



Development of Specific DNA Barcodes for the Dinophyceae Family Kareniaceae and Their Application in the South China Sea

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Species from the family Kareniaceae (Dinophyceae) frequently cause harmful algal blooms (HABs), with serious ecological impacts and risks to human safety and aquaculture activities in coastal waters worldwide. However, due to their small size, lack of morphological divergence, and low abundance during non-bloom periods, the diversity within this family is not well understood. By comparing the commonly used molecular markers, the Internal Transcribed Spacer (ITS) region was found to have an appropriate mutation rate to distinguish three of the most common genera (*Karenia*, *Karlodinium*, and *Takayama*) within the Kareniaceae family and different geographical strains of Kareniaceae. Specific primers targeting the ITS region of *Karenia*, and the other primers specific to the genera *Karlodinium* and *Takayama*, were designed. Specificity of the primers was tested using 17 strains of Kareniaceae species and 15 non-target species. Representative Kareniaceae species could be successfully detected even at low concentrations of target DNA template with a limit of detection of 3.2 pg. The primers were also assessed using high-throughput sequencing with two environmental samples from the South China Sea (SCS). Analysis of the reads identified as Kareniaceae species revealed a high diversity and the existence of unreported Kareniaceae species in the SCS. In conclusion, the newly developed molecular barcodes specifically detected Kareniaceae species in the field and will provide technical support for the effective warning and monitoring of Kareniaceae HABs.

Keywords: *Karenia*, *Karlodinium*, DNA barcode, species diversity, *Takayama*, ITS region

INTRODUCTION

The family Kareniaceae (Gymnodiniales, Dinophyceae) was described in 2005, with the following characteristic features: a straight or sigmoid apical groove on the cell epicone and fucoxanthin and its derivatives as diagnostic pigments (Bergholtz et al., 2005). It is widely accepted that the family comprises three genera *Karenia*, *Karlodinium*, and *Takayama*. Recently, three genera, namely *Asterodinium*, *Gertia*, and *Shimiella* were added to Kareniaceae (Benico et al., 2019; Takahashi et al., 2019; Ok et al., 2021), but these additions are controversial due to morphological differences and the absence of fucoxanthin or the derivatives.

Species of *Karenia*, *Karlodinium*, and *Takayama* often cause harmful algal blooms (HABs), increasing in frequency, intensity, and distribution worldwide (Yang et al., 2001; Brand et al., 2012; Place et al., 2012; Li et al., 2019; Yang et al., 2021). For example, Kareniaceae HABs have occurred for more than 20 years in China since their first detection in the Pearl River Estuary in 1998 (Cui et al., 2009). More than 90 cases of *Karenia mikimotoi* blooms have been reported along China's coastline (Lin et al., 2010; Liang, 2012; Lu et al., 2014; Liu et al., 2015; Lv et al., 2019). Kareniaceae species often form intense HABs with cell densities more than 10^6 cells L^{-1} , and when the bloom collapses, the decomposition of the dense cell biomass causes hypoxia and threatens the health of marine life. Additionally, Kareniaceae species produce toxins and harmful substances that affect marine zooplankton, shellfish, fish, and humans. For example, *K. mikimotoi* produces cytotoxins, hemolytic toxins, glycolipid toxins, various polyunsaturated fatty acid toxins (Yasumoto et al., 1990; Arzul et al., 1995; Gentien et al., 2007), as well as hemolytic toxins and cytotoxins associated with massive mortalities of fish, shellfish, and invertebrates (Gentien, 1998; Mitchell and Rodger, 2007; Brand et al., 2012; Kim, 2010). *Karenia brevis*, another well-known HAB species, produces the neurotoxic brevetoxins that cause respiratory distress in humans (Xu et al., 2010; Errera and Campbell, 2011; Poli et al., 2000; Naar et al., 2007; Fleming et al., 2011). *Karlodinium veneficum* produces karlotoxins which has been associated with fish mortality (Deeds et al., 2002; Xu et al., 2012; Xu et al., 2014; Dai et al., 2014). The development of a hypoxic environment and the production of toxins mean these species can have detrimental impacts on both coastal ecosystems and marine aquaculture development. Economic losses caused by *K. mikimotoi* blooms were approximately US\$370 million in China from 2005 to 2017 (China Marine Disaster Bulletin 2005-2017, SOAC). In 2012, a *K. mikimotoi* bloom decimated farmed abalone and caused financial losses exceeding US\$330 million in the coastal waters of Fujian Province (Li et al., 2017).

When the family Kareniaceae was first described, 21 species were recorded (Bergholtz et al., 2005; Wang et al., 2018). To date, 37 species have been described (11 *Karenia* spp., 15 *Karlodinium* spp., seven *Takayama* spp., two *Asterodinium* spp., one *Gertia* sp. and one *Shimiella* sp.) and updated in the AlgaeBase database (<https://www.algaebase.org/>) (Guiry and Guiry, 2022; De Salas et al., 2008; Li and Shin, 2018; Luo et al., 2018; Benico et al., 2019;

Takahashi et al., 2019; Cen et al., 2020; Ok et al., 2021). Among them, nine species of *Karenia*, six species of *Karlodinium* and one species of *Takayama* are considered as HAB species based on the HAB taxon list (Lundholm et al., 2009), as detailed in **Table S1**. There is increasing evidence that multiple Kareniaceae species co-occur or co-dominate in a bloom event. For example, in 2021 a massive HAB in the Pacific coast of eastern Hokkaido, Japan was dominated by *K. selliformis*, with other Kareniaceae dinoflagellates (*K. longicanalis*, *K. mikimotoi*, *Karlodinium* sp., *Takayama* cf. *acrotrocha*, *T. tuberculata* and *Takayama* sp.) also present (Iwataki et al., 2022). A *Karenia* bloom in Hong Kong waters in 2016 was dominated by the species *K. mikimotoi* and *K. papilionacea*, with *K. selliformis* and *K. longicanalis* also present in low abundance (Lin et al., 2020). Five species of *Karenia* (*K. brevis*, *K. mikimotoi*, *K. papilionacea*, and *K. selliformis* and one unknown *Karenia* species) were isolated and identified in a *Karenia* bloom along the west coast of Florida from 2007 to 2008 (Wolny et al., 2015). Due to the production of diverse toxins and impacts from multiple species, accurate species identification from environmental samples are crucial for early warning systems and the management of Kareniaceae blooms in coastal waters.

Morphological features are traditionally used for the identification of bloom-forming species (Richlen et al., 2008; Smayda, 2010). However, many Kareniaceae species are morphologically similar, and distinguishing them using microscopy is virtually impossible. Additionally, Kareniaceae species are unarmored and fragile, meaning they are easily destroyed during sampling and preservation processes. It is increasingly urgent and necessary to develop a reliable, sensitive method for the simultaneous detection of multiple species of Kareniaceae in the field.

DNA sequence data can provide useful auxiliary information for the classification of microalgae. Various DNA markers (e.g., the 18S ribosomal DNA (rDNA), 28S rDNA, and Internal Transcribed Spacer (ITS) regions) have been used for the detection and identification of HAB species, including Kareniaceae species. For example, using DNA sequence data, *K. digitata* was revised to *Karl. digitatum*, and recently two new species, *Karl. zhouanum* and *Karl. elegans*, have been described based on morphological characteristics, pigment composition, and 28S rDNA sequences (Luo et al., 2018; Cen et al., 2020). The ITS region has been proven as a good DNA barcode region for dinoflagellate species (Litaker et al., 2007; Stern et al., 2012); however, it cannot resolve species from some genera such as *Alexandrium*, *Symbiodinium*, *Gymnodinium*, *Heterocapsa*, *Prorocentrum* and *Karenia* (Litaker et al., 2007; Stern et al., 2012). In addition, species-specific quantitative assays, such as quantitative PCR (qPCR) and loop-mediated isothermal amplification-lateral flow dipstick (LAMP-LFD), that target the ITS region of Kareniaceae species have been developed (Yuan et al., 2012; Huang et al., 2020). However, no specific DNA marker has been developed for the whole Kareniaceae family, which has hindered the investigation of species diversity in the natural environments. Studies have shown that compared with 18S rDNA and 28S rDNA, the ITS and ribulose-bisphosphate carboxylase (*rbcl*) regions of the family Kareniaceae are more genetically variable,

and they have been extensively used as targets for interspecific identification (Garcés et al., 2006; Al-Kandari et al., 2011; Toldrà et al., 2018). However, further studies investigating the ITS region of Kareniaceae is necessary to develop effective molecular markers for the detection of diversity within the family.

In this study, the variation rates of the 18S rDNA V1-V9 region, 28S rDNA D1-D2 region, and ITS region of Kareniaceae species were compared. The ITS region, with a relatively high variation, was selected as the target region for the design of molecular markers. Two pairs of specific primers were designed based on the ITS region of Kareniaceae species: one pair for the genus *Karenia*, and the other for the genera *Karlodinium* and *Takayama*. Both pairs of primers were highly sensitive with a low limit of detection. Specificity of the primers was tested using 17 strains of Kareniaceae species and 15 non-target species. The developed molecular markers were then applied using high-throughput sequencing metabarcoding to determine the diversity of Kareniaceae in the South China Sea (SCS).

