Detection of *Gambierdiscus* and *Fukuyoa* single cells using recombinase polymerase amplification combined with a sandwich hybridization assay

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Abstract

Dinoflagellates of the genera *Gambierdiscus* and *Fukuyoa* are known to produce several bioactive compounds including the potent neurotoxic ciguatoxins (CTXs), which are able to accumulate in fish and through the food web. When humans ingest fish contaminated with CTXs, it can result in an intoxication named ciguatera. Although not all the currently recognized species are able to produce toxins, *G. australes* and *G. excentricus* have been highlighted to be the most abundant and toxic among the species present in the Atlantic. Even though genus *Gambierdiscus* and *Fukuyoa* are endemic to tropical areas, recently their presence was recorded in subtropical and temperate regions. In this work, the development of three molecular assays for the detection of the *Gambierdiscus* and *Fukuyoa* genera and for *G. australes* and *G. excentricus* species, based on the combination of recombinase polymerase amplification with detection via hybridization, is successfully described. Furthermore, a remarkable limit of detection of a single cell was achieved. Additionally, six different species have been used to check the ability of each primer set to give an amplified product, even in presence of potentially interfering non-target DNAs. Therefore, these developments provide a rapid and cost-effective strategy for detection of both genera and two of the most toxic species, which will undoubtedly contribute to reliable screening of samples and ciguatera risk assessment, guaranteeing seafood safety and protection of human health.

Keywords

Gambierdiscus, *Fukuyoa*, ciguatera, recombinase polymerase amplification (RPA), species-specific molecular assay, single-cell detection

Declarations

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Conflicts of interest/Competing interests

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

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Authors' contributions

GG: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing

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1. Introduction

Marine dinoflagellates are well-established as toxin producers, and have thus attracted the attention of researchers worldwide, with the epibenthic genera *Gambierdiscus* and *Fukuyoa* being of particular interest due to their ability to produce the potent neurotoxic ciguatoxins (CTXs) (Chinain et al. 2010; Yasumoto et al. 2000; Yogi et al. 2011), maitotoxins (MTXs) (Holmes and Lewis 1994; Murata et al. 1993; Pisapia et al. 2017b), and other bioactive compounds (Nagai et al. 1992; Satake et al. 1993; Watanabe et al. 2013). When *Gambierdiscus* and *Fukuyoa* cells are grazed by herbivorous and detritivorous fish, these toxins accumulate through the food web, potentially reaching humans and causing one of the most common foodborne diseases, known as ciguatera (Begier et al. 2006; Larsson et al. 2019; Lewis 2001; Smith et al. 2017). The presence of *Gambierdiscus* and *Fukuyoa* in tropical areas is well known (Lewis 2001; Stewart et al. 2010; Vandersea et al. 2012). However, in the past decade, *Gambierdiscus* and *Fukuyoa* have been recorded in subtropical and temperate regions, such as the Canary Islands (Fraga and Rodriguez 2014; Fraga et al. 2011; Litaker et al. 2017), Madeira(Kaufmann and Böhm-Beck 2013), the Mediterranean Sea (Aligizaki and Nikolaidis 2008; Aligizaki et al. 2009; Laza-Martínez et al. 2016; Tudó et al. 2018), the Gulf of Mexico(Gómez et al. 2015; Litaker et al. 2017; Litaker et al. 2009), Japan (Nishimura et al. 2014), Brazil (Gómez et al. 2015; Laza-Martínez et al. 2016) and the coast of North Carolina(Litaker et al. 2017; Litaker et al. 2009). The reason behind the increase of these new findings is still unclear. Whether this is due to a worldwide expansion of these genera or because more intense samplings have been performed in the last few years, global warming has most certainly played and will continue to play a role in favor of their proliferation. This will create changes in the diversity and distribution of *Gambierdiscus* and *Fukuyoa* species, resulting in the spread of those species in new areas and potentially increasing the occurrence of ciguatera.

