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Comparative approaches to the taxonomy of the genus *Galdieria* Merola (Cyanidiales, Rhodophyta)

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Abstract – Ecophysiological, morphological, ultrastructural, and phylogenetic methods were used to study nine strains of *Galdieria* isolated from different geographical regions in order to clarify the taxonomy and evolution of the genus and to understand the phylogeny of the Cyanidiales. The studied characters were often conserved in all of the isolates. Confocal laser scanning microscopy (CLSM) observations confirmed the presence of a single plastid and allowed its 3-D reconstruction. Phylogenetic analysis showed that all *Galdieria* isolates but one (*G. maxima*) form a strongly supported monophyletic lineage within the Cyanidiales. *G. maxima* is closely related to *Cyanidioschyzon merolae* and its taxonomic status needs to be revised. The Cyanidiales is an evolutionarily distinct group within the Rhodophyta. In spite of the uniform morphology among *Galdieria* strains, analysis of *rbc*L sequences showed moderate to high levels of sequence divergence within this genus. Apart from *G. maxima*, which is from Kunashir, Russia, the two other Russian isolates, *G. daedala* and *G. partita* grouped together with the remaining *Galdieria* taxa forming three separate clades. The *Galdieria* clades reflected the geographic site of origin of the isolates. On the basis of these data, we suggest that *Galdieria* (excluding *G. maxima*) represents a species complex. The limited sampling makes it difficult, however, to recommend changes to the existing classification system. We suggest that a broad and systematic study of *Galdieria* is required to understand fully the evolutionary history and taxonomy of this genus.

Cyanidiales / *Galdieria* / *rbc*L sequence data / taxonomy / thermoacidic environments / unicellular red algae

Résumé — Approches comparatives de la taxinomie du genre *Galdieria* Merola (Cyanidiales, Rhodophyta). Des techniques écophysiologique, morphologique, ultrastructurale, et phylogénétique ont été utilisées pour étudier neuf souches de *Galdieria* originaires de différentes régions géographiques afin de clarifier la taxinomie et l'évolution du genre, et de comprendre la phylogénie des Cyanidiales. Les caractères étudiés sont souvent conservés chez tous les isolats. Les observations effectuées avec un microscope à balayage laser confocal (CLSM) confirment la présence d'un seul plaste et permettent la représentation en 3-D.

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L'analyse phylogénétique montre que tous les isolats de *Galdieria* sauf un (*G. maxima*) forment, au sein des Cyanidiales, une lignée monophylétique fortement soutenue. *G. maxima* est étroitement lié à *Cyanidioschyzon merolae* et sa position taxinomique doit être révisée. Les Cyanidiales constituent un groupe évolutif distinct à l'intérieur des Rhodophyta. En dépit de la morphologie uniforme des souches de *Galdieria*, l'analyse des séquences du gène *rbc*L montre que celles-ci présentent des divergences moyennes à fortes, à l'intérieur du genre. *G. maxima*, provenant de Kunashir, Russie, n'appartient pas à l'ensemble formé par les autres *Galdieria* ; dans cet ensemble, les deux autres isolats de Russie, *G. daedala* et *G. partita* se regoupent et les taxa restants de *Galdieria* forment trois clades séparés. Les clades de *Galdieria* reflètent l'origine géographique des isolats. Sur la base des ces résultats, nous proposons que *Galdieria* (sauf *G. maxima*) représente un complexe d'espèces. Cependant, l'échantillonage limité ne permet pas de recommander des changements dans le système de classification actuel. Nous suggérons qu'une large étude systématique soit entreprise afin de comprendre pleinement l'évolution et la taxinomie de ce genre.

algues rouges unicellulaires / Cyanidiales / *Galdieria* / séquences du gène *rbc*L / taxinomie / environnements thermoacides

INTRODUCTION

Galdieria Merola is a genus that includes unicellular red algae occurring worldwide in thermoacidic environments (Seckbach, 1994, 1999). This taxon was separated from the related genus *Cyanidium* Geitler primarily on the basis of morphological differences such as cell size, number of endospores, presence of one or several mitochondria, and presence of a vacuole (Merola *et al.*, 1981). *Galdieria* was thought to be monospecific and its single species *G. sulphuraria* (Galdieri) Merola broadly distributed until Sentsova (1991) recognized three new species, *G. partita*, *G. daedala*, and *G. maxima*. The latter two species have only been found in acidic sites in Kunashir Island (Russia). Two strains, both ascribed to *G. partita*, have also been isolated from acidic soils of Kamchatka Peninsula (Russia) and one from Yellowstone Park (USA, Sentsova, 1991). Since the erection of these three species, several attempts have been made to clarify the relationship between *Cyanidium* and *Galdieria* (Ott & Seckbach, 1994; Gross, 1999; Albertano *et al.*, 2000; Cozzolino *et al.*, 2000). In a systematic revision of acidophilic red algae, Ott & Seckbach (1994) retained the four species of *Galdieria* but suggested that they belong to the genus *Cyanidium*. Alternatively, Albertano *et al.* (2000) have taken a more conservative approach, retaining the genus *Galdieria* after amending the diagnosis and confirming all the previously described species.

The four species of *Galdieria* are not easily distinguishable because of intra- and interspecific variation in the critical morphological features used for their identification. Until now, the key characters include cell size, endospore number and, primarily, the shape and number of plastids. According to the original descriptions, *G. sulphuraria* has a single, parietal, cup-like plastid (Merola *et al.*, 1981), *G. partita* has a single to several parietal or lobed plastids in mature and old cells, *G. daedala* has one to few multilobed plastids, and *G. maxima* possesses two or more parietal, lobed to oval plastids (Sentsova, 1991). However, the morphological description of the *Galdieria* plastid is based on light microscopy and, although some ultrastructural data have been reported for *G. sulphuraria*, a detailed comparative study of plastid cytomorphology or a three-dimensional

(3D) reconstruction has never been attempted in order to confirm the relevance of this character as a diagnostic feature at the species level.

Karyological studies of Cyanidiales show a chromosome number of 2 for the genus *Galdieria*, which distinguishes these taxa from *C. caldarium* (Tilden) Geitler (n = 5, Muravenko *et al.*, 2001). In the last decade, molecular approaches have been used successfully to understand phylogenetic relationships in the red algae (Freshwater *et al.*, 1994; Ohta, 1997; Oliveira *et al.*, 2000). Recently, the taxonomy of *Galdieria* and related genera was evaluated on the basis of *rbc*L gene sequence data (Cozzolino *et al.*, 2000). These data show *Galdieria* to be distinct from *Cyanidium* and to be divided in two clades, one including *Galdieria* strains from Italy and the U.S.A. (strains from California and Yellowstone Park), and the other containing the Russian isolates, along with a strain of *G. sulphuraria* from Mount Lawu (Java, Indonesia). The phylogenetic analysis of *rbc*L in Müller *et al.*, (2001) showed *Cyanidium*, *Galdieria*, and *Cyanidioschyzon* to form a strongly supported monophyletic lineage within the red algae and supported a long evolutionary separation of the Cyanidiales from other red algae. This result has been robustly supported in a recent plastid 5-gene phylogenetic analysis of the major red algal orders (Yoon *et al.,* 2002).

