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Erratic calcareous deposits: Biotic formation insights and biomineralising bacterial strain isolation

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ABSTRACT

The present study investigated the contribution of microbial communities in producing "living stones" and the suitability of these clasts as sources of microorganisms with biomineralisation abilities. The calcareous samples were analysed for their microbial community (16S rRNA gene metabarcoding and culturable approach) and *in vitro* regeneration tests. Scanning electron microscopy and Energy Dispersive Spectroscopy (SEM-EDX) were applied to investigate microbial aggregation structures and footprints in natural and *in vitro* samples. The metabarcoding unveiled amplicon sequence variants (ASVs) assigned to lineages with biomineralisation abilities (*e.g.*, Proteobacteria and Actinobacteriota). The culturable approach resulted in nineteen calcifying isolates with diverse morphological, metabolic, and mineral precipitation properties. Based on mineralising properties, *Stenotrophomonas maltophilia, Lysinbacillus fusiformis*, and *Microbacterium ginsengiterrae* were identified at the molecular level. *In vitro* regeneration tests and SEM-EDX analyses confirmed the active role of the endogenous microorganisms in forming these "living stones". These findings allow us to hypothesise an essential role of microbial precipitation in forming these "living stones", previously described as of abiotic origin. The current study findings provide a solid scientific foundation for future investigations. The obtained bacterial isolates and their potential applications in bioremediation, construction, and cultural heritage restoration demonstrate the direct applicability of our study in sectors involving biomaterials application.

Statement of significance: We studied some "living stones" that can be found worldwide and whose origin is still not completely understood. Geologists have not yet fully explained the origin of these inorganic structures that grow in size over time. The results obtained from our microbiological investigations allowed us to discover that microorganisms play a crucial role in forming these masses. In the investigations of the structures and microbial communities within the stones, we identified specific bacteria that actively contribute to forming minerals and isolated bacteria that can form biominerals. These findings deepen our understanding of natural processes involved in the formation of these structures and show their potential for several applications (*e.g.*, building materials or cultural heritage preservation).

1. Introduction

Calcareous deposits (*i.e.*, concretions, clasts, nodules, limestone, or caliche) comprise calcium carbonate ($CaCO_3$) and other minerals such as iron oxides and silica. These deposits are commonly present in sedimentary environments, often compact and mostly spherical, and can vary from a few millimetres to several meters in diameter [1]. Various microbial activities can induce their formation via the precipitation of calcium carbonate (*i.e.*, photosynthesis, ammonification, denitrification, organic acid conversion, urea hydrolysis, and sulphate reduction) [2]. During precipitation, organisms can secrete one or more metabolic products (CO_3^{2}), which react with ions (Ca^{2+}) in the environment, forming calcium carbonate crystals [3]. Biomineralisation can form hydrated crystalline phases such as monohydrocalcite, hexahydrocalcite, amorphous calcium carbonate and different $CaCO_3$ phases such as calc, aragonite, and vaterite. The precipitate is formed in three stages. The first stage involves the formation of amorphous calcium carbonate (unstable and highly soluble). The second phase consists of the formation of vaterite (still unstable). The last phase forms stable calcite [4].

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Microbial autotrophic or heterotrophic pathways can induce $CaCO_3$ precipitation. Autotrophic pathways leading to $CaCO_3$ precipitation include the activities of methanogenic archaeobacteria, anoxygenic photosynthetic bacteria, and cyanobacteria. These bacteria use CO_2 from the calcium ion-rich environment as a carbon source [5]. Heterotrophic pathways linked to the nitrogen cycle involve amino acid ammonification, dissimilatory nitrate reduction, and urea degradation. These reactions raise pH, and the carbonate-bicarbonate balance shifts toward carbonate ions [5]. In the heterotrophic pathways linked to the sulphur cycle, sulphate is reduced to sulphide. The metabolic CO_2 is released by sulphate-reducing bacteria that raise the pH and cause calcium carbonate precipitation [6]. The surface structure of bacterial cells plays an important role, serving as sites of ion adsorption. Bacterial surfaces serve as nucleation centres for crystal growth, facilitated by the high surface-to-volume ratio and interaction with the surrounding environment. Because the surface is rich in negatively charged functional groups, it acts as an interface for ion exchanges, precipitating metal and mineral ions [2].

Numerous bacterial species participate in calcium biogeochemical cycles and precipitate carbonates in various environments (*e.g.*, soil, saline, and freshwater systems) [7]. Bacteria participate in the creation of several calcium carbonate sediments, deposits, and rocks [8–11]. From these structures, the isolation of biotechnologically valuable bacteria can also be achieved. Biomineralising bacteria application is progressing in various fields, including construction and bioremediation [12]. Carbonatogenic bacteria are being researched to produce "bio--bricks" with higher compressive strength, water absorption, salt resistance, and fire resistance than standard ones [13]. They are also used in construction to restore cementitious mortar, consolidate sandy matrices and limestone material, and fill pores and cracks in concrete [14]. Several techniques have advanced in recent years to reduce degradation and repair various fractures by using calcifying bacteria (*Bacillus, Pseudomonas, Acinetobacter*, and *Micrococcus* [15] together with nutrients to reduce the time of deposition and solidification [6]. Recent research demonstrated that some microorganisms involved in calcium biomineralisation could also sequester other chemical pollutants from solution, and some of these appear to be effective in various contaminated matrices such as water, industrial effluents, soil, and sediments [16].

This study focused on “living stones” calcareous deposits sampled from three sites, one located in Romania and two in Italy. Over a century ago, the Romanian geologist Gheorghe Murgoci coined the term Trovants to describe these concretionary sandstone formations found; the geological reserve at Costesti, Romania, contains a diverse collection of Trovants in size and shape [17]. We hypothesised a biogenic origin and an active biomineralising microbial community due to their unusual, rounded shapes and growing sizes. Therefore, we sought to study the possible microbial involvement in the erratic mass formation and the suitability of these deposits as an isolation source of biomineralising bacteria. A multidisciplinary geomicrobiological approach was used to study the samples collected. The microbial involvement in the erratic mass formation was studied by microbiota community characterisation by DNA extraction and 16S rRNA gene metabarcoding. Saline suspensions of the samples were obtained to study the *in vitro* regeneration. Natural and *in vitro* regenerated samples were also investigated for their composition by SEM-EDX analysis. Deposits were also processed to isolate biomineralising bacteria. Isolated strains were identified by bacteriological tests and 16S rRNA gene barcoding and phylogenetic analysis.

