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Algal Research

Nuclear and Organellar Phylogenies Provide Insights into Evolutionary History and Species Boundaries in *Galdieria*

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Abstract:	Exploration of life in extreme environments allows the discovery of organisms with extraordinary biotechnological potential. Hot springs, characterised by extreme conditions (pH < 2; Temperature > 40°C; high concentrations of metals), present a formidable challenge for most life forms, yet support a few specialised archaea, bacteria, and eukaryotes, including the unicellular red alga <i>Galdieria</i>. Beyond their ecological relevance, members of the order Galdieriales emerged as promising platforms for biotechnological applications, including metal and rare earth elements (REEs) recovery, bioremediation under extreme conditions, and sustainable biomass production. Previous phylogenetic studies based on plastid and nuclear genes suggested a complex genetic structure within <i>Galdieria</i>, with evidence for multiple diverging clades within the species <i>G. sulphuraria</i>. To resolve these enigmatic relationships, we employed a genome-scale approach, integrating plastid, mitochondrial, and nuclear data from <i>Galdieria</i> strains collected across diverse geographical locations. Our comprehensive phylogenetic analysis revealed divergence of mitochondrial, plastid, and nuclear genomes into at least seven distinct and well-supported clades; alongside i) independent evolutionary trajectories of these genomic lineages, and ii) incongruent interspecies relationships among the three genomes, reflecting complex evolutionary dynamics. Synonymous and non-synonymous substitution analyses further underscored differential evolutionary pressures on the three cellular genomes acting across the <i>Galdieria</i> species, with strong purifying selection in plastid genes and more relaxed selection in mitochondrial and nuclear genomes. In this context, the genetic and evolutionary framework presented here provides a crucial basis for understanding the molecular determinants underlying the exceptional metabolic versatility and stress tolerance that make Galdieriales attractive candidates for applied biotechnology.

MI and **SCLL**: Conceptualization, Data curation, Formal analysis, Methodology, Validation, Visualization, Writing – original draft and Writing – review & editing. **MI** and **SCLL** contributed equally to this work. **SJ**: Methodology and Formal analysis. **DJ**: Methodology, Formal analysis, Supervision. **AP**: Investigation. **EDG**: Investigation. **CC**: Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing. **SJD**: Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing. **LCMM**, **JPJC**, **GAF** and **HSY**: Writing – review & editing.

Nuclear and Organellar Phylogenies Provide Insights into Evolutionary History and Species Boundaries in *Galdieria*.

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Abstract

Exploration of life in extreme environments allows the discovery of organisms with extraordinary biotechnological potential. Hot springs, characterised by extreme conditions (pH < 2; Temperature > 40°C; high concentrations of metals), present a formidable challenge for most life forms, yet support a few specialised archaea, bacteria, and eukaryotes, including the unicellular red alga *Galdieria*. Beyond their ecological relevance, members of the order Galdieriales emerged as promising platforms for biotechnological applications, including metal and rare earth elements (REEs) recovery, bioremediation under extreme conditions, and sustainable biomass production. Previous phylogenetic studies based on plastid and nuclear genes suggested a complex genetic structure within *Galdieria*, with evidence for multiple diverging clades within the species *G. sulphuraria*. To resolve these enigmatic relationships, we employed a genome-scale approach, integrating plastid, mitochondrial, and nuclear data from *Galdieria* strains collected across diverse geographical locations. Our comprehensive phylogenetic analysis revealed divergence of mitochondrial, plastid, and nuclear genomes into at least seven distinct and well-supported clades; alongside i) independent evolutionary trajectories of these genomic lineages, and ii) incongruent interspecies relationships among the three genomes, reflecting complex evolutionary dynamics. Synonymous and non-synonymous substitution analyses further underscored differential evolutionary pressures on the three cellular genomes acting across the *Galdieria* species, with strong purifying selection in plastid genes and more relaxed selection in mitochondrial and nuclear genomes. In this context, the genetic and evolutionary framework presented here provides a crucial basis for understanding the molecular determinants underlying the exceptional metabolic versatility and stress tolerance that make Galdieriales attractive candidates for applied biotechnology.

KEYWORDS

Galdieria mesoamericana, Galdieriales, phylogenomics, purifying and positive selection, taxonomy.

1. Introduction

The genus *Galdieria* comprises a unique group of polyextremophilic microalgae capable of colonising environments marked by extreme acidity (pH 0.8–2), high temperatures (up to 56 °C), and elevated concentrations of heavy, precious, and rare earth metals. Such resilience positions *Galdieria* as a distinctive lineage within the subphylum Cyanidiophytina (phylum Rhodophyta), providing an important model for extremophile biology, while offering significant potential for biotechnological applications [1,2]. Species of the genus *Galdieria*, and in particular *Galdieria sulphuraria*, [3], have been successfully employed in diverse applied contexts, including the biosorption and selective recovery of precious and rare earth elements from acidic solutions [4–8], wastewater treatment and bioremediation [9], heterotrophic growth on industrial and agro-industrial residues for sustainable biomass production [10], and the identification of bioactive compounds with antiviral properties [11]. Despite these advances, the genetic and evolutionary bases underlying such remarkable physiological plasticity remain poorly understood. A comprehensive investigation of the genomic features and evolutionary history of Galdieriales is therefore essential to rationally exploit their metabolic capabilities and to support future strain improvement and biotechnological applications.

The genus was established in 1981 with the description of its type species, *Galdieria sulphuraria* [3], isolated from acidic hot springs in Pisciarelli, Naples, Italy. Initially monotypic, *Galdieria* was placed in the newly created family Galdieraceae. *G. sulphuraria* was described as a rounded unicellular alga, with diagnostic traits including average cell size, production of autospores, and the presence of a multilobed chloroplast [3]. It was considered closely related to *Cyanidium caldarium* (Tilden) L. Geitler 1933 [12], sharing a cosmopolitan distribution across extremely acidic environments in North and South America, Eurasia, and Zealandia (Ott and Sechbach 1994). A hallmark of *Galdieria* species is their metabolic versatility: they can grow photoautotrophically or heterotrophically, utilising a broad range of substrates even at very low pH. This adaptability makes them particularly promising for large-scale cultivation and biotechnological applications [13,14]. Recent research highlights their potential in

bioresource exploitation, where their capacity to withstand and metabolically adapt to hostile environments provides a foundation for innovative applications [8,9,15–18].

In the years following the description of *Galdieria sulphuraria*, three additional species, *G. partita* Sentsova ex Pollio et al. 2023, *G. daedala* Sentsova ex Pollio et al. 2023 and *G. maxima* Sentsova ex Pollio et al. 2023 [19], were reported from acidic habitats of the Kamchatka Peninsula and Kuril Islands (Russia). A subsequent revision by Albertano et al. (2000) [20] placed the genus within Phragmonemataceae and recognised all four species. However, morphological circumscription relied mainly on plastid number and shape, and it soon became evident that diagnostic traits were insufficient to reliably distinguish species, as in many coccoid microalgae [19,20].

Later studies confirmed this taxonomic uncertainty [21], while plastid multigene analyses suggested that *Galdieria* is more species-rich than initially thought, with diversity shaped by geographical isolation [22]. A putative fifth species, *G. phlegrea* Yoon et al. 2023, was proposed from Pisciarelli, Naples, Italy, sympatric with *G. sulphuraria* but occupying distinct ecological niches: humid rocks near acidic springs (40–48 °C, pH 1.0–1.5) for *G. sulphuraria*, and drier fissures and pebbles (25–28 °C, pH 1.0–2.0) for *G. phlegrea* [22,23]. A major taxonomic revision reorganized the phylum Rhodophyta into the subphyla, Cyanidiophytina and Rhodophytina. Within this framework, the order Cyanidiales was assigned to the subphylum Cyanidiophytina, with the genus *Galdieria* placed in the newly redefined family Galdieriaceae [24]. Phylogenetic studies since then have revealed deep divergence within the genus, shaped by fragmented distributions, limited dispersal, and long-term vicariant evolution [25,26]. More recently, molecular analyses have uncovered additional lineages, treated as Operational Taxonomic Units [27], with divergence driven by habitat heterogeneity and low gene flow [28]. These findings suggest that ongoing molecular investigations will likely expand the number of recognised *Galdieria* taxa, enriching our understanding of their evolutionary history.

A recent study, supported by plastid and mitochondrial genomic analyses, has proposed a taxonomic reassessment of *Galdieria*, confirming the presence of deep genetic divergence

107 within this genus [29]. According to Park et al. (2023), *Galdieria* presently encompasses seven
 108 species, namely *G. sulphuraria*, *G. phlegrea*, *G. yellowstonensis* [29], *G. jawensis* [29] and
 109 the three Russian species, considered by the authors as *nomina nuda*, but recently validated
 110 by Del Guacchio et al. in 2023 [30]. The species of the genus seem to be geographically
 111 confined to different continents, although numerous putative *G. sulphuraria* strains have been
 112 isolated in the last decades throughout the acidic environments of different continents [28,31].
 113 The implications of this research suggest that many *Galdieria* isolates collected worldwide
 114 may actually represent distinct species.
 115 Here, we analysed the phylogenetic relationships among *Galdieria* strains collected from
 116 Europe, Asia, North America, and Central-South America, using whole-genome data. By
 117 examining the distribution, gene functions, and evolutionary histories of these strains, we have
 118 crafted a comprehensive view of the genetic and evolutionary dynamics within the genus. Our
 119 study leverages the high-resolution maps of plastid, mitochondrial, and nuclear genomes we
 120 generated, alongside in-depth phylogenetic analyses and comparisons of synonymous and
 121 non-synonymous substitution rates. This research underscores a significant shift from
 122 traditional gene-level analysis to a more comprehensive pan-genome-level approach,
 123 essential for accurately delineating the intricate relationships and evolutionary pressures at
 124 play across different *Galdieria* species. Notably, the mitochondrial, plastid, and nuclear
 125 genomes exhibit strikingly independent evolutionary trajectories, underscoring the complex
 126 genomic landscape of this lineage. This revealed i) the partitioning of the three genomic
 127 compartments into at least seven distinct and well-supported clades; ii) genome-specific
 128 modes of evolution; and iii) deeply incongruent interspecies relationships among the three
 129 genomes, reflecting a level of complexity that could not have been foreseen. What began as
 130 an effort to clarify phylogenomic structure instead exposed a profoundly multifaceted
 131 evolutionary narrative, an unforeseen finding that reshapes how we interpret organellar and
 132 nuclear evolution in extremophilic red algae.

