

1 **Visualization of Loki- and Heimdallarchaeia (Asgardarchaeota) by fluorescence *in situ***
2 **hybridization and catalyzed reporter deposition (CARD-FISH)**

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4 *Running title: CARD-FISH visualization of Asgardarchaeota*

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22 **Competing Interests**

23 The authors declare no competing financial interests in relation to the work described.

24 **Abstract**

25 Metagenome-assembled genomes (MAGs) of Asgardarchaeota are starting to be recovered
26 from a variety of habitats, broadening their environmental distribution and providing access to
27 the genetic makeup of this archaeal lineage. Despite their singular phylogenetic position at
28 the base of the eukaryotic tree of life, the morphology of these bewildering organisms
29 remains a mystery. In order to visualize this elusive group, we applied a combination of
30 CARD-FISH and epifluorescence microscopy on coastal hypersaline sediment samples,
31 using specifically designed CARD-FISH probes for Heimdallarchaeia and Lokiarchaeia
32 lineages and provide the first visual evidence for both these groups. Here, we show that
33 while Heimdallarchaeia are characterized by a uniform cellular morphology typified by central
34 DNA localization, Lokiarchaeia display a plethora of shapes and sizes that likely reflect their
35 broad phylogenetic diversity and ecological distribution.

36

37 **Main text**

38 The discovery of the Asgardarchaeota not only revealed the closest archaeal lineage to the
39 eukaryotic ancestor, but even more unexpectedly demonstrated that the descendants of the
40 same archaeal lineage are still with us today (1-4). While Thor- and Lokiarchaeia have
41 anaerobic lifestyles, Heimdallarchaeia appear capable of facultative aerobic metabolism, also
42 possessing at least three types of light-activated rhodopsins (5). Despite the overall growing
43 appreciation of these remarkable microbes, a pressing concern is that not a single member
44 of Asgardarchaeota has yet been seen. We chose to tackle this issue by using CARD-FISH
45 and epifluorescence microscopy techniques that have been previously successfully used for
46 visualization of a wide array of microbes (6-9).

47 In order to design specific probes for a morphological characterization of Asgardarchaeota,
48 we manually optimized the alignment of all 16S rRNA gene sequences classified as
49 Asgardarchaeota in ARB (10) using the latest SILVA database (SSURef_NR99_132) (11)

50 and constructed a RAxML tree (GTR-GAMMA model, 100 bootstraps (12)) for all high-quality
51 near full-length sequences (Supplementary Figure 1). All attempts to construct a general
52 probe targeting all Asgardarchaeota sequences (n=935) failed, likely due to the high diversity
53 of this super-phylum. The impossibility to design such broad-range probes is not surprising,
54 as similar difficulties have been reported for other diverse prokaryotic groups (e.g.
55 Proteobacteria (8)). Even the widely used 'general' archaeal probe ARCH915 (13) is
56 unspecific in this regard, overlapping only partially with Asgardarchaeota (80% coverage).
57 This 'general' archaeal probe covers 85% of all archaea, 88.8% Lokiarchaea, but fails to
58 detect most Heimdallarchaeia (only 13% targeted) and is thus not sufficiently reliable to
59 detect Asgardarchaeota. Consequently, we decided to design specific probes for Heimdall-
60 and Lokiarchaeia. Two probes and a set of competitors (14) were constructed targeting
61 93.4% of sequences affiliated to lineage loki1, the larger of the two specific branches of
62 Lokiarchaeia, and 97% of Heimdallarchaeia (Supplementary Figure 1; Supplementary Tables
63 1, 2; for details see Supplementary methods).

64 We tested these probes in sediment samples from two sites from where recently several
65 Asgardarchaeota genomes were recovered by metagenomics (Lakes Tekirghiol and Amara,
66 South Eastern Romania) (5). Seven sediment layers (0-70 cm, in 10-cm ranges) were
67 recovered from Lake Tekirghiol, and the top 10 cm was sampled in Lake Amara. Samples
68 were fixed with formaldehyde, treated by sonication, vortexing and centrifugation to detach
69 cells from sediment particles (15) and aliquots were filtered onto white polycarbonate filters
70 (0.2 μm pore size, Millipore). CARD-FISH was conducted as previously described with
71 fluorescein labelled tyramides (15). Filters were counterstained with DAPI and inspected by
72 epifluorescence microscopy (for details see Supplementary methods).

73 Both phyla were rare and appeared completely absent below depths of 40 cm. All observed
74 Heimdallarchaeia were similar in cell size (2.0 ± 0.5 μm length x 1.4 ± 0.4 μm width, n=23) and
75 of conspicuous shape with DNA condensed (0.8 ± 0.2 x 0.5 ± 0.2 μm) at the center of the cells
76 (Figure 1, Supplementary Figures 2, 4, 6), which is rather atypical for prokaryotes. In

77 contrast, Lokiarchaeia presented diverse shapes and sizes and we could distinguish at least
78 three distinct morphotypes. The most common Lokiarchaeia were small-medium sized, ovoid
79 cells ($2.0\pm 0.5 \times 1.4\pm 0.3 \mu\text{m}$, $n=30$, Figure 2 a-c, Supplementary Figure 3, 4, 5) that were
80 found in different depth layers in Lake Tekirghiol (0-10 cm, 10-20 cm, 20-30 cm) and in the
81 top 10 cm sample from Lake Amara. A single large round cell ($3.8 \times 3.6 \mu\text{m}$, Figure 2 d-f)
82 with bright fluorescence signal and condensed DNA at the center was detected in Lake
83 Amara, and large rods/filaments ($12.0\pm 4.3 \times 1.4\pm 0.5 \mu\text{m}$, $n=6$, Figure 2 g-i, Supplementary
84 Figures 3, 4, 5) with filamentous, condensed DNA ($10.2\pm 4.8 \times 0.6\pm 0.1 \mu\text{m}$) were present at
85 30-40 cm sediment depth in Lake Tekirghiol and in 0-10 cm depth in Lake Amara. The
86 variety of Lokiarchaeia morphologies most likely reflects the higher sampling of the
87 phylogenetic diversity within this phylum. Precise quantification was hampered by the very
88 low abundances in the analyzed samples, which corresponds well to low recoveries of 16S
89 rRNA reads from metagenomes (Supplementary Figure 6)(4). Consequently, it is difficult to
90 draw firm conclusions on sediment depth preferences or rule out additional morphotypes of
91 Lokiarchaeia.

92 During microscopic inspections, we carefully checked for potential non-specific or
93 autofluorescent signals at wavelengths not interfering with the probe signal and found no
94 overlap for any of the inspected cells. A set of negative controls were conducted to rule out
95 false-positive signals due to unspecific binding of dye or nucleic acid components of probes
96 by using a nonspecific probe (NON338, Supplementary Figure 7)(16). To avoid false-positive
97 signals from cellular peroxidases, we performed additional control experiments including the
98 CARD reaction only (without probes). All these control treatments resulted in low, unspecific
99 background signals (comparable to those obtained in samples with probes for
100 Asgardarchaeota), but no obvious staining of cells (Supplementary Figures 4, 7). Further
101 evidence of specificity was seen in all cells hybridized with the Heimdallarchaeia probe, both
102 the shapes and staining patterns coupled to DAPI were remarkably consistent.

