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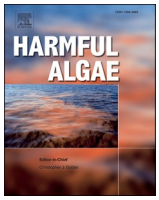

Original Article

Intense blooms of *Phaeocystis globosa* in the South China Sea are caused by a unique "giant-colony" ecotype

Qing-Chun Zhang [a,b,d], Chao Liu [a,c], Jin-Xiu Wang [a,c], Fan-Zhou Kong [a,b,d], Zhuang Niu [a,c], Ling Xiang [a,c], Ren-Cheng Yu [a,b,c,d,*]

[a] CAS Key Laboratory of Marine Ecology and Environmental Sciences, Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China
[b] Laboratory for Marine Ecology and Environmental Science, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266071, China
[c] University of Chinese Academy of Sciences, Beijing 100049, China
[d] Center for Ocean Mega-Science, Chinese Academy of Sciences, Qingdao 266071, China

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ABSTRACT

The haptophyte *Phaeocystis globosa*, an important causative agent of harmful algal blooms globally, exhibits varying morphological and physiological features and high genetic diversity, yet the relationship among these has never been elucidated. In this study, colony sizes and pigment profiles of 19 *P. globosa* isolates from the Pacific and Atlantic Oceans were determined. Genetic divergence of these strains was analyzed using the chloroplast *rbcS-rpl27* intergenic spacer, a novel high-resolution molecular marker. Strains could be divided into four genetic clades based on these sequences, or two groups based on colony size and the identity of diagnostic pigments (19′-hexanoyloxyfucoxanthin, hex-fuco, and 19′-butanoyloxyfucoxanthin, but-fuco). Three strains from the South China Sea (SCS), all belonging to the same genetic clade, have unique biological features in forming giant colonies and possessing but-fuco as their diagnostic pigment. Based on these findings, we propose that these SCS strains should be a unique "giant-colony" ecotype of *P. globosa*. During the period 2016-2021, more than 1000 *rbcS-rpl27* sequences were obtained from 16 *P. globosa* colony samples and 18 phytoplankton samples containing solitary *P. globosa* cells in the SCS. Phylogenetic analysis indicated that >95% of the sequences from *P. globosa* colonies in the SCS were comprised of the "giant-colony" ecotype, whereas the genetic diversity of solitary cells was much higher. Results demonstrated that intense blooms of *P. globosa* featuring giant colonies in the SCS were mainly caused by this giant-colony *P. globosa* ecotype.

1. Introduction

The haptophyte *Phaeocystis globosa* Scherffel is considered a causative species of harmful algal blooms (HAB) (Medlin et al., 1994; Vaulot et al., 1994), due to its adverse effects on marine environments and ecosystems (Davidson and Marchant, 1992). Outbreaks of *P. globosa* usually occur in eutrophic coastal waters in the form of colonies, which contain thousands of cells embedded in a polysaccharide envelope (Zingone et al., 1999; Jacobsen, 2002). The North Sea in the Atlantic Ocean and East and Southeast Asia coastal waters in the Pacific Ocean are two typical regions where a large number of *P. globosa* blooms have been documented (e.g., Riegman and vanBoekel, 1996; Schoemann et al., 2005; Smith et al., 2014). In September 1997, a bloom of *P. globosa* was recorded for the first time in coastal waters of the South China Sea (SCS) that covered an area > 3,000 km^2 and led to economic losses exceeding 7.5 million US dollars (Chen et al., 1999). Since then, *P. globosa* blooms have been recorded almost every year in the SCS (Qi et al., 2004; Shen et al., 2018). Recently, intense blooms of *P. globosa* in the Beibu Gulf, SCS, posed a potential threat to the routine operation of a nuclear power plant and the marine ecosystem (Yu et al., 2017).

P. globosa blooms in the SCS have been characterized by a number of unique features (Qi et al., 2004; Smith et al., 2014), such as the formation of giant colonies with a maximum size of ca. 3 cm (Qi et al., 2004; Shen et al., 2004; Doan et al., 2010; Smith et al., 2014; He et al., 2019). In contrast, *P. globosa* colonies generally range from 10 μm to 3 mm in temperate coastal waters of the North Sea (Rousseau et al., 1990; Smith et al., 2014). Additionally, *P. globosa* strains isolated from the SCS can produce hemolytic compounds, leading to detrimental effects on

* Corresponding author at: CAS Key Laboratory of Marine Ecology and Environmental Sciences, Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China.
E-mail address: rcyu@qdio.ac.cn (R.-C. Yu).

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fisheries and the aquaculture industry (Shen et al., 2004; Peng et al., 2005). Two pigments derived from fucoxanthin (fuco), i.e., 19'-hexanoyloxyfucoxanthin (hex-fuco) and 19'-butanoyloxyfucoxanthin (but-fuco), are considered marker pigments for *Phaeocystis* spp. (Zapata et al., 2004). Both were detected in phytoplankton samples collected prior to the formation of *P. globosa* blooms in the Beibu Gulf, southeastern China, but but-fuco was determined to be the major diagnostic pigment during these blooms (Wang et al., 2019). It is noteworthy that *P. globosa* strains isolated from the SCS have high genetic diversity and differ in their composition of marker pigments (Hu et al., 2019; Wang et al., 2019; Zhang et al., 2020a).

Cumulative evidence suggests that *P. globosa* could maintain a high level of genetic diversity, and the strains isolated from different geographical regions have variable morphological and physiological features, including colony size, pigment profile, temperature for optimal growth and cell toxicity (e.g., Vaulot et al., 1994; Zapata et al., 2004; Medlin and Zingone, 2007; Smith et al., 2014). Current molecular markers, however, cannot effectively resolve the intraspecific genetic diversity of *P. globosa*. The 18S rDNA sequence provides inadequate intraspecies resolution and only allows discrimination among different *Phaeocystis* species. The 28S rDNA and internal transcriber spacer (ITS) regions, in contrast, obscure the accurate phylogenetic relationships of *P. globosa* due to substantial intracellular sequence divergence (Lange et al., 2002; Chen et al., 2003; Medlin and Zingone, 2007; Hu et al., 2019; Zhang et al., 2020a). Recently, the chloroplast intergenic spacer, characterized by a rapid evolutionary rate and little intra-genome variation, was proposed as the target to resolve phylogeographic relationships of *P. globosa* (Zhang et al., 2020a). Two high-resolution molecular markers that targeted the chloroplast intergenic spacer were used to identify and track *P. globosa* strains isolated from different locations worldwide (Song et al., 2020; Zhang et al., 2020a). Among them, Zhang et al. (2020a) developed the high-resolution molecular marker based on chloroplast-encoded *rbcS-rpl27* spacer, and effectively distinguished the intraspecific phylogeographic relationships of *P. globosa* isolated from eight different sites in the Pacific and Atlantic.

In the present study, the colony size and pigment composition of 19 *P. globosa* strains isolated from different sites in the Pacific and Atlantic Oceans were examined, and their genetic relationship was investigated based on the chloroplast *rbcS-rpl27* intergenic spacer using a high-resolution molecular marker specific to the genus *Phaeocystis* (Zhang et al., 2020a). The relationship between colony size, marker pigment and genetic diversity of *P. globosa* was established in free-living solitary cells and colonial specimens collected from 2015 to 2021 in the SCS. These were further analyzed to determine the causative species of intense *P. globosa* blooms in the SCS.

2. MATERIALS AND METHODS

2.1. Collection of P. globosa strains

Nineteen strains of *P. globosa* were used in this study, including 12 strains isolated from coastal Pacific waters and 7 strains from the Atlantic coast (Table 1, Fig. 1). The strains were cultured in L1 cultural media (Guillard and Hargraves, 1993) and in the laboratory at 20 ± 1°C under a light intensity of 100 μE $m^{-2}s^{-1}$ and a 14 h:10 h light:dark cycle. After 7-10 days, approximately 100 ml and 20 ml of exponentially growing cultured algae were filtered on GF/F glass fiber filters (Millipore, USA) and HTTP polycarbonate membranes (Millipore, USA, with 0.40 μm pore diameter), respectively, at a 40 kPa vacuum level. The filtered samples were then stored in a freezer at -80°C for subsequent pigment and DNA extraction.

