Real-Time PCR for the Detection and Quantification of *Geodermatophilaceae* from Stone Samples and Identification of New Members of the Genus *Blastococcus*†

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Real-time PCR (RT-PCR) technology was used for the specific detection and quantification of members of the family *Geodermatophilaceae* in stone samples. Differences in the nucleotide sequences of the 16S rRNA gene region were used to design a pair of family-specific primers that were used to detect and quantify by RT-PCR DNA from members of this family in stone samples from different geographical origins in Spain. These primers were applied later to identify by PCR-specific amplification new members of the family *Geodermatophilaceae* isolated from the same stone samples. The diversity and taxonomic position of the wild-type strains identified from ribosomal sequence analysis suggest the presence of a new lineage within the genus *Blastococcus*.

The microbial diversity in stones and monument surfaces has been extendedly studied according to different approaches (colony and cell morphology, physiology, protein patterns, and 16S rRNA gene sequences studies) and bacterial strains have been repeatedly described as isolated from different surfaces of stones and monuments (3, 25), karstic caves (5), mountain soils (12), and biodeteriorated wall paintings (6). These studies demonstrated the occurrence of different groups of heterotrophic bacteria, including many species of the order *Actinomycetales*. These actinomycetes demonstrated a great taxonomic diversity and, in spite of a predominance of isolates of the genus *Streptomyces*, members of the genera *Nocardia*, *Rhodococcus*, and *Geodermatophilus* have also been described (5).

The family *Geodermatophilaceae* (18) includes the three genera *Geodermatophilus* (10), *Blastococcus* (1), and *Modestobacter* (12). Some strains of the genus *Geodermatophilus* have been isolated from stone and monument surfaces in the Mediterranean basin, although many of them originate from extreme cryptolithic environments (3, 24). The presence and diversity of *Geodermatophilaceae* in the environment was previously described after a 16S rRNA gene restriction analysis confirmed the presence of three well-separated clusters of the genera *Geodermatophilus*, *Blastococcus*, and *Modestobacter* (24, 25).

In recent years, 16S rRNA gene PCR primers have been extensively used for the molecular identification of microorganisms at different taxonomic levels (11, 15, 19, 20) and more specifically of different families and genera of actinomycetes (13, 16, 17). Two probes were recently described for the specific detection of *Geodermatophilaceae* and *Modestobacter* in

rock surfaces by using fluorescence in situ hybridization (22). Alternatively, the development of a real-time PCR (RT-PCR) with specific primers could provide a quantitative method to detect the presence of members of these taxa in any given environmental sample.

We have focused here on the development of family-specific primers to detect by RT-PCR the presence of members of the family *Geodermatophilaceae* in stones and associated saxicolous lichen samples. Two different geographical origins in Spain were sampled to evaluate the presence of this microbial group: the Central Mountain System in Madrid, with plutonic and metamorphic rocks covered with sedimentary rocks and the limestone outcrops from San Vicente beach in Mallorca island from Balearic islands. We discuss the occurrence of members of *Geodermatophilaceae* in both geological systems.

MATERIALS AND METHODS

Bacterial strains. New isolates were isolated from stones from two Spanish mountain areas (Table 1). Isolates were grown at 28°C on YMG (0.4% glucose, 0.4% yeast extract, and 1.0% malt extract) agar or LM medium (10).

Isolation of stone-inhabiting bacteria. Stone samples were taken from lichen colonized and lichen-free surfaces in each sampling site. At each site, two to five rock faces separated by 20 to 200 m and in different orientations were sampled. The stone surface was disinfected with 96% ethanol to reduce the influence of dust and airborne spores. Samples for isolation plates were prepared by chipping or scrapping with a sterile knife and chisel blade to remove small superficial fragments from 10- to 20-cm² area from the outer layer of the field collected rock. Fragments were pulverized in a sterile mortar.

Genomic DNA extraction. Total genomic DNAs from the type strains used in the present study were recovered and purified as previously described (8).

Stone DNA extraction. Total DNA extraction from lichen-colonized and lichen-free stone surfaces was adapted from a previously described method (26). Stone particles were (250 mg) frozen in liquid nitrogen, ground, and suspended in 0.5 ml of 0.4% (wt/vol) skim milk solution (Difco). Stone particles were sedimented by centrifugation for 10 min at 17,500 × *g*. Supernatants (300 μl) were mixed with 200 μl of extraction buffer (0.66% sodium dodecyl sulfate, 0.31 M NaCl, 110 mM potassium acetate [pH 5.1]), vortexed, and added to 500 μl of water-saturated phenol. After extraction, supernatants (400 μl) were recovered by centrifugation (15 min, 17,500 × *g*), and total DNA was precipitated overnight at −20°C by addition of 1 ml of ethanol. After a 15-min centrifugation at 17,500 ×