The goal of this study was to use various methodological approaches with *Galdieria* strains collected from acidic sites of North America, Asia, and Europe and, in particular, with the four strains used to describe the species of *Galdieria* and three isolates from Yellowstone National Park, California, and Java. These taxa were studied to provide a more detailed understanding the genus *Galdieria*. Comparative studies have been carried out on the following sets of characters: (i) ecophysiological response to pH, salt stress and ability of growth on glucose, (ii) cell size and morphology, size and number of endospores, (iii) 3D morphology of the plastid, (iv) cell ultrastructure. We sequenced plastid *rbc*L from representative members of the Cyanidiales to infer their evolutionary relationships.

MATERIAL AND METHODS

The algal strains utilized were *Galdieria sulphuraria* (Galdieri) Merola strain 002 (authentic strain, Naples, Italy), 009 (Viterbo, Italy), 063 (Agrigento, Italy), and 074 (mount Lawu, Java, Indonesia) from the algal collection of the Dipartimento di Biologia vegetale (Naples, Italy) (Pinto *et al.*, 1992), *Galdieria partita* Sentsova strain IPPAS P500 (authentic strain, Kamtchatka, Russia), *Galdieria daedala* Sentsova strain IPPAS P508 (authentic strain, Kunashir, Russia), and *Galdieria maxima* Sentsova strain IPPAS P507, (authentic strain, Kunashir, Russia) from the Culture Collection of Microalgae of the Institute of Plant Physiology, Saint Petersburg (Russia), *Galdieria sulphuraria* strain 107-79 (= CCAP 1355/1, Lemonade springs, California, U.S.A.) and *Galdieria sulphuraria* strain 108-79 (Yellowstone National Park, U.S.A.) from the Sammlung von Algenkulturen, Pflanzenphysiologisches Institut der Universität Göttingen (SAG, Schlösser, 1994). Other members of the Cyanidiales that were included in the phylogenetic analyses are *Cyanidioschyzon merolae* (strains 201 Java and 001 Naples) and *Cyanidium caldarium* RK1 (Glockner *et al.*, 2000). The algae were grown in Erlenmeyer flasks (1000 ml) containing 500 ml of modified Allen medium with $(NH_4)2SO_4$ 0.25 gL^{-1} as a nitrogen source (Allen, 1968) and the pH was adjusted

to 1.5 by adding H_2SO_4. The cultures, bubbled by air, were maintained in a temperature-controlled room at 37°C on a plexiglass shaking apparatus (Shihira & Krauss, 1965) under a photon irradiance of 150 mmol photons m^{-2} s^{-1} with continuous light provided by cool-light fluorescent lamps (Philips TLD30w/55). Cell density of the algal cultures was assessed at 550 nm with a Bausch & Lomb Spectronic 20 colorimeter.

Ecophysiological tests

All the tests were carried out in 100 ml Erlenmeyer flasks containing 50 ml of modified Allen medium at pH 1.5, except for the different pH tests, which were done at the same culture conditions as specified above. For the mixotrophic and heterotrophic growth tests, glucose (2.5 mM) was added to the medium. For the salt tolerance tests, the algae were grown at different concentrations of NaCl (2, 4, 6, 8 and 10%). For the tests dealing with the pH limits for growth, H_2SO_4 was added at different concentrations in modified Allen medium to obtain final pH values of 0, 0.5, 0.7, 1.5, 3.0, 5.0, 6.0, and 7.0. During the experiments, the pH value was monitored daily in each flask. For growth tests on nitrate as the sole source of N, algal cultures, previously centrifuged (5000 rpm x 10 min) and washed twice with nitrogen-free medium, were inoculated in Allen medium containing 3 mM $NaNO_3$. In all the experiments, a control for each *Galdieria* strain containing only modified Allen medium at pH 1.5 was also tested. After sterilisation by autoclaving, the flasks were inoculated with several drops of enrichment cultures in exponential growth phase to have a cell density of 0.003 units (corresponding to 100.000 cells ml^{-1}). Growth in each flask was followed daily by measuring the cell density (as previously described). Ecophysiological tests were carried out in triplicate for each strain and were repeated three times. Specific growth rates were calculated for each individual flask by linear regression of logarithmic cell density data obtained during the experiments. The results were evaluated on the basis of the average of three tests and the relative standard error was never higher than 5%.

Morphological observations

Algal samples of each *Galdieria* species were observed with a Leitz microscope equipped with Nomarski interference optics and the number of endospores and the size of 100 cells of each strain were measured with a micrometer eyepiece. The observations were made at different stages of the life cycle, either on cells in full exponential phase, or on cells in late stationary phase of growth. In order to detect differences between the shape and number of plastids of the different strains of *Galdieria*, observations were made using a Zeiss CLSM 410 Axiovert (Zeiss Microscopy facility at Stazione Zoologica A. Dohrn, Naples). For each strain, 20 cells ranging from the smallest to the largest size previously recorded were observed to study the different morphology of the plastid at an early and mature stage. Images were obtained with 63 × 1.2 NA and with Zeiss CLSM software. Plastids were examined with a He/Ne laser at 488 nm; stacks of digital images were obtained for each sample along Z-axis, each 0.3 mm. Acquired images were used in 3D reconstruction with the Metaview software of Crisel Instruments (Rome). The images were deblurred using the blind deconvolution algorithm of AutoDeblur (Crisel Instruments) by selecting the power acceleration method and 20 iterations. This method enhances sharpness and clarity in 3D data by compensating computationally for out-of-focus haze and blur (Holmes *et al.*, 1995). Then, using the AutoVisualize 3D software (Crisel Instruments), different projections (e.g., maximum and sum projection) were obtained for each sample and a rotated projection was performed in order to show particular features.

Transmission electron microscopy

As widely known in the literature, the Cyanidiales are very difficult to observe with TEM. Their thick proteinaceous cell wall retards good fixation and the final result is generally not of the quality found when studying other red algae. That being stated, algae from cultures in late exponential phase of growth were harvested by centrifugation (5000 rpm × 10 min). The cells were previously treated with 0.1% (w/v) pepsin (3 h at 4 °C) and then fixed in a mixture of 4% glutaraldehyde + 2% formaldehyde, freshly prepared from paraformaldehyde, in 0.05 M Na-phosphate buffer (PB) pH 7.2 for 15 minutes at 45 °C. The algae were then harvested after centrifugation (5000 rpm × 5 min), washed in PB and resuspended in 1% osmium tetroxide (aqueous solution) overnight at 4 °C. After centrifugation, cells were rinsed with distilled water for 30 min and suspended in 2% agar. Small blocks of agar-suspended algae were dehydrated with an ethanol series and embedded in Spurr's resin. Ultrathin sections were cut with a diamond knife, sequentially stained with 2% uranyl acetate for 10 min and 1.33% Pb citrate for 10 min and observed with a Philips CM12 electron microscope at 50 kV.