2.2. Phytoplankton sampling

To determine the genetic diversity of *P. globosa* solitary cells in the phytoplankton communities in both bloom and non-bloom areas, a total

Table 1
Phaeocystis globosa strains used in this study, their sampling date and site and GenBank *rbcS-rpl27* sequence.

Strain	Source for culture	Sampling date	Sampling area	GenBank ID of *rbcS-rpl27* sequence	Reference
MEL42	from a colony	2015	Beibu Gulf, South China Sea, Pacific Ocean	MW981522	this study
MEL43	from a colony	2018	Beibu Gulf, South China Sea, Pacific Ocean	MN935489	(Zhang et al., 2020a)
MEL44	from a cell	2017	Beibu Gulf, South China Sea, Pacific Ocean	MN935490	(Zhang et al., 2020a)
MEL45	-	-	Pearl River Estuary, South China Sea, Pacific Ocean	MN935491	(Zhang et al., 2020a)
MEL47	from a single cell	2017	Quanzhou coast, East China Sea, Pacific Ocean	MN935492	(Zhang et al., 2020a)
MEL68	from a colony	2018	Beibu Gulf, South China Sea, Pacific Ocean	MW981524	this study
MEL69	from a colony	2017	Beibu Gulf, South China Sea, Pacific Ocean	MN935493	(Zhang et al., 2020a)
MEL70	from a cell	2019	Beibu Gulf, South China Sea, Pacific Ocean	MW981525	this study
MEL71	from a cell	2019	Sansha Bay, East China Sea, Pacific Ocean	MN935494	(Zhang et al., 2020a)
MEL78	from a colony	2019	Vietnam, Pacific Ocean	MW981526	this study
TIO393	from a colony	2016	Daya Bay, South China Sea, Pacific Ocean	MW981523	this study
CCMP628	-	1965	Caribbean Sea, Atlantic Ocean	MN935496	(Zhang et al., 2020a)
CCMP629	-	1982	Gulf of Mexico, Atlantic Ocean	MN935497	(Zhang et al., 2020a)
CCMP1524	-	1992	Gulf of Thailand, Pacific Ocean	MN935498	(Zhang et al., 2020a)
CCMP2754	-	2003	North America, Atlantic Ocean	MN935499	(Zhang et al., 2020a)
RCC678	-	2000	North Sea, Atlantic Ocean	MW981528	this study
RCC736	-	1991	North Sea, Atlantic Ocean	MN935501	(Zhang et al., 2020a)
RCC1736	-	1991	North Sea, Atlantic Ocean	MW981527	this study
RCC2055	-	2003	English Channel, Atlantic Ocean	MN935500	(Zhang et al., 2020a)

"-" unknown information

of 18 phytoplankton samples without any colony of *P. globosa* were collected from coastal waters of the Beibu Gulf and Guangdong Province, two regions with high frequency of *P. globosa* blooms in the SCS (Fig. 2 and Table S1). Approximately 1 liter of surface seawater was collected from sampling sites P1, P4 and K1 in December 2015 and sieved through a 20 μm Mesh with no additional pressure to remove *P. globosa* colonies and microplankton. The filtrate was re-filtered through HTTP membranes (0.40 μm pore diameter, Millipore, USA) under a vacuum of 40 kPa. Using the sampling protocol described above, phytoplankton samples were also collected from sites ZN1-2, ZN1-4, ZN1-5, ZN1-6, ZN2-5, ZN3-1, ZN4-3, ZN4-4, ZN5-2 and P4 in the Beibu Gulf in January, February and March 2019, site ZJ along the Zhanjiang coast, Guangdong Province, in November 2018, and January and March 2019, and sites A14 and B9 adjacent to the Pearl River estuary, Guangdong Province, in January 2020. Phytoplankton samples for DNA extraction were kept at -80°C.

2.3. Sampling of colonies

A total of 16 colonial samples were collected from coastal waters of the Beibu Gulf and Guangdong Province in 2016, 2019 and 2021 (Fig. 2 and Table S1). In January 2016, ~10 liters of surface seawater were collected from three sampling sites P1, P4 and K1. Giant colonies (diameter >1 cm) were washed in a beaker containing sterile seawater to remove attached contaminants (Fig. S1a). A total of 20 cleaned colonies with diameters ≥5 mm were selected and from each sample site and were acted as a sample of colonies filtered through a HTTP membrane (0.40 μm pore diameter, Millipore, USA) at a vacuum level of 40 kPa. In January 2019, colonies were collected using a plankton net (mesh size 76 μm) from four sites P1, ZN4-1, ZN4-3 and ZN4-4. Ten colonies with diameters ≥5 mm were selected from each sample site (Fig. S1b) and again filtered through a HTTP membrane (0.40 μm pore diameter, Millipore, USA), and were acted as a sample of colonies. In February 2019, colonies were collected from three sites, ZN1-5, ZN2-3 and ZN4-3. Single colony with diameters ≥5 mm was collected and filtered through a HTTP membrane (0.40 μm pore diameter, Millipore, USA), and was acted as a sample of colony. In January 2021, a *P. globosa* bloom occurred in Dapeng Bay, Guangdong Province. Three single colonies measuring 1 cm in diameter (Fig. S1c) were collected from site S3 in Dapeng Bay (Fig. 2 and Table S1). Colony samples were kept at -80°C for DNA extraction.

2.4. Measurement of colony size of cultured Phaeocystis globosa strains

To determine the difference in the colony sizes formed by the geographical strains of *P. globosa*, approximately 20−40 large colonies of each *P. globosa* strain at the exponential growth phase were collected and placed in a 12-well plate. The colony diameter was then measured under an inverted microscope with 50 × magnification. Giant colonies produced by strains MEL42, MEL70 and TIO393 were placed in a culture dish and measured directly with a ruler. Average diameter values of the top 10 large colonies were determined for each *P. globosa* strain.

2.5. Pigment analysis of cultured Phaeocystis globosa strains

Pigments of cultured *P. globosa* strains were extracted and analyzed using a high-performance liquid chromatography (HPLC) method developed by Zapata et al. (2000). Details of the protocol can be found in Wang et al. (2019). Briefly, 1.4 ml of 95% methanol were used to extract pigments, and 100 μl of 8′-apo-β, ψ-carotaldehyde (apocarotenal) was added as the internal standard (IS). Pigment separations were achieved using a Waters Symmetry C8 column connected to a Waters E2695 HPLC system with binary gradient elution. The detection wavelength was 440 nm using a Waters 2998 diode array detector, and the spectrum between 300−750 nm was recorded. The detected pigments were identified and quantified by co-chromatography using commercial standards (DHI Water and Environment, Denmark).

