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**A SPOTLIGHT ON *RAD52* IN *CYANIDIOPHYTINA* (*RHODOPHYTA*): A
RELIC IN ALGAL HERITAGE**

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Abstract

RAD52 (RAD52) protein catalyzes the pairing between two homologous DNA sequences double-strand break repair and meiotic recombination, mediating RAD51 loading onto single-stranded DNA ends, and initiating homologous recombination and catalyzing DNA annealing. This article reports for the first time the presence of RAD52 homologs in the thermo-acidophilic Cyanidiophyceae whose genomes have undergone extensive sequencing. Database mining, phylogenetic inference, prediction of protein structure and evaluation of gene expression were performed in order to determine the functionality of RAD52 protein in Cyanidiophyceae. Our findings support that RAD52 gene and protein have an ancient origin, though it has been subsequently lost in all green algae and land plants. Its current function in Cyanidiophytina could be related to stress damage response for thriving in hot and acidic environments as well as to the genetic variability of these algae, in which – conversely to extant Rhodophyta - sexual mating was never observed.

Keywords RAD52, Homologous recombination, Cyanidiophytina, *Galdieria*, extremophiles

Introduction

Cyanidiophytina are unicellular red algae living in volcanic and post volcanic areas, where temperatures rise above 50°C, and high sulphuric acid concentrations, generated by the oxidation of sulphur gaseous emissions, greatly reduce the pH to values (pH 0.5-3.0) prohibitive for the majority of eukaryotic life forms [1–6]. The class includes three genera, the walled *Galdieria* (*G. sulphuraria*, *G. phlegrea*, *G.*

maxima) and *Cyanidium* (*C. caldarium*, *C. chilense*) and the naked *Cyanidioschyzon* (*C. merolae*).

The long evolutionary history of Cyanidiophytina began around 1.5 BYA ([7–9], before the formation of the supercontinent Rodinia (1.3-0.9 BYA), which resulted in an increase in volcanic activity that would have favored the diversification and dispersal of these thermoacidophilic algae [7–9].

According to Gross and Bhattacharya [10], the rising oxygenic atmosphere would have exerted a selective pressure for efficient repair of ROS/UV-damaged DNA, driving ultimately the evolution of sex, through cell-cell fusions, chromosome movement, and emergence of the nuclear envelope, with the concurrent evolution of meiosis and eukaryogenesis.

The occurrence of meiotic genes is not only related to genetic variation but it is also involved in DNA repair [11]: one of the most threatening forms of DNA damage is the break of the double helix (DSB), as both strands of the DNA duplex are impaired simultaneously. The RAD52 epistasis group is implicated in various cellular processes, such as recombinational repair and chromosome pairing in meiosis, thus guaranteeing the genome integrity; in particular, the RAD52 protein catalyzes the pairing between two homologous DNA sequences double-strand break repair and meiotic recombination mediating the loading of RAD51 onto single-stranded DNA ends, and thereby initiating homologous recombination and catalyzing DNA annealing [12] RAD52 is recruited to the Replication Protein A (RPA)-single-stranded DNA nucleoprotein complex, formed upon DSB induction and exonucleolytic ends resection, and mediates its replacement by RAD51. RAD51 then catalyzes strand invasion and D-loop formation. Eventually, RAD52 may assist in capturing the second DNA end and promote its annealing to the D-loop, thus leading to the formation of a Holliday junction [13].

RAD52 Epistasis Group also includes RAD50, RAD51, RAD54, RAD55, RAD57, RAD59, RDH54, MRE11; they all cooperate in the process of homologous recombination, playing an essential role in the mitotic and meiotic cell cycles, also affecting the response to DNA damaging agents [12]. Homologues of the RAD52 group of genes have been identified in many eukaryotes, including animals and fungi [14] and in some cases in prokaryotes [15] indicating high conservation of the recombinational repair pathway. The lack of RAD52 in the vast majority of photosynthetic protists, sexuated or not, is intriguing, considering its role in homologous recombination process and its relatively high conservation across eukaryotes. Even more unexpected is the presence of this key gene in the asexual red algae *G. sulphuraria* and *C. merolae* genomes along with its absence in other available genomes from sexuated Rhodophyta such as *Porphyra* and *Chondrus*. The present paper displays the characterization of RAD52 homologs in *Galdieria sulphuraria* genomes. The correspondence of the homologs to yeast and animal of the RAD52 proteins was also provided. An in-depth sequence analysis of this protein from 17 *Galdieria* strains was performed in order to delineate its evolutionary relationship and phyletic horizon in available genomes. To exclude a relictic nature of RAD52 sequences in *Galdieria*, selective pressures acting on the sequences were detected by analysis of non-synonymous nucleotide substitutions over the number of synonymous substitutions (Ka/Ks) [16–18]. The phylogenetic analyses were combined with preliminary gene expression data on *Galdieria* in order to verify the increasing of RAD52 mRNA expression during saline stress inducing DSBs.

