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Fungi as Architects of the Rimstone Dams in Huanglong, NSD, Sichuan, China

Jie Xie¹ · Gary Strobel² · Wei-Fang Xu¹ · Jie Chen¹ · Hui-Shuang Ren¹ · De-Jun An³ · Brad Geary⁴

Abstract The Huanglong park area of the Sichuan Province of China is a unique scenic area of the world. It is known for its thousands of aquamarine-colored pools that are formed behind naturally formed rimstone dams of travertine (calcite) along a cold water stream. The travertine, based on its crystalline structural analysis, is of biological origin. This makes sense since the temperature of the waters of Huanglong varies from 5 to 7 °C and thus geochemical crystallization does not occur as it does in other locations around the world possessing thermal pools whose structures are primarily formed through cooling processes. Fungi and bacteria were discovered associated with both leaves associated with the calcite dams as well as in the older parts of well-established dams. Several species of *Phytium*, a phycomycete and an endophyte, accounted for over 45 % of all of the fungi successfully isolated from the well-established dam samples and at least 85 % in the floating leaf samples. *Saprolegnia* spp. (Phycomycetes) along with *Phoma* spp. (Ascomycetes) were noted along with *Mortierella* sp. as other dam-associated fungi. The fungal hyphae observed on dead leaf material as well as in the calcite dams directly served as nucleation points for the formation of

crystalline CaCO₃. Eventually, these crystals grow large enough to fuse to make calcite plates which form the main structural feature of all of the travertine dams in this area. Interestingly, each of the individual crystals associated with the dams has an associated hole in its core where a fungal hypha used to reside as observed by scanning electron microscopy. While diatoms were present in the analysis, they too seem to contribute to the structure of the dams but in a minor way. The only bacteria isolated from the older dam of this aquatic environment were *Pseudomonas* spp. and their role in dam formation is uncertain. Huanglong is a unique and beautiful place, and the water features present in this area can definitely be attributed to those fungal architects that encourage calcite crystal formation.

Keywords Aquatic fungi · Travertine · Calcium carbonate · Crystals · Pools · China

Introduction

Only a few areas in the world are known to possess naturally formed travertine (calcite) rimstone dams holding back beautiful blue pools of cold water (5–7 °C). This is in sharp contrast to several hot water travertine water formations in such places as Yellowstone National Park, USA; Badab-e Surt, Iran; and Pamukkale, Turkey. The cold water travertine areas are in Band-e Amir, Afghanistan, and certainly the most impressive is the Huanglong Natural Scenic District of Sichuan, China. The latter area is so impressive that it was declared a World Heritage Site by United Nations Educational, Scientific and Cultural Organization (UNESCO) in 1992. It is famous for the colorful travertine landscape, diverse biological ecosystems, and an extensive system of beautifully colored pools created by travertine dams varying in height from several

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centimeters up to 5 m. The site of travertine deposition in Huanglong Valley is located in Songpan County of Northwestern Sichuan Province (32° 45' N, 103° 50' E). It is also home to many endangered animal species including the giant panda and the Sichuan golden snub-nosed monkey [1].

The water in the discharge area of Huanglong is extremely high in calcium carbonate concentrations and this is apparently due to the dissolution of the deep basement limestone rock through which the water in this area originates and flows [2]. This is a critical observation since this calcium carbonate serves as the source material for the construction of the calcite (travertine) rimstone dams of Huanglong. However, this condition alone is not enough to account for the dramatic appearance of nearly perpendicular walls made of impenetrable layers of calcite making up the structure of each dam at Huanglong. Overall, it appears that a combination of geochemical, mineralogical, and microbiological factors contribute to the formation of calcium carbonate crystals in this natural cold aquatic environment (5–7 °C). It is well understood, however, that there are significant differences between biogenic calcite and geologically formed calcite. Thus, if biogenic factors have taken part in the processes of travertine formation this can be ascertained by examining the crystal structure of the calcite. It turns out that the calcite in Huanglong dams is a typical anisotropic lattice distortion, which means that biogenic factors have contributed to its formation [3]. However, there is little information on how any life form may have contributed to travertine dam formation of Huanglong [4]. Recently, the influence of diatoms on travertine dam formation at the Huanglong Natural Scenic District has been studied but no conclusive information on the direct involvement of diatoms in the direct formation of the calcite structure of the dams has been provided [3]. However, it is possible that other microorganisms might be in the formation of Huanglong travertine dams. These include aquatic microbes such as filamentous fungi and bacteria that have not been explored or even considered as playing a role in dam formation. Yet it is known that microbes, especially filamentous fungi and some bacteria, can serve as nucleation sites for calcite crystal formation *in vitro* [5–9].

Several interesting observations were made in September of 2015, by the first two authors of this report. As leaves were falling from the deciduous trees and floating on the surface of the cold flowing waters in the Huanglong area, they could each be observed supporting mats of stringy filamentous fungi. These leaves then were literally stopped in their movement as they hit each travertine dam wall and became immobile. The idea that these leaves and their respective microflora were contributing to dam formation was then hypothesized. Samples of leaves, dam structures, and other features of the area were taken and analyzed by a myriad of methods. Overall, it became obvious that the travertine rimstone dams of Huanglong

were formed, in part, by the fungal hyphae associated with the dead leaves floating on the cold calcite saturated waters of this area. The numerous hyphae each serve as a nucleation point sources for calcite crystal formation which ultimately results in the rapid growth of the crystals and the eventual fusing of structures to form larger calcite plates. Thus, this report shows which fungi, bacteria, and other microbial forms were recorded from the waters and features of Huanglong and indicates how these organisms contribute to one of the most beautiful and unique places on this earth.

