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Sponge Cytogenetics — Mitotic Chromosomes of Ten Species of Freshwater Sponge

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Porifera (sponges) are the most basal phylum of extant metazoans. To gain insight into sponge genome construction, cytogenetic analysis was performed for ten freshwater sponge species of six genera, using conventional Giemsa staining, chromosome banding, and fluorescence in-situ hybridization. The karyotypes were very similar among the ten species, exhibiting a diploid chromosome number of 2*n*=46 or 48, and usually consisted of microchromosomes with one or two pairs of large chromosomes. The 18S-28S rRNA genes were localized to a single pair of microchromosomes in two *Ephydatia* species. Hybridization signals of the telomere $(TTAGGG)_n$ sequences were observed at the ends of metaphase chromosomes. The genome sizes of *Ephydatia fluviatilis* and *Ephydatia muelleri* were estimated by flow cytometric analysis as about 0.7 pg per diploid complement. These freshwater sponge species appear to represent a fairly homogeneous group with respect to karyotypes.

Key words: Porifera, freshwater sponge, chromosome banding, fluorescence in-situ hybridization, FISH, genome size

INTRODUCTION

There are 5,000 to 10,000 known living species classified in the phylum Porifera (sponges) (Margulis and Schwartz, 1998). They are generally sessile, filter-feeding animals that possess several cell types arranged in distinct layers, but they do not develop true tissues or organ-grade structures (Simpson, 1984; reviewed in Müller et al., 2004). They are considered to possess primitive characteristics of multicellular organisms, and molecular phylogenetic analyses have confirmed that sponges are the most basal extant metazoans (e.g., Müller, 1995). Numerous genetic studies on sponges have recently investigated the evolutionary process of the gene structure and multiple gene families associated with the acquisition of multicellularity (Iwabe et al., 1996; Müller, 1998; Patthy, 2003; Larroux et al., 2007).

Cytological studies would contribute to our knowledge of the genomic construction of sponges, but few studies have been performed. Early investigations using section preparation methods reported the mitotic/meiotic chromosome number of several species (listed in Makino, 1951). The karyotypes of *Spongilla lacustris* (*2n*=18) and *Suberites domuncula* (*2n*=32) were recently determined using an air-drying technique (Imsiecke et al., 1993; Imsiecke et al., 1995); however, no detailed chromosomal constitutions have been studied.

Twenty-five freshwater sponge species, representing 11 genera of the family Spongillidae (Demospongiae, Haplosclerida), have been reported in Japan (Masuda, 1998), including several cosmopolitan species (Manconi and Pronzato, 2002). Most produce gemmules, a kind of dormant structure, which can hatch in vitro and form an entirely new body within a few days. The culture system for gemmules favors chromosome preparation, because it is effective in identifying the cell cycle (Rozenfeld, 1974) and reduces the contamination from other organisms usually existing in the porous sponge body.

Here, we investigated the cytogenetic characteristics of 10 freshwater sponge species to increase our knowledge of the chromosomal construction and genomic organization of sponges, and the evolutionary relationships among the species examined. We made chromosome preparations with germinated cells and compared chromosome numbers and configurations. To further characterize the chromosomes, several banding and fluorescence in-situ hybridization

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(FISH) techniques were applied to the chromosomes of *E. fluviatilis*. The genome sizes of two *Ephydatia* species were also estimated by flow cytometry.

MATERIALS AND METHODS

Sponge materials

The following ten species were used in this study: *Ephydatia fluviatilis, Ephydatia muelleri, Eunapius fragilis, Eunapius* sp., *Radiospongilla cerebellata, Radiospongilla sendai, Trochospongilla pennsylvanica*, and *Heterorotula multidentata* (collected from Kamiike (KAM) in the Okayama Prefectural Nature Conservation Center), *Spongilla lacustris* (collected from Otome-hama in Shiga Prefecture), and *Spongilla alba* (Shinji-ko in Shimane Prefecture). *Ephydatia fluviatilis* and *Ephydatia muelleri* were also collected from Tonegawa (TON) in Chiba Prefecture and from Tsukisamu-gawa (TSU) in Hokkaido, respectively. Gemmules were stored at 4°C in the dark until use.

