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Rapid TaqMan-Based Quantification of Chlorophyll *d*-Containing Cyanobacteria in the Genus *Acaryochloris*

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Reports of the chlorophyll (Chl) *d*-containing cyanobacterium *Acaryochloris* have accumulated since its initial discovery in 1996. The majority of this evidence is based on amplification of the gene coding for the 16S rRNA, and due to the wide geographical distribution of these sequences, a global distribution of *Acaryochloris* species was suggested. Here, we present a rapid, reliable, and cost-effective TaqMan-based quantitative PCR (qPCR) assay that was developed for the specific detection of *Acaryochloris* species in complex environmental samples. The TaqMan probe showed detection limits of ~10 16S rRNA gene copy numbers based on standard curves consisting of plasmid inserts. DNA from five *Acaryochloris* strains, i.e., MBIC11017, CCMEE5410, HICR111A, CRS, and Awaji-1, exhibited amplification efficiencies of >94% when tested in the TaqMan assay. When used on complex natural communities, the TaqMan assay detected the presence of *Acaryochloris* species in four out of eight samples of crustose coralline algae (CCA), collected from temperate and tropical regions. In three out of these TaqMan-positive samples, the presence of Chl *d* was confirmed via high-performance liquid chromatography (HPLC), and corresponding cell estimates of *Acaryochloris* species amounted to 7.6×10^1 to 3.0×10^3 per mg of CCA. These numbers indicate a substantial contribution of Chl *d*-containing cyanobacteria to primary productivity in endolithic niches. The new TaqMan assay allows quick and easy screening of environmental samples for the presence of *Acaryochloris* species and is an important tool to further resolve the global distribution and significance of this unique oxyphototroph.

Chlorophyll (Chl) *d* was first reported in 1943 in extracts of macrophytic algae (1), but the inability to reproducibly sample Chl *d* in nature and a report suggesting that Chl *d* might be an artifact of the extraction process (2) impeded further research. Thus, it was surprising when Chl *d* was rediscovered in 1996 in the cyanobacterium *Acaryochloris marina* (3). *Acaryochloris* now forms its own genus with seven described strains (4–9). The large amount of Chl *d* within these oxygenic phototrophs (>95% of cellular Chl) indicates a profound involvement in light harvesting and ecological niche occupation, and *A. marina* has indeed exchanged almost all of its Chl *a* (the usually predominant photopigment in oxyphototrophs) with Chl *d* (10, 11). Surprisingly, this exchange includes both of its reaction centers in photosystem I (PSI) and probably almost all of PSII (12, 13). The possession of Chl *d* enables *A. marina* to harvest the near-infrared radiation part (NIR; 700 to 740 nm) of the solar spectrum and grants it the ability to grow in light microhabitats depleted of visible wavelengths (VIS) (14–16). Since its rediscovery, Chl *d* has repeatedly appeared on a global scale, often by indirect evidence in molecular microbial surveys that lead to the detection of 16S rRNA gene sequences related to *Acaryochloris* species (reference 15 and references therein). Chl *d* was also directly observed in pigment extracts separated by high-performance liquid chromatography (HPLC) (6, 16, 17) and in studies employing advanced spectral/microscopic imaging (7, 14–16, 18). The geographical locations with evidence of Chl *d*-like pigments and/or 16S rRNA signatures are widespread and encompass such different habitats as epilithic biofilms in Antarctica (19), Mayan ruins (20), high-altitude lakes in Bolivia (21), mangroves (7), macroalgae (6), stromatolites (9), and endolithic niches on coral reefs (16). Many of these environments are characterized by a limited amount of VIS and enrichment of NIR, favoring growth and proliferation of Chl *d*-containing phototrophs. The majority of these studies provide evidence of *Acaryochloris* species based on microbial community surveys, which are relatively elaborate and time-consuming to perform. A primer-based *Acaryochloris* sp. detection method is already available (22, 23) but requires the use of denaturing gel gradient electrophoresis (DGGE) and subsequent sequencing, making such an approach laborious, costly, and less sensitive. As an alternative, TaqMan probes have been applied in medical microbial studies and have advanced species or group-specific detection of microbes in biofilms (24–26). Here, we report on the development of a TaqMan-based quantitative PCR (qPCR) assay, targeting an *Acaryochloris* sp.-specific region of the 16S rRNA gene allowing for the rapid and stringent detection and quantification of *Acaryochloris* species in environmental DNA extracts.