Materials and Methods

Sampling

Two different types of travertine sample were collected from Zhengyancai pool of Huanglong Natural Scenic District (32° 44' 26" N, 103° 49' 51" E). A soft sample (HL1) was collected from the edge of the rimstone pool. It was yellow to brown and appeared as a little Chinese dragon in the pool. The hard sample (HL2) was collected by scraping the crusty surface of a well-established calcite dam. Each sample was about 10 g. Yet another sample consisted of leaves supporting massive fungal growth caught on the dam face in the same area were collected (HL3). The samples were immediately transported back to the Southwest University in a sterile plastic sampling bottle and stored at 4 °C until processed and examined.

Morphological Observations, Scanning Electron Microscopy, and Edax

In order to acquire detailed information about the morphological features of the Huanglong travertine, light microscopic observations were performed on the soft sample (HL1) and hard sample (HL2). The samples were transferred and mixed in the sterile water on the slide and then examined with an OLYMPUS FV1200 laser scanning microscope, and the samples were photographed. Samples were also prepared for scanning electron microscopy (SEM). The SEM samples were slowly dehydrated in ethanol and then critical point dried, coated with gold palladium, and examined with an FEI Helios Nanolab 600 dual beam instrument. The SEM was also used to do elemental analysis of the individual crystals associated with fungal hyphae using an Edax energy dispersive X-ray analyzer on the SEM [10].

Microbial Isolation and Storage

In this study, efforts were made to investigate the microbial biodiversity associated with the dams forming the aquamarine-colored pools of the Huanglong Valley using

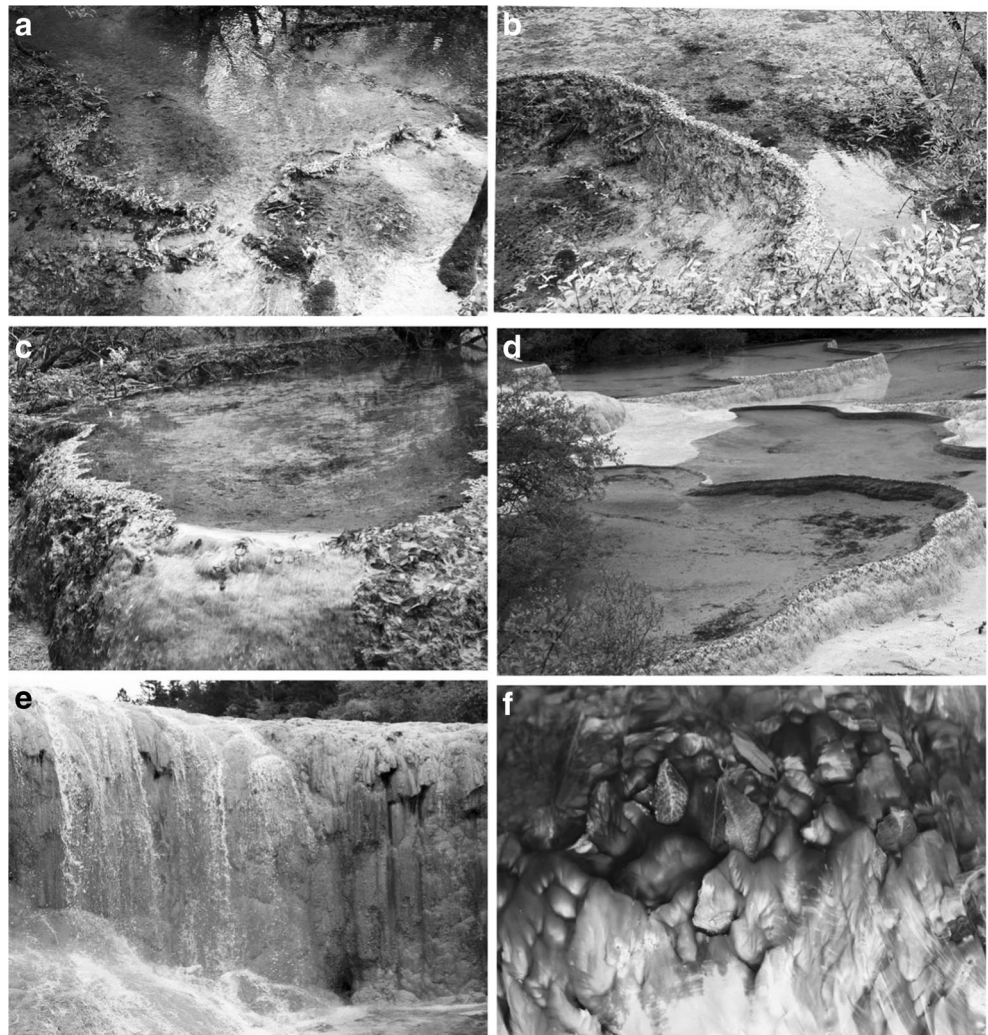
a culture-dependent method rather than a strictly exclusive metagenomics approach. The following procedures were used to acquire the microbes associated with the soft sample (HL1), hard sample (HL2), and leaf (HL3) which were collected from the travertine pools. Firstly, the sample was cut into small fragments in sterilized petri dish with surgical blades and then aseptically transferred to the plate surface of oatmeal agar (OA), potato dextrose agar (PDA), water agar (WA), and nutrient agar (NA). After several days of incubation at 22 °C, hyphal tips of the developing fungi were aseptically removed and placed on the plate of PDA. For the bacteria, a small amount of sample was obtained from the edge of the lawn and then streaked onto the PDA medium. Thus, pure cultures were obtained. The isolated fungi were preserved by growing a pure fungal culture on sterile barley seeds and storing the seeds at −70 °C. Fresh bacterial liquid cultures were maintained in 15 % glycerol. All individual cultures were appropriately labeled and stored at the author's lab at the State Key Laboratory of Silkworm Genome Biology,

College of Biotechnology, Southwest University, Chongqing 400715, People's Republic of China.

