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ORIGINAL PAPER

Cryptic dispersal of Cyanidiophytina (Rhodophyta) in non-acidic environments from Turkey

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Abstract

Cyanidiophytina are a group of polyextremophilic red algae with a worldwide, but discontinuous colonization. They are restricted to widely dispersed hot springs, geothermal habitats, and also some human-altered environments. Cyanidiophytina are predominant where pH is prohibitive for the majority of eukaryotes (pH 0.5–3). Turkey is characterized by areas rich in volcanic activity separated by non-volcanic areas. Here we show that Cyanidiophycean populations are present in thermal baths located around Turkey on neutral/alkaline soils. All known genera and species within Cyanidiophytina were detected in Turkey, including *Galdieria phlegrea*, recorded up to now only in Italian Phlegrean Fields. By phylogenetic analyses, Turkish *G. sulphuraria* strains are monophyletic with Italian and Icelandic strains, and with Russian *G. daedala* strains. *G. maxima* from Turkey clustered with Icelandic, Kamchatka, and Japanese populations. The discovery of Cyanidiophytina in non-acidic Turkish soils raises new questions about the ecological boundaries of these extremophilic algae. This aids in the understanding of the dispersal abilities and distribution patterns of this ecologically and evolutionarily interesting group of algae.

Keywords Extremophiles · Cyanidophytina · Phylogeny · Population structure · rbcL · Biodiversity

Introduction

Cyanidiophytina (Rhodophyta) are a group of red unicellular algae highly adapted to the environmental extremes offered by volcanic regions. These environments often support temperatures above 50 °C and have high sulfuric acid concentrations that result in acidic pH levels prohibitive for most eukaryotes (Albertano et al. 2000; Brock 1978; Pinto et al. 2003; Pinto et al. 2007; Cennamo and Ciniglia 2017). The interest in global biodiversity and distribution patterns of thermoacidophilic Cyanidiophyceae populations led to numerous explorations of volcanic regions both in and outside of Europe, such as Italy, Iceland, USA, New Zealand, and Japan. In this, molecular approaches were successfully used to assess the level of biodiversity in this group (Ciniglia et al. 2004; Yoon et al. 2004, 2006; Toplin et al. 2008, Ciniglia et al. 2014). This provided a hypothesis of the origin and dispersal routes of *Galdieria maxima* and *G. sulphuraria* in populations from Iceland and northeastern Asia. Cyanidiophytina mobility is still poorly understood.

A novel estimate of species richness of Cyanidiophyceae has recently come from the analysis of

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thermoacidophilic communities from aquatic and non-aquatic volcanic sites in Taiwan (Hsieh et al. 2015). The habitats so far explored, in search of polyextremophilic algae, have usually been characterized by strong acidity, as pH range is considered a greater constraint on the growth of Cyanidiophytina than temperature range. Thus, many explorations have focused on acidic geothermal areas (Brock 1978; Toplin et al. 2008; Hsieh et al. 2015). Currently, the genus *Cyanidium* encompasses two main species. These are *C. caldarium* (Tilden) Geitler, a polyextremophilic alga adapted to acidic and hot springs and fumaroles, usually rich in heavy metals, and *C. chilense*, a hypogean, neutrophilic (pH around 7.0) and mesophilic (20–25 °C) alga discovered in several caves worldwide (Schwabe 1936; Friedmann 1964; Skuja 1970; LeClerc et al. 1983; Azúa-Bustos et al. 2009; Darienko and Hoffmann 2010; Del Rosal et al. 2015; Cennamo et al. 2012; Ciniglia et al. 2017). The phylogenetically distinct thermoacidophilic *C. caldarium* and the neutrophilic and mesophilic *C. chilense* are clearly separated on the basis of both molecular and ecophysiological characters (Ciniglia et al. 2004). These findings suggest that other Cyanidiophytina could have a much wider distribution than those considered so far. This prompted us to search for alternative ecological niches, such as non-acidic environments.

In this study, we report on our new explorations of seven thermal baths located in Turkey and report the presence of Cyanidiophycean populations on neutral/alkaline soils. Anatolian volcanism is a consequence of convergence occurring between Afro-Arabian and Eurasian plates and it can be considered as a bridge between the geothermal areas of Europe and Asia. This zone is characterized by deposits of andesitic and rhyolitic lava, alternating with black and clastic sedimentary rocks, resulting from the solidification of mud mixed with water (Pearce et al. 1990). Although Turkey is still geologically active, intense volcanic activity has not been recorded for a number of years; Turkish volcanism varies from mildy alkaline volcanoes, such as Nemrut, to calc-alkaline/alkaline volcanoes, such as Ararat and Tendurek (Pearce et al. 1990).

The chemical composition of rocks collected in our sampling areas was determined by X-ray diffraction. Next a culture-dependent approach combined with rbcL gene sequencing was employed to characterize the phylogenetic positioning of algal diversity of the Cyanidiophycean populations we isolated from Turkey. We also added all of the available rbcL gene sequences from a wide geographic range, to refine the population structure and molecular variance. Then, we explored the geographical distribution of global genetic variation in different species and genera of Cyanidia.

Materials and methods

X-ray diffraction (XRD)

XRD was performed on the mineralogical phases of substratum inorganic components occurring in the algal biofilms. XRD patterns were collected in the 3°–90° 2θ range, according to the step scanning procedure with Co radiation on a Miniflex Diffractometer (Rigaku, Japan). The tube operated at 30 kV and 15 mA, and the counting time was 3600 s. The identification of mineralogical phases was performed with a search/match on the Joint Committee on Powder Diffraction Standards.

Sample collection, isolation and cultivation

Environmental samples were collected from seven Turkish thermal stations located in the south eastern, north eastern, and south western peninsula: (1) Cermik-Diyarbakir, (2) Biloris-Siirt; (3) Güçlükonak-Şirnak; (4) Nemrut crater lake-Bitlis; (5) Agri-Diyadin; (6) Kula-Manisa; (7) Germencik-Aydin (Fig. S1). For each station, samples were collected where algae were present either superficially or covered by crystals, crumbly soil, and mud layers, respectively (Fig. 1). The samples were collected from different microenvironments, such as the surface of the crystals, around the granules of crumbly soil and between the layers of mud (Fig. 1). Temperatures were measured with a digital thermometer (Field Environmental Instruments, Pittsburgh, Pennsylvania, USA). pH was measured with a portable pH meter (Hanna Instruments, Padova, Italy) and with pH strips (Macherey–Nagel Bethelem, USA).

