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Article

샷건 시퀀싱을 활용한 한반도 남부 석회동굴에서의 미생물 다양성 연구: 건습환경과 시료 종류에 따른 차이

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Exploring microbial diversity in South Korean caves through shotgun sequencing: contrasting dry and wet environments, swabbing versus sediment sampling

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요약: 동굴은 흔히 외부환경의 영향으로부터 격리되어 다양하면서도 독특한 생태계와 박테리아 군집을 이룬다. 박테리아 군체 사이의 다양성은 동굴의 지리적 위치에 따라 크게 좌우될 수 있으나 동굴 입구로부터의 깊이, 빛, 습도, 영양공급 정도 및 기저 환경 등 동굴 내부에서의 세밀한 환경 조건에도 영향을 받게 된다. 이에 따라 동굴 미생물은 특수한 대사 경로를 발달시킴으로써 극한환경 조건에 적응해왔다. 이번 연구에서는 동굴환경 조건에 대한 잠재적 지시자로서의 미생물 분류군을 확인하기 위해 남한 지역의 5개 동굴로부터 채취한 시료들의 군유전체 염기서열을 분석하였다. 이를 위해 독립적 샷건 시퀀싱 플랫폼인 Illumina와 Oxford Nanopore technologies가 쇼트 및 롱 리드를 판독하는 데 적용되었다. 디옥시리보핵산 시료는 습윤, 건조, 계절적 습윤환경으로 각각 분류할 수 있는 동굴 내부 지점들로부터 암석표면 면봉긁기 방식과 퇴적물 채취 방식으로 확보하였다. 입구로부터의 깊이와 기저 환경(암석 또는 퇴적물) 조건을 중심으로 내부 미생물군 조성의 원인을 해석하였다. 연구 결과, 상대적으로 건조환경에 해당하는 바람굴 및 심복굴에서는 *Actinobacteria* 문에 속하는 종들이 우세하게 나타난 반면, 습윤환경으로 분류된 온달굴, 산지당굴, 서대굴에서는 *Proteobacteria* 문에 속하는 종들이 우세한 것으로 나타났다. 또한, 비교란 암흑대에서의 미생물 군집은 입구 인근 구역에 비해 *Gammaproteobacteria* 문이 우점하는 특징을 보인다.

주요어: 동굴 군유전체학, 남한, 샷건 시퀀싱

ABSTRACT: Caves host bacterial communities that represent diverse and unique ecosystems, often isolated from external influences. Diversity among bacterial colonies can vary greatly depending on the cave's geographical location but it is also linked to the environmental conditions inside the cave such as a depth gradient, light and humidity levels, nutrients availability and substrate conditions. Microbes in caves have adapted to live under extreme conditions and because of that have often developed unique metabolic pathways. In this present work, metagenomics sequence data from five South Korean caves are analyzed with the main objective of identifying microbial taxa as potential proxies for cave environmental conditions. Two independent shotgun sequencing platforms have been used targeting short and long reads through Illumina and Oxford Nanopore technologies respectively. The DNA samples come from swabs of rock surfaces or sediments inside these Korean caves that can be classified as wet, dry or seasonally wet. The internal depth gradient as well as different substrates (swabs and sediments) have also been considered for the interpretation of the composition of the internal microbiota. Dry environments like those from Baram and Simbok Caves have a high abundance of species belonging to the phylum *Actinobacteria*, compared to wetter caves like

Ondal, Sanjidang and Seodae where *Proteobacteria* are instead more abundant. In dark and undisturbed sectors of the caves, the microbial colonies are dominated by *Gammaproteobacteria* compared to areas near the entrance.

Key words: cave metagenomics, South Korea, shotgun sequencing

1. Introduction

Caves are considered oligotrophic environments due to their limited nutrient and energy sources to support life. Nevertheless bacteria, which constitute the major portion of cave biodiversity, can survive and thrive in this type of environment by modifying their metabolic strategies and microbial communities are also key organisms in the subterranean food web and in decompositions processes (Graening and Brown, 2003). In karstic environments, bacteria are affected by the geology of the surroundings and by the presence of specific geochemical elements and can utilize inorganic compounds for their energetic sustainment (Barton, 2006; De Mandal *et al*., 2017). Trophic levels and light intensity define the different zonal gradients within a cave and influence the distribution and abundance of the microbiota on the different substrates (Ghosh *et al*., 2017).

The cave's interior can be categorized into four primary zones based on light penetration: 1) the entrance zone; 2) the twilight zone, characterized by diminishing light until; 3) the transition zone, which remains dark but still experiences variations in temperature and moisture; and 4) the deep zone, where darkness is absolute, humidity is high, and the temperature remains constant (Ghosh *et al*., 2017).

Since microorganisms need to survive in low nutrient environments, they can use minerals and inorganic matter for catalyzing reactions (Dong *et al*., 2020) and are also involved in many in-cave mineralization processes. For example, calcifying bacteria promote carbonate precipitation (Cacchio *et al*., 2003), possibly in the form of speleothems (Cacchio *et al*., 2004), and/or catalyze the development of crystalline fabrics in stalagmites via metabolic processes or, intrinsically, via water evaporation and CO_2 degassing (Franchi and Frisia, 2020).

On the other hands, heterotrophic microorganisms, including certain bacterial groups and fungi, thrive in caves on the abundant organic matter of substrates like guano, utilizing organic carbon as a source of energy (Dimkić *et al*., 2021). Since cave substrates are abundant in inorganic molecules such as iron (Parker *et al*., 2022), manganese (Carmichael *et al*., 2013), ammonia (Martin-Pozas *et al*., 2023), methane (Webster *et al*., 2022) and sulphur (Jones *et al*., 2016), chemolithoautotrophic organisms are particularly abundant in caves.

To overcome growth limiting factors, microorganisms establish sophisticated, mutualistic networks. This strategy enables a greater number of organisms to survive and grow in the extreme conditions of caves. On cave walls and speleothems, multispecies biofilms consisting of algae/cyanobacteria, bacteria, or fungi, promoting nutrient circulation enabling diverse organisms to thrive in this harsh environment. Conversely, some bacteria favour competition over cooperation by producing secondary metabolites that suppress the growth of nearby microorganisms, particularly fungi (Roldán *et al*., 2009; Jaroszewicz *et al*., 2021; Kosznik-Kwaśnicka *et al*., 2021).

The main groups described in the cave ecosystem are *Actinobacteria*, *Proteobacteria*, *Bacteriodetes, Acidobacteria, Firmicutes* (Kato *et al*., 2024). Actinobacteria are able to capture CO_2, which is highly abundant in the cave air and in turn promoting the precipitation of calcium carbonate and the emergence of speleothems (Cuezva *et al*., 2012).

Proteobacteria, the second largest phyla in caves, thrive in sediments, water, and rocks and can metabolize methane as their sole carbon and energy source (Zhu *et al*., 2021).

Moreover, heterotrophic bacteria in caves can be pathogenic and may contribute to emerging diseases, such as the Firmicutes phylum that thrive in substrates with high organic content, like close to waste disposal (Tomova *et al*., 2013) in caves frequently visited by people.

In this study we describe microbial communities from different South Korean caves and explore whether there is a correlation to different environmental conditions. Particularly, we wanted to identify possible differences in microbial composition between dry and wet caves and along the depth-gradient inside the caves. Two types of cave have been chosen: caves not accessible to the public and touristic show-caves, to inspect whether human disturbances impact the composition of bacterial communities.

Different sampling methods can yield varying insights

into microbial communities; therefore two types of samples were selected, cave sediments and swabs of surfaces. Swabbing is often used to collect surface-associated microbes, providing information on microbial colonization patterns on cave walls and formations. In contrast, sediment sampling captures microbes residing within the cave substrates.

To have a better insight into the metagenomic reconstructions, shotgun metagenomic DNA sequencing based on two independent methods have been used: PCR-free long-reads sequencing with an Oxford Nanopore Technologies (ONT) MinION sequencer using sediment samples only and short-reads sequencing using an Illumina sequencing platform applied to only swabs. ONT MinION Technology major drawback is the high error rate compared to other sequencing technologies nevertheless, this has been consistently improved over the years with the release of new improved flow cells and more accurate basecalling software (Ciuffreda *et al*., 2021). At the current time of this work, the read accuracy is at 97%. The Illumina platforms, while performing better in terms of read accuracy and error rate (> 99%), when use short read lengths (150-300 bp) make it difficult to resolve complex genomic regions, such as those containing repetitive elements or large structural variants (Chiang and Dekker, 2020).