2. Methods

2.1. Sampling sites

The first sampling site was an unprotected area near the Romanian “Muzeul Trovanților” (Costesti, 45°08′15.6″N, 24°04′09.5″E). At this site, two shape types were collected: round Trovants (CMT) and long Trovants (CLT). The other sampling sites were Italian locations where "living stones"-like formations were observed: Caccuri (39°13′32.5″ N 16°45′32.4″ E) and Monte Mario (41°92′19″ N; 12°45′28″ E) (SC and RC, respectively). Three replicates were collected per site using sterile tools. Samples were placed in DNase-free plastic bags and kept at 4–10 °C in polystyrene containers for the transfer. Samples were immediately processed once in the laboratory. Rock fragments were collected with a sterile chisel under a laminar flow hood. The fragments were reduced to fine powders with sterile mortar and pestle. Unused fragments were stored at −80 °C for further analyses.

2.2. 16S rRNA gene metabarcoding

The NucleoSpin® Soil kit (Macherey Nagel, Germany) manufacturer’s instructions were followed for genomic DNA extraction using 500 mg of powdered samples obtained as described in Section 2.1. For each sample, the extraction was repeated three times. Extracted samples were spectrophotometrically and fluorometrically evaluated using a Nanodrop spectrophotometer (Thermo Scientific™) and a Qubit fluorometer (Thermo ScientificTM), employing Qubit dsDNA HS Assay Kit (Invitrogen™) to determine DNA content and purity. The replicates were mixed in an equimolar proportion and sequenced on the Mi-Seq Illumina platform, using the approach previously reported [11] (Bio--Fab Research, Italy). The reads were examined for quality and counted after they had been filtered. QIIME2 (qiime2–2020.2 version) was used to assemble ASV (Amplicon Sequence Variant) using the DADA2 plugin [18]. The V3-V4 specific area was taken from the 16S file retrieved from the SILVA 138 database (https://www.arb-silva.de/, accessed in October 2021) and used to train the classifier using the fit-classifier-naive-bayes plugin. For the taxonomy assignment, a 97 % similarity was used.

2.3. In vitro regeneration tests

All experiments were conducted using natural silica sand (Tegolaia Srl, Treviso, Italy) sieved at 0.5 mm, sterilised in autoclave for 20 min at 120 °C, and oven-dried at 100 °C for 72 h. Natural commercial bottled oligomineral water (Ca^{2+}, 20.4 mg L^{-1}; Na^{+}, 1.9 mg L^{-1}; SiO_2, 5.4 mg L^{-1}; HCO_3^- 57.4 mg L^{-1}; NO_3^-, 1.5 mg L^{-1}; Mg^{2+}, 1.8 mg L^{-1}; K^{+}, 1.6 mg L^{-1}; SO_4^{2-}, 16.1 mg L^{-1}; NO_2^-, < 0.002 mg L^{-1}; F^-, 0.2 mg L^{-1}) was used to set up the experiments. Containers were one-third filled with sand. Water was added up to half the container. Fine powders obtained in Section 2.1 were homogenised in a bag mixer with saline solution (8.5 %) for 1 hour. The liquid phase of the resulting suspension was used to inoculate the systems. The inoculated systems were incubated at 28 °C for three months and at 4 °C for seven months. Experiments were carried out in triplicate. A control (without saline inocula) was also set up for each system.

2.4. SEM-EDX analyses

SEM observations were carried out to investigate the microstructural morphology of natural samples (*i.e.*, rock fragments collected as described in Section 2.1) and bioliths and find the presence of microbial structures. EDX analysis allowed the microbe-matrix associations and elemental distribution characterisation. The list of the samples investigated by SEM-EDX is described in Table 1. The sand used for the *in vitro* experiments was also studied. All the samples were uniformly spread directly on adherent tabs and analysed without sputtering. SEM observations were performed with a ZEISS Gemini SEM 500 equipped with EDX Microanalysis, OXFORD Aztec Energy with INCA X-ACT PELTIER COOLED ADD detector. SEM-EDX imaging was performed in the back-scattered electron mode at low vacuum (20 Pa), a 15 kV high voltage, and a working distance of 8.5 mm.

Table 1
Natural concretions were sampled for SEM-EDX analysis.

Sample ID	Sample origin	Sample shape	Sample size	Fragment portion
CMTO	Romania	Round	Medium (7 cm)	Outer layer
CMTI				Brittle nucleus*
CLTO		Long	large (25 cm)	Outer layer
CLTI				Inner part**
SCO	Caccuri	Round	large (25 cm)	Outer layer
SCI				Inner part**
RCO	Monte Mario	Long	large (25 cm)	Outer layer
RCI				Inner part**

In the Table: Romanian samples, round Trovants (CMT) and long Trovants (CLT), Caccuri (SC), Monte Mario (RC); outer, surface of the sample; inner, core of the sample; * 3–4 cm depth; **5–7 cm depth.

2.5. Isolation of microorganisms

Fine powders were homogenised in a bag mixer with saline solution (8.5 %) for 1 hour. Serial dilutions were plated in Petri dishes containing B-4 medium [19]. Plates were incubated at 28 °C for 7 days. Several colony transfers provided pure isolates. The different characteristics related to colony and crystal morphology were monitored during growth. For each isolate, shape, thickness, margin, colour, size, transparency, texture, and surface were visually studied. In the case of calcification, crystals are described according to their size, shape, and quality (three quality levels expressed through the symbols +, ++, and +++ were defined), presence of aggregates, and position (on the colony, in proximity or far away from it). Gram staining and catalase tests were performed for bacteriological classification in Gram-positive or Gram-negative and catalase-positive or catalase-negative to identify species of bacteria.

