

## Cyanidiophyceae in Iceland: plastid *rbcL* gene elucidates origin and dispersal of extremophilic *Galdieria sulphuraria* and *G. maxima* (Galdieriaceae, Rhodophyta)

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**ABSTRACT:** The Cyanidiophyceae are a group of unicellular organisms that diverged from ancestral red algae around 1.3 billion years ago. Present-day species are restricted to hot springs and geothermal habitats from around the world. Because of discontinuous geothermal environments, the distribution patterns and dispersal modes of the cyanidiophycean species are poorly understood. Iceland is the third largest island in the Atlantic Ocean and has intense underground volcanic activity that generates broad hydrothermal areas with different ecological conditions that are excellent for thermoacidophilic microfloral development. We analyzed populations to address the Icelandic cyanidiophycean biodiversity and dispersal. A global *rbcL* phylogeny showed two main populations inhabiting Iceland, *Galdieria sulphuraria* and *G. maxima*. Their areas of distribution are not completely superimposed because they coexisted only in New Zealand, Kamchatka (Russia), Japan, and Iceland. Because of the strong monophyly of Icelandic species with Japanese and Russian species, we hypothesized an origin and dispersion of Icelandic *G. sulphuraria* and *G. maxima* from northeastern Asia. On the basis of network analysis of *rbcL* haplotypes, it is likely that the southwestern region of Iceland is the diversity center of both *G. sulphuraria* and *G. maxima*.

**KEY WORDS:** Cyanidiophyceae, Dispersal, *Galdieria*, Population structure

### INTRODUCTION

Studies and experimental data about microbial biogeography have contributed to contrasting indigenous and ubiquity models. The individual abundance of microbial species, along with their small size, metabolic plasticity, and spore-forming ability, are features that argue in favor of microbial ubiquity and suggest that microbes are not affected by geographic or ecological dispersal (Fenchel & Finlay 2004), summarized as “everything is everywhere” (Baas-Becking 1934). However, this model collides against the evidence that so many free-living microorganisms exhibit biogeographic patterns, which modifies the statement to “everything is everywhere but the environment selects” (De Wit & Bouvier 2008), which accounts for spatial variation in microbial diversity (Foissner 2006). Neither the indigenous nor the ubiquity model can be rejected (Ragon *et al.* 2012), but neither model satisfactorily applies *ex ante* to all microorganisms. For example, extremophile dispersal is much more hampered than dispersal for nonextremophiles because of the stringent ecological requirements for extremophiles.

Cyanidiophyceae are an example of “worldwide-but-discontinuous” colonization because they inhabit acidic (pH 0–4) and thermal sites (25–55°C). They reproduce asexually and do not produce resting spores during their life cycle, as observed both in natural populations and in

laboratory conditions. Using molecular phylogenetic analyses, five lineages of Cyanidiophyceae have been described: (1) the *Cyanidium caldarium* (Tilden) Geitler clade with a low intraspecific level of molecular diversity; (2) the *Cyanidioschyzon merolae* DeLuca, Taddei & Varano clade; (3) the *Galdieria maxima* Sentsova clade with global distribution; (4) a mesophilic *Cyanidium* clade including taxa from nonthermal and nonacidic sites; and (5) the *Galdieria* clade with a negligible morphological diversity, including a cosmopolitan *G. sulphuraria* (Galdieri) Merola, two Russian species *G. daedala* Sentsova and *G. partita* Sentsova, and *G. phlegrea* Pinto, Ciniglia, Cascone & Pollio, an Italian endemic retrieved only at acidic sites (Pinto *et al.* 2003, 2007; Ciniglia *et al.* 2004, 2005; Yoon *et al.* 2006; Toplin *et al.* 2008; Skorupa *et al.* 2013). To date, studies show that worldwide hydrothermal environments are inhabited by the cyanidiophycean species; however, the distribution patterns for individual species are still unclear. All cyanidiophycean species have a simple morphology with few diagnostic features, and therefore it is almost impossible to identify taxa by naked eye or even with a microscope.

Iceland is the third largest island in the Atlantic Ocean, with an area of 103,106 km<sup>2</sup>, and it is located at the juncture of the North Atlantic and Arctic oceans. Iceland is entirely south of the Arctic Circle and located near Greenland (290 km) and the European Faroe Islands (420 km). The Mid-Atlantic Ridge (MAR) crosses Iceland from the southwest to the northeast, and throughout Iceland’s 16-million-year (Ma) geological history (Geirsdottir *et al.* 2007), volcanic activities have been mostly confined to relatively narrow

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volcanic zones that are the subaerial expression of the MAR. Around 35 volcanic systems are present in Iceland, and they are located near the junction of the eastern volcanic zone and the northern volcanic zone in central Iceland (Sturkell *et al.* 2006). The intense underground volcanic activities generate numerous and extensive hydrothermal areas with different ecological conditions (i.e. hydrothermal vents in the form of geysers, hot lakes, boiling mud, solphataras) that are excellent for the growth of a thermoacidophilic microflora. Therefore, Iceland, as a remote volcanic island, is an excellent experimental site to analyze biodiversity and dispersal pattern(s) of the cyanidiophycean species.

To improve our understanding on biogeography of Cyanidiophyceae, we studied the origin, dispersal patterns, and diversity of Iceland populations of the cyanidiophycean species using molecular phylogenetic analyses based on the *rbcL* marker. All available *rbcL* gene sequences from worldwide acidothermal populations were added to discern the potential founder population(s) for the Iceland cyanidiophycean species. The geographic distribution and the dispersal route of different Icelandic cyanidiophycean populations have been tested by the network analysis using *rbcL* haplotypes.

