Cyanobacterial symbionts diverged in the late Cretaceous towards lineage-specific nitrogen fixation factories in single-celled phytoplankton

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The unicellular cyanobacterium UCYN-A, one of the major contributors to nitrogen fixation in the open ocean, lives in symbiosis with single-celled phytoplankton. UCYN-A includes several closely related lineages whose partner fidelity, genome-wide expression and time of evolutionary divergence remain to be resolved. Here we detect and distinguish UCYN-A1 and UCYN-A2 lineages in symbiosis with two distinct prymnesiophyte partners in the South Atlantic Ocean. Both symbiotic systems are lineage specific and differ in the number of UCYN-A cells involved. Our analyses infer a streamlined genome expression towards nitrogen fixation in both UCYN-A lineages. Comparative genomics reveal a strong purifying selection in UCYN-A1 and UCYN-A2 with a diversification process ~91 Myr ago, in the late Cretaceous, after the low-nutrient regime period occurred during the Jurassic. These findings suggest that UCYN-A diversified in a co-evolutionary process, wherein their prymnesiophyte partners acted as a barrier driving an allopatric speciation of extant UCYN-A lineages.
Sybiontic relationships involving diazotrophic microorganisms, that is, those capable of converting dissolved dinitrogen gas into ammonia, are of relevant interest in marine biogeochemistry because they represent major sources of fixed nitrogen, a limiting nutrient for primary production in the world's oceans. As such, identifying these interactions is essential for understanding the role of symbiosis in biogeochemical cycles. Fortunately, the application of novel approaches such as high-throughput sequencing and single-cell genomics has greatly accelerated the pace of microbial symbiosis research. This is notable in the case of *Candidatus Atelocyanobacterium thalassa* (UCYN-A), a unicellular diazotrophic cyanobacterium, and its partner, a single-celled eukaryotic alga of the class Prymnesiophyceae. Prymnesiophytes as well as UCYN-A are abundant and widely distributed members of the marine plankton and represent ecologically relevant players in carbon and nitrogen cycles. The streamlined genome of UCYN-A and the striking lack of genes encoding the photosystem II complex, the Calvin/Benson/Bassham cycle for carbon fixation, as well as other essential pathways such as the tricarboxylic acid cycle, hinted at a symbiotic lifestyle. UCYN-A is now known to establish a mutualistic relationship based on the exchange of fixed carbon and nitrogen with two different cell-sized prymnesiophyte partners, the unicellular alga *Braarudosphaera bigelowii* (7–10 μm) and an uncultured closely related prymnesiophyte (1–3 μm).

Phylogenomic analyses have demonstrated the monophyly of UCYN-A within the marine cyanobacteria clade that includes *Crocosphaera* sp. and *Cyanophora* sp. clades. Phylogenetic analysis of the UCYN-A nitrogenase gene (nifH) sequences, a common marker used to address the diversity of N₂-fixing microorganisms, distinguished at least three distinct UCYN-A clades: UCYN-A1, UCYN-A2 and UCYN-A3. Comparative genomics revealed that UCYN-A1 and UCYN-A2 lineages share largely syntenic genomic structures, suggesting that both lineages diverged after genome reduction from a common ancestor. Yet, their time of evolutionary divergence and evolutionary pressures remain unknown. It has been suggested that these two variants could be adapted to different niches, that is, coastal waters (B. bigelowii) and open ocean (its closely related prymnesiophyte), but this ecological differentiation was recently ruled out. Although the two prymnesiophyte partners could follow different ecological strategies, the partner fidelity has never been tested in this symbiotic system, and therefore we cannot assume a similar ecological niche for their symbionts. Comparative gene expression studies could help to disentangle the ecological distinction of these two UCYN-A lineages but they are scarce and solely focused on the nifH gene expression without showing a clear differentiation in lineage-specific patterns.

By designing and applying new probes in double catalysed reporter deposition fluorescence in situ hybridization (CARD-FISH), we identified the specific symbiotic associations at the UCYN-A lineage level in samples from South Atlantic waters from the Tara Oceans expedition, where we had previously verified significant abundances of the prymnesiophyte partners. The new probes allowed us to differentiate both symbiotic systems that resulted to vary in the number of UCYN-A cells involved. The coupled analyses of metagenomes and metatranscriptomes from surface and deep chlorophyll maximum (DCM) depths that encompassed four different plankton size fractions distinguish different prymnesiophyte partners based on difference in cell sizes captured in different size fractions, complementing and extending the results obtained by CARD-FISH. Gene expression was explored in the two UCYN-A lineages to decipher whether distinct lineages, in association with distinct partners, exhibit different expression patterns. Finally, we investigated the evolutionary pressures acting on UCYN-A1 and UCYN-A2 lineages by comparative genomic analyses and performed phylogenomic analyses to estimate the age divergence of the two symbiotic lineages. Our findings support a symbiont–host co-evolutionary scenario in the marine environment originating from a single ancestral symbiotic event in the late Cretaceous from which at least two different UCYN-A lineages diversified to become lineage-specific nitrogen fixation factories in their prymnesiophyte partners. Together, these investigations improve our understanding of the relevance of co-evolutionary processes in marine ecosystems and the ecological significance of N₂-fixing symbiosis in the marine biogeochemical cycles.