Phylogenetic studies

Cells of each *Galdieria* strain were harvested by centrifugation (6000 rpm × 5 min) and total DNA was extracted from liquid cultures following the procedure described in Doyle & Doyle (1987), with slight modifications (*i.e.*, a higher cell lysis temperature was used, 75 °C). The *rbc*L coding region was amplified using PCR and the following primers: RBCL1For: 5'-ATGTCTCAATCAATAGAAGAAAAATC-3', RBCL2Rev: 5'-TAGTACCGCAGCAGGATCTATAACA-3'. Internal primers were used to complete both strands of the sequence from each taxon: ELRev: 5'-AACAACTGTCCAT GTAGC-3', RBCL1Rev: 5'-AGCACGTGCATACATTTCTTCCATAG-3', RubX:5'-ATTT GGTAGACCTTTACTTGGTTGTA-3', RubY: 5'-TAGCACCAGCTTGTATTCCATCTGG-3', RBCL2For: 5'-GGTGGTATTCATGCAGGACAAATGCAT-3'. PCR amplifications were done for 30 cycles in a Perkin-Elmer Cetus 9600 thermocycler using the following conditions: 1 min denaturation at 94 °C, 1 min annealing at 55 °C, and 45 sec extension at 72 °C. Samples were denatured for 5 min at 94 °C before the beginning of the first cycle. The extension time was increased to 3 sec/cycle and the extension time was extended to 7 min at the end of the last cycle. PCR fragments, purified using Microcon 100 microconcentrators (Amicon, Denvers, MA, U.S.A.), were ligated into pUC18 (Pharmacia Biotech, Uppsala, Sweden) and then seven clones, for each taxa, were sequenced by using universal M13 primers and a modification of the Sanger dideoxy method (Sanger *et al.*, 1977) implemented in a double strand DNA cycle sequencing system with fluorescent dyes. Sequence reactions were then loaded into a 373A Applied Biosystems Automated DNA sequencer Applied Biosystems, Foster City, CA, U.S.A.). The clones' sequences were aligned in order to obtain a consensus sequence for each strain.

The *rbc*L coding regions of the Cyanidiales were aligned with the homologous sequence in the red algae and proteobacterial outgroups (Delwiche & Palmer, 1996). All orders of the red algae, except the Erythropeltidales, were sampled to assess the phylogenetic position of the Cyanidiales. A minimum evolution (ME) tree was built using LogDet distances with random stepwise addition of taxa (10 rounds) and rearranged with the tree bisection-reconnection (TBR) branch-swapping algorithm (PAUP*; Swofford, 2002). Bootstrap support for nodes in the tree was calculated using the ME-LogDet analysis (2000 replications, each with 10 rounds of taxon addition). We also did a ME analysis using GTR + Γ + I

distances. The parameter estimates for this model were estimated using PAUP and a starting ME tree built with HKY-85 distances. Ten heuristic searches with random-addition-sequence starting trees and TBR branch swapping were used to find the optimal trees. To test the stability of monophyletic groups in the ME-GTR trees, 2,000 bootstrap replicates were analyzed with the DNA data set. Bayesian inference with Metropolis-coupled Markov chain Monte Carlo (MCMCMC) sampling of trees was also used with the *rbc*L amino acid data (JTT + Γ model) to calculate posterior probabilities for different groups (Huelsenbeck & Ronquist, 2001). A total of 500,000 MCMCMC generations were generated in each of five different runs with random starting trees. These runs resulted in essentially identical consensus trees.

RESULTS

Ecophysiological data

As shown in Tab. 1, the tolerance to low or high pH was almost identical in all the algae tested. All the strains tolerated pH levels ranging from 0.5 to 6.0. Only the strains P500 (*G. partita*) and P507 (*G. maxima*) had an upper growth limit at pH 5.0. Salt tolerance was also similar among the strains tested. Some grew up to 4 % (strains 074, 107-79 and 108-79) or at 5 % NaCl (strains P500, P507 and P508). The only exception was *G. sulphuraria*, strain 002 (Naples), that was able to grow at 8 % NaCl and survived to 10 % NaCl. All the isolates could grow heterotrophically and mixotrophically on glucose. When they were grown on glucose in the light and dark, the plastids did not lose their chlorophyll (except strain 074 that bleached in the dark). *G. sulphuraria* strain 002 was the only strain not able to utilize NO_3 as a nitrogen source; all the other *Galdieria* strains grew either using ammonium or nitrate.

Table 1. Ecophysiological characters of *Galdieria* strains tested at 37 °C. Heterotrophic, mixotrophic and NO_3 tests were conducted at pH 1.5

Species	*Strain*	*Growth limits*[a]			*Heterotrophy*[b]	*Mixotrophy*[c]	*NO_3 Utilization*[d]
		pH		*NaCl*			
		low	high	(%)			
G. sulphuraria	002	0.5	6.0	10	+ green	+	–
G. sulphuraria	074	0.5	6.0	4	+ white	+	+
G. sulphuraria	107-79	0.5	6.0	4	+ green	+	+
G. sulphuraria	108-79	0.5	6.0	4	+ green	+	+
G. daedala	P508	0.5	6.0	5	+ green	+	+
G. partita	P500	0.5	5.0	5	+ green	+	+
G. maxima	P507	0.5	5.0	5	+ green	+	+

[a] The lowest or the highest pH at which the alga still grows after 30 days in laboratory tests; the highest NaCl concentration at which the alga still grows after 30 days in laboratory tests.

[b,c] Algal growth (+) in Allen medium added with 2.5 mM of glucose in dark or light conditions respectively; white or green indicate if the cells bleached or not.

[d] Algal growth (+) in ammonium free Allen medium added with $NaNO_3$ (3mM).

Table 2. Cell diameter (μm) and number of endospores of seven strains of *Galdieria*. Sizes of vegetative cells (V) and sporangia (S) were measured during both the exponential (Ex) and stationary (St) phases of growth.

Species	*Strain*	*Ex*		*St*		*Endospore number*
		V	*S*	*V*	*S*	
G. sulphuraria	002	2.5-8.0	5.0-10.5	3.5-8.0	6.0-10.5	2-4-8-16
G. sulphuraria	074	4.5-14.0	8.0-16.5	4.0-18	7.0-18.5	2-4-8-16
G. sulphuraria	107-79	4.0-9.0	6.0-9.5	4.0-9.5	6.5-12.0	2-4-8
G. sulphuraria	108-79	4.5-13.0	7.0-15.0	4.0-14.5	6.5-16.0	2-4-8
G. daedala	P508	2.5-9.0	6.0-9.5	2.5-9.5	6.0-13.0	2-4-8-16
G. partita	P500	2.5-8.0	6.5-9.5	2.5-9.5	6.0-10.5	2-4-8
G. maxima	P507	6.0-14.0	6.0-14.0	7.0-14.0	10.5-16.5	2-4-8

