Cytological Evidence of Cell-Nuclear Genome Size of a New Ultra-Small Unicellular Freshwater Green Alga, “Medakamo hakoo” strain M-hakoo 311

I. Comparison with Cyanidioschyzon merolae and Ostreococcus tauri

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Summary Ultra-small unicellular algae provide information on the basic cellular mechanisms and essential genes that support the lives of photosynthetic eukaryotes, including higher plants. We have discovered the smallest free-living photosynthetic picoeukaryote to date, a green alga, “Medakamo hakoo” (provisional name) strain M-hakoo 311, which was isolated from freshwater. Based on its pigment composition, M. hakoo belongs to the Chlorophyta. Its cell size and nuclear genome size were compared with those of the primitive red alga Cyanidioschyzon merolae, which lives in freshwater, and with the green alga Ostreococcus tauri, which lives in seawater. The minor and major axes of M. hakoo, C. merolae and O. tauri in the G1 phase were 0.73 and 0.98 μm, 1.2 and 2.37 μm, and 0.76 and 0.96 μm, respectively. The cell size of M. hakoo was thus very similar to that of O. tauri. The nuclear genome sizes of the three algae in the G1 phase were examined using the video-intensified photon-counting system (VIMPCS). The nuclear genome size of C. merolae has previously been determined as 16.5 Mbp by sequencing. Using that value as a standard, the genome sizes of M. hakoo and O. tauri were determined as 9.2 Mbp and 20 Mbp, respectively.

Key words Ultra-small alga, Chlorophyte M. hakoo, Cyanidioschyzon merolae, Ostreococcus tauri, Genome size, Cytochemistry.

Small algae are advantageous in the study of organelle biology because they frequently contain the minimum set of essential organelles. These include the three double-membrane-bound organelles (cell nucleus, mitochondrion, and plastid), and the four single-membrane-bound organelles [endoplasmic reticulum (ER), Golgi apparatus, peroxisome (microbody), and lysosome] that are required to fulfill the functions of eukaryotic cells and that proliferate by division (Matsuzaki et al. 2004, Kuroiwa et al. 1994, Yagisawa et al. 2009, Fujiwara et al. 2010, Imoto et
In addition, the cell division of algae can be synchronized to light and dark cycles as was first shown for *Chlorella* by Tamiya *et al.* (1961).

To understand the basic mechanisms of organelle division, we previously sought an ultra-small unicellular red alga, whose division of organelles could be synchronized. Cells of the red alga *Cyanidioschyzon merolae* are smaller than those of *Chlorella*, and the cell division is simpler. The double- and single-membrane-bound organelles in *C. merolae* divide synchronously with cell division in light and dark cycles (Suzuki *et al.* 1994, Terui *et al.* 1995, Imoto *et al.* 2011, 2013). We determined that the mitochondrion, plastid and peroxisome in *C. merolae* divide using the plastid-dividing apparatus (PD ring, PD machinery), mitochondrion-dividing apparatus (MD ring, MD machinery) and peroxisome-dividing apparatus (POD ring, POD machinery), respectively (Kuroiwa 1982, Kuroiwa *et al.* 1998, Imoto *et al.* 2013). Although *C. merolae* contains the smallest genome of the known algae as shown using VIMPCS, its cell sizes are more than twice as large as those of the green alga *Ostreococcus tauri* (Kuroiwa *et al.* 2004). Thus, to further study the fundamental mechanisms of eukaryotic origin, multiplication and growth of organelles, it would be desirable to identify an ultra-small alga in which the nucleus has a smaller genome and from which organelles can be isolated easily.

In this study, we have isolated a novel green alga, “Medakamo hakoo” (provisional name) strain M-hakoo 311, from freshwater. To characterize *M. hakoo* we analyzed its nuclear genome by using cytological DNA analysis [DAPI-staining and a video-intensified microscope photon-counting system (VIMPCS)]. VIMPCS is a traditional method to examine quantitatively and qualitatively the DNA of cell nuclei (Kuroiwa *et al.* 1984). Using this method, we determined the number of chromosomes in *Saccharomyces cerevisiae* (16) (Kuroiwa *et al.* 1984) and in *Chlamydomonas reinhardtii* (18) (Aoyama *et al.* 2008). VIMPCS (Suzuki *et al.*1992, Takahashi *et al.* 1993, Toda *et al.*1995), pulse-field gel electrophoresis (PFGE) (Takahashi *et al.* 1993, Maleszka 1993) and sequencing methods (Matsuzaki *et al.* 2004, Barbier *et al.* 2005, Nozaki *et al.* 2007) were used to determine the genome sizes of *C. merolae*, *Cyanidium caldarium* and *Galdieria sulphuraria*, and reveal the differences in genome size among algae. For example, the DNA content of *C. caldarium* is about twice that of *C. merolae*. As some bands in PFGE overlap, the apparent genome size determined by that method is smaller than the actual genome size (Matsuzaki *et al.* 2004). The same is often true for sequenced algal genomes because not all copies of the genes, or the non-coding regions, are sequenced. Only the *C. merolae* genome has been 100% sequenced including all copies of all the genes (Matsuzaki *et al.* 2004, Nozaki *et al.* 2007). The genome size of *C. merolae* determined by sequencing was similar to that found by VIMPCS and PFGE. Therefore, in this work, we examine the amount of genomic DNA in nuclei of *M. hakoo* using VIMPCS and compare the data with those for *C. merolae* (as a standard) and *O. tauri*.

