TROPICAL LIFE SCIENCES RESEARCH

Molecular Detection of Harmful Raphidophyte *Chattonella subsalsa* Biecheler by Whole-Cell Fluorescence *in situ* Hybridization Assay

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Submitted: 15 February 2022; **Accepted:** 11 August 2022; **Early view:** 7 September 2022

To cite this article: Winnie Lik Sing Lau, Sing Tung Teng, Hong Chang Lim, Kieng Soon Hii, Sandric Chee Yew Leong, Chui Pin Leaw, Po Teen Lim (in press). Molecular detection of harmful raphidophyte *Chattonella subsalsa* Biecheler by whole-cell fluorescence *in situ* hybridization assay. *Tropical Life Sciences Research.*

Highlights

- Two strains of the harmful raphidophyte *Chattonella subsalsa* were established from the Johor Strait.
- Whole-cell fluorescence *in situ* hybridization (FISH) assay targeting *C. subsalsa* cells was developed based on the nucleotide sequences of the LSU rDNA and ITS2.
- The species-specific probes developed showed specificity toward the target cells, thus having the potential in detecting this harmful microalga in the environment.

Accepted author version posted online: 7 September 2022

Molecular Detection Of Harmful Raphidophyte *Chattonella subsalsa* Biecheler by Whole-Cell Fluorescence *in situ* Hybridization Assay

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Running head: Molecular detection of harmful Chattonella subsalsa

Abstract. Species of the genus *Chattonella* (Raphidophyceae) are a group of marine protists that are commonly found in coastal marine waters. Some are known as harmful microalgae that form noxious blooms and cause massive fish mortality in finfish mariculture. In Malaysia, blooms of *Chattonella* have been recorded since the 1980s in the Johor Strait. In this study, two strains of *Chattonella* were established from the Strait, morphological examination revealed the characteristics resembling *Chattonella subsalsa*. The molecular characterization further confirmed the species' identity as *C. subsalsa*. To precisely detect the cells of *C. subsalsa* in the environment, a whole-cell fluorescence *in situ* hybridization (FISH) assay was developed. The species-specific oligonucleotide probes were designed *in silico* based on the nucleotide sequences of the large subunit (LSU) and internal transcribed spacer 2 (ITS2) of the ribosomal RNA gene (rDNA). The best candidate signature regions in the LSU-rRNA and ITS2-rDNA were selected based on the hybridization efficiency and probe parameters. The probes were synthesized as biotinylated probes and tested by tyramide signal amplification-FISH (FISH-TSA). The results showed the specificity of the probes toward the target cells. FISH-TSA is proven to be a potential tool in the detection of harmful algae in the environment and could be applied in the harmful algal monitoring program.

Keywords: *Chattonella*, harmful algal bloom, oligonucleotide probe, ribosomal DNA, fluorescence *in situ* hybridization (FISH)

INTRODUCTION

Harmful algal bloom (HAB), a phenomenon commonly referred to as “red tide”, occurs when the harmful microalgae grow in high biomass in the water column and cause severe impacts including food poisoning syndromes in humans after consuming the algal toxins-contaminated seafood, and massive mortality of marine organisms (Hoagland et al. 2002). Among the shellfish poisonings, paralytic shellfish poisoning has been the focus in Malaysia, as most human intoxication cases were attributed to this form of shellfish poisoning (Lim et al. 2012, Usup et al. 2012, Yñiquez et al. 2020). Several causative dinoflagellates, *Pryodinium bahamense* Plate, *Alexandrium tamiyavanichii* Balech, *A*. *minutum* Halim, and *Gymnodinium catenatum* Graham have been documented throughout the Malaysian waters (Leaw et al. 2005, Lim et al. 2007). Nonetheless, other algal-originated incidents such as massive fish kills in aquaculture farms have been recorded in Malaysia (Lim et al. 2012, 2014, Teng et al. 2016, Yñiquez et al. 2020, Lum et al. 2021). Most of these events have been associated with the marine harmful dinophytes (dinoflagellates) such as *Margalefidinium polykrikoides* (Margalef) Gómez, Richlen & Anderson, *Noctiluca scintillans* (Macartney) Kofoid & Swezy, *Karlodinium australe* Salas, Bolch & Hallegraeff (Lim et al. 2014, Teng et al. 2016, Yñiquez et al. 2020).

Among the harmful microalgae, several groups of raphidophytes have been recognized as harmful to marine organisms (Lum et al. 2021). Members in the genus *Chattonella* Biecheler are among those that have caused severe damage to the aquaculture industries in many coastal countries (e.g., Japan: Okaichi 2003, Imai & Yamaguchi 2012). The first record of *Chattonella* bloom has been reported in Malabar Coast, India; while the most severe fish kill event was recorded in Harima-Nada, the Seto Inland Sea, Japan in the summer of 1972 (Imai & Yamaguchi 2012). In Malaysia, the occurrence was first documented in 1983 along the Johor Strait (Maclean 1989).

Conventionally, light microscopy has been used to identify morphological characteristics of *Chattonella* species. The species are unicellular, bi-flagellated, and pigmented with golden brown to greenish in some species depending on the fucoxanthin content (Klöpper et al. 2013). In general, species in *Chattonella* are differentiated based on the cell size, cell shape, presence of hyaline posterior tail, and mucocysts (Hara & Chihara 1982, Hara et al., 1994, Bowers et al. 2006). Diversity in the morphology of *Chattonella*, however, is high, even within the same species. Often, molecular characterization using gene markers such as ribosomal RNA genes (rDNA) is required to aid species recognition (Bowers et al., 2006, Demura et al. 2009). Among the species of *Chattonella*, *C*. *antiqua* (Hada) Ono, *C*. *marina* (Subrahmanyan) Hara & Chihara, *C*. *ovata* Hara & Chihara (also referred to as *C*. *marina* complex *sensu* Demura et al. 2009), and *C*. *subsalsa* Biecheler have been reported to cause HABs that associated with massive farmed-fish mortality and impacting the economy of affected countries worldwide (Hiroishi et al. 2005, Edvardsen & Imai 2006, Imai et al. 2006, Imai & Yamaguchi 2012, Lum et al. 2021).