Progress in the field has underlined the existence of 18 species of *Gambierdiscus* (Chinain et al. 1999; Fraga et al. 2011; Jang et al. 2018; Kretzschmar et al. 2019; Litaker et al. 2009; Nishimura et al. 2014; Rhodes et al. 2017) and 3 species of *Fukuyoa* (Gómez et al. 2015). Whilst only some species have been demonstrated to be toxic (*F. paulensis, G. australes, G. caribaeus, G. excentricus, G. pacificus, G. polynesiensis* and *G. toxicus*) (Chinain et al. 2010; Fraga et al. 2011; Gaiani et al. 2020; Litaker et al. 2017; Longo et al. 2019; Pisapia et al. 2017a; Rhodes et al. 2014; Rossignoli et al. 2020; Sibat et al. 2018), the ability to detect these genera and discriminate among *Gambierdiscus* and *Fukuyoa* species in field samples is of utmost interest. Light microscopy (LM) and electron microscopy allow the identification and discrimination between *Gambierdiscus* and *Fukuyoa* genera, but species identification is almost impossible using those methods. Therefore, the use of genetic sequencing techniques is practically mandatory for the correct identification of *Gambierdiscus* and *Fukuyoa* cells (Bravo et al. 2019). As an alternative approach to save time and resources, molecular assays, based mainly on the use of quantitative polymerase chain reaction (qPCR), have appeared for the identification and quantification of *Gambierdiscus* and *Fukuyoa* genera or species. These assays have been demonstrated to detect *G. belizeanus*, *G. caribaeus*, *G. carpenteri*, *G. carolinianus*, *G. ruetzleri* and *Gambierdiscus* sp. ribotype 2 (Vandersea et al. 2012), *G. australes*, *G. scabrosus*, *Gambierdiscus* sp. type 2 and *Gambierdiscus* sp. type 3 (Nishimura et al. 2016), *Gambierdiscus/Fukuyoa* and *F. paulensis* (Smith et al. 2017), *G. excentricus* and *G. silvae* (Litaker et al. 2019) and *G. lapillus* (Kretzschmar et al. 2019).

PCR-based methods require the use of a thermocycler, often laboratory based, and can require several hours to perform, with this delay resulting in a lengthy period between sampling and the

analysis of results. Isothermal DNA amplification techniques may overcome these limitations. These techniques facilitate rapid DNA amplification at a constant temperature, requiring less time and power than conventional PCR. Although handheld PCR-based devices are commercially available, the use of a constant temperature for DNA amplification could simplify the hardware. Among isothermal DNA amplification techniques, recombinase polymerase amplification (RPA) is very convenient as it does not require any initial denaturation step, can be carried out at 22 – 45 °C without any need for tight temperature control, only requires two primers, and can be completed in 15-30 min. In this work, we exploited the use of primers modified with short oligonucleotide tails, which result in double-stranded DNA (dsDNA) amplicons fringed with single-stranded DNA (ssDNA) tails, avoiding the need for denaturation of the amplified products to generate ssDNA for detection by hybridization. The detection is achieved using a sandwich hybridization assay (SHA), where specific surface-anchored thiolated capture probes are complementary to one of the amplicon tails and an enzyme-labelled reporter probe is complementary to the tail in the other extreme (Fig. 1). Despite its undeniable advantages, this approach has been barely used for the detection of marine toxic dinoflagellates (Toldrà et al. 2019a; Toldrà et al. 2019b; Toldrà et al. 2018).

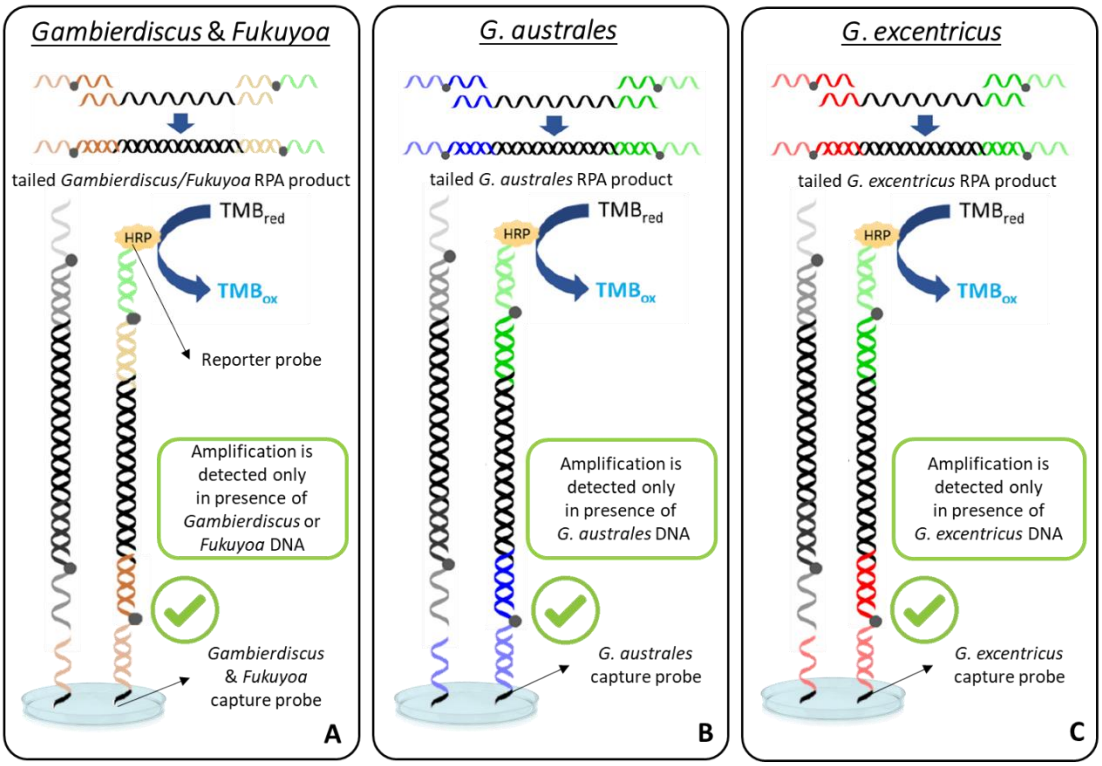

Fig. 1 The three systems used in this work. (A) *Gambierdiscus* & *Fukuyoa* primer set amplifies DNA from all the species of both genera and it does not detect DNA from other species. (B) *G. australes* and (C) *G. excentricus* primer sets amplify only their target DNA and they do not detect non-target species or genera