103 While tempting, in absence of strong supporting evidence it would be too premature to
104 conclude whether the condensed DNA, particularly in Heimdallarchaeia cells is indicative of a
105 proto-nucleus. Microscopic images of bacterial cells with apparently eukaryotic-features have
106 been misinterpreted before, e.g. in the case of the phylum Planctomycetes (17).

107 Examining these first images of Asgardarchaeota, we could not avoid to note the unusual
108 happenstance of their naming with shapes and ecology. Their initial baptism after
109 mythological characters from Norse mythology (Odin, Thor, Loki and Heimdall (3, 4)) has
110 been unusually prescient (at least for Loki- and Heimdallarchaeia). The many-faced Loki
111 lives up to the name under microscopic scrutiny (Figure 2, Supplementary Figure 3), and
112 Heimdallarchaeia (Heimdall, 'the one who illuminates the world'), appear to be if not light-
113 bringer, at least light-sensitive (5). The unexpected parallels extend even further, Heimdall as
114 gatekeeper between the realms of Midgard and Asgard, reprises the role in a phylogenetic
115 reincarnation as a transition of the archaeal proto-eukaryotes from the dark, anoxic domain
116 of Loki to the sun-lit, oxic world of present day eukaryotes (5).

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127 **Competing Interests**

128 The authors declare no competing financial interests in relation to the work described.

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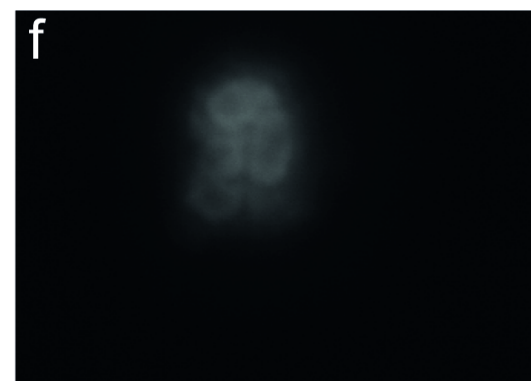
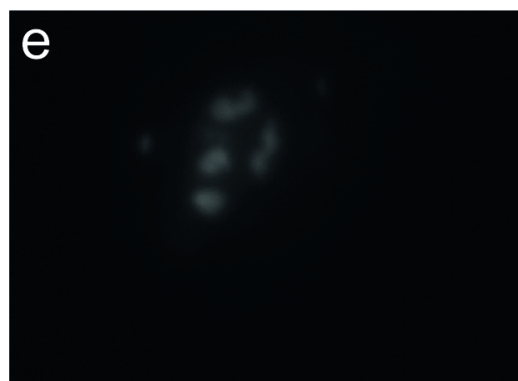
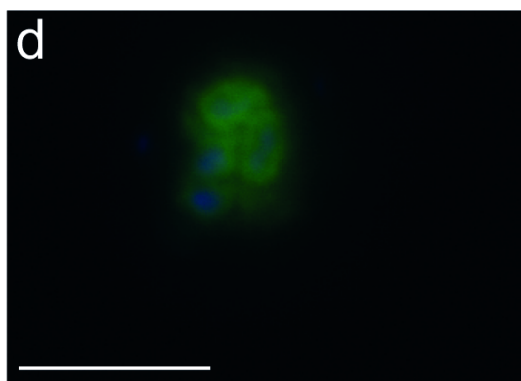
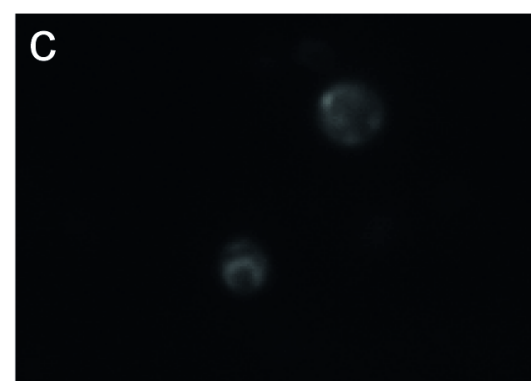
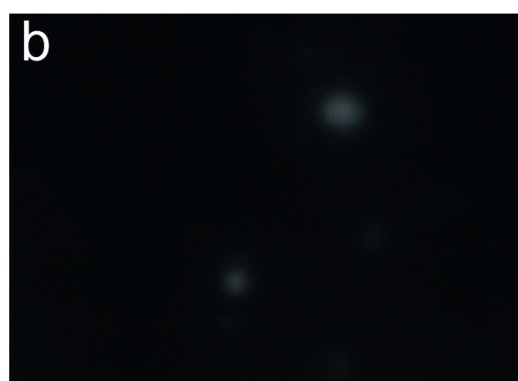
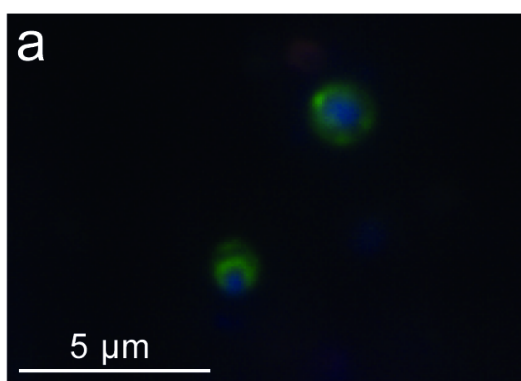
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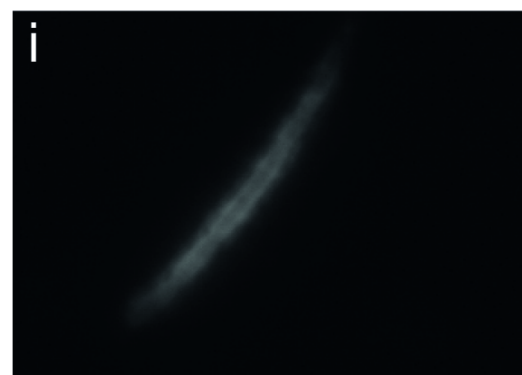
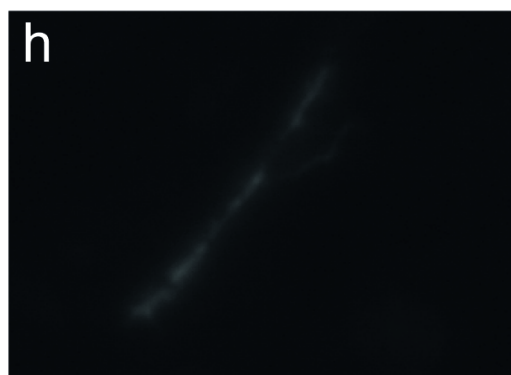
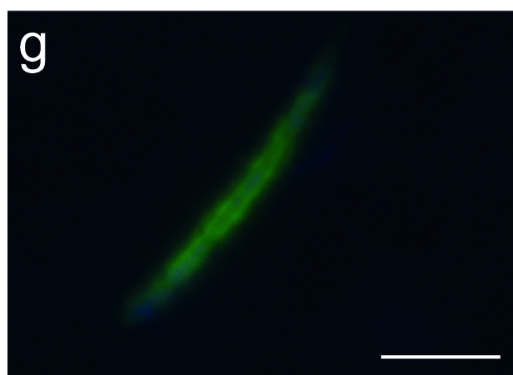
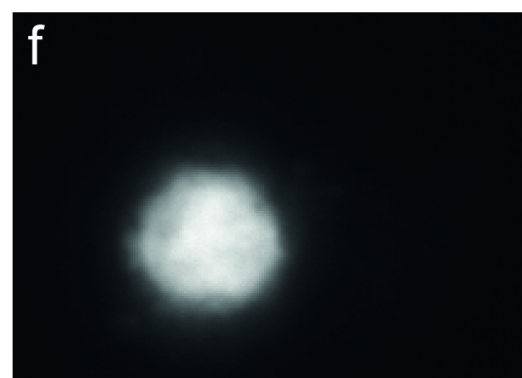
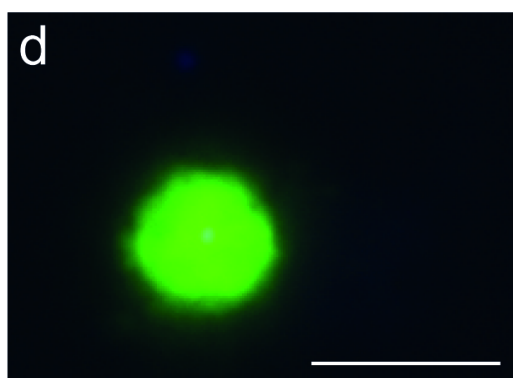
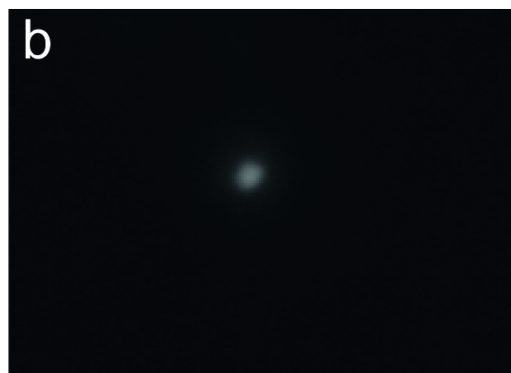
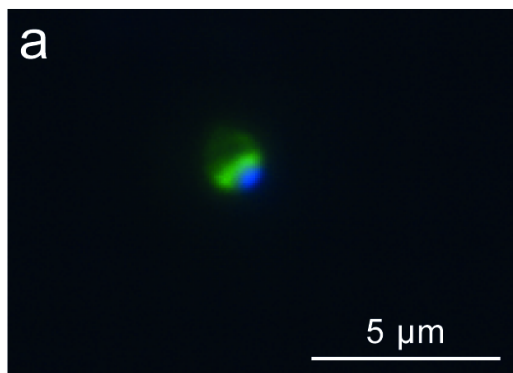
178 **Figure legends**