In the Johor Strait that shared between Malaysia and Singapore, the occurrence of *Chattonella* has often been reported from the monitoring and research studies of both countries (e.g., Khoo and Wee 1997, Leong et al. 2015, Tan et al. 2016, Kok et al. 2019, Liow et al. 2019). Morphological plasticity in the species, however, has hampered precise species recognition, particularly in the preserved environmental samples, where the cells tend to deform, and the morphology deteriorated after fixation (Katano et al. 2009). This

often leads to species misidentification. Alternative approaches such as molecular techniques (Bower et al. 2006, Stacca et al. 2016), therefore, could be explored to overcome the limitation. In this study, a whole-cell tyramide signal amplification-fluorescence *in situ* hybridization (FISH-TSA) was developed to detect the harmful raphidophyte *Chattonella subsalsa*. The ribosomal RNA-targeted species-specific probes were designed *in silico* and applied in the assay.

MATERIALS AND METHODS

Algal Cultures and Morphological Observation

Live plankton samples were collected from the Johor Strait using a 20 µm-mesh plankton net and vertically hauled in subsurface seawater (<5 m) during high tide. The micropipetting technique was used to isolate the targeted cells. Cultures were established and grown in f/2 medium (Guillard & Ryther 1962) with a salinity of 30, 25 ± 0.5°C, under a light intensity of 100 µmol photons m^{-2} s^{-1}, with a 12:12 h light: dark photoperiod.

Morphological observation of cell shape and chloroplast was performed using an Olympus IX51 research microscope (Olympus, Tokyo, Japan). To observe the nuclear position, cells were first stained with the DAPI-nuclei stain and then examined under ultraviolet light with a UV filter set. Digital images were captured with an Olympus DP72 digital camera (Olympus, Tokyo, Japan).

Genomic DNA Extraction, rDNA Amplification and sequencing

Genomic DNA of *Chattonella* cultures was extracted as described in Leaw et al. (2010). In brief, the mid-exponential cells from 200 mL of cultures were harvested by centrifugation (1100 ×*g*, 1 min). The cell pellets were rinsed with ddH_2O and resuspended in 10× NET lysis buffer (5 M NaCl, 0.5 M EDTA, 1 M Tris-HCl, pH 8) and 1% sodium dodecyl sulfate. The mixture was incubated at 65°C and subsequently extracted with chloroform: isoamyl alcohol (24:1) and phenol: chloroform: isoamyl alcohol (25:24:1). The genomic DNA was then precipitated by adding absolute ethanol and 3 M sodium acetate (pH 5). The DNA pellet was then rinsed with cold 70% ethanol. Finally, the DNA pellet was dissolved in 30 µL of TE buffer (10 mM Tris-HCl, pH 7.4 and 1 mM EDTA, pH 8) and stored at -20°C until further analysis.

The large subunit (LSU) rDNA was amplified using a pair of universal primers: D1R (5´-ACC CGC TGA ATT TAA GCA TA-3´) and D3Ca (5´-ACG AAC GAT TTG CAC GTC AG-3´) (Scholin et al. 1994); while the internal transcribed spacer (ITS) region was amplified using the primer pairs: ITSA (5´-GTA ACA AGG THT CCG TAG GT-3´), ITSB (5´-AKA TGC TTA ART TCA GCR GG-3´) (Adachi et al. 1994), or the primer pair, ITSFC (5´-TAG AGG AAG GTG AAG TCG-3´), ITSFR (5´-TTA CTA GGG GAA TCC GAG-3´) designed in this study. The 25 µL PCR mixtures contained 1× PCR buffer, 2 mM $MgCl_2$, 0.02 µM of each primer, 0.2 mM of each dNTP, 2.5 U *Taq* polymerase (Invitrogen, Life Technologies, USA), and 20–100 ng μL^{-1} of genomic DNA. The amplification was performed by using an Artik 5020 thermal cycler (Thermos Scientific, USA). The amplicons were further purified by the QIAquick purification kit (QIAGEN, Germany,

Hilten) and single-pass DNA sequencing was performed on an ABI 3700XL automated DNA sequencer (Applied Biosystems, USA), with both strands sequenced.

Phylogenetic Analyses

Taxon sampling was performed by retrieving the LSU and ITS-rDNA nucleotide sequences of *Chattonella* species in the NCBI GenBank nucleotide database (Table S1). The sequences of *Heterosigma akashiwo* were used as outgroup. The newly obtained *C. subsalsa* sequences in this study and the retrieved sequences were multiple aligned using the program MUSCLE (https://www.ebi.ac.uk/Tools/msa/muscle/). Phylogenetic inferences of the aligned datasets were performed by Phylogenetic Analysis Using Parsimony* (PAUP*) v4.0b10 (Swofford 2003) and MrBayes v3.1.2 (Huelsenbeck & Ronquist 2001) as described in Leaw et al. (2016).

***In silico* rRNA-Targeted Oligonucleotide Probe Design**

The rDNA sequences of *Chattonella* species retrieved from GenBank and SILVA (http://www.arb–silva.de/) public databases were used to identify potential signature regions by using the PROBE_DESIGN tool of the ARB program package (Ludwig et al. 2004). The parameters for probe design included probe length, percentage of GC content, melting temperature (T_m), and self-complementary (Kumar et al. 2005). The probe candidates were selected for both target and probe sequences and were displayed in a result list (Kumar et al. 2005; Table S2, S3). The selected probe candidates were then evaluated using the PROBE Match tool (PMT) of ARB. The oligonucleotide sequences were then subjected to extensive specificity tests through BLAST comparisons against nucleotide databases of non-target sequences. The candidate sequences that complemented the region of target sequences with at least one mismatch in other non-target sequences were chosen (Hugenholtz et al. 2002). BLAST was also used to confirm that the sequences were transcribed in the correct orientation (Hugenholtz et al. 2002). The selected probes satisfying the *in silico* experimental constraints were then synthesized as a biotinylated probe (IDT Inc., Singapore).

