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FIRST RECORD OF THE SPATIAL ORGANIZATION OF THE NUCLEOSOME-LESS CHROMATIN OF DINOFLAGELLATES: THE NONRANDOM DISTRIBUTION OF MICROSATELLITES AND BIPOLAR ARRANGEMENT OF TELOMERES IN THE NUCLEUS OF *GAMBIERDISCUS AUSTRALES* (DINOPHYCEAE)[1]

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Dinoflagellates are a group of protists whose exceptionally large genome is organized in permanently condensed nucleosome-less chromosomes. In this study, we examined the potential role of repetitive DNAs in both the structure of dinoflagellate chromosomes and the architecture of the dinoflagellate nucleus. Non-denaturing fluorescent in situ hybridization (ND-FSH) was used to determine the abundance and physical distribution of telomeric DNA and 16 microsatellites (1- to 4-bp repeats) in the nucleus of *Gambierdiscus australes*. The results showed an increased relative abundance of the different microsatellite motifs with increasing GC content. Two ND-FISH probes, $(A)_{20}$ and $(AAT)_5$, did not yield signals whereas the remainder revealed a dispersed but nonrandom distribution of the microsatellites, mostly in clusters. The bean-shaped interphase nucleus of *G. australes* contained a region with a high density of trinucleotides. This nuclear compartment was located between the nucleolar organizer region (NOR), located on the concave side of the nucleus, and the convex side. Telomeric DNA was grouped in multiple foci and distributed in two polarized compartments: one associated with the NOR and the other peripherally located along the convex side of the nucleus. Changes in the position of the telomeres during cell division evidenced their dynamic distribution and thus that of the chromosomes during dinomitosis. These insights into the spatial organization of microsatellites and telomeres and thus into the nuclear architecture of *G. australes* will open up new lines of research into the structure and function of the nucleosome-less chromatin of dinoflagellates.

***Key index words: Gambierdiscus*; microsatellites; NOR; nuclear architecture; telomeres**

***Abbreviations*: DAPI, 4′-6′-diamidino-2-phenylindole; ND-FISH, non-denaturing fluorescence in situ hybridization; NOR, nucleolar organizer region**

Eukaryotic genomes contain large amounts of repetitive DNA sequences, grouped in different classes. The class comprising repetitive DNA sequences arranged in tandem (e.g., ribosomal DNA [rDNA], protein-coding multigene families, and satellite DNA) includes microsatellites, also known as short tandem repeats or simple sequence repeats. The nucleotide sequence repeats that make up these simple segments of DNA typically range in length from one to six base pairs. Microsatellites are among the most abundant type of tandem repeat present in the genomes of prokaryotes and eukaryotes, in both coding and non-coding regions (Tautz and Renz 1984).

The development of high-throughput next-generation sequencing has allowed the microsatellite fraction of many species to be characterized at the molecular level, including determinations of the frequency and genomic location of individual microsatellite motifs. At the chromosomal level, the

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abundance and distribution of repeated sequences can be precisely analyzed by physical mapping using fluorescent in situ hybridization (FISH). FISH uses simple-sequence oligonucleotides as probes and its introduction was a breakthrough in investigations of the presence and distribution of microsatellite types in plants as well as animals, including the chromosomal origin, organization, structure, function, and evolution (e.g., Cuadrado and Jouve 2011, Carmona et al. 2013, Ruiz-Ruano et al. 2015, Jiang et al. 2021). The role of microsatellite motifs in nuclear processes such as the regulation of transcription, the organization of chromatin, meiotic recombination, the regulation of RNA, genome size, and the cell cycle has been discussed in many publications (reviewed in Bagshaw 2017). Moreover, microsatellites have been implicated in the pathogenesis of several human diseases, via the unstable expansion of particular classes of repeats (Rohilla and Gagnon 2017). Thus, a precise understanding of the distribution and differential abundance of microsatellites in eukaryotic genomes can shed light on their functional role from an evolutionary perspective. Telomeres are specialized structures located at the ends of linear chromosomes. They typically consist of short tandem repeats bounded by multiple telomeric interacting proteins (reviewed in Shay and Wright 2019). For example, the telomere DNA of vertebrates contains the hexanucleotide repeat TTAGGG, while the tandem repeats of insects are even shorter. While telomeres are not microsatellites stricto sensu (as microsatellites are distributed throughout an organism's genome), telomeres can be arguably considered as a type thereof.

Dinoflagellates are a large monophyletic phylum comprising >2,000 species of flagellated protists, thus representing one of the largest groups of eukaryotes. They are important marine primary producers, major contributors to harmful algal blooms, but also essential symbionts of coral reefs (Saldarriaga and Taylor 2017). Both biologically and evolutionarily, dinoflagellates are an enigma because their genomes are the largest in the domain Eukarya (~ 1–250 Gbp; Hou and Lin 2009) and at some point in time their nucleosomes were lost. Dinoflagellate chromosomes are permanently condensed and their DNA fibers are organized as cholesteric liquid crystals (Chow et al. 2010) stacked in rows of parallel nested arches stabilized by metal cations and structural RNA (Costas and Goyanes 2005). The adaptive value of this chromosomal configuration and how the dinoflagellate genome is organized into functional chromosomes are poorly understood despite several decades of research (Moreno Diaz de la Espina et al. 2005, Gornik et al. 2019). However, these unique features of the dinoflagellate genome can be exploited to study genome organization, gene expression, and genome evolution as part of the growing field of 4D nucleosome research (Cremer et al. 2018).

