An INDEL genomic approach to explore population diversity of phytoplankton

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Abstract (258 words)

Background: Although metabarcoding and metagenomic approaches have generated large datasets on worldwide phytoplankton species diversity, the intraspecific genetic diversity underlying the genetic adaptation of marine phytoplankton to specific environmental niches remains largely unexplored. This is mainly due to the lack of biological resources and tools for monitoring the dynamics of this diversity in space and time.
Results: To gain insight into population diversity, a novel method based on INDEL markers was developed on *Bathycoccus prasinos* (Mamiellophyceae), an abundant and cosmopolitan species with strong seasonal patterns. Long read sequencing was first used to characterise structural variants among the genomes of six *B. prasinos* strains sampled from geographically distinct regions in the world ocean. Markers derived from identified insertions/deletions were validated by PCR then used to genotype 55 *B. prasinos* strains isolated during the winter bloom 2018-2019 in the bay of Banyuls-sur-Mer (Mediterranean Sea, France). This led to their classification into eight multi-loci genotypes and the sequencing of strains representative of local diversity, further improving the available genetic diversity of *B. prasinos*. Finally, selected markers were directly tracked on environmental DNA sampled during 3 successive blooms from 2018 to 2021, showcasing a fast and cost-effective approach to follow local population dynamics.
Conclusions: This method, which involves (i) pre-identifying the genetic diversity of *B. prasinos* in environmental samples by PCR, (ii) isolating cells from selected environmental samples and (iii) identifying genotypes representative of *B. prasinos* diversity for sequencing, can be used to comprehensively describe the diversity and population dynamics not only in *B. prasinos* but also potentially in other generalist phytoplankton species.

Background

Marine phytoplankton, including picoeukaryotic algae, is responsible for a large fraction of primary production [1]. In temperate regions, the abundance and diversity of the phytoplankton is often seasonal and occurs in bursts, as algal blooms. *Per se*, blooms have a large impact on global primary production and therefore the understanding of the genetic basis of phytoplankton adaptation to seasonal niches and the effects of ocean warming on phytoplankton blooms are of the utmost importance.

Meta-ribosomal barcoding on the nuclear or plastidial 18/16S rRNA gene has opened the access to massive data in time and space and has accelerated the study of phytoplankton species and genera diversity in natural communities. However, metabarcoding approaches do not provide information on intraspecific genetic variations. Assessing only interspecific diversity leads to the underestimation of the diversity of the populations. Equally, a single isolate cannot represent the diversity of a population. Since natural selection acts on variation among individuals within populations, it is essential to incorporate both intra and interspecific trait variability into community ecology [2]. Indeed, Raffard et al. [3] demonstrated that intraspecific variation has significant ecological effects across a large set of species, confirming a previous estimate based on a more restricted species set [4]. Furthermore, it has been shown that diversity within species is rapidly decreasing, making them more homogeneous and highlighting the need to preserve intraspecific variations since this intraspecific diversity reinforces the overall population stability in the face of environmental change [4].

In diatoms, intraspecific variations play a key role in the response of species to several important environmental factors such as light, salinity, temperature and nutrients [5]. Modelling approaches suggest that intraspecific variation extends bloom periods by providing variability in competitive interactions between species under changing conditions, and contributes to fitness in temporary microhabitats. Intraspecific genetic diversity allows local adaptation by optimising physiological responses, allowing local populations to be exceptionally fit and competitive in their respective habitat.

Since an assembly of genotypically diverse individuals constitutes a population, methodological approaches have been developed in order to determine the genetic variation among individuals [6]. One of the main challenges is the difficulty of isolating a sufficient number of individuals for classical diversity analyses while ensuring representativity of diverse subpopulations. Historically, sequence-based intraspecific markers correspond to small nucleotide repeats such as microsatellites [7], chloroplastic [8] and mitochondrial [9] genes and a few nuclear genes that were applied to several hundreds of isolated individuals. The development of Randomly Amplified Polymorphic DNA (RAPD) markers [10] and more recently of Restriction-site-Associated DNA sequencing techniques (RADseq) [11] allowed the discovery and genotyping of thousands of genetic markers for any given species at relatively low-cost. Some of these approaches have been used to analyse the diversity of populations during algal blooms [12]. Recently, the dramatic increase of the number of sequenced genomes led to large-scale diversity studies with large sets of nuclear genes or whole genome comparisons. However, most of these approaches require the isolation of a large number of individuals. As a consequence, the intraspecific diversity remains poorly documented in marine phytoplankton.

Widely distributed from the equator to arctic and antarctic poles with a marked seasonality in temperate and polar regions, picoeukaryotes belonging to the *Mamiellales* order (*Bathycoccus*, *Ostreococcus* and *Micromonas*) are abundant and have a cosmopolitan presence, illustrating a high capacity for adaptation to a wide range of contrasting environments [13–16]. Novel, rapid and cheap sequencing technologies have given access to *Mamiellales* interspecific diversity by metagenomic [16,17] and metatranscriptomic approaches [18]. However, to date, very little information is available on intraspecific diversity of *Bathycoccaceae*, with the exception of *Ostreococcus tauri* from mediterranean lagoons [19] . Unlike *O. tauri*, which is usually not detectable in publicly available metagenomes, *Bathycoccus sp.* is the most cosmopolitan *Mamiellophyceae* genus. Although *Bathycoccus*

sp. is abundant in the world Ocean, Metabarcoding of 18S ribosomal RNA (V9 region) does not allow the identification of *Bathycoccus* species [20,21]. Through metagenomic analysis, *Bathycoccus* genus was divided into two species, the polar and temperate *Bathycoccus prasinos* type B1 genome [13,22] and the tropical *Bathycoccus calidus* type B2 genome [21,23,24]. Thus the cosmopolitan nature of *Bathycoccus* from poles to equator is due to the combination of both B1 and B2 species.

In the bay of Banyuls, *Bathycoccus* and *Micromonas* bloom yearly from November to April, *B. prasinos* being one of the most abundant species [15,25]. The highly reproducible yearly occurrence of *B. prasinos* in the Banyuls bay during the last decade [15] raises the question of the persistence of a *B. prasinos* population adapted to the bay or of a variation of the population structure each year. In addition, since outside of the bloom period *Mamiellales* are virtually absent from the bay, is the *B. prasinos* bloom initiated by an uptake of resident “resting cells” in the sediment or by a fresh input carried by North western Mediterranean currents along the Gulf of Lion? At present no resting stages that can act as inoculum of subsequent blooms have been described for *B. prasinos*.