Tyramide Signal Amplification-fluorescence *in situ* Hybridization

Cells were fixed with Lugol iodine solution (~1%) and transferred to a glass slide that was pre-fixed with 2% HistoGrip™ (Invitrogen, Life Technologies, USA) (Breininger & Baskin 2000). The fixed cells were air-dried and later rinsed twice with 5× SET hybridization buffer (10% Nonidet) and allowed to stand in the buffer for 3 min (Chen et al. 2008). Then, the probe was added to the slide containing the cells. The slide was incubated in a dry bath at 58°C for 30 min. After incubation, the slide was washed twice with a 5× SET buffer.

Subsequently, 1% blocking reagent was added and incubated at room temperature for 30 min. Streptavidin-horseradish peroxidase (HRP) solution was added to the slide and incubated at room temperature for 30 min. The glass slide was then washed with phosphate buffer saline (PBS) that was pre-heated at 37°C. The tyramide working solution (TSA kit with Alexa Fluor® 488 Tyramide; Molecular Probe®, Life Technologies, USA) was then added to the slide in the dark and incubated at room temperature for 10 min. The slide was rinsed again in PBS to remove excess tyramide

working solution. The universal UniC probe (positive control) (5´-/5Biosg/ GWA TTA CCG CGG CKG CTG-3´) and UniR probe (negative control) (5´-/5Biosg/ CAG CMG CCG CGG TAA TWG-3´) were used as controls (Lebaron et al. 1997).

The slides were then observed under an Olympus IX51 microscope equipped with a filter set (470–490 nm excitation and 510–550 nm emission) under UV light. Digital images were captured with an Olympus DP72 digital camera (Olympus).

RESULTS

Species Identification

Two strains of *C. subsalsa* from the Johor Strait were established and used in this study. Cells of the two strains showed similar morphology, with cell dimensions of 36.6 ± 2.9 µm long and 20.5 ± 4.5 µm wide (*n* = 50). Under LM, cells are oval, pear-liked in shape, which is similar to other *C. subsalsa* reported previously (Fig. 1). There are two sub-equal, hetero-dynamic flagella at the anterior of the cells (Fig. 1A). The flagella can only be observed in the living cells. The cells contain many golden-brown chloroplasts, which appear barrel-shaped (Fig. 1B). The nucleus is large and appears oval in shape, located at the middle of the cell (Fig. 1D).

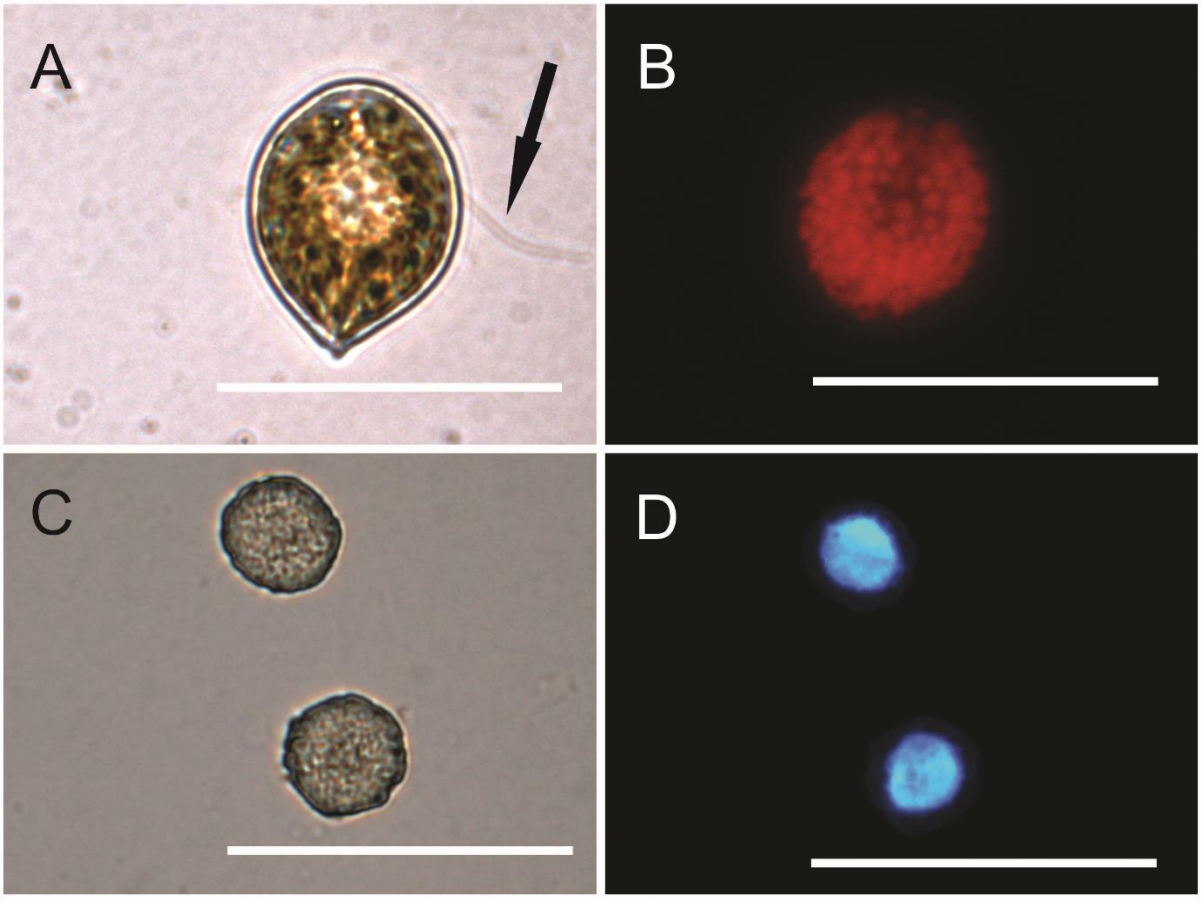

Figure 1. Light and epi-fluorescence micrographs of *Chattonella subsalsa*. (A) Cell with flagellum observed (arrow). (B) Auto-fluorescence micrograph of cell showing the chloroplast. (C–D) Cells with the DAPI-stained nuclei showing the position of nuclei. Scale, 50 μm.