In recent years, dinoflagellate transcriptomes and gene expression data have become increasingly available, but due to the huge size and the complexity of the dinoflagellate genome, current technologies have failed to provide complete genomic sequences for dinoflagellates. Available draft genome sequences of dinoflagellates are mostly restricted to the smallest genomes, those of species in the symbiotic family Symbiodiniaceae (1–5 Gbp), but these are usually partial and not assembled at the chromosomal resolution, with the exception of some chromosomes in *Fugacium kawagutti* (Li et al. 2020) and the 94 chromosome-scale scaffolds covering <1 Gb of the genome of *Symbiodinium microadriaticum* (Nand et al. 2021). However, most dinoflagellates are not symbionts and are free-living, either as plankton or sand dwellers. The genomes of these species are very large, 10–80 times larger than that of humans, and distributed across hundreds of chromosomes (LaJeunesse et al. 2005, Lin 2011). Whether this high DNA content is related to polyploidy is unclear. In the few free-living dinoflagellates analyzed so far, the DNA repeats are mostly in the form of highly tandem repeats and they may account for more than half of the genome, a proportion substantially higher than in Symbiodiniaceae. Moreover, the repeat composition in the genomes of free-living dinoflagellates differs from that of the smaller genomes of Symbiodiniaceae, largely due to the prevalence of microsatellites (González-Pech et al. 2021). For example, 58% of the genome of *Alexandrium ostenfeldii* consists of repeats, of which a significant fraction (13%) is composed of microsatellites (Jaeckisch et al. 2011). In *Polarella glacialis,* ~68% of its genome is made up of repetitive DNA, including 25% microsatellites (Stephens et al. 2020). Yet, to date, nearly nothing is known about how repeated sequences become distributed within the permanently condensed chromosomes of dinoflagellates. Thus, a still-open question about the overall nature of dinoflagellate genomes: Are tandem repeats, including microsatellite motifs, clustered, or evenly spread throughout the genome?

In this study, we investigated the significant fraction of microsatellites in the dinoflagellate genome with respect to chromosomal organization and nuclear architecture. We began using nondenaturing fluorescence in situ hybridization (ND-FISH) to determine the abundance and distribution of a set of 16 microsatellites motifs (mono-, di-, tri-, and tetranucleotide repeats) in the dinoflagellate *Gambierdiscus australes*. In addition, we used a simple heptanucleotide repeat sequence, the consensus plant telomeric sequence TTTAGGG, which is also present in several dinoflagellates species (Alverca et al. 2007), to examine the intranuclear arrangement of telomeres during the cell cycle, again using *G. australes* as the model organism. Species of the *Gambierdiscus* genus are marine dinoflagellates that

produce ciguatoxins, which are responsible for causing ciguatera fish poisoning in humans via the consumption of contaminated fish. *Gambierdiscus australes* was chosen for several reasons. First, it divides well in culture under laboratory conditions. Second, recent evidence does not support polyploidy as accounting for its DNA content (Cuadrado et al. 2021) and flow cytometry has demonstrated a genome size of ~32 Gbp (Kohli et al. 2015), including large amounts of repetitive DNA. Third, DNA staining using the fluorescent dye 4′-6′-diamidino-2-phenylindole (DAPI) reveals the nucleolus (Cuadrado et al. 2021), the largest compartment in the nucleus of eukaryotic cells.

MATERIALS AND METHODS

The study was performed using strain VGO1360 of *Gambierdiscus australes*, sourced from La Gomera Island (Canary Islands) and obtained by isolating a single cell. The strain is maintained at the Culture Collection of Harmful Microalgae of the Spanish Institute of Oceanography (CCVIEO, https://vgohab.com/coleccion-de-cultivos/) and is available upon request.

Culture conditions, cell fixation, and slide preparation. *Gambierdiscus australes* was cultured in 100-mL Erlenmeyer flasks filled with 50 mL of K/2 medium (Keller et al. 1987) without silicates and prepared with seawater from Ría de Vigo, Spain. The medium was adjusted to a salinity of 32 by the addition of sterile distilled water. Cultures were incubated at 20°C under an irradiance of ~90 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} and a photoperiod of 12:12 h L:D (light:dark), as previously described (Rodríguez et al. 2017).

The cells in 12 mL of culture were harvested at the beginning of the light phase by centrifugation at 1,200*g* for 10 min. The pellets were dissolved in 3 mL of hypotonic solution (KCL 0.075 M) and incubated at 37°C for 30 min, during which time the cells enlarged, allowing the adequate spreading of their nuclei/chromosomes. After the second round of centrifugation under the same conditions, the pellets were treated with 5 mL of Liquinox and gently agitated at room temperature (RT) to remove the theca. Finally, the pellets were fixed in 2 mL of ethanol:acetic 3:1 (v/v) for at least 24 h and stored at 4°C.

Each slide was prepared using 50 µL of fixed cells as described above. After centrifugation for 10 min to remove the fixative, the cells were resuspended in a drop of 45% acetic acid and squashed with a coverslip onto clean microscope slides. The coverslips were removed by freezing and the slides air-dried.

