Article
Phylogenomic Analyses and Molecular Signatures Elucidating the Evolutionary Relationships amongst the Chlorobia and Ignavibacteria Species: Robust Demarcation of Two Family-Level Clades within the Order Chlorobiales and Proposal for the Family Chloroherpetonaceae fam. nov

Sarah Bello 1, Mohammad Howard-Azzeh 2, Herb E. Schellhorn 3 and Radhey S. Gupta 1,*

1 Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, ON L8N 3Z5, Canada; bellos1@mcmaster.ca
2 Department of Population Medicine, Ontario Veterinary College, University of Guelph, Guelph, ON N1G 2W1, Canada; mhowarda@uoguelph.ca
3 Department of Biology, McMaster University, Hamilton, ON L8S 4K1, Canada; schell@mcmaster.ca
* Correspondence: gupta@mcmaster.ca

Abstract: Evolutionary relationships amongst Chlorobia and Ignavibacteria species/strains were examined using phylogenomic and comparative analyses of genome sequences. In a phylogenomic tree based on 282 conserved proteins, the named Chlorobia species formed a monophyletic clade containing two distinct subclades. One clade, encompassing the genera Chlorobaculum, Chlorobiurn, Pelodictyon, and Prosthecochloris, corresponds to the family Chlorobiaceae, whereas another clade, harboring Chloroherpeton thalassium, Candidatus Thermochlorobacter aerophilum, Candidatus Thermochlorobacteriaceae bacterium GBChlB, and Chlorobium sp. 445, is now proposed as a new family (Chloroherpetonaceae fam. nov). In parallel, our comparative genomic analyses have identified 47 conserved signature indels (CSIs) in diverse proteins that are exclusively present in members of the class Chlorobia or its two families, providing reliable means for identification. Two known Ignavibacteria species in our phylogenomic tree are found to group within a larger clade containing several Candidatus species and uncultured Chlorobi strains. A CSI in the SecY protein is uniquely shared by the species/strains from this “larger Ignavibacteria clade”. Two additional CSIs, which are commonly shared by Chlorobia species and the “larger Ignavibacteria clade”, support a specific relationship between these two groups. The newly identified molecular markers provide novel tools for genetic and biochemical studies and identification of these organisms.

Keywords: phylogenomic and comparative genomic analyses; conserved signature indels (CSIs); molecular signatures; class Chlorobia and the families Chlorobiaceae and Chloroherpetonaceae; Ignavibacteria; uncultured species/strains related to Chlorobia/Ignavibacteria

1. Introduction

Members of the class Chlorobia, also known as green sulfur bacteria, were, until recently, comprised of a group of strictly anaerobic, photosynthetic bacteria [1] that occupy a very narrow environmental niche consisting of anoxic aquatic settings in stratified lakes, where sunlight can penetrate [1–4]. These bacteria use reduced sulfur compounds as reductants and electron source to fix CO$_2$ using the reverse TCA cycle [4–6]. Based on their branching in phylogenetic trees for the 16S rRNA gene and FMO protein sequences, these bacteria have been placed in a separate phylum Chlorobiota, consisting of a single class “Chlorobia” containing a single order Chlorobiales and a single family Chlorobiaceae [3–5,7–10]. According to the List of Prokaryotic Names with Standing in Nomenclature (LPSN) server [11], the
family Chlorobiaceae contains six genera (Ancalochloris, Chlorobaculum, Chlorobium, Chloroherpeton, Pelodictyon, and Prosthecochloris) [4,5,12,13]. However, there is no information available for the sole species Ancalochloris perfilielvi from the genus Ancalochloris [14] and most of the species from the family Pelodictyon have been transferred into the genus Chlorobium [12,15]. According to the LPSN server, the family Chlorobiaceae now contains 15 species (excluding synonyms and non-validly published species) [11]. However, most of these species were described prior to 2008 [11] and since then, except for a few Candidatus species [16,17], no new Chlorobiaceae species have been identified. Until 2013, members of the phylum Bacteroidetes (recently renamed Bacteroidota) [18] were the closest relatives of the Chlorobia species [3,5,12,19,20] and together with the Fibrobacteres they formed the Fibrobacteres–Bacteroidetes–Chlorobia (FBC) superphylum [20,21]. However, subsequent studies have led to the identification of two new species, viz., Melioribacter roseus [22,23] and Ignavibacterium album [7], which, based on phylogenetic trees for several gene/protein sequences, are considered the closest relatives of Chlorobia species [7,23,24]. Unlike the Chlorobia species, all of which are strictly anaerobic and obligate photolithotrophs [3–6], Ignavibacterium as well as Melioribacter are moderately thermophilic, facultatively anaerobic, and obligate chemoorganotrophs [7,23–26]. In earlier phylogenetic trees based on the 16S rRNA gene and other genes/proteins, a clade comprising I. album and M. roseus was separated from Chlorobia species by a long branch [7,23]. This led to the placement of these two species into a new phylum, recently renamed Ignavibacteriota [18], containing a single class Ignavibacteria and a single order Ignavibacteriales [23,24], which is a sister taxon of the Chlorobia (Chlorobiota) [23,24]. In addition to these two Ignavibacteria species, analyses of metagenomic sequences from several thermophilic habitats have led to the reconstruction of genomes for several uncultivated organisms that are related to Chlorobia–Ignavibacteria species [25–27]. Based on their genome sequences, some of these organisms are hypothesized to have biochemical–physiological characteristics overlapping with the properties of either members of the class Chlorobia or members of the class Ignavibacteria (i.e., aerobic phototrophs possessing genes for bacteriochlorophyll biosynthesis and related proteins) [25–27].

Due to rapid advances in genome sequencing technology, and because of several major genomic sequence projects [28–30], whole genome sequences are now available for most of the species from the Chlorobia/Ignavibacteria groups. In addition, sequence information is also available for several unnamed Chlorobia species in the NCBI database (https://www.ncbi.nlm.nih.gov/genome/) (accessed on 15 January 2022) [31]. The available genomes provide a comprehensive resource for more reliably understanding the evolutionary relationships amongst these organisms based on multiple independent genomic approaches. Based on genome sequences, robust phylogenetic trees can be constructed based on a large dataset of genes/proteins, exhibiting a high degree of statistical support at different taxonomic levels [32–35]. Indeed, phylogenetic analyses based on 120 proteins that are ubiquitously found in different micro-organisms have led to the creation of a Genome Taxonomy database (GTDB), which has become an important reference resource for the classification of prokaryotic organisms [32,36]. In the GTDB taxonomy (https://gtdb.ecogenomic.org/) (accessed on 30 May 2022), members of the classes Chlorobia and Ignavibacteria exhibit considerable genetic diversity, indicating that more detailed studies are needed to clarify the evolutionary relationships and taxonomy of these organisms.

With the aim of clarifying the evolutionary relationships amongst Chlorobia/Ignavibacteria species, we have carried out phylogenomic and comparative genomic studies on the genome sequences of these species. To this end, we have constructed phylogenetic trees for these two groups of species based on concatenated sequences for several sets of conserved proteins. These trees, along with the GTDB taxonomy [32] and phylogenetic trees reported in earlier studies [25–27], provide a phylogenetic framework for understanding the evolutionary relationships among the Chlorobia species and their relationship to the class Ignavibacteria. In parallel, we have also performed extensive studies on protein sequences from the Chlorobia/Ignavibacteria genomes to identify molecular signatures, consisting of conserved signature indels (CSIs), that are uniquely shared by species from the major clades of Chloro-
Bacteria/Ignavibacteria observed in our phylogenetic trees. Molecular synapomorphies, such as CSIs, that are specific for a given group of organisms, provide strong evidence, independently of phylogenetic trees, for the genetic cohesiveness and common ancestry of a given group of organisms [21,37–41]. Hence, these molecular markers provide reliable means for the delineation of specific clades in molecular terms, and they have proven useful for taxonomic purposes [35,39,42,43]. Results presented here show that the named Chlorobia species (order Chlorobiales) form a strongly supported clade in trees based on different genes/proteins. The distinctness of this clade is independently strongly supported by 33 CSIs that are present in diverse proteins that are uniquely shared by the members of this class/order. Furthermore, members of the class Chlorobia (order Chlorobiales) form two distinct clades, which can be reliably distinguished from each other based upon several identified CSIs that are exclusively shared by the members of these two clades. One of these clades, encompassing all Chlorobiales genera except Chloroherpeton, corresponds to the revised family Chlorobiaceae, whereas a second clade grouping together Chloroherpeton thalassium and some Candidatus Thermochlorobacter strains/isolates and an uncultured Chlorobiun sp. 445 (we will be referring to this group as thermophilic phototrotophores), is now proposed as a new family (Chloroherpetonaceae fam. nov). Results presented here also show that the class Ignavibacteria is genetically highly diverse and that several uncultured species, currently referred to as “Chlorobi bacterium” as well as some Candidatus species, are also related to this class/phyllum of bacteria. Our work has also identified two new CSIs, which are uniquely shared by most of the Chlorobia and Ignavibacteria species, providing further evidence that these two groups of organisms are closely related.

2. Materials and Methods

2.1. Construction of Phylogenetic Trees

Protein sequences were downloaded for different available genomes from named Chlorobia species as well as several unnamed/uncultured Chlorobi species that are denoted by specific numbers in the NCBI genome database [31]. In addition, the sequences for I. album and M. roseus and some Candidatus species (viz., Cand. Thermochlorobacter aerophilum [27], Cand. Thermochlorobacteriaceae bacterium GBChlB, Cand. Kapabacteria thiocyanatum, and Cand. Kryptonium thompsoni), which according to the GTDB taxonomy are related to the Chlorobia/Ignavibacteria taxa, were also downloaded. In addition, genome sequences for several Bacteroidetes/Fibrobacteres species (viz., Rhodothermus (R.) marina, Salinibacter (S.) ruber, Cytophaga (C.) aurantiaca, Bacteroides (B.) fragilis, and Fibrobacter (F.) succinogenes) were also downloaded to serve as outgroups in phylogenetic analysis. Using these genome sequences, a phylogenetic tree was constructed based on concatenated sequences of 282 conserved proteins that are a part of the phyloeco marker set for the FBC superphylum [20]. Two additional phylogenetic trees were constructed for these species based on the concatenated sequences for Gyrase A and Gyrase B proteins, as well as UvrD and PolA proteins, which are highly conserved proteins commonly employed for phylogenetic analysis [38].