Phylogenetic Inferences of LSU and ITS rDNA

A total of 23 LSU rDNA sequences and 30 sequences of ITS of *Chattonella* were retrieved from the GenBank nucleotide database. Both LSU and ITS rDNA datasets yielded identical tree topologies for maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI); the BI tree is shown in Fig. 2. The trees revealed two monophyletic clades with strong support values (MP/ML/BI, 100/100/1); one clade comprised species in the *C*. *marina* complex: *C. marina* var. *antiqua, C. marina* var. *marina, C. minima, C. marina* var. *ovata*, while the other clade comprised only taxa from *C. subsalsa*. Both LSU and ITS phylogenetic trees showed that *C. subsalsa* strains (CtSg01 and CtSg02) in this study were grouped with other *C. subsalsa* and formed a distinct clade that separated from the strains of *C. marina* complex.

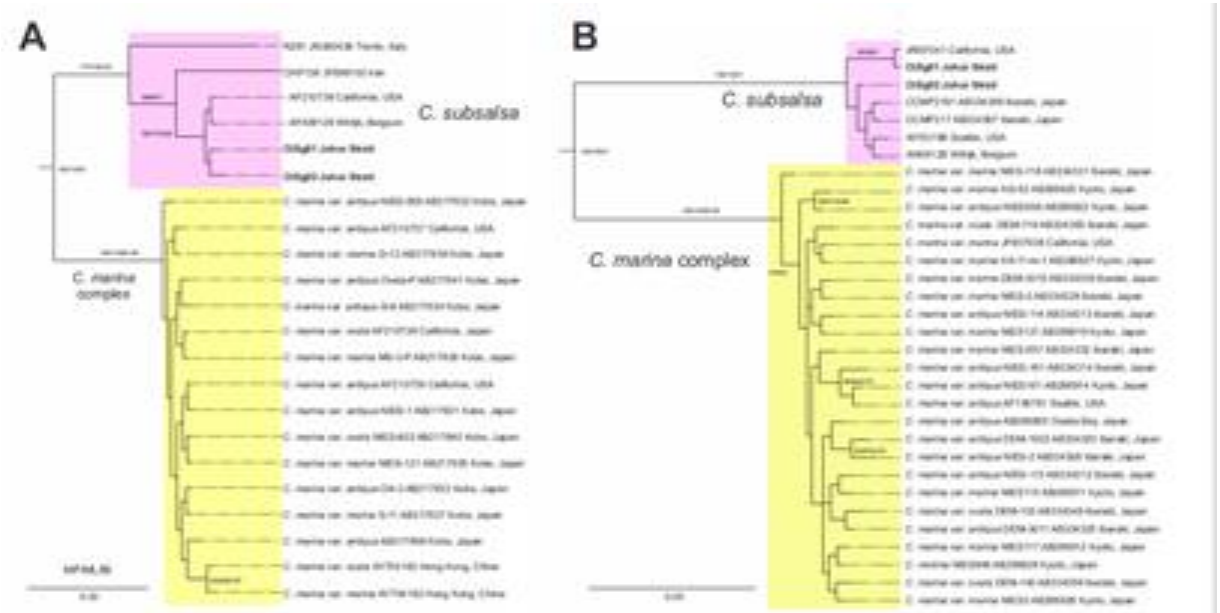

Figure 2 The Bayesian inference of the LSU rDNA dataset (A) and ITS dataset (B) of *Chattonella* species. Outgroups were not shown. Strains of *C*. *subsalsa* obtained in this study are in boldface.

Species-Specific Oligonucleotide Probes of *Chattonella subsalsa*

LSU rRNA signature region and probe

In the first run, a total of 21 candidate sequences of the potential signature regions in the LSU rDNA of *C. subsalsa* were detected from a 730-nucleotide length (Table S2). At least one mismatch was found between the related species, such as *C. marina* var. *antiqua* and *C. marina.* The probes selected *in silico* by ARB contained 18 bases; with GC contents in the range of 50 to 70%. Several of them showed the Gibb energy (ΔG°) greater than -14 kcal/mol, indicative of secondary structure formation (Table S2). A confirmatory test of the probe specificity was performed by blasting in the nucleotide database. The Blastn results showed that the probes selected were not specific to *C. subsalsa* where the probes matched diatom species with 100% coverage and 100% identity.

Therefore, a second attempt of *in silico* analysis was performed with a slight modification of the signature regions. A total of seven candidate sequences were chosen (Table S3). The length of the probes was in the range of 19–23 bases, longer than the first run, to ensure the presence of GC complementary pairs at the start and end of the probe sequences. Subsequently, the parameters of the probes were determined and the specificity of the probes was evaluated through Blastn search. Out of the seven probe candidates, Probe set 7 (5´-GGG GAA UCC GGG UUG GUU UC-3´) was selected (Fig. 3) based on the high GC content (60%), the lowest Gibb energy (ΔG° = -20.2 kcal mol^{-1}), and lower melting point (58.6°C) in contrast to other probes (Table S3). The sequence was further synthesized as a biotinylated probe to perform the FISH assay in the later analysis. The probe was designated as L-S-C.sub-0039-a-A-20 following the Probe Nomenclature (cf. Alm et al. 1996).