Probes and labeling. Table 1 shows the 17 oligonucleotide probes used in this study. Either biotin or DY-547 (red) was incorporated at their ends. Based on the number of nucleotides per repeat, microsatellites can be classified as mono-, di-, tri-, tetra-, penta-, and hexanucleotides. In addition, they can be grouped according to their motif, which may be overlapping or complementary. For example, the probe $(AAG)_5$ allows the detection of $(AAG)_n$, $(AGA)_n$, and $(GAA)_n$ in different reading frames, and (CTT)n, $(TCT)_n$, and $(TTC)_n$ in the complementary strand. Thus, all possible classes of trinucleotide units can be detected using just ten oligonucleotides. Similarly, the homonucleotides A_{20} and C_{20} can be used to detect the four mononucleotide microsatellites (arrays of A/T and C/G, respectively). All possible dinucleotide repeats can be grouped into four motifs, AC, AG, AT, and CG, but only two oligonucleotide probes $(AC)_{10}$ and $(AG)_{10}$ are employed. The two other dinucleotide repeats, poly AT and poly CG, cannot be analyzed accurately using hybridization techniques, because the oligo probes used to detect them are self-complementing. Tetranucleotide microsatellites can be grouped into 30 motifs. Only GACA and GATA were analyzed in this study, as in many eukaryotes these motifs are present in large numbers in the genome. Telomeres were detected using the oligo $(CCCTAAA)_3$, hereafter referred to as the telomeric probe. Probe pTa71, containing 45S rDNA from *Triticum aestivum*, was used to locate 45S rDNA by FISH (Figueroa et al. 2014). The probe was labeled with digoxigenin-11-dUTP using a nick translation kit from Roche according to the manufacturer's instructions.

ND-FISH, microscopy, and imaging. ND-FISH was performed as described in Cuadrado and Jouve (2010). Briefly, slides prepared as described above were incubated at 24°C in a humidified chamber for 2 h with 30 µL of hybridization buffer containing 2 pm of the oligo probe in 2×SSC. The slides were then washed by immersion in 4×SSC/0.2% $Tween^{20}$ and agitated for 10 min at RT. Biotin was detected by incubating the slides with streptavidin-Cy3 (Sigma–Aldrich) in 5% (w/v) BSA for 1 h at 37°C. The slides were then rinsed for 10 min in 4×SSC/0.2% $Tween^{20}$ at RT, stained with DAPI, and mounted in antifade solution (Vector Laboratories).

Analyses were conducted using an epifluorescence Axiophot Zeiss microscope. The images were captured with a cooled CCD camera (Zeiss), merged, and optimized for best contrast and brightness using Adobe Photoshop functions that treated all pixels in the image equally.

RESULTS

Physical mapping of microsatellites in Gambierdiscus australes. The most common nuclear morphology was a bean-shaped nucleus with a convex and a concave border (Figs. 1 and 2, A–C). This nuclear shape is seen in non-dividing cells and in the interphase of vegetative cells undergoing division, as reported in several dinoflagellate species, including *Gambierdiscus australes* (Bravo et al. 2014). The brighter DAPI-stained area on the concave border corresponded to the nucleolar organizer region (NOR), where chromosomes bearing 45S rDNA are located (Fig. 2A; Cuadrado et al. 2021). According to our results and for descriptive purposes, in addition to the NOR we divided the bean-shaped nucleus into two compartments: a convex region, referred to herein as the cortex and a middle region, referred to herein as the medulla, located between the NOR and cortex and defined by the presence of microsatellites at high density (Fig. 1, K and L).

The abundance and distribution of 16 microsatellite motifs, including mono-, di-, tri-, and tetranucleotides, in the nuclei of *Gambierdiscus australes* were investigated using ND-FISH (Table 1). The most abundant motifs were identifiable by their brighter signals. According to their nuclear distribution of those signals, two main patterns were distinguished: (1) dispersed microsatellites (Fig. 1E) and (2) clustered at specific regions beyond the dispersed microsatellites (Fig. 1F) and ranging in size from small to large depending on their abundance (Fig. 1D).

TABLE 1. Oligos used as probes in the ND-FISH experiments.

	Probe	Sequence	5′ and 3′ modifications	GC content (%)
Mononucleotides	$(A)_{20}$	AAAAAAAAAAAAAAAAAAAA	Biotin	0
	$(C)_{20}$	CCCCCCCCCCCCCCCCCCCC	Biotin	100
Dinucleotides	$(AC)_{10}$	ACACACACACACACACACAC	Biotin	50
	$(AG)_{10}$	AGAGAGAGAGAGAGAGAGAG	Biotin	50
Trinucleotides	$(AAC)_5$	AACAACAACAACAAC	Biotin	33.33
	$(AAG)_5$	AAGAAGAAGAAGAAG	Biotin	33.33
	$(AAT)_5$	AATAATAATAATAAT	Biotin	0
	$(ACC)_5$	ACCACCACCACCACC	Biotin	66.67
	$(ACG)_5$	ACGACGACGACGACG	DY-547	66.67
	$(ACT)_5$	ACTACTACTACTACT	Biotin	33.33
	$(AGC)_5$	AGCAGCAGCAGCAGC	Biotin	66.67
	$(AGG)_5$	AGGAGGAGGAGGAGG	DY-547	66.67
	$(ATC)_5$	ATCATCATCATCATC	Biotin	33.33
	$(CCG)_5$	CCGCCGCCGCCGCCG	DY-547	100
Tetranucleotides	$(GACA)_5$	GACAGACAGACAGACAGACA	Biotin	50
	$(GATA)_5$	GATAGATAGATAGATAGATA	Biotin	50
Telomeric DNA	$(CCCTAAA)_3$	CCCTAAACCCTAAACCCTAAA	DY-547	42.86

The homonucleotide probes A_{20} and C_{20} were used to detect arrays of the mononucleotides A/T and C/G, respectively (Fig. 1, A and B). No signals were obtained with A_{20} even after long image-capture exposure times (Fig. 1A). By contrast, C_{20} signals were dispersed throughout the nucleus, except at the NOR. The highest signal density was in the medulla, and a signal cluster was seen in the outer cortex (Fig. 1B).