RESULTS AND DISCUSSION

RAD52 origin and distribution

103 RAD52 gene homolog was identified in *G. sulphuraria* 074 genome (Gasu_26690,
 104 Accession number M2XIH5). To support the identification of RAD52 homologs
 105 within the genome of all analyzed taxa, a phylobayesian inference on protein
 106 sequences was built (Fig. 1). Analyses showed that all the algal aminoacid sequences
 107 were strongly supported as homologs of RAD52 excluding then being with RAD59
 108 paralog; by the survey of the sequences, RAD52 appears to be sporadically distributed
 109 both among bacteria and eukaryotes. RAD52 protein is commonly present in
 110 Bacteria; among phototrophic bacteria, RAD52 was confirmed only for
 111 *Synechococcus* sp. (Cyanophyta), and clusterized with significant posterior
 112 probability (0.99) with *Spirochaete*, *Hyphomicrobium denitrificans* and
 113 *Phaeomarinobacter ectocarpus*. Non-ambiguous blast hits included also Haptophyta
 114 (*Emiliania huxleyi*), and Heterokontophyta (*Ectocarpus siliculosus*, *Phaeodactylum*
 115 *tricornutum*, *Thalassiosira oceanica*, *Thalassiosira pseudonana*).
 116 Within the phylogenetic tree, cyanidophycean RAD52 proteins formed a moderately
 117 supported clade with the red algal group of Florideophyceae (*Gelidium*,
 118 *Gracilariopsis* and *Calliarthron*), as sister clade of the RAD52 from Heterokonts
 119 (*Phaeodactylum tricornutum*, *Thalassiosira oceanica*, *Thalassiosira pseudonana*),
 120 with *Ectocarpus* positioned outside of this branch. Noteworthy, all these algal phyla
 121 evolved through a secondary endosymbiosis in which a primary red algal cell would
 122 have been acquired by a eukaryotic lineage [19]. Previous phylogenetic analyses
 123 supported for a monophyletic origin of the plastids in cryptophytes, haptophytes and
 124 heterokonts. According to Oliveira and Bhattacharya [20], the



Fig. 1. RAD52 homologs, rooted with the RAD52 paralogs outgroup. 140 aligned amino acid sites from 54 taxa were analyzed; this consensus topology derived from >21.000 trees, $\alpha = 1.86$ ($1.45 < \alpha < 2.28$), $pI = 7.269E-3$ ($7.4239E-8 < pI < 0.0217$) and $\ln L = -8952.79$.

plastids of heterokonts would be most closely related to members of *Cyanidium-Galdieria* group, and not directly related to cryptophytes and haptophyte plastids, thus suggesting for these last an independent origin from different members of Bangiophycidae [20].

According to our investigations, the homology search for RAD52 in green algal genomes gave no results, as well as for Land Plants, Glaucophyta and Euglenophyta. However, the databases of protein, genomic, and transcribed (EST) sequences from the NCBI queried by Samach et al (2011) would have provided the evidence of RAD52-like proteins in several plants (monocotids and dicotids), as well as in some ferns and in filamentous (*Spyrogira pratensis*) and multicellular chlorophytes (*Chara vulgaris*). A gene duplication would have occurred according to Samach et al. [21] genome investigations: the green protists *S. pratensis* and *C. vulgaris* would possess only the paralog RAD52-1, whilst the gene would be lacking in Stramenopiles, Rhodophytes and unicellular Chlorophytes.

The level of similarity among RAD52 *G. sulphuraria* sequences ranged from 72 to 100%; the clustering reflects the phylogeny built on rbcL genes [5]: *G. sulphuraria* from Euroasiatic geothermal sites clusterized in an independent lineage (posterior probability= 0.89), but forming two well supported separate subclades: subclade I, including *G. sulphuraria* from Java and Russia (bp= 100%); subclade II, including both *G. sulphuraria* from Taiwan and *G. sulphuraria* from Iceland (bp= 100%). A second lineage included American accessions of *G. sulphuraria* clusterizing with Japanese and New Zealand strains, but into two well supported subclades (Fig. 2).

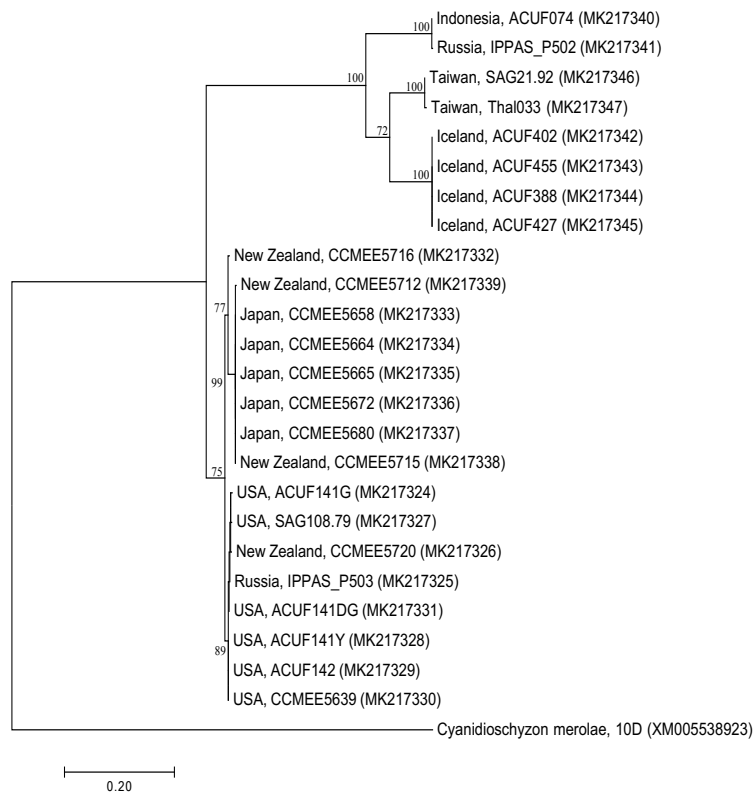


Fig. 2. Maximum likelihood tree for 24 newly sequenced *Galdieria* Rad52 gene. Only bootstrap values > 60% were reported.