The construction of the phylogenetic trees was done using an internally developed pipeline described by Adeolu et al. (2016) [40]. Briefly, the CD-HIT program and the profile Hidden Markov Models (HMMs) of the proteins that are part of the FBC–phyloeco set [34] were used to search for homologs of these proteins in the input genomes. The search parameters used required that the selected homologs of different proteins shared a minimum of 50% sequence identity and sequence length, and they were found in at least 80% of the input genomes. The Clustal Omega algorithm [44] was used to generate multiple sequence alignments (MSAs) of these protein families. The aligned protein families were trimmed with TrimAI [45] to remove poorly aligned regions before concatenation of the other core proteins. The final concatenated sequence alignments of these proteins used for tree construction consisted of 89,743 aligned amino acids. Based on this sequence alignment, an initial tree was constructed using FastTree 2 [46] based on the Whelan and Goldman model of protein sequence evolution [47] and optimized using RAxML 8 [48] based on the Le and Gascuel model of protein sequence evolution [49]. SH-like statistical support values
(which are similar to bootstrap scores) were calculated for each branch node using RAxML 8 [48]. The resultant phylogenetic tree was drawn using MEGA X [50]. Sequence alignment of the 282 core proteins was also used to calculate the pairwise average amino acid identity (AAI) [51] for the species belonging to the order Chlorobiales. In addition, based on genome sequences for the Chlorobia species, the pairwise percentage of conserved proteins (POCP) between different species was also determined [52,53].

We also constructed a 16S rRNA gene tree for the Chlorobia and Ignavibacteria species based on sequences obtained from the SILVA ribosomal RNA [54] and the NCBI genome database (https://www.ncbi.nlm.nih.gov/ (accessed on 25 March 2022)). The sequences were aligned using the MUSCLE program in MEGA-X [50]. The non-conserved regions as well as regions with gaps were removed, leaving 1269 aligned positions in the final dataset. A maximum-likelihood phylogenetic tree based on this dataset was created using MEGA X [50], employing the Tamura–Nei model [55] based on 100 bootstrap replicates.

2.2. Identification of Conserved Signature Indels

The identification of conserved signature indels (CSIs) was carried out as described in earlier work [56,57]. Briefly, BLASTp searches using the NCBI non-redundant database were carried out on all proteins from the genomes of Chlorobium limicola and Chloroherpeton thalassium. Based on these BLASTp searches, protein sequences were obtained for 8–10 divergent Chlorobia/Ignavibacteria species (generally including representative species from all three main groups of interest, i.e., Chlorobiaceae, Chloroherpeton, and Ignavibacteria) as well as 8–10 species from other bacterial taxa (generally belonging to different families/orders of Bacteroidetes). The multiple sequence alignments (MSAs) of different proteins were created using ClustalX 2.1. However, other programs can also be used for the creation of multiple sequence alignments and will yield similar results [56,57] (unpublished results). Multiple sequence alignments were visually examined for insertions or deletions of fixed length that were present in conserved regions (i.e., flanked on both sides by minimally 4–5 conserved/identical amino acids (aa) in the neighbouring 40–50 aa) and shared by only the Chlorobia/Ignavibacteria species. The query sequences of interest containing the identified conserved indels and their flanking 30–50 aa (generally beginning and ending with a stretch of completely conserved amino acid residues) were reblasted against the NCBI non-redundant (nr) database and the top 250–500 hits were examined. Based on these BLASTp searches, conserved indels that were specifically shared by all or most of the species from the different main clades of Chlorobia/Ignavibacteria were identified and further formatted using SIG_CREATE and SIG_STYLE programs (available from Gleans.net (accessed on 25 March 2022) [56]. Due to space constraints, sequence information is presented in the main figures for only a limited number of representative species. However, unless otherwise stated, the CSIs described here are shared by and are exclusive to the indicated groups of Chlorobia/Ignavibacteria and absent in all other bacterial homologues in the top 250–500 BLASTp hits examined. More detailed information for different CSIs is provided in the Supplemental Figures.

3. Results

3.1. Phylogenetic Analysis of the Chlorobia/Ignavibacteria Species Based on Genome Sequences

The genome sequences for 36 available Chlorobia/Ignavibacteria species in the NCBI database were used to construct a rooted maximum-likelihood phylogenetic tree for these organisms based on concatenated sequences for 282 conserved proteins. The proteins used for tree construction are from the phyloeco set for the FBC group of bacteria and they are conserved and widely distributed in these bacteria [34]. The tree also includes sequences for some outgroup species (viz., R. marina, S. ruber, C. aurantiaca, and B. fragilis) and it was rooted using the sequence for F. succinogenes. The resulting maximum-likelihood distance tree based on 100 bootstrap replicates is shown in Figure 1. All major nodes in this phylogenomic tree are supported by 100% SH-support values (like bootstrap values), indicating that the evolutionary relationships observed here are robust.
for tree construction are from the phyloeco set for the FBC group of bacteria and they are conserved and widely distributed in these bacteria [34]. The tree also includes sequences for some outgroup species (viz., *R. marina*, *S. ruber*, *C. aurantiaca*, and *B. fragilis*) and it was rooted using the sequence for *F. succinogenes*. The resulting maximum-likelihood distance tree based on 100 bootstrap replicates is shown in Figure 1. All major nodes in this phylogenomic tree are supported by 100% SH-support values (like bootstrap values), indicating that the evolutionary relationships observed here are robust.

**Figure 1.** A bootstrapped maximum-likelihood tree for different genome-sequenced *Chlorobia/Ignavibacteria* and related species/strains based on concatenated sequences for 282 conserved proteins that are core proteins for these species. Statistical support values for different branches are indicated on the nodes. The tree was rooted using the sequences from the species *Fibrobacter succinogenes*. The type strains of different species are marked by the superscript T. Some species clades observed in this tree are marked.

In addition to the tree shown in Figure 1, we have also constructed phylogenetic trees for these species using concatenated sequences for GyrA and GyrB proteins as well as PolA and UvrD proteins. The results for these are shown in Supplementary Figures S1 and S2. The branching pattern as well as the grouping of species into different clades in these two trees are identical to that seen in Figure 1.

The branching pattern of species shown in Figure 1 (as well as Figures S1 and S2) is similar to that reported by Roy et al. [25]. Based on this tree, several inferences regarding the evolutionary relationships among the *Chlorobia/Ignavibacteria* species/strains can be drawn. First, all named *Chlorobia* species formed a strongly supported clade (labeled the class *Chlorobia* or the order *Chlorobiales* clade), which is separated from a clade harboring *I. album*, *M. roseus*, and several other uncultured species/strains by a long branch. Second,
the Chlorobiales clade comprises several distinct clades. One of these subclades groups together species from the genera Chlorobaculum, Chlorobium, Pelodictyon, and Prosthecochloris, whereas the second subclade consists of the species Chlororherpeton thalassium [58] and three uncultured thermophilic photoheterotrophic organisms related to Candidatus Thermochlorobacter aerophilum [25,27]. We have designated these two clades as the family Chlorobiaceae and the family Chlororherpetonaceae fam. nov., respectively. Third, within the Chlorobiaceae clade, species from the genera Chlorobaculum and Prosthecochloris group together, supporting the monophyly of these taxa. However, the genus Chlorobium is not monophyletic due to branching within Pelodictyon phaeoclathratiforme, indicating that this latter species is misclassified [12]. Fourth, the second major clade observed in Figure 1 consists of the species I. album and M. roseus, two Candidatus species (viz., Cand. Kapabacterium thiocyanatum [59] and Cand. Kryptonium thompsoni [60]), and several uncultured organisms annotated as Chlorobi bacterium (OLB4, OLB5, OLB6, OLB7, and NCICIL-2). We have designated this clade as the “larger Ignavibacteria clade”. Unlike the Chlorobia species clade, this larger Ignavibacteria clade shows greater genetic diversity, and it is made up of several subclades separated by short branches. We also constructed a phylogenetic tree for Chlorobia/Ignavibacteria species/strains based on 16S rRNA gene sequences (Figure 2).

![Phylogenetic tree](image)

**Figure 2.** Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences for the type strains of all Chlorobi species. The tree was rooted using the sequences for S. ruber and R. marinus. Accession numbers of the 16S rRNA gene sequences are given within the bracket after species names in the tree. The main identified clades in this tree are marked.

Unlike the phylogenomic tree (Figure 1), the 16S rRNA gene tree is characterized by lower bootstrap scores for several branches. In spite of its somewhat poor resolution, all named Chlorobia species formed a distinct clade within this tree. Within this clade, the clade corresponding to the family Chlorobiaceae is strongly supported. However, unlike the core protein tree, this tree did not group together C. thalassium with Candidatus Thermochlorobacter aerophilum and Chlorobium sp. 445 and the latter two species branched more deeply than C. thalassium. Similar branching of C. thalassium and thermophilic phototrophic organisms has also been previously observed [25]. However, the 16S rRNA sequences from thermophilic organisms have higher G+C content, which may, artefactually, lead to more
deeper branching of thermophilic organisms in the phylogenetic tree [61]. Additionally, in the 16S rRNA tree a clade grouping the two Ignavibacteria species with several unnamed Chlorobi bacterium (OLB4, OLB5, OLB6, and NICIL-2) and the two Candidatus species was not observed. These strains/species instead formed several clusters some with very long branches, branching at different positions in between the clades for Chlorobia and outgroup species.

Genome sequences for Chlorobia species were also used to calculate a pairwise matrix of percentage of conserved proteins (POCP) between different species/genomes. The POCP provides a whole-genome-based method for assessing the similarity and differences between species from related taxa [52,53]. It has been suggested that a POCP matrix is more useful than an average amino acid identity (AAI) matrix for discrimination of taxa at or above the genus rank [53]. In Figure 3, we show the POCP matrix for members of the class Chlorobia.

![POCP matrix](image)

**Figure 3.** Pairwise comparison matrix showing the averages of the percentage of conserved proteins (POCP) between different genome-sequenced Chlorobia species. Genome pairs sharing higher POCP values are shaded more darkly (red). The regions of the matrix corresponding to the species from the two families are labeled.

As seen from this matrix, based on the POCP values, different species/strains from the order Chlorobiales are clearly separated into two clades corresponding to the families Chlorobiaceae and Chlorotherpetonaceae. In terms of POCP values, the members of these two clades show no overlap and they are thus clearly distinct. In addition, a matrix based on pairwise AAI values between different Chlorobia species was also calculated based on core proteins using an internally developed pipeline [40]. This matrix is shown in Figure S3. While the AAI values for species within these two families were in the range of 0.72–0.87 (for Chlorobiaceae) and 0.65–0.80 (for Chlorotherpetonaceae), the AAI values for interfamily comparisons ranged from 0.67 to 0.70. Although there is no established threshold value for the demarcation of family-level taxa based on AAI [51], the observed differences between the intrafamily and interfamily AAI values supported the overall distinction between the members of these two family-level clades.