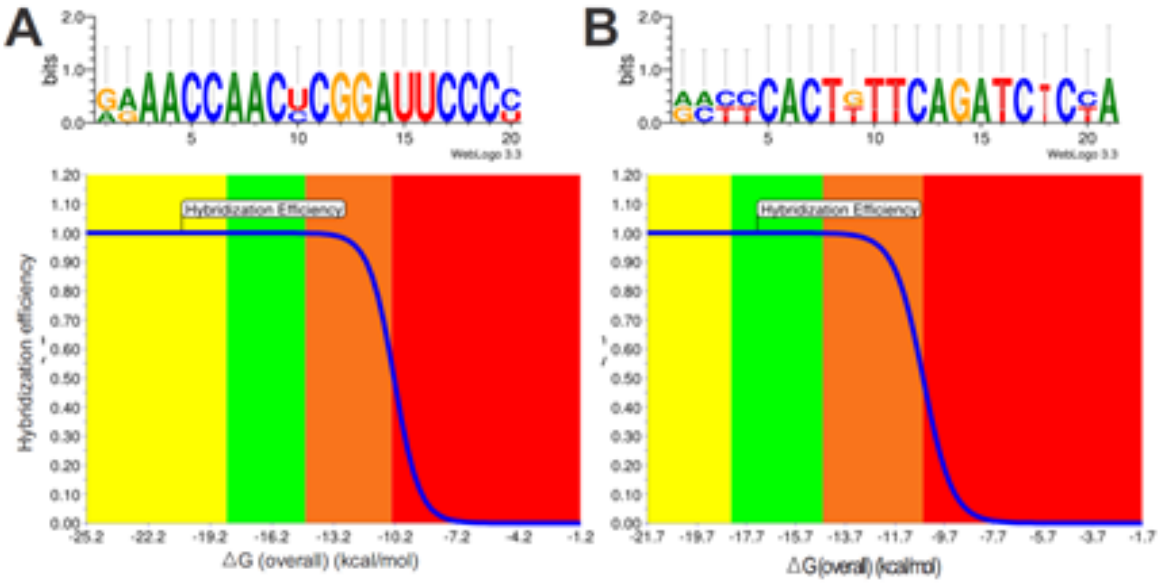

Figure 3 Signature sequences of *Chattonella subsalsa* identified in this study (sequence direction, 5´→3´), (A) L-S-C.sub-0039-a-A-20 and (B) L-S-C.sub-0219-a-A-21; and their hybridization efficiency curves.

ITS2 rRNA signature region and probe

The ITS2 region of the rDNA was used to design a species-specific probe as it is more specific at the species level than the LSU rDNA. In this study, ten candidate sequences of *C. subsalsa* were determined from a 262-bp length ITS2-rDNA complete sequence;

the sequences that are expected to identify the target are listed in Table S4. The candidate sequence length was in the range of 18 to 21 bases. These candidate sequences were then subjected to specificity analysis by performing BLAST comparisons against the nucleotide databases and the results showed that there was no match to other non-target species. Among the ten candidate sequences (Table S4), the probe set 10 (5´-TGG AGA TCT GAA CAG TGA GG- 3´) was chosen because it exhibited lower $\Delta G°$, which is -16.7 kcal mol^{-1}, comprised of 52.4% of GC pair, with a 100% hybridization efficiency. Most importantly, the probe is unique to *C. subsalsa*, and a total of six mismatches were found in the sequence when compared to other non-target species (Fig. 3B). This ITS2 probe was designated as I-S-C.sub-0219-a-A-21 and synthesized as a biotinylated probe for later hybridization experiments.

Tyramide signal amplification-fluorescence in situ hybridization (FISH-TSA)

The FISH-TSA assay with the biotinylated-labeled probes was tested on the clonal cultures of *C. subsalsa*. The species *Heterosigma akashiwo* was used as the non-target species. When treated with the positive-control eukaryotic-universal UniC probe, the hybridized cells of *C. subsalsa* and *H. akashiwo* showed bright green fluorescence signals (Fig. 4). When *C. subsalsa* cells were hybridized with the *C. subsalsa* LSU-rRNA and ITS-rDNA species-specific probes, lime-green fluorescent signals were observed (Fig. 4). In contrast, when the cells were treated with the negative-control UniR probe, it showed chartreuse-yellow fluorescence with low intensity (Fig. 4). When the *C. subsalsa* species-specific probes were tested on *H. akashiwo* cells, chartreuse-yellow fluorescent signals were observed, indicating of negative results (Fig. 5).

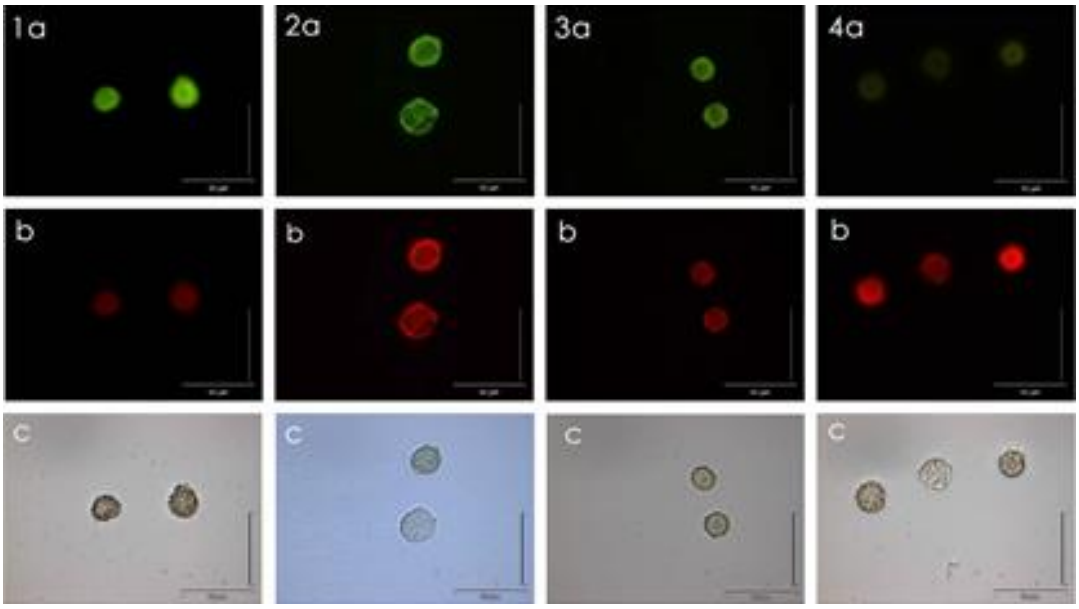

Figure 4. Micrographs of *Chattonella subsalsa* cells treated with UniC positive control (1a), LSU-rRNA probe (2a), ITS2-rDNA probe (3a), and UniR negative control (4a). Cells with chloroplast autofluorescence (1b–4b), LM (1c–4c).

