

Short Communication

Occurrence of homospermidine and thermospermine as a cellular polyamine in unicellular chlorophyte and multicellular charophyte green algae

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In order to consider the phylogenetic significance of cellular polyamine profiles in the early evolution of eukaryotes, cellular polyamines from the eleven lower eukaryotic taxa, *Apicomplexa*, *Cercozoa*, *Chlorophyta*, *Ciliophora*, *Cryptophyta*, *Dinophyta*, *Euglenozoa*, *Glaucophyta*, *Haptophyta*, *Heterokonta* (*Stramenopiles*) and *Rhodophyta*, have been analyzed (Hamana, 2008; Hamana and Matsuzaki, 1982, 1985a; Hamana and Niitsu, 2006; Hamana et al., 1990, 2004a, b). The three phototrophic taxa, the (sub)kingdom *Chloroplastida* (formerly *Viridiplantae*) including a green algal division (phylum), *Chlorophyta* and the two phyla *Glaucophyta* and *Rhodophyta*, have plastids from the primary endosymbiosis of a phototrophic cyanobacterium and are located together in the *Archaeplastida* (unranked) (Adl et al., 2012; Becker, 2012; Finet et al., 2010; Inouye, 2006; Leliaert et al., 2012; MCC-NIES Catalogue, 2013; NCBI website, 2013). Distributions of triamines, such as norspermidine and spermidine, and tetra-amines, such as norspermine and spermine, were almost phylogenetically specific among the eleven taxa; furthermore, a part of their polyamine profiles seems to be correlated to their evolutionary secondary or tertiary endo-

symbiotic process. Within the eleven taxa, homospermidine was found only in multicellular rhodophyte red algae as a major polyamine (Hamana and Niitsu, 2006), whereas triamine was widespread in cyanobacteria (Hamana et al., 1983, 1988, 2008; Hosoya et al., 2005) and in land plants including mosses and ferns (Hamana and Matsuzaki, 1985b; Hamana et al., 1988; Shaw et al., 2010). Thermospermine, an isomer of spermine found as a usual, minor tetra-amine in higher land plants (Fuell et al., 2010; Hamana et al., 1992, 1994, 1996, 1998, 2000; Minguet et al., 2008; Otsuka et al., 2005; Takano et al., 2012), has never been found in the lower eukaryotic eleven taxa.

Another green algal division, the *Charophyta*, plus the embryophytes (the *Embryophyta*, unranked) make up the (phylum) *Streptophyta*, which is a monophyletic group (Adl et al., 2012; Becker, 2012; Finet et al., 2010; Leliaert et al., 2012). The ancestor of all green algae evolved into two major groups. Multicellular charophyte green algae evolutionally transited to the ancestor of early land plants in the *Chloroplastida* (*Chlorophyta* plus *Charophyta* and/or *Streptophyta*). However, taxonomic ranks of the *Charophyta*, *Streptophyta* and *Phragmoplastophyta* are conflicting (Adl et al., 2012).

In the present study, polyamines of an additional 27 green algae belonging to the *Chlorophyta* and *Charophyta* were newly analyzed. High-performance liquid chromatography (HPLC) and a packed column meth-

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od of gas chromatography (GC) were used in our previous polyamine analyses; however, identification of minor polyamine components was insufficient, so a high-performance GC equipped with a capillary column was used in the present study. This is the first report on the occurrence of homospermidine and thermospermine in unicellular chlorophyte green algae and on the polyamine analysis of multicellular charophycean green algae in which novel tetra-amines, aminopropylhomospermidine and canavalline, were found in addition to homospermidine and thermospermine.