2.6. Genomic DNA extraction, PCR amplification and sequencing

The total genomic DNA of cultured *P. globosa* strains and field samples was extracted using the modified method described by Winnepenninckx et al. (1993), and stored at -20°C until analysis. The *rbcS-rpl27* intergenic spacer of *P. globosa* strains was amplified using a pair of specific primers: *rbcS-rpl27*-F (5'-GAAGCATACGCACCAAGC-3') and *rbcS-rpl27*-R (5'-CAGCCAGTAAAGGCAGGT-3') (Zhang et al., 2020a); PCR was conducted under the amplification system and conditions indicated by Zhang et al. (2020a). The targeted DNA bands were cut from the PCR products in a 1% agar gel, purified, and sequenced from both ends by Sangon Biotech (Shanghai). The forward and reversed

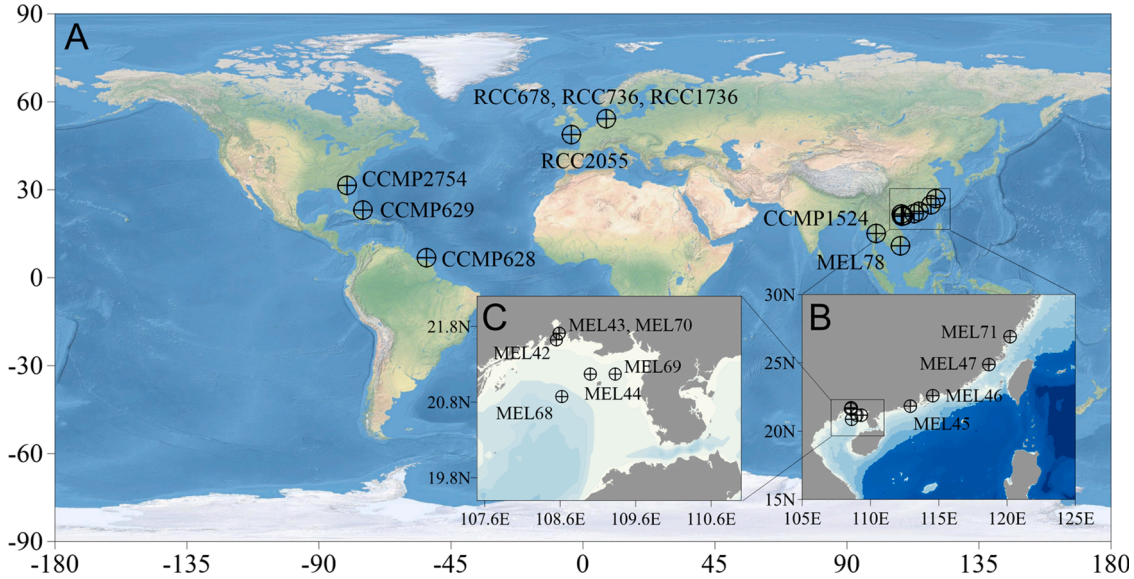

Fig. 1. Maps showing the isolated source of cultured *Phaeocystis globosa* strains (A: world map, B: inset map of the East and South China Seas, and C: of the Beibu Gulf. Strains RCC736 and RCC1736 were collected from the North Sea, Atlantic Ocean (refer to website: http://www.roscoff-culture-collection.org/ for details)

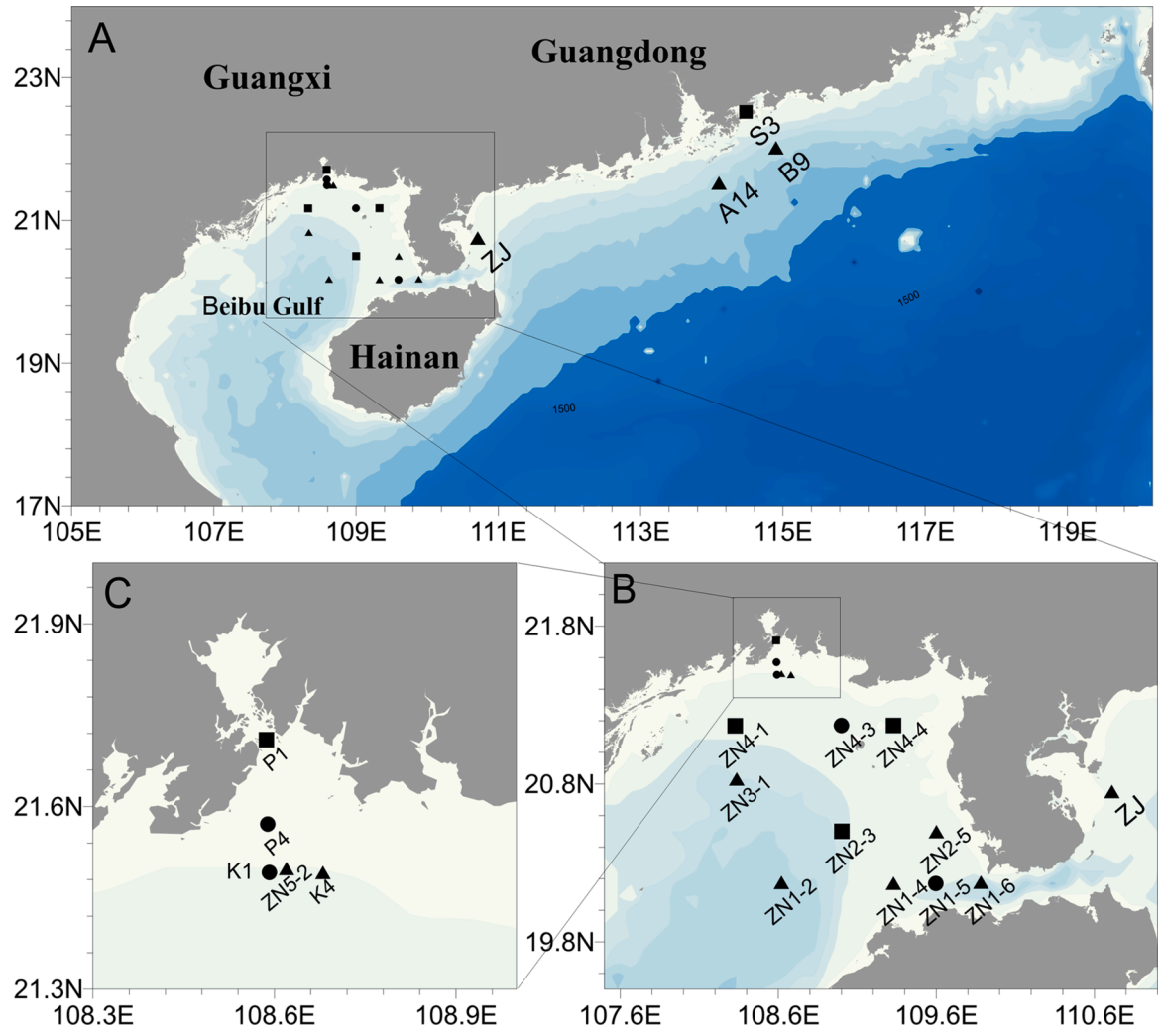

Fig. 2. Maps (A and inset maps B and C) showing sampling sites of phytoplankton and *Phaeocystis globosa* colonies in the South China Sea (see Table S1): black triangles and black squares indicate sampling sites for phytoplankton and *P. globosa* colonies, respectively; black circles indicate sampling sites for both phytoplankton and colony samples).

fragments obtained were assembled with Vector NTI 11.5.3 ContigExpress (ThermoFisher, USA).

2.7. Clone libraries of field samples

Clone libraries of field samples were constructed using purified PCR fragments. These were cloned into Blunt simple cloning vectors (Trans com., China), and then transformed into *Trans*1-T1 competent *Escherichia coli* cells (Trans Com., China). The latter were plated onto LB ampicillin plates containing a final concentration of 100 μg ml^{-1} ampicillin. A total of 20−50 colonies were randomly selected from each plate and sequenced from both ends using the specific primer pairs *rbc*S-*rpl*27-F (5'-GAAGCATACGCACCAAGC-3') (Zhang et al., 2020a) and *rbc*S-*rpl*27-3R (5'-ATTGTTGAGTTTACGACGGATA-3') designed as for this study. PCR was conducted under the amplification system and conditions indicated by Zhang et al. (2020a). The resulting forward and reversed fragments were assembled using Invitrogen 11.0 ContigExpress (ThermoFisher, USA).

