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Absolute quantification of chromosome copy numbers in the polyploid macronucleus of *Tetrahymena thermophila* at the single-cell level

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Abstract

Amitosis is widespread among eukaryotes, but the underlying mechanisms are poorly understood. The polyploid macronucleus (MAC) of unicellular ciliates divides by amitosis, making ciliates a potentially valuable model system to study this process. However, a method to accurately quantify the copy number of MAC chromosomes has not yet been established. Here, we used droplet digital PCR (ddPCR) to quantify the absolute copy number of the MAC chromosomes in *Tetrahymena thermophila*. We first confirmed that ddPCR is a sensitive and reproducible method to determine accurate chromosome copy numbers at the single-cell level. We then used ddPCR to determine the copy number of different MAC chromosomes by analyzing individual *T. thermophila* cells in the G1 and the amitotic (AM) phases. The average copy number of MAC chromosomes was 90.9 at G1 phase, approximately half the number at AM phase (189.8). The copy number of each MAC chromosome varied among individual cells in G1 phase and correlated with cell size, suggesting that amitosis accompanied by unequal cytokinesis causes copy number variability. Furthermore, the fact that MAC chromosome copy number is less variable among AM-phase cells suggests that the copy number is standardized by regulating DNA replication. We also demonstrated that copy numbers differ among different MAC chromosomes and that interchromosomal variations in copy number are consistent across individual cells. Our findings demonstrate that ddPCR can be used to model amitosis in *T. thermophila* and possibly in other ciliates.

KEYWORDS

amitotic cell division, ciliated protozoa, copy number variation, droplet digital PCR, polyploidy

AMITOSIS is a simple type of cell division in which nuclear genetic materials are distributed to daughter cells in the absence of a mitotic spindle. This process occurs not only in primitive eukaryotes such as ciliates and amoeba (Gicquaud & Tremblay, 1991; Prescott, 1994) but also in a wide variety of higher eukaryotic organisms,

including plants, insects, fish, and mammals (Miller, 1980; Nagata & Ma, 2004; Nakahara, 1917; Wang et al., 2010). Furthermore, recent studies indicate that amitosis has an important role in tumorigenesis and in the regeneration of tissue-specific stem cells (Lucchetta & Ohlstein, 2017; White-Gilbertson et al., 2020). However,

despite its widespread occurrence and functional importance in eukaryotes, the evolutionary significance and molecular mechanisms underlying amitosis have been poorly explored.

Ciliates are unicellular eukaryotes that contain a diploid germline micronucleus (MIC) and a polyploid somatic macronucleus (MAC). The MIC is analogous to other eukaryotic nuclei and divides by mitosis and meiosis, whereas the MAC of most ciliates divides by amitosis. Genetic evidence indicates that during amitosis homologous chromosome copies of the parental MAC randomly segregate to daughter nuclei (Doerder et al., 1992). This can generate variation in chromosome numbers among individual cells, with repeated divisions yielding offspring that are homozygous for a given locus via phenotypic assortment. Theoretical models indicate that this unique feature of amitosis can provide adaptive advantages in ciliates resembling those of sex, e.g. through preventing the accumulation of deleterious mutations (Doerder, 2014; Zhang et al., 2021). Therefore, ciliate MACs are a powerful system to study the mechanism and evolution of amitosis. An essential prerequisite for our further understanding of amitosis is the ability to accurately determine MAC chromosome copy numbers, but this is lacking for most ciliates, including some model species.

Droplet digital polymerase chain reaction (ddPCR) is a method of quantifying the abundance of target DNA within a sample. In ddPCR, DNA molecules are randomly distributed into tens of thousands of nanodroplets, each of which represents an independent PCR system to amplify target DNA to the end-point. The copy number of a target DNA molecule is calculated based on the ratio of positive to total droplets using Poisson statistics (Hindson et al., 2011). Compared with real-time quantitative PCR, ddPCR is more sensitive and has a greater tolerance to PCR inhibitors or differing PCR efficiencies, and enables accurate DNA quantitation without external calibrators (reviewed in Quan et al., 2018).

The model ciliate, *Tetrahymena thermophila* is a well-established experimental system for studying cellular and molecular biology, epigenetics, genomics, ecology, and evolution (Greider & Blackburn, 1985; Mochizuki et al., 2002; Wang et al., 2020; Xiong et al., 2019; Yan et al., 2021; Zufall et al., 2013). The *T. thermophila* MIC contains five pairs of large metacentric chromosomes (Hamilton et al., 2016); in contrast, the most recent complete genome assembly showed that the mature polyploid MAC is composed of 181 acentric chromosomes, including one highly amplified rDNA minichromosome (Sheng et al., 2020; Wang, Wang, et al., 2021). MAC chromosomes differentiate from MIC chromosomes during sexual conjugation via chromosome fragmentation, deletion of internal eliminated sequences (IES), and polyploidization (Orias et al., 2011). Assortment kinetics of several phenotypic markers suggest that there are

approximately 45 haploid assorting units (i.e. 45 copies) for each MAC locus at G1 phase (Allen & Nanney, 1958; Doerder et al., 1977, 1992). On the other hand, cytochemical data indicate that the G1 MAC contains about 23 times as much DNA as the diploid MIC (Doerder & Debault, 1975; Woodard et al., 1972), and genome assembly data suggest that the MIC genome is approximately 1.5 times larger than the MAC genome (Hamilton et al., 2016); based on these data, the estimated chromosome copy number in G1 MAC is approximately 70 (Yao et al., 2014). Therefore, the copy number of *T. thermophila* MAC chromosomes remains under debate.

This study aimed to use ddPCR to quantify the copy numbers of *T. thermophila* MAC chromosomes at single-cell resolution. For this, we first assessed the accuracy, sensitivity, and reproducibility of this method. We then determined the copy number of different MAC chromosomes in individual *T. thermophila* cells, both at G1 phase and at amitotic (AM) phase when DNA replication is complete. G1-phase cells were obtained by starvation and confirmed using flow cytometry and 5-ethynyl-2'-deoxyuridine (EdU) labeling. We also examined variations in chromosome copy numbers between and within individual cells. Finally, we discuss discrepancies between our current estimates and previous estimates of the copy number of *T. thermophila* MAC chromosomes, and propose possible explanations.

MATERIALS AND METHODS

Cell culture and isolation of single cells at G1 and AM phases

The *T. thermophila* strain used in this study was generated by genomic exclusion crosses (Wang, Fu, et al., 2021) between strains SB210 and B* VII (obtained from the *Tetrahymena* Stock Center at Cornell University). Cells were grown to log-phase (3×10^5 cells/ml) in SPP medium (1% proteose peptone, 0.2% glucose, 0.1% yeast extract, 0.003% sequestrene) at 30°C, with shaking at 135 rpm. To obtain synchronized G1-phase cells, cells were then washed and transferred to starvation conditions (10 mM Tris-HCl buffer, pH 7.4; Cameron & Jeter, 1970; Doerder & Debault, 1975). We confirmed G1 arrest by flow cytometry and EdU labeling (see below). To obtain AM-phase (amitotic) cells, starved cells were transferred into SPP medium to restart cell division, followed by selection of cells with the characteristic “peanut” shape of dividing cells. The MACs of peanut-shaped cells have completed DNA replication (Cameron & Jeter, 1970; McDonald, 1962). Individual G1- and AM-phase cells were isolated using a mouth pipette under microscopy and transferred to a PCR tube, as previously described (Chen et al., 2019). All isolated cells were stored at -80°C before analysis.