Gemmule culture

Gemmules were washed with distilled water and treated with 1% hydrogen peroxide for 4 min to reduce bacterial contamination before the start of incubation. Sponges were raised at 25°C in plastic dishes containing natural mineral water (NMW) (House Foods Co., Osaka, Japan) under semi-dark conditions. The gemmules of *Spongilla alba,* a sponge that inhabits low-salinity brackish lakes, were cultured in 1/8 artificial sea water containing 0.5% marine salt (Tetra Marin; TetraWerke, Melle, Germany).

Chromosome preparation

Sponges in the choanocyte differentiation phase (about 2–3 days after germination) were exposed to colcemide (1 μg/ml) for 6 hr or colchicine (2 μg/ml) for 24 hr before harvest. The sponges were washed twice with magnesium- and calcium-free medium (MCFM) (Curtis and Van de Vyver, 1971), treated with 0.25% trypsin/MCFM for 5 s, and then suspended in MCFM by gentle pipetting. The cells were collected by low centrifugation (1500 rpm, 7 min), and treated with 3.7 mM KCl for 20 min at room temperature. The suspension was then fixed in methanol/acetic acid (3:1) and spread onto clean glass slides by an air-drying method. The slides were stained with conventional Giemsa solution (pH 6.8) using a standard method. The chromosome number was determined by counting more than 20 satisfactory metaphase spreads for each species.

Chromosome banding

To obtain replication bands, 1.31 mM (100 μg/ml) hydroxyurea (Sigma) was added to the cultures 24 hr after the gemmules hatched, and the cell culture was continued for 17 hr to synchronize the cell cycle (Rozenfeld and Rasmont, 1976). The cells were then washed three times with NMW and kept in culture for 43 hr. The medium was then changed to NMW containing BrdU (25 μg/ml), and the cell culture was continued for 2.5 hr. Colcemid (1 μg/ml) was added to the medium, and the cells were cultured for another 2.5 hr before harvest. Replication-banded chromosomes were obtained using the fluorochrome-photolysis-Giemsa (FPG) technique (Perry and Wolff, 1974). Chromosomes were stained with Hoechst 33258 (1 μg/ml) for 5 min, heated at 65°C for 3 min in 2X saline-sodium citrate buffer (SSC), and exposed to UV for 5 min. The slides were then stained with Giemsa solution.

Chromosome G-banding was performed using the trypsin-Giemsa technique with slight modification (Seabright, 1971). Slides were treated with 0.025% trypsin solution for 30 s at 4°C and dipped in 10% fetal bovine serum (FBS)/phosphate-buffered saline (PBS) for 10–15 s. The slides were then rinsed in distilled water, fixed in methanol, and stained with Giemsa solution.

FISH mapping

FISH analysis was performed as described previously (Matsuda and Chapman, 1995) using a biotin-labeled human 18S-28S rDNA probe (provided by the Japanese Cancer Research Resource Bank) and digoxigenin-labeled telomere $(TTAGGG)_7$ and $(TAACCC)_7$ probes. After hybridization, the slides were incubated with FITC-avidin (Roche Diagnostics) and mouse monoclonal anti-digoxigenin clone DI-22 ascites fluid (Sigma-Aldrich) labeled with Cy3 by using a Cy3 Ab Labeling Kit (Amersham Biosciences) to stain the rDNA probe and the telomere repeat probe, respectively. The chromosome slides were counterstained with propidium iodide (PI) or 4', 6-diamidino-2-phenylindole-dihydrochloride (DAPI). Dgital FISH images were captured by using a cooled CCD camera (MicroMAX 782Y, Princeton Instruments) mounted on a Leica DMRA microscope and analyzed with the 550CW-QFISH application program (Leica Microsystems Imaging Solutions).

