

Short Communication

Cellular polyamines of phototrophs and heterotrophs belonging to the lower eukaryotic phyla Cercozoa, Euglenozoa, Heterokonta and Metamonada

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Analysis of cellular polyamine components in the lower eukaryotic phyla, the phylum Euglenozoa, Dinophyta, Cryptophyta, Haptophyta, Heterokonta, Apicomplexa, Ciliophora, Cercozoa, Chlorophyta, Glaucophyta and Rhodophyta, have been studied in order to consider the phylogenetic significance of cellular polyamine distributions in early evolution of eukaryotes (Hamana and Matsuzaki, 1982, 1985; Hamana and Niitsu, 2006; Hamana et al., 1990, 2004a, b, 2006a). In the eleven phyla, the three phototrophic phyla Glaucophyta, Rhodophyta and Chlorophyta, have plastids from the primary endosymbiosis of an oxygenic phototrophic prokaryote, cyanobacterium (blue-green alga), and multicellular species evolved within the latter two (Baldauf, 2003; Bhattacharya et al., 2004; Cavalier-Smith, 1998; Dyall et al., 2004; Falkowski et al., 2004; Inouye, 2006; NCBI website, 2007; Rodriguez-Ezpeleta et al., 2005). The other eight phyla include heterotrophs evolved without the endosymbiosis, phototrophs from secondary or tertiary endosymbiotic plastids, and heterotrophs evolved after the loss of primary endosymbiotic plastids (Baldauf, 2003; Bhattacharya et al., 2004; Cavalier-Smith, 1998; Falkowski et al., 2004; NCBI website, 2007; Rodriguez-

Ezpeleta et al., 2005). Among the eight phyla, multicellular species evolved within the phylum Heterokonta. The distribution patterns of three triamines, norspermidine, homospermidine and spermidine, and two tetraamines, norspermine and spermine, were almost phylum-specific among the eleven phyla. Furthermore, a part of their polyamine components seems to be correlated to their evolutionary endosymbiotic process.

The other non-photosynthetic, unicellular, lower eukaryotes evolved without the endosymbiosis of cyanobacteria are located within the five taxa (phyla), Metamonada, Acanthamoebidae, Lobosea, Entamoebidae and Percolozoa (Cavalier-Smith, 2002, 2003). Norspermidine, homospermidine and norspermine have never been detected in *Trichomonas* or *Giardia* belonging to the phylum Metamonada, *Acanthamoeba* belonging to the phylum Acanthamoebidae, *Amoeba* belonging to the phylum Lobosea, *Entamoeba* belonging to the phylum Entamoebidae, or five species of the phylum Percolozoa (Gillin et al., 1984; Hamana and Niitsu, 2006; Hamana et al., 2006a).

In the present study, cellular polyamines of several non-photosynthetic, heterotrophic genera known in the two phyla Euglenozoa and Cercozoa (Mullner et al., 2001), 26 unicellular phototrophs distributed in nine classes of the phylum Heterokonta and two heterotrophic trepomonads belonging to the phylum Metamonada were newly analyzed. Therefore, cellular polyamine distribution profiles in the all of 16 lower eu-

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karyotic taxa (phyla) were demonstrated by the present study on 37 unicellular eukaryotic strains, in addition to our previous studies.

Micro eukaryotic organisms were supplied from ATCC, MBIC and NIES (see Table 1), and cultivated using the culture conditions designed by the culture collections (ATCC Cultures, Protozoa and Algae, 2007;

Kasai et al., 2004; MBIC Strain Catalog Algae, 2006). *Euglena clara* was grown phototrophically in AF-6 (NIES) at 20°C in the light. *Astasia longa* was cultured heterotrophically in ATCC medium 351 plus 0.1% sodium acetate, at 20°C, in the dark. *Diplonema ambulator* was grown in ATCC medium 1728 containing heat-inactivated horse serum, at 2°C. *Bodo saltans* and

Table 1. Cellular polyamine concentrations in lower eukaryotes.