179 **Figure 1.** CARD-FISH imaging of Heimdallarchaeia hybridized with probe heim-526. The left
180 panels (a, d) display overlay images of probe signal (green), DAPI staining (blue) and
181 autofluorescence (red), the middle panels (b, e) DAPI staining of DNA, the right panels (c, f)
182 CARD-FISH staining of proteins. Individual microphotographs of autofluorescent objects are
183 not displayed because of low intensities and no interference with probe signals (see Figure
184 S4). The scale bar (5 μm) in the left images applies to all microphotographs. Additional
185 images of Heimdallarchaeia can be found in Supplementary Figure S2.

186

187 **Figure 2.** CARD-FISH imaging of Lokiarchaeia hybridized with probe loki1-1184. Three
188 different morphotypes are displayed: (a-d) small-medium sized ovoid cells, (d-f) large round
189 cell, and (g-i) large filamentous cells. The left panels (a, d, g) display overlay images of probe
190 signal (green), DAPI staining (blue) and autofluorescence (red), the middle panels (b, e, h)
191 DAPI staining of DNA, the right panels (c, f, i) CARD-FISH staining of proteins. Individual
192 microphotographs of autofluorescent objects are not displayed because of low intensities and
193 no interference with probe signals (see Figure S4). The scale bar (5 μm) in the left images
194 applies to all microphotographs. Additional images of Lokiarchaeia can be found in
195 Supplementary Figure S3.





Visualization of Loki- and Heimdallarchaeia (Asgardarchaeota) by fluorescence *in situ* hybridization and catalyzed reporter deposition (CARD-FISH)

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Supplementary methods and additional references

Supplementary Figure 1: RAxML tree of 16S rRNA genes of Asgardarchaeota with target hits of probes loki1-1184 and heim-562.

Supplementary Figure 2: CARD-FISH images of Heimdallarchaeia (probe heim-526).

Supplementary Figure 3 CARD-FISH images of two distinct morphotypes of Lokiarchaeia (probe loki1-1184).

Supplementary Figure 4: Images of Heimdallarchaeia and Lokiarchaeia at 400 x magnification with inserts taken at 1000 x magnification.

Supplementary Figure 5: Cell sizes of Heimdallarchaeia and two different morphotypes of Lokiarchaeia.

Supplementary Figure 6: Abundance estimation of recovered 16S rRNA reads affiliated with Heimdallarchaeia and Lokiarchaeia from metagenomes from Loki's castle, Lake Tekirghol and Lake Amara.

Supplementary Figure 7: Control treatments with nonsense probe NON338 and the CARD reaction only without using a probe.

Supplementary methods

During *in silico* testing of the designed probes for specificity and group coverage, we identified two sequences that behaved aberrantly, i.e. could theoretically be regarded as outgroup hits for probe loki1-1184 (GU363076 and EU731577, **Supplementary Table 2**). However, these were discarded after closer examination because of pintail values of 20 and 0, respectively, indicating a chimeric origin (1). Pintail values are measures of chimeric nature for rRNA sequences in the databases, a value closer or equal to 100 indicating a non-chimeric sequence (1). Additional outgroup candidates for probe loki1-1184 (KU351219, AY133348, JQ817340, **Supplementary Table 2**) were initially located within Heimdallarchaeia in the basic tree provided by SILVA, but turned out to belong to Lokiarchaeia after alignment optimizations and RAxML tree reconstruction.