## MATERIAL AND METHODS

To assess the pattern of the cyanidiophycean diversity occurring within and between the geothermal areas in Iceland, environmental samples were collected from five Icelandic geothermal stations in southwestern Iceland: Gunnuhver (SWI.GU), Nesjavellir (SWI.NE, Fig. 1), Seltun (SWI.SE, Fig. 2); southeastern Iceland: Landmannalaugar (SEI.LA, Fig. 3); and northeastern Iceland: Viti (NEI.VI, Fig. 4; Table S1). The number of samples collected in each site was directly proportional to the wideness of the site and to the variety of contrasting environments. Gunnuhver is a narrow geothermal area of steaming fumaroles and boiling mud pools, with no water springs and geysers. Nesjavellir is located on a geothermal rift outlying from the much bigger North Atlantic rift (Fig. 1). Krisuvik is a geothermal area situated on the Reykjanes peninsula in the middle of the fissure zone on the MAR. It is a wide area consisting of several geothermal fields, including Seltun, which contains solfataras, fumaroles, mud pots, and hot springs (Fig. 2). Samplings in SEI were carried out in the natural geothermal hot springs of Landmannalaugar (Figs 3, 5, 6), a region near the volcano Hekla. NEI in the Myvatn region is characterized by Krafla caldera, and Viti is one of its craters characterized only by steaming fumaroles and boiling mud pools (Fig. 4).

The distance between stations varied from 32.8 to 612 km, with no geothermal continuity among the stations. For each station, samples were collected where algae were present either superficially or covered by sulphur crystals, crumbly soil, and mud layers (Fig. 5). Temperature was measured for each sample with a digital thermometer (Field Environmental Instruments, Pittsburgh, Pennsylvania, USA), and pH was measured with a portable pH meter (Hanna Instru-

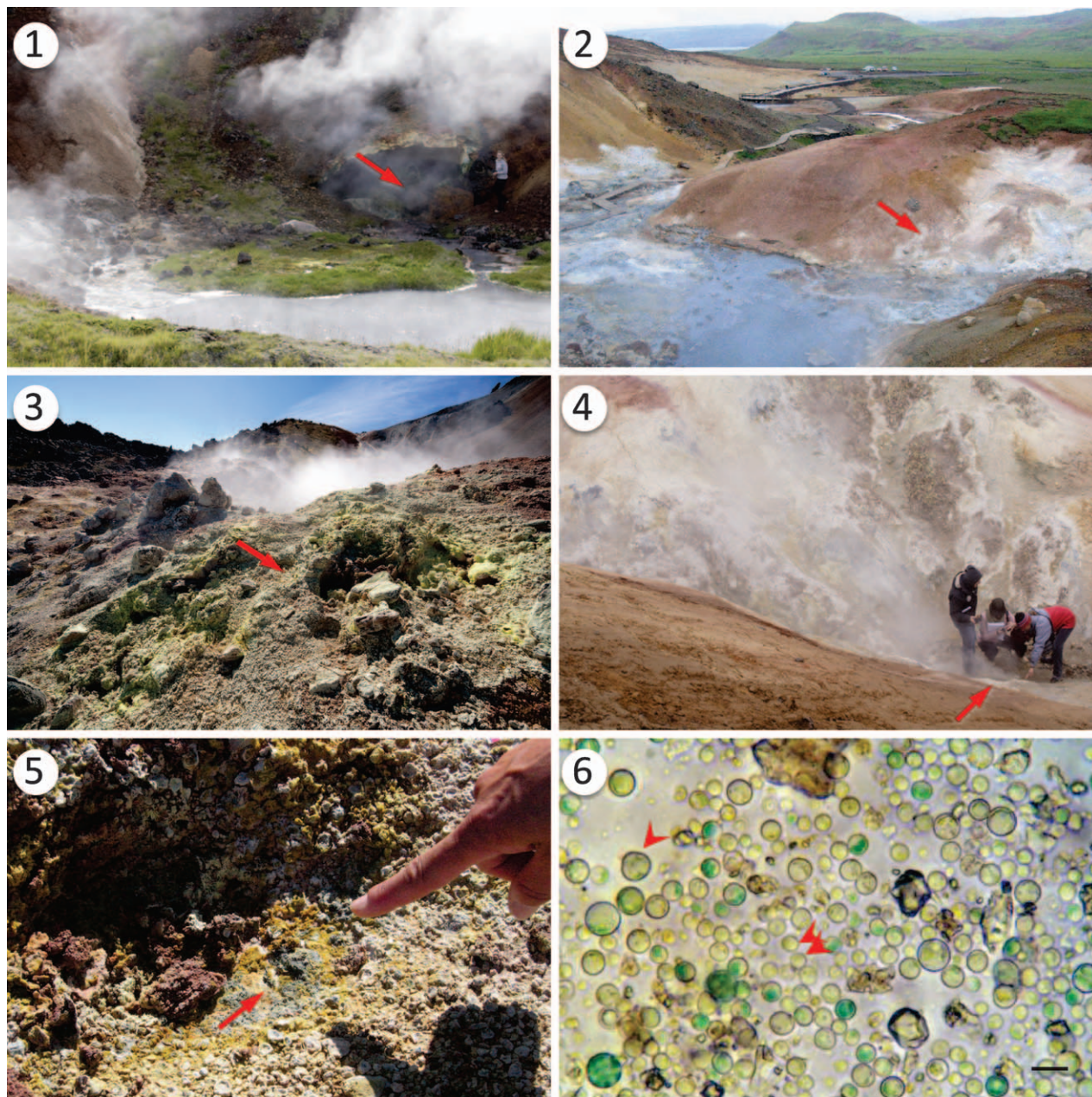
ments, Padova, Italy) along with pH indicator strips. Environmental information was summarized in Table S1.

Cyanidiophycean mats were collected by scraping the surface, kept in sterile tubes, and refrigerated until their use. To obtain monoclonal cultures, each sample was serially diluted in Allen's medium (Allen & Stanier 1968) at pH 1.5 by using multiwell plates (see Fig. 6). Maximum dilution enrichments were also plated on the agar medium at pH 1.5. Single colonies were picked up from the plates and suspended in liquid medium. 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, [www.acuf.net](http://www.acuf.net)) (Table S2). Cultures are available upon request to the authors.