Sampling location, coordinates, pH, temperature, and habitat for each sampling site are summarized in Table 1. All samples were collected by scraping the mineral substratum and these were stored in sterile tubes. To obtain monoclonal cultures of each sample, serial dilutions were performed in a specific medium for Cyanidiophytina (Allen's medium, pH 1.5, Allen and Stanier 1968); multiwell plates were used for the isolations. Maximum dilution enrichments were also streak-plated onto Allen's medium supplemented with agar. Single colonies were chosen from each plate and suspended in liquid Allen's medium. Cultures in both tubes and plates were grown at 37 °C under continuous fluorescent light. All isolates were numbered and stored in the Algal Culture Collection of University Federico II of Naples (ACUF, http://www.acuf.net). Cultures are available upon request to the authors.

Algal samples were inspected using a light microscope (Nikon Eclipse E800 equipped with Nomarski interference), to visualize strains grown in Allen's medium.

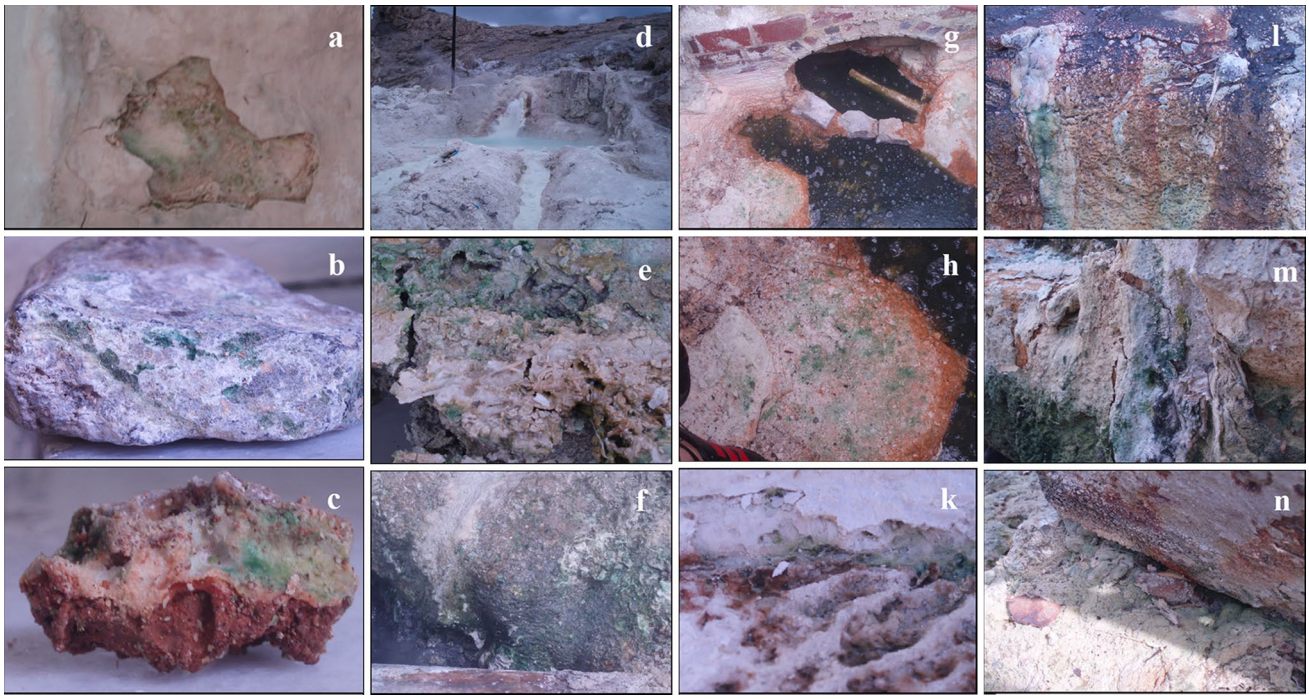

Fig. 1 Pictures of some sampling points from the Turkish thermal areas for Cyanidiophytina. **a** Cermik, Southeastern Turkey; **b**, **c** endolithic growth of Cyanidiophytina in Germencik, Southwestern Turkey; **d–f** Agri, Diyadin, Northeastern Turkey; **g**, **h**, Kula Manisa, Southwestern Turkey; **i** Saart, Manisa, Southwestern Turkey; **j–l** Salihli, Manisa, Southwestern Turkey

Table 1 Location, codes, habitat, pH, temperature and main minerals of sampling sites in Turkey

Location, region (coordinates)	Sampling location code	Habitat	pH	*T* (°C)	Minerals
Cermik, Diyarbakir Southeast Turkey (38°8′16″N, 39°28′3″E)	SET.CE	Thermal bath, on the wall inside and outside the hammam	7	24.6	Quartz, pyroxenes, dolomites
Biloris, Siirt Southeast Turkey (37°56′7″N, 41°56′12″E)	SET.BI	Thermal bath, on the wall, inside the hammam	7	25.8	Quartz, pyroxenes, dolomites
Gü.lükonak, Şirnak Southeast Turkey (37°28′10″N, 41°54′39″E)	SET.GU	Thermal bath, on the wall inside the hammam	1	54	Quartz, feldspars, gypsum
Nemrut crater lake East Turkey (38°37′33″N, 42°14′44″E)	CET.NE	Fumaroles	6.7	32–46	Quartz, feldspars, gypsum
Agri, Diyadin Northeast Turkey (39°32′26″N, 43°40′57″E)	NET.DI	Fumaroles, hot spring, hot pool, hot soil	6.5	45	Quartz, pyroxenes, dolomites
Kula, Manisa Southwest Turkey (38°32′45″N, 28°38′48″E)	SWT.KU	Hot soil–hot pool	5	41	Quartz, feldspars, miche, calcyte
Germencik, Aydin Southwest Turkey (37°52′15″N, 27°35′58″E)	SWT.GE	Hot spring	5.8	27	Quartz, feldspars, miche, calcyte

DNA extraction, gene amplification and sequencing

For DNA extraction, algal cells were suspended in a specific buffer (DNeasy Plant Mini Kit, Qiagen, Santa Clarita, CA, USA) and ground with glass beads using a Mini-BeadBeater (BioSpec, Bartlesville, OK, USA) operated at 13,000 revolutions per min for 5 min. Total genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Santa Clarita, CA, USA). Four degenerate primers were used to amplify the rbcL gene from isolated samples (Ciniglia et al. 2004). The resultant products were purified with the QIAquick PCR purification kit (Qiagen) and used for direct sequencing using the BigDyeTM Terminator Cycle Sequencing Kit 3.1 (PE-Applied Biosystems, Norwalk, CT, USA) and an ABI-3500 XL at the Microgem Laboratory (Naples, Italy). Forward and reverse electropherograms were assembled and edited using

the program Chromas Lite v.2.1 (http://www.technelsium.com.au/chromas.html).

