Microsatellite markers for the dinoflagellate Gambierdiscus caribaeus from high-throughput sequencing data

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Members of the benthic dinoflagellate genus Gambierdiscus are the causative agents of ciguatera fish poisoning worldwide. Ciguatera outbreaks appear to be more common in recent years and new incidences are reported from unprecedented regions. To investigate Gambierdicus population dynamics, connectivity, and dispersal routes, we developed microsatellite markers for *Gambierdiscus caribaeus*, a globally distributed species that is common at our study site at St. Thomas in the US Virgin Islands. We used high-throughput partial genome sequencing along with an existing transcriptome for microsatellite discovery. Screening of contigs with less than three times coverage resulted in 558 (partial genome) and 33 (transcriptome) candidate microsatellites. Four primer pairs from the partial genome and three from the transcriptome successfully amplified polymorphic microsatellites in multiplexed PCR reactions. The seven markers were tested on 150 *G. caribaeus* strains isolated monthly from August 2013 to July 2015 at St. Thomas, USVI. The numbers of alleles per locus varied between 3 and 14, and the allele diversity ranged from 0.214 to 0.899 in this dataset. These newly developed microsatellites will enable studies of population structure, connectivity, and dispersal in *G. caribaeus* and can give new insights into the expansion of ciguatera outbreaks worldwide.

Keywords: Caribbean, Dinoflagellate, *Gambierdiscus*, Microsatellites, Population genetics

This file is the Author's accepted version of a manuscript published in the Journal of Applied Phycology (2017)29:1927 with the identifier DOI 10.1007/s10811-017-1076-8. The final publication is available at link.springer.com.

1. Introduction

Members of the benthic dinoflagellate genus *Gambierdiscus* produce potent toxins (Bagnis et al. 1980; Satake et al. 1993), which cause ciguatera fish poisoning worldwide (Van Dolah 2000; Chateau-Degat et al. 2005). The incidence and range of ciguatera appears to be increasing (Hallegraeff 2010; Kohli et al. 2015a), similar to many other HAB phenomena, with outbreaks being reported from new regions. Best estimates indicate that more than 50,000 people are globally affected every year making it the most prominent and widespread problem in the world that is caused by harmful algae (Fleming et al. 1998; Van Dolah 2000). The toxins are moved through the food web as the epiphytic microalgae are consumed together with macroalgae by herbivorous fish, which are later eaten by carnivorous fish and humans. Ciguatera fish poisoning is very difficult to predict, as ciguatoxin concentrations vary significantly over time and space due to changes in environmental conditions like nutrient concentrations and sea surface temperature (Parsons et al. 2010). Furthermore, physiological differences between *Gambierdiscus* species and populations might as well impact ciguatoxin concentration and the duration of outbreaks (Litaker et al. 2010). However, the population structure of *Gambierdiscus* species has not been studied yet due to the lack of appropriate genetic markers. Moreover, investigating dispersal routes, connectivity among different outbreak sites, and population dynamics are currently also not possible because of this methodological problem.

Microsatellites are highly polymorphic, tandem repeat motifs of 16 bases that can be found in both coding and noncoding regions of all prokaryotic and eukaryotic genomes (Tautz 1989; Weber and May 1989). These markers have frequently been applied in population genetic studies of many other microalgal species (e.g., Erdner et al. 2011; Dia et al. 2014; Godhe et al. 2016). Microsatellites are conventionally detected by screening genomic libraries with appropriate probes, which is a long and labor-intensive process (Zane et al. 2002). High-throughput sequencing now allows to rapidly sequence large parts of the genome and transcriptome, even in dinoflagellates that have highly redundant and highly recombined genomes (Lin 2011) of huge size (*Gambierdiscus*, approx. 30 Gbp; Kohli et al. 2015b). The raw data from these high-throughput sequencing platforms can be screened for a wide variety of genetic markers. This approach can facilitate and accelerate the discovery of novel microsatellites allowing population genetic analyses of nonmodel organisms.