For DNA extraction, algal cells both from isolated and from environmental samples were ground with glass beads using a Mini-BeadBeater (BioSpec, Bartlesville, Oklahoma, USA) operated at 13,000 revolutions per min for 1 min. Total genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Santa Clarita, California, USA). Four degenerate primers were used to amplify the *rbcL* gene from environmental samples and from isolates (Ciniglia *et al.* 2004). The polymerase chain reaction (PCR) products were purified with the QIAquick PCR purification kit (Qiagen) and used for direct sequencing using the BigDye™ Terminator Cycle Sequencing Kit (PE-Applied Biosystems, Norwalk, Connecticut, USA) and an ABI-3100 at the Center for Comparative Genomics at the University of Iowa (Iowa City, Iowa, USA).

Forward and reverse electropherograms were assembled and edited using the program Chromas Lite v.2.1 ([www.techneium.com.au/chromas.html](http://www.techneium.com.au/chromas.html)). Published sequence data (e.g. Ciniglia *et al.* 2004, Toplin *et al.* 2008, Skorupa *et al.* 2013) were obtained from GenBank ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). Each DNA sequence was aligned with published available sequence, then refined on the basis of translated amino acid sequence manually using Se-AL v.2.0a11 (Sequence Alignment Editor, <http://evolve.zoo.ox.ac.uk>). No gaps or indels have been incorporated in the alignments. Newly determined sequences were available from the GenBank; final alignments are available by request to HSY.

DNA polymorphism levels within locations and regions were summarized in haplotype diversity ( $h$ ) and nucleotide diversity ( $\pi$ ) with standard deviation. Population expansions were assessed by neutrality tests implemented in DnaSP v.5.10.01 (Librado & Rozas 2009) for Tajima's  $D$  (Tajima 1989) and the  $F^*$  of Fu & Li (1993). Population genetic analyses were conducted using ARLEQUIN v.3.5.1.3 (Excoffier & Lischer 2010), following basic analytic scheme as described in Yoon *et al.* (2014). A hierarchical analysis of molecular variance (AMOVA) framework was used to estimate three molecular variance components, such as among the regions (SWI, SEI, and NEI), among locations within each region, and within each location. Significance of the AMOVA was validated using random permutations of sequences among populations ( $\Phi$  statistics). Gene genealogies were estimated with the minimum spanning network (MSN) in ARLEQUIN and statistical parsimony network (SPN) in TCS v.1.21 (Clement *et al.* 2000). In TCS cladogram estimations, we checked the connections among

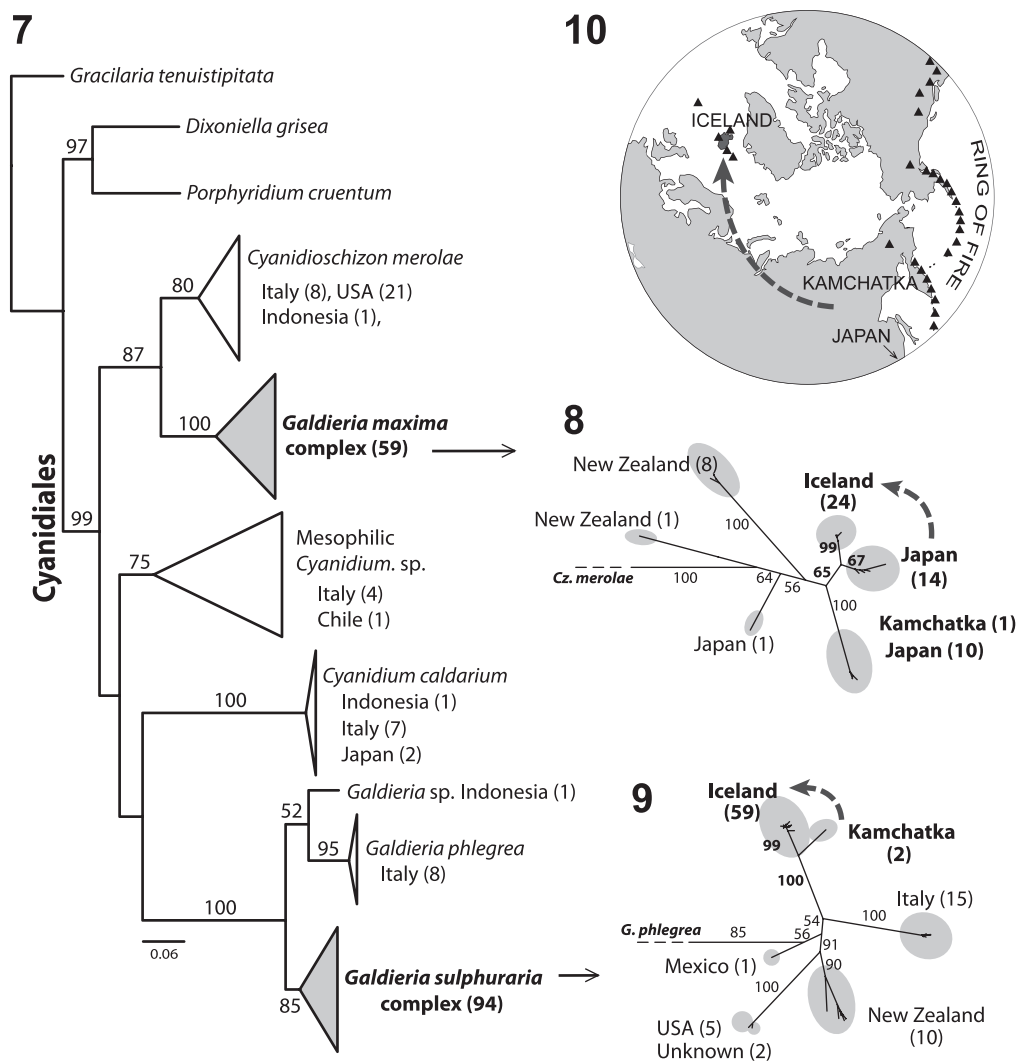


**Figs 1–6.** Sampling stations from five Icelandic geothermal regions for the Cyanidiophyceae. Arrows indicate collection site from the stations.  
**Fig. 1.** Nesjavellir of Thingvellir, southwestern Iceland (SWI.NE).  
**Fig. 2.** Seltun of Krisuvik, southwestern Iceland (SWI.SE).  
**Fig. 3.** Landmannalaugar of Hekla, southeastern Iceland (SEI.LA).  
**Fig. 4.** Viti of Krafla, northeastern Iceland (NEI.VI).  
**Fig. 5.** Closer look at the Landmannalaugar collection site.  
**Fig. 6.** Light microscopic image of the Cyanidiophyceae collected from Landmannalaugar. Single arrowhead indicates tentatively identified *Galdieria maxima*; double arrowheads indicate possible *G. sulphararia* with smaller cell size. Scale bar = 10  $\mu$ m.