South Korean cave systems have not received much international scientific attention. International peer-reviewed studies on South Korean caves have so far focused on the microbial diversity found in moonmilk encrustations from Baeg-nyong Cave (Park *et al*., 2020), biota found in caves from the Gangwon and Chungcheongbuk provinces (Lee *et al*., 2020; Chang *et al*., 2021) and speleothem-based paleoclimate reconstructions by Jo *et al*. (2010, 2014, 2017). With this paper we aim to add to the knowledge about South Korea as a region of interest for cave systems based environmental reconstructions. Specifically, this work has been intended for paving the way to paleoenvironmental research using stalagmites as a source of ancient DNA (aDNA) and explore their potential as biological paleoarchives. Since the preservation of the aDNA is favored in deposits deeper inside caves (Stahlschmidt *et al*., 2019), it is crucial to understand the diversity of the local microbial communities and how the in-cave depth gradient plays a role. Some of the caves studied here showed a clearer microbial gradient compared to others but they all seem to agree that relatively drier surfaces are characterized by a preponderance of microbes belonging to the phylum *Actinobacteria* (also known as *Actinomycetes*) also confirmed by previous studies (Vardeh *et al*., 2018; Park *et al*., 2020).

2. MATERIALS AND METHODS

2.1. Geographical context and description of the caves

The present work examines the microbial communities of five South Korean caves from the central area of the country (Fig. 1) that have been divided into three categories: dry, seasonally wet, and permanently wet.

2.1.1. Wet cave

Ondal Cave (ON) located in Danyang-gun, Chungcheongbuk-do (37°03.32'N, 128°29.27'E), is a show-cave open all year round to the public excluding during the monsoon season, when it can get flooded (Fig. 1). Like most Korean caves, Ondal does not extend to any considerable depth being developed only for less than 1 km. Air parameters monitoring has been performed in four occasions between November 2020 and April 2022 and showed a constant temperature of ~13℃ in the inner parts of the cave and high CO_2 pressure, ranging from 460 ppmv up to 1100 ppmv due to seasonal ventilation processes. The accessible level of the cave corresponds to the phreatic zone with the subterranean river constantly flowing inside the cave and occasionally inundating parts of the cave. The cave is extremely well decorated with a wide range of deposits.

2.1.2. Dry caves

Simbok Cave (SB) (36°47.13'N, 127°57.76'E) is a small cave located in Goesan-gun, Chungcheongbuk-do in central South Korea (Lee *et al*., 2020). It is formed by horizontal passages accessible to humans in a standing position. During the time of the visiting and the sampling (October 2020), the cave was mostly dry. Hibernating bats were also observed hanging from the ceiling. For this cave no planimetry has been found but its length can be approximated to be nearly 500 meters.

Baram Cave (BA) (37°20.07'N, 128°29.13'E) ("wind cave" in Korean) is in the Gangwon province but further inland compared to Seodae Cave, in the Pyeongchang-gun county (Kim *et al*., 2018; Chang *et al*., 2021). The cave is formed by a single long and horizontal duct (~350 m long),

which is quite wide and has high ceiling allowing an easy traversing. Towards the end of the cave, red-clay deposits pile up along the edges of the pavement and in the interstices. At the time of the visit, the cave was dry and excluding a few stalactites and broken stalagmites (only a couple of speleothems noticed) the cave is not decorated with any dripstones, and the bare bedrock is exposed. In some part of the cave, it is possible to observe some anticline fold structures and fault planes.

2.1.3. Seasonally wet caves

Seodae Cave (SD) is a cave in Gangwon province located in the southern part of the Seokbyeongsan Formation (37°57'N, 128°94'E) (Choi *et al*., 2003) (Fig. 1). The access to the cave is technical challenging and requires rope maneuvers. The structure of the system is quite complex with a combination of vertical and horizontal passages that extend for almost 800 meters. At the time of the exploration, the cave was dry, and no speleothem formations have been observed but during the summer monsoon season the drippings restore, suggesting a fast discharge rate of the vadose layer and seasonally modulated environmental conditions.

Sanjidang Cave (SJ) (37°49.04'N, 128°54.23'E) has multi-leveled, narrow and short (~ 520 meters) passages that mostly develop as a deep crack in the bedrock and "crevasses" obstruct the passing along the conduit (Fig. 1). The cave is peculiar because of coralloid deposits that entirely cover the walls in the deep sectors. At the time of the expedition, no air flows have been perceived by the participants, but the nature of coralloid speleothems indicates the occurrence of some sort of air flow or aerosols combined to active dripping (Vanghi *et al*., 2017), which most likely occurs during the wet season in summer.

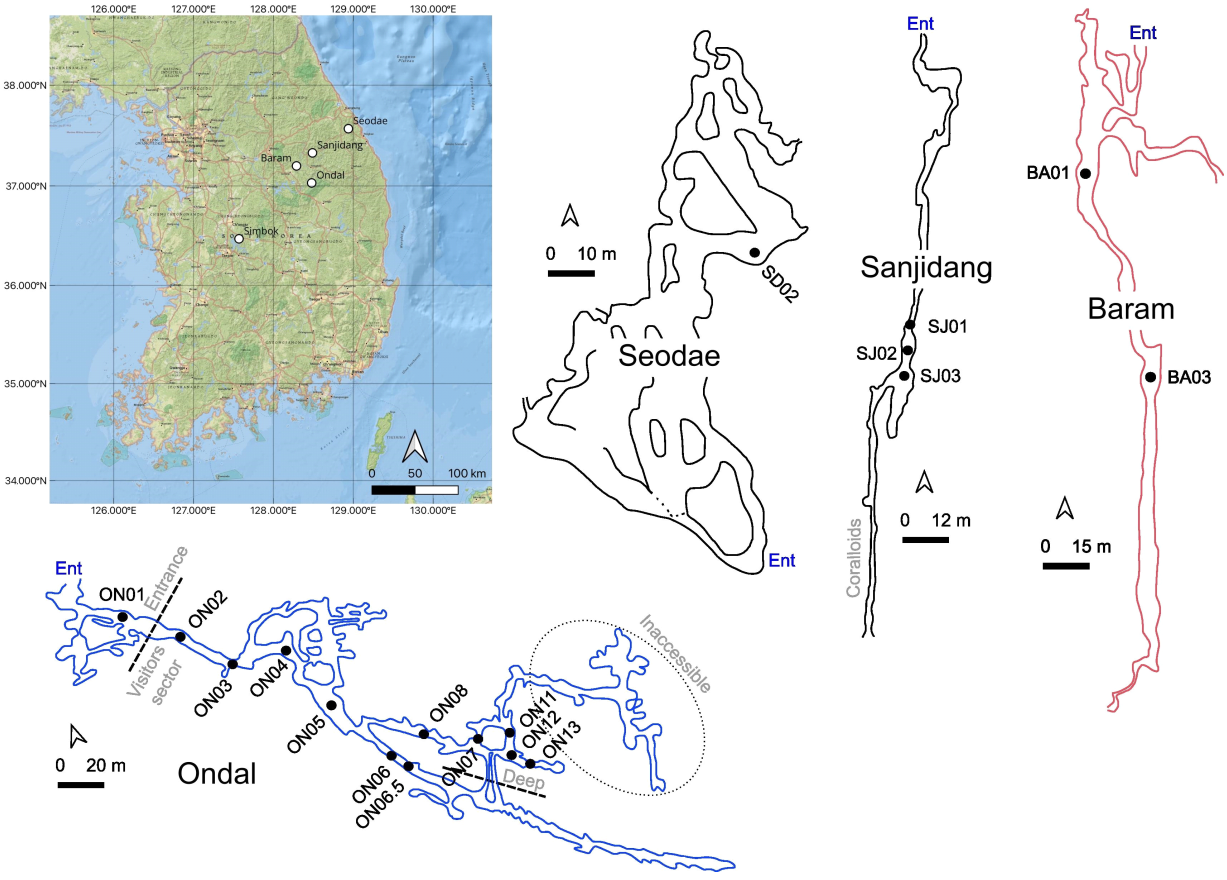

Fig. 1. Cave maps with their geographical locations in the Korean peninsula (map from ESRI National Geographic, obtained using QGIS software). Blue outline for Ondal Cave (wet cave), red outline for Baram Cave (dry cave) and black outline for Seodae and Sanjidang Caves (seasonally wet caves). Simbok Cave map is unreported therefore is has been excluded from this figure. Maps have been modified from the originals. Surveyors: Kyoung-nam Jo and Moo-Yeol Lee (Seodae Cave map), Ki-Bok Kim and Sang-Ho Kim (Baram Cave map), Korea Cave Research Institute (Sanjidang and Ondal Cave maps).

