

Polyamine distribution profiles in unicellular and multicellular red algae (phylum Rhodophyta) —Detection of 1,6-diaminohexane, aminobutylcadaverine, canavalmine and aminopropylcanavalmine—

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To consider the phylogenetic significance of cellular polyamine distribution profiles in multicellular red-algal evolution, polyamines procured through acid extraction from 27 additional red algae species (29 strains) including 18 species (19 strains) of marine multicellular species (seaweeds) were newly analyzed by HPLC and HPGC-MS, and compared with 21 previously analyzed red-algal polyamine profiles. In the unicellular thermoacidophilic order Cyanidiales, *Cyanidium* and *Cyanidioschyzon* contained putrescine, spermidine and spermine, and *Galdieria* contained norspermidine and norspermine in addition to the three polyamines. Freshwater/marine unicellular *Porphyridium* contained putrescine and spermidine, furthermore, spermine was found in some *Porphyridium* species. In the marine unicellular orders Dixonellales, Rhodellales and Stylonematales, the presence of putrescine, spermidine, norspermidine, spermine and norspermine was detected. Of the five polyamines, *Bulboplastis* containing thermospermine instead of spermine, has a novel algal polyamine profile. In the freshwater macroalgal orders Thorelales, Batrachospermales and Compsopongonales, the presence of putrescine, spermidine, homospermidine and spermine was abundant. In red seaweeds belonging to seven orders of the class Bangiophyceae or Florideophyceae, 1,3-diaminopropane, putrescine, cadaverine, spermidine, norspermidine, homospermidine, spermine, norspermine and thermospermine were widely distributed. 1,6-Diaminohexane was distributed within 12 species, aminobutylcadaverine was found in *Gelidium* and *Hynea*, and canavalmine and aminopropylcanavalmine were detected in *Meristotheca*, as unusual algal polyamines. The distribution of norspermidine and norspermine was particular in marine unicellular species. Homospermidine was a special polyamine member for freshwater multicellular species. 1,6-Diaminohexane and penta-amines were found in seaweeds in addition to common red-algal polyamines. The polyamine profiles of red seaweeds distinguished them from those of green seaweeds (ulvophytes) and brown seaweeds.

Key words: aminobutylcadaverine, aminopropylcanavalmine, canavalmine, diamino-hexane, polyamine, red algae, Rhodophyta

INTRODUCTION

As a contribution to advances in cellular polyamine biosynthetic diversity in the evolution of algae (Fuell *et al.*, 2010; Minguet *et al.*, 2008; Shaw *et al.*, 2010; Schweikert & Burritt, 2015), we have analyzed polyamines of various algae which evolved after primary, secondary and tertiary symbiotic pro-

cesses (Hamana, 2008; Hamana & Matsuzaki, 1982, 1985; Hamana & Niitsu, 2006; Hamana *et al.*, 1990, 2004a, 2004b, 2013, 2016b, 2017, 2018). The four algal phyla Glaucophyta, Rhodophyta, Chlorophyta and Streptophyta have plastid(s) as a result of the primary endosymbiosis of a phototrophic cyanobacterium, which evolved independently without secondary and tertiary endosymbiosis, and are located together in the Archaeplastida (unranked). Glaucophytes, such as *Cyanophora* and *Glaucocystis* species, evolved in a primitive unicellular form, which contained putrescine and spermidine alone as their cellular poly-

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amines (Hamana & Matsuzaki, 1985; Hamana & Niitsu, 2006). Unicellular green algae evolved into colony forming green algae and multicellular ulvophytes (green seaweeds) within the phylum Chlorophyta. Multicellular charophytes belonging to the phylum Streptophyta transited to the ancestor of early land plants. It has been shown that the distribution profiles of norspermidine, homospermidine, norspermine, spermine, thermospermine and pentaamines are significant within multicellular green algal evolution (Hamana *et al.*, 2004b, 2013, 2018).