haplotypes with 90, 95, and 99% confidence limits, respectively. Asymmetric gene flows between regions were estimated using MIGRATE-N v.2.4.1 (Beerli & Felsenstein 2001, Beerli 2006). We used a Bayesian search strategy as described in Yang *et al.* (2008).

Maximum likelihood (ML) analyses were performed using the RAxML v.7.2.8 (Stamatakis 2006). The best likelihood tree was estimated under the general time reversible substitution with gamma-distributed rate heterogeneity (G

model. Automatically optimized subtree pruning and re-grafting branch rearrangement and rapid hill-climbing tree search method (default setting) was used for ML tree construction. One thousand bootstrap analyses (MLB) were performed using the same program option. Alternative relationships were evaluated using the approximately unbiased (AU) and Shimodaira–Hasegawa (SH) tests (Shimodaira 2002) implemented in CONSEL v.0.1k (Shimodaira & Hasegawa 2001). The test was performed with 100,000



**Figs 7–10.** Maximum likelihood tree of Cyanidiophyceae and hypothetical dispersal routes giving rise to the Icelandic *Galdieria* species.

**Fig. 7.** Simplified phylogeny of the Cyanidiophyceae inferred from maximum likelihood analysis using RAXML based on *rbcL* sequences. The original ML tree is shown in Fig. S1. Bootstrap support values are indicated near nodes. Number in the parentheses represents the number of isolates with *rbcL* sequences. Scale bar = number of substitutions per nucleotide.

**Fig. 8.** Unrooted ML tree of *Galdieria maxima* strains. Number in parentheses represents number of culture isolates.

**Fig. 9.** Unrooted ML tree of the *Galdieria sulphuraria* strains. Number in parentheses represents number of culture isolates.

**Fig. 10.** A hypothetical dispersal route (arrow) that led to colonization on Iceland. Triangles indicate volcanos.

bootstrap replicates using the same evolutionary models as used in the best ML tree search.

## RESULTS

A total of 1191 base pairs of 215 *rbcL* sequences was aligned, including 83 Iceland cyanidiophycean species (Table S2), 129 cyanidiophycean *rbcL* sequences available from GenBank, and three outgroup taxa (Figs 7–9, S1). The *rbcL* phylogeny identified only two cyanidiophycean species, *Galdieria sulphuraria* and *G. maxima*, from Iceland. Members of all the other cyanidiophycean lineages were not encountered in the analysis. Fifty-nine isolates of *G. sulphuraria* from Iceland (Figs 9, S1) were grouped together with *G. daedala* IPPAS P508 and *G. partita* IPPAS P500

from Kamchatka with strong bootstrap support (100% MLB). This clade was the sister group to isolates from Italy ( $n = 15$ ), New Zealand ( $n = 10$ ), Mexico ( $n = 1$ ), and the United States ( $n = 7$ ). Twenty-four Icelandic *G. maxima* isolates (Figs 8, S1) were grouped with Japanese ( $n = 25$ ), Kamchatka ( $n = 1$ ), and New Zealand ( $n = 9$ ) isolates with 100% bootstrap support.

The *rbcL* of 59 *Galdieria sulphuraria* revealed 12 variable sites (1%) with 12 haplotypes (haplotypes A–L; Table 1). Twelve variable positions were evenly distributed along the alignment and each codon position (i.e. first codon position, 769, 802, 805, and 1072; second codon position, 806, 827, 860, and 1136; and third codon position, 300, 447, 1011, and 1137). All substitutions occurred on the first and second codon positions, and half of the third codon position (positions 447 and 1137) showed nonsynonymous substitution, whereas only two substitutions that occurred

**Table 1.** Condensed *rbcL* alignment and haplotype distributions for *Galdieria sulphuraria* and *G. maxima* from Iceland. Only haplotype positions are included. Dots indicate that nucleotides are identical to haplotype A. Asterisk (\*) indicates synonymous change.

Haplotype	Nucleotide position in the <i>rbcL</i> alignment (codon position)											
<i>Galdieria sulphuraria</i>	300 (3)	447 (3)	769 (1)	802 (1)	805 (1)	806 (2)	827 (2)	860 (2)	1011 (3)	1072 (1)	1136 (2)	1137 (3)
A	A	T	C	G	G	G	G	A	A	G	A	A
B	.	.	.	.	.	.	.	.	G*	.	.	.
C	.	.	A	.	.	.	.	.	.	.	.	.
D	.	.	.	.	A	.	.	.	.	.	.	.
E	G*	.	.	.	.	.	.	.	.	.	.	.
F	.	.	.	.	.	.	.	.	.	.	T	T
G	.	G	.	.	.	.	.	.	.	.	.	.
H	.	G	.	A	.	.	.	.	.	.	.	.
I	.	G	.	.	.	.	.	G	.	.	.	.
J	.	G	.	.	A	A	.	.	.	.	.	.
K	.	G	.	.	.	.	.	.	.	T	.	.
L	.	G	.	.	.	.	A	.	.	T	.	.
<i>Galdieria maxima</i>	150 (3)	388 (1)	410 (2)	817 (1)	892 (3)	893 (2)	895 (1)					
M	C	C	A	A	A	T	G					
N	.	.	.	C	.	.	.					
O	.	.	T	.	.	.	.					
P	.	.	.	.	T*	G	A					
Q	A*	T*	.	.	.	.	.					

on the third position (positions 300 and 1011) were the synonymous change. Twenty-three sequences (one partial sequence excluded) of *G. maxima* revealed seven variable sites (0.6%) that defined five haplotypes (haplotypes M–Q; Table 1). Seven variable positions included three first-codon positions (positions 380, 817, and 895), two second-codon positions (positions 410 and 893), and two third-codon positions (positions 150 and 892). Four substitutions at positions 410, 817, 893, and 895 were nonsynonymous changes.