2.8. Phylogenetic analysis of cultured Phaeocystis globosa strains

Sequences of the *rbc*S-*rpl*27 intergenic spacers from *P. globosa* Pg-G (A) and *Phaeocystis antarctica* strain CCMP1374 were selected from their chloroplast genomes (NC021637, JN117275) downloaded from GenBank. Together with the sequences of the *rbc*S-*rpl*27 intergenic spacers obtained in this study and the previous study (Zhang et al., 2020a), a maximum-likelihood tree was built using the Mega 10 variance estimation method with bootstrap replications of 1000, and the best-fitting nucleotide substitution model of Tamura 3-parameter (T92)+Gamma Distributed (G) was selected (Kumar et al., 2018). A Bayesian tree was constructed using MrBayes 3.2.6 (Ronquist et al., 2012) with 5,000,000 generations and 100 sampling frequencies, and the best-fitting nucleotide substitution model of HKY+F+G4 was selected using ModelFinder (Kalyaanamoorthy et al., 2017). *P. antarctica* strain CCMP1374 (JN117275) was used as the outside group.

2.9. Genetic diversity of field-sampled Phaeocystis globosa

Sequences of the *rbc*S-*rpl*27 intergenic spacers from *P. globosa* strains, as well as those derived from field samples of phytoplankton and colonies, were aligned using ClustalW. Maximum-likelihood and Bayesian trees were built with the same best-fitting nucleotide substitution models as described in section 2.8, using *P. antarctica* strain CCMP1374 (JN117275) as the outside group. Based on genetic diversity results for field samples, the genetic distances of the sequences in field samples and cultured strains within and between the genetic clades of *P. globosa* were estimated by the Mega 10 pairwise distance method with 1000 bootstrap replications (Kumar et al., 2018).

3. Results

3.1. Colony size and pigment profile of cultured Phaeocystis globosa strains

The colony size of 19 cultured *P. globosa* strains (Table 2) showed significant differences ($p < 0.05$). No colonies were found in the cultures of strains RCC678, RCC736, and RCC1736. Colonies with diameters <1 mm occasionally appeared in cultures of strains CCMP2754 and CCMP1524. Among the 14 colony-forming strains, three SCS strains (MEL42, MEL70 and TIO393) isolated from coastal waters of the Beibu Gulf and Guangdong Province formed giant colonies with maximum diameters of 1.1−1.4 cm, while the other 11 strains produced only small colonies with a maximum diameter ranging from 0.2 mm to 3.0 mm.

Pigment profiles of the 19 *P. globosa* strains also appeared to differ (Fig. 3 and Table 2). The major pigments detected in *P. globosa* included chlorophyll *a* (Chl *a*), chlorophyll c_3 (Chl c_3), chlorophyllide *a* (chlide *a*), methyl chlorophyllide *a* (me-chlide *a*), fuco, but-fuco, hex-fuco, 19′-hexanoyloxy-4-ketofucoxanthin (4k-Hex-fuco), diadinoxanthin (diad), diatoxanthin (diat), and *β,β*-carotene (*ββ*-Car) (Fig. 3). The three stains (MEL42, MEL70 and TIO393) isolated form the SCS contained only but-fuco as their diagnostic pigment, while no hex-fuco was detected, whereas both pigments were detected in the other 16 strains. Additionally, the three strains isolated from the SCS had higher ratios of light-protecting pigments (such as diad and diat) to Chl *a* than the other strains.

3.2. Phylogenetic relationship of cultured Phaeocystis globosa strains

Phylogenetic analysis revealed high genetic diversity among different geographical strains of *P. globosa* based on the *rbcS-rpl27* intergenic spacer sequences of 19 strains collected in this study and our previous study (Zhang et al., 2020a), and the sequence of a strain isolated from Atlantic North Sea, Pg-G(A), downloaded from the GenBank (Fig. 4). Twenty strains of *P. globosa* were divided into two groups, and each was further divided into two clades. Within the first group, Clade I showed considerable divergence and could be further divided into three subclades. All the *P. globosa* strains in Clade Ia were isolated from East and Southeast Asia, including MEL47 isolated from the ECS, five SCS strains (MEL43, MEL44, MEL45, MEL68 and MEL69), the Vietnamese strain MEL78, and a Thai strain CCMP1524. Clade Ia strains clustered with two Central American strains CCMP628 and CCMP629 (Clade Ib) and strain RCC2055 (Clade Ic) from the English Channel, Atlantic Ocean, to jointly constitute Clade I. Strain CCMP2754 from North America and strain MEL71 from the ECS together constituted Clade II. Within the second group, Clade III contained four strains, RCC678, RCC736, RCC1736 and Pg-G(A) isolated from the North Sea, Atlantic Ocean, among which RCC678 displayed little genetic difference from the other three strains. Strains MEL42, MEL70 and TIO393 from the SCS jointly constituted Clade IV.

3.3. Diversity of Phaeocystis globosa from the South China Sea

A total of 1179 sequences of *rbcS-rpl27* intergenic spacer were obtained from 16 colony samples of *P. globosa* and 18 phytoplankton samples without any *P. globosa* colony (Table S2). The genetic diversity of *P. globosa* in field samples was much higher than that in cultured *P. globosa* strains, and an additional clade (Clade V) was found in field samples. Additionally, much higher genetic variation was revealed within Clade I (Fig. 5). In addition to the 3 subclades established based on 11 *P. globosa* strains, four additional subclades (Clades Id, e, f, and g) were added based on sequences derived from field samples. In these, a total of 280 sequences were clustered in Clade I, including 40 sequences in Clade Ia, 67 sequences in Clade Ib, 12 sequences in Clade Id, 8 sequences in Clade Ie, 12 sequences in Clade If, and 141 sequences in Clade Ig. Clade II contained 22 sequences derived from field samples, but no sequences were assigned to Clade III. Most of the field-derived sequences were clustered in two subclades of Clade IV. Clades IVa and IVb contained 796 and 17 sequences, respectively. Existence of the additional Clade V was supported by 64 sequences from field samples.

Genetic distances within and between different clade(s) were estimated based on the genetic divergence of the sequences derived from field samples (Table 3). The maximum intraclade genetic distances in Clades I, II and V were exceeded 0.026 and were much higher than the values of 0.0025 and 0.0087 in Clades III and IV, respectively. Clade III contained only four strains from the North Sea, which may lead to the lowest genetic distance within this clade. Clade IV also had low intraclade genetic variation, unlike Clades 1, II and V that co-occur in the SCS.

Among the field-derived *rbcS-rpl27* intergenic spacer sequences (Table 4), 559 sequences were cloned from 18 phytoplankton samples collected from the SCS. Within these samples, 252 sequences (45%) belonged to Clade I, 22 sequences (3.9%) belonged to Clade II, 221 sequences (40%) belonged to Clade IV and 64 sequences (11%) were ascribed to Clade V. The remaining 620 sequences were cloned from 16 *P. globosa* colony samples. A large percentage (95%, 592 sequences) of

Table 2
Maximum colony size and pigment ratios of 19 strains of *Phaeocystis globosa* in this study (see text for pigment abbreviations).