The availability of an assay capable of detecting the presence of all species of the genera *Gambierdiscus* and *Fukuyoa* can provide information of the general composition of a field sample. Additionally, since *G. excentricus* and *G. australes* have consistently demonstrated the ability to produce toxic compounds (Chinain et al. 1999; Gaiani et al. 2020; Pisapia et al. 2017a; Rhodes et al. 2014; Rossignoli et al. 2020), and their range is rapidly expanding (Hoppenrath et al. 2019; Rodríguez et al. 2017), a rapid assay for their simultaneous and discriminable detection will be helpful in assessing the risk of a ciguatera outbreak. Herein, we present the development

of three molecular assays based on the RPA-SHA strategy for the detection of the *Gambierdiscus* & *Fukuyoa* genera, and the *G. australes* and *G. excentricus* species. The specificity of the assays has been characterized using clonal cultures of different *Gambierdiscus* species (*G. australes*, *G. balechii*, *G. belizeanus*, *G. caribaeus* and *G. excentricus*) and *F. paulensis*, as well as *Coolia monotis*, *Ostreopsis* cf. *ovata* and *Prorocentrum lima* as non-target genera. The detection and identification of single cells from clonal cultures has been evaluated. Finally, the amplification capacity of the three different primer sets and the discrimination ability of the species-specific primer sets have been proved with several mixtures of DNA from six different target species.

2. Material and methods

2.1. Microalgal cultures

Several microalgal strains were used in this work, obtained from IRTA collection (IRTA-SMM) and the Culture Collection of Microalgae of the Instituto Español de Oceanografía (CCVIEO) in Vigo, Spain (VGO) (Table 1). Clonal cultures were grown in polystyrene flasks containing 500 mL of modified ES medium (Provasoli 1968) prepared with filtered and autoclaved seawater from L'Ametlla de Mar, Spain (salinity adjusted at 36). Cultures were maintained at 24 ± 1 °C under a photon flux rate of 100 µmol m^{-2} s^{-1} with a 12:12h light:dark regime. Once the cultures reached the late exponential phase (*ca.* 21 days), 5 µL containing single cells were isolated from some of them. Isolations were performed under the microscope with the aid of a micropipette. The 5-µL drop containing the cell was stored in PCR tubes at -20 °C until DNA extraction. Culture aliquots were fixed with 3% Lugol's iodine and counted using a Kolkwitz chamber (Hydro-Bios, Altenholz, Germany) under an inverted light microscope (Leica DMIL, Spain), following the Sedgwick-Rafter method (Greeson 1977). Additionally, microalgal pellets were obtained by harvesting the entire culture volume in 50 mL tubes and centrifuging at 2,500 rpm for 25 min (Allegra X-15R, Beckman Coulter, Brea, USA). Supernatants were discarded and tubes were stored at -20 °C until DNA extraction.

Table 1. Microalgae strains used in this study.

