



A time travel story: metagenomic analyses decipher the unknown geographical shift and the storage history of possibly smuggled antique marble statues

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Abstract

In this study, three possibly smuggled marble statues of an unknown origin, two human torsos (a female and a male) and a small head, were subjected to molecular analyses. The aim was to reconstruct the history of the storage of each single statue, to infer the possible relationship among them, and to elucidate their geographical shift. A genetic strategy, comprising metagenomic analyses of the 16S ribosomal DNA (rDNA) of prokaryotes, 18S rDNA of eukaryotes, as well as internal transcribed spacer regions of fungi, was performed by using the Ion Torrent sequencing platform. Results suggest a possible common history of storage of the two human torsos; their eukaryotic microbiomes showed similarities comprising many soil-inhabiting organisms, which may indicate storage or burial in land of agricultural soil. For the male torso, it was possible to infer the geographical origin, due to the presence of DNA traces of *Taiwania*, a tree found only in Asia. The small head displayed differences concerning the eukaryotic community, compared with the other two samples, but showed intriguing similarities with the female torso concerning the bacterial community. Both displayed many halotolerant and halophilic bacteria, which may indicate a longer stay in arid and semi-arid surroundings as well as marine environments. The microbiomes retrieved from each statue showed to be very specific, but some individual members showed to be biological markers for the geographical regions through which the pieces traveled and for the conditions under which they were stored.

Keywords Metagenomics · Microbiome · Cultural heritage objects · Geographical shifts · Storing conditions

Introduction

Each piece of art harbors a specific and absolute unique pile of information about its own history. Every step in the history of an object leaves its own genetic information: the plant from which a canvas or paper was made, the organic binder used to prepare a paint, the animal from which a parchment was made, and the hands by which an object was manufactured and

through which it was passed during its history (Bower et al. 2010; O’Sullivan et al. 2016). In addition to the information given by the material, each object carries a specific microbiome (Teasdale et al. 2017), mainly consisting of bacteria and fungi. The microflora again can be used as an indicator for the geographical and climatic regions through which a piece of art traveled, the conditions under which it might have been stored, or the soil in which it might have been buried. Microbes—as humans—leave a specific mark on and in an object: their unique fingerprint represented by DNA (Burger et al. 2000).

Nowadays, using high-throughput molecular methods, it is possible to extract and to analyze even smallest traces of DNA from an object, to amplify these fragments, to visualize, and by the help of bioinformatics and international databases, to assign single molecules of DNA to a certain plant, a bacterium, and a fungus or even to humans (Fu et al. 2013). The compilation of these data results in an individual “biological pedigree.” The biochemical and molecular methods applied

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are the same that are in use in forensics—for example to identify an offender—or in medicine, where the microbiome of the human skin is currently in the focus of research (Schommer and Gallo 2013).

To date, molecular analyses applied on cultural heritage studies have mainly focused on the identification of microbial communities associated with specific biodeterioration phenomena displayed by different materials. Such studies have enabled the implementation of conservative and disinfecting treatments as well as the monitoring during and after the conservation treatments of valuable objects (Sterflinger and Piñar 2013). Recently, the ongoing scientific and technological progress has led to metagenomics, metatranscriptomics, metaproteomics, and metabolomics, which are giving a more complete overview of the present microorganisms, their activity, and the expressed proteins and produced metabolites in a specific environment. These state-of-the-art methodologies are now developing at a very fast pace, and next-generation sequencing (NGS) methods are becoming applicable in the field of cultural heritage (Adamiak et al. 2017, 2018), allowing to answer the two most basic questions in microbial ecology: “who is out there?” and “what are they doing?”

Nevertheless, the biological information contained in pieces of art has the potential to answer many other interesting questions that may arise when dealing with cultural heritage objects. The state-of-the-art genetic analyses can, for instance, provide amazing insights into the geographical origins of artifacts (geolocation), the selection of materials at the time of manufacturing, the storage conditions, the species composition of materials, and the use history of an object (Bower et al. 2010; Teasdale et al. 2017). These answers may help to understand many open questions in a variety of fields, such as archeology, history, restoration, philology, and last but not least, criminology. In some cases, curators and custom officers are confronted with valuable smuggled objects of an unknown origin. In these cases, analyses that could enable to make a grouping of some of the objects, as well as to infer the conditions of their storage and their geographical shift, could help to reconstruct the history of these objects, a kind of time travel.

In this study, three possibly smuggled marble statues of an unknown origin were temporarily deposited in the Museum of Art History (KHM) in Vienna, Austria. Curators and conservators of the museum put many efforts to infer the origin and the possible relationship among the three statues through petrological and morphological analyses, without success. Therefore, they considered the possibility of constituting an interdisciplinary team, recruiting microbiologists and bioinformaticians, in order to include a genetic strategy, which could provide novel insights into these questions. Metagenomic analyses of the 16S ribosomal DNA (rDNA) of prokaryotes, 18S rDNA of eukaryotes, as well as internal transcribed spacer (ITS) regions of fungi were performed by using the Ion Torrent sequencing platform, and the generated

data were used to infer the storage history and the geographical shift of the samples, as well as to consider the possible relationship among them.

Material and methods

Sampling

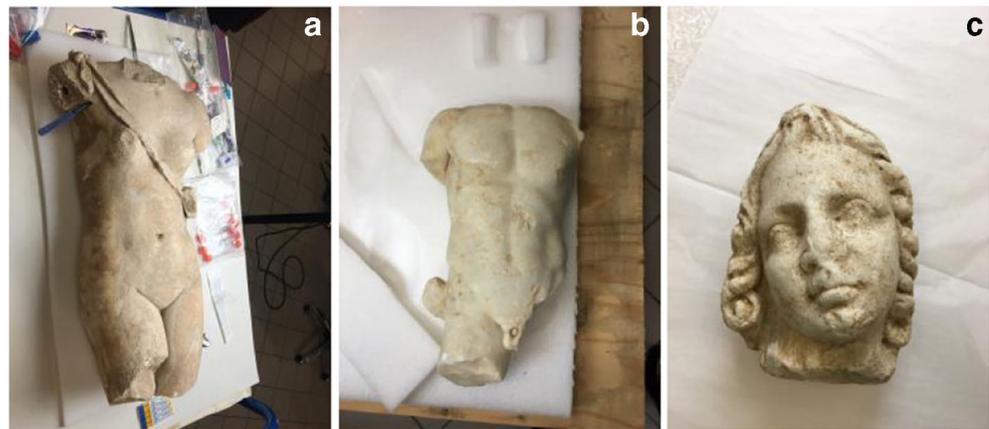
Three statues of unknown origin, two of them representing human torsi, one female (S1) and one male (S2), and the third one representing a small young girl head (S3), were deposited in the KHM, Vienna, Austria (Fig. 1). Samples were taken from the surface of the three statues with the help of sterile scalpels and needles by gently scratching. Approximately 40–60 mg of material, mainly powdered material, dust, dirt, and even some textile fibers, was collected independently from four different points of each statue without damaging the marble stone. The material collected from each point was immediately placed into sterile Eppendorf tubes. Later, in the laboratory, the material collected from each point of the same statue was pooled in order to obtain about 160–250 mg of material for DNA extraction analyses per statue. DNA extraction was performed at the next day of sampling.

Quality assurance (QA), in order to avoid any kind of contamination or bias, was performed by using only high-quality reagents for DNA extraction, including single-use manufactured vials to avoid cross-contamination as well as high-quality reagents for down steps, as PCR amplification and sequencing. All reagents used for library preparation and sequencing are tested and single packet/reaction, to avoid contaminations. In addition, the steps of (1) DNA extraction and amplification, (2) the DNA library preparation, and (3) the sequencing were performed in three separated laboratories dedicated only and exclusively to one of the mentioned steps. DNA extraction was performed in a laminar flow bank only used for ancient DNA (aDNA) preparations, and PCR reactions were performed in a PCR workstation equipped with UV lamps.

DNA extraction

Total DNA was isolated directly from the powdered material collected and pooled from each statue (160–250 mg/statue, as mentioned above) using the FastDNA SPIN Kit for soil (MP Biomedicals, Illkirch, France) as recommended by the manufacturers. The DNA concentrations were assessed by using the Qubit 2.0 fluorometer (Thermo Fisher Scientific, MA, USA), which uses fluorescent dyes to determine the concentration of nucleic acids, with the Qubit dsDNA HS Assay Kit.

Fig. 1 The three antique marble statues. **a** S1, female torso. **b** S2, male torso. **c** S3, small head



PCR amplification

All PCR reactions were executed in a Bio-Rad C1000 Thermal Cycler. PCR reactions performed to amplified eukaryotes and fungi were carried out with the 2× PCR Master Mix from Promega (Vienna, Austria) (50 units/ml of Taq DNA Polymerase supplied in an appropriate reaction buffer (pH 8.5), 400 μM dNTPs, 3 mM MgCl₂) diluted to one time, to which 12.5 pmol/μl of each primer (stock 50 pmol/μl; VBC-Biotech, Vienna, Austria) and 400 μg/ml BSA (stock 20 mg/ml; Roche Diagnostics GmbH, Basel, Switzerland) were added.

Quality control (QC) was performed in order to exclude the possibility of cross-contamination, and in each PCR reaction, a negative control was included (no DNA template).

For the amplification of the eukaryotic 18S rDNA, fragments targeting the V4 region were amplified with the primers 528F (GCGGTAATTCAGCTCCAA) and 706R (AATCCRAGAATTTACCTCT). The thermocycling program was as follows: 5-min denaturation at 95 °C, followed by 35 cycles of 1-min denaturation at 95 °C, 1-min annealing at 53 °C, and 1-min extension at 72 °C. Five-minute denaturation at 72 °C was performed as a final extension step. Reactions were performed in 2× 50 μl, and a 3.5-μl DNA template was added to each reaction tube. The duplicate amplicons of each sample (2× 50 μl) were pooled, and 7 ml was visualized on 2% (w/v) agarose gels, stained in an ethidium bromide solution (10 mg/ml), and documented using a UVP documentation system (Bio-Rad Transilluminator; Universal Hood, CA, USA). The remaining PCR reaction was purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany).

For the amplification of fungal ITS regions, fragments of 450–600 bp, corresponding to the ITS1 and the ITS2 regions, and the 5.8S ribosomal RNA (rRNA) gene situated between them were amplified with the primer pairs ITS1 forward and ITS4 reverse (White et al. 1990). The thermocycling program was as follows: 5-min denaturation at 95 °C, followed by 35 cycles of 1-min denaturation at 95 °C, 1-min annealing at 55 °C, and 1-min extension at 72 °C. Five-minute

denaturation at 72 °C was performed as a final extension step. Thereafter, the obtained PCR products were further amplified in a nested PCR using the forward primer ITS1 and the reverse primer ITS2. The cycling scheme was the same as before, but the annealing *T* was 58 °C, and the reactions were performed in 2× 50 μl volume containing each 3.5 μl of template DNA. The duplicate amplicons of each sample (2× 50 μl) were pooled and visualized as mentioned above. The remaining PCR reaction was purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany).

