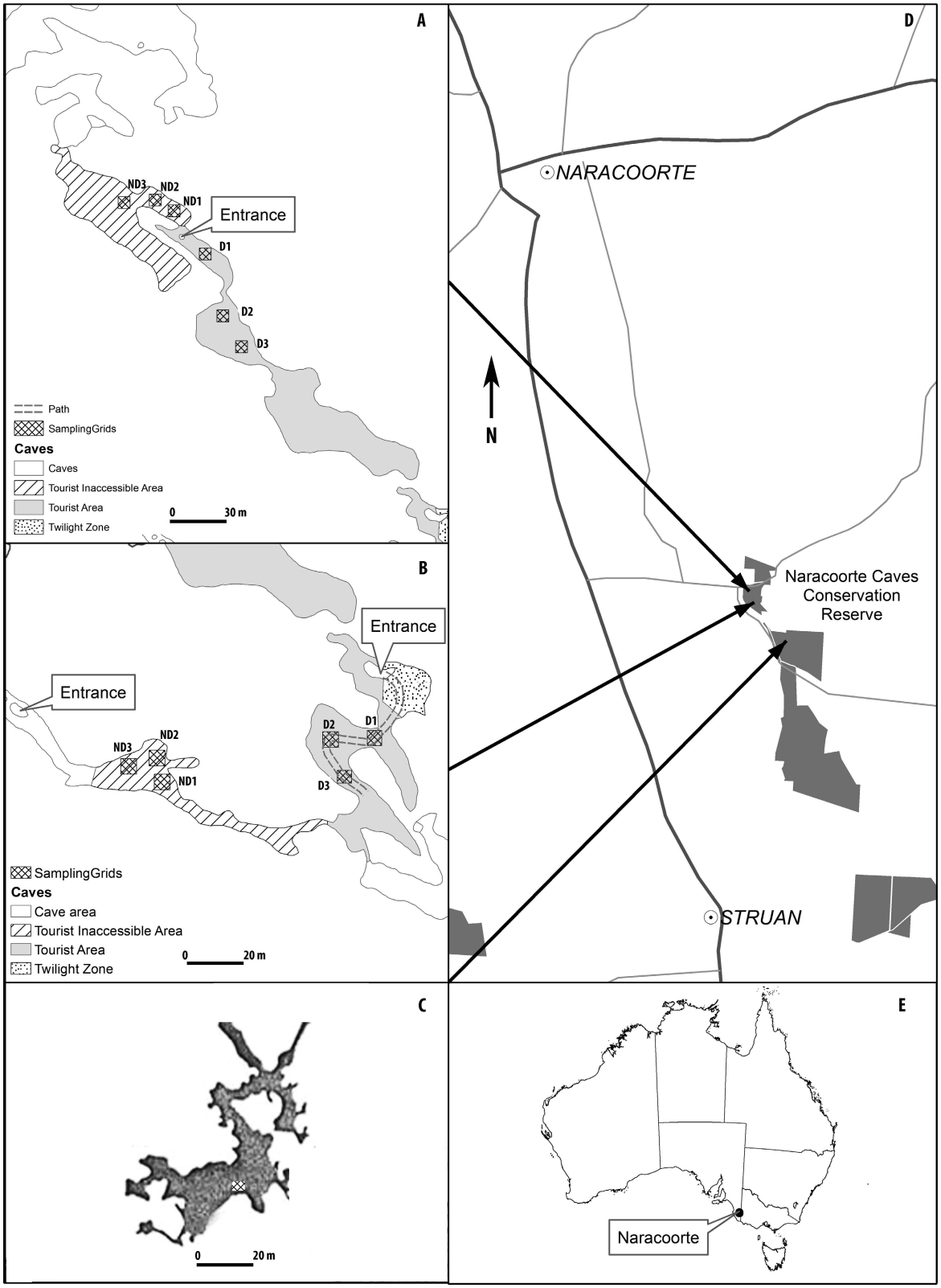

FIG. 1. Naracoorte caves studied and approximate areas where samples were taken. A = Alexandra Cave, B = Stick Tomato Cave, C = Strawhaven Cave, D = location of Naracoorte Caves and E = Naracoorte in Australia. D1–D3 = tourist accessible areas, while ND1–ND3 = closed to tourist. Sampling grids and shaded areas (C) are not drawn to scale and are approximate sampling points.