MATERIALS AND METHODS

Microalgal Culture

Thirty-three strains of microalgae (Table 1) were used in this study: 18 strains of *Karenia*, *Karlodinium*, and *Takayama*, and

15 other non-target strains. All the strains were cultured in L1 medium and maintained at 20 °C under a photon flux density of 75 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and a 14:10 h light-dark cycle. Approximately 50 mL of culture was collected at exponential stage from each microalgal strain and centrifuged at 5000 $\times g$ for 10 min. Cell pellets were stored at -80 °C until DNA extraction.

Sample Collection

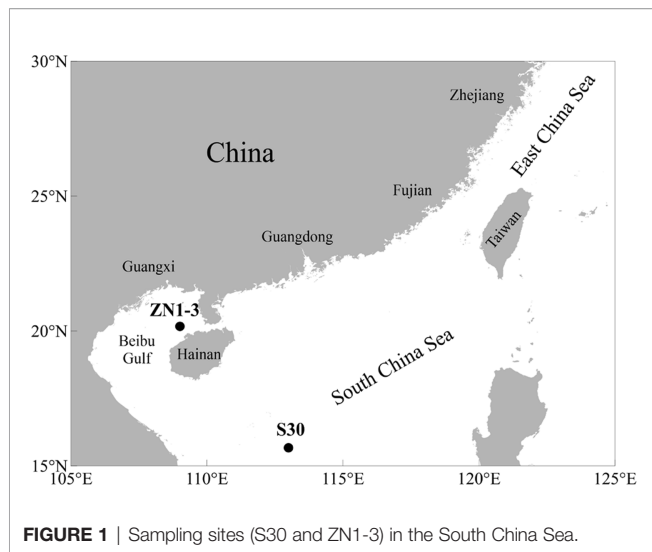
Two phytoplankton samples were collected from the sampling sites (ZN1-3 and S30) in the South China Sea (SCS) in February 2019 and June 2020, respectively (Figure 1). Approximately 1 L of surface phytoplankton sample was filtered using sieves (mesh size 200 μm) to remove zooplankton and then filtered using an HTPP membrane (pore diameter 0.40 μm , Millipore, USA) under a vacuum of 40 kPa. The filtered membranes were stored in a freezer at -80 °C until DNA extraction.

DNA Extraction, PCR Amplification, and Sequencing

Total genomic DNA of microalgal cultures and environmental samples was extracted using the CTAB method (Winnepeninckx et al., 1993) with the following modifications: incubation with CTAB buffer at 65 °C for 1 h; centrifugation at 4 °C and 12,500 g; and isopropyl alcohol to precipitate the DNA. The extracted

TABLE 1 | Microalgal strains used in this study.

Species	Strain	Class	Geographic origin
<i>Karenia mikimotoi</i>	KM201	Dinophyceae	Fujian coastal waters, East China Sea (ECS)
<i>K. mikimotoi</i>	KM2019	Dinophyceae	Fujian coastal waters, ECS
<i>K. selliformis</i>	CAWD79	Dinophyceae	Coastal waters of New Zealand (NZ)
<i>K. brevisulcata</i>	CAWD82	Dinophyceae	NZ coastal waters
<i>K. papilionacea</i>	CAWD91	Dinophyceae	NZ coastal waters
<i>K. bicuneiformis</i>	CAWD92	Dinophyceae	NZ coastal waters
<i>K. brevis</i>	CAWD122	Dinophyceae	Florida, USA
<i>Karlodinium veneficum</i>	Kv-LYG1	Dinophyceae	Haizhou Bay, Yellow Sea (YS)
<i>Karl. veneficum</i>	Kv-LYG2	Dinophyceae	Haizhou Bay, YS
<i>Karl. veneficum</i>	Kv-LYG5	Dinophyceae	Haizhou Bay, YS
<i>Karl. veneficum</i>	Kv-lu	Dinophyceae	Zhejiang coastal waters, ECS
<i>Karl. ballantinum</i>	TIO1456	Dinophyceae	Fujian coastal waters, ECS
<i>Karl. zhouanum</i>	GM8	Dinophyceae	Xiangshan Bay, ECS
<i>Takayama tasmanica</i>	TIO1452	Dinophyceae	Xiamen Port, ECS
<i>T. tasmanica</i>	CAWD115	Dinophyceae	NZ coastal waters
<i>T. xiamenensis</i>	TX-LYG2	Dinophyceae	Haizhou Bay, YS
<i>T. xiamenensis</i>	TX-LYG3	Dinophyceae	Haizhou Bay, YS
<i>T. xiamenensis</i>	C2-M-3	Dinophyceae	Haizhou Bay, YS
<i>Alexandrium pacificum</i>	MEL2	Dinophyceae	South China Sea (SCS)
<i>Prorocentrum obtusidens</i>	MEL15	Dinophyceae	Fujian coastal waters, ECS
<i>Chattonella</i> sp.	MEL39	Rhaphidophyceae	YS
<i>Heterosigma akashiwo</i>	MEL40	Rhaphidophyceae	Jiaozhou Bay, YS
<i>Aureococcus anophagefferens</i>	MEL51	Pelagophyceae	Bohai Sea
<i>Gymnodinium catenatum</i>	MEL11	Dinophyceae	Fujian coastal waters, ECS
<i>Amphidinium carterae</i>	MEL28	Dinophyceae	ECS
<i>Scrippsiella acuminata</i>	MEL30	Dinophyceae	ECS
<i>Skeletonema costatum</i>	MEL32	Mediophyceae	YS
<i>Phaeodactylum tricornutum</i>	MEL34	Bacillariophyceae	Jiaozhou Bay, YS
<i>Cylindrotheca closterium</i>	MEL36	Bacillariophyceae	YS
<i>Phaeocystis globosa</i>	MEL45	Haptophyceae	Beibu Gulf, SCS
<i>Isochrysis galbana</i>	MEL55	Chrysophyceae	YS
<i>Chlorella</i> sp.	MEL56	Chlorophyceae	YS
<i>Akashiwo sanguinea</i>	MEL93	Dinophyceae	YS



DNA was stored at -80°C until further analysis. The 18S rDNA V1-V9 region and 28S rDNA D1-D2 region of five strains (Kv-LYG1, Kv-LYG5, Kv-lu, KM2019, KM201) of Kareniaceae, and ITS region of 15 strains (Kv-LYG1, Kv-LYG2, Kv-LYG5, Kv-lu, KM2019, KM201, TIO1456, GM8, TIO1452, CAWD79, CAWD82, CAWD91, CAWD92, CAWD115, CAWD122) of Kareniaceae (Table S2), were PCR amplified with universal primers for eukaryotes (Table 2). PCR was performed with a final volume of 20 μL containing 10 μL $2 \times$ Taq PCR MasterMix (Tiangen, China), 1 μL of genomic DNA, 0.8 μL of each primer at 10 μM , and 7.4 μL sterilized deionized water. Amplifications were performed with an initial denaturation temperature of 95°C for 3 min, followed by 35 cycles of 95°C for 20 s; 53°C (18S rDNA), 52°C (28S rDNA), and 59°C (ITS) for 30 s; 72°C for 100 s; and a final elongation at 72°C for 7 min. The PCR products were separated by electrophoresis on 1% agarose gel, and images were obtained using a gel imaging system (Bio-Rad, USA). Targeted DNA bands were purified by quick DNA extraction using a SteadyPure PCR DNA Purification Kit AG21003 (AG, China) and then sequenced by Sangon Biotech (Shanghai, China) and assembled using Vector NTI Advance 11.5.3 ContigExpress.

Clone Library Construction and Sequencing

Clone libraries of 18S rDNA, 28S rDNA, and ITS were constructed using purified PCR fragments to determine the polymorphism of target genes. The purified PCR fragments were cloned into pMD 18-T vectors (Takara), and the vectors were transformed into Takara *Escherichia coli* Competent Cells DH5 α , which were placed on LB ampicillin plates for cultivation (37°C , 16-18 h). The positive clones were screened by colony PCR, and at least three clones were sequenced by Sangon Biotech (Shanghai, China). Next, BLASTn analysis was performed on the obtained target sequences, ensuring the validity of Kareniaceae species. Finally, the obtained sequences of the Kareniaceae species in this study were submitted to GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>).

Genetic Distance Analysis

Kareniaceae sequences (18S rDNA region, 28S rDNA region, and ITS region) were downloaded from GenBank (Table S3). Together with the sequences obtained in this study, the genetic distances of the different target sequences were estimated by MEGA7 using the pairwise distance method with bootstrap replications of 1000 (Kumar et al., 2016).

Phylogenetic Analysis of ITS Sequences of Kareniaceae Species

Sixty-two ITS sequences from the genera *Karenia*, *Karlodinium*, *Takayama*, *Asterodinium*, *Gertia* and *Shimiella*, comprising 43 sequences downloaded from GenBank (Table S3) and 19 sequences obtained in this study (Table S2), were used to construct the phylogenetic tree. ITS sequence from *Gymnodinium catenatum* was used as outgroup. All sequences were aligned using ClustalW, and a maximum-likelihood tree was built using the MEGA7 variance estimation method with 1000 bootstrap replications and the best-fitting nucleotide substitution models of GTR (Nei and Kumar, 2000; Kumar et al., 2016). Bayesian analysis was performed using MrBayes 3.2.6 (Ronquist et al., 2012) with generations of 20,000 and sampling frequencies of 1,000, and the best-fitting nucleotide substitution models of GTR+F+I+G4 were selected using Modelfinder (Kalyaanamoorthy et al., 2017).