Support for functional homology of RAD52 protein in Cyanidiophytina

The structure of RAD52 from Cyanidiophyceae was modeled on the base of the N-terminal domain of human RAD52 [22]. In Figs. 3 and 4 results from Selecton analysis are reported and related to information gained by I-Tasser. Results are shown concerning M8 model. Ka/Ks ratio was never higher than 1, evidencing that no divergent selection was detectable on analysed fragments. Values by MEC model were not substantially different (data not shown). The longest conserved sequence was made up of 36 residues that constitute 2 α -helix lining in the inner surface of the DNA binding groove of the protein. Many other highly conserved residues were in the first three β -sheets that constitute the outer surface of the DNA binding groove. In β -sheets, conserved residues were flanked by non-conserved ones. All five AA (I4, M9, Q59, K60 and V63) predicted as DNA binding by I-Tasser had highly conserved pattern (evidenced by a yellow square in Fig.3 and a yellow halo in Fig. 4d, e). For these residues, posterior probability evidenced a confidence interval for Ka/Ks estimated between 2.60E-05 and 3.50E-01 for I4 and between 3.20E-04 and 2.40E-01 for all the others. Residues evidenced by a red square in Fig. 3 and a yellow in Fig. 4d,e are those predicted as DNA binding sites by Kagawa [22] (K129, R130 and R133) and were highly conserved as well. The second part of the sequence, not involved in the DNA binding groove formation, seemed not to be under purifying selection during *Galdieria* speciation. In Fig. 4c, the predicted model by I-Tasser was shown, based on Singleton et al. [23] partial model for human RAD52 (Fig. 4a). All these features supported the functional homology between RAD52 from Cyanidiophyceae and the known RAD52 protein. To evaluate the functionality of RAD52 and its role in repairing DNA damage by inducing homologous recombination, the gene expression profile of RAD52 of *G. maxima* under salt-stressed conditions was analyzed using real-time quantitative PCR (qPCR). RNAs

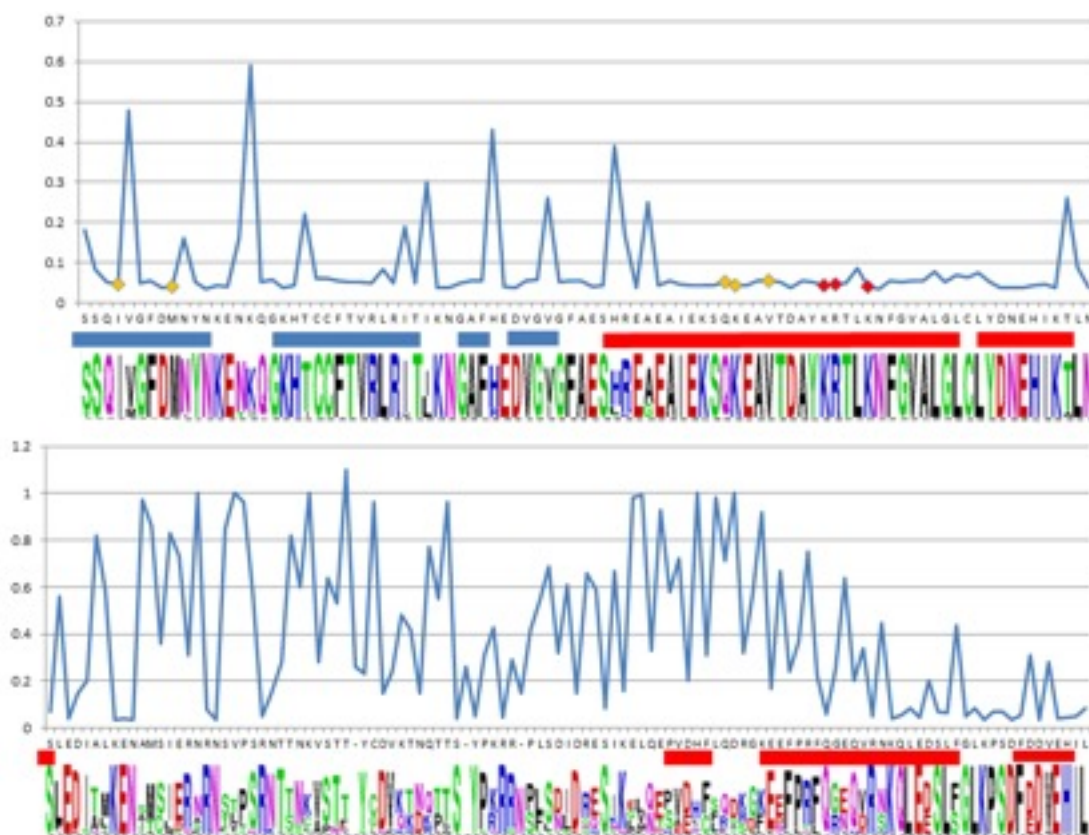


Fig. 3. Point value of Ka/Ks ratio along amino acid sequence indicated by the Web-
 logo graphics. Values gained under M8 model. Amino acid participating in a β -sheet
 formation are underlined in blue, while α -helix are underlined in red. All the five AA
 (I4, M9, Q59, K60 and V63) predicted as DNA binding by I-Tasser are evidenced by
 a yellow square on the diagram. Residues evidenced by a red square on the diagram
 are those predicted as DNA binding sites by Kagawa [22] (; K129, R130 and R133)