2.6. 16S gene barcoding and phylogenetic analysis

Calcifying bacterial isolates with fast crystal precipitation were subjected to molecular characterisation by 16S barcoding (Microbion, Verona, Italy). Using the local base alignment search (BLAST) and sequence identity values greater than 99.8 %, sequences were compared to those in the NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/; accessed 3 May 2022) genetic database. Clustal W was used to align sequences, and a phylogenetic tree was constructed using the Maximum Likelihood method. *Pirellula staleyi* (Accession: NR_119,130.1) was used as an outgroup, and a Maximum Likelihood bootstrap analysis was performed with RAxML [20], using MegAlign Pro 17 to repeat the data matrix 1000 times (DNASTAR, Lasergene, Madison, Wisconsin, USA).

3. Results

3.1. 16s rRNA gene metabarcoding

The metagenomic sequencing results showed similarities and differences among the samples. As shown in Table 2, the outer samples had higher diversity and more individuals than the inner ones. CMTO had the highest number of ASVs, but low diversity indexes were observed in these samples. RCO had the highest biodiversity, followed by RCI. Regarding community composition, common representative taxa were identified at the highest taxonomic levels, but these similarities were less pronounced at lower levels. Fig. 1 summarises the composition of the microbial communities at the phylum and genus levels. Specifically, all samples shared the presence of ASVs assigned to Proteobacteria (syn. Pseudomonadota) and Actinobacteriota at the phylum level. However, their abundances varied within samples, ranging from 7 to 70 % for Proteobacteria and 23–72 % for Actinobacteriota. The Firmicutes (syn. Bacillota) assignments were only found in the outer samples. Acidobacteriota and Gemmatimonadota were exclusively described in SCI and RI, respectively. Cyanobacteria were abundant in CLTI, while Verrucomicrobiota were found exclusively in RCI. Only the association with the *Sphingomonas* genus was ubiquitous at the genus level. Most samples contained ASVs assigned to 0319–6G20, *Acinetobacter*, and *Caulobacter*, but these lineages were not found in CLTI, RCO, and RCI samples. Unknown lineages were found in all the samples except for CMTO. As shown in Table 3, uncultured and unknown assignments were associated with Proteobacteria and Actinobacteriota lineages.

3.2. SEM-EDX analyses of natural samples

SEM-EDX micrographs obtained from natural samples are shown in Fig. 2. Elemental analyses of samples revealed the prevalence of Si, clearly indicating the siliceous nature of the matrix in contrast to the calcareous nature of the filling and covering concretions of calcium (Ca). Aluminum (Al), iron (Fe), magnesium (Mg), and potassium (K) were also present. The comparison of the different maps obtained by EDX analysis showed a more diverse composition of the outer samples, with the siliceous structure embedded in different Ca structures. The Italian Caccuri sample presented Ca aggregated of greater size with the presence of Fe and Mg. The Romanian sample showed thinner Ca concretions with Al inclusions. The presence of organic inclusions was also detected in this sample.

Fig. 3 shows the micrographs obtained from the investigations of Romanian samples. From the panels of outer samples, it is possible to observe the presence of loose deposits with calcified microbial biofilms for both round and long shapes. Inner sections showed a different structure, with small carbonate particles. Moreover, the long sample (CLTI) was characterised by a more compact structure than the round structure (CMTI). All micrographs show the presence of perforations, holes, and calcified filaments that may be associated with the trace of fossil cellular forms. These biosignatures are included in overlapping sedimentary layers to form multilayer cemented structures.

Fig. 4 shows the micrographs of the observations of Italian samples. As described for Romanian samples, the observations of the outer and inner sections underlined the presence of calcified filamentous bacteria and microbial biofilms, filling multi-layered structures incorporated in the concretion with bacterial imprints. The calcified microbial structures in these samples were more filamentous than those in the Romanian sample.

3.3. In vitro regeneration tests and SEM-EDX analyses of bioliths

Laboratory regeneration tests resulted in the formation of bioliths. Table 4 reports the summary of the characteristics of the bioliths regenerated. The biolith sizes were 2–3.5 mm, and shapes were round,

Table 2
Alpha-diversity metrics obtained for natural samples.

	CMTO	CMTI	CLTO	CLTI	SCO	SCI	RCI	RCO
Taxa	221	87	209	100	158	148	120	219
Number of ASVs	34,287	4581	12,931	9583	17,489	6093	2675	4065
Simpson 1-D	0.59	0.80	0.87	0.93	0.84	0.95	0.97	0.99
Shannon H'	2.04	2.87	3.61	3.34	3.16	3.76	4.26	4.98
Chao-1	221	87	209	100	158	148	120	219