TABLE 2 | Summary of primers used in this study.

Primer	Specificity	Sequence (5'-3')	Target region	Reference
LH2	Universal to Eukaryote	AGGTGAACCTGCGGAAGGATC	ITS1-5.8S-ITS2	Zheng et al. (2009a, 2009b)
Dlam	Universal to Eukaryote	CCTGCAGTCGACAKATGCTTAARTTCAGCRGG	ITS1-5.8S-ITS2	Zheng et al. (2009a, 2009b)
Euk328f	Universal to Eukaryote	ACCTGGTTGATCCTGCCAG	18S V1-V9	Guillou et al. (2002)
Euk329r	Universal to Eukaryote	TGATCCTTCYGCAGGTTCCAC	18S V1-V9	Guillou et al. (2002)
D1R	Universal to Eukaryote	ACCCGCTGAATTTAAGCATA	28S D1-D2	Scholin et al. (1994)
D2C	Universal to Eukaryote	CCTTGGTCCGTGTTCAAGA	28S D1-D2	Scholin et al. (1994)
KareF2	Specific to <i>Karenia</i>	ATTGTGMACCAYCTKMTGT	ITS1-5.8S-ITS2	This study
KareR1	Specific to <i>Karenia</i>	ACAAGWTGACAGTGGCATG	ITS1-5.8S-ITS2	This study
KarITaF1	Specific to <i>Karlodinium</i> and <i>Takayama</i>	AYTGTGAMCYWCTTYGTGAG	ITS1-5.8S-ITS2	This study
KarITaR2	Specific to <i>Karlodinium</i> and <i>Takayama</i>	ACMCMTGACAGCACCAAG	ITS1-5.8S-ITS2	This study

Design, Optimization, and Validation of Kareniaceae Specific Primers

Design of Kareniaceae Specific Primers

Multiple alignments were performed between the obtained 19 sequences of the ITS region in this study (Table S2) and the downloaded sequences (Table S3), using Vector NTI Advance 11.5.3. Based on the alignment results, two specific primer pairs for the family Kareniaceae were designed using Primer 5: primers specific for the genus *Karenia* (KareF2 and KareR1) and primers specific for the genera *Karlodinium* and *Takayama* (KarlTaF1 and KarlTaR2) (Table 2). The primers were synthesized commercially by Sangon Biotech (Shanghai, China).

Optimization of PCR Reaction System

The volume of PCR reaction mixture was 20 μL : 1 μL genomic DNA (approximately 10 ng μL^{-1}), 4 μL 5 \times PCR Buffer, 1.6 μL dNTP, 0.4 μL TransStart FastPfu Fly DNA Polymerase (TransGen, China), 0.4 μL of forward and reverse primers (10 μM), and 12.2 μL sterilized deionized water. To optimize the PCR reaction, gradient PCR was first used to determine the optimal amplification temperature by using genomic DNA of *K. mikimotoi*, *Karl. veneficum*, and *T. xiamenensis* as templates, respectively. The reactions were performed on a TaKaRa PCR Thermal Cycler (Takara, Japan): 95°C for 2 min; followed by 35 cycles of 15 s at 95°C, 20 s at 46–61 °C, and 15 s at 72°C; and a final extension at 72°C for 5 min. The amplification products (approximately 400–440 bp) were separated using 1.5% AGE. The brightness of electrophoresis bands were used as criteria to choose the appropriate annealing temperature.

Limit of Detection

The cell concentration of microalgal cultures of *K. mikimotoi*, *Karl. veneficum* and *T. xiamenensis* were determined using an optical microscope and 15 mL of culture was filtered to extract total genomic DNA. DNA was quantified using a Qubit 4 fluorometer (Thermo, USA). The relationship between the cell number and DNA concentration was recorded in Table 6. The optimal assay conditions were then used to determine the primer sensitivity with 5-fold gradient diluted DNA solutions (10 ng μL^{-1} –0.128 pg μL^{-1}) of the *K. mikimotoi*, *Karl. veneficum*, and *T. xiamenensis* as DNA templates.

Test of Specificity

The specificity of the primer pairs was evaluated using the online Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) and by running the optimized assays with genomic DNA template from the target species (*K. mikimotoi*, *Karl. veneficum* and *T. xiamenensis*) and 15 non-target microalgal strains (Table 1). The resulting PCR products were analyzed using 1.5% AGE.

Applicability of the Two Molecular Markers

Seventeen strains of *Karenia* (seven strains, six spp.), *Karlodinium* (six strains, three spp.), and *Takayama* (four strains, two spp.) (Table 1), and two field samples (S30 and

ZN1-3) collected from the SCS were selected to test the applicability of the developed method. The genomic DNA of the Kareniaceae strains and field samples was extracted using the method described above. PCR reactions were conducted under the optimised conditions.

Application of the Barcodes in the South China Sea

To further verify the reliability of the developed molecular markers, the amplified products of the field samples (S30 and ZN1-3) were sequenced using high-throughput Illumina sequencing using the Novaseq 6000 system with 250 bp paired-end reads. The target DNA fragments were purified by quick DNA extraction using a SteadyPure PCR DNA Purification Kit AG21003 (AG, China), and the purified PCR products of each sample were quantified using a Qubit 4 fluorometer (Thermo, USA). Four sequencing libraries (two samples, two primer pairs) were constructed using the VAHTS Universal DNA Library Prep Kit for IlluminaV3 (ND607) and VAHTSTM DNA Adapters set1 (N801). Paired-end raw reads were preprocessed to generate high-quality clean reads by using fastp software (Version 0.20.1), including removal of barcode and adapter sequences, reads less than 200 bp, and low-quality filtering. Cleaned reads were assembled to generate high-quality tags (Tag refers to a contig of an F read and the associated R read, with the length around 440–450bp) using QIIME2 (Version 2021.4).

Two hundred and forty-eight ITS sequences from Kareniaceae species downloaded from GenBank and nineteen ITS sequences obtained in this study were used to create a reference database. The GenBank database was searched using each species of Kareniaceae and the full name or abbreviation of the target gene (e.g., 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA). Sequences were then aligned and trimmed to obtain the target region. Sequences with obvious alignment errors and only partial ITS sequences were removed. The homologous sequences were searched using local BLAST, with each clean tag sequence using the reference database. Homologous sequences with a similarity above 98% were clustered into an operational taxonomic unit (OTU) using QIIME2 (Version 2021.4). The mapping rarefaction curve for sampling depth verification to evaluate whether the sequencing volume was sufficient to cover all groups in the sample, and the diversity of the two samples, was analyzed with QIIME2. OTUs obtained by clustering were annotated to obtain detailed information of the two samples.

RESULTS

Selecting the Target Region for the Molecular Marker

Sequences of the ITS region were successfully amplified from 15 strains of *K. mikimotoi*, *K. brevisulcata*, *K. selliformis*, *K. bicuneiformis*, *K. brevis*, *K. papilionacea*, *Karl. veneficum*,

Karl. ballantinum, *Karl. zhouanum*, and *T. tasmanica*. Sequences of the 18S rDNA V1-V9 and 28S rDNA D1-D2 regions were successfully amplified from five strains of *K. mikimotoi* and *Karl. veneficum*. All sequences obtained in this study were deposited in GenBank (accession numbers: MW750282-MW750293, MZ323882, MZ675742-MZ675744, MZ676047-MZ676050, and OK093375-OK093386; **Table S2**). The genetic distances of sequences of the 18S rDNA V1-V9, 28S rDNA D1-D2, and ITS regions of Kareniaceae species obtained from this study and GenBank were analyzed.

The genetic distances between the sequences of the 18S rDNA V1-V9, 28S rDNA D1-D2, and ITS regions were 0.000-0.025, 0.000-0.199, and 0.000-0.323, respectively (**Tables 3–5**). The interspecific genetic distances of 18S rDNA V1-V9 and 28S rDNA D1-D2 were relatively small and were lower than those of ITS. The 18S rDNA and 28S rDNA sequences from different geographical strains of *K. mikimotoi* and *Karl. veneficum* were almost identical within a species, and the intraspecific distances of

the ITS varied between 0.003 and 0.007. These results suggested that the ITS region was better suited as a molecular marker for the family Kareniaceae than the 18S rDNA and 28S rDNA.

Phylogenetic Relationship of Different Strains of Kareniaceae Species

Kareniaceae species were divided into two reciprocal sister groups, one contained *Karenia* and *Asterodinium*, and the other *Karlodinium*, *Takayama*, *Gertia* and *Shimiella*. (**Figure 2**). Within the latter, *Shimiella* formed a separate clade from *Karlodinium*, *Takayama* and *Gertia*. The latter clade was further divided into two subclades, and one subclade contained *Gertia*, *Takayama*, and *Karl. decipiens*, and the other the remaining *Karlodinium* species. Several Kareniaceae species showed intraspecific variation in their ITS marker, for example, the nine strains of *Karl. veneficum* showed slight variation (**Figure 2**). The results suggested that *Karlodinium* and *Takayama* have closer phylogenetic relationships than

TABLE 3 | Genetic distances between sequences of the 18S rDNA V1-V9 region of Kareniaceae species.