**Materials and methods**

**Materials**

*M. hakoo* strain M-hakoo 311 was isolated from freshwater in the outer moats of the Imperial Palace in Tokyo. Samples of *C. merolae* 10D were from the hot spring algae collection provided by Dr. Pint, Naples University (Toda *et al.* 1995). We obtained *O. tauri* NIES-2673 cells from the Microbial Culture Collection at the National Institute for Environmental Studies, Tsukuba, Japan, and confirmed its identity from the base sequence of the 18S rRNA gene. The alga was identical to *O. tauri* strain RCC 116, as reported previously (Kuroiwa *et al.* 2004)

**Pigment extraction and TLC-profiles**

For preparative TLC, *C. merolae*, *C. reinhardtii* and *M. hakoo* cell pellets (500 mg dried weight) frozen in liquid nitrogen were pulverized with silica gel and extracted with 1 mL of diethyl
ether. The extracts were developed on a TLC silica gel 60 aluminum plate (Merck, Darmstadt, Germany) with a solvent system of hexane/acetone/water (7:3:1, v/v) for 20 min.

**VIMPCS after DAPI staining**

The minor and major axes of cells were measured by phase contrast microscopy and light microscopy. *M. hakoo* and *C. merolae* cells in stationary phase were mixed, put on a slide glass, and squashed slightly after staining with DAPI. In the same way, *M. hakoo* and *O. tauri* cells in stationary phase were mixed and put on a slide glass after staining with DAPI. The samples were observed using an Olympus BHF epifluorescence microscope (Olympus, Tokyo) and the DNA contents of the nuclei in 10–30 cells were measured by a VIMPCS system according to methods described previously (Kuroiwa 1982). The mean values are reported.

**Results and discussion**

To classify *M. hakoo*, pigments were extracted and characterized using thin-layer chromatography (TLC), and the profiles were compared with those of extracts from *C. merolae* (red alga) and *C. reinhardtii* (green alga) (Fig. 1). *C. merolae* contained chlorophylls *a* and *β*-carotene, but lacked chlorophyll *b*, neoxanthin and violaxanthin. This result was in agreement with a previous report that used high-pressure liquid chromatography (HPLC) (Cunningham et al. 2007) and suggests that *C. merolae* may contain the simplest assortment of chlorophylls and carotenoids in any eukaryotic photosynthetic organism. Pigments identified in *C. reinhardtii* and *M. hakoo* included chlorophylls *a* and *b*, *β*-carotene, neoxanthin and violaxanthin. These results suggest that *M. hakoo* belongs to the green algal phylum Chlorophyta.

Figure 2 shows phase contrast/fluorescence and fluorescence images of both *M. hakoo* and *C.
merolae in the same field after staining with DAPI. The remarkable difference in cell size is due to the volumes of the chloroplasts (Fig. 2a,b). In C. merolae, the long chloroplast, which has a characteristic image during G₁ phase, occupied the whole cell (Kuroiwa et al. 1998). The spherical M. hakoo cells were smaller than C. merolae cells. Previously we reported that the cell sizes of C. merolae and O. tauri are 1.5μm and 0.76μm, respectively, while the genome sizes of C. merolae and O. tauri are 16.5 Mbp and 20 Mbp, respectively (Kuroiwa et al. 2004). In measurements made in this work (Table 1), the minor and major axes of M. hakoo cells, C. merolae and O. tauri were 0.73 and 0.98μm, 1.2 and 2.37μm, 0.76 and 0.96μm, respectively. Despite the difference in their environments, fresh water and sea water, and their phylogenetic classification, green alga and prasinophyceae alga, the cell size of M. hakoo and O. tauri was very similar and thus it was difficult to distinguish between these two algae on that basis. However, the M. hakoo cells were

Fig. 2. Phase contrast/fluorescence (a) and fluorescence images (b–f) of chlorophyte M. hakoo (short arrows in a, e–f) and C. merolae cells (long arrows in a, c–f) after staining with DAPI. a–e and f were excited by UV and green light, respectively. a, b and e, f were the same fields. The cell-nuclei, mitochondrial nuclei (nucleoids) and chloroplast nuclei (nucleoids) are visualized in C. merolae (b) and M. hakoo (enlarged figure in b). M. hakoo cells are smaller than those of C. merolae, and the fluorescence intensities of M. hakoo cell nuclei (short arrows in a, c–f) are lower than those of C. merolae nuclei (long arrows in a, c–f). One chloroplast nucleus can be seen in each daughter chloroplast in dividing cells (c, f). Cell nuclei, mitochondrial nuclei and chloroplast nuclei emit blue-white fluorescence after excitation by UV light. Chloroplasts emit red autofluorescence (f). n, cell nuclei; m, mitochondrial nuclei (mt-nucleoids); c, chloroplast nuclei (ct-nucleoids). Scale bar in (a) indicates 1μm.
Table 1. Cellular size (long and short diameter) of *M. hakoo*, *C. merolae* and *O. tauri*.