Madison, Wisconsin). However a nested PCR approach was used for the analysis of DNA extracted from sediments. The first reaction was carried out with ITS1 and ITS4 primers, while ITS1F-GC and ITS2 primers were used for the second nested reaction. The initial reactions for ITS1 and ITS4 were as described in Anderson and Parkin (2007), while the nested reaction with ITS1F-GC (forward primer) and ITS2 (reverse primer) was carried out with 1 µL of the amplicon from the ITS1 and 4 reaction and the same thermocycling conditions used for ITS1 and 4. The negative control of the ITS1 and 4 reaction also was used as a template to eliminate the possibility of carryover contamination.

Denaturing gradient gel electrophoresis analysis.—The PCR products from genomic amplifications of ITS regions were analyzed with Universal Mutation Detection System (Bio-Rad Inc., Hercules, California) with 9% polyacrylamide gels (the ratio of acrylamide to bisacrylamide was 37 : 1). Fungal communities were profiled by DGGE analysis of ITS1F-GC and ITS2 amplicons with a denaturing gradient of 42–52% and electrophoresed at 60 V for up to 20 h at 60 C. DGGE gels were silver- (Girvan et al. 2003) or SYBR gold-stained, scanned and saved as tiff. files with Epson Expression 700 Pro and used for subsequent analysis with Phoretix 1D advanced analysis package (Phoretix Ltd, UK). Bands were excised from SYBR gold-stained gels for fungal identification because it was impossible to recover them from silver-stained DGGE gels.

Band excision and purification.—Dominant bands (numbered) on fungal DGGE gels were excised aseptically with sterile scalpel and incubated in 100 µL elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDT and 0.1% SDS) overnight at 70 C. The eluted DNA was re-amplified with the appropriate primer pair (ITS1F-GC and ITS2) and subject to further rounds of DGGE with a narrower gradient range, band excision and further PCR (as described before) to ascertain the purity of the excised bands. The excised band was sequenced only when its purity was validated (the band was not a mixture of sequences).

DGGE bands and microbial isolate sequencing.—PCR amplicons of purified DGGE bands and genomic extractions from fungal cultures were cleaned with the Wizard® SV Gel and PCR system (Promega, Madison, Wisconsin) according to the manufacturer's protocol. DNA quantification before sequencing was carried out with NANODROP 1000 spectrophotometer (Thermo Scientific, Madison, Wisconsin). Sequencing was carried out with the BigDye® Terminator 3.1 cycle sequencing kit (Applied Biosystems) in accordance with the manufacturer's protocol on an ABI Prism 3730 Genetic Analyzer. The sequence data from the different amplicons were trimmed and aligned with Sequencher 4.1.4 software (Gene Codes Corp., Ann Arbor, Michigan) and the consensus sequence data was submitted to GenBank. Similarity searches were generated with BLASTN from GenBank (http://www.ncbi.nlm.nih.gov) and sequences from the database that matched the unknown sequence data submitted were aligned with CLUSTAL W2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) module and used to calculate pairwise evolutionary distances between the sequences. Phylogenetic trees were constructed and viewed with the maximum likelihood algorithm and TreeDyne in PhyML (Dereeper et al. 2008). Thirty-five newly obtained sequences from culture and DGGE based analysis (JF429658–JF429692) were deposited at GenBank. The sequence alignment files also were deposited at TreeBASE (S11262) http://www.treebase.org/treebase-web/home.html.

Statistical analysis.—The digitized image was analyzed with Phoretix 1D advanced analysis package (Phoretix Ltd., UK) with the relatedness of the microbial communities being expressed as similarity clusters with the unweighted pair-group method with mathematical averages (UPGMA). Shannon diversity values (H') was calculated from DGGE profiles as described by Girvan et al. (2003) with the formula $H' = -\sum P_i \text{ LN } P_i$. Pareto Lorenz (PL) curves were used to estimate evenness and functional organization within the microbial community. Bands were ranked first from high to low based on their intensities and the cumulative normalized bands (numbers) used as 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 degrees (Marzorati et al. 2008). Where necessary data were transformed and statistical significance was determined in replicate samples comparison within caves by either *t*-test or analysis of variance (ANOVA) and Tukey tests (Sigma Stat 2.03, Systat, London, UK).