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TABLE 1. Geographical origin of stone samples used in this study, detection of *Geodermatophilaceae* using primers Geosp2 and Geosp1, and concentration of total DNA

Sample	Geographical origin	Stone type	Total DNA[b]	RT-PCR amplification[a] (Geosp2/Geosp1)
1A	La Cabrera (Madrid)	Granite (stone)	5.69	1
1B	La Cabrera (Madrid)	Granite (lichen)	15.17	1
2A	La Cabrera (Madrid)	Granite (stone)	29.58	1
2B	La Cabrera (Madrid)	Granite (lichen)	59.28	1
3	Cala S. Vicente (Mallorca)	Limestone	9.40	0
4	Cala S. Vicente (Mallorca)	Limestone	69.99	1
5A	El Atazar (Madrid)	Slate (stone)	41.99	1
5B	El Atazar (Madrid)	Slate (lichen)	89.21	1

[a] Amplification results: 1, positive amplification; 0, absence of amplification products.
[b] Values are expressed as ng of DNA/mg of stone.

g, DNA was washed with 75% cold ethanol and finally dissolved in 100 μl of sterile distilled water. DNA was quantified by spectrometry at 260 nm. The initial yields of total stone DNA is indicated in Table 1. The concentrations of total stone DNA from all of the samples were normalized to 1 ng/μ land serially diluted from 1 to 10^{-5} ng/μl.

Design of oligonucleotide primers. Sequence comparison and analysis were carried out by using programs from the University of Wisconsin GCG package (version 7.2, 1994). GenBank 16S rRNA/DNA sequences were used to design the primers Geosp2 (5′-TCCAAGAAATTGGTGCTA-3′) and Geosp1 (5′-CAG TTGTKGCCCAGAGAC-3′; reverse primer) shown in Fig. 1. Alignments of the 16S region were performed by using the multiple alignment software CLUSTAL W (21) to determine the regions only conserved among *Geodermatophilaceae* species from which the family-specific primers were derived. The genus specificity of oligonucleotides was tested against all DNA sequences available in GenBank with the FASTA program. The melting temperature (T_m) was estimated by using the formulae of Thomas and Dancis and the Lathe formulae (20). Relative T_m values obtained using 0.3 M as a standard salt concentration helped to design pairs of primers with similar high melting temperatures. The presence of primer dimers was discarded by analysis of the melting curve in RT-PCR using iCycler IQ software (Bio-Rad Laboratories, Hercules, CA). The designed oligonucleotides were supplied by Eurogentec (Liege, Belgium).

RT-PCR amplification. DNA preparations were used as a template DNA for the *Taq* polymerase. Reactions were performed in a final volume of 50 μl containing a 0.2 mM concentration of each of the four deoxynucleoside triphosphates (Roche), 0.1 μM concentrations of each primer, 10 μl of the extracted DNA (1/100 dilution) corresponding to 1 ng of total DNA, 0.5 U of *Taq* polymerase (Appligene) in its recommended reaction buffer, and 1 μl of a 1:10,000 dilution of SYBR Green Dye I. Controls without bacterial DNA were included for each PCR experiment. Amplifications were performed in a iCycler IQ detection system (Bio-Rad) according to the following profile: 40 cycles of 30 s at 93°C, 30 s at 49°C, and 2 min at 72°C, followed by 10 min at 72°C. Amplified products were confirmed by observation of a single melting peak and the presence of a unique band of the expected size in a 1.2% agarose gel stained with ethidium bromide.

Quantification of template DNA was performed by interpolation in a standard curve of threshold cycle (C_T) values generated by amplification of known concentrations of genomic DNA of *Geodermatophilus obscurus* DSM43160[T] and *Blastococcus aggregatus* DSM4725[T]. Amplification efficiencies (E) were calculated by the formula E = $10^{(1/m)} - 1$, where *m* is the slope of the standard curve (7).

A
Correlation Coefficient: 0.917   Slope: -2.384   Intercept: 17.813   Y = -2.384 X + 17.813
PCR Efficiency: 162.7 %

□ Soil sample DNA
◦ Serial dilution from *G. obscurus* DNA

B
Correlation Coefficient: 0.985   Slope: -2.962   Intercept: 19.570   Y = -2.962 X + 19.570
PCR Efficiency: 117.6 %