Algal strain	Diameter of top 10 large colonies (mm)	Hex-fuco/ But-fuco	Chl c_3/ Chl *a*	Me-chlide *a*/Chl *a*	But-fuco/ Chl *a*	Fuco/ Chl *a*	4k-Hex-fuco/Chl *a*	Hex-fuco/ Chl *a*	Diad/ Chl *a*	Diat/ Chl *a*	*ββ*-Car/ Chl *a*
MEL43	1.44−2.40	3.038	0.050	0.025	0.016	0.756	0.053	0.049	0.081	0.003	0.014
MEL44	1.40−1.96	4.875	0.120	0.063	0.025	0.875	0.169	0.122	0.073	0.008	0.022
MEL45	0.84−0.98	1.125	0.000	0.053	0.023	0.834	0.042	0.026	0.046	0.003	0.018
MEL68	1.04−1.66	2.744	0.041	0.030	0.041	0.988	0.079	0.112	0.072	0.000	0.009
MEL69	0.68−0.82	3.975	0.003	0.039	0.016	0.826	0.066	0.064	0.046	0.003	0.042
MEL47	2.40−3.00	4.423	0.081	0.015	0.023	0.719	0.102	0.101	0.054	0.012	0.000
MEL71	0.35−1.02	4.920	0.000	0.000	0.034	1.200	0.037	0.165	0.149	0.027	0.000
MEL78	0.42−0.68	0.394	0.011	0.000	0.095	1.818	0.065	0.037	0.091	0.012	0.033
CCMP628	1.10−2.80	5.734	0.064	0.019	0.027	0.736	0.151	0.155	0.052	0.009	0.018
CCMP629	1.20−1.66	7.626	0.054	0.000	0.022	0.575	0.087	0.165	0.034	0.003	0.018
CCMP1524	/	8.720	0.053	0.000	0.041	0.514	0.099	0.356	0.032	0.000	0.000
CCMP2754	/	3.864	0.000	0.000	0.085	1.118	0.235	0.328	0.201	0.014	0.022
RCC2055	0.12−0.20	11.775	0.025	0.013	0.015	0.514	0.057	0.171	0.044	0.000	0.019
RCC678	/	0.285	0.000	0.003	0.016	0.315	0.003	0.005	0.026	0.012	0.218
RCC736	/	1.019	0.001	0.000	0.036	1.295	0.024	0.036	0.063	0.009	0.035
RCC1736	/	2.389	0.005	0.000	0.081	0.934	0.149	0.193	0.135	0.044	0.018
MEL42	10.0−13.0	0.000	0.005	0.000	0.105	1.316	0.000	0.000	0.257	0.019	0.127
MEL70	4.50−11.0	0.000	0.023	0.067	0.102	1.195	0.000	0.000	0.102	0.019	0.000
TIO393	10.5−14.0	0.000	0.020	0.030	0.278	1.674	0.000	0.000	0.239	0.049	0.053

/ *P. globosa* strains occasionally form colonies in the laboratory.

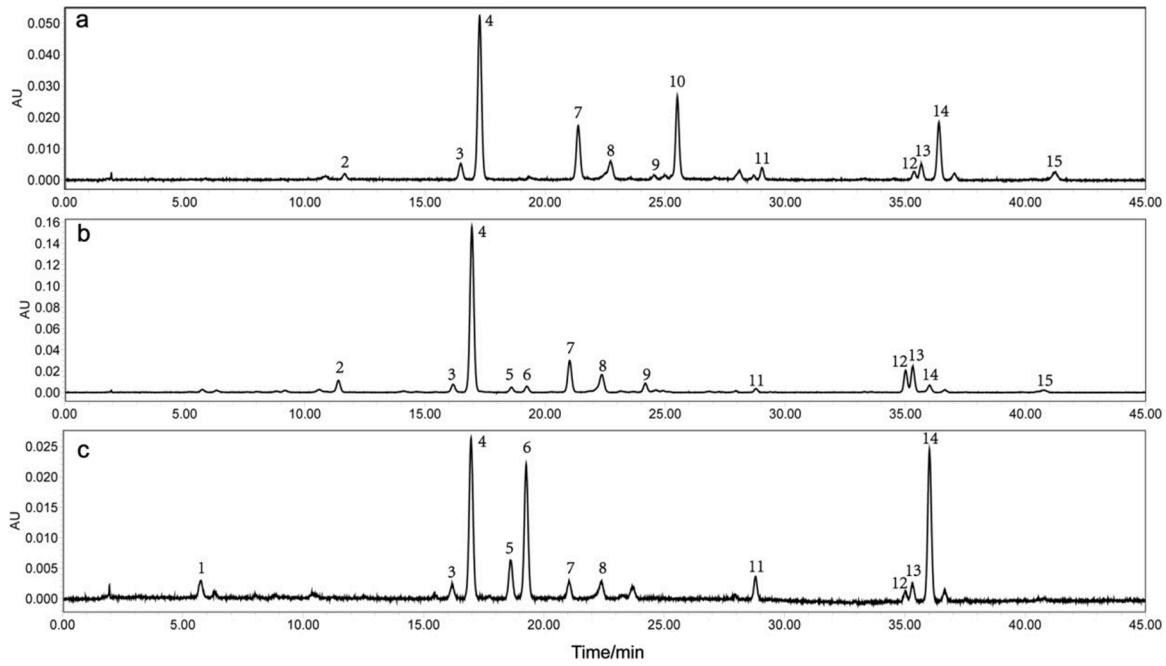

Fig. 3. Chromatograms of three representative *Phaeocystis globosa* strains (a. MEL42, b. RCC736, and c. CCMP1524); pigment composition indicated by number: 1. Chl c_3; 2. chlide *a*; 3. but-fuco; 4. fuco; 5. 4k-hex-fuco; 6. hex-fuco; 7. diad; 8. unknown carotenoid; 9. diat; 10. unknown carotenoid; 11. IS; 12, 13. Chl *a* derivatives; 14. Chl *a*; and 15. *ββ*-car

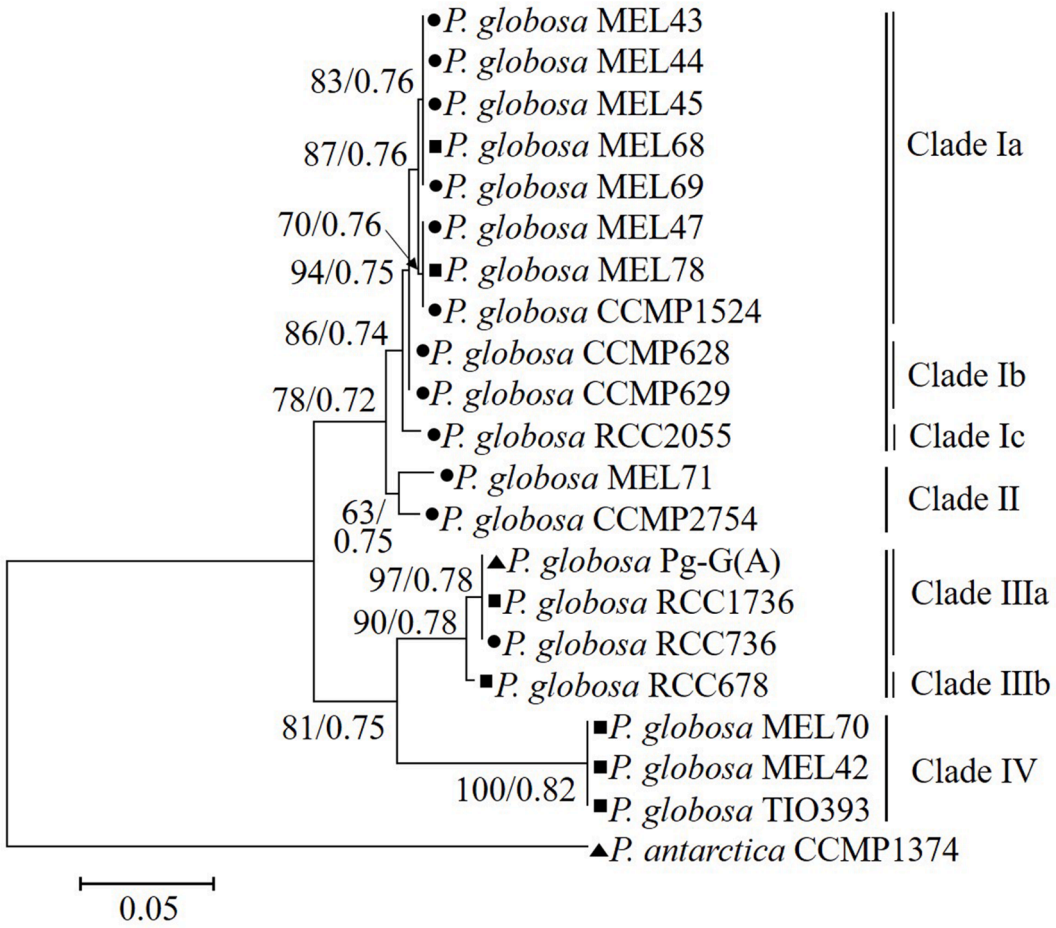

Fig. 4. Phylogenetic tree established for 20 strains of *Phaeocystis globosa* based on the *rbc*S-*rpl27* intergenic spacer. Numbers indicate bootstrap values from maximum likelihood (left) and Bayesian (right) analyses. The sequences with squares were obtained by this study, and those with circles were referred from Zhang et al., (2020a), and those with triangles were downloaded from the GenBank.