**Results and Discussion**

**Partner fidelity of the two UCYN-A lineages.** Microscopic *in situ* identification of different UCYN-A lineages as well as their prymnesiophyte partners by specific CARD-FISH staining is crucial to determine the specificity of their relationships. The CARD-FISH method has been successfully applied to identify unicellular diazotrophic cyanobacteria as well as specifically targeting the UCYN-A clade. However, to our knowledge there was not any reported probe to distinguish UCYN-A at the lineage level. We designed a competitor probe to be used with the UCYN-A732 probe to distinguish UCYN-A1 and UCYN-A2 lineages (Fig. 1a–c; Supplementary Table 1). Similarly, we designed two probes to distinguish the two prymnesiophyte partners, *B. bigelowii* (UBRAD069 probe) and the closely related prymnesiophyte (UPRYM69 probe) (Fig. 1a–c; Supplementary Table 1). The UCYN-A732 probe, in the absence of its competitor, labelled UCYN-A cells inside either *B. bigelowii* or the closely related prymnesiophyte partner (Fig. 1a,c). However, when the UBRADO69 probe was applied with the UCYN-A732 probe together with its competitor, UCYN-A cells were unlabelled or labelled when accompanying *B. bigelowii* or the closely related prymnesiophyte partner, respectively (Fig. 1b). It has been proposed that smaller UCYN-A cells are associated with smaller prymnesiophyte cells and vice versa, indicating different growth stages. However, those findings were interpreted from microscopic observations of the UCYN-A symbiosis detected with the general prymnesiophyte PRYM02 and UCYN-A732 (without its competitor) probes, that is, without the ability to distinguish UCYN-A1 and UCYN-A2 cells. The results presented here show that both prymnesiophyte partners are phylogenetically closely related but distinct species, and therefore we suggest that the observed differences in cell sizes of prymnesiophyte partners reflect distinct species rather than different growth stages of the same species. These results demonstrate that UCYN-A lineages display partner fidelity with their prymnesiophyte partners, being *B. bigelowii* and the closely related prymnesiophyte in specific association with UCYN-A2 and UCYN-A1 lineages, respectively.

**The number of UCYN-A cells per partner is lineage specific.** Previous studies have shown that the prymnesiophyte partners can harbour one or two UCYN-A cells, pointing to a coupling between the prymnesiophyte cell division and the number of symbiotic cells, at least for UCYN-A1 (ref. 9). In our samples, only one UCYN-A1 cell per prymnesiophyte cell was detected (Fig. 1a,b). By contrast, *B. bigelowii* carried a symbiosome-like compartment with a variable but higher number of UCYN-A2 cells (~3–10 cells) (Fig. 1b,c). This structure was observed both attached to the host and in a free state, as an entity composed of several UCYN-A2 cells enclosed by a common envelope (Fig. 1c). In a previous study, the UCYN-A2 cells found
in *B. bigelowii* were separated from the *B. bigelowii* cytoplasm by a single membrane, likely a perisymbiont membrane, and the envelope of the UCYN-A2 itself consisted of three layers, possibly an outer membrane, a peptidoglycan wall and a plasma membrane. Although UCYN-A1 and UCYN-A2 are very similar in terms of gene content, the genes involved in cell wall

Figure 1 | Partner specificity and variation of UCYN-A lineages with plankton size fraction. (a–c) Epifluorescence microscopy images with the double-CARD-FISH assay showing the specificity of symbiont-host pairs and (d–f) fragment recruitment of UCYN-A lineages in size-fractionated metagenomes from surface waters collected in station TARA_078. (a–c) Left panels correspond to the 4',6-diamidino-2-phenylindole signal (blue-labelled DNA); right panels correspond to the combined signal of the prymnesiophyte-specific probes (green-labelled host under blue light excitation) and the UCYN-A probe (red-labelled symbiont under green-light excitation). (a) UCYN-A1 with its prymnesiophyte partner; (b) the two UCYN-A symbiotic pairs, indicating the specific labelling of UCYN-A1 (upper) and *B. bigelowii* (lower) with their specific partners, the small prymnesiophyte closely related to *B. bigelowii* and UCYN-A2 respectively; (c) *B. bigelowii* with UCYN-A2. The inset in c shows the detail of non-associated UCYN-A2 cells within a common symbiotic structure. Prymnesiophyte partners are indicated by arrow heads. Scale bar in a represents 5 µm and this scale is shared in a–c except in the inset of c where it indicates 2 µm. (d–f) On the left side, recruitment of metagenomic reads using UCYN-A1 and UCYN-A2 genomes as reference. Reads are plotted as red (UCYN-A1) or blue (UCYN-A2) dots depending on the closest hit genome, representing the covered genome positions (x axis) and the % of identity with the closest reference (y axis). A horizontal grey line set at 95% indicates the threshold for reads representing members of the same population as the reference genome. On the right side, histograms represent the number of recruited reads, in logarithmic scale, by UCYN-A1 (red) or UCYN-A2 (blue) genomes in intervals of 1% identity, from 100 to 70% identity.
biogenesis and cell shape determination appear to be only present in UCYN-A2, suggesting clear structural differences associated with its host. Therefore, our observations hint at different symbiotic organizations: while the UCYN-A1 lineage has one or two separated cells per host, the UCYN-A2 lineage may harbour up to 10 cells per prymnesiophyte partner cell within a common symbiotic structure.