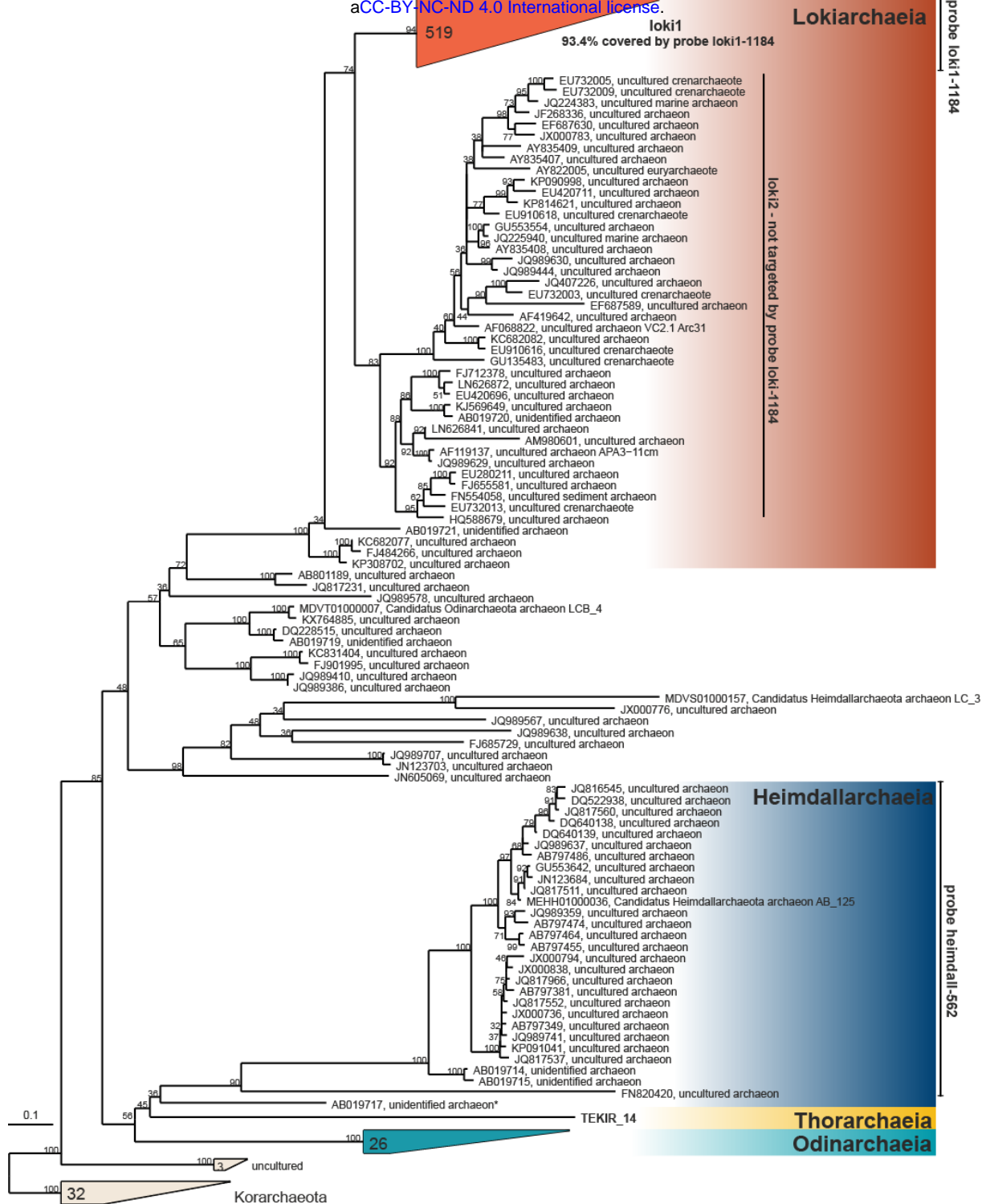
To be absolutely certain to avoid false-positive signals, i.e., to target no other organisms, we designed a set of competitor oligonucleotides that bind specifically to those rRNA sequences that have a single mismatch with our probes (2). We designed three distinct competitor probes for Heimdallarchaeota and two for Lokiarchaeota (**Supplementary Table 1**). Each competitor was used in the same concentrations as the CARD-FISH probes in order to prevent non-specific binding. The usage of specific probes together with competitors has been previously shown to work very well for visualizing cell-morphology and enumeration and was applied numerous times (3-7). Probes were tested *in silico* (8) and in the laboratory with different formamide concentrations in the hybridization buffer until stringent conditions were achieved (**Supplementary Table 1**).

We tested these probes in sediment samples from two sites from where recently several Asgardarchaeota genomes were recovered by metagenomics (Lakes Tekirghiol and Amara, Romania) (9). Sediment sampling was performed using a custom mud corer on 22 April 2018 at 12:00 in Tekirghiol Lake, Romania, (44°03.19017 N, 28°36.19083 E) and Amara Lake (44°36'N, 27°20'E, 23 April 2018, 14:00). Seven sediment layers (0-70 cm, in 10-cm ranges) were sampled in Lake Tekirghiol, and the top 10 cm was sampled in Lake Amara. Samples were fixed with formaldehyde for 1 h and washed three times with 1 x PBS, with centrifugation at 16000 g for 5 min between washes, and a final resuspension in a 1:1 mixture of PBS and ethanol. A treatment of sonication (20 sec, minimum power) on ice, vortexing and centrifugation to detach cells from sediment particles was applied (10) and aliquots diluted with PBS were filtered onto white polycarbonate filters (0.2 µm pore size, Millipore). CARD-FISH was conducted as previously described with fluorescein labelled tyramides and included permeabilization steps with lysozyme and achromopeptidase and an inactivation step for cellular peroxidases with methanol (10). Filters were counterstained with DAPI and inspected by epifluorescence microscopy (Zeiss Imager.M1) with filter sets for DAPI (filter set 01: BP 365/12, FT 395, LP 397), fluorescein (filter set 10: BP 450-490, FT 510, BP 515-565), and autofluorescence (filter set 15: BP 546/12, FT 580, LP 590). Micrographs of CARD-FISH stained cells were recorded with a highly sensitive charge-coupled device (CCD) camera (Vosskühler) at fixed exposure times (70 and 100 ms for DAPI, 100 and 200 ms for CARD-FISH and 100 and 400 ms for autofluorescence for magnifications of 400 x and 1000 x respectively) and cell sizes were estimated with the software LUCIA (Laboratory Imaging Prague, Czech Republic).