Phylogenetic analyses

A total of 81 new rbcL sequences were obtained in this present study from our Turkish samples, and these were integrated with the 255 available rbcL sequences available at GenBank (Table S1). All sequences were aligned with published sequence data (Ciniglia et al. 2004; Toplin et al. 2008; Skorupa et al. 2013; Ciniglia et al., 2014; Hsieh et al. 2015), using BioEdit Sequence Alignment Editor (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). No gaps or indels have been incorporated in the alignment. Newly determined sequences are all available on NCBI GenBank (Table S1). Maximum likelihood (ML) phylogenetic analysis of rbcL was performed using the GTR + Γ + I model implemented in RAxML software (Stamatakis et al. 2008). Statistical support for each branch was obtained from 1000 bootstrap replications using the same substitution model and RAxML program settings. Bayesian analyses (BA) were performed for combined and individual datasets with MrBayes v.3.1.1 (Ronquist and Huelsenbeck 2003) using the Metropolis-coupled Markov chain Monte Carlo (MC3) with the GTR + Γ + I model. For each matrix, one million generations of two independent runs were performed with sampling trees generated every 100 generations. The burn-in period was identified graphically by tracking the likelihoods at each generation to determine whether they reached a plateau. Seven red algal taxa belonging to Bangiophyceae and Stylonematophyceae were chosen as outgroup taxa, being the closest relatives to Cyanidiophytina.

An estimate of genetic diversity was carried out using DNASP v.5.10.01 (Librado and Rozas 2009). For each population, the following statistics were computed: haplotype (h) (Nei 1987) and nucleotide diversities (π) (Nei 1987), with standard deviation. Population expansion was assessed by neutrality test (Tajima 1989; Fu and Li 1993) and mean number of pairwise differences (symbol) (Tajima 1983).

To assess population differentiation, pairwise F_{st} values were calculated as the pairwise genetic differentiation (pairwise F_{st} statistics) in ARLEQUIN version 3.5.2.2 (Excoffier and Lischer 2010) based on 50,000 permutations ($P < 0.05$). The isolation-by-distance was tested using a Pearson correlation in R, testing for a positive correlation between pairwise geographic distance (in km) and F_{st} average pairwise differences.

Results

Soil and rock samples at the Anatolian volcanism region were surveyed in search of Cyanidiophytina species. Table 1 shows the location of the sampling sites, temperature and pH, along with the type of substratum for each sampling station. In all of the examined samples, quartz and potassium feldspars were the main minerals found, followed by calcyte (Kula Manisa and Germencik), pyroxenes and dolomites (Agri-Dyadin, Cermik-Dyiarbakir and Biloris-Sirt), and gypsum (Gucklukonak-Sirnak). These Turkish sites had mostly neutral pHs (Table 1). Despite this, all collected samples had Cyanidiophycean. We were successful in isolating cultures at all sites using Allen's medium at pH 1.5. Cyanidiophycean cultures grew abundantly, suggesting that although adapted to neutral soil, these microalgae were acid tolerant. The same medium was used to obtain single colonies, and axenic cultures were deposited at the Algal Collection of University Federico II (ACUF, http://www.acuf.net).

The identification of different genera and species in the Cyanidiophytina has previously been difficult, as there are few unequivocal morphological features to distinguish between them, and furthermore, there is homoplasy between some lineages. Thus, to identify the algal species been studied, molecular tools were used. For this, we first generated 491 base pairs of rbcL sequence for the different isolates. These were aligned, including the 81 new Turkish Cyanidiophycean isolates (Table S1), and the existing 168 Cyanidiophycean rbcL sequences available from GenBank. These strains originated from Japan, Iceland, Italy, Kuril Islands, Kamchatka, USA, New Zealand, and seven outgroup taxa. rbcL phylogeny identified five Cyanidiophycean taxa from Turkey: *Galdieria sulphuraria, Galdieria maxima, Galdieria phlegrea, Cyanidium caldarium*, and *Cyanidioschyzon merolae* (Fig. 2). The inferred RAxML tree based on rbcL dataset showed several well-supported sublineages within *G. sulphuraria* and *G. maxima* clades. *G. sulphuraria* included at least five sublineages, including one defined by a New Zealand population (Fig. 2, subclade S1) and another with a USA population (Fig. 2, subclade S2). Accessions nested in an independent lineage, separable as two well-supported subclades (posterior probability/bootstrap: New Zealand subclade, 1/97; USA subclade, 1/98). We noted that 10 Turkish specimens grouped within *G. sulphuraria* and this was in two different subclades, 7 nesting with the Italian strains (Fig. 2, subclade S3; posterior probability/bootstrap 1/100), and 3 with the Icelandic strains along with the Russian *G. daedala* strain (Fig. 2, subclade S5; posterior probability/bootstrap 1/69). The sequences from Taiwan clustered with *G. partita* from Russia. Together the relations were clearly resolved with high statistical confidence.

The *G. maxima* assemblage included four subgroups reported in M1 to M4. Turkish specimens of *G. maxima* ($n = 40$) clustered in two well-supported, different subclades, 13 of which clustered with Icelandic specimens (Fig. 2, subclade M1), 27 nesting with conspecific strains from Japan, Taiwan, and the Russian *G. maxima* authentic strain (Fig. 1, subclade M2). rbcL sequences from Taiwan

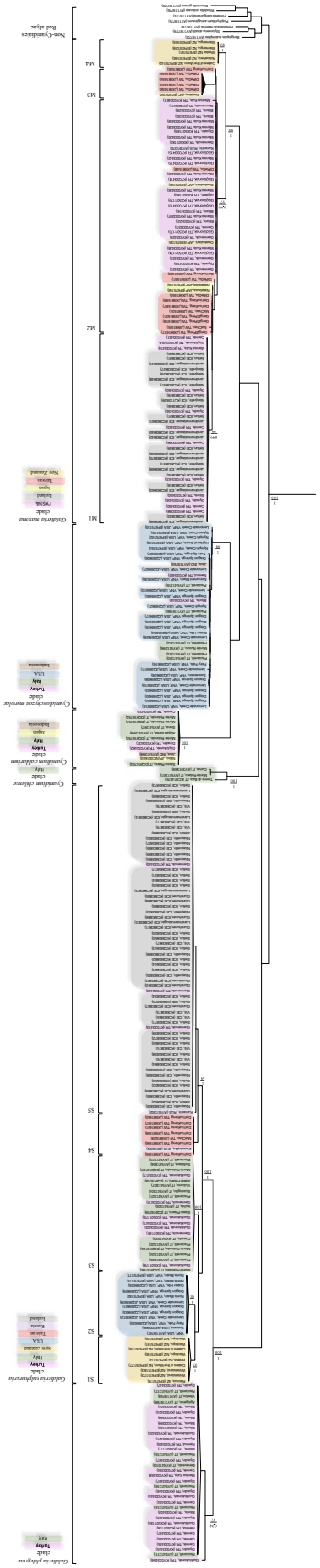

Fig. 2 Consensus Bayesian tree of Cyanidiophytina based on rbcL sequences. The Bayesian posterior probability and maximum likelihood (RAxML) bootstrap values (MLBT) are shown above the branches. Dashes indicate support values < 50%

and Japan both grouped into two subclades M2 and M3. *G. maxima* sequences from New Zealand did not group with any of conspecific strains collected from other locations, as in *G. sulphuraria* (Fig. 2, subclade M4).

