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Anthropogenic Influences on the Diversity of Fungi Isolated from Caves in Kentucky and Tennessee

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ABSTRACT.—Caves are unique habitats and important components of their ecosystems. Caves are also vital to local economies because they serve as tourist attractions. Cave habitats appear to host diverse communities of fungi. In this study we explore associations between levels of human disturbance and the diversity of fungi in four caves in Kentucky and Tennessee. Species isolated from cave soils were cultured at 10 C (cave temperature) and room temperature. The results show that fungal diversity is low in heavily trafficked sites, increases in moderately visited sites and peaks at low disturbance levels. No fungi were cultured from sites that had very rarely or never been entered before we sampled. Species were counted using the morphological species concept. Fungi from the most diverse site were also characterized using sequence data of the ITS locus. Using this method the fungi were identified as species of *Bionectria*, *Cadophora*, *Fusarium*, *Hypocrea*, *Mortierella*, *Paraconiothyrium*, *Penicillium* and *Podospora*.

INTRODUCTION

Fungi are critical components of ecosystems, functioning as decomposers, mutualists and pathogens. Caves shelter species whose entire life cycles are completed within a cave, as well as troglophiles; species that live in caves but regularly leave them, for example bats and insects.

A number of studies have explored the diversity of fungal species growing in caves (Kuehn and Koehn, 1991; Pitzalis *et al.*, 1991; Cunningham *et al.*, 1995; Gunde-Cimerman *et al.*, 1998; Kim *et al.*, 1998; Koilraj *et al.*, 1999; Taylor, Chavez-Tapica *et al.*, 2000; Canaveras *et al.*, 2001; Dupont *et al.*, 2007; Hsu and Agoramoorthy, 2001; Benoit *et al.*, 2004; Grishkan *et al.*, 2004; Roldan *et al.*, 2004; Macalady *et al.*, 2007; Santamaria and Faille, 2007), but little work has been done to determine whether human activities in caves influence the diversity of cave fungi. Research from across the globe suggests that the most common cave fungi are ascomycetes. Zygomycetes and basidiomycetes are found as well, although the diversity in terms of numbers of species for these phyla is not as great. The numbers of species found varies widely, from about a dozen to almost 70 species, depending on the location and focus of the study.

Most species isolated from caves are soil saprophytes; patterns of spatial diversity and substrate specificity vary by study. The greatest diversities are generally found near the entrances to caves, probably because many species are carried to cave entrances by animals or wind, or because these species can grow on the organic debris found at cave entrances (Cunningham *et al.*, 1995; Koilraj *et al.*, 1999; Hsu and Agoramoorth, 2001; Roldan *et al.*,

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2004). Other fungal species appear to be opportunistic and are brought to caves by troglophiles or by the terrestrial streams that flow underneath the ground and through caves (Kuehn and Koehn, 1991; Pitzalis *et al.*, 1991; Cunningham *et al.*, 1995; Taylor, Chavez-Tapica *et al.*, 2000; Dupont *et al.*, 2007; Santamaria and Faille, 2007). Some species are specific to a particular host or substrate within the cave, for example cave crickets or beetles (Benoit *et al.*, 2004, Santamaria and Faille, 2007, *see also* Gunde-Cimerman *et al.*, 1998). Data collected from caves near the Dead Sea in Israel suggest that other species are cosmopolitan and found not only within caves, but in nearby terrestrial habitats (Grishkan *et al.*, 2004). Most studies identify species using a morphological species concept. Counts that use morphology as a guide may not give accurate species numbers, because a single species may have several morphologies, and similar looking morphologies may in fact be different genetic species (Taylor, Jacobson *et al.*, 2000).

Our research compares the diversity of fungi cultured from caves with high, moderate or minimal levels of human disturbance. Humans can easily introduce new species to caves but may also harm the species already growing there. We conducted our research in caves in Kentucky and Tennessee, using both protected and unprotected caves and sites that are heavily visited, somewhat visited or rarely visited by people. Species were counted using a morphological species concept, but we also used DNA sequences of the ITS locus (Gardes and Bruns, 1993) to supplement the morphological data.