For the amplification of the prokaryotic 16S rDNA, the DNA was amplified using the Ion 16S Metagenomics Kit (Thermo Fisher Scientific, MA, USA). The kit includes two primer sets that selectively amplify the corresponding hyper-variable regions of the 16S region in bacteria (primer set V2-4-8 and primer set V3-6,7-9). After amplification, the reactions obtained from each sample with the two primer sets were pooled and mixed with Agencourt AMPure XP reagent (Beckman Coulter, CA, USA) for purification, using a magnetic rack as described by the manufacturers.

The concentration of all the purified amplicons (prokaryotes, fungi, and eukaryotes) was assessed with the Qubit 2.0 Fluorometer (Thermo Fisher Scientific, MA, USA).

Library construction and quantification

DNA libraries were constructed using the Ion Plus Library Kit for the AB Library Builder System (Thermo Fisher Scientific, MA, USA) following the library preparation protocol for short amplicons, provided by the manufacturers. The amplicons were processed as barcoded libraries, by using the P1 adapter and the barcoded A adapters provided in the Ion Xpress Barcode Adapter Kit. The adapters were diluted according to the amount of DNA input, as recommended by the manufacturers.

The resulting fungal and eukaryotic DNA libraries were subsequently quantified by qPCR using the Ion Library Quantitation Kit (Thermo Fisher Scientific, MA, USA) and the prokaryotic DNA libraries using the Ion Universal Library

Quantitation Kit (Thermo Fisher Scientific, MA, USA). Reactions were performed as described by the manufacturers, to calculate the dilution factor of each library for a final concentration of 50 pmol. After individual quantitation, the barcoded libraries of each sample (amplified with prokaryote-, eukaryote-, and fungus-specific primers) were pooled in equimolar amounts to ensure equal representation of each barcoded library in the sequencing run.

Template preparation and sequencing

Template preparation was performed by the Ion Chef System (Thermo Fisher Scientific, MA, USA) using the Ion PGM Hi-Q Chef Kit and the sequencing by using the Ion Personal Genome Machine (PGM) using the Ion PGM Hi-Q Sequencing Kit with the Ion 318 Chip v2 following the instructions of the manufacturers (Thermo Fisher Scientific, MA, USA). All barcoded libraries amplified with the prokaryote primer sets were pooled in one chip, and all barcoded libraries amplified with eukaryote- and fungus-specific primers were pooled in a second chip.

Data analyses

Raw reads were trimmed and filtered with cutadapt (Martin 2011). After trimming, only reads longer than 100 nt and containing at least one primer were kept. The prokaryotic reads were first grouped based on their hypervariable region (HVR) of origin. The HVR was inferred by running cmscan (Nawrocki and Eddy 2013) with the prokaryotic 16S rRNA model from Rfam (Kalvari et al. 2018) on each read and comparing the start and end position of the match with the corresponding HVR position in the Rfam model. Reads mapping outside of the HVR were discarded while the other reads were trimmed and filtered with cutadapt similar to what was done for the eukaryotic and fungal reads.

Taxonomy assignment was made with DADA based on the protocol published in Callahan et al. (2016). For the prokaryotic reads, the protocol was applied on each group of HVR separately as well as on all HVR at the same time.

Geographical assignment

For each genus found in the samples, the geographic spread was plotted by fetching information from the Global Biodiversity Information Facility. Geographical assignment of the species was done with the help of the R *rgbif* package (Chamberlain and Boettger 2017).

Nucleotide sequence accession number

The nucleotide sequences were deposited in the GenBank database under the accession number PRJNA506995:

- Statue 1 (SAMN10477674): fungi1 (SRX5063102), RUN: FungiTO1.fastq (SRR8245025); bacterial1 (SRX5063103) RUN: bac1RfamAllClean.fastq (SRR8245024); eukaryote1 (SRX5063105) RUN: Euk4TrimmedOnly.fastq (SRR8245022)
- Statue 2 (SAMN10477675): fungi2 (SRX5063107), RUN: FungiTO2.fastq (SRR8245020); bacteria2 (SRX5063104) RUN: bac2RfamAllClean.fastq (SRR8245023); eukaryote2 (SRX5063106) RUN: Euk5TrimmedOnly.fastq (SRR8245021)
- Statue 3 (SAMN10477695): fungi3 (SRX5063108) RUN: FungiTO3.fastq (SRR8245019); bacteria3 (SRX5063109) RUN: bac3RfamAllClean.fastq (SRR8245018); eukaryote3 (SRX5063110) RUN: Euk6TrimmedOnly.fastq (SRR8245017)

Data availability

All data generated or analyzed during this study are included in this published article.

Results and discussion

In this study, we investigated the microbiomes of three antique marble statues of unknown origin. Two different theories arose, the first arguing that the statues might have been found under the rubble of an old building and the second arguing that they could have a criminal background of being smuggled. In order to provide novel insights into the origin of the three statues, we applied a genetic strategy including metagenomic analyses, by using the Ion Torrent sequencing platform, to test the suitability and the potential of the strategy to retrieve the maximal genetic information contained on the surface of the statues. The resulting information was used to reconstruct the history of the storage of each single object, to infer the possible relationship among the three statues, and to elucidate their geographical shift. To this end, DNA libraries, targeting seven variable regions of the 16S rDNA fragments of prokaryotes, as well as a variable region of the 18S rDNA of eukaryotes and the ITS regions of fungi, were performed from each statue and further analyzed. To our knowledge, this is the first metagenomic approach in cultural heritage studies targeting so many regions in one single sample. The results showed to be very complex, and the challenge in this study, as in other “omics” studies, was the interpretation of the results. Bioinformatic pipelines were optimized in order to get results from lower taxonomic levels, as genus and species, what is not always possible when using universal primers.

DNA extraction, yield, and amplification procedure

The DNAs were directly extracted from the pooled powdered material collected from each statue and yielded a concentration of 11.7 ng/μl, 0.054 ng/μl, and 0.5 ng/μl for S1–S3, respectively. DNAs were subjected to PCR analyses using all the three primer sets (prokaryotes, eukaryotes, and fungi) described in the section “[Material and methods](#).” All PCR reactions showed positive results when using the eukaryote-specific primer pair, the fungal ITS-specific primer pair, as well as the Ion 16S Metagenomics Kit.

Sequencing analyses

Two sequencing runs were carried out; the first run was performed with the three pooled barcoded libraries derived from the amplicons obtained with the Ion 16S Metagenomics Kit of all three samples, generating 4,307,464 total reads, with an average of 1,305,936 reads per sample (supplementary Table 1). The second run was performed with the six pooled barcoded libraries derived from the amplicons obtained with eukaryote- and fungus-specific primers of all three samples, generating 4,195,209 total reads, with an average of 585,818 reads per sample (supplementary Table 2).

Eukaryotic community

The DNAs obtained from the three statues were processed for eukaryotic and fungal metagenomic analyses, respectively. The obtained DNA libraries were barcoded and pooled as mentioned in the section “[Material and methods](#)” and were sequenced in a single run, generating a total of 830 Mb.

Results derived from the eukaryotic DNA libraries revealed similarities between the two torsos (S1 and S2), both displaying a low biodiversity concerning the amount of detected phyla, namely Ascomycota, Basidiomycota, and Embryophyta (or land plants) (Fig. 2). In contrast, the small head (S3) showed a much higher diversity and some other additional phyla were detected in lower proportions (Figs. 2 and 3a). Even if the main targeted organisms in all three samples were fungi, it is worth noting that those phyla present in lower relative proportions showed to be important biological markers for specific environments.

The phylum Chlorophyta (green algae) accounted for 8% of the eukaryotic sequences retrieved from S3, and this phylum was negligible in the other samples (Fig. 4). Members belonging to three of the four recognized classes within the Chlorophyta were identified in S3: the freshwater or terrestrial *Chlorophyceae* (97%) and *Trebouxiophyceae* (3%), as well as the *Ulvophyceae* (0.5%). The latter group is best known as marine macroalgae (green seaweeds) in coastal ecosystems (Cocquyt et al. 2010). Within the *Chlorophyceae* class, the genera *Heterochlamydomonas* (82%), *Fasciculochloris*

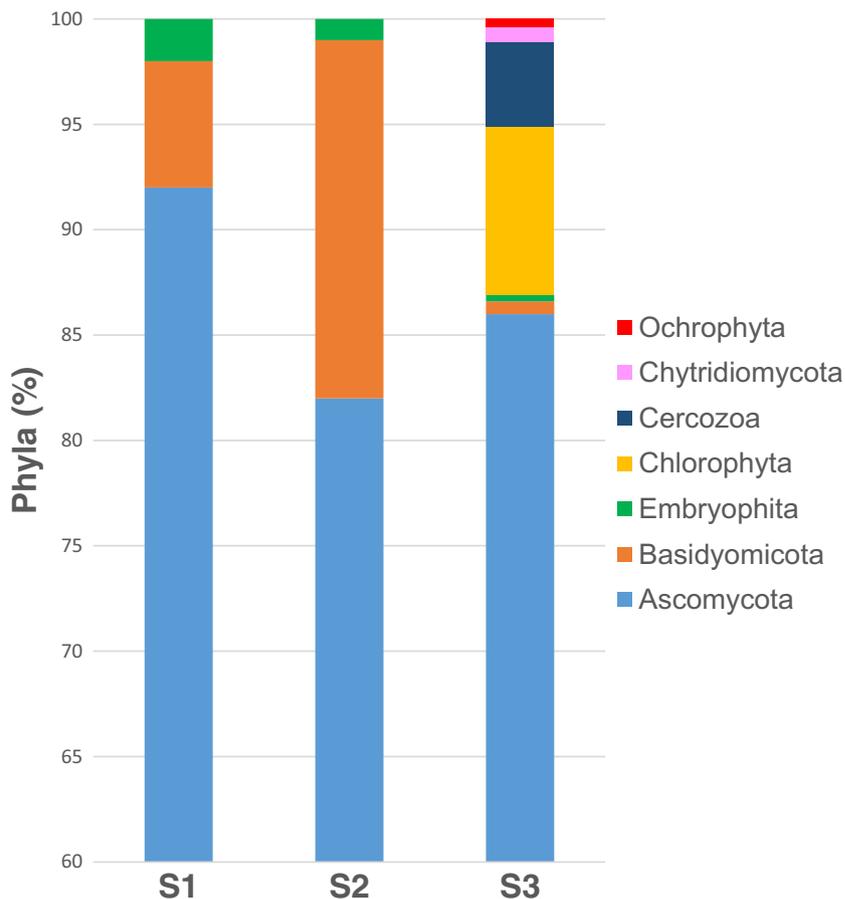
(3%), *Chlorosarcinopsis* (3%), *Chlorosarcina* (4%), and *Chlorococcum* (5%) were identified. All of them have been described as terrestrial species (McLean and Trainor 1965), but some of them are also found in freshwater and marine environments and as aerial isolates. *Chlorococcum* sp. has been previously described as one of the dominant organisms contributing to biodeterioration of stone monuments in Korea (Klochkova et al. 2006). Within the *Trebouxiophyceae* class, *Trebouxia* was the dominant genus. Members of this genus are the most widespread photobionts in lichens but are only sporadically found in soil or bark outside of a lichen. They all appear to be desiccation tolerant; i.e., they can survive drying to water contents of below 10% (Carniel et al. 2015) and, thus, are a good indicator for an osmophilic environment.