Flow cytometry and EdU labeling

Flow cytometry and EdU labeling assays were used to follow cell cycle progression and evaluate whether cells had entered G1 phase after starvation treatment. Flow cytometry was carried out essentially as described previously (Morrison et al., 2005). Briefly, 1.5-ml cells were harvested from a 50-ml starved culture (cell density, 2×10^5 cells/ml) at 0, 2, 4, 8, 16, and 24 h, resuspended in 13.5 ml cold 75% ethanol, and incubated for 2 h at 4°C to fix the cells. Fixed cells were sedimented at 150 g for 2 min and stained for 30 min in a 1-ml solution of 0.1% Triton X-100, 0.02 mg/ml propidium iodide, and 0.2 mg/ml RNase A. Cell fluorescence was measured on a CytoFLEX S flow cytometer (Beckman Coulter) and data were analyzed using FlowJo_V10 software (Becton, Dickinson).

EdU specifically labels S-phase nuclei via incorporation into nascent DNA stands. The EdU labeling assay was performed using Click-iT EdU Imaging Kits (Thermo Fisher Scientific, Cat. No. C10337) according to the manufacturer's protocol. Briefly, 1-ml cells were collected after 0, 2, 4, 8, 16, and 24 h of starvation and incubated with 100 μ M EdU at 30°C for 30 min. Cells were then fixed by incubation with 3.7% formaldehyde for 30 min at room temperature and then washed twice with 10 mM Tris-HCl buffer (pH 7.4). After permeabilization with 0.5% Triton X-100, cells were incubated with 100- μ l Click-iT reaction cocktail for 30 min in the dark. Images were collected using laser scanning confocal microscopy (Leica TCS SP8, Leica Microsystems) at 400X magnification. DAPI (4',6-diamidino-2-phenylindole) staining was performed to label all cell nuclei.

Cell lysis

Single cells at G1 or AM phase were lysed using the REDExtract-N-Amp Tissue PCR Kit (Sigma) according to the manufacturer's protocol, except that incubations at 55°C for 10 min and then 95°C for 5 min were performed using a thermal cycler. The final volume of cell lysates was 2 μ l.

ddPCR workflow

All ddPCR procedures were conducted using the QX200 Droplet Digital PCR System according to the manufacturer's instructions (Figure S1). Briefly, each ddPCR reaction mixture contained a final concentration of 1 \times ddPCR Supermix for Probes (no dUTP) (Bio-Rad), 250 nM each primer, 125 nM each probe, 2 μ l cell lysate or DNA sample, and ddH₂O to a final volume of 20 μ l. The entire reaction mix was separated into droplets using a QX200 droplet generator (Bio-Rad). After droplet formation, PCR was performed at 95°C for 5 min; 40

cycles of 95°C, 53–58°C (depending on the primer pair), and 72°C for 30 s; and then 98°C for 10 min. PCR products were stored at 4°C. Fluorescence was measured using a QX200 droplet reader (Bio-Rad). The fluorescence threshold for positive droplets was determined by comparison with nontemplate negative controls (lysis buffer or ddH₂O). In addition, ddPCR reactions with fewer than 15,000 droplets were excluded from further analysis. ddPCR data were analyzed using QuantaSoft software to calculate the copy number of each target.

Genomic DNA extraction and PCR amplicons preparation

Total genomic DNA was isolated from approximately 4×10^5 cells as previously described (Gorovsky et al., 1975). PCR amplification of the unique MAC-destined sequence (MDS) on the MAC chromosome chr_005 was carried out using genomic DNA as the template and specific primers. The PCR amplicon was purified using 2% agarose gel electrophoresis and a FastPure Gel DNA Extraction mini kit (Code No. DC301–01, Vazyme Biotech). We then measured the concentration of the amplicon with a Qubit dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific, Cat. No. Q33231) (O'Hara et al., 2019) and determined the copy numbers using an online calculator (<https://www.technologynetworks.com/tn/tools/copynumbercalculator>).

Evaluation of DNA quantification by ddPCR

We used serial dilutions of the PCR amplicon (see above) and cell lysates to evaluate the performance of the ddPCR assay for DNA quantification. For each PCR amplicon, 10-fold serial dilutions of the sample were prepared in four replicates. For cell lysates, 64 G1-phase cells were isolated and lysed as described above, and then twofold serial dilutions of lysate samples containing 0.5–8 cells were prepared in four replicates.

Quantification of MAC chromosome copy number

We randomly selected 15 non-rDNA MAC chromosomes (three from each of the five MIC chromosomes) for copy number quantification using ddPCR (Figure S2). For this, we first identified a unique region on each MAC chromosome through a BLAST search to use as a proxy for each chromosome and then designed primers and probes for these regions. Because the MAC differentiates from the MIC, these unique MDS are common to both the MAC and the MIC. Therefore, to remove the background signal from MIC sequences, primers/probes were designed to target 15 unique IES from the five MIC

chromosomes, i.e. three MIC-specific sequences for each chromosome. The final copy number of each MAC chromosome was calculated from the copy number of the MDS minus the copy number of the IES. To simultaneously measure the copy numbers of MDS and IES in a single ddPCR reaction (i.e. duplex ddPCR), the MDS and IES probes were labeled with different fluorescence signals (FAM and VIC). Table S1 lists the combinations of MDS and IES primer–probe sets used for the 15 MAC chromosomes.

Analysis of cell size

Single live cells at G1 phase were picked onto glass slides and images were obtained at 400X magnification using an Olympus BX43 microscope. The cells were immediately transferred into PCR tubes and stored at -80°C for ddPCR analysis. Cell length and width were measured from the images using Image J (<https://imagej.net/software/fiji/>).

Statistical analysis

Statistical tests were performed using SPSS v16.0. Linear regression analyses were carried out using GraphPad Prism 8.0.

RESULTS

Evaluation of the ddPCR assay

To evaluate the accuracy and sensitivity of the ddPCR assay in quantifying MAC chromosome copy numbers, PCR amplicons (sequences amplified from genomic

DNA) from a unique MDS region on a specific MAC chromosome (chr_005) were prepared and 10-fold serial dilutions (ranging from about 3 to 30,000 molecules per reaction) were tested. As shown in Figure 1A, the copy numbers detected by ddPCR could be fitted to a slope of almost equal to 1 ($R^2 = 0.993$) by linear regression analysis, indicating that the ddPCR assay can accurately quantify the absolute copy number of target molecules. Moreover, the ddPCR assay was shown to have a dynamic range from several copies to tens of thousands copies of the target molecule per reaction (Figure 1A).

We next evaluated the performance of the ddPCR assay in directly quantifying *T. thermophila* MAC chromosome copies. For this, both the MDS region and a unique IES (MIC-specific) sequence proximity to the MDS region were examined by ddPCR in the same reaction using probes labeled with different fluorescence dyes, and then the IES copy number was subtracted from the MDS sequence copy number. Results from twofold serial dilutions of cell lysates (0.5–8 cell equivalents per reaction) showed significant linearity, $R^2 = 0.998$ ($p < 0.0001$), with low coefficient of variation (CV) values of about 10% (Thulesius et al., 2011), even for samples equivalent to half a cell (Figure 1B). These results demonstrate that our ddPCR-based method can reliably quantify the copy number of *T. thermophila* MAC chromosomes in single-cell lysates.