The *G. phlegrea* clade was formed by Turkish ($n = 26$) and Italian ($n = 8$) isolates. This was strongly supported by high posterior probability/bootstrap values of 0.94/97%. *C. caldarium* from Turkey ($n = 3$) were closely related to all other isolates with 100% bootstrap value.

Only two Turkish isolates were found to be closely related to *C. merolae*. The low level of intraspecific variation recorded in *C. merolae* did not generate any subclusterization associated to geographic populations. Our phylogenetic tree conformed to previously reported monophyly of Cyanidiophyceae (posterior probability, 1; ML LogDet bootstrap = 100%) (Fig. 2) (Ciniglia et al. 2004). However, by adding the rbcL sequences from the new Turkish isolates, at least six lineages within the class were indicated by the high bootstrap values, instead of the previously reported four lineages (Ciniglia et al. 2004, 2014). These six independent lineages were grouped in different monophyletic clades (Fig. 1), namely: (1) *C. merolae* (posterior probability 1/bootstrap, 99%); (2) *G. maxima* (1/99), sharing a common ancestor with *C. merolae*, but with strong evidence of molecular divergence between them; (3) the mesophilic lineage of *C. chilense* (1/100; Ciniglia et al., 2017); (4) *C. caldarium* (1/100), clearly phylogenetically divergent from the mesophilic *C. chilense* (Yang et al., 2016); (5) *G. sulphuraria* (1/100) and (6) *G. phlegrea* (0.94/97), as sister clades (1/100).

Genetic diversity and population differentiation

Next, an analysis of genetic diversity within and between populations of Cyanidiophyceae was performed using DNAsp, which provides an estimate of the extent of genetic variation between individuals belonging to the same geographic population and between different populations. Results are listed in Table 2. We excluded *C. caldarium* from the analysis because of the low number of haplotypes and their restricted geographic distribution. A total of 159 haplotypes were recovered from 459 individuals analyzed and 149 (95.5%) of the haplotypes were private, i.e., unique to a single locality. The highest values of average sequence divergences were recorded for *G. sulphuraria* ($K = 19.47$), and *G. maxi*ma ($K = 17.37$), with a high level of haplotype diversity, as well (*G. sulphuraria*, hd, 0.83 ± 0.028; *G. maxima*, hd, 0.956 ± 0.006).

In *G. sulphuraria*, the analysis of genetic diversity was performed on 136 partial sequences of rbcL with 80 polymorphic sites and 33 different haplotypes (only two haplotypes were shared by Italy and Turkey and by Taiwan and Russia). The highest levels of haplotype diversity were found

Table 2 Statistics of rbcL haplotypes for the Turkish cyanidiophycean strains

Phylotype	*n*	*v*	*N*	*K*	*h*	π	Tajima	Fu and Li *F**
G. sulphuraria								
All	136	80	33	19.47	0.83 ± 0.028	0.0426 ± 0.00356	− 0.35987	− 0.42141
Italy	15	8	7	1.29	0.724 ± 0.121	0.00283 ± 0.0019	− 1.744	− 1.992
USA	13	5	4	1.2	0.6 ± 0.131	0.00269 ± 0.00157	− 0.84	− 1
Turkey	10	39	3	17.13	0.6 ± 0.131	0.0375 ± 0.00482	− 1.17	1.3426
Taiwan	27	39	6	6.83	0.732 ± 0.054	0.015 ± 0.0035	− 1.405	0.637
Iceland	59	6	7	0.267	0.224 ± 0.072	0.00058 ± 0.000115	− 1.95362*	− 3.337**
New Zealand	10	8	6	2.022	0.867 ± 0.085	0.0044 ± 0.002	− 1.23	− 1.43
G. maxima								
All	245	161	100	17.3721	0.956 ± 0.006	0.038 ± 0.0012	− 1.38	− 5.416**
Turkey	40	43	8	8.8	0.652 ± 0.069	0.02 ± 0.0033	− 0.52	− 2.2
Japan	23	34	8	10.52	0.861 ± 0.039	0.023 ± 0.00345	− 0.302	− 0.815
Iceland	24	4	3	0.3333	0.163 ± 0.0098	0.00073 ± 0.00051	− 1.88381*	− 2.796*
Taiwan	149	108	80	17.08	0.957 ± 0.009	0.0373 ± 0.00067	− 0.6142	− 5.3**
New Zealand	7	44	4	13.05	0.81 ± 0.13	0.028 ± 0.0059	− 1.58	− 1.836
C. merolae								
All	44	19	19	2.0296	0.918 ± 0.022	0.00443 ± 0.00219	− 1.73184	− 3.456**
Turkey	2	1	2	1	1 ± 0.5	0.0028 ± 0.00109	–	–
USA	35	17	17	2.2454	0.934 ± 0.021	0.0049 ± 0.00053	− 1.515	− 2.89*
Italy	5	2	3	0.8	0.7 ± 0.2	0.00175 ± 0.00066	− 0.97	− 0.95
G. phlegrea								
All	34	26	7	2.3244	0.458 ± 0.104	0.0051 ± 0.00272	− 2.3221**	− 3.4274**
Italy	8	4	2	1	0.250 ± 0.180	0.0022 ± 0.0016	− 1.5347	− 1.7974
Turkey	26	24	6	2.72	0.517 ± 0.113	0.006 ± 0.0028	− 2.20**	− 3.267* *

n sample size, *v* variable sites, *N* number of haplotypes, *h* haplotype diversity, *K* average number of pairwise nucleotide differences, π nucleotide diversity

Significance *$P < 0.05$; **$P < 0.10$

in the samples from New Zealand (hd = 0.867), Italy, and Taiwan (hd = 0.724 and 0.732). An average value of haplotype diversity was recorded in Turkey (hd = 0.600), despite the degree of nucleotide diversity higher than any other population ($\pi = 0.0375$). Iceland exhibited comparatively lower values of these indices (hd = 0.224; $\pi = 0.0006$).