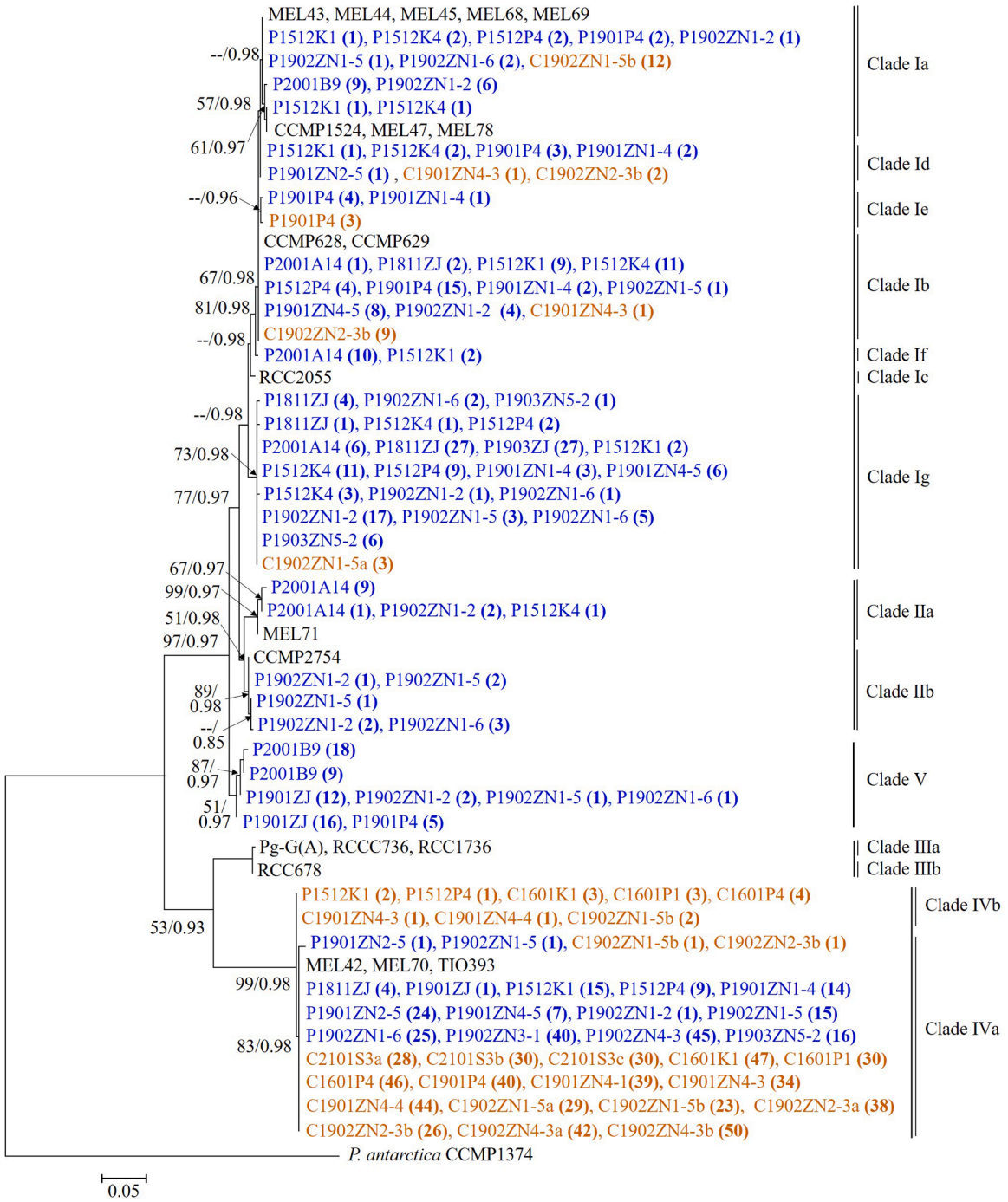

Fig. 5. Phylogenetic tree based on *rbcS-rpl27* intergenic spacers of 20 cultured *Phaeocystis globosa* strains and 1179 sequences derived from 16 *P. globosa* colony samples and 18 phytoplankton samples collected from the South China Sea. Numbers indicate bootstrap values obtained from maximum likelihood (left) and Bayesian (right) analyses. The former <50 was omitted. Sample names with black, blue and brown fonts mean the cultured strains of *P. globosa*, phytoplankton samples and colony samples, respectively. Sequence numbers of samples belonging to the clade/subclade are indicated by the boldface number in parentheses following the sample name.

Table 3
Genetic distances within and between different *Phaeocystis globosa* clades.

	Clade I	Clade II	Clade III	Clade IV	Clade V
Clade I	0–0.0263*				
Clade II	0.0190–0.0514	0–0.0270*			
Clade III	0.1265–0.1438	0.1341–0.1537	0–0.0025*		
Clade IV	0.1636–0.1859	0.1730–0.1992	0.1428–0.1517	0–0.0087*	
Clade V	0.0276–0.0451	0.0308–0.0613	0.1144–0.1417	0.1444–0.1680	0–0.0271*

* Minimum and maximum values of intra-clade genetic distances

Table 4
Number (N) and percentage of sequences in field samples assigned to different clades.

Clade	I		II		III		IV		V	
	N	%	N	%	N	%	N	%	N	%
Phytoplankton samples	252	45	22	4	0	0	221	40	64	11
Colony samples	28	5	0	0	0	0	592	95	0	0

the sequences belonged to Clade IV, while a small percentage (5%, 28 sequences) belonged to Clade I. These 28 sequences within the Clade I were only detected in four colony samples (C1901ZN4-3, C1902ZN1-5a, C1902ZN1-5b and C1902ZN2-3b).

4. Discussion

4.1. Diversity of morphological, physiological and genetic features of *Phaeocystis globosa*

Haptophyte *Phaeocystis* spp. have been extensively studied because some of them lead to intense HABs in many areas worldwide, including the coastal areas of the north Atlantic, the North Sea, the Norwegian Sea, the Dutch coastal waters, the Arabian Sea, and the East and Southeast Asian including China, Vietnam, Thailand (Lancelot et al., 1987; Cadée, 1996; Qi et al., 2004; Schoemann et al., 2005; Doan et al., 2010; Rousseau et al., 2013; Smith et al., 2014). Unlike *Phaeocystis antarctica* and *Phaeocystis pouchetii* that prevail in Antarctic and Arctic waters, respectively, *P. globosa* is primarily distributed in temperate and tropical oceans as a result of divergence from cold-water species 30-60 million years ago (Schoemann et al., 2005; Medlin and Zingone, 2007). It has been proposed that diverse geographic patterns in temperate and tropical oceans have been driving the evolution and diversification of morphological and physiological characteristics within the *P. globosa* taxon (Vaulot et al., 1994; Lange et al., 2002;Chen et al., 2003).