In the present study, we used high-throughput partial genome sequencing along with an existing transcriptome to generate seven microsatellite markers for *Gambierdiscus caribaeus*. The contigs in both datasets

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were screened for candidate microsatellites, for which we designed primer pairs. Successful amplification and polymorphism of the markers were tested in two rounds, first with visual confirmation using gel electrophoresis and later with accurate genotyping using capillary electrophoresis. Finally, the microsatellite primers were tested in 150 *G. caribaeus* strains, which were collected monthly over 2 years at St. Thomas, USVI.

2. Material and Methods

2.1. Screening of microsatellites in high-throughput sequencing reads

DNA from one strain of *Gambierdiscus caribaeus* was extracted using the CTAB protocol (Winnepenninckx et al. 1993). The partial genome was sequenced using Illumina MiSeq paired-end sequencing at the Genome Sequencing and Analysis Facility of the University of Texas at Austin. These reads were quality checked, trimmed (error probability 0.05), and merged (FLASH 1.2.9, Magoč and Salzberg 2011; minimum overlap 15 bp, maximum mismatch density 0.075) in Geneious 8.1.7 (Kearse et al. 2012). Reads from bacteria, archaea, and viruses were identified and removed from both merged and unmerged reads using blastn and the NCBI database. All further analyses were performed on the remaining eukaryotic reads, which were assembled using Geneious 8.1.7. The consensus sequences of all contigs were exported from Geneious for further analyses. The coverage of all contigs was calculated by multiplying the number of reads in each contig with the average read length (377 bp) and dividing this by the contig length. Only contigs with coverage of less than 3x were considered for microsatellite development to avoid duplicated genomic regions with potential polymorphisms.

In addition to the de-novo-assembled genomic contigs, a transcriptome from *G. caribaeus* consisting of 107,034 contigs, provided by K. Pitz and D. Anderson (Woods Hole Oceanographic Institution), was used for microsatellite screening. FASTA files of genomic and transcriptomic contigs were loaded into phobos v3.3.12 (Mayer 2006) for microsatellite screening using the imperfect search mode. Repeat units of two and three base pairs were targeted, while at most two successive Ns were allowed in a repeat, and 50 nucleotides from the flanking regions were reported in the output file. All other parameters were left at default.

Microsatellites that were part of the same very long, imperfect repeat on a contig were removed from the dataset. Furthermore, microsatellites with quality thresholds below 94%, less than 50 nucleotides in both flanking regions, and repeat length below 15 bp and above 250 bp were deleted as well. Primers for the remaining microsatellites were designed online in BatchPrimer3 (You et al. 2008).

2.2. Primer testing

Fifty-one microsatellite primer pairs designed from the genomic contigs and 13 primer pairs from the transcriptome dataset were synthesized at Integrated DNA Technologies (Coralville, IA, USA). Primers were initially tested on two different *G. caribaeus* strains in 20 µL PCR reactions using the following protocol with primer pair-specific annealing temperatures: 1x PCR buffer, 0.25 mM dNTPs, 5 µM $MgCl_2$, 1 µL DMSO, 10 µg BSA, 0.4 µM of each primer, 2 U Taq DNA polymerase, and 1 µL template; 2 min 94 °C, (35 cycles) 1 min 94 °C, 40 s X °C, 1 min 72 °C, and 5 min 72 °C. PCR products were visualized on a 1.5% agarose gel using GelRed (Biotium, Inc., Fremont, CA, USA). For few microsatellites with multiple bands due to misamplification, new primers were designed using the online program Primer3Plus (Untergasser et al. 2007).