Microbial Biodiversity Analysis

The microbial samples were individually analyzed by amplified the ITS sequences of fungal DNA and 16S rDNA sequences of bacterial DNA. Genomic DNA was extracted from the colony grown on PDA medium after about 1 week. DNA templates were prepared with the Prepman Ultra Sample Preparation Reagent (Applied Biosystems, USA), according to the manufacturer's guidelines. The phylogenetic analysis of fungi was carried out by acquisition of ITS-5.8S ribosomal gene sequence using the polymerase chain reaction (PCR) with universal ITS primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3')/ITS4 (5'-TCCTCCGCTTATTGATATGC-3') or ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3')/ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [11, 12]. The PCR conditions were set as follows: initial denaturing at 94 °C for

Fig. 1 The cold water pools of Huanglong in Sichuan Province of China. **a** The initiation of pool formation by the accumulation of leaves and other organic debris deposited in an arching manner in the stream bed. **b** A nearly perpendicular calcite wall formed by calcite deposition to form a dam. **c** A more mature calcite dam that is over a meter in height. **d** An intricate system of calcite dams in the midst of the stream bed. **e** A giant wall calcite dam (est. 3 m) over which a water falls has developed. **f** A biological mat located at the top of a calcite dam containing filamentous fungal filaments, bacteria, and algal forms (mainly diatoms). Leaf material supports microbial growth



5 min, followed by 30 cycles of denaturing at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 45 s, and a final extension at 72 °C for 10 min. The phylogenetic analyses of bacteria were carried out by acquisition of 16S rDNA ribosomal gene sequence using PCR with the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1500R (5'-GGTTACCTTGTTACGACTT-3') [13]. The PCR conditions were as follows: initial denaturing at 95 °C for 4 min, followed by 30 cycles of denaturing at 94 °C for 30 s, annealing at 50 °C for 45 s, extension at 72 °C for 60 s, and a final extension at 72 °C for 8 min. PCR products were purified and sequenced by Sangong Biotech (Shanghai) Co., Ltd. The amplified sequences were carried on the phylogenetic analyses on NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). According to the blast results, the microbial population distribution was assayed at the genus level with designations to the genus level of

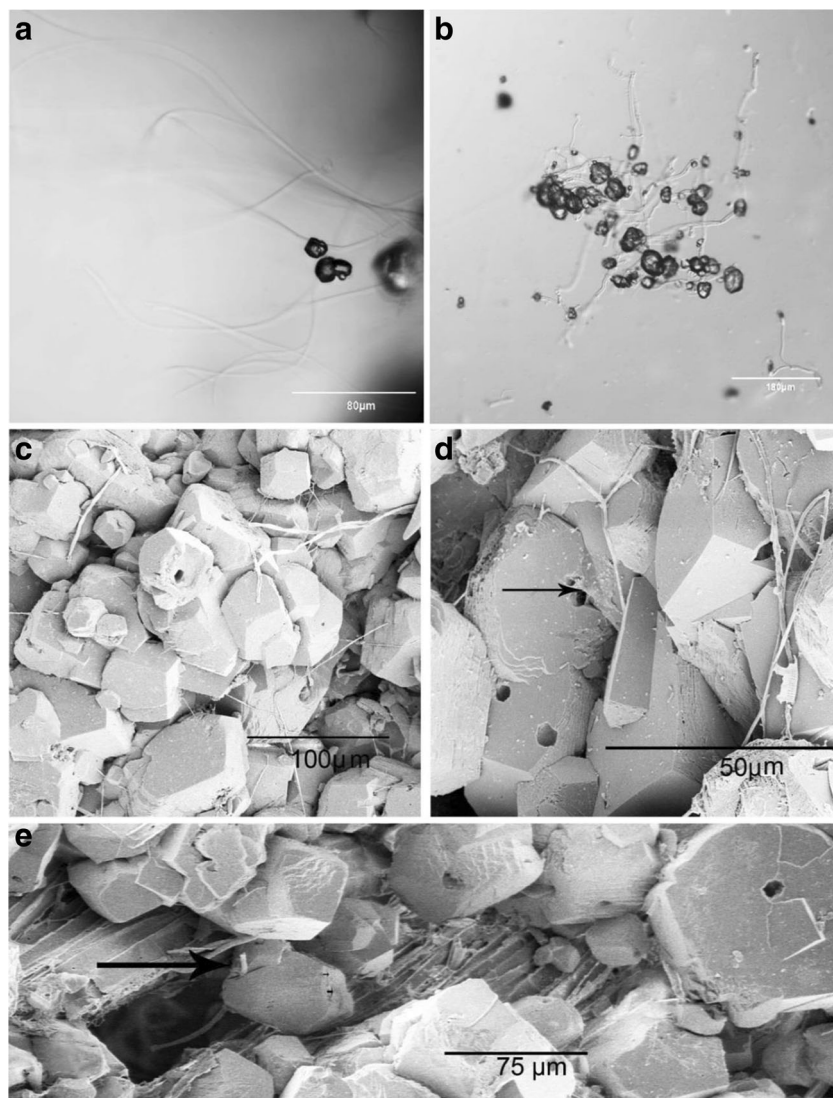
microbes having at least a 95 % or greater match to the GenBank database. Furthermore, all sequences were submitted to the GenBank. Accession numbers of the strains were from KX378872 to KX378962.