Genetic distance was represented as F_{st} for each pairwise combination of populations, based on rbcL marker. The value of inter-populational pairwise genetic differentiation, F_{st} (5 populations of *G. sulphuraria* analyzed: USA, Italy, Turkey, New Zealand, and Iceland) was significantly high (0.7788, $P < 0.05$). F_{st} ranges from 0 to 1; F_{st} of 0 indicates panmixy with high interbreeding between populations, while a value of F_{st} of 1 means that the populations are fixed and do not interbreed. When considering the genetic differentiation between two populations, F_{st} values ranged from low (0.14) to high (0.97) (Table 3). The lowest level of genetic differentiation was recorded between Turkey and Italy, which were also the closest populations geographically (1950 km). However, high genetic divergences were found between the furthest and the closest *G. sulphuraria* populations, such as Taiwan and USA (0.97, 12254 km), USA and Iceland (0.91, 5719 km), Italy and USA (0.85, 8622 km), New Zealand and Iceland (0.844, 17215 km), Italy and Iceland (0.839, 3247 km), and New Zealand and Italy (0.71, 18,559 km). We next investigated the potential for isolation-by-distance (IBD) via statistical tests of correlations to weigh the contribution of geographic distance in the population structure. The correlation between genetic and geographic distances based on rbcL was weakly positive, but not statistically significant in *G. sulphuraria*, as shown in Fig. 3 ($R = 0.264$, $P = 0.333$). This thus rejected an isolation-by-distance model from these data.

In examinations of 245 *G. maxima* partial rbcL (434 bp) sequences, these contained 161 polymorphic sites and 100 haplotypes (Table 2). There was a high level of detected diversity (hd = 0.956). Haplotype and genetic diversity of rbcL in Turkish populations, calculated from 40 sequences and 8 haplotypes were 0.652 ± 0.069 (hd) and 0.02 ± 0033 (π) in 43 polymorphic sites. The highest genetic diversity was found in the Taiwanese population, where among 149 individuals, 80 haplotypes and 108 parsimony informative

Table 3 Matrix of pairwise estimates of F_{st} between pairs of populations of *G. sulphuraria* and *G. maxima*

G. sulphuraria	ICE	ITA	NZE	TWN	TUR	USA
ICE	***	0.839	0.844	0.87	0.55	0.91
ITA		***	0.71	0.88	0.14	0.85
NZE			***	0.91	0.511	0.831
TWN				***	0.67	0.97
TUR					***	0.68
USA						***

G. maxima	ICE	JAP	TWN	TUR
ICE	***	0.4	0.64	0.67
JAP		***	0.21	0.06
TWN			***	0.56
TUR				***

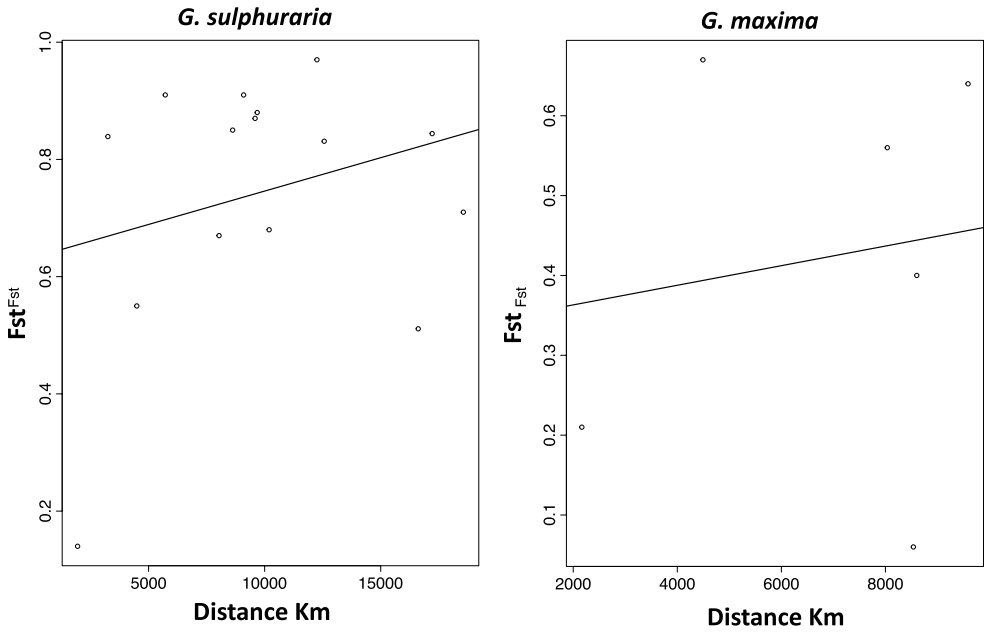

Fig. 3 Correlation among genetic divergence and geographic distance. Each point represents a single pairwise comparison between seven isolated populations. Regression lines show relationships between genetic divergence and geographic distance (*G. sulphuraria*, $R=0.245$, $P=0.333$; *G. maxima*, $R=0.145$, $P=0.763$)

sites showed high haplotype diversity (0.957 ± 0.009) with low genetic polymorphism ($\pi = 0.0373 \pm 0.00067$). The Japanese population was the highest in both diversities ($hd = 0.861 \pm 0.039$; $\pi = 0.023 \pm 0.00345$). This resulted from 23 sequences, 8 haplotypes, and 34 polymorphic sites. The level of haplotype and nucleotide diversity for the New Zealand population was calculated on the few sequences available (7 individuals, 4 haplotypes, $hd = 0.81 \pm 0.13$, $\pi = 0.028 \pm 0.006$). The 24 Icelandic sequences showed a lower haplotype and nucleotide diversity ($hd = 0.163 \pm 0.0098$; $\pi = 0.00073 \pm 0.00051$). In the neutrality test of *G. maxima*, Tajima *D* and Fu and Li were both significantly negative for the Icelandic samples ($D = -1.88381$; $F = -2.796$ Table 2). However, all samples from the other regions showed negative values of Tajima *D*, but without statistical significance of Tajima and Fu and Li, except for Taiwan samples showing strong significantly negative values of *F* (Table 2).

The inter-populational genetic differentiation, F_{st} calculated on 5 *G. maxima* populations (Turkey, Japan, Iceland, New Zealand, and Taiwan) was 0.55. However, the highest similarity in genetic structure calculated between two populations was accounted for the geographically closest populations, Japan and Taiwan ($F_{st} = 0.162$). Low levels of genetic

differentiation were also found between Turkey and Taiwan ($F_{st}=0.287$) and Turkey and Japan ($F_{st}=0.257$), despite the significant geographic distances between them. The highest F_{st} value was exhibited between Iceland and New Zealand, areas geographically far apart. A weakly positive correlation between genetic and geographic distances was detected for *G. maxima*, although it was not significant ($R=0.145$; $P=0.763$, Fig. 3).