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TABLE 1 The *Acaryochloris*-specific primer pair and TaqMan probe used in this study[a]

Primer or probe	Sequence (5′→3′)	T_m (°C)	Position (bp)
Forward primer	CTGCAATCTGAACTGAGGCT	57.89	358–377
Reverse primer	TTACGCTCTGGGCTACACA	58.34	442–460
TaqMan probe	CGCGAGTTGGCAGCTCTTTGTCCTAACC	70.40	403–430

[a] Annealing temperatures are based on thermodynamic calculations as implemented in primer3. Locations of the primers and probe are given as the base position in the alignment in Fig. 1.

MATERIALS AND METHODS

Origin and preparation of samples. The *Acaryochloris* strains MBIC110771 (3), CCMEE5410 (5), Awaji-1 (6), CRS (8), and HICR111A (4) were grown in 500-ml glass Erlenmeyer flasks with 200 ml BG11 medium (salinity of 30) in a shaking incubator at 28°C (100 rpm; see Fig. S1 in the supplemental material). Near-infrared radiation (NIR) was provided by narrow-band light-emitting diodes (LEDs) (L720-04AU, 700 to 740 nm, centered at 720 nm; Epitex Inc., Japan) at an irradiance of 20 to 40 μmol photons m^{-2} s^{-1} over a 12:12-h light-dark shift cycle. Absolute irradiance measurements of NIR were done with a calibrated spectroradiometer (Jaz ULM-200; Ocean Optics, Dunedin, FL, USA). For analysis, 6 ml of dense *Acaryochloris* sp. cell culture was spun down, and DNA was extracted using the FastDNA kit for soil (MP Biomedicals, France) using the manufacturer's standard protocol.

Environmental samples for testing the new method consisted of small pieces of crustose coralline algae (CCA) broken off the substratum and collected from eight different marine sites around the globe at depths of <5 m (see Table 2). The sampling depth was informed by a previous study showing that the abundance of *Acaryochloris* was negatively correlated with increasing depth due to the strong NIR absorption in water (16). After collection, all CCA samples were directly submerged in RNAlater (Ambion, Applied Biosystems, USA), incubated at 4°C overnight in complete darkness, and either frozen at −20°C or kept at ambient temperature. Samples stored in RNAlater were removed from tubes with sterile forceps, weighted, and crushed in bleach-cleaned and sterilized mortars. The resulting powder was immediately processed using the FastDNA SPIN kit for soil (MP Biomedicals), with one additional bead-beating cycle. For HPLC analysis, the same CCA samples, stored in RNAlater, were used as input material for pigment extraction.

Soil and sponge samples, used for determining assay-specific inhibition, originated from Hygum (Denmark) and the aquarium of the Museum of National History (Paris, France). All DNA was eluted in molecular-grade water, and the DNA was quantified using a Qubit system (Invitrogen, Life Technologies Europe) and stored at −20°C until further use. All template DNA to be tested for the presence of *Acaryochloris* species was normalized to 5 ng μl^{-1}.