To assess the intraspecific diversity of *B. prasinos* worldwide and locally in the bay of Banyuls, we developed an efficient method to isolate strains together with whole genome sequencing by Oxford Nanopore Technology (ONT) in order to identify Structural Variations (SV) in *B. prasinos* genomes. Diversity markers designed from INDEL (insertion or deletion of bases in the genome of an organism) were used to genotype *B. prasinos* strains and populations from environmental samples.

Methods

Algal strains and culture conditions

World-wide *B. prasinos* strains were obtained from the Roscoff Culture Collection (RCC) centre (See Supplementary Table 1, Additional File 1). Strain RCC1105 from which the current *B. prasinos* reference genome originated [22] was lost and replaced by its clonal culture RCC4222. The strains were cultivated in 100 mL flasks in filtered artificial seawater ($1x10^{-3}$ M TrisHCl pH 7.2, 24.55 g/L NaCl, 0.75 g/L KCl, 4.07 g/L MgCl2 6H2O, 1.47 g/L CaCl2 2H2O, 6.04 g/L MgSO4 7H2O, 0.21 g/L NaHCO3, 0.00138 g/L NaH2PO4, 0.075 g/L NaNO3, $1.28x10^{-6}$ g/L H2SeO3) supplemented with trace metals and vitamins B1 (300 nM) and B12 (1 nM). Cultures were maintained under constant gentle agitation in an orbital platform shaker (Heidoph shaker and mixer unimax 1010). Sunlight irradiation curves recreating realistic light regimes at a chosen latitude and period of the year were applied in temperature-controlled incubators (Panasonic MIR-154-PE).

Cell isolation

Surface water was collected at 3 metre depth at SOLA buoy in Banyuls bay, North Western Mediterranean Sea, France (42°31′N, 03°11′E) approximately every week from December, 2018 to March, 2019 ; November, 2019 to March, 2020 and October, 2020 to April, 2021. Two ml aliquots were used to determine the quantity and size of phytoplankton by flow cytometry. For the 2018/2019 bloom, 50 ml were filtered through a 1.2-µm pore-size acrodisc (FP 30/1.2 CA-S cat N° 10462260 Whatman GE Healthcare Sciences) and used to inoculate 4 culture flasks with 10 ml of filtrate each. The sea water was supplemented by vitamins B1 and B12, NaH2PO4, NaNO3 and metal traces at the same final concentration as artificial sea water (ASW), antibiotics (Streptomycin sulfate and Penicillin at 50 µg/ml) were added to half of the cultures. The cultures were incubated under light and temperature conditions similar to those during the sampling date for 3-4 weeks. The presence of picophytoplankton was analysed by a

150 BD Accuri C6 flow cytometer. Generally, superior results were obtained without antibiotics.
151 Cultures containing at least 90% of picophytoplankton with only residual nanophytoplankton
152 were used for plating on agarose (0.21%). Colonies appearing after 10 days were hand-picked
153 and further cultured in 2 ml ASW in deepwell plates (Nunc, Perkin Elmer, Hessen, Germany)
154 for 10 days. Cells were cryopreserved at this stage. (See Supplementary Figure 1, Additional
155 File 2) Circa 500 clones were cryopreserved. At the same time, DNA extraction and PCR were
156 performed in order to identify *B. prasinos* strains.

157 DNA extraction, genome sequencing, assembly and PCR 158 amplification

159 For PCR analysis, total DNA was extracted from 4 ml *B. prasinos* cell cultures according to the
160 Plant DNAeasy Qiagen protocol. For whole genome sequencing by Oxford Nanopore
161 Technology (ONT), DNA was extracted by a CTAB method from 100 ml culture principally
162 based on Debladis et al. [26]. ONT libraries were prepared using the Rapid Barcoding
163 Sequencing kit (SQK-RBK004) and deposited on R9.4 flow cells for sequencing run. For
164 environmental samples, 5 litres of seawater at SOLA 3 metre depth were passed through 3
165 microns and 0.8 micron pore filters. DNA from cells collected on the 0.8 micron filters were
166 extracted using the Plant DNAeasy Qiagen protocol with the addition of a proteinase K
167 treatment in the AP1 buffer. PCR was performed using the Red Taq polymerase Master mix
168 (VWR) with the required primers (See Supplementary Appendix 1, Additional File 3) and
169 corresponding DNA. For sequencing, the PCR products were purified using the NucleoSpin
170 Gel and PCR Clean-up kit (Macherey-Nagel reference 740609.50) and sent to GENEWIZ for
171 Sanger sequencing.
172
173 Raw ONT Fast5 data were basecalled using Guppy 4.0.15 (https://nanoporetech.com) and the
174 HAC model, and QC performed using NanoPlot 1.38.1 [27]. All reads with a QPHRED higher
175 than 8 were retained and subjected to genome assembly using Flye 2.8 [28] under standard

176 options for ONT data. Raw assemblies were then polished with 3 turns of Racon 1.4.3 with
177 standard options [29] after mapping of raw reads on the previous round sequence using
178 minimap2 2.24 (-ax map-ont mode) [30]. Final scaffolding was performed using Ragoo 1.1 [31]
179 upon the original *B. prasinos* reference genome (GCA_002220235.1) [22]. Final QC of
180 assemblies was performed using QUAST 5.0 [32].

181 Variant calling and size control

182 Draft assemblies were aligned to *B. prasinos* reference genome using MUMmer 4.0.0 [33]
183 nucmer (options: –maxmatch -c 500 -b 500 -l 100). Alignments were then filtered for identity
184 (>90%) and length (>100bp) using delta-filter from MUMmer 4.0.0 (options: -m -i 90 -l 100).
185 Variant calling on the resulting delta files was performed with SVanalyzer 0.36
186 (https://github.com/nhansen/SVanalyzer) SVrefine subcommand, and resulting VCFs were
187 merged with bcftools merge 1.18 [34].
188 Size control on VCF was performed using a set of home-made Python scripts available at
189 https://forge.ird.fr/diade/genomecodes under GPLv3 licence.

190 Determination of Growth Rates

191 Cells isolated during December, 2018, January and February, 2019 in Banyuls bay were used
192 in this experiment. For each culture condition the cell number was determined by flow
193 cytometry daily, for 9 days. The growth rate was determined as Ln(N)/dT, where N is the cell
194 concentration per ml and T the time (days). The determination of the maximal growth rate
195 (μmax) and subsequent statistical analysis were conducted in accordance with the
196 methodology described in Guyon et al. [35].