Table 1. Information about the samples (tot. 21) that have been sequenced using MinION sequencing technology.

Sample ID	Cave	Sample type	Sampling site	Distance from Entrance (mt)	Show-cave	DNA concentration (ng/μl)	Reads tot	Filtered reads tot	N50*	% Classified reads**
ON01.a	Ondal	sediments	entrance	20	yes	71.2	134974	132734	3983	41.5
ON01.b	Ondal	sediments	entrance	20	yes	20.4	396599	389646	4762	67
ON02	Ondal	sediments	visitors sector	60	yes	67	160295	157733	3056	41
ON03.a	Ondal	sediments	visitors sector	80	yes	40.2	140689	138744	2136	39
ON03.b	Ondal	sediments	visitors sector	80	yes	38.8	721979	711311	2113	67
ON04.a	Ondal	sediments	visitors sector	100	yes	86	472232	464715	2015	40
ON04.b	Ondal	sediments	visitors sector	100	yes	57.6	90256	88841	2506	67.5
ON05.a	Ondal	sediments	visitors sector	140	yes	77.8	823262	810147	1991	39
ON05.b	Ondal	sediments	visitors sector	140	yes	54	75510	74350	3586	69
ON06.a	Ondal	sediments	visitors sector	200	yes	30.8	171628	168986	3155	40
ON06.b	Ondal	sediments	visitors sector	200	yes	39.6	516431	507480	2647	71
ON07.a	Ondal	sediments	deep sector	280	yes	18.3	526222	518762	2124	42
ON07.b	Ondal	sediments	deep sector	280	yes	25	574787	565609	3010	71.5
ON08.a	Ondal	sediments	deep sector	260	yes	26.8	494254	486783	2453	41.5
ON08.b	Ondal	sediments	deep sector	260	yes	25	1250995	1230120	4284	73
ON11.a	Ondal	sediments	deep sector	460	yes	75.6	574651	565431	3690	47
ON11.b	Ondal	sediments	deep sector	460	yes	104	90607	89220	5176	71
ON11.c	Ondal	sediments	deep sector	460	yes	82.2	383086	376741	2954	71
SB01	Simbok	sediments	entrance	5	no	62.2	33922	33375	6100	42
SJ01	Sanjidang	sediments	deep sector	126	no	36.2	655944	646067	2775	40
SJ03	Sanjidang	sediments	deep sector	126	no	26	696469	685123	3564	71

*N50 represents the weighted median, such that 50% of the contigs equal to or larger than the specified value. **Viruses excluded.

2.2. Samples collection and DNA extraction

For each cave, around 20 grams of sediment were collected using a steel spatula decontaminated with bleach between different sampling actions and put into 50 ml polypropylene tubes. Sterile cotton tip swabs were used to sample areas of speleothem surfaces (5 cm^2). To maximize the capture of the microbial diversity inside the cave, different speleothem deposits were swabbed, such as stalagmites, stalactites and flowstones. After swabbing the surface, each swab was placed into 2 ml vials filled with Zymo DNA/RNA shield buffer. The wooden sticks of each swab were then broken by hand making sure not to touch the cotton head submerged in the buffer, and the vials were finally closed with a screwtop. Once in the laboratory, the sediment samples were stored at -20℃ while the tubes with the swabs in DNA/RNA shield buffer were stored in the fridge at 5℃.

A total of 40 samples were collected at different depths inside the caves and named with two letters referring to the cave name and a number gradually increasing with increasing depth inside in the cave. For example, ON01 corresponds to a sample that was taken from a location inside Ondal Cave closer to the entrance compared to ON07 that was taken at a greater distance. Total number and labelling of the samples for each cave are indicated in Table 1 and 2.

Some samples have biological replicates that have been indicated with a small letter (“a”, “b” or “c”) after the number. In this study, biological replicates refer to samples removed from the same tube of sediment/soil or to a “twin” sterile swab used to swab the same portion of speleothem surface during the cave expeditions but that have been extracted and sequenced in the labs at different moments in time.

DNA, from a total of 21 sediment samples, was extracted in the metagenomics laboratory of Pusan National University (PNU) from 500 mg of sediment using FastDNA SPIN Kit for soil (MP Biomedicals, United States) following

Table 2. Information about the samples (tot. 19) that have been sequenced using Illumina sequencing technology.

Sample ID	Cave	Sample type	Sampling site	Distance from Entrance (mt)	Show-cave	Reads tot	N50*	% Classified reads**
BA01.a	Baram	swab	deep sector	67.5	no	29382625	150	53
BA01.b	Baram	swab	deep sector	67.5	no	38100922	150	53
BA03	Baram	swab	deep sector	187.5	no	36735344	150	52
SJ01	Sanjidang	swab	deep sector	96/520	no	34639577	150	50
SJ02	Sanjidang	swab	deep sector	114	no	23013185	150	53
SJ03.a	Sanjidang	swab	deep sector	126	no	26082292	150	49
SJ03.b	Sanjidang	swab	deep sector	126	no	27479738	150	50
ON01	Ondal	swab	entrance	20	yes	43562101	150	49
ON06	Ondal	swab	visitors sector	200	yes	695845	150	43
ON06.5	Ondal	swab	visitors sector	205	yes	40500193	150	51.5
ON07	Ondal	swab	deep sector	280	yes	32347034	150	52
ON08	Ondal	swab	deep sector	260	yes	25941217	150	51
ON11	Ondal	swab	deep sector	460	yes	68557549	150	54
ON12.a	Ondal	swab	deep sector	470	yes	9589367	150	82
ON12.b	Ondal	swab	deep sector	470	yes	10470816	150	82.5
ON13.a	Ondal	swab	deep sector	480	yes	13683081	150	83.5
ON13.b	Ondal	swab	deep sector	480	yes	8459675	150	83.5
SD02.a	Seodae	swab	deep sector	80	no	26389411	150	52
SD02.b	Seodae	swab	deep sector	80	no	32185113	150	52

*N50 represents the weighted median, such that 50% of the contigs equal to or larger than the specified value. **Viruses excluded.

the manufacturer's indication. The DNA has been quantified using a ThermoFisher Qubit-4 fluorometer and the purity defined by ThermoFisher Nanodrop spectrophotometer. 19 swab samples were sent to Zymo Research Corp., Irvine (CA, USA) laboratories and DNA extracted using ZymoBIOMICS-96 MagBead DNA Kit (Zymo Research, Irvine, CA).

2.3. Long-read sequencing

The DNA extracted in the PNU lab have been sequenced using the ONT MinION sequencer. Prior to the library preparation all samples have undergone an additional AMPure XP magnetic beads purification to increase the 260/230 values and remove contaminants, such as ethanol, that have been used during the DNA extraction step. For each sample, at least 1 µg of DNA has been used to prepare the sequencing library with the PCR-free SQK-LSK109 ligation kit (ONT) according to the manufacturer's instructions. The libraries were loaded on FLO-MIN106 flow cells (R9.4.1 sequencing chemistry) inserted on the MinION device and basecalling was carried out using MinKNOW operating software (ONT) in fast basecalling mode.

In all cases, negative controls were included to assess the level of bioburden during the wet-lab procedures. We have tried to replicate each samples using both the technologies but due to low DNA yield during the extraction step, for some samples this has not been possible.

MinION raw reads' barcodes were trimmed on both ends using the default filtering options of MinKNOWN (ONT) and the Qscore cut-off value was set to 7 or 9.

2.4. Short-read sequencing

The DNA extracted at Zymo Research Corp. laboratories were prepared for sequencing using Nextera DNA Flex Library Prep Kit (Illumina, San Diego, CA), that only requires reduced PCR cycles, with up to 100 ng DNA input and using internal dual-index 8 bp barcodes with Nextera adapters. The platform used for sequencing was NovaSeq 6000 (Illumina, San Diego, CA).

Illumina raw paired-end mode (150 nt) sequence reads were manually trimmed using Trimmomatic-0.33 (Bolger *et al*., 2014) to remove adapters: 6 bp window size for the trimming and a quality cutoff of 20. Reads with size lower than 70 bp were removed. Overrepresented G-poly-

mers and duplicate reads were removed using bbduk and seqkit, respectively (Shen *et al*., 2016; Bushnell *et al*., 2017). Paired-end reads were merged using Pear (Zhang *et al*., 2014).