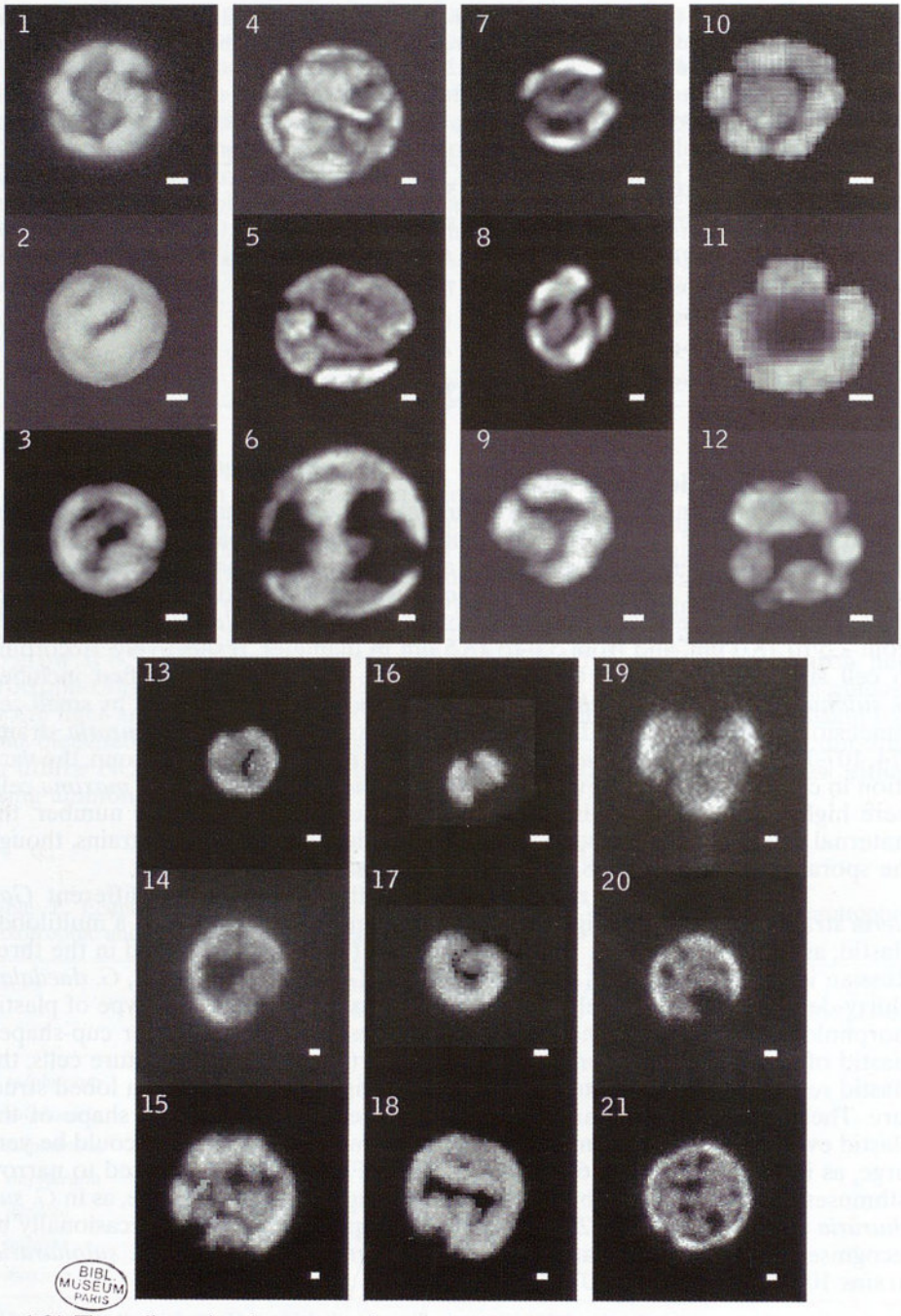
Morphological observations

Cell sizes and the endospore number of the seven strains of *Galdieria* are shown in Tab. 2. The range of cell diameters measured for each strain is reported both for the exponential growth phase and the late stationary phase. Generally, each strain showed larger dimensions of vegetative cells during the stationary phase. Vegetative cells and sporangia of the different *Galdieria* strains ranged from 2.5 to 18.0 μm, and from 5.0 to 18.5 μm in diameter, respectively. According to cell size, two groups of *Galdieria* could be distinguished: the first included *G. sulphuraria* 002, *G. daedala*, and *G. partita*, and was characterized by small cell dimension down to 2.5 μm. The second group consisted of *G. sulphuraria* strains 074, 107-79, 108-79 and *G. maxima*. Among the strains of the latter group, the variation in cell size was not relevant, even though the lower limits of *G. maxima* cells were higher than those of the other isolates. Regarding endospore number, the maternal cell generally included from 2 to 8 endospores in all the strains, though the sporangia of strains P508, 074, and 002 contained 16 endospores.

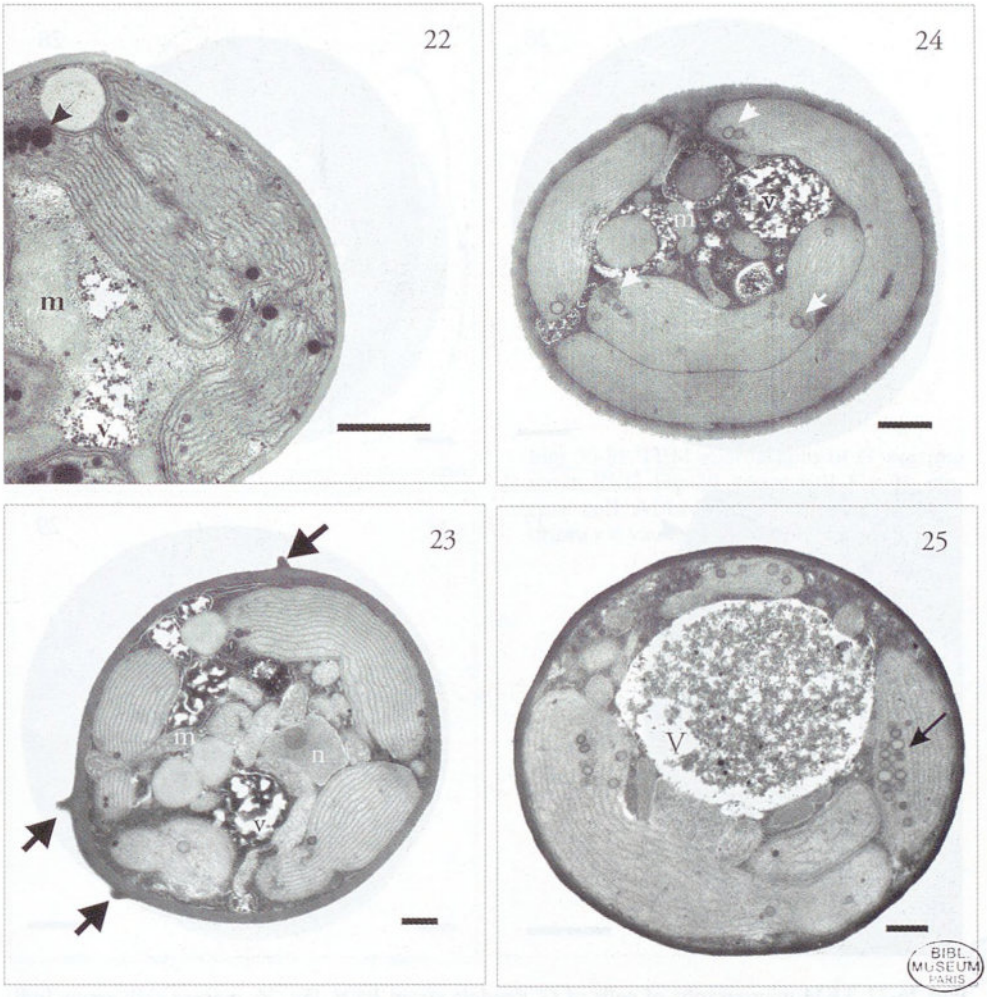
Three-dimensional reconstructions of the plastid of the different *Galdieria* strains are shown in Figs 1-21. Most frequently, cells contained a multilobed plastid, as in the different *G. sulphuraria* strains (Figs 1-3, 15, 19) and in the three Russian isolates (Figs 4-6, *G. maxima*; Figs 7, 8, *G. partita;* Figs 10-12, *G. daedala*). Thirty-degree rotations of cells around x and y axes confirmed this type of plastid morphology (not shown). The image analysis resolved the girdle- or cup-shaped plastid of *Galdieria* endospores and young cells (Figs 13, 16). In mature cells, the plastid remained single, located near the cell periphery and showed a lobed structure. The number of lobes and their width varied, and the overall shape of the plastid evolved to a more complex arrangement with cell age. Lobes could be very large, as in *G. maxima* (Fig. 6) and *G. daedala* (Figs 10-12), or reduced to narrow isthmuses which occupy the bulk of the cell, giving a reticulated shape, as in *G. sulphuraria* strain 108.79 (Figs 20, 21). A girdle shaped plastid could occasionally be recognised in adult cells, particularly in *G. partita* (Fig. 9) and *G. sulphuraria*, strains 107-79 (Fig. 14) and 074 (Figs 17, 18).