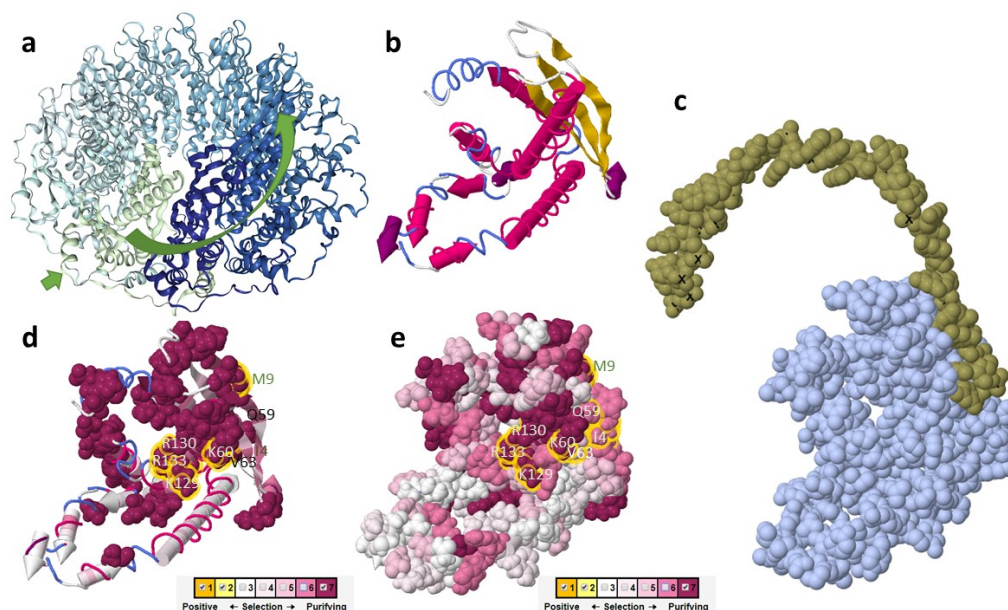


Fig. 4. Three-dimensional representation of the structure predicted by I-TASSER integrated with Selecton results; *a*, structure of human RAD 52 is reported with the DNA binding groove evidenced and chains represented in different colours; *b*, structure predicted by I-Tasser for the reference sequence used in the Selecton analysis; *c*, DNA binding site as predicted by I-Tasser; *d*, Selecton results in M8 model reported on the predicted structure, 3D structures are represented as cartoons with only strongly negatively selected sites highlighted. DNA binding AA are highlighted with yellow halos; *e*, Selecton results in M8 model reported on the predicted structure, 3D structures are represented as spacefill. DNA binding AA are highlighted with yellow halos.

were extracted at multiple points (3,6 and 12 hours) from *G. maxima* cells under sub-lethal and lethal NaCl (0,95M and 1,25M). RAD52 mRNA transcription levels increased after salt-exposition at 1.25M NaCl with a significant up-regulation at 12 hours whereas at 0.95M NaCl the fold increase was higher compared to the control up to 6 hours exposition but then a drastic decrease is observed after 12 hours (Fig. 5). Accordingly with our expectations, RAD52 gene is present and plays an important role in *Galdieria*. The observation of functional conserved residues in a RAD52 protein alignment showed that the catalytic activity of the protein may be conserved not only in *Galdieria* but also in the other related algal organisms.

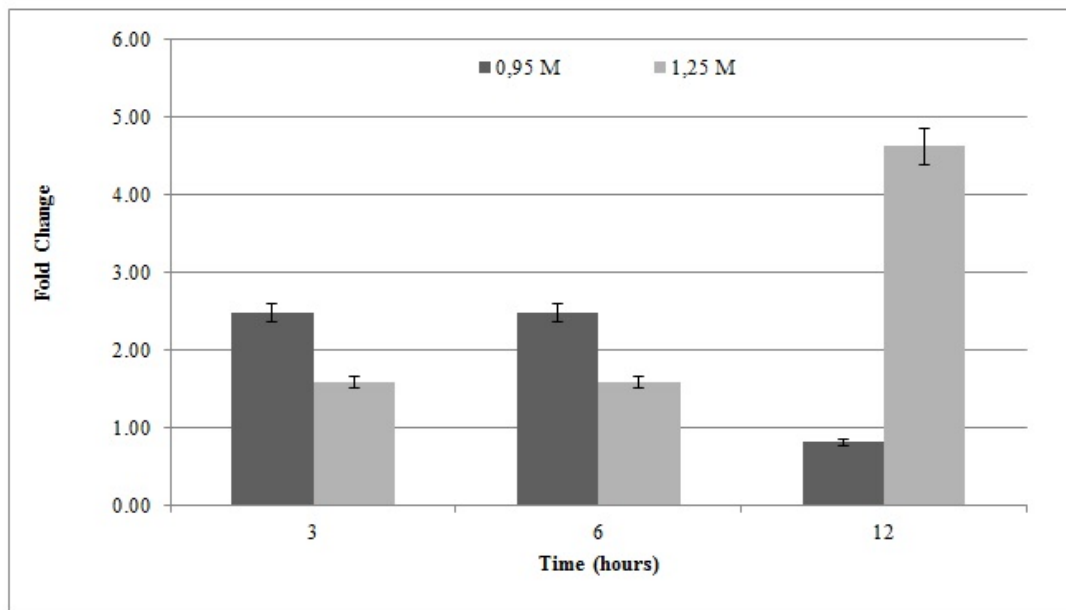


Fig. 5 .RAD52 gene expression in *G. sulphuraria* ACUF 074 cells cultured under 0.95 M (dark grey bars) and 1.25M (light grey bars) NaCl. The mRNA levels were normalized with respect to the level of mRNA for the reference genes (EF1 α and H2B). Bars show means \pm SE from three independent experiments (n=3).

Putative role of RAD52 protein in Cyanidiophytina

The findings herewith reported show RAD52 homologs in the polyextremophilic red algae Cyanidiophyceae; the conservation of predicted structures and of the amino acid residues implicated in DNA binding strongly supports the hypothesis of a common function between RAD52 from Cyanidiophyceae and the N-terminal domains of RAD52 from previously described proteins. Cyanidiophyceae are likely to be the oldest eukaryote with a RAD52 protein, in which it surely co-operates in DNA damage response and maybe in other meiosis-like mechanism of genetic variability (not shown); although RAD52 protein is lost for the most part in algae, it looks to be conserved in algal lineages derived from an event of secondary endosymbiosis involving a red alga, in which probably the ancestral RAD52 gene of the internalized rhodophyte was re-arranged and conserved. Because of its key role in DNA repair mechanism, RAD52 could have been retained as a relic heritage in some photosynthetic eukaryotes still living in primordial-like environments, while lost in others, even in closely related Rhodophyta with intricate life cycles. Being RAD52 gene crucial in meiotic machinery as well, its presence is probably also a hint for looking at sexual behavior in putatively asexual Cyanidiophytina, inhabiting in Archean environments where eukaryogenesis and meiosis co-evolved to reduce the injuries in DNA of a rising oxygen atmosphere.