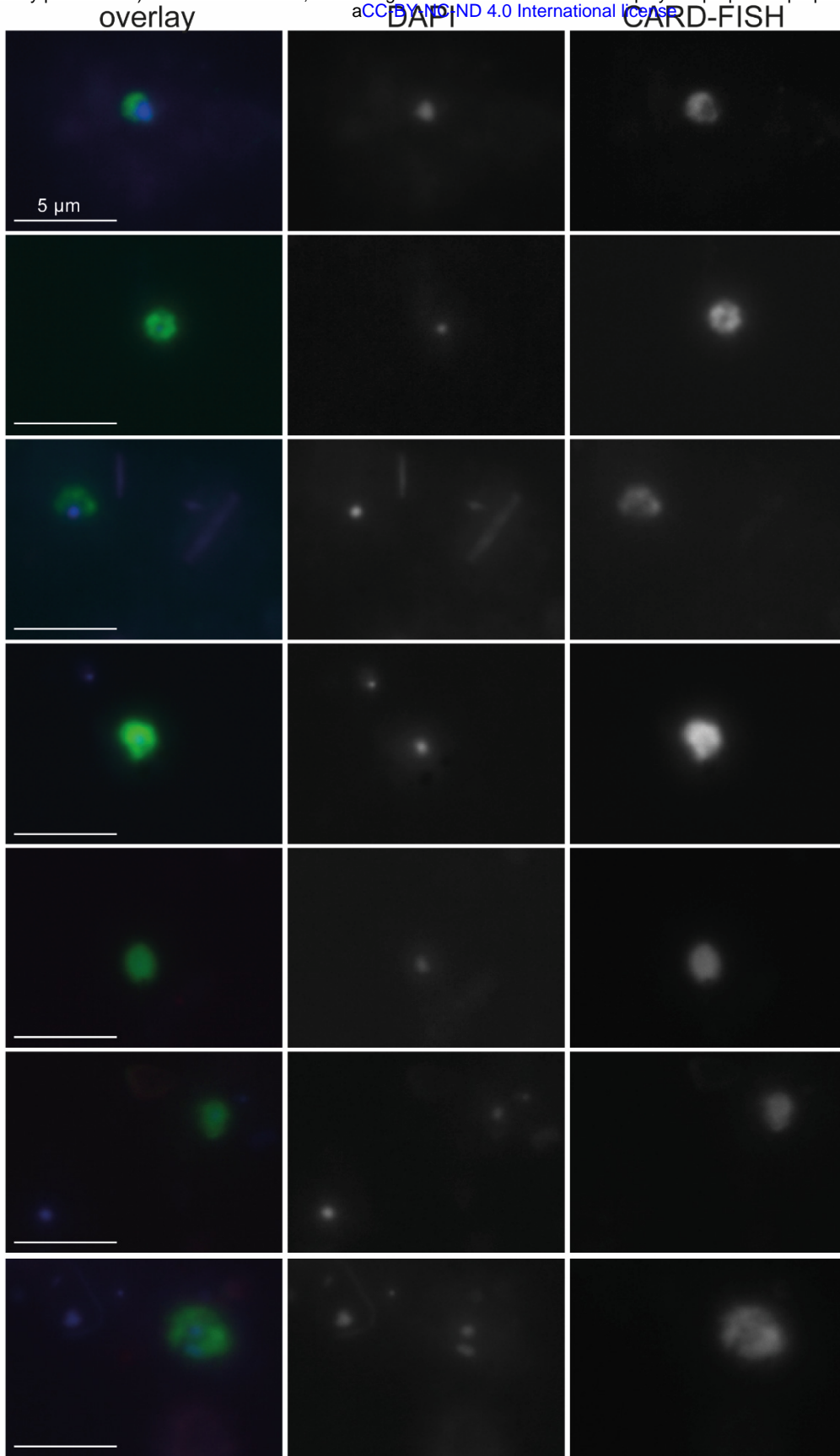
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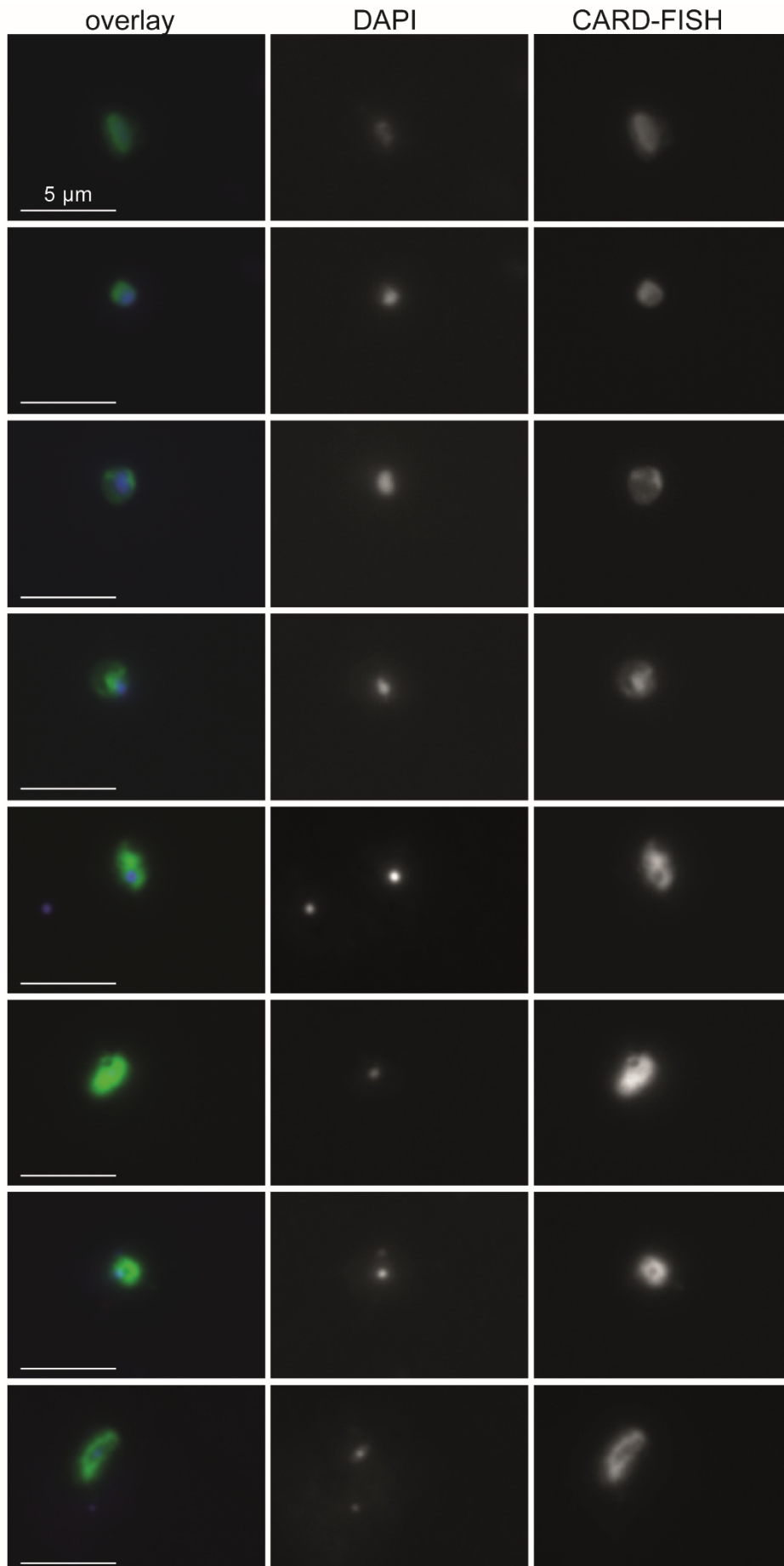


Supplementary Figure 1: RAxML tree (GTR-GAMMA model, 100 bootstraps) of 16S rRNA genes of Asgardarchaeota with target hits of probes loki1-1184 and heim-562. Branches with bootstrap supports <30% were collated to multifurcations. The number of sequences is given for collapsed branches; an asterisk indicates the sequence not targeted by probe heim-562.