Organism	N	Polyamines (μmol/g wet weight)									
		Dap	Put	Cad	NSpd	Spd	HSpd	NSpm	Spm	Agm	
Phylum Euglenozoa											
Class Euglenidea (Euglenophyta, Euglenophyceae)											
<i>Euglena gracilis</i> IAM E-6	(c)	0.01	0.14	0.18	0.57	0.93	—	0.02	0.03	—	
<i>Euglena viridis</i> IAM E-11	(b)	—	1.11	0.31	0.17	0.85	—	—	0.06	—	
<i>Euglena mutabilis</i> NIES-286	(c)	0.04	0.42	—	0.12	0.20	—	—	—	—	
<i>Euglena clara</i> NIES-253*	3	0.02	0.32	—	0.54	0.84	—	0.04	—	—	
		±0.01	±0.04	—	±0.06	±0.06	—	±0.01	—	—	
<i>Eutreptiella gymnastica</i> NIES-381	(c)	—	0.03	—	0.15	0.05	—	0.04	0.02	—	
<i>Phacus agilis</i> NIES-387	(c)	0.50	1.05	—	0.20	0.39	—	0.04	0.02	—	
<i>Trachelomonas</i> sp. Gunma*	(a)	0.23	0.27	—	0.17	0.27	—	0.40	—	—	
<i>Astasia longa</i> (1204-17/B) ATCC 12896	2	—	0.02	0.06	0.03	0.27	—	—	0.04	—	
Class Diplonemea											
<i>Diplonema ambulator</i> (X3 Smith) ATCC 50223	1	—	0.02	0.29	—	0.02	—	—	—	—	
Class Kinetoplastea											
<i>Trypanosoma cruzi</i> (MAR 6)	(c)	—	—	0.01	—	0.67	—	—	0.16	0.02	
<i>Leishmania major</i> (MHOM)	(c)	—	0.10	—	—	1.30	—	—	—	—	
<i>Bodo saltans</i> NIES-1439*	1	—	0.02	0.02	—	0.40	—	—	0.07	—	
<i>Dimastigella trypaniformis</i> (CH-AX) ATCC 50331	1	—	0.15	—	—	0.50	—	—	—	—	
Phylum Cercozoa											
Class Chlorarachniophyceae (Chlorarachniophyta)											
<i>Chlorarachnion reptans</i> NIES-624	(c)	—	0.40	—	0.30	0.35	—	0.05	0.15	—	
<i>Chlorarachnion</i> sp. MBIC 10857*	3	—	0.25	—	0.20	0.20	—	—	—	—	
			±0.05		±0.02	±0.02					
Class Cercomonadida											
<i>Cercomonas bodo</i> (DE-1) ATCC 50366*	1	—	0.34	0.15	—	0.90	—	—	—	—	
<i>Massisteria marina</i> (CS1982) ATCC 50266*	2	—	1.30	0.02	—	0.22	0.03	—	—	—	
Class Thaumatomonadida											
<i>Thaumatomastix</i> sp. NIES-1443	1	—	0.02	—	—	0.45	—	—	—	—	
Phylum Heterokonta (Heterokontophyta, Stramenopiles)											
Class Chrysophyceae											
<i>Ochromonas danica</i> IAM CS-4	(b)	—	0.97	0.96	—	0.42	—	—	0.02	—	
<i>Ochromonas minuta</i> IAM CS-5	(b)	—	0.28	1.43	—	0.63	—	—	0.04	—	
<i>Poterioochromonas malhamensis</i> IAM CS-1	(b)	—	0.54	0.20	—	0.10	—	—	—	—	
<i>Sarcinochrysis marina</i> MBIC 10482	1	—	0.12	0.10	—	0.35	—	—	—	—	
Class Raphidophyceae											
<i>Chattonella antiqua</i> NIES-86	2	—	—	—	—	0.20	—	—	—	—	
<i>Chattonella marina</i> NIES-118	1	—	—	—	—	0.20	—	—	—	—	
<i>Chattonella ovata</i> NIES-603	1	—	—	—	—	0.22	—	—	—	—	
<i>Fibrocapsa japonica</i> NIES-1303	1	—	—	—	—	0.30	—	—	—	—	
<i>Heterosigma akashiwo</i> NIES-9	3	—	—	—	—	0.25	—	—	—	—	
						±0.04					
<i>Gonyostomum semen</i> NIES-1380*	1	—	—	—	—	0.14	—	—	—	—	
<i>Olisthodiscus luteus</i> NIES-15	1	—	—	—	—	0.14	—	—	—	—	
Class Bacillariophyceae											
<i>Phaeodactylum tricornutum</i> IAM B-14	(b)	—	0.40	0.05	0.60	0.35	—	0.20	—	—	
<i>Nitzschia palea</i> IAM B-18	(b)	0.04	1.08	0.10	0.01	0.08	—	—	—	—	
<i>Nitzschia closterium</i> IAM B-16	(c)	—	0.15	—	0.03	0.08	—	—	—	—	