UCYN-A lineages vary in different plankton size fractions. A total of eight marine metagenomes from stations TARA_078 and TARA_076 were analysed to assess the distribution of UCYN-A lineages in several plankton size fractions (0.2–3, 0.8–5, 5–20 and >0.8 µm) of the microbial assemblages in surface and DCM waters (Table 1). We used the two UCYN-A genomes sequenced to date as reference genomes in the fragment recruitment of these metagenomic samples (Table 1). Because of the UCYN-A partner fidelity displayed by double CARD-FISH (see above), metagenomic sequence reads from UCYN-A lineages should vary with size fraction as predicted by the different cell sizes of the prymnesiophyte partners. The sequence reads from the UCYN-A1 lineage were primarily present in surface waters within the size fraction range of the small prymnesiophyte partner (0.2–3, 0.8–5 and >0.8 µm; Table 1). Almost 100% of the UCYN-A1 genome was recovered in each of the metagenomes from these size fractions in the two stations. Likewise, UCYN-A1 sequence reads were poorly represented in the 5–20 µm size fraction (~10% of genome recovery; Fig. 1d–f; Table 1). On the other hand, in TARA_078, the UCYN-A2 sequence read distribution in surface waters was consistent with the B. bigelowii cell size, that is, UCYN-A2 reads were nearly absent in the 0.2–3 µm size fraction metagenomes, but were more abundant in the 0.8–5, 5–20 and >0.8 µm fractions. In all these larger fractions, the UCYN-A2 reached high genome recovery values (90%, 76% and 99%, respectively), except for the >0.8 µm fraction in TARA_076 where UCYN-A2 was virtually absent (Fig. 1d–f, Table 1). In the >0.8 µm size fraction, UCYN-A1 was approximately nine times more abundant than UCYN-A2 in TARA_078 (Table 1). Likewise, in the same station, the small prymnesiophyte partner was more abundant than UCYN-A2 in TARA_078 (Table 1). Likewise, in the same station, the small prymnesiophyte partner was more abundant than UCYN-A2 in TARA_078 (Table 1).

Another interesting finding was that most of the metagenomic (and metatranscriptomic) reads mapping to the UCYN-A1 or UCYN-A2 genomes had very high sequence identities (>99%) to their respective reference genome; Fig. 1d–f), which suggests an extremely low microdiversity within populations that were sampled from geographically distant regions in the Pacific (ALOHA and SIO) and South Atlantic Oceans (this study). The size-fractionated sampling strategy combined with the metagenomic analyses reported in this study will be also important to uncover the genomic pool of new UCYN-A lineages, such as UCYN-A3, to identify the lineage-specific distribution of UCYN-A populations and to set the cell size range of their partners, a first step for their identification.

UCYN-A expression is streamlined to fuel nitrogen fixation. The analyses of seven size-fractionated metatranscriptomes from two stations (TARA_078 and TARA_076) and depths (surface and DCM) allowed for the first time a whole-genome transcription profiling of these widely distributed diazotrophic cyanobacteria (Table 1). In surface waters, UCYN-A1 transcripts were in general more abundant than those from UCYN-A2, except in the 5–20 µm size fraction (TARA_078) in which the latter were dominant (Table 1). The gene expression of 1,131 and 1,179 protein-coding genes in UCYN-A1 (Supplementary Data 1) and UCYN-A2 (Supplementary Data 2), respectively, were examined. In both lineages, the nitrogen fixation operon, including the nifH gene, was the most highly expressed gene-cluster accounting for a quarter of the total transcripts (Fig. 2a,b). In the >0.8 µm size fraction (TARA_078), despite UCYN-A1 being more abundant than UCYN-A2, the expressed nifH transcripts per cell were almost two times higher for UCYN-A2 (648.33) than for UCYN-A1 (396.60; Supplementary Data 1 and 2). It is well known that biological nitrogen fixation

| Table 1 | Fragment recruitment (FR) of UCYN-A lineages. |
|---------|-----------------------------------------------|
| Station | Depth | Sample | Fraction (µm) | Sequencing depth (reads) | FR (reads) | Genome recovery (%) |
| 76      | SRF   | MG     | 0.2–3        | 177,019,968          | 188,088    | 99.30               | 0.14          |
| 76      | SRF   | MT     | 0.2–3        | 18,908,305          | 25,340     | 21.35               | 0.37          |
| 76      | SRF   | MG     | >0.8         | 73,651,199          | 54,776     | 98.61               | 1.35          |
| 76      | SRF   | MT     | >0.8         | 10,283,396          | 12,143     | 8,018               | 0.59          |
| 76      | DCM   | MG     | >0.8         | 115,099,936         | 848        | 9.00                | 0.03          |
| 76      | DCM   | MT     | >0.8         | 12,998,358          | 76         | 0.49                | 0.02          |
| 78      | SRF   | MG     | 0.2–3        | 155,580,203         | 842,234    | 99.94               | 13.95         |
| 78      | SRF   | MT     | 0.2–3        | 13,151,362          | 133,693    | 46.61               | 0.99          |
| 78      | SRF   | MG     | 0.8–5        | 105,731,269         | 980,895    | 99.81               | 90.24         |
| 78      | SRF   | MT*    | 5–20         | 139,070,786         | 1,182      | 10.14               | 76.47         |
| 78      | SRF   | MG     | >0.8         | 97,646,287          | 292        | 1.76                | 34.69         |
| 78      | SRF   | MT*    | 5–20         | 163,575,710         | 719,803    | 99.32               | 99.03         |
| 78      | SRF   | MG     | >0.8         | 44,613              | 9,415      | 30.77               | 11.51         |
| 78      | DCM   | MG     | >0.8         | 86,446,300          | 1,358      | 13.32               | 0.48          |
| 78      | DCM   | MT     | >0.8         | 10,659,304          | 82         | 0.71                | 0.07          |

DCM, deep chlorophyll maximum; MG, metagenome; MT, metatranscriptome; SRF, surface.

