Genome Size of the Ultrasmall Unicellular Freshwater Green Alga, *Medakamo hakoo* 311, as Determined by Staining with 4′,6-Diamidino-2-phenylindole after Microwave Oven Treatments: II. Comparison with *Cyanidioschyzon merolae*, *Saccharomyces cerevisiae* (*n, 2n*), and *Chlorella variabilis*

Tsuneyoshi Kuroiwa¹,²*, Mio Ohnuma²,³, Yuuta Imoto²,⁴, Osami Misumi²,⁵, Noriko Nagata¹, Isamu Miyakawa⁶, Masahiro Fujishima⁶, Fumi Yagisawa⁷ and Haruko Kuroiwa¹,²

¹Department of Chemical and Biological Science, Faculty of Science, Japan Women’s University, 2–8–1 Mejirodai, Bunkyo-ku, Tokyo 112–8681, Japan
²Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Gobancho, Chiyoda-ku, Tokyo 102–0076, Japan
³Institute of Technology, Hiroshima College, 4272–1 Higashino, Osakikamijima-cho, Toyota-gun, Hiroshima 725–0231, Japan
⁴Medical Institute of Bioregulation, Kyushu University, 3–1–1 Maidashi, Higashi-ku, Fukuoka 812–8582, Japan
⁵Department of Applied Molecular Bioscience, Graduate School of Medicine, Yamaguchi University, 1677–1 Yoshiida, Yamaguchi 753–8512, Japan
⁶Department of Environmental Science and Engineering, Graduate School of Science and Engineering, Yamaguchi University, 1677–1 Yoshiida, Yamaguchi 753–8512, Japan
⁷Instrumental Research Center, University of the Ryukyus, 1 Senbaru, Nishihara-cho, Nakagami-gun, Okinawa 903–0213, Japan

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Summary  Eukaryotic cells contain three types of nuclei: the cell nucleus, the mitochondrial nuclei (nucleoids), and plastid nuclei (nucleoids). Here, we report that the chlorophyte strain *Medakamo hakoo* 311 has the smallest cell nucleus genome among the free-living eukaryotes analyzed to date. The size of the nuclear genome was analyzed by staining with 4′,6-diamidino-2-phenylindole (DAPI), followed by fluorimetry using a videointensified microscope photon-counting system (VIMPCS) and fluorescence microscopy. The exact genome size of *M. hakoo* remains elusive because of the low permeability of the thick algal cell wall to DAPI. To improve the DAPI staining method for *M. hakoo*, we added a microwave oven heating step. We examined the genome sizes of *M. hakoo*, *Cyanidioschyzon merolae*, *Saccharomyces cerevisiae* (*n, 2n*), and *Chlorella variabilis* ¹N by DAPI staining, which included a microwave oven heating step, followed by fluorimetry with the VIMPCS. The new method of DAPI staining that included a microwave oven heating step improved the staining efficiency of *C. variabilis* and *M. hakoo* nuclei, and slightly improved the staining efficiency of *S. cerevisiae* and *C. merolae* nuclei. Using well-stained samples and the cell nucleus genome size of *C. merolae* (16.5 Mb) as the standard, the cell nucleus genome sizes of *M. hakoo*, *S. cerevisiae* (*n*), and *C. variabilis* were determined as 9.2, 20.2, and 35.9 Mb, respectively. The results are consistent with previous reports that *M. hakoo* cell has the smallest genome among the free-living eukaryotes evaluated to date.

Key words  Ultrasmall genome size, *Medakamo hakoo*, *Saccharomyces cerevisiae*, *Chlorella variabilis*, DAPI staining, Microwave oven.

The primitive red alga *Cyanidioschyzon merolae* has many advantages for studying organelle biology because of its very small nuclear genome (16.5 Mb). Its genome includes 4775 protein encoding genes on 20 chromosomes, the fewest genes among eukaryotes analyzed so far. Also, *C. merolae* has a minimum set of organelles: three types of double-membrane-bound organelles (the
cell nucleus, mitochondrion, and plastid), and four types of single-membrane-bound organelles (endoplasmic reticulum (ER), Golgi apparatus, peroxisome (microbody), and lysosome). These organelles are essential for fulfilling the functions of eukaryotic cells and all of them proliferate by division (Kuroiwa et al. 1994, Matsuzaki et al. 2004, Yagisawa et al. 2009, Fujiwara et al. 2013, Imoto et al. 2013). In addition, the cellular and organelle divisions of *C. merolae* can be completely synchronized by light and dark cycles (Terui et al. 1995, Fujiwara et al. 2013, Imoto et al. 2013).

