Rapid TaqMan-based Quantification of Chlorophyll d-containing Cyanobacteria in the Genus *Acaryochloris*

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Abstract

Reports of the chlorophyll (Chl) d-containing cyanobacterium Acaryochloris sp. in various habitats 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 sp. was suggested. Here we present a rapid, reliable and cost-effective TaqMan-based qPCR assay that was developed for the specific detection of Acaryochloris sp. in complex environmental samples. The 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 sp. 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 HPLC and corresponding cell estimates of Acaryochloris sp. amounted to 7.6 x 10^1 - 3.0 x 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 sp. and is an important tool to further resolve the global distribution and significance of this unique oxyphototroph.
Introduction

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 high amount of Chl d within these oxygenic phototrophs (>95% of cellular Chl’s) 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 (PS) I and probably almost all of PSII (12, 13). The possession of Chl d enables *A. marina* to harvest the near-infrared part (NIR, 700-740 nm) of the solar spectrum and grants it the ability to grow in light microhabitats depleted of visible wavelengths (VIS) (14–16). Since its re-discovery, Chl d has 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* sp. (15 and references herein). Chl d was also directly observed in pigment extracts separated by 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) as well as 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 majorities of these studies provide evidence of *Acaryochloris* sp. 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 qPCR assay, targeting an Acaryochloris sp. specific region of the 16S rRNA gene allowing for the rapid and stringent detection and quantification of Acaryochloris sp. in environmental DNA extracts.

**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 media (salinity of 30) in a shaking incubator at 28°C (100 rpm, see Fig.S1). Near infrared radiation (NIR) was provided by narrow band LEDs (L720-04AU, 700-740 nm, centered at 720nm, Epitex Inc., Japan) at an irradiance of 20-40 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 extracted using the FastDNA for soil kit (MP Biomedicals, France) using the manufacturers standard protocol.

Environmental samples for testing the new method consisted of small pieces of crustose coralline algae (CCA) broken of the substratum and collected from eight different marine sites around the globe at depths <5 m (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 hereafter either frozen at -20°C or kept at ambient temperature. Samples stored in RNAlater were removed from tubes with a 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 National, 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, USA) and stored at -20°C until further use. All template DNA to be tested for the presence of Acaryochloris sp. 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 Tₘ of 70.4°C (see Table 1). Probe specificity was confirmed using the check probe program implemented in the ribosomal database project (28), the testProbe tool implemented in the SILVA database (29) and by performing a nucleotide blast (BLASTn). The probe was 5’- 6-carboxyfluorescein-N-hydroxysuccinimide 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’-CTGCAATCTGAACCTGAGGCT-3’ and AcmRv’ (5’- TTACGCTCTGGCTACACA-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, DK) on a LightCycler 96 real-time PCR system (Roche Diagnostics A/S, Hvidovre, Denmark). After testing, primers were found to give optimal amplification results at final concentrations of (AcmFv’) 900 nmol and (AcmRv’) 900 nmol per 25 µL reaction 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 µL 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 plastic ware. 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 PCRmaster 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, USA) following
the manufacturers recommendations. Clones were grown in LB media with the addition of Kanamycin (50 µg mL⁻¹), and plasmids extracted using the Qiagen mini-prep kit (Qiagen Nordic, Sweden). Hereafter, the plasmid insert was sequenced to confirm the correct insert (Macrogen, Seoul, 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 & concentrator kit (Zymo-research, CA, USA). The plasmid concentration was quantified using the Qubit system (Invitrogen, Life Technologies Europe, USA) and copy numbers were calculated using the Thermo-Scientific copy number calculator (http://www.thermoscientificbio.com/webtools/copynumber/). The plasmids were diluted into a copy number ranging from 10⁸-10⁹ 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 non-template control (NTC).

qPCR inhibition test

DNA used for subsequent inhibition testing was extracted according to the above mentioned protocol from two samples, (i) Danish soil and (ii) and the marine sponge Haliclona sp. An aliquot of 5 ng µL⁻¹ DNA from (i) and (ii) was spiked with 5ng µL⁻¹ of DNA extracted from the A. marina type strain MBIC11017. Two microliter of the mix was used as template DNA in the TaqMan assay and the resulting Ct values were used to determine % inhibition as described in (32).
HPLC based pigment analysis:

For HPLC analysis, intact CCA samples from around the globe (Table 2) were crushed in cleaned mortars and re-suspended in cold acetone:methanol (7:2 by vol). The pellet was sonicated for 15s, on ice using a Soniprep 150 sonicator (MSE, UK). The cells were incubated for 2 min on ice in complete darkness, centrifuged at 13,000 x g and the supernatant 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 50-100 µL onto an Ascentis C18 column (dimensions: 4.6x 250mm, Sigma-Aldrich, DK). 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 vol) and solvent B (methanol:acetonitrile:ethyl acetate, 39:31:30 by vol) in a gradient comprised of 40% solvent B at time of injection, a linear increase to 100% B at 60 min and back to 40% B in 3 minutes. Flow rate was kept constant at 1 mL min⁻¹ and the column at a temperature of 30°C. Photopigments were identified manually by detecting absorption from the resulting HPLC chromatograms as stated in (33) (Fig. S2).