Seq. ID	1	2	3	4	5	6	7	8	9	10	11	12
1												
2	0.008											
3	0.007	0.002										
4	0.007	0.002	0.000									
5	0.007	0.002	0.000	0.000								
6	0.010	0.004	0.003	0.003	0.003							
7	0.004	0.004	0.003	0.003	0.003	0.006						
8	0.011	0.006	0.005	0.005	0.005	0.008	0.007					
9	0.020	0.022	0.021	0.021	0.021	0.024	0.019	0.025				
10	0.019	0.019	0.019	0.019	0.019	0.021	0.016	0.023	0.008			
11	0.019	0.019	0.019	0.019	0.019	0.021	0.016	0.023	0.008	0.001		
12	0.018	0.019	0.018	0.018	0.018	0.021	0.015	0.022	0.008	0.001	0.001	
13	0.021	0.022	0.021	0.021	0.021	0.023	0.019	0.025	0.014	0.012	0.012	0.011

1: *K. bicuneiformis* (HM067002); 2: *K. brevis* (EF492501); 3-6: *K. mikimotoi* (AF022195, KU314866, EF492505, OK093378); 7: *K. papilionacea* (HM067005); 8: *K. selliformis* (HM067007); 9: *Karl. zhouanum* (MG365891); 10-12: *Karl. veneficum* (JN986577, OK093376, OK093377); 13: *T. acrotrocha* (HM067010).

TABLE 4 | Genetic distances between sequences of the 28S rDNA D1-D2 region of Kareniaceae species.

Seq. ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1																
2	0.044															
3	0.044	0.000														
4	0.044	0.000	0.000													
5	0.027	0.045	0.045	0.045												
6	0.040	0.045	0.045	0.045	0.040											
7	0.002	0.045	0.045	0.045	0.029	0.042										
8	0.157	0.169	0.169	0.169	0.160	0.199	0.159									
9	0.164	0.170	0.170	0.170	0.153	0.191	0.167	0.011								
10	0.140	0.156	0.156	0.156	0.135	0.162	0.142	0.076	0.071							
11	0.138	0.161	0.161	0.161	0.135	0.167	0.141	0.051	0.051	0.081						
12	0.151	0.169	0.169	0.169	0.138	0.173	0.153	0.072	0.065	0.082	0.089					
13	0.151	0.169	0.169	0.169	0.138	0.173	0.153	0.072	0.065	0.082	0.089	0.000				
14	0.151	0.169	0.169	0.169	0.138	0.173	0.153	0.072	0.065	0.082	0.089	0.000	0.000			
15	0.151	0.169	0.169	0.169	0.138	0.173	0.153	0.072	0.065	0.082	0.089	0.000	0.000	0.000		
16	0.154	0.166	0.166	0.166	0.140	0.170	0.156	0.095	0.093	0.100	0.058	0.121	0.121	0.121	0.121	
17	0.136	0.152	0.152	0.152	0.127	0.156	0.139	0.076	0.069	0.083	0.037	0.098	0.098	0.098	0.098	0.018

1: *K. longicanalis* (MT754556); 2-4: *K. mikimotoi* (AY355460, KT860560, OK093380); 5: *K. papilionacea* (AB623225); 6: *K. selliformis* (KT020848); 7: *K. longicanalis* (KJ508368); 8: *Karl. digitatum* (MN134476); 9: *Karl. armiger* (DQ114467); 10: *Karl. ballantinum* (EF469232); 11: *Karl. decipiens* (LC521288); 12-15: *Karl. veneficum* (DQ114466, KU314867, OK093385, OK093386); 16: *T. acrotrocha* (DQ656115); 17: *T. tasmanica* (MZ358880).

TABLE 5 | Genetic distances between sequences of the ITS region of Kareniaceae species.

Seq. ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23		
1																									
2	0.109																								
3	0.151	0.123																							
4	0.121	0.050	0.110																						
5	0.121	0.046	0.110	0.003																					
6	0.121	0.050	0.110	0.000	0.003																				
7	0.127	0.100	0.155	0.091	0.096	0.091																			
8	0.116	0.073	0.122	0.085	0.085	0.085	0.094																		
9	0.139	0.112	0.010	0.099	0.099	0.099	0.143	0.112																	
10	0.235	0.219	0.243	0.242	0.234	0.242	0.203	0.226	0.249																
11	0.298	0.253	0.238	0.251	0.259	0.251	0.224	0.250	0.259	0.041															
12	0.284	0.299	0.319	0.300	0.310	0.300	0.269	0.299	0.319	0.098	0.112														
13	0.234	0.254	0.255	0.253	0.261	0.253	0.210	0.262	0.249	0.160	0.156	0.148													
14	0.233	0.251	0.253	0.251	0.258	0.251	0.209	0.260	0.248	0.158	0.154	0.147	0.007												
15	0.237	0.249	0.244	0.243	0.251	0.243	0.212	0.269	0.244	0.080	0.084	0.074	0.141	0.140											
16	0.303	0.311	0.322	0.313	0.323	0.313	0.278	0.293	0.322	0.125	0.141	0.020	0.163	0.162	0.100										
17	0.254	0.247	0.253	0.270	0.261	0.270	0.257	0.267	0.253	0.078	0.085	0.062	0.127	0.126	0.060	0.077									
18	0.253	0.245	0.266	0.267	0.259	0.267	0.255	0.265	0.251	0.077	0.094	0.070	0.137	0.136	0.068	0.086	0.007								
19	0.254	0.247	0.253	0.270	0.261	0.270	0.257	0.267	0.253	0.078	0.085	0.062	0.127	0.126	0.060	0.077	0.000	0.007							
20	0.300	0.306	0.316	0.313	0.323	0.313	0.273	0.305	0.316	0.130	0.126	0.028	0.158	0.157	0.105	0.028	0.079	0.087	0.079						
21	0.222	0.230	0.256	0.242	0.250	0.242	0.217	0.233	0.235	0.149	0.157	0.149	0.075	0.074	0.141	0.166	0.133	0.132	0.133	0.144					
22	0.250	0.245	0.245	0.258	0.266	0.258	0.250	0.258	0.225	0.175	0.170	0.172	0.099	0.098	0.164	0.192	0.133	0.132	0.133	0.156	0.021				
23	0.241	0.236	0.261	0.247	0.255	0.247	0.227	0.233	0.241	0.166	0.162	0.156	0.083	0.082	0.157	0.174	0.138	0.136	0.138	0.152	0.020	0.031			

1: *K. bicuneiformis* (MZ675743); 2: *K. brevis* (MZ675744); 3: *K. longicanalis* (MF781068); 4-6: *K. mikimotoi* (LC055227, HM807314, MW750286); 7: *K. papilionacea* (FJ823565); 8: *K. selliformis* (HM067008); 9: *K. longicanalis* (FJ823566); 10: *Karl. armiger* (AJ557024); 11: *Karl. australe* (KJ670420); 12: *Karl. ballantini* (MW750292); 13: *Karl. decipiens* (MN134481); 14: *Karl. digitatum* (MG738210); 15: *Karl. elegans* (MT161380); 16: *Karl. gentienii* (LC521290); 17-19: *Karl. veneficum* (EF036540, AJ557028, MW750289); 20: *Karl. zhouanum* (MG738207); 21: *T. acrotrocha* (HM067011); 22: *T. helix* (MN961148); 23: *T. tasmanica* (MZ676050).

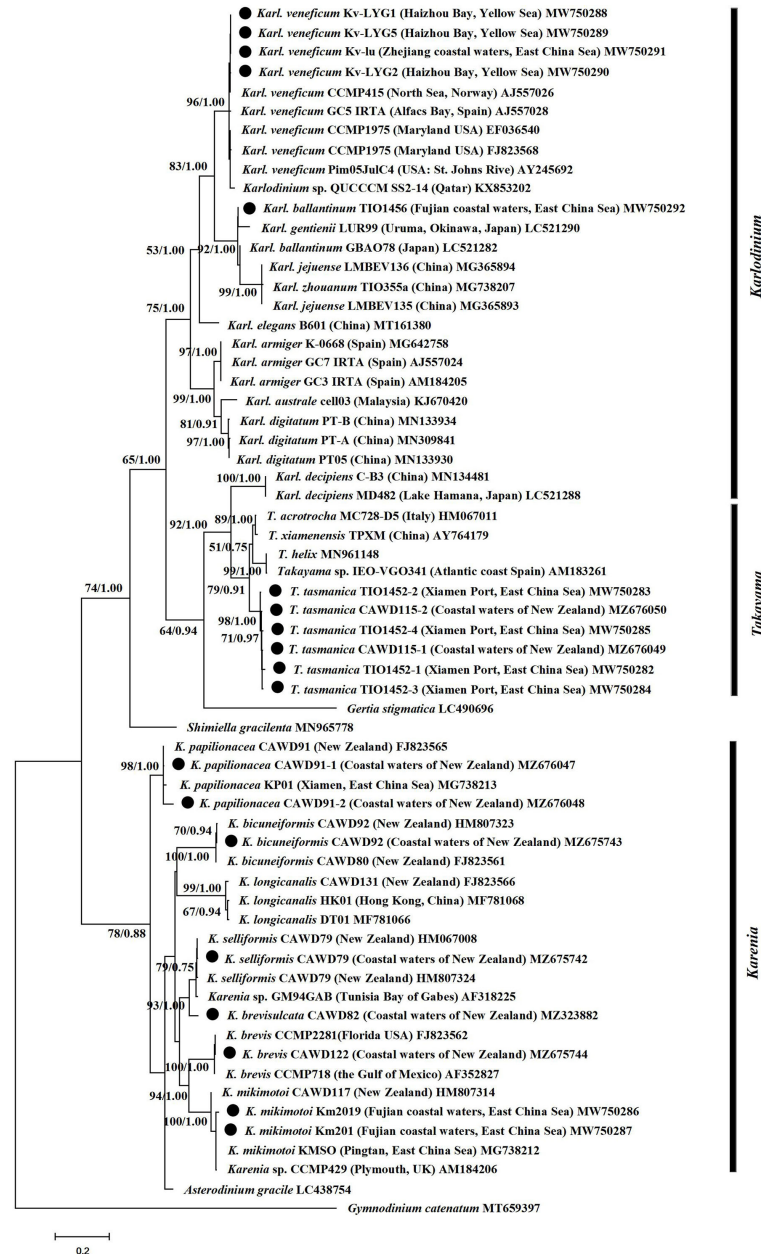


FIGURE 2 | Phylogenetic tree of Kareniaceae species based on ITS sequences. Sequences with a circle were obtained in this study. Values at nodes suggests statistical support values (ML bootstrap values/Bayesian posterior probability). Only bootstrap values >50% and posterior probabilities >0.5 are shown.