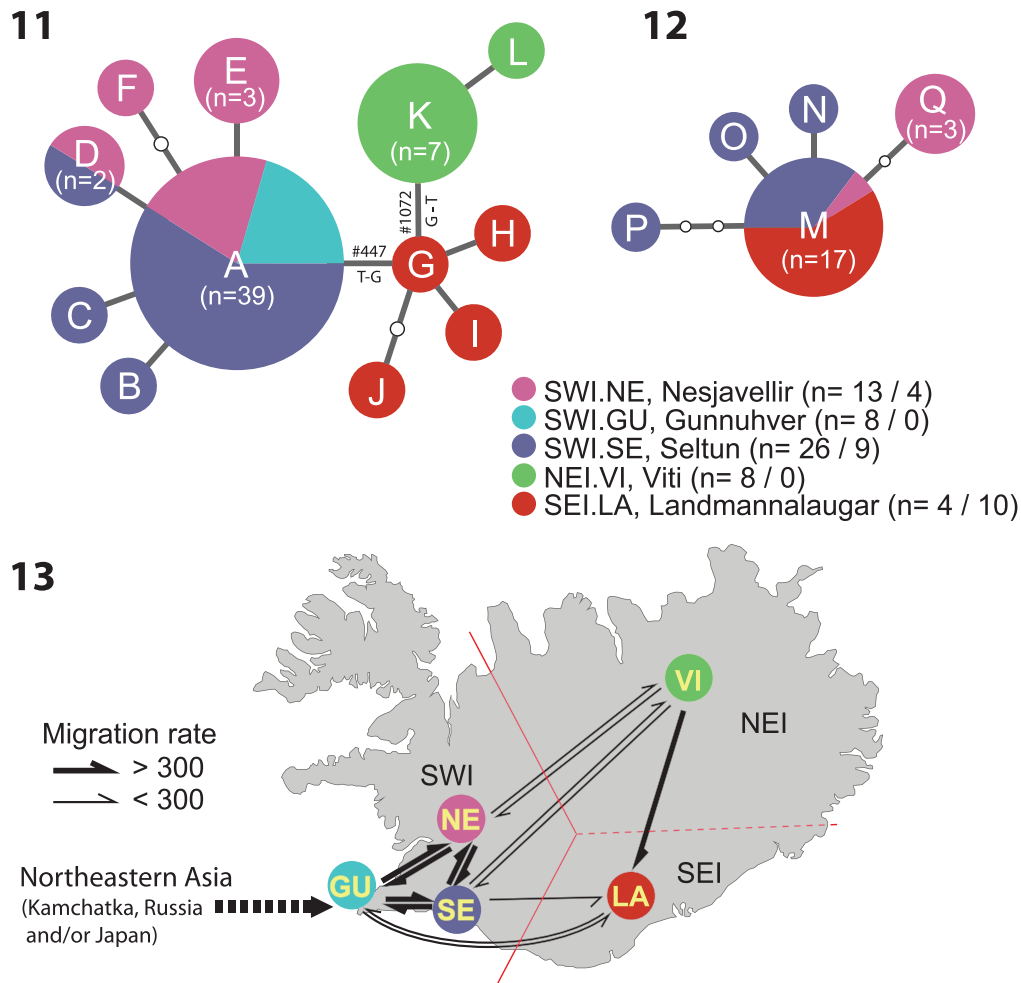
MSNs of *Galdieria sulphuraria* and *G. maxima* based on *rbcL* sequence data are shown in Fig. 11 and Fig. 12 respectively. The SPN showed basically the same relationship, except one unresolved connection between haplotypes D and J, which was clearly separated in MSN. Thus, we processed subsequent analyses and interpretations on the basis of the MSN. In the *G. sulphuraria* network (Fig. 11), 12 haplotypes (A–L) were geologically structured for three regions (SWI, NEI, SEI). Each region included exclusive haplotypes: haplotypes A–F were only found in SWI, haplotypes G–J were found in SEI, and haplotypes K and L were found in NEI, respectively (Table 2). Haplotype A from Seltun, Gunnuhver, and Nesjavellir (SWI) populations showed the highest outgroup weight probability (0.325 on the basis of SPN; 95% connection limit level) with the highest frequency ( $n = 39$ ) than any other haplotypes (B–L; outgroup weight probability = 0.003–0.311,  $n = 1–7$ ) and positioned in the center of the network (Fig. 11). Most haplotypes were connected with their neighbor haplotypes by one mutation step, however, between haplotypes A and F; haplotypes G and J required one missing haplotype for connections. Two *rbcL* mutations were used as diagnostic characters for regional populations: position 447 (T–G substitution; nonsynonymous mutation between aspartic acid and glutamic acid) for the west vs east populations, and position 1072 (G–T substitution; nonsynonymous mutation between alanine and serine) for the northeast vs southeast populations (Figs 11, 13). In the *G. maxima* network, five haplotypes (M–Q)

were found (Fig. 12). Haplotype M from Landmannalaugar, Seltun, and Gunnuhver populations showed the highest outgroup weight probability (0.864) and frequency ( $n = 17$ ) than the rest of the haplotypes (N–Q; outgroup weight probability = 0.027–0.068;  $n = 1–3$ ).