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, CCME5410, CRS, Awaji-1 and HICR111A, to determine the assay-specific detection limit and qPCR efficiencies. Lastly, DNA from crustose coralline algae samples was used in the assay to detect and quantify the potential presence of *Acaryochloris sp.* in environmental samples.
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, Table S1). 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 sp.* being the primary target (100% homology) and four other uncultured bacteria demonstrating similar e-values (6 x 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 Tₘ’s of 60°C, short fragment size (≈102 bp) and standard 6-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 1x10¹ to 1x10⁶, yielded high correlation coefficients (R² > 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 (< 1x10¹) or higher (> 1x10⁶) copy numbers was possible but resulted in non-optimal 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 (~0.7-0.9%) and almost complete recovery of the spiked DNA.
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 100%), while similar or lower amplification efficiencies were determined for strain Awaji-1 (~100%), CRS (~96%) and HICR111A (~94%, see Table S1).

**Detection of *Acaryochloris sp.* 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 sp.* (Table 2). Four of these samples harbored considerable amounts of 16S rRNA gene copy numbers originating from *Acaryochloris sp.*, with concentrations of $2.5 \times 10^1$-$1.1 \times 10^3$ per 5 ng of input DNA. The following CCA-samples were within the detection limit of the assay: Australia ($1.1 \times 10^3$ copies), Spain ($2.4 \times 10^2$), Red Sea ($6.6 \times 10^2$) and Croatia ($2.5 \times 10^1$). 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 US (Peaks Island) and Thailand (Similian Islands) did not contain detectable amounts of *Acaryochloris sp.* Weight normalized (per mg of input material) copy numbers in CCAs were higher and resulted in the following concentrations (see Table 2): Australia ($3.0 \times 10^3$ copies mg$^{-1}$), Red Sea ($1.7 \times 10^3$ copies mg$^{-1}$), Spain ($1.6 \times 10^3$ copies mg$^{-1}$) and Croatia ($7.6 \times 10^1$ copies mg$^{-1}$).

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

We developed a rapid and stringent screening assay for *Acaryochloris sp.* 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 when compared to MBIC1107 (94% vs. 100%, respectively). Lower amplification efficiencies due to probe-target mismatching and template complexity has 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 ~60-69 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 sp.* (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 sp.* are necessarily linked to the co-occurrence 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 254 *ssball1* from stromatolites (9) and a symbiotic strain (*Candidatus* Acaryochloris bahamiensis nov. sp.) 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* sp./Chl *d***

Based on chromatography, imaging and 16S rRNA gene analysis, Chl *d*-containing cyanobacteria, and specifically *Acaryochloris* sp. appear to be almost ubiquitously distributed around the globe (16). The wide distribution of *Acaryochloris* sp. has been partially attributed to the large genome inherent to all known strains (7.88-8.37 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 strain 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* sp., highlight the niche-specific adaptations occurring in these microenvironments. Chl *d* enables growth under NIR, a wavelength range mainly used 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* sp. 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, 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* sp. in CCAs 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* sp. 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 $3.81 \times 10^1$ (Croatia), $8.21 \times 10^2$ (Spain), $8.90 \times 10^3$ (Red-Sea) and $1.51 \times 10^3$ (Australia) per mg CCA. Previous estimates of the relative abundance of *Acaryochloris* sp. in tropical environments ranged from 1.3-14% 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 $\sim 10^5$ cells g$^{-1}$ *Acaryochloris* sp. 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 ($8.90 \times 10^2$-$1.51 \times 10^3$). Considering that CCAs are almost ubiquitously found on coral reefs and very common in temperate shallow subtidal/intertidal habitats, the contribution of *Acaryochloris* sp. to the endolithic microbiome could be considerable. Our HPLC data supports this further as three out of four samples tested positive for *Acaryochloris* sp. also contained Chl d. The remaining sample,
originating from Croatia, contained the least amount of *Acaryochloris* sp. cells (~3.81 x 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* sp. is most likely influenced by physico-chemical 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* sp./Chl d can as such only provide an integrated signal (within their specific limit of detection) and do not provide information concerning the micro-distribution 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.

**Acknowledgements**

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References


Figure legends:

Figure 1: Alignment of the 16S rRNA gene from six strains of *Acaryochloris* sp. with indicated binding sites for the primers (Acm Fv' and Acm Rv') and TaqMan probe used in this study. The downward pointing arrow indicates the probe binding site containing a single mismatch towards *Acaryochloris* strain HICR111A.

Figure 2: Sensitivity and amplification efficiencies of the *Acaryochloris*-specific TaqMan assay. Cycle-threshold (Ct) values were determined by qPCR amplification of *A. marina* MBIC11017 16S rRNA gene fragments within linearized plasmid vectors of known concentrations (10⁰-10⁶ copies). All measurements were performed in technical triplicates and displayed as the mean Ct with SD (not visible due to small deviations). Using the primer pair and TaqMan probe developed in this study the detection limit of the assay is ~10¹ (indicated by the dotted line). The calculated PCR amplification efficiency was 94.6%, as derived from the slope of the standard curve (R²>0.99).
Table legends:

Table 1: The *Acaryochloris*-specific primer pair and TaqMan probe used in this study. Annealing temperatures are based on thermodynamic calculations as implemented in Primer3. Location of the primers and probe is given as the base position in the alignment in Fig. 1.

Table 2: Crustose coralline algae (CCA) samples collected from different geographic and climatic zones. 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).
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<table>
<thead>
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<th>Primer and TaqMan probe</th>
<th>Sequence (5'-3')</th>
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<td>Reverse Primer</td>
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<table>
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<th>Sample ID</th>
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<th>Climatic origin</th>
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<th>16S rRNA gene copies mg⁻¹</th>
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