Ultrastructural observations

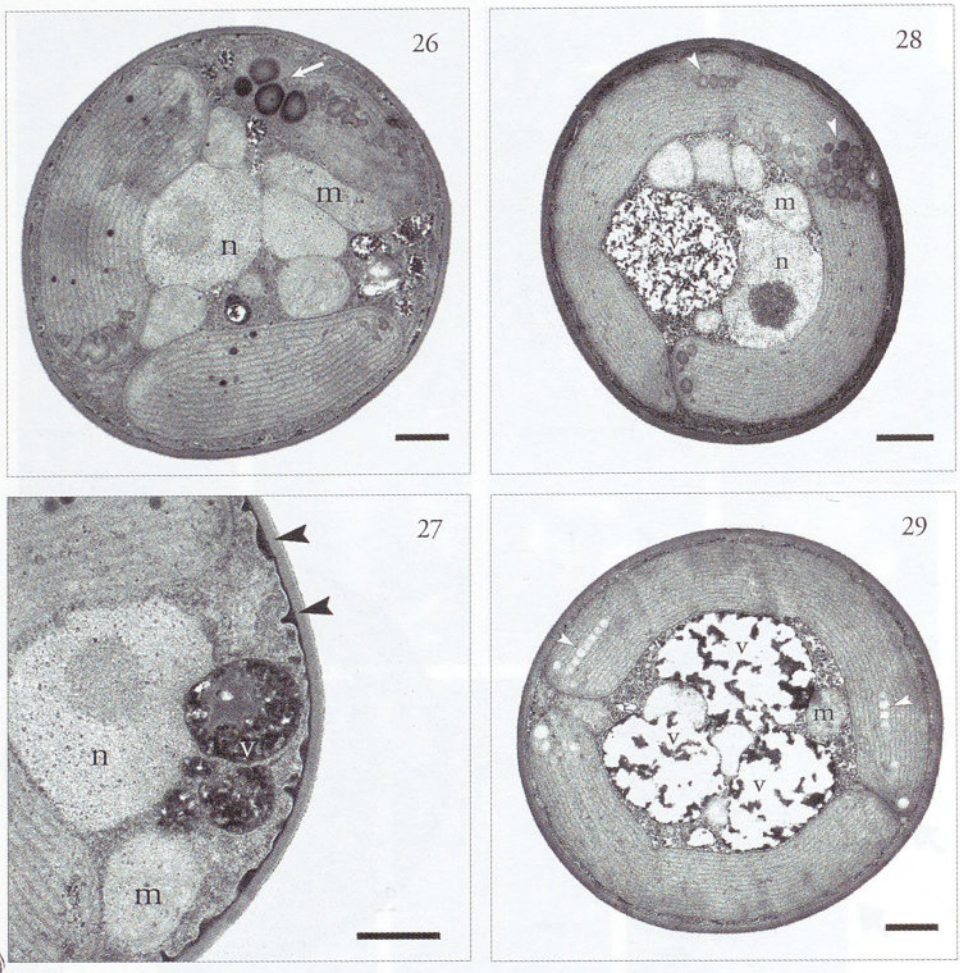
The cell wall of all *Galdieria* strains (Figs 22-31) ranged from 50-350 nm in thickness and was characterised by the presence of loosely arranged fibrils. This variation in thickness could be dependent on pepsin digest used to obtain an



Figs 1-21. Three-dimensional reconstruction of plastids of different *Galdieria* strains obtained by the deblurring of CLSM images. Figs 1-3, *Galdieria sulphuraria*, strain 002. Figs 4-6, *G. maxima*, strain P507. Figs 7-9 *G. partita*, strain P500. Figs 10-12, *G. daedala*, strain P508. Figs 13-15, *G. sulphuraria,* strain 107-79. Figs 16-18, *G. sulphuraria*, strain 074. Figs 19-21, *G. sulphuraria*, strain 108-79. Scale bars = 1 μm

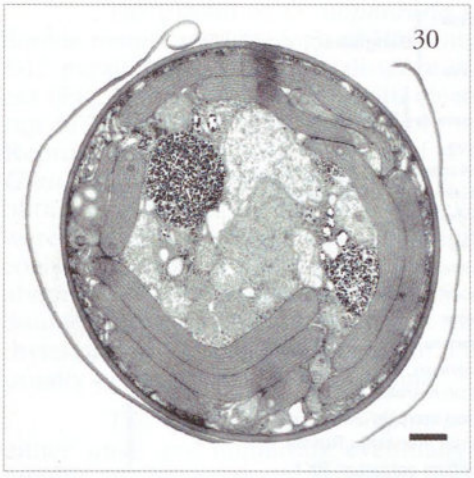


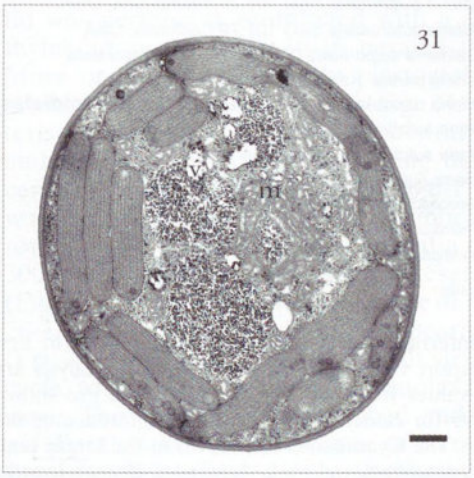
Figs 22-25. Sectioned cells of different strains of *G. sulphuraria*. Fig. 22, mature cell of *G. sulphuraria* strain 002. Fig. 23, *G. sulphuraria* strain 108-79; arrows show the conical protrusions of the outermost layer of the cell wall. Fig. 24, *G. sulphuraria* strain 107-79; arrows indicate plastoglobules. Fig. 25, *G. sulphuraria* strain 074; plastoglobules (arrow). Abbreviations. M = mitochondrion; n = nucleus; v = vacuole. Scale bars = 1 μm



Figs 26-27. TEM micrographs of cells of *G. daedala* strain P508. Fig. 26, mature cell; arrow indicates osmiophilic globules in the plastid stroma. Fig. 27, a detail of a mature cell showing the protrusions of the inner layer of the cell wall (arrowheads). Abbreviations: m = mitochondrion; n = nucleus; v = vacuole. Scale bars = 1 μm.

Figs 28-29. TEM micrographs of cells of *G. partita* strain P500. Fig. 28, young cell; arrowheads show plastoglobules grouped or forming rows interspersed within intertylakoidal space. Fig. 29, mature cell showing a different number of vacuoles; arrowheads indicate rows of plastoglobules. Abbreviations: m = mitochondrion, n = nucleus, v = vacuole. Scale bars = 1 μm.





Figs 30-31. TEM micrographs of *G. maxima* strain P507. Fig. 30, young cell; Fig. 31, mature cell. Abbreviations: m = mitochondrion; v = vacuole. Scale bars = 1 µm.