Freshwater and marine micro green algae supplied from NIES, NBRC and Iwakuni City Microlife Museum (ICMM) were cultivated axenically in the light (14 h/24 h) at 20–25°C using the media C, C-agar, ESM, MG and Daigo's IMK-Sea (IMK, Nissui Pharm. Co., Tokyo, Japan, dissolved in seawater) designed by NIES (Kasai et al., 2004; MCC-NIES Catalogue, 2013) or NBRC (NBRC Catalogue, 2013), Daigo's IMK-SP (IMK plus Artificial Seawater SP) and 0.1% HYPONeX (Hyponex · Japan, Osaka, Japan) (Hyp). The freshwater charophycean algae *Nitella* and *Chara* were purchased from Mitunokusa Co., Osaka, Japan, and Kagaku-club Co., Shiga, Japan, respectively. Micro algae (0.01–10 g wet weight) harvested at the early stationary phase from five agar slants, 100–200 ml cultures (for HPLC alone) or 2–20 L cultures (for HPLC and GC), and a 50–100 g wet weight of charophycean algae, were homogenized in 5% perchloric acid (PCA). The PCA extract was subjected to a column of a cation-exchange resin, Dowex 50WX8 (1 cm I.D. × 3 cm or 3 cm I.D. × 1 cm) to concentrate polyamines and then analyzed by HPLC on a Hitachi L6000 using a column of cation-exchange resin, Hitachi 2619F (4 mm I.D. × 50 mm) using post-labeled fluorometry after heating with *o*-phthalaldehyde (Hamana, 2002; Hamana et al., 1995). After heptafluorobutyrylation of the concentrated polyamines, GC on a Shimadzu GC-17A and GC-mass spectrometry (GC-MS) on a JEOL JMS-700, equipped with a capillary column of Inert Cap 1MS (0.32 mm I.D. × 30 m) (GL Sciences) were operated by a flame ionization detector (Niitsu et al., 1993; Hamana et al., 2005). Molar concentrations of cellular polyamines per gram of wet weight of the starting pellets of algae, estimated from the HPLC and GC analyses using authentic polyamine standards, are shown in Table 1.

Chlorophyta (consisting of nine classes, as shown in Table 1): In the class *Trebouxiophyceae*, it has been known that norspermidine (and norspermine) are dis-

tributed in non-photosynthetic achlorophyllous *Prototheca* and *Helicosporidium* species (similar to a *Chlorella* species that lacked a photosynthetic ability (Inouye, 2006)) as well as the typical photosynthetic genera *Ankistrodesmus* and *Mymecia* (Hamana et al., 2004a).

Norspermidine was found as a major polyamine in *Botryococcus braunii*, a unicellular green alga producing oil and forming a cell cluster, and was found as a minor polyamine in *Micractinium pusillum*, as shown in the present study (Table 1). *B. braunii* NIES-2199 was characterized as a green alga containing a high level of agmatine. Spermine and thermospermine were detected as a minor polyamine in *B. braunii* NIES-836 by GC-MS (Fig. 1). Classification of the genus *Chlorella* is conflicting, and the new genera *Auxenochlorella*, *Parachlorella* and *Pseudochlorella* were proposed (MCC-NIES Catalogue, 2013; NCBI website, 2013). Although polyamines of three green algae of the *Chlorella* group were newly analyzed and added into Table 1, the chemotaxonomic significance of their polyamine profiles is not clear.

Sixteen previously analyzed genera (*Chlamydomonas*, *Chlorogonium*, *Desmodesmus*, *Eudorina*, *Gonium*, *Haematococcus*, *Hydrodictyon*, *Polytoma*, *Polytomella*, *Scenedesmus*, *Volvox*, etc.) of the class *Chlorophyceae* ubiquitously contained norspermidine (Hamana et al., 2004a). The occurrence of a significant amount of spermine in *Chlorogonium elongatum* has been reported (Hamana et al., 2004a), as cited in Table 1. In addition to norspermidine, spermidine and spermine, thermospermine and homospermidine were detected in *Chlorogonium capillatum*, as shown in Table 1. Spermine and thermospermine were identified by GC-MS (Fig. 1). The polyamine levels at the log phase (cultured for 4 days) decreased at the stationary phase (cultured for 8 days) in the alga (Table 1). *Eudorina* as well as *Gonium* and *Volvox* (the motile order *Volvocales*) are colony (coenobium)-forming green algae. *Eudorina illinoisensis* contained spermine as a minor polyamine (Hamana et al., 2004a), as cited in Table 1. In the present study, spermine and thermospermine were detected as a minor polyamine in another *Eudorina* species, *E. cylindrica* (Fig. 1).