Results

The Huanglong Area

The Huanglong Gou—Valley of the Yellow Dragon—is an outstanding karst landscape within the southern part of the Min Shan range which runs from the eastern Qinghai-Tibetan plateau down to the Sichuan basin in SW China. The Huanglong section of the reserve covers the catchment of 22 tributaries of the upper Fujiang River which has its source in the Snow Mountain Ridge. The Huanglong Valley is one of these tributaries. The slopes above the valleys are

Fig. 2 Fungi serving as to nucleate calcite crystal formation from samples taken in Huanglong, China. **a** Hard calcite wall surface sample (sample HL-2) was taken for observation and some hyphae were associated with individual crystals. **b** Surface samples taken from the soft calcite wall surface (sample HL-1), showing many fungal hyphae each with a corresponding calcite crystal. **c** The wall is composed of a myriad of fused calcite crystals each in the range of 10–100 µm. **c, d** Careful examination of each crystal reveals the presence of a small hole 3–4 µm in diameter which is the exact diameter of a fungal hyphae (*arrow*). **e** A broken fungal hypha is seen emerging from a hole in a calcite crystal (*arrow*)



forested and steep. Above the tree line, the site is surrounded by mountains; their strata tilted into jagged peaks, with several glaciers. The pyramidal summit of Xuebaoding (Snow Treasure Peak) is permanently snow-covered and carries the easternmost glacier in China.

The 3.6-km-long Huanglong Valley consists of clusters of over 3300 brightly colored travertine pools varying in color depending upon the temperature of the water in each pool to a certain extent (Fig. 1). For instance, some of the warmer more stagnant pools support green algal growth and are varying from yellowish to green while the majority of the pools are cold and are aquamarine varying to deep blue in color (see cover of this issue of ME) (Fig. 1). In the area are terraces, travertine shoals, water rapids, pools, and waterfalls. There are many caves in the valley. Each of these features is basically related to a geological source of calcite which originates in the tilted rock strata of the area. It is comprised of largely carbonate Paleozoic deposits over 4000 m thick and Mesozoic deposits of at least 1000 m thick with a variety of sedimentary rocks in a cataclastic sedimentation formation [14]. The water flowing over and through this area contains saturating amounts of CaCO_3 [14].

Central to a study of the microbiology of the Huanglong Valley and its travertine pools is an understanding of the botany of the area. Huanglong lies close to the intersection of four floristic regions: Eastern Asia, Himalaya, and the subtropical and tropical zones of the Palaearctic. It is situated at the transition between the eastern damp forest and the zone of mountainous

coniferous woods with meadow grassland and shrubs of Qinghai-Tibetan Plateau. More than 1500 higher plants are recorded for the site. About 65.8 % of the site is forest covered, with much of the remainder being above the tree line [15, 16]. Thus, the Huanglong area hosts a number of species of *Acer*, mixed with *Alnus nepalensis*, *Juglans sigillata*, and *Betula alnoides*. There are three major spruce species, two fir species as well as a larch species—*Larix potaninii*. Most interestingly, the area is home to the most diverse species of *Rhododendron* in the world, i.e., 16 species which is critical to this study since the leaves of this plant possess a thick impervious cuticle, readily float on water and serve as a food reservoir for fungi and bacteria. Besides these species, the area also is home to a myriad of orchids, several threatened species of spruce, larch, magnolia, and cedar. Oddly, the area also supports two bamboo species (*Fargesia denudata* and *F. scabrida*) that are important food plants for indigenous wild populations of the giant panda [15].

Travertine Pools and Their Formation

The saga begins with the fall season and the shedding of leaves from deciduous trees of the types mentioned above. As the wind blows, the leaves fall from the nearby forests of Huanglong to the cold stream below. The leaves float on the water surface and become lodged on obstacles in their path in the form of branches, other leaves, or quiet water that lead to a their deposit—usually in an arching manner as a function of water flow