<table>
<thead>
<tr>
<th></th>
<th><em>M. hakoo</em></th>
<th><em>C. merolae</em></th>
<th><em>O. tauri</em></th>
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<tbody>
<tr>
<td>Long diameter (μm)</td>
<td>0.98±0.14</td>
<td>2.37±0.47</td>
<td>0.96±0.18</td>
</tr>
<tr>
<td>Short diameter (μm)</td>
<td>0.73±0.08</td>
<td>1.2±0.18</td>
<td>0.76±0.10</td>
</tr>
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Mean±SD, n>30.

Fig. 3. Phase contrast (a, d, g) and fluorescence images (b, c, e, f, h, i) of *O. tauri* cells (short arrows in b, e, h) and *C. merolae* cells (long arrow in b) during G₁ (small cells in b, e) and M phases (delta-shaped cells in e and h), after staining with DAPI. Chloroplasts emit red autofluorescence after excitation with green light (c, f, i). a–c, d–f, and g–i are the same fields. The fluorescence intensities of *O. tauri* cell nuclei (short arrow-G₁ in b) are higher than those of *C. merolae* nuclei (long arrow in b). In *O. tauri*, the fluorescence intensities of cell nuclei during M phase are higher than those of cell nuclei during G₁. The intensities of DAPI-stained cell nuclei of *M. hakoo* (arrow head in j) are darker than those of *O. tauri* cells (short arrow in j) in the same field. Scale bar in (a) indicates 1 μm.
characterized by the image of three double-membrane-bound and DNA containing organelles: a spherical cell nucleus, a small spherical chloroplast with one small chloroplast nucleus (ct-nucleoid) and one mitochondrion with small mitochondrial nucleus (mt-nucleoid) (Fig. 2b). In the dividing cell, one chloroplast nucleoid was observed in each daughter chloroplast (Fig. 2c–f). In general, chloroplasts and mitochondria in algae and higher plants contain more than 20 copies of the organelle genome (Kuroiwa 1982). One chloroplast nucleoid and one mitochondrial nucleoid, as we observed here in *M. hakoo*, is unusual. Because the dividing chloroplast was U-shaped, division seemed to progress and finish in the spherical cells with hard cell walls (Fig. 2e, f).

In *O. tauri* cells, chloroplast and mitochondrial nucleoids were obvious (Fig. 3a–i). The phase contrast and fluorescence images of *O. tauri* cells after DAPI staining were compared with those of *C. merolae* previously (Kuroiwa et al. 2004). We demonstrated that the cell nuclei of *O. tauri* and *C. merolae* contained 19 Mbp and 16.5 Mbp, respectively, although total sequencing showed the genome size of *O. tauri* was 12.56 Mbp (Derelle et al. 2006). Therefore, although the *C. merolae* cell is larger than the *O. tauri* cell, it contains less nuclear DNA. The dividing-cell nucleus of *O. tauri* contained about two-fold more DNA than the cell nuclei in the spherical or football-like cells during G1 phase (Fig. 3a–i). As the dividing *O. tauri* cells appear to be delta-shaped (Fig. 3g–i), the system of division is different from *M. hakoo* (Fig. 2e,f). We are currently examining the mode and process of division in both organisms.

The intensities of DAPI-stained cell nuclei of *M. hakoo* are darker than those of *O. tauri* cells (Fig. 3j), suggesting that the cell nuclei of *M. hakoo* contain less DNA than *O. tauri*, which has heretofore been regarded as the world’s smallest free-living eukaryote. We therefore measured the fluorescence intensity of *M. hakoo*, *C. merolae* and *O. tauri* cell-nuclei using VIMPCS after staining with DAPI. The genome size of *C. merolae*, 16.5 Mbp (Matsuzaki et al. 2004, Nozaki et al. 2007), was used as a standard. As a result, the genome sizes of *M. hakoo* and *O. tauri* were estimated as 9 Mbp and 20 Mbp, respectively (Table 2). Further work is required to determine the precise size of *M. hakoo* genome.

To date, identified green algae with ancestral features [small genome size, tiny cell size (ca. 1 μm), single mitochondrion and chloroplast] have been marine picoeukaryotes such as *Ostreococcus* sp. (Derelle et al. 2002) and *Micromonas* sp. (Worden et al. 2009). In this study, we showed that a freshwater chlorophyte, *M. hakoo*, is characterized by the smallest cell size yet identified and a genome half the size of that in *O. tauri*. A complete genome sequence of *M. hakoo* will offer valuable insight into the fundamental mechanisms of a free-living eukaryote, and the environment where a cyanobacterium was captured by a heterotrophic protist and incorporated as an endosymbiont, giving rise to the first eukaryotic alga.

### References


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