*A protocol that selectively sequenced RNA sequences with poly(A) tails was conducted.
Figure 2 | Genome expression in UCYN-A1 and UCYN-A2 lineages. (a) Metatranscriptome recruitment at the surface of the TARA_078 station of UCYN-A1 (0.2–3 µm fraction) and UCYN-A2 (5–20 µm fraction) transcripts. Transcripts are plotted as black dots representing the covered genome positions and the % of identity with the closest reference. A horizontal grey line set at 95% identity shows the threshold used to count the number of times, or coverage, that a gene was expressed. The most expressed genes in both lineages are highlighted. (b) Relative contribution of nitrogen fixation operon, FOF1-ATP synthase operon, cytochrome b6f and PSI genes to the total UCYN-A transcripts contribution in surface samples; percentages are indicated. (c) Transcript counts of nitrogen fixation operon versus those of ATP synthase (triangle), cytochrome b6f (square) and PSI (open circle) transcripts. All of these transcripts were significantly correlated (P<10^-5) and regression lines, regression equations and R^2 values are indicated in the figure.

has a high energetic cost (16 mol of ATP to generate 2 mol of ammonia). Notably, the F0F1-ATP synthase operon and genes encoding for the cytochrome b6f complex and photosystem I complex (PSI) were highly transcribed and positively correlated (P<10^-5, N=6, linear regression analysis) with the nitrogen fixation operon transcript abundances (Fig. 2c). These findings suggest that the generation of reducing power and the ATP synthesis could be coupled to fuel the nitrogen fixation process in UCYN-A. Likewise, UCYN-A2 might have higher nitrogen fixation rates per cell than UCYN-A1 based on the higher number of nifH transcripts per cell. It is reasonable to assume that the differences in nifH gene expression between the UCYN-A lineages could simply reflect the differences in the cell size of their partners with differential nutrient requirements for growth. In addition, it has been indirectly demonstrated that the nitrogen fixation of UCYN-A supports the CO2 fixation of its prymnesiophyte partner18. Therefore, we hypothesize that the larger B. bigelowii host cell would meet its larger N nutrient requirements by partnering with a larger number of UCYN-A2 symbiotic cells.

Nitrogen-fixing microorganisms, and particularly cyanobacteria, should protect their nitrogenase from inactivation by oxygen. The absence of the ability to use photosystem II that evolves O2 explains why UCYN-A appears to fix N2 and express the nitrogenase genes during the day19. However, its association with an oxygen-evolving partner could make the nitrogenase enzyme in UCYN-A not completely safe from oxygen. We observed that the sufB gene (cysteine desulfurase), involved in the assembly or repair of oxygen-labile iron–sulfur clusters under oxidative stress, was highly transcribed with an oxygen-evolving partner could make the nitrogenase enzyme in UCYN-A not completely safe from oxygen. We observed that the sufB gene (cysteine desulfurase), involved in the assembly or repair of oxygen-labile iron–sulfur clusters under oxidative stress, was highly transcribed (Supplementary Data 1 and 2). It may be that UCYN-A requires high expression level of sufB genes to repair the nitrogenase enzyme from oxygenic inactivation, suggesting then a similar role than for the peroxidase genes found in their
To analyse the selection pressure and evolution of the protein-coding genes, we calculated the Ka/Ks ratio for 887 protein-coding genes shared by the UCYN-A1 and UCYN-A2 genomes (Supplementary Data 3). The Ka/Ks ratio indicates purifying selection and ratios <1 point to purifying selection while ratios >1 point to positive selection. We found that 873 out of the 887 protein-coding genes were under purifying selection (Ka/Ks <1) but were not statistically well supported (P >0.05). Purifying selection means that synonymous mutations are maintained, while non-synonymous mutations are continuously removed from the population. We did not detect signs of large-scale positive selection, that is, no apparent strong adaptation to novel niches in UCYN-A lineages, suggesting that the evolutionary forces for niche adaptation would act on the prymnesiophyte partners rather than on UCYN-A. Our results are consistent with the fact that UCYN-A lacks the same major pathways and proteins that are absent in UCYN-A1 (ref. 12), indicating then that the symbionts were genetically adapted to their hosts before they were separated by speciation.

The age of divergence for UCYN-A1 and UCYN-A2 lineages was calculated by phylogenomic and Bayesian relaxed molecular clock analyses (Fig. 3; Supplementary Table 2). Our results indicate that UCYN-A1 and UCYN-A2 lineages diverged around 91 Myr ago, that is, during the late Cretaceous. In agreement, B. bigelowii has a fossil record extending back to the late Cretaceous (ca. 100 Myr ago)23, reported from neritic and pelagic sediments, for example, in lower Paleogene sediments immediately above the K/Pg mass extinction level as well as in the Oligocene Diversity Minimum24,25. In the Jurassic, between 190 and 100 Myr ago, nutrient availability in the ocean was lower than at any point during the last 550 Myr ago26. It is therefore likely that the symbiotic relationship between the common ancestor of UCYN-A1 and UCYN-A2, and a *Bracharodosphaera*-related species was established by the late Cretaceous to cope with extremely low-nutrient conditions and a generalized oligotrophy in marine surface waters, as it has been recognized for other symbiotic system such as the Acantharia–*Phaeocystis* symbiosis27.