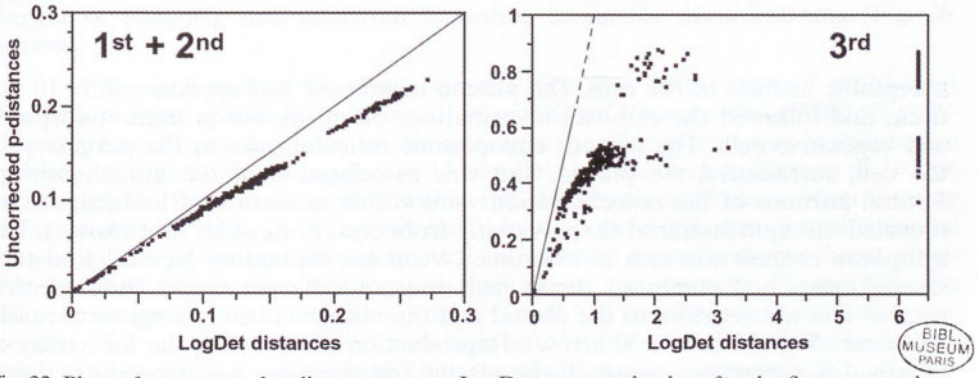


Fig. 32. Plots of uncorrected p-distances versus LogDet-corrected values for the first + second or only third codon positions of *rbc*L in the red algae. The lines representing a 1:1 ratio of p versus LogDet distances are shown. In this analysis, the bending of the line at higher uncorrected distances indicates mutational saturation.

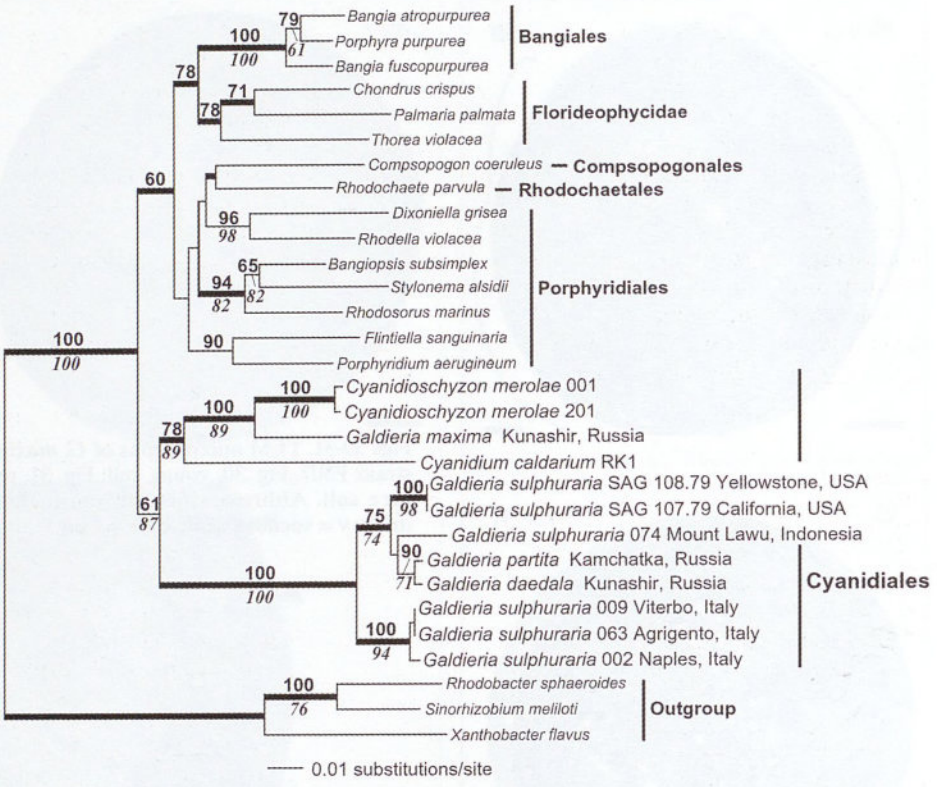


Fig. 33. Phylogenetic relationships of red algae inferred from a ME-LogDet analysis of the first + second codon positions of *rbc*L. Bootstrap support values from the ME-LogDet analysis are shown above the branches, whereas bootstrap values from the ME-GTR analysis are shown below the branches. Posterior probabilities ≥ 0.95 for clades from a Bayesian inference of the amino acid data are shown as the thick branches. The Cyanidiales are shown in the larger text. This tree is rooted on the branch leading to the proteobacterial *rbc*L sequences. Branch lengths are proportional to the number of substitutions per site (see legend).

acceptable fixation of the cells. The plasma membrane was approximately 10 nm thick, and followed the cell wall invaginations often present in both endospores and vegetative cells. The smooth endoplasmic reticulum ran at the periphery of the cell, surrounded the plastid, and was associated with the mitochondrion. Several portions of the mitochondrion were visible in sections. Rhodamine stain revealed the mitochondrial shape varying from cord to net-like (not shown). The cytoplasm content was rich in ribosomes. Vacuoles, stained by Neutral Red (not shown) contained numerous dense inclusions of different types. The eccentric nucleus was always close to the plastid and the mitochondrion. Golgi were rarely observed. (Figs 26 arrow, 30 arrow). Reproduction occurred by the formation of usually 4-8 endospores, in which the plastid and the mitochondrion showed few lobes. Before the formation of daughter cell walls, the space between adjacent spores and the mother cell wall was filled with electron-dense material and vesicles (Fig. 29).

The plastid of *G. sulphuraria* strain 002 (Fig. 22) was multilobed with a double membrane envelope of 10-12 nm. A girdle thylakoid of 15 nm surrounded 8-12 parallel thylakoids at a distance of 50 nm, plastoglobules were usually present (Fig. 22, arrow), together with electron-dense aggregates of large dimensions (up to 700 nm in diameter) possibly tannins in the plastid stroma (not shown). Reproduction was by formation of 4-16 endospores (Fig. 22). The cell wall of *G. sulphuraria* strain SAG 108.79 (Fig. 23) occasionally showed conical protrusion of the outermost layer in vegetative cells (Fig. 23, arrows). One to several vacuoles were present in the cytoplasm containing either small and numerous, eventually coalescing, dense spherical inclusions, or bundles of concentrically arranged fibrils about 0.5-0.7 μm in diameter (Fig. 25 arrow). The plastid was multilobed with a double membrane envelope, with a girdle thylakoid surrounding 12-16 parallel thylakoids at a distance of 50-70 nm. Plastoglobules, 60-200 nm in diameter, were usually present in the plastid stroma.