2.5. Bioinformatics analysis

Both Illumina and MinION cleaned and filtered reads were classified using 1.18.1 Kaiju (Menzel *et al*., 2016) in Greedy mode with 3 mismatches allowed against a subset of NCBI BLAST nr database (downloaded in April 2021) containing all proteins belonging to known Archaea, Bacteria, Viruses, Fungi, and some microbial Eukaryotes.

After the alignment to the database, the reads were filtered to remove reads with ambiguous taxa annotations and to exclude taxa with a mean less than 1 (counts) across all samples.

Read counts were processed for statistical analyses using functions in Phyloseq 4.2 R package (McMurdie and Holmes, 2013). Alpha diversity measures were carried out on clean and filtered data using Shannon index. Beta diversity was studied using non-metric multidimensional scaling (NMDS) and applying the Bray-Curtis distance using vegan R-packages.

3. Results

3.1. MinION samples taxonomic composition

The taxonomical classification led to a mean of 55% of successfully classified reads (Table 1). After filtering for taxa less than 2% to reduce the chances of false positive predictions, the total number of taxa decreased from 37,498 to 15,377. The taxonomy results were then normalized to 100%.

Among the taxa accounting for more than 2% of abundance, the most prominent ones are *Proteobacteria* and *Actinobacteria* (also known as *Actinomycetes* or *Actinomycetota*) followed by *Acidobacteria*, *Planctomycetes*, *Chloroflexi*, *Candidatus Rokubacteria* and *Verrucomicrobia* (Fig. 2).

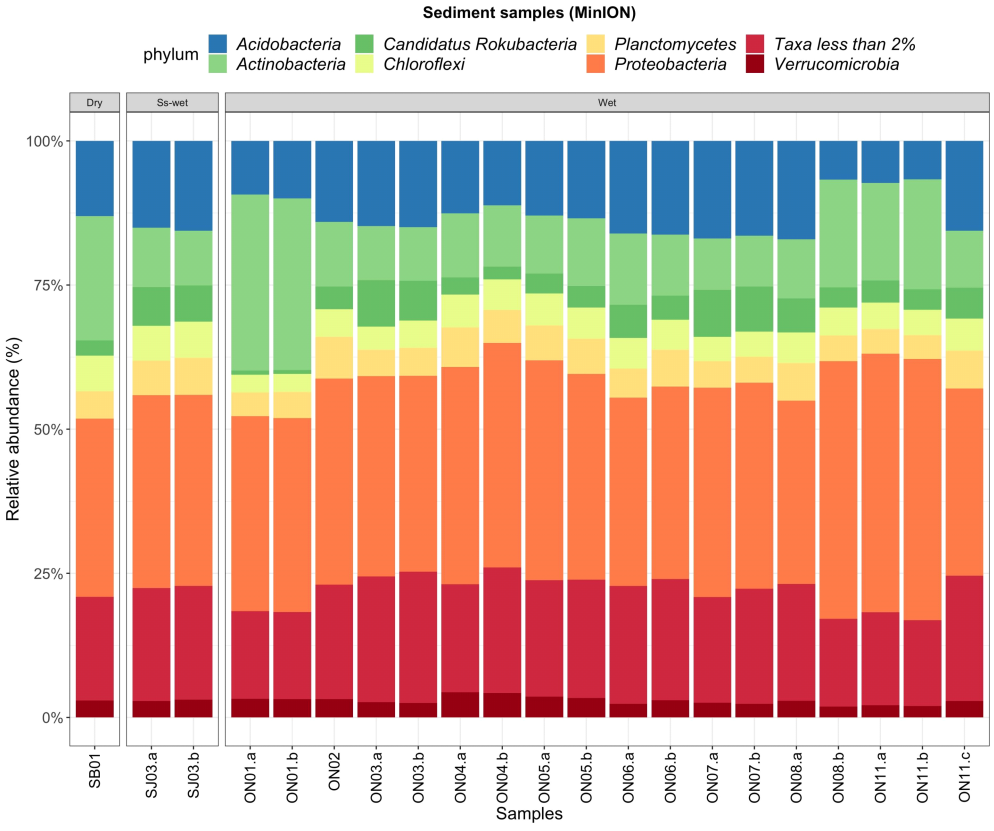

Fig. 2. Bar-plot of the relative abundances of the most common phyla found in the different caves and sequenced using MinION Nanopore technology. The graph below the bar-plot shows the ratio between the major two phyla: *Proteobacteria* and *Actinobacteria*. The samples are grouped by type of environment: dry, seasonally wet ("Ss-wet") and permanently wet ("Wet") caves and the soil. SB = Simbok Cave, SJ = Sanjidang Cave, ON = Ondal Cave.

The microbial distributions of each cave are comparable. These communities are predominantly formed by Proteobacteria accounting for 36% and 33% and 30% of the total for Ondal, Sanjidang and Simbok Caves respectively. *Actinobacteria* presence along Ondal and Sanjidang Caves maintains stable on average at 13% and 9% respectively but in the samples taken near the entrance (ON01) and in deep sectors (ON8, ON11.a and ON11.b) it increases to on average 30% and 18% respectively. Similarly, Simbok Cave hosts relatively more *Actinobacteria* 21% of the total microbial composition. On a species level this phylum is dominated by a generic species of *Actinobacteria* and there is not a clear distinction between the three caves, although Ondal wet Cave shows a slight increase of the species *Pseudonocardiales* with respect to dry and seasonally wet caves (Fig 3a). *Acidobacteria* are a minor phylum: ranging from 12% in Ondal Cave, 13% in Simbok Cave and 15% in Sanjidang Cave.

Proteobacteria is the dominant phylum found across all samples (Fig. 2). Among this phylum the most dominant species belong to the class of *Deltaprotobacteria* (also known as *Mixococcota*) and *Betaprotobacteria* (Fig. 3b). *Betaprotobacteria* show an increase in seasonally-wet and wet environments as well as the *Gammaproteobacteria* belonging to the *Chromatiales* species. On the contraty, *Alphaproteobacteria* are more abundant in dry environments compared to seasonally-wet and wet environments. Inside Ondal Cave sediment samples were collected from different locations (Fig. 4). Near the entrance of Ondal Cave, *Betaprotobacteria* and *Alphaproteobacteria* species are higher compared to other sites of the cave. Deep sections are mainly composed by the species *Chromatiales* and species of *Deltaproteobacteria* whereas species of *Betaprotobacteria* show a decrease compared to sectors

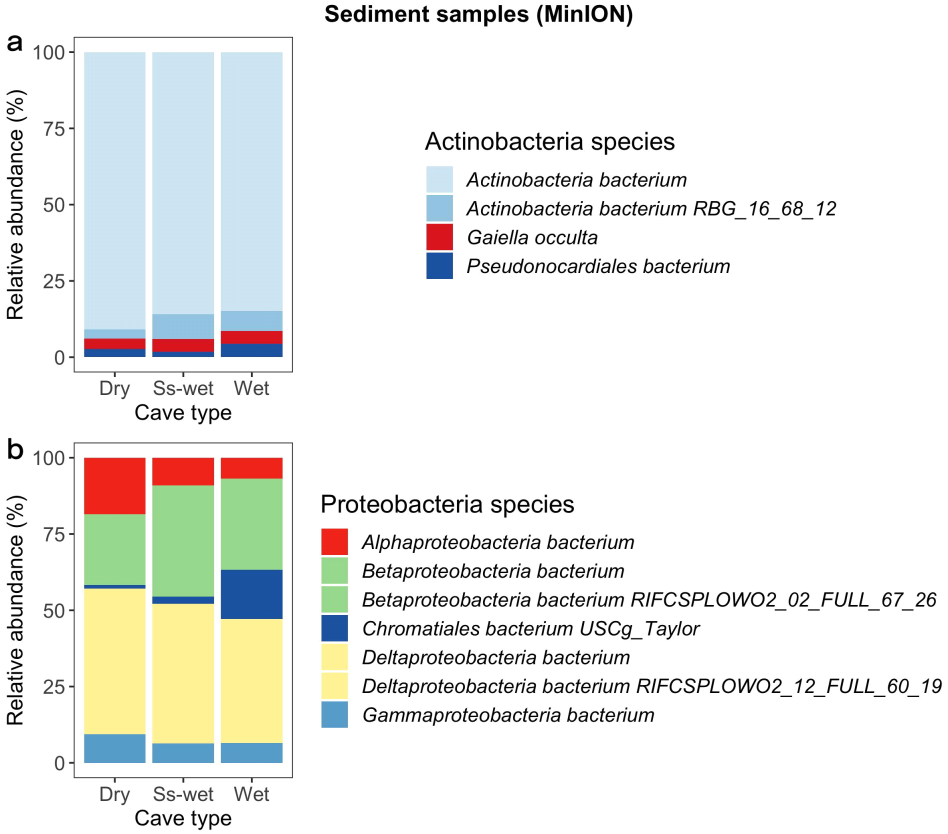

Fig. 3. Compositional barplots normalized to 100% of main *Proteobacteria* species and *Actinobacteria* species from inside the caves grouped into dry, seasonally wet (Ss-wet) and wet groups and sequenced with nanopore MinION. Soil samples have been excluded from these plots. The two species of *Actinobacteria bacterium*, *Betaproteobacteria bacterium* and *Deltaproteobacteria bacterium* have been assigned with same colors (pale blue, green and yellow) because they represent generic species of that specific phylum. (Taxa filtering is based on a mean relative abundance greater than 15% and 30% for *Actinobacteria* species and *Proteobacteria* species respectively).

near the entrance.