RESULTS

Chemical characteristics of cave sediments.—The organic matter content of sediment was higher in the areas accessible to tourists (Stick Tomato 5.2 ± 0.72%, Alexandra 3.1 ± 0.13%) than in areas closed to tourists (Stick Tomato 4.5 ± 1.42%, Alexandra 3.0 ± 0.12% and Strawhaven 4.7 ± 0.79%). The sediment pH ranged from neutral (6.8) to slightly basic (7.8) in all the caves (Stick Tomato, tourist accessible 6.7 ± 0.012, tourist inaccessible 7.8 ± 0.012; Alexandra, tourist accessible 7.0 ± 0.03 and tourist inaccessible 7.0 ± 0.03; and Strawhaven, 7.2 ± 0.06). The moisture content of the sediments was variable within and among caves (Stick Tomato, tourist accessible 3.7 ± 0.23%, tourist inaccessible 12.7 ± 2.80%; Alexandra, tourist accessible 6.3 ± 0.20% and tourist inaccessible 6.4 ± 0.12%; and Strawhaven, 16.2 ± 0.58%.

Microbial community profiling, diversity and evenness in selected caves.—Fungal community analysis of the sediment samples was carried out to evaluate fungal diversity in tourist-accessible and tourist- inaccessible areas. ITS1 and 2 gene analysis of fungi was carried out on DGGE. There were substantial differences in the fungal community banding patterns between