Interestingly, RAD52 sequences demonstrated to have undergone purifying selection on all the part of the sequence involved in interaction with ssDNA and dsDNA. As expected, mutations in such sites may reduce fitness and are therefore more likely to be removed from the population (purified sites) [24]. In the remaining part of the sequence, instead, several K, R and Y residues are conserved, interspersed in a variable amino acidic context. As evidenced in human, these parts of the sequence are responsible of the globular structure of each module of RAD52 and of the interactions

between modules. In such regions of the protein, a certain sequence variability is compatible with the maintaining of the function.

MATERIAL AND METHODS

Bioinformatics and phylogenetic analysis

RAD52 nucleotide sequences of *G. sulphuraria* 074 (Java, Indonesia) and *Cyanidioschyzon merolae* 10D (Japan) were retrieved from genome databases [25,26] (<http://www.ncbi.nlm.nih.gov/genbank>) while 24 additional unannotated nucleotide sequences of RAD52 from different *Galdieria* strains (10 *G. sulphuraria*, 14 *Galdieria* sp.) were obtained by MySeq Illumina data. RAD52 from *C. merolae* 10D was retrieved from genome database and used as outgroup. For DNA extraction used for Illumina, DNA was extracted by resuspending a stationary phase algal paste with DNA extraction buffer [27]. DNA was incubated for 1 hr at 65 °C, centrifuged and the supernatant was precipitated by the addition of 1:1 isopropanol. The resultant pellet was suspended in Qiagen buffer PB, then applied to a miniprep column and washed according to manufacturers' details. DNA was eluted by adding pre-heated elution buffer provided by Quiagen to the column in 4 sequential elution steps. The sequencing was carried out as reported by Willing et al.[28]. After trimming, Illumina MiSeq reads were assembled using Spades v3.1 [29].

RAD52 amino acid sequences were searched using the National Center for Biotechnology Information (NCBI, <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) by querying protein, genomic and EST sequences on BLAST. A total of 45 RAD52 protein sequences from different organisms including algae, fungi, animals and bacteria were recruited, and used to generate a multiple sequence alignment, together with 9 RAD59 protein sequences as an outgroup. Among Cyanidiophytina, RAD52 protein sequences were retrieved from genome databases of *G. sulphuraria* 074 (Java,

Indonesia), *Cyanidioschyzon merolae* 10D (Japan) (Tables 1, 2)
(<http://www.ncbi.nlm.nih.gov/genbank>); [25,26] and *G. phlegrea* [30].
Phylogenetic inference of the evolutionary relationships of RAD52 from
Cyanidiophyceae and its homologs obtained from public databases was used to verify
the orthology of the protein; multiple alignment of amino acid sequences was
performed by ClustalW [31], trimmed and adjusted by eye. Only unambiguously
aligned amino acid sites were used for phylogenetic analyses. RAD52 phylogeny was
rooted by outgroup by using a RAD52 paralogue, RAD59. Bayesian analyses (BA)
were performed for combined and individual datasets with MrBayes v.3.1.1 [32]
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 burnin period
was identified graphically by tracking the likelihoods at each generation to determine
whether they reached a plateau.
Maximum likelihood (ML) phylogenetic analysis was performed using the GTR + Γ
+ I model implemented in RAxML software [33]. Statistical support for each branch
was obtained from 1000 bootstrap replications using the same substitution model and
RAxML program settings. The RAD52 evolutionary history of *Galdieria* strains was
inferred using Maximum likelihood (ML) method, based on Hasegawa-Kishino-Yano
model [34]. A discrete gamma distribution was used to model evolutionary rate
differences among sites. Bootstrap analyses were performed as previously described.

Taxa	GenBank ID
RAD52	
<i>Albugo candida</i>	635369772
<i>Albugo laibachii</i>	325180256
<i>Aphanomyces invadans</i>	673048395
<i>Arcobacter butzleri</i>	315478862
<i>Blastomyces gilchristii</i>	261192601
<i>Bos taurus</i>	528951193
<i>Calliarthron tuberculosum</i>	SRP005182
<i>Campylobacter curvus</i>	516863234
<i>Campylobacter showae</i>	489037738
<i>Candidatus Phaeomarinobacter ectocarpii</i>	918662481
<i>Cyanidioschyzon merolae</i>	544217672
<i>Danio rerio</i>	66269435
<i>Ectocarpus siliculosus</i>	298704860
<i>Emiliana huxleyi</i>	551599108
<i>Encephalitozoon cuniculi</i>	85014303
<i>Entamoeba histolytica</i>	67476176
<i>Entamoeba invadens</i>	471202697
<i>Entamoeba nuttali</i>	672809564
<i>Galdieria sulphuraria</i> IPPAS P507	
<i>Galdieria sulphuraria</i> IPPAS P503	MK21733250
<i>Galdieria</i> sp. ACUF074	MK217340
<i>Gallus gallus</i>	730466
<i>Gracilaria chorda</i>	NBIV01000177
<i>Homo sapiens</i>	863018
<i>Hyphomicrobium denitrificans</i>	505409238
<i>Kuraisha capsulata</i>	584391207
<i>Mus musculus</i>	261824011
<i>Naegleria gruberi</i>	290981385
<i>Phaeodactylum tricornutum</i>	219126773
<i>Phytophthora nicotianae</i>	970651832
<i>Phytophthora parasitica</i>	566015423
<i>Plasmopara halstedii</i>	953492183
<i>Rhizopus microsporus</i>	729702307
<i>Saprolegnia diclina</i>	669164116
<i>Saprolegnia parasitica</i>	813177361
<i>Schizophyllum commune</i>	302678737
<i>Schizosaccharomyces pombe</i>	19112088
<i>Spirochaeta</i> sp.	917473204
<i>Synechococcus</i> sp.	494162898