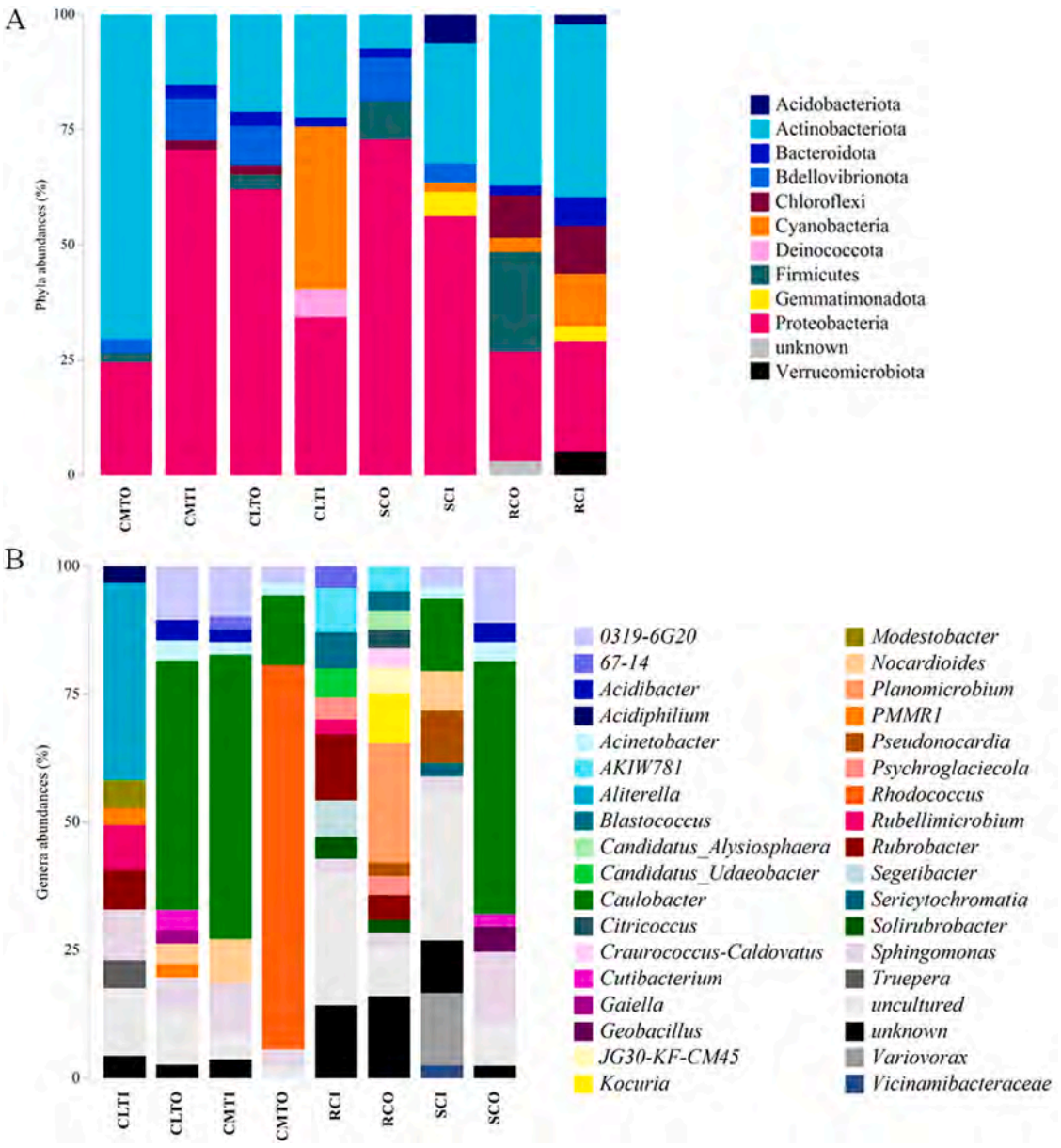

Fig. 1. Taxonomy barplots showing the abundances (%) of main phyla (A) and genera (B) in each sample (cut off 2 %).

except for those regenerated from the Long Romanian sample (CLT), which were of an elongated shape. SEM observation of the control showed the presence of sand particles of different sizes and clean surfaces. EDX analysis (EDX elemental chromatogram) has confirmed the composition of aluminium silica with the presence of Na and Mg in small amounts. K, chlorine (Cl), Fe, and Ca were also present in minor amounts. Fig. 5 also summarises the results obtained using/via/through SEM-EDX analyses of *in vitro* regenerated samples. SEM observations of the bioliths showed aggregated sand particles and the presence of abundant microbial structures. Sand particle aggregation was made possible by the activity of microbial biofilms that formed calcified bridges between silica particles and fasciculate crystals. EDX analysis allowed us to identify the presence of carbonates inside the bridges with a differential ability of the inocula to aggregate several other elements. Within Romanian and Caccuri samples, Ca was associated with K to form the bridges. Meanwhile, for Monte Mario samples, Ca was associated with P and sodium (Na).

3.4. Isolation of microorganisms

Nineteen isolates displayed biomineralisation abilities. Seven isolates were obtained from the Romania sample, five from Caccuri, and seven from Monte Mario. 63 % of the strains were Gram-negative, and 90 % of the colony types identified were of a circular shape and smooth, transparent texture, while some of the colonies obtained from the Caccuri sample were crusty and opaque. The crystals formed within the colonies were primarily of round and regular shapes. There was a change in shape related to the time required for their production, indicating the possible presence of crystallisation phases. Most colonies distributed crystals internally and externally, moving them away even from the very edge of the colony. However, Monte Mario isolates deposited crystals only internally. Caccuri isolates formed precipitates after 24–48 h. For the other isolates, 3–6 days were necessary to produce aggregates. Crystal precipitation by strains isolated from the initial samples was faster for strains S3, TR1, TR2, T3, R6 and TR8 than the other thirteen strains obtained from isolation.

Table 3
Taxonomic assignments of uncultured and unknown lineages on the family level.

Taxa	CMTO	CMTI	CLTO	CLTI	SCO	SCI	RCO	RCI
Uncultured								
Acidobacteriota	0.0	0.0	9.5	0.0	2.5	10.9	0.0	2.3
Actinobacteriota	16.8	20.4	35.1	51.5	14.1	21.8	27.8	31.9
Armatimonadota	0.4	0.0	2.3	0.0	0.0	0.9	0.0	2.4
Bacteroidota	2.4	2.9	4.5	0.0	5.8	0.8	0.0	0.5
Chloroflexi	0.9	0.0	1.6	0.0	4.2	0.0	3.7	1.6
Cyanobacteria	0.0	0.0	0.0	1.0	0.0	0.3	13.3	21.4
Firmicutes	2.6	3.2	2.8	0.0	5.4	0.3	6.1	0.0
Gemmatimonadota	1.8	0.0	1.0	0.0	3.7	16.6	1.6	1.5
Myxococcota	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.7
Patescibacteria	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.6
Planctomycetota	1.1	0.0	1.4	0.0	0.0	0.5	1.6	0.0
Proteobacteria	73.5	73.5	40.9	47.5	62.1	47.9	31.7	36.9
unknown Bacteria	0.0	0.0	0.7	0.0	2.1	0.0	14.2	0.0
Verrucomicrobiota	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Unknown								
Actinobacteriota	20.2	17.7	43.1	26.3	53.1	50.8	31.4	56.2
Bacteroidota	3.8	0.0	0.0	0.0	3.0	0.0	0.0	0.0
Chloroflexi	0.0	0.0	8.1	0.0	0.0	0.0	4.6	0.0
Firmicutes	0.0	7.8	0.0	0.0	15.0	0.0	10.0	0.0
Gemmatimonadota	0.0	0.0	0.0	0.0	0.0	0.0	2.6	2.2
Myxococcota	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.9
Proteobacteria	76.0	74.5	45.1	73.7	20.2	49.2	28.3	39.7
unknown Bacteria	0.0	0.0	3.7	0.0	8.7	0.0	23.1	0.0