### 3.2. Identification of Molecular Markers Specific for the Main Clades of Chlorobia Species

Results of our phylogenomic studies show that the named Chlorobia species form a strongly supported clade. Furthermore, within it, two family-level clades are observed. However, several uncultured species whose genome sequences are available have been
annotated as Chlorobi bacterium (OLB4, OLB5, OLB6, OLB7, and NICIL-2) branched outside of the main Chlorobia clade. Instead of grouping with the Chlorobiales, these sequences showed a closer relationship to the two Ignavibacteria species. Therefore, it is important to employ other means to reliably demarcate the family Chlorobia and its two family-level clades. With this objective, we have performed detailed comparative studies on protein sequences from Chlorobia genomes to identify molecular markers consisting of CSIs that are uniquely shared by members from the main clades of Chlorobia species. As noted in the introduction, CSIs in gene/protein sequences that are specifically shared by members of a given clade provide an important class of molecular markers for evolutionary and taxonomic studies [21,35,37,41,62,63]. Our analyses of protein sequences from Chlorobia/Ignavibacteria genomes have identified 50 new CSIs that are specific for the different main clades of these bacteria, providing important means for reliably demarcating these clades in molecular terms. The group-specificities and some characteristics of the identified CSIs are described below. Of the identified CSIs, 33 CSIs present in diverse proteins are commonly shared by all or most Chlorobia species for whom genome sequences are available. One example of a CSI specific for the class Chlorobia, as demarcated based on phylogenetic studies (Figures 1 and 2), is presented in Figure 4.

Sequence information for the 32 other CSIs that are also specific for the class Chlorobi (order Chlorobiales) is presented in Figures S4–S35 and some of their characteristics are summarized in Table 1.

### Table 1. Conserved signature indels specific for members of the order Chlorobiales *a,b*

| Protein Name                                      | Accession/GI Number | Figure No. | Indel Size | Indel Position |
|---------------------------------------------------|----------------------|------------|------------|----------------|
| Phosphoribosylformylglycinamidine synthase II     | WP_041465690         | Figure 4   | 5 aa ins   | 270–320        |
| Glycyl-tRNA synthetase                           | 78185909             | Figure 54  | 1 aa ins   | 248–314        |
| RNA polymerase sigma-32 subunit                   | 194337394            | Figure 55  | 12 aa ins  | 24–79          |
| Uroporphyrinogen decarboxylase                    | 189347814            | Figure 56  | 3 aa ins   | 284–333        |
| Glutamyl-tRNA reductase                          | 194336061            | Figure 57  | 1 aa ins   | 211–250        |
| Glutamate-1-semialdehyde aminotransferase         | 21674908             | Figure 58  | 1 aa ins   | 60–109         |
| fructose-1,6-bisphosphate aldolase, class II      | 189500266            | Figure 59  | 11 aa del  | 233–282        |
| A major facilitator superfamily protein           | 193212482            | Figure S10 | 1 aa ins   | 325–365        |
| DNA polymerase III subunit alpha                  | 78187124             | Figure S11 | 2–4 aa ins | 294–340        |
| Acetyl-CoA carboxylase carboxyltransferase subunit alpha | 119356086           | Figure S12 | 6 aa ins   | 103–137        |
| N-acetyl-alpha-D-glucosaminyl L-malate synthase BshA | 193214252         | Figure S13 | 3 aa ins   | 88–132         |
| Serine hydroxymethyltransferase                   | 193215659            | Figure S14 | 2 aa ins   | 228–280        |
| Deoxyribonucleotide triphosphate pyrophosphatase  | 193215546            | Figure S15 | 3 aa ins   | 94–144          |
| Polynucleotide phosphorylase/polyadenylase        | 189346136            | Figure S16 | 1 aa ins   | 37–69           |
| Polynucleotide phosphorylase/polyadenylase        | 189346136            | Figure S17 | 17 aa ins  | 264–335        |
| 7-carboxy-7-deazaguanine synthase QueE             | 500067752            | Figure S18 | 3–7 aa ins | 119–164        |
| DNA polymerase III subunits gamma and tau         | 193213861            | Figure S19 | 2 aa ins   | 47–93           |
| DNA polymerase III subunits gamma and tau         | 193213861            | Figure S20 | 4 aa ins   | 220–268         |
| Cytidylate kinase                                 | 21673125             | Figure S21 | 4 aa ins   | 146–199        |
| 4-hydroxy-3-methylbut-2-enyl diphosphate reductase| 194333263            | Figure S22 | 2–5 aa ins | 264–311        |
| molecular chaperone DnaK                          | 193214979            | Figure S23 | 1 aa del   | 70–119          |
| Tryptophanyl-tRNA synthetase                      | 78188056             | Figure S24 | 8 aa ins   | 16–59           |
| Tryptophanyl-tRNA synthase                        | 493409794            | Figure S25 | 5 aa ins   | 153–204         |
| Succinyl-CoA synthetase subunit alpha             | 193216377            | Figure S26 | 7 aa ins   | 31–87           |
| A Glu/Idh/MocA family oxidoreductase              | 21673819             | Figure S27 | 4 aa ins   | 2–48            |
Table 1. Cont.

| Protein Name                                      | Accession/GI Number | Figure No. | Indel Size | Indel Position |
|--------------------------------------------------|---------------------|------------|------------|----------------|
| A Gfo/Idh/MocA family oxidoreductase              | 21673819            | Figure S28 | 2 aa ins   | 209–249        |
| Ribonuclease R                                    | 193213033           | Figure S29 | 7–8 aa ins | 176–223        |
| Ribonuclease R                                    | 193213033           | Figure S30 | 4–5 aa ins | 558–608        |
| Ribonuclease R                                    | 193213033           | Figure S31 | 4 aa ins   | 640–720        |
| Bifunctional 5,10-methylene-THF dehydrogenase     | 78186789            | Figure S32 | 2 aa ins   | 128–161        |
| Ribonuclease R                                    | 78187149            | Figure S33 | 2 aa ins   | 5–46           |
| Ribonuclease R                                    | 78187419            | Figure S34 | 1 aa ins   | 55–106         |
| DNA mismatch repair protein *                     | 193213813           | Figure S35 | 1 aa del   | 599–644        |

* Except for an isolated exception all of these CSIs are specific for the Chlorobia/Chlorobia species. a For some CSIs, homologs were not found in all Chlorobia species.

Our analyses have also identified multiple CSIs that are specific for the two family-level clades within the order Chlorobiales. Figure 5 presents a partial sequence alignment of the protein polyphosphate kinase-1 highlighting a one-amino-acid insert in a conserved region (boxed) that is exclusively shared by the members of the family Chlorobiales.

The polyphosphate kinase CSI in Figure 5 is commonly shared by all species/strains that are a part of the Chlorobiaceae family (see Figure 1), but it is absent in members of the family Chloroherpetonaceae as well as all other deeper branching species/strains including the Ignavibacteria species, unnamed Chlorobi bacteria, as well as different outgroup bacteria. In addition to this CSI, seven other CSIs identified in this study within other protein sequences are also specific for members of the family Chlorobiaceae. Sequence information for these other CSIs is presented in Figures S36–S42 and some of their characteristics are summarized in Table 2. Based on these CSIs, members of the family Chlorobiaceae can be clearly distinguished in molecular terms from all other bacteria.

Table 2. Conserved Signature Indels Specific for Members of the families Chlorobiaceae and Chloroherpetonaceae *a.

| Protein Name                                      | Accession/GI Number | Figure No. | Indel Size | Indel Position | Specificity |
|--------------------------------------------------|---------------------|------------|------------|----------------|-------------|
| Polyphosphate kinase                             | WP_012466203        | Figure 5   | 1 aa ins   | 25–65          | Family Chlorobiaceae |
| Peptide chain release factor 3                   | 193212508           | Figure S36 | 1 aa del   | 400–447        |             |
| Peptidase U32                                    | 493410285           | Figure S37 | 6 aa ins   | 260–316        |             |
| MiaB-like tRNA modifying protein                 | 145220314           | Figure S38 | 5 aa ins   | 329–382        |             |
| Molecular chaperone HtpG                         | 145219831           | Figure S39 | 3 aa ins   | 259–314        |             |
| DegT/Dnrl/FryC1/StrS aminotransferase            | 193212751           | Figure S40 | 1 aa del   | 111–156        |             |
| biogenesis of lysosome-related organelles complex 1 subunit 2 | 194336576   | Figure S41 | 1 aa del   | 238–279        | Family Chlorobiaceae |
| DNA gyrase subunit A                             | 194335275           | Figure S42 | 1 aa ins   | 234–268        |             |
| UDP-glucose/GDP-mannose dehydrogenase family protein | WP_012498759 | Figure 6   | 2 aa ins   | 287–330        |             |
| hypoxanthine phosphoribosyltransferase           | P1408526            | Figure S43 | 1 aa ins   | 57–94          | Family Chloroherpetonaceae |
| dihydrodiol dehydrogenase                        | P1408610            | Figure S44 | 1 aa ins   | 30–64          |             |
| SDR family oxidoreductase                        | WP_012499003        | Figure S45 | 4 aa ins   | 236–280        |             |
| RecQ family ATP-dependent DNA helicase           | WP_012499407        | Figure S46 | 2 aa ins   | 164–203        |             |
| alkaline phosphatase family protein              | RFM24133            | Figure S47 | 5 aa ins   | 33–78          |             |

* Except for an isolated exception, all of these CSIs are specific for the members of the indicated families. a For some CSIs, homologs were not identified in all members of a given family.
Figure 4. Partial sequence alignment of the protein phosphoribosylformylglycinamidine synthase II showing a five-amino-acid insertion (boxed) in a conserved region that is exclusively shared by all species/strains from the class Chlorobia (Figure 1). This insert is lacking in Ignavibacteria as well as other Candidatus and Chlorobi bacteria strains that do not group with the Chlorobia clade. The dashes (-) in this and all other sequence alignments indicate identity with the amino acids on the top line. Gaps in sequence alignment indicate that no amino acid is present in that position. Accession numbers for different sequences are indicated in the second column and the position of this sequence fragment within the protein is indicated above the sequences.
Figure 5. Excerpts from the sequence alignment of the protein polyphosphate kinase highlighting a one-amino-acid insertion (boxed) in a conserved region that is specifically shared by species/strains that are part of the family Chlorobiaceae (Figure 1). This insert is not found in members of the family Chloroherpetonaceae, class Ignavibacteria, other outgroup bacteria, as well as in other Candidatus and Chlorobi bacteria strains that do not group with the Chlorobia clade.

Six other CSIs identified in this work are specific for members of the family Chloroherpetonaceae. In Figure 6, we show a partial sequence alignment of the protein UDP-glucose GDP-mannose dehydrogenase, where a two-amino-acid insert is present in a conserved region (boxed) that is exclusively found in members of the family Chloroherpetonaceae but not found in any other Chlorobia/Ignavibacteria species or other bacteria. Sequence information for five other CSIs that are also specific for members of the family Chloroherpetonaceae is presented in Figures S43–S47 and some of their characteristics are summarized in Table 2. These CSIs provide strong and independent evidence for the distinctness of the family Chloroherpetonaceae from other Chlorobiales species and provide reliable means for the identification/demarcation of this clade.
Figure 6. Partial sequence alignment of the protein UDP-glucose/GDP-mannose dehydrogenase showing a two-amino-acid insertion (boxed) in a conserved region that is exclusively shared by all species/strains that are part of the proposed family Chloroherpetonaceae (Figure 1). This insert is not found in members of the family Chlorobiaceae, class Ignavibacteria, as well as other Candidatus and Chlorobi bacteria strains that do not group with the Chlorobia clade. Sequence information for five other CSIs specific for the family Chloroherpetonaceae is presented in Figures S43–S47 and Table 2.