2. Material and Methods

2.1 Strains and cultures

Galdieria strains were harvested from the Algal Collection of the University of Naples (www.acuf.net), the Culture Collection of Autotrophic Organisms (<https://ccala.butbn.cas.cz/>), the Collection of Microorganisms from Extreme Environments, the Institute of Plant Physiology, Russian Academy of Sciences (<http://en.cellreg.org/Collection-IPPAS.php>), the Culture Collection of Algae at Göttingen University (<https://www.uni-goettingen.de/en/culture+collection+of+algae+%28sag%29/184982.html>), and the Tung-Hai Algal Lab Culture Collection (http://algae.thu.edu.tw/lab/?page_id=42) (Figure 1, Table S1). All strains were isolated by streaking three times the colonies across agar plates, and a single colony was used to create a master stock. The final strains were inoculated in Allen medium at pH 1.5 [32] and cultivated at 37°C under continuous fluorescent illumination of 45 $\mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$.

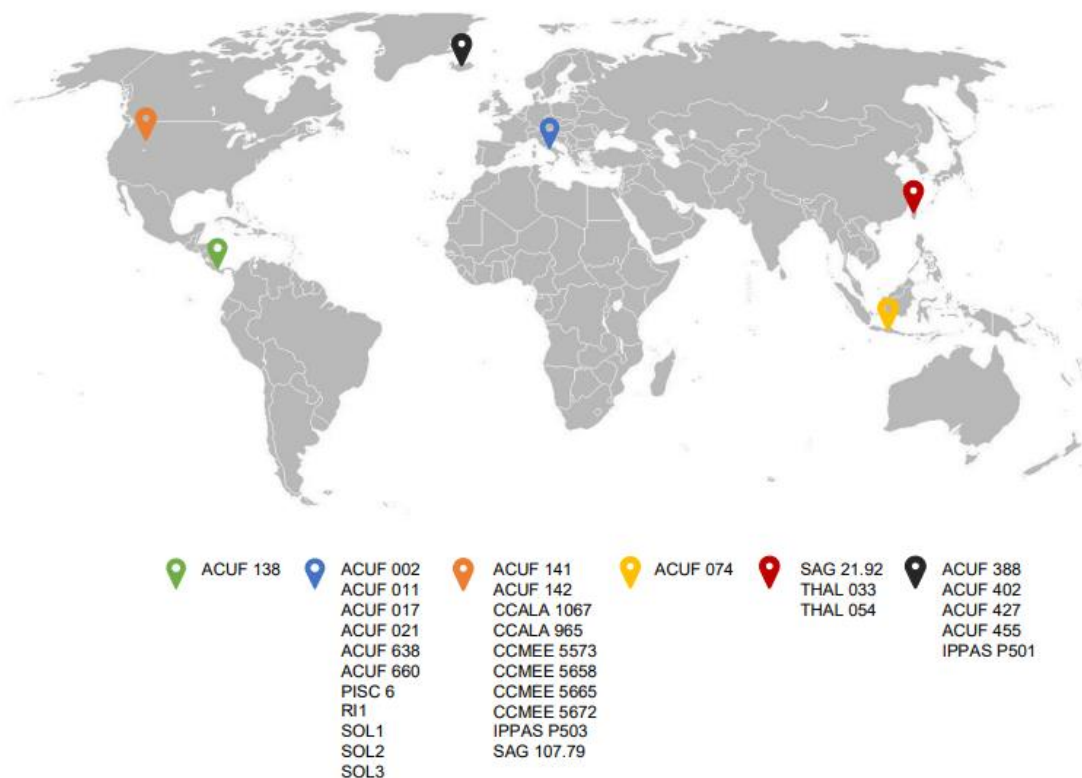


Figure 1. Worldwide distribution of Galdieriales strains used in this study. Details of the collection sites, along with the sample source and corresponding reference are listed in Table S1.

2.2 DNA preparation and sequencing

Samples were collected from stock solutions by centrifugation. Total genomic DNA was respectively extracted using a modified SDS-EDTA protocol (Table S2) followed by a clean-up step performed with DNeasy Plant Mini Kits (Qiagen, Hilden, Germany). DNA quality and concentration were assessed using a Nanodrop photospectrometer ND-1000 (Thermo Fisher Scientific, Massachusetts, U.S.A).

Library preparations were performed with NEBNext® Ultra™ II DNA Library Prep Kit for Illumina Sequencing (Ipswich, Massachusetts, USA), according to the manufacturer's instructions. Libraries were then sequenced with Illumina MiSeq (Illumina, San Diego, CA, USA) and the resulting reads were trimmed with Trimmomatic v 0.39 [33] and assembled using Spades v3.1 [34]. Assembly quality was assessed using a range of stats shown in Table S3. To obtain one single scaffold for plastid and mitochondrial genomes, respective contigs were

identified by comparing known proteins from the reference genome, strain ACUF 074 (KJ700460 for mitochondria and NC_024665 for plastid), using TBLASTN from BLAST+ version 2.2.27[35]. The extracted contigs were re-assembled into one single scaffold using Ragout v. 2.3, employing the organelle genomes from strain ACUF 074 as the reference [36].

2.3 RNA preparation and sequencing

G. sulphuraria ACUF 017, *G. partita* THAL 033, *G. javensis* ACUF 074, *G. yellowstonensis* SAG 107.79, *G. mesoamericana* ACUF 138 and *G. daedala* ACUF 427 were grown under a 12h/12h light/dark cycle under $42 \mu\text{mol m}^{-2} \text{s}^{-1}$ at 37°C and 130 rpm on an orbital shaker. To enhance the diversity of transcripts detected, the cell cultures were grown under different physiological conditions (Table S5). Cells were then ground into a fine powder with a pestle and a drill in the presence of liquid nitrogen. RNA was isolated and cleaned using the Monarch Total RNA Miniprep Kit (New England BioLabs, T2010S), and the RNAs quality and concentration were assessed using a Nanodrop photospectrometer ND-1000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA). All RNAs were treated with DNaseI and pooled together by strain, considering the relative concentration of each sample. RNA quality and concentration were then assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA).

RNA library preparation and sequencing were performed at Novogene (UK) Company Limited (Cambridge). Library preparation was performed using NEB Next® Ultra™ RNA Library Prep Kit (NEB, San Diego, CA, USA), employing AMPure XP Beads to purify the products of the reactions during the library prep. Poly-a mRNA was isolated using poly-T oligo-attached magnetic beads, then fragmented through sonication and enriched into 250-300bp fragments. The purified mRNA was converted to cDNA and subjected to adaptor ligation. The barcoded fragments were finally multiplexed and run on the Illumina Novaseq 6000 (s4 flow cell) to acquire 20 million read pairs per sample using the 150bp PE sequencing mode.

2.4 Gene prediction and annotation

Plastid and mitochondrial scaffolds were annotated with GeSeq [37] using *G. javensis*, strain 074W, using the NCBI Reference Sequence Database [38]. Ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs) were annotated using ARAGORN [39] and tRNAscan-SE v2.0 [40]. Finally, the annotated genomes were visualised using OrganellarGenomeDraw v1.2 [41]. Gene prediction was performed using the program AUGUSTUS [42] with parameters trained from *G. yellowstonensis* strain ACUF 074. Untrimmed RNA sequencing reads were aligned to their Illumina assemblies using the STAR aligner v. 2.7.3 [43]. Alignments were filtered with AUGUSTUS v. 3.3.3 filterBAM and converted to AUGUSTUS hints with bam2hints using the defaults [44]. The annotation was performed with AUGUSTUS using the generated hints and de novo gene prediction. Coding sequences with over 94% identity were removed with CD-HIT v. 4.8.1 to remove redundant gene sequences [45]. Predicted amino acid sequences were generated with EMBOSS transeq v. 6.6.0 [46] with a minimum ORF length of 40. Sequences without start codons and with stop codons contained in the sequence were removed. All the annotated genomes were deposited into the GenBank database (Table S6).

2.5 Phylogenetic analysis

We first inferred an *rbcL* phylogenetic tree to identify Galdieriales species by retrieving all available *rbcL* Cyanidiophyceae sequences from NCBI, yielding a total of 273 *rbcL* sequences. Maximum likelihood (ML) phylogenetic analysis and bootstrap methods were conducted using IQ-TREE with 10,000 ultrafast bootstrap replications and applying the model test option. Four non-cyanidales red alga taxa were chosen as an outgroup: *Porphyra umbilicalis* and *Pyropia haitanensis* as Bangiophyceae, *Chondrus crispus* and *Gracilariopsis chorda* as Florideophyceae. Plastid and mitochondrial CDSs were retrieved from the assemblies using tblastn [35] and Geseq [37], using the reference genomes of *G. yellowstonensis*, strain ACUF 074. Nuclear gene sequences of strain ACUF 074 (NCBI) were filtered to exclude mitochondrial and plastid

genes. For ortholog identification, genome-specific BLAST databases were generated, and nuclear genes were queried using BlastN (v2.10.0) [35]. Relative hit scores were clustered by gene (heat map, Fig. S1). Genes with mean similarity scores >0.4 were retained for downstream phylogenetic analyses.