Although the polyamines of 21 red algae located in the two subphyla Cyanidiophytina and Rhodophytina of the phylum Rhodophyta (Yoon *et al.*, 2006) (Table 1) have been analyzed, thermospermine and pentaamines have never been detected, however, one red seaweed was included in the previous studies (Hamana & Matsuzaki, 1982, 1985; Hamana & Niitsu, 2006; Hamana *et al.*, 1990), as cited in Table 1.

As shown in Table 1, three new freshwater unicellular thermoacidophilic red algae belonging to the order Cyanidiales (class Cyanidiophyceae) have been isolated in Japan and Italy. Four freshwater/marine unicellular red algae belonging to the order Porphyridiales (class Poryphyridiophyceae) and two new marine unicellular red algae belonging to the order Dixonellales, Rhodellales (class Rhodellophyceae) or Stylonematales (class Stylonematophyceae) have been collected in Japan. Two new freshwater multicellular (macro) red algae belonging to the order Thoreaales, Batrachospermales (class Florideophyceae) or Compsopogonales (class Compsopogonophyceae) have been identified as endangered red algae in Japan. On the other hand, many marine multicellular (macro) red algae belonging to the order Bangiales (class Bangiophyceae), Creamiales, Halymeniales, Gelidiales, Gracilariales or Gigartinales (class Florideophyceae) are considered edible seaweed in Japan.

Recently, the subphylum Rhodophytina was divided into the two subphyla Proteorhodophytina (consisting of the classes Poryphyridiophyceae, Compsopogonophyceae, Rhodellophyceae and Stylonematophyceae) and Eurhodophytina (consisting of the classes Bangiophyceae and Florideophyceae) (Muñoz-Gómez *et al.*, 2017) (Table 1).

To reveal the phylogenetic, morphologic and growth-related environmental significance of cellular polyamine profiles in red-algal evolution, and furthermore, to compare the polyamine profiles of red

seaweeds with those of green seaweeds and brown seaweeds, a polyamine analysis of an additional 29 red-algal samples including 19 red seaweeds (as shown in Table 1) was performed in the present study.

MATERIALS AND METHODS

The red algal strains supplied from the Microbial Culture Collection at the National Institute for Environmental Studies (MCC-NIES) and the Biological Resource Center, National Institute of Technology and Evaluation (NBRC) were cultivated phototrophically in the light (10–14 h/24 h) at 20–25°C (at 40°C for *Cyanidium* and *Galdieria*) using 1–10 l of liquid medium designed by MCC-NIES (<http://mcc.nies.go.jp>, 2017) and NBRC (<https://www.nite.go.jp/en/nbrc>, 2017). The cultures of the non-axenic unicellular *Porphyridium aeruginosum* NIES-1957 and *Bulboplastis apyrenoidosa* NIES-2742 contained some bacterial contaminants, and so the red algae were purified by sonication in an ultrasonic washing machine following differential centrifugation. The dry and wet red-algal seaweeds “Susabinori” (*Pyropia yezoensis*), “Narawa-susabinori” (*P. yezoensis* form. *narawaensis*), “Kaigara-amanori” (*P. teruipeddalis*), “Uppuruinori” (*P. pseudolinearis*), “Egonori” (*Campylaeophora hypnaeoides*), “Matsunori” (*Polyopes affinis*), “Makusa” (“Tengusa”) (*Gelidium elegans*), “Ogonori” (*Gracilaria vermiculophylla*), “Yumigata-ogonori” (*G. arcuata*), “Fukuro-funori” (*Gloiopeltis furcata*), “Ma-funori” (*G. tenax*), “Aka-tosakanori” (*Meristotheca papulosa*), “Akaba-ginnansou” (*Mazzaella japonica*), “Kotoji-tsunomata” (*Chondrus elates*), “Tsunomata” (*C. ocellatus*), “Ibaranori” (*Hypnea charoides*), “Isodantsu” (*Caulacanthus ustulatus*) and “Dulse” (*Palmaria palmata*) were purchased from food markets in the habitats in Japan or Korea (Table 1).