Starvation treatment induces G1 arrest in *Tetrahymena thermophila* cells

To obtain cells at G1 phase (of the MAC cycle, unless otherwise stated) for MAC chromosome copy number quantification, previous studies used the starvation method by transferring growing cells to nonnutrient

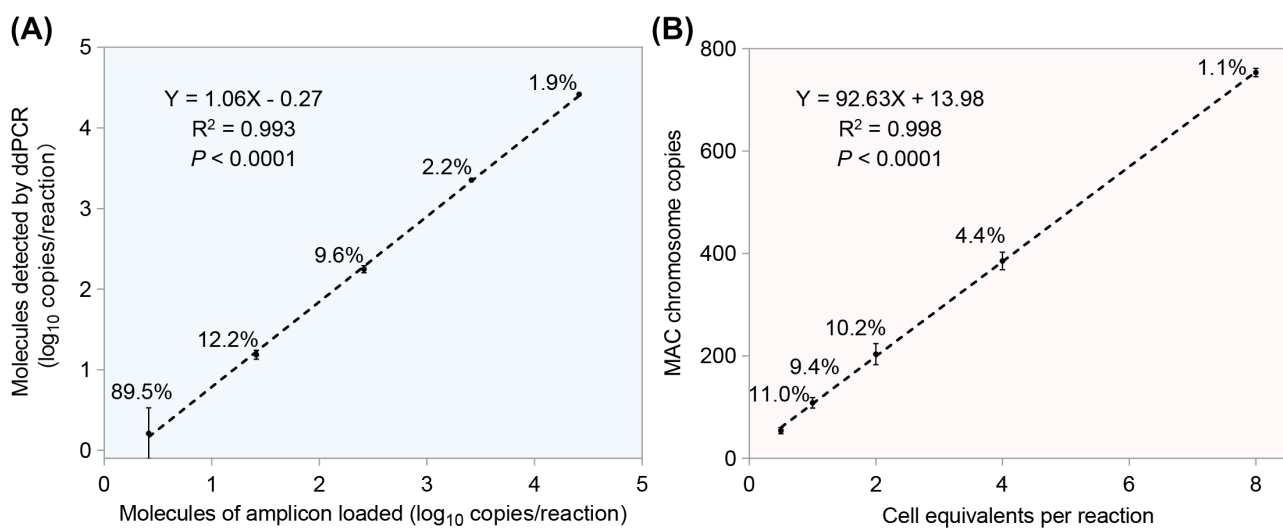


FIGURE 1 Evaluation of the ddPCR assay. (A) Absolute quantification of PCR amplicons in a 10-fold dilution series. (B) Absolute quantification of MAC chromosomes in a twofold dilution series of cell lysate. Data points represent the mean of four replicates and error bars show the standard deviation. Percentages indicate the CV of the mean

medium (Cameron & Jeter, 1970; Doerder & Debault, 1975). To validate this method and determine the time needed for the whole cell population to enter G1 phase, we followed cell cycle progression by flow cytometric measurement of cellular DNA content at different time points after transfer to starvation condition. There was a broad distribution of DNA content during the first 2 h of starvation (Figure 2A), suggesting that the population contained cells at various phases of the cell cycle. However, with prolonged starvation, the distribution of DNA content gradually became narrower and shifted toward the lowest value (Figure 2A). After 16 h of starvation, the distribution remained unchanged, indicating that almost all cells had entered G1 phase; they stayed in G1 phase until at least 24 h after starvation.

We also used EdU labeling assay (to stain S-phase nuclei) to visualize cell cycle progression during starvation. The proportion of cells with an EdU-positive MAC decreased rapidly from 38% to 4% during the first 4 h of starvation and reached almost 0% at 16 h (Figure 2B,C), indicating that most cells had exited S phase by 16 h post starvation. This corresponds to the time point at which most cells are in the G1 phase based on their DNA content (Figure 2A).

DNA replication does not occur synchronously between MAC and MIC: the MIC enters S phase directly after undergoing mitosis (Dorder, 1979; Flickinger, 1965). We confirmed this by refeeding the starved cells and observing cytokinesis (i.e. peanut-shaped cells) by EdU labeling: in these cells, the MAC had not yet entered S phase, whereas the MIC had already undergone DNA replication (Figure 2D). Because starvation causes cells to arrest at the MAC G1 phase and MIC G2 phase (Dorder & Debault, 1975), DNA replication should cease earlier in the MAC than in the MIC. Consistent with this, upon starvation the rate of reduction was slower in cells possessing an EdU-positive MIC than in those with an EdU-positive MAC; the S-phase MIC did not become undetectable until 24 h after transfer to starvation conditions (Figure 2C). Together, these results indicate that starvation for 24 h can be used to prepare a homogeneous population of cells in which the MAC is in the G1 phase and the MIC is in the G2 phase.

Quantification of MAC chromosome copy numbers in single cells

We randomly selected 15 of the 180 non-rDNA MAC chromosomes and quantified their copy numbers by single-cell ddPCR. Copy numbers of the adjacent IES in the MIC were quantified to enable correction for the copy number of the MIC MDS sequence (Figure S2). We isolated both G1-phase (starvation for 24 h) and AM-phase (peanut-shaped; Figure 2D) single cells for the ddPCR assay. Results from at least 24 individual cells for each

chromosome at each cell cycle phase are summarized in Figure 3 and Table S2. Copy numbers of the 15 MAC chromosomes were relatively uniform, with means ranging from 71.2 to 104.2 (Figure 3A). This is consistent with high-throughput sequencing data that showed that copy numbers of all 180 non-rDNA chromosomes are maintained at similar levels (Eisen et al., 2006; Sheng et al., 2020). Moreover, the mean chromosome copy numbers of AM-phase cells were almost twice the value in G1-phase cells (Figure 3B), indicating that our method can precisely quantify MAC chromosome copy numbers. By pooling all single-cell ddPCR data from 15 MAC chromosomes at each phase, we found that *T. thermophila* cells had an average chromosome copy number of 90.9 in G1 phase (95% confidence interval (CI) = [88.1, 93.6]) and 189.8 in AM phase (95% CI = [185.4, 194.2]). We also use the MIC chromosome counts as a standard reference to calibrate MAC chromosome copy numbers in G1 phase because each starved cell contains four copies of each MIC chromosome. To obtain robust MIC chromosome counts, we pooled 15 starved cell for each ddPCR reaction. MAC chromosomes chr_076 and chr_170 were selected for calibration, and the MDS primer–probe set for each MAC chromosome and corresponding IES primer–probe set (Table S1) were used to simultaneously quantify the copies of MAC and MIC chromosomes. After calibration, the copy numbers of chromosomes chr_076 and chr_170 were 100.5 and 81.7 (Figure S3), which are very close to the values (97.5 and 76.4, respectively; Table S2) estimated by single-cell ddPCR assay.

Copy number of individual MAC chromosomes varies between cells

We next examined intercell copy number variation for each MAC chromosome. As shown in Figure 4A, the copy number of MAC chromosome chr_005 detected in different single cells varied widely, from 51 to 178. The other chromosomes showed similar variability in copy number (Figures S4 and S5). To exclude the possibility that this was due to a technical artifact, we pooled and lysed a specific number of cells at G1 phase (starved for 24 h), and aliquoted the cell lysates into one-cell equivalent samples for ddPCR analysis. The detected copy numbers of chromosome chr_005 among the duplicate samples were clearly more homogeneous than those for individual single cells (Figure 4B). Moreover, the CV in copy number for the duplicate samples was only 12.1% (Figure 4C), which is close to the intrinsic variation of the ddPCR method (Figure 1B) but remarkably lower than the CV for individual single cells (35.4%). These results suggest that the detected copy number variation among different cells is not due to a technical artifact but reflects genuine intercellular variations in MAC chromosome copy number.