□ Soil sample DNA
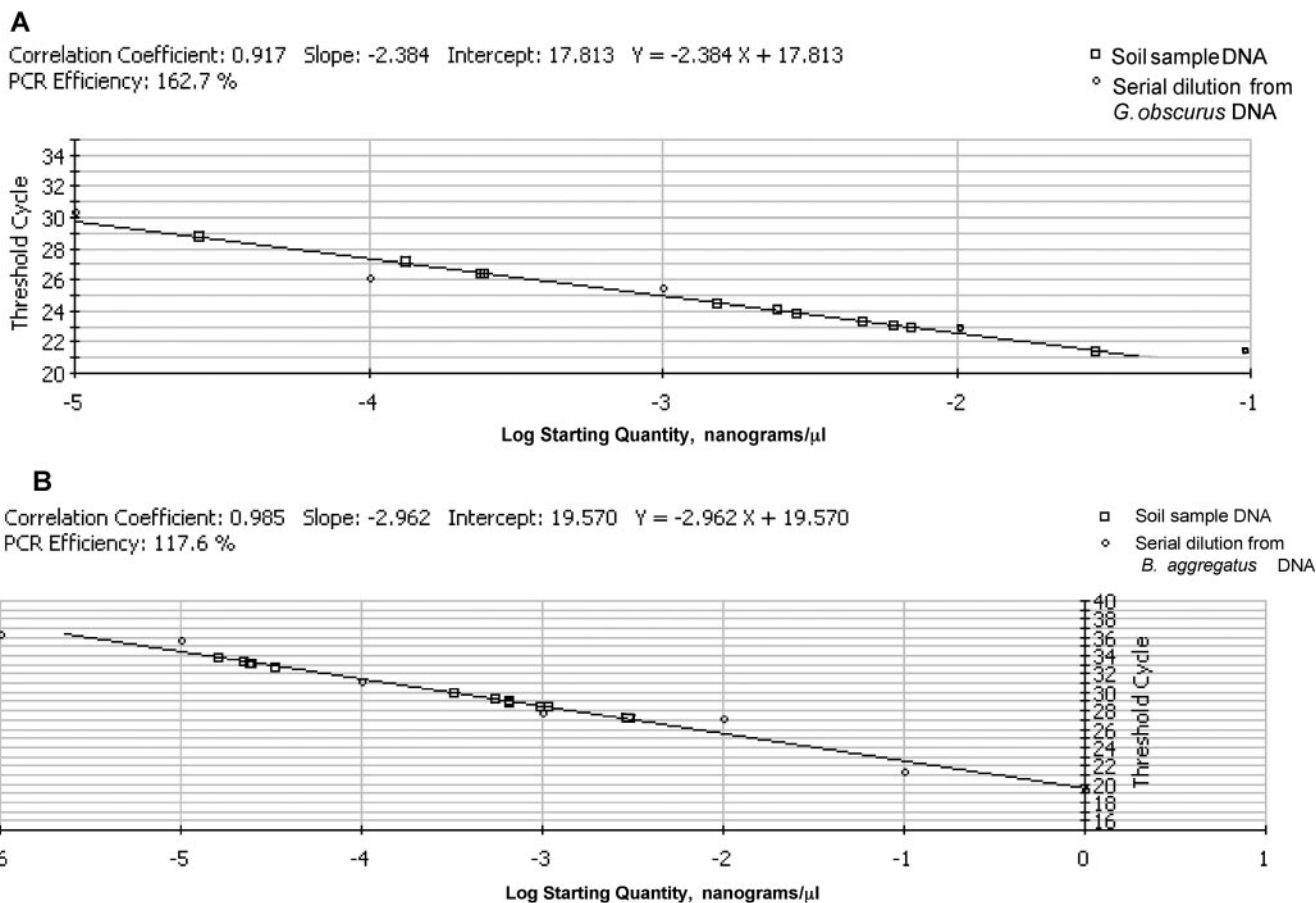◦ Serial dilution from *B. aggregatus* DNA

FIG. 1. Standard curve for the log starting concentration of serial dilutions of DNA from *Geodermatophilus obscurus* DSM43160[T] (A) and *Blastococcus aggregatus* DSM4725[T] (B) versus the C_T value.

TABLE 2. RT-PCR quantification of *Geodermatophilaceae* DNA in serial dilutions of stone DNA samples
using the primers Geosp2 and Geosp1[a]

Sample	DNA quantification (ng/μl)					
	Group A at dilution:			Group B at dilution:		
	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}
1A (stone)	2.18×10^{-3}	7.50×10^{-4}	NA	3.22×10^{-3}	3.43×10^{-4}	NA
1B (lichen)	9.70×10^{-3}	9.52×10^{-5}	NA	6.24×10^{-3}	1.17×10^{-4}	NA
2A (stone)	1.66×10^{-2}	8.40×10^{-4}	NA	7.06×10^{-3}	6.27×10^{-4}	NA
2B (lichen)	3.40×10^{-3}	1.18×10^{-4}	NA	3.25×10^{-3}	8.42×10^{-5}	NA
3 (limestone)	NA	NA	NA	NA	NA	NA
4 (limestone)	7.78×10^{-3}	1.31×10^{-4}	NA	3.74×10^{-3}	4.55×10^{-4}	NA
5A (stone)	1.34×10^{-3}	NA	NA	2.49×10^{-3}	NA	NA
5B (lichen)	1.46×10^{-3}	NA	NA	1.17×10^{-3}	NA	NA

[a] Standard curves were established using serial dilutions of DNA from *Geodermatophilus obscurus* DSM43160[T] (group A) and DNA from *Blastococcus aggregatus* DSM4725[T] (group B). NA, no amplification.