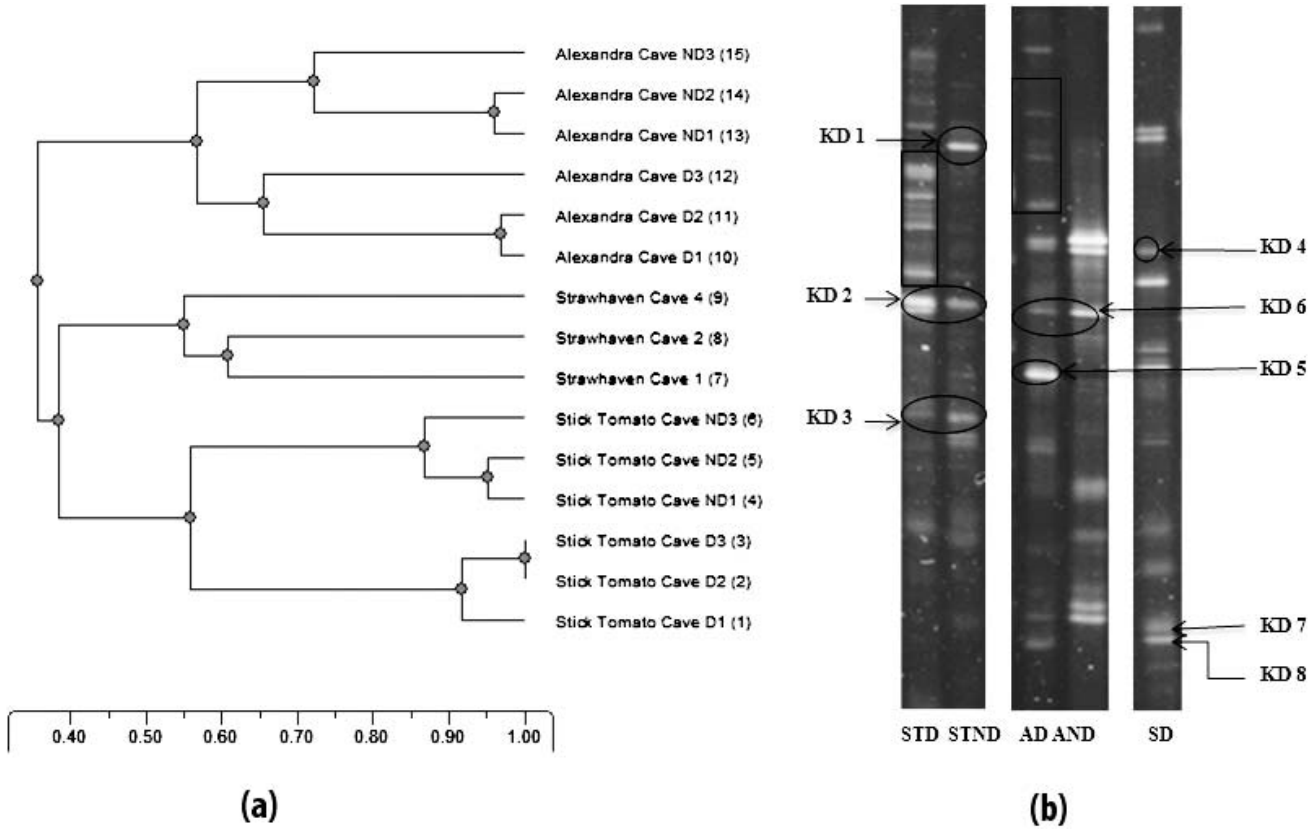

FIG. 2. Cluster analysis via UPGMA (Dice Sorensen's similarity index) of amplified ITS based DGGE profiles of fungal communities in selected caves from Naracoorte Park (A) and community profiles (B) from which bands have been excised and identified (circled). Bands peculiar to tourist accessible areas are boxed. Dendrograms generated with TL120 analysis package (Non-Linear Dynamics, Durham, United States of America). FIG. 2A, B. D1–D3 = replicate samples from tourist accessible areas, while ND1–ND3 = areas open to tourist. Scale is indicative of levels of similarity. FIG. 2B. STD = Stick Tomato tourist accessible, STND = Stick Tomato tourist inaccessible; AD = Alexandra tourist accessible; AND = Alexandra tourist inaccessible; and SD = Strawhaven Caves. STD, STND, AD, AND and SD were composite lanes consisting of all the replicates in each respective sample.

tourist-accessible and tourist-inaccessible areas of Stick Tomato Cave and to a lesser extent in Alexandra Cave (FIG. 2). In all caves there was significantly greater fungal diversity (H') in tourist-accessible areas than in tourist-inaccessible areas (t-test, $P < 0.05$) (TABLE I). Pareto Lorenz curve distribution patterns were used to assess the evenness of the fungal communities based on the cumulative number of bands and intensities detected on the DGGE profiles. The fungal community Parento Lorenz values were very similar in these two areas of the caves (48–55%), which indicated a highly even community profile (FIG. 3).

TABLE I. Shannon diversity values (H') determined from DGGE profiles of PCR amplified ITS regions of fungi from tourist accessible and inaccessible areas of Naracoorte Caves

	Shannon diversity values (H')	
Caves	Tourist accessible	Tourist inaccessible
Stick Tomato	2.83 ± 0.03[a]	2.53 ± 0.04[b]
Alexandra	2.92 ± 0.06[a]	2.64 ± 0.07[b]
Strawhaven	NA	2.68 ± 0.11

For tourist accessible and inaccessible areas in each cave samples [a] and [b] are significantly different.

Identification of microbial groups.—Two strategies were employed to determine the general species variety and evaluate whether there were differences in species composition of tourist-accessible and tourist-inaccessible sites. The first strategy involved determining the sequence identities of culturable microbial species. Thirty-two isolates were selected and purified based on colonial morphological characterizations from mixed culture plates and sequenced. All sequenced isolates belonged to division Ascomycota. The most commonly isolated culture (> 50% phenotype detected) irrespective of sampling area had sequences most similar to the genus *Trichurus*,

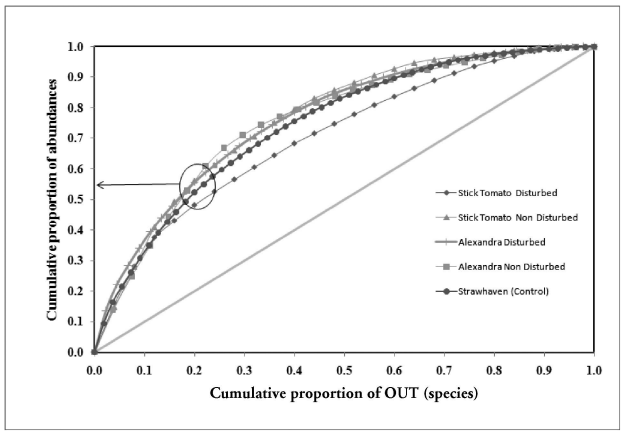

FIG. 3. Pareto-Lorenz distribution curves generated from ITS regions based DGGE profiles of tourist accessible and inaccessible microbial community in selected caves from Naracoorte Park. The straight line at 0.2 x is plotted to obtain Pareto values. Arrow indicates average Pareto Lorenz values for tourist accessible and inaccessible microbial communities. The percentage values are indicative of levels of functional organization, while 45 degree diagonal represents the perfect evenness of a community.