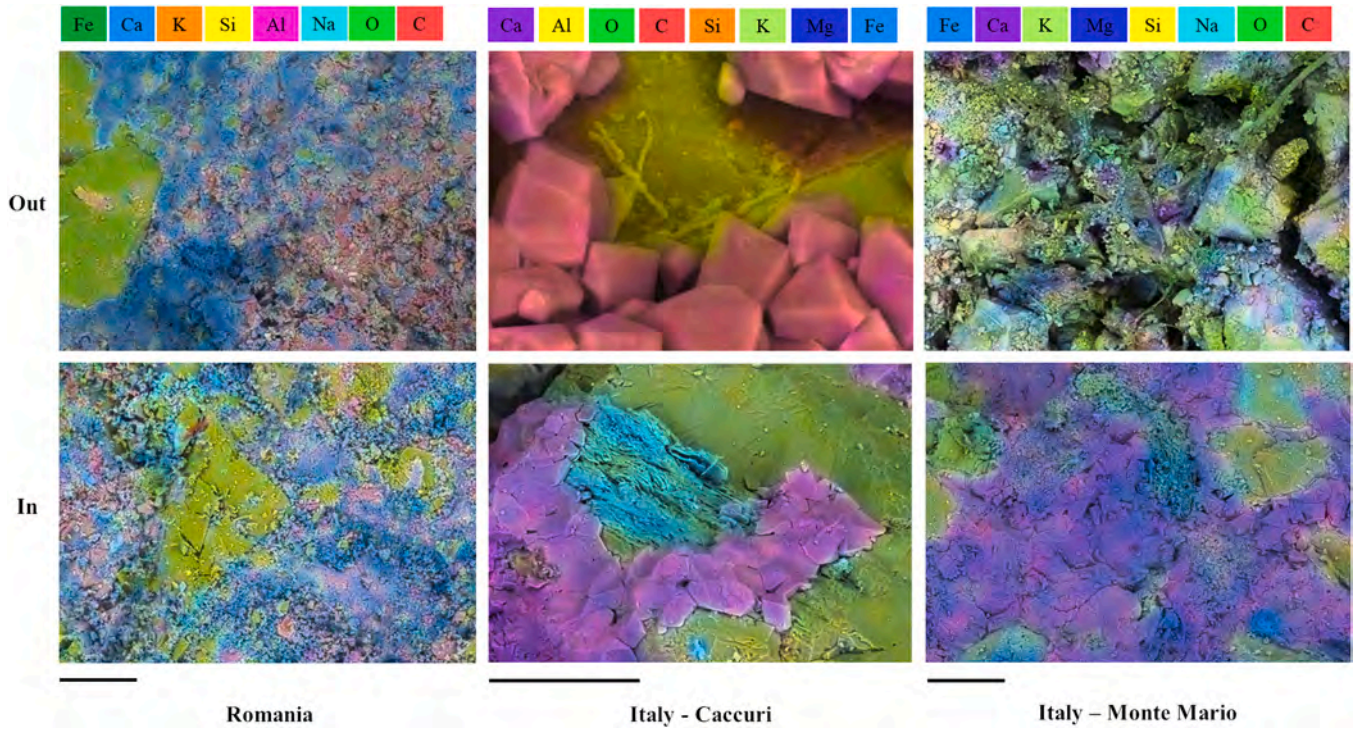

Fig. 2. Elemental map distribution. In each column, the elemental distribution is shown for the outer (top) and inner (bottom) samples collected from each sampling site (one in Romania and two in Italy). For the same column, colour keys are provided above. Scale bars 100 μm.

3.5. 16S barcoding and phylogenetic analysis

The six strains with interesting crystal deposition abilities were further characterised by 16S barcoding. The phylogenetic analysis allowed us to associate bacterial isolates to several species belonging to *Microbacterium, Lysinbacillus*, and *Stenotrophomonas* genera. Namely, isolate S3 was identified as *Microbacterium ginsengiterreae*, isolate TR1 was identified as *Lysinbacillus fusiformis*, and TR2, T3, R6, TR8 were identified as *Stenotrophomonas maltophilia* (identity percentage higher than 99.5 %). Fig. 6 shows the phylogenetic tree obtained for the bacterial isolates.

4. Discussion

Calcium carbonate precipitation is the primary ability that allows microorganisms to be involved in many geological processes, such as the formation of limestone [21]. This important biogeochemical role is enabled by the microbial ability to alter the macroenvironment through autotrophic and heterotrophic pathways. These pathways lead to an increase in pH and a accumulation of inorganic carbon in solution and