MATERIALS AND METHODS

SAMPLE SITES

Our samples were collected from four caves in Kentucky and Tennessee: Dogwood Cave (Edmonson County, KY), Diamond Caverns (Edmonson County, KY), Lost Creek Cave (White County, TN) and Cumberland Caverns (Warren County, TN) (Table 1). Dogwood Cave is a small cave owned by the non-profit National Speleological Society, adjacent to Mammoth Cave National Park and also hydrologically connected to Mammoth Cave (Larry Johnson, pers. comm.). The total length of the cave is about 1000 ft and access is limited because of its remote and unmarked location on private property. Diamond Caverns is a privately owned show cavern in Park City, Kentucky that is hydrologically connected to the Mammoth Cave system. Up to 16 tours of 20 or more people may take place each day during the tourist season. People have been visiting the attraction since its discovery in 1859. Walkways and electric lighting have been installed (Sides, 2006). Lost Creek Cave is located in a rural residential area in Tennessee. There are no restrictions to access, and it is a popular site for local cavers. There is evidence of litter around the entrance and in other parts of the cave. Like many caves in the area, it was used for saltpeter mining in the 18th and 19th centuries (Larry Johnson and Jim Hodson, pers. comm.). Cumberland Caverns is a privately owned cave outside of McMinnville, Tennessee with over 32 mi of mapped passage. It was first discovered in 1810 and saltpeter mining took place from 1812 through the Civil War period. In the early 1900s, the cave opened as a tourist attraction, and National Speleological Society surveys began in 1945. Tours are given daily throughout the year and hundreds of people may pass through in a single day (Cumberland Caverns, 2008). *See* Table 1 for descriptions of the specific sampling sites within each cave.

We collected soil from 11 sites of the four caves. Each site was categorized as having ''high,'' ''middle,'' ''low'' or ''very low'' human disturbance levels based on estimated visitation. High traffic sites were located in commercial caves near the tourist trails (Diamond Caverns and Cumberland Caverns), or where recent human presence was evident through footprints or trash (Dogwood Cave and Lost Creek Cave). Medium and low impact

TABLE 1.—Description of cave sites

Cave	Disturbance level	Distance and entrance	Soil type
Dogwood Cave	Low	Side room. 500 ft from entrance, accessed through short crawlway.	Moist with crickets and bats present.
Dogwood Cave	Moderate	1000 ft from entrance in main cave passage. Near pit-dome structure.	Moist, close to water flow.
Diamond Caverns	Moderate (a)	Small hill 1500 ft from entrance and 200 ft from tourist trail.	Muddy and claylike.
Diamond Caverns	Moderate (b)	1000 ft from entrance and 50 ft from tourist trail.	Muddy pit, was used for saltpeter mining in 19th century.
Diamond Caverns	High	100 ft from entrance, directly off the tourist trail.	Muddy soil; guides often stand there.
Lost Creek Cave	High	300 ft from cave entrance next to trail.	Hardpacked, but moist dirt. Litter present.
Lost Creek Cave	Moderate	1500 ft from entrance in upper level of cave.	Sandy and dry. Area of saltpeter mining, equipment still present.
Lost Creek Cave	Low	3500 ft from entrance, dead end trail in upper level of cave.	Muddy with some litter present about 400 ft away.
Cumberland Caverns	High	50 ft from tourist trail, ½ mile within cave.	Dry, grainy soil.
Cumberland Caverns	Very Low (a)	Edge of large room ½ mile behind tourist trail.	Moist, dense soil.
Cumberland Caverns	Very Low (b)	Small room, accessed by moving rocks from entrance in small passage.	Moist, clay-like soil. Possibly previously unvisited.

sites were located further from tourist trails (Diamond Caverns and Cumberland Caverns) or further from entrances and main trails (Dogwood Cave and Lost Creek Cave). The very low disturbance level sites in Cumberland Caverns had not been visited for many years, or possibly never before by humans (James Hodson and Larry Johnson, pers. comm.). We could not sample across the entire spectrum of disturbance levels for any individual cave, either because particular disturbance levels do not exist within a particular cave or because of restricted access to some sites.

Soil samples from Diamond Caverns, Dogwood Caverns and Lost Creek Caverns were collected 21–29 Jan. 2008. Samples from Cumberland Caverns were taken on 8 Mar. 2008. Cultures were made within 1 wk of collection.