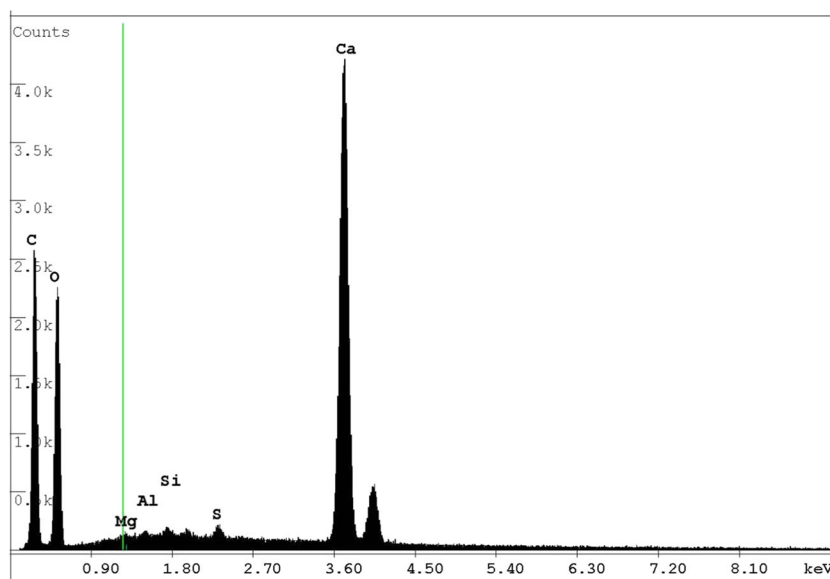


Fig. 3 X-ray data set collected by the SEM on an Oxford Instrument energy dispersive X-ray microanalysis instrument on sample HL-1 (biological mat-like material). The beam was focused on a calcite crystal that had formed on a fungal hypha. The data set shows the major elemental peaks with net integrations (counts) of carbon at 131.5,

oxygen at 129.2, and calcium at 382.3. The keV is on the Y-axis. Other elements present are indicated on the graph. In another experiment done on HL-2 (hard surface of a calcite dam sample), the beam also focused on a calcite crystal and yielded virtually the same result

Table 1 Polygenetic analysis of Huanglong microbial isolates based on rDNA partial sequences

Taxon	Loci used	Strain number	Accession number	Source	Isolation area
<i>Botryotinia</i> sp.	ITS	HL 1-1	KX378872	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Botryotinia</i> sp.	ITS	HL 1-2	KX378873	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Botrytis</i> sp.	ITS	HL 1-3	KX378874	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Botrytis</i> sp.	ITS	HL 1-4	KX378875	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Mucor</i> sp.	ITS	HL 1-5	KX378876	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Phoma</i> sp.	ITS	HL 1-6	KX378877	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Pythium</i> sp.	ITS	HL 1-7	KX378878	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Pythium</i> sp.	ITS	HL 1-8	KX378879	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Pythium</i> sp.	ITS	HL 1-9	KX378880	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Saprolegnia</i> sp.	ITS	HL 1-10	KX378881	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Saprolegnia</i> sp.	ITS	HL 1-11	KX378882	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Saprolegnia</i> sp.	ITS	HL 1-12	KX378883	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Saprolegnia</i> sp.	ITS	HL 1-13	KX378884	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Saprolegnia</i> sp.	ITS	HL 1-14	KX378885	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Saprolegnia</i> sp.	ITS	HL 1-15	KX378886	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Saprolegniaceae</i> sp.	ITS	HL 1-16	KX378887	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Saprolegniaceae</i> sp.	ITS	HL 1-17	KX378888	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Mortierellales</i> sp.	ITS	HL 1-18	KX378889	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Arthrinium</i> sp.	ITS	HL 2-1	KX378907	Hard travertine sample of Huanglong pool	Sichuan, China
<i>Bjerkandera</i> sp.	ITS	HL 2-2	KX378908	Hard travertine sample of Huanglong pool	Sichuan, China
<i>Cladosporium</i> sp.	ITS	HL 2-3	KX378909	Hard travertine sample of Huanglong pool	Sichuan, China
<i>Phoma</i> sp.	ITS	HL 2-4	KX378910	Hard travertine sample of Huanglong pool	Sichuan, China
<i>Pythium</i> sp.	ITS	HL 2-5	KX378911	Hard travertine sample of Huanglong pool	Sichuan, China
<i>Pythium</i> sp.	ITS	HL 2-6	KX378912	Hard travertine sample of Huanglong pool	Sichuan, China
<i>Pythium</i> sp.	ITS	HL 2-7	KX378913	Hard travertine sample of Huanglong pool	Sichuan, China
<i>Pythium</i> sp.	ITS	HL 2-8	KX378914	Hard travertine sample of Huanglong pool	Sichuan, China
<i>Pythium</i> sp.	ITS	HL 2-9	KX378915	Hard travertine sample of Huanglong pool	Sichuan, China
<i>Saprolegnia</i> sp.	ITS	HL 2-10	KX378916	Hard travertine sample of Huanglong pool	Sichuan, China
<i>Psilogonium</i> sp.	ITS	HL 2-11	KX378917	Hard travertine sample of Huanglong pool	Sichuan, China
<i>Pythium</i> sp.	ITS	HL 3-1	KX378939	Leaf of Huanglong travertine pool	Sichuan, China
<i>Pythium</i> sp.	IT	HL 3-2	KX378940	Leaf of Huanglong travertine pool	Sichuan, China
<i>Pythium</i> sp.	ITS	HL 3-3	KX378941	Leaf of Huanglong travertine pool	Sichuan, China
<i>Pythium</i> sp.	ITS	HL 3-4	KX378942	Leaf of Huanglong travertine pool	Sichuan, China
<i>Pythium</i> sp.	ITS	HL 3-5	KX378943	Leaf of Huanglong travertine pool	Sichuan, China
<i>Phytophthium</i> sp.	ITS	HL 3-6	KX378944	Leaf of Huanglong travertine pool	Sichuan, China
<i>Pythium</i> sp.	ITS	HL 3-7	KX378945	Leaf of Huanglong travertine pool	Sichuan, China
<i>Aeromonas</i> sp.	16S rDNA	HL 1-19	KX378890	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Bacillus</i> sp.	16S rDNA	HL 1-20	KX378891	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Erwinia</i> sp.	16S rDNA	HL 1-21	KX378892	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Hafnia</i> sp.	16S rDNA	HL 1-22	KX378893	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 1-23	KX378894	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 1-24	KX378895	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 1-25	KX378896	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 1-26	KX378897	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 1-27	KX378898	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 1-28	KX378899	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 1-29	KX378900	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 1-30	KX378901	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 1-31	KX378902	Soft travertine sample of Huanglong pool	Sichuan, China