Results

INDELs diversity of selected *Bathycoccus prasinos* strains

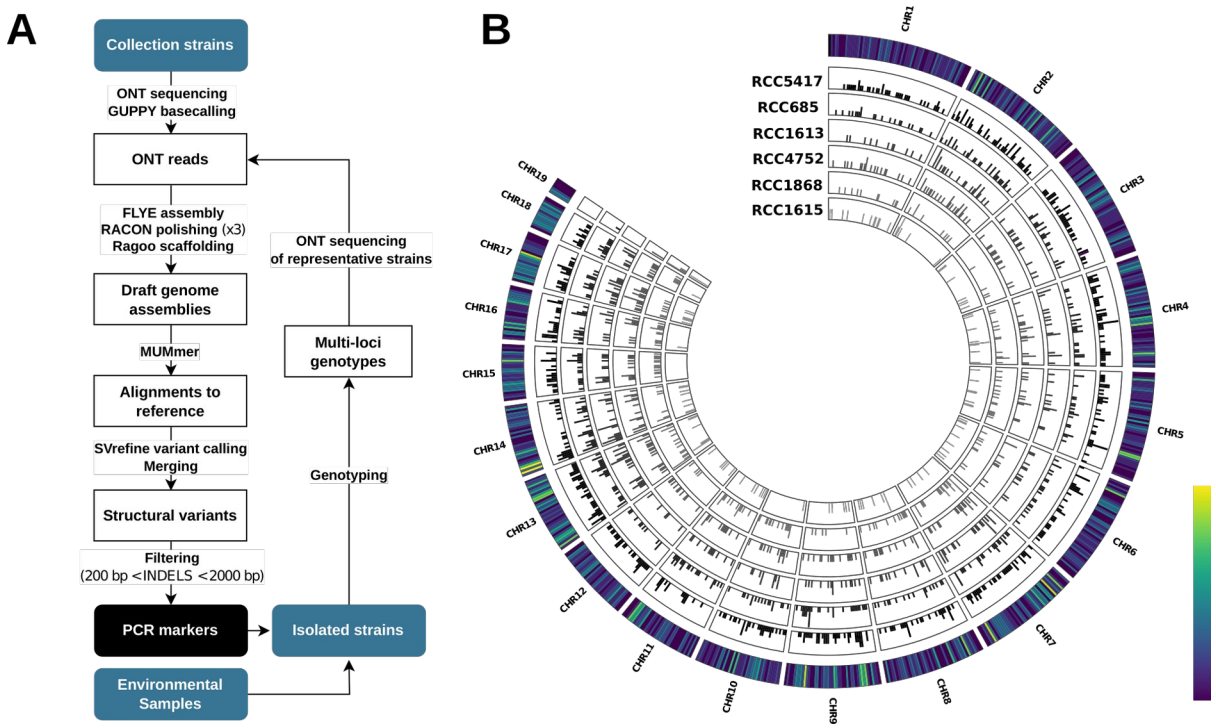

Figure 1 : Structural variant identification in collection strains of B. prasinos. (A) Summary of the sequencing and analysis strategy for the identification of INDELs structural variations and the design of PCR diversity markers. (B) Distribution frequency of identified SV along a 20Kb sliding window on nuclear chromosomes. Each inner circle corresponds to the distribution of SV within a sequenced strain. The outer circle corresponds to the cumulative frequency of all sequenced strains.

With the aim of creating a genetic resource of natural variants for *B. prasinos*, we first undertook a search for genetic markers of diversity that could be used to differentiate isolates from natural communities (Fig. 1A). From the few strains available in the Roscoff culture collection, we examined the genetic diversity of *B. prasinos* and selected geographically dispersed strains, so that the markers could potentially be used locally as well as worldwide. Oxford Nanopore Technology (ONT) was used to sequence the genomes of 6 selected strains spread along a latitudinal gradient from the Baffin bay (67° N) to the Mediterranean Sea (40° N) (See Supplementary Table 1, Additional File 1). After individual *de novo* assembly, each genome was compared to the reference genome of the RCC1105 strain from the Banyuls bay

218 [22] to identify 200 to 2,000 bp long INDELs. Selective amplification of these variable size
219 INDELs by PCR generates different size DNA fragments that can be used to genotype new
220 isolated strains as well as to track population diversity in environmental samples. Once
221 sequenced, new genotypes further enrich the marker resource that could be used
222 subsequently for genotyping *B. prasinos* isolates (Fig. 1A).
223
224 A total of 1,346 unique SV were identified, the contribution of each strain being highly variable
225 (Table 1). Strain RCC5417 from the Baffin bay was the most geographically distant from the
226 reference strain, and also the most diverse with 536 SV of size > 200 bp identified. Conversely,
227 RCC1615, with only a 4X sequencing depth, made the smallest contribution to SV
228 identification up to 10,000 bp but it was the main contributor to larger SVs (>10,000 bp), with 73
229 SV detected. This may be due to the overall lower assembly contiguity and quality of this strain
230 genome (Table 1; See Supplementary Table 1, Additional File 1). Despite these disparities, SV
231 were evenly distributed along *B. prasinos* nuclear genome, with the exception of the outlier
232 chromosome 19 [22], for which few variations were identified, most likely because of the
233 hypervariable structure of this chromosome (Fig. 1B). In the case of the big outlier
234 chromosome 14, no contrast in variant identification could be detected with other nuclear
235 chromosomes.