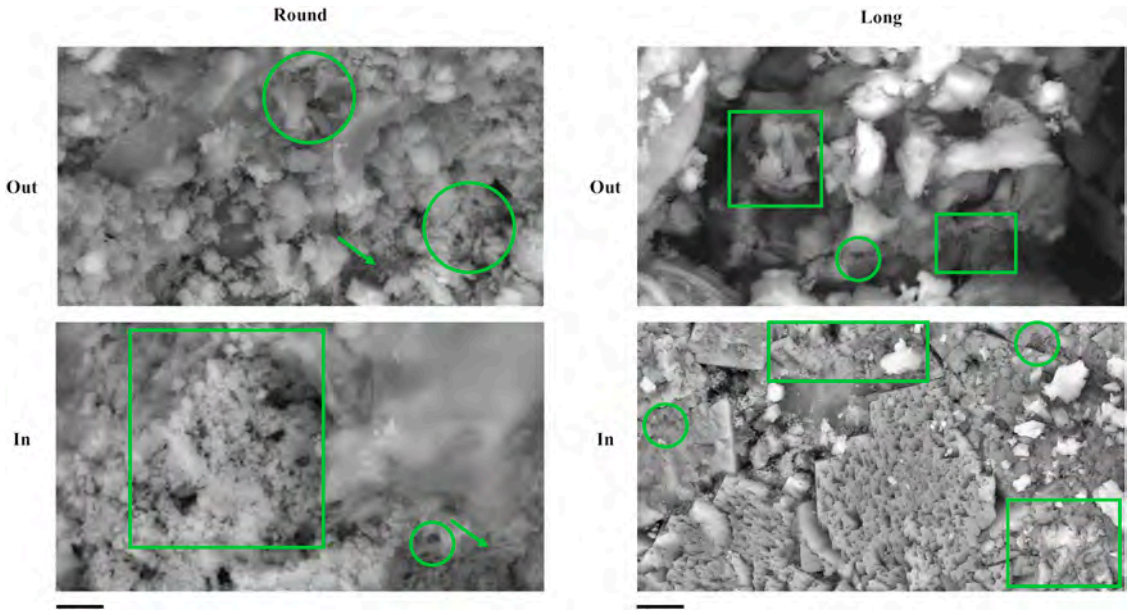

Fig. 3. SEM micrographs were obtained for round (left) and long (right) Romania samples. Scale bars: 1 μm. In the panels are highlighted borings and holes (with circles), filaments (with arrows), and calcified bacterial aggregated (squares/rectangles).

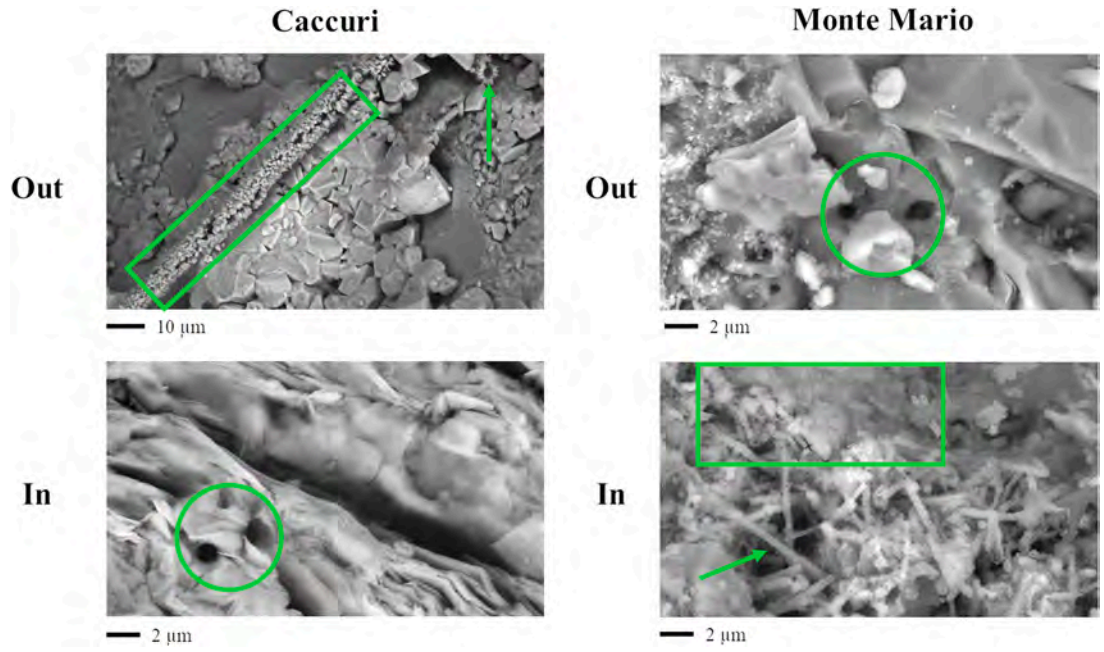

Fig. 4. SEM micrographs were obtained for outer (A) and inner (B) Cuccari and Monte Mario samples. In the panels are highlighted borings and holes (with circles), filaments (with arrows), and calcified bacterial aggregated (squares/rectangles).

Table 4
Characteristics of the bioliths regenerated *in vitro*.

In vitro samples	Inoculum origin	Size (mm)
Romania	Round Trovant	2.5
	Long Trovant	3.5
Caccuri	Round Trovant	3
Monte Mario	Long Trovant	2

precipitation of $CaCO_3$ in an environment rich in calcium [22]. Furthermore, the bacterial cell, having negative charges on the external surface, can bind calcium and act as a crystallisation nucleus [23]. The present study examined the role of microbial communities, specifically bacteria, in forming "living stones".

Geologists have formulated numerous hypotheses on the origin and formation of "living stones". These structures are considered erratic carbonate clasts of variable size, present in a substrate different from the surrounding geomorphology [24]. Several hypotheses emerged, including the action of rivers, exogenous forces, explosions, erosion, and sedimentation processes, which would have caused these stones to move away from their place of origin, glacial transportation being the most plausible [25]. The literature provides many other indications, both in Italy and abroad, of similar concretions of uncertain origin [24,26]. The most recent article describes Romanian formations as high porosity