Taxa	GenBank ID
RAD52	
<i>Thalassiosira oceanica</i>	397635710
<i>Thalassiosira pseudonana</i>	220968365
<i>Vittaforma corneae</i>	667640414
<i>Wickerhamomyces ciferrii</i>	754409763
RAD59	
<i>Bos taurus</i>	61864423
<i>Chrysochromulina sp.</i>	922864786
<i>Gallus gallus</i>	45383087
<i>Guillardia theta</i>	551643257
<i>Homo sapiens</i>	21717826
<i>Kluyveromyces lactis</i>	49643317
<i>Mus musculus</i>	13385116
<i>Pan troglodytes</i>	55645233
<i>Saccharomyces cerevisiae</i>	6320144

Table 1. Accession numbers of RAD52 aminoacidic sequences used in this study

Strain	Strain code	Accession number
<i>Galdieria sulphuraria</i>	ACUF141G	MK217324
	ACUF141Y	MK217328
	ACUF141DG	MK217331
	ACUF142	MK217329
	ACUF388	MK217344
	ACUF402	MK217342
	ACUF427	MK217345
	ACUF455	MK217343
	SAG108.79	MK217327
	SAG21.92	MK217346
<i>Galdieria</i> sp.	IPPAS_P503	MK217325
	CCMEE5720	MK217326
	CCMEE5639	MK217330
	CCMEE5716	MK217332
	CCMEE5658	MK217333
	CCMEE5664	MK217334
	CCMEE5665	MK217335
	CCMEE5672	MK217336
	CCMEE5680	MK217337
	CCMEE5715	MK217338
	CCMEE5712	MK217339
	ACUF074	MK217340
	IPPAS_P502	MK217341
	THAL033	MK217347
<i>Cyanidioschyzon merolae</i>	10D	XM_005538923

Table 2. Accession number of RAD52 nucleotide sequences from Cyanidiophyceae used in this study

2.2 *In silico* protein structure analysis

The Selecton 2.4 Server (<http://selecton.tau.ac.il/>) was used to detect selection affecting specific sites. The server program measures the Ka/Ks rate on each amino acid residue [35–37]. Both M8 and MEC models were used. In M8 model, each substitution that implies a different coded amino-acid is considered as non synonymous, by contrast the mechanistic empirical combination model (MEC) takes into account the differences between amino acid replacement probabilities, expanding a 20×20 amino acid replacement rate matrix (such as the commonly used JTT matrix) into a 61×61 sense-codon rate matrix. Confidence interval of Ka/Ks values at each site were determined by posterior probability. The I-Tasser server (<http://zhanglab.ccmb.med.umich.edu/I-TASSER>) was used to predict the 3D structure of the domain and to map DNA binding sites especially conserved on the examined sequences. A multi-alignment representation was draft by using WebLogo application (<http://weblogo.berkeley.edu/logo.cgi>) and FirstGlance in Jmol was used to visualize the 3D structure (<http://bioinformatics.org/firstglance/fgj/index.htm>).

Rad52 gene expression under salt stress

The functionality of RAD52 gene was also investigated by analyzing the gene expression profile of the selected meiotic gene under osmotic stress conditions; *G. sulphuraria* ACUF 074 was maintained in liquid culture in Allen medium [38], pH 1.5 at 37°C under a continuous irradiance of $60 \mu\text{mol photons.m}^{-2}\text{s}^{-1}$. When in exponential growth stage, the culture was supplemented with different NaCl concentrations (0.16-2.5M). The growth rate was monitored until the stationary phase and evaluated spectrophotometrically at 550nm. All test were prepared in triplicate. Two NaCl stressed *G. sulphuraria* cultures with a sub-lethal (0,95M) and a lethal (1,25M) salt concentration were then used to evaluate RAD52 mRNA levels after 3, 6

and 12 hours from the salt addition. A qRT-PCR assay was performed on *G. sulphuraria* ACUF 074. Total RNA was isolated by PureLink RNA Mini Kit (Thermo Fisher Scientific, Waltham, MA USA), according to the manufacturer's instructions. The RNA concentration was quantified by measuring the absorbance at 260 nm using a Jasco V-530 UV/VIS spectrophotometer (Tokyo, Japan). The purity of all of the RNA samples was assessed at an absorbance ratio of OD260/280 and OD260/230, while its structural integrity was checked by agarose gel electrophoresis. Only high-quality RNA with OD 260/280 and OD 260/230 >2 was used for subsequent steps. Single-stranded cDNA was synthesized from 100 ng of total RNA using an SuperScript® VILO™ cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA USA), according to the manufacturer's instructions. EF1α and H2B were used as housekeeping genes [39]. The amplification efficiency of each gene was determined using a pool representing all of the cDNA samples. First, all of the primers were examined by end-point PCR, all of the chosen target were expressed, and specific amplification was confirmed by a single band of appropriate size in a 2% agarose gel after electrophoresis. In a second step, the pool was used to generate a five-point standard curve based on a ten-fold dilution series. The amplification efficiency (E) and correlation coefficient (R²) of the primers were calculated from the slope of the standard curve according to the equation [40]:

$$E(\%) = (10^{(-1 / \text{slope})} - 1) \times 100$$

Quantitative Real-time-PCR was performed using a CFX Connect Real-time PCR Detection System (Bio-Rad, Milan, Italy) to analyse the specific expression of each reference/target gene. cDNA was amplified in 96-well plates using the SsoAdvanced™ SYBR® Green Supermix (Bio-Rad, Milan, Italy), 15 ng of cDNA and 300 nM specific sense and anti-sense primers in a final volume of 20 µl for each well. Thermal cycling was performed, starting with an initial step at 95°C for 180 s,

followed by 40 cycles of denaturation at 95°C for 10 s and primer-dependent annealing for 30 s. Each run was completed with a melting curve analysis to confirm the specificity of amplification and lack of primer dimers.