To understand the origin and the mechanisms of the proliferation of the plant cell and the seven types of membrane-bound organelles contained within, we searched for an ultrasmall, unicellular, fresh-water green alga with a small nuclear genome and the minimum set of seven organelles whose divisions could be synchronized, like those of *C. merolae*. We identified the novel green alga, the chlorophyte *Medakamo hakoo*, as one of the candidates. The cells of this fresh-water alga are approximately 0.8 µm in diameter. Thin-layer chromatography profiles clarified that *M. hakoo* is a Chlorophyte (Kuroiwa et al. 2015). Its genome size was estimated as 9.2 Mb (Kuroiwa et al. 2015) by traditional 4',6-diamidino-2-phenylindole (DAPI) staining, using the 100%-complete sequence of *C. merolae* (16,546,747 b) (Matsuzaki et al. 2004, Nozaki et al. 2007) as the standard.

Analyses of the ultrastructure of *M. hakoo* by electron microscopy showed that each cell contains one nucleus, one mitochondrion, and one chloroplast containing many starch grains, with all the organelles enclosed by a multi-layered cell wall (Fig. 1). Because its thick cell wall interferes with the photon count obtained using the video-intensified microscope photon-counting system (VIMPCS) after DAPI staining, its estimated genome size is likely to be smaller than its actual genome size. To improve the effectiveness of DAPI staining, we used a microwave oven heating method that was originally developed for the retrieval of antigens from paraffin-em-

Fig. 1. Electron micrographs of *M. hakoo* cells. At low magnification, many cells appear to be 1 µm or smaller in diameter. (a). Cells contain one nucleus, one mitochondrion, and one chloroplast with many starch grains (st in b), all enclosed by a multi-layered cell wall (1–5 in c) and a cell membrane (6 in c). N, cell nucleus; M, mitochondrion; C, chloroplast. Scale bars=500 nm.
bedded tissue in immunohistochemical staining (Shi et al. 1991). The addition of this microwave oven treatment to the DAPI staining method effectively eliminated the effect of the thick cell walls, and allowed DAPI to stain the cell nucleus.

**Materials and methods**

**Materials**

*M. hakoo* 311 was obtained from the outer moats of the Imperial Palace in Tokyo (Kuroiwa et al. 2015). Samples of *C. merolae* 10D were isolated by researchers in our laboratory (Toda et al. 1995) from the hot spring algae collection provided by Prof. Pinto (Naples University). *Saccharomyces cerevisiae* laboratory strains BY4741 and BY4743 were used as haploid and diploid strains, respectively, and they were provided by Miyakawa’s laboratory (Yamaguchi University). *Chlorella variabilis* strain 1N was provided by Fujishima’s laboratory (Yamaguchi University, Kodama and Fujishima 2007).

**Fluorescence microscopy and DAPI staining**

**Traditional DAPI staining**

A 3-µL aliquot of culture medium of *M. hakoo* 311, *C. merolae*, or *C. variabilis*, or of solution of a pellet resuspended in medium, was placed on a slide glass, and then 3-µL 1% (v/v) glutaraldehyde was added to the drop before adding 3-µL DAPI (1 µg mL⁻¹ in TAN buffer; 17% sucrose, 20mM Tris–HCl, 0.5 mM EDTA, 1.2 mM spermidine, 7mM 2-mercaptopethanol and 0.4 mM PMSF) (Toda et al. 1995, Momoyama et al. 2003). The mixture was covered with a cover slip and squashed slightly against filter paper. Finally, 3-µL 1 µg mL⁻¹ DAPI was added to the edge of the cover slip. After 30 min, the samples were observed.

**Microwave oven heating for DAPI staining**

We used a MITSUBISHI microwave oven (model ROMD2, Tokyo, Japan). A 1.5 mL solution of *M. hakoo*, *C. merolae*, *S. cerevisiae*, or *C. variabilis* cells was added to a 10 mL tube, which was placed in a 100 mL cup and then positioned in the center of the microwave oven plate. The samples were heated for 3, 10, 20, or 30 s at 200 W. All samples were stained for 5 min according to a standard method (Toda et al. 1995).