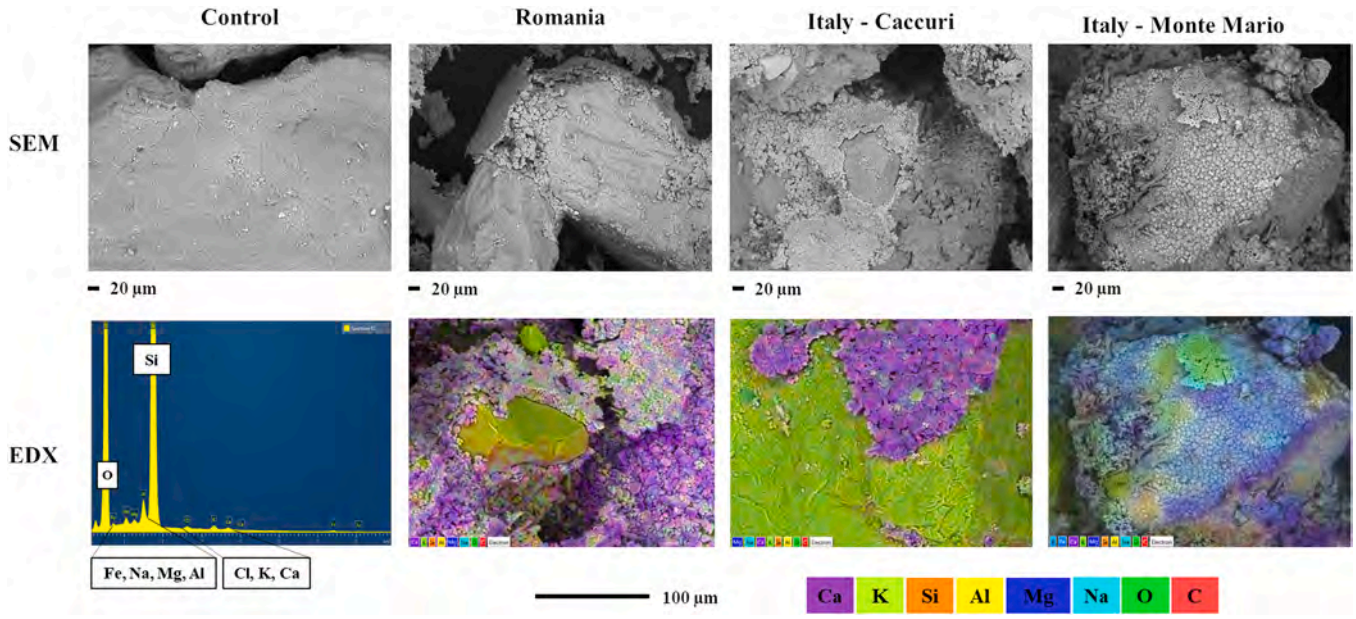

Fig. 5. SEM micrographs and EDX maps were obtained from *in vitro* regenerated samples. Scale bars: SEM, 20 µm; EDX, 100 µm.

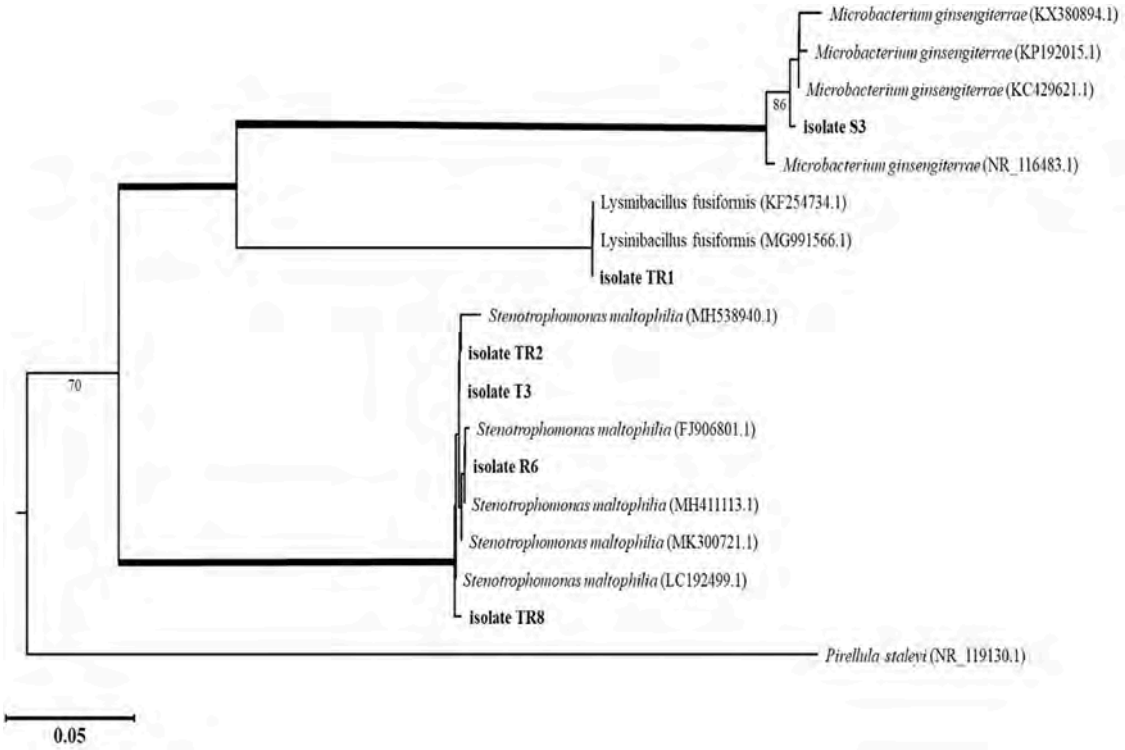

Fig. 6. Phylogenetic analysis of bacterial isolates compared with other isolates (GenBank accession numbers shown in brackets). The Maximum likelihood method was used with a bootstrap consensus tree (from 1000 replicates to represent the distance). Pirellula staleyi (Accession: NR_119,130.1) was used as an outgroup. The number of substitutions per nucleotide site for a unit of branch length is given in the scale bar (0.05).

spherical sandstone concretions developed around a fossil [27].

Based on the results obtained in this study, we propose that microorganisms actively participate in forming these "living stones" erratic deposits. These microorganisms can combine CO_2 absorbed from the atmosphere with the calcium ion dissolved in the waters that permeate the sandy matrix, producing carbonate that cements the interstices between the silicates [28]. This hypothesis is supported by integrating results obtained from metagenomic sequencing, SEM-EDX analysis, and isolation of calcifying bacterial strains.

The 16S rRNA gene sequencing underlined the presence of key similarities among the samples. Beyond the specific microbial communities linked to the diverse environmental conditions of the sampling sites, the presence of the biomineralising phyla was observed: Actinobacteriota [29], Firmicutes (particularly the genus *Bacillus* [30]), Cyanobacteria (photosynthetic) [31], Verrucomicrobiota [32,33], Gemmatimonadota [34], and Proteobacteria [35] (particularly

Acinetobacter [36], *Caulobacter* [37], and *Sphingomonas* [38]).