TaqMan probe design, testing, and amplification conditions. A TaqMan hydrolysis probe was designed based on a unique region of the 16S rRNA gene sequence found in the *A. marina* type strain MBIC11017. The probe sequence was determined using the probe design tool implemented in the software package ARB (27). Additional bases were added to obtain optimal annealing temperatures and to avoid unspecific fluorophore quenching at 5′, yielding a 28mer hybridization probe with a melting temperature (T_m) of 70.4°C (Table 1). Probe specificity was confirmed by using the check probe program implemented in the ribosomal database project (28), by using the testProbe tool implemented in the SILVA database (29), and by performing a nucleotide blast (BLASTn). The probe was 5′-6-carboxyfluorescein-*N*-hydroxysuccinimode ester (FAM) and 3′-TQ2 labeled (Biomers, Ulm, Germany). Based on the probe target site on the 16S rRNA gene sequence, primer sets were designed in primer3 (30) to obtain a 102-bp fragment with the probe being located approximately in the middle of the fragment. NCBI primer blast against all sequenced bacterial genomes (as of January 2014) was used to determine the most stringent, *Acaryochloris*-targeting primer pair. The following primer pair was chosen for subsequent qPCR-based assays: AcmFv′ (5′-CTGCAATCTGAACTGAGGCT-3′) and AcmRv′ (5′-TTACGCTCTGGGCTACACA-3′; Table 1). Primer concentrations and annealing temperatures were optimized on diluted DNA obtained from *A. marina* MBIC11017 using the TaqMan Environmental 2.0 master mix (Applied Biosystems, Life Technologies Europe, Denmark) on a LightCycler 96 real-time PCR system (Roche Diagnostics A/S, Hvidovre, Denmark). After being tested, primers were found to give optimal amplification results at final concentrations of 900 nmol for AcmFv′ and 900 nmol for AcmRv′ per 25 μl reaction mixture at an annealing temperature of 60°C. The probe concentration used throughout all experiments was 200 nM in a reaction volume of 25 μl. One microliter of (5 ng μl^{-1}) template DNA was used in subsequent amplification reactions under the following amplification conditions: hot start at 95°C for 10 min followed by 45 cycles of 95°C for 10 s and 60°C for 30 s. All qPCR experiments were set up in a bleach-cleaned laminar flow hood using PCR-grade plasticware. To account for pipetting bias, all amplifications were performed in technical triplicates.

TaqMan standard curves. Plasmid standards for qPCR were prepared by amplifying the 16S rRNA gene fragment using AcmRv′ and AcmFv′ in conjunction with the PCR master mix (Promega, Madison, WI, USA) and DNA originating from the *A. marina* MBIC11017. The resulting 102-bp fragment was purified on an agarose gel, excised using a sterile scalpel, purified (QiaexII; Qiagen Nordic, Sweden), and cloned into the TOPO-TA cloning vector (Invitrogen, Life Technologies Europe) by following the manufacturer's recommendations. Clones were grown in LB medium with the addition of kanamycin (50 μg ml^{-1}), and plasmids were extracted using the Qiagen miniprep kit (Qiagen Nordic, Sweden). Here, the plasmid insert was sequenced to confirm the correct insert (Macrogen, Seoul, South Korea) and then linearized using the NotI restriction enzyme (New England BioLabs, Ipswich, MA, USA), in order to avoid template overestimation due to plasmid supercoiling (31). Complete linearization was confirmed on an agarose gel, and the linearized plasmid was cleaned and concentrated using the DNA clean and concentrator kit (Zymo Research, California, USA). The plasmid concentration was quantified using the Qubit system (Invitrogen, Life Technologies Europe), and copy numbers were calculated using the Thermo-Scientific copy number calculator. The plasmids were diluted into a copy number ranging from 10^8 to 10^0 in molecular biology-grade water for subsequent use as qPCR standard templates. Plasmid standards were immediately aliquoted in a laminar flow hood and stored at −80°C until subsequent use. Plasmid standards were run in each assay together with a nontemplate control (NTC).

qPCR inhibition test. DNA used for subsequent inhibition testing was extracted according to the above-mentioned protocol from two samples, Danish soil (sample i) and the marine sponge *Haliclona* sp. (sample ii). An aliquot of 5 ng μl^{-1} DNA from samples i and ii was spiked with 5 ng μl^{-1} of DNA extracted from the *A. marina* type strain MBIC11017. Two microliters of the mix was used as the template DNA in the TaqMan assay, and the resulting threshold cycle (C_T) values were used to determine the percent inhibition as described in reference 32.