order Microascales (TABLE II, FIG. 4A). Other fungal cultures belonged to orders Hypocreales, Capnodiales and Eurotiales (FIG. 4A). Some isolates belonging to genera *Fusarium, Hypocreales, Trichoderma* and *Neonectria* (KS00-44, 43, 18, 21 and 22) were detected only in regions of Stick Tomato Cave not open to tourists, while *Aspergillus* sp. was peculiar to the areas open to tourists. Isolates from other caves also are provided (TABLE II).

The second strategy involved the excision and sequencing of prominent bands from the DGGE profiles of the caves. Detailed examination of the fungal community profile (FIG. 2A, B) showed that there were many fungal bands unique to the areas open to tourists of Stick Tomato and Alexandra. Unfortunately, despite repeated attempts at band excision and purification, the identities of most of these bands could not be resolved. Difficulties with band excision and identification have been reported by others and are thought to be dependent on the complexity and resolution of the original profile and also whether a single band on the gel was a mixture of sequence types (Anderson et al. 2003). Sequence

TABLE II. Summary and location of fungal species partial ITS sequences obtained from isolated cultures and DGGE profiles in selected caves in Naracoorte Park

Isolate code	Cave	Division (or subdivision)	Nearest taxon	Similarity (%)
KS0001	All except SC and AND	Ascomycota	*Trichurus* sp. FJ571495	97
KS00-02, 04, 05, 06, 07, 08, 14, 16, 17, 19, 24, 27, 29, 41, 48, 49, 50, 51	All except SC and AND	Ascomycota	*Trichurus* sp. EF540758	97–100
KS0044	STND	Ascomycota	*Fusarium oxysporum* EF495230	100
KS0043	STND	Ascomycota	*Hypocrea koningii* EF417481	98
			Trichoderma longibrachiatum FJ462745	98
KS0018	STND	Ascomycota	*Trichoderma longibrachiatum* FJ462745	98
KS0021	STND	Ascomycota	*Neonectria macrodidyma* AM419075	99
KS0022	STND	Ascomycota	*Penicillium citreonigrum* FJ904848	96
KS0012	STND	Ascomycota	*Penicillium camemberti* FJ025142	99
KS0025, 0033	STND, AND	Ascomycota	*Penicillium glabrum* DQ682590	95
KS0047	STD	Ascomycota	*Aspergillus aureolatus* EF652501	88
KS0010	STD	Ascomycota	*Penicillium* sp. DQ 779784	99
KS0035	AD	Ascomycota	*Cladosporium cladosporioides* EU622924	100
KS0039	AD	Ascomycota	*Penicillium griseolum* EF422848	99
KS0037	AD	Ascomycota	*Penicillium restrictum* AY373928	99
KD6	AD & AND	Ascomycota	*Cladosporium* sp. FJ790290	98
KD5	AD	Basidiomycota	*Thanetophorus cucumeris* EU244842	93
KD3	AD & AND STD & STND	Ascomycota	*Neonectria macrodidyma* AM419076	99
KD2	STD & STND	Basidiomycota	*Cryptococcus magnus* AF190009	99
KD1	STD & STND	Ascomycota	*Doratomyces* sp. FJ025216	93
KD8	SC	Ascomycota	*Doratomyces* sp. AJ608985	98
KD7	SC	Ascomycota	*Fusarium oxysporum* FJ478116	98
KD4	SC	Ascomycota	*Doratomyces* sp. FJ025216	91