The phylogeny shown was estimated based on 135 proteins from 57 taxa. Three calibration points (black circles) were used for the tree presented and were treated as soft bounds. The root of the tree was set with a maximum age of 2,700 Myr ago and a maximum age of divergence for 62 remaining genes (Supplementary Data 3). The 14 remaining genes (Supplementary Data 3) were consistent with the fact that UCYN-A2 lacks the same major pathways and proteins that are absent in UCYN-A1 (ref. 12), indicating then that the symbionts were genetically adapted to their hosts before they were separated by speciation.

UCYN-A diverged during the late Cretaceous. Our findings on partner fidelity in UCYN-A point to the hypothesis of symbiont-host co-evolution44. To analyse the selection pressure and evolution of the protein-coding genes, we calculated the number of synonymous or silent (Ks) and non-synonymous (Ka, inducing amino-acid change) nucleotide substitutions20,21 for 887 protein-coding genes shared by the UCYN-A1 and UCYN-A2 genomes (Supplementary Data 3). The Ka/Ks ratio may offer important clues about the selection pressure where ratios <1 indicate purifying selection and ratios >1 point to positive selection22. We found that 873 out of the 887 protein-coding genes were under purifying selection (P <0.05, codon-based Z-test) (Supplementary Data 3). The 14 remaining genes also presented Ka/Ks <1 but were not statistically well supported (P >0.05). Purifying selection means that synonymous mutations are maintained, while non-synonymous mutations are continuously removed from the population. We did not detect signs of large-scale positive selection, that is, no apparent strong adaptation to novel niches in UCYN-A lineages, suggesting that the evolutionary forces for niche adaptation would act on the prymnesiophyte partners rather than on UCYN-A. Our results are consistent with the fact that UCYN-A lacks the same major pathways and proteins that are absent in UCYN-A1 (ref. 12), indicating then that the symbionts were genetically adapted to their hosts before they were separated by speciation.

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The phylogeny shown was estimated based on 135 proteins from 57 taxa. Three calibration points (black circles) were used for the tree presented and were treated as soft bounds. The root of the tree was set with a maximum age of 2,700 Myr ago and a maximum age of 2,320 Myr ago. Divergence time for the ancestor of cyanobacteria UCYN-A1 and UCYN-A2 (highlighted with a grey box) is given with the corresponding values for the posterior 95% confidence intervals in Supplementary Table 2.
harbour a variable number (up to 10) of UCYN-A2 cells, while the small prymnesiophyte partner harboured only one or two UCYN-A1 cells, it is reasonable to think that a larger nutrient acquisition could be linked to a larger number of symbionts. Indeed, the whole-genome expression patterns suggested that the metabolic investment in UCYN-A1 and UCYN-A2 is mainly focused on the nitrogen fixation machinery. Our evolutionary analysis revealed that UCYN-A1 and UCYN-A2 were genetically adapted to their prymnesiophyte partners before UCYN-A speciation (purifying selection) but, on the contrary, the prymnesiophyte partners seem to follow different ecological strategies, suggesting a speciation process under positive selection. Our results suggest that the partner fidelity shown by UCYN-A lineages together with the speciation in the common ancestor of B. bigelowii and Its closely related prymnesiophyte may have forced an allopatric speciation of UCYN-A1 and UCYN-A2 populations in the late Cretaceous. Comparative genome analysis of the two prymnesiophyte partners would clarify whether these two algal species underwent positive selection through evolution by adaptation to novel niches. As revealed by methyl phylegetic analysis, it seems that now all UCYN-A lineages, such as UCYN-A3, and prymnesiophyte (or not prymnesiophyte) partner will help to understand the evolutionary relationships of N₂-fixing cyanobacterial symbionts and the extent of their ecological relevance on marine biogeochemical cycles.

The present study offers new insights into the marine nitrogen-fixing UCYN-A symbiosis by disentangling the partner fidelity, host–symbiont organization and size distribution, gene expression and evolution of UCYN-A1 and UCYN-A2 lineages. These results demonstrate that specific UCYN-A symbiotic pairs co-exist without cross-symbiotic partnerships. The fact that its distribution occupies new plankton size fractions accordingly to the host size should be considered in global nitrogen fixation models. The number of UCYN-A1 and UCYN-A2 cells involved in this symbiosis differs and appears to be a conserved phylogenetic trait. Remarkably, about a quarter of the UCYN-A transcripts were from nitrogen fixation genes, highlighting the importance of nitrogen fixation in this symbiosis. Our results present further evidences of a host and symbiont co-evolution scenario in the marine environment, probably derived from a single ancestral symbiotic event wherein at least two different lineages diversified in the late Cretaceous. Investigation of N₂-fixing cyanobacterial symbionts and their partners should provide clues for discovering new ecological compartments for nitrogen fixation that would increase our understanding of the nitrogen cycle in the ocean.