**Electron microscopy**

A suspension of *M. hakoo* was fixed for 4h in 1% (v/v) glutaraldehyde buffered in sodium cacodylate, pH 7.2, rinsed in the same buffer, post-fixed overnight in 1% osmium tetroxide, dehydrated in a graded ethanol series followed by propylene oxide, and then embedded in Spurr’s resin. Ultrathin sections were cut and stained with 5% (w/v) uranyl acetate and lead citrate, and observed under a JEM 1200 EXS electron microscope (JEOL, Tokyo, Japan).

**Results and discussion**

Previously, traditional DAPI staining and photon counting by VIMPCS estimated the nuclear genomes of *M. hakoo* and *Ostreococcus tauri* to be 9.2 and 19.0 Mb, respectively. To obtain more information about *M. hakoo*, we compared the size of its cell nucleus genome with that of *S. cerevisiae*. Figure 2 shows fluorescence images of both *S. cerevisiae* (n) and *S. cerevisiae* (2n) after traditional staining with DAPI. When observed
under a fluorescence microscope, the typical spherical cell nuclei of diploid \(S. \text{cerevisiae} \ (2n)\) were larger and brighter than those of haploid \(S. \text{cerevisiae} \ (n)\). The ratio of the intensity of the cell nucleus between diploid and haploid was 2.0 (Table 1). This result confirmed that the assay detected the actual genome size. Therefore, we used this assay to compare the cell nucleus genome size of \(S. \text{cerevisiae}\) to that of \(M. \text{hakoo}\).

We found that the cell nucleus, mitochondrial nuclei (nucleoids), and chloroplast nuclei (nucleoids) of \(M. \text{hakoo}\) cells were not stained by applying weak squashing pressure during the staining procedure. Instead, firmer pressure and longer staining (\(\geq\)30 min) were essential to clearly stain these organelles. Figure 3 shows fluorescence images of both \(M. \text{hakoo}\) and \(S. \text{cerevisiae}\) in the same field after traditional DAPI staining. In some cells of \(M. \text{hakoo}\), multiple nuclei or nucleoids of organelles were observed. Mitochondrial nuclei were often observed in \(S. \text{cerevisiae}\) cells. The fluorescence intensity of the cell nucleus of \(S. \text{cerevisiae}\) was stronger than that of \(M. \text{hakoo}\). The data supported these observations, because the fluorescence intensity of \(S. \text{cerevisiae}\) haploid nuclei was 2.14 times that of \(M. \text{hakoo}\) cell nuclei and its genome size was estimated as 19.7 Mbp.

### Table 1. Amount of DNA in cell nucleus genome of haploid (\(n\)) and diploid (\(2n\)) \(S. \text{cerevisiae}\).

<table>
<thead>
<tr>
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<th>(S. \text{cerevisiae} \ (n))</th>
<th>(S. \text{cerevisiae} \ (2n))</th>
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<tr>
<td>Photon count</td>
<td>(58.1 \times 10^3 \pm 10.5 \times 10^3)</td>
<td>(117.6 \times 10^3 \pm 28.6 \times 10^3)</td>
</tr>
<tr>
<td>Genome size ratio</td>
<td>1</td>
<td>2.0</td>
</tr>
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</table>

Mean\(\pm SD\), \(n=27\) (\(S. \text{cerevisiae}\) haploid), 22 (\(S. \text{cerevisiae}\) diploid). Typical spherical cell nuclei of haploid and diploid were examined. Data are mean photon counts from whole area per 0.5 s after DAPI staining.

### Table 2. Amount of DNA in cell nucleus genome of \(S. \text{cerevisiae}\) haploid (\(n\)) and \(M. \text{hakoo}\) estimated after traditional DAPI staining.

<table>
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<th></th>
<th>(S. \text{cerevisiae} \ (n))</th>
<th>(M. \text{hakoo})</th>
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<tr>
<td>Photon count</td>
<td>(128.0 \times 10^3 \pm 24.9 \times 10^3)</td>
<td>(59.9 \times 10^3 \pm 9.3 \times 10^3)</td>
</tr>
<tr>
<td>Genome size ratio</td>
<td>2.1</td>
<td>1</td>
</tr>
</tbody>
</table>

Mean\(\pm SD\), \(n=39\) (\(M. \text{hakoo}\)), 20 (\(S. \text{cerevisiae}\)). Cells of \(S. \text{cerevisiae}\) and \(M. \text{hakoo}\) in the same field were examined. All data are mean photon counts from whole area per 0.5 s after DAPI staining.