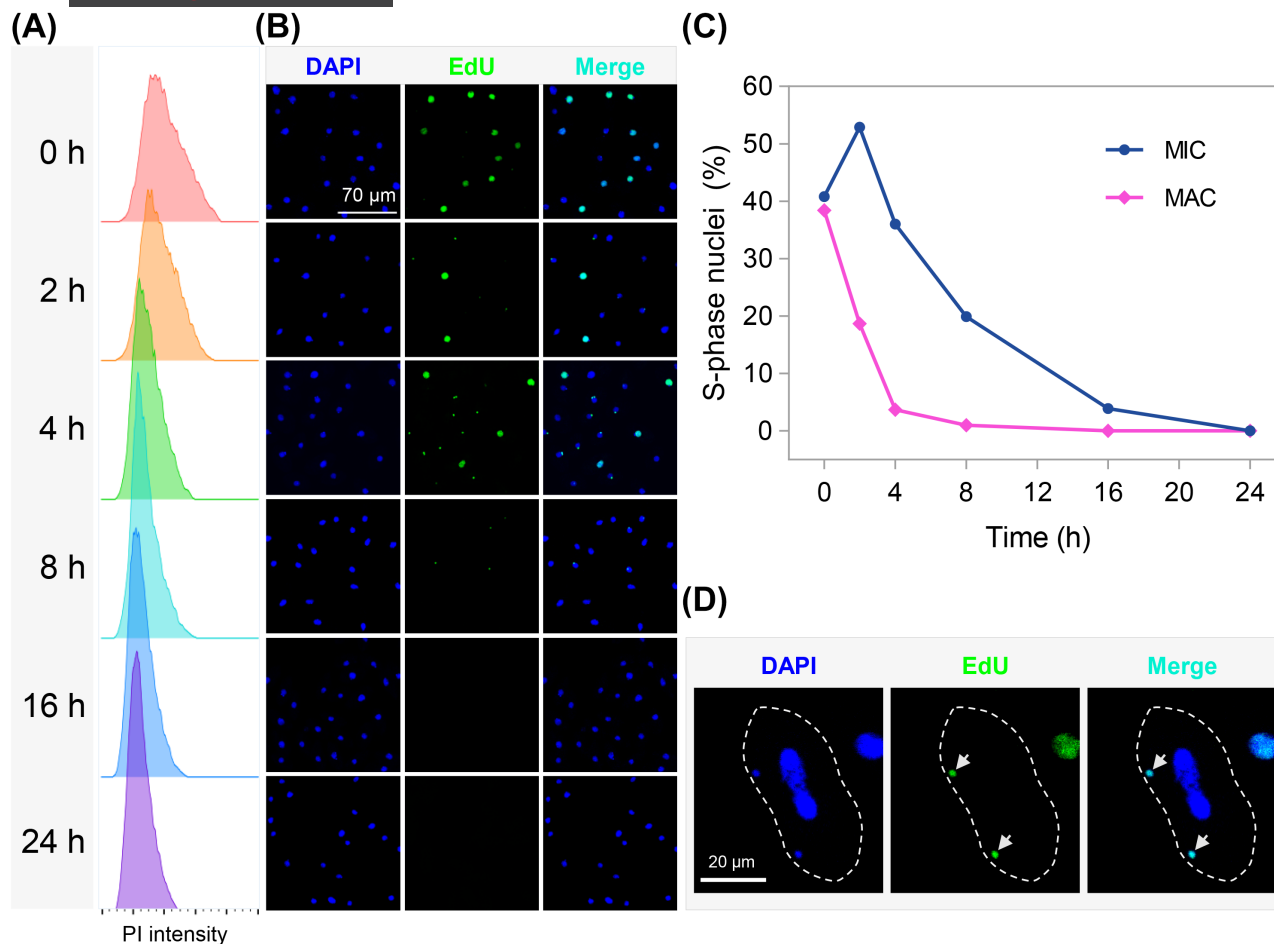


FIGURE 2 *Tetrahymena thermophila* cell cycle progression during starvation treatment and features of AM-phase cells. (A) Time course of the flow cytometry profile of *T. thermophila* cells under starvation conditions. The x-axis shows the propidium iodide (PI) signal (DNA content) and the y-axis shows the number of events detected (total number of events = 20,000). (B) Fluorescence microscopy images of *T. thermophila* cells labeled with DAPI and EdU at different time points during starvation treatment. DAPI stains all nuclei (blue) and EdU labels nuclei undergoing DNA synthesis (green). (C) Time course showing the percentage of MACs and MICs in S phase during starvation treatment. (D) AM-phase cells with the typical peanut shape. Note that EdU signals can be detected in the MICs of AM-phase cells (arrows)

Variation in MAC chromosome copy number is caused by unequal cytokinesis

Random segregation of MAC chromosomes by amitosis has long been suggested as a cause of variation in MAC chromosome copy number among cells within a population (Doerder, 1979). The other (major) source of MAC chromosome copy number variation could be unequal cytokinesis in which two daughter cells differ in their size and unevenly inherit the parental MAC DNA content. Indeed, a recent study reported that MAC DNA content correlates with cell size (Liu et al., 2021). Therefore, to explore whether copy numbers of MAC chromosomes also vary with cell size, we randomly isolated individual live cells (starved for 24 h), measured their length and width by microscopy imaging, and then analyzed the copy numbers of MAC chromosome chr_005 in each cell by ddPCR. Copy numbers of this chromosome in individual cells positively correlated with both cell length (Figure 4D) and cell width (Figure 4E). These data

support our hypothesis that variation in MAC chromosome copy number is a consequence of unequal cytokinesis. However, the modest correlation between copy number and cell size ($r \approx 0.60$) suggests that random chromosome segregation during amitosis also plays a significant role in MAC chromosome copy number variation.

Variation in MAC chromosome copy number is buffered by regulating DNA replication

Despite large variations in the copy number of MAC chromosomes at G1 phase, overall MAC chromosome copy numbers are maintained at about 90. Therefore, an active mechanism must exist to buffer the variation in MAC chromosome copy number caused by amitosis. We speculated that this may involve regulating DNA replication prior to the next amitosis. To test this possibility, we examined intercell copy number variation for each MAC chromosome at AM phase. Indeed, we found that intercell copy number