Wet red algae harvested at the stationary phase in our laboratory (1–10 g wet weight) and the red seaweeds rehydrated and washed by an ultrasonic washing machine (100–200 g wet weight), were homogenized in 5% perchloric acid (PCA). The PCA extract was subjected to a column containing a cation-exchange resin, Dowex 50WX8 (3 cm I.D. ×1 cm or 1 cm I.D. ×3 cm), to concentrate the polyamines. The polyamines were eluted with 6M HCl and then analyzed with by high-performance liquid chromatography (HPLC) on a Hitachi L6000 using a column

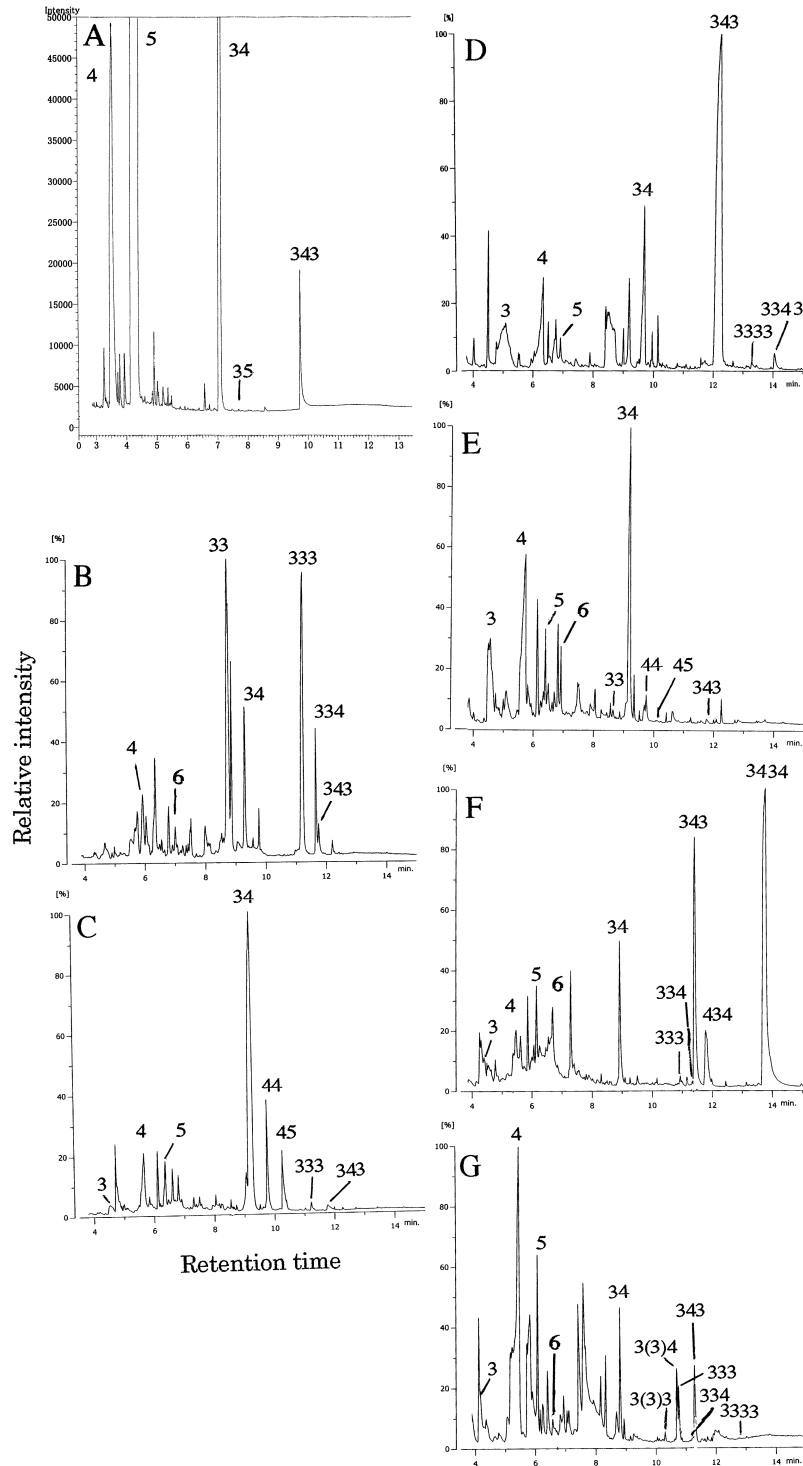


Fig. 1 HPGC analysis of the concentrated polyamine fraction of *Porphyridium purpureum* NIES-2138 “Chinorimo” grown in the presence of 1 mM cadaverine (A), *Bulboplastis apyrenoidosa* NIES-2742 No. 3 (B), *Gelidium crinale* “Makusa” (“Tengusa”) (Kumamoto) (C), *Gloiopeltis tenax* “Ma-funori” (D), *Hypnea charoides* “Ibaranori” (E), *Meristotheca papulosa* “Aka-tosakanori” No. 2 (F), and *Mazzaella japonica* “Akaba-ginnansou” No. 1 (G) on SHIMADZU GC-17A (A) or JEOL JMS-700 (B-G). Several non-polyamine peaks assigned by MS spectra were detected in the chromatograms. Abbreviations for polyamines are shown in Table 1.

of cation-exchange resin, Hitachi 2619F (=Hitachi 2720) (4 mm I.D. ×50 mm), using post-labeled fluorometry after heating with *o*-phthalaldehyde (Hamana *et al.*, 2016a, 2016b). After the heptafluorobutyrylation (HFB) of concentrated polyamine samples, HPGC on a SHIMADZU GC-17A and HPGC-mass spectrometry (HPGC-MS) on a JEOL JMS-700, equipped with a capillary column of Inert Cap IMS (0.32 mm I.D. ×30 m, df 0.25 μm) (GL Sciences) were performed at the column temperatures of 90°C–(16°C/min)–280°C and 120°C–(16°C/min)–280°C, respectively (Hamana *et al.*, 2016a, 2016b; Niitsu *et al.