In the genera *Nephroselmis* of the class *Nephroselmophyceae*, *Monomastix* of the class *Mamiellophyceae*, *Tetraselmis* of the class *Chlorodendrophyceae* and *Pynamimonas* of the class *Prasinophyceae*, previously analyzed, norspermidine and norspermine were not detect-

ed (Hamana et al., 2004a). A species of the class *Nephroselmidophyceae* and five species of the class *Prasinophyceae*, newly analyzed in the present study, contained norspermidine (Table 1). In the class *Pedinophyceae*, *Marsupiomonas* and *Pedinomonas* species contained diaminopropane, norspermidine and

norspermine (Table 1). In the class *Ulvoephyceae*, norspermidine and/or norspermine was found in *Monostroma* and *Oltmannsiellopsis* species (Fig. 1 and Table 1), as well as two *Ulothrix* species previously reported (Hamana et al., 2004a).

Charophyta (consisting of six classes, as shown in

Table 1. Cellular polyamine concentrations of 27 green algae newly analyzed in the present study and related 17 green algae previously analyzed (cited as a or b) belonging to the divisions *Chlorophyta* and *Charophyta*.

Organism	Medium (Ref.)	Polyamines (μmol/g wet weight)												Can	Agm
		Dap	Put	Cad	N Spd	Spd	H Spd	N Spm	Spm	T Spm	AH Spd				
Division <i>Chlorophyta</i>															
Class <i>Trebouxiophyceae</i>															
<i>Botryococcus braunii</i> NIES-2199	C-agar	—	0.04	—	0.48	0.08	—	—	—	—	—	—	—	—	0.70
	C	0.05	0.15	—	1.20	0.25	—	—	—	—	—	—	—	—	0.75
NIES-836	Hyp	—	0.96	—	1.10	1.21	—	—	0.02	0.01	—	—	—	—	0.02
<i>Micractinium pusillum</i> NIES-151	C	—	0.27	—	0.05	0.75	—	—	—	—	—	—	—	—	—
	Hyp	—	0.10	—	—	0.70	—	—	—	—	—	—	—	—	—
<i>Chlorella sorokiniana</i> IAM C-212	A-3(b)	—	0.15	0.04	0.03	0.65	—	—	—	—	—	—	—	—	—
<i>Chlorella vulgaris</i> IAM C-27	A-10(a)	—	1.89	0.09	0.05	2.18	—	—	—	—	—	—	—	—	—
IAM C-207	A-14(b)	—	0.37	0.02	—	0.20	—	—	0.02	★	—	—	—	—	—
	A-4(b)	—	0.32	0.05	—	0.47	—	—	0.02	★	—	—	—	—	—
<i>Auxenochlorella protothecoides</i> IAM C-150	A-4(b)	—	—	0.03	—	0.50	—	—	0.02	★	—	—	—	—	—
IAM C-206	A-4(b)	—	0.15	0.02	0.85	0.20	—	—	—	—	—	—	—	—	—
NIES-2164 (=IAM C-624)	Hyp	—	—	—	—	0.51	—	—	—	—	—	—	—	—	—
<i>Parachlorella kessleri</i> IAM C-531	A-10(b)	—	1.40	—	—	0.64	—	—	—	—	—	—	—	—	—