Species	Strain	Sampling location and year	GenBank accession number	Sequenced region	Source
G. australes	IRTA-SMM-13_07	Selvagem Grande Island, Portugal, 2013	KY564320	D1-D3	Reverté et al. (2018)
G. australes	IRTA-SMM-13_17	Selvagem Grande Island, Portugal, 2013	KY564328	D1-D3	Reverté et al. (2018)
G. australes	IRTA-SMM-16_286	Lanzarote, Spain, 2016	MT119197	D8-D10	Gaiani et al. (2020)
G. australes	IRTA-SMM-17_164	Menorca, Spain, 2017	MG708120	D8-D10	Tudó et al. (2018)
G. balechii	VGO920	Manado, Indonesia, 2007	KX268469	D8-D10	Fraga et al. (2016)
G. belizeanus	IRTA-SMM-13_19	La Réunion, France, 2013	MW350058	D8-D10	This study
G. belizeanus	IRTA-SMM-17_421	El Hierro, Spain 2017	MT379471	D8-D10	Tudó et al. (2020a)
G. caribaeus	IRTA-SMM-17_03	El Hierro, Spain 2017	MT119203	D8-D10	Gaiani et al. (2020)
G. excentricus	IRTA-SMM-17_01	Gran Canaria, Spain, 2017	MT119198	D8-D10	Gaiani et al. (2020)
G. excentricus	IRTA-SMM-17_126	Gran Canaria, Spain, 2017	MT119199	D8-D10	Gaiani et al. (2020)
G. excentricus	IRTA-SMM-17_407	La Gomera, Spain, 2017	MT119200	D8-D10	Gaiani et al. (2020)
G. excentricus	IRTA-SMM-17_428	La Gomera, Spain, 2017	MT119201	D8-D10	Gaiani et al. (2020)
G. excentricus	IRTA-SMM-17_432	La Gomera, Spain, 2017	MT119202	D8-D10	Gaiani et al. (2020)
G. excentricus	VGO791	Tenerife, Spain, 2004	JF303066; JF303075	D1-D3; D8-D10	Fraga et al. (2011)
F. paulensis	IRTA-SMM-17_206	Mallorca, Spain, 2017	MT119204	D8-D10	Tudó et al. (2020b)
F. paulensis	IRTA-SMM-17_211	Menorca, Spain, 2017	MT119205	D8-D10	Tudó et al. (2020b)
F. paulensis	IRTA-SMM-17_220	Menorca, Spain, 2017	MT119206	D8-D10	Tudó et al. (2020b)
F. paulensis	VGO1185	Ubatuba, Brazil, 2013	KM886379	18S; D1-D4; ITS	Gómez et al. (2015)
C. monotis	IRTA-SMM-16_285	Formentera, Spain, 2016	MW328563	ITS	This study
O.* cf. *ovata	IRTA-SMM-16_133	Catalonia, Spain, 2016	MH790463	ITS	Toldrà et al. (2019b)
P. lima	IRTA-SMM-17_47	Lanzarote, Spain, 2017	MW328564	ITS	This study

2.2. DNA extraction

Extraction of genomic DNA from microalgal pellets was performed using a bead beating system and the phenol/chloroform method (Toldrà et al. 2019a). Briefly, cell pellets were re-suspended in 200 µL of lysis buffer (1 M NaCl, 70 mM Tris, 30 mM EDTA, pH 8.6) and moved to an extraction tube containing zirconium beads (0.5 mm in diameter). Subsequently, 25 µL of 10% *w*/*v* DTAB and 200 µL of chloroform were added for cellular disruption using a Bead Beater-8 (BioSpec, Bartlesville, USA) for 45 s at full speed. Disrupted cells were then centrifuged at 2,300 rpm for 5 min (Eppendorf 5415D, Hamburg, Germany), the aqueous phase was transferred to a fresh tube and DNA was extracted using standard phenol/chloroform method (Sambrook et al. 1989). Precipitation of the DNA was obtained by the addition of 2 volumes of absolute ethanol and 0.1 volumes of 3 M sodium acetate (pH 8.0). The DNA was rinsed with 70% *v*/*v* ethanol and then dissolved in 50 µL of molecular DNAse/RNAse-free water. Extracted DNA samples (50 µL) were quantified and checked for their purity using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Madrid, Spain). Extracted DNA was stored at -20 °C until analysis.

Extraction of genomic DNA from single microalgal cells was performed using an Arcturus® PicoPure® DNA Extraction Kit (Thermo Fisher Scientific, Madrid, Spain) following the manufacturer's instructions. Briefly, 155 µL of reconstitution buffer were added to one of the kit vials containing Proteinase K and mixed. Once the pellet had been dissolved, 15 µL of the obtained solution were added to each tube containing a single cell. DNA extraction was performed with a Nexus Gradient Thermal Cycler (Eppendorf, Spain) at 65 °C for 3 h and then 95 °C for 10 min. Extracted DNA was stored at -20 °C until analysis (Tudó et al. 2018).

2.3. Primers and probes

Primers were designed within the D8-D10 (*Gambierdiscus & Fukuyoa*) and D1-D3 (*G. australes* and *G. excentricus*) regions of the 28 S LSU ribosomal DNA (rDNA) gene and synthesized by Biomers (Ulm, Germany). Three primer sets were used: one for *Gambierdiscus* & *Fukuyoa* genera, one for *G. australes*, and one for *G. excentricus*. *G. australes* and *G. excentricus* primer sets shared the same reverse primer. Primers were subsequently modified with oligonucleotide tails to enable direct detection of the RPA product. Each primer set had its individual cognate capture probe, which hybridizes with the corresponding primer tail. The reporter probe was common among all primer sets and hybridizes with all primer tails. The primers are between 23 and 26 bp long and amplify a product of around 150 bp. Tails and probes were tested using Multiple Primer Analyser Software (Themo Fisher Scientific) to confirm absence of cross-reactivity with primers and target sequences. The primers and probes used are listed in Table 2.

Table 2. Primers with tails and probes used in this study. Tails are underlined.