*, 2014, as shown in Fig. 1). Mass spectra of the HFB derivatives of all the polyamines detected in HPGC-MS were obtained for their identification (Hamana *et al.*, 2018). Molar concentrations of cellular polyamines per gram of wet weight of the starting algae were estimated from the HPLC analysis, and two HPGC analyses were performed using authentic polyamine standards and are shown in Table 1. Polyamine extraction from seaweeds was quantitatively difficult, so the concentration values were unstable. Previous polyamine data for 21 red algae analyzed with standard GC with a packed column in 1982–2006 are cited in Table 1.

RESULTS AND DISCUSSION

Polyamines of thermoacidophilic unicellular red algae

Thermoacidophilic unicellular red algae (order Cyanidiales) constitute three genera *Cyanidium*, *Cyanidioschyzon* and *Galdieria* and are unique with a green colored red alga, single or few mitochondria and chloroplasts, and optimum growth at 40–50°C and pH 3–4 (Inouye, 2007; Miyagishima *et al.*, 2017). Polyamines of a new strain of *Cyanidium caldarium* “Ideyukogome” and a new species of *Galdieria*, *G. partita*, were analyzed in the present study. Four strains of *Cyanidium caldarium* and a strain of *Cyanidioschyzon melorae* “Shianidioshizon” always contained putrescine (4), spermidine (34) and spermine (343) (Table 1) (Hamana & Niitsu, 2006; Hamana *et al.*, 1990). *G. partita* as well as *G. sulphuraria* contained norspermidine (33) and norspermine (333) in addition to putrescine, spermidine and spermine (Table 1) (Hamana & Matsuzaki, 1985; Hamana *et al.*, 1990). A small amount of thermospermine (334) in addition to spermine was possible in the *Galdieria* species containing norspermine as a major polyamine. *Galdieria* belongs to a lineage far from that