For primer pairs that were successfully tested with the two strains, the extent of polymorphism was assessed by analysis of allele sizes in 16 *G. caribaeus* strains. To avoid the cost associated with synthesizing fluorescently labeled primers for each locus during the testing phase, we used two-step PCRs that incorporated M18-tailed forward primers during the first step and then universal M18 primers with 5' FAM label in the second step (Schuelke 2000). In the first step, the fragments were amplified in 35-cycle 10 µL PCR reactions optimized for the original primer combinations using the modified forward primers with M18 tail (GTAAAACGACGGCC AGTNNNNNN). Reactions contained 1x PCR buffer, 0.25 mM dNTPs, 5 µM MgCl2, 0.5 µL DMSO, 5 µg BSA, 0.5 µM of each primer, 1.25 U Taq DNA polymerase, and 1 µL template. The second PCR step consisted of a 10-cycle 20 µL PCR reaction optimized for the 5' FAM-labeled M18 primer (1x PCR buffer, 0.25 mM dNTPs, 5 µM MgCl2, 1 µL DMSO, 10 µg BSA, 0.4 µM FAM M18 forward primer, 0.15 µM reverse primer, 2 U Taq DNA polymerase, and 5 µL PCR product). The final PCR products were analyzed via capillary electrophoresis at the Genomics Core Lab, Texas AM University, Corpus Christi.

Microsatellite peaks were scored in Geneious 8.1.7 based on a LIZ 600 size standard. Eight microsatellites (four from the partial genome and four from the transcriptome) successfully amplified polymorphic microsatellites. The forward primers of this final set of microsatellites were synthesized with 5' fluorophores (FAM, VIC, NED, PET) at Applied Biosystems (USA). These markers were tested in various combinations in multiplexed PCR reactions. The final reaction conditions are detailed in Table 1.

The newly developed microsatellite markers were used to genotype 150 *G. caribaeus* strains isolated monthly from August 2013 to July 2015 at St. Thomas in the US Virgin Islands. DNA from these isolates was extracted using the DNeasy tissue kit from Qiagen (Germany). Allele frequencies and genetic diversity were calculated in the software GenoDive 2.0b27 (Meirmans and Van Tien-

deren 2004). To assess clonal diversity, clones were assigned based on a stepwise mutation model in GenoDive. Furthermore, missing data were replaced by average allele length and a threshold of two bases was chosen to distinguish clones. Genotypic linkage disequilibrium between pairs of loci was calculated with the web version of GenePop (Rousset 2008) using 1000 dememorizations, 100 batches, and 1000 iterations per batch.

Table 1. Composition of master mixes for multiplexed PCR reactions using primers labeled with fluorophores. Primer1 = G.caribT5, G.caribT7, G.carib3, or Gcarib4; Primer2 = G.carib1 or G.caribT6; Primer3 = G.carib2

Reagents	40 µL rxn	30 µL rxn	20 µL rxn
dd H_2O	to 40 µL	to 30 µL	to 20 µL
PCR buffer	2x	2x	1x
dNTPs (mM)	0.3	0.3	0.25
$MgCl_2$ (mM)	1.25	1	0.5
DMSO (µL)	2	1.5	1
BSA (µg μL^{-1})	0.5	0.5	0.5
Primer1 fwd (µM)	0.3	0.37	0.4
Primer1 rev (µM)	0.3	0.37	0.4
Primer2 fwd (µM)	0.25	0.3	
Primer2 rev (µM)	0.25	0.3	
Primer3 fwd (µM)	0.2		
Primer3 rev (µM)	0.2		
Taq DNA polymerase (U)	5	3.75	2
Template (µL)	1	1	1

3. Results and discussion

Microsatellite markers are powerful tools for population genetic studies, as they display high mutation rates and can differentiate closely related populations (Haasl and Payseur 2011; Vieira et al. 2016). Due to this high sensitivity, these markers are suitable for studying population genetic structure on small regional scales and over short evolutionary time spans. Here, we present newly developed microsatellite markers for the epiphytic dinoflagellate species *G. caribaeus*. In total, 1,581,568 forward and reverse reads (each 300 bp) were generated from the genomic DNA. The software FLASH merged 832,550 reads, while 748,720 reads remained unmerged. After removal of prokaryotic sequences, 883,048 eukaryotic reads totaling 215 Mbp remained, which represent approximately 0.7% of the genome of *G. caribaeus* (based on estimations for Gambierdiscus australes and Gambierdiscus belizeanus in Kohli et al. 2015b). These reads were assembled to 103,561 contigs ranging from 51 to 21,104 bp in length, with 75,214 reads not assembled. Of the 103,561 assembled contigs, 87,397 contigs had less than 3x coverage, and the mean length of these contigs was 580.8 bp, while transcriptomic contigs had a mean length of 390.3 bp.