References

1. Ciniglia, C.; Yoon, H.S.; Pollio, A.; Pinto, G.; Bhattacharya, D. Hidden biodiversity of the extremophilic Cyanidiales red algae. *Mol. Ecol.* **2004**, *13*.
2. Pinto, G.; Ciniglia, C.; Cascone, C.; Pollio, A. Species Composition of Cyanidiales Assemblages in Pisciarelli (Campi Flegrei, Italy) and Description of *Galdieria Phlegrea* SP. NOV. In: Springer, Dordrecht, 2007; pp. 487–502.
3. Ciniglia, C.; Yang, E.C.; Pollio, A.; Pinto, G.; Iovinella, M.; Vitale, L.; Yoon, H.S. Cyanidiophyceae in Iceland: plastid *rbc L* gene elucidates origin and dispersal of extremophilic *Galdieria sulphuraria* and *G. maxima* (Galdieriaceae, Rhodophyta). *Phycologia* **2014**, *53*, 542–551.
4. Cennamo, P.; Ciniglia, C. The algal diversity in the Phlegrean Fields (Campania, Italy) archeological districts: an overview. *Upl. - J. Urban Planning, Landsc. Environ. Des.* **2017**, *2*, 97–106.
5. Iovinella, M.; Eren, A.; Pinto, G.; Pollio, A.; Davis, S.J.; Cennamo, P.; Ciniglia, C. Cryptic dispersal of Cyanidiophytina (Rhodophyta) in non-acidic environments from Turkey. *Extremophiles* **2018**, *22*, 713–723.
6. Eren, A.; Iovinella, M.; Yoon, H.S.; Cennamo, P.; de Stefano, M.; de Castro, O.; Ciniglia, C. Genetic structure of *Galdieria* populations from Iceland. *Polar Biol.* **2018**, *41*.
7. Yang, E.C.; Boo, S.M.; Bhattacharya, D.; Saunders, G.W.; Knoll, A.H.; Fredericq, S.; Graf, L.; Yoon, H.S. Divergence time estimates and the evolution of major lineages in the florideophyte red algae. *Sci. Rep.* **2016**, *6*,

- 453 21361.
- 454 8. Müller, K.M.; Oliveira, M.C.; Sheath, R.G.; Bhattacharya, D. Ribosomal DNA
455 phylogeny of the Bangiophycidae (Rhodophyta) and the origin of secondary
456 plastids. *Am. J. Bot.* **2001**, *88*, 1390–1400.
- 457 9. Yoon, H.S.; Hackett, J.D.; Pinto, G.; Bhattacharya, D. The Single, Ancient
458 Origin of Chromist Plastids. *J. Phycol.* **2002**, *38*, 40–40.
- 459 10. Gross, J.; Bhattacharya, D. Uniting sex and eukaryote origins in an emerging
460 oxygenic world. *Biol. Direct* **2010**, *5*, 53.
- 461 11. Argueso, J.L.; Westmoreland, J.; Mieczkowski, P.A.; Gawel, M.; Petes, T.D.;
462 Resnick, M.A. Double-strand breaks associated with repetitive DNA can
463 reshape the genome. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 11845–50.
- 464 12. Symington, L.S. Role of RAD52 epistasis group genes in homologous
465 recombination and double-strand break repair. *Microbiol. Mol. Biol. Rev.* **2002**,
466 *66*, 630–70, table of contents.
- 467 13. Mortensen, U.H.; Bendixen, C.; Sunjevaric, I.; Rothstein, R. DNA strand
468 annealing is promoted by the yeast Rad52 protein. *Proc. Natl. Acad. Sci. U. S.*
469 *A.* **1996**, *93*, 10729–34.
- 470 14. Iyer, L.M.; Koonin, E. V; Aravind, L. Classification and evolutionary history
471 of the single-strand annealing proteins, RecT, Red β , ERF and RAD52. *BMC*
472 *Genomics* **2002**, *3*, 8.
- 473 15. Aravind, L.; Walker, D.R.; Koonin, E. V. Conserved domains in DNA repair
474 proteins and evolution of repair systems. *Nucleic Acids Res.* **1999**, *27*, 1223–
475 1242.
- 476 16. Kreitman, M. METHODS TO DETECT SELECTION IN POPULATIONS
477 WITH APPLICATIONS TO THE HUMAN. *Annu. Rev. Genomics Hum.*
478 *Genet.* **2000**, *1*, 539–559.

- 479 17. MacCallum, C.; Hill, E. Being Positive about Selection. *PLoS Biol.* **2006**, *4*,
- 480 e87.
- 481 18. Roth, C.; Liberles, D.A. A systematic search for positive selection in higher
- 482 plants (Embryophytes). *BMC Plant Biol.* **2006**, *6*, 12.
- 483 19. Keeling, P.J. The endosymbiotic origin, diversification and fate of plastids.
- 484 *Philos. Trans. R. Soc. B Biol. Sci.* **2010**, *365*, 729–748.
- 485 20. Oliveira, M.C.; Bhattacharya, D. PHYLOGENY OF THE
- 486 BANGIOPHYCIDAE (RHODOPHYTA) AND THE SECONDARY
- 487 ENDOSYMBIOTIC ORIGIN OF ALGAL PLASTIDS. *J. Phycol.* **2000**, *36*,
- 488 52–52.
- 489 21. Samach, A.; Melamed-Bessudo, C.; Avivi-Ragolski, N.; Pietrokovski, S.;
- 490 Levy, A.A. Identification of plant RAD52 homologs and characterization of the
- 491 *Arabidopsis thaliana* RAD52-like genes. *Plant Cell* **2011**, *23*, 4266–79.
- 492 22. Kagawa, W.; Kurumizaka, H.; Ishitani, R.; Fukai, S.; Nureki, O.; Shibata, T.;
- 493 Yokoyama, S. Crystal Structure of the Homologous-Pairing Domain from the
- 494 Human Rad52 Recombinase in the Undecameric Form. *Mol. Cell* **2002**, *10*,
- 495 359–371.
- 496 23. Singleton, M.R.; Wentzell, L.M.; Liu, Y.; West, S.C.; Wigley, D.B. Structure
- 497 of the single-strand annealing domain of human RAD52 protein. *Proc. Natl.*
- 498 *Acad. Sci. U. S. A.* **2002**, *99*, 13492–7.
- 499 24. D., G. *Fundamental of Molecular Evolution*; Sinauer Press, Ed.; 2000;
- 500 25. Schönknecht, G.; Chen, W.-H.; Ternes, C.M.; Barbier, G.G.; Shrestha, R.P.;
- 501 Stanke, M.; Bräutigam, A.; Baker, B.J.; Banfield, J.F.; Garavito, R.M.; et al.
- 502 Gene transfer from bacteria and archaea facilitated evolution of an
- 503 extremophilic eukaryote. *Science* **2013**, *339*, 1207–10.
- 504 26. Matsuzaki, M.; Misumi, O.; Shin-i, T.; Maruyama, S.; Takahara, M.;