Flow cytometry

The amount of DNA per nucleus was measured by flow cytometric analysis. Cell suspensions were fixed in cold 70% ethanol followed by RNase treatment (0.25 mg/ml) for 30 min at 37°C, and then stained with PI (50 μg/ml) for 30 min at 4°C. The stained cells were filtrated with a 40-μm nylon mesh and used for flow cytometry with FACSCanto (Becton Dickinson), and analyzed by FACSDiVa software (Becton Dickinson). Four independent measurements were made for each species, and at least 10,000 cells were counted per experiment. As an internal standard, mouse lymphocytes were isolated from the spleen of male C57BL/6J mice according to a technique used for lymphocyte culture (Matsuda et al., 1992). The nuclear DNA content of the sponges was calibrated against the genome size of male C57BL/6J mice (6.03 pg DNA per nucleus; Capparelli et al., 1997).

RESULTS

Karyotypes

The chromosome numbers and chromosome morphology of ten sponge species representing six genera were determined by conventional Giemsa staining. Fig. 1 shows a typical metaphase spread of *Ephydatia fluviatilis*. The chromosomes were relatively small (0.5–2.5 μm), consisting

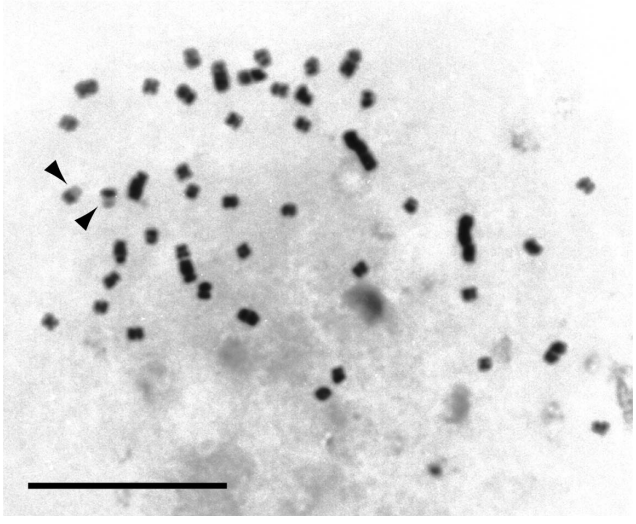


Fig. 1. Giemsa-stained mitotic metaphase spread prepared from germinated cells of *Ephydatia fluviatilis* (KAM). Arrowheads indicate the secondary constriction. Scale bar represents 10 μm.

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of mostly microchromosomes. The distributions of chromosome numbers in the metaphase spreads in 12 populations of the 10 species are shown in Table 1. The number of cells with aberrant chromosome numbers was within the normal range.

The karyotypes of two species of the genus *Ephydatia, E. fluviatilis* (Fig. 2a) and *E. muelleri* (Fig. 2b), had a diploid chromosome number of *2n*=46 and showed almost identical morphologies. The first pair consisted of distinctively large (~2.5 μm) submetacentric chromosomes. The other chromosomes comprised a large number of microchromosomes, and the size variation was sequential (0.5–1.5 μm), showing a continuous distribution from the largest to smallest chromosome. The position of the centromere was hardly determined for the majority of microchromosomes; however, suitably contracted spreads showed that they mainly consisted of submetacentric and/or metacentric chromosomes. Secondary constrictions were localized in the proximal region of two acrocentric chromosomes. We compared the karyotypes between two different populations of *E. fluviatilis* collected from Tone-gawa (TON) and Kami-ike (KAM), and between *E. muelleri* collected from Tsukisamu-gawa (TSU) and Kami-ike. *Ephydatia fluviatilis* collected from Tone-gawa showed heteromorphic secondary constrictions: one occurred at the interstitial position and the other in the proximal region of acrocentric microchromosomes (Fig. 2a'); in the Kami-ike samples (Fig. 2a), secondary constrictions were homomorphic. Other morphological variations were not detected in the karyotypes of *E. fluviatilis* or *E. muelleri* by conventional Giemsa staining.