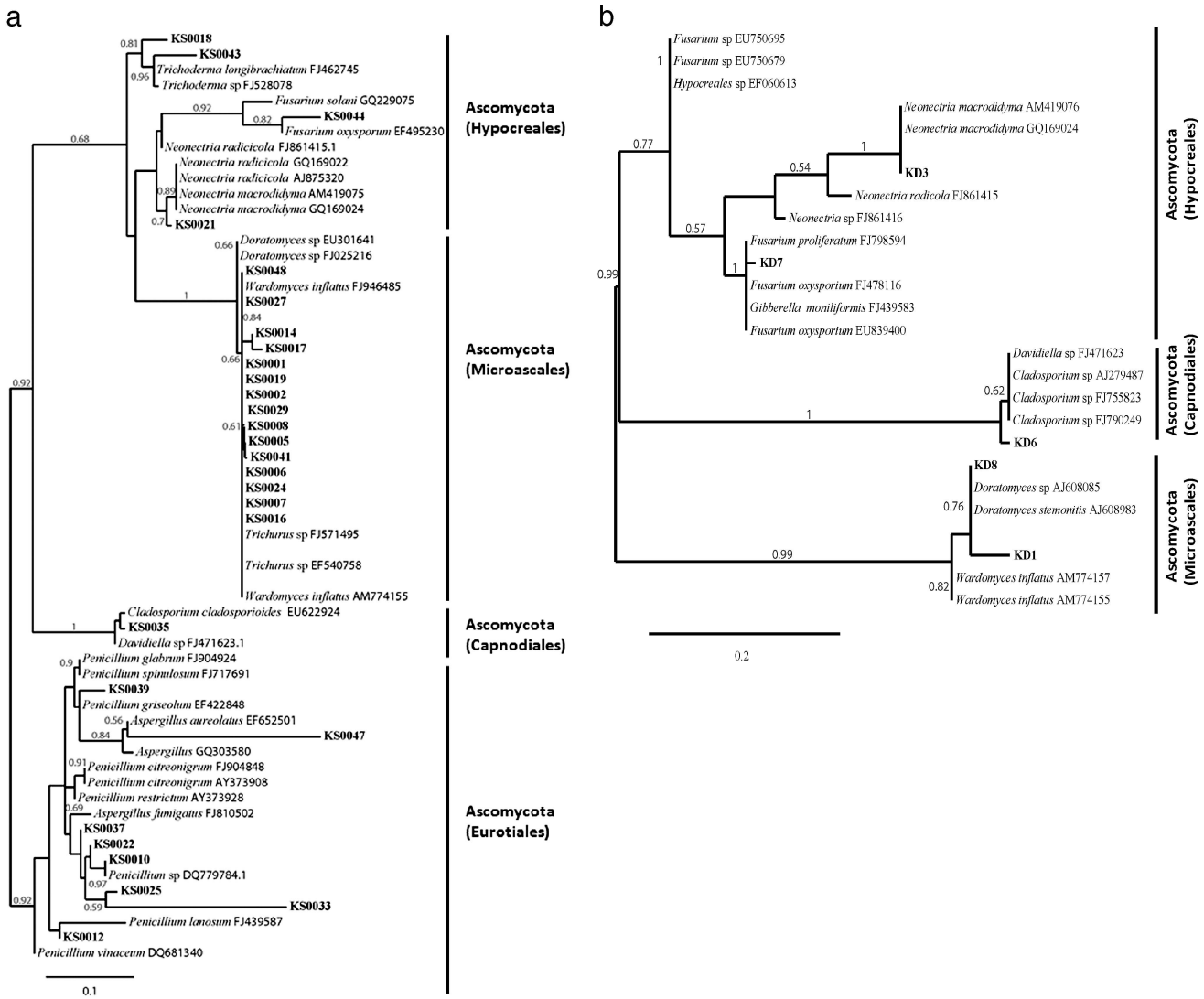

FIG. 4. Phylogenetic tree showing the distribution of cave phylotypes derived from pure fungal isolates (a) and some excised ITS DGGE bands (b) across fungal divisions. A. Tree was constructed from an alignment of 350–460 nucleotide positions. B. Tree was constructed from an alignment of 180–210 nucleotide positions. Distances were calculated with the maximum likelihood model in PhyML. Only partial sequences that could be aligned were used for both tree and bootstrap values >50 are shown. Bar = 0.2 substitutions per site.

analysis of excised DGGE bands whose identities were resolved showed most of the bands belonged to phylum Ascomycota (FIG. 2B, TABLE II). Most of the sequences also were assigned putatively to orders Microascales, Capnodiales and Hypocreales after phylogenetic analysis (FIG. 4B).