P. globosa has a complex haploid-diploid life cycle involving free-living solitary cells and colonial cells (Cariou et al., 1994; Rousseau et al., 1994, 2007, 2013; Vaulot et al., 1994; Peperzak et al., 2000). The colony size of *P. globosa* differs significantly between the two regions with high frequency of *P. globosa* blooms, the temperate North Atlantic, and tropical and subtropical Southeast Asian waters. The colony size of *P. globosa* usually ranges from 10 μm to 3 mm in the North Atlantic; whereas *P. globosa* usually forms giant colonies (>1 cm in diameter) in East and Southeast Asia (Rousseau et al., 1990; Madhupratap et al., 2000; Qi et al., 2004; Lancelot et al., 2009; Doan et al., 2010; Smith et al., 2014). In the present study, five *P. globosa* strains could not generate colonies or occasionally formed only small colonies in our laboratory. Originally, these strains also took the form of solitary cells at the National Center for Marine Algae and Microbiota (NCMA, formerly Culture Collection of Marine Phytoplankton (CCMP), Bigelow, Laboratory for Ocean Sciences, Maine, USA, and the Roscoff Culture Collection (RCC), France. Most likely, they did not grow under optimum growth conditions or a lack of zooplankton grazing pressure or maintaining of haploid lifestyle resulted in the colonies gradually becoming smaller and even ceasing to form colonies under culture conditions (Jakobsen and Tang, 2002; Tang, 2003). The remaining 14 colony-forming strains were readily divided into two groups based on their colony diameter. The three strains isolated from the SCS (MEL42, MEL70 and TIO393) could form giant colonies >1.0 cm in diameter, and the other 11 strains produced small colonies <3.0 mm in maximum diameter. Among these, three giant-colony forming strains (MEL42, MEL70 and TIO393) and five small-colony forming strains (MEL43, MEL44, MEL45, MEL68 and MEL69) coexist in the SCS. Many prior studies have demonstrated that the formation and enlargement of *P. globosa* colonies are triggered by various environmental factors, including light, temperature, nutrients, and grazing by heterotrophic dinoflagellates and zooplankton (Jakobsen and Tang, 2002; Tang, 2003; Sazhin et al., 2007; Wang et al., 2010a; Wang et al., 2011). The effects of such factors, however, could not reasonably account for the fact that *P. globosa* strains with such different colony size coexist in the SCS.

P. globosa isolates from different geographic regions have different pigment profiles (Vaulot et al., 1994; Zapata et al., 2004), which is supported by results of the present study (Table 2). Both hex-fuco and but-fuco, the two diagnostic pigments for this species, have been detected in some geographical strains, but other geographical strains contain only one or neither of the two marker pigments (Buma et al., 1991; Jeffrey and Wright, 1994; Vaulot et al., 1994; Breton et al., 2000; Antajan et al., 2004; Zapata et al., 2004; Seoane et al., 2009; Wang et al., 2019). In the present study, both hex-fuco and but-fuco were detected in 16 strains of *P. globosa*, excluding the three SCS strains (MEL42, MEL70 and TIO393) containing only but-fuco. In general, environmental factors and life cycle stage can influence both pigment content and composition, but rarely influence the pigment content composition (Buma et al., 1991; van Leeuwe and Stefels, 1998; Schluter et al., 2000; DiTullio et al., 2007; van Leeuwe et al., 2014). A previous study found that colonial and solitary cells of four *P. globosa* strains (MEL42, MEL44, MEL45 and CCMP628) shared the same pigment composition, but differed in pigment content (Wang et al., 2019). Our subsequent experiments further confirmed that nutrients, light, and temperature do not alter the pigment composition in colonial and solitary cells (unpublished data). In addition to the differences in colony sizes, *P. globosa* strains with different pigment profiles coexist in the same geographic zone. Thus, these differences are difficult to attribute to the role of environmental factors and life cycle stage.

Genetic diversity within the *P. globosa* has been previously demonstrated (Medlin et al., 1994; Chen et al., 2003; Lange et al., 2002; Medlin and Zingone, 2007; Qu et al., 2008; Liu et al., 2010; Decelle et al., 2012; Hu et al., 2019). However, the lack of high-resolution molecular markers has hindered the effective resolution of genetic divergence among different geographic strains. Common ribosomal molecular markers, including 18S rDNA, 28S rDNA and ITS, have not been successful in distinguishing different *P. globosa* strains (Chen et al., 2003; Lange et al., 2002; Hu et al., 2019; Zhang et al., 2020a). Other molecular markers such as *rbc*L and *psb*A in the chloroplast coding region have been too conserved to be used as molecular markers to examine the genetic diversity within *P. globosa* (Yang et al., 2004; Decelle et al., 2012). Recently, two high-resolution chloroplast markers, *pgcp*1 and *rbc*S-*rpl*27, were proposed to characterize the genetic diversity of *P. globosa* (Song et al., 2020; Zhang et al., 2020a). Among the available options, the primer pair designed for the *rbc*S-*rpl*27 intergenic spacer is specific to the genus *Phaeocystis* (Zhang et al., 2020a). Thus, this primer pair can amplify the *rbc*S-*rpl*27 intergenic spacers of *P. globosa* from the phytoplankton community containing solitary and colonial *P. globosa* cells.

For this study, we collected 19 strains of *P. globosa* from coastal waters of East and Southeast Asia, Europe, and America, and 16 *P. globosa* colony samples and 18 phytoplankton samples from the SCS. Their intraspecific phylogenetic relationship and genetic diversity have been determined based on more than 1000 *rbc*S-*rpl*27 intergenic spacer sequences. Cultured *P. globosa* strains exhibited remarkable intraspecies diversity, and *P. globosa* in field-collected phytoplankton samples had an even higher genetic diversity and a more complex phylogenetic relationship. Reflecting high intra-clade genetic variation, Clades I and II contained 302 environmental sequences and 14 cultured strains, suggesting that these two clades are cosmopolitan in coastal waters of the Pacific and Atlantic Oceans. In contrast, Clade III contained only four North Sea strains, suggesting that it is more likely a local clade confined

to the North Sea, in agreement with results of (Song et al., 2020). As the largest clade, Clade IV contained three strains (MEL42, MEL70 and TIO393) and 813 environmental sequences from the SCS; however, it exhibited very low intra-clade genetic variation, indicating that Clade IV is not globally distributed, although further supporting evidence is required from analysis of additional *P. globosa* strains and field samples. To date, no culture in Clade V has been established.

This study revealed remarkable differences in genetic diversity between colonies and solitary cells of *P. globosa* in the SCS. The *rbc*S-*rpl*27 sequences in 18 phytoplankton samples collected from 2015 to 2020 were grouped into Clades I, II, IV and V, indicating that solitary *P. globosa* cells have much higher genetic divergence in the SCS than colonies. Sequences within Clades I and IV accounted for 45% and 40%, respectively, of the total sequences derived from phytoplankton samples. These findings indicate that the two clades are dominant in the SCS, which may explain the successful isolation of Clade I and IV strains from the SCS. Colony samples only contained sequences from these two clades. Among them, Clade IV accounted for 95% of total sequences; a small number of Clade I sequences were only found in four samples (C1901ZN4-3, C1902ZN1-5a, C1902ZN1-5b and C1902ZN2-3b). Samples collected in 2016 were carefully and repeatedly washed with sterile seawater in the laboratory to remove cells attached to the surface of the colonies. Other colony samples collected in 2019 and 2021, however, were not similarly treated due to difficulties in sample preparation *in situ*. Thus, a low proportion of sequences in Clade I detected in the four colony samples could be a result of contamination by solitary Clade I cells. Therefore, it can be readily inferred that giant colonies were mainly formed by *P. globosa* Clade IV in the SCS. In this study, the number of cultured strains from different global regions was still limited, and solitary and colony samples of *P. globosa* were only collected from the SCS. Therefore, inclusion of additional strains and field samples may ultimately reveal an even higher genetic diversity in *P. globosa*.

Based on the relationship between genetic diversity, colony size and pigment profile in this study, it was found that all *P. globosa* strains in Clades I, II and III form small colonies, and both hex-fuco and but-fuco serve as their diagnostic pigments. Although cultures of three strains (RCC678, RCC736 and RCC1736) isolated from the North Sea did not form colonies in this study, it can be concluded that North Sea strains of *P. globosa* also form small colonies based on prior field studies in this region (Rousseau et al., 1990; Smith et al., 2014). Three strains (MEL42, MEL70 and TIO393) isolated from the SCS, which were grouped in Clade IV, could form cm-sized giant colonies and possessed only but-fuco as the diagnostic pigment. Phylogenetic analysis of 16 giant colony samples collected from the SCS over the past six years (2016-2021) were also dominated by Clade IV cells. Only but-fuco was detected in colony samples from the Beibu Gulf in 2016 and 2019 and from Dapeng Bay in 2021 (Fig. S2). Thus, existing evidence supports the finding that *P. globosa* cells capable of forming giant colonies belong to a distinct genetic clade with but-fuco as their marker pigment in the SCS.