In addition to the largest chromosome pair, *Spongilla lacustris* (*2n*=46) (Fig. 2c) and *Spongilla alba* (*2n*=48) (Fig. 2d) contained one pair and two pairs of relatively larger submetacentric microchromosomes, respectively.

The karyotypes of *Eunapius fragilis* (*2n*=46), *Eunapius* sp. (*2n*=46), *Radiospongilla cerebellata* (*2n*=48), *Radiospongilla sendai* (*2n*=46), and *Trochospongilla pennsylvanica* (*2n*=46) comprised a single pair of large chromosomes and 22 or 23 pairs of microchromosomes, and showed marked similarities among species (Fig. 2e, f, g, h, i). *Heterorotula multidentata* (*2n*=46) was characterized by two pairs of medium-sized submetacentric chromosomes instead of a single pair of large chromosomes (Fig. 2j).

The karyotype of *T. pennsylvanica* was characterized by heteromorphism of the first pair of chromosomes, in which the length of the short arms was different between the two chromosomes (Fig. 2i). Heteromorphism of the largest chromosome pair was also found in *E. muelleri* (Fig. 2b), *H. multidentata* (Fig. 2j), and *E. fluviatilis* (Fig. 3b), but not consistently.

Replication banded karyotype

Replication R-banded patterns were analyzed for four metaphase spreads of *E. fluviatilis* (TON) (Fig. 3a). The method used in this study yielded almost identical banding patterns between metaphase spreads, although replication bands were obtained in few cells (~10%). Incorporation of BrdU into DNA elongated chromosomes slightly and made it possible to arrange relatively larger microchromosomes (chromosomes 2–7) into pairs. The replication bands were not clear in smaller chromosomes, but differences in the staining pattern indicated asynchronous replication timing between microchromosomes.

G-banded karyotype

The G-banded karyotype of *E. fluviatilis* (TON) is shown in Fig. 3b, and the order of microchromosomes does not correspond with the order shown in the R-banded karyotype. The G-banded pattern was not clear, but allowed us to distinguish several pairs of similarly sized chromosomes.

Table 1. The distribution of chromosome counts in ten freshwater sponge species.

Species	Number of chromosomes								
	<42	43	44	45	46	47	48	49	Total
Genus *Ephydatia*									
(a) *E. fluviatilis* (TON)	4	3	5	10	87 (75.7[a])	3	3	–	115
(a') *E. fluviatilis* (KAM)	–	1	1	3	30 (81.1)	1	1	–	37
(b) *E. muelleri* (TSU)	3	4	3	13	93 (77.5)	3	1	–	120
(b') *E. muelleri* (KAM)	–	–	2	5	28 (77.8)	1	–	–	36
Genus *Spongilla*									
(c) *S. lacustris*	–	–	2	3	18 (78.3)	–	–	–	23
(d) *S. alba*	–	–	–	1	4	1	19 (73.1)	1	26
Genus *Eunapius*									
(e) *E. fragilis*	–	–	1	1	20 (87.0)	1	–	–	23
(f) *E.* sp.	–	–	1	3	16 (76.2)	–	1	–	21
Genus *Radiospongilla*									
(g) *R. cerebellata*	–	–	1	–	1	–	20 (90.9)	–	22
(h) *R. sendai*	–	1	1	1	18 (81.8)	–	1	–	22
Genus *Trochospongilla*									
(i) *T. pennsylvanica*	–	–	2	–	18 (85.7)	1	–	–	21
Genus *Heterorotula*									
(j) *H. multidentata*	1	1	2	4	24 (72.7)	1	–	–	33

[a] Percent of cells with a modal diploid chromosome number.