CULTURES

This study focused on culturable species. At each site, approximately 2 tbsp of soil were collected using a sterile plastic spoon. Each sample was placed in a sterile plastic vial. Plastic gloves were worn throughout the process and a fresh pair was used at each site.

Initially, 5 gm of each soil sample were mixed with 15 mL of a sterile 5% saline solution. Serial dilutions were made by adding 100 µL of the original dilution to a new 1 mL quantity

of saline solution, and the procedure was repeated for each sample to make an original dilution and three serial dilutions for each site.

One hundred and fifty microliter aliquots of every dilution were plated on separate Petri dishes. All samples were grown on a Czapek-Dox medium (Bucknova *et al.*, 2007). Six mL of 5 mg/mL solutions of both tetracycline and streptomycin were mixed with the medium to prevent bacterial contamination. Once fungi began growing on the plates, each distinct morphology was subcultured onto a different Petri dish. Individual morphologies were determined by visual inspection of the hyphae, color and texture of cultures (Summerell *et al.*, 2003). All samples were grown at 10 C, approximately cave temperature, for 3 mo. Additional morphologies were found on plates that had been left at room temperature for two nights. These plates had not been opened since the original plating of soil samples and these morphologies were not the result of contamination from laboratory fungi. After this discovery, another subset of soil samples from each cave site were plated and cultured at room temperature for 2 mo. The soil samples had been stored in vials in dry, dark cabinets. All samples were grown in darkness, to simulate cave conditions.

MOLECULAR METHODS

To understand whether cultures with similar morphologies were the same or different species, sequences were taken from morphologically identical cultures of different cave sites. To get a sense of the genetic diversity and identity of species growing at our most species rich site, we also sequenced the ITS locus from every morphological species collected from the low impact Lost Creek Caverns site, the most diverse of the 11 sites.

To extract DNA from the fungal samples, approximately 50 mg of lyophilized tissue of each culture was placed in a 2.0 ml microcentrifuge tube with 4–5 3 mm glass beads and ground using a MiniBeadbeater-8 (BioSpec Products) set at 3/4 speed for 1 min. Four hundred microliters of CTAB were added to each sample and then each tube was heated in a warm bath and subsequently frozen on dry ice, to further macerate the tissue. Thaw/freeze cycles were repeated twice. Afterwards, the samples were placed in a 70 C water bath for an additional 45 min. Then 1 mL of chloroform was added and the vials were tipped upside down and back for 10 min, and centrifuged for 10 min. The rest of the procedure followed the Qiagen DNeasy Plant Mini Kit protocol starting at step 6 (Qiagen Sciences).

All initial PCR products were obtained with the following recipe: 7.9 µL water, 5 µL of buffer, 2 µL dNTPs, 2 µL MgCl, 5 µL of a PCR enhancer, 1 µL each of primers ITS1F and ITS4 (Gardes and Bruns, 1993), 0.1 µL GoTaq (Promega) and 1 µL of genomic DNA. PCR reactions were amplified in a BioRad iCycler using the following parameters: 5 min at 95 C, followed by 35 cycles of 1 min at 95 C, 55 min at 55 C, 45 min at 72 C, and then a final elongation of 7 min at 72 C.

All PCR products were cleaned using the Promega Wizard Geneclean kit following manufacturer's specifications. Sequences were obtained using an Applied Biosystems 3730 sequencer, following Pringle laboratory protocols (Pringle *et al.*, 2009). All sequence traces were edited using the program Sequencher version 4. (Gene Codes).

All sequences were compared to sequences in the National Center for Biotechnology Information (NCBI) public database. Sequences were entered in the Nucleotide BLAST program and data matches were catalogued. Sequences isolated from different cultures that gave identical BLAST results were aligned by hand and compared using McClade version 4.05 OSX (Madison and Madison, 2002).