The phylum Cercozoa showed to be present only in S3, with a relative abundance (4% of eukaryotes). This phylum embraces numerous ancestrally biciliate zooflagellates, euglyphid, and other amoebae (Cavalier-Smith and Chao 2003). The Cercozoa contribute to a great biomass in soil (Urich et al. 2008) but are also abundant in every marine habitat. A high percentage of these sequences could not be identified at lower taxonomic levels (data not shown). However, 29% of the sequences affiliated with members of the family *Cercomonadidae*, which are found in soil, salt, brackish, and fresh water (Mylnikov and Karpov 2004); 3% with members of the order *Glissomonadida*; and 1% with the *Marimonadida*, namely with the genus *Pseudopirsonia*, which are marine, naked, ancestrally gliding amoeboid flagellates and flagellates (Howe et al. 2011).

Some additional eukaryotic phyla were detected only in S3, as the phyla Ochrophyta and Chytridiomycota (0.3% and 0.8% of total eukaryotic reads, respectively). Even though they were detected in very low proportions, they represented important ecological markers in this sample. The phylum Ochrophyta is a major line of eukaryotes, most of them algae, which are a primary component of plankton (Patterson 1989). Several genera were identified, such as *Xanthonema*, *Spumella*, *Eustigmatos*, and *Chlamydomyxa*, all reported to be common in fresh waters. The phylum Chytridiomycota, often called chytrids, are mainly aquatic organisms (Barr 1990; Zhang et al. 2016), found in peats, bogs, rivers, ponds, springs, and ditches. The presence of these two phyla in S3 supports the suspect that this sample was in contact with water or even a marine environment.

Other sequences retrieved in very low proportion, but reinforcing the hypothesis that S3 was in contact with water, are sequences related with the *Dinophyceae* class of the dinoflagellates (0.06% of eukaryotic reads). Dinoflagellates constitute one of the main groups of marine and freshwater protists (Gómez 2012). In terms of a number of species, dinoflagellates are one of the largest groups of marine eukaryotes, and some species are endosymbionts of marine animals. In addition, we detected some parasites, as members of the *Ichthyosporaea* class (0.04% of eukaryotes), namely the genus

Fig. 2 Relative abundance of the eukaryotic community at the phylum level. S1, female torso; S2, male torso; S3, small head



Ichthyophonus, which species are known to be unicellular eukaryotic parasites of marine and freshwater fishes (Ragan

et al. 1996), and members of the *Tubulinea* of the Amoebozoa (0.03% of eukaryotes), which are free-living amoebae (FLA).

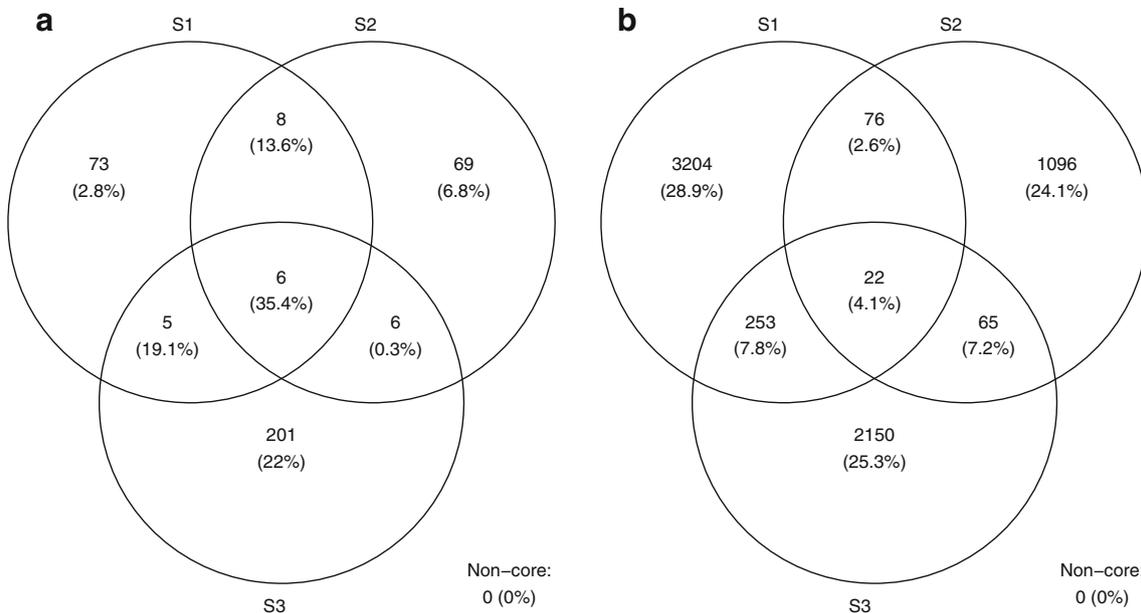


Fig. 3 Venn diagrams showing the OTUs shared by all three samples. S1, female torso; S2, male torso; S3, small head. **a** OTUs derived from eukaryotic DNA libraries. **b** OTUs derived from prokaryotic DNA libraries

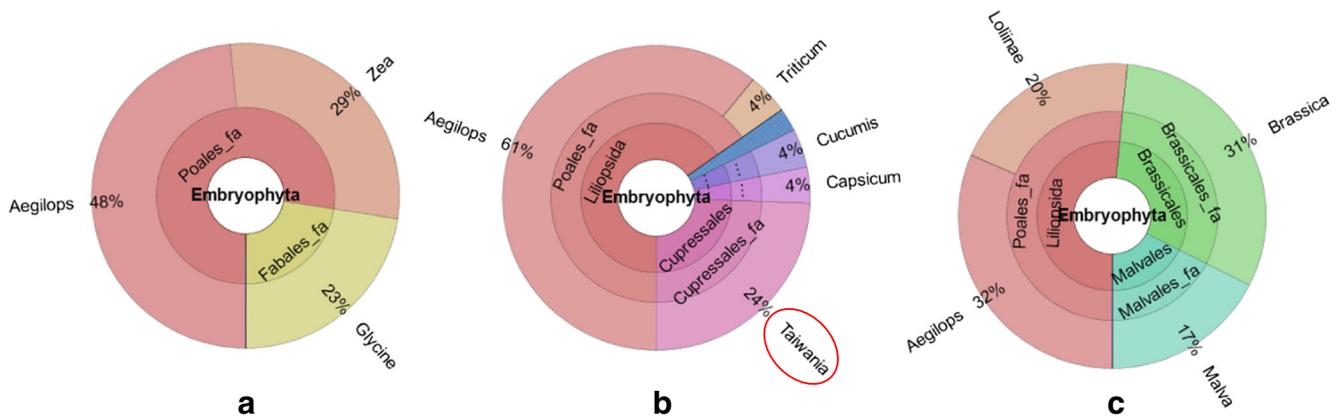


Fig. 4 Krona charts displaying the relative abundance of Embryophyta (land plants) at the genus level. **a** S1, female torso. **b** S2, male torso. **c** S3, small head

Within this group, we identified the genus *Vermamoeba*, which infects various organisms, as freshwater fishes (Milanez et al. 2017).

The Embryophyta (or land plants) showed to be present in all three samples in relatively low proportions (2%, 1%, and 0.3% of eukaryotes for S1–S3, respectively), but they represented important biological markers. In fact, all samples showed signs of storage in contact with agricultural soils, where the most detected land plants were crop plants.

The order *Poales* was dominant in all three samples (77%, 65%, and 52% of Embryophyta in S1–S3, respectively) (Fig. 4), and it was possible to identify plants of the genus *Aegilops* in all samples (48%, 61%, and 32% of Embryophyta in S1–S3, respectively), which are commonly known as goat-grasses. The statue S1 was also in contact with other crop plants, as members of the *Zea* genus (29%). This genus includes large grasses, such as *Zea mays*, commonly known as maize or corn, which is one of the most important crops for human societies all over the world. In addition, we could detect in this statue DNA of plants of the *Glycine* genus (order *Fabales*, 23%), of which the best-known species is the soybean. The majority of the *Glycine* species are found only in Australia, but the soybean's native range is in East Asia.

In S2, we could detect DNA from some other crop plants, such as the genus *Triticum*, or common wheat (4%), which are widely cultivated for their cereal grains and are a worldwide staple food. In addition, this sample revealed traces of DNA from plants of the genus *Capsicum* (or peppers) of the family *Solanaceae* (4%), which are cultivated worldwide, but with the highest production in East Asia. Also DNA of plants of the genus *Cucumis* was found in this sample (4%), which is the major group of angiosperms and includes gherkins, melons, and cucumbers, as examples.

S3 differed from the other two samples and showed the presence of one of the largest subtribes of temperate grasses, namely plants of the genus *Lolium* (20%). In addition, plants of the genus *Brassica* (order *Brassicales*, 31%), which are informally known as cabbages, or mustard plants, were

detected in this sample. The genus *Brassica* is known for its important agricultural and horticultural crops and includes species and varieties commonly used for food, such as broccoli, cauliflower, cabbage, choy sum, rutabaga, and turnip, and some seeds used in the production of canola oil and the condiment mustard. The genus is native to Western Europe, the Mediterranean, and temperate regions of Asia. Finally, 17% of sequences of Embryophyta were related to the order *Malvales*, namely to the genus *Malva*. This genus consists of about 25–30 species of herbaceous annual, biennial, and perennial plants and is widespread throughout the temperate, subtropical, and tropical regions of Africa, Asia, and Europe.