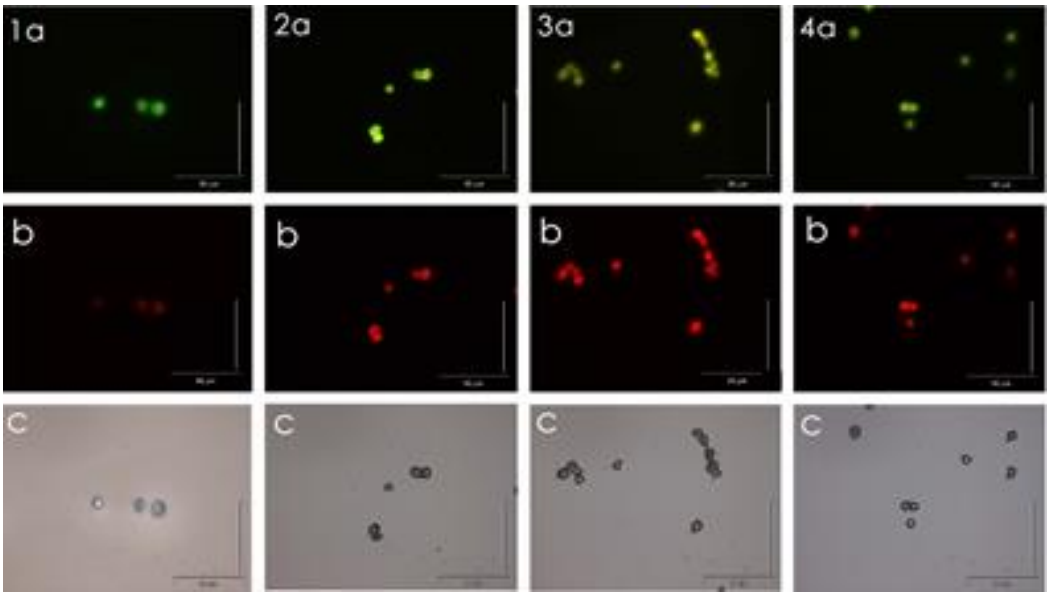

Figure 5. Micrographs of *Heterosigma akashiwo* cells treated with UniC positive control (1a), LSU-rRNA probe (2a), ITS2-rDNA probe (3a), and UniR negative control (4a). Cells with chloroplast autofluorescence (1b–4b), LM (1c–4c).

DISCUSSION

In this study, two species-specific oligonucleotide probes in the LSU-rRNA and ITS2-rDNA were developed to detect the harmful raphidophyte *Chattonella subsalsa*. The probes were applied in the assay of whole-cell fluorescence *in situ* hybridization (FISH) for species detection. The region of LSU-rRNA gene was chosen owing to its universally conserved region while exhibiting some taxon-specific variable regions (Amann & Ludwig 2000). However, the results of the specificity analysis on the LSU-rRNA selected sequences showed cross identity with other *Chattonella* species and diatom species. Therefore, a more taxon-specific rDNA region, the ITS2-rDNA has been selected to design the species-specific probe of *C. subsalsa*.

The biotinylated probes developed in this study have been tested on the *C. subsalsa* cells through the assay of FISH-TSA. The technique of FISH has been widely used in identifying HAB species such as *Pseudo-nitzschia* spp., *Alexandrium* spp., and *Karenia brevis* (Davis) Hansen & Moestrup (Miller & Scholin 1998, Chen et al. 2008). The method, however, has been shown to exhibit less sensitivity when observed under an epi-fluorescence microscope (Lecuyer et al. 2008). The efficiency of FISH, therefore, has been improved by tyramide signal amplification (TSA) to obtain a better resolution in the FISH application (Lecuyer et al. 2008). FISH-TSA is a protocol that enables the detection with a very small probe by signal amplification (Schriml et al. 1999). The biotinylated probes have been designed to achieve the enzymatic action of HRP as they provided strong enzymatically amplified signals and improved the resolution (Kerstens et al. 1995).

In this study, both LSU-rRNA and ITS2-rDNA probes of *C. subsalsa* exhibited positive green-fluorescent signals when hybridized to the cells of *C. subsalsa*. Generally, ITS2-rDNA probe does not give whole-cell fluorescence as it was only hybridized to the

nucleus of the cells. However, cells of *C. subsalsa* that were applied with the ITS2-rDNA probe showed almost whole-cell fluorescence owing to its large nucleus as shown in Fig. 1.

To confirm the specificity of the probes, both *C. subsalsa* species-specific probes were tested with the non-target species *H. akashiwo*. The results showed that *H. akashiwo* was observed as light-yellow fluorescence when tested with the ITS2-rDNA probe, as like the negative control. This showed that the ITS2-rDNA probe was specific only to *C*. *subsalsa*. But when tested with the LSU-rRNA probe, it showed yellow-green fluorescence that was difficult to evaluate if the result was positive or negative. It is thus suggested that the ITS2-rDNA probe is better than the LSU-rRNA probe in detecting *C*. *subsalsa*.

The assay of FISH-TSA was applied on microscope glass slides throughout the study. This method has been previously described in Chen et al. (2008) that applied to *H. akashiwo* cells. The cell harvesting procedures such as centrifugation and filtration that were previously applied to the armoured dinophyte *Alexandrium* and the diatom *Pseudo-nitzschia* (Miller & Scholin 1998) were less suitable in this case as cells tend to burst when undergone centrifugation or filtration.

Several factors affect the efficiency of FISH-TSA. Physiological growth conditions of the cells are among the factors that affect FISH-TSA detection (Sako et al. 2004, Chen et al. 2008). Kim et al. (2004) reported that higher fluorescent intensity has been observed in the exponentially grown cells than those in the stationary phase. The low fluorescent intensity of the cells was likely due to the decreasing rRNA content in stationary-phased cells (Anderson et al. 1999).

To conclude, the species-specific oligonucleotide probe of *C. subsalsa* was successfully designed in the ITS2-rDNA region. The results of this study revealed that the ITS2 probe was more specific as compared to the LSU probe. The strong fluorescent signal in FISH-TSA also proves its efficiency in detecting harmful algal species from the environmental samples. Future field applications should be carried out to further evaluate the feasibility of this assay for HAB monitoring purposes.