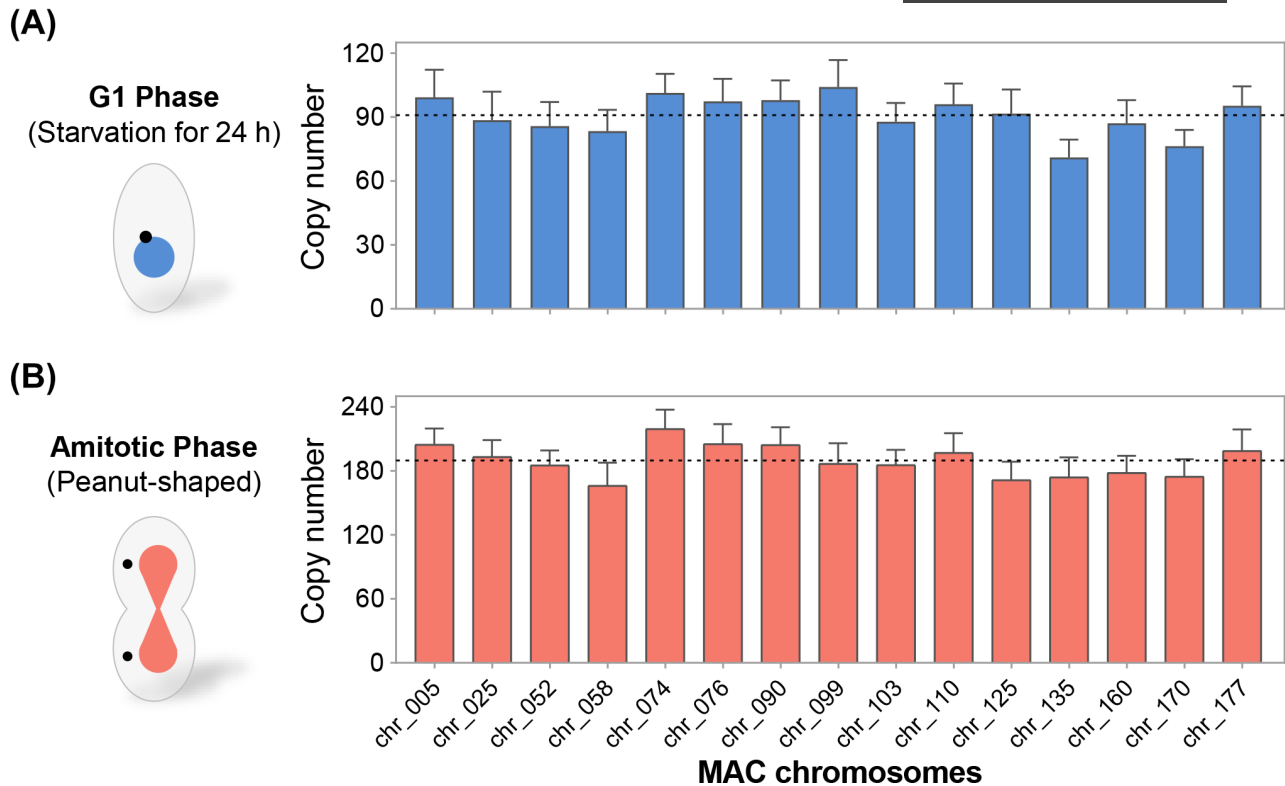


FIGURE 3 ddPCR analysis of individual G1- and AM-phase cells. Copy numbers of 15 MAC chromosomes at G1 phase (A) and at AM phase (B). Error bars represent the 95% CIs of the means. Dashed lines represent the mean chromosome copy numbers in all cells analyzed at each cell cycle phase

variation in 13 of the 15 MAC chromosomes examined was much smaller at AM phase than at G1 phase (Figure 4F), suggesting that copy number variation can be reduced by modulating DNA replication in the MAC.

Copy number variation in different MAC chromosomes

We showed that copy numbers are highly variable across different MAC chromosomes (Figure 3). However, this result might be a sampling artifact caused by intercellular heterogeneity (Figure 4) or technical variables. Therefore, to critically test for copy number variation between MAC chromosomes, we aimed to simultaneously quantify the copies of two different MAC chromosomes within a single G1 phase cell (Bodenbender et al., 1992; Doerder, 1979). For this, we examined chr_076 and chr_170 for which we had previously measured copy numbers above and below, respectively, the average value for 15 chromosomes (Figure 3).

We next designed one additional primer set targeting another unique MDS sequence on each chromosome (Table S1) and performed ddPCR with two primer sets targeting either the same chromosome (Figure 5A,B) or the two different chromosomes (Figure 5C,D) by labeling the primer sets with different fluorescent dyes. We found that the two primer sets targeting different amplicons in

the same chromosome detected very similar copy numbers for both chromosomes (no significant difference; paired t -test, $p > 0.05$), although the intercell variation in copy number was large (Figure 5A,B). In contrast, both combinations of two primer sets amplifying sequences in different chromosomes showed consistently higher copy numbers for chr_076 than for chr_170 in individual cells (Figure 5C,D; paired t -test, $p < 0.0001$). Altogether, our results demonstrate that there are indeed variations in copy number between different MAC chromosomes.

DISCUSSION

In this study, we present the first use of ddPCR methodology to quantify the absolute copy numbers of different MAC chromosomes in the model ciliate *T. thermophila*. Our results show that ddPCR is a sensitive method to reliably measure the number of MAC chromosome copies in *T. thermophila* at the single-cell level (Figure 1). In addition, ddPCR has a linear detection range from several copies to tens of thousands of copies of target molecules (Figure 1A), enabling its use in quantifying a wide range of MAC chromosome copy numbers; therefore, it may be applicable in most ciliate species (Spring et al., 2013; Xu et al., 2012). Furthermore, ddPCR can be used to directly quantify chromosome copy numbers in single-cell lysates (Figure 1B). This is especially important for investigating

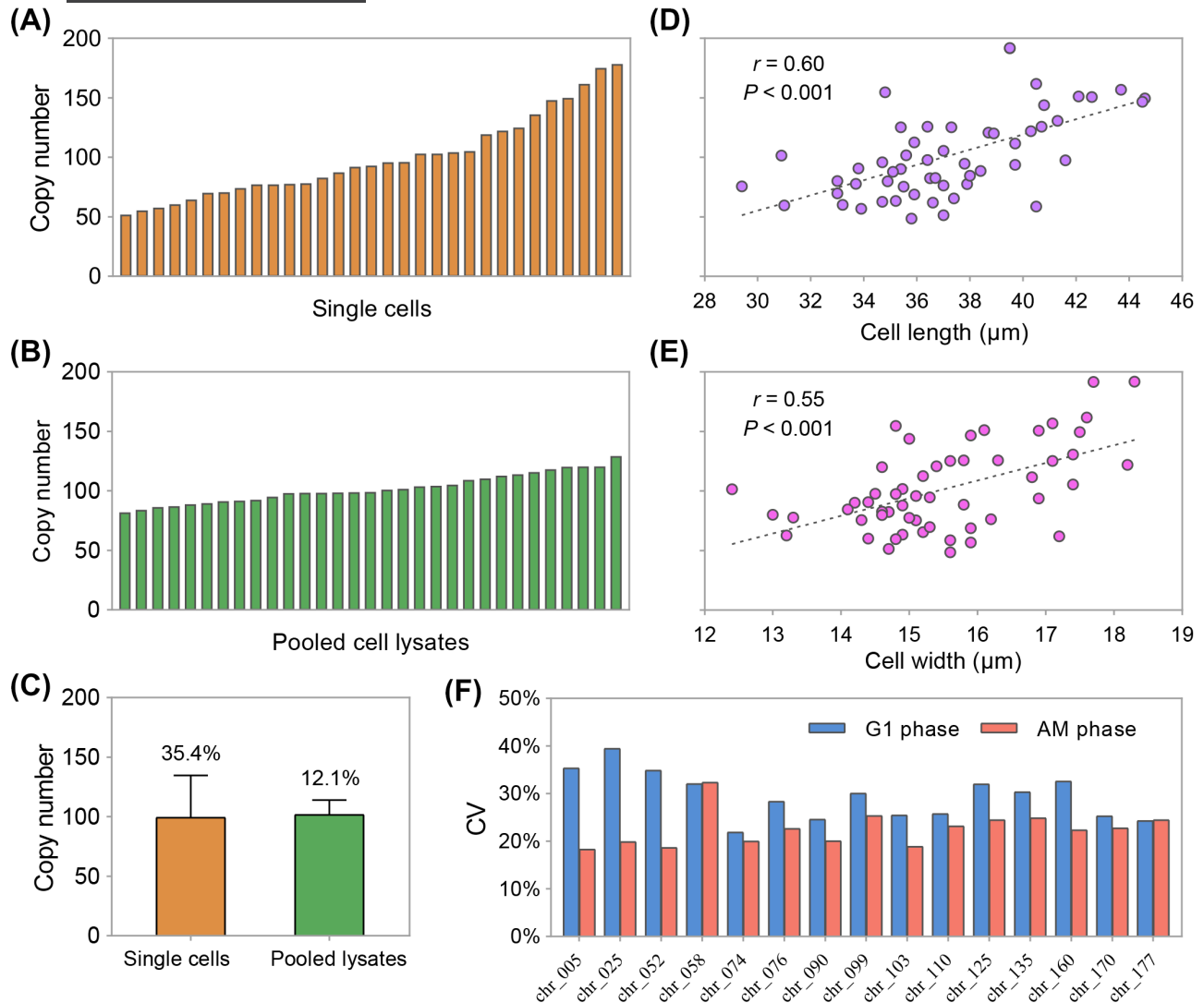


FIGURE 4 Variations in chromosome copy number among single cells. (A and B) Chromosome copy numbers in single cells (A) and one-cell equivalent lysate samples (B). (C) Bars show the mean copy numbers from single-cell and cell lysate samples ($N = 31$), error bars show the standard deviation, and percentages show the CVs of the means. (D and E) Correlations between chromosome copy numbers and cell length (D) and width (E). r represents the Pearson correlation coefficient. (F) Intercell copy number variation was much smaller at AM phase than at G1 phase. CV, coefficient of variation

nonmodel ciliates, for most of which culture conditions in the laboratory have not been established.