Despite extensive sampling, current and previous molecular analysis has to date only identified 44 rbcL sequences from *C. merolae*. The majority belonged to individuals spread across the American territories, as few sequences were detected in the Turkish or Italian samples, and no sequences have yet been detected in Taiwanese samples. The analysis revealed the presence of 19 polymorphic sites, generating 19 haplotypes. The two most frequently represented were shared by the Turkish, Italian, and American samples. Genetic haplotype diversity was estimated using all of the isolates and gave results of 0.918 ± 0.022, with a very low degree of nucleotide diversity, namely $\pi=0.00443 \pm 0.00219$ (Tajima, -1.73184; Fu and Li, -3.456). This indicates the absence of geographical population structuring. This was also shown by the low level of the overall genetic differentiation ($F_{st}=0.05$). We could not perform correlation test for *C. merolae*, as well as for *G. phlegrea* and *C. caldarium*, because of the limited number of accessions and populations available for the analysis.

Discussion

Cyanidia are the most abundant photosynthetic protists found in extremely acidic, sulfur-rich environments that are close to active volcanoes (Brock 1978; Ciniglia et al. 2004; Skorupa et al. 2013; Toplin et al. 2008). Until now, Cyanidia have been isolated mainly in solfataras (Italy, Iceland, Japan, New Zealand, Yellowstone National Park, and Taiwan), where the condensation of sulfur dioxide and hydrogen sulfide produces crystals of sulfur subsequently oxidized to sulfuric acid resulting in acidification.

Turkey is characterized by collision volcanism, varying from mildly alkaline volcanoes, such as Nemrut, to calc-alkaline/alkaline ones, such as Mount Ararat (Pearce et al. 1990). Residual volcanic activity in Turkey explains the presence of many geothermal spots, with neutral and subneutral pH values, due to the limited presence of sulfuric acid. The main minerals detected in the areas explored were quartz, feldspars, calcite, and dolomites (Table 1). Narrow and thin biofilms of Cyanidia were detected in Turkish thermal baths, mostly in hypolithic and endolithic conditions.

Most of the species isolated from Anatolia were highly acidotolerant organisms, able to survive in a wide range of pH conditions (*Galdieria maxima, Galdieria phlegrea*, and *Cyanidium caldarium* between 1 and 7, *Galdieria sulphuraria* between 1 and 5.8). However, all species and strains, regardless of the ecological features of the sampling sites, remained well-suited to acido-thermal or at least acidic growth conditions. One exception is represented by *Cyanidium chilense* (=cave *Cyanidium*, Schwabe 1936, 1942; Hoffman 1994; Ciniglia et al. 2017), which represents a separate monophyletic lineage within Cyanidiophytina, including several strains dispersed worldwide. It appears to be limited to cave habitats where pH and temperature are not extreme, and is unable to proliferate in laboratory conditions. Cyanidiophytina are thus abundant in mesophilic areas of Turkey, but are still adapted to thrive under acido-thermal environment.

According to Doemel and Brock (1971), the occurrence of *C. caldarium* in non-thermal habitat was frequent, being recorded in aquatic habitats between 20 and 55 °C and on soils at temperature between 10 and 55–57 °C. Pinto (1993) similarly reported the presence of *C. caldarium*, *G. sulphuraria*, and *C. merolae* in more than 100 hydrothermal sites around Italy. These were not only in acidic hot springs, but also in acidic non-thermal ones, such as the sulfur mines. Recently, Hsieh et al. (2015) identified a novel mesophilic *Cyanidium* clade from non-thermal, but acidic sites in Taiwan, thus supporting the frequent occurrence of Cyanidiophytina in geothermal environments not necessarily in high temperature conditions (Gross et al. 2002).

Lowell and Castenholtz (2013) tested the ability of several *Cyanidium* to lower the external pH from 6 to more acidic values. They confirmed that many *Cyanidium* obtained from Yellowstone, Japan, Philippines, and New Zealand hot springs could acidify their growth environment. This suggested the importance of this process as survival strategy in confined environments, such as microbial mats, interstitial soil spaces, and endolithic niches. These algae appear to harbor adaptive responses to survive the non-ideal conditions during their dispersal, helped by wind flow, air particles, or birds. Despite the limited tolerance to desiccation and the absence of resting spores for Cyanidiophytina (Gross et al. 2002), the ability to lower the pH outside the cell would render them able to survive in non-acidic environments. This could potentially serve as a connection between the thermoacidic locations as a mechanism of long-distance migration (Brock 1978; Gross 1999).

The molecular investigations on new Cyanidiophycean isolates revealed the presence of all representatives of this class of microalgae, namely *G. sulphuraria*, *G. phlegrea*, *G. maxima, C. merolae*, and *C. caldarium* on hydrothermal soils around Turkey. The new rbcL sequences were mostly attributed to *G. phlegrea* and *G. maxima*, while *G. sulphuraria*, *C. merolae*, and *C. caldarium* sequences were rarely detected. Turkey is the first site in which all these species have been collected in one local. For example, in

Italy *G. maxima* has not yet been detected, while all other thermoacidophilic communities sampled to date have an incomplete number of species and strains (Toplin et al. 2008; Skorupa et al. 2013, Hsieh et al. 2015).

Of remarkable interest is the detection of *G. phlegrea* in almost all of the sampling stations from Turkey, recorded to now only in one Italian area located within the Phlegrean Fields (Naples, Italy), adapted to relatively dry areas and to dim light (Ciniglia et al. 2004; Pinto et al. 2007). *G. phlegrea* possesses interesting ecophysiological traits, exhibiting maximal growth at 25 °C, which is lower than *G. sulphuraria* at 38 °C. It is known that amongst Rhodophyta, all Cyanidiophytina encountered an extensive reduction of their genome. It has been proposed that this is an adaptation strategy to stressful environmental conditions. *G. phlegrea* have regained genes through horizontal gene transfer, suggested as an ameliorative strategy for adaptation to specific environmental niches (Qiu et al. 2013).

Genomic analyses revealed that *G. phlegrea* and *G. sulphuraria* belong to different taxa, since the protein divergences between them are comparable to the protein-divergence distances between humans and teleosts (Qiu et al. 2013). The rbcL sequences of Turkish *G. sulphuraria* isolates showed the highest genetic variability both in terms of haplotype diversity and in nucleotide diversity, followed by Taiwanese conspecific specimens. *G. sulphuraria* strains from Turkey clustered in two separate lineages, the former including Italian isolates, the latter including Icelandic strains. This finding suggests that there have been at least two separate introductions from Turkey in Western Europe; the levels of inter-populational genetic differentiation suggested a dispersal ability significantly higher between Turkey and Italy than between Turkey and Iceland, which would be consistent with a correlation between genetic and geographic distance.