Table 1 (continued)

Taxon	Loci used	Strain number	Accession number	Source	Isolation area
<i>Pseudomonas</i> sp.	16S rDNA	HL 1-32	KX378903	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Rahnella</i> sp.	16S rDNA	HL 1-33	KX378904	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Rahnella</i> sp.	16S rDNA	HL 1-34	KX378905	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Ewingella</i> sp.	16S rDNA	HL 1-35	KX378906	Soft travertine sample of Huanglong pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 2-12	KX378918	Hard travertine sample of Huanglong pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 2-13	KX378919	Hard travertine sample of Huanglong pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 2-14	KX378920	Hard travertine sample of Huanglong pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 2-15	KX378921	Hard travertine sample of Huanglong pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 2-16	KX378922	Hard travertine sample of Huanglong pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 2-17	KX378923	Hard travertine sample of Huanglong pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 2-18	KX378924	Hard travertine sample of Huanglong pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 2-19	KX378925	Hard travertine sample of Huanglong pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 2-20	KX378926	Hard travertine sample of Huanglong pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 2-21	KX378927	Hard travertine sample of Huanglong pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 2-22	KX378928	Hard travertine sample of Huanglong pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 2-23	KX378929	Hard travertine sample of Huanglong pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 2-24	KX378930	Hard travertine sample of Huanglong pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 2-25	KX378931	Hard travertine sample of Huanglong pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 2-26	KX378932	Hard travertine sample of Huanglong pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 2-27	KX378933	Hard travertine sample of Huanglong pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 2-28	KX378934	Hard travertine sample of Huanglong pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 2-29	KX378935	Hard travertine sample of Huanglong pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 2-30	KX378936	Hard travertine sample of Huanglong pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 2-31	KX378937	Hard travertine sample of Huanglong pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 2-32	KX378938	Hard travertine sample of Huanglong pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 3-8	KX378946	Leaf sample of Huanglong travertine pool	Sichuan, China
<i>Hafnia</i> sp.	16S rDNA	HL 3-9	KX378947	Leaf sample of Huanglong travertine pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 3-10	KX378948	Leaf sample of Huanglong travertine pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 3-11	KX378949	Leaf sample of Huanglong travertine pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 3-12	KX378950	Leaf sample of Huanglong travertine pool	Sichuan, China
<i>Hafnia</i> sp.	16S rDNA	HL 3-13	KX378951	Leaf sample of Huanglong travertine pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 3-14	KX378952	Leaf sample of Huanglong travertine pool	Sichuan, China
<i>Hafnia</i> sp.	16S rDNA	HL 3-15	KX378953	Leaf sample of Huanglong travertine pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 3-16	KX378954	Leaf sample of Huanglong travertine pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 3-17	KX378955	Leaf sample of Huanglong travertine pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 3-18	KX378956	Leaf sample of Huanglong travertine pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 3-19	KX378957	Leaf sample of Huanglong travertine pool	Sichuan, China
<i>Ewingella</i> sp.	16S rDNA	HL 3-20	KX378958	Leaf sample of Huanglong travertine pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 3-21	KX378959	Leaf sample of Huanglong travertine pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 3-22	KX378960	Leaf sample of Huanglong travertine pool	Sichuan, China
<i>Pseudomonas</i> sp.	16S rDNA	HL 3-23	KX378961	Leaf sample of Huanglong travertine pool	Sichuan, China
<i>Ewingella</i> sp.	16S rDNA	HL 3-24	KX378962	Leaf sample of Huanglong travertine pool	Sichuan, China

Placement at the genus level is based on at least 95 % identity of the listed genera to that in GenBank

(Fig. 1a–c). The calcite dams eventually become larger resulting in numerous deep pools or even water falls (Fig. 1d, e). The dams in this area are formed mainly by biological processes associated with the degradation

of the leaf material and the formation of fungal hyphae. It is surmised that the leaves carrying endophytic fungi or becoming infested with other aquatic fungi begin to accumulate on the upper dam surface forming a

biomatrix or biomat with these leaves being the base material (Fig. 1f). Eventually, the fungal hyphae serve as sites for the nucleation of calcite crystals (Fig. 2a, b). The calcite crystals merge into larger plates and form much more substantial calcite structural walls that act as dams for the calcite pools (Fig. 2c–e).