Table 1. Continued.

Organism	N	Polyamines ($\mu\text{mol/g}$ wet weight)								
		Dap	Put	Cad	NSpd	Spd	HSpd	NSpm	Spm	Agm
<i>Synedra acus</i> Gunma*	(a)	—	1.60	—	0.02	0.56	0.04	—	—	—
<i>Achnanthes subconstricta</i> NIES-330	1	—	0.24	—	0.02	0.15	—	—	—	—
<i>Achnanthes minutissimum</i> NIES-71	2	—	0.26	—	—	0.12	—	—	—	—
<i>Eucampia zodiacus</i> NIES-1049	1	—	0.10	—	—	0.20	—	—	—	—
<i>Melosira granulata</i> NIES-333	2	—	0.27	—	0.02	0.25	—	—	—	—
<i>Skeletonema costatum</i> NIES-323	1	—	0.10	—	0.05	0.25	—	—	—	—
Class Phaeophyceae										
<i>Sargassum thunbergii</i> Gunma*	(a)	0.10	0.37	0.18	0.03	0.01	—	—	—	—
<i>Sargassum fulvellum</i> Gunma*	(a)	—	1.40	0.34	0.01	0.01	—	—	0.01	0.01
<i>Acinetospora crinita</i> NIES-548	1	—	—	—	0.05	0.25	—	—	—	—
Class Xanthophyceae										
<i>Botrydiopsis arrhiza</i> NIES-621	1	—	—	—	0.35	0.20	—	0.08	—	—
<i>Botrydium granulatum</i> NIES-622	1	—	0.10	0.26	0.02	0.70	—	—	—	—
<i>Ophiocytium capitatum</i> NIES-1011	1	—	0.16	0.34	—	0.14	—	—	—	—
Class Eustigmatophyceae										
<i>Nannochloropsis oculata</i> IAM ST-4	(c)	—	0.65	—	—	1.32	—	—	—	—
<i>Nannochloropsis granulata</i> MBIC 10054	1	—	0.63	—	—	0.60	—	—	—	—
<i>Nannochloropsis oceanica</i> MBIC 10090	1	—	0.10	—	—	0.30	—	—	—	—
<i>Nannochloropsis salina</i> MBIC 10063	1	—	0.15	—	—	0.31	—	—	—	—
<i>Vischeria punctata</i> IAM X-4	(b)	0.02	0.26	0.62	0.13	0.95	—	—	0.03	—
<i>Vischeria stellata</i> IAM X-5	(b)	—	0.04	0.45	0.13	0.88	—	—	—	—
Class Pelagophyceae										
<i>Pelagomonas calceolata</i> NIES-1003	1	—	0.10	—	—	0.15	—	—	—	—
Class Pinguiphyceae										
<i>Glossomastix chrysolasta</i> NIES-1002	1	—	0.15	0.02	—	0.10	—	—	—	—
<i>Phaeomonas parva</i> MBIC 10503	1	—	—	—	—	0.10	—	—	—	—
<i>Pinguiochrysis pyriformis</i> MBIC 10872	1	—	0.10	—	—	0.22	—	—	—	—
<i>Polypodochrysis teissieri</i> MBIC 10541	1	—	0.10	—	—	0.30	—	—	—	—
Class Schizocladiphyceae										
<i>Schizocladia ischiensis</i> NIES-1044	1	—	—	0.15	—	0.17	—	—	—	—
Phylum Metamonada										
Superclass Eopharyngia										
Class Trepomonadea										
<i>Hexamita</i> sp. NIES-1440*	1	—	0.36	0.20	—	0.06	—	—	—	—
<i>Trepomonas</i> sp. NIES-1444*	1	—	0.07	0.07	—	0.03	—	—	—	—
<i>Giardia intestinalis</i> (formerly <i>G. lamblia</i>)	(f)	—	9.60	—	—	9.57	—	—	0.77	—
Superclass Parabasalia										
Class Trichomonadea										
<i>Trichomonas vaginalis</i> ATCC 30010	(d)	—	1.45	0.25	—	0.04	—	—	0.02	—
Wheat grain**										
(supplemented for the culture of <i>Bodo</i> and <i>Thaumatococcus</i>)										
Rice grain**										
(supplemented for the culture of <i>Hexamita</i> and <i>Trepomonas</i>)										
<i>Escherichia coli</i> IAM 12119***		—	0.10	0.75	—	0.17	—	—	—	—
(used for the culture of <i>Dimastigella</i> , <i>Cercomonas</i> and <i>Massisteria</i>)										