236 **Table 1: Number of structural variations identified in selected strains of *B. prasinos.***
237 Individual loci corresponds to the cumulated number of variable genomic positions without duplicates.
238

Size range

	200 - 2000 bp	2000 - 10000 bp	> 10000 bp	**Total**
RCC5417	371	125	40	**536**
RCC685	210	131	28	**369**
RCC4752	146	77	28	**251**
RCC1613	147	66	29	**242**
RCC1868	85	66	38	**189**
RCC1615	18	16	73	**107**
Individual loci	765	385	196	**1346**

Search for intraspecific diversity markers

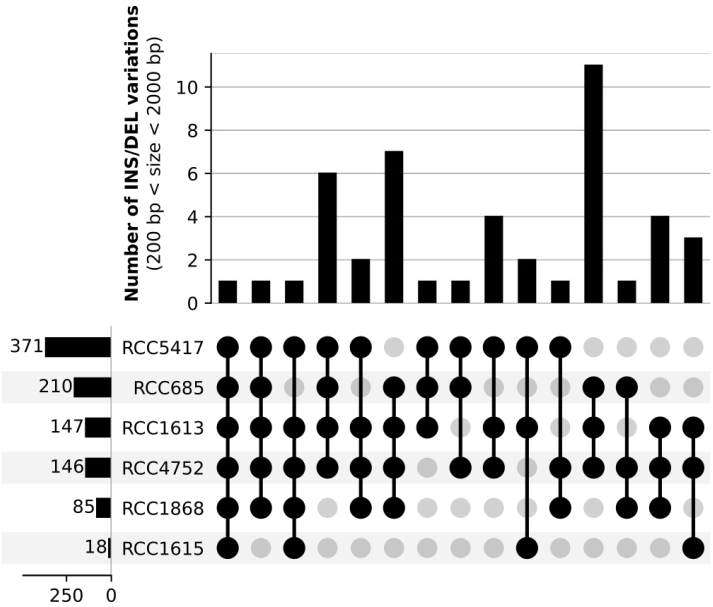

Figure 2 : Distribution of structural variations shared by three or more strains. The vertical barplot represents the number of structural variations between 200 and 2000 bp detected only within the corresponding group of strains. The horizontal barplot represents the number of structural variations detected within the corresponding strain.

To select putative PCR markers of *B. prasinos* diversity several criteria were considered. The size of the amplified fragment had to be between 0.2 and 2 Kb and sufficiently different among genomes to be unambiguously visualised on agarose gel after amplification with a single set of primers. In addition, selected INDEL SV had to be found in the genome of at least three strains. The aim of this selection was to identify the most divergent markers among the largest available genetic diversity of *B. prasinos*, with the expectation that some of these variations could potentially be found and tracked in local communities (Fig.1 A). In total, 765 unique SV with sizes ranging between 0.2 and 2 Kb were identified (Table 1). However, only 44 of them were found in at least three strains, thus meeting our criteria as putative markers (Fig. 2). Manual analysis of INDEL flanking sequences was performed to identify conserved sequences that could be used to design primers for PCR amplification of INDELS. Four markers were selected for experimental validation of INDELs by PCR (Fig. 3) (See Supplementary Appendix 2, Additional File 3).

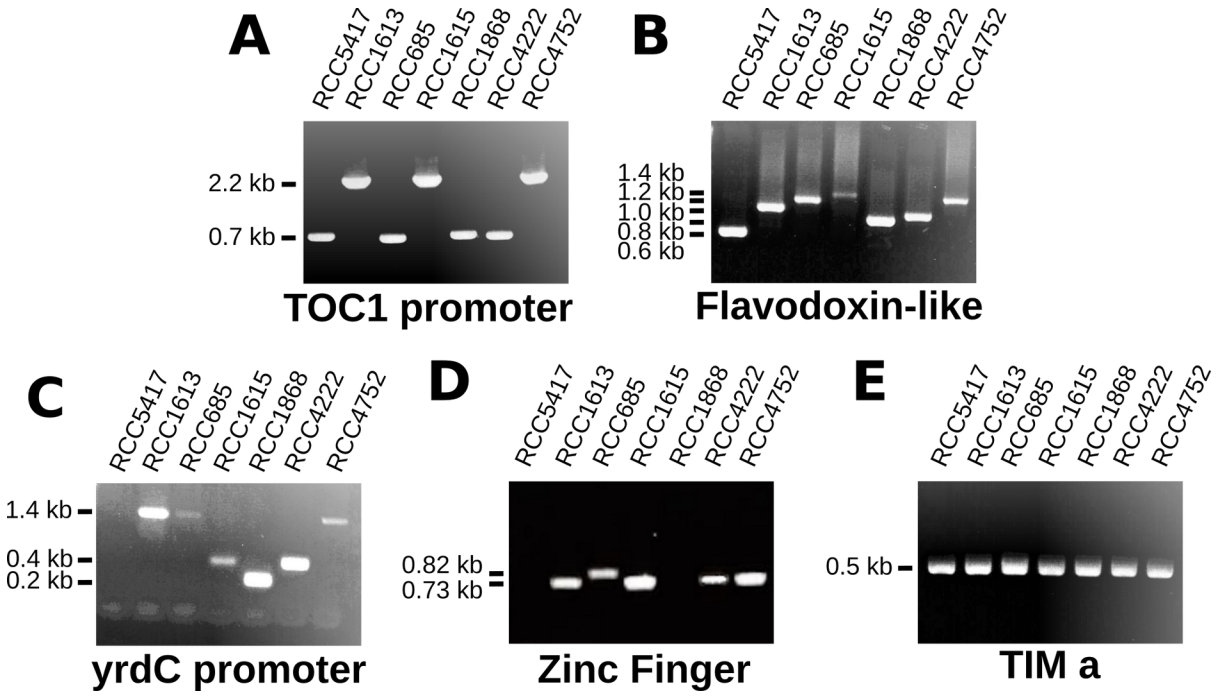

Figure 3 : Diversity markers in collection strains. PCR amplification of diversity markers (A) TOC1 promoter, (B) Flavodoxin-like, (C) yrdC promoter, (D) Zinc Finger, (E) TIMa. Variations in amplification efficiency are due to mismatches between primer sets and genomes.

The "TOC1 promoter" marker, located on chromosome 17 (RCC1105: NC_023992.1:308225..308959) corresponds to an INDEL variation in the intergenic region upstream of gene Bathy17g01510, a homolog of circadian clock gene TOC1. This diversity marker is bi-allelic with amplified fragments of 700 or 2,200 bp in size (Fig. 3A).

The "Flavodoxin-like" marker, located on chromosome 3 (RCC1105: NC_024006.1:379834..380504) corresponds to an INDEL variation in the gene Bathy03g02080 which encodes a protein containing amino stretches repeats of various lengths. This marker shows 5 alleles producing amplified fragment lengths of 600, 800, 1000, 1,200 and 1,400 bp (Fig. 3B).

The "yrdC promoter" marker, located on chromosome 1 (RCC1105: NC_024008.1:800915..801320) corresponds to an INDEL variation in the promoter region of a yrdC domain-containing protein of unknown function (Bathy01g04300). In the tested strains, this diversity marker is tri-allelic and shows amplified fragments of 200, 400 or 1,400 bp.