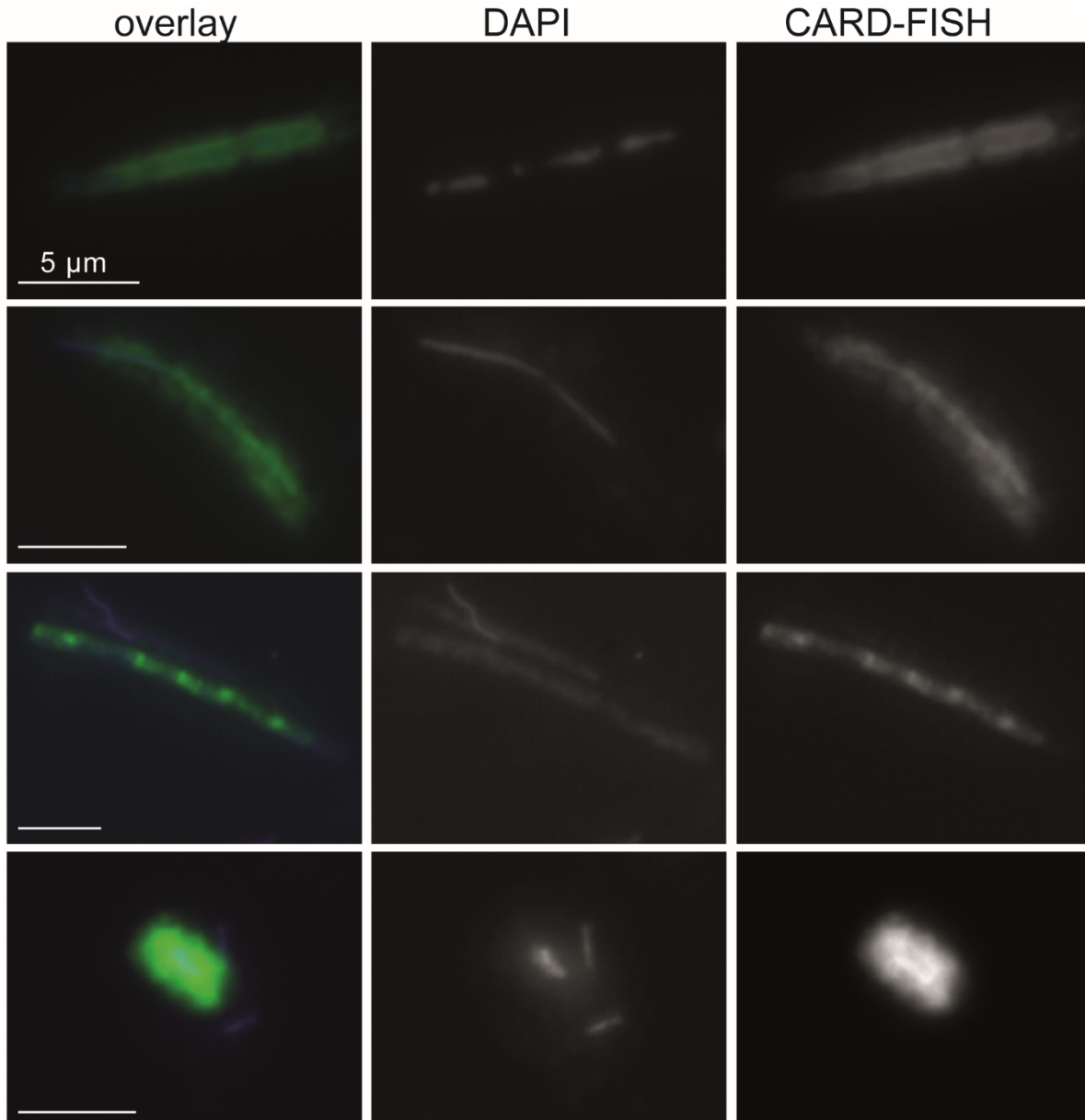


Supplementary Figure 2: CARD-FISH images of Heimdallarchaeia (probe heim-526). The left panels display overlay images of probe signal (green), DAPI staining (blue) and autofluorescence (red), the middle panels DAPI staining of DNA, the right panels CARD-FISH staining of proteins. Individual microphotographs of autofluorescent objects are not displayed because of low intensities and no interference with probe signals.