Dap, diaminopropane; Put, putrescine; Cad, cadaverine; NSpd, norspermidine; Spd, spermidine; HSpd, homospermidine; NSpm, norspermine; Spm, spermine; Agm, agmatine; N, number of pooled or individual samples; —, below the detection limit (0.005 $\mu\text{mol/g}$ wet weight of cells); IAM, IAM Culture Collection, Center for Bioinformatics, Institute of Molecular and Cellular Biosciences, The University of Tokyo, Tokyo, Japan; NIES, Microbial Culture Collection at the National Institute for Environmental Studies, Tsukuba, Japan; ATCC, American Type Culture Collection, Manassas, Virginia, USA; MBIC, Marine Biotechnology Institute Culture Collection, Kamaishi, Iwate, Japan; Gunma, isolated from ponds in Gunma Prefecture. (a) cited from Hamana and Matsuzaki (1982); (b) Hamana and Matsuzaki (1985); (c) Hamana et al. (2004b); (d) Hamana et al. (2006 a); (e) Hamana et al. (2004a); (f) Gillin et al. (1984) (nmol/mg protein). *Non-axenic unialgal (uniprotozoal) strain. Others were axenic unialgal (uniprotozoal) strain. ** Polyamines were extracted by PCA from 10 ml of the medium supplemented with wheat grains or rice grains. ***Polyamines were extracted by PCA from the packed heat-killed cells.

Thaumatomastix sp. were grown in URO (a synthetic medium) +wheat grains (NIES) and seaURO+wheat grains (NIES), respectively, at 15°C. *Dimastigella trypaniformis* was axenically cultured in Sonneborn's *Paramecium* Medium (ATCC 802 medium) supplemented with heat-killed *Escherichia coli* IAM 12119 cells (ATCC 1865 medium) at 20°C. *Cercomonas bodo* and *Massisteria marina* were grown with *E. coli* IAM 12119 in ATCC 802 medium and ATCC seawater 802 medium (ATCC 1526 medium), respectively, at 20°C. Phototrophic heterokonta were grown in the media, MES, AF-6 or Csi (NIES, MBIC) in the light, at 15–20°. Two marine monads, *Hexamita* sp. and *Trepomonas* sp., were cultivated in UYTS (URO+yeast extract, tryptone, horse serum)+rice grains (NIES) at 20°C.