Karenia, and demonstrated variation between strains at species level. The phylogenetic analyses could distinguish between and within species of the family Kareniaceae.

Design, Optimization, and Verification of the Primers

Based on the alignment results of the ITS sequences from Kareniaceae species, developing a primer pair that would amplify all species from the whole family was difficult. Because

the genera *Karlostinium* and *Takayama* had a close phylogenetic relationship, the *Karlostinium*-*Takayama*-specific primer pair (KarlTaF1 and KarlTaR2) was designed in a relatively conserved region covering *Karlostinium* and *Takayama* ITS sequences, whereas *Karenia*-specific primer pair (KareF2 and KareR1) was designed to target the ITS region of *Karenia* spp. independently. Genomic DNA extractions from *K. mikimotoi*, *Karl. veneficum*, and *T. xiamenensis* (approximately 10 ng μL^{-1}) were used as DNA templates to optimize the annealing

temperature ranging from 46 to 61°C. The PCR results showed that 49°C was the most suitable annealing temperature for both primer pairs (**Figure 3A**).

Total genomic DNA was extracted from 15 mL of cultures of *K. mikimotoi*, *Karl. veneficum*, and *T. xiamenensis*, respectively (**Table 6**). The sensitivity of the designed primers was verified using serially diluted genomic DNA as DNA template. The PCR results showed that the amplified bands weakened as the DNA content decreased. The presence of target microalgae could be detected when PCR tubes contained at least 3.2 pg of target DNA, corresponding to 0.17 cells of *T. xiamenensis*, 0.26 cells of *K. mikimotoi*, and 0.60 cells of *Karl. veneficum*, demonstrating that the assays were sensitive and had a low detection limit (**Figure 3B**).

Primer-BLAST was used to evaluate the specificity of the primers and the results showed that the designed primers have good specificity. Then, strains of *K. mikimotoi*, *Karl. veneficum*, *T. xiamenensis*, and 15 non-target microalgal cultures were used as positive and negative controls, respectively, to test the specificity of the designed primers under the optimized experimental conditions. PCR results showed that only the genomic DNA of *K. mikimotoi*, *Karl. veneficum*, and *T. xiamenensis* (positive controls) had amplification bands at the expected size 400–440 bp (**Figure 3C**, lane 16–18), and other microalgae (negative controls) had no amplification bands at approximately 400–440 bp (**Figure 3C**). The KarlTa primer pair amplified only *Karlodinium* and *Takayama*, but not *Karenia* and the Kare primer pair only amplified *Karenia*, but not *Karlodinium*/*Takayama*. The findings indicated that both primer pairs were specific to their respective targets, and the combination of both primer pairs were specific to all species from the family Kareniaceae.

Applicability of the Two Primer Pairs

The results of PCR reactions using 17 unialgal strains of Kareniaceae species as DNA templates showed that the KarlTa primer pair could amplify all species of *Takayama* and *Karlodinium* in this study (samples No. 1–10, 10 strains, 5 spp.), comprising *Karl. veneficum*, *Karl. ballantinum*, *Karl. zhouanum*, *T. tasmanica*, and *T. xiamenensis*, but could not amplify the seven strains of *Karenia* (**Figure 3D**). By contrast, the Kare primer pair could amplify all species of *Karenia* in this study (samples No. 11–17, 7 strains, 6 spp.), including *K. mikimotoi*, *K. selliformis*, *K. brevisulcata*, *K. papilionacea*, *K. bicuneiformis*, and *K. brevis*, but could not amplify the 10 strains of *Takayama* and *Karlodinium*. The results implied that both molecular markers were perfectly applicable to their respective target genera and had the potential to be used for natural phytoplankton communities.

Application of the Metabarcodes for the Detection Of Field Sample

The two primer pairs were further validated for use with field samples, and both the SCS samples could be specifically amplified with the two primer pairs (**Figure 4**). The high-throughput sequencing results of the SCS samples using the

two primer pairs as barcode were shown in **Table 7**. The raw data of the four libraries constructed from the SCS samples were 633,600, 849,090, 773,514, and 549,038 reads, and after quality filtering more than 90% of the reads remained, indicating that the obtained sequencing data were reliable and could be used for subsequent analyses.

After bioinformatic processing, the assembled high-quality tags were 289,603, 270,601, 346,279, and 74,723. The homologous ITS sequences of Kareniaceae species obtained through BLAST analysis of the self-built reference database of Kareniaceae species were 280,600, 233,985, 338,924, and 46,626, accounting for 96.9%, 86.5%, 97.9%, and 62.4% of the assembled high-quality tags. These homologous sequences were then clustered into 2665, 1341, 872, and 509 OTUs with a similarity above 98%. The rarefaction curves of the OTUs were prepared for all samples (**Figure S1**), and the number of OTUs in each sample flattened with an increase in sequencing quantity, indicating that the sequencing depth was sufficient to reflect the species diversity in the samples.

For the analysis of the different Kareniaceae species in the two SCS samples, the OTU annotation results were analysed according to different identity levels (**Figure 5**). Differences between the known genera of Kareniaceae were within 90% sequence identity (**Table S4**), so sequences with identity greater than 90% were considered as homologs of the Kareniaceae family. 93% of OTUs had an identity greater than 90%, demonstrating reads from the family Kareniaceae dominated the two samples. 3% of the OTUs had identity greater than 98% and a total of fifteen known Kareniaceae species and one *Brachidinium* species were identified with high certainty. Sample S30 contained seven *Karenia* spp., five *Karlodinium* spp., and two *Takayama* spp. Sample ZN1-3 contained six *Karenia* spp., four *Karlodinium* spp., two *Takayama* spp., and one *Brachidinium* sp. (**Figures 6A, B**). These results displayed that Kareniaceae species composition was different between the two samples and the species diversity in sample S30 was slightly higher than that in ZN1-3. The dominant species in sample S30 were *K. papilionacea* (13.0%) and *Karl. veneficum* (4.4%), and those in the coastal sample ZN1-3 were *K. papilionacea* (10.8%), *K. mikimotoi* (5.6%), *B. capitatum* (6.6%) and *Karl. veneficum* (2.0%) (**Table S5**).

The representative sequences of the 329 OTUs (7%) with identity ranging from 80 to 90% were subjected to additional BLAST analyses in the GenBank Database, and sequences with the highest identity for OTU were pooled and analyzed (**Table 8**). The results showed that 266 OTUs had the closest relationship with the species from the family Kareniaceae. For the other 60 unknown hits (Uncultured eukaryotes), the second and third highest identities were Kareniaceae species (**Table S6**).

The alpha-diversity indices of the two SCS samples were analyzed using QIIME2. The coverage rates of sequencing results were greater than 98.2%, indicating that the sequencing results were effective and reliable. The Ace and Chao1 indices and the Simpson and Shannon indices reflected the species abundance and species diversity in the samples, respectively. The four