HPLC-based pigment analysis. For HPLC analysis, intact CCA samples from around the globe (Table 2) were crushed in cleaned mortars and resuspended in cold acetone-methanol (7:2 by volume). The pellet was sonicated for 15 s on ice using a Soniprep 150 sonicator (MSE, United Kingdom). The cells were incubated for 2 min on ice in complete darkness and centrifuged at 13,000 × *g*, and the supernatant was filtered through a Minisart 0.2-μm-pore-size filter (Sartorius, Germany). Ammonium acetate (15 μl; 1.0 M) was added to the extracts to further improve pigment resolution before subsequent injection of 50 to 100 μl onto an Ascentis C_{18} column (dimensions, 4.6 by 250 mm; Sigma-Aldrich, Denmark). Pigment separation was performed on an Agilent 1100 Infinity HPLC machine (Agilent Technologies, Santa Clara, CA, USA) equipped with an 1100 Infinity diode array detector for the detection of compound-specific absorption wavelengths. CCA extracts were run with solvent A (methanol-acetonitrile-water, 42:33:25 by volume) and solvent B (methanol-acetonitrile-ethyl acetate, 39:31:30 by volume) in a gradient comprised of

TABLE 2 CCA samples collected from different geographic and climatic zones[a]

Sample ID	Geographic origin	Climatic origin	Chl *d* present	16S rRNA gene copies mg^{-1}
CCA-Peaks	Peaks Island, ME, USA	Temperate	−	0
CCA- Red Sea	Red Sea, Saudi Arabia	Tropical	+	1,781
CCA- South Korea	Changwon, South Korea	Temperate	−	0
CCA- Hanstholm	Hanstholm, Denmark	Temperate	−	0
CCA-Australia	Heron Island, Australia	Tropical	+	3,019
CCA- Thailand	Similian Islands, Thailand	Tropical	−	0
CCA-Croatia	Mljet Island, Croatia	Temperate	−	76
CCA- Spain	St. Felioux, Spain	Temperate	+	1,643

[a] Extracted DNA from the samples was subsequently used in the *Acaryochloris*-specific TaqMan assay presented in this study. *Acaryochloris*-specific copy numbers were derived from plasmid standards containing the 16S rRNA gene fragment and normalized to milligram of input material. All CCA samples were tested for the presence of Chl *d* via HPLC (see Fig. S2 in the supplemental material).

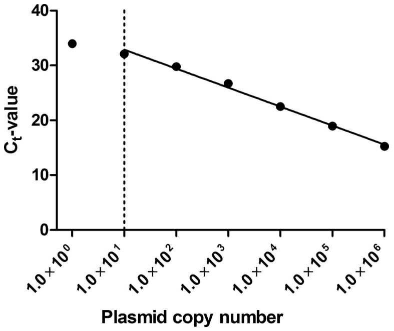

FIG 2 Sensitivity and amplification efficiencies of the *Acaryochloris*-specific TaqMan assay. Threshold cycle (C_T) values were determined by qPCR amplification of *A. marina* MBIC11017 16S rRNA gene fragments within linearized plasmid vectors of known concentrations (10^0 to 10^6 copies). All measurements were performed in technical triplicates and displayed as the mean C_T with standard deviations (not visible due to small deviations). Using the primer pair and TaqMan probe developed in this study, the detection limit of the assay is $\sim10^1$ (indicated by the dotted line). The calculated PCR amplification efficiency was 94.6%, as derived from the slope of the standard curve ($R^2 > 0.99$).

40% solvent B at the time of injection, a linear increase to 100% B at 60 min, and back to 40% B in 3 min. Flow rate was kept constant at 1 ml min^{-1}, and the column was kept at a temperature of 30°C. Photopigments were identified manually by detecting absorption from the resulting HPLC chromatograms as stated in reference 33 (see Fig. S2 in the supplemental material).

RESULTS

A unique, *Acaryochloris* sp.-specific region of the 16S rRNA gene was identified using the ARB software package and used to design a TaqMan-based qPCR assay (see Fig. 1). The assay was tested on plasmid standards and five different *Acaryochloris* sp. strains, i.e., MBIC11017, CCMEE5410, CRS, Awaji-1, and HICR111A, to determine the assay-specific detection limit and qPCR efficiencies. Lastly, DNA from crustose coralline alga samples was used in the assay to detect and quantify the potential presence of *Acaryochloris* species in environmental samples.