The abundance and distribution of two dinucleotide repeats, AG/GA/CT/TC and AC/CA/GT/TG, were investigated using oligonucleotides $(AG)_{10}$ and $(AC)_{10}$, respectively. For both repeats, several clusters of signals of different sizes and intensities were detected (Fig. 1, C and D). Larger, more intense clusters of the AC repeats mostly occurred in the cortex (Fig. 1C), and those of the AG repeats in the medulla, in close proximity to the NOR (Fig. 1D).

All trinucleotide microsatellite motifs could be analyzed using 10 oligonucleotide probes: $(AAC)_5$, $(AAG)_5$, $(AAT)_5$, $(ACC)_5$, $(ACG)_5$, $(ACT)_5$, $(AGC)_5$, $(AGG)_5$, $(ATC)_5$, and $(CCG)_5$. No $(AAT)_5$ signals were obtained, even after long image-capture exposure times. The other trinucleotide probes yielded characteristic motif-dependent distribution patterns differing in their intensities, indicative of differences in the abundances of the respective trinucleotide motifs in the genome of *Gambierdiscus australes*. $(AAC)_5$ was mostly dispersed in the medulla and cortex but did not occur in the NOR (Fig. 1E). Similarly, $(AAG)_5$ was dispersed throughout the nucleus, except in the NOR; however, in contrast to $(AAC)_5$, it formed signal clusters of different sizes and intensity (compare Fig. 1, E and F). Five trinucleotide motifs, ACC, ACG, ATC, AGG, and CCG (Fig. 1, G, H, K and M), were present in abundance, scattered within the medulla. The motifs $(ACC)_5$, $(ATC)_5$, and $(AGG)_5$ were detected as clusters of different sizes in the medulla. In addition, many clusters of $(ACC)_5$ and $(ATC)_5$ signals differing in size and intensity, including larger $(ACC)_5$ signals, were seen in the cortex. Although $(AGC)_5$ (Fig. 1I) and $(ACT)_5$ (Fig. 1J) signals were clearly present in the medulla, mostly near the NOR, they were of weaker intensity than the signals of the AAC, AAG, ACC, ACG, ATC, AGG, and CCG probes (i.e., all other signal-yielding trinucleotide repeats). In addition, in all nuclei examined, a large cluster of AGC signals was present in the outer cortex (Fig. 1I) together with a cluster of ACT signals in the medulla, in close proximity to the NOR (Fig. 1J).

Finally, for the two tetranucleotide repeats, $(GACA)_4$ (Fig. 1N) and $(GATA)_4$ (Fig. 1O), a dispersed but uniform pattern of low-intensity signals were observed in both the cortex and medulla but only after very long exposure times. These repeats were also represented by well-defined signals that clustered in a few locations in the medulla, at the periphery of the NOR.

Visualization of telomeres and 45S rDNA during cell division. Both the oligo $(CCCTAAA)_3$, complementary to the guanosine-rich telomeric DNA strand, and the clone probe pTa71 were used to target 45S rDNA in two-color FISH experiments. This allowed us to track the position of the telomeres during cell division and to analyze their distribution in relation to the NOR. Figure 2 shows the sequence of nuclear transformations during dinomitosis in *Gambierdiscus australes*. A key feature of this process is that the nuclear envelope never breaks down, such that the chromosomes are never free in the cytoplasm. Figure 2, A–D is a representative image showing the distribution of telomeres and 45S rDNA in the nuclei of non-dividing or interphase cells. As noted above, the nucleus of these cells is typically bean-shaped or slightly U-shaped (Fig. 2A) with 45S rDNA, the genetic component of the nucleolus, clustered in the concave region (Fig. 2B). As shown