Nevertheless, the most relevant finding when analyzing the DNA of land plants was the relative abundance of DNA (24% of total reads of land plants) of the genus *Taiwania* in S2. *Taiwania*, with the single living species *Taiwania cryptomerioides*, is a large coniferous tree in the cypress family *Cupressaceae*. It is native and endemic to Eastern Asia, growing in the mountains of central Taiwan and locally in Southwest China, adjoining Myanmar, and northern Vietnam (according to the Global Biodiversity Information Facility). The outliers outside Asia are located in botanical gardens (supplementary Fig. 1). This tree was in very high demand in the past for timber, particularly for temple building and making furniture and coffins (“Chinese coffin tree”). Therefore, the species was decreasing and, nowadays, has legal protection in China and Taiwan (Hayata 1906). The detection of DNA of *Taiwania* tree on the surface of S2 enabled the geolocation of this statue, as staying or being transported through Asia.

Fungal community

As mentioned in the section “Eukaryotic community,” the main targeted organisms in the eukaryotic 18S rDNA libraries were fungi, accounting for 98% and 99% of the total reads for S1 and S2, respectively, and a lower proportion (86.6%) for S3. The phylum Ascomycota accounted for 92%, 82%, and

86%, while the phylum Basidiomycota represented 6%, 17%, and 0.6% of all reads in the eukaryotic DNA libraries for S1–S3, respectively. It is worth mentioning that data derived from 18S rRNA sequence analyses, even if showing a higher diversity of fungi than ITS analyses, are not variable enough to infer identification at taxonomic levels lower than genus. Therefore, analyses of fungal ITS regions were implemented to identify fungal species.

Ascomycota Within the Ascomycota, it was possible to infer similarities, but also differences at lower taxonomic levels among the three samples (Fig. 5). The order *Eurotiales* (97%, 93%, and 90% of Ascomycota reads for S1–S3, respectively), with the genus *Aspergillus*, showed to be dominant in all three samples. ITS analyses enabled to identify fungi at the species level. The species *Aspergillus sydowii* was present in all three samples. This species is a saprophytic fungus found mainly in soil, but it has been recently proven to be present in dust and indoor environments (Visagie et al. 2014). *Aspergillus proliferans* was present in S1 and S2; this species has been widely found in indoor environments and food (Chen et al. 2017). *Aspergillus penicillioides* was present only in S1. This fungus is typically found on substrates with low water activity, such as dried food. It is also known as a causal agent of foxing on paper and books and of brown spots on ancient Egyptian paintings (Koestler 2003). *Aspergillus ruber* was present only in S2. This fungus has been described as plant pathogen, i.e., in red peppers (Ham et al. 2016), and their presence in S2 may correlate with the presence of plants of the genus *Capsicum* (or peppers) also detected in this sample. S3

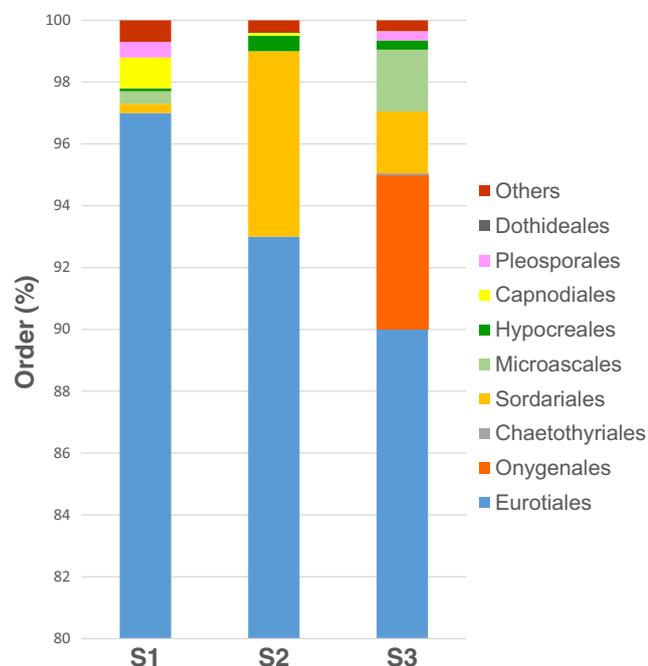


Fig. 5 Relative abundance of the Ascomycota community at the order level. S1, female torso; S2, male torso; S3, small head

differed from the other two samples, and the dominant species was *Aspergillus microcysticus*, which was isolated from a Savannah soil in Somalia (Samson et al. 2011). Members of the genus *Penicillium* were detected surprisingly only in S1, with the species *Penicillium glandicola* and *Penicillium citrinum*. These species have been detected growing in association with plants, such as cereals (Vinokurova et al. 2003), which were also identified as dominant land plants in this sample. Nevertheless, both species have been recently identified as causative agents of the biodeterioration on rock surfaces (Ogórek et al. 2016; Boniek et al. 2017).

The order *Onygenales* was negligible in S1 and S2 but accounted for 6% of the Ascomycota in S3 (Fig. 5), in which fungal members of the family *Gymnoascaceae* were all identified. This family includes species of some genera (as *Gymnoascus*) that have been found in sediments of salt lakes, compost, and cornfield soils and described as halophilic fungi (Zhou et al. 2016). Interestingly, only S3 showed the presence of members of the order *Chaetothyriales*, even though in a very low proportion (0.03% of *Eurotiomycetes* class).

Members of the order *Sordariales* showed to be represented in all three samples (Fig. 5) but dominated in S2 (0.3%, 6%, and 2% of Ascomycota reads for S1–S3, respectively). ITS analyses revealed that all members of this order belonged to the family *Chaetomiaceae*, with *Chaetomium* as the dominant genus in S1 and S3. *Chaetomium* species are capable of colonizing various substrates and are well known for their ability to degrade cellulose. The species *Chaetomium murorum* was identified in S1; this species has been identified in geographically widely distributed indoor environments (Wang et al. 2016). In contrast, the genus *Thielavia* with the species *Thielavia basicola* dominated in S2. This species is a phytopathogenic fungus, known to attack the roots of at least 100 angiosperm plants (Alam et al. 2012), as members of the family *Cucurbitaceae*, which correlates with some of the land plant sequences retrieved from this sample.

Members of the order *Microascales* could be identified in S1 and S3 (0.4% and 2% of Ascomycota) but were absent in S2 (Fig. 5). ITS analyses allowed identifying the species *Microascus verrucosus* in S1. The genus *Microascus* comprises species commonly isolated from soil, decaying plant material, and indoor environments, but few species are also recognized as opportunistic pathogens.

The order *Hypocreales* was detected in all three samples, accounting for 0.1%, 0.5%, and 0.3% of Ascomycota (Fig. 5), with *Gibberella* and *Fusarium* as the dominant genera. Both genera comprise a large variety of plant pathogens (rice and grain) with worldwide distribution. *Gibberella intricans* was identified only in S1, which is an opportunistic pathogen of durians (Sivapalan et al. 1998). The durian is the fruit of several tree species belonging to the genus *Durio*, abundant in Southeast Asia. *Fusarium* sp. and, in less proportion, *Acremonium* sp. were identified only in S3. *Acremonium*

contains many species that are saprophytic, which was isolated from dead plant material and soil. The presence of members of the order *Hypocreales* in all three samples seems to correlate with the presence of plants and as normal inhabitants of soils.

The order *Capnodiales* was present in S1 and S2 and made up 1% and 0.1% of Ascomycota, respectively (Fig. 5). ITS sequencing allowed to infer lower taxonomic levels and showed differences between the samples. The genus *Cladosporium*, with species of *Cladosporium halotolerans* and *Cladosporium sphaerospermum*, was detected only in S1. Both species have been found in hypersaline aqueous habitats worldwide and are described as halo- or osmotolerant as a recurrent feature. Both species have been isolated from hypersaline water (Zalar et al. 2007). This finding is in line with the many halotolerant-halophilic bacteria detected in this sample. In contrast, the genus *Vermiconia* of the *Extremaceae* family, with the species *Vermiconia calcicola*, was identified in S2. This species is an extremotolerant rock-inhabiting black fungus, which was isolated and described for the first time in marble monuments in Italy (Isola et al. 2016). Surprisingly, in S3, members of the order *Capnodiales* were detected only when analyzing ITS regions, and two species, namely *V. calcicola* and *Extremus antarcticus*, were identified. The latter species was originally isolated from rock samples in the Antarctica (Quaedvlieg et al. 2014) but also isolated and identified as a rock-inhabiting black fungus in Italian monumental sites (Isola et al. 2016).

The order *Pleosporales* accounted for 0.5%, 0.01%, and 0.3% of Ascomycota in S1–S3, respectively (Fig. 5). Further analyses of ITS regions allowed identifying *Alternaria alternata* as the dominant species in S1, in addition to *Alternaria chlamydospora*. *Alternaria alternata* causes leaf spot and other diseases on over 380 host species of plant. Interestingly, *A. chlamydospora* is primarily found in salty soils (Mouchacca 1973). Some additional species were identified in S1 in lower proportions, such as *Didymella urticicola*, *Phoma saxea*, *Stagonosporopsis* sp., and *Preussia* sp. All of them are plant pathogenic, saprobic, and endophytic species (Chen et al. 2015). S2 and S3 showed much less diversity inside this order, being *Phoma saxea* the only species identified in S2 and as the dominant species in S3.

Deeper analyses of 18S rDNA sequences enabled to detect the presence of other fungal taxa, even if in very low proportions. In this way, members of the order *Dothideales* represented 0.01% of the Ascomycota in S2; members of the *Pezizomycetes* class were identified in S1 and S3 (0.3% and 0.07% of Ascomycota, respectively). The genus *Pseudombrophila* was detected in S1, which is found on dead wood, soil, or rotten plant remains and on substrates often contaminated with urine by animals. In contrast, in S3, all sequences affiliated with the genus *Tuber*. The true truffles belong to this genus, one of the few ectomycorrhizal Ascomycetes, which are highly valued as delicacies (Mello

et al. 2006). In addition, members of the *Saccharomycetales* were detected in S2 and S3 (0.2% and 0.03% of Ascomycota, respectively). The genus *Cyberlindnera* with the species *Cyberlindnera jadinii* was detected only in S2. This fungus has been used since the early 1900s as fodder yeast for livestock and as a dietary supplement for humans.

Basidiomycota Within the Basidiomycota, the majority of the sequences affiliated with members of the *Agaricomycetes* class (99% of Basidiomycota for S1 and S2 and 78% for S3, data not shown), in which it was not possible to infer lower taxonomic levels in S1 and S2, but in sample S3. The latter sample showed the presence of relatives of the genera *Pleurotus* and *Pluteus* (order *Agaricales*) commonly called oyster, abalone, or tree mushrooms. In addition, S3 showed that 22% of the Basidiomycota sequences were affiliated with the order *Malasseziales* (*Exobasidiomycetes* class), namely with species of the genus *Malassezia*. This genus comprises yeast species that are part of the normal skin microbiota, but some species can also be involved in skin disorders (Velegraki et al. 2015). *Malassezia* spp. have not yet been cultured from the environment; nevertheless, metagenomic studies have identified *Malassezia* phylotypes from marine habitats (Amend 2014).