STATISTICS

We used JMP version 5.0.1a (SAS Institute) to analyze data on species numbers. Species were grouped according to the cave they were isolated from and according to disturbance

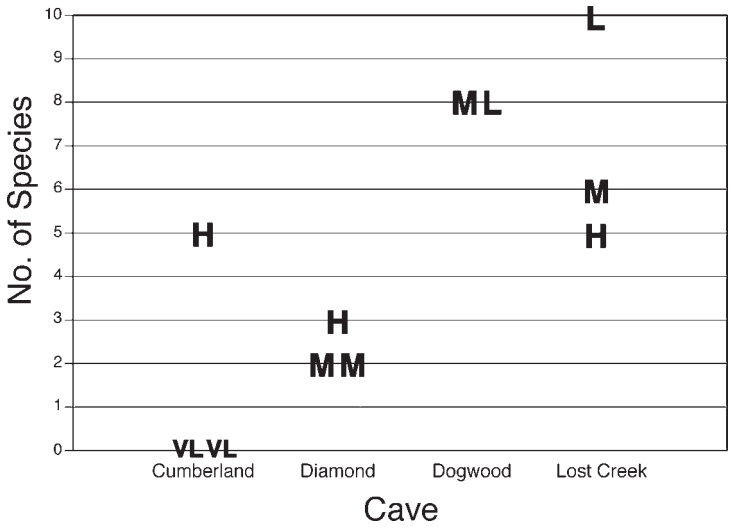

FIG. 1.—The number of morphological species isolated from different cave systems when soil dilutions were grown at 10 C (cave temperature). A summary of the number of morphological species cultured at 10 C from each cave. Each letter or set of letters plotted on the figure represents a site within the cave labeled below, and the letters H, M, L, VL are abbreviations for ''high,'' ''moderate,'' ''low'' and ''very low'' levels of disturbance. In two cases two sites within one cave system housed the same number of species, and in these cases two symbols are placed next to each other on a single line

level. No cave offered the complete spectrum of disturbance levels and so a full statistical treatment that analyzed both cave identity and disturbance level simultaneously was not possible. We did separate analyses using either cave identity or disturbance level as the treatment, and we interpret our results with caution. We used a one-way or single factor ANOVA to test the hypotheses that species numbers differed according to cave identity, or according to disturbance level. We conducted analyses on the total number of species, the number of species that grew at 10 C (cave temperature) and the number of species that grew at room temperature. We calculated Jaccard coefficients of similarity by hand.

RESULTS

Cave identity does not appear to have a strong influence on species richness. When grouped by cave, cave identity was not a statistically significant predictor of the number of species for either total species ($n = 11$, $F_{3,7} = 0.46$, $P = 0.72$) or for species isolated at room temperature ($n = 11$, $F_{3,7} = 0.25$; $P = 0.86$). However, for species grown at 10 C, differences in diversity were statistically significant ($n = 11$; $F_{3,7} = 6.06$; $P = 0.02$). Dogwood Cave is the most diverse cave system, followed by Lost Creek Cave, Diamond Caverns and Cumberland Caverns (Fig. 1). Because it was not possible to collect samples of every disturbance level from every cave, we are unsure whether there is a genuine trend to more diversity of cold temperature fungi in Dogwood and Lost Creek caves. The lack of sampling from highly disturbed and very low disturbance sites in these caves could be driving the appearance of greater species richness in these systems (*see* below).

Jaccard coefficients of similarity among pairs of caves were low and ranged between 0 and 0.074. The maximum number of species shared by any two caves was four; this comparison was between Lost Creek Cave and Diamond Caverns.

Species richness does appear to be correlated to disturbance level. When grouped by disturbance level, medium and low impact sites had significantly greater species numbers than highly disturbed or very low disturbance sites, for both total numbers of species ($n = 11$, $F_{3,7} = 4.10$; $P = 0.06$) and species grown at 10 C ($n = 11$, $F_{3,7} = 6.00$; $P = 0.02$) (Fig. 2). Although the species richness from samples cultured at room temperature was not significantly different at the different sites ($n = 11$, $F_{3,7} = 1.31$, $P = 0.35$), there is a slight trend toward greater numbers of species at intermediate levels of disturbance (Fig. 2). The very low impact sites had no fungal growth at any temperature. The moderately disturbed sites had totals of 7–18 species; 2–10 species at 10 C and 1–16 species at room temperature. Low impact sites generally had the greatest diversities with totals of 9–26 species; 8–10 species at 10 C and 8–16 species at room temperature.

As expected, we found that morphological species counts are probably underestimates of true species numbers. Five sets of apparently identical morphological species isolated from different sites each have different sequences, and may be different genetic species (Table 2). Five sets had similar ITS sequences, and are perhaps different strains of the same species. Only one set of identical morphological species had identical sequences.