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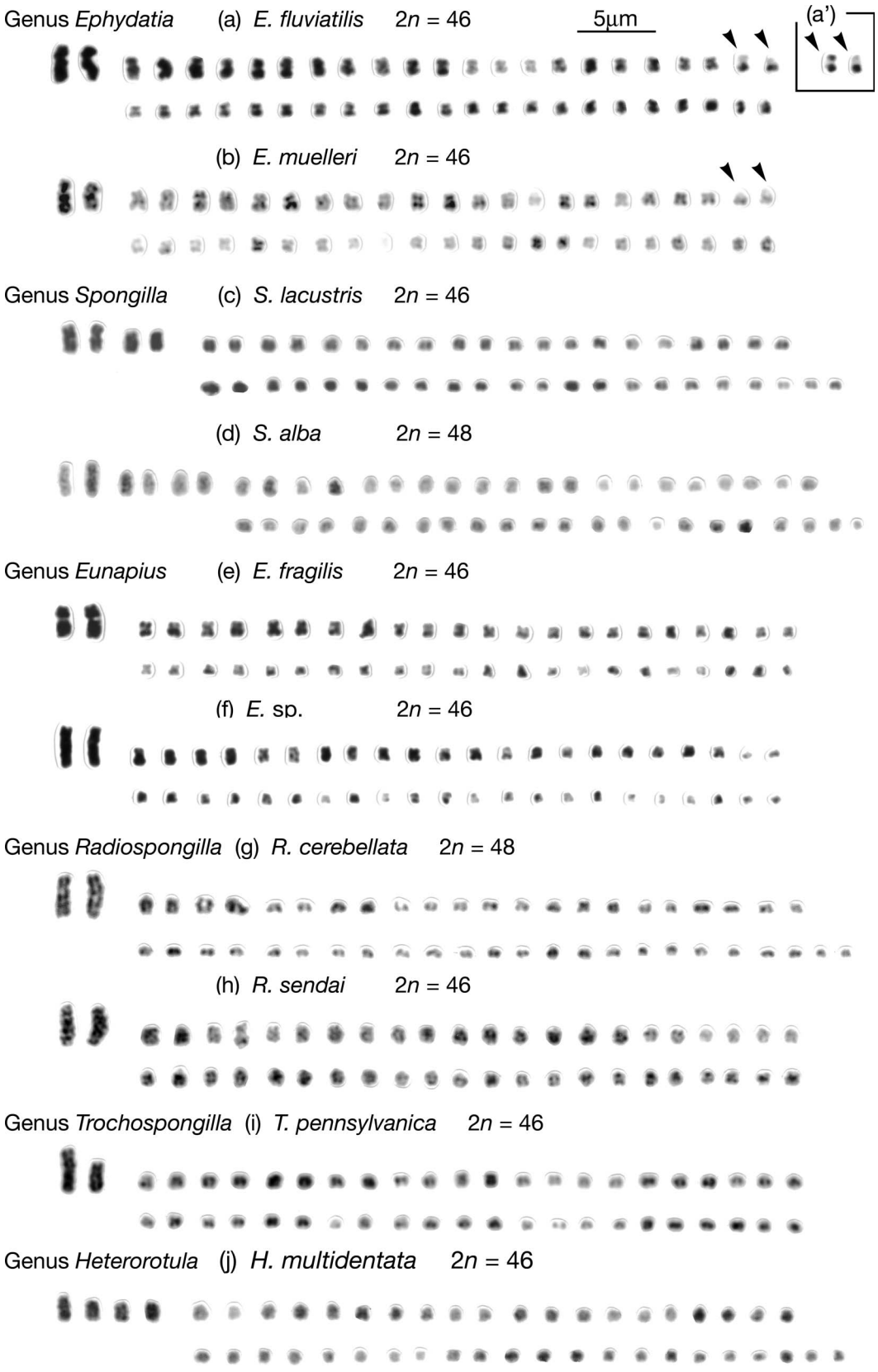


Fig. 2. Conventional Giemsa-stained karyotypes of 10 species of freshwater sponge, representing six genera. **(a)** *Ephydatia fluviatilis* (KAM). **(a')** A pair of *Ephydatia fluviatilis* (TON) chromosomes with secondary constrictions. Arrowheads indicate the secondary constrictions. **(b)** *Ephydatia muelleri* (TSU). **(c)** *Spongilla lacustris*. **(d)** *Spongilla alba*. **(e)** *Eunapius fragilis*. **(f)** *Eunapius* sp. **(g)** *Radiospongilla cerebellata*. **(h)** *Radiospongilla sendai.* **(i)** *Trochospongilla pennsylvanica.* **(j)** *Heterorotula multidentata*. Scale bar represents 5 μm.