3.2. Illumina samples taxonomic composition

The taxonomical classification led to a mean of 55% of successfully classified reads (Table 1). After filtering for taxa less than 2% to reduce the chances of false positive predictions, the total number of taxa decreased from 49,917 to 34,778. The taxonomy results were then normalized to 100%.

The swabs, like the sediment samples sequenced using the Nanopore MinION (section 3.1), mostly show the presence of *Proteobacteria* and *Actinobacteria* phyla (Fig. 5) showing an almost equal distribution of these two phyla. In Baram Cave samples, the dominant phylum is *Actinobacteria* accounting on average for the 65% of the total, whereas *Protobacteria* only accounts for the 11%. In Ondal Cave, *Actinobacteria* and *Protobacteria* abundances are comparable, being on average 35% and 34% of the total respectively. In Seodae and Sanjidang Caves, half of their microbial composition is formed by *Proteobacteria*, and only 13% on average is formed by *Actinobacteria*. The third most abundant phylum, *Acidobacteria*, has a constant abundance of less than 10% among all the swab samples from all the caves.

A clear distinction in the composition of *Proteobacteria* and *Actinobacteria* species is noticeable between dry caves compared to wet and seasonally-wet caves (Fig. 6). Wet and seasonally-wet cave swab colonies mainly host the species *Chromatiales* (Phylum *Gammaproteobacteria*), whereas dry cave samples are mostly composed by species of *Beta-* and *Deltaproteobacteria* (Fig. 6b).

Regarding *Actinobacteria*, all the three categories of caves are different (Fig. 6a). Wet caves mostly host the species *Pseudocardiales*, for the 75% of the total abundance.

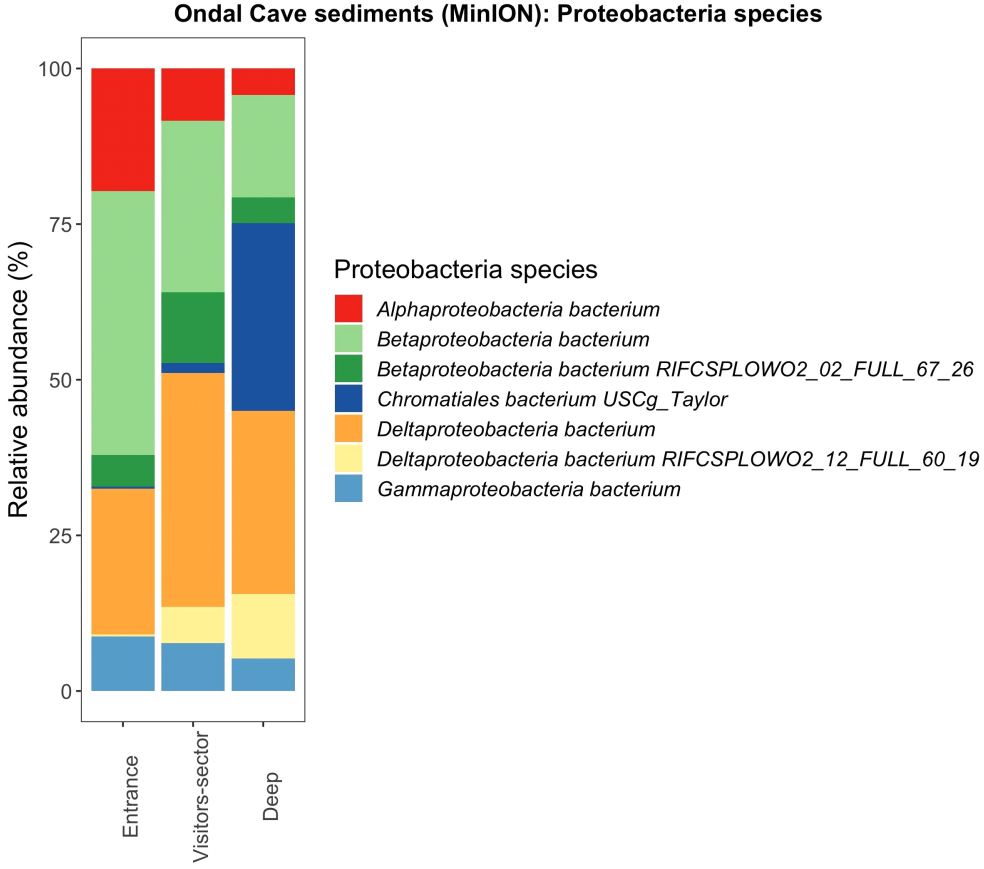

Fig. 4. Compositional bar plots normalized to 100% of main *Proteobacteria* species for the samples from Ondal Cave sequenced with nanopore MinION. Samples are grouped depending on the site inside the cave where they have been collected: entrance, visitors sector and deep sector. The two species of *Betaproteobacteria bacterium* and *Deltaproteobacteria bacterium* have been assigned with same colors (green and yellow) because they represent generic species of that specific phylum. (Taxa filtering is based on a mean relative abundance greater than 30%).

Seasonally-wet cave specimen are almost entirely composed by the species *Actinomycetia*, while the dry caves present a more heterogenous microbial composition formed by the same amount (40%) of *Pseudonocardiales* and *Rubrobacteraceae* species and the rest by the species *Actinomycetia*.

Inside Ondal Cave sediment samples have been collected from different locations (Fig. 7). Near the entrance of the cave and in the sector where visitors are allowed in, the microbial composition is heterogeneous and mainly formed by species of *Delta-*, *Alpha-* and *Proteobacteria*. Deep sections are mainly dominated by the species *Chromatiales*.

3.3. Alpha diversity

The Illumina NovaSeq platform generated an average of 27,320,423 raw reads per sample compared to nanopore sequencing that produced an average of 437,510 raw reads per sample (Table 1 and 2). The ONT technology can analyze long fragments of DNA therefore, whereas the DNA sequenced with the NovaSeq had a fixed length of 150 bp, MinION reads have different lengths and their N50 values (weighted median) range from 1991 bp to 6100 bp (Table 1).

The diversity index was calculated on cleaned and filtered data. Considering the different sequencing depths obtained with the two different sequencing technologies, alpha diversity measures are discussed separately.

In general, the communities analyzed using Illumina Novaseq show similar diversity values of the communities analyzed with the MinION (Table 3 and 4). In Ondal Cave, deep-room communities have lower values compared to the entrance. The Shannon values from this study are higher compared to the values from another South Korean cave that ranged between 3 and 4.9 (Park *et al.*, 2020), however they are similar to the values of cave microbiomes described by Ortiz *et al.* (2013) (USA) and