The ploidy level of the *T. thermophila* MAC was first estimated by Allen and Nanney (1958) using assortment kinetics of a phenotypic marker across amitotic cell divisions. By mathematically modeling this assortment process, they proposed that the MAC genome is composed of 45 independently assorting genetic units (Allen & Nanney, 1958; Schensted, 1958). Originally, these units were assumed to be diploid, like MIC chromosomes. However, later cytochemical experiments to measure the ratio of DNA content between the MAC and MIC (23:1 at G1 phase) suggested that the amount of MAC DNA is sufficient for only 45 haploid units (Doerder & Debault, 1975; Woodard et al., 1972), based on the assumption of little difference in size between MAC and MIC genomes. However, the current version of the assembled MIC

genome (157 Mb) is approximately 1.5 times larger than the MAC genome (103 Mb) (Hamilton et al., 2016; Wang, Wang, et al., 2021), corresponding to approximately 70 copies of MAC chromosomes at G1 phase. In the present study, we quantified 15 of the 180 non-rDNA MAC chromosomes and obtained an average copy number at G1 phase of approximately 90 (Figure 2). This agrees well with the estimate of Allen and Nanney (1958) if the assorting units are diploid. Whether these 90 chromosome copies are assembled into 45 assorting units during amitotic division needs to be rigorously tested. We propose to use molecular markers, such as genome-wide single-nucleotide polymorphic sites (Chen et al., 2019), rather than phenotypic markers to study assortment kinetics by tracking changes in their frequency during successive amitotic divisions through sequencing (Vitali et al., 2021). This could directly reveal how many assorting

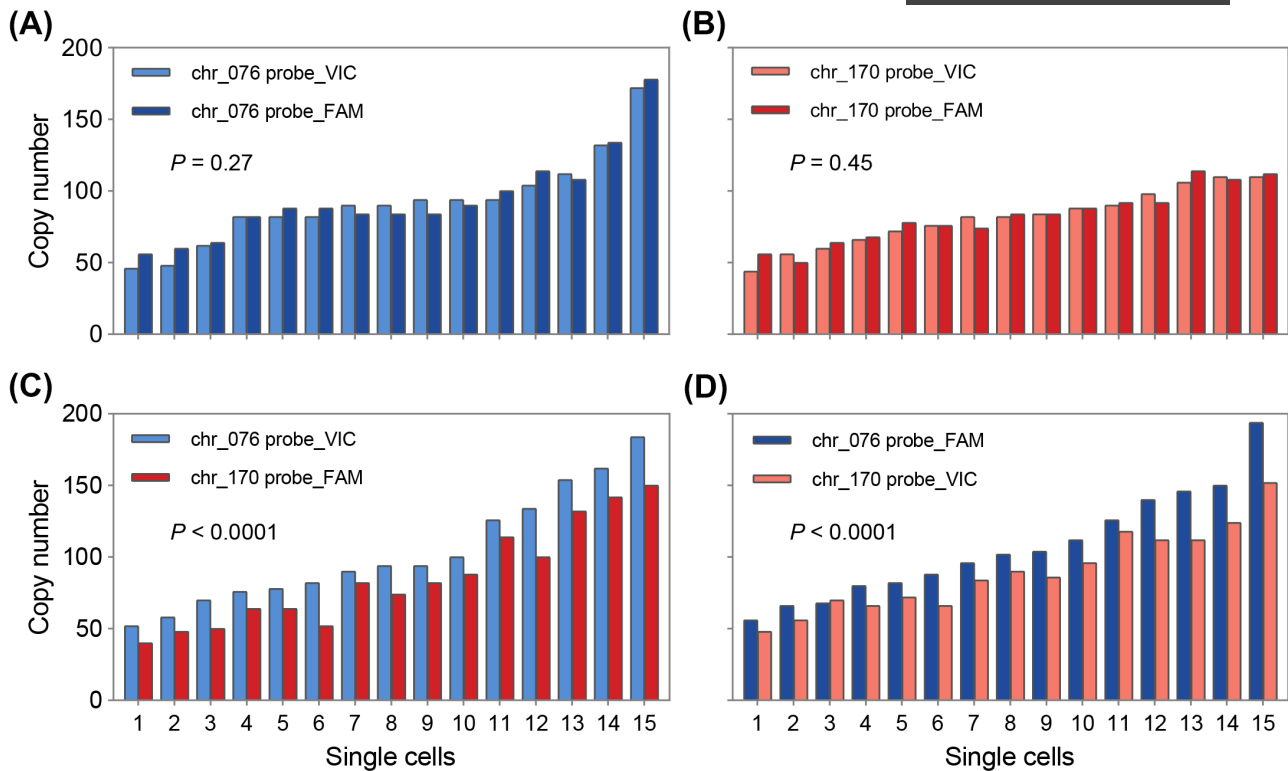


FIGURE 5 Interchromosomal variation in copy number within single cells. (A and B) Quantification of the copy numbers of two MDS regions on the same MAC chromosome, chr_076 (A) or chr_170 (B), using fluorescent probes labeled with either FAM or VIC. (C and D) Quantification of the copy numbers of two MDS regions on different MAC chromosomes, chr_076 (C) and chr_170 (D), using fluorescent probes labeled with either FAM or VIC. Paired *t*-test was performed for each panel

units are assembled from the 90 genomic or allelic copies for each MAC chromosome.

This study also questions the currently estimated genome size of the MIC (157 Mb). If the 15 MAC chromosomes we randomly selected are good representatives of all MAC chromosomes, then the MIC genome should be about twice as large as the MAC genome, based on the ratio of DNA content between the MAC and MIC (23:1 at G1 phase) and the MAC genome size (103 Mb) and ploidy (90.9 at G1 phase), i.e. $(103 \times 90.9)/(23 \times 2) \approx 204$ Mb. To resolve this discrepancy, MIC genome assembly needs to be refined using long-read sequencing technology, as was done for MAC genome assembly (Sheng et al., 2020; Wang, Wang, et al., 2021), because the current version of the assembled MIC genome contains many gaps that are likely caused by repetitive sequences (Hamilton et al., 2016).

In all ciliates except for the primitive karyorelicts, the MAC divides amitotically during asexual growth without mitotic spindles or functional centromeres. A major consequence of amitosis is the unequal distribution of DNA molecules between the two daughter nuclei, which tends to increase the variance in MAC DNA content among progeny (Blackburn & Karrer, 1986; Doerder, 1979; Doerder & Debault, 1978; Seyfert, 1977). Indeed, through determining the copy numbers of different MAC chromosomes at the single-cell level, we demonstrated

that amitotic cell divisions can cause intercellular and interchromosomal copy number variation in *T. thermophila* (Figures 4 and 5). Therefore, in the absence of a compensatory regulatory mechanism for copy number control, a long period of amitotic cell division is expected to cause an imbalance in aneuploid chromosome numbers, which would eventually lead to cell senescence and death (Bell, 1982). Our findings in this study that different chromosomes have similar copy numbers across cells suggest that a coordinated mechanism controls chromosome copy numbers (Figure 3). However, we also found stable pattern in chromosome copy number across individual cells (Figure 5), suggesting that there might also be a specific mechanism to independently count the number of each chromosome.