DISCUSSION

Survey of the fungal community.—The use of culture-based techniques for surveying microbial communities in caves is not unusual despite its limitations (Hawksworth and Rossman 1997) and when properly applied can give useful information on microorganisms (Bastian et al. 2009a, Stomeo et al. 2009). Several culture-independent techniques, such as PCR, DGGE, cloning and sequencing, also have been used to study cave microflora distribution (Dupont 2007, Bastian et al. 2009b). Because DGGE will detect only the dominant 1% bacterial population (Murray et al. 1996, Muyzer et al. 1993) the use of both techniques in this study should ensure a more accurate picture of caves microbial diversity.

Culture-dependent and independent analysis showed that group Ascomycota was predominant in control caves and in both tourist accessible and inaccessible areas of the other caves. This probably indicated that members of this group played impor-

tant roles in the ecosystem of these caves. Members of the Ascomycota group, such as *Fusarium, Penicillium, Trichoderma*, have been isolated from other caves in Japan, France and Spain (Docampo et al. 2010, Dupont et al. 2007, Kiyuna et al. 2008, Nagai et al. 1998). Therefore their isolation was not unusual. However the predominance of *Trichurus* (Microascales) among the culturable isolates was interesting. While it was possible that the suite of culture media used for fungal isolation preferentially might have selected for this genus, the fact that phylogenetically close *Doratomyces* and *Wardomyces* (both genera and *Trichurus* belong to order Microascales) were detected on DGGE profiles suggested that its presence was genuine and not due to media bias. Although Microascales such as *Doratomyces* have been isolated frequently from other cave systems (Nagai et al. 1998, Novakova 2009), *Trichurus* are found commonly in compost instead of in caves because of their cellulolytic and lignolytic enzymes (Hart et al. 2003). However apart from this study *Trichurus* also has been detected in rodent fecal material in cave sediments (Jurado et al. 2010).

The detection of these saprophytic Microascales in show and control caves also indicated that their presence might not have been due primarily to tourism or cave configuration but could have been related to the presence of animal droppings in cave sediments. Cave animals such as bats and possums, which are plentiful at Naracoorte, are important sources of organic matter via their bodies and droppings. These droppings are thought to support the growth of Microascales and other cave saprophytes (Jurado et al. 2010, Novakova 2009). While possums (and their droppings) are found in the three caves, bats (<50) are found only in some parts of Stick Tomato. However none of the caves is fully closed; small gaps and openings are used readily by animals. It therefore was possible that cave animals (especially possums) and cave arthropods (Jurado et al. 2008) that are not limited by tourist-related designations or access could have brought these Microascales from other caves, spreading them throughout the systems. Human activities also could have contributed to the spread of fungi in some of the caves studied (especially in the show caves) (Docampo et al. 2010). Nevertheless the specific reason for the predominance of *Trichurus* sp remains to be elucidated. In addition these small animals and insects might have contributed to the high organic matter observed in some of the caves. Other potential exogenous source of organic matter could have been floods, especially in Stick Tomato Cave, which might have led to the introduction of plant materials at cave entrances from where they probably spread to other parts of the caves by small animals and visitors.

Other fungal genera, such as *Fusarium, Neonectria, Penicillium, Aspergillus, Cladosporium* detected in these studies, are known soil and cave saprophytes (Kiyuna et al. 2008, Mulec et al. 2002, Stomeo et al. 2009). Saprophytic fungi are crucial to cave ecosystems because some bacteria and insects can feed on saprophytic fungal mycelia and spores (Culver 1982). Fungal decomposition of organic matter (animal and insects bodies and feces) also releases nutrients back into the ecosystem, which then can be used by other microorganisms such as autotrophic bacteria. Therefore thriving fungal groups (predominantly Ascomycota) supported vital ecosystems in the caves.