280 Amplification efficiency was lower in strains RCC1615, RCC685 and RCC4752 and no
281 amplification was obtained for strain RCC5417 (Fig. 3C).
282
283 Finally, the "Zinc finger" marker, located on chromosome 15 (RCC1105:
284 NC_023994.1:446402..447132), corresponds to variation in the number of Zinc finger repeats
285 encoded in the gene Bathy15g02320. This marker is biallelic and shows amplified fragments of
286 730 or 810 bp. No amplification was obtained for strain RCC5417 and RCC1868 (Fig.3 D).
287
288 The best sets of primers designed for markers “yrdC promoter” and “Zinc finger” show
289 differences in amplification efficiencies between strains due to the lack of conservation in PCR
290 primer sites in these strain genomes. However, the sequences of all amplified INDELs were
291 verified by sequencing and correspond to the expected amplification.
292
293 A fifth additional marker, named "TIMa", was designed to amplify a 530 bp fragment. This
294 marker was selected because it is located in the BOC region of chromosome 14 at the border
295 between a conserved region and an outlier region putatively involved in the definition of mating
296 types in other *Bathycocacceae* (RCC1105: NC_023995.1:564926..565456) [19]. As expected
297 this marker was amplified in the 6 genomes which would correspond to the same putative
298 mating type (Fig 3E).

299 Isolation and identification of *Bathycoccus prasinos* Multi Loci 300 Genotypes in the Banyuls bay

301 Surface water was collected weekly at the SOLA buoy in Banyuls bay from December the 3rd,
302 2018 to March the 19th, 2019. During this period, the sea temperature ranged between 15.87°C
303 in December and 10.68°C in February. We implemented a protocol to isolate *B. prasinos* cells
304 based on filtration through 1.2 µm pore-size filters to remove larger cells of nanoplankton. This
305 pore size allows the passage of *B. prasinos* cells (which size is estimated to be of 1.5 µm) and

306 most importantly eliminates the potential larger predators. After a period of acclimation of two
307 weeks in natural sea water supplemented with nitrate, phosphate and vitamins B1 and B12,
308 cells were isolated by plating in low melting agarose supplemented sea water. Out of more
309 than 400 colonies, only light green-yellow coloured colonies were picked and sub-cultured
310 subsequently. In order to identify putative *B. prasinos* strains, amplification of a fragment of the
311 LOV-HK gene (Bathy10g02360) was performed. These primers did not amplify LOV-HK
312 sequences in other *Mamiellales* (*Ostreococcus* or *Micromonas*). The identity of these clones
313 was further confirmed by ribotyping (amplification of a 2kb ribosomal DNA fragment followed
314 by sequencing) (See Supplementary Appendix 1, Additional File 3). In total, 55 *B. prasinos*
315 isolates were recovered for nine sampling dates (See Supplementary Table 2, Additional File
316 1).
317
318 The five markers, including the four diversity markers and the TIMa marker were used in
319 combination to distinguish the different isolates of *B. prasinos* into Multi Loci Genotypes
320 (MLG). Eight MLG were identified in Banyuls' strains, MLG1 and MLG2 being dominant with 30
321 and 15 isolated strains respectively (Table 2). These two dominant MLG were distinguished
322 only by the TIMa marker, which was amplified only in MLG1. For yrdC promoter marker, the
323 200 bp allele was dominant with an amplification in 54 isolates. The 1,400 bp allele, identified
324 in worldwide strain, was not detected in the Banyuls bay strain. For the TOC1 promoter
325 marker, the 1,200 bp allele was dominant with an amplification in 51 isolates. For the
326 Flavodoxin-like marker, the 1,200 bp allele was dominant with an amplification in 49 isolates.
327 From the 6 remaining isolates, 3 alleles could be identified, including a new 1,600 bp allele;
328 however the 1,000 bp allele was not detected. Finally, for the Zinc finger marker, the dominant
329 allele was the 730 bp one, amplified in 52 isolates.
330
331 **Table 2: Marker characterisation of multi-loci genotypes in *B. prasinos* strains isolated from**
332 **Banyuls bay.** Marker values correspond to amplified sizes in base pairs.
333

Marker

29

	yrdC	TOC1	Flavodoxin	Zinc finger	TIMa	Number of isolates
MLG1	200	2200	1200	730	530	30
MLG2	200	2200	1200	730	n.d.	15
MLG3	200	2200	800	730	530	4
MLG4	200	700	1200	730	530	2
MLG5	200	2200	1200	820	n.d.	1
MLG6	200	700	1200	820	n.d.	1
MLG7	400	700	1400	820	530	1
MLG8	200	2200	1600	730	530	1

n.d. : Not Detected

334

335 The genotypic diversity observed by the MLG classification was also correlated with biological
336 and physiological diversities of the strains. Indeed, by monitoring the growth response of
337 representative strains under different temperature and light conditions corresponding to those
338 observed at different parts of the bloom in the Banyuls bay, we showed clear differences in
339 growth rate between MLG and conditions. Specifically, there were notable variations between
340 strains from MLG1/MLG2 (isolated in December/January) and other tested MLG (isolated in
341 February) (See Supplementary Figure 2, Additional File 2).

342 Diversity of INDEL markers

343 Seven isolates corresponding to 6 MLG were selected for whole genome sequencing and
344 assembly following the methodology described in Figure 1A. The genome assemblies
345 revealed that the two MLG, which failed to amplify the TIMa marker, have a different haplotype
346 of the chromosome 14 outlier region. A complementary marker called "TIMb" was designed
347 from these genome assemblies, corresponding to a 1,300 bp amplified fragment. TIMb was
348 detected in isolates for which TIMa was not amplified (See Supplementary Figure 3, Additional
349 File 2).