Calculation of 16S rRNA gene molecules. To determine the number of molecules of 16S rRNA gene amplified by RT-PCR, we estimated the weight of the 587-bp *Geodermatophilaceae*-specific amplification fragment in 3.99×10^5 Da (587 bp × 680 Da/bp). To calculate the number of nanomoles per microgram, we used the following calculation: 10^3 ng/μg × [1 nmol/(3.99 × 10^5 ng)] = 2.5 × 10^{-3} nmol/μg. The number of molecules per microgram was determined as follows: [(6.022 × 10^14 molecules)/nmol] × 2.5 × 10^{-3} nmol/μg = 1.505 × 10^12 molecules/μg = 1.505 × 10^9 molecules/ng).

Cloning of PCR fragments from stone samples. Amplification products with the primers Geosp2 and Geosp1 from stone samples and lichens were cloned by using the TOPO cloning Kit (Invitrogen Life Technologies).

DNA sequencing. PCR primers 27f and 1525r (9) were used for the amplification of the 16S rRNA gene genes of the wild-type isolates. The 1,500 bp. PCR products were purified and used as a template in sequencing reactions with the primers 27f, 357f, 530f, 926f, 1392f, 1525r, 1110r, 685r, and 357r (9). *Geodermatophilaceae* amplification fragments from stone samples cloned into pCRTOPO vector were sequenced by using M13 forward and reverse primers (TOPO Cloning Kit; Invitrogen Life Technologies). Amplified and cloned DNA fragments were sequenced by using an ABI Prism Dye terminator cycle sequencing kit (Amersham Biosciences).

Sequence analysis. Sequences were assembled by using the GCG Fragment Assembly System (Program Manual for the Wisconsin Package, version 8). Alignments of the 16S sequences were performed by using the multiple alignment program CLUSTAL W (22). The phylogenetic analysis was completed with 16S rRNA gene sequences of type species of actinomycetes available in GenBank. The data were resampled with 1,000 bootstrap replicates (4) by using the heuristic search option of PAUP (21). The percentage of bootstrap replicates that yielded each grouping was used as a measure of statistical confidence. A grouping found on 95% of the bootstrap replicates was considered statistically significant.

The GenBank accession numbers for the 16S rRNA gene sequences of *Blastococcus* sp. isolates CIBE-G1, CIBE-G2, CIBE-G3, CIBE-G4, CIBE-G5, CIBE-G6, CIBE-G7, CIBE-G8, CIBE-G9, CIBE-G10, CIBE-G11, CIBE-G12, CIBE-G13, and CIBE-G14 are AY903267 to AY903280, respectively. The accession numbers for the amplified partial 16S region from stone samples GEO1A-10, GEO1A-9, GEO1A-4, GEO1A-1, GEO1A-3, GEO1A-2, GEO1A-7, GEO1A-6, GEO1A-8, and GEO1A-5 are AY903281 to AY903290, respectively.

RESULTS AND DISCUSSION

PCR primers for the family *Geodermatophilaceae*. The design of the two PCR specific primers for members of the family *Geodermatophilaceae* was based on the analysis of conserved sequences in the 16S rRNA gene in all members of the family. Primers Geosp2 (18 bp, forward) and Geosp1 (18 bp, reverse) hybridize, respectively, in positions 156 to 174 and positions 725 to 743 (2) of the 16S rRNA gene (see supplemental material). Sequence alignment shows a total identity with members of the genus *Geodermatophilus*. The species *Modestobacter multiseptatus* shows a single mismatch with one of the primers.

Two *Blastococcus* species (*Blastoccoccus aggregatus* DSM4525[T] and *Blastococcus saxobsidens* BC444[T], BC542, BC521, BC517, and BC512) show three mismatches with the sequence of the primer Geosp1 and one mismatch with the sequence of the primer Geosp2 (see supplemental material). This region is not so well conserved in other genera, where more than five mismatches can be observed. We have validated the primer pair Geosp2-Geosp1 with DNA extracted from three different subspecies of *Geodermatophilus obscurus* and from *Blastococcus aggregatus* DSM4525[T] obtaining in all cases the expected family-specific amplification fragment of 587 bp. No amplification was obtained when the primers were also tested with members of the families *Pseudonocardiaceae*, *Micromonosporaceae*, *Actinosynnemataceae*, *Nocardiaceae*, and *Streptomycetaceae*. Our data show that the primer pair Geosp2-Geosp1 allows the specific identification of all of the tested strains belonging to the family in spite of the small differences observed in their sequences.