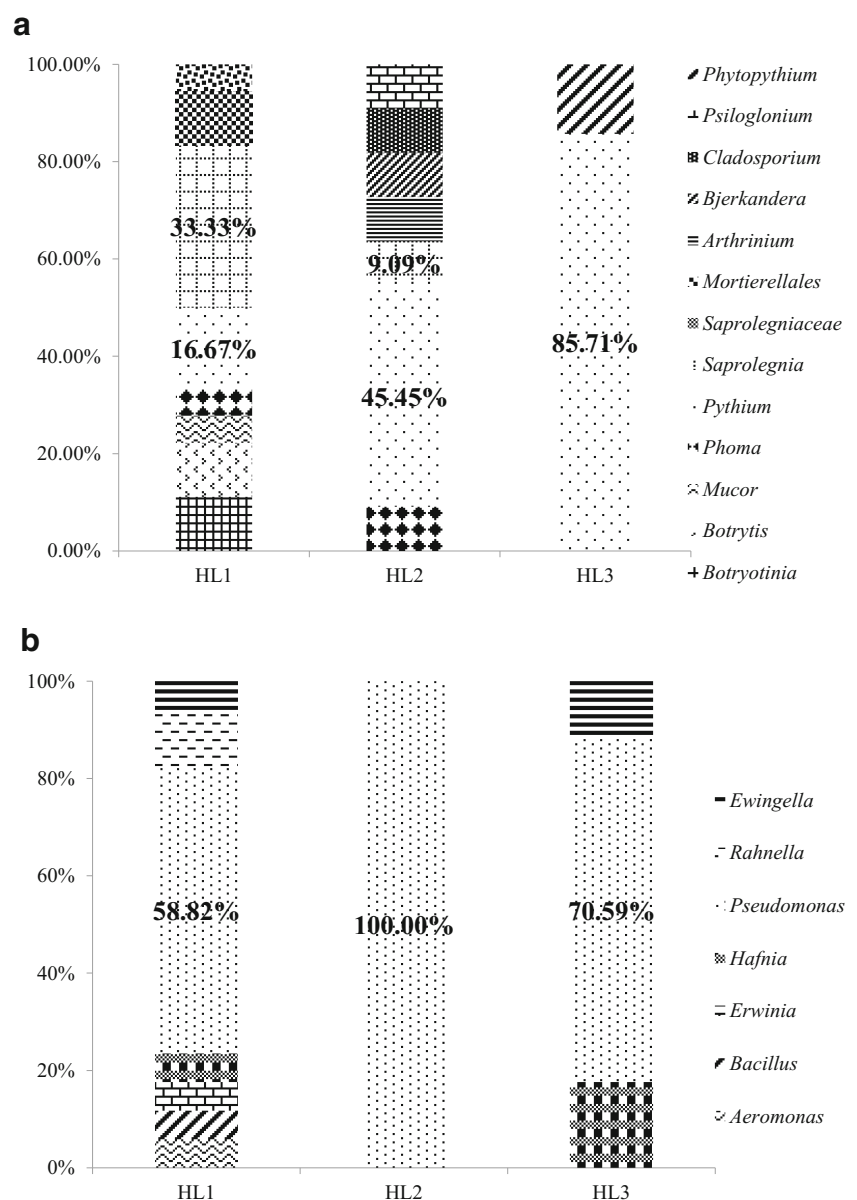
Morphological Features and Analysis of the Fungal Associated Crystals

Using Edax energy dispersive X-ray analyzer on the SEM, it was possible to do elemental analysis of the crystals forming on fungal hyphae taken directly from the travertine dam sites, i.e., samples HL1 and HL2. The data showed the major presence of calcium, oxygen, and carbon with trace amounts of

silicon, aluminum, magnesium, and sulfur (Fig. 3). The data are consistent with the crystals being composed mainly of calcite or calcium carbonate. The X-ray dispersive analyses of both samples were nearly identical (Fig. 3).

The crystals associated with samples HL1 and HL2 were examined by SEM they have the typical appearance of calcite crystals, and in this case, they are anisotropic when viewed with plane polarized light and this suggests that their origin is biological. Also, of great interest is the appearance of one or more discrete holes (and traces thereof) in virtually every calcite crystal that was examined by SEM (Fig. 2c, d). The diameter of the holes varied from 3 to 4 μm which approximates the diameter of the hyphal strands commonly seen associated with the holes in light and SEM observations (Fig. 2a–d). In the SEM, it is worthy to note that some compression of the

Fig. 4 Analysis of the microbial communities of Huanglong travertine samples at the genus level. HL1 is the soft sample, HL2 is the hard sample, and HL3 is the leaf. **a** Fungal analysis. **b** Bacterial analysis



fungus hyphae did occur as a result of the high vacuum in the chamber but holes with associated hyphae have been noted as exemplified by a fungal hypha, as observed by SEM, to be associated with a hole in a calcite crystal (Fig. 2e).

Fungal Diversity of Huanglong Travertine Pool and the Fungal Architects

There were at least 18 individual fungal isolates made from sample HL1 and were distributed among eight different fungal genera with a domination of *Saprolegnia* spp. (33.33 %) and *Pythium* spp. (16.67 %) which are both phycomycetes (water molds) (Table 1, Fig. 4a). In the case of HL2, there were 11 fungal isolates and they were distributed among seven genera with *Pythium* spp. being dominant and accounting for 45.45 % of the isolates. The remainder of the isolates was represented by *Saprolegnia* spp. and a variety of others including *Phoma* sp. and *Mortierella* sp. (Table 1, Fig. 4a). Furthermore, seven isolates were obtained from sample HL 3 and they were dominated

by *Pythium* spp. (85.71 %) (Table 1, Fig. 4a). The cultural characteristics of the major fungi isolated from the travertine samples HL1, HL2, and HL3 are distinct and characteristic for these genera. *Pythium* spp. having hyaline hyphae and producing a rapid growth on PDA (covering a 90-mm petri dish within 9 days) resulting in a whitish-erumpent mycelium (Fig. 5a–c) while *Saprolegnia* spp. resembles *Pythium* sp. but with a plain, non-erumpent surface (Fig. 5d, e). *Phoma* sp. on the other hand makes a brownish-black mycelium having a whitish halo and commonly associated with the mycelium are pycnidia (fruiting bodies) (Fig. 5f). Confirmation of the identity of each fungal isolate was further made by comparing its ITS sequence data with that in GenBank. In each case, at least a 95 % match was found and all sequence information was deposited in GenBank.