In addition, other basidiomycetes detected in very low proportion allowed inferring additional differences among the three samples, as members of the *Microbotryomycetes* class, only detected in S1 (0.6% of Basidiomycota). ITS analyses further enabled to assign these sequences to the genus *Rhodotorula*, namely to *Rhodotorula kratochvilovae*. *Rhodotorula* is common environmental yeast isolated from different ecosystems and environments, including sites with extreme conditions. In contrast, members of the order *Ustilaginales* (0.5% of Basidiomycota) were detected only in S2. Members of this order are plant pathogens and are known as the smut fungi, which are the causal agent of corn smut disease (Stirnberg and Djamei 2016). Their presence in this sample may be associated with the detected corn plants (*Zea*).

Prokaryotic community

First, it is worth mentioning that in this study, we applied for the first time in cultural heritage studies a metagenomic strategy implying the amplification of seven of the nine variable regions of the 16S rDNA of prokaryotes, simultaneously. The Ion 16S Metagenomics Kit (Thermo Fisher Scientific) was used. This kit takes advantage of using two primer pools to amplify seven of the nine variable regions of the prokaryotic 16S rDNA (V2, V3, V4, V6, V7, V8, and V9). The results (supplementary Fig. 2) clearly highlighted that some variable regions overestimate specific phyla, as it was very evident for the V9 region, which almost uniquely amplified the phylum *Proteobacteria*. In contrast, other variable regions

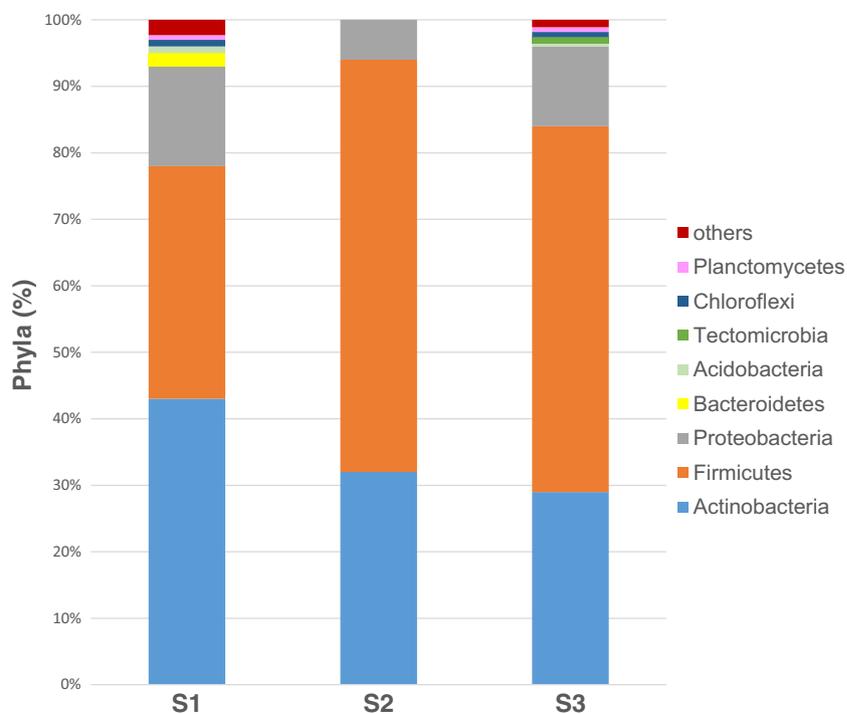
underestimated or even completely missed specific phyla, as the V4, which was not targeting the phylum Planctomycetes in S3 (supplementary Fig. 2c). This fact is of great relevance, because the V4 region is the one that is standardly used in most of the metagenomic approaches performed with the Illumina sequencing platform. In summary, these results show that the targeting of specific variable regions of the 16S rDNA gene can influence the results and remark the necessity to target more than one variable region simultaneously, in order to diminish the bias caused when analyzing single hypervariable regions of the 16S rDNA (Fouhy et al. 2016).

All three samples were processed for 16S rDNA metagenomic sequencing, generating a total of 774 Mb. Computational analyses were performed, merging the results obtained from all targeted variable regions, and revealed 22 bacterial phyla in all three marble samples. Nevertheless, only eight phyla showed to count for at least 0.5% of the total reads in at least one sample. At the level of phyla, differences could be observed among the three samples (Figs. 3b and 6); members of the Actinobacteria phylum showed to be dominant in S1, representing 43% of the total sequences and 32% and 29% of total sequences for S2 and S3, respectively. In contrary, the Firmicutes phylum dominated in S2 and S3, with 62% and 55% of the total sequences, respectively, representing 35% of sequences for S1. The Proteobacteria phylum showed to be also representative in all three samples (15%, 6%, and 12% for S1–S3, respectively). Interestingly, the phyla Bacteroidetes and Acidobacteria accounted for 2% and 1% of the total reads only in S1, and the phylum Tectomicrobia accounted for 1% of total reads only in S3. The phylum Chloroflexi accounted

for 1% and 0.7% of total reads for S1 and S3, respectively, and the phylum Planctomycetes for 0.7% of total reads in S1 and S3, and the latter phyla were negligible in S2.

Results derived from prokaryotes supported those obtained from eukaryotes, and sequences derived from all samples showed strong evidence of contact with agricultural soils. All samples showed high abundance of Actinobacteria (Fig. 6). Members of this phylum are widespread in soils throughout the world and are successful colonizers of different extreme environments. However, important differences were observed among the three samples concerning the biodiversity of this phylum (supplementary Fig. 3). S2 showed the lowest biodiversity within the Actinobacteria, and only members of the order *Micromonosporales* (76% of Actinobacteria), *Frankiales* (17% of Actinobacteria), and *Micrococcales* (2% of Actinobacteria) were represented with relatively high abundance in this sample. Within the order *Micromonosporales*, the genus *Micromonospora* dominated, accounting for 49% of all Actinobacteria, followed by the genera *Plantactinospora* (10%) and *Verrucosipora* (3%). Bacteria belonging to the *Micromonospora* genus have been widely isolated from mangrove soils, where they represent high populations (Hong et al. 2009). The genus *Plantactinospora* represents mainly bacteria associated with plant tissues, such as endophytic Actinomycetes which was isolated from *Camptotheca acuminata* Decne, a Chinese traditional medicine plant endemic to Southwest China (Zhu et al. 2012). Species of the genus *Verrucosipora* have been isolated from peat bog and deep-sea sediments but also from mangrove sediments in China (Xi et al. 2012). Within the *Frankiales*, the only

Fig. 6 Relative abundance of the bacterial community at the phylum level. S1, female torso; S2, male torso; S3, small head



detected genus was *Blastococcus* (16% of Actinobacteria). Bacteria of this genus are commonly isolated from the surface of marble and calcareous stones (Urzi et al. 2004).

In contrast, S1 and S3 showed a much higher biodiversity concerning the phylum Actinobacteria. At least seven orders could be detected within the Actinobacteria class in all two samples with relatively high abundance, at least in one of them. Some orders were present in only one sample, such as the order *Corynebacteriales*, which made up 23% of the phylum Actinobacteria in S1, which were almost negligible in the other two samples (supplementary Fig. 3). The *Corynebacterium_1* group showed to be dominant (17% of Actinobacteria). Members of this group have been described mainly in swine manure (Song et al. 2017). At the species level, it was possible to identify the species *Corynebacterium maris* (5% of Actinobacteria), which are halotolerant bacteria isolated for the first time from the coral *Fungia granulosa* in the Gulf of Eilat, Israel (Ben-Dov et al. 2009), and in lower proportion in *Corynebacterium efficiens*, which have been isolated from soil and vegetables (Fudou et al. 2002). Two additional genera were detected within this order in S1, namely *Mycobacterium* and *Dietzia* (each representing 2% of Actinobacteria), and both were isolated from aquatic and terrestrial habitats, as well as from clinical material (Koerner et al. 2009).

Bacteria of the order *Micrococcales* dominated in S1 (16% of the phylum Actinobacteria) but were also present in S2 and S3 (2% and 5% of Actinobacteria, respectively). The genera *Brachybacterium* and *Kocuria* were the most dominant in S1 (each 3% of Actinobacteria), followed by the genera *Citricoccus* and *Curtobacterium* (each 1%), in addition to other genera with relatively lower proportions as *Arthrobacter* and *Pseudarthrobacter* (both 0.7% of Actinobacteria) as well as *Agromyces* (0.5%). Species of the genus *Brachybacterium* have been isolated from poultry deep litter, stool samples, soils, sediments, and seawater (Collins et al. 1988). Bacteria belonging to all abovementioned genera are typically species found in soils. *Kocuria* and *Citricoccus* have been widely isolated from soils, and some species are halotolerant and alkalitolerant (Li et al. 2005; Wang et al. 2015). The *Curtobacterium* genus has a global distribution with a predominant presence in soil ecosystems and is described mainly in association with plants, especially in the phyllosphere (Chase et al. 2016). *Agromyces* are soil actinomycetes with formation of true mycelia (Zgurskaya et al. 1992). In contrast, the most dominant genus in S3 was *Arthrobacter*, with the species *Arthrobacter crystallopoietes*. This species has been described as desiccation-resistant bacteria, and this capability may explain their numerical dominance in soil (Boylan 1973).

Bacteria of the order *Streptomycetales* were present in relatively high abundance in S1 (14% of the phylum Actinobacteria) and S3 (9%). The genus *Streptomyces* dominated in both samples (13% and 8%, respectively). Bacteria belonging to this genus are ubiquitous in soils and marine

sediments and are commonly found in the rhizosphere or inside plant roots (Rey and Dumas 2017). The species *Streptomyces albiacialis* was detected only in S1, which is a thermotolerant and halotolerant *Streptomyces* (Kuznetsov et al. 1992). In addition, it was also possible to detect in a very low proportion members of the genus *Streptacidiphilus*. The genus *Streptacidiphilus* is composed of acidophilic actinomycetes and has a major role in the turnover of organic matter in acidic habitats (Kim et al. 2003).

The order *Streptosporongiales* was better represented in S1 and S3 (6% and 7% of the phylum Actinobacteria, respectively), with the genus *Nocardopsis* as dominant in S1 and *Actinomadura* in S3. *Nocardopsis* is a bacterial genus that produces some antimicrobial compounds and occurs mostly in saline and alkaline soils (Bennur et al. 2014). Bacteria of the genus *Actinomadura* are commonly isolated from soils (Zhao et al. 2015).