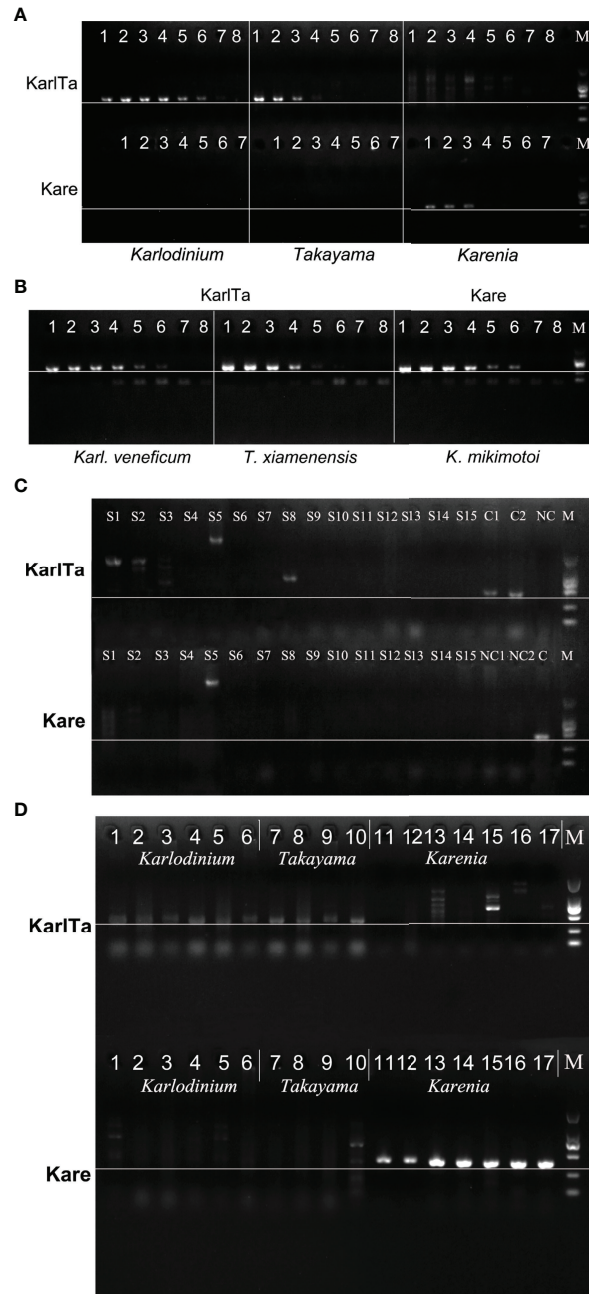


FIGURE 3 | Sensitivity and specificity of KarlTa and Kare primer pairs. **(A)**: Gel electrophoresis of annealing temperature gradient of KarlTa and Kare primer pairs (KarlTa, 1: 47 °C; 2: 49 °C; 3: 51 °C; 4: 53 °C; 5: 55 °C; 6: 57 °C; 7: 59 °C; 8: 61 °C; M: DL2000 DNA marker; Kare, 1: 46 °C; 2: 48 °C; 3: 50 °C; 4: 52 °C; 5: 54 °C; 6: 56 °C; 7: 58 °C; M: DL2000 DNA marker. The horizontal line indicates the correct size of target bands). **(B)**: Gel electrophoresis of sensitivity of KarlTa and Kare primer pairs (1: 10 ng, 2: 2 ng, 3: 0.4 ng, 4: 0.08 ng, 5: 16 pg, 6: 3.2 pg, 7: 0.64 pg, 8: 0.128 pg of DNA template content; M: DL2000 DNA marker. The horizontal line indicates the correct size of target bands). **(C)**: Gel electrophoresis of specificity of KarlTa and Kare primer pairs (S1: *A. pacificum*; S2: *P. obtusidens*; S3: *Chattonella* sp.; S4: *H. akashiwo*; S5: *A. anophagefferens*; S6: *G. catenatum*; S7: *A. carterae*; S8: *S. acuminata*; S9: *S. costatum*; S10: *P. tricornutum*; S11: *C. closterium*; S12: *P. globosa*; S13: *I. galbana*; S14: *Chlorella* sp.; S15: *A. sanguinea*; C1 (KarlTa) & NC1 (Kare): *Karl. veneficum*; C2 (KarlTa) & NC2 (Kare): *T. xiamenensis*; NC (KarlTa) & C (Kare): *K. mikimotoi*; M: DL2000 DNA marker. The horizontal line indicates the correct size of target bands). **(D)**: Validation results of KarlTa and Kare primers with laboratory samples (*Karlodinium* (1-6): 1-4: *Karl. veneficum* Kv-LYG1, Kv-LYG2, Kv-LYG5, and Kv-lu; 5: *Karl. ballantinum* TIO1456; 6: *Karl. zhouanum* GM8; *Takayama* (7-10): 7-9: *T. xiamenensis* TX-LYG2, TX-LYG3, C2-M-3; 10: *T. tasmanica* CAWD115; *Karenia* (11-17): 11-12: *K. mikimotoi* KM2019, KM201; 13: *K. selliformis* CAWD79; 14: *K. brevisulcata* CAWD82; 15: *K. papilionacea* CAWD91; 16: *K. bicuneiformis* CAWD92; 17: *K. brevis* CAWD122; M: DL2000 DNA marker. The horizontal line indicates the correct size of target bands).

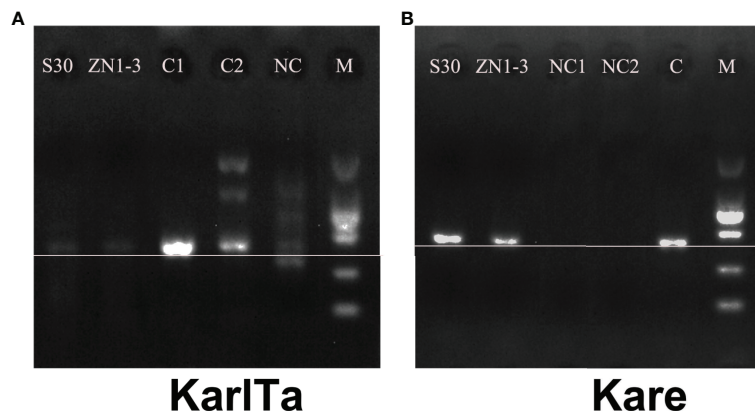


FIGURE 4 | PCR results of specific primers in field samples (**A**: KarlTa; **B**: Kare). (Field sample S30; Field sample ZN1-3; C1 (**A**) & NC1 (**B**): *Karl. veneficum*; C2 (**A**) & NC2 (**B**): *T. xiamenensis*; NC (**A**) & C (**B**): *K. mikimotoi*; M: DL2000 DNA marker. The horizontal line indicates the correct size of target bands).

indices in sample S30 were higher than those in sample ZN1-3 (Table 9), suggesting that the abundance and diversity of Kareniaceae species in sample S30 were higher than those in sample ZN1-3.

DISCUSSION

Kareniaceae species are widely distributed in global oceans, and many form recurrent HABs in coastal waters (De Salas et al., 2008; Brand et al., 2012). Kareniaceae species can produce harmful compounds and toxins that can threaten coastal ecosystems and aquaculture (Mooney et al., 2007; Zhang et al., 2008; Brand et al., 2012; Holland et al., 2012; Fowler et al., 2015). Due to difficulties with morphological identification including small and fragile cells and the absence of distinguishable features, accurately identifying species in the family Kareniaceae from the environment is difficult. In this study, we developed a molecular method for the rapid detection of Kareniaceae species with high sensitivity and specificity. This technique shows great promise for understanding the diversity of Kareniaceae and for the early warning and monitoring of HABs.

Feasibility of the Specific Barcodes for Kareniaceae Species Detection

To date, molecular identification and detection methods for HABs have largely focused on species-specific molecular assays (Gray et al., 2003; Zhang et al., 2008; Al-Kandari et al., 2011; Toldrà et al., 2018; Huang et al., 2020; Wang et al., 2020) or

universal eukaryotic molecular markers (Scorzetti et al., 2009; Zheng et al., 2009a; Marie et al., 2010; Yuan et al., 2012; Wu et al., 2015; Li et al., 2018). Kareniaceae species are generally present at a low cell density before a bloom occurs, which is usually undetectable or underestimated using universal eukaryotic primers (Gentien et al., 2005; Liu et al., 2020). Notably, as new species of Kareniaceae are regularly discovered, and the tendency that Kareniaceae HABs are not limited to a single species is becoming increasingly apparent, meaning that the successful application of species-specific primers for the early warning and monitoring of Kareniaceae HABs can be difficult and of limited use. Recently, some researchers have reported that family-level specific primers were useful to reveal the diversity, distribution and abundance of Dinophyceae species (Wietkamp et al., 2019), suggesting a type of “universal” barcode specific to the family Kareniaceae could be developed to identify various potential HAB species from environmental water samples.

Various molecular markers have been reported to detect and identify Kareniaceae HAB species, generally based on the 28S rDNA, 18S rDNA, and ITS regions (Zheng et al., 2009a; Yuan et al., 2012; Luo et al., 2018; Huang et al., 2020). These genes are arranged in tandem high copy number and thus are easily obtained using PCR amplification. In addition, many sequences from these regions are available from public sequence databases, making it easy to compare relative sequences of target species for the development of molecular methods. In this study, the comparison of genetic distances between sequences from the 18S rDNA, 28S rDNA, and ITS regions of Kareniaceae species showed that the ITS had greater variation than 18S rDNA and 28S rDNA regions. Phylogenetic

TABLE 6 | Cell numbers and DNA concentration of the target algae.