The metabarcoding analyses also showed the differences among the samples collected from the outer and inner sections. We found a greater diversity in outer samples in line with previous reports. Generally, surfaces harbour more diverse microbial communities due to their significant exposure to external factors and availability of nutrients [39]. The other differences displayed among the different samples were linked to the existing conditions during the establishment and survival of microbial communities on rocks, directly influenced by the physical and chemical properties of the stones, such as their mineral composition, permeability, and pore structure [40]. Environmental factors like water availability and nutrient sources also play essential roles [41].

SEM-EDX analyses provided a further understanding of the structure and elemental composition of the samples. The contrasting siliceous nature of the matrix with the calcareous concretions underlined complex biogeochemical interactions driving the formation of mineralised structures. The different distribution of the minor elements Al, Fe, Mg, and K is lined to the diverse geochemical backgrounds and mineralogical compositions. These mineral characteristics have also been reported in similar studies [42,43] and our previous research [44–46]. The numerous calcified microbial biofilms and filamentous structures, particularly their fossil-like filaments and imprints, observed by SEM confirmed that microbial activity preserves geological features over time [47], providing convincing evidence of the contribution of biomineralisation processes in forming these clasts. In the literature, filamentous bacteria, particularly members of *the Actinobacteria* phylum, were reported to be possibly involved in the genesis of calcareous *speleothems* [48]. *Actinobacteria* could be engaged in $CaCO_3$ precipitation through (i) active transport of calcium ions across the cellular membrane by $Ca^{2+}/2H^{+}$ antiporter system (ChaA), (ii) nitrogen cycle-related pathways (ureolysis, ammonification and dissimilatory reduction of nitrate), and (iii) reversible hydration of CO_2, as shown by silico search to identify genetic predispositions for carbonate precipitation in *Streptomyces* [49].

The potential of inocula obtained from the natural samples to induce the formation of bioliths was demonstrated by *in vitro* regeneration tests, which confirmed the microbial role in the formation of natural clasts. The different ability of inocula to aggregate elements such as Ca, K, phosphorus (P), and Na highlights the specificity of microbial-mediated and induced mineralisation processes. The successful isolation and characterisation of calcifying bacteria also underlined this aspect. The phylogenetic analysis of isolated strains identified key species within the *Microbacterium, Lysinbacillus*, and *Stenotrophomonas* genera, characterised by a wide environmental adaptation and metabolic versatility [50–54].

The biomineralization abilities of strains belonging to the *Microbacterium, Lysinibacillus*, and *Stenotrophomonas* genera have been widely reported. *Microbacterium* spp. are known to produce calcite and vaterite, two polymorphs of calcium carbonate, through their metabolic activities [55]. These bacteria can induce carbonate precipitation, which has potential applications in construction materials, environmental remediation, and carbon sequestration [56]. For example, *Microbacterium* sp. GM-1, produced urease and induced calcium carbonate precipitation in a metabolic process [57]. Additionally, *Microbacterium* spp. have been implicated in the formation of stromatolites, layered carbonate structures constructed by microbes [2] and the biomineralization of hydroxyapatite, a calcium phosphate mineral with biomedical applications [58].

Similarly, *Lysinibacillus* spp. have been reported to induce the formation of hydroxyapatite, a calcium phosphate mineral [59]. *Lysinbacillus fusiformis* is reported as an ureolytic calcium carbonate producer [59–61]. However, strains belonging to this genus facilitate biomineralization through the secretion of extracellular polymeric substances and the modulation of local pH and ion concentrations [60]. For instance, *Lysinibacillus* sp. WH [62] *Lysinibacillus boronitolerans* YS11 [63] were found to form calcite through their metabolic activities and via non-ureolytic process.

Stenotrophomonas spp. have been observed to precipitate a range of minerals, including calcium carbonates, and inhabiting stone monuments [64]. *Stenotrophomonas* spp. initiate biomineralization through the binding of calcium ions to cell surfaces and the subsequent nucleation and growth of mineral phases [65]. *Stenotrophomonas maltophilia*, has also been described for its ability to form calcium carbonate crystals of different sizes [66].

The rapid crystal precipitation catalyzed by specific strains suggests potential applications in bioengineering and biotechnology, where controlled biomineralisation processes are desirable [67]. Isolates with mineralisation abilities can be used, for example, to produce self-healing concrete [68] for construction purposes, in bioremediation applications [56], as soil [69] consolidating agents [70], cultural heritage [71], and in a wide range of biomaterials with multiple applications (*e.g.*, biomedical [72]).

5. Conclusions

The current study investigated the “living stones” erratic deposits sampled in three different Romanian and Italian locations using molecular, microscopic and culturing approaches. Metagenomic sequencing, SEM-EDX analysis, and culturable approaches were combined to comprehensively understand the microbial diversity community structure and mineralisation processes. SEM-EDX analyses revealed the presence of biosignatures, elemental compositions, and associations, suggesting a past biomineralisation activity. 16S rRNA gene sequencing results underlined the presence of biomineralising bacterial communities. Laboratory regeneration tests confirmed the ability of natural inocula to form bioliths. These results demonstrate an active microbial community inhabiting these calcareous structures that can increase their dimensions over time. This aspect was also shown by isolating bacterial strains with calcifying abilities and metabolic versatility, which proves these structures can serve as useful sources of microbes with interesting potential biotechnological and bioengineering applications. Future research could enhance understanding of the metabolic pathways involved in the formation of "living stones”; these aspects may be unveiled by integrating microbiological studies with geological characterisations (*e.g.*, metagenome functions - from bioinformatic predictions to RNA analysis - and deposit characterizations - from texture to Raman Microscopy). In addition, future studies might be directed towards clarifying the potential of isolated strains in biotechnological and bioengineering contexts (*i.e.,* genetic, metabolic, biochemical and physiological properties).

Data availability

The data that support the findings of this study are available upon request from the corresponding authors.

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CRediT authorship contribution statement

Beatrice Farda: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Amedeo Mignini:** Writing – original draft, Visualization, Investigation, Formal analysis. **Rihab Djebaili:** Investigation, Formal analysis, Data curation. **Paola Cacchio:** Writing – original draft, Visualization, Investigation. **Maddalena Del Gallo:** Writing – review & editing, Validation, Conceptualization. **Marika Pellegrini:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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