The order *Propionibacteriales* accounted for 3% and 7% of the phylum Actinobacteria in S1 and S3, respectively. The most represented genera in both samples were *Nocardioides* and *Microtholatus*, and both genera are common bacteria in soils (Kämpfer et al. 2010; Wang et al. 2017). Finally, the order *Pseudonocardiales* accounted for 4% and 6% of the phylum Actinobacteria in S1 and S3, respectively, in which *Pseudonocardia* is the dominant genus. In both samples, it was possible to detect the species *Pseudonocardia zijingensis*, which was isolated from a soil sample collected from Zijing Mountain, Yunnan Province, China (Huang et al. 2002).

Members of the *Thermoleophilia* class were detected in S1 and S3 (24% and 23% of the phylum Actinobacteria, respectively). Uncultivated bacteria of the *Solirubrobacterales* Elev-16S-1332 group as well as cultivated bacteria of the genera *Solirubrobacter* and *Gaiella* showed to be dominant in both samples, which are bacteria commonly isolated from soil (Singleton et al. 2003) and water. In addition, members of the class *Acidimicrobiia* were detected in both samples (3% of the phylum Actinobacteria in each sample) where it was possible to infer only the genus *Lamia*, which members have been isolated from marine environments (Kurahashi et al. 2009). Curiously, members of the *Rubrobacteria* class were only detected in a relatively high proportion (23% of the phylum Actinobacteria) in S3, where it was possible to identify only the genus *Rubrobacter*. Unfortunately, it was not possible to infer at the species level, but species of this genus have been widely detected in terrestrial habitats, as stone monuments usually subjected to salt weathering (Otlewska et al. 2017) as well as in marine environments, and some of them are known to be halotolerant, thermotolerant, and radiotolerant (Egas et al. 2014).

Interestingly, members of other phyla found in significant proportions only in S1 strongly indicated that the soil being in contact with this statue must have been from animal farm. For example, members of the phylum Bacteroidetes were present only in this sample, accounting for 2% of bacteria (Fig. 6),

with mainly species of *Bacteroides*. Members of *Bacteroides* make up the most substantial portion of the mammalian gastrointestinal flora and play a fundamental role in the processing of complex molecules to simpler ones in the host intestine (Wexler 2007); the species *Bacteroides barnesiae* was identified, which has been isolated from the cecum of a healthy chicken (Lan et al. 2006). In addition, members of the family *Rikenellaceae*, with the *Rikenellaceae_RC9_gut* group and the genus *Alistipes*, as well as members of the family *Prevotellaceae* were identified. Members of these two families are often found in the gastrointestinal tract of different animals and humans (Rosenberg 2014). Other bacteria that could be indicators of a contact with soils of animal farms belonged to the phylum Acidobacteria, accounting for 1% of bacteria in S1 (Fig. 6), which are among the most common bacteria in soils worldwide. Nevertheless, the main Acidobacteria group detected in S1 was subgroup 6, which members are related to those detected in pasture soils (Navarrete et al. 2015).

In addition, sequences related to the phylum Firmicutes, retrieved in significant proportions only from S1, confirmed the suspicion that this statue must have been in contact with animal farm soil (supplementary Fig. 3). Sequences affiliated to the order *Clostridiales* were found in a relatively high proportion (8% of Firmicutes). Within this order, the most dominant members comprised the *Christensenellaceae_R-7* group, which includes uncultured bacteria mainly found in rumen, as well as members of the genera *Romboutsia*, *Intestinibacter*, and *Clostridium* and of the families *Ruminococcaceae* and *Lachnospiraceae*, all of them were identified in the gut microbiota of human and other animals (Gerritsen et al. 2014; Meehan and Beiko 2014). Sequences related to the order *Lactobacillales* were also detected in high proportion in this sample (12% of Firmicutes). The most detected genus was *Lactobacillus* (3% of Firmicutes), which are a major part of the group of lactic acid bacteria (LAB) and in humans and constitute a significant component of the microbiota at a number of body sites, such as the digestive, urinary, and genital systems. At lower taxonomic levels, it was possible to identify the species *Lactobacillus kitasatonis*, *Lactobacillus reuteri*, *Lactobacillus pontis*, and *Lactobacillus salivarius*, among the other unidentified species. Several genera within the family *Carnobacteriaceae* were also detected, such as *Atopostipes* and *Carnobacterium* (each 3% of Firmicutes). At the level of species, the only one known species (*Atopostipes suicloacale*) was identified in this study. This species has been previously isolated from an underground swine manure storage pit (Cotta et al. 2004). Members of the genus *Carnobacterium* are ubiquitous LAB, which are isolated from cold and temperate environments, and predominate in a range of foods, including fish, meat, and some dairy products (Leisner et al. 2007). Other members of the family *Enterococcaceae* could be detected even in low proportions (0.2% of Firmicutes), with the species *Enterococcus cecorum*, which is known as a commensal in the intestines of mammals

and birds, but it has been described as an emerging pathogen in poultry industry worldwide (Dolka et al. 2017). Sequences related to the order *Erysipelotrichales* (Firmicutes), which species are known to be common in the gut microbiome of animals, were also detected in this sample (4% of Firmicutes), with the genus *Turicibacter* as the sole representative (Auchtung et al. 2016).

In contrast, S2 and S3 showed to have a lower biodiversity of Firmicutes and were dominated by members of the order *Bacillales* (supplementary Fig. 3), which represent 97% and 82% of Firmicutes, while this order accounted for 76% of Firmicutes in S1. Nevertheless, the detected genera showed to be different among the samples. S2 and S3 showed a strong dominance of *Bacillus* (39% and 23% of Firmicutes, respectively). At the level of species, *Bacillus timonensis* and *Bacillus subtilis* were detected in S2. *Bacillus timonensis* has been detected in human fecal flora (Kokcha et al. 2012), and *B. subtilis* is commonly found in the gastrointestinal tract of ruminants and humans and is widely used as a probiotic feed supplement for farm animals (Wang et al. 2018). In addition, this species collectively inhabits the rhizosphere, where it contributes to the promotion of plant growth (Hirooka 2014).

In S3, it was possible to detect a wider range of species of *Bacillus*. Some of them accounted for relatively high proportions, such as *Bacillus niacini* and *Bacillus longiquaesitum*, both isolated from soils (Nagel and Andreesen 1991), but the latter one was also commonly isolated from microbial communities associated with truffles (Mello et al. 2013). It is worth noting that traces of DNA of truffles were identified in this sample (genus *Tuber*). Other species were detected in this sample, such as *Bacillus nealsonii*, which produces endospores that are resistant to UV, gamma-radiation, salinity, and desiccation (Venkateswaran et al. 2003). In lower proportions, *Bacillus humi*, *Bacillus benzoovorans*, and *Bacillus pumilus* were detected as well, all of them most commonly isolated from soils (Heyrman et al. 2005) and known to be plant growth-promoting bacilli (Yadav et al. 2011).

The genus *Anoxybacillus* was additionally detected in S2 (2% of Firmicutes). Many species of this genus are known to be alkaliphilic and thermophilic and commonly occur in geothermal springs, manure, and milk processing plants (Yamamoto et al. 2006). Other detected genera in S3 were *Fictibacillus* (2% of Firmicutes) and *Falsibacillus* (1%), both commonly isolated from marine environments (Dastager et al. 2014).

In contrast, S1 showed a much lower proportion of *Bacillus* (7% of Firmicutes) and the only identified species was *Bacillus alkalitelluris*, an alkaliphilic bacterium isolated from soil in Korea (Lee et al. 2008). The genus *Oceanobacillus* was also detected in this sample in a relatively high proportion (4% of Firmicutes). *Oceanobacillus* species have been isolated from diverse environmental samples, including deep-sea sediment cores and salt fields; fermented shrimp paste samples, soy sauce production equipment; and traditional Korean fermented food. We could identify some species, such as

Oceanobacillus iheyensis, an extremely halotolerant species isolated from deep-sea sediment (Lu et al. 2001); *Oceanobacillus profundus*, a halotolerant bacterium, isolated from the deep surface of a sediment core in Korea (Kim et al. 2007a); and *Oceanobacillus picturae*, isolated for the first time from a mural painting (Heyrman et al. 2003). Other genera identified in lower proportions (0.9–0.2% of Firmicutes), such as *Virgibacillus*, *Falsibacillus*, *Fictibacillus*, *Pontibacillus*, *Terribacillus*, and *Marinococcus*, further supported the hypothesis that statue 1 was in contact with a saline environment, in which many of the species of all detected genera were able to grow in moderate concentrations of NaCl and were isolated from saline environments.

S1 showed actually a higher proportion of members of the *Planococcaceae* family (39% of Firmicutes), which belong to the genus *Planomicrobium* (32% of Firmicutes). At the level of species, *Planomicrobium okeanoikoites* showed to be dominant. This species has been isolated from the marine muds and from soils in China (Nakagawa et al. 1996). Other species detected in lower proportions were *Planomicrobium glaciei* (Zhang et al. 2009), which was isolated from a glacier in Northwest China, and *Planomicrobium chinense* (Dai et al. 2005), which was isolated from a coastal sediment of the Eastern China Sea, China. Both species are able to growth at moderate salinities (0–10% NaCl). The genera *Planococcus* and *Sporosarcina* were also detected, but in lower proportions (1% of Firmicutes). Members of the *Planococcaceae* were less dominant in S2 and S3 (3% and 10% of Firmicutes, respectively), and the main genera detected in these samples differed from those in S1. Bacteria of the genus *Domibacillus* were dominant in both samples (1% and 5% of Firmicutes in S2 and S3, respectively), in addition to *Sporosarcina* (0.2% and 2% of Firmicutes, respectively). At the level of species, *Sporosarcina soli* was detected in S2 and *Sporosarcina koreensis* in S3. Both species were isolated for the first time from soils in Korea, and both tolerate moderate (5–7%) NaCl concentrations (Kwon et al. 2007). The genus *Chungangia* (1% of Firmicutes) was only present in S2. Other genera detected in lower proportions in S3 were *Paenisporosarcina*, *Lysinibacillus*, and *Bhargavaea*.

Members of the *Staphylococcaceae* were present in relatively high abundance only in S1 (14% of Firmicutes) which were negligible in the other two samples. The genus *Salinicoccus* showed to be dominant (10% of Firmicutes) in this sample. It was possible to identify the species *Salinicoccus kekensis*, a moderate halophilic bacterium isolated from a salt lake in China (Gao et al. 2010), as well as other species, such as *Salinicoccus halodurans*, *Salinicoccus kunmingensis*, and *Salinicoccus roseus*, which were isolated from saline soils and a salt mine in China (Chen et al. 2007; Wang et al. 2008). The genus *Jeotgalicoccus* accounted, in addition, for 3% of Firmicutes in S1. Bacteria of this genus are halotolerant to halophilic bacteria. The species *Jeotgalicoccus psychrophilus* was identified, which was

isolated from the Korean fish sauce jeotgal (Yoon et al. 2003). The genus *Staphylococcus* was also found in this sample, even if in less proportion, with the species *Staphylococcus saprophyticus*. This species is part of the normal human flora that colonizes the perineum, rectum, urethra, cervix, and gastrointestinal tract but has also been found as a common gastrointestinal flora in pigs and cows (Ehlers and Merrill 2018).