Ciniglia et al. (2014) previously hypothesized that the northeastern Asian populations of *Galdieria* would be the potential donor of Icelandic *G. sulphuraria* populations, because of the occurrence of the Russian species *G. daedala* within the same clade, alongside some Turkish accessions. The strong monophyly among Turkey, Iceland, and Russian strains, along with the highly divergent haplotypes associated with Turkish accessions, would be consistent with Turkey in being a center of *G. sulphuraria* diversification and dispersal to Western European sites. A similar pattern was found in the *G. maxima* clade; Turkish isolates strictly grouped both with Icelandic and with Japanese and Taiwanese accessions, along with the Russian haplotype *G. maxima* IPPAS P507. In the present study, the combination of high haplotype and low nucleotide diversity is a signature of a rapid population expansion from a small effective population size (Avise 2000); Tajima's *D* test and Fu's *F*s tests, applied to find out the population expansion, were both negative in all cases; this indicates excess of the rare mutations in populations, thus supporting the hypothesis of recent population expansions within Cyanidiophytina.

The discovery of Cyanidiophyceae in Turkey confirms the cosmopolitan distribution of these algae, despite the peculiar ecological requirements that are present in discontinuous and distant habitats. The worldwide distribution of extremophiles has been demonstrated also for *Sulfolobus*, an archea inhabiting the geothermal sulfuric springs at $T > 70$ °C and strongly acidic pH, isolated in several hot springs throughout Northern hemisphere (Brock et al. 1972; Zuo et al. 2015). It is intriguing for these extremophiles, such as the Cyanidiophytina, to understand how they can survive long-distance dispersal, through inhospitable environments, without tolerating desiccation and without producing resistance spores.

We examined the population structure in *G. sulphuraria*, *G. maxima*, *G. phlegrea*, and *C. merolae*, measuring F_{st}, a parameter that provides a measure of population differentiation based on genetic variance between the populations. Pairwise comparisons between strains grouped by region have produced different results in the Cyanidiophycean taxa. Large, significant F_{st} values across the hydrothermal locations were recorded in *G. sulphuraria* and *G. maxima* suggesting a high level of genetic differentiation, and a reduction in dispersal ability of the individuals. However, in *G. maxima* low F_{st} values were recorded among the Asiatic populations, indicating that there is at least a small level of genetic differentiation between them, and a substantial level of gene flow. Perhaps this was due to the contiguity of the geothermal areas, being located on the Ring of Fire. Within *C. merolae* and *G. phlegrea,* F_{st} values were not significantly different from zero ($F_{st} = 0.05$ and 0.013, respectively), indicating that populations from different geothermal springs were not genetically differentiated, suggesting a frequent gene flow among the geothermal springs. *G. phlegrea* populations to date have only been identified in Turkey and Italy, and it is intriguing that even in *G. sulphuraria*, the lowest level of genetic differentiation was recorded between the same populations. This supports the hypothesis of gene flow between Turkey and Italy. The level of genetic divergence of *G. phlegrea* was much lower than that observed in *G. sulphuraria* and in *G. maxima. G. phlegrea* has a restricted areal of dispersal, because of its peculiar adaptation to dry habitats, such as rock fissures, chasmoendolithic and cryptoendolithic environments. These habitats were very frequently encountered in Turkey, and are preferred by *G. phlegrea* in spite of fumaroles and hot springs.

Significant levels of genetic divergence were reported for other extremophilic microorganisms, such as in populations of *Sulfolobus solfataricus* where gene flow among different geothermal stations is limited (Whitaker et al. 2003). However, while in *S. solfataricus* the global population structure is mainly ascribed to isolation-by-distance,

in Cyanidiophytina, namely in *G. sulphuraria* as well as in *G. maxima*, gene flow and species dispersal among populations was not found to increase with the geographic distance. This is notable as there was no significant positive correlation between genetic and geographic distance. For an extremophile, hot springs may be considered as island-like habitats occurring as clusters in globally distant regions. For an extremophilic organism to thrive in such conditions, they must adapt to drastically different conditions from the surrounding habitat through which they would have to disperse (Ramette and Tiedje 2007). As such, it would be expected that geographical isolation might be an important component in the diversification of microextremophiles (Papke et al. 2003), as already observed in *S. solfataricus* (Whitaker et al. 2003). In stark contrast, our results suggest that for Cyanidiophyceae, their growth requirements limit dispersal, but do not prevent it. The discovery of such a high number of Cyanidiophycean species and strains from global explorations is helpful to better delineate ecological boundaries. Moreover, the phylogenetic analyses strongly support the reconstruction of the relationships between the 6 lineages recovered. For this purpose, sequencing of the whole rbcL gene as well as additional markers, such as the nuclear small and large subunit rDNA genes (SSU and LSU), concatenated with rbcL, should result in a substantial improvement in phylogenetic resolution.

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References

Albertano P, Ciniglia C, Pinto G, Pollio A (2000) The taxonomic position of *Cyanidium*, *Cyanidioschyzon* and *Galdieria*: an update. Hydrobiologia 433:137–143

Allen MM, Stanier RY (1968) Selective isolation of blue-green algae from water and soil. J Gen Microbiol 51:203–209

Avise JC (2000) Phylogeography: the history and formation of species. Harvard University Press, Cambridge

Azúa-Bustos A, González-Silva C, Mancilla RA, Salas L, Palma RE, Wynne JJ, McKay CP, Vicuña R (2009) Ancient photosynthetic eukaryote biofilms in an Atacama Desert coastal cave. Microb Ecol 58:485–496

Brock TD (1978) The genus *Cyanidium*. In: Starr PM (ed) Thermophilic microorganisms and life at high temperatures. Springer, New York, pp 255–301

Brock TD, Brock KM, Belly RT, Weiss RL (1972) *Sulfolobus*: a new genus of sulfur-oxidizing bacteria living at low pH and high temperature. Arch Mikrobiol 84:54–68

Cennamo P, Ciniglia C (2017) The algal diversity in the Phlegrean Fields (Campania, Italy) archeological districts. UPLanD 2(2):97–106

Cennamo P, Marzano C, Ciniglia C, Pinto G, Cappelletti P, Caputo P, Pollio A (2012) A survey of the algal flora of anthropogenic caves of Campi Flegrei (Naples, Italy) archeological district. J Cave Karst Stud 74(3):243–250

Ciniglia C, Yoon HS, Pollio A, Pinto G, Bhattacharya D (2004) Hidden biodiversity of the extremophilic Cyanidiales red algae. Mol Ecol 13:1827–1838

Ciniglia C, Yang EC, Pollio A, Pinto G, Iovinella M, Vitale L, Yoon HS (2014) Cyanidiophyceae in Iceland: plastid rbcL gene elucidates origin and dispersal of extremophilic *Galdieria sulphuraria* and *G. maxima* (Galderiaceae, Rhodophyta). Phycologia 53(6):542–551

Ciniglia C, Pinto G, Pollio A (2017) *Cyanidium* from caves: a reinstatement of *Cyanidium chilense* Schwabe (Cyanidiophytina, Rhodophyta). Phytotaxa 295(1):86–88