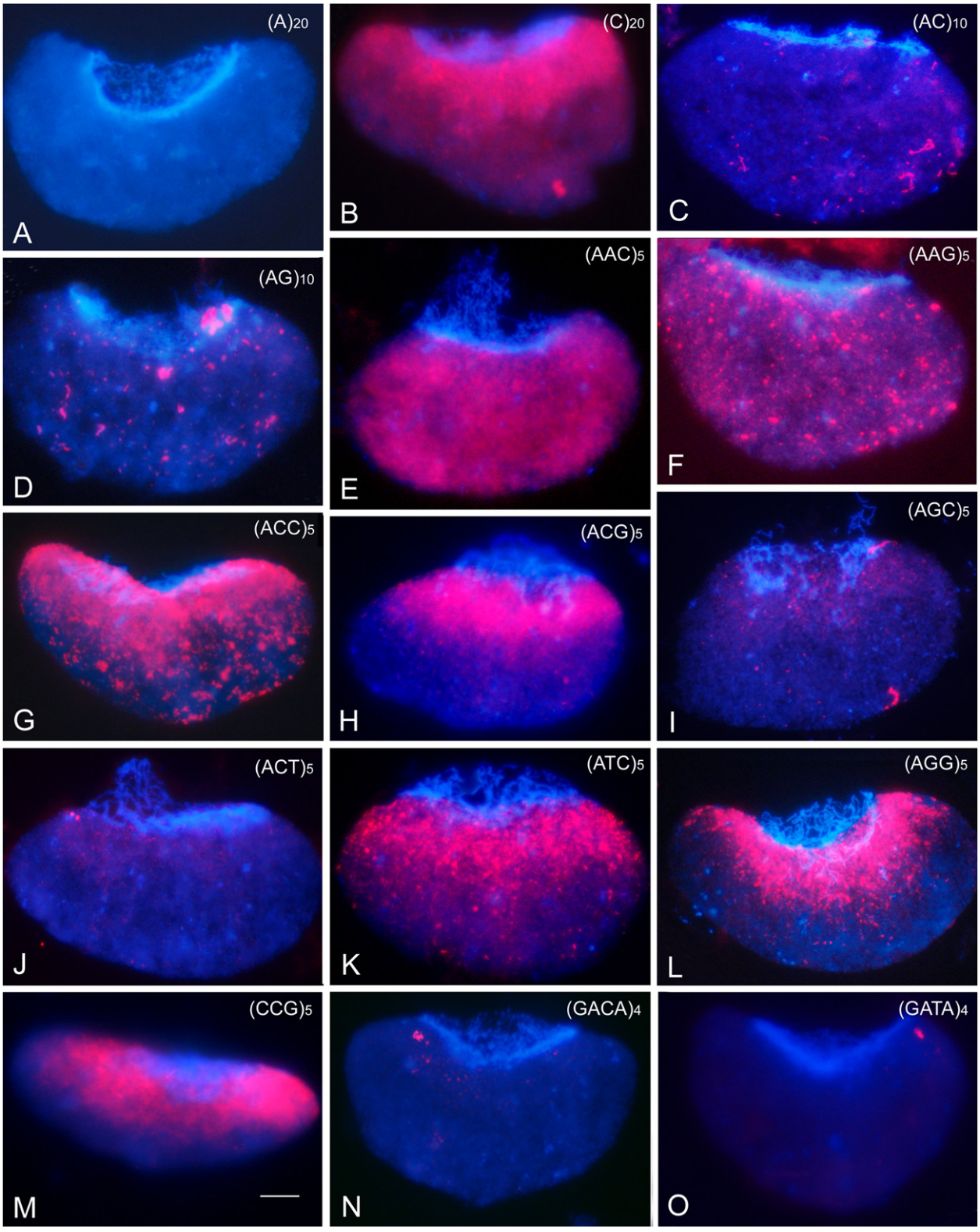

FIG. 1. ND-FISH and DAPI staining show the motif-dependent distribution of the indicated microsatellite probes in the interphasic nuclei of *Gambierdiscus australes*. The NOR is easily observed based on the strong DAPI signature (DAPI+) of the chromosomes bearing 45S rDNA (Cuadrado et al. 2021). Note that every microsatellite is unique in its FISH pattern depending on its abundance and distribution. The dispersed pattern can go from the complete absence of FISH signals (A) to general brightness dispersed throughout the nucleus, except at the NOR (E) or found at specific regions (H). The dispersed pattern, ranging in the number of clusters (compare G and I), includes both large (F) and small clusters (K). Scale bar in panel M applicable to all panels = 10 μm. [Color figure can be viewed at wileyonlinelibrary.com]