Members of the *Paenibacillaceae* were present in relatively high proportions in all three samples, with the dominance of the genus *Paenibacillus* (2%, 12%, and 5% of Firmicutes, respectively). *Paenibacillus* spp. are found in soil, often associated with plant roots. These rhizobacteria promote plant growth and can be exploited for use in agriculture. Nevertheless, some differences were observed among the three samples concerning the detected species. *Paenibacillus alginolyticus* and *Paenibacillus harenae* were detected in S1 and S3. The first one was isolated from soils and the second one from desert sand in China (Jeon et al. 2009). In S2, the detected species were *Paenibacillus lautus*, which has been isolated from soil and human intestinal tract, and *Paenibacillus glucanolyticus*, which is known to degrade cellulose, hemicellulose, and lignin (Mathews et al. 2014). The genus *Cohnella* was also detected in all three samples (0.5%, 0.2%, and 5% of Firmicutes, respectively). Members of this genus are highly cellulolytic bacteria, which can be found in a variety of habitats. At the level of species, it was possible to detect *Cohnella saccharovorans* only in S3. This species was isolated from ginseng soils (Choi et al. 2016). Additional genera were detected in S1, such as *Saccharibacillus*, with the species *Saccharibacillus deserti*, which was isolated from desert soil in China (Sun et al. 2016).

Members of the *Alicyclobacillaceae*, with the genus *Effusibacillus*, were detected in relatively high abundance only in S2 (6% of Firmicutes). Unfortunately, it was not possible to infer at the level of species. The genus *Exiguobacterium* was detected only in S1 (2% of Firmicutes), in which it was possible to identify the species *Exiguobacterium acetylicum*. This species has been isolated from soils and expressed multiple plant growth promotion attributes such as phosphate solubilization (Selvakumar et al. 2010). Finally, concerning the phylum Firmicutes, some sequences affiliated with uncultivated bacilli in S2 and S3, as the uncultivated clone TSCOR001-H18 (2% of Firmicutes sequences in each sample), which has been detected on rice paddy soils in Japan (Ishii et al. 2009).

Proteobacteria was the third phylum most represented in all three samples (15%, 6%, and 12% of total reads for S1–S3, respectively; see Fig. 6). Nevertheless, some differences were observed among the three samples (supplementary Fig. 3). The *Alphaproteobacteria* class was dominant on S1 and S3, accounting for 40% and 76% of Proteobacteria, respectively, whereas it accounted for only 18% of Proteobacteria reads of S2. Some additional differences were noticed at lower phylogenetic levels. The order *Rhizobiales* dominated on S1 (13% of Proteobacteria)

and accounted for 4% and 8% of the Proteobacteria in S2 and S3, respectively. Within this order, the genera *Mesorhizobium* (3% of Proteobacteria), *Bradyrhizobium*, and *Neorhizobium* (both 0.6% of Proteobacteria) were present in S1, all of them were nitrogen-fixing endosymbionts associated with roots of legumes. It is worth mentioning that members of the *Fabales* (leguminous plants) were detected in this sample. Another dominant genus in S1 was *Devosia* (1% of Proteobacteria). Species of *Devosia* are commonly isolated from rhizospheric soils (Mohd Nor et al. 2017). In contrast, the dominant genus in S2 and S3 was *Microvirga*. This genus comprises mainly soil bacteria, and some species are root nodule symbiotic bacteria that fix nitrogen in different plant hosts (Ardley et al. 2012).

The *Sphingomonadales* were present in S1 and S2 (9% and 2% of Proteobacteria, respectively) and almost negligible in S3. The most dominant genus in both samples was *Sphingomonas*. Members of this genus are pigmented bacteria found in various environments, including both aqueous (freshwater and seawater) and terrestrial habitats, plant root systems, clinical specimens, and others. Nevertheless, it was possible to infer differences at the level of species. *Sphingomonas mucosissima* and *Sphingomonas jaspsi* were identified in S1, being the first species isolated from biological soil crust collected from sandy arid soils (Reddy and Garcia-Pichel 2007), and *S. jaspsi* was isolated from freshwater samples collected in Japan (Asker et al. 2007). In contrast, *Sphingomonas kaistensis* and *Sphingomonas humi* were detected in S2, both previously isolated from soils in South Korea (Kim et al. 2007b; Yi et al. 2010).

The *Rhodobacterales* accounted for 6%, 7%, and 3% of Proteobacteria in S1–S3, respectively. Samples S1 and S2 showed certain similarities, as the most dominant genus in both samples was *Paracoccus*. *Paracoccus siganidrum* was identified in both samples, and this species was isolated from the fish gastrointestinal tract (Liu et al. 2013). In addition, *Paracoccus sanguinis* was identified only in S1 and *Paracoccus yeei* only in S2. Both species have been identified as clinical specimens (McGinnis et al. 2015). The genus *Rubellimicrobium* was also identified in both samples. Species of this genus are reddish bacteria commonly isolated from soils (Riedel et al. 2014). In contrast, in S3, the genus *Amaricoccus* showed to be dominant. Species of this genus have been isolated from activated sludge (Maszenan et al. 1997).

Members of the *Caulobacterales* were detected in S1 and S2, but absent in S3. The most dominant genus in both samples was *Brevundimonas*, and it was possible to identify *Brevundimonas vesicularis* in S1. *Brevundimonas* spp., and in particular *B. vesicularis*, are emerging global opportunistic pathogens (Ryan and Pembroke 2018).

Sample S3 differed significantly from the other two samples, concerning the Alphaproteobacteria group, and the dominant order was the *Rhodospirillales* (65% of Proteobacteria), of which most reads match the *Rhodospirillales* MSB-1E8 group (53% of

Proteobacteria). Members of this group are all uncultivated bacteria, which have been found on the microbiome of seabirds and corals (Grottoli et al. 2018). The genus *Skermanella* was also dominant in this sample (5% of Proteobacteria). It was not possible to infer at the level of species, but species of this genus have been identified as airborne bacteria in Asian dust, as well as isolated from freshwater and soils in extreme environments, as deserts in China (Zhang et al. 2015). In addition, the *Candidatus Alysiosphaera* group and the genus *Craurococcus* accounted each for 3% of Proteobacteria in this sample, and the latter one was isolated from soils (Saitoh et al. 1998). This order accounted for 9% and 3% of Proteobacteria in S1 and S2, respectively. The group *Rhodospirillales* MSB-1E8 was also identified in S1, but in lower proportion.

The *Betaproteobacteria* class showed to be dominant in S2 (51% of Proteobacteria) and accounted for 21% and 6% of Proteobacteria in S1 and S3, respectively (supplementary Fig. 3). However, some differences were observed at the family and genus levels, being the *Oxalobacteraceae*, with the genus *Massilia* dominant on S1. Species of *Massilia*, even if known as pathogens, have been also isolated from soils in China (Zhang et al. 2006). The family *Burkholderiaceae*, with the genus *Ralstonia*, dominated on S2. Species of this genus are well-known plant pathogens, and their significant dominance in S2 may correlate with the presence of plants detected in this sample. Sample S2 showed the highest biodiversity of *Betaproteobacteria*, and additional genera, such as *Aquabacterium*, *Pelomonas*, and *Achromobacter*, were identified. Species of all these genera have been isolated from water (fresh and marine) and soils. Sample S3 comprises also species of *Ralstonia*, in addition to members of the family *Nitrosomonadaceae*. All cultivated representatives of the last family are lithoautotrophic ammonia oxidizers that exert control over nitrification by oxidizing ammonia to nitrate. They therefore play major roles in the control of the nitrogen cycle in terrestrial, freshwater, and marine environments.

The *Gammaproteobacteria* class accounted for 33% and 27% of Proteobacteria in S1 and S2, respectively, and for significantly less proportion in S3 (3%). Members of the *Pseudomonadales* were present in S1 and S2, with the genus *Pseudomonas* being dominant in S1 and the genus *Acinetobacter* in S2. *Pseudomonas* are commonly isolated from soils. The species *Pseudomonas pseudoalcaligenes*, detected in S1, has been isolated from plant rhizospheres and is an alkaliphilic cyanide-degrading bacterium (Luque-Almagro et al. 2011). In addition, the genus *Alkanindiges*, with the species *Alkanindiges illinoisensis*, an alkane-degrading bacterium isolated from oilfield soils (Bogan et al. 2003), was detected only S1.

The order *Enterobacterales* showed to be relevant only in S1 (12% of Proteobacteria), less represented in S2 (3% of Proteobacteria), and absent in S3. Many members of this order are a normal part of the gut flora found in the intestines of humans and other animals. The significant presence of these

bacteria in S1 supports, once more, the hypotheses that this statue was in contact with farm soil. The genus *Pantoea* was dominant in S1 and S2. The species *Pantoea agglomerans* and *Pantoea ananatis* were identified in S1. *P. agglomerans* is a ubiquitous bacterium commonly isolated from plant surfaces, seeds, fruit, and animal or human feces. *P. ananatis* is a bacterium with the ability to colonize and interact with hosts in both the plant and animal kingdoms and was found in a wide range of natural environments, as part of the epiphytic and endophytic flora of various plant hosts, and in the insect gut (De Maayer et al. 2014). In addition, members of *Enterobacter*, *Escherichia/Shigella*, *Cronobacter*, and *Salmonella* were detected only in S1 and *Buttiauxella* only in S2.

The order *Xanthomonadales* accounted for 5% of Proteobacteria in S1 and was very low in S2 and S3 (0.5% and 0.8% of Proteobacteria, respectively). The genera *Steroidobacter*, with the species *Steroidobacter flavus*, and *Acidibacter* were identified in S1 and S3. The detected *S. flavus* is a microcystin-degrading strain, isolated from forest soil in China (Gong et al. 2016). The genus *Acidibacter* was recently described as acidophilic, mesophilic, and capable of reducing ferric iron (Falagán and Johnson 2014). In addition, the genera *Stenotrophomonas* and *Lysobacter* were identified in S1 and S2. The genus *Lysobacter* accommodates non-fruiting, gliding bacteria commonly isolated from soils. At lower taxonomic levels, the species *Lysobacter niastensis* was identified. This species was originally isolated from greenhouse soil cultivated with cucumber in Korea (Weon et al. 2007). It is worth noting that plants of the genus *Cucumis* were detected in S2. Within the genus *Stenotrophomonas*, *Stenotrophomonas maltophilia* was detected. This species is ubiquitous in aqueous environments, soil, and plants, but it is also an emerging multidrug-resistant global opportunistic pathogen (Brooke 2012).