Our analysis has also identified one CSI that is commonly shared by most of the species/strains that are a part of the larger Ignavibacteria clade. Sequence information for this CSI consisting of a two-to-three amino acid insert in the protein preprotein translocase subunit SecY is shown in Figure 7. This CSI, in addition to the known Ignavibacteria species/strains, is also commonly shared by specific Candidatus species and unnamed Chlorobi bacterium strains that group with the Ignavibacteria clade in our core protein tree. However, this CSI is absent in all Chlorobiales species and the outgroup bacteria examined.
Figure 7. Partial sequence alignment of the protein preprotein translocase subunit SecY showing a two-to-three amino acid insertion (boxed) in a conserved region that is specifically shared by most of the species/strains that are a part of the larger *Ignavibacteria* clade (Figure 1). This insert is not found in members of the class *Chlorobia* or different outgroup bacteria examined, indicating that the genetic change giving rise to this CSI likely occurred in a common ancestor of the *Ignavibacteria* clade of species/strains.

Lastly, in phylogenetic trees, members of the class *Ignavibacteria* branch in the proximity of *Chlorobia* species (Figure 1) [25–27]. Our work has also identified two CSIs that are uniquely shared by all *Chlorobia* species and different *Ignavibacteria* species/strains. Figure 8 shows a partial sequence alignment of the protein methionine t-RNA ligase, where a four-amino-acid insert is present in a conserved region that is commonly shared by most species/strains from these two groups, but it is not present in different Bacteroides species examined or other closely related bacterial phyla in the top 500 BLASTp hits.
Figure 8. Partial sequence alignment of the protein methionine t-RNA ligase showing a four-amino-acid insertion (boxed) in a conserved region that is specifically shared by all Chlorobia species and most of the species/strains from the larger Ignavibacteria clade (Figure 1). However, this insert is not found in any other outgroup bacteria within the top 500 BLASTp hits. Sequence information for one additional CSI in the protein tRNA-dihydrouridine synthase that is also commonly shared by the species/strains from these two classes is presented in Figure S48.

The CSI shown in Figure 8 in addition to different Chlorobia and Ignavibacteria species is also shared by several Candidatus species, which group within the larger Ignavibacteria clade in our phylogenomic tree (Figure 1). However, several Chlorobi bacterium (viz., OLB5, OLB6, and NICIL-2), which also group within the larger Ignavibacteria clade (Figure 1), do not share this CSI, indicating that the grouping together of all these species/strains is not confirmed by the identified CSI. Sequence information for another CSI, consisting of a one-amino-acid deletion in the protein tRNA-dihydrouridine synthase that is commonly
shared by various Chlorobia and Ignavibacteria species/strains, is presented in Figure S48. This CSI is specific for the Chlorobia and Ignavibacteria species/strains, as homologs of this protein were not detected in other Candidatus species or Chlorobi bacterium strains.

4. Discussion

Members of the class Chlorobia constitute one of the seven discontinuous lineages of prokaryotic organisms that can carry out bacteriochlorophyll and chlorophyll-based photosynthesis [42,64,65]. As these bacteria are primarily found in a narrow environmental niche consisting of anoxic aquatic settings in stratified lakes, where sunlight can penetrate, identification and culturing of these bacteria are often not easy [3–5]. Thus, it is important to better understand the evolutionary relationships amongst these and related bacteria in addition to developing reliable means for their identification. Based on earlier work, members of the class Ignavibacteria are thought to be the closest relatives of Chlorobia [23–27,66]. Genome sequences are now available for most of the named Chlorobia and Ignavibacteria species as well as several other related uncultured species. In the present work, we have used these genome sequences to elucidate the evolutionary relationships among these organisms through several genome-sequence-based approaches. The approaches used to examine their evolutionary relationships include: (i) construction of a phylogenetic tree based on concatenated sequences of 282 core proteins from their genomes (Figure 1); (ii) construction of phylogenetic trees based on concatenated sequences of PolA–UvrD proteins (Figure S1), GyrA–GyrB proteins (Figure S2), and 16S rRNA gene sequences (Figure 2); (iii) determination of pairwise comparison matrices for Chlorobia species based on POCP (Figure 3) and AAI (Figure S3); and (iv) detailed analyses of protein sequences from Chlorobia/Ignavibacteria species, which have identified 50 novel conserved CSIs that are specific for different clades of these organisms. The CSIs in protein sequences result from rare genetic changes [21,41,56]. Hence, the shared presence of these molecular synapomorphies by a given group of species provides strong evidence that the species from that clade shared a common ancestor exclusive of other organisms and thus are specifically related to each other [21,41,56]. Additionally, earlier work on CSIs provides evidence that these molecular markers possess a high degree of predictive ability to be found in other unidentified or uncharacterized members of these clades [35,37,63].

Based on the results presented here, several consistent inferences can be drawn concerning the evolutionary relationships among Chlorobia/Ignavibacteria species. First, the results presented here confirm that the named Chlorobia species form a strongly supported clade, which can be reliably distinguished from Ignavibacteria and other bacteria by phylogenomic analysis and by 33 identified CSIs in diverse proteins that are uniquely shared (synapomorphies) by the members of this clade. The results from phylogenetic studies and clade specificities of the identified CSIs also reveal that several uncultured organisms that are referred to in the NCBI database as Chlorobi bacterium (OLB4, OLB5, OLB6, OLB7, and NICIL-2) are not related to the class Chlorobia and thus they are misclassified (or incorrectly annotated) as Chlorori bacterium. Based on the grouping of these uncultured strains with the larger Ignavibacteria clade, rather than with the clade for Chlorobia species, it is suggested that in future these sequences should be referred to as the Ignavibacteria bacterium OLB4, OLB5, OLB6, OLB7, and NICIL-2. Second, the results presented here provide compelling evidence that the class Chlorobia or the order Chlorobiales comprises two distinct clades. The first of these clades grouping together different species from the genera Chlorobaculum, Chlorobium, Pelodictyon, and Prosthecococcus corresponds to the family Chlorobiaceae. The second clade harbors the species Chlororherpeton thalassium, Candidatus Thermochlorobacter aerophilum, Candidatus Thermochlorobacteriaceae bacterium GBChlB, and an unnamed Chlorobium sp. 445. Separation of the members of the order Chlorobiales into two family-level clades is also observed in other phylogenetic studies including the GTDB taxonomy [4,25,32]. Furthermore, the species from these two clades can also be distinguished from each other in pairwise matrices based on whole-genome comparison of POCP and AAI. However, the strongest evidence that the species from these two clades are
distinct from each other is provided by our identification of eight and six CSIs in diverse proteins, respectively, that are exclusively shared by the members of these two clades. These CSIs provide a novel and unambiguous means for the identification and demarcation of the members of these two clades in molecular terms. Based on the clear distinction seen between the members of these two clades, based on phylogenetic and molecular sequence-based characteristics, we propose that the species from the second clade consisting of Chloroherpeton-related organisms be referred to as a new family Chloroherpetonaceae fam. nov.

It should be noted that the species from the two main clades of Chlorobiales exhibit interesting clade-specific differences in growth, biochemical, and physiological characteristics [4,5,25–27,67,68]. Some of these differences are noted in Figure 9, which also summarizes the results from this study.

**Figure 9.** A conceptual diagram summarizing the evolutionary relationships among members of the phylum Chlorobi based on phylogenetic analysis and specific identified molecular signatures (synapomorphies). The numbers of CSIs that are specific for each clade or species-grouping are noted on the respective nodes. The species/genera present in each clade are listed underneath each label. The physiological and biochemical traits unique to each clade are listed on the right-hand side of the figure. Other notes: 1 These species have yet to be isolated and grown in pure culture; 2 Chlorobium ferroxidans is the sole exception; 3 Subunits NuoEFG missing from the sole copy in all Chlorobiaceae (11 subunits); Chloroherpeton thalassium NuoEFG missing from only one copy (14 and 11 subunits); Candidatus Thermochlorobacter aerophilum (12 and 11 subunits); Ignavibacterium album (14 and 11 subunits); 4 Inferred from genome sequences of Chlorobium sp. 445, Candidatus Thermochlorobacter aerophilum, and Candidatus Thermochlorobacteriaceae bacterium GBChlB; 5 Observations by light and electron microscopy suggested that I. album was not motile and lacked flagella (Iino et al., 2010 [7]). However, the I. album genome contains a nearly complete set of genes for flagella along with genes for chemotaxis and signal transduction. Melioribacter roseus is motile only during exponential growth, using flagella to move through water, but loses its flagella and mobility during stationary growth.
As noted in Figure 9, members of the family Chlorobiaceae are uniformly anaerobic, nonmotile, and photolithoautotrophic bacteria that use reduced sulfur compounds as reductants and electron source to fix CO₂. They contain reverse dissimilatory sulfate reduction (rDsr) system genes to oxidize sulfur to sulfite as well as sulfur oxidation genes (soxXAYZB, soxEF) [25,67,68]. In contrast, members of the proposed Chloroherpetonaceae family exhibit important differences in their biochemical and physiological characteristics. Among these, Chloroherpeton thalassium is an anaerobic photolithotroph like the members of the family Chlorobiaceae. However, this species differs from the other Chlorobiaceae species in that it lacks the genes for the reverse dissimilatory sulfate reduction (rDsr) system as well as the sulfur oxidation genes (soxXAYZB, soxEF) needed to oxidize sulfur to sulfite. It also exhibits flexing and gliding motility. All other members of this clade are uncultured species that, based on their genome sequences, have been inferred to be aerobic photoheterotrophs, containing genes for photosynthetic reaction centers and bacteriochlorophyll biosynthesis, that likely use photo-assimilated acetate and propionate as carbon sources [25–27].