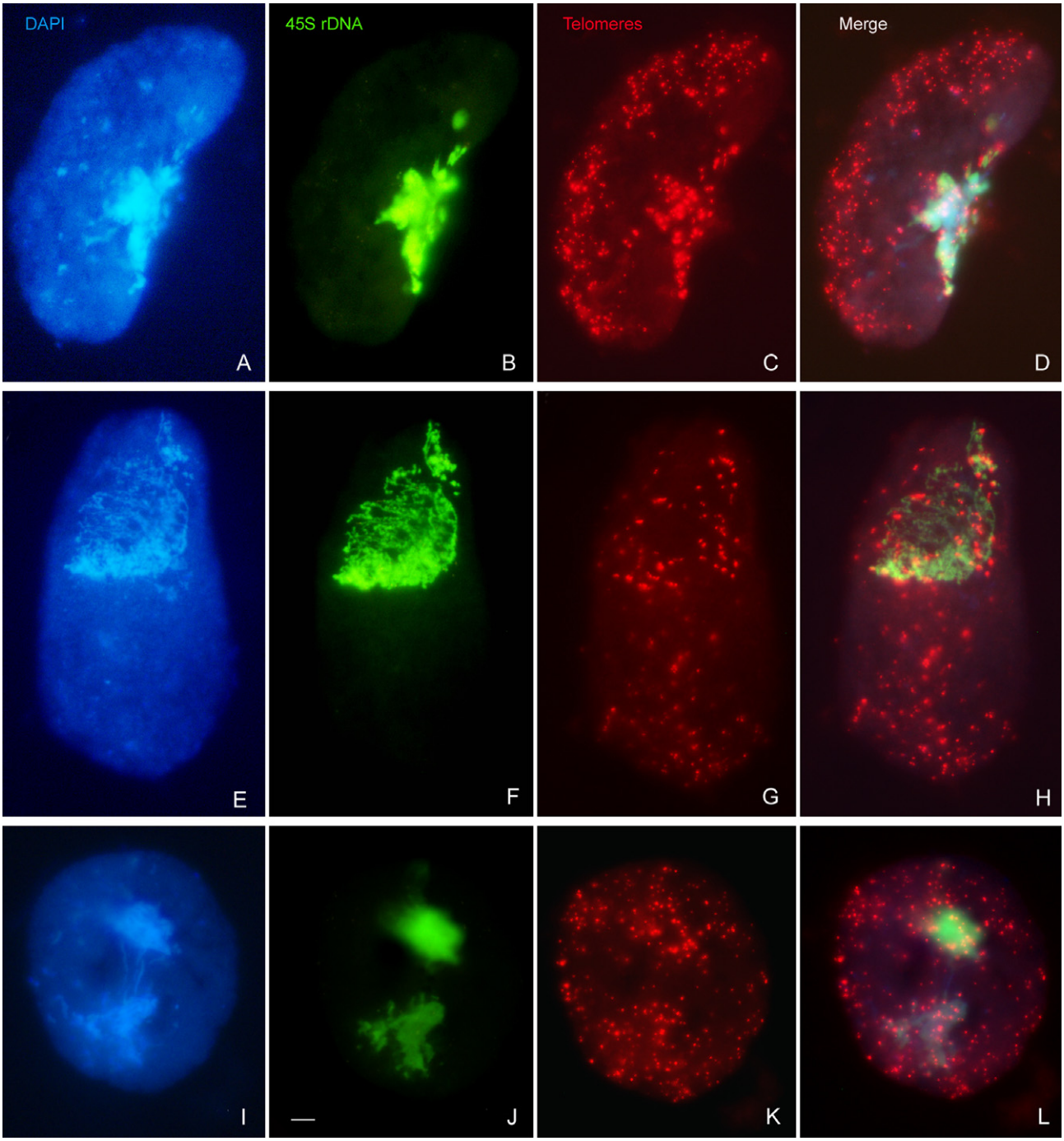

FIG. 2. FISH showing the distribution of 45S rDNA and telomeric DNA in the DAPI-stained nuclei of *Gambierdiscus australes*. The large morphological differences between the nuclei seen in A, E, and I correspond to the different phases of the cell cycle (see text for details). Note that the bipolarized distribution of the telomeric DNA in interphasic nuclei (C) changes with the onset of dinomitosis (G and K). In contrast to the bean-shaped nucleus in A–D, the morphology of the nucleus in E–H is consistent with that of a cell in the earliest mitotic stages. The nucleus in with the sister chromatids were migrating toward opposite poles of the cell prior to completion of nuclear division is shown in I–L. Scale bar in panel J applicable to all panels = 10 μm. [Color figure can be viewed at wileyonlinelibrary.com]

in Figure 2C, telomeric signals of different size and intensity were localized in two-polarized foci, one in connection with the 45S rDNA signals, and hence in close association with the NOR (Fig. 2D), and the other opposite the NOR, at the nuclear periphery all along the convex region of the bean-shaped nucleus and apparently associated with the nuclear envelope. This bipolarized organization persisted until the onset of dinomitosis. The nuclear morphology of the cells changed during division,

from roundish to elongated (Fig. 2, E–H). Nuclear division proceeded with the constriction of the nucleus at its equatorial region, followed by the formation of two daughter nuclei before cell division was completed (data not shown). The morphology of the elongated nucleus shown in Figure 2, E–H is consistent with that of a cell just prior to anaphase. In contrast to the typical interphasic bean-shaped nucleus, in which the NOR is located in the concave region, in the earliest mitotic stages, the 45S rDNA occupied different positions (Fig. 2E). As suggested by the different 45S rDNA signal patterns (compare Fig. 2, B and F), the onset of dinomitosis was marked by changes in chromatin condensation, at least in the NOR. In addition, during dinomitosis, the telomere pattern differed from that seen in interphase, as the telomeres lose their bipolar organization (Fig. 2G). The typical metaphase of other eukaryotic cells, in which the chromosomes become arranged at the equatorial plate before the onset of anaphase, has never been observed in dinoflagellates. An anaphase nucleus, identified by the presence of two NORs of the same size, is shown in Figure 2, I–L. At this stage, the sister chromatids migrated to opposite poles of the cell prior to the completion of nuclear division. During the segregation of the sister chromatids and the formation of two NORs, the telomeres became dispersed throughout the nucleus (Fig. 2K).

DISCUSSION

Microsatellite motifs differ in their abundance and nuclear location. Genomic databases have been used to analyze microsatellite occurrence, abundance, and location in hundreds of genomes. Nevertheless, because whole-genome sequences of dinoflagellates are not yet available, genome-wide characterization of microsatellites in dinoflagellate nuclei is not possible (e.g., Srivastava et al. 2019). Thus, as an alternative to an extensive analysis of microsatellites by a computer-based screening of DNA sequence databases, we searched for the presence of microsatellites in situ using ND-FISH to detect nuclear regions harboring clusters of microsatellite repeats. Our study is the first thorough in situ analysis of microsatellites in dinoflagellates. The high density of microsatellites detected in the nucleus of *Gambierdiscus australes* suggests that the amplification and dispersion of microsatellite motifs are among the processes underlying genome expansion and evolution in this and potentially other dinoflagellates.

Studies that used sequence databases to explore microsatellites in species ranging from protists to mammals have shown considerable differences in the absolute and relative frequencies of the respective motifs. Likewise, we found large variations in the abundance and distribution of microsatellites in the nucleus of *Gambierdiscus australes* (32 Gbp; Kohli et al. 2015). The GC-content of the analyzed microsatellites ranged from 0% to 100% (see Table 1). Microsatellites of a minimum target sequence size or, in the case of short loci, of high density are required for FISH signal positivity. Accordingly, the absence of in situ signals obtained with the $(A)_{20}$ and $(AAT)_5$ probes suggests a very low abundance of poly-A and trimers of AAT, that is, motifs with a 0% GC-content (Table 1), in the *G. australes* genome. This was in contrast to the other microsatellite motifs analyzed, especially poly C and trimers of CCG, motifs with a 100% CG content and present in high abundance. These results are consistent with the bias toward a higher GC-content of the genomic sequences and transcriptomes of free-living dinoflagellates (Jaeckisch et al. 2011, Williams et al. 2017). Unlike the genomes of protists, plants, and animals other than green algae, which contain a large proportion of poly-A stretches, in the *G. australes* genome the predominant mononucleotide is C. There were no differences in the overall abundance of microsatellite motifs with a 33.3% GC-content (e.g., AAC, AAG, or ATC) and those with a 66.6% GC-content (e.g., ACC, ACG, or AGG) in *G. australes*.