Methods

Sample choice. From a total of 243 metagenomes from 68 globally distributed stations from Tara Oceans expedition⁴⁸, the abundance of UCYN-A based on 16S rRNA (UBRADO69 and UPRYM69 probes) was verified manually to remove chimeras and sequences with ambiguities (466 sequences were kept). A maximum likelihood phylogenetic tree was built using RAxML⁴⁹ with 100 trees for both topological and bootstrap analyses and visualized with iTol⁵⁰ (Supplementary Fig. 1). The newly designed probe UBRADO69 targeted B. bigelowii, while probe UPRYM69 targeted the closely related prymnesiophyte partner (Supplementary Table 1). UBRADO69 and UPRYM69 probes differed in only one position, and required a competitor to avoid unspecific hybridizations. Therefore, the labelled probe UBRADO69 was used in combination with the unlabelled UPRYM69 oligonucleotide for the detection of B. bigelowii, and vice versa for the detection of the closely related prymnesiophyte partner (Supplementary Table 1). Two helpers, helper-A PRYM and helper-B PRYM69, were designed to improve the hybridization process for both probes (Supplementary Table 1). The UCYN-A732 probe designed against UCYN-A by targeting the 16S rRNA was used to label UCYN-A2 sequences (Supplementary Table 1). The specificity of the new probes was checked with the online tool ProbeCheck (http://www.cme.msu.edu/RDP/) and by searching in the GenBank database (http://www.ncbi.nlm.nih.gov/index.html) to detect potential matching sequences in non-target groups.

CARD-FISH assay and epifluorescence microscopy. A preliminary double-hybridization assay using the universal haptotrope PRYM02 probe⁵¹ and UCYN-A732 was first applied to check whether the partner of UCYN-A in our sample belong to class Prymnesiophyceae. To specifically target the different UCYN-A lineages belonging to the same prymnesiophyte partner, we designed a second CARD-FISH assay that would allow us to distinguish UCYN-A732 from the unlabelled UCYN-A symbiotic system.

Nucleic acid extractions and sequencing. Surface and DCM seawater samples collected by Tara Oceans’ station 76 and 78 in the South Atlantic Ocean (TARA_076 and TARA_078) for metagenomic sequencing were size fractionated.
For surface samples, metagenomes from two and four fractions were analysed in TARA_076 (0.2–3 and >0.8 μm) and TARA_078 (0.2–3, 0.8–5, 5–20 and >18 μm), respectively. For DCM samples, metagenomes from one fraction were analysed in TARA_076 (>0.8 μm) and TARA_078 (>0.8 μm). Seawater samples for metatranscriptomic sequencing used also several size fractions. For surface samples, metatranscriptomes from two and three fractions were analysed in TARA_076 (0.2–3 and >0.8 μm) and TARA_078 (0.2–3, 5–20 and >0.8 μm), respectively. For DCM samples, metatranscriptomes from one fraction were analysed in TARA_076 (>0.8 μm) and TARA_078 (>0.8 μm). DNA and RNA extraction protocols for the different size fractions and metagenome sequencing are described in refs 31–33.

cDNA synthesis and sequencing. For 0.2–3 μm and >0.8 μm filters, bacterial rRNA depletion was carried out on 240–500 ng total RNA using Ribo-Zero Magnetic Kit for Bacteria (Epigenex, Madison, WI). The Ribo-Zero depletion protocol was modified to be adapted to low RNA input amounts.42 Depleted RNA was used to synthesize complementary DNA (cDNA) with SMARTer Stranded RNA-Seq Kit (Clontech, Mountain View, CA).42 For >0.8-μm filter from TARA_078, cDNA was synthesized starting from 30 ng total RNA using SMARTer Ultra Low RNA Kit (Clontech) by oligo(dT) priming, following the manufacturer protocol. Full-length double-stranded cDNA was fragmented to a 150–600 bp size range using the E210 Covaris instrument (Covaris Inc., USA). Then, fragments were end-repaired and 3′-adenylated, and ligated to Illumina adaptors using NEBNext Sample Reagent Set (New England Biolabs, Ipswich, MA). Fragments were PCR-amplified using Illumina adapter-specific primers and purified. All metatranscriptomic libraries were quantified by qPCR using the KAPA Library Quantification Kit for Illumina Libraries (KapaBiosystems, Wilmington, MA) and library profiles were assessed using the DNA High Sensitivity LabChip kit on an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). Libraries were sequenced on Illumina HiSeq2000 instrumentation at DNA Technologies, San Diego, CA. cDNA from TARA_076 were sequenced using Illumina HiSeq2500/Miseq, whereas samples from the Gymnosphaera and Cyanozoa were sequenced using Illumina HiSeq3000 to a total depth of 100x coverage.