Detection and quantification of *Geodermatophilaceae* DNA in stone surfaces by specific amplification. The detection limits of the primers Geosp2 and Geosp1 was established in standard curves representing the threshold cycles (C_T) versus the log of 10-fold serial dilutions (1 to 10^{-5} ng/μl) of DNA from *Geodermatophilus obscurus* DSM43160[T] and *Blastococcus aggregatus* DSM4725[T] (Fig. 1). A strong linear relationship between C_T and the log of the starting copy number was demonstrated with both *G. obscurus* DSM43160[T] DNA and *B. aggregatus* DSM4725[T] DNA standard curves (correlation coefficients $r^2 \geq 0.917$ and $r^2 \geq 0.985$; amplification efficiencies [E] = 162.7% and [E] = 117.6%, respectively). The lowest estimated amount of DNA detected by PCR is in both cases in the range of 10^{-5} ng/μl. However, the C_T values generated by amplification of the minimal level of DNA using both standard curves shows differences with lower values for *G. obscurus* DNA ($C_T = 30$) than for *B. aggregatus* DNA ($C_T = 36$) (Fig. 1).

The family-specific primers were applied to detect the presence of *Geodermatophilaceae* in DNA from stone samples obtained from two different geographic origins in Spain. Total DNAs were purified from the surface of five stone samples, including three lichen colonized and five lichen-free surfaces and diluted to a final concentration of 1 ng/μl. A specific amplification fragment was detected in all of the samples with the exception of the limestone sample three from Mallorca

TABLE 3. Calculation of total 16S rRNA content and total cell
counts per milligram of stone

Sample	No. of 16S rRNA genes (molecules/mg of stone)	Total bacterial cells/mg of stone
1A (stone)	6.56×10^6	6.56×10^6–3.93×10^7
1B (lichen)	2.92×10^7	2.92×10^7–1.75×10^8
2A (stone)	5.01×10^7	5.01×10^7–3.06×10^8
2B (lichen)	1.24×10^7	1.24×10^7–7.55×10^6
3 (limestone)	NA[a]	NA
4 (limestone)	2.34×10^7	2.34×10^7–1.40×10^8
5A (stone)	4.04×10^6	4.04×10^6–2.42×10^7
5B (lichen)	4.40×10^6	4.40×10^6–2.64×10^7

[a] NA, no amplification.

Island (Table 1). Serial dilutions of the template DNA samples (10^{-1} to 10^{-11} ng/μl) were tested in RT-PCR experiments to evaluate the concentration range of *Geodermatophilaceae* DNA in crude stone DNA extracts by extrapolation in both standard curves (Table 2). In all cases, the minimal concentration of total stone DNA required to obtain an amplification product was ca. 10^{-2} ng/μl, and no significant differences were observed between the stone DNAs and stone with lichen DNA samples (Table 2). The levels of *Geodermatophilaceae* DNA detected in total stone DNA samples as deduced from the quantitative amplification of serial dilutions show similar concentrations and range between 10^{-2} and 10^{-3} ng/μl.

The quantification data of *Geodermatophilaceae* DNA in stone DNA serial dilutions using *G. obscurus* and *B. aggregatus* DNA standard curves show results within the same concentration range. In both analysis, the minimal amount of *Geodermatophilaceae* DNA detected is ca. 10^{-4} ng/μl (Table 2). However, a higher concentration of *Geodermatophilaceae* DNA were observed in a 10^{-1} ng of DNA/μl dilution of stone sample 2A, where 1.66×10^{-2} and 7.06×10^{-3} ng/μl were estimated by using, respectively, the *Geodermatophilus obscurus* DSM43160[T] and *Blastococcus aggregatus* DSM4725[T] standard curves (Table 3).

When we compared the amplification data from crude DNA, the C_T values generated by amplification of the minimal level of *Geodermatophilaceae* DNA using both standard curves show differences between both analysis, with lower values in *G. obscurus* curve (C_T = 29 to 30) than in *B. aggregatus* curve (C_T = 32 to 34). However, in spite of the lack of linearity observed in some DNA dilutions, in most of the cases DNA quantification data derived from the *B. aggregatus* DNA standard curve are smaller than those obtained from the same stone DNA dilutions with the *G. obscurus* standard curve. The lack of specificity using DNA from *B. aggregatus* can be due to the lack of total identity of the primer pair Geosp2-Geosp1 with the se-

quence of the 16S rRNA region of *B. aggregatus* (see supplemental material).

To evaluate the effect of nonspecific DNA on the detection level of our primers in amplification reactions with crude stone DNA preparations, we performed a competition experiment where *Blastococcus aggregatus* DSM4725[T] DNA was added to a DNA preparation of stone sample 3 where no amplification products were detected with primers Geosp1 and Geosp2. The presence of endogenous bacterial DNA in the stone sample was confirmed by RT-PCR experiments with the universal primers 1497f and 115r directed to conserved sequences at the end and beginning of the 16S and 23S rRNA genes, respectively (9). Serial dilutions of total stone DNA (10^{-1} ng/μl) containing *B. aggregatus* DNA (10^{-2} ng/μl) were tested in real-time PCR experiments to evaluate the concentration range of *B. aggregatus* DNA detected in these conditions. The minimal concentration of *B. aggregatus* DNA is 1.17×10^{-4} ng/μl, whereas this stone sample dilution contains 10^{-3} ng/μl *B. aggregatus* DNA showing the 10-fold reduction in the detection levels in crude stone samples (Table 4).