Name	Sequence (5'-3')
***G. australes* Reverse primer**	GTT TTC CCA GTC ACG AC-C3-ATG CAT AAC TCT TCA TTG CCA GTA G
***G. excentricus* Reverse primer**	TCT ACA GGC TCG TAT ATG TA-C3-AGC TTG GGT CAC AGT GCA ACA GAG
***G. australes & G. excentricus* Forward primer**	TGT AAA ACG ACG GCC AGT-C3-TGC TGC ATG YGG AGA TTC TTT YYT KG
***Gambierdiscus* & *Fukuyoa* Forward primer**	ATA GGC TGG TTC GTA ATC GG-C3-GAY NCG GAC AAG GGG AAT CCG AC
***Gambierdiscus* & *Fukuyoa* Reverse primer**	TGT AAA ACG ACG GCC AGT-C3-GAG AGT CAT AGT TAC TCC CGC CG
***G. australes* capture probe**	GTC GTG ACT GGG AAA ACT TTT TTT TTT TTT TT-C3-thiol
***G. excentricus* capture probe**	TAC ATA TAC GAG CCT GTA GAT TTT TTT TTT TTT TT-C3-thiol
***Gambierdiscus* & *Fukuyoa* capture probe**	CCG ATT ACG AAC CAG CCT ATT TTT TTT TTT TTT TT-C3-thiol
Reporter probe	HRP-ACT GGC CGT CGT TTT ACA

2.4. Recombinase Polymerase Amplification (RPA)

DNA was amplified with RPA using the TwistAmp Liquid Basic kit (TwistDx Ltd, San Diego, USA). Each reaction contained: 3.1 µL of DNAse/RNAse-free water, 4.5 µL of dNTPs at 1.8 mM, 25 µL of rehydration buffer, 5 µL of Basic E-mix, 2.5 µL of Core reaction mix, 1.2 µL of each primer at 480 nM, 5 µL of genomic DNA at 1 ng/µL or solution with DNA extracted from single cells, and finally 2.5 µL of 14 mM magnesium acetate was used to initiate the RPA reaction. The total volume for each reaction was 50 µL. Non-target controls (NTCs, only DNAse/RNAse-free water) were included in the experimental design. Samples were isothermally amplified for 30 min at 37 °C. Following amplification, RPA products were purified using a GeneJet PCR purification kit (Thermo Fisher Scientific, Madrid, Spain) following the manufacturer´s instructions, ending with 50 µL of DNA in TE buffer after the final elution step.

2.5. Sandwich hybridization assay (SHA)

Three assays were developed: one for the detection of the genera *Gambierdiscus* & *Fukuyoa,* one for the detection of the species *G. australes* and a last one for the detection of the species *G. excentricus.* In these assays, the amplicons obtained in the respective RPA reactions were incubated with the corresponding surface-anchored thiolated capture probe. Thiolated capture probes were prepared in PBS (pH 7.4, 100 mM phosphate, 150 mM NaCl) at a concentration of 500 nM, and 50 µL of this solution were added to the wells of a maleimide-coated plate (Pierce maleimide-activated microtitre plates from Thermo Fisher Scientific, Madrid, Spain) and incubated overnight at 4 °C on a microplate shaker under gentle agitation. Blocking of the non-functionalised maleimide groups was performed in two different steps, first via the addition of 200 µL of 100 µM 6-mercapto-1-hexanol in Milli-Q water and secondly 200 µL of 5% *w*/*v* skimmed milk in PBS. Subsequently, 45 µL of RPA product was added to the microtiter wells, followed by addition of 50 µL of 10 nM HRP-conjugated reporter probe in PBS containing 0.05% *v*/*v* Tween-20 (PBS-Tween). Three washing steps were performed between each step. All incubations, except for capture probe immobilization, were performed at room temperature for 30 min on a microplate shaker under gentle agitation. Finally, 100 µL of TMB liquid substrate were added and after 10 min, the absorbance was measured at 620 nm using a Microplate Reader KC4 (BIO-TEK Instruments, Winooski, USA). Gene 5 software was used to collect and evaluate the data.

2.6. Molecular identification

To identify microalgae at species level, which had already been recognized at genus level by light microscopy as either *Gambierdiscus* or *Fukuyoa*, the D8-D10 domain of the 28S LSU rDNA gene was amplified by PCR using the pair of primers FD8/RB (5'-GGATTGGCTCTGAGGGTTGGG-3' and 5'- GATAGGAAGAGCCGACATCGA-3') (Chinain et al. 1999). For microalgae cells other than *Gambierdiscus* and *Fukuyoa*, the D2C (5'-CCTTGGTCCGTGTTTCAAGA- 3') (Chomérat et al. 2010), and D1R (5'- ACCCGCTGAATTTAAGCATA-3') (Scholin et al. 1994) primers were used. In both cases, the 25 µL reaction mixtures contained 600 µM dNTP, 2 mM $MgCl_2$, 0.2 µM of each primer, 1 U of Taq polymerase, 5% DMSO and 2 µL of template DNA at 1 ng/µL. For *Gambierdiscus* and *Fukuyoa* cells, the protocol included 45 cycles of amplification (95 °C for 30 s, 60 °C for 45 s and 72 °C for 30 s, followed by an elongation of 10 min at 72 °C) (Gaiani et al. 2020). For the other microalgae, the protocol includes 45 cycles of amplification (94 °C for 30 s, 54 °C for 30 s and 72 °C 4 min, followed by an elongation of 5 min at 72 °C) (Chomérat et al. 2010). Amplifications were carried out in a Nexus Gradient Thermal Cycler (Eppendorf Iberica, Madrid, Spain). PCR reactions were checked using agarose gel electrophoresis and PCR products were then purified with a QIAquick PCR Purification Kit (Thermo Fisher Scientific, Madrid, Spain). Bidirectional sequencing was performed by Sistemas Genómicos, LLC (Valencia, Spain). Sequence reads were edited using BioEdit v7.0.5.2 (Hall 1999) and a consensus sequence for each read was obtained. Sequences were aligned using MAFFT v.7 (Rozewicki et al. 2019) and the phylogenetic relationships were inferred by Maximum Likelihood (ML) using RaxML v.8 (Stamatakis 2014) and Bayesian Inference (BI) using Mr. Bayes v.3.2.2 (Huelsenbeck and Ronquist 2001). GenBank codes for all the sequences used in this work are listed in Table 1.