TaqMan hydrolysis probe. Based on the testProbe tool (SILVA) and the probeCheck tool (ARB), the designed TaqMan probe was closely matching all five *Acaryochloris* strains, with HICR111A having one mismatch within the 28mer probe and all other strains having 100% identity (Fig. 1; see Table S1 in the supplemental material). No matches to other organisms were found to occur when the probe was aligned with a single mismatch against the entire SILVA/ARB database. Two mismatches increased the number of false positives to four (both databases), while three mismatches resulted in 60 (SILVA) and 69 (ARB) false positives, many of which were assigned as uncultured bacteria in the databases. Performing a nucleotide blast (BLASTn) with the 28mer hybridization probe resulted in *Acaryochloris* species being the primary target (100% homology) and four other uncultured bacteria demonstrating similar E values (6×10^{-6}).

Primers and the TaqMan probe were tested and designed to operate under standard amplification conditions, which can be employed in most qPCR machines, i.e., primer T_m of 60°C, short fragment size (102 bp), and standard FAM fluorophores in conjunction with common quenchers (Table 1). TaqMan-based qPCR assays were initially performed on linearized plasmids containing the 16S rRNA gene fragment. Dilutions of the plasmids, ranging from 1×10^1 to 1×10^6, yielded high correlation coefficients ($R^2 > 0.99$), good PCR efficiency (94.6%, based on a slope of −3.458), and minimal detection limits of ~10 copy numbers (Fig. 2). Detection of lower ($<1 \times 10^1$) or higher ($>1 \times 10^6$) copy numbers was possible but resulted in nonoptimal amplification efficiencies and increasing errors in the technical replicates. Consequently, these standards were omitted in quantification assays performed on environmental samples. Spiking of notoriously difficult samples (DNA from a marine sponge and soil from DK) with DNA from *A. marina* MBIC11017 yielded no significant inhibition (~0.7 to 0.9%) and almost complete recovery of the spiked DNA.

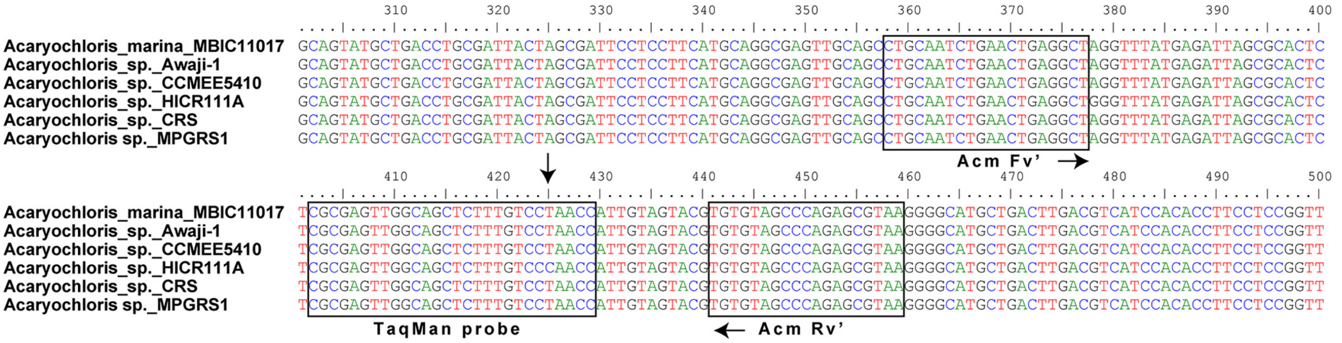

FIG 1 Alignment of the 16S rRNA gene from six strains of *Acaryochloris* species with indicated binding sites for the primers (AcmFv′ and AcmRv′) and TaqMan probe used in this study. The downward-pointing arrow indicates the probe binding site containing a single mismatch toward *Acaryochloris* sp. strain HICR111A.

Amplification efficiencies were calculated for the *Acaryochloris* strains HICR111A, CCMEE5410, CRS, and Awaji-1 and compared to strain MBIC11017, for which 100% efficiency was assumed. Strain CCMEE5410 showed a higher (~106%) amplification efficiency than strain MBIC11017 (assumed to be 100%), while similar or lower amplification efficiencies were determined for strain Awaji-1 (~100%), CRS (~96%), and HICR111A (~94%) (see Table S1 in the supplemental material).