The most diverse site was the minimally disturbed site in Lost Creek Cave (Table 1). It had a total of 26 morphologically identified species. We have made tentative identifications based on comparisons between our sequences and sequences found in the NCBI database. These are not definitive species identifications, but allow us to provide some information about the taxonomic identity of our cultures. The data suggest that the genera isolated from this site include species of *Fusarium* (5), *Bionectria* (4), *Penicillium* (3) and single isolates of *Paraconiothyrium*, *Podospora*, *Hypocrea*, *Cadophora* and *Mortierella* (Table 3). *Bionectria*, *Cadophora*, *Mortierella*, *Paraconiothyrium*, *Penicillium* and *Podospora* were cultured at 10 C. *Bionectria* was also cultured at room temperature, as were *Fusarium* species and the *Hypocrea*. Five additional isolates had their closest matches to ''uncultured soil specimens'' and remain unidentified. Four specimens did not yield DNA that could be sequenced.

In some cases different morphologies had closest matches to the same sequence (Table 3). In these cases we compared the sequences from the morphologies to each other; in only one case were the two sequences in fact identical. However, because we do not have data from other genetic loci for these morphologies we cannot be sure that these morphological species are the same genetic species. These cultures may be morphologically different strains of the same genetic species, or may be different genetic species that are identical at this locus. In general, sequences of different morphologies that had closest matches to the same GenBank sequence were not identical and had multiple polymorphisms.

DISCUSSION

Anthropogenic activity in caves appears to influence fungal diversity in complex ways: sites with no human disturbance appear to have no or very few fungi, but sites that are heavily visited also have low species richness. Diversity rises with moderate levels of disturbance and peaks at minimally disturbed sites.

The numbers of species that grow at 10 C may serve as the best metric of human impacts on cave ecosystems because caves are cool habitats and these are the species that may naturally grow in caves. Their presence may influence the health of the local ecosystem, for