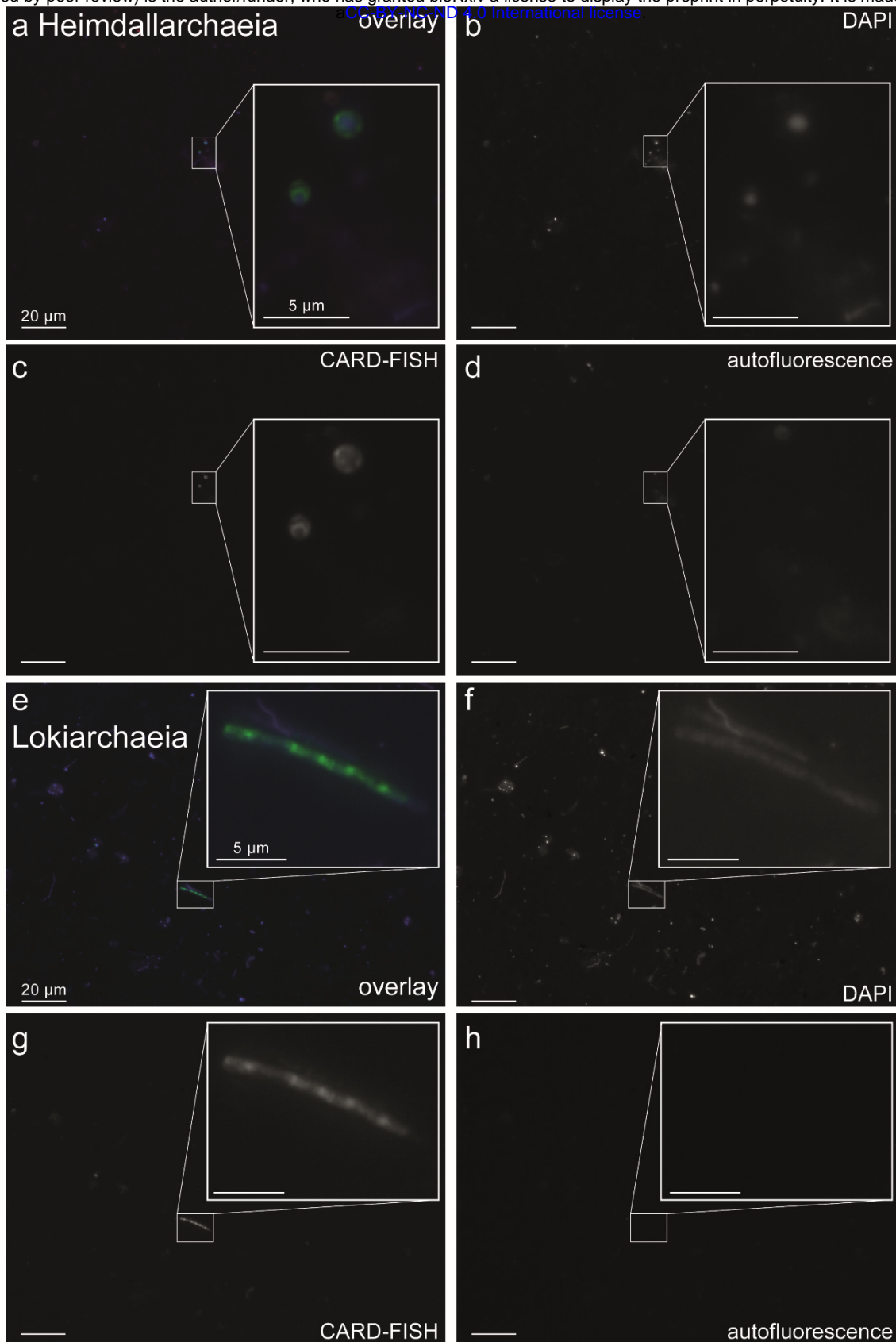
a medium-sized ovoid cells



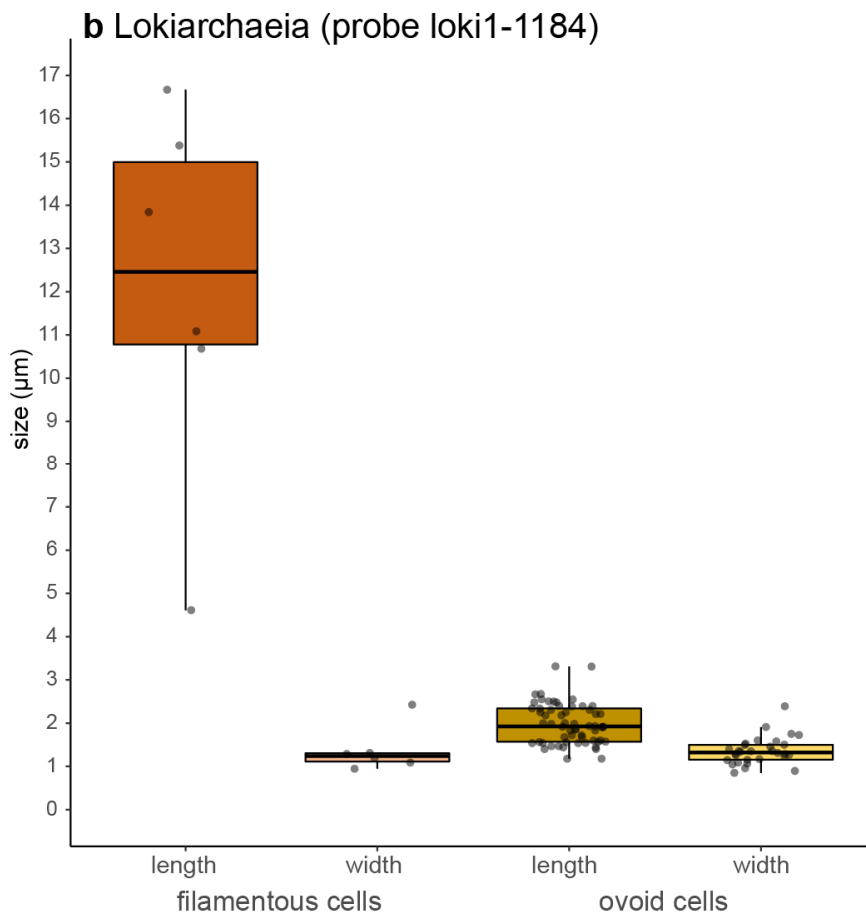
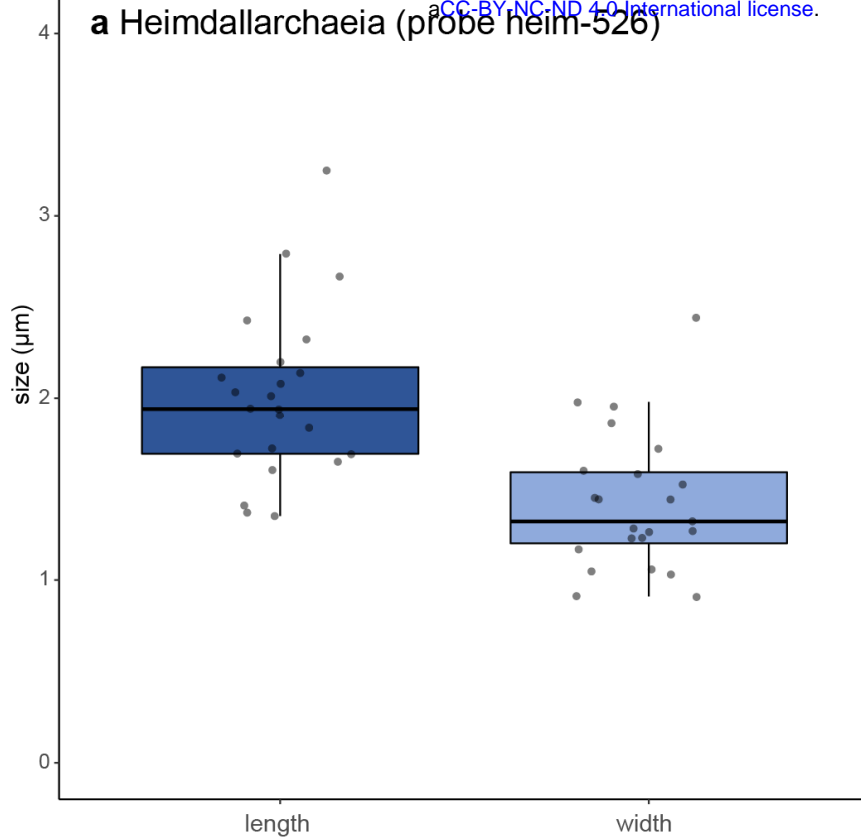
b filamentous cells



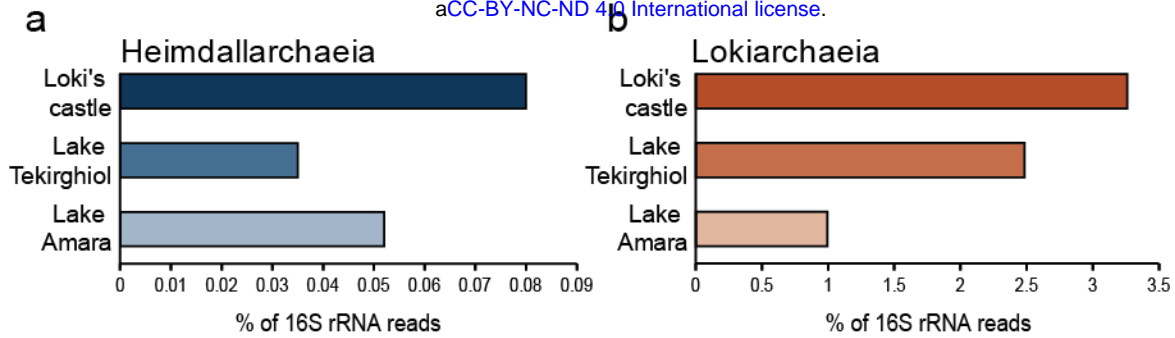
Supplementary Figure 3. CARD-FISH imaging of two distinct morphotypes of *Lokiarchaeia* (probe loki1-1184), (a) medium-sized ovoid cells and (b) filamentous cells. The left panels display overlay images of probe signal (green), DAPI staining (blue) and autofluorescence (red), the middle panels DAPI staining of DNA, the right panels CARD-FISH staining of proteins. Individual microphotographs of autofluorescent objects are not displayed because of low intensities and no interference with probe signals. The scale bar (5 μm) in the left images applies to all microphotographs.