Based on the biochemical and physiological characteristics of Chloroherpeton, which overlap with those of Chlorobiaceae, it has been suggested [25,27] that this species/genus should be placed into a separate family distinct from the other Cand. Thermochlorobacter-related photoheterotrophs. However, based on considerations discussed in this work, we propose to keep all members of this clade in one family rather than two separate families. The main reasons for doing this are as follows. In the different phylogenetic trees constructed in this work and in earlier studies [25,32], Chloroherpeton groups reliably with the Cand. Thermochlorobacter-related photoheterotrophs. Six CSIs identified in this work are also uniquely shared by these two groups, providing strong evidence that the species from these two groups are specifically related. On the other hand, no CSI was identified that was commonly shared by only the Chloroherpeton and Chlorobiaceae. The GTDB taxonomy, which now provides a widely used reference resource for prokaryotic taxonomy, also places these species into a single family. If we place Cand. Thermochlorobacter-related photoheterotrophs into a separate family, it will not be possible to publish it as a valid family in the List of Prokaryotic Names with Standing in Nomenclature [11], as it does not contain any cultured species. However, in future work, if additional information becomes available for this clade of species, including an isolated cultured species for the Cand. Thermochlorobacter-related photoheterotrophs, the possibility of dividing the proposed family Chloroherpetonaceae into two families could be re-evaluated.

In contrast to the monophyly and reliable demarcation of the clades corresponding to the order Chlorobia (class Chlorobia) and the two families within this order, our results indicate that the class Ignavibacteria is genetically highly diverse, and it is difficult at present to unambiguously delineate this taxon either in phylogenetic terms or by means of identified molecular markers. The class Ignavibacteria presently contains only two named species (M. roseus and I. album), which have been placed into two separate families within the order Ignavibacteriales [7,23,24]. However, unlike the class Chlorobia, where very few new species have been identified in the past 10–15 years, in the same period several Candidatus and other uncultured species/strains have been described that branch in the proximity of two Ignavibacteria species (Figures 1a and 2) [17,27,59,60]. In the phylogenomic tree constructed in this work, the two Ignavibacteria species are part of a larger clade that includes several Candidatus species and uncultured Chlorobi species/strains. The GTDB taxonomy also indicates that these Candidatus and uncultured species/strains branch in the proximity of Ignavibacteria [32]. Although branching in a phylogenetic tree is affected by large numbers of variables, and can sometimes be misleading [69–71], a specific grouping or relationship of the species/strains that are a part of the larger Ignavibacteria clade is also independently supported by our identification of a CSI in the SecY protein that is uniquely shared by most of the species/strains from this clade (Figure 7). Two additional CSIs identified in this work, which are commonly shared by different Chlorobia species and several species/strains from this larger Ignavibacteria clade, provide further support that these specific Candidatus and uncultured species/strains are related to these bacteria.
These observations suggest that the circumscription of the class *Ignavibacteria* should be expanded to include these other *Candidatus* and uncultured *Chlorobi* species/strains (i.e., corresponding to the large *Ignavibacteria* clade (Figure 1)). However, we refrain from making a formal proposal, because the number of uncultured *Candidatus* species/strains related to this group is rapidly expanding in the public databases. Thus, more detailed future studies on *Ignavibacteria* and related species/strains need be conducted to develop a more precise and informative classification scheme for this group/class of bacteria, which will likely lead to its division into several different order/family-level taxa.

The present study has identified many molecular markers (CSIs) that are exclusively found in members of different clades within the *Chlorobia/Ignavibacteria* groups of bacteria. Extensive earlier work on CSIs specific for other prokaryotic taxa provides compelling evidence that these molecular markers possess a high degree of predictive ability to be found in other members from these clades whose genome sequences are currently unavailable, as well in novel and uncultured species that are related to these specific clades [39,57,62,63,72,73]. Due to the presence of these CSIs in conserved regions of different genes/proteins, they provide a novel means for the identification of other species from these taxa by either in silico analysis of genomic sequences (based on BLASTp searches checking for the presence or absence of these molecular sequences) or experimental means using PCR-based assays [62,74–76]. Most of the novel species/strains related to the *Chlorobia/Ignavibacteria* taxa reported in recent years consist of uncultured species that have been identified based on analyses of metagenomic sequences from diverse geological habitats [17,27,59,60]. In this context, the molecular markers described here, which are highly specific for these groups of bacteria, should prove helpful in the identification and characterization of other novel species/strains related to these taxa. Lastly, earlier work on CSIs provides evidence that these molecular characteristics are functionally important for the group of organisms for which they are specific, and several of these CSIs found in key chlorophyll–bacteriochlorophyll biosynthesis proteins and core centre proteins have provided important insights into the evolutionary relationships among different groups/phyla of photosynthetic bacteria [77–80]. Currently, very few reliable characteristics are known that are specific for the *Chlorobia/Chlorobiales* species [3,4]. Therefore, to incorporate the information for the CSIs that are specific for the order *Chlorobiales* and the family *Chlorobiaceae*, we also provide emended descriptions of these taxa. In addition, we provide a formal description of the class *Chlorobia* as this has not yet been validly published [11]. The descriptions of the emended and novel taxa are given below.

**Description of the Class Chlorobia class nov. (Garrity and Holt 2001, 601EP)**

*Chlorobia* (Chlo.ro’bi.a. N.L. neut. n. *Chlorobium*, genus of the class, changing ending to denote a class; N.L. neut. pl. n. *Chlorobia*, class of the genus *Chlorobium*).

The class *Chlorobia* contains a single order (*Chlorobiaceae*) [8] and the description of this class is the same as that given below for the order *Chlorobiaceae*.

The type order is *Chlorobiaceae* Gibbons and Murray 1978 (Approved Lists 1980).

**Emended Description of the order Chlorobiaceae Gibbons and Murray 1978 (Approved Lists 1980)**

The order is composed of two families: *Chlorobiaceae* and *Chloroherpetonaceae*. Members of this order form a monophyletic clade in phylogenetic trees based on 16S rRNA gene sequences and trees based on several individual proteins (viz., Fmo, GyrA, GyrB, PolA, RecA, and UvrD) and large datasets of concatenated protein sequences. The order comprises species that are anaerobic and strict photolithoautotrophs, which use reduced sulfur compounds as an electron source to fix CO$_2$, as well as aerobic photoautotrophic organisms, which likely use photo-assimilated acetate and propionate as carbon sources. Species from this phylum generally contain genes encoding for photosynthetic reaction centers and bacteriochlorophyll biosynthesis. The members of this order can be reliably distinguished from all other bacteria based on the shared presence of 33 conserved signature indels (CSIs) in diverse proteins (Table 1) that are uniquely shared by the members...
of this order. The CSIs specific for the order Chlorobiales are found in the following proteins: a major facilitator superfamily protein, a radical SAM-domain-containing protein, a Gfo/Idh/MocA family oxidoreductase, an acetyl-CoA carboxylase carboxyltransferase alpha subunit, a DNA mismatch repair protein, bifunctional 5,10-methylene-tetrahydrofolate dehydrogenase, cytidylyl kinase, deoxyribonucleotide triphosphate pyrophosphatase, a DNA polymerase III alpha subunit, DNA polymerase III subunits gamma and tau, fructose-1,6-bisphosphate aldolase (class II), glutamate-1-semialdehyde aminotransferase, glutamyl-tRNA reductase, glutamate-1-semialdehyde aminotransferase, glycyl-tRNA synthetase, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase, lactoylglutathione lyase, molecular chaperone DnaK, phosphoribosylformylglycinamidine synthase II, polynucleotide phosphorylase/polyadenylase, ribonuclease R, an RNA polymerase sigma-32 subunit, serine hydroxymethyltransferase, succinyl-CoA synthetase subunit alpha, thiazole synthase, tryptophanyl-tRNA synthetase, uroporphyrinogen decarboxylase, N-acetyl-alpha-D-glucosaminyl L-malate synthase BshA, and 7-carboxy-7-deazaguanine synthase QueE. The type genus of this order is Chlorobium Nadson 1906 [81] (Approved Lists 1980).

Emended Description of the family Chlorobiaceae Copeland 1956 (Approved Lists 1980)

The family Chlorobiaceae contains the type genus Chlorobium [8,81] and the following validly published genera: Ancalochloris [8,14], Chlorobaculum [12], Pelodictyon Lauterborn [8], and Prosthecochloris [8,14]. The description of this family is partially based on that given by Imhoff [4]. Members of this family are non-motile photolithoautotrophs and they grow under anoxic conditions in the presence of limited light using reduced sulfur compounds as an electron source to fix CO₂. Thiosulfate, hydrogen, and ferrous iron can also be used as reductants. Most of the members of this family have been isolated from anoxic aquatic settings in stratified lakes, where sunlight can penetrate. All members of this family except Chlorobium ferroxidans possess a dissimilatory sulfite reductase (DSR) system. Members of this family also form a monophyletic clade in phylogenetic trees based on 16S rRNA gene sequences and trees constructed based on large datasets of concatenated protein sequences. The members of this order can be clearly distinguished from members of the family Chloroherpetonaceae and all other bacteria based on the shared presence of eight conserved signature indels (CSIs) found in diverse proteins (listed in Table 2) that are uniquely shared by the members of this family. The proteins containing the CSIs specific for this family are as follows: biogenesis of lysosome-related organelles complex 1 subunit 2, DegT/DnrJ/EryC1/StrS aminotransferase, DNA gyrase subunit A, molecular chaperone HtpG, MiaB-like tRNA modifying protein, peptidase U32, peptide chain release factor 3, and polyphosphate kinase. The type genus of this order is Chlorobium Nadson 1906 [81] (Approved Lists 1980).

Description of the family Chloroherpetonaceae fam. nov.

Chloroherpetonaceae (Chlo.ro.her’pe.to.na.ce’ae. N.L. neut. n. Chloroherpeton, type genus of the family; -aceae ending to denote a family; N.L. fem. pl. n. Chloroherpetonaceae the Chloroherpeton family).

This family contains the type genus Chloroherpeton and another Candidatus genus Thermochlorobacter. In addition, two uncultured organisms with assembled genomes known by the names Chlorobium sp. 445 and Candidatus Thermochlorobacteriaceae GBChlB are also members of this family. Like the Chlorobiaceae family, members of this family contain type-I reaction centers and chlorosomes. The sole cultured species from this family is Chloroherpeton thalassium, which like members of the Chlorobiaceae family is a photolithoautotroph that grows under anoxic conditions in the presence of light using reduced sulfur compounds as an electron source to fix CO₂. However, unlike Chlorobiaceae species, which are nonmotile, cells of Chloroherpeton thalassium exhibit gliding mobility. In contrast to Chloroherpeton, the Candidatus species Thermochlorobacter aerophilum is an aerobic photoheterotroph that cannot oxidize sulfur compounds, cannot fix N₂, and does not fix CO₂ autotrophically [27]. It is considered to have gliding and flexing motility and two copies of the type-1 NADH dehydrogenase complex similarly to Chloroherpeton
**Microorganisms** 2022, 10, 1312

*Chlorobiaceae*. Members of this family are characterized by their lack of a dissimilatory sulfite reductase (DSR) system, which is a protein present in all members of *Chlorobiales* except for *Chlorobium ferroxidans*. Members of this family form a monophyletic clade in a phylogenetic tree based on concatenated sequences of core proteins from the genomes of *Chlorobiaceae* species. The members of this family are also clearly differentiated from the *Chlorobiaceae* species in a pairwise matrix of the percentage of conserved proteins in the genomes. Furthermore, members of this family can be reliably distinguished from members of the family *Chlorobiaceae* and all other bacteria based on the shared presence of six conserved signature indels (CSIs) found in diverse proteins (Table 2) that are uniquely shared by members of this family. The proteins containing the CSIs specific for this family are: alkaline phosphatase family protein, dihydrolipoyl dehydrogenase, hypoxanthine phosphoribosyltransferase, SDR family oxidoreductase, RecQ family ATP-dependent DNA helicase, and UDP-glucose/GDP-mannose dehydrogenase family protein.