The current evidence indicates that ciliate species use different mechanisms to regulate chromosome copy number. In *T. thermophila* and *Paramecium tetraurelia*, copies of all MAC chromosome (except for the rDNA minichromosome) are maintained at relatively equal numbers, indicating the existence of a coordinated regulation mechanism (Eisen et al., 2006; Le Mouel et al., 2003). In *Euplotes crassus*, amplification of each chromosome is regulated individually, so each chromosome may have a different number of copies (Baird & Klobutcher, 1991). Moreover, for MAC chromosomes in *Oxytricha trifallax*, variations in copy number were shown to positively

correlate with gene expression levels across chromosomes (Xu et al., 2012). Furthermore, a recent study in *Paramecium bursaria* found low variation in copy number for chromosomes harboring housekeeping genes, whereas chromosomes with high copy number variations often include environmental response or species-specific genes (Cheng et al., 2020). In addition, evidence from *T. thermophila* rDNA minichromosome analyses indicate that RNA expression may be involved in rDNA copy number control (Larson et al., 1991). The exceptional accuracy and high sensitivity of ddPCR (Figure 1B) will allow us to address whether and how MAC chromosome copy number is modulated in different environmental conditions in *T. thermophila* and in other ciliates.

Our study found that in *T. thermophila*, the MAC chromosome copy number is controlled at least partly at the level of DNA replication (Figure 4F). Therefore, during DNA replication, cells must have a MAC chromosome counting mechanism by which cells can distinguish and separately count each MAC chromosome to maintain copy numbers at similar levels, while allowing a degree of plasticity in copy number between different chromosomes. We acknowledge that the starvation method may not be optimal to synchronize *Tetrahymena* cells at MAC G1 phase because upon starvation cells in G2 phase do not always undergo division. In addition, DNA degradation may occur in cells that are undergoing active replication when transferred to starvation conditions. These opposing effects can slightly increase copy number variation in G1 cells (Figure 4F). For future works, centrifugal elutriation may be a good alternative method to synchronize growing cells in MAC G1 phase (Liu et al., 2021). However, G1 MACs should be purified by flow cytometry when using this method because chromatin exclusion bodies often occur in growing cells, and these can confound the quantification of MAC chromosome copy number (Bodenbender et al., 1992). In addition to DNA replication, MAC chromosome copy number might also be regulated at the level of chromosome segregation, which also requires a MAC chromosome counting mechanism. We believe that the ddPCR-based method for copy number quantification established in this study will help in identifying the MAC chromosome counting mechanism.

In summary, we used ddPCR methodology to accurately quantify the copy number of MAC chromosomes, which is a longstanding issue in *Tetrahymena* biology. We expect that application of this method to other species could make ciliates a useful model to address fundamental questions on the genetics and evolution of amitosis.

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REFERENCES

- Allen, S.L. & Nanney, D.L. (1958) An analysis of nuclear differentiation in the selfers of *Tetrahymena*. *The American Naturalist*, 92, 139–160.
- Baird, S.E. & Klobutcher, L.A. (1991) Differential DNA amplification and copy number control in the Hypotrichous Ciliate *Euplotes Crassus*. *The Journal of Protozoology*, 38, 136–140.
- Bell, G. (1982) *The masterpiece of nature: the evolution of sex and the genetics of sexuality*. Berkeley, CA: University of California Press.
- Blackburn, E.H. & Karrer, K.M. (1986) Genomic reorganization in ciliated protozoans. *Annual Review of Genetics*, 20, 501–521.
- Bodenbender, J., Prohaska, A., Jauker, F., Hipke, H. & Cleffmann, G. (1992) DNA elimination and its relation to quantities in the macronucleus of *Tetrahymena*. *Developmental Genetics*, 13, 103–110.
- Cameron, I.L. & Jeter, J.R. (1970) Synchronization of the cell cycle of *Tetrahymena* by starvation and refeeding. *The Journal of Protozoology*, 17, 429–431.
- Chen, K., Wang, G.Y., Xiong, J., Jiang, C.Q. & Miao, W. (2019) Exploration of genetic variations through single-cell whole-genome sequencing in the model ciliate *Tetrahymena thermophila*. *Journal of Eukaryotic Microbiology*, 66, 954–965.
- Cheng, Y.H., Liu, C.F.J., Yu, Y.H., Jhou, Y.T., Fujishima, M., Tsai, I.J. et al. (2020) Genome plasticity in *Paramecium bursaria* revealed by population genomics. *BMC Biology*, 18. <https://doi.org/10.1186/s12915-020-00912-2>
- Doerder, F.P. (1979) Regulation of macronuclear DNA content in *Tetrahymena thermophila*. *The Journal of Protozoology*, 26, 28–35.
- Doerder, F.P. (2014) Abandoning sex: multiple origins of asexuality in the ciliate *Tetrahymena*. *BMC Evolutionary Biology*, 14. <https://doi.org/10.1186/1471-2148-14-112>
- Doerder, F.P., Deak, J.C. & Lief, J.H. (1992) Rate of phenotypic assortment in *Tetrahymena thermophila*. *Developmental Genetics*, 13, 126–132.
- Doerder, F.P. & Debault, L.E. (1975) Cytofluorimetric analysis of nuclear DNA during meiosis, fertilization and macronuclear development in the ciliate *Tetrahymena pyriformis*, syngen 1. *Journal of Cell Science*, 17, 471–493.
- Doerder, F.P. & Debault, L.E. (1978) Life-cycle variation and regulation of macronuclear DNA content in *Tetrahymena Thermophila*. *Chromosoma*, 69, 1–19.
- Doerder, F.P., Lief, J.H. & Debault, L.E. (1977) Macronuclear subunits of *Tetrahymena thermophila* are functionally haploid. *Science*, 198, 946–948.
- Eisen, J.A., Coyne, R.S., Wu, M., Wu, D.Y., Thiagarajan, M., Wortman, J.R. et al. (2006) Macronuclear genome sequence of the ciliate *Tetrahymena thermophila*, a model eukaryote. *PLoS Biology*, 4, 1620–1642.
- Flickinger, C.J. (1965) The fine structure of the nuclei of *Tetrahymena pyriformis* throughout the cell cycle. *Journal of Cell Biology*, 27, 519–529.
- Gicquaud, C. & Tremblay, N. (1991) Observations with Hoechst staining of amitosis in *Acanthamoeba Castellani*. *The Journal of Protozoology*, 38, 221–224.
- Gorovsky, M.A., Yao, M.C., Keevert, J.B. & Pleger, G.L. (1975) Isolation of micro- and macronuclei of *Tetrahymena pyriformis*. *Methods in Cell Biology*, 9, 311–327.