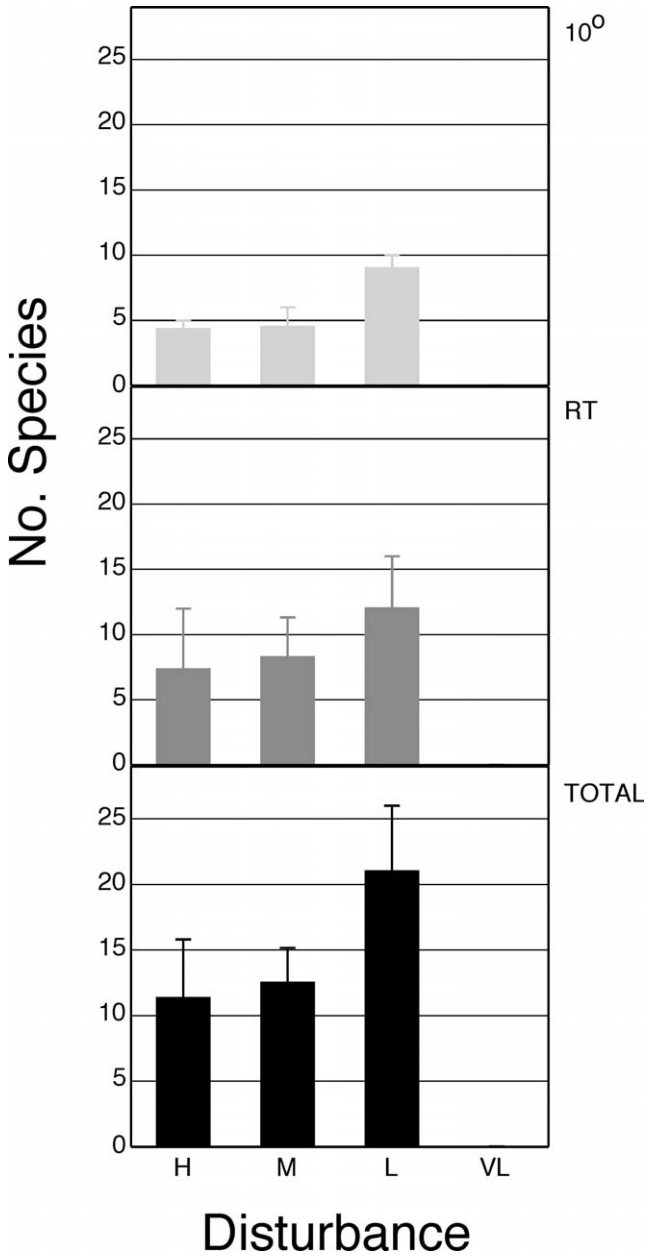

FIG. 2.—Impacts of human disturbance on species richness of fungi (using a morphological species concept). This figure shows the average number of species found at each disturbance level, ''high,'' ''moderate,'' ''low'' and ''very low.'' Bars are standard errors. Numbers were calculated from the data of all sites in all caves. The top graph shows species cultured at 10 C (cave temperature), the middle graph portrays species cultured at room temperature and the bottom graph includes the average total species cultured at each impact level

TABLE 2.—Comparing sequences of morphologically identical groups of species

Morphological species/ specimen no.	Cave/impact level	Temp.*	First match in NCBI database	Accession number of match	Maximum identity between sequences (%)	Query coverage (%)	Sequence length
A/LCC143	Lost Creek/Low	RT	*Fusarium* spp.	EU750695.1	99	100	574
A/LCB14	Lost Creek/Mod.	RT	*Fusarium solani*	AM412635.1	98	98	574
A/DIAA13	Diamond/Mod.	RT	uncultured soil fungus	DQ420780.1	98	100	562
B/DIAA59	Diamond/Mod.	RT	*Sordariomycete* sp.	EU680539.1	99	99	574
B/DWB14	Dogwood/Mod.	RT	*Bionectriaceae* sp.	EF672316.1	98	98	570
C/DIAB2	Diamond/Mod.	RT	*Bionectria ochroleuca*	AF106532.1	98	99	600
C/DWB18	Dogwood/Mod.	RT	Ascomycete sp.	AJ279488.1	99	98	562
C/LCC153	Lost Creek/Mod.	RT	*Bionectria ochroleuca*	AF106532.1	99	99	583
D/LCC75	Lost Creek/Low	10°C	uncultured ascomycete	AM901807.1	99	99	582
D/DIAA14	Diamond/Mod.	RT	*Penicillium commune*	AF236103.1	99	99	582
E/DIAC21	Diamond/High	10°C	*Trichurus* sp.	EF540758.1	98	94	632
E/DWA20	Dogwood/Low	10°C	*Doratomyces stemonitis*	AJ608983.1	98	94	610

* = Room temperature

example as parasites and decomposers of cave insects, including beetles and crickets (Kuehn and Koehn, 1991; Gunde-Cimerman *et al.*, 1998; Santamaria and Faille, 2007).

In contrast, the fungi that grow only at room temperature are unlikely to grow in caves. The room temperature specimens may suggest which spores can reach a cave, but it is also possible that in smaller caves, for example Dogwood Cave, room temperature fungi do play a role in maintaining the ecosystem. These shallow caves receive large quantities of debris from outside the cave ecosystem, and room temperature fungi could decompose this material if temperatures within these smaller systems are warmer than the typical temperatures in larger caves. Common fungal decomposers have been found in entrance regions and shallow caves by other researchers (Koilraj *et al.*, 1999; Hsu and Agoramoorthy, 2001; Roldan *et al.*, 2004).

We isolated no fungi from the two sites with minimal human visitation, both of which were located within Cumberland Caverns. This suggests that many fungi found in caves are carried into the systems from outside sources, or from other parts of the cave system, although not necessarily by humans. The lack of fungi at these sites suggests that some sort of disturbance is necessary to carry spores into a cave. An unvisited or very rarely visited site might not have a source of spores.