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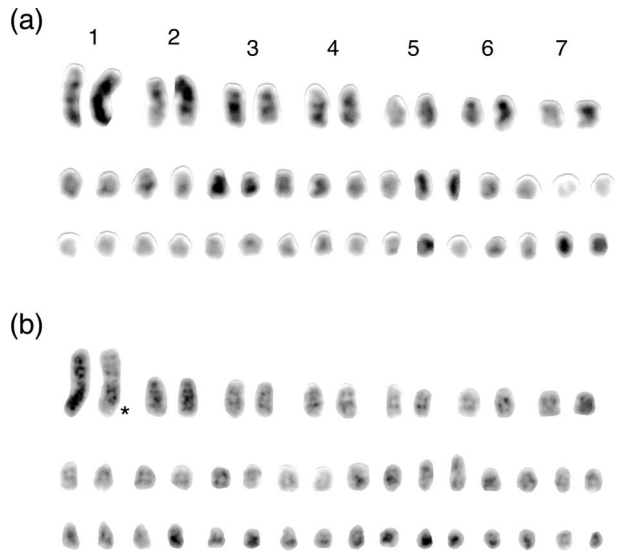


Fig. 3. R- and G-banded karyotypes of *E. fluviatilis* (TON). **(a)** Replication R-banded karyotype. **(b)** G-banded karyotype. Asterisk indicates a G-negative region of the largest chromosome.

The distal end of one of the largest chromosomes was G-negative in this karyotype, but this band was not obtained consistently.

FISH mapping of the 18S-28S ribosomal RNA genes and telomere $(TTAGGG)_n$ sequences

FISH analysis was performed to determine the chromosomal locations of the 18S-28S ribosomal RNA gene clusters and the telomere repeat sequences for *E. fluviatilis* (Fig. 4a, b) and *E. muelleri* (data not shown). Hybridization signals of the rRNA genes were localized to two microchromosomes in both species. In *E. fluviatilis* (TON), the signals were heteromorphic, which corresponded to the characteristic of the secondary constrictions observed for the Giemsa-stained karyotype (Fig. 2a'). Hybridization signals of telomeric $(TTAGGG)_n$ sequences were observed at chromosomal ends, with some variation in intensity between chromosomes (Fig. 4b). In the PI (Fig. 4a) and AT-specific DAPI staining (Fig. 4c), all chromosomes fluoresced with similar intensity.

DNA content

Flow cytometry was performed with PI-stained cells of *E. fluviatilis* (TON) and *E. muelleri* (TSU). An example of a fluorescence pulse-high histogram is shown in Fig. 5. The lowest peak of *E. fluviatilis* cells was considered to be a remnant of gemmule components. The peak value of *E. fluviatilis* cells (EF) was almost one-eighth that exhibited by mouse lymphocytes (ML). Comparative estimation with known values of mouse lymphocytes yielded a nuclear DNA content of 0.77±0.02 (mean±SD) pg for *E. fluviatilis* and 0.73±0.03 pg for *E. muelleri*.

DISCUSSION

Chromosomal preparations of freshwater sponge species belonging to the family Spongillidae were prepared using a gemmule culture system, and karyotypes were characterized by conventional Giemsa staining and chromosome banding. The ten species representing six genera exhibited similar diploid chromosome numbers and chromosome morphologies. Eight species, *E. fluviatilis*, *E. muelleri*, *S. lacustris, E. fragilis*, *E.* sp., *R. sendai, T. pennsylvanica*, and *H. multidentata*, had a diploid number of *2n*=46. *Spongilla alba* and *R. cerebellata* had a diploid number, *2n*=48, possessing an additional pair of microchromosomes.