350

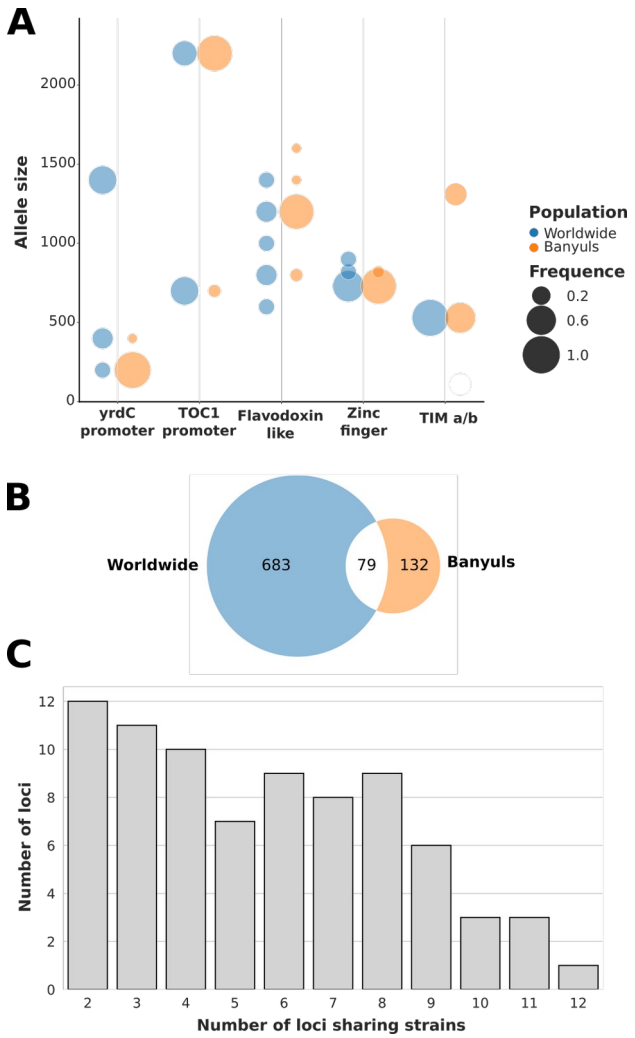

Figure 4 : Worldwide and local diversity in marker alleles and variant loci
(A) Sizes and frequencies in marker alleles within worldwide strains (n=6) and local strains from the Banyuls Bay (n=55). (B) Specific and shared variant loci (200 < size < 2000 bp) between at least one worldwide sequenced strains (n=6) and one sequenced strains from the Banyuls Bay(n=7). (C) Distribution frequency of the 79 shared variant loci (200 < size < 2000 bp) in the 13 sequenced strains.

Comparative analysis of the polymorphism in our markers between worldwide strains and newly isolated strains revealed that all five markers were at least biallelic in the Banyuls local population (Fig. 4A). Furthermore, new alleles were identified through the genotyping of Banyuls bay isolates for Flavodoxin-like and TIMa/b markers, thus extending the diversity observed of our markers. The pipeline of INDEL identification was applied to the seven strains sequenced from the Banyuls bay (Fig. 1A). This led to the detection of 211 unique INDELs of sizes ranging between 0.2 and 2 Kb (Fig. 4B). Within these loci, 79 corresponding to 37% of

loci diversity in the Banyuls bay, were shared with worldwide strains (Fig. 4B). On average, each variant locus was found in 5.6 strains across the two populations (Fig. 4C).

Determination of allelic variants in environmental samples

The identification of dominant MLG during the 2018/2019 bloom raises the question of their yearly or occasional prevalence in the Banyuls bay. However, isolating strains is highly time consuming and not appropriate to follow population dynamics at higher frequency. To overcome this, our diversity markers were directly tracked on environmental DNA samples during 3 successive blooms from 2018 to 2021. Five litres of seawater were sampled once a week and filtered between 3 and 0.8 µm. DNA extracted from 0.8 µm filters was used for PCR analysis of presence/absence variations in alleles of yrdC promoter, TOC1 promoter and TIMa/TIMb markers (Table 3). Flavodoxin-like and Zinc finger markers could not be used on complex environmental DNA samples due to the presence of high background.

The initiation of the 2019/2020 bloom was defined by the exclusive presence of the 200 bp allele of yrdC promoter marker from the third week of November to the third week of December. This was followed from January to March by the unambiguous detection of both the 200 bp and the 400 bp alleles. During this bloom, TIMa and TIMb markers were both amplified in all samples, with the exception of the second week of March, during which only the TIMa marker was detected.

Table 3: Marker alleles detection in environmental DNA samples from successives *B. prasinos* blooms in the Banyuls bay. YrdC promoter (A : 200bp, B : 400bp), TOC1 promoter (A : 2200bp, B: 700bp), TIM a/b (a : 530 bp, b : 1300bp). Missing datapoints are represented by empty cells.

	Oct				Nov				Dec				Jan				Feb				Mar				Apr	
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2
yrdC promoter																										
2018 - 2019																A,B	A,B	A,B	A,B	A,B	A	A,B	A,B	A,B		
2019 - 2020							A	A	A	A	A		A,B	A,B		A,B	A,B	A,B	A,B	A,B	A,B	A,B				
2020 - 2021	A,B	A,B		A,B	n.d.		n.d.		A,B	A,B	A,B		A,B		A,B	A,B	A,B	A,B	A,B	A,B	A,B	A,B	A,B	A,B	A,B	A,B
TOC1 promoter																										
2018 - 2019																A,B	A,B	A,B	A,B	A,B		A,B	A,B	A,B		
2019 - 2020								A,B	A,B	A,B	A,B		A,B	A,B		A,B	B	A,B	A,B	A,B	A,B	A,B				
2020 - 2021	A,B	A,B		n.d.	n.d.		n.d.		A,B	A,B	A,B		A,B		A,B	A,B	A,B	A,B	A,B	A,B	A,B	A,B	A,B	A,B	A,B	A,B
TIM a/b																										
2018 - 2019																a,b	a,b	a,b	a,b	a,b	a,b	a,b	a,b	a,b		
2019 - 2020							a,b	a,b	a,b	a,b	a,b		a,b	a,b		a,b	a,b	a,b	a,b	a,b	a,b	a				
2020 - 2021	a	a		a	n.d.		n.d.		a	a,b	a		a,b		a,b	a,b	a,b	a,b	a,b	a,b	a,b	a,b	a,b	a,b	a,b	a,b

n.d. : Not Detected

389 Several distinctions can be done with the 2020/2021 bloom, the first one being that no
390 amplification was detected in November samples, an observation consistent with the overall
391 decrease in picophytoplankton abundance (See Supplementary Figure 4, Additional File 2).
392 For yrdC promoter marker, the 400 bp allele was observed in October and December, hence
393 between one and three months earlier than in the 2019/2020 bloom. In addition, only TIMa was
394 amplified in October and December 2020. Both alleles of the TOC1 promoter marker were
395 amplified in nearly all samples where *B. prasinos* could be detected and were therefore not
396 informative in this context (Table 3). From the study of these successive blooms, we were able
397 to conclude that their onset differed in both chronology and population diversity. Furthermore,
398 we demonstrated that population diversity changes both within and between blooms at a given
399 site and that diversity markers, designed from the worldwide diversity, could be used to
400 successfully follow these local population dynamics.