Cave identity was important for the diversity of species growing at cave temperature (10 C), but not for the total number of species or room temperature species. This suggests that there may be an inherently variable diversity of ''naturally'' occurring fungi among the different cave systems. Cumberland Caverns is an example of a cave that may have high diversity of species. However, within this pattern the highest impact sites tended to be less diverse and the general trend to greater diversity in moderately disturbed sites held (Figure 1). Because we could not sample across the spectrum of disturbance levels for any

TABLE 3.—Genetic diversity of morphospecies isolated from the minimally disturbed site of Lost Creek Caverns

Sample no.*	Temp.	First match	Accession no. of match	Maximum identity (%)	Query coverage (%)	Sequence length
LCC35	10°C	*Paraconiothyrium sporulosum*	AB303549.1	99	100	583
LCC45	10°C	*Podospora* sp.	EU273519.1	99	97	539
LCC107	10°C	uncultured ascomycete	EU490130.1	99	100	677
∧LCC111	10°C	*Bionectria ochroleuca*	AF106532.1	99	99	567
LCC81	10°C	*Cadophora melinii*	DQ404351.1	96	61	865
LCC75[c]	10°C	*Penicillium expansum*	DQ339562.1	99	99	582
LCC93[c]	10°C	*Penicillium expansum*	DQ339562.1	100	100	581
LCC103	10°C	*Penicillium expansum*	DQ339562.1	99	94	647
LCC16	RT**	*Fusarium* spp.	AJ279478.1	99	100	556
LCC32	RT	*Fusarium* spp.	EU750687.1	99	99	585
LCC115	RT	uncultured ascomycete	AM901807.1	100	99	584
LCC118[b]	RT	uncultured ascomycete	AM901807.1	99	99	582
LCC128	RT	uncultured ascomycete	AM901807.1	100	99	561
LCC71[b]	RT	uncultured ascomycete	AM901807.1	99	99	584
∧LCC126	RT	*Bionectria ochroleuca*	AF106532.1	99	99	571
LCC128	RT	*Bionectria ochroleuca*	AF106532.1	100	99	575
LCC133[d]	RT	*Bionectria ochroleuca*	AF106532.1	98	99	564
LCC153[d]	RT	*Bionectria ochroleuca*	AF106532.1	99	99	583
LCC136[e]	RT	*Fusarium* spp.	EU750695.1	99	99	550
LCC143[e]	RT	*Fusarium* spp.	EU750695.1	99	99	574
LCC155	RT	*Fusarium solani*	AM412635.1	98	98	547
LCC69	RT	*Hypocrea virens*	EU280090.1	99	97	610

* the ∧ indicates identical genetic sequences; letters indicate similar, but not identical, sequences
** RT = Room temperature

single cave system, we cannot disentangle the effects of cave identity from disturbance. The highly trafficked site of Cumberland Caverns produced 20 species in culture; however, we could not sample at moderate or low impact sites and cannot provide definitive data on the fungal diversity of Cumberland Caverns at these kinds of sites.

Most of the species we identified using genetic techniques are soil saprophytes or plant pathogens. *Mortierella* and *Penicillium*, which grew at 10 C, are commonly found decomposers. Interestingly, *Fusarium*, another common soil fungus, was only cultured at room temperature and is therefore unlikely to grow within the cave. Lost Creek Cave may be especially open to colonization because a river runs from the hills outside through the cave. This river may partially explain the high diversity at Lost Creek Cave's minimally disturbed site, and explain how plant pathogens have entered the system. For example, *Hypocrea* is a genus usually found on decaying wood, bark and leaves. It only grew at room temperature and that suggests that it is not a ''natural'' part of the cave habitat; perhaps the river carried it to this site. However, *Paraconiothyrium*, a plant pathogen, was cultured at 10 C. Because there are no living plants within the cave, it is not clear what *Paraconiothyrium* does within the cave. It may simply have a physiological capacity to grow at 10 C, even if it can't complete its life cycle within the cave.

As tourist attractions, caves are an important part of the local economies in Kentucky and Tennessee, and have been for several centuries. Caving is also a popular pastime for many local residents and their families. Our results do not suggest a need for caves to be closed to

human access. The lowest and highest diversity sites were often within the same cave system, for example Lost Creek Cave, suggesting that the impacts of human disturbance are highly localized. It seems that if heavy human traffic is present in some parts of a cave system, diversity will persist in other parts of the cave. If necessary, some parts of a single cave system could be closed to humans, while others remain open. The greatest damage to cave systems is probably done by the reckless and careless cavers who leave waste, and damage cave formations. Rather than complete closures, sensible management with controlled access that limits litter and damage in and around caves may be the best conservation policy.

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