The vacuoles of *G. sulphuraria* strain SAG 107.79 (Fig. 24) contained either small and numerous, eventually coalescing, dense spherical inclusions, or bundles of concentrically arranged fibrils about 0.5-0.7 μm in diameter. The plastid was peripheral, multilobed with a double membrane envelope, with a girdle thylakoid surrounding 10-16 parallel thylakoids at a distance of about 50 nm. More or less electron-dense plastoglobules, up to 400 nm in size, were usually present in the plastid stroma (Fig. 24, arrows). One to several vacuoles characterised the cells of *G. sulphuraria* strain 074 (Fig. 25) and contained either small and numerous, eventually coalescing, dense spherical inclusions or bundles of concentrically arranged fibrils about 0.8-1 μm in diameter (not shown). The plastid was multilobed with a double membrane envelope, and a girdle thylakoid surrounding 10-16 parallel thylakoids at a distance of 50-70 nm. Plastoglobules, up to 300 nm in diameter, were usually present, sometimes in rows, in the plastid stroma (Fig. 25, arrow). The innermost layer of the cell wall of *G. daedala* strain P508 (Figs 26-27) had a dense osmiophilic appearance and protruded towards the protoplast (Fig. 27 arrowheads). This cell wall structure was present in all stages of the life cycle, whereas the number of vacuoles decreased from the juvenile to the mature stage. The plastid was multilobed and a peripheral thylakoid surrounded 10-15 parallel thylakoids at a distance of 50 nm. Plastoglobules were interspersed within the interthylakoidal space. Osmiophilic globules of bigger or irregular size appeared sometime inside the plastid stroma (Fig. 31, arrow). Plastoglobules, of bigger or irregular size, appeared sometime inside the plastid stroma (Fig. 26, arrow).

The cell wall of *G. partita* strain P500 (Figs 28-29) showed protrusions towards the protoplast. The number of vacuoles of *G. partita* strain P500 (Figs 28-29) decreased from the early to the mature stage. The plastid was multilobed, a peripheral thylakoid surrounded 10-15 parallel thylakoids at a distance of 50 nm. Plastoglobules were interspersed within the interthylakoidal space often forming rows or clusters (Figs 28-29, arrowheads). In vegetative cells, the plastid occupied almost all the peripheral part of the cell and showed a more tubular shape, possibly because of the size and central position of the vacuole. Furthermore, in *G. maxima* strain P507 (Figs 30-31) the cell wall had protrusions towards the protoplast. The number of vacuoles was high both in the juvenile and in the mature stage. The plastid was multilobed. The several chloroplast lobes present a parallel arrangement and a girdle thylakoid surrounded 5-10 parallel thylakoids at a distance of 70 nm. Several plastoglobules were interspersed within the interthylakoidal space, often forming rows (Fig. 40, arrowheads).

Phylogenetic data

The *rbc*L coding regions were straightforward to align within the Cyanidiales and between this group and other red algae. Analysis of the three codon positions showed, however, extreme nucleotide content bias among the taxa. Nucleotide contents at third positions, which are expected to most clearly express genomic bias, were highly skewed within the red algae and the proteobacterial outgroups. The outgroups showed significantly depressed A + T content, whereas *Galdieria* spp. (except *G. maxima*) and *Cyanidium* spp. had extremely high A + T contents (Tab. 3). First and second codon positions showed this phenomenon to a lesser extent. To account for this obvious bias in the sequence data, we built trees using minimum evolution and LogDet distances. The LogDet transformation is useful because it is applicable even when lineages vary in nucleotide frequencies (Lockhart *et al.*, 1994). LogDet distances were calculated for data sets in which either first + second or only third *rbc*L codon positions were considered. Saturation curves were plotted of the LogDet-corrected versus uncorrected 'p' distances (Fig. 32). This analysis showed generally that the first + second positions contained useful evolutionary signal regarding all taxa in the data set, whereas the third positions were saturated and would be of little use for inferring deeper phylogenetic relationships. A ME-LogDet tree of the first + second codon positions of *rbc*L is shown in Fig. 33. This analysis provides weak to moderate support (ME-LogDet = 61 %, ME-GTR = 87 %) for the monophyletic origin of the Cyanidiales as a sister group of the remaining red algae (see also Müller *et al.*, 2001). A 5-gene (16S rRNA, *psa*A, *psb*A, *rbc*L, *tuf*A) analysis of red algal plastid genes also significantly supports Cyanidiales monophyly (Yoon *et al.*, 2002). We are, therefore, confident of the separate evolutionary histories of the Cyanidiales and other red algae as shown in Fig. 33.

All *Galdieria* spp., except for *G. maxima*, are resolved as a closely related clade that is distinct from *Cyanidioschyzon merolae* and *Cyanidium caldarium*. Surprisingly, *G. maxima* is positioned with *Cyanidioschyzon merolae* and is clearly not closely related to the other two Russian *Galdieria* strains. This result was also found in small subunit rDNA trees (Gross *et al.*, 2001). The interrelationships of the other *Galdieria* taxa reflect their geographic origins. The two Russian isolates form a monophyletic group as do the two American strains and the three from Italy (Naples, Viterbo, and Agrigento). The Mount Lawu strain appears to form a separate lineage. Our data suggest, therefore, that there are up to four different

Table 3. Average nucleotide frequencies of a partial rbcL coding sequence (1215 nt) from the Cyanidiales and other members of the Rhodophyta. The number of sequences in each phylogenetic group is shown in the brackets. The extreme nucleotide contents are shown in bold.

	1st + 2nd codon positions				*3rd codon position*			
Taxon	*A*	*T*	*G*	*C*	*A*	*T*	*G*	*C*
Cyanidium (1)	29.1	26.0	27.2	17.7	**40.2**	**30.9**	**13.3**	**15.6**
Cyanidioschyzon (2)	27.9	26.3	27.4	18.4	35.9	37.9	11.2	15.0
Galdieria maxima (1)	29.0	26.3	27.2	17.5	33.8	35.3	13.6	17.3
Galdieria spp. (8)	31.0	25.0	26.8	17.5	**44.7**	**39.7**	**8.6**	**7.0**
Non-Cyanidiales red algae (15)	28.7	25.1	27.9	18.3	34.0	45.0	8.5	12.5
Outgroup (3)	26.7	21.5	29.2	22.7	**3.9**	**7.2**	**36.0**	**52.9**

Galdieria clades in the *rbc*L tree, with *G. maxima* representing a separate taxon that needs to be reclassified either as a member of *Cyanidioschyzon* or as a separate genus. Preliminary analyses of plastid *psb*A sequences are consistent with this result (Yoon and Bhattacharya unpublished data). The *psb*A analyses, however, show greater levels of sequence divergence between the two SAG strains (108.79 and 197.79) and between them and *G. partita* than found with *rbc*L. The Russian taxa (*G. daedala*, *G. partita*) are clearly distinct from the other *G. sulphuraria* strains in both *rbc*L and *psb*A analyses.

DISCUSSION

The combined use of physiological and biochemical traits has been successfully adopted in the taxonomic treatment of coccoid green algae such as *Chlorella* and *Scenedesmus* (Kessler *et al.* 1997; Huss *et al.* 1999) and has been valuable for resolving often controversial issues regarding the attribution of strains to different species. In the case of unicellular red algae, Merola *et al.* (1981) utilized physiological and biochemical characters to separate *Cyanidum* from *Galdieria*. Our results indicated that traits such as pH and NaCl tolerance, growth on nitrate, and growth on glucose in the dark and in the light do not allow one to distinguish between *Galdieria* species. All the strains grew on glucose heterotrophically and mixotrophically, and the differences observed in the other selected characters were strain-specific. The strain 002 of *G. sulphuraria* from Campi Flegrei (Italy) was the most peculiar, being able to grow at NaCl values much higher (8 %) than the other strains. Musacchio *et al.* (1976) reported high halotolerance (8-12 %) in 35 *Galdieria* strains (sub *Cyanidium caldarium* form B) isolated from different Italian acidic sites, confirming this peculiarity for the Italian isolates. Strain 002 is also the only isolate not capable of growing on nitrate. This peculiarity has already been reported by Gross (1999) who showed, in a series of experiments carried out at a temperature of 25 °C, that it was not possible to use these and other biochemical traits, such as the occurrence of linolenic acid, to assign 20 isolates to the genus *Galdieria* or *Cyanidium*.