16S rRNA content and total cell counts. Our data show the relative low frequency of DNA sequences from members of *Geodermatophilaceae* in the stone samples studied, suggesting their minor presence in these environments. To evaluate the presence of *Geodermatophilaceae* cells in stones we have estimated that the number of 16S rRNA gene molecules in crude stone DNA preparations range from 10^6 to 10^7 molecules/ng of crude stone DNA (10^{-1} ng/μl) (Table 3). The highest 16S rRNA gene content was observed in stone sample 2A from La Cabrera, with 5.01×10^7 molecules/ng (Table 3).

The number of 16S rRNA genes per genome reported in *Actinomycetales* is still limited to a small number of genera, and it has been shown to vary from one to six copies as described in *Streptomyces* and *Thermomonospora* species (14, 27; rRNA Operon Copy Number Database [http://rrndb.cme.msu.edu]). Given the lack of information regarding the number of 16S rRNA genes per genome in *Geodermatophilaceae*, we have considered a range of one to six genes per genome to calculate the number of cells per milligram of stone (Table 3). Total cell counts in stone samples ranged from a high of 5.01×10^7 to 3.06×10^8 (sample 2A) to a low of 4.04×10^6 to 2.42×10^7 (sample 5B) cells/mg of stone using DNA from *G. obscurus* in the standard curve (dilution of 10^{-1} ng/μl) (Table 3).

These results confirm the ability of these pairs of primers to detect the presence of *Geodermatophilaceae* directly from crude stone DNA extracts prior to the microbial isolation, and this is the first study detecting and quantifying this taxon in the environment using quantitative PCR.

TABLE 4. RT-PCR quantification of *Geodermatophilaceae* DNA in serial dilutions of stone DNA from sample 3A
using the primer pairs 1497f-115r and Geosp2-Geosp1

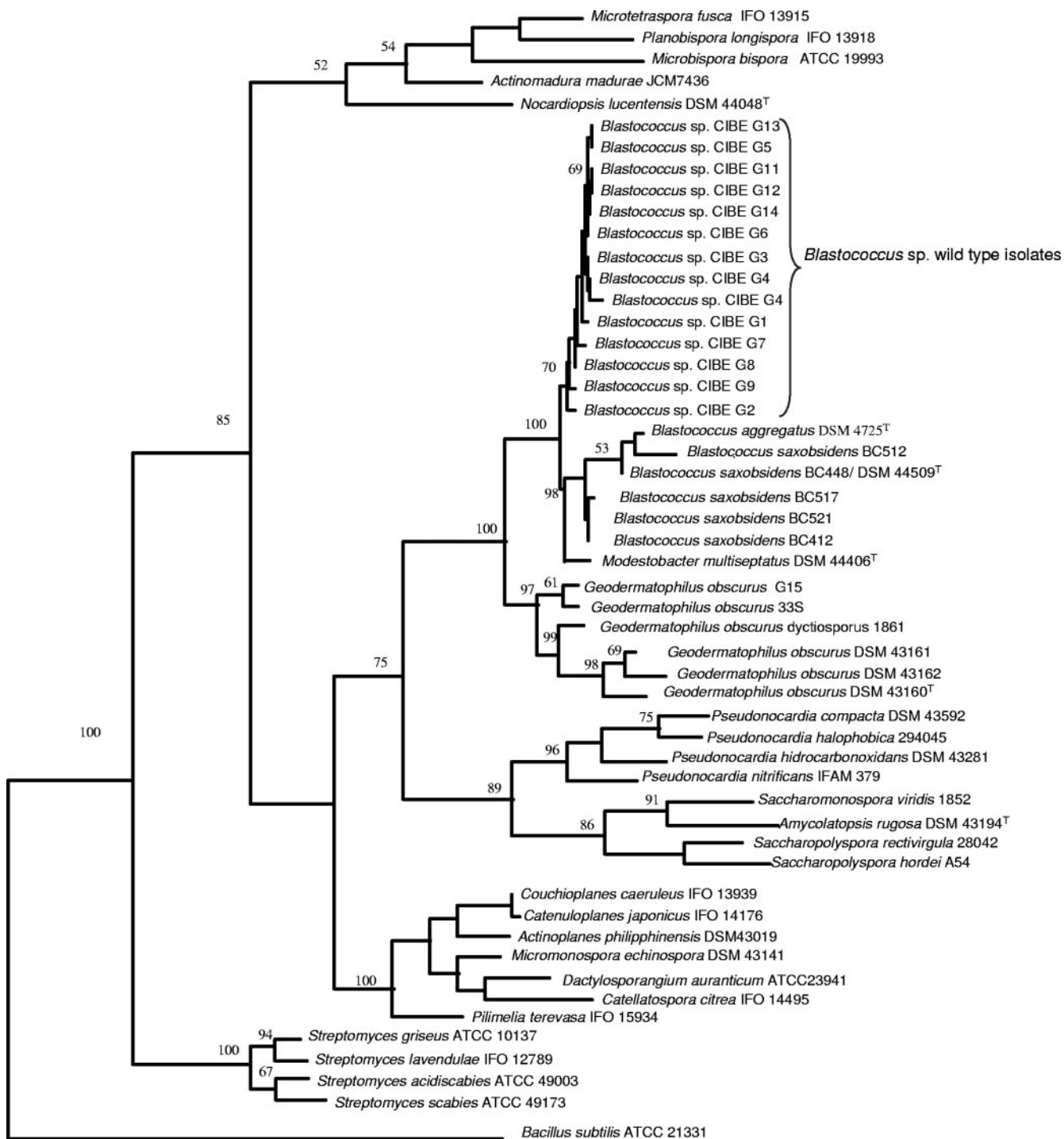| Sample	Primer pair	DNA concn (ng/μl) at dilution:			
		10^{-1}	10^{-2}	10^{-3}	10^{-4}
3A	1497f-115r	5.67×10^{-1}	4.57×10^{-2}	7.98×10^{-3}	5.17×10^{-4}
3A	Geosp2-Geosp1	NA[a]	NA	NA	NA
3A + DNA	Geosp2-Geosp1	6.24×10^{-3}	1.17×10^{-4}	NA	NA