ACKNOWLEDGEMENTS

This work was supported by the Malaysian Ministry of Higher Education LRGS [LRGS/1/2020/UMT/01/1/3] and HiCOE [IOES-2014C] to PT Lim.

AUTHOR CONTRIBUTIONS

Lau WLK: Writing – draft, Investigation. Teng ST: Methodology, Visualization. Lim HC: Methodology. Hii KS: Formal analysis. Leong SCY: Writing – review & editing, Resources. Leaw CP: Conceptualization, Writing – review & editing, Validation, Supervision. Lim PT: Writing – review & editing, Conceptualization, Resources, Funding acquisition, Supervision.

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Supplementary Materials

Table S1. LSU-rDNA (D1–D3) and ITS sequences of *Chattonella* species that were used in this study, with strain, location, GenBank accessions, and references. * Sequence with direct submission.

Species	Strain	LSU Accession	ITS Accession	Location	References
C. marina var.*antiqua*	–	–	AF136761	Seattle, USA	Connell, L. 1999
	–	AF210737	–	California, USA	Tyrrell et al. 1999*
	–	AB217868	–	Kobe, Japan	Tanabe et al. 2005*
	NIES–558	AB217632	–	Kobe, Japan	Tanabe et al. 2005
	Ovata–P	AB217641	–	Kobe, Japan	Tanabe et al. 2005*
	NIES–1	AB217631	–	Kobe, Japan	Tanabe et al. 2005*
	G–8	AB217634	–	Kobe, Japan	Tanabe et al. 2005*
	OA–3	AB217633	–	Kobe, Japan	Tanabe et al. 2005*
	NIES558	–	AB286922	Kyoto, Japan	Kamikawa et al. 2007
	NIES161	–	AB286914	Kyoto, Japan	Kamikawa et al. 2007
	NIES–161	–	AB334314	Ibaraki, Japan	Demura & Kawachi. 2007*
	NIES–114	–	AB334313	Ibaraki, Japan	Demura & Kawachi. 2007*
	NIES–113	–	AB334312	Ibaraki, Japan	Demura & Kawachi. 2007*
	NIES–2	–	AB334308	Ibaraki, Japan	Demura & Kawachi. 2007*
	–	–	AB286905	Osaka Bay, Japan	Kamikawa et al. 2007
	DEM–3011	–	AB334325	Ibaraki, Japan	Demura & Kawachi. 2007*
	DEM–1002	–	AB334320	Ibaraki, Japan	Demura & Kawachi. 2007*
C. marina	KG–52	–	AB286920	Kyoto, Japan	Kamikawa et al. 2007
	–	AF210739	–	California, USA	Tyrrell et al. 1999*
	MS–3–P	AB217639	–	Kobe, Japan	Tanabe et al. 2005*
	G–12	AB217638	–	Kobe, Japan	Tanabe et al. 2005*
	S–11	AB217637	–	Kobe, Japan	Tanabe et al. 2005*
	KA–11–m–1	–	AB286927	Kyoto, Japan	Kamikawa, R. 2007
	NIES3	–	AB286926	Kyoto, Japan	Kamikawa, R. 2007
	NIES121	–	AB286919	Kyoto, Japan	Kamikawa et al. 2007
	NIES117	–	AB286912	Kyoto, Japan	Kamikawa et al. 2007
	NIES115	–	AB286911	Kyoto, Japan	Kamikawa et al. 2007

	DEM–3015	–	AB334339	Ibaraki, Japan	Demura & Kawachi. 2007*
	NIES–557	–	AB334332	Ibaraki, Japan	Demura & Kawachi. 2007*
	NIES–118	–	AB334331	Ibaraki, Japan	Demura & Kawachi. 2007*
	NIES–3	–	AB334328	Ibaraki, Japan	Demura & Kawachi. 2007*
	NIES–121	AB217635	–	Kobe, Japan	Tanabe et al. 2005*
	–	AY704162	–	Hong Kong, China	Cheung et al. 2004*
	S–11	AB217637	–	Kobe, Japan	Tanabe et al. 2005*
	–	–	JF907038	California, USA	Band–Schmidt et al. 2011*
C. minima	NIES848	–	AB286928	Kyoto, Japan	Kamikawa et al. 2007
C. marina var. *ovata*	DEM–140	–	AB334359	Ibaraki, Japan	Demura & Kawachi. 2007*
	NIES–603	AB217640	–	Kobe, Japan	Tanabe et al. 2005
	DEM–119	–	AB334355	Ibaraki, Japan	Demura & Kawachi. 2007*
	DEM–103	–	AB334349	Ibaraki, Japan	Demura & Kawachi. 2007*
	–	AF210738	–	California, USA	Tyrrell et al. 1999*
C. marina var. *ovata*	–	AY704163	–	Hong Kong, China	Cheung et al. 2004*
C. sp. R281	–	JN390438	–	Trento, Italy	D'Alelio. 2011*
C. subsalsa	–		AF153196	Seattle, USA	Connell, L. 2000
	–	AF409126	AF409126	Wilrijk, Belgium	Ben et al. 2002
	–	AF210736	–	California, USA	Tyrrell et al. 1999*
	CCMP217	–	AB334367	Ibaraki, Japan	Demura & Kawachi. 2007*
	–	–	JF907041	California, USA	Band–Schmidt et al. 2011*
	CCMP2191	–	AB334368	Ibaraki, Japan	Demura & Kawachi. 2007*

Table S2. First *in silico* selection of the LSU-rRNA probe signature regions of *Chattonella subsalsa*, with probe length constraint to 18 bases and their parameters.