Darienko T, Hoffmann L (2010) Subaerial algae and cyanobacteria from the archaeological remains of Carthage (Tunisia), including the record of a species of *Cyanidium* (Rhodophyta). Algol Stud 135(1):41–60

Del Rosal Y, Jurado V, Roldán M, Hernández Mariné M, Sáiz-Jiménez C (2015) *Cyanidium* sp. colonizadora de cuevas turísticas. In: Moreno Oliva M, Rogerio-Candelera MA, López Navarrete JT, Jolín VH (eds) Estudio y Conservación del Patrimonio Cultural. Actas, Universidad de Málaga, Malaga, pp 170–173

Doemel WN, Brock TD (1971) The physiological ecology of Cyanidium caldarium. J Gen Microbiol 67:17–32

Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Res 10(3):564–567

Friedmann I (1964) Progress in the biological exploration of caves and subterranean waters in Israel. Int J Speleol 1:29–33

Fu YX, Li WH (1993) Statistical tests of neutrality of mutations. Genetics 133:693–709

Gross W (1999) Revision of comparative traits for the acid- and thermophilic red algae *Cyanidium* and *Galdieria*. In: Seckbach J (ed) Enigmatic microorganisms and life in extreme environments. Kluwer Academic Publisher, London, pp 439–446

Gross W, Oesterhelt C, Tischendorf G, Lederer F (2002) Characterization of a non-thermophilic strain of the red algal genus *Galdieria* isolated from Soos (Czech Republic). Eur J Phycol 37(3):477–482

Hoffman L (1994) *Cyanidium*-like algae from caves. In: Seckbach J (ed) Evolutionary pathways and enigmatic algae: *Cyanidium caldarium* (Rhodophyta) and related cells. Kluwer, Dordrecht, pp 175–182

Hsieh CJ, Zhan SH, Lin Y, Tang SL, Liu SL (2015) Analysis of rbcL sequences reveals the global biodiversity, community structure, and biogeographical pattern of thermoacidophilic red algae (Cyanidiales). J Phycol 51(4):682–694

Leclerc JC, Couté A, Dupuy P (1983) Le climat annuel de deux grottes et d'une eglise du Poitou, ou vivent des colonies pures d'algues sciaphiles. Cryptogam Algol 4(1–2):1–19

Librado P, Rozas J (2009) DNASp v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25:1451–1452

Lowell C, Castenholtz RW (2013) The lowering of external pH in confined environments by thermo-acidophilic algae (class: cyanidiophyceae). Environ Microbiol Rep 5(5):680–684

Nei M (1987) Molecular evolutionary genetics. Columbia University Press, New York, pp 287–326

Papke RT, Niels B, Ramsing NB, Bateson MM, Ward DM (2003) Geographical isolation in hot spring cyanobacteria. Environ Microbiol 5(8):650–659

Pearce JA, Bender JF, De Long SE, Kidd WSF, Low PJ, Guner F, Saroglu Y, Yilmaz Y, Moorbath S, Mitchell JG (1990) Genesis of collision volcanism in Eastern Anatolia, Turkey. J Volcanol Geotherm Res 44:189–229

Pinto G (1993) Acid-tolerant and acidophilic algae from Italian environments. Plant Byosis 127:400–406

Pinto G, Albertano P, Ciniglia C, Cozzolino S, Pollio A, Yoon HS, Battacharya D (2003) Comparative approaches to the taxonomy of genus *Galdieria* Merola (Cyanidiales) Rhodophyta. Cryptogam Algol 24(1):13–22

Pinto G, Ciniglia C, Cascone C, Pollio A (2007) Species composition of Cyanidiales assemblages in Pisciarelli (Campi Flegrei, Italy) and description of *Galdieria phlegrea* sp.nov. In: Seckbach J (ed) Algae and cyanobacteria in extreme environments. Springer, Dordrecht, pp 489–502

Qiu H, Price DC, Weber APM, Reeb V, Yang EC, Lee JM, Kim SY, Yoon HS, Bhattacharya D (2013) Adaptation through horizontal gene transfer in the cryptoendolithic red alga *Galdieria phlegrea*. Curr Biol 23(19):865–866

Ramette AN, Tiedje JM (2007) Multiscale responses of microbial life to spatial distance and environmental heterogeneity in a patchy ecosystem. PNAS 104(8):2761–2766

Ronquist F, Huelsenbeck JP (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574

Schwabe GH (1936) Über einige Blaualgen aus dem mittleren und südlichen Chile. Verhandlungen des Deutschen Wissenschaftlichen Vereins zu Santiago de Chile 3:113–174

Schwabe GH (1942) Über das thermalbad Kusatu. Mitteilungen Der Deutschen. Gesellschaft for Natur- und Völlkerkunde Ostasiens. Otto Harrassowitz, Tokyo, Leipzig, Band XXIII(Teil C):C41–C42

Skorupa DJ, Reeb V, Castenholz RW, Bhattacharya D, McDermott TR (2013) Cyanidiales diversity in Yellowstone National Park. Lett Appl Microbiol 57:459–466

Skuja H (1970) Alghe cavernicole nelle zone illuminate delle Grotte di Castellana (Murge di Bari). Le Grotte d'Italia 4:193–202

Stamatakis A, Hoover P, Rougemont J (2008) A rapid bootstrap algorithm for the RAxML web-servers. Syst Biol 75(5):758–771

Tajima F (1983) Evolutionary Relationship of DNA sequences in finite populations. Genetics 105(2):437–460

Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123(3):585–595

Toplin JA, Norris TB, Lehr CR, McDermott TR, Castenholz RW (2008) Biogeographic and phylogenetic diversity of thermoacidophilic Cyanidiales in Yellowstone National Park, Japan, and New Zealand. Appl Environ Microbiol 74:2822–2833

Whitaker RJ, Grogan DW, Taylor JW (2003) Geographic barriers isolate endemic populations of hyperthermophilic archaea. Sci Rep 301(5635):976–978

Yang EC, Boo SM, Bhattacharya D, Saunders GW, Knoll AH, Fredericq S, Graf L, Yoon HS (2016) Divergence time estimates and the evolution of major lineages in the florideophyte red algae. Sci Rep 6:21361

Yoon HS, Hackett JD, Ciniglia C, Pinto G, Bhattacharya D (2004) A molecular timeline for the origin of photosynthetic eukaryotes. Mol Biol Evol 21:809–818

Yoon HS, Ciniglia C, Wu M, Cameron JM, Pinto G, Pollio A, Bhattacharya D (2006) Establishment of endolithic population of extremophilic Cyanidiales (Rhodophyta). BMC Evol Biol 6:78

Zuo G, Xu Z, Hao B (2015) Phylogeny and taxonomy of Archaea: a comparison of the whole-genome-based CVTree approach with 16S rRNA sequence analysis. Life (Basel) 5:949–968