4.2. Intense blooms in the South China Sea are caused by the unique "giant-colony" ecotype of Phaeocystis globosa

In the SCS, an unusual characteristic of *P. globosa* blooms is the formation of giant colonies (>1 cm in diameter) (Madhupratap et al., 2000; Qi et al., 2004; Doan et al., 2010; Liu et al., 2015), which have attracted a great deal of interest (Smith et al., 2014; Shen et al., 2018). Various explanations for their formation, including the effects of nutrients, temperature, and zooplankton grazing, have been proposed (Jakobsen and Tang, 2002; Tang, 2003; Sazhin et al., 2007; Wang et al., 2010a; Wang et al., 2011). The present study demonstrated that intense blooms of *P. globosa* in the SCS are caused by Clade IV of *P. globosa*, which is distinct from other coexisting genetic clades.

Colony formation triggered by various environmental factors is an important growth and survival strategy of *P. globosa* (Verity and Smetacek, 1996; Jakobsen and Tang, 2002; Wang et al., 2010b, 2011). However, enlargement of colony size is also associated with substantial adverse impacts on the growth and survival of *P. globosa*. In general, phytoplankton sinking rates are 1 m d^{-1} or less (Kiorboe et al., 1996); however, an average sinking rate of 189 m d^{-1}, ranging from 29.3 to 516 m d^{-1} has been measured for giant colonies (≈1 cm) of *P. globosa* in Vietnamese coastal waters (Smith et al., 2014). These extremely high sinking rates will remove giant colonies from the euphotic zone within several hours, indicating that giant colony blooms of *P. globosa* can only be confined to shallow waters with strong mixing to sustain a high *P. globosa* biomass in the euphotic zone (Smith et al., 2014; Liu et al., 2015). Additionally, giant colonies may decrease the nutrient uptake capability of cells due to the presence of a diffusive boundary layer formed by the colonial envelope (Ploug et al., 1999), because the ion diffusion coefficients and low molecular-weight proteins are orders of magnitude lower. Smith et al. (2014) concluded that the colony envelope restricts the flux of nutrients to the intracolonial fluid. These limiting effects of the colonial envelope on nutrient uptake by *Phaeocystis* colony cells are reflected by the higher half-saturation constants of nutrient uptake for colonial than solitary cells (Ploug et al., 1999), and by the significantly slower growth of colony cells compared to free-living solitary cells (Veldhuis and Admiraal, 1987). Additionally, the carbon contribution of mucus associated with colonies to total carbon ranges from 63 to 95%, suggesting that a large amount of photosynthetic carbon is required to produce the mucoid sheath (Smith et al., 2014; Liu et al., 2015; Zhang et al., 2020b). To reduce the adverse effects of forming large colonies, it has been proposed that *P. globosa* giant colonies should be confined to nearshore, turbulent and nutrient-rich waters, as evidenced by the occurrence of giant colonies in East and Southeast Asian coastal waters (Chen et al., 1999; Smith et al., 2014; Yuan et al., 2019).

Selection pressure operating on phytoplankton presumably forces a balance between growth rate, and protection against predators and pathogens (Hamm, 2000). Colonial cells of *Phaeocystis* exhibit a lower growth rate than free-living solitary cells under nutrient-deficient conditions, and even under eutrophic conditions (Veldhuis and Admiraal, 1987; Schoemann et al., 2005), which is in agreement with results of the present study. Maximum growth rates (MGRs) of giant-colony forming strains (MEL42 and MEL70) do not exceed 0.3 d^{-1}, are < 0.31−0.36 d^{-1} in *P. globosa* cultures (MEL44 and MEL71) that form small colonies, and 0.50-0.63 d^{-1} in *P. globosa* cultures (CCMP2754 and RCC736) that do not form colonies in nutrient-sufficient laboratory cultures. Living in nutrient-rich nearshore environments, *P. globosa* cells must face high grazing pressure and are susceptible to infection by bacteria and viruses. The unique biological structure of the *Phaeocystis* colony has been considered an energetically inexpensive mechanical defense strategy to reduce the susceptibility to grazers due to size mismatch and make the cells in the colony inaccessible to many bacteria and viruses due to the small pore size of colony envelope measuring <4.4 nm (Hamm et al., 1999; Hamm, 2000; Tang, 2003; Tang et al., 2008; Wang et al., 2015). The reduction in grazing pressure and pathogenic infection may compensate for the reduction in the nutrient supply during colony formation (Ploug et al., 1999). Additionally, our studies found that cells of the giant-colony forming strain MEL42 (Clade IV) produce more hemolytic toxins than strain MEL44 in Clade I. Moreover, cells of strain MEL42 exert more adverse impacts on the survival of the zooplankters *Brachionus frutensis* and *Artemia* sp. than strain MEL44 (Clade I) and strain MEL71 (Clade II) (unpublished data). This may further allow Clade IV *P. globosa* a defense against predation. These results also supported a previous finding that giant-colony forming *P. globosa* in the SCS had high ichthyotoxic and hemolytic properties (Shen et al., 2004; Peng et al., 2005). Thus, giant colony-forming *P. globosa* have evolved complex survival strategies to adapt to the nearshore environment, which may account for the frequent occurrence of *P. globosa* blooms in the SCS.

Another unique feature of bloom-forming *P. globosa* in the SCS is the production of but-fuco, as its diagnostic pigment. Fucoxanthin and its two derivatives, hex-fuco and but-fuco, are important light-harvesting

pigments (Papagiannakis et al., 2005), and but-fuco has an additional photoprotective function (Stolte et al., 2000). Furthermore, high concentrations of light-protecting pigments, such as diadinoxanthin and diatoxanthin, are detected in the cells of giant-colony forming strains. The relatively high content of light-protecting pigment present in giant-colony forming *P. globosa*, which often float in shallow coastal waters, may thus protect them from intense light in the shallow coastal water.

An ecotype is defined as a population of a species that survives as a distinct group through environmental selection and isolation and that is comparable to a taxonomic subspecies (Turesson, 1922; Michael et al., 2005). The present study demonstrated that giant-colony forming *P. globosa* belong to the Clade IV, which is distinct from other genetic clades and exhibits lower intra-clade variation. The genetically independent Clade IV associated with other unique morphological and physiological features, such as the formation of giant colonies containing more light-protecting pigments, adverse effects on zooplankton survival, and strong hemolytic properties, support the classification of Clade IV *P. globosa* as a unique ecotype. The intense blooms of giant colony forming-*P. globosa* in the SCS, indeed, are mainly caused by this unique giant-colony ecotype of *P. globosa*.

5. Conclusions

In this study, colony sizes and the composition of marker pigments from 19 strains of *P. globosa* isolated from Pacific and Atlantic coastal waters were determined. Their genetic divergence was analyzed based on a new chloroplast molecular marker. Results showed that morphological and physiological features and genetic profiles were diverse among different geographic *P. globosa* strains, and a unique giant-colony ecotype of *P. globosa* was distributed in the SCS, that generated blooms of giant colonies and a specific pigment profile. Analysis of the genetic diversity of 16 colony forming samples of *P. globosa* and 18 phytoplankton samples indicated that solitary *P. globosa* cells were characterized by high genetic diversity and were divided into five genetic clades. However, colony cells belonged to a distinct genetic clade of giant-colony *P. globosa* distributed in the SCS. Thus, the intense blooms of *P. globosa* in the SCS are caused by a unique "giant-colony" ecotype possessing the unique diagnostic pigment but-fuco.

Supporting information

Additional supporting information may be found online in the Supporting Information section.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that cdocould have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hal.2022.102227.

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