401 Discussion

402 We aimed to develop a time and cost effective method to produce a biological resource and
403 characterise the intraspecific diversity of the marine microalga *B. prasinos*. In recent years, the
404 cost of high-throughput sequencing has fallen sharply, favouring blind sequencing approaches
405 without prior genotyping [19]. However, the isolation of new strains from complex microbial
406 communities is time consuming, requiring multiple steps from sample isolation in remote areas
407 of the ocean, to incubations, shipment and isolation by subculturing on agarose plates.
408 Moreover, the incubation step can lead to the amplification of strains that, without genotyping,
409 could be sequenced multiple times, while missing rare genotypes that contribute to the fitness
410 of a species under changing environmental conditions. Given the bottleneck steps of strain
411 isolation and whole genome sequencing in complex microbial communities, we turned to PCR
412 amplification as a potentially effective approach for (i) preliminary screening of environmental
413 samples to retain and incubate only those containing *B. prasinos* cells, (ii) identifying incubates
414 containing *B. prasinos* for subsequent isolation of strains, (iii) genotyping and identifying the

415 main MLGs prior to sequencing. These first steps of screening by PCR could be potentially
416 performed during oceanographic cruise to guide and optimise sample selection and culturing
417 on boats. Remarkably, our protocol proved efficient and selective for *B. prasinos* isolation
418 since *Ostreococcus* and *Micromonas* strains were not obtained even though *Micromonas* is as
419 abundant as *B. prasinos* in the Banyuls bay in winter [15]. This is all the more important as flow
420 cytometry cannot distinguish the *Bathycoccus* genus from other *Mamiellales*.

421 Design of diversity markers from INDELs structural variations

422 While PCR, combined with DNA fragment size estimation by gel electrophoresis, has already
423 been used to follow intraspecific diversity [12], this approach requires the specific amplification
424 of size-varying loci. This leads to challenges such as identifying putative marker loci that vary
425 in size, are sufficiently conserved to be representative of subpopulations and can be found in
426 conserved regions for which PCR primers can be designed. To address this, we explored the
427 utility of INDEL variations within coding sequences, which offer higher stability in clonal
428 populations compared to SNP and microsatellite markers, and are therefore good candidates
429 for the design of size markers to track intraspecific diversity [36,37]. Furthermore, information
430 on such INDELs variation and on repeated/low complexity sequences are now becoming
431 easily available through the leveraging of the recent ONT and PacBio long read sequencing
432 technologies, providing access to structural variations and marker designs previously
433 overlooked [31,38,39].
434
435 By long read sequencing a selection of only six geographically distinct strains available in
436 culture collection, we were able to obtain a first picture of the genetic diversity of *B. prasinos*. T
437 he implemented genome assembly pipeline provided the draft genomes required for
438 comparison and identification of structural variations. This resulted in the initial identification of
439 683 INDEL variation candidates, including 44 meeting our criteria for the design of diversity
440 markers. While each candidate marker must be validated experimentally, this demonstrates
441 the effectiveness of long reads whole genome sequencing for the identification of diversity

442 marker loci using a limited biological resource. Four loci were selected for PCR validation,
443 resulting in the diversity markers Flavodoxin-like, TOC1 promoter, yrdC promoter and Zinc
444 finger. All markers were successfully amplified in a majority of strains even though a lack of
445 conservation in PCR primer sites resulted in uneven amplification signals for yrdC promoter
446 and Zinc finger markers. As such, nucleotide diversity remains a limiting factor for the design of
447 universal markers when considering phylogenetically distant strains. Nonetheless, the pipeline
448 developed in this study, by systematically mapping all INDEL and iteratively integrating them in
449 the database, provides an extensive resource to identify novel markers (Fig. 1a; Fig. 4).
450
451 As expected, fewer alleles were detected in the local Mediterranean population for the
452 designed markers, but genetic diversity was still observed for all of them. Since all markers
453 correspond to structural variations either within coding sequences or regulatory sequences,
454 this raises the question of whether they play a role in local adaptation. For instance, in the case
455 of the central circadian clock TOC1, the variation was found in the promoter which contains a
456 key *cis*-regulatory Evening element that is essential for the circadian regulation of TOC1 [40].
457 However, no clear pattern of allele distribution was identified neither in the worldwide strains
458 nor in the sampled Banyuls population. The potential role of these INDELs in adaptation would
459 therefore require additional experimental investigation.

460 Genotyping and phenotyping in local populations of *B. prasinos*

461 The five diversity markers developed in this study were tested in combination on 55 freshly
462 isolated Mediterranean strains of *B. prasinos*. This resulted in their classification into eight
463 multi-loci genotypes. This highlights, firstly, the efficiency of our protocol for isolating *B.*
464 *prasinos* strains from complex seawater samples and its effectiveness in preserving genotypic
465 diversity. This also includes the TIMa/b markers which were manually designed from
466 assembled genomes to detect different haplotypes of the big outlier chromosome 14 (BOC) as
467 described by Blanc-Mathieu et al. [19] in *Ostreococcus tauri*. This marker combination allowed
468 us to confirm the existence of at least two distinct haplotypes of the BOC in the *B. prasinos*

469 subpopulation from the Banyuls bay, and thus can be used to distinguish these putative mating
470 types in newly isolated strains and environmental samples.
471
472 A high level of intraspecific diversity has been reported in the few studies describing the
473 genetic diversity of blooming phytoplankton populations [41–44]. For example, in diatoms
474 (although not directly comparable as microsatellite markers have a higher mutation rate than
475 other regions of the genome), more than 600 individuals were genotyped using microsatellite
476 markers, and it was estimated that the blooming population was comprised of at least 2400
477 different genotypes [41]. Therefore, the observation of eight MLG in *B. prasinos* mediterranean
478 population is most likely an underestimation due to the low number of markers used and their
479 limited polymorphism. However, further improvement in the accuracy of our marker set can be
480 achieved in an iterative way: through the identification of new size alleles (as exemplified by
481 the 1,600 bp allele of the flavodoxin-like marker identified in the Banyuls population), or by the
482 sequencing and integration of newly isolated strains distinguished by our original set of primer,
483 further increasing the number of candidate loci. In this study, the integration of 7 newly
484 sequenced genomes from the Banyuls bay nearly doubled the number of putative universal
485 markers and provided population specific variants that could be used in local subpopulations
486 tracking.
487
488 The first physiological characterization of selected strains revealed distinct growth responses,
489 the strains isolated in December and January showing reduced growth under lower
490 temperature (MLG1 and MLG2) and short photoperiod (MLG1) which suggest that these
491 strains may correspond to seasonal ecotypes. Before concluding whether the different MLG
492 classes correspond to different ecotypes, it will be necessary to characterise the physiological
493 response of more strains, in particular those belonging to each of the dominant MLG1 and
494 MLG2 classes.