of *Cyanidium* and *Cyanidioschyzon* in the order Cyanidiales (Yoon *et al.*, 2006). The higher levels of spermine, a tetra-amine, in the unicellular thermoacidophiles grown at 40–50°C compared with other unicellular red algae grown at 20–25°C seems to be related to the high optimum growth temperature of the thermoacidophiles (Hamana *et al.*, 1990). The acidophilic property of the thermoacidophiles appears to be unaffected in the cellular polyamine profiles.

On the other hand, spermine has not been found in unicellular glaucophyte algae (phylum Glaucophyta) in which putrescine and spermidine were found as the polyamine components (Hamana & Niitsu, 2006), and in the primitive unicellular green algae belonging to the class Prasinophyceae of the phylum Chlorophyta in which norspermidine and homospermidine (44) in addition to putrescine and spermidine were the major polyamines (Hamana *et al.*, 2013, 2018). Three different polyamine synthetic abilities were distributed independently in the early evolution of the three primitive unicellular algal lineages (phyla) after the primary endosymbiosis.

Polyamines of the freshwater/marine unicellular red alga *Porphyridium*

Salt tolerant *P. sordidum*, *P. purpureum* and *Porphyridium* sp. NIES-1032 contained putrescine (4), spermidine (34) and spermine (343). The freshwater *P. aerugineum* analyzed in the present study contained putrescine and spermidine but lacked in spermine. The spermine level varied within the salt tolerant *Porphyridium* species, however, the relation between the level and their salt tolerance is unclear. When *P. purpureum* NIES-2138 and *P. aerugineum* NIES-1957 were cultured in the presence of 1 mM cadaverine in the medium, a small amount of aminopropylcadaverine (35) was detected in the former but not in the latter, indicating that there was aminopropylation to the supplied cadaverine (5). However, this was at very low levels in the red algae. Aminobutylcadaverine (45) was not produced from the supplemented cadaverine.

In the order Porphyridiales containing a single chloroplast, although *Porphyridium purpureum* (formerly *P. cruentum*) “Chinorimo” NIES-2138 and NIES-2140 were isolated from a terrestrial hot spring in Kagoshima, Japan and land soil in the Tohoku region, Japan, respectively, they showed salt tolerance and good growth in a marine algal medi-

um (NIES-ESM medium) that was also used for marine *P. sordidum* MBIC 10454 and *Porphyridium* sp. NIES-1032 isolated in the Pacific Ocean, Okinawa, Japan. *Porphyridium aerugineum* NIES-1957 was isolated from a land soil in Nepal and is a blue-green colored red alga likely related to the thermoacidophilic red algae belonging to the order Cyanidiales (Gantt *et al.*, 1968), and it thrives in a freshwater algal medium (NIES-C medium). These findings suggest that the marine species are more primitive phylogenetically than the freshwater species within the genus *Porphyridium*.

Polyamines of marine unicellular red algae

Marine unicellular red algae are located in the orders Dixoniales (class Rhodellophyceae), Rhodellales (class Rhodellophyceae) and Stylonematales (class Stylonematophyceae). Norspermidine (33) (and norspermine (333)) was detected in six marine species belonging to the three orders, and their polyamine profiles were distinguished from those of *Porphyridium* species (order Prophyridiales of class Porphyridiophyceae). Newly analyzed *Corynoplatis japonica* (Yokoyama *et al.*, 2009) contained norspermidine as a major polyamine. Although few analytical data were obtained, the same distribution profile of polyamines was suggested within the classes Rhodellophyceae and Stylonematophyceae.