In the neutrality tests of *Galdieria sulphuraria*, Tajima's  $D$  and  $F^*$  of Fu and Li were both significantly negative for the pooled SWI samples ( $D = -1.865$  and  $F^* = -2.470$ , Table 3); however, all samples from the other regions (NEI and SEI) and locations of SWI (i.e. Gunnuhver, Nesjavellir, and Seltun) showed negative values of Tajima's  $D$  but without statistical significance ( $P > 0.05$ ). Seltun (SWI region) and overall *G. sulphuraria* showed significant negative values of  $F^*$  of Fu and Li, but Tajima's  $D$  was not statistically significant. None of the values for Tajima's  $D$  and  $F^*$  of Fu and Li of *G. maxima* sites (regions and locations) was significantly different from zero (Table 3).

The genetic structure of the *Galdieria sulphuraria* population in Iceland was determined by hierarchical AMOVA (Table 4). The *rbcL* AMOVA revealed strong (significant  $\Phi$  index,  $P < 0.05$ ) population subdivisions among regions, among locations within each region, and within each location (Table 4). Molecular variation based on sequence divergence using the Kimura 2 parameter model was larger among regions (78.65%) than among locations within regions (0.81%) and within locations (20.54%).

Estimated gene flow based on the *rbcL* data of *Galdieria sulphuraria* in Iceland revealed relatively high levels of gene flow ( $M > 300$ ) within three sites (Gunnuhver, Nesjavellir, and Seltun) of SWI region, and from Viti of NEI region to Landmannalaugar of SEI region (see bold arrows in Fig. 13). The mode value of  $M$  was lower than 300 between Nesjavellir–Viti, Seltun–Viti, Gunnuhver–Landmannalaugar, and Seltun–Landmannalaugar (thin arrows in Fig. 13), whereas there was no gene flow between Gunnuhver and Viti, from Landmannalaugar to Viti, and from Landmannalaugar to Seltun.



**Figs 11–13.** Analyses of *rbcL* haplotypes and the estimated migration routes for cyanidialean red algae in Iceland. Haplotypes are colored according to the collection locations. For Figs 11, 12, circle sizes with numbers reflect the number of specimens sharing the same haplotype; small open circles represent missing haplotypes, and the bar between haplotypes indicates a single base substitution; numbers beside collection locations indicate number of isolates.

**Fig. 11.** *Galdieria sulphuraria* haplotype network comprised of 12 haplotypes (A–L). Substitutions characterizing transitions between three haplotypes (A, G, and K) are indicated with position number in the nucleotide alignment.

**Fig. 12.** *Galdieria maxima* haplotype network comprised of five haplotypes (M–Q).

**Fig. 13.** Estimated migration rates among sample locations. All possible asymmetric gene flows between locations were estimated using the Bayesian strategy. Bold arrows indicate higher migration rates between populations (> 300); thin arrows indicate lower migration rates between populations (< 300). The dotted line between northeast Iceland (NEI) and southeast Iceland (SEI) indicates genetic connectivity based on the high rate of gene flow from Viti (VI) to Landmannalaugar (LA).

**DISCUSSION**

Extensive Cyanidiophyceae sampling from different world-wide acidothermal areas is helping to build the dispersal

areas of each taxon and to investigate their diversity, dispersion, and distribution. In particular, acidothermal sites worldwide do not always have similar and overlapping thermoacidophilic algal communities. An interesting two-

**Table 2.** Number of haplotypes of *Galdieria sulphuraria* and *G. maxima* from each sample site [southwest Iceland, Gunnuhver site (SWI.GU); southwest Iceland, Seltun site (SWI.SE); southwest Iceland, Nesjavellir site (SWI.NE); northeast Iceland, Viti site (NEI.VI); southeast Iceland, Landmannalaugar site (SEI.LA)].

Species	<i>Galdieria sulphuraria</i>												<i>Galdieria maxima</i>							
	A	B	C	D	E	F	G	H	I	J	K	L	Sum	M	N	O	P	Q	Sum	
Haplotype																				
SWI.GU	8												8							
SWI.SE	23	1	1	1									26	6	1	1	1		9	
SWI.NE	8			1	3	1							13	1					3	4
NEI.VI												7	1						8	
SEI.LA							1	1	1	1			4	10					10	
Sum	39	1	1	2	3	1	1	1	1	1	7	1	59	17	1	1	1	3	23	

**Table 3.** Summary statistics of *rbcL* haplotypes for the Icelandic cyanidiophycean strains; *n* = sample size, *v* = variable sites, *N* = number of haplotypes, *h* = haplotype diversity,  $\pm s$  = standard deviation,  $\pi$  = nucleotide diversity, and N.A. = data not available. Asterisk (\*) indicates statistically significant difference ( $P < 0.05$ ). Sampling location codes: GU = Gunnuhver, NE = Nesjavellir, NEI = northeast Iceland, SE = Seltun, SEI = southeast Iceland, SWI = southwest Iceland.

Taxa region	Location code	<i>n</i>	<i>V</i>	<i>N</i>	<i>h</i> $\pm$ <i>s</i>	$\pi$ $\pm$ <i>s</i>	Tajima's <i>D</i> test	<i>F</i> * test of Fu and Li	
<i>Galdieria sulphuraria</i>	SWI	all	47	6	6	0.311 $\pm$ 0.087	0.0003 $\pm$ 0.0001	-1.865*	-2.470*
		GU	8	0	1	0	0	N.A.	N.A.
		SE	26	3	4	0.222 $\pm$ 0.106	0.0002 $\pm$ 0.0001	-1.734	-2.708*
		NE	13	4	4	0.603 $\pm$ 0.131	0.0007 $\pm$ 0.0002	-1.167	-1.482
	NEI	VI	8	1	2	0.250 $\pm$ 0.180	0.0002 $\pm$ 0.0002	-1.055	-1.204
	SEI	LA	4	4	4	1.000 $\pm$ 0.177	0.0017 $\pm$ 0.0005	-0.780	-0.721
Total		59	12	12	0.552 $\pm$ 0.075	0.0009 $\pm$ 0.0002	-1.723	-3.053*	
<i>Galdieria maxima</i>	SWI	all	13	7	5	0.692 $\pm$ 0.119	0.0013 $\pm$ 0.0004	-1.206	-1.538
		SE	9	5	4	0.583 $\pm$ 0.183	0.0009 $\pm$ 0.0004	-1.678	-2.039
		NE	4	2	2	0.500 $\pm$ 0.265	0.0009 $\pm$ 0.0005	-0.710	-0.604
	SEI	LA	10	0	1	0	0	N.A.	N.A.
	Total		23	7	5	0.451 $\pm$ 0.121	0.0008 $\pm$ 0.0003	-1.646	-2.171