Set	Target sequence (5'–3')	Probe sequence (5'–3')	GC content (%)	ΔG° (kcal/mol)	Hybridization efficiency	T_m (°C)	position in alignment (5'–3')
1	CUUGAAACACGGGACCAA	UUGGUCCCGUGUUUCAAG	50	–7.2	0.0376	52.8	711–728
2	GUCUUGAAACACGGGACC	GGUCCCGUGUUUCAAGAC	55.6	–11.4	0.9697	53.4	709–726
3	UCUUGAAACACGGGACCA	UGGUCCCGUGUUUCAAGA	50	–14	0.9995	53.8	710–727
4	UGAAACACGGGACCAAGG	CCUUGGUCCCGUGUUUCA	55.6	–17.3	1	55.1	713–730
5	UUGAAACACGGGACCAAG	CUUGGUCCCGUGUUUCAA	50	–14.5	0.9998	52.8	712–729
6	CGUCUUGAAACACGGGAC	GUCCCGUGUUUCAAGACG	55.6	–13.4	0.9987	50.9	708–725
7	CCGUCUUGAAACACGGGA	UCCCGUGUUUCAAGACGG	55.6	–12.3	0.9928	55	707–724
8	CCCGUCUUGAAACACGGG	CCCGUGUUUCAAGACGGG	61.1	–11.9	0.9853	56.3	706–723
9	GAAAAGAAACCAACCCGG	CCGGGUUGGUUUCUUUUC	50	–18.1	1	51.5	34–51
10	GGAAAAGAAACCAACCCG	CGGGUUGGUUUCUUUUCC	50	–17.2	1	51.5	33–50
11	AAACCAACCCGGAUUCCC	GGGAAUCCGGGUUGGUUU	55.6	–17.1	1	55.4	40–57
12	AACCAACCCGGAUUCCCC	GGGGAAUCCGGGUUGGUU	61.1	–18.1	1	57.8	41–58
13	AACCCGGAUUCCCCUAGU	ACUAGGGGAAUCCGGGUU	55.6	–16.1	1	55.3	45–62
14	ACCAACCCGGAUUCCCCU	AGGGGAAUCCGGGUUGGU	61.1	–18	1	59	42–59
15	ACCCGGAUUCCCCUAGUA	UACUAGGGGAAUCCGGGU	55.6	–15.3	0.9999	54.4	46–63
16	AGAAACCAACCCGGAUUC	GAAUCCGGGUUGGUUUCU	50	–15.9	1	52.3	38–55
17	CAACCCGGAUUCCCCUAG	CUAGGGGAAUCCGGGUUG	61.1	–16.3	1	54.9	44–61
18	CCAACCCGGAUUCCCCUA	UAGGGGAAUCCGGGUUGG	61.1	–16.6	1	56.6	43–60
19	CCCGGAUUCCCCUAGUAA	UUACUAGGGGAAUCCGGG	55.6	–13	0.9976	53	47–64
20	CCGGAUUCCCCUAGUAAC	GUUACUAGGGGAAUCCGG	55.6	–13.1	0.9978	51.6	48–65
21	**GAAACCAACCCGGAUUCC**	**GGAAUCCGGGUUGGUUUC**	**55.6**	**–16.2**	**1**	**53.6**	**39–56**

Table S3. Second *in silico* selection of the LSU-rRNA probe signature regions of *Chattonella subsalsa* and their parameters.

Set	Target Sequence (5'–3')	Probe Sequence (5'–3')	GC Content (%)	ΔG° (kcal/mol)	Hybridization efficiency	T_m (°C)	Length (bp)	Position in alignment (5'–3')
1	CCAACCCGGAUUCCCCUAG	CUAGGGGAAUCCGGGUUGG	63.2	–18.4	1	57.4	19	43–61
2	GAACCAACCCGGAUUCCCC	GGGGAAUCCGGGUUGGUUC	63.2	–19	1	58.5	19	39–59
3	GAAAAGAAACCAACCCGGAUUC	GAAUCCGGGUUGGUUUCUUUUC	45.5	–19.7	1	54.5	22	34–55
4	CCAACCCGGAUUCCCCUAGTAAC	GUUACUAGGGGAAUCCGGGUUGG	56.5	–20.2	1	59.1	23	43–65
5	CCAACCCGGAUUCCCCUA	UAGGGGAAUCCGGGUUGG	61.1	–16.6	1	56.6	18	43–60
6	CCCGGAUUCCCCUAGUAACGG	CCGUUACUAGGGGAAUCCGGG	61.9	–19	1	59.1	21	47–67
7	**GAAACCAACCCGGAUUCCCC**	**GGGGAAUCCGGGUUGGUUUC**	**60**	**–20.2**	**1**	**58.6**	**20**	**39–58**

Table S4 *In silico* selection of the ITS2-rDNA probe signature regions of *Chattonella subsalsa* and their parameters.

Set	Target sequence (5'–3')	Probe sequence (5'–3')	GC (%)	ΔG° (kcal/mol)	Hybridization efficiency	T_m (°C)	Length (bp)	Position in alignment (5'–3')
1	CCGCCTCACTGTTCAGAT	ATCTGAACAGTGAGGCGG	55.6	–8.2	0.0864	54.6	18	217–234
2	CCTCACTGTTCAGATCTC	GAGATCTGAACAGTGAGG	50	–12.3	0.985	49.1	18	220–237
3	CGCCTCACTGTTCAGATC	GATCTGAACAGTGAGGCG	55.6	–9.6	0.4953	52.9	18	218–235
4	CTCACTGTTCAGATCTCC	GGAGATCTGAACAGTGAC	50	–12	0.9773	49.4	18	221–238
5	GCCTCACTGTTCAGATCT	AGATCTGAACAGTGAGGC	50	–12.8	0.9934	51.4	18	219–236
6	GGTGGCTCTGCCGCCTCACT	AGTGAGGCGGCAGAGCCACC	70	–13.8	0.9985	65.0	20	207–226
7	GTGGCTCTGCCGCCTCACTG	CAGTGAGGCGGCAGAGCCAC	70	–12.7	0.992	63.5	20	208–227
8	TGGCTCTGCCGCCTCACTGT	ACAGTGAGGCGGCAGAGCCA	65	–13.9	0.9987	64.1	20	209–228
9	CCGCCTCACTGTTCAGATCTC	GAGATCTGAACAGTGAGGCGG	57.1	–11.5	0.9443	57.4	21	217–237
10	**GCCTCACTGTTCAGATCTCCA**	**TGGAGATCTGAACAGTGAGGC**	**52.4**	**–16.7**	**1**	**56.5**	**21**	**219–239**