3. Results and discussion

3.1. RPA-SHA specificity

The *Gambierdiscus* & *Fukuyoa* primer set was tested with genomic DNA from all the strains used in this work, since the objective was to assess the ability of the system to detect both genera. The *G. australes* and *G. excentricus* primer sets were tested with their target genomic DNA, as well as with genomic DNA from other *Gambierdiscus* species (*G. balechii*, *G. belizeanus* and *G. caribaeus*) and *F. paulensis*. Additionally, all primer sets were tested with genomic DNA from other genera (*C. monotis, O.* cf. *ovata* and *P. lima*) and with NTC.

Since previous works (Toldrà et al. 2019a; Toldrà et al. 2019b) have demonstrated that the purification of RPA products is not always required, a trial without a purification step was first performed. However, there were no differences between samples with target DNA and with non-target genera, non-target species or NTC, with all of them showing very high absorbance values. Thus, despite the fact that the purification step is time consuming, it was included in the procedure to remove undesirable proteins and residual primers, and to avoid non-specific signals.

Results for the RPA-SHA using the three primer sets are shown in Fig. 2. The thresholds to discriminate between positive and negative results (i.e. the limits of detection, LODs) were defined as the absorbance values of the NTC plus 10-fold their standard deviations. As can be observed, the *Gambierdiscus* & *Fukuyoa* system provided positive responses (> 0.196 Abs. units) for all *Gambierdiscus* species (*G. australes*, *G. excentricus*, *G. balechii*, *G. belizeanus* and *G. caribaeus*) and for *F. paulensis*, and no response from *C. monotis, O.* cf. *ovata* and *P. lima*. The

G. australes system provided positive responses (> 0.136 Abs. units) for this species and no responses from all others. Finally, the *G. excentricus* system also provided positive responses (> 0.090 Abs. units) only for the strains belonging to this species.

Comparing the three assays, absorbance values are higher in the system for the detection of the genera *Gambierdiscus* & *Fukuyoa*. This fact could be attributed to a better efficiency provided by primers during the RPA and/or the SHA. It can also be observed that within the same assay, not all strains provide the same absorbance value, this effect being more evident in the system for the detection of *G. excentricus*. This is likely due to the differences in the rDNA copy number of the samples analyzed. The rDNA copy number can vary between species, strains, geographic origins, and even cell growth phases, and thus sample harvesting times (Galluzzi et al. 2010; Kretzschmar et al. 2019; Nishimura et al. 2016; Vandersea et al. 2012).

All these results confirm the specificity of the primers and the RPA-SHAs for their respective targets. The three systems are not affected by non-target DNA of microalgae that share the same ecological niche as *Gambierdiscus* and *Fukuyoa*, making them suitable for the screening of field samples where microalgae of other genera will be present.