The *Salinisphaerales* showed to be present only in S3, with members of the *Salinisphaeraceae* family. Unfortunately, it was not possible to infer lower taxonomic levels. This family comprises two genera: *Salinisphaera* and *Halophibacterium*. All members of the family are halophilic or halotolerant and have been isolated from marine/oceanic and high-salinity environments (Vetriani et al. 2014).

The *Deltaproteobacteria* accounted for 14% of Proteobacteria in S3, in contrast to the lower proportions detected in S1 and S2 (6% and 4%, respectively). Members of the orders *Desulfurellales* and *Myxococcales* accounted each for 6% of Proteobacteria in S3. It is worth mentioning that members of *Desulfurellales* were negligible in S1 and S2. This order comprises exclusively the family *Desulfurellaceae*. Members of this family are all strictly anaerobic and moderately thermophilic bacteria, found mostly in warm-hot sulfur-containing anoxic environments, such as hydrothermal springs and sediments, and are often in association with microbial mats (Greene 2014). All the reads matched with the uncultured genus *Desulfurellaceae* H16,

previously detected in sediments of lakes (Ruuskanen et al. 2018). Members of the order *Myxococcales* were also represented in S1 and S2 (5% and 3% of Proteobacteria). The genera *Haliangium* and *Sorangium* were present in all three samples, with the first one being dominant in S1 and the second in S2 and S3. Species of *Haliangium* are known as marine myxobacteria and are halotolerant (Albataineh and Stevens 2018). The genus *Sorangium*, with the detected species *Sorangium cellulorum*, plays an important role in soil ecology by its ability to degrade cellulosic materials. In addition, S1 showed the presence of the *Myxococcales* Blrii 41 group, which comprises uncultivated bacteria that have been identified in rice field soils (Hori et al. 2007) as well as the genera *Vulgatibacter*, *Anaeromyxobacter*, and *Polyangium*, all of them being commonly isolated from soils and rhizosphere, and specifically from saline-alkaline soils (Zhang et al. 2013b). These genera were also present in S3, but absent in S2. The order *Bdellovibrionales* accounted for 1% of Proteobacteria in S3 and was present at lower proportions in S1 and S2. In S1 and S3, this order was composed exclusively of members of the OM27 clade, which comprises uncultivated bacteria involved in nutrient cycles in the ocean (Orsi et al. 2016).

Some additional phyla were detected in S1 and S3 but were absent in S2 (Fig. 6). Even though they were detected in low proportions, they represented important ecological markers in these two samples. The phylum Chloroflexi (1% and 0.8% of total reads in S1 and S3, respectively) contains bacteria with various metabolic features and is one of the most common and diverse bacterial phyla in sponges with many sponge-specific lineages (Schmitt et al. 2011). Nevertheless, it is worth noting that all detected sequences belonging to this phylum affiliated with uncultivable microorganisms of the no-rank groups Gitt-GS-136, TK10, S085, JG30-KF-CM45, and KD4-96. These groups have been detected in bat guano (De Mandal et al. 2015) and in manure- and fertilizer-treated soils (Zhang et al. 2013a); thus, they seem to be biological markers indicating a contact with agricultural land.

The phylum Planctomycetes (0.7% of total reads in both S1 and S3) comprises mainly aquatic bacteria found in samples of brackish, marine, and fresh waters. The family *Planctomycetaceae* accounted for 83% of Planctomycetes in both samples; within this family, it was possible to identify some genera in both samples, such as *Gemmata*, *Zavarzinella*, and *Singulisphaera*, all of them being isolated from acid bogs, swamps, and wetlands, and the genus *Planctomyces* was detected only in S3. The family *Tepidisphaeraceae* was present in both samples. Members of this family have been isolated from microbial mats (Kovaleva et al. 2015).

Finally, the phylum Tectomicrobia was detected only in S3 (1% of bacteria; see Fig. 6). This phylum consists of uncultured members comprising at least three discrete phylogenetic clades. The largest one encompasses all *Enttheonella* sequences sensu stricto, and in this study, half of the reads matching this phylum belonged to the candidate genus

Entotheonella, which are widely distributed in marine sponges and seawater (Wilson et al. 2014).

Giving answers to the questions posed

The complex data obtained in this metagenomic study show that all three statues own a very specific microbiome, with a biodiversity that differs with respect to all groups studied (bacteria, fungi, plants, protozoa, etc.). Nevertheless, their microbiomes enable to answer some of the posed questions:

Were the statues stored individually or together during their recent history? Moreover, could all three objects have been stored under building dust and rubble?

A period of common storage in the recent past can be accepted cautiously only for the two torsi (S1 [female] and S2 [male]), but not for the girl's head (S3), which differs considerably from the other two objects mainly in terms of eukaryotes. Nevertheless, in terms of bacteria, S1 and S3 harbor intriguing similarities, such as the presence of many halotolerant and halophilic bacteria, which could indicate a common origin in the past.

There is no definitive indication that all three pieces would have been stored together for a longer period of time under house dust or building rubble, as previously supposed by custom officers. Typical indicators for this environment would be species of *Aspergillus* and *Penicillium* (Pitt and Hocking 2009). Very common *Aspergillus* species described in indoor include *Aspergillus calidoustus*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus penicillioides*, *Aspergillus restrictus*, *Aspergillus versicolor*, *Aspergillus westerdijkiae*, and more recently, *A. sydowii* (Visagie et al. 2014). Among the *Penicillium* species, commonly reported species from indoor surveys include *Penicillium expansum*, *Penicillium digitatum*, *Penicillium italicum*, *Penicillium brevicompactum*, *Penicillium chrysogenum*, *Penicillium commune*, *Penicillium glabrum*, *Penicillium olsonii*, *Penicillium oxalicum*, and *Penicillium rubens*.

In our study, *Penicillium* spp. were only detected in S1, but completely absent in S2 and S3. Furthermore, the identified species *P. glandicola* and *P. citrinum* are commonly growing in association with plants, such as cereals. Thus, their presence is more in line with the hypothesis that statue S1 was stored in an agricultural soil. In addition, some species of *Aspergillus* could be markers of house dust, such as the identified *A. proliferans* in S1 and S2, which has been widely found in indoor environments, and *A. penicillioides* only in S1, which is typically found in indoor air and house dust and on substrates with low water activity. Nevertheless, these species were not the dominant ones among the identified Aspergilli. The most dominant species in samples S1 and S2 was *A. sydowii*, which has been identified in indoor environments and dust in countries of Asia and South America, but not in

European countries (Visagie et al. 2014), where it is mainly found as a saprophytic fungus in soils. In S2, *A. ruber*, which is a plant pathogen, was relevant. Therefore, a common storage in a household or in building rubble is not considered the most plausible hypothesis for the statues in the recent past. The data strongly pointed out that most probably both torsi were stored or even buried temporarily together in agricultural soils or even animal farm soils.

In contrast, the girl's head differed considerably from the other two samples and clearly showed a primarily longer stay in arid and semi-arid surroundings, but in any case, a contact or even its submersion in seawater. The fungal and eukaryotic flora differs considerably from the other two objects.

What is the long-term storage history of the three statues?

To answer this question, we have focused on the following biological markers that strongly point out some specific environments where the statues could have been stored:

- i. Biological markers indicating a prolonged contact with agricultural soils, such as land plants, plant-associated and phytopathogenic fungi, as well as typical soil and plant-associated bacteria (found in all three samples; see supplementary Table 3).
- ii. Biological markers indicating a storage in courtyard, stables, or animal farm, such as those bacteria which are typical inhabitants of the gastrointestinal tract of animals, rumen, or manure (mainly in S1, but also in low proportion in S2; see supplementary Table 4).
- iii. Biological markers indicating a contact with saline or arid environments, such as halotolerant and halophilic fungi and bacteria isolated from desert soils, sandy arid soils, saline soils, or even salt mines (in S1 and S3; see supplementary Table 5)
- iv. Biological markers indicating a contact with aquatic and/or marine environments, including members of the Chlorophyta (green algae), Ochrophyta (brown seaweeds), amoebflagellates, and flagellates of the phylum Cercozoa, Dinophyceae (Dinoflagellates), and some fungi of the phylum Chytridiomycota, indicating an aquatic environment in S3. In addition, some bacteria indicated contact with aquatic environments for S1 and S3 (see supplementary Table 6).

Is it possible to infer a geographical origin or a geographical shift?

The eukaryotic community of objects S1 and S2 is similar and can allow the conclusion that both objects have been stored and/or transported together in the recent past. Both objects have references to a stay in China or contact with materials

from China (e.g., wood of packaging/containers/packaging crates). In S2, the detection of *Taiwania* sequences allowed a real geolocation of this sample, because this tree is native and endemic to Eastern Asia. The additional geolocation of an object due to the detection of bacteria uniquely or frequently isolated from one country may be used carefully, due to the ubiquity of bacteria in different geographical locations with similar environmental conditions. Nevertheless, it is important to point out that many of the identified bacteria in this study, mainly in S1, were described in environmental samples in Asia and mainly in China.

Conclusion

Our data showed the potential of the applied genetic strategy to answer interesting questions that may arise when dealing with cultural heritage objects and that may not be related with the state of conservation, but more with the history of the object. It is worth mentioning that the challenge was to retrieve, among the immense amounts of data, those referring to unique groups of organisms that could be considered as specific of one sample or as biological markers of a specific environment.

All three statues showed each a very specific microbiome. Nevertheless, each of the kingdoms analyzed gave hints for the following environments, however with some differences between the three statues: (a) a contact with arid or saline environment, (b) a period spent in agricultural soil and/or animal farm, and (c) a period spent in fresh, brackish, or sea water. A specialty was the clear affiliation of the male torso with a tree endemic to Taiwan.

Summarizing and based on the genetic fingerprints of the three objects, conclusions can be drawn about the storage of the objects: The female and male torsos both indicate storage in agricultural soils, with the female torso being the most probably stored even in an animal farm. The eukaryotic communities of both objects are similar and can allow the conclusion that both objects have been stored or transported together in the recent past. Both objects have references to a stay in China or contact with materials from China (e.g., packaging/containers/packaging crates). The girl's head differs considerably from the two other samples concerning the eukaryotic community but shows intriguing similarities with the female torso concerning the bacterial community, displaying many halotolerant and halophilic bacteria. This fact may indicate a longer stay in arid and semi-arid surroundings as well as in marine environments in the past.

In summary, these strategies offer an immense potential and reveal more information than we could have imagined before. Nevertheless, it is important to note that these techniques pose a challenge concerning the interpretation of the immense amount of data generated.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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