[a] NA, no amplification.

FIG. 2. Phylogenetic tree showing the position of the wild-type strains of the family *Geodermatophilaceae* derived from 16S rRNA gene analysis by using the maximum-parsimony method in PAUP 4.0. The numbers above the branches indicate the bootstrap percentage from 1,000 replicates that was used as a measure of statistical confidence. GenBank accession numbers of sequences from all of the species included in the analysis are as follows: X92359 (*Geodermatophilus obscurus* G16), X92357 (*Geodermatophilus obscurus* DSM 43162), X92355 (*Geodermatophilus obscurus* DSM 43161), X92356 (*Geodermatophilus obscurus* DSM 43160), L46061 (*Geodermatophilus obscurus dictyosporus* 33S), L46020 (*Geodermatophilus obscurus* G15), Y18646 (*Modestobaster multiseptatus* DSM 44406), AJ315674 (*Blastococcus saxobsidens* BC412), AJ316573 (*Blastococcus saxobsidens* BC521), AJ316572 (*Blastococcus saxobsidens* BC517), AJ316571 (*Blastococcus saxobsidens* BC448/DSM 44509[T]), AJ316570 (*Blastococcus saxobsidens* BC512), X97888 (*Nocardiopsis lucentesis* U0297), D85497 (*Streptoalloteichus hindustans* IFO15115), X53191 (*Kibdelosporangium aridum* 2030), Z38017 (*Saccharomonospora azurea* K161), AF139830 (*Saccharomonospora glauca* KCTC 3673), Z38021 (*Saccharomonospora viridis* 1852), AF051342 (*Amycolatopsis rugosa* DSM 43194[T]), U9334 (*Saccharopolyspora hirsuta* ATCC 27865), X53197 (*Saccharopolyspora hordei* A54),

Cloning and sequence analysis of PCR products from stone DNA. To confirm the specificity of the PCR primers, we cloned and sequenced the amplification fragments obtained by using the primers Geosp1 and Geosp2 from the stone sample 1A from La Cabrera Mountains. Ten transformants were randomly selected, and the sequences obtained from the 587-bp amplification fragments (positions 133 to 720 of 16S rRNA gene region) showed a high conservancy among the sequences, with nucleotide similarity ranging between 99.0 to 99.87%. FastA analysis of cloned sequences shows as closest match sequences of type species of the genera *Geodermatophilus* and *Blastococcus* (99% similarity), therefore validating the specificity of the primers for the detection of members of *Geodermatophilaceae* in stone samples.