Brownish *Bulboplastis apyrenoidosa* NIES-2742 containing a single chloroplast was isolated from the mangroves of Iriomote Isl., Okinawa, Japan (Kushibiki *et al.*, 2012) and contained a high level of norspermidine, spermidine (34), norspermine and thermospermine (334), and a low levels of spermine (343), indicating its preferential aminopropylation to the aminopropyl moieties of norspermidine and spermidine to produce norspermine and thermospermine, respectively, in the red alga. The same polyamine profile was obtained in acid extracts from the three different cultures No. 1, 2 and 3 (Table 1). This polyamine profile containing of thermospermine as a major polyamine is unique among all previously analyzed algae. 1,6-Diaminohexane was detected as a minor polyamine in two of the three *Bulboplastis* samples.

Polyamines of freshwater macro red algae

The freshwater multicellular red algae “Chisuzunori”, “Kawamozuku” and “Ohishisou” that grow in rivers

or lakes in Japan are known to be endangered. The 10 species of this group, belonging to two orders of the class Florideophyceae and an order of the class Compsopogonophyceae (Table 1), were analyzed in both previous studies and present work. They ubiquitously contained homospermidine (44) in addition to putrescine (4), spermidine (34) and spermine (343). Norspermidine (33) was found in four of the 10 species, whereas norspermine (333) was not found in any of the species analyzed. The ubiquitous occurrence of homospermidine in multicellular red algae suggests that homospermidine synthetic abilities have been coupled with multicellular evolution within the freshwater macro red algae.

Unusual polyamines of red seaweeds

Although the macro red-algal seaweeds purchased from food markets were washed in artificial seawater by sonication, the seaweeds were contaminated by other marine microorganisms. However, the unusual polyamines found in the seaweeds have never been detected in various marine bacteria. 1,6-Diaminohexane (6) was widely distributed within the red seaweeds. Although its biosynthetic pathway is unknown, this diamine is also widely distributed in brown seaweeds (Hamana *et al.*, 2017) but not in green seaweeds (Hamana *et al.*, 2018). On the other hand, thermospermine (334) is found in many freshwater multicellular green algae, green seaweeds and brown seaweeds as a major polyamine, and is rich in the thallic species (Hamana *et al.*, 2017, 2018). Thermospermine was detected sporadically in the red seaweeds as a minor polyamine.

Since *Gelidium elegans* “Makusa” (“Tengusa”) is rich in agar, acid extraction of the whole polyamine fraction from the alga and concentration of polyamines on ion-exchange resin were difficult to achieve, so the polyamines were analyzed in only one sample of four polyamine extractions from the Kumamoto sample. Polyamine analysis of the Shizuoka sample was performed for only one sample among three polyamine extractions. Aminobutylcadaverine (45) was found in the Kumamoto sample in which homospermidine (44) was at a high level, but not detected in the Shizuoka sample in which homospermidine was at a low level. *Hypnea charoides* “Ibaranori” contained aminobutylcadaverine (45) as a minor polyamine. *Campylaeophora hypnaeoides* “Egonori”, *Gloiopeltis furcata* “Fukuro-funori” and *Mazzaella japonica* “Akabaginnansou” were rich in cadaverine (5) but poor in

homospermidine (44), however, aminobutylcadaverine was not detected in them. Aminobutylcadaverine has been found in a unicellular green alga, *Westella* sp. DBT, however, the alga was rich in cadaverine and homospermidine (Hamana *et al.*, 2018).

Two tertiary branched triamines N^4 -aminopropyl norspermidine (3(3)3) and N^4 -aminopropyl spermidine (3(3)4) were detected in *Mazaella japonica* (No. 1) as a minor polyamine. Although the two branched triamines were not detected in the red-algal sample No. 2 and its detection was not replicable, the triamines have also been found in a green alga, *Chlorogonium complex* (Hamana *et al.*, 2018).