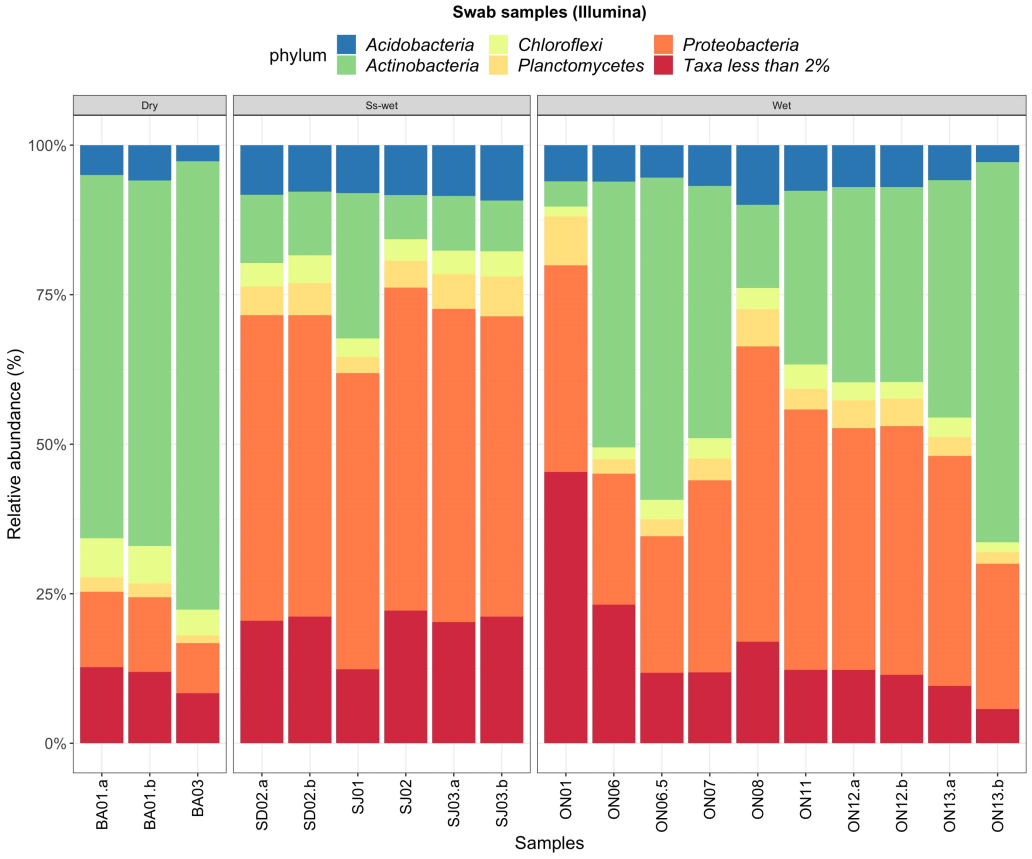

Fig. 5. Bar-plot of the relative abundances of the most common phyla found in the different caves and sequenced using with Illumina NovaSeq. The graph below the bar-plot shows the ratio of the major two phyla: *Proteobacteria* and *Actinobacteria*, grouped by type of environment: dry, seasonally wet ("Ss-wet") and permanently wet ("Wet") caves and the soil. BA = Baram Cave, SD = Seodae Cave, SJ = Sanjidang Cave, ON = Ondal Cave.

Wiseschart *et al*. (2019) (Thailand), Addesso *et al*. (2021) (Italy).

3.4. Beta diversity

Ondal Cave's bacterial communities grouped by sites: entrance, visitors sector and deep sector, were compared among each other to determine their similarity using non-metric multidimensional scaling (NMDS) (Supplementary Fig. 1). For the samples sequenced with MinION, the ordination analyses show three distinct groups of samples. Even though the samples collected near the entrance well separate from the other samples, 4 out of 7 samples collected in the deep sections of the cave fall within the group of the samples collected in the visitor sector. Illumina samples show three distinct groups in the NMDS space, and the samples cluster depending on the cave site where they were collected.

4. Discussion

This study aimed at testing whether cave-dwelling microbial communities are sensitive to environmental conditions inside South Korean caves, given their restricted availability of nutrients and the type of substrate. Therefore, since the main purpose of this study is to describe the biodiversity of the samples as a function of their surrounding environment, shotgun sequencing can be more appropriate compared to the 16S sequencing approach.

Shotgun sequencing, in contrast to the 16S ribosomal RNA sequencing method, can classify down to the species level while also aiming to estimate the relative abundances of the taxa within a sample (Tovo *et al*., 2020). Other advantages of the shotgun method compared to the 16S-amplicons approach include the mitigation of PCR biases, since it does not rely on the amplification of spe-

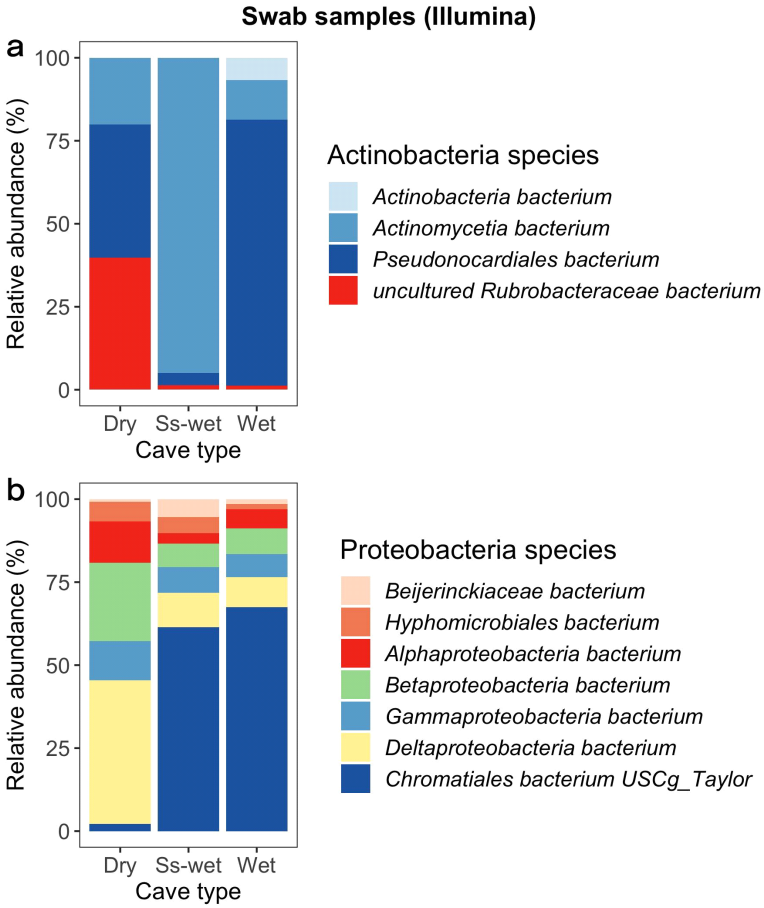

Fig. 6. Compositional barplots normalized to 100% of main *Proteobacteria* species and *Actinobacteria* species (using cutoff at average abundance of 0.55%) from inside the caves grouped into dry, seasonally wet (Ss-wet) and wet groups and sequenced with Illumina NovaSeq. (Taxa filtering is based on a mean relative abundance greater than 30% for both *Actinobacteria* species and *Proteobacteria* species).

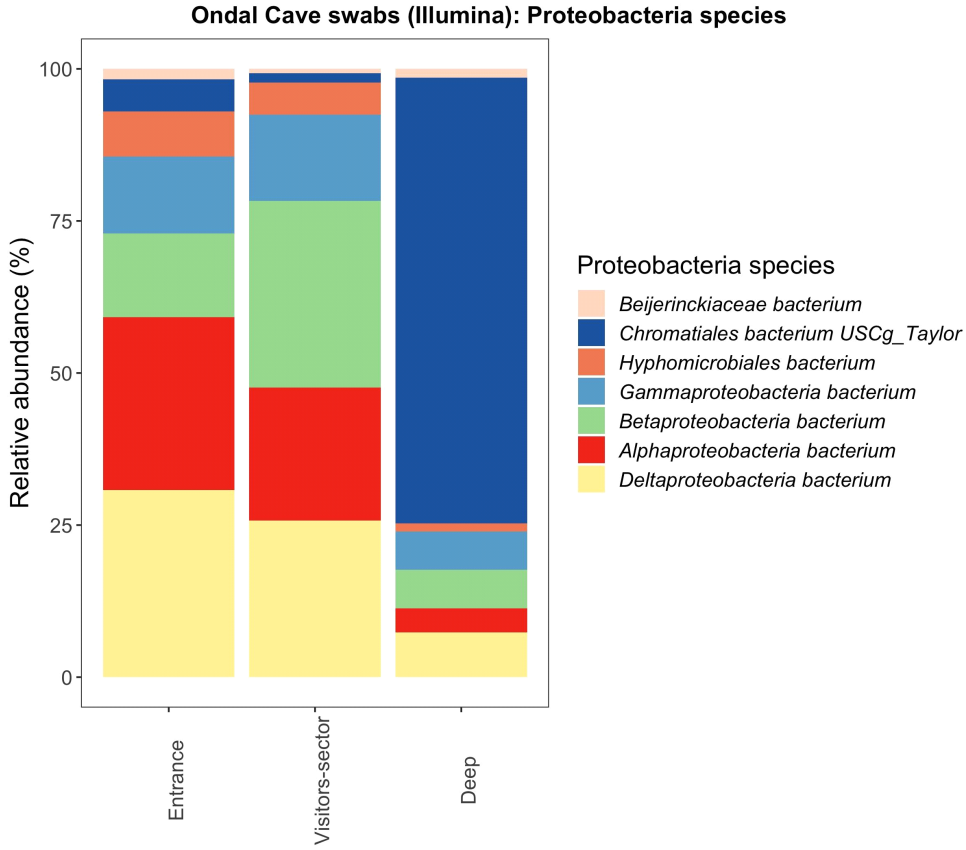

Fig. 7. Compositional bar plots normalized to 100% of main *Proteobacteria* species for the samples from Ondal Cave sequenced with Illumina NovaSeq. Samples are grouped depending on the site inside the cave where they have been collected: entrance, visitors sector and deep sector. (Taxa filtering is based on a mean relative abundance greater than 30%).

cific DNA target regions and the size of the database (Logares *et al*., 2014). In addition, the shotgun approach relies on larger and more comprehensive databases compared the amplicon homologue.