Tracking intraspecific diversity directly in environmental samples

Isolation of *B. prasinos* strains is too laborious and costly to be performed systematically on a large scale. For this reason, we attempted to assess intraspecific diversity in environmental samples during three consecutives annual blooms of *B. prasinos* in the Banyuls bay. Although flavodoxin-like was the most resolutive marker in isolated strains, PCR on environmental DNA yielded a smear on electrophoresis gel, possibly due to high polymorphism. Three diversity markers, TIMa/b, yrdC and TOC1 promoter, were suitable for amplifying environmental DNA, highlighting local population dynamics within and between bloom events. While this approach based on PCR amplification provides only qualitative data at this stage, approaches based on quantitative PCR could be developed to track the dynamics of genetic diversity in *B. prasinos* blooms directly from environmental samples [45]. As such, by accessing a level of structural diversity hardly obtainable through metagenomic short read mapping, it is complementary with approaches of metagenomic characterisation through SNP, as proposed by Da silva et al. [46] .

Variations in interannual presence/absence of alleles raise the question of the nature of the highly reproducible yearly occurrence of *B. prasinos* in the Banyuls bay. Seasonal blooms may result either from re-activation of “dormant/survivor” cells from the water column (whose genetic fingerprint will determine the genetic profile of the next bloom) or by yearly *de novo* seeding by cells carried by the north Mediterranean current along the Gulf of Lion. At first glance, our preliminary qualitative results are in favour of the introduction of a new population rather than “resurrection” of cells from the previous bloom, since the allelic composition is distinct between the end of a bloom and the onset of the next (Table 3). By monitoring the temporal population structures of the dinoflagellate *Alexandrium minutum* in two estuaries in France, Dia et al. [44] showed that interannual genetic differentiation was greater than intra-bloom differentiation. Alternation of genotypes/populations has also been observed with diatoms in the dominance of one of the two sympatric populations of *Pseudonitzschia*

522 *multistriata*, which could be due either to environmental factors favouring one population over
523 the other or intrinsic factors coupled to the obligate sexual life cycle of *P. multistriata* [47]. Thus
524 the observed fluctuations in allele frequencies could equally be the result of new inoculum from
525 currents or sexual reproduction. Even though sexual reproduction has not been demonstrated
526 in *B. prasinos*, there is genomic evidence that it may occur [48], and potential mating types
527 were identified in our study (TIMa/TIMb markers). Sexual recombination generates new
528 combinations of alleles, whereas clonality favours the spread of the fittest genotype through
529 the entire population [44]. Erdner et al. [49] propose for *A. fundyense* that mitosis is the primary
530 mode of multiplication during blooms, whereas mating is triggered presumably in response to
531 unfavourable conditions at the end of blooms, with vegetative cells not overwintering in the
532 water column. Knowing that (i) *B. prasinos* blooms are followed by severe bottlenecks between
533 one bloom and the next [15], (ii) allele occurrences were different between the end of one
534 bloom and the onset of the next, and (iii) structural markers are very stable in mitotic dividing
535 cells, the hypothesis of rare vegetative cells remaining in the water column between the
536 blooms is unlikely, except if those remaining cells were produced by sexual reproduction. The
537 alternative hypothesis of new strains brought by current is however still equally probable. To
538 test these hypotheses, more quantitative approaches based on real time quantitative PCR, or
539 direct mapping of INDEL markers on the 7 year metagenomic time series in the Banyuls bay
540 could potentially be used [50].

541 Conclusions

542 In this paper, we describe the development of a new type of diversity markers based on INDEL
543 structural variants obtained by long read sequencing. After validation on freshly isolated
544 individuals, markers were used *in situ* on environmental samples. Whole genome sequencing
545 of the new MLG refined a marker resource that could be further used to guide strain isolation
546 and genotype new strains from the world ocean. In addition, the assessment of the
547 physiological performances of genotyped strains suggest the presence of seasonal ecotypes

548 in the Banyuls bay. Finally, PCR on environmental samples provided insight into the genetic
549 diversity of *B. prasinos* seasonal blooms.
550 This INDEL marker resource represents a new tool for grasping the maximum diversity of
551 newly isolated *B. prasinos* with the long term goal of identifying putative molecular
552 mechanisms involved in adaptation to environmental niches in this cosmopolitan genus. The
553 marker resource could also potentially be used to check culture purity or to follow specific
554 genotypes in competition experiments in culture or in microcosms. The developed pipeline
555 aimed at optimising sequencing efforts and building a collection of sequenced genomes
556 representative of *B. prasinos* intraspecific diversity. This pipeline could easily be applied to
557 other cosmopolitan and cultivable planktonic species that are available in collection, whether
558 they are related to *B. prasinos* (e.g. *Ostreococcus* RCC809, *Micromonas commoda*,
559 *Micromonas pusilla*) or share similar genome features.

560 List of abbreviations

561 ASW : Artificial Sea Water
562 BOC : Big Outlier Chromosome
563 INDEL : Insertion/Deletion
564 ONT : Oxford Nanopore Tech
565 PCR : Polymerase Chain Reaction
566 RCC : Roscoff Culture Collection
567 SV : Structural Variation

568 Declarations

Availability of data and materials

Whole genome sequences and basecalled reads of the strains sequenced in this study as well as variant calling outputs are available at Zenodo https://doi.org/10.5281/zenodo.11395682. Strains isolated in Banyuls are available on request. All codes are available at https://forge.ird.fr/diade/genomecodes under the GPLv3 licence.

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Author contribution

MD, FYB and FS conceived the work and acquired funding. MD extracted high molecular weight genomic DNA. CM and MD performed the ONT sequencing. LD and FS performed bioinformatic analysis. MD designed and validated the diversity markers. PS analysed the seawater samples by flow cytometry. MD and VV isolated Banyuls strains during the winter bloom, genotyped by MD and JCL. MD and JCL determined the diversity of seawater samples. MD, LD and FYB wrote the article. JCL and FS took part in the critical reviews. All authors approved the final version for submission.

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