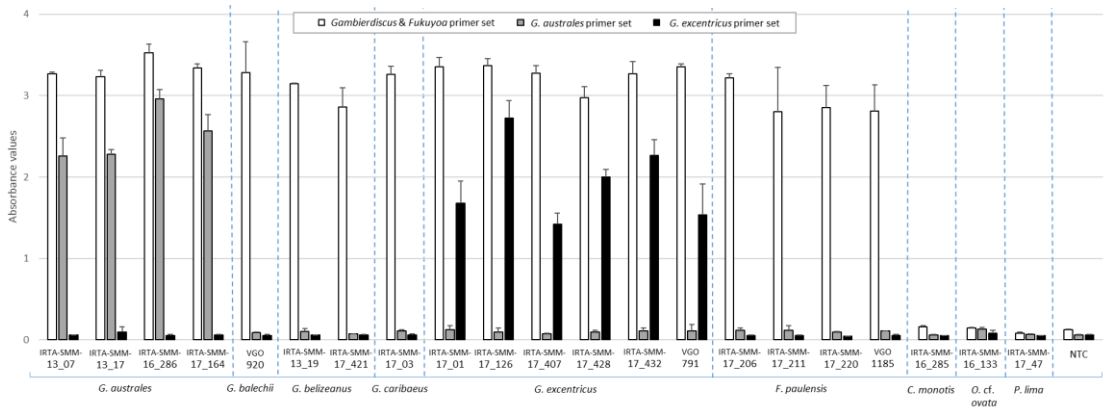

Fig. 2 RPA-SHA experiments using genomic DNA extracted from different genera and species and the *Gambierdiscus* & *Fukuyoa* primer set (white), the *G. australes* primer set (grey) and the *G. excentricus* primer set (black). Experiments were performed in triplicate and bars indicate standard deviations. Vertical dashed lines separate species and/or genera

3.2. Detection of single cells

The purpose of this work was to provide a method for the identification of *Gambierdiscus* & *Fukuyoa* genera, and *G. australes* and *G. excentricus* species, rather than a method for the quantification of cell abundances. Nevertheless, the LOD of the technique plays an important role in such identification. When working with clonal cultures, the number of cells used to extract the DNA is not usually a problem, but in field samples, a robust identification system requires the ability to detect even a single cell. Thus, to assess the sensitivity of our systems, DNA was extracted from single cells isolated from the clonal cultures of *Gambierdiscus* and *Fukuyoa* listed in Table 3, and the three RPA-SHAs were performed simultaneously with aliquots of the same extract. Results showed positive responses (above the respective threshold) in the presence of target DNA and no responses from non-target genera or NTC. These results demonstrate the successful detection and identification of individual cells and confirm the specificity already observed in the previous section. In fact, a unique single cell extract was used for the three RPA-SHAs, so the assays are able to detect even less than 1 cell. This is not

surprising since the rDNA copy number per cell in *Gambierdiscus* species has been reported to be as high as 4,560-21,500 (Vandersea et al. 2012), or even up to 3,197,000 (Nishimura et al. 2016), probably due to the large cell size and high amount of genomic DNA. Thus, our approach allows the discrimination of the presence/absence of a single cell belonging to the target genera/species.

Table 3. Results of the RPA-SHAs performed with DNA extracted from single cells of clonal cultures. Experiments were performed in duplicate.

Species	Strain	*Gambierdiscus* & *Fukuyoa* primer set	*G. australes* primer set	*G. excentricus* primer set
G. australes	IRTA-SMM-13_07	+	+	-
G. australes	IRTA-SMM-16_286	+	+	-
G. balechii	VGO920	+	-	-
G. belizeanus	IRTA-SMM-13_19	+	-	-
G. belizeanus	IRTA-SMM-17_421	+	-	-
G. caribaeus	IRTA-SMM-17_03	+	-	-
G. excentricus	IRTA-SMM-17_126	+	-	+
G. excentricus	IRTA-SMM-17_407	+	-	+
F. paulensis	IRTA-SMM-17_206	+	-	-
F. paulensis	IRTA-SMM-17_211	+	-	-

Sign + (plus) indicates the detection of amplified product and the sign – (minus) indicates the absence.

3.3. Detection of DNA combinations

In order to assess the ability of the *Gambierdiscus* & *Fukuyoa* primer set to amplify target DNA in the presence of different species, 10 different DNA combinations composed by the target species (five *Gambierdiscus* and one *Fukuyoa*) were tested with the RPA-SHA system (Table 4). All the combinations were prepared at a total DNA concentration of 1 ng/µL, and all the species within each combination were at the same concentration. The results obtained (Fig. 3A) demonstrated the ability of the system to amplify and detect the amplicons without any false positives (combination 2). Differences in the absorbance values at 620 nm for each combination were observed. Unlike the results shown in Fig. 2, where the strains used for the mixtures provided absorbance values between 2.8 and 3.5, in this experiment some of the combinations provided lower values, with combinations 3 and 4 giving the lowest signals, which could be attributable to the use of microalgal pellets with different rDNA copy number. Nevertheless, since the discrimination between positive and negative results is clear, this experiment shows the reliability and applicability of the RPA-SHA system for *Gambierdiscus* & *Fukuyoa*.

The same DNA combinations were then tested with the *G. australes* (Fig. 3B) and the *G. excentricus* (Fig. 3C) primer sets, to assess their ability to amplify the corresponding target DNA when mixed with others. The results obtained showed absorbance values higher than the LOD only for the combinations where the target DNA was present. In both cases, absorbance values were lower than in Fig. 2 (3.0 for *G. australes* IRTA-SMM-16_286 and 1.5 for *G. excentricus* VGO791), as expected since 1 ng/µL was the total DNA concentration of the mixtures (i.e. the DNA concentrations for *G. australes* IRTA-SMM-16_286 and *G. excentricus* VGO791 were between 0.16 and 0.5 ng/µL, depending on the combination). It is evident that DNA concentration is not the only crucial parameter, and the presence of non-target DNA may also be playing an important role. Nevertheless, even if the presence of non-target DNA may cause steric hindrance and inhibit the efficiency of the RPA, the experiments demonstrate the robustness of the RPA-SHA systems.