Fragment recruitment analysis from omics data sets. BLAST + v2.2.25 was used to recruit metagenomic and metatranscriptomic reads similar to the two UCYN-A genomes sequenced up to date43,44 using default parameter values, except for the following: -perc_identity 50, -evalue 0.0001. Metagenomic/metatranscriptomic reads were aligned to the reference genomes of UCYN-A1 and UCYN-A2 (Supplementary Table 2). We applied the gene positions to count the number of metatranscripts covering each gene. We also used the gene positions to estimate the number of reads for each gene families and pathways. Metatranscript counts), and (ii) by metagenomic read counts, for each UCYN-A gene (in this case, we also normalized by reads coverage). For each UCYN-A gene, we calculated the number of reads that passed the mapping filters. We then calculated the difference between the number of reads for each gene in the two UCYN-A genomes, allowing for 2.5% on each side for an upper and lower bound. In MCMCTree, LSU, SSU and rpoCl were selected as separate loci and branch lengths were estimated in BEAST.12 We used the HKY85 (ref. 50) model of nucleotide evolution based on 16S rRNA (1,546 characters), SSU (1,546 characters) and LSU (1,548 characters). In PhyloBayes46, we used the CAT-GT replacement model of nucleotide evolution. For all analyses, we used fixed values for the birth–death process model \( \lambda = 1 \) and \( \rho = 0 \). Analyses were performed at least twice to ensure convergence of the MCMC, although only one analysis is reported. For all age calibrations, both minimum and maximum bounds were soft and specified by uniform distributions between the maximum/minimum time constraints with 2.5% tail probabilities above/below these limits allowing for molecular data to correct for conflicting fossil information55. To check whether analyses had converged, we used Tracer v1.5.0 (http://beast.bio.ed.ac.uk/Tracer). For the cyanobacterial root, 2,700 Myr ago52 and 2,320 Myr ago53 (the rise in atmospheric oxygen) were set as the maximum and minimum age, respectively. Other fossils exhibiting unique morphological features were assigned to well-supported groups such as the Nostocales47 and the clade containing two Pleurocapsa genomes (PCC 7319 and PCC 7327) in the Pleurocapsales45.