The type genus for this family is *Chlorotherpeton* Gibson et al. 1985 [13,82].

**Supplementary Materials:** The following supporting information can be downloaded at: [https://www.mdpi.com/article/10.3390/microorganisms10071312/s1](https://www.mdpi.com/article/10.3390/microorganisms10071312/s1), Figure S1. A bootstrapped maximum-likelihood tree for different genome-sequenced *Chlorobia/Ignavibacteria* and related species/strains based on concatenated sequences of UvrD and PolA proteins. Figure S2. A bootstrapped maximum-likelihood tree for different genome-sequenced *Chlorobia/Ignavibacteria* and related species/strains based on concatenated sequences of GyrA and GyrB proteins. Figure S3. AAI matrix indicating the pairwise percentage average amino acid identities based on core proteins for the type species of different genera within the order *Chlorobiaceae*. Figure S4. Partial sequence alignment of the protein glycine-tRNA synthetase, showing a 1 aa insertion (boxed) that is specific for the order *Chlorobiaceae*. Figure S5. Partial sequence alignment of the protein RNA polymerase sigma factor RpoD/SigA, showing a 12 aa insertion (boxed) that is exclusive to all members belonging to the order *Chlorobiaceae*. Figure S6. Partial sequence alignment of the protein uroporphyrinogen decarboxylase, showing a 3 aa insertion (boxed) that is exclusive to all members belonging to the order *Chlorobiaceae*. Figure S7. Partial sequence alignment of the protein glutamate-1-semialdehyde 2,1-aminomutase, showing a 1 aa insertion (boxed) that is exclusive to all members belonging to the order *Chlorobiaceae*. Figure S8. Partial sequence alignment of the protein glutamate-1-semialdehyde 2,1-aminomutase, showing a 1 aa insertion (boxed) that is found in all members belonging to the order *Chlorobiaceae*. Figure S9. Partial sequence alignment of the protein class II fructose-1,6-bisphosphatase aldolase, showing an 11 aa deletion (boxed) that is found in all members belonging to the order *Chlorobiaceae*. Figure S10. Partial sequence alignment of the protein major facilitator superfamily transporter, showing a 1 aa insertion (boxed) that is specific for members of the order *Chlorobiaceae*. Figure S11. Partial sequence alignment of the protein DNA polymerase III subunit alpha, showing a 2–4 aa insertion (boxed) that is specific for all members belonging to the order *Chlorobiaceae*. Figure S12. Partial sequence alignment of the protein acetyl-CoA carboxylase carboxyltransferase subunit alpha, showing a 6 aa insertion (boxed) that is found in all members belonging to the order *Chlorobiaceae*. Figure S13. Partial sequence alignment of the protein N-acetyl-alpha-D-glucosaminyl L-malate synthase BshA, showing a 3 aa insertion (boxed) that is found in all members belonging to the order *Chlorobiaceae*. Figure S14. Partial sequence alignment of the protein serine hydroxymethyltransferase, showing a 2 aa insertion (boxed) that is found in all members belonging to the order *Chlorobiaceae*. Figure S15. Partial sequence alignment of the protein deoxyribonucleotide triphosphate pyrophosphatase, showing a 3 aa insertion (boxed) that is found in all members belonging to the order *Chlorobiaceae*. Figure S16. Partial sequence alignment of the protein polynucleotide phosphorylase/polyadenylase, showing a 1 aa insertion (boxed) that is found in all members belonging to the order *Chlorobiaceae*. Figure S17. Partial sequence alignment of the protein polynucleotide phosphorylase/polyadenylase, showing a 17 aa insertion (boxed) that is found in all members belonging to the order *Chlorobiaceae*. Figure S18. Partial sequence alignment of the protein 7-carboxy-7-deazaguanine synthase QueE, showing a 3–7 aa insertion (boxed) that is found in all members belonging to the order *Chlorobiaceae*. While other *Chlorobia* species contain a 7 aa insertion, *Chlorobaculum* species have a 3 aa insertion in this position. Figure S19. Partial sequence alignment of the protein DNA polymerase III subunits gamma and tau, showing a 2 aa insertion (boxed) that is found in all members belonging to the order *Chlorobiaceae*. Figure S20. Partial sequence alignment of the protein DNA polymerase III subunits gamma and tau, showing a 4 aa insertion (boxed) that is shared by all members of the order *Chlorobiaceae*.
found in all members belonging to the order Chlorobiales. Figure S21. Partial sequence alignment of the protein cytidylic kinase, showing a 4 aa insertion (boxed) that is found in all members belonging to the order Chlorobiales. Figure S22. Partial sequence alignment of the protein 4-hydroxy-3-methylbut-2-enyl diphosphate reductase, showing a 2–5 aa insertion (boxed) that is found in all members belonging to the order Chlorobiales. Figure S23. Partial sequence alignment of the protein molecular chaperone DnaK, showing a 1 aa deletion (boxed) that is found in all members belonging to the order Chlorobiales. Figure S24. Partial sequence alignment of the protein tryptophanyl-tRNA synthetase, showing an 8 aa insert (boxed) that is found in all members belonging to the order Chlorobiales. Figure S25. Partial sequence alignment of the protein tryptophanyl-tRNA synthetase, showing a 5 aa insert (boxed) that is found in all members belonging to the order Chlorobiales. Figure S26. Partial sequence alignment of the protein succinyl-CoA synthetase subunit alpha, showing a 7 aa insert (boxed) that is found in all members belonging to the order Chlorobiales. Figure S27. Partial sequence alignment of the protein Gfo/Idh/MocA family oxidoreductase, showing a 4 aa insert (boxed) that is found in all members belonging to the order Chlorobiales. Figure S28. Partial sequence alignment of the protein Gfo/Idh/MocA family oxidoreductase, showing a 2 aa insert (boxed) that is found in all members belonging to the order Chlorobiales. Figure S29. Partial sequence alignment of the protein ribonuclease R, showing a 7–8 aa insert (boxed) that is found in all members belonging to the order Chlorobiales. Figure S30. Partial sequence alignment of the protein ribonuclease R, showing a 4–5 aa insert (boxed) that is found in all members belonging to the order Chlorobiales. Figure S31. Partial sequence alignment of the protein ribonuclease R, showing a 4 aa insert (boxed) that is found in all members belonging to the order Chlorobiales. Figure S32. Partial sequence alignment of the protein bifunctional 5,10-methylene-tetrahydrofolate dehydrogenase, showing a 2 aa insert (boxed) that is found in all members belonging to the order Chlorobiales. Figure S33. Partial sequence alignment of the protein lactoylglutathione lyase, showing a 2 aa insert (boxed) that is found in all members belonging to the order Chlorobiales. Figure S34. Partial sequence alignment of the anti-sense, showing a 1 aa insert (boxed) that is found in all members belonging to the order Chlorobiales. Figure S35. Partial sequence alignment of the protein DNA mismatch repair protein (MutS), showing a 1 aa deletion (boxed) that is found in all members belonging to the order Chlorobiales. Figure S36. Partial sequence alignment of the protein peptide chain release factor 3, showing a 1 aa deletion (boxed) that is specific for members of the family Chlorobiaceae. Figure S37. Partial sequence alignment of the protein U32 family peptidase, showing a 6 aa insertion (boxed) that is specific for members of the family Chlorobiaceae. Figure S38. Partial sequence alignment of the protein like tRNA modifying protein, showing a 5 aa insertion (boxed) that is specific for members belonging to the family Chlorobiaceae. Figure S39. Partial sequence alignment of the protein tRNA dihydrouridine synthase, showing a 1 aa insertion (boxed) that is found in all members belonging to the order Chlorobiales. Figure S40. Partial sequence alignment of the protein tRNA dihydrouridine synthase, showing a 1 aa insertion (boxed) that is found in all members belonging to the order Chlorobiales. Figure S41. Partial sequence alignment of the biogenesis of lysosome-related organelles complex 1 subunit 2 protein, showing a 1 aa deletion (boxed) that is specific for members of the family Chlorobiaceae. Figure S42. Partial sequence alignment of the protein DNA gyrase subunit A, showing a 1 aa insertion (boxed) that is specific for members belonging to the family Chlorobiaceae. Figure S43. Partial sequence alignment of the protein hypoxanthine phosphoribosyltransferase, showing a 1 aa insertion (boxed) that is specific for members of the family Chlorobiaceae. Figure S44. Partial sequence alignment of the protein hypoxanthine phosphoribosyltransferase, showing a 1 aa insertion (boxed) that is exclusively shared by all members of the family Chlorobiaceae. Figure S45. Partial sequence alignment of the protein dihydrolipoamide dehydrogenase containing a 1 aa insertion (boxed) that is specific for members of the family Chlorobiaceae. Figure S46. Partial sequence alignment of the protein SDR family oxidoreductase, showing a 4 aa insertion (boxed) that is specific for members of the family Chlorobiaceae. Figure S47. Partial sequence alignment of the protein RecQ family ATP-dependent DNA helicase, showing a 2 aa insertion (boxed) that is specific for members of the family Chlorobiaceae. Figure S48. Partial sequence alignment of the protein tRNA dihydrodouridine synthase DusB, showing a 1 aa insertion (boxed) that is specific for members of the order Chlorobiales and the “larger Ignavibacteria clade”.

**Author Contributions:** Conceptualization, R.S.G.; data curation, S.B.; formal analysis, S.B. and M.H.-A.; funding acquisition, R.S.G.; methodology, S.B., M.H.-A. and R.S.G.; project administration, R.S.G.; resources, R.S.G. and H.E.S.; software, R.S.G.; supervision, R.S.G.; validation, S.B. and R.S.G.;
writing—original draft, R.S.G.; writing—review & editing, R.S.G. and H.E.S. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was supported by a Discovery Research Grant (RGPIN-2019-06397), an Alliance Research Grant from the Natural Science and Engineering Research Council of Canada (HES and RSG), and an ORF grant from the Ontario Ministry of Research, Innovation and Science.

**Data Availability Statement:** The data presented in this study are available in a publicly accessible repository ([https://www.ncbi.nlm.nih.gov/genome/](https://www.ncbi.nlm.nih.gov/genome/), accessed on 10 January 2022) and the Supplementary Material.