way “middle-ground” model (Azeem Jadoon *et al.* 2013) takes into account both the ubiquity and the indigenous model. When global dispersal is hampered by low transportability, tolerance, and adaptability, the microbial population is considered to be moderately endemic. According to cosmopolitanism view, all microbes have the ability to scatter ubiquitously but are limited to definite ecological conditions. Consequently, similar microbial communities colonize similar habitats (Azeem Jadoon *et al.* 2013). Within Cyanidiophyceae, there exist cosmopolitan species, moderately cosmopolitan species, and potentially endemic species. *Galdieria sulphuraria* would represent a cosmopolitan thermoacidophilic species, being retrieved in all volcanic area throughout the world (Ciniglia *et al.* 2004; Toplin *et al.* 2008; Skorupa *et al.* 2013). It is potentially ubiquitous where its peculiar environmental requirements are guaranteed. *Galdieria sulphuraria* shows the widest distribution, from the Indo-Australian plate (New Zealand), the volcanic areas of Mexico and the United States, the volcanic areas of Japan, and the peninsulas of Kunashir and Kamchatka (Russia). The area reflects perfectly “the Ring of Fire” (see Fig. 10). It is found widely in Europe including Italy (Ciniglia *et al.* 2004). It is plausible to assume that the Aleutian Islands, which act as a bridge between the North American plate and the Eurasian plate, represent a continuity in sulphur emissions that may have favored the dispersion of *G. sulphuraria* in North America.

The failure in detection of *Galdieria maxima* in the United States and in Italy would give a reliable mean of a species moderately cosmopolitan (Azeem Jadoon *et al.* 2013), as for *Cyanidium caldarium* (i.e., Indonesia, Italy, Japan) and *Cyanidioschyzon merolae* (i.e. Indonesia, Italy, United

States), whereas *G. phlegrea* should be defined as an endemic species, being retrieved only in Italian hot springs, suggesting that transportability, tolerance, and adaptability are much more difficult for these strains. However, we cannot still rule out the presence of this species in other acidothermal areas over the world because one isolate from the archipelago of Indonesia (Jave, strain DBV 074) was grouped together with *G. phlegrea*, although its bootstrap support value is low (52%).

This study demonstrated that only two species, *Galdieria sulphuraria* and *G. maxima*, inhabit Iceland (Fig. S1). Newly generated *rbcL* sequences, along with all available published data, reveal that these two species coexist only in New Zealand, Kamchatka, Japan, and Iceland. Although it is not easy to determine the founder of Icelandic *Galdieria* species, our *rbcL* phylogeny showed a strong monophyly of Icelandic *G. sulphuraria* with Kamchatka populations (known as *G. daedala* and *G. partita* but as a population of *G. sulphuraria* in our phylogenetic trees, see Figs 9, S1). A similar pattern is found for the *G. maxima* clade. Icelandic isolates strictly grouped with Japanese isolates (i.e. Fig. S1, a clade of 14 Japanese isolates from strain CCME 5675 down to strain CCME 5673), as well as a clade of 19 Japanese isolates and the Russian *G. maxima* strain IPPAS P507 (Figs 8, S1). Taken together, it is highly likely that the northeastern Asian populations were the potential donor of Icelandic *G. sulphuraria* and *G. maxima* populations. Because Iceland originated from volcanic activities 16 Ma (Geirsdottir *et al.* 2007), the Icelandic cyanidiophycean populations were dispersed throughout the Arctic basin (Fig. 10) sometime after 16 Ma. It would be interesting to find Cyanidiophyceae in Greenland because Greenland moved away from the European continent as

**Table 4.** Hierarchical analysis of molecular variation (AMOVA) for *Galdieria sulphuraria* from five locations in Iceland.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	$\Phi$ statistic	<i>P</i>
Among regions	2	17.521	0.8393	78.65	$F_{CT} = 0.7865$	0
Among locations within regions	2	0.677	0.0086	-0.81	$F_{SC} = 0.0378$	0.043
Within locations	54	11.837	0.2192	20.54	$F_{ST} = 0.7946$	0
Total	58	30.034	1.0671	99.99		

part of the Paleocene explosive volcanism in the northeastern Atlantic region and became the closest land mass to Iceland (Knox & Morton 1988). To date, however, there is no report of Cyanidiophyceae from Greenland.

The Cyanidiophyceae are aerophytic algae, and wind flow and air particles can be considered as the most reasonable means of transport for Cyanidiophyceae, allowing them to reach reasonably long distances in a short time (Proctor 1959, Toplin *et al.* 2008, Boo *et al.* 2010). Interestingly, however, only two cyanidiophycean species have been detected in Iceland. Therefore, it is likely that not all cyanidiophycean species are durable with regard to temperature, wind velocity, relative humidity, and rainfall, which play an important role in shaping the structure and composition of most aerophytic algal communities (Sharma *et al.* 2006). For example, Ciniglia and colleagues demonstrated that *Galdieria sulphuraria* (referred as *Galdieria-A* in Ciniglia *et al.* 2004) has a wider distribution within diverse environmental conditions (i.e. humidity) than *G. phlegrea* (as *Galdieria-B*). Also, *Cyanidium caldarium* was distributed in endolithic and interlithic microhabitats and *Cyanidioschyzon merolae* was dominant in a humid hot spring and ditch (Ciniglia *et al.* 2004). Furthermore, the richness and the diversity of the atmospheric algal population are strongly dependent on the process of aerosolization (i.e. release from the source and the vertical uplift), which has strong implications for dispersal limitations (Sharma & Singh 2010).