- 505 Miyagishima, S.; Mori, T.; Nishida, K.; Yagisawa, F.; Nishida, K.; et al.
 506 Genome sequence of the ultrasmall unicellular red alga *Cyanidioschyzon*
 507 *merolae* 10D. *Nature* **2004**, *428*, 653–657.
- 508 27. Davis, A.M.; Iovinella, M.; James, S.; Robshaw, T.; Dodson, J.H.; Herrero-
 509 Davila, L.; Clark, J.H.; Agapiou, M.; McQueen-Mason, S.; Pinto, G.; et al.
 510 Using MinION nanopore sequencing to generate a de novo eukaryotic draft
 511 genome: preliminary physiological and genomic description of the
 512 extremophilic red alga *Galdieria sulphuraria* strain SAG 107.79 - White Rose
 513 Research Online Available online: <http://eprints.whiterose.ac.uk/105094/>
 514 (accessed on Dec 18, 2018).
- 515 28. Willing, E.-M.; Rawat, V.; Mandáková, T.; Maumus, F.; James, G.V.;
 516 Nordström, K.J.V.; Becker, C.; Warthmann, N.; Chica, C.; Szarzynska, B.; et
 517 al. Genome expansion of *Arabis alpina* linked with retrotransposition and
 518 reduced symmetric DNA methylation. *Nat. Plants* **2015**, *1*, 14023.
- 519 29. Bankevich, A.; Nurk, S.; Antipov, D.; Gurevich, A.A.; Dvorkin, M.; Kulikov,
 520 A.S.; Lesin, V.M.; Nikolenko, S.I.; Pham, S.; Prjibelski, A.D.; et al. SPAdes: A
 521 New Genome Assembly Algorithm and Its Applications to Single-Cell
 522 Sequencing. *J. Comput. Biol.* **2012**, *19*, 455–477.
- 523 30. Qiu, H.; Price, D.C.; Weber, A.P.M.; Reeb, V.; Yang, E.C.; Lee, J.M.; Kim,
 524 S.Y.; Yoon, H.S.; Bhattacharya, D. Adaptation through horizontal gene transfer
 525 in the cryptoendolithic red alga *Galdieria phlegrea*. *Curr. Biol.* **2013**, *23*, R865-
 526 6.
- 527 31. Larkin, M.A.; Blackshields, G.; Brown, N.P.; Chenna, R.; McGettigan, P.A.;
 528 McWilliam, H.; Valentin, F.; Wallace, I.M.; Wilm, A.; Lopez, R.; et al. Clustal
 529 W and Clustal X version 2.0. *Bioinformatics* **2007**, *23*, 2947–2948.
- 530 32. Ronquist, F.; Huelsenbeck, J.P. MrBayes 3: Bayesian phylogenetic inference

531 under mixed models. *Bioinformatics* **2003**, *19*, 1572–1574.

532 33. Stamatakis, A.; Hoover, P.; Rougemont, J. A Rapid Bootstrap Algorithm for
533 the RAxML Web Servers. *Syst. Biol.* **2008**, *57*, 758–771.

534 34. HASEGAWA, M.; YANO, T.; KISHINO, H. A new molecular clock of
535 mitochondrial DNA and the evolution of hominoids. *Proc. Japan Acad. Ser. B*
536 *Phys. Biol. Sci.* **1984**, *60*, 95–98.

537 35. Nielsen, R.; Yang, Z. Likelihood models for detecting positively selected
538 amino acid sites and applications to the HIV-1 envelope gene. *Genetics* **1998**,
539 *148*, 929–36.

540 36. Yang, Z.; Bielawski, J.P. Statistical methods for detecting molecular
541 adaptation. *Trends Ecol. Evol.* **2000**, *15*, 496–503.

542 37. Stern, A.; Doron-Faigenboim, A.; Erez, E.; Martz, E.; Bacharach, E.; Pupko, T.
543 Selecton 2007: advanced models for detecting positive and purifying selection
544 using a Bayesian inference approach. *Nucleic Acids Res.* **2007**, *35*, W506–
545 W511.

546 38. Allen, M.M. & Stainer, R.Y. Selective isolation of blue-green algae from water
547 and soil. *J Gen Microbiol* **1968**, *51*, 203–209.

548 39. Carfagna, S.; Bottone, C.; Cataletto, P.R.; Petriccione, M.; Pinto, G.; Salbitani,
549 G.; Vona, V.; Pollio, A.; Ciniglia, C. Impact of sulfur starvation in autotrophic
550 and heterotrophic cultures of the Extremophilic Microalga *Galdieria Phlegrea*
551 (Cyanidiophyceae). *Plant Cell Physiol.* **2016**, *57*.

552 40. Radonić, A.; Thulke, S.; Mackay, I.M.; Landt, O.; Siegert, W.; Nitsche, A.
553 Guideline to reference gene selection for quantitative real-time PCR. *Biochem.*
554 *Biophys. Res. Commun.* **2004**, *313*, 856–862.

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