Fig. 3. Fluorescence micrographs of haploid (arrows in a, b) and diploid cells (arrows in c–f) of \(S. \text{cerevisiae}\) and \(M. \text{hakoo}\) (arrowsheads in a–f). Spherical cell nuclei are visible in \(S. \text{cerevisiae}\) and mitochondrial nuclei are visible as background around cell nuclei. Small cell nuclei are present in \(M. \text{hakoo}\). Chloroplast nuclei and mitochondrial nuclei are located near cell nuclei (arrowheads, a–f). Some cells are daughter cells after division (a, b, c). Nuclei of \(S. \text{cerevisiae}\) are brighter than those of \(M. \text{hakoo}\). a–f show the same magnification. Scale bar=1 \(\mu m\).
Fig. 4. Percentage of cell nuclei of *S. cerevisiae*, *C. merolae*, *C. variabilis*, and *M. hakoo* stained with DAPI after microwave oven treatments. All (100%) cell nuclei of *C. merolae*, *S. cerevisiae*, *C. variabilis*, and *M. hakoo* were stained after 3-, 3-, 10-, and 30-s treatments, respectively. –○–, *S. cerevisiae*; –▲–, *C. merolae*; –□–, *C. variabilis*; –■–, *M. hakoo*.

Table 3. Amount of DNA in cell nucleus genome of *M. hakoo*, *C. merolae*, *S. cerevisiae* (n), and *C. variabilis* estimated after DAPI staining including a microwave heating step.

<table>
<thead>
<tr>
<th></th>
<th><em>C. merolae</em></th>
<th><em>M. hakoo</em></th>
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<tbody>
<tr>
<td>Photon counts</td>
<td>16.5 × 10^3 ± 1.4 × 10^3</td>
<td>64.6 × 10^3 ± 1.1 × 10^3</td>
</tr>
<tr>
<td>Genome size (Mb)</td>
<td>16.5</td>
<td>9.2</td>
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<tr>
<th></th>
<th><em>S. cerevisiae</em> (n)</th>
<th><em>M. hakoo</em></th>
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<tr>
<td>Photon counts</td>
<td>20.2 × 10^3 ± 3.6 × 10^3</td>
<td>6.7 × 10^3 ± 6.7 × 10^3</td>
</tr>
<tr>
<td>Genome size (Mb)</td>
<td>20.2</td>
<td>9.2</td>
</tr>
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</table>

Mean±SD, a. n=40 (*C. merolae*), 30 (*M. hakoo*), b. n=22 (*S. cerevisiae* haploid), 21 (*M. hakoo*), c. 23 (*C. variabilis*), 20 (*M. hakoo*). Cells of *M. hakoo* and *S. cerevisiae*, *M. hakoo* and *C. merolae*, *M. hakoo* and *C. variabilis*, in the same field were examined. Data are mean photon counts from whole area per 0.2 s after DAPI staining.

Fig. 5. Fluorescence (a, c, e) and phase contrast/fluorescence images (b, d, f) of mixed cells of *C. merolae* and *M. hakoo*. a–b, c–d, and e–f show the same fields. Several small cells of *M. hakoo* (arrowheads in a–f) located around cell nucleus bound to chloroplast of *C. merolae* (arrows in a–f). Cell nuclei of *C. merolae* were larger and brighter than those of *M. hakoo*. By microwave oven treatment, cell nuclei became clear, whereas mitochondria and chloroplast nuclei became ambiguous. Scale bar=1 μm. a–f are the same magnification.
We determined the effects of adding a microwave oven heating step to the DAPI staining procedure on the cell nuclei of *M. hakoo*, *C. merolae*, *S. cerevisiae*, and *C. variabilis* (Fig. 4). The cell nuclei and mitochondrial nuclei of *C. merolae* and *S. cerevisiae* and the chloroplast nuclei of *C. merolae* were stained well with DAPI after a 3-s microwave oven treatment. Although the cell nuclei of *C. merolae* and *S. cerevisiae* were not affected by the 20–30 s microwave oven treatments, the DAPI-stained mitochondrial and chloroplast nuclei were deformed and faded after these longer periods of microwave oven heating. For cells of *M. hakoo* and *C. variabilis*, the microwave heating step significantly increased the effectiveness of DAPI staining (Fig. 4). Almost all cell nuclei were stained after 10-s microwave treatments for *C. variabilis*, and after 30-s microwave treatments for *M. hakoo* (Fig. 4). In general, the stainability of the cell nuclei of these organisms was unchanged by increasing the microwave treatment to 30 s, but the fluorescence signals from mitochondrial and chloroplast nuclei and chlorophyll became weaker when the microwave treatment exceeded 10 s. Therefore, *C. merolae*, *S. cerevisiae*, *C. variabilis*, and *M. hakoo* cells were subjected to 3-, 3-, 10-, and 30-s microwave treatments, respectively, before mixing and DAPI staining. The fluorescence intensities of cell nuclei were stronger in *C. merolae* (Fig. 5), *S. cerevisiae* (Fig. 6), and *C. variabilis* (Fig. 7) than in *M. hakoo*. The statistical data were consistent with these observations (Table 3) and were similar to the data obtained by traditional DAPI staining (Fig. 3, Table 2).