The choice of the Kaiju taxonomic classification algorithms has been reasoned on the proteins database used by the program, which are much more conserved with respect to nucleotide sequences (Menzel *et al*., 2016).

Cave bacterial consortia are influenced by the geology of the area (Zhu *et al*., 2019) and the geochemical characteristics of the surface but despite being geographically distant, the structure of the microbial communities from all these five South Korean caves is consistent with what is considered the core cave microbiome: *Alphaproteobacteria*, *Betaproteobacteria*, *Deltaproteobacteria*, *Gammaproteobacteria*, *Chloroflexi*, *Planctomycetales*, *Bacteroidetes*, *Firmicutes*, *Acidobacteria*, *Actinobacteria*, *Nitrospirae*, *Gemmatimonadetes* and *Verrucomicrobia* (Hershey and Barton, 2018) (Fig. 2 and 5). The source of these organ-

Table 3. Alpha diversity indexes for group of samples sequenced using MinION.

Cave	Type	Site	Shannon
Ondal	sediment	entrance	7.55
Ondal	sediment	visitors sector	6.8
Ondal	sediment	deep	6.4
Simbok	sediment	entrance	6.6
Sanjidang	sediment	deep	6.7

Table 4. Alpha diversity indexes for group of samples sequenced using Illumina technology.

Cave	Type	Site	Shannon
Baram	swab	deep	6.5
Sanjidang	swab	deep	6.9
Ondal	swab	entrance	6.9
Ondal	swab	visitors sector	7.2
Ondal	swab	deep	6.3
Sodae	swab	deep	6.6

isms is generally attributed to the soil microbiota transported inside the cave by vertical infiltration or surface streams, air currents or other animals (Kováč, 2018). Variations in the composition of microbial communities, both in the outside soil and in the dripping water, are also likely influenced by seasonal environmental parameters. Particularly Rasche *et al*. (2011) and Yun *et al.* (2016), showed that with high temperatures the abundance of *Alphaproteobacteria*, *Acidobacteria* and *Verrucomicrobia* decreases, whereas Betaproteobacteria increase. However, once in the cave, the selective pressure of this oligotrophic environment further sorts the bacterial species changing the structure of the original community of microbes (Ortiz *et al*., 2013; Lavoie *et al*., 2017) and even filtering contaminant species. The case of Lechuguilla Cave is emblematic as its native bacterial communities remained dominated by the endemic species despite being contaminated by humans' urine commensal species introduced during some exploration trips (Johnston *et al*., 2012).

The most abundant bacteria in all the caves studied in this work are *Proteobacteria* and *Actinobacteria*, which are also the most common phyla found in many cave environments (Ortiz *et al*., 2013; Yun *et al*., 2016; de Mandal *et al*., 2017; Lavoie *et al*., 2017; Wiseschart *et al*., 2019; Zhu *et al*., 2019). Both phyla are ubiquitous in nature and *Proteobacteria* also constitute the largest and most diverse group of the *Bacteria* domain (Kersters *et al*., 2006).

Among the *Proteobacteria* phylum, bacteria belonging to the *Gammaproteobacteria* taxonomical class have been found in high percentages in the swab samples (Fig. 6 and 7). This agrees with Macalady *et al*. (2006)'s study, who found this type of *Proteobacteria* particularly dominant in biofilms in an Italian cave as well as with Jurado *et al*. (2020) work who mostly found *Gammaproteobacteria* in vermiculations from an Alpine cave. Similarly, in another cave in Slovenia, microbial communities from the walls were mostly dominated by members of *Gammaproteobacteria* (Pašić *et al*., 2009).

Proteobacteria have an important role in caves' nitrogen cycle. Subphyla like *Alpha-* and *Beta- Proteobacteria* play critical role in the fixation of nitrogen, where N_2 is fixated as ammonia (NH_3) so that it can be utilized by other organisms, and they are also involved in the processes of nitrification and denitrification. Other subphyla like *Gamma-* and *Delta- Proteobacteria* are instead involved in other biogeochemical cycles, including sulfur and carbon cycles. They contribute to the carbon cycle by breaking down organic matter into simpler molecules that can be utilized by other microorganisms, or they take part in the process of sulphate reduction where sulphur gets recycled making it available for other biological processes, which is critical in anaerobic environment like caves. These processes are important in maintaining the balance of sulphur and nitrogen in cave ecosystems (Holmes *et al*., 1999; Ojeda *et al*., 2019; Knief *et al*., 2003; Macalady *et al*., 2006, 2008; Zhu *et al*., 2021, 2022). Some are also crucial in the formation of biofilms on cave surfaces (Engel *et al*., 2004) and in the processes of calcification of speleothems (Banks *et al*., 2010).

Actinobacteria have been found in association with pigment-forming communities on the walls of certain caves that are regularly visited by tourists suggesting that this phylum thrives in anthropized environments (Cuezva *et al*., 2012; Porca *et al*., 2012; Vautrin *et al*., 2021). Moreover, some of its species have also presented some pathogenic potential and health concerns for cave visitors (Jurado *et al*., 2010). Other species instead dominate pristine caves taking advantage of their ability of degrading complex compounds present in the cave, such as in nitrate reduction processes or to fix CO_2 (Severino *et al*., 2019). Cave *Actinobacteria*, are also considered a potential source of novel bioactive compounds (Axenov-Gibanov *et al*., 2016; Adam *et al*., 2018).

The sediment species communities from all the caves in this study, show an increase of *Acidobacteria* species (Fig. 2) compared to the swabs where their presence is minimal (Fig. 5). This is a very common phylum of bacteria usually present in soils low in nutrients and often observed associated to *Proteobacteria* (Kielak *et al*., 2016).

At a species level, the most dominant taxa found on the walls of the caves from this study are *Chromatiales* (*Gammaproteobacteria*), *Pseudonocardiales* and *Actinomycetia* (Fig. 6 and 7). Similarly, the communities belonging to sediments from remote parts of the caves, showed an increase in *Chromatiales Gammaproteobacteria* (Fig. 4).

4.1. Distribution patterns of the major species in the cave niches

Chromatiales (*Gammaproteobacteria*), also known as

purple sulfur bacteria, have been found to be one of the core microbes in many other cave environments around the world, such as in South Korea (Park *et al*., 2020), Spain, Czech Republic and Slovenia (Porca *et al*., 2012), Italy (D'Angeli *et al*., 2019), Brazil (Marques *et al*., 2019) and in an Azorean lava cave (Riquelme *et al*., 2015). Despite it does not surprise to find this species on wet rock surfaces (Imhoff, 2006), these are phototrophic organisms hence light is a selective factor for their development (Rosenberg *et al*., 2006). It is, therefore, unexpected to find *Chromatiales* at high percentages in deep sections of Ondal, Seodae and Sanjidang Caves that are not illuminated by natural nor artificial lights (Fig. 4 and 7). A similar result occurred to Marques *et al.* (2019), who found *Chromatiales* dominating the bacterial communities in aphotic zones of a Brazilian cave and interpreted its presence for being associated to the nitrogen cycle and ammonia oxidation processes. Nitrogen-based metabolism is indeed commonly found in bacteria adapted to environments scarce in nutrients (Ortiz *et al*., 2014; Reboleira *et al*., 2022). Zhu *et al*. (2019) also presumed that nitrogen is an important source of nutrients for microbes living in caves and that can decrease the overall species richness in favor of only certain dominant taxa, like *Chromatiales* indeed. This is consistent with soil cover above karst systems being generally saturated in N (Wen *et al*., 2016; Chen *et al*., 2019) that can leach inside the cave through seepage. Therefore, the presence of this species that have been found in seasonally-wet and wet caves could be mainly related to relatively high humid levels in the environment and nitrogen availability.