Species	Cell numbers	Total DNA concentration (ng)	DNA concentration in cell ($\mu\text{g cell}^{-1}$)
<i>K. mikimotoi</i>	217500	2688	12.4
<i>Karl. veneficum</i>	454500	2394	5.3
<i>T. xiamenensis</i>	180000	3300	18.3

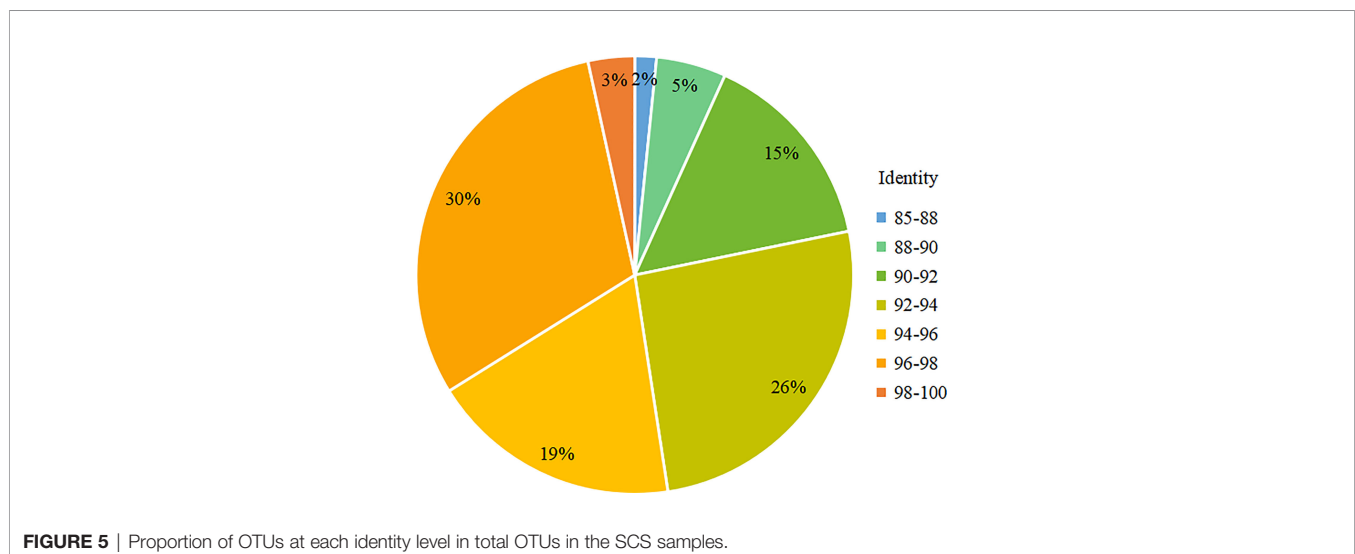
TABLE 7 | Quality control results of the Illumina sequencing data.

Sample ID	S30-Kare	S30-KarITa	ZN1-3-Kare	ZN1-3-KarITa
raw data (reads)	633600	849090	773514	549038
quality control (reads)	621682	793664	747052	498662
quality control/raw data (%)	98.1	93.5	96.6	90.8
Assemble (tags)	289603	270601	346279	74723
BLAST (tags)	280600	233985	338924	46626
BLAST/assembly (%)	96.9	86.5	97.9	62.4
Clustered OTUs	2665	1341	872	509

analyses of the ITS sequences showed that currently classified Kareniaceae species were clustered into three clades that included the three core genera of Kareniaceae. Variation in the ITS region which corresponds to different geographical isolates is a common phenomenon in Kareniaceae species. For example, fourteen different geographical strains of *K. mikimotoi* clustered into four groups based on differences in the ITS region (Al-Kandari et al., 2011). Therefore, the ITS region could be used as a tool for geographical and taxonomic inference at both the intraspecific and the interspecific level. Additionally, according to the phylogenetic analyses in this study, the genera *Karlodinium* and *Takayama* clustered together and were quite distinct from the *Karenia* genus, which has also been reported in other studies (Huang et al., 2017; Toldrà et al., 2018; Elleuch et al., 2020), indicating the difficulty in developing a single molecular marker covering the whole family. However, we were able to design two primer pairs targeting the ITS region, one for *Karenia* and the other for *Karlodinium* and *Takayama*. The simultaneous use of both primer pairs allowed the successful amplification and detection of Kareniaceae species.

The specificity of molecular tools for the detection of harmful algae from environmental samples is crucial. Some detection methods based on pigment types and optical techniques have been developed to identify and detect Kareniaceae species, each with apparent advantages and disadvantages. Monitoring using pigment analysis by high-

performance liquid chromatography (HPLC) has been proposed based on the biomarker pigment gyroxanthin-diester (Richardson and Pinckney, 2004), but this pigment is found in all Kareniaceae species (De Salas et al., 2003; De Salas et al., 2005) and is not exclusive to Kareniaceae, therefore, species-specific detection is not provided. An optical plankton detector (OPD, BreveBuster) developed for *K. brevis* relies on the unique absorption to detect its presence in a mixed phytoplankton community (Kirkpatrick et al., 2000), which exhibits high specificity for *K. brevis*, but cannot detect other Kareniaceae species. Current monitoring programs often use light microscopy to detect and enumerate toxic microalgae, but this method is time-consuming, requires substantial taxonomic expertise, and is based on morphological characteristics, which, in some cases, are insufficient for identification at the genus or species level. For example, identification of cells from the genus *Karlodinium* is particularly difficult and cells can easily be misidentified (e.g., as *Gymnodinium*, *Karenia*, *Heterocapsa*, and *Ansanella*) due to their unarmored morphology and a lack of distinct features (Shao et al., 2004; Bergholtz et al., 2005; Garcés et al., 2006; Jeong et al., 2014). The two assays designed in this study were specific for Kareniaceae species and could easily detect *Karenia* and *Karlodinium/Takayama* species. The specificity of the assays was verified against strains of other common HAB species, including dinoflagellate species closely related to Kareniaceae present in the coastal waters of China,



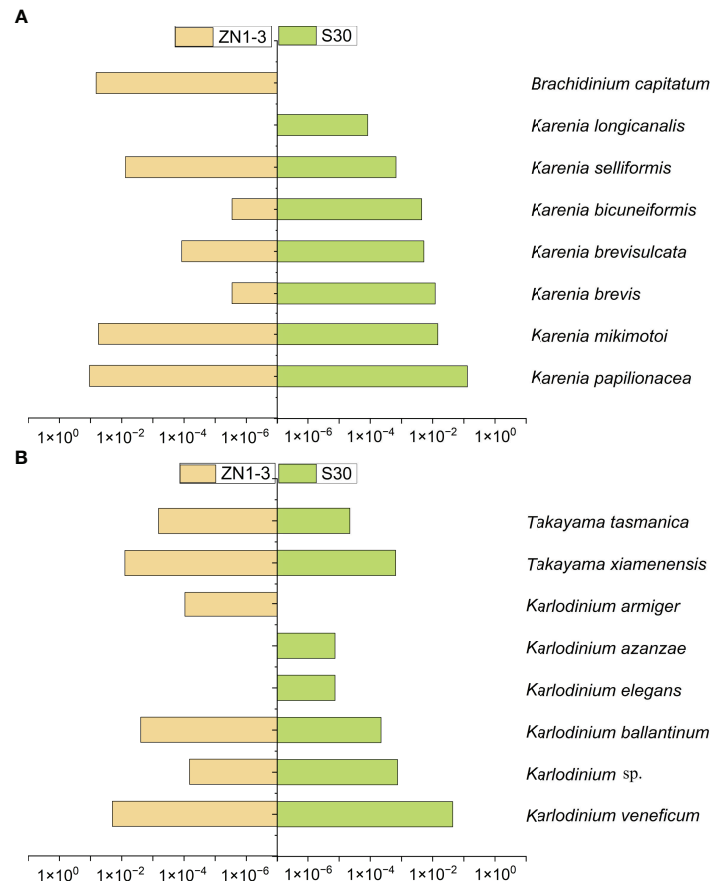


FIGURE 6 | Comparison of the statistics OTUs annotation results between sample ZN1-3 and S30. **(A)**: the tags proportion of the annotated as *Karenia* species in the two SCS samples. **(B)**: the tags proportion of the annotated as *Karlodinium* and *Takayama* species in the two SCS samples. (Column length represents the tags proportion of the annotated Kareniaceae species with more than 98% identity in the total assembled Kareniaceae tags).

and demonstrated their high specificity to Kareniaceae species (Table 1).

The primer pairs were also tested against environmental samples collected from the SCS using a high-throughput sequencing approach. Bioinformatic analyses of the Kare primers showed that 96.7% (S30) and 97.9% (ZN1-3) of high-quality tags were identified as belonging to the genus *Karenia*, indicating the high specificity of primers. To successfully amplify *Karlodinium* and *Takayama* species from the environmental samples (potentially due to low cell densities), the number of PCR cycles was increased and the number of

reads that could be identified as *Karlodinium/Takayama* was much lower (85% for S30 and 62.4% for ZN1-3). Further BLASTN analyses were carried out on sequences that could not be successfully classified in the ZN1-3-KarlTa library, showed that only a small number could be aligned with the sequences in GenBank and most of them failed to match any sequences, which could be caused by low cell concentrations and the excess PCR cycles.

In recent years, studies have described new genera in the Kareniaceae family, including *Asterodinium*, *Gertia* and *Shimiella* (Benico et al., 2019; Takahashi et al., 2019;

TABLE 8 | BLAST results of representative sequences of 329 OTUs with the identity less than 90%.

Results of BLAST	OTUs
uncultured eukaryote	60
<i>Karenia</i> . spp.	215
<i>Karlodinium</i> . spp.	45
<i>Brachidinium capitatum</i>	3
<i>Takayama acrotrocha</i>	1
<i>Asterodinium gracile</i>	5
total	329

TABLE 9 | Results of the diversity analysis of samples from the SCS.