References
1. Picozzi, D. et al. Dinitrogen fixation in the world’s oceans. Biogeochemistry 57/58, 47–98 (2002).
2. McFall-Ngai, M. Are biologists in ‘future shock’? Symbiosis integrates biology across domains. *Nat. Rev. Microbiol.* 6, 789–792 (2008).
3. Martinez-Garcia, M. et al. Unveiling in situ interactions between marine protists and bacteria through single cell sequencing. *ISME J.* 6, 703–707 (2012).
4. Thompson, A. W. et al. Unicellular cyanobacterium symbiotic with a single-celled eukaryotic alga. *Science* 337, 1456–1459 (2012).
5. Montoya, J. P. et al. High rates of N₂ fixation by unicellular diazotrophs in the oligotrophic Pacific Ocean. *Nature* 430, 1027–1032 (2004).
6. Jardillier, L., Zubkov, M. V., Pearlman, J. & Scanlan, D. J. Significant CO₂ fixation by small prymnesiophytes in the subtropical and tropical northeast Atlantic Ocean. *ISME J.* 4, 1180–1192 (2010).
7. Cabello, A. M. et al. Global distribution and vertical patterns of a prymnesiophyceae-cyanobacteria obligate symbiosis. *ISME J.* 10, 693–706 (2015).
8. Zehr, J. P. & Kudela, R. M. Nitrogen cycle of the open ocean: from genes to ecosystems. *Annu. Rev. Mar. Sci.* 3, 197–225 (2011).
9. Tripp, H. J. et al. Metabolic streaming in an open-ocean nitrogen-fixing cyanobacterium. *Nature* 464, 90–94 (2010).
10. Bombar, D. et al. Comparative genomics reveals surprising divergence of two closely related strains of uncultivated UCYN-A cyanobacteria. *ISME J.* 8, 2530–2542 (2014).
11. Hage, N., Norum, M., Koga, M. & Horiguchi, T. Discovery of an endosymbiotic nitrogen-fixing cyanobacterium UCYN-A in Braarudosphaera bigelowii (Prymnesiophyceae). *PLoS ONE* 8, e61749 (2013).
12. Thompson, A. P. et al. Genetic diversity of the unicellular nitrogen-fixing cyanobacteria UCYN-A and its prymnesiophyte host. *Environ. Microbiol.* 16, 3238–3249 (2014).
13. Krupke, A. et al. In situ identification and N₂ fixation rates of uncultivated cyanobacteria populations. *Syst. Appl. Microbiol.* 36, 259–271 (2013).
14. Le Moal, M., Collin, H. & Bisgala, I. C. Intriguing diversity among dinoflagellate picooplankton along a Mediterranean transect: a dominance of thistoza. *Biogeosciences* 8, 827–840 (2011).
15. Krupke, A. et al. Distribution of a consortium between unicellular algae and the N₂ fixing cyanobacterium UCYN-A in the North Atlantic Ocean. *Microbiol. Biogeochemistry* 16, 3135–3167 (2014).
16. Krupke, A. et al. The effect of nutrients on carbon and nitrogen fixation by the UCYN-A-Haptophyte symbiosis. *ISME J.* 9, 1658–1674 (2015).
17. Zehr, J. P. Nitrogen fixation by marine cyanobacteria. *Trends Microbiol.* 19, 162–173 (2011).
18. Li, W. H. Unbiased estimation of the rates of synonymous and nonsynonymous substitution. *J. Mol. Evol.* 36, 99–99 (1993).
19. Hurst, L. D. The Ka/Ks ratio: diagnosing the form of sequence evolution. *Trends Genet.* 18, 486–487 (2002).
20. McDonald, I. H. & Kreitman, M. Adaptive protein evolution at the Adh locus in Drosophila. *Nature* 351, 652–654 (1991).
21. Bown, P. R. (ed). *Coccolithophores: From Molecular Process to Palaeoceanographic Implications.* 17–26 (1999).
22. McDonald, I. H. & Kreitman, M. Adaptive protein evolution at the Adh locus in Drosophila. *Nature* 351, 652–654 (1991).
23. Bown, P. R. (ed). *Coccolithophores: From Molecular Process to Global Impact* (eds Thierrin, H. R. & Young, J. R.) 481–508 (Springer, 2004).
26. Cardenas, A. L. & Harries, P. J. Effect of nutrient availability on marine origination rates throughout the Phanerozoic eon. Nat. Geosci. 3, 430–434 (2010).
27. Decelle, J. et al. An original mode of symbiosis in open ocean plankton. Proc. Natl Acad. Sci. USA 109, 18000–18005 (2012).
28. Kneip, C., Lockhart, P., Voss, C. & Maier, U.-G. Nitrogen fixation in eukaryotes - new models for symbiosis. BMC Evol. Biol. 7, 55 (2007).
29. Nakayama, T. et al. Spheroid bodies in rhopalodiacean diatoms were derived from a single endosymbiotic cyanobacterium. J. Plant Res. 124, 93–97 (2011).
30. Karsenti, E. et al. A holistic approach to marine eco-systems biology. PLoS Biol. 9, e1001177 (2011).
31. Sunagawa, S. et al. Structure and function of the global ocean microbiome. Science 348, 1261359 (2015).
32. Logares, R. et al. Metagenomic 16S rDNA Illumina tags are a powerful alternative to amplicon sequencing to explore diversity and structure of microbial communities. Environ. Microbiol. 16, 2659–2671 (2013).
33. De Vargas, C. et al. Ocean plankton. Eukaryotic plankton diversity in the sunlit ocean. Science 348, 1261605 (2015).
34. Pesant, S. et al. Open science resources for the discovery and analysis of Tara Oceans Data. Sci. Data 2, 150023 (2015).
35. Guillou, L. et al. The Protist Ribosomal Reference database (PR2): a catalog of unicellular eukaryote Small Sub-Unit rRNA sequences with curated taxonomy. Nucleic Acids Res. 41, D597–D604 (2013).
36. Katoh, K., Misawa, K., Kuma, K. & Miyata, T. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. 30, 3052–3060 (2002).
37. Stamatakis, A. RAxML-V1-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22, 2688–2690 (2006).
38. Letunic, I. & Bork, P. Interactive Tree Of Life (iTOl): an online tool for interpretation of metatranscriptomic data. Bioinformatics 23, 127–128 (2007).
39. Letunic, I. & Bork, P. Interactive Tree Of Life v2: online annotation and display of phylogenetic trees made easy. Nucleic Acids Res. 39, W475–W478 (2011).
40. Simon, N. et al. Oligonucleotide probes for the identification of three algal groups by dot blot and fluorescent whole-cell hybridization. J. Eukaryot. Microbiol. 47, 76–84 (2000).
41. Pernthaler, A., Pernthaler, J. & Amann, R. Sensitive multi-color fluorescence in situ hybridization for the identification of environmental microorganisms. Mol. Microbiol. 16, 2613–2627 (2004).
42. Alberts, A. et al. Comparison of library preparation methods reveals their impact on interpretation of metatranscriptomic data. BMC Genomics 15, 912 (2014).
43. Caro-quintero, A. & Konstantinidis, K. T. Bacterial species may exist, metagenomics reveal. Environ. Microbiol. 14, 347–355 (2011).
44. Blank, C. E. & Sánchez-Baracaldo, P. Timing of morphological and ecological innovations in the cyanobacteria - a key to understanding the rise in atmospheric oxygen. Geobiology 8, 1–23 (2010).
45. Yang, Z. PAML 4: phylogenetic analysis by maximum likelihood. Mol. Biol. Evol. 24, 1586–1591 (2007).
46. Lartilhot, N., Lepage, T. & Blanquart, S. PhyloBayes 3: a Bayesian software package for phylogenetic reconstruction and molecular dating. Bioinformatics 25, 2286–2288 (2009).
47. Drummond, A. J., Ho, S. Y. W., Phillips, M. J. & Rambaut, A. Relaxed phylogenetics and dating with confidence. PLoS Biol. 4, 699–710 (2006).
48. Sánchez-Baracaldo, P., Ridgwell, A. & Raven, J. A. A neoproterozoic transition in the marine nitrogen cycle. Curr. Biol. 24, 562–567 (2014).
49. Lepage, T., Bryant, D., Philippe, H. & Lartilhot, N. A general comparison of relaxed molecular clock models. Mol. Biol. Evol. 24, 2669–2680 (2007).
50. Hasegawa, M., Kishino, H. & Yano, T. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. 22, 160–174 (1985).
51. Yang, Z. & Rannala, B. Bayesian estimation of species divergence times under a molecular clock using multiple fossil calibrations with soft bounds. Mol. Biol. Evol. 23, 212–226 (2006).
52. Brooks, I. J., Buick, R., Summons, R. E. & Logan, G. A. A reconstruction of Archean biological diversity based on molecular fossils from the 2.78 to 2.45 billion-year-old Mount Bruce Supergroup, Hamersley Basin, Western Australia. Geochim. Cosmochim. Acta 67, 4321–4335 (2003).
53. Bekker, A. et al. Dating the rise of atmospheric oxygen. Nature 427, 117–120 (2004).
54. Tomitani, A., Knoll, A. H., Cavanaugh, C. M. & Ohno, T. The evolutionary diversification of cyanobacteria: molecular-phylogenetic and palaeontological perspectives. Proc. Natl Acad. Sci. USA 103, 5442–5447 (2006).
55. Zhang, Y. & Golubic, S. Endolithic microfossils (cyanophyta) from early Proterozoic stromatolites, Hebei, China. Acta Micropaleontol. Sin. 4, 1–3 (1987).