Tourist-accessible versus tourist-inaccessible areas.—Substantial differences were observed in culturable fungal species diversity (TABLE II) of tourist-accessible and inaccessible areas (in Stick Tomato Cave) with a variety of *Fusarium, Trichoderma* and *Neonectria* species being isolated only from inaccessible areas. Analysis of the culturable community also showed that only *Aspergillus* was unique to areas open to tourists in Stick Tomato. Some caves with heavily trafficked sites have been reported to have lower culturable fungal diversity compared to the high diversity at low disturbance sites (Shapiro and Pringle 2010). Culturable microbial diversity could decrease as the effects of humans rise, indicating a negative correlation between tourist number and culturable microbial diversity (Ikner et al. 2007). Molecular analysis with PCR-DGGE technique also showed substantial differences in the band phylotypes (DGGE profile) and banding patterns (cluster analysis) of fungal communities in the tourist-accessible and inaccessible areas of Stick Tomato. The cluster analysis also showed that the sites in each cave formed a distinct cluster with one another that was different from the clusters formed by the sites in other caves. This implied that differences observed between tourist-accessible and inaccessible sites of each cave (within cave variation) were alterations in the original community instead of the replacement of this community by a new one. Unlike in culturable fungal diversity, molecular analysis indicated an increase in fungal diversity (Shannon diversity values derived from DGGE) in tourist-accessible areas of both Stick Tomato and Alexandra caves compared to inaccessible areas and the control Strawhaven Cave. The different conclusions obtained from different methods might be related to the bias associated with these methods. For example more than 90% of microorganisms are not culturable with culture-based analysis being biased toward the comparatively fewer cultur-

able groups (unlike the use of molecular tools that allows for the detection of both the culturable and non-culturable microbial groups). However both methods showed that there were differences in the fungal communities of tourist-accessible and inaccessible areas (especially in Stick Tomato). Despite these differences, the fungal community was highly even (PL values) in both areas, and this indicated that the community was relatively stable in all caves.

Tourism can cause changes in microbial diversity and distribution because visitors introduce microorganisms. Tourists also introduce a variety of substances, such as hair, dead skin cells, sweat and skin oils, which are potential nutrients for cave microorganisms (Barton 2006, Ikner et al. 2007, Jablonsky et al. 1993). However it was difficult to conclude that the changes in culturable fungal diversity observed in this study in Stick Tomato Cave (a high impact cave) were due primarily to tourism because this trend was not observed in Alexandra (another high impact cave). It therefore was possible that other factors, such as cave configuration (open access or close), use and some environmental factors such as organic matter content, could have contributed to the differences observed in the fungal population of Stick Tomato.

Stick Tomato Cave is fully open to tourists and sometimes used for adventure caving, while Alexandra Cave has secure doors and all tours are guided. Therefore there is a greater risk of the entry of exogenous materials and organisms in Stick Tomato Cave than in the other caves. Environmental factors, such as pH, air flow, sampling area heterogeneity, spatial variability of microbial population, sediment moisture content, organic matter input and type and location, also can influence microbial distribution (Northup et al. 2000, Portillo et al. 2009). Fungal spores and their mycelia are readily distributed by air, small animals and insects (Bastian et al. 2009b, Dupont et al. 2007, Jurado et al. 2008), and this factor might affect fungal diversity and distribution in caves.

In conclusion this study has shown that the fungal community in Naracoorte Caves was dominated by members of the Ascomycota, with Microascales being frequently isolated. Substantial differences in the fungal diversity and distribution also were observed in tourist-accessible and inaccessible areas of Stick Tomato as determined by both culture-dependent and independent assays. However because the same trend (except in DGGE-based Shannon diversity measurements) was not observed in Alexandra Cave it was possible that differences observed in Stick Tomato were not due primarily to tourism. Cave use, configuration and environmental factors could have been responsible for some of the differences. Further investigations therefore are needed to quantify the effect of these factors on fungal community structure in Stick Tomato Cave. The significance of some the fungal genera detected, such as *Fusarium, Cladosporium*, on formations (rocks and speleothems) needs to be investigated further. The fact that we were not able to conclusively link the differences observed in tourism does not necessarily mean tourism did not have any effect on fungal diversity in the Naracoorte Caves. It was possible that tourism effects were transient in the caves studied (and therefore missed) or more prevalent in caves that were not part of this study. We therefore recommend further investigations of other show caves and sampling in different seasons, times of the day and specific tourist events for the prevalence of fungal spores or specific fungal groups to identify any tourism-related effects on cave microbial diversity (Docampo et al. 2010).

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