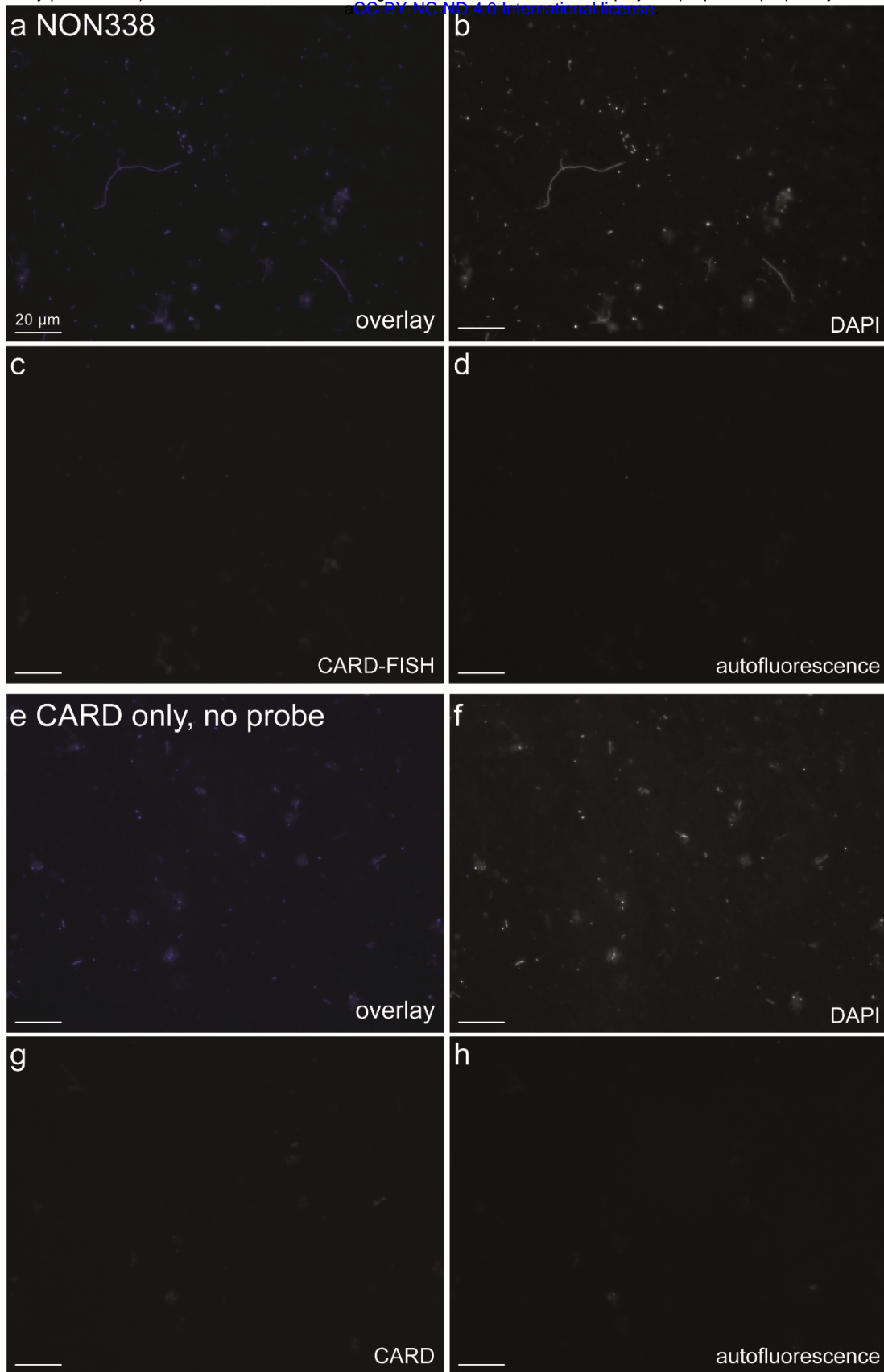
Supplementary Figure 4: Images of Heimdallarchaeia (a-d) and Lokiarchaeia (e-h) at 400 x magnification with inserts taken at 1000 x magnification. (a, e): Overlay images of probe signal (green), DAPI staining (blue) and autofluorescence (red); (b, f): DAPI staining; (c, g): CARD-FISH staining; (d, h): autofluorescence. Images were recorded at fixed exposure times (70 or 100 ms for DAPI, 100 or 200 ms for CARD-FISH and 100 or 400 ms for autofluorescence for 400 x or 1000 x magnification, respectively).



Supplementary Figure 5: Cell sizes (lengths and widths) of (a) Heimdallarchaeia (n=23) and (b) two different morphotypes of Lokiarchaeia (filaments, n=6; small-medium sized ovoid cells, n=30).



Supplementary Figure 6: Abundance estimation of recovered 16S rRNA reads affiliated with Heimdallarchaeia (a) and Lokiarchaeia (b) from metagenomes from Loki's castle, Lake Tekirghiol and Lake Amara. Shotgun metagenomes from Amara (SRA7615342) and Tekirghiol (SRA7614767) lakes, as well as the published SRX684858 from Loki's castle were subsampled to 20 million sequences. Each subset was queried for putative RNA sequences against the non-redundant SILVA SSURef_NR99_132 database, that was clustered at 85% sequence identity. Identified putative 16S rRNA sequences (e-value < 1e-5) were screened using SSU-ALIGN. Resulting bona fide 16S rRNA sequences were compared by blastn (e-value < 1e-5) against the curated SILVA SSURef_NR99_132 database. Matches with identity $\geq 80\%$ and alignment length ≥ 90 bp were considered for downstream analyses. Sequences assigned to Loki- and Heimdallarchaeia were used to calculate abundances for these taxa in their originating environments.



Supplementary Figure 7: Control treatments with (a-d) nonsense probe NON338 and (e-h) the CARD reaction only without using a probe. Overlay images of probe signal (green), DAPI staining (blue) and autofluorescence (red); (b, f): DAPI staining; (c, g): CARD-FISH or CARD only staining; (d, h): autofluorescence. Images were recorded at fixed exposure times (70 ms for DAPI, 100 ms for CARD-FISH and 100 ms for autofluorescence).