The other dominant species is *Pseudonocardiales* bacterium (*Actinobacteria*), found at relatively high percentages in the swab communities from Ondal and Baram Caves (Fig. 6), which are wet and dry caves respectively despite being very low in all the sediment samples (Fig. 3). This species of bacteria is commonly found in caves, and it has been proposed as being an indicator of specialized limestone bacterial communities (Zhu *et al*., 2019) and it was also found to be the major constituent in microbial biofilms covering Paleolithic paintings in some Spanish caves (Stomeo *et al*., 2008).

The presence of members belonging to *Pseudonocardiaceae* has been described as part of the core microbiome of an unmarked cave environment (Porca *et al*., 2012; Riquelme *et al*., 2015; Lavoie *et al*., 2017; Buresova-Faitova *et al*., 2022). In that regard, we can understand the increased counts of *Pseudonocardiales* bacterium in the undisturbed Baram Cave or in the non-touristic sectors of Ondal Cave (Fig. 6). Despite Seodae and Sanjidang being both pristine caves, the presence of this species of *Actinobacteria* is very low. This could be interpreted because these caves mostly host species of *Proteobacteria*, at the expenses of *Actinobacteria*, that are linked to consistent availability of Dissolved Organic Carbon (DOC) and high humidity. This order of bacteria, when living in the soil, degrades aromatic compounds and converts organic litter into humic acids. By transporting aromatic compounds, drip waters contribute to feed and sustain the community of this type of bacteria inside caves (Marques *et al*., 2019; Turrini *et al*., 2020). The same can be said for Ondal Cave that in terms of water availably is more similar to Seodae and Sajindang Caves compared to Baram cave.

Actinobacteria have also been interpreted as indicators of bare dry rock surfaces or inactive speleothems (Vardeh *et al*., 2018). Considering that Baram Cave is also high in other *Actinobacteria* species, such as *Rubrobacteraceae* bacterium (Fig. 6), this microorganisms could be considered as a proxy of a dry cave environment. The cave was indeed ventilated and dry at the time of the visit, with no speleothem formations and the bare bedrock is exposed for all its length. Crucially, *Rubrobacterales*, is an order of bacteria highly desiccation-resistant that generally grows after an active microbial mat passes to be inactive (Vardeh *et al*., 2018). This also agrees with Baram Cave relatively lower abundance of *Proteobacteria* that are associated to highly available organic matter and relatively wet and humid environments.

Cave walls, compared to lose and porous sediments, are better substrates to form biofilms and bacterial colonies where dissolved nutrients from the topsoil can be provided by dripping water as demonstrated by the presence in the swabs of cave wall dweller species such as *Hyphomicrobiales* (*Alpha-Proteobacteria*) (Jurado *et al*. 2022) and *Actinomycetia* (Groth and Saiz-Jimenez, 1999; Pašić *et al*., 2009; Farda *et al*., 2022), which are absent in the sediments.

In general, it is possible to notice that the distribution of the phyla in the two types of substrates is different

(Fig. 2 and 5). In the sediments, the phyla abundances are more constantly distributed compared to the swabs where they fluctuate more likely reflecting more specialized colonies due to limited nutrients input from the outside environment (Zhu *et al*., 2019).

In addition, examining the distribution of microbes within cave surfaces like stalagmite or flowstone layers provides an additional method to interpret paleoclimate data obtained from speleothem archives. Embracing a multidisciplinary scientific approach to climate science, metagenomics could complement the analysis of stable isotope records (^{18}O and ^{13}C), trace element distribution and the petrographic study of crystalline fabrics (Fairchild and Baker, 2012).

5. Conclusions

The microbial composition identified for the South Korean caves investigated in this study agrees with the core microbiome for karstic environments (Hershey and Barton, 2018). The more common bacteria found in these colonies belong to the phyla *Actinobacteria* and *Proteobacteria*. In general, *Proteobacteria* dominate communities highly exposed to nutrients and animals. Natural sources of organic matter in caves are DOC and colloidal organics from the soil layers above, then transported by subterranean waters, either dripping or floodings. The second source of nutrients comes from plants and animals (Ikner *et al*., 2007). *Proteobacteria* that comprise *Gamma-*, *Beta-*, *Alpha-* and *Deltaproteobacteria* classes, are largely widespread thanks to their capacity of degrading a broad spectrum of organic materials and thus to survive in hostile and nutrients-deprived environments, like caves (Addesso *et al*., 2021). In either way, *Protobacteria* not only are associated to high source of organic matter but also to relatively wet and humid environments where nutrients can be easily transported around (Pašić *et al*., 2009).

In our study we found high abundances of *Proteobacteria* for all the well decorated caves with active dripping like in the case of Ondal Cave or seasonally wet, during the summer monsoons, caves like Sanjidang and Seodae Caves (Fig. 2 and 5). The only caves with relatively low percentages of *Proteobacteria* are Baram and Simbok Caves that were dry and dusty during the exploration and lack speleothem deposits (Fig. 2 and 5). The major pattern of *Proteobacteria* distribution observed in these caves is a relative increase of *Gammaproteobacteria* in the deep sectors contrary to *Alpha-* and *Beta- Proteobacteria* that show a decrease and can be potentially interpret as due to a major nitrogen availability in these parts of the caves, that would need to be confirmed with a metabolic pathways study. A major presence of *Actinobacteria* relative to *Proteobacteria* is here interpreted as a proxy of a relatively dry environment, like in the case of Baram (Fig. 5) and Simbok (Fig. 2) Caves. Whether the relative abundance of *Rubrobacteraceae* and *Chromatiales* species of bacteria can be regarded as proxy for dry and wet cave conditions respectively, needs to be further explored in the future. In summary, specific microhabitats play a crucial role in controlling the distribution of microbial communities even in the face of an overall resemblance in microbial communities amid geographically distant cave systems.

Data availability statement

The datasets generated and/or analysed in this study are available in the Sequence Read Archive (SRA), hosted by the National Center for Biotechnology Information (NCBI), available with accession number PRJNA946675.

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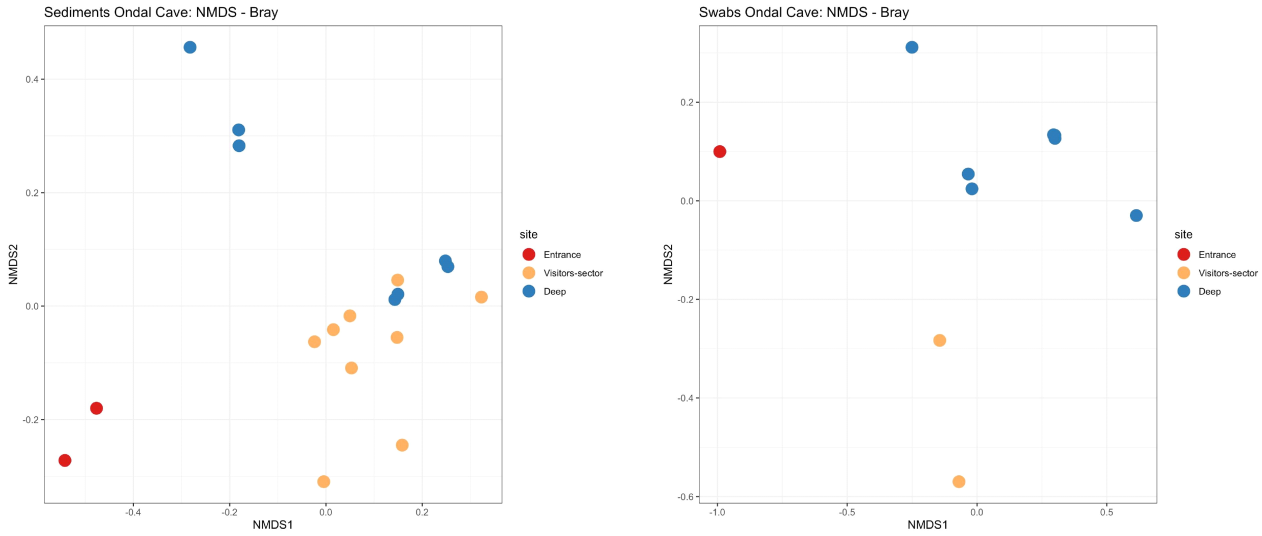

Supplementary Fig. 1. Non-metric multidimensional scaling (NMDS) analysis of Ondal Cave's microbial communities for both the sediments and the swabs, grouped by sites inside the cave.