Bacteria Associated with the Pool Samples

In sample HL1, there were seven genera of bacteria with *Pseudomonas* being the dominant one (58.82 %) from 17 isolates (Table 1, Fig. 4b). However, in HL2, the only bacterium isolated was the genus *Pseudomonas* (Table 1, Fig. 4b). Thus, it appeared as if the maturation of the calcite crystals, as in HL2, there was a marked reduction in the bacterial diversity of the sample which is in contrast to a sample having rapid crystal growth as per HL1. Furthermore, of the 17 bacteria isolates that were isolated from the leaves associated with the travertine pool, the genus *Pseudomonas* was still the one that dominated (70.59 %) (Table 1, Fig. 4b).

Conclusions

While ITS sequence technology and cultural identity work provided information on the various fungal and bacterial genera associated with the calcite dams and floating leaves, these data were not substantial enough to provide information on species identity. However, it appears that the phycomycetous fungi, i.e., *Pythium* and *Saprolegnia* were the dominant fungi recovered from each sample type (Fig. 4). And it seems that multiple species of these fungi are present but more work will be needed to substantiate this observation. Fungi, of this type, are commonly considered as water molds and have a preference for substrates that are aquatically based [17]. Other fungal species were recovered from the three sample types including *Phoma* sp., *Mucor* sp., *Botrytis* sp., and others.

It also appears that other microbial forms are associated with the rimstone calcite dams and these include diatoms (observed in some SEMs) and bacteria. Bacteria were found in each of the three samples with uneven distribution of genera. Interestingly, the most frequently isolated bacterial genus was *Pseudomonas*. It is not understood if the bacteria make a significant impact upon the development of the structure of the calcite dams. However, it is the case that each of the

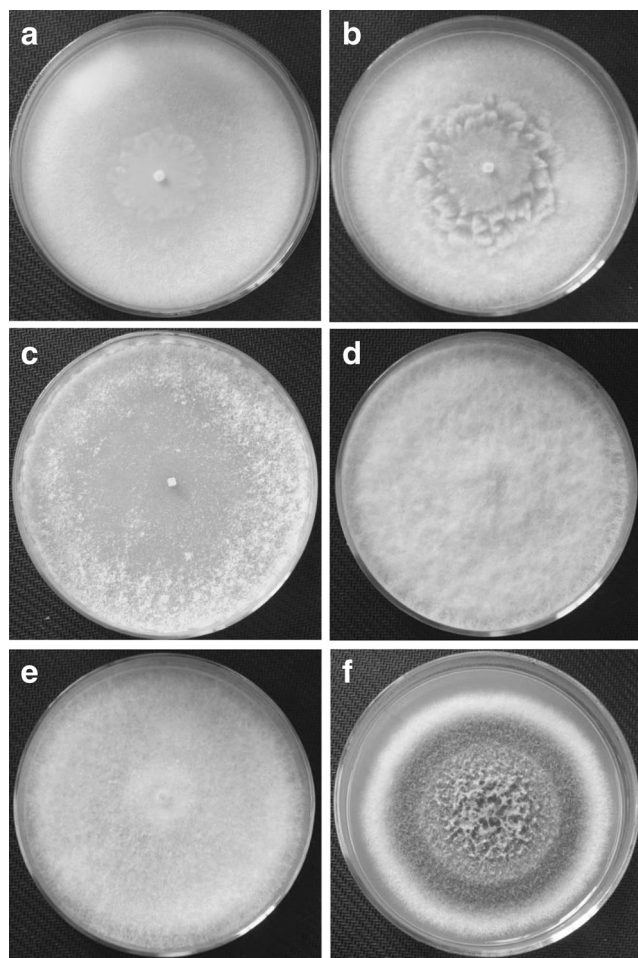


Fig. 5 Individual fungal cultures grown for 9 days on PDA. They were each isolated from Huanglong samples HL1, HL2, and HL3. They are each represented by the strain acquisition number as per Table 1. **a** *Pythium* spp. 1–7. **b** *Pythium* spp. 2–5. **c** *Pythium* spp. 3–7. **d** *Saprolegnia* spp. 1–15. **e** *Saprolegnia* spp. 2–10. **f** *Phoma* spp. 1–6

pseudomonad has significant antifungal activity towards the fungal species associated with the calcite dams as well as other plant-associated fungi (Xie unpublished). This antifungal activity may in some way help regulate fungal development so as to have appropriate fungal growth as a function of available nutrient supply.

The main thesis of the paper for the involvement of fungi as architects of the dams at Huanglong seems to have substantial support via the data provided in this report. That is, fungal hyphae are seen on virtually every leaf floating in the valley stream. Examination of the hyphae on these leaves reveals the presence of crystals forming on these hyphae (Fig. 2). Eventually, the crystals fuse into the matrix of the dam and individual holes exist in each crystal indicating the location of the individual fungal hypha. The fusion of all of the crystals results in massive calcite dams at Huangong (Fig. 1). The fact that the crystals are calcite is strongly supported by the SEM-Edax study showing the presence of the elements making up CaCO_3 (Fig. 3). The mechanism of calcite crystal formation is likely regulated by the presence of calcium-binding proteins on the hyphal surfaces [18, 19]. While microbial induced calcite-crystal formation has been noted in vitro, this appears to be the first and most noteworthy example of microbial induced calcite dam formation in nature.

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