The orthologous CDS sequences for each gene were then aligned using MUSCLE 3.8.31 [47], and the resulting alignments were uploaded to Gblocks v 0.91 b [48] to remove poorly aligned regions applying the options -t = d -b5 = h. For the nuclear alignments, genes represented by <40% of the original gene alignment length were excluded; the remaining gene sequences were concatenated using Gblocks v 0.91 b [48]. The final plastid, mitochondrial and nuclear alignments comprised 116936 bp, 13609 bp, and 5,212,746 bp DNA positions, respectively. Four red algal taxa from Florideophyceae and Bangiophyceae were chosen as outgroup taxa (Table S1). ML analyses were performed with IQ-Tree v. 1.6.9 [49], using the best substitution model estimated under the partition scheme selected by the program (- spp, -m TEST). Phylogenetic trees were inferred by applying 10000 ultrafast bootstrap replicates UFBoot [50] for the branch statistical support.

2.6 Consensus Network Analysis and Estimation of Gene Concordance Factors

Phylogenies of nuclear (3532 genes), plastid (124), and mitochondrial (17) genomes across 43 taxa were imported into SplitsTree v5.0.0_alpha [51,52] to generate a consensus network [53]. The Consensus Network methods was used (default options) to obtain 116 splits [50], and the Splits Network Algorithm method was used (default options) to generate a splits network with 290 nodes and 516 edges [54]. Gene concordance factors (gCF) were estimated with IQ-TREE[55] using reference species trees from concatenated datasets and single-locus trees with model selection.

2.7 Estimation of non-synonymous to synonymous substitutions ratio

For each core species, protein-coding gene sequences and translations were retrieved, with the longest transcript used when multiple variants were present. Protein sequences were aligned using MUSCLE v3.8.31 [47] and converted to codon alignments with PAL2NAL [56]. Nonsynonymous (dN), synonymous (dS), and dN/dS (ω) values were estimated in CODEML, PAML v4 [57]. Average ω values were obtained with the M0 model, while M7 (beta distribution, no positive selection) and M8 (allowing $\omega > 1$) were compared by likelihood ratio tests. Genes under selection were screened for hydrolase activity and signal peptides using UniProt Consortium [58] and SignalP5.0 [59].

2.8 Comparative genome analysis

Plastid and mitochondrial scaffolds obtained from the re the re-assembly step were aligned using the ProgressiveMauve algorithm [60] and applying the default settings. The predicted amino-acid sequences of nuclear Galdieriales were clustered into orthologous groups using OrthoFinder (version 2.3.11) software [61]. Orthologs were identified and clustered by an all-versus-all protein comparison with predicted proteins of the core 7 species along with extremophile *Cyanidioschyzon merolae* and mesophilic *Porphyridium purpureum*.

3. Results

3.1 Taxonomy and Nomenclature

The present study confirmed the split of *Galdieria* into more genetic lineages and confirmed their establishment as species. According to both organellar and nuclear phylogenies, strain ACUF 138, originally collected from San Salvador and previously identified as *G. sulphuraria*, formed a highly supported clade, separated from the other *Galdieria* species. The separation of this strain, also supported by the high nucleotide divergence percentage, prompted us to

describe ACUF 138 as a new species, namely *Galdieria mesoamericana*. sp. nov. The species was described as follows:

Class Cyanidiophyceae A.Merola 1982. Giorn Bot.Ital. 115: 191.

Order Galdieriales H.S.Yoon, S.I.Park, C.H.Cho & R.A.Andersen 2023 J. Phycol.

Family Galdieriaceae A.Merola 1982. Giorn Bot.Ital. 115: 193.

Genus *Galdieria* Merola 1982. Giorn Bot. Ital. 115: 193.

Galdieria mesoamericana M. Iovinella, S.C.L. Lock, L.C.M. Mackinder, J.P.J. Chong, G.A. Feichtinger, S. James, D. Jeffares, A. Pollio, E. Del Guacchio, H.S. Yoon, C. Ciniglia, S.J. Davis.

Description: Cells spherical, 2.5 to 8 µm in diameter; with a cell wall and a single multi-lobed chloroplast; reproduction by 2-8 autospores per mother cell; the strain grows at temperatures between 15 and 55 °C under laboratory conditions. Phototrophic and facultative heterotrophic. DNA sequences are distinct and unique (NCBI BioSample ID = SAMN33241377).

Illustration: Figure 2

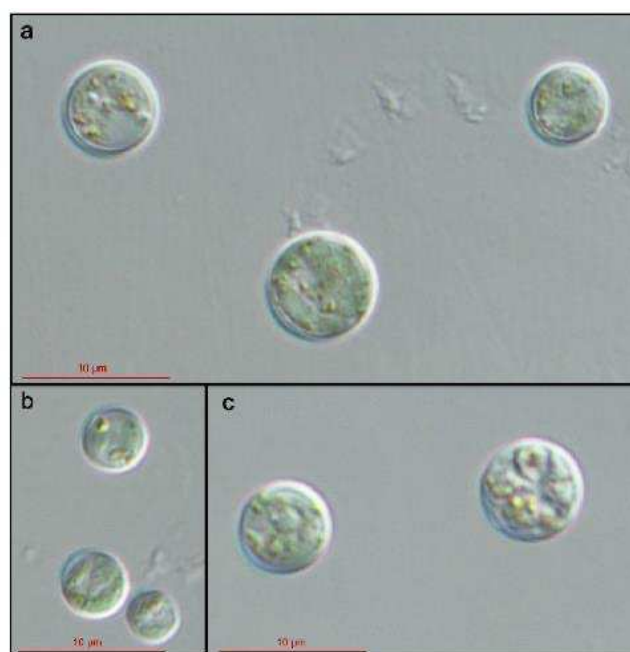


Figure 2. Light micrographs of different *Galdieria mesoamericana* from San Salvador life cycle stages: Vegetative cells (a,b) and sporangium (c). Scale bar = 10 µm.

Holotype: No. NAP0006291, deposited in the Naples Botanical Garden herbarium (NY), Naples, Italy, cells from culture strain ACUF 138 preserved in a metabolically inactive state in a 4% glutaraldehyde solution.

Type locality: Playon de Ahuachapan, El Salvador (13°55'00.0"N 89°50'00.0"W), from hot springs at pH between 1.7-2.0, and temperature in the range of 28-42 °C.

Etymology: The species name refers to Central America, where the sample was collected in 1975 by P. De Luca, R. Gambardella and A. Merola.

3.2 Phylogenetic analysis

Preliminary phylogenetic analyses, carried out using *rbcL*, highlighted the monophyly of Cyanidiophyceae, with clades that largely correspond to the currently known orders (Figure S2). The Cyanidioschyzonales (*Cyanidioschyzon merolae* [62], and *Cyanidiococcus yangmingshanensis* [63]) resulted as a sister group to Galdieriales, which includes *G. phlegrea* [23], *G. sulphuraria* [3], *G. yellowstonensis* [29], *G. javensis* [29], *G. partita* [30], *G. daedala* [30], and the new herewith established *G. mesoamericana* Iovinella et al. 2025 (see later sections for its taxonomic details). In the second branch of the phylogenetic tree, Caverniculales (*Cavernicola chilense* [29], *Sciadococcus taiwanensis* [29], *Gronococcus sybilensis* [29]) and Cyanidiales (*Cyanidium caldarium* [12]) were sister clade (Figure S2). For a more detailed analysis of the evolutionary history of *Galdieria* species, representative strains of each *rbcL* clade (38 *Galdieria* red-labelled strains, Figure S2) were chosen for the subsequent analysis, generating high-quality draft genomes to assess their genetic structure at a genome level (Figure 3).

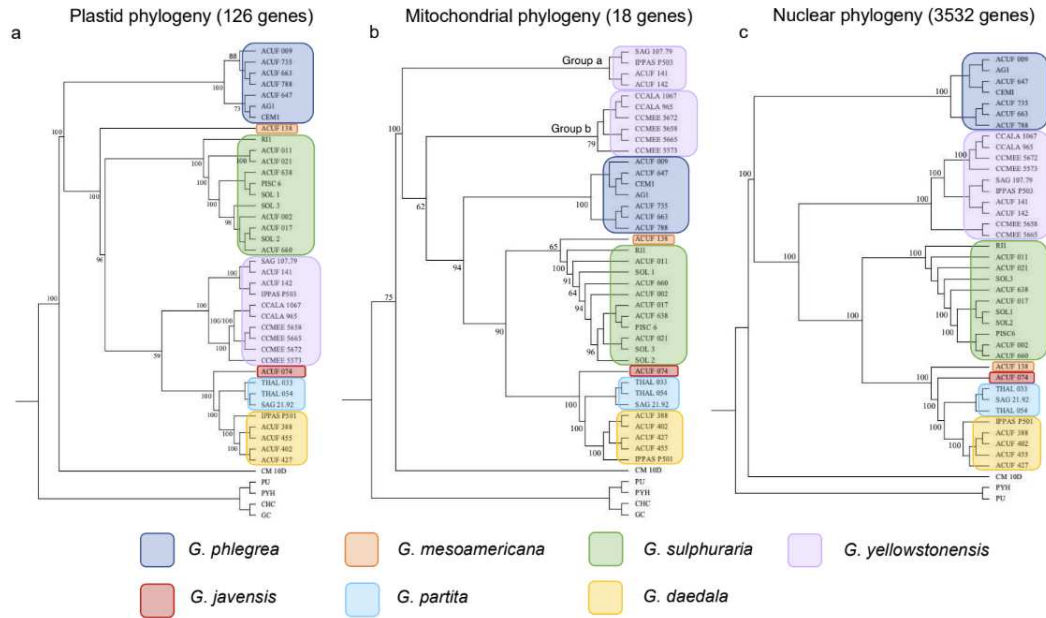


Figure 3. Comparison of the plastid (a), mitochondrial (b) and nuclear (c) Species trees of Cyanidiophyceae. The phylogenies were inferred from Maximum Likelihood (ML) analysis using the concatenated plastid, mitochondrial and nuclear CDSs, and the partition scheme for the best substitution model. Ultrafast bootstrap (UFBoot) support values are indicated near nodes. Only bootstrap values $\geq 50\%$ are shown. Different color boxes represent different *Galdieria* species and include strains from different geographic locations: blue box = *G. phlegrea* from Mediterranean areas; orange box = *G. mesoamericana* from San Salvador; green box = *G. sulphuraria* from Mediterranean areas; purple box = *G. yellowstonensis* from the Atlantic region plus US; red box = *G. javensis* from Java island; light blue box = *G. partita* from Taiwan; Yellow box = *G. daedala* from Icelandic and Russian regions; CHC = *C. crispus*; GC = *G. chorda*; PU = *P. umbilicalis*; PYH = *P. haitanensis*; CM 10D = *C. merolae*, strain 10D; detailed collection site information are listed in Table S1.

Both organellar and nuclear genome analyses confirmed the monophyletic origin of both Cyanidiophyceae (100% UFBoot) and Galdieriales (100% UFBoot; Figure 3). Plastid phylogenomics, based on 126 concatenated genes, supported the deeply diverging *G. phlegrea* clade, whose concatenated sequences differed by 18% from the other species (Table 1). The phylogenetic analysis based on nuclear data confirmed the separation of this species, with a nucleotide dissimilarity of 40% (Figure 3c; Table 3), unlike the mitochondrial data, which identified *G. phlegrea* as more closely related to the other species (Figure 3b; Table 2).

Table 1. Hamming dissimilarity percentage among the Galdieriales species. The analyses was performed over the total length of the concatenated alignment used to infer the plastid species phylogeny.

	<i>Galdieria phlegrea</i>	<i>Galdieria mesoamericana</i>	<i>Galdieria sulphuraria</i>	<i>Galdieria yellowstonensis</i>	<i>Galdieria javensis</i>	<i>Galdieria partita</i>	<i>Galdieria daedala</i>
<i>Galdieria phlegrea</i>	-						
<i>Galdieria mesoamericana</i>	18%	-					
<i>Galdieria sulphuraria</i>	18%	10%	-				
<i>Galdieria yellowstonensis</i>	18%	10%	9%	-			
<i>Galdieria javensis</i>	18%	9%	9%	8%	-		
<i>Galdieria partita</i>	18%	9%	9%	8%	5%	-	
<i>Galdieria daedala</i>	18%	10%	9%	8%	6%	3%	-

Table 2. Hamming dissimilarity percentage among the Galdieriales species. The analyses was performed over the total length of the concatenated alignment used to infer the mitochondrial species phylogeny.

	<i>Galdieria phlegrea</i>	<i>Galdieria mesoamericana</i>	<i>Galdieria sulphuraria</i>	<i>Galdieria yellowstonensis</i> (group a)	<i>Galdieria yellowstonensis</i> (group b)	<i>Galdieria javensis</i>	<i>Galdieria partita</i>	<i>Galdieria daedala</i>
<i>Galdieria phlegrea</i>	-							
<i>Galdieria mesoamericana</i>	15%	-						
<i>Galdieria sulphuraria</i>	15%	10%	-					
<i>Galdieria yellowstonensis</i> (group a)	17%	14%	14%	-				
<i>Galdieria yellowstonensis</i> (group b)	17%	15%	15%	1%	-			
<i>Galdieria javensis</i>	15%	10%	9%	13%	13%	-		
<i>Galdieria partita</i>	15%	10%	9%	13%	13%	7%	-	
<i>Galdieria daedala</i>	15%	10%	10%	13%	13%	8%	7%	-

Table 3. Hamming dissimilarity percentage among the Galdieriales species. The analysis was performed over the total length of the concatenated alignment used to infer the nuclear species phylogeny.

	<i>Galdieria phlegrea</i>	<i>Galdieria mesoamericana</i>	<i>Galdieria sulphuraria</i>	<i>Galdieria yellowstonensis</i>	<i>Galdieria javensis</i>	<i>Galdieria partita</i>	<i>Galdieria daedala</i>
<i>Galdieria phlegrea</i>	-						
<i>Galdieria mesoamericana</i>	40%	-					
<i>Galdieria sulphuraria</i>	39%	26%	-				
<i>Galdieria yellowstonensis</i>	42%	31%	29%	-			
<i>Galdieria javensis</i>	39%	25%	23%	29%	-		
<i>Galdieria partita</i>	39%	24%	22%	28%	16%	-	
<i>Galdieria daedala</i>	39%	24%	22%	28%	16%	11%	-

Both organelle and nuclear phylogenomic confirmed with high confidence the emergence of *G. mesoamericana* ACUF 138 (100% UFBoot; Figure 3a,c), even though the mitochondrial analysis identified this species as a sister clade of the other *Galdieria* species (59.4% UFBoot; Figure 3b), while the nuclear ones highlighted its more close relationship with *G. javensis*, *G. partita*, and *G. daedala* (100% UFBoot; Figure 3c). This taxonomical incongruence is confirmed by the nucleotide dissimilarity that reached 10% for plastid data (Table 1), 15% for mitochondrial data (Table 2) and 31% for nuclear ones (Table 3).

The following divergent event in plastid phylogeny (100% UFBoot) generated the *G. sulphuraria* clade, whose populations colonised the acido-thermal habitats surrounding the Mediterranean Sea (Spain, Italy and Turkey, Figure 3a). All strains belonging to this clade shared a common ancestor with an intra-level nucleotide divergence of less than 5% from each other (data not shown), while the whole population showed a 9-18% nucleotide dissimilarity with all other species (Figure 3a, Table 1). Based on the mitochondrial phylogeny, *G. sulphuraria* clustered together in a clade, along with *G. mesoamericana*, strain ACUF 138, even if small divergences were highlighted at an intrapopulation level. The separation of these

populations was supported by 100% UFBoot, and the mean percentage of nucleotide divergence was 10%, with higher values (15%) when comparing it with the early diverged American strains (Figure 3b, Table 2). Conversely, the ML tree of nuclear genes identified an early divergence of the Mediterranean populations, which separated from the others by around 22-29% (Table 3).

Based on plastidial and nuclear data, all the *G. yellowstonensis* strains obtained from the Culture Collection of Microorganisms from Extreme Environments (CCMEE) and the Culture Collection of Autotrophic Organisms (CCALA), gathered in a large clade (100% UFBoot, Figure 3a,c). This group is characterised by low intrapopulation genetic dissimilarity and a high percentage of divergence from the other lineages. The mitochondrial phylogeny highlights two different diverging events resulting in an early separation of all the populations from the American areas (Figure 3b). The first segregation occurrence resulted in one monophyletic subgroup (*G. yellowstonensis*, Figure 3b, group a) well supported by the statistical analyses (100% UFBoot, Figure 3b). The phylogenetic tree revealed the subsequent separation of strains from CCMEE and CCALA algal collections (*G. yellowstonensis*, Figure 3b group b). This separation was not supported only by UFBoot statistics (44% UFBoot), and neither the percentage of dissimilarity among them (1%) confirms the separation of the lineage in two different groups (Table 2).

When considering the phylogenetic relationships between *G. javensis*, *G. partita*, *G. daedala*, and *G. sulphuraria* (100% UFBoot; Figure 3a,c), both organelle and nuclear genomes showed an identical topology with the lowest dissimilarities percentages (Tables 1,3). Thus, while the mitochondrial genomes provided compelling evidence for early diverging events within *G. yellowstonensis*, the discrepancies supporting these separations underscore the complexity of their evolutionary history.

3.3 Consensus Network Analysis and Estimation of Gene Concordance Factors.

Concordance factors were measured for each node on the resolved species tree for all genomes and compared with discordance factors, which relate to the proportion of genes supporting a different node resolution (gDF). The number of gene trees that supported each branching event (gCF) is shown in Figure 4, along with the consensus network of the mitochondrial, plastid, and nuclear splits tree.

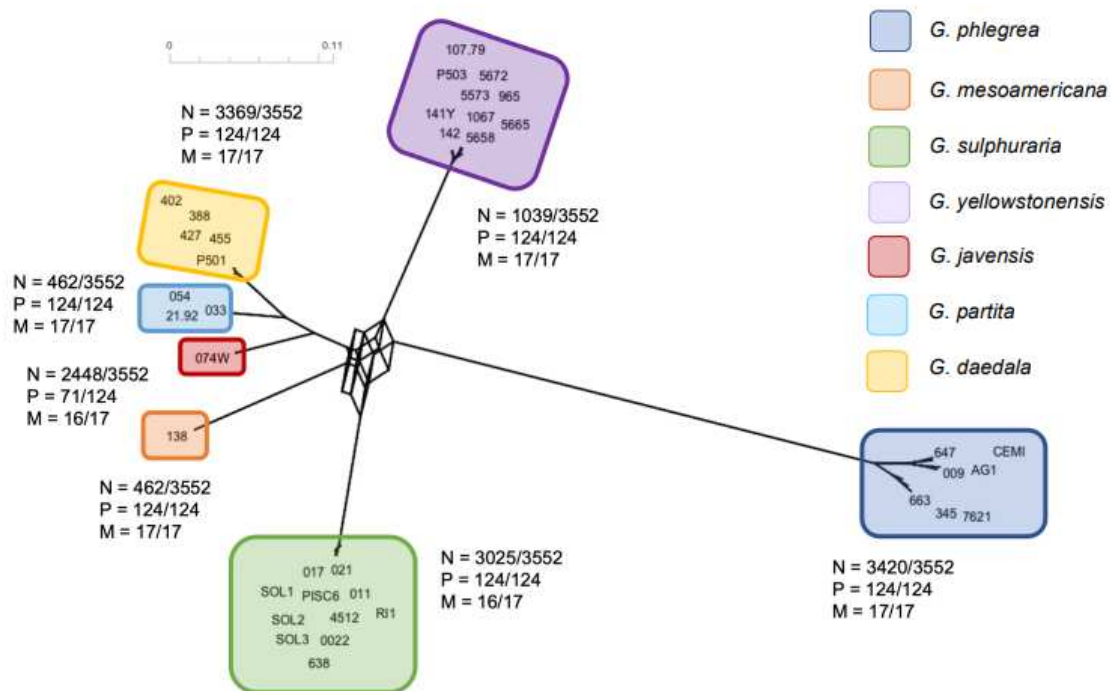


Figure 4. Consensus network of the nuclear, plastid and mitochondrial phylogenies using 38 *Galdieria* strains, containing 116 splits with 290 nodes and 516 edges. The number of nuclear, plastid and mitochondrial gene trees that support each lineage in the species tree topology are indicated near the lineages, collected from gene concordant (gCF) analysis.

Based on the concatenation of the plastid, mitochondrial and nuclear genomes, the network showed a clear and distinct separation of the seven species supported by most organellar and nuclear genes. The differences in topology that appear in the seven species between the nuclear, plastid, and mitochondrial are also highlighted (Figure 4). Consensus networks from all plastids, mitochondrial, and nuclear genes highlighted the monophyletic clustering and parallel evolution of the *Galdieria* species (*G. phlegrea*, *G. mesoamericana*, *G. sulphuraria*,

G. javensis, *G. daedala*, and *G. partita*) as already revealed by the species phylogeny, except for *G. yellowstonensis*. According to the mitochondrial genome, this species diverged in two clades, well supported by the gene phylogenies. Gene concordance factors on the concatenated nuclear tree highlighted significant support for *G. sulphuraria*, *G. partita*, and *G. daedala* species (more than 3000 nuclear genes over the total). *G. yellowstonensis* shows discrete support from the individual gene trees (2448/3552), while less than 30% of gene trees support the monophyly of *G. mesoamericana* and *G. javensis* (Figure 4).

3.4 Evolutionary rates

The following investigation aims to understand the high divergence between the seven species highlighted in the previous analysis. Therefore, one *Galdieria* strain from each of the seven identified species was selected to represent each group in further analysis. These were as follows: ACUF 009 (*G. phlegrea*), ACUF 138 (*G. mesoamericana*), ACUF 017 (*G. sulphuraria*), ACUF 074 (*G. javensis*), SAG 107.79 (*G. yellowstonensis*), THAL 033 (*G. partita*), and ACUF 427 (*G. daedala*). Substitution rates were measured between the seven *Galdieria* species, including 126 plastid, 17 mitochondrial and 1947 nuclear-encoded genes shared by all the species (Table 4).

Table 4. Substitution rates measured between the seven *Galdieria* species, including 126 plastid, 17 mitochondrial and 1947 nuclear-encoded genes shared by all the species.

Genomes	Substitution per site		
	Mitochondrial	Plastid	Nuclear
Synonymous sites			
Average (SD)	1.93 (0.71)	1.64 (0.38)	2.74 (2.32)
CV	0.37	0.23	0.85
Nonsynonymous sites			
Average (SD)	0.22 (0.22)	0.1 (0.07)	0.36 (0.25)
CV	1.00	0.70	0.69
dN/dS			
Average (SD)	0.42 (1.36)	0.07 (0.05)	0.13 (0.07)
CV	3.24	0.71	0.54
Ratios of nonsynonmyous rates			
nuclear:mito	1.636363636		
nuclear:plastid	3.6		

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383 Nucleotide substitution rates in *Galdieria* are higher in the nuclear genome than in the
384 mitochondrial and plastid ones (Table 4). Synonymous site divergence was found to be
385 essentially saturated in mitochondrial, plastid, and nuclear genes, with estimates of 1.9, 1.6,
386 and 2.7 substitutions per site, respectively (Table 4), meaning that the synonymous
387 divergences are essentially too large to be estimated accurately. Nonsynonymous site
388 divergence is much less, with average rates of 0.22 (mitochondria), 0.1 (plastid), and 0.36
389 (nuclear) substitutions/site (Table 4).

390 The rates of substitution at nonsynonymous sites (dN) were lower in all three compartments
391 0.22 (0.22), 0.1 (0.07), and 0.36 (0.25), but were, on average, 1.6 and 3 times greater for the
392 nucDNA relative to the mtDNA and ptDNA. The dN/dS ratio, which can be used to gauge the
393 intensity and directionality of selection, differed across the three genomes (Table 4). The
394 average for plastid, nuclear, and mitochondrial genomes is 0.07 (0.05), 0.13 (0.07), and 0.42
395 (1.36), which is consistent with the most robust purifying selection acting on the plastid
396 genome. Whilst the mitochondrial genome is presented with the highest average dN/dS ratio
397 when looking at the SD, caused by the *nad6* gene having an above average ratio and skewing
398 the adjusted mitochondrial dN/dS 0.09 (0.08), then substitution rates at non-coding sites were
399 also highest in the nuclear genes (Table 4). Figure 5a plots the plastid, mitochondrial, and
400 nuclear genes' dN vs dS. Most genes have dN <1 and dS <10.

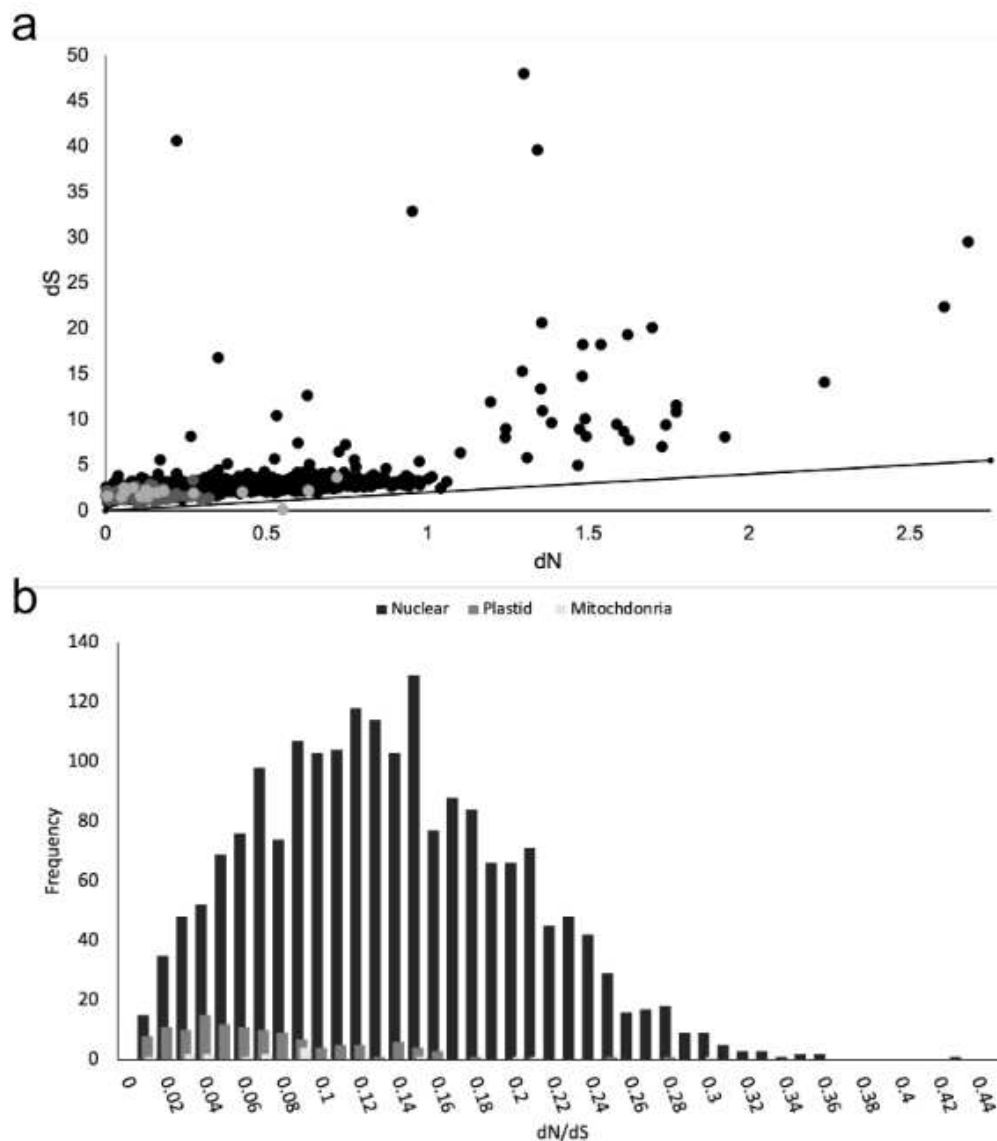


Figure 5. (a) Pairwise omega (dN/dS) values. This graph shows pairwise dN vs dS values for *Galdieria* nuclear, mitochondrial and plastid genes. The line is dN/dS = 0.5. (b) Histogram showing the distribution of ω for each of the nuclear, mitochondrial and plastid genes.

Additionally, Figure 5b shows the distribution of ω to be normal, and most genes have $\omega \leq 0.2$, as expected. For all genes, the ratio of substitutions is never above 0.43. The dN and dS values were analysed using different models to assess any gene under positive selection. This resulted in two mitochondrial genes (*nad2* and *nad6*), three plastid genes (*gltB*, *psaA* and *ycf80*) and 288 nuclear genes (Table S6) showing positive selection that would require further investigation.

3.5 Comparative genome analysis

3.5.1 General Features of *Galdieria* Genomes

The total number of genes in each *Galdieria* genome varied along with genome size (Table 5). *G. sulphuraria* was found to have the largest genome and contained the highest number of genes, while strain *G. partita* contained the lowest number of genes. *C. merolae* has the lowest number of genes but a genome larger than 5/6 (7) of the *Galdieria* species.

Table 5. Galdieriales seven nuclear genomes (*G. phlegrea*, *G. mesoamericana*, *G. sulphuraria*, *G. javensis*, *G. partita*, *G. daedala*) and two outgroups (*C. merolae* and *P. purpureum*) assembly and orthologs analysis statistics. Genomes were annotated using AUGUSTUS and orthogroups assessed using OrthoFinder.

Strain/species	Genome Assembly (Mb)	Number of Protein-Coding Genes	GC Content (%)	Number of orthogroups containing species	Percentage of orthogroups containing species	Number of genes in species-specific orthogroups
<i>Galdieria phlegrea</i>	13.82	5,439	35.85	4,532	71.65	56
<i>Galdieria mesoamericana</i>	13.7	6,046	39.34	4,783	76	86
<i>Galdieria sulphuraria</i>	19.75	6,445	38.72	4,865	77.3	24
<i>Galdieria yellowstonensis</i>	14.54	6,147	37.67	4,722	75	145
<i>Galdieria javensis</i>	15.04	5,876	37.85	4,869	77.3	36
<i>Galdieria partita</i>	14.21	5,092	40.25	4,482	71.2	7
<i>Galdieria daedala</i>	12.93	5,776	37.91	4,818	76.5	15
<i>Cyanidioschyzon merolae</i>	16.42	4,803	54.94	3,309	52.6	142
<i>Porphyridium purpureum</i>	19.67	9,898	55.8	4,051	64.3	2473

The *P. purpureum* genome size was similar to *G. sulphuraria*, strain ACUF 017, but contained over 3000 more genes. Across the *Galdieria* genomes, the average GC content was 38.23%, lower than the values from *C. merolae* (54%) and *P. purpureum* (55%). Cluster analysis on the nine species (7 *Galdieria* species, plus *C. merolae* and *P. purpureum*) identified 6298 orthologous clusters. Analysis of these clusters suggested that, on average, across the *Galdieria* genomes, 75% of orthogroups contain proteins from the species, compared to 52% from *C. merolae* and 64% from *P. purpureum* (Table 5). Among the set of homologous genes, there were 313 single-copy orthologs in *Galdieria*. This ranged across the species, with *G. partita* containing the lowest species-specific orthogroups (7 genes) and *G. sulphuraria* with

the highest ones (145 genes). *C. merolae* contained 142 species-specific genes, and *P. purpureum* contained 2473 (Table 5). *Galdieria* organelle genomes are more conserved and smaller (13% of reduction for the plastid genome and 40% for the mitochondrial one) than non-*Galdieria* red algae. Most of *Galdieria* plastid genomes demonstrated a partition in four distinct zones (LSC, SSC, and two inverted repeats) and the presence of the second inverted region (Figure S3a-b). Both plastid and mitochondrial genome synteny demonstrated highly conserved structure among *Galdieria* species with some minor variations (Figure S4, S5).

4. Discussion

This study set out to refine the phylogenomic structure of *Galdieria* using newly generated high-resolution plastid, mitochondrial, and nuclear genomes. While our primary aim was taxonomic clarification and evolutionary resolution across this red algal group, the resulting data unexpectedly revealed a far more complex evolutionary scenario. Notably, the three genomes - mitochondrial, plastid, and nuclear - exhibit remarkably independent trajectories and incongruent phylogenetic relationships. These findings point to a dynamic evolutionary landscape shaped by genome-specific forces, challenging the assumption of a unified evolutionary signal across compartments. Here, we integrate these unexpected discoveries with past literature and provide a critical reassessment of *Galdieria* diversity, genome evolution, and adaptation.

In the last decade, molecular phylogenetic studies indicated an ever-increasing number of new cryptic species, genera, and orders within the class Cyanidiophyceae. In this study, preliminary phylogenetic results focused on the partial *rcbL* sequence from 277 Cyanidiophyceae species confirmed the orders established and described in 2023 [29]. The identified orders in question are: 1) Cyanidiales F.D.Ott, 2009. J. Cramer, Berlin; 2) Cyanidioschyzonales F.D.Ott, 2009. J. Cramer, Berlin; 3) Cavernulicolales H.S.Yoon, S.I.Park, C.H.Cho, & R.A.Andersen ordo nov; 4) Galdieriales H.S.Yoon, S.I.Park, C.H.Cho & R.A.Andersen ordo nov [29].

Moreover, the rbcL phylogeny highlighted the Galdieriales as the order with the highest number of identified species and genetic diversity. Based on past research, this order includes the genus *Galdieria* and 7 species: 1) *G. sulphuraria* (Galdieri) A. Merola 1982; 2) *G. daedala* nom. nud.; 3) *G. maxima* nom. nud.; 4) *G. partita* nom. nud.; 5) *G. javensis* H.S. Yoon, S.I. Park, & R.A. Andersen 2023; 6) *G. phlegrea* H.S. Yoon, S.I. Park, & C. Ciniglia 2023; 7) *G. yellowstonensis* H.S. Yoon, S.I. Park, & T. McDermott 2023 [29,64]. *G. daedala*, *G. maxima*, and *G. partita* [19] were nominated as nom. nud. due to the lack of a designated type as required by the Art. 40, International Code of Nomenclature or ICN (Turland et al. 2018). The original names of these species, established by Sentsova in 1991, were recently validated by Del Guacchio et al. in 2023 after a deep discussion on the nomenclatural issues regarding the order Galdieriales and morphophysiological analysis of the strains [65]. The authors established the species holotypes after preservation in 4% formalin of the living strains isolated and sent to the University of Naples Algal collection (ACUF) by O. Ju. Sentzova. The species were nominated as follows: 1) *G. daedala* Sentzova ex Pollio, De Natale & Del Guacchio; 2) *G. maxima* Sentzova ex Pollio, De Natale & Del Guacchio; 3) *G. partita* Sentzova ex Pollio, De Natale & Del Guacchio.

Among the Russian species, *G. maxima* IPPAS P507 has a controversial history of inconsistency between morphological and phylogenetic features and has been for long misapplied because of its erroneous genetic relationship with *Cyanidioschyzon merolae* [22,25,28,66]. The inconsistency of the taxonomic position of *G. maxima*, hypothetically due to a culture contamination [63], requires a more thorough and comprehensive analysis of the species, which should include the study of both morphological and molecular traits that will be addressed in a separate study. Due to this, in the present paper, *G. maxima* was momentarily excluded by the genetic/genomic analysis.

The phylogenetic tree based on the rbcL sequences provided in this study highlights the existence of a new well-supported clade (100% UFBoot), currently including only two *Galdieria* strains, both originating from Central America, ACUF 135 from Mexico and ACUF 138 from San Salvador. We here propose the establishment of a new *Galdieria* species, named as *G.*

mesoamericana, with morphophysiological traits overlapping those of all other Galdieriales, hoping to strengthen this clade with new sampling from Central and Southern America.

The phylogenetic relationships within *Galdiera* were assessed by comparing the nuclear pangenome (3,532 genes) with the plastid (126 genes) and mitochondrial (18 genes) datasets. While all genomic compartments agreed with our *rbcL* analysis (Figure S2) regarding the broad clustering of the primary species (*G. phlegrea*, *G. mesoamericana*, *G. sulphuraria*, *G. yellowstonensis*, *G. javensis*, *G. partita* and *G. daedala*), the genomic data provided significantly higher resolution for the internal branch topologies. Specifically, the mitochondrial multi-gene approach resolved finer intraspecific relationships and internal subclade divergences that were not highlighted in the single-gene *rbcL* phylogeny. Moreover, the nuclear pangenome resolved the deep evolutionary position of *G. phlegrea* as the earliest diverging lineage within the genus with maximal statistical support. This higher resolution revealed incongruent evolutionary trajectories between the organelle and nuclear genomes. This incongruence manifested as topological conflicts among the trees regarding the internal branching order within specific lineages, alongside pronounced differences in branch lengths that indicate divergent substitution rates across the three genomic compartments. Based on the endosymbiotic theory, plastid, and mitochondria originated as a result of two separate absorption events of cyanobacteria and α -proteobacteria from unicellular eukaryotes [67,68]. The close relationship of a symbiont with the host gave new functionalities to the latter, such as aerobic respiration and/or photosynthesis. The genomes could have evolved differently as well; it is well established that mitochondrial genomes, for example, have been widely evolving due to gene transfer from the symbiont to the nucleus and the loss of redundant genes [67]. Moreover, the uncertainty of the exact root topology could be possibly due to saturated mutations [69]. Even though this is not unexpected for photosynthetic eukaryotes, evidence of the relative mutation rates among organelle and nuclear genomes, resulting in their different evolution, was already stated in plants, green and red algae [70,71]. Furthermore, high variability of the dN/dS ratios across all *Galdiera* genes would have confirmed that the organelle and nuclear genes could be subjected to different evolutionary

forces (Figure 5a). dN/dS ratios were calculated for each gene pair across *Galdieria* species, expressed as mean values (Figure 5a; Table 4). Results indicated stronger purifying selection in plastid genes (0.07) compared to mitochondrial (0.42) and nuclear (0.13) genomes. Such variability reflects differential evolutionary pressures, where highly conserved proteins (e.g., histones; Peterson & Laniel 2004) contrast with more variable membrane transporters [72,73]. dN/dS analyses, valuable for detecting biochemical/ecological constraints or adaptive proteins [74], identified 288 nuclear genes under positive selection. Coefficient of variation analyses further highlighted higher variability in dN across all genomes.

In Galdieriales, along with the high substitution rates, the exchange of genetic material between organisms, known as horizontal gene transfer (HGT), profoundly influences the phylogenetic relationship among *Galdieria* species and their adaptation to extreme environments [75]. Acquired genes are mainly involved in the adaptation of the species to harsh environments, including metal and xenobiotic resistance/detoxification, cellular oxidant reduction, carbon and aminoacid metabolism, osmotic resistance and salt tolerance, non-metabolic and uncertain functions [76–79]. Hybridisation, recombination, and gene duplication are further essential events contributing to genetic variation in populations and, consequently, to the incongruence between Gene and Species trees [80].

4.1 Galdieriales taxonomic remarks

The order Galdieriales is herein emended following recent taxonomic revisions [29]. It includes a single genus, *Galdieria*, comprising seven species (*G. phlegrea*, *G. mesoamericana*, *G. sulphuraria*, *G. yellowstonensis*, *G. javensis*, *G. partita*, and *G. daedala*), described through integrated morphophysiological and molecular approaches. Species display variable cell sizes (3–11 µm), net-like mitochondria, vacuoles, and autosporic reproduction with 4–32 autospores [3,20,29]. All grow photo-, mixo-, and heterotrophically, exploiting >50 carbon sources [81]. Phylogenies consistently support Galdieriales as a monophyletic clade with species-level subclades [28,31,76].

Galdieria genomes are notably compact (~10 Mb), among the smallest reported for eukaryotes, consistent with adaptation to extreme environments [82]. Genome size is shaped by mutation rates [82,83] and correlates with metabolic efficiency, growth, and development [84,85]. Reduced genome size has been linked to enhanced growth and fitness in algae [86], while population size dynamics further constrain genome evolution through purifying selection [87]. The genome size reduction was also highlighted in the organelle genomes and shared by all the species, along with a similar number of CDS and GC content; all these features were consistent with previous findings [75]. All the mitochondrial genomes diverged only by a long non-coding sequence, the exact functionality of which is unknown. Non-coding DNA may have essential functions in transcriptional and translational regulation or may be the original site of DNA replication [88]. For instance, the longest non-coding and most variable region in animal mitochondrial DNA is identified as the Control Region (CR) and comprises a third strand of DNA, creating a D-loop [88]. This region has a strand-specific bias in vertebrates and is involved in the asymmetric DNA replication mechanism [76,89].

Galdieria plastid genomes reflected a similar structure with those from Glaucophyta, green algae, land plants, and from all eukaryotic algae with red algal-derived plastid genomes [75]. Thus, it is possible that this region was present in the common ancestor and was then lost or rearranged in those lineages that diverged afterwards. The plastid genome feature that can distinguish the *G. mesoamericana* is the presence of one single region, which includes the ribosomal RNAs (Figure S2).

5. Conclusion

The genetic patterns identified in this study provide an evolutionary framework that is highly relevant in light of the growing biotechnological interest in Galdieriales, reinforcing its value as a model extremophile.. Given the thermophilic preference of *Galdieria* (optimum ~42 °C), their historical dispersal across non-thermal regions remains enigmatic; however, the extent of nonsynonymous divergence suggests ancient separation and long-term isolation of lineages.

Although morphological traits and organellar genome structures vary only slightly among species, phylogenetic analyses reveal deep divergence into cryptic lineages evolving independently. Patterns of synonymous and nonsynonymous substitutions further indicate heterogeneous selective pressures among strains. High mutation rates, horizontal gene transfer, and recombination are likely the major evolutionary forces shaping genetic diversity within *Galderia*. Our data support recognition of a new Central American species, *Galdieria mesoamericana*. While its genomic features broadly align with the established Galdieriales range, its elevated nucleotide and organellar diversity justify species-level distinction. Nonetheless, limitations must be acknowledged, including sampling bias and reliance on overlapping morphological traits that complicate taxonomic resolution. Expanding sampling in unexplored acidic habitats, coupled with functional analyses of positively selected genes and transcriptomic studies under stress conditions, will deepen understanding of adaptive mechanisms. The genetic and evolutionary insights presented here represent a critical step toward identifying molecular targets for strain selection and genetic engineering, ultimately facilitating the development of optimised Galdieriales-based platforms for industrial and environmental biotechnology.

DATA AVAILABILITY

The data is set to be released upon publication. NCBI BioprojectID PRJNA929444 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA929444>)

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COMPETING INTEREST

None declared.

AUTHORS CONTRIBUTIONS

MI and **SCLL**: Conceptualization, Data curation, Formal analysis, Methodology, Validation, Visualization, Writing – original draft and Writing – review & editing. **MI** and **SCLL** contributed equally to this work. **SJ**: Methodology and Formal analysis. **DJ**: Methodology, Formal analysis, Supervision. **AP**: Investigation. **EDG**: Investigation. **CC**: Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing. **SJD**: Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing. **LCMM**, **JPJC**, **GAF** and **HSY**: Writing – review & editing.

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ACUF 138



ACUF 002
ACUF 011
ACUF 017
ACUF 021
ACUF 638
ACUF 660
PISC 6
RI1
SOL1
SOL2
SOL3



ACUF 141
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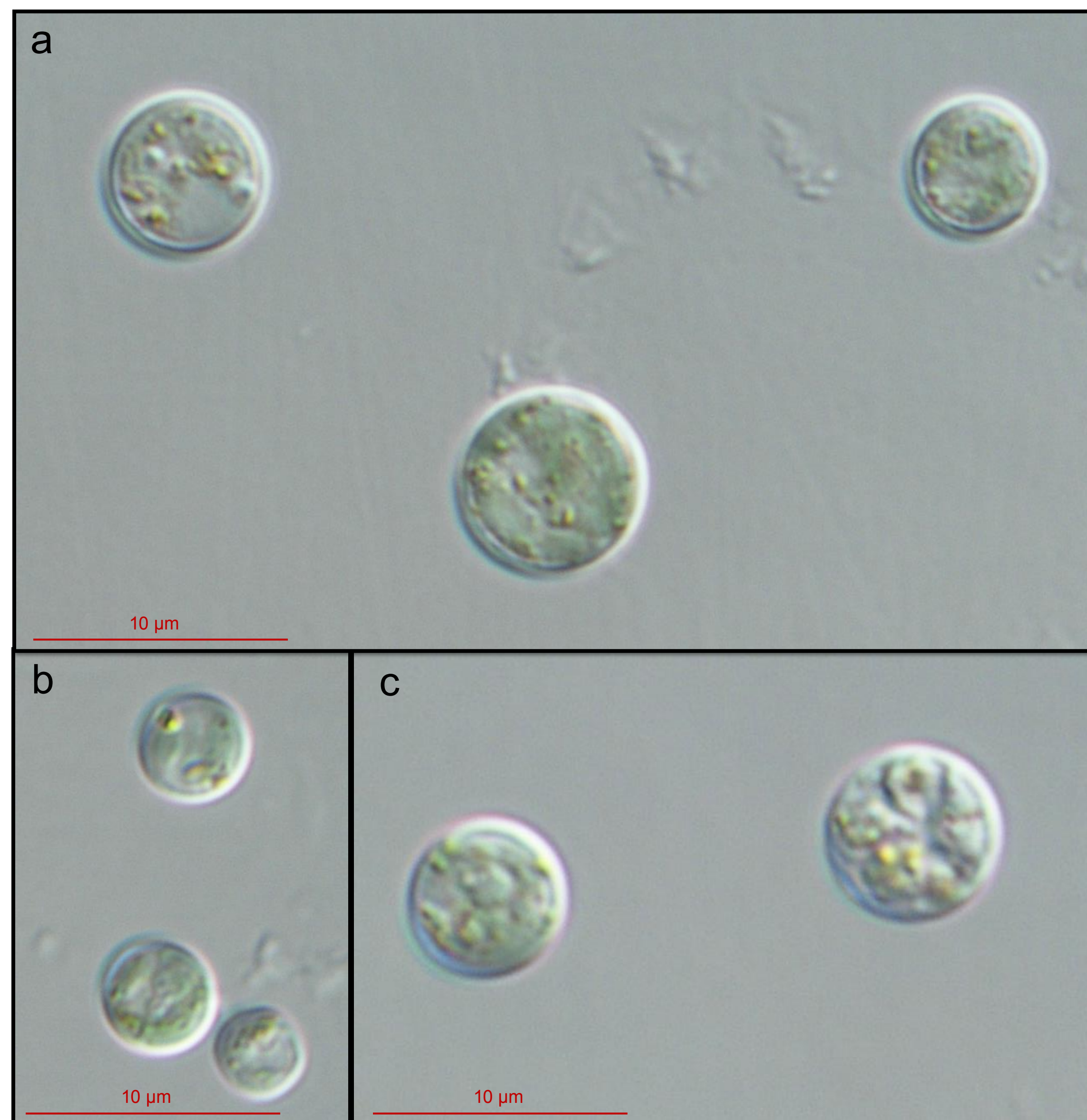
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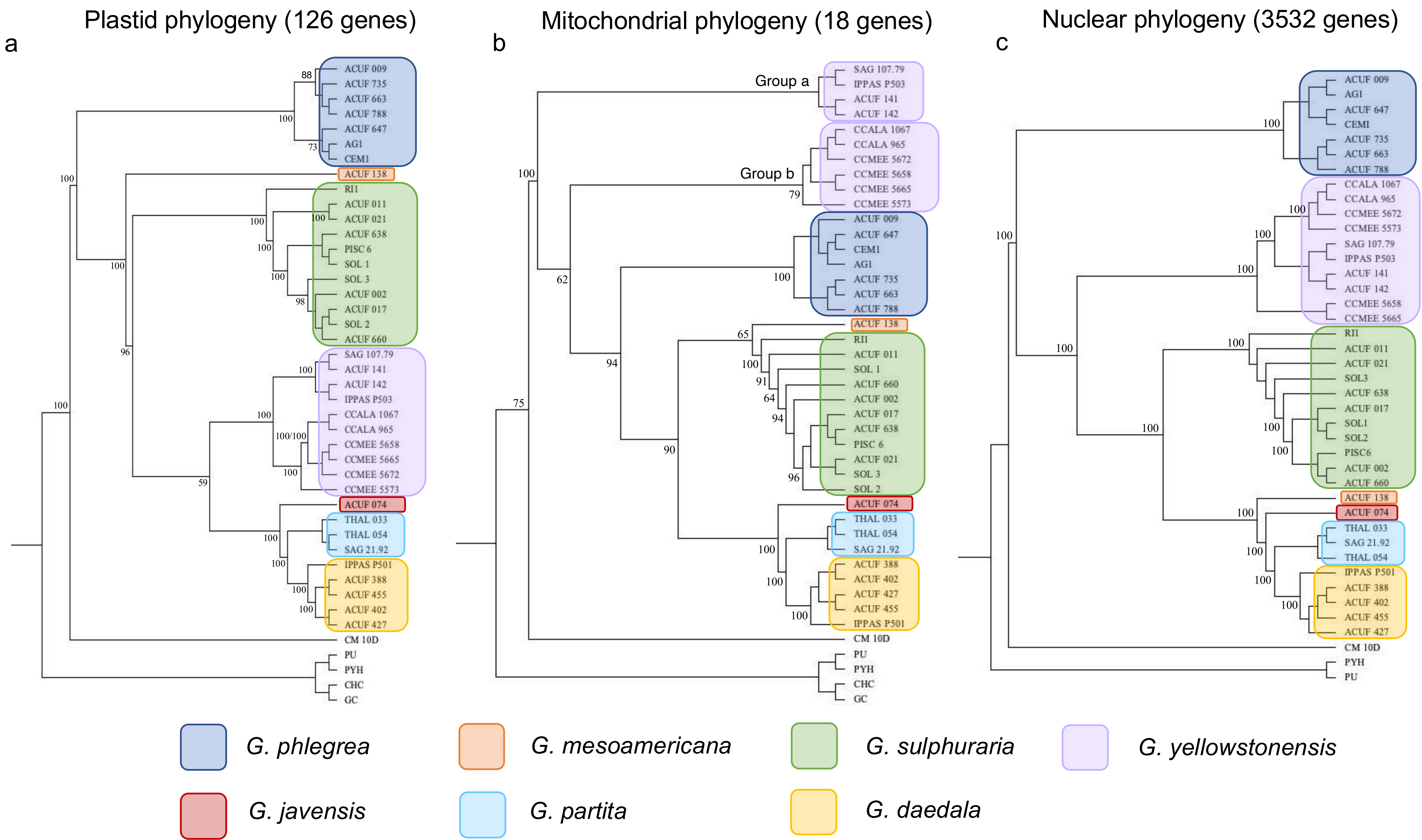


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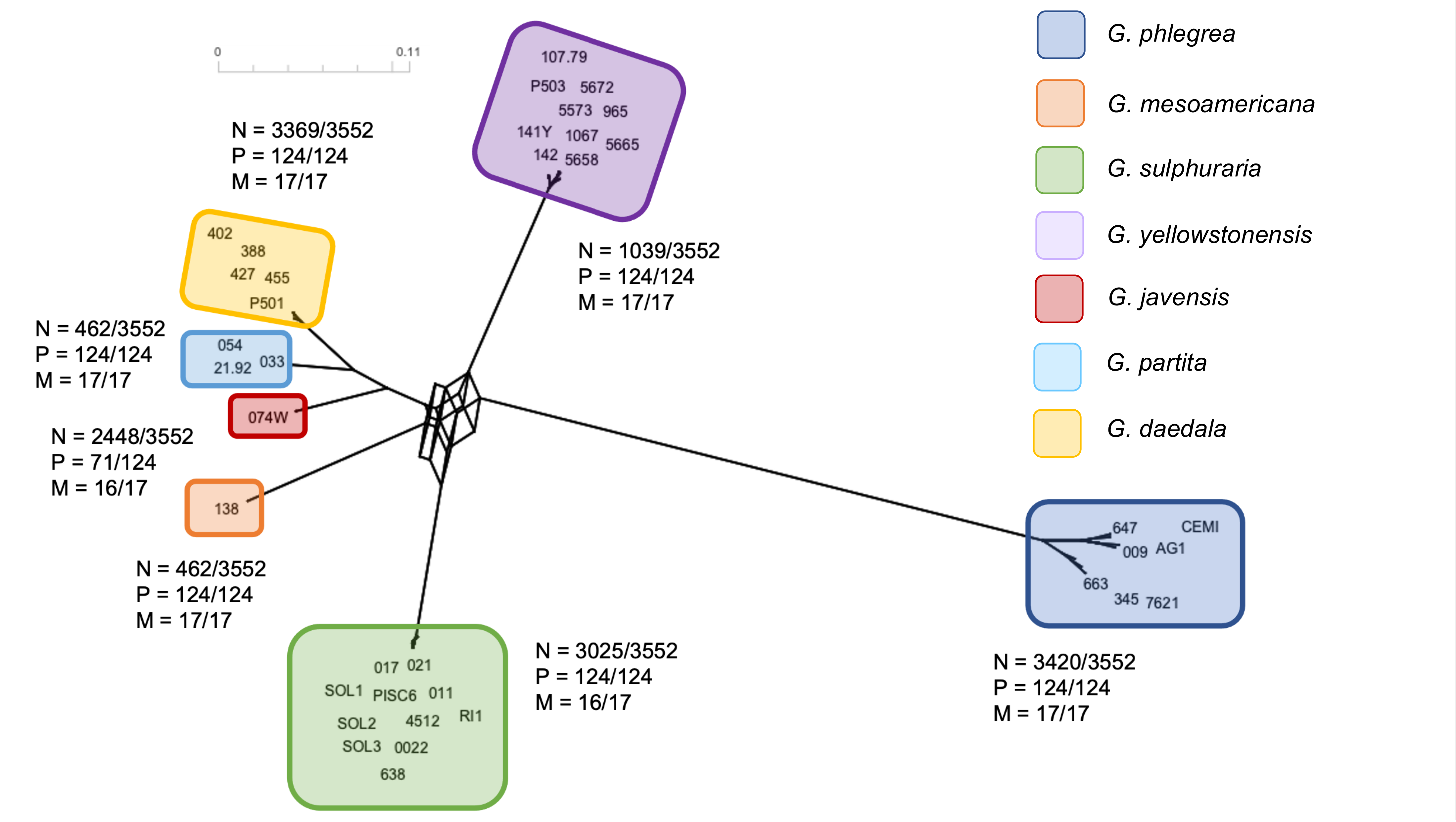
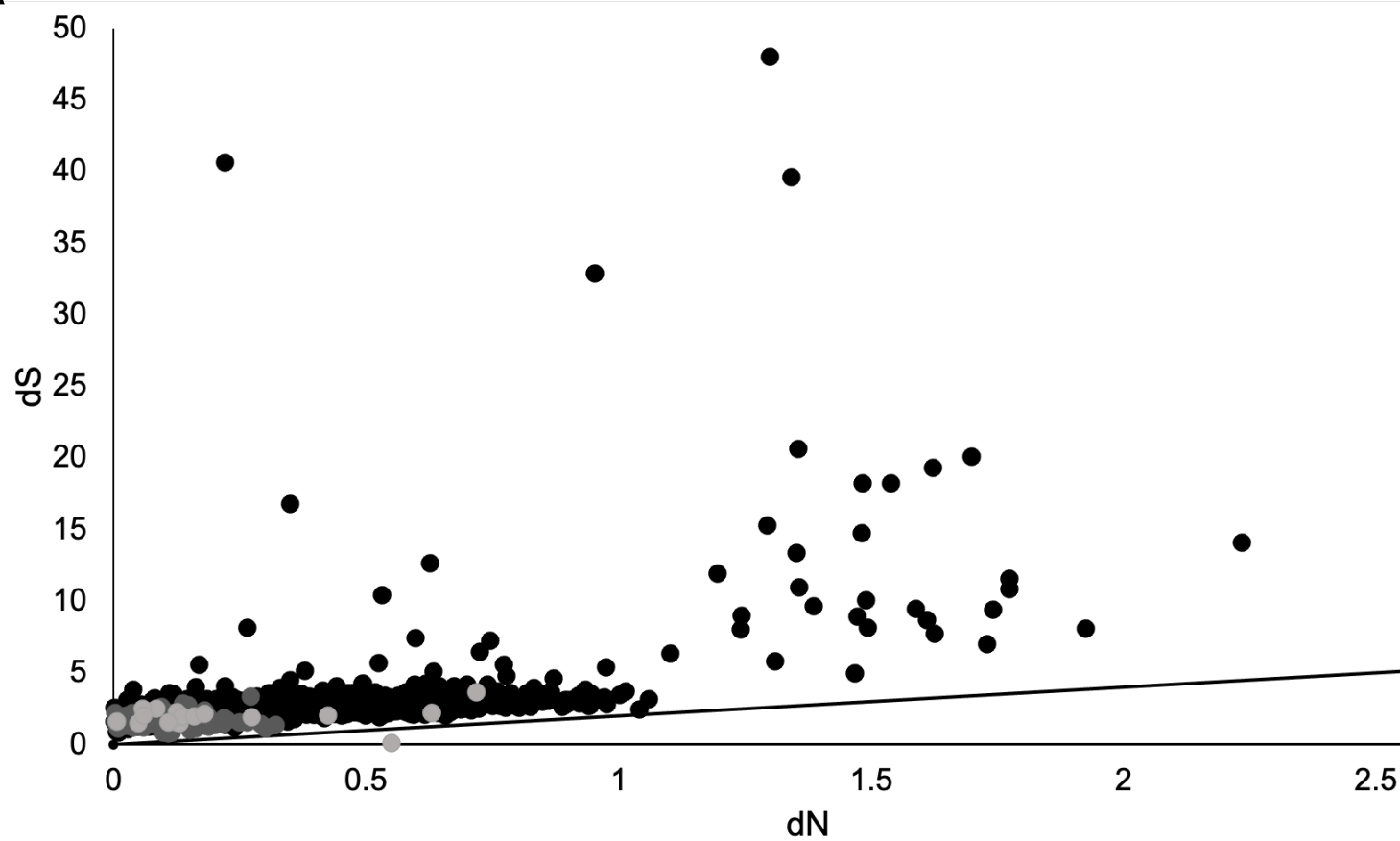


Figure 5

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