A remakable and common feature of the karyotypes of the species examined was that they consisted of microchromosomes with one pair of large chromosomes. *Heterorotula multidentata* has two pairs of medium-sized submetacentrics instead of one large pair. Comparison of the two medium-sized submetacentric pairs of *H. multidentata* with the large chromosome pair of other species suggests that the ancestral type of the large chromosome pair may have been rearranged into two pairs of medium-sized chromosomes in this species. The highly conserved karyotypes suggest genetic homogeneity in the Spongillidae, supporting a relatively

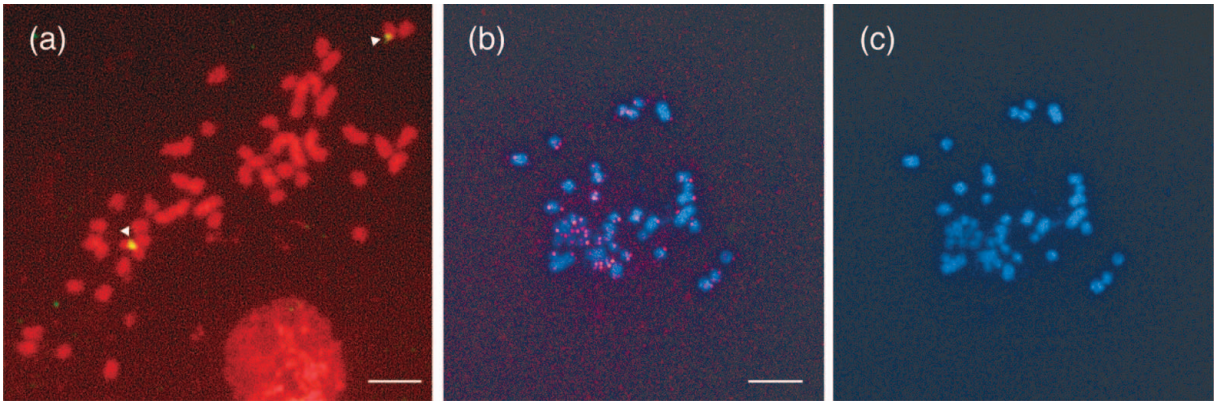


Fig. 4. FISH pattern of the ribosomal RNA genes and telomere $(TTAGGG)_n$ repeat sequences in *E. fluviatilis* (TON). **(a)** PI-stained metaphase spread hybridized with biotin-labeled 18S-28S ribosomal DNA as a probe. **(b)** DAPI-stained metaphase spread hybridized with the Cy3-labeled TTAGGG repeat sequence as a probe and **(c)** DAPI staining of the same metaphase. Arrowheads show the 18S-28S ribosomal DNA signals. Scale bar represents 5 μm.

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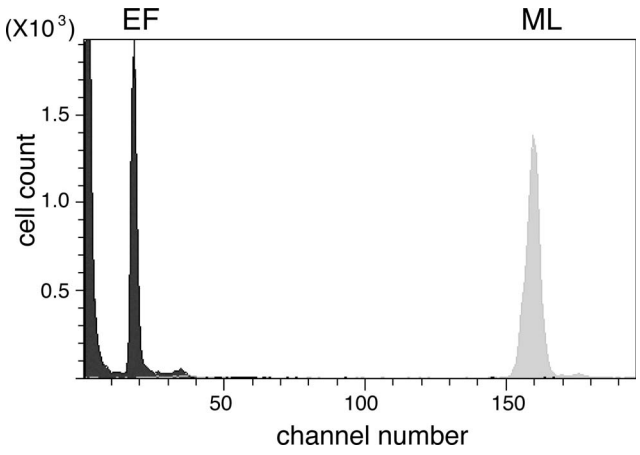


Fig. 5. DNA histogram obtained by flow cytometry of *E. fluviatilis* (TON) cells (EF) and mouse lymphocyte cells (ML). The horizontal axis indicates the intensity of fluorescence, and the vertical axis displays the number of cells. EF cells include a small population of cells with a corresponding tetraploid fluorescence value.

recent origin of freshwater sponges (Peterson and Butterfield, 2005; Müller et al., 2007). The ancestral karyotype of freshwater sponges may have comprised 44 or 46 microchromosomes and one pair of large chromosomes. The additional chromosomal pair in *S. alba* and *R. cerebellata*, with 2*n*=48, is thought to have resulted from an independent duplication and/or fission event in each lineage. The chromosomal characterization in this study, however, was not sufficient to allow a detailed discussion of evolutionary processes leading to the karyotypes of these sponge species.