Our results indicate that trinucleotide repeats constitute a larger proportion of the microsatellites in the genome of *Gambierdiscus australes* than either mono- or AC and AG dinucleotide repeats. In most eukaryotes, especially higher vertebrates such as birds and mammals, mononucleotides as well as AC and AG dinucleotides are the most common microsatellites (e.g., Song et al. 2021). The exception is in green algae and some of the protists and fungi analyzed by Srivastava et al. (2019), in which trinucleotides accounted for a large proportion of the motif types and were second in abundance only to hexamers. In a genome survey of *Polarella glacialis* (the first draft genome assembled for a free-living dinoflagellate), Stephens et al. (2020) found that microsatellites consisting of AAC repeats were the most prominent, covering ~19% of the genome (~7 Gbp; LaJeunesse et al. 2005). AAC was also very common in *G. australes*. The fact that these two species belong to two phylogenetically distant orders (Suessiales and Gonyaulacales, respectively) suggests that AAC is a genome signature of free-living dinoflagellates.

Of the 30 possible tetranucleotide motifs, only GACA and GATA, both of which are present in abundance in plants and animals (e.g., Vosman and Arens 1997, Srivastava et al. 2008), were analyzed in *Gambierdiscus australes*. While neither microsatellite was as common as most of the trinucleotide repeats, whether this is the case for other tetranucleotides motifs remains to be determined. Jaeckisch et al. (2011), in their analysis of random clones from the genome of the dinoflagellate *Alexandrium ostenfeldii*, found that the tetranucleotides CACG, CATG, and CGTG were present in abundance.

The non-random genomic distribution of microsatellites has long been interpreted as evidence of their functional role (Katti et al. 2001).

Accumulations of microsatellites are frequently found at heterochromatic sites in specialized regions of the chromosome, such as the centromere (Cuadrado and Jouve 2007a, Cuadrado et al. 2008) and telomeres (Hatanaka et al. 2002), but also on specific chromosomes (e.g., sex chromosomes; Poltronieri et al. 2013). In other cases, microsatellites colonize the euchromatin, especially in intergenic regions. At the single-gene level, mononucleotide and dinucleotide motifs are more abundant in non-coding regions (e.g., 5′-UTRs, introns), and trinucleotide motifs in coding regions, where their presence would not disrupt the coding frame (Tóth et al. 2000). Our results indicate that the NOR of *Gambierdiscus australes* is devoid of microsatellites, at least those analyzed in this study. This is in contrast to other species, in which the intergenic spacers of 45S rDNA contain microsatellites. In primates including humans, FISH experiments showed that GACA and AG colocalize with 45S rRNA genes (Nanda et al. 1991, Cuadrado and Jouve 2007b). In *G. australes*, we found a nonrandom nuclear location of some microsatellites, including a high enrichment of poly C and especially the trinucleotide repeats ACC, ACG, ATC, AGG, and CCG; the presence of the latter defined the nuclear compartment referred to herein as the medulla. While microsatellites have frequently been used as molecular markers in dinoflagellates studies, including a study of *Gambierdiscus* (Sassenhagen and Erdner 2017), the chromosomal distributions of only four microsatellite motifs (AC, AG, GACA, and GATA) in three species of the genus *Karenia* have been reported. Those motifs were scattered over spherically shaped nuclei, with a particularly high density of AG repeats in one chromosome of *Karenia selliformis* and in one chromosome of *Karenia mikimotoii* (Cuadrado et al. 2019). Whether the preferential enrichment of trinucleotide microsatellites in a compartment around the NOR is peculiar to the nucleus of dinoflagellates, the genus *Gambierdiscus*, or only *G. australes* remains to be determined. Nonetheless, the biased distribution of microsatellites suggests that the different motifs play different roles in the nuclear architecture of *G. australes*, if not that of other dinoflagellates.

In higher eukaryotes, euchromatin and heterochromatin are separated in different nuclear regions. Heterochromatin consists of highly tandem-repeated DNA (satellite DNA, including microsatellites) organized as multiple copies of a repeat sequence (ranging in length from a few base pairs to >1 kb) over megabase-long arrays. In eukaryotes, heterochromatin generally localizes at the nucleolar and nuclear peripheries and is associated with the centromeric and/or the sub-telomeric region of chromosomes. Interest in the analysis of nuclear architectures arises from the recognition that the spatial organization of chromatin, such as the chromatin domain, associations of chromatin with nuclear structures, and chromosomal territories, affects genome function (e.g., Cremer and Cremer 2010, Bickmore and Steensel 2013, Dekker and Mirny 2016). An important aspect of the nucleolus is that it creates a large domain within the nucleus from which RNA polymerase II is absent (Fedoriw et al. 2012). In animal and plant cells, the nucleolus is surrounded by a shell of densely packed, transcriptionally silent, and late-replicated heterochromatin (reviewed in Bizhanova and Kaufman 2021). However, heterochromatin has not been described in the nuclei of dinoflagellates. Lacking a distinct centromere, dinoflagellate chromosomes are instead characterized by nucleofilaments that remain condensed throughout the cell cycle and by the absence of longitudinal chromosomal differentiation in Q, G, or C bands (Soyer-Gobillard et al. 1999). Whether the specific enrichment of trinucleotide repeats is of functional significance and whether the medulla of the *Gambierdiscus australes* nucleus plays an important role in essential genome functions are open questions. The aim of the 4D nucleome network is to elucidate the role that nuclear organization plays in nuclear function by investigating the spatial and temporal organization of both the human and the mouse nucleus (https://commonfund.nih.gov/4Dnucleome; Cremer et al. 2015). Ours is the first study to identify a subnuclear compartment enriched in microsatellites, especially in trinucleotide motifs, in the nucleosome-less nuclei of a dinoflagellate. This result suggests that the origin of genome compartmentalization, including the nucleosome-less genome organization of dinoflagellates, can be traced back to Protista.