Detection of *Acaryochloris* species and Chl *d* in environmental samples. Eight different environmental samples, i.e., flakes of crustose coralline algae (CCA), were investigated for the presence of *Acaryochloris* species (Table 2). Four of these samples harbored considerable amounts of 16S rRNA gene copy numbers originating from *Acaryochloris* species, with concentrations of 2.5×10^1 to 1.1×10^3 per 5 ng of input DNA. The following CCA samples were within the detection limit of the assay: Australia (1.1×10^3 copies), Spain (2.4×10^2), Red Sea (6.6×10^2), and Croatia (2.5×10^1). The remaining CCA samples had copy numbers that were below the detection limit of the assay; specifically, samples from South Korea (Changwon), Denmark (Hanstholm), the Northeastern United States (Peaks Island), and Thailand (Similian Islands) did not contain detectable amounts of *Acaryochloris* species. Weight-normalized (per mg of input material) copy numbers in CCAs were higher and resulted in the following concentrations (see Table 2): Australia, 3.0×10^3 copies mg^{-1}; Red Sea, 1.7×10^3 copies mg^{-1}; Spain, 1.6×10^3 copies mg^{-1}; and Croatia, 7.6×10^1 copies mg^{-1}.

HPLC of photopigment extracts from the CCA samples revealed the cooccurrence of Chl *d* in three out of four samples that were previously found to contain *Acaryochloris* species (Table 2). No detectable amounts of Chl *d* were found in the CCA sample originating from Croatia, containing 2.5×10^1 copies of *Acaryochloris* species as determined by the TaqMan assay.

DISCUSSION

We developed a rapid and stringent screening assay for *Acaryochloris* species in environmental samples. The assay showed good recovery of 16S rRNA gene copies of *A. marina* MBIC11017 when mixed with DNA extracted from notoriously difficult, inhibitor-containing samples such as sponges and soil. Five different strains of *Acaryochloris* were targeted by the TaqMan probe in a relatively conserved, *Acaryochloris*-specific region. Strain HICR111A was the only strain displaying a single mismatch within the 28mer nucleotide probe, and this strain also exhibited the lowest amplification efficiency compared to that of MBIC1107 (94% compared to 100%, respectively). Lower amplification efficiencies due to probe-target mismatching and template complexity have been reported before (34), showing TaqMan binding to occur at >1 mismatches. Therefore, we cannot completely dismiss the possibility for unspecific binding of the probe to low-complexity target templates with more mismatches. *In silico* alignments of the TaqMan probe against the curated SILVA/ARB database revealed increasing numbers of target species when increasing numbers of mismatches were allowed. Permitting three mismatches, the amount of target species was increased to ~60 to 69, and many of these species were of unknown origin and uncultured. In addition to the known *Acaryochloris* strains, a nucleotide (BLASTn) analysis of the hybridization probe revealed four other sequences displaying 100% homology to the 28mer probe. Interestingly, these sequences originated from DNA surveys of shallow aquatic environments (algae, corals), a habitat preferentially occupied by *Acaryochloris* species (15). We hypothesize that many of the uncultured species targeted by the TaqMan probe could belong to the genus *Acaryochloris* and carry Chl *d* as their major photopigment. Still, not all findings of *Acaryochloris* species are necessarily linked to the cooccurrence of Chl *d*. An *Acaryochloris* sp. strain was, e.g., recently obtained from oil-utilizing communities and reported to not contain Chl *d* (35). Also, other *Acaryochloris* strains have been reported, such as *Acaryochloris* sp. MPGRS1 isolated from mangrove pneumatophores (7), strain *ssball1* from stromatolites (9), and a symbiotic strain ("*Candidatus* Acaryochloris bahamiensis" sp. nov.) residing in the tissue of didemnid ascidians (18). Due to absence of 16S rRNA gene data and/or cultures, we cannot say whether these novel strains are targeted by our probe with the same efficacy as the ones tested herein. We recommend that quantification of *Acaryochloris* with the new TaqMan probe should preferentially be combined with chromatographic methods to confirm the actual presence of Chl *d*.