' <i>Chlorella</i> ' <i>ellipsoidea</i> IAM C-87	A-4(a)	0.63	0.74	0.14	0.08	2.07	—	0.08	—	—	—	—	—	—	—
' <i>Chlorella</i> ' <i>saccharophila</i> IAM C-169	A-14(b)	—	0.04	—	—	0.18	—	—	—	—	—	—	—	—	0.15
NIES-640	C	—	0.56	—	0.02	0.13	—	0.02	—	—	—	—	—	—	—
	Hyp	—	1.10	—	0.04	0.20	—	0.03	—	—	—	—	—	—	—
NBRC 102700	IMK-SP	—	0.04	—	—	0.44	—	—	—	—	—	—	—	—	0.05
Class <i>Chlorophyceae</i>															
<i>Chlorogonium elongatum</i> IAM C-295	A-7(b)	—	1.00	—	0.35	0.55	—	—	0.10	★	—	—	—	—	—
<i>Chlorogonium capillatum</i> ICMM strain (S)	Hyp	—	0.01	0.03	0.02	0.56	0.05	—	0.16	★	—	—	—	—	—
(=ATCC 50936) (L)	Hyp	—	0.55	—	0.30	0.43	0.20	—	0.23	0.03	—	—	—	—	—
<i>Eudorina illinoisensis</i> IAM C-596	A46(b)	—	0.75	—	0.20	0.33	—	—	0.04	★	—	—	—	—	—
<i>Eudorina cylindrica</i> NIES-722	AF-6	—	1.20	—	1.20	1.02	—	—	0.02	0.01	—	—	—	—	—
Class <i>Nephroselmidophyceae</i>															
<i>Nephroselmis pyriformis</i> NBRC 102849	IMK-SP	0.18	0.25	—	0.24	0.38	—	—	—	—	—	—	—	—	0.30
Class <i>Mamiellophyceae</i>															
<i>Monomastix minuta</i> NIES-255	C(b)	—	0.01	—	—	0.30	—	—	—	—	—	—	—	—	—
Class <i>Chlorodendrophyceae</i>															
<i>Tetraselmis cordiformis</i> NIES-18	C(b)	—	0.75	—	—	0.10	—	—	—	—	—	—	—	—	—
Class <i>Prasinophyceae</i>															
<i>Mantoniella squamata</i> NIES-1409	IMK-SP	—	0.54	—	0.10	0.07	—	—	—	—	—	—	—	—	0.24
<i>Micromonas pusilla</i> NIES-1411	IMK-SP	—	0.10	—	0.17	0.05	—	—	—	—	—	—	—	—	0.20
<i>Micromonas pusilla</i> NBRC 102743	IMK-SP	—	0.10	—	0.10	0.54	—	—	—	—	—	—	—	—	—
<i>Pseudoscurfieldia marina</i> NIES-1419	IMK-SP	—	0.40	—	—	0.20	—	—	0.05	★	—	—	—	—	—
<i>Prasinococcus capsulatus</i> NBRC 102962	IMK-SP	0.05	0.05	—	0.05	0.15	—	—	—	—	—	—	—	—	0.05
<i>Prasinoderma coloniale</i> NBRC 102841	IMK-SP	—	0.05	—	—	0.25	—	—	—	—	—	—	—	—	—
<i>Prasinopapilla vacuolata</i> NBRC 102950	IMK-SP	—	0.02	—	—	0.42	—	—	—	—	—	—	—	—	—
<i>Pyramimonas aurea</i> NBRC 102947	IMK-SP	0.04	0.05	—	0.05	0.20	—	—	—	—	—	—	—	—	—
Class <i>Pedinophyceae</i>															
<i>Pedinomonas minor</i> NIES-363	C	0.56	0.05	—	0.20	0.20	—	—	—	—	—	—	—	—	—
<i>Marsupiomonas</i> sp. NIES-1410	IMK-Sea	0.72	0.04	0.25	0.20	0.12	—	0.03	—	—	—	—	—	—	0.01