The results obtained with the combination trials demonstrated the ability of the *Gambierdiscus* & *Fukuyoa* primer set to amplify target DNA in the presence of different target species, and the capacity of the *G. australes* and *G. excentricus* primers sets to discriminate the corresponding target DNA in a mixture with DNA from other species. These achievements can be considered as

a step forward to the applicability of the systems to screen field samples, even though further studies, beyond the current work, are needed.

Table 4. DNA combinations.

Species	Strain	1	2	3	4	5	6	7	8	9	10
G. australes	IRTA-SMM-16_286	+	-	+	+	-	+	-	-	+	-
G. excentricus	VGO791	+	-	+	-	+	+	-	-	-	+
G. balechii	VGO920	+	-	+	+	+	-	+	+	+	+
G. belizeanus	IRTA-SMM-17_421	+	-	+	+	+	-	+	+	+	+
G. caribaeus	IRTA-SMM-17_03	+	-	+	+	+	-	+	+	+	+
F. paulensis	VGO1185	+	-	-	+	+	-	-	+	-	-

Sign + (plus) indicates presence of the species and sign - (minus) indicates absence.

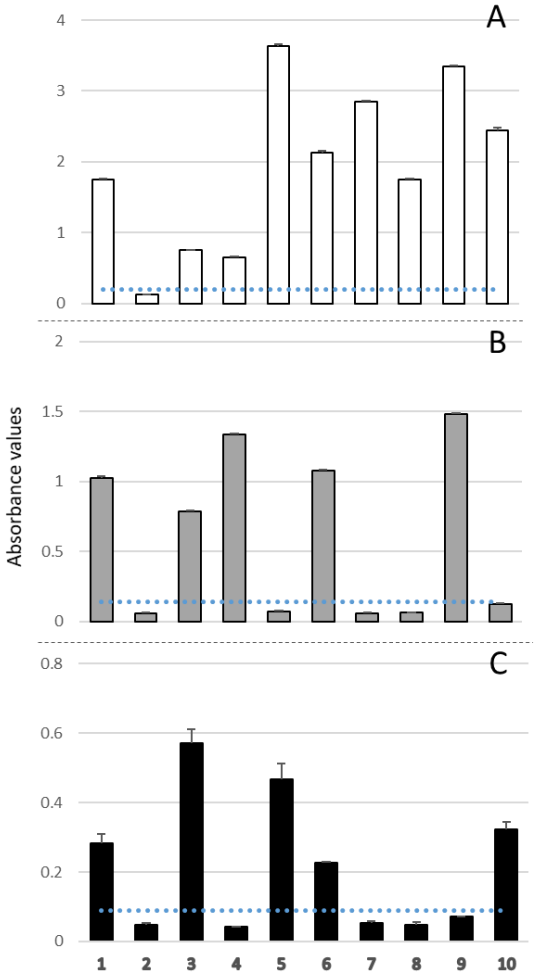

Fig. 3 RPA-SHA experiments using combinations of genomic DNA extracted from different genera and species and the (A) *Gambierdiscus* & *Fukuyoa* primer set (white), (B) the *G. australes* primer set (grey) and (C) the *G. excentricus* primer set (black). Dotted lines represent the LOD for each system. Experiments were performed in triplicate and bars indicate standard deviation

4. Conclusions

This work reports the successful development and application of the RPA-SHA system for the detection of microalgae of the genera *Gambierdiscus* and *Fukuyoa,* and the discrimination between the species *G. australes* and *G. excentricus*. The method showed a high specificity for the target species and a sufficient LOD for identification of a single cell. Furthermore, the ability of the *Gambierdiscus* & *Fukuyoa* primer set to amplify target DNA in the presence of different species was demonstrated, together with the discriminable capacity of the species-specific primer sets (*G. australes* and *G. excentricus*).

This approach, applied for the first time to microalgae of the genera *Gambierdiscus* and *Fukuyoa*, has several advantages. Firstly, the ability to discriminate these genera from other microalgae is extremely helpful, because the assay is more rapid than traditional light microscopy and does not require taxonomical experts to screen samples. Furthermore, unlike microscopy techniques, the strategy can discriminate between the species *G. australes* and *G. excentricus*, which are known CTX producers. Additionally, the ability to detect a single cell is of extreme importance to avoid false negatives due to the LOD. Moreover, the RPA-SHA system is versatile, and with additional primer optimization, can be utilized for the detection of other toxic microalgal species.

Therefore, the summation of the achievements obtained demonstrate the robustness of the developed system, although further studies are needed to test the applicability for screening of field samples. Undoubtedly, the inclusion of the RPA-SHA system in monitoring programs would be useful to assess the risk of ciguatera, to predict possible outbreaks and consequently to preserve human health.

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