Although the karyotypes are generally similar in the ten species, each genus has several unique characteristics. They can be divided roughly into two groups: a group containing species possessing relatively larger microchromosomes (*Ephydatia*, *Spongilla*), and a group without such chromosomes (*Eunapius, Radiospongilla, Trochospongilla, Heterorotula*). This classification is thought to reflect their phylogenetic relationships, because it is largely correlated with the morphological classification based on gemmule coat layer structures characterized by electron microscopy (Masuda, 1998). Molecular data on these species are required to define their phylogenetic interrelationships.

It was difficult to identify each chromosome using the usual banding methods (G-, R-, or fluoro-staining) due to their small size; however, the replication banding technique was helpful for identifying several pairs of chromosomes. The low heterogeneity of the base composition and the low copy number of highly repetitive sequences in the genomic constitution probably caused the low resolution of chromosome bands in the sponges. Reassociation analysis of nuclear DNA for *Geodia cydonium* indicated that the sponge genome mostly consisted of single-copy DNA (Bartmann-Lindholm et al., 1997). No SINEs/transposable elements were identified by genomic DNA-DNA hybridization and random nucleotide sequencing of the *Suberites domuncula* genome (Breter et al., 2003).

We mapped the vertebrate-type telomere $(TTAGGG)_n$ sequence (Meyne et al., 1989) and the 18S-28S rDNA to metaphase chromosomes of *Ephydatia* species by using the FISH method. There was variation in the intensity of the hybridization signals of $(TTAGGG)_n$ at the ends of chromosomes. This may have been due to differences in the copy numbers of the telomere repeat sequences between chromosomes. As previously reported by Traut et al. (2007) and Sakai et al. (2007), conservation of the telomere sequences in basal metazoans, including sponge species, leads us to propose that the $(TTAGGG)_n$ repeat is the evolutionary origin of telomere sequences in metazoans. The chromosomal location and copy number of the ribosomal RNA gene clusters (nucleolar organizer regions, NORs) are useful cytogenetic markers to search for phylogenetic relationships among different species. Polymorphism in the chromosomal location of the rRNA gene cluster, which corresponded to that of the secondary constrictions observed with Giemsa staining, was detected in the population of *E. fluviatilis*.

The amount of nuclear DNA in *E. fluviatilis* was estimated to be about 0.77 pg per diploid complement, which corresponds to about 0.37×10^9 bp per haploid complement. This value seems appropriate for lower invertebrates and the small chromosomal configuration of sponges. Genome size has been examined in several sponge species. Mirsky and Ris (1951) measured the genome size of *Dysidea crawshagi* (0.11 pg/cell) using Feulgen densitometry. Imsiecke et al. (1995) recorded larger genome sizes for *G. cydonium* (3.3 pg/cell) and *S. domuncula* (3.7 pg/cell). The presence of a large number of genes in the genome of sponges, therefore, was predicted on the basis of the genome size of *G. cydonium* (Breter et al., 2003). These results suggest the possibility that there is large variation in genome size among sponge species, although all belong to the same class, Demospongiae. This variation could be partially explained by methodological differences, but we need to consider the long divergence time of the class Demospongiae. Extant Porifera are usually classified into three classes (Demospongiae, Hexactinellida and Calcarea), and recent phylogenetic studies suggest that sponges may be paraphyletic (Collins, 1998; Medina et al., 2001; Peterson and Butterfield, 2005). Extensive examination of several species from other classes is required to accurately assess the genome size of sponges and the characteristics of their chromosomes as a whole.

We developed several techniques for the karyotyping and genome mapping of freshwater sponges in this study. These cytological techniques should be useful to study the evolution of genome organization in sponges.

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