Table 1. Continued

Organism	Medium (Ref.)	Polyamines (μmol/g wet weight)												
		Dap	Put	Cad	N Spd	Spd	H Spd	N Spm	Spm	T Spm	AH Spd	Can	Agm	
Class <i>Ulvophyceae</i>	IMK-SP	0.95	0.04	0.15	0.35	0.10	—	0.03	—	—	—	—	0.01	
<i>Blidingia minima</i> NIES-1837	IMK-Sea	—	0.04	—	—	0.20	—	—	—	—	—	—	—	
<i>Monostroma oxyspermum</i> NBRC 103673	IMK-SP	—	0.10	—	0.10	0.30	—	—	—	—	—	—	—	
<i>Oltmannsiellopsis viridis</i> NIES-360	ESM	0.03	0.02	—	—	0.72	—	0.02	0.01	—	—	—	0.02	
	IMK-SP	—	0.02	—	0.01	0.58	—	0.20	0.04	—	—	—	—	
Class <i>Palmophyllales</i>	No polyamine data													
Division <i>Charophyta</i>														
Class <i>Mesostigmatophyceae</i>														
<i>Mesostigma viride</i> NIES-296	C(b)	—	0.90	—	—	0.45	—	—	—	—	—	—	—	
Class <i>Chlorokybophyceae</i>														
<i>Chlorokybus</i> sp. NIES-160	C-agar	—	0.02	—	0.24	0.50	—	—	—	—	—	—	—	
Class <i>Klebsormidiophyceae</i>														
<i>Klebsormidium flaccidum</i> IAM C-117	A-4(b)	—	—	—	—	0.60	—	—	—	—	—	—	—	
<i>Interfilum paradoxum</i> IAM C-161	A-4(b)	—	—	—	0.05	0.80	—	—	—	—	—	—	—	
Class <i>Zygnematophyceae</i>														
<i>Pleurotaenium ehrenbergii</i> IAM C-468	A-18(b)	0.35	0.75	0.10	0.35	0.45	—	—	—	—	—	—	—	
<i>Pleurotaenium cylindricum</i> NIES-306	MG	0.05	0.02	—	—	0.25	—	—	—	—	—	—	—	
Class <i>Coleochaetophyceae</i>	No polyamine data													
Class <i>Charophyceae</i>														
<i>Nitella flexilis</i>		0.04	0.21	—	0.02	1.28	0.28	0.04	0.14	0.01	0.01	0.01	0.01	
<i>Chara braunii</i>		0.02	0.90	—	0.20	7.15	0.53	0.28	0.10	0.07	0.01	—	—	
<i>Chara australis</i>		—	—	—	0.01	1.04	0.30	0.02	0.02	—	—	0.03	—	

Dap (3) (abbreviations for the number of methylene chain units between N), diaminopropane; Put (4), putrescine; Cad (5), cadaverine; NSpd (33), norspermidine; Spd (34), spermidine; HSpd (44), homospermidine; NSpm (333), norspermine; Spm (343), spermine; TSp (334), thermospermine; AHSpd (344), aminopropylhomospermidine; Can (434), canavalmine (aminobutylspermidine); Agm, agmatine; ATCC, American Type Culture Collection, Manassas, Virginia, USA; IAM, IAM Culture Collection, Institute of Molecular and Cellular Biosciences, The University of Tokyo, Tokyo, Japan (IAM algal collections have been transferred into NIES, 2000); ICMM, Iwakuni City Microlife Museum, Iwakuni, Yamaguchi, Japan; NIES, National Institute for Environmental Studies, Tsukuba, Japan; NBRC, Biological Resource Center, National Institute of Technology and Evaluation, Kisarazu, Japan; —, not detected (<0.005). Data were obtained from a single sample. Quotation marks show that the scientific name has not been validated. Reference (Ref.) (a), cited from Hamana and Matsuzaki (1982). (b), cited from Hamana et al. (2004a). (S) and (L) in *C. capillatum*, harvested at the stationary phase and the logarithmically growing (log) phase, respectively. Medium is shown in the text. ★, Analysis of thermospermine by GC or GC-MS was not performed.