The software Phobos identified 345,538 microsatellites in the genomic contigs and 427,333 microsatellites in the transcriptomic contigs. After sorting of candidate microsatellites by quality and length, 422 markers from the genomic contigs and 858 markers from the transcriptomic contigs remained. BatchPrimer3 designed 558 primers pairs for microsatellites in the selected genomic contigs and 33 primer pairs in the selected transcriptomic contigs. Quality filtering of candidate microsatellites and primer sequences was essential for reducing the number of markers that had to be tested in the laboratory. For instance, many of these primer pairs targeted the same microsatellites. Furthermore, dinoflagellate genomes contain long, imperfect repeats (Jaeckisch et al. 2011) and, therefore, primers that were surrounded by microsatellites had to be manually excluded from the dataset. Thus, many repeats in the genomic and transcriptomic contigs had to be disregarded due to low quality and overlapping repeat and primer sequences. The amplification of 51 primer pairs from the partial genome and 13 primer pairs from the transcriptome was tested afterwards in several PCR reactions on a limited set of *G. caribaeus* strains. In total, eight microsatellites, four from the genomic contigs and four from the transcriptome, successfully amplified fragments of correct length. Unsuccessful amplification of many primer pairs might be due to incorrect assembly of genomic reads or mutations in the primer binding sites. After testing on the large set of *G. caribaeus* strains, one microsatellite from the transcriptome was excluded from the study due to high number of null alleles. The three remaining transcriptomic microsatellites were located in genes, which are currently not annotated. One gene, in which microsatellite G.caribT5 is found, shows a high similarity to the predicted protein PFB0615c in Plasmodium falciparum.

Primers were also designed for microsatellites in unassembled genomic reads and contigs with 3 to 10x coverage. However, primers designed for microsatellites in these sequences did not successfully amplify any fragments. Therefore, we recommend focusing the screening for markers on contigs with low coverage.

Three of the successful microsatellites consisted of dinucleotide repeats, while four consisted of tri-nucleotide repeats (Table 2). Guanine and cytosine were very common in the repeats, reflecting the high GC-content (approx. 59%) in the genome of the dinoflagellate Gambierdiscus. Product sizes ranged from 90 to 180 bp due to on average short contigs (580.8 and 390.3 bp). The microsatellites G.carib1, G.carib2, and G.caribT5 could be amplified together in one PCR reaction, likewise G.caribT6 and G.caribT7 could be amplified in duplex (Table 2). The remaining microsatellites G.carib3 and G.carib4 were amplified individually. Annealing temperatures ranged from 52 to 60 °C in the different PCR reactions.

Similar numbers of microsatellites were developed for other dinoflagellate species in different laboratories (Frommlet and Iglesias-Rodriguez 2008; Lundholm et al. 2014; Minter et al. 2015) with the exception of higher numbers of markers for the genus Alexandrium (Nagai et al. 2004, 2006, 2014; Sehein et al. 2016). Fur-

Table 2. Annealing temperature, PCR product size, repeat motif, and primer sequence of the microsatellites markers for *G. caribaeus*. "T" in primer name indicates location in transcriptome

Name	Annealing T	Product size	Repeat	Dye	Multiplex	Primer sequence
G.carib1-fwd	60	89-113	CA	NED	A	ACGACCCCGTGGCATACT
G.carib1-rev					A	ATGGTTGATGTGGCTACTTGC
G.carib2-fwd	60	117-135	TC	PET	A	ACAAAGCGGAAAGTGTCA
G.carib2-rev					A	CCATGTGAGACAGAGAGAGAG
G.carib3-fwd	52	107-122	CTT	FAM	C	GAGTTCAGACTTGTGCTCTTC
G.carib3-rev					C	CAGATGTGGGAGTCGATG
G.carib4-fwd	54	122-150	CT	FAM	D	TCTCTGGACTCGCTGCAC
G.carib4-rev					D	TTAAAGCAGAAGACTGCATACG
G.caribT5-fwd	60	170-182	GCA	VIC	A	GTCAATTGGTGGCCTGTT
G.caribT5-rev					A	GATGTGTCGCTTGTCCTCTT
G.caribT6-fwd	54	106-112	GAC	PET	B	CCAATACCGATAGCACTGG
G.caribT6-rev					B	CCCTCTTGAAGAATGAAGC
G.caribT7-fwd	54	113-128	GCC	NED	B	GCTACAGTCACACATTCAGGA
G.caribT7-rev					B	GCTTGCACACGTAGAAGTAGT