Endolithic *Acaryochloris* species/Chl *d*. Based on chromatography, imaging, and 16S rRNA gene analysis, Chl *d*-containing cyanobacteria, and specifically *Acaryochloris* species, appear to be almost ubiquitously distributed around the globe (16). The wide distribution of *Acaryochloris* species has been partially attributed to the large genome inherent to all known strains (7.88 to 8.37 Mbp), possibly providing genomic plasticity to cope with a wide range of environmental conditions (36, 37). Such microenvironmental genome imprinting has been elegantly proven for strains MBIC11017 and CCMEE5410, which contain strain-specific mechanisms to alleviate iron starvation and heavy metal toxicity, respectively (5, 37). Recent findings of endolithic Chl *d* (16), attributable to *Acaryochloris* species, highlight the niche-specific adaptations occurring in these microenvironments. Chl *d* enables growth under NIR, a wavelength range used mainly in habitats that encounter very little to no VIS due to the absorbance of overlying layers of phototrophs (13, 14). As to other ecological advantages of using NIR, it has been suggested that *Acaryochloris* species can avoid a considerable amount of light-induced stress by using NIR instead of VIS for oxygenic photosynthesis (8).

So far, members of the genus *Acaryochloris* are the only known Chl *d*-containing phototrophs, and yet their global distribution and relative contribution to primary production is barely known. The new TaqMan assay now allows easy screening of environmental samples from a variety of geographical locations and environments for the presence of *Acaryochloris*. In a first application of the new assay, *Acaryochloris*-related sequences were detected in 4 out of 8 samples of CCA from widely separated habitats (see Table 2): coral reefs in the Red Sea (i) and Australia (ii) and the rocky intertidal zone in Spain (iii) and Croatia (iv). Our findings of *Acaryochloris* species in CCA samples from temperate and subtropical biomes corroborates previous findings of Chl *d* in surface sediments in the arctic/temperate oceanic environments (17) and reports of *Acaryochloris* species in the tropics (15, 16). Available genomic information for strain MBIC11017 and CCMEE5410 reveals that both possess two copies of the 16S rRNA SSU gene, while for the remaining strains, the exact copy numbers are as of yet unknown. Based on 16S rRNA gene copy numbers found in the two genomes, we estimate that cell concentrations in the environmental samples range between 3.81×10^1 (Croatia), 8.21×10^2 (Spain), 8.90×10^2 (Red Sea), and 1.51×10^3 (Australia) per mg CCA. Previous estimates of the relative abundance of *Acaryochlo-*

ris species in tropical environments ranged from 1.3 to 14% of the entire microbial community depending on sample location and depth (15, 16). As no weight normalization was done in the latter studies, we can only hypothesize about exact numbers of cells being present. However, a recent study found ~10^5 cells g^{-1} *Acaryochloris* species in (subtropical) oil-associated communities (corresponding to 10^3 cells mg^{-1}) (35). Such a cell density resembles our concentration estimates in CCAs from the Red Sea and Australia (8.90×10^2 to 1.51×10^3). Considering that CCAs are almost ubiquitously found on coral reefs and very common in temperate shallow subtidal/intertidal habitats, the contribution of *Acaryochloris* species to the endolithic microbiome could be considerable. Our HPLC data support this further, as three out of four samples tested positive for *Acaryochloris* species also contained Chl *d*. The remaining sample, originating from Croatia, contained the least amount of *Acaryochloris* sp. cells (~3.81×10^1), and we conclude that the Chl *d* concentration apparently was too low to be detected in our specific HPLC setup. Also, the CCA samples used in the HPLC and TaqMan assays may not originate from the exact same sampling site; hence, local differences in cell distribution would affect the outcome of the respective detection methods. The microscale distribution of *Acaryochloris* species is most likely influenced by physicochemical parameters such as light and O_2, often resulting in a relatively "patchy" distribution of Chl *d* as revealed by imaging systems (15, 16). Bulk analysis tools such as TaqMan probing and HPLC for the detection of *Acaryochloris* species/Chl *d* can as such provide only an integrated signal (within their specific limit of detection) and do not provide information concerning the microdistribution patterns within naturally occurring biofilms; such information requires more complicated microenvironmental analysis (38). Nevertheless, we believe that the new *Acaryochloris*-specific TaqMan assay will greatly accelerate the search for known and additional strains of these unique phototrophs in a wide range of hitherto unexplored environments.

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