Table 1): Sporadic distribution of norspermidine was shown within the genera *Chlorokybus* of the class *Chlorokybophyceae*, *Interfilum* of the class *Klebsormidiophyceae* and *Pleurotaenium* of the class *Zygnematophyceae* (Table 1). Spermidine, norspermidine, homospermidine, norspermine, spermine and thermospermine were found in *Nitella flexilis*, *Chara braunii* and *Chara australis* of the class *Charophyceae* (Table 1). Minor tetra-amines were identified and determined in the present large-scale polyamine analysis by GC and GC-MS (Fig. 1). It is suggested that the two unusual tetra-amine isomers, an aminopropyl derivative of ho-

mospermidine, aminopropylhomospermidine, and an aminobutyl derivative of spermidine, canavalmine (aminobutylspermidine), appeared first in multicellular charophycean green algae. Although *Nitella* and *Chara* non-axenically grown were washed with sterile water before polyamine analysis, contamination of the two minor tetra-amines derived from other microorganisms is not excluded completely. However, the two tetra-amines have been found in mushrooms (Hamana et al., 2005) and higher land plants (Hamana et al., 1992, 2000; Otsuka et al., 2005) but have never been found in ordinary bacteria, cyanobacteria, protists or

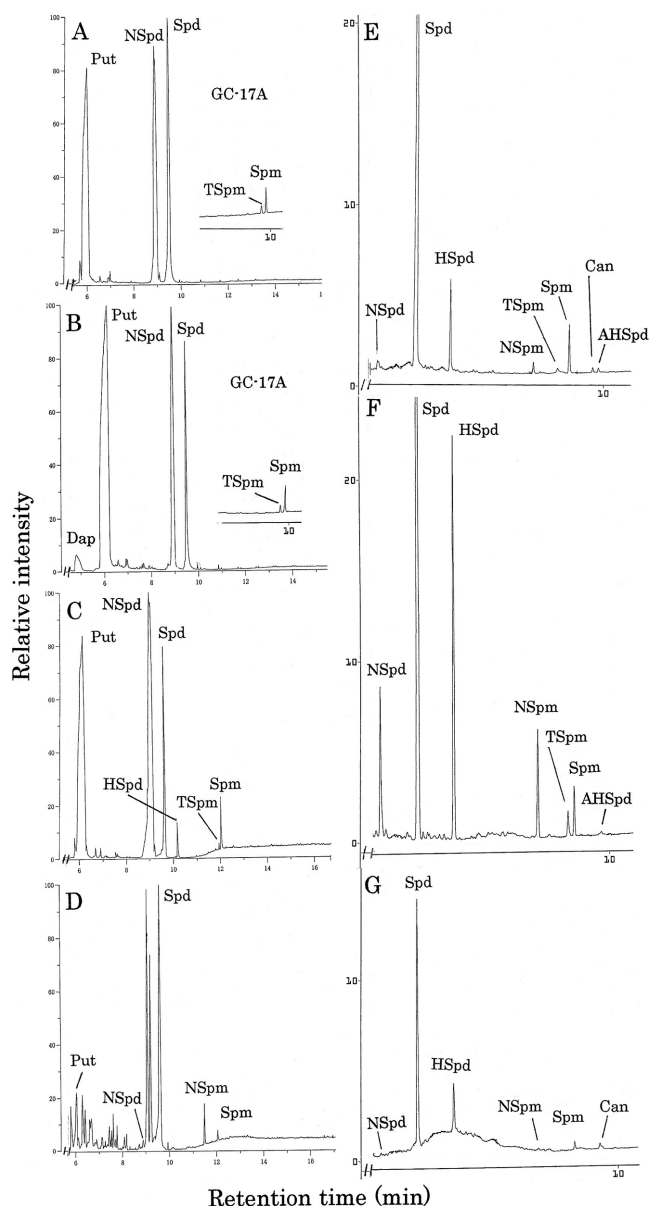


Fig. 1. GC analysis of cellular polyamines of the freshwater unicellular green algae *Botryococcus braunii* NIES-836 (A), *Eudorina cylindrica* NIES-722 (B) and *Chlorogonium capillatum* ICMM (C), the marine unicellular green alga *Oltmannsiellopsis viridis* NIES-360 (D), the freshwater multicellular charophycean green algae *Nitella flexilis* Osaka, Japan (E), *Chara braunii* Shiga, Japan (F) and *Chara australis* Shiga, Japan (G) on a JEOL JMS-700 at the column temperature 90–16°C/min–280°C (A, B, C, D) or a Shimadzu GC-17A at the column temperature 120–16°C/min–280°C (E, F, G).

A part is shown in the GC chromatograms. Another chromatogram by GC-17A (intensity $\times 10$) is added in Figs. A and B. Agmatine is destroyed during GC analysis. Abbreviations for polyamines are given in Table 1.

unicellular algae.

Polyamine distribution profiles: Putrescine and/or spermidine was ubiquitously distributed, whereas diaminopropane, cadaverine and agmatine were sporadically distributed within the green algal divisions *Chlorophyta* and *Charophyta*. Triamines such as norspermidine and homospermidine and tetra-amines such as norspermine, spermine and thermospermine found in several green algae are phylogenetically important polyamine components.