![Image](image_url)

**Fig. 6.** Fluorescence (a, c, e) and phase contrast/fluorescence images (b, d, f) of mixed cells of *C. merolae* and *S. cerevisiae* (a, b), and *S. cerevisiae* and *M. hakoo* (c–f). a–b, c–d, and e–f show the same fields. Cell nuclei of *S. cerevisiae* (long arrows in a–f) were larger and brighter than those of *C. merolae* (short arrows in a, b) and *M. hakoo* (arrowheads in c–f). By microwave oven treatment, cell nuclei became clear, whereas mitochondria and chloroplast nuclei became ambiguous. Scale bar=1 µm. a–f are the same magnification.

Using the cell nucleus genome size of *C. merolae* as the standard, the size of the cell genome of *M. hakoo*, *S. cerevisiae* (*n*), and *C. variabilis* was estimated as 9.2, 20.2, and 35.9 Mb, respectively. The value obtained for the genome size of *M. hakoo* did not change when the microwave oven treatment was included in the DAPI staining method (9.2 Mb, compared with 9.2 Mb estimated using the traditional DAPI staining method). The microwave oven treatment seemed to be useful for the rapid observation of cell nuclei in various organisms.

Next, we compared the cell nucleus genome sizes of microorganisms estimated by sequencing and DAPI staining. Sequencing analyses have estimated the cell
nucleus genome sizes of *C. merolae*, *S. cerevisiae* (*n*), *C. variabilis*, and *Ostreococcus lucimarinus* as 16.5 Mb (Matsuzaki et al. 2004, Nozaki et al. 2007), 12 Mb (Goffeau et al. 1996), 43 Mb (Blanc et al. 2010), and 13 Mb (Derelle et al. 2006), respectively. On the other hand, when the photon counting data of *C. merolae* was used as the standard, the genome of *S. cerevisiae* (*n*) and *O. tauri* was estimated to be 20.2 Mb (Table 3) and 19.0–30 Mb (Kuroiwa et al. 2004, 2015), respectively. In addition, the stained nuclei of *S. cerevisiae* (*n*) and *O. tauri* were larger than those of *C. merolae*. *C. merolae* is the only eukaryotic organism with a fully sequenced genome, as it contains extremely few repeats. There are clear gaps between the sequence data and cytogenetic data. As pointed out by Nozaki et al. (2007), all previously reported eukaryotic cell nucleus genome sequences except that of *C. merolae* are incomplete, especially those with highly repeated units at the ends of chromosomes. Repetitive DNA is essential for genome function, and may contribute to the diversity of isoforms and to evolution. Consequently, complete chromosomal structures are fundamental for understanding eukaryotic cells. Therefore, comparing the amount of repetitive DNA between *C. merolae* and *M. hakoo* by genome sequencing will be useful for future research aimed at understanding the origins of algae.

The red alga *C. merolae* and the green alga *M. hakoo* have ultrasmall cell nucleus genomes, despite their different growth environments and phylogenetic classifications. Like *C. merolae* cells (Kobayashi et al. 2002), *M. hakoo* cells have one chloroplast with one centrally located chloroplast nucleus, similar to a cyanobacterium. A centrally located chloroplast nucleus is thought to be a characteristic of primitive algae (Kuroiwa et al. 1994). Interestingly, the mitochondrial and chloroplast nuclei (nucleoids) in *M. hakoo* cells were much smaller than those in *C. merolae* cells, suggesting that the chloroplast and mitochondrial genomes in *M. hakoo* have a minimum number of gene copy.

Because the 100%-complete sequences of the cell nucleus genome and the mitochondrial and chloroplast genomes of *C. merolae* are now freely available, com-
paring the complete cell nucleus genomes of *C. merolae* and *M. hakoo* will offer valuable insights into the fundamental mechanisms of cell proliferation in free-living eukaryotes.

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