Canavalmine (434) has been detected in some green algae as a minor polyamine (Hamana *et al.*, 2013, 2018). Both aminopropyl canavalmine (3434) and canavalmine were found as major polyamines in two samples of *Meristotheca papulosa* “Aka-tosakanori”. Although canavalmine and the aminopropylated canavalmine, aminopropyl canavalmine, have been found in some leguminous seeds (Hamana *et al.*, 1992; Matsuzaki *et al.*, 1990) and sea cucumber (Hamana *et al.*, 1991), this is the first known report of a major occurrence of canavalmine and aminopropyl canavalmine in algae.

Caldopentamine (3333) and thermopentamine (3343), probably produced from norspermine and spermine, respectively, were detected in some red seaweeds, but homocaldopentamine (3334), which is produced from thermospermine, was not detected in the red seaweeds. The three penta-amines caldopentamine, thermopentamine and homocaldopentamine have been found in green and brown seaweeds containing norspermine, spermine, thermospermine and canavalmine, whereas aminopropyl canavalmine has not been detected (Hamana *et al.*, 2017, 2018).

Brown seaweeds belonging to the class Phaeophyceae of the phylum Heterokontophyta, evolved after the secondary symbiosis of a red alga within stramenopiles, and are unique among heterokontophytes in developing into multicellular forms with differentiated tissues. The distribution of the long linear diamine, 1,6-diaminohexane, penta-amines and hexa-amines has been observed in 12 typical brown seaweed species (Hamana *et al.*, 2017). On the other hand, the macro green algae belonging to the classes Ulvophyceae and Charophyceae were not present in 1,6-diaminohexane and hexa-amines,

which contained small amounts of penta-amine (Hamana *et al.*, 2018). As shown in the present study, 1,6-diaminohexane was found in the most of red seaweeds except for *Meristotheca papulosa* “Aka-tosakanori”, but no significant amounts of penta-amine and hexa-amine were detected. The polyamine profiles of red seaweeds resemble to those of green seaweeds more closely than those of brown seaweeds.

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単細胞および多細胞の紅藻類（紅色植物門）のポリアミン構成
— 1,6-ジアミノヘキサン，アミノブチルカダベリン，カナバルミン，アミノプロピルカナバルミンの検出 —

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細胞内ポリアミン構成と多細胞紅藻類の系統進化との関連性を調べるため，大型多細胞の海藻 18 種（19 株）を含む 27 種（29 株）の紅藻類から酸抽出したポリアミンを HPLC と HPGC-MS にて追加分析し，既分析の紅藻 21 種のポリアミン構成と比較した．好熱好酸性単細胞の Cyanidiales 目では，*Cyanidium* と *Cyanidioschyzon* がプトレスシン，スベルミジン，スベルミンを含有し，*Galdieria* は，これらに加えて，ノルスベルミジンとノルスベルミンを有していた．単細胞の淡水産および海産 *Poryphyridium* では，プトレスシンとスベルミジンで，種によりスベルミンを含有していた．海産単細胞性の Dixoniellales 目，Rhodellales 目，Stylonematales 目では，プトレスシン，スベルミジン，スベルミンに，ノルスベルミジンとノルスベルミンが加わった．*Bulboplastis* はこの 5 ポリアミンの内のスベルミンに代わってサーモスベルミンを主要ポリアミンの一つとして含む特徴があった．淡水産多細胞 Thoreales 目，Batrachospermales 目，Compsopogonales 目では，プトレスシン，スベルミジン，スベルミンにホモスベルミジンが追加される種が優勢であった．Bangiophyceae 綱と Florideophyceae 綱の 7 目に属する海藻では，1,3-ジアミノプロパン，プトレスシン，カダベリン，スベルミジン，スベルミン，ノルスベルミジン，ノルスベルミン，ホモスベルミジン，サーモスベルミンが広く分布していた．1,6-ジアミノヘキサンが海藻 12 種に検出され，アミノブチルカダベリンが *Gelidium* と *Hypnea* に，カナバルミンとアミノプロピルカナバルミンが *Meristotheca* に検出された．これら紅藻のポリアミン構成は系統進化的には異なる緑藻（アオサ藻）や褐藻のポリアミン構成とは異なっていた．