The species of *Galdieria* have been separated mainly on the basis of light microscopy observations. Some of the morphological features used by Sentsova (1991) to distinguish between *Galdieria* species, such as cell size and number of endopores per sporangium, did not seem to be consistent in identifying different *Galdieria* taxa. The strains of *G. sulphuraria* showed a wide variability in cell and sporangium diameters within the size range (2.5-10.5 µm) of strain 002 comparable to those of *G. partita* and *G. daedala*. On the other hand, strain 074 (Java, Indonesia) had a minimum cell size of 4.5 µm but cell diameters more similar or larger than those of *G. maxima* in the stationary growth phase. De Luca *et al.* (1975) found remarkable differences in cell dimension of 18 *G. sulphuraria* strains coming from different Italian locations and reported that large cells usually occurred when the pH of the culture medium was lower than 2.0. A possible influence of pH on cell dimensions could, in fact, explain some discrepancies between our data and Sentsova's observations, since in our experimental conditions, the medium was maintained at pH 1.5, the optimum pH for *Galdieria*, whereas the Russian strains were grown at pH 2.0-2.2 (Sentsova, 1991).

All *Galdieria* isolates produced sporangia with no more than 16 spores each. Giant sporangia containing 32 endospores were reported for the strain 002 *G. sulphuraria* (Merola *et al.*, 1981), but were not observed in our culture conditions. This is in agreement with the findings of De Luca & Taddei (1970), who described very large sporangia (up to 25 mm diameter) containing 32 endospores only for the field samples from Pisciarelli, the locality from which strain 002 had been isolated. However, since this trait has been observed only for one strain, it should not to be used as a taxonomic marker until field observations are available for the other *Galdieria* localities. The shape and number of plastids played a key role in the taxonomical treatment proposed by Sentsova (1991). *Galdieria maxima* has been described as the only species containing more than one plastid (also in young cells), whereas a single plastid of different shape has been described for *G. daedala* and *G. partita*. However, senescent cells of the latter species were reported to contain several plastids (Sentsova, 1991). The 3D images of *Galdieria* plastids were obtained with Z series images, gathered with CLSM and reconstructed by maximum likelihood deconvolution, and revealed that all the strains always had a single plastid either in juvenile or in mature cells. The shape of the *Galdieria* plastid changed during the life cycle, being a more-or-less complex, always parietal, reticular system of tubular branches, the number of which increases in mature cells. The orientation of the branches varied within the cytoplasm and was associated with slight differences in plastid shape, which seemed to be strain-specific but not useful as a taxonomic marker.

Looking at the various ultrastructural features, the cell walls showed a similar structure and variation in thickness in the species observed. The cell wall of *G. sulphuraria* was described in detail by other authors, who also reported thickness ranging from 20 to 500 nm (Mercer *et al.*, 1962; Bailey & Staehelin, 1968; Staehelin, 1968; Castaldo, 1970; Selyakh *et al.*, 1982; Kuroiwa *et al.*, 1994) and a very hard consistency (Ohta *et al.*, 1992). As for the trilaminate plasma membrane, its thickness ranged between 10-15 nm in all the species, in agreement with the observations reported for other red algae (Pueschel, 1990).

In all the species, the plastid appeared multilobed in mature cells and enclosed 5-16 unstacked thylakoids running in parallel within the plastid matrix. The parallel arrangement of the 2-3 chloroplast lobes in *G. maxima* (Figs 30-31) was distinctly different from the other species, consistent with its distant evolutionary relationship with *Galdieria* spp. (see Fig. 33). The presence of a peripheral thylakoid is in agreement with the observations on several other unicellular Rhodophyta (Broadwater & Scott, 1994). Plastoglobules are common in unicellular red algae (Pueschel, 1990) and their number and type of organization have been used to distinguish between species of *Rhodella* (Deason *et al.*, 1983). In the case of *Galdieria*, the same organization of plastoglobules was observed in all *Galdieria* species. The absence of the pyrenoid can be regarded as a derived character (Ueda, 1994), distinguishing this group of acidophilic unicells from the other unicellular red algae (Broadwater & Scott, 1994). The occurrence of Golgi in *G. sulphuraria* has been known for several decades (Castaldo 1968, 1970) and is confirmed by our study. The presence of a vacuole is peculiar for the genus. It was observed in *G. sulphuraria* and in the Russian species of *Galdieria* (Sentsova, 1994), as well as in other unicellular red algae (Broadwater & Scott, 1994). In spite of the reports of floridean starch floridoside in *G. sulphuraria* (De Luca & Moretti, 1983; Reed, 1983; Frederick, 1987), storage polysaccharides were not observed in our strains. However, the nature of cell inclusions visible inside the vacuoles still needs clarification. One possibility is small polyphosphate bodies based on their ultrastructure (Simon, 1986).

The results previously obtained with the *rbc*L analysis of Cozzolino *et al.* (2000) and in other studies (Freshwater *et al.*,1994; Muller *et al.*, 1998; Patwary *et al.*, 1998), and in the present work clearly indicate a common evolutionary origin and high sequence identity among the two Russian species (*G. daedala* and *G. partita*) and a separate origin for *G. maxima*. The former two taxa may be a single species or a recently diverged species complex that trace their origin to an ancestral Russian strain, whereas *G. maxima* may be a member of *Cyanidioschyzon* or a new genus. Additional sequence data will be needed to distinguish between these possibilities. The remaining *Galdieria* strains form three independent clades with moderate to strong bootstrap support. It is conceivable that all four clades represent distinct species because of the relatively high divergence of their *rbc*L sequences. Alternatively, the sequence divergence may reflect the peculiar population structure of this genus. The apparent absence of sexual reproduction and isolation of different populations may lead to rapid inter-population sequence divergence, in particular if population size is small or if new populations are established through rare colonization events involving a few cells. Brock (1978) and Gross (1999) suggested that low dessication tolerance and a very reduced resistance to neutral pH could confine *Galdieria* to low pH environments, resulting in high divergence between geographically isolated populations. In this regard, the clustering of clades in Fig. 33 suggests that geographic origin is a better indicator of *Galdieria* ancestry than the present taxonomy (e.g., the two Russian strains are monophyletic as are the two American and three Italian isolates).

Our results cannot, as yet, convincingly confirm or refute the existing taxonomy of the genus *Galdieria*. Considering the difficulties in defining the species concept in asexual organisms (Hey, 2001; Mishler & Budd, 1990), we favour retaining the present classification system until more sequence data is available. In the future, it will be necessary to focus on the genetic population structure throughout the geographic range of these species/strains and to analyse within- and between- population sequence differences to get an accurate picture of the species diversity in this group (work in progress).

To summarize, the taxonomy of unicellular Rhodophyta at the species level is based on clearly recognizable characters such as plastid colour, in the case of *Porphyridium* (Ott, 1972), or on differences in mitosis and cytokinesis, which have been used to distinguish between *Rhodella* species (Scott & Broadwater, 1990). The results obtained in the present work indicate that characters such as cell size and number and shape of plastids do not allow a clear separation of the *Galdieria* species. *Galdieria* strains appear to be physiologically uniform. The molecular sequence analyses, however, reveal a surprising level of divergence between the different *Galdieria* strains suggesting that more such data will likely allow the resolution of the taxonomy of this group.

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