Identification of wild-type isolates and evaluation of their diversity. One of the purposes of the design of the primers was the rapid detection of members of the family among the strains that are isolated from the environment and that share morphological traits with the family *Geodermatophilaceae*. We have applied these pairs of primers to the rapid PCR identification of 14 wild-type strains isolated from stone sample 1A (La Cabrera Mountain, Madrid, Spain) that exhibited morphological characteristics of *Geodermatophilaceae*. All of the isolates were dark-brown pigmented and exhibited a sparse growth as irregularly shaped coccoid cell aggregates. The tentative taxonomic identification of the wild-type isolates was confirmed in all cases by real-time PCR with *Geodermatophilaceae*-specific primers (data not shown). To confirm the genus assignment of our isolates, we determined the almost complete nucleotide sequence of the 16S rRNA gene of the 14 strains. In all of the cases we found that the annealing sequence of each pair of primers was highly conserved among the strains, supporting the specificity of the primers. The alignment shows high homology with the sequence of the forward primer Geosp2 (one mismatch) and reverse primer Geosp1 (two mismatches) (see supplemental material). Among the strains assigned to the family *Geodermatophilaceae* the level of sequence similarity for the complete 16S rRNA gene sequence ranges from 98.9 to 99.86%, indicating the high relatedness of this group of isolates obtained from the same environment. For all of the isolates the closest sequence match was observed with species of the genus *Blastococcus* (sequence similarity levels 97.1 to 99.2%), and lower sequence homologies were observed with other members of the family, such as species of the genus *Geodermatophilus* (96.7 to 96.4%) and the genus *Modestobacter* (97.%).

The sequences from cloned amplification fragments of stone sample 1A DNA and the homologous region of the 16S rRNA gene of the 14 sequenced wild-type strains present a high sequence similarity that ranged between 98 and 96.5%. The high sequence conservation of the amplified region makes it impossible to distinguish at the genus level among members of the family, and therefore these results do not exclude the presence of members of other genera of the family in the stone sample.

A phylogenetic tree based on complete 16S rRNA gene sequences of the wild-type isolates was built by using the maximum-parsimony method (Fig. 2) showing the inter- and intraspecific relationships of the wild-type strains to reference strains of the family *Geodermatophilaceae*, such as the genera *Geodermatophilus*, *Blastococcus*, and *Modestobacter* and other members of the order *Actinomycetales*. The topology of the tree confirms previous works where the monophyletic lineage of the family *Geodermatophilaceae* is supported by 100% of bootstrap value (24). All of the wild-type strains in our study cluster with 70% of bootstrap value and are closely related within the same group with species of the genus *Blastococcus*. This close relationship to members of this genus is highly supported by the bootstrapping values (100%). Nevertheless, the presence of this cluster clearly distinct from the cluster grouping the remaining species of *Blastococcus* highly supported by a 98% of bootstrap could suggest the existence of a new species within the genus *Blastococcus* that will have to be investigated further given the different morphological features exhibited in culture. Previous works on stones biodiversity have noted that, whereas strains of the genus *Geodermatophilus* are very rare, strains of *Modestobacter* are very common on stone surfaces from different climates and strains of the genus *Blastococcus* could be found on rock surfaces in the Mediterranean basin (25). Our data support the view that the interspecific diversity of the genus *Blastococcus* is higher than the number of species described at the moment, being the surface of rock samples and the microenvironment that is associated valuable sources for isolation of new members of the genus.

In the present study we have presented the design and application of the new pair of family-specific primers as a simple method for the rapid identification of new members of the family *Geodermatophilaceae* using RT-PCR. Our results with reference strains, as well as the high degree of conservancy observed among the sequences of the wild-type isolates, validate the specificity of this new pair of primers. The genetic diversity of the wild-type strains obtained from stone samples supports the usefulness of these tools for the specific detection and tentative family assignment of new isolates of these taxa. Further studies with these primers could reveal the extreme value of these tools to determine the presence and distribution of new members of this family in alternative ecological niches not yet explored for this bacterial group.

AF061976 (*Saccharopolyspora rectivirgula* 28042), Z14111 (*Pseudonocardia halophobica* 294045), X55609 (*Pseudonocardia nitrificans* IFAM 379), AJ252826 (*Pseudonocardia hidrocarboxidans* DSM 43281), X76959 (*Pseudonocardia compacta* DSM 43592), X93187 (*Actinoplanes philippinensis* DSM 43019), D85479 (*Couchioplanes caeruleus* IFO 13939), D85476 (*Catenuloplanes japonicus* IFO 14176), X92597 (*Micromonospora echinospora* DSM 43141), U58528 (*Dactylosporangium aurantiacum* ATCC 23491), D85477 (*Catellatospora citrea* IFO 14495), D86946 (*Pilimelia terevasa* IFO 15934), X97888 (*Nocardiopsis lucentesis* DSM 44048^T 14626), U48973 (*Microtetraspora fusca* IFO 13915), D85494 (*Planobispora longispora* IFO 13918), U83912 (*Microbispora bispora* ATCC 19993), U58257 (*Actinomadura madurae* JCM 7436), AB041132 (*Streptomyces acidiscabies* ATCC 49003), D63862 (*Streptomyces scabies* ATCC 49173), Y15501 (*Streptomyces griseus* ATCC 10137), D85116 (*Streptomyces lavendulae* IFO 12789), and AB018487 (*Bacillus subtilis* ATCC 21331).

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