Sample ID	S30-Kare	S30-KarlTa	ZN1-3-Kare	ZN1-3-KarlTa
Coverage (%)	0.982	0.992	0.995	0.992
Ace index	2189	1149	697	1107
Chao1 index	2146	1153	637	1102
Shannon index	6.470	4.711	4.172	4.081
Simpson index	0.971	0.919	0.909	0.882

Ok et al., 2021), as well as *Brachidinium*, of which the classification is still under debate. Alignments of ITS sequences from the new genera and the two molecular markers showed that the Kare primer pair differed by only 1-2 bases from the ITS sequences of *Asterodinium* and *Brachidinium*, but differed by 4-11 bases from those of *Gertia* and *Shimiella*, implying that the Kare primer pair can potentially amplify *Asterodinium* and *Brachidinium*, but is not likely to amplify *Gertia* and *Shimiella*. The KarlTa primers are 5-10 bases different from the ITS sequences these four genera, and are specific to *Karlodinium* and *Takayama*. High-throughput sequencing analyses verified the existence of at least fifteen known Kareniaceae species and one *Brachidinium* species, and homologous sequences to *Asterodinium* with low identity, indicating that the barcodes developed in this study can likely distinguish species of *Karenia*, *Karlodinium*, *Takayama*, *Brachidinium* and *Asterodinium* from the environmental samples.

The sensitivity of molecular assays is another critical parameter that must be evaluated for field detection because it determines the applicability of a method to the early detection and timely warning for HABs. The detection limit for the developed gyroxanthin-based approach by HPLC was below 5000 cells L⁻¹ of *K. brevis* in estuarine waters (Richardson and Pinckney, 2004), and the OPD method had no clear minimum detection limit (Kirkpatrick et al., 2000). Molecular detection methods have been developed for specific species of Kareniaceae, including real-time nucleic acid sequence-based amplification with internal control RNA (IC-NASBA) (Ulrich et al., 2010) and qPCR assays (Yuan et al., 2012) for *K. mikimotoi*. These methods had a detection limit of one and five cells, respectively. In this study, the lower limit of detection for the developed detection methods was 3.2 pg genomic DNA which corresponded to 0.17-0.60 cells of target species. Although the copy number of the ITS gene may vary between different species or strains, which may create difficulty for absolute quantification, methods targeting ribosomal regions including the ITS provide a very low limit of detection, especially compared with the detection method based on pigment or optical technology (Toldrà et al., 2018; Elleuch et al., 2020).

With the rapid development of DNA sequencing technology and the continuous improvement of bioinformatics analysis methods, metabarcoding analyses has significantly improved and are widely used in ecological studies of phytoplankton communities. The global distribution of marine chlorophyte algae was analyzed by the international Ocean Sampling Day Consortium through

simultaneous sampling of 141 sites worldwide and subsequent sequencing of 18S rDNA V4 region (Tragin and Vaultot, 2018). In addition, the Earth Microbiome Project, an international cooperative project, has carried out many metabarcoding analyses of marine phytoplankton communities (Thompson et al., 2017). Metabarcoding has promoted the study of HABs in various marine ecosystems in China since its development (Lin et al., 2014; Chen et al., 2016; Zhang et al., 2017; Stern et al., 2018) and revealed many previously overlooked nano- and pico-sized HAB species, which cannot be adequately identified and distinguished by light microscopy. For example, metabarcoding analysis based on the amplification and sequencing of the 18S rDNA V4 region confirmed that the causative species of brown tide in the Bohai Sea was *Aureococcus anophagefferens* (Zhang et al., 2012). Metabarcoding analysis of *rbcL* gene revealed the phytoplankton biodiversity in Jiaozhou Bay (Liu et al., 2004), and metabarcoding analysis of ITS2 revealed the diversity of symbiotic algae in coral reefs (Qin et al., 2019; Chen et al., 2019). However, Kareniaceae species are rarely or only occasionally detected by metabarcoding analyses, potentially due to their low abundance before bloom development. For instance, only *Karl. veneficum* was detected among eight species of harmful dinoflagellates in Jiaozhou Bay via metabarcoding analysis (Liu et al., 2020), meaning the diversity of Kareniaceae species is often underestimated. In our study, at least 18 known species of Kareniaceae were detected in one sample by metabarcoding using the developed specific ITS primers. This method allowed for the rapid and accurate detection of low-density Kareniaceae species from field samples and as an early warning system for monitoring of HABs caused by Kareniaceae species.

Application of the Developed Molecular Markers in the SCS and its Implications

The developed detection method was applied to environmental samples collected in two areas of the SCS. Both samples were amplified using the two designed primer pairs, indicating that species of different genera of Kareniaceae existed simultaneously in these two areas of the SCS. The PCR productions of the two samples were further verified by high-throughput sequencing, and 4531 OTUs with identity greater than 90% were obtained from the two samples, implying substantial diversity in the family Kareniaceae from the SCS. A total of 15 known Kareniaceae species were detected in the two samples. Among them, nine species have been previously recorded in the coastal waters of China. New records for the region were *K. brevisulcata*,

K. bicuneiformis, *Karl. ballantinum*, *Karl. azanzae*, and *Karl. armiger* (Table S7). The results suggest primers developed in this study can contribute to the discovery of Kareniaceae species that have not been reported previously in the coastal waters of China and would advance early warning and monitoring systems for Kareniaceae HABs.

High-throughput sequencing of amplicons derived from two SCS samples demonstrated that significant differences were observed in species composition between the sample S30 and sample ZN1-3. More known Kareniaceae species were found in the sample S30 and some possible reasons for this phenomenon are variations in hydrologic, chemical, and ecological environments. Notably, sea surface temperature and salinity differed between the two sampling sites, which were 30.1 °C and 33.94 in the sample S30, while 23.1°C and 33.20 in the sample ZN1-3. Higher temperatures could stimulate the growth of various dinoflagellate species (Gobler et al., 2017). Concentrations of different nutrients were relatively low and stable at site S30 in the SCS central waters, which was suitable for the co-existence of multiple species. By contrast, the environmental conditions of coastal waters are relatively unstable and changeable under the impact of human activities on the coastline, which can lead to the proliferation of one or some specific species. This hypothesis seems to be consistent with the previous results, that is, eutrophication processes in inshore areas caused by nutrient pollution from terrestrial inputs not only affects the scale of algal blooms but also changes the diversity and dominant groups (Anderson et al., 2002; Beusen et al., 2013; Glibert, 2017). Additionally, some species of *Karenia*, *Karlodinium*, and *Takayama* have been confirmed to be mixotrophs (organisms can combine phototrophy and phagotrophy) (Berge et al., 2012; Brand et al., 2012; Zhang et al., 2013; Jeong et al., 2016; Lim et al., 2018). To some degree, mixotrophic species can be beneficial in the coastal waters containing a high abundance of dissolved organic matter, and pico- and nano-phytoplankton.

With recent increased research on the causative species of HABs, many new Kareniaceae species have been described and identified, and some known species have been revised. For example, *Karl. corsicum* was revised from *Gyr. corsicum* (Siano et al., 2009), and several new species, including *T. xiamenensis*, *Karl. gentienii*, and *Karl. elegans* were published by Gu et al. (2013); Nézan et al. (2014) and Cen et al. (2020), respectively. In this study, all obtained sequences from the SCS field samples and the sequences of known Kareniaceae species were analyzed using phylogenetic methods, with *Amphidinium carterae* as the outgroup (Figure S3). The phylogenetic tree showed that all environmental sequences clustered together, and were far away from *A. carterae*. These sequences constituted 4860 OTUs, including 4531 OTUs with identity greater than 90% to sequences from species in the family Kareniaceae and 329 OTUs with identity between 80-90% to the family Kareniaceae (Table 8, S6). The identity of sequences between Kareniaceae genera was approximately 90% (Table S4), indicating that the 329 OTUs with 80-90% identity do not belong to the known

Kareniaceae genera. Representative sequences of seven OTUs within the 329 OTUs were randomly selected and aligned with the ITS sequences of known Kareniaceae species and other non-Kareniaceae species in the order Gymnodiniales. The both ends of OTU sequences were highly variable over 10 to 20 bases and the middle region was highly conserved (Figure S2). These selected OTUs were more similar to known Kareniaceae species in GenBank than non-Kareniaceae species, even when compared to the related *Gymnodinium* and *Gyrodinium* species in the order Gymnodiniales. These results strongly suggest the existence of unknown diversity within the family Kareniaceae.

CONCLUSIONS

In this study, two molecular assays with a low detection limit and strong specificity were developed for the rapid detection of Kareniaceae species. The advantages of this method for monitoring of Kareniaceae blooms are its simple operation, short detection time, and easy-to-obtain results. Moreover, high-throughput sequencing metabarcoding using the two primer pairs revealed the existence of several Kareniaceae species and the high diversity of Kareniaceae in the SCS. These approaches will enable further research into the diversity and geographical distribution of Kareniaceae along the coast of China and worldwide.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI [accession: MW750282-MW750293, MZ323882, MZ675742-MZ675744, MZ676047-MZ676050, and OK093375-OK093386].

AUTHOR CONTRIBUTIONS

QZ and LQ conceived and designed the study. WZ and QZ finished the experiment and draft of this manuscript. LQ and KS revised the manuscript. QZ and KS provided the algae. QL assisted in DNA extraction and experiment preparations. CL and XY assisted in sample collection and results analysis. All authors contributed to the article and approved the submitted version. There is no conflict of interest in submission of this manuscript, which has been approved by all authors for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2022.851605/full#supplementary-material>

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