thermore, the ratio of applicable microsatellites to the amount of tested markers in our study (partial genome 7.8%, transcriptome 23%) is comparable to the success rate of other studies that use high-throughput sequencing (Nagai et al. 2014; Minter et al. 2015). Thus, this study shows that screening of high-throughput sequencing data represents an efficient alternative to traditional methods for microsatellite development such as dual-suppression PCR and compound SSR PCR, which are usually very labor intensive and time consuming (Nagai et al. 2014).

Using 150 *G. caribeaus* strains from the US Virgin Islands, the amount of null alleles at each marker ranged between 8 and 35%(Table 3). Amplification success could potentially be improved by further purification of DNA extracts. GenoDive identified 111 unique algal strains in the dataset based on the microsatellite alleles, which resulted in a clonal diversity of 0.74 (G/N). No linkage disequilibrium was detected among all pairs of microsatellites, thus, the loci were independent from each other. To our knowledge, this is the first time that microsatellite markers were developed from both genomic and transcriptomic contigs. An initial analysis regarding the allele frequency and diversity of these markers showed several distinct differences between the genomic and transcriptomics microsatellites. Genomic microsatellites had more alleles (6- 14) than transcriptomic markers (3-5), which resulted also in higher allele diversity of microsatellites from the partial genome (total range 0.214-0.899, Table 3). Fewer alleles and lower allele diversity in microsatellites from the transcriptome likely indicate higher selection pressure on these regions, which limits the amount of mutations. Therefore, evolutionary processes like genetic drift might have less impact on these markers in comparison to microsatellites that are located in noncoding genomic regions. However, transcriptomic microsatellites could potentially be linked to genes under selection and might highlight evolutionary processes like local adaptation.

The newly developed microsatellites markers will enable studies of population structure, connectivity and dispersal in the dinoflagellate *G. caribaeus* in regions impacted by ciguatera like the Greater Caribbean Region. Furthermore, the distribution of *G. caribaeus* in both the Atlantic and Pacific Ocean allows global population genetic analyses with the new microsatellites. The application of genomic and transcriptomic microsatellites might identify diverse population genetic patterns in *G. caribaeus* in the future and could indicate differences in evolutionary drivers over spatial and temporal scales.

Table 3. Number of alleles, repeat size in bp, allele diversity, and amount of null alleles in the seven microsatellite loci from 150 tested *G. caribeus* strains

Marker	No. alleles	Repeat size (bp)	Total heterozygosity	Percent null
G.carib1	12	4-28	0.764	17.3
G.carib2	9	16-34	0.788	20.7
G.carib3	6	9-24	0.56	16.7
G.carib4	14	16-44	0.899	35.3
G.caribT5	4	9-21	0.346	8.7
G.caribT6	3	12-18	0.214	25.3
G.caribT7	5	12-27	0.327	26

4. Acknowledments

We thank Kathleen Pitz and Donald Anderson from Woods Hole Oceanographic Institution for providing the transcriptome of *Gambierdiscus caribeaus*. Yesid Lozano-Duque greatly contributed to the isolation, cultivation, and DNA extraction of *Gambierdiscus* strains from St. Thomas. We are grateful for the constructive comments from two anonymous reviewers on this manuscript. This article is a result of research funded by the National Oceanic and Atmospheric Adminis-

tration Center for Sponsored Coastal Ocean Research ECOHAB program under award no. NA11NOS4780061 to the University of Texas Marine Science Institute (D.L.E.). This is publication #ECOHAB881.

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