Living organisms at the early stationary stage were harvested and then washed with Artificial seawater SP (Wako Chemicals, Tokyo) or PBS (Nissui Pharmaceutical Co., Tokyo) by centrifugation at $1,500 \times g$. Packed cells were homogenized in an equal volume of cold 1 M perchloric acid (HClO_4) (Hamana and Matsuzaki, 1982, 1985). Polyamines of the whole HClO_4 extract were analyzed by high-performance liquid chromatography (HPLC) on a column of cation-exchange resin in a Hitachi L6000 high-speed liquid chromatograph (Hamana et al., 1995). Cellular concentrations of polyamines estimated by HPLC are shown in Table 1. The algae used for polyamine analysis were harvested from a large (200–1,000 ml) or a few repeats of cultivation, and major polyamine components were determined without statistical analysis. In the present study, the concentrations under the lower limit (0.005 $\mu\text{mol/g}$ wet weight of alga) were defined as absence. The levels of about 0.01–0.02 were minor components. When organisms were cultivated in polyamine-containing media or with bacteria or seeds as a food, it was important to distinguish polyamines intracellularly synthesized from those incorporated from the media. The polyamine data on non-axenic unialgal strains cultured photosynthetically were useful, because contaminants were negligible under a microscopic observation; therefore, the polyamines derived from contaminants might be excluded in the algal polyamine samples. Algal samples collected from fields and the axenic cultures of the purified alga showed the same polyamine patterns (Hamana and Matsuzaki, 1982; Hamana et al., 1990). Furthermore, the same polyamine components and similar concentrations were observed in the two cultures using axenic strains of blue-green algae

and its non-axenic strains (Hosoya et al., 2005).

The phylum Euglenozoa comprises the three classes, Euglenidea (Euglenophyta, Euglenophyceae), Diplonemea and Kinetoplastea. Non-photosynthetic (osmotrophic) *Astasia longa*, heterotrophically grown in the dark, contained putrescine, cadaverine, norspermidine, spermidine and spermine, similar to the polyamine profiles of the photosynthetic euglenidea, *Euglena*, *Eutreptiella*, *Phacus* and *Trachelomonas* species. A colorless euglena, *A. longa*, has reduced chloroplasts. It has been demonstrated that the polyamine distribution profile of *Euglena gracilis* was not altered when the organism was cultivated phototrophically in the light and heterotrophically in the dark (Hamana et al., 2004b). These results suggest that the occurrence of both norspermidine and spermidine as the major triamines is ubiquitous within the class Euglenidea. The diamines, diaminopropane and putrescine, as the precursor for the triamines were widely spread within the class. Distribution of the tetra-amines, norspermine and spermine, which are produced from the two triamines was sporadic within the class, indicating their low cellular concentration levels.

Non-photosynthetic, achlorophyllous, colorless *Polytoma*, *Polytomella*, *Prototheca* and *Helicosporidium* species containing degenerated chloroplasts, belonging to the phylum Chlorophyta, as well as typical green algae, contained norspermidine (and norspermine) (Hamana et al., 2004a). A green unicellular paramecium, *Paramecium bursaria* belonging to the phylum Ciliophora and a multicellular green hydra, *Chlorohydra viridisima* belonging to the phylum Cnidaria, are carrying symbiotic *Chlorella* species (Chlorophyta), and contained norspermidine probably derived from the symbiotic chlorella (Hamana et al., 2004b, 2006b).

A diamine, cadaverine, which is produced from L-lysine by lysine decarboxylase but cannot be used for triamine and tetra-amine syntheses, was found sporadically in some algal species. The phylogenetic significance of the detection of cadaverine is not clear.

Diplonema ambulator belonging to the class Diplonemea, and *Bodo saltans* and *Dimastigella trypaniformis* belonging to the class Kinetoplastea, grown heterotrophically in the dark, contained putrescine and spermidine, but the three organisms lacked norspermidine and norspermine. Since spermine was produced in the trypanosoma cultures of six *Trypanosoma* species (Hamana et al., 2004b), the spermine detect-

ed in *B. saltans* seems to be synthesized in the cells. Although the synthetic ability of spermine is demonstrated in the classes Diplonemea and Kinetoplastea, there is no doubt that norspermidine and norspermine are not produced in the heterotrophic euglenozoa belonging to the two classes. It is suggested that the cellular occurrence of norspermidine and norspermine phylogenetically correlate with the presence of endosymbiotic plastids (chloroplasts) among the three classes of the phylum Euglenozoa.