The bipolar arrangement of telomeres in Gambierdiscus australes. Among the most well-known repetitive DNA sequences are those that, together with DNA-binding proteins, cap the end of linear chromosomes and are responsible for chromosomal integrity. In most organisms, telomeres consist of many repeats of a simple sequence added by telomerase. Telomeres of *Gambierdiscus australes* and other dinoflagellates species share the plant telomeric consensus sequence (TTTAGGG; Alverca et al. 2007, Lin et al. 2015). In our study, using the same probe and FISH conditions as applied to detect the telomeres of several plant species with a range of genome sizes (Cuadrado et al. 2009); we observed that the genome of *G. australes* is composed of many hundreds of telomeric repeats. Telomere sequences 25–80 kb in length have been reported in the dinoflagellate *Karenia papilionacea* and are the longest among unicellular eukaryotes (Fojtová et al. 2010).

Telomeres are found at different nuclear locations depending on the species, cell type, and cell cycle phase (Armstrong et al. 2001). For example, in many organisms, they contribute to the bouquet configuration of meiotic prophase chromosomes, where they promote the pairing and recombination of homologous chromosomes (Scherthan et al.

2000). In the interphase nucleus of *Gambierdiscus australes*, the telomeres assumed a bipolarized focal distribution, with one of the two subset in clear association with the nucleolus. In plants with small genomes, such as *Arabidopsis thaliana*, the centromeres are located at the nuclear periphery and the telomeres congregate around the nucleolus (Roberts et al. 2009). In yeast (Bystricky et al. 2005), *Drosophila* (Hochstrasser et al. 1986), and many plants with larger genomes, such as wheat or barley, interphase chromosomes exhibit a Rabl organization, with the telomeres and centromeres at opposite poles of the nucleus (reviewed by Cremer et al. 2006). Dinoflagellates are unique among eukaryotes, as their chromosomes lack centromeres and never directly contact the cytoplasmic spindles (Gavelis et al. 2019). Instead, it is the ends of the chromosomes themselves that mediate chromosomal segregation. Electron microscopy studies have shown that the tips of dinoflagellate chromosomes are closely opposed to the nuclear envelope, to which their filaments attach. Chromosomal segregation into two daughter chromatids begins following telomere attachment to the nuclear envelope and proceeds along the chromosomal axis toward the opposite end, with first a Y-shaped and then a V-shaped configuration (Soyer-Gobillard and Dolan 2015). Moreover, dividing dinoflagellate chromosomes contain a differentiated region at one chromosomal end, consisting of two spherically shaped structures tightly attached to each other; these may correspond to the remnant of an ancient kinetochore predecessor (Costas and Goyanes 2005). The bipolar arrangement of the telomeres in *G. australes* and its similarity to the arrangement found in species with large genomes and chromosomes in the Rabl arrangement suggests that the peripheral localization of the telomeres along the convex region of the *G. australes* nucleus is important for genome organization and serves as the starting point of chromosomal segregation. In dividing cells of *G. australes*, during the separation of the sister chromatids and their migration to opposite poles, the telomeres move such that at the end of the cell cycle (anaphase), prior to the formation of two daughter cells, they are widely dispersed throughout the nucleus.

The most prominent nuclear domain apart from the chromosomes is the nucleolus. The nucleolus is the site of rRNA transcription, processing, and ribosomal assembly but it has also been implicated in many other cellular functions (reviewed in Kalinina et al. 2018), including the maintenance of telomerase activity and thus immortality in species ranging from *Saccharomyces cerevisiae* to humans (Yuan and Tong 2018). The ultrastructural organization and composition of the functional subdomains of the nucleolus demonstrate that the nucleolus of dinoflagellates is very close to those of ciliates and plants (Geraud et al. 1991, Moreno Diaz de la Espina et al. 2005), but in contrast with most eukaryotes, the nucleolus of dinoflagellates do not disassemble during the cell cycle (Soyer-Gobillard and Geraud 1992). The frequent association of plant telomeres with the nucleolus suggests a functional link between these structures (Pontvianne et al. 2016). The surprisingly high density of telomeric DNA associated with the NOR of *Gambierdiscus australes* implies that the organization of telomeres within the nucleus and their association with the NOR are features of the dinoflagellate nucleus. However, the forces that determine telomere position in interphase nuclei are thus far unknown.

CONFLICT OF INTEREST

The authors have no competing interests to declare that are relevant to the content of this article.

AUTHOR CONTRIBUTIONS

Á. Cuadrado: Conceptualization (equal); formal analysis (lead); funding acquisition (supporting); investigation (equal); methodology (lead); writing – original draft (lead); writing – review & editing (lead). **R. I. Figueroa:** Conceptualization (equal); funding acquisition (lead). **M. Sixto:** Data curation (equal); formal analysis (equal). **I. Bravo:** Data curation (equal); funding acquisition (supporting); investigation (equal). **A. De Bustos:** Conceptualization (equal); data curation (equal); funding acquisition (supporting); investigation (equal).

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