**Conflicts of Interest:** The authors declare no conflict of interest.

**References**

1. Pfennig, N. Chlorobium. In *Bergey’s Manual of Systematics of Archaea and Bacteria*; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2015. [CrossRef]
2. Pfennig, N. Chlorobium phaeobacteroides nov. spec. and C. phaeovibrioides nov. spec., two new species of green sulfur bacteria. *Arch. Mikrobiol.* **1968**, *63*, 224–226. [CrossRef]
3. Imhoff, J.F.; Thiel, V. Phylogeny and taxonomy of Chlorobiaceae. *Photosynth. Res.* **2010**, *104*, 123–136. [CrossRef] [PubMed]
4. Imhoff, J.F. The Family Chlorobiaceae. In *The Prokaryotes—Other Major Lineages of Bacteria and the Archaea*, 4th ed.; Rosenberg, E., De Long, E.F., Lory, S., Stackebrandt, E., Thompson, F., Eds.; Springer: Berlin/Heidelberg, Germany, 2014. [CrossRef]
5. Overmann, J. The Family Chlorobiaceae. In *The Prokaryotes*, 3rd ed.; Springer: New York, NY, USA, 2003.
6. Kondratieva, E.N.; Pfennig, N.; Truper, H.G. The Phototrophic Prokaryotes. In *The Prokaryotes*, 2nd ed.; Balows, A., Truper, H.G., Dworkin, M., Harder, W., Schleifer, K.H., Eds.; Springer-Verlag: New York, NY, USA, 1992; Volume 1, pp. 312–330.
7. Iino, T.; Mori, K.; Uchino, Y.; Nakagawa, T.; Harayama, S.; Suzuki, K. *Ignavigibacterium album* gen. nov., sp. nov., a moderately thermophilic anaerobic bacterium isolated from microbial mats at a terrestrial hot spring and proposal of Ignavigibacter classis nov., for a novel lineage at the periphery of green sulfur bacteria. *Int. J. Syst. Evol. Microbiol.* **2010**, *60*, 1376–1382. [CrossRef]
8. Skerman, V.B.D.; McGowan, V.;neath, P.H.A. Approved lists of bacterial names. *Int. J. Syst. Bacteriol.* **1980**, *30*, 225–420. [CrossRef]
9. Truper, H.G.; Pfennig, N. The Family Chlorobiaceae. In *The Prokaryotes*, 2nd ed.; Balows, A., Truper, H.G., Dworkin, M., Harder, W., Schleifer, K.H., Eds.; Springer: New York, NY, USA, 1992; pp. 3583–3592.
10. Gibbons, N.E.; Murray, R.G.E. Proposals Concerning the Higher Taxa of Bacteri. *Int. J. Syst. Evol. Microbiol.* **1978**, *28*, 1–6. [CrossRef]
11. Parte, A.C. LPSN—The List of Prokaryotic Names with Standing in Nomenclature. *Int. J. Syst. Evol. Microbiol.* **2018**, *68*, 1825–1829. [CrossRef] [PubMed]
12. Imhoff, J.F. Phylogenetic taxonomy of the family Chlorobiaceae on the basis of 16S rRNA and fmo (Fenna-Matthews-Olson protein) gene sequences. *Int. J. Syst. Evol. Microbiol.* **2003**, *53*, 941–951. [CrossRef] [PubMed]
13. Gibson, J.; Pfennig, N.; Waterbury, J.B. Chloroherpeton thalassium gen. nov. et spec. nov., a non-filamentous, flexing and gliding green sulfur bacterium. *Arch. Microbiol.* **1984**, *138*, 96–101. [CrossRef]
14. Gorlenko, V.M.; Lebedeva, E.V. New green sulfur bacteria with apophyses. *Mikrobiologiia* **1971**, *40*, 1035–1039.
15. Alexander, B.; Andersen, J.H.; Cox, R.P.; Imhoff, J.F. Phylogeny of green sulfur bacteria on the basis of gene sequences of 16S rRNA and of the Fenna-Matthews-Olson protein. *Arch. Microbiol.* **2002**, *178*, 131–140. [CrossRef]
16. Panwar, P.; Allen, M.A.; Williams, T.J.; Haque, S.; Brazendale, S.; Hancock, A.M.; Paz‐Espino, D.; Cavichioli, R. Remarkably coherent population structure for a dominant Antarctic Chlorobium species. *Microbiomes* **2021**, *9*, 231. [CrossRef] [PubMed]
17. Tsuji, J.M.; Tran, N.; Schiff, S.L.; Venkiteswaran, J.J.; Molot, L.A.; Tank, M.; Hanada, S.; Neufeld, J.D. Anoxygenic photosynthesis and iron-sulfur metabolic potential of Chlorobia populations from seasonally anoxic Boreal Shield lakes. *ISME J.* **2020**, *14*, 2732–2747. [CrossRef] [PubMed]
18. Oren, A.; Garrity, G.M. Valid publication of the names of forty-two phyla of prokaryotes. *Int. J. Syst. Evol. Microbiol.* **2021**, *71*, 005056. [CrossRef] [PubMed]
19. Gupta, R.S.; Lorenzini, E. Phylogeny and molecular signatures (conserved proteins and indels) that are specific for the Bacteroidetes and Chlorobii species. *BMC Evol. Biol.* **2007**, *7*, 71. [CrossRef]
20. Gupta, R.S. The Phylogeny and Signature Sequences characteristics of Fibrobacters, Chlorobi and Bacteroidetes. *Crit. Rev. Microbiol.* **2004**, *30*, 123–143. [CrossRef]
21. Gupta, R.S. Impact of genomics on the understanding of microbial evolution and classification: The importance of Darwin’s views on classification. *FEMS Microbiol. Lett.* **2016**, *40*, 520–533. [CrossRef]
22. Kadnikov, V.V.; Mardanov, A.V.; Podosokorskaya, O.A.; Gavrilo, S.N.; Kublanov, I.V.; Beletsky, A.V.; Bonch-Osmolovskaya, E.A.; Ravin, N.V. Genomic analysis of Melioribacter roseus, facultatively anaerobic organotrophic bacterium representing a novel deep lineage within Bacteriodetes/Chlorobi group. *PLoS ONE* **2013**, *8*, e53047. [CrossRef]
23. Podosokorskaya, O.A.; Kadnikov, V.V.; Gavrilov, S.N.; Mardanov, A.V.; Merkel, A.Y.; Karnachuk, O.V.; Ravin, N.V.; Bonch-Osmolovskaya, E.A.; Kublanov, I.V. Characterization of Melioribacter roseus gen. nov., sp. nov., a novel facultatively anaerobic thermophilic cellulolytic bacterium from the class Ignavibacteria, and a proposal of a novel bacterial phylum Ignavibacteria. *Environ. Microbiol.* 2013, 15, 1759–1771. [CrossRef]

24. Iino, T. Ignavibacteria. In *Berger’s Manual of Systematics of Archaea and Bacteria*; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2018; p. 1. [CrossRef]

25. Roy, C.; Bakshi, U.; Rameez, M.J.; Mandal, S.; Haldar, P.K.; Pyne, P.; Ghosh, W. Phylogenomics of an uncultivated, aerobic and thermophilic, photoheterotrophic member of Chlorobia sheds light into the evolution of the phylum Chlorobi. *Comput. Biol. Chem.* 2019, 80, 206–216. [CrossRef]

26. Hiras, J.; Yu, W.Y.; Eichorst, S.A.; Simmons, B.A.; Singer, S.W. Refining the phylum Chlorobi by resolving the phylogeny and metabolic potential of the representative of a deeply branching, uncultivated lineage. *ISME J.* 2016, 10, 835–843. [CrossRef]

27. Liu, Z.; Klatt, C.G.; Ludwig, M.; Rusc, D.B.; Jensen, S.I.; Kuhl, M.; Ward, D.M.; Bryant, D.A. ‘Candidatus Thermochlorobacter aerophilum’, an aerobic chlorophilophototrophic member of the phylum Chlorobi defined by metagenomics and metatranscriptomics. *ISME J.* 2012, 6, 1869–1882. [CrossRef] [PubMed]

28. Mukherjee, S.; Seshadri, R.; Varghese, N.J.; Eloe-Fadrosh, E.A.; Meier-Kolthoff, J.P.; Goker, M.; Coates, R.C.; Hadjithomas, M.; Pavloveoulos, G.A.; Paez-Espino, D.; et al. 1003 reference genomes of bacterial and archaeal isolates expand coverage of the tree of life. *Nat. Biotechnol.* 2017, 35, 676–683. [CrossRef] [PubMed]

29. Whitman, W.B. Genome sequences as the type material for taxonomic descriptions of prokaryotes 1. *Syst. Appl. Microbiol.* 2015, 38, 217–222. [CrossRef]

30. Wu, L.; McCluskey, K.; Desmth, P.; Liu, S.; Hideaki, S.; Yin, Y.; Moriya, O.; Itoh, T.; Kim, C.Y.; Lee, J.S.; et al. The global catalogue of microorganisms 10K type strain sequencing project: Closing the genomic gaps for the validly published prokaryotic and fungi species. *Gigascience* 2018, 7, gky026. [CrossRef] [PubMed]

31. Sayers, E.W.; Agarwala, R.; Bolton, E.E.; Brister, J.R.; Canese, K.; Clark, K.; Connor, R.; Fiorini, N.; Funk, K.; Hefferon, T.; et al. Database resources of the National Center for Biotechnology Information. *Nucleic Acids Res.* 2019, 47, D23–D28. [CrossRef]

32. Parks, D.H.; Chuvochina, M.; Waite, D.W.; Rinke, C.; Skarshewski, A.; Chaumeil, P.A.; Hugenholtz, P. A standardized bacterial taxonomy based on genome phylogeny substantially revises the tree of life. *Nat. Biotechnol.* 2018, 36, 996–1004. [CrossRef]

33. Rokas, A.; Williams, B.L.; King, N.; Carroll, S.B. Genome-scale approaches to resolving incongruence in molecular phylogenies. *Nature* 2003, 425, 798–804. [CrossRef]

34. Wu, D.; Jospin, G.; Eisen, J.A. Systematic identification of gene families for use as “markers” for phylogenetic and phylogeny-driven ecological studies of bacteria and archaea and their major subgroups 1. *PLoS ONE* 2013, 8, e77033. [CrossRef] [PubMed]

35. Gupta, R.S.; Patel, S.; Saini, N.; Chen, S. Robust demarcation of 17 distinct *Mycobacterium* gen. nov. and *Tuberculosis* gen. nov. and *Mycobacterium* gen. nov. and *Alkalitaleobacillus* gen. nov. Int. J. Syst. Evol. Microbiol. 2020, 70, 5753–5798.