Within the phylum Cercozoa, *Cercomonas bodo* and *Massisteria marina* belonging to the class Cercomonadida and *Thaumatomastix* sp. belonging to the class Thaumatomonadida contained putrescine, cadaverine and spermidine, and putrescine and spermidine, respectively. Norspermidine was not found in the three non-photosynthetic cercozoa. On the other hand, norspermidine was found as a major polyamine in the photosynthetic *Chlorarachnion* species containing secondary endosymbiotic plastids, belonging to another class, Chlorarachniophyceae (Chlorarachniophyta). It is speculated that presence of endosymbiotic plastids correlate to the synthetic ability of norspermidine (and norspermine) within the phylum Cercozoa. The polyamine profiles of *Chlorarachnion* are similar to those found in the class Euglenidea.

It has been known that phototrophic cyanobacteria, as a primary endosymbiotic prokaryote into primitive unicellular eukaryotes, contained spermidine and/or homospermidine as the major polyamines (Hamana et al., 1983; Hosoya et al., 2005). Norspermidine has never been detected in living cyanobacteria. Primary symbiotic processes seem to not correlate directly to the polyamine profiles found in cyanobacteria. Homospermidine was found in *Massisteria marina* (a cercomonas) alone in the present study. This triamine was found in several species of the phylum Rhodophyta (Hamana and Niitsu, 2006) but has never been detected in the algae belonging to other phyla.

Major species of the phylum Heterokonta (Heterokontophyta, Stramenopiles) are phototrophic and contain secondary or tertiary endosymbiotic plastids; however, colorless lineages have been known in the phylum. Polyamines of various photosynthetic heterokonts distributed in the nine classes of the phylum were analyzed in the present study. Putrescine, cadaverine and spermidine were major in the three species of the class Chrysophyceae. The class Raphidophyceae was poor in cellular polyamine components and

its cellular concentration levels. Although a large scale culture (1 L) was carried out and whole polyamines were extracted from the packed cells, spermidine alone was detected in all seven species of the class Raphidophyceae analyzed here. Within nine diatoms belonging to the class Bacillariophyceae and six algae belonging to the class Phaeophyceae or Xanthophyceae, norspermidine as well as spermidine was widely distributed. In the class Eustigmatophyceae, cadaverine and norspermidine were found in the two *Vischeria* species, but not detected in the four *Nannochloropsis* species. The occurrence of norspermidine was not found in the six species located in the class Pelagophyceae, Pinguicophyceae or Schizocladiophyceae. Heterogeneity on the distribution of cadaverine and norspermidine were observed among the nine classes of the phylum Heterokonta. These results suggest that the presence of norspermidine and norspermine in heterokonts does not relate to the photosynthetic ability of their endosymbiotic plastids. Polyamine analyses of colorless, non-photosynthetic heterokonts are expected.

In the phylum Metamonad, it has been known that amitochondrial parasitic *Trichomonas* (a parabasalid) of the superclass Parabasalia contained putrescine, cadaverine, spermidine and spermine and amitochondrial parasitic *Giardia* (a diplomonad) of the superclass Eopharyngia contained putrescine, spermidine and spermine. However, spermine was incorporated from culture media and back-converted to spermidine in these organisms (Gillin et al., 1984; Hamana et al., 2006a; Yarlett et al., 2000). As shown in this study, non-parasitic heterotrophic *Hexamita* (a trepomonad) and *Trepomonas* species (a trepomonad) belonging to the superclass Eopharyngia contained putrescine, cadaverine and spermidine. Norspermidine and norspermine, widespread in phototrophic lower eukaryotic algae, have never been detected in the phylum Metamonad, nor in the four unicellular heterotrophic phyla Entamoebidae, Percolozoa, Acanthamoebidae and Lobosea or the lower eukaryotes located in the phyla Mycetozoa and Microsporidia (Hamana and Niitsu, 2006; Hamana et al., 2006a).

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