Norspermidine has never been detected in prokaryotic cyanobacteria, roughly separated into the spermidine-dominant type and homospermidine-dominant type (Hamana et al., 1983, 1988, 2008; Hosoya et al., 2005). The widespread occurrence of norspermidine in the *Chlorophyta* and *Charophyta*, suggests that its synthetic ability was obtained without the primary endosymbiosis of a cyanobacterium.

Homospermidine had been detected as a minor polyamine in *Scenedesmus* sp., *Stigeoclonium* sp. and *Codium* sp. belonging to the *Chlorophyta* and *Spirogyra* sp. and *Nitella* sp. belonging to the *Charophyta*, which were collected in the field (Hamana and Matsuzaki, 1982). Homospermidine was also found in a non-axenic halophilic chlorophycean green alga, *Dunaliella* sp. (Hamana et al., 1988) by HPLC alone. In the present study with GC and GC-MS, homospermidine was found clearly to be a major polyamine in a unicellular chlorophycean green alga, *C. capillatum*, axenically grown and in the three multicellular charophycean green algae. Distribution of homospermidine in green algae after the primary endosymbiosis and before *Chlorophyta/Charophyta* (*Streptophyta*) split in the *Chloroplastida* is suggested. Many cyanobacteria contained homospermidine (Hamana et al., 2008); however, triamine has never been detected in the various protists which evolved without primary endosymbiosis (Hamana, 2008; Hamana et al., 2004b, 2006).

Although we could not detect thermospermine in the three *Chlamydomonas* species (Hamana and Matsuzaki, 1982; Hamana et al., 2004), a tentative study on the occurrence of thermospermine in a *Chlamydomonas* species has been published (Takehi et al., 2012). The occurrence of thermospermine as a precursor of homocaldopentamine is suggested in the unicellular flagellates *Chattonella* and *Heterosigma* (the class *Raphidophyceae* of the phylum *Heterokonta*) containing homocaldopentamine (Nishibori et al., 2009). Putative spermine synthase from a unicellular diatom,

Thalassiosira pseudonana (the class *Bacillariophyceae* of the phylum *Heterokonta*), was identified as thermospermine synthase (Knott et al., 2007). These findings and the present report indicate that thermospermine as well as spermine are distributed in green algae belonging to the *Chlorophyta* and *Charophyta* and yellow algae belonging to the phylum *Heterokonta*. The minor occurrence of thermospermine and spermine in some thermophilic and halophilic cyanobacteria (Hamana et al., 2008) supports its wide distribution as a minor polyamine component within photosynthetic eukaryotic algae evolved after the primary endosymbiosis.

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