- Greider, C.W. & Blackburn, E.H. (1985) Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts. *Cell*, 43, 405–413.
- Hamilton, E.P., Kapusta, A., Huvos, P.E., Bidwell, S.L., Zafar, N., Tang, H.B. et al. (2016) Structure of the germline genome of *Tetrahymena thermophila* and relationship to the massively rearranged somatic genome. *Elife*, 5. <https://doi.org/10.7554/eLife.19090>
- Hindson, B.J., Ness, K.D., Masquelier, D.A., Belgrader, P., Heredia, N.J., Makarewicz, A.J. et al. (2011) High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. *Analytical Chemistry*, 83, 8604–8610.
- Larson, D.D., Umthun, A.R. & Shaiu, W.L. (1991) Copy number control in the *Tetrahymena* macronuclear genome. *The Journal of Protozoology*, 38, 258–263.
- Le Mouel, A., Butler, A., Caron, F. & Meyer, E. (2003) Developmentally regulated chromosome fragmentation linked to imprecise elimination of repeated sequences in *Paramecia*. *Eukaryotic Cell*, 2, 1076–1090.
- Liu, Y.Q., Nan, B., Niu, J.H., Kapler, G.M. & Gao, S. (2021) An optimized and versatile counter-flow centrifugal elutriation workflow to obtain synchronized eukaryotic cells. *Frontiers in Cell and Developmental Biology*, 9. <https://doi.org/10.3389/fcell.2021.664418>
- Lucchetta, E.M. & Ohlstein, B. (2017) Amitosis of polyploid cells regenerates functional stem cells in the *Drosophila* intestine. *Cell Stem Cell*, 20, 609–620.
- McDonald, B.B. (1962) Synthesis of deoxyribonucleic acid by micro- and macronuclei of *Tetrahymena pyriformis*. *Journal of Cell Biology*, 13, 193–203.
- Miller, R.H. (1980) Amitosis and endocytogenesis in the fruit of *Malus sylvestris*. *Annals of Botany*, 46, 567–575.
- Mochizuki, K., Fine, N.A., Fujisawa, T. & Gorovsky, M.A. (2002) Analysis of a piwi-related gene implicates small RNAs in genome rearrangement in *Tetrahymena*. *Cell*, 110, 689–699.
- Morrison, T.L., Yakisich, J.S., Cassidy-Hanley, D. & Kapler, G.M. (2005) TIF1 represses rDNA replication initiation, but promotes normal S phase progression and chromosome transmission in *Tetrahymena*. *Molecular Biology of the Cell*, 16, 2624–2635.
- Nagata, T. & Ma, H. (2004) Electron microscopic radioautographic study on protein synthesis in amitotic hepatocytes of the aging mouse. *Medical Electron Microscopy*, 37, 62–69.
- Nakahara, W. (1917) Preliminary note on the nuclear division in the adipose cells of insects. *Anatomical Record*, 13, 81–86.
- O'Hara, R., Tedone, E., Ludlow, A., Huang, E., Arosio, B., Mari, D. et al. (2019) Quantitative mitochondrial DNA copy number determination using droplet digital PCR with single-cell resolution. *Genome Research*, 29, 1878–1888.
- Orias, E., Cervantes, M.D. & Hamilton, E.P. (2011) *Tetrahymena thermophila*, a unicellular eukaryote with separate germline and somatic genomes. *Research in Microbiology*, 162, 578–586.
- Prescott, D.M. (1994) The DNA of ciliated protozoa. *Microbiological Reviews*, 58, 233–267.
- Quan, P.L., Sauzade, M. & Brouzes, E. (2018) dPCR: a technology review. *Sensors*, 18. <https://doi.org/10.3390/s18041271>
- Schensted, I.V. (1958) Model of subnuclear segregation in the macronucleus of ciliates. *American Naturalist*, 92, 161–170.
- Seyfert, H.M. (1977) Short G1 period is correlated with low macronuclear DNA contents in *Tetrahymena*. *Experimental Cell Research*, 108, 456–459.
- Sheng, Y.L., Duan, L.L., Cheng, T., Qiao, Y., Stover, N.A. & Gao, S. (2020) The completed macronuclear genome of a model ciliate *Tetrahymena thermophila* and its application in genome scrambling and copy number analyses. *Science China Life Sciences*, 63, 1534–1542.
- Spring, K.J., Pham, S. & Zufall, R.A. (2013) Chromosome copy number variation and control in the ciliate *Chilodonella uncinata*. *PLoS One*, 8, e56413.
- Thulesius, H.L., Cervin, A. & Jessen, M. (2011) Can we always trust rhinomanometry? *Rhinology*, 49, 46–52.
- Vitali, V., Rothering, R. & Catania, F. (2021) Fifty generations of amitosis: tracing asymmetric allele segregation in polyploid cells with single-cell DNA sequencing. *Microorganisms*, 9(9), 1979.
- Wang, B., Liu, Y., Chen, X. & Fan, Z. (2010) Amitosis-like nuclear division in erythrocytes of triploid rainbow trout *Oncorhynchus mykiss*. *Journal of Fish Biology*, 76, 1205–1211.
- Wang, G.Y., Chen, K., Zhang, J., Deng, S.J., Xiong, J., He, X.L. et al. (2020) Drivers of mating type composition in *Tetrahymena thermophila*. *Genome Biology and Evolution*, 12, 2328–2343.
- Wang, G.Y., Fu, L., Xiong, J., Mochizuki, K., Fu, Y.X. & Miao, W. (2021) Identification and characterization of base-substitution mutations in the macronuclear genome of the ciliate *Tetrahymena thermophila*. *Genome Biology and Evolution*, 13. <https://doi.org/10.1093/gbe/evaa232>
- Wang, G.Y., Wang, S., Chai, X.C., Zhang, J., Yang, W.T., Jiang, C.Q. et al. (2021) A strategy for complete telomere-to-telomere assembly of ciliate macronuclear genome using ultra-high coverage Nanopore data. *Computational and Structural Biotechnology Journal*, 19, 1928–1932.
- White-Gilbertson, S., Lu, P., Jones, C.M., Chiodini, S., Hurley, D., Das, A. et al. (2020) Tamoxifen is a candidate first-in-class inhibitor of acid ceramidase that reduces amitotic division in polyploid giant cancer cells—Unrecognized players in tumorigenesis. *Cancer Medicine*, 9, 3142–3152.
- Woodard, J., Kaneshiro, E. & Gorovsky, M.A. (1972) Cytochemical studies on the problem of macronuclear subnuclei in *Tetrahymena*. *Genetics*, 70, 251–260.
- Xiong, J., Yang, W.T., Chen, K., Jiang, C.Q., Ma, Y., Chai, X.C. et al. (2019) Hidden genomic evolution in a morphospecies—The landscape of rapidly evolving genes in *Tetrahymena*. *PLoS Biology*, 17, e3000294.
- Xu, K., Doak, T.G., Lipps, H.J., Wang, J.M., Swart, E.C. & Chang, W.J. (2012) Copy number variations of 11 macronuclear chromosomes and their gene expression in *Oxytricha trifallax*. *Gene*, 505, 75–80.
- Yan, G.X., Yang, W.T., Han, X.J., Chen, K., Xiong, J., Hamilton, E.P. et al. (2021) Evolution of the mating type gene pair and multiple sexes in *Tetrahymena*. *Iscience*, 24. <https://doi.org/10.1016/j.isci.2020.101950>
- Yao, M.C., Chao, J.L. & Cheng, C.Y. (2014) Programmed genome rearrangements in *Tetrahymena*. *Microbiology Spectrum*, 2. <https://doi.org/10.1128/microbiolspec.MDNA3-0012-2014>
- Zhang, H., West, J.A., Zufall, R.A. & Azevedo, R.B.J.b. (2021) Amitosis confers benefits of sex in the absence of sex to *Tetrahymena*. *bioRxiv*, 794735.
- Zufall, R.A., Dimond, K.L. & Doerder, F.P. (2013) Restricted distribution and limited gene flow in the model ciliate *Tetrahymena thermophila*. *Molecular Ecology*, 22, 1081–1091.

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