Another possible dispersal method is by birds (e.g., waterfowl, gulls). Because cyanidiophycean species generally have a weak desiccation tolerance, dispersal either within moist acidic mud on bird feet and feathers or within bird acidic digestive tracks is possible, as suggested for dispersal of other algae (Proctor 1959). However, the major water-bird migration routes for the East Atlantic Flyway (Iceland) and the East Asian Flyway (Kamchatka/Japan) do not overlap (Boere & Stroud 2006).

#### Dispersal of the Icelandic *Galdieria sulphuraria* and *G. maxima*

*Galdieria sulphuraria* network analysis shows the origin center for species as well as the dispersal route within Iceland. Half of the haplotypes ( $N = 6$ ) are limited to the southwestern region and do not overlap the haplotypes from the southeast and northeast regions. A recent study showed that there was a relationship between diversity and colonization time (Wiens *et al.* 2009). For example, a high diversity reflects the long evolutionary history for regional organisms and a low diversity reflects the relatively recent colonization in the region. Our network analyses show that the southwestern region is the diversity center for *G. sulphuraria* because this region has the highest number of haplotypes. Similarly, a pattern was found for *G. maxima*: southwestern Seltun and Nesjavellir show high haplotype diversity (Fig. 12), whereas southeastern Landmannalaugar has only one haplotype (M), perhaps representing a recent dispersal. Although *G. sulphuraria* sampling was biased [SWI ( $n = 47$ ), NEI ( $n = 8$ ), SEI ( $n = 4$ )], this could represent actual diversity because haplotype numbers were not correlated with the numbers of samples (i.e. number of

haplotypes vs number of samples = 1/8 in SWI,SE, 2/8 in NEI,VI, and 4/4 in SEI,LA, see Fig. 11 and Table 2). To better understand the population structure, equal sampling and a greater number of individuals (i.e. > 30) are required for future studies.

Migration analysis provided a similar result. High bidirectional migration rates between three southwest sites of Gunnhver, Nesjavellir, and Seltun represent a high linkage of populations within the southwest region (Fig. 13). Conversely, there is a recent single directional migration rate from Viti (northeast) to Landmannalaugar (southeast) despite the long distance (c. 300 km) between two sites. On the basis of coalescent theory, *Galdieria sulphuraria* colonized first on the southwestern region (hypothetical ancestral haplotype is abundant), then dispersed to southeast and northeast regions. Our network and migration analysis suggests that *G. sulphuraria* dispersed from southwest to northeast and southeast direction (Figs 11, 13); however, we could not rule out another dispersal route such as southwest–southeast–northeast direction, which is not rejected ( $\alpha = 0.01$  level) in alternative hypothesis tests ( $\Delta\ln L = 7.1$ ,  $P = 0.031$  in AU test, and  $P = 0.08$  in SH test).

Icelandic glaciation may provide a clue for understanding the dispersal paths of the species. Stratigraphic and sedimentological studies showed that Iceland has experienced more than 20 glaciation events during the last 4–5 Ma (Geirsdóttir *et al.* 2007). The oldest glacial deposit, 4.6–4.7 Ma, was found in the Skaftafell area in the southeast (Helgason & Duncan 2001). The other glacial deposits were formed in the north volcanic zone of the Tjörnes peninsula c. 2.5 Ma (Geirsdóttir & Eriksson 1994) and in the west volcanic zone of the Hreppar Formation about 2.1–2.2 Ma (Geirsdóttir *et al.* 1993). The last major glaciation ended approximately 15,000–10,000 years ago, and the last deglaciation occurred in Borgarfjörður located in the southwest of Iceland (Ingólfsson *et al.* 1997). Although full glaciation covered nearly all of Iceland, part of the southwest remained ice free (Andrews *et al.* 2000; Jennings *et al.* 2000), and these ice-free regions may have provided refugia for the cyanidiophycean red algae. Alternatively, cyanidiophycean algae in Iceland may have arrived from northeastern Asia as a postglacial dispersal event. Because of short branch lengths between the Iceland population and the two Russian populations of *G. sulphuraria*, a relationship that also exists for the Iceland and Japanese populations of *G. maxima*, it is possible that the divergences of two Icelandic cyanidiophycean species occurred in relatively recent times (e.g. after deglaciation; divergence time estimation is not available). This postglacial dispersal hypothesis suggests that the two Icelandic cyanidiophycean species migrated and colonized new Icelandic sites during the last 10,000–15,000 years.

According to the literature (Thordarson & Larsen 2007), currently the most active volcanic systems in Iceland, producing 80% of the verified eruptions, are located in the southeastern zone (Hekla), including the area around our Landmannalaugar sampling site. The wide, intensive, and continuous volcanic activity may explain the low haplotypic diversity in the southeast volcanic zone (e.g. Landmannalaugar) if the continuous eruptions destroy populations and a small seed population repeatedly provides cells for new settlement. In contrast, the other volcanic zones such as the



southwest volcanic zones (Reykjanes) and northeast volcanic zone (Krafla) produced only 9% and 5%, respectively, of the verified eruptions (Thordarson & Larsen 2007). Currently, Krafla is an active volcano, with the last eruptions occurring in 1984. Our sampling sites in the northeast and southwest of Iceland are part of recent and active Icelandic volcanic systems. These southwest and northeast volcanic regions are not vigorous volcanic eruptions but continuously provide thermoacidic habitats that are colonized by the Cyanidiophyceae. The oldest colonization of *Galdieria* occurred in the more stable southwest volcanic zone, whereas on the basis of neutrality tests, the southeast region experienced population expansion only recently. Taken together, all analyses support the hypothesis that *G. sulphuraria* and *G. maxima* migrated from the southwest to northeast and southeast of Iceland.

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#### SUPPLEMENTARY DATA

Supplementary data associated with this article can be found online at <http://dx.doi.org/10.2216/PH14-032.1.s1>.

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