36. Hugenholtz, P.; Chuvochina, M.; Oren, A.; Parks, D.H.; Soo, R.M. Prokaryotic taxonomy and nomenclature in the age of big sequence data. *ISME J.* 2021, 15, 1879–1892. [CrossRef]

37. Gupta, R.S. Microbial Taxonomy: How and Why Name Changes Occur and Their Significance for (Clinical) Microbiology. *Clin. Chem.* 2021, 68, 134–137. [CrossRef] [PubMed]

38. Patel, S.; Gupta, R.S. A Phylogenomics and Comparative Genomic Framework for Resolving the Polyphyly of the Genus *Bacillus*: Proposal for six new genera of *Bacillus* species, *Peribacillus* gen. nov., *Cytobacillus* gen. nov., *Mesobacillus* gen. nov., *Neobacillus* gen. nov., *Metabacillus* gen. nov. and *Alkalitaleobacillus* gen. nov. *Int. J. Syst. Evol. Microbiol.* 2020, 70, 406–438.

39. Gupta, R.S.; Lo, B.; Son, J. Phylogenomics and Comparative Genomic Studies Robustly Support Division of the Genus *Mycobacterium* into an Emended Genus *Mycobacterium* and Four Novel Genera. *Front. Microbiol.* 2018, 9, 67. [CrossRef] [PubMed]

40. Adeolu, M.; Alnajar, S.; Naushad, S.; Gupta, R.S. Genome-based phylogeny and taxonomy of the ‘Enterobacteriales’: Proposal for *Enterobacteriales* ord. nov. divided into the families *Enterobacteriaceae*, *Erwiniaeae* fam. nov., *Pectobacteriaceae* fam. nov., *Yersiniaceae* fam. nov., *Hafniaceae* fam. nov., *Morganellaceae* fam. nov., and *Budviciaceae* fam. nov. *Int. J. Syst. Evol. Microbiol.* 2016, 66, 5575–5599.

41. Rokas, A.; Holland, P.W. Rare genomic changes as a tool for phylogenetics. *Trends Ecol. Evol.* 2000, 15, 454–459. [CrossRef]

42. Gupta, R.S. Molecular signatures for the main phyla of photosynthetic bacteria and their subgroup. *Photosynth. Res.* 2010, 104, 357–372. [CrossRef]

43. Gupta, R.S.; Chander, P.; George, S. Phylogenetic framework and molecular signatures for the class Chloroflexi and its different clades; proposal for division of the class Chloroflexia class. nov. [corrected] into the suborder Chloroflexineae subord. nov., consisting of the emended family Oscillochloridaceae and the family Chloroflexaceae fam. nov., and the suborder Roseiflexineae subord. nov., containing the family Roseiflexaceae fam. nov. *Antonie Van Leeuwenhoek* 2013, 103, 99–119.

44. Sievers, F.; Wilm, A.; Dineen, D.; Gibson, T.J.; Karplus, K.; Li, W.; Lopez, R.; McWilliam, H.; Remmert, M.; Soding, J.; et al. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol.* 2011, 7, 539. [CrossRef]

45. Capella-Gutierrez, S.; Silla-Martinez, J.M.; Gabaldon, T. trimAl: A tool for automated alignment trimming in large-scale phylogenetic analyses. *Bioinformatics* 2009, 25, 1972–1973. [CrossRef]
46. Price, M.N.; Dehal, P.S.; Arkin, A.P. FastTree 2—approximatively maximum-likelihood trees for large alignments. *PLoS ONE* 2010, 5, e9490. [CrossRef]

47. Whelan, S.; Goldman, N. A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. *Mol. Biol. Evol.* 2001, 18, 691–699. [CrossRef] [PubMed]

48. Stamatakis, A. RAXML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 2014, 30, 1312–1313. [CrossRef] [PubMed]

49. Le, S.Q.; Gascuel, O. An improved general amino acid replacement matrix. *Mol. Biol. Evol.* 2008, 25, 1307–1320. [CrossRef] [PubMed]

50. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol. Biol. Evol.* 2018, 35, 1547–1549. [CrossRef]

51. Thompson, C.C.; Amaral, G.R.; Campeao, M.; Edwards, R.A.; Polz, M.F.; Dutilh, B.E.; Ussery, D.W.; Sawabe, T.; Swings, J.; Thompson, F.L. Microbial taxonomy in the post-genomic era: Rebuilding from scratch? 3. *Arch. Microbiol.* 2015, 197, 359–370. [CrossRef]

52. Gupta, R.S. Distinction between Borrelia and Borreliella is more robustly supported by molecular and phenotypic characteristics than all other neighbouring prokaryotic genera: Response to Margos’ et al. “The genus Borrelia reloaded” (*PLoS ONE* 13(12): e0208432). *PLoS ONE* 2019, 14, e0221397. [CrossRef]

53. Qin, Q.L.; Xie, B.B.; Zhang, X.Y.; Chen, X.L.; Zhou, B.C.; Zhou, J.; Oren, A.; Zhang, Y.Z. A proposed genus boundary for the newly proposed genus *Eubacterium*. *FEMS Microbiol. Lett.* 2017, 366, 1–8. [CrossRef]

54. Yilmaz, P.; Parfrey, L.W.; Yarza, P.; Gerken, J.; Pruesse, E.; Schweer, T.; Peplies, J.; Ludwig, W.; Glockner, F.O. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Res.* 2014, 42, D630–D641. [CrossRef]

55. Tamura, K.; Nei, M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.* 1993, 10, 512–526. [CrossRef]

56. Gupta, R.S. Identification of Conserved Indels that are Useful for Classification and Evolutionary Studies. In *Bacterial Taxonomy, Methods in Microbiology Volume 41*; Goodfellow, M., Sutcliffe, I.C., Chun, J., Eds.; Elsevier: Amsterdam, The Netherlands, 2014; Volume 41, pp. 153–182.

57. Naushad, H.S.; Lee, B.; Gupta, R.S. Conserved signature indels and signature proteins as novel tools for understanding microbial phylogeny and systematics: Identification of molecular signatures that are specific for the phytopathogenic genera *Dickeya*, Pectobacterium and Brenneria. *Int. J. Syst. Evol. Microbiol.* 2014, 64, 366–383. [CrossRef]

58. Gibson, J. Chloroherpeton. In *Bergey’s Manual of Systematics of Archaea and Bacteria*; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2015. [CrossRef]

59. Kantor, R.S.; van Zyl, A.W.; van Hille, R.P.; Thomas, B.C.; Harrison, S.T.; Banfield, J.F. Bioreactor microbial ecosystems for thiocyanate and cyanide degradation unravelled with genome-resolved metagenomics. *Environ. Microbiol.* 2015, 17, 4929–4941. [CrossRef] [PubMed]

60. Price, M.N.; Dehal, P.S.; Arkin, A.P. FastTree 2—approximately maximum-likelihood trees for large alignments. *PLoS ONE* 2010, 5, e9490. [CrossRef]

61. Holkenbrink, C.; Barbas, S.O.; Mellerup, A.; Otaki, H.; Frigaard, N.U. Sulfur globule oxidation in green sulfur bacteria is dependent on the dissimilatory sulfite reductase system. *Microbiology* 2011, 157, 1229–1239. [CrossRef]

62. Felsenstein, J. Inferring Phylogenies; Sinauer Associates, Inc.: Sunderland, MA, USA, 2004.

63. Baldauf, S.L. Phylogeny for the faint of heart: A tutorial. *Trends Genet.* 2003, 19, 345–351. [CrossRef]

64. Gupta, R.S. Protein phylogenies and signature sequences: A reappraisal of evolutionary relationships among archaeabacteria, eubacteria, and eukaryotes. *Microbiol. Mol. Biol. Rev.* 1998, 62, 1435–1491. [CrossRef] [PubMed]
72. Barbour, A.G.; Adeolu, M.; Gupta, R.S. Division of the genus Borrelia into two genera (corresponding to Lyme disease and relapsing fever groups) reflects their genetic and phenotypic distinctiveness and will lead to a better understanding of these two groups of microbes (Margos et al. (2016) There is inadequate evidence to support the division of the genus Borrelia. Int. J. Syst. Evol. Microbiol. doi: 10.1099/ijsem.0.001717). Int. J. Syst. Evol. Microbiol. **2017**, **67**, 2058–2067. [CrossRef] [PubMed]

73. Gupta, R.S. Protein signatures (molecular synapomorphies) that are distinctive characteristics of the major cyanobacterial clades. *Int. J. Syst. Evol. Microbiol.* **2009**, **59**, 2510–2526. [CrossRef] [PubMed]

74. Gao, B.; Gupta, R.S. Conserved indels in protein sequences that are characteristic of the phylum Actinobacteria. *Int. J. Syst. Evol. Microbiol.* **2005**, **55**, 2401–2412. [CrossRef] [PubMed]

75. Ahmod, N.Z.; Gupta, R.S.; Shah, H.N. Identification of a *Bacillus anthracis* specific indel in the yeAC gene and development of a rapid pyrosequencing assay for distinguishing *B. anthracis* from the *B. cereus* group. *J. Microbiol. Methods* **2011**, **87**, 278–285. [CrossRef]

76. Wong, S.Y.; Paschos, A.; Gupta, R.S.; Schellhorn, H.E. Insertion/deletion-based approach for the detection of Escherichia coli O157:H7 in freshwater environments. *Environ. Sci. Technol.* **2014**, **48**, 11462–11470. [CrossRef]

77. Singh, B.; Gupta, R.S. Conserved inserts in the Hsp60 (GroEL) and Hsp70 (DnaK) proteins are essential for cellular growth. *Mol. Genet. Genom.* **2009**, **281**, 361–373. [CrossRef] [PubMed]

78. Khadka, B.; Gupta, R.S. Identification of a Conserved 8 aa Insert in the PIP5K Protein in the Saccharomycetaceae family of Fungi and the Molecular Dynamics Simulations and Structural Analysis to investigate its Potential Functional Role. *Proteins* **2017**, **85**, 1454–1467. [CrossRef]

79. Khadka, B.; Adeolu, M.; Blankenship, R.E.; Gupta, R.S. Novel insights into the origin and diversification of photosynthesis based on analyses of conserved indels in the core reaction center proteins. *Photosynth. Res.* **2016**, **131**, 159–171. [CrossRef]

80. Gupta, R.S. Molecular Markers for Photosynthetic Bacteria and Insights into the Origin and Spread of Photosynthesis. *Adv. Bot. Res.* **2013**, **66**, 37–66.

81. Nadson, G.A. The morphology of inferior Algae. III. Chlorobium limicola Nads., the green chlorophyll bearing microbe. *Bull. Du Jard. Bot. St. Petersbourg* **1906**, **6**, 190.

82. Gibson, J.; Pfennig, N.; Waterbury, J.B. In Validation list no. 17. Validation of publication of new names and new combinations previously effectively published outside the IJSB. *